T Lymphocyte Immunophenotyping: 14-Color Flow Cytometry Panel Design Using the Attune NxT Flow Cytometer and Super Bright Fluorescent Dyes

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Abstract

The Invitrogen™ eBioscience™ Super Bright polymer dyes represent a suite of bright fluorophores excited by the violet laser (405 nm). Optimized for use in flow cytometry, the Super Bright dyes allow for expanded use of violet laser excitation and promote streamlined multicolor panel design. Due to their inherent brightness, detection of cell populations with low abundance targets is possible. A 14-color panel used to characterize human T-cell lymphocytes is presented, with information on differentiation profiles, activation or exhaustion status, and co-stimulation activity. Human peripheral blood mononuclear cells (PBMC) collected from a normal donor was tested in stimulated and unstimulated cells. Acquisition and analysis was performed using the Invitrogen™ Attune™ NxT Flow Cytometer four laser system with V6 configuration, which has the ability to detect six violet-excited fluorophores. The results show well characterized cell populations, with expected changes between unstimulated and stimulated PBMC *ex vivo*. Correction for spectral overlap was performed using standard instrument auto-compensation procedures. The unique acoustic focusing technology of Attune NxT Flow Cytometer enables rapid and accurate detection with sensitivity at high flow rates without compromise of data quality, as demonstrated with this 14-color data set. The Attune NxT Flow Cytometer with V6 configuration is designed to accommodate a wide variety of experimental conditions and combined with the Super Bright dyes provides expanded choice for panel design.

Background

This 14-color panel comprehensively characterizes T-cell phenotypic markers that identify differentiation profiles, activation, exhaustion status, and co-stimulatory activity. Instead of using the conventional intracellular markers for cytokines (e.g., IFNγ) and/or transcription master regulator (e.g., T-bet), only surface markers are used. To detect T-helper1 (T_h1) and the subsets T_h2 and T_h17, we used the surface markers: CD45RA, CD196 (CCR6), CD183 (CXCR3), and CD194 (CCR4) to identify these three populations.[1] By eliminating the need for fixation and permeabilization procedures, the sample preparation is simplified and streamlined, the sample process time is shortened, and cell loss due to multiple wash steps is reduced.

Regulatory T cells (Treg) play a crucial role in the maintenance of self-tolerance, and alterations in their frequencies can often indicate autoimmune, allergic and/or inflammatory reactions. The intracellular marker FoxP3 can be used together with CD25 to identify Treg population ($CD3^+CD4^+CD25^+FoxP3^+$i.c.).[2] It has recently been reported that surface markers CD25 and CD127 can be used to detect the Treg population ($CD3^+CD4^+CD25^+CD127^-$)[3], simplifying and streamlining sample preparation.

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), the inducible co-stimulator. Interaction of CD279 (PD-1) and its ligands results in inhibition of T cell proliferation and cytokine secretion. CD279 is an immunoregulatory (checkpoint) receptor expressed on T cells, some B cells and myeloid cells. CD279 expression is used to identify T cells as exhausted and/or quiescent. CD223 (LAG-3) is an immune checkpoint receptor, and negatively regulates cellular proliferation, activation, and homeostasis of T cells, and plays a role in Treg suppressive activity.[4] This panel examines the expression of CD134, CD278, CD279, and CD223 in a T cell panel in stimulated PBMC as compared to unstimulated PBMC from the same normal donor.

Method

PBMCs were prepared from whole blood using density gradient separation, and stimulated using two different methods:

Method 1) Dynabeads™ Human T-Activator CD3/CD28 for a 5-day stimulation, with unstimulated cells and a whole blood sample also tested. To aid in gating cell populations, isotpye controls were prepared. For method 1, two Super Bright antibody conjugates were used in the panel.

Method 2) Concanavalin A at 5 µg/mL for a 3-day stimulation, with unstimulated cells also tested. Fluorescence-Minus-One (FMO) controls were prepared for marker boundary placement.[5] For method 2, four Super Bright antibody conjugates were used in the panel.

Both methods used the same targets and antibody clones. Samples were labeled with the optimal titer for each conjugate, using 5µl Super Bright Staining Buffer per sample. Single color compensation controls were prepared using the AbC™ Total Antibody Compensation and ArC™ Amine-Reactive Compensation Bead kits. Correction for spectral overlap was performed using standard instrument auto-compensation procedures.

Samples were acquired on the Attune NxT V6 instrument at 200 µl/minute flow rate. Data were analyzed using the Attune NxT V6 v2.6 software, gating strategy and comparison of stimulated and unstimulated cells was performed for each panel.

Conclusion

The results show well characterized cell populations, with expected changes between unstimulated and stimulated PBMC *ex vivo* in the increased percentage of $CD3^+CD4^+CD25^+CD127^-$ Treg population as well as expression of Th1, Th2, Th17 populations, and activation/exhaustion of immunoregulatory populations. The Attune NxT with V6 configuration along with the Super Bright antibody conjugates expand the utility of the violet laser, and allow for streamlined panel design.

References

1. J Immunol. 2010 Feb1;184(3):1604–1616
2. Nat Rev Immunol. 2010 Jul;10(7):490-500
3. Inflammation. 2012 Dec;35(6):1773-80
4. Immunity. 2004 Oct;21(4):503-13
5. Cyto A. 2006; 69:1037-1042

Laser	Attune NxT V6 Detector	Bandpass	Target	Clone	fluorophore
Blue 488 nm	BL1	530/30	CD134 (OX40)	ACT-35	FITC
	BL2	695/40	CD45RA	HI100	PerCP-Cy5.5
Red 637 nm	RL1	670/14	CD183 (CXCR3)	CEW33D	eFluor 660
	RL2	720/30	CD8	RPA-T8	Alexa Fluor 700
	RL3	780/60	viability	NA	L/D Fixable Near-IR
Violet 405 nm	VL1	450/40	CD196 (CCR6)	R6H1	Super Bright 436
	VL2	525/50	CD127	A019D5	Brilliant Violet 510
	VL3	610/20	CD4	SK3	Super Bright 600
	VL4	660/20	CD25 *method 1	BC96	Brilliant Violet 650
			CD3 *method 2	OKT3	Super Bright 645
	VL5	710/50	CD3 *method 1	OKT3	Brilliant Violet 711
			CD25 *method 2	BC96	Super Bright 702
	VL6	780/60	CD279 (PD-1)	EH12.1	Brilliant Violet 786
Yellow 561 nm	YL1	585/22	CD278 (ICOS)	ISA-3	rPE
	YL2	620/15	CD223 (LAG-3)	3DS223H	PE-eF 610
	YL3	780/60	CD194 (CCR4)	1G1	PE-Cy7

Figure 1. Attune NxT V6 instrument configuration with multicolor panel showing antibody target, clone and fluorophore. Method 1 utilizes two Super Bright and four Brilliant Violet antibody conjugates, while Method 2 utilizes four Super Bright and two Brilliant Violet antibody conjugates.

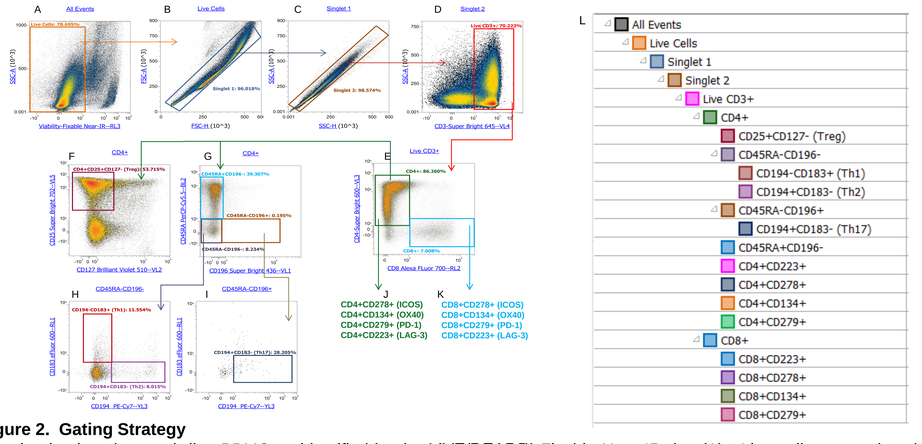
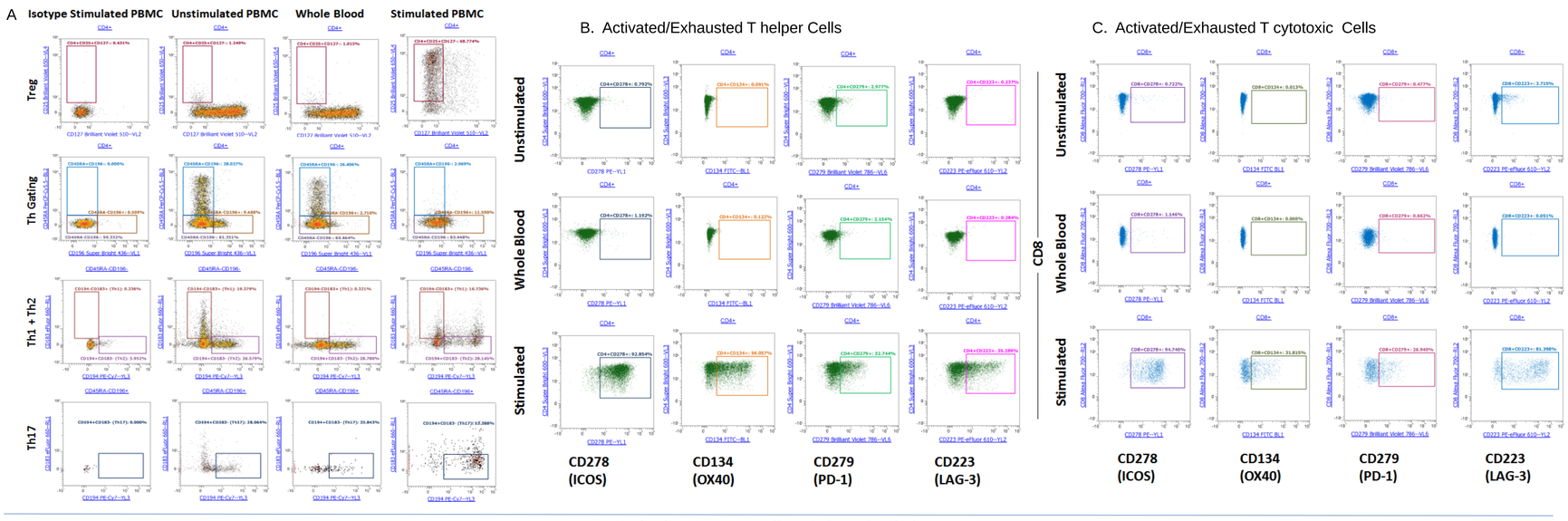


Figure 2. Gating Strategy
A region is placed around live PBMC as identified by the LIVE/DEAD™ Fixable Near-IR dye (A) . Live cells are analyzed through sequential singlet gating (B,C). A region is then placed on the CD3+ population (D) for gating on CD4+ and CD8+ populations (E). The CD4+ population is used to gate on CD127 vs. CD25 (F), for CD45RA vs. CD196 (G) and CD278, CD134, CD279 and CD223 populations (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 (K). The entire gating strategy is displayed in hierarchical format using the Attune NxT V6 v2.6 software for easy visualization (L).



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		CD3/CD28 DynaBeads 5 day Stimulation		Concanavalin A 3 day Stimulation	
Immunophenotype	Population Significance	Unstimulated PBMC % positive	Stimulated PBMC % positive	Unstimulated PBMC % positive	Stimulated PBMC % positive
CD3+CD4+CD45RA-CD196-CD183+CD194-	T_h1 cells	19.28	16.74	16.74	10.88
CD3+CD4+CD45RA-CD196-CD183-CD194+	T_h2 cells	26.58	28.15	3.79	6.43
CD3+CD4+CD45RA-CD196+CD183-CD194+	T_h17 cells	28.06	53.39	18.79	28.21
CD3+CD4+CD25+CD127-	T_{reg} cells	1.25	68.77	0.92	51.63
CD3+CD4+CD223+	LAG-3+ exhausted T_h cells	0.24	35.29	0.79	39.84
CD3+CD8+CD223+	LAG-3+ exhausted T_c cells	3.72	81.4	0.17	37.3
CD3+CD4+CD134+	OX-40+ activated T_h cells	0.09	56.06	1.08	21.26
CD3+CD8+CD134+	OX-40+ activated T_c cells	0.01	31.82	0.23	11.16
CD3+CD4+CD278+	ICOS+ activated T_h cells	0.79	92.85	0.27	18.65
CD3+CD8+CD278+	ICOS+ activated T_c cells	0.72	94.74	0.76	14.59
CD3+CD4+CD279+	PD-1+ exhausted/quiescent T_h cells	2.98	32.74	1.36	16.12
CD3+CD8+CD279+	PD-1+ exhausted/quiescent T_c cells	0.48	26.94	1.24	13.17

Figure 3. Using the gating strategy outlined in figure 2, dual parameter plots are shown for Unstimulated and Stimulated PBMC across both methods: Treg, Th1, Th2, and Th17 populations (A, D), Activated or Exhausted T Helper Populations (B,E) and Activated or Exhausted T Cytotoxic Populations (C,F).
The percent positive of the unstimulated and stimulated populations are summarized (G) for each of the populations examined.