

Staining Cell Surface Antigens for Flow Cytometry

Research Use Only

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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 4°C in the dark. *Do not freeze.*
2. Prior to use, quick spin the antibody vial to recover the maximum volume.
3. Except where noted in the protocol, all staining should be done on ice or at 4°C with minimal exposure to light.
4. Modifications relevant to staining with eFluor® nanocrystal (NC) reagents are noted in the general protocol by **bold print**.

Useful websites

Mario Roederer's Home Page (<http://www.drmm.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: 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)
- Affinity Purified Anti-Mouse CD16/32 (eBioscience Cat. No. [14-0161](#))
- Affinity Purified Human FcγR-Binding Inhibitor (eBioscience Cat. No. [14-9161](#))
- eBioscience® Flow Cytometry Staining Buffer (eBioscience Cat. No. [00-4222](#))
- **eFluor® NC Flow Cytometry Staining Buffer** (eBioscience Cat. No. [00-3222](#))
- 7-AAD Viability Staining Solution (eBioscience Cat. No. [00-6993](#)) or Propidium Iodide Staining Solution (eBioscience Cat. No. [00-6990](#))

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Experimental Procedure

1. Prepare cells as described in Section I – Cell Preparation. **For staining panels that include nanocrystal reagents, cells should be resuspended in eFluor® NC Flow Cytometry Staining Buffer so that the final cell concentration is 2×10^7 /ml.**
2. [Optional] Block non-specific Fc-mediated interactions.
 - a) For mouse cells: Pre-incubate the cells with 0.5-1 µg of purified anti-mouse CD16/CD32 per 100 µl for 20 minutes on ice prior to staining.
 - b) For human cells: Pre-incubate the cells with 20 µl of Affinity Purified Human FcγR-Binding Inhibitor per 100 µl for 20 minutes on ice prior to staining.
3. Aliquot 50 µl of cell suspension to each tube or well.
4. Combine the recommended quantity of each primary antibody in an appropriate volume of Flow Cytometry Staining Buffer (**eFluor® NC 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. Vortex gently to mix.

For directly conjugated antibodies:
5. Incubate for at least 30 minutes in the dark on ice or at 4°C.

For purified antibodies:
6. Incubate for at least 60 minutes in the dark on ice or at 4°C.

Note: 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 (**eFluor® NC Flow Cytometry Staining Buffer**). Use 2 ml for tubes or 200 µl/well for microtiter plates. Pellet the cells by centrifugation at 300-400 xg at 4°C for 5 minutes. 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, the cells may be resuspended in Flow Cytometry Staining Buffer (**eFluor® NC Flow Cytometry Staining Buffer**).

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 Flow Cytometry Staining Buffer (**eFluor® NC Flow Cytometry Staining Buffer**) to the cells and incubate for 15-30 minutes in the dark on ice or at 4°C.
10. Wash the cells by adding Flow Cytometry Staining Buffer (**eFluor® NC Flow Cytometry Staining Buffer**). Use 2 ml for tubes or 200 µl/well for microtiter plates. Pellet the cells by centrifugation at 300-400 x g at 4°C for 5 minutes. Repeat for a total of two washes, discarding supernatant between washes.
11. Resuspend stained cells in Flow Cytometry Staining Buffer (**eFluor® NC Flow Cytometry Staining Buffer**).
12. [Optional] Add a viability dye, either 7-AAD (5 µl/sample) or PI (5 µl/sample) to each sample to exclude dead cells from analysis.
13. Acquire data on a flow cytometer.

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Protocol B: Human Lysed Whole Blood

General Notes

1. eBioscience offers two solutions for preparing whole blood samples for analysis by flow cytometry. **eBioscience® 1X RBC Lysis Buffer** simply lyses the red blood cells in the sample leaving live cells for analysis while **1-step Fix/Lyse Solution** both lyses the red blood cells and fixes the sample.
2. This protocol is written such that whole blood is stained for flow cytometry prior to RBC lysis. Alternatively, whole blood can be lysed in bulk prior to staining using a ratio of 2 ml of lysing solution (**eBioscience® 1X RBC Lysis Buffer** or **1-step Fix/Lyse Solution**) for every 100 µl of blood.
3. If cells will be prepared in bulk prior to staining with **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.

Materials

- eBioscience® 1X RBC Lysis Buffer (eBioscience Cat. No. [00-4333](#)) or 1-Step Fix/Lyse Solution (eBioscience Cat. No. [00-5333](#))
- 12x75 mm round bottom test tubes
- Primary antibodies (directly conjugated or purified)
- Secondary reagents, if necessary (for indirect staining)
- Affinity Purified Human FcγR-Binding Inhibitor (eBioscience Cat. No. [14-9161](#))
- eBioscience® Flow Cytometry Staining Buffer (eBioscience Cat. No. [00-4222](#))
- **eFluor® NC Flow Cytometry Staining Buffer** (eBioscience Cat. No. [00-3222](#))

Experimental Procedure

1. Aliquot 100 µl of whole blood to each tube or well.
2. [Optional] Pre-incubate the cells with 20 µl of Affinity Purified Human FcγR-Binding Inhibitor per 100 µl of blood for 20 minutes on ice prior to staining.
3. Combine the recommended quantity of each primary antibody in an appropriate volume of Flow Cytometry Staining Buffer (**eFluor® NC Flow Cytometry Staining Buffer**) so that the final volume of antibody mix is 50 µl. Add to cells and vortex gently to mix.
4. Incubate for 20-30 minutes in the dark on ice or at 4°C.
Note: Some antibodies require non-standard incubation conditions that will be noted on the technical data sheet provided with the antibody).
5. Without washing cells, add 2 ml of room temperature 1X RBC Lysis Buffer or 1X 1-Step Fix/Lyse Solution to each tube (200 µl/well for microtiter plates) and gently pipet up and vortex briefly.

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Incubate in the dark at room temperature for 15 minutes. Do not exceed 15 minutes of incubation with RBC Lysis Buffer.

6. Centrifuge samples at 300-400 x *g* at 4°C for 5 minutes, discard supernatant.
7. Wash the cells twice with Flow Cytometry Staining Buffer (**eFluor® NC Flow Cytometry Staining Buffer**). Use 2 ml for tubes or 200 µl/well for microtiter plates. Pellet the cells by centrifugation at 300-400 x *g* at 4°C for 5 minutes.

For directly conjugated antibodies:

8. If all of the antibodies used for staining were directly conjugated to a fluorochrome, the cells may be resuspended in Flow Cytometry Staining Buffer (**eFluor® NC Flow Cytometry Staining Buffer**)

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 Flow Cytometry Staining Buffer (**eFluor® NC Flow Cytometry Staining Buffer**) to the cells and incubate for 15-30 minutes in the dark on ice or at 4°C.
10. Wash the cells with Flow Cytometry Staining Buffer (**eFluor® NC Flow Cytometry Staining Buffer**). Use 2 ml for tubes or 200 µl/well for microtiter plates. Pellet the cells by centrifugation at 300-400 x *g* at 4°C for 5 minutes. Repeat for a total of two washes, discarding supernatant between washes.
11. Resuspend stained cells in Flow Cytometry Staining Buffer (**eFluor® NC Flow Cytometry Staining Buffer**).
12. [Optional] For cells lysed with 1X RBC Lysis buffer, add a viability dye, either 7-AAD (5 µl/sample) or PI (5 µl/sample) to each sample in order to exclude dead cells from analysis.
13. Acquire data on a flow cytometer.