Immunofluorescent Staining of Mouse and Rat Leukocytes

I. Procedure

- Harvest cells from tissue, preparing a single cell suspension. Red blood cells may be removed by lysis or density gradient: Red blood cells from murine peripheral blood or a spleen cell suspension can be lysed using BD Pharmingen’s PharM Lyse™ (Cat. No. 35221E) solution. Add 2.0 ml of 1X Lysing Solution to the spleen cell suspension or per 200 µl of murine peripheral blood. Gently vortex immediately after adding the lysing solution. Incubate at room temperature, protected from light, for 15 min. Centrifuge 200 x g for 5 min. Carefully aspirate and dispose of supernatant, without disturbing the pellet. Resuspend pellet in 1X cold wash buffer (PBS/0.1% NaN3/1.0% fetal bovine serum). Centrifuge at 350 x g for 5 min. Finally, resuspend cell pellet to a concentration of 2 x 107 cells/ml (i.e., 106 cells per 50 µl).
- Dilute primary mAbs (e.g., unconjugated, biotinylated, or fluorochrome-conjugated mAbs) to predetermined optimal concentrations (see Staining Tips) in wash buffer and deliver to the wells of a U-bottom microtiter plate in a volume of 50 µl.
- Deliver 106 cells in 50 µl to each well already containing 50 µl of mAb (or 50 µl wash buffer for negative controls). Mix by gently vortexing or tapping.
- Incubate at 4°C for 20-40 min in the dark.
- Wash 2X with 200 µl wash buffer (or 3X if a biotin-conjugated primary antibody is used). After each centrifugation, 350 x g for 5 min, aspirate wells or flick plate to remove supernatant. Vortex gently or tap plate to loosen pellet prior to adding next wash or diluted secondary reagent.
- If a second-step reagent is needed, resuspend cell pellet in 100 µl of appropriately diluted secondary reagent (e.g., fluorochrome-conjugated avidin, streptavidin, anti-Ig allotype, anti-Ig isotype, polyclonal anti-Ig). For example, dilute antibody to ~1 µg per 100 µl in wash buffer and add this to each well containing the loosened cell pellet.
- Incubate at 4°C for 20-40 min in the dark.
- Wash 2X with 200 µl wash buffer, as in Step 5. Use 100 µl wash buffer to transfer cell pellets to 0.4 ml aliquots of wash buffer (final concentration ~106 cells in 0.5 ml) in tubes appropriate for flow cytometer. Acquire sample data on flow cytometer as soon as possible after staining. (Please see Staining Tip 5.)

II. Staining Tips

- Determine optimal concentrations (brightest staining/lowest background) of each primary and secondary antibody by titrating, in a preliminary experiment, between 1.0 µg and 0.1 µg antibody per 100 µl wash buffer for 106 cells.
- When performing multi-color labeling, directly-conjugated mAbs can be added simultaneously, rather than sequentially. For instrument set-up, please see description in "Procedure for Setting Compensation for Multi-Color Flow Cytometric Analysis".
- For reducing FcgII/IIIR-mediated antibody binding (or binding of Sav-PE or Sav-Cy-chrome) which could contribute to background, the use of anti-mouse CD32/CD16 (Mouse Fc Block™; Cat No. 01241A/D) or anti-rat CD32 (Rat Fc Block™; Cat. No. 22881A/D) is recommended. Fc Block™ can be added to cells (~0.25 µg per million cells, 3 - 5 min, 4°C) and need not be washed out prior to addition of primary mAb. It is important to verify that no secondary reagent will bind the Fc Block™. Please see description in "The Uses of Fc Block™ in Immunophenotyping of Mouse and Rat Leukocytes".
- For very low-density cell surface markers (e.g., cytokine receptors), a three-step protocol may amplify the staining: use purified primary antibody (steps 2-4 of above procedure), biotinylated anti-Ig for the 2nd step (steps 6-7, above), and fluorochrome-conjugated avidin or streptavidin as the 3rd step (repeat steps 6-7). We find that SAv-PE and SAv-Cy-Chrome™ are "brighter" than FITC conjugates and may provide even better discrimination of low-density antigens, especially in the presence of Fc Block™, for mouse cells. (Please see Staining Tip 3.)
- We have found that freshly-isolated leukocytes and cell lines may wait for analysis in wash buffer at 4°C, without fixation, for up to 18 hr post-staining, without loss of viability. Activated lymphocytes may lose viability rapidly, and data should be collected within 5 hr post-staining. To preserve cell integrity beyond these time limits, paraformaldehyde fixation may be necessary; however, it is possible that the quality of staining may be diminished by such fixation. We do not recommend fixation of stained cells, except when the possibility of exposure to biohazardous material exists.
- Every experiment must include controls. Negative controls are samples of the same cell population treated
exactly as the test sample, but with the omission or modification of one of the staining steps. Examples of
negative controls are unstained cells, cells exposed to the 2nd step reagent alone, or cells exposed to isotype
controls which are the same isotype and format (e.g., purified, biotin or fluorochrome) as the primary antibody
and titrated in parallel. For multi-color staining, single-color stained controls should be included. To identify
markers on an unknown or novel cell type, positive controls (i.e., cells which are known to express the antigen
of interest) should be included in each experiment and should be handled exactly as the test samples.

Protocols

The Uses of BD Fc Block™ in Immunophenotyping of Mouse or Rat Leukocytes

FcγII and FcγIII low-affinity receptors for complexed IgG (Fc receptors or FcR) are designated CD32 and
CD16, respectively, and are expressed on many cell types, including B lymphocytes, NK cells,
granulocytes, monocytes, macrophages, and platelets. Some antibody preparations may bind via their Fc
portions to FcR-bearing cells, resulting in high "background" staining.

Mouse BD Fc Block™ is a purified rat IgG2b anti-mouse CD16/CD32 monoclonal antibody, while Rat BD
Fc Block™ is a purified mouse IgG1 anti-rat CD32 monoclonal antibody. Mouse and Rat BD Fc Block™
can be used to block the Fc-mediated adherence of antibodies to mouse and rat FcR, respectively.
Furthermore, it has been reported that the Fc domain of the anti-mouse CD16/CD32 mAb 2.4G2 binds the
FcγI receptor (CD64) and blocks this additional FcR. Since Mouse and Rat BD Fc Block™ are antigen-
specific mAbs, anti-mouse CD16/CD32 and anti-rat CD32, they may also be used as primary staining
reagents for detection of mouse and rat FcR-bearing cells, respectively.

Pre-incubation of mouse or rat cell suspensions with the respective BD Fc Block™ for several minutes,
prior to staining with specific antibodies, assures that any observed staining is due to the interaction of
the antigen-binding portion of the antibody with an antigen on the cell surface. The following figures
illustrate typical applications of Mouse and Rat BD Fc Block™.
Experiments using unconjugated rat anti-mouse primary antibodies or mouse anti-rat primary antibodies in the presence of BD Fc Block™ must be carefully designed. Second-step reagents must be chosen which will not cross-react with BD Fc Block™, rat IgG₂b or mouse IgG₁. BD Biosciences Pharmingen offers a selection of isotype-specific anti-rat and anti-mouse immunoglobulin mAbs which may be used for this purpose.

References: