Studying Cell Signaling with the BD Accuri™ C6 Flow Cytometer

Technical Bulletin

Contents

Introduction

Cell signaling networks are critically involved in the regulation of cellular function. Cell survival, growth, and differentiation are tightly regulated through phosphorylation and dephosphorylation of key proteins in signaling cascades. Studies of protein phosphorylation can be useful for identifying informative biomarkers.

Many common techniques for measuring protein phosphorylation, such as Western blot, ELISA, and bead- and array-based technologies, require cell lysis. However, lysate-based approaches can miss the differences among individual cells within a mixed population. For subset analysis, cell enrichment or sorting is required, which can change the functional response of the cell. Beyond these limitations, lysate-based analysis can be complicated and time-consuming, especially for complex pathways or heterogeneous samples.

Flow cytometry provides a fast, accurate, and powerful method for studying cell signaling. Using BD Phosflow™ technology, researchers can stain cells with antibodies against surface and intracellular proteins to assess protein phosphorylation simultaneously in multiple cell populations of interest, including rare cells. This technique allows scientists to study cell signaling in near-native conditions, reducing the potential for artifacts. Analysis at the single-cell level allows the detection of heterogeneous signaling responses, which cannot be distinguished by lysate-based approaches.

Paired with BD Phosflow technology, the BD Accuri™ C6 flow cytometer provides a complete flow cytometry solution for cell signaling analysis. Equipped with a blue laser, a red laser, two light scatter detectors, and four fluorescence detectors, the BD Accuri C6 can analyze up to four colors right on the benchtop. Compact design, fixed alignment, pre-optimized detector settings, and intuitive software result in a system that is simple to use. For walkaway convenience, the optional BD CSampler™ accessory offers automated sampling from 24-tube racks or multiwell plates. For deeper analysis, data files can be exported into Cytobank, FCS Express™, FlowJo™, or other flow cytometry analysis programs.

This technical bulletin presents an overview of the BD Phosflow methodology, along with sample cytokine signaling data from the BD Accuri C6 using two BD Phosflow cell activation kits. Although the examples focus on cytokine signaling, the kits can be used to assess other signaling pathways as well, including cellular responses to lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA).

Figure 1. The BD Accuri C6 flow cytometer system.

The instrument weighs just 13.6 kg (30 lb). Exterior dimensions (H x W x D) are 27.9 x 54.6 x 41.9 cm (11.0 x 21.5 x 16.5 in.) with fluid tanks in place.

Cytokine signaling (JAK/STAT pathway)

Cytokine signaling is critical for regulation of immunity and inflammation. Some cytokines, such as IL-2, promote cell-mediated immunity by driving the expansion of T cells, while others, such as IL-6 and IFN-α, act as pro-inflammatory mediators in innate and adaptive immune responses.

Cytokine signaling is mediated primarily through the JAK/STAT pathway (Figure 2). The pathway is initiated when a cytokine binds to its cognate receptor at the cell surface, which activates the Janus kinases (JAKs) constitutively associated with the receptor. Activated JAKs phosphorylate the cytoplasmic tail of the cytokine receptor, creating docking sites for the Signal Transducers and Activators of Transcription (STATs), and then, in turn, phosphorylating the STATs at specific tyrosine residues. Phosphorylated STATs dimerize and translocate to the nucleus, where they bind to promoter regions of target genes and initiate transcription. Different STAT family members (such as Stat1, 3, 5, and 6) are preferentially activated by particular cytokines in different cell subsets.

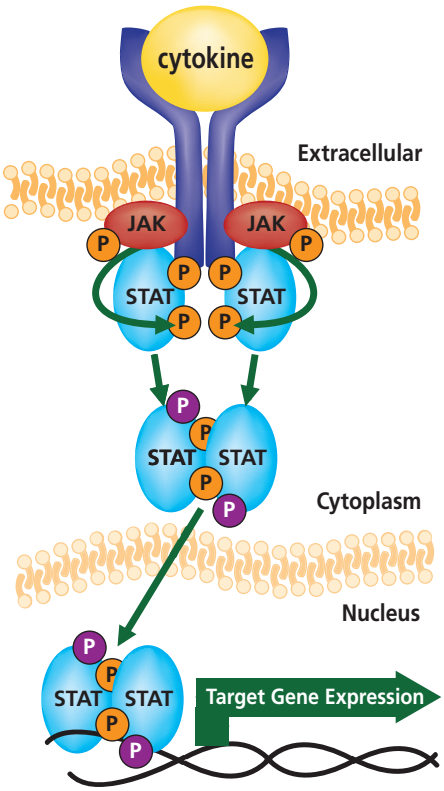

Figure 2. The JAK/STAT pathway.

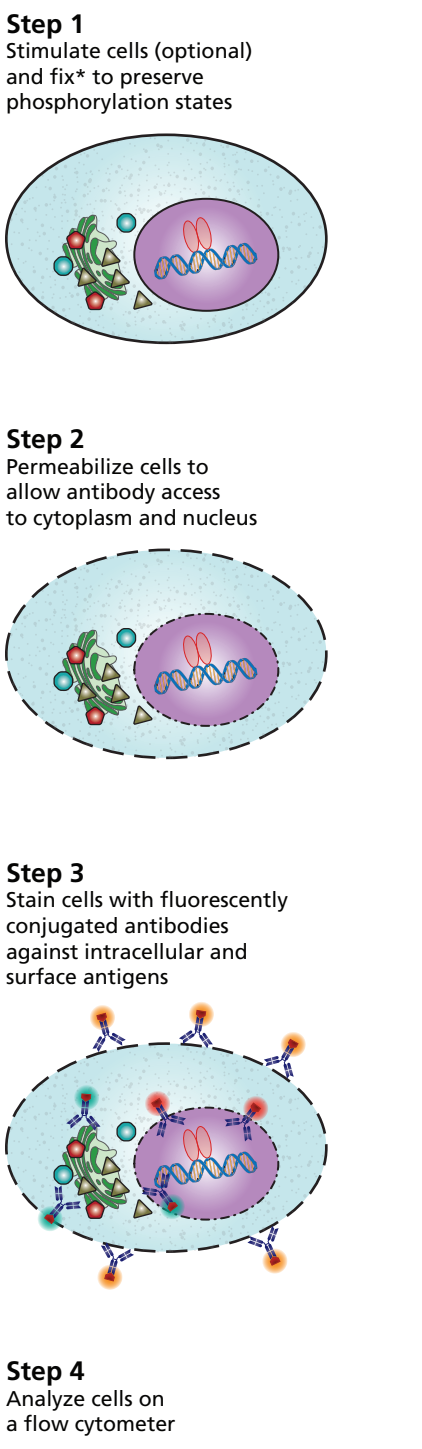

Figure 3. BD Phosflow standard protocol.

**If working with whole blood, spleen, or other samples containing erythrocytes, red blood cells (RBCs) may be lysed during fixation using BD Phosflow™ Lyse/Fix Buffer (Cat. No. 558049).*

BD Phosflow protocol

The basic protocol for preparing and analyzing samples with BD Phosflow involves four steps as shown in Figure 3. First, cells of interest are stimulated with cytokines or other treatments to induce a signaling response, and fixed to preserve their phosphorylation states. In Step 2, the cells are permeabilized using one of four BD Phosflow™ permeabilization buffers chosen to match the samples and desired epitopes. Next, the cells are stained with antibodies to the intracellular and cell surface antigens of interest, and finally they are analyzed on a flow cytometer.

For higher throughput, cells can be stimulated, fixed, permeabilized, and stained in 96-well plates. Plated samples can then be acquired and analyzed using the BD CSampler accessory.

Because phosphorylation is a transient and sensitive process, the development of BD Phosflow had to overcome significant technical and scientific challenges. Buffers, for example, must fix the cellular proteins rapidly and efficiently (to maintain their phosphorylation state) and permeabilize both the cellular and nuclear membranes (to allow antibodies to enter) yet leave cell surface antigens intact. In the end, multiple buffer systems were developed that optimize detection of different sample types, signaling proteins, and surface proteins.

Optimal detection depends on determining the correct sample preparation method for the particular phosphoprotein of interest. Phosphoproteins are often found inside the cell nucleus and may be bound in complexes with other proteins and/or nucleic acids. Accessing these epitopes often requires harsher permeabilization methods. For example, upon activation, STAT proteins rapidly dimerize around the phosphotyrosine (pY) residue and move into the nucleus. Because the pY epitope is in the middle of a dimer, a harsh permeabilization buffer such as BD Phosflow™ Perm Buffer III (methanol-based) or BD Phosflow™ Perm Buffer IV (harsh detergent-based) is needed to gain access to it. A mild buffer such as BD Phosflow™ Perm/Wash Buffer I (saponin-based) is insufficient. In contrast, the phosphoserine (pS) epitope resides outside of the dimerization domain (although inside the nucleus), and both mild and harsh buffers allow it to be detected.

Although harsh buffers can open up access to phosphoepitopes, they can also adversely affect surface antigens. For this reason, it is sometimes advantageous to stain for surface markers before fixing and permeabilizing the cells. However, harsh buffers also affect certain fluorochromes if used after staining, so researchers should evaluate each case individually.

Finally, because phosphorylation events are transient, it is important to identify the optimal time point after stimulation to assess a particular signaling response. Again, the best time to fix the samples depends on the type of cells, stimulant, and phosphoepitopes of interest.

To optimize detection of particular phosphoproteins and surface markers of interest, researchers can find recommended buffers, detailed protocols, and other intracellular flow cytometry resources in BD Phosflow™ kits and online at **bdbiosciences.com** under Resources.

Simultaneous analysis of signaling responses in multiple leucocyte subsets

A major advantage of analyzing phosphoproteins using flow cytometry is the ability to measure signaling responses in individual cell types within a mixed population, such as whole blood, without isolating the cells beforehand. In Figure 4, the BD Phosflow™ Human Monocyte/NK Cell Activation Kit was used to assess signaling responses to IL-6 stimulation of monocytes and B, T, and NK cells. The kit uses fluorochrome-labeled antibodies to CD markers (CD14 and CD19 Alexa Fluor® 488, CD16 and CD56 PE, and CD3 PE-Cy™7), excited by the blue laser and detected in the FL1, FL2, and FL3 channels, to differentiate among cell types. An antibody to Stat3 (pY705) conjugated to Alexa Fluor® 647, which is excited by the red laser and detected in FL4, assesses the signaling responses of all four cell types.

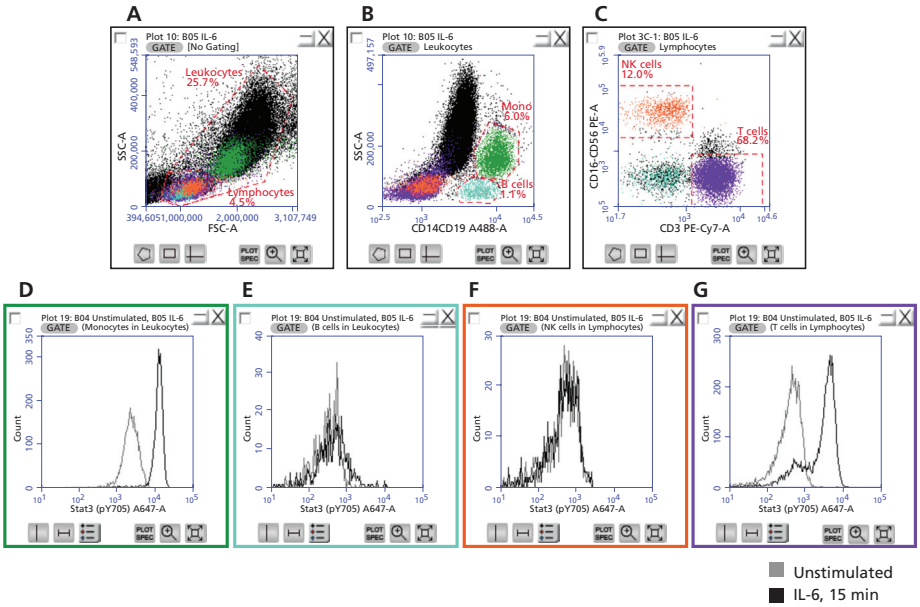

Figure 4. Differences in phospho-signaling responses to IL-6 across human leucocyte subsets.

Human whole blood was stimulated with 100 ng/mL of recombinant human IL-6 for 15 minutes at 37°C and fixed, permeabilized, and stained using the BD Phosflow Human Monocyte/NK Cell Activation Kit (Cat. No. 562089). Data was acquired on a BD Accuri C6 flow cytometer using BD Accuri™ C6 software. ***A.*** *Leucocyte and lymphocyte populations were gated using light scatter.* ***B.*** *Within the leucocyte gate, monocytes ($CD14^+$, green) and B cells ($CD19^+$, cyan) were identified using a combination of light scatter and fluorescence.* ***C.*** *Within the lymphocyte population, NK cells ($CD16/56^+CD3^-$, orange) and T cells ($CD3^+CD16/56^-$, purple) were identified on a dual-fluorescence plot.* ***D–G.*** *Using histogram plots gated on each cell subset, the Stat3 (pY705) staining profiles for unstimulated and stimulated cells were overlaid to assess responses to IL-6 stimulation.*

Results: *Analysis of Stat3 (pY705) phosphorylation responses to IL-6 revealed that only monocytes* ***(D)*** *and a subset of T cells* ***(G)*** *responded to IL-6 stimulation (compared to unstimulated controls). B cells* ***(E)*** *and NK cells* ***(F)*** *did not phosphorylate Stat3 in response to stimulation with IL-6.*

Results showed that only monocytes and a subset of T cells responded to stimulation with IL-6 by phosphorylating Stat3. To tease out which T-cell subpopulations were responding, researchers could use the BD Phosflow™ Human T Cell Activation Kit, as illustrated in Figure 6 later in this paper.

BD Phosflow technology allows rapid processing of multiple samples, facilitating informative analyses across multiple samples, time points, and stimulus concentrations. In Figure 5, responsiveness to varying concentrations of IFN-α was examined in multiple leucocyte subsets, using the same gating strategy as in Figure 4. In this case, the data was exported into Cytobank software, which highlights fold changes in responsiveness.

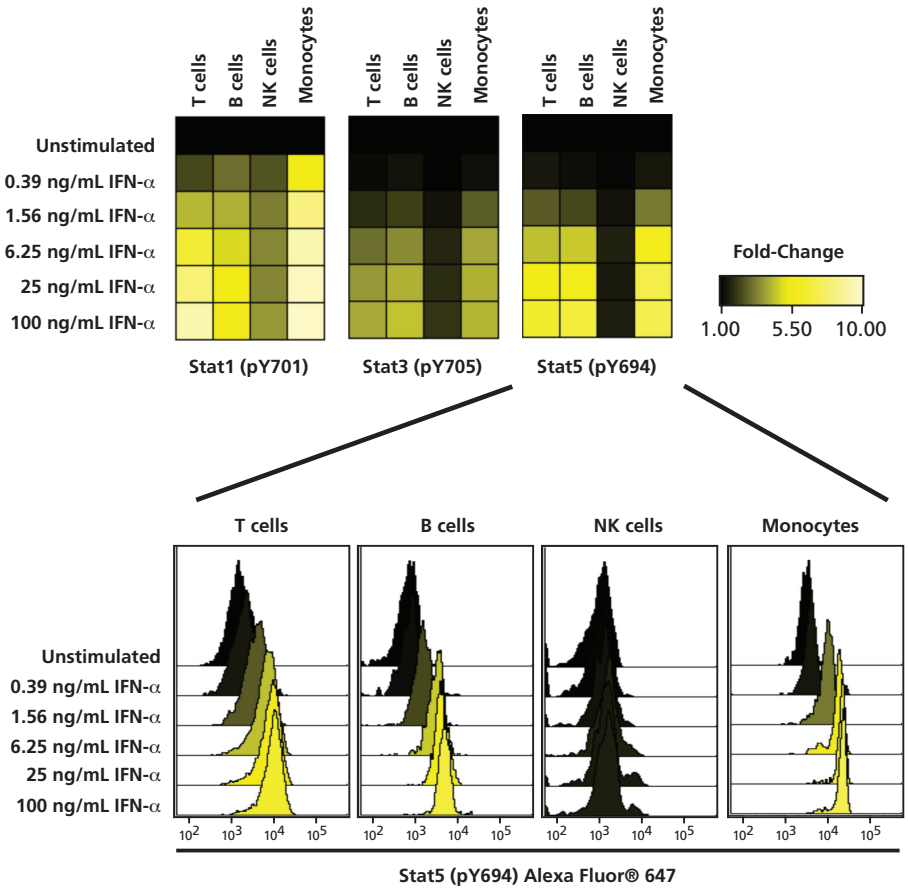

Figure 5. Dose-dependent increases in STAT phosphorylation in response to IFN-α stimulation.

Human whole blood was stimulated with the indicated concentrations of recombinant human IFN-α (0.39 to 100 ng/mL) for 15 minutes at 37°C and fixed, permeabilized, and stained using the BD Phosflow Human Monocyte/NK Cell Activation Kit (Cat. No. 562089). Separate samples were stained to assess Stat1 (pY701), Stat3 (pY705), and Stat5 (pY694) response. Data was acquired on a BD Accuri C6 flow cytometer and analyzed using Cytobank software. Leucocyte subpopulations were identified as described in Figure 4. Median fluorescence intensity values for Stat1, Stat3, and Stat5 staining were used to calculate a fold-change value for each kind of phosphorylation event in each leucocyte subpopulation. Responses to different doses of IFN-α were plotted as heatmaps (top) and histogram overlays (bottom).

Results: *Analysis of Stat1 (pY701), Stat3 (pY705), and Stat5 (pY694) phosphorylation revealed differences across leucocyte subsets in responsiveness to IFN-α. Monocytes responded most robustly to IFN-α stimulation, while NK cells demonstrated minimal response to IFN-α.*

The data examples in Figures 4 and 5 both used the BD Phosflow Human Monocyte/NK Cell Activation Kit, which includes BD Phosflow Perm Buffer IV, a harsh detergent-based buffer. Harsh permeabilization buffers allow optimal detection of STAT phosphorylation, although they disrupt some surface marker epitopes, including CD14, CD16, CD19, and CD56. To prevent disruption, surface marker stains were performed prior to cellular fixation and permeabilization.

An alternative harsh buffer, methanol-based Perm Buffer III, can damage protein fluorochromes such as PE and PE-Cy7 if cells are stained for surface markers prior to permeabilization. In contrast, Perm Buffer IV does not damage any of the fluorochromes used in the kit if cells are stained prior to fixation, allowing resolution of all leucocyte populations of interest.

Detection of heterogeneous signaling responses

Unlike lysate-based approaches to cell signaling analysis, flow cytometry can resolve signaling responses at the single-cell level, and thus can provide information about both the intensity of phosphorylation and the proportion of cells responding. These heterogeneous signaling responses, in which different cells of the same type respond differently, can be the subject of further analysis or follow-up studies.

In Figure 6, $CD4^+$ T cells demonstrated a heterogeneous response to IL-2, while $CD8^+$ T cells demonstrated a more homogeneous response, particularly at high IL-2 concentrations.

The data example in Figure 6 used the BD Phosflow Human T Cell Activation Kit, which includes BD Phosflow Perm Buffer III, a highly concentrated methanol-based buffer. Perm Buffer III is the recommended starting buffer for new BD Phosflow studies, since it allows optimal detection of many phosphoepitopes. Although Perm Buffer III can disrupt some surface marker epitopes, the antibodies in the kit allow the detection of CD3, CD4, and CD8 following permeabilization with Perm Buffer III.

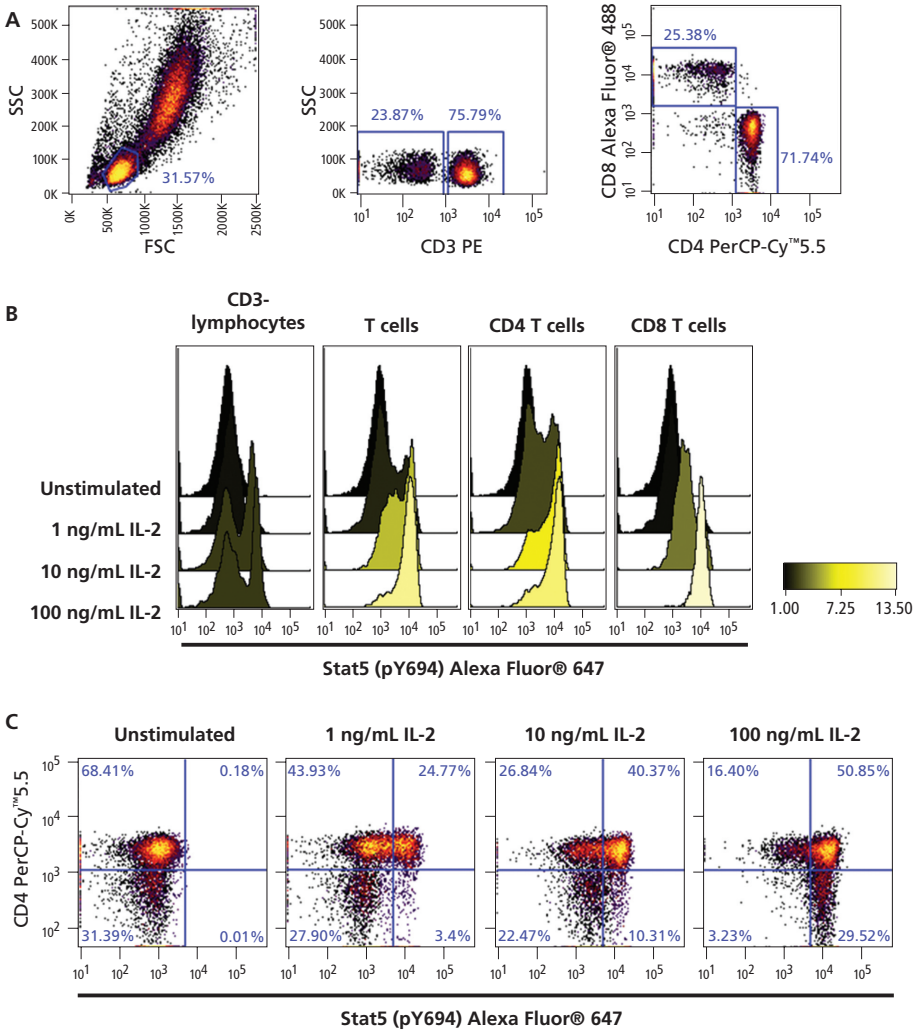

Figure 6. Heterogeneous IL-2 responses in CD4 and CD8 T cell populations.

Human whole blood was stimulated with varying concentrations of recombinant human IL-2 for 15 minutes at 37°C and fixed, permeabilized, and stained using the BD Phosflow Human T Cell Activation Kit (Cat. No. 560750). Data was acquired on a BD Accuri C6 flow cytometer and analyzed using Cytobank software. ***A.*** *The lymphocyte population was identified by light scatter. Within the lymphocyte gate, T cells were identified as CD3⁺ cells. Within the T cell gate, CD4⁺ and CD8⁺ T cells were identified on a dual-parameter fluorescence plot.* ***B.*** *Histogram overlays for each subpopulation were colored based on the fold change in Stat5 (pY694) median fluorescence intensity (MFI) in response to each dose of IL-2.* ***C.*** *Signaling response heterogeneity was further analyzed by plotting CD4 vs Stat5 (pY694) for CD3⁺ T cells and applying quadrant gates to assess the percentage of CD4⁺ or CD4⁻ T cells responding to each concentration of IL-2.*

Results: *IL-2 induced a dose-dependent increase in Stat5 (pY694) phosphorylation in T cells and in a subpopulation of CD3⁻ lymphocytes. Compared to CD8 T cells, a larger subpopulation of CD4 T cells responded at the lowest concentration of IL-2. However, a subpopulation of CD4 T cells remained unresponsive to IL-2 at the highest concentration, whereas the majority of CD8 T cells responded strongly.*

Tips for BD Phosflow signaling studies on the BD Accuri C6

To achieve optimal cell signaling results using BD Phosflow, it's important to follow time and temperature recommendations for fixation, permeabilization, and staining. If you're using a BD Phosflow activation or analysis kit as exemplified in this paper, you'll find recommended reagents and buffers, along with detailed protocols, right in the kit. If you're using individual BD Phosflow reagents, make sure to consult the recommended protocols and other intracellular flow cytometry resources at bdbiosciences.com under Resources.

As emphasized throughout this paper, a major strength of using flow cytometry is its ability to resolve cell signaling responses at the single-cell level. This also means, however, that detection is limited to the amount of phosphoprotein present within a single cell. Because of this, not every phosphorylation event that can be detected by Western blot will be detectable by flow cytometry.

To maximize detection of phosphorylation events, we recommend using a bright fluorophore (such as Alexa Fluor® 647 or PE) for the phosphoepitope of interest. Because phosphoepitope staining is relatively weak in comparison to many surface markers, it is best to allocate brighter fluorophores to phosphoepitopes and low-density surface markers, and dimmer fluorophores to high-density surface markers. On the BD Accuri C6 standard configuration, PE is excited by the blue laser and detected in the FL2 channel, while Alexa Fluor® 647 is excited by the red laser and detected in the FL4 channel.

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