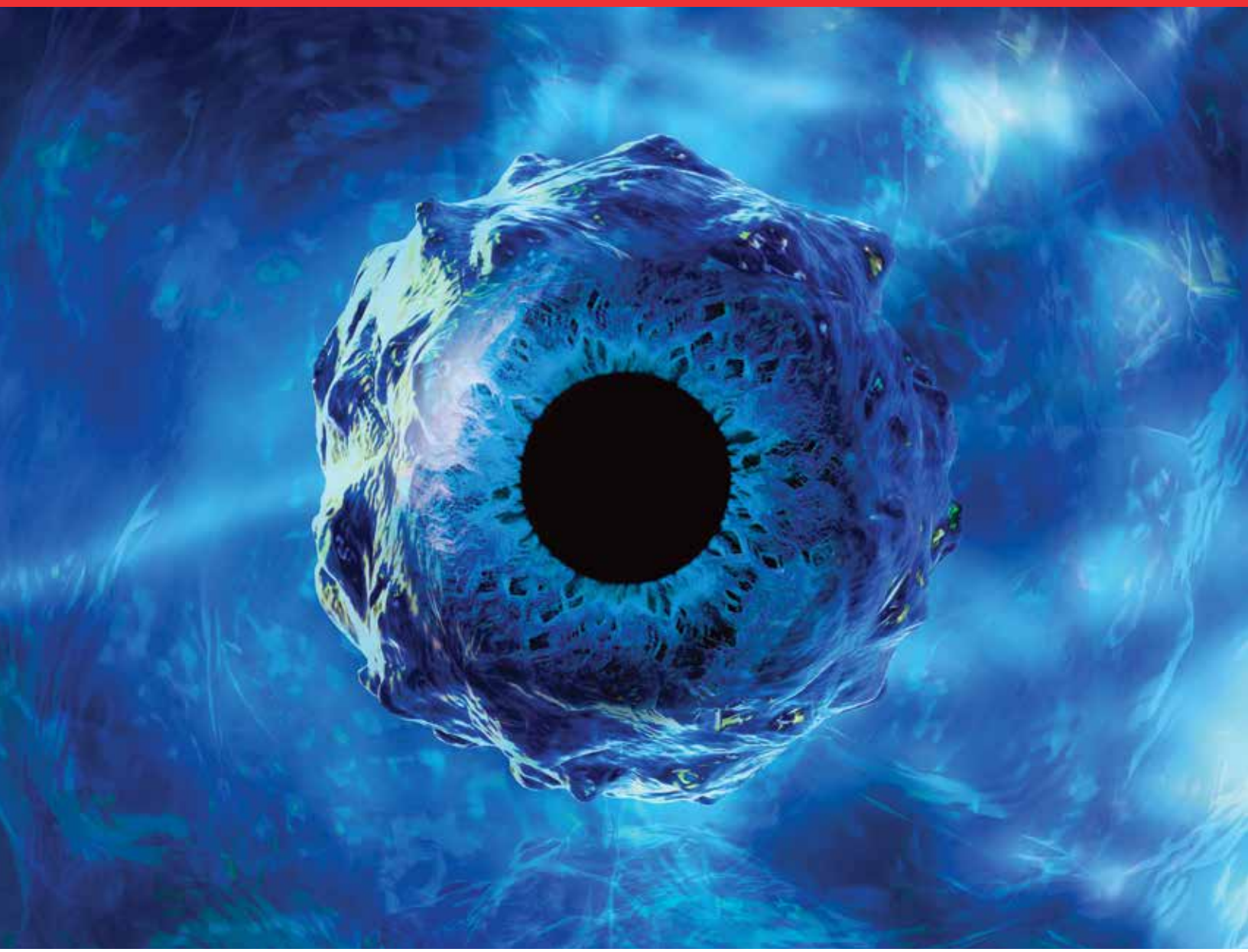
invitrogen



Intracellular flow cytometry

Understand the cells from a different perspective

ThermoFisher
SCIENTIFIC

The ability to stain and detect intracellular molecules opens the door for both identification of distinct cell subsets as well as further characterization of what is going on in the cell. Modifications to the basic flow cytometry cell surface staining protocols enables simultaneous detection of surface molecules, intracellular antigens, cell signaling phosphorylation events, and RNA transcripts at the single-cell level. This increased level of profiling allows for a better understanding of the biology and greater discovery.

Contents

Non-nuclear intracellular staining

The staining of intracellular proteins for flow cytometry analysis requires additional steps and different buffers than traditional cell surface antibody staining. The cells require fixation to crosslink the proteins and stabilize the cell membrane, as well as permeabilization to allow the antibodies access to the intracellular antigens. The Invitrogen™ eBioscience™ intracellular fixation and permeabilization buffers are designed for optimal detection of cytoplasmic proteins and secreted proteins residing in organelles and vesicles.

While the appropriate stimulation conditions and kinetics of protein production are dependent upon the cell type and the particular protein being assayed, secreted proteins such as cytokines and chemokines are generally expressed at low levels in resting cells. Their expression must be induced and secretion of the protein blocked to allow for their detection by flow cytometry. While PMA (phorbol 12-myristate 13-acetate) and ionomycin (calcium ionophore) are often used in combination to induce cytokine production, more specific stimulation or cell-type activation of agonistic antibodies against cell receptors, such as CD3 and CD28 for T lymphocytes, are a great option.

It is often necessary to block the secretory pathway of the expressed proteins to allow for accumulation of the proteins of interest. This is commonly achieved with brefeldin A that blocks protein transport at the endoplasmic reticulum or with monensin that blocks protein transport at the Golgi apparatus. Invitrogen™ eBioscience™ Cell Stimulation Cocktails contain the stimulants PMA and ionomycin with or without the inclusion of protein transport (secretory pathway) inhibitors at a ready-to-use concentration.

For detection and analysis, a diverse portfolio of high-quality Invitrogen™ eBioscience™ antibodies are available with multiple options for conjugated dyes.

For details on cytokine stimulation protocols, see pages 6 and 7.

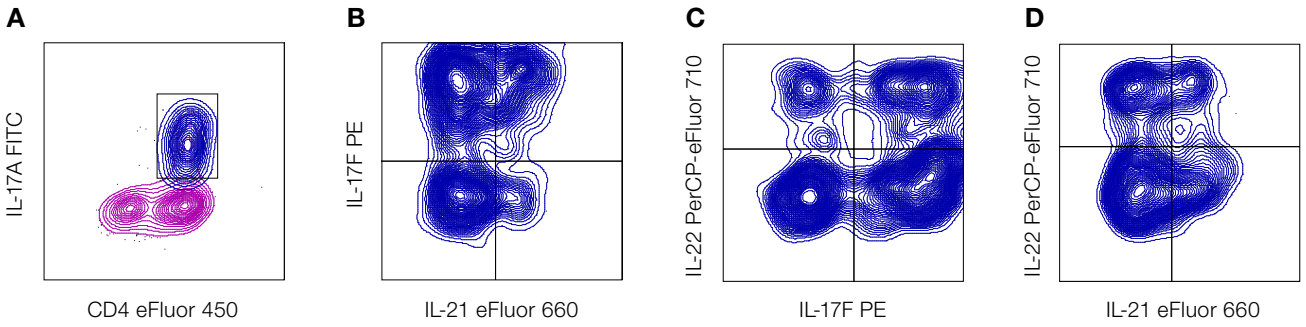


Figure 1. Human Th17 cytokine staining panel in CD4⁺ T cells. Th17-polarized CD4⁺ PBMCs were stimulated for 5 hours with Cell Stimulation Cocktail (plus protein transport inhibitors) (Cat. No. 00-4975) and then intracellularly stained with the Invitrogen™ eBioscience™ Human Th17 Cytokine Staining Panel (Cat. No. 88-8419). Lymphocytes were gated for staining with Invitrogen™ eBioscience™ **(A)** CD4 eFluor™ 450 (Cat. No. 48-0049) and IL-17A FITC (Cat. No. 11-7179) antibodies and then analyzed for staining with Invitrogen™ eBioscience™ **(B)** IL-21 eFluor™ 660 (Cat. No. 50-7219), **(C)** IL-17F PE (Cat. No. 12-7169), and **(D)** IL-22 PerCP-eFluor™ 710 (Cat. No. 46-7229) antibodies.

Table 1. Ready-to-use reagents for cell stimulation and transport inhibition.

Description	Cat. No.
Cell Stimulation Cocktail (500X)	00-4970
Cell Stimulation Cocktail (plus protein transport inhibitors) (500X)	00-4975
Protein Transport Inhibitor Cocktail (500X)	00-4980
Brefeldin A Solution (1,000X)	00-4506
Monensin Solution (1,000X)	00-4505
Concanavalin A (Con A) Solution (500X)	00-4978
Lipopolysaccharide (LPS) Solution (500X)	00-4976
Phytohemagglutinin-L (PHA-L) Solution (500X)	00-4977
Anti–Human CD3 Functional Grade Monoclonal Antibody (clone OKT3)	16-0037
Anti–Human CD28 Functional Grade Monoclonal Antibody (clone CD28.2)	16-0289
Anti–Mouse CD3e Functional Grade Monoclonal Antibody (clone 145-2C11)	16-0031
Anti–Mouse CD28 Functional Grade Monoclonal Antibody (clone 37.51)	16-0281

Cytokine antibodies

Table 2. Invitrogen™ eBioscience™ cytokine antibodies.*

Antigen	Clone	Cat. No.	Purified	Functional grade	Biotin	Violet laser: eFluor™ 450	Blue laser: FITC or Alexa Fluor™ 488	Blue laser: PerCP-Cyanine5.5	Blue laser: PerCP-eFluor™ 710	Green, yellow-green lasers: PE	Green, yellow-green lasers: PE-eFluor™ 610	Green, yellow-green lasers: PE-Cyanine5	Green, yellow-green lasers: PE-Cyanine5.5	Green, yellow-green lasers: PE-Cyanine7	Green, yellow-green lasers: Alexa Fluor™ 532	Red laser: APC or eFluor™ 660	Red laser: Alexa Fluor™ 700	Red laser: APC-eFluor™ 780
Mouse																		
G-CSF	9B4CSF	7353		■												■		
GM-CSF	MP1-22E9	7331	■	■			■			■						■		
IFNγ	XMG1.2	7311	■	■	■	■	■	■		■	■			■		■		
IL-1α	ALF-161	7011	■	■			■			■								
IL-1β pro-form	NJTEN3	7114				■	■		■	■				■		■		■
IL-2	JES6-5H4	7021	■	■	■	■	■	■		■				■		■		
IL-4	11B11	7041	■	■			■		■	■				■		■		
IL-5	TRFK5	7052	■	■						■								
IL-6	MP5-20F3	7061	■	■		■	■		■	■								
IL-9	RM9A4	8091														■		
IL-10	JES5-16E3	7101	■	■			■	■		■						■	■	
IL-12 p35	4D10p35	7352														■		
IL-12/IL-23 p40	C17.8	7123		■	■	■	■	■		■				■		■		
IL-13	eBio13A	7133	■	■		■	■		■	■	■			■		■		
IL-17A	eBioMM17F3	7173		■														
IL-17AF	B8KN8R	9171								■						■		
IL-17F	eBio18F10	7471					■		■	■						■		
IL-21	mhalx21	7213								■						■		
IL-22	1H8PWSR	7221							■	■								
IL-23 p19	fc23cpg	7023					■		■							■		
IL-27 p28	MM27-7B1	7285		■					■	■								
LAP	TW7-16B4	9821							■	■				■				
TL1A	Tandys1a	7911							■									
TNFα	MP6-XT22	7321	■	■		■	■		■	■				■		■		
Human																		
G-CSF	8F5CSF	7351								■						■		
GM-CSF	GM2F3	7356								■						■		
IFNγ	4S.B3	7319	■		■	■	■	■		■	■			■		■	■	■
IL-1α	364/3B3-14	7118	■				■											
IL-1β	CRM56	7018	■	■			■			■								
IL-1RA	CRM17	7015					■											
IL-2	MQ1-17H12	7029	■			■	■		■	■	■			■		■		
IL-4	MP4-25D2	7048	■	■	■													
IL-5	TRFK5	7052	■	■						■								
IL-6	MQ2-13A5	7069	■	■		■	■		■	■				■		■	■	
IL-8 (CXCL8)	8CH	8088				■			■							■		
IL-9	MH9A4	7097				■										■		
IL-10	JES3-9D7	7108	■	■		■	■			■				■		■		
IL-12 p35	SNKY35	7359														■		
IL-12/IL-23 p40	C8.6	7129	■	■	■	■	■			■						■		
IL-13	85BRD	7136					■											
IL-17A	eBio64CAP17	7178	■	■						■						■		
IL-17AF	20LJS09	9179				■	■			■								
IL-17F	SHLR17	7169		■					■	■				■		■		
IL-17FF	WU24A4P	9178							■							■		
IL-21	eBio3A3-N2	7219	■							■						■		
IL-22	22URTI	7229				■	■		■	■				■		■		
IL-23 p19	eBio473P19	7238	■															
IL-27 EBI3 subunit	ebic6	7358							■	■						■		
IL-27 p28	3D1p28	8277														■		
IL-31	31SNEZE	9319														■		
LAP	FNLAP	9829				■			■	■				■		■		
TL1A	Tandys1a	7911							■									
TNFα	MAb11	7349	■		■	■	■	■		■				■		■	■	
TNFβ	359-81-11	7327			■					■						■		

* Table is not exhaustive. Search the full catalog of flow cytometry antibodies at **thermofisher.com/flowantibodies**

Table 3. Mouse cytokine stimulation guide.

Mouse cytokine	Cell source	Activation	Incubation time	Restimulation	Intracellular block	Clone (antibody)
GM-CSF	Mouse spleen	Con A (3 µg/mL) (2 d)/IL-2 (20 ng/mL) + IL-4 (20 ng/mL) (3 d)	2 d/3 d	Anti-CD3 (10 µg/mL immobilized) + anti-CD28 (2 µg/mL soluble) (5 hr)	Brefeldin A	MP1-22E9
IFNγ	Mouse spleen	Con A (3 µg/mL) (2 d)/IL-2 (20 ng/mL) + IL-4 (20 ng/mL) (3 d)	2 d/3 d	Anti-CD3 (10 µg/mL immobilized) + anti-CD28 (2 µg/mL soluble) (5 hr)	Brefeldin A	XMG1.2
IL-1α	Mouse PEC	mIFNγ (100 ng/mL) (2 hr)/LPS (100 ng/mL) (22 hr)	2 hr/22 hr	–	Brefeldin A	ALF-161
IL-1β	Mouse PEC	LPS (100 ng/mL) (22 hr)	22 hr	–	Monensin	NJTEN3
IL-2	Mouse spleen	Con A (3 µg/mL) (2 d)/IL-2 (20 ng/mL) + IL-4 (20 ng/mL) (3 d)	2 d/3 d	Anti-CD3 (10 µg/mL immobilized) + anti-CD28 (2 µg/mL soluble) (5 hr)	Brefeldin A	JES6-5H4
IL-4	Mouse spleen	Th2 polarized	6 d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) (5 hr)	Brefeldin A	BVD6-24G2, 11B11
IL-5	Mouse splenic CD4	Con A (3 µg/mL) (2 d)/IL-2 (20 ng/mL) + IL-4 (20 ng/mL) (3 d)	2 d/3 d	Anti-CD3 (10 µg/mL immobilized) + anti-CD28 (2 µg/mL soluble) (5 hr)	Brefeldin A	TRFK5
IL-6	Mouse PEC	LPS (100 ng/mL) (22 hr)	22 hr	–	Monensin	MP5-20F3
IL-10	Mouse spleen	Con A (3 µg/mL) (2 d)/IL-2 (20 ng/mL) + IL-4 (20 ng/mL) (3 d)	2 d/3 d	Anti-CD3 (10 µg/mL immobilized) + anti-CD28 (2 µg/mL soluble) (5 hr)	Brefeldin A	JES5-16E3, JES5-2A5
IL-12/IL-23 (p40)	Mouse PEC	LPS (100 ng/mL) (22 hr)	22 hr	–	Brefeldin A	C17.8
IL-13	Mouse spleen	Th2 polarized	6 d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) (5 hr)	Brefeldin A	eBio13A
IL-17A	Mouse spleen	Th17 polarized	6 d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) (5 hr)	Monensin	eBio17B7
IL-17F	Mouse spleen	Th17 polarized	6 d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) (5 hr)	Monensin	eBio18F10
IL-21	Mouse spleen	Th17 polarized	9 d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) (5 hr)	Monensin	FFA21
IL-22	Mouse spleen	Th17 polarized	12 d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) (5 hr)	Brefeldin A	IL22JOP
IL-23 p19	Mouse bone marrow	mGM-CSF (40 ng/mL)	8 d	LPS (1 µg/mL) (24 hr)	Monensin	fc23cpg
TNFα	Mouse spleen	Con A (3 µg/mL) (2 d)/IL-2 (20 ng/mL) + IL-4 (20 ng/mL) (3 d)	2 d/3 d	Anti-CD3 (10 µg/mL immobilized) + anti-CD28 (2 µg/mL soluble) (5 hr)	Brefeldin A	MP6-XT22, TN3-19

Annotations: mouse PEC = mouse thioglycolate-elicited peritoneal macrophages; con A = concanavalin A; LPS = lipopolysaccharide; PMA = phorbol myristate acetate; 2 d = 2-day culture; 3 d = 3-day culture; 5 hr = 5-hour culture.

Table 4. Human cytokine stimulation guide.

Human cytokine	Cell source	Activation	Incubation time	Restimulation	Intracellular block	Clone (antibody)
G-CSF	PBMC	LPS (1 µg/mL)	24 hr	–	Monensin	8F5CSF
GM-CSF	PBMC	PMA (30–50 µg/mL) /Ionomycin (1 µg/mL)	5 hr	–	Monensin	BVD2-21C11
IFNγ	PBMC	PMA (30–50 µg/mL) /Ionomycin (1 µg/mL)	5 hr	–	Brefeldin A	4S.B3
IL-1α	PBMC	LPS (1 µg/mL)	24 hr	–	Monensin	364/3B3-14, CRM8
IL-1β	PBMC	LPS (100 µg/mL)	4 hr	–	Brefeldin A	CRM56
IL-1RA	PBMC	LPS (100 µg/mL)	24 hr	–	Brefeldin A	CRM17
IL-2	PBMC	PMA (30–50 µg/mL) /Ionomycin (1 µg/mL)	4–6 hr	–	Brefeldin A	MQ1-17H12
IL-4	PBMC	PMA (30–50 µg/mL) /Ionomycin (1 µg/mL)	4–6 hr	–	Brefeldin A	8D4-8
IL-5	CD4	Th2-polarizing cultures	6 d	PMA (50 µg/mL) + Ionomycin (1 µg/mL) (5 hr)	Brefeldin A	TRFK5, JES1-5A10
IL-6	PBMC	LPS (100 µg/mL)	24 hr	–	Brefeldin A	MQ2-13A5
IL-9	CD4	Th2-polarizing cultures	6 d	PMA (50 µg/mL) + Ionomycin (1 µg/mL) (5 hr)	Monensin	MH9A4
IL-10	CD4	Th2-polarizing cultures	6 d	PMA (50 µg/mL) + Ionomycin (1 µg/mL) (5 hr)	Monensin	JES3-9D7
IL-12/IL-23 (p40)	PBMC	hIFNγ (100 µg/mL) (2 hr)/LPS (100 µg/mL) (22 hr)	2 hr/22 hr	–	Brefeldin A	C8.6
IL-13	CD4	Anti-CD3 (10 µg/mL, immobilized) + anti-CD28 (2 µg/mL, soluble) + IL-2 (10 µg/mL) + IL-4 (20 µg/mL) (2 d); IL-2 (10 µg/mL) + IL-4 (20 µg/mL) (3 d)	2 d/3 d	PMA (5 µg/mL) + Ionomycin (500 µg/mL) (4 hr)	Brefeldin A	PVM13-1
IL-17A	PBMC	Th17-polarizing cultures	6 d	PMA (50 µg/mL) + Ionomycin (1 µg/mL) (5 hr)	Brefeldin A	eBio64CAP17, eBio64DEC17
IL-17F	PBMC	Th17-polarizing cultures	6 d	PMA (50 µg/mL) + Ionomycin (1 µg/mL) (5 hr)	Brefeldin A	SHLR17
IL-21	PBMC	PMA (30–50 µg/mL)/ Ionomycin (1 µg/mL)	4–7 hr or 12–18 hr	–	Brefeldin A	eBio3A3-N2
IL-22	CD4	Th17-polarizing cultures	6 d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) (5 hr)	Brefeldin A	IL22JOP
IL-23 p19	PBMC	hGM-CSF (40 µg/mL) + hIL-4 (40 µg/mL)	6 d	LPS (1 µg/mL) (24 hr)	Monensin	23dcdp
MCP-1/CCL2	PBMC	LPS (1 µg/mL)	24 hr	–	Monensin	2H5, 5D3-F7
RANTES/CCL5	PBMC	LPS (1 µg/mL)	24 hr	–	Monensin	VL1
TNFα	PBMC	PMA (30–50 µg/mL) /Ionomycin (1 µg/mL)	5 hr	–	Brefeldin A	MAb11
TNFβ	PBMC	Th1-polarizing cultures	6 d	PMA (50 µg/mL) + Ionomycin (1 µg/mL) (5 hr)	Monensin	359-81-11

Chemokine antibodies

Table 5. Invitrogen™ eBioscience™ chemokine antibodies.*

Antigen	Clone	Cat. No.	Purified	Functional grade	Biotin	Violet laser: eFluor 450	Blue laser: FITC or Alexa Fluor 488	Blue laser: PerCP-Cyanine5.5	Blue laser: PerCP-eFluor 710	Green, yellow-green lasers: PE	PE-eFluor 610	PE-Cyanine5	PE-Cyanine5.5	PE-Cyanine7	Alexa Fluor 532	Red laser: APC or eFluor 660	Alexa Fluor 700	APC-eFluor 780
Mouse																		
CCL2 (MCP-1)	2H5	7096	■	■	■		■			■								
CCL3 (MIP-1α)	DNT3CC	7532							■	■						■		
CXCL9 (MIG)	MIG-2F5.5	3009								■						■		
Human																		
CCL2 (MCP-1)	5D3-F7	7099	■							■						■		
CCL3 (MIP-1α)	CR3M	9706					■			■								
CCL4 (MIP-1β)	FL34Z3L	7540							■							■		
CCL5 (RANTES)	VL1	9905														■		
CCL7 (MCP-3)	OLGMASCE	7077								■						■		
CCL8 (MCP-2)	DWZEE	9789														■		
CXCL1 (GROα)	KTYFLF	7515														■		
CXCL10 (IP-10)	4NY8UN	9744							■	■								

* Table is not exhaustive. Search the full catalog of flow cytometry antibodies at **thermofisher.com/flowantibodies**

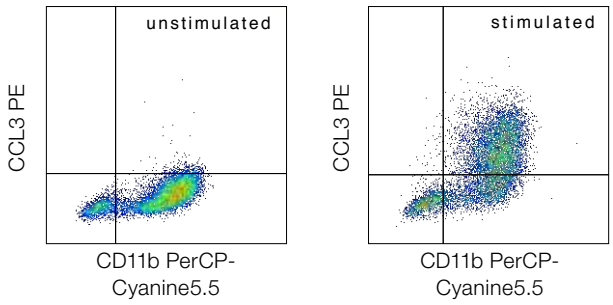


Figure 2. Staining of mouse CCL3 (MIP-1α) in macrophages. BALB/c thioglycolate-elicited peritoneal macrophages were unstimulated (left) or stimulated for 5 hours with LPS (Cat. No. 00-4976) (right) in the presence of Invitrogen™ eBioscience™ Protein Transport Inhibitor Cocktail (Cat. No. 00-4980). Cells were intracellularly stained with Anti–Mouse CD11b PerCP-Cyanine5.5 (Cat. No. 45-0112) and Anti–Mouse CCL3 (MIP-1α) PE (Cat. No. 12-7532) using the Invitrogen™ eBioscience™ Intracellular Fixation & Permeabilization Buffer Set (Cat. No. 88-8824) and protocol. Total viable cells, as determined by the Invitrogen™ eBioscience™ Fixable Viability Dye eFluor™ 450 (Cat. No. 65-0863), were used for analysis.

Effector molecule antibodies

Table 6. Invitrogen™ eBioscience™ effector molecule antibodies.*

Antigen	Clone	Cat. No.	Purified	Functional grade	Biotin	Violet laser: eFluor 450	Blue laser: FITC or Alexa Fluor 488	Blue laser: PerCP-Cyanine5.5	Blue laser: PerCP-eFluor 710	Green, yellow-green lasers: PE	Green, yellow-green lasers: PE-eFluor 610	Green, yellow-green lasers: PE-Cyanine5	Green, yellow-green lasers: PE-Cyanine5.5	Green, yellow-green lasers: PE-Cyanine7	Green, yellow-green lasers: Alexa Fluor 532	Red laser: APC or eFluor 660	Red laser: Alexa Fluor 700	Red laser: APC-eFluor 780
Mouse																		
Granzyme A	GzA-3G8.5	5831				■			■	■				■		■		
Granzyme B	NGZB	8898				■	■		■	■				■		■		
IDO	mIDO-48	9473							■							■		
NOS2 (iNOS)	CXNFT	5920	■				■			■	■			■		■		
Perforin	eBioOMAK-D	9392	■				■			■						■		
Human																		
Granulysin	eBioDH2 (DH2)	8828								■								
Granzyme A	CB9	9177												■		■		
Granzyme B	GB11	8899								■								
Granzyme K	G3H69	8897							■							■		
Granzyme M	4B2G4	9774														■		
IDO	eyedio	9477							■	■						■		
Myeloperoxidase (MPO)	MPO455-8E6	1299	■			■	■			■								
PARP (Cleaved)	HLNC4	6668	■			■	■			■								
Perforin	dG9 (delta G9)	9994	■				■		■	■				■		■		

* Table is not exhaustive. Search the full catalog of flow cytometry antibodies at **thermofisher.com/flowantibodies**

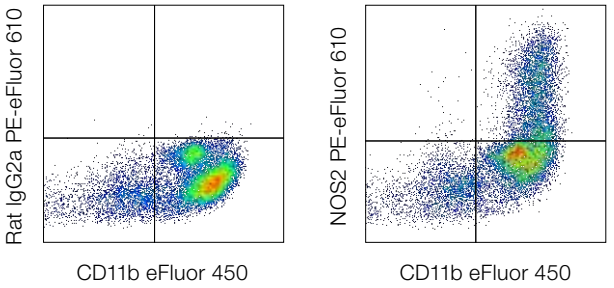


Figure 3. Staining of mouse NOS2 (iNOS) in stimulated thioglycolate-elicited peritoneal exudate cells. Mouse thioglycolate-elicited peritoneal macrophages were stimulated overnight with LPS (Cat. No. 00-4976), then surface stained with the Invitrogen™ eBioscience™ Anti–Mouse CD11b eFluor™ 450 conjugate (Cat. No. 48-0112), followed by fixation and permeabilization with the Intracellular Fixation & Permeabilization Buffer Set (Cat. No. 88-8824) and protocol. The cells were then intracellularly stained with the Invitrogen™ eBioscience™ Rat IgG2a K Isotype Control PE-eFluor™ 610 (Cat. No. 61-4321 [left]) or Anti–Mouse NOS2 PE-eFluor™ 610 (Cat. No. 61-5920 [right]) conjugate. Total viable cells, as determined by the Invitrogen™ eBioscience™ Fixable Viability Dye eFluor™ 506 (Cat. No. 65-0866), were used for analysis.

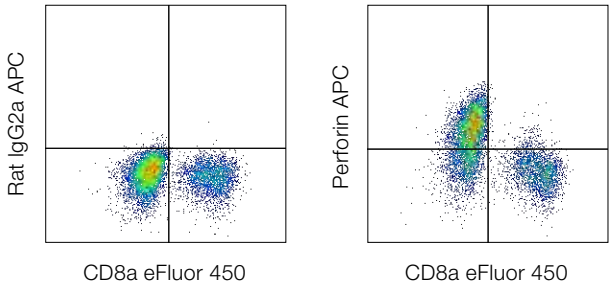


Figure 4. Staining of mouse perforin in stimulated splenocytes. BALB/c splenocytes were stimulated for 4 days with Invitrogen™ eBioscience™ Mouse IL-2 Recombinant Protein (Cat. No. 14-8021). Cells were harvested and stained with the Invitrogen™ eBioscience™ Anti–Mouse CD8a eFluor™ 450 conjugate (Cat. No. 48-0081) followed by fixation and permeabilization using the Intracellular Fixation & Permeabilization Buffer Set (Cat. No. 88-8824) and protocol. Cells were subsequently stained intracellularly with Rat IgG2a K Isotype Control APC (Cat. No. 17-4321 [left]) or Anti–Mouse Perforin APC (Cat. No. 17-9392 [right]). Cells in the lymphocyte gate were used for analysis.

Other cytoplasmic antibodies

Table 7. Other Invitrogen™ eBioscience™ cytoplasmic antibodies.*

Antigen	Clone	Cat. No.	Purified	Functional grade	Biotin	Violet laser: eFluor 450	Blue laser: FITC or Alexa Fluor 488	Blue laser: PerCP-Cyanine5.5	Blue laser: PerCP-eFluor 710	Green, yellow-green lasers: PE	Green, yellow-green lasers: PE-eFluor 610	Green, yellow-green lasers: PE-Cyanine5	Green, yellow-green lasers: PE-Cyanine5.5	Green, yellow-green lasers: PE-Cyanine7	Green, yellow-green lasers: Alexa Fluor 532	Red laser: APC or eFluor 660	Red laser: Alexa Fluor 700	Red laser: APC-eFluor 780
Mouse																		
CD63	NVG-2	0631							■					■		■		
CD79a	24C2.5	0791	■				■									■		
CD107a (LAMP-1)	eBio1D4B	1071	■		■	■	■		■	■						■		
CD107b (LAMP-2)	eBioABL-93	1072	■		■		■									■		
CD152 (CTLA-4)	UC10-4B9	1522	■		■					■						■		
CD289 (TLR9)	M9.D6	9093	■		■		■											
Cytochrome C	6H2	6601	■				■											
Notch1	mN1A	5785	■		■					■								
IκBα	MFRDTRK	9036								■								
Themis	1TMYS	5918								■						■		
ZAP-70	1E7.2	6695	■		■		■			■						■		
Human																		
CD63	H5C6	0639				■				■				■		■		
CD68	eBioY1/82A	0689	■		■		■			■				■				
CD79a	HM47	0792						■	■	■								■
CD107a (LAMP-1)	eBioH4A3	1079	■		■	■	■		■	■	■	■				■		
CD107b (LAMP-2)	eBioH4B4	1078	■		■		■									■		
CD152 (CTLA-4)	14D3	1529	■	■	■				■	■								
CD208 (DC-LAMP)	31B	2089														■		
CD217 (IL-17Ra)	424LTS	7917		■					■							■		
CD247 (CD3ζ)	6B10.2	2479							■	■								
CD289 (TLR9)	eB72-1665	9099	■							■						■		
Cytochrome C	6H2	6601	■				■											
FREB (FCRLA, FcRX)	N28.1	5847	■															
LAT	LAT.10-17	9967	■							■						■		
Notch1	mN1A	5785	■		■					■								
IκBα	MFRDTRK	9036								■								
SAP	XLP-1D12	9787								■						■		
SYK	4D10.1	6696	■							■						■		
ZAP-70	1E7.2	6695	■		■		■			■						■		

* Table is not exhaustive. Search the full catalog of flow cytometry antibodies at **thermofisher.com/flowantibodies**

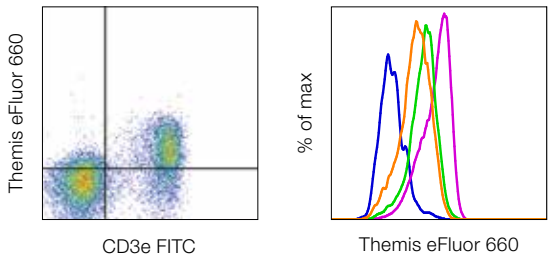


Figure 5. Staining of mouse themis in double-negative, double-positive, CD8⁺ single-positive, and CD4⁺ single-positive thymocytes. Left: Surface staining of mouse splenocytes with Anti–Mouse CD3e FITC (Cat. No. 11-0031) was followed by intracellular staining with the Invitrogen™ eBioscience™ Anti–Mouse Themis eFluor™ 660 conjugate (Cat. No. 50-5918), using the Intracellular Fixation & Permeabilization Buffer Set (Cat. No. 88-8824) and protocol. Cells in the lymphocyte gate were used for analysis; quadrant lines are based on isotype control. Right: Mouse thymocytes were surface stained with Anti–Mouse CD4 FITC (Cat. No. 11-0042), Invitrogen™ eBioscience™ Anti–Mouse CD8a eFluor™ 450 (Cat. No. 48-0081), Anti–Mouse CD25 PerCP-Cyanine5.5 (Cat. No. 45-0251), and Anti–Human/Mouse CD44 PE-Cyanine7 (Cat. No. 25-0441) conjugates, followed by staining with the Fixable Viability Dye eFluor 506 (Cat. No. 65-0866). The cells were then intracellularly stained with Anti–Mouse Themis eFluor 660 conjugate (Cat. No. 50-5918), using the Intracellular Fixation & Permeabilization Buffer Set (Cat. No. 88-8824) and protocol. Single, viable cells in the $CD4^-$ $CD8^-$ $CD25^-$ $CD44^+$ double-negative (blue histogram), $CD4^+$ $CD8^+$ double-positive (pink histogram), $CD8^+$ single-positive (green histogram), or $CD4^+$ single-positive (orange histogram) gates were used for analysis.

Nuclear staining

Transcription factors

Transcription factors are DNA-binding proteins that regulate gene expression by modulating the synthesis of messenger RNA. A greater understanding of the expression and regulation of transcription factor activity in immune cells may reveal new cell types and novel therapeutic opportunities.

The detection of transcription factors in populations at low frequencies is challenging when using other protein detection methods such as western blotting. This is further complicated when determining the cellular transcription factor expression in heterogeneous cell populations. Fortunately, flow cytometry permits the detection of transcription factors within discrete immune cell subsets among a heterogeneous population and provides a powerful approach to analyzing an immune response. Refer to the following section on intracellular staining buffers prior to any transcription factor staining analysis.

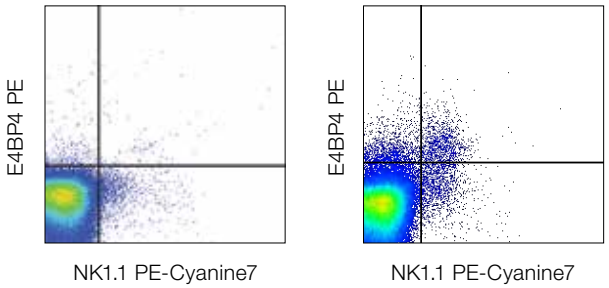


Figure 6. Staining of mouse E4BP4 (NFIL3) in stimulated NK cells. Mouse splenocytes unstimulated (left) or stimulated overnight with Invitrogen™ eBioscience™ Mouse IL-15/IL-15R Complex Carrier-Free Recombinant Protein (Cat. No. 34-8152 [right]) were stained with Anti–Mouse NK1.1 PE-Cyanine7 (Cat. No. 25-5941) and Anti–Mouse E4BP4 (NFIL3) PE (Cat. No. 12-5927). Intracellular staining for E4BP4 was performed using the Invitrogen™ eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523) and protocol. Cells in the lymphocyte gate were analyzed.

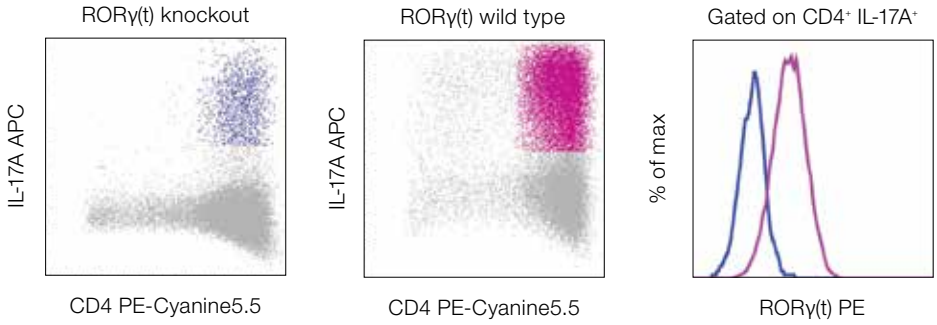


Figure 7. Identification of Th17 cells by flow cytometric detection of RORγ(t). $CD4^+$ T cells were sorted from RORγ(t)-deficient (left plot) or wild type (middle plot) mouse spleen and lymph nodes, cultured in Th17-polarizing conditions for 3 days, and stained with Anti–Mouse CD4 PE-Cyanine5.5 (Cat. No. 35-0042), Anti–Mouse IL-17A APC (Cat. No. 17-7177), and Anti–Human/Mouse RORγ(t) PE (Cat. No. 12-6988). The histogram shows staining of RORγ(t), gated on $CD4^+$ $IL\text{-}17A^+$ events from RORγt-deficient mice (blue line) and wild type mice (pink line) (right plot). Cells in the lymphocyte gate were used for analysis. Data provided courtesy of Dr. Littman, New York University.

Transcription factor/nuclear antibodies

Table 8. Invitrogen™ eBioscience™ mouse transcription factor/nuclear antibodies.*

Mouse																			
						Violet laser	Blue laser			Green, yellow-green lasers						Red laser		
Antigen	Clone	Cat. No.	Purified	Functional grade	Biotin	eFluor 450	FITC or Alexa Fluor 488	PerCP-Cyanine5.5	PerCP-eFluor 710	PE	PE-eFluor 610	PE-Cyanine5	PE-Cyanine5.5	PE-Cyanine7	Alexa Fluor 532	APC or eFluor 660	Alexa Fluor 700	APC-eFluor 780
AHR	4MEJJ	5925					■			■						■		
Aiolos	8B2	5789	■							■						■		
AIRE	5H12	5934	■				■									■		
Bcl6	BCL-DWN	5453							■	■						■		
β-Catenin	15B8	2567	■				■			■						■		
BrdU	BU20A	5071	■			■	■		■	■						■		
c-Maf	sym0F1	9855							■							■		
c-Rel	1RELAH5	6111	■							■						■		
E4BP4 (NFIL3)	S2M-E19	5927								■								
Egr2	erongr2	6691								■						■		
EOMES	Dan11mag	4875	■			■	■		■	■	■			■		■		
Eos	ESB7C2	5758								■						■		
Foxp3	FJK-16s	5773	■		■	■	■	■		■	■	■	■	■	■	■	■	
Foxp3	150D/E4	4774	■				■			■						■		
GATA-3	TWAJ	9966	■						■	■						■		
Helios	22F6	9883				■	■		■	■	■					■		
IκBζ	LK2NAP	6801	■						■									
IRF4	3E4	9858	■			■	■		■	■						■		
IRF8	V3GYWCH	9852							■	■						■		
Ki-67	SolA15	5698	■		■	■	■		■	■	■			■		■		
Nanog	eBioMLC-51	5761	■				■									■		
Nur77	12.14	5965	■				■		■	■								
Oct3/4	EM92	5841	■				■			■						■		
Pax5	1H9	9918	■							■						■		
PLZF	Mags.21F7	9320					■			■								
Sox2	Btjce	9811	■				■									■		
RORγ(t)	B2D	6981	■						■	■	■					■		
Runx1	RXDMC	9816								■								
T-bet	eBio4B10	5825	■					■		■				■		■		
TdT	19-3	5846								■								
ThPOK	2POK	5928								■								
TOX	TXRX10	6502	■							■						■		

* Table is not exhaustive. Search the full catalog of flow cytometry antibodies at **thermofisher.com/flowantibodies**

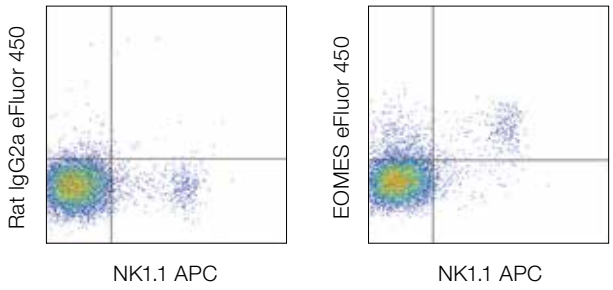


Figure 8. Staining of mouse EOMES in splenocytes. C57Bl/6 splenocytes were surface-stained with Anti–Mouse NK1.1 APC (Cat. No. 17-5941), followed by fixation and permeabilization using the Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523) and protocol. Cells were intracellularly stained with the Invitrogen™ eBioscience™ Rat IgG2a K Isotype Control eFluor™ 450 (Cat. No. 48-4321 [left]) or Anti–Mouse EOMES eFluor™ 450 (Cat. No. 48-4875 [right]) conjugate. Cells in the lymphocyte gate were used for analysis.

Table 9. Invitrogen™ eBioscience™ human transcription factor/nuclear antibodies.*

Human						Violet laser	Blue laser			Green, yellow-green lasers						Red laser		
Antigen	Clone	Cat. No.	Purified	Functional grade	Biotin	eFluor 450	FITC or Alexa Fluor 488	PerCP-Cyanine5.5	PerCP-eFluor 710	PE	PE-eFluor 610	PE-Cyanine5	PE-Cyanine5.5	PE-Cyanine7	Alexa Fluor 532	APC or eFluor 660	Alexa Fluor 700	APC-eFluor 780
AHR	FF3399	9854	■						■	■				■		■		
BATF	MBM7C7	9860							■	■						■		
Bcl2	10C4	6992	■			■								■		■		
Bcl6	BCL-UP	9880							■							■		
β-Catenin	15B8	2567	■				■			■						■		
BrdU	BU20A	5071	■			■	■		■	■						■		
c-Maf	sym0F1	9855							■							■		
E4BP4 (NFIL3)	MABA223	9812								■								
Egr1	HEGR1DS	9851							■	■								
EOMES	WD1928	4877					■		■	■	■					■		
Foxp3	150D/E4	4774	■				■			■						■		
Foxp3	236A/E7	5774	■		■	■	■			■				■		■		
Foxp3	PCH101	4776	■		■	■	■	■		■	■		■	■	■	■	■	
GATA-3	TWAJ	9966	■						■	■						■		
Helios	22F6	9883				■	■		■	■						■		
IκBζ	hft2nap	9853								■								
IRF4	3E4	9858	■			■	■		■	■						■		
IRF5	ALYSCLN	9698														■		
IRF8	V3GYWCH	9852							■	■						■		
Ki-67	20Raj1	5699	■		■	■	■		■	■	■			■		■		
OCT3/4	EM92	5841	■				■			■						■		
Pax5	1H9	9918	■							■						■		
PCNA	PC10 (3F81)	9910	■		■		■			■								
PLZF	Mags.21F7	9320					■			■								
Sox2	Btjce	9811	■				■									■		
Survivin	STLALYV	9176	■				■		■	■						■		
RORγ(t)	AFKJS-9	6988	■							■						■		
Runx1	RXDMC	9816								■								
T-bet	eBio4B10	5825	■					■		■				■		■		
TdT	19-3	5846								■								
TOX	TXRX10	6502	■							■						■		

* Table is not exhaustive. Search the full catalog of flow cytometry antibodies at **thermofisher.com/flowantibodies**

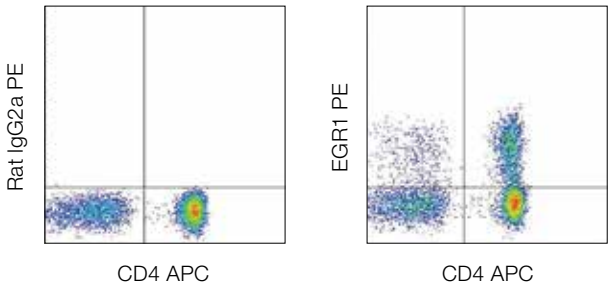


Figure 9. Staining of human Egr1 in activated T cells. Normal human peripheral blood cells were stimulated with Human CD3 Functional Grade, Purified Antibody (Cat. No. 16-0037) for 2 hours. Cells were then intracellularly stained with Anti–Human CD4 APC (Cat. No. 17-0047) and Rat IgG2a K Isotype Control PE (Cat. No. 12-4321 [left]) or Anti–Human EGR1 PE (Cat. No. 12-9851 [right]), using the Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523) and protocol. Cells in the lymphocyte gate were used for analysis.

Intracellular staining buffer selection

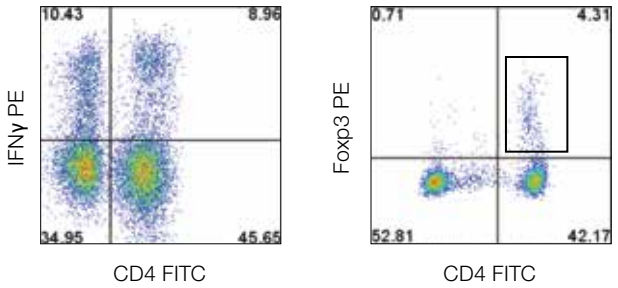
The selection of fixation and permeabilization buffer systems has a significant impact on the quality and accuracy of your flow cytometry data when performing intracellular staining. The location of the target proteins within the cell is an important consideration in the selection of the appropriate buffer, and Invitrogen™ eBioscience™ buffer sets are optimized based on the types and activation state of the the proteins, such as the nuclear proteins, cytoplasmic, and secreted proteins you are looking to detect in your analysis. For example, to obtain optimal staining of a transcription factor, the Foxp3/Transcription Factor Staining Buffer Set is recommended. However, secreted proteins such as cytokines and chemokines work best with the Intracellular Fixation & Permeabilization Buffer Set. When staining proteins that localize to different regions of a cell, the correct buffer choice becomes more challenging. Each antibody should be optimized independently to validate the staining pattern. The following chart provides general rules to choose the appropriate buffer system.

Need more help selecting the appropriate buffer?
Visit **thermofisher.com/icflowbufferguide** to see an intracellular staining buffer selection guide to determine buffer compatibility with an intracellular antigen.

Intracellular staining protocols at-a-glance

Cytoplasmic staining (cytokines)	Nuclear staining (transcription factors) -OR- Nuclear and cytoplasmic (cytokines and transcription factors)
Stain surface proteins	Stain surface proteins
Fix cells	Fix + permeabilize cells
Permeabilize cells	Wash
Wash	Stain proteins
Stain proteins	
Intracellular Fixation & Permeabilization Buffer Set (Cat. No. 88-8824) Kit components: IC Fixation Buffer Permeabilization Buffer (10X)	**Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523) Kit components:** Fixation/Permeabilization Concentrate Fixation/Permeabilization Diluent Permeabilization Buffer (10X)

Foxp3/Transcription Factor Staining Buffer Set



Intracellular Fixation & Permeabilization Buffer Set

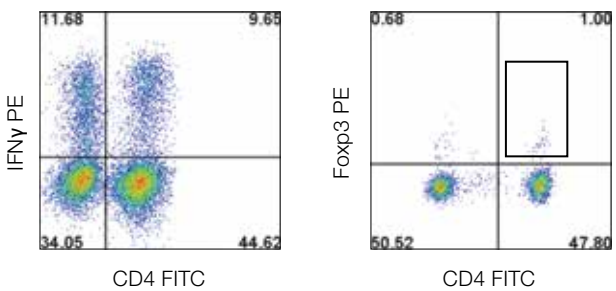


Figure 10. Evaluating buffer system performance for anti-Foxp3 and INFγ antibody staining. Cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523 [left]) and the Intracellular Fixation & Permeabilization Buffer Set (Cat. No. 88-8824 [right]) followed by intracellular staining with Anti–Human CD4 FITC (Cat. No. 11-0042) and Anti–Human IFNγ PE (Cat. No. 12-7311) or Anti–Human Foxp3 PE (Cat. No. 12-5773). Cells in the lymphocyte gate were used for analysis.

Clone performance after fixation and permeabilization

Surface staining after fixation is possible, but is dependent on the effects of fixation on each antibody's epitope. Furthermore, not every clone against the same target will perform similarly following fixation and permeabilization. The table below contains information regarding the performance of different clones after using the Invitrogen™ eBioscience™ Intracellular (IC) Fixation & Permeabilization Buffer Set (Cat. No. 88-8824), or after using IC Fixation Buffer (Cat. No. 00-8222) followed by treatment with methanol. Please note that staining after fixation may allow intracellular stores of protein to also be stained with the antibody.

Table 10. Staining performance of different clones after fixation and permeabilization.

Human				
Antigen	Clone	Live cells before IC fixation	After IC Fixation & Perm. Buffer Set	After IC Fixation Buffer/ methanol
CD3	OKT3	+++	++	+
	UCHT1	+++	+++	+++
	SK7	+++	++*	+++
	HIT3a	+++	+++*	ND
CD4	OKT4	+++	+*	+/–
	RPA-T4	+++	+++*	+
	SK3	+++	+++	+
CD5	UCHT2	+++	+++	+++
CD8	OKT8	+++	+/–	–
	RPA-T8	+++	+++	+/–
	SK1	+++	+++*	+/–
CD8b	SIDI8BEE	+++	++	++
CD11a	HI111	+++	+++	+
CD11b	CBRM1/5	+	–	+++
	ICRF44	++	–	++
CD11c	3.9	++	+*	++
CD14	61D3	+++	+++*	++
CD19	HIB19	+++	++*	+/–
	SJ25C1	+++	++	ND
CD20	2H7	+++	+++	–
CD25	BC96	+	–	+/–
	CD25-4E3	++	++	ND
CD27	O323	+++	+*	+
CD28	CD28.2	+	–	ND
CD31	WM59	+++	++*	+++
CD33	p67.6	+++	+++*	+++
CD38	HB7	++	–	++
	HIT2	++	–	++
CD40	5C3	++	–	++
CD44	IM7	+++	+++	+++
CD45	2D1	+++	+++	+++
	HI30	+++	+++	+++
CD45RA	HI100	+++	+++	+++
CD45RO	UCHL1	+++	+++	++
CD62L	DREG-56	++	–	++
CD56	TULY56	++	+	ND
	CMSSB	++	+/–*	–
CD57	TB01	++	++	++
CD62L	DREG56	++	–	ND
CD69	FN50	+++	++	++
CD80	2D10.4	+	–	+
CD83	HB15e	++	++	++
CD86	IT2.2	+	+*	+
CD94	HP-3D9	++	++	++
CD95	DX2	+	–	+
CD127	RDR5	++	+*	++
CD161	HP-3G10	++	++*	+
CD223	3DS223H	++	++	ND

Mouse				
Antigen	Clone	Live cells before IC Fixation	After IC Fixation & Perm. Buffer Set	After IC Fixation Buffer/ methanol
CD3	145-2C11	++	+	+/–
	500A2	+++	+++*	+++
	17A2	+++	+++	+++
CD4	GK1.5	+++	++*	+++
	RM4-5	+++	+++*	+++
CD8	53-6.7	+++	++	++
CD11b	M1/70	++	++	++
CD11c	N418	+++	+++	++
CD19	1D3	+++	++*	–
	MB19-1	++	–	–
CD24	M1/69	+++	+++	++
CD25	PC61.5	+	–	+
	3C7	++	–	+
	7D4	++	++	+
CD39	24DMS1	+	–	++
CD44	IM7	++	++*	++
CD45	30-F11	+++	+++	+++
CD45R (B220)	RA3-6B2	+++	++	+++
CD49b	DX5	++	–	+
CD69	H1.2F3	++	–	–
CD185 (CXCR5)	SPRCL5	++	–	–
CD304	3DS304M	++	++	++
GR-1	RB6-8C5	+++	+++	++
NK1.1	PK136	++	+	+
IgD	11-26c	+++	+++*	++
IgM	II/41	++	++*	++
TCRβ	H57-597	++	++*	++
Rat				
CD25	OX39	+	–	ND
Canine				
CD4	ykix302.9	+++	+	ND
CD5	ykix322.3	+++	–	ND
CD8a	ycate55.9	+++	++	ND
CD44	ykix337.8	+++	++	ND
CD45	ykix716.13	+++	++	ND
CD45R	ykix753.22	+++	++	ND
CD90	ykix337.217	+++	++	ND
MHC class II	ykix334.2	+++	+++	ND

* Longer fixations are detrimental to staining for these clones.
ND = not determined

Phosphospecific flow cytometry

Validated for success

Flow cytometric analysis of phosphorylated proteins using phosphospecific antibodies provides researchers with an effective way to elucidate signaling cascades in individual cells. Invitrogen™ eBioscience™ phosphospecific antibodies for flow cytometry have been validated* in a variety of samples, conditions, and platforms to give scientists the confidence that these antibodies will perform robustly and reliably.

Validation includes:

- Pathway-specific testing
- Cell type–specific testing
- Application testing: western blot, ELISA, and immunochemistry
- Performance in different intracellular fixation/permeabilization buffers
- Mouse and human cross-reactivity testing

Pathway-specific testing

Phosphospecific staining is observed only in cells in which the pathway of interest has been activated.

Cell type–specific testing

Phosphospecific staining is observed only in cell types in which the protein is expressed.

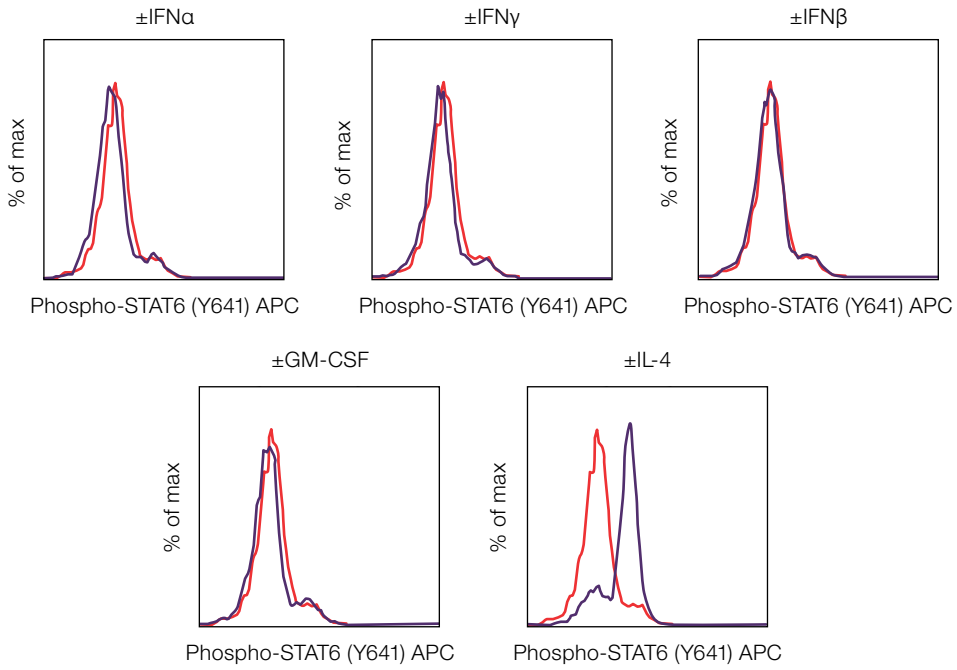


Figure 11. Staining of phospho-STAT6 in U937 cells stimulated with a variety of cytokines. Intracellular staining of untreated (red histogram) or treated (purple histogram) U937 cells with Anti–Human/Mouse Phospho-STAT6 APC (Cat. No. 17-9013). Cells were treated with cytokines, as indicated.

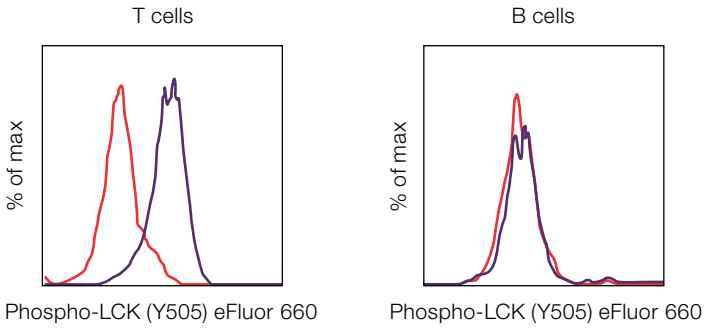


Figure 12. T cell–specific staining of phospho-LCK (Y505) in stimulated PBMCs. Human PBMCs were left untreated (red histogram) or were treated with hydrogen peroxide–activated sodium pervanadate (purple histogram) for 5 minutes at 37°C. Cells were then intracellularly stained with Anti–Human/Mouse Phospho-LCK (Y505) eFluor™ 660 (Cat. No. 50-9076), Anti–Human CD3 PE (Cat. No. 12-0037), and Anti–Human CD19 FITC (Cat. No. 11-0199) conjugates. As expected, LCK phosphorylation is observed only in T cells but not in B cells.

* The use or any variation of the word "validation" refers only to research use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. It does not ensure that the product(s) was validated for clinical or diagnostic uses.

Application testing

Western blotting confirms the presence of protein at the appropriate molecular weight in treated cells and its absence in untreated cells. Whenever possible, inhibitors are used to ensure specificity.

Performance in various intracellular fixation and permeabilization buffers

To maximize compatibility with other antibody staining, each phosphospecific antibody is tested in three different intracellular flow cytometry buffer systems: Intracellular Fixation & Permeabilization Buffer Set (Cat. No. 88-8824), Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523), and Intracellular Fixation Buffer (Cat. No. 00-8222) followed by methanol permeabilization. The recommended buffer system(s) are noted on the Technical Data Sheet for each antibody.

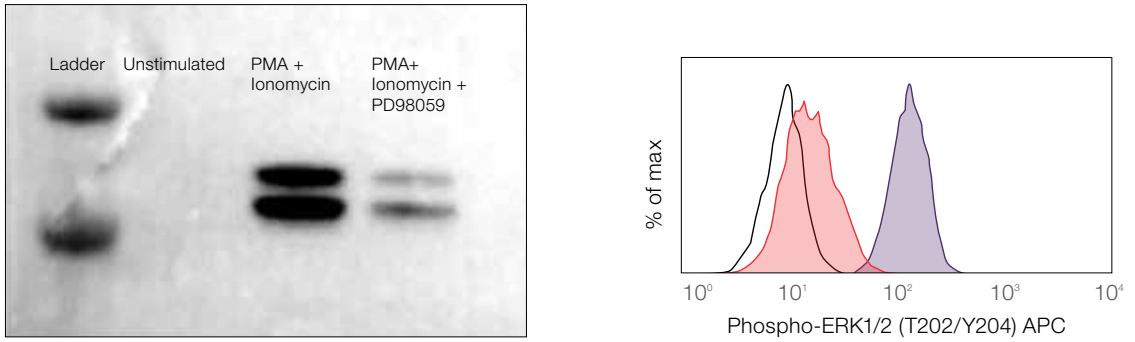


Figure 13. Comparison of western blotting versus flow cytometry. Left: Western blotting of reduced lysates from Jurkat cells unstimulated (left lane), stimulated with PMA and Ionomycin (middle lane), or stimulated with PMA and Ionomycin in the presence of the MEK1/2 inhibitor PD98059 (right lane) using Anti–Human/Mouse Phospho-ERK1/2 (T202/Y204) (Cat. No. 14-9109). Right: Intracellular staining of Jurkat cells that were unstimulated (black histogram), stimulated with PMA and Ionomycin (purple histogram), or stimulated with PMA and Ionomycin in the presence of the MEK1/2 inhibitor PD98059 (red histogram) with Anti–Human/Mouse Phospho-ERK1/2 (T202/Y204) APC (Cat. No. 17-9109).

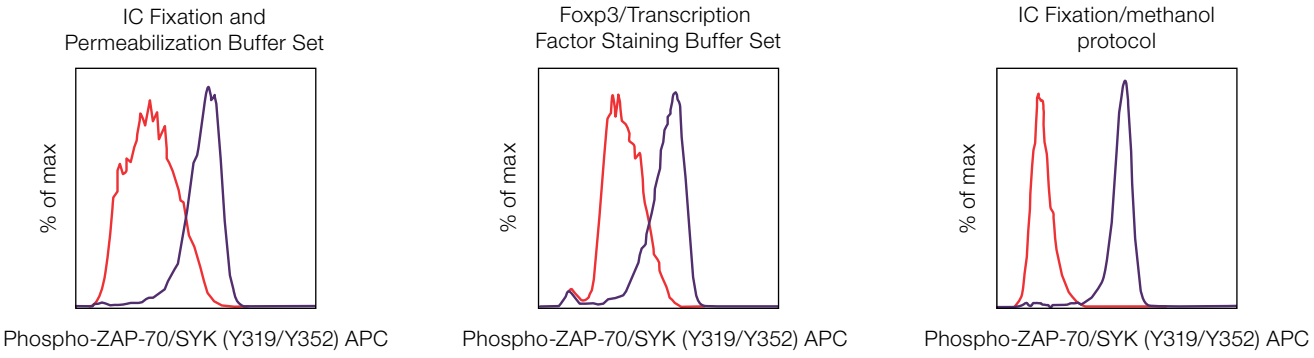


Figure 14. Performance of Phospho-ZAP-70/SYK (Y319/Y352) APC in various intracellular fixation and permeabilization buffers. Jurkat cells were untreated (red histogram) or were treated (purple histogram) with hydrogen peroxide–activated sodium pervanadate for 5 minutes at 37°C. Cells were then divided and either fixed and permeabilized with Intracellular Fixation & Permeabilization Buffer Set (Cat. No. 88-8824 [left]), Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523 [middle]), or IC Fixation Buffer (Cat. No. 00-8222) followed by methanol permeabilization (right). Cells were then intracellularly stained with Anti–Human/Mouse Phospho-ZAP-70/SYK (Y319/Y352) APC (Cat. No. 17-9006) followed by flow cytometric analysis.

Mouse and human cross-reactivity testing

Each phosphospecific antibody is tested in human and mouse cells to determine species cross-reactivity. Species specificity of phosphospecific antibodies is always indicated in the name of the antibody and on the Technical Data Sheet.

Multiple fluorochromes for flow cytometry antibody panel configurations

We offer a comprehensive portfolio of antibody conjugates with the flexibility and freedom to design an experiment within the parameters of your current panel configuration.

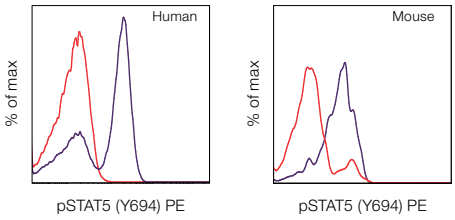


Figure 15. Mouse and human phospho-STAT5 (Y694) cross-reactivity. Left: Intracellular staining of untreated (red histogram) or 15-minute IL-2–treated (purple histogram) human Th2-polarized CD4$^+$ cells with Anti–Human/Mouse Phospho-STAT5 (Y694) PE (Cat. No. 12-9010). Right: Intracellular staining of untreated (red histogram) or 15-minute GM-CSF–treated (purple histogram) mouse thioglycolate-elicited peritoneal exudate cells with Anti–Human/Mouse Phospho-STAT5 (Y694) PE (Cat. No. 12-9010). CD11c$^+$ cells in the large scatter population were used for analysis.

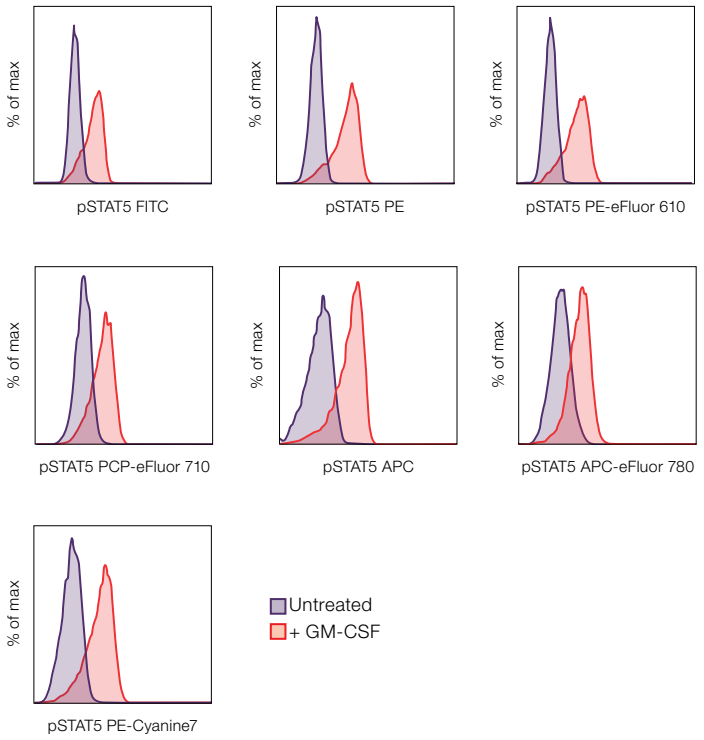


Figure 16. Comparison of anti-pSTAT5 (Y694) formats. Intracellular staining of untreated (purple histogram) or 15-minute Human GM-CSF Recombinant Protein–treated (Cat. No. 14-833 [red histogram]) U937 cells with Anti–Human/Mouse Phospho STAT5 (Y694 [clone SRBCZX]) conjugated to different formats using the IC Fixation/methanol protocol. Formats are indicated on the x-axis.

Table 11. Invitrogen™ eBioscience™ phosphospecific antibodies.*

Target	Clone	Cat. No.	Hu X-reactivity	Ms X-reactivity	Purified	eFluor 450	FITC	PerCP-eFluor 710	PE	PE-eFluor 610	PE-Cyanine7	APC or eFluor 660	APC-eFluor 780	IC Fix/Perm	Foxp3 buffer set	IC Fix/MeOH
p4E-BP1 (T36/T45)	V3NTY24	9107	■	■								■		++	-	+++
pAKT (S473)	SDRNR	9715	■	■								■		++	++	+++
pBTK/ITK (S551/Y511)	M4G3LN	9015	■	■	■			■	■			■		++	++	+++
pERK1/2 (T202/Y204)	MILAN8R	9109	■	■	■			■	■	■		■		-	-	+++
pH2AX (S139)	CR55T33	9865	■	■	■			■	■			■		-	+++	+++
pHistone H3 (S28)	HTA28	9124	■	■								■		++	+	+++
pIκBα (S32/S36)	RILYB3R	9035	■	■								■		+++	-	++
pLCK (Y505)	SRRCHA	9076	■	■				■				■		++	++	+++
pMCL-1 (S159)	RBCERNR	9038	■	■					■			■		++	+	+++
pmTOR (S2448)	MRRBY	9718	■	■				■	■			■		++	++	+++
pNFκB p65 (S529)	B33B4WP	9863	■					■	■			■		+++	-	+++
pS6 Ribosomal (S235/S236)	cupk43k	9007	■	■	■			■	■					++	++	+++
pSLP-76 (Y128)	HNDZ55	9037	■									■		+++	-	+++
pSrc (Y418)	SC1T2M3	9034	■	■	■			■	■			■		++	++	+++
pSTAT1 (Y701)	KIKSI0803	9008	■		■	■			■			■		-	-	+++
pSTAT3 (Y705)	LUVNKLA	9033	■	■					■			■		-	-	+++
pSTAT4 (Y693)	4LURPIE	9044	■	■					■			■		-	-	+++
pSTAT5 (Y694)	SRBCZX	9010	■	■			■	■	■	■	■	■	■	-	-	+++
pSTAT6 (Y641)	CHI2S4N	9013	■	■				■	■	■		■		-	-	+++
pSYK (Y348)	moch1ct	9014	■	■					■			■		++	++	+++
pTyrosine	pY20	5001	■	■	■	■		■				■		++	++	+++
pZAP-70/SYK (Y319/Y352)	n3kobu5	9006	■	■				■	■			■		++	++	+++

* Table is not exhaustive. Search the full catalog of flow cytometry antibodies at **thermofisher.com/flowantibodies**

Gene expression by flow cytometry

PrimeFlow RNA Assay

The Invitrogen™ PrimeFlow™ RNA Assay reveals the dynamics of RNA and protein expression within individual cells, facilitating superior analysis of their correlation as the cells change over time or in response to stimulation. This novel assay uses fluorescent *in situ* hybridization (FISH) to enable simultaneous detection of as many as three RNA transcripts in a single cell using a standard flow cytometer. The PrimeFlow RNA Assay is compatible with cell surface and intracellular staining, using common flow cytometry fluorochromes. The assay is based upon proven and well-published Invitrogen™ ViewRNA™ assays designed for microscopic analysis of RNA in cells and tissues that combine paired oligonucleotide probe design with branched DNA (bDNA) signal amplification to robustly detect gene expression at the single-cell level.

Coupling RNA expression with protein detection on a flow cytometer generates multiparametric data in heterogeneous cell populations and offers in-depth and high-content details at the single-cell level. In contrast, microarrays and sequencing can provide comprehensive gene expression data in bulk sample preparations; however, the analysis of bulk samples can mask the individual effects of unique cellular subsets. Using the PrimeFlow RNA Assay, specific cell populations may be analyzed for unique transcript expression levels or cell subsets evaluated over time to determine transcriptional regulation and protein expression simultaneously. Such unique and valuable insights are highly applicable to answering previously unanswerable questions and have broad implications for advancing research across multiple fields of biology.

- Observe the heterogeneity of gene expression in millions of single cells
- Correlate RNA and protein within the same cell
- Detect noncoding RNA in cell subsets
- Evaluate viral RNA expression in infected cells
- Analyze mRNA expression levels when antibody is unavailable

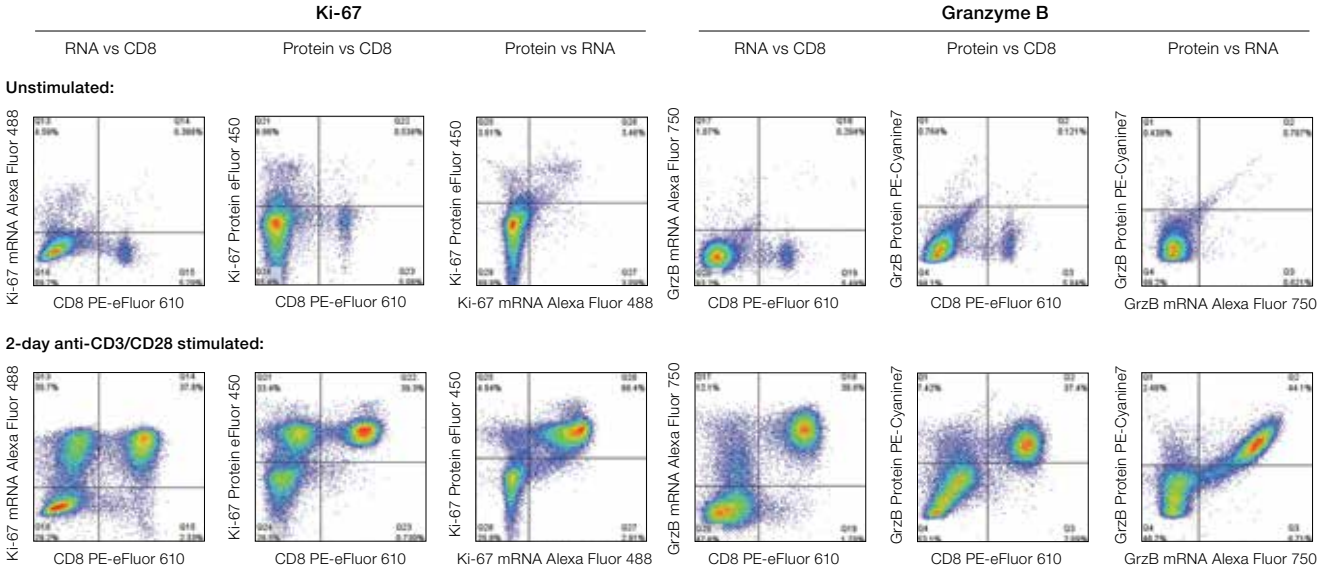


Figure 17. The PrimeFlow RNA Assay in action. C57Bl/6 splenocytes were unstimulated (top row) or stimulated for 2 days with Anti–Mouse CD3 and CD28 Functional Grade Purified antibodies (Cat. No. 16-0031 and Cat. No. 16-0281 [bottom row]) and in the presence of Protein Transport Inhibitor Cocktail (Cat. No. 00-4980) for the last 3 hours of culture, followed by analysis using the PrimeFlow RNA Assay (Cat. No. 88-18001). Cells were fixed and permeabilized using the PrimeFlow RNA Assay buffers and protocol, then intracellularly stained with the Invitrogen™ eBioscience™ Anti–Mouse CD8a PE-eFluor™ 610 (Cat. No. 61-0081), Anti–Mouse Ki-67 eFluor™ 450 (Cat. No. 48-5698), and Anti–Mouse Granzyme B PE-Cyanine7 (Cat. No. 25-8898) conjugates. Cells were then hybridized with the Invitrogen™ Type 6 Mouse Granzyme B Alexa Fluor™ 750 (Cat. No. VB6-16522), Type 4 Mouse Ki-67 Alexa Fluor™ 488 (Cat. No. VB4-16518), and Type 1 Mouse β-actin Alexa Fluor™ 647 (Cat. No. VB1-10350) target probes.

PrimeFlow RNA Assay technology

Fluorescent *in situ* hybridization (FISH) is a powerful technique that allows specific localization of ribonucleic acid targets in fixed cells. The basic premise of the application relies on detecting nucleic acids through sequential hybridization of nucleic acid probes that provides gene expression information at a single-cell level. Traditional FISH techniques are generally limited by high background and low sensitivity due to nonspecific binding and inefficient signal amplification.

The PrimeFlow RNA Assay incorporates a proprietary oligonucleotide probe set design and branched DNA (bDNA) signal amplification technology to analyze RNA transcripts by flow cytometry. bDNA technology provides a unique approach to RNA detection and signal amplification by amplifying the reporter signal rather than the target sequence (e.g., PCR) for consistent results, a common problem for PCR-based assays.

In the PrimeFlow RNA Assay, target-specific probe sets contain 20 to 40 oligonucleotide pairs that hybridize to the target RNA transcript. Signal amplification is achieved through specific hybridization of adjacent oligonucleotide pairs to bDNA structures, formed by Pre-amplifiers, Amplifiers, and fluorochrome-conjugated Label Probes, resulting in excellent specificity, low background, and high signal-to-noise ratios (Figures 18 and 19).

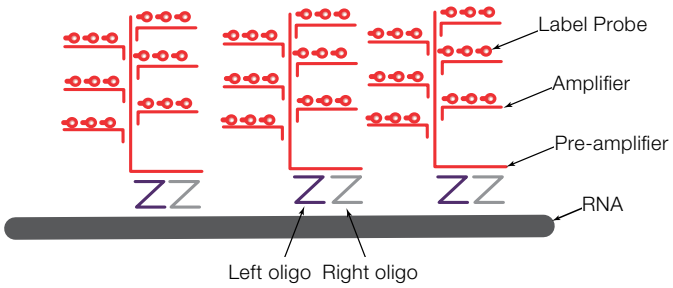


Figure 18. Branched DNA (bDNA) probe design principle.

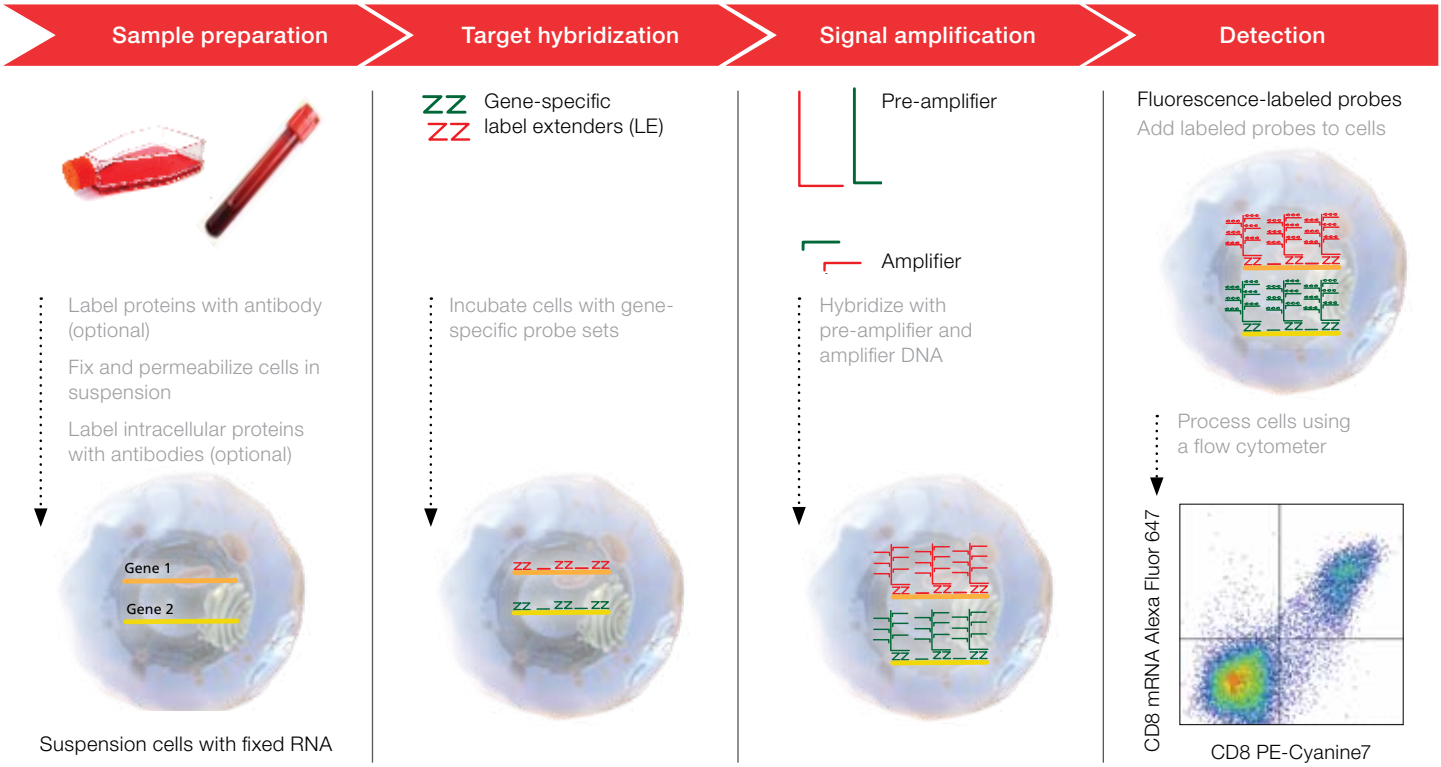


Figure 19. The PrimeFlow RNA Assay workflow. The assay workflow contains several steps: antibody staining; fixation and permeabilization, including intracellular staining, if desired; followed by target hybridization with a target-specific probe set containing 20 to 40 oligonucleotide pairs.

Intracellular antibody staining protocols

Protocol A: Intracellular (cytoplasmic) proteins

The following protocol allows the simultaneous analysis of cell surface molecules and intracellular antigens at the single-cell level. In this protocol, fixation is followed by permeabilization resulting in the creation of pores in the cell membrane that require the continuous presence of the permeabilization buffer during all subsequent steps to allow antibodies to have access to the cytoplasm of the cell as well as allowing unbound antibody out of the cell. Thus, all intracellular staining must be done in the presence of the permeabilization buffer. This protocol is recommended for the detection of cytoplasmic proteins, cytokines, or other secreted proteins in individual cells.

For the detection of nuclear proteins such as transcription factors, please see Protocol B: Intracellular (nuclear) proteins (next page). For detection of some phosphorylated signaling molecules such as MAPK and STAT proteins, it may be preferential to use Protocol C, below.

Materials

- 12 x 75 mm round bottom test tubes
- Invitrogen™ eBioscience™ Fixable Viability Dyes eFluor™ 455UV, 450, 506, 520, 660, and 780 (Cat. No. 65-0868, 65-0863, 65-0866, 65-0867, 65-0864, and 65-0865)
- Directly conjugated antibodies specific for intracellular proteins
- Invitrogen™ eBioscience™ Intracellular Fixation & Permeabilization Buffer Set (Cat. No. 88-8824)
- Invitrogen™ eBioscience™ Flow Cytometry Staining Buffer (Cat. No. 00-4222)
- Invitrogen™ eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitors) (500X) (Cat. No. 00-4975), Protein Transport Inhibitor Cocktail (500X) (Cat. No. 00-4980), Brefeldin A Solution (Cat. No. 00-4506), or Monensin Solution (Cat. No. 00-4505)

Buffer and solution preparation

- Prepare a 1X working solution of Permeabilization Buffer by diluting the 10X concentrate with distilled water prior to use. You will need 8.5 mL of Permeabilization Buffer for each sample.

Experimental procedure

1. Prepare cells of interest for evaluation of intracellular proteins. Refer to Best Protocols: “Cell Preparation for Flow Cytometry” on our website.
2. To eliminate potential artifacts due to dead-cell contamination, we recommend the use of a fixable viability dye to allow the exclusion of dead cells from analysis.
3. Stain cell surface antigen(s) as described in Best Protocols “Staining cell surface antigens”.
4. After the last wash, discard the supernatant and pulse vortex the sample to completely dissociate the pellet. Typically about 100 µL residual volume remains.
5. Fix the cells by adding 100 µL of IC Fixation Buffer and pulse vortex.
6. Incubate tubes in the dark at room temperature for 20–60 minutes.
7. Without washing, add 2 mL of 1X Permeabilization Buffer to each tube.
8. Centrifuge samples at 300–400 x *g* at room temperature for 5 minutes, then discard the supernatant.
9. Resuspend the cell pellet in 2 mL of 1X Permeabilization Buffer.
10. Centrifuge samples at 300–400 x *g* at room temperature for 5 minutes, then discard the supernatant.
11. Resuspend the cells in 100 µL of 1X Permeabilization Buffer. Add the recommended amount of fluorochrome-conjugated antibody for detection of intracellular antigen(s) to cells and incubate in the dark at room temperature for 20–60 minutes.
12. Add 2 mL of 1X Permeabilization Buffer to each tube.

13. Centrifuge samples at 300–400 x *g* at room temperature for 5 minutes, then discard the supernatant.

14. Add 2 mL of Flow Cytometry Staining Buffer to each tube.

15. Centrifuge samples at 300–400 x *g* at room temperature for 5 minutes, then discard the supernatant.

16. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer and acquire samples on a flow cytometer.

Protocol B: Intracellular (nuclear) proteins

The following protocol allows the simultaneous analysis of cell surface molecules and intracellular antigens, including nuclear antigens, at the single-cell level. This protocol combines fixation and permeabilization into a single step. This protocol is recommended for the detection of nuclear antigens such as transcription factors, but is also useful for the detection of many cytokines. For compatibility of the Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523) with cytokine antibodies, please see our Intracellular Staining Buffer Selection Guide online: **thermofisher.com/icflowbufferguide**

Materials

- 12 x 75 mm round bottom test tubes or 96-well V or U bottom plate
- Fixable Viability Dyes eFluor 455UV, 450, 506, 520, 660, and 780 (Cat. No. 65-0868, 65-0863, 65-0866, 65-0867, 65-0864, and 65-0865)
- [Optional] Normal Mouse Serum (Cat. No. 24-5544)
- [Optional] Normal Rat Serum (Cat. No. 24-5555)
- Directly conjugated antibodies specific for intracellular proteins
- Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523)
- Flow Cytometry Staining Buffer (Cat. No. 00-4222)

Buffers and solution preparation

- Prepare a fresh Foxp3 Fixation/Permeabilization working solution by diluting Foxp3 Fixation/Permeabilization Concentrate (1 part) with Foxp3 Fixation/Permeabilization Diluent (3 parts). You will need 1 mL of the Fixation/Permeabilization working solution for each sample.
- Prepare a 1X working solution of Permeabilization Buffer by diluting the 10X concentrate with distilled water prior to use. You will need 8.5 mL of Permeabilization Buffer for each sample, if staining in tubes.

Experimental procedure in tubes

1. Prepare cells of interest for evaluation of intracellular proteins. Refer to Best Protocols: “Cell Preparation for Flow Cytometry” on our website.

2. To eliminate potential artifacts due to dead-cell contamination, we recommend the use of a Fixable Viability Dye to allow the exclusion of dead cells from the analysis.

3. Stain cell surface antigen(s) as described in Best Protocols “Staining cell surface antigens” protocol.

4. After the last wash, discard the supernatant and pulse vortex the sample to completely dissociate the pellet.

5. Add 1 mL of Foxp3 Fixation/Permeabilization working solution to each tube and pulse vortex.

6. Incubate in the dark at 4°C or room temperature for 30–60 minutes. (Mouse samples can be incubated for up to 18 hours at 4°C in the dark.)

7. Without washing, add 2 mL of 1X Permeabilization Buffer to each tube.

8. Centrifuge samples at 300–400 x *g* at room temperature for 5 minutes, then discard the supernatant.

9. [Optional] Repeat steps 7–8.

10. Resuspend pellet in 100 µL of 1X Permeabilization Buffer. This is typically the residual volume after decanting.

11. [Optional] Block with 2% normal mouse/rat serum by adding 2 µL directly to the cells. Incubate at room temperature for 15 minutes.

12. Without washing, add the recommended amount of fluorochrome-conjugated antibody for detection of intracellular antigen(s) to cells and incubate in the dark at room temperature for at least 30 minutes.

13. Add 2 mL of 1X Permeabilization Buffer to each tube.

14. Centrifuge samples at 300–400 x *g* at room temperature for 5 minutes, then discard the supernatant.

15. Add 2 mL of 1X Permeabilization Buffer or Flow Cytometry Staining Buffer to each tube.

16. Centrifuge samples at 300–400 x *g* at room temperature for 5 minutes, then discard the supernatant.

17. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer and acquire samples on a flow cytometer.

Experimental procedure in a 96-well plate

1. Prepare cells of interest for evaluation of intracellular proteins. Refer to Best Protocols: "Cell Preparation for Flow Cytometry" on our website.

2. To eliminate potential artifacts due to dead-cell contamination, we recommend the use of a Fixable Viability Dye to allow the exclusion of dead cells from the analysis.

3. Stain cell surface antigen(s) as described in Best Protocols "Staining Cell Surface Antigens".

4. After the last wash, discard the supernatant and pulse vortex the sample to completely dissociate the pellet.

5. Add 200 µL of Foxp3 Fixation/Permeabilization working solution to each well. It is ideal to add the solution such that the cells are fully resuspended in the solution. Pipetting is an option.

6. Incubate in the dark at room temperature for 30–60 minutes. (Mouse samples can be incubated for up to 18 hours at 4°C in the dark).

7. Centrifuge samples at 300–400 x *g* at room temperature for 5 minutes, then discard the supernatant.

8. Add 200 µL 1X Permeabilization Buffer to each well.

9. Centrifuge samples at 300–400 x *g* at room temperature for 5 minutes, then discard the supernatant.

10. Repeat steps 8–9.

11. Resuspend pellet in residual volume and adjust volume to about 100 µL with 1X Permeabilization Buffer.

12. [Optional] Block with 2% normal mouse/rat serum by adding 2 µL directly to the cells. Incubate at room temperature for 15 minutes.

13. Without washing, add the recommended amount of fluorochrome-conjugated antibody for detection of intracellular antigen(s) to cells and incubate in the dark at room temperature for at least 30 minutes.

14. Add 200 µL of 1X Permeabilization Buffer to each well.

15. Centrifuge samples at 300–400 x *g* at room temperature for 5 minutes, then discard the supernatant.

16. Add 200 µL of 1X Permeabilization Buffer or Flow Cytometry Staining Buffer to each well.

17. Centrifuge samples at 300–400 x *g* at room temperature for 5 minutes, then discard the supernatant.

18. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer and acquire samples on a flow cytometer.

Protocol C: Fixation/methanol (phosphospecific proteins)

The following protocol allows for the simultaneous analysis of cell surface molecules and some intracellular phosphorylated signaling proteins. In this protocol, fixation is followed by treatment of cells with methanol. For phosphoprotein detection, the appropriate stimulation conditions and kinetics of phosphorylation will vary depending on the cell type and the particular signaling event being assayed. For example, to induce phospho-STAT1 (Y701) phosphorylation, macrophages can be activated with IFNγ or IFNα, while phospho-ERK1/2 (T202/Y204) is induced in T cells in response to PMA (a phorbol ester, a protein kinase C activator) or anti-CD3 antibodies.

General Notes

- Fluorochrome-conjugated antibodies can be used to stain surface proteins for the purpose of immunophenotyping cells that will be further analyzed for phosphorylated proteins; however, additional considerations for staining are warranted:
  - Antibody staining for surface markers on live cells has been shown to alter expression of signaling proteins due to possible stimulation/suppression of signaling events. Because of this, surface staining is not recommended prior to cell stimulation. Instead, stain surface proteins at the same step as the intracellular protein staining. Antibody clones to surface proteins that will recognize fixed epitopes will need to be evaluated and used. Refer to page 13 for antibodies that we have tested; otherwise, performance will need to be determined empirically.
  - If surface staining is required prior to fixation, in step 5 (due to epitope destruction), cells may be stained with fluorochrome-conjugated antibodies before the fixation/methanol steps only if the fluorochromes are resistant to methanol exposure.
- For adherent cells, we recommend fixing the cells (step 5) in the plates/well. After fixation, scrape cells or treat with an EDTA solution to harvest and then continue with the protocol. Trypsin can be used if you are not staining for surface antibodies or you know your surface protein is resistant to tryspin digestion.

Methanol-resistant fluorochromes	Methanol-sensitive fluorochromes
Alexa Fluor 488	PE
eFluor 660	PE-tandems
Alexa Fluor 647	PerCP
eFluor 450	PerCP-tandems
FITC	APC
	APC-tandems

Materials

- 12 x 75 mm round bottom test tubes or 96-well round or V-bottom microtiter plates
- Primary antibodies (directly conjugated)
- Flow Cytometry Staining Buffer (Cat. No. 00-4222)
- IC Fixation Buffer (Cat. No. 00-8222)
- 90–100% methanol (HPLC grade)
- [Optional] Fc Receptor Block: Mouse CD16/CD32 Antibody (Cat. No. 14-0161) or Human Fc Receptor Binding Inhibitor, Purified (Cat. No. 14-9161)

Experimental procedure

1. Prepare cells of interest for stimulation in appropriate media.
2. Count cells and resuspend in appropriate media at 1–5 x 10^6 cells/mL.
3. Stimulate cells at 37°C with appropriate treatment for desired time point(s). Remember to incubate untreated cells at 37°C as a negative control.
4. [Optional] If surface staining is needed prior to fixation (in step 5), stain cell surface antigen(s) as described in Best Protocols “Staining Cell Surface Antigens” using antibodies conjugated to methanol-resistant fluorochromes.
5. At the end of the stimulation period, fix cells to stop stimulation by adding an equal volume of IC Fixation Buffer directly to cells and vortex.
6. Incubate cells in the dark at room temperature for 10–60 minutes.

7. Centrifuge cells at 600 x *g* at room temperature for 4–5 minutes, then discard supernatant.

8. Resuspend the cell pellet in residual volume and add 1 mL of ice-cold 90–100% methanol, vortex, and incubate at 4°C or on ice for at least 30 minutes.

NOTE: Once in methanol, cells can be stored at –20°C for up to 4 weeks.

9. Wash cells with an excess volume of Flow Cytometry Staining Buffer.

10. Centrifuge cells at 600 x *g* at room temperature for 4–5 minutes, then discard supernatant.

11. Resuspend cells at 1 x 10^6 cells/mL in Flow Cytometry Staining Buffer.

12. Aliquot 1 x 10^6 cells (100 µL) into separate flow tubes.

13. [Optional] Cells can be blocked for nonspecific Fc receptor–mediated binding using Mouse CD16/CD32 Antibody or Human Fc Receptor Binding Inhibitor, Purified prior to staining.

14. Add the recommended amount of fluorochrome-conjugated antibody to each tube and incubate in the dark at room temperature for 30–60 minutes.

NOTE: If needed, surface staining and intracellular staining can be performed simultaneously. Please refer to the table on page 13 for antibody clones that will stain cells after fixation and methanol treatment.

15. Add 2 mL of Flow Cytometry Staining Buffer and centrifuge at 600 x *g* at room temperature for 4–5 minutes. Discard supernatant.

16. Repeat step 15.

17. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer and acquire samples on a flow cytometer.

Experimental procedure in a 96-well plate

1. Prepare cells of interest for stimulation in the appropriate media.

2. Count cells and resuspend in appropriate media at 1–5 x 10^6 cells/mL.

3. Add 100 µL of appropriate treatment to wells in a 96-well plate.

4. Add 100 µL of cells to wells and stimulate cells at 37°C for desired time point(s). Remember to incubate untreated cells at 37°C as a negative control.

5. [Optional] If surface staining is needed prior to fixation (in step 5), stain cell surface antigen(s) as described in Best Protocols “Staining Cell Surface Antigens” using antibodies conjugated to methanol-resistant fluorochromes.

6. At the end of the stimulation period, fix cells to stop stimulation by adding 200 µL of IC Fixation Buffer directly to wells.

7. Incubate plate in the dark at room temperature for 10–60 minutes.

8. Centrifuge plate at 600 x *g* at room temperature for 4–5 minutes, then discard supernatant.

9. Resuspend the cell pellets in residual volume and add 100 µL of ice-cold 90–100% methanol to wells, vortex, and incubate plate at 4°C or on ice for at least 30 minutes.

NOTE: Once in methanol, cells can be stored at –20°C for up to 4 weeks.

10. Add 200 µL Flow Cytometry Staining Buffer. Centrifuge cells at 600 x *g* at room temperature for 4–5 minutes, then discard supernatant.

11. Repeat step 10.

12. [Optional] Cells can be blocked for nonspecific Fc receptor–mediated binding using Mouse CD16/CD32 Antibody or Human Fc Receptor Binding Inhibitor, Purified prior to staining.

13. Add the recommended amount of fluorochrome-conjugated antibody to each well and incubate in the dark at room temperature for 30–60 minutes.

NOTE: If needed, surface staining and intracellular staining can be performed simultaneously. Please refer to the table on page 13 for antibody clones that will stain cells after fixation and methanol treatment.

14. Add 200 µL of Flow Cytometry Staining Buffer and centrifuge at 600 x *g* for 4–5 minutes.

15. Repeat step 14.

16. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer and acquire samples on a flow cytometer.

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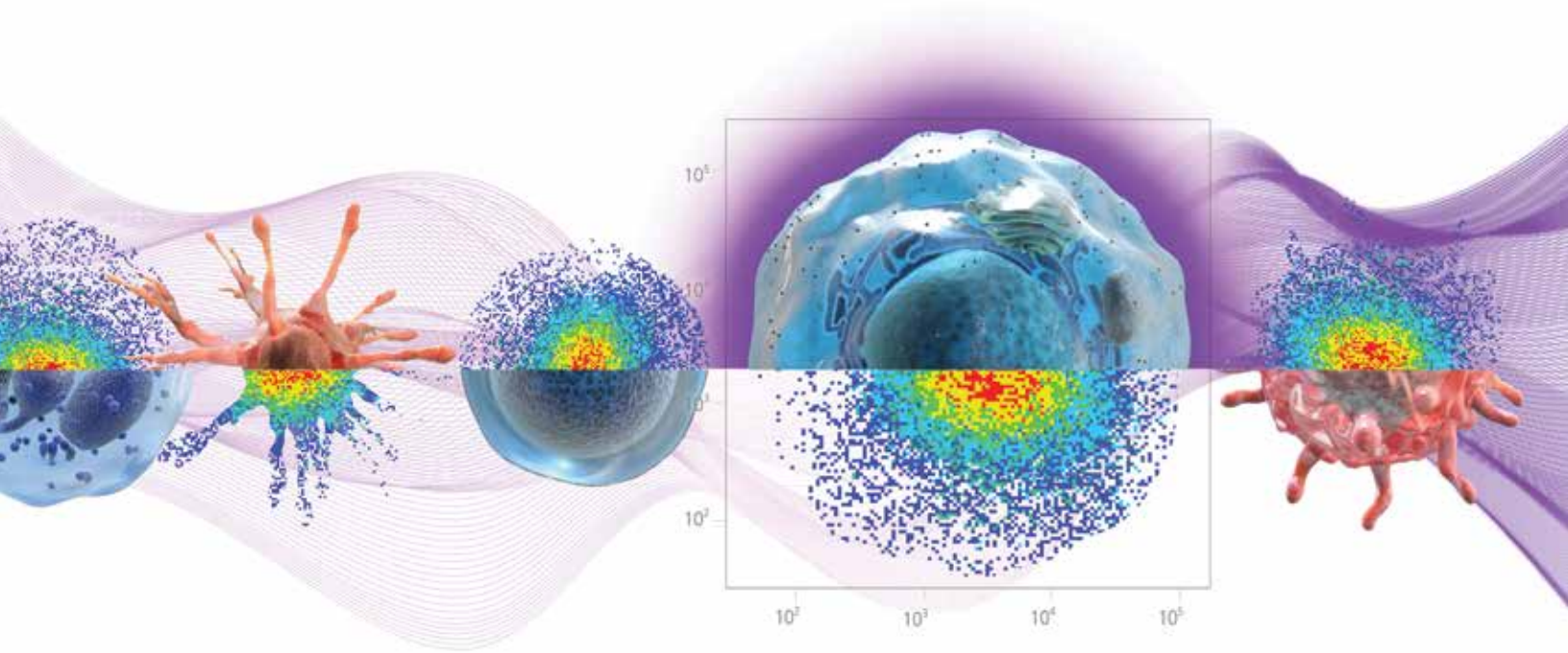

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