

Monitoring Intracellular Phosphorylation Events by Flow Cytometry: Experiment #1: Human Cell Line

Materials

- U937 cell line
 - ATCC CRL-1593.2
- Human interferon-gamma (IFN- γ) and interleukin-4 (IL-4)
 - BD # 554617 and BD # 554605
- Formaldehyde: 16% solution in water, ampules
 - Electron Microscopy Sciences (# 15710)
- 100% 4°C methanol
- Staining media:
 - PBS
 - 0.5% BSA
 - 0.02% NaN₃
- Phospho-specific antibodies
 - Becton Dickinson
 - Phospho-Stat1 (Y701) clone 4a
 - Alexa Fluor 488 (BD# 612596)
 - Phospho-Stat6 (Y641) clone 18
 - Alexa Fluor 647 (BD # 612601)
- 5 ml FACS tubes
- Culture/Stimulation Media:
 - RPMI
 - 10% FBS
 - PSG

Methods

Stimulation:

Sample	1	2	3	4	5
Stimulus	None	None	IFN- γ	IL-4	IFN- γ + IL-4
Concen.	---	---	10 ng/ml	10 ng/ml	10 ng/ml; 10ng/ml

1. Suspend cells at $\sim 1 \times 10^6$ cells/mL in culture media, and warm to 37°C in a tissue culture incubator (5% CO₂). Aliquot 1 ml ($\sim 1 \times 10^6$ cells) per stimulation condition into FACS tubes.
2. Add IFN- γ and/or IL-4 at a final concentration of 10 ng/mL (e.g. 10 μ L of a 1 μ g/mL cytokine stock).
3. Vortex to mix. Incubate for 15 min at 37 °C, 5% CO₂.

Fixation and Permeabilization:

1. Add formaldehyde directly to the cells at a final concentration of 1.5% (1:10 dilution of the 16% stock; $\sim 100 \mu$ l for 1ml sample), and vortex to mix. Do this

- quickly for all samples. *The media will turn yellow.*
2. Incubate for 10 min at room temperature.
 3. Pellet the cells by centrifugation at 500g for 5 min at 4°C. *Do not add any staining media prior to the spin; it is bad for the methanol permeabilization step.*
 4. Decant supernatant, and vortex to thoroughly resuspend cells in the residual buffer. Then add 4°C MeOH (about 1 ml per 1×10^6 cells) and vortex.. *The addition of methanol to a pellet of cells will result in severe clumping and cell loss.*
 5. Permeabilize at 4°C for 10 min, -20°C overnight, or -80°C longer than overnight. *Signal decreases slightly with long permeabilization periods. The permeabilization is the best place to take an overnight break (rather than after the staining).*

Staining and Analysis:

Sample	1	2	3	4	5
Stain	none	pStat1-Alexa488 + pStat6-Alexa647			

1. Add 3 ml staining media to samples. Pellet the cells as in Step 3.
2. Wash the pellet with ~3 ml staining media. Pellet.
3. Resuspend cells at $\sim 1 \times 10^7$ /ml (100 μ l for 1×10^6 cells) in staining media.
4. Pipet 100 μ l of each sample into a new tube. *Identical staining volumes are critical for consistent staining between samples.*
5. Add the indicated antibodies (20 μ l each) to the samples. *In order to achieve consistent staining, make up a cocktail of the antibodies needed for all the samples in one vial, then distribute the antibodies to the samples.*
6. Incubate for 30 min at room temperature.
7. Wash with 3 ml staining media.
8. Analyze on FACSCalibur style instrument with 488 and 633 nm laser lines.
 - a. Set PMTs so that unstained cells appear in the lower quadrant of both channels.

Expected Results:

- Unstained/Unstimulated: lowest staining in both channels.
- Unstimulated: low staining in both channels, slightly higher than the unstained sample.
- IFN- γ stimulation: high pStat1 staining. IFN- γ is a strong inducer of pStat1. pStat6 staining should remain as in sample 2. Fold change in pStat1 (calculated as [median fluorescence intensity (MFI) of stimulated cells]/[MFI of unstimulated cells]) should be 10-fold or greater.
- IL-4 stimulation: high pStat6 staining, low pStat1 staining (as in sample 2). IL-4 induces pStat6 selectively. Fold change in pStat6 should be ~ 5 -fold.
- Dual stimulation: high pStat1 and pStat6 staining. Fold changes should be identical to those observed in samples 3 and 4.

