INSPIRE™

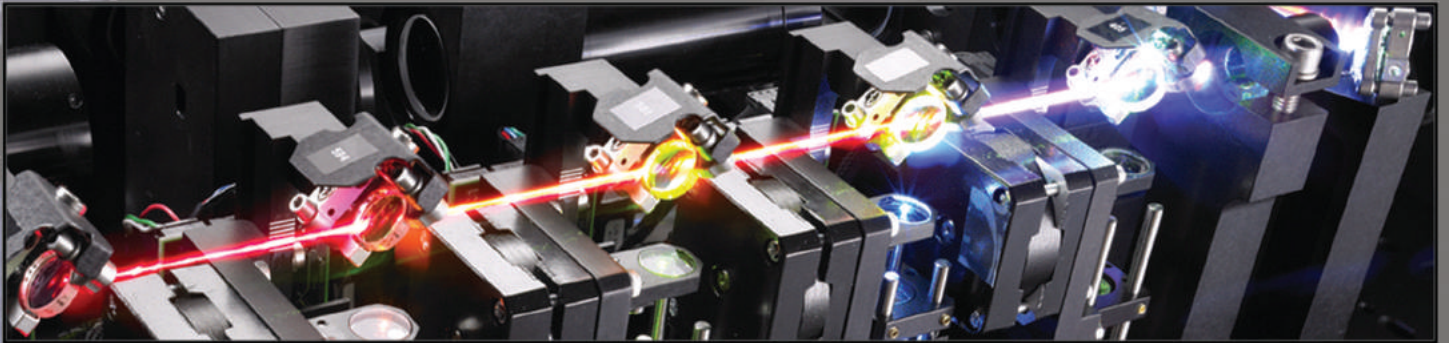## ImageStreamX MKII Software
User's Manual

INSPIRE ®

ImageStreamX® System Software
User's Manual

Part Number: 780-01286-01 Rev: B

For updates log in at www.amnis.com

Patents and Trademarks

Amnis technologies and products are protected under one or more of the following U.S. patents: 6211955; 6249341; 6256096; 6473176; 6507391; 6532061; 6563583; 6580504; 6583865; 6608680; 6608682; 6618140; 6671044; 6707551; 6763149; 6778263; 6875973; 6906792; 6934408; 6947128; 6947136; 6975400; 7006710; 7009651; 7057732; 7079708; 7087877; 7190832; 7221457; 7286719; 7315357; 7450229; 7522758, 7567695, 7610942, 7634125, 7634126, 7719598, 7889263, 7925069,8005314, 8009189, 8103080, 8131053.

Additional U.S. and corresponding foreign patent applications are pending.

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All other trademarks are acknowledged.

Disclaimers

The screen shots presented in this manual may vary in appearance from those on your computer, depending on your display settings.

The Amnis® ImageStream® cell analysis system is for research use only and not for use in diagnostic procedures.

Technical Assistance

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Seattle, WA 98119

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Toll free: 800-730-7147

www.amnis.com

Table of Contents

Chapter 1: Information and Safety

This section covers safety information for operating the Amnis ImageStream^X® multispectral imaging flow cytometer. Anyone who operates the ImageStream^X should be familiar with this safety information. Keep this information readily available for all users.

The safety information consists of the following areas:

- General Information and Safety
- Explanation of Symbols
- Electrical Safety
- Laser Safety
- Biological Safety

General Information and Safety

The ImageStream^X imaging flow cytometer is manufactured by Amnis Corporation and has a rated voltage of 100–240 VAC, a rated frequency of 50/60 Hz, and a rated current of 3 A. The years of construction were 2004–2014, and the product contains CE Marking.

Environmental conditions: This instrument was designed for indoor use at an altitude of less than 2000 m; at a temperature from 5°C through 25°C; and at a maximum relative humidity of 80%, non-condensing. During instrument operation the ambient temperature should be maintained within +/- 2°C. The mains power supply may not fluctuate more than +/- 10% and must meet transient over voltage category (II). The instrument is evaluated to Pollution Degree 2.

Noise level: The noise level of the ImageStream^X is less than 70 dB(A).

Weight: 160 kg.

Ventilation: Provide at least 3 inches of clearance behind the instrument to maintain proper ventilation.

Disconnection: To disconnect the instrument from the power supply, remove the plug from the socket outlet—which must be located in the vicinity of the machine and in view of the operator. Do not position the instrument so that disconnecting the power cord is difficult. To immediately turn the machine off (should the need arise), remove the plug from the socket outlet.

Transportation: The ImageStream^X relies on many delicate alignments for proper operation. The machine may be moved only by an Amnis representative.

Cleaning: Clean spills on the instrument with a mild detergent. Using gloves clean the sample portal and sample elevator with a 10% bleach solution. Dispose of waste using proper precautions and in accordance with local regulations.

Preventative maintenance: The ImageStream^X contains no serviceable parts. Only Amnis-trained technicians are allowed to align the laser beams or otherwise repair or maintain the instrument. The instrument fluidic system is automatically sterilized after each day's use. This reduces the occurrence of clogging. Tubing and valves are replaced by Amnis service personnel as part of a routine preventive maintenance schedule.

Access to moving parts: The movement of mechanical parts within the instrument can cause injury to fingers and hands. Access to moving parts under the hood of the ImageStream^X is intended only for Amnis service personnel.

Protection impairment: Using controls or making adjustments other than those specified in this manual can result in hazardous exposure to laser radiation, in exposure to biohazards, or in injury from the mechanical or electrical components.

FCC compliance: This equipment has been tested and found to comply with the limits for a Class A digital device, pursuant to part 15 of the FCC rules. These limits

were designed to provide reasonable protection against harmful interference when the equipment is used in a commercial environment. This equipment generates, uses, and can radiate radio-frequency energy and, if not installed and used in accordance with the instruction manual, can cause harmful interference to radio communications. The operation of this equipment in a residential area is likely to cause harmful interference—in which case the user will be required to correct the interference at the user's own expense.

Declaration of Conformity

DECLARATION OF CONFORMITY

IN ACCORDANCE TO ISO/IEC GUIDE 67
FOR A
Cellular Analyzer

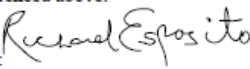|	
MANUFACTURER:	Amnis Corporation
	645 Elliott Avenue W, Suite 100
	Seattle, WA 98119
	Phone: 206.576.6865
MODEL NUMBER:	ImageStream X Mk II
REPORT #:	582624/02 and F2LQ3326-02S
DIRECTIVES:	EMC Directive (2004/108/EC) & Low Voltage Directive (2006/95/EC)

STANDARDS:
- Electrical Equipment for Measurement, Control and Laboratory Use – EMC Requirements, Part 1: General Requirements, EN 61326-1:2006 edition
- Industrial, Scientific, and Medical Equipment – Radio-Frequency Disturbance Characteristics – Limits and Methods of Measurement, CISPR 11:2009 edition
- Information Technology Equipment – Radio Disturbance Characteristics – Limits and Methods of Measurement, EN 55022:1998 CISPR 22:1997 edition
- Electromagnetic Compatibility (EMC) – Part 3-2: Limits – Limits for Harmonic and Emissions (Equipment Input Current ≤16 A per phase), IEC 61000-3-2:2009 edition
- EMC - Part 3-3: Limits - Limitations of voltage changes, voltage fluctuations and flicker, in public low-voltage supply-systems, for equipment with rated current <=16 A per phase and not subject to conditional connections; IEC 61000-3-3:1995 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 2: Electrostatic Discharge Immunity Test, EN 61000-4-2:2008 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 3: Radiated, Radio-Frequency, Electromagnetic Field Immunity Test, EN 61000-4-3:2008 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 4: Electrical Fast Transient/Burst Immunity Test, EN 61000-4-4:2004 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 5: Surge Immunity Test, EN 61000-4-5:2005 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 6: Immunity to Conducted Disturbances, Induced by Radio-Frequency Fields, EN 61000-4-6:2008 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 8: Power Frequency Magnetic Field Immunity Test, EN 61000-4-8:2001 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 11: Voltage Dips, Short Interruptions, and Voltage Variations Immunity Test, EN 61000-4-11:2004 edition
- Safety Requirements for Electrical Equipment for Measurement, Control, and Laboratory Use Part 1: General Requirements, EN 61010-1:2008, 2nd edition

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	Damascus, MD 20872 USA	Hillsboro, OR 97124 USA

The Cellular Analyzer, Model ImageStream X is in effective conformance to the Directives and Standards referenced above.

Authorized by: *Richard Esposito*

Date: 23 August 2012

Name: Richard Esposito

Title: Quality Assurance Officer

Explanation of Symbols

Table 1:

Label	Location	Hazard
	Waste tank	Risk of exposure to transmissible biological disease.
	Power supply cover	Risk of injury by electric shock.
	Power supply	Protective earth ground.
	Inside surface of hood	Risk of exposure to hazardous laser radiation.
	Interior, side panels near release mechanisms and next to hood latch release	Risk of exposure to hazardous laser radiation.
	On the back of the instrument	No laser radiation is accessible to the user during normal instrument operation.

Electrical Safety

Equipment ratings: The ImageStream^X is rated to the following specifications: 100–240 VAC, 50/60 Hz, and 3A.

Electrical hazards are present in the system, particularly in the main power supply. To protect against electrical shock, you must connect the instrument to a properly grounded receptacle in accordance with the electrical code that is in force in your region.

Sécurité Electronique

Alimentation: 100–240 V altenatif, 50/60 Hz, 3A.

Les hazards électrique se trouvent dans l'appareil, surtout prés de la source d'alimentation. Pour éviter les choks électriques, introduire la lame le plus large de la fiche dans la borne correspondante de la prise et pousser à fond.

Laser Safety

The ImageStream^X is a Class 1 laser device and complies with the U.S. FDA Center for Devices and Radiological Health 21 CFR Chapter 1, Subchapter J. No laser radiation is accessible to the user during normal instrument operation. When the hood is opened, interlocks on the hood turn the lasers off.

The ImageStream^X may have the following lasers:

Table 1:

Wavelength	Maximum Power
370-380 nm	30 or 85 mW
400-413 nm	150 mW
483-493 nm	200 mW or 400 mW *(high power option)*
558-562 nm	200 mW
592-593 nm	300 mW
635-647 nm	150 mW
720-740 nm	50 mW
775-800 nm	100 mW
815-840 nm	180 mW

The following laser warning label appears on the inside surface of the hood:

The following laser warning label appears on the interior side panels near release mechanisms and next to hood latch release.

Caution: Using controls, making adjustments, or performing procedures other than those specified in this manual may result in hazardous exposure to laser radiation.

Sécurité Laser

L'ImageStreamX c'est une appareil au laser, Classe I, qui se conforme à U.S. FDA Center for Devices and Radiological Health 21 CFR Chapitre 1, subchapitre J. Aucune radiations laser sont accessible a l'utilsateur pendant le fonctionnement normal. Quand le capot est ouvert, les enclenchements eteindents les lasers.

ImageStreamX peut avoir les lasers suivants:

Longueurd'opnde	La Puissance Maximale
370-380 nm	30 or 85 mW
400-413 nm	150 mW
483-493 nm	200 mW or 400 mW (high power option)
558-562 nm	200 mW
592-593 nm	300 mW
635-647 nm	150 mW
720-740 nm	50 mW
775-800 nm	100 mW
815-840 nm	180 mW

Les etiquettes d'avertissement suivantes sont placeés dans l'interior du capot:

Les etiquettes d'avertissement suivantes sont placeés dans L'Intérieur, de panneaux latéraux près de mécanismes de libération et à côté du loquet de fermeture de capot.

Avertissement: L'utilisation des commandes ou les rendement des procedures autres que celle preciseés aux presentes peuvent provoquer une radioexposition dangereuse.

Biological Safety

Biohazards: The Image Stream is rated at BSL1. Do not load or flush samples containing infectious agents without first exposing the sample to inactivating conditions. It is recommended that samples be fixed in 2% paraformaldehyde for at least 10 minutes before running the samples on the ImageStream^X.

The use, containment and disposal of biologically hazardous materials are required to be in accordance with Personnel Protective Equipment Directive 93/95/E and are the responsibility of the end user. Follow all local, state, and federal biohazard-handling regulations for disposal of the contents of the waste reservoir.

Prevent waste-reservoir overflow by emptying the container when the waste indicator indicates that it is full.

Run the instruments sterilize routine after each day's use. Note that this procedure has not been proven to result in microbial sterility.

Sécurité BiologiqueBiorisques:

L'image Stream est évalué à un niveau de sécurité biologique L1. Ne pas acquérir ou vider des échantillons contenant des agents infectieux sans les avoir inactivés. Il est recommandé que les échantillons soient fixés dans du paraformaldéhyde 2% pendant au moins 10 minutes avant d'acquérir des échantillons avec l'ImageStreamX.

L'utilisation, le confinement et l'élimination des matériels biologiques dangereux sont tenus d'être en conformité avec les normes de sécurité relatives au laboratoire et de la directive 93/95/E et restent sous la responsabilité de l'utilisateur. Respectez la réglementation en vigueur pour le traitement et l'élimination des déchets dans des réservoirs prévus à cet effet.

Prévenir l'accumulation des déchets en vidant le réservoir lorsque l'indicateur indique qu'il est plein. Stériliser les instruments de routine après chaque journée d'utilisation. Notez que cette procédure ne garantit pas la stérilité vis à vis des microbes.

Spare Parts List

The instrument contains no serviceable parts. Only Amnis-trained technicians are allowed to repair, maintain, and set up the alignment of the laser beams.

Chapter 2: Introduction to the ImageStream^X®

The Amnis ImageStream^X is a bench top multispectral imaging flow cytometer designed for the acquisition of up to 12 channels of cellular imagery. By collecting large numbers of digital images per sample and providing numerical representation of image-based features, the ImageStream^X combines the per cell information content provided by standard microscopy with the statistical significance afforded by large sample sizes common to standard flow cytometry. With the ImageStream^X system, fluorescence intensity measurements are acquired as with a conventional flow cytometer; however, the best applications for the ImageStream^X take advantage of the system's imaging abilities to locate and quantitate the distribution of signals on or within cells or between cells in cell conjugates.


The Amnis ImageStream^X system includes the ImageStream^X multispectral imaging flow cytometer and the INSPIRE^{TM} and IDEAS^{TM} software applications.

The INSPIRE software is integrated with the ImageStream^X and is used to run the instrument. INSPIRE also provides tools for configuring the ImageStream^X, defining cell parameters, and collecting data files for image analysis. The IDEAS software is used for spectral compensation, image analysis and statistical analysis of the images acquired by the ImageStream^X multispectral imaging flow cytometer.

Technology Overview

The ImageStream^X acquires up to twelve images simultaneously of each cell or object including brightfield, scatter, and multiple fluorescent images at rates of up to 5000 objects per second. The time-delay-integration (TDI) detection technology

used by the ImageStream^X CCD camera allows up to 1000 times more signal to be acquired from cells in flow than from conventional frame imaging approaches. Velocity detection and autofocus systems maintain proper camera synchronization and focus during the process of image acquisition. The following diagram illustrates how the ImageStreamX works.
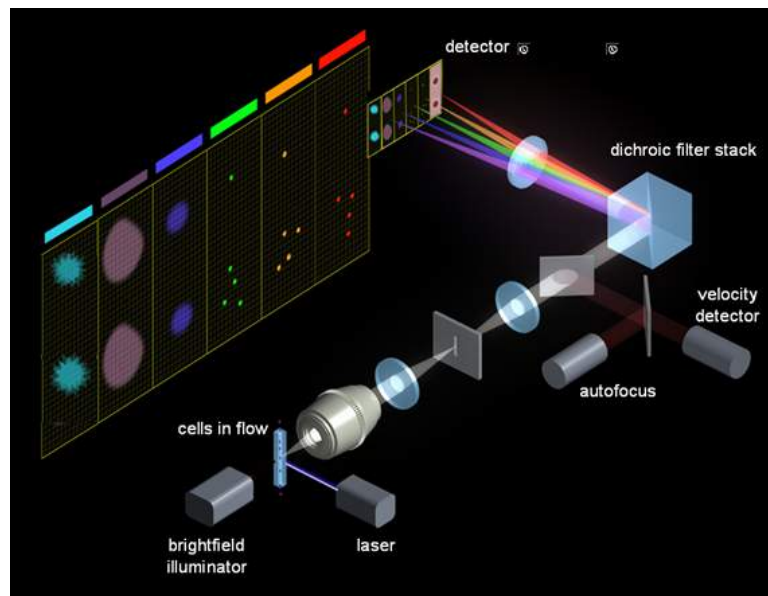
Hydrodynamically focused cells are trans-illuminated by a brightfield light source and orthogonally by laser(s). A high numerical aperture (NA) objective lens collects fluorescence emissions, scattered and transmitted light from the cells. The collected light in optical space intersects with the spectral decomposition element. Light of different spectral bands leaves the decomposition element at different angles such that each band is focused onto 6 different physical locations of one of the two CCD cameras with 256 rows of pixels. As a result, each cell image is decomposed into six separate sub-images on each CCD chip based on a range of spectral wavelengths. Up to 12 images are collected per object with a two camera system.

The CCD camera operates in TDI (time delay integration) mode that electronically tracks moving objects by moving pixel content from row to row down the 256 rows of pixels in synchrony with the velocity of the object (cell) in flow as measured by the velocity detection system. Pixel content is collected off the last row of pixels. Imaging in this mode allows for the collection of cell images without streaking and with a high degree of fluorescence sensitivity. TDI imaging combined with spectral decomposition allows the simultaneous acquisition of up to 12 spectral images of each cell in flow.

Chapter 3: Operating the ImageStream^X® Using INSPIRE^{TM}

This chapter describes the operation of the ImageStream^X system using the INSPIRE software. Daily operation involves an initial calibration and testing of the system using SpeedBeads and ASSIST, followed by sample runs and data acquisition, and finally sterilization of the system to prepare for use the following day. Optimizing instrument setup for sample runs is also described here in detail.

- Fluidics
- INSPIRE User Interface
- Daily Operations
- Data Acquisition
- Daily Shutdown Procedure
- Optional upgrades

Fluidics

Sterilizer, Cleanser, and Debubbler

These recommended reagents have been formulated to optimize the performance of the ImageStream^X seals, valves, syringes, and lines. The use of the recommended reagents is required for proper operation of the instrument. The Sterilizer, Cleanser, and Debubbler reagents are used in the Sterilize and Debubble scripts.

Reagent	Name	Source*	Catalog #
Cleanser	Coulter Clenz®	Beckman Coulter	8546929
Debubbler	70% Isopropanol	Millipore	1.37040
Sterilizer	0.4-0.7% Hypochlorite	VWR	JT9416-1
Sheath	PBS, Ca^{++}Mg^{++} free	Millipore	BSS-1006-B (1X) 6506- (10X)
Rinse	deionized water		

*   provided for information only, other sources of the same reagent may be used.

Waste Fluid

The waste bottle holds all of the fluids that have been run through the ImageStream^X, and can hold up to 1600 ml. Add 160 ml of bleach to the empty waste tank. It is recommended that the waste bottle contain 10% bleach when full.

Sheath Fluid

Two bottles are provided: one labeled Sheath to be filled with phosphate buffered saline (PBS with no surfactants) for running samples and one labeled Rinse to be filled with de-ionized (DI) water for rinsing the instrument during shutdown. Fluid is drawn from these bottles into the sheath and flush syringe pumps. The sheath pump controls the speed of the core stream and the size of the core stream diameter. The flush pump is used to clean and flush the system and alternating with the sheath pump also controls the core.

INSPIRE User Interface

The user interface is divided into 3 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 changed under the Layout menu. Status information is displayed along the bottom of the window.
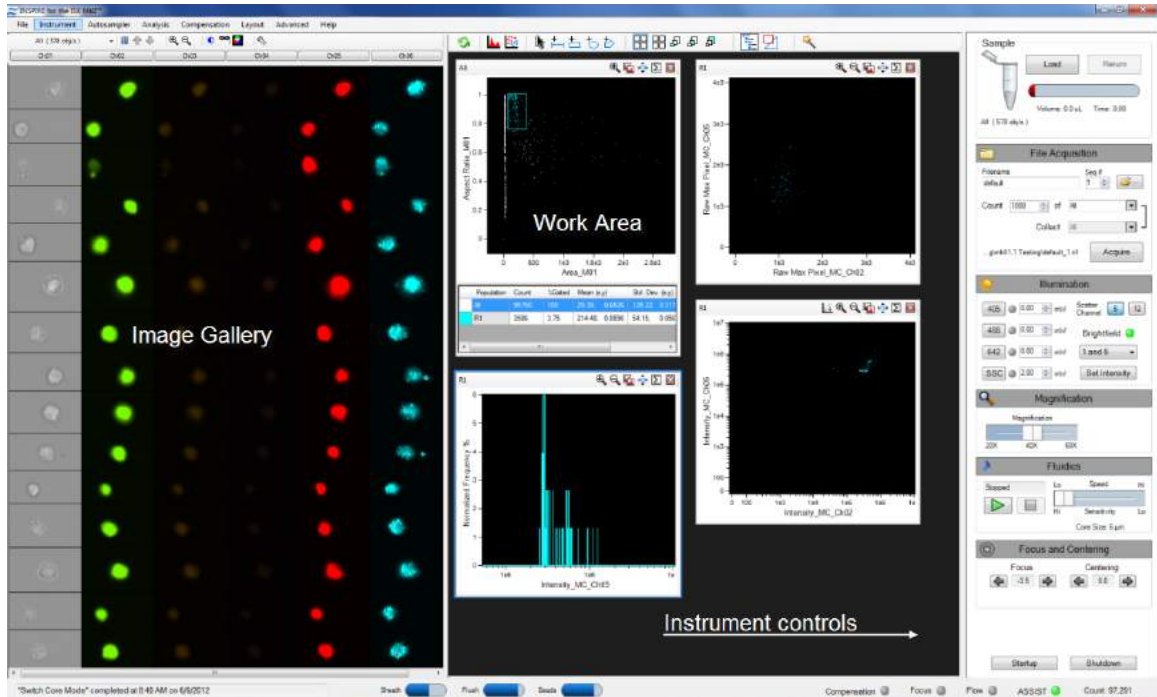
The Image Gallery

Images are displayed in the image gallery during setup and acquisition.

Image Gallery Tools

Icon	Name	Description
All	All	Select the population to view
▐▐	Pause	Pause/Resume the display
⬆ ⬇	Up/Down	Move up or down in the image gallery while paused
🔍	Zoom in	Enlarges the imagery
🔍	Zoom out	Resets the zoom
◐	Display	Opens the display settings window
∞	Mask	Displays the segmentation mask on the images
🔴	Color	Turns pseudocolor ON/OFF
🔧	wrench	Tools to measure pixel intensity of displayed images
Ch01 Ch02	channel name	Click to change selection of channels to view/acquire

Setting the Image Display Properties

There are two methods for setting the display of the imagery. The display properties wizard will adjust the display as the images are being collected. The display can also be set manually by the following procedure. Note that these settings adjust the monitor display and do not change the pixel intensities of the images collected.

Using the wizard

1   Click on the wand 🪄 next to the analysis area tools and select the Display Settings Wizard.

2   Select the channels to set and click Finish.

The wizard will adjust the display for a channel with greater than 75 Raw Max Pixel values using the Min and Max pixels from the images being collected. If

there are no positive events in a channel the default settings (35-750) are used. The manual settings may be used for channels with rare events since there may not be enough data for the wizard.

Manual method

1 Click on the Display Settings tool ◑ to open the window.

2 Select the channel by clicking on the channel name.

3 To change the channel name, type a new name. To change the channel color click on the color box.

4 To set the display mapping adjust the right and left green bars in the graph. You will adjust the Display Intensity settings on the graph (the Y Axis), to the Pixel Intensity (the X axis). The range of pixel intensities is 0-4095 counts. The display range is 0-255. The pixel intensities shown in gray are gathered from the images coming through in the specific channel and updates with every 10 images. Updates to the adjustments can be visualized in the image gallery.

At each intensity on the X Axis of the graph, the gray histogram shows the number of pixels in the image. This histogram provides you with a general sense of the range of pixel intensities in the image. The dotted green line maps the pixel intensities to the display intensities, which are in the 0-255 range.

Manual setting is done by Click-dragging the vertical green line on the left side (crossing the X Axis at 0) allows you to set the display pixel intensity to 0 for all intensities that appear to the left of that line. Doing so removes background noise from the image.

Click-dragging the vertical green line on the right side allows you to set the display pixel intensity to 255 for all intensities that appear to the right of that line.

If a non-linear mapping occurs by moving the yellow crosshair, click 'Set Linear Curve' to return to a linear transformation.

Note: Changing the display properties does not change the pixel intensity data. They are for display purposes only.
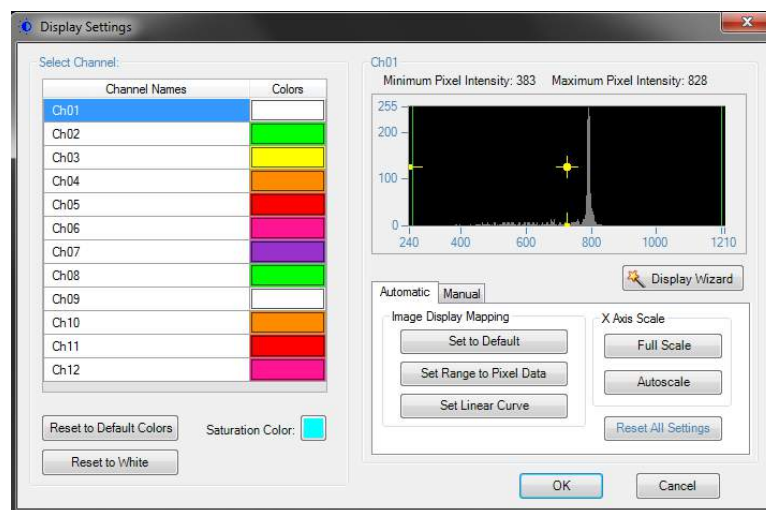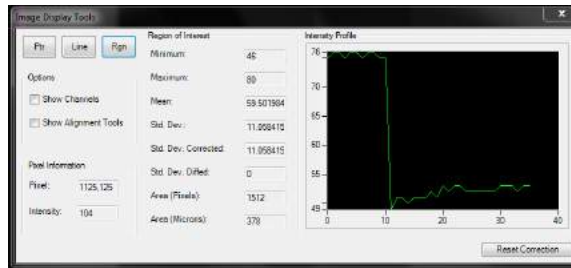
Image Display Tools

- **Ptr, Line, Rgn:** Buttons that allow interrogation of pixel information of a single point (Ptr), a line, or a region (Rgn) of the imagery.
- **Pixel Information** box: Displays the selected Pixel (x,y) coordinates and its Intensity value.
- **Region of Interest** box: Displays the Minimum, Maximum and Mean pixel intensity values, their standard deviation (Std. Dev.), and the Area of the drawn region.
- **Intensity Profile:** Plot of horizontal pixel number vs. Mean pixel intensity for the drawn region.

The Analysis Area

Graphs are displayed in the analysis area during setup or acquisition. Regions can be drawn on the graphs to create populations. The functionality of the analysis area is the same as in IDEAS. Refer to the IDEAS user manual for further information on graphs, regions and populations.

Analysis Area Tools
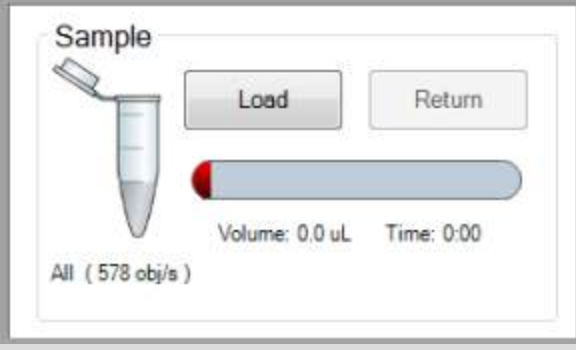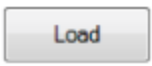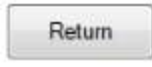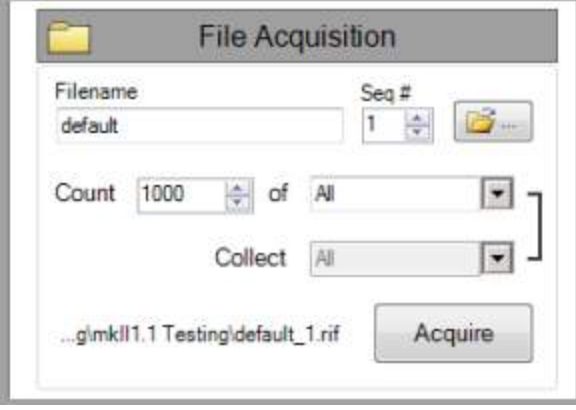
Icon	Name	Description
	Reset	Refreshes the graphs with incoming data
	Histogram	Opens the histogram graph tool
	Scatter Plot	Opens the bivariate scatter plot tool
	Pointer	Reset cursor to pointer
	Line region	Draw a line region on a histogram
	Rectangle region	Draw a rectangular region on a scatterplot
	Oval region	Draw an oval region on a scatterplot
	Polygon region	Draw a polygon region on a scatterplot
	Select All	Selects all plots in analysis area
	Tile	Tiles the graphs in the analysis area to fill the space
	Size Plots	Sets size of selected plots to small, medium or large
	Populations	Opens the population manager
	Regions	Opens the region manager
	Wizards	Opens the list of wizards

The Instrument Control Panel

The instrument control panel provides tools to control instrument operation, data acquisition and status.

Sample Load Return Volume: 0.0 uL Time: 0:00 All (578 obj/s)	In the Sample section you can load a sample or return a sample. Sample time remaining is displayed when a sample is running.
Load	Loads the sample
Return	Returns the sample
File Acquisition Filename Seq # default 1 Count 1000 of All Collect All ...g\mkII1.1 Testing\default_1.rif Acquire	In the File Acquisition section you can type in a custom filename, set the sequence #, choose the data file folder, type the number of events and choose the population to collect.
Acquire	Begin Acquisition
Filename	Type the filename
Seq#	Choose the beginning sequence number
(folder icon)	Navigate to the folder to save the data
Count	Enter the number of events to collect in the box.
of	Choose the population to collect in the dropdown menu.
]]	Collect a different population while counting the first population. Click on the bracket to break the link and select population.

Illumination 405 · 0.00 mW · Scatter Channel 6 12 488 · 0.00 mW · Brightfield ● 642 · 0.00 mW · 1 and 9 ▾ SSC · 2.00 mW · Set Intensity	In the Illumination section you can turn laser and brightfield illumination on or off and set intensities.Select the scatter channel, either 6 or 12. All lasers have variable power and are defined by their excitation bandwidth.
405 · 0.0 mW	405nm laser excitation - currently set to OFF and 0 mW of power.
488 · 60.0 mW	488nm laser excitation- currently set to ON at 60 mW of power.
642 · 150.0 mW	642nm laser excitation- currently set to ON at 150 mW of power.
SSC · 2.00 mW	SSC (side scatter) laser excitation- currently set to OFF at 2.00 mW of power. SSC is produced from a dedicated 785 nm laser.
Brightfield ● 1 and 9 ▾	Brightfield illumination is shown as ON in channels 1 and 9.
Set Intensity	Sets the Intensity of the brightfield to 800 counts.
Magnification & EDF Magnification ☐ EDF 20X 40X 60X	Select the magnification. Note: this is optional equipment.Turn EDF on by checking the box.
Fluidics Running · Lo Speed Hi ▷ ■ · Hi Sensitivity Lo Core Size: 6 µm	Adjust the speed and sensitivity for the run. (see table below for an explanation of speed versus sensitivity)
▷	Run fluidics.
■	Stop fluidics.

Lo Speed Hi Hi Sensitivity Lo	Speed and Sensitivity are inversely related. See the table below for more information of speed, sensitivity and resolution.
Focus and Centering Focus ← 0.2 → Centering ← 0.0 →	Focus and Centering can be adjusted using the right and left arrows.
Startup	Runs the startup script.
Shutdown	Runs the shutdown script and sterilizes the system.

Speed versus Sensitivity

The table below shows the pixel resolution, relative sensitivity and approximate time to collect 10,000 obects at a concentraion of 1×10^7 objects per ml at different magnifications and speed settings. In order to run at a higher speed the rows on the camera are binned, this means the signal is collected in 1, 2 or 4 rows of the CCD at a time.

Objective	Speed	Bin	Pixel Resolution	Sensitivity	Core Diameter	Velocity mm/sec	Sample obj/ml	Cells/Sec	Time to 10,000
20x	Low	1x	1 x 1 um²	High	10um	55	1×10^{7th}	200	54 sec
	Med	1x	1 x1 um²	Med	10um	110	1×10^{7th}	360	28 sec
	High	2x	1 x 2 um²	Low	10um	220	1×10^{7th}	720	14 sec
40x	Low	1x	.5 x .5 um²	High	10um	55	1×10^{7th}	200	50 sec
	Med	2x	.5 x 1 um²	Med	10um	110	1×10^{7th}	360	29 sec
	High	4x	.5 x 2 um²	Low	10um	220	1×10^{7th}	720	14 sec
60x	Low	1x	.3 x .3 um²	High	7um	40	1×10^{7th}	60	160 sec
	Med	2x	.3 x .6 um²	Med	7um	55	1×10^{7th}	75	135 sec
	High	4x	.3x1.2 um²	Low	7um	110	1×10^{7th}	150	68 sec

Note: Binning refers to the number of pixel rows used to collect the data.
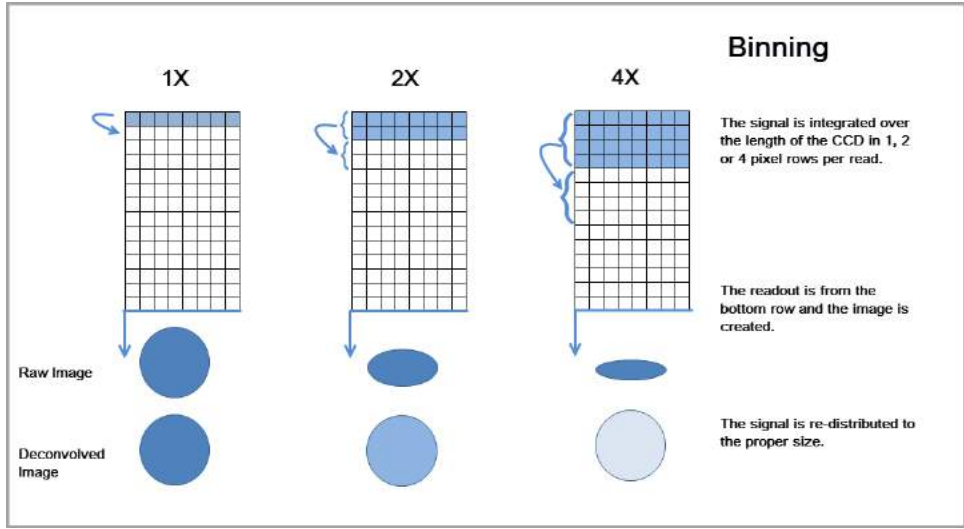
Bottom task bar:

Status buttons are displayed at the bottom of the INSPIRE window.

"Switch Core Mode" completed at 8:40 AM on 6/6/2012 "Flush Lock and Load Sample" running Acquiring 060612 X197 60X LPS_2.rif...	Describes the current script
Sheath Flush Beads	Level indicator for pumps
Compensation	Green indicates compensation is being applied to the Intensity feature. Note that imagery and other features are not compensated.
ASSIST	Yellow- calibrations and tests not run Red- one or more calibrations or tests failed Green- all calibrations and tests have passed

Menu Bar

The menu bar is located in the upper-left portion of the INSPIRE screen. It consists of these four menus:

- **File** menu: Load and save instrument setup templates. A template contains instrument settings that can be predefined and loaded to simplify the instrument setup process.
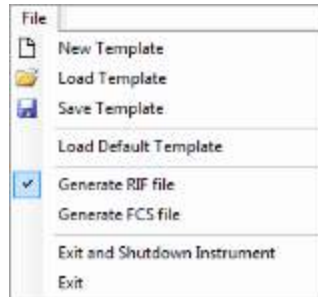
  - **New Template:** Create a new template.
  - **Load Template:** Browse for and open saved templates.
  - **Save Template:** Save your settings as a template for future use. Template file names are appended with the suffix .ist. They are saved in the INSPIRE Data folder.
  - **Load Default Template:** Loads factory settings.
  - **Generate RIF file:** Check to save a Raw Image File during acquisition.
  - **Generate FCS file:** Check to save a Flow Cytometry Standard file during acquisition.
  - **Exit and Shutdown Instrument:**: Turns off the instrument control system exits INSPIRE and shuts down.
  - **Exit:**: Exits INSPIRE.
- **Instrument** menu: Run the ImageStream^X camera and instrument-specific fluidic scripts (automated fluidic routines).
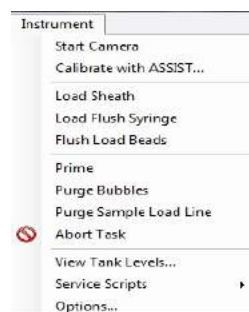
  - **Start/Stop Camera:**: Starts or Stops imaging.
  - **Calibrate with ASSIST:** Opens the Calibrations and Tests window.

– **Load Sheath:** Fills the sheath syringe with sheath fluid and an air bubble that facilitates stable flow.

– **Flush Load Beads:** Flushes the bead syringe and reloads beads from the bead tube.

– **Load Flush Syringe:** Fills the flush syringe with sheath fluid.

– **Prime:** Pushes sample and beads into the flow cell.

– **Purge Bubbles**: Removes air bubbles from the flow cell by filling the flow cell with air then filling the sheath line and pump with debubbler and rinsing the flow cell. The sheath syringe is then refilled with sheath and the bubble trap, lines and flow cell are filled with sheath.

– **Purge Sample Load Line**: Flushes the sample load line with debubbler to remove bubbles formed during sample loading.

– **Abort Task:** Stops the current script.

– **View Tank Levels::** Opens the fluid level window.

– **Service Scripts::** For field service personnel only.

– **Options::**
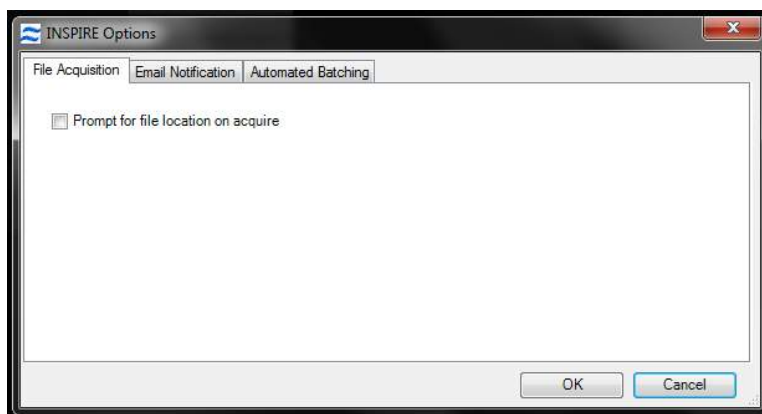
• **Autosampler menu:** Access autosampler controls.
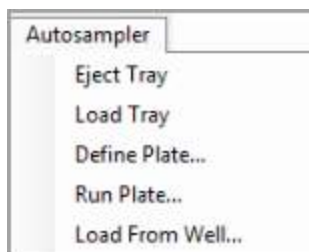
– **Eject Tray:** Opens the door of the autosampler and extends the tray for the 96 well plate.

– **Load Tray:** Retracts the plate tray back into the instrument and closes the door.

– **Define Plate:** Opens the plate definition dialog.

– **Run Plate:** Starts the autosampler run as defined by the plate definition.

– **Load From Well:** Allows a single sample load from a well plate.

–

- **Analysis** menu: Access the Feature, Population and Region Managers. Functionality is the same as for IDEAS. Refer to the IDEAS user manual for more information.
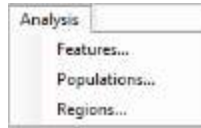
- **Features:** Opens the Feature Manager. Features can be renamed or new combined features can be created.
- **Populations:** Opens the Populations Manager. View,edit or delete populations.
- **Regions:** Opens the Regions Manager. View, edit or delete regions.

  Note: See IDEAS User manual for more information.

- **Compensation** menu: View, edit or create a new compensation matrix.
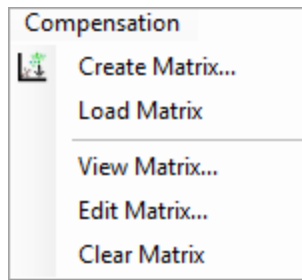
- **Create Matrix:** Opens the compensation wizard.
- **Load Matrix:** Applies compensation to the Intensity features.
- **View Matrix:** Opens the compensation matrix values table.
- **Clear Matrix:** Stops applying compensation of Intensity features.
- **Layout** menu:
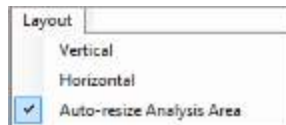
- **Vertical:** View the image gallery and analysis area side by side.
- **Horizontal:** View the image gallery and analysis area top and bottom.
- **Auto-resize Analysis Area:** When selected automaticaly adjusts the separator between the image gallery and analysis area when images are added or removed from the view.
- **Advanced:**menu: For field service personnel only.
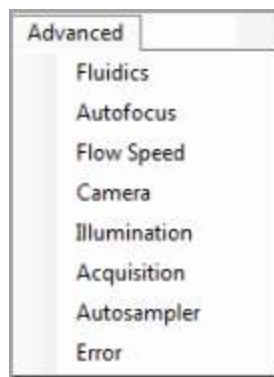
– **About ImageStream^X:** Access the current INSPIRE version number with the About ImageStream^Xoption.

–

Daily Operations

Turning on the ImageStream^X:

This section describes how to prepare the ImageStream^X for use. The ImageStream^X is usually left on with INSPIRE launched, but the following instructions also describe how to turn the ImageStream^X on if the power is off.

Note: If the ImageStream^X power is on and INSPIRE is already launched, go directly to step 4.

1   Press the green power button inside the front door of the ImageStream^X to turn on the instrument and start the computer.
2   Log on with the user name (Amnis) and password (is100).
3   Launch the INSPIRE software and by double-clicking the **INSPIRE** icon on the desktop.

Preparing to run and calibrating the ImageStream

4   Fill the rinse bottle with deionized water and the sheath bottle with PBS. Ensure the SpeedBead reagent is loaded on the bead port and is well mixed. The beads are automatically mixed while the instrument is in use. If the instrument has been idle for a long period, remove the bead vial and vortex. Refer to the following compatibility chart to choose the appropriate Sheath fluid.

Sample Solution	Sheath Fluid	Acceptable
PBS	PBS	Yes
PBS	Water	Yes*
PBS/Surfactant	PBS	Yes
PBS/Surfactant	Water	No
Water	PBS	Yes
Water	Water	Yes
Water/Surfactant	PBS	No
Water/Surfactant	Water	Yes

* Cells in PBS run with water sheath will swell.

5   Empty the waste tank. Push on the quick-disconnect buttons to remove the tubing from the waste tank. Add 160 ml of bleach to the Waste bottle. The final volume of waste when full will be approximately 1600 ml and therefore the final bleach concentration for a full waste tank will be 10% bleach. It is recommended that the waste be emptied every day and fresh bleach added before Startup.
6   Click **Startup** This script fills the system with sheath and flushes out all of the old sheath or rinse that was in the system. The sample line is prepared by loading 50 µl of air into the uptake line. Beads are loaded into the bead pump from the 15 ml conical tube. The Initializing fluidics window opens showing the progress bar. The box is checked by default to run ASSIST after the fluidics are ready. Uncheck the box if you wish to start ASSIST later.
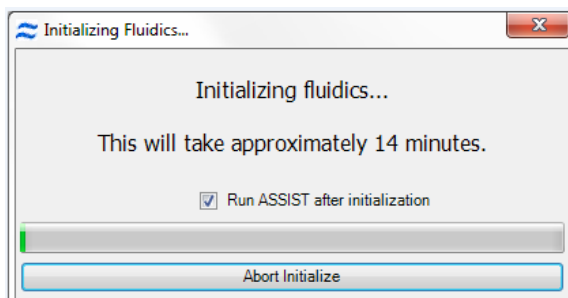
7   Click **Start All Calibrations and Tests** in the calibration window if ASSIST was not run in the previous step.

8   Center the core stream images (if necessary) by laterally moving the objective under **Focus and Centering**. Centering is adjusted by pressing right or left arrows to center images.

9   The event rate should be 800-1000 events per second. (If not, see Chapter 5: Troubleshooting)

10 When the calibrations and tests have passed the ASSIST status light will change to green. Close the Calibrations window.

11 Instrument calibrations may also be run individually by selecting a particular procedure under **Calibrations** or **Tests**. Next to each calibration or test button is a green or red rectangle. If the procedure fails, it turns red. If a procedure fails, repeat it. If it fails twice, see **Chapter** 5:See "Chapter 5: Troubleshooting" on page 77or call the Amnis Service department. For more information on the individual calibrations and tests, refer to theFigure , "ASSIST Calibrations," on page 55 in chapter 4.

Note: If adjustments are made to the instrument in order to pass an individual ASSIST calibration or test, it is important to re-run all calibrations and tests in order to record the current settings after adjustments.
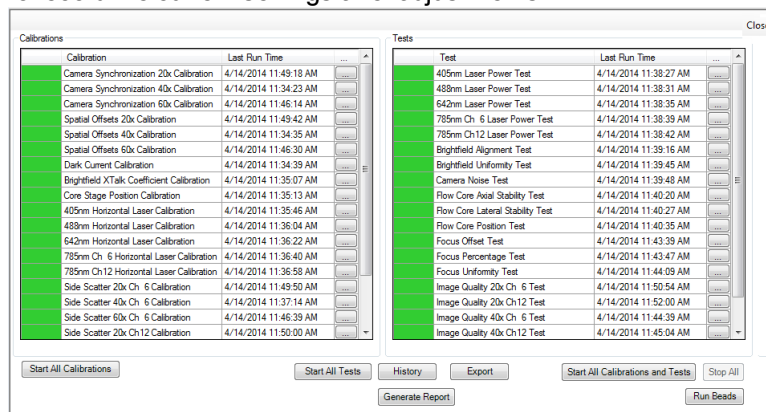
Data Acquisition

After the ImageStream^X system is calibrated, you are ready to acquire experiment data files. The sample is loaded into the sample pump. Beads and sample are injected into the flow cell to form a single core stream that is hydrodynamically focused in front of the imaging objective. The beads are used by the system to keep the autofocus and camera synchronized during the sample run, while the objects from the sample are saved to the data file. To use the Autosampler for unattended operation see Using the Autosampler.

Refer to the ImageStream Sample Preparation Guide for experimental set-up recommendations. Use compatible sample solutions from the table below.

Sample Solution	Sheath Fluid	Acceptable
PBS	PBS	Yes
PBS	Water	Yes*
PBS/Surfactant	PBS	Yes
PBS/Surfactant	Water	No
Water	PBS	Yes
Water	Water	Yes
Water/Surfactant	PBS	No
Water/Surfactant	Water	Yes

Sample order:

Samples from an experiment are typically run in the following order:

- Experimental sample with the brightest stains to set the sensitivity for the run
- 10% bleach to wash out DNA dye followed by PBS
- Using the compensation wizard or manually setting parameters
  - Single color fluorescence controls (no DNA dye) NO BF or SSC
  - Single color DNA dye control NO BF or SSC
- The rest of the experimental samples with DNA dye

Note: compensation controls may be collected after experimental files if desired.

Loading and running the sample:

1 In the file menu, choose **Load Default Template**.

   Note: Application-specific instrument settings can be saved in a template and used to facilitate instrument setup, but it is recommended that you verify the appropriateness of the settings for the specific experimental run. The default template has a set of Raw Max Pixel scatterplots for all channels helpful for setting laser powers.

2 Press **Load**, and load an aliquot of the brightest sample in the experiment, that fluoresces with each fluorochrome used. It is critical that you run this sample first to establish the instrument settings. (DO NOT change laser settings for the experiment once established on this sample.)

When prompted place sample vial with 20-200 ul into the sample loader.
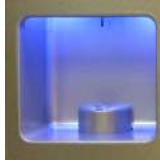
3   Choose the objective under Magnification (optional)
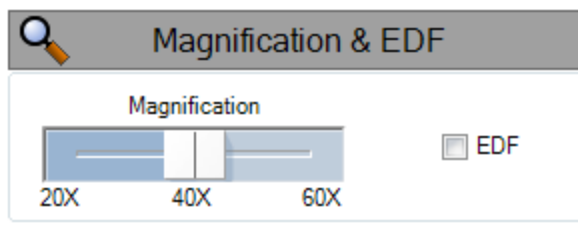
4   Select **EDF** collection if desired. See Using EDF for details.
5   Turn on each laser used in the experiment by clicking on the wavelength. Set the laser powers so each fluorochrome has Raw Max Pixel Intensities between 100 and 4000 counts, as measured in scatterplots or histograms of the appropriate channels and there is no saturation.
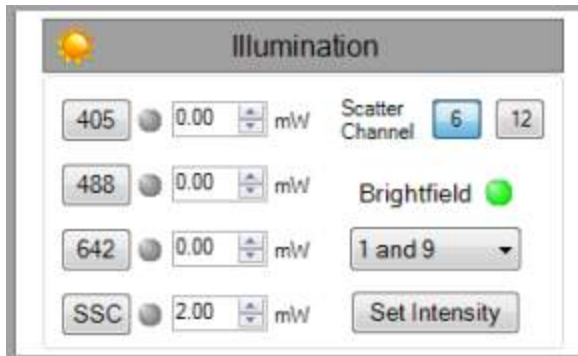
6   Select the channels to be collected by clicking on a channel name. This opens a window where channel selection can be set by checking the box next to the channel name.

7   See Setting the Image Display Propertiesfor Setting the Image Display.

8   Select Brightfield channels. Default is Ch1 for a 6 channel system; Ch1 and 9 for a 12 channel system. Click **Set Intensity**.

9   Create graphs to gate on cells of interest.

Recommended: Scatterplot of Area versus Aspect Ratio of Brightfield to gate on cells and eliminate debris. Scatterplots or Histograms of Intensity for channels used in the experiment. Scatterplots of Raw Max Pixel to observe any saturation.

To identify objects for inclusion in or exclusion from the acquiring data file the following features in any channel are available:

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

- • **Uncompensated Intensity:** The integrated intensity of the entire object image; the sum of all pixel intensities in an image, background subtracted.

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

Collecting and saving the data files

Once the sample is running and the ImageStream^X is properly set up, you are ready to acquire the data as a raw image file (.rif) and/or an FCS file. The .rif contains uncompensated pixel data along with instrument settings and ASSIST information in a modified TIFF format. The file includes only those objects defined by the population selected in the acquisition section.
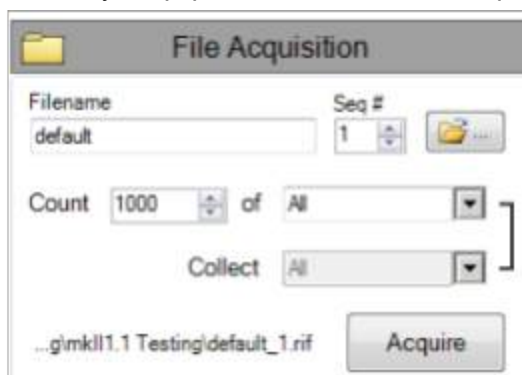
10 Enter the file name.

The number in the **Sequence #** box is appended to the file name, followed by the **.rif or .fcs**extension. The sequence number increases by 1 with each successive data acquisition. Files collected with BF off will be appended with noBF and files collected with EDF enabled will be appended with EDF in the file names. File names must be 256 or fewer characters in length, including the path and file extension. In addition, file names cannot contain the following characters: \,/,:,*,<,>, or |.

Note: to collect a .fcs file at the same time as a .rif file choose the .fcs option under the File menu.

11 Browse to select an existing folder or to create a new folder in which to save the files.

12 Enter the number of cells you want to acquire in the box after **Count** and select the population from the dropdown. To count one population but limit the collection to another population, click on the bracket to break the link and enable the Collect box.

13 Click on the **Acquire** button to begin acquiring a data file:
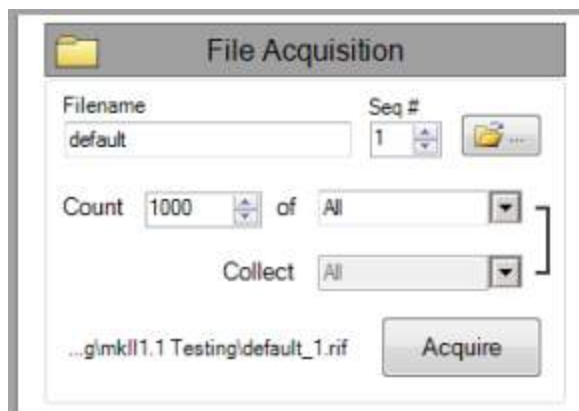
14 The data file(s) are automatically saved in the selected folder once the desired number of objects are collected.

To prematurely stop acquisition click [⬛] . The system prompts you to either discard the acquired data or to save the collected data in a file. The acquisition can be paused and resumed by clicking [ⅠⅠ] .

15 Once acquisition finishes, either load the next sample or return the remaining sample.

Note: If the next sample has no nuclear dye and follows a DNA intercalating dye-stained sample, **Load** a solution of 10% bleach and then **Load** PBS to ensure that residual dye does not stain the subsequent samples.

16 Change the file name for the next sample and continue collecting samples..

17 Repeat for each sample.

18 When finished running the experiment samples or after setting the template, run single color compensation controls with the same laser settings as the experimental samples with the exception of the scatter laser 785 which turns off in compensation mode.

19 Click [🪄] in the analysis tools section and choose Compensation to begin

The compensation wizard will set up the instrument for compensation controls that must be collected with brightfield and scatter (785 nm laser) OFF and every channel to be collected. Keep all laser powers the same as for the experimental samples.

Follow the prompts in the wizard to collect all compensation control files:

- Click Load or if a compensation control sample is already running, click Next.
- Place the tube on the uptake port and Click OK.
- Click Next when sample is running.
- Verify the channel for compensation.
- Draw a region on the Uncompensated Intensity scatter plot if not all cells are positive to define the positive population. View the population in the Image Gallery and choose this population to collect.
- Name the file and choose the path to save the data.

- Click Collect File. The compensation coefficients are calculated. The compensation coefficients and an Intensity scatter plot using the coefficients is displayed.
- Click OK on the Acquisition Complete popup window.
- Click Load to continue with the next single color control sample or click Return (optional).
- Repeat the previous steps for each compensation sample. For each sample reset the Image gallery population to view All and then create an appropriate population for each sample. Note, the R1 gate can be moved for each sample.
- Click Exit when done and Save the coefficients to a compensation matrix file.

The beginning template is restored and the saved matrix is used to compensate the Intensity feature. Note that other features are not compensated. The compensation can be cleared if desired from the compensation menu. Scatter plots can be made with the feature Uncompensated Intensity to compare with and without compensation.

20 Continue to collect experimental files.

21 Click Shutdown when done. See Daily Shutdown Procedure

Optional settings

Squelching Debris

Some samples have an abundance of small particulate debris. These can be eliminated from collection by gating or by using Squelch to reduce the sensitivity of object detection. As opposed to gating debris away from cells, squelching debris can prevent INSPIRE crashes related to overburdening the computer processor with an abnormally high event rate. Squelch should only be used if the rate of total objects per second reaches 4000. Squelch values range from 0 to 100; increasing the value decreases object detection sensitivity.

1   Choose All in the image gallery.
2   Observe the relative proportion of cell to debris images appearing in the imaging area and the event rate (**Total/Sec** under **Acquisition Status**).
3   On the **Advanced Setup -Acquisition** tab, increase the **Squelch** value until the observed proportion of cells to debris increases in the imaging area.
4   Observe the **Total/Sec** event rate on the **Setup** tab under **Acquisition Status**. If it is still greater than 500, repeat step 2.

Setting ImageStreamX Speed and Sensitivity

The optimal operating speed is set at the factory for each instrument and is approximately 60 mm/sec. This speed corresponds to the highest resolution setting (shown below) with a pixel size of 0.5 µm at 40 X magnification. In order to collect images at higher speed, the rows on the camera can be binned. Image collection speed is inversely related to image resolution (sensitivity).

The table below shows the pixel resolution, relative sensitivity and approximate time to collect 10,000 obects at a concentraion of 1 x 10^7 objects per ml at different magnifications and speed settings. In order to run at a higher speed the rows on the camera are binned, this means the signal is collected in 1, 2 or 4 rows of the CCD at a time.

Objective	Speed	Bin	Pixel Resolution	Sensitivity	Core Diameter	Velocity mm/sec	Sample obj/ml	Cells/Sec	Time to 10,000
20x	Low	1x	1 x 1 um^2	High	10um	55	1x10^{7th}	200	54 sec
	Med	1x	1 x1 um^2	Med	10um	110	1x10^{7th}	360	28 sec
	High	2x	1 x 2 um^2	Low	10um	220	1x10^{7th}	720	14 sec
40x	Low	1x	.5 x .5 um^2	High	10um	55	1x10^{7th}	200	50 sec
	Med	2x	.5 x 1 um^2	Med	10um	110	1x10^{7th}	360	29 sec
	High	4x	.5 x 2 um^2	Low	10um	220	1x10^{7th}	720	14 sec
60x	Low	1x	.3 x .3 um^2	High	7um	40	1x10^{7th}	60	160 sec
	Med	2x	.3 x .6 um^2	Med	7um	55	1x10^{7th}	75	135 sec
	High	4x	.3x1.2 um^2	Low	7um	110	1x10^{7th}	150	68 sec

Note: Binning refers to the number of pixel rows used to collect the data.

Binning

The signal is integrated over the length of the CCD in 1, 2 or 4 pixel rows per read.

The readout is from the bottom row and the image is created.

The signal is re-distributed to the proper size.
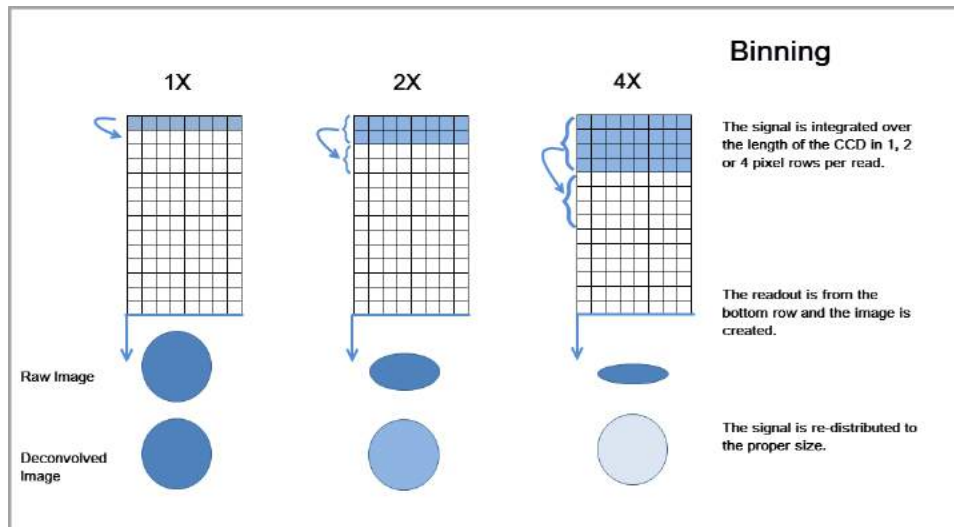
Daily Shutdown Procedure

This procedure sterilizes the system and leaves it with pumps empty and water in the fluidic lines. The instrument is left on with INSPIRE running.

1 Fill the Rinse, Cleanser, Sterilizer, and Debubbler bottles if necessary.
2 Empty the Waste bottle.
3 Remove any tubes from the uptake ports.
4 Click **Shutdown**

   Note: This procedure automatically turns off all illumination sources and rinses the entire fluidic system with water, sterilizer, cleanser, de-bubbler, and then water again. The sterilizer is held in the system for ten minutes to ensure de-contamination. It takes about 45 minutes of unattended (walk-away) operation to complete.

Optional upgrades

Using EDF

Extended depth of field (EDF) is a novel technique used in a variety of applications including FISH spot counting where having the entire cell in optimal focus is critical to obtaining accurate results.

First images are acquired with the EDF element in place. The data is automatically deconvolved using the EDF kernel from the calibration prior to analysis using IDEAS. Calibration of the element is done by the Amnis engineer wheninstalled and should be repeated by Amnis service when any optical changes are made to the instrument.

To collect a data file using the EDF element

1   Set instrument settings for the experiment.
2   Check the box 'EDF' in the instrument control panel.
3   Adjust any regions being collected to accommodate using EDF.
4   The calibration kernels saved during the last EDF calibration will be appended to the file and the file name will be appended with -EDF.

EDF image of a small bead showing characteristic L-shaped pattern

Cell images as they appear on the instrument with (left pair) or without (right pair) EDF element in place. Raw Max Pixel values indicated in upper left

General characteristics of using EDF

- The EDF element spreads all points of light within a cellular image into consistent L-shaped patterns. When EDF images are opened in ideas, the data is deconvolved to create an image of the entire cell projected simultaneously in focus.
- During acquisition and before deconvolution, images will appear blurred into characteristic L-shaped patterns and raw max pixel values will be lower with EDF than with standard mode collection.
- Compensation controls for EDF data can be collected with or without the EDF element in place.
- When analyzing data in IDEAS, after the deconvolution process there will be more light per pixel than in non-deconvolved imagery. Therefore, raw max pixel values may exceed 1023 (for the IS100 instrument) or 4095 (for the ISX). As long as the images did not saturate the camera during acquisition, these pixel values are valid.
- Object, Morphology and System Masks will be smaller in EDF mode.
- Focus gating is not required. However if there are blurred events due to streaking, these can be removed from the analysis using a focus gate.

- • EDF images exhibit increased texture due to higher resolution. • Brightfield imagery is not as crisp in EDF mode as in standard mode.
- • An in-depth discussion of EDF can be found in the following reference: *Cytometry Part A (2007) 71A:215-231*

Using MultiMag

The MultiMag option includes 2 additional objective lenses. The 20X lense is useful for very large objects that do not fit into the field of view of the 40X objective such as cardiomyocytes or epithelial cells. The pixel size using the 20X objective is 1 square micron. The 60X objective provides a higher magnification for small objects. The pixel size using the 60X objective is 0.33 microns.

To collect a data file using the 20X or 60X object

Objective	Field of view	Pixel size	Depth of field	NA
40X	60 um	0.5 um	4 um	0.75
20X	120 um	1 um	8 um	0.5
60X	40 um	0.33 um	2.5 um	0.9

The optional objective can be chosen by selecting the button under Magnification. When using the 60X obective the core velocity will be reduced to 40 mm/sec instead of the normal 60 mm/sec used during 40X or 20X acquisition. See the section on the Speed versus Sensitivityfor more information on resolution at different magnifications.

Using the Autosampler

To enable high throughput experiments and unattended operation the autosampler option includes upgraded fluidics, software and an imbedded tray for loading of samples in a 96 well plate format.

Prior to running the plate, a plate definition is created that assigns instrument settings to the wells, names to the output files, and parameters to include in a well plate report that is generated once the plate has completed. While the plate is running, the user may be notified of any errors encountered via email. The instrument can also sterilize at the completion of the plate.

Workflow:

- Create Instrument Setting Template(s) (.ist) to be used for your plate. To do this, run an experimental sample manually with all of the fluorescence dyes to be used in the experiment (see INSPIRE Setup Quick Start Guide). Save each relevant template.
- Create a Well Plate Definition (.def) that assigns instrument settings to wells, names to the sample output files, and parameters to include in the plate report (see procedure below).
- Add 75 ul samples to the 96 well plate, cover with Sigma-Aldrich X-Pierce Film (XP-100, Cat # 2722502) and load the plate into the autosampler.
- Run the plate (see procedure below).

Access to AutoSampler operations is found under the AutoSampler menu.

From this menu you may:
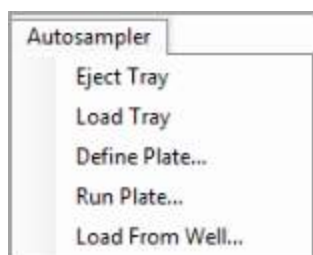
- Extend or retract the tray
- Create a plate definition
- Run a plate
- Run a single well from a plate

Define the plate:

The steps required to create a Well Plate Definition (.def) and run a plate are outlined below:

1 Choose **Define Plate** from the Autosampler menu to open the Well Plate Definition window.
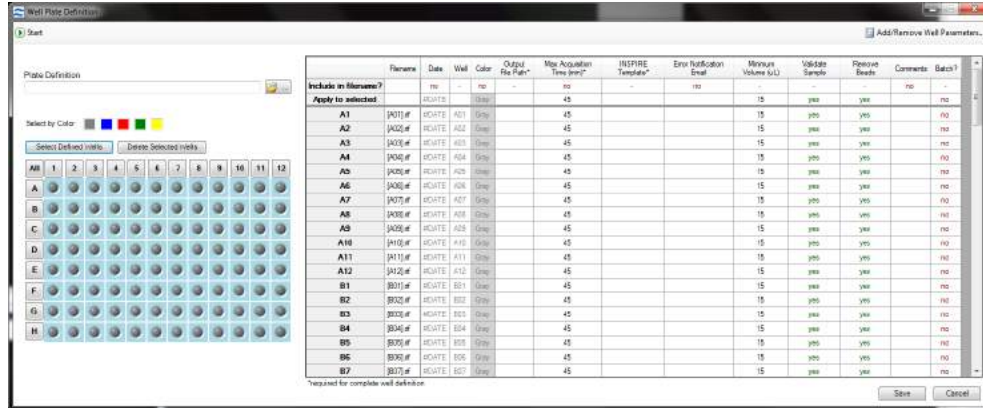
2 Begin to create a new definition or you may browse for a previously saved definition (to edit) by clicking on the folder icon.

3 **Name** the plate definition.

4 At a minimum, each well requires an Output File Path, Max Acquisition Time, and Template File in order to be considered 'defined'. Other parameters can be added to the definition in the next step.

5 Choose the parameters you would like to use.

   • Click **Add/Remove Well Parameters** to choose the parameters you want to report for the wells.

Add or Remove Columns (left dialog)

- ☐ Standard Parameters
  - ☑ Date
  - ☑ Well
  - ☑ Color
  - ☑ Output File Path
  - ☑ Max Acquisition Time (min)
  - ☑ INSPIRE Template
  - ☑ Error Notification Email
  - ☑ Minimum Volume (uL)
  - ☑ Validate Sample
  - ☑ Remove Beads
  - ☑ Comments
  - ☑ Batch?
  - ☐ Dose
  - ☐ Time
- ☐ Custom Parameters
- ☐ Illumination Parameters
- ☐ Imaging Parameters

Enter custom column name here

[Add Custom Column] [Delete]

[OK] [Cancel]

Add or Remove Columns (right dialog)

- ☐ Standard Parameters
- ☐ Custom Parameters
- ☐ Illumination Parameters
  - ☐ BF Channel
  - ☐ 405 Power
  - ☐ 405 SSC Blocker
  - ☐ 488 Power
  - ☐ 488 SSC Blocker
  - ☐ 561 Power
  - ☐ 561 SSC Blocker
  - ☐ 594 Power
  - ☐ 594 SSC Blocker
  - ☐ 642 Power
  - ☐ 642 SSC Blocker
  - ☐ 785 Power
  - ☐ 785 SSC Blocker
- ☐ Imaging Parameters
  - ☐ Binning
  - ☐ Magnification

Enter custom column name here

[Add Custom Column] [Delete]

[OK] [Cancel]

There are several categories of parameters that may be chosen as a group or individually. See the list of parameters above. Check or uncheck the desired parameters. The user can also define custom parameters. Expand the category to see the individual parameters. To delete a custom parameter, select it and use the delete key. Click OK when done

- It is highly recommended to leave the validate sample option 'Yes'. See notes below regarding validating samples.
- To include a parameter in the file name, click in the box below the column heading (make sure it says 'yes').
- Columns can be re-ordered by click/drag.
- Batching of the data into IDEAS may be done if a compensation matrix and template exist for the experiment.
- Click OK when finished adding or removing parameters.

6   Define the wells. Select wells to define by clicking a) individually (orCtrl click / shift click for multi-select), b) rows or columns, c) color, d) the 'Select Defined' button or e) All. In this example column 1 is selected.

Note: Selecting and defining wells with shared parameters first and then refining the definition for sets of wells makes it easier to organize the definition.
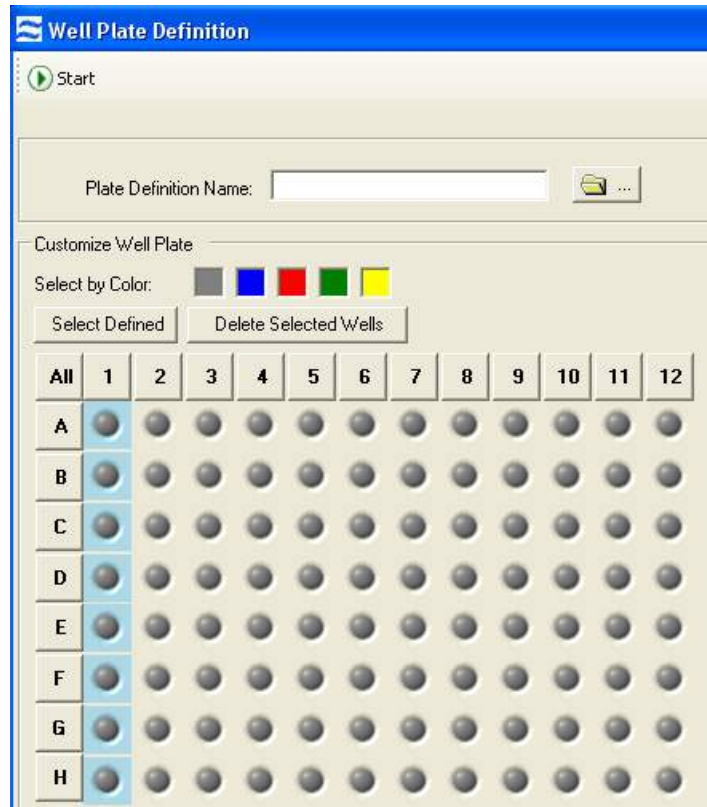
7   You can edit values for some of the Custom and many of the Standard parameters. You can do this for all selected wells or for individual wells.

For example, if you want to collect with Max Acquisition Time 10 minutes for the selected wells, type 10 in the 'Apply to selected' box below the Max Acquisition Time heading. If you want to only apply this to a single well, type this value in the box corresponding to that well.
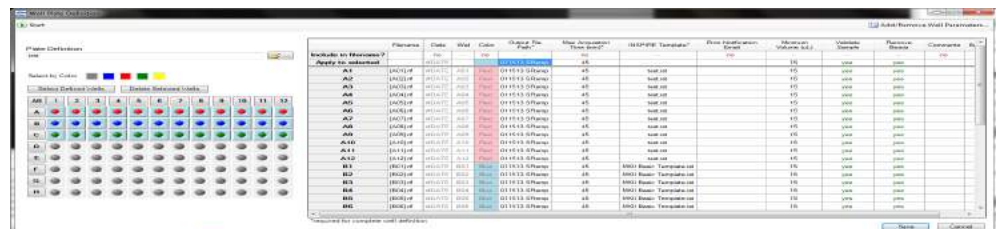
8   Highly recommended- select **Error notification Email** from the list of standard parameters and type in the user email address in the 'Apply to Selected' box.

9   When done click **Save**.

- A warning may be displayed if there are undefined or partially defined wells. Select Yes to return to plate definition or No to continue.
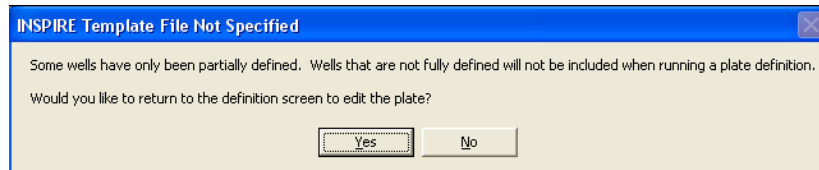
Start the autosampler

10 Click **Start** to run the plate. The Auto Sampler Unattended Operation window opens with the Plate Definition you just saved.

- If you wish to choose a different Definition, browse for it by clicking on the folder icon.
- If you want to edit the Plate Definition, click 'Edit This Plate' and you will be taken to the Well Plate Definition window.
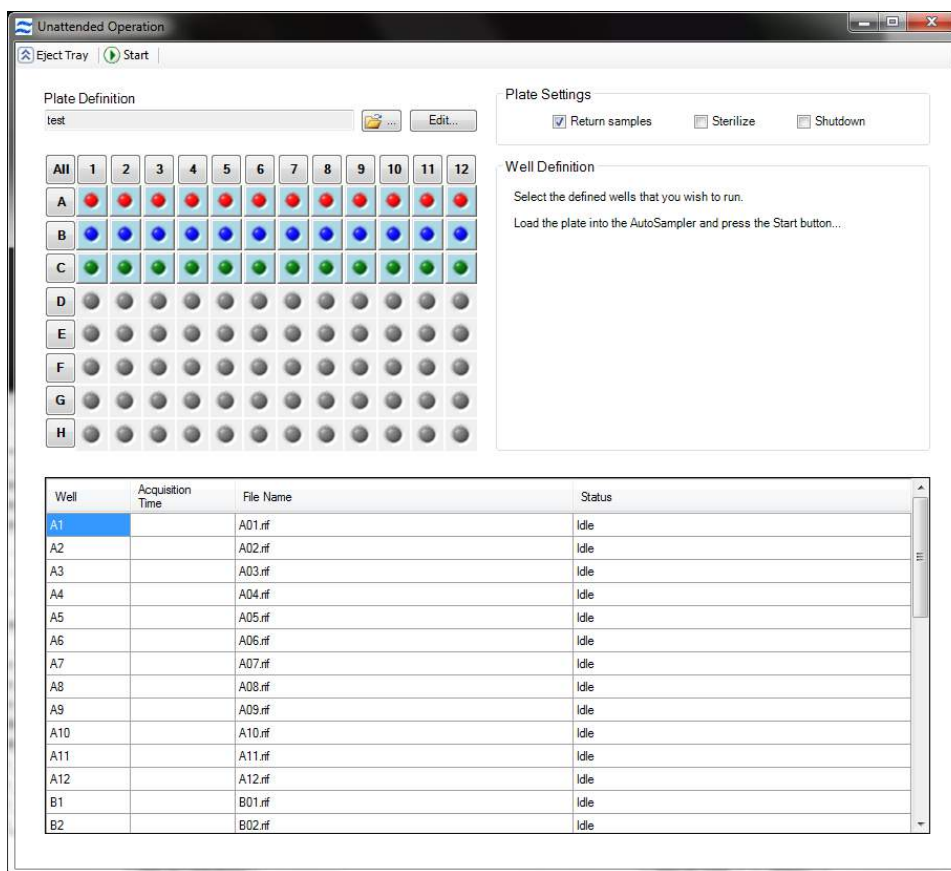
11 Check or uncheck the boxes **Return samples, Sterilize, Shutdown**.

Note that these boxes may be checked or unchecked while the plate is running and the operation will apply after the current sample is finished.

12 **Select** the wells to run (they will appear in the list).

13 Click **Eject Tray** to extend the plate nest.

14 Add at a minimum 75 ul samples to the 96 well plate, cover with Sigma-Aldrich X-Pierce Film (XP-100, Cat # 2722502) and place your plate on the tray with well A1 positioned at the upper left corner.

15 Click **Start** to begin.

16 The Status column will be updated for each well as it is run. For each sample, the instrument performs the following in sequence : 1) Load, 2) Validation (flow speed CV, focus, brightfield intensity object rate, 3) Data Acquisition, 4) Result (success or error).

During a run:

- You may stop the plate at any time by clicking the Stop button. This does not initiate sterilize (even if the 'Sterilize after running plate' box is checked).

- Should the sheath tank or beads reservoir become empty or the waste tank full during a run, an alert will be sent to the email entered in the well plate definition. Acquisition will pause until the user intervenes.

- If an error occurs on a well, the sample is returned, an alert is sent to the email address entered in the well plate definition, and the autosampler moves on to the next well.

- If the same error occurs on three consecutive wells, the autosampler aborts the plate and sterilizes the instrument (if the 'Sterilize after running plate' box is checked)

Report:

17 A well plate report .txt file will be saved (to the folder designated in the Output File Path of the plate definition) at the end of the run either when it was stopped manually or completed the entire plate.

18 If batching was included in the well plate definition the data files will be processed using the IDEAS compensation matrix and templates designated. All of the .cif , .daf and statistics report .txt files will be saved to the designated output file path.

Notes:

Email notifications:

The parameter 'Error Notification Email' may be selected. Provide the email address for notification. In order for this to work, the system must be enabled as follows.

- From the Instrument menu choose Options.
- Select "Email Notification" tab.
- Your IT department must provide the following informationand add firewall exceptions for INSPIRE and this port.
  - SMTP Host
  - SMTP Port
  - Login
  - Password
  - To
  - From

- Cc (optional)
- Choose whether to enable SSL
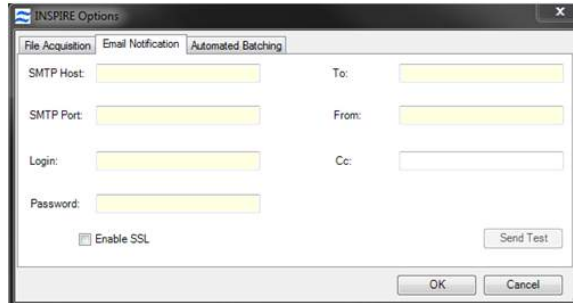
If the email notifications do not work, have your IT department make sure the messages are not being blocked by firewalls or other network security settings. Also, try enabling/disabling the SSL check box.

Sample validation:

When using the autosampler, each sample load is validated to ensure good imagery is collected for the sample.

First the core speed and rough synchrony is checked to make sure it is close enough to proceed and this requires Brightfield (if the template has BF off then BF must be turned on). This step occurs whether validation is ON or OFF in the plate definition.

It is highly recommended that the validation is 'Yes' in the plate definition for every well.

When the validation is 'Yes' for the well, the object rate and synchrony is then tested and must pass some predetermined limits. Focus adjustment is enabled. This ensures good imagery is collected.

Finally, if BF was turned ON for the first step then it is turned OFF again for the current template.

Experimental design

This chapter includes the Sample Preparation Guide which is a guideline to designing the ImageStream experiment.

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 (2x107 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.

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.

Fluorochrome charts

6 channel system

Ch	Band (nm)	Excitation Laser (nm)								Used	Ch
		375	405	488	561	592	642	730	785		
1	435-505 (457/45)	*DAPI, BV421, AF350, Hoechst, PacBlue, CascadeBlue,	*DAPI, BV421, AF405, Hoechst, PacBlue, CascadeBlue, , eFluor450, DyLight405, CFP, LIVE/DEAD Violet								1
2	505-560 (533/55)	*BV510, PacOrange, CascadeYellow, AF430, eFluor525, QD525,	*BV510, PacOrange, CascadeYellow, AF430, eFluor525, QD525,	FITC, AF488, GFP, YFP, DyLight488, PKH67, Syto13, SpectrumGreen, LysoTrackerGreen, MitoTrackerGreen							2
3	560-595 (577/35)	*QD565, QD585, eFluor565	*QD565, QD585, eFluor565	PE, PKH26, DSRed, mOrange, CellMask/CellTracker/ SYTOX Orange, Cy3	PE, AF546, Cy3*, DyLight550, PKH26, DSRed, SpctmOrng, MTOrng						3
4	595-642 (610/30)	*QD625, eFluor625, BV605	*QD625, eFluor625, BV605	PE-TexRed, ECD, PE-AF610, PI, RFP, QD625, eFluor625	AF568, Cy3*, PE-TexRed, ECD, TexRed, PE-AF610, RFP, mCherry, PI	TexRed, AF594, DyLight594, mCherry, SpectrumRed, PI,					4
5	642-745 (702/85)	*QD705, eFluor700, BV711	*QD705, eFluor700, BV711	PE-Cy5, PE-AF647, 7AAD, PerCP, PerCP-Cy5.5, DRAQ5, QD705, eFluor650, FuraRedlo, DRAQ5*, LDS 751	PE-Cy5, PE-AF647, DRAQ5*, 7AAD, LDS751	AF647, AF660, AF680, APC, Cy5, DyLight649, PE-AF647, PE-Cy5, DRAQ5*	APC, AF647, AF660, AF680, DRAQ5, Cy5, DyLight649, DyLight680, PE-AF647, PE-Cy5, PerCP, PerCP-Cy5.5				5
6	745-780 (762/35)	*QD800, BV786	*QD800, BV786	PE-Cy7, PE-AF750, QD800	PE-Cy7, PE-AF750	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, PE-Cy7, PE-AF750	SSC		6

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 during an experiment. Values listed are assuming 405, 488, and 642 laser excitation.

1 laser (488): ideal dyes are BF, AF488, PE, PE-TxRed, PE-Cy5, SSC
2 laser (488,642): ideal dyes are BF, AF488, PE, PE-TxRed, AF647, SSC
3 laser (405,488,642): ideal dyes are DAPI, AF488, PE, PE-TxRed, AF647, SSC/BF

12 channel system

Ch	Band (nm)	375***	405	488	561	592	642	730	785	Used	Ch
		Excitation Laser (nm)									
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, Cascade Blue, AF350	*DAPI, BV421, AF405, Hoechst, PacBlue, Cascade Blue, eFluor450, DyLight405, CFP, LIVE/DEAD Violet								7
8	505-570 (537/65)	*BV510, PacOrange, Cascade Yellow, AF430,	*BV510, PacOrange, Cascade Yellow, 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

Chapter 4: Instrument Calibrations and Tests

ASSIST Tab

ASSIST (**A**utomated **S**uite of **S**ystemwide ImageStream^X**T**ests) is a suite of calibrations and tests for critical subsystems operating within the ImageStream^X. ASSIST performs specific calibrations and tests, measuring, evaluating and storing thousands of values to ensure all subsystems are operating within normal limits. ASSIST permanently logs results for all tests and flags any parameters that are beyond specified limits. It is run daily using SpeedBeads to ensure optimal performance of the ImageStream^X.

A calibration is a sequence of operations designed to measure and set internal parameters that are used to operate a subsystem. Calibrations are used to optimize performance of a subsystem or place it in predefined state. After a calibration is performed, it is tested to determine whether the calibration values are within a prescribed range. A test is a sequence of operations designed to measure the performance of a specific subsystem. The calibration and test values and acceptable ranges are listed on the ASSIST display tab. A failed calibration or test is flagged with a red box. The history of any calibration or test can be viewed by clicking on the box to the right of the specific item.

Utilities are calibrations used by service technicians.

Run ASSIST daily to optimize the performance of the ImageStream^X.

To run ASSIST calibrations and tests:

1   Click **Start All Calibrations and Tests** to run all standard calibrations and tests.
2   Optional: Click **Run Beads** to begin running beads without starting calibrations or tests.
3   To run one calibration or test, click on an individual calibration or test and click Run.
4   To stop a calibration or test click **Stop** or **Stop All** if Start All was chosen.
     A calibration or test will be flagged red if it fails.
     If a calibration or test fails, run that calibration or test individually and if it fails again call or email Amnis service.
     Note: Calibrations and tests do not run in order. 40X Calibrations are completed before changing magnifications to run 20X and 60X calibrations.
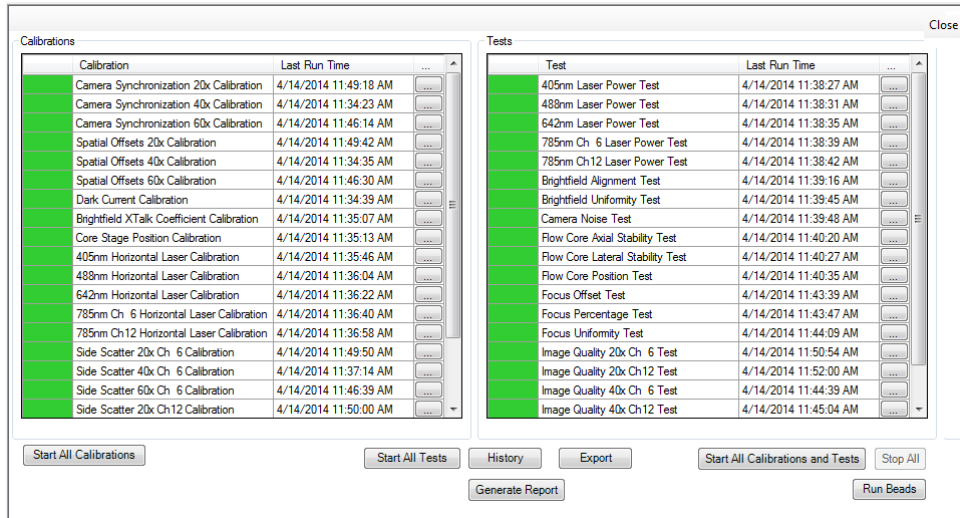
Reporting ASSIST data

ASSIST calibration and test results are stored in a database that can be reported.

To view the last result of a specific calibration or test click on the button to the right of that item or click on the History button and choose the routine and date you wish to view.

To create a report for all calibrations and tests click on the Generate Report button. A window opens that allows you to choose the dates for the report.
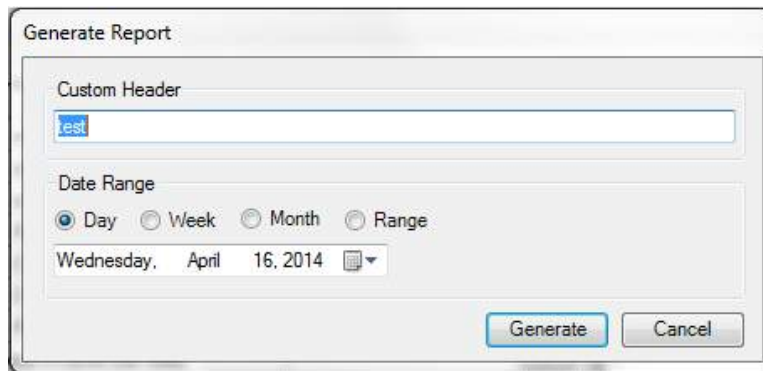
ASSIST Calibrations

The calibrations in the current suite are described in detail below.

Camera Synchronization Calibration

Measures and stores a magnification calibration (camera synch) factor relating the Flow Speed Detection frequency and the camera clock rate. This factor is used to maintain synchronization between the moving imagery projected onto the camera surface and the electronic charge resulting from that imagery. Proper synchronization helps ensure crisp image collection.
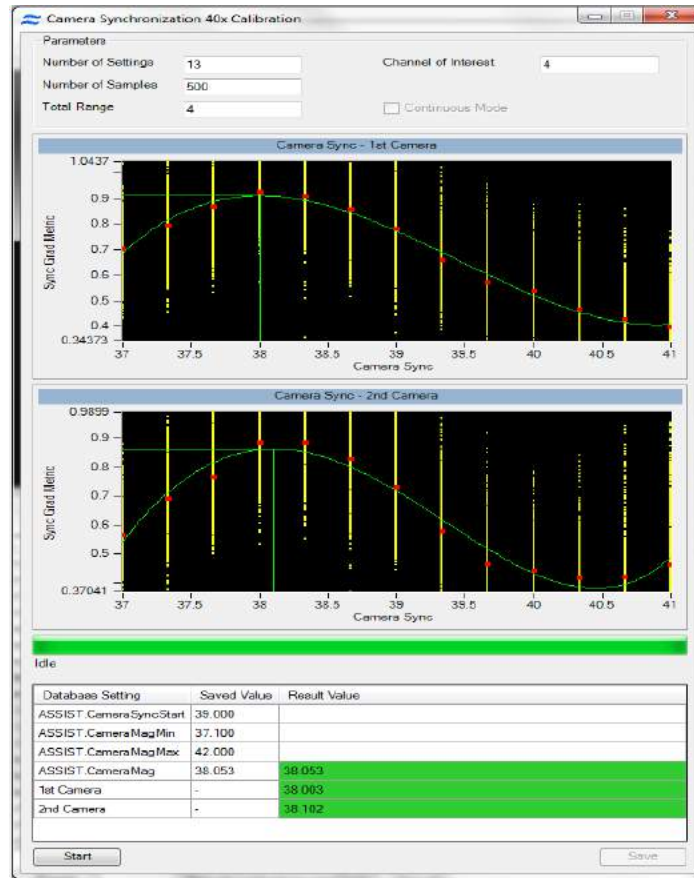
As shown in the figure above, the camera synch calibration measures SpeedBead ellipticity at numerous discrete camera synch settings and plots the camera synch setting (horizontal axis) versus the ellipticity (vertical axis). It then generates the best fit curve for a 4th order polynomial through the data and determines the horizontal location (camera synch) of the peak of the curve. The peak occurs where the SpeedBeads appear round. This setting is then stored and used for all subsequent image acquisitions. The result and the limits for the calibration are shown below the list when the calibration is selected. Please note that Camera Synchronization Calibrations will be done for each magnification present in the system.

Spatial Offsets Calibration

Measures and stores 12 calibration factors for the vertical and horizontal registration of each spectral channel of the ImageStream^X. Many assays that are run on the ImageStream^{X}quantify the spatial relationships between molecules located within cells of interest. To accurately perform these measurements and to accurately perform spectral compensation of image data, the ImageStream^{X}must maintain sub-pixel spatial registry between channels.
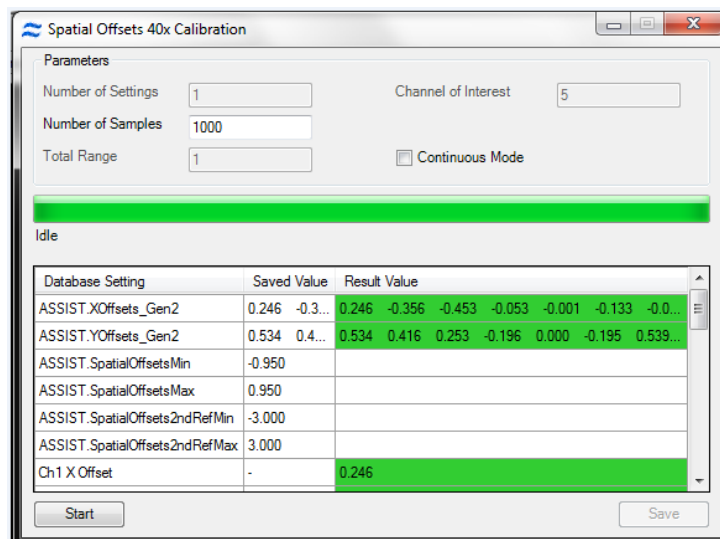

The SpatialOffsets calibration commands the brightfield system to illuminate all 6 channels simultaneously and collects imagery from 1000 SpeedBead objects in each of the six channels (6000 images total). It then performs a two-axis autocorrelation between the imagery from channels 1-5 with the imagery from channel 6. Autocorrelation is an accurate algorithmic technique that identifies the point at which two images exhibit the highest degree of overlap. The autocorrelation results in a vertical and horizontal coordinate for each image correlation. These values are then processed to determine the mean coordinates to bring each channel into spatial registry with channel 6, and therefore with each other. The values on the ASSIST tab are reported as the number of pixels required to bring each channel into perfect spatial registry when the raw image file (.rif) file is processed to generate the compensated image file (.cif) file. Values exceeding 0.95 pixel are flagged as errors and will require manual intervention to realign the filter stack assembly. The result and the limits for the calibration are shown below the list when the calibration is selected. Please note, if the 12 channel option is present, this calibration will illuminate and calibrate all 12 channels.

Dark Current Calibration

Measures and stores 3072 offset values corresponding to pixel columns in the TDI camera. Every pixel in a CCD detector is an individual sensor with its own sensitivity characteristics. In the absence of any light, each pixel emits a signal,

known as dark current. Although the statistical variation of any given pixel over time is less than one count, the mean dark current signal generated by any pixel may vary as much as several counts from a different pixel in the array. When the ImageStream^X is measuring very dim signals, even one count difference between pixels can be critical. Therefore, a Dark Current calibration factor is stored for each pixel column. This factor is added to or subtracted from each pixel in the .rif file during .cif creation to normalize detector variation. In the .cif, each pixel is calibrated so that in the absence of light, its signal is 30 counts.
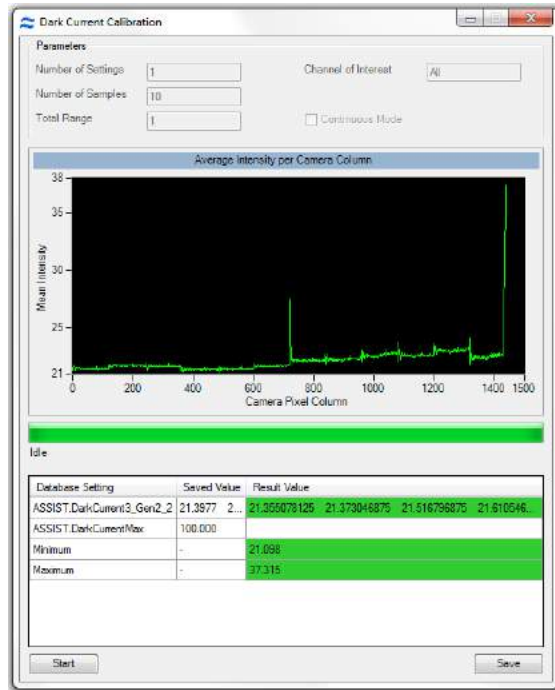

The Dark Current calibration commands the system to turn off the excitation laser and brightfield illumination. The system then measures the mean signal value of each camera column from 1000 rows of data per column. The difference between this value and 30 counts is stored for subsequent correction. When the camera is operated at different stage settings (32, 64, 128, 256 stages) the dark current characteristics of a column of pixels can change. Therefore, values for all stage settings are stored (total of 3072 values). INSPIRE automatically appends the calibration values appropriate for the stage settings used during acquisition to the .rif file. The values reported on the ASSIST tab indicate the maximum variation detected from all test conditions. The result and the limits for the calibration are shown below the list when the calibration is selected. If the 12 channel options is installed the Dark Current calibration will be simultaneously performed for both cameras.

## Brightfield Crosstalk Coefficient Calibration

The brightfield cross talk calibration measures the amount of spectral leakage between channels using the brightfield illuminator. This calibration illuminates each channel individually and characterizes how much light leakage is present in the

remaining five channels. The purpose of this calibration is two fold. First, the spectral leakage values are used to spectrally correct the imagery in IDEAS by removing any Brightfield light leakage from the other five channels. The second purpose is to ensure that the spectral characteristics of the instrument remain constant over time. The Brightfield cross talk calibration will simultaneously calibrate leakage from all eleven channels if the 12 channel option is installed in the instrument.
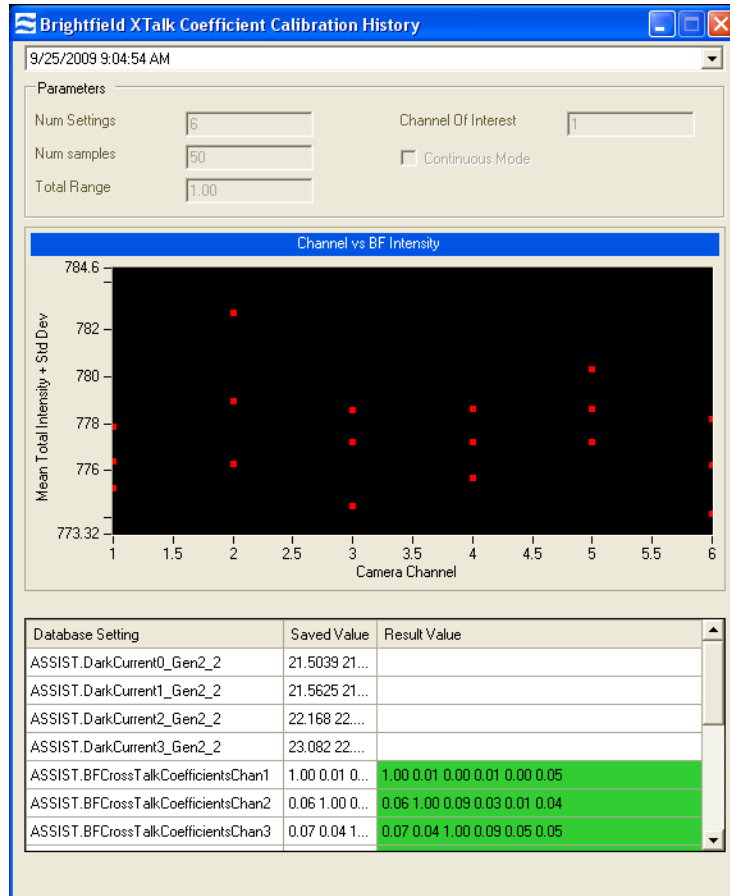

Core Stage Position Calibration

The alignment of the stage in the X direction is controlled so that the position of the core is centered in the field. This calibration finds the core position using the X centroid position of the SpeedBeads and calculates an offset from the factory setting and sets the position of the stage in the X dimension.
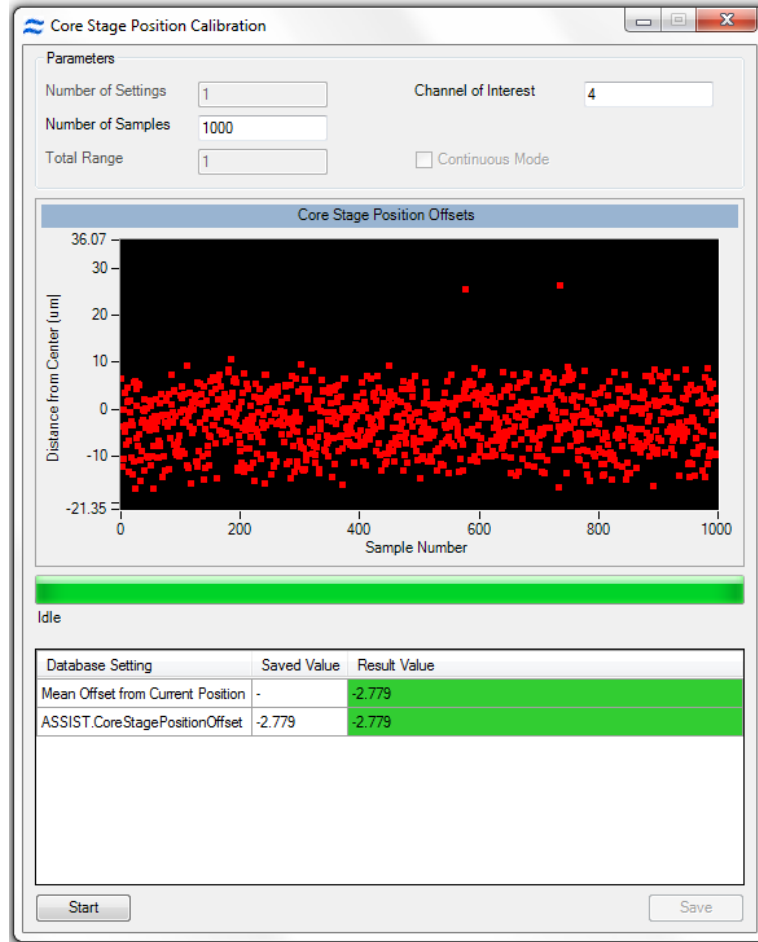
Horizontal Laser Calibrations

The alignment of each laser in the ImageStream^X is automatically controlled to ensure optimal performance via the Horizontal Laser Calibration. The calibration routine sweeps the horizontal position of the laser across the flow stream. At each of 15 predefined intervals during the sweep, 1000 SpeedBead images are collected and analyzed to determine the intensity of each bead. The median intensity for each position is then plotted and fit to a fourth order polynomial. The peak height of the polynomial is then determined. This position is the point where the peak intensity of the Gaussian laser beam intersects the center of the flow core. This position provides both the highest intensity for illuminating the core stream and the point with the lowest coefficient of variation. This position is stored for each laser and used as the default position during subsequent assays.
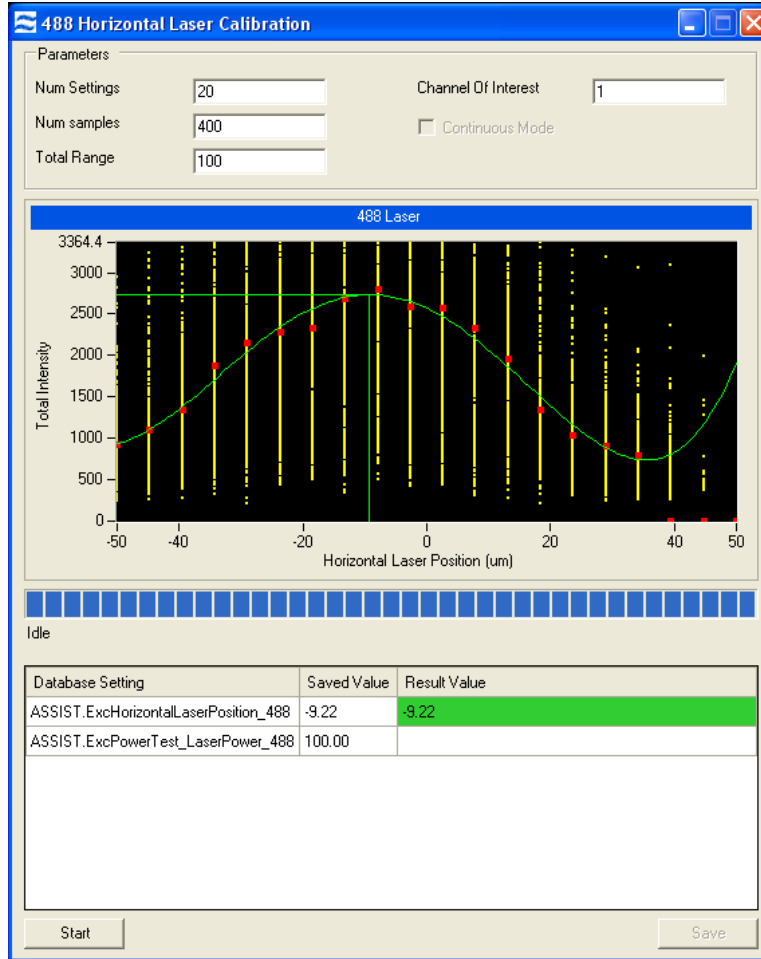
The result for the calibration are shown below the list when the calibration is selected.

Side Scatter Calibration

The purpose of this calibration is to set the power of the 785nm laser. The calibration routine consists of measuring SpeedBead intensities at a predefined power setting and then actively adjusting the power to achieve 7200 counts of light per bead. This calibration ensures a consistent intensity for subsequent ASSIST testing and also ensures a consistent starting position for scatter laser power when analyzing cells.
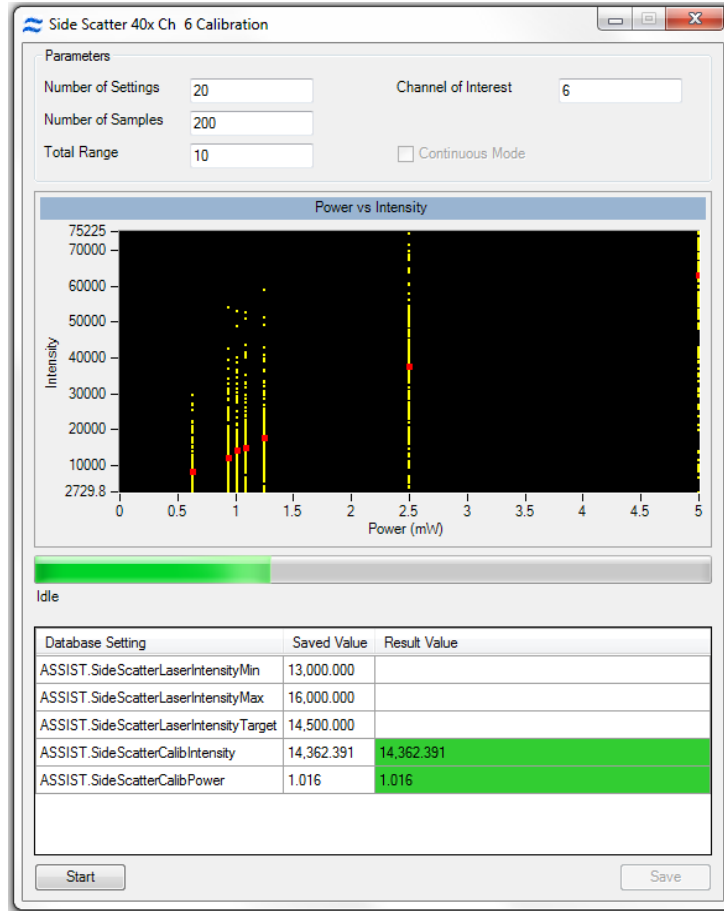
Retro Calibration

The ImageStream^X uses a retro illumination scheme to maximize the amount of light incident on the cell. The vast majority of light incident on the core stream passes through the stream and through cells and other particulates in the stream. The retro illumination system captures this light and redirects it back on to the core stream to double to the total amount of light incident on cells in the stream.

In this calibration, the retro reflective system is panned in manner nearly identical to the Horizontal Laser Calibration. Using the same technique, the optimal position of the retroreflection system is determined to maximize intensity and reduce measurement variation.
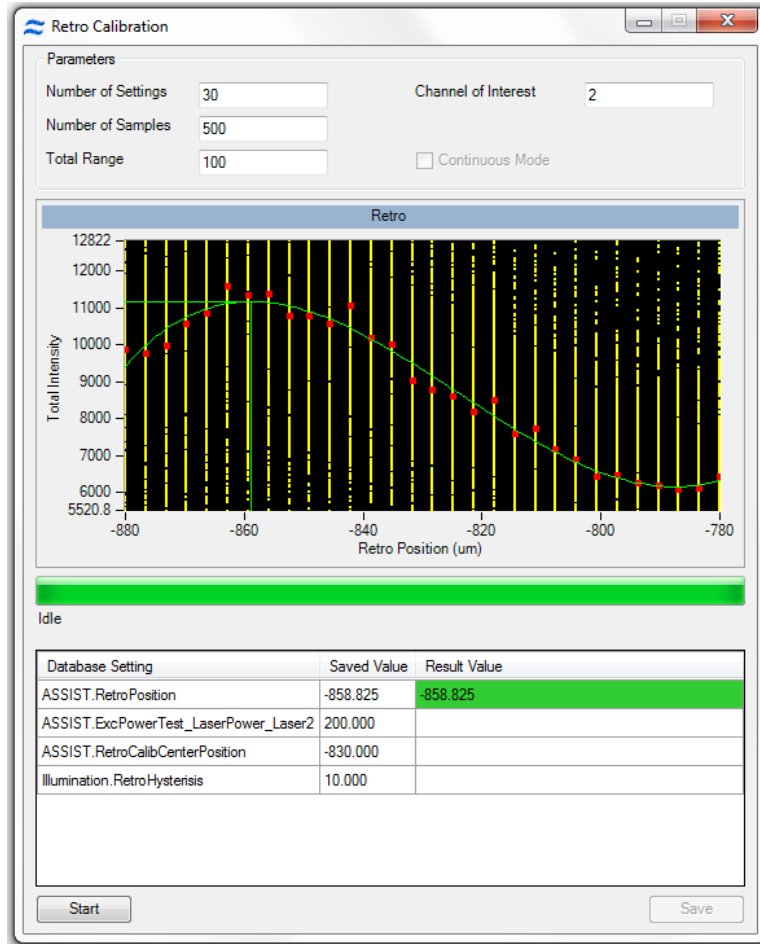
Autosampler Nest Calibration

The ImageStream^X autosampler runs a self calibration. This calibration verifies that the sipper can self-calibrate and find the home position. If the calibration fails or is not run the autosampler will not run.
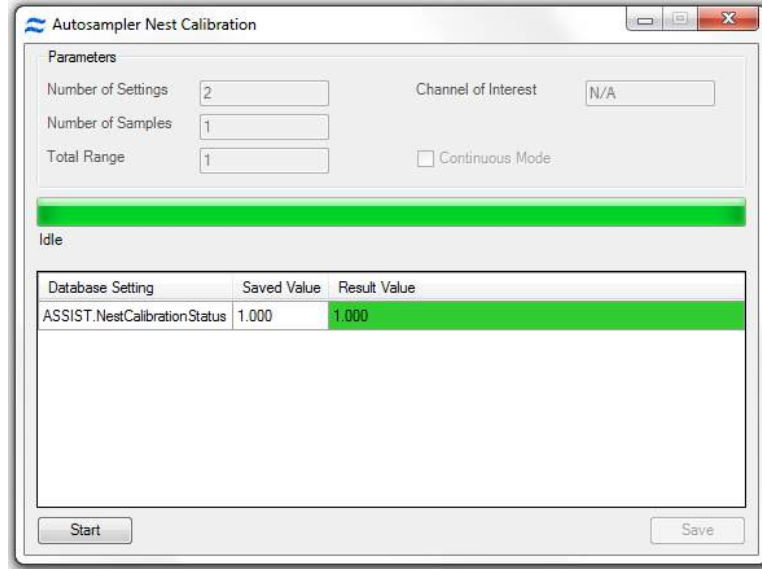
ASSIST Tests

A test is a sequence of operations designed to measure the performance of a specific subsystem. When a test is performed one or more test parameters are generated and evaluated against predefined limits. The test results and acceptable limits are listed on the ASSIST display tab. Values outside of accepted limits are highlighted with a light red background. ASSIST allows complete automated operation of all tests as well as the ability to invoke a single test by clicking a button. The four tests in the current suite are described in detail below.

Excitation Laser Power Tests

The power of each excitation laser present in the system is measured and tested against limits by quantifying the amount of light scattered from SpeedBeads. The instrument is configured specifically to test each laser by adjusting classifiers, setting stage selections and inserting the proper neutral density filters into the collection path. The test compares the mean signal strength acquired from each laser and compares it to radiometric ally calibrated signal strengths collected during the manufacturing process. The intensity of each laser is stored in the database.
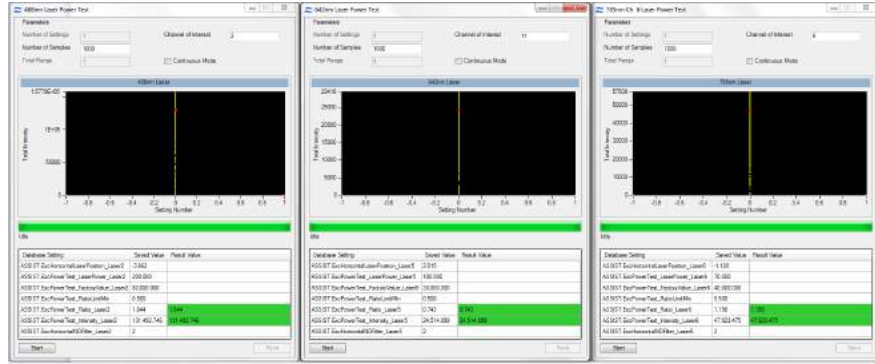
The results and limits of the test are shown below the list when the test is selected.

BF Intensity Selection Test

Verifies the BF intensity calibration for each BF mode. The image intensity must reach 200 within 20 iterations. If this test fails, the user should run the BF Intensity Selection Calibration individually and then re-run the test.
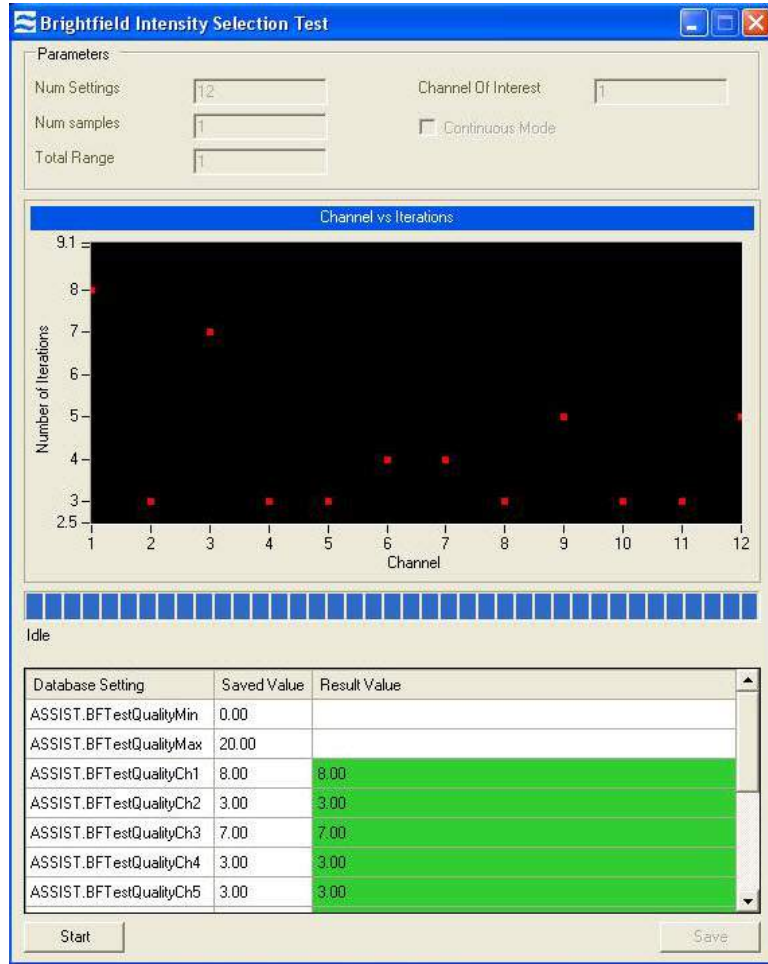
The results and limits of the test are shown below of the list when the test is selected.

BF Uniformity Test

Measures the static and temporal uniformity of illumination in all brightfield channels, channels 1 through 6 (1-12 if the Twelve Channel option is installed). Non-uniformities in illumination can affect segmentation and the accuracy of photometric absorbance measurements made in the brightfield channel. Non-uniformities can be caused by misaligned illumination and collection path elements, degradation of pixel responsiveness and electronic noise. The brightfield uniformity test measures the response from each pixel column the illumination and collection systems are providing a uniform photometric response.
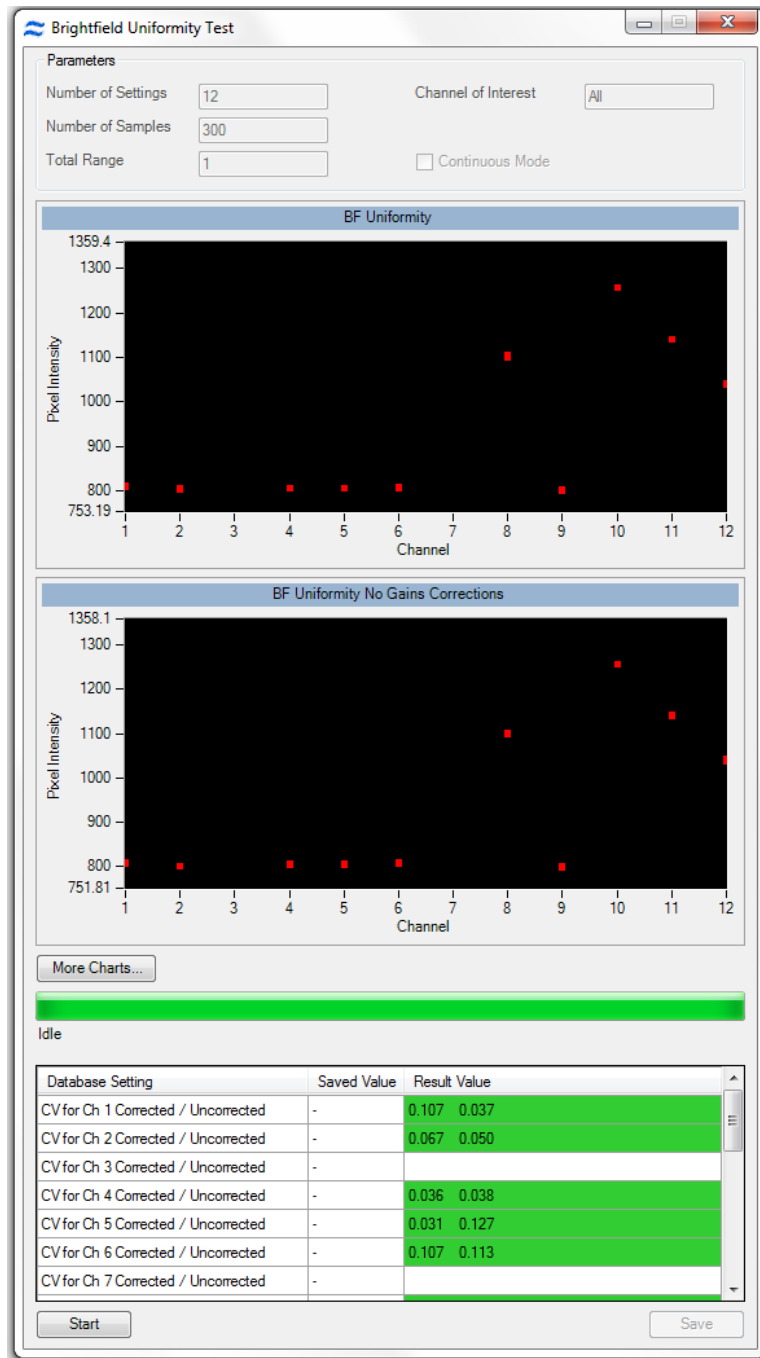
Camera Noise Test

The electronic noise is measured with no illumination to the CCD in two successive frames. The fluctuation is measured on a pixel by pixel basis.
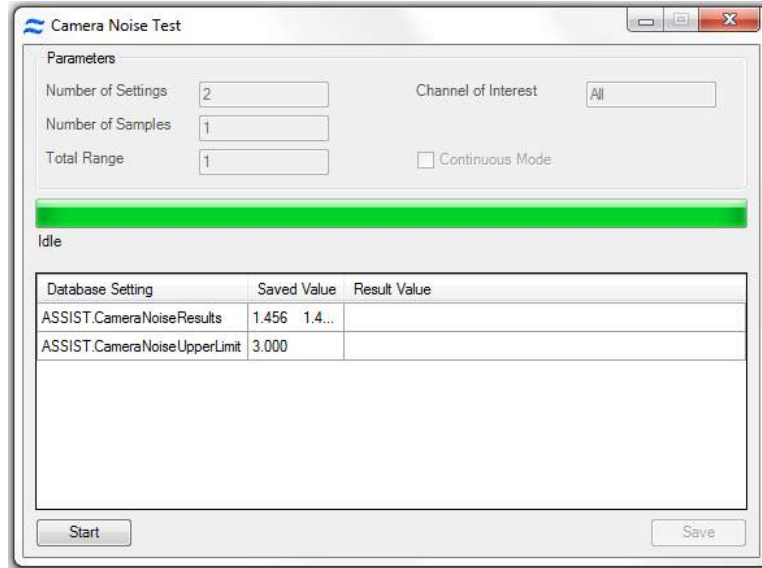
Flow Core Axial Stability Test

Measures the stability of the core stream velocity over time. Measures the variation in the speed of the core stream as a percentage of the mean sample speed. The ImageStream^X is designed to automatically sterilize, cleanse and purge air from its fluidics systems after every day of operation. Improper sterilization, contaminants, partially clogged fluidic lines, air bubbles or non-homogenous sheath solution can lead to excessive sample speed variation. Although the ImageStream^X very accurately measures the sample speed to synchronize camera line rate with cell movement on the detector, excessive speed variation can lead to small amounts of desynchronization. The flow core axial stability test verifies that the fluidic system is operating within normal limits, thereby providing the collection system with hydrodynamically focused objects traveling at a consistent speed for proper image synchronization.
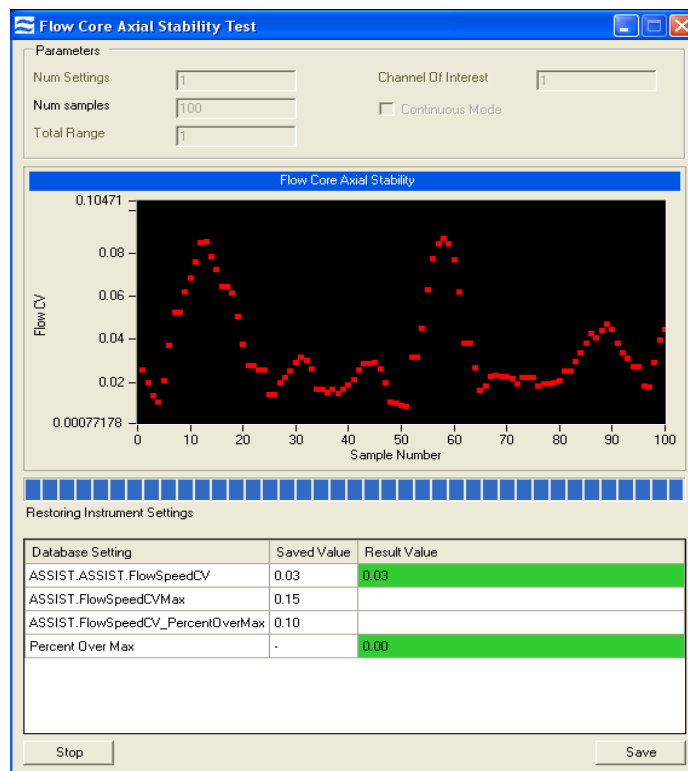
The flow core axial stability test plots 100 flow speed sample intervals, each of which consists of an average velocity measurement of approximately 50 SpeedBeads thereby measuring the speed of approximately 5000 SpeedBeads. The test computes a running average of all measurements which is listed under results on the pop up window and ensures that no more than 5% of all measurements exceed a 0.15% speed variation. This ensures that synchronization is maintained between the imagery and the camera to better than a fraction of a pixel. Test results are stored in the ASSIST database. The results and limits of the test are shown below the list when the test is selected.

Flow Core Lateral Stability Test

Provides a statistical characterization of the stability of the core in the direction lateral to flow. The test computes the centroid position of approximately 3000 SpeedBeads. During the test a histogram of bead centroid position is plotted in the test window. When the test is complete, the standard deviation of bead centroid position (in pixels) is printed in the test window.
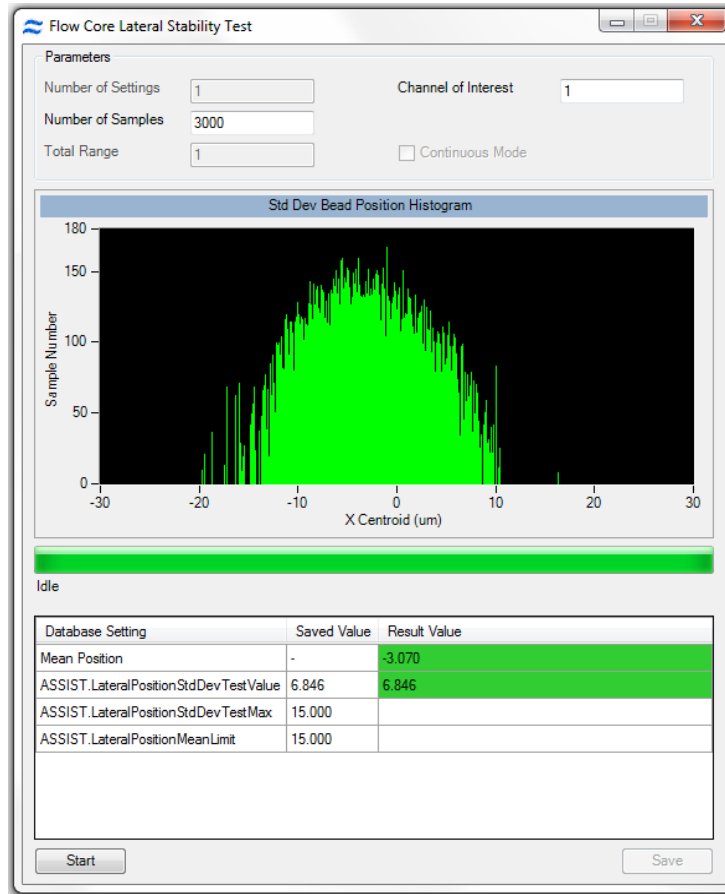
Contaminated sheath, obstructions, air or improper pump function may broaden the core which can reduce focus consistency and increase variation in intensity measurements. This flow core lateral stability ensures the core is operating as designed with minimal variation. Failure to pass this test is indicative of at least one of the issues listed above.

The result and the limits for the calibration are shown below the list when the calibration is selected.

Flow Core Position Test

Measures the position of the core relative to its ideal position within the flow cuvette. The ImageStream^X uses sheath flow to hydrodynamically focus objects within a precise region in the cuvette. Improper sheath solution, protein buildup, micro-bubbles and other factors can alter the position of the core within the cuvette. If this occurs, the photometric and morphological measurement repeatability may degrade. This test measures the current core position and compares it to the ideal location of the core as determined in the manufacturing process. The deviation from the ideal position is reported in microns and stored in the ASSIST database.
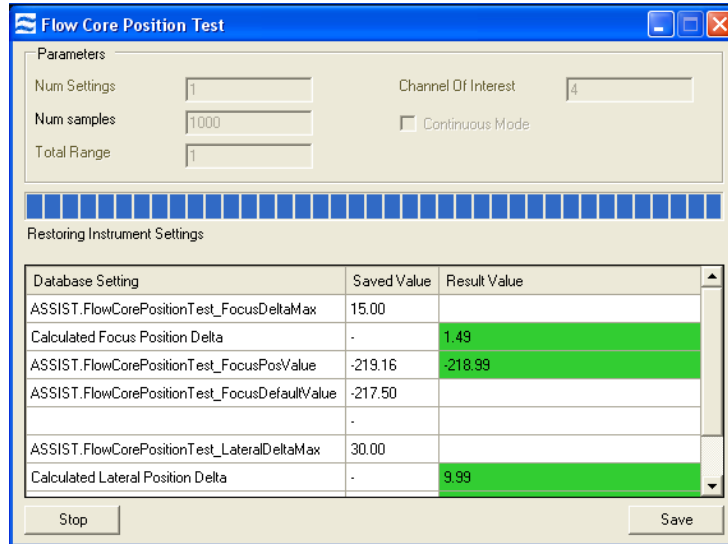
The result and the limits for the calibration are shown below the list when the calibration is selected.

Focus Offset Beads Test

Measures the offset between the focus determined by the AFFS system and location of the peak response of the Image Collection system. This test performs a pan through focus while simultaneously collecting SpeedBead focus data from the AFFS system and SpeedBead image data from the image collection system. The AFFS data are processed to find the zero crossing (point of no defocus) and the image data are processed to determine the peak response (point of highest spatial resolution). Both sets of data are plotted as a function of Z position along the horizontal axis. The AFFS zero crossing and image collection system peak response are indicated vertical lines and numerical results are reported to the Focus Offset test tab. The difference (in microns) between these two positions is determined and compared against predetermined limits and stored in the ASSIST database. If the MultiMag option is installed, a focus offset test will be performed for each magnification.
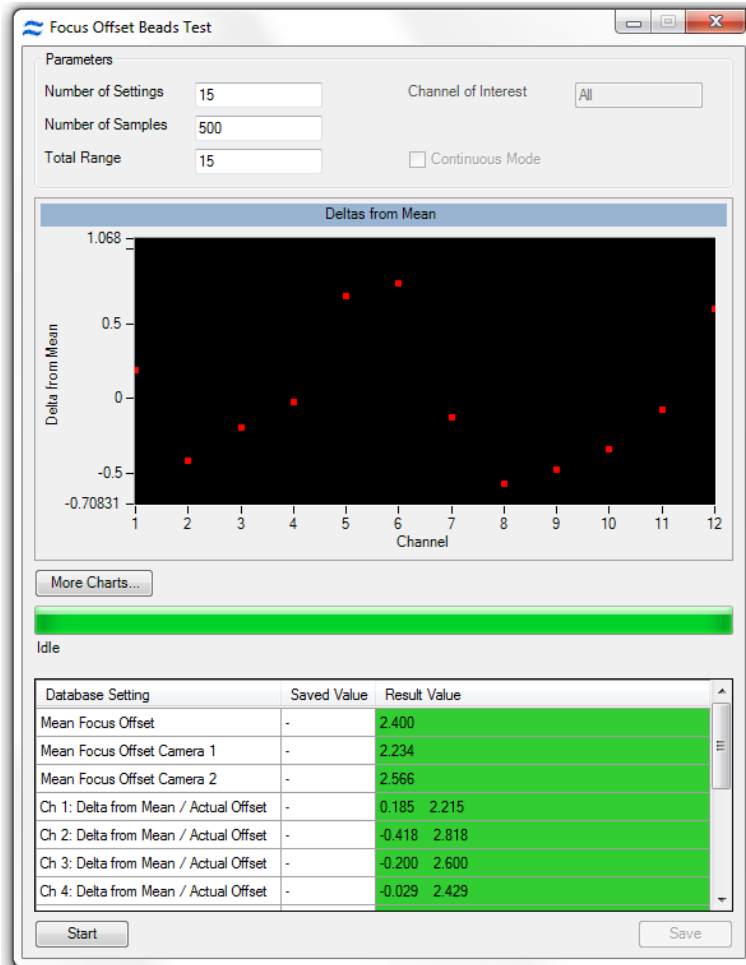
Focus Percentage Test

Measures the percentage of SpeedBeads in focus and sets a limit of 90%.
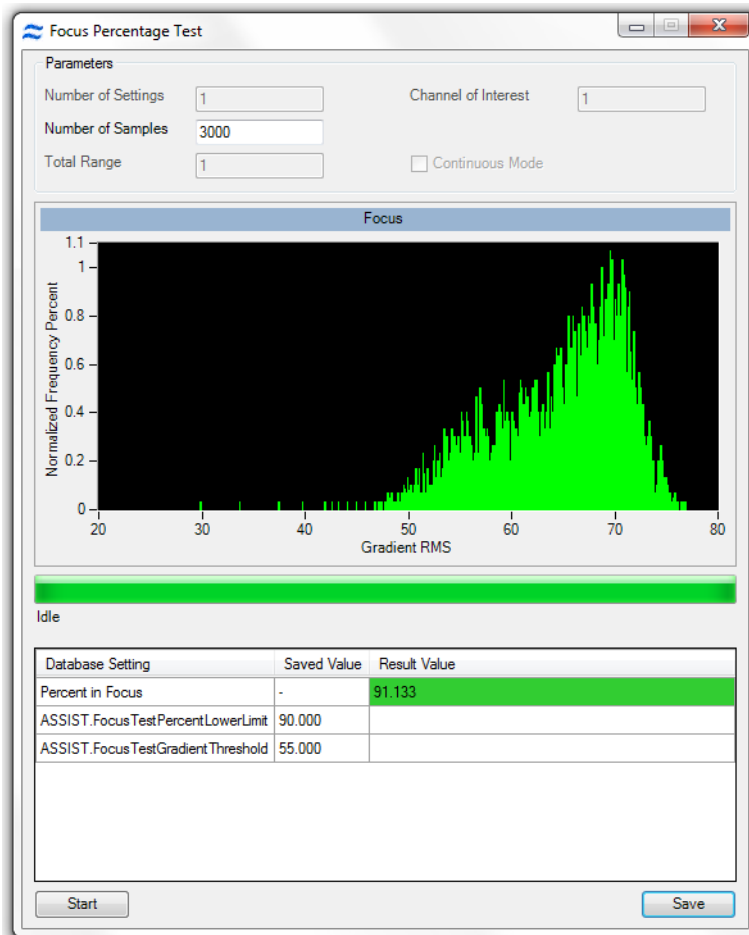
Focus Uniformity Test

Measures the best focus position for every channel and then calculates the difference of each channel from the mean for all channels. The tolerance for focus uniformity ensures that all channels are in optimal focus.
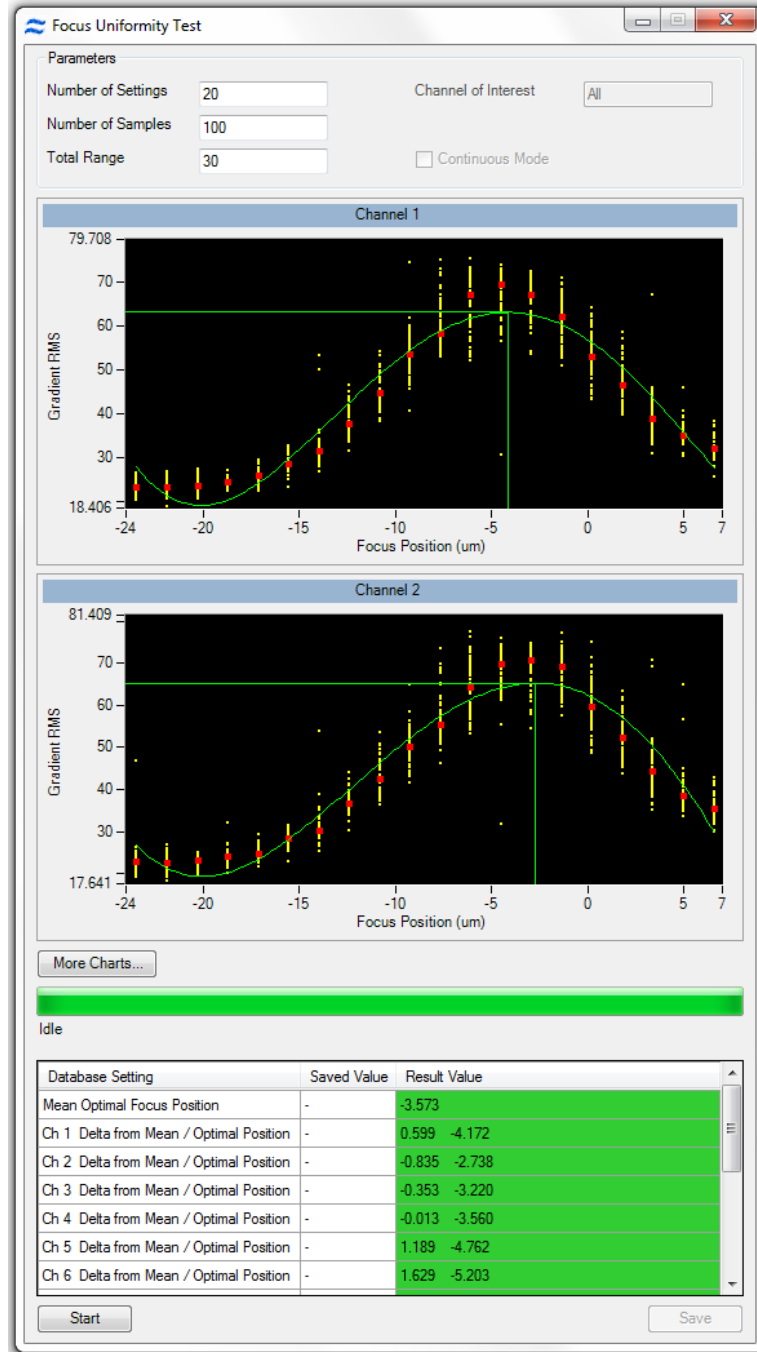
Image Quality Ensquared Energy Test

Measures the ability of the optical system to resolve fine details in the image using ensquared energy ratio. The optics term **ensquared energy** refers to a measure of concentration of energy in an optical image when quantifying image sharpness for digital imaging cameras using pixels. The ensquared energy ratio is one of several parameters often used in the design of high resolution optical systems to characterize their performance. In this ASSIST test, the ensquared energy ratio of a 3x3 pixel array centered within an 11x11 pixel array is determined and compared against predetermined limits. The test is designed to measure the optical quality of the image independent of focus, lateral core stability, and axial core stability. During the test approximately 5000 SpeedBead images are collected over a range of focus positions. The imagery is analyzed during collection by computing the ensquared energy ratio in each image. The ensquared energy for each image at each focus location is shown in a plot. The mean ensquared energy for each focus position is noted as a dark blue data point for each focus position. The ensquared energy for the top 2% of all imagery is computed and indicated as a dark blue data point on the plot. This result is tested against predetermined limits and reported on the Collection Image Quality test tab and in the popup window. This value is stored in the ASSIST database.

A highly magnified composite image of the top 2% of all images is also generated and displayed on the popup window. Each small square of light is an individual pixel approximately 0.5 microns on a side (in object space). This image generally shows a small amount of "flair" on the right hand side. This is due to light scatter from the far side of the SpeedBead which is approximately 1um in diameter.
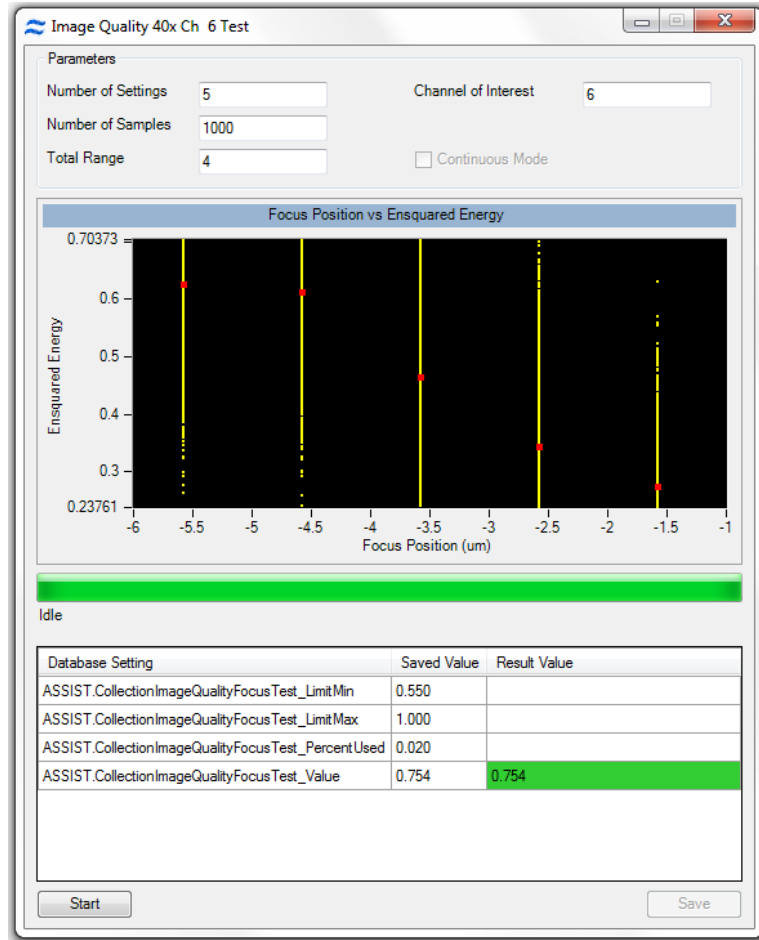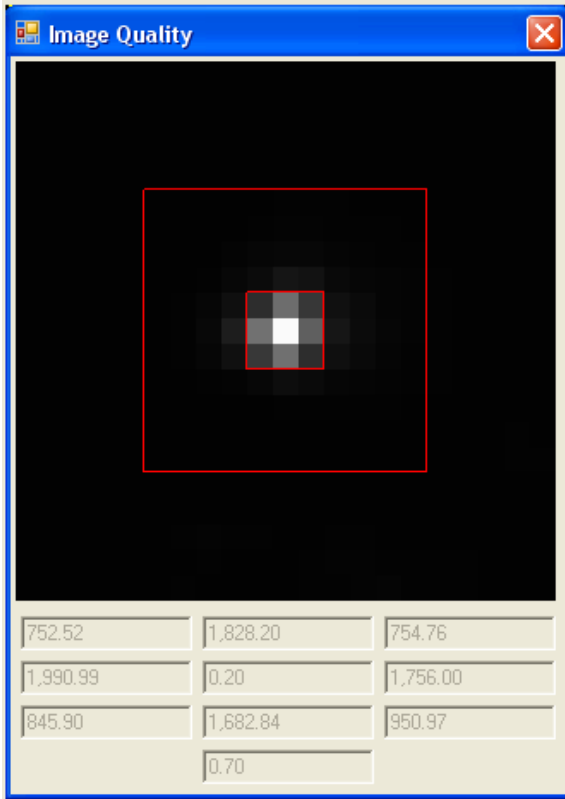
The test also reports regional scores which are not tested against limits. The scores include the energy ratios for line profiles in the horizontal and vertical axes, displayed at the bottom of the regional score grid, and summed energy values for the horizontal, vertical and diagonal directions radiating outward from the center of the image. The summed energy values are displayed in a 3 x 3 array. The value in the center of the array is the ensquared energy ratio for the single pixel in the center of the image. If the MultiMag option is installed, and ImageQuality Ensquared energy test will be performed for each magnification.

Chapter 5: Troubleshooting

This section is designed to help you troubleshoot the operation of the ImageStreamX-Mark II. If additional assistance is required, contact the Amnis service department.

System

- Unstable fluidics (Air or clog in system)
- Fluidics respond sluggishly
- Event rate slows over time
- Event rate is slower than expected
- Cross-contamination from previous samples
- Erroneous fluid level indicator
- Instrument will not pass ASSIST
- Compensation wizard fails to complete

Software

- INSPIRE appears to freeze
- INSPIRE Fails to launch
- Plots fail to update, or update slowly
- Data file fails to collect

Image

- No images
- Imaging and acquisition rate is erratic, or appears frozen
- Objects appear streaked
- Objects are not centered in the channel
- Objects are rotating in the core stream
- Objects are out of focus or distorted
- Objects are cropped
- The two brightfield images are not of the same cell
- Images appear pixelated or larger than normal
- Objects appear larger or smaller than normal
- Not all 12 channels are being displayed
- Images have incorrect colors

Intensity

- Fluorescence imagery appears too dim
- Fluorescence is too bright, images have a contrasting color or appear flat

- One channel saturates while the others do not
- Scatter is too dim or bright or changes over time
- Large variation in brightfield intensity levels
- Brightfield intensity level sets incorrectly

System

Symptom	Possible Causes	Recommended Solutions
Unstable fluidics (Air or clog in system)	Air bubbles in the sample	Make sure a sufficient sample volume is used. To clear the air bubble: Run the purge bubbles script.
		Detergents and foaming agents (such as FBS) can cause bubbles to form in the lines. If these buffers are causing air in the system remove them from the sample and resuspend in dPBS. Run the purge bubbles script.
	Air bubbles in fluid lines	Run the sterilize script, followed by the startup script. Load calibration beads and verify the system runs normally.
	Clog in fluid lines	Filter the sample with a 70um nylon cell strainer. Run the sterilize script, followed by the startup script. Load calibration beads and verify the system runs normally.
	Sample is too concentrated	Clumpy and viscous samples cause cavitation in the fluidic lines and create bubbles. Dilute the sample to 1×10^7 cells/ml and strain the cells through a 70um nylon mesh. Run the purge bubbles script.
	Inappropriate sheath solution	Verify the sheath is dPBS. De-gas the sheath as appropriate. Third party sheath buffers cannot be used.
	Fluid lines are leaking	With the system powered down look for leaking sheath. Verify the fluid lines mount snuggly into position. Call Amnis service.
	SpeedBeads fail to run	Verify the beads will run by returning any sample, going to fluidics section and press stop, then run. Next go to the advanced drop down, select flow speed, and check that the red and black histograms have tight CVs at the appropriate core velocity. To view bead images select the All population and check include beads.

Symptom	Possible Causes	Recommended Solutions
Fluidics respond sluggishly	Air buffer in the sheath syringe is not correct	The sheath syringe should contain 2-4 ml of air to buffer the movement of the pump's microstepper motor. If too little air is present run the "start-up" script.
	Fluid lines are leaking	With the system powered down look for leaking sheath. Verify the fluid lines mount snuggly into position. Call Amnis service.
Event rate slows over time	Cells have settled in the lines	Cells settle in the lines after 45-60 minutes of running, resulting in a drop in cell event rate. Stop and save the acquisition. Return the remaining sample, restore the sample volume to 30ul and re-load the sample to continue acquisition. Data can then be appended together in IDEAS.
	There is a clog or air bubble in the system	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
	Sample syringe is empty	Load a fresh sample
	Sheath syringe is empty	Load sheath, then go to the instrument drop down and run prime.
	Fluid lines are leaking	With the system powered down look for leaking sheath. Verify the fluid lines mount snuggly into position. Call Amnis service.
Event rate is slower than expected	Sample concentration is low	Make sure the sample concentration is between 10^7 and 10^8 cells/ml. Lower concentrations can be used but this will decrease the cells/second.
	Core is off center	Cropped images will be eliminated from data acquisition and if enough of the images are cropped the event rate can appear lower than normal. Normally this is due to air in the system. Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
	Insufficient	Turn the appropriate lasers on. Set the

Symptom	Possible Causes	Recommended Solutions
	illumination	785 SSC laser to 40mw. Set the laser powers to maximum and decrease them to prevent pixel saturation.
	Cells are not displayed due to over clipping.	For large diameter cells go to the advanced drop down, select acquisition and check the box labeled keep clipped objects.
Cross-contamination from previous samples	DNA dye from previous sample is labeling current sample	DNA dyes must be thoroughly flushed from the sample lines, to prevent residual dye from labeling subsequent samples. Load a sample of 10% bleach followed by a PBS wash, to remove all traces of the DNA dye in the instrument, or run the sterilize script (~30min).
	Cells from the previous sample are appearing in current sample	This suggests a minor clog. Load a sample of 10% bleach followed by a PBS wash to remove most contaminating cells, or run the sterilize script (~30min).
Erroneous fluid level indicator	Tank has moved away from the sensor	Open the buffer compartment and move the tank closer to the sensor until the fluid level indicator is correct.
	Sensor is broken	Power down and power up the instrument, if this does not fix the problem, call Amnis service.
Instrument will not pass ASSIST	Incorrect template loaded	Go to the file drop down and select "load default template". Re-run ASSIST.
	SpeedBeads fail to run	Verify the beads will run by returning any sample, going to fluidics section and press stop, then run. Next go to the advanced drop down, select flow speed, and check that the red and black histograms have tight CVs at the appropriate core velocity. To view bead images select the All population and check include beads.
	SpeedBeads are not running properly	The particles must be running >1000 events per second, and without significant clumping. If the beads are diluted or clumped, try running a fresh

Symptom	Possible Causes	Recommended Solutions
		tube of beads. If the problem persists there may be a fluidics issue, see the Flow rate stops or slows over time section.
	Calibration and/or test failure	Tests may fail if the system is reloading sheath, or failed to set up properly. Re-run the test by clicking in the box next to the test, and pressing the start button in the popup window. If the test fails three times in a row, call Amnis service.
	Focus adjustor calibration failure	Verify brightfield is working properly.
	Frame Offset calibration failure	Verify brightfield is working properly.
	Spatial Offsets calibration failure	Verify brightfield is working properly.
	Dark Current calibration failure	Make sure the excitation lasers are off and brightfield is blocked. Completely power down the instrument and power back up to re-run the test.
	Brightfield XTalk calibration failure	Verify brightfield is working properly, and that spatial offsets passed.
	Horizontal Laser Calibration failure	Verify the laser turns on and can set power properly. Completely power down the instrument and power back up to re-run the test. Verify spatial offsets passed.
	Retro Calibration failure	Verify the laser turns on and can set power properly. Verify spatial offsets and frame offsets passed.
	Side Scatter Calibration failure	Verify the 785 SSC laser turns on and can set power properly. Completely power down the instrument and power back up to re-run the test. Verify spatial offsets passed.
	Laser Power test failure	Verify the laser turns on and can set power properly. Completely power down the instrument and power back up to re-run the test. Verify spatial offsets and frame offsets passed.

Symptom	Possible Causes	Recommended Solutions
	Brightfield alignment test failure	Verify brightfield is working properly.
	Brightfield uniformity test failure	Verify brightfield is working properly.
	Camera noise test failure	Verify camera can image properly. Completely power down the instrument and power back up to re-run the test.
	Flow Core Axial Stability test failure	Verify the reagent buffers are full. Run the sterilize script followed by the startup script, and re-run the test. See solutions for unstable fluidics.
	Flow Core Lateral Stability test failure	See solution above ([Flow core Axial stability test](#)).
	Flow Core Position test failure	See solution above ([Flow core Axial stability test](#)).
	Focus Percentage test failure	See solution above ([Flow core Axial stability test](#)).
	Focus Uniformity test failure	See solution above ([Flow core Axial stability test](#)).
	Image Quality test failure	See solution above ([Flow core Axial stability test](#)).
Compensation wizard fails to complete	The region to collect was set incorrectly	In the wizard verify that 1,000 of "All" cells (or of a region drawn on the appropriate population) are being collected.
	Too many objects are being collected	Set the events to acquire less than 1,000.
	Cells are not fluorescent	Make sure that the compensation control sample has more than 10% positive events, and are as bright as possible. IgG capture beads or a cell line stained with a single fluorochrome may be used for comp controls.
	Cells are stained with more than one fluorochrome	Compensation controls must be a sample with a single fluorochrome label in a single tube. Each fluorochrome must be run separately.

Software

Symptom	Possible Causes	Recommended Solutions
INSPIRE appears to freeze	Camera is not running	Click Run/Setup.
	If the camera is already running	Click Stop then Run/Setup
	Imaging is paused	Click Resume.
	No objects in the current image view mode	In the cell view area, select the all population.
	A script is running	Wait until the script completes, or if necessary, click Abort Script to prematurely stop the operation.
	The INSPIRE application has crashed	Open the Windows Task Manager by pressing <Ctrl + Alt + Del>. Click the Applications tab. If INSPIRE is 'Not Responding', select the INSPIRE task and click End Now. Restart the INSPIRE application by double clicking the icon on the desktop. If the program restarts, make sure the lasers and brightfield lamp are turned on and then re-establish the core stream. If the application does not start, use the Windows Task Manager to end the INSPIRE task again. Shut the instrument and computer down from the Start menu. Then turn on the instrument as described. If a crash occurs during the day, a complete shutdown is recommended at the end of the day, before running sterilize.
INSPIRE fails to launch	Splash screen is not responding	On the keyboard press Ctrl-Alt- Delete, open the task manager, select INSPIRE and press end task. Wait 60 seconds and try restarting INSPIRE.
	Loss of communication between the computers and instrument.	Shut down the computer, and power off the instrument. Verify all computers are off. Power on the instrument and the computer, wait 5 min and try launching INSPIRE.

Symptom	Possible Causes	Recommended Solutions
Plots fail to update, or update slowly	Computer resources are being over used	Close all third party software.
	Too many plots in the template	For optimal plot update rates limit the number of plots to 15.
	Parent population has no qualifying events	Right click on the plot, select graph properties, and change the selected population to "all" or a population that has qualifying events.
	Plots are scaled incorrectly	In the plot tool bar, press the – magnifying glass and rescale the plot.
Data file fails to collect	No events qualify for the region	Make sure there are events going into the collection region by viewing that region in the image gallery and updating the acquisition collection population appropriately.
		Verify the cell concentration is appropriate. 1x10^7 cells/ml is ideal.
	Computer hard drive is full	Verify the computer hard drive has sufficient room to save the data file. To do this go to Start / Computer / right click on properties and a pie chart showing how much disk space is available is displayed. Backup and delete data to free up disk space.
	Data file collected rapidly	Some samples have high concentrations and acquire faster than the display rate. Check the destination folder and see if the raw data was collected.
	File directory was lost	Collecting data over a downed network or changing the name of the destination folder will cause the instrument to lose the data directory. Verify the data destination folder is accessible using the browse button in the Acquisition Settings section.
	No .rif or .fcs file was created	Go to the file drop down menu and check Generate .rif and or .fcs file.

Image

Symptom	Possible Causes	Recommended Solutions
No Images	Camera is not running	Click Run/Setup.
	If the camera is already running	Click Stop to stop the camera, and then click Run/Setup.
	Imaging is paused	Click Resume.
	Displayed region is incorrect	In the cell view area, select the all population.
	Insufficient illumination	Turn the appropriate lasers on. Set the 785 SSC laser to 40mw. Set the laser powers to maximum and decrease them to prevent pixel saturation.
		Make sure the brightfield lamp is turned on and click Set Intensity.
	Core stream is outside the objective's field of view	Manually find the core stream. In the focus and centering section, move core track left or right to find the core.
	Computer resources are being over used	Close all third party software.
Imaging and acquisition rate is erratic, or appears frozen	Sample concentration is low	Make sure the sample concentration is between 10^7 and 10^8 cells/ml. Lower concentrations can be used but this will decrease the cells/second.
	Region being viewed has few or no cells	In the cell view area, select the all population, or readjust regions to include more cells.
	Insufficient illumination	Turn the appropriate lasers on. Set the 785 SSC laser to 40mw. Set the laser powers to maximum and decrease them to prevent pixel saturation.
		Make sure the brightfield lamp is turned on and click Set Intensity.

Symptom	Possible Causes	Recommended Solutions
	The sample is too concentrated	The process of object detection can safely handle up to 4000 objects per second. The maximum sample concentration is 4-5x10^8 cells per ml, with the recommended concentration 1-10 x10^7 cells per ml. To decrease the event rate, dilute the sample.
	The sample has an excessive amount of debris	Use a region to eliminate the debris from the data file, or prepare a fresh sample.
	Computer resources are being overused	Exit all third party programs.
Objects appear streaked	Camera is not tracking the cell velocity accurately	Verify brightfield is working normally and rerun ASSIST using calibration beads. See solutions for unstable fluidics.
	SpeedBeads fail to run	Verify the beads will run by returning any sample, going to fluidics section and press stop, then run. Next go to the advanced drop down, select flow speed, and check that the red and black histograms have tight CVs at the appropriate core velocity. To view bead images select the All population and check include beads.
	There is only one channel of brightfield	Verify that there are two brightfield channels. Check in the image display properties that 1 and 9 are active, verify that brightfield is emitting in channels 1 and 9.
Objects are not centered in the channel	Lateral deviation of the core stream due to air or clog in the system	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
	Autofocus and centering is not tracking properly	In the Focus and Centering section, adjust focus and centering left or right, until the images are centered and in optimal focus.

Symptom	Possible Causes	Recommended Solutions
Objects are rotating in the core stream	Core stream position is grossly off-center within the flow cell due to air or clog in the fluidics	The core tracking and focus tracking should not change significantly from day to day. If either value changes radically, objects may rotate due to interactions with the sheath. An off-center core stream is caused by air or clogs in the fluidic system. Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
Objects are out of focus	Camera line rate is incorrect	Re-run the focus adjuster and frame offset calibration in ASSIST, and verify it passes.
	Excessive core stream variation due to air or clog in the fluidics	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
	Core stream is moving too fast for the camera	Allow the system to settle for 60 seconds after loading a sample. Collect data once imagery looks good.
	Autofocus is not tracking properly	In the Focus and Centering section, adjust focus and centering, left or right, until the images are centered and in optimal focus.
	Data is binned	Turn off bin mode by selecting fluidics, and set the slider bar to low speed high sensitivity. Verify this change in the advanced drop down by selecting camera and verifying a 1x bin mode.
	EDF is on	In the magnification and EDF section uncheck the EDF checkbox.
Objects are cropped	Lateral deviation of the core stream due to air or clog in the system	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
	Autofocus and centering is not tracking properly	In the Focus and Centering section, adjust focus and centering left or right, until the images are centered and in optimal focus.
	Incorrect	In the magnification and EDF section

Symptom	Possible Causes	Recommended Solutions
	Magnification	choose a lower magnification.
The two brightfield images are not of the same cell	Frame offset is incorrect	Run SpeedBeads by returning any sample and then sopping and running fluidics. Load the default template and verify brightfield is in channel 1 and 9 at 800 counts of background. Open ASSIST re-run the frame offset calibration routine, and verify it passes.
	Illumination is grossly misaligned	Call service, and verify that the illumination pathways are in proper alignment.
	Cross correlation is incorrect	Run SpeedBeads by returning any sample and then sopping and running fluidics. Load the default template and verify brightfield is in channel 1 and 9 at 800 counts of background. Open ASSIST, re-run the cross correlation utility, and verify it passes.
Images appear pixelated or larger than normal	Image gallery zoom is active	Use the – magnifying glass to zoom out and restore the native image size.
Objects appear larger or smaller then normal	Incorrect magnification	In the magnification and EDF section choose an appropriate magnification for your cell type.
Not all 12 channels are being displayed	Image gallery zoom is active	Use the – magnifying glass to zoom out and restore the native image size.
	Channel is not activated	To activate a channel for acquisition, click on the channel column heading (i.e. Ch2) and check the "collected" check box to save that channel.
Images have incorrect colors	Image gallery display is set up incorrectly	Click on the channel column heading (i.e. Ch2) and set the display and channel color for the channel.

Intensity

Symptom	Possible Causes	Recommended Solutions
Fluorescence imagery appears too dim	Image display settings are set too low	Increase the image display gain by clicking on the channel column, selecting the appropriate channel, and moving the right green handle bar to a smaller value, or the brightest pixel in the histogram. To set the display background to black move the left green handle bar to the dimmest pixel in the histogram.
	Sample did not label well	Look at the sample with a fluorescent microscope.
	Insufficient illumination	Turn the appropriate lasers on. Set the laser powers to maximum and decrease them to prevent pixel saturation.
		If the probing protocol results in dim staining, sensitivity of the instrument can be increased by changing the fluidics speed to Lo / Hi sensitivity mode.
	Core stream position is grossly off-center within the flow cell due to air or clog in the fluidics	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
	Excitation laser is misaligned	Run calibration particles on the Flow Sight. Load the default template. Open ASSIST, re-run the laser alignment calibration for the appropriate laser line, and verify it passes.
Fluorescence is too bright, images have a contrasting color or appear flat	Image display settings are set too high	Decrease the image display gain by moving the right hand green handle bar to a higher value for the appropriate camera channel.
	Instrument sensitivity is set too high	Decrease the excitation laser power to prevent pixel saturation. Saturation is indicated in the image gallery by pixels colored in a contrasting color

Symptom	Possible Causes	Recommended Solutions
		(generally red or white).
		Set the brightfield intensity to 800 counts by pressing "Set Intensity".
	The sheath syringe is empty	Load sheath, then go to the instrument drop down and run prime.
	There is a clog or air bubble in the system	Run the Purge Bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
One channel saturates while the others do not	Instrument sensitivity is not optimized	The best instrument setup maximizes the dynamic range of fluorescence signal, while at the same time avoiding image pixel saturation (which cannot be compensated). In general decreasing the laser powers until no pixels saturate.
	Probing protocol requires better stain balance	Reduce the concentration of the stain that produces the saturating signal so that all probes can be simultaneously imaged without excessive saturation.
	Excessive fluorescent dye is left in the sample buffer.	Some DNA dyes are required to run with the sample to stain properly, however if too much dye is in solution it can cause the core stream to fluoresce. It's important to balance the concentration of these dyes so that the cells can be imaged properly. Typically the concentrations in "Current Protocols in Cytometry" should work.
Scatter is too dim or bright or changes over time	Instrument is experiencing large temperature variation	Allow the instrument to warm up by running for 15 minutes.
		Direct a fan toward the back of the instrument to dissipate excess heat, or move the system to a temperature controlled environment.
	Laser power set too high or low	Increase or decrease the 785 SSC laser power.

Symptom	Possible Causes	Recommended Solutions
	Core stream position is grossly off-center within the flow cell due to air or clog in the fluidics	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
Large variation in brightfield intensity levels	Large flow speed variation due to air	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
	Light source delivering variable output	Power down and power up the instrument, if this does not fix the problem, call Amnis service.
Brightfield intensity level sets incorrectly	Intensity set before desired flow speed has been achieved	Allow the system to stabilize after loading a sample, and then click Set Intensity.
	Movable optics are out of position	Power down and power up the instrument, if this does not fix the problem, call Amnis service.

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