A 40-Color High Dimensional Flow Cytometry Panel to Evaluate Cellular Immune Responses on Various T-Cell Subsets from Cryopreserved Human Peripheral Blood Mononuclear Cells

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BACKGROUND

With the ongoing outbreak of the coronavirus disease COVID-19 in recent years, there has been increased interest into better understanding an individual's cellular immunity to COVID-19 or any other vaccine or gene therapeutic agent. A combination of ICS and AIM detection simultaneously by flow cytometry assay would be expected to provide a comprehensive understanding of the frequency, polyfunctionality, and compartmentalization of the antigen specific T cell responses.

Herein, a 40-color high-dimensional flow cytometry (HDFCM) panel was developed to assess the functions of various T cell subsets in response to vaccination or therapeutic drug treatment. The assay involves short-term re-stimulation of peripheral blood mononuclear cells with peptides, peptide pools, or whole proteins in the presence of transport inhibitors to detect intracellular cytokines (IFNγ, TNFα, IL-2, IL-8). Simultaneously, activation-induced markers (CD25, CD69, CD137, CD134, CD154, CD152, CD279, CD278, CD38, CD39, HLA-DR) are assessed.

PANEL INFORMATION

Specificity	Fluorochrome	Vendor	Cat #	Clone	Purpose
Zombie NIR	***Zombie NIR***	***BioLegend***	***423105***	***NA***	***Exclusion marker***
CD45	Spark UV387	BioLegend	304085	HI30	Leukocyte marker
CD19	RB545	BD Biosciences	569195	SJ25C1	B cell marker
CD14	AF647	BioLegend	301818	M5E2	Monocyte marker
CD3	SB436	Thermofisher	62-0036-42	SK7	T cell marker
CD8	PerCP	BioLegend	344708	SK1	T cell marker
CD4	Spark Plus UV395	BioLegend	344627	SK3	T cell marker
TCR γ/δ	RB613	BD Biosciences	759644	B1	TCR γ/δ cell marker
TCR Vα7.2	BV711	BioLegend	351731	3C10	MAIT marker
CD161	BV480	BD Biosciences	746305	DX12	MAIT/Th17 marker
CD56	BUV563	BD Biosciences	612929	NCAM16.2	NK cell marker
CD16	Pacific Blue	BioLegend	302024	3G8	NK/monocyte cell marker
CD196(CCR6)	APC	BioLegend	353415	G034E3	T cell marker
CD185 (CXCR5)	PE Cy5	BioLegend	356951	J252D4	Tfh cell marker
CD183(CXCR3)	RB780	BD Biosciences	755404	1C6	Th1 marker
CD194(CCR4)	BV785	BioLegend	359447	L291H4	Th2 marker
Foxp3	**PE Cy5.5**	**Thermofisher**	**35-4776-42**	**PCH101**	**Treg cell marker**
CD45RA	BUV805	BD Biosciences	568330	HI100	T cell differentiation
CD27	Spark NIR685	BioLegend	302856	O323	T cell differentiation
CD197(CCR7)	BV750	BioLegend	353254	G043H7	T cell differentiation
CD95	RY586	BD Biosciences	568443	DX2	T cell differentiation
CD28	APC H7	BD Biosciences	561368	CD28.2	T cell differentiation
IL-8	**PE CF594**	**BD Biosciences**	**563531**	**G265-8**	**Chemokine**
TNFa	**BV650**	**BD Biosciences**	**563418**	**Mab11**	**Cytokines (Th1)**
IL-2	**RB705**	**BD Biosciences**	**570624**	**MQ1-17H12**	**Cytokines (Th1)**
IFNg	**PE**	**BD Biosciences**	**554701**	**B27**	**Cytokines (Th1)**
Perforin	**BV421**	**BioLegend**	**353307**	**B-D48**	**Cytotoxic marker**
Granzyme B	**BV510**	**BD Biosciences**	**563388**	**GB11**	**Cytotoxic marker**
Ki-67	**R718**	**BD Biosciences**	**566963**	**B56**	**Proliferation marker**
CD154(CD40L)	**BUV615**	**BD Biosciences**	**752859**	**24-31**	**AIM**
CD152(CTLA4)	**PE Fire 640**	**BioLegend**	**369638**	**BNI3**	**AIM**
CD38	BUV496	BD Biosciences	612947	HIT2	AIM
CD278(ICOS)	BUV661	BD Biosciences	741664	DX29	AIM
CD137 (4-1BB)	BUV737	BD Biosciences	568348	4B4-1	AIM
CD39	BV605	BD Biosciences	742522	TU66	AIM
CD25	BB515	BD Biosciences	567319	BC96	Treg cell marker/AIM
CD69	SBB580	Bio-Rad	MCA2806SBB580	FN50	AIM
CD134 (OX40)	BB700	BD Biosciences	745957	L106	AIM
HLA-DR	RB744	BD Biosciences	757050	L243	AIM
CD279 (PD-1)	PE Cy7	BioLegend	329917	EH12.2H7	Tfh cell marker/AIM

Table 1: All markers used in the panel. ***Italics and bold font*** refer to the steps performed before cell surface staining. **Bold font** refers to intracellular/intranuclear staining.

SAMPLES

- Cryopreserved healthy donor PBMCs were sourced from BioIVT and performed in accordance with the human resources ethics committee.
- Cryopreserved healthy donor PBMCs were stimulated with PMA & Ionomycin, or CytoStim in the presence or the absence of transport inhibitors for the indicated time, if applicable.

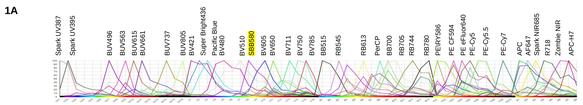
FLUORESCHROME SELECTIONS

Marker-Fluorochrome Combination (MFC)

	UV352		Violet		B488		YG561		R640	
Emission	Marker	Fluorochrome	Marker	Fluorochrome	Marker	Fluorochrome	Marker	Fluorochrome	Marker	Fluorochrome
370	CD45	Spark UV 387								
395	CD4	Spark Plus UV395								
420			Perforin	BV421						
440			CD3	Super Bright 436						
450			CD16	Pacific Blue						
480			CD161	BV480						
500	CD38	BUV496	Granzyme B	BV510	CD25	BB515				
500										
520					CD19	RB545				
550										
570	CD56	BUV563					IFNg	PE		
570										
580					CD60	StarBright Blue580	CD95	RY586		
600	CD154(CD40L)	BUV615	CD39	BV605	TCR γδ	RB613	IL8	PE CF594		
600										
660	CD278(ICOS)	BUV661	TNFa	BV650			CD152(CTLA4)	PE-Fire640	CCR6	APC
680					CD8	PerCP	CD185 (CXCR5)	PE-Cy5	CD14	AF647
690					CD134 (OX40)	BB700			CD27	Spark NIR 685
700			TCR Vα7.2	BV711	IL-2	RB705			Ki-67	R718
730	CD137 (4-1BB)	BUV737					Foxp3	PE-Cy5.5	Zombie NIR	Zombie NIR
750			CD197	BV750	HLA-DR	RB744				
780			CCR4	BV785	CXCR3	RB780	CD279 (PD-1)	PE-Cy7	CD28	APC-H7
800	CD45RA	BUV805								
# of Fluorochromes	8		10		9		7		6	

Table 2: The final selected fluorochromes paired with each marker in the panel are shown in this fluorochrome optical layout.

1A



1B

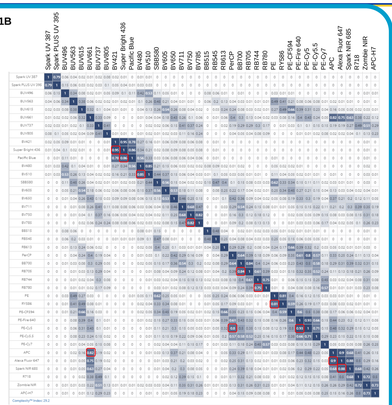
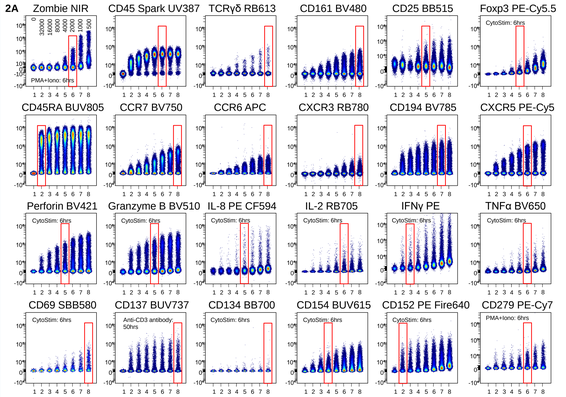


Figure 1A. Spectral signatures of 40 selected fluorochromes visualized using the Cytek Full Spectrum Viewer on the Cytek Cloud platform. SBB580 was highlighted since it showed the peak channel at B5 in our instrument. **Figure 1B.** Similarity Index Matrix and Complexity Index for selected fluorochromes using the Cytek Cloud platform.

ANTIBODY TITRATIONS AND FULL PANEL ASSESSMENT

2A



2B

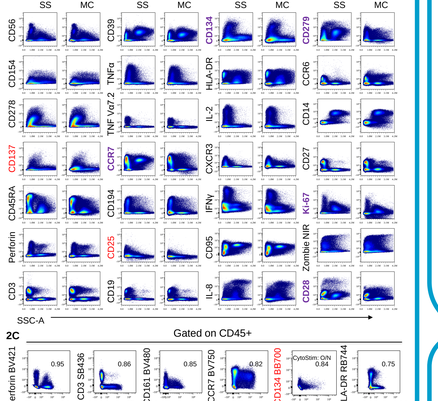


Figure 1A. Representative Antibody Titrations. All reagents in this panel were titrated using cryopreserved PBMCs with appropriate conditions. All titrations (except Zombie NIR as indicated in the plot) started with 5 μL/test in 2-fold serial dilutions, ranging from 5 μL per test to 0.08 μL per test, and ended with an FMO control. **Figure 1B.** Representative comparison between SS and MC staining for each marker. **Figure 1C.** Two fluorochromes with a Similarity Index ≥ 0.75 are plotted against each other. Plots are generated from unstimulated PBMCs (unless indicated otherwise in the plot) and gated on CD45+ cells. The Similarity Index for each plot is indicated in the plot.

FULL PANEL ANALYSIS

3A

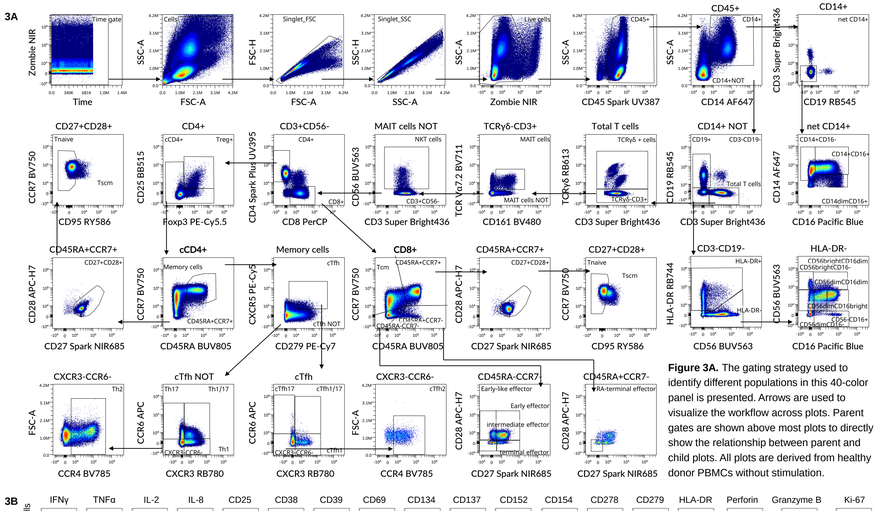


Figure 3A. The gating strategy used to identify different populations in this 40-color panel is presented. Arrows are used to visualize the workflow across plots. Parent gates are shown above most plots to directly show the relationship between parent and child plots. All plots are derived from healthy donor PBMCs without stimulation.

3B

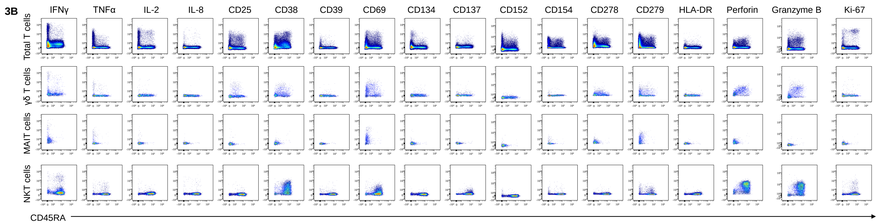


Figure 3B. The expression of functional markers on total T cells, γδ T cells, MAIT cells, and NKT cells is shown in the representative figures. A mixed sample was generated by combining unstimulated PBMCs, PMA & Iono-stimulated PBMCs (incubated overnight in the presence of transport inhibitors), CytoStim-stimulated PBMCs (incubated overnight in the presence of transport inhibitors), and CytoStim-stimulated PBMCs (cultured for more than 2 days without transport inhibitors), just prior to flow cytometry staining.

CONCLUSION

- This 40-color high-dimensional flow cytometry (HDFCM) panel (Complexity Index: 29.2) is currently to our knowledge the largest available, capable of assessing a wide range of T-cell functional markers, including cytokines/chemokines, activation-induced markers, cytotoxicity markers, and proliferation markers across different T-cell subsets in human PBMCs. It also simultaneously evaluates the immunophenotyping and function of monocytes, B cells, and NK cells in cryopreserved PBMCs.
- This HDFCM panel was developed and optimized to detect every marker with optimal resolution and can be used as a foundation for investigating the cellular immune response in various contexts of use.
- The data featured in this poster is part of a manuscript currently under review for publication.