Optimizing Intracellular Flow Cytometry

Detection of Cytokines, Transcription Factors, and Phosphoprotein by Flow Cytometry

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23-14876-00

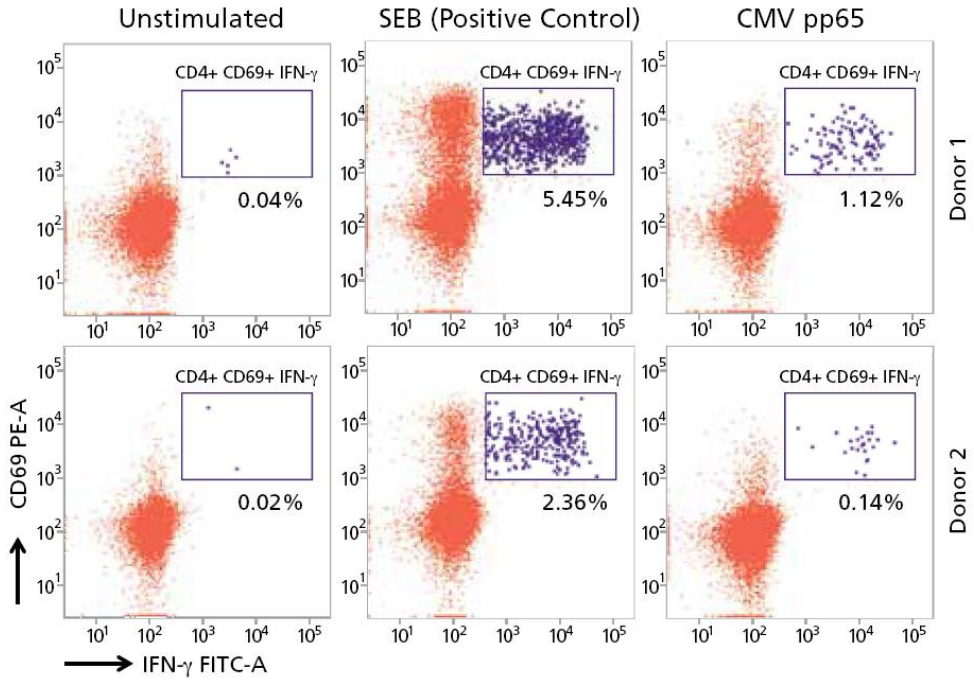
Outline

- Basic principles of intracellular flow cytometry
- Detection of cytokines
- Detection of transcription factors
- Detection of phosphoprotein
- Combining techniques

Applications of Intracellular Flow Cytometry

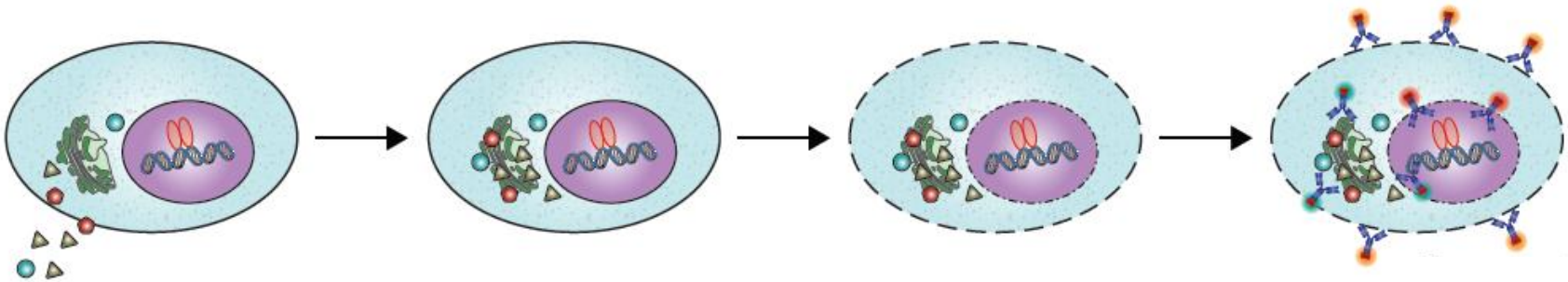
- Identification/phenotyping of cell populations
- Study of cellular signaling, function, and differentiation
- Simultaneous analysis of multiple proteins
- Analysis of frequency *and* magnitude of responses within heterogeneous samples

Applications of Intracellular Flow Cytometry



- Human whole blood was stimulated with staphylococcal enterotoxin B or cytomegalovirus pp65 for 6 hours in the presence of Brefeldin A.
- Cells were fixed, permeabilized, and stained using the BD FastImmune™ 3-color CD4 intracellular cytokine detection kit.
- Cells were analyzed on a BD FACSVerse™ flow cytometer.

Overview of Intracellular Staining



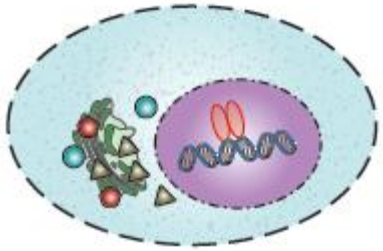
Treat with protein transport inhibitor (for cytokine staining only)

Fix and permeabilize cells

Stain cells

Flow cytometry analysis

Optimal Conditions for Intracellular Staining Depend on Epitope Accessibility



- To access intracellular antigens, cells must be fixed and permeabilized.
- Different permeabilization conditions favor the detection of different types of epitopes.
  - Cytokines (once trapped inside the cell) are accessible using gentle conditions.
  - Transcription factors and phosphoproteins often require stronger permeabilization buffers.
  - Cellular fixation and permeabilization conditions can have adverse effects on surface antigens or fluorochromes.

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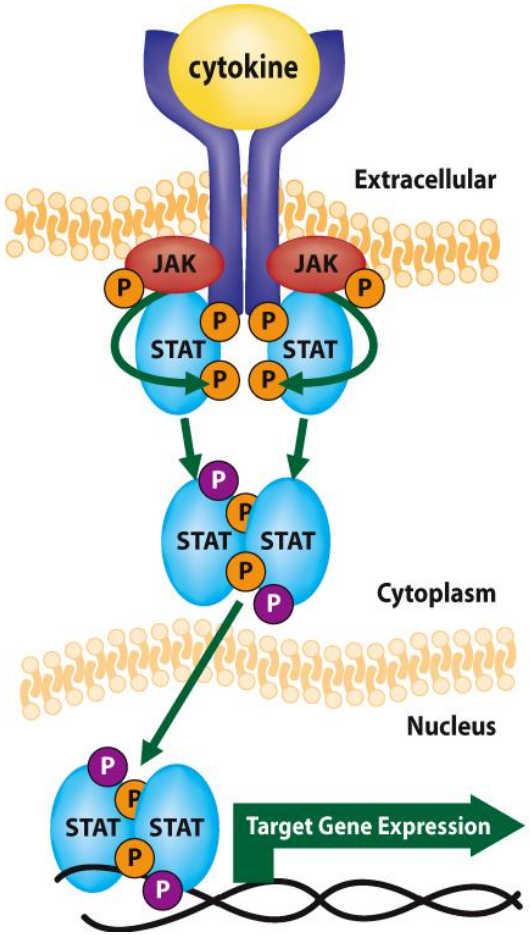
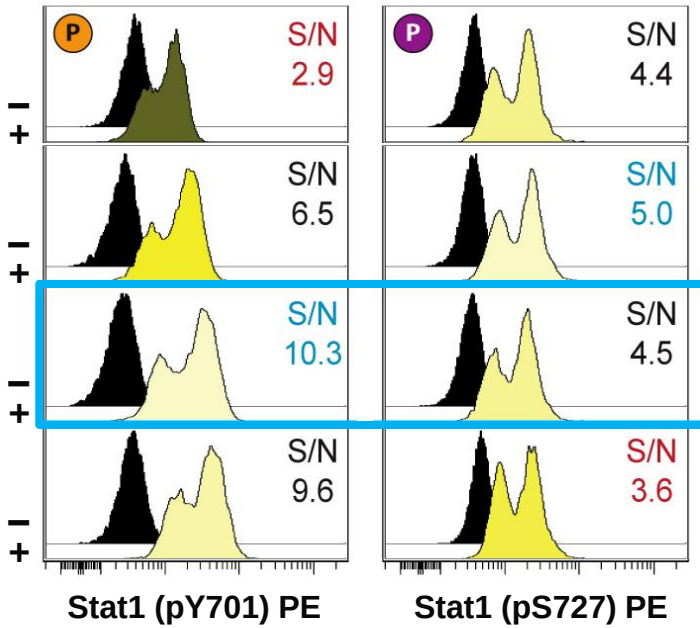
Optimal Conditions for Intracellular Staining Depend on Epitope Accessibility





Mild Detergent (Saponin) Perm/Wash Buffer I

Methanol: Low Conc. Perm Buffer II

Methanol: High Conc. Perm Buffer III

Harsh Detergent Perm Buffer IV (1X)

- Human PBMCs were left untreated (–) or were activated (+) with human IFN-α (Stat1 pY701) or PMA (Stat1 pS727).
- Cells were fixed using BD Cytofix™ fixation buffer and permeabilized using BD Phosflow™ perm buffer I, II, III, or IV prior to staining.

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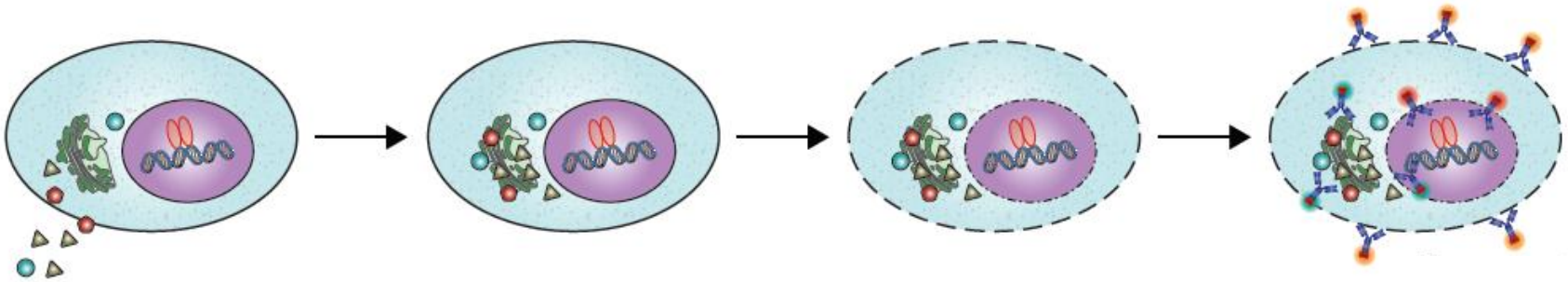
Detection of Cytokines by Flow Cytometry



Treat with protein transport inhibitor (for cytokine staining only)

Fix and permeabilize cells

Stain cells

Flow cytometry analysis

- Because cytokines are secreted proteins, they must be trapped inside the cell using a protein transport inhibitor.
- BD Cytofix/Cytoperm™ buffer is recommended for detection of cytokines by flow cytometry.
- Surface markers are usually stained prior to fixation and permeabilization.

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Protein Transport Inhibitors for Cytokine Detection by Flow Cytometry

Monensin (BD GolgiStop™) and Brefeldin A (BD GolgiPlug™) inhibitors are commonly used to trap cytokines inside the cell for analysis.

- Work by slightly different mechanisms
  - Monensin prevents protein secretion by interacting with the Golgi transmembrane Na^{++}/H^{+} transport.
  - Brefeldin A redistributes intracellularly produced proteins from the cis/medial Golgi complex to the endoplasmic reticulum.
- Different inhibitors may work better for detection of different cytokines.

Species	Cytokines	Transport Inhibitor
Human	IL-1α, IL-6, IL-8, TNF-α	Monensin
Human	IFN-γ, IL-2, IL-10, IL-12, MCP-1, MCP-3, MIG, MIP-1α, RANTES	Either monensin or brefeldin A
Mouse	IL-6, IL-12, TNF-α	Brefeldin A
Mouse	GM-CSF, IL-3, IL-4, IL-5, IL-10	Monensin
Mouse	IFN-γ, IL-2	Either monensin or brefeldin A

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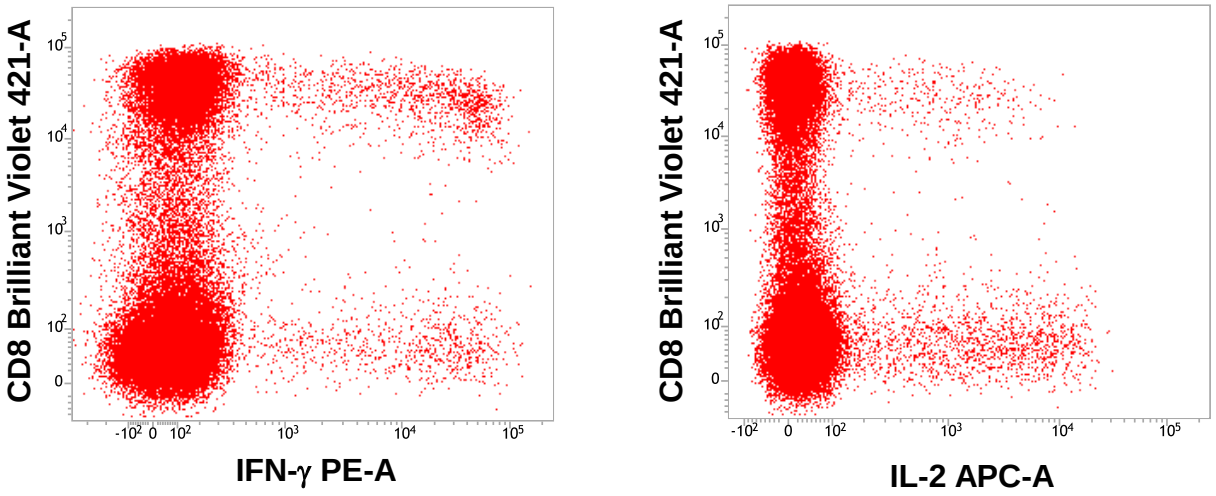
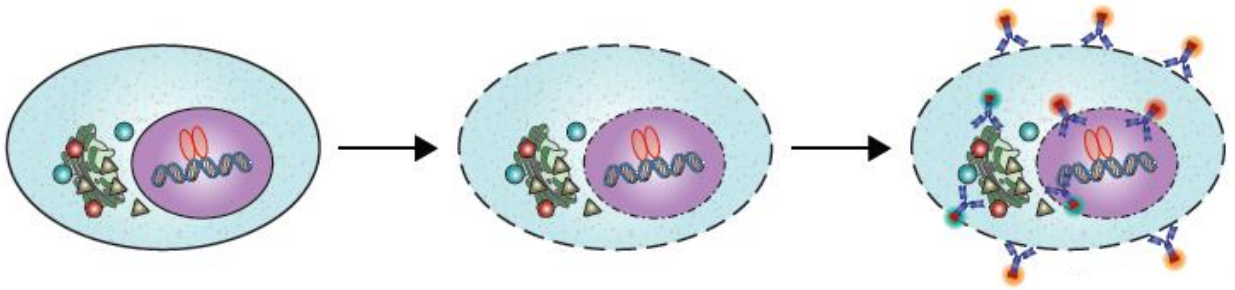
Example 1: IFN-γ and IL-2 Production in $CD8^+$ Cells



- Human PBMCs were stimulated with staphylococcal enterotoxin B for 6 hours in the presence of Brefeldin A.
- Cells were fixed and permeabilized using the BD Cytofix/Cytoperm buffer system.
- Cells were stained with CD3 FITC, CD4 PerCP-Cy™5.5, CD8 BD Horizon™ Brilliant Violet™ 421, IFN-γ PE, and IL-2 APC.
- Cells were analyzed on a BD FACSVerse flow cytometer.

Detection of Transcription Factors by Flow Cytometry

- Transcription factors are proteins that bind to specific DNA sequences and regulate gene expression.
- BD Pharmingen™ transcription factor buffer is the recommended starting buffer.
  - Compatible with staining of most surface markers (stained before or after cellular permeabilization) and cytokines

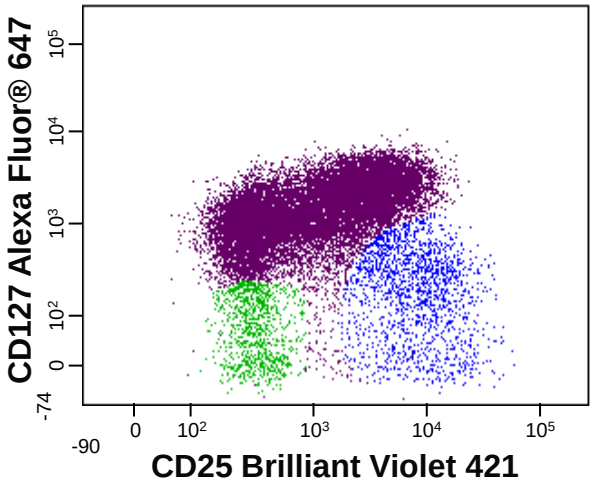


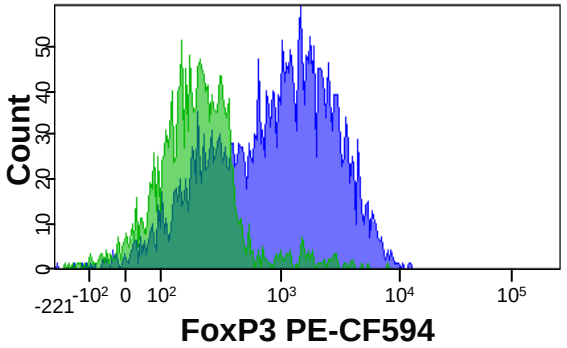
Fix and permeabilize cells

Stain cells

Flow cytometry analysis

Example 2: Detection of FoxP3 Regulatory T Cells (Tregs)

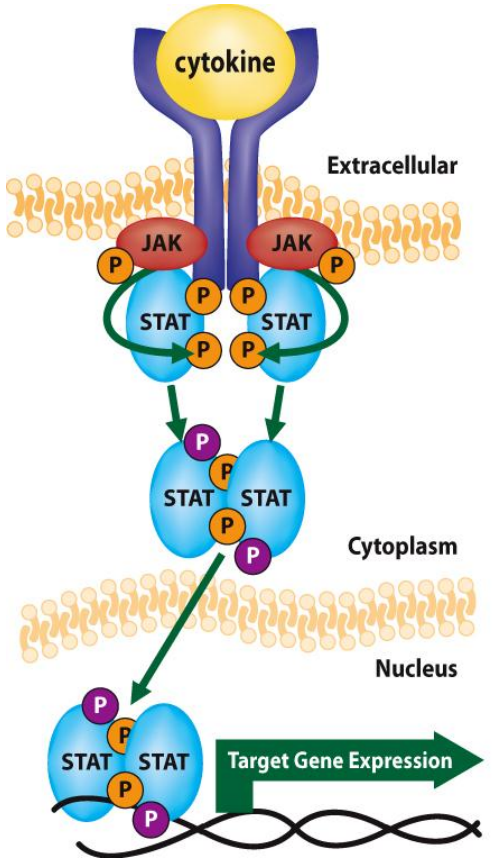




- Tregs are a subset of T cells that regulate the immune response by suppressing the activity of other T cells.
- Human PBMCs were stained for surface markers CD4 FITC, CD25 Brilliant Violet 421, and CD127 Alexa Fluor® 647.
- After washing, cells were fixed and permeabilized with the BD Pharmingen transcription factor buffer set and stained with FoxP3 PE-CF594.
- Data was acquired on a BD FACSVerse flow cytometer.

Detection of Phosphoprotein by Flow Cytometry (BD Phosflow)

- Proteins are phosphorylated in response to many types of stimuli including cytokines and small molecules.
- Protein phosphorylation is transient; cells must be fixed quickly to maintain phosphoepitopes.
- Perm buffer III is the recommended starting buffer for most BD Phosflow applications.
  - Perm buffer III is a harsh denaturing buffer.
  - Other perm buffers are available.



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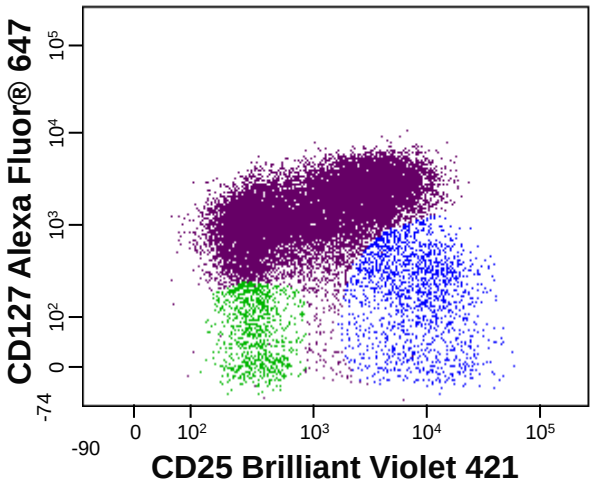
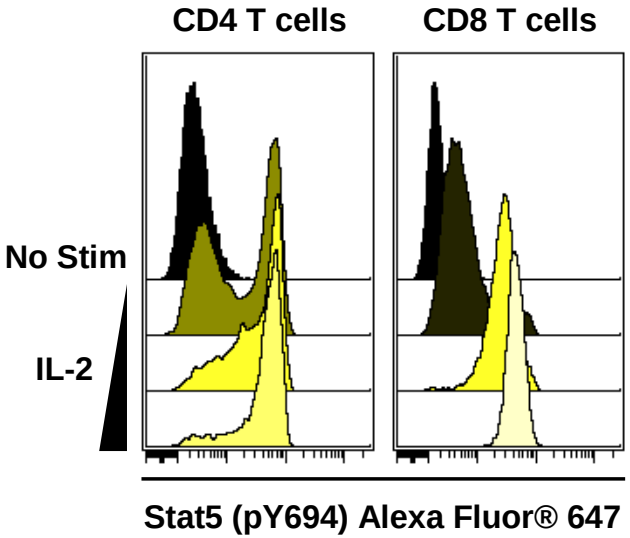
Considerations for Phosphospecific Flow Cytometry

- Stimulation kinetics: most phosphorylation events occur very rapidly
- Controls: Unlike isotype controls, unstimulated cells take into account basal phosphorylation and the unique background characteristics of each antibody
- Expression level of signaling protein of interest
- Perm buffer III can impact surface marker staining performed before or after fixation and permeabilization
  - The BD FACSelect™ buffer compatibility resource lists buffer compatibility for many popular markers. (http://www.cytobank.org/facselect/)

Example 3: Enhanced IL-2 Sensitivity of Tregs

- Stimulation by IL-2 leads to Stat5 (pY694) phosphorylation in most human T cells.
- Tregs express large amounts of the IL-2 receptor alpha chain (CD25). Do they respond differently to treatment with IL-2?





Human whole blood was stimulated with 1, 10, or 100 ng/mL of IL-2 for 15 min prior to fixation, permeabilization, and staining with the BD Phosflow™ T-cell activation kit.

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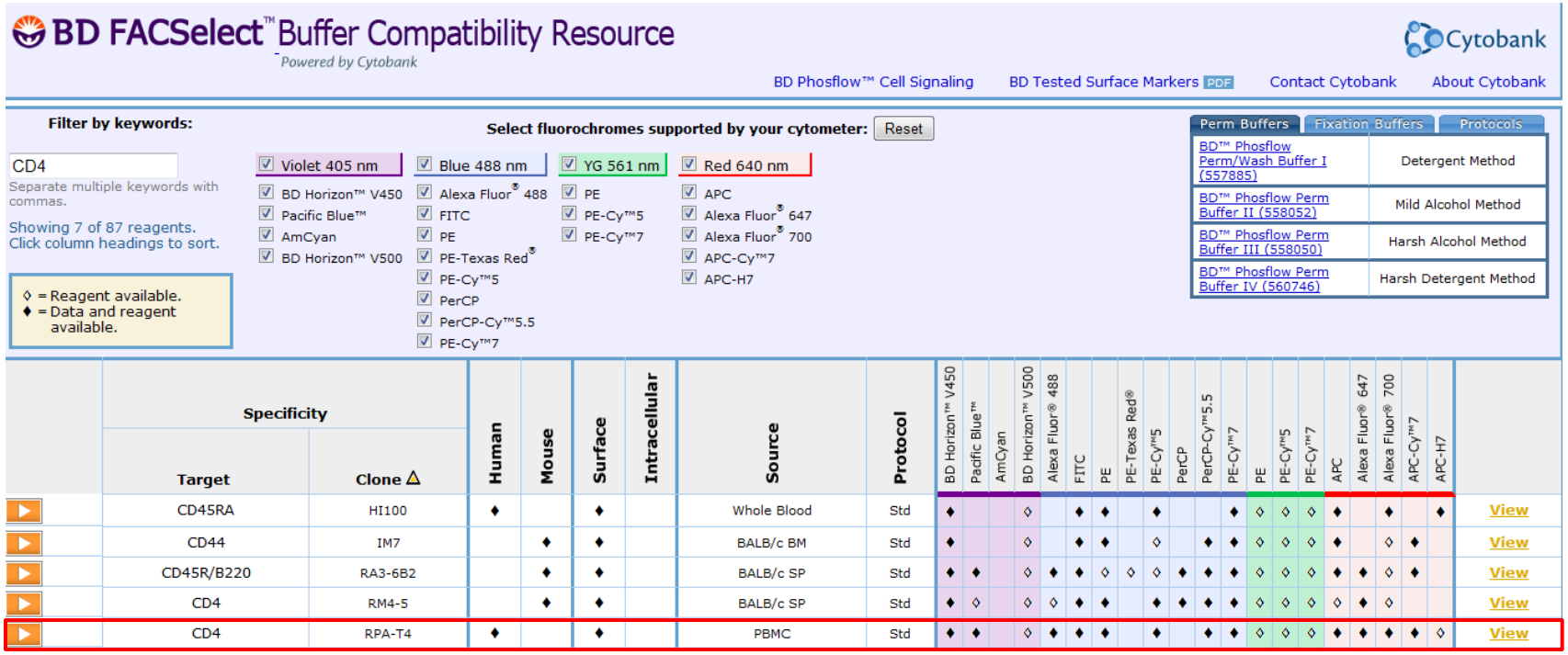
Determination of Buffer Compatibility

- T-cell subsets were identified using CD4 PerCP-Cy5.5, CD8 APC-Cy™7, CD25 Brilliant Violet 421, and CD127 Alexa Fluor® 647.
- To determine compatibility and recommended staining conditions for perm buffer III, the BD FACSelect buffer compatibility resource was used.



BD FACSelect™ Buffer Compatibility Resource

Powered by Cytobank

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BD Phosflow™ Cell Signaling | BD Tested Surface Markers PDF | Contact Cytobank | About Cytobank

Filter by keywords:

CD4

Separate multiple keywords with commas.

Showing 7 of 87 reagents.
Click column headings to sort.

◊ = Reagent available.
♦ = Data and reagent available.

Select fluorochromes supported by your cytometer: Reset

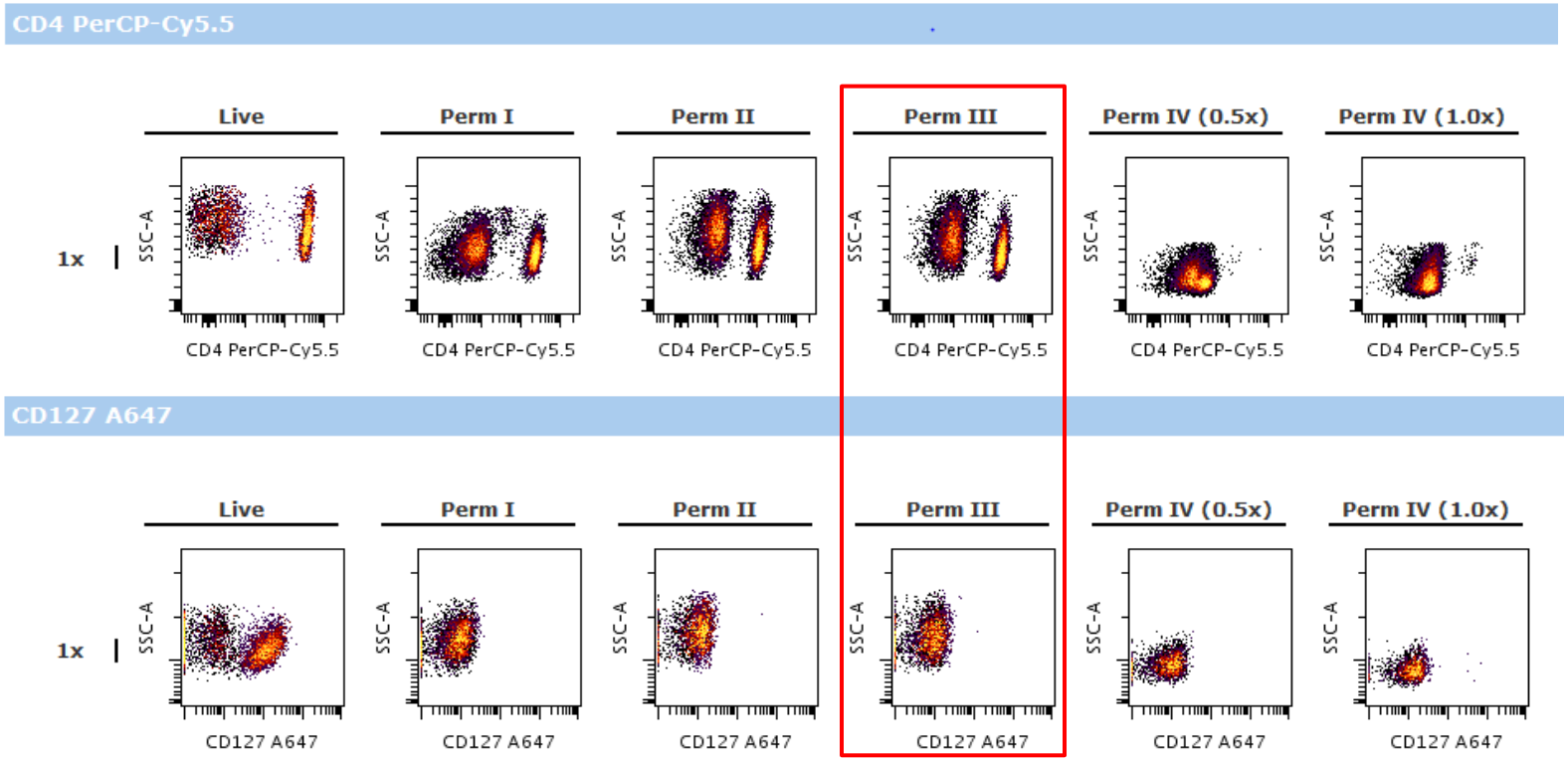
- Violet 405 nm: BD Horizon™ V450, Pacific Blue™, AmCyan, BD Horizon™ V500
- Blue 488 nm: Alexa Fluor® 488, FITC, PE, PE-Texas Red®, PE-Cy™5, PerCP, PerCP-Cy™5.5, PE-Cy™7
- YG 561 nm: PE, PE-Cy™5, PE-Cy™7
- Red 640 nm: APC, Alexa Fluor® 647, Alexa Fluor® 700, APC-Cy™7, APC-H7

Perm Buffers | Fixation Buffers | Protocols

BD™ Phosflow Perm/Wash Buffer I (557885)	Detergent Method
BD™ Phosflow Perm Buffer II (558052)	Mild Alcohol Method
BD™ Phosflow Perm Buffer III (558050)	Harsh Alcohol Method
BD™ Phosflow Perm Buffer IV (560746)	Harsh Detergent Method

	Specificity								BD Horizon™ V450	Pacific Blue™	AmCyan	BD Horizon™ V500	Alexa Fluor® 488	FITC	PE	PE-Texas Red®	PE-Cy™5	PerCP	PerCP-Cy™5.5	PE-Cy™7	PE	PE-Cy™5	PE-Cy™7	APC	Alexa Fluor® 647	Alexa Fluor® 700	APC-Cy™7	APC-H7	
	Target	Clone △	Human	Mouse	Surface	Intracellular	Source	Protocol																						
▶	CD45RA	HI100	♦		♦		Whole Blood	Std	♦			◊		♦	♦		♦			♦	◊	◊	◊	♦		♦		♦	View
▶	CD44	IM7		♦	♦		BALB/c BM	Std	♦			◊		♦	♦		◊		♦	♦	◊	◊	◊	♦		◊	♦		View
▶	CD45R/B220	RA3-6B2		♦	♦		BALB/c SP	Std	♦	♦		◊	♦	♦	◊	◊	♦	♦	♦	♦	◊	◊	◊	♦	♦	◊	♦		View
▶	CD4	RM4-5		♦	♦		BALB/c SP	Std	♦	◊		◊	◊	♦	♦		♦	♦	♦	♦	◊	◊	◊	◊	♦	◊			View
▶	CD4	RPA-T4	♦		♦		PBMC	Std	♦	♦		◊	♦	♦	♦		♦		♦	♦	◊	◊	◊	♦	♦	♦	♦	◊	View

Determination of Buffer Compatibility (continued)

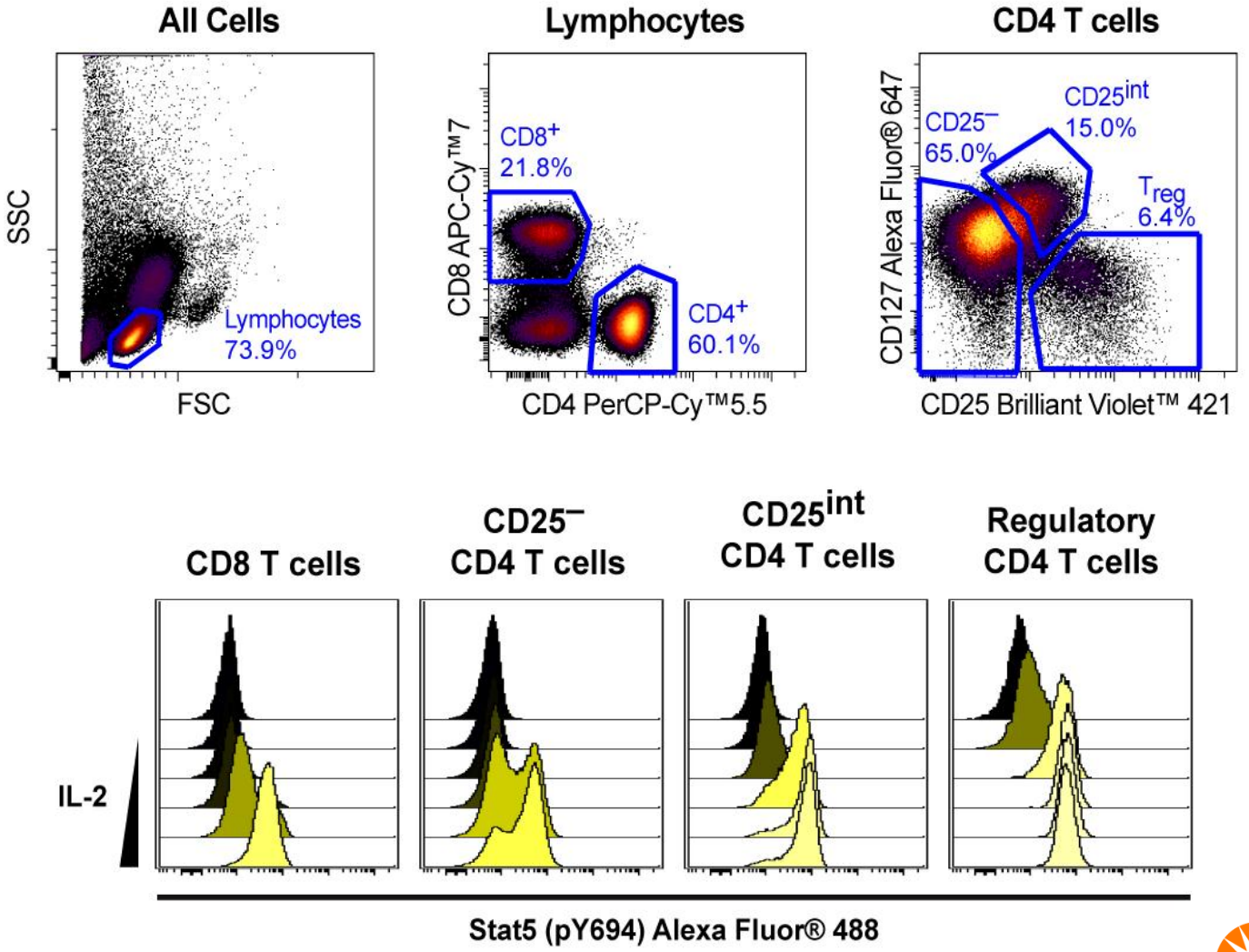


- CD4 is compatible with perm buffer III and other buffers.
- CD127 is not compatible with post-permeabilization staining.
  - Use an alternative protocol with CD127.

Example 3: Enhanced IL-2 Sensitivity of Tregs (continued)

- Human PBMCs were stained with CD127 Alexa Fluor® 647 during a 15-minute stimulation with 0-, 0.01-, 0.1-, 1-, 10-, or 100-ng/mL doses of recombinant IL-2.
- Cells were fixed using BD Cytofix fixation buffer and permeabilized using perm buffer III.
- Cells were then stained with Stat5 (pY694) Alexa Fluor® 488, CD4 PerCP-Cy5.5, CD8 APC-Cy7, and CD25 Brilliant Violet 421.
- Samples were acquired using a BD LSRFortessa™ flow cytometer and analyzed using Cytobank software.

Example 3: Results



Considerations when Combining Different Intracellular Techniques

- Timing of signaling responses
  - Signaling responses such as protein phosphorylation may have ended before others such as cytokine expression begin.
- Buffer selection
  - Need to select markers and fluorochromes compatible with the permeabilization method needed.
  - May need to try multiple buffers.
- Staining protocols
  - Staining surface markers prior to cell permeabilization may be necessary.

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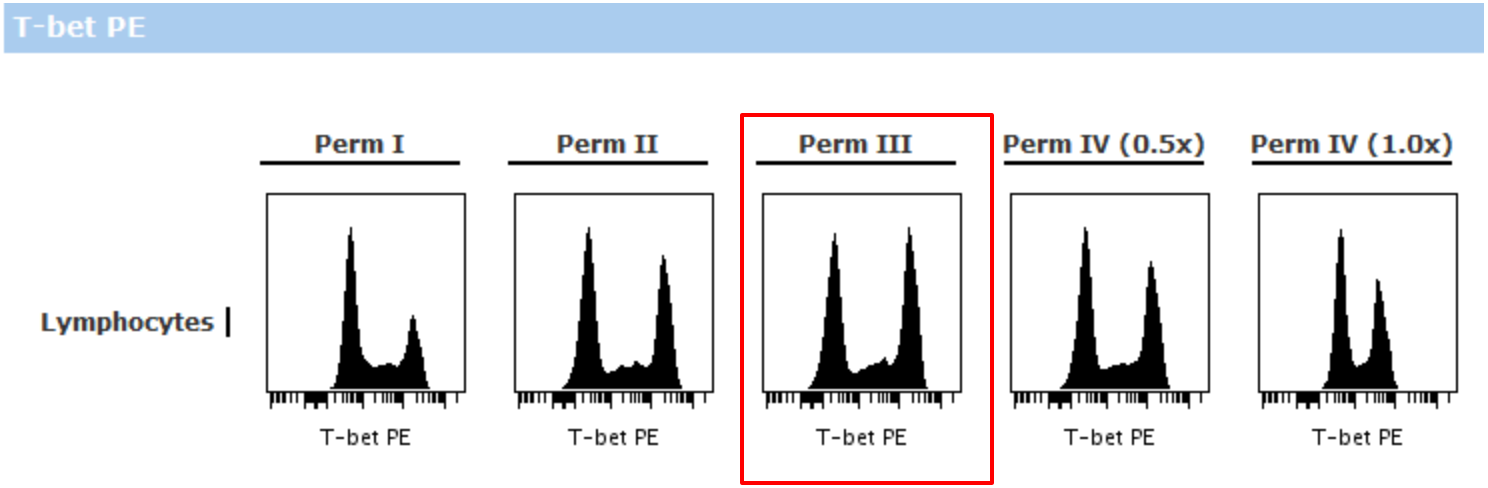
Example 4: IL-2 Response in Th1-Like and Non-Th1 Effector Memory CD4$^+$ T Cells

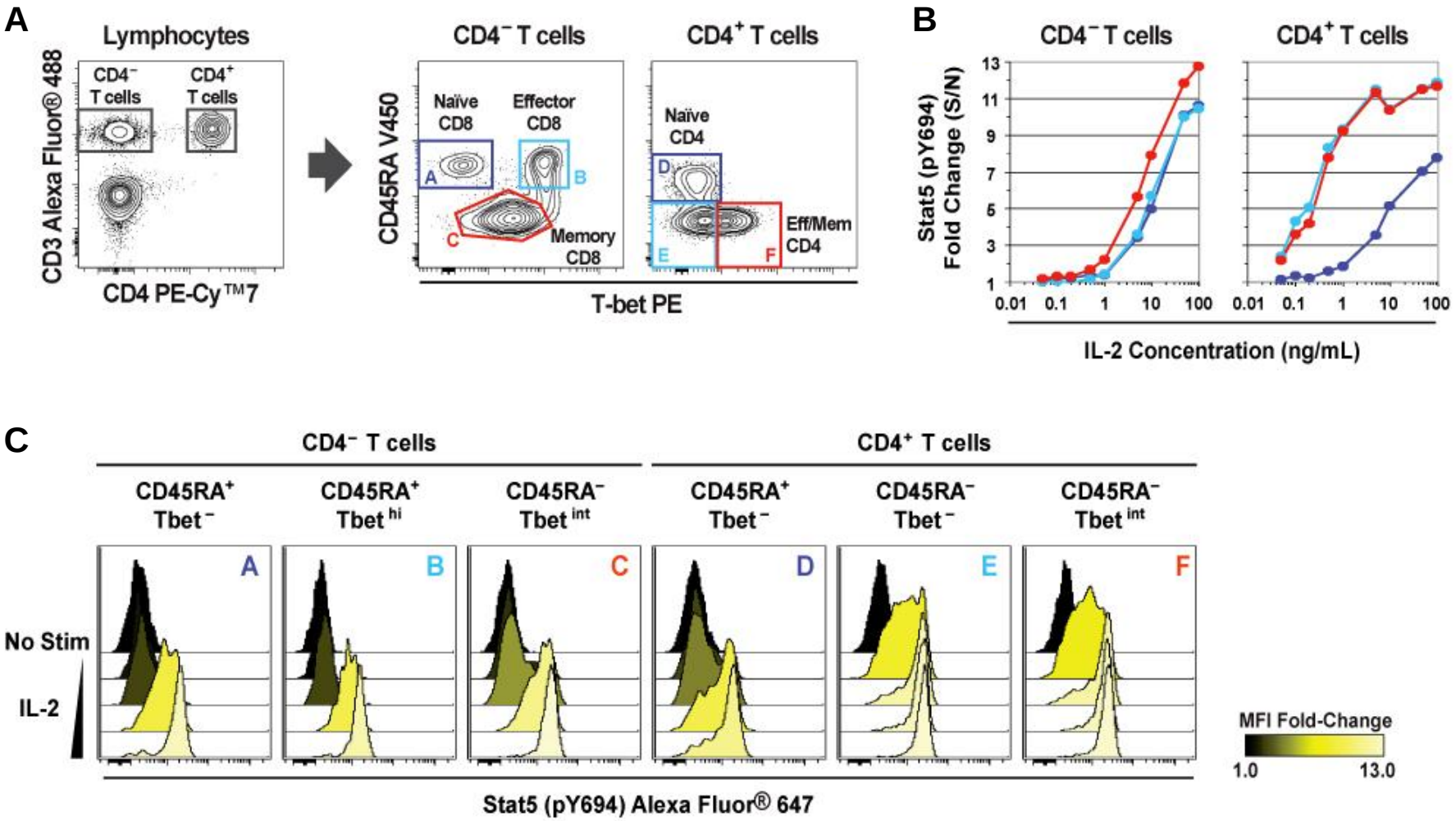
- In this experiment, T-bet was used to identify Th1-like cells.
  - T-bet is a transcription factor that controls the expression of IFN-γ.
- The T-bet antibody is compatible with perm buffer III.



Example 4, continued

- Human whole blood was stimulated with various concentrations of IL-2 (0.05–100 ng/mL) for 15 min.
- Cells were fixed with BD Phosflow™ lyse/fix buffer and permeabilized with perm buffer III.
- Cells were stained with CD3 Alexa Fluor® 488, CD4 PE-Cy7, CD45RA V450, T-bet PE, and Stat5 (pY694) Alexa Fluor® 647.
- Samples were acquired using a BD™ LSR II flow cytometer and analyzed with Cytobank software.

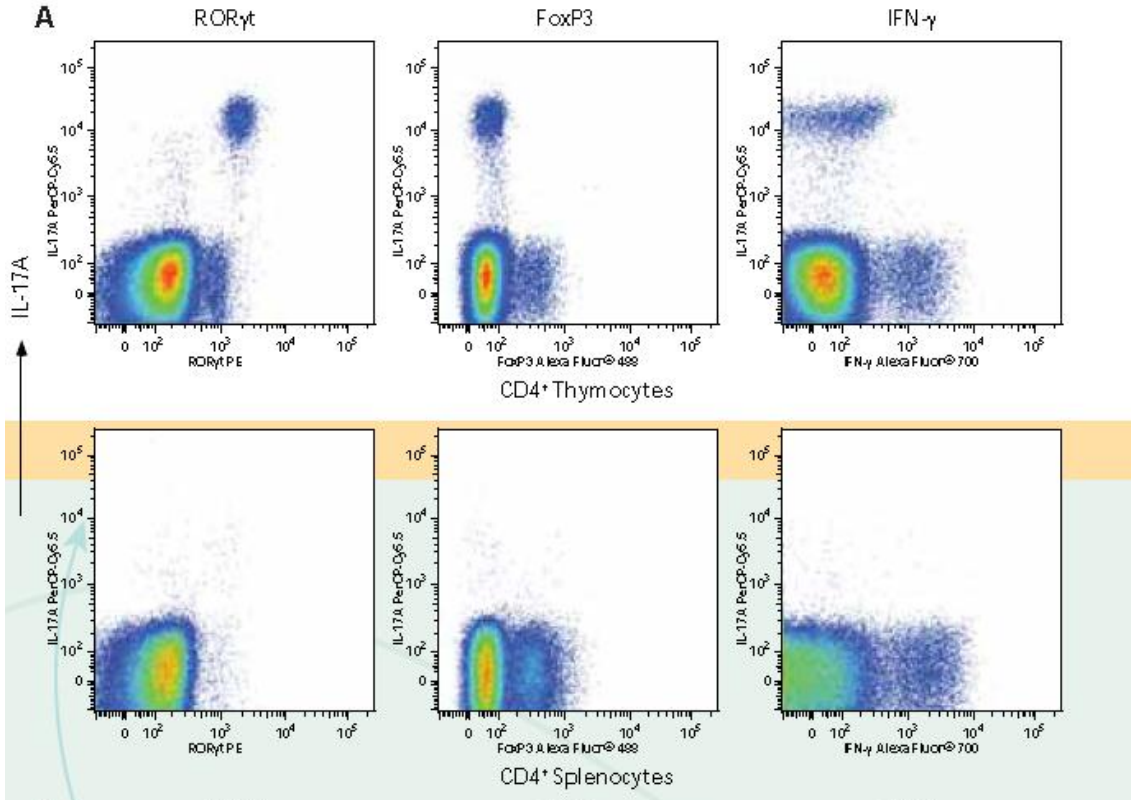
Example 4: Results



Example 5: Phenotypic Analysis of Th17 Cells from Mouse Spleen and Thymus

- RORγT is important for the secretion of IL-17 and the maintenance of $CD4^+CD8^+$ thymocytes.
- Cells isolated from BALB/c thymus and spleen were surface stained with fluorescently labeled antibodies to surface markers CD44, CD62L, CD196, and appropriate isotype controls.
- Cells were fixed and permeabilized with the BD Pharmingen transcription factor buffer set.
- Cells were then stained with antibodies to transcription factors RORγT and Foxp3 as well as cytokines IL-17A and IFN-γ.

Example 5: Results



Summary and Conclusions

- Intracellular flow cytometry is a powerful technique for the study of cellular signaling, function, and differentiation within subpopulations of cells.
- Different buffers work best for particular applications due to the biochemistry and cellular localization of the antigen.
  - BD Cytofix/Cytoperm (Cat. No. 554722) for cytokines
  - BD Pharmingen transcription factor buffer set (Cat. No. 562574) for transcription factors as well as transcription factors combined with cytokines
  - BD Phosflow™ perm buffer III (Cat. No. 558050) for phosphoprotein detection

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