Immunofluorescent Staining of Rat Thymocytes

I. Procedure

1. Harvest cells from tissue, preparing a single cell suspension to a final concentration of 2 x 10^7 cells/ml (i.e., 10^6 cells per 50 µl).

2. Dilute primary mAbs (e.g., unconjugated, biotinylated, or fluorochrome-conjugated mAbs) to predetermined optimal concentrations (see Below) in wash buffer (PBS/0.1% NaN_3/1.0% fetal bovine serum) and transfer a volume of 50 µl to a flow tube.

3. Transfer 10^6 cells in 50 µl to each tube already containing 50 µl of mAb (or 50 µl wash buffer for negative controls). Mix by gently vortexing or tapping.

4. Incubate at 4°C for 20-40 min in the dark.

5. Wash 2X with 200 µl wash buffer. After each centrifugation, 350 x *g* for 5 min, remove the supernatant. Vortex gently or tap the tube to loosen pellet prior to adding next wash. Use 500 µl wash buffer to resuspend the cell pellets (final concentration ~10^6 cells in 0.5 ml).

6. Acquire sample data on flow cytometer as soon as possible after staining. (Please see Staining Tips below)

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 10^6 cells.

- When performing multi-color labeling, directly-conjugated mAbs can be added simultaneously, rather than sequentially.

- 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 BD Fc Block™; Cat No. 553141/553142) or anti-rat CD32 (Rat BD Fc Block™; Cat. No. 550270/550271) is recommended. BD 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 BD Fc Block™.

- 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-BD Cy-Chrome™ are "brighter" than FITC conjugates and may provide even better discrimination of low-density antigens, especially in the presence of BD Fc Block™, for mouse cells.

- 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.

Protocol and Staining Tips modified from BD Bioscience Immunocytometry