Intracellular antibody staining protocols

Protocol A: 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 as well as allowing unbound antibody out 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.

For the detection of nuclear proteins such as transcription factors, please see **Protocol B: Intracellular (nuclear) proteins** (next page). 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
- Fixable Viability Dyes eFluor® 455UV, 450, 506, 520, 660 and 780 (cat. no. 65-0868, 65-0863, 65-0866, 65-0867, 65-0864, 65-0865)
- Directly conjugated antibodies specific for intracellular proteins
- Intracellular Fixation and Permeabilization Buffer Set (cat. no. 88-8824)
- Flow Cytometry Staining Buffer (cat. no. 00-4222)
- Cell Stimulation Cocktail (plus protein transport inhibitors) (500X) (cat. no. 00-4975) or Protein Transport Inhibitor Cocktail (500X) (cat. no. 00-4980) or Brefeldin A Solution (cat. no. 00-4506) or Monensin Solution (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' on our website.*
2. 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 analysis.
3. Stain cell surface antigen(s) as described in Best Protocols 'Staining cell surface antigens' protocol.
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 300-400 x 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 300-400 x 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-conjugated 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 300-400 x 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 300-400 x 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.

Protocol B: 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 (cat. no. 00-5523) with cytokine antibodies, please see our **Buffer Compatibility Chart online: ebioscience.com/resources/application/flow-cytometry/intracellular-staining-buffer-guide.htm.**

Materials

- 12x75 mm round bottom test tubes or 96 well V or U bottom plate
- Fixable Viability Dyes eFluor® 455UV, 450, 506, 520, 660 and 780 (cat. no. 65-0868, 65-0863, 65-0866, 65-0867, 65-0864, 65-0865)
- [Optional] Normal Mouse Serum (cat. no. 24-5544)
- [Optional] Normal Rat Serum (cat. no. 24-5555)
- Directly conjugated antibodies specific for intracellular proteins
- Foxp3/Transcription Factor Staining Buffer Set (cat. no. 00-5523)
- Flow Cytometry Staining Buffer (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' on our website.*
2. 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
3. Stain cell surface antigen(s) as described in Best Protocols 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 300-400 x g at room temperature for 5 minutes, then discard the supernatant.
9. [Optional] Repeat Steps 7-8.
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 300-400 x 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 300-400 x 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' on our website.*
2. 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
3. Stain cell surface antigen(s) as described in Best Protocols '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 300-400 x 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 300-400 x 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 300-400 x 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.
17. Centrifuge samples at 300-400 x 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.

Protocol C: Fixation/Methanol (phospho-specific proteins)

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 Anti-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. Antibody clones to surface proteins that will recognize fixed epitopes will need to be evaluated and used. *Refer to Page 13 for antibodies tested at eBioscience; otherwise performance will need to be determined empirically.*

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

Methanol Resistant Fluorochromes	Methanol 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 an EDTA solution to harvest and then continue with the protocol. Trypsin can be used if you are not staining for surface antibodies or you know your surface protein is resistant to tryspin digestion.

Materials

- 12 x 75 mm round bottom test tubes or 96-well round or V-bottom microtiter plates
- Primary antibodies (directly conjugated)
- Flow Cytometry Staining Buffer (cat. no. 00-4222)
- IC Fixation Buffer (cat. no. 00-8222)
- 90-100% Methanol (HPLC grade)
- [Optional] Fc Receptor Block: Anti-Mouse CD16/CD32 Purified (cat. no. 14-0161) or Human Fc Receptor Binding Inhibitor Purified (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' using 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 x g at room temperature for 4-5 minutes, then discard supernatant.

8. Resuspend the cell pellet in residual volume and add 1 mL of 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 x g at room temperature for 4-5 minutes, then discard supernatant.
11. Resuspend cells at $1x10^6$ 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 receptor-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-conjugated antibody to each tube and incubate in the dark at room temperature for 30-60 minutes.

NOTE: If needed, surface staining and intracellular staining can be performed simultaneously. Please refer to Page 13 table for antibody clones that will stain cells after fixation and methanol treatment.

15. Add 2 mL of Flow Cytometry Staining Buffer and centrifuge at 600 x g at room temperature for 4-5 minutes. Discard supernatant.
16. Repeat step 15.
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 stimulation in appropriate media.
2. Count cells and resuspend in appropriate media at $1\text{-}5x10^6$ cells/mL.
3. Add 100 µL of appropriate treatment to wells in a 96-well plate.
4. Add 100 µL of 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.
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' using 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 x g 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. Add 200 µL Flow Cytometry Staining Buffer. Centrifuge cells at 600 x g at room temperature for 4-5 minutes, then discard supernatant.
11. Repeat step 10.
12. [Optional] Cells can be blocked for nonspecific Fc Receptor-mediated binding using Anti-Mouse CD16/CD32 Purified or Human Fc Receptor Binding Inhibitor Purified prior to staining.
13. Add the recommended amount of fluorochrome-conjugated antibody to each well and incubate in the dark at room temperature for 30-60 minutes.

NOTE: If needed, surface staining and intracellular staining can be performed simultaneously. Please refer to table on page 13 for antibody clones that will stain cells after fixation and methanol treatment.

14. Add 200 µL of Flow Cytometry Staining Buffer and centrifuge at 600 x g for 4-5 minutes.
15. Repeat step 14.
16. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer and acquire samples on a flow cytometer.