Investigation of problematic dye combinations to inform optimal panel design in spectral cytometry

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Abstract:

High complexity flow cytometry is becoming increasingly adopted as spectral analyzers and sorters can support detection of greater than 30-40 parameters. While experimental design and panel building can be straightforward for small and even mid-sized conventional and spectral panels, high parameter panel building requires special considerations.

Reaching the full capability of any cytometer requires the user to build a complex panel with all the available detectors in use. While smaller panels can rely on techniques such as separation of fluorophores based on laser excitation and emission peaks and selection of more spectrally clean dyes, increasingly complex higher parameter panels often require the use of problematic fluorophore combinations. Fluorophore combinations can be categorized as problematic for several reasons, including but not limited to; high spectral similarity, overlapping peak channel detection, high cross-laser excitation, and relative brightness.

We present data intended to help inform the selection of fluorophores when building a complex panel. To address this, we designed mini panels composed of problematic fluorophore combinations to investigate which properties of such combinations contributed most significantly to overall panel usability, as well as to make recommendations for which fluorophores can be used together successfully. Data collected from both conventional and spectral cytometers showed that some combinations fail to resolve target populations or cause insurmountable compensation or unmixing errors, while others can be used together effectively. Thus, strategic dye selection can improve panel resolution as well as increase the number of detectable parameters, serving to maximize cytometer potential.

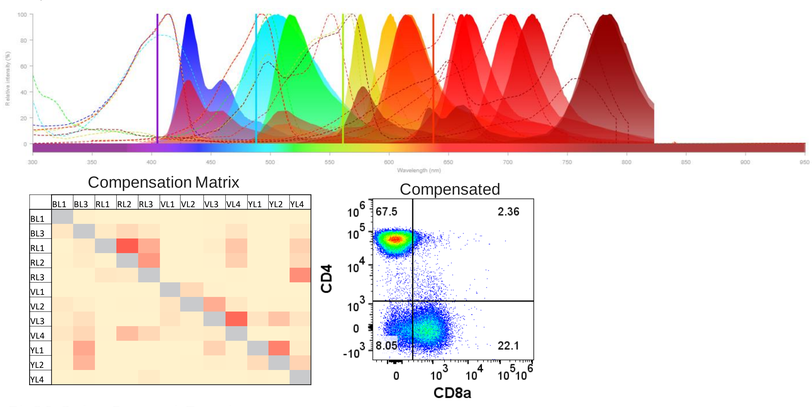
Introduction:

Spectral and conventional flow cytometry are not as different as they may seem. They utilize the same hardware, the same math, and the same biology just viewed in a different context. Where conventional cytometry is focused on the peak excitation and emission of fluorophores and the relevant detectors, spectral cytometry uses an expanded field of detectors to look at the full spectrum of the fluorophore. The full spectrum view of the fluorophore yields a spectral signature, a unique set of fluorescent intensities in all detectors which helps visualize not only peak emission, but secondary peaks and cross-laser excitation. This novel view of the fluorophore, combined with the increase in detectors, enables larger parameter panels and thus deeper biological interrogation. Spectral panel building relies highly on the spectral signature of each dye, creating a complexity and similarity index to inform optimal dye combinations. However, how do we know where the new limits are?

In these experiments we tested fluorophore combinations that were predicted to be problematic based on observations from building high parameter panels. Selected combinations included highly similar, spectrally bunched, and highly cross-excited fluors. These combinations were then evaluated based on the ability to resolve biologically relevant populations as well as metrics such as changes in Mean Fluorescent Intensity, Separation Index, and population percentages.

Figure 1: Panel Evaluation Conventional vs Spectral

A) 14 Color Conventional Panel



B) 36 Color Spectral Panel

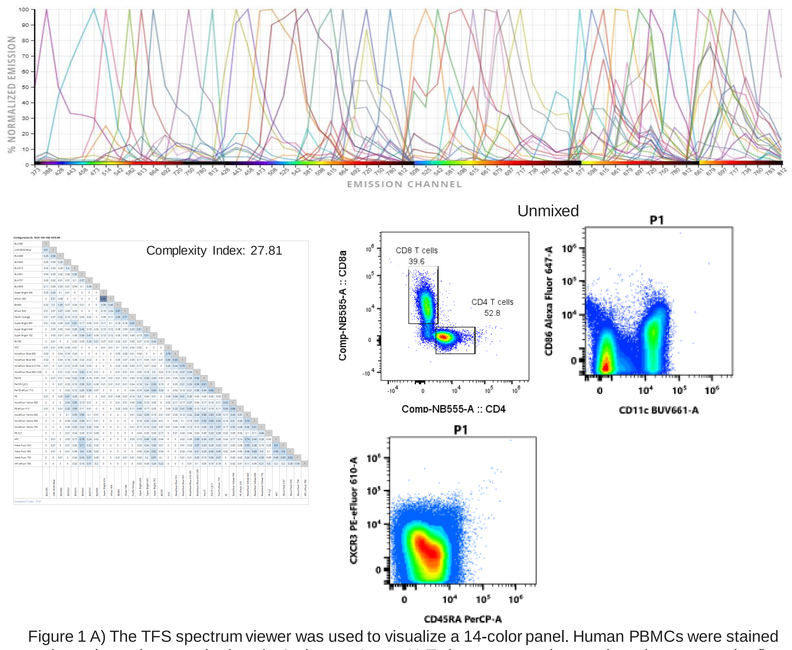


Figure 1 A) The TFS spectrum viewer was used to visualize a 14-color panel. Human PBMCs were stained and panel was then acquired on the Invitrogen Attune NxT, the compensation matrix and representative flow plot are displayed. B) The Cytek spectrum viewer was used to visualize the spectrum of a 35-color panel. The theoretical Similarity and Complexity Indices were also calculated. The Complexity Index is 27.81. Human PBMCs were stained with the panel then acquired on the Cytek Aurora and representative flow plots are displayed.

Hypothesis:

Current mathematical models and metrics do not effectively predict data distortion or panel usability (or lack thereof) when problematic dye combinations are used in high parameter flow. We hypothesize that features of the data including, distorted negative populations and changes in SI, can be used to assess which combinations of dyes can be used together successfully in a complex panel.

Results:

Figure 2: What Makes a Fluorophore Combination Problematic? The Good, the Bad, and the Ugly

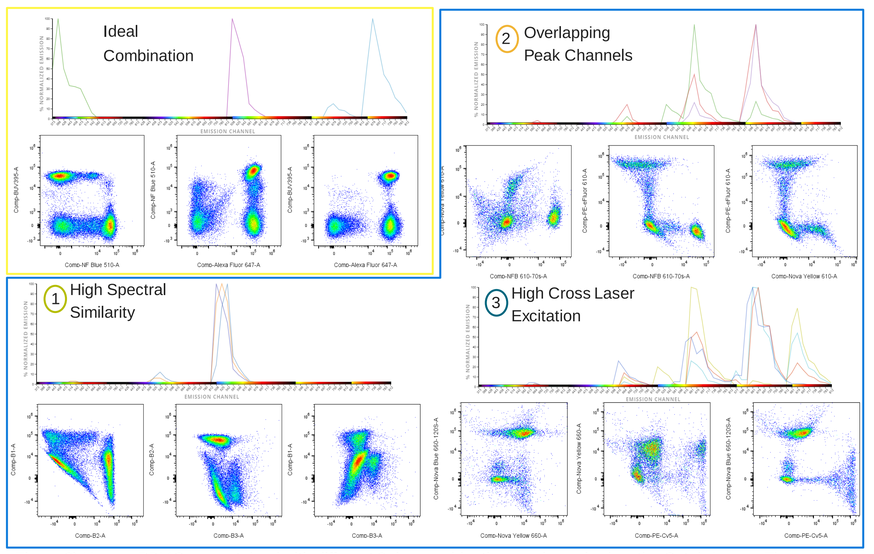


Figure 2) Human PBMC were stained with different 2 dye combinations and acquired on the Cytek Aurora. The panels were visualized with the Cytek Spectrum viewer and the spectra are displayed here. Dot plots showing the issues caused by the fluorescent characterizes of the panel are also displayed.

Figure 3: Features of Problematic Data

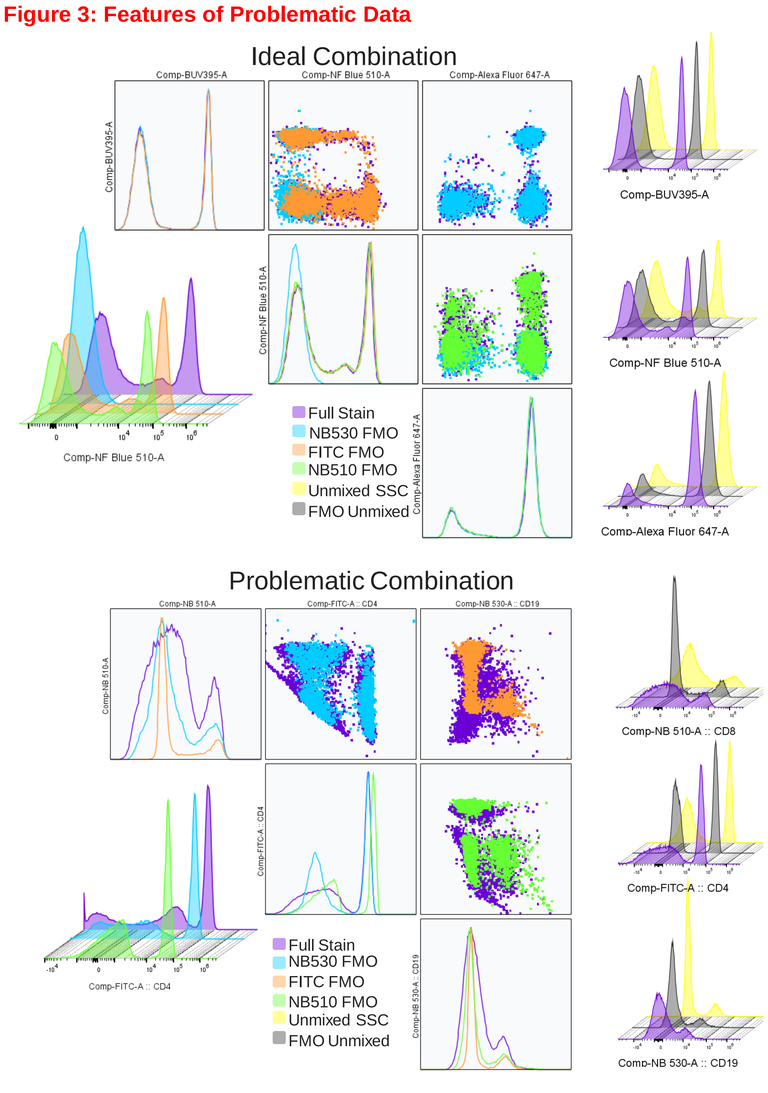


Figure 3 Human PBMCs were stained with an ideal and problematic fluor combination, respectively. Data was acquired on the Cytek Aurora and unmixed in Spectroflo. The overlayed histograms and dot plots represent the unmixed single control (yellow), the unmixed fluorescence minus ones (blue, orange, and green), and the unmixed full stained (purple) in the fluor's primary channel.

Figure 4: Changes in MFI and Stain Index, but not Complexity or Similarity Index, Correlate with Combination Usability

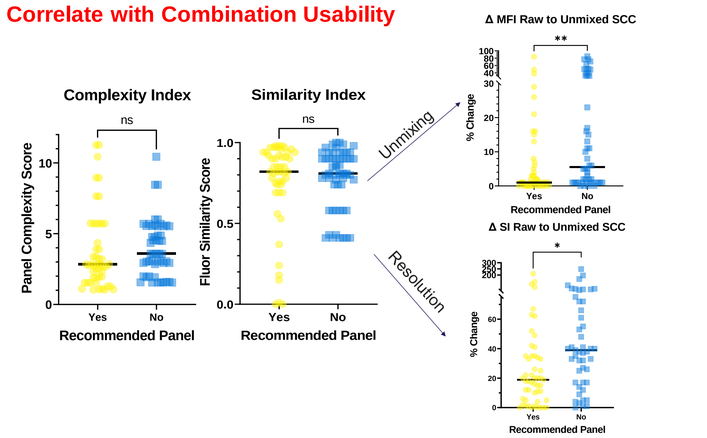
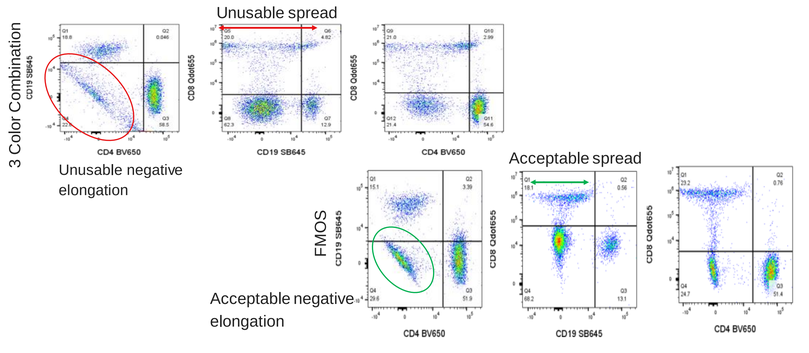


Figure 4) Data from all fluor combinations (n=99) was combined and analyzed in GraphPad Prism. All comparisons were analyzed using an unpaired, two-tailed t-test. There was not a statistically significant difference in the Complexity or Similarity Index between recommended combinations and unrecommended combinations (p=.558, p=.450). However, a significant difference was observed between the Mean Fluorescence Intensity of the positive populations in the raw to unmixed single-color controls (p=.0085). A significant difference was also observed between the change in Separation Index of the raw to unmixed single-color controls (p=.036)

Conclusion:

As the field of cytometry expands there is a renewed need to find metrics and methods to define the boundaries of what constitutes good panel design. It is essential to understand which highly similar fluorophores can be used together successfully, and more importantly, *why* these combinations can be used together while others can't. Currently the tools used to make this decision are the Complexity and Similarity Indices, which use a scoring system to determine if fluorophore combinations can be successfully unmixed. These tools are very useful but appear to break down in a crowded spectral space and their values do not correctly predict panel usability. Figure 4 shows that neither Complexity nor Similarity Index correctly predict whether a problematic fluorophore combination can be used successfully. However, we demonstrate that both the change in MFI between the positive populations of the raw single color control (SCC) and unmixed SCC and the change in SI between the raw SSC and the unmixed full stain have a stronger correlation with overall panel usability. This increased predictive power likely results from the ability to capture features of the data, such as negative spread, elongation, and heteroskedasticity of populations, which are not accounted for in other analyses. Based on this data, we make recommendations for which fluorophore combinations should be used in tight spectral spaces and describe features of your data that can indicate that a panel will be problematic



Fluorophore Options	Recommended Combination
Brilliant Violet 421, eFluor 450, Super Bright 436	Brilliant Violet 421/eFluor 450
Super Bright 645, Brilliant Violet 650, Qdot655	Super Bright 645/Brilliant Violet 650
Super Bright 702, Qdot705, Brilliant Violet 711	Super Bright 702/Brilliant Violet 711
Brilliant Violet 780, Super Bright 786, Qdot800	Brilliant Violet 780/Qdot800
NovaFluor Blue 510, NovaFluor Blue 530, FITC	NovaFluor Blue 510/NovaFluor Blue 530
NovaFluor Blue 530, Alexa Fluor 532, NovaFluor Blue 555	NB530/NB555
PE-Alexa Fluor 610, NovaFluor Yellow 660, PE-Cyanine 5, NovaFluor Blue 660-120S	PE-Alexa Fluor 610/NovaFluor Yellow 660
NovaFluor Blue 610-70S, NovaFluor Yellow 610, PE-eFluor 610	NovaFluor Blue 610-70S/PE-eFluor 610
Alexa Fluor 660, NovaFluor Red 685, Alexa Fluor 700	Alexa Fluor 660/Alexa Fluor 700
Alexa Fluor 647, NovaFluor Red 660, APC	Alexa Fluor 647/NovaFluor Red 660

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