**ImageStream experimental and sample preparation guide**

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In spite of the similarity between conventional flow cytometry and imaging flow cytometry, there are a few important sample preparation and experimental design tips that may be detrimental to the results of your ImageStream experiments.

1. **Recommended final cell concentration and volume:** 2-5 million cells in 50 µL PBS/2%FBS in a 1.5 mL siliconized eppendorf tube.
2. **Protocols:** In general, any established labelling protocol used for flow cytometry will work with the ImageStream (1, 2). However, we highly recommend that you stain your cells on ice in the presence of azid to reduce non-specific capping of antibody. Capping may affect the ability to analyze your data.
3. **Titration:** Remember to titrate your antibodies (3). Titrate preferably to a 2-log difference between positive and negative population and a Raw Max Pixel value between 200 and 4095.
4. **Choice of fluorochromes:** Choose fluorochromes that are excited by the lasers either the 405nm, 488nm, 561nm, or 642nm laser. Be aware that the 488nm and 561nm lasers are co-linear. Likewise, the 405nm and 642nm lasers are co-linear. Use the chart on FACS.au.dk or use a spectra viewer (e.g. reference 4) that will help you plan which dyes will work the best.

Note: We have very poor experience with the BV785/BV786 fluorochrome and do not recommend the use of this specific dye.

1. **Fixation:** If fixation is desired, thoroughly fix cells with 1% PFA or 0.9% formalin on ice for 20 min.
2. **Number of samples:** We recommend that you limit the number of samples.
   * positive and negative biological controls (5)
   * compensation controls (6)
   * experiment samples
3. **Compensation:** Have samples of cells (or beads) each labelled with a single colour for each fluorochrome used in your experimental samples (i.e. FITC only, PE only, etc.) (6). Compensation on cells will give the best result on the ImageStream. Using a different cell type than the experimental cell type (but with similar autofluorescence) and a different antibody with your experimental fluorochrome may be a better solution than using beads for compensation on the ImageStream (this does not apply for tandem-dyes).
4. **Cell aggregation:** Minimize aggregation problems by straining the sample through a 70 µm nylon mesh strainer, and/or by using an [anti-clumping buffer containing EDTA](https://facs.au.dk/fileadmin/user_upload/Sticky_cell_buffer_2.docx) prior to fixation. Cell aggregates may:
   * cause air bubbles in the instrument
   * impair focus and thus the quality of your data
   * slow down event rate

**References**

1. [FACS Core Facility Guidelines, Antibody Staining using a 96 well plate](https://facs.au.dk/facscorefacilityguidelines/#:~:text=Antibody%20Staining%20using%20a%2096%20well%20plate)
2. [FACS Core Facility Guidelines, Antibody Staining of Intracellular Antigens](https://facs.au.dk/facscorefacilityguidelines/#:~:text=Antibody%20Staining%20of%20Intracellular%20Antigens)
3. [FACS Core Facility Guidelines, Titration in Flow Cytometry](https://facs.au.dk/facscorefacilityguidelines/#:~:text=Titration%20in%20Flow%20Cytometry)
4. https://fluorofinder.com/spectra-viewer/
5. [FACS Core Facility Guidelines, Controls in Flow Cytometry](https://facs.au.dk/facscorefacilityguidelines/)
6. [FACS Core Facility Guidelines, Compensation in Flow Cytometry](https://facs.au.dk/facscorefacilityguidelines/)