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An automated approach to high-parameter spectral flow cytometry assay validation using a novel AI/ML platform to assess the precision and stability of predefined biomarker endpoints in the context of a 48-color pan-immune profiling panel.

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Abstract

Analytical method validation for flow cytometry assays is essential to generate reliable and reproducible data that can be used to inform decision-making and drive translation of scientific findings into clinical applications. It is critical to confirm that flow cytometry assays perform as intended, with assessments of precision and stability being well-established parameters of method validation. High-parameter cytometry panels with a myriad of predefined biomarker endpoints introduce unique challenges in validating these parameters. Analysis of these complex data sets using traditional approaches, such as manual gating and spreadsheet based calculations of summary statistics, can be excessively cumbersome and resource intensive.

We designed and optimized a 48-color **P**an-**I**mmune **P**rofiling assay (PIP-01) to measure surface proteins indicative of activation, differentiation, and function across all major circulating mononuclear cells with excellent resolution, yielding hundreds of phenotypic and functional biomarker endpoints. Here we present a novel approach to high-dimensional flow cytometry assay precision and stability analysis using an innovative end-to-end AI/ML platform. We outline our validation strategy, consisting of cryopreserved PBMC from 5 healthy donors with inter- and intra-assay precision measured across automated and manual assay execution protocols, and specimen stability assessed across multiple timepoints. Our logic-based endpoints gating scheme utilizes a modular approach to define terminal gates based on functional marker expression, grouping surface protein measurements into discrete Boolean modules for activation, differentiation, trafficking, co-stimulation, cell health, and immune checkpoints based on current literature. Analysis of these data is performed using an automated, robust AI/ML platform capable of delivering rapid biomarker analysis with no limitation on the number of endpoints or volume of data. Our novel approach of pairing high-parameter cytometry assay validation with automated endpoint analysis allows for robust and rapid evaluation of assay precision and stability criteria.

Methods

Ozette's 48-color full spectrum cytometry assay was designed to provide comprehensive phenotypic and functional readouts for all major circulating immune cell subsets found in PBMC, providing deep characterization of the state of the immune system with hundreds of phenotypic endpoints.

A **fit-for-purpose** validation strategy was developed to assess the precision and specimen stability of our assay. Intra- and inter-assay precision were measured by testing 5 healthy donors with 3 technical replicates using either manual or automated methods (Figure 1). Specimen stability was assessed by isolating fresh PBMC from 6 healthy donor blood samples collected into ACD tubes. Samples were held at room temperature over a four day time course to assess the potential impact of variable shipping times of incoming samples. PBMC were isolated and split for fresh testing and cryopreservation at each time point, with all cryopreserved samples being run at the end of the study after a minimum of 7 days stored in liquid nitrogen (Figure 2).

Gating of precision data was performed using the Ozette Endpoints™ analysis pipeline according to a pre-defined endpoints gating strategy measuring 172 discrete endpoints (Figure 3). Ozette Discovery™ was employed for specimen stability analysis to elucidate the expression of surface proteins and corresponding immune phenotypes most affected by post-collection hold time and cryopreservation.

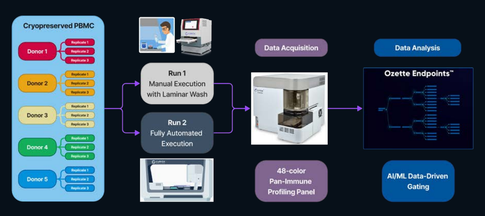

Figure 1: Assay Precision Study Design Schematic

Cryopreserved PBMC from 5 healthy donors were assayed in triplicate over two runs on separate days using using either a conventional manual pipetting method paired with the Curiox Laminar Wash™ HT2000 system, or a fully automated method using the Curiox AUTO1000 platform. Samples were stained with a 48-color pan-immune profiling panel and acquired the same day on a 5-laser Cytek Aurora full spectrum cytometer. Multicolor files were unmixed using single color reference controls and analyzed according to the gating strategy shown in Figure 3 using Ozette Endpoints™ technology.

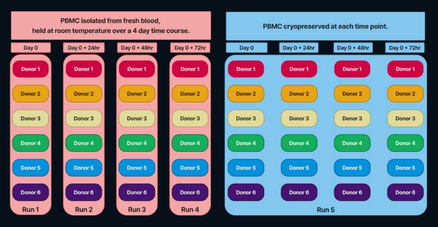

Figure 2: Specimen Stability Study Design Schematic

Fresh blood was drawn from 6 healthy donors into a series of ACD collection tubes to assess whole specimen stability over a 72-hour time course. Blood samples assayed after Day 0 were held at room temperature protected from light for the duration of the study.

At each timepoint, PBMC were isolated, counted, and split, with half of the cells put directly into our 48-color pan-immune profiling assay, and the other half cryopreserved and transferred to to liquid nitrogen storage. Cryopreserved samples were assayed in a single batch after being stored in liquid nitrogen for a minimum of 7 days.

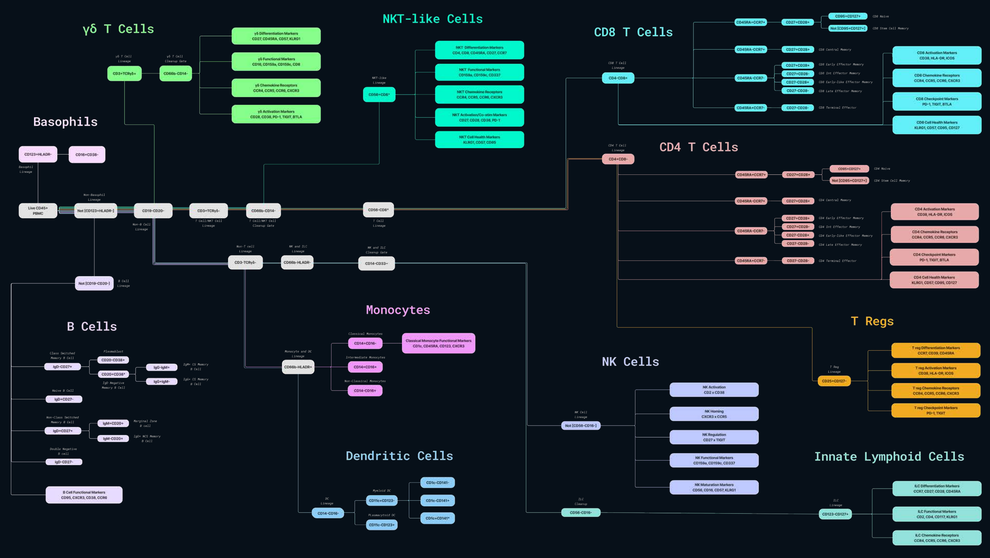

Figure 3: PIP-01 Gating Schematic for Ozette Endpoints™

Hierarchical gating strategy for pre-defined phenotypic and functional endpoints in Ozette's 48-color PIP-01 assay. Leveraging canonical markers of major cell lineages, our logic-based gating strategy ensures accuracy and avoids redundancy in measurements of lineage-specific endpoints of all major circulating immune cell types.

Terminal endpoint readouts are grouped into functional modules indicative of broad categories of biological significance which can be multiplexed using Boolean logic, to expand the analysis to hundreds of possible endpoints.

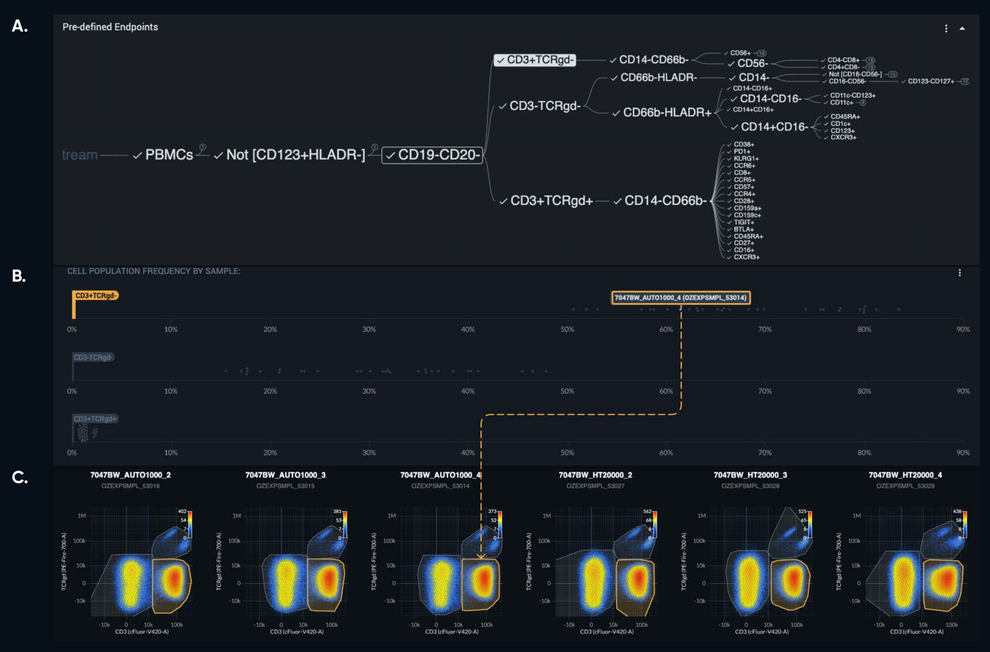

Figure 4: Interactive Ozette Endpoints™ Dashboard

Representative view of PIP-01 precision data. CD3+TCRγδ- events are selected, and plot displays have been filtered on a single donor with one file highlighted in the bee swarm plot.

A: Clicking on a node of interest in the dynamic gating tree expands downstream nodes, facilitating navigation through different nodes in the tree.

B: Bee swarm plots show the distribution of the events in the selected node and accompanying gates across all samples.

C: Data-driven gates generated by our proprietary AI/ML technology are displayed for the selected node, ensuring full transparency of gate placements.

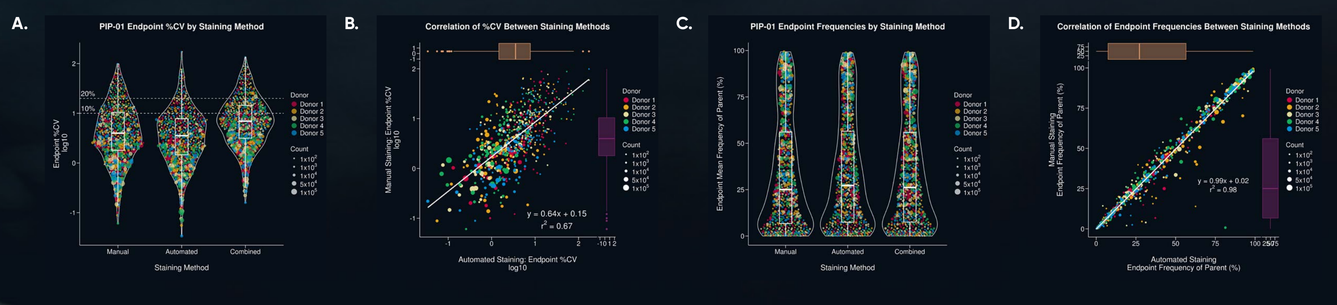

Figure 5: Statistical Analysis Summary of PIP-01 Precision Data Gated Using Ozette Endpoints™

Data points are colored by Donor, and sized according to the number of gated events. Figures were generated in R from the Ozette Cytoverse GatingSet and Ozette Endpoints™ count matrix using the tidyverse suite of R packages.

A: Violin plots comparing the distribution of intra-assay precision of pre-defined endpoints for manual and automated methods, and the inter-assay precision of the combined runs measured by %CV. Endpoint %CV distributions overlap significantly across methods, with a median of <10% CV across all endpoints for both intra- and inter-assay precision.

B: Correlation of intra-assay precision between staining methods shows that endpoint count heavily influences the precision across methods, with the automated staining method generally achieving greater intra-assay precision (slope < 1).

C: Violin plots comparing the distribution of endpoint parental frequencies between staining methods. Parental frequency distributions overlap significantly across methods.

D: Correlation of intra-assay population frequencies between staining methods reveals a strong positive correlation (r^2 = 0.98). The choice of staining method does not impact the accuracy of the computed population frequencies.

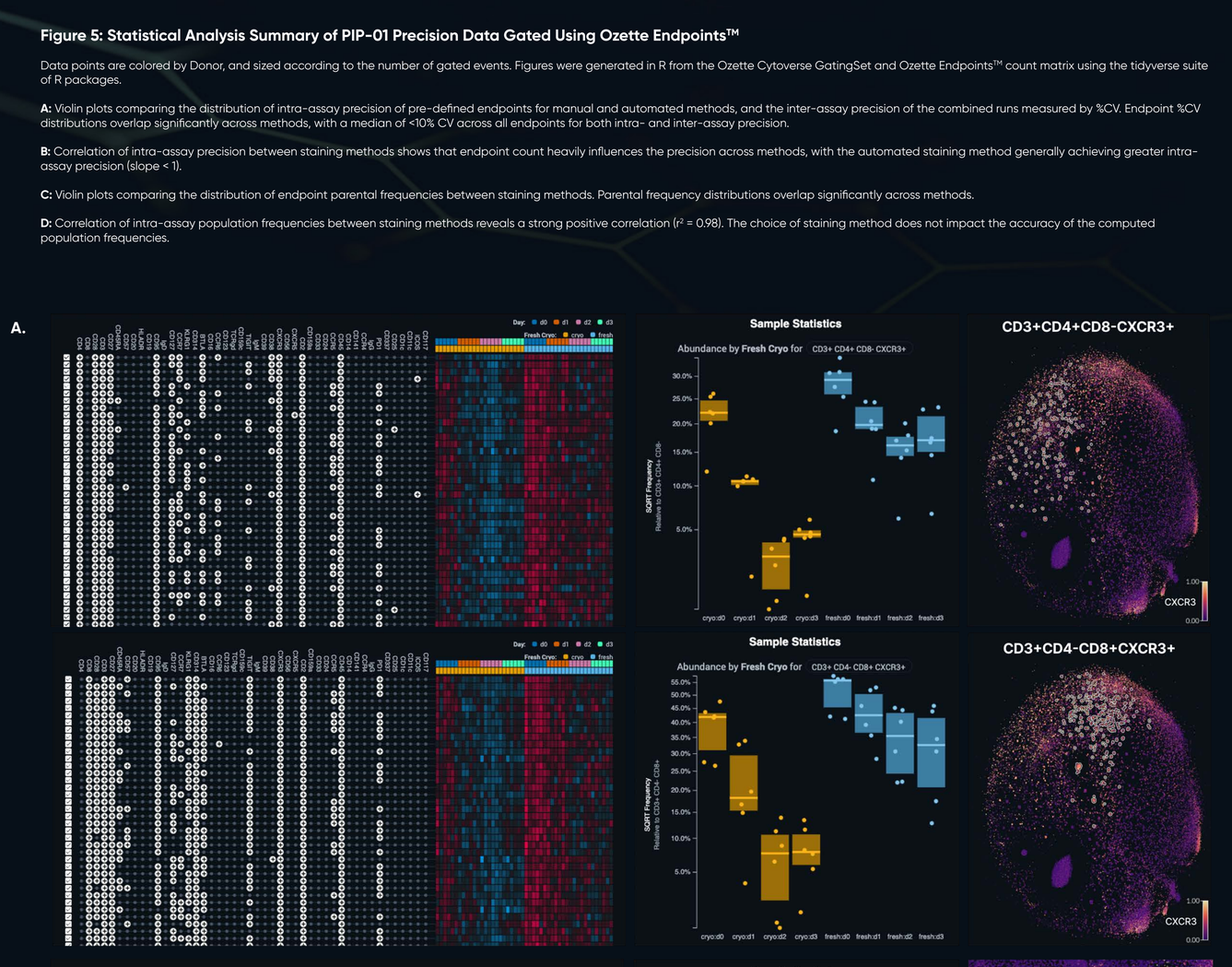

Figure 6: Ozette Discovery™ Dashboard - Whole Specimen Stability

Representative views of the interactive Ozette Discovery™ dashboard show examples of phenotypes adversely affected by post-collection hold time and cryopreservation.

A: CXCR3 expression was found to be the most heavily impacted measurement by both post-collection hold time and cryopreservation. Heat map visualizations display the most significantly affected CD4+ (n=148) and CD8+ (n=213) T cell phenotypes expressing CXCR3. Bar graphs show the frequency of CXCR3+ phenotypes relative to all CD4+ or CD8+ phenotypes found to be robust across all samples. Single-cell embedding visualizations colored by phenotypes expressing CXCR3 highlight the location of the affected phenotypes in 2-dimensional space.

B: A population of CD19+IgD-CD27+CD20-CD38+ events, commonly associated with a plasmablast phenotype (1), was also found to be affected by both hold time and cryopreservation. Representative data-driven gating thresholds are displayed on bivariate backgating plots, bar graphs show the frequency of CD20-CD38+ events relative to total IgD-CD27+ B cells, and the location of the affected phenotype is highlighted in magnified single-cell embedding visualizations colored by phenotypes expressing IgD or CD38.

CD33 high myeloid cells were gated out prior to running the above analysis, improving the resolution of lymphocyte measurements by mitigating the spreading error introduced by a bright CD33 signal, and reducing the background resulting from monocyte autofluorescence. CD33 high events were analyzed separately (data not shown).

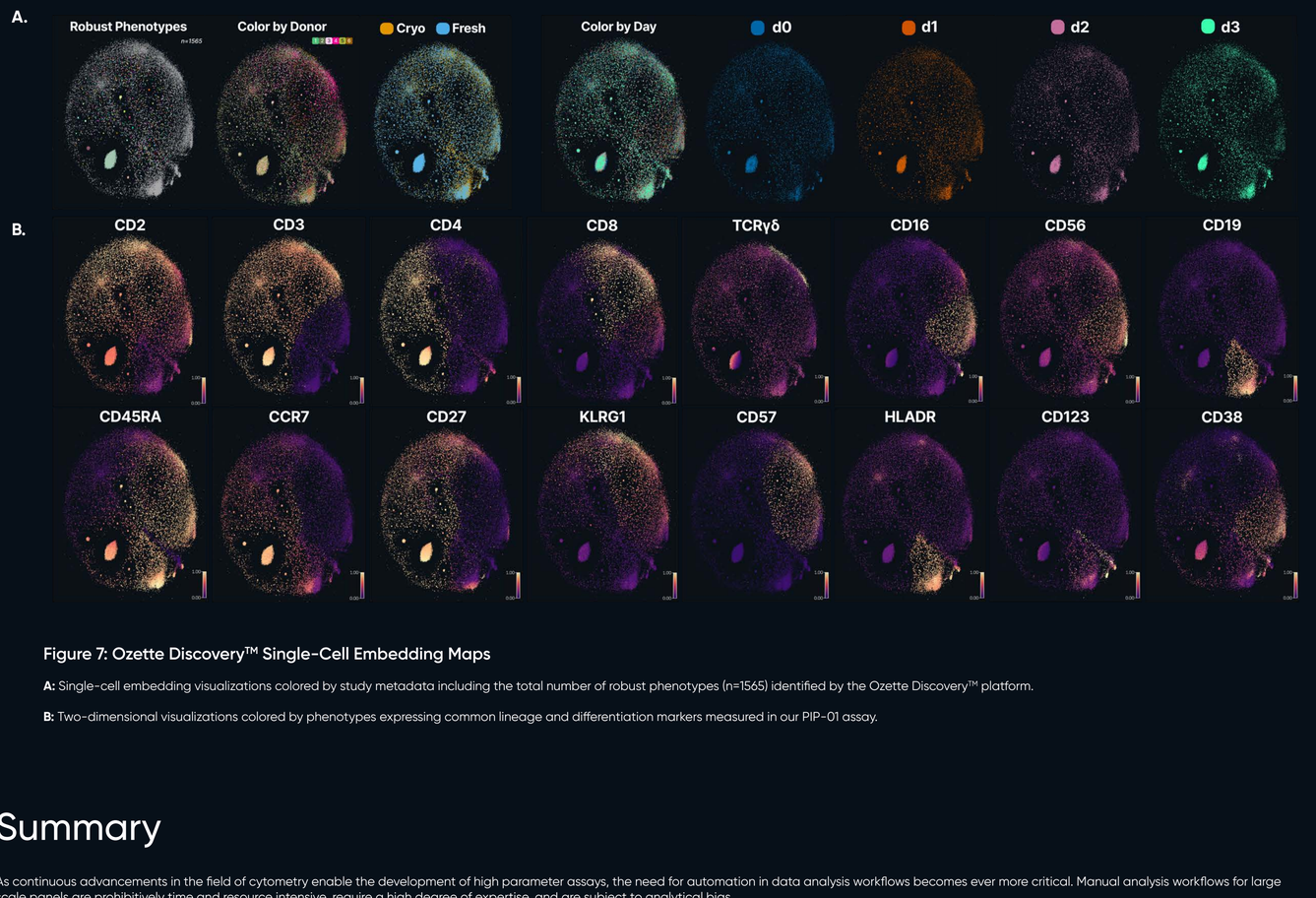

Figure 7: Ozette Discovery™ Single-Cell Embedding Maps

A: Single-cell embedding visualizations colored by study metadata including the total number of robust phenotypes (n=1565) identified by the Ozette Discovery™ platform.

B: Two-dimensional visualizations colored by phenotypes expressing common lineage and differentiation markers measured in our PIP-01 assay.

Summary

As continuous advancements in the field of cytometry enable the development of high parameter assays, the need for automation in data analysis workflows becomes ever more critical. Manual analysis workflows for large scale panels are prohibitively time and resource intensive, require a high degree of expertise, and are subject to analytical bias.

Per CLSI guidance (2), a desirable target for assay precision is ≤10% CV. Given the intended use of PIP-01 in biomarker discovery and research applications only, higher imprecision is acceptable for less-abundant cell populations or populations that are not based on bimodally expressed markers (≤20% CV). Downstream statistical analysis of PIP-01 validation data gated using Ozette Endpoints™ shown in Figure 5 demonstrates excellent intra- and inter-assay precision with the majority of endpoint measurements falling below a 10% CV threshold for both manual and automated methods across all pre-defined endpoints. As expected endpoint measurements for gates with lower event counts are generally less precise.

Our method validation strategy includes a comprehensive assessment of whole specimen stability of PBMC isolated from whole blood drawn into ACD collection tubes over a four day time course. In cases where fresh blood will be shipped to the Ozette Lab for our vertically integrated Assay-to-Insights offering, our understanding of phenotypes affected by shipping time and/or cryopreservation is critical. Here we provide examples of T and B cell subsets that are adversely affected by both conditions. These data will be used to guide logistical considerations for future partnerships with the Ozette Lab.

The power and speed of the Ozette Endpoints™ and Ozette Discovery™ platforms is astounding, allowing us to gain an understanding of the precision of our assay and the stability of representative samples in a matter of days, rather than weeks to months of manual analysis. The interactive Endpoints™ dashboard provides full transparency of data-driven gating decisions and facilitates exploration of the data set before exporting for downstream analysis. Surface protein expression and associated phenotypes affected by post-collection hold time and cryopreservation were quickly elucidated through the Ozette Discovery™ dashboard, providing critical insights for future partnerships and for our general understanding of the stability of PBMC immune subset measurements.

References

1. Tâm D. Quách, Nely Rodríguez-Zhurbenko, Thomas J. Hopkins, Xiaoti Guo, Ana María Hernández, Wentian Li, Thomas L. Rothstein; Distinctions among Circulating Antibody-Secreting Cell Populations, Including B-1 Cells, in Human Adult Peripheral Blood. J Immunol 1 February 2016; 196 (3): 1060–1069. https://doi.org/10.4049/jimmunol.1501843
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