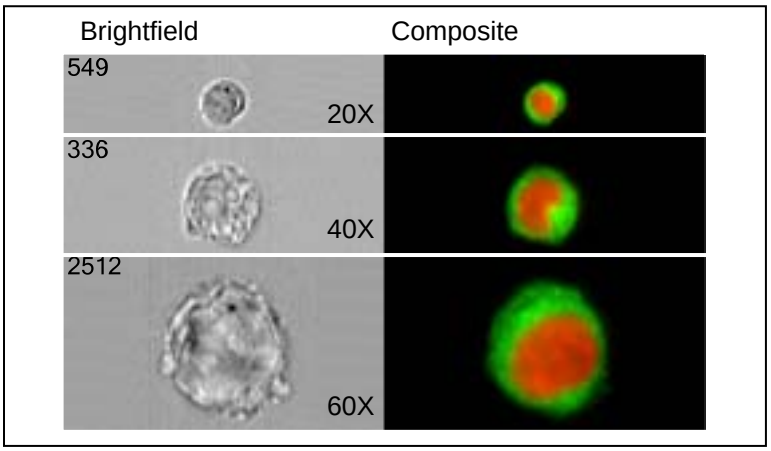
Sample Preparation Guide

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 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 particle size should be less than 120um using 20x magnification, 60um using 40x, and 40um using 60x. Images below are THP1 cells (~15um diameter) labeled with FITC NFkB and Draq5.



2. **Final Sample Concentration and Volume:** At least 1 million cells in 50 μL ($2x10^7$ cells/ml) in PBS/2%FBS in a 1.5mL siliconized microcentrifuge tube. Will run ~400 cells per second on low speed.

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 siliconized polypropylene tubes when possible.

4. **Choice of Fluorochromes:** Choose fluorochromes that are excited by the lasers in your ImageStream (405,488,642nm are most common). Use the chart on p.3 or look online for a spectra viewer that will help you plan which dyes will work the best.

5. **Compensation:** Have a sample of cells each labeled with a single-color for each fluorochrome used (i.e. FITC only cells, PE only cells, etc.).

6. **Cell Aggregation:** Minimize aggregation problems by straining the sample through a 70um nylon mesh strainer, or by using an anti-clumping buffer such as EDTA or Accumax prior to fixation.

7. **Fixation:** If fixation is desired, thoroughly fix cells with 1% PFA on ice for 20 min.

8. **Number of samples:** No more than 30 total for feasibility experiments. Please limit the samples to the following; Positive and Negative ***biologic controls***, ***compensation controls***, and ***experiment samples***.

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9. **Brightness of Stain and Stain Balancing:** Quantifying the location and distribution of signals in an image is a demanding task that requires optimized labeling. Below are a few suggestions to help design the experiment:
    - Try to achieve at least a full log shift in fluorescence, as measured by FACS.
    - Use the brightest dye for the antigen with the smallest copy number.
    - The brightness of probes can be independently controlled by changing the laser power. However, data quality is enhanced when the brightness levels of all probes excited off a single laser 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.

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Fluorochrome Chart

		Excitation Laser (nm)										
Ch	Band (nm)	375***	405	488	561	592	642	730	785	Used	Ch
1	**435-480** (457/45)	***375 & 405*** ***Ch1/Ch9 BF or *DAPI, BV421, AF350,*** Hoechst, PB	**BRIGHTFIELD**								1
2	**480-560** (528/65)	*BV510, PacOrange, QD525,		**FITC, AF488, GFP**, YFP, DyLight488, PKH67, Syto13, SpectrumGreen, LysoTrackerGreen, MitoTrackerGreen,							2
3	**560-595** (577/35)	*QD565, QD585, eFluor565		**PE**, PKH26, Cy3, DSRed, CellMask/CellTracker/SYTOX Orange	**PE, AF546**, DyLight550, PKH26, DSRed, Cy3, SpctmOrange						3
4	**595-642** (610/30)	**Ch4/Ch10 BF or *QD625,** eFluor625, BV605		**Ch4/Ch10 BF or PE-TexRed*, ECD***, PE-AF610*, PI*, RFP, QD625*, eFluor625*	**Ch4/Ch10 BF or AF568***, DyLight594*, PE-TxRed*, ECD*, PE-AF610*, RFP, mCherry*						4
5	**642-745** (702/85)	*QD705, eFluor700, BV711		**PE-Cy5*, PE-AF647***, 7AAD*, PerCP*, PerCP-Cy5.5*, eFluor650*, FuraRedlo, Draq5*, LDS751*,	**PE-Cy5*, PE-AF647***, 7AAD*, Draq5*, LDS751*,						5
6	**745-780** (762/35)	*QD800, BV786		**PE-Cy7*, PE-AF750***,	**PE-Cy7*, PE-AF750***,				**SSC**		6
7	**435-505** (457/45)	***375 No 405*** ***DAPI, BV421,*** Hoechst, PacBlue, CascadeBlue, AF350	***DAPI, BV421, AF405,** Hoechst, PacBlue, CascadeBlue, eFluor450, DyLight405, CFP, LIVE/DEAD Violet								7
8	**505-570** (537/65)	***BV510**, PacOrange, CascadeYellow, AF430,	***BV510,** PacOrange, CascadeYellow, AF430, eFluor525, QD525								8
9	**570-595** (582/25)	**Ch1/Ch9 BF or** *QD565, QD585	**Ch1/Ch9 BF or** *QD565, QD585, eFluor565	**BRIGHTFIELD**							9
10	**595-642** (610/30)	**Ch4/Ch10 BF or** *QD625, eFluor625, BV605	**Ch4/Ch10 BF or** *QD625, eFluor625, BV605			**Ch4/Ch10 BF or TexRed*, AF594***, DyLight594*, mCherry*					10
11	**642-745** (702/85)	*QD705, eFluor700, BV711	*QD705, eFluor700, BV711			**APC, AF647, AF660**, Cy5, DyLight649, DRAQ5*	**AF647, AF660, AF680**, APC, Cy5, DyLight649, DyLight680, Draq5* PE-AF647*, PE-Cy5*, PerCP*, PerCP-Cy5.5*				11
12	**745-780** (762/35)	*QD800, BV786	*QD800, BV786			**APC-Cy7, APC-AF750,** APC-H7, APC-eFluor750	**APC-Cy7, APC-AF750**, APC-H7, APC-eFluor750, Cy7, AF750, DyLight750, PE-Cy7*, PE-AF750*	**AF750,** Cy7, DyLight750	**SSC**		12

Recommended dyes (based on optimal excitation and detection channels) are in boldface.
*Many dyes will excite by more than one laser, and this can increase cross camera compensation.
**Channel bandpass may change depending on which lasers are on. Values listed are assuming 405,488, and 642 excitation.
***375 laser is aligned to Ch1 if the system also has a 405 laser, if not its aligned to Ch7. In cases where Ch1 is used for 375 excited dyes brightfield should be placed in Ch4 and Ch10.
1 laser (488): ideal dyes are AF488, PE, PE-TxRed, PE-Cy5, PE Cy7, SSC-Ch12,
2 laser (488,642): ideal dyes are AF488, PE, PE-TxRed, SSC-Ch6, and AF647, APC Cy7
3 laser (405,488,642): ideal dyes are AF488, PE, PE-TxRed, SSC-Ch6, and DAPI, AF647, APC Cy7

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