Luminex®
complexity simplified.

16-Color Immunophenotyping of Major Lymphocyte and Myeloid Subsets in Human PBMCs and Whole Blood Using the Amnis® CellStream® Flow Cytometer

Christine E. Probst, Prabhjoat Chana, Haley R. Pugsley, Raymond Kong, Phil Morrissey
Luminex Corporation, Seattle, WA, USA

Introduction

A 16-color immunophenotyping panel was developed using the CellStream® Flow Cytometer to simultaneously quantify major lymphoid and myeloid populations, including monocytes, basophils, myeloid and plasmacytoid dendritic cells, natural killer (NK) cells, B cells, cytotoxic-T and T-helper cells within human PBMCs and whole blood. T cells were further characterized into well-defined subsets, including naïve, effector, effector memory, central memory, activated, and regulatory T cells. The CellStream System enabled high-parameter flow cytometric analysis with exceptional population resolution using the Amnis-patented Time-delayed Integration (TDI) charge-coupled device (CCD) detection system.

Materials and Methods

Sample Preparation

Human PBMCs or whole blood from healthy donors (AllCells) were stained using a 16-color panel adapted from OMIP-042 (**Table 1**).[1, 2]

	Specificity	Fluorochrome	Clone	Purpose
1	Live/Dead	Violet	N/A	Viability
2	CD4	BUV496	SK3	CD4 T Cells
3	CD56	APC-R700	5.1H11	NKs
4	HLADR	APCCy7	L243	DCs
5	CD123	BV421	6H6	pDCs
6	CD20	V500	L27	B Cells
7	CD8	BV570	RPA-T8	CD8 T Cells
8	CD25	BV605	BC96	Treg
9	CD16	BV650	3G8	Monocytes
10	CD14	BV785	M5E2	Monocytes
11	CD45RA	AF488	HI100	Naïve/memory
12	CD38	PE	HIT2	Activation
13	CD3	BB700	HIT3a	T Cells
14	CCR7	PECF594	150503	Central/effector
15	CD11c	PeCy5	3.9	mDCs
16	CD127	PeCy7	A019D5	Treg

Table 1. Reagents used for 16-color panel.

The staining protocol used was as follows: A fluorescent antibody cocktail was prepared by mixing 2 µL of each fluorescent conjugate and adding BD Brilliant Stain Buffer to a final volume of 50 µL per sample. Cryopreserved PBMCs were thawed and resuspended by dropwise addition of prewarmed resuspension media, washed, and incubated for 15 minutes with fixable LIVE/DEAD™ violet stain (ThermoFisher Scientific). Following another wash, 1x10^6 cells were incubated for 25 minutes with the fluorescent antibody cocktail, washed, and resuspended into 200 µL of wash buffer (0.5% FBS, 2 mM EDTA, PBS). Whole blood was centrifuged, serum was removed, the sample was incubated for 15 minutes with fixable LIVE/DEAD™ violet stain, and then washed again. White blood cells (WBCs) were counted, and a volume containing 1x10^6 WBCs were incubated for 25 minutes with the fluorescent antibody cocktail. Red blood cells (RBCs) were lysed using FACS™ Lysing Solution (BD Biosciences), the sample was then washed, fixed in 1% formalin, and resuspended into 200 µL of wash buffer.

Data Acquisition

A minimum of 100k events were acquired on a 7-laser CellStream System in 'fast' mode. The FSC, 375, 405, 488, 561, and 642 nm lasers were set to 100% (SSC, 532, and 730 nm lasers were turned off). Debris was eliminated using a FSC threshold. The channels used for each dye are shown in **Table 2**. Compensation controls for each fluorescent antibody were prepared using antibody-capture compensation beads and 5,000 events were collected for each compensation control. Compensation was calculated using CellStream Analysis Software, and data was analyzed using FCS Express 6 to demonstrate compatibility between the CellStream System and third-party software platforms.

		Excitation			
		375/642	405/SSC	488/730	FSC/532/561
Emission	456/51	Live/Dead	BV421		FSC
	528/46	BUV496	V500	AF488	
	583/24		BV570		PE
	611/31		BV605		PECF594
	702/87	APC-R700	BV650	BB700	PECy5
	773/56	APCCy7	BV785		PECy7

Table 2. Amnis® CellStream® channels used for 16-color panel.

Results

Figure 1 shows the gating strategy to identify major lymphoid and myeloid subsets within human PBMCs (**i**) and human whole blood (**ii**). Single WBCs were selected using FSC vs. FSC Aspect Ratio (the gate coordinates were determined by backgating the lymphocyte population) (**A**), followed by live cell selection (Live/Dead Violet-) (**B**). From the live cell population, a plot of CD3 vs. CD20 identified B cells (CD3-/CD20+), T cells (CD3+/CD20-), and non-B/T cells (CD3-/CD20-) (**C**). Non-B/T cells were subset using HLADR vs. CD14 into monocytes (CD14+), dendritic cells (DCs: CD14-/HLADR+), and remaining granulocyte/natural killer cells (Gr/NK: CD14-/HLADR-) (**D**). A CD56 vs. CD123 bivariate was then used to resolve basophils (CD56-, CD123+) and natural killer cells (NK: CD56+/CD123-) (**E**). NK cells were further subset using CD56 vs. CD16 into 16+NK, 56^{lo}16^{lo}NKs, and 56^{hi}15^{lo}NKs (**F**). Monocytes (MO) were further classified using CD16 vs. HLADR into classical MO (HLADR^{hi}/CD16^{lo}), non-classical MO (HLADR^{hi}/CD16^{hi}), and the subset containing MDSCs (HLADR^{lo}/CD16^{lo}) (**G**). Finally, CD11c vs. CD123 was used to differentiate DCs into plasmacytoid DCs (pDCs: CD11c-/CD123+) and myeloid DCs (mDCs: CD11c+/CD123-) (**H**).

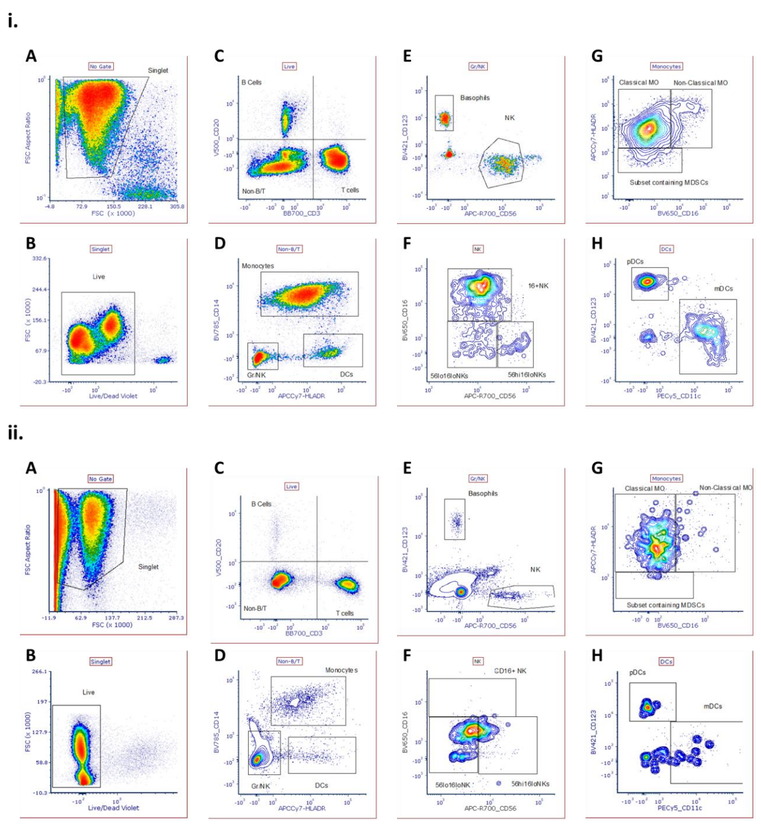

Figure 1. Identification of lymphoid and myeloid populations within human PBMCs (i) and whole blood (ii).

Figure 2 shows the gating strategy to identify T cell subsets within human PBMCs (**i**) and human whole blood (**ii**). T cells (CD3+/CD20-) were identified (**A**) and phenotyped as CD4 T-helper cells (CD4+/CD8-) and CD8 Cytotoxic T cells (CD4-/CD8+) (**B**). A plot of CD127 vs. CD25 was used to identify CD4 T-regulatory cells (Treg: CD127-/CD25+) (**C**), while CD38 vs. HLADR was used to identify activated CD4 cells (CD38+/HLADR+) and non-activated CD38hi CD4 cells (CD38+/HLADR-) (**D**). CCR7 vs. CD45RA characterized CD4 (**E**) and CD8 (**F**) as naïve (Tnaive: CCR7+/CD45RA+), effector (Teff: CCR7-/CD45RA+), central memory (Tcm: CCR7+/CD45RA-), and effector memory (Teff: CCR7-/CD45RA-).

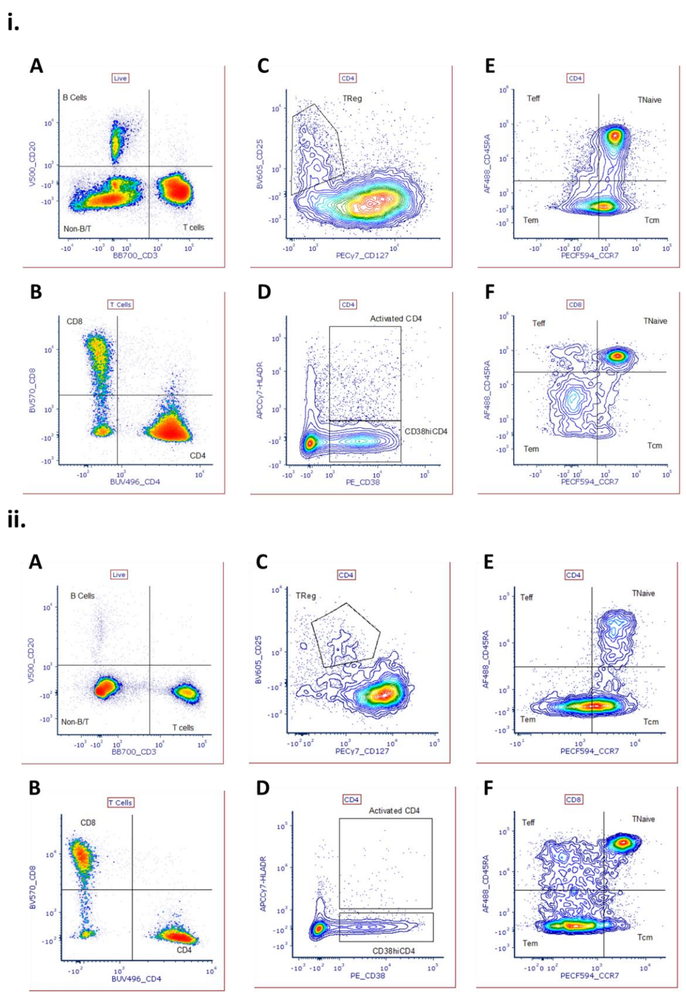

Figure 2. Identification of T cell subsets within PBMC (**i**) and whole blood (**ii**).

Conclusions

In this study, the CellStream Flow Cytometer was used to immunophenotype human PBMCs and whole blood for major lymphoid, myeloid, and T cell subsets using a 16-color panel. All expected populations were resolved, including populations identified by dim markers such as T-regulatory cells.

The CCD-TDI detector did not impact the appearance of flow cytometric data compared to traditional PMT detectors, allowing the CellStream Platform to be easily adopted by flow users. In summary, the CellStream Flow Cytometer offers high sensitivity combined with high-parameter capability, making it an excellent platform for deep immunophenotyping and resolution of low-expression antigens.

References

1. Mahnke Y, Chattopadhyay P, Roederer M. Publication of optimized multicolor immunofluorescence panels. Cytometry A 2010;77A:814-818.
2. Staser K, Eades W, Choi J, Karpova D, DiPersio J. OMIP-042: 21-color flow cytometry to comprehensively immunophenotype major lymphocyte and myeloid subsets in human peripheral blood. Cytometry A. 2018;93(2):186-189.