

Characterize Immune Cell Populations using Flow Cytometry

Benefits of Flow Cytometry

Flow cytometry is particularly useful in immunology and immuno-oncology studies and offers the following advantages over other protein-detection methods:

- Ideal for analyzing complex intracellular signaling cascades and cellular responses to treatment in a heterogeneous sample (eg, tumor tissue)
- More efficient analysis and conservation of precious samples
- Signaling levels of single-cell subpopulations can be quantified (as opposed to a pool of cell lysates), allowing visualization of infrequent signaling events
- Highly flexible, allowing multiplexed analysis of phenotype and functional characteristics of cell populations
- Populations of cells exhibiting a range of defined characteristics can be identified, quantified, and subdivided in just a few hours, making flow cytometry the ideal technique for the generation of robust datasets in a very short timeline

Introduction

Both flow cytometry and western blotting rely on the use of antibodies to selectively and specifically identify proteins of interest in a given sample. As a classic technique, western blotting has been used for several decades to identify the presence of unique antigens in cell and tissue samples in a qualitative and semi-quantitative manner. While western blotting certainly has a crucial place in protein detection, here we focus on flow cytometry and the benefit provided by the ability to perform multiplexed single-cell level analyses. Cell Signaling Technology (CST) is here to help ensure a smooth transition from western blotting to flow cytometry and to enable you to maximize your data collection from precious samples.

Flow cytometry allows you to use a combination of antibodies to immunophenotype heterogeneous cell populations and to detect intracellular signaling states, post-translational protein modifications, and functional characteristics, all in one experiment. Therefore, unlike western blotting, flow cytometry can identify, quantify, and subdivide populations of cells exhibiting a range of defined characteristics in just a few hours, making flow cytometry the ideal technique for the generation of robust datasets in a very short timeline.

In particular, flow cytometry is a widely used tool in immuno-oncology studies, where assessing tumor responses to various drug treatments is essential. This is because analysis of drug-driven changes in tumor samples that comprise a heterogeneous mixture of cancer, stromal, and immune cells requires the ability to capture unique responses from all represented cell types. Therefore, the use of flow cytometry allows researchers to rapidly and effectively identify specific responses to treatment and pinpoint critical cell types within the tumor microenvironment, in turn expediting and expanding drug screening efforts.

In addition, unlike western blotting, which requires a large number of cells for protein isolation and analysis, flow cytometry is much better suited for the multivariate assessment of samples containing a small number of cells—for example, patient-derived tissue samples and primary cells that exist in very limited quantities. This is also true when isolating and analyzing a particular subtype of immune cells from a mixed population. For example, when using peripheral blood to isolate T cells or B cells, there may be as few as 2,500 CD4⁺ T cells and 1,000 B cells for every 10,000 peripheral blood mononuclear cells (PBMCs). Using western blot analysis, the signal for protein changes in this small subset of cells may be imperceptible within the noise of a mixed population, and due to the limited amount of lysate from such a small number of isolated cells, flow cytometry would be the superior method.

Another advantage to flow cytometry is its ability to analyze signaling states in cells within a heterogeneous sample to better identify unique biological processes that are indicative of cellular function or dysfunction. For example, based on the expression and phosphorylation status of several intracellular proteins and cell-surface receptors, researchers can determine the differentiation and activation states of a lymphocyte and assess how these parameters change when treatment is applied.

Flow Cytometry Methods

Selecting Targets

Selecting targets for the development of an appropriate antibody panel is of the utmost importance for the success of a flow cytometry experiment. For example, when investigating T cell activation, cell population markers and signaling targets should be carefully chosen to selectively identify activated T cells. Specifically, phenotyping markers such as CD3 (pan T cell) and CD4 (helper or regulatory T cell) can be multiplexed with phospho-SLP-76 (activated T cell) to specifically identify activated T lymphocytes.

In addition to selecting the appropriate marker, selecting antibody clones that are validated for flow cytometry is also important. In this experiment characterizing activated T cells, we chose the clones UCHT1, RPA-T4, and D7S1K for CD3, CD4, and phospho-SLP-76, respectively (**Table 1**).

As a leader in antibody development, CST offers a wide array of flow-validated antibodies that can be combined to identify the many different immune cells and their functional states.

Table 1 - Antibody panel for the analysis of activated T cells.

Product #	Target	Description	Clone	Fluorophore
#19881	CD3	T cell phenotyping marker	UCHT1	APC
#66182	CD4	T helper cell phenotyping marker	RPA-T4	PE-Cy [®] 7
#90247	Phospho-SLP-76 (Ser376)	T cell activation signaling	D7S1K	Alexa Fluor [®] 488
#72086	Dead Cells	Negative cells are live cells; positive cells are dead cells		Ghost Dye [™] Violet 540 Viability Dye

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Selecting Fluorophores

Careful selection of distinct fluorophores that minimize spectral overlap and are compatible with the lasers in your flow cytometer is important for maximizing the multiplexing potential of the chosen antibodies. If spectral overlap occurs, fluorescence compensation using a mathematical correction of the signal overlap between channels of emission spectra is required.

As with primary antibodies for panel development, CST offers conjugated antibodies in a variety of fluorophores. In the examples above, we chose Violet 540, APC, PE-Cy[®]7, and Alexa Fluor[®] 488 (**Table 1**).

In the example experiment, the specific fluorophores chosen for the antibody conjugates were selected to minimize spectral overlap and, therefore, did not require compensation.

Additional Selection Markers

Discrimination between live and dead cells is a critical step to exclude false positives in your cell populations when analyzing flow cytometry results. Processing, freezing, and/or treating the cells, such as for fixation and permeabilization, can induce cell death, and dead cells will not exhibit proper signaling. As such, a live-dead viability stain should be included in addition to the antibody panel in order to gate on live cells and exclude the dead cells (**Figure 1**).

Important Considerations

It is important to note that a fixation and permeabilization protocol is required for antibodies to access intracellular signaling cascades; however, not all antibodies to extracellular phenotyping markers will function appropriately in those conditions. Since flow cytometry allows for multiplexed detection of intracellular and extracellular proteins, be sure to check the protocol from the antibody manufacturer, or test and optimize your protocol to make sure you select the correct combination of antibodies and reagents for your experimental needs.

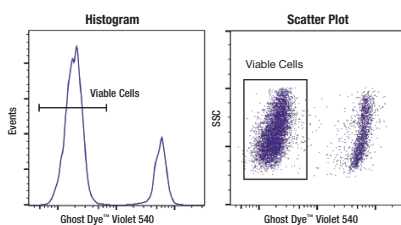


Figure 1: Representative side scatter vs forward scatter of gated live cells, with dead cells excluded, using Ghost Dye[™] Violet 540 Viability Dye depicted as histogram (left panel) and scatter plot (right panel).

Flow Cytometry Results

Dot Blot vs Histogram Shift

Flow cytometry data are represented either as a scatter plot or a histogram shift (**Figure 1**).

Scatter plots convey protein expression levels and data corresponding to individual cells, while histogram shifts demonstrate more population-level changes such as mean expression in a subgroup of cells.

How to Gate on Specific Subpopulations

Gates are placed around populations of cells with common characteristics. Forward and side scatter of the data represent unique properties, such as size and granularity. Subclasses of cells can be gated based on these parameters as well as on phenotypic biomarker expression detected with the antibody conjugates. For example, to selectively analyze activated T cells in our experiment, we place sequential gates around the following subpopulations of cells (**Figure 3**):

1. Cells showing a small forward and side scatter are characteristic of lymphocytes.
2. Of the cells selected in **Gate 1**, viable cells are identified by detection with the live/dead stain Ghost Dye[™]. Live cells will be low or negative for the fixable dye.
3. Of the cells selected in **Gate 2**, cells with CD3 expression are detected by an antibody conjugate.
4. Of the cells selected in **Gate 3**, cells with CD4 expression are detected by an antibody conjugate.

Flow Cytometry Data vs Western Blot Data

Because flow cytometry provides a readout on individual cells or an enriched subpopulation within a heterogeneous sample, it can detect subtle changes in protein expression or modifications over time. For example, by isolating and enriching the CD4⁺ T cell population in our western blot experiment, the fold change in phospho-SLP-76 from 0 to 2 min (**Figure 4A**, Lanes 3 and 4) is far more pronounced than the fold change of phospho-SLP-76 in total PBMCs (**Figure 4A**, Lanes 1 and 2). The complex protocol necessary to enrich for CD4⁺ T cells for the purpose of western blot analysis may take days, depending on your sample size. In contrast, one flow cytometry experiment can quickly enrich for CD4⁺ T cells and examine phosphorylation of key activation readouts in just a few hours (**Figure 4B**).

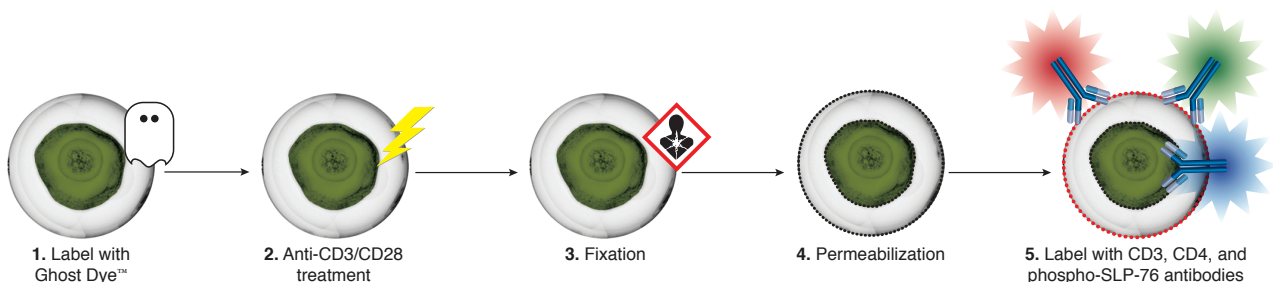


Figure 2: Steps for setting up a basic flow cytometry experiment, which includes viability staining and both cell population marker and intracellular signaling target identification. Detailed protocols for Ghost Dye[™] labeling are available at cst-science.com/ghostflow and product-specific protocols are available on each product page.

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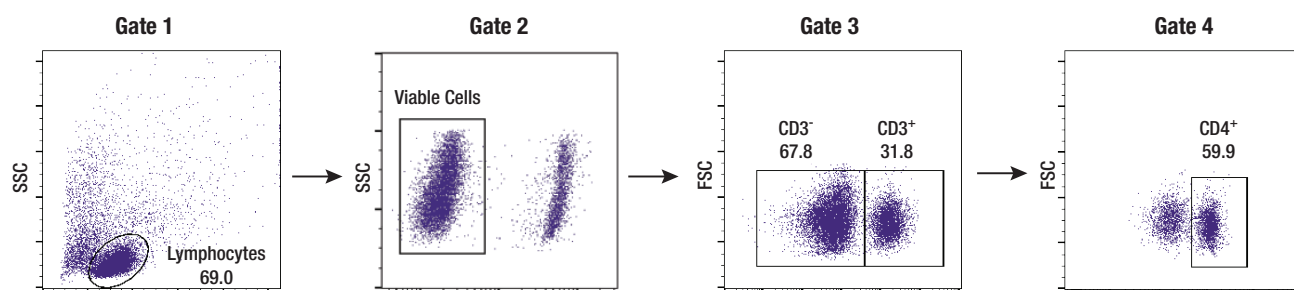


Figure 3: Example gating scheme. Forward scatter vs side scatter (**Gate 1**), live cells (**Gate 2**), CD3 gated cells (**Gate 3**), and CD4 gated cells (**Gate 4**).

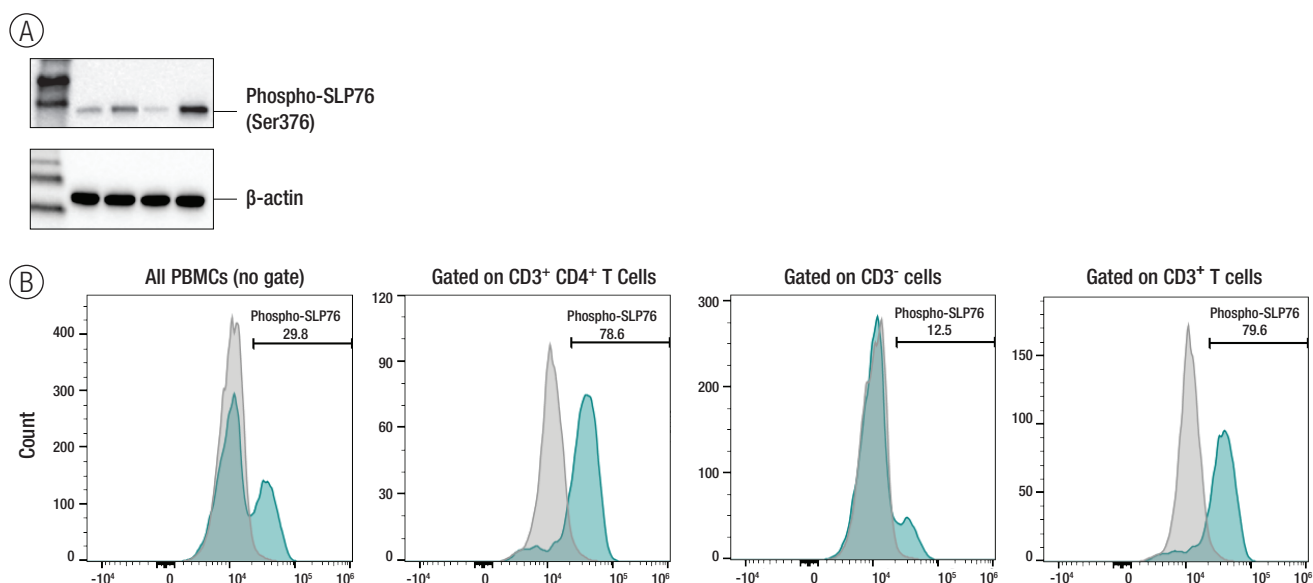


Figure 4: Comparison between western blot and flow cytometry. **A.** Western blot: Lane 1 = PBMCs treated with control; Lane 2 = PBMCs treated with CD3/CD28; Lane 3 = CD4-enriched cells treated with control; Lane 4 = CD4-enriched cells with CD3/CD28. **B.** Histogram plot showing phospho-SLP-76 staining in mixed PBMCs (left), CD3⁺CD4⁺ double-gated cells (center, left), CD3⁻ gated cells (center, right), and CD3⁺ gated cells (right). The percentage of phospho-SLP-76-positive cells in each gated population is indicated in the upper right-hand corner of each histogram plot.

Conclusions

The characterization of multiple phenotypic and signaling parameters is essential for immuno-oncology studies, and flow cytometry enables the performance of multiplexed single-cell based analysis, where western blot does not. Using flow cytometry and well-designed antibody panels can increase throughput and expedite drug development, because each experiment can identify changes in a wide array of important biological processes.

As a leading producer of high-quality antibodies, CST can provide you with ready-to-use or custom-made flow-validated antibody conjugates in a variety of fluorophores that are designed to reproducibly detect expression levels of cytokines, transcription factors, phosphorylated proteins, and epigenetic modifiers. All of these parameters can be

analyzed using flow cytometry and can shed light on signal transduction and other cellular processes that are happening beneath the surface in disease states and in response to treatment. In addition, our comprehensive technical support and educational assistance are available for the set-up, optimization, and follow-through of your flow cytometry experiment to help you painlessly and quickly transition from western blotting to flow cytometry and ensure the success of all your flow cytometry experiments in order to streamline your immunology or immuno-oncology research.

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Technical Support

At CST, providing exceptional customer service and technical support are top priorities. Our scientists work at the bench daily to produce and validate our antibodies, so they have hands-on experience and in-depth knowledge of each antibody's performance. In the process, these same scientists generate valuable reference information that they use to answer your questions and help troubleshoot your experiment by phone or email.

For questions about how to customize your protocol using our full catalog of nearly 1000 antibodies approved for IHC, please contact technical support: Visit www.cellsignal.com/support or call 1-877-678-8324.

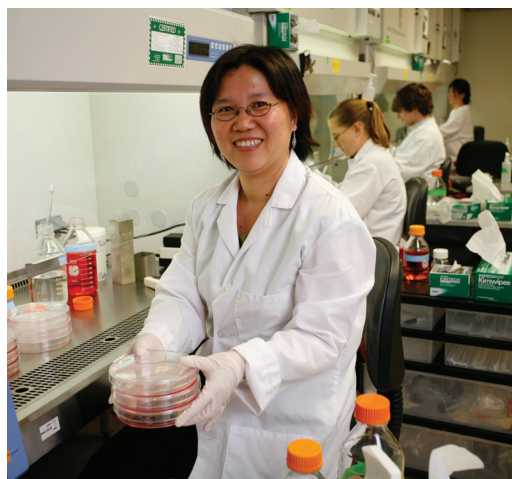


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