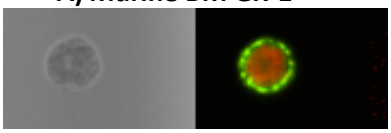
Experimental Design and Sample Preparation

Experimental Design: General guideline – The ImageStream System provides high information content data for individual cells and cell populations within heterogeneous samples. In addition to providing standard fluorescent signal strength data like a flow cytometer, the ImageStream system quantifies the specific location and distribution of signals within cells. Further, it can measure photometric absorbance and quantify morphology at any location within the cell. Therefore, the best applications take advantage of these capabilities to discriminate subtle changes within individual cells or cell populations.

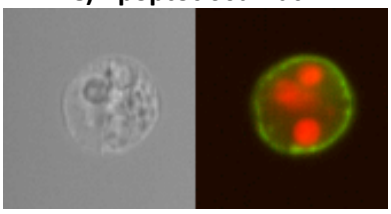
1. **Choice of cell type:** Cells to be analyzed can be either adherent or non-adherent, but should be adaptable to flow and have a diameter inferior to 40µm for the 60X magnification (< 60µm for the 40X and < 120µm for the 20X)

A) Murine BM GR-1+

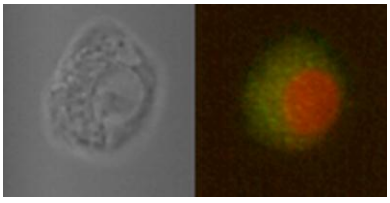


B) Murine BM CD19-PE+

C) Apoptotic Jurkat



D) A-549 (NFκB FITC)



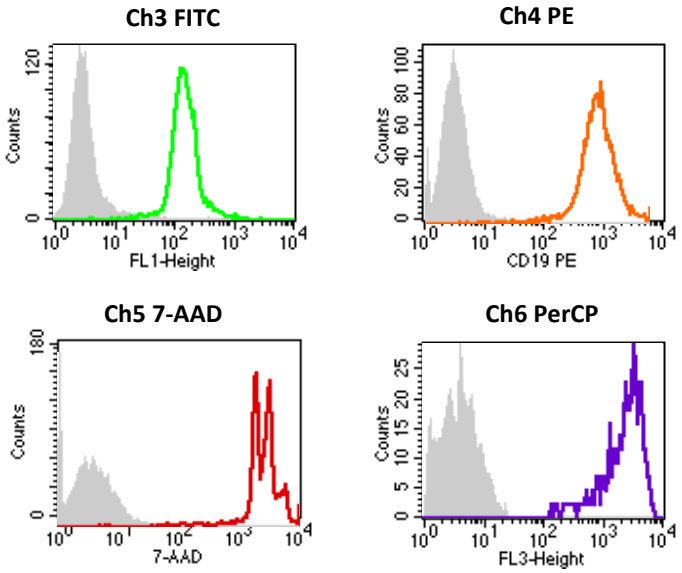
2. **Final sample concentration:** Ideal – $5x10^7$ to 10^8 cells/ml. Can image as low as $2x10^7$ cells/ml. We routinely run samples at 10^8 cells/ml to increase throughput. Lower concentrations increase collection time.

3. **Scale:** We typically image 10,000 cells per sample. The speed at which the sample is acquired depends on the cell concentration.
   File size vary based on the number of cells and parameters saved. It is possible to turn off unused parameters to reduce file size.

4. **Final sample volume:** A minimum of 60µl in a 1.5ml Eppendorf tube.

5. **Choice of fluorochromes:** see "ImageStreamX information" pdf.

6. **Brightness of stain:** In terms of pure sensitivity, the ability of the ImageStream system to detect and quantify fluorescent stains (using 488nm excitation) has been carefully measured in head to head trials with industry standard flow cytometers. Depending upon the distribution of signals, the size of the objects being measured and the wavelength of fluorescence, the ImageStream is as much as 10X more sensitive than the most sensitive of these devices. However, quantitation of the location and distribution of signals is a far more demanding task than pure measurement of signal strength. Therefore, to ensure the best results for localization and distribution applications, Amnis has developed some rough guidelines using standard flow cytometric measurements for signal strength of stained and non-stained cells. These are as follows; a one decade shift in Ch3 (FL1), a two decade shift in Ch4 (FL2) and a three decade shift in Ch5 and Ch6 (FL3 and 4). The following flow cytometer histograms show intensity levels for surface of nuclear stains that would be ideal for localization applications.



7. **Controls:** For spectral compensation it is important to have unlabeled cells, and cells labeled with a single color positive control for each fluorochrome used (i.e. FITC only cells, PE only cells, etc.).

8. **Comparative data:** Take microscope images and run samples on a flow cytometer before sending samples to Amnis to prevent ambiguity of results.

Sample Preparation:

1. **Protocols:** In general any established labeling protocol should suffice. See "Current Protocols in Cytometry" for general labeling techniques. Stain cells on ice when possible to reduce non-specific capping of antibody. Include a sample list with the number of cells per tube, fluorochromes, and expected results of the staining pattern.

2. **DNA dyes:** If using a DNA intercalating dye such as 7-AAD it is best to permeabilize the membrane of fixed cells with 0.1% triton X-100 and leave the dye in suspension during the run to prevent it from leaching out.

3. **Fixation:** Thoroughly fix cells (ie. 1% PFA at RT for 20 minutes) if applicable.

4. **Optimal Focus and Core Stability:** The ImageStreamX uses 250 micron diameter tubing and is designed to produce and operate with a well-controlled and stable hydrodynamically focused core. Large cell aggregates approaching or exceeding half the tubing diameter can lead to erratic and unstable core behavior. Therefore, we strongly advise de-aggregation of clumps as a final step by straining the sample through a 70 micron nylon mesh strainer. If this is not available, 5 plunges through a 25 gauge syringe may be substituted for straining. If the sample is still aggregated, we suggest an anti-clumping buffer such as EDTA or Accumax.

5. **Final sample Volume and Concentration:** $5x10^7$ to 10^8 cells/ml in 100 µl in a 1.5 ml Eppendorf tube.