Comprehensive CD4 characterization

of novel and established fluorochromes facilitates data-driven in silico panel design and optimization in high-parameter cytometry applications.

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BACKGROUND

Maximizing resolution of cellular subsets of interest through effective panel design is a critical step in flow cytometry assay development. Recent advancements in fluorochrome chemistry across the industry have given rise to a new wave of high-performance dyes enabling improved resolution in cytometry applications. To effectively utilize these innovations in high-parameter assays it is important to understand their interaction with established dyes and their impact on measurement sensitivity in the context of a given panel.

Iterative panel optimization experiments are resource intensive and may limit fluorochrome assignments to commercially available reagents, as custom conjugations can be cost prohibitive in the panel design phase of assay development. Existing open source tools offer some insights into the expected performance of a proposed panel though metrics such as cosine similarity, stain index reduction, and Complexity Index™; however, these tools do not allow visualization of potential spillover and unmixing spreading error, nor do they elucidate the effective resolution of a given measurement in the context of the full panel. To address this gap, we have characterized 125 novel and established fluorochromes using anti-CD4 conjugated reagents on a 5-laser Cytek Aurora instrument, and built a reference database of emission spectra and tools to quickly estimate panel performance through computational analysis, generating metrics and visualizations to characterize of the interaction of a given set of dyes. This, combined careful consideration of antigen density and co-expression, provides critical insights into the potential sensitivity of all measurements in a proposed panel prior to performing costly and time consuming wet lab experiments.

In this poster we highlight the utility of our in silico approach to panel design through a case study focusing on the modification of our 48-color Pan-Immune Profiling panel (PIP-01), substituting four recently released Real Blue™ dyes for their corresponding Brilliant Blue™ analogs used in the original panel configuration.

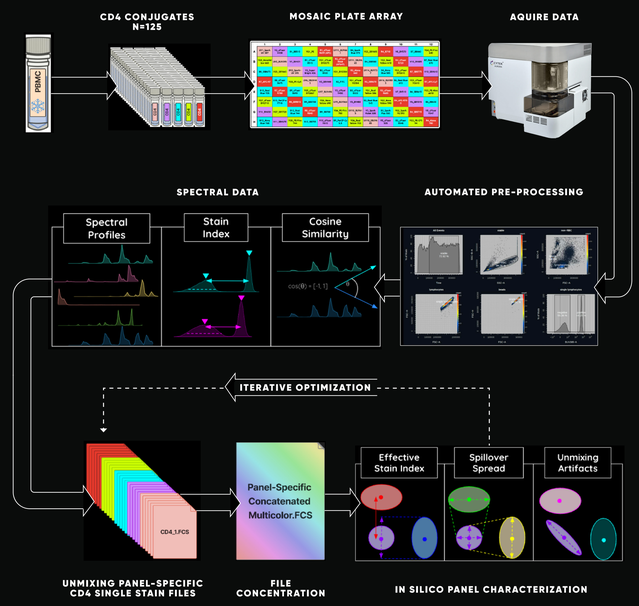

FIGURE 1

Characterization of 125 unique CD4-conjugated fluorochromes

Performed by staining cryopreserved PBMC, employing a mosaic plating strategy to buffer against potential cross contamination between wells with similar dyes. Commonly used viability dyes were also characterized using heat-killed PBMC (not shown). Single stain CD4 data were acquired on a 5-laser Cytek Aurora, pre-processed using an automated gating pipeline, and compiled into an internal database. After careful consideration of antigen density, expected co-expression, and direct conjugate availability for the markers to be measured in the panel, individual CD4 files for the proposed dyes in the panel are unmixed and concatenated to create a surrogate multi-stain file. Effective Stain Index (ESI), spillover spreading error, and unmixing artifacts (manifesting as tilted negative spread) are then quantified and incorporated into a suite of panel performance metrics. Concatenated data may also be visualized using N x 1 layouts, where any individual dye is plotted against all other fluorochromes in the proposed panel to assess its potential impact on panel performance.

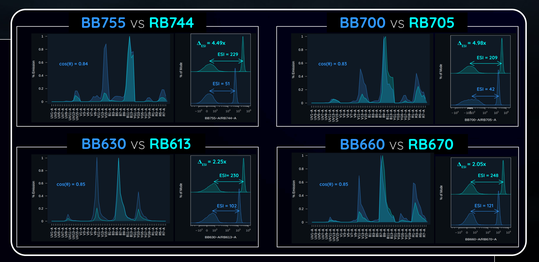

FIGURE 2

Novel dye resolution impact assessment

Shows improved performance in the context of Ozette's 48-color Pan-Immune Profiling panel (PIP-01). Overlaid spectra of Brilliant Blue™ (BB) dyes and Real Blue™ (RB) alternatives show a reduction in cross-laser excitation across the spectrum, with the most dramatic difference observed in violet laser excitation. Effective stain indices (ESI) are calculated using concatenated unmixed files from the 48 dyes used in PIP-01 in the context of either the original panel configuration with BB dyes, or a modified panel configuration using the four RB alternatives.

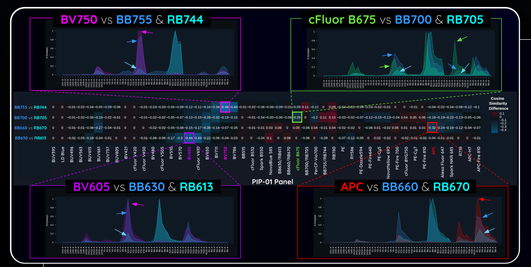

FIGURE 3

Cosine similarity comparison

Heatmap illustrates the difference in similarity across all dyes used in PIP-01. Overlaid spectral signatures of BB vs RB replacements against an impacted PIP-01 dye highlight a significant reduction in cross-laser excitation with RB dyes. An overall reduction in cosine similarity was observed across all dyes, resulting in a lower panel complexity with RB substitutions.

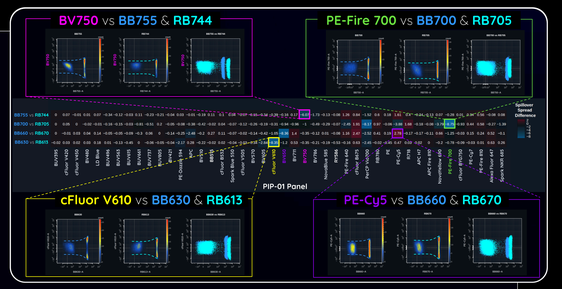

FIGURE 4

Spillover spreading error

Heatmap visualization facilitates the identification of increases or decreases in the magnitude of spread into impacted measurements when using BB vs RB dye substitutions in the context of PIP-01. Spillover spreading error (SSE) propagation is correlated with signal intensity; however, RB744™, RB705™, and RB613™ impart less SSE into the highlighted measurements despite demonstrating a brighter signal. Conversely, RB670™ imparts a larger degree of SSE into PE-Cy5; thus, careful consideration of antigen density and coexpression is needed for this measurement.

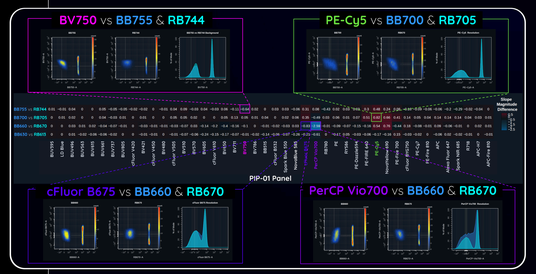

FIGURE 5

Identification of unmixing artifacts

Is achieved through computation of the slope of the negative population in bivariate dye combinations in the context a given panel. Tilting of the negative population is associated with impaired resolution for dim antigens [1]; thus, reduced tilting in either direction is desirable to increase resolution by minimizing background spread into other dyes. In this figure, we interrogate the change in the magnitude of the slope of the negative population for all markers in our PIP-01 panel against BB dyes used in the original panel or against their corresponding RB substitutions. Heatmap representation indicates a change in slope magnitude with negative values indicating a reduction in slope (closer to zero) or an increase slope (values further from zero). Concatenated CD4 data allows Nx1 visualization to confirm the impact of unmixing artifacts in any particular dye combination. Panels show unmixed single stain files highlighting examples of differential tilting of the negative population identified through this metric, and their potential impact on resolution.

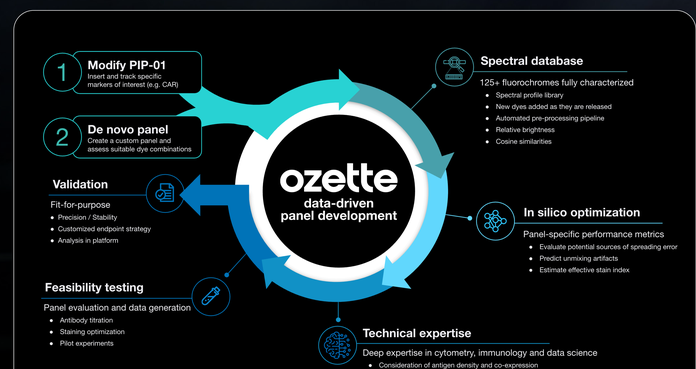

FIGURE 6

Panel development flywheel

Demonstrates how dye characterization and in silico optimization tools integrate with Ozette's data driven approach to panel modification and de novo panel development workflows.

DISCUSSION

Ozette's data-driven approach to panel design enables efficient development of high-parameter cytometry assays through iterative in silico optimization prior to costly and time consuming wet lab experiments. Our comprehensive spectral database of 125 commercially available fluorochromes is regularly expanded to include novel dyes as they are released, ensuring the ability to evaluate the potential impact of these reagents in new and existing panels. Pairing deep technical expertise and an understanding of antigen density and coexpression with panel-specific performance metrics, we are able to quickly and empirically identify optimal fluorochrome assignments for a given set of markers.

A continuing focus on the improvement of fluorochrome chemistry across the industry will enable larger panels and superior resolution; however, the characterization of how these dyes may perform in a given panel, whether used for panel improvement or expansion, should not be overlooked. The ability to gain insight into the impact on effective measurement resolution in silico enables informed decision making before investing resources on an endeavor that may result in only marginal improvement, or worse, an adverse impact on a critical measurement.

While the data presented here focus on conventional unmixing methodology, we plan to expand this tool set to incorporate Ozette Resolve™ unmixing workflows to allow direct comparisons and quantify the improved resolution afforded by our novel adaptive method.

REFERENCES & ETHICS

References:

1. Peter L. Mage, Andrew J. Konecny, Florian Mair. Measurement and prediction of unmixing-dependent spreading in spectral flow cytometry panels. Pre-print.

Acknowledgements:

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Ethics Approval:

All donors provided informed consent using Institutional Review Board (IRB) approved consent forms.