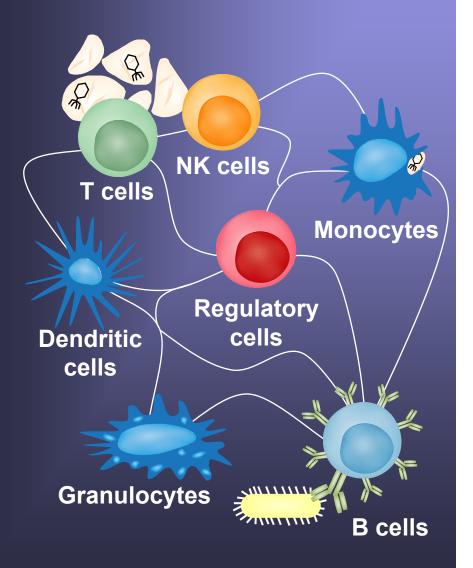


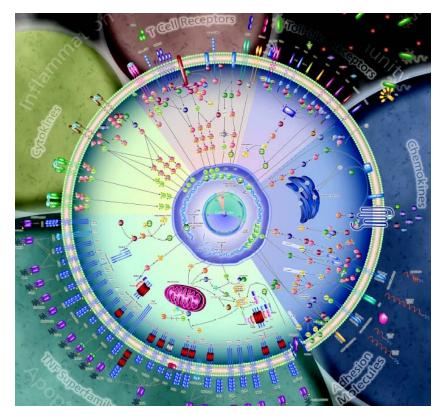
live healthy lives

Analyzing Protein Phosphorylation Pathways in Heterogeneous Samples

Erika O'Donnell Cell Signaling Research BD Biosciences, San Diego, CA December 1, 2011

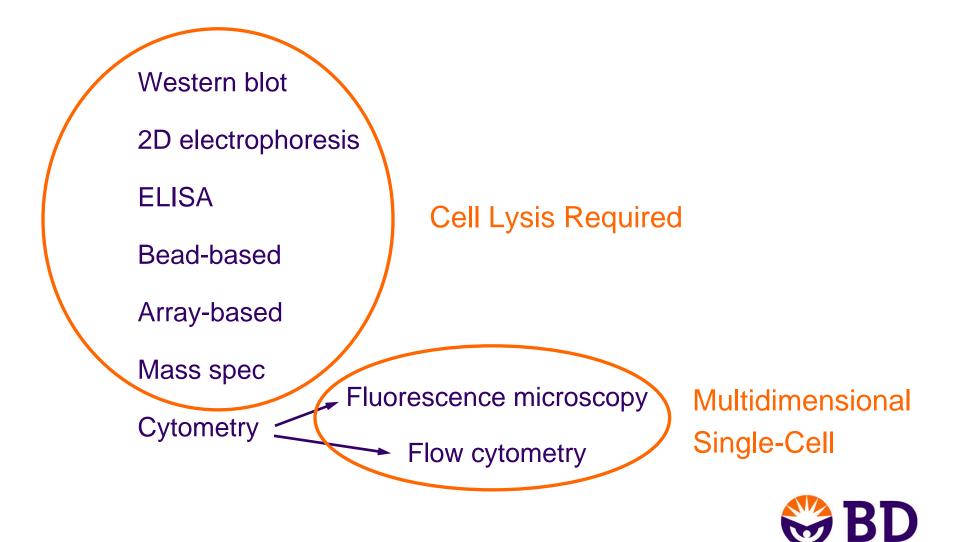
A Critical Role for Cell Signaling in Communication within the Immune System







Many Tools Are Available Today for Studying Signal Transduction

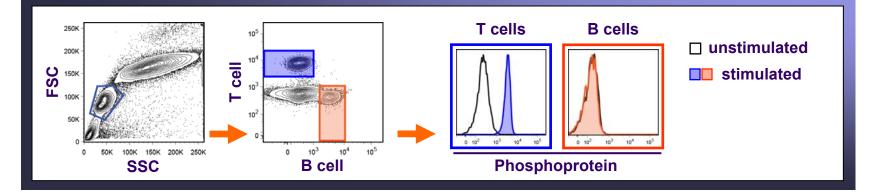


Many Tools Are Available Today for Studying Signal Transduction

Fluorescence Microscopy

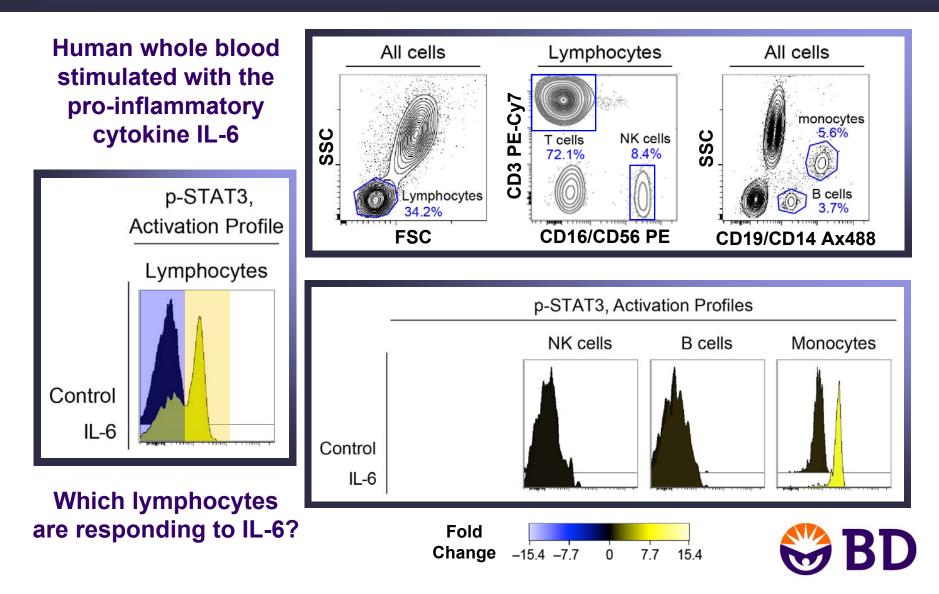


Flow Cytometry

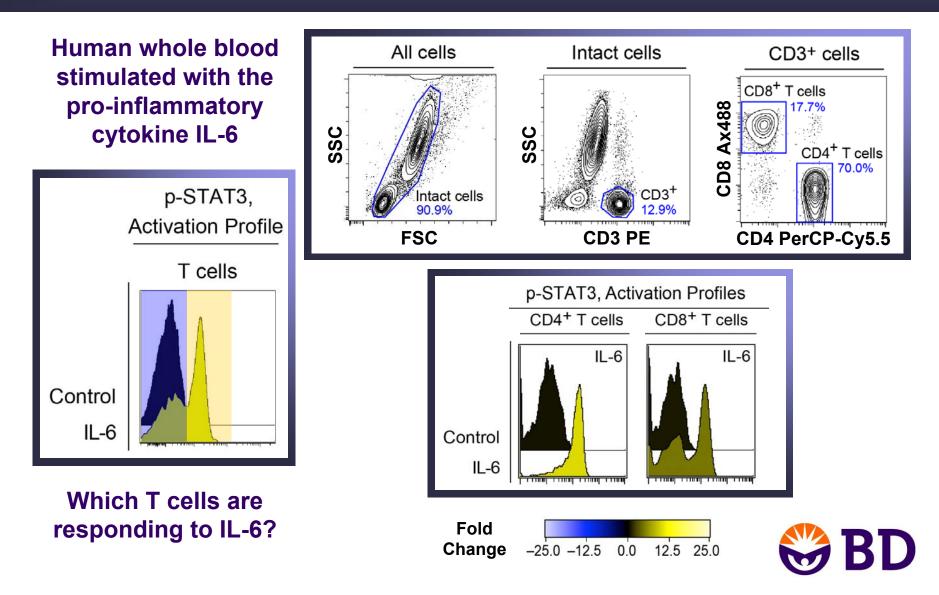




Monocyte / NK Cell Activation Kit: Signaling Responses in Human Leucocyte Subsets



T Cell Activation Kit: Measuring Signaling Responses in CD4 and CD8 T Cells



Standard Protocol for Analyzing Protein Phosphorylation by Flow Cytometry

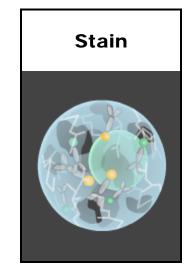


 Permeabilize

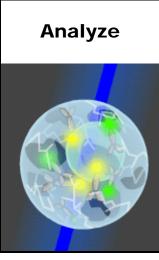
Step 1 Stimulate cells (optional) and fix to preserve phosphorylation states

Step 2 Permeabilize cells to allow antibody access to cytoplasm and nucleus

If working with whole blood, spleen, or other erythrocyte-containing samples, RBC lysis can be performed during fixation using BD Phosflow™ Lyse/Fix Buffer



Step 3 Stain cells with fluorescently conjugated antibodies against phosphoepitopes, surface markers, and other targets of interest



Step 4 Analyze cells on a properly set up flow cytometer



Elements Required for Successful Analysis of Cell Signaling at the Single-Cell Level

- Phospho-specific antibodies validated for flow cytometry
 - Specific
 - High S/N
 - Consistent
- Optimized buffer systems for fixation and permeabilization
 - Different buffer options for different sample types and phosphoepitopes
- Strategy for identification of cell populations of interest
 - Compatibility with fixation and permeabilization buffers
 - Optimization of staining conditions

- Viable and healthy samples
 - Ex vivo stimulation to trigger phospho-signaling networks
 - Detection of altered basal phosphorylation states
- Robust results
 - Careful panel design and instrument setup
 - Consistent staining
- Data analysis tools

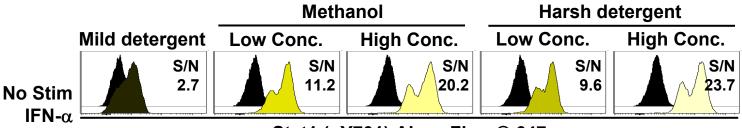


bdbiosciences.com/phosflow



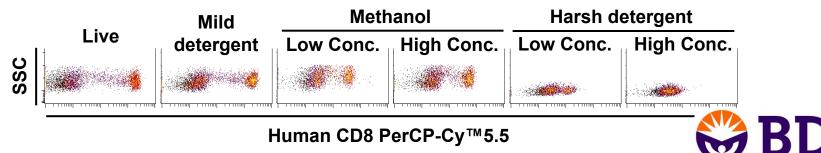
Fixation and Permeabilization: Selecting the Best Protocol

- Multiple fixation and permeabilization buffers are available
 - Appropriate buffer choice is critical for successful detection of phosphoproteins, surface markers, and other proteins of interest (eg, transcription factors, cell cycle or apoptosis proteins, etc.)
- Harsh, denaturing conditions favor detection of some phosphoproteins



Stat1 (pY701) Alexa Fluor® 647

• Fixation and permeabilization can adversely affect the detection of some surface markers, with harsh buffers causing more severe effects



Selecting Fixation and Permeabilization Buffers: Fixation

- Formaldehyde-based fixation prior to permeabilization provides optimal phosphoprotein detection and FSC/SSC resolution
 - Formaldehyde stability and concentration are critical
 - Use a source recommended in established protocols
- Sample type determines fixative choice

Sample Type	Fixative
Whole blood, spleen, or other erythrocyte-containing samples	BD Phosflow Lyse/Fix Buffer
PBMCs, cell lines, etc.	BD Cytofix™ Fixation Buffer

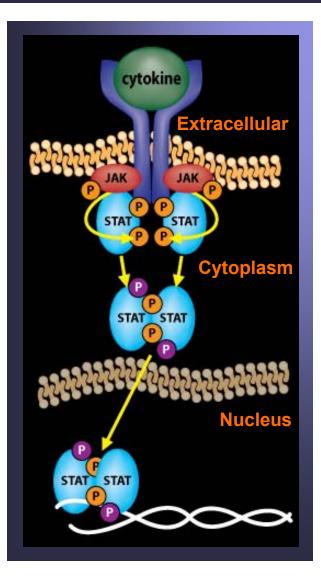


Selecting Fixation and Permeabilization Buffers: Permeabilization

	BD Phosflow	Perm Buffers	
Perm/Wash Buffer I • Mild detergent (saponin) method • Easiest on cell- surface markers • Adequate for detection of nuclear and	 Perm Buffer II Mild alcohol method (low conc. methanol) Few cell-surface markers lost Good for intracellular staining 	 Perm Buffer III Harsh alcohol method (high conc. methanol) Some cell-surface markers lost Best for many intracellular markers 	 Perm Buffer IV Harsh detergent method Some cell-surface markers lost Best for Stat pY and certain other intracellular markers
cytoplasmic proteins but suboptimal for Stat pY detection		 Most similar to Nolan lab method Recommended starting protocol 	 May result in greater cell loss than other buffers

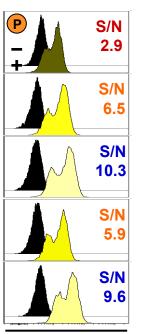


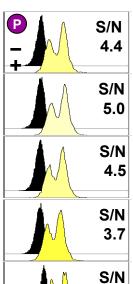
Selecting Fixation and Permeabilization Buffers: Permeabilization



- Phosphoprotein detection requirements:
 - Access to cytoplasmic and/or nuclear proteins
 - Some phosphoepitopes favor harsh, denaturing permeabilization buffers

3.6





Mild Detergent (Saponin) Perm/Wash Buffer I

Methanol – Low Conc. Perm Buffer II

Methanol – High Conc. Perm Buffer III

S/NHarsh Detergent – Low Conc.3.7Perm Buffer IV (0.5X)

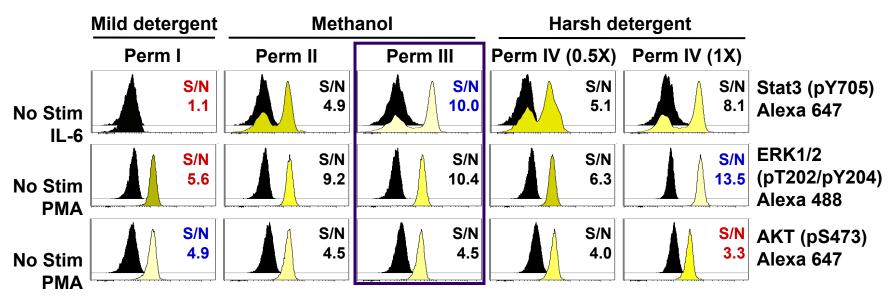
Harsh Detergent – High Conc. Perm Buffer IV (1X)

Stat1 (pY701) PE Stat1 (pS727) PE

Human PBMCs activated with IFN- α (pY701) or PMA (pS727) for 15 min and fixed with BD Cytofix



Permeabilization Buffer Selection Impacts Phosphoprotein Staining

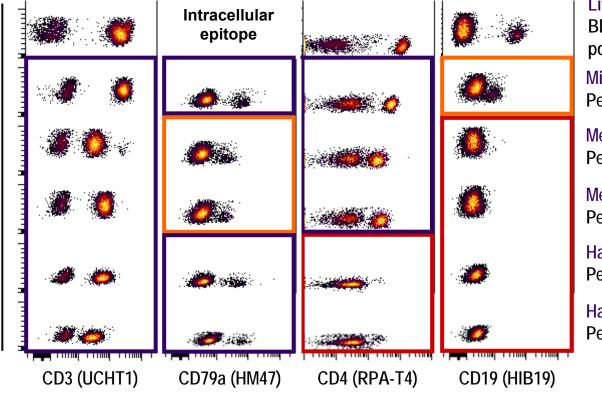


Human whole blood (Stat3) or PBMCs (ERK and AKT) activated with IL-6 or PMA for 15 min and fixed with BD Phosflow Lyse/Fix Buffer (whole blood) or BD Cytofix Buffer (PBMCs)

- Harsh permeabilization buffers provide superior staining of many, but not all, phosphoepitopes
 - High-concentration methanol (Perm III) and harsh detergent buffer (Perm IV)
- Vast majority of BD Phosflow[™] antibodies work with Perm Buffer III, although some yield superior staining with other buffers (eg, CREB pS133 and IκBα antibodies work best with Perm Buffer II)

Permeabilization Buffer Selection Impacts Surface Marker Resolution

- Perm/Wash Buffer I usually has the mildest effects on surface marker staining
- Different effects of methanol-based (Perm II and III) vs harsh-detergent (Perm IV) buffers on different epitopes



SSC

Human whole blood fixed with BD Phosflow Lyse/Fix Buffer, permeabilized, and stained with PerCP-Cy™5.5–conjugated antibodies

Live Stain BD FACS[™] Lysing Solution post-stain

Mild Detergent (Saponin) Perm/Wash Buffer I

Methanol – Low Conc. Perm Buffer II

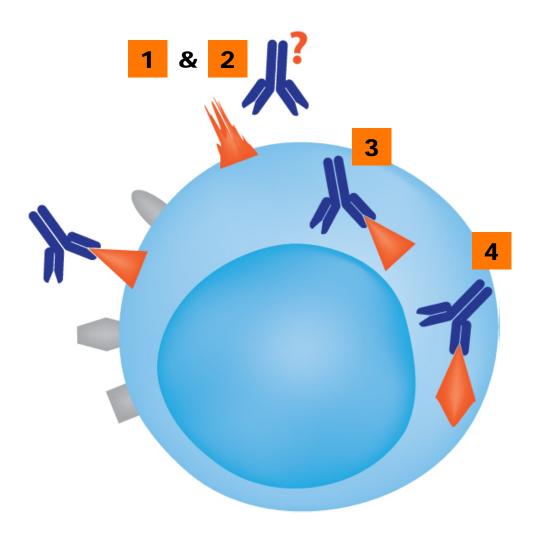
Methanol – High Conc. Perm Buffer III

Harsh Detergent – Low Conc. Perm Buffer IV (0.5X)

Harsh Detergent – High Conc. Perm Buffer IV (1X)



Why is Resolution of Surface Marker Stains Reduced in Permeabilized Cells?



Fixation covalently modifies surface marker epitopes, preventing antibody binding

2 Harsh permeabilization buffers denature epitopes, preventing antibody binding

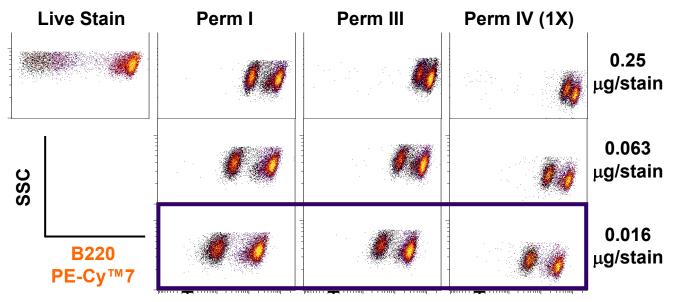
3 Permeabilization allows antibodies access to intracellular stores of antigen

Permeabilization opens up access to epitopes that were inaccessible during antibody screening, increasing nonspecific background



Antibody Titration Can Improve Surface Marker Resolution

Post-perm staining of anti-mouse B220 antibody is optimal at a concentration far below that used for live cell stains



BALB/c mouse spleen cells fixed with BD Phosflow Lyse/Fix Buffer, permeabilized, and stained

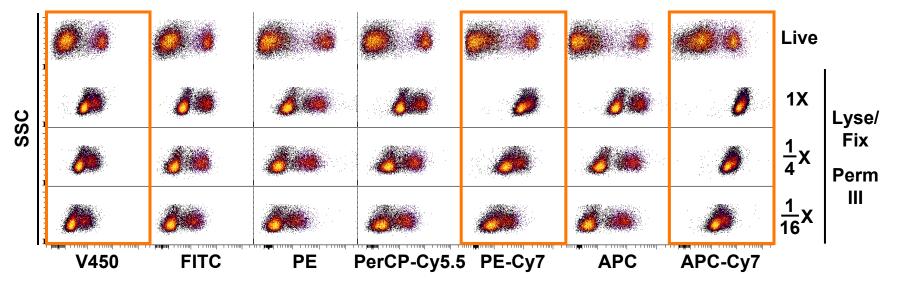


4 Permeabilization opens up access to epitopes that were inaccessible during antibody screening, increasing nonspecific background



Post-Perm Staining Success May Differ for Different Fluorescent Conjugates

Very high background staining and/or low signal can prevent some fluorophore conjugates of an antibody from working well in post-permeabilization stains



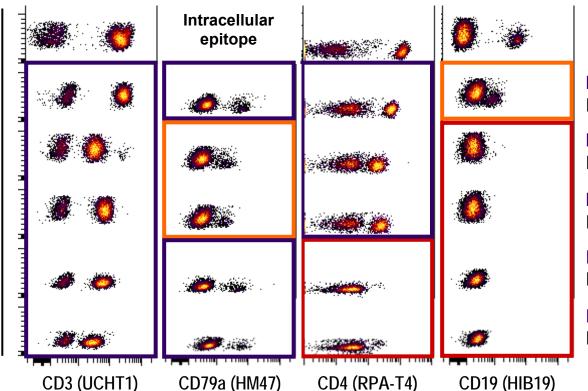
BALB/c mouse spleen cells fixed with BD Phosflow Lyse/Fix Buffer, permeabilized, and stained with various fluorophore conjugates of anti-TCRβ antibody (H57-597)



4 Permeabilization opens up access to epitopes that were inaccessible during antibody screening, increasing nonspecific background



Permeabilization Buffer Selection Impacts Surface Marker Resolution



Live Stain BD FACS Lysing Solution post-stain

Mild Detergent (Saponin) Perm/Wash Buffer I

Methanol – Low Conc. Perm Buffer II

Methanol – High Conc. Perm Buffer III

Harsh Detergent – Low Conc. Perm Buffer IV (0.5X)

Harsh Detergent – High Conc. Perm Buffer IV (1X)

Human whole blood fixed with BD Phosflow Lyse/Fix Buffer, permeabilized, and stained with PerCP-Cy5.5–conjugated antibodies



SSC

2 Harsh permeabilization buffers denature epitopes, preventing antibody binding



Differential Effects of Fix/Perm on Epitopes: Antibody Clone Choice is Important

Antibodies to Human Cell-Surface Markers Tested for BD Phosflow Protocols

Specificity	Clone	Fluorochrome	Protocol I Detergent method	Protocol II Mild alcohol method	Protocol III Harsh alcohol method	Protocol IV Detergent method
		APC	+	+	+	+
		APC-Cy™7	+	+	-	+
		FITC	+	+	+	+
	SK7	PE	+	+	+	+
		PE-Cy™7	+	+	+	+
		PerCP	+	+	+/-	-
		PerCP-Cy™5.5	+	+	+	+
		Alexa Fluor® 488	+	+	+	+
		Alexa Fluor® 647	+	+	+	+
		Alexa Fluor® 700		+	+/-	_
		APC	+	+	+	+
Human CD3	UCHT1	FITC	+	+	+	+
	UCHII	BD Horizon™ V450			+	+
		Pacific Blue™		+	+	+
		PE	+	+	+	+
		PE-Cy™5	+	+	+	+
		PE-Cy7	+	+	+	+
		APC	+	-	-	+/-
	UIT2	FITC		-	-	
	HIT3a	PE	+	-	-	-
		PE-Cy5	+		-	-
	6024	PE-Cy7	+		-	+/-
	SP34	PerCP	+	+	+	-



Fixation covalently 1 modifies surface marker epitopes, preventing antibody binding

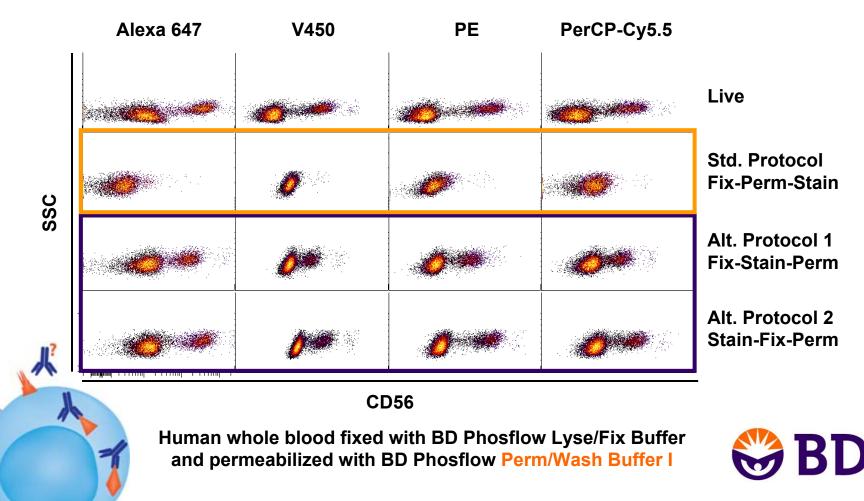


Harsh permeabilization buffers denature epitopes, preventing antibody binding



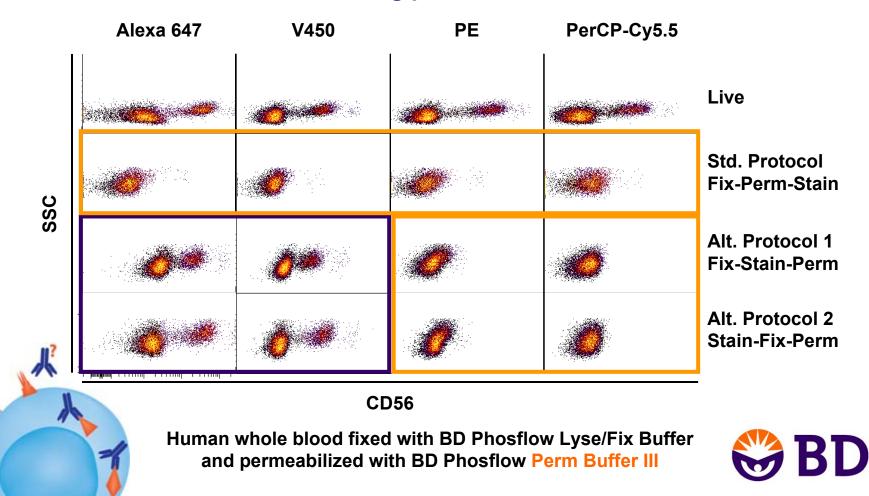
Alternative Staining Protocols Can Improve Surface Marker Resolution

Resolution of some surface markers can be improved by staining before permeabilization (Alt. Protocol 1) or before fixation (Alt. Protocol 2)



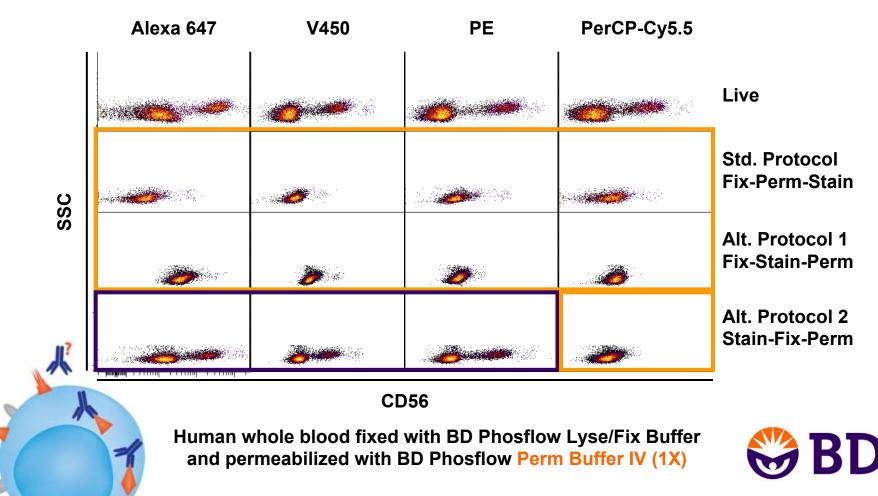
Fluorophore Choice for Alternative Staining Protocols: Perm Buffer III

Protein fluorophores are destroyed by exposure to methanol-containing permeabilization buffer



Fluorophore Choice for Alternative Staining Protocols: Perm Buffer IV

Many fluorophores are damaged by the harsh detergent-containing BD Phosflow Perm Buffer IV, but fixation stabilizes some stains



BD FACSelect[™] Buffer Compatibility Resource

Goal:

• To create a resource to facilitate the design of multiparameter staining panels for simultaneous analysis of intracellular and surface marker proteins

Approach:

• Generate data for key intracellular and surface marker specificities using available fluorochromes and various fixation and permeabilization protocols

Variables:

- Sample types: Human whole blood & PBMCs, murine splenocytes, & bone marrow
- Surface and intracellular specificities in all available fluorochromes
- Fixatives: BD Phosflow Lyse/Fix Buffer (human whole blood, mouse cells) or BD Cytofix Fixation Buffer (human PBMCs, cell lines)
- Permeabilization buffers: BD Phosflow Perm Buffers I–IV (IV at 1X and 0.5X conc.)
- Antibody concentrations: Three-point titrations of all surface marker antibodies
- Surface marker staining protocols: Standard Protocol (Fix-Perm-Stain), Alternative Protocol 1 (Fix-Stain-Perm), and Alternative Protocol 2 (Stain-Fix-Perm)



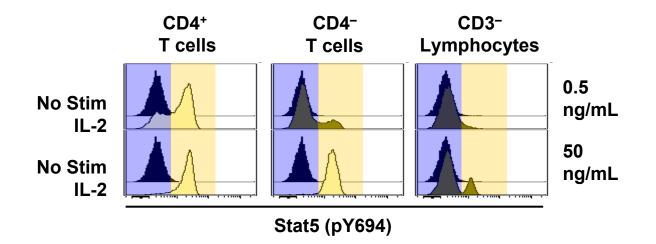
BD FACSelect[™] Buffer Compatibility Resource

۲	BD FACSelect	Buffer Com	pa	ati	bil	ity	Resourc	e																(3	C	ytc	ban	ık
		rowcrcu by Cytobunk			BD P	host	flow™ Cell Signal	ling	E	3D T	est	ed S	urfa	ace	Mar	kers	PD	F	0	ont	act	Cyt	:oba	nk		Abo	ut C	/tobai	nk
3 Separa comma Showi Click c	ate multiple keywords with as. ng 28 of 87 reagents. olumn headings to sort.	✓ BD Horizon™ V450 ✓ Pacific Blue™ ✓ AmCyan ✓ BD Horizon™ V500 ✓ BD Horizon™ V500	BI AI FI PE PE PE	ue exa TC : :-Te: :-Cy erCP	488 r Fluor ×as R ™5	nm •* 48	✓ YG 561 nr	n C		PC lexa	⊢Fluo ⊢Fluo Cy™	nm pr [®] 6 pr [®] 7 7	547				Per BD ^m (557 BD ^m Buff BD ^m BD ^m BD ^m	1 Ph n/W 1885 1 Ph er II 1 Ph er II	<u>ash</u>) ((55 (1 (5 (1 (5 osflo	<u>w</u> Buff 805 w P 5805	erm 2) erm 50) erm			Mi Hai	ld A	rgent Icohi Alcot	t Met ol Me nol M	hod thod ethod Method	-1
	Specific Tarqet	ity Clone ∆	Human	Mouse	Surface	Intracellular	Source	Protocol	BD Horizon™ V450	Pacific Blue™	AmCyan	BD Horizon™ V500	Alexa Fluor® 488	FITC	FE	PE-Texas Red®	PE-Cy TM 5	PerCP	PerCP-Cy [™] 5.5	PE-Cy™7	Ц	PE-Cy ^{™5}	PE-Cy TM 7			Alexa Fluor® 700	APC-CY ^{IM} 7	ARC-II/	
	CD3e	145-2C11		٠	٠		BALB/c SP	Std					٠	•	٠		\$	٠	٠	•	۵	۵	٥	•			•	Vi	iew
	CD3	17A2		٠	٠		BALB/c SP	Std	٠					٠	٠		٥		٠	٠	٥	٥	۵		•	<u>ه</u>	•	Vi	iew
	p38 MAPK (pT180/pY182)	36/p38 (pT180/pY182)	٠			٠	Whole Blood	Std		٠			٠		٠				٠	٠	٥		٥		٠			⊻i	iew
	p38 MAPK (pT180/pY182)	36/p38 (pT180/pY182)	٠			٠	PBMC	Std		٠			٠		٠				٠	٠	٥		٥		٠			Vi	<u>iew</u>
	р38 МАРК (рТ180/рҮ182)	36/p38 (pT180/pY182)		٠		٠	BALB/c SP	Std		٠			٠		٠				٠	٠	٥		٥		•			Vi	iew
	Stat4 (pY693)	38/p-Stat4	٠			٠	PBMC	Std					٠		٠				٠		٥				•			⊻i	<u>iew</u>
	Stat4 (pY693)	38/p-Stat4	٠			٠	Whole Blood	Std					٠		٠				٠		٥				•			Vi	<u>iew</u>



Designing a Multicolor Phosflow Staining Panel

Heterogeneous Threshold for IL-2 Responsiveness within Lymphocyte Subpopulations in Human Whole Blood



Does IL-2 responsiveness differ between naïve, effector, and memory T cells?

Need to simultaneously stain CD3, CD4, CD45RA, CD45RO, T-bet, and Stat5 (pY694) in IL-2–stimulated human whole blood cells



Step 1: Check Buffer Compatibility for Intracellular Antibodies

₿	BD FACSel	ect [™] Bu			obank	•	,																	ę				bank
						BD Ph	nosflow™ Cell Sig	naling)	BD	Te	sted	Su	rfac	e Ma	arke	rs 🖪	DF	C	ont	act (Cyto	ban	k	A	bout	: Cyt	tobank
Filter b	Filter by keywords: Select fluorochromes supported by your cytometer: Reset Perm Buffers Fixation Buffers Protocols BD ^m Phosflow BD Phosflow Protocols Protocols Protocols																											
stat5		Violet 4	05 nr	n	V	Blue	488 nm 🛛 🛛	YG 56:	1 nm		V F	Red	640	nm			Pe	rm/W	ash I		er I			De	eterg	gent M	Meth	od l
with corr	eparate multiple keywords ith commas.																											
Click co	Click column headings to ort. W AmCyan W PE W PE-Cy™7 W Alexa Fluor 700 BD Horizon™ V500 V PE-Texas Red [®] V APC-Cy™7 Harsh Alcohol Method																											
sort.	sort.																											
♦ = D																												
	Specifici	ty	_		g	Intracellular	a	lo	on™ V450	ue™		on™ V500	Jor® 488			s Red®		/™5.5						Jor® 647	uor® 700	۲.	Ι	
			Human	Mouse	Surface	ntrac	Source	Protocol	BD Horizon™	Pacific Blue™	AmCyan	BD Horizon™	Alexa Fluor®	FITC	믭	PE-Texas	PE-Cy ^{IN5}	PerCP PerCP-Cv™	PE-Cy TM 7	믭	PE-Cy™5	РЕ-СУ™7	APC	Alexa Fluor®	Alexa Fluor®	APC-Cy™7	APC-H7	
	Target	Clone 🛆	-	2	S		••	-	8	ď.	A	6	∢	E.	۵.	۵.	۵.		-		٩		∢	∢	∢	A	∢	
	Stat5 (pY694)	47	•			•	PBMC	Std		٠			٠		٠			•	•	\$		\$		٠				<u>View</u>
	Stat5 (pY694)	47	•			٠	Whole Blood	Std		٠			٠		٠			•	•	\$		٥		٠				<u>View</u>
N	Stat5 (pY694)	47		٠		٠	BALB/c SP	Std		٠			٠		٠			•	•	\$		٥		٠				<u>View</u>
				-		_		-	-																			-



Stat5 (pY694) Antibody Works Well with Perm III or Perm IV (0.5x or 1x)

Stat5 (pY694) (47)

BD Cytobank

Back to FACSelect

bout Stat5 (pY694) (47)

Protein Name: Stat5 (pY694) Clone: 47 Isotype: IgG1 Reactive species: Human, Mouse Host species: Mouse Protocol: <u>details</u> Experiment Cell Source: Human Whole Blood Cytometer used: FACSCantoII

Conjugates Shown

 Pacific Blue™
 [BD]

 Alexa Fluor® 488
 [BD]

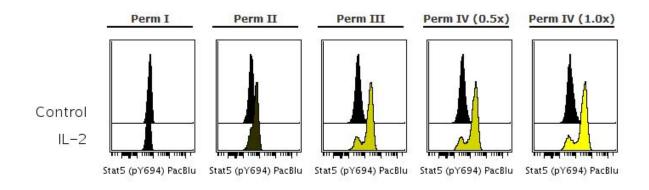
 PE
 [BD]

 PerCP-Cy5.5
 [BD]

 PE-Cy™7
 [BD]

 Alexa Fluor® 647
 [BD]

tat5 (pV604) DacBlue



Perm I Perm II Perm III Perm IV 0.5x Perm IV 1.0x



Calculated Fold of Medians by First Row using X-Axis channel(s): Use Panel/Channel Values

	Perm 1	Perm II	Perm III	Perm IV (0.5x)	Perm IV (1.0x)
Control	1.0	1.0	1.0	1.0	1.0
IL-2	0.84	1.93	5.06	5.21	6.15

😂 BD

View in Cytobank Jun

Jump to Gating Hierarchy

Back to Top

Step 1: Check Buffer Compatibility for Intracellular Antibodies

😌 B	D FAC			ffel red by		pank	patibility D Phosflow™ Cell S					este	d Si	urfa	ce M	arke	rs P	DF	c	Cont	act	Cyte	obar				, 	bank /tobank
tb Separate with comm Showing Click colu sort. ♦ = Rea ♦ = Dat	keywords: multiple keywor nas. 2 of 87 reage mn headings t agent available ta and reagen ailable.	rds Vic nts. to V bD Pac Am V BD	Horiz Horiz Cific B	05 nn :on™ \ lue™	1 /450	V E	Alexa Fluor [®] 488 🔽 ITC 🗸	YG 56 PE PE-Cy PE-Cy	i1 n ™5		 ✓ ✓ ✓ ✓ ✓ ✓ 	APC Alex Alex APC	C ka Fl	uor [®] ™7	n 647 700		BD Per (55 BD BU BD BD BD	rm B ™ Ph 7885 7885 7885 7885 7885 7885 7885 788	iosflo (ash 5) iosflo I (55 iosflo II (5 iosflo	<u>ow</u> Buff 5805 5805 5805 5805	<u>erm</u> 2) 50)		on B	D Mil Har	eter Id Al	gent coho lcoh	Met Me ol Me	hod thod ethod Method
	Spec Target	ificity Clone ∆	Human	Mouse	Surface	Intracellular	Source	Protocol	BD Horizon [™] V450	Pacific Blue™	AmCyan	BD Horizon™ V500	Alexa Fluor® 488	FITC	PE	PE-Texas Red®	PerCP	PerCP-Cy TM 5.5	PE-Cy TM 7	PE	PE-Cy ^{™5}	PE-Cy™7	APC	Alexa Fluor [®] 647	Alexa Fluor [®] 700	APC-Cy ^{TN} 7	APC-H7	
	T-bet	O4-46	٠			٠	Whole Blood	Std	٠				٠		٠			•		٥				٠				<u>View</u>
ζ m	T-bet	04-46	٠			٠	PBMC	Std	٠				٠		٠			•		۰				٠				<u>View</u>



T-bet Antibody Also Works with Perm III or Perm IV (0.5x or 1x)

T-bet (04-46)



About T-bet (04-46

Protein Name: T-bet Clone: 04-46 Isotype: IgG1, k Reactive species: Human, Mouse Host species: Mouse Protocol: <u>details</u> Experiment Cell Source: Human Whole Blood Cytometer used: FACSCantoII

Conjugates Shown

 V450
 IBD]

 Alexa Fluor®
 488
 IBD]

 PE
 IBD]

 PerCP-Cy5.5
 IBD]

 Alexa Fluor®
 647
 IBD]

Back to FACSelect

T-bet V450

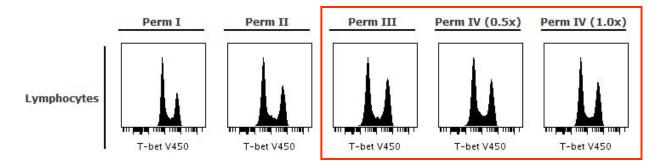
Perm

Perm II

Perm III

Perm IV 0.5x

Perm IV 1.0x

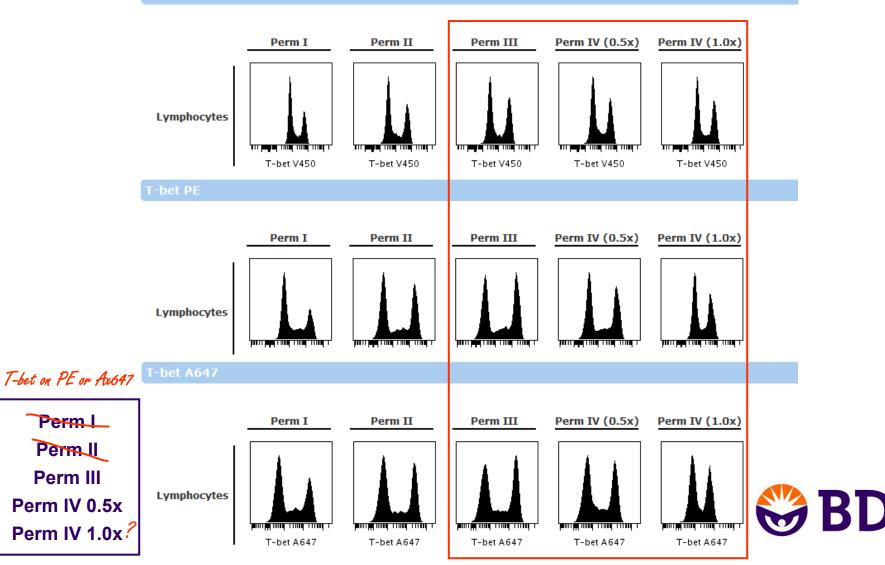


Calculated Raw values of statistic using X-Axis channel(s): Use Panel/Channel Values
Perm I Perm II Perm III Perm IV (0.5x) Perm IV (1.0x)
Lymphocytes
2.6
3.48
3.57
3.28
3.16
View in Cytobank
Jump to Gating Hierarchy
Back to Top



Best T-bet Resolution with Brightest Fluorophores and Perm III or IV (0.5x)

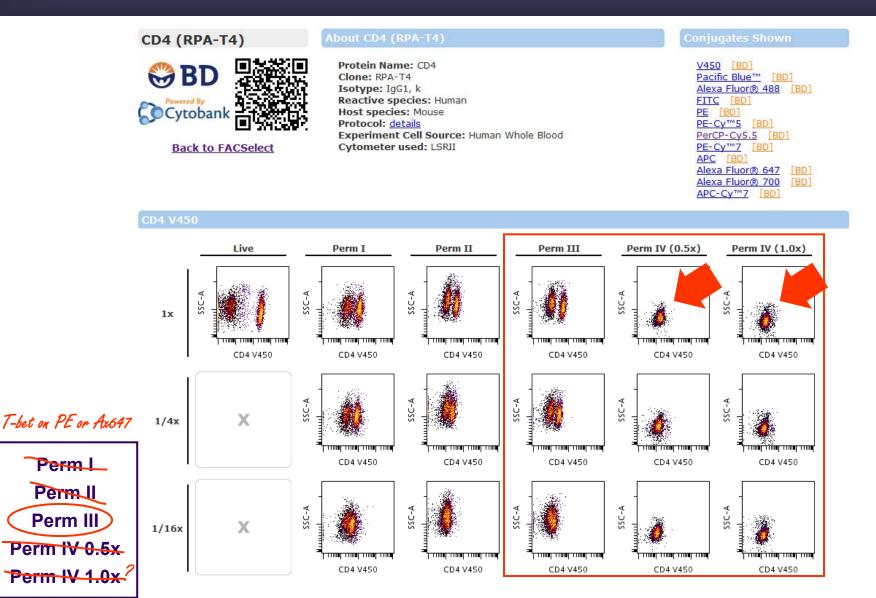
T-bet V450



Step 2: Check Buffer Compatibility for Surface Marker Antibodies

😂 E	BD FACSe		uffe vered				oatibility R	esc	DUI	°C(Э														(3	C	yto	bank
				.,.,			Phosflow™ Cell Sig	naling)	BC	Te	steo	d Su	rfac	e M	arke	ers	PDF		С	onta	act (Cyto	bar	ık	A	bou	It Cy	tobank
Filter b	y keywords:	Select flu	orocl	nrom	es si	uppo	rted by your cyto	meter	: F	Rese	et							Perm				Fix	atio	on B	uffe	rs	F	roto	cols
cd4		Violet	405 r	nm	V	Blu	ie 488 nm 🛛 🔽	YG 56	1 nm	1	V F	Red	640) nm			Ē	8 <u>D™</u> 9erm 5578	/Wa			er I			D	eter	gent	Met	hod
Separate with corr	e multiple keywords imas.	BD Ho					xa Fluor® 488 👿				V /							3D™ Buffe	Pho	sflo	w Pe	erm			Mil	d Ale	coho	l Me	thod
	g 7 of 87 reagents. umn headings to	Pacific		м				PE-Cy [*] PE-Cy*					a Flu a Flu					BD™	Pho	sflo	w Pe	erm			Han	sh A	lcoh	ol M	ethod
sort.	unin neadings to	V BD Ho	rizon™	[™] V50	0 🔽	_	-Texas Red [®] -Cv™5						-Cy™	™7				Buffe BD™	Pho	sflo	w Pe	erm							
	Reagent available.																												
♦ = D	> = Reagent available. Image: CP-Cy™5.5 > = Data and reagent available. Image: CP-Cy™5.5 Image: CP-Cy™7 Image: CP-Cy™7																												
						ular			" V450	Σ		∾ V500	0 488			®p			ŝ						647	700			
	Specific	city	E	e	e	cell	e	0	izon"	Blue™	c	rzon™	luor			as Re	ណ្		Cy™5.	۲,		ហ្	47		luor®	luor®	Ĺνι/		
	Target	Clone 🛆	Human	Mouse	Surface	Intracellular	Source	Protocol	BD Horizon™	Pacific Blue™	AmCyan	BD Horizon™	Alexa Fluor®	FITC	빒	PE-Texas Red®	PE-Cy™5	PerCP	PerCP-Cy™	РЕ-Су™7	Ы	PE-Cy ^{IN5}	РЕ-СУ™7	APC	Alexa Fluor®	Alexa Fluor®	APC-Cy™7	APC-H7	
	CD45RA	HI100	•	-	•		Whole Blood	Std	•	_	-	۵		•	•	-	•	-	-	•	0	6	6	•	-	•		•	View
	CD44	IM7		•	•		BALB/c BM	Std	•			• •		•	•		•		•	•	۰ ۵	0	0	•		•	•	·	View
	CD45R/B220	RA3-6B2		•	•		BALB/c SP	Std	•	•		٥	•	•	\$	٥	\$	•	٠	•	\$	٥	٥	•	٠	\$	•		View
	CD4	RM4-5		٠	٠		BALB/c SP	Std	٠	٥		٥	٥	٠	٠		٠	٠	٠	٠	٥	٥	٥	٥	٠	٥			View
	CD4	RPA-T4	٠		٠		PBMC	Std	٠	٠		٥	٠	٠	٠		٠		٠	٠	٥	٥	٥	٠	٠	٠	٠	٥	<u>View</u>
	CD4	RPA-T4	٠		٠		Whole Blood	Std	٠	٠		٥	٠	٠	٠		٠		٠	٠	٥	٥	٥	٠	٠	٠	٠	٥	<u>View</u>

CD4 Clone RPA-T4 is Not Compatible with Perm Buffer IV



Step 3: Confirm Compatibility of All Antibodies with Selected Buffer

Specificity	Clone	Compatible with Perm Buffer III?
Stat5 (pY694)	47	\checkmark
T-bet	O4-46	✓
CD3	UCHT1	✓
CD4	RPA-T4	✓
CD45RA	HI100	✓
CD45RO	UCHL1	\checkmark

😂 B	D FAC	Sel	ect™	Bu Power			bank	patibility D Phosflow™ Cell
Filter by	keywords:		Select	fluor	ochr	omes	supp	oorted by your c
with come Showing Click colu sort. ♦ = Re ♦ = Da	multiple keywo mas. 2 of 87 reage umn headings agent availabl ta and reagen ailable.	nts. to e.	🗹 An	Horiz	on™ \ ue™	/450		
	Spec Target	cificity Cloi	ne 🛆	Human	Mouse	Surface	Intracellular	Source
	CD45RO	UC	HL1	•		٠		Whole Blood
<u>_</u>	CD3	UC	HT1	•		•		Whole Blood

T-bet on PE or Ax647



<u>Note</u>: If some surface markers are incompatible with the chosen buffer system, alternative staining protocols may be useful. Be aware of fluorophore choice considerations.



Step 4: Select an Appropriate Conjugate for Each Antibody

Specificity	Clone	Compatible with Perm Buffer III?	Fluorophore	Optimal Concentration
Stat5 (pY694)	47	\checkmark	Ax647	
T-bet	O4-46	✓	PE	
CD3	UCHT1	✓	Ax488	
CD4	RPA-T4	✓	PE-Cy7	
CD45RA	HI100	✓	V450	
CD45RO	UCHL1	✓	PerCP-Cy5.5	

T-bet on PE or Ax647



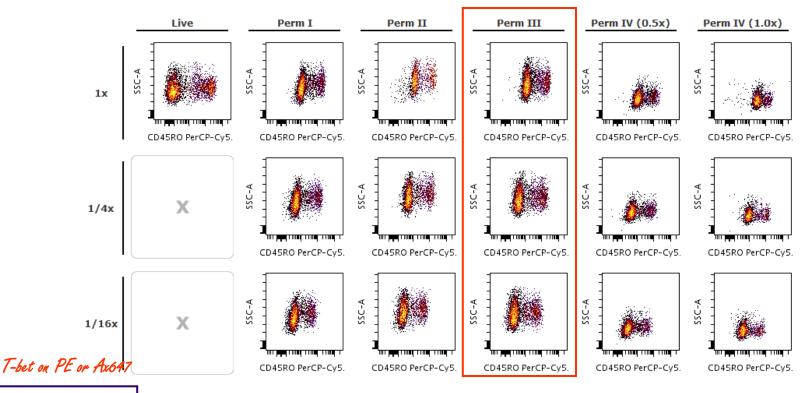
General Principles for Panel Design:

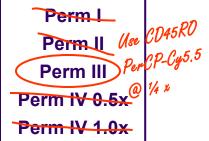
- Brightest fluorophores should be used for phospho-specific antibodies and other dim or important stains
- Try to avoid spectral overlap in channels used for phosphoprotein detection



Step 5: Identify the Optimal Concentration for Each Antibody

CD45RO PerCP-Cy5.5





Calculated Raw values of statistic using X-Axis channel(s): Use Panel/Channel Values

1x	5.93	3.28	3.71	3.32	2.88	2.2
1/4x	X	3.7	3.65	4.01	3.45	2.96
1/16x	Х	3.15	3.54	3.84	3.28	3.04



Step 5: Identify the Optimal Concentration for Each Antibody

Specificity	Clone	Compatible with Perm Buffer III?	Fluorophore	Optimal Concentration
Stat5 (pY694)	47	\checkmark	Ax647	Test Size
T-bet	O4-46	✓	PE	Test Size
CD3	UCHT1	✓	Ax488	1x
CD4	RPA-T4	✓	PE-Cy7	1x
CD45RA	HI100	✓	V450	1x
CD45RO	UCHL1	✓	PerCP-Cy5.5	1⁄4 X

T-bet on PE or Ax647

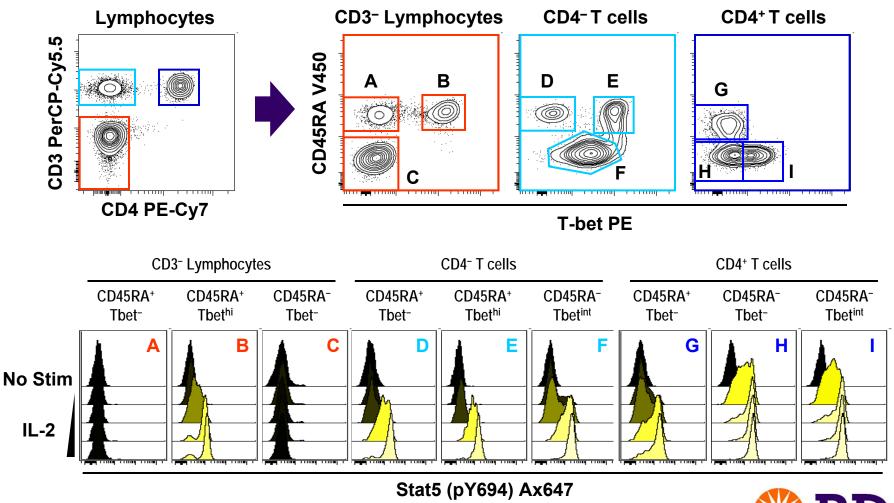


General Principles for Panel Design:

- Brightest fluorophores should be used for phosphospecific antibodies and other dim or important stains
- Try to avoid spectral overlap in channels used for phosphoprotein detection



Step 6: Stimulate, Fix, Perm, Stain, and Get Great Data





Helpful Resources

BD FACSelect™ Buffer Compatibility Resource cytobank.org/facselect BD Phosflow™ Website bdbiosciences.com/phosflow

BD FACSelect[™] Multicolor Panel Designer bdbiosciences.com/paneldesigner

Manage, analyze, and share flow cytometry data over the web with Cytobank cytobank.org

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

Alexa Fluor is a registered trademark of Molecular Probes, Inc.

Cy is a trademark of Amersham Biosciences Corp. Cy dyes are subject to proprietary rights of Amersham Biosciences Corp and Carnegie Mellon University and are made and sold under license from Amersham Biosciences Corp only for research and in vitro diagnostic use. Any other use requires a commercial sublicense from Amersham Biosciences Corp, 800 Centennial Avenue, Piscataway, NJ 08855-1327, USA.

Cytobank and the Cytobank Logo are property of Cytobank Inc. \circledast 2011 Cytobank Inc., All rights reserved.

BD, BD Logo and all other trademarks are property of Becton, Dickinson and Company. © 2011 BD



Thank You!

BD Biosciences - San Diego

Guo-Jian Gao Xiao Wang Xiang Dong Ji Xiao-Wei Wu Ai-Li Wei Andrea Nguyen Dennis Sasaki Paul Waterman Mette Ejrnaes John Apgar Chad Sisouvanthong David Ernst Christian Carson Jurg Rohrer Lori Anderson Cynthia Lane Olaf Zoellner Sun-Min Lee Sue Reynolds Mervi Reunanen Tika Ransaw

<u>Cytobank</u> Nikesh Kotecha Chris Covey Stephanie Huang Peter Krutzik Jonathan Irish **BD Biosciences - San Jose**

Maria Suni Skip Maino

