Intracellular Staining
(BD Biosciences Protocol)

Materials:
Staining medium (SM) [1X HBSS; 2% (v/v) calf serum; 10mM NaN_3, 10 mM HEPES, pH 7.2]
BD Cytofix/Cytoperm Kit [554714]*
Sterile filtered calf serum (CS) [0.45μm TC sterile filtered]
Gey's solution
0.1% Trypan Blue
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. A considerable amount of cell loss will occur during the multiple washes and spins. The CS is used to underlay the cell suspension during washes. For peripheral blood lymphocytes (PBLs) use 1-8 X 10^5 cells/tube *ie.* whatever you can get.

1) If desired, first perform staining for extracellular markers according to the FACS staining protocol.

Optional: block EC epitopes with unconjugated antibody of choice if investigating the intracellular levels of proteins that are expressed on the cell surface (ex: TCR, CD4. 8 etc.)

2) Wash cells in SM and collect them by centrifugation at 400x*g* for 5 minutes at 4ºC (1500 rpm in Beckmann GS6-KR). Aspirate supernatant.

3) Fix and permeabilize cells by re-suspending cell pellet in 200μl of BD Cytofix/Cytoperm Buffer™ per tube. Incubate cells for 20-30 minutes on ice.

4) Wash cells in 1ml 1xPerm Wash Buffer™ per sample (this buffer comes as a 10x solution, should be diluted to 1x using ddH_2O prior to the experiment. You can store any unused portions at 4°C). Collect cells by centrifugation at 400x*g* for 5 minutes at 4ºC. Aspirate supernatant.

5) Perform intracellular staining by re-suspending the cell pellet in 50μl of the appropriate antibody dilution <u>in 1xPerm Wash Buffer™</u>. Incubate 20-30 minutes on ice, light protected.

6) Wash cells in 1ml 1xPerm Wash Buffer™ per sample. Collect them by centrifugation at 400x*g* for 5 minutes at 4ºC. Aspirate supernatant.

7) Resuspend cells in 500μl SM, filter through Nitex mesh and acquire samples on the cytometer. DO NOT add PI to the SM used in this step, since your cells are now permeabilized and will all be PI positive.

Note: you can store cells at 4ºC, protected from light for subsequent analysis on the cytometer. It is recommended that fixed samples be run on the cytometer within 1 week, preferably the next day. Autofluorescence tends to increase and sample quality generally declines with long-term storage of fixed samples.

*These buffers can be purchased separately too: BD Cytofix (contains 4% paraformadehyde), cat # 554655 and BD Perm/Wash (contains saponin and FBS), cat # 554723.

***Note*:** works well with all fluorochromes, including PE and APC based tandem conjugates. The optimal antibody concentrations for intracellular stains tend to be lower than for the same antibody used for extracellular stains. As always, the optimal antibody concentration for your specific application should be pre-determined by running a titration series – see our related "Antibody Titration protocol".