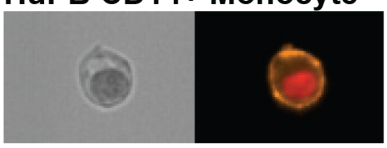
Sample Preparation Guide

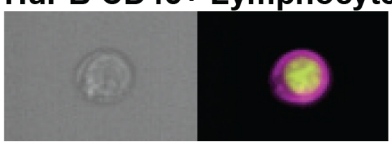
Experimental Design: The ImageStream system can quantify the intensity, specific location, and distribution of signals within tens of thousands of cells per sample. The system can perform most any standard flow cytometric assay, but the best applications take advantage of the technology's imaging capabilities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

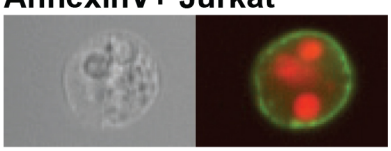
HuPB CD14+ Monocyte



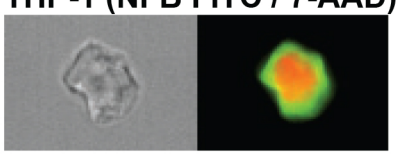
HuPB CD45+ Lymphocyte



AnnexinV+ Jurkat



THP-1 (NFB FITC / 7-AAD)



2. **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.

3. **Choice of Fluorochromes:** Choose fluorochromes from the table on **page 3** that are excited by the lasers in our ImageStream (405, 488, 561, and/or 642 nm). 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 indicated in boldface.

   Please note that this is not a complete list of all of the fluorochromes that will work on the ImageStream. Please consult Amnis before choosing other dyes.

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

5. **Cell Aggregation:** Please de-aggregate your samples before running. A 40 micron filter is best for single cells; if you are running large cells or you are looking for cell-cell interaction you can use a 70 micron filter. If sample aggregation is a problem, use an anti-clumping buffer such as EDTA or Accumax prior to fixation.

amnis

800.730.7147 www.amnis.com

Sample Preparation Guide

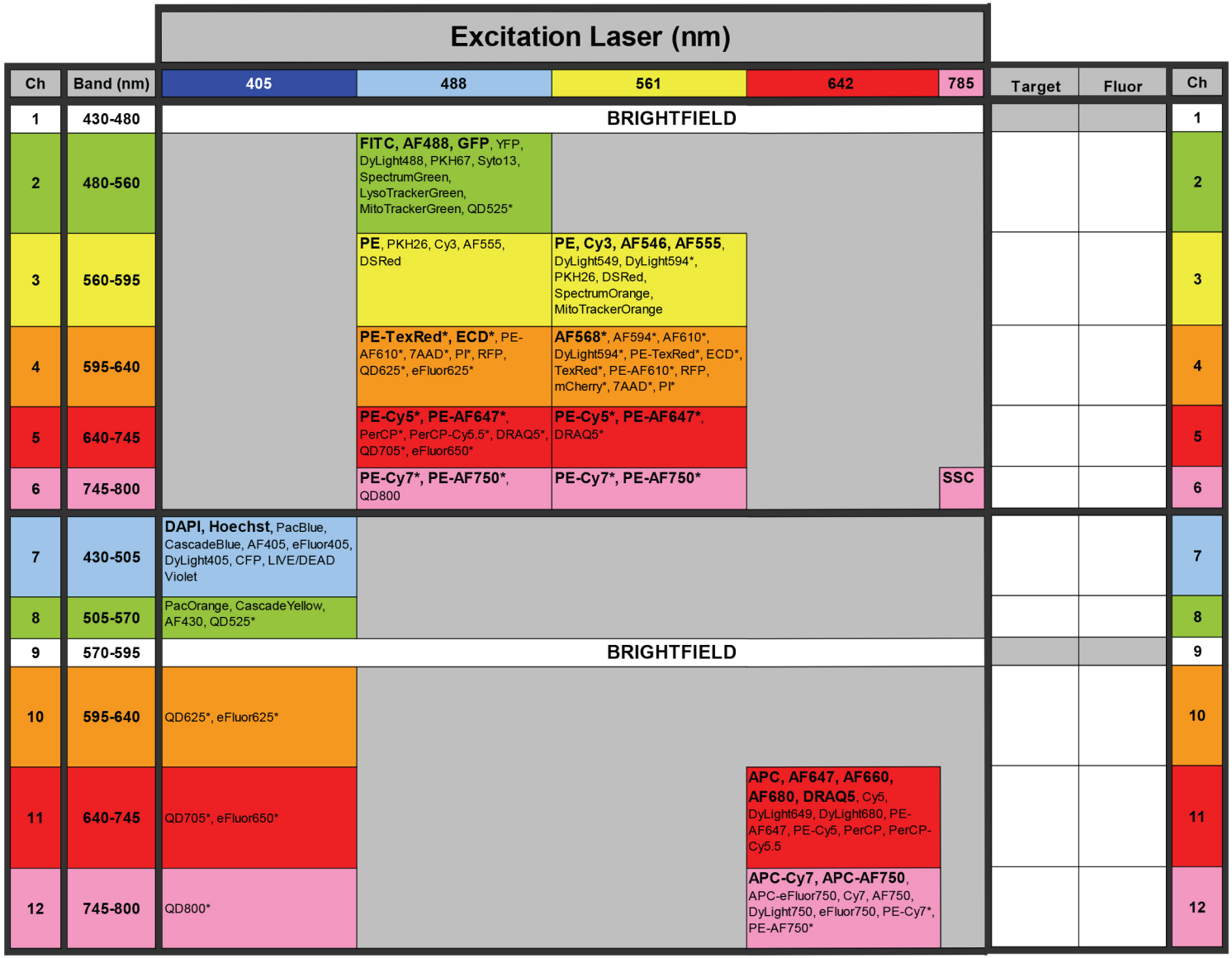
6. **Brightness of Stain and Stain Balancing:** The sensitivity of the ImageStream is comparable to a flow cytometer. However, quantifying the location and distribution of signals in an image is a more demanding task than the measurement of simple signal strength. Therefore, follow these guidelines for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

7. **Fixation:** The Image Stream is rated BSL1. Do not run any samples containing infectious agents without first exposing the sample to inactivating conditions. **All samples must be fixed.** 2% paraformaldehyde for at least 10 minutes before running the samples is recommended,

8. **Final Sample Concentration and Volume:** At least 1 million cells in 50 µL ($5x10^7$ cells/ml) of protein containing buffer (ie PBS/2%FBS) in a 1.5mL siliconized microcentrifuge tube (Sigma T4816) Sample volume can be as low as 15µL and as high as 200µL.

ImageStreamX Fluorochrome Chart



Ch	Band (nm)	Excitation Laser (nm) 405	488	561	642	785	Target	Fluor	Ch
1	430-480	BRIGHTFIELD							1
2	480-560		**FITC, AF488, GFP**, YFP, DyLight488, PKH67, Syto13, SpectrumGreen, LysoTrackerGreen, MitoTrackerGreen, QD525*						2
3	560-595		**PE**, PKH26, Cy3, AF555, DSRed	**PE, Cy3, AF546, AF555**, DyLight549, DyLight594*, PKH26, DSRed, SpectrumOrange, MitoTrackerOrange					3
4	595-640		**PE-TexRed*, ECD***, PE-AF610*, 7AAD*, PI*, RFP, QD625*, eFluor625*	**AF568***, AF594*, AF610*, DyLight594*, PE-TexRed*, ECD*, TexRed*, PE-AF610*, RFP, mCherry*, 7AAD*, PI*					4
5	640-745		**PE-Cy5*, PE-AF647***, PerCP*, PerCP-Cy5.5*, DRAQ5*, QD705*, eFluor650*	**PE-Cy5*, PE-AF647***, DRAQ5*					5
6	745-800		**PE-Cy7*, PE-AF750***, QD800	**PE-Cy7*, PE-AF750***		**SSC**			6
7	430-505	**DAPI, Hoechst**, PacBlue, CascadeBlue, AF405, eFluor405, DyLight405, CFP, LIVE/DEAD Violet							7
8	505-570	PacOrange, CascadeYellow, AF430, QD525*							8
9	570-595	BRIGHTFIELD							9
10	595-640	QD625*, eFluor625*							10
11	640-745	QD705*, eFluor650*			**APC, AF647, AF660, AF680, DRAQ5**, Cy5, DyLight649, DyLight680, PE-AF647, PE-Cy5, PerCP, PerCP-Cy5.5				11
12	745-800	QD800*			**APC-Cy7, APC-AF750**, APC-eFluor750, Cy7, AF750, DyLight750, eFluor750, PE-Cy7*, PE-AF750*				12

Dyes with an * are excited by at least one laser directed to camera1 and another directed to camera2. The channel that the dye will appear brightest in depends on the relative laser powers used. Recommended dyes (based on optimal excitation and detection channels) are in boldface. QD565 and QD585 are not included because their primary fluorescence appears in the Ch9 Brightfield reference image.

amnis