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Whole Blood Processing for Measurement of Signaling Proteins by Flow Cytometry

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ABSTRACT

Signal transduction pathways link external stimuli with cellular responses, which normally regulate cell proliferation, death, and differentiation. The study of signal transduction was revolutionized through the development of phospho-specific antibodies that recognize proteins only when they are phosphorylated at specific sites. As discussed by Nolan and co-workers (*UNIT 6.20*), one of the unique features of flow cytometry is its ability to perform correlated measurements of multiple phosphorylation states at the single cell level. This provides insight into the complexity of signaling networks that is not obtained by standard biochemical techniques. Furthermore, in combination with other phenotypic markers, flow cytometry can measure alterations in signaling pathways in subpopulations of cells. This clearly has wide potential for studying disorders of the hematopoietic and immune systems. *Curr. Protoc. Cytom.* 46:9.27.1-9.27.19. © 2008 by John Wiley & Sons, Inc.

Keywords: whole blood fixation/permeabilization • signal transduction • phospho-specific antibody • pharmacodynamics • human leukemias

INTRODUCTION

The emphasis in this unit is on whole blood processing for clinical applications. The techniques were developed by the authors independently of the work done by Nolan and co-workers (see *UNIT 6.20*), with the main objective being pharmacodynamic monitoring of molecular-targeted anticancer agents. Pharmacodynamics deals with the effects of drugs in the patient. In the context of molecular cancer therapeutics, the main pharmacodynamic issues are establishing whether the drug dose schedule is achieving optimum effect on its target, the elucidation of drug resistance mechanisms, and the study of drug interactions. These questions are critical to the long-term goal of individualized treatment programs.

Pharmacodynamic monitoring requires whole-blood processing to maintain the drug/target equilibrium, as well as the preservation of phenotypic markers, including light scatter and surface immunofluorescence used for the identification of relevant subpopulations of cells. These technical challenges are solved using the techniques described in this unit. In addition to allowing pharmacodynamic monitoring during early-phase clinical trials involving molecular-targeted agents, the use of whole blood processing allows the identification of constitutively activated signaling pathways in acute leukemia patients, and this method can thus address the question of whether resistance to standard chemotherapy agents is associated with aberrant activation of signaling pathways. Although currently considered research applications, the methods can be readily developed further for routine clinical applications.

In this unit, we have used examples drawn from our own work examining interactions between ERK and PI3-kinase/Akt pathways in activating the S6 ribosomal protein in acute myeloid leukemia patients (see Fig. 9.27.1). The application is based on the use of

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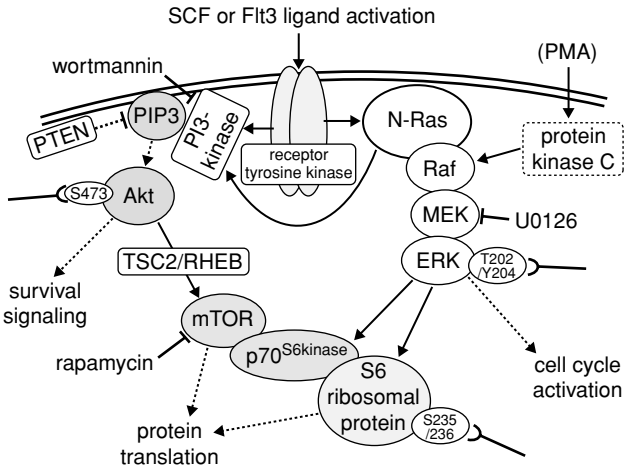

Figure 9.27.1 Schematic representation of important signaling pathways in adult acute myeloid leukemia (AML) that are described in this unit. AML cells can respond to stimulation of receptor tyrosine kinases by stem cell factor (SCF) or Flt3 ligand, resulting in the activation of the ERK pathway and/or the PI3 kinase pathway. The diagram depicts specific phosphorylation sites detected by antibodies (S473, T202/Y204, S235/236) that specifically detect phospho-epitopes on ERK, Akt, and the S6 ribosomal protein used in these studies, as well as inhibitors used to block phosphorylation of ERK (UO126), or Akt (wortmannin or LY 294002).

multiple tubes of whole blood that are either acutely activated by growth factors—Flt3 ligand or the c-Kit ligand stem cell factor (SCF)—or pre-incubated with pharmacological inhibitors of PI3-kinase (LY294002 or wortmannin), mTOR (rapamycin), or MEK (U0126). At specific time points, the samples are fixed using formaldehyde, subjected to red cell lysis using Triton X-100, and stained with combinations of antibodies to specific phosphorylation sites on ERK, Akt, and the S6 ribosomal protein. The protocol gives excellent preservation of light scatter and surface phenotypic markers (see Chow et al., 2005), thus allowing analysis of subpopulations including rare (<1%) subsets of cells. From this relatively straightforward application, we develop a platform that identifies constitutively activated signaling via PI3-kinase/Akt and ERK pathways, as well as monitors the pharmacodynamic effects of a wide range of clinically important agents, including selective inhibitors of the Flt3 and c-Kit receptors, PI3-kinase, and the ERK pathway.

The sample preparation protocols described in this section are robust and broadly applicable to the study of signaling pathways in whole blood samples; however, it should be emphasized that the analysis of signaling pathways by flow cytometry is a new and rapidly evolving application, and the examples described here should be taken as illustrative rather than as a comprehensive catalogue of laboratory techniques. Furthermore, it should be noted that the list of available antibodies is rapidly growing and that some of the newer products give much better signal-to-noise ratios compared to the earlier products used in flow cytometry applications. In addition to Cell Signaling Technology/Beckman Coulter and BD Pharmingen, there are several companies that now provide high-quality monoclonal antibodies that can be used for flow cytometry applications, and it is strongly recommended that up-to-date listings of products be reviewed prior to selecting antibodies for specific applications.

CAUTION: Follow all appropriate guidelines and regulations for the use and handling of human-derived materials.

STRATEGIC PLANNING

Selecting a CD Marker

These protocols are primarily for whole blood or bone marrow samples. It is important to initially identify a cell surface marker (e.g., CD markers) that can be used to study the cell population of interest. For example, in acute myeloid leukemia (AML) the CD marker of choice is CD34 or, when absent, CD45 can be used. For the studies of drug responsiveness in the treatment of solid tumors, surrogate markers can be used to measure drug responsiveness in peripheral blood cells (e.g., CD3 positive cells; see Chow et al., 2001). If preliminary clinical flow data is not available, it will be necessary to perform preliminary immunophenotyping studies to identify useful markers.

It should be noted that the percentage of cells positive for the selected marker or markers (more than one CD marker can be used) impacts the volume of blood necessary to obtain a significant number of events for subsequent analysis of phosphoprotein expression patterns. For instance, in order to analyze 1000 cells of interest (e.g., blast cells), 10,000 cells should be accumulated for a sample containing 10% target cells, while for a sample containing only 0.1% target cells, a minimum of 1×10^6 cells should be accumulated.

Measuring steady state levels of phosphoproteins is usually not sufficiently informative, and signaling pathways in patients may be aberrant. Agonists and inhibitors are used to map out signaling pathways and to identify predominant pathways activated (or suppressed) in individual patients. In addition, this approach is used to determine potential therapeutic targets or to monitor the effects of specific drug treatments. After determining the CD marker that best identifies the population of interest (e.g., blast cells), additional tubes are used to measure phosphoprotein expression patterns in this population.

Strategy for Analyzing the MAPK and PI3K/Akt Pathways in AML

The overall strategy for our approach in studying the MAPK and PI3K/Akt pathways in AML patient samples is depicted in Figure 9.27.1. Both the MAPK and PI3K pathways may be stimulated via the SCF or Flt3 receptor. We measure MAPK activation by monitoring phosphorylation of threonine 202 and tyrosine 204 on ERK 1/2, and PI3K activation by monitoring phosphorylation of serine 473 on Akt using appropriate monoclonal antibodies. By treating individual tubes with phorbol myristate acetate (PMA) and/or the MEK inhibitor U0126, it is possible to determine if the MAPK pathway is activated (and can be inhibited) in an individual patient. We also monitor the activation status of the S6 ribosomal protein (phosphorylation of serine 235 and serine 236). As shown in Figure 9.27.1, S6 can be activated by either mTOR (through Akt) or ERK. In our experience, some AML patients activate S6 via Akt, while others activate S6 through ERK (Chow et al., 2006), which has implications on how individual AML patients might be treated with different therapies.

Sample Preparation

It is important to prepare and test all of these reagents on appropriate cell types. For instance, SCF stimulates the Akt pathway in CD34 positive cells. In normal blood there are no CD34 positive cells, hence we use an AML cell line to validate SCF stimulation of this pathway. Suspension cultures are recommended, as preparation of monolayers or tissues for phospho-specific intracellular staining is problematic due to potential changes in signaling caused by cell isolation. Control cells (both positive and negative) for flow cytometry are prepared under the appropriate treatments (i.e., specific pathway stimulation and/or inhibition) and can be frozen for subsequent staining and analysis. Cells are frozen at a maximum of 2×10^7 cells per milliliter in freezing medium (see Reagent and Solutions), using 0.5-ml aliquots or other suitable volume

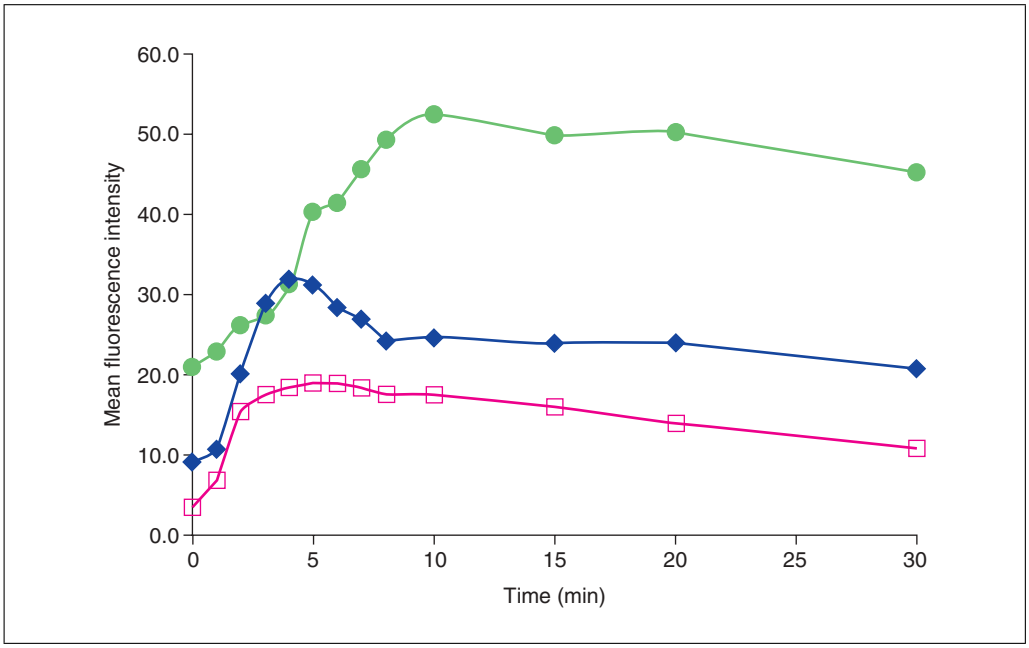

Figure 9.27.2 Time course for activation of p-S6 (circles), p-ERK (diamond) and p-Akt (boxes) in the M2 acute myeloid leukemia (AML) cell line following stem cell factor (SCF) treatment in vitro. M2 cells at 37°C were treated by the addition of an optimal concentration of SCF. At the indicated times, an aliquot of the cell suspension was fixed (0.1% formaldehyde for 10 min), permeabilized (absolute methanol at −20°C; see Jacobberger et al., 2003), washed, and incubated with antibodies to p-S6, or p-Akt, or p-ERK.

for staining. Clinical samples can also be prepared (fixed and permeabilized) and then stored in freezing medium at −20°C for subsequent staining. Cells stored in freezing medium can be maintained at −20°C for prolonged time periods without significant loss of phospho-epitope staining intensity (Krutzik and Nolan, 2003; Chow et al., 2005).

Since agonist-induced activation of signaling pathways generally results in sequential activation (phosphorylation) and inactivation (dephosphorylation) of specific members of one or more pathways, it is essential to perform dose-time response titrations to determine the concentration and time required to achieve peak signal for specific phospho-epitopes (see Fig. 9.27.2). In addition, for the assessment of a specific targeted agent or inhibitor, it is necessary to perform in vitro drug titrations using appropriate target cells (e.g., samples from untreated patients with the same disease) to establish the effective drug concentration range and IC_{50}. A reasonable rule of thumb is to use ∼10× more stimulus (agonist) and/or inhibitor when using whole blood compared with optimal concentrations for tissue culture cells, due to the effects of serum protein binding of many agonists or inhibitors.

Reagent Preparation

Working solutions of stimuli and inhibitors should be prepared so that 1 µl per 100 µl blood gives the desired final concentration. These are stored in ≤100-µl aliquots for easy thawing and manipulation. In addition, small aliquots allow for good management and economy, as some reagents may be unstable or have short shelf lives. Reagents should be tested regularly to assure confidence in the results obtained. PMA stimulation of whole blood is routinely used to generate maximum p-ERK and p-S6 signals, providing readily available positive controls for titration of antibody conjugates directed against these targets.

Table 9.27.1 Schedule for Addition of Inhibitors, Agonists, Fixative, and Lysis/Permeabilization Buffer in a Representative Experiment[a]

					Addition time[b]		
Tube	Stain[c]	Inhibitors	Agonist	Agonist	10% formaldehyde	Lysis/permeabilization buffer[d]	Wash buffer
1	Antibody cocktail	U0126 + LY294002	None	NA	30:30	40:30	1:10:30
2	Antibody cocktail	None	SCF	25:20	30:20	40:20	1:10:20
3	Antibody cocktail	None	SCF	25:10	30:10	40:10	1:10:10
4	Antibody cocktail	None	PMA	20:00	30:00	40:00	1:10:00
5	Antibody cocktail	None	None	NA	29:50	39:50	1:09:50
6	CD45-PC7 only	None	None	NA	29:40	39:40	1:09:40

[a]The data presented in Figure 9.27.3 were obtained from an experiment scheduled as indicated in this table.
[b]Times are given in min:sec.
[c]Antibody cocktail for this experiment was CD45-PC7, p-ERK-Alexa 488, and p-Akt-Alexa 647 in diluent buffer.
[d]The concentration of Triton X-100 in this buffer is 0.1165%.
Abbreviations: NA, not applicable; PMA, phorbol myristate acetate; SCF, stem cell factor.

Prepare sufficient mixtures of antibodies for all necessary experimental points (see experiment scheduling under Time Considerations and in Table 9.27.1), including additional material to accommodate pipetting loss.

Staining Strategies

For the measurement of both cell surface (CD) and phospho-specific epitopes, direct antibody-fluorophore conjugates are preferred to indirect staining. In addition to reducing time and eliminating extra washes and cell loss, staining with directly conjugated antibodies allows use of multiple antibodies from the same host directed against different epitopes in the same tube. As a general strategy, brightly fluorescent conjugates, e.g., phycoerythrin (PE), are used for weak signals, and weakly fluorescent conjugates are used for higher-density epitopes.

For quantitative comparisons, samples should be stained at the same cell concentration (usually 500,000 cells per test) and for the same amount of time (usually 20 to 30 min at room temperature). Preferably, sample staining and flow cytometric analysis should be done on the same day. After antibody staining, samples are maintained at 4°C in the dark until analyzed. Alternatively, stained samples can be resuspended in analysis buffer containing 0.1% paraformaldehyde, stored at 4°C in the dark, and analyzed within 24 hr.

Antibody Conjugates

Antibody conjugates are selected according to the instrumentation available. Commercially available antibodies to phospho-epitopes are currently available as direct conjugates only for use with the 488-nm laser (e.g., Alexa Fluor 488 or PE conjugates) or the 635-nm laser (e.g., Alexa Fluor 647).

Sample Filtration

Since flow cytometry interrogates single cells and clumps of cells can clog the flow cell, sample filtration is recommended to remove aggregates. For samples with significant cell clumping (e.g., bone marrow samples), filter cells through a 70-μm nylon cell strainer before fixation and cell staining.

Instrument Setup

The specific details for instrumentation setup, compensation, and analysis will vary depending on the type of flow cytometer used (colinear versus spatially separate laser beams) and the specific fluorochrome combinations used. That said, we have previously demonstrated that by using a standardized protocol for instrument setup and establishing appropriate compensation, both the interlaboratory and intralaboratory reproducibility of an assay employing both cell surface and cytoplasmic (signaling pathway) epitope analysis are greatly enhanced (Shankey et al., 2006). For further details on instrument setup, see *UNIT 9.22*.

Treatment Times

Incubation times with inhibitors and Triton X-100-containing lysis/permeabilization buffer are not as critical as agonist stimulation time and duration of formaldehyde treatment. The latter require careful attention, and prior preparation and scheduling is required to provide appropriate critical timing intervals.

BASIC PROTOCOL 1

WHOLE BLOOD PROCESSING FOR MEASUREMENT OF SIGNALING PROTEINS BY FLOW CYTOMETRY

This protocol is designed to measure one or more intracellular signal transduction epitopes using whole blood or bone marrow specimens. This approach also allows the use of peripheral blood cells as surrogate markers for pharmacodynamic monitoring of signal transduction pathway inhibitors for solid tumors (Chow et al., 2001). Anticoagulated blood is treated with agonists and/or specific pathway inhibitors (see Fig. 9.27.1) or used untreated and then fixed with formaldehyde and permeabilized with Triton X-100. Samples can either be stained for analysis at this point or frozen in freezing medium at –20°C for subsequent batch staining and analysis. Alcohol treatment before staining significantly improves the signal/noise of some intracellular phospho-epitopes such as p-ERK (Chow et al., 2005). Alcohol treatment to unmask intracellular phospho-epitopes is covered in Alternate Protocol 1. Typically, cells are stained with a CD marker(s) in addition to one or more phospho-specific antibodies (see Strategic Planning) and run on a flow cytometer equipped with one or more (488 nm and 635 nm) lasers, depending on the fluorophores used.

Materials

Anticoagulated blood or bone marrow in appropriate tubes: e.g., 6-ml lithium heparin vacutainer tubes (BD Biosciences, cat. no. 367886) *or* 3-ml K_2EDTA vacutainer tubes (BD Biosciences, cat. no. 367856)

Inhibitor working solutions, for example:
- 10 mM U0126 (MEK 1/2 inhibitor; Cell Signaling Technology) in 100% absolute methanol; store up to 6 months at −20°C
- 50 mM Ly 294002 (Calbiochem) in 100% anhydrous ethanol: store up to 6 months at −20°C
- 0.1 mg/ml rapamycin solution (see recipe)

Agonist working solutions, for example
- 40 μM phorbol myristate acetate (PMA; Sigma) in 100% anhydrous ethanol (store up to 1 year at −20°C)
- Stem cell factor (SCF) working solution (see recipe)

10% (v/v) formaldehyde, methanol free (Polysciences): store up to 6 months at room temperature in the dark

Lysis/permeabilization buffer (see recipe), 37°C

Wash buffer: 4% (v/v) fetal bovine serum (FBS) in Dulbecco's phosphate-buffered saline, calcium and magnesium free (CMF-DPBS): sterilize by passing through a 0.22-μm filter and store at 4°C

Freezing medium (see recipe), optional

Antibodies (conjugated to fluorescent labels) to CD (cell surface) markers (see Strategic Planning), for example:
CD45-PE Cy7 (clone J.33; Beckman Coulter)
CD3-PE (clone UCHT1, Beckman Coulter)
CD34-PE (clone 581; Beckman Coulter)
CD45-PE (clone J.33; Beckman Coulter)
CD117-PE (clone 104D2D1; Beckman Coulter)

Antibodies (conjugated with fluorescent labels) to intracellular phospho-epitopes (see Strategic Planning), for example:
p-ERK (T202/Y204) clone E10-Alexa 488 *or* -Alexa 647 (Beckman Coulter)
p-Akt (S473) clone 193H12-Alexa 488 *or* -Alexa 647 (Cell Signaling Technology)
p-S6 (S235/S236; Cell Signaling Technology)

Diluent buffer: 4% (v/v) FBS/0.1% (w/v) NaN_3 /CMF-DPBS

Analysis buffer: 0.1% (w/v) paraformaldehyde/CMF-DPBS

12 × 75–mm polypropylene tubes and racks

6-in. cotton-tip swabs

Repeater pipettor, recommended

Flow cytometer

Fix whole blood cells

1. Set up the schedule for treating appropriate samples with agonists and/or inhibitors. Label 12 × 75–mm polypropylene tubes and place in an appropriate rack.

   See Table 9.27.1 and additional comments under the Time Considerations section for information on scheduling the addition of multiple agonists and/or inhibitors to the sample.

2. Using universal precautions, pipet 100 μl of blood or bone marrow into the bottoms of the tubes.

3. Use a cotton swab to remove blood from the sides of the tubes to eliminate potential contamination of the sample with unfixed cells.

4. Add inhibitor working solutions to appropriate tubes. Mix each tube by vortexing at medium speed, returning it to the rack when mixed.

   Addition of different agonists and/or inhibitors is up to the individual reader. Thus, the timing for different experiments will vary according to experimental design.

5. After all inhibitors are added, vortex all tubes and incubate at 37°C.

   Start the timer after tubes have been placed into the incubator. The timing of incubation with inhibitors does not appear to be critical, as long as sufficient time is provided (generally 30 min or less).

6. Add 1 μl agonist working solution to each tube as per experiment schedule.

   In the cases where agonist is not added, we suggest that there is no need for the "mock addition" of DPBS, as the added volume is very small and time is critical.

7. At the appropriate times, add 65 μl of 10% formaldehyde to the tube.

8. Vortex well and return to the rack. Incubate each tube exactly 10 min at room temperature.

Lyse red blood cells and permeabilize white blood cells

9. Pipet 1 ml lysis/permeabilization buffer (prewarmed to 37°C) into each tube (after exactly 10 min incubation with formaldehyde), vortex vigorously, and return to the rack. Incubate 20 min at room temperature.

   The overall scheduling of an experiment determines the time points that are used to add different reagents. The amount of time formaldehyde is in a tube, before addition of Triton X-100, is critical. The amount of time the lysis/permeablization buffer (with Triton X-100) is in each tube is less critical. The amount of time each sample tube is exposed to inhibitor and/or agonist depends on the specific inhibitor and/or agonist used.

   Prewarming the lysis/permeabilization buffer facilitates red blood cell lysis. Note that in this technique, fixative is not removed before the addition of the red blood cell (RBC) lysis/white blood cell (WBC) permeabilization reagent. Previous studies have demonstrated that optimal fixation and lysis/permeabilization is obtained by adding lysis/permeablization buffer to whole blood sample while leaving fixative in the sample.

10. At the appropriate time point (20 min after addition of lysis/permeabilization buffer), add 1 ml cold (4°C) wash buffer to each tube.

11. Centrifuge all tubes 3 min at 1000 × *g*, room temperature.

12. Aspirate and discard the supernatant.

    There may be some residual red blood cells in the pellet at this point. This will typically be removed by subsequent washes. A heavy red blood cell pellet at this point indicates incomplete RBC lysis.

13. Vortex the tubes to loosen the pellets and then add 2 ml wash buffer. Incubate 15 min at room temperature.

14. Vortex the tubes and centrifuge 3 min at 1000 × *g*, room temperature.

    Incomplete RBC lysis is patient related and may be affected by the individual clinical situation. An extra wash with wash buffer is required for a very small fraction of patient samples, when RBC lysis is not observed.

15a. *For later batch analysis of frozen samples:* Aspirate the supernatant and vortex the cell pellets. Add 0.5 ml cold freezing medium to each tube, cap, and vortex. Store up to 1 year at −20°C. Stain and analyze samples as described in Alternate Protocol 1.

15b. *For immediate staining and analysis of samples:* Remove as much supernatant as possible and proceed to step 16 below.

   It is important to have a minimal amount of fluid remaining in the tubes when antibodies are subsequently added. Significant amounts of residual fluid changes the final concentration of all antibodies used at the staining step. This could significantly decrease the immunofluorescence of target epitopes.

Simultaneously label intracellular and surface epitopes

The technique employed in this protocol allows antibodies to react with CD epitopes on the cell surface or in the cytoplasm, in addition to phospho-epitopes after cell fixation and permeabilization, resulting in the staining of cell surface plus any cytoplasmic CD epitopes. An alternate technique (see Basic Protocol 2) employs cell surface marker (CD) staining of whole blood samples before sample fixation and permeabilization, which limits the analysis to epitopes expressed on the cell surface.

16. Add antibody cocktail containing all cell surface (e.g., CD34, CD45) plus anti-phospho-epitope (intracellular) antibodies to the cell pellet at optimal concentrations.

    All antibodies should be used at an optimal concentration (determined by prior titrations).

Antibodies are added directly to the washed cell pellets; there is no need to resuspend the cells first.

The antibodies indicated in the materials list are representative for our studies of adult AML. Protocol users should feel free to substitute other CD or phospho-specific antibodies according to what they are studying.

17. Add wash buffer to a final volume of 100 µl. Mix to resuspend the cell pellet.
18. Incubate 20 min at room temperature protected from exposure to light.
19. Add 2 ml cold wash buffer to each tube and centrifuge 3 min at 1000 × *g*, room temperature.
20. Aspirate and discard the supernatant. Resuspend the pellet in 0.5 ml analysis buffer. Maintain at 4°C for analysis on the flow cytometer.

    Samples may be stored in analysis buffer at 4°C protected from light for 24 hr with little change in light scatter characteristics or changes in fluorescence intensity of antibody-labeled epitopes.

21. Analyze on a flow cytometer (see Strategic Planning).

MEASUREMENT OF SIGNALING PROTEINS BY FLOW CYTOMETRY USING FROZEN, FIXED CELLS

ALTERNATE PROTOCOL 1

Cells can be activated and/or inhibited, fixed, permeabilized, and stored frozen for subsequent analysis (Basic Protocol 1, step 15a). This approach is particularly attractive for studies which require accumulation and analysis of multiple samples over multiple time points, allowing simultaneous staining and analysis of all samples under identical conditions. Note that in this approach, the cells are fixed, permeabilized, and frozen before staining, resulting in the analysis of CD epitopes to both cell surface and cytoplasmic epitopes. Because some clinical situations can require the differentiation of cell surface versus cytoplasmic epitope expression, this general approach will not be useful in such situations.

Additional Materials *(also see Basic Protocol 1)*

Frozen, fixed cells (Basic Protocol 1, step 15a)

1. Thaw individual tubes containing frozen, fixed cells at room temperature or at 37°C.
2. To each tube, add 2 ml cold wash buffer, vortex, and centrifuge 3 min at 1000 × *g*, room temperature.
3. Aspirate and discard the supernatant and proceed with sample staining and analysis by flow cytometry as described in Basic Protocol 1, steps 16 to 21.

ALCOHOL UNMASKING OF PHOSPHO-EPITOPES

ALTERNATE PROTOCOL 2

Studies of the impact of formaldehyde fixation and Triton X-100 permeabilization/RBC lysis have demonstrated that treatment with alcohol can enhance the immunofluorescence signals of some intracellular phospho-epitopes (Chow et al., 2005). These studies show that the ratio of p-ERK immunofluorescence in PMA stimulated versus nonstimulated (control) samples was highest with 90% methanol treatment post fixation and Triton X-100 permeabilization, and lower without methanol treatment. This pattern is consistent with epitope unmasking by alcohol (Jacobberger, 2000). The p-ERK epitope (phospho-Thr 202/Tyr 204-p44/42 ERK) can also be unmasked by treatment with high salt, urea, acid, or heat, but none of these produced the level of unmasking of methanol, and each of these was associated with degradation of light scatter and decrease in staining

intensity of some surface epitopes (e.g., CD3, CD19). Samples prepared using 50% cold methanol treatment after formaldehyde/Triton treatment demonstrated good preservation of light scatter and surface immunophenotypic patterns, similar to those obtained using a commercial whole blood/RBC lysing system (Chow et al., 2005). In our experience, some intracellular phospho-epitopes demonstrate significant improvement in positive staining intensity following cold alcohol treatment, while others show no significant improvement.

Additional Materials *(also see Basic Protocol 1)*

50% methanol/0.85% NaCl: store at −20°C

Unmask phospho-epitopes with alcohol

1. Carry out steps 1 to 12 in Basic Protocol 1. Place the cell pellet on ice, resuspend the cells in 1 ml cold (4°C) 50% methanol/0.85% NaCl, and incubate 10 min on ice.
2. Centrifuge the tubes 3 min at 1000 × *g*, room temperature.
3. Decant the supernatant by inverting the tube or aspirate and discard any remaining supernatant.
4. Resuspend the cells with 1 ml wash buffer, 4°C, mix vigorously, and centrifuge 3 min at 1000 × *g*, room temperature.
5. Decant or aspirate and discard the supernatant.
6. Repeat step 4.
7. After centrifugation, remove all tubes from centrifuge and decant or aspirate and discard the supernatant. Remove as much supernatant as possible and dry residual fluid droplets on the sides of tubes, using a cotton swab.

Label intracellular and surface epitope

As in Basic Protocol 1, the technique employed below results in the staining of cell surface plus any cytoplasmic CD epitopes, in addition to staining cytoplasmic phospho-epitopes. In order to stain cell surface epitopes only, refer to Basic Protocol 2.

8. Add antibody cocktail containing all cell surface (e.g., CD 34, CD45) and anti-phospho-epitope (intracellular) antibodies to the cell pellet.

   All antibodies should be used at an optimal concentration (determined by prior titrations).

9. Add wash buffer to final volume of 100 µl and mix to resuspend the cell pellet. Incubate at room temperature for 30 min, protected from exposure to light.
10. Add 3 ml cold (4°C) wash buffer to each tube. Centrifuge each tube 3 min at 1000 × *g*, room temperature.
11. Decant or aspirate and discard the supernatant.
12. Wash all tubes by adding 2 ml wash buffer, 4°C, mixing vigorously, and centrifuging 3 min at 1000 × *g*, room temperature. Decant or aspirate and discard the supernatant. Repeat.
13. Resuspend cells in 1 ml analysis buffer and maintain at 4°C for analysis on the flow cytometer.

    Samples may be stored in analysis buffer at 4°C, protected from light, for 24 hr with little change in light scatter characteristics or changes in fluorescence intensity of antibody labeled epitopes.

14. Analyze on a flow cytometer (see Strategic Planning).

STAINING CELL SURFACE MARKERS (CD) BEFORE FIXATION AND PERMEABILIZATION

BASIC PROTOCOL 2

This protocol is very similar to Basic Protocol 1, with the exception that only cell surface epitopes are stained, although the method allows for subsequent fixation, permeabilization, and staining of any intracellular epitopes. Basic Protocol 1 offers a number of advantages, including simplicity and the ability to readily store fixed and frozen cells for subsequent analysis of both CD and phospho-epitope expression. Basic Protocol 2 offers the advantage of limiting the analysis to CD epitopes expressed on the cell surface, a situation that is important in identifying important cellular populations in some cancers. The technique is outlined below. For further technical details and background information, please refer to *UNIT 9.22*.

Materials

Anticoagulated blood or bone marrow in appropriate tubes: e.g., 6-ml lithium heparin vacutainer tubes (BD Biosciences) and 3-ml K_2EDTA vacutainer tubes (BD Biosciences)

Antibodies (conjugated to fluorescent labels) to CD (cell surface) markers (see Strategic Planning), for example:
- CD3-PE (clone UCHT1; Beckman Coulter)
- CD34-PE (clone 581; Beckman Coulter)
- CD45-PE (clone J.33; Beckman Coulter)
- CD117-PE (clone 104D2D1; (Beckman Coulter)

Inhibitor working solutions, for example:
- 10 mM U0126 (MEK 1/2 inhibitor; Cell Signaling Technologies) in 100% absolute methanol; store up to 6 months at −20°C
- 50 mM Ly 294002 (Calbiochem) in 100% anhydrous ethanol; store up to 6 months at −20°C
- 1 mg/ml rapamycin solution (see recipe)

Agonist working solutions, for example:
- 40 μM phorbol myristate acetate (PMA; Sigma) in 100% anhydrous ethanol; store up to 1 year at −20°C
- Stem cell factor (SCF) working solution (see recipe)

10% (v/v) formaldehyde, methanol free (Polysciences): store up to 6 months at room temperature in the dark

Lysis/permeabilization buffer (see recipe), 37°C

Wash buffer: 4% (v/v) fetal bovine serum (FBS) in Dulbecco's phosphate-buffered saline, calcium and magnesium free (CMF-DPBS): sterilize by passing through a 0.22-μm filter and store at 4°C

Freezing medium (see recipe), optional

Antibodies (conjugated to fluorescent labels) to intracellular phospho-epitopes (see Strategic Planning), for example:
- p-ERK (T202/Y204) clone E10-Alexa 488 *or* -Alexa 647 (Beckman Coulter)
- p-Akt (S473) clone 193H12-Alexa 488 *or* -Alexa 647 (Cell Signaling Technology)
- p-S6 (S235/S236; Cell Signaling Technology)

Diluent buffer: 4% (v/v) FBS/0.1% (w/v) NaN_3 /CMF-DPBS

Analysis buffer: 0.1% (w/v) paraformaldehyde/CMF-DPBS

12 × 75–mm polypropylene tubes and racks

6-in. cotton-tip swabs

Repeater pipettor, recommended

Flow cytometer

Label cell surface receptors

1. Label one 12 × 75–mm polypropylene tube for untreated (control) sample, and one 12 × 75–mm polypropylene tube for each treatment condition (agonist +/- inhibitor; see Table 9.27.1).
2. Place 100 µl whole blood or bone marrow into each tube.
3. Using a cotton swab, carefully remove any excess blood from the sides of the tubes.
4. Add cell surface antibody mix.

   The total volume of antibodies added at this point should be kept to a minimum, ideally not exceeding 100 µl for all surface markers.
5. Gently mix blood and surface antibody mixture and incubate 20 min at room temperature.

   It is important to validate the immunoreactivity of the particular antibody-conjugate used with this assay format (adding fixative to whole blood-antibody suspension), and to determine the optimal antibody concentrations used with this protocol.

   Optimal antibody concentrations for incubation before fixation and permeabilization may be different than those used following Basic Protocol 1.

   Since some surface markers are integral components of signaling pathways, it is important to be aware that labeling specific surface epitopes before cell fixation could potentially activate or inhibit specific downstream signaling pathways. It is important that preliminary experiments are performed to establish the impact of labeling specific surface markers with the anti-CD antibodies on the target phospho-epitopes used in these assays.
6. Add inhibitors to appropriate tubes, gently mix, and incubate at 37°C.
7. Add agonists as per experimental schedule. Protect from exposure to light.

Fix cells

8. At the appropriate times, add 65 µl of 10% formaldehyde to the tube.
9. Vortex well and return to the rack. Incubate each tube exactly 10 min at room temperature.

Permeabilize cells and lyse RBC

10. Pipet 1 ml lysis/permeabilization buffer (prewarmed to 37°C) into each tube (after exactly 10 min incubation with formaldehyde), vortex vigorously, and return to the rack. Incubate 20 min at room temperature.

    The overall scheduling of an experiment determines the time points that are used to add different reagents. The amount of time formaldehyde is in a tube, before addition of Triton X-100, is critical. The amount of time the lysis/permeablization buffer (with Triton X-100) is in each tube is less critical. The amount of time each sample tube is exposed to inhibitor and/or agonist depends on the specific inhibitor and/or agonist used.

    Prewarming the lysis/permeabilization buffer facilitates red blood cell lysis. Note that in this technique, fixative is not removed before the addition of the RBC lysis/WBC permeabilization reagent. Previous studies have demonstrated that optimal fixation and lysis/permeabilization is obtained by adding lysis/permeablization buffer to whole blood sample while leaving fixative in the sample.
11. At the appropriate time point (20 min after the addition of lysis/permeabilization buffer), add 1 ml cold (4°C) wash buffer to each tube.
12. Centrifuge all tubes 3 min at 1000 × *g*, room temperature.

13. Aspirate and discard the supernatant.

    There may be some residual red blood cells in the pellet at this point. They will typically be removed by subsequent washes. A heavy red blood cell pellet at this point indicates incomplete RBC lysis.

14a. *For later batch analysis of frozen samples:* Vortex the cell pellets. Add 0.5 ml cold freezing medium to each tube, cap, and vortex. Store up to 1 year at −20°C. Stain and analyze samples as described in Alternate Protocol 1.

14b. *For immediate staining and analysis of samples:* Remove as much supernatant as possible and proceed to step 15 below.

    It is important to have a minimal amount of fluid remaining in the tubes when antibodies are subsequently added. Significant amounts of residual fluid changes the final concentration of all antibodies used at the staining step. This could significantly decrease the immunofluorescence of target epitopes.

Label intracellular antigens

15. Add antibody cocktail containing only the intracellular anti-phospho-epitope-specific antibodies to the cell pellet at optimal concentrations.

    All antibodies should be used at an optimal concentration (determined by prior titrations).

    Antibodies are added directly to the washed cell pellets; there is no need to resuspend the cells first.

    The antibodies indicated in the materials list are representative for our studies of adult AML. Protocol users should feel free to substitute other CD or phospho-specific antibodies according to what they are studying.

16. Add diluent buffer to a final volume of 100 µl. Mix to resuspend the cell pellet.

17. Incubate 20 min at room temperature, protected from exposure to light.

18. Add 2 ml cold wash buffer to each tube and centrifuge 3 min at 1000 × *g*, room temperature.

19. Remove tubes from the centrifuge and aspirate supernatant. Resuspend in 0.5 ml analysis buffer. Maintain at 4°C for analysis on the flow cytometer.

    Samples may be stored in analysis buffer at 4°C, protected from light, for 24 hr with little change in light scatter characteristics or changes in fluorescence intensity of antibody-labeled epitopes.

20. Analyze on a flow cytometer (see Strategic Planning).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Freezing medium

Slowly pour 5 ml glycerol from the original bottle directly into a 50-ml, sterile, graduated screw-cap, polypropylene tube. Add 10 ml FBS using a pipet. Add 35 ml RPMI 1640. Cap tube and mix thoroughly by inverting. Store up to 1 year at −20°C. Prior to use, thaw freezing medium at room temperature or 37°C. Cool on ice before adding to cells for freezing. Refreeze leftover medium.

The final concentration is 10% (v/v) glycerol/20% (v/v) FBS in RPMI-1640.

This procedure avoids viscosity problems encountered with glycerol.

Lysis/permeabilization buffer

Dilute 0.233 ml of 10% Triton X-100 to a final volume of 10 ml with Dulbecco's phosphate-buffered saline, calcium and magnesium free (CMF-DPBS). Prepare enough to allow 1 ml lysis/permeabilization buffer for every tube. Store up to 1 month at room temperature. Immediately prior to use, preheat to 37°C.

The final concentration of Triton X-100 is 0.1165% (v/v).

Rapamycin solution, 0.1 mg/ml

Stock solution: Prepare 1 mg/ml rapamycin (Calbiochem) in DMSO.

Working solution: Dilute to 0.1 mg/ml in DMSO. Dispense into 100-µl aliquots and store up to 1 year at −20°C.

Stem cell factor (SCF) solution

Stock solution: Prepare 100 µg/ml stem cell factor (SCF; Calbiochem) in Dulbecco's phosphate-buffered saline, calcium and magnesium free (CMF-DPBS). Dispense into 5-µl aliquots and store up to 1 year at −20°C. Avoid refreezing after thawing.

Working solution: Dilute stock solution to 10 µg/ml with CMF-DPBS immediately before use.

COMMENTARY

Background Information

This application was specifically developed to analyze phospho-epitope expression in clinical (whole blood, bone marrow, etc.) samples with the goal of developing and validating methodologies to facilitate real-time monitoring of individual leukemia patients' responses to therapies. While a number of published reports have described the analysis of phospho-epitope expression in tissue culture cells or in isolated mononuclear cells from blood or bone marrow, we have focused on the use of whole blood or bone marrow samples from clinical specimens. Our primary impetus for developing a whole blood assay is based on the concern (with supporting data) that, in assays using isolated cells, either stimuli or inhibitors could be removed during ex vivo processing (e.g., ficoll/hypaque separation of peripheral blood mononuclear cells), resulting in a change of the basal or steady state signaling that was present in vivo. In addition, the use of a preliminary separation step has the potential to lose important cell populations from the intact specimen. Another concern is that phosphorylation states are transient in many normal signaling cascades (see Chow et al., 2001), and we hypothesized that an optimal technique for assessment of in vivo signaling pathways should include fixation of the cells as soon as possible after removal from the individual.

Thus, we set out to develop a technique that would provide fixation of the intracellular phospho-epitopes as rapidly as possible after sample acquisition, without the need for additional processing and separation of cell populations. While formaldehyde fixation provides rapid fixation of phospho-epitopes, its addition to whole blood or bone marrow presents the subsequent problem of inhibiting red blood cell lysis, a step that greatly facilitates subsequent flow cytometric analysis. Our solution to these problems is to use Triton X-100, providing a critical concentration of Triton to a sample containing RBCs in the presence of formaldehyde (and after a critical prior fixation period for white blood cells), which results in both RBC lysis and permeabilization of white blood cells with out any significant loss of white blood cell populations (see Chow et al., 2005).

Many clinical flow cytometry applications presently rely on the use of light scatter measurements to identify important cell populations. As such, one important goal in our development of a whole blood fixation, permeabilization, and RBC lysis technique is to maintain light scatter signatures of white cell populations. The initial whole blood technique developed by Chow et al. (2001) used rapid hypotonic lysis to remove RBC's followed by alcohol treatment to permeabilize WBCs, which resulted in poor resolution of monocytes from either lymphocytes or granulocytes. We have carefully measured the impact of the formaldehyde/Triton X-100 technique described here on both forward-scatter and side-scatter (orthogonal) signals, comparing these to light scatter of WBC populations

prepared using a relatively gentle RBC lysis technique (Q-Prep, Beckman Coulter). The results of these studies indicate that although the separation of WBC populations by light scatter is reduced following formaldehyde/Triton X-100 treatment, the populations remain separate and distinct (Chow et al., 2005).

While developing this whole blood technique, we observed that the dynamic range of expression, comparing unstimulated or inhibited levels of some phospho-epitopes with levels seen in stimulated cells, was quite small (MFI difference between 3-fold and 5-fold). For example, the levels of p-ERK measured in whole blood samples with and without PMA stimulation using formaldehyde/Triton X-100 was generally in the 5-fold range (gating on normal lymphocytes). In contrast, our prior experience using the hypotonic RBC lysis followed by alcohol permeabilization routinely demonstrated a 20-fold difference in the MFI of PMA stimulated versus unstimulated normal lymphocytes (Chow et al., 2001). These results suggested that, for some phospho-epitopes, alcohol treatment could be providing an epitope "unmasking" step (see Jacobberger, 2000). Thus, for some phospho-epitopes, measured changes in expression levels may be significantly enhanced following treatment with cold 50% methanol (Alternate Protocol 2). At present, it is not possible to offer any comprehensive guideline regarding enhancement of specific phospho-epitope expression following cold alcohol treatment, and it is recommended that empirical testing be undertaken when investigating a new phospho-epitope or a different type of cell.

Although alcohol treatment may provide an important benefit of "unmasking" the expression of some phospho-epitopes, it has the potential to significantly change light scatter properties, due to cell dehydration caused by the hypotonic environment. We have carefully measured this (Chow et al., 2005) and demonstrated that cold alcohol (up to ~50% methanol) following formaldehyde/Triton X-100 treatment results in little further light scatter change. This is likely due to the fact that the white blood cells are first fixed with a concentration of cross-linking fixative that limits further shape and/or size changes following exposure to cold alcohol. One additional advantage that alcohol treatment has is that it allows short-term storage of fixed and permeabilized samples at −20°C without significant loss in either cell number or phospho-epitope expression (Chow et al., 2005; Krutzik and Nolan, 2003).

Fixation and permeabilization of cells before labeling with antibodies to some CD markers can create several problems. In addition to simultaneously labeling both cell surface and cytoplasmic epitopes, this approach also exposes all epitopes to concentrations of cross-linking fixative that can potentially reduce or eliminate epitope recognition by some antibodies. As we (Chow et al., 2005) and others (Krutzik and Nolan, 2003) have demonstrated, the immunoreactivity of CD19 can be significantly reduced by prior formaldehyde treatment. For this reason, we have included Basic Protocol 2, which allows staining of cell surface epitopes in whole blood samples prior to treatment with formaldehyde. Before using this Protocol, it is critical that optimal concentrations of antibodies to cell surface epitopes are first established (see Shankey et al., 2006). The presence of excess (unbound) antibodies could result in nonspecific fixation of the antibody to a cell that actually lacks the appropriate epitopes on its surface.

Critical Parameters

Reagents

In our experience, some laboratories have difficulties reproducing our results due to problems caused by reagents. The formaldehyde used must be reagent (EM) grade and must be free of any methanol (an additive frequently used to stabilize formaldehyde). While some reports have commented that the formaldehyde used must be "fresh" or used within a few months of opening the reagent, our experience suggests that 10% formaldehyde solution is stable for months when stored at room temperature (20° to 25°C), avoiding exposure to light. An additional difficulty seems to arise when laboratories try to use 100% Triton X-100 solution to prepare the working solution. It is difficult to accurately dilute 100% Triton X-100. In addition, the 100% solution appears to oxidize during storage at room temperature. For this reason, we recommend the use of a stock Triton X-100 solution (10%) that is stored under nitrogen.

Antibodies to specific phospho-epitopes are becoming widely available through a number of commercial sources. Although we have stressed in this unit the use of directly conjugated antibodies, direct conjugates are not available for many phospho-epitopes. While we have used indirect staining techniques, these can become cumbersome for applications requiring staining with multiple antibodies.

Fixation and permeabilization

Timing of the formaldehyde fixation step used in Basic Protocols 1 and 2 is critical. Exposure of blood cells for a shorter time period results in loss of white blood cells, while longer time periods result in incomplete RBC lysis (and subsequent clumping if the alcohol unmasking step is used). Similarly, the final concentration of fixative in each tube is critical. The techniques used here appear to be somewhat sensitive to the overall protein concentration in the sample. Our experience has demonstrated that using the protocols detailed here with isolated cells (e.g., PBMC or cells suspended in buffer) results in poor cell recovery and poor access of antibodies to cytoplasmic epitopes.

The time period between the addition of Triton X-100 and the addition of wash buffer is not critical in our experience, and periods from 20 to 30 min generally provide similar results. The final concentration of Triton X-100 is important, and some care must be taken in the preparation of the lysis/permeabilization buffer. Prewarming this buffer to 37°C immediately before use has been shown to facilitate RBC lysis, particularly in patient samples from individuals with a variety of hematological malignancies.

Fluorophore strategies and compensation

In our experience, the dynamic range of phosphoprotein signaling (e.g., difference between negative and positive populations) is not large, frequently 10-fold or less (see Fig. 9.27.3, last row of histograms on right). For this reason, attention to planning the antibody-fluorophore combinations used and to the use of appropriate compensation is important. Commercially available antibodies to phospho-epitopes are generally limited to Alexa 488, phycoerythrin (PE), and Alexa 647 conjugates. The strategy used to stain specific cell populations, based on surface marker expression, depends on the number of different specificities that will be used (e.g., CD34, CD45, and CD117 for AML) and on the instrument/laser configuration used. It is generally not advisable to use a brightly staining cell surface marker in a fluorophore with an emission wavelength close to the emission of the fluorophore used for a phospho-epitope (e.g., brightly staining PE epitope used with a phospho-epitope labeled with Alexa 488). For instruments with colinear red (635 nm) and blue (488 nm) lasers, we generally use a single CD marker to identify the blast cell population. As shown in the results presented in Figure 9.27.3, the CD 45-PECy7 shows minimal fluorescence emission into the PMT used to detect Alexa 647. For situations where both a PE-labeled cell surface marker and an Alexa 647-labeled antibody to a phospho-protein are used in the test, compensation for signal spillover of PE into the Alexa 647 channel can be significant (in the 30% to 40% range), necessitating the use of an appropriate single color compensation tube (see Fig. 9.27.3).

Troubleshooting

Two problems that we have encountered using clinical samples for studies of intracellular phospho-epitopes include incomplete lysis of red blood cells and incomplete fixation of white blood cells. These problems can be related to the fixation and/or lysis/permeabilization steps used in the assay. In some cases, use of formaldehyde that contains certain additives used to stabilize the fixative (e.g., alcohols) can result in incomplete WBC fixation and/or destruction of sensitive epitopes. For this reason, we recommend the use of EM grade formaldehyde. In addition, the type of Triton X-100 used has been shown to impact WBC permeabilization and RBC lysis, as previously discussed in Critical Parameters.

For the analysis of relatively straightforward samples (e.g., whole blood) where target populations can be readily identified using a single surface marker, it is frequently advisable to analyze samples stained with anti-phospho-epitope antibodies individually, using side scatter (see Fig. 9.27.3, fourth and fifth columns of histograms from left). These types of analyses are useful to compare compensated versus non-compensated files in order to determine if the compensation used is appropriate.

The issue of negative and positive controls is important in the analysis of signal transduction pathways in clinical samples. As previously noted, the dynamic range of these signals (difference between negative and positive state in any cell in a population) is generally 10-fold or less, making the determination of the negative signal level very important. In our experience, normal whole blood samples have internal negative control cell populations, i.e., cells which have either little or no phosphorylation of signal transduction epitopes. For example, peripheral blood T lymphocytes ($CD3^+$ cells) in normal individuals have basal levels of the phospho-epitopes used in studies of signal transduction

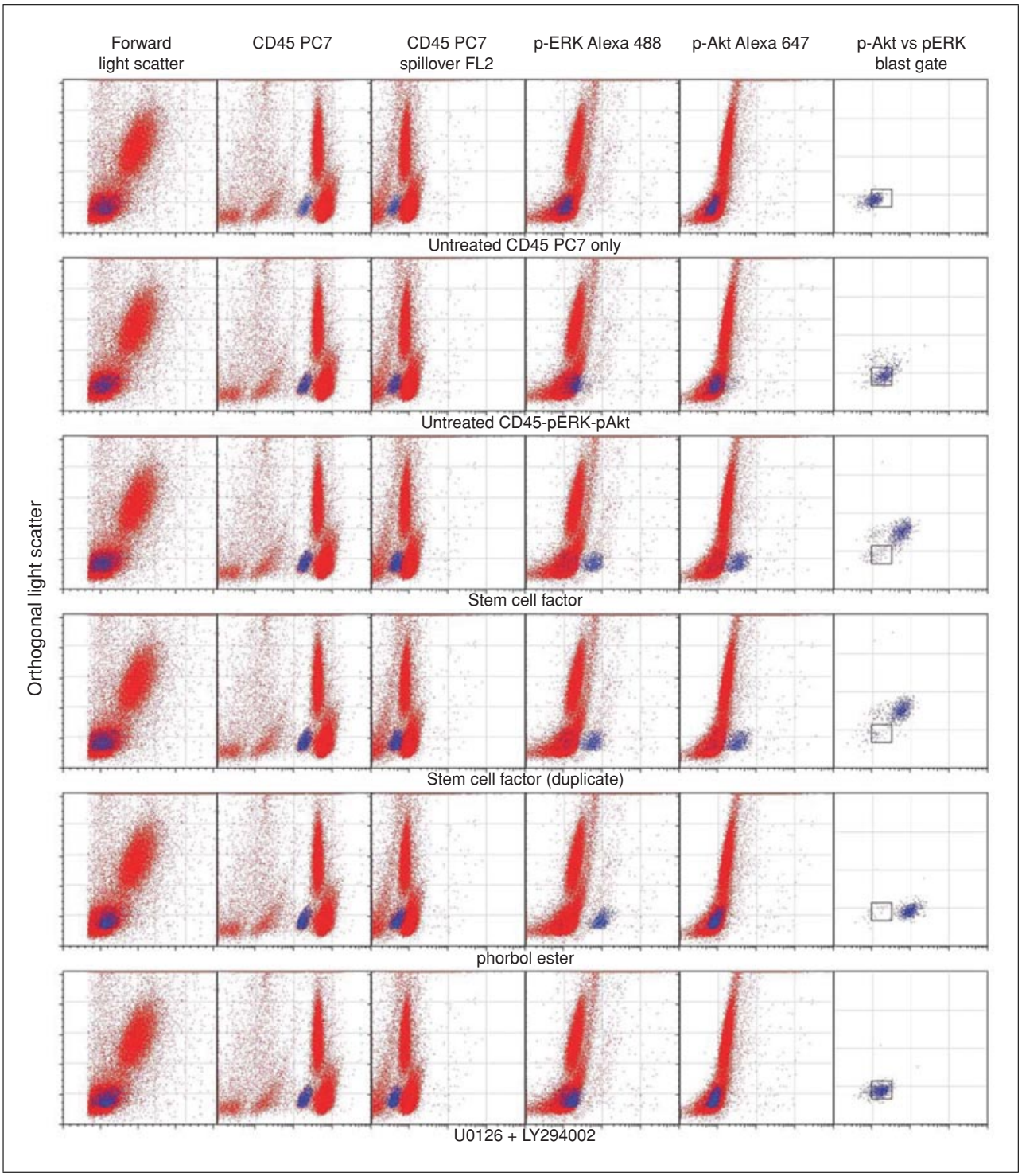

Figure 9.27.3 Results of a representative experiment demonstrating signaling through ERK and Akt for a whole blood sample from an acute myeloid leukemia (AML) patient. The blast cell population, based on CD45 (low) versus side-scatter (orthogonal) gate (second column of histograms) is shown as black dots in all histograms. From top to bottom, rows of panels represent untreated (no agonist or inhibitor) sample stained with CD45 only, untreated sample stained with CD45 plus p-ERK plus p-Akt (second row), sample stimulated with stem cell factor (duplicate samples in third and fourth rows), sample stimulated with PMA (phorbol myristate acetate; fifth row), and sample treated with both p-ERK (UO126) and p-Akt (LY294002) inhibitors (without stem cell factor stimulation; sixth row) and showing no further reduction of either phosphoprotein. The last column of histograms on the right represent levels of p-ERK-Alexa 488 (ordinate) vs. p-Akt-Alexa 647 (abscissa) in the blast cell population, based on CD45 versus side-scatter (orthogonal gating) for samples treated (or not) as described above.

pathways noted in this section (p-ERK, p-Akt and p-S6). Here, basal levels refers to either no or very low levels of expression, a distinction that is very difficult to make using flow cytometry (as well as many other detection techniques). Normal whole blood samples can be stimulated in vitro to stimulate phosphorylation of ERK, S6, as well as other phosphoepitopes (although transiently). In this sense, the unstimulated sample provides a biological negative control, while optimally stimulated whole blood samples provide the positive control.

In clinical samples, we have similarly used internal cell populations to provide references for negative and positive expression levels for specific phospho-epitopes. In whole blood samples from AML patients, the T cell populations in unstimulated samples (and/or samples treated with specific pathway inhibitors) provide internal negative control populations for the AML blast cell population. Similarly, normal cell populations in clinical samples that have been treated with specific agonists (e.g., PMA), can provide positive controls for specific signaling pathways (e.g., p-ERK levels in PMA treated T lymphocytes). For a further discussion on the use of internal positive and negative control cell populations, see *UNIT 9.22* and Shankey et al. (2006).

Time Considerations

Even for experiments that are relatively straightforward (using only one or two different patient samples) the timing for the addition of inhibitors, agonists, fixation and lysis/permeabilization can be challenging. For this reason, we recommend that the scheduling of all steps in the experiment be worked out in advance of starting the experiment. An example of the scheduling required for a representative experiment employing a single AML sample is shown in Table 9.27.1. This experiment uses only one cell surface marker (CD45-PECy7) to mark the AML blast cell population, plus the intracellular markers anti-p-ERK-Alexa 488 and p-Akt-Alexa 647. As shown, one tube is stained with CD45-A + PECy7 only, to determine compensation needed for the Alexa 488 and Alexa 647 channels. Whole blood warmed to 37°C is added to the six tubes used for this experiment, inhibitor (here a mixture of the p-ERK inhibitor U0126 plus the p-Akt inhibitor LY294002) is added to tube 1 (only), all tubes are placed in a 37°C bath, and the timer is started. After 20 min incubation, PMA is added to tube 4 (at 25 min 10 sec), SCF is added to tube 3, and so on (as indicated in Table 9.27.1). The timings indicated in the table provide optimal PMA stimulation (10 min) and SCF stimulation (5 min) and the timing of all six tubes for the addition of fixative (10 min incubation for each tube) and subsequent addition of lysis/permeabilization reagent (30 min incubation for each tube). Obviously, the analysis of multiple patient samples and/or the use of additional agonists and inhibitors significantly increases the complexity of the experiment, and careful planning and scheduling of the experiment will facilitate its execution.

Anticipated Results

Analysis of whole blood from an AML patient

Typical results for the analysis of a whole blood sample from an AML patient are presented in Figure 9.27.3. Light scatter profiles (forward-scatter versus side-scatter histograms at left) are maintained using this fixation and permeabilization technique, allowing the discrimination of lymphocytes, monocytes, and granulocytes. For the sample shown here, we have used CD45 (PE Cy7 conjugate) to identify the blast population (Fig. 9.27.3, second and third columns of histograms). The levels of phosphorylated ERK or Akt, measured in the blast gate (Fig. 9.27.3 histograms at far right), change minimally when comparing the results with a tube containing only CD45-PE Cy7 (Fig. 9.27.3, top row of histograms) to that with CD45-PE Cy7 plus p-ERK-Alexa 488 and p-Akt-Alexa 647 (Fig. 9.27.3, second row of histograms). Following stimulation in vitro with stem cell factor, the measured levels of both p-ERK and p-Akt increase (Fig. 9.27.3, third and fourth rows), and as demonstrated by comparing the third and fourth rows of Figure 9.27.3, the levels of activation in the blast cell population following SCF stimulation in vitro are highly reproducible. In contrast to SCF, phorbol (PMA) stimulation in vitro results in the activation of p-ERK (through protein kinase C) but not p-Akt (Fig. 9.27.3, fifth row). Finally, the simultaneous addition of the ERK inhibitor UO126 and the Akt inhibitor Ly294002 before stimulation in vitro with SCF results in levels of p-ERK and p-Akt in the blast population (Fig. 9.27.3, last row of histograms) similar to that measured in unstimulated blast cells.

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