Intracellular Staining Methods and Notes:

Below are two intracellular staining methods. It is important that cells are fixed with paraformaldehyde before they are permeabilized as cells will lyse without fixation during the permeabilization process.

Saponin method

1. Fix cells in 4% paraformaldehyde in PBS at room temperature for 15 min.
2. Wash in PBS/2% FBS/3 mM azide twice.
3. Permeabilize in PBS/0.1% saponin/10% FBS RT for 15 min.
4. Incubate with Ab in saponin buffer for 30 min at room temperature.
5. Wash in saponin 3 times.
6. Do second and third steps same as first, if necessary.

Tween method

1. 1% paraformaldehyde in PBS room temperature for 15 min.
2. Wash in PBS/2% FBS twice.
3. Permeabilize in 0.2% Tween for 15 min at 37°C.
4. Incubate in Ab in 0.1% Tween in PBS/2% FBS for 30 min on ice.
5. Wash in 0.1% Tween times 3.
6. Repeat as necessary.

Becton Dickinson makes a Perm/Wash buffer (Cat. # 554723) that works quite well for intracellular staining. BD also offers two kits with protein transport inhibitors, Cytofix/Cytoperm Plus Kit with BD GolgiStop™ protein transport inhibitor (containing monensin; Cat. # 554715) and BD Cytofix/Cytoperm Plus™ Kit with BD GolgiPlug™ protein transport inhibitor (containing brefeldin A; Cat. # 555028). Monensin and brefeldin A act as cytokine secretion inhibitors and are both available from Sigma.

eBioscience has a handy web page (http://us.ebioscience.com/resources/best-protocols/flow-cytometry/flow-cytometry-intracellular-staining-quick-guide.htm) that describes how best to stimulate, block secretion of and clones used to stain most intracellular cytokines.

Intracellular Staining Notes from Purdue Cytometry Email Discussion List: (http://www.cyto.purdue.edu/hmarchiv/index.htm)

For most intracellular staining experiments, we start with surface staining and then fix, permeabilize, and then do the intracellular staining. The exceptions to this order of events is when you are looking at things that are time sensitive, such as phospho-ERK, where you want to fix at specific time points to stop the

reaction. Also, some surface Abs are fine on fixed cells, some do not work. Unfortunately, there is only one way to know – try it.

I am not sure how you do your staining; I like to do it in 96-well plates, but most people use FACS tubes. They both work. 96-well plates are easier than tubes because you can see the pellets better. Tubes are faster because your wash volumes can be much larger and thus, you need fewer washes.

Surface staining (if doing surface + intracellular staining)
For standard intracellular staining, start with your normal surface staining protocol. We use 1 x 10^6 cells in 100 μl in staining buffer (see below). The concentration of the Ab will vary from Ab to Ab and is best determined by titrating each Ab individually. It is a pain, but it is the best way.

After surface staining (30 min on ice), wash the cells as usual (3 times with 200 μl in plates, or 1-2 times with 2 ml in tubes).

Fix the cells
Bring cells up in 1% paraformaldehyde in PBS room temperature for 15 min.
We keep a 4% stock in the fridge and dilute to 1% in PBS.
I like to have the paraformaldehyde at RT before adding, but it probably does not really matter. Wash away the paraformaldehyde. (2 washes, either plates or tubes)

Permeabilize the cells
Re-suspend cells in 100 μl 0.2% Tween in PBS for 15 min at 37°C.
This is probably the most variable part of the protocol and for some reason; different cells and Ab behave differently. If it does not work, you can try 0.1% saponin or try extending the permeabilization time. Increasing the detergent concentration may help, but also may ruin the cells – hard to say.

Intracellular staining
For intracellular staining, we add the antibodies to 0.1% Tween in PBS/2% FBS. Stain 10^6 cells in 100 μl buffer. The Ab concentration will vary, depending on the Ab.
Incubate 30 min on ice.
Wash in 0.1% Tween in PBS/2% FBS (3 times in plates, 2 times in tubes).
Washing permeabilized cells can be a bit dangerous; they do not pellet very well. I usually spin them for a few extra minutes each time (normally I do 1200 rpm x 5 min; here I would do 1200 rpm x 8 min). People who use tubes like to pour off the supernatant. This is a good way to lose your cells here. I strongly recommend aspirating off most, not all, the supernatant. I usually leave 25-50 μl. This is where I particularly like using plates to stain.

After the wash, bring cells up in 1% paraformaldehyde to fix and store at 4°C. The percentage of paraformaldehyde likely does not matter.