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Staining Cell Surface Antigens for Flow Cytometry

Research Use Only

Introduction

Flow cytometry is a useful tool for simultaneously measuring multiple physical properties of individual particles (such as cells). Cells pass single-file through a laser beam. As each cell passes through the laser beam, the cytometer records how the cell or particle scatters incident laser light and emits fluorescence. Using this flow cytometric analysis protocol, one can perform a simultaneous analysis of surface molecules at the single-cell level.

General Notes

1. For optimal performance of fluorochrome conjugated antibodies, store vials at 2-8°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 at 2-8°C with minimal exposure to light.
4. If you do need to store your samples that were stained with antibodies conjugated to organic fluorochromes, we recommend you complete your staining protocol and fix your samples with IC Fixation Buffer (cat. 00-8222) (100 µL of sample with 100 µL of IC Fixation Buffer) or 2 mL of 1-step Fix/Lyse Solution (cat. 00-5333). Cells can be stored in these buffers for up to 3 days in the dark at 2-8°C.
    - We have observed minimal impact on brightness or FRET efficiency/compensation when using the IC Fixation (cat. 00-8222) or 1-step Fix/Lyse Solution (cat. 00-5333). Differences in fixation buffer quality can affect fluorochrome brightness or FRET efficiency.
    - Fixation of tandem dyes, such as APC-eFluor® 780 and PE-Cy7, does not significantly increase the amount of compensation required from the APC or PE detector, respectively. Some generalizations regarding fluorophore performance after fixation can be made, but clone specific performance should be determined empirically.

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.

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Protocol A: Cell suspensions

Materials

- 12x75 mm round bottom test tubes or 96-well round bottom microtiter plates
- Primary antibodies (directly conjugated or purified)
- Secondary reagents, if necessary (for indirect staining)
- Anti-Mouse CD16/CD32 Purified (Cat. No. 14-0161)
- Human Fc Receptor Binding Inhibitor Purified (Cat. No. 14-9161)
- Flow Cytometry Staining Buffer (Cat. No. 00-4222)
- [Optional] Viability solutions: 7-AAD Viability Staining Solution (Cat. No. 00-6993), Propidium Iodide Staining Solution (Cat. No. 00-6990), or Fixable Viability Dyes eFluor® 450, 506, 660 and 780 (Cat. No. 65-0863, 65-0866, 65-0864, 65-0865)

Experimental Procedure

1. Prepare cells as described in Cell Preparation for Flow Cytometry Protocols found in our Best Protocols section. Consider the use of Fixable Viability Dyes before beginning the antibody staining protocol (Refer to Viability Staining Protocol in our Best Protocols section).
2. [Optional] Block non-specific Fc-mediated interactions.
    a) For mouse cells: Pre-incubate the cells with 0.5-1 µg of Anti-Mouse CD16/CD32 Purified per 100 µL for 10-20 minutes at 2-8°C or room temperature prior to staining.
    b) For human cells: Pre-incubate the cells with 20 µL of Human Fc Receptor Binding Inhibitor Purified per 100 µL for 10-20 minutes at 2-8°C or room temperature prior to staining.
3. Aliquot 50 µL of cell suspension (from $2x10^5$-10^8) to each tube or well.
4. Combine the recommended quantity of each primary antibody in an appropriate volume of Flow Cytometry Staining Buffer so that the final staining volume is 100 µL (i.e. 50 µL of cell sample + 50 µL of antibody mix) and add to cells. Pulse vortex gently to mix.

For directly conjugated antibodies:

5. Incubate for at least 30 minutes at 2-8°C or on ice. Protect from light.
   ***Note**: Antibody binding kinetics are temperature dependent. Staining on ice may require longer incubation times. Furthermore, some antibodies require non-standard incubation conditions that will be noted on the technical data sheet provided with the antibody.*

For purified antibodies:

6. Incubate for at least 60 minutes at 2-8°C or on ice.
   ***Note**: Antibody binding kinetics are temperature dependent. Staining on ice may require longer incubation times. Furthermore, some antibodies require non-standard incubation conditions that will be noted on the technical data sheet provided with the antibody.*

7. Wash the cells by adding Flow Cytometry Staining Buffer. Use 2 mL for tubes or 200 µL/well for microtiter plates. Pellet the cells by centrifugation at 400-600x*g* for 5 minutes

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at room temperature. Repeat for a total of two washes, discarding supernatant between washes.

For directly conjugated antibodies:

8. If all of the antibodies used for staining were directly conjugated to a fluorochrome, continue with Step 11.

For purified or biotin-conjugated antibodies:

9. If purified or biotin-conjugated primary antibodies were used, add the appropriate fluorochrome-labeled second step reagent diluted in 100 µL of Flow Cytometry Staining Buffer to the cells and incubate for at least 30 minutes at 2-8°C or on ice. Protect from light.
   ***Note**: Antibody binding kinetics are temperature dependent. Staining on ice may require longer incubation times. Furthermore, some antibodies require non-standard incubation conditions that will be noted on the technical data sheet provided with the antibody.*

10. Wash the cells by adding Flow Cytometry Staining Buffer. Use 2 mL for tubes or 200 µL/well for microtiter plates. Pellet the cells by centrifugation at 400-600x*g* for 5 minutes at room temperature. Repeat for a total of two washes, discarding supernatant between washes.
11. [Optional] If cells were not prelabeled with a Fixable Viability Dye in Step 1, stain samples with a viability dye, according to the Viability Staining Protocol in our Best Protocols section for the viability dye of your choice.
12. [Optional] Resuspend cells in 100 µL of Flow Cytometry Staining and add 100 µL of IC Fixation Buffer or 2 mL of 1-step Fix/Lyse Solution. Cells may be stored in this buffer for up to 3 days at 2-8°C in the dark.
13. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer.
14. Acquire data on a flow cytometer.

Protocol B: Human Lysed Whole Blood

General Notes

1. eBioscience offers two solutions for preparing whole blood samples for analysis by flow cytometry.
   - **eBioscience® 10X RBC Lysis Buffer (Multi-species)** simply lyses the red blood cells in the sample leaving live leukocytes for analysis.
   - **1-step Fix/Lyse Solution (10X)** both lyses the red blood cells and fixes the sample.
2. This protocol is written such that whole blood is stained for flow cytometry before RBC lysis. Alternatively, whole blood can be lysed in bulk before staining using a ratio of 2 mL of lysing solution [**eBioscience**® 10X RBC Lysis Buffer (Multi-species) (diluted to 1X) or **1-step Fix/Lyse Solution** (diluted to 1X)] for every 100 µL of blood. Refer to Red Blood Cell Lysis Protocol for a detailed protocol.
   ***Note**: If lysed whole blood cells will be prepared before staining using the **1-step Fix/Lyse Solution**, you will need to confirm that the antibodies in your staining panel recognize fixed epitopes on the antigens of interest. Please refer to our Antibody Fixation Considerations table online for antibody clone performance following fixation/permeabilization.*

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Materials

- eBioscience® 10X RBC Lysis Buffer (Multi-species) (Cat. No. 00-4300) or 1-step Fix/Lyse Solution (10X) (Cat. No. 00-5333).
  - Before using, the 10X RBC Lysis Buffer (Multi-species) must be diluted to 1X by adding 1 part buffer with 9 parts room temperature reagent grade water.
  - Before using, the eBioscience 1-step Fix/Lyse Solution (10X) must be diluted to 1X by adding 1 part buffer with 9 parts room temperature reagent grade water.
- 12x75 mm round bottom test tubes
- Primary antibodies (directly conjugated or purified)
- Secondary reagents, if necessary (for indirect staining)
- Human Fc Receptor Binding Inhibitor Purified (Cat. No. 14-9161)
- Flow Cytometry Staining Buffer (Cat. No. 00-4222)
- [Optional] Viability solutions: 7-AAD Viability Staining Solution (Cat. No. 00-6993), Propidium Iodide Staining Solution (Cat. No. 00-6990), or Fixable Viability Dyes eFluor® 450, 506, 660 and 780 (Cat. No. 65-0863, 65-0866, 65-0864, 65-0865)

Experimental Procedure

1. Aliquot 100 µL of whole blood to each tube.
2. [Optional] Pre-incubate the cells with 20 µL of Human Fc Receptor Binding Inhibitor Purified per 100 µL of blood for 10-20 minutes at 2-8°C or room temperature prior to staining.
3. Combine the recommended quantity of each primary antibody in an appropriate volume of Flow Cytometry Staining Buffer so that the final volume of antibody mix is 50 µL. Add to cells and pulse vortex gently to mix. Incubate for 20-30 minutes at room temperature. Protect from light.
   ***Note**: Antibody binding kinetics are temperature dependent. Staining on ice may require longer incubation times. Furthermore, some antibodies require non-standard incubation conditions that will be noted on the technical data sheet provided with the antibody.*

For directly conjugated antibodies:

4. If all of the antibodies used for staining were directly conjugated to a fluorochrome, continue with Step 6.

For purified or biotin conjugated antibodies:

5. If purified or biotin conjugated primary antibodies were used, wash cells with 2 mL of Flow Cytometry Staining Buffer. Pellet the cells by centrifugation at 400-600x*g* for 5 minutes at room temperature. Add the appropriate fluorochrome-labeled second step reagent diluted in 100 µL of Flow Cytometry Staining Buffer to the cells and incubate for 15-30 minutes at 2-8°C or on ice. Protect from light. Continue with Step 6.
   ***Note**: Antibody binding kinetics is temperature dependent. Staining on ice may require longer incubation times. Furthermore, some antibodies require non-standard incubation conditions that will be noted on the technical data sheet provided with the antibody.*

6. Without washing cells, add 2 mL of freshly prepared 1X RBC lysing solution prepared as indicated above and pulse vortex briefly. Incubate for 10-20 minutes at room temperature. Protect from light.
   ***Note**: Do not exceed 20 minutes of incubation with RBC Lysis Buffer.*

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7. Centrifuge samples at 400-600x*g* for 5 minutes at room temperature, discard supernatant.
8. Wash the cells twice with 2 mL of Flow Cytometry Staining Buffer. Pellet the cells by centrifugation at 400-600x*g* for 5 minutes at room temperature.
9. [Optional] For cells lysed using the 10X RBC Lysis buffer, dead cells may be excluded from analysis using a viability dye. Follow the Viability Staining Protocol in our Best Protocols section for the viability dye of your choice. Viability dyes may not be used on cells lysed using the 1-Step Fix/Lyse Solution as fixation can cause permeabilization of the cells.
10. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer.
11. Acquire data on a flow cytometer.

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