Leveraging AI/ML to analyze the effect of leukocyte isolation and cryopreservation on surface protein expression in peripheral blood samples in the context of a 48-color full spectrum cytometry pan immune profiling panel

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Abstract

Isolation and cryopreservation of peripheral blood mononuclear cells (PBMC) can detrimentally affect the detection of certain surface epitopes by flow cytometry. This can lead to inaccurate measurements of circulating immune cell subsets in peripheral blood. Here we employ a 48-color pan immune profiling panel and a proprietary Artificial Intelligence/Machine Learning (AI/ML) data analysis pipeline to assess the variance in marker expression and immune cell phenotype abundance between RBC-lysed whole blood, freshly isolated PBMC, and cryopreserved PBMC in three healthy donor subjects across three longitudinal blood draws. We describe the development and optimization of the 48-color panel, including our in-silico design approach leveraging cytometer characterization data generated using CD4-conjugated antibodies, as well as our automated assay execution methodology using laminar wash technology. Data from this study were analyzed using a novel unbiased computational algorithm that exhaustively discovers and annotates specific and robust cellular subsets, highlighting key differences between groups based on marker expression and abundance[1]. The results of this study demonstrate the power and speed of the Ozette Endpoints™ and Ozette Discovery™ platforms, and expand our knowledge of the effects of isolation and cryopreservation on PBMC in healthy donor blood.

Methods

Our 48-color full spectrum cytometry assay has been designed to provide comprehensive phenotypic and functional readouts for all major circulating immune cell subsets, maximizing our understanding of the state of the immune system.

To ensure optimal resolution of all markers in a panel of this size, we first performed an extensive characterization of our 5-laser Cytek Aurora instrument using CD4 antibodies conjugated to all compatible fluorochromes. By concatenating single-stained CD4 files from this experiment, we were able to visualize potential spreading error from any given signal into all other measurements and the relative signal intensity of each dye. Leveraging these data and following best practice panel design guidance[2,3], we mitigated the impact of severe spreading error by assigning markers of distinct immune cell lineages to highly overlapping dye combinations.

Our staining methodology used the Curiox Auto1000 system, which integrates the Hamilton Nimbus liquid handling robotic platform with automated laminar wash technology. This system significantly streamlined our staining workflow, provided increased cell retention and viability, and allowed for greater assay precision by removing inter-operator variability.

The proof-of-principle study presented here was designed to highlight the power and speed of the Ozette Endpoints™ and Ozette Discovery™ pipelines, and elucidate the effect of different biospecimen processing techniques and cryopreservation on PBMC.

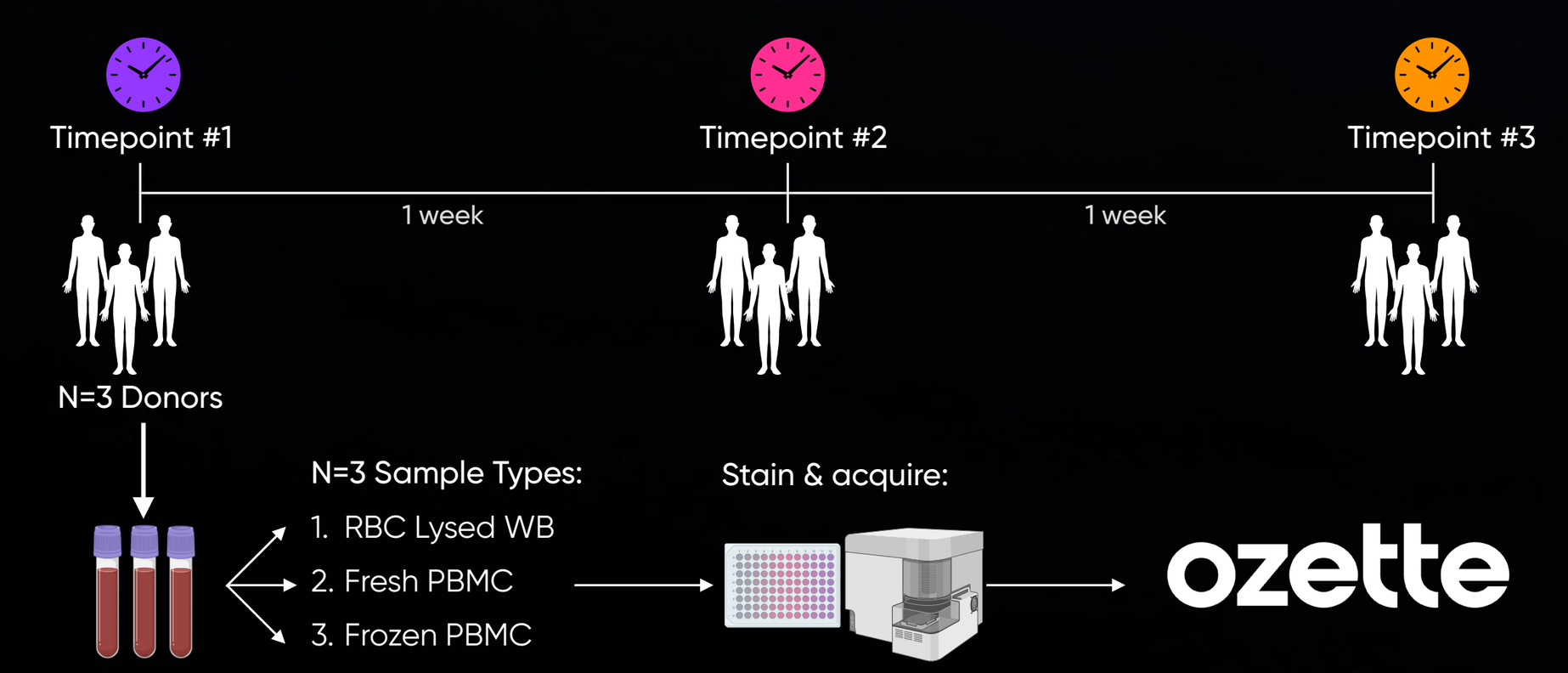

Figure 1: Peripheral blood collected from three healthy donor subjects across three timepoints was processed the day of collection to generate three sample types. Fresh RBC-lysed whole blood (WB) and freshly isolated PBMC were stained immediately post processing. Frozen PBMC samples were cryopreserved for one week prior to thawing and staining. Data generated were delivered directly into Ozette's computational analysis pipeline to measure pre-defined phenotypic immune endpoints and explore fully-annotated and comprehensive cell populations.

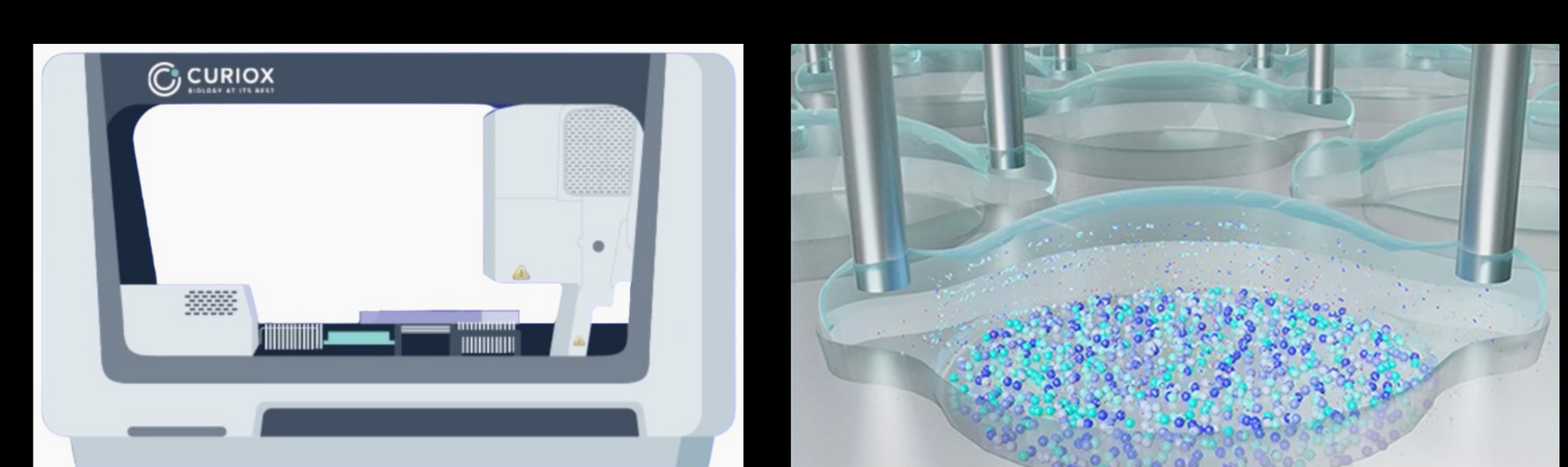

Figure 2: Ozette's state-of-the-art immunology lab is equipped with the Curiox Auto1000 system that integrates the Hamilton Nimbus liquid handling robotic platform with fully automated laminar wash technology. Cells settle by gravity, followed by cycles of aspirating and dispensing wash buffer to initiate a gentle laminar flow across the well, leaving the settled cells undisturbed.

Spillover spreading error characterization using CD4 conjugated antibodies

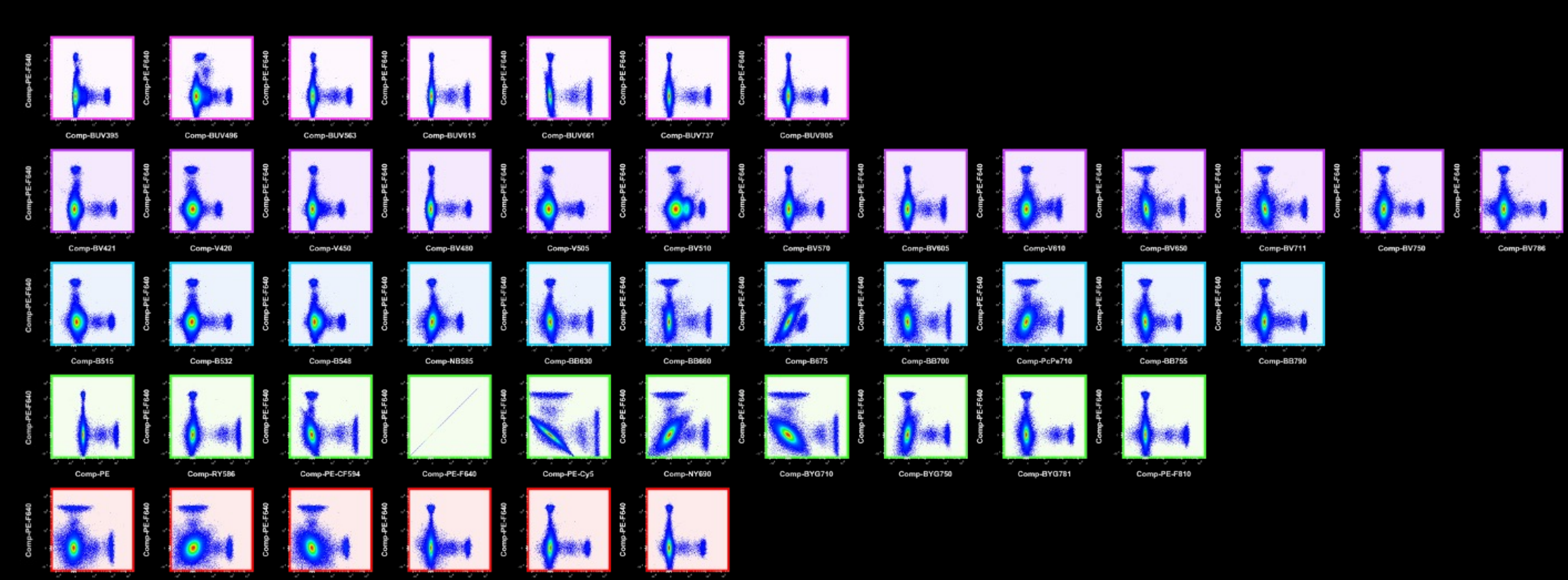

Figure 3: Nx1 plots of a concatenated file comprised of 47 CD4 single-stained reference controls allows visualization of the spreading error introduced by PE-Fire640 into 46 adjacent measurements, the spreading error introduced by 46 adjacent signals into the PE-Fire640 measurement, and the relative signal intensity of each dye in the panel.

Deep insights into the state of the immune system can be achieved using Ozette's high-parameter profiling assay

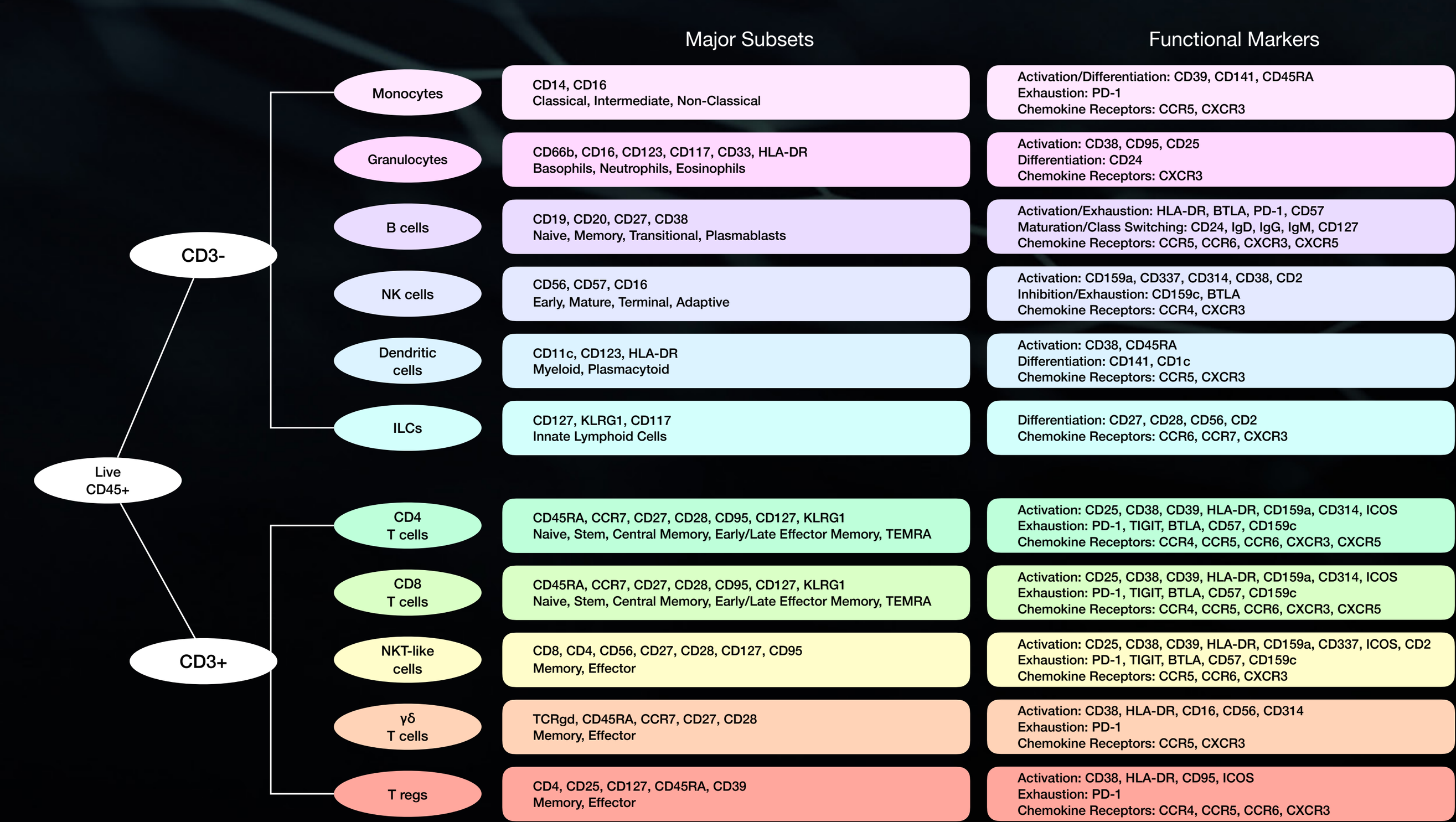

Figure 4: Ozette's 48-color full spectrum flow cytometry panel was designed to provide comprehensive characterization of all major circulating immune cell subsets. Cell surface markers listed in the figure note their phenotypic and functional association with major cell lineages. Data generated from this panel paired with Ozette's computational analysis technology allows for a rapid and robust snapshot of the state of the immune system in peripheral blood.

ozette endpoints™

Ozette Endpoints™ uses pre-defined gating strategies to deliver robust computational analysis pipelines

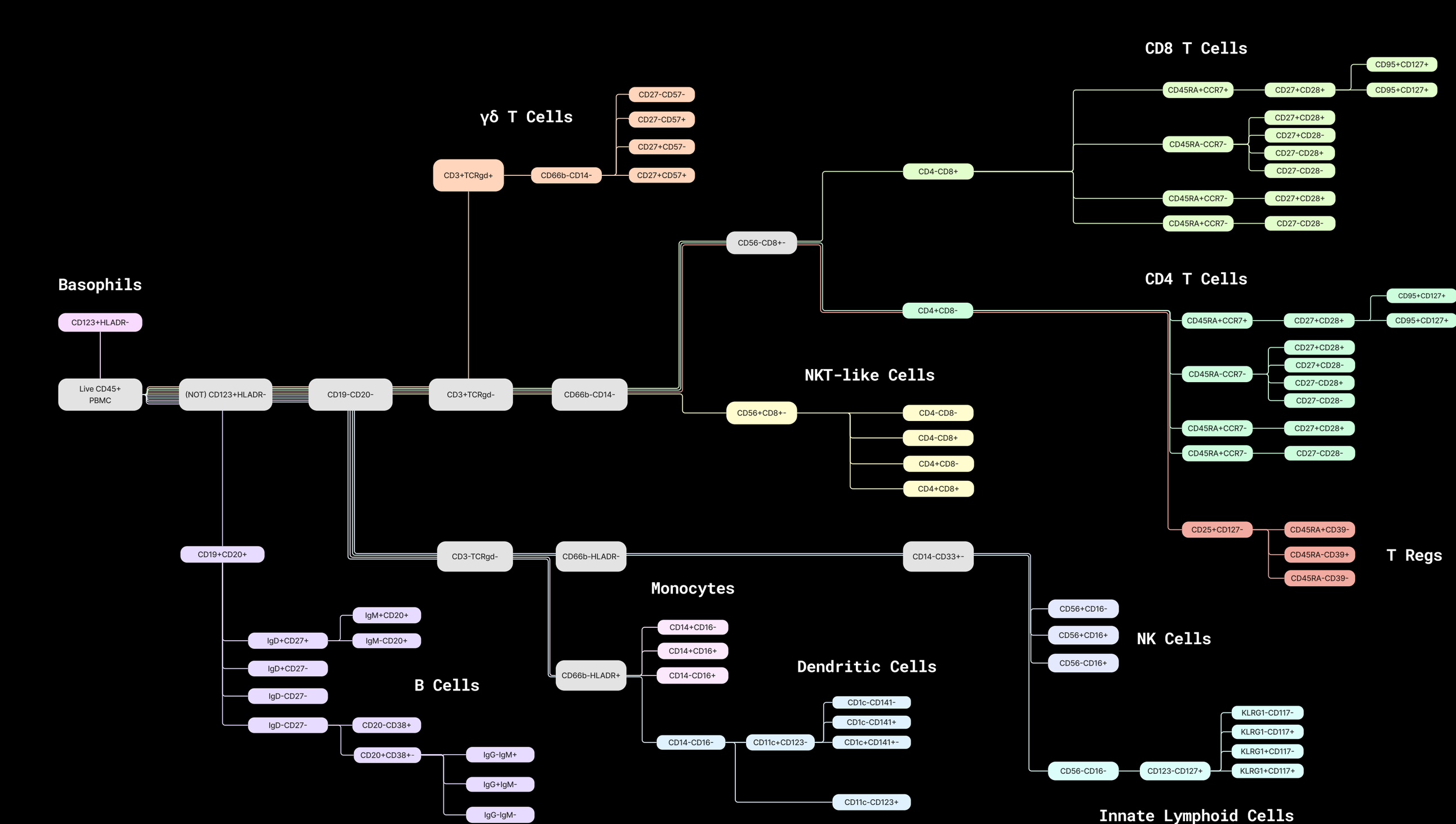

Figure 5: Gating tree schematic for the 82 pre-defined endpoints tracked using Ozette's 48-color pan-immune profiling panel. Our logic-based exclusionary gating strategy uses multiple lineage polishing gates to ensure accurate measurements of phenotypic endpoints and avoids redundant measurements. Seamless integration into the Ozette Endpoints™ pipeline allows for rapid and robust computational analysis of biomarker endpoints data, reducing analytical bias, and moving from assay to insights in a fraction of the time.

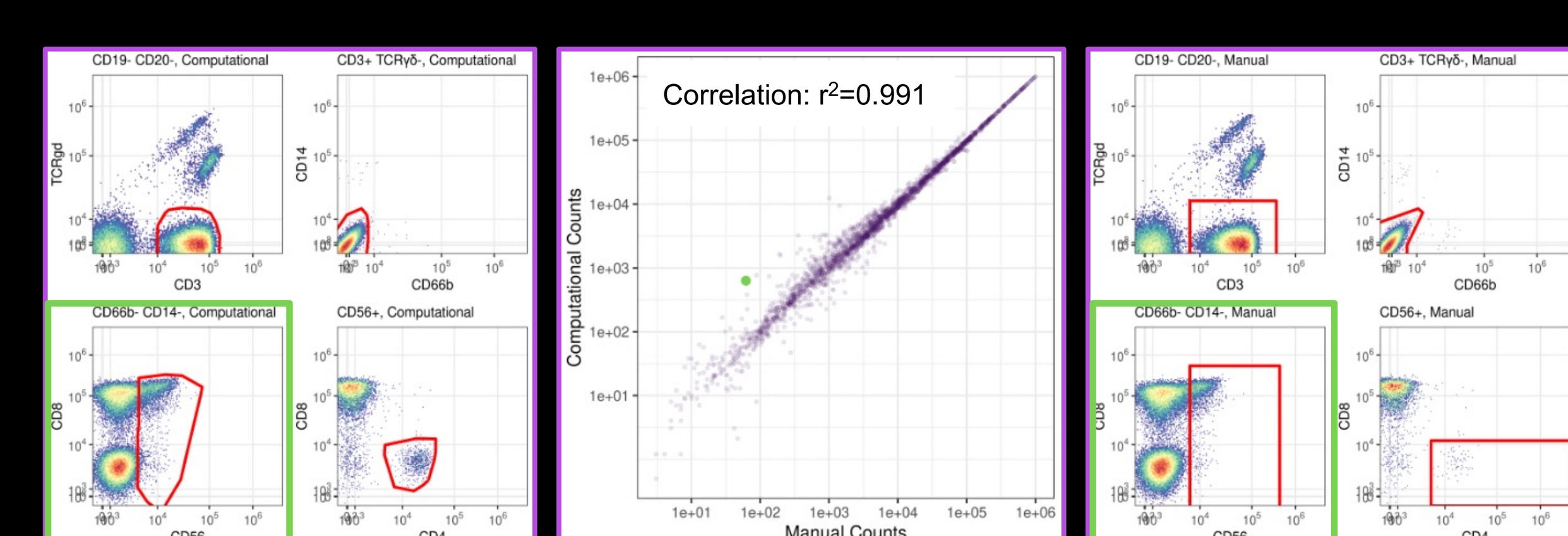

Figure 6: A strong correlation in counts between computational gating (using the Ozette Endpoints™ pipeline) and manual gating was observed across 82 pre-defined phenotypic endpoints across all healthy donor peripheral blood samples included in this study. Gating differences highlighting a non-concordant data point (outlined in green) are shown to demonstrate minor discrepancies in gate placement for a dim signal (CD56 expression on CD3+ cells).

ozette discovery™

Ozette Discovery™ uses machine-learning computational technologies to fully annotate all cell populations and allows rapid insights into complex high-dimensional data

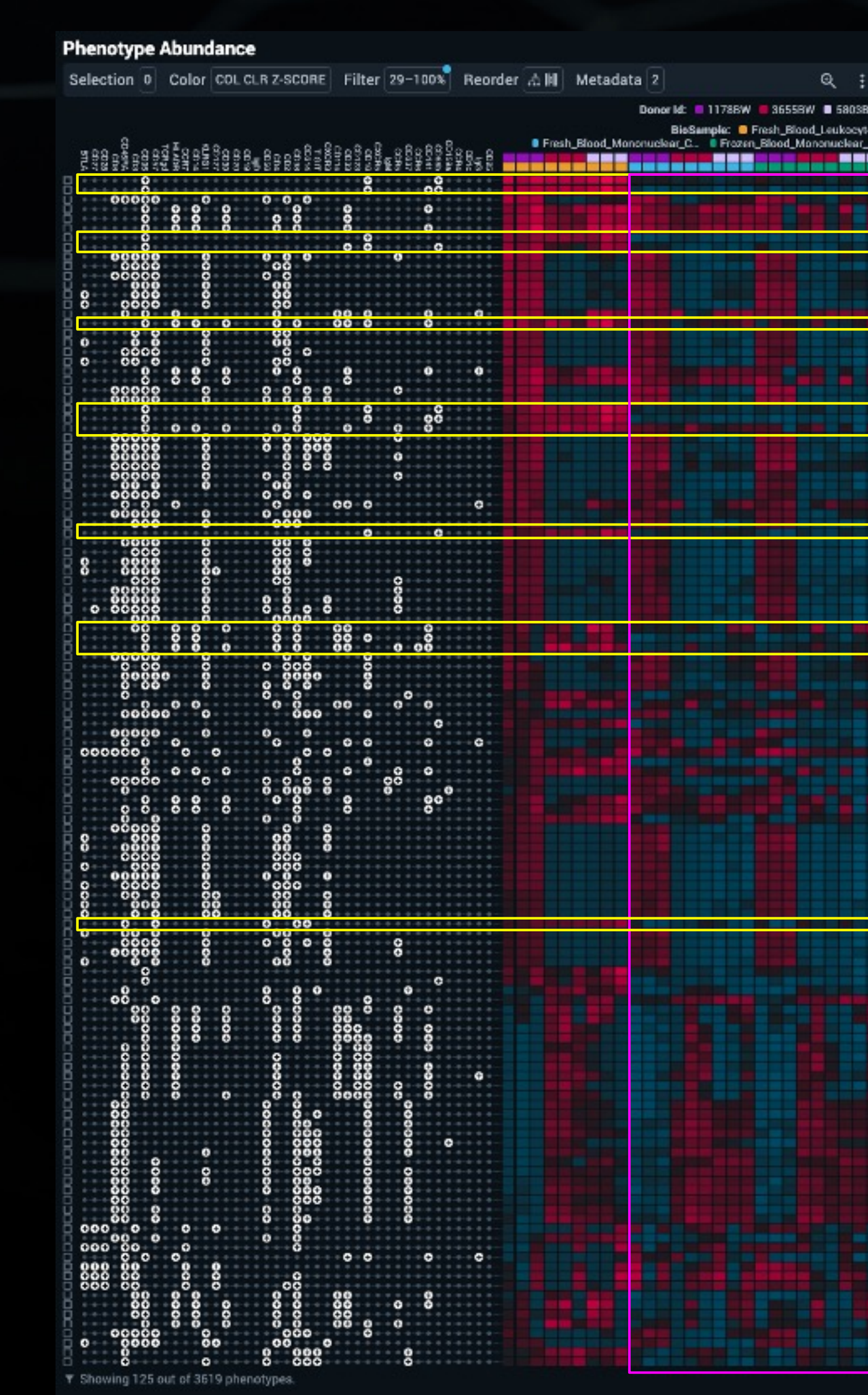

Figure 7: Comparison of high-variance phenotypes between RBC-lysed whole blood, fresh PBMC and frozen PBMC samples.

A dashboard image showing 125 out of 3619 robust cell phenotypes identified by the Ozette Discovery™ pipeline. The heat map is ordered by 1) sample type (fresh RBC-lysed whole blood in orange, fresh PBMC in blue, frozen PBMC in green), then by 2) donor ID, and clustered hierarchically. As expected, some immune cell phenotypes were differentially abundant between whole blood compared to gradient isolated PBMC, in particular populations expressing CD66b+, CD16+ or CD14+ (yellow boxes), while immune cell phenotypes were relatively conserved between fresh versus frozen PBMC (pink box).

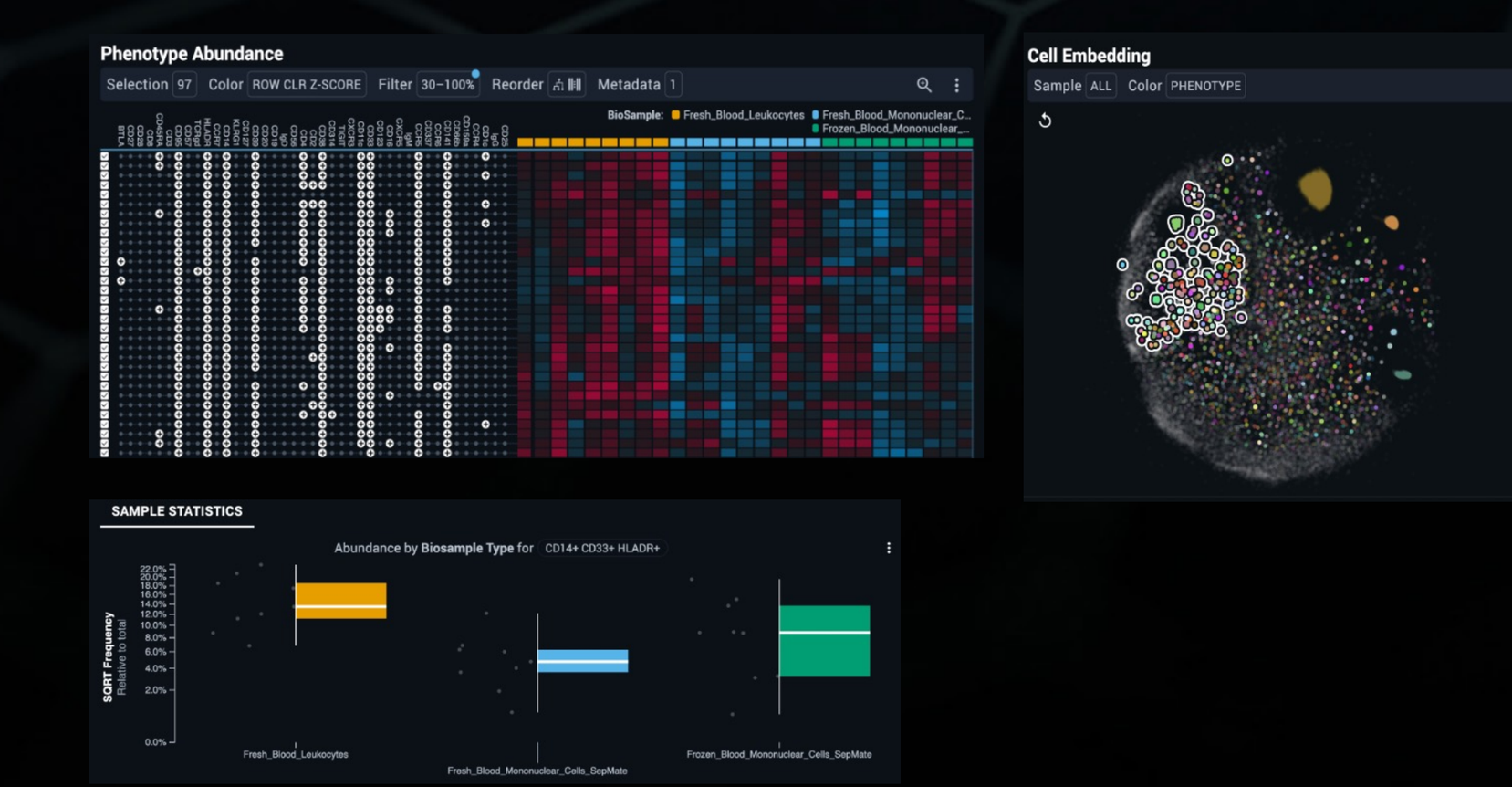

Figure 8: Differential abundance of Lineage-CD14+CD33+HLA-DR+ monocytes between sample processing methods.

A dashboard image of the Ozette Discovery™ platform heat map (left) filtered on Lineage-CD14+CD33+HLA-DR+ illustrates a high degree of variance in monocytic subsets between RBC-lysed whole blood (orange) and gradient isolated PBMC (blue or green). Selected robust phenotypes are highlighted in the interactive cell embedding map (right) and frequencies are displayed in the associated bar chart.

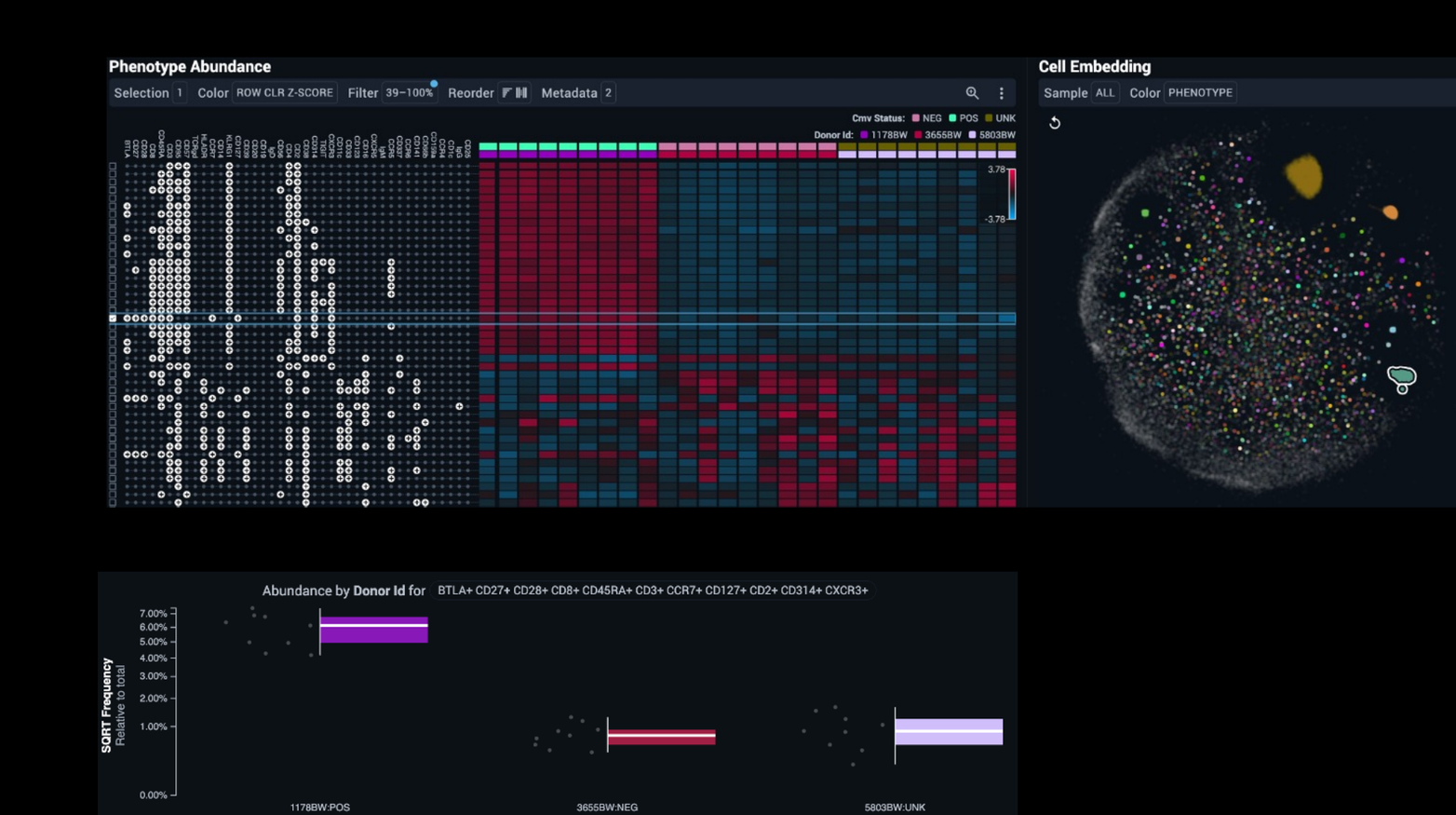

Figure 9: Clustering by CMV status metadata reveals a distinct donor-specific immune profile.

A dashboard image of the Ozette Discovery™ platform heat map (left) ordered by donor ID and CMV status. Clustering phenotypes by differential abundance revealed a subset of immune cell phenotypes unique to the CMV-positive donor (green) compared to the CMV-negative donor (pink) or the donor of unknown status (gold). The phenotypes observed included cells of T, NK and NKT-like lineage with expression of markers associated with activation and/or terminal differentiation. A representative robust phenotype is selected in the heat map (blue box) and highlighted in the cell embedding map (right, circled island). Corresponding frequencies of the selected CD8+ phenotype relative to total viable PBMC across the three donors are shown in the associated bar chart.

Summary

Ozette Panel Design Strategy

- Extensive CD4 characterization provides the ability to readily visualize the effective spread from any given signal into any measurement of interest and proves to be a valuable tool for high-dimensional flow cytometry panel design, agnostic of antibody vendor.

Endpoints

- A strong correlation was achieved between Ozette's computational analysis pipeline and conventional gating measuring 82 pre-defined phenotypic endpoints across all healthy donor peripheral blood samples included in this study.

Discovery

- The Ozette Discovery™ pipeline identified 3619 robust immune phenotypes with the highest variance in abundance observed between RBC-lysed whole blood and gradient isolated PBMC. This variance was largely driven by differential expression of CD66b, CD14 and CD16. In particular, Lineage-CD14+CD33+HLA-DR+ monocytes appeared significantly more abundant in RBC-lysed whole blood compared to PBMC. Interestingly, phenotypic abundance was relatively conserved between fresh versus frozen PBMC samples.
- We observed a distinct donor-specific immune profile when clustering data by CMV status. This immune profile consisted of differentially abundant T, NK and NKT-like cells expressing markers associated with activation and terminal differentiation; however, this observation would need to be verified in an appropriately powered study.

References

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3. Mair F and Tyznik AJ. High-Dimensional Immunophenotyping with Fluorescence-Based Cytometry: A Practical Guidebook. In: McCoy, Jr, J. (eds) Immunophenotyping. *Methods in Molecular Biology*, 2019.