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The path to legendary discovery™

Cell Surface Immunofluorescence Staining Protocol

Harvest Tissue or Cells:

1. Obtain desired tissue (*e.g*. spleen, lymph node, thymus, bone marrow) and prepare a single cell suspension in Cell Staining Buffer (BioLegend Cat. #420201). If using *in vitro* stimulated cells, simply resuspend previously activated cultures in Cell Staining Buffer and proceed to Step 2.
2. Add Cell Staining Buffer up to ~15 ml and centrifuge at 350 x *g* for 5 minutes, discard supernatant.

Lyse Red Cells:

3. If necessary (*e.g*. spleen), dilute 10X Red Blood Cell (RBC) Lysis Buffer (BioLegend Cat. #420301) to 1X working concentration with DI water and resuspend pellet in 3 ml 1X RBC Lysis Buffer. Incubate on ice for 5 minutes.
4. Stop cell lysis by adding 10 ml Cell Staining Buffer to the tube. Centrifuge for 5 minutes at 350 x *g* and discard supernatant.
5. Repeat wash as in step 2.
6. Count viable cells and resuspend in Cell Staining Buffer at 5-10 x 10^6 cells/ml and distribute 100 µl/tube of cell suspension (5-10 x 10^5 cells/tube) into 12 X 75 mm plastic tubes.

Block Fc-Receptors:

7. Reagents that block Fc receptors may be useful for reducing nonspecific immunofluorescent staining. In the mouse, purified anti-mouse CD16/CD32 antibody specific for Fcγ R III/II (BioLegend Cat. #101302, clone 93) can be used to block nonspecific staining of antibodies. In this case, block Fc receptors by pre-incubating cells with 5-10 µg/ml purified anti-CD16/32 on ice for 10 minutes. In the absence of an effective/available blocking antibody for human and/or rat Fc receptors, an alternative approach is to pre-block cells with excess irrelevant purified Ig from the same species and same isotype as the antibodies used for immunofluorescent staining.

Cell-Surface Staining with Antibody:

8. Add appropriately conjugated fluorescent, biotinylated, or purified primary antibodies at predetermined optimum concentrations (*e.g*. anti-CD3-FITC, anti-CD4-Biotin, and anti-CD8-APC) and incubate on ice for 15-20 minutes in the dark.
9. Wash 2X with at least 2 ml of Cell Staining Buffer by centrifugation at 350 x *g* for 5 minutes.

10. If using a purified primary antibody, resuspend pellet in residual buffer and add previously determined optimum concentrations of anti-species immunoglobulin fluorochrome conjugated secondary antibody (*e.g*. FITC anti-mouse Ig) and incubate in the dark for 15-20 minutes.

    If using a biotinylated primary antibody, resuspend cell pellet in residual buffer and add previously determined optimum concentrations of fluorochrome conjugated Streptavidin (SAv) reagent (*e.g*. SAv-PE, BioLegend Cat. # 405204) and incubate on ice for 15-20 minutes in the dark.

11. Repeat step 9.

12. Resuspend cell pellet in 0.5 ml of Cell Staining Buffer and add 5 µl (0.25 µg)/million cells of 7-AAD Viability Staining Solution (BioLegend Cat. #420403) to exclude dead cells. Note, BioLegend does not recommend use of 7-AAD with either PE-Cy5 or PE-Cy7 antibody conjugates.

13. Incubate on ice for 3-5 minutes in the dark.

14. Analyze with a Flow Cytometer.

Immunofluorescent Staining of Whole Blood:

1. Add predetermined optimum concentrations of desired fluorochrome conjugated, biotinylated, or purified primary antibodies to 100 µl of anti-coagulated whole blood.

2. Incubate at room temperature for 15-20 minutes in the dark.

3. Dilute 10X Red Blood Cell (RBC) Lysis Buffer (BioLegend Cat. #420301) to 1X working concentration with DI water. Warm to room temperature prior to use. Add 2 ml of 1X RBC lysis solution to whole blood/antibody mixture. Incubate at room temperature for 10 minutes.

4. Centrifuge at 350 X *g* for 5 minutes, discard the supernatant.

5. Wash 1X with at least 2 ml of Cell Staining Buffer by centrifugation at 350 x *g* for 5 minutes.

6. If using a purified primary antibody, resuspend pellet in residual buffer and add a previously determined optimum concentration of anti-species immunoglobulin fluorochrome conjugated secondary antibody (*e.g*. FITC anti-mouse Ig) and incubate in the dark for 15-20 minutes.

    If using a biotinylated primary antibody, resuspend cell pellet in residual buffer and add a previously determined optimum concentration of fluorochrome conjugated Streptavidin (SAv) reagent (*e.g*. SAv-PE, BioLegend Cat. # 405204) and incubate for 15-20 minutes in the dark.

7. Repeat step 5.

8. Resuspend cells in 0.5 ml Cell Staining Buffer or 0.5 ml 2% paraformaldehyde-PBS fixation buffer

9. Analyze with a Flow Cytometer.

Key Reference: *Current Protocols in Cytometry (John Wiley & Sons, New York)*, Unit 6 *Phenotypic Analysis*.

Reagent List:

Cell Staining Buffer (BioLegend Cat. #420201)
Red Cell Lysis Buffer (BioLegend Cat. #420301)
7-AAD Viability Staining Solution (BioLegend Cat. #420403)
TruStain FcX™ (anti-CD16/32, BioLegend Cat. #101319)