



# INSPIRE™ ImageStream®X MkII System User's Manual



**RUO**

For Research Use Only. Not for use in diagnostic procedures.

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# Chapter 1: System and Software Introduction

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## Introduction

Read the entire manual before using the system and software.

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## Instrument Overview

The ImageStream®X MkII instrument 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 MkII 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 MkII system, fluorescence intensity measurements are acquired as with a conventional flow cytometer; however, the best applications for the ImageStream®X MkII 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 ImageStream®X MkII system includes the ImageStream®X MkII multispectral imaging flow cytometer and the INSPIRE™ and IDEAS® software applications.

## Software Overview

The INSPIRE™ software is integrated with the ImageStream®X MkII and is used to run the instrument. INSPIRE also provides tools for configuring the ImageStream®X MkII, 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 MkII multispectral imaging flow cytometer.

## Consumables



### Sterilizer, Cleanser, and Debubbler

These recommended reagents have been formulated to optimize the performance of the ImageStream®X MkII 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 <sup>++</sup> Mg <sup>++</sup> free	Millipore	BSS-1006-B (1X) 6506- (10X)
Rinse	deionized water		

This information is 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 MkII, 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.

Two bottles are provided:

- Sheath — To be filled with phosphate buffered saline (PBS) with no surfactants for running samples.
- Rinse — To be filled with deionized (DI) water for rinsing the instrument during shutdown.

Fluid is drawn from these two 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.

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## Luminex Technical Support

Contact Luminex Technical Support by telephone in the U.S. and Canada by calling: 1-877-785-2323

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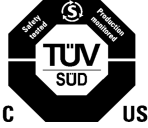



Email: [support@luminexcorp.com](mailto:support@luminexcorp.com)

Additional information is available on the website. Search on the desired topic, navigate through menus. Also, review the website's FAQ section. Enter <http://www.luminexcorp.com> in your browser's address field.

This manual can be updated periodically. To ensure that you have a current version, contact Technical Support.

# Symbols Glossary

You may encounter these symbols throughout this manual. They represent warnings, conditions, identifications, instructions, and regulatory agencies.

Symbol	Meaning	Symbol	Meaning	Symbol	Meaning
	Date of manufacture		Serial number		WEEE Symbol
	Use-by date		Temperature limit		Manufacturer
	Catalog Number		Caution		Consult instructions for use
	For Research Use Only. Not for use in diagnostic procedures		Contains Sufficient for <n> Tests	-	-
	Laser warning		Batch Code		Biological risks
	Hand Crush / Force From Above		Caution, possibility of electric shock		Caution, hot surface
	Alternating Current		TÜV SÜD NRTL Certification Mark		Stand-by Power on/off
	Protective earth; protective ground		Keep Dry	-	-

# Chapter 2: Regulatory and Safety Considerations

## Description

The ImageStream®X MkII is a bench top multispectral imaging flow cytometer designed for the acquisition of up to 12 channels of cellular imagery and fluorescence intensity. By collecting large numbers of digital images per sample and providing numerical representation of image-based features, the ImageStream®X MkII combines the per cell information content provided by standard microscopy with the statistical significance afforded by large sample sizes common to standard flow cytometry. The ImageStream®X MkII system is for research use only and is not for use in diagnostic procedures. The ImageStream®X MkII is for indoor laboratory and professional use only.

## Safety Testing and Certifications

The ImageStream®X MkII System has been tested by an Occupational Safety and Health Administration (OSHA) Nationally Recognized Testing Laboratory (NRTL) which is also an Accredited Certification Organization by the Standards Council of Canada.

## Regulatory and Safety Labels

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 the hood latch release:





The following laser warning label appears on the exterior of the back panel of the instrument:



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## Warnings and Precautions

In any situation that you encounter a symbol shown below, consult this manual or other Luminex documentation to determine the nature of the potential hazard and any necessary actions you should take.

### General Safety

The ImageStream®X MkII imaging flow cytometer is manufactured by Luminex 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.

### Mechanical Safety



The system has parts that move during operation. Risk of personal injury is present. The moving parts present pinching and crushing hazards. Access to moving parts under the hood of the ImageStream®X MkII is intended only for Luminex service personnel.

### Electrical Safety

Equipment ratings: The ImageStream®X MkII 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 alternatif, 50/60 Hz, 3A.



Les dangers électrique se trouvent dans l'appareil, surtout près de la source d'alimentation. Pour éviter les chocs 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 MkII 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.

Complies with IEC 60825-1:2014-05 Ed.3.0

The ImageStream<sup>X</sup> MkII may have the following lasers:

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



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'ImageStream<sup>X</sup> 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'utilisateur pendant le fonctionnement normal. Quand le capot est ouvert, les enclenchements eteindents les lasers.

ImageStreamX peut avoir les lasers suivants:

Longueur d'onde	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



L'utilisation des commandes ou les rendement des procédures autres que celle précisée aux présentes peuvent provoquer une radioexposition dangereuse.

## Biohazard/Biological Safety

The ImageStream®X MkII 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 MkII.



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

## Sécurité Biologique Biorisques:

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'ImageStream®X MkII.



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.

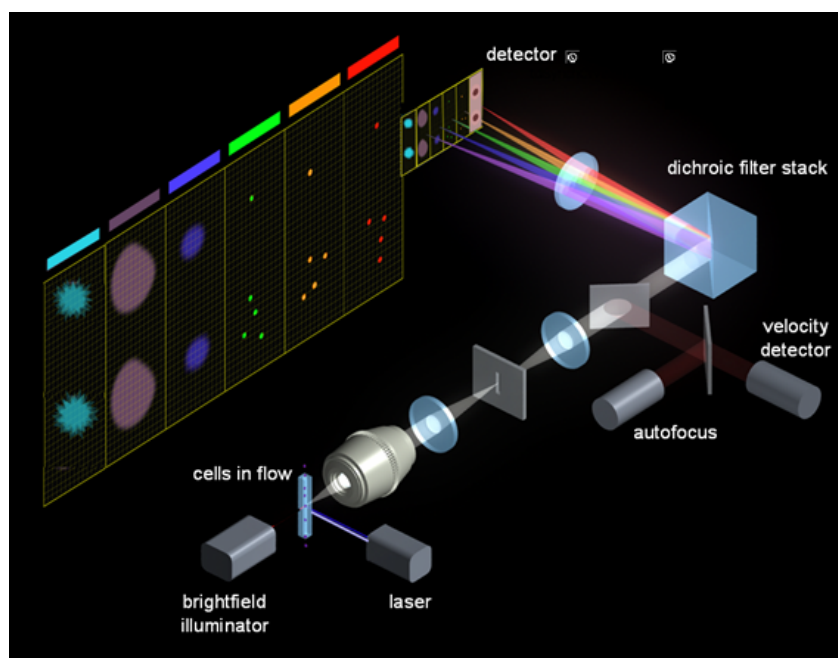
## FCC Compliance

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.

# Chapter 3: Performance Specifications and System Components

## General System Overview

The ImageStream<sup>X</sup> MkII acquires up to twelve images simultaneously of each cell or object including brightfield, scatter, and multiple fluorescent images at rates of up to 1000 objects per second. The time-delay-integration (TDI) detection technology used by the ImageStream<sup>X</sup> MkII 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 ImageStream<sup>X</sup> MkII works.



Hydrodynamically focused cells are trans-illuminated by a brightfield light source and orthogonally by a 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.

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## Environmental Conditions

- Indoor laboratory and professional use only.
- Altitude: less than 2000 m (6562 ft.) above mean sea level
- Operating temperature and humidity: 5°C to 25°C (41°F to 77°F) at a maximum relative humidity of 80%, non-condensing. During instrument operation, the ambient temperature should be maintained within  $\pm 2^\circ\text{C}$
- Pollution degree: 2

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## Performance Specifications

### General System Specifications

- Weight: 160 kg (353 lbs)
- Produces sound pressure levels below 70 db(A).

# Laser Specifications

## Fluorochrome Charts

Table 1. 6 Channel System

		Excitation Laser (nm)						
Ch	Band (nm)	375	405	488	561	592	642	785
1	435-505 (457/45)	<b>*DAPI, BV421, AF350,</b> Hoechst, PacBlue, CascadeBlue,	<b>*DAPI, BV421, AF405,</b> Hoechst, PacBlue, CascadeBlue, eFluor450, DyLight405, CFP, LIVE/DEAD Violet					
2	505-560 (533/55)	<b>*BV510,</b> PacOrange, CascadeYellow, AF430, eFluor525, QD525,	<b>*BV510,</b> PacOrange, CascadeYellow, AF430, eFluor525, QD525,	<b>FITC, AF488, GFP,</b> YFP, DyLight488, PKH67, Syto13, SpectrumGreen, LysoTrackerGreen, MitoTrackerGreen				
3	560-595 (577/35)	<b>*QD565,</b> QD585, eFluor565	<b>*QD565, QD585,</b> eFluor565	<b>PE, PKH26, DSRRed,</b> mOrange, CellMask/CellTracker/SYTOX Orange, Cy3	<b>PE, AF546,</b> Cy3*, DyLight550, PKH26, DSRRed, SpctmOrg, MTOrg			
4	595-642 (610/30)	<b>*QD625,</b> eFluor625, BV605	<b>*QD625, eFluor625,</b> BV605	<b>PE-TexRed, ECD, PE-AF610, RFP, QD625,</b> eFluor625	<b>AF568,</b> Cy3*, PE-TexRed, ECD, TexRed, PE-AF610, RFP, mCherry, PI	<b>TexRed, AF594,</b> DyLight594, mCherry, SpectrumRed, PI,		
5	642-745 (702/85)	<b>*QD705,</b> eFluor700, BV711	<b>*QD705, eFluor700,</b> BV711	<b>PE-Cy5, PE-AF647,</b> 7AAD, PI*, PerCP, PerCP-Cy5.5, DRAQ5, QD705, eFluor650, FuraRedlo, DRAQ5*, LDS 751	<b>PE-Cy5, PE-AF647,</b> DRAQ5*, 7AAD, LDS751	<b>AF647, AF660,</b> AF680, APC, Cy5, DyLight649, PE-AF647, PE-Cy5, DRAQ5*	<b>APC, AF647, AF660, AF680, DRAQ5, Cy5,</b> DyLight649, PE-AF647, PE-Cy5, PerCP, PerCP-Cy5.5	
6	745-785 (765/40)	<b>*QD800,</b> BV786	<b>*QD800, BV786</b>	<b>PE-Cy7, PE-AF750,</b> QD800	<b>PE-Cy7, PE-AF750</b>	<b>APC-Cy7, APC-AF750,</b> APC-H7, APC-eFluor750	<b>APC-Cy7, APC-AF750, APC-H7 APC-eFluor750, Cy7, AF750, DyLight750,</b> PE-Cy7, PE-AF750	<b>SSC</b>

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 (or BV421), AF488, PE, PE-TxRed, AF647, SSC/BF

Table 2.12 Channel System

		Excitation Laser (nm)						
Ch	Band (nm)	375***	405	488	561	592	642	785
1	435-480 (457/45)	<b>375 &amp; 405***</b> Ch1/Ch9 BF or *DAPI, BV421, AF350, Hoechst, PB						
2	480-560 (528/65)	*BV510, PacOrange, QD525,		FITC, AF488, GFP, YFP, DyLight488, PKH67, Syto13, SpectrumGreen, LysoTrackerGreen, MitoTrackerGreen,				
3	560-595 (577/35)	*QD565, QD585, eFluor565		PE, PKH26, Cy3, DSRed, CellMask/CellTracker/SY TOX Orange	PE, AF546, DyLight550, PKH26, DSRed, Cy3, SptcmOrange			
4	595-642 (610/30)	Ch4/Ch10 BF or *QD625, eFluor625, BV605		Ch4/Ch10 BF or PE-TexRed*, ECD*, PE-AF610*, RFP, QD625*, eFluor625*	Ch4/Ch10 BF or AF568*, DyLight594*, PE-TxRed*, ECD*, PE-AF610*, RFP, mCherry*			
5	642-745 (702/85)	*QD705, eFluor700, BV711		PE-Cy5*, PE-AF647*, 7AAD*, PI* PerCP*, PerCP-Cy5.5*, eFluor650*, FuraRedLo, Draq5*, LDS751*,	PE-Cy5*, PE-AF647*, 7AAD*, PE-AF647*, 7AAD*, Draq5*, LDS751*,			
6	745-785 (765/40)	*QD800, BV786		PE-Cy7*, PE-AF750*,	PE-Cy7*, PE-AF750*,			SSC
7	435-505 (457/45)	<b>375 No 405***</b> *DAPI, BV421, AF405, Hoechst, PacBlue, CascadeBlue, eFluor450, DyLight405, CFP, LIVE/DEAD Violet AF350						
8	505-570 (537/65)	*BV510, PacOrange, CascadeYellow, AF430,	*BV510, PacOrange, CascadeYellow, AF430, eFluor525, QD525					
9	570-595 (582/25)	Ch1/Ch9 BF or *QD565, QD585	Ch1/Ch9 BF or *QD565, QD585, eFluor565					
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*		
11	642-745 (702/85)	*QD705, eFluor700, BV711	*QD705, eFluor700, BV711			APC, AF647, AF660, Cy5, DyLight649, DyLight680, Draq5* PE-DRAQ5* APC, Cy5, DyLight649, DyLight680, Draq5* PE-AF647*, PE-Cy5*, PerCP*, PerCP-Cy5.5*	AF647, AF660, AF680, APC, Cy5, DyLight649, DyLight680, Draq5* PE-AF647*, PE-Cy5*, PerCP*, PerCP-Cy5.5*	
12	745-785 (765/40)	*QD800, BV786	*QD800, BV786			APC-Cy7, APC-AF750, APC-H7, APC-eFluor750	APC-Cy7, APC-AF750, APC-H7, APC-eFluor750, PE-Cy7*, PE-AF750*	SSC

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 (or BV421), AF647, APC Cy7



## Electronics Specifications

- Rated voltage: 100-240 VAC
- Rated frequency: 50/60 Hz
- Rated current: 3 A
- The main power supply may not fluctuate more than  $\pm 2^{\circ}\text{C}$ .
- The main power supply must meet transient over voltage category (II).

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## System Components

The three system components of the ImageStream®X MkII System are software, hardware, and consumables.

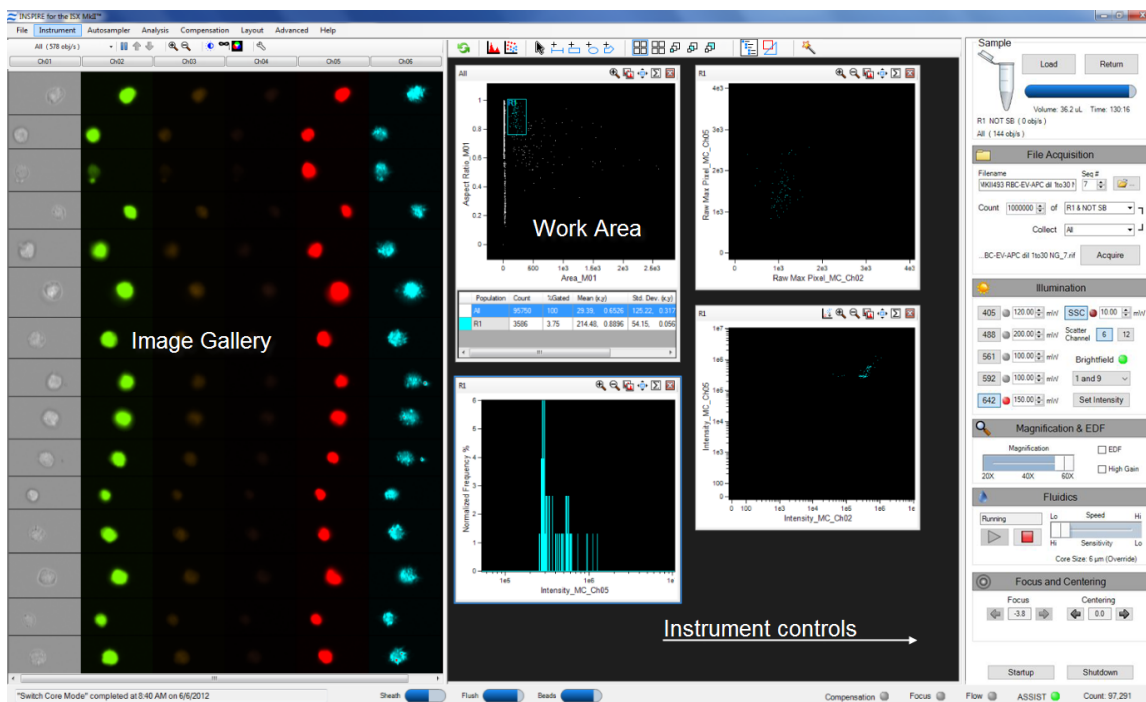
### Software Components

The INSPIRE™ software is integrated with the ImageStream®X MkII and is used to run the instrument. INSPIRE also provides tools for configuring the ImageStream®X MkII, 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 MkII multispectral imaging flow cytometer.

## INSPIRE™ User Interface

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



## Hardware Components

The ImageStream®X MkII System includes the following:

- ImageStream<sup>X</sup> MkII multispectral imaging flow cytometer

## Consumable Components

### SpeedBeads Calibration Reagent

The SpeedBead reagent is integral to the operation of the ImageStream®X MkII system, and is run with each sample. SpeedBeads are used to test and calibrate the instrument's illumination, optical, camera and fluidic systems at the beginning of each day. In addition, run-time information from the SpeedBeads is used by the ImageStream<sup>X</sup> MkII to maintain continuous synchronization between the camera and the sample flow rate and to automatically focus on the sample core stream. SpeedBead imagery is excluded from image acquisition files automatically by turning on the bead classifier. The SpeedBead reagent comes in 15 ml conical tubes and is loaded onto the bead port behind the fluidics door on the front of the instrument.

## Required Laboratory Reagents

These recommended reagents have been formulated to optimize the performance of the ImageStream®X MkII 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 <sup>++</sup> Mg <sup>++</sup> free	Millipore	BSS-1006-B (1X) 6506- (10X)
Rinse	deionized water		

This information is provided for information only; other sources of the same reagent may be used.

**NOTE:** 10% bleach may be substituted for Sterilizer. Follow the manufacturer's recommendation on the use of diluted bleach. In general, this means preparing a fresh 1:10 dilution daily for applications that require complete disinfection. This includes but is not limited to running samples containing live bacteria, yeast, or virus particles on a regular basis. For most general use, replacing the sterilizer with a fresh 1:10 dilution of bleach once a week is sufficient.

**NOTE:** In this manual, "Bleach" refers to a solution of 8% ± -2% NaOCl (Sodium Hypochlorite) in water. Diluted bleach 1:10 refers to a solution with a final NaOCl concentration of 0.8% ± -0.2%. Stock solutions of concentrated bleach should be stored in dark, airtight bottles at temperatures below 68 °F (20 °C) for no more than 6 months.

## Subsystems

The ImageStream®X MkII system includes four essential subsystems: electrical, fluidic, optical, and laser.

### Electrical Subsystem

The electrical subsystem of the ImageStream®X MkII system is comprised of the computer and all the electronics used to operate the syringes, valves, power supplies and other components that help the system operate.

There are two system computers: one running Linux OS and the other running Windows. The Linux OS computer runs the ImageStream®X MkII components and the frame-grabber. The Windows computer is the system computer the user interfaces with to customize the instrument set up for their experiment. Data analysis is also performed on the Windows computer using the IDEAS® image analysis software.

### Fluidics Subsystem

The fluidics subsystem of the ImageStream®X MkII system includes waste fluid, sheath fluid, sterilizer, cleanser, and debubbler. The fluidics system uses a series of syringes and stepper-driven motors to move fluids throughout the instrument. The syringes help to start up the system, load the samples, drive samples through the flowcell during data acquisition, and sterilize the system using the onboard cleaning reagents.

Two bottles are provided:

- Sheath — To be filled with phosphate buffered saline (PBS) with no surfactants for running samples.
- Rinse — To be filled with deionized (DI) water for rinsing the instrument during shutdown.

Fluid is drawn from these two 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.

## Waste Fluid

The waste bottle holds all of the fluids that have been run through the ImageStream®X MkII, 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.

## Optical Subsystem

The optical system starts at the excitation lasers, which are directed through a series of beam-shaping optics to align the lasers collinearly at the flowcell, where they excite fluorescence. The fluorescence is then collected by either a 20x, 40x or 60x imaging objective and projected to the spectral decomposition element, where each color is spectrally separated to discrete regions on the CCD camera. To maximize photonic sensitivity and minimize image blurring, the camera is operated in time delay integration (TDI) mode and allows the system to collect images at up to 0.3  $\mu\text{m}$  per pixel resolution with minimal object blur. Up to 12 image channels are collected simultaneously at a rate of up to 5,000 cells per second and stored in the computer database.

## 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.

## 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 Luminex® engineer when installed and should be repeated by Luminex service when any optical changes are made to the instrument.

- 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.
- 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

## High Gain Functionality

The High Gain (HG) functionality allows users to adjust the camera gain, effectively doubling the photonic sensitivity of the system in all channels simultaneously. This functionality was developed to allow users to collect Extracellular Vesicles (EVs) and other submicron-sized particles more accurately at 60x magnification; however, it can be activated with other system settings. When High Gain is activated, the camera is gained up, and object detection is optimized for small particles. It can be used on any sample where increased photonic sensitivity is required.

- High Gain is available on ImageStream®X MkII systems with updated cameras and the multi-mag option.
- High Gain was developed to image EVs and small particles and is optimized to work with the 60x magnification.
- Because camera gain amplifies signal, users may notice a small increase in the background; however, the sensitivity will nearly double.
- When first applying HG, the brightfield image will be twice as bright. Click the brightfield Set Intensity button to return the BF background to 800 counts.
- High Gain also modifies object detection by lowering the detection threshold and optimizing the mask to identify submicron particles.
- Calibrations specific to data files collected using High Gain will automatically be applied to those files only.

## MultiMag

The MultiMag option includes two additional objective lenses. The 20X lens 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 0.1 square microns.

## Laser Subsystem

The ImageStream®X MkII has a number of laser configurations. The base system has a 200mw, 488nm laser and a 785nm dedicated SSC laser, with options to add each of the following lasers: 375nm, 405nm, 488nm, 561nm, 592nm, and 642nm. The 200mw, 488nm laser can also be replaced with a 400mw high-powered 488nm laser.

The brightfield system uses a series of colored LED lights and allows the user to place brightfield in any of the spectral channels to accommodate different combinations of fluorochromes. For 12-channel systems, brightfield images must be collected on both Camera 1 and Camera 2 so the system can precisely align all 12 images during analysis. Collecting brightfield in channels 1 and 9 is the default setting on 12-channel systems, but can be adjusted to any paired channels to accommodate different combinations of fluorochromes.

# Chapter 4: Preparing the System

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## Power on the System and Log into the Software


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

## Set the Image Display Properties

There are two methods for setting the display of the imagery: automatically or manually. The display properties wizard will automatically adjust the display as the images are being collected. The display can also be set manually.


**NOTE:** These settings adjust the monitor display and do not change the pixel intensities of the images collected.

### Automatically Set the Image Display Properties

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 adjusts 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.

### Manually Set the Image Display Properties

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.

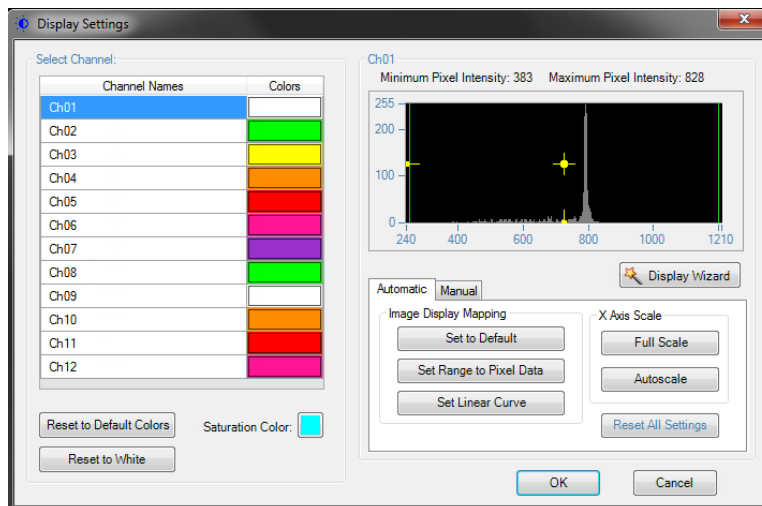
- To set the display mapping, adjust the right and left green bars in the graph. You will adjust the **Display Intensity** settings on the graph (the Y Axis) to the **Pixel Intensity** (the X axis). The range of pixel intensities is 0-4095 counts. The display range is 0-255. The pixel intensities shown 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.

- Click and drag the vertical green line on the left side (crossing the X Axis at 0) to manually set the display pixel intensity to 0 for all intensities that appear to the left of that line. This removes background noise from the image.
- Click and drag the vertical green line on the right side 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.



## Prepare the ImageStream®X MkII System

1. 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. Choose the appropriate sheath fluid using the Acceptable Sheath Fluid chart.

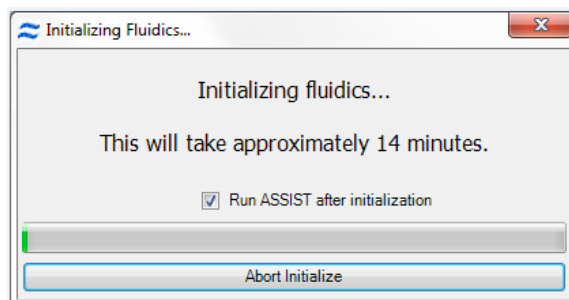
Sample Solution	Sheath Fluid	Acceptable
PBS	PBS	Yes
PBS	Water	Yes*
PBS/Surfactant	PBS	Yes
PBS/Surfactant	Water	No
Water	PBS	Yes
Water	Water	Yes
Water/Surfactant	PBS	No
Water/Surfactant	Water	Yes

\*Cells in PBS run with water sheath will swell.

2. Empty the waste tank and add fresh bleach.
  - a. Push on the quick-disconnect buttons to remove the tubing from the Waste tank.
  - b. Empty the Waste tank.
  - c. Add 160 mL of bleach to the Waste tank.

**NOTE:** The final volume of waste when full will be approximately 1600 mL. Therefore, the final bleach concentration for a full waste tank will be 10% bleach. Luminex® recommends that the waste be emptied every day and fresh bleach added before Startup.
3. 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.

**NOTE:** The **Run ASSIST after initialization** box is checked by default to run ASSIST after the fluidics are ready. Uncheck the box if you wish to start ASSIST later.



4. Click **Start All Calibrations and Tests** in the calibration window if ASSIST was not run in the previous step.
5. Center the core stream images (if necessary) by laterally moving the objective under **Focus and Centering**. Adjust centering by pressing the right or left arrows.
6. The event rate should be 800-1000 events per second.



- When the calibrations and tests have passed the ASSIST, status light will change to green. Close the **Calibrations** window.
- 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, call Luminex Technical Support.

**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.

Calibrations		Tests	
Calibration	Last Run Time	Test	Last Run Time
Camera Synchronization 20x Calibration	7/15/2020 8:58:17 AM	405nm Laser Power Test	7/15/2020 8:42:56 AM
Camera Synchronization 40x Calibration	7/15/2020 8:32:38 AM	488nm Laser Power Test	7/15/2020 8:43:05 AM
Camera Synchronization 60x Calibration	7/15/2020 8:54:11 AM	561nm Laser Power Test	7/15/2020 8:43:12 AM
Core Stage Position Calibration	7/15/2020 8:32:47 AM	592nm Laser Power Test	7/15/2020 8:43:25 AM
Spatial Offsets 20x Calibration	7/15/2020 8:58:53 AM	642nm Laser Power Test	7/15/2020 8:43:32 AM
Spatial Offsets 40x Calibration	7/15/2020 8:33:06 AM	Brightfield Alignment Test	7/15/2020 8:44:18 AM
Spatial Offsets 60x Calibration	7/15/2020 8:54:30 AM	Brightfield Uniformity Test	7/15/2020 8:44:59 AM
Dark Current Calibration High Gain	7/15/2020 8:33:21 AM	Camera Noise Test	7/15/2020 8:45:04 AM
Dark Current Calibration	7/15/2020 8:33:27 AM	Flow Core Axial Stability Test	7/15/2020 8:45:41 AM
Brightfield XTalk Coefficient Calibration	7/15/2020 8:34:08 AM	Flow Core Lateral Stability Test	7/15/2020 8:45:53 AM
Brightfield XTalk High Gain Coefficient	7/15/2020 8:34:58 AM	Flow Core Position Test	7/15/2020 8:46:05 AM
405nm Horizontal Laser Calibration	7/15/2020 8:35:54 AM	Focus Offset Test	7/15/2020 8:49:28 AM
488nm Horizontal Laser Calibration	7/15/2020 8:36:40 AM	Focus Percentage Test	7/15/2020 8:49:41 AM
561nm Horizontal Laser Calibration	7/15/2020 8:37:25 AM	Focus Uniformity Test	7/15/2020 8:50:35 AM
592nm Horizontal Laser Calibration	7/15/2020 8:38:16 AM	Image Quality 20x Ch. 6 Test	7/15/2020 9:00:37 AM
642nm Horizontal Laser Calibration	7/15/2020 8:39:01 AM	Image Quality 20x Ch.12 Test	7/15/2020 9:02:00 AM
SSC Ch. 6 Horizontal Laser Calibration	7/15/2020 8:39:47 AM	Image Quality 40x Ch. 6 Test	7/15/2020 8:51:13 AM
SSC Ch.12 Horizontal Laser Calibration	7/15/2020 8:40:32 AM	Image Quality 40x Ch.12 Test	7/15/2020 8:51:47 AM

History      Start All Calibrations and Tests      Stop All  
Generate Report      Run Beads

## Run ASSIST Calibrations and Tests



Reagents can contain sodium azide as a preservative. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. It is also highly toxic and rates a safety data sheet (SDS) health hazard 4. On disposal, flush drains with a generous amount of cold water to prevent azide buildup. Consult the manual guideline "Safety Management No. CDC-22, Decontamination of Laboratory Sink Drains to Remove Azide Salts" (Centers for Disease Control, Atlanta, Georgia, April 30, 1976)

- Click **Start All Calibrations and Tests** to run all standard calibrations and tests.
- Optional: Click **Run Beads** to begin running beads without starting calibrations or tests.
- To run one calibration or test, click on an individual calibration or test and click **Run**.
- 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.

6. If a calibration or test fails, run that calibration or test individually and if it fails again, call or email Luminex® service.

**NOTE:** Calibrations and tests do not run in order. 40X Calibrations are completed before changing magnifications to run 20X and 60X calibrations.

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

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

History      Start All Calibrations and Tests      Stop All

Generate Report      Run Beads

## View Calibration or Test Results

View the latest result of a specific calibration or test using one of two methods:

- Click on the button to the right of the test or calibration you want to view the results of.
- Click on the **History** button and choose the routine and date you want to view.

## Create a Calibration or Test Report

ASSIST calibration and test results are stored in a database that can be reported. To create a report for all calibrations and tests, complete the following steps:

1. Click the **Generate Report** button. The **Generate Report** window opens.
2. (Optional) Set a custom header for the report.
3. Set a **Date Range** for the report using the radio buttons and the date drop-down menu.
4. Click **Generate**. The custom report generates.

# Chapter 5: Running the Assay

## Load and Run the Sample

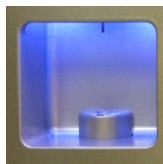
1. In the file menu, choose **Load Default Template**.

**NOTE:** Application-specific instrument settings can be saved in a template and used to facilitate instrument setup, but Luminex® recommends 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.

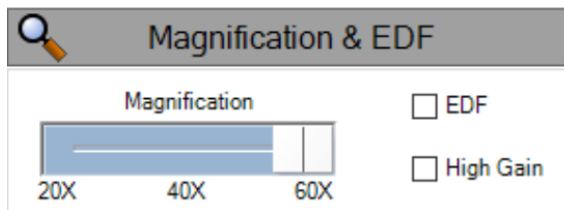
2. Press **Load** and load an aliquot of the brightest sample in the experiment that fluoresces with each fluorochrome used. It is critical that you run this sample first to establish the instrument settings.

**NOTE:** DO NOT change laser settings for the experiment once established on this sample.

3. When prompted, place sample vial with 20-200 µL into the sample loader.

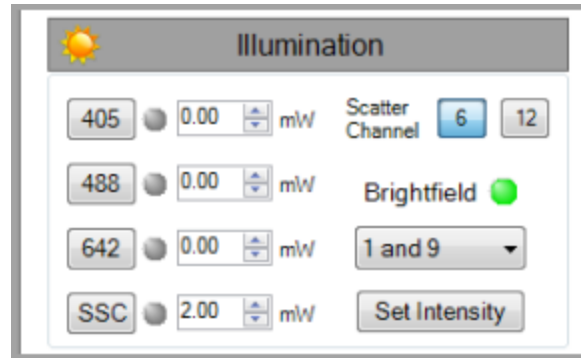


4. In the **Magnification & EDF** window, choose the objective under **Magnification** (optional).

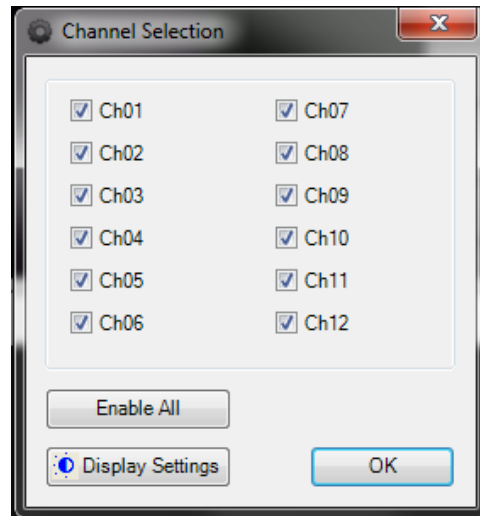


5. (Optional) Select the **EDF** checkbox if you want extended depth of field (EDF) collection.
6. (Optional) Select the **High Gain** checkbox if you want High Gain (HG) collection.
7. Click on the wavelength button to turn on each laser used in the experiment.

- Set the laser powers so there is no saturation, and each fluorochrome has Raw Max Pixel Intensities between 100 and 4000 counts, as measured in scatterplots or histograms of the appropriate channels. Click the **Set Intensity** button when finished.



- Click a channel name to open the **Channel Selection** window.
- (Optional) Click **Display Settings** to set the image display properties.
- Select the box next to the channel name to enable collection of the specified channel. When you have selected all channels to be collected, click **OK**.



- Select **Brightfield** channels. Default is Ch1 for a 6 channel system; Ch1 and 9 for a 12 channel system. Click **Set Intensity**.
- Create graphs to gate on cells of interest.

**NOTE:** Luminex recommends the following graphs:

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

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

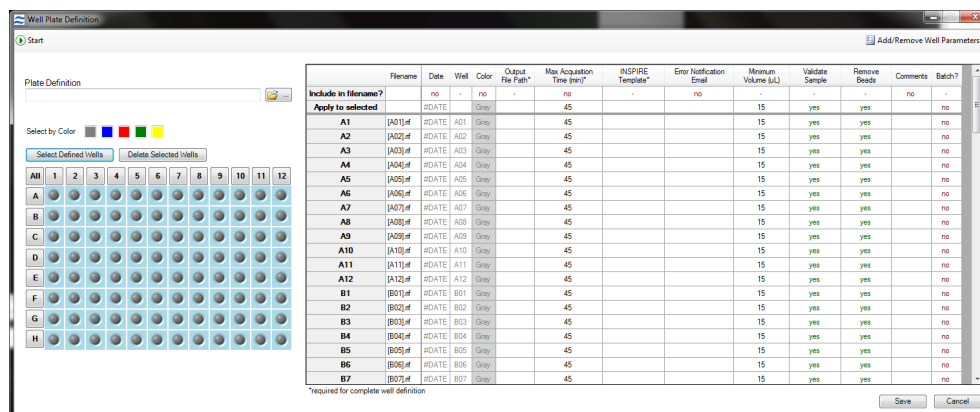
- **Area:** The number of pixels in an image reported in square microns.
- **Aspect Ratio:** The Minor Axis divided by the Major Axis is a measure of how round or oblong an object is. See below for the definitions for Major and Minor Axis.
- **Background Mean:** The average pixel intensity of the background 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.

**NOTE:** See the IDEAS® User Manual for more details on features and graphing.

## Define the Plate

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

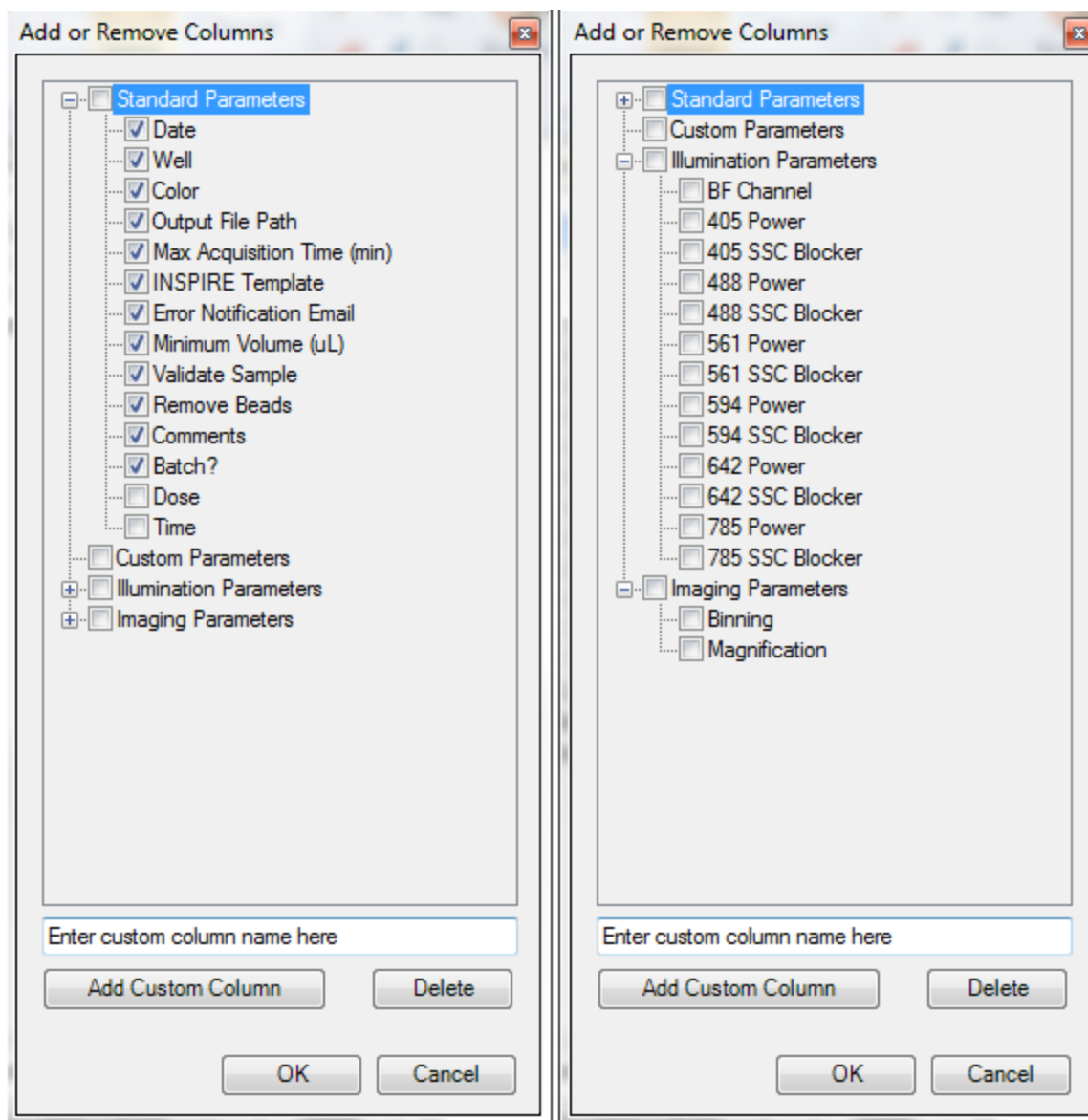
1. Choose **Define Plate** from the Autosampler menu to open the **Well Plate Definition** window.



2. Create a new definition. Alternately, click the folder icon to browse for a previously saved definition to edit.
3. **Name** the plate definition.

**NOTE:** 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.

4. Choose the parameters you would like to use. Click **Add/Remove Well Parameters** to choose the parameters you want to report for the wells.

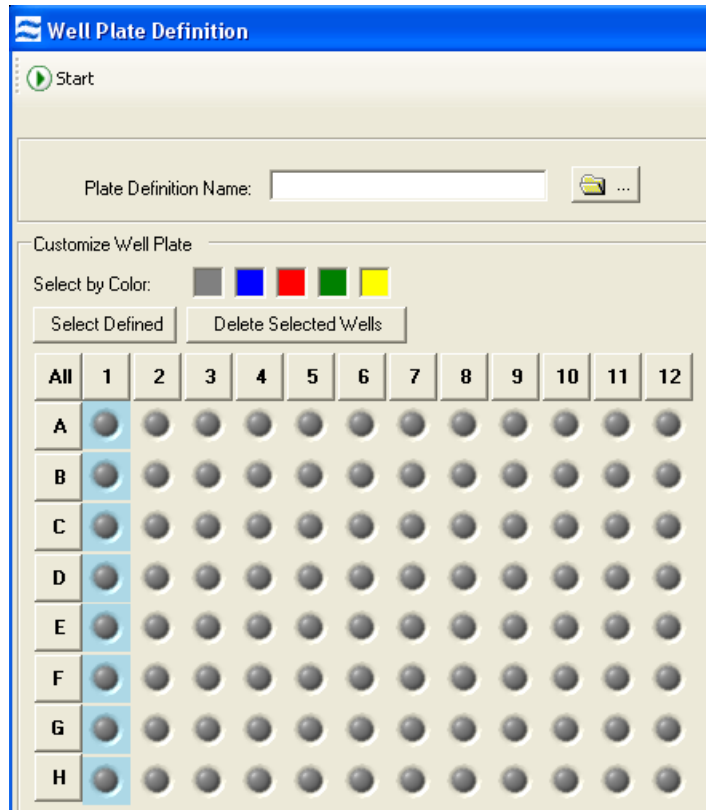


There are several categories of parameters that may be chosen as a group or individually. See the list of parameters above. Check or uncheck the desired parameters. The user can also define custom parameters. Expand the category to see the individual parameters. To delete a custom parameter, select it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

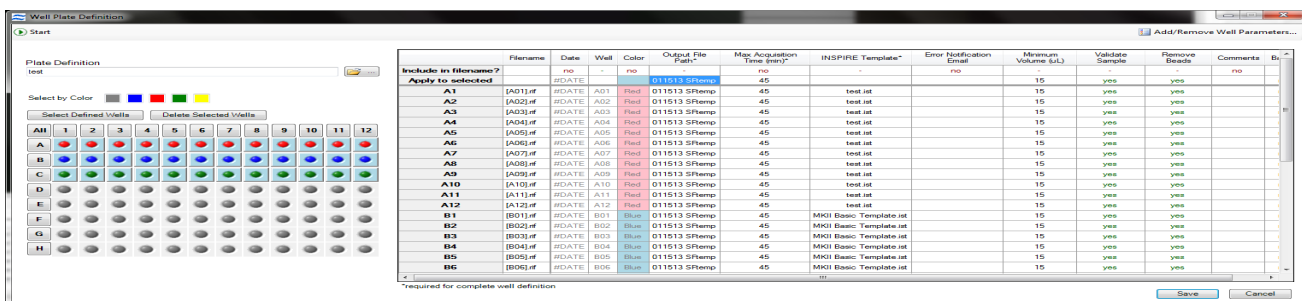
- It is highly recommended to leave the Validate Sample option 'Yes'. See notes below regarding validating samples.
- To include a parameter in the file name, click in the box below the column heading (make sure it says 'yes').
- Columns can be re-ordered by click/drag.
- Batching of the data into IDEAS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

- Define the wells. Select wells to define by clicking a) individually (or Ctrl 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.



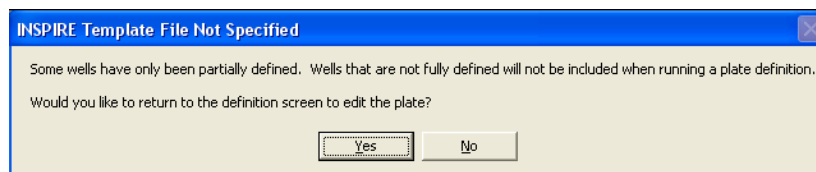
- You can edit values for some of the Custom and many of the Standard parameters. You can do this for all selected wells or for individual wells.
- For example, if you want to collect with Max Acquisition Time 10 minutes for the selected wells, type 10 in the 'Apply to selected' box below the Max Acquisition Time heading. If you want to only apply this to a single well, type this value in the box corresponding to that well.



- 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**.

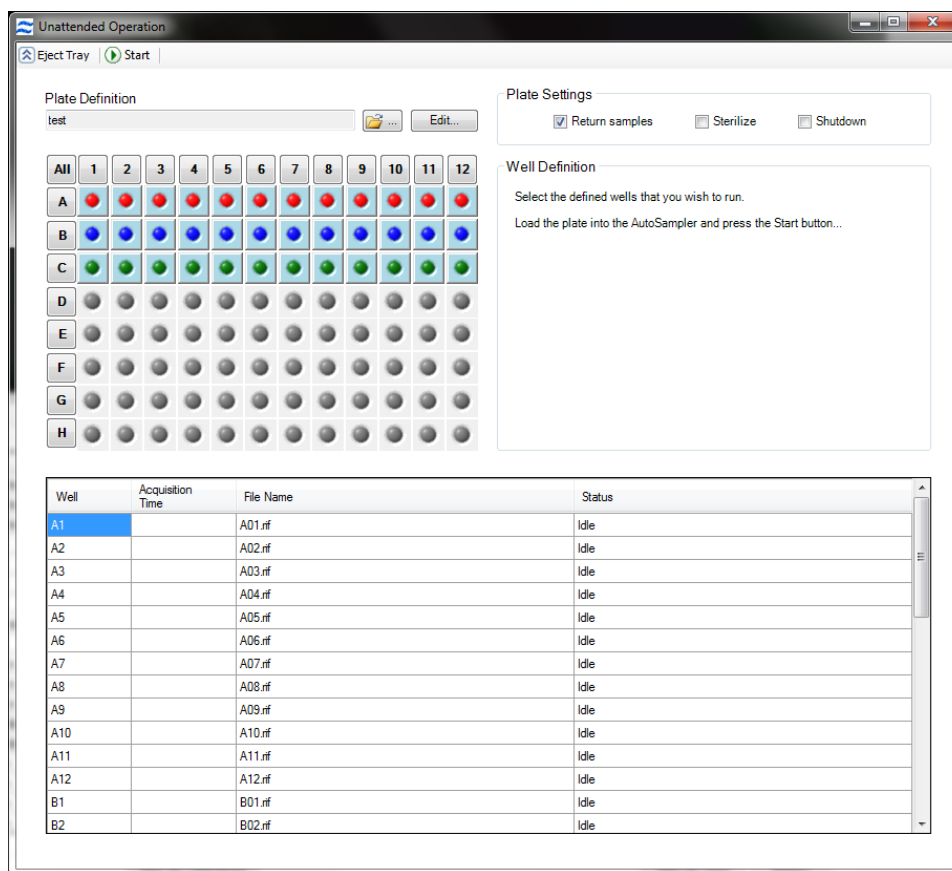
**NOTE:** A warning may be displayed if there are undefined or partially defined wells. Select Yes to return to plate definition or No to continue.



## Start the AutoSampler

1. 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 Plate Definition, browse for it by clicking on the folder icon.
- If you want to edit the Plate Definition, click **Edit** and you will be taken to the Well Plate Definition window.



2. Check or uncheck the boxes **Return samples**, **Sterilize**, **Shutdown**.

**NOTE:** 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.

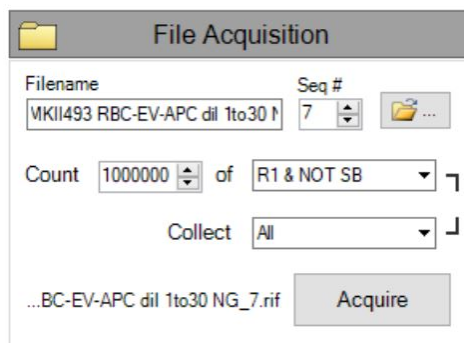
3. **Select** the wells to run (they will appear in the list).  
 4. Click **Eject Tray** to extend the plate nest.



5. Add at a minimum 75  $\mu$ L samples to the 96 well plate, cover with Sigma-Aldrich X-Pierce Film (XP-100, Cat # 2722502) to minimize evaporation and allow the membrane piercing probe to access the sample. Place your plate on the tray with well A1 positioned at the upper left corner. Luminex® recommends using round bottom (U-bottom) plates to facilitate mixing. The user has the option to use deep well plates accommodating up to 2 mL.
6. Click **Start** to begin.
7. The Status column will be updated for each well as it is run. For each sample, the instrument performs the following in sequence: 1) Mixing and resuspension of sample, 2) Load, 3) Validation (flow speed CV, focus, brightfield intensity, object rate), 4) Data Acquisition, 5) Result (success or error).

## Collect and Save Data Files


Once the sample is running and the ImageStream®X MkII 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.





1. Enter the file name in the **Filename** field.
2. Enter the sequential number in the **Seq #** field.

**NOTE:** The sequential number 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.

3. Click the folder icon to select a folder where data acquisition files will be saved. You can also create a new folder to save acquisition files in.
4. Enter the number of cells you want to acquire in the **Count** field and select the population from the adjacent drop-down.
5. (Optional) To count one population but limit the collection to another population, click on the bracket to break the link and enable the **Collect** drop-down.
6. Click **Acquire** to begin acquiring a data file. The data file(s) are automatically saved in the selected folder once the desired number of objects are collected.
7. Click  to prematurely stop acquisition. The system prompts you to either discard the acquired data or to save the collected data in a file.

8. Click  to pause acquisition while running or to resume acquisition when paused.
9. 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.
10. Change the file name for the next sample and continue collecting samples until you have acquired data for all the desired samples.
11. 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.
12. Click  in the analysis tools section and choose **Compensation** to launch the **Compensation Wizard**.

**NOTE:** The Compensation Wizard sets the instrument for compensation controls that must be collected with bright-field and scatter (785 nm laser) OFF and every channel set to be collected. Keep all laser powers the same as for the experimental samples.
13. Follow the prompts in the Compensation Wizard to collect all compensation control files:
  - a. Click **Load**. Alternately, if a compensation control sample is already running, click **Next**.
  - b. Place the tube on the uptake port and click **OK**.
  - c. Click **Next** when sample is running.
  - d. Verify the channel for compensation.
  - e. If not all cells are positive, draw a region on the **Uncompensated Intensity** scatter plot to define the positive population. View the population in the Image Gallery and choose this population to collect.
  - f. Name the file and choose the path to save the data.
  - g. Click **Collect File**. The compensation coefficients are calculated. The compensation coefficients and an Intensity scatter plot using the coefficients displays.
  - h. Click **OK** on the **Acquisition Complete** popup window.
  - i. Click **Load** to continue with the next single color control sample or click **Return** (optional).
  - j. Repeat the previous steps for each compensation sample. For each sample, reset the Image gallery population to view **All**, then create an appropriate population for each sample.

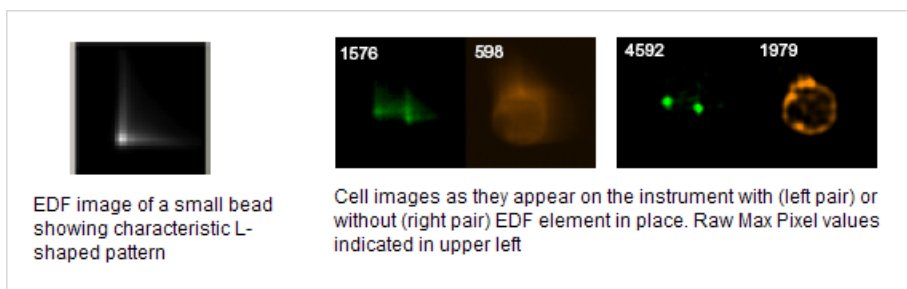
**NOTE:** The R1 gate can be moved for each sample.
  - k. Click **Exit** when done with the Compensation Wizard.
14. Save the coefficients to a compensation matrix file. The beginning template is restored and the saved matrix is used to compensate the Intensity feature.

**NOTE:** 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.
15. Continue to collect experimental files.
16. Click **Shutdown** when done.

## Collect a Data File Using the EDF Element

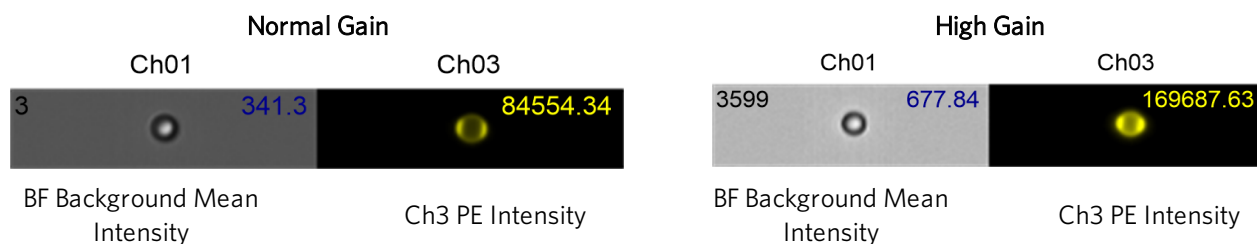
1. Set instrument settings for the experiment.
2. Check the **EDF** box in the instrument control panel.
3. Adjust any regions being collected to accommodate using EDF.

- The calibration kernels saved during the last EDF calibration will be appended to the file and the file name will be appended with "-EDF."



## Collect a Data File Using High Gain

- Set instrument settings for the experiment.
- Select the **High Gain** box in the instrument control panel.
- Adjust laser powers to optimize signal and minimize saturation.
- Adjust any regions being collected to accommodate the increased sensitivity.
- Use the Brightfield **Set Intensity** button to set the background mean intensity to ~800.
- High Gain calibrations will automatically be applied to files collected with HG active.
- Acquire samples as usual.



## Collect a Data File Using the MultiMag 20X or 60X Objective Lens

Objective	Field of view	Pixel size	Depth of field	NA
40X	60 $\mu\text{m}$	0.5 $\mu\text{m}$	4 $\mu\text{m}$	0.75
20X	120 $\mu\text{m}$	1 $\mu\text{m}$	8 $\mu\text{m}$	0.5
60X	40 $\mu\text{m}$	0.33 $\mu\text{m}$	2.5 $\mu\text{m}$	0.9

Select the button under Magnification to choose an optional MultiMag objective lens. When using the 60X objective, the core velocity will be reduced to 40 mm/sec instead of the normal 60 mm/sec used during 40X or 20X acquisition.

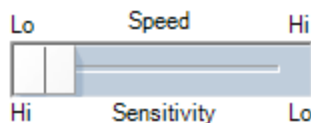
## Remove Debris

Some samples have an abundance of small particulate debris. Debris 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, increase the **Squelch** value until you achieve the desired results..

## Set ImageStream®X MkII Speed and Sensitivity

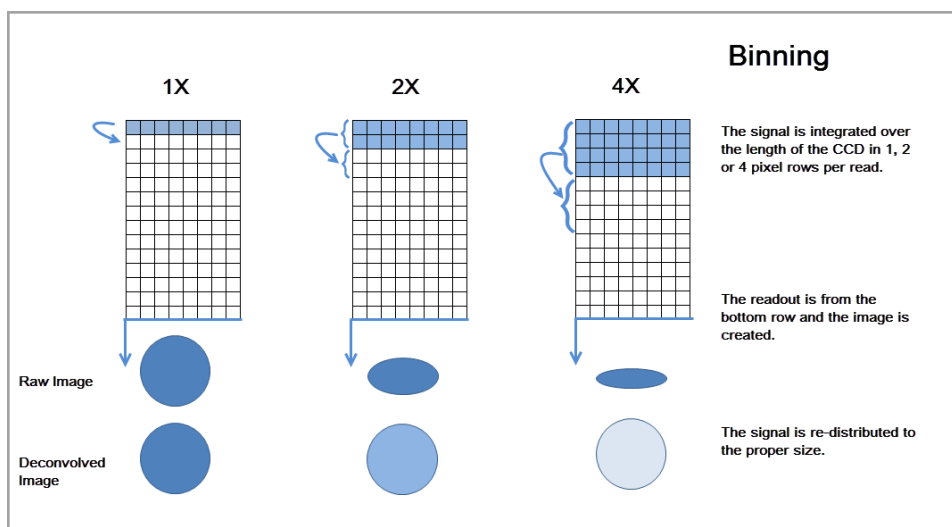
The optimal operating speed is set at the factory for each instrument and is approximately 60 mm/sec. This speed corresponds to the highest resolution setting with a pixel size of 0.5  $\mu\text{m}$  at 40 X magnification. In order to collect images at higher speed, the rows on the camera can be binned. 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 objects at a concentration of  $1 \times 10^7$  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 $\mu\text{m}^2$	High	10 $\mu\text{m}$	55	$1 \times 10^{7\text{th}}$	200	54 sec
	Med	1x	1 x 1 $\mu\text{m}^2$	Med	10 $\mu\text{m}$	110	$1 \times 10^{7\text{th}}$	360	28 sec
	High	2x	1 x 2 $\mu\text{m}^2$	Low	10 $\mu\text{m}$	220	$1 \times 10^{7\text{th}}$	720	14 sec
40x	Low	1x	.5 x .5 $\mu\text{m}^2$	High	10 $\mu\text{m}$	55	$1 \times 10^{7\text{th}}$	200	50 sec
	Med	2x	.5 x 1 $\mu\text{m}^2$	Med	10 $\mu\text{m}$	110	$1 \times 10^{7\text{th}}$	360	29 sec
	High	4x	.5 x 2 $\mu\text{m}^2$	Low	10 $\mu\text{m}$	220	$1 \times 10^{7\text{th}}$	720	14 sec
60x	Low	1x	.3 x .3 $\mu\text{m}^2$	High	7 $\mu\text{m}$	40	$1 \times 10^{7\text{th}}$	60	160 sec
	Med	2x	.3 x .6 $\mu\text{m}^2$	Med	7 $\mu\text{m}$	55	$1 \times 10^{7\text{th}}$	75	135 sec
	High	4x	.3x1.2 $\mu\text{m}^2$	Low	7 $\mu\text{m}$	110	$1 \times 10^{7\text{th}}$	150	68 sec

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



## Shut Down the System

This procedure sterilizes the ImageStream®X MkII 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 deionized (DI) 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.

# Chapter 6: Troubleshooting

This section is designed to help you troubleshoot the operation of the ImageStream®X MkII. If additional assistance is required, contact Luminex® technical support.

# System

## Unstable Fluidics (Air or Clog in 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 70 µm 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 $1 \times 10^7$ cells/mL and strain the cells through a 70 µm 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 snugly into position. Call Luminex technical support.
-	SpeedBeads fail to run	Verify the beads will run by returning any sample, going to the fluidics section, and pressing 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.

## Fluidics Respond Sluggishly

Symptom	Possible Causes	Recommended Solutions
Fluidics respond sluggishly	Air buffer in the sheath syringe is not correct	The sheath syringe should contain 2 mL to 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 snugly into position. Call Luminex® technical support.

## Event Rate Slows Over Time

Symptom	Possible Causes	Recommended Solutions
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.
-	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 snugly into position. Call Luminex service.



## Event Rate is Slower than Expected

Symptom	Possible Causes	Recommended Solutions
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.
-	Insufficient illumination	Turn the appropriate lasers on. Set the 785 SSC laser to 40 mW. 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

Symptom	Possible Causes	Recommended Solutions
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 (~30 min).
-	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

Symptom	Possible Causes	Recommended Solutions
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 Luminex service.

## Instrument Will Not Pass ASSIST

Symptom	Possible Causes	Recommended Solutions
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 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 Luminex 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.

Symptom	Possible Causes	Recommended Solutions
-	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.
-	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..
-	Flow Core Lateral Stability test failure	See the Flow Core Lateral Stability test failure solution..
-	Flow Core Position test failure	See the Flow Core Lateral Stability test failure solution.
-	Focus Percentage test failure	See the Flow Core Lateral Stability test failure solution.
-	Focus Uniformity test failure	See the Flow Core Lateral Stability test failure solution.
-	Image Quality test failure	See the Flow Core Lateral Stability test failure solution.

## Compensation Wizard Fails to Complete

Symptom	Possible Causes	Recommended Solutions
<b>Compensation wizard fails to complete</b>	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

## INSPIRE™ Appears to Freeze

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

Symptom	Possible Causes	Recommended Solutions
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™.

## Plots Fail to Update or Update Slowly

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

Symptom	Possible Causes	Recommended Solutions
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. $1 \times 10^7$ 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: 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

## No Images

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 40 mW. 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

Symptom	Possible Causes	Recommended Solutions
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 40 mW. 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.
-	The sample is too concentrated	The process of object detection can safely handle up to 4000 objects per second. The maximum sample concentration is $4-5 \times 10^8$ cells per ml, with the recommended concentration $1-10 \times 10^7$ 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

Symptom	Possible Causes	Recommended Solutions
Objects appear streaked	Camera is not tracking the cell velocity accurately	Verify brightfield is working normally and rerun ASSIST using calibration beads.
-	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

Symptom	Possible Causes	Recommended Solutions
Objects are not centered in the channel. Images may be 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.
-	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.

## Objects are Rotating in the Core Stream

Symptom	Possible Causes	Recommended Solutions
Objects are rotating in the core stream	Core stream position is grossly off-center within the flowcell 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.

## Objects are Out of Focus

Symptom	Possible Causes	Recommended Solutions
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.
-	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

Symptom	Possible Causes	Recommended Solutions
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.
-	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 Magnification	In the magnification and EDF section choose a lower magnification.

## Images Appear Pixellated or Larger Than Normal

Symptom	Possible Causes	Recommended Solutions
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.

## Not All 12 Channels are Being Displayed

Symptom	Possible Causes	Recommended Solutions
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

Symptom	Possible Causes	Recommended Solutions
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

## Fluorescence is Too Bright, Images Have Contrasting Color or Appear Flat

Symptom	Possible Causes	Recommended Solutions
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 (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.

## Fluorescence Imagery Appears Too Dim

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 flowcell due to air or clog in the fluidics	Run the purge bubbles script from the instrument drop-down menu.
-	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.

## One Channel Saturates While the Others Do Not

Symptom	Possible Causes	Recommended Solutions
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 Too Bright or Changes Over Time

Symptom	Possible Causes	Recommended Solutions
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.
-	Core stream position is grossly off-center within the flowcell due to air or clog in the fluidics	Run the purge bubbles script from the instrument drop-down menu.

## Large Variation in Brightfield Intensity Levels

Symptom	Possible Causes	Recommended Solutions
Large variation in brightfield intensity levels	Large flow speed variation due to air	Run the purge bubbles script from the instrument drop-down menu.
-	Light source delivering variable output	Power down and power up the instrument, if this does not fix the problem, call Luminex service.

## Brightfield Intensity Level Sets Incorrectly








Symptom	Possible Causes	Recommended Solutions
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 Luminex service.



# Chapter 7: Maintaining the System

## General Maintenance Precautions

Observe the following general maintenance precautions:

	Personnel who use, maintain, or clean the ImageStream®X MkII System should be trained in standard laboratory safety practices and should follow those practices when handling the instrument.
	Samples and waste fluid can contain biohazardous material. Where exposure to biohazardous material, including in an aerosol form, exists, follow appropriate biosafety procedures, use personal protective equipment (PPE), and use ventilation devices.
	Do not place your hand inside the module openings at any time. Do not place any object other than the cartridge or the Cartridge Removal Tool into the module openings.
	Avoid contact with moving parts. Disconnect the instrument from the power source when the procedure instructs you to do so.
	Adhere to standard laboratory safety practices when handling hazardous, toxic, or flammable reagents and chemicals. Consult the package insert for the assay you are running and the Safety Data Sheet (SDS) for more information. Contact <i>Luminex Technical Support</i> when in doubt about compatibility of cleaning and decontamination agents or materials.
	Refer to the applicable SDS regarding safe handling practices for any spills.
	Wear appropriate personal protective equipment (PPE) when handling parts that come into contact with potentially biohazardous samples.

---

# Daily Maintenance

## Shut Down the System

This procedure sterilizes the ImageStream®X MkII 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 deionized (DI) 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.

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# Preventative Maintenance

The ImageStream®X MkII contains no serviceable parts. Only Luminex®-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 Luminex service personnel as part of routine preventative maintenance.

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# As Needed Maintenance

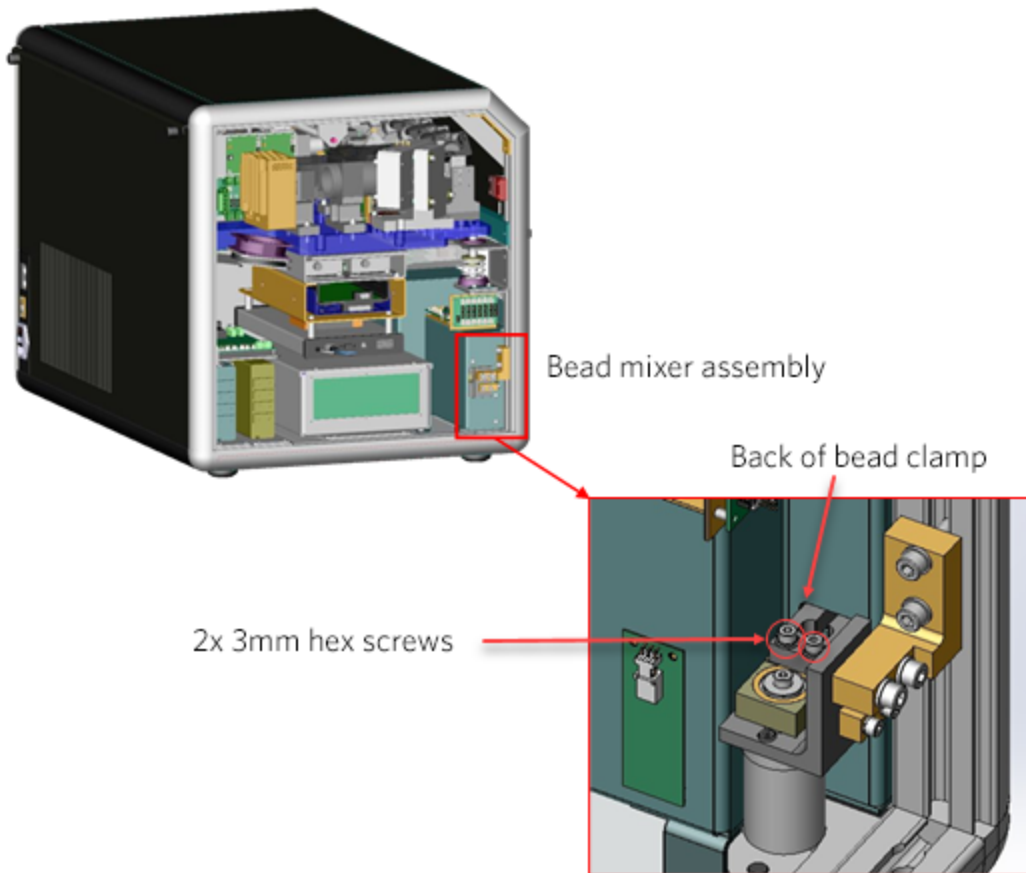
## Replace the Bead Clamp

The bead tube mixer is an essential part of proper operation and calibration of the ImageStream®X MkII. It assures that the SpeedBead® reagent is maintained at the proper concentration when being used by the system. On rare occasions, this clamp may break and need replacing. Replacement parts can be obtained by contacting your Luminex service representative or by email at [support@luminexcorp.com](mailto:support@luminexcorp.com). The part can be easily be installed following these instructions.

The only tool required is a 3mm hex wrench.



1. Power down the ImageStream<sup>X</sup> MkII and unplug it from the wall.
2. Remove the bead vial and set it aside. Do not remove the bead line or alter the bead vial.
3. Remove the left panel.
  - a. Use the side of a 3mm hex wrench to pull the hood lock back and open the hood. The hood lock is located on the underside of the hood seam protrusion.
  - b. Push the left panel lock down and angle the side panel to lift it out.
4. Locate the bead mixer assembly.



5. Remove the broken bead by removing the two 3mm screws and pulling the broken bead clamp through the bulk-head.
6. Position the new bead clamp and tightly reinstall the bead clamp screws.
7. Replace the side panel and close the hood.
8. Verify the clamp is secure by powering up the system and checking the bead tube mixes normally on startup.

## Sterilize the System

The ImageStream®X MkII instrument's fluidic system is automatically sterilized after each day's use. This reduces the occurrence of clogging.

## Disconnect the System

1. Remove the plug from the socket outlet to disconnect the instrument from the power supply.

**NOTE:** The socket outlet must be located in the vicinity of the instrument and in full view of the operator. Do not position the instrument so that disconnecting the power cord is difficult.

**NOTE:** To immediately turn the instrument off (if necessary), remove the plug from the socket outlet.

## Clean the Instrument

1. Clean spills on the exterior of the ImageStream®X MkII with a mild detergent.
2. Using gloves, clean the sample portal and sample elevator with a 10% bleach solution.
3. Dispose of waste using proper precautions and in accordance with local regulations.

## Chapter 8: Storing the System

Before storing the ImageStream®X MkII, shut down the system. The ImageStream<sup>X</sup> MkII has a thorough automatic shutdown procedure that is initiated by pressing the shutdown button, typically at the end of the day. The shutdown procedure will first flush the system with water, run detergent through the fluidics lines, followed by the sterilizer, debubbler and finally, water. The system then turns itself off, with water filling the fluidics lines, allowing you to safely store the system.

Luminex recommends that the shutdown procedure be run once per month to maintain the system fluidics.

# Chapter 9: Shipping and Decontaminating the System

Luminex® offers an instrument relocation service so the ImageStream®X MkII system can be safely uninstalled, transported and installed in a new location. Luminex can also help in arranging long distance shipping of the system.

Please contact Luminex Technical Support at [support@luminexcorp.com](mailto:support@luminexcorp.com) for assistance with shipping and decontaminating the ImageStream®X MkII system.

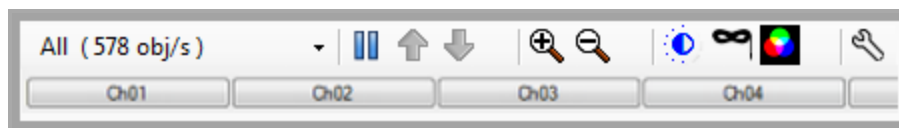
# Chapter 10: Software Functionality

## INSPIRE™ Software

### The Image Gallery

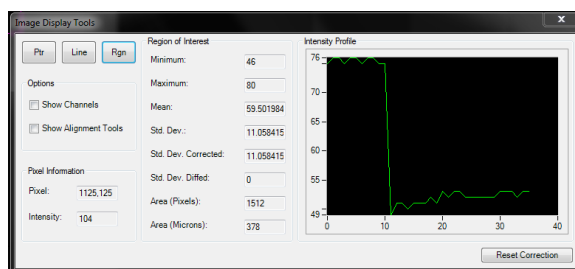
Images are displayed in the image gallery during setup and acquisition.

#### Image Gallery Tools



Icon	Name	Description
	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
	Channel Name	Click to change selection of channels to view/acquire

## 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.



## Analysis Area Tools

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.



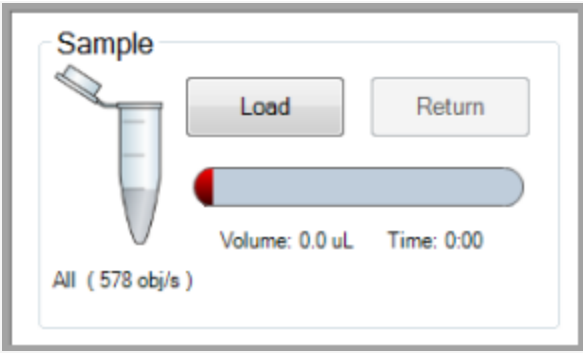

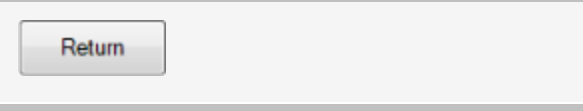
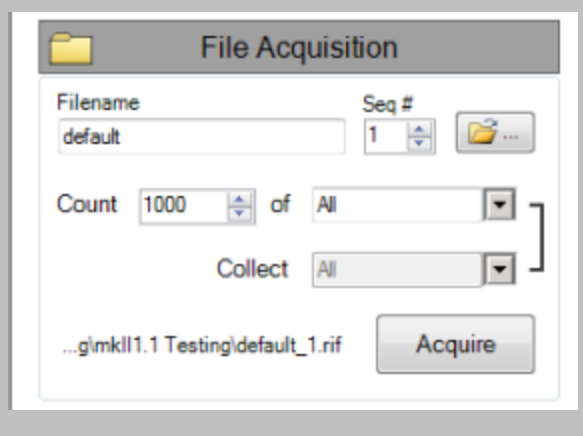
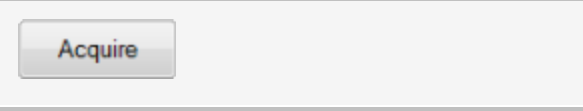

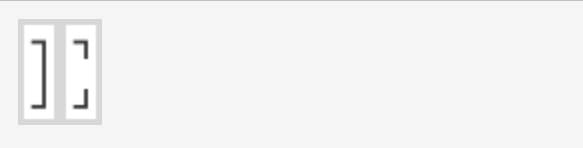
Icon	Name	Description
	Reset	Refreshes the graphs with incoming data
	Histogram	Opens the histogram graph tool
	Scatter Plot	Opens the bivariate scatter plot tool
	Pointer	Reset cursor to pointer
	Line region	Draw a line region on a histogram
	Rectangle region	Draw a rectangular region on a scatterplot
	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

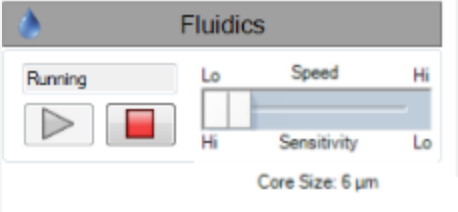


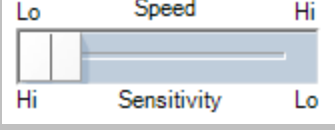
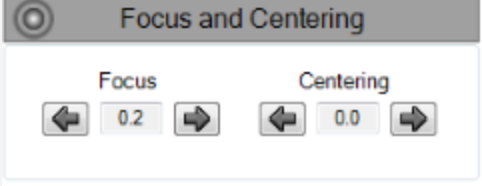
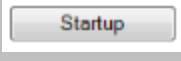
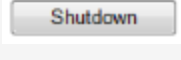
The Instrument Control Panel is divided into several functional sections:

- Sample:** Includes a pipette icon, 'Load' and 'Return' buttons, a progress bar, and status information: 'Volume: 36.2 uL Time: 130:16', 'R1 NOT SB ( 0 obj/s)', and 'All ( 144 obj/s)'.
- File Acquisition:** Features a folder icon, 'Filename' (VKII493 RBC-EV-APC dil 1to30 t) and 'Seq #' (7) fields, 'Count' (1000000) and 'of' (R1 & NOT SB) dropdowns, 'Collect' (All) dropdown, a file path, and an 'Acquire' button.
- Illumination:** Contains laser power controls for 405 (120.00 mW), 488 (200.00 mW), 561 (100.00 mW), 592 (100.00 mW), and 642 (150.00 mW) lines. Includes 'SSC' (10.00 mW) and 'Brightfield' (green indicator) settings, 'Scatter Channel' (6) and '1 and 9' dropdowns, and a 'Set Intensity' button.
- Magnification & EDF:** Shows a 'Magnification' slider (20X to 60X) and checkboxes for 'EDF' and 'High Gain'.
- Fluidics:** Includes a 'Running' status indicator, 'Speed' and 'Sensitivity' sliders (Lo to Hi), and 'Core Size: 6 μm (Override)'.
- Focus and Centering:** Features 'Focus' (value: -3.8) and 'Centering' (value: 0.0) controls with directional arrows.

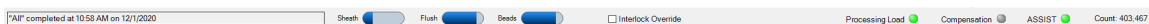
At the bottom of the panel are 'Startup' and 'Shutdown' buttons.

	<p>In the <b>Sample</b> section, you can load a sample or return a sample.</p> <p>Sample time remaining is displayed when a sample is running.</p>
	<p>Loads the sample</p>
	<p>Returns the sample</p>
	<p>In the <b>File Acquisition</b> 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.</p>
	<p>Begin Acquisition</p>
<p>Filename</p>	<p>Type the filename</p>
<p>Seq#</p>	<p>Choose the beginning sequence number</p>
	<p>Navigate to the folder to save the data</p>
<p>Count</p>	<p>Enter the number of events to collect in the box.</p>
<p>of</p>	<p>Choose the population to collect in the drop-down menu.</p>
	<p>Collect a different population while counting the first population. Click on the bracket to break the link and select population.</p>

	<p>In the <b>Illumination</b> 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.</p>
	<p>405nm laser excitation - currently set to OFF and 0 mW of power.</p>
	<p>488nm laser excitation- currently set to ON at 60 mW of power.</p>
	<p>642nm laser excitation- currently set to ON at 150 mW of power.</p>
	<p>SSC (side scatter) laser excitation- currently set to OFF at 2.00 mW of power. SSC is produced from a dedicated 785 nm laser.</p>
	<p>Brightfield illumination is shown as ON in channels 1 and 9.</p>
	<p>Sets the Intensity of the brightfield to 800 counts.</p>
	<p>Select the magnification.</p> <p><b>NOTE:</b> This is optional equipment. Turn EDF on by checking the EDF box.</p>
	<p>Select high gain mode.</p> <p><b>NOTE:</b> This is optional functionality. Turn high gain on by checking the <b>High Gain</b> box.</p>

 The Fluidics control panel features a 'Running' indicator, a play button, a red stop button, and two sliders for 'Speed' and 'Sensitivity'. The 'Speed' slider is at the 'Lo' end and the 'Sensitivity' slider is at the 'Hi' end. Below the sliders, it displays 'Core Size: 6 µm'.	Adjust the speed and sensitivity for the run. See "Set ImageStream®X MkII Speed and Sensitivity" for more information on speed, sensitivity and resolution.
 A play button icon.	Run fluidics.
 A red square stop button icon.	Stop fluidics.
 A detailed view of the Speed and Sensitivity sliders. The Speed slider is positioned towards the 'Lo' end, and the Sensitivity slider is positioned towards the 'Hi' end.	Speed and Sensitivity are inversely related. See "Set ImageStream®X MkII Speed and Sensitivity" for more information on speed, sensitivity and resolution.
 The Focus and Centering control panel includes a target icon, 'Focus' controls with left and right arrows and a '0.2' value, and 'Centering' controls with left and right arrows and a '0.0' value.	Focus and Centering can be adjusted using the right and left arrows.
 A button labeled 'Startup'.	Runs the startup script.
 A button labeled 'Shutdown'.	Runs the shutdown script and sterilizes the system.

## Bottom Task Bar



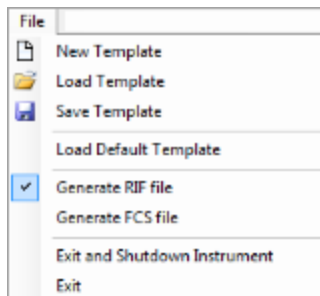
Status buttons are displayed at the bottom of the INSPIRE window.

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

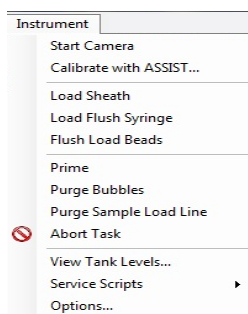
## Menu Bar

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

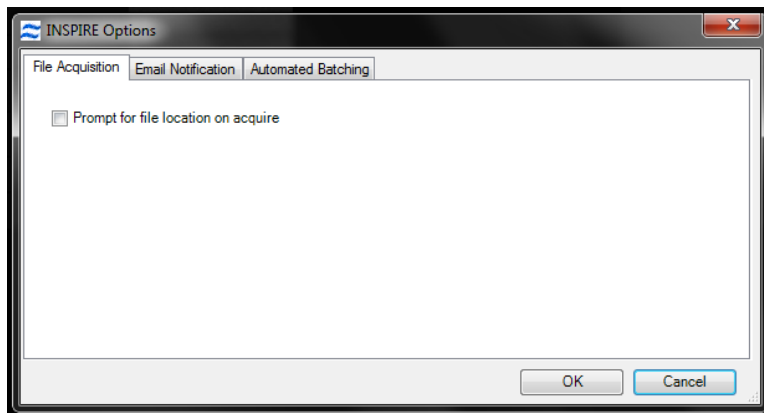
- **File** menu: Load and save instrument setup templates. A template contains instrument settings that can be pre-defined and loaded to simplify the instrument setup process.



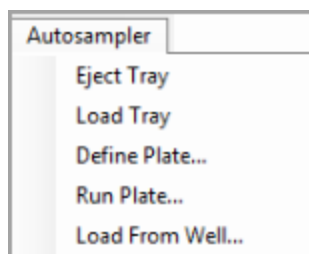
- **New Template:** Create a new template.
  - **Load Template:** Browse for and open saved templates.
  - **Save Template:** Save your settings as a template for future use. Template file names are appended with the suffix .ist. They are saved in the INSPIRE Data folder.
  - **Load Default Template:** 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 MkII 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 flowcell.
- **Purge Bubbles:** Removes air bubbles from the flowcell by filling the flowcell with air then filling the sheath line and pump with debubbler and rinsing the flowcell. The sheath syringe is then refilled with sheath and the bubble trap, lines and flowcell 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:**



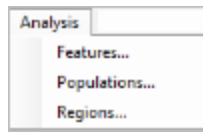
- **Autosampler menu:** Access autosampler controls.



- **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.



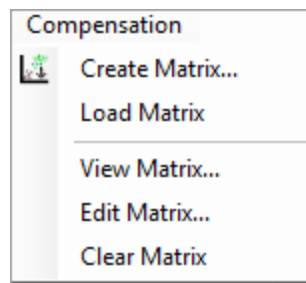
- **Analysis** menu: Access the Feature, Population and Region Managers. Functionality is the same as for IDEAS. Refer to the IDEAS user manual for more information.



- **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.

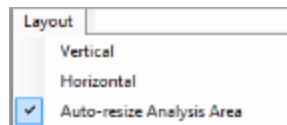
**NOTE:** See IDEAS User Manual for more information about Analysis options.

- **Compensation** menu: View, edit or create a new compensation matrix.



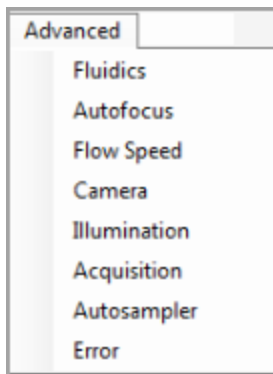
- **Create Matrix:** Opens the compensation wizard.
- **Load Matrix:** Applies compensation to the Intensity features.
- **View Matrix:** Opens the compensation matrix values table.
- **Clear Matrix:** Stops applying compensation of Intensity features.

- **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 automatically 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<sup>X</sup> MkII**: Access the current INSPIRE version number with the About ImageStream<sup>X</sup> MkII option.

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## ASSIST Tab

ASSIST (**A**utomated **S**uite of **S**ystemwide ImageStream<sup>X</sup> **T**ests) is a suite of calibrations and tests for critical subsystems operating within the ImageStream<sup>®X</sup> MkII. 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<sup>X</sup> MkII.

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<sup>X</sup> MkII.

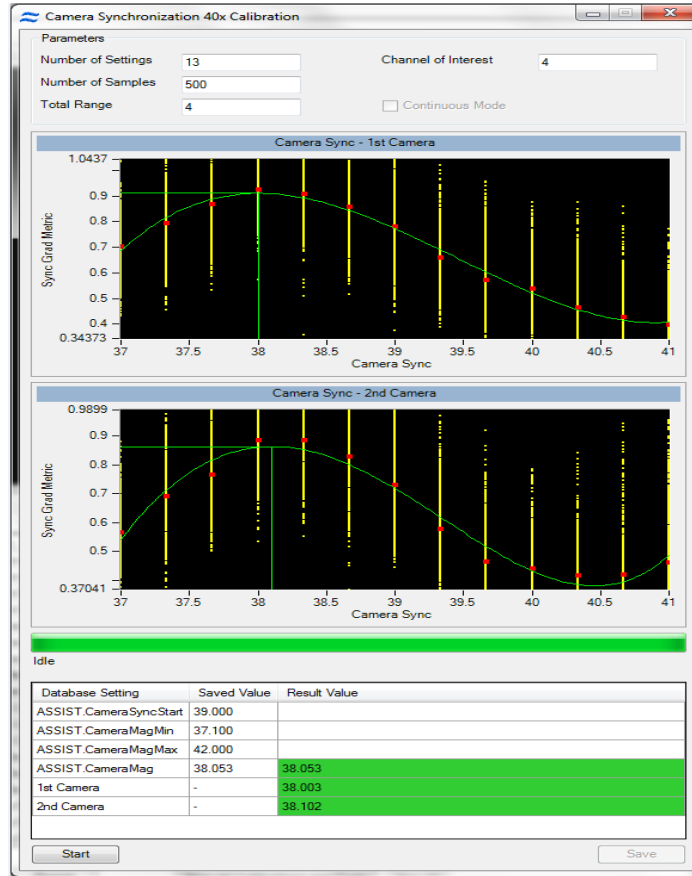
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## ASSIST Calibrations

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

# Camera Synchronization Calibration

This calibration measures and stores a magnification calibration (camera sync) 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.

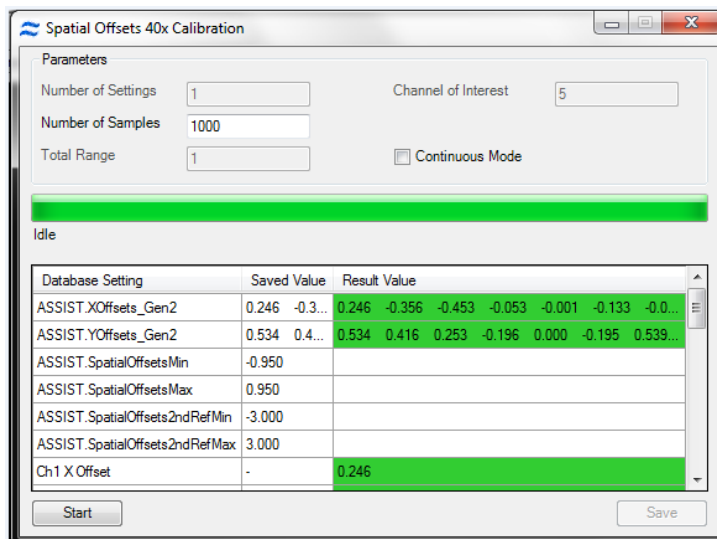


As shown in the figure above, the camera sync calibration measures SpeedBead® ellipticity at numerous discrete camera sync settings and plots the camera sync setting (horizontal axis) versus the ellipticity (vertical axis). It then generates the best fit curve for a 4th order polynomial through the data and determines the horizontal location (camera sync) 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.

**NOTE:** Camera Synchronization Calibrations will be done for each magnification present in the system.

## Spatial Offsets Calibration

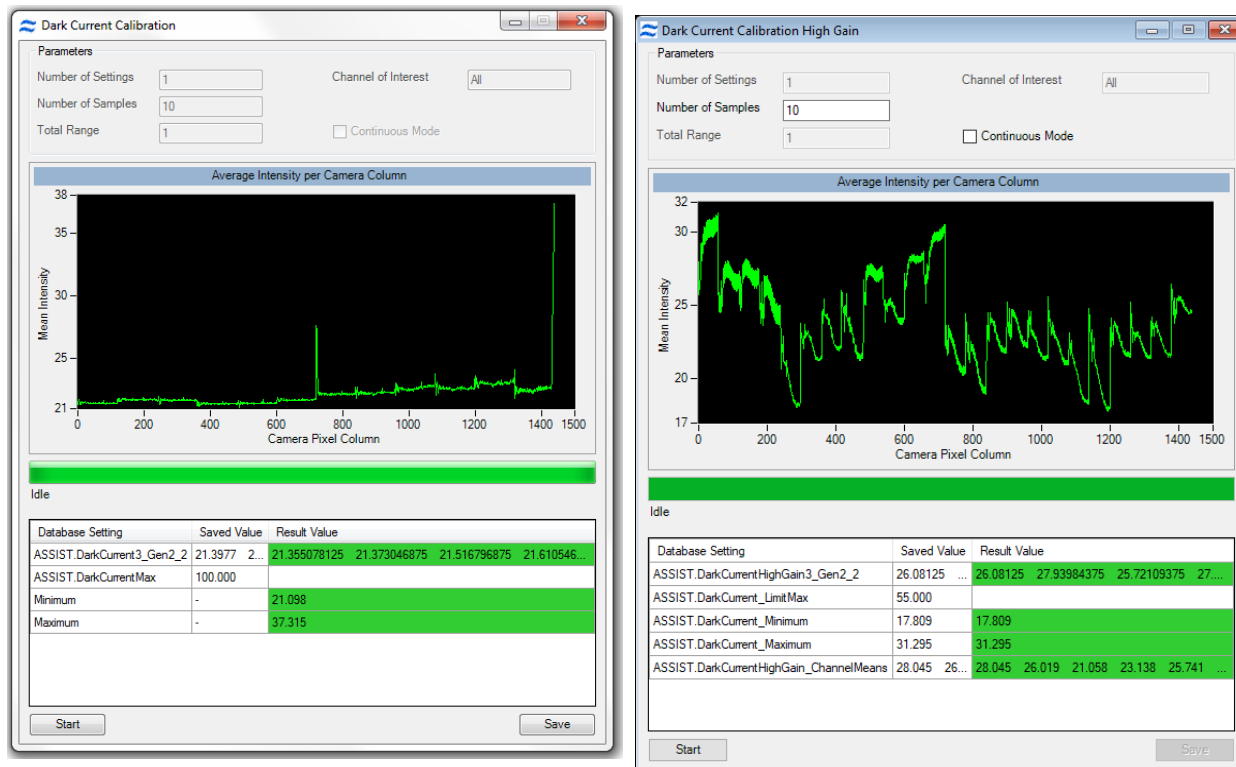
Measures and stores 12 calibration factors for the vertical and horizontal registration of each spectral channel of the ImageStream®X MkII. Many assays that are run on the ImageStream®X MkII quantify the spatial relationships between molecules located within cells of interest. To accurately perform these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



The Spatial Offsets 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 auto-correlation 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 auto-correlation 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

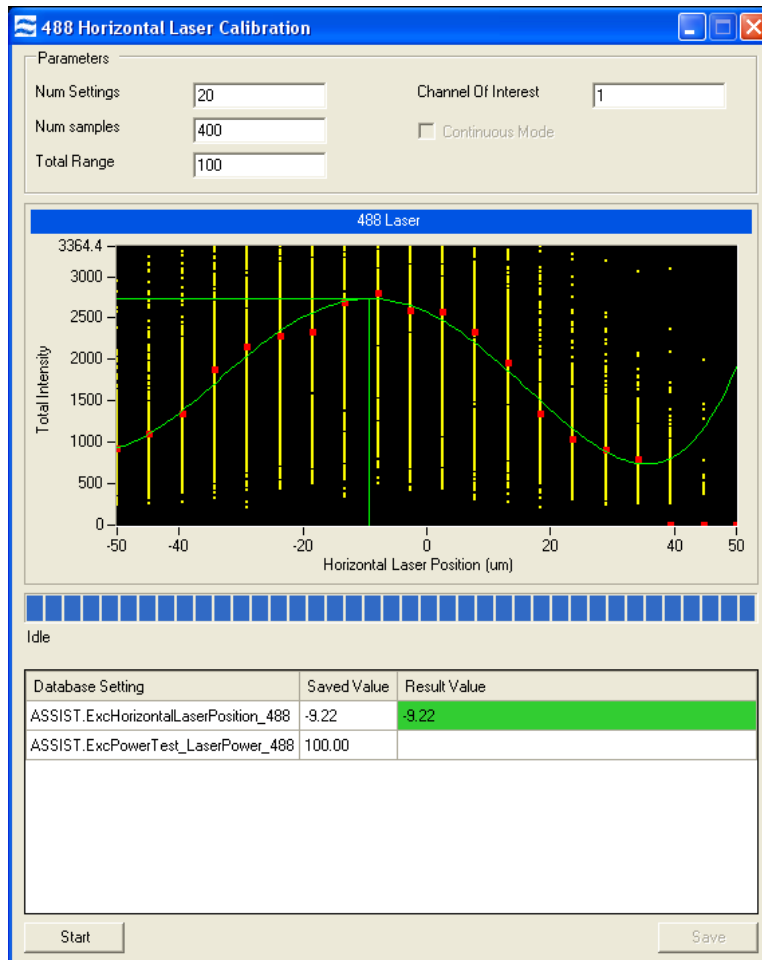
Measures and stores 3072 offset values corresponding to pixel columns in the TDI camera. Every pixel in a CCD detector is an individual sensor with its own sensitivity characteristics. In the absence of any light, each pixel emits a signal, known as dark current. Although the statistical variation of 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 MkII 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. For systems with high gain available, it is run both without high gain active and with high gain active.



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 option is installed, the Dark Current calibration will be simultaneously performed for both cameras.

## Horizontal Laser Calibration

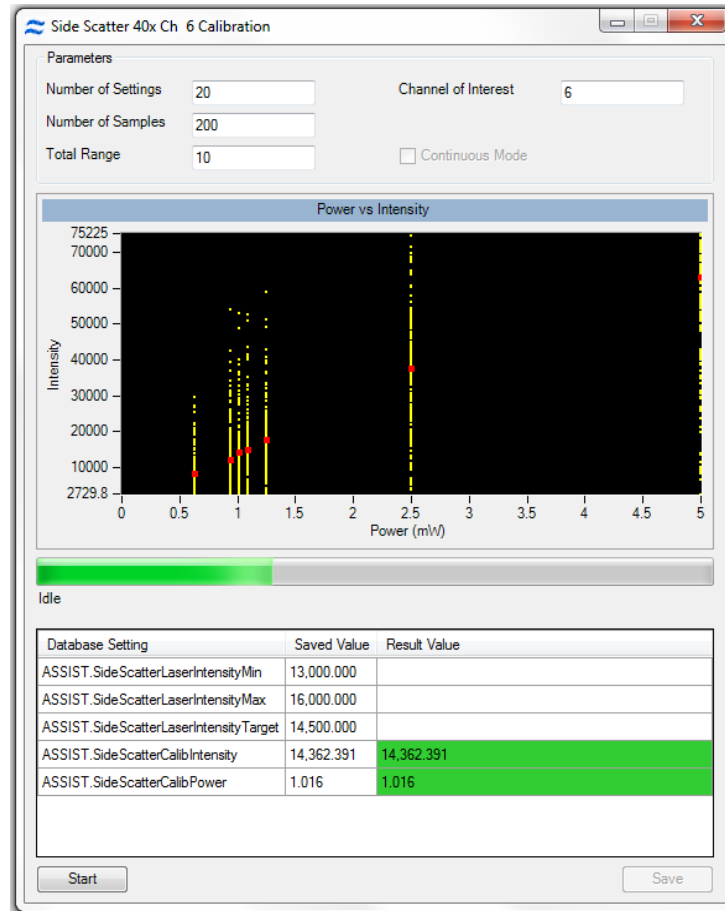
The alignment of each laser in the ImageStream®X MkII is automatically controlled to ensure optimal performance via the Horizontal Laser Calibration. The calibration routine sweeps the horizontal position of the laser across the flow stream. At each of 15 predefined intervals during the sweep, 1000 SpeedBead® 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

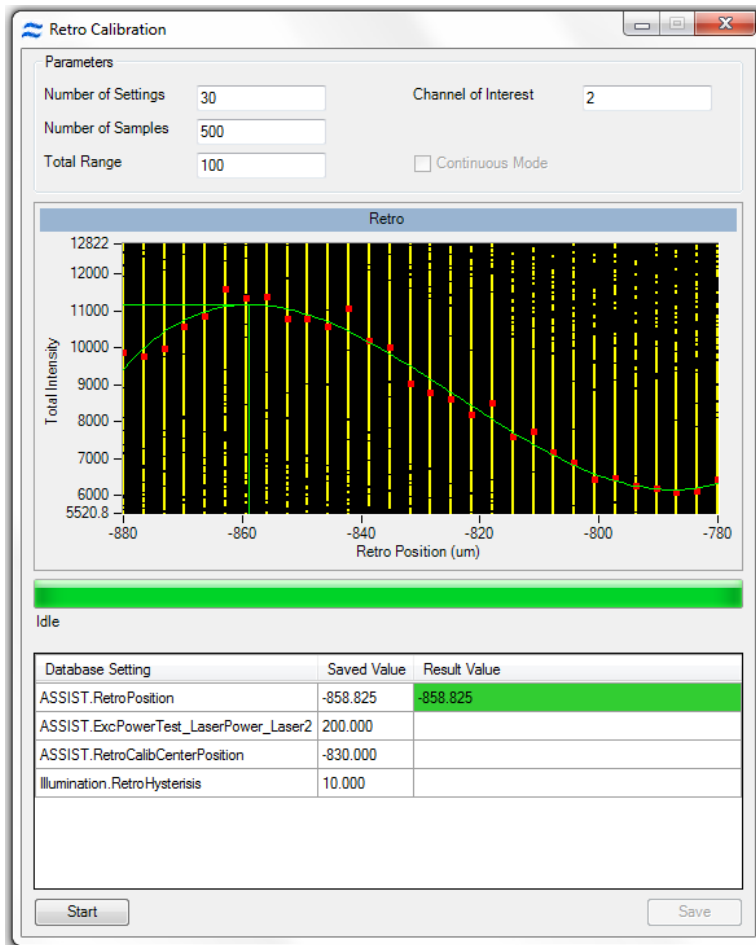
The purpose of this calibration is to set the power of the 785nm laser. The calibration routine consists of measuring SpeedBead® intensities at a predefined power setting and then actively adjusting the power to achieve 7200 counts of light per bead. This calibration ensures a consistent intensity for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



## Retro Calibration

The ImageStream®X MkII 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 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 MkII 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.

Autosampler Nest Calibration

Parameters

Number of Settings: 2  
Number of Samples: 1  
Total Range: 1  
Channel of Interest: N/A  
 Continuous Mode

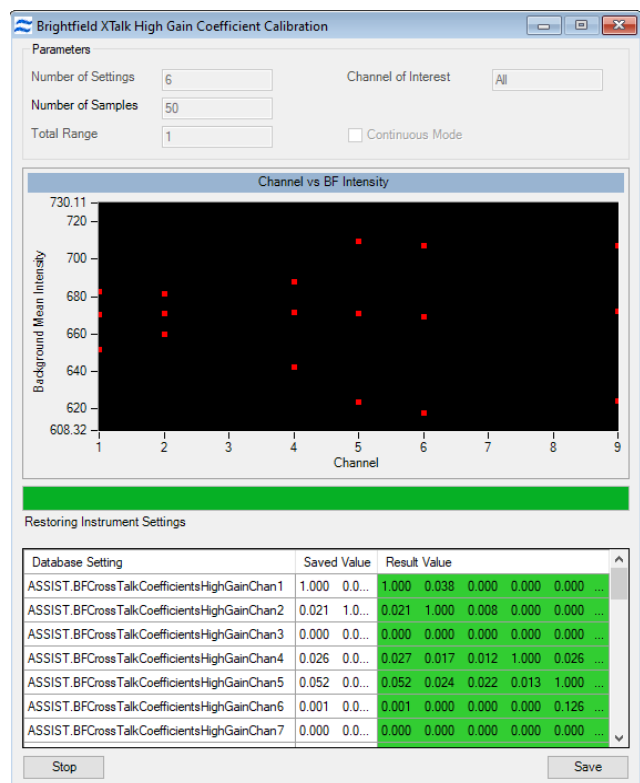
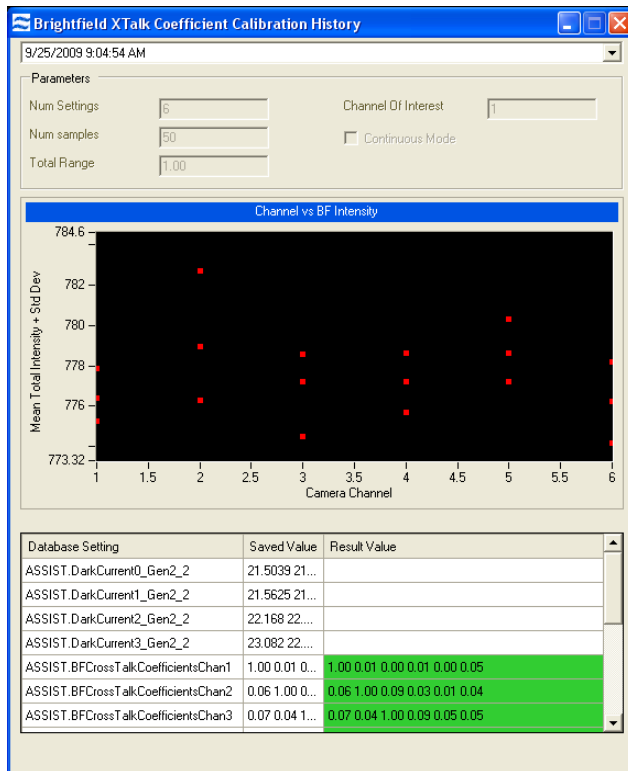
Idle

Database Setting	Saved Value	Result Value
ASSIST.NestCalibrationStatus	1.000	1.000

Start Save

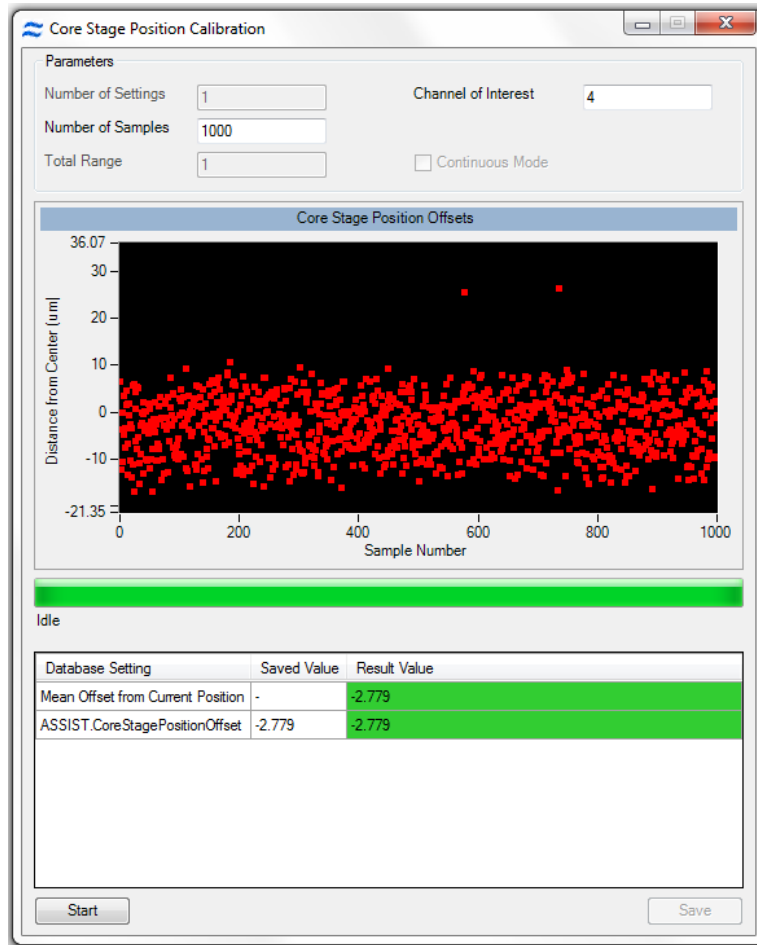
# Brightfield Crosstalk Coefficient Calibration

The brightfield cross talk calibration measures the amount of spectral leakage between channels using the brightfield illuminator. For systems with high gain available, the calibration runs both without high gain active and with high gain active. 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, calculates an offset from the factory setting, and sets the position of the stage in the X dimension.



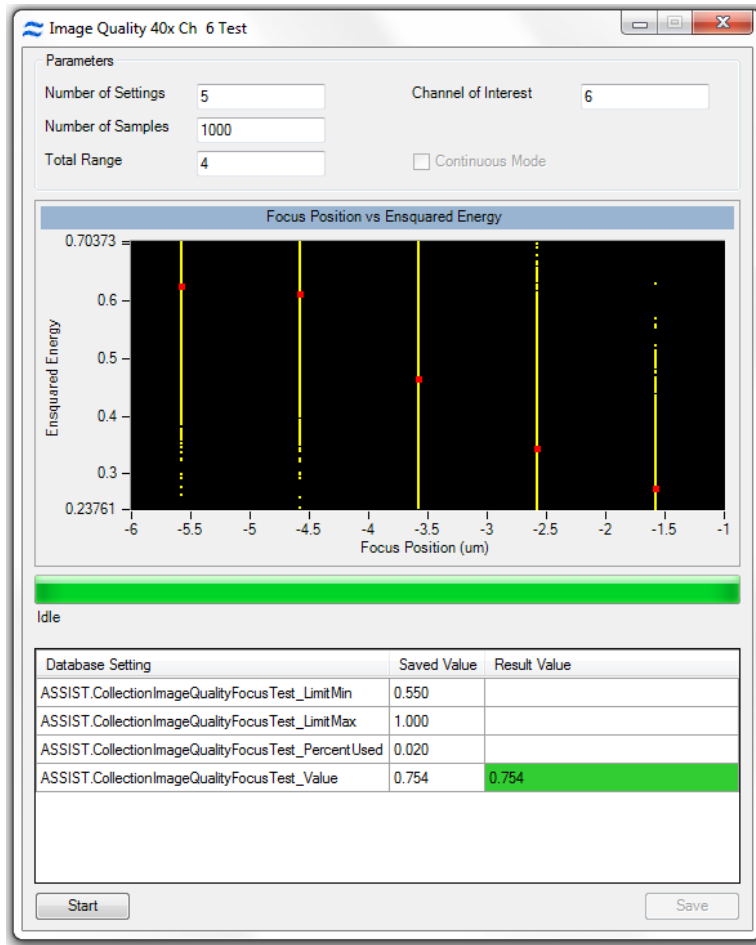
## ASSIST Tests

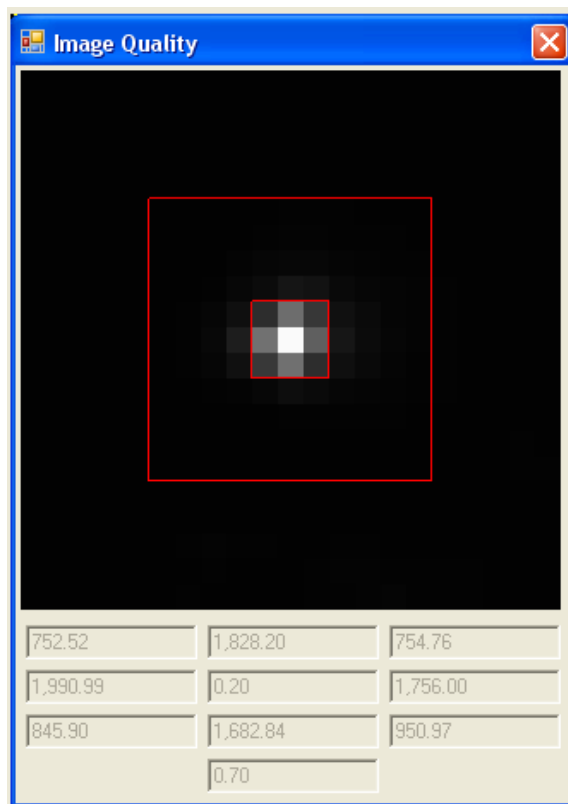
A test is a sequence of operations designed to measure the performance of a specific subsystem. When a test is performed, one or more test parameters are generated and evaluated against predefined limits. The test results and acceptable limits are listed on the ASSIST display tab. Values outside of accepted 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 eleven tests in the current suite are described in detail below.

## Image Quality Ensquared Energy Test

This 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 1 µm in diameter.

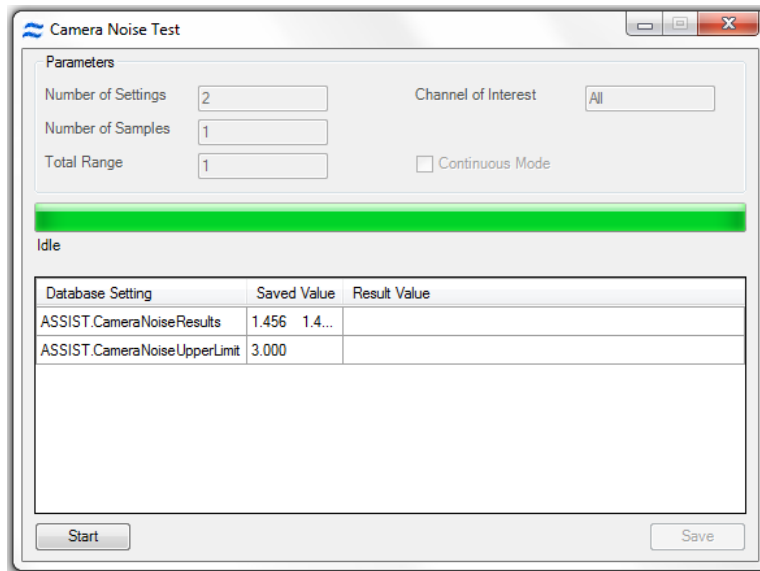




The test also reports regional scores which are not tested against limits. The scores include the energy ratios for line profiles in the horizontal and vertical axes, displayed at the bottom of the regional score grid, and summed energy values for the horizontal, vertical and diagonal directions radiating 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, the Image Quality Ensquared energy test will be performed for each magnification.

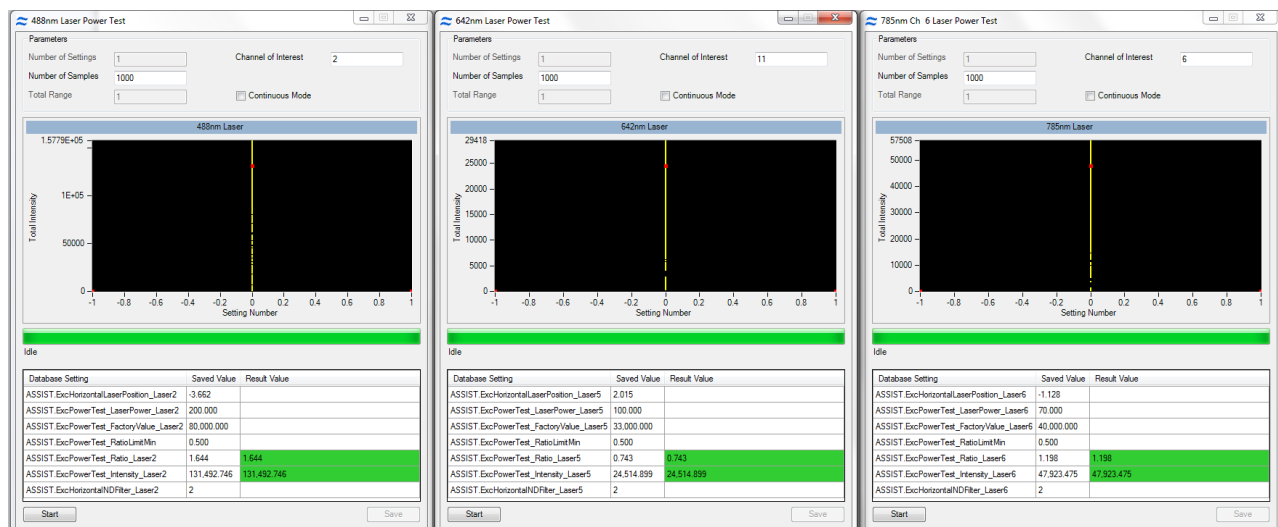
## Camera Noise Test

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.



## Excitation Laser Power Tests

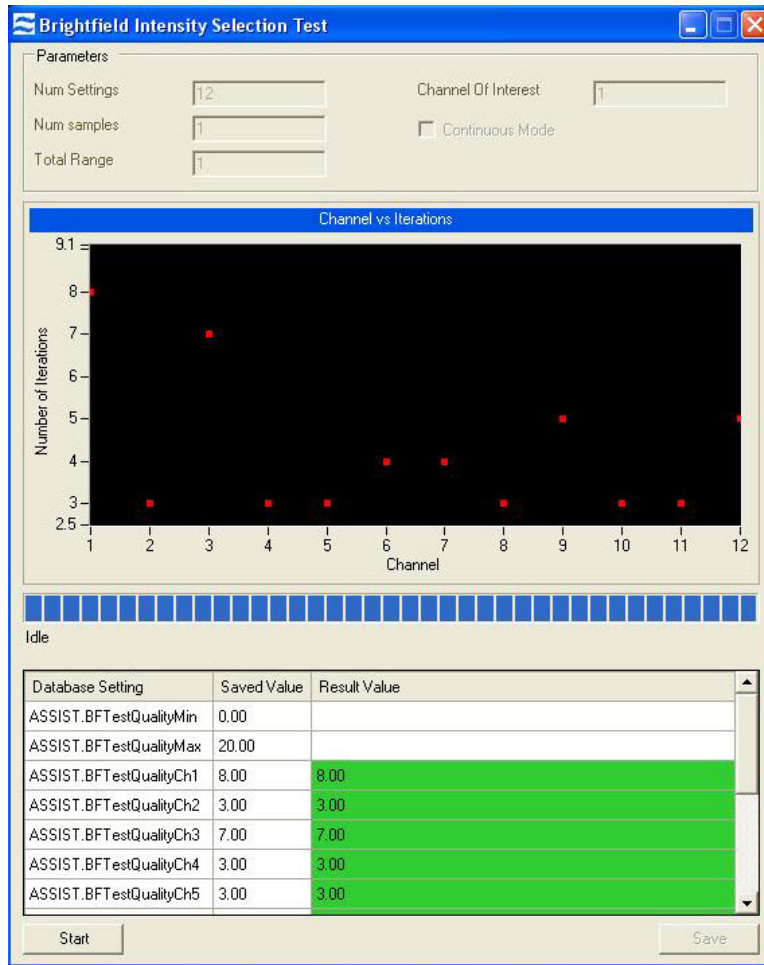
The power of each excitation laser present in the system is measured and tested against limits by quantifying the amount of light scattered from SpeedBeads. The instrument is configured specifically to test each laser by adjusting classifiers, setting stage selections and inserting the proper neutral density filters into the collection path. The test compares the mean signal strength acquired from each laser to radiometrically 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.

## Brightfield 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.

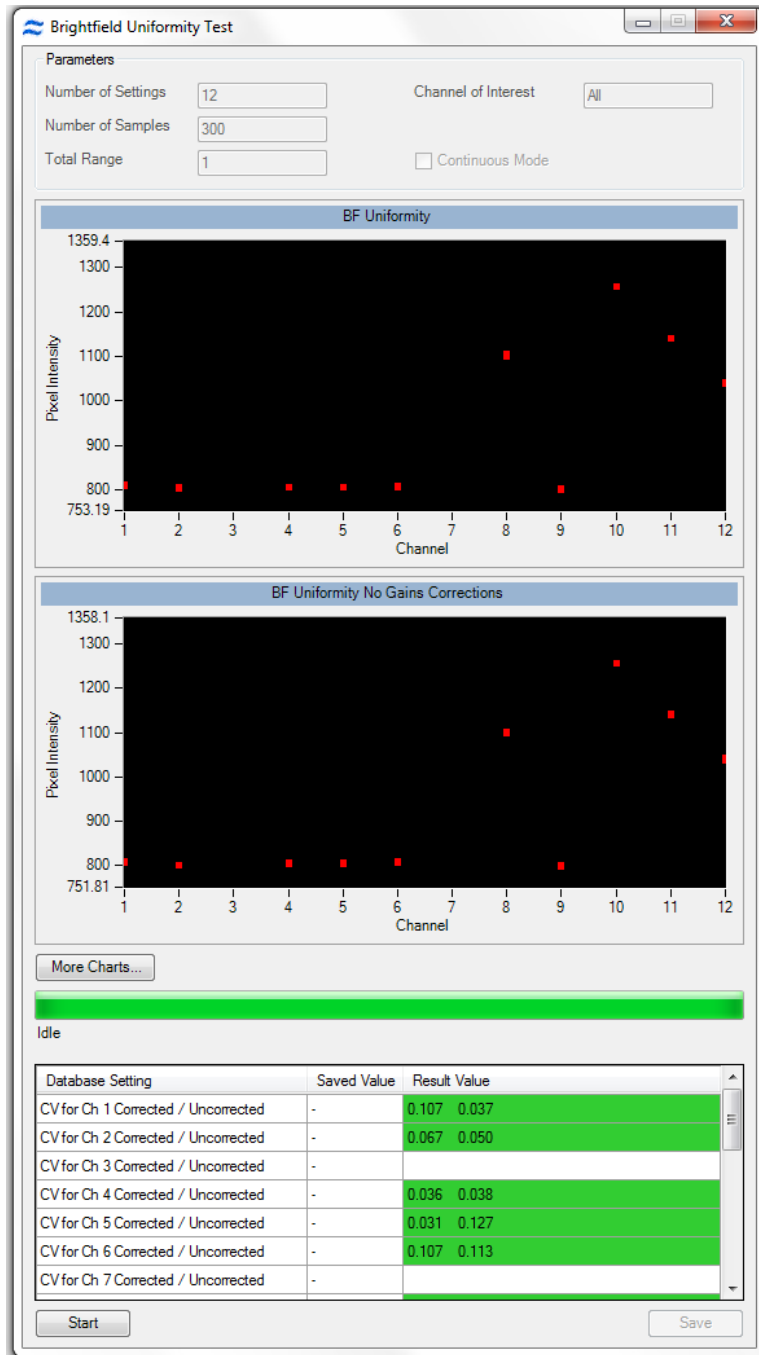


The results and limits of the test are shown below the list when the test is selected.



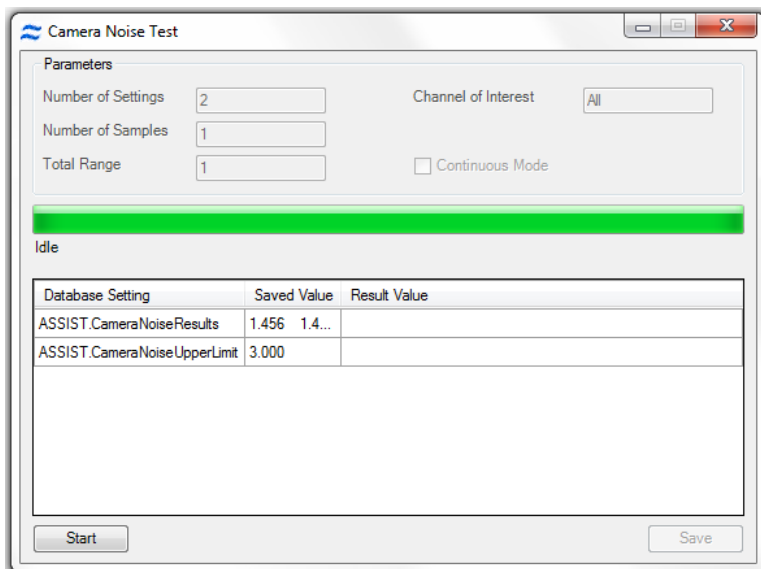
## BF Uniformity Test

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 to ensure the illumination and collection systems are providing a uniform photometric response.



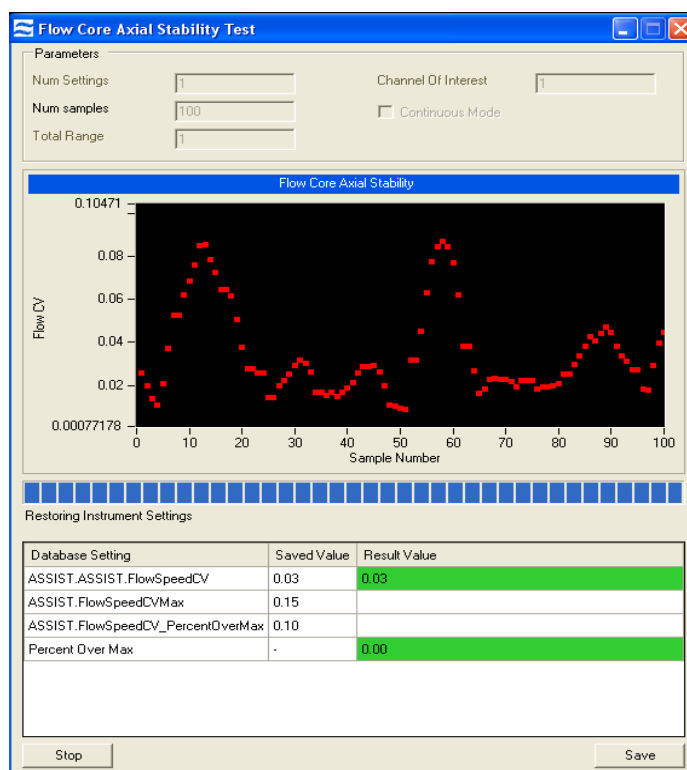
## Camera Noise Test

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

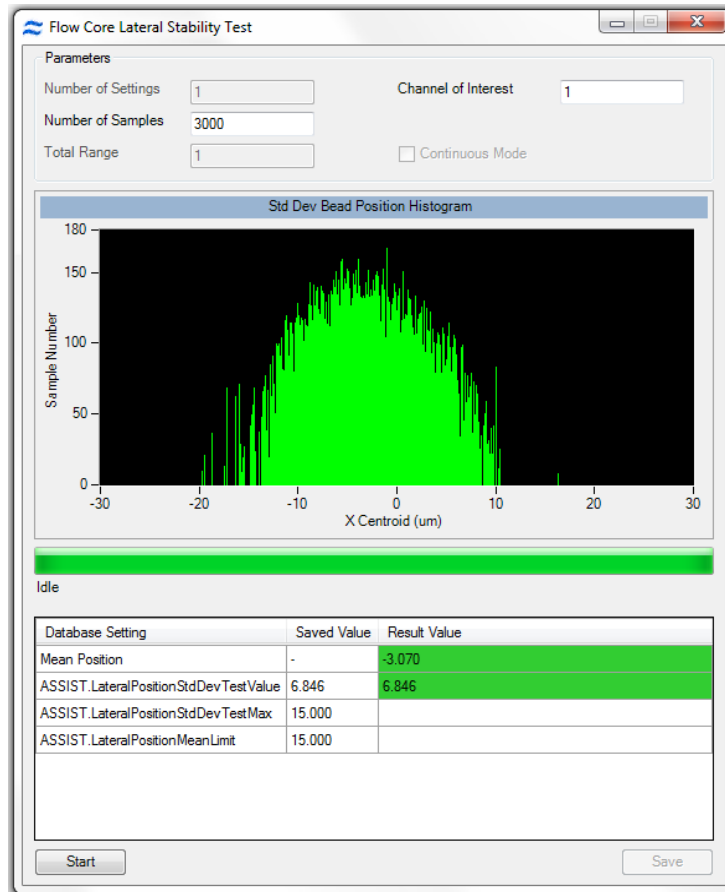
Measures the stability of the core stream velocity over time. Measures the variation in the speed of the core stream as a percentage of the mean sample speed. The ImageStream®X MkII is designed to automatically sterilize, cleanse and purge air from its fluidics systems after every day of operation. Improper sterilization, contaminants, partially clogged fluidic lines, air bubbles or non-homogenous sheath solution can lead to excessive sample speed variation. Although the ImageStream®X MkII 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 hydro-dynamically 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

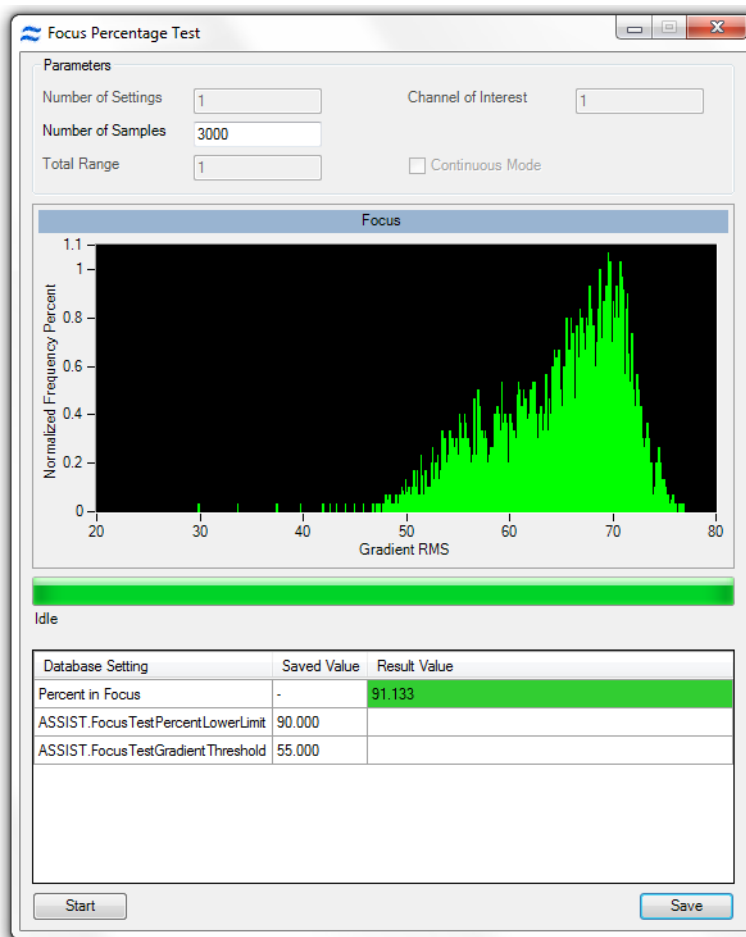
Measures the position of the core relative to its ideal position within the flow cuvette. The ImageStream®X MkII uses sheath flow to hydrodynamically focus objects within a precise region in the cuvette. Improper sheath solution, protein buildup, micro-bubbles and other factors can alter the position 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.

Database Setting	Saved Value	Result Value
ASSIST.FlowCorePositionTest_FocusDeltaMax	15.00	
Calculated Focus Position Delta	-	1.49
ASSIST.FlowCorePositionTest_FocusPosValue	-219.16	-218.99
ASSIST.FlowCorePositionTest_FocusDefaultValue	-217.50	
	-	
ASSIST.FlowCorePositionTest_LateralDeltaMax	30.00	
Calculated Lateral Position Delta	-	9.99

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

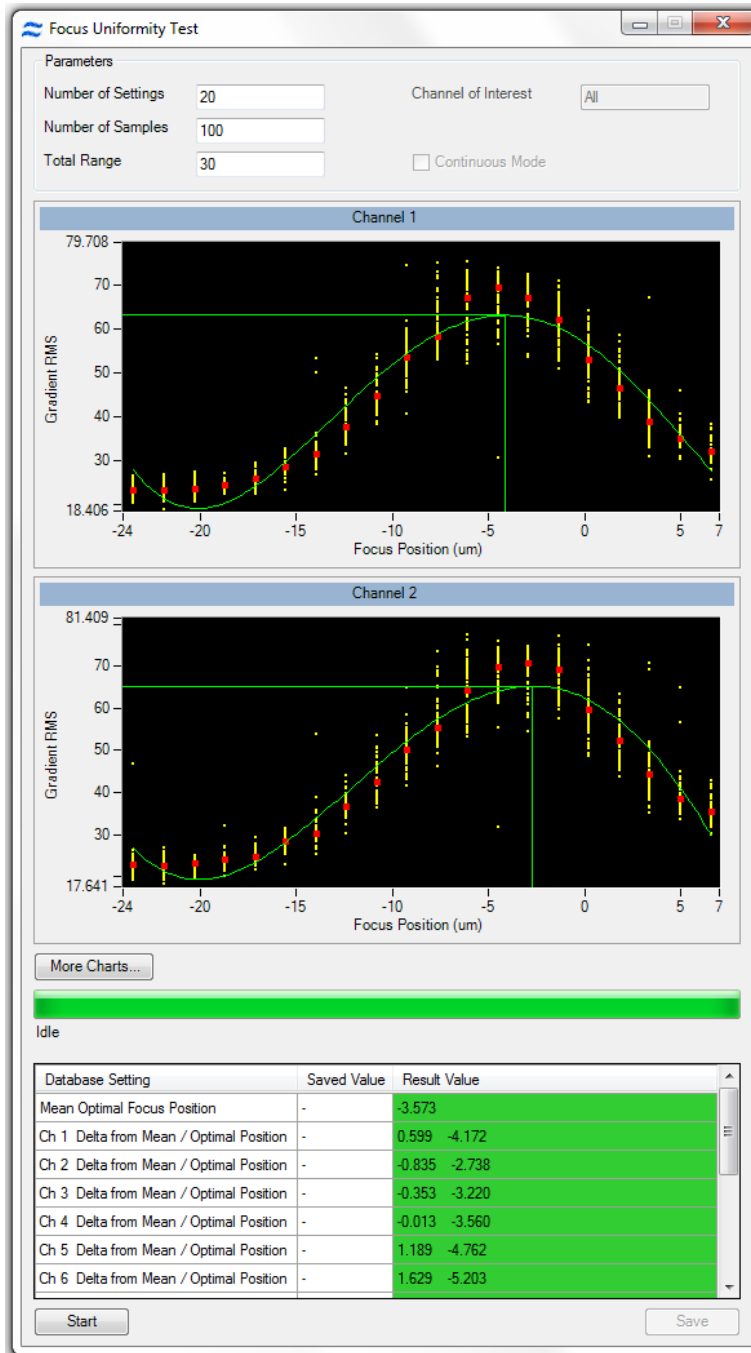
## Focus Percentage Test

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



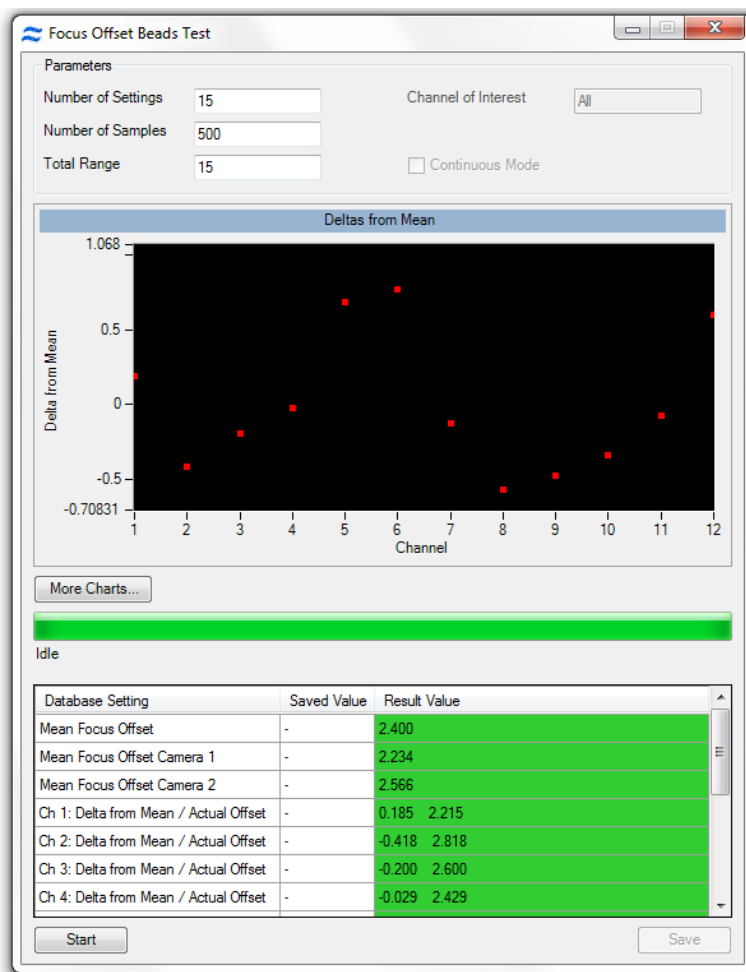
# Focus Uniformity Test

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.



## Focus Offset Bead 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 pre-determined limits and stored in the ASSIST database. If the MultiMag option is installed, a focus offset test will be performed for each magnification.



## 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.



## Autosampler 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 Bright-field (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.

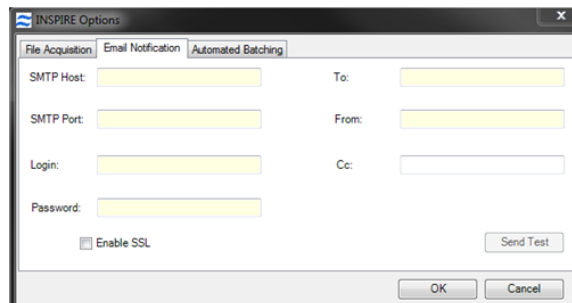
When the validation is 'Yes' for the well, the object rate and synchrony is then tested and must pass some pre-determined limits. Focus adjustment is enabled. This ensures good imagery is collected.

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

## Setup 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.

1. From the **Instrument** menu, choose **Options**.
2. Select the **Email Notification** tab.
3. Enter the following information. Your IT department must provide this information and 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.

## AutoSampler Report

- 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 is stopped manually or when it has completed the entire plate.
- 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.

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