Chapter 9

Phospho Flow Cytometry Methods for the Analysis of Kinase Signaling in Cell Lines and Primary Human Blood Samples

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Abstract

Phospho-specific flow cytometry, or phospho flow, measures the phosphorylation state of intracellular proteins at the single cell level. Many phosphorylation events can be analyzed simultaneously in each cell, along with cell surface markers, enabling complex biochemical signaling networks to be resolved in heterogeneous cell populations. The method has been applied to many diverse areas of biology, including the characterization of signaling pathways in normal immune responses to antigenic stimulation and microbial challenge, alteration of signaling networks that occur in cancer and autoimmune diseases, and high-throughput, high-content drug discovery. In this chapter, we provide detailed experimental protocols for performing phospho flow in cell lines, Ficoll-purified peripheral blood mononuclear cells, and whole blood. These protocols are applicable to both human and murine samples. We also provide methods for the validation of surface marker antibodies for use in phospho flow. Finally, we discuss data analysis methods, in particular, how to quantify changes in phosphorylation and how to visualize the large data sets that can result from experiments in primary cells.

Key words: Phospho flow cytometry, Signaling, Cytokine, Cell-based, Primary cells, Drug discovery

1. Introduction

Phospho-specific flow cytometry, or phospho flow, takes advantage of the two key traits of flow cytometry: multiparameter measurements and single-cell resolution (1, 2). By measuring ten or more fluorescent parameters for each individual cell that runs through the cytometer, phospho flow enables researchers to measure multiple kinase signaling pathways in heterogeneous cell populations such as peripheral blood. This has allowed our laboratory and others to explore the normal signaling responses of the immune

Teresa S. Hawley and Robert G. Hawley (eds.), *Flow Cytometry Protocols*, Methods in Molecular Biology, vol. 699,
DOI 10.1007/978-1-61737-950-5_9,

system in response to foreign challenges (3–5), the signaling changes that occur in disease states such as autoimmunity and cancer (6–9), and to perform high-content drug screening in both cell lines and primary cells (10, 11).

Like any other method, phospho flow cytometry requires practice to perform it accurately and reproducibly. In this chapter, we progress from a simple cell line experiment to more complex experiments in blood samples. After having taught hundreds of researchers how to perform phospho flow, we have learned that the key to success is in following this progression from simple to complex, without skipping steps.

The largest difficulty faced by novice users is attempting to do too much in their first experiments (for instance, trying to analyze phospho-protein levels in a cell population that requires five surface markers to define and only comprises 0.3% of the total population of cells). Therefore, in our laboratory, all new researchers are taught to perform a simple phospho flow experiment using the U937 cell line (see Subheading 3.1). The basic steps include cell stimulation, fixation, permeabilization, and staining (12). The U937 cell line is easy to grow in suspension, responds robustly to stimulation, and provides consistent results. In this experiment, only phospho-proteins are analyzed, and compensation is not required on the flow cytometer. This simplifies the experiment and allows the researcher to focus on performing the phospho flow method rather than setting up the cytometer. Once mastered, the U937 cell line experiment gives the researcher confidence that they can measure intracellular signaling events by flow cytometry, and provides the basis for performing more advanced experiments in primary samples.

In the subsequent sections, we add levels of complexity to the simple cell line experiment. In the PBMC experiment (see Subheading 3.2), surface markers are analyzed in addition to the intracellular proteins. In this way, cell types of interest (such as T cells, B cells, and monocytes) can be identified and analyzed biochemically. It is important to note that the choice of surface marker antibodies, as well as the titration and validation for use in this platform, must be considered carefully. In the whole blood experiment (see Subheading 3.3), signaling is measured directly in whole blood without first purifying the mononuclear cells by Ficoll separation. This enables measurements in the most physiologically relevant context, and is particularly appealing for pharmacodynamic monitoring of drugs directly in patient samples, or for diagnostic stratification of disease states.

We then present a slightly modified method, which we term "sequential staining," whereby surface markers that are difficult to analyze after permeabilization are stained prior to cell permeabilization (see Subheading 3.4). Although useful for staining surface antigens, this method has limitations as to the fluorophores that can be used.

Finally, we present a basic outline of how to analyze the data obtained with phospho flow (see Subheading 3.5). Unlike traditional flow cytometry, which typically compares percentages of cells in a particular gate, phospho flow is a quantitative method that compares the fluorescence intensity of a population before and after stimulation with a cytokine or other molecule. Here, we provide simple equations and ways to visualize the data, which can quickly become overwhelming in the light of the number of cell types and phospho-proteins that can be analyzed simultaneously in one sample.

2. Materials

1. Cells of interest: cell line (e.g. U937, Jurkat, THP-1, and Ramos), Ficoll-purified human peripheral blood mononuclear cells (PBMCs), and human whole blood.
2. Culture media for cells of interest: typically RPMI containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (RPMI-10).
3. Stimuli: recombinant human IFN-γ, IL-4, IL-6, IL-10 (BD Biosciences, San Jose, CA); lipopolysaccharide (LPS) (Sigma–Aldrich, St. Louis, MO).
4. Fixative:
   (a) For experiments using cell lines or purified PBMCs: 16% formaldehyde in water in sealed ampules (EM grade from Electron Microscopy Sciences, Hatfield, PA).
   (b) For experiments in whole blood: Lyse/Fix buffer (#558049; BD Biosciences).
5. Permeabilization reagent: 100% methanol.
6. Staining medium: PBS with 0.5% BSA and 0.02% sodium azide.
7. Antibodies:
   (a) Phospho-specific antibodies: pStat1 Alexa Flour 647 (Ax647) (clone 4a), pStat6 Ax488 (clone J71-773.58.11), pStat3 Ax488 (clone 49), and p-p38 Ax647 (clone 36) (BD Biosciences).
   (b) Surface marker antibodies: CD3 PE-Cy7 (UCHT-1), CD4 Pacific Blue (RPA-T4), CD20 PerCP-Cy5.5 (H1), and CD33 PE (P67.6) (BD Biosciences).
8. Tubes or plates:
   (a) When processing smaller numbers of samples, 12 × 75 mm polystyrene (5 mL capacity) BD Falcon tubes (FACS tubes) are suggested.

(b) When processing large numbers of samples or performing experiments in volumes less than 200 μL, 96-well V-bottom deep block plates (2 mL capacity, polypropylene) (#40002-012; VWR Scientific, West Chester, PA) are more convenient.

9. CO_2 incubator, 37°C.
10. Swinging bucket centrifuge with tube and plate carriers (capable of ~500× *g* force).
11. Flow cytometer equipped with 488- and 633-nm lasers (e.g. BD FACSCalibur; BD Biosciences) for U937 experiment; and 405-, 488-, and 633-nm lasers for PBMC and whole blood experiments (e.g. BD LSRII; BD Biosciences).
12. Analysis software: we suggest using Cytobank, web-based software for storing, sharing, analyzing, and visualizing flow cytometry data sets (accessible at http://www.cytobank.org).

3. Methods

The phospho flow method can be broken down into the following six steps:

1. Stimulation
   Cells of interest, which can be a cell line, or primary cells from a mouse or human, are treated with various molecules that might affect cell signaling such as cytokines, small molecule drugs, or growth factors. Cells can also be obtained directly from patients and tested without further treatment to examine "basal" signaling, but we have found that probing the cells with stimuli better reveals signaling changes in disease states.
2. Fixation
   Cells are fixed with formaldehyde in order to stop signaling and phosphorylation events as rapidly as possible. Formaldehyde is a cross-linking agent that enters the cell and reacts with amine groups on proteins. This binding can lead to protein–protein cross-linking but more importantly arrests enzymatic activity and halts cellular metabolism. Some signaling events, particularly on proteins that are proximal to the cell membrane such as Syk, ZAP70, and PLCγ, can decay within 15 min of induction. Therefore, cells must be fixed without washing, in order to preserve the most accurate representation of the phosphorylation state.
3. Permeabilization
   Cells are permeabilized with methanol so that antibodies against intracellular proteins can enter the cells and stain their

target antigens. Although different permeabilization reagents are available, methanol, or other similar denaturing agents, is required for staining the Signal Transducer and Activator of Transcription (Stat) proteins which are critical to nearly all cytokine-mediated signaling pathways. In addition, we have found methanol to be the most universal reagent, working for the largest number of phospho-specific antibodies.

4. Staining
   Cells are stained with antibodies specific to the phosphorylated form of intracellular signaling proteins. These antibodies do not bind to the non-phosphorylated form, and therefore, the amount of antibody binding to a cell directly correlates to the amount of phosphorylation on the target protein. Primary blood samples must also be stained with antibodies against surface antigens to identify cell types of interest. These surface antibodies must be carefully validated for use in permeabilized cells, as many show greatly increased background staining when exposed to the massive number of protein and nucleic acid epitopes that are present inside the cell, but not on the cell surface. If necessary, cells can be surface stained after fixation, but prior to permeabilization, in a method called sequential staining, outlined below.
5. Acquisition
   Cells are acquired on a flow cytometer, and the fluorescence intensity of each antibody binding to each cell is measured.
6. Analysis
   Phospho flow data analysis requires quantitative comparisons between samples to determine the amount of protein phosphorylation induced after stimulation, or in a particular disease state. This differs from typical surface phenotyping experiments done with flow cytometry, where the percentage of positive cells is the main metric. Therefore, in phospho flow analysis, the fluorescence intensity of the phospho-antibody staining is compared to a control, and a "fold change" value is calculated. Data sets produced with phospho flow can become extremely large due to the multiparametric nature of the experiments. For instance, a typical experiment might measure five stimulation conditions in five cell populations, across five different signaling proteins. This experiment, which might only require about ten samples to be run on the cytometer, would yield 125 data points. Therefore, we often display data in heatmap format, as a tool to summarize the data for overall visualization. It is important to remember that each point in a heatmap is representative of thousands of individual cells, requiring the researcher to examine samples with interesting signaling phenotypes more closely.

3.1. U937 Cell Line

The U937 cell line experiment is the simplest phospho flow experiment that all new users should perform (12). In this experiment, cells are stimulated with two cytokines, IFN-γ and IL-4. The cells are then fixed with formaldehyde, permeabilized with methanol, washed, and stained with phospho-specific antibodies. Because the cell line is extremely consistent in its response profile, users can optimize their technique by repeating the experiment until the expected pattern of phosphorylation is observed, and the shifts in phospho-protein levels are adequate.

1. Grow U937 cell line to ~0.5 to 1×10^6 cells/mL in RPMI-10 (5 mL is required for this experiment).
2. Quickly place 1 mL of cells in five FACS tubes, numbered 1–5 (see Note 1).
3. Quickly add the cytokines IFN-γ and IL-4 at 10 ng/mL final concentration, in the order listed below (see Notes 2 and 3).

Tube 1	2	3	4	5
None	None	IFN-γ	IL-4	IFN-γ + IL-4

4. Incubate the cells for 15 min at 37°C in a 5% CO_2 incubator.
5. Quickly add 100 µL of 16% formaldehyde (final concentration of ~1.5%) (see Note 4).
6. Keep the tubes at room temperature for 10 min.
7. Centrifuge the samples ($500 \times g$, 4°C, 5 min).
8. Decant the supernatant by inverting and flicking the tubes, leaving the pellet of cells at the bottom.
9. Resuspend the cell pellet in the residual medium (normally 30–70 µL) by vortexing briefly for 3–5 s or shaking the tubes vigorously by hand.
10. Add 1 mL of ice cold methanol to the resuspended cells.
11. Vortex the cells for 3–5 s.
12. Place the tubes on ice for 15–30 min (see Note 5).
13. Add 3 mL of staining medium on top of the methanol.
14. Repeat steps 7–9.
15. Add 4 mL of staining medium to the cell pellet.
16. Repeat steps 7–9.
17. Add 80 µL of staining medium to the pellet. With the residual volume typically left after decanting the supernatant, each tube should have 110–150 µL of suspension at this point.
18. Transfer 80 µL of the cell suspension into a fresh set of five FACS tubes, numbered A1–5 (see Note 6).

19. Stain the cells by adding 20 μL each of pStat1 Ax647 and pStat6 Ax488 phospho-specific antibodies to tubes A2–5, and add 40 μL of staining medium to tube A1 as a control. The total volume will be 120 μL. Tube A1 serves as an unstained control useful for instrument setup and comparison of phospho-protein staining intensities.
20. Vortex to mix.
21. Stain for 30 min to 1 h at room temperature (see Note 7).
22. Add 3 mL of staining medium.
23. Repeat steps 7–9.
24. Add 100 μL of staining medium. Keep the samples cold until acquisition (see Note 8).
25. Run the samples on a flow cytometer. Use the unstained control to set the instrument PMTs for Ax488 (FL1 with 530/30 bandpass filter on FACSCalibur) and Ax647 (FL4 with 661/16 bandpass filter) so that the cells appear in the lower quadrant of each parameter. Because Ax488 and Ax647 are spectrally distinct and are excited by the 488- and 633-nm lasers, respectively, compensation is not required.
26. Acquire 10,000 events for each sample. Expected results are shown in Fig. 1. IFN-γ induces pStat1, while IL-4 induces pStat6. The combination stimulation should induce both phospho-proteins.

3.2. Primary Human PBMC

Experiments in primary cells are more complex than the U937 cell line experiment in that surface markers must also be used to identify cell types within the heterogeneous PBMC sample. It is absolutely critical to validate the surface marker antibodies for use in permeabilized cells. Many antibodies that work well in live cell staining do not separate the appropriate cell populations once the cells have been fixed and permeabilized. See Subheading 3.4 for more information regarding surface marker antibodies used in phospho flow.

In addition, primary samples first require isolation, freezing, and thawing of the PBMCs, all of which can affect signaling responses. The method outlined below is used routinely in our laboratory for clinical samples.

3.2.1. Preparation and Freezing of PBMCs

Note: If PBMCs are to be prepared immediately before the experiment, skip to Subheading 3.2.2, step 9.

1. Prepare PBMCs from whole blood by Ficoll purification.
2. Resuspend the cells at approximately ten million cells per mL in ice-cold FBS containing 10% DMSO (the freezing medium).
3. Aliquot 1 mL of cells per cryovial.

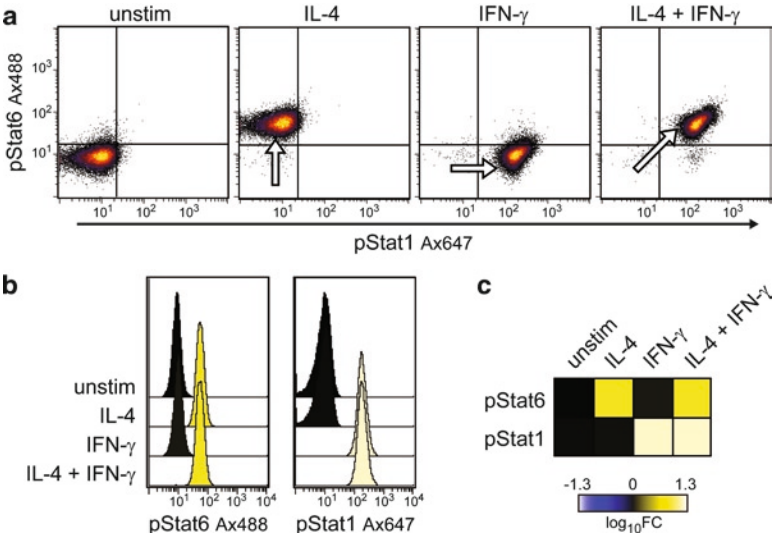

Fig. 1. Sample data from U937 cell line experiment. U937 cells were left unstimulated, or were treated with 10 ng/mL of IFN-γ, IL-4, or the combination of both IFN-γ and IL-4. Cells were fixed, permeabilized, and stained with phospho-specific antibodies against Stat1 (pY701) and Stat6 (pY641). The pStat6 antibody was conjugated to Ax488, while the pStat1 antibody was conjugated to Ax647, enabling simultaneous detection by flow cytometry. Cells were first gated based on forward and side scatter characteristics to eliminate cellular debris from the analysis. (**a**) Two-dimensional density plot showing stimulation of Stat6 phosphorylation with IL-4, and Stat1 phosphorylation with IFN-γ. Adding both cytokines induced both phosphorylation events. (**b**) Histogram overlay representation of the data. (**c**) Heatmap representation of the data. Histograms and heatmaps are colored (in the electronic version) according to the fold change (FC) of phosphorylation relative to the unstimulated sample, with lighter shades indicating a positive fold induction. Data analysis (including heatmap generation) was performed with Cytobank software.

4. Freeze slowly (1°C/min) in –80°C freezer (see Note 9).
5. Transfer to liquid nitrogen the next day for long-term storage.

3.2.2. Phospho Flow in PBMCs

1. Remove a vial of frozen PBMCs from liquid nitrogen.
2. Thaw quickly (1 min) in 37°C water bath.
3. Pipette the cells into 25 mL of RPMI-10 at room temperature in a 50-mL conical tube.
4. Centrifuge the samples (500 × *g*, room temperature, 5 min).
5. Aspirate or decant the supernatant into 10% bleach solution (see Note 10).
6. Tap the tube onto the benchtop to dislodge the cell pellet.
7. Add 25 mL of RPMI-10 and pipette up and down to break any cell clumps (see Note 11).

8. Repeat steps 4–6.
9. Add 5 mL of RPMI-10 and pipette up and down to break any cell clumps. The cell concentration should be at ~2×10^6/mL at this point.
10. Place the cells in 37°C 5% CO_2 incubator for 1 h (see Note 12).
11. (Perform steps 11 and 12 as quickly as possible to avoid cooling the cells.) Place 1 mL of cells into five FACS tubes labeled 1–5.
12. Add the following stimuli.

Tube 1	2	3	4	5
None	None	IL-6 (50 ng/mL)	IL-10 (50 ng/mL)	LPS (1 μg/mL)

Note: At this point in the experiment, the protocol of the U937 experiment in Subheading 3.1, from step 4 onward, is followed. The only change in the PBMC experiment is that regarding the choice of antibodies added during the staining step.

13. Incubate the cells for 15 min at 37°C in a 5% CO_2 incubator.
14. Quickly add 100 μL of 16% formaldehyde (final concentration of ~1.5%).
15. Keep the tubes at room temperature for 10 min.
16. Centrifuge the samples (500 × *g*, 4°C, 5 min).
17. Decant the supernatant by inverting and flicking the tubes, leaving the pellet of cells at the bottom.
18. Resuspend the cell pellet in the residual medium (normally 30–70 μL) by vortexing for a few seconds or shaking the tubes vigorously by hand.
19. Add 1 mL of ice-cold methanol to the pellet.
20. Vortex the cells for 3–5 s to suspend them in the methanol.
21. Place the tubes on ice for 15–30 min (see Note 5).
22. Add 3 mL of staining medium on top of the methanol.
23. Repeat steps 16–18.
24. Add 4 mL of staining medium to the cell pellet.
25. Repeat steps 16–18.
26. Add 50 μL of staining medium to the pellet. With the residual volume typically left after decanting the supernatant, each tube should have 80–120 μL of the suspension at this point.
27. Transfer 50 μL of the cell suspension into a fresh set of five FACS tubes, numbered A1–5 (see Note 6).

28. Create the antibody cocktail below and add 80 µL to tubes A2–5 (total volume will be 130 µL). Add 80 µL of staining medium to tube A1. Antibody cocktail should be made such that there is enough for at least one extra sample, to ensure having enough for all samples and accommodating small pipetting errors. Here, antibody cocktail is prepared for five samples, although only four are stained.

Antibody	Volume per sample (µL)	Total volume (µL)
CD3 PE-Cy7	10	50
CD4 PacBlu	5	25
CD20 PerCP-Cy5.5	20	100
CD33 PE	5	25
pStat3 Ax488	20	100
p-p38 Ax647	20	100
Total	80	400

29. Vortex to mix.
30. Stain for 1 h at room temperature (see Note 7).
31. Add 3 mL of staining medium.
32. Repeat steps 16–18.
33. Add 100 µL of staining medium. Keep the samples cold until acquisition (see Note 8).
34. Prepare proper compensation controls for each fluorophore being used in the experiment.
35. Acquire compensation controls and samples on a flow cytometer. Acquire at least 50,000 events per sample. Expected results are shown in Fig. 2. IL-6 and IL-10 induce Stat3 phosphorylation in many cell types, while LPS induces p38 phosphorylation in monocytes only.

3.3. Primary Human Whole Blood

Experiments in whole blood enable measurements to be made in the most physiologically relevant environment for human samples. Unlike Ficoll-purified PBMC samples, whole blood contains neutrophils and granulocytes (creating a much different forward vs. side scatter plot), red blood cells, and all of the protein factors present in the serum. This provides a more "normal" context for cell signaling to occur. However, the presence of the massive number of red blood cells complicates flow cytometry and requires that the cells be lysed prior to analysis.

In this method, the red blood cells are lysed simultaneously as the white blood cells are fixed, using a Lyse/Fix buffer. This extra step allows stimulation to occur in the whole blood and enables

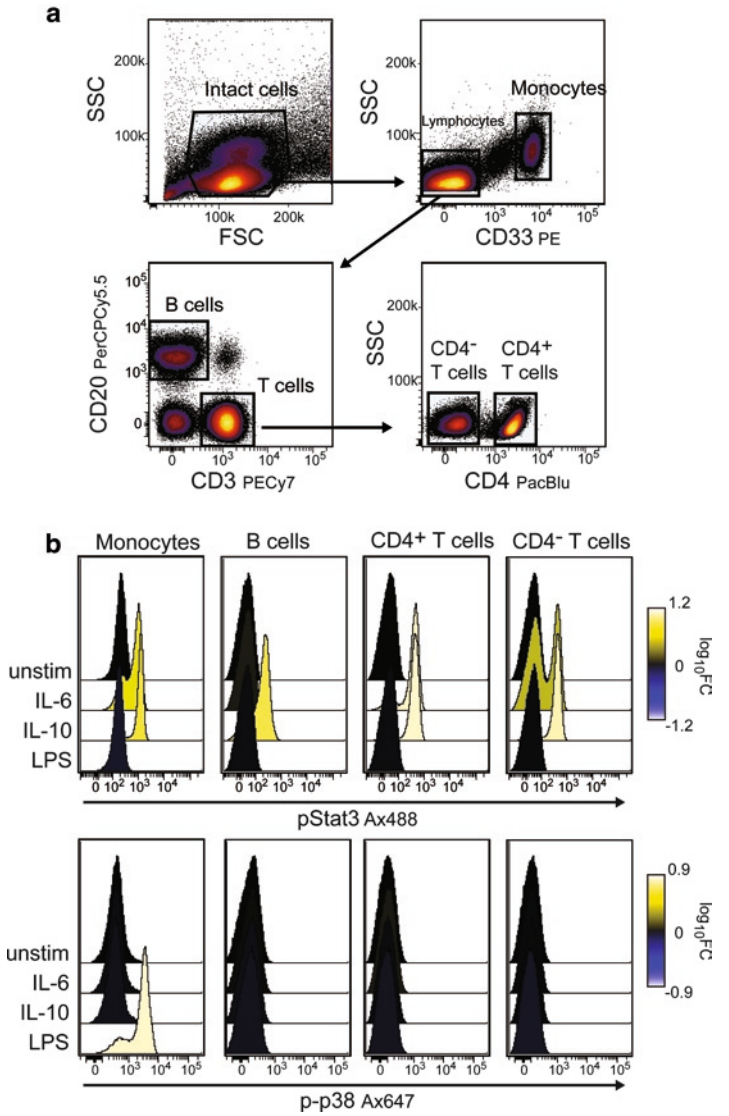

Fig. 2. Sample data from PBMC experiment. PBMCs were left unstimulated or were treated with IL-6, IL-10 (both 50 ng/mL), or LPS (1 μg/mL). Cells were fixed, permeabilized, and stained with phospho-specific antibodies against Stat3 (pY705) and p38 (pT180/Y182) conjugated to Ax488 and Ax647, respectively. (**a**) Identification of cellular subsets by surface gating. Intact cells were identified based on forward and side scatter characteristics. Intact cells were then gated based on CD33 expression, with $CD33^{hi}$ cells representing monocytes, and $CD33^{-}$ cells representing lymphocytes. Lymphocytes were separated into B- and T-cell populations based on CD20 and CD3 expression, respectively. Finally, the T-cell population was divided into $CD4^{+}$ and $CD4^{-}$ (or $CD8^{+}$) subsets. (**b**) Histogram analysis of phosphorylation levels in gated cellular subsets. For each of the populations identified in (**a**), the levels of Stat3 and p-p38 were analyzed for each of the four treatment conditions. IL-6 induced Stat3 phosphorylation in monocytes, $CD4^{+}$ and $CD4^{-}$ T cells, but not B cells. IL-10 induced Stat3 phosphorylation in all the cell types analyzed. LPS-induced p-p38 levels in monocytes only. Histograms are colored (in the electronic version) according to fold change in phosphorylation relative to the unstimulated control (using Cytobank software).

rapid termination of the stimulation reaction at the desired time point. Once the red blood cells are lysed, the samples are treated just as PBMC samples or the U937 cell line.

Note: Prior to starting the experiment, prepare 1× Lyse/Fix buffer by diluting 5× buffer with purified water and warming to 37°C in a water bath.

1. Obtain human whole blood drawn into heparin tube (typically green top) (see Note 13).
2. Place in 37°C water bath for 30 min to ensure blood is warm for stimulation (see Note 14).
3. Aliquot 200 µL of blood into five FACS tubes, labeled 1–5 (see Note 1).
4. Add stimuli as follows:

Tube 1	2	3	4	5
None	None	IL-6 (50 ng/mL)	IL-10 (50 ng/mL)	LPS (1 µg/mL)

5. Incubate for 15 min at 37°C.
6. Add 4 mL of 1× Lyse/Fix buffer (prewarmed to 37°C).
7. Mix thoroughly by inverting the tube with cap ten times or by pipetting up and down ten times (see Note 15).
8. Incubate for 15 min at 37°C in a waterbath or incubator.
9. Centrifuge the samples (500 × *g*, 4°C, 5 min).
10. Aspirate or decant the supernatant.
11. Vortex the cells for 3–5 s to dislodge the cell pellet in the residual volume.
12. Add 4 mL of ice-cold PBS (see Note 16).
13. Repeat steps 9–11.
14. Add 1 mL of ice-cold methanol.
15. Place the samples on ice for 15–30 min (see Note 5).
16. Add 3 mL of staining medium on top of the methanol.
17. Repeat steps 9–11.
18. Add 4 mL of staining medium to the cell pellet.
19. Repeat steps 9–11.
20. Add 50 µL of staining medium to the pellet. With the residual volume typically left after decanting the supernatant, each tube should have 80–120 µL of cell suspension at this point.
21. Transfer 50 µL of the cell suspension into a fresh set of five FACS tubes, numbered A1–5 (see Note 6).
22. Create the antibody cocktail below and add 80 µL to tubes A2–5 (total volume will be 130 µL). Add 80 µL of staining

medium to tube A1. Sample A1 serves as an unstained control for cytometer setup. Antibody cocktail should be made so as to have enough for at least one extra sample, to ensure having enough for all samples and accommodating small pipetting errors (in this case, enough cocktail should be prepared for five samples).

Antibody	Volume per sample (μL)	Total volume(μL)
CD3 PE-Cy7	10	50
CD4 PacBlu	5	25
CD20 PerCP-Cy5.5	20	100
CD33 PE	5	25
pStat3 Ax488	20	100
p-p38 Ax647	20	100
Total	80	400

23. Incubate for 1 h at room temperature.
24. Add 4 mL of staining medium to the cells.
25. Repeat steps 9–11.
26. Add 100 μL of staining medium to the cells.
27. Acquire 100–200,000 events per sample on the flow cytometer. Expected results are shown in Fig. 3. The results are nearly identical to the PBMC experiment. However, the presence of neutrophils and granulocytes adds many large cells to the forward versus side scatter plots, and makes gating of the monocytes slightly more difficult. Monocytes are high for CD33 expression, while neutrophils and granulocytes are intermediate (with the suggested clone of CD33 antibody). Monocytes have a much more robust response to LPS, so accurate gating is important for observing maximal induction.

3.4. Surface Marker Antibody Validation and Sequential Staining

Perhaps the most important, and most difficult, part of phospho flow is the proper identification of cellular subsets (e.g. T cells, B cells, and monocytes), within heterogeneous samples such as peripheral blood. This is accomplished by staining antigens (e.g. CD3, CD20, and CD33) present on the cell surface of each cell subset. Surface staining can be performed at several points in the phospho flow protocol, each with its advantages and disadvantages, summarized in Table 1.

In our laboratory, most "surface" staining is performed on fixed/permeabilized cells with antibodies that have been carefully validated for use in this protocol. This enables us to stain all markers, both intracellular and surface, simultaneously,

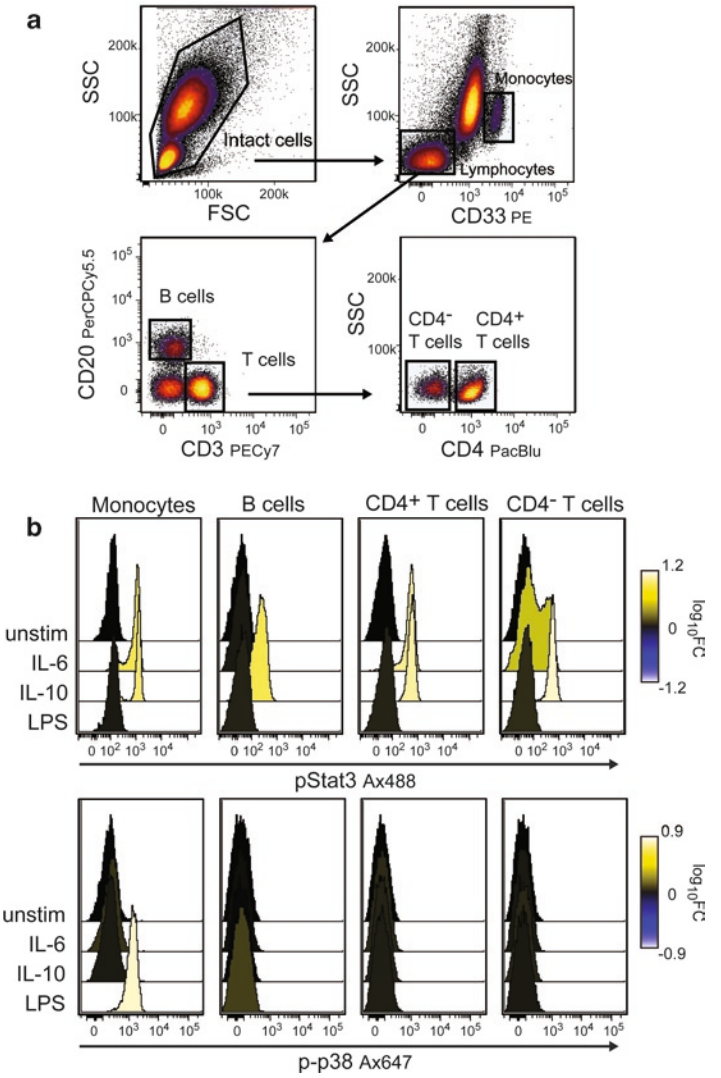

Fig. 3. Sample data from experiment in whole blood. Whole blood was obtained and treated as in the PBMC experiment. (**a**) The gating strategy in whole blood is the same as that for the PBMC experiment shown in Fig. 2. Note, however, that whole blood has a large population of neutrophils, which are high in the side scatter dimension and have intermediate expression of CD33 (with this antibody clone). Typical PBMC isolation procedures remove neutrophils; therefore, this population is unique to whole blood experiments. (**b**) Histogram overlay analysis of signaling in whole blood. The results are very similar to stimulation in PBMC, shown in Fig. 2.

greatly simplifying the protocol. This is also the most flexible way to stain samples, because they can be stored in methanol for months at a time, and then stained with different antibody cocktails as the research project develops and new hypotheses need to be tested.

Table 1
Comparison of different surface staining strategies for phospho flow

Surface stain timing	Advantages	Disadvantages
On live cells, before or during stimulation of cells	Use standard Ab titrations	Limited in fluorophore choices May affect signaling by activating/blocking surface receptors
On fixed cells, before permeabilization	Use standard Ab titrations Most Abs stain similar to live cells	Limited in fluorophore choices Adds extra steps in protocol Some antigens blocked by fixative
On fixed/permeabilized cells	Simplest protocol, all Abs added at same time Works for any fluorophore Quickest protocol Flexibility to stain any surface markers at later time	Some Abs have higher background in permeabilized cells Some Abs do not bind to their antigen once denatured by methanol Requires re-titration of Abs to get optimal titer

Table 2
Fluorophore selection for sequential staining technique

Can be used	Cannot be used
Small molecule dyes (e.g. Pacific Blue, FITC, Alexa dyes, and DyLight dyes)	Protein fluorophores (e.g. PE, PerCP, and APC[a])
Quantum dots	Protein tandem dyes (e.g. PE-TR, PE-Cy5, PerCP-Cy5.5, and APC-Cy7)

[a]APC conjugates are not completely quenched by methanol treatment, but lose over 90% of their signal. Therefore, Ax647 conjugates are recommended for this detection channel for sequential staining

However, some antibodies do not work when staining fixed/permeabilized cells. In these cases, the background staining may increase dramatically, or positive staining may decrease. In either case, one can no longer resolve the positive population. To solve this problem, we adopt a "sequential staining" approach, where stimulated cells are fixed, stained for these difficult surface antigens, then permeabilized, and stained for intracellular epitopes. Note, however, that fluorophore choices are limited in the sequential staining protocol (see Table 2). Protein fluorophores such as PE, PerCP, and APC are denatured by methanol and lose their fluorescence if used in sequential methods. Therefore, antibodies must be conjugated to small molecule fluorophores such as FITC, the Alexa dyes, and DyLight dyes, or to Quantum dots, for use in the sequential methods.

With proper testing and validation, nearly all surface antigens can be stained effectively in the phospho flow protocol.

3.4.1. Surface Marker Validation for Use After Methanol

1. Prepare or thaw 10×10^6 PBMCs.
2. Resuspend at 2×10^6 cells/mL in prewarmed, 37°C RPMI-10.
3. Place 2.5 mL (5×10^6) of cells into two FACS tubes.
4. Tube 1 (live cells): Place on ice. Proceed to step 6.
5. Tube 2 (fixed/permeabilized cells): these steps are the same as Subheading 3.1, steps 5–12.
   (a) Add 250 µL of 16% formaldehyde.
   (b) Incubate for 10 min at room temperature.
   (c) Pellet the cells by centrifugation (500 × *g*, 4°C, 5 min).
   (d) Decant or aspirate the supernatant.
   (e) Vortex 3–5 s to resuspend the cells in residual volume.
   (f) Add 1 mL of ice-cold methanol.
   (g) Incubate on ice for 15–30 min.
6. Add 2–3 mL of staining medium.
7. Centrifuge the samples (500 × *g*, 4°C, 5 min).
8. Decant the supernatant by inverting and flicking the tubes, leaving the pellet of cells at the bottom.
9. Resuspend the cell pellet in the residual medium (normally 30–70 µL) by vortexing for 3–5 s or shaking the tubes vigorously by hand.
10. Add 4 mL of staining medium.
11. Repeat steps 7–9.
12. Resuspend the cells by adding 500 µL of staining medium to each tube.
13. Aliquot 100 µL of cells into five tubes each for live and fixed/permeabilized cells.
14. Add varying amounts of the surface marker antibody being tested. Titrate from high to low concentration. Begin with ~1 µg/mL of antibody, or at the manufacturer's recommended dilution. Threefold dilutions work well, and cover a large enough range to obtain a good titer for most antibodies (most monoclonal antibodies have optimal titers between 10 ng/mL and 1 µg/mL).
15. Analyze on the flow cytometer.
16. Compare staining in live cells versus fixed/permeabilized cells. For proper validation, antibody must show (see Note 17):
    (a) Same percentage of cells in both cases.
    (b) Adequate resolution, or separation, of positive cells from negative populations.

17. If antibody does not stain proper percentage of cells, or lacks resolution/separation, sequential staining may be preferred.

3.4.2. Sequential Staining

1. Prepare and stimulate cells as in Subheadings 3.2 and 3.3.
2. Fix cells with 100 µL of 16% formaldehyde per 1 mL of cells (1.5% final concentration).
3. Centrifuge the samples (500 × *g*, 4°C, 5 min).
4. Decant the supernatant by inverting and flicking the tubes.
5. Resuspend the cell pellet in the residual medium.
6. Wash the cells by adding 4 mL of staining medium.
7. Centrifuge the samples (500 × g, 4°C, 5 min).
8. Resuspend the cells at ~1×10^6 cells/100 µL in staining medium.
9. Transfer 100 µL of cells into fresh tubes.
10. Stain with the appropriate dilution of surface marker antibodies (typically the same amount as that used for live cell staining). Important note: antibodies must be conjugated to small molecule fluorescent dyes or quantum dots for sequential staining. Antibodies conjugated to protein fluorophores such as PE, PerCP, and APC will lose fluorescence when treated with methanol (see Note 18 and Table 2).
11. Incubate for 30 min on ice.
12. Wash the cells by adding 3 mL of staining medium.
13. Centrifuge the samples (500 × *g*, 4°C, 5 min).
14. Decant the supernatant by inverting and flicking the tubes.
15. Resuspend the cell pellet in the residual medium.
16. Add 1 mL of ice-cold methanol per mL of starting cell volume.
17. Incubate for 15–30 min on ice.
18. Repeat steps 12–15 twice to wash methanol from cells.
19. Resuspend the cells at ~1×10^6 cells/100 µL in staining medium.
20. Transfer to fresh FACS tube.
21. Stain the samples with phospho-specific antibodies and any surface marker antibodies conjugated to PE, PerCP, or APC (and their tandems), which have been validated for use in fixed/permeabilized cells.
22. Incubate for 1 h at room temperature.
23. Wash the cells and analyze on flow cytometer as above.

3.5. Data Analysis in Phospho Flow

Phospho flow data analysis is somewhat different than typical flow cytometry experiments, because different samples must be compared quantitatively to a control sample. In phospho flow, one

wants to measure the amount of phosphorylation induced by treating a sample with a particular cytokine, or the change in phosphorylation associated with a diseased sample versus that in a normal sample.

Different cell populations within the sample are first gated based on their surface marker staining. The median fluorescence intensity is then calculated for the phospho-specific antibody channel for each population. Medians, as opposed to means, are utilized to avoid the effects of outliers. However, it is important to be cognizant of your data. Medians may not be appropriate for a bimodal distribution, for instance. Comparisons are made within cell types under different conditions, e.g. stimulated versus unstimulated B cells. However, it is difficult to compare between different cell types, e.g. B cells versus monocytes, due to differences in background binding of phospho-specific antibodies, as well as autofluorescence differences due to cell size/shape.

Our laboratory has developed web-based software, called Cytobank, for storing, sharing, analyzing, and visualizing flow cytometry data sets. In particular, Cytobank is well-suited to analyze phospho flow data with its ability to create heatmaps and histogram overlays without requiring third party software. In fact, the steps outlined below can all be performed automatically within Cytobank, eliminating the need for spreadsheet programs. All figures in this chapter were generated using Cytobank.

1. During or after acquiring data on the flow cytometer, compensate data with appropriate compensation controls.
2. Draw gates around cell populations of interest. A standard gating method is to first gate on intact cells based on their forward and side scatter characteristics.
3. Then, use the other fluorescent parameters to identify cell types of interest. For instance, $CD3^+$ cells are T cells, $CD20^+$ cells are B cells, and $CD33^+$ cells are monocytes.
4. For each population that has been gated, calculate the median fluorescence intensity (MFI) of the phospho-protein channel (typically Alexa 488 or Alexa 647).
5. Apply this gating and statistic to all the samples that were acquired.
6. Export or copy/paste the MFI values for each population into a spreadsheet program.
7. Calculate the fold change in phosphorylation induced by each particular stimulation or treatment (e.g. cytokine) with the following equation:

$$\text{Fold change} = \text{MFI}_{\text{stimulated}} / \text{MFI}_{\text{unstimulated.}}$$

(a) This equation simply compares the MFI value of the stimulated samples to the MFI value of the control/unstimulated sample.

(b) If there is no change in phosphorylation upon stimulation, then fold change = 1.

(c) If the phospho-specific antibody staining intensity doubles upon stimulation, then fold change = 2.

8. For visualizing the data (and to accommodate negative changes), it is useful to represent no change in phosphorylation as zero. Therefore, we often calculate the $\log_2$ or $\log_{10}$ fold change (Table 3):

$$\text{Log}_2\text{fold change} = \log_2\left(\text{MFI}_{\text{stimulated}} / \text{MFI}_{\text{unstimulated}}\right).$$

(a) Here, if no change in phosphorylation is observed upon treatment, $\log_2\text{FC} = 0$.

(b) If the staining intensity doubles, $\log_2\text{FC} = 1$.

(c) If the staining intensity is halved, $\log_2\text{FC} = -1$.

9. The $\log_2\text{FC}$ values can now be exported to heatmap analysis software, often used for DNA microarray analysis. A positive change is often represented as yellow, and a negative change as cyan. No change is represented as black (see Fig. 4). This allows rapid visual identification of stimulations or conditions that lead to a change in phosphorylation levels. However,

Table 3
Sample calculations from PBMC experiment (Subheading 3.2)

		pStat3 median	Fold change	Log_2FC	Log_{10}FC
Monocytes	Unstim	204.6	1.00	0.00	0.00
	IL-6	708.9	3.46	1.79	0.54
	IL-10	1172.2	5.73	2.52	0.76
	LPS	165.5	0.81	−0.31	−0.09
B cells	Unstim	29.2	1.00	0.00	0.00
	IL-6	32.9	1.13	0.17	0.05
	IL-10	190.5	6.53	2.71	0.81
	LPS	28.0	0.96	−0.06	−0.02
CD4+ T cells	Unstim	28.1	1.00	0.00	0.00
	IL-6	393.0	13.99	3.81	1.15
	IL-10	406.4	14.47	3.85	1.16
	LPS	27.1	0.96	−0.05	−0.02
CD4− T cells	Unstim	31.0	1.00	0.00	0.00
	IL-6	83.0	2.68	1.42	0.43
	IL-10	393.5	12.71	3.67	1.10
	LPS	30.3	0.98	−0.03	−0.01

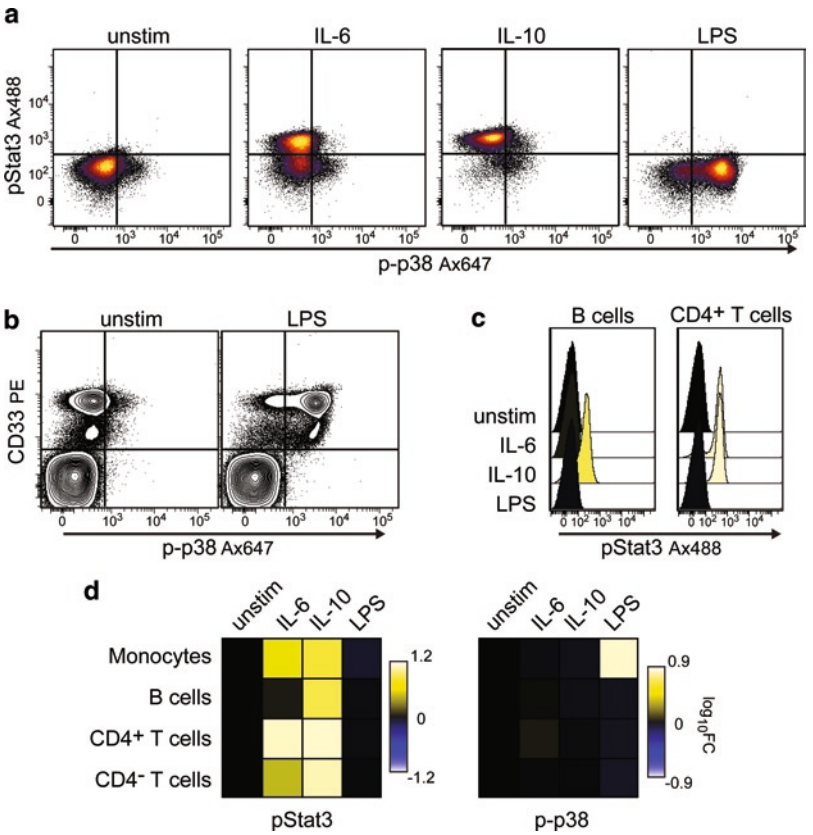

Fig. 4. Data visualization methods for phospho flow. Many methods are available for viewing changes in phosphorylation, and the optimal method should be chosen based on the level of detail required. For instance, heatmaps provide a broad overview of phosphorylation trends when performing large signaling profiling experiments. Histogram overlays provide more detail and enable the identification of heterogeneous, or bimodal, signaling responses. Two-dimensional contour and density plots provide the most detailed analysis level; however, it is difficult to view/analyze many two-dimensional plots. Therefore, a common workflow is to proceed from heatmaps, through histogram overlays, and finally down to the two-dimensional plot level. (**a**) Two-dimensional density plot showing levels of two phospho-proteins against each other. Data are shown for the monocyte subset in PBMC. (**b**) Two-dimensional contour plot showing a surface marker, CD33 versus a phospho-protein, p-p38. This enables the identification of specific CD33-expressing subsets that respond to LPS treatment. (**c**) Histogram overlay analysis of B-cell and $CD4^+$ T-cell phosphorylation of Stat3 in response to treatments in PBMC. (**d**) Heatmap analysis of the PBMC experiment in Fig. 2, enabling rapid identification of both cell subsets and treatments that lead to phosphorylation of either Stat3 or p38. Data analysis performed with Cytobank software.

since only the median is utilized, it is critical to examine the responding samples to determine whether all of the cells in a population responded (unimodal), or if only a fraction responded (bimodal or multimodal peaks). Multimodal peaks typically indicate that a particular population contains sub-populations of cells that need to be identified with more surface markers.

10. As mentioned above, Cytobank, a software suite developed in our laboratory, is able to perform all of these calculations for the user. In addition, Cytobank enables visualization of the data in heatmap and histogram overlay format (Fig. 4).

4. Notes

1. Because biochemical events inside cells are highly temperature dependent, perform all operations outside the incubator as quickly as possible to avoid drops in temperature. Although cell lines such as U937 cells respond well even at lower temperatures, some primary cell types will be affected more dramatically by slight changes in temperature.
2. In these experiments, the cytokine stimulations are added at doses that are 10–50 times above the EC_{50} concentration, which for most cytokines is in the range of 0.1–1 ng/mL. This ensures complete receptor binding and stimulation of the cells.
3. Because cytokines are proteins, it is best to avoid multiple freeze/thaw cycles or storage of cytokine stocks at 4°C. Therefore, we typically prepare stock solutions at 10 µg/mL (1,000×), and freeze dozens of aliquots at −80°C. Each aliquot is then thawed before an experiment and discarded after that experiment (not re-frozen). This ensures consistent stimulation and dose–response experiments.
4. After adding formaldehyde, typical media such as RPMI will turn yellow temporarily. After about 1 min, the original color will return. This is normal and is an easy way to monitor if formaldehyde was added correctly to all the tubes/wells.
5. If samples need to be stored for longer periods of time, they must be stored at colder temperatures. For storage up to 6 h, placing in a −20°C freezer is acceptable. For overnight storage, or storage up to 6 months, place the samples at −80°C. We have analyzed samples stored at −80°C in methanol for over 6 months and found phospho-protein and surface antigen staining to be nearly identical to that of the same samples analyzed after only a few days of storage.
6. One of the most critical parameters to getting consistent staining between samples is to have the same sample volume. However, after decanting the supernatant, the residual volume left in FACS tubes or plates can vary from 30 to 70 µL depending on how skillfully the researcher decants. Therefore, we recommend transferring samples to a new set of tubes, so that all samples have identical volume. Antibodies should be

titrated at this sample volume to obtain the optimal concentration of antibody. Not having the same sample volume will lead to "wobble" in the median fluorescence staining intensity between samples, and poor statistics. The number of cells in the sample volume does not affect staining intensity significantly when the cell concentration varies less than threefold. If some samples contain ten times more or less cells, then variation in staining intensity may be observed, depending on the antibody being used.

7. Staining can be done in the dark if the researcher prefers, but we have not found any significant difference between staining on the benchtop in typical laboratory lighting versus staining in the dark or by covering the tube rack.
8. Samples can be stored on ice for several hours before acquisition on the flow cytometer. In our laboratory, we have stored stained samples overnight at 4°C and acquired the next day, with results nearly identical to those of samples run immediately. However, this should be tested empirically with different antibodies, since they may have different off-rate kinetics.
9. Freezing the cells slowly is important for viability upon thawing. Several manufacturers produce slow freezing devices for –80°C freezers, including Nalgene-NUNC. These devices are typically plastic containers that insulate the cryovials with isopropanol to produce slower temperature changes.
10. Human primary blood cells should always be treated as potentially biohazardous until the cells have been fixed with formaldehyde. Therefore, the supernatant from live cells should be treated as though it contains infectious agents, and should be poured into bleach before proper disposal.
11. Cell clumping is usually caused by lysis of cells and the release of their DNA into the medium, and this DNA aggregates with cell surface proteins to cause larger cellular clumps. Addition of DNAse to the RPMI-10 used for washing the cells can decrease cell clumping.
12. Cells must be warmed to 37°C to obtain optimal stimulation. We have found that a 1 h rest time allows ample time for warming, but avoids artifacts of increased basal phosphorylation that are often observed with longer resting periods, such as 2–6 h.
13. Phospho flow experiments have been performed successfully on blood drawn into heparin and EDTA as anticoagulants. Heparin tubes have performed most consistently and, therefore, have become our standard tubes. Citrate tubes have shown slightly higher variability in cellular responses, although this has not been tested exhaustively. The blood draw tube that is

chosen should be used throughout the entire study, to avoid any differences the anticoagulant may produce.

14. Human blood should be treated as potentially biohazardous at all times until adequately fixed and/or bleached.
15. After adding the Lyse/Fix buffer to the blood, the tubes must be mixed thoroughly to ensure complete red blood cell lysis. The Lyse/Fix buffer simultaneously lyses red blood cells and fixes the white blood cells. Inadequate mixing may lead to large red blood cell pellets and heterogeneity between samples. The samples should be mixed until the solution is bright red and mostly clear. Cloudiness represents incomplete lysis. Red blood cells appear on the flow cytometer as events that are slightly smaller than the lymphocytes, and can often make lymphocyte identification difficult. Therefore, this step is critical to the whole blood phospho flow method, and should be focused on.
16. The PBS wash is performed to remove as much of the red blood cell debris as possible after the lysis step.
17. In most cases, staining of surface antigens after fixation and permeabilization results in increased backgrounds and/or decreases in positive signals. This leads to an overall decrease in separation between positive cell events and negative cell events. Often, using lower concentrations of antibody is more optimal in permeabilized cells because this leads to lower background staining (13).
18. Protein fluorophores such as PE, PerCP, and APC are denatured by methanol and, therefore, lose fluorescence. APC is not completely quenched, but loses ~90% of its brightness upon methanol treatment. Therefore, with some well-separated signals, APC may be usable in sequential staining methods. In some of our studies, we have observed that using 70% ethanol as the permeabilization reagent may retain protein fluorophore fluorescence and also lead to sufficient permeabilization for good phospho-protein staining. Therefore, with optimization, it may be possible to use these protein fluorophores in sequential staining protocols.

References

1. Krutzik, P. O., Irish, J. M., Nolan, G. P., and Perez, O. D. (2004) Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications. *Clin Immunol* **110**, 206–21.
2. Schulz, K. R., Danna, E. A., Krutzik, P. O., and Nolan, G. P. (2007) Single-cell phospho-protein analysis by flow cytometry. *Curr Protoc Immunol* Chapter 8, Unit 8.17.
3. Hotson, A. N., Hardy, J. W., Hale, M. B., Contag, C. H., and Nolan, G. P. (2009) The T cell STAT signaling network is reprogrammed within hours of bacteremia via secondary signals. *J Immunol* **182**, 7558–68.
4. O'Gorman, W. E., Dooms, H., Thorne, S. H., Kuswanto, W. F., Simonds, E. F., Krutzik, P. O., Nolan, G. P., and Abbas, A. K. (2009) The initial phase of an immune response

functions to activate regulatory T cells. *J Immunol* **183**, 332–9.

5. Krutzik, P. O., Hale, M. B., and Nolan, G. P. (2005) Characterization of the murine immunological signaling network with phosphospecific flow cytometry. *J Immunol* **175**, 2366–73.
6. Hale M. B., Krutzik, P. O., Samra, S. S., Crane, J. M., and Nolan, G. P. (2009) Stage dependent aberrant regulation of cytokine-STAT signaling in murine systemic lupus erythematosus. *PLoS One* **4**, e6756.
7. Irish, J. M., Czerwinski, D. K., Nolan, G. P., and Levy, R. (2006) Altered B-cell receptor signaling kinetics distinguish human follicular lymphoma B cells from tumor-infiltrating nonmalignant B cells. *Blood* **108**, 3135–42.
8. Kotecha, N., Flores, N. J., Irish, J. M., Simonds, E. F., Sakai, D. S., Archambeault, S., Diaz-Flores, E., Coram, M., Shannon, K. M., Nolan, G. P., and Loh, M. L. (2008) Single-cell profiling identifies aberrant STAT5 activation in myeloid malignancies with specific clinical and biologic correlates. *Cancer Cell* **14**, 335–43.
9. Irish, J. M., Kotecha, N., and Nolan, G. P. (2006) Mapping normal and cancer cell signalling networks: towards single-cell proteomics. *Nat Rev Cancer* **6**, 146–55.
10. Krutzik, P. O., Crane, J. M., Clutter, M. R., and Nolan, G. P. (2008) High-content single-cell drug screening with phosphospecific flow cytometry. *Nat Chem Biol* **4**, 132–42.
11. Krutzik, P. O. and Nolan, G. P. (2006) Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. *Nat Methods* **3**, 361–8.
12. Krutzik, P. O. and Nolan, G. P. (2003) Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry A* **55**, 61–70.
13. Krutzik, P. O., Clutter, M. R., and Nolan, G. P. (2005) Coordinate analysis of murine immune cell surface markers and intracellular phosphoproteins by flow cytometry. *J Immunol* **175**, 2357–65.