June 2014

Simultaneous Measurement of Cell Surface Markers with FoxP3 and Stat5 (pY694) in IL-2–Stimulated Human PBMCs using BD Cytofix™ Fixation Buffer and BD Phosflow™ Perm Buffer III

Reagents Used

Mouse Anti-Stat5 (pY694) Alexa Fluor® 488, Clone 47 (Cat. No. 612598)

Mouse Anti-Human FoxP3 Alexa Fluor® 647, Clone 259D/C7 (Cat. No. 560045) or Clone 236a/E7 (Cat. No. 561184)

Mouse Anti-Human CD3 BD Horizon™ V450, Clone UCHT1 (Cat. No. 560365)

Mouse Anti-Human CD4 PerCP-Cy™5.5, Clone RPA-T4 (Cat. No. 560650)

Mouse Anti-Human CD25 PE, Clone M-A251 (Cat. No. 555432)

BD Cytofix™ Fixation Buffer (Cat. No. 554655)

BD Phosflow™ Perm Buffer III (Cat. No. 558050)

BD Pharmingen™ Stain Buffer (FBS) (Cat. No. 554656)

Recombinant Human IL-2 (Cat. No. 554603)

Procedural Notes

Detailed procedures for peripheral blood mononuclear cell (PBMC) preparation, reagent preparation, stimulation, fixation, permeabilization, and staining are described in Protocol III of the *BD Phosflow™ Protocols for Human PBMCs.*

Cells

Human PBMCs were freshly prepared, washed, and resuspended at ~2 x 10^6 cells/mL in complete media.

Stimulation

Cells were either untreated or stimulated with recombinant human IL-2 (100 ng/mL) for 15 minutes in a 37°C water bath.

Fixation and Permeabilization

Cells were fixed using BD Cytofix Fixation Buffer and permeabilized using BD Phosflow Perm Buffer III as described in Protocol III of the *BD Phosflow™ Protocols for Human PBMCs*.

Staining with Antibodies for Intracellular and Cell Surface Markers

Cells were stained for 60 minutes at room temperature with the recommended or determined optimal concentration of each fluorochrome-conjugated antibody.

Antibody titration is critical for successful post-permeabilization staining of CD markers and other cell surface antigens. For more information, see the BD FACSelect™ Buffer Compatibility Resource (**http://cytobank.org/facselect/**) and the Tested Surface Markers chart (**http://www.bdbiosciences.com/documents/antibodies_human_cellsurface_marker.pdf**).

The optimal concentration of each antibody used in this protocol is listed as follows. The 1X concentration represents cells stained with the recommended test size for live cell stains (see product Technical Data Sheets for test size information). The 1/2X or 1/4X concentrations represent cells stained with 1/2 or 1/4 the antibody recommended for live cell stains. Mouse Anti-Human FoxP3 antibodies were also titrated to achieve optimal staining in cells permeabilized using BD Phosflow Perm Buffer III.

Mouse Anti-Stat5 (pY694) Alexa Fluor® 488, Cat No. 612598, 1X

Mouse Anti-Human FoxP3 Alexa Fluor® 647, Cat No. 560045, 1/2X

Mouse Anti-Human FoxP3 Alexa Fluor® 647, Cat. No. 561184, 1X

Mouse Anti-Human CD3 BD Horizon V450, Cat. No. 560365, 1X

Mouse Anti-Human CD4 PerCP-Cy5.5, Cat. No. 560650, 1/4X

Mouse Anti-Human CD25 PE, Cat. No. 555432, 1X

Flow Cytometric Analysis of Stained Cell Samples

Flow cytometric analysis of the samples and controls was performed on a BD™ LSR II flow cytometer equipped with three lasers: a 488-nm blue laser, a 633-nm red laser, and a 405-nm violet laser. For each cell sample, ~100,000 events were collected in the lymphocyte gate. Prior to sample collection, fluorescence compensation settings were established using single-color BD™ CompBead control samples and the BD FACSDiva™ software compensation procedure.

Figure 1 shows a representative example of FoxP3 and Stat5 (pY694) signal analysis in stimulated human PBMCs analyzed using this protocol. Figure 2 shows an alternative gating strategy using antibodies against CD3, CD4, CD25, and CD127.

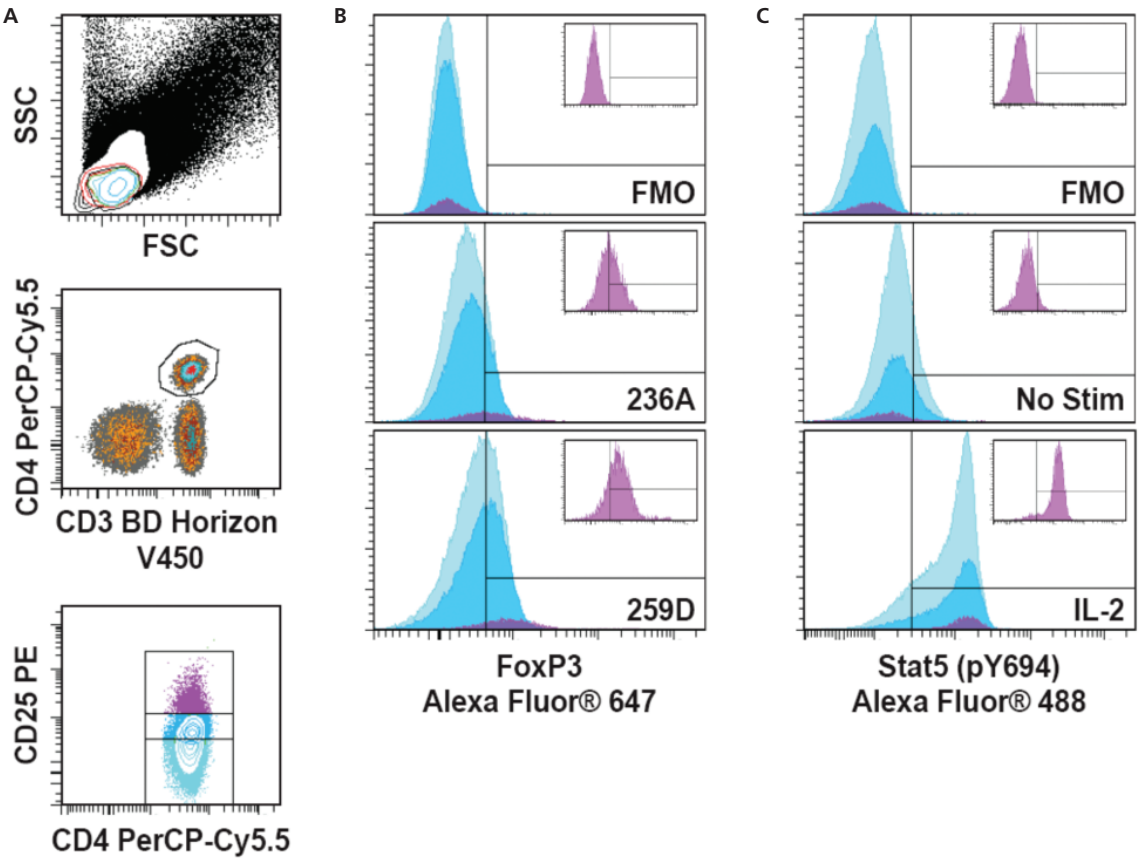

Figure 1

Human PBMCs were either not treated or stimulated with 100 ng/mL of IL-2 for 15 minutes at 37°C, followed by fixation with BD Cytofix Fixation Buffer, permeabilization with BD Phosflow Perm Buffer III, and staining with optimal concentrations of antibodies against CD3, CD4, CD25, FoxP3 (clone 259D or 236A), and Stat5 (pY694).

A. Identification of CD4 T-cell subsets. Lymphocytes were identified based on scatter characteristics, and CD4 T cells were identified as $CD3^{+}CD4^{+}$ lymphocytes. CD25 staining was used to identify regulatory T cells ($CD25^{++}$; purple) and other CD4 T-cell subpopulations ($CD25^{int}$ and $CD25^{-}$; dark and light blue, respectively).

B. FoxP3 signal in CD4 T-cell subsets, gated based on CD25 expression. As expected, most of the FoxP3 signal was observed in the $CD25^{++}$ CD4 T-cell population, with some variation in the percentage of expression among different samples. Within the same sample, there was no difference in FoxP3 signal between unstimulated cells and IL-2–stimulated cells (data not shown for the unstimulated condition). The performance of two anti-FoxP3 clones (259D vs 236A) was comparable within the same donor at the optimal concentration of each clone, yielding similar percentages of FoxP3 signal within the $CD25^{++}$ gate ($n = 4$).

The performance of the FoxP3 259 clone in the BD Phosflow Perm Buffer III condition was comparable to the performance in the BD Pharmingen™ Human FoxP3 Buffer Set (Cat. No. 560098) in terms of percentage of FoxP3 signal in the $CD25^{++}$ population (data not shown). However, the resolution between $FoxP3^{-}$ vs $FoxP3^{+}$ cells was substantially reduced using BD Phosflow Perm Buffer III, relative to the resolution observed when using the BD Pharmingen Human FoxP3 Buffer Set.

C. Stat5 (pY694) signal in CD4 T-cell subsets, gated based on CD25 expression. As expected, Stat5 was phosphorylated within the majority of CD4 T cells after IL-2 stimulation. Compared to unstimulated cells, Stat5 (pY694) signal intensity (measured by median fluorescence intensity) was 6.2-fold higher in IL-2–stimulated cells. In all samples tested ($n = 4$), the percentage of IL-2–responsive cells was lower within the $CD25^{-}$ CD4 T cells than within cells expressing higher levels of the CD25 chain of the IL-2R complex.

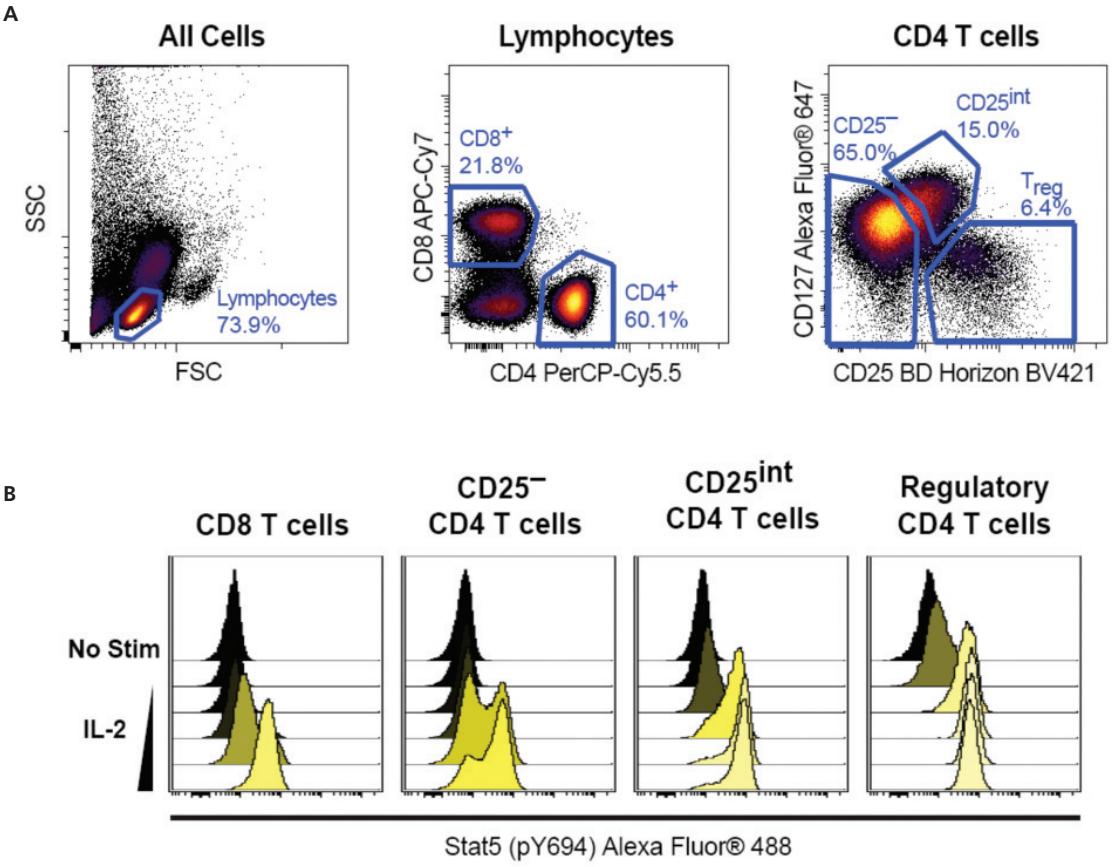

Figure 2

Human PBMCs were stained with Alexa Fluor® 647 Anti-Human CD127 (Cat. No. 558598) during a 15-minute stimulation with 0, 0.01, 0.1, 1, 10, or 100 ng/mL doses of recombinant human IL-2. See *BD Phosflow™ Alternative Protocol 2: Stain–Fix–Perm* for more information. Cells were fixed using BD Cytofix Fixation Buffer, permeabilized using BD Phosflow Perm Buffer III, and stained with Alexa Fluor® 488 Mouse Anti-Stat5 (pY694) (Cat. No. 612598), PerCP-Cy5.5 Mouse Anti-Human CD4 (Cat. No. 560650), APC-Cy™7 Mouse Anti-Human CD8 (Cat. No. 557760), and BD Horizon™ BV421 Mouse Anti-Human CD25 (Cat. No. 562442). Samples were analyzed using a BD LSRFortessa™ flow cytometry system, and CD4 T-cell subsets were identified as shown (A).

B. Enhanced IL-2 sensitivity in regulatory T cells (Tregs). Compared to other T cells, Tregs phosphorylate Stat5 in response to much lower concentrations of IL-2.