A 45-color Panel for Comprehensive Immunophenotyping of Human Leukocytes

Ming Lei[1], Peifang Ye[1], Xiaohuan Wang[1], Yan Lu[1], Garret Guenther[2], Xiaoyu Zhang[2], Nan Li[2]
[1]Agilent Biosciences (Hangzhou) Co., Ltd., China, [2]Agilent Technologies, Inc., USA

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Introduction

Agilent has developed NovoCyte Opteon™ spectral flow cytometer, which consisting of up to five lasers (349nm, 405nm, 488nm, 561nm, and 637nm) and 73 detectors (70 fluorescence detection channels, FSC, BSSC, and VSSC).

Taking OMIP-069[1] panel as a backbone, a 45-color immuno-phenotyping panel was designed and optimized for the Agilent NovoCyte Opteon™ spectral flow cytometer.

Five additional biomarkers were added for broader research applications **(Table 1)**. These biomarkers are CD31, CD45RO, CD69, CD33 and LAG-3. The addition of CD31 and CD45RO enables the identification of recent thymic emigrants (RTEs) within the CD4+ T cells. CD69 is an early activation marker that is upregulated on T cells upon stimulation. LAG-3 enables the analysis of exhausted T cells together with PD-1. CD33 allows the phenotyping of DCs and monocytes.

Fluorochromes for four markers in OMIP-069 were repla ced **(Table 1)** for optimized results.

Experimental

Information on sample preparation and data analysis

Samples were prepared by sequential staining using PBMCs, data were analyzed using NovoExpress (Opteon) software (v2.0.0) for 2D plots, and FlowJo software (v10.10.0) for high dimensional analysis (UMAP v4.0.4, FlowSOM v4.0.0).

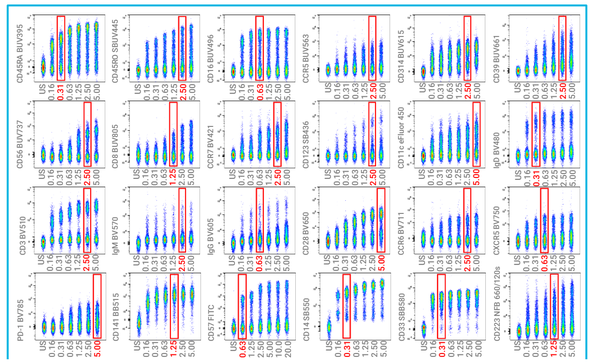
Marker	Fluorochromes	Marker	Fluorochromes	Marker	Fluorochromes
CD45RA	BUV395	CD141	BB515	CD159a	APC
CD45RO	**SBUV445**	CD57	FITC	CD1c	AF647
CD16	BUV496	CD14	Spark Blue 550	CD19	SNIR685
CCR5	BUV563	**CD33**	**SBB580**	**CD127**	**SR718**
CD314	BUV615	**CD223**	**NFB660/120s**	**Viability**	**Zombie NIR**
CD39	BUV661	CD45	PerCP	CD27	APC-H7
CD56	BUV737	CD2	PerCP-Cy5.5	CD38	APC-Fire 810
CD8	BUV805	TCRγδ	PerCP-eF710		
CCR7	BV421	**CD69**	**SBB765**		
CD123	SB436	**CD31**	**SBB810**		
CD11c	eF450	CD159c	PE		
IgD	BV480	**CD20**	**Spark YG 593**		
CD3	BV510	CD337	PE-Dazzle 594		
IgM	BV570	**CD4**	**CF594**		
IgG	BV605	CD24	PE-AF610		
CD28	BV650	CD95	PE-Cy5		
CCR6	BV711	CD25	PE-AF700		
CXCR5	BV750	CXCR3	PE-Cy7		
PD-1	BV785	HLA-DR	PE-Fire 810		

Text in red type:
five added biomarkers

Text in blue type:
modified fluorochromes

Abbreviation:
SBUV=StarBright UltraViolet
SB=Super Bright
SBB=StarBright Blue
NFB=NovaFluor Blue
eF=eFluor
AF=Alexa Fluor
SNIR=Spark NIR
SR=Spark Red

Table1. Antibodies used for 45-color panel.



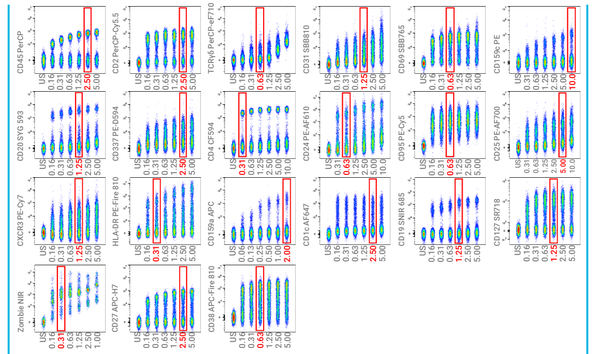
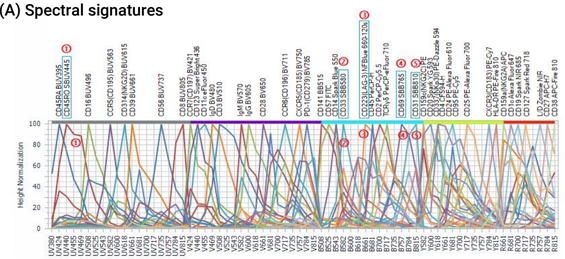


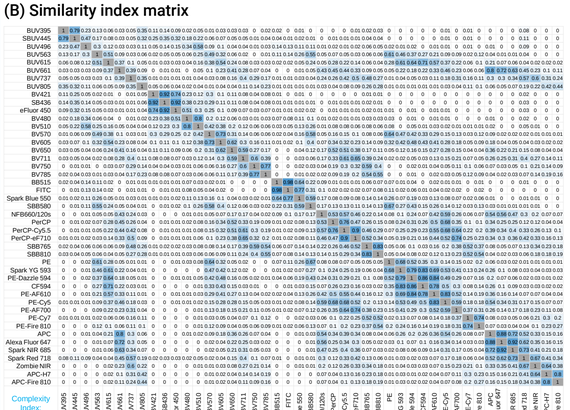
Figure 1. **Antibody titrations for the 45-color panel.** All antibodies were titrated over six concentrations with a starting concentration ranging from 1 to 20 µL per 100 µL staining volume. The optimal titer was chosen based on the Stain Index values (i.e., choosing a higher Stain Index while considering a lower titer to minimize the spreading error) and is indicated by the red box in the plot.

Spectral signatures and Similarity Index Matrix

(A) Spectral signatures



(B) Similarity index matrix



Complexity Index: 55.38

Figure 2. **Spectral signatures and Similarity Index Matrix for the 45-color panel on the NovoCyte OpteonTM flow cytometer. (A)** Spectral signatures. The five added dyes filled in spectral gaps between the current repertoire of fluorochromes. **(B)** Similarity Index Matrix. The Complexity Index is 55.38, which is 1.66 points higher than 53.72, based on published data[1].

Results and Discussion

Gating strategy

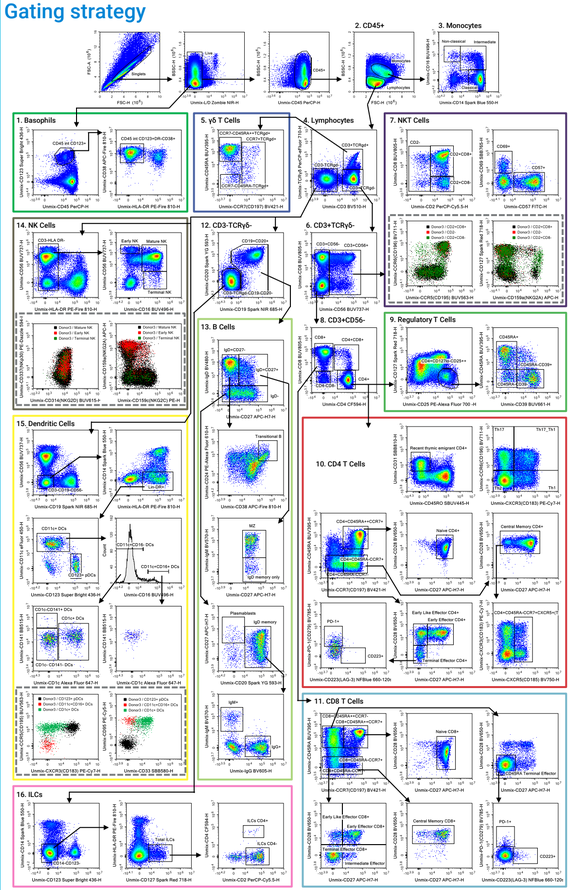
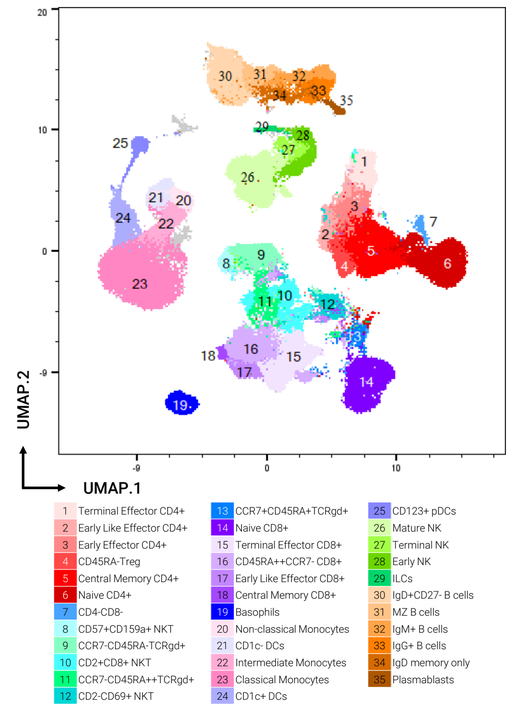


Figure 3. **Gating strategy to identify the main immune cell subsets.** All leukocyte subsets can be clearly distinguished by manual gating, including **(1)** Basophils, **(3)** Monocytes, **(5)** γδT cells **(7)** NKT cells, **(9)** Tregs, **(10)** CD4 T cells, **(11)** CD8 T cells **(13)** B cells, **(14)** NK cells, **(15)** Dendritic cells, **(16)** lymphoid cells (ILCs). All data presented here are derived from frozen PBMCs of one healthy donor.

High dimensional analysis

(A) FlowSOM cluster over UMAP



(B) UMAP across donors

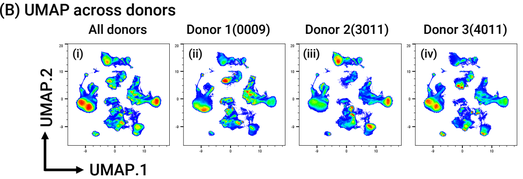


Figure 4. **High dimensional analysis.** After platelets, debris, doublets and dead cells were excluded, the cells in the live CD45+ gate from each donor are reduced to 500,000 events and concatenated into one file, then UMAP and FlowSOM are performed separately based on all markers except CD45 and live/dead dye. (A) UMAP with FlowSOM cluster overlay. (B) UMAP results across donors: (i) All donors, (ii) Donor 1(0009), (iii) Donor 2(3011), and (iv) Donor 3(4011).

Conclusions

- We successfully added more dyes to the original OMIP-069 panel to be able to detect more biomarkers.
- All immune subsets could still be accurately and clearly distinguished without affecting the dimensionality reduction and clustering result of the original markers even though more highly over-lapping fluorochromes were included in this 45-color panel.
- The results demonstrate the performance and high accuracy of the Agilent NovoCyte Opteon as a spectral flow cytometer.
- Researchers have more choices and can easily generate highly complex flow cytometry panels up to 45 colors in a single tube to address their biological questions.

Reference

[1] Park LM, Lannigan J, Jaimes MC. OMIP-069: Forty-Color Full Spectrum Flow Cytometry Panel for Deep Immunophenotyping of Major Cell Subsets in Human Peripheral Blood. Cytometry A 2020;97:1044–1051.

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