Protocol for Phospho-Flow Cytometry Preparation

(Provided by Donald J McGuire and Dr. Chander Raman)

Phospho Flow Methanol perm

Cell stimulation

Do your regular cell stimulation procedure

Fix and Perm

- Remove supernatant
- Fix in fixation buffer 15 min @ RT (BD Cytofix™ buffer, Cat# 554655 or Biolegend #420801. They are both a stabilized 4% paraformaldehyde)
- Centrifuge **1000 rpm** 5 min remove supernatant and resuspend in cold 90% methanol (10% PBS) (for STATs)(70% for most other) incubation 15 min **4°C**

Fix and Perm cell culture (with live/dead staining)

- Remove supernatant
- Fix in fixation buffer diluted 1:10 (0.4% paraformaldehyde) in PBS 7 min @ RT
- Centrifuge **1000 rpm** 5 min remove supernatant
- Live/dead stain with a fixable live/dead 15 min 4C (eBioscience Fixable Viability Dye eFluor® 660, 1:500 or eBioscience Fixable Viability Dye eFluor® 780, 1:1000)
- Centrifuge **1000 rpm** 5 min remove supernatant
- Fix in fixation buffer (4% paraformaldehyde) 7 min @ RT
- Centrifuge **1000 rpm** 5 min remove supernatant and resuspend in cold 90% methanol (10% PBS) (for STATs)(70% for most other) incubation 15 min **4°C**

Staining

- In Standard FACS buffer
- Centrifuge **1000 rpm** 5 min remove supernatant and resuspend in Fc block (Ab clone 2.4G2 or rat serum could be used) 1:400 15 min at **4°C**

- Centrifuge **1000 rpm** 5 min remove supernatant and
- Resuspend in 50 ul 1^{o} Ab 1:100-1:400 for 1 hr 4^{o}C (p-STAT1 or p-STAT3 1:400; Cell signaling)
- Centrifuge **1000 rpm** 5 min remove supernatant and wash with FACS buffer
- Wash 3x
- Stain with A488 conjugated Goat anti Rabbit 2^{o} Ab (Jackson ImmunoResearch Laboratories) 1:500 and other cells markers eg CD4 and CD25 (Note: Methanol perm effects epitopes and dyes. Thus, antibody specific optimization of staining is required. CD25 staining is weaker when done after fix and perm PerCP or Cy5.5 and Cy7 do not work if done before methanol perm. BD collaborated with Cytobank and has validated a large number of Ab clones and dyes for different conditions, please see additional links below)
- Wash 3x
- Centrifuge **1000 rpm** 5 min remove supernatant and wash
- Wash 3X
- Run flow

Additional useful information

1. **Techniques for Phospho Protein Analysis** available from *BD Biosciences* http://www.bd.com/resource.aspx?IDX=17718

2. **Phospho-Ab chart for titration and FACSelect™ Buffer Compatibility** available from *Cytobank* (this is a highly recommended resource) http://www.cytobank.org/facselect/

3. **FACSelect™ Buffer Compatibility** provided by *BD Biosciences* http://www.bdbiosciences.com/documents/BD_FACSelect_BufferCompatabilityResource_ProdInfoSheet.pdf