Signaling flow cytometry: One-step staining for phenotyping and functional characterization of immune cells

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INTRODUCTION

Understanding how signaling pathways in immune cells are modulated by various perturbations can reveal potential therapeutic targets and uncover mechanisms of therapeutic response. Immune cells are typically observed in the context of a heterogeneous mixture of cells, and techniques that enable single cell analysis, such as flow cytometry, are essential. However, observing changes in pathway activation by flow cytometry can be challenging as antibodies used for immune phenotyping are generally validated in live cell assays, while antibodies targeting signaling readouts usually require fixation and permeabilization. Fixation and/or permeabilization can impact the functionality of antibodies that are normally used on live cells (Fig. 1), so identifying antibodies that maintain functionality on fixed/permeabilized samples is critical for enabling single-step immunostaining.

METHODS

To simplify multiplexing with signaling and phenotyping readouts, we tested many commonly used immune phenotyping antibodies in live cell flow vs. multiple fixation and permeabilization protocols. We compiled the data into a Protocol Compatibility Table (Table 1) to enable design of flow cytometry panels that include both phenotyping and signaling antibodies used as a single staining antibody mix on cells following fixation and permeabilization. To illustrate this functionality, we designed and generated data for two different panels on fixed, permeabilized cells. One panel was designed to enable observation of signaling pathway activation downstream of the B cell receptor and included the antibody conjugates listed in Table 2. This panel was tested as a single staining mix following 4% formaldehyde fixation and 100% methanol permeabilization of human PBMCs untreated or treated with anti-human IgM (20 µg/mL, 5 min). Another panel was designed to enable observation of STING activation among myeloid cell subsets and included the antibodies listed in Table 3. This panel was tested as a single staining mix following 4% formaldehyde fixation and 0.3% Triton™ X-100 permeabilization of human PBMCs untransfected or transfected with 2',3'-cGAMP (sodium salt) (10 µg/mL, 3 hr).

CONCLUSIONS

We observed that fixation and permeabilization of cells differentially impacts performance of antibody clones. However, we were able to identify multiple antibody clones for established phenotyping markers that perform effectively following various fixation and permeabilization methods. Antibody panels that incorporate both phenotyping antibodies targeting extracellular epitopes and antibodies targeting intracellular signaling readouts may be used as a single staining mix following fixation and permeabilization. With one panel, we were able to quantify phospho-Syk (Tyr525/Tyr526) and phospho-CD79A (Tyr182) at the single cell level in primary human B cells. With the other panel, we were able to observe differences in STING activation between populations of primary human myeloid cells. This study demonstrates that understanding antibody protocol compatibility can alleviate technical hurdles encountered when combining signaling readouts and phenotyping markers and enable design of a flow cytometry experiment with a simplified protocol.

REFERENCES

www.cst-science.com/FlowTable

Table 1: Antibody Protocol Compatibility

Antibody Clone Information			Protocol Compatibility Testing				Available Conjugates (Emission spectra (nm) listed above fluorophore name)															
							Violet Laser		Blue Laser							Red Laser						
			Protocols				450	455	520	520	578	667	678	695	785	660	668	710	719			
Antibody	Epitope Location	Species Reactivity	Live	Fix, Meth Perm	Fix, Triton Perm	FoxP3 Fix Perm	violet Fluor 450	Pacific Blue™	FITC	Alexa Fluor® 488	PE	PE-Cy®5	PerCP	PerCP-Cy®5.5	PE-Cy®7	APC	Alexa Fluor® 647	red Fluor 710	Alexa Fluor® 700			
Aiolos (D1C1E)	IC	H, M	●	●	●	●				94568	21799						10896		59612			
Phospho-Akt (Ser473) (193H12) Rabbit mAb	IC	H, M, R	●	●	●	●				2336							2337					
Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb	IC	H, M, R, Hm, Mk, Dm, Z, B	●	●	●	●				4071	5315				88106	11962	4075					
Phospho-Akt (Thr308) (D25E6)	IC	H, M, R, Mk	●	●	●	○				43506	13842						48646					
BCL6 (D4I2V)	IC	H	●	●	●	○				26556	84882						41194					
Bcl-xL (54H6)	IC	H, M, R, Mk	●	●	●	●				2767	13835				81965	12099	86387					
Cleaved Caspase-3 (Asp175) (D3E9)	IC	H, M	●	●	●	●		8788		9603	12768						9602					
CD3 (17A2)	EC	M	●	●	●	●	38527		86603		28306					24265		26582				
CD3 (UCHT1)	EC	H	●	●	●	●	61347		86774		46233			31767	62670	19881		44330				
CD4 (RM4-5)	EC	M	●	●	●	●	92599		96127		26589			49482	38341	82116		75508				
CD4 (RPA-T4)	EC	H	●	●	●	●	26755		48705		55927			46699	66182	78292						
CD8α (2.43)	EC	M	●	●	●	●	17083		35467		56984			72461	87922	64786						
CD8α (RPA-T8)	EC	H	●	●	●	●	25109		55397		88829			75003	27442	64915		86135				
CD11b/ITGAM (M1/70)	EC	H, M	●	●	●	●	70078		24442		24965	16538		85601		41249		55274				
CD11c (3.9)	EC	H	●	●	●	●	97473		69627		56025				80342	36268		42847				
CD11c (N418) Hamster mAb	EC		●	●	●	●																
CD14 (61D3)	EC	H	●	●	●	●	82944		29943		59896			99811	44947	36377		64342				
CD16 (3G8)	EC	H	●	●	●	●					82004				23290							
CD19 (1D3)	EC	M	●	●	●	○	54508		53343		82168			43145	27221	39831		72880				
CD19 (Intracellular Domain) (D4V4B)	IC	H, M	●	●	●	○				70418	58960						16344					
CD20 (2H7)	EC	H	●	●	●	●	88560		87491		26137			72374	50665	69077		38268				
CD24 (M1/69)	EC	M	●	●	●	○	9705				90378					68390						
CD25/IL2-Rα (BC96)	EC	H	○	●	●	●									43212	20164						
CD27 (O323)	EC	H	●	●	●	○	70511		35379		55584				66120	42404						
CD28 (CD28.2)	EC	H	●	●	●	●			15666		27826			25544	82076	73906						
CD34 (ICO115) Mouse mAb	EC	H	●	●	○	○					79253											
CD38 (HIT2)	EC	H	●	●	●	●	24713		90005		90775			10284		73977						
CD44 (IM7)	EC	H, M	●	●	●	●	53289		75122		88151	94170		38200	43675	80813		63882				
CD45 (30-F11)	EC	M	●	●	●	●	83090		62307		47742				60943	41104		99856				
CD45 (HI30)	EC	H	●	●	●	●	74292		86532		89492			32125	28418	98228		39170				
CD45RA (HI100)	EC	H	●	●	○	○	72177		23672		96979				78564	37558	97321					
CD45R/B220 (RA3-6B2)	EC	H, M	●	●	●	●	67084			34399		93992	16935		17299	82922	98126		82984			
CD56/NCAM1 (MY31)	EC	H	●	●	●	○			67184			73521				42804	51997					
CD62L/L-Selectin (MEL-14)	EC	M	●	●	●	○			76378			32908			90088	61885	46592		60310			
Phospho-CD79A (Tyr182) (D1B9)	IC	H	●	●	●	○				52821		14948					29742					
CD68 (D4B9C)	EC	H	○	●	●	●				24850		79594										
CD69 (H1.2F3) Hamster mAb	EC	M	●	○	●	○																
CD71 (D7G9X) XP® Rabbit mAb	EC	H	●	●	○	○						82582										
CD74 (D5N3I) XP® Rabbit mAb	EC	H	●	●	●	○				99916		55084										
CD86/B7-2 (GL-1)	EC	M	●	○	○	●			99879			60712				11527	84393					
CD161/NK1.1 (PK136)	EC	M	●	●	●	●			28486			45378			88957	78652	35986		19809			
CD161/KLRB1 (HP-3G10)	EC	H	○	●	●	●	26540		67086			83130			26217		51113					
Phospho-CREB (Ser133) (87G3)	IC	H, M, R	●	●	●	○				9187		14228					14001					
Phospho-CREB (Ser133) (87G3) Rabbit mAb	IC	H, M, R	●	●	●	●				9187		14228					14001					
CTLA-4 (D4E9I)	EC	H	●	●	○	○				15162		15132										
EOMES (D8D1R)	IC	H	●	●	●	●						17984					42582					
FoxP3 (3G3)	IC	M	●	●	●	●						65210				10680	41442					
F4/80 (BM8.1)	EC	M	●	●	○	●	40781		52267			64763			88154	80380	86007		94009			
GATA-3 (D13C9)	IC	H, M	●	●	●	○						13411										
Granzyme B (D2H2F)	IC	H, M	●	●	●	●				33359		65563					50590					
Helios (D8W4X)	IC	H, M	●	●	●	○				56424		29360					65432					
Cleaved PARP (Asp214) (D64E10)	IC	H, Mk	●	●	●	●				9148		8978					6987					
Phospho-PLCγ1 (Tyr783) (D6M9S)	IC	H, M	●	●	●	○				25678		14461					88717					
Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E)	IC	H, M, R, Mk, Mi, Sc	●	●	●	○		8520		4803		5316				34411	14733	4851		27036		
Phospho-S6 Ribosomal Protein (Ser240/244) (D68F8) XP® Rabbit mAb	IC	H, M, R, Mk	●	●	●	●				5018		14236						5044				
Phospho-SLP-76 (Ser376) (D7S1K)	IC	H	●	●	●	○		83768		90247		16318						40887				
Phospho-SLP-76 (Ser376) (E3G9U) XP® Rabbit mAb	IC	H, M	●	●	●	●																
Phospho-Stat1 (Tyr701) (58D6)	IC	H, M	●	●	●	●				9174		8062						8009				
Phospho-Stat2 (Tyr690) (D3P2P)	IC	H, R	●	●	●	●				68826		77366						90740				
Phospho-Stat3 (Tyr705) (D3A7)	IC	H, M, R, Mk	●	●	●	●				4323		8119						4324				
Phospho-Stat3 (Ser727) (D3Q3C)	IC	H	●	●	●	●				58978		87544						71958				
Phospho-Stat4 (Tyr693) (D2E4)	IC	H	●	●	●	●						13223										
Phospho-Stat5 (Tyr694) (C71F5)	IC	H, M	●	●	●	●				3939		5387						9365				
Phospho-Stat5 (Tyr694) (D47E7)	IC	H, M	●	●	●	●						14603										
Phospho-Stat6 (Tyr641) (D8S9Y)	IC	H, M	●	●	●	●						99902						10205				
Phospho-STING (Ser365) (D1C4T)	IC	M	●	●	●	○																
Phospho-STING (Ser366) (D8K6H)	IC	H	●	●	●	○				41622								43499				
Phospho-Syk (Tyr525/526) (C87C1)	IC	H	●	●	●	○				4349		6485						12081				
Phospho-TBK1/NAK (Ser172) (D52C2)	IC	H, M	●	●	●	○				14586		13498						14590				
T-bet/TBX21 (D6N8B)	IC	H	●	●	●	○				14298								14307				
TCF1/TCF7 (C63D9)	IC	H, M	●	●	●	●		9066		6444		14456						6709				
TNF-α (D2D4)	IC	M	●	●	●	●				87509												
Phospho-Zap-70 (Tyr319)/Syk (Tyr352) (65E4)	IC	H, M	●	●	●	○				73382		14791						82975				
Phospho-4E-BP1 (Thr37/46) (236B4)	IC	H, M, R, Mk, Dm	●	●	●	○				2846		7547						5123				

Table Key: IC: Intracellular EC: Extracellular ● Recommended ● Decreased Performance ● Not Recommended ○ Not Tested

Species Reactivity Key: H—Human M—Mouse R—Rat Mk—Monkey B—Bovine Pg—Pig Mi—Mink Dm—D. melanogaster Z—Zebrafish Sc— S. cerevisiae

Figure 1: Protocol Comparison Examples

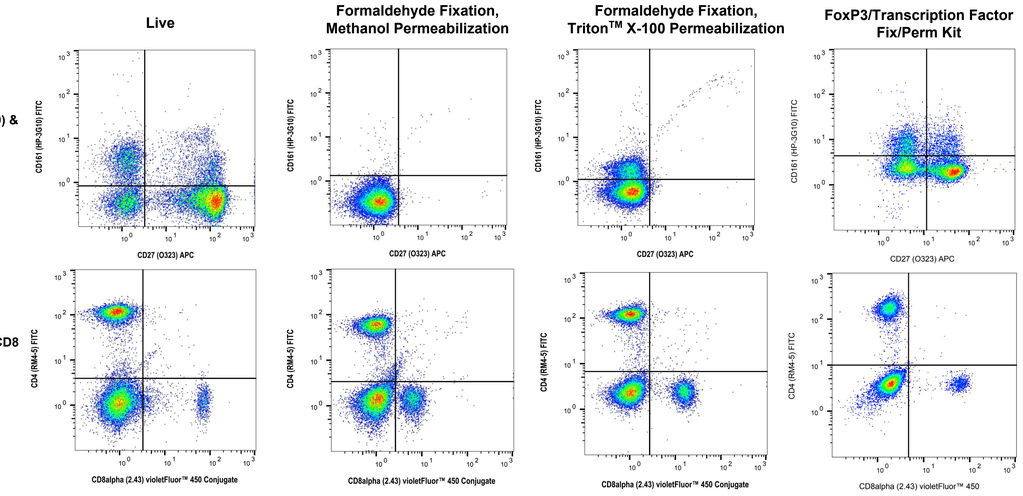

Figure 1: Flow cytometric analysis of human PBMCs (top row) or mouse splenocytes (bottom row) stained with various CD marker clones. Cells were stained either as live cells or following fixation/permeabilization with formaldehyde/methanol, formaldehyde/Triton™ X-100, or the FoxP3/Transcription Factor Fixation/Permeabilization Kit.

Table 2: B Cell Signaling Panel Antibody Selection

							Violet Laser		Blue Laser							Red Laser						
			Protocols				450	455	520	520	578	667	678	695	785	660	668	710	719			
Antibody	Epitope Location	Species Reactivity	Live	Fix, Meth Perm	Fix, Triton Perm	FoxP3 Fix Perm	violet Fluor 450	Pacific Blue™	FITC	Alexa Fluor® 488	PE	PE-Cy®5	PerCP	PerCP-Cy®5.5	PE-Cy®7	APC	Alexa Fluor® 647	red Fluor 710	Alexa Fluor® 700			
CD45 (HI30)	EC	H	●	●	●	●	74292		86532		89492			32125	28418	98228		39170				
CD19 (Intracellular Domain) (D4V4B)	IC	H, M	●	●	●	○				70418	58960						16344					
Phospho-CD79A (Tyr182) (D1B9)	IC	H	●	●	●	○				52821	14948						29742					
Phospho-Syk (Tyr525/526) (C87C1)	IC	H	●	●	●	○				4349	6485						12081					

Figure 2: B Cell Signaling Panel Results

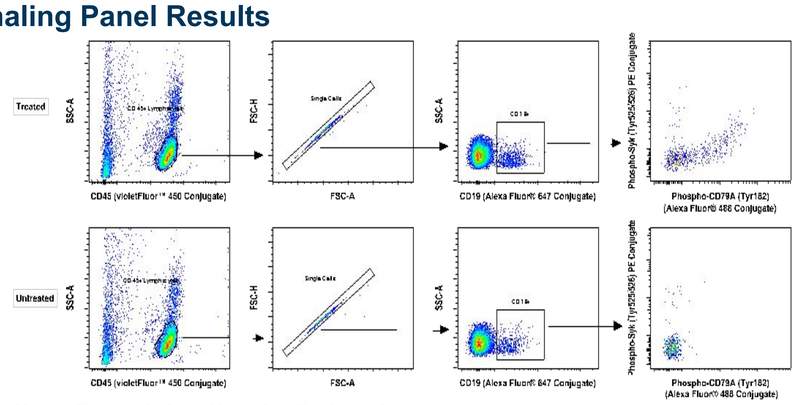

Figure 2: Analysis of human PBMCs treated with anti-human IgM (20 µg/mL, 5 min; top row) or untreated (bottom row), fixed with 4% formaldehyde and permeabilized with 100% methanol, and stained after fixation and permeabilization with the panel of antibody conjugates in Table 2.

Table 3: STING Signaling Panel Antibody Selection

							Violet Laser		Blue Laser							Red Laser						
			Protocols				450	455	520	520	578	667	678	695	785	660	668	710	719			
Antibody	Epitope Location	Species Reactivity	Live	Fix, Meth Perm	Fix, Triton Perm	FoxP3 Fix Perm	violet Fluor 450	Pacific Blue™	FITC	Alexa Fluor® 488	PE	PE-Cy®5	PerCP	PerCP-Cy®5.5	PE-Cy®7	APC	Alexa Fluor® 647	red Fluor 710	Alexa Fluor® 700			
CD45 (HI30)	EC	H	●	●	●	●	74292		86532		89492			32125	28418	98228		39170				
CD11b/ITGAM (M1/70)	EC	H, M	●	●	●	●	70078		24442		24965	16538		85601		41249		55274				
HLA-DR (L243)	EC	H	●	●	●	●			54126		78397		17634					46861				
CD14 (61D3)	EC	H	●	●	●	●	82944		29943		59896			99811	44947	36377		64342				
CD16 (3G8)	EC	H	●	●	●	●					82004				23290							
Phospho-STING (Ser366) (D8K6H)	IC	H	●	●	●	○				41622							43499					

Figure 3: STING Signaling Panel Results

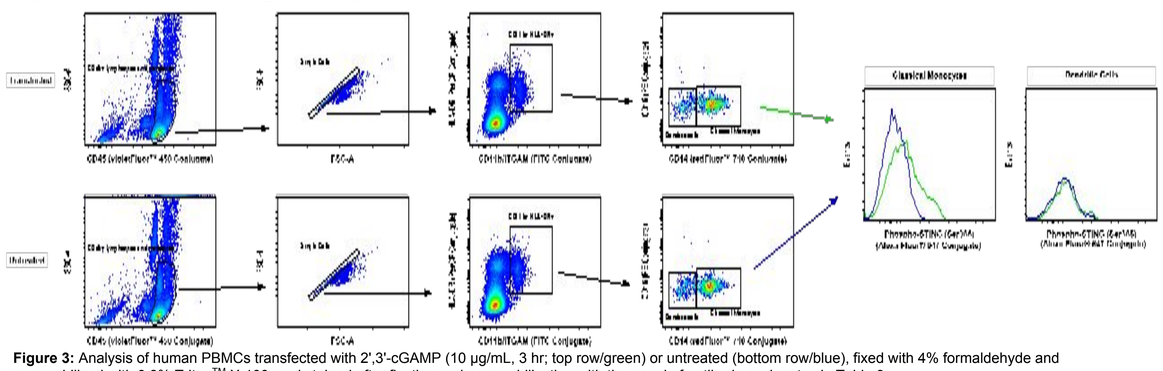

Figure 3: Analysis of human PBMCs transfected with 2',3'-cGAMP (10 µg/mL, 3 hr; top row/green) or untreated (bottom row/blue), fixed with 4% formaldehyde and permeabilized with 0.3% Triton™ X-100, and stained after fixation and permeabilization with the panel of antibody conjugates in Table 3.

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