Table of Contents

1. Introduction to Imaging Flow Cytometry

Imaging flow cytometry is a unique technique that allows users to simultaneously collect flow cytometry data as well as single cell images analogous to fluorescence microscopy. This technique is helpful in three main areas. The first type of experiments that benefit from Imaging Flow Cytometry are those focused on **statistical interpretation of fluorescent imaging event**s such as Autophagy and related fluorescent particle up-take, Co-localization, Nuclear translocation, Development of Immune Synapses, and Cell cycle analysis among others. The second type of experiments that benefit from Imaging Flow Cytometry are those focused on **validating fluorescently tagged antibodies of novel targets**, these applications include the studies of a range of cells from microbes to large mammals where flow cytometry has not historically been done. One specific example is analysis of white blood cells in bronchial alveolar lavage from horses as a novel animal model of asthma. A third application for Imaging Flow Cytometry is the **study of exosomes**. Due to their small size it is difficult to directly analyze exosomes in traditional flow cytometry, but the addition of an imaging component helps separate true exosome signal from masking signal due to debris or electronic noise.

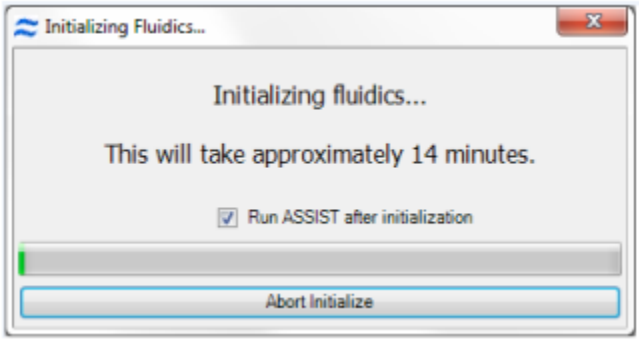
2. Fluidics

- ***SpeedBeads***: monitor and synchronize the flow of the sample and maintain focus and core tracking
- ***Sterilizer*** = FACSClean
- ***Cleanser*** = Coulter Clenz
- ***Debubbler*** = 70% Isopropyl alcohol
- ***Rinse*** = Deionized Water (diH_2O)
- ***Sheath*** = 1X PBS (Phosphate-buffered saline), 0.1 nm filtered

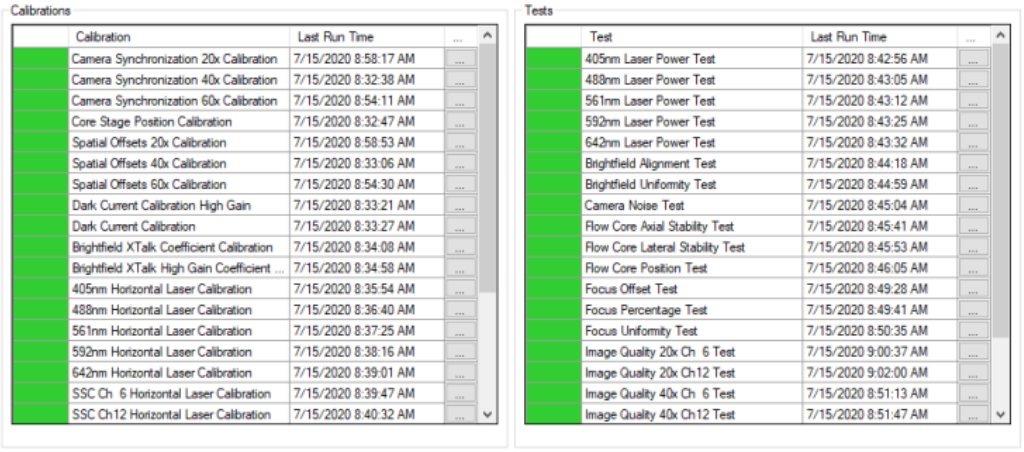
Note: The Flow core can provide each of the required buffers, as well as 0.1nm bottle-top filters. Please check with core staff if you need help replenishing any of the fluids.

3. Startup

- To power on the instrument you need to first turn on the Linux computer (far left) wait 2-3 minutes, then turn on the ImageStream instrument (middle) wait 2-3 minutes, then finally turn on the Windows computer. This sequence is necessary for the instrument to correctly communicate with the two workstations.
- Next launch ISX to open the INSPIRE software.
- This will open a DOS prompt that will take several minutes to load the INSPIRE software. Once the GUI is open the Startup process can start.
- First Person of the day will click on the STARTUP button assuring that the Run Assist radio box is selected this will run the Startup and ASSIST programs



  - ***Startup-*** *This automated* Initializes fluidics by flushing sheath and loading beads ~14min
  - ***ASSIST*** (**A**utomated **S**uite of **S**ystemwide **I**mage**S**tream **T**ests) after initialization" Calibration and testing using SpeedBeads ~20min
  - Assure that all the ASSIST tests have run (Date is updated) and passed (Green Box). If any of the set-up tests failed click on that specific test to re-run. If problems persist please contact Core Staff.


Calibrations

Calibration	Last Run Time
Camera Synchronization 20x Calibration	7/15/2020 8:58:17 AM
Camera Synchronization 40x Calibration	7/15/2020 8:32:38 AM
Camera Synchronization 60x Calibration	7/15/2020 8:54:11 AM
Core Stage Position Calibration	7/15/2020 8:32:47 AM
Spatial Offsets 20x Calibration	7/15/2020 8:58:53 AM
Spatial Offsets 40x Calibration	7/15/2020 8:33:06 AM
Spatial Offsets 60x Calibration	7/15/2020 8:54:30 AM
Dark Current Calibration High Gain	7/15/2020 8:33:21 AM
Dark Current Calibration	7/15/2020 8:33:27 AM
Brightfield XTalk Coefficient Calibration	7/15/2020 8:34:08 AM
Brightfield XTalk High Gain Coefficient ...	7/15/2020 8:34:58 AM
405nm Horizontal Laser Calibration	7/15/2020 8:35:54 AM
488nm Horizontal Laser Calibration	7/15/2020 8:36:40 AM
561nm Horizontal Laser Calibration	7/15/2020 8:37:25 AM
592nm Horizontal Laser Calibration	7/15/2020 8:38:16 AM
642nm Horizontal Laser Calibration	7/15/2020 8:39:01 AM
SSC Ch 6 Horizontal Laser Calibration	7/15/2020 8:39:47 AM
SSC Ch12 Horizontal Laser Calibration	7/15/2020 8:40:32 AM

Tests

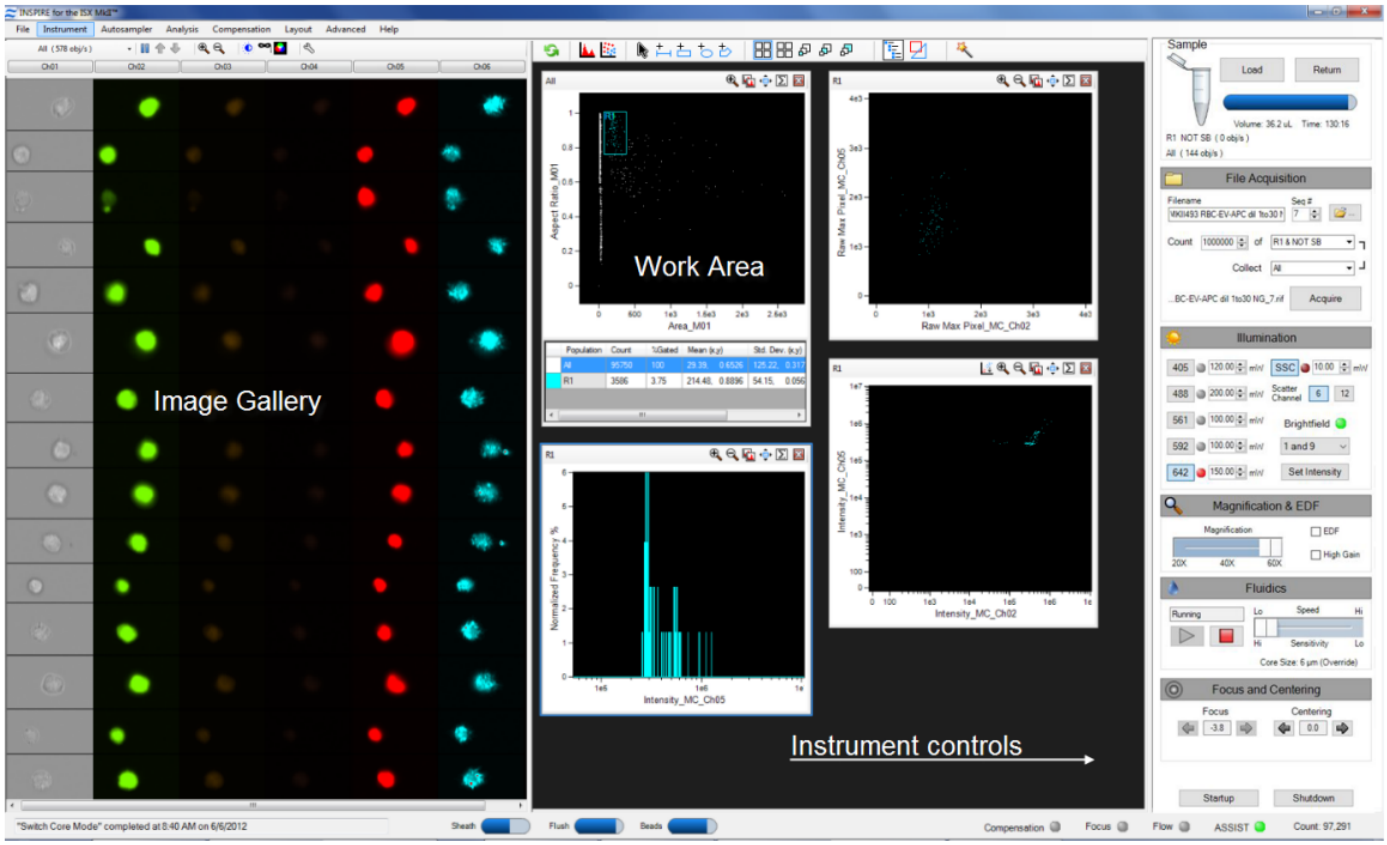
Test	Last Run Time
405nm Laser Power Test	7/15/2020 8:42:56 AM
488nm Laser Power Test	7/15/2020 8:43:05 AM
561nm Laser Power Test	7/15/2020 8:43:12 AM
592nm Laser Power Test	7/15/2020 8:43:25 AM
642nm Laser Power Test	7/15/2020 8:43:32 AM
Brightfield Alignment Test	7/15/2020 8:44:18 AM
Brightfield Uniformity Test	7/15/2020 8:44:59 AM
Camera Noise Test	7/15/2020 8:45:04 AM
Flow Core Axial Stability Test	7/15/2020 8:45:41 AM
Flow Core Lateral Stability Test	7/15/2020 8:45:53 AM
Flow Core Position Test	7/15/2020 8:46:05 AM
Focus Offset Test	7/15/2020 8:49:28 AM
Focus Percentage Test	7/15/2020 8:49:41 AM
Focus Uniformity Test	7/15/2020 8:50:35 AM
Image Quality 20x Ch 6 Test	7/15/2020 9:00:37 AM
Image Quality 20x Ch12 Test	7/15/2020 9:02:00 AM
Image Quality 40x Ch 6 Test	7/15/2020 8:51:13 AM
Image Quality 40x Ch12 Test	7/15/2020 8:51:47 AM

  - Speed beads will run continuously following the Startup**. Remember to stop the Fluidics after the Start-up** if you will not be using the Instrument for some time.

- If you let staff know in advance, we will try to run the Startup process before your appointment. If the instrument is already started, ***Start Fluidics*** to ensure Focus and Centering is valid using the SpeedBeads

4. INSPIRE User Interface

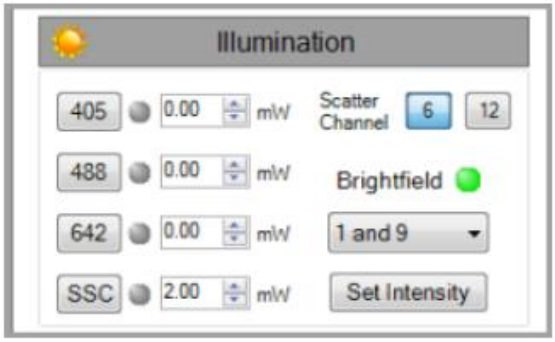
The user interface is divided into three areas: the image gallery where channel images are displayed, a work area where graphs of features are displayed, and the controls section where the instrument is controlled. The layout of the Image Gallery and Analysis area can be vertical or horizontal, and the layout can be changed under the Layout menu. Status information is displayed along the bottom of the window.



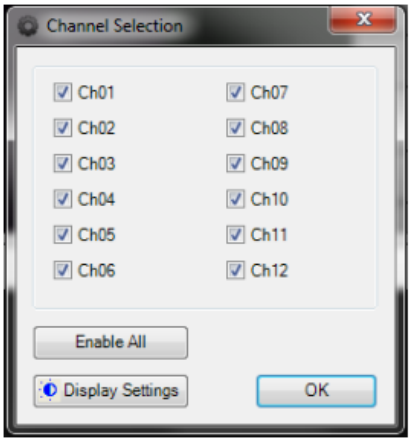
5. Set-up for Data Acquisition

- To start a data acquisition click on File > ***Load default template*** or experiment template.
- ***File Acquisition***: set path and number of objects to collect
    a. Always save to the Desktop initially then copy to the J: Drive ONYEN folder
- ***Magnification***: select the 20X, 40X or 60X objective.
    a. If viewing internal components of the cell, need to run 60X objective
    b. 20X= 1 um/pixel
    c. 40X= 0.5 um/pixel
    d. 60X= 0.3 um/pixel

- ***Illumination***: turn on the appropriate lasers.
    a. Begin with all lasers at max output (hover over input box to view range of power available per laser)
    b. Keep SSC power between 3-5 mV



- ***(Optional) - Channel Selection***: Click on any of the channels at the top of the gallery display to open the Channel selection window. Pick the appropriate detector channels for your experiment. Make sure you have selected your Brightfield channels. Default Brightfield channels are Ch01 and Ch09.



- ***Fluidics***: Select appropriate Speed/sensitivity for your experiment
    a. If viewing internal components of the cell, need to run on Lo speed/Hi sensitivity
- **Set-up plots to check the laser powers**
    a. The goal is to **maximize signal** and **prevent saturation**.
    b. Check each detector channel to assure that your fluorescent signal is in range. Unlike a traditional flow cytometer the ImageStream uses CCD cameras and off-scale signal needs to be checked at the pixel level rather than total detector intensity.
        i. For each detector in use make a plot with ***Raw Max Pixel_MC_ChX by Area_MChX*** to determine if any pixels are saturated. Max output is 4096.

c. Be careful to closely look at axis labels as the scales will automatically update. This can be overridden by R-click Graph> Graph properties> Scaling > Manual > Maximum= 4096 (make sure scale is linear for Raw Max Pixel)

- ***Load*** a fully-stained sample on the uptake port.
  a. Click the **Load** button, this will trigger the sample port to first close and do a flush; then open. At this point the User can place the sample in the uptake port.
  b. Note 15 uL is the minimum amount of liquid uptake
  c. Decrease Laser powers as needed to avoid Pixel saturation.
- Next create a gating scheme for your Experiment. Please carefully consider the type of analysis you intend to run as this will help guide your experiment design and gating scheme.
- Create plots and gates to identify the cells to collect:
  a. Cells in Focus: Gradient RMS_M01_Ch01 (Use Brightfield or Channel of choice)
  b. Singlets: Area_M01 vs. Aspect Ratio_M01 (Use Brightfield or Channel of choice)
  c. Phenotyping (signal intensity): use Intensity_MC_ChX
- ***Compensation*** > Create Matrix… or click on the Wizards icon () and follow the prompts.
  a. Note: Single stained samples must be collected WITHOUT brightfield/SSC channels
  b. Collect at least 1000 positive events
    i. Compensation wizard is looking for ONLY positive events, so it is preferable to save only events in the positive gate defined.
  c. Run PBS between samples if worried about residual sample
  d. Run DNA dye last
  e. Beads are not sufficient to compensate, must use cells
- Collect all experimental samples (return the remaining sample).
- File > Save Template.

6. Shutdown

- Between users:
  a. FACSClean (3 minutes)
  b. diH_2O (3 minutes)
- Last user
  a. Click ***Shutdown*** (sterilizes the instrument ~43min)
  b. Select “Shutdown after sterilize” (powers off all system components)
  c. Do not exit program

Note: Sample concentration $1x10^7$-$1x10^8$/mL.
Note: BF usually is set to ch1&9, DF (ch6 – SSC) should be between 3-5 mW, and single cells are visualized with a BF Area vs. Aspect Ratio.
Note: If a sample without DNA dye follows a sample with DNA dye, Load FACSClean followed by 1X PBS for a minute each.

7. Available Image Features for Analysis in INSPIRE & IDEAS

Use the following features (available in any channel) to identify objects for inclusion in or exclusion from the acquiring data file:

1. **Area:** The number of pixels in an image reported in square microns.
2. **Aspect Ratio**: The Minor Axis divided by the Major Axis is a measure of how round or oblong an object is. Seeb below for the definitions for Major and Minor Axis.
3. **Background Mean**: The average pixel intensity of the background pixels.
4. **Gradient RMS**: The average slope spanning three pixels in an image. This feature measures image contrast or focus quality.
5. **Intensity**: The integrated intensity of the entire object image; the sum of all pixel intensities in an image, background subtracted.
6. **Major Axis**: The longest dimension of an ellipse of best fit.
7. **Mean Pixel**: The average pixel intensity in an image, background subtracted.
8. **Minor Axis**: The shortest dimension of an ellipse of best fit.
9. **Object Number**: The serial number of an object.
10. **Raw Centroid X**: The center of the object in the X dimension of the frame.
11. **Raw Centroid Y**: The center of the object in the Y dimension of the frame.
12. **Raw Max Pixel**: The intensity value of the brightest pixel in an image (no background subtraction).
13. **Raw Min Pixel**: The intensity value of the dimmest pixel in an image (no background subtraction).
14. **Time**: The object's time value in seconds.
15. **Uncompensated Intensity**: The integrated intensity of the entire object image; the sum of all pixel intensities in an image, background subtracted.

Note: See the IDEAS User Manual for more details on features and graphing.

8. Data Analysis Tips

- Sample Export File Types:
  - ***a) .rif = raw image file***
  - b) .ctm = compensation matrix
  - c) .cif = compensated image file
  - ***d) .daf = data analysis file***
  - e) .ast = template file

i) Use for batch Analysis

- F1 in IDEAS will bring up user manual; type to search

9. References

- ImageStreamX ® System Software User's Manual Version Mark II, January 2013.

10. Revisions

SOP Version Number	Date	Tracked Changes (clearly list changes made & why)	Employee
UT SOP004.2	1/25/2023	General Updates throughout to aid in user training	Ayrianna Woody
UT SOP004.3	4/11/2025	Added some more images from the Cytek User Manual	Robert Immormino