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Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications

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Abstract

Analysis of protein phosphorylation with flow cytometric techniques has emerged as a powerful tool in the field of immunological signaling, allowing cellular subsets in complex populations to be analyzed accurately and rapidly. In this review, we examine the development of phospho-epitope, or phospho-specific, flow cytometry and the premises upon which the technique is based. Phospho-specific flow cytometry is compared to traditional biochemical methods, and its advantages, such as single cell analysis, multiparameter data acquisition, rapid protocols, and the ability to analyze rare cell subsets, are detailed. We also discuss the many technical considerations that must be addressed when developing new antibodies or analyzing new epitopes including antigen accessibility, stability of the phospho-epitope, fluorophore selection, surface phenotype integrity, and antibody suitability for staining epitopes inside fixed and permeabilized cells. The methods that have been used to date are described in light of these technical considerations. The importance of developing bioinformatic platforms in parallel with these techniques is emphasized due to the large, multiparameter datasets that are rapidly accumulated and which require more efficient data viewing and complex clustering methods than currently available for flow cytometric data. Finally, we discuss the potential clinical applications of phospho-specific flow cytometry in analyzing immune cell development and antigen-specific immune responses, as well as pharmacodynamic profiling of disease states or drug efficacy and specificity against particular signaling proteins.

Keywords: Intracellular; Phosphorylation; Multiparameter; Phospho-specific antibodies; Activation; Drug screening; Disease characterization

Introduction

Flow cytometry has become an indispensable tool in clinical and basic immunological research due to its ability to distinguish subsets in heterogeneous populations of cells. Recently, major advances have been made in both flow cytometry machinery and applications, expanding the number of possible simultaneous analysis parameters to 13 or more [1,2]. With more parameters available, researchers have begun to identify more well-defined and biologically interesting subsets of lymphocytes in human and murine samples based upon surface epitope staining. Although surface staining may be an effective means of characterizing cells, it does not provide information about the functional responses of those cells to stimuli that are immediately reflective of intracellular events. Even in cases where the marker used is a cytokine receptor or receptor tyrosine kinase, levels of the antigen do not always correlate with cellular response to the specific ligand [3]. Therefore, methods have been developed to characterize cells by levels of intracellular epitopes: cytokines, DNA, mRNA, enzymes, hormone receptors, cell cycle proteins, and of particular interest to this review, phosphorylated signaling molecules.

Analysis of DNA, mRNA, and cell cycle proteins by flow cytometry is frequently used to determine the proliferative status of cells, important in studies of cancer and stem cells. Levels of proteins such as Bcl-2 or p53 have expanded phenotypic analysis of tumor samples to include resistance to apoptosis. However, most of these indicators are relatively static and are often the culmination of rapid cellular signaling events triggered by extracellular stimuli. Therefore, the characterization of more immediate outcomes

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of signaling such as phosphorylation events on discrete signaling proteins within kinase cascades would be very helpful. Elucidating the functional responses of cells and characterizing intracellular epitopes may clarify the role of cells in the immune system in ways that are not possible by surface marker analysis alone. Cell types that appear homogeneous by surface characterization have been found to be heterogeneous based on their intracellular signaling responses to various stimuli.

Measurement of phosphorylation states of specific proteins has recently been simplified by the production of phospho-specific antibodies against particular phospho-epitopes on proteins, eliminating the need to perform immunoprecipitations and follow by blotting with generic phospho-tyrosine, serine, and threonine antibodies. In the flow cytometry platform, these phospho-specific antibodies are coupled to fluorophores to allow fluorescent detection and provide a way to perform multiple analyses simultaneously. Briefly, phospho-epitope flow cytometry is performed by stimulating cells, then fixing, permeabilizing, and staining with fluorophore-coupled phospho-specific antibodies. Data concerning signaling events are collected for each individual cell and can be correlated to surface marker expression or other phospho-epitopes. Because more than 13 parameters can be collected for each cell, the connections that can be drawn between particular cell types and their cognate signaling pathways using this technique cannot be determined by traditional biochemical methods. For example, a heterogeneous population of lymphocytes might appear to respond uniformly to a cytokine when probed by Western blotting techniques. However, with phospho-specific flow cytometric methods, one often finds that only one particular subset of lymphocytes, perhaps B cells or T cells, responds to the stimulus while the others do not. In this way, examining all the cell types present in a sample individually can clarify changes that appear small in the whole population.

In this review, we will examine how flow cytometry has been used to analyze various intracellular epitopes and discuss the premises upon which phospho-specific flow cytometric analysis is based. The advantages that flow cytometry provides relative to standard biochemical and immunological techniques and some of the critical technical considerations pertinent to the technique will be reviewed. Next, we will discuss the various techniques that have been applied for measurement of phospho-epitope levels by flow cytometry and new bioinformatics analysis programs that are being developed to analyze the large amounts of data being generated. Finally, we will explore the existing and potential applications of phospho-specific flow cytometry to clinical settings, including characterization of immune system development and signaling, antigen-specific T-cell responses, drug screening, and disease phenotyping. In many ways, this field is still in development with only a few published papers on the subject. Thus, this review seeks to provide an update on the current state of the art, keeping in mind the fact that considerable work is still being done to refine and optimize the techniques for emerging applications.

Flow cytometry as a tool for intracellular antigen analysis

A wide variety of intracellular epitopes has recently been analyzed by flow cytometry (Table 1). Intracellular cytokine staining has gained appreciation in recent years because of its ability to positively identify cytokine-producing cells. Other reviews cover this topic more thoroughly, but we would like to emphasize two of the difficulties encountered during cytokine staining as they are similar to phospho-epitope staining in some cases [4]. First is the issue of signal intensity when detecting cytokines in cells. Initial analyses were plagued by low signal-to-noise ratios and prompted the use of Golgi-disrupting agents such as brefeldin A and monensin [5,6]. These agents effectively inhibit secretion of cytokines and increase their intracellular levels. This technique increased staining intensities, but one cannot overlook the artificial nature of "plugging up" cytokine release and paracrine and autocrine signaling that the cytokines normally elicit. A second major difficulty that was overcome early in the development of cytokine staining was the specificity of particular antibodies when used to stain epitopes within fixed and permeabilized cells. It was found that certain clones of monoclonal antibodies (mAbs)

Table 1
Intracellular antigens analyzed by flow cytometry

Antigen	Reference
Cytokines	
IL-2, IL-4, IFN-γ, TNF-α, etc.	[4]
Cell cycle or apoptosis	
Ki67, PCNA	[67]
p53	[68,69]
DNA	[35,70]
Bcl-2	[71,72]
Caspases	[73,74]
Viral particles	
Rabies	[75]
HIV	[76]
Enzymes or receptors	
Cox	[77]
Estrogen receptor	[78]
Phospho-proteins	
Akt	[34]
MEK, ERK	[23]
c-Jun, p38	[32]
MAPKs, Tyk-2	[33]
MAPKs, Stat1,5,6	[24]
Stat1	[3,36]
Stat4	[37]
Histone H3, Rb	[38,39]

provided more specific and intense staining and these were adopted for widespread use [7]. In a parallel fashion, phospho-specific mAbs must be carefully screened for optimal measurement of cellular signaling events in the context of fixed and permeabilized cells.

Although diverse intracellular epitopes have been stained for flow cytometric analysis, a common trait among the protocols is the need to optimize and tailor fixing and staining procedures for the specific epitope in question. Localization, conformation or structure, accessibility by antibodies, and stability of the epitope all play critical roles in necessary procedures. For example, cytokines that accumulate in the Golgi apparatus or leak into the cytoplasm can be accessed by relatively mild permeabilization reagents such as saponin, while some nuclear antigens, such as DNA, are best measured after alcohol dehydration. The conformation of the antigen is also critical, depending on the structure of the immunogen used to develop the mAb. Antibodies raised against peptides may recognize their epitope more efficiently when it is in the denatured state, while Abs raised against whole proteins may be optimal for binding to fully natured proteins. These considerations will be examined in the context of phospho-specific staining below.

Premise of phospho-specific flow cytometry

Phospho-specific analyses of any kind, flow cytometry and Western alike, are based on the premise that the phosphorylation state of a particular protein correlates with its biological status [8,9]. For kinases, phosphorylation typically enhances their enzymatic activity, propagating signals downstream [10–13]. In the mitogen-activated protein (MAP) kinase cascade, signaling begins at the cell surface and is passed from a MAP kinase kinase kinase (MEKK) to a MAP kinase kinase (MEK) to a MAP kinase and finally to a transcription factor. Each member of this cascade is activated by phosphorylation by the upstream member. Phosphorylation of transcription factors often increases DNA-binding affinity or alters their conformation to cause dimerization and DNA binding. In the Janus kinase-Signal transducer and activator of transcription (JAK-Stat) cascade, dimerization of cytokine receptors leads to the activation of JAKs which then phosphorylate Stats in their dimerization domains [14–16]. The dimerized Stats enter the nucleus and activate transcription. In other cases, phosphorylation provides docking sites for other proteins to bind and localize to specific intracellular locations, such as phosphorylation of tyrosine motifs on receptor tyrosine kinases [8]. Though phosphorylation often leads to ‘‘positive’’ activity, in some cases such as for the T cell protein Lck, phosphorylation inhibits enzymatic activity, and it is a dephosphorylation event by a phosphatase that causes Lck to signal [17].

To measure phosphorylation events uniquely, antibodies that are specific to the phosphorylated form of a protein must be raised. This is typically done by using short phosphorylated peptide immunogens coupled to carrier proteins. Thus, mAbs specific for different phospho-residues within the same signaling protein can be made, providing insight into which residues are important for particular signaling events. Phospho-specificity can be confirmed by comparing resting versus stimulated cells, treating samples with phosphatases before analysis, competing with phosphorylated peptides versus nonphosphorylated ones, and normalizing phospho-protein levels to total protein content.

In most cases, phosphorylation is a transient, reversible event that is indicative of the activation status of signaling proteins. Therefore, by measuring the phosphorylation state of proteins by flow cytometry, one can determine which signaling cascades are used in response to specific stimuli such as cytokines or growth factors, the kinetics of signaling, and the downstream targets that are transcribed. In addition, comparing diseased cells to healthy samples can easily identify aberrant signaling events, a trait that is useful for phenotyping cancers and immune disorders.

The advantages of flow cytometry for phospho-epitope analysis

Flow cytometry is an extremely powerful multiparameter method for analyzing phospho-specific epitopes that has many advantages over Western blotting or ELISAs. But it is clear that measuring intracellular antigens by flow cytometry is not a simple process; the approach requires extensive optimization of both the protocol and reagents used (see Technical considerations for phosphorylation state analysis below). To maintain accuracy and semiquantitative results, the flow cytometric methods must be compared to traditional methods such as Western blotting and ELISAs to provide cross-platform validation. However, once the techniques have been shown to yield the same results experimentally, flow cytometry provides many benefits when compared to traditional methods. A comparison of Western blotting and flow cytometry is summarized in Table 2 and is explained in detail below.

Single-cell analysis

A critical and often overlooked advantage of phospho-epitope analysis by flow cytometry is the capability to measure events in single cells. The flow cytometer is unique in analyzing single cells based on their fluorescence properties, rivaled only by microscopy techniques (which are limited in the number of the cells that can be analyzed). Westerns and ELISAs measure epitopes from whole populations of cells and may therefore not recognize characteristics of signaling events such as bistability, where cells show an all-or-none response to a particular stimulus [18–20]. In fact, these events are often erroneously quantified by traditional methods because there is no way of separating

Table 2
Comparison of phospho-specific flow cytometry and traditional techniques (Western blotting)

Western blot	Flow cytometry
Population analysis	**Single cell analysis**
Obtain average value of multiple cells	Collects data for each individual cell
Homogeneous sample	**Heterogeneous cell types**
Limited to cultured or purified cells	Complex primary samples, that is, immune cells
One parameter	**Multiparameter**
Obtain data sets individually	Correlate multiple markers simultaneously
Large number of cells	**Small number rare subsets**
Requires in vitro derived cultures of rare cells	Direct analysis of rare cell types (i.e., DC)
Time consuming for large sample sets	**Rapid and scalable**
Not amenable to large screening efforts	Performed in 96-well plates in parallel
Protein size and Ab specificity	**Ab must be validated**
Ab selectivity for target is clearly visible	Ab must have high affinity and selectivity

cells based on their responsiveness (Fig. 1). To obtain results specific to one cell type (not single cells), traditional methods require cell sorting or depletion of heterogeneous cell populations before analysis, both of which are time consuming and put cells through artificial conditions.

Multiparameter analysis

Another major advantage of flow cytometry is its multiparameter nature, that is, the ability to measure several fluorescent parameters simultaneously on each individual cell (see Fig. 4 below). Western blotting takes the average of many cells and can only provide one data point for that group of cells. The parameters being measured in the flow cytometry techniques can include surface markers, intracellular antigens, or a combination of both. With current developments in flow cytometric machinery, 13 or more dimensions or colors can be analyzed [1]. Analysis of immune cells absolutely requires both single-cell and multiparameter analysis to differentiate among the cell types present in peripheral blood or other tissues. With flow cytometry, one can analyze B cells versus T cells, diseased or cancerous cells versus healthy cells, cells in one stage of development versus those in another stage, without any prior cell sorting or depletion. This provides rich information as immediate comparisons can be drawn among cell types, and results are obtained in the same experiment, eliminating interexperiment variability.

How can this be applied to intracellular signaling events? Multidimensional analysis could allow several signaling cascades or members of one particular cascade to be analyzed simultaneously. Thus, one may be able to profile diseases based on their signaling states or by comparing their response to stimuli to normal, healthy cells. Indeed, many cancers are characterized by overexpressed or constitutively active signaling molecules [21,22]. This approach of using flow cytometry to analyze most of the possible signaling pathways pertinent to a disease state or specific response to stimulus can be termed "FACS proteomics". Cell-based drug screening may also be performed with flow cytometry, reducing the biases and false positives produced by in vitro assays, and providing simultaneous monitoring of several signaling cascades to determine drug specificity [23]. Correlation of surface markers with intracellular signaling events, or signaling events with one another, could produce insights into immune cell roles and the intricacies of signaling that are impossible to observe without monitoring events simultaneously in single cells. Entirely novel connections may be drawn between particular cell types and their signaling mechanisms, furthering understanding of how the immune system responds to challenge.

Rare subsets, small numbers of cells

Flow cytometry is also advantageous compared to traditional methods because of its ability to analyze rare subsets of cells within complex populations. In this laboratory,

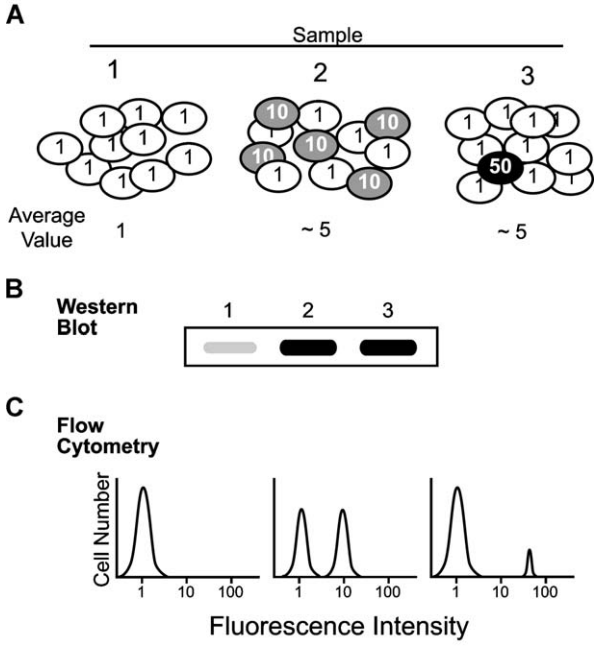

Fig. 1. The advantages of single cell analysis. (A) In this hypothetical experiment, three samples are obtained that contain a protein of interest at 1, 10, or 50 copies per cell as indicated. The average number of protein molecules per cell is 1 for sample 1, and 5 for both samples 2 and 3. (B) When these cell populations are analyzed by Western blotting, samples 2 and 3 will show darker bands but will appear identical to one another. (C) When the samples are stained for the protein with fluorescently labeled antibodies and analyzed by flow cytometry, however, one can clearly see that sample 2 contains cells in two distinct populations that are equally represented, while in sample 3, only about 1 in 10 cells has an elevated level of protein. This kind of heterogeneity in the samples could be due to different cell types (i.e., immune cells), or because of all-or-none type signaling responses.

techniques have been advanced such that one can routinely analyze cell types that comprise less than 1% of the total cell population, such as B cell development precursors. By allowing analysis of rare cell subsets in heterogeneous populations, flow cytometry provides a unique platform to monitor signaling events in environments that best simulate those in vivo, that is, in the presence of many cell types. Methods for purification of rare cell populations for conventional biochemical analysis can be expensive due to long sorting times or magnetic separation reagents, and can lead to inadvertent changes in signaling or protein status during cell purification.

Rapid protocols, simple visualization

Optimized procedures for staining phospho-specific epitopes are extremely rapid and flexible [24]. First, with suitable application of the protocols, one can freeze signaling events at nearly any time point for later staining. Second, it is possible to stain many samples (in a 96-well plate format) and rapidly measure their intracellular signaling events. For instance, one can stimulate peripheral blood lymphocytes with various cytokines, then measure phosphorylation of MAP kinases such as ERK, p38, and JNK or Stat transcription factors in several different cell types (T cells, B cells, NK cells, etc). This level of information is beyond the scope of Western analysis or ELISAs. Finally, flow cytometry data are easy to visualize and compare among samples. Histogram layouts are useful for direct comparison between two samples or cell types while two-dimensional dot and contour plots allow one to visualize small shifts in fluorescence that would not be discernible on a one-dimensional histogram.

Statistical significance

Because flow cytometry acquires data for individual cells, the technique is inherently rich in statistical properties such as population means, medians, standard deviations, and coefficients of variation. In addition, characteristics such as peak shapes (i.e., Gaussian or uniform peaks versus slanted or skewed peaks) may be indicative of small changes that are not easily discernible when comparing mean or median values. Thus, a combination of population statistics and peak characterization may help to make flow cytometric analysis of phosphorylation levels more informative than that obtained by Western blotting. In addition, as discussed below, new methods for data presentation and clustering will allow multidimensional data to be displayed in ways that are simple to interpret, allowing complex associations to be drawn between cell types and their cognate signaling cascades.

Thus, flow cytometry is a unique platform for analysis of phospho-epitopes within cells of the immune system. Multidimensional, single cell measurements will provide researchers with unique insights into signaling events of the immune system that are not easily ascertained with traditional techniques.

Technical considerations for phosphorylation state analysis

Although some of the considerations that must be taken into account when attempting to stain phospho-epitopes inside cells were discussed briefly above, we will examine and discuss them more specifically here.

Antigen accessibility

A technical question that must be addressed is whether phospho-epitopes will be accessible for antibody binding. Unlike typical protein epitopes, phospho-specific epitopes are often buried by protein–protein interactions, such as SH2 domains of receptor tyrosine kinases [8], or lie within active sites that are bound to the substrate. After fixing cells with crosslinking reagents such as formaldehyde, these epitopes may be buried within protein interfaces. Experimental evidence suggests, however, that with proper permeabilization reagents, most antigens can be measured efficiently [24]. In particular, alcohol permeabilization seems to be well suited to the preparation of cells and proteins of interest for phospho-epitope analysis.

Cellular localization

Similarly, the location of the epitope within the cell may limit its accessibility. Nuclear antigens such as Stat transcription factors and phosphorylated MAP kinases (ERK, p38, JNK) may require different fixation and permeabilization techniques than antigens in the cytosol or at the plasma membrane such as PLC-γ, Raf, or Zap-70. Thus, various protocols must be screened for different classes of phospho-epitopes and protein families. It is currently unclear whether one general protocol will suffice for the large majority of phospho-proteins. However, alcohol permeabilization techniques, discussed below, appear to work more widely than other detergent-based methods [24]. Subcellular localization of antigens is difficult with flow cytometric techniques and is better performed with microscopy or cellular fractionation and Western blotting.

Stability of phospho-epitopes

Because of the transient nature of intracellular signaling events, fixation techniques used for phospho-specific analysis must be rapid and efficient at freezing signaling to prevent dephosphorylation or further phosphorylation. Lysis buffers used for Western blotting typically include phosphatase inhibitors, but do not block kinase activity (often because in vitro kinase assays are to follow). If possible, fixation conditions for phospho-specific flow cytometry

would eliminate the need to use any inhibitors to avoid potential artifacts that could result from blocking one-half of the kinase/phosphatase balance. It is also crucial to address the stability of phospho-epitopes in staining buffers to avoid degradation during the protocol. Stability is of particular concern when applying the techniques to patient samples that have been cryo-preserved because of the effects of freezing on both surface and intracellular epitopes. It will be necessary to analyze the effects of cryo-preservation and if necessary, to modify preparative steps to improve later staining of phospho-epitopes and surface markers. Most phospho-proteins that have been analyzed in our laboratory appear to be stable in methanol for up to a month with some degradation being seen over time (one might expect different phospho-epitopes to vary in their stability).

Antibody selection

As mentioned for cytokine staining, it is clear to us that phospho-specific flow cytometry requires careful selection and screening of antibodies to find those that stain the antigen of interest most efficiently and specifically. Many antibodies that work extremely well for Western blotting do not perform as well when put in the context of fixed and permeabilized cells during flow cytometric analysis. In addition, different antibody clones against the same peptide immunogen can show disparate staining levels when used for flow cytometry (unpublished data). Therefore, one must use caution when attempting to stain phospho-epitopes for flow cytometric analysis because negative results may not represent a lack of phosphorylation but rather a lack of epitope availability—and therefore a lack of antibody binding. Positive controls with well-established stimulation conditions, such as, PMA stimulation of ERK, must be used to assess antibody binding affinity and specificity. There may also be cases where phospho-epitope analysis of a particular protein will simply not be possible because of intracellular localization, buried epitopes, or low affinity antibodies.

Fluorophore selection

To take advantage of the multidimensional aspect of flow cytometry by measuring multiple signaling events simultaneously, one must conjugate the phospho-specific mAbs to fluorophores to create primarily labeled mAbs. Two step procedures employing a fluorescently labeled secondary antibody cannot be used for more than one epitope at a time because the mAbs are nearly always mouse IgG_1. When choosing a fluorophore for conjugation, two characteristics must be met: first, the fluorophore's absorbance spectrum must match the laser line used in the cytometer and its emission must fall within detection filter sets, and second, the fluorophore cannot interfere with antibody binding characteristics or permeability through the fixed cell structure. Thus, large protein fluorophores like PE or APC may slow mAb entry into cells and affect its binding characteristics. Though PE conjugates have worked in some cases, we have found that small molecule fluorophores such as FITC, Alexa 488, and Alexa 647 provide the best staining characteristics as long as fluorophore-to-protein ratios are carefully controlled. Commercialization of these reagents will eliminate independent user variability. Extensive discussion of fluorophore uses and applications in flow cytometry can be found elsewhere [25–27].

Maintenance of surface and light scatter properties

Perhaps one of the most difficult technical aspects of staining phospho-epitopes is to maintain surface staining and scatter properties. The major advantage of flow cytometry is its ability to differentiate cell types in immunological samples based on their surface staining properties, that is, CD3 for T cells, CD19 for B cells, and so on. Therefore, while protocols are being refined during an experiment for phospho-specific staining, one must constantly monitor the effects of the staining regimen on maintaining surface antigen recognition. Some protocols that provide excellent staining of phospho-epitopes decrease staining levels of particular surface antigens, while preservation of surface epitopes leads to weak phospho-staining. This balance between surface and intracellular epitopes must be kept in mind while attempting to stain particular antigens. As an example, new tetramer-staining techniques that are indispensable to antigen-specific T cell analysis have been reported to be sensitive to certain types of fixation and cell handling techniques [28–30]. Thus, before application of such tools to phospho-epitope analysis, methods will need to be developed that maintain TCR–MHC interaction. However, we are finding that with proper sequential staining steps more than 90% of surface antigens can be stained with optimal measurement of intracellular epitopes.

Empirical properties of forward and side scatter light are used to distinguish cell types in the absence of any surface staining (lymphocytes are distinctly smaller than granulocytes and monocytes for example). But scatter properties of cell populations can change upon cell fixation and permeabilization [24]. With proper "before and after" fixation or permeabilization staining, these changes can be noted and gating adjusted to accommodate slight variations in outcome. However, this will require revisions to standard scatter-based gating. Our laboratory relies more upon surface staining than cell size, as the procedures employed for phospho-epitope staining alter certain cell types more than others.

Methods for intracellular phospho-epitope detection

The general technique for staining phospho-epitopes for flow cytometry is outlined in Fig. 2. Briefly, a biological cell sample is taken, or a stimulus such as a cytokine or small

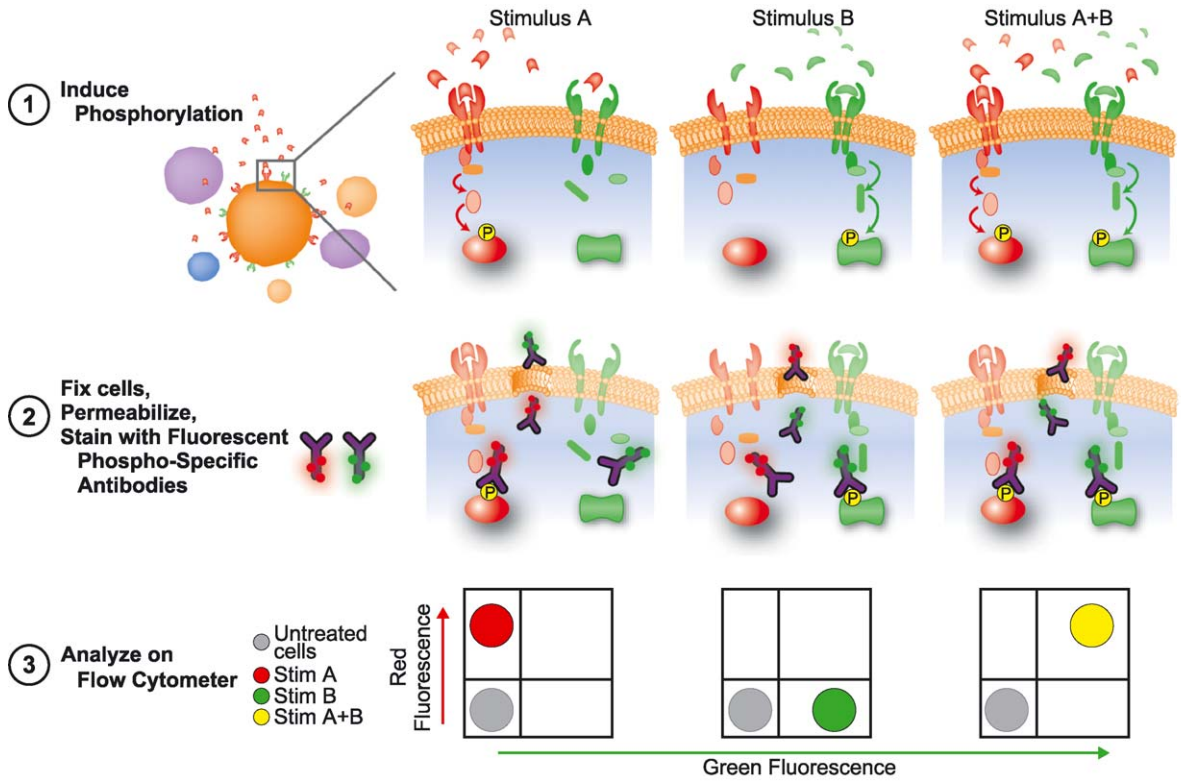

Fig. 2. General phospho-protein staining technique for flow cytometry. (Step 1) A heterogeneous sample of cells is treated with two different stimuli, A and B (i.e., cytokines, growth factors, drugs, inhibitors), to induce distinct signaling cascades and phosphorylation of two target proteins. A third sample is treated with both stimuli simultaneously to induce phosphorylation of both proteins of interest. (Step 2) The cells are then fixed, permeabilized, and stained with fluorophore-conjugated phospho-specific antibodies to the phosphorylated (and typically active) forms of the two proteins (surface markers can also be stained during this step with appropriate antibodies and fluorophore combinations). (Step 3) Finally, the cells are analyzed on a flow cytometer with two or more fluorescence channels. Because the antibodies bind only to the phosphorylated form of the proteins, an increase in fluorescence correlates with an increase in phosphorylation. Therefore, stimulus A produces an increase in red fluorescence because the red protein is phosphorylated. The combination of stimuli A and B induces phosphorylation of both proteins making the cells both green and red fluorescent. This technique can also be applied to patient samples to help characterize aberrant signaling events that occur during disease progression or determine the efficacy of signaling pathway-specific drugs in vivo. In this case, samples must be isolated from patients and immediately subjected to fixation and permeabilization conditions that will maintain phospho-epitope integrity.

molecule is applied to cells that are then fixed with a crosslinking reagent (typically formaldehyde) and permeabilized with detergents (Triton X-100, saponin) or alcohol (ethanol, methanol). Cells are then stained with phospho-specific antibodies that have been conjugated to different fluorophores and the cells are analyzed by flow cytometry. The technique shown illustrates application of an external stimulus to cells; however, one can modify the protocol to preserve and analyze phospho-epitope levels in samples taken directly from patients to determine disease-specific characteristics. Although the protocols that have been used to stain phospho-epitopes for flow cytometry differ from one another, they have relied on two primary permeabilization reagents, saponin or methanol.

Saponin permeabilization

Saponin is a mixture of terpenoid molecules and glycosides typically derived from the bark of the Quillaja tree that permeabilizes cells by interacting with cholesterol present in the cell membrane [31]. This creates pores in the plasma membrane that are large enough for the entry of fluorophore-conjugated antibodies. Because intracellular proteins can leak out of saponin-treated cells, they must first be exposed to a crosslinking reagent such as formaldehyde to fix proteins and nucleic acids into a cohesive unit within the cell. Saponin has become the detergent of choice for cytokine staining, and several groups have utilized it for permeabilization in phospho-epitope staining protocols [4,32–34]. It is typically used at concentrations from 0.1% to 0.5%, similar to cytokine-staining procedures. Because saponin is derived from a complex mixture of molecules, different lots vary considerably according to the manufacturer and natural source. Therefore, saponin lots should be titrated for optimal efficacy.

Methanol permeabilization

Alcohol permeabilization has typically been used for the analysis of DNA by flow cytometry [35], but recently has been applied to phospho-epitope staining as well [3,23,24,36–39]. It is thought that alcohols fix and permea-

bilize cells by dehydrating them and solubilizing molecules out of the plasma membrane. Proteins may be made more accessible to antibodies during the process and cells are permeabilized to a greater extent than with saponin, allowing efficient access to nuclear antigens. Both of these traits may be advantageous for phospho-epitope analysis because of the development of antibodies against short phosphopeptides and the large number of phospho-protein targets in the nucleus. We are finding that a combination of formaldehyde and methanol is advantageous in almost all cases and provides a broadly applicable method (Fig. 3, [24]). In particular, this combination provides superior results for nuclear antigens such as Stat transcription factors. However, some phospho-epitopes may still benefit from saponin permeabilization or modifications to the methanol protocol, differences that will be clarified by further development of the technique.

Current limitations

Though flow cytometry possesses all of the advantages that were discussed above, it also has some limitations. First, flow cytometry does not readily produce data concerning localization of antigens within cells, an attribute generally unique to microscopic techniques or cellular fractionation and Western blotting (though methods are being developed to determine differential fluorescence across cells by some modifications of flow techniques that can be correlated to subcellular localization). Second, the signal-to-noise ratio when detecting low-abundance signaling proteins may be too small in some flow cytometry experiments. Western blotting, because it utilizes enzymatic amplification, is capable of measuring small amounts of protein with sufficient signal-to-noise ratios because one does not have concerns about antigen accessibility and

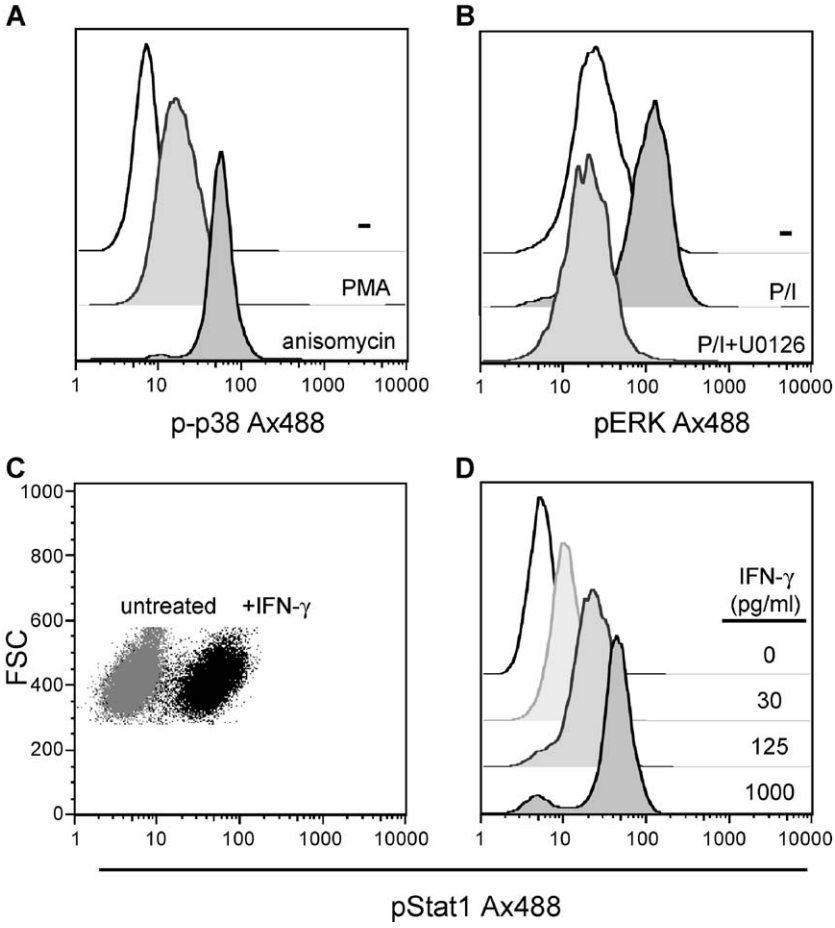

Fig. 3. Diverse applications of phospho-specific flow cytometry. (A) Multiple stimuli: Jurkat T cells were treated with PMA or anisomycin and analyzed for phospho-p38 levels after formaldehyde fixation and methanol permeabilization (Ax = Alexa Fluor). The different levels of p-p38 produced by the two stimuli are indicative of their ability to induce p38 activity. Open histograms represent unstimulated cells while filled plots indicate treated samples. (B) Inhibitor studies: Jurkat cells were treated with PMA and ionomycin (P/I), or with the MEK inhibitor U0126 before the addition of P/I. The inhibitor completely blocked phosphorylation of ERK, as one would expect from inhibition of the upstream kinase. (C) Dot plot layout: U937 cells were left untreated (gray) or stimulated with IFN-γ (black) and analyzed for phospho-Stat1 levels. This method of visualization allows two markers to be compared simultaneously and correlations to be drawn between the two, unlike histograms which are limited to one dimensional analysis (see Fig. 4 for more examples). (D) Titration studies: U937 cells were treated with increasing amounts of IFN-γ and measured for phosphorylation of Stat1. It is clear that titration data can be analyzed reliably by flow cytometry. Coupling titration experiments with inhibitor studies provides a novel cell-based platform for drug screening.

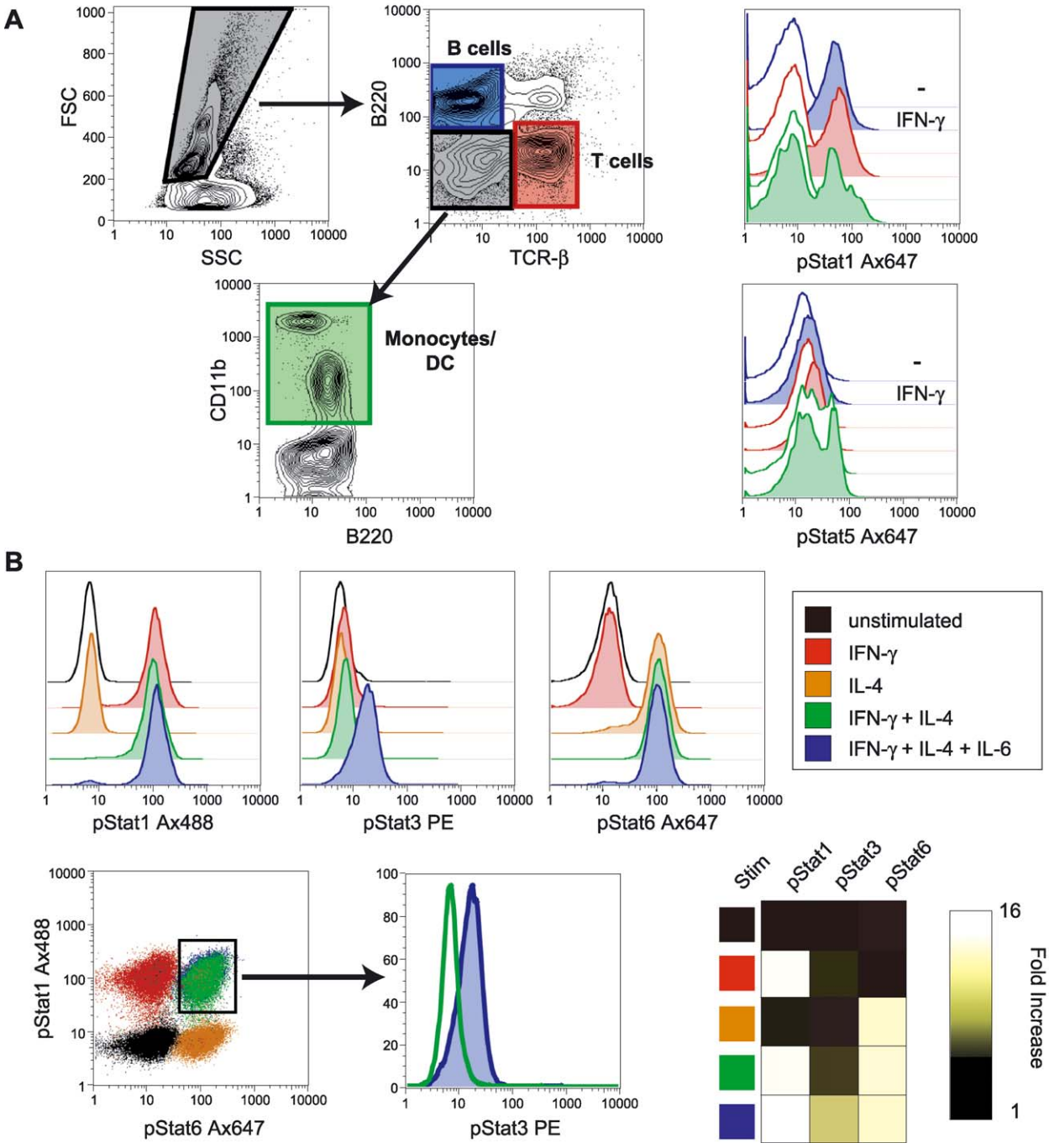

Fig. 4. Multidimensional analyses with phospho-specific flow cytometry. (A) Surface markers with phospho-epitope staining. Murine splenocytes were subjected to IFN-γ stimulation (filled histograms) or left unstimulated (open histograms), then stained with B220, TCR-β, and CD11b to distinguish B cells (blue), T cells (red), and monocytes or dendritic cells (green), respectively. The cell types were simultaneously analyzed for induction of Stat1 and Stat5 phosphorylation with phospho-specific antibodies. B cells and T cells showed clear Stat1 responses to IFN-γ, but the CD11b-positive population was heterogeneous in its response. Only minor inductions of phospho-Stat5 are seen. (B) Multiple kinases: U937 cells were treated with IFN-γ, IL-4, and IL-6 in the combinations shown. The cells were then analyzed for pStat1, pStat3, and pStat6 simultaneously after fixation and permeabilization. The top panel shows histograms of each channel individually and clearly shows the expected induction of Stat1 with IFN-γ, Stat3 with IL-6, and Stat6 with IL-4. When plotted in two dimensions (lower left panel), two samples appear coincidentally in the pStat1/pStat6 positive quadrant. However, when one analyzes these samples for pStat3, only the sample treated with IL-6 shows an induction. Therefore, samples that appear homogeneous within two dimensions can be separated clearly with simultaneous staining in three dimensions. Such correlations are not possible with Western blotting. The lower right panel is a representation of the data generated by a FACS analysis tool being developed in our laboratory. Each row represents a different stimulus, and each column represents a phospho-protein. The color of each block is indicative of the fold change in median fluorescence intensity in that channel. The data are easily visualized and compared without needing to plot all 15 samples. Larger screening experiments will require this form of analysis.

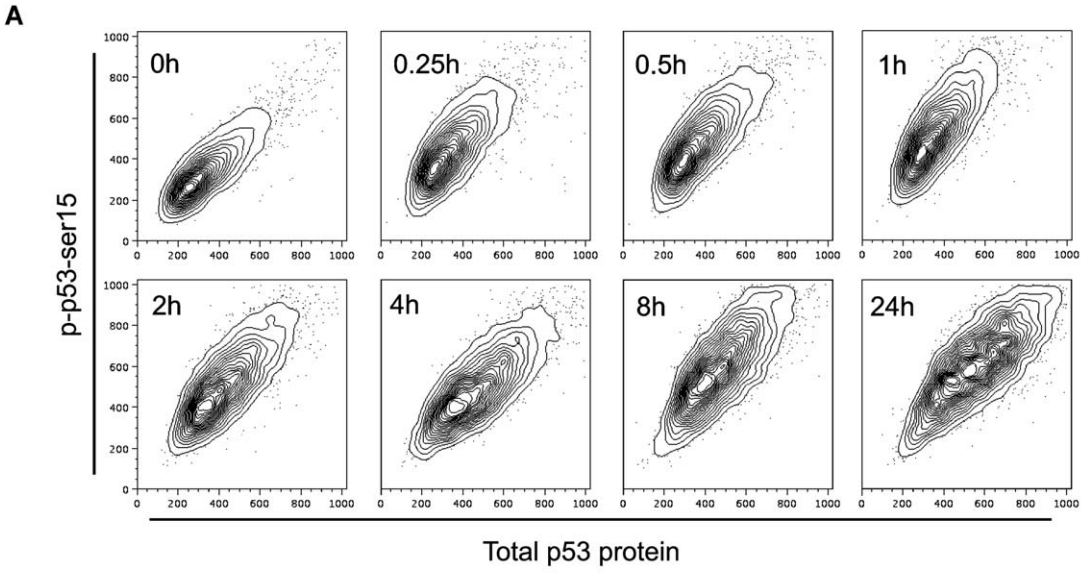

Fig. 5. Analysis of p53 phosphorylation and total protein levels shows p53 phosphorylation at serine15 precedes accumulation of total p53 protein. (A) GM0536 lymphoblastoid cells were treated with 8 Gy of gamma irradiation and p53 phosphorylation at serine15 and total p53 protein levels were monitored over time following irradiation and compared on a per cell basis. (B) Quantitation of the change in median fluorescence (log_2 converted) of the population over time showed rapid induction of phosphorylation in the first hour followed by a more gradual accumulation of total p53 protein. Similar analyses can be performed on cancerous cells to determine p53 status and activation.

autofluorescence. Interestingly, there are approaches to amplify fluorescent signals using enzymatic deposition of reactive fluorophore species known as tyramide signal amplification. Such methods may help obtain signals from low-abundance epitopes or enhance small shifts in fluorescence seen upon stimulation of phosphorylation. Current work in our laboratory is focused on understanding the quantitative aspects of cytometry and its correlation with Western blotting and ELISAs. The development of rigorous statistical methods applicable to flow cytometry is also necessary to effectively obtain quantitative results. Thus, small changes in fluorescence, that is, a two-fold increase in phospho-epitope staining, still need verification as to their relevance, but we have observed several cases where small shifts are significant and highly reproducible. We expect that normalization controls, wherein one antibody targets a non-phospho epitope and a second targets the phospho epitope, will allow for better measures of real changes in phosphorylation versus changes in absolute protein levels.

Data analysis

One of the difficulties that will be encountered in phospho-epitope flow cytometry is the analysis of the large amount of data that will be generated. Experiments can be extremely rich in information, providing details on surface staining and intracellular signaling simultaneously. To take advantage of these data fully, we have been developing new software platforms that allow rapid analysis of large datasets so that ‘‘positive hits’’ can easily be identified among hundreds of samples. Visualization of the data will be enhanced by advanced median analysis and data conversion to simple graphical representations (see examples in Figs. 4 and 5). In addition, data can easily be exported to more rigorous statistical programs or to clustering software already available for genomics and other applications. We feel that development of bioinformatic resources must match the rapid improvements in flow cytometry to analyze large datasets properly and bring confidence to small, repeatable fold changes.

Clinical applications of phospho-specific flow cytometry

With the ability to monitor signaling events that occur in unique subsets of cells, phospho-specific flow cytometry is particularly well suited for the analysis of immune system development and response, as well as drug screening and pharmacodynamic monitoring of patients in clinical trials. By understanding molecular events that occur in disease

samples or in response to various drugs, researchers and clinicians will be able to tailor therapies for particular patients based on their responsiveness. Such ''patient-specific'' treatment may become possible with this technique because it allows analysis to easily be carried out within a day of sample collection and provides a wide range of information including cell type representation and functional signaling levels. We have summarized some of the possible applications of phospho-specific flow cytometry to immune system characterization and pharmacodynamic monitoring in Table 3, and will discuss them in detail below.

Characterization of immune systems

Immune systems have typically remained outside the scope of biochemical analysis because of cell type heterogeneity. Phospho-specific flow cytometry opens this field to interrogation by discriminating cell types of interest via their surface markers (Fig. 4). The development of the immune system and the generation of immune responses against particular cytokines or antigens can thus be analyzed at the level of intracellular signaling events, providing new insight into extremely complex processes.

Table 3
Clinical applications of phospho-specific flow cytometry

Immune system characterization
Immune cell development: monitoring phospho-signature of developing T, B, or other lineage specific cells to correlate intracellular activities with stages of cellular differentiation.
Disease state profiling: combining tetramer staining with intracellular signal assessment to study antigen-specific T cells in viral or bacterial infections. This offers the potential to monitor lymphocyte subsets for responses under acute and chronic infections.
Monitoring lymphocyte populations in disease murine models or patients, such as blood borne leukemias, or autoimmune diseases, such as rheumatoid arthritis, to correlate phospho-signatures with disease manifestation.
Biochemical signatures of rare cell populations (dendritic cells, naive and memory effector cells, stem cells) that cannot be analyzed by conventional biochemical techniques.
Multidimensional assessment of cell signaling networks to understand cell function. Identification of signaling thresholds and connections among disparate signaling cascades.
Monitoring virally infected cells for altered function and intracellular signaling.
Characterizing immune cell response patterns to cytokines and extracellular stimuli.
Pharmacodynamic monitoring and drug screening
Intracellular kinase screens for rapid identification of specific inhibitors or modulators of target kinases.
Drug screening in primary cells to determine subset-specific efficacy and side effects.
Target validation of compound specificity by analyzing multiple intracellular pathways simultaneously.
Clinical trials: monitoring particular compounds for their effects during drug treatment on cellular populations of interest.
Identification of phospho-epitopes on kinases as diagnostic indicators of disease progression by correlating intracellular biochemical differences with additional clinical parameters.
Phospho-epitope analysis during vaccination protocols to monitor efficacy at the cellular level.

Immune system development

To determine the effects of a particular cytokine, IFN-γ, on neonatal macrophages, a recent study used antibodies against the dimerizing phospho-epitope of Stat1 [3]. Previous work had shown that neonatal cells possessed similar levels of the IFN-γ receptor to adult cells, but that their antimicrobial responses differed [40]. By examining Stat1 responses to IFN-γ, the authors showed that neonatal cells respond less strongly than adult cells, providing a reasonable hypothesis as to why neonatal cells responded poorly. It is interesting to note that the authors used a commercial fixation or permeabilization kit but followed with methanol treatment. The study exemplifies the novel approach that flow cytometry provides by helping to elucidate differences that were unexplainable by phenotypic analysis or overall cellular response to a stimulus.

Cytokines and growth factors dictate both the development and differentiation of immune cells. Because nearly all cytokines signal through JAK-Stat pathways, analysis of Stat phosphorylation is critical to understanding how cytokines exert their effects and how they modulate gene transcription. Therefore, our laboratory has expanded the number of phosphorylated Stat epitopes available to flow cytometric analysis to Stats 1, 5, and 6 [24], as well as Stat3 (Fig. 4, in preparation). In parallel, we are working to maintain surface staining to discriminate immune cell subsets that respond to particular cytokines. In this way, we are finding subset-specific responses to cytokines that are unexpected and are not accessible to traditional biochemical methods. We soon hope to profile cytokine responses globally both in vitro and in vivo using flow cytometric methods.

Such applications of phospho-specific flow cytometry may elucidate signaling pathways that are important for the development of the immune system as well as other organs in the body. By using mouse models of various diseases, one can monitor signaling events that occur during embryonic development to analyze differences from healthy mice. Aberrant signaling pathways can thus be identified and targeted for pharmaceutical intervention, perhaps reducing the effects of developmental abnormalities.

Antigen-specific signaling

The possibility of observing signaling events as they occur in vivo in response to natural ligands is extremely powerful and will increase understanding of immunity at the molecular level. In one case, transgenic T cells with T-cell receptors specific to chicken ovalbumin that had been transferred into syngeneic mice were challenged with the ovalbumin peptide and monitored for c-Jun and p38 phosphorylation [32,41]. The authors performed experiments both in vitro and in vivo and noted differences between the two, with in vivo injection of the peptide producing a

larger shift in phosphorylation staining for c-Jun and p38. In vitro experiments in cell lines and isolated lymphocytes did not generate large shifts when phospho-epitopes were visualized by flow cytometry, but did show the expected results. Because the authors were able to remove tissues from mice after stimulation and still maintain phosphorylation levels, one can imagine performing similar experiments removing tumors or drawing blood samples for phenotypic characterization of clinically relevant samples. Indeed, the power of flow cytometry lies in the ability to monitor small subsets in complex populations (as was the case with transgenic T cells that had been transferred into syngeneic mice at less than 1% of the total population), overcoming a limitation that has been hampering analysis of signaling by traditional techniques.

Application of phospho-epitope analysis to elucidate signaling pathways that are activated in antigen-specific T cells under both acute and chronic phases of infections, as well as during drug treatment, may provide some insight into the biochemical differences between effector and memory T cells during infection. For instance, it is now possible to identify antigen-specific T cells by flow cytometry through the development of soluble peptide–MHC complexes known as tetramers [42,43]. Tetramer technology for MHC class I has advanced the functional assessment of CD8+ cytotoxic T cells significantly [44,45]. New tetramer tools, such as MHC class II–peptide complexes and CD1d-galactoceramide complexes, make it possible to enumerate MHC class II restricted T cell and natural killer T cell responses, respectively [46]. Using these tools, studies of immune responses to viral infection indicate that there are host–pathogen-specific differences in antigen responses in models that include murine lymphocytic choriomeningitis [47], influenza virus [48], murine herpesvirus [49], listeria monocytogenes [50], and vesicular stomatitis virus[51]. Tetramer technologies have also been useful in studying human viral infections and antigen-specific responses in HIV [52,53], Hepatitis B and C [54], Epstein–Barr virus [55], and cytomegalovirus [56] infections. Using tetramer staining in conjunction with other surface and cytokine reagents by flow cytometry to study antigen-specific T cells from human and murine viral models has increased understanding of antigen-specific T cell dynamics, surface immunophenotype, distribution, and function. Application of phospho-epitope analysis to elucidate signaling pathways that are activated in antigen-specific T cells under both acute and chronic phases of infections, as well as during drug treatment, may provide some insight into the biochemical differences between effector and memory T cells during a viral infection or during vaccination. Correlating such intracellular biochemical differences with additional clinical parameters may provide diagnostic indicators of disease progression.

Finally, early work from our group coupled complex surface staining with intracellular phospho-epitope analysis to monitor MAP kinase (ERK, JNK, p38) and Akt response in naive T cells after T cell receptor engagement with CD3/CD28 crosslinking and IL-2 stimulation [33]. Phospho-specific antibodies were verified by Western blotting and compared to flow methods, showing a strong correlation between the two techniques. In addition, inhibitors were used to block the pathways in question to validate phospho-specificity. The measurement of multiple kinases along with several surface markers provided rich insight into T cell receptor signaling and differences among naive T cell subsets. This work was the first to expand beyond four colors and shows that extremely rare cell types may be identified with surface characterization and subsequently analyzed for multiple signaling pathways in response to physiological stimuli. Such complexity will be required for researchers interested in stem cell development or bone marrow disease because several surface markers are required for proper identification of these cell types.

Pharmacodynamic monitoring and drug screening

With recent advances in drug development against particular molecular targets, it will become increasingly important to determine the specificity of these drugs in patient samples to test whether the effects seen in vitro also occur in vivo and if the drug's molecular efficacy matches expectations. Analysis of signaling in patient samples or murine models will provide new information regarding the pharmacodynamic profile of drugs that does not rely upon long-term analysis of disease progression, but rather immediate determination of drug efficacy against a particular target. In addition, disease progression can be analyzed by using phospho-specific markers as indicators of disease status. Phenotyping of tumor samples via phospho-epitopes may develop into a novel field of flow cytometry proteomics (FACS proteomics) by analyzing active cellular processes and finding novel targets of therapy.

Inhibitor screening and efficacy

Drug screening is typically performed in vitro in cell-free systems that are amenable to high-throughput robotics and analysis platforms. However, recently cell-based screens have gained interest because they eliminate positive hits that are not cell-permeable or do not reach their target appropriately within cells [57,58]. Phospho-epitope analysis in cells treated with compound libraries can simultaneously determine the efficacy of a compound against a particular target and its specificity for that target relative to other signaling molecules (see Fig. 3 for example). For example, in the generation of tyrosine kinase inhibitors, it would be ideal to inhibit pathways that are overly active in oncogenic settings but not those normally occurring in healthy cells. One can readily perform such a screen with phospho-epitope flow cytometry on cell culture models or in vivo tumor models. This analysis can be continued throughout drug development and into clinical trials to assess specific activity of the drug and determine the cell types that are

affected. Refinement of the drug based on in vivo phospho-epitope levels will be invaluable in developing designer drugs that are effective and nontoxic.

Cell-based assays and analysis of clinical samples can help to clarify differences seen between biochemical screens and drug effects in vivo. An example is the compound Gleevec that not only blocks the activity of BCR-ABL, the constitutively active kinase that precipitates chronic myelogenous leukemia (CML), but also two other closely related tyrosine kinases, PDGF-R and the receptor for stem cell factor, c-kit [59,60]. Knocking out the genes for PDGF-R and c-Kit in mice results in embryonic death; however in CML patients, Gleevec normalized the uncontrolled proliferation of blood cells [61]. The lack of major toxicity exhibited by Gleevec, as might be expected from a compound that blocks such critical receptors as PDGF-R and c-kit, suggests substantial differences between drug actions in vivo and in vitro. Analysis of signaling cascades from patient samples could be achieved via phospho-specific flow cytometry and would eliminate the need to obtain pure tumor cell samples because of the ability to discriminate healthy from diseased cells.

Such studies would benefit from direct analysis of whole blood samples without requiring purification of peripheral blood mononuclear cells (PBMC) or sorted fractions of cells, both of which can modify signaling cascades through cell handling. In addition, purification removes immune cells from their natural environment that includes red blood cells and serum proteins. Recent work showed that with a formaldehyde–methanol combination, signaling events could be measured after direct stimulation of whole blood [23]. Not only is this a more physiologically relevant stimulation, it also provides a rapid method of assessment with results being obtained a few hours after blood withdrawal. The authors showed the utility of phospho-epitope staining by monitoring the effects of MAP kinase inhibitors on the phosphorylation of MEK and ERK. By inhibiting Raf activity, which is upstream of both MEK and ERK, no phosphorylation was induced by PMA. However, when MEK was inhibited, ERK phosphorylation alone was blocked. Such exquisite step-wise monitoring of MAP kinase cascades, or any other cascade, will improve development of kinase inhibitors specific to particular pathways or particular kinases that are aberrant in disease states. Pharmacodynamic monitoring in whole blood samples has not been possible previously and will undoubtedly become an important part of clinical trials on kinase inhibitors.

Pharmacodynamic profiling

Another application of phospho-specific flow cytometry is in profiling disease states via their signaling status and response to particular compounds. Correlation of phospho-epitope signatures to the progression of a disease may aid in developing therapies tailored to patients that are in the early or late stages of a disease. For example, various tyrosine kinase receptors including Flt-3, PDGF-R, EGF-R, and HER2 have been correlated with disease severity and prognosis in leukemias and breast cancer and are targets of drug therapy [21,62]. It is also known that several intracellular molecules are indicators of actively dividing cancers such as p53 and cyclin D1 [63,64]. In conjunction with clinical collaborators, our group is using phospho-epitope flow cytometry to understand acute myelogenous leukemia (AML) by measuring levels of five of the relevant phospho-epitopes of p53 (Fig. 5 illustrates p53 staining). Such work is providing invaluable insights that can be correlated to the efficacy of treatment in particular patients. Although several activation markers do exist, including those mentioned above, what often remains unknown is the role that these molecules play in disease progression. Methods currently used, such as cDNA microarrays and protein arrays, provide information about abundance of molecules but not their activity. Proteins that are present in low concentrations may play large roles in disease progression if they are constitutively active, a trait that can only be characterized by phospho-specific analysis. It is thus critical to correlate levels of proteins with their activity in particular disease states.

One study of interest examined levels of phosphorylated Akt in cells that had been selected for their resistance to camptothecin and etoposide, two agents that typically induce apoptosis (the latter being used as a chemotherapy clinically) [34]. The authors showed that total Akt levels were similar in the resistant and parental cell line, but that more phosphorylated (and active) Akt was present in the resistant line. These results were obtained by Western blot and flow cytometric analysis with two different antibodies. Treatment with specific PI3K inhibitors decreased the levels of phospho-Akt, adding an extra control for phospho-specificity and pointed to possible therapeutic intervention. Because Akt plays a large role in mediating cell growth and survival, it is an important marker for proliferating neoplasms and may help to classify patient samples into more or less severe stages of disease. In addition, Akt is an attractive target for drug development because inhibitors may block uncontrolled proliferation [65,66]. This example serves to show the necessity of measuring total protein levels as well as their relative activity to avoid misinterpretation of a molecule's role in disease progression.

We envision that phospho-epitope analysis by flow cytometry will be applicable to tumor masses, biopsies, and tissue-derived samples after suitable technical advances in preparation of solid masses for flow. This will give clinical researchers an entirely novel tool in the fight against cancer and immune system diseases by allowing them to first profile aberrant signaling, and then to analyze the efficacy and specificity of therapies both before and during clinical trials. As more phospho-epitope specific antibodies are developed and validated for flow cytometry, patient samples can be screened to find possible leads for drug development and further research into the causes of particular diseases.

Conclusion

Phospho-protein analysis by flow cytometric techniques is a unique method for analyzing signaling in cell types that are too rare or few in number for typical biochemical analyses. Because of its multidimensional nature, several kinases can be profiled simultaneously to produce disease or stimulus-specific phenotypic characterizations. Incorporation of surface markers into staining protocols allows different cell types to be analyzed concurrently in the same sample, reducing experimental time and interexperiment variability. Researchers must be aware of the many considerations relevant to phospho-epitope staining outlined above, including the availability of antibodies to specific epitopes, the rapid and reversible nature of signaling events, and the suitability of antibodies for the flow cytometry platform. However, novel and exciting applications of the technique to in vivo and whole blood models as well as complex subpopulations of peripheral blood make it an attractive new field of study. We expect that development of phospho-epitope staining and its use in FACS proteomics will dramatically improve analysis of signaling in the immune system and provide insight into the functional roles of immune cells that do not rely upon long-term differentiation events. It is also clear that the technique will become invaluable to pharmacodynamic monitoring of drugs in both screening and clinical trial settings. Intracellular analysis of proteins and phospho-epitopes may therefore expand the field of proteomics to complex populations of cells typically outside the scope of traditional biochemical analysis.

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