FLOW CYTOMETRY PROTOCOL FOR INTRACELLULAR TARGETS USING ALCOHOL

The following flow cytometry protocol for staining intracellular molecules using alcohol to permeabilize cell membranes has been developed and optimized by Bio-Techne. For best results, use 1 x 10^6 cells per 100 µL of sample. Individual experimental designs for flow cytometry must be optimized, including antibody dilution and incubation time. For low cell density or poorly expressed intracellular targets, Single-Cell Westerns may be useful. Learn more about Milo™ here. *Please read the protocol in its entirety before starting.*

Materials

- 1 X PBS (0.137 M NaCl, 0.05 M NaH^2PO^4, pH 7.4)
- Bovine Serum Albumin to make 1% BSA in PBS
- -20 °C Methanol (keep on ice during procedure)
- Intracellular Staining Kit (Novus Biologicals, Cat No. NBP2-29450) which includes 10X Permeabilization Buffer, 1000X Brefeldin A (inhibitor), 1X Fixation Buffer, and 1X Staining Buffer or equivalent products
- Deionized water to dilute stock Permeabilization Buffer to 1X
- Fc Receptor Blocking Reagents (These include Fc receptor blocking antibodies or IgG solutions)
- 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
- Trypan Blue
- FACS Tubes (5 mL round-bottom polystyrene tubes)
- Pipettes with appropriate tips
- Centrifuge
- Vortex

Sample Preparation

Sample Type	Suggestions
Cells in Suspension	Suspended cells should be thoroughly washed with cold PBS to remove residual growth factors from cell culture media. After removing media remnants, use cells suspended in PBS to 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.*

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Methods

1. Harvest your cells (see Sample Preparation for guidance).
2. Add 2 mL of PBS with a pipette to wash cells. Centrifuge at 1,300 RPM (500 x g maximum) and 4°C for 5 minutes, decanting the supernatant. Wash 3 times.
3. Using a small aliquot, create a 1:1 Trypan Blue exclusion stain to count cells using a hemocytometer.

   TIP: Staining of surface antigens may be done at this point. Cell viability staining should be done prior to cell fixation.

4. Add 500 µL of cold 1X Fixation Buffer into FACS tubes required for your experiment. Aliquot up to 1 x 10^6 cells/100 µL (i.e. 5 x 10^6 cells) and vortex to mix. *A separate set of cells should be stained with an isotype control antibody as a negative control.*
5. Incubate the cells at room temperature for 10 minutes. Gently vortex intermittently to maintain a single cell suspension.
6. Add 2 mL of PBS. Centrifuge cells at 1,300 RPM and 4°C for 5 minutes. Decant the Fixation Buffer.
7. Resuspend cell pellet in 900 µL of cold methanol and incubate for 30 minutes at 4°C. Gently vortex intermittently to maintain a single cell suspension.
8. Centrifuge cells at 1,300 RPM and 4°C. Decant the methanol.
9. Remove methanol remnants with 2 mL of PBS. Centrifuge at 1,300 RPM (500 x g maximum) and 4°C for 5 minutes. Discard supernatant.
10. Resuspend each cell pellet in 150 µL of 1X Permeabilization Buffer. Add 1 µg blocking IgG per 1 x10^6 cells and let stand for 15 minutes at room temperature. *Do NOT wash excess blocking IgG from this reaction and keep cells in the presence of Permeabilization Buffer during intracellular staining.*
11. Add 5-10 µL of conjugated antibody (or a previously titrated amount) per 1 x 10^6 cells and vortex. Incubate cells for 30 minutes at room temperature protected from light. Gently vortex intermittently to maintain a single cell suspension.
12. Wash cells using 2 mL of Permeabilization Buffer. Centrifuge at 1,300 RPM (500 x g maximum) and 4°C for 5 minutes. Decant the supernatant.
13. Resuspend cells in 200 – 400 µL of 1X Staining Buffer for final flow cytometric analysis.