Flow Cytometry Staining Protocol
Intracellular Staining

Stocks

- Ionomycin - 2.5 mg/ml in DMSO - stored -80°C
- 10µl stock — 40µl Clone Medium = diluted ionomycin (500µg/ml)
- Calbiochem cat #542400
- PMA - 2.5 mg/ml in DMSO - stored -80°C
- 10µl stock — 490µl Clone Medium = diluted PMA (50µg/ml)
- Calbiochem cat #407952
- Monensin - 2mM in ETOH - stored -20°C - Sigma cat#M5273
- Good IX PBS - stored room temperature - lab prepared
- FBS - stored -20°C - Gemini Bio Products cat #100-106
- NaN3 - 20% - stored room temperature - lab prepared
- CytoFix/CytoPerm Kit: - stored 4°C - Pharmingen cat#2075KK
- DNase — 1mg/ml in PBS — stored -20°C — Sigma cat#D5025
- EMA — stored -20°C - Molecular Probes cat#E1374
- Para-formaldehyde - 10% - stored -20°C - lab prepared

Clone Medium

- RPMI 1640 w/o L-glutamine (500ml)
- 10% FCS (50ml)
- 2mM L-glutamine (5ml)
- 10mM Hepes (5ml)
- 25ug/ml gentamicin (1ml)
- 50uM 2-ME (0.5ml)
- 2U/ml IL-2 (0.85ml)

Stock Solutions

- FCS (HyClone)
- L-glutamine - 200mM
- Hepes buffer - 1M
- gentamicin sulfate - 12.5mg/ml
- 2-ME - 50mM

- IL-2 - 1200U/ml

Stimulating Medium

- 50µl diluted ionomycin (50µg/ml)
- 5µl diluted PMA (0.5µg/ml)
- 445µl Clone Medium
- 0.5ml total volume - use immediately

(Hint: Add 485 µl Clone Medium to ionomycin vial; add 490 µl Clone Medium to PMA vial, mix and transfer 5µl of diluted PMA to ionomycin vial yielding the final solution.)

Stain Medium

- 1L Good 1X PBS
- 30ml FBS (3%)
- 2ml NaN3 stock (0.04%)
- approx 1L total volume - store 4°C - 6 months

PBS-A

- 1L Good 1X PBS
- 2ml NaN3 stock (0.04%)
- approx 1L total volume - store RT - 1 year

DNAse Solution

- PBS-A containing 10% stock DNAse (0.1mg/ml)
- (1ml per sample - make an extra dose)
- Must be made fresh.

EMA Staining Mix

- Stain Medium + 0.04% EMA
- (250µl per sample - make an extra dose)
- Must be made fresh - protect from light at all times.

CytoFix/CytoPerm: Pharmingen

- Cytofix/Cytoperm: pre-prepared
- Perm/Wash Buffer: dilute 1:10 in ddH2O

Final Fixing Buffer

- 1ml para-formaldehyde stock (1%)
- 9ml Stain Medium
- 10ml total volume - use immediately - light sensitive

Isolation/Preparation of Spleen Cells

- Collect spleens from mice and place each in a small petri dish containing 5ml Clone Medium.
- Isolate lymphocytes using the syringe puncture method.
- Wash and lyse rbcs with ACT.
- Wash with Clone Medium and resuspend in 2ml Clone Medium.
- Place exactly 1ml in one well of a 12 well plate.
- Add an additional 1ml of Clone Medium to the well.
- Can store spleen suspensions in 12 well plate overnight in refrigerator.
- Use remaining spleen cells for phenotype FACS if desired - wash with Staining Buffer to remove Clone Medium.

Stimulation of Spleen Cells

Prepare Stimulating Medium

- Add 10µl Stimulating Mix per 1ml of cell suspension (ie 20µl/well). **Note:** final concentrations should be 5ng/ml PMA and 500ng/ml ionomycin.
- Incubate at 37°C for 3 hours.
- Add 1µl Monensin stock per 1ml cell suspension (ie 2µl/well). Note: final concentration should be 1µM Monensin.
- **Remember to thaw out Th1 and Th2 positive cells to treat along with spleen cells in the following steps.**

- Place cells into FACS tubes containing 1ml Stain Medium and centrifuge at 1200rpm for 5 min.
- Resuspend in 1ml DNAse Solution.
- Incubate in 37°C water bath for 10 minutes.
- **Remove approximately 10ul of cells from one sample's cell pellet to be used for unstained and single color controls**
- Prepare EMA staining mix - protect from light.
- Resuspend each sample in 250µl EMA Staining Mix.
- Incubate on ice PROTECTED FROM LIGHT for 15 minutes.
- Expose to fluorescent light for 10 minutes - place on bench under bench-top fluorescent light - approx. 12-18 inches away.
- Wash in 3ml Stain Medium.
- Resuspend each pellet in 200µl CytoFix/CytoPerm solution.
- Incubate in refrigerator or on ice PROTECTED FROM LIGHT for 20 minutes.
- Wash 2 times in 1ml Perm/Wash Buffer.
- Resuspend cell pellet in 50µl Perm/Wash Buffer X number of rxns.
- Remove some cells for EMA single color control.
- Distribute 50µl into individual tubes or wells.
- Add antibody mixes to appropriate wells.
- Incubate in refrigerator for 30-60 minutes.
- Wash 2x with CytoPerm Wash Buffer.
- Resuspend in 50µl Final Fixing Buffer.
- Place cell suspension in labeled FACS tubes containing 100-200µl Stain Medium prior to running on analyzer.