FACS Staining Method for Cell Surface Proteins

Staining medium (SM) (recipe below)
Sterile filtered calf serum (CS) [0.45μm TC sterile filtered stored TC fridge]
1X Gey's solution (recipe below)
0.1% Trypan Blue
1 mg/ml propidium iodide (PI) [in ddH_2O] (store frozen in 50 ul aliquots – can thaw & refreeze many times)
Nitex mesh, 85-μm mesh size
4 ml conical tubes [Diamed STK 8550]
5 ml round bottom tubes [Falcon 2052]
Antibodies as required for experiment

Sample Note:
For each FACS sample use 0.5-2x10^6 cells/tube. Up to 4x10^6 cells/sample can be used in a 50 μl staining volume, especially if analyzing rare cell populations. If larger cell numbers are stained, see Note (c) below. For peripheral blood lymphocytes (PBLs) use 1-8 X 10^5 cells/tube *ie.* whatever you can get.

Protocol:
1. Prepare antibody dilutions in SM based on a total staining volume of 50 μl diluted Ab per sample tube. Calculate total volume of antibody solution needed based on number of samples plus one for each stain.
   Note that before using an antibody in an experiment, the optimal antibody concentration for your application should be determined by staining a test cell sample with serial dilutions of the antibody. See Note (e) below.

2. (a) For *ex vivo* cells, dissect desired tissue (usually lymphoid tissue) from animal and place on ice in a tissue culture dish with ice-cold SM.

   (b) Make single cell suspension. Place tissue on pre-wet steel mesh screen that is sitting in a 35-mm tissue culture dish. Mince tissue with scissors and push through steel screen using the plunger from a 3 ml syringe. Wash remaining cells through screen with ice-cold SM. Transfer cell suspension from tissue culture dish to a 15-ml Falcon 2096 tube on ice. Keep cells on ice throughout procedure.

3. For cultured cells, collect cells by decanting media and cells into a 15-ml Falcon 2096 or 50-ml Falcon 2070 tube. Keep cells on ice throughout procedure.
   Exceptions apply for certain cultured cell lines. eg. *293T cells should be kept at room temperature as they tend to die with prolonged incubation on ice. Centrifugation, counting and staining of these cells should be done at room temperature. Staining times should be kept to a minimum to avoid changes in observed phenotype due to metabolism during staining.*

4. Underlay cell suspension with 0.5 ml CS. Collect cells by centrifugation at 400x*g* for 5 minutes at 4ºC (1500 rpm in Beckmann GS6-KR).
   The calf serum underlay helps make a more compact cell pellet and gets rid of some dead cells and cellular debris.

5. Aspirate supernatant.

Optional step:

6. Lyse red blood cells by re-suspending cell pellet in 1X Gey's solution. Incubate at room temperature 2-5 minutes. Add 10 ml SM, mix well and filter through Nitex. Underlay with 1-2 ml calf serum.
   Incubation longer than 5 minutes should be avoided as this will lead to lysis of nucleated cells. Gey's solution should be used at a concentration of approximately $2x10^7$ cells/ml. For one mouse spleen, resuspend pellet in 4-5 ml Gey's solution. For the bone marrow from one mouse, resuspend cell pellet in 2 ml Gey's solution. Thymocytes and lymph node cells do not need to be lysed, if tissues have been dissected properly. Thymocytes are sensitive cells, avoid lysis step if possible.

7. Collect all cells by centrifugation at 400 x*g* for 5 minutes at 4ºC. Aspirate supernatant.

8. Re-suspend cell pellets in appropriate volume of SM (try to approximate a final concentration of $1\text{-}5x10^7$/ml).

9. Count live cells by trypan blue exclusion in 0.1% Trypan blue on a hemacytometer.

10. Aliquot appropriate number of cells (see *Sample Note* above) into pre-labeled 4 mL FACS staining conical tubes. Add 0.5 mL of SM to each tube and underlay with 0.3 ml CS. Collect cells by centrifugation at 400x*g* for 5 minutes at 4ºC.
    The calf serum underlay helps make a more compact cell pellet, gets rid of some dead cells and cellular debris, makes the supernatant easier to aspirate without loss of cells, and also leaves less residual fluid behind that can further dilute the antibody solution.

11. **Completely** aspirate supernatant. Resuspend cell pellet in 50 μL 1º Ab stain in each tube. Incubate for 20-40 minutes on ice. Protect from light if fluorochrome-labeled 1º antibodies are being used.
    Residual fluid can further dilute the antibody solution and adversely affect your staining results.

12. Add 0.5 mL SM to each tube (*ie.* 10 staining volumes) and underlay with 0.3 mL of CS using $5^3/_4$" pasteur pipettes.

13. Collect cells by centrifugation at 400x*g* for 5 minutes at 4ºC.

14. **Completely** aspirate supernatant. Resuspend cell pellet in 50 μl 2º antibody. Protect from light. Incubate for 20-25 minutes on ice. If biotinylated 1º antibodies were used, incubate avidin-fluorochrome conjugate 2º stage for 30 minutes on ice.
    Residual fluid can further dilute the antibody solution and adversely affect your staining results.

15. Repeat wash steps 13 & 14.

16. Aspirate supernatant. Resuspend cell pellet in 0.5 mL of SM containing 1 μg/mL PI. Filter through Nitex screens (to remove clumps and debris) into labeled 5 mL round-bottom FACS tubes (Falcon 2052).

17. Collect data on flow cytometer. If this step is to be delayed more than 2-3 hours after staining is complete, they can be fixed in 1.6-4% paraformaldehyde and stored at 4^0C overnight or for a few days. However, be sure to use SM without PI in Step 17. See the related protocol "Fixing Stained Cells").

Buffers:

Staining Media (SM)

1X HBSS with Ca^{2+}/Mg^{2+}; 2% calf serum; 10mM HEPES, pH 7.2; 10 mM NaN_3 (to prevent microbial growth). *For some cell types it may be preferable to use HBSS without Ca^{2+}/Mg^{2+}.* Store at 4ºC.

Gey's Solution

Stock A: 35.0 g NH_4Cl; 1.85 g KCl; 1.13 g $Na_2HPO_4 \cdot 7H_2O$; 0.12 g KH_2PO_4; 5.0 g glucose
Bring to 1 L with ddH_2O and autoclave.
Stock B: 0.42 g $MgCl_2 \cdot 6H_2O$; 0.14 g $MgSO_4 \cdot 7H_2O$; 0.34 g $CaCl_2$
Bring to 100 ml with ddH_2O and autoclave.
Stock C: 2.25 g $NaHCO_3$
Bring to 100 ml with ddH_2O and autoclave.
1X Gey's Solution: 20 parts Stock A; 5 parts Stock B, 5 parts Stock C; 70 parts sterile ddH_2O. If not prepared aseptically, add 10 mM NaN_3 (to prevent microbial growth). Store at 4ºC.

0.1% Trypan Blue

Dilute 0.4% Trypan blue (Sigma, T-8154) 1:4 in 1X PBS, pH 7.2, with 5mM NaN_3. Filter through a 0.2 μm filter.

Notes:

(a) If using NaN_3 in SM, make sure to keep cells cold as the azide kills cells at room temperature.

(b) If cells are being used in subsequent *in vivo* or *in vitro* assays, leave out the azide, filter sterilize all solutions and handle cells aseptically. Nitex can be autoclaved in sealed pouches. Alternatively, sterile cell strainers (Falcon #2350; 70μm mesh) can be used if dealing with limited sample numbers.

(c) To scale up staining, keep cells at 4-10 x 10^7cells/ml. Wash with 5-10x staining volume, and underlay with 0.5-l mL CS. After last wash, filter through nylon mesh and adjust cell density to 1-10 x 10^6cells/mL in SM with PI.

(d) If you are troubled by high levels of non-specific background staining, try removing antibody aggregates: airfuge reagents (concentrated unconjugated, FITC, TR, or biotin conjugates) for 10 min. For APC/PE conjugates -> microfuge for 10 min in cold room. Always keep Ab stocks on ice and in the dark if using directly conjugated preps.

(e) For each 1º or 2º Ab, the saturating concentration must be determined empirically. A 3-fold diltution series is often most useful (*eg.* $^1/_{50}$, $^1/_{150}$, $^1/_{450}$, etc.) with appropriate cells. Antibody vendors often recommend a concentration to use, but in our experience these are usually

excessive and often not optimal. Depending on cell frequency and antigen expression levels, different tissues may require different antibody concentrations. Staining should always be done with Ab at 2X saturation. For further information, consult our Antibody Titration Protocol.

References:

1. Holmes, K., Lantz, L.M., Fowlkes, B.J., Schmid, I., and Giorgi, J.V. (2001)Preparation of Cells and Reagents for Flow Cytometry. Coligan *et al*. (Eds.) *In* Current Protocols in Immunology, pp. 5.3.1-5.3.24. John Wiley & Sons, Inc.

2. Strober, W. (1997). Common Immunologic Techniques – Monitoring Cell Growth. Coligan *et al*. (Eds.) *In* Current Protocols in Immunology, pp. A.3A.1-A.3A.2. John Wiley & Sons, Inc.