GENERAL CELL STAINING PROTOCOL FOR FLOW CYTOMETRY

1) Except for cells grown in culture, cells obtained directly from tissues must first be resolved to a single cell suspension by means of mechanical dissociation (mincing, grinding between the ends of two frosted glass slides, dounce homogenization, steel mesh sieving, passage through a fine-bore syringe tip etc.) or enzymatic digestion (collagenase, DNAse, neutral protease disruption of stromal cells or pepsin digest of solid tissue). Mechanical dissociation of spleen and thymus is effective in releasing lymphocytes but is often damaging towards larger, blastic cells and usually leaves dendritic and stromal cells behind in the debris. These cells are obtained through gentle enzymatic digestion.

Prior to staining, cells must then be purified by at least one of several procedures to enable analysis on the flow cytometer without damage to the flow cell and permit staining without excessive background. This will invariably include some combination of density gradient centrifugation, positive or negative selection procedure (panning, complement-mediated cell lysis) straining through nylon-mesh and/or red-cell lysis where necessary. (Prior to the BD FACScan, earlier instruments mandated nylon mesh screening s the smaller flow cells on these machines could easily become clogged by clumped or aggregated cells and often this could take months and hundreds of dollars to repair!)

2) Wash purified cells 1X in staining buffer. (A suitable buffer will be isotonic and buffered to neutrality, will cushion the cells against damage during centrifugation, block non-specific staining, prevent capping of bound antibody and will block Fc receptor binding.)

e.g. formulations:

.05 M Tris buffered Saline pH 7.4
1% normal sera of the animal for which the primary antibody is derived or 0.1mg/ml Ig of such animal.
2% Fetal Calf Serum
0.1% Azide

or: Phospate buffered Saline
2% Bovine Serum Albumin
0.1% Azide
(will not block Fc receptor binding)

3) Adjust concentration of cell suspension to at least 5 x 10^6 cells/ml. Cells should be greater than 90% viable as determined by trypan blue exclusion. Determine # of samples necessary for experiment and aliquot (1 x 10^5 – 1 x 10^6 cells/sample, preferably the higher value if possible) to U bottom microtiter well or plastic centrifuge tube.

4a) For staining in microtiter plates. Spin plate for 3 minutes at 100 x g (usually 600 rpm) at 8^{o}C. Flick plate or remove fluid from wells by suction and immediately resuspend pellet in 100ul of 1st reagent, appropriately diluted to previously determined titration point in staining buffer (primary antibody directly labeled (e.g. with FITC) if performing direct-labeling, primary antibody biotinylated or unlabeled if performing indirect staining.) Incubate 30' at 4^{o}C. (in the dark if fluorescent labeled)

5a) Wash 3X. (Spin, flick, resuspend in S.B.) After last wash, flick and resuspend in 100ul of secondary reagent (e.g. FITC labeled Goat anti-mouse Ig or Avidin-FITC also pre-titered) and repeat step 4 if performing indirect staining, otherwise simply resuspend in 100ul of staining buffer and transfer to Falcon 2052 tube (this is the only tube that fits the FACScan)

containing 3-500ul of staining buffer. Keep samples on ice and perform FACS analysis. If samples cannot be run immediately fix them in 1% formaldehyde or 3% paraformaldehyde in PBS for 15 minutes at 4^{o}C, wash 1X in staining buffer (samples are left in fix by most researchers but this writer has found a great deal of cell clumping without fixative removal), store in fridge in the dark for later analysis.

4a) **For staining in tubes.** Centrifuge at 300 x g (usually 1000 rpm) for 5 minutes at 8^{o}C. Carefully aspirate supernatant from cell pellet and resuspend pellet in 100ul of 1st reagent in staining buffer as above. Incubate and wash as above for direct or indirect procedures.

Important Things to Remember:

1) Both primary and secondary antibodies or avidin reagents must be tittered on known positive and negative cell populations prior to use in actual experiments.

2) Controls that are necessary:

- **a. Negative** (no stain whatsoever)
- **b. or Negative** (secondary reagent added only or similarly labeled non-specific primary antibody, ideally of the same isotype as the specific antibody you will be using)
- **c. Negatives and Positives** for each <u>different</u> primary antibody, each different animal, each different strain or species, and each different cell population used in your experiment, when possible.

3) For two and three color analysis compensation controls must be run to compensate for spectral overlap. These consist of one sample each stained with each fluorescent reagent separately and a control containing both colors; e.g. if two-color analysis is performed with FITC and phycoerythrin then samples stained with FITC alone and PE alone and a sample certain to be positive for both colors should be run.