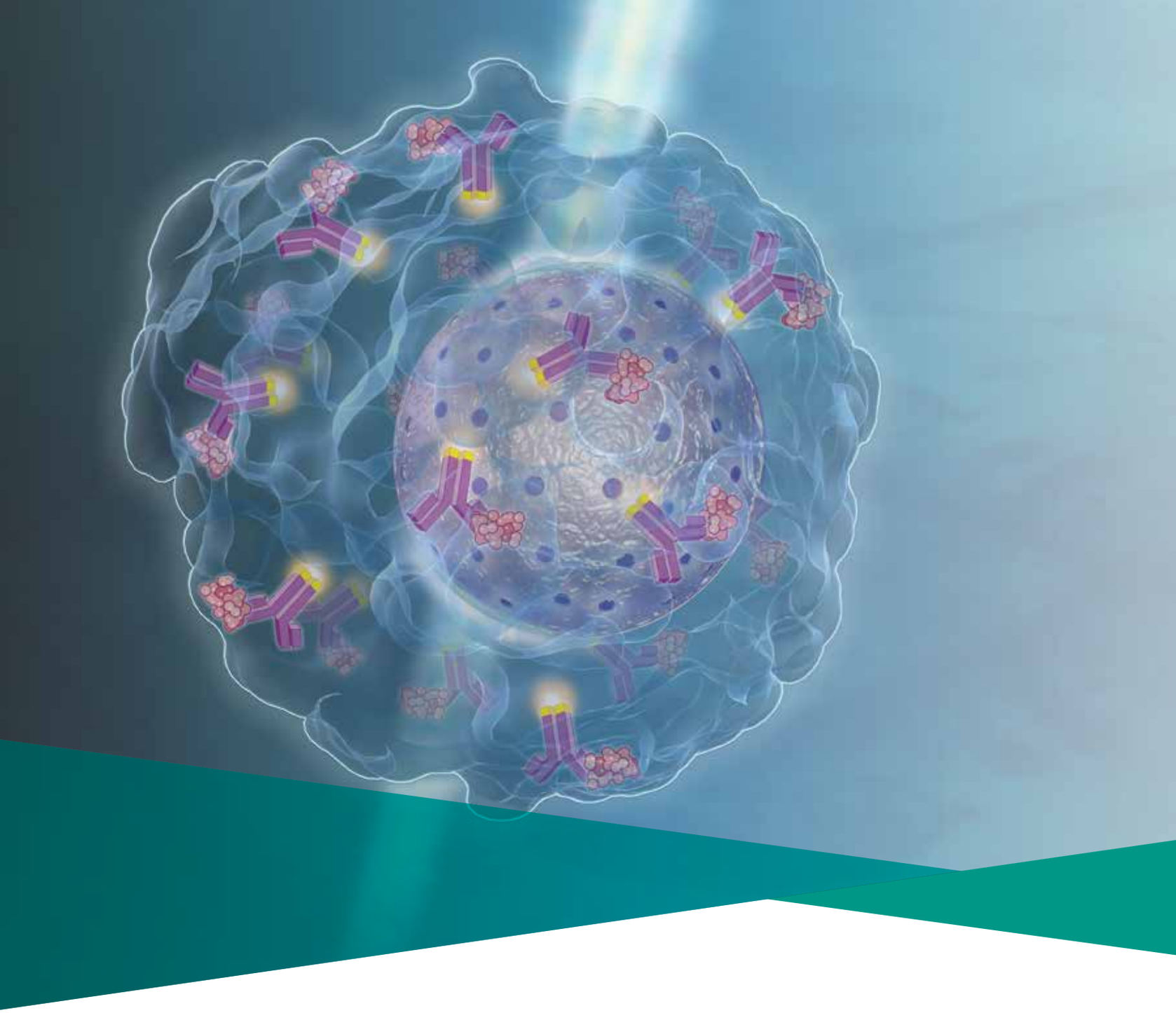


# Intracellular flow cytometry

## Multiparameter analysis of cytokine, transcription factor and phosphoprotein expression by flow cytometry

BD

# Intracellular flow cytometry

Intracellular flow cytometry is a powerful technique for the identification of cell types and the analysis of signaling and functional responses within cell lines and heterogeneous cell samples.

For some cell types, such as Th17 and regulatory T cells (Tregs), definitive identification depends on the combined use of surface and intracellular markers such as cytokines or transcription factors.

Intracellular flow cytometry also provides rich information concerning cellular function and signaling responses. Fluorescent antibodies specific for cell surface markers can be combined with markers of apoptosis, proliferation and protein phosphorylation to determine which cell subsets respond to various stimuli or treatments. The combined use of multiple markers decreases data acquisition time and conserves precious samples, since more parameters can be measured on a per-cell basis.

While Western blot and other methods are useful for the examination of single proteins expressed by entire cell populations, flow cytometry allows the detection of multiple proteins simultaneously at the level of individual cells.

BD provides fluorochrome-conjugated antibodies, buffers, kits and protocols to facilitate intracellular flow cytometry. BD antibodies are tested in biologically relevant model systems. These established tools enable new discoveries in fields such as immunology, inflammation and stem cell biology.

This brochure provides an overview of general techniques for intracellular flow cytometry and of specific methods for detection of cytokines and inflammatory mediators, transcription factors, phosphoproteins and combinations of these target molecules.

*From basic research to drug screening, BD Life Sciences carries high-quality reagents in the latest formats to examine cytokine, transcription factor and phosphoprotein expression within a heterogeneous population of cells.*

# Accessing antigens inside the cell

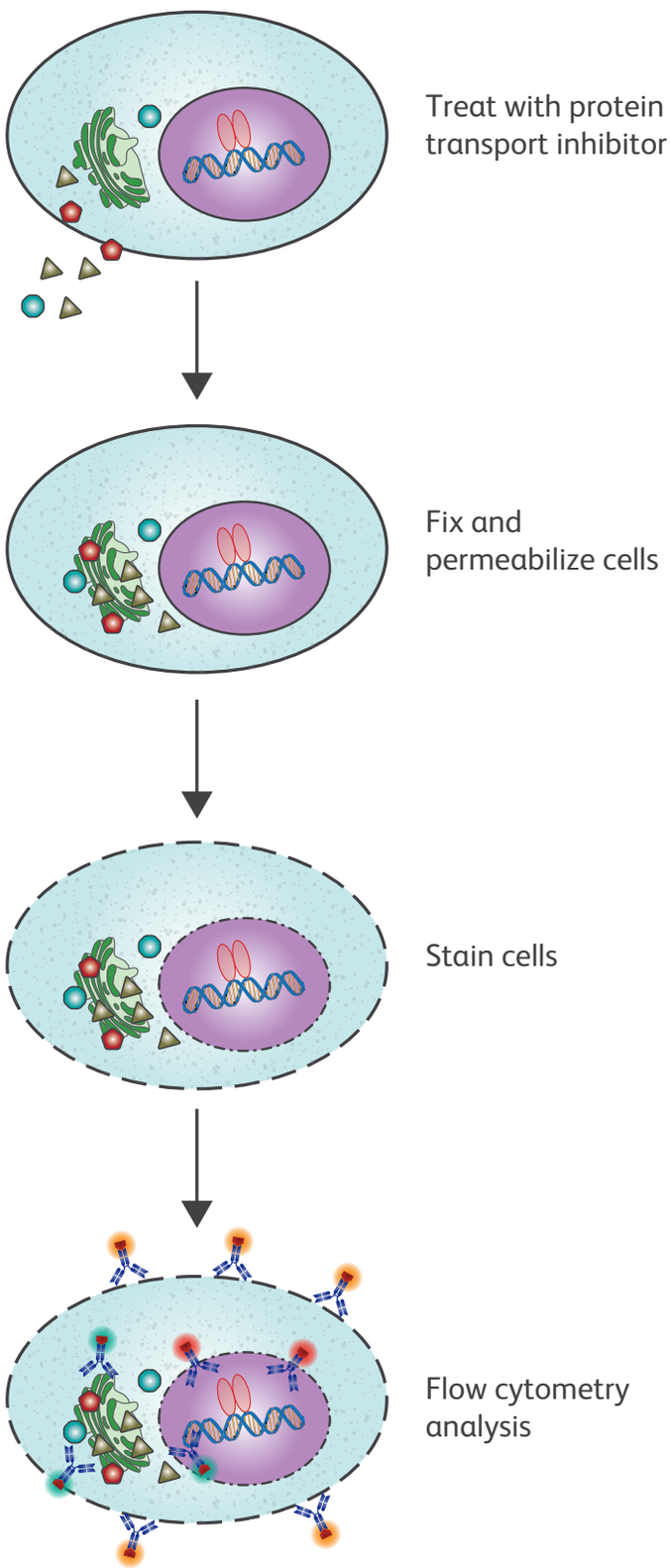
## Fundamentals of intracellular staining

Multicolor flow cytometry is a powerful technique for the analysis of intracellular proteins that are expressed by specific cell types. For many cell types such as Th17 cells, the combined use of cell surface and intracellular markers is necessary for definitive phenotypic identification. Simultaneous analysis of surface markers and signaling proteins can be used to characterize the nature of signaling responses within specific target cells.

While techniques for cell surface staining are relatively standard, optimal staining for intracellular markers often depends on the biology of the target protein. Depending on the protein's location inside the cell, association with other molecules and its stability, different cell preparation and staining methods are recommended. Cytokines, for example, are typically secreted proteins. However, if they are trapped inside the cell, they can be stained as intracellular proteins using protein transport inhibitors such as BD GolgiStop™ (containing monensin) or BD GolgiPlug™ (containing brefeldin A). Cytokines are relatively accessible using the gentle fixation and permeabilization afforded by BD Cytofix/Cytoperm™ fixation and permeabilization solution.

In contrast to cytokines, transcription factors often are localized inside the nucleus and bound to DNA and other proteins. Phosphorylation of some proteins, such as Stat5, results in dimer formation that masks the phosphorylated epitope of interest. Also, intracellular phosphatases can quickly dephosphorylate these proteins. Therefore, after treatment, cells must be quickly fixed and subjected to stronger permeabilization conditions to allow the antibody to enter the nucleus and access the epitope within disrupted molecular complexes.

The permeabilization technique used can negatively impact the detection of cell surface and other intracellular antigens. The same techniques that allow access to the nucleus and open up DNA / protein or protein / protein complexes can often denature cell surface antigens, preventing their detection by antibodies. While detection of different intracellular proteins might require different conditions, the basic principles are the same: cells are fixed and permeabilized and then stained intracellularly with fluorescent antibodies.



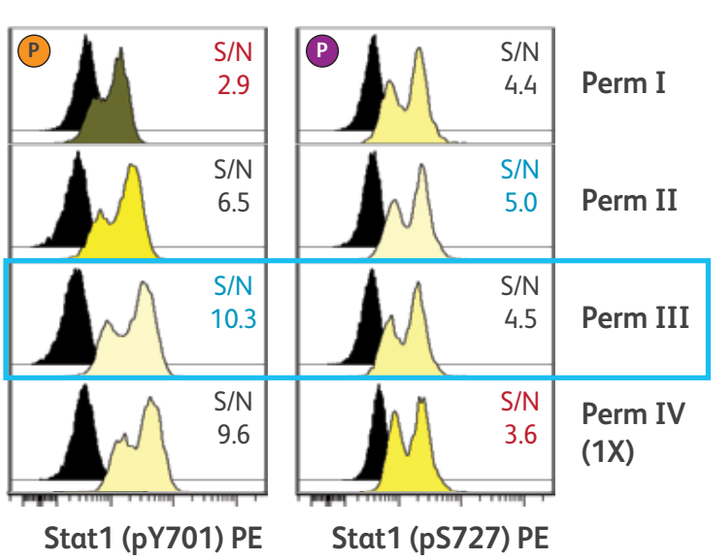

**Basic principles of intracellular staining.**
Cells are fixed and permeabilized (symbolized by dashed line membrane), stained, and then analyzed by flow cytometry. For studies of secreted proteins, cells are first treated with a protein transport inhibitor to allow accumulation of the target protein inside the cell.

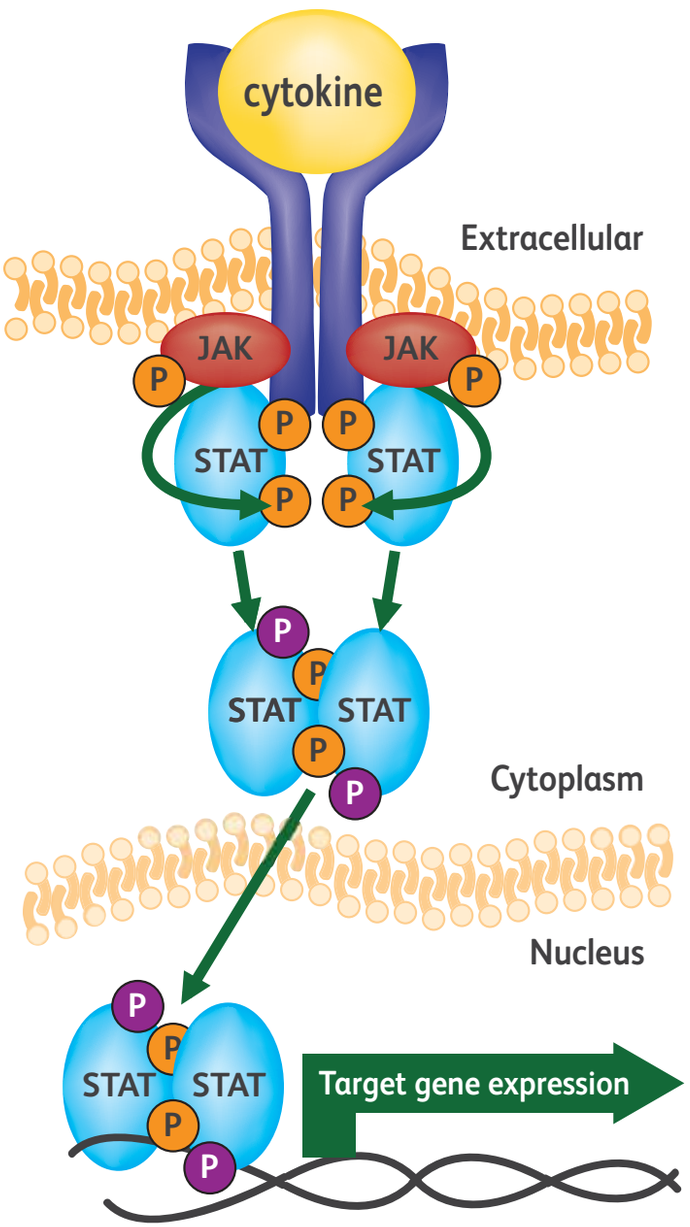
**BD tools for intracellular flow cytometry**
To facilitate intracellular flow cytometry assays, BD has developed several kits, buffers and protocols. In addition, many fluorescent antibodies specific for key cell surface markers have been tested in several buffer systems to save researchers' time, samples and money. Commonly used BD buffers for particular applications include:

**Detection of cytokines**
BD Cytofix/Cytoperm fixation / permeabilization solution (Cat. No. 554722) is suitable for staining most cytokines and cell surface markers. This buffer system can also be used in staining of some transcription factors and other intracellular proteins. This buffer system contains mild detergents along with a formaldehyde-based fixative.

**Transcription factors**
BD Pharmingen™ transcription factor buffer set (Cat. No. 562574/562725) is designed for the staining of transcription factors alone or in combination with cell surface markers and cytokines. This buffer system contains mild detergents along with a formaldehyde-based fixative.

**Detection of phosphorylated protein**
BD Phosflow™ perm buffer III (Cat. No. 558050) is the recommended permeabilization buffer for phosphoepitope detection by flow cytometry. Perm buffer III is a harsh alcohol-based buffer. Alternative permeabilization buffers also are available to accommodate particular experimental requirements. The BD Pharmingen™ Transcription Factor Phospho Buffer Set (563239/565575) is a new formulation that combines the power of the BD Pharmingen Transcription Factor buffer with phosphorylated protein detection.




STAT phosphotyrosine epitopes are obscured by dimerization within activated cells.

**Optimal cell permeabilization conditions vary by epitope location.**
Optimal permeabilization conditions are determined by the accessibility of the epitope within the cell.

The phosphotyrosine 701 epitope (orange) is located within the Stat protein dimer, while the phosphoserine 727 epitope is located outside the dimerization regions. For the phosphotyrosine 701 site, a harsher buffer such as perm buffer III is required for staining. To induce protein phosphorylation, human peripheral blood mononuclear cells (PBMCs) were either 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 with fluorescent phosphospecific antibodies.

# Tools and techniques for cytokine staining

## Tools to analyze cytokine-expressing cells

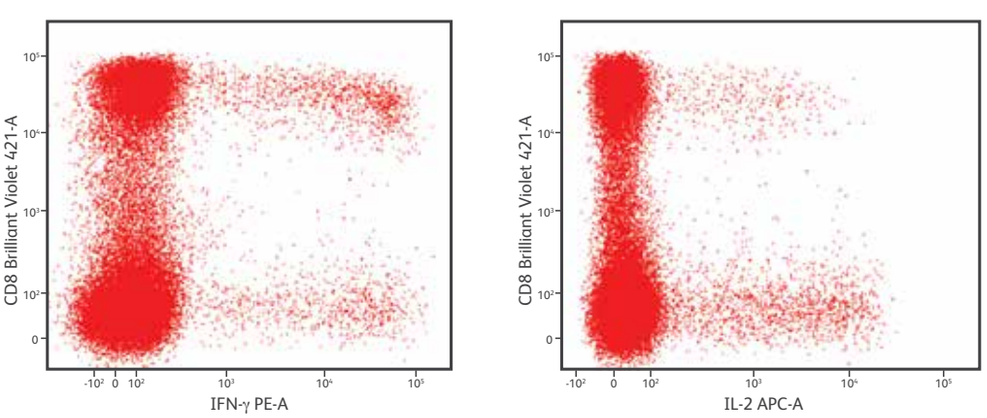
Various activated cell types can secrete cytokines, chemokines and other inflammatory mediators such as perforin and granzymes as part of an immune or inflammatory response. Methods such as ELISA and BD™ Cytometric Bead Array (CBA) measure secreted proteins produced by entire cell populations. In contrast, intracellular flow cytometry allows the analysis of cytokines and other inflammatory mediators produced by individual, phenotypically identified cell types within cell populations of interest.

Intracellular flow cytometry makes it possible to easily determine if the cytokine production by an activated cell population is the result of a few cells producing large amounts of cytokine or a large population of cells producing small quantities of cytokine per cell. Moreover, intracellular flow cytometry makes it possible to easily measure multiple cytokines simultaneously, to identify polyfunctional cells.[1] Intracellular cytokine staining is also useful for a variety of studies including B- and T-cell differentiation and plasticity.

Since cytokines typically are secreted proteins, they must first be trapped inside the cell by using a protein transport inhibitor. The two most commonly used protein transport inhibitors are monensin (BD GolgiStop) and brefeldin A (BD GolgiPlug). Monensin prevents protein secretion by interacting with the Golgi transmembrane $Na^{++}/H^{+}$ transport, while brefeldin A redistributes intracellularly produced proteins from the cis / medial Golgi complex to the endoplasmic reticulum.[2] As a result, the best choice of protein transport inhibitor varies by cytokine and by species. See Table 1.

**Table 1. Recommended protein transport inhibitor for cytokines by species**

| Species | Cytokines | Transport inhibitor |
|---|---|---|
| Human | IL-1α, IL-6, IL-8, TNF-α | Monensin |
| Human | IFN-g, 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 |



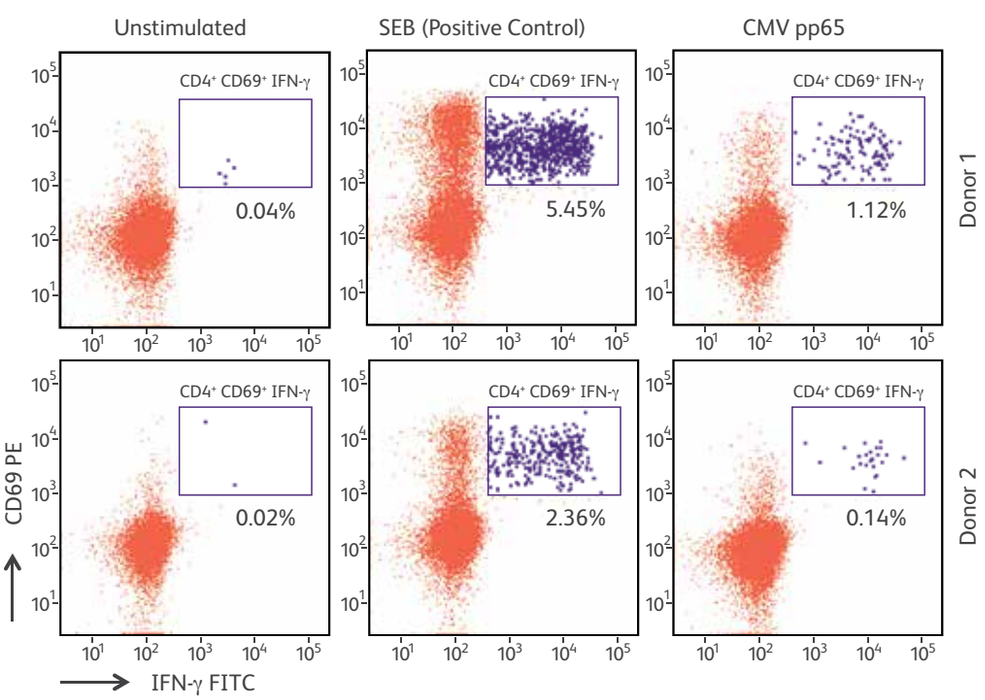

**IFN-γ and IL-2 production in CD8⁺ cells.**
PBMCs were stimulated with staphylococcal enterotoxin B for 6 hours in the presence of brefeldin A. After stimulation, cells were fixed and permeabilized using the BD Cytofix/Cytoperm buffer system. Cells were stained with the following fluorescent antibodies: CD3 FITC, CD4 PerCP-Cy™5.5, CD8 Brilliant™ Violet 421, IFN-γ PE, and IL-2 APC. Cells were then washed. Finally, cells were analyzed using a BD FACSVerse™ flow cytometer.

BD has simplified the detection of cytokines and cell surface markers with well established kits, buffer systems and rich panels of fluorescent antibodies. Antibodies to surface markers and cytokines conjugated to a variety of fluorochromes are available. This allows for flexibility in staining panel design, supporting high content multicolor flow cytometric analyses to gain the most data from precious cell samples.

BD FastImmune™ kits contain tested cocktails of fluorescent antibodies, an appropriate protein transport inhibitor, compatible buffers and a detailed protocol for the optimal preparation, staining and detection of cytokine-producing cells from whole blood. Onboard assays on the BD FACSVerse flow cytometer system further simplify the process.

BD Cytofix/Cytoperm buffer has been cited in thousands of publications for the analysis of cytokine-producing cells by flow cytometry. Researchers select the protein transport inhibitor best suited to their cytokine of interest and use the BD Cytofix/Cytoperm buffer system to fix, permeabilize, and stain their cells for flow cytometric analysis.


BD FastImmune CD4 intracellular cytokine detection kit.




**Antigen-specific IFN-γ production by cytomegalovirus (CMV) pp65-stimulated CD4⁺CD69⁺ T lymphocytes.** Two-color flow cytometric dot plots show IFN-γ vs CD69 expression by CD4 T cells that were either unstimulated (left panels), SEB-stimulated (as positive controls, center panels), and CMV pp65-stimulated (right panels) samples from two donors. Human whole blood was stimulated in the presence of brefeldin A before fixing, permeabilizing, and staining using the BD FastImmune™ 3-color CD4 intracellular cytokine detection kit. Data was acquired using a BD FACSVerse flow cytometer and a BD FACSuite™ software research assay.

# Detection of transcription factors and regulators by flow cytometry

## Regulators of cell differentiation

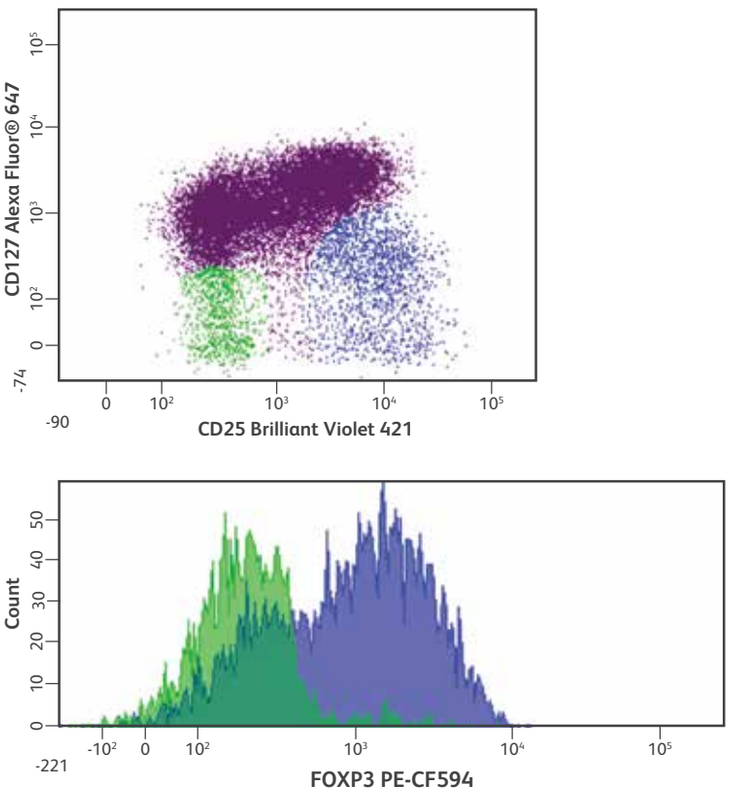
Intracellular flow cytometry enables the detection of transcription factors and associated proteins within heterogeneous cell populations. The simultaneous analysis of multiple markers allows for the determination of critical time points, markers and frequencies of cells moving along a particular differentiation pathway.

Transcription factors are proteins that bind to DNA and other proteins to regulate gene expression. They play key roles in cellular development and differentiation. Examples include FoxP3 for Treg differentiation and Sox17 for definitive endoderm.

FoxP3 is considered to be the master regulator of Tregs. Like many transcription factors, FoxP3 binds to thousands of genes, resulting in the up- or down-regulation of gene expression necessary for Treg function.[3] SATB1, a genome organizer, is repressed by FoxP3, preventing the induction of T-effector cytokines, including IL-4 and IFN-γ.[4]

Similarly to intracellular cytokine staining, transcription factor detection using flow cytometry requires cellular fixation and permeabilization. Transcription factors are typically located in the nucleus bound to DNA and other proteins. Depending on the nature of the target molecule epitopes, different fixation and permeabilization buffers might be necessary.

The fixation and permeabilization of cells can compromise cell surface marker staining, which makes the choice of compatible buffers even more critical. To further enable easy transcription factor detection, BD has developed the BD Pharmingen transcription factor buffer set. This buffer system is strong enough to allow access to most intracellular antigens while maintaining the detectability of most cell surface markers.



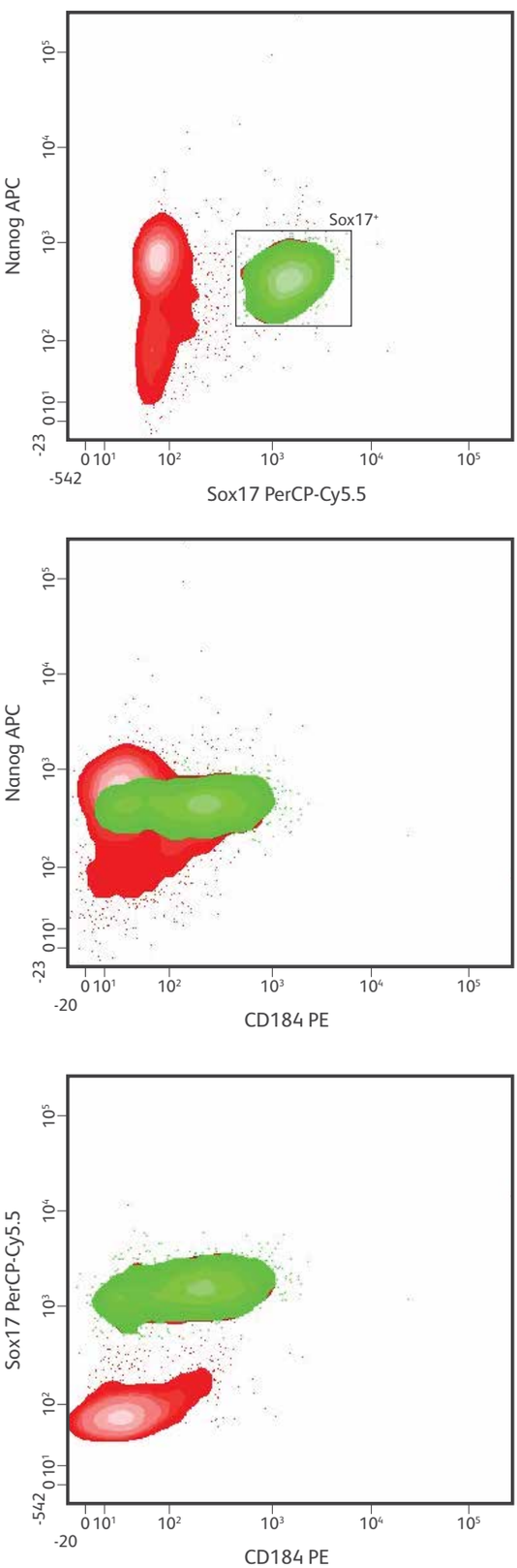

**Detection of human Tregs by intracellular flow cytometry.**
**Human PBMC**s were stained for surface markers using the following fluorescent antibodies: CD4 FITC, CD25 Brilliant Violet 421, and CD127 Alexa Fluor® 647. After washing, the cells were fixed and permeabilized using the BD Pharmingen transcription factor buffer set. Cells were then stained intracellularly with FoxP3 PE-CF594, washed, and acquired on a BD FACSVerse flow cytometer equipped with a PE-CF594 optional filter mirror unit. Gated Tregs (colored blue) have a $CD4^+$ $CD25^{high}$ $CD127^{dim}$ $FoxP3^+$ phenotype.

**Convenient kits for the study of stem cell differentiation**

As pluripotent stem cells differentiate into different cell types, expression of transcription factors and other proteins changes. In mammalian embryonic development, the definitive endoderm generates the liver, pancreas and intestine.[5–7] During specification into definitive endoderm, levels of the transcription factors Sox17 and FoxA2 and the cell surface marker CD184 (CXCR4) increase, while pluripotency markers such as Nanog and Sox2 decrease.[8]

Multicolor flow cytometry is an excellent method for determining the relative numbers of cells expressing markers of interest. This is useful for the study of cell differentiation pathways and specifically for the optimization, quantification and comparison of differentiation protocols and the differentiation potentials of different cells.

To facilitate the study of transcription factors in stem cells, BD has developed several kits for the detection of key stem-cell–specific transcription factors including the BD Stemflow™ human pluripotent stem cell transcription factor analysis kit (Cat. No. 560589), the BD Stemflow™ mouse pluripotent stem cell transcription factor analysis kit (Cat. No. 560585), the BD Stemflow™ human neural cell lineage analysis kit (Cat. No. 561526) and the BD Stemflow™ human definitive and pancreatic endoderm analysis kit (Cat. No. 562496). These kits contain optimized antibodies and buffer systems to characterize pluripotent stem cells and allow tracking of the differentiation of pluripotent stem cells into respective lineages.




**Definitive endoderm differentiation of H9 hESCs.**
H9 human embryonic stem cells (hESCs) (WiCell, Madison, WI) were differentiated to definitive endoderm for three days according to a protocol in D'Amour et al.[8] Differentiated cells were analyzed by flow cytometry using components of the BD Stemflow human definitive and pancreatic endoderm analysis kit and CD184 PE. As cells differentiated, levels of the pluripotency marker Nanog decreased, and levels of definitive endoderm markers Sox17 (green) and CD184 increased. Flow cytometry was performed on a BD™ LSR II flow cytometry system.

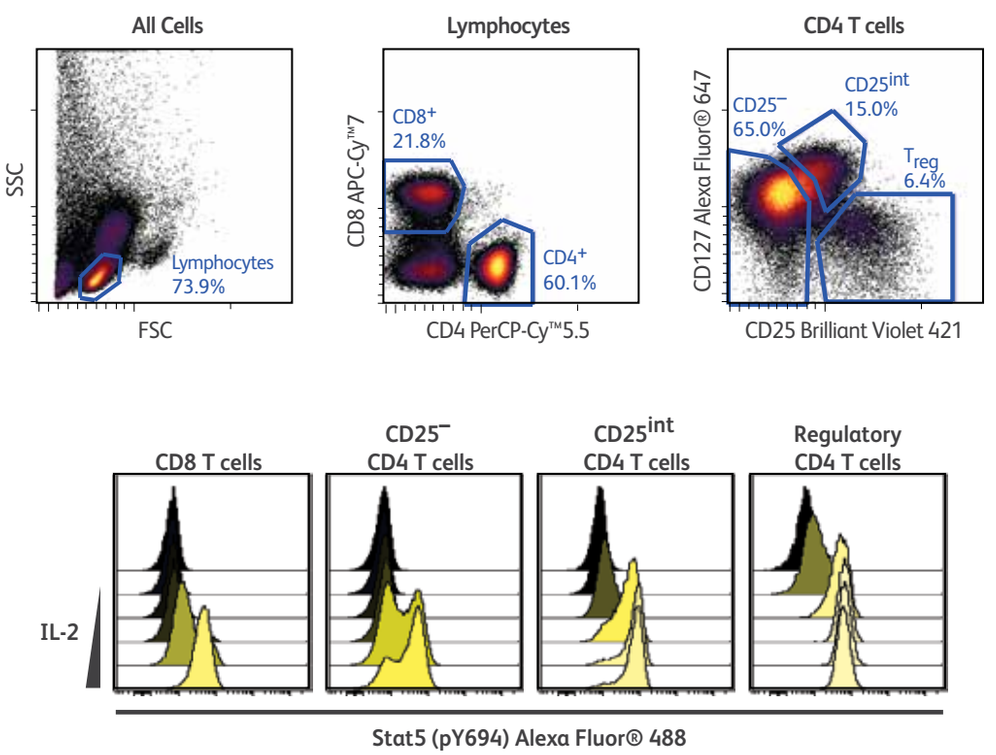
# Detection of phosphorylated proteins by flow cytometry

## Tools for the study of cell signaling

With the ability to collect data at the level of individual cells, flow cytometry offers several advantages for cell signaling studies. Unlike lysate-based approaches, flow cytometry facilitates the detection and analysis of heterogeneous signaling responses. Thus, it is possible to distinguish between a robust protein phosphorylation response within a small population of cells versus a smaller but more homogeneous response. With the addition of fluorescent antibodies specific for cell surface markers to cell subsets within complex cell mixtures such as whole blood, signaling responses mediated by protein phosphorylation can be detected. Moreover, rare cell populations can be uncovered without pre-enrichment of the cells. As a result, rich data is obtained from limited cell samples.

BD Phosflow™ products consist of a system of buffers and fluorescent monoclonal antibodies optimized for the flow cytometric detection of intracellular signaling molecules and specific post-translational modifications. To detect specific phosphorylated epitopes by flow cytometry, cells are fixed to maintain the phosphorylated state of signaling proteins and then permeabilized to allow antibodies to enter cells and specifically bind to target proteins. Flow cytometric analysis of stained cells can then capture a "snapshot" of intracellular signaling and protein phosphorylation.

Protein phosphorylation is transient by nature and heavily regulated by protein phosphatases. Appropriate stimulation time points and prompt inactivation of phosphatases are required for most methods of phosphoprotein detection, including Western blot analysis. Likewise, cell samples for flow cytometric analysis must be quickly fixed to maintain phosphoepitopes. Another important consideration is the level of phosphoprotein expression. Certain protein phosphorylation events can be difficult to detect at the single-cell level, particularly when cells express low levels of a particular signaling protein or exhibit incomplete phosphorylation of the site of interest. BD offers high-quality antibodies directly conjugated to bright fluorochromes to greatly improve the intracellular detection of phosphoproteins.

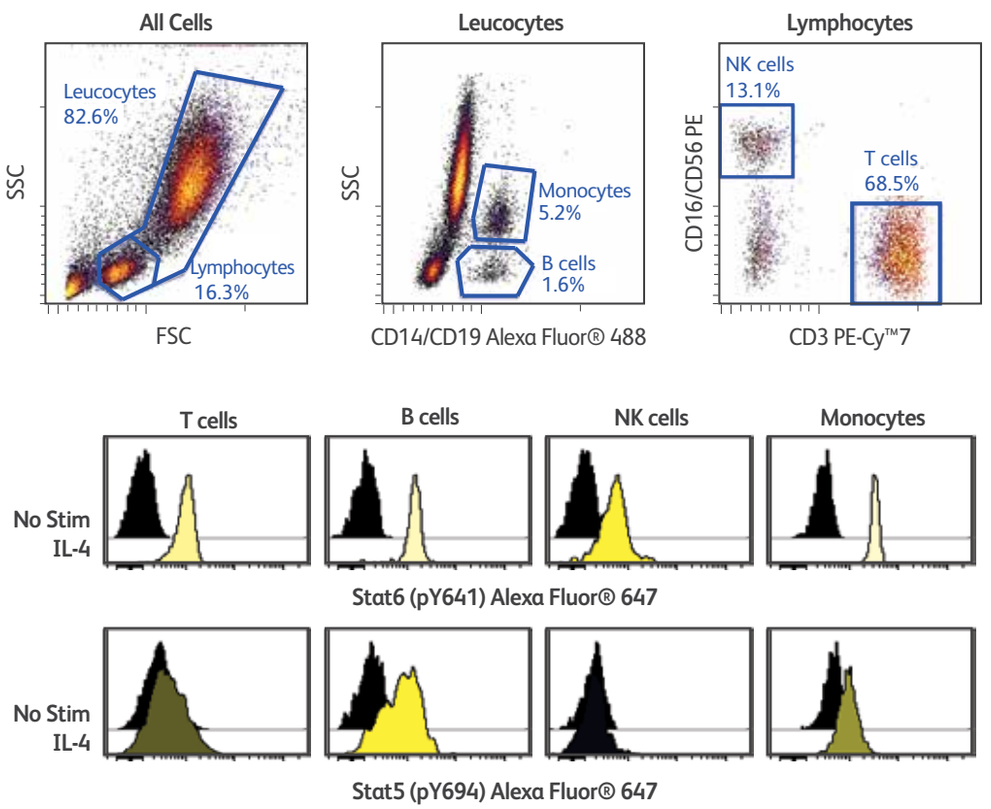



**Enhanced IL-2 sensitivity of Tregs.**
Human PBMCs were stained with Alexa Fluor® 647 Anti-Human CD127 antibody during a 15-minute stimulation with 0-, 0.01-, 0.1-, 1-, 10-, or 100-ng / mL doses of recombinant human IL-2. Cells were fixed using BD Cytofix fixation buffer, permeabilized using BD Phosflow perm buffer III and stained with fluorescent antibodies specific for Stat5 (pY694), CD4, CD8 and CD25. Samples were acquired using a BD LSRFortessa™ flow cytometry system and analyzed using Cytobank software. CD4 T-cell subsets were identified as shown. Compared to other T cells, Tregs phosphorylate Stat5 in response to much lower concentrations of IL-2.

Multiparameter flow cytometry offers key advantages for the detection and analysis of intracellular signaling within cells and cell subsets in complex mixtures. Since cell signaling studies often examine cellular responses to treatment with stimulatory or inhibitory molecules, unstimulated or untreated cells often provide the best controls for evaluating background staining. Unlike an immunoglobulin isotype control, an unstimulated cell control takes into account the unique background characteristics of each antibody as well as the basal phosphoprotein expression levels within the cells of interest.

Cellular permeabilization is required to expose phosphorylated epitopes. Although multiple buffer systems are available, BD Phosflow perm buffer III is recommended for most applications. The BD Biosciences website contains useful information about the performance of various BD cell surface marker and intracellular antibodies in different buffer conditions and staining protocols.

BD Phosflow kits provide useful tools for analyzing the signaling responses elicited by cells of the adaptive and innate immune systems. These kits include fluorescent antibody cocktails specific for cell surface markers to identify specific leucocyte subsets as well as fluorescent antibodies to key phosphoproteins. In addition, the kits provide compatible buffers, positive and negative control cells and detailed protocols. The BD Phosflow™ human monocyte / NK cell activation kit (Cat. No. 562089) allows the simultaneous study of protein phosphorylation in B cells, T cells, monocytes and NK cells from human whole blood, while the BD Phosflow™ human T-cell activation kit (Cat. No. 560750) is useful for the study of phosphoprotein responses in CD4 versus CD8 T cells.




**Stat5 and Stat6 signaling responses to IL-4 differ among human leucocyte subsets.**
Human whole blood was stimulated with human IL-4 and fixed, permeabilized and stained using the BD Phosflow human monocyte / NK cell activation kit. Samples were acquired and analyzed by flow cytometry using a BD LSRFortessa™ cell analyzer, and then analyzed using Cytobank software. Leucocyte subsets were identified as shown. Analysis of Stat6 (pY641) and Stat5 (pY694) phosphorylation responses to IL-4 revealed that all four leucocyte subsets responded to IL-4 by phosphorylating Stat6. In contrast, Stat5 responses varied considerably among cell types, with NK cells not showing a detectable Stat5 phosphorylation response.

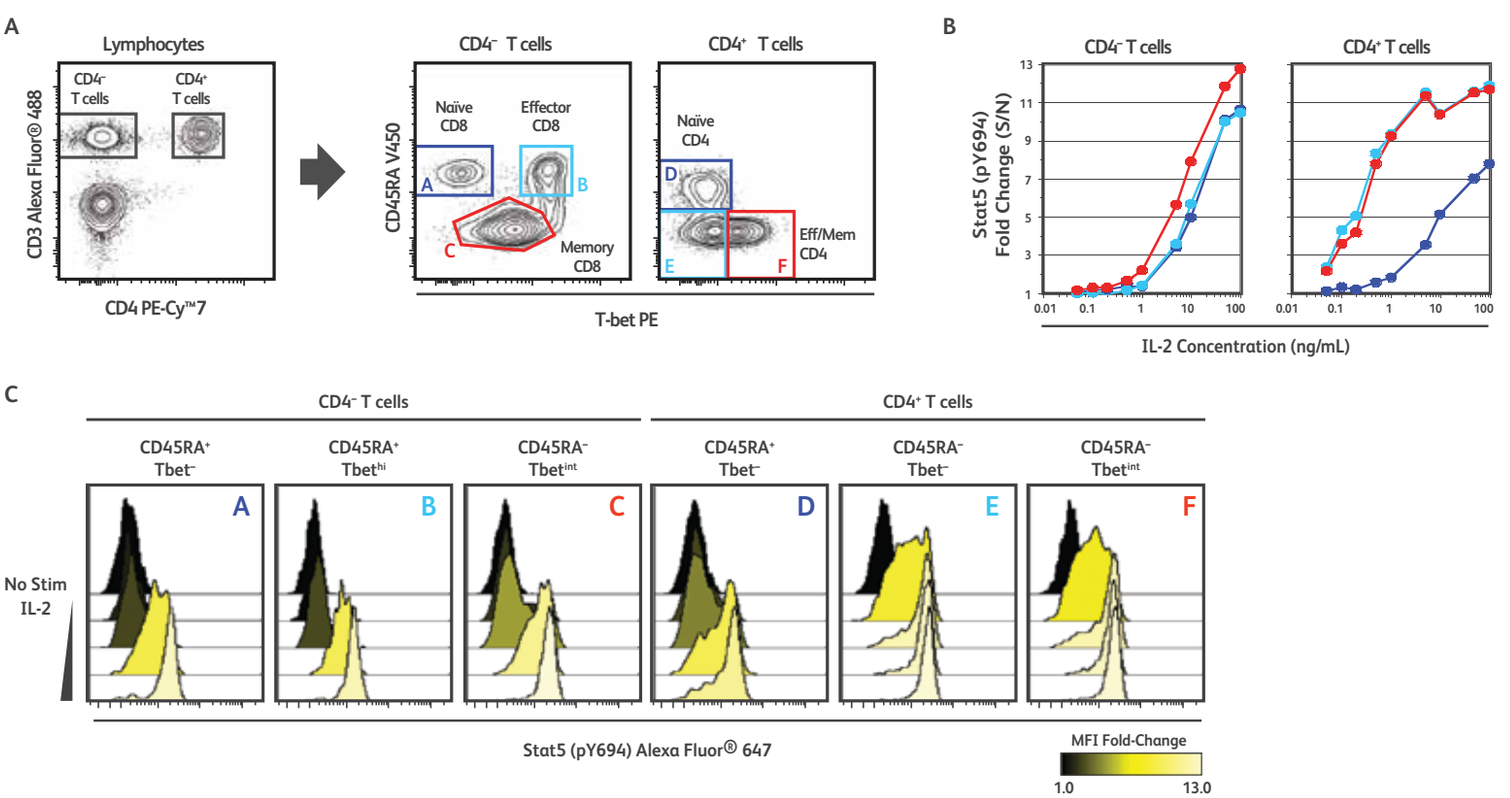
# Combining intracellular markers to maximize results from precious samples

## Fundamentals of intracellular staining

While other techniques can measure either cytokines, transcription factors or phosphorylated proteins separately, intracellular flow cytometry enables the measurement of multiple intracellular markers simultaneously at the single-cell level. This method provides data on signaling responses, differentiation states and other cellular events. The combined use of fluorescent antibodies specific for cell surface and intracellular markers enables high resolution comparative analyses of the phenotypic and functional differences within multiple cell types across samples.

In the example shown, cells from human whole blood were analyzed for Stat5 phosphorylation induced by IL-2 stimulation. Different populations of naïve, effector and memory T cells were identified using phenotypic markers. In addition to cell surface markers, phenotyping was aided by analysis of T-bet, a signature transcription factor involved in interferon-γ production and known to be expressed in NK cells, CD8 effector T cells, and CD4 T helper 1 (Th1) cells.[9,10]

In this experiment, differences in IL-2 sensitivity were observed across the different cell types. For instance, Th1-like and non-Th1 effector / memory CD4 T-cell subsets (populations F and E, respectively) responded to stimulation with very low concentrations of IL-2, whereas naïve CD4 T cells (population D) and naïve CD8 T cells (population A) required higher doses of IL-2 to induce Stat5 phosphorylation.




**IL-2 dose-response in T-cell subsets.**
Human whole blood was stimulated with various concentrations of human recombinant IL-2 (0.05–100 ng/mL) for 15 minutes. Cells were fixed with BD Phosflow™ lyse/fix buffer, permeabilized with BD Phosflow perm buffer III and stained with fluorescent antibodies specific for CD3, CD4, CD45RA, T-bet and Stat5 (pY694). Samples were analyzed using a BD LSR II flow cytometry system with Cytobank software. T-cell subsets were identified as shown.

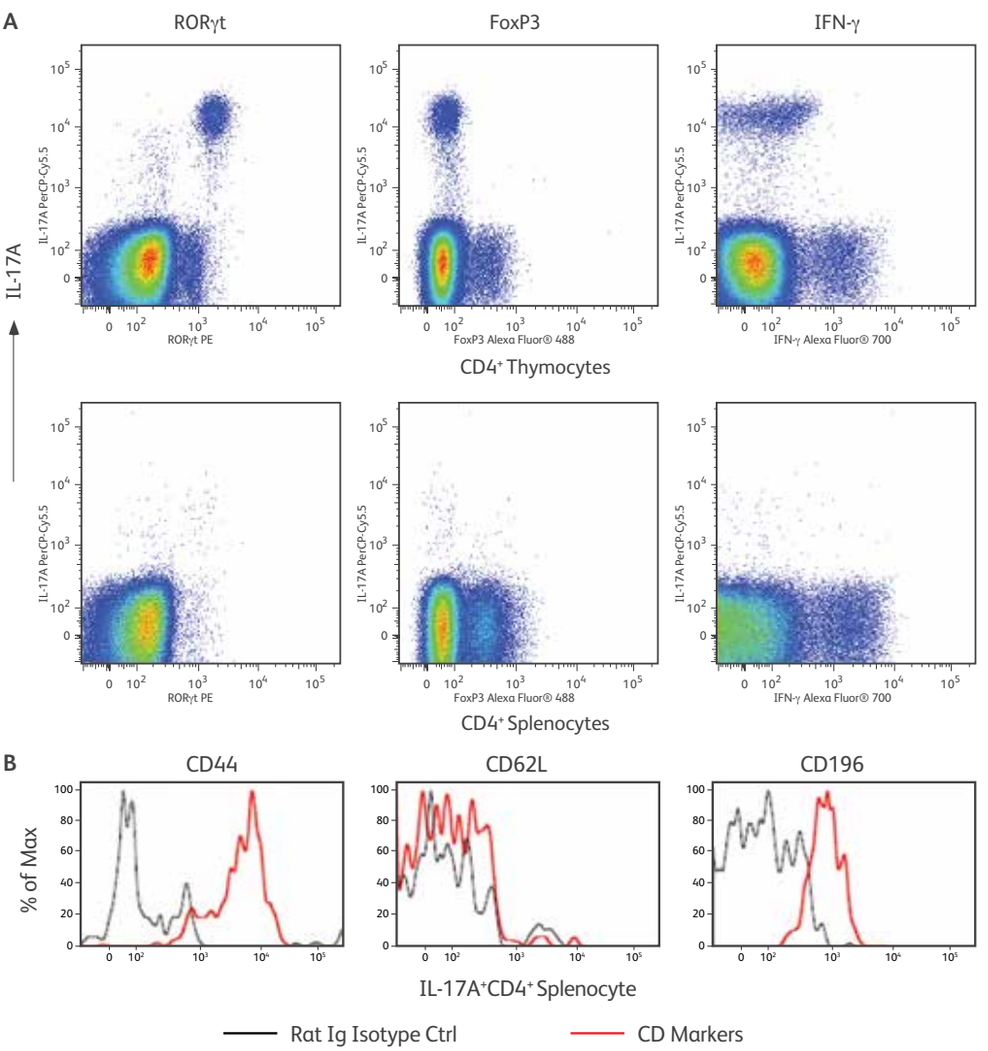
**Simultaneous detection of cytokines, transcription factors, and surface markers to characterize mouse Th17 cells**

The simultaneous measurement of the expressed levels of multiple transcription factors, cytokines and surface markers by cells within the same sample is very useful for the study of T helper (Th) cell differentiation and function. RORγt is the signature transcription factor for Th17 cells. It is important for the secretion of IL-17 and the maintenance of $CD4^+CD8^+$ double-positive thymocytes.[11,12]

In the experimental results shown, cells were isolated from BALB/c mouse thymus and spleen. Thymocytes or splenocytes were surface stained with fluorescent antibodies specific for CD44, CD62L, CD196 or appropriate immunoglobulin isotype controls. Cells were fixed and permeabilized with the BD Pharmingen transcription factor buffer set and then intracellularly stained with fluorescent antibodies specific for RORγt, Foxp3, IL-17A and IFN-γ.

In the top panel, the cellular expression of IL-17A is compared with the expression of RORγt, Foxp3 (Treg transcription factor) and IFN-γ (Th1 cytokine). As expected in thymocytes, there were many $IL\text{-}17A^+$ $ROR\gamma t^+$ double-positive cells, while there was essentially no co-expression of IL-17A with Foxp3 or IFN-γ. Splenocytes expressed very little IL-17A.

In the bottom panel (B), further analysis revealed that IL-17A–producing cells from spleen expressed the surface markers CD44 and CD196 (CCR6) but not CD62L, providing additional information on the phenotype of $IL\text{-}17A^+$ cells.




**Phenotypic analysis of Th17 cells from BALB/c thymus and spleen.**

Thymocytes or splenocytes were surface stained with fluorescent antibodies specific for CD44, CD62L, CD196 or appropriate immunoglobulin isotype controls. Cells were fixed and permeabilized with the BD Pharmingen transcription factor buffer set, then intracellularly stained for RORγt, Foxp3, IL-17A and IFN-γ.

# Services and support

BD Biosciences instruments and reagents are backed by a world-class service and support organization with unmatched flow cytometry experience. Our integrated approach combines flow cytometry instrumentation with trusted, certified reagents and advanced applications. The BD Biosciences tools enable our customers to discover more and obtain the most complete picture of cell function, and at the same time experience improved workflow, ease of use and optimal performance.

Researchers come to BD Biosciences not only for quality products, but as a trusted lab partner. Our repository of in-depth, up-to-date knowledge and experience is available to customers through comprehensive training, application and technical support and expert field service.

For example, our website, **bdbiosciences.com**, contains useful tools such as the multicolor panel designer and spectrum viewer. Links can also be found to the BD FACSelect™ buffer compatibility resource.

**Technical application support**

BD Biosciences technical application support specialists are available to provide field- or phone-based assistance and advice. Expert in a diverse array of topics, BD technical application support specialists are well equipped to address customer needs in both instrument and application support.

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