Jonathan Irish is an Assistant Professor in the Department of Cancer Biology at Vanderbilt University (my.vanderbilt.edu/irishlab). He is expert at both mass cytometry and phospho-specific flow cytometry. We asked Dr. Irish if he had any advice for one of our CyTOF users who is new to phospho-flow protocols and looking for any helpful tips, especially regarding epitope stability when fixing with methanol. We thought it might be helpful to share his reply:

My main piece of advice*: unless it's part of the experimental question, don't even try to stain surface markers post methanol fixation!

For CyTOF work, we use a multi-step staining protocol. In signaling experiments, we typically stain for surface markers after the cells have been fixed so that detection of cell identity does not alter signaling. However, since many surface marker target epitopes are no longer detectable following harsh permeabilization conditions (e.g. >90% methanol), we prefer to stain surface targets after a short fix of 1.6% para-formaldehyde (PFA) for 5 minutes at room temperature (this is the fix step that stops signaling in the phospho-flow protocol). In this protocol, surface staining occurs following stimulation/fixation and prior to the methanol (MeOH) step, which additionally fixes and permeabilizes the cells. For more information, see Table I and Fig. 2 in Krutzik et al., *J Immunol* 2005 and Krutzik and Nolan *Cytometry A* 2003 (references below).

Usually a short PFA-fix doesn't destroy the target epitope and detection of surface markers is acceptable (although not as good as staining pre-fixation), although there are exceptions. We recommend titrating antibodies in the exact conditions that they will be used on mixed populations of positive and negative control cells. Also, for certain intracellular targets – especially transcription factors – permeabilization with Saponin or Triton can yield superior staining.

Usually the staining workflow post stimulation and fixation should move from mild to harsh permeabilization conditions:

Quick fix (1.6% PFA, 5' @ 23 °C) **> Staining Round 1** (surface epitopes) **> Gentle Perm** (e.g. saponin) **> Staining Round 2** (e.g. transcription factors) **> Harsh Perm** (e.g. MeOH) **> Staining Round 3** (e.g. phospho-proteins, nucleic acid stain)

We haven't observed any impact on detection of the signal for CyTOF metal-labeled tags be impacted by any of the perm conditions above.

Resources for information on specific epitopes regarding permeabilization sensitivity include:

1) BD FACSelect:
http://www.cytobank.org/facselect/
Shows titration and perm condition data for human and mouse; links to a comprehensive table.

2) Published phospho-flow papers (see below).

DVS Sciences, 639 N. Pastoria Ave
Sunnyvale, CA 94085-2917
www.DVSsciences.com

A few other tips:
1) With intracellular work, less is more. If an antibody clone is "supposed to work" but one is having trouble getting it to stain controls, usually it's because of over-staining / too much background. See Figure 2 in Krutzik et al. (below).

2) If an antibody works by immunofluorescence, then that clone will likely work by fluorescent flow or mass cytometry using the same fix/perm conditions.

3) Always titrate!! (even if someone else says an antibody clone works best at "x" dilution / concentration)

a) Make sure to have a mix of positive and negative cells in the same tube for the titration (see Fig.2 in the Krutzik et al. paper)
b) Use a cell type that is easy to work with and has known expression. Do not use "special/rare/ interesting" cells for titration.
c) Use a "good channel". For especially tricky epitopes or low abundance targets, consider titrating first with an outstanding fluorochrome on fluorescence flow before mass cytometry (using the same antibody clone).
d) It may be necessary to titrate multiple clones under multiple perm conditions if an intracellular epitope is something new or unusual.

References:

Krutzik PO, Clutter MR, Nolan GP. J Immunol 2005 Aug 15; 175(4):2357-65
Krutzik PO and Nolan GP. Cytometry A 2003; 55A:61–70

* Caveat: These are observations that we've found to work over time in our experiments, but there may be other techniques that work just as well.

DVS Sciences, 639 N. Pastoria Ave
Sunnyvale, CA 94085-2917
www.DVSsciences.com