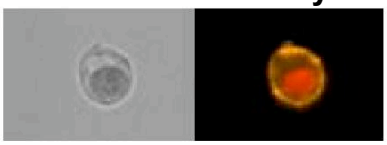
2 camera STD

Sample Preparation Guide

Experimental Design: The ImageStream system can quantify the intensity, specific location, and distribution of signals within tens of thousands of cells per sample. The system can perform most any standard flow cytometric assay, but the best applications take advantage of the technology's imaging capabilities to discriminate subtle morphologic or signal distribution changes within individual cells and cell populations.

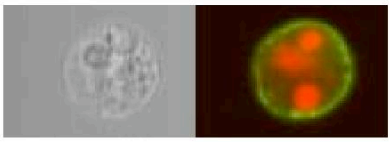
1. **Choice of Cell Type:** The cell/particle size should be **less than 45 microns** in diameter. The system can analyze a wide variety of cell types and applications. Example imagery is shown below:

HuPB CD14+ Monocyte

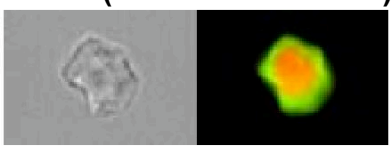


HuPB CD45+ Lymphocyte

AnnexinV+ Jurkat



THP-1 (NFB FITC / 7-AAD)



2. **Choice of Fluorochromes: Choose fluorochromes from the table below that are excited by the lasers in your ImageStream** (405, 488, 561 and 658 nm). Note: a more detailed chart on page 4 includes laser excitation information for each dye. **Channel 1/9 are always brightfield**. **SSC imagery may be placed into channel 6** if desired. Dyes with **an *** are excited by at least one laser directed to camera 1 and another directed to camera 2. **For these dyes, the channel that the dye will appear brightest in depends on the relative laser powers used**. Recommended dyes are in boldface.

Ch	Band (nm)	Fluorochromes
1	430-480	Brightfield
2	470-560	**FITC**, **AF488**, **GFP**, YFP, Syto13, SpecGreen, Kaede Green, mVenus
3	560-595	**PE**, **AF546**, Cy3, DSRed
4	595-660	**PE-TexasRed***,ECD*, 7-AAD*, PI*, Kaede Red, mCerulean, mKate
5	660-745	PE-Cy5*, PE-AF647*, PerCP-Cy5.5*, PE-Ax700
6	745-800	**SSC**, PE-Cy7*, PE-AF750*, PE-eFluor780
7	430-505	**DAPI**, Hoechst, AF405, PacBlue, CasBlue, CFP, Dyecycle Violet, SYTOX Blue
8	505-570	PacOrange, AF430, CasYellow, LIVE/DEAD Aqua
9	570-595	Brightfield
10	595-660	**TexasRed***, AF594*
11	660-745	**AF647, AF660,** APC, Cy5, DRAQ5*, Dyecycle Ruby, Alexa 700, SYTOX Red
12	745-800	**APC-Cy7**, APC-AF750, APC-eFluor780

Please note that this is not a complete list of all of the fluorochromes that will work on the ImageStream.

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3. **Protocols:** In general, any established labeling protocol used for flow cytometry will work with the ImageStream (see *Current Protocols in Cytometry* for general labeling techniques). Stain cells on ice in the presence of azide when possible to reduce non-specific capping of antibody. Use polypropylene tubes, preferably siliconized, to process samples.

4. **Brightness of Stain and Stain Balancing:** The sensitivity of the ImageStream is comparable to a flow cytometer. However, quantifying the location and distribution of signals in an image is a more demanding task than the measurement of simple signal strength. Therefore, follow these guidelines for the highest possible data quality:

   - Adjust your staining protocols to achieve at least a full log shift over background, as measured on a standard flow cytometer.
   - Use the brightest fluorochrome (ie AlexaFluor 488 or PE) for the antigen with the smallest copy number.
   - The sensitivity of the instrument to different fluorochrome probes can be independently controlled. However, data quality is significantly better when the reagents used in an experiment that are excited with the same laser are titrated such that the brightness levels of all probes are balanced to within a log of each other. Probe balancing avoids the saturation of bright stains when they are combined with dim stains in the same sample.

5. **Controls and Samples:** Please be sure to provide

   - Single color and unlabeled controls in separate tubes
   - Positive biologic control
   - Negative biologic control
   - Experimental samples

6. **Cell Aggregation:** We strongly advise de-aggregation of clumps as a final step before straining the sample through a 70 micron nylon mesh strainer. If sample aggregation is a problem, we suggest using an anti-clumping buffer such as EDTA or Accumax prior to fixation.

7. **Fixation:** If fixation is desired, thoroughly fix cells (i.e. 1% PFA on ice for 20 minutes).

8. **Final Sample Concentration and Volume:** At least 1 million cells in 60 µL ($2x10^7$ cells/ml) of protein containing buffer (ie PBS/2%FBS) in a 1.5mL siliconized microcentrifuge tube (Sigma T4816).

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9. Documentation:

- Detailed description(s) of the sample preparation protocols.
- Be sure to assess final cell concentrations of samples before you bring them to FCRC.
- IMPORTANT: To verify sample quality upon receipt, please bring microscope images and flow cytometry data if you've acquired previously.