T lymphocyte immunophenotyping

14-color flow cytometry panel featuring Super Bright antibody conjugates.

Here we present a 14-color immunophenotyping panel used to characterize human T lymphocytes. This analysis takes advantage of the diverse collection of fluorescent antibodies available, including those labeled with Invitrogen™ eBioscience™ Super Bright polymer dyes, a suite of exceptionally bright fluorophores excited by the violet laser. Optimized for use in flow cytometry, the Super Bright dyes allow for expanded use of violet laser excitation, promote streamlined multicolor panel design, and enable detection of low-abundance cell targets.

Recently expanded to include 6 fluorescence detectors for the violet laser (405 nm), the Invitrogen™ Attune™ NxT Flow Cytometer is capable of detecting multiple Super Bright dyes in a single immunophenotyping panel, greatly expanding the number of fluorescent parameters that can be simultaneously measured. The unique acoustic-assisted hydrodynamic focusing technology of the Attune NxT cytometer enables rapid, accurate, and sensitive detection at high flow rates without compromising data quality, as demonstrated with this 14-color data set (Figure 1).

Summary of methods and panel development

Human peripheral blood mononuclear cells (PBMCs) were prepared from whole blood using density gradient separation, and then stimulated by treating with 5 µg/mL concanavalin A for 3 days. Unstimulated cells were also tested, and fluorescence-minus-one (FMO) controls were prepared for marker boundary placement [1]. Cell samples were labeled with the optimal titer for each antibody conjugate, and single-color compensation controls were prepared using the Invitrogen™ AbC™ Total Antibody Compensation Kit for antibody conjugate compensation and Invitrogen™ ArC™ Amine-Reactive Compensation Bead Kit for compensation of the Invitrogen™ LIVE/DEAD™ Fixable Dead Cell Stain.

Table 1 shows the specifications for the 14-color T cell immunophenotyping panel, including the Attune NxT V6 instrument configuration and the panel reagents. The T cell phenotypic markers used in this panel identify differentiation profiles, activation, exhaustion status, and co-stimulatory activity. Instead of using conventional intracellular markers

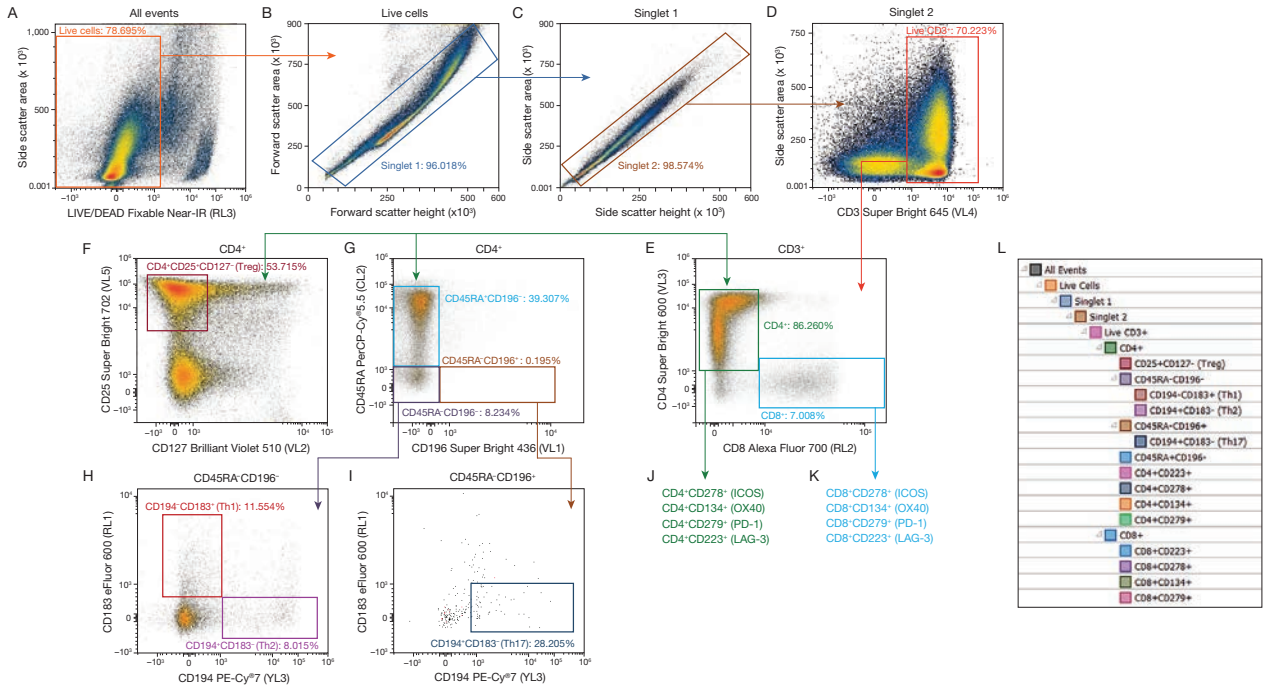

Figure 1. Gating strategy for the 14-color T lymphocyte immunophenotyping panel using the Attune NxT V6 Flow Cytometer. A region is placed around live peripheral blood mononuclear cells (PBMCs), as identified by the Invitrogen™ LIVE/DEAD™ Fixable Near-IR Dead Cell Stain **(A)**. Live cells are analyzed through sequential singlet gating **(B, C)**, and the resulting CD3⁺ population **(D)** is further delineated by gating on CD4⁺ and CD8⁺ populations **(E)**. The CD4⁺ population is used to gate on CD25 vs. CD127 **(F)**, CD45RA vs. CD196 **(G)**, and CD278, CD134, CD279, and CD223 populations (data not shown) **(J)**. The CD45RA⁻CD196⁻ population from **(G)** is gated on CD183 vs. CD194 **(H)**. The CD45RA⁻CD196⁺ population from **(G)** is gated on CD183 vs. CD194 **(I)**. The CD8⁺ population from **(E)** is used for gating CD278, CD134, CD279, and CD223 populations (data not shown) **(K)**. The entire gating strategy is displayed in hierarchical format using the Invitrogen™ Attune™ NxT V6 v2.6 software for easy visualization **(L)**.

for cytokines (e.g., IFN-γ) or transcription master regulators (e.g., T-bet), only surface markers were selected for this panel so that fixation and permeabilization steps were not required. This streamlined and shortened the sample preparation process, while also reducing cell loss due to multiple wash steps. For example, to detect T helper 1 (Th1), Th2, and Th17 subpopulations, we used the surface markers CD45RA, CD196 (CCR6), CD183 (CXCR3), and CD194 (CCR4) [2]. Likewise, T regulatory (Treg) cells, often detected with the surface marker CD25 and the intracellular marker FoxP3, were instead identified using only surface markers CD25 and CD127 [3,4]. Upon activation or in diseases such as hematologic malignancies, T cells up-regulate expression of activation markers such as CD25, CD134 (OX40), and CD278 (ICOS, inducible co-stimulator). Expression of CD279 (PD-1), an immune checkpoint receptor, is used to identify T cells as exhausted or quiescent. Another immune checkpoint receptor, CD223 (LAG-3), negatively regulates cellular proliferation, activation, and homeostasis of T cells, and plays a role in Treg suppressive activity [5]. Table 2 lists the T cell subpopulations and corresponding immunophenotypes identified by this panel.

Table 1. Specifications for the 14-color T cell immunophenotyping panel, including the Attune NxT V6 instrument configuration and the panel reagents (antibody target, clone, and fluorophore).

Laser	Attune NxT V6 detector	Bandpass filter (nm)	Target	Clone	Fluorophore	Cat. No.
Blue 488 nm	BL1	530/30	CD134 (OX40)	ACT-35	FITC	11-1347-42
	BL2	695/40	CD45RA	HI100	PerCP-Cy®5.5	45-0458-42
Red 637 nm	RL1	670/14	CD183 (CXCR3)	CEW33D	eFluor 660	50-1839-42
	RL2	720/30	CD8	RPA-T8	Alexa Fluor 700	56-0088-42
	RL3	780/60	Viability	NA	LIVE/DEAD Fixable Near-IR	L34976
Violet 405 nm	VL1	450/40	CD196 (CCR6)	R6H1	Super Bright 436	62-1969-41
	VL2	525/50	CD127	A019D5	Brilliant Violet 510	NA
	VL3	610/20	CD4	SK3	Super Bright 600	63-0047-42
	VL4	660/20	CD3	OKT3	Super Bright 645	64-0037-42
	VL5	710/50	CD25	BC96	Super Bright 702	67-0259-42
	VL6	780/60	CD279 (PD-1)	EH12.1	Brilliant Violet 786	NA
Yellow 561 nm	YL1	585/22	CD278 (ICOS)	ISA-3	PE	12-9948-42
	YL2	620/15	CD223 (LAG-3)	3DS223H	PE-eFluor 610	61-2239-42
	YL3	780/60	CD194 (CCR4)	1G1	PE-Cy®7	NA

Table 2. Summary of results from the 14-color T cell immunophenotyping panel.

		Percent (%) positive cells after 3-day stimulation with control or concanavalin A	
Immunophenotype	T cell subpopulation*	Unstimulated PBMCs	Stimulated PBMCs
$CD3^+CD4^+CD45RA^-CD196^-CD183^+CD194^-$	Th1 cells	16.74	10.88
$CD3^+CD4^+CD45RA^-CD196^-CD183^-CD194^+$	Th2 cells	3.79	6.43
$CD3^+CD4^+CD45RA^-CD196^+CD183^-CD194^+$	Th17 cells	18.79	28.21
$CD3^+CD4^+CD25^+CD127^-$	Treg cells	0.92	51.63
$CD3^+CD4^+CD223^+$	LAG-3+ exhausted Th cells	0.79	39.84
$CD3^+CD8^+CD223^+$	LAG-3+ exhausted Tc cells	0.17	37.30
$CD3^+CD4^+CD134^+$	OX40+ activated Th cells	1.08	21.26
$CD3^+CD8^+CD134^+$	OX40+ activated Tc cells	0.23	11.16
$CD3^+CD4^+CD278^+$	ICOS+ activated Th cells	0.27	18.65
$CD3^+CD8^+CD278^+$	ICOS+ activated Tc cells	0.76	14.59
$CD3^+CD4^+CD279^+$	PD-1+ exhausted/quiescent Th cells	1.36	16.12
$CD3^+CD8^+CD279^+$	PD-1+ exhausted/quiescent Tc cells	1.24	13.17

*Th = T helper cells. Treg = T regulatory cells. Tc = T cytotoxic cells.

Data acquisition and analysis

Samples were acquired on the recently introduced 4-laser Attune NxT V6 Flow Cytometer, which has the ability to detect 6 violet laser–excited fluorophores, with a flow rate of 200 µL/minute. Data were analyzed using Attune NxT v2.6 software. Corrections for spectral overlap were performed using standard instrument auto-compensation procedures. The results show well-characterized cell populations, with the expected differences between unstimulated and stimulated PBMCs *ex vivo* (Table 2).

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References

1. Maecker HT, Trotter J (2006) *Cytometry A* 69:1037–1042.
2. Gosselin A, Monteiro P, Chomont N et al. (2010) *J Immunol* 184:1604–1616.
3. Sakaguchi S, Miyara M, Costantino CM et al. (2010) *Nat Rev Immunol* 10:490-500.
4. Yu N, Li X, Song W et al. (2012) *Inflammation* 35:1773–1780.
5. Huang CT, Workman CJ, Flies D et al. (2004) *Immunity* 21:503–513.