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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. In this protocol, cells are first stained for surface antigens as in the surface antigen staining protocol, then fixed with formaldehyde to stabilize the cell membrane and permeabilized with the detergent saponin to allow anti-cytokine antibodies to stain intracellularly. *In vitro* stimulation of cells is usually required for detection of cytokines by flow cytometry since cytokine levels are typically too low in resting cells. Stimulation of cells with the appropriate reagent will depend on the cell type and the experimental conditions. For example, to stimulate T cells to produce IFN-γ, TNF-α, IL-2, and IL-4, a combination of PMA (a phorbol ester, 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.

Note: The optimal stimulation condition for induction of a given cytokine is variable and must be determined empirically. For example, the best time for detection of IL-6-producing cells by human LPS-activated monocytes is 6 hours, whereas detection of IL-10 requires stimulation for at least 24 hours.

In contrast to detection of secreted cytokines by ELISA, it is necessary to block secretion of cytokines with protein transport inhibitors, such as Monensin or Brefeldin A, during the last few hours of the stimulation for detection of intracellular cytokines by flow cytometry. It is advised that investigators evaluate the use and efficacy of different protein transport inhibitors in their specific assay system.

The fixation and permeabilization buffers used for intracellular staining can have varying effects. eBioscience antibodies are optimized for use with the Foxp3 Staining Buffer Set (eBioscience Cat. No. 00-5523) or IC Fixation Buffer (eBioscience Cat. No. 00-8222) and Permeabilization Buffer (10X) (eBioscience Cat. No. 00-8333). 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, quick 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 and may increase non-specific background staining. Including extra protein such as BSA or FCS in the staining buffer can help reduce the non-specific background. We also recommend the use of the Fixable Viability Dyes to help in 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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Useful websites

Mario Roederer's Home Page (http://www.drmr.com/compensation/index.html)
Mario Roederer is a key opinion leader in the field of flow cytometry.

Purdue University Cytometry Laboratories (http://www.cyto.purdue.edu/index.htm)
Flow Cytometry based public forum maintained by the Purdue University.

Protocol A: Two-step protocol for 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. This results in the creation of pores in the cellular 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. This mandates that all intracellular staining be done in the presence of the permeabilization buffer. This protocol is recommended for detecting 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 *in vitro* stimulation of cells, it is necessary to block secretion of cytokines with protein transport inhibitors, such as the Monensin or Brefeldin A Solution, during the final hours of the stimulation protocol. For the detection of nuclear proteins such as transcription factors, please see **Protocol B** below.

Materials

- 12x75 mm round bottom test tubes
- [Optional] Fixable Viability Dyes eFluor 450, 506, 660 and 780 (eBioscience Cat. No. 65-0863, 65-0866, 65-0864, 65-0865)
- Directly conjugated antibodies specific for intracellular proteins
- IC Fixation Buffer (eBioscience Cat. No. 00-8222)
- Permeabilization Buffer (10X) (eBioscience Cat. No. 00-8333)
- 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 BestProtocols: '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

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(See BestProtocols: 'Staining Dead Cells with eBioscience Fixable Viability Dyes' staining protocol for instructions for use).

3. Stain cell surface antigen(s) as described in BestProtocols 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. Typically about 100 µL residual volume remains.
5. Fix the cells by adding 100 µL of IC Fixation Buffer while vortexing the tube. Minimize vortexing time.
6. Incubate in the dark at room temperature for 20 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 to each tube. Add the recommended amount of fluorochrome-labeled antibody for detection of intracellular antigen to cells and incubate in the dark at room temperature for 20 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 data on a flow cytometer.

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

The following protocol allows the simultaneous analysis of cell surface molecules and intracellular antigens at the single-cell level. This protocol combines fixation and permeabilization into a single step and does not require continuous exposure to permeabilization buffer for intracellular staining. This protocol is recommended for detecting nuclear antigens such as transcription factors but is also useful for detecting many cytokines. For compatibility of the Foxp3 Fixation/Permeabilization buffer 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
- [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 Fixation/Permeabilization Concentrate and Diluent (eBioscience Cat. No. 00-5521)
- Flow Cytometry Staining Buffer (eBioscience Cat. No. 00-4222)
- Permeabilization Buffer (10X) (eBioscience Cat. No. 00-8333)

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.

Experimental Procedure

1. Prepare cells of interest for evaluation of intracellular proteins. Refer to BestProtocols: '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 BestProtocols: 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 BestProtocols 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 4°C or room temperature for 30-60 minutes in the dark. (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.

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9. [Optional] Repeat Steps 7-8.
10. Resuspend pellet in 100 µL of 1X Permeabilization buffer.
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 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 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 data on a flow cytometer.

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