

Optimization of a 40-Color High-Dimensional Flow Cytometry Panel to Simultaneously Evaluate Activation-Induced Markers and Cytokines in Human Peripheral Blood Mononuclear Cells. Lifeng Tian, John Kolman

BACKGROUND

With the increasing requirements for monitoring cellular immune responses during clinical trials, particularly with the advancement of gene therapy and vaccine modalities, we previously developed a comprehensive 40-color high-dimensional flow cytometry (HDFCM) panel (Complexity Index: 29.2) to assess various cellular immune responses across a broad array of immune populations. The assay involved the overnight re-stimulation of peripheral blood mononuclear cells (PBMC) with a peptide pool or superantigen, in the presence of transporter inhibitors, to detect intracellular cytokines or chemokines (TNFα, IFNγ, IL-2, IL-8). Additionally, activation-induced markers (AIM) (CD25, CD69, CD137, CD134, CD154, CD152, CD279, CD278, CD38, CD39, HLA-DR), cytotoxic markers (Granzyme B, Perforin), and the proliferation marker Ki-67 were included.

In this poster, we further optimized this comprehensive panel to address the impact of the presence of transport inhibitor(s) during the stimulation process on the detection of AIM and other immunophenotyping markers.

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 Va7.2	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. Blue font refers to intracellular/intranuclear staining after optimization.

SAMPLES

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

QPS, Newark, Delaware, USA



. Expression levels of each marker in the absence or presence of transport inhibitors (no inhibitor, with Brefeldin A, with Monensin, with both Brefeldin A and Monensin) are shown. Stimulation conditions are indicated in the plot, if applicable. The expression of TCR Va7.2 (Light Green) was affected by CytoStim stimulation (data not shown). Markers shown in Red (AIMs) and Purple (Immunophenotyping markers) indicate that their expression was affected by the presence of transport inhibitor(s). * indicates markers that were tested further.

FIGURE 2. COMPARISON OF AFFECTED MARKERS ON CELL SURFACE AND **INTRACELLULAR STAINING**



Figure 2. Expression levels of each affected marker were assessed by both cell surface (first 4 columns) and intracellular staining (the last 4 columns), in the absence or presence of transport inhibitors (no inhibitor, with Brefeldin A, with Monensin, with both Brefeldin A and Monensin). Stimulation conditions are indicated in the plot, if applicable. Markers shown in Red indicate that their expression could be obviously rescued by intracellular staining. Markers shown in **Dark Blue** indicate that their expression can not be rescued by intracellular staining. Markers shown in Green indicate that the performance of these marker-fluorochrome combinations in the intracellular staining was suboptimal.

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.

• In summary, we further optimized the previously developed 40-color HDFCM panel by switching several markers from cell surface staining to intracellular staining and reducing the concentration of the transport inhibitor Monensin during the stimulation procedure to maintain better cell viability and detect as many markers as possible.



Lifeng Tian Email: lifeng.tian@qps.com

FIGURE 3. OPTIMIZATION OF THE CONCENTRATION OF TRANSPORT INHIBITOR PMA & CytoStin peptide pool stimulation lonomvci stimulation lonomycin XXXXXXX D19 10⁴ IIII 104 D28

1 2 3 4 5 6 7 8 9 Figure 3. Expression levels of each affected marker were assessed in the following order: no inhibitor, 1x Brefeldin A, 0.5x Brefeldin A, 0.25x Brefeldin A, 0.125x Brefeldin A, 1x Monensin, 0.5x Monensin, 0.25x Monensin, 0.125x Monensin. The expression levels of markers in Dark Teal were restored by intracellular staining. Markers in Red indicated that expression levels were dramatically affected by Brefeldin A, and remined relatively stable in 0.125x Monensin. Markers in **Black** indicated that expression levels were comparable in both 0.125x Monensin and 0.125x Brefeldin A.

88888888

10¹

10*

104



Figure 4A. The gating strategy used to identify major populations in this 40-color panel is presented. Arrows are used to visualize the workflow across plots. Plots are derived from unstimulated PBMCs. **4B**. Comparison of expression levels on representative functional markers between 1x Monensin and 0.125x Monensin. Plots are derived from CytoStim stimulated PBMCs.