A 55-Color Panel for Comprehensive Immunophenotyping of Human Leukocytes with the Agilent NovoCyte Opteon Spectral Flow Cytometer

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Introduction

A 55-color immuno-phenotyping panel was designed and optimized for the Agilent NovoCyte Opteon spectral flow cytometer.

Taking OMIP-102 panel as a backbone,[1] five additional biomarkers (TCRαβ, CD95, CD223, CXCR5 and CD33) were added for broader research applications (burnt orange in Table 1). TCRαβ specifically enables discrimination of αβT cell subsets from γδT cells; CD95 facilitates the identification of stem cell memory T (T-SCM) cells; CD223 supports the analysis of exhausted T cells in combination with PD-1; CXCR5 allows quantification and functional evaluation of $CD4^+CXCR5^+$ T follicular helper (Tfh) cells; and CD33 is used to evaluate the maturity and activation status of myeloid cells. Furthermore, fluorochromes for ten markers were replaced or optimized to enhance assay performance (light blue in Table 1).

Recent studies have demonstrated that extending the staining time from the conventional 30 to 60 minutes to overnight can significantly improve experimental resolution, reduce gating bias, conserve antibody consumption, lower reagent costs, and minimize batch-to-batch variability.[2,3] To take full advantage of these merits, we thus assessed overnight staining in the 55-color panel and performed a side-by-side comparison with the conventional staining.

Experimental

Information on sample preparation and data analysis

Samples were prepared by sequential staining in appropriate buffers using cryopreserved PBMCs, data were analyzed using NovoExpress software (v2.2.0) for 2D plots, with fluorescence minus five (FM5) applied as the gating control, and OMIQ platform for high dimensional analysis.

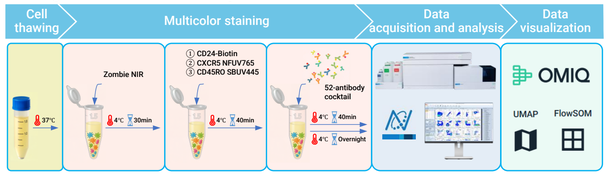

Figure 1. Workflow overview for conventional and overnight staining.

Marker	Fluorochromes	Stain	Titer (50µL staining volume) 40-min staining	Titer (50µL staining volume) Overnight staining
n/a	Zombie NIR	n/a	1:500	n/a
CD45RO	SBUV445	1	1:20	n/a
CD24	Biotin	1	1:320	n/a
CXCR5	NovaF V800	1	1:10	n/a
CD45RA	Spark UV 387	2	1:40	1:80
CD40	BUV395	2	1:20	1:80
CD27	BUV496	2	1:160	1:320
CD56	BUV563	2	1:160	1:640
CD141	BUV615	2	1:320	1:640
CD2	Qdot 605	2	1:80	1:200
Streptavidin	Qdot 625	2	1:320	1:320
CD303	BUV661	2	1:80	1:1000
CD86	BUV737	2	1:160	1:320
TCRαβ	NovaF UV765	2	1:20	1:80
CD45	BUV805	2	1:160	1:160
CD161	BV421	2	1:20	1:80
IgM	SB436	2	1:80	1:200
CD1c	Pacific Blue	2	1:320	1:5120
CD28	BV480	2	1:80	1:320
CD11c	BV510	2	1:160	1:320
CD19	Spark V500	2	1:160	1:320
CD3	BV570	2	1:20	1:40
CCR7	BV605	2	1:10	1:20
CD69	BV650	2	1:160	1:1280
CD4	Qdot 705	2	1:160	1:320
FcεRIα	BV711	2	1:40	1:200
CD103	BV750	2	1:160	1:2560
CD8	BV785	2	1:320	1:1280
CD25	BB515	2	1:40	1:160
CD57	FITC	2	1:80	1:400
CD14	RB545	2	1:80	1:160
CD33	SBB580	2	1:160	1:320
BTLA	RB613	2	1:40	1:200
CD11b	PerCP	2	1:40	1:80
CD223	RB670	2	1:20	1:40
IgG	BB700	2	1:640	1:5120
TCRγδ	RB705	2	1:20	1:80
CD127	RB744	2	1:80	1:80
TIGIT	RB780	2	1:80	1:320
IgD	PerCP-Fire 806	2	1:80	1:320
NKP46	PE	2	1:160	1:320
CD20	Spark YG 593	2	1:80	1:100
CD95	PE-Dazzle594	2	1:80	1:160
CXCR3	PE-Fire 640	2	1:80	1:160
CD123	PE-Cy5	2	1:40	1:160
ICOS	PE-Cy5.5	2	1:80	1:320
CD16	PE-AF700	2	1:320	1:640
CCR4	PE-Fire744	2	1:2560	1:12800
PD1	PE-Cy7	2	1:160	1:640
HLA-DR	PE-Fire 810	2	1:320	1:640
Vα24-Jα18	APC	2	1:80	1:160
TCR Vα7.2	AF647	2	1:160	1:320
KLRG1	SNIR 685	2	1:40	1:160
CD39	R718	2	1:160	1:640
CD163	APC-Cy7	2	1:40	1:160
CD38	APC-Fire 810	2	1:320	1:640

Table 1. Antibody reagents, staining groups, and varied titers were used in conventional(40-min) and overnight staining workflow.

Results and Discussion

Antibodies titrations

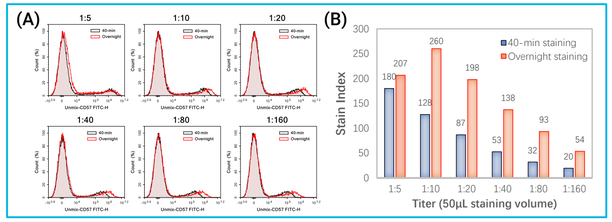

Figure 2. Titration of CD57 FITC antibody on human PBMCs. CD57 FITC antibody was titrated over six concentrations (starting at 10 µL per 50 µL staining volume) under two staining conditions: 40-min and overnight staining. **(A)** Overlaid histograms showing improved resolution with overnight staining. **(B)** Stain index quantification confirms that overnight staining significantly increases resolution across all dilutions. All presented data are representative results from the full antibody panel titration experiments.

Spectral signatures and Similarity Index Matrix visualized by NovoExpress software (55 fluorochromes + 1AF)

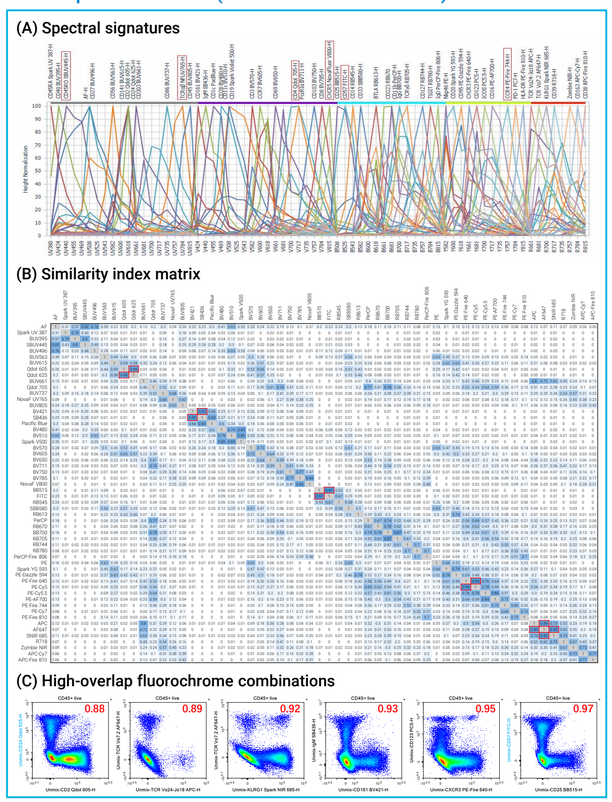

Figure 3. Spectral signatures and Similarity Index Matrix for the 55-color panel. **(A)** Spectral signatures. The fluorochromes added additional (red box) filled in spectral gaps between the current repertoire of fluorochromes. **(B)** Similarity Index Matrix. The panel complexity index (48.78) outperforms previously published 45-color panel (55.38). **(C)** Dot plots of fluorochrome pairs with spectral similarity index ≥ 0.88, all combinations were well unmixed in this 55-color panel.

Results and Discussion

Gating strategy

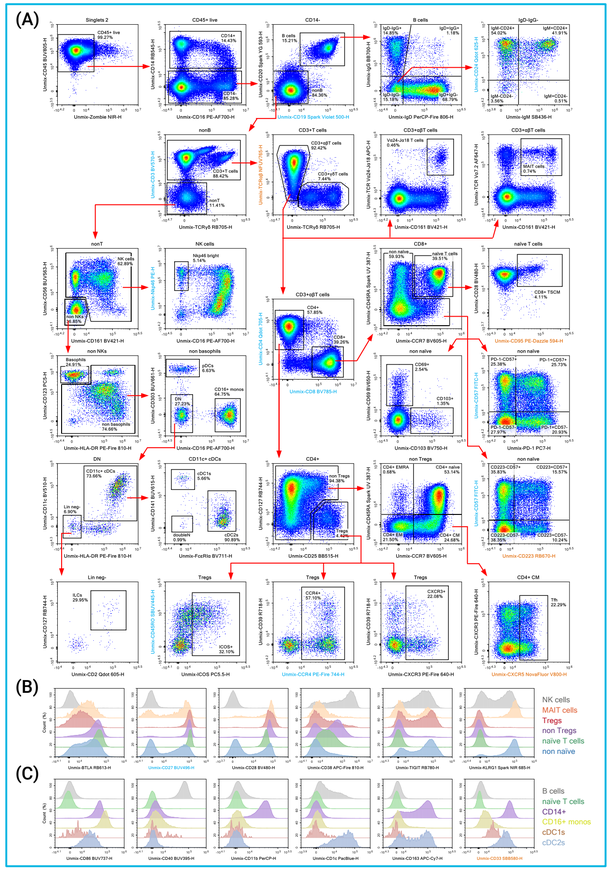

Figure 4. Gating strategy for identification of major immune cell subsets. **(A)** All leukocyte subsets were clearly resolved via manual gating, including Monocytes, B cells, γδT, Vα24-Jα18 T, iNKT, Tregs, $CD4^+$ T cells, $CD8^+$ T cells, T-SCM cells, Tfh cells, NK cells, Basophils, Dendritic cells, lymphoid cells(ILCs). **(B and C)** Histogram overlays. All data shown here were prepared by overnight staining.

Evaluation of population resolution

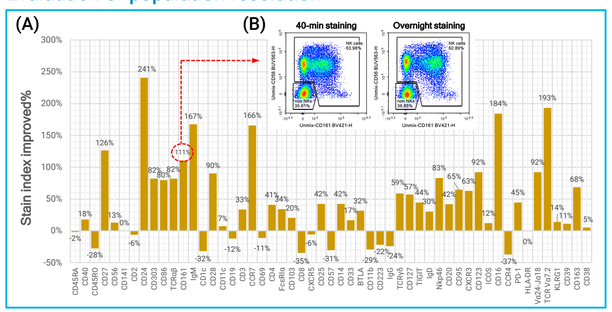

Figure 5. Improved population resolution via overnight staining. **(A)** Percent improvement in stain index relative to conventional staining, most markers benefited from overnight staining. **(B)** Representative plots of NK cells showing improved resolution.

Results and Discussion

High dimensional analysis

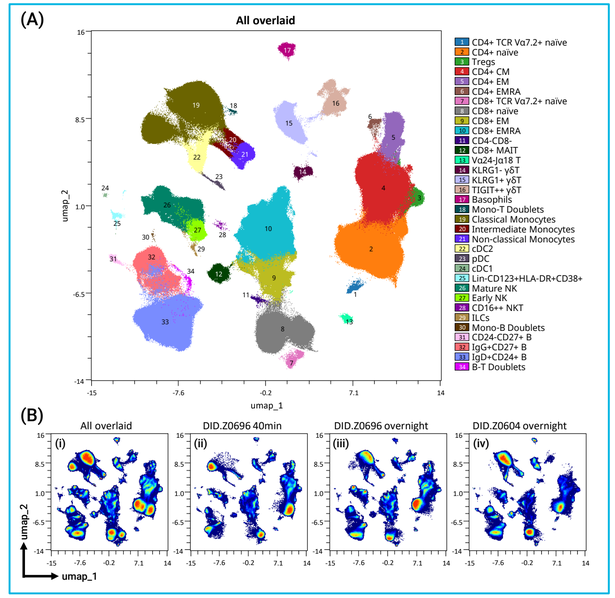

Figure 6. High dimensional analysis. After platelets, debris, doublets and dead cells were excluded, the cells in the live CD45+ gate from each sample are reduced to 500,000 events, then UMAP and FlowSOM are performed separately based on all markers except CD45 and Zombie NIR. **(A)** UMAP with FlowSOM cluster overlay. **(B)** UMAP results across donors with varied staining condition: (i) All samples, (ii) Donor 1 (40-min staining), (iii) Donor 1 (overnight staining), and (iv) Donor 2 (overnight staining).

Conclusions

- We successfully developed a 55-color panel to detect more biomarkers by both conventional and overnight staining protocols.
- All immune subsets could be accurately and clearly distinguished even though more highly overlapping fluorochromes were included in this 55-color panel.
- The results demonstrate the performance and accuracy of full spectrum NovoCyte Opteon and NovoExpress unmixing software.
- Researchers have more choices and can easily generate highly complex flow cytometry panels up to 55 colors in a single tube to address their biological questions.

Reference

[1] Konecny, A.J.; Mage, P.L.; Tyznik, A.J.; Prlic, M.; Mair, F. OMIP-102: 50-color phenotyping of the human immune system with in-depth assessment of T cells and dendritic cells. *Cytometry Part A.* **2024**, *105*(6), 430–436.

[2] Whyte, C.E.; Tumes, D.J.; Liston, A.; Burton, O.T. Do more with less: Improving high parameter cytometry through overnight staining. *Current Protocols.* **2022**, *2*, e589.

[3] Neubauer, F.; Spittler, A.; Berger, A.; Wisgrill, L. Optimization of a 40-Color Spectral Flow Cytometry Panel for Human Blood Immunophenotyping: Impact of Blood Volume, Sample Preservation, and Overnight Staining. *Cytometry Part A*, **2025**, *107*(12), 837–853.