Immunophenotyping and cell isolation with high-parameter spectral flow cytometry

High-parameter multicolor flow cytometry to identify and characterize specific T, B, NK and dendritic subpopulations in peripheral blood

Features

- High-resolution immunophenotyping of cell profiles through unbiased characterization on a multidimensional cell analysis platform (UMAP)
- Analysis of variation in immune cell subsets among different donors using a 33-color spectral flow cytometry panel
- Subsequent isolation of specific cell populations after live spectral unmixing.

Immunophenotyping is a widely used technique in the field of flow cytometry for characterizing and diagnosing a diverse range of diseases, including hematological cancers and immunodeficiency disorders. In recent years, there have been remarkable advancements in flow cytometry technologies, enabling considerable expansion in the number of parameters utilized in immunophenotyping panels. The inclusion of more markers provides for a more comprehensive and in-depth characterization of immune cell populations. Notably, the advancement in spectral flow cytometry affords sensitive and simultaneous detection of a large number of discrete immune subsets to provide unprecedented insights into their cellular composition and functions, particularly when analyzing clinical research samples.

There are several well-designed and optimized multicolor immunophenotyping panels available for characterization of leukocytes, including T cells, B cells, NK cells and specific subsets of dendritic cells. As the number of panel parameters increases, considerations for panel design and experimental planning become more intricate. Determining the optimal combination of fluorochromes to minimize spectral complexity and ensuring appropriate single-color controls for spectral unmixing are critical aspects that need to be carefully addressed. In this study, we present a 33-color panel for analysis of circulating human immune cells using spectral flow cytometry. More specifically, this panel was developed on a BD FACSymphony™ A5 SE Cell Analyzer using BD Biosciences fluorochrome-conjugated antibodies for optimal resolution of cell subsets. This panel can be directly transferred to a cross-standardized BD FACSymphony™ S6 SE Cell Sorter with matched configuration, facilitating the physical isolation of target cell populations of interest.

Protocol

Fresh human PBMCs were derived from whole blood obtained from healthy volunteer donors. PBMCs were isolated by Ficoll™ gradient centrifugation and resuspended in DPBS (1X). The staining solution comprised a cocktail of antibodies as per the specified test volumes (Table 1) suspended in BD Horizon™ Brilliant Stain Buffer Plus. Cells were stained by first prestaining the freshly isolated PBMCs with antibodies against TCRγδ, CD185 and CD197 for 10 min at 37 °C. The antibody cocktail was then added to the prestained PBMCs and the mixture was incubated for 30 min at room temperature in the dark. Single-stained controls were processed in parallel with the full panel.

Cells were washed twice using cold BSA wash buffer (PBS and 0.01% NaN_3). Cells were resuspended in wash buffer and stored on ice, protected from light, until acquisition. Viability dye 7-AAD was added at the specified test volume 5 min before acquisition on the flow cytometer. Optimized gain settings were derived for the BD FACSymphony™ A5 SE Cell Analyzer to generate Application Settings. Application Settings were then applied to maintain consistent fluorescence intensity values across experiments.

This panel can be run on an equivalently configured instrument after standardization with target values to achieve similar performance.

Laser	Marker	Fluorochrome	Primary Assigned Detector	Catalog #	Clone	Compensation Control	Test Size (µL or µg / test)
UV	CD45RA	BUV395	UV379	740315	5H9	PBMCs	0.06 µg
	Auto F	Cells	UV446	–	–	PBMCs	–
	CD45RO	BUV496	UV515	749888	UCHL1	PBMCs	0.5 µg
	CD16	BUV563	UV585	748851	3G8	PBMCs	0.5 µg
	CD185 / CXCR5	BUV661	UV660	741559	RF8B2	PBMCs	0.06 µg
	CD28	BUV737	UV736	612815	CD28.2	PBMCs	0.25 µg
	CD127	BUV805	UV809	748486	HIL-7R-M21	PBMCs	0.5 µg
Violet	CD25	BV421	V427	567485	BC96	PBMCs	2.5 µL
	CD20	V450	V450	561164	L27	PBMCs	2.5 µL
	CD62L	BV480	V470	566174	DREG-56	PBMCs	0.125 µg
	CD122 / IL2-RB	BV510	V510	747741	Mik-β3	Beads	2.5 µL
	IgG	BV605	V595	563246	G18-145	Beads	2.5 µL
	CD56	BV650	V660	564057	NCAM16.2	PBMCs	0.125 µg
	CD303	BV711	V710	748002	V24-785	Beads	1.25 µL
	KLRG1	BV750	V750	753692	Z7-205.rMAb	PBMCs	1.25 µL
	CD123	BV786	V785	564196	7G3	Beads	0.125 µg
Blue	CD57	FITC	B510	555619	NK-1	PBMCs	0.03 µg
	CD4	RB545	B537	569183	SK3	PBMCs	0.06 µg
	CD11c	BB630	B602	Custom	B-LY6	PBMCs	0.5 µg
	CD27	BB660	B660	Custom	M-T271	PBMCs	0.0125 µg
	CD45	PerCP	B675	340665	2D1	PBMCs	10 µL
	CD279 / PD-1	BB700	B710	566460	EH12.1	PBMCs	2.5 µL
	CD3	BB755	B750	Custom	UCHT1	PBMCs	0.025 µg
	CCR7 / CD197	RB780	B810	568748	2-L1-A	PBMCs	1 µg
Yellow Green	CD19	PE	YG585	555413	HIB19	PBMCs	5 µL
	CD158	RY586	YG602	753232	HP-MA4	Beads	0.125 µg
	CD14	PE-CF594	YG660	562335	MφP9	PBMCs	0.125 µg
	CD95	PE-Cy5	YG670	559773	DX2	PBMCs	20 µL
	7-AAD	–	YG730	559925	–	PBMCs (10% heat killed)	5 µL
	TCRγδ	PE-Cy7	YG780	655434	11F2	PBMCs	1.25 µL
Red	HLA-DR	APC	R675	559868	TU36	PBMCs	20 µL
	IgD	R718	R730	567993	IA6-2	PBMCs	0.125 µg
	CD8	APC-H7	R780	641409	SK1	PBMCs	0.03 µg

Table 1. Instrument configuration and reagent selection. This 33-color panel allows identification and sorting of specific human peripheral blood mononuclear cell (PBMC) populations. Careful evaluation of single-color controls identified superior performance in spectral unmixing using BD® CompBeads Particles in place of cell based controls for five colors (BV510, BV605, BV711, BV786 and RY586) in this panel.

Cell analysis results

The BD FACSymphony™ A5 SE Cell Analyzer is an advanced flow cytometry instrument equipped with five lasers (Ultraviolet, Violet, Blue, Yellow-Green, and Red) and 48 fluorescent detectors, allowing full emission profiling of fluorochromes. BD FACSDiva™ Software provides two powerful options for data analysis: compensation and spectral unmixing. In this study, we assembled a 33-color broad immunophenotyping panel for classification of classical immune cells.

Due to donor variation and frequency of targeted cell populations, careful consideration was given to identify PBMCs or beads as compensation controls. The panel's performance highlights the instrument's high sensitivity detectors, and the assignment of bright fluorochromes enables resolution of markers expressed at variable or low levels among circulating PBMC populations.

Using UMAP dimensional reduction and clustering analysis, immunophenotypic variation among three healthy donors was evaluated in this study. Detection of rare populations was achieved by recording a minimum of 500,000 leukocytes and then excluding doublets, inviable cells and non-relevant cell lineages through analysis using FlowJo™ v10.9.0 Software (Figures 1 and 2).

Figure 1. Identification of classical human immune cell subsets circulating in human blood

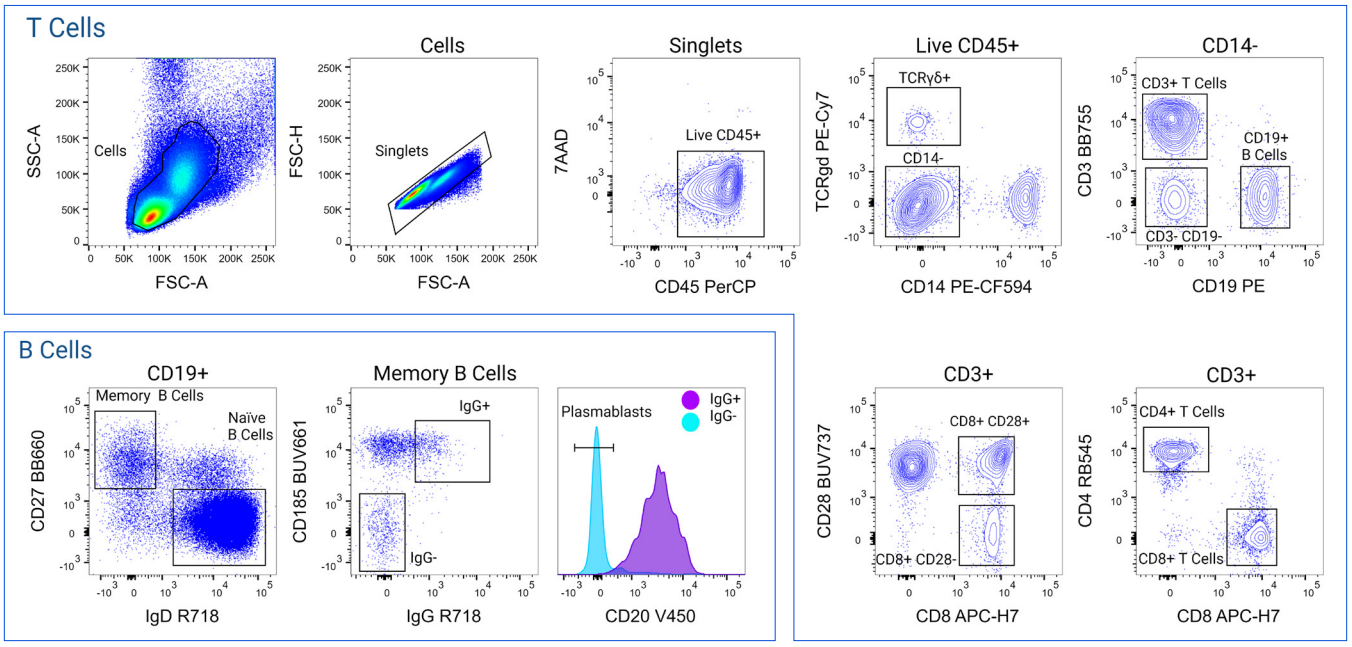
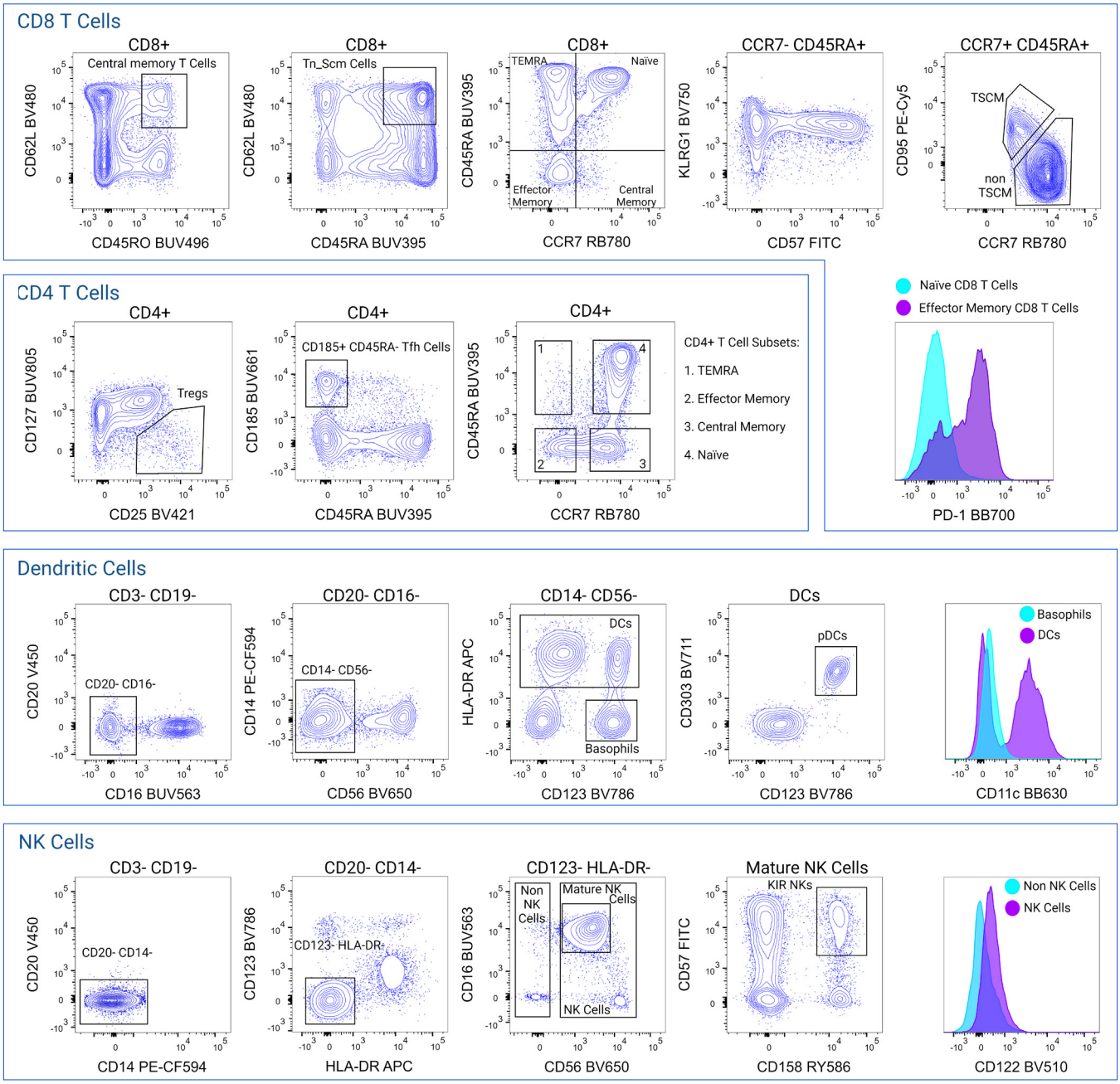
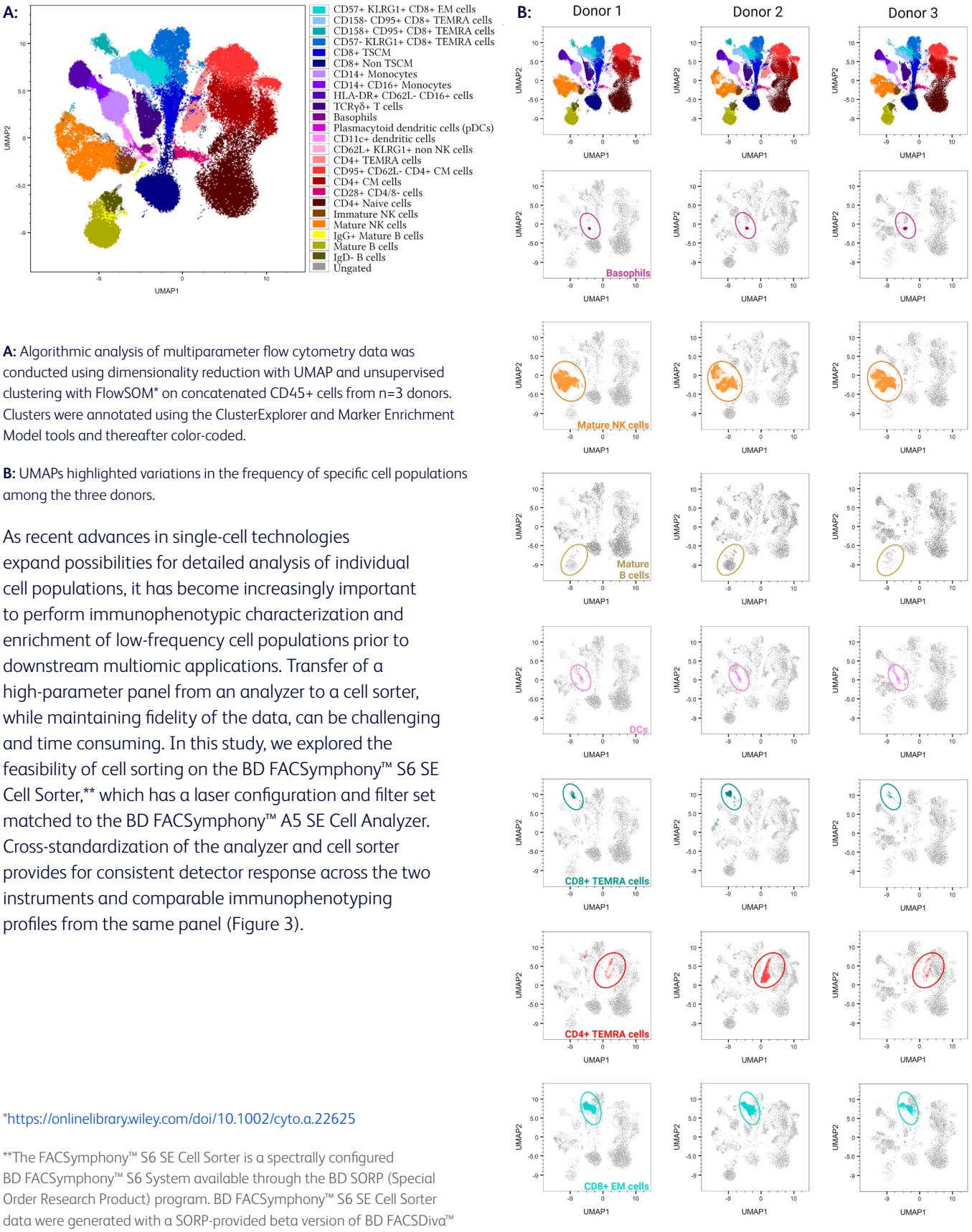


Figure 1. Identification of classical human immune cell subsets circulating in human blood (continued)



T cells, B cells, DCs and NK cells were gated on CD45+ cells by excluding 7AAD+ inviable cells and doublets. B cells were identified as CD19+ followed by identification of Naïve/Mature CD27-IgD+ B cells. Plasmablasts (CD27+ CD20-) were identified by assessing IgD- IgG- CD185- B cells. Classical T cells were identified as either CD4+, CD8+ or TCRγδ+, followed by identification of well-characterized T cell subsets based on the expression of CD62L and CD45RA or CD45RO (central memory and TN/SCM naïve and stem cell memory T cells), CD45RA and CCR7 (naïve, central memory, effector memory and terminally differentiated effector memory cells), CD95 and CCR7 (stem memory T cells TSCM), CD127 and CD25 (Tregs), and CD185 and CD45RA (T follicular helper cells). PD1 expression was assessed in CD8+ naïve and effector memory cells, and KLRG1 expression was assessed in CD8+ TEMRA cells. Classical DCs were identified as CD3- CD19- CD20- CD16- CD14- CD56- HLA-DR+, followed by identification of the pDC subset exclusively as CD303+CD123+. Basophils were identified as CD3- CD19- CD20- CD16- CD14- CD56- HLA-DR- CD123+. NK cells were identified as CD3- CD19- CD20- CD14- CD123- HLA-DR-. Mature and immature NK cells were distinguished based on the expression of CD16 and CD56 followed by identification of KIR-NK cells as CD57+CD158+ mature NK cells. NK cells and non-NK cells were assessed for the expression of CD122.

Figure 2. High-dimensional UMAP analysis of multiparameter flow cytometry data for identification of donor variable cell subsets for cell sorting



A: Algorithmic analysis of multiparameter flow cytometry data was conducted using dimensionality reduction with UMAP and unsupervised clustering with FlowSOM* on concatenated CD45+ cells from n=3 donors. Clusters were annotated using the ClusterExplorer and Marker Enrichment Model tools and thereafter color-coded.

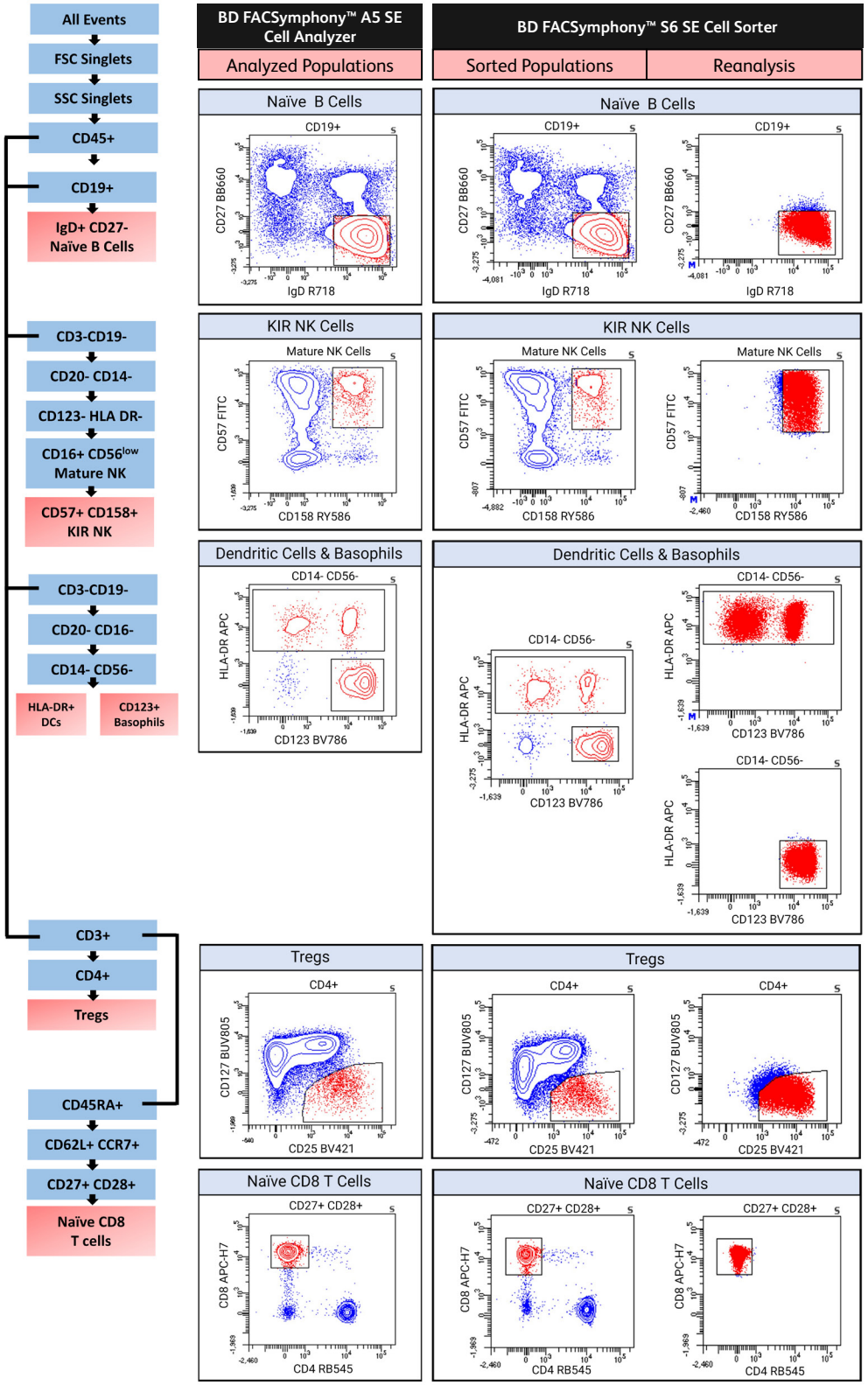
B: UMAPs highlighted variations in the frequency of specific cell populations among the three donors.

As recent advances in single-cell technologies expand possibilities for detailed analysis of individual cell populations, it has become increasingly important to perform immunophenotypic characterization and enrichment of low-frequency cell populations prior to downstream multiomic applications. Transfer of a high-parameter panel from an analyzer to a cell sorter, while maintaining fidelity of the data, can be challenging and time consuming. In this study, we explored the feasibility of cell sorting on the BD FACSymphony™ S6 SE Cell Sorter,** which has a laser configuration and filter set matched to the BD FACSymphony™ A5 SE Cell Analyzer. Cross-standardization of the analyzer and cell sorter provides for consistent detector response across the two instruments and comparable immunophenotyping profiles from the same panel (Figure 3).

*https://onlinelibrary.wiley.com/doi/10.1002/cyto.a.22625

**The FACSymphony™ S6 SE Cell Sorter is a spectrally configured BD FACSymphony™ S6 System available through the BD SORP (Special Order Research Product) program. BD FACSymphony™ S6 SE Cell Sorter data were generated with a SORP-provided beta version of BD FACSDiva™ Software. (Diva 9.8_2023_05_11_03_00).

Figure 3. Transfer of the 33 color panel from the BD FACSymphony™ A5 SE Cell Analyzer to the BD FACSymphony™ S6 SE Cell Sorter for cell isolation



Following cross-standardization, the panel was transferred to the BD FACSymphony™ S6 SE Cell Sorter. Representative data from the same sample demonstrate the comparable phenotypes of cell populations analyzed on the BD FACSymphony™ S6 SE Cell Sorter. Reanalysis of the sorted target populations, on the BD FACSymphony™ S6 SE Cell Sorter, reveals fidelity to the sort regions as gated on the parent population.

Conclusion

In this study, we described a 33-color spectral panel that accomplished resolution of specific T, B, NK and DC cell populations in donor samples. Subsequent comparison of donor immune cell profiles was performed through unbiased characterization on a multidimensional cell analysis platform (UMAP). Additionally, we demonstrated the feasibility of transferring this high-parameter panel from an analyzer to a sorter, allowing the sorting of specific subsets based on live spectral unmixing using BD FACSDiva™ Software.

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