Flow Cytometry Staining Protocol

Experimental reagents

1. Cell Staining Buffer: 0.5% BSA in 1x PBS solution
2. BSA (Biofroxx; 4240GR100)
3. 1x PBS (Biosharp; BL601A)
4. Ficoll (Cytiva; 17144003)
5. Sodium citrate anti-coagulation sample tube
6. 4% paraformaldehyde solution (Beyotime; P0099-500 mL)
7. Triton X-100 (Beyotime; P0096-500 mL)
8. RPMI 1640 (Gibco; C11875500BT)
9. Trypan blue (Gibco;15250061)
10. Blocking Buffer (BioLegend;422301)
11. Trypsin/EDTA solution (Procell; PB180219)

Protocol Steps

- Cell collection and processing

1. For cryopreserved PBMCs:

    1) Thaw vials of PBMCs briefly in a 37℃ water bath till 80% ice thawed
    2) slowly drip PBMC suspension to 10 mL pre-warmed RPMI 1640 medium and mix cells 5-6 times by inverting tube
    3) centrifuge for approximately 5 minutes at 400x g
    4) Resuspend cell pellet in a small volume of warm RPMI 1640 medium
    5) Perform a viable cell count using Trypan blue
    6) Block PBMC in Blocking Buffer for 30 min at room temperature
    7) Dilute cells to a concentration of 2.5E6 - 1E7 live lymphocytes/mL using Cell Staining Buffer.

2. For adherent cell culture:

   1) Remove culture medium from culture vessel
   2) Rinse cells in pre-warmed 1x PBS to remove residual culture medium
   3) Lift cells with trypsin/EDTA solution for 3-10 minutes at room temperature
   4) Collect cultured cells in a 15 mL or 50 mL centrifuge tube and count
   5) Centrifuge cells at 400x g for 5 minutes and remove supernatant
   6) Resuspend cell pellet with 1 mL of Cell Staining Buffer
   7) Centrifuge cells at 400x g for 3 minutes and remove supernatant
   8) Repeat steps 6) and 7) one time
   9) Resuspend cells were then resuspended with 1 mL of Cell Staining Buffer.

3. For suspension cell culture:

   1) Gently break cell clumps by aspiration to generate single cell suspension
   2) Collect cultured cells in a 15 mL or 50 mL centrifuge tube and count cell number
   3) Centrifuge cells at 400x g for 5 minutes and remove supernatant
   4) Resuspend cell pellet with 1 mL of Cell Staining Buffer
   5) Centrifuge cells at 400x g for 3 minutes and remove supernatant
   6) Repeat steps 6) and 7) one time
   7) Resuspend cells were then resuspended with 1 mL of Cell Staining Buffer.

4. For whole blood sample:

   1) Collect whole blood in sodium citrate anti-coagulation sample tube
   2) Gently inverting tube 5-6 times to mix blood sample with sodium citrate thoroughly
   3) store at room temperature for no more than 8 h prior to use.

5. For fresh PBMCs:

   1) Collect whole blood in sodium citrate anti-coagulation sample tube
   2) Gently inverting tube 5-6 times to mix blood sample with sodium citrate thoroughly
   3) Prepare PBMCs from whole blood using Ficoll gradient separation or other commercially available methods

4) Resuspend PBMCs at 2.5E6-1E7 cells/mL with Cell Staining Buffer.

- Stimulation of cells (Optional) (immune cells often require special stimulation to express certain cytokines or increase expression level of cytokines so they can be detectable using intracellular flow cytometry)

1. Prepare cells using the protocols described in “Cell collection and processing”.
2. Resuspend cells at 2-3E6/mL in proper culture medium with or without fetal bovine serum.
3. Split cells equally to two groups, one group for stimulation, one group for non-stimulation control.
4. Prepare cell stimulation master mix and Brefeldin A at 100x - 1000x for easy dilution. Always prepare a slight excess of each mix.
5. Add desired volume of cell stimulation mix to stimulation and non-stimulation groups respectively.
6. Continue culturing cells for needed duration of stimulation, typically from a few hours to a few days at 37℃.
7. Add Brefeldin A to a final concentration of 3-5 μg/mL about 1-2 hours before ending stimulation for both stimulation and non-stimulation groups. Brefeldin A will retain more cytokines in cells to enhance intracellular staining. Incubation time with Brefeldin A can be optimized if experiment result is not ideal.
8. Harvest cells for subsequent flow cytometry staining.

- Cell surface staining Steps

1. The cells collected earlier were divided into 96-well plates in Cell Staining Buffer , each well 50 μL of 3E5-5E5 cells.
2. If FcR blocking is needed for FcR-expressing cells, one can add additional FcR blocking reagents to incubate with cells for 0.5-1 hour. Blocking regents are typically 2-5% of normal rabbit serum and mouse serum, or FcR blocking antibodies.

3. Primary antibody incubation:
    1) Add 50 µL Cell Staining Buffer with primary antibody at 0.5-10 µg/mL into each well,Primary antibody concentration may need optimization for best result. (Please note that combined with the added cell sample, the total incubation volume is 100 µL)
    2) Gently rotating staining plates for 20 min at room temperature.
    3) If the primary antibody is fluorescently conjugated, avoid light during staining and go straight to step 6 after staining.
4. Pellet cells at 400x g for 5 minutes and remove supernatant.
5. Resuspend cells in 200 µL Cell Staining Buffer.
6. Pellet cells at 400x g for 5 min and remove supernatant.
7. Secondary antibody incubation:
    1) Resuspend cells in 100 µL Cell Staining Buffer supplemented with appropriate concentration of fluorescent secondary into each well.
    2) Incubate plates in the dark at room temperature for 20 min.
8. Centrifuge cells at 400x g for 5 minutes and remove supernatant.
9. Washing:
    1) resuspend cells in 200 µL Cell Staining Buffer
    2) pellet cells at 400x g for 5 minutes and remove supernatant
    3) Repeat 1) and 2) twice.
10. Resuspend cells in 200 µL Cell Staining Buffer
11. Analyze cells using flow cytometer

- Intracellular staining

1. Collect cells
2. The cells collected earlier were transferred into microtube or 15 mL conical tube in Cell Staining Buffer.
3. Dead/alive cell staining:

1) Add appropriate amount of Zombie dye following manufactural manual to cell suspension.
2) incubate in the dark at room temperature for 15 min.
3) Centrifuge cells at 400x g for 5 minutes and remove supernatant.
4) Resuspend cell pellet with 200 µL Cell Staining Buffer.
5) Centrifuge cells at 400x g for 5 min and remove supernatant.
6) Repeated steps 4 and 5 once.

4. Cell fixation:
   1) Resuspend cells with 4% paraformaldehyde solution (1E7 cells/mL)
   2) Rotate cells gently for 15 min at room temperature in the dark
   3) Pellet cells at 400x g for 5 minutes and decant supernatant
   4) Wash cells once with 1 mL Cell Staining Buffer.
5. Cell permeabilization and blocking
   1) Permeabilize cells with 0.1-0.5% Triton X-100 in Cell Staining Buffer, incubated for 15 min in the dark
   2) Centrifuge cells at 400x g for 5 minutes and remove supernatant
   3) Resuspend cells in Cell Staining Buffer supplemented with FC receptor block reagent
   4) Incubate cells at room temperature for 1 hour in the dark
   5) Centrifuge cells at 400x g for 5 minutes and remove supernatant
   6) Resuspend cell in Cell Staining Buffer.
6. Distribute cells evenly into 96-well plate，each well 50 µL with 3E5-5E5 cells/well.
7. Primary antibody incubation:
   1) Add 50 µL Cell Staining Buffer with primary antibody at 0.5-10 µg/mL into each well. Primary antibody concentration may need optimization for best result.(Please note that combined with the added cell sample, the total incubation volume is 100 µL)
   2) Gently rotating staining plates for 30 min at room temperature.
   3) If the primary antibody is fluorescently conjugated, avoid light during staining and go

straight to step 8 after staining.

8. Pellet cells at 400x g for 5 minutes and remove supernatant.
9. Resuspend cells in 200 µL Cell Staining Buffer.
10. Pellet cells at 400x g for 5 min and remove supernatant.
11. Secondary antibody incubation:
    1) Resuspend cells in 100 µL Cell Staining Buffer supplemented with appropriate concentration of fluorescent secondary into each well.
    2) Incubate plates in the dark at room temperature for 30 min.
12. Centrifuge cells at 400x g for 5 minutes and remove supernatant.
13. Washing:
    1) resuspend cells in 200 µL Cell Staining Buffer
    2) pellet cells at 400x g for 5 minutes and remove supernatant
    3) Repeat 1) and 2) twice.
14. Resuspend cells in 200 µL Cell Staining Buffer
15. Analyze cells using flow cytometer

- **Note**：These methods provide general procedures. It is highly recommended that you refer to detailed product information provided by manufacturers.