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Staining Intracellular Antigens for Flow Cytometry

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Introduction

A modification of the basic immunofluorescent staining and flow cytometric analysis protocol can be used for the simultaneous analysis of surface molecules and intracellular antigens at the single-cell level by flow cytometry. Typically, cells are fixed with formaldehyde to stabilize the cell membrane, and then permeabilized with detergent or alcohol to allow antibodies against intracellular antigens access to stain intracellularly.

When staining proteins inside the cell, it is important to consider their location as this may dictate the protocol and buffer system that will perform optimally. For example, nuclear proteins and many secreted proteins work well with the eBioscience Foxp3/Transcription Factor Staining Buffer Set (eBioscience Cat. No. 00-5523), while secreted proteins such as cytokines and chemokines work well with the Intracellular Fixation and Permeabilization Buffer Set (eBioscience Cat. No. 88-8824). Lastly, there are several phosphorylated signaling proteins that may not work in the two previously-mentioned buffer systems but will work with the Fixation/Methanol Protocol. Information about performance and preferred buffers is noted on the specific product's Technical Data Sheet. Please contact Technical Support (tech@ebioscience.com) for more information.

General Notes

1. For optimal performance of fluorochrome-conjugated antibodies, store vials at 4°C in the dark. *Do not freeze.*
2. Prior to use, quickly spin the antibody vial to recover the maximum volume. We do not recommend vortexing the antibody vial.
3. Except where noted in the protocol, all staining should be done on ice or at 4°C with minimal exposure to light.
4. The fixation and permeabilization steps that are required for the detection of intracellular antigens may alter the light scatter properties of cells and may increase non-specific background staining. Including extra protein such as BSA or FCS in the staining buffer may help reduce non-specific background. We also recommend the use of the Fixable Viability Dyes to help eliminate dead cells during the analysis.
5. As fixation and permeabilization will impact the brightness of eFluor nanocrystals, we recommend using a minimum fixation and permeabilization time followed by immediate analysis for optimal results. Some generalizations regarding nanocrystal performance after fixation can be made, but clone-specific performance should be determined empirically.

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Protocol A: Two-step protocol: intracellular (cytoplasmic) proteins

The following protocol allows the simultaneous analysis of cell surface molecules and intracellular antigens at the single-cell level. In this protocol, fixation is followed by permeabilization resulting in the creation of pores in the cell membrane that require the continuous presence of the permeabilization buffer during all subsequent steps to allow antibodies to have access to the cytoplasm of the cell. Thus, all intracellular staining must be done in the presence of the permeabilization buffer. This protocol is recommended for the detection of cytoplasmic proteins, cytokines, or other secreted proteins in individual cells following activation *in vitro* or *in vivo*. For cytokine detection, the appropriate stimulation conditions and kinetics of cytokine production will vary depending on the cell type and the particular cytokine being assayed. For example, to stimulate T cells to produce IFN-γ, TNF-α, IL-2, and IL-4, a combination of PMA (a phorbol ester, a protein kinase C activator) and Ionomycin (a calcium ionophore) or anti-CD3 antibodies can be used. To induce IL-6, IL-10, or TNF-α production by monocytes, stimulation with lipopolysaccharide (LPS) can be used. For *in vitro* stimulation of cells, it is necessary to block secretion of cytokines with protein transport inhibitors, such as Monensin or Brefeldin A Solution, during the final hours of the stimulation protocol. It is advised that investigators evaluate the use and efficacy of different protein transport inhibitors in their specific assay system.

For the detection of nuclear proteins such as transcription factors, please see **Protocol B** below. For detection of some phosphorylated signaling molecules such as MAPK and STAT proteins, it may be preferential to use **Protocol C**, below.

Materials

- 12x75 mm round bottom test tubes
- [Optional] Fixable Viability Dyes eFluor 450, 506, 660, or 780 (eBioscience Cat. No. 65-0863, 65-0866, 65-0864, 65-0865)
- Directly conjugated antibodies specific for intracellular proteins
- Intracellular Fixation and Permeabilization Buffer Set (eBioscience Cat. No. 88-8824)
- Flow Cytometry Staining Buffer (eBioscience Cat. No. 00-4222)
- Cell Stimulation Cocktail (plus protein transport inhibitors) (500X) (eBioscience Cat. No. 00-4975) or Protein Transport Inhibitor Cocktail (500X) (eBioscience Cat. No. 00-4980) or Brefeldin A Solution (eBioscience Cat. No. 00-4506) or Monensin Solution (eBioscience Cat. No. 00-4505).

Buffer and solution preparation

- Prepare a 1X working solution of Permeabilization Buffer by diluting the 10X concentrate with distilled water prior to use. You will need 8.5 mL of Permeabilization Buffer for each sample.

Experimental Procedure

1. Prepare cells of interest for evaluation of intracellular proteins. Refer to Best Protocols: 'Cell Preparation for Flow Cytometry'.
2. [Optional] To eliminate potential artifacts due to dead cell contamination, we recommend the use of a Fixable Viability Dye to allow the exclusion of dead cells from the analysis (See Best Protocols: 'Staining Dead Cells with eBioscience Fixable Viability Dyes' staining protocol for instructions for use).
3. Stain cell surface antigen(s) as described in Best Protocols for antibodies conjugated to organic fluorochromes: 'Staining cell surface antigens' protocol.

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4. After the last wash, discard the supernatant and pulse vortex the sample to completely dissociate the pellet. Typically about 100 µL residual volume remains.
5. Fix the cells by adding 100 µL of IC Fixation Buffer and pulse vortex.
6. Incubate tubes in the dark at room temperature for 20-60 minutes.
7. Without washing, add 2 mL of 1X Permeabilization Buffer to each tube.
8. Centrifuge samples at 300-400x*g* at room temperature for 5 minutes, then discard the supernatant.
9. Resuspend the cell pellet in 2 mL of 1X Permeabilization Buffer.
10. Centrifuge samples at 300-400x*g* at room temperature for 5 minutes, then discard the supernatant.
11. Resuspend the cells in 100 µL of 1X Permeabilization Buffer. Add the recommended amount of fluorochrome-labeled antibody for detection of intracellular antigen(s) to cells and incubate in the dark at room temperature for 20-60 minutes.
12. Add 2 mL of 1X Permeabilization Buffer to each tube.
13. Centrifuge samples at 300-400x*g* at room temperature for 5 minutes, then discard the supernatant.
14. Add 2 mL of Flow Cytometry Staining Buffer to each tube.
15. Centrifuge samples at 300-400x*g* at room temperature for 5 minutes, then discard the supernatant.
16. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer and acquire samples on a flow cytometer.

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Protocol B: One-step protocol: intracellular (nuclear) proteins

The following protocol allows the simultaneous analysis of cell surface molecules and intracellular antigens, including nuclear antigens, at the single-cell level. This protocol combines fixation and permeabilization into a single step. This protocol is recommended for the detection of nuclear antigens such as transcription factors but is also useful for the detection of many cytokines. For compatibility of the Foxp3/Transcription Factor Staining Buffer Set (eBioscience Cat. No. 00-5523) with cytokine antibodies, please see our Buffer Compatibility chart online: http://www.ebioscience.com/resources/application/flow-cytometry/antibody-fixation-considerations.htm.

Materials

- 12x75 mm round bottom test tubes or 96 well V or U bottom plate
- [Optional] Fixable Viability Dyes eFluor 450, 506, 660 and 780 (eBioscience Cat. No. 65-0863, 65-0866, 65-0864, 65-0865)
- [Optional] Normal Mouse Serum (eBioscience Cat. No. 24-5544)
- [Optional] Normal Rat Serum (eBioscience Cat. No. 24-5555)
- Directly conjugated antibodies specific for intracellular proteins
- Foxp3/Transcription Factor Staining Buffer Set (eBioscience Cat. No. 00-5523)
- Flow Cytometry Staining Buffer (eBioscience Cat. No. 00-4222)

Buffers and solution preparation

- Prepare fresh Foxp3 Fixation/Permeabilization working solution by diluting Foxp3 Fixation/Permeabilization Concentrate (1 part) with Foxp3 Fixation/Permeabilization Diluent (3 parts). You will need 1 mL of the Fixation/Permeabilization working solution for each sample.
- Prepare a 1X working solution of Permeabilization Buffer by diluting the 10X concentrate with distilled water prior to use. You will need 8.5 mL of Permeabilization Buffer for each sample, if staining in tubes.

Experimental Procedure in tubes

1. Prepare cells of interest for evaluation of intracellular proteins. Refer to Best Protocols: 'Cell Preparation for Flow Cytometry'.
2. [Optional] To eliminate potential artifacts due to dead cell contamination, we recommend the use of a Fixable Viability Dye to allow the exclusion of dead cells from the analysis (See Best Protocols: Protocol C: 'Staining Dead Cells with eBioscience Fixable Viability Dyes' staining protocol for instructions for use).
3. Stain cell surface antigen(s) as described in Best Protocols for antibodies conjugated to organic fluorochromes: 'Staining cell surface antigens' protocol.
4. After the last wash, discard the supernatant and pulse vortex the sample to completely dissociate the pellet.
5. Add 1 mL of Foxp3 Fixation/Permeabilization working solution to each tube and pulse vortex.
6. Incubate at in the dark at 4°C or room temperature for 30-60 minutes. (Mouse samples can be incubated for up to 18 hours at 4°C in the dark).
7. Without washing, add 2 mL of 1X Permeabilization Buffer to each tube.
8. Centrifuge samples at 300-400x*g* at room temperature for 5 minutes, then discard the supernatant.
9. [Optional] Repeat Steps 7-8.

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10. Resuspend pellet in 100 µL of 1X Permeabilization Buffer. This is typically the residual volume after decanting.
11. [Optional] Block with 2% normal mouse/rat serum by adding 2 µL directly to the cells. Incubate at room temperature for 15 minutes.
12. Without washing, add the recommended amount of fluorochrome-conjugated antibody for detection of intracellular antigen(s) to cells and incubate in the dark at room temperature for at least 30 minutes.
13. Add 2 mL of 1X Permeabilization Buffer to each tube.
14. Centrifuge samples at 300-400x*g* at room temperature for 5 minutes, then discard the supernatant.
15. Add 2 mL of 1X Permeabilization Buffer or Flow Cytometry Staining Buffer to each tube.
16. Centrifuge samples at 300-400x*g* at room temperature for 5 minutes, then discard the supernatant.
17. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer and acquire samples on a flow cytometer.

Experimental Procedure in 96 Well Plate

1. Prepare cells of interest for evaluation of intracellular proteins. Refer to Best Protocols: 'Cell Preparation for Flow Cytometry'.
2. [Optional] To eliminate potential artifacts due to dead cell contamination, we recommend the use of a Fixable Viability Dye to allow the exclusion of dead cells from the analysis (See Best Protocols: Protocol C: 'Staining Dead Cells with eBioscience Fixable Viability Dyes' staining protocol for instructions for use).
3. Stain cell surface antigen(s) as described in Best Protocols for antibodies conjugated to organic fluorochromes: 'Staining cell surface antigens' protocol.
4. After the last wash, discard the supernatant and pulse vortex the sample to completely dissociate the pellet.
5. Add 200 µL of Foxp3 Fixation/Permeabilization working solution to each well. It is ideal to add the solution such that the cells are fully resuspended in the solution. Pipetting is an option.
6. Incubate in the dark at room temperature for 30-60 minutes. (Mouse samples can be incubated for up to 18 hours at 4°C in the dark).
7. Centrifuge samples at 300-400x*g* at room temperature for 5 minutes, then discard the supernatant.
8. Add 200 µL 1X Permeabilization Buffer to each well.
9. Centrifuge samples at 300-400x*g* at room temperature for 5 minutes, then discard the supernatant.
10. Repeat Steps 8-9.
11. Resuspend pellet in residual volume and adjust volume to about 100 µL with 1X Permeabilization Buffer.
12. [Optional] Block with 2% normal mouse/rat serum by adding 2 µL directly to the cells. Incubate at room temperature for 15 minutes.
13. Without washing, add the recommended amount of fluorochrome-conjugated antibody for detection of intracellular antigen(s) to cells and incubate in the dark at room temperature for at least 30 minutes.
14. Add 200 µL of 1X Permeabilization Buffer to each well.
15. Centrifuge samples at 300-400x*g* at room temperature for 5 minutes, then discard the supernatant.
16. Add 200 µL of 1X Permeabilization Buffer or Flow Cytometry Staining Buffer to each well.

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17. Centrifuge samples at 300-400x*g* at room temperature for 5 minutes, then discard the supernatant.
18. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer and acquire samples on a flow cytometer.

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Protocol C: Two-step protocol: Fixation/Methanol

The following protocol allows for the simultaneous analysis of cell surface molecules and some intracellular phosphorylated signaling proteins. In this protocol, fixation is followed by treatment of cells with Methanol. For phospho-protein detection, the appropriate stimulation conditions and kinetics of phosphorylation will vary depending on the cell type and the particular signaling event being assayed. For example, to induce phospho-STAT1 (Y701) phosphorylation, macrophages can be activated with IFNγ or IFNα, while phospho-ERK1/2 (T202/Y204) is induced in T cells in response to PMA (a phorbol ester, a protein kinase C activator) or CD3 antibodies.

General Notes

1. Fluorochrome-conjugated antibodies can be used to stain surface proteins for the purpose of immunophenotyping cells that will be further analyzed for phosphorylated proteins, however, additional considerations for staining are warranted.

    - Antibody staining for surface markers on live cells has been shown to alter expression of signaling proteins due to possible stimulation/suppression of signaling events. Because of this, surface staining is not recommended prior to cell stimulation. Instead, stain surface proteins at the same step as the intracellular protein staining. Please note that some proteins will also have intracellular pools, in addition to surface localization, which should be considered. Antibody clones to surface proteins that will recognize fixed cells/epitopes will need to be evaluated and used. Refer to http://us.ebioscience.com/resources/application/flow-cytometry/clone-performance-after-fix-perm.htm in Technical Support Resources.

    - If surface staining is required prior to the fixation step in Step 5 (due to epitope destruction), cells may be stained with fluorochrome-conjugated antibodies before the Fixation/MeOH steps only if the conjugated fluorochromes are resistant to methanol exposure.

MeOH Resistant Fluorochromes	MeOH Sensitive Fluorochromes
Alexa Fluor 488	PE
eFluor 660	PE-tandems
Alexa Fluor 647	PerCP
eFluor 450	PerCP-tandems
FITC	APC
	APC-tandems

2. For adherent cells, we recommend fixing the cells (Step 5) in the plates/well. After fixation, scrape cells or treat with EDTA solution to harvest and continue with protocol. Trypsin can be used if you are not staining for surface antibodies or you know your surface protein is resistant to tryspin digestion.

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Materials

- 12x75 mm round bottom test tubes or 96-well round or V- bottom microtiter plates
- Primary antibodies (directly conjugated)
- eBioscience Flow Cytometry Staining Buffer (Cat. No. 00-4222)
- eBioscience IC Fixation Buffer (cat. 00-8222)
- 90-100% Methanol (HPLC grade)
- Optional Fc Block: Anti-Mouse CD16/CD32 Purified (eBioscience Cat. No. 14-0161) or Human Fc Receptor Binding Inhibitor Purified (eBioscience Cat. No. 14-9161)

Experimental Procedure

1. Prepare cells of interest for stimulation in appropriate media.
2. Count cells and resuspend in appropriate media at ~1-5x10^6 cells/mL.
3. Stimulate cells at 37°C with appropriate treatment for desired time point(s). Remember to incubate untreated cells at 37°C as a negative control.
4. [Optional] If surface staining is needed prior to fixation (in Step 5), stain cell surface antigen(s) as described in Best Protocols 'Staining Cell Surface Antigens' for antibodies conjugated to methanol-resistant fluorochromes.
5. At the end of the stimulation period, fix cells to stop stimulation by adding an equal volume of IC Fixation Buffer directly to cells and vortex.
6. Incubate cells in the dark at room temperature for 10-60 minutes.
7. Centrifuge cells at 600*xg* at room temperature for 4-5 minutes, then discard supernatant.
8. Resuspend the cell pellet in residual volume and add 1 mL ice-cold 90-100% methanol, vortex, and incubate at 4°C or on ice for at least 30 minutes.
   NOTE: Once in methanol, cells can be stored at ≤-20°C for up to 4 weeks.
9. Wash cells with an excess volume of Flow Cytometry Staining Buffer.
10. Centrifuge cells at 600*xg* at room temperature for 4-5 minutes, then discard supernatant.
11. Resuspend cells at 1x10^7 cells/mL in Flow Cytometry Staining Buffer.
12. Aliquot 1x10^6 cells (100 µL) into separate flow tubes.
13. [Optional] Cells can be blocked for nonspecific Fc-mediated binding using Anti-Mouse CD16/CD32 Purified or Human Fc Receptor Binding Inhibitor Purified prior to staining.
14. Add the recommended amount of fluorochrome-labeled antibody to each tube and incubate in the dark at room temperature for 30-60 minutes.
    NOTE: If needed, surface staining and intracellular phospho staining can be performed simultaneously. As not all antibody clones will bind to a fixed epitope, please refer to online table for antibody clones that will stain cells after fixation and methanol treatment.
15. Wash cells with 2 mL Flow Cytometry Staining Buffer and centrifuge at 600*xg* at room temperature for 4-5 minutes. Repeat step 15.
16. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer and acquire samples on a flow cytometer.

Experimental Procedure in 96 Well Plate

1. Prepare cells of interest for stimulation in appropriate media.
2. Count cells and resuspend in appropriate media at ~1-5x10^6 cells/mL.
3. Add 100 µL appropriate treatment to wells in a 96 well plate.
4. Add 100 µL cells to wells and stimulate cells at 37°C for desired time point(s). Remember to incubate untreated cells at 37°C as a negative control.

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5. [Optional] If surface staining is needed prior to fixation (in Step 5), stain cell surface antigen(s) as described in Best Protocols 'Staining Cell Surface Antigens' for antibodies conjugated to methanol resistant fluorochromes.
6. At the end of the stimulation period, fix cells to stop stimulation by adding 200 µL of IC Fixation Buffer directly to wells.
7. Incubate plate in the dark at room temperature for 10-60 minutes.
8. Centrifuge plate at 600*xg* at room temperature for 4-5 minutes, then discard supernatant.
9. Resuspend the cell pellets in residual volume and add 100 µL ice-cold 90-100% methanol to wells, vortex, and incubate plate at 4°C or on ice for at least 30 minutes.
   NOTE: Once in methanol, cells can be stored at ≤-20°C for up to 4 weeks.
10. Wash cells with 200 µL Flow Cytometry Staining Buffer.
11. Centrifuge cells at 600*xg* at room temperature for 4-5 minutes, then discard supernatant.
12. Repeat steps 10 and 11.
13. [Optional] Cells can be blocked for nonspecific Fc-mediated binding using Anti-Mouse CD16/CD32 Purified or Human Fc Receptor Binding Inhibitor Purified prior to staining.
14. Add the recommended amount of fluorochrome-labeled antibody to each well and incubate for in the dark at room temperature 30-60 minutes.
    NOTE: If needed, surface staining and intracellular phospho staining can be performed simultaneously. As not all antibody clones will bind to a fixed epitope, please refer to online table for antibody clones that will stain cells after fixation and methanol treatment.
15. Wash wells with 200 µL Flow Cytometry Staining Buffer and centrifuge at 600*xg* for 4-5 minutes. Repeat step 15.
16. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer and acquire samples on a flow cytometer.

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