A predictive toolkit for evaluating single-color controls in spectral flow cytometry

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Abstract

Understanding immunologically relevant cellular phenotypes within models necessitates the use of high-dimensional flow cytometry panels.

Spectral flow cytometry supports these advancements but is critically dependent on single-color controls (SCCs) for accurate unmixing. Current compensation beads employed as SCCs may introduce unmixing errors. Our research activities endeavor to refine spectral flow cytometry by optimizing and statistically evaluating SCC performance to predict and mitigate unmixing errors in panels.

We engineered a comprehensive 33-color pseudo-panel to systematically compare various SCCs. Rather than bulk unmixing, we executed isolated unmixing of distinct fluorochromes substituted with bead SCCs to pinpoint sources of unmixing errors. By leveraging advanced robotic process automation and devising a novel statistical scoring system, we quantified unmixing errors across extensive datasets. This approach minimized subjectivity through the creation of a "Matrix of Unmixability," which evaluates each SCC's efficacy in unmixing bi-variate fluorochrome combinations within a complex panel.

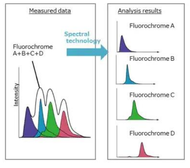
Using this tool, we then developed 20-color immunophenotyping panels with predictable unmixing error reproducibility and strategies to circumvent them. This advancement allows for the conservation of precious cells as SCCs while concurrently reducing bead-induced unmixing errors conserving researchers' time and resources.

Introduction

Spectral flow cytometry analysis involves the deconvolution of different spectral signatures of each fluorophore in a multicolor experiment using their representative single-color spectra as a reference. It is critical that these single-color controls (SCCs) accurately represent the fluorophores in the multicolor experimental samples.

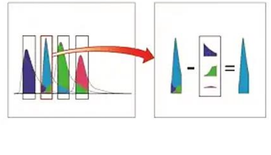
Spectral Unmixing

SCC must accurately determine spectral **fingerprint** of each fluorophore



Conventional Compensation

SCC must accurately determine spectral **overlap** of each fluorophore



Compensation Beads, such as Invitrogen™ UltraComp eBeads™ Plus, are useful tools for conventional compensation and spectral unmixing, particularly 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

However, subtle variations in the spectral signatures of SCCs on compensation beads compared to those observed in actual cell or experimental samples can lead to errors in spectral unmixing. These discrepancies can produce misleading results, potentially leading to incorrect conclusions. Predicting these unmixing errors is challenging, and there are limited resources available to address this issue.

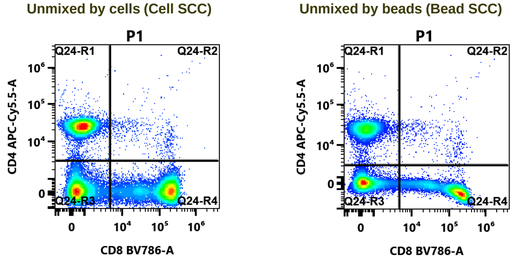


Figure 1. Unmixing errors can occur when using compensation beads to unmix experimental cell data

Materials and Methods

Invitrogen™ UltraComp Spectral eBeads™

The microenvironment following fluorophore-antibody conjugate staining can affect the stability of emission characteristics. To optimize performance specifically for spectral flow cytometry, we evaluated various surface chemistry modifications to better align with the spectral properties of experimental samples. This research resulted in the development of UltraComp Spectral eBeads, a product better tailored to meet the requirements of spectral flow cytometry.

Single color controls

Spectral unmixing errors from spectral mismatching of compensation beads were assessed using 33 fluorescent dyes covering the full spectral range with excitation by all five lasers on the Cytek Aurora and evaluated various dye technology platforms such as small organic dyes, protein-based dyes, tandem dyes, Phiton-based NovaFluors, and polymer-based dyes. Dyes were conjugated to CD4 on human peripheral blood cells, UltraComp eBeads Plus, and UltraComp eBeads Spectral. CD4 was chosen for its high antigen density and clear staining pattern, making it excellent for comparing dye performance. Additionally, brighter staining is more sensitive to unmixing errors caused by spectral mismatching.

Results - Workflow

Unmixing performance was evaluated by comparing the unmixing results between using all cell-based single-color controls and using cell-based single-color controls with a single bead-based single-color control replacement for each fluorescent dye. Cell based unmixing was use as the standard for correct unmixing. We believed that unmixing individual fluorophores would allow a more precise understanding of which fluorophore-bead pairings could potentially lead to unmixing errors in cell samples.

Unmixing iterations used robotic process automation

The physical process of unmixing one individual dye single-color controls to a bead-based control can be time consuming, especially for larger panels, so a robotic process automation was utilized. Using a UiPath software package, individual dyes were exchanged, unmixed, and .fcs files saved for further evaluation.

Statistics evaluated using R and Excel

Unmixing errors can be identified subjectively by looking for instances where the cell-stained single-color control exhibited unwanted fluorescent signals in other fluorescent dye channels. Typically, a subject matter expert would need to evaluate multiple NxNs to determine where unmixing errors might occur if a bead-based single-color control was used.

Subjective evaluation of potential unmixing errors by a subject matter expert can be a time-consuming process, and different evaluators may reach different conclusions. To address this, we explored and implemented a more rapid and objective process by incorporating R, R packages from Bioconductor, and Excel to evaluate potential unmixing errors. Using these tools, we were able to predict unmixing errors in multi-color panels more efficiently. Other unmixing errors were not evaluated.

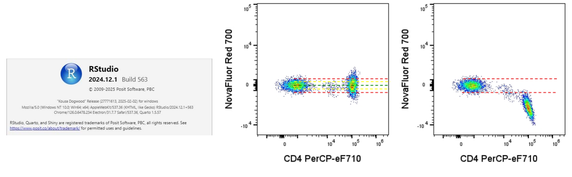


Figure 2. R-studio used to: (1) perform singlet and lymphocyte gating (2) gate positive and negative events (3) calculate and save MFI values of interest for positive and negative populations and save to .csv files

UC+	BUV563	AF532	Percp-eF710	PE	APC	NF Red 700
BUV563		Y	G	Y	G	G
AF532	G		Y	R	G	G
Percp-eF710	G	G		R	R	Y
PE	Y	R	Y		G	G
APC	G	G	R	Y		R
NF Red 700	G	G	R	G	R	

Spectral	BUV563	AF532	Percp-eF710	PE	APC	NF Red 700
BUV563		G	G	Y	G	G
AF532	G		Y	G	G	G
Percp-eF710	Y	Y		Y	R	G
PE	Y	Y	G		Y	Y
APC	G	G	Y	G		R
NF Red 700	G	G	R	G	Y	

Table 1. Calculated MFI values were then input into an excel file that compared MFI and created an "matrix of unmixability", which is a table to represent unmixing errors seen with single color controls on CD4. Selected fluorophores are shown above. (G = Green: very similar median; Y = Yellow, small difference in median; R = Red, larger differences in median)

Summary of unmixing errors with UltraComp Plus and UltraComp Spectral

There are ~25-40% fewer unmixing errors with UltraComp Spectral compared to Ultracomp Plus when evaluating 33 fluorescent dyes as single-color controls.

Summary UC+	
Green	587
Yellow	294
Red	175

Summary Spectral	
Green	723
Yellow	233
Red	100

Severe Unmixing Errors Summary		
Dye excitation	UC+	Spectral
Ultra Violet (5 dyes)	9	6
Violet (11 dyes)	23	17
Blue (6 dyes)	48	29
Yellow-Green (6 dyes)	53	31
Red (5 dyes)	42	17

Table 2. Left: General summary of unmixing errors for Ultracomp Plus and Ultracomp Spectral. Right: Table presents differences in unmixing errors based on primary laser excitations of dyes. Improvement was observed across all laser lines, with the most noticeable improvements in dyes excited by the red laser.

Results – Panel

Unmixing performance can vary widely due to the combinations of fluorophores and the specificities assigned to them. These combinations can sometimes introduce unexpected unmixing errors, reduce the number of errors, or diminish the degree of an error. Brighter staining from more fluorescently intense dyes and higher antigen density targets tends to be more sensitive to small differences in spectral signatures between single-color controls and experimental samples. To further evaluate the data and insights generated from single-color control experiments, a multi-parameter immunophenotyping panel was created. Unmixing errors due to the bead-based single-color controls were predicted and confirmed in the multi-parameter experiments.

Panel datal was unmixed by using either all single-color controls stained on cells, or single-color control stained with compensation beads.

UV2 (387)	CD3 BUV395	V1 (428)	CD25 BV421	B2 (525)	CD4 AF488	YG1 (577)	CD127 PE	R1 (660)	CD28 APC
UV6 (473)	Live/Dead Blue	V3 (458)	CD45RA eF450	B3 (542)	CD8 AF532	YG7 (720)	CCR7 PE-Cy5.5	R3 (697)	CD19NFR 700
UV9 (581)	CD27 BUV563	V7 (542)	CD16 eF506	B7 (660)	CD14 NFB 660	YG9 (780)	CD11c PE-Cy7	R7 (783)	CD38 APC-eF780
UV14 (750)	CD56 BUV737	V10 (615)	IgD SB600	B10 (717)	TCRgd PerCP-eF710				
UV16 (812)	HLA-DR BUV805	V15 (780)	CD123 BV786						

Table 3: A general immunophenotyping panel was created using the fluorophores employed to evaluate Ultracomp Spectral and Ultracomp Plus unmixing errors.

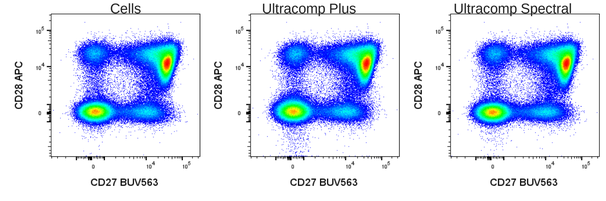


Figure 3: Single-color evaluation of Ultracomp Plus and Ultracomp Spectral suggests that there will be no spectral unmixing error between BUV563 and APC. In a 20-color panel, no unmixing error was observed between these two fluorophores as predicted based on the CD4 single-color experiment.

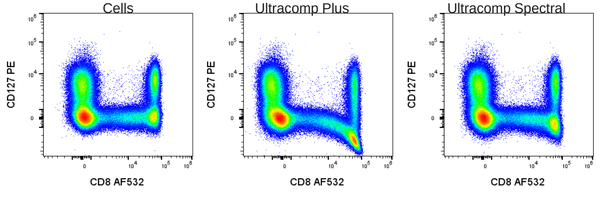


Figure 4: Single-color evaluation of Ultracomp Plus suggests that there may be a major unmixing error when used as a single-color control to unmix AF532 against PE (middle plot). A less noticeable unmixing error is predicted when using Ultracomp Spectral (right plot). In a 20-color panel, unmixing errors were predicted based on the CD4 single-color experiment for these two fluorophores.

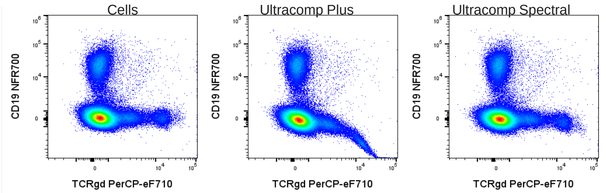


Figure 5: Single-color evaluation of Ultracomp Plus suggests that there may be a major unmixing error when used as a single-color control to unmix PerCP-eF710 against NovaFluor Red 700 (middle plot). Single-color evaluation of Ultracomp Spectral also suggests a major unmixing error; however, only a minor unmixing error was observed (right plot). Though a single-color experiment involving CD4 resulted in a large unmixing error when using Ultracomp Spectral for PerCP-eF710, it's degree of unmixing error was less than Ultracomp Plus (data not shown). When unmixing a less abundant antigen, TCRgd, the slight difference in spectral signature appeared to be close enough to reduce observable unmixing error when using Ultracomp Spectral.

Conclusions

- Identifying potential unmixing errors when using single-color control particles can be a subjective and time-consuming process that requires constant human attention. This poster highlights a more rapid and objective process utilizing robotic process automation, R scripts, and Excel. The robotic process automation enabled the creation of dozens of unmixing matrices from single-color controls that were bead-based without continuous human input. Subsequently, an R-based script analyzed FCS files and calculated the medians of the unmixed data. Finally, the data was transferred to an Excel file, where the medians were analyzed to create a table that identified unmixing errors between fluorophores.
- Data generated from an experiment using only single-color controls to identify unmixing errors can be used to predict unmixing errors in multi-parameter panels. However, it is important to consider that unmixing errors can be amplified or diminished depending on the choice of antigens and fluorophores. Nevertheless, this information can still be useful in predicting which fluorophores will likely need a cell-based single-color control and which will be compatible using a bead-based single-color control.
- A new single-color stain control bead, Ultracomp Spectral, was developed specifically for spectral unmixing. There is an estimated improvement of 30-40% fewer minor and major unmixing errors compared to Ultracomp Plus when tested on 33 fluorescent dyes. An additional dozen or so dyes were also tested (data not shown), and in all tested cases, there was an improvement in spectral unmixing.

Acknowledgements

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