FlowMetric™

Introduction to Phosphoflow

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Phosphoflow cytometry assays allow us to measure the phosphorylation state of intracellular proteins with single-cell precision. Using carefully designed phosphoflow panels, multiple phosphorylation events can be simultaneously analyzed in different cells types within the same sample.

Such detailed analysis of cell signaling pathways has many applications in clinical research, particularly in the fields of infectious diseases, oncology and autoimmune conditions, and has been used to support pharmacodynamic profiling of disease states and drug efficacy/safety against particular disease-associated signaling proteins. More recently, phosphoflow techniques are becoming a valuable tool for researchers developing immuno-oncology applications by providing critical mechanistic insights (Wu et. al. 2010).

Key Applications of Phosphoflow Cytometry.

Protein phosphorylation is a functional modification to cytokines, transcription factors and other proteins involved in cellular signaling and metabolic processes, and phosphoflow cytometry can profile several aspects of this process in a single sample, including the analysis of cytokines or other phosphorylated proteins including within cells. Approximately 13,000 human proteins have sites that can become phosphorylated to regulate their activity. These included membrane proteins (receptors, ion channels, transporters), cytosolic enzymes, cell cycle signaling proteins, protein degradation associated proteins, histones, nuclear transcription factors and signaling network associated proteins.

In general, phosphoflow is applied to measure changes in the phosphorylation status of key intracellular signaling molecules such as STATs, MAP Kinases, as well as many other kinases whose activities may be tracked in different cell types. Unlike other approaches such as ELISA or reverse protein arrays to examine phosphorylation states, phosphoflow is uniquely able to discriminate between responses in individual cells and discrete cell subtypes within a heterogeneous sample. Such insights have enabled researches to study how pathways interact in real time and identify correlations in phosphorylation events across pathways (examples include Irish et al. 2006; Perez et. al. 2002; Johnson and Lapadat, 2002).

Furthermore, studies examining multiple active kinase states have identified key differences in signaling responses in heterogenous samples compared with isolated cellular subtypes (Perez and Nolan, 2002). Such research has clearly demonstrated the importance of coordination between intracellular signaling molecules, intercellular interactions and local cellular environment on the fates and responses of different immune cell types (Krutzik et. al. 2005). In fact, this underpins one of the key values of phosphoflow cytometry, in that it makes it feasible to simultaneously analyze different populations without the need to isolate cell types and so maintain the cellular interaction mechanisms.

Considerations for Performing Phosphoflow.

Since protein phosphorylation is a transient state, in vitro assay design must be optimized to measure phosphorylation accurately. Specialized buffers and reagents are used to preserve the phosphorylation state of intracellular proteins during processing and staining steps, all while maintaining fluorescent antibody staining. Rapid fixation of cell samples for phosphoflow is a critical first step. Secondly, the antibody-conjugates for staining must have access to their intracellular targets and this requires effective cell permeabilization. In addition, antibody conjugates for cell surface markers should be selected based on their ability to bind to epitopes that have been denatured by the fixation process.

Fixation with formaldehyde and permeabilization with methanol is a generally used method, and optimization around length of and temperature of incubation times can also have an effect based on the stability of the phosphoepitopes. Small fluorophores such as Alexa Fluor™ 488, and Alexa Fluor™ 647 are preferred for phosphoflow applications since they do not impede access to the intracellular epitopes.

After several years in the development of phosphoflow methods, commercial kits that are optimized for different phoshoflow applications and targets are now available. However, there is still a lack of suitable antibody reagents able to withstand the harsh fixation and permeabilization conditions required for phosphoflow analysis. Standardization of methods will help to ensure consistency and reproducibility of these studies across research teams. As this methodology becomes more wide-spread and complex, novel data analysis tools, such as tSNE, will certainly be helpful in effectively examining these datasets and identifying rare cell events more readily?

Phosphoflow promises enormous potential for studying the immune system and the complex responses in disease states. But it can be a challenging experimental system to optimize, and as such, it is important to empirically test and validate your system to ensure the highest quality of phosphoflow analysis.

Case Study

In the example below, we examined key phosphoproteins within lymphocyte subsets without prior isolation from whole blood. Fresh whole blood from normal healthy donors was stimulated with either hIL-6 or hIL-2 at a concentration of 100ng/mL for 15 minutes at 37˚C. The phosphoflow process to measure STAT-3 and STAT-5 included the lysis and fixation, washing and subsequent permeabilization of the cells for 30 minutes at 4˚C. The cells were then stained for the surface markers CD3, CD4, CD8 and CD19 for one hour at room temperature, before acquisition on a BD Fortessa™. The plots show the CD4+ and CD8+ T cell signaling profiles for each of the phosphorylation markers, STAT-3 and STAT-5. As expected, the CD19+ B-cells were negative for these markers.

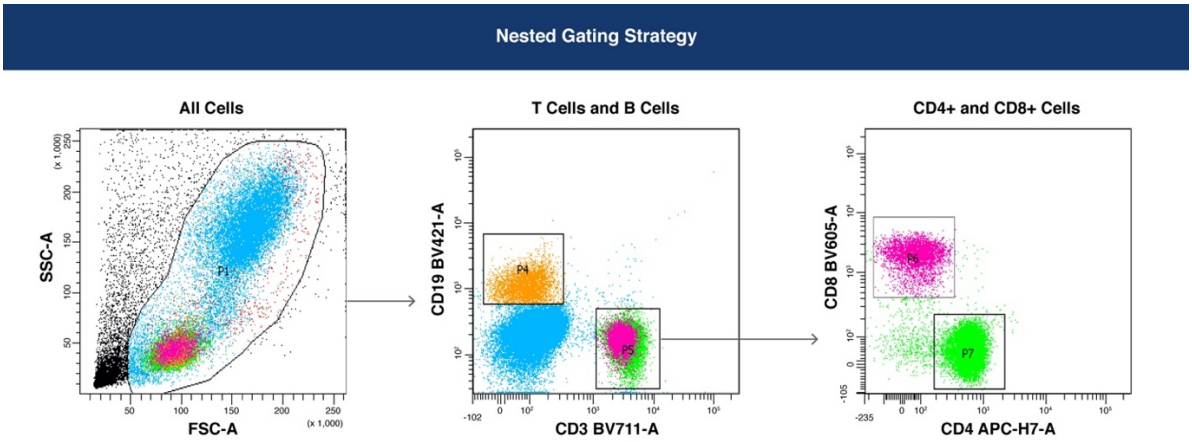

Figure 1. The first plot shows a light scatter gate used to identify peripheral blood cell populations. Next, a CD3+ and CD19+ population is identified. Finally, CD8+ and CD4+ T Cell populations are identified within the CD3+ population.

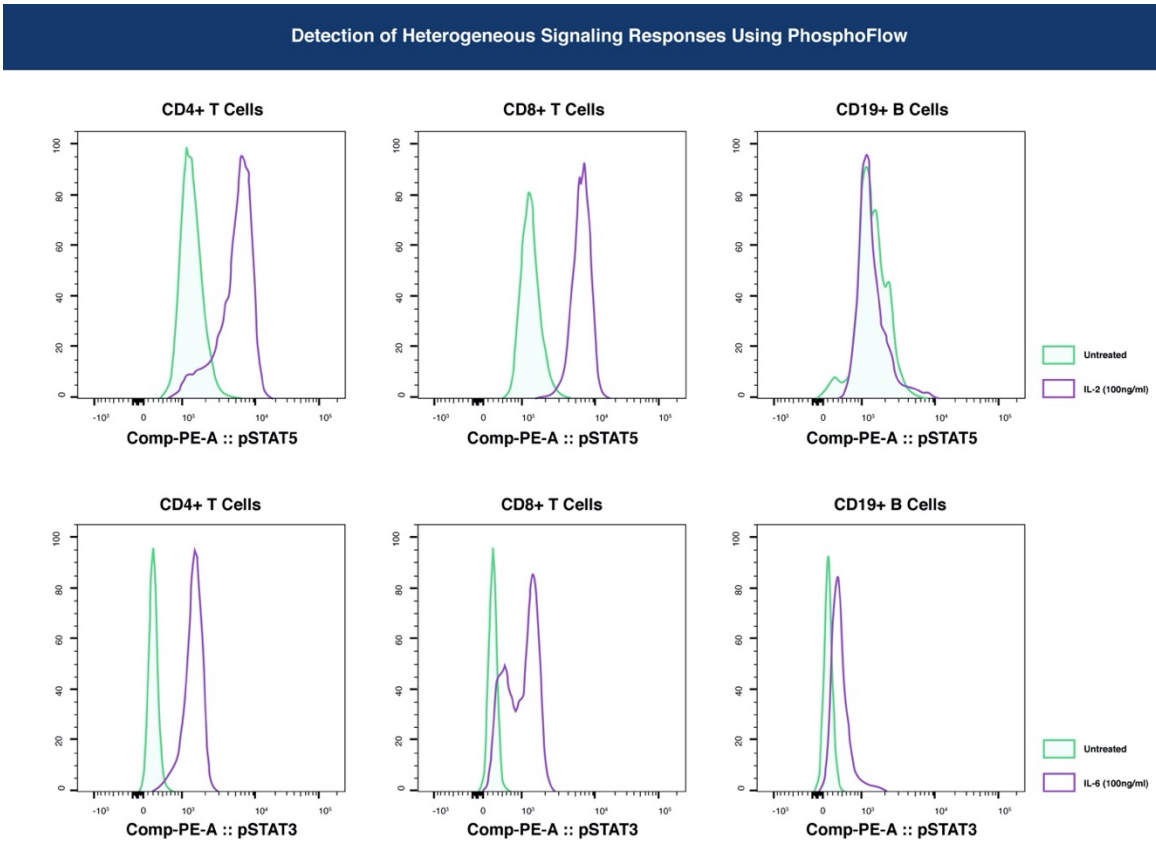

Figure 2. Activation profiles for CD4+ and CD8+ T cells as well as CD19+ B cells. The data are displayed as histogram overlays with untreated cells. These histograms show signaling responses of each phosphorylation marker, STAT-3 and STAT-5, induced by the corresponding response modifier, hIL-6 (100ng/mL) for STAT-3 and hIL-2 (100ng/mL) for STAT-5.

References:

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