

Analysis of Protein Phosphorylation Using Multiparametric Flow Cytometry

Introduction

Caprion offers a wide range of GLP/GCLP-compliant services to characterize cell populations and immune response in support of vaccine and large molecule drug development efforts. Long a leader in mass spectrometry-based proteomics services, Caprion has more recently expanded its immune monitoring capabilities with the addition of the ImmuneCarta multiparametric flow cytometry platform.

The ImmuneCarta technical team has ten years of experience in providing services to the research community and offers the unique combination of highly multiparametric flow cytometry under GLP/GCLP for both pre-clinical and clinical studies. Capabilities of the team are very broad and include assays to monitor PD-1 and related immune exhaustion processes as well as BD Phosflow™ technology, capable of interrogating multiple intracellular phosphoproteins and cell surface markers simultaneously to develop an information-rich view of cell signaling pathways in discrete subpopulations of cells.

Our clients have been especially interested in interrogating multiple phosphoproteins simultaneously as a way to study activation levels of an entire cell signaling pathway and thereby assess therapeutic response to a new drug or treatment intervention. This paper describes ImmuneCarta's use of the Phosflow platform to measure the phosphorylation state of multiple signaling proteins in subpopulations of circulating cells. Studies presented here include basal level assessment of phosphorylation levels as well as phosphoprotein analysis following *in vitro* stimulation. By generating a complete picture of cell signaling pathway activation reactions in distinct cell subpopulations, Caprion's protein phosphorylation services help maximize the efficiency of our clients' therapeutic development programs.

Methodology

The Phosflow platform is compatible with fresh whole blood and cell lines as well as fresh or cryopreserved peripheral blood mononuclear cell (PBMC) samples. Steps involved for both basal level and *in vitro* stimulated phosphoprotein analysis of all sample types are summarized in Figure 1.

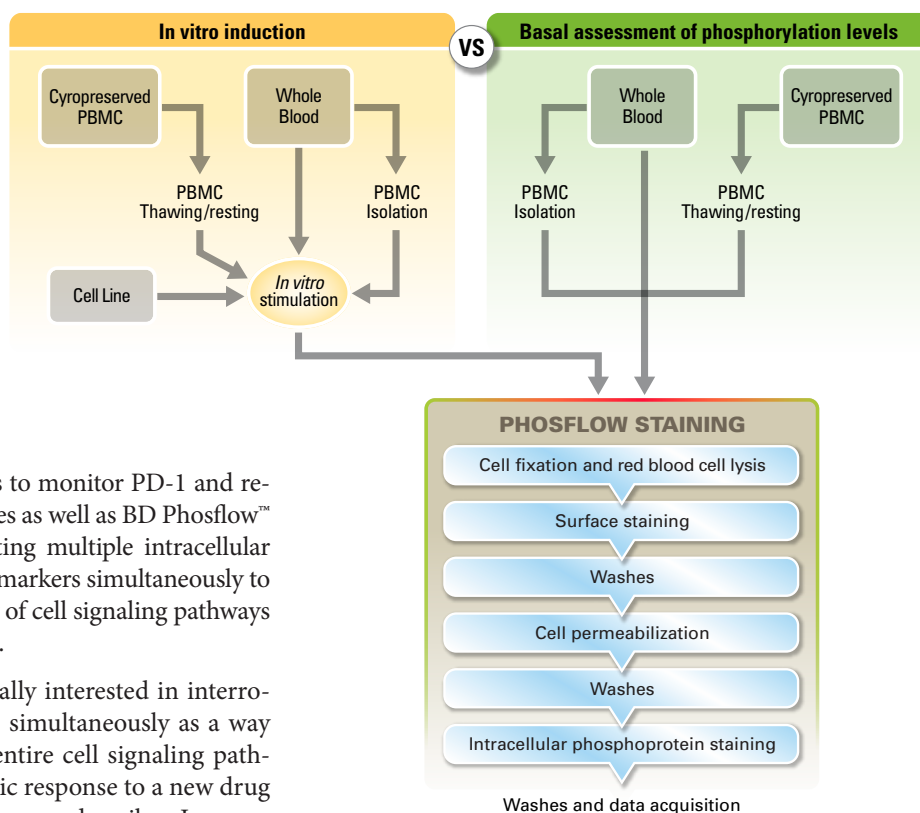


Figure 1
Overall Phosflow methodology

For the *in vitro* stimulation portion of this study, cryopreserved PBMCs from a healthy donor were thawed and allowed to rest overnight. On the following day, PBMCs were incubated 10 minutes with PMA (100 nM), ionomycin (5 µg/mL), and IL-2 (100 µg/mL) and then fixed. An unstimulated control was generated simultaneously. Cells were then

washed several times and resuspended in a labeling buffer before incubating with antibodies directed against human cell surface antigens for 30 minutes at 4°C. Following surface staining, PBMCs were washed and permeabilized in a cold methanol-based buffer for 30 minutes on ice. PBMCs were again washed and then resuspended in a staining buffer before undergoing incubation with a cocktail of 2 fluorochrome-conjugated antibodies directed against specific phosphoresidues for 1 hr at 4°C. Three different antibody cocktails were used: cocktail #1: anti-pERK1/2 and anti-pAkt, cocktail #2: anti-pNF-kB and anti-pMEK1/2, cocktail #3: anti-pSTAT5 and anti-pS6. Following staining of phosphoproteins, PBMCs were washed and analyzed on a BD™ LSR II flow cytometer. A hierarchical gating strategy was applied to all samples in order to direct the phosphoprotein analysis toward CD45+CD3+CD5+CD19-CD20-T cells.

Assessment of basal level protein phosphorylation was conducted using whole blood samples collected in BD Vacutainer® CPT™ Cell Preparation Tubes from which PBMCs were immediately isolated. PBMCs were fixed 10 minutes at 37°C (without any prior *in vitro* stimulation), washed several times and resuspended in a staining buffer before incubating 30 minutes at 4°C with antibodies directed against human B cell surface antigens. Following surface staining against CD45, CD3, CD19, CD5 and CD20, PBMCs were washed and permeabilized in a cold methanol-based buffer for 30 minutes on ice. PBMCs were again washed and resuspended in a staining buffer before undergoing incubation with a panel of 3 fluorochrome-conjugated antibodies directed against specific phosphoresidues: anti-pSyk, anti-pAKT and anti-pS6. Following staining of phosphoproteins, PBMCs were washed and analyzed on a BD LSR II flow cytometer.

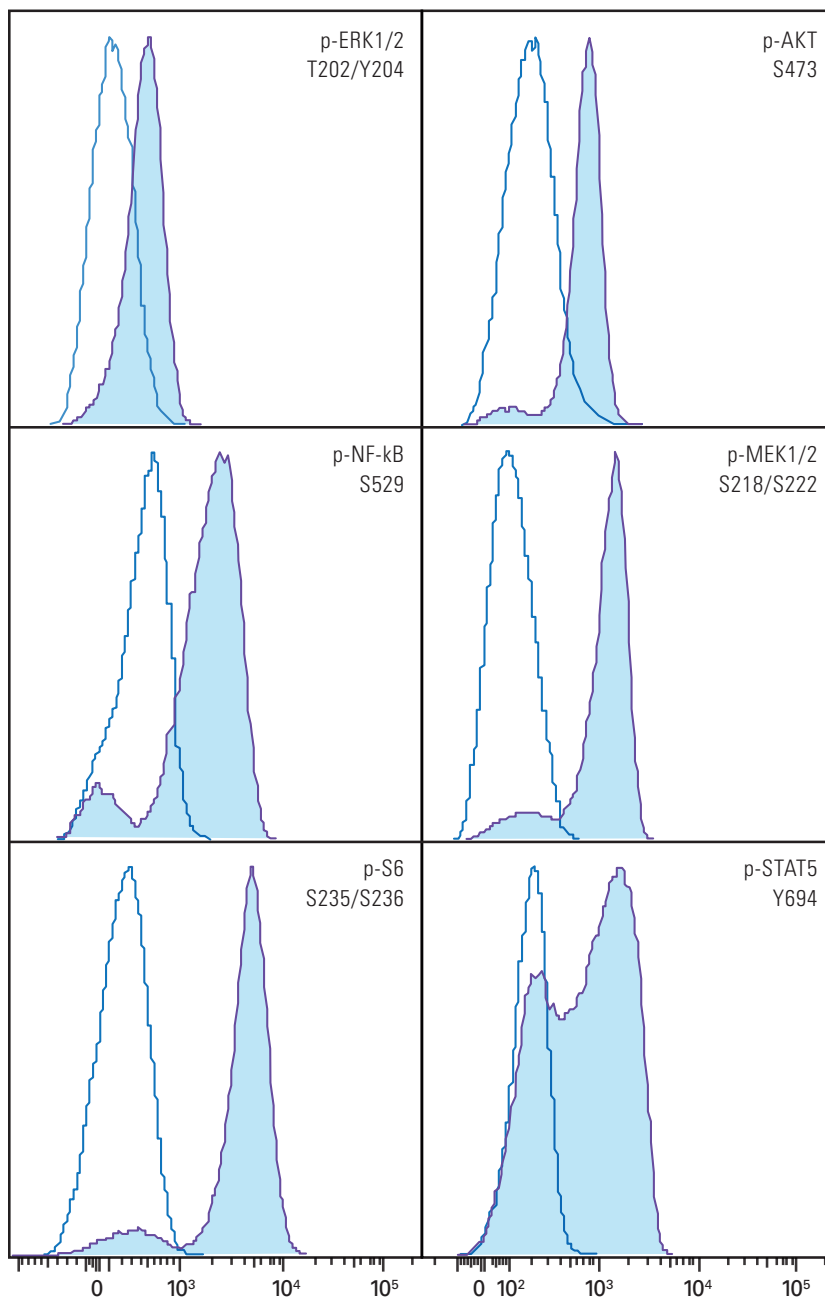


Figure 2

Simultaneous detection of phosphoproteins in human CD45+CD3+CD5+ T cells (CD19-CD20-) following *in vitro* stimulation with PMA, ionomycin and IL-2 (shaded histograms) or in unstimulated controls (white histograms). All histograms are pre-gated on CD45+CD3+CD5+CD19-CD20-cells.

For both the *in vitro* stimulation and basal level studies, data analysis was conducted using FlowJo software and a hierarchical gating strategy was applied to all samples in order to direct the phosphoprotein analysis toward B lymphocytes.

Results and Discussion

In vitro-induced phosphorylation

Flow cytometric fluorescent intensity (FI) data presented in Figure 2 indicates that cryopreserved PBMCs that were stimulated *in vitro* exhibited marked increases in phosphorylation for all six phosphoproteins assayed when compared to unstimulated control cells.

Basal levels of phosphorylation

Phosphoprotein analysis of unstimulated clinical sample cells detects the basal level of protein phosphorylation. Assessment of basal levels leads to an understanding of the complex phosphorylation equilibrium that exists *in vivo*. By contrast, phosphorylation analysis done on the same clinical specimens following *in vitro* stimulation with PMA, ionomycin and IL-2 serves to identify blockages in, or over-activation of, specific cell signaling pathways. Additional types of polyclonal (PHA, LPS, anti-CD3/CD28) and oligoclonal (peptide) stimulators can also be utilized *in vitro*.

In the unstimulated assessment of basal levels of protein phosphorylation, significant differences in MFIs were observed for all 3 phosphoproteins assayed; untreated B

cell lymphoma subjects exhibited increases in MFI when compared to healthy donors - as shown in Figure 3.

Hierarchical and Boolean gating of phosphoprotein expression

One of the benefits of the ImmuneCarta flow cytometry platform is that it enables the analysis of discrete populations of cells within complex samples like whole blood and PBMCs. Caprion uses a hierarchical gating approach when the cell subset of interest is known. More complex flow cytometry data can be analyzed using a Boolean gating approach when the phosphorylated forms of proteins studied are well-separated from their non-phosphorylated counterparts, which generates a protein phosphorylation “signature” that can facilitate subsequent data-mining and biomarker discovery efforts.

Related Applications

In addition to projects like the one described above, Caprion leverages the Phosflow platform to provide many other types of protein phosphorylation services, some of which are detailed below.

Simultaneous measurement of protein phosphorylation and cell surface molecules

By using a combination of antibodies specific for cell surface markers and for intracellular or intranuclear phosphoproteins, it is possible to generate information-rich views of cell signaling pathways in discrete populations of cells without a preliminary cell sorting step. Caprion can

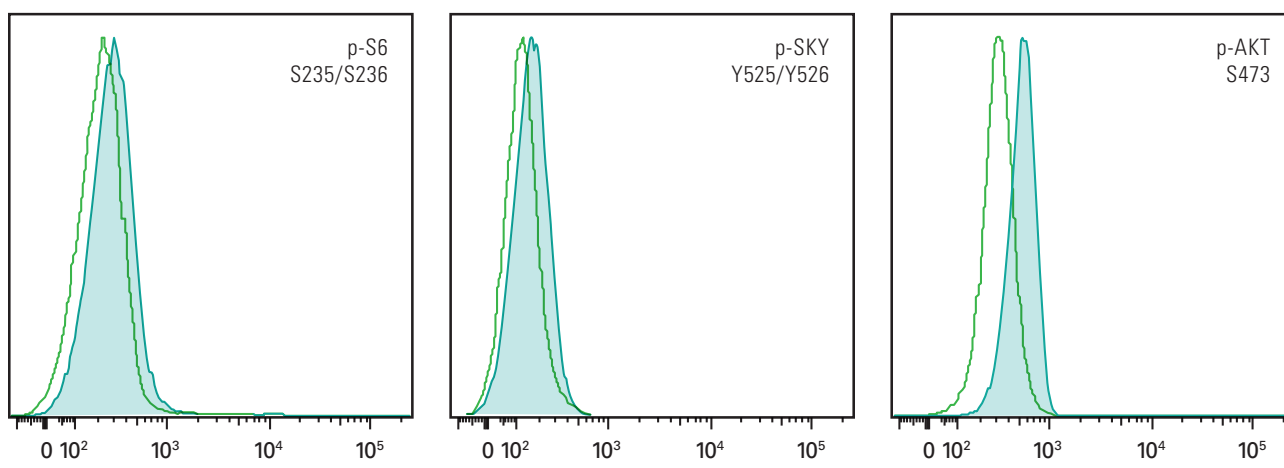


Figure 3

Comparative analysis of Syk, AKT and S6 basal phosphorylation levels in a healthy donor (white histogram) as opposed to an untreated B cell lymphoma subject (shaded histogram).

perform these multiplex assays using just 100 µl of whole blood or the equivalent of 10⁶ PBMCs.

Phosphorylation analysis in multiple species

Drawing from an ever-expanding menu of commercially-available flow cytometry reagents and antibodies, the ImmuneCarta team is capable of interrogating protein phosphorylation states in samples from a wide array of species. It is thus possible to assay phosphoproteins in tandem with cell surface molecules in both mouse and rat specimens.

Compound screening using protein phosphorylation as a functional read-out

Caprion's protein phosphorylation assay services enable drug discovery scientists to better understand highly complex signaling pathways and to determine the effect of a disease or a stimulus on the pathway of interest under near-native conditions. Kinases are recognized as "highly-druggable" targets for a host of therapeutic indications (including cancers, CNS disorders, pain, inflammatory diseases, immune function disorders, cardiotoxicity, and diabetes), so ImmuneCarta's line of Phosflow assays is ideally suited to screen compounds for their ability to modulate phosphorylation levels of specific signaling proteins.

Conclusions

Phosflow assays conducted by Caprion's ImmuneCarta group offer many benefits over more conventional testing technologies. Unlike Western blotting methods, Phosflow can be used to identify and analyze phosphoprotein signaling in single cells through the use of multiple cell surface markers. Compared to Western blotting and immunoprecipitation tests that take several days to complete, the Phosflow platform can generate complex signaling pathway data within a single day, providing a richer dataset in a fraction of the time. This fast turnaround time is fueled by Caprion's ability to assay as many as 18 parameters and 14 colors simultaneously, a feat made possible by nearly a decade of experience designing high-dimensional flow cytometry assays and antibody cocktails for phosphoprotein analysis.

All of Caprion's ImmuneCarta assays are conducted in strict accordance with FDA GLP regulations and published OECD GCLP guidelines - under which studies are planned, conducted, monitored, reported and archived. Our scientists are also experienced in conducting client-specific protocols upon request.

Caprion provides a full complement of consultative study planning, custom assay development/validation, sample management and data interpretation services to support preclinical and clinical trials. We have the capacity and knowledge necessary to receive and process samples in a wide range of collection vessels and formats from anywhere in the world. Our scientific experts can train clinical sites to properly prepare, store and ship viable PBMCs or can perform PBMC isolation in our own labs using a density-based cell separation approach.

For all of the above reasons, protein phosphorylation studies contracted out to Caprion's ImmuneCarta flow cytometry team help streamline the investigation of cell signaling pathways in support of preclinical through Phase II vaccine, small molecule, and biologic/biosimilar drug development efforts.

References

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