

Spectral flow cytometry meets REAfinity® Recombinant Antibodies

A robust, ready-to-go panel for high-dimensional immune profiling

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Background

Spectral flow cytometry is rapidly transforming immunophenotyping by enabling the simultaneous analysis of more markers than ever before. This high-parameter approach provides new insights into complex cell populations, uncovering hidden cell subsets and previously undetectable biological patterns. This is especially critical when working with rare cell types or limited sample material, where every cell counts. However, designing a robust, high-dimensional panel is a complex task that requires careful marker selection.

High-parameter panels start with high-performance reagents

Our REAfinity Recombinant Antibodies are engineered to deliver superior specificity, minimal background signal, and unmatched lot-to-lot consistency, enabling accurate analysis and reproducible results. Every antibody is thoroughly validated for flow cytometry to provide optimal performance and compatibility with multicolor panels.

When paired with our Vio® and Vio Bright Dyes, known for their exceptional brightness and distinct spectral signatures, REAfinity Recombinant Antibodies deliver clear population separation and high-quality, reliable data for even the most complex panels.

In this study, we developed a backbone panel of REAfinity Recombinant Antibodies, optimized for spectral cytometry of human peripheral blood mononuclear cells (PBMCs). While profiling PBMCs is essential for studying immune responses in infection, cancer, and autoimmunity, applying spectral flow cytometry enables deeper, high-resolution immune profiling in a single, streamlined assay.

Methods

Panel design

The 13-antibody panel of REAfinity Recombinant Antibodies was carefully chosen to enable broad and detailed immunophenotyping of human PBMCs (table 1). Capturing both lineage-defining and differentiation markers, this panel allows for the identification of 20 major immune cell subsets (table 2), offering the flexibility for further customization depending on the research focus. The conjugation with Vio and Vio Bright Dyes ensures optimal resolution of each marker in a spectral flow setting, providing a strong backbone for immune profiling in both supervised and unsupervised analysis.

All 13 antibodies included in the panel were individually titrated to determine their optimal staining concentrations, ensuring maximal signal intensity, minimal background, and ideal spectral resolution.

Markers	Conjugates	Titration	Channel	Clone	Order no.
CD56	Vio Bright V423	1/80	V2	REA196	130-126-299
CD45RA	VioBlue®	1/160	V3	REA1047	130-117-743
CD14	VioGreen™	1/20	V7	REA599	130-110-525
CD16	Vio Bright V600	1/50	V10	REA423	130-131-159
CD19	Vio Bright B515	1/40	B1	REA675	130-113-650
CD8	PerCP-Vio 700	1/40	B10	REA734	130-110-682
CD197 (CCR7)	PE	1/40	YG1	REA546	130-119-583
CD127	PE-Vio 615	1/80	YG3	REA614	130-123-027
CD27	PE-Vio 770	1/80	YG9	REA499	130-113-641
CD38	APC	1/200	R1	REA572	130-113-429
CD25	Vio Bright R667	1/40	R2	REA570	130-114-542
CD4	Vio Bright R720	1/40	R4	REA623	130-127-378
CD3	APC-Vio 770	1/80	R7	REA613	130-113-136

Table 1: Panel design for immunophenotyping of human PBMCs by spectral flow cytometry using REAfinity Recombinant Antibodies.

Staining of PBMCs

For each experiment (n = 4), 1×10^6 thawed PBMCs from healthy donors were used. Dead cell staining was performed using Zombie UV™ Fixable Viability Kit (BioLegend) for 10 minutes at 4 °C in the dark. Alternatively, DAPI can be used for viability assessment (data not shown). Cells were then washed with 1 mL of PBS and centrifuged at 400 × g for 5 minutes. To improve the binding specificity of tandem dye–conjugated antibodies and reduce background signal, cells were incubated with Tandem Signal Enhancer for 10 minutes at 4 °C. After a second wash under the same conditions, cells were incubated with the 13-antibody panel mix (table 1) for 15 minutes, at 4 °C in the dark. Following a final washing step (1 mL PBS, 400 × g for 5 minutes), cells were resuspended in 150 µL of PBS and kept in the dark until acquisition.

Spectral flow cytometry

Stained cells were analyzed on the 5-laser Cytek® Aurora spectral flow cytometer (Cytek Biosciences) at the Flow Cytometry Core Facility of Saint-Louis Hospital. Supervised and unsupervised analyses were performed using the FlowJo™ Software (version 10.8.1).

Supervised analysis

In the supervised approach, manual gating was performed. PBMCs were sequentially gated to exclude debris, time-based artifacts, doublets (via FSC-A/H and SSC-A/H), and dead cells (Zombie UV$^+$), resulting in a clean population of viable singlets for downstream immunophenotyping. Immune cell subsets were identified based on the marker expression profiles outlined in table 2.

Unsupervised analysis

For the unsupervised analysis, two algorithm-driven tools were applied:

- **Uniform manifold approximation and projection (UMAP):** reduces high-dimensional data into a 2D or 3D map, uncovering biological patterns or relationships between cells.
- **Flow self-organization map (FlowSOM):** clusters similar cells together based on their marker expression. These clusters might reflect distinct cell types or states.

Markers	Cell subpopulation
Natural killer (NK) cells	
$CD3^-CD56^+$	NK[1]
Monocytes ($CD14^+$)	
$CD14^{hi}CD16^+$	Intermediate[2]
$CD14^{hi}CD16^-$	Classical[2]
$CD14^+CD16^{hi}$	Non-classical[2]
T cells ($CD3^+$)	
$CD3^+CD8^+$	CD8 T
$CCR7^+CD45RA^-$	Central memory CD8 T (CD8 CM)[3]
$CCR7^+CD45RA^+$	Naive CD8 T (CD8 N)[3]
$CCR7^-CD45RA^+$	Terminal effector CD8 T (CD8 TE)[3]
$CD3^+CD4^+$	CD4 T
$CCR7^+CD45RA^-$	Central memory CD4 T (CD4 CM)[3]
$CCR7^+CD45RA^+$	Naive CD4 T (CD4 N)[3]
$CCR7^-CD45RA^+$	Terminal effector CD4 T (CD4 TE)[3]
$CD25^+CD127^{lo}$	Regulatory T (Treg)[4]
$CD25^+CD127^{lo}CD45RA^+$	Naive Treg[4]
$CD3^+CD56^+$	NKT[1]
B cells ($CD19^+$)	
$CD27^+CD38^{int}$	Memory B (Bm)[5]
$CD27^+CD38^{hi}$	Antibody-secreting cells (ASC)[5]
$CD27^-CD38^+$	Naive B (Bn)[5]

Table 2: Immune cell subpopulations characterized by surface marker expression profiles.

Results

REAfinity Recombinant Antibodies and Vio Dyes are fully compatible with spectral flow cytometry

To validate the compatibility of REAfinity Recombinant Antibodies and Vio Dyes with spectral flow cytometry, we assessed staining quality, background levels, and spectral performance. Titration was essential to achieving clear separation between positive and negative populations, minimizing spillover, and sustaining optimal spectral resolution on the 5-laser Cytek Aurora system. To further confirm signal accuracy and discard unmixing artifacts, we performed fluorescence minus one (FMO) and fluorescence minus laser (FML) controls. No spectral overlap or unmixing artifacts were observed, confirming the robust compatibility of our titrated antibody–dye combinations with high-parameter spectral cytometry workflows.

Supervised analysis enables clear identification of the 20 immune cell subsets

Using supervised analysis through manual gating, we successfully identified 20 distinct immune cell populations within PBMCs, based on the expression of the 13 markers. The panel enabled precise resolution of major lymphoid and myeloid subsets, including NK cells, monocytes, T cells, and B cells, as well as functionally relevant subpopulations such as naive, central memory, effector memory, and regulatory T cells (fig. 1). REAfinity Recombinant Antibodies conjugated to Vio and Vio Bright Dyes provided high sensitivity and clean population separation, allowing confident detection of rare or low-abundance populations without background interference. These results validate the 13-antibody panel as a robust backbone for high-dimensional immune profiling of PBMCs by spectral flow cytometry.

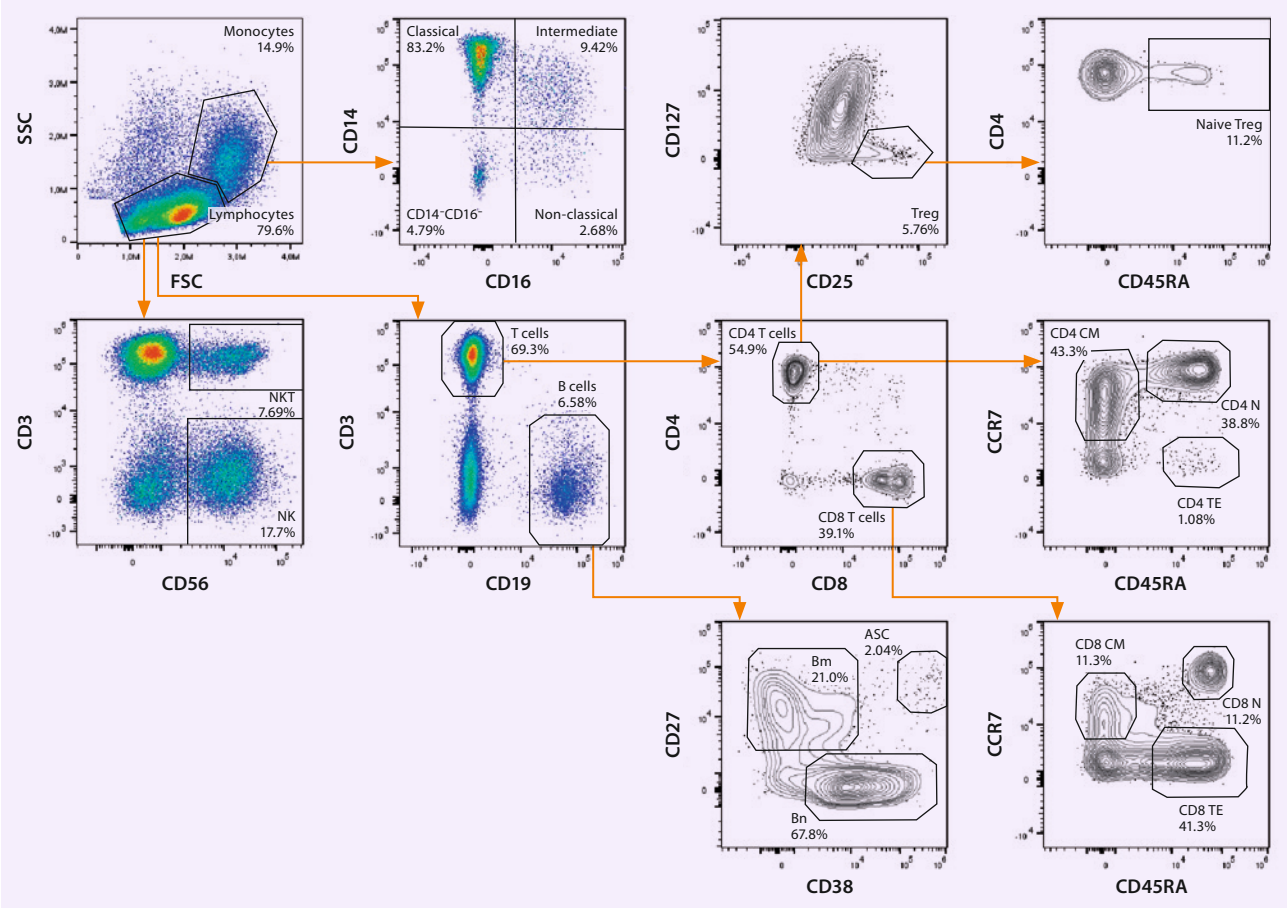


Figure 1: Manual gating strategy for immunophenotyping of human PBMCs by spectral flow cytometry.

Unsupervised clustering reveals cell subsets mapped to known immune profiles

To assess the power of unsupervised high-dimensional analysis, we applied UMAP for dimensionality reduction and FlowSOM for clustering. These algorithms enabled unbiased visualization and classification of immune subpopulations in a data-driven manner, without predefined gating strategies.

UMAP projections revealed well-separated clusters that mapped onto known immunophenotypic profiles, while FlowSOM clustering validated the identity and reproducibility of these populations. The 13-antibody panel provided sufficient resolution to resolve major cell lineages (monocytes, T, and B cells) and distinguish subtle phenotypic differences within those, such as naive, memory, and activated subsets. Importantly, even rare and low-abundance subsets, such as natural killer and regulatory T cells, were accurately resolved, highlighting the sensitivity and performance of REAfinity Recombinant Antibodies combined with Vio and Vio Bright Dyes.

Conclusions

The developed 13-antibody backbone panel using REAfinity Recombinant Antibodies conjugated to Vio and Vio Bright Dyes showed excellent compatibility with spectral flow cytometry, serving as a robust and ready-to-use tool for high-dimensional immunophenotyping of human PBMCs:

- **High sensitivity and optimal performance**
  Titrated conjugates showed clear spectral signal resolution, enabling reliable detection of both abundant and rare cell subsets with minimal background.
- **Flexible, high-quality foundation**
  Pretitrated and validated for immunophenotyping of PBMCs, this panel streamlines experimental setup while providing the flexibility to expand panels with markers tailored to your research needs.
- **Robust across analysis**
  Both manual gating and unsupervised analysis enabled clear identification of the distinct immune subsets, confirming the panel's biological relevance and analytical power.
- **Maximal efficiency, minimal risk of error**
  Pretitrated REAfinity Recombinant Antibodies and ultra-bright Vio Dyes minimize the need for additional concentration adjustments and reduce variability, saving time and improving reproducibility.

Together, REAfinity Recombinant Antibodies and Vio Dyes deliver the resolution and reliability needed to fully harness the analytical power of spectral flow. This panel offers a strong, validated starting point for both routine and exploratory immune profiling, enabling researchers to confidently tackle high-dimensional flow cytometry with precision and efficiency.

References

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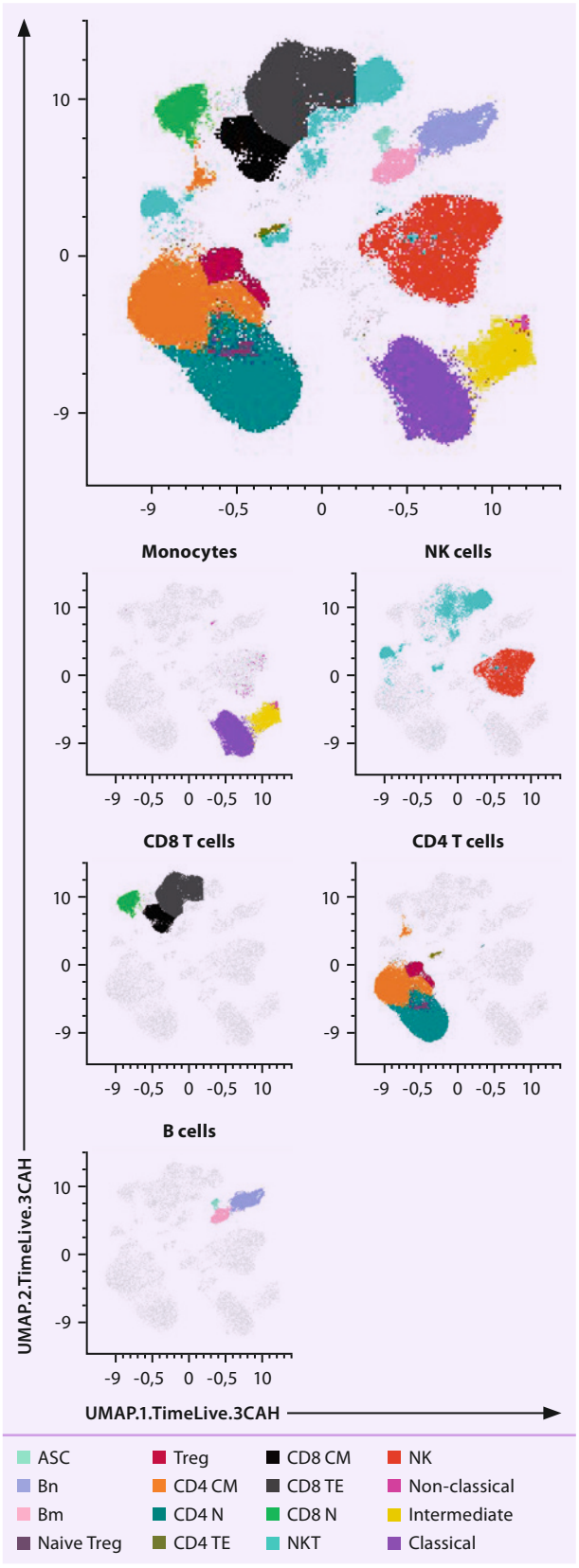


Figure 2: Unsupervised analysis using UMAP for dimensionality reduction and FlowSOM for clustering.

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