FLOW CYTOMETRY PROTOCOL FOR EXTRACELLULAR MEMBRANE-ASSOCIATED PROTEINS

Immunophenotyping suspended cells based on membrane-associated antigens is the most common use for flow cytometry. Membrane proteins are readily accessible to the antibody, therefore permeabilization steps are not required. This flow cytometry protocol for staining extracellular antigens has been developed and optimized by Bio-Techne. Individual experimental designs for flow cytometry must be optimized, including antibody dilution, incubation time and temperature (i.e. some receptors internalize with warmer temperatures). If you are concerned about target internalization, do all incubation and centrifugation steps at 4°C. For best results, use 1 x 10^6 cells per 100 µL of staining buffer in each sample tube. If performing surface and intracellular staining on the same sample, surface staining should be performed prior to permeabilization treatments as these may decrease surface antigen availability. *Please read the protocol in its entirety before starting.*

Materials

- Fc Receptor Blocking Reagents (These include Fc receptor blocking antibodies or IgG solutions)
- **Optional:** Red Blood Cell Lysis Buffer (Novus Biologicals, Cat No. NBP2-29442), Human Lysis Buffer (10X) (R&D Systems, Cat No. FC-002), OR Mouse Lysis Buffer (10X) (R&D Systems, Cat No. FC-003)
- Flow Cytometry Staining Buffer (R&D Systems, Cat No. FC-001) or an equivalent solution containing BSA and sodium azide
- Primary Antibodies
- Isotype Control Antibodies
- **Recommended viability dye:** DAPI, Novus Biologicals, Cat No. NBP2-31156; Propidium Iodide, Novus Biologicals, Cat No. NBP2-31155; or 7-AAD, Novus Biologicals, Cat No. NBP2-29446
- 1 X PBS (0.137 M NaCl, 0.05 M NaH2PO4, pH 7.4)
- Bovine Serum Albumin to make 0.5% BSA in PBS as wash buffer
- Trypan Blue
- FACS Tubes (5 mL round-bottom polystyrene tubes)
- Pipettes with appropriate tips
- Centrifuge
- Vortex

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Sample Preparation

Sample Type	Suggestions
Cells in Suspension	After removing as much media as possible from suspended cells, add cold PBS to remove residual growth factors from cell culture media. After washing media remnants, use cells suspended in PBS and proceed with washing in Step 2.
Adherent Cells	After removing media from adherent cells, add cold PBS to remove residual growth factors from cell culture media. • Harvest cells with a 1% BSA solution in PBS and then proceed with washing in Step 2. • Adherent cell lines may require 0.5 mM EDTA to facilitate removal and then washed according to Step 2. *Exposure time with EDTA should be minimal.*
Tissue	To prepare tissues for flow cytometry, mechanical and/or enzymatic disaggregation is required. • First, mince the tissue into small sections that expose the cells and suspend in PBS. *Enzymatic digestion may be required after mincing the tissue, but digestion buffer will be tissue type dependent.* • Next, pass the minced tissue suspension through a fine gauge needle several times until all cells are fully in suspension. *If you experience resistance, exchange needle with a larger gauge to dissociate cells first.*
Whole Blood	Collection of whole blood into commercially available anticoagulant-treated collection tubes (EDTA or heparin) is recommended if peripheral blood cells are required for staining. • To separate the cells from a plasma supernatant, centrifuge for 10 minutes at 1,000–2,000 x g at 4°C. Carefully remove the plasma from atop the cell pellet using a pipette. Resuspend the cell pellet in 2 mL of a 0.5% BSA in PBS wash buffer to remove plasma sample contaminants before any staining occurs (see Step 2).

Methods

1. Harvest your cells (see Sample Preparation for guidance).
2. Add 2 mL of 0.5% BSA in PBS wash buffer with a pipette to wash the cells. Centrifuge at 1,300 RPM (500 x g maximum) at room temperature (RT) for 5 minutes, decanting the supernatant. Wash a total of three times.

   Optional: If using whole blood, a Red Blood Cell (RBC) lysis step is required at this point. This will remove RBCs allowing for identification of lymphocytes, granulocytes, and monocytes by flow cytometry. You may use RBC Lysis Buffer, Human Lysis Buffer (1X) or Mouse Lysis Buffer (1X) diluted in Flow Cytometry Staining Buffer. Add 2 mL of desired 1X Lysis Buffer, gently vortex and incubate 10 minutes at RT protected from light to lyse RBCs. Vortex intermittently. Centrifuge at 1,300 RPM (500 x g maximum) at RT for 5 minutes to pellet cells. Resuspend in 2 mL of 0.5% BSA in PBS wash buffer with a pipette to wash the cells of any remaining lysis buffer. Centrifuge at 1,300 RPM (500 x g maximum) at RT for 5 minutes to pellet cells. Discard supernatant.

3. Using a small aliquot, count the cells. Count cells using a hemocytometer and a 1:1 trypan blue exclusion stain to determine cell viability before starting.

   TIP: Staining of surface antigens may be done at this point.

4. Add 100 µL of Flow Cytometry Staining Buffer into FACS tubes required for your experiment. Aliquot up to 1 x 10^6 cells

per 100 µL. *A separate set of cells should be prepared as a negative control alongside samples.*

5. Add 1 µg blocking IgG per 1 x 10^6 cells, gently vortex and let stand for 15 minutes at RT. *Do NOT wash excess blocking IgG from this reaction.*
6. Add 5-10 µL of conjugated antibody (or a previously determined amount) per 1 x 10^6 cells and gently vortex. *For unconjugated antibodies, be sure to check the data sheet for any appropriate concentrations validated for use in flow cytometry. 1 µL of primary antibody per 1 x 10^6 cells is a good starting point.* Incubate cells for 30 minutes protected from light at RT. Gently vortex intermittently to maintain a single-cell suspension.

   TIP: If an unconjugated primary antibody was used, incubation with an appropriate secondary antibody is required. Remove any unbound conjugated antibody by washing the cells ONCE using 2 mL Flow Cytometry Staining Buffer, centrifuging at 1,300 RPM (500 x g maximum) at RT for 5 minutes to pellet cells. After washing cells to remove the primary antibody, resuspend the cell pellet in 100 µL of Flow Cytometry Staining Buffer. Add the recommended volume of secondary antibody and incubate 30 minutes protected from light. Gently vortex intermittently to maintain a single-cell suspension.

7. Remove any unbound conjugated antibody by washing cells with 2 mL Flow Cytometry Staining Buffer. Centrifuge at 1,300 RPM (500 x g maximum) for 5 minutes, decanting the supernatant.
8. Resuspend the cells in 200 – 400 µL of Flow Cytometry Staining Buffer for final flow cytometric analysis.

   Recommended: To assess cell viability, staining using products DAPI, PI or 7-AAD should be performed after antibody staining is complete. Do NOT wash cells after this reaction. Cells must remain in the buffer during acquisition, so add dyes approximately 15 minutes prior to analysis.