ImageXpress® Ultra
Confocal High Content Screening System

User Guide

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Molecular Devices

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Contents

Foreword 9
Purpose of this Manual 9
Conventions 9
Safety Information 10
Safety Labels 11
Warning Labels 13
Protective Housing 13
Interlock Failure 14
Non-interlocked Panels 14
Laser Safety Information 14
High-Voltage Hazard 16
Moving Parts 16
Fuses 16
Power Supply 16
Lifting Hazard 17
Maintenance and Service 17
Hazardous Material Precautions 17
Obtaining Support 18

Chapter 1 Introduction 19
ImageXpress Ultra System Overview 19
System Installation 20

Chapter 2 Quick Start Guide 21
Turning on the ImageXpress Ultra System 21
Starting the Software 22
Logging AxoTrace Messages to a .txt File 23
Setting up and Running Plate Acquisition 25
Turning off the ImageXpress Ultra System 25

Chapter 3 Imaging System Overview 27
Fluorescence Imaging 27
Confocal Imaging 28
Electronics 29
Imaging Optics 29
Excitation Lasers 29
Beam Combiners 30
Beam Expander 30
Galvanometer 30
Relay Lens 31
Main Beam Splitter 31
Objective Lenses 32
Tube Lens 32
Motorized Iris 33
Emission Filters 33
Typical ImageXpress Ultra System Filter Cube Configurations 35
Photomultiplier Tubes 37
Objective (Z) Stage 37
Motorized Z Stage 37
Motorized Objective Changer 37
Sample X-Y Stage 38
Sample 38
Plate Holder and Plate Clamp 38
Motorized X-Y Stage 38
Autofocus Laser 38

Chapter 4 Post-Installation and Testing 39
Verifying Device Settings in the Meta Imaging Series Administrator 39
Verifying Acquisition Settings in the Meta Imaging Series Administrator 50
Verifying and Backing Up Settings in the MetaXpress Software 51
Verifying and Backing Up Magnification Settings 52
Verifying and Backing Up Calibration Settings 53
Verifying the Laser Autofocus Sensor 54
Verifying the Plate Reference Point (A1 Center) 57

Verifying Plate Types 61
Adding Pre-configured Plate Types 61
Confirming Laser Autofocus Settings for Plate Files 62
Verifying Shading Correction Files 62

Chapter 5 Guidelines for Planning Experiments 63

Planning High-Content Experiments 63
Obtaining Quality Images 64
Additional Guidelines for Planning Experiments 65
Sample Preparation 65
Laser Power 65
Objective Choice 67
Use of Different Fluorophores 68
Dyes and Applications for the ImageXpress Ultra System 68
Incompatible Probes 76
Plate Selection 77

Chapter 6 Setting Up Plate Acquisition 79

Overview 80
Screening Menu 80
Plate Acquisition and Control dialog 81
Plate Acquisition Setup dialog 82
Creating Experiments 83
Naming the Experiment 86
Configuring Binning, Averaging, and Scan Lines Settings 87
Binning Overview 87
Scan Lines and Averaging Overview 89
Combined Binning and Averaging 89
Determining Plate Optical Thickness and Plate Bottom Variation 91
Configuring Plates 91
Optical Thickness and Laser Focus 94
Configuring the Wells to Visit on a Plate 95
Configuring the Sites to Visit on a Plate 96
Configuring Cell Counting 100
Setting Multiple Timepoints to Acquire Images 102

Defining Protocol Settings 105
Generating Shading Correction Files 105
PMT Gain Overview 114
Configuring Autofocus Settings 118
Configuring Laser Autofocus 118
Configuring Scans 121
Running Journals During Acquisition 125
Configuring the Display Settings 127
Selecting a Data Analysis Method 129
Reviewing the Plate Acquisition Settings 131
Saving Acquisition Settings 133

Chapter 7 Maintenance 135

User Safety Instructions 135
Filter Cubes 135
Changing Filter Cubes 136
Objectives 140
Correct Objective Placement 140
Changing Objectives 140
Configuring Objective Offsets and Parfocality 145
Entering Objective Offset and Parfocality Values 159
Cleaning Objectives 164
Adjusting the Spherical-Aberration Correction Collar on Objectives 165
Using Oil-Immersion Objectives 167
Cleaning the ImageXpress Ultra System 168

Chapter 8 Robotic Plate Handling 169

CRS Catalyst Express Robot Hardware 169
CRS Catalyst Express Robot User Procedures 169
Loading Plates onto the CRS Catalyst Express Robot . . . 169

Appendix A Objectives Compatible with the ImageXpress Ultra System 173

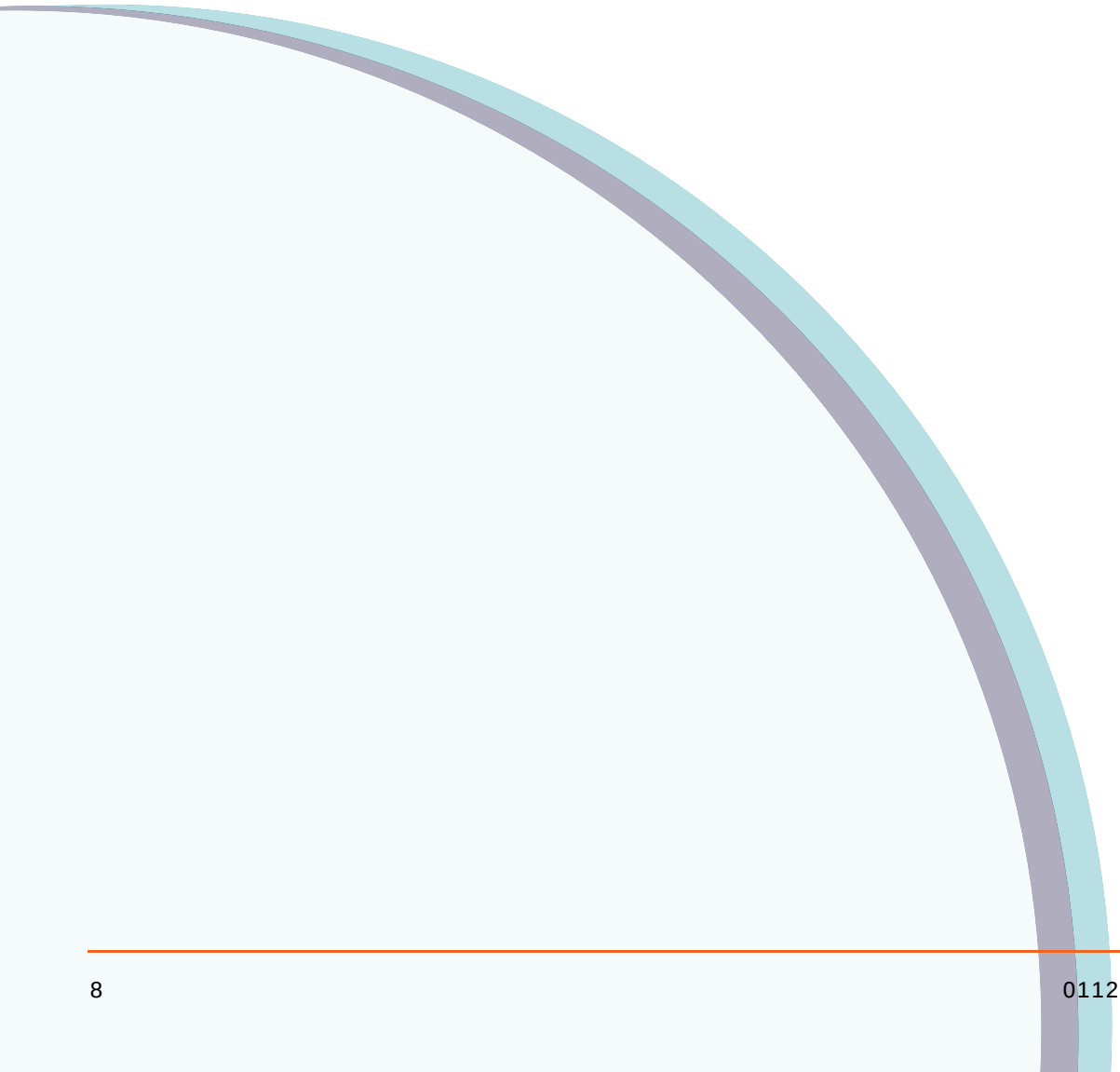
Selecting a Beam Expander for an Objective 176

Appendix B Site Requirements 177

ImageXpress Ultra System Specifications 177

Appendix C Electromagnetic Compatibility (EMC) 179
REGULATORY INFORMATION FOR CANADA (ICES/NMB-001:2006) 179
ISM EQUIPMENT CLASSIFICATION (Group 1, Class A) . . 179
INFORMATION FOR THE USER (FCC NOTICE) 179

Index 181



Foreword

This foreword contains information on the *ImageXpress® Ultra Confocal High Content Screening System User Guide* and the text conventions used. It also includes important safety information.

Purpose of this Manual

This User Guide details the technical features and specifications of the ImageXpress® Ultra System instrument and software. It also provides installation, configuration, maintenance and troubleshooting procedures. To operate the ImageXpress Ultra System, only follow the procedures in this User Guide. Using procedures other than those specified within this guide can result in hazardous conditions or injury or damage to the instrument.

Conventions

Make sure that you follow the precautionary statements presented in this guide. The conventions used in this guide are:

WARNING! Indicates a possibility of severe or fatal injury to the user or other persons if the precautions are not observed.

CAUTION! Indicates that damage to the instrument, loss of data, or individual injury could occur if the user fails to comply with the advice given.

Note: Emphasizes significant information in a procedure or description.

Tip! Provides useful information or shortcuts, but the information is not essential to complete a procedure.

Safety Information

Before using or servicing the ImageXpress Ultra System, you must be familiar with the operation and potential hazards of the instrument. You should read, understand, and obey all safety precautions. Failure to follow these safety precautions could result in serious personal injury or damage to the instrument. Warnings in this document and labels on the instrument use international symbols.

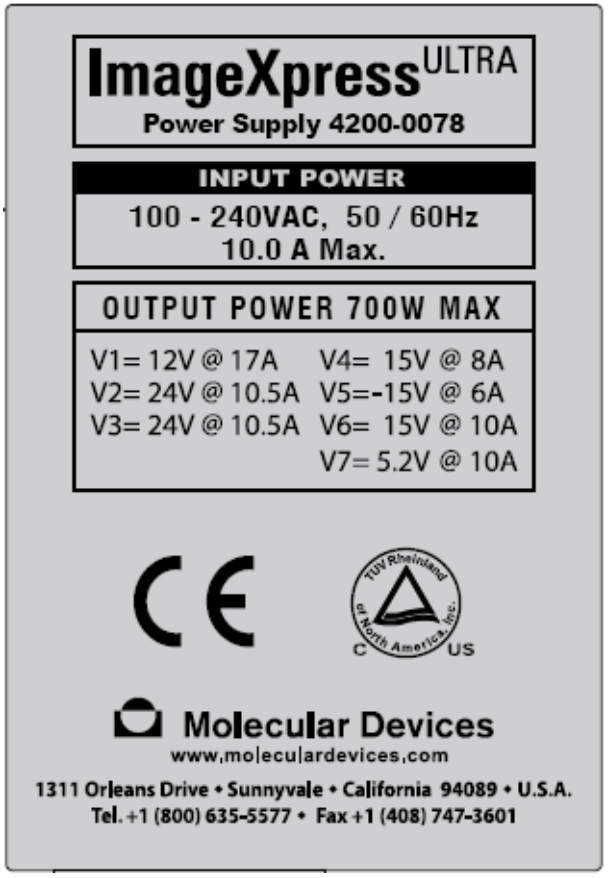
The operator of the ImageXpress Ultra System must be trained in the correct operation of the instrument and in the safety procedures. Making adjustments or performing procedures other than those specified in this guide might result in hazardous exposure to laser light, high voltage, hot surfaces or moving parts. Exposure to these hazards can cause severe or fatal injury.

Note: These safety practices are intended to supplement your national and local health and safety regulations and laws. The information provided covers instrument-related safety regarding the operation of the instrument. The information does not cover every safety procedure that should be practised. Ultimately, you and your organization are responsible for compliance with national and local EHS (Environmental Health and Safety) legal requirements and for maintaining a safe laboratory environment.

Safety Labels

If the safety label on the instrument becomes illegible or is missing for any reason, contact Technical Support for a free replacement label. While waiting for a replacement label, copy the label below and attach a copy of the label to the instrument.

The following safety label appears on the power supply.



Warning Labels

Table F-1 displays the safety symbols used on the ImageXpress® Ultra System and in this User Guide.

Table F-1 Warning Labels

Safety Symbol	Description	Safety Symbol	Description
	Electric Shock Hazard		Warning
	Laser Hazard		Toxic Chemical Hazard
	Lifting Hazard		Puncture Hazard

Protective Housing

The protective outer housing and instrument panel interlocks are designed to protect you from exposure to laser light, high voltage, hot surfaces or moving parts.

WARNING! Do not defeat the interlocks, open the protective housing, or try to gain access to the interior of the instrument through any other openings, unless specifically instructed by one of the user procedures outlined in this manual. Read each procedure carefully and follow all outlined safety precautions. Incorrectly opening the outer protective housing can damage the instrument components and result in hazardous exposure to laser light, high voltage, hot surfaces or moving parts.

Interlock Failure

There are three safety interlocks on the automated (top) door. Do not operate this instrument with the door open.

WARNING! Do not disable any of the interlocks. When the automated (top) door is opened, the interlocks trigger both the laser light source and the motion control electronics to turn off to prevent hazards associated with laser emission or moving parts.

The interlocks have failed if the focus lasers, scanning lasers, or PMTs (photomultiplier tubes) remain on after the automated (top) door is opened.

It is unsafe to continue using the ImageXpress® Ultra System under these conditions. Contact Molecular Devices Technical Support immediately.

Non-interlocked Panels

There are several other panels on the instrument that are intended for use by field service personnel and are not interlocked. All non-interlocked service panels are secured to the protective housing with screws that require a special tool to remove.

WARNING! Make sure the instrument is powered off and the power cable is unplugged if you are instructed to remove non-interlocked panels. Do not operate or access the interior of this instrument with any covers or panels removed.

Laser Safety Information

For safe laser operation:

- The system must be installed and serviced by an Molecular Devices FSE (field service engineer).
- All instrument panels must be in place on the instrument while the instrument is operating.
- Do not remove safety labels or disable safety interlocks.

With interlocks in place, the ImageXpress Ultra System is a Class 1 laser device. There are multiple Class 3b lasers embedded within the unit, which the user cannot and should not attempt to access. Table F-2 displays the embedded laser classification and power.

Table F-2 Embedded Laser Classification and Power

Nominal Wavelength	Typical Power	Maximum Laser Power	Duration	Embedded Laser Class
405 nm	20 mW	200 mW	Continuous Wave	Class 3b
488 nm	15 mW	150 mW	Continuous Wave	Class 3b
532 nm	20 mW	300 mW	Continuous Wave	Class 3b
561 nm	15 mW	300 mW	Continuous Wave	Class 3b
635 nm	20 mW	100 mW	Continuous Wave	Class 3b
690 nm	20 mW	100 mW	Continuous Wave	Class 3b

During normal operation, you can safely place samples in the loading area without exposure to laser power levels above Class 1.

WARNING! LASER HAZARD. Maintenance and servicing of the lasers must only be done by Molecular Devices trained personnel. Invisible Class 3b laser radiation is accessible with the covers removed and the interlocks defeated. Approved safety eye protection must be worn. Eye protection must be rated for use with the emitted wavelength.

WARNING! OCULAR HAZARD. Never look directly into the laser beam. Do not remove the instrument panels. Lasers can burn the retina causing permanent eye injury or blindness.

WARNING! BURN HAZARD. Do not operate the lasers when they cannot be cooled by the cooling fan. An overheated laser can cause severe burns if it comes into contact with the skin. Always wear laser safety goggles appropriate for the emitted wavelength.

High-Voltage Hazard

There are no user-accessible high-voltage electronics found inside the ImageXpress Ultra System. Do not attempt to open or service the instrument, apart from through the sample loading door, while the system is powered on. Do not attempt to open or service the power supply at any point.

Moving Parts

The ImageXpress® Ultra System contains moving parts that can cause injury. Under normal conditions, the instrument is designed to protect you from these moving parts. The interlocks and protective housing are designed so you cannot access the moving parts during a scan.

WARNING! Do not try to gain access to the interior of the instrument unless specifically instructed to do so. The moving parts inside the instrument can cause injury. Do not operate this instrument with covers or panels removed.

Fuses

The instrument power supply contains a circuit-breaker switch with a trip point of 18.75 amps.

Power Supply

The instrument has two cables running from the instrument to the external power supply (input voltage range is from 100 to 240 VAC, 50/60 Hz, 2 A).

WARNING! Make sure the power supply cable is unplugged before accessing any part of the instrument. Failure to do so can result in serious harm.

Lifting Hazard

WARNING! Do not attempt to lift or move the instrument without assistance. The instrument weighs approximately 220 pounds (100 kg).

CAUTION! Moving your instrument can disrupt sensitive optical alignments. Contact Technical Support to schedule an FSE (field service engineer) to help with moving your instrument. Your warranty or service contract will not cover problems caused during or as a result of shipment or relocation.

Maintenance and Service

User service and maintenance is strictly limited to the procedures outlined in this manual. The majority of serviceable components are accessed through the interlocked panels described above and as such must be accessed by a Molecular Devices FSE. If there is a problem or you have questions, contact Molecular Devices Technical Support.

Hazardous Material Precautions

Use standard laboratory procedures and cautions when working with chemicals.

WARNING! Always follow the manufacturer's precautions when working with chemicals. Molecular Devices is not responsible or liable for any damages caused by, or as a consequence of, the use of any hazardous material.

Obtaining Support

Part of effective communication with Molecular Devices is determining the channels of support for the ImageXpress Ultra System, including the MetaXpress® High Content Image Acquisition and Analysis Software. Molecular Devices provides a wide range of support:

1. Documentation — Check the manuals that are included on the installation media and the help that is available within the MetaXpress Software. Help for an active dialog box can be accessed by pressing the [F1] key.
2. Online knowledge base — The knowledge base has links to technical notes, software upgrades, newsletters, manuals, and other resources. Visit the Molecular Devices Support web page at www.moleculardevices.com/support and follow the links to the knowledge base.
3. Technical Support —

   Phone: Contact Technical Support at (800)-635-5577 (U.S. only) or +1 408-747-1700.

   Online: Visit www.moleculardevices.com/support and follow the links in the knowledge base to the Technical Support Request Form to send an e-mail to a group of experienced Technical Support representatives.

   Please have the system ID number, system serial number, software version number, and the system owner's name available when you call.

   - To find your system ID number, from the Help menu, select About MetaXpress. The dialog that appears displays your system ID number.
   - The system serial number is located on your instrument.
4. Additional support resources include:
   - Nikon web-based microscopy course — http://www.microscopyu.com
   - The Molecular Probes handbook — http://www.probes.invitrogen.com offers advice on fluorescent probes and can help you determine if there are better stains available for your analysis.

   The following sites offer filter information:

   - http://www.chroma.com
   - http://www.semrock.com
   - http://www.omegafilters.com

Introduction

1

ImageXpress Ultra System Overview

The ImageXpress® Ultra Confocal High Content Screening System is an integrated high throughput confocal cellular imaging and analysis system that is designed for rapid, automated screening of fluorescently labeled biological samples in microplates. The ImageXpress Ultra system consists of:

- Imaging instrument
- MetaXpress® High Content Image Acquisition and Analysis Software

The core hardware component of the imaging system is a custom-designed, fully automated, point-scanning confocal microscope, with rapid autofocus and precision sample movements. With the ImageXpress Ultra System, you can acquire large numbers of high-resolution images in the shortest possible time. All key optical and mechanical elements are motorized, with asynchronous command execution, thereby allowing complete real-time control of the instrument configuration through the MetaXpress Software interface.

When used with the powerful imaging capabilities of the MetaXpress Software, the instrument becomes an extremely flexible and programmable device, ideally suited for user-defined high-speed automated assays. Key features of the instrument include:

- Two to four solid-state lasers for efficient, specific sample illumination from 405 nm to 635 nm
- Four photomultiplier tubes (PMTs) for simultaneous or sequential multiwavelength scanning
- Fast laser autofocus system with a precision motorized Z (focus) stage, dedicated 690 nm laser, and fast optical sensor
- User-adjustable detection pinhole
- Precision motorized X-Y (sample) stage
- High-quality user-changeable Nikon objectives in a four-position linear selector. The first position must be occupied by the 10X Plan Fluor objective which is used for system calibration and can be used for imaging.
- User-changeable emission filter sets in a three-position slider
- Selectable averaging to increase the signal-to-noise ratio of the images
- User-configurable scan length for variable sized images
- Five self-aligning main beam splitters that users can select in the MetaXpress Software

- Operation and configuration by integrated MetaXpress Software
- Plate-handling robot (optional) with automated barcode reader

System Installation

The ImageXpress Ultra System is shipped fully configured, and is installed at your site by a Molecular Devices FSE (field service engineer). The base system includes the imaging unit, host computer, and toolkit.

Make sure that the power cord from the ImageXpress Ultra System power supply is connected to a 100 VAC to 240 VAC power source. The ImageXpress Ultra System host computer is shipped with the MetaXpress Software already installed, and the instrument is connected to the power supply host computer during installation.

There are four main connections, excluding power cords:

- Two power supply cables to the instrument
  - For the lasers
  - For all other components
- Two USB 2.0 data cables between the instrument and the acquisition computer
  - For device control
  - For image acquisition and transfer

Quick Start Guide

2

This chapter describes how to turn on the ImageXpress® Ultra System and log in to the MetaXpress® Software. All procedures assume that the ImageXpress Ultra System has been installed and configured by an Molecular Devices FSE (field service engineer).

The following topics are included in this chapter:

- Turning on the ImageXpress Ultra System on page 21
- Starting the Software on page 22
- Logging AxoTrace Messages to a .txt File on page 23
- Setting up and Running Plate Acquisition on page 25
- Turning off the ImageXpress Ultra System on page 25

Turning on the ImageXpress Ultra System

To turn on the ImageXpress Ultra System

1. Turn on the power switch on the front of the ImageXpress Ultra System power supply control box.
2. Turn on the power switch on the computer.
3. Turn on the power switch on the LCD monitor.
4. After the computer has started and Windows is running, log in to Windows using the user name and password provided by your system administrator.

CAUTION! Do not log in to your system as Guest unless you are specifically instructed to do so by your system administrator. Many functions require administrator-level privileges to operate correctly.

Starting the Software

To start the MetaXpress Software application

1. Click **Start > Programs > MetaXpress > MetaXpress**.
2. If you are required to select a user name before the **Welcome to MetaXpress** dialog is displayed then you are in multi-user mode, and you must select a user name and then click **OK** to continue.
3. In the **Welcome to MetaXpress** dialog, in the **Data Source** list, select the data source to connect to.

4. In the **Login Name** and **Password** fields, type the name and password, and then click **OK**.

   For versions 4.0 and 5.0 of the MetaXpress Software, the default User Login Name is **MolDev**, the default System Administrator Login Name is **sa**, and the default password for both is **moldev**. For earlier versions of the software, the default User Login Name and password was **mdc**. You might need to log in using **mdc** if you had an earlier version of the software on your instrument workstation. If needed, you can change the password by clicking **Change Password**.

5. In the **Group** dialog, select one of the groups that your particular login has been assigned to and click **OK**.

   The MetaXpress Software starts and initializes the various components of the ImageXpress Ultra System. If you receive error messages when the system is initializing, make sure that all hardware connections are plugged in and fully seated. Close the software, turn off the instrument, wait for 10 seconds, turn on the instrument, and open the software.

Note: If hardware errors persist, contact Molecular Devices Technical Support and make sure you provide: the serial number of the instrument, the MetaXpress Software version (see the Help in the software), MetaXpress ID (on the MetaXpress dongle or see the Help in the software), screenshot of any error message, and the saved AxoTrace output.

Logging AxoTrace Messages to a .txt File

AxoTrace tracks the hardware status of the ImageXpress Ultra System and logs all the hardware activities of the system. If the error occurs downstream of the initial cause, AxoTrace provides a log that Molecular Devices Technical Support can use to trace the error back to the original cause and pinpoint the problem. Use the procedure below if a reproducible hardware error occurs. Follow the AxoTrace setup procedure and record the steps that lead to the error. If the error results in the instrument not starting up, not responding, or the error cannot be reproduced, contact Molecular Devices Technical Support immediately.

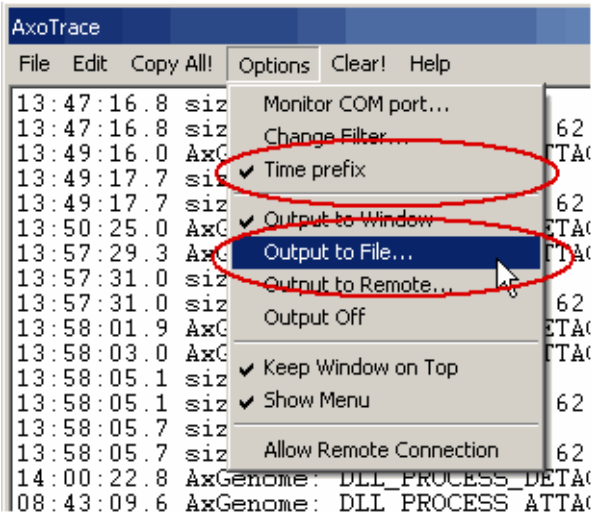
To log AxoTrace messages to a .txt file

1. Open the MetaXpress Software and **AxoTrace** starts automatically.

   The AxoTrace icon appears in the toolbar.

2. Close the MetaXpress Software.
3. Double-click the **AxoTrace** icon in the toolbar.

4. In the **AxoTrace** window, click **Options > Output to File** and make sure that the **Time prefix** option is selected.



5. Click **File > Save As** and in the **Save As** dialog that appears, in the **Name** field, navigate to the location where you want to save the log file, type the file name (for example, axotrace log.txt) and then click **Save** to save the log file.
6. Close the **AxoTrace** dialog and then start the MetaXpress Software.
7. Reproduce the error and after the error is reproduced, close the MetaXpress Software.
8. Send the AxoTrace log as an e-mail attachment to Molecular Devices Technical Support.

Setting up and Running Plate Acquisition

This procedure assumes that the ImageXpress Ultra System and the MetaXpress High Content Image Acquisition and Analysis Software have been properly installed and configured by the Molecular Devices FSE and your system administrator.

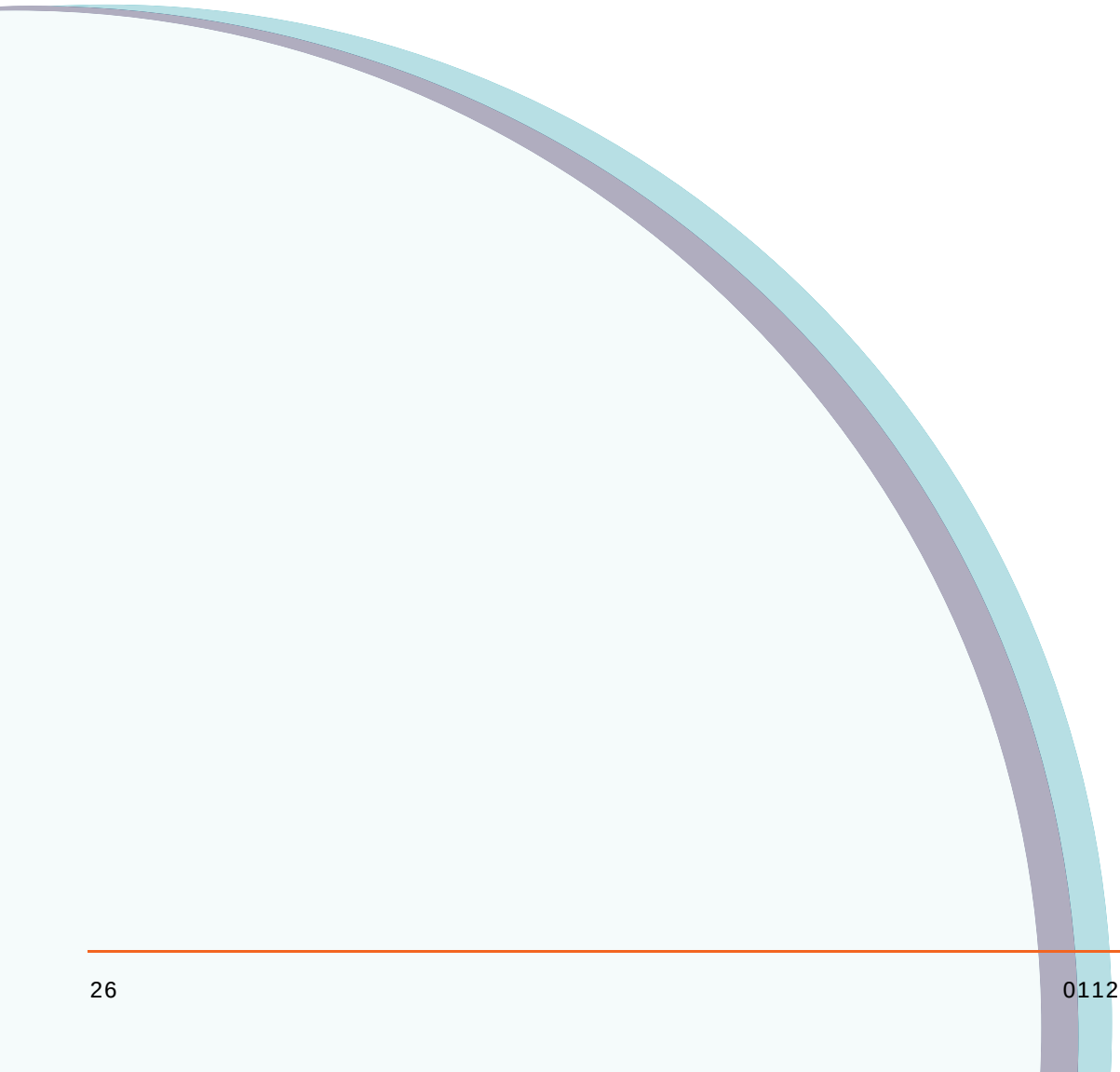
To set up and run plate acquisitions

1. Turn on the ImageXpress Ultra System and start the MetaXpress Software.
2. Click **Screening > Plate Acquisition Setup**.
3. In the **Plate Acquisition Setup** dialog, complete each tab with the appropriate settings for your experiment, working from the top tab to the bottom tab. See Setting Up Plate Acquisition on page 79.
4. After you have completed the information in all the tabs and are on the final **Summary** tab, click **Acquire Plate** to acquire a plate of images using the settings.

Turning off the ImageXpress Ultra System

To turn off the ImageXpress Ultra System

1. Close the MetaXpress Software. You will be prompted to save any open images.
2. Click **Start > Shutdown > Shutdown to** shut down the computer.
3. Turn off the power switch on the LCD monitor.
4. Turn off the power switch on the front of the ImageXpress Ultra System power supply control box.



Imaging System Overview

3

This chapter provides information on imaging systems. The following topics are included:

- Fluorescence Imaging on page 27
- Confocal Imaging on page 28
- Electronics on page 29
- Imaging Optics on page 29
- Objective (Z) Stage on page 37
- Sample X-Y Stage on page 38
- Autofocus Laser on page 38

Fluorescence Imaging

Fluorescence is a phenomenon observed in certain species of molecules (fluorochromes, or dyes) in which photons of a specific wavelength are absorbed (excitation), and as a result a very short time later photons are emitted at a longer wavelength (emission). The use of fluorescence imaging in biological applications stems from the ability to conjugate fluorescent molecules with biologically active probe molecules, so that the application of the combined dye/probe molecule (fluorophore) to the specimen highlights the specific substances or regions to which the probe is targeted.

By attaching different probes to a set of dye molecules with different excitation and emission spectra, you can stain a specimen with multiple fluorophores, and either simultaneously or sequentially image different structures or substances within the same specimen. Usually sequential imaging is required when the excitation and emission spectra of selected fluorophores are significantly overlapped. The absorption and emission peaks for each dye in a given environment are physical characteristics of that molecule, and their specific properties determine the initial selection of the optical components to be used, for example, excitation lasers, emission filters, and beam splitter.

Confocal Imaging

In standard wide-field microscopy, fluorescent objects above or below the focal plane are seen as out-of-focus objects and increase the background fluorescence. Confocal microscopy rejects out-of-plane signals by using point-source illumination and a pinhole aperture ahead of the detector.

A confocal microscope is neither more sensitive nor faster than a wide-field microscope. The main advantage of confocal imaging is the rejection of unwanted signal and the resulting improved resolution and reduced background. By changing the size of the pinhole in front of the detector you can achieve a suitable balance between the degree of confocality and the sensitivity and speed of the instrument. A smaller pinhole gives better axial resolution, while a larger pinhole increases the collection efficiency resulting in higher sensitivity.

Figure 3-1 Schematic of the ImageXpress Ultra System shows the internal components of the system.

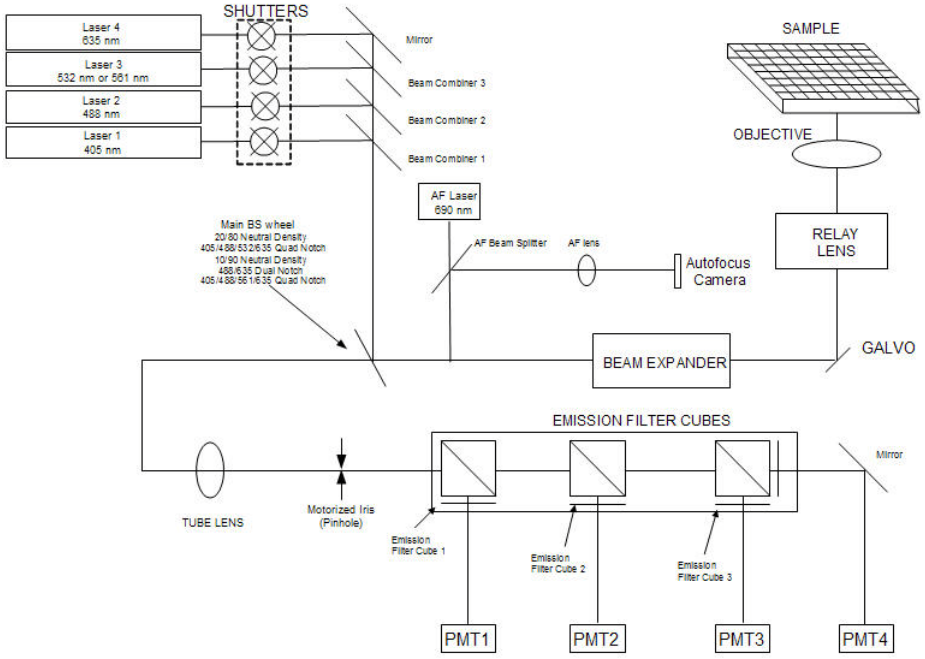


Figure 3-1 Schematic of the ImageXpress Ultra System

Electronics

The ImageXpress Ultra System includes:

- External power supply with two DC cables
- USB 2.0 port and cable to computer for device control
- USB 2.0 port and cable to computer for image transfer

Imaging Optics

This section provides information on components of imaging optic systems.

- Excitation Lasers on page 29
- Beam Combiners on page 30
- Beam Expander on page 30
- Galvanometer on page 30
- Relay Lens on page 31
- Main Beam Splitter on page 31
- Objective Lenses on page 32
- Tube Lens on page 32
- Motorized Iris on page 33
- Emission Filters on page 33

Excitation Lasers

In the ImageXpress Ultra System the sample is illuminated by any combination of up to four pre-installed solid-state excitation lasers. These lasers have variable power output, controllable through the MetaXpress Software. Shutters protect samples from laser illumination while not in use. The lasers have a minimum expected lifetime of 5000 hours.

You can configure the system to use one laser at a time (sequential scanning), a combination of lasers (simultaneous scanning), or a combination of sequential and simultaneous scanning. For example, you can complete an initial scan with the 405 nm laser only and then do a second scan with the 488 nm and 635 nm lasers simultaneously.

A combination of the following five lasers might be included in your ImageXpress Ultra System.

- 405 nm (optional)
- 488 nm (standard)
- 532 nm (optional)
- 561 nm (optional)
- 635 nm (standard)

Note: Either the 532 or 561 nm laser (but not both) can be installed in the instrument.

Beam Combiners

Beam combiners are used to combine the illumination light provided by the excitation lasers that are required for simultaneous imaging of multiple channels.

Beam Expander

The ImageXpress Ultra System achieves its best resolution when the entire pupil of the objective is used for emission and excitation. The 1x, 2x, and 3x beam expanders in the system have dual purpose:

- Expansion of the excitation beam diameter
- Contraction of the emission beam diameter

Because of the beam expanders, the ImageXpress Ultra System can work with various types of objectives with the greatest possible efficiency.

Galvanometer

A mirror reflecting light at a variable angle is a key component of many scanning microscopes. The mirror delivers the excitation light to a specified point on the sample. The fluorescent signal acquired at different positions of the mirror is reconstructed by the software into a fluorescent image of the sample. In the ImageXpress Ultra System, the motion of the galvanometer provides data for the X-axis (horizontal) of the image.

The ImageXpress Ultra System uses a state of the art galvanometer as a variable angle mirror. An optimal combination of mirror size, flatness, rigidity, and operating speed guarantees the best resolution and image acquisition rate.

Relay Lens

The relay lens is a complex optical system that redirects the angularly scanning excitation laser beam (scanned by the galvanometer) into the objective pupil while keeping the beam centered in the pupil.

Main Beam Splitter

One of the five pre-installed beam splitters separates the excitation and the emission pathways in the ImageXpress Ultra System. All five are mounted on a precision-motorized wheel that is controlled by the MetaXpress Software. When a beam splitter is selected, the main beam splitter wheel self-aligns for optimal image conditions. This precision self-alignment can take as long as 15 seconds. Only one beam splitter per experiment can be selected to maintain high-speed image acquisition.

The main beam splitter reflects excitation light and transmits emission light. The following five beam splitters are included in the standard configuration of the ImageXpress Ultra System:

- 80/20 neutral density (80% transmission efficiency, 20% reflection)
- 405/488/532/635 quad notch
- 90/10 neutral density (90% transmission efficiency, 10% reflection)
- 488/635 dual notch
- 405/488/561/635 quad notch

Both beam splitter 80/20 and 90/10 are neutral density separators. They deliver only 20% or 10% respectively, of the incident laser light to the sample, and 80% or 90% of the fluorescent light to the detector but with no limitation on the spectrum of the fluorescent light. The main disadvantage of neutral density separators is that a significant portion of the laser light reflected from the sample is mixed with fluorescent light and is delivered to the detector. You must take special precautions to block this reflected light, requiring sophisticated emission filters. See Emission Filters on page 33.

The other beam splitters produce spectral separation. Each three digit number in the filter name denotes the central wavelength of the narrow band (approximately 10 to 15 nm) of light being reflected by the beam splitter. For example, the 488/635 dual notch beam splitter reflects 488 ±5 nm and 635 ±5 nm light to the sample and at the same time transmits other wavelengths from the sample to the detector.

Spectral separation beam splitters deliver nearly 100% of laser light to the sample and collect fluorescent light with nearly 100% efficiency, except in those wavelength ranges knocked out by notches. In addition, these notches greatly diminish the presence of laser light reflected from the sample in the fluorescent light delivered to the detector.

In the MetaXpress Software, you cannot select lasers that are incompatible with the selected beam splitter. For example, the 532 nm laser option is unavailable if either the 488/635 dual notch or 405/488/561/635 quad notch is selected.

Objective Lenses

The selected objective lens focuses excitation light on to the sample, and collects fluorescence light emitted by the sample. Objectives are classified according to the optical correction, flatness of field, numerical aperture, and working distance. Before choosing additional objectives to use with the system, it is important to consider the types of plates that you are imaging. The plate material (plastic or glass) and plate thickness are major considerations when choosing an objective. You can configure the ImageXpress Ultra System with a large number of high-quality standard Nikon objectives from the CF160 series. Molecular Devices offers a wide range of objectives to suit your experimental needs. For a complete list of objectives, see Appendix A: Objectives Compatible with the ImageXpress Ultra System on page 173.

The 10X Plan Fluor objective is standard on all ImageXpress Ultra Systems. It is installed in position 1 of the objective changer and must never be removed from this position. The position 1 placement of this objective is critical for galvanometer calibration and laser autofocus configuration. This objective is also available for standard imaging.

Certain objectives have adjustable spherical-aberration correction collars for imaging through thick substrates such as the plastic or glass bottom of most microplates. For details on how to adjust them, see Adjusting the Spherical-Aberration Correction Collar on Objectives on page 165.

Note: Setting these correction collars is critical for the optimization of the image quality.

Tube Lens

The relay lens and beam expander reduce the diameter of the collimated light from the objective by a factor of 3 to 9 times, depending on the beam expander selected. The tube lens focuses the reduced diameter collimated beam onto the aperture (iris) in front of the detector. The combined effect of these components is to collect the in-focus fluorescence signal while rejecting the out-of-focus (non-collimated) fluorescence signal.

Motorized Iris

In confocal imaging, the diameter of the iris (pinhole) determines the amount of out-of-focus fluorescent light that reaches the detector. Increasing the iris diameter allows more signal (higher sensitivity) but decreases the degree of confocality.

In the ImageXpress Ultra System, the iris is motorized, and its diameter can be changed from 1 mm to 8 mm using the MetaXpress Software.

Emission Filters

In the ImageXpress Ultra System, the emission filters are located in sets of filter cubes.

When imaging the illuminated sample, you must collect emission photons only from the target fluorophore, rejecting reflected or scattered excitation light, any light from other dyes, and autofluorescence from the sample and substrate. This is accomplished by placing a filter in the emission path called the emission filter. In the ImageXpress Ultra System, the emission filters have an additional role of separating out the signal from multiple fluorophores and delivering them to separate PMTs.

The ImageXpress Ultra System uses custom-designed Semrock filters. It contains three rack positions and each rack contains a position for three Nikon TE2000 cubes. The emission filter in front of the PMT filters the light to that PMT. Emission filter cubes can either be bandpass, for maximum specificity, or longpass to maximize the amount of emission light collected.

Note: While standard Nikon TE2000 cubes are used, the component positioning within these cubes is not the same as in an epifluorescence microscope.

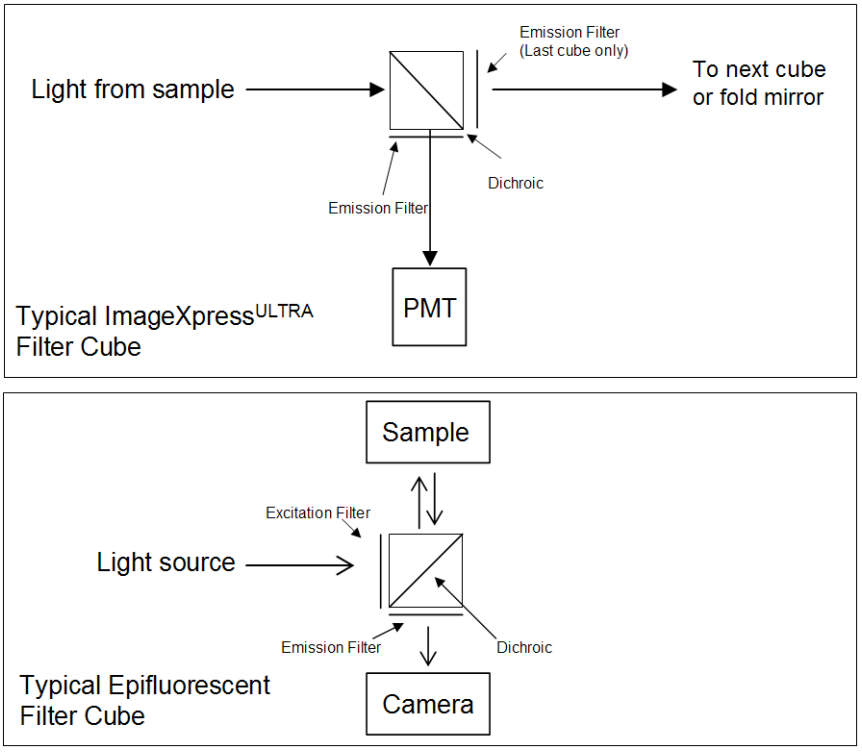
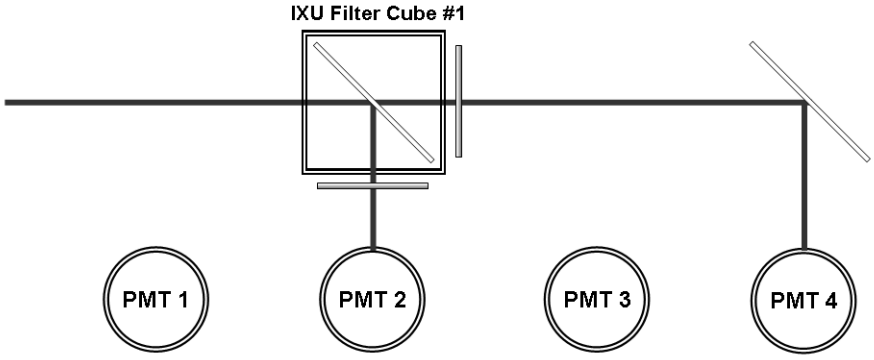


Figure 3-2 Comparison of ImageXpress Ultra System and Epifluorescent filter cubes

Typical ImageXpress Ultra System Filter Cube Configurations

The figures below show the typical configurations for the filter cubes and their position in the ImageXpress Ultra System.

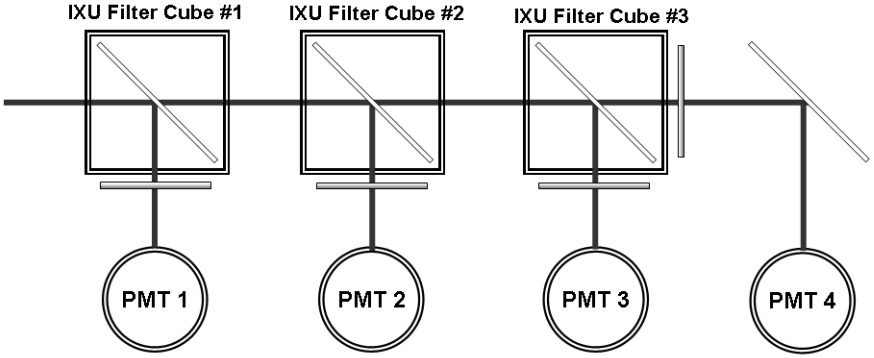
Standard Filter Set Configuration
488 / 635 (2 color)



PMT	Excitation	Dye	Emission	Dichroic (LP)
1				
2	488	GFP	538/84	650
3				
4	635	DRAQ5	692/40	

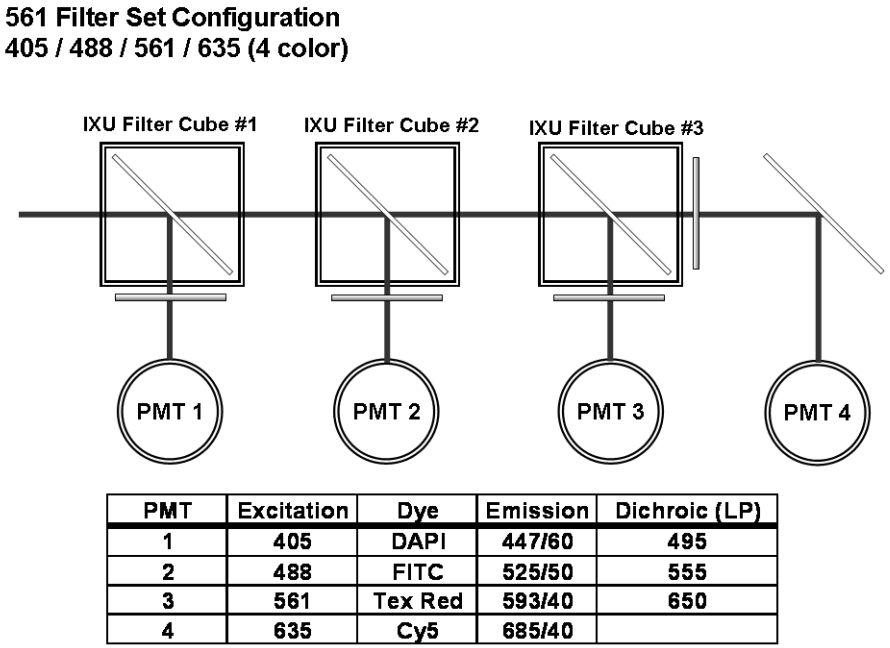
Figure 3-3 Standard Filter Set Configuration

532 Filter Set Configuration
405 / 488 / 532 / 635 (4 color)



PMT	Excitation	Dye	Emission	Dichroic (LP)
1	405	DAPI	447/60	495
2	488	FITC	514/17	555
3	532	Cy3	585/40	650
4	635	Cy5	685/40	

Figure 3-4 532 Filter Set Configuration



PMT	Excitation	Dye	Emission	Dichroic (LP)
1	405	DAPI	447/60	495
2	488	FITC	525/50	555
3	561	Tex Red	593/40	650
4	635	Cy5	685/40	

Figure 3-5 561 Filter Set Configuration

Photomultiplier Tubes

In the ImageXpress Ultra System, the PMTs (photomultiplier tubes) are used as the photodetectors. The standard configuration uses four PMTs and the PMT gain is individually controlled by the PMT voltage.

Objective (Z) Stage

Motorized Z Stage

The Z stage position is measured by a precision linear encoder and has better than 100 nm resolution.

Motorized Objective Changer

The ImageXpress Ultra System has a four-position objective changer allowing you to select the required objective using the software. Only the selected objective will be moved by the motorized Z stage.

Sample X-Y Stage

Sample

The plate holder is designed for scanning multiwell plastic and glass-bottom microplates in standard ANSI (SBS) 1-2004 through ANSI (SBS) 4-2004 formats, but can accommodate other plate formats that have standard microplate footprint dimensions. For example, glass slides can be imaged using a slide adapter included in the tool kit. Optimal image quality depends on plate flatness and optical clarity.

Plate Holder and Plate Clamp

A spring-loaded mechanical clamp holds the sample plate securely in the plate holder. The clamp automatically opens when the X-Y stage moves to the load/unload position, and automatically closes when the X-Y stage moves the plate into position for imaging. The ImageXpress Ultra System is configured so that the A1 position is placed next to the plate clamp, although this can be reversed if required.

Motorized X-Y Stage

The X-Y stage position is measured by precision linear encoders and has better than 100 nm resolution. The X-Y stage controls the position of the sample plate above the optical path. In addition, in the ImageXpress Ultra System, the Y-stage provides the motion required for the Y-axis (vertical) scan direction of the imaging.

Autofocus Laser

A red (690 nm) diode laser projects a beam on to the sample plate. Reflections of this laser beam from the bottom of the microplate and from the plate–sample interface are imaged by a dedicated, fast camera. The ImageXpress Ultra System uses these reflections to autofocus as the sample plate moves from well to well.

Post-Installation and Testing

4

After the installation of the ImageXpress® Ultra System is complete, the system is ready for initial testing and use.

This chapter includes the following topics:

- Verifying device settings in the Meta Imaging Series® Administrator
  - X-Y stage
  - Objectives
  - Filter cube sets
- Verifying camera settings in the Meta Imaging Series Administrator
- Verifying settings in the MetaXpress® Software
  - Magnification settings
  - Calibration settings
  - Laser autofocus sensor
  - Plate settings
  - Shading correction files

Verifying Device Settings in the Meta Imaging Series Administrator

You must check that the ImageXpress Ultra System components are properly configured using the Meta Imaging Series Administrator software and the MetaXpress Software. All hardware and software configuration settings are implemented in the Meta Imaging Series Administrator software. You cannot run the Meta Imaging Series Administrator and the MetaXpress Software at the same time.

Tip! For additional information on any of the dialogs in the Meta Imaging Series Administrator, press the F1 key to access Help.

Before using the ImageXpress Ultra System, you must verify the device settings for the hardware components:

- To verify the ImageXpress Ultra System X, Y, and Z settings on page 40
- To verify the objective settings on page 44
- To verify the beam expander settings on page 46
- To verify the pinhole settings on page 46
- To verify the filter cube settings on page 47
- To verify the beam splitter settings on page 49

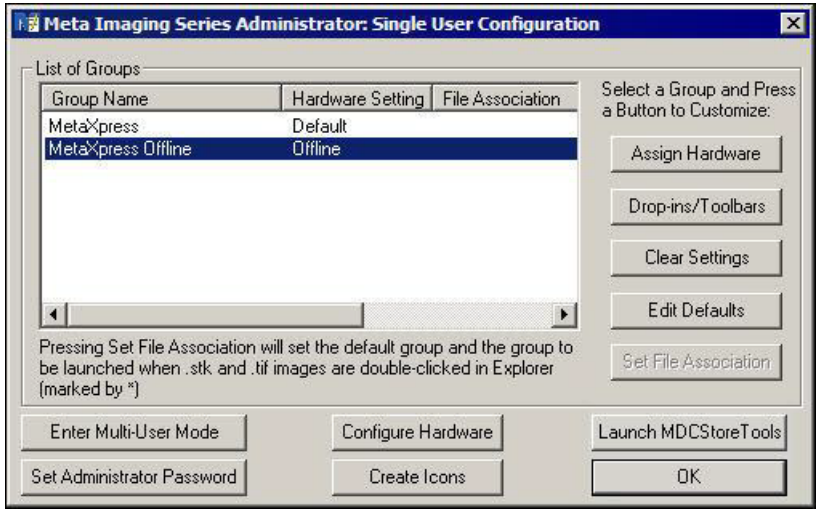
To verify the ImageXpress Ultra System X, Y, and Z settings

1. Turn on the ImageXpress Ultra System but do not start the MetaXpress Software. See Turning on the ImageXpress Ultra System on page 21.
2. Click **Start > Programs > MetaXpress > Meta Imaging Series Administrator**.

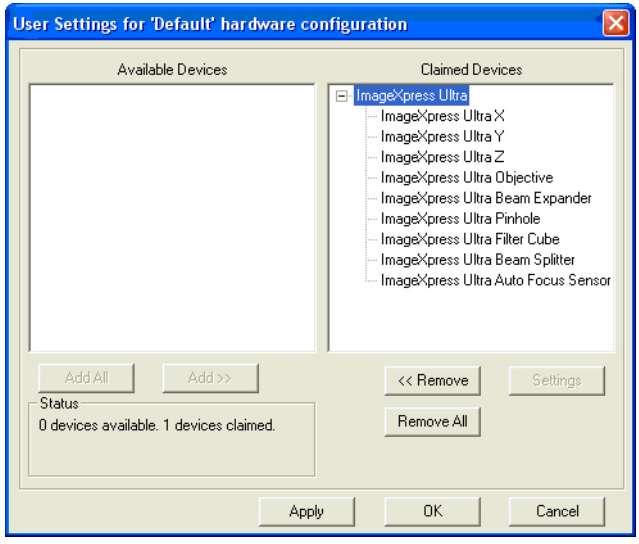
Tip! For additional information on any of the dialogs in the Meta Imaging Series Administrator, press the F1 key to access the Help for the active dialog. This includes accessing information on the single-user and multi-user modes.

3. In the **Meta Imaging Series Administrator** dialog, in the **List of Groups** field, select **MetaXpress** and then click **Configure Hardware**. There is a 30 to 45 second delay while the system initializes the components.

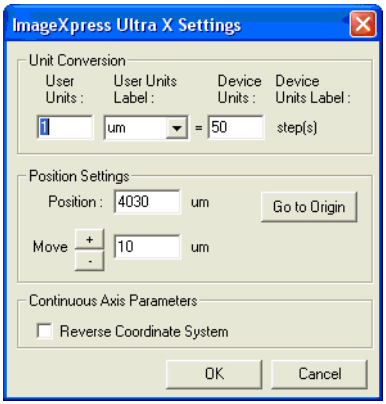
CAUTION! It is critical that the ImageXpress Ultra System is powered on before clicking Configure Hardware. Failure to do so can result in the loss of critical system settings. If Configure Hardware is selected with the system turned off, do not click OK. Use Cancel to avoid losing the critical system settings.



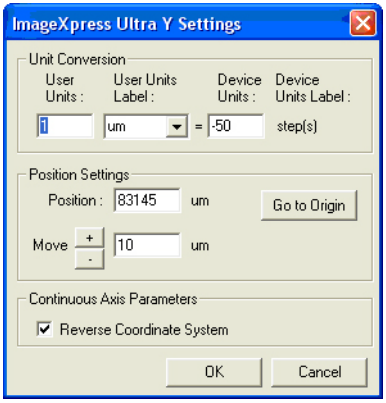
4. **In the Configure Hardware dialog,** click **Configure Devices**.
5. **In** the **User Settings for Default hardware configuration** dialog that appears, in the **Claimed Devices** field, select **ImageXpress Ultra X** and then click **Settings**.



6. In the **ImageXpress Ultra X Settings** dialog, in the **Device Units** field, verify that the value is **50** and that the **Reverse Coordinate System** check box is clear.



7. In the **Position Settings** section, in the **Move** field, type **10000** to increase the step size.
8. In the **Move** field, click the + icon and confirm by sound that the stage responds to the control. Verify that the X-stage position has been updated and that no error messages appear.
9. Click **OK**.
10. **In** the **User Settings for hardware configuration** dialog that appears, in the **Claimed Devices** field, click **ImageXpress Ultra Y** and then click **Settings.**
11. In the **ImageXpress Ultra Y Settings** dialog, in the **Device Units** field, verify that the value is **-50** and that the **Reverse Coordinate System** check box is selected.



12. In the **Position Settings** section, in the **Move** field, type **10000** to increase the step size.
13. Click the + icon and confirm by sound that the stage responds to the control and also verify that the Y-stage position has been updated and that no error messages appear.
14. Click **OK** and then in the **User Settings for hardware configuration** dialog that appears, in the **Claimed Devices** field, click **ImageXpress Ultra Z** and then click **Settings.**
15. In the **ImageXpress Ultra Z Settings** dialog, in the **Device Units** field, verify that the value is **50**.
16. In the **Position Settings** section, in the **Move** field, type **1000** to increase the step size.
17. Click the + icon and confirm by sound that the Z Motor responds to the control and also verify that the Z-stage position has been updated and that no error messages appear.

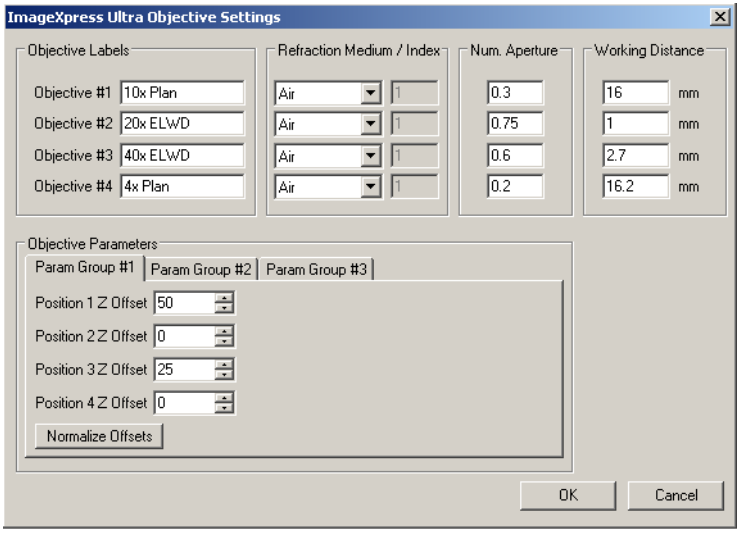
Note: Do not exceed a Z position of 5000 µm during this verification procedure.

18. Lower the Z Motor to **0** µm and then click **OK**.

To verify the objective settings

1. **In** the **User Settings for hardware configuration** dialog, in the **Claimed Devices** field, select **ImageXpress Ultra Objective** and then click **Settings**.
2. In the **ImageXpress Ultra Objective Settings** dialog, confirm that the objectives in the **Objective** fields, and the values in the **Num. Aperture** fields match each objective on your system.

Note: The first character of each objective label name must be its numerical magnification value. If there is no objective in that position, use a number at the beginning, for example, 4-Empty. The NA (numerical aperture) values are written on each objective. See Table A-1 Nikon Objectives Compatible with the ImageXpress Ultra System on page 173 for the NA value for each objective.

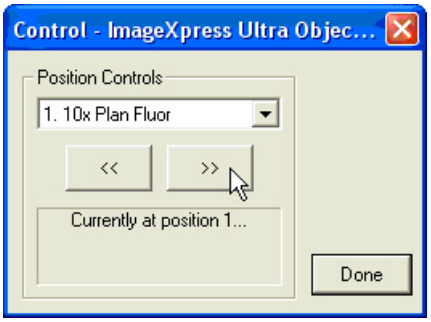


Note: The 10X Plan Fluor objective must always be in position #1. This objective must be in this position for the system to perform calibration prior to plate acquisition.

3. In the **Objective Parameters** section, click the **Param Group #1** tab. This tab contains the Z offset positions in microns for the objectives. Confirm that these are valid numbers and all but one is greater than 0.

   See Configuring Objective Offsets and Parfocality on page 145 to determine the offset values.

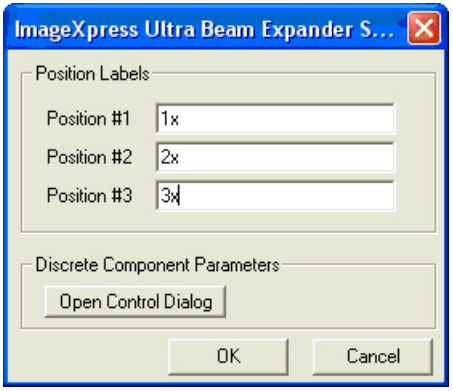
4. Click the **Param Group #2** tab and then click **Open Control Dialog**.
5. In the **Control – ImageXpress Ultra Objective** dialog, click the arrow buttons and confirm by sound that the objective changer is moving and also verify that the objective position has been updated and that no error messages appear.



6. Click **Done** and then click **OK** to close the Objective Settings dialog.

To verify the beam expander settings

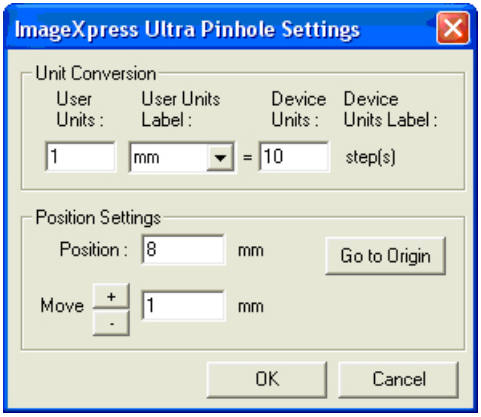
1. **In** the **User Settings for hardware configuration** dialog, in the **Claimed Devices** field, select **ImageXpress Beam Expander** and then click **Settings**.
2. In the **ImageXpress Ultra Beam Expander Settings** dialog, confirm that the positions listed in the **Position Labels** section are correct.



3. Click **Open Control Dialog** and in the **ImageXpress Ultra Beam Expander Settings** dialog, click the arrow buttons to confirm that the beam expander changer is responding to the program by verifying that the beam expander positions have been updated and that no error messages appear.
4. Click **Done** and then in the **ImageXpress Ultra Beam Expander Settings** dialog click **OK** to close it.

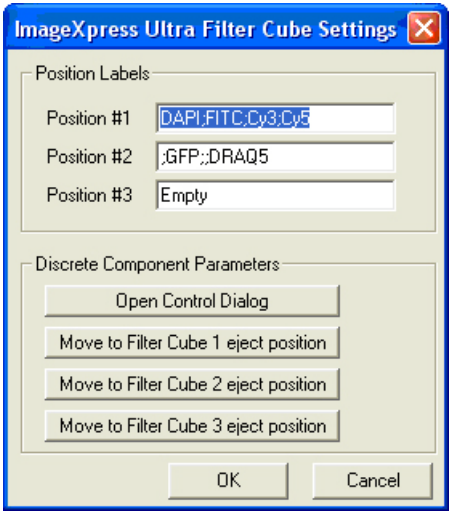
To verify the pinhole settings

1. **In** the **User Settings for hardware configuration** dialog, in the **Claimed Devices** field, select **ImageXpress Pinhole** and then click **Settings**.
2. In the **ImageXpress Ultra Pinhole Settings** dialog, verify that the value in the **Device Units** field is **10**.



To verify the filter cube settings

1. **In** the **User Settings for hardware configuration** dialog, in the **Claimed Devices** field, select **ImageXpress Filter Cube** and then click **Settings**.



2. In the **Position Labels** section, confirm that the **Position** fields match the filter cubes on your system.

Note: The format for the filter names is Wavelength1;Wavelength2; Wavelength3;Wavelength4.

Each entry must be separated by a semicolon. If a filter cube is missing from any filter cube assemblies, leave it blank between the semicolons.

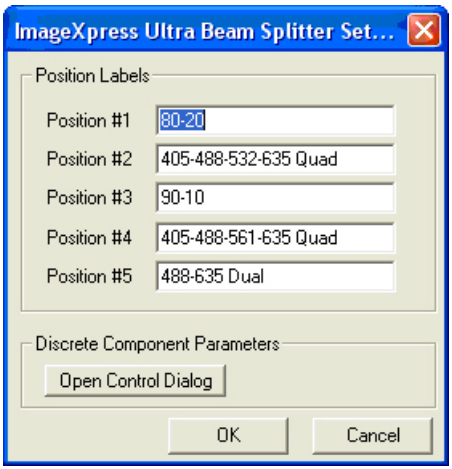
3. In the **Discrete Component Parameters** section, click **Open Control Dialog**.
4. In the **Control – ImageXpress Filter Cube** dialog, click the arrow buttons and confirm by sound that the filter cubes are responding to the program and also verify that the emission filter set position has been verified and no error messages appear.

WARNING! Ambient light will damage the PMTs. Do not open the filter cube access door while the system is powered on.

5. Click **Done** and then in the **ImageXpress Ultra Filter Cube Settings** dialog, click **OK** to close it.

To verify the beam splitter settings

1. **In** the **User Settings for hardware configuration** dialog, in the **Claimed Devices** field, select **ImageXpress Beam Splitter** and then click **Settings**.



2. In the **ImageXpress Ultra Beam Splitter Settings** dialog, confirm that the positions listed in the Position Labels field are correct.
3. In the **Discrete Component Parameters** section, click **Open Control Dialog**.
4. In the **Control - ImageXpress Ultra Beam Splitter Settings** dialog, verify that the main beam splitter position has been updated and that no error messages appear.

Note: The main beam splitter wheel automatically self-aligns so there is a slight delay of up to 15 seconds when changing beam splitters.

5. Click **Done** and then click **OK** to close the dialog.

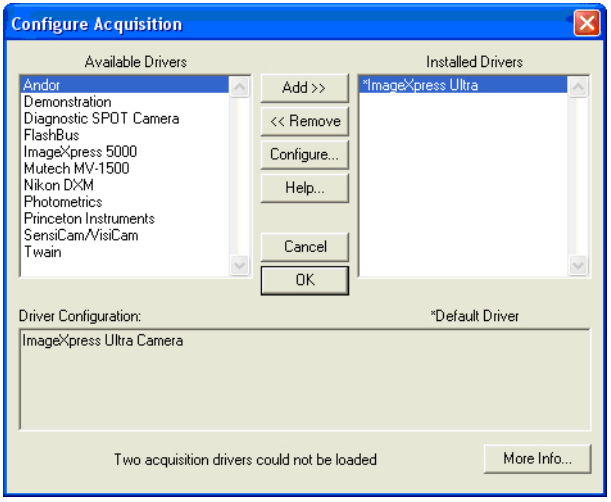
Verifying Acquisition Settings in the Meta Imaging Series Administrator

Complete the following procedure to make sure that the ImageXpress Ultra System acquisition driver is installed.

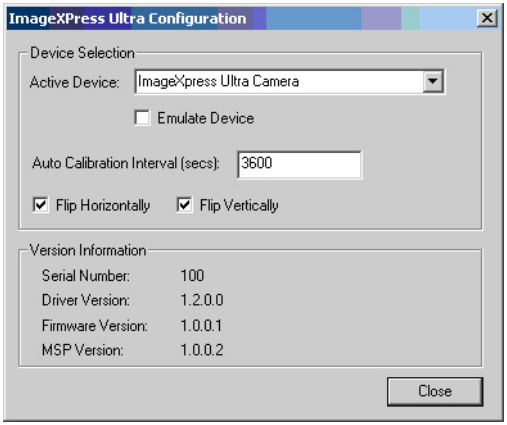
Note: Although the ImageXpress Ultra System uses PMTs (photomultiplier tubes) instead of a camera, the PMTs are treated as a camera by the MetaXpress Software.

To verify acquisition settings

1. Click **Start > Programs > MetaXpress > Meta Imaging Series Administrator**.
2. In the **Meta Imaging Series Administrator** dialog, click **Configure Hardware**.
3. In the **Configure Hardware** dialog, click **Configure Acquisition**.
4. In the **Configure Acquisition** dialog, in the **Installed Drivers** field, make sure that **ImageXpress Ultra** is listed. If it is not listed, select it from the **Available Drivers** field and then click **Add**.



5. Click **Configure** and then in the **ImageXpress Ultra Configuration** dialog confirm that the **Flip Horizontally** and **Flip Vertically** check boxes are selected.



6. In the **Auto Calibration Interval** field, check that the value is set to **3600** (the default value) or to the appropriate setting for your instrument.

Note: The Auto Calibration Interval field refers to the minimum time interval in seconds between the automatic calibrations of the galvanometer at the beginning of plate acquisition.

7. In the **Version Information** section, confirm that the serial number, driver, firmware, and MSP versions are listed. If there are no values for these fields, make sure that the cable set up is correct from the ImageXpress Ultra System to the computer.
8. Click **OK** to close the **ImageXpress Ultra Configuration Camera Driver** dialog, and then click **OK** to close the **Meta Imaging Series Administrator** dialog.

Verifying and Backing Up Settings in the MetaXpress Software

After confirming hardware settings in the Meta Imaging Series® Administrator, check the following in the MetaXpress Software:

- Magnification settings
- Calibration settings

During the verification process it is recommended that you backup these settings so that you can restore the settings if they are lost.

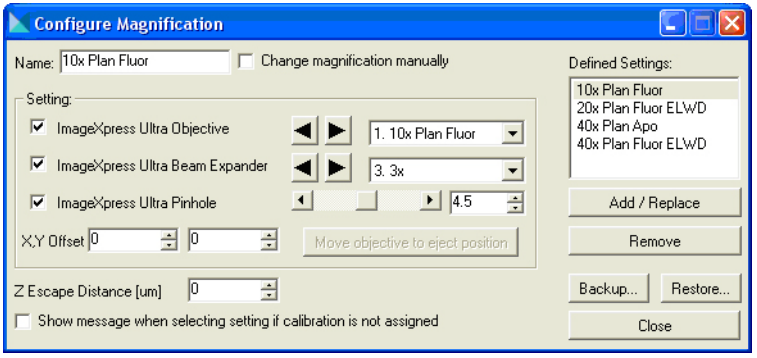
Verifying and Backing Up Magnification Settings

You must confirm the magnification settings for the ImageXpress Ultra System objectives.

Tip! For additional information about any of the dialogs in MetaXpress Software, press the F1 key to access the Help for the active dialog.

To verify and back up the magnification settings

1. Open the MetaXpress Software and log in to the database.
2. Click **Devices > Configure Magnification**.
3. In the **Configure Magnification** dialog, in the **Settings** section, make sure that the **ImageXpress Objective**, **Beam Expander**, and **Pinhole** check boxes are selected and the fields are completed.



4. In the **X, Y Offset** fields make sure that the offsets are set for the objective (for the 10X Plan Fluor objective, the offset is 0).
5. In the **Z Escape Distance** field, make sure the value is **0**.
6. In the **Defined Settings** field, make sure that each objective in your system is listed.
7. Click **Backup**.
8. In the **Backup All Magnification Settings** dialog, select a name and location for the backup file and then click **Save**. The default location is **C:\MX5\app\mmproc\DATA**.

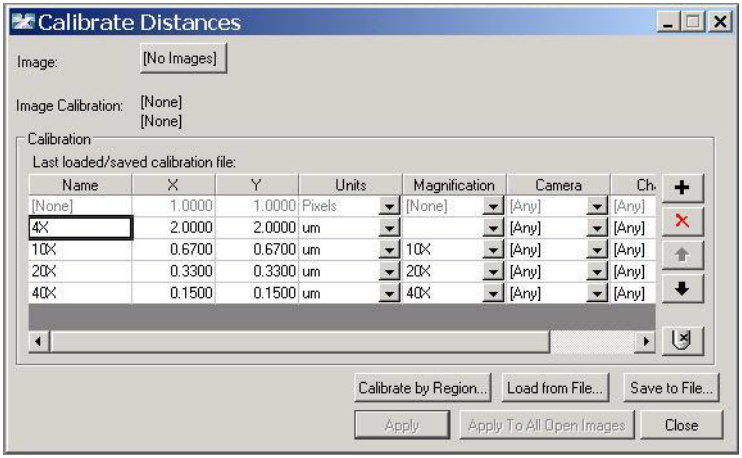
Tip! Restore the settings by clicking Restore and then choosing the saved file.

9. Click **Close**.

Verifying and Backing Up Calibration Settings

To verify and backup calibration settings

1. In the MetaXpress software, click **Measure > Calibrate Distances**.
2. In the **Calibrate Distances** dialog, click the **Setup** tab and in the **Calibrations** field, confirm that the calibration settings match the objective settings in the **Configure Magnification** dialog. Make sure the appropriate magnification setting is selected for each calibration. See Table A-1 Nikon Objectives Compatible with the ImageXpress Ultra System on page 173 for the distance calibrations for specific objectives.



3. Click **Save to File**.
4. In the **Save Spatial Calibrations** dialog that appears, select a name and location for the backup file and then click **Save**. The default location is **C:\MX5\app\mmproc\DATA**.

Tip! Restore the settings by clicking Load from File and then choosing the saved file.

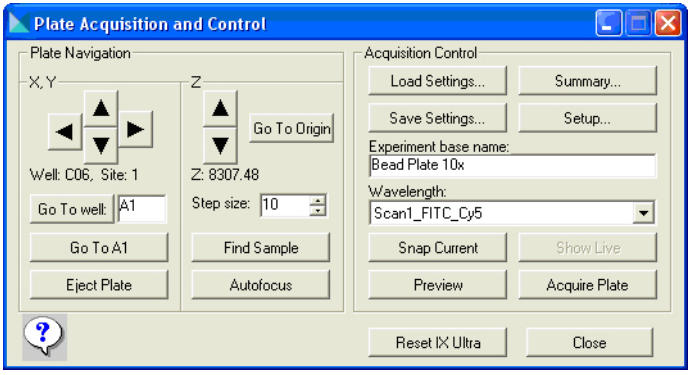
5. Click **Close**.

Verifying the Laser Autofocus Sensor

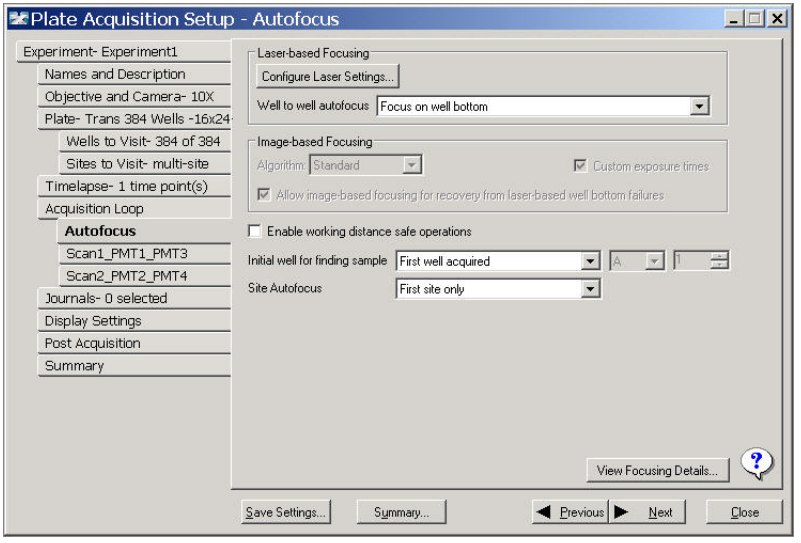
This procedure uses a bead plate to test that the laser autofocus sensor is enabled and functional.

To verify the laser autofocus response

1. In the MetaXpress Software, click **Screening > Plate Acquisition Setup**.
2. Click the **Plate** tab and in the **Plate name** field, select **Bead Plate**.
3. Click the **Objective and Camera** tab and then select the **10X Plan Fluor** objective.
4. Click **Screening > Plate Acquisition and Control**.
5. In the **Plate Acquisition and Control** dialog, click **Eject Plate** to move the stage to the load position.



6. Load the bead plate and then click **Load Plate**.
7. Click **Go To A1** to move the stage to the A1 position.
8. In the **Plate Acquisition Setup** dialog, click the **Autofocus** tab.

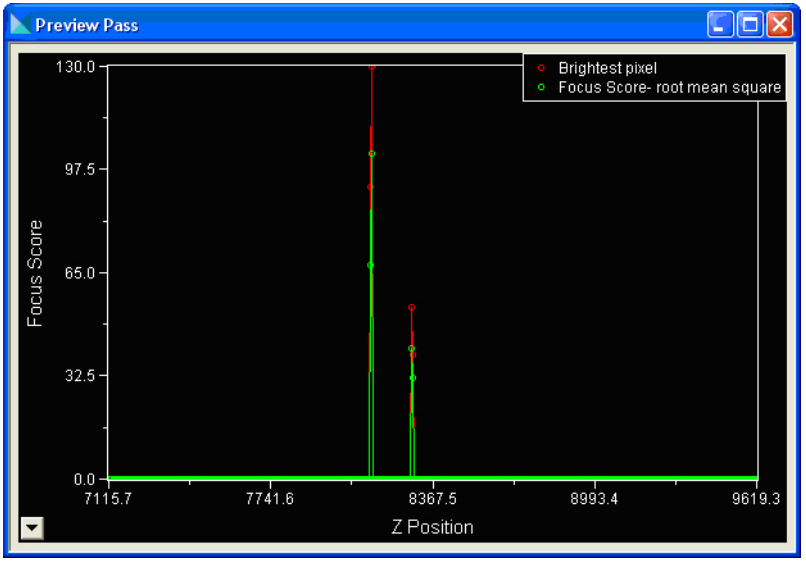


9. Click **Configure Laser Settings**

The Configure Laser Autofocus Settings dialog appears.

10. Click **Preview Pass**.

The Preview Pass dialog appears displaying a graph of focus intensities versus Z position.



Note: If the Preview Pass window contains at least one peak, the laser autofocus sensor is enabled and functional. If the Preview Pass window does not contain any peaks, make sure that the plate is properly seated, increase the Exposure value in the Configure Laser Autofocus Settings dialog, and then try again. If the chart still does not contain a peak, contact Technical Support and report the issue. For more information on the Preview Pass window, see To configure laser autofocus on page 118.

11. In the **Configure Laser Autofocus Settings** dialog, click **Close**.

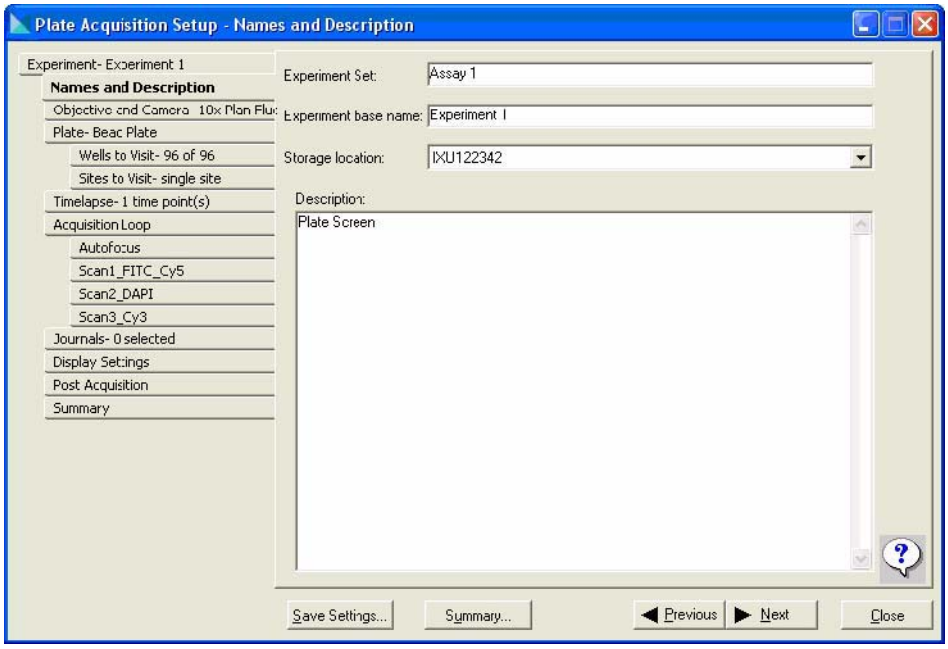
Verifying the Plate Reference Point (A1 Center)

You need the metal X/Y calibration plate/slide holder that ships with the ImageXpress Ultra System to complete this procedure.

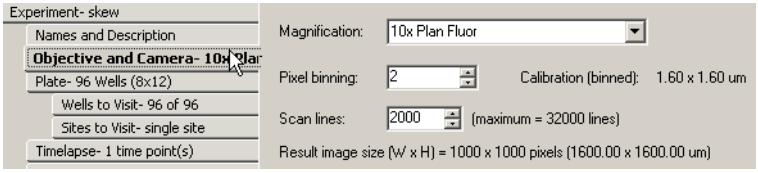
To set the plate reference point (A1 center)

1. In the MetaXpress Software, click **Screening > Plate Acquisition Setup**.

   The Plate Acquisition Setup dialog appears.

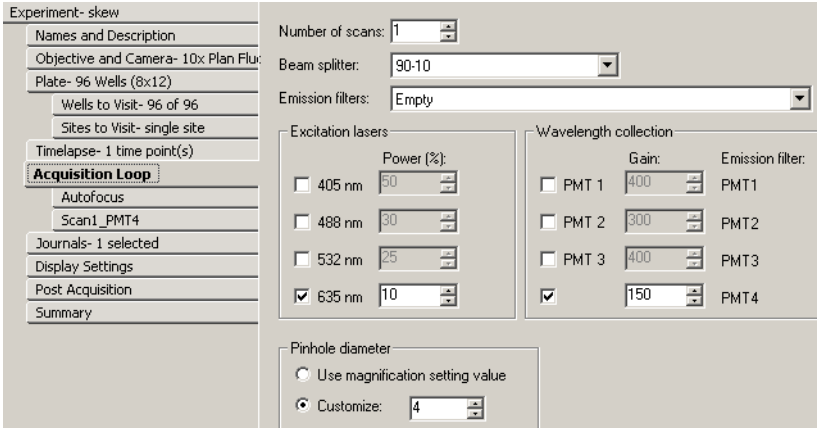


2. Click the **Objective and Camera** tab and then do the following:
   - In the **Magnification** field, select **10X Plan Fluor**.
   - In the **Pixel Binning** field, type **2.**
   - In the **Scan lines** field, type **2000**.

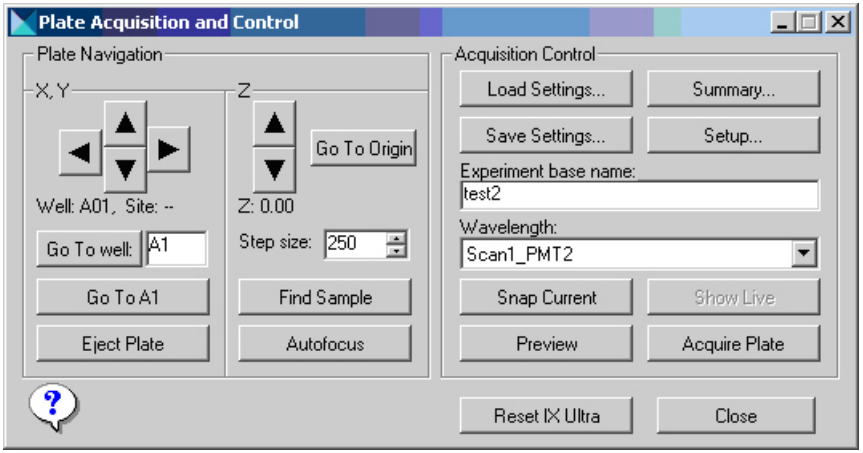


3. Click the **Plate** tab.
4. In the **Plate name** field, click **96 Wells (8×12)**.
5. Click the **Sites to Visit** tab and select **Single site**.
6. Click the **Acquisition Loop** tab and in the **Emission filters** field, click **Empty**, in the **Beam splitter** field, click **90-10** to create a reflectance channel. In a system with no empty filter sets, you must remove one of the filter sets to perform this operation.

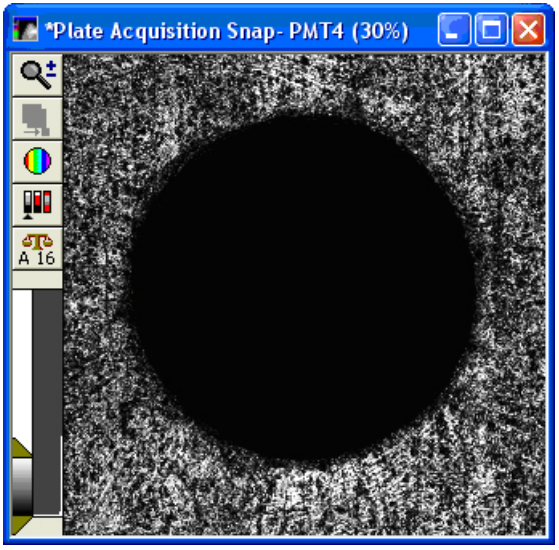
WARNING! Turn off the ImageXpress Ultra System when removing emission filter sets.



7. In the **Excitation Lasers** section, in the **635 nm Power** field, type **10**.
8. In the **Wavelength collection** section, in the **PMT4 Gain** field, type **150**.
9. Click **Screening > Plate Acquisition and Control**.
10. In the **Plate Acquisition and Control** dialog, in the **Step size** field, type **250**.



11. Click **Eject Plate** to move the stage to the load position.
12. Load the metal X/Y calibration plate and make sure that the notch in the plate is in the A1 position on the stage.
13. Click **Load Plate** to load the plate.
14. Click **Go To A1** to move the stage to the A1 position.
15. In the **Plate Navigation** section, adjust Z until it is at 6000 µm above the bottom position (typically the bottom position is indicated as 0 µm).
16. In the **Step size** field, type **20** µm.
17. Click **Snap Current**.
18. Use the Z control arrows to step the Z motor (reducing the step size as you get closer to focus if needed) and then click **Snap Current** until the A1 calibration hole comes into focus.



19. Verify that the hole is visually centered in the field of view. If it is not, or if you cannot find the hole, contact Technical Support.

Note: The A1 Calibration pinhole will always fit in the field-of-view of the ImageXpress Ultra System with a 10X objective (as long as the image is 2000 scan lines).

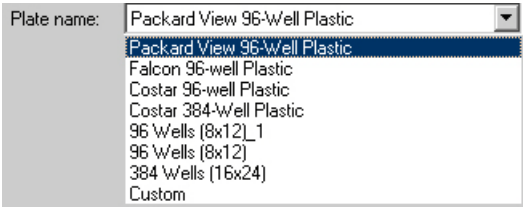
20. In the **Plate Acquisition and Control** dialog, click **Eject Plate** to eject the X/Y calibration plate.

Verifying Plate Types

You must verify that the preconfigured plate type files included with the MetaXpress Software are available from the Plate Acquisition Setup dialog.

To verify plate types

1. In the MetaXpress Software, click **Screening > Plate Acquisition Setup** and then click the **Plates** tab.
2. Click the **Plate name** list to view the available plate type files and check that a number of custom plates are available.



Adding Pre-configured Plate Types

If there are no plate types listed other than the three defaults, 96 Wells (8×12), 384 Wells (16×24), and Custom, then the preconfigured plate type files are not loaded and you must add them.

To add pre-configured plate types

1. Insert the MetaXpress Software installation USB flash drive into the computer.
2. In the **MetaXpress Installation** screen that appears, click **Explore Installation Folders / Files**.
3. Open the **Plates** folder.

   This folder contains the preconfigured plate type files (.plt).
4. Copy the plate files to the **Plates** directory of the MetaXpress Software installation directory. The default location is **C:\MX5\Plates**.

   These files appear in the Plate name list in the Plates tab of the Plate Acquisition Setup dialog.
5. As the plate files are Read-only after they are copied from the flash drive, you must turn off the Read-only attribute by selecting all the plate files that you copied to the **Plates** directory (Shift + click to select multiple continuous items), right-click the selected files, and then select **Properties**.

6. In the **Properties** dialog, in the **Attributes** section, clear the **Read-only** check box and then click **OK**.

Confirming Laser Autofocus Settings for Plate Files

Just before each site is acquired during plate acquisition, the laser autofocus system automatically moves the vertical (Z-axis) position of the objective to a point where the bottom of the well is in focus. Before using a plate file, confirm that the laser autofocus settings are optimal for the plate. You must prepare the plate that you are going to test by putting water or buffer similar to the buffer you will be using for real experiments in several of the wells.

If you are using an objective with a correction collar, make sure that the correction collar is set appropriately for the plate you are using. See Adjusting the Spherical-Aberration Correction Collar on Objectives on page 165.

Use the Laser Autofocus Wizard, available on the Plate tab, to confirm laser autofocus settings. The wizard walks you through the process. Additional information about the settings that the wizard calculates is available in the online help, in the topic “Configure Laser Autofocus Settings - Dialog Box Options.”

Note: The Laser Autofocus Wizard calculates measurements as accurately as possible. Some manual verification and adjustment of the settings may be necessary to optimize the results, particularly for thin-bottom plates. Contact Technical Support if you need assistance.

Verifying Shading Correction Files

Shading correction files are required for each objective and filter combination and must be generated whenever an objective or filter is replaced or added to the system. There will also be a weaker effect on shading of the beam expander setting. Shading correction files must be generated with a pinhole diameter of 1 mm. These files are applicable to images generated with other pinhole settings. The image scan length of the shading files must exceed that of your acquired image for effective shading correction to be applied. See Generating Shading Correction Files on page 105.

To use shading correction images during plate acquisition

1. In the **Plate Acquisition Setup** dialog, in the **Acquisition Loop** tab, select the **Perform shading correction** check box.
2. In the **Scan** tab, select a shading correction file for each active PMT.

Guidelines for Planning Experiments

5

This chapter provides information on:

- Planning High-Content Experiments on page 63
- Obtaining Quality Images on page 64
- Additional Guidelines for Planning Experiments on page 65

Planning High-Content Experiments

When acquiring fluorescence images in a screening environment, follow a basic set of rules and guidelines to acquire quality images.

As with any biological assay, you must evaluate the assay conditions required to obtain a meaningful result. Include in your sample preparation both negative and positive controls so you can judge the validity of your assay and optimize experimental conditions. Molecular Devices recommends using a nuclear stain such as DRAQ5, Hoechst 33342 or DAPI to stain your cells, since this stain can be used for image segmentation as part of your analysis.

While image enhancement tools and options are available in the MetaXpress Software, it is difficult to analyze a poor quality image. Starting with quality images ensures that your image data is more meaningful, and yields more information. Part of assay optimization includes the image analysis of positive and negative controls to make sure that the imaging conditions are sufficient to distinguish these cases.

The following are some criteria to consider for attaining the highest possible high-content assay results:

- cell lines
- treatment conditions
- staining conditions
- dyes
- fixation conditions
- microplate choice
- media
- laser lines and filters
- objectives
- binning
- averaging

Obtaining Quality Images

The following conditions or situations can interfere with obtaining the highest quality images.

Table 5-1 Conditions that Interfere with Quality Images

Condition	Description and Possible Corrective Actions
Optics are degraded by dust, dirt, fingerprints, or oil contamination	If you detect any contamination on your objectives, you should inform your system administrator, who can take steps to clean the optics and correct the problem.
Uneven background	The first step to correct an uneven background is to check for uneven illumination. If the illumination is uneven during fluorescence acquisition, contact your system administrator.
Poor quality microplates	Not all microplates are the same quality. The composition of the bottom of the microplate must be of optical quality, or the images can be degraded. For fluorescence imaging, microplates with black well sides and flat clear bottoms usually work the best. Plastic-bottom plates are usually more uneven and distort light more than glass-bottom plates.
Incorrect microplates	If you are screening multiple plates, make sure that all plates are the same type and from the same manufacturer. Well spacing can vary slightly and plate bottom thickness can vary significantly from one manufacturer to another. It is not possible to continuously change the plate settings during a single experiment run.
Plate bottom quality degraded by dust, dirt, fingerprints, or oil contamination on the microplate	Since the laser measures the reflection from the bottom of the plate or from the bottom of the well, interference with reflections caused by dust particles, dirt, fingerprints, and scratches affects the performance of the autofocus. Clean the bottom of the plate using lens tissue and an optical cleaning solution to improve the autofocus. Dust, dirt, fingerprints, or oil contamination on the bottom of the microplate can also reduce image quality.

Additional Guidelines for Planning Experiments

Sample Preparation

Media with a refractive index similar to that of the microplate (for example, media containing glycerol) can make it difficult to focus on the bottom of the well due to the weak reflection of the autofocus laser beam. Media containing dyes such as Phenol Red increase the background fluorescence in the images. Therefore, a clear buffer such as PBS (phosphate buffered saline) is recommended. You must have sufficient volume in the wells so that the fluid is 2.5 mm above the sample. In addition, avoid bubbles in the sample, for both image quality and autofocus performance.

Laser Power

Note: Increasing the laser power increases the brightness of acquired images, however, excessive laser power has the potential to saturate the dye and photobleach your sample without increasing image brightness.

To find the optimal laser power for your sample

1. Acquire the same image from a typical sample at different laser powers (starting with low powers first).
2. Plot fluorescence intensity versus the laser power. The resulting graph provides an estimate of the safe range of illumination intensities (that is, intensities that are below the saturation level of the dye).

The following figures show three examples of such an experiment. The figures show the average GFP fluorescence intensity of the non-saturated portion of the image as a function of the percent of maximum power of the 488 nm laser set in the software. A significant deviation from the linear behavior (decreasing slope) indicates that there is a diminishing advantage to further increasing the laser power.

The first experiment (Figure 5-1) shows that the intensity is low even at 100% laser power. The second experiment (Figure 5-2) shows intensity being maximized at close to 60% laser power. The third experiment (Figure 5-3) shows a system where increasing laser power to 40% gives maximum intensity, and to 50% and above causes a reduction in intensity.

The plots serve as an example and appropriate laser power ranges vary for different optical configuration and dye combinations. For optimal data quality, run similar calibration experiments with the specific objective, beam expander, beam splitter, plate type, and assay type that you intend to use.

Note: Laser intensities below 10% are not stable and are not accepted by the instrument firmware.

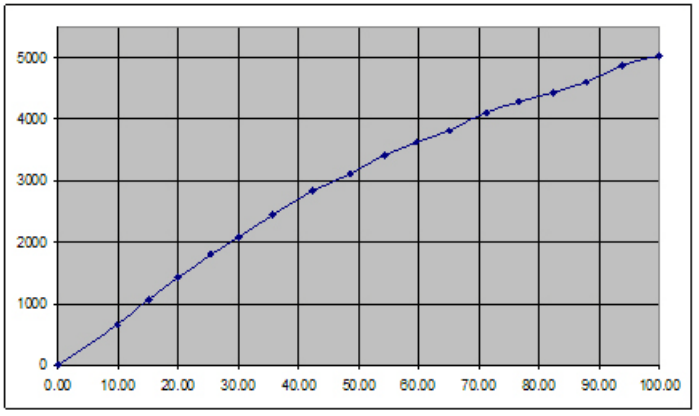


Figure 5-1 Objective:10X Plan Fluor, expander – 3x, beam splitter – 20/80. PMT voltage = 550 V

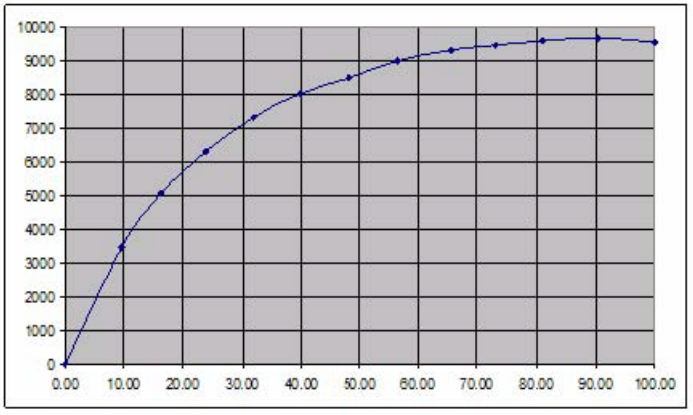


Figure 5-2 Objective – 20X ELWD, expander – 2x, beam splitter – 488/635 dual notch. PMT voltage = 550 V

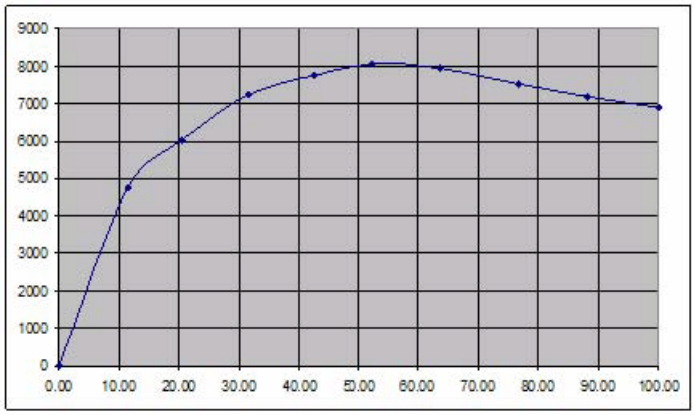


Figure 5-3 Objective – 40X ELWD, expander – 1x, beam splitter – 488/635 dual notch. PMT voltage = 600 V

Objective Choice

The choice of objective determines the magnification, the depth of field, and the brightness of the image. A key attribute of the objective is the numerical aperture (NA). With the magnification constant, brightness increases quickly with the increasing NA.

Higher NA objectives produce a sharper picture due to a narrower depth of field. This can be an advantage if some of your objects are in different Z positions. However, higher NA short working distance objectives cannot reach the outer rows and columns of some multiwell plates because of the plate's skirt height, causing the edge of the objective to collide with the plate skirt or with the ribs connecting the plate skirt to the well region.

Imaging through a thick-bottom plate (for example, 0.5 to 1 mm) is also degraded with high NA objectives unless they have suitable spherical-aberration correction collars. The Nikon ELWD (extra long working distance) objectives are suitable for focusing through thick plates. See Appendix A: Objectives Compatible with the ImageXpress Ultra System on page 173 for the Nikon objectives compatible with the ImageXpress Ultra System.

Use of Different Fluorophores

Individual fluorophores have unique characteristics that help determine their best use. Some fluorophores provide brighter intensities and require low laser power, while others do not bleach as quickly.

There also might be toxicity issues with some cell types or bleed-through issues between pairs of fluorophores.

These factors should be considered when choosing a fluorophore.

Dyes and Applications for the ImageXpress Ultra System

The following tables detail the dyes and applications for each laser that you can use in the ImageXpress Ultra System. The probes that have been validated on the ImageXpress Ultra System by Molecular Devices are also indicated in the tables. Other probes that are listed in the tables are predicted to work to some extent with the indicated lasers but their performance on the system cannot be guaranteed.

Note: The list of potential dyes and applications for each laser is not intended to be an exhaustive list of all possible probes.

Table 5-2 405 Laser

Dyes	Applications	Detection theoretically possible with Emission Filters	Validated on the ImageXpress Ultra System by Molecular Devices
DAPI	DNA (nuclear) staining	Yes (417-447 filter)	Yes
Hoecht 33342	DNA (nuclear) staining	Yes (417-447 filter)	Yes
Alexa 405	Secondary antibody or streptavidin staining	Yes (417-447 filter)	No
Alexa 430	Secondary antibody or streptavidin staining	Yes (525/50 filter, others possible)	No

Table 5-2 405 Laser (cont'd)

Dyes	Applications	Detection theoretically possible with Emission Filters	Validated on the ImageXpress Ultra System by Molecular Devices
Cyan fluorescent protein (CFP) (not optimal for 405 laser)	Live cell protein dynamics, FRET with YFP	Yes (525/50 filter, others possible)	No
Blue fluorescent protein	Live cell protein dynamics	Yes (417-447 filter)	No
Pacific Blue	Secondary antibody or streptavidin staining	Yes (417-447 filter)	No
Lucifer Yellow	Neuronal tracing	Yes (525/50 filter, others possible)	No
CellTrace calcein violet 405	Live cell identification	Yes (417-447 filter)	No
LIVE/DEAD Fixable Violet Dead Cell Stain	Dead cell identification	Yes (417-447 filter)	No
LIVE/DEAD Fixable Aqua Dead Cell Stain	Dead cell identification	Yes (525/50 filter, others possible)	No
Photoactivatable GFP (PA-GFP)	Live cell protein dynamics	Not Applicable (405 laser used for activation only)	No

Table 5-3 488 Laser

Dyes	Applications	Detection theoretically possible with Emission Filters	Validated on the ImageXpress Ultra System by Molecular Devices
GFP	Live cell protein dynamics	Yes (500-520 or 525/50 or 496-580)	Yes
EGFP	Live cell protein dynamics	Yes (500-520 or 525/50 or 496-580)	Yes
FITC	Secondary antibody or streptavidrin staining	Yes (500-520 or 525/50 or 496-580)	Yes
Alexa 488	Secondary antibody or streptavidrin staining	Yes (500-520 or 525/50 or 496-580)	Yes
BODIPY FL	Coupled to various small compounds	Yes (500-520 or 525/50 or 496-580)	Yes
Calcein	Live cell identification	Yes (500-520 or 525/50 or 496-580)	No
Yellow fluorescent protein (YFP)	Live cell protein dynamics, FRET with CFP	Yes (525/50 filter or 496-580)	No
Oregon green	Fluorescein analog	Yes (500-520 or 525/50 or 496-580)	No
Rhodamine green	Secondary antibody or streptavidrin staining	Yes (500-520 or 525/50 or 496-580)	No
Acridine Orange	Dye (nuclear) staining	Yes (500-520 or 525/50 or 496-580)	No
7-AAD (7-Aminoactino mycin D)	Dye (nuclear) staining	Yes (685/40)	No
Ethidium Bromide	Dye (nuclear) staining	Yes (565-605 or 593/40)	No

Table 5-3 488 Laser (cont'd)

Dyes	Applications	Detection theoretically possible with Emission Filters	Validated on the ImageXpress Ultra System by Molecular Devices
SYTOX Green	Dye (nuclear) staining	Yes (500-520 or 525/50 or 496-580)	No
BCECF	Intracellular pH measurements	Yes (500-520 or 525/50 or 496-580)	No
Calcium Green	Intracellular Ca2+ measurements	Yes (500-520 or 525/50 or 496-580)	No
Carboxy-SNARF (R)-1-AM pH 6	Intracellular pH measurements	Yes (565-605 or 593/40)	No
JC-1	Mitochrondrial membrane potential	Yes (565-605 or 593/40)	No
DiO	Membrane staining for cell tracking	Yes (500-520 or 525/50 or 496-580)	No
Fluo-3	Intracellular Ca2+ measurements	Yes (500-520 or 525/50 or 496-580)	No
NBD-C6	Golgi staining	Yes (500-520 or 525/50 or 496-580)	No

Table 5-4 532 Laser

Dyes	Applications	Detection theoretically possible with Emission Filters	Validated on the ImageXpress Ultra System by Molecular Devices
Cy3	Secondary antibody or streptavidin staining	Yes (565-605)	Yes
Rhodamine	Secondary antibody or streptavidin staining	Yes (565-605)	Yes
TRITC	Secondary antibody or streptavidin staining	Yes (565-605)	Yes
MitoTracker Orange	Mitochondrial staining in live cells	Yes (565-605)	Yes
Alexa 532	Secondary antibody or streptavidin staining	Yes (565-605)	Yes
Alexa 546	Secondary antibody or streptavidin staining	Yes (565-605)	No
Alexa 555	Secondary antibody or streptavidin staining	Yes (565-605)	No
Alexa 610-RPE	Secondary antibody or streptavidin staining	Possibly (565-605)	No
R-phycoerythrin (R-PE)	Secondary antibody or streptavidin staining	Yes (565-605)	No
Calcium Orange	Intracellular Ca2+ measurements	Yes (565-605)	No

Table 5-4 532 Laser (cont'd)

Dyes	Applications	Detection theoretically possible with Emission Filters	Validated on the ImageXpress Ultra System by Molecular Devices
DiI	Membrane staining for cell tracking	Yes (565-605)	No
Propidium Iodide (PI)	DNA (nuclear) staining in dead cells	Yes (565-605)	No
POPO-3	DNA (nuclear) staining	Yes (565-605)	No
PO-PRO-3	DNA (nuclear) staining in dead cells	Yes (565-605)	No
TAMRA	Oligonucleotide labelling	Yes (565-605)	No
Ethidium homodimer (LIVE/DEAD)	DNA (nuclear) staining in dead cells	Yes (565-605)	No

Table 5-5 561 Laser

Dyes	Applications	Detection theoretically possible with Emission Filters	Validated on the ImageXpress Ultra System by Molecular Devices
TRITC	Secondary antibody or streptavidin staining	Yes (593/40)	Yes
Texas Red	Secondary antibody or streptavidin staining	Yes (593/40)	Yes
Mitotracker Red	Mitochondrial staining in live cells	Yes (593/40)	Yes
DsRed	Live cell protein dynamics	Yes (593/40)	Yes

Table 5-5 561 Laser (cont'd)

Dyes	Applications	Detection theoretically possible with Emission Filters	Validated on the ImageXpress Ultra System by Molecular Devices
Alexa 555	Secondary antibody or streptavidin staining	Yes (593/40)	Yes
Alexa 568	Secondary antibody or streptavidin staining	Yes (593/40)	No
Alexa 594	Secondary antibody or streptavidin staining	Yes (593/40)	No
Alexa 610 (not optimal)	Secondary antibody or streptavidin staining	No	No
Alexa 610-RPE	Secondary antibody or streptavidin staining	No	No
R-phycoerythrin (R-PE)	Secondary antibody or streptavidin staining	Yes (593/40)	No
Dil	Membrane staining for cell tracking	Yes (593/40)	No
Calcium Crimson	Intracellular $Ca2+$ measurements	Yes (593/40)	No
LysoTracker Red	Lysosome staining in live cells	Yes (593/40)	No
Rhodamine Red	Secondary antibody or streptavidin staining	Yes (593/40)	No
Nile Blue (not optimal)	Lipid staining in fixed cells	No	No

Table 5-6 635 Laser

Dyes	Applications	Detection theoretically possible with Emission Filters	Validated on the ImageXpress UltraSystem by Molecular Devices
DRAQ5	DNA (nuclear) staining	Yes (685/40)	Yes
Cy5	Secondary antibody or streptavidin staining	Yes (685/40)	Yes
TO-PRO-3	DNA (nuclear) staining in dead cells	Yes (685/40)	Yes
Alexa 633	Secondary antibody or streptavidin staining	Yes (685/40)	No
Alexa 635	Secondary antibody or streptavidin staining	Yes (685/40)	No
Alexa 647	Secondary antibody or streptavidin staining	Yes (685/40)	Yes
Cy5.5	Secondary antibody or streptavidin staining	Yes (685/40), not optimal	No
BODIPY 630/650	Protein conjugation	No	No
DiD	Membrane staining for cell tracking	Yes (685/40)	No
CellMask Deep Red	Plasma membrane staining	Yes (685/40)	No

Incompatible Probes

The following table details some probes that might not work well with any of the available lasers.

Table 5-7 Incompatible Probes

Dyes and Applications	Notes
Any QDots	Fluorescent lifetime is much slower than the point scanning
CellMask Blue	
Marina Blue	
LysoTracker Blue	
Indo-1	
Hoechst 33258	
DiR	
Alexa 750	
Alexa 790	
Alexa 350	

Plate Selection

There are numerous types of multiwell plates available from a variety of vendors. Molecular Devices recommends that you determine the plates to use for your screening experiments based on the guidelines in the following table.

Table 5-8 Plate Selection Guidelines

Guideline	Description
Cell Compatibility	Verify that your cells are compatible with the plate material. Given the wrong surface, some cells might not bind and will act in an unusual manner, such as rounding up or migrating to the edges of the well. Plastic plates tend to show higher background staining as compared to glass plates.
Fluorescence background	There is a difference in auto fluorescence between glass and plastic. In addition, there can be up to a five-fold difference in auto fluorescence among plates from different manufacturers. If your signal-to-background ratio is low, try other brands of plates as a first step in troubleshooting. **Note:** Black plates with clear bottoms are superior to all-clear plates.
Plate skirt height	If you are using a high NA or short working distance objective you should use a plate with a short skirt height (the height is the difference between the edge of the plate and the bottom surface of the wells). If the skirt height is too high, you might not be able to image the outermost parts of a plate (the outermost rows and columns on a 384-well plate or some sites on the outermost rows and columns on a 96-well plate). **Note:** The skirt height referred to is not the same as the flange height described in the ANSI (SBS) standard.
High-magnification image clarity	When using high magnifications, there are significant differences in clarity between standard plastic plates, optically clear plastic plates, and glass-bottom plates.
Plate flatness and reproducibility of the Z pattern	A truly flat plate is faster to scan than an uneven plate because the autofocus search range can be made smaller. The reproducibility of a plate allows you to set tighter focus ranges for a specific plate type. This reduces the amount of focusing needed and speeds up acquisition. The primary impact of plate flatness on throughput is the typical variation from one well to a neighboring well (rather than the total plate flatness). In general, glass plates are flatter than plastic plates.

Table 5-8 Plate Selection Guidelines (cont'd)

Guideline	Description
Outside edge of the plate	If you use a plate-handling robot, some types of plates do not work well with the fingers supplied with the robot and might require custom fingers from the robotics vendor to work correctly.
Plate-to-plate reproducibility	Plates can vary in their behavior from one manufacturing lot to another. Your assay will perform better if the plates do not vary significantly.
Plate bottom thickness	The plate bottom thickness must be compatible with a range of working distances for the specific objective being used. See Table A-1 Nikon Objectives Compatible with the ImageXpress Ultra System on page 173.

Setting Up Plate Acquisition

6

Before configuring an experiment, it is important to become familiar with the tools used in the MetaXpress® High Content Image Acquisition and Analysis Software. The foundation of the MetaXpress Software is MetaMorph® Software. The MetaMorph Software contains numerous dialogs for image acquisition, processing, and analysis. However, the majority of the dialogs available are not needed for a typical MetaXpress Software plate acquisition. This chapter explains the dialogs and procedures required to configure a screening experiment.

Note: For more information on any MetaXpress Software dialog, press the F1 key to access the Help in the MetaXpress Software application.

The chapter contains the following:

- Overview on page 80
- Creating Experiments on page 83
- Naming the Experiment on page 86
- Configuring Binning, Averaging, and Scan Lines Settings on page 87
- Determining Plate Optical Thickness and Plate Bottom Variation on page 91
- Configuring Plates on page 91
- Optical Thickness and Laser Focus on page 94
- Defining Protocol Settings on page 105
- Configuring Autofocus Settings on page 118
- Running Journals During Acquisition on page 125
- Configuring the Display Settings on page 127
- Selecting a Data Analysis Method on page 129
- Reviewing the Plate Acquisition Settings on page 131
- Saving Acquisition Settings on page 133

Overview

This section describes the main MetaXpress Software components that you will use when you are configuring the ImageXpress® Ultra System for plate acquisition.

Screening Menu

The Screening menu provides access to all plate acquisition-specific dialogs:

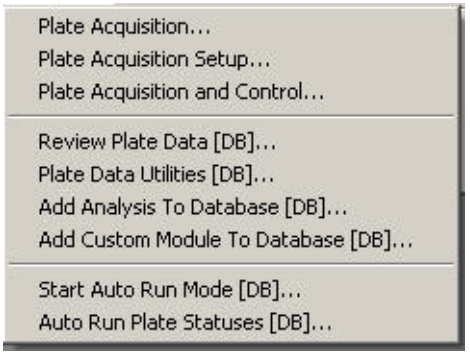


Plate Acquisition and Control dialog

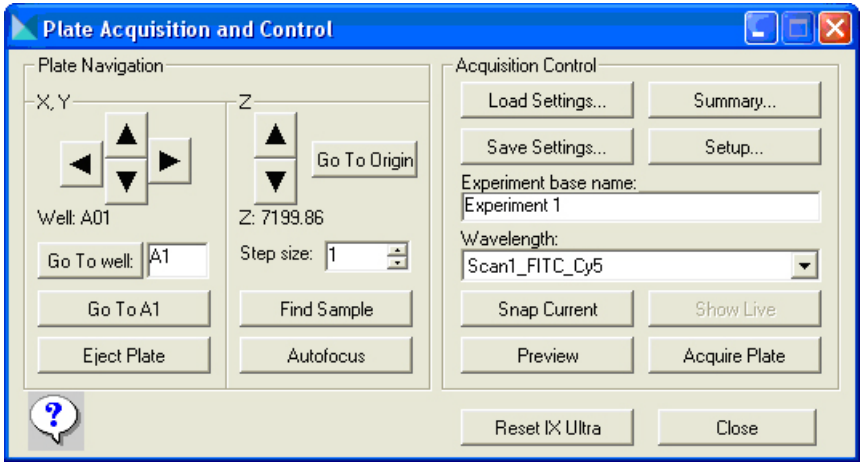
The Plate Acquisition and Control dialog provides two primary functions:

- Plate Navigation provides manual control of some of the movable physical components of the ImageXpress Ultra System.
- Acquisition Control enables you to prepare and start a plate acquisition.

In the Plate Navigation section, use the X, Y arrow buttons on the left to move the plate manually from one well to another on the X and Y axes.

If sites are enabled, the currently selected site is displayed below the arrow buttons along with the selected well. When you move from well to well, the selected site will remain the same.

To change the site selection, use the site selection buttons on the Plate Acquisition tool bar, or the Sites to Visit tab fields of the Plate Acquisition Setup dialog.



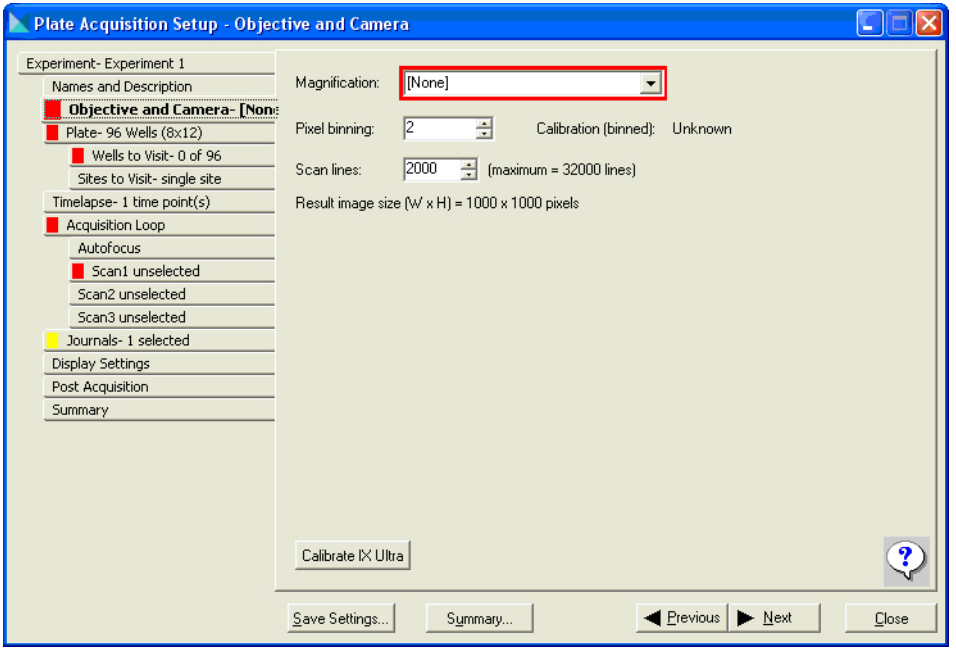
The Acquisition Control section groups commands that can also be found in other dialogs. These controls enable you to save settings, load settings that have previously been saved, open the Plate Acquisition Setup dialog, specify a new experiment base name, choose the wavelength that you want to use, snap an image using the current wavelength, preview the current selection of wells, and acquire an entire plate or all wells selected on the plate.

Plate Acquisition Setup dialog

To acquire data using the ImageXpress Ultra System, you must configure the settings in the Plate Acquisition Setup dialog. The Plate Acquisition Setup dialog is organized in a “top-to-bottom” structure designed to guide you through the process of setting up your MetaXpress Software configuration in the correct order.

The Plate Acquisition Setup dialog is a multi-tabbed dialog in which the number of visible tabs can change according to the options that you have selected and the number of wavelengths that you are acquiring. Each tabbed section of the dialog is dedicated to a specific type of setting.

When configuring the Plate Acquisition Setup dialog, settings might be highlighted either in yellow or red. A yellow highlight can mean that an optional field is not filled in or could indicate a minor error. A red highlight means that a required field is either not filled in or contains invalid data that should be changed. These visual reminders help when configuring an experiment.

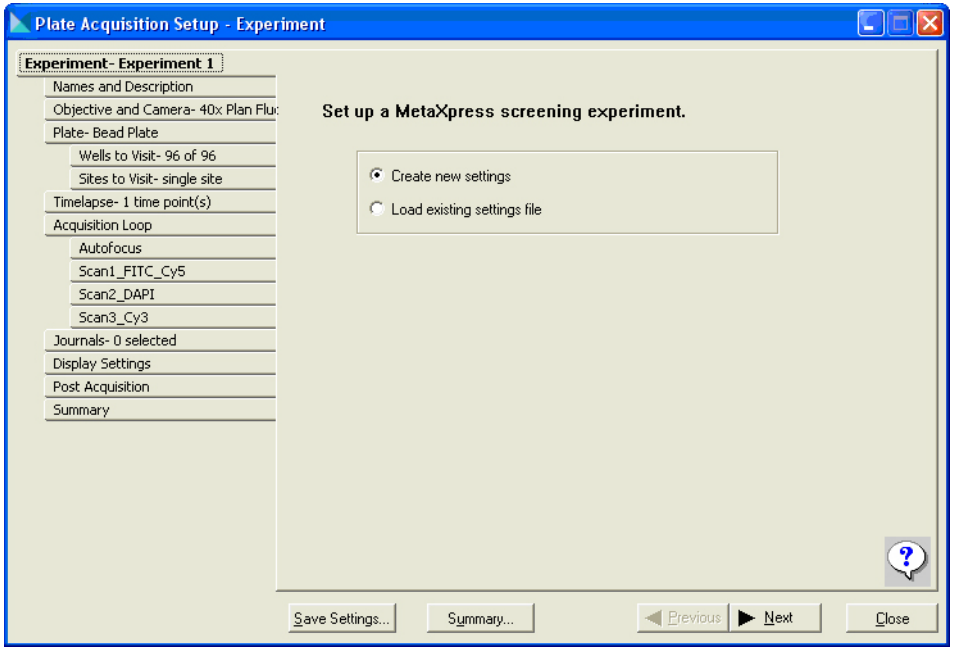


Creating Experiments

You can configure new settings for your experiment or create experiments using an existing settings file.

To create a new experiment

1. In the **Plate Acquisition Setup** dialog, click the **Experiment** tab and then click **Create new settings**.
2. Complete the information in all the tabs in the **Plate Acquisition Setup** dialog following the order of the tabs.

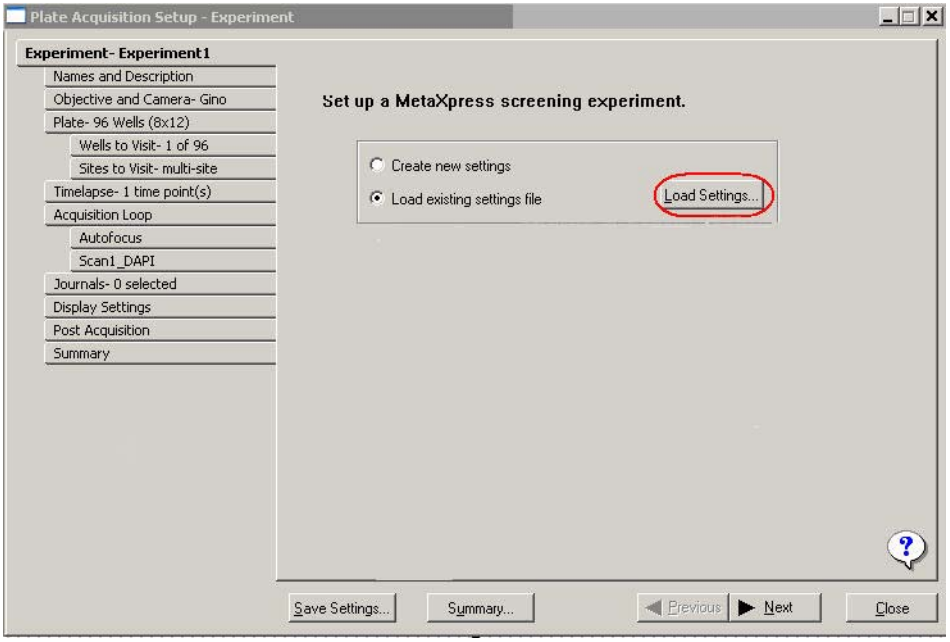


To create an experiment using an existing settings file

You can load a settings file that was saved to the database or that was saved outside the database, for example, on the desktop or a network drive.

1. Select **Load existing settings** file.

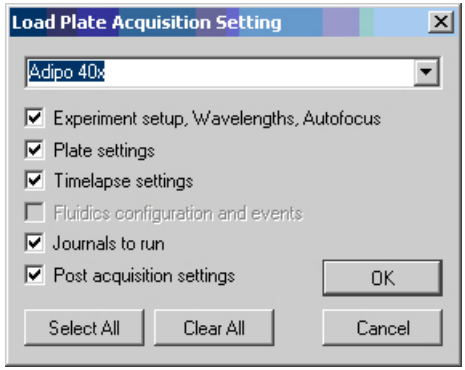
   The Load Settings button is displayed.



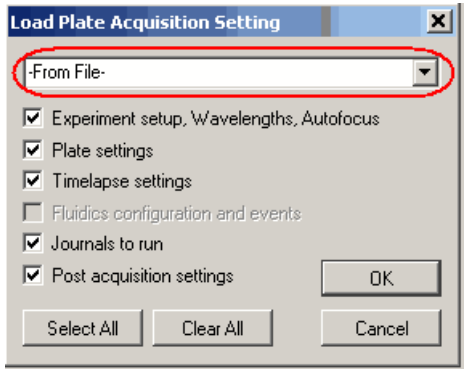
2. Click **Load Settings**.

   The Load Plate Acquisition Setting dialog appears.

3. If you are using a settings file (.hts) that was saved to the database, select the required file and then click **OK**. The file loads from the database and the **Load Plate Acquisition Setting** dialog closes.



4. If you are loading a settings file that was saved outside the database, select **From File** and then click **OK**. You are prompted to navigate to the required settings file, select it and then click **Open**. The settings file loads and the Load Screen Acquisition dialog closes.



Note: You can load all components or selected components of a saved settings file.

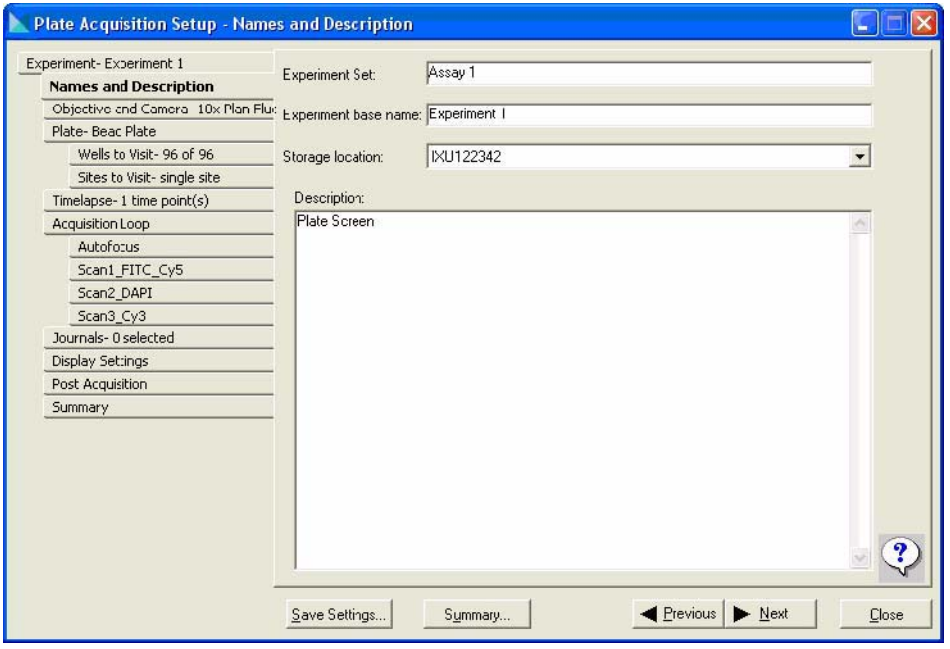
5. Click **Summary** to review and confirm your settings.

Naming the Experiment

After you have selected your settings file or created a new settings file, you must designate a name and description for your experiment, and choose the storage location for your experiment data.

To add an experiment set name, base name, and description

1. In the **Experiment Set** field, type a name to sort and group experiments.



2. In the **Experiment base name** field, type a base file name for all image files in the experiment.
3. In the **Storage Location** field, select where screening images are saved. If a location is not available or you are unsure about where to store the images, contact your system administrator.

Note: Image storage locations are configured using the MDCStoreTools utility. If a location does not appear in this list, add it to the list using the MDCStoreTools utility. See the *MDCStoreTools User Guide* included on the MetaXpress Software installation USB flash drive and in the Molecular Devices knowledge base at www.moleculardevices.com/Support.html (follow the links to the knowledge base).

4. In the **Description** field, type information about the experiment set. This is stored with the image information in the database.

Configuring Binning, Averaging, and Scan Lines Settings

For each magnification setting that you have defined, you can edit the camera binning and the number of scan lines. These settings enable you to improve either the image acquisition speed or the image quality. If sufficient light is available, lower camera binning increases image resolution.

Binning Overview

Binning of an image can be beneficial for low intensity images, since it increases the signal-to-noise ratio and reduces the image file size. In this process, you lose resolution since you are grouping pixels together. For example, a 2 × 2 binning will combine the signals of four pixels into a single pixel, resulting in a reduction of your image from 100% maximum size to 25% of its maximum size. Image acquisition time is inversely proportional to the binning number (2x binning generates an image twice as fast as no binning).

The ImageXpress Ultra System is a scanning system and binning is implemented in a different fashion than in a CCD camera.

Horizontal scan lines in the ImageXpress Ultra System are acquired with the internal galvanometer scanning mirror. Each scan line is composed of a series of very fast (several ns) 8-bit samples. Sets of 32 sequential 8-bit samples are summed to 13-bit numbers. Each 13-bit number is left-shifted 3-bits to become an 16-bit number, representing a pixel. Each scan line has 2000 pixels.

Each image is composed of a series of horizontal scan lines. The test sample is moved in the vertical (Y) direction by the XY sample stage, allowing the acquisition of a user-selected number of scan lines (up to 32,000).

With 2x binning selected, 64 sequential 8-bit samples are summed for each pixel in the horizontal direction, resulting in 1000 pixels in each scan line. The 64 summed samples make a 14-bit number, which is shifted 2 bits to become a 16-bit number. For greater than 2x binning, the number of samples summed together is correspondingly increased, with the final pixel value always being represented as a 16-bit number. Each line has 2000 pixels divided by the amount of binning.

The size of each pixel in an image is:

(Binning * 16mm) / (2000 * Objective Magnification)

For example, an image acquired with a 40X objective and 2x binning will have (2 * 16 mm) / (2000 * 40) = 0.4 um pixels. The pixels are always square.

The size of the scanned sample region is independent of binning. The specified number of scan lines is divided by the amount of binning, while the Y-stage speed is multiplied by the amount of binning.

The galvanometer frequency is fixed at 1 KHz and scanning is done bi-directionally. The total acquisition time for an image is the number of scan lines multiplied by 0.5 ms.

The following set of conditions will be obtained with a 40X objective with or without binning, where 2000 scan lines have been specified:

Table 6-1 Binning Conditions

Binning	Scan Lines	Pixel Size (µm)	Image Size (pixels)	Image Size (MB)	Scanned Region (µm)	Scan Time (ms)
1x (no binning)	2000	0.2 x 0.2	2000 x 2000	8	400 x 400	1000
2x	1000	0.4 x 0.4	1000 x 1000	2	400 x 400	500
4x	500	0.8 x 0.8	500 x 500	0.5	400 x 400	250

The scanned region in a scanning system is comparable to the field-of-view in an imaging system.

While binning in a CCD camera increases the apparent brightness of an image, it will not do so in the ImageXpress Ultra System. However, there is a signal-to-noise reaction benefit of binning because the random noise level will be reduced due to the increased number of samples assigned to each pixel. See Combined Binning and Averaging on page 89.

Scan Lines and Averaging Overview

The number of scan lines refers to the movement of the stage in the Y direction only. The maximum value is 32,000 lines. The width of the image is fixed at 2,000 pixels when no binning is performed. The Scan lines value and the Pixel binning value determine the final size of the acquired image as shown in the Result image size field.

Increasing the number of times each line is scanned increases the signal-to-noise ratio in the resulting image at the cost of an overall increased scan time and potentially increased photo-bleaching. After you reach the maximum safe illumination intensity and the maximum recommended PMT voltage (750V) signal averaging is a way to increase the signal-to-noise ratio. Increasing the number of scan lines also increases the size of the image as well as the field of view. For lower magnification objectives, large scans might result in images that span multiple wells. With long scans, the quality of the images becomes even more dependent on plate flatness. Long scans can be beneficial when imaging large continuous structures such as extended neurite outgrowths.

Averaging in the ImageXpress Ultra System is accomplished by slowing the motion of the vertical (Y) stage during acquisition. For 2x averaging, the stage moves at half-speed. Pixels in each scan line are still acquired in the same fashion as without averaging. However, pairs (or sets for greater than 2x averaging) of pixels in adjacent scan lines are averaged with the result assigned to a single pixel. Therefore, averaging has a similar signal-to-noise effect as binning but without the reduction in resolution and image size.

Combined Binning and Averaging

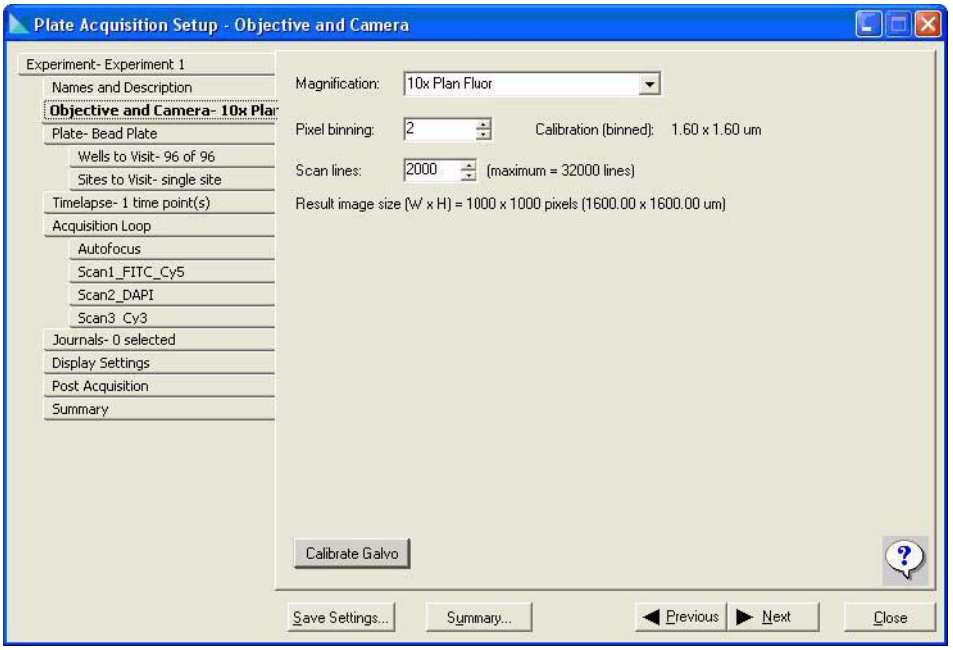
The closest ImageXpress Ultra System equivalent to 2 x 2 binning in a CCD camera-based system is 2x binning and 2x averaging. In this case, the scanned region and scan time are unchanged but each pixel is assigned the collected light for a 2 x 2 pixel region. The conditions obtained with combined binning and averaging are shown in the table below for a 40X objective where 2000 scan lines have been specified.

Table 6-2 Combined Binning and Averaging Conditions

Binning	Averaging	Scan Lines	Pixel Size (µm)	Image Size (pixels)	Scanned Region (µm)	Scan Time (ms)
1x (none)	1x (none)	2000	0.2 x 0.2	2000 x 2000	400 x 400	1000
2x	2x	1000	0.4 x 0.4	1000 x 1000	400 x 400	1000

To change the binning and scan lines settings

1. In the **Magnification** list, select the magnification setting to use.



Note: The settings available in the list were created in the Configure Magnification dialog in the Devices menu. Magnification settings have a calibration and assign X and Y offset values and a Z escape distance to a specific objective. You must assign a calibration to the magnification setting using the Calibrate Distances dialog. See Verifying and Backing Up Settings in the MetaXpress Software on page 51.

2. In the **Pixel Binning** field, select the amount of binning.
3. In the **Scan lines** field, select the number of scan lines used to acquire the image.
4. Click **Calibrate Galvo** to calibrate the system data acquisition timing to eliminate the pixel shift between successive scan lines. This calibration also occurs automatically before a plate is acquired.

Note: The frequency of the automatic calibration is set in the ImageXpress Ultra System Camera Settings dialog in the Meta Imaging Series Administrator.

Determining Plate Optical Thickness and Plate Bottom Variation

Molecular Devices strongly recommends that you use the Laser Autofocus Wizard, available on the Plate tab, to obtain plate bottom measurements. Although plate manufacturers generally provide reliable plate and well dimensions, you need to calculate the plate bottom measurements, such as average thickness and maximum variation in thickness of the entire plate. The Laser Autofocus Wizard walks you through steps to automatically calculate plate bottom settings as well as exposure times for each objective. Additional information about the settings that the wizard calculates is available in the application help, in the topic “Configure Laser Autofocus Settings - Dialog Box Options.”

Note: The Laser Autofocus Wizard calculates measurements as accurately as possible. Some manual verification and adjustment of the settings may be necessary to optimize the results, particularly for thin-bottom plates. Contact Technical Support if you need assistance.

All plate bottom values are optical thickness (also known as reduced thickness) measurements, as measured using the objective. The values are not physical thickness measurements.

Configuring Plates

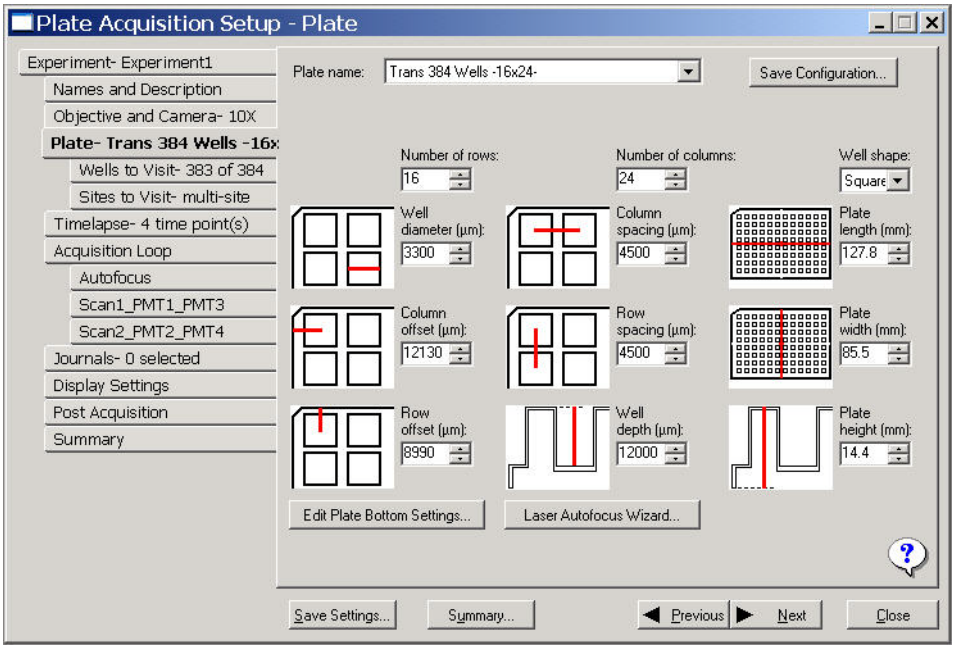
You must provide the plate dimensional information required to control the X, Y, and Z movements of the ImageXpress Ultra System.

Correct plate dimensional specifications ensure that:

- The imager will not make any movements that might create a potentially hazardous situation.
- The laser-based autofocusing is as accurate as possible.

The MetaXpress Software has pre-defined plate types that have been configured. If you use an existing plate type file, you must verify the accuracy of the optical bottom thickness and the bottom variation on your plates. These parameters are critical and can vary from one manufacturing lot to another. In addition, plate manufacturers can change plate parameters without changing plate names.

Note: The Laser Autofocus, optical thickness, and variance settings for each plate must be optimized on your instrument.



If the plate type you are using is not listed in the Plate name field, you should use the Laser Autofocus Wizard available on the Plate tab to assign and save correct plate values for the plate type. The options available depend on what is selected in the Plate name list.

To configure plates

1. In the **Plate name** field, select the plate size that corresponds to your plate, or select **Custom** to specify a non-standard plate configuration. The values in the remaining boxes are pre-filled according to what you select.

   If you select 96 Wells (8×12) or 384 Wells (16×24), only the following options will be available for configuration: Well shape, Well depth, Plate height, Optical Thickness, and Bottom Variation.

   If you select Custom, all options will be available for configuration, including Number of columns and Number of rows.
2. In the **Well shape** field, select a square or circular well shape. The graphics of the plate are updated to reflect your selection.
3. Click **Laser Autofocus Wizard** and follow the steps that the wizard provides to calculate plate bottom measurements.

   The optical thickness is a value in microns that is the average measured thickness for the well bottom using the Laser Autofocus. It is proportional but not equal to the physical thickness of the bottom plate.

   The bottom variation is a value in microns that is the maximum variation in Z direction between adjacent well bottoms.

Note: The Laser Autofocus Wizard calculates measurements as accurately as possible. Some manual verification and adjustment of the settings may be necessary to optimize the results.

4. If required, in the **Well depth** and **Plate height** fields, change the values.

   Correct Well depth and Plate height values are required for autofocusing. These values can be obtained from the manufacturer.
5. If you selected **Custom**, complete the fields as required.
6. After you have configured the plate settings, click **Save Configuration** to type a name for your setting and save the plate configuration. Configurations that have been saved are then available in the Plate name list.

Note: Adjust any objectives with correction collars to match the plate type.

Optical Thickness and Laser Focus

A plate bottom typically consists of plastic or glass material. The transitional point between the plastic or glass and air or liquid is referred to as an interface. The laser in the laser autofocus system reflects off both interfaces: first at the plastic-to-air interface (when looking from the bottom of the plate through the plastic or glass material) at the bottom of the plate, and then at the air-to-plastic or liquid-to-plastic interface at the bottom of the well. The Z position difference between these two interfaces is called the optical thickness (also referred to as reduced thickness).

When the system performs a focus search with the laser, it moves from bottom to top, looking for the first laser spot that it can locate. This represents the bottom of the plate. Then the Z motor moves the objective by a percentage of the optical thickness and performs another search for the second laser spot. This is the interface of plastic-to-air or plastic-to-liquid. Correctly specifying the optical thickness is important since it ensures proper location of the second interface and thus accurate focusing.

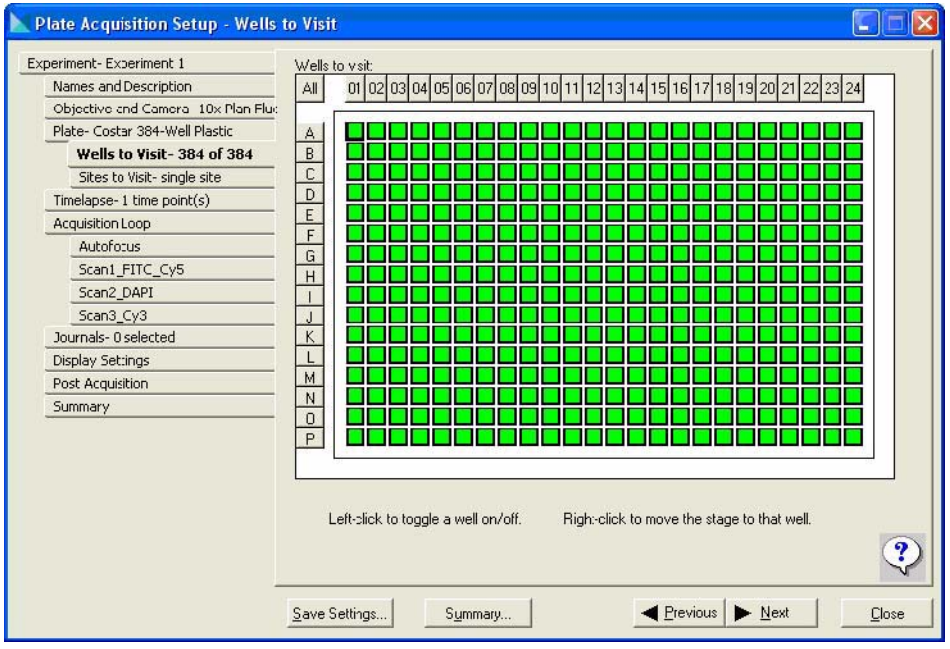
After the instrument has found both interfaces, you can set it to perform a search for only the second interface. The search range used is based on the plate bottom variation that you enter in the plate definition. The variation between neighboring wells must be accurately determined for this to be effective.

Configuring the Wells to Visit on a Plate

You can configure the wells to acquire on your plate and enable acquisition from multiple sites per well. The Wells to Visit tab displays a graphical representation of the type of plate that you are using, based on the settings that you made on the Plate tab.

To configure the wells to visit on a plate

1. Click the **Wells to visit** tab and then click individual wells to select or clear each well.
2. Click lettered buttons to select or clear an entire row.
3. Click numbered buttons to select or clear an entire column.
4. Click **All** in the upper-left corner to select or clear all wells on the plate.
5. Right-click a well to move the stage to the well.



Configuring the Sites to Visit on a Plate

You can specify the acquisition of more than one location in a well and configure the vertical and horizontal spacing between sites in an image. Both positive and negative values can be entered to add a space between images (using positive values) or to overlap images using negative values.

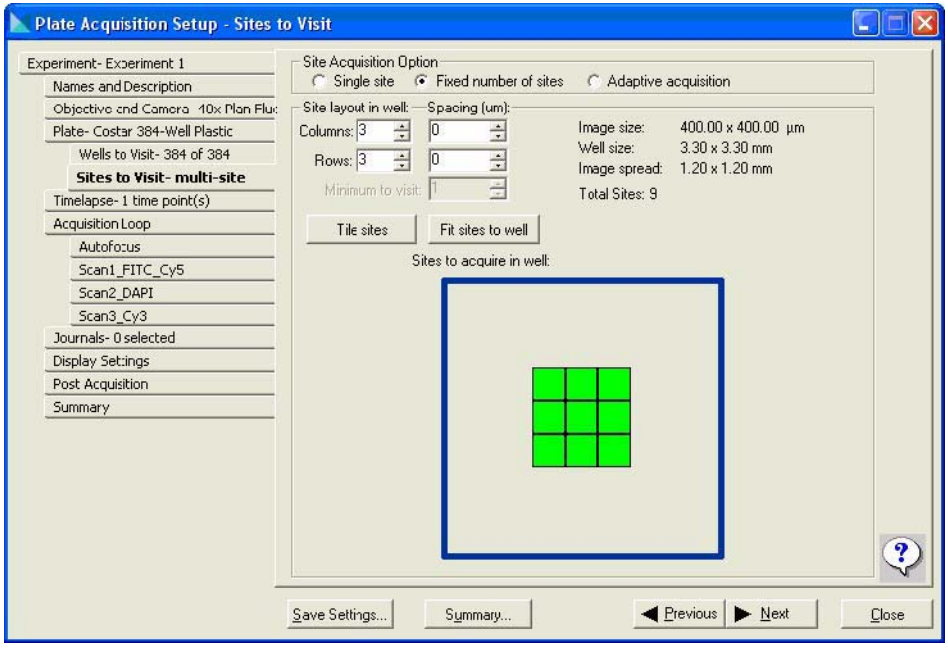
The number of sites to acquire in a well depends on the following factors that can influence data quality:

- size of the well
- objective magnification
- image size (number of scan lines)
- distribution of sample material in the well
- type of plate
- location of the material in the well that you want to acquire
- amount of spacing that you require between each acquisition location

Focusing options for multiple sites are set on the Autofocus tab. See Configuring Laser Autofocus on page 118.

To configure the sites to visit on a plate

1. In the **Columns** and **Rows** fields, specify the number of columns and rows (for example, 2 columns and 2 rows to acquire up to four sites; 3 columns and 3 rows to acquire up to nine sites).

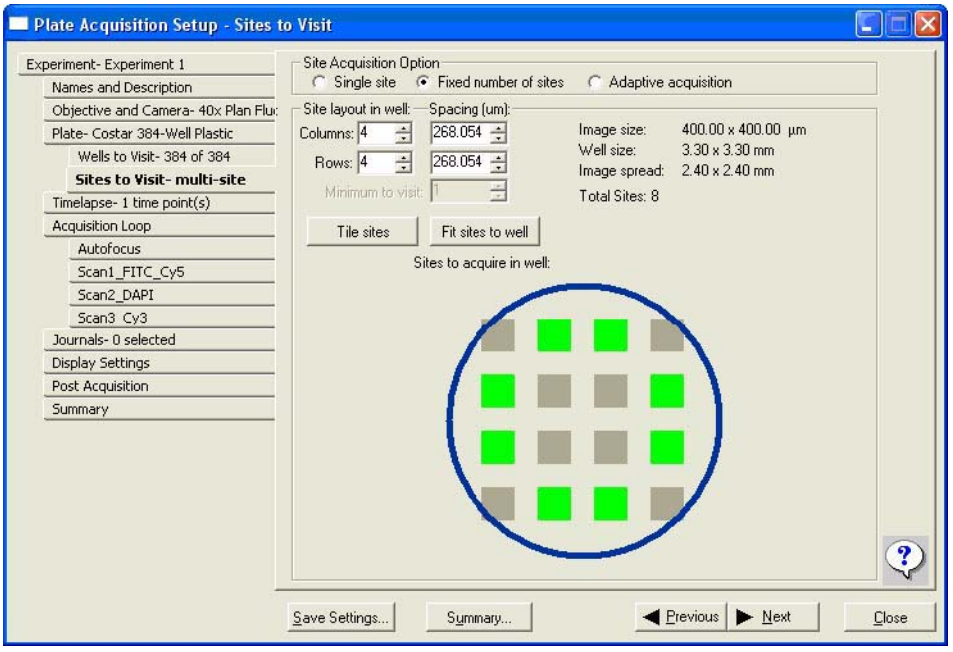


Note: The maximum number of columns and rows that can be configured is 45 × 35.

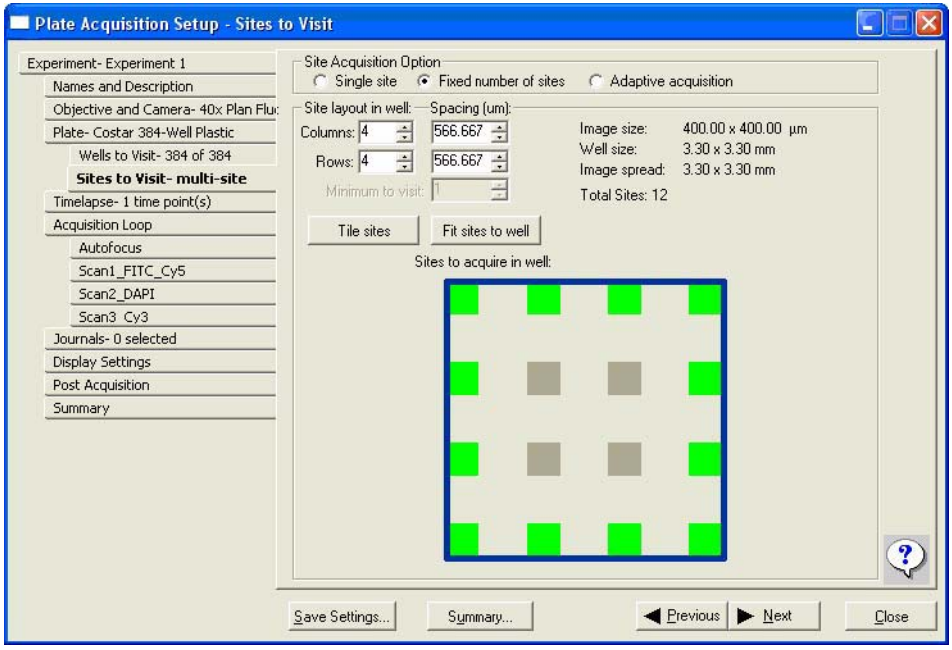
2. Click **Tile Sites** to configure the sites with zero spacing between adjacent sites.
3. Click **Fit sites to well** to maximize the spacing between the sites within the well.
4. In the graphical display, left-click individual sites to turn off any sites that you do not want to acquire, or to turn on any sites that are turned off. You can also click and drag to select or clear a rectangular block of sites. Right-click a site to move the stage to that site.

Examples

Configure the **Sites to Visit** tab as shown in the screenshot to acquire images at the edges of a round well configured with 16 sites.



Configure the **Sites to Visit** tab as shown in the screenshot to acquire images at the edges of a square well configured with 16 sites.



5. In the **Spacing (µm)** lists, select the X and Y values of the spacing between images to include a distance between adjacent sites.

Note: A negative value results in overlapping data between adjacent images. The overlapping data can be useful if the images are stitched together for image analysis.

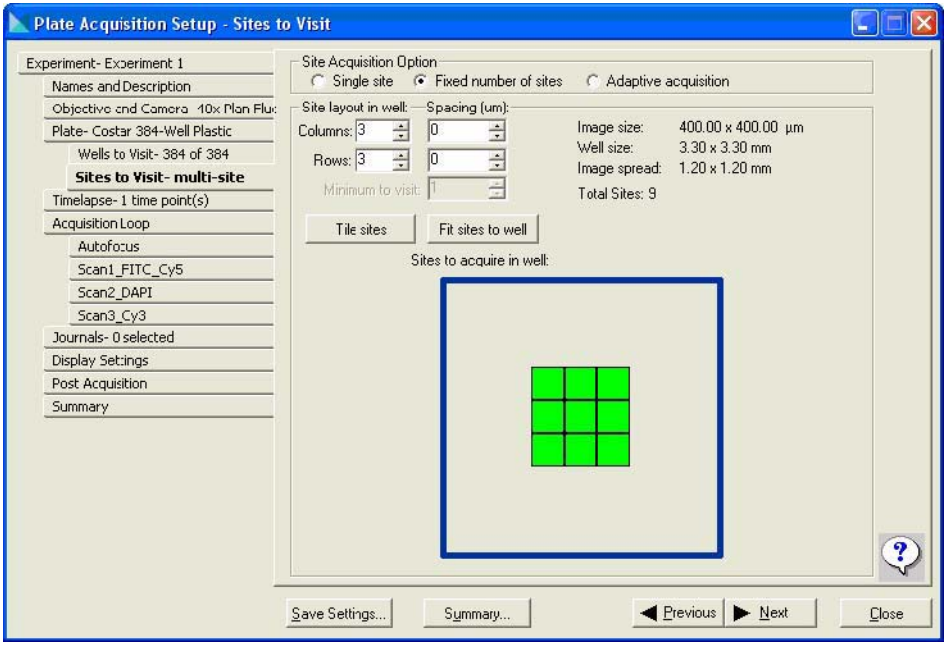
6. In the **Sites to acquire in well** section, view the current image size, well size, and image spread values on the tab. These values update to reflect changes in configuration.

Configuring Cell Counting

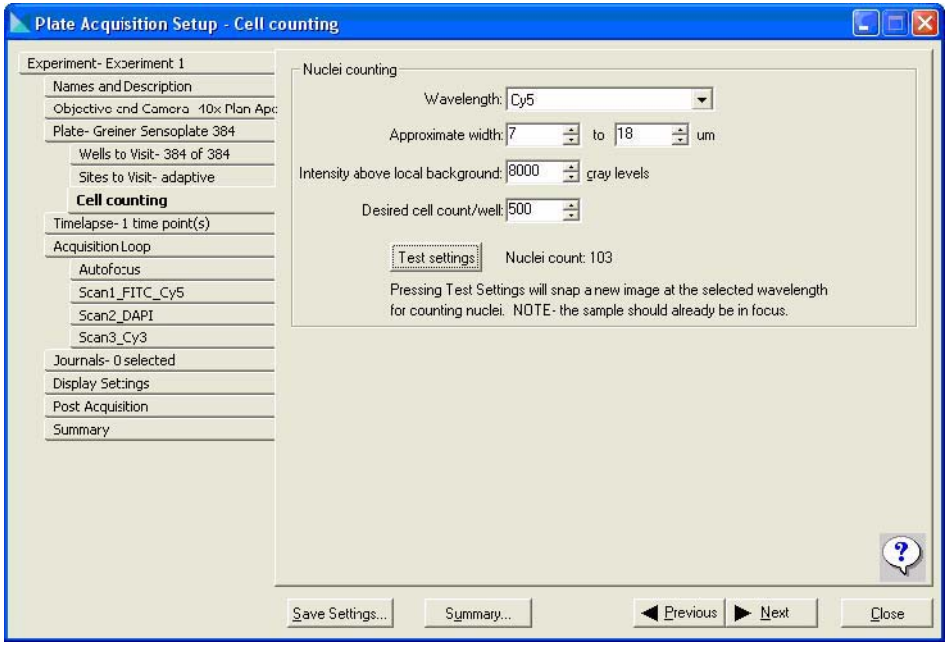
The Adaptive Acquisition option in the Sites to Visit tab is a computational algorithm that analyzes the cell number on the fly during sample acquisition, which increases the chances of collecting valid data in every well. Adaptive Acquisition can also significantly reduce acquisition time for multiwavelength acquisition requiring a minimum number of cells per well or for samples with differing conditions across the plate.

To configure cell counting

1. In the **Sites to Visit** tab, in the **Site Acquistion Option** section, click **Adaptive acquisition**.



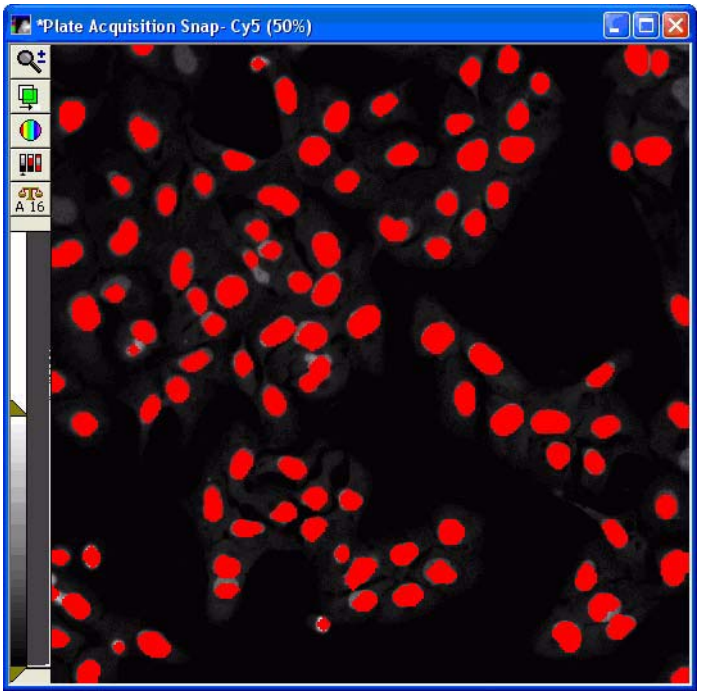
2. In the **Cell Counting** tab, in the **Wavelength** list, select the channel to use for cell counting.



3. In the **Approximate width** fields, select the approximate values for the width of the nuclei.
4. In the **Intensity above local background** field, select the intensity of the nuclei.

5. Click **Test settings** to optimize your settings on a representative site as shown in the following screenshot. You should already be in focus at that location and in an area on your sample that has a representative number of cells.

Note: Test Settings snaps an image and counts the cells using the current settings, displaying both an analysis overlay and the total number of cells counted in the image.



Setting Multiple Timepoints to Acquire Images

You can set the loop order to use when acquiring images at multiple timepoints, the number of timepoints to acquire, and the total time required for all timepoints.

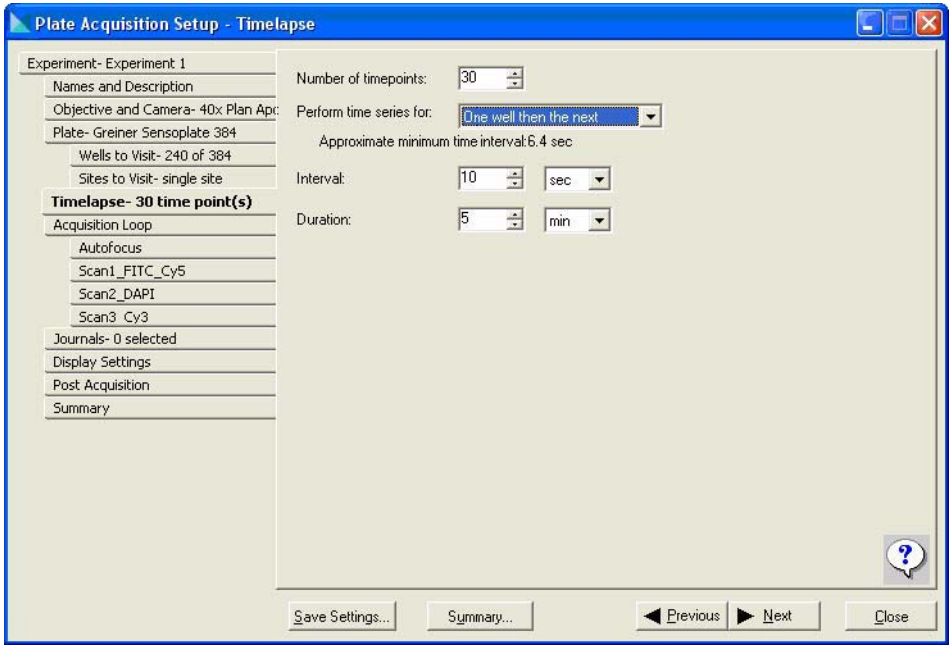
The values in the Number of Timepoints, Interval, and Duration fields have the following relationship:

Number of Timepoints × Interval = Duration

To configure multiple timepoints to acquire images

1. In the **Timelapse** tab, in the **Number of timepoints** field, select the required number. If you change any of the values, other fields are updated as required.

Note: If you are not using multiple timepoints in your acquisition, in the Number of timepoints list, select 1 and click Next to move to the next tab.



2. In the **Perform time series for** list, select a loop order to use when acquiring images at multiple timepoints. The following options are available:

   Table 6-3 Perform time series options

Option	Description
One well, then the next	A set of wavelength images is acquired at each site in the well at each time point. Use this option for rapid acquisition from a well if multiple sites are selected. All sites are acquired per time point.
One row, then the next	All the images in one row of wells are collected at each time point. After the series is collected the next row is acquired. This option requires a longer time interval because all the wells in a row are acquired.
One column, then the next	All the images in one column of wells are collected at each time point. After the series is collected, the next column is acquired.
All selected wells	Every well selected for acquisition is acquired at each time point. The well selection is determined at the start of the first acquisition. This option is suitable for a long time-lapse assay.

3. In the **Interval** fields, select the amount of time and the time unit (for example, ms, sec, min, hr) between the start of acquisition at one time point to the start of acquisition at the next time point.

   If the time interval is shorter than the length of time required to acquire the images, the next acquisition occurs as soon as possible after the previous acquisition is complete. The Interval field can be set to 0 to acquire images as fast as possible. If the interval is set to 0, the duration is set according to the approximate minimum time, and the Duration field is disabled.

Note: No warning is given if the acquisition time is longer than the specified interval. Under these circumstances, the actual duration is longer than the duration time shown in the Duration field.

4. If required, in the **Duration** field, select the amount of time for the entire time series. The Duration value is the result of multiplying the number of timepoints by the interval.

   The Number of timepoints list automatically updates as the Duration list is changed.

Note: If timelapse imaging is enabled, additional options for autofocus are available on the Autofocus tab. See Configuring Laser Autofocus on page 118. Collection of specific scans at different timepoints can be configured on the tab for the scan of interest.

Defining Protocol Settings

In the Acquisition Loop tab, you can define the protocol for your experiment. Use this tab to configure the following settings:

- Number of scans to perform
- Beam splitter position
- Active emission filter set
- Available lasers and laser power settings (the beam splitter position determines which lasers are available for selection)
- Available PMTs and PMT gain settings
- Pinhole diameter
- Activation of shading correction during acquisition

Generating Shading Correction Files

A shading correction file is required for each laser, filter, and objective combination. The following table details the optimal combination for lasers and filters.

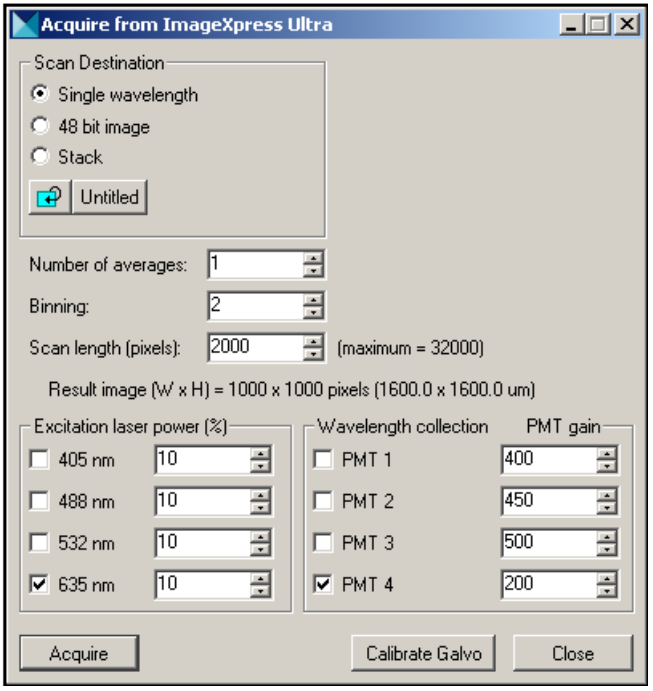
Table 6-4 Shading Correction File Filter and Laser Combinations

Filter	Beam splitter	Laser	Plate
DAPI	Quad	405	Green
FITC	Quad	488	Pink
GFP	Dual	488	Pink
Cy3	Quad	532	Pink
Texas Red	Quad	561	Pink
DRAQ5	Dual	635	Chroma Blue slide
Cy5	Quad	635	Chroma Blue slide

Note: The shading correction file will only work with images that have an equal or shorter scan length. If the scan length is longer than 2000, create the shading correction file with the appropriate scan length.

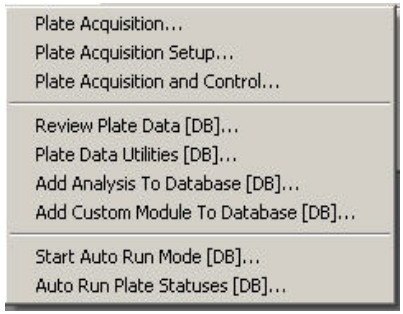
To generate a shading correction file

1. In the MetaXpress Software, click **Acquire > Acquire from ImageXpress Ultra**.
2. In the **Acquire from ImageXpress Ultra** dialog, click **Calibrate** or **Calibrate Galvo** (depending on the MetaXpress Software version that you have installed).

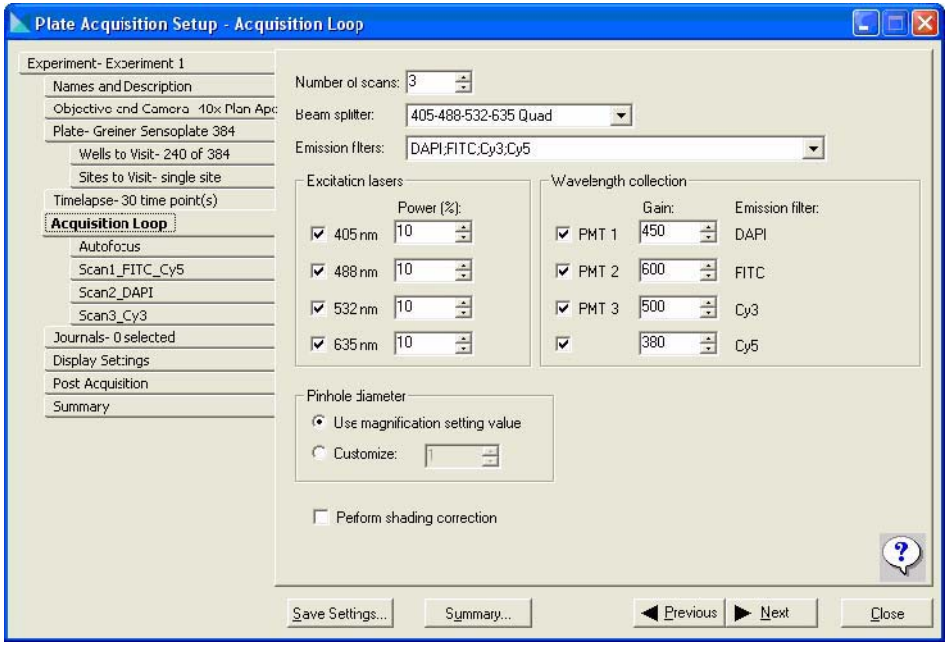


3. Open the top door, insert the required plate (or slide placed in the metal slide holder) into the IXU plate holder and then select the appropriate objective. If the objective has a correction collar, set this to the lowest possible value. See Shading Correction File Filter and Laser Combinations on page 105.

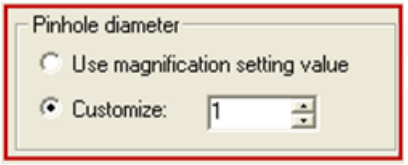
4. Click **Screening > Plate Acquisition Setup**.



5. In the **Plate Acquisition Setup** dialog, click the **Acquisition Loop** tab, and then select the beam splitter and emission filter set.

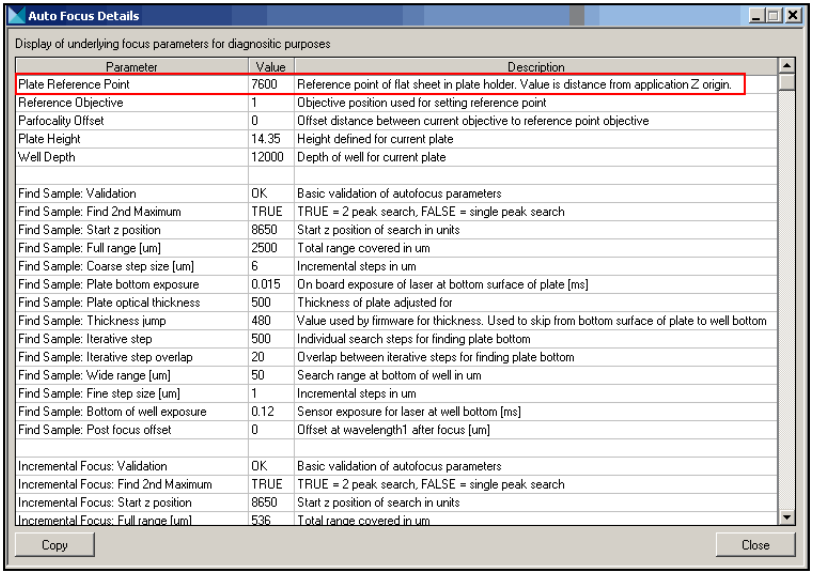


6. In the **Pinhole Diameter** section, in the **Customize** field, select **1** mm.



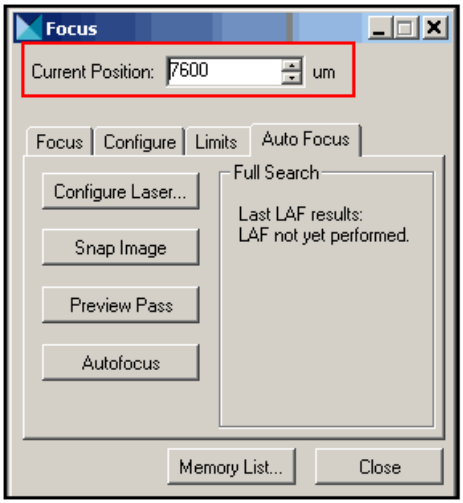
7. Click the **Autofocus** tab and then click **View Focusing Details**.
8. In the **View Focusing Details** dialog, look up the value after Plate Reference Point. Calculate the Z value using:

   Plate Reference Point – 3000 = Z value.



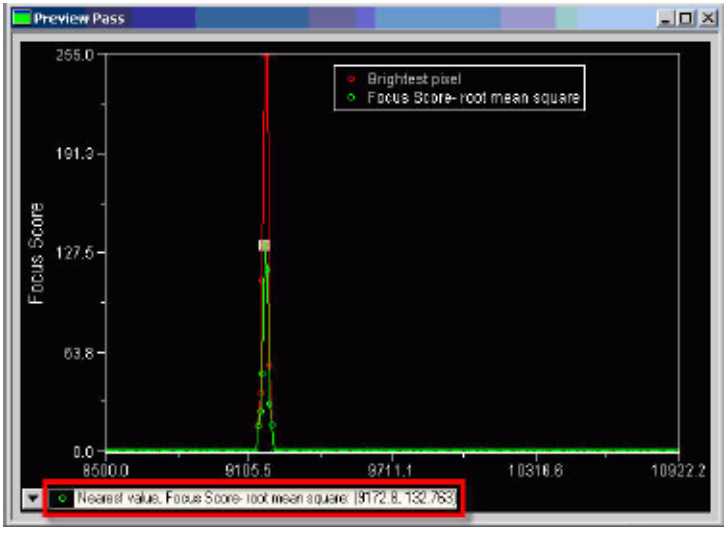
9. In the **Focus** dialog, click **Devices > Focus**.

10. In the **Current Position** dialog, type the Z value calculated in the previous step.



11. In the **Plate Acquisition Setup** dialog, in the **Autofocus** tab, click **Configure Laser Settings**.
12. In the **Configure Laser Autofocus Settings** dialog, in the Preview Pass section, select **Start from current position** and then click **Preview Pass**.
13. If there is no peak, in **Focus** dialog, increase the value by 2000 higher than the value calculated and repeat steps 9 to 12. If there is still no peak, increase the value again by 2000 and repeat. If there is still no peak make sure that the plate is still positioned correctly in the instrument.

14. When you see a peak, or two peaks, in the **Preview Pass** dialog, click the trace line at the top of the green line of the first peak. The X value information is located in the bottom left as highlighted in the screen shot. This is the Z position of the bottom plate. In the example, the current Z position value is 9172.8.

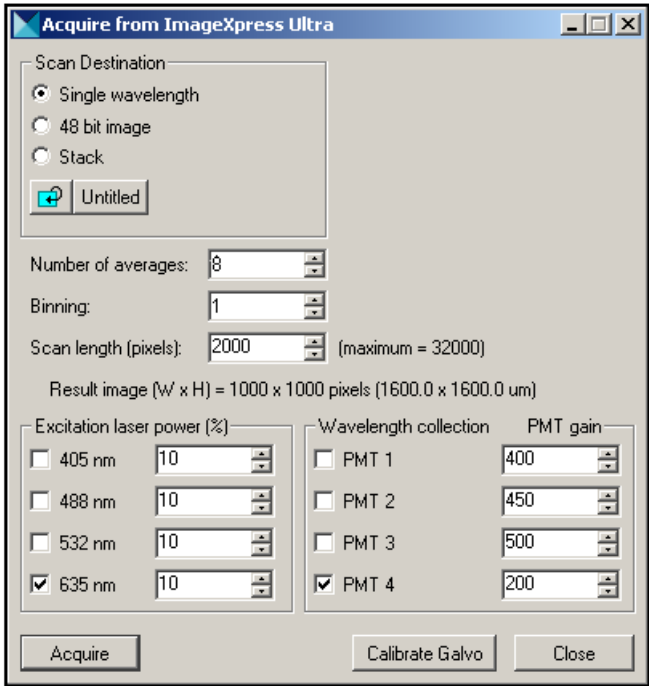


15. Click **Devices > Focus** and in the **Focus** dialog, type the Z-position value (rounded off) that was calculated in the previous step.
16. Click **Acquire > Acquire from ImageXpress Ultra**.

17. In the **Acquire from ImageXpress Ultra** dialog, edit the fields:

Table 6-5 Acquisition settings

Field	Value
Scan Destination section	Single wavelength
Number of Averages	8
Binning	1
Scan Length	2000



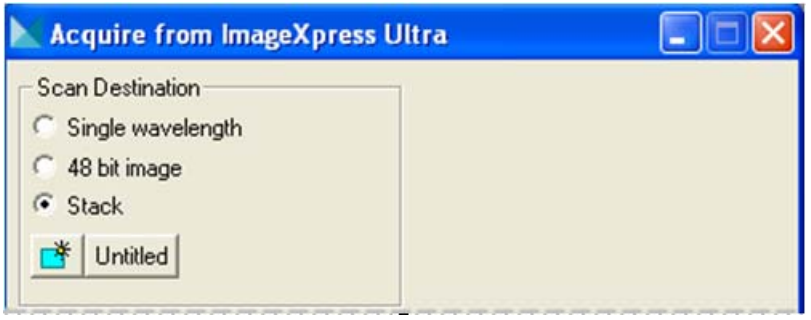
18. In the **Excitation laser power** and **Wavelength collection** sections, select the required laser power and wavelength.

19. Click **Acquire** and adjust the laser power and PMT gain to get an image around 75% saturation with a 16-bit scale.

Tip! Start the laser at 10% and the PMT at 650. If required, lower the PMT or increase the percentage of laser power.

20. Close the acquired images without saving.

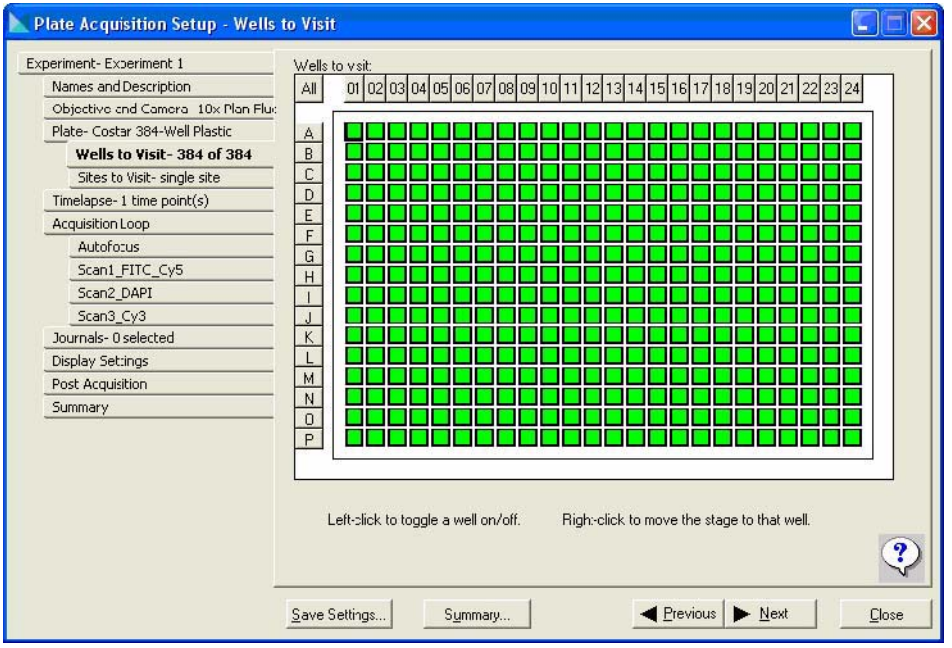
21. In the **Acquire from ImageXpress Ultra** dialog, in the **Scan Destination** section, click **Stack**.



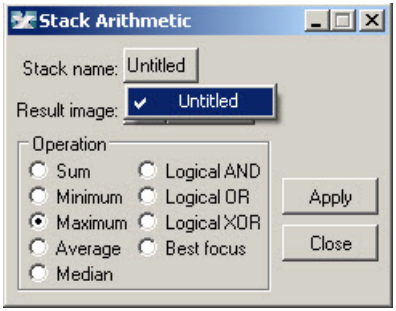
22. Click **Screening > Plate Acquisition Setup**.

23. In the **Plate Acquisition Setup** dialog, click the **Plate** tab and select the 96 well plate.

24. Click the **Wells to visit** tab and then right click well **D05**.



25. In the **Acquire from ImageXpress Ultra** dialog, click **Acquire**.
26. Repeat steps 23 and 24 for wells **D06**, **D07**, **D08**, **E05**, **E06**, **E07** and **E08**.
27. Click **Process > Stack Arithmetic**.



28. In the **Stack Arithmetic** dialog, select the stack name (same as the Scan destination name in the **Acquire from ImageXpress Ultra** dialog).
29. In the **Operation** section, click **Average** and then click **Apply**.
30. Click the image (same name as Result image in the Stack Arithmetic dialog).
31. Click **File > Save As** and navigate to the **C:\MX5\Shading** directory. You can store the file in a different directory.
32. Use the following format to name the file: **Shading_Objective_Laser_Filter.tif**.

    For example, if the objective and camera is named 20X ELWD and the laser selected is 405, then the image name should be Shading_20X_ELWD_405_dapi.tif.
33. Close the image.
34. If required, change the objective and laser and repeat the procedure starting at step 3.

Note: The shading image is dependent on laser/PMT/objective/filters/beamsplitter/pinhole/beam expander settings so it is best practice to acquire images with the same configuration as your experiment. If the shading image is smaller than your acquired image, then the shading will not work properly and you will need to acquire new shading images.

PMT Gain Overview

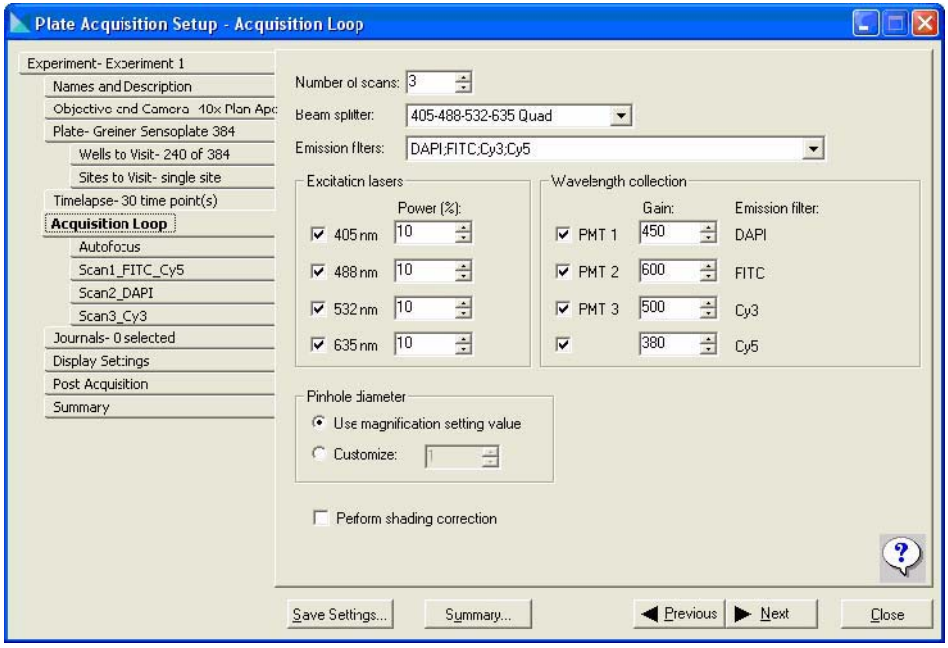
You can select separate gain values for each PMT. Increasing the PMT gain yields a brighter image, but also results in more background noise.

The typical operational voltage range for PMTs is between 300 and 750 V. Avoid using voltages outside this range:

- If you use less than 300 V and still have a very bright signal (partial saturation), your fluorescent/reflected light is too bright and you are permanently damaging the PMTs photocathode. Reduce the illumination intensity (laser power). You can further reduce the illumination intensity by 5 or 10 times by switching to one of the neutral density beam splitters.
- Voltages above 750 V do not damage the PMT but are not recommended. Increasing the PMT gain in this range increases the signal amplitude, but it also increases the noise and so does not improve the signal-to-noise ratio. Increase the illumination intensity (if the laser power is still below dye saturation level), or increase averaging. Signal intensity averaging is a common way to improve signal-to-noise ratio.

To configure the protocol settings

1. In the **Number of scans** field, select the total number of sequential scans to perform at a single stage location. Eight is the maximum number of scans.



2. In the **Beam splitter** field, select the beam splitter to use to determine which Excitation lasers are available for the acquisition. Select a beam splitter based on the combination of wavelengths you require.

   Table 6-6 Beam splitter options

Beam splitter	Description
Quad 405/488/532/635	Reflects 405 nm, 488 nm, 532 nm, and 635 nm to the sample. Transmits spectral bands between these lines and above 635 nm from the sample to the detector.
Quad 405/488/561/635	Reflects 405 nm, 488 nm, 561 nm, and 635 nm to the sample. Transmits spectral bands between these lines and above 635 nm from the sample to the detector.
Dual	Reflects 488 nm and 635 nm to the sample and transmits between these wavelengths and above 635 nm from the sample to the detector. This is optimized for GFP and DRAQ5.
80/20	Transmits 80% of light and reflects 20%. This is flexible but not efficient for illumination.
90/10	Transmits 90% of light and reflects 10%. This is flexible but not efficient for illumination.

3. In the **Emission filters** field, select the filter cube to use. This setting determines which emission filters are used with each PMT in the Wavelength collection fields.
4. In the **Excitation lasers** field, select the lasers to use and adjust the power between 10% and 100%. To set the illumination level below 10%, use higher laser power and the 80/20 or 90/10 beam splitter. To reduce the amount of sample damage from laser illumination (photobleaching and/or phototoxicity), maintain the laser power levels below 50% with the quad and dual beam splitters. If the images are dim, adjust the PMT gain prior to increasing the laser power. It is highly recommended to start with a setting of 10% power and only increase if required.

Note: The shot noise from the laser is a larger contributor to noise than the dark noise in the PMT, and it increases with the square root of the laser intensity. The signal-to-noise ratio, however, is not significantly affected by the PMT gain. Therefore, when you start an experiment, set the laser power to 10% and the PMT gain to 700. If the image is saturated, turn the PMT down. If the image is not bright enough (though focused), then turn the laser up.

5. In the **Wavelength collection** section, select the **PMT** check boxes to use and adjust the gain for each PMT if required. It is highly recommended to maintain the PMT gain at or below 750. Higher PMT gain settings result in excessively noisy images. You can select separate gain values for each PMT. Increasing the PMT gain yields a brighter image but also results in more background noise.
6. In the **Pinhole diameter** section, click **Customize** to use a custom pinhole diameter and then type a value (millimeters). This controls the amount of collected fluorescence that is transmitted to the detectors. Smaller pinhole diameters produce better axial resolution (optical sectioning) at the expense of lower light collection efficiency.
7. Select the **Perform shading correction** check box to enable shading correction.

   Correction images must exist for each wavelength used to apply shading correction during acquisition. The shading correction file used for each wavelength is selected in the Scan tabs. The ImageXpress Ultra System has a pre-defined set of shading correction files available.

Note: Shading is an artifact intrinsic to all imaging systems that can come from the objective or other optics. Shading should be addressed in the hardware first, and if no hardware issues exist then, subsequently by using the available shading correction within the software. The system has been aligned to minimize shading artifacts but minor shading will still result from the intrinsic property of the optical components.

Configuring Autofocus Settings

You can configure the autofocus settings using a combination of three different tabs in the Plate Acquisition Setup dialog and in additional dialogs within the MetaXpress Software. Your system administrator must define certain autofocus settings in advance to simplify the daily autofocus procedures required when setting up an experiment.

Use the following tabs and dialogs to configure autofocus:

- Plate Acquisition Setup dialog
  - Acquisition Loop tab
  - Autofocus tab
  - Scan tab
- Laser Autofocus Wizard on the Plate tab
- Focus dialog (optional)
  - Focus tab (optional)
  - Autofocus tab (optional)
- Configure Laser Autofocus dialog (optional)

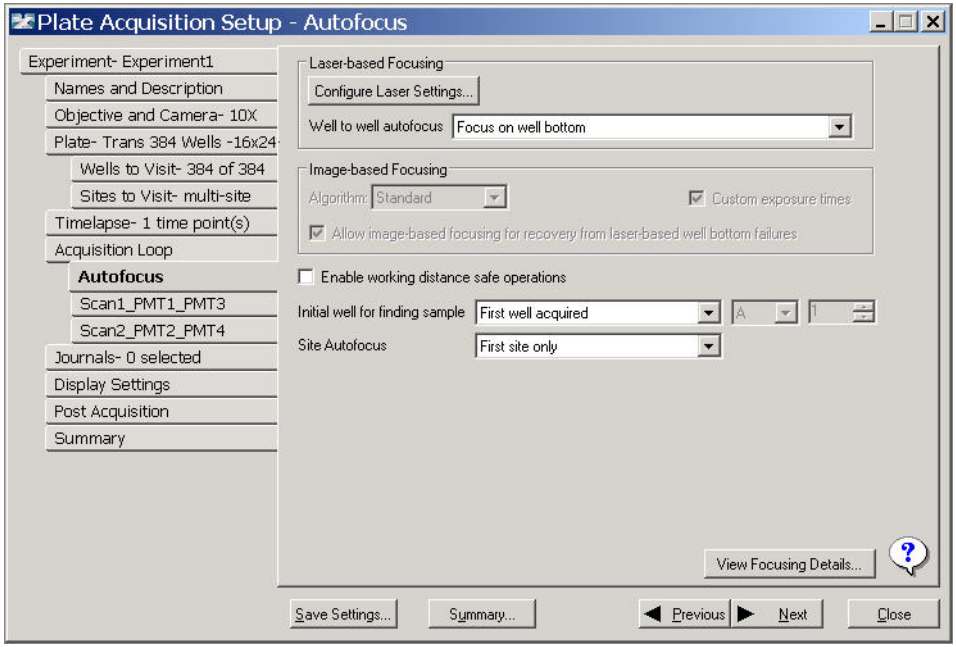
Configuring Laser Autofocus

After the laser autofocus settings have been completed correctly, they typically do not need to be modified.

To configure laser autofocus

1. In the MetaXpress Software, click **Screening > Plate Acquisition Setup**.
2. In the **Plate Acquisition Setup** dialog, click the **Wells to Visit** tab.
3. Right-click on the well to use to set up and verify the focus accuracy.
4. Click the **Autofocus** tab and in the **Site Autofocus** section, if sites are enabled on the Sites tab, select the site pattern to use:
   - First site only—Autofocuses in the top left site in the well.
   - Center of well only—Autofocuses in the center of the well.
   - All sites—Autofocuses for each site.

Note: For closely-spaced sites and lower magnifications, it is best practice to focus once per well to save time. For higher magnifications or sites that are spaced farther apart, it is best practice to focus on all sites.



5. In the **Timelapse Autofocus** section, if timelapse acquisition is enabled on the **Timelapse** tab, choose the focusing pattern to use.
   - First timepoint only—Autofocuses prior to acquisition at the first timepoint. Subsequent timepoints are acquired at the same Z position. This is useful for fast kinetic responses.
   - All timepoints—Autofocuses prior to acquisition at every timepoint. This is recommended for longer timelapse experiments.
   - Every Nth timepoint—Autofocuses at the specified timepoint interval.
6. In the **Laser-based Focusing** section, in the **Well to well autofocus** field, select **Focus on plate bottom only, then offset by bottom thickness** to focus only on the bottom of the plate and then use the bottom thickness value to focus on your sample.

Note: This option offsets the focus by the bottom thickness of the plate as defined in the Plate tab and is suitable for lower magnification objectives and slides. Select this option if you are using a uniform thickness glass-bottom plate and a liquid medium. The focus is performed with the strong bottom surface reflection and then the high precision Z stage is used to offset the focus for the plate bottom thickness.

7. In the **Laser-based Focusing** section, in the Well to well autofocus field, select **Focus on plate and well bottom** if you are using thin-bottom plates. With this option, the autofocus always searches for both peaks in every well. This is recommended if the plate bottom variation is at least half the thickness of the plate bottom.

Note: This operation mode is slower but ensures more accurate focusing on warped plates.

8. On the **Autofocus** tab, click **Configure Laser Settings** to confirm the settings that will be used for autofocusing, including exposure times. If you need to change the exposure values, on the **Plate** tab, click **Laser Autofocus Wizard** and follow the steps that the wizard provides for measuring exposure times. Additional information about the settings that the wizard calculates is available in the online help, in the topic “Configure Laser Autofocus Settings - Dialog Box Options.”

Note: The Laser Autofocus Wizard calculates measurements as accurately as possible. Some manual verification and adjustment of the settings may be necessary to optimize the results.

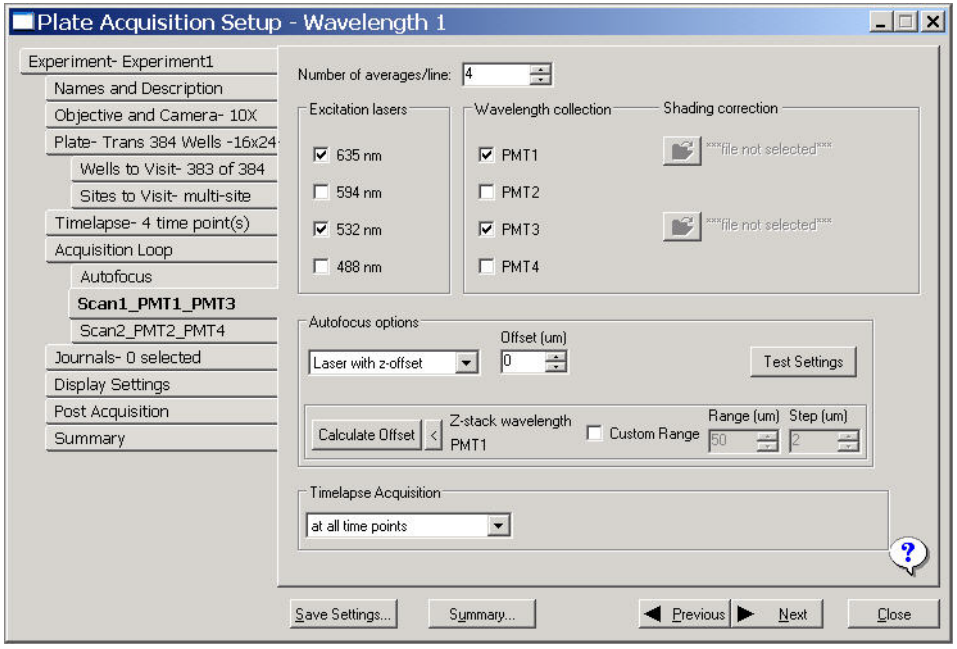
9. On the **Autofocus** tab, click **View Focusing Details** to view all the settings that will be applied to your autofocus procedures.

Configuring Scans

Use the Scan tabs to configure the lasers and illumination to use for each scan. The total number of Scan tabs depends on the number of scans selected in the Acquisition Loop tab.

To configure the lasers for each scan

1. In the **Plate Acquisition Setup** dialog, click the **Scans** tab and in the **Number of averages/line** field, click the number of times to scan each line. Increasing this value can improve results for samples with low fluorescence but increases the total acquisition time. See Scan Lines and Averaging Overview on page 89.



2. In the **Excitation lasers** section, select which lasers to use for the scan.

   The lasers available are determined by what is selected in the Beam splitter and Excitation lasers fields of the Acquisition Loop tab.

3. In the **Wavelength collection** section, select the filter and PMT combinations to use for the scan.

   The filters available are determined by what is selected in both the Emission filters and Wavelength collection fields of the Acquisition Loop tab.

4. Optionally, in the **Autofocus options** section, in the **Offset** field, adjust the Z offset used during autofocusing. To use the Calculate Offset feature, which automatically determines the appropriate Z motor offset distance for each scan as defined on the Plate Acquisition Setup tab, follow Steps 5-12.

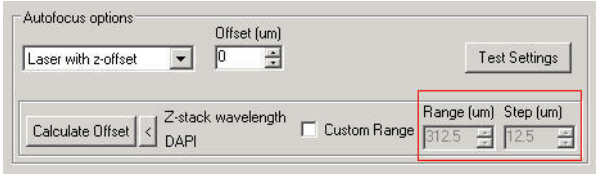
   For the first Scan tab, the offset is the distance between the bottom of the well and the in-focus plane. For the second and subsequent Scan tabs, the offset is the difference between the focus position of the Z motor using the first scan and the focus position of the Z motor using the second or subsequent scan. As a simple example, suppose the position of the Z motor when the sample is in focus using Scan 1 is 500 and the position of the Z motor when the sample is in focus using Scan 2 is 700. The difference between the two positions is 200, so the offset for Scan 2 is 200.

   Before using the Calculate Offset feature:

   - Configure the plate parameters using all of the tabs above the Acquisition Loop tab.
   - Configure all laser autofocus parameters using either the Laser Autofocus Wizard (available from the Plate tab) or the Configure Laser Autofocus Settings dialog (available from the Autofocus tab).
   - Ensure that the signal/noise is set so that the system can focus appropriately (adjust the exposure time if necessary to correct a dim or saturated signal).

5. On the first Scan tab, in the **Autofocus options** section, select **Laser with z-offset**.

   The default range and step size that will be used to calculate the offset is based on the selected magnification setting. For example:

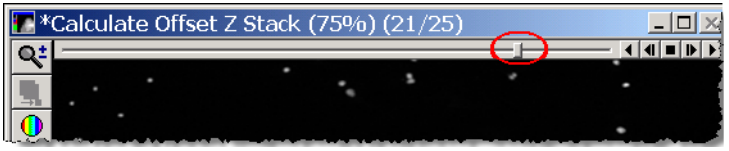


The software uses the range and step size to determine the number of planes to acquire (the number of planes acquired is equal to approximately the range divided by the step size).

6. Optionally, to acquire fewer or more planes than the default number of planes (25), select the **Custom Range** check box and type new values for the range and step size of the Z stack. You can acquire as many planes as needed.
7. Click **Calculate Offset**.

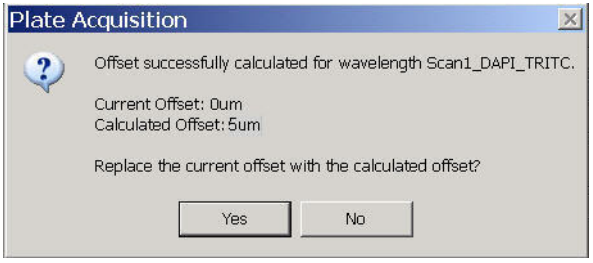
   The MetaXpress software acquires a Z stack of images and displays the following message:

8. Use the slider at the top of the Z stack image window to navigate to the most in-focus image. For example:



9. With the most in-focus image displayed, click **OK**.

   The software calculates the offset and a message appears:



The offset can be a negative value.

10. To replace the current offset with the newly calculated offset, click **Yes**.
11. Optionally, to save the Z stack, click **File>Save As**.

Tip! The Test Settings button focuses on the sample and snaps an image using the current settings for the selected wavelength.

12. On the second and subsequent Scan tabs, in the Autofocus options section, select **Z-offset from W1** and repeat Steps 6-11.
13. If you specified multiple timepoints in the **Timelapse** tab, set the image collection intervals to use for each wavelength.

Table 6-7 Image Collection Intervals

Option	Description
All timepoints	Acquires an image for each timepoint for this wavelength.
At start	Acquires an image at this wavelength for the first timepoint only.
At start and end	Acquires images at this wavelength for the first and last timepoints only.
Every nth timepoint	Acquires an image at this wavelength at the selected timepoint interval beginning at the first timepoint.

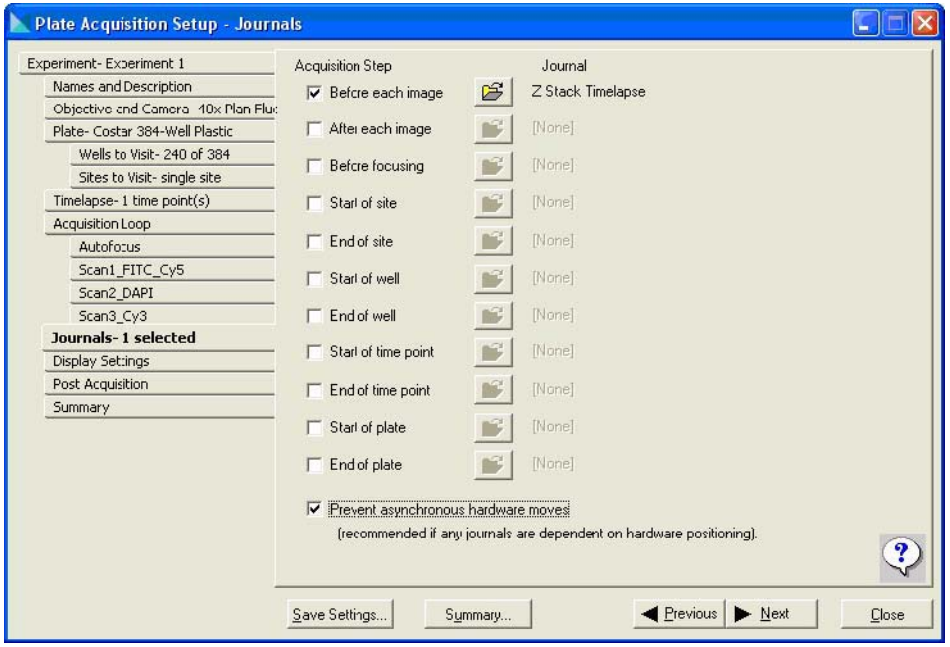
Running Journals During Acquisition

Use the Journals tab to configure specific journals to run during different stages of acquisition.

Note: If you do not need to run any journals during the acquisition, click Next to move to the next tab.

To configure running journals during acquisition

1. In the **Plate Acquisition Setup** dialog, click the **Journals** tab.



2. Select the check box next to an acquisition step to run a journal.

Table 6-8 Acquisition Steps to Run Journals

Acquisition Steps	Description
Before each image	Runs only during the acquisition loop, after the illumination is set and focusing is done.
After each image	Runs only during the acquisition loop, after scanning is complete and before images are saved.
Before focusing	Runs only during the acquisition loop, before focus evaluation begins.
Start of site	Runs only during the acquisition loop, before any images are acquired from each site.
End of site	Runs only during the acquisition loop, after all images have been acquired from each site.
Start of well	Runs only during the acquisition loop, at the beginning of each well, before any images are acquired from a well.
End of well	Runs only during the acquisition loop, at the end of each well, after all images have been acquired from a well.
Start of timepoint	Runs only during the acquisition loop, at the beginning of each timepoint, before any images are acquired for a timepoint.
End of timepoint	Runs only during the acquisition loop, at the end of each timepoint, after all images have been acquired for a timepoint.
Start of plate	Runs after the stage is moved to the find sample position, but before the find sample action is performed.
End of plate	Runs after the last acquisition for a plate is complete.

3. Click the folder icon next to the selected acquisition step.

   The Select Screen Acquisition Journal dialog opens with the contents of the Journals folder displayed by default.

4. Select the journal to run at the selected acquisition step and then click **Open**.

Note: If the journal is not located in the Journals folder, navigate to the folder where it is located, select it and then click Open.

5. Repeat steps 1 to 3 as required to assign journals to run at additional acquisition points.
6. If any of the journals you run move hardware (for example, change Z position), select **Prevent asynchronous hardware moves**. The journals run correctly but slow down acquisition.

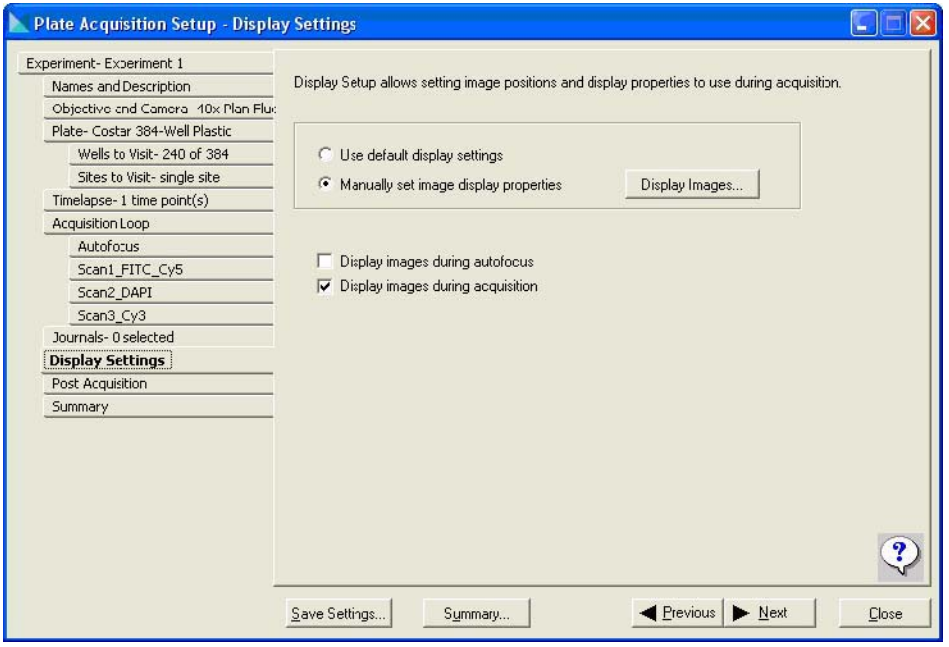
Configuring the Display Settings

You can configure the MetaXpress Software desktop appearance during acquisition. You can choose to use the default display settings or create custom settings. The pre-defined display settings tile and autoscale all acquired images, and make sure that the status dialog is unobstructed.

Note: If you do not need to change the display settings, make sure that Use default display settings is selected and then click Next to move to the next tab. Changing the display does not change the saved data.

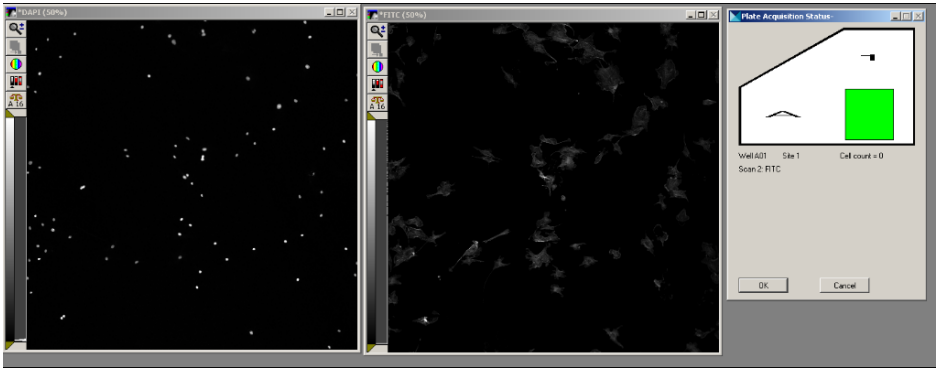
To change the default display settings

1. In the **Plate Acquisition Setup** dialog, click the **Display Settings** tab and then click **Manually set image display properties**.



2. Click **Display Images**.

   The Screening Status dialog opens and an image is acquired for each configured wavelength.



3. Change the configuration of the display by arranging the image windows and dialogs. You can change the location, size, zoom, scaling, gamma, and LUT of images. These new settings are saved and used during acquisition.

Note: The **Display images during autofocus** option shows images during image autofocus. The ImageXpress Ultra System does not use image autofocus so this option does not affect the display properties.

Selecting a Data Analysis Method

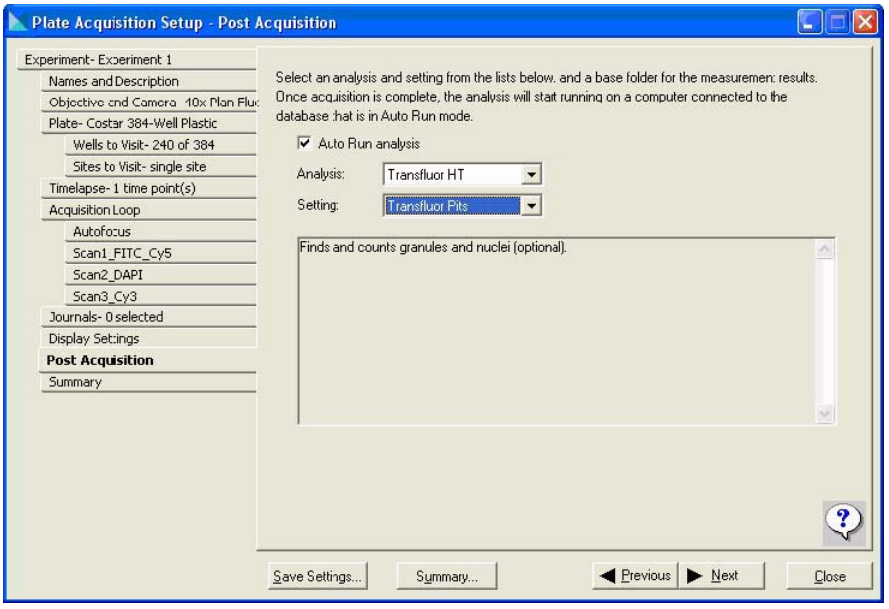
You can choose a specific analysis to run on a data set after the acquisition is complete. The data set is added to the Auto Run queue for analysis by an offline computer system running the MetaXpress Software set to Auto Run mode. You can select from a list of saved settings from any application modules or journal assays saved to the database.

The list of available assays and settings is the same list that is in the Assay tab of the Review Plate Data dialog.

Note: If you do not want to automatically run post-acquisition analysis, make sure that the **Auto Run analysis** check box is not selected and click Next to move to the next tab.

To select an analysis method to automatically run after acquisition

1. In the **Plate Acquisition Setup** dialog, click the **Post Acquisition** tab and then select the **Auto Run analysis** check box.



2. In the **Analysis** list, select the assay (Application module or Journal Assay) to run after acquisition.
3. In the **Setting** list, select a settings file.

   The field below the Setting list displays a description of the settings if one exists.
4. You can configure and save settings in the Review Plate Data dialog.

Reviewing the Plate Acquisition Settings

After you have completed all the steps to set up the plate acquisition, you can review a list of all current settings for the acquisition, save the settings to a file, and start acquiring images.

The information in the Summary tab is identical to the information displayed when you click the Summary button at the bottom of the dialog.

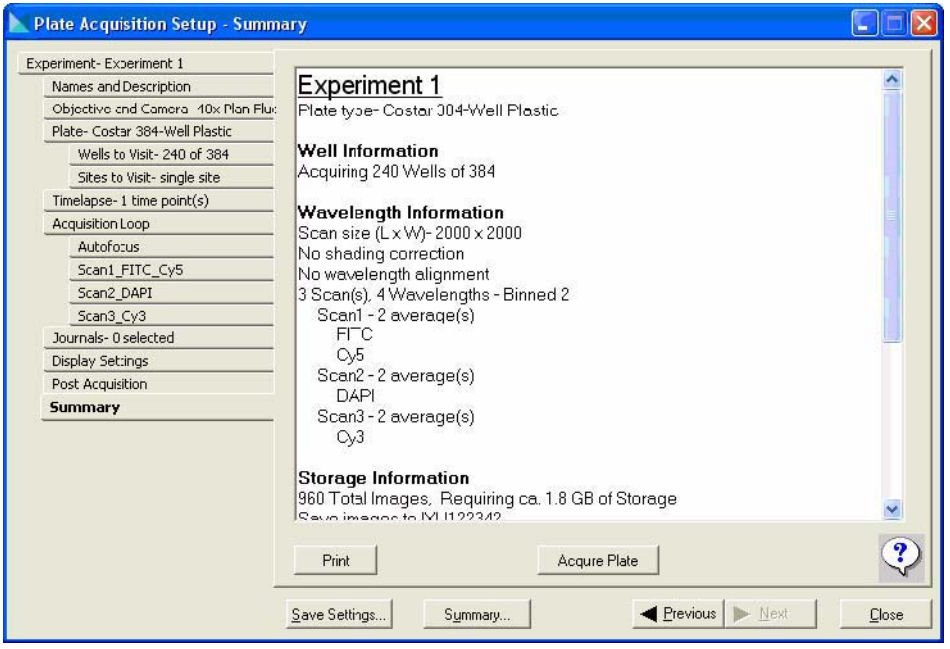
Use the Plate Acquisition and Control dialog or the Plate Acquisition toolbar to change the stage or Z position, or snap an image to test the current settings before starting the acquisition.

If an error dialog opens after you click Acquire Plate, the most likely cause is a configuration error. Read the text in the dialog to determine the error.

After your acquisition is complete and the images have been saved to the database, you can use the Review Plate Data dialog to view the images and set up analysis.

To review the plate acquisition settings file and start acquisition

1. In the **Plate Acquisition Setup** dialog, click the **Summary** tab and then click **Save Settings.**



2. In the **Save Plate Acquisition Settings** dialog, save the current settings to a file on the local hard drive or to the database.
3. Click **Print** to open the Print Setup dialog and then print the settings summary.
4. In the **Summary** tab, click **Acquire Plate** to acquire images from a plate based on the settings completed in the Plate Acquisition Setup dialog.
5. The **Plate Acquisition Setup** dialog closes and the **Screen Status** dialog opens.

   Each image appears briefly on the MetaXpress Software desktop as it is acquired and saved to the database.

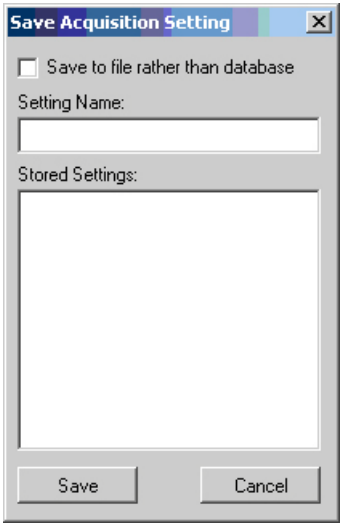
   After the last image is acquired and saved, the Screen Status dialog closes and the Plate Acquisition Setup dialog opens.
6. In the **Plate Acquisition Setup** dialog, click **Close**.

Saving Acquisition Settings

After you configure the plate acquisition, save the settings in the Plate Acquisition Setup dialog so that they can be reused. You can save the acquisition settings to a file on a local or network drive or to the database.

To save the current plate acquisition settings

1. In the MetaXpress Software, click **Screening** and then one of the following:
   - Plate Acquisition and Control
   - Plate Acquisition
   - Plate Acquisition Setup
2. Depending on your selection, in the dialog that appears, click **Save Settings**.
3. In the **Save Acquisition Setting** dialog, select the **Save to file rather than database** check box to save the settings file on the local computer.



4. Click **Save** and in the **Screen Acquisition Settings** dialog, in the **File name** field, type the name of a new settings file or select a listed settings file name to overwrite an existing settings file and then click **Save**.

5. In the **Save Acquisition Setting** dialog, clear the **Save to file rather than database** check box to save the settings file to the database.

Note: It is recommended to save to files rather than a database for a system that has multiple users as it is easier to organize settings files. Saving to files is required for running specific settings with a plate-loading robot.

6. In the **Setting Name** field, type the name of the new settings file or select a listed settings file from the **Stored Settings** field to overwrite an existing settings file in the database and then click **Save**.

Maintenance

7

This chapter contains information and procedures on how to maintain the ImageXpress® Ultra System. It includes:

- User Safety Instructions on page 135
- Filter Cubes on page 135
- Objectives on page 140
- Configuring Objective Offsets and Parfocality on page 145
- Cleaning the ImageXpress Ultra System on page 168

User Safety Instructions

It is important to strictly observe the following safety information to avoid personal injury or damage to your equipment during user service and maintenance:

- Close the MetaXpress Software.
- Make sure that the power supply for the ImageXpress Ultra System is turned off and the power cable is unplugged.
- Disconnect the USB connection to the hardware server (host) PC, and turn off any attached peripherals, such as the robot plate-loading arm.
- Access only the user-serviceable components inside the enclosure. Avoid contact with other components as they can be damaged or knocked out of alignment.
- Keep liquids, vapor, and dust away from the interior of the instrument. Do not attempt to clean inside the enclosure.
- Do not leave the interlocked access panels open for extended periods of time.
- Make sure all components and access panels are replaced before restarting the instrument.

Filter Cubes

If you replace or add to any of the optical components in the factory-standard ImageXpress Ultra System, you must:

- Change the component within the instrument.
- Update the software to reflect the new hardware configuration.

Changing Filter Cubes

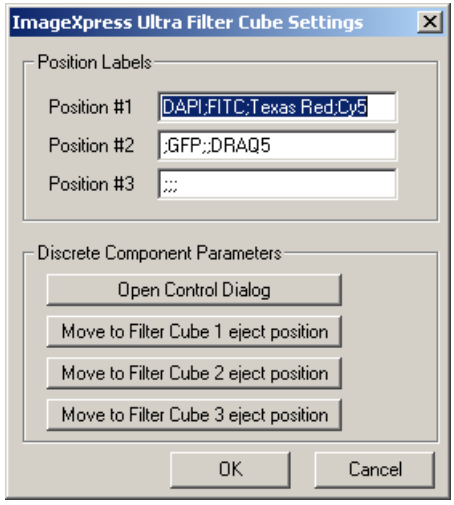
The filter cubes in the ImageXpress Ultra System are mounted in racks in a three-position slider within the instrument enclosure. Each rack holds up to three filter cubes. Filter cubes and the optical components they hold are delicate components, and special care is required when handling them.

WARNING! Before you access the emission filter changer compartment, turn off the ImageXpress Ultra System.

CAUTION! Wear powder-free gloves when you change the filter cubes to prevent skin oils from damaging the optical coatings. Do not touch the optical coatings.

To change the filter cubes

1. Click **Start > Programs > MetaXpress > Meta Imaging Series Administrator**.
2. In the **Meta Imaging Series Administrator** dialog that appears, in the **List of Groups** field, select **MetaXpress** and then click **Configure Hardware**.
3. Click **Configure Devices** and then click **ImageXpress Ultra Filter Cube Changer**.
4. Click **Settings**.
5. Click **Eject Filter Cubes** for the desired filter cube rack.



6. Close the **Meta Imaging Series Administrator** software.
7. Turn off the ImageXpress Ultra System instrument at the main power switch, located on the external power supply.
8. Lift the small panel up to access the filter cube changer. The panel is located on the side of the instrument.

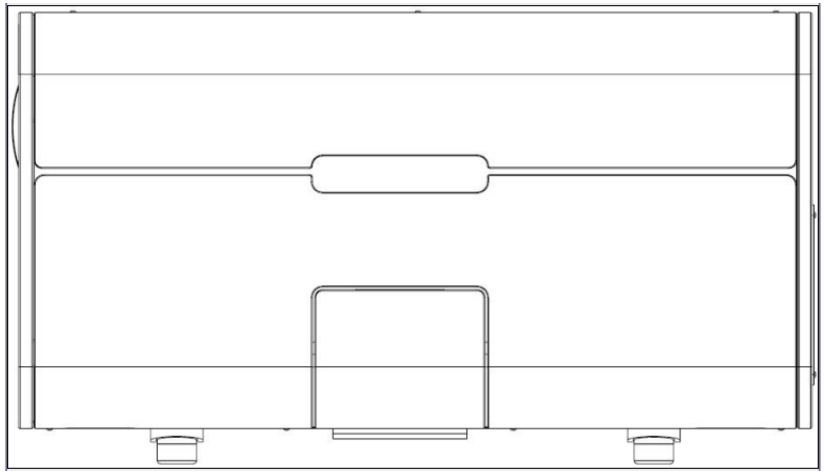


Figure 7-1 ImageXpress Ultra System filter door

9. Slide out the desired filter cube rack cassette.

There might be some slight resistance.

Note: Filter cube rack 1 is the furthest one on the left.

10. Remove the filter cube cassette.

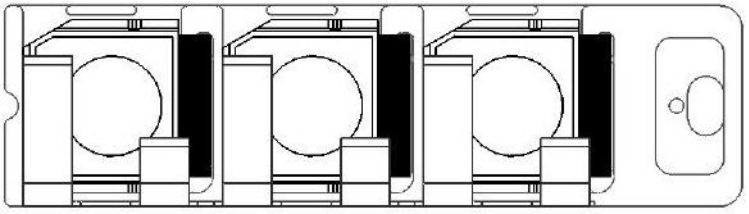


Figure 7-2 ImageXpress Ultra System filter cube cassette (below)

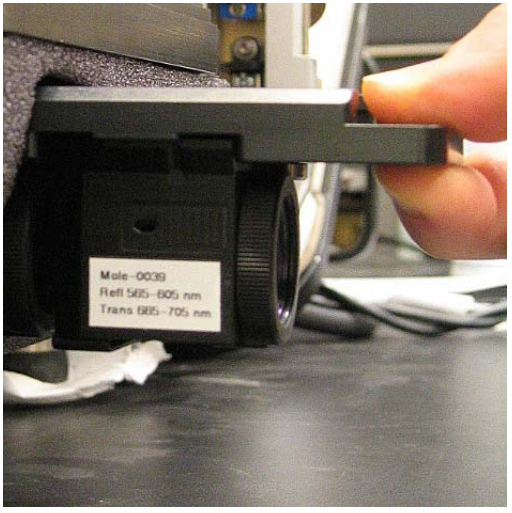


Figure 7-3 ImageXpress Ultra System filter cube cassette (side view)

11. Slightly tilt the filter cube to lift it over the raised edge of the filter cube cassette, and pull it out to remove it.

12. Slide the new filter cube into place, tilting it to insert it. Check the orientation of the cube matches the others to make sure that it is firmly seated.
13. Carefully line up the filter cube cassette with the changer and then push the filter cube cassette back into place until the latch engages.

CAUTION! Do not continue if you feel resistance while replacing the filter cube cassette. Remove and check it to make sure that it is lined up correctly with the changer.

14. Turn on the instrument at the main power switch located on the external power supply.
15. Click **Start > Programs > MetaXpress > Meta Imaging Series Administrator**.
16. In the **Meta Imaging Series Administrator** dialog, click **Configure Hardware** and then click **Configure Devices**.

Tip! For additional information about any of the dialogs in the Meta Imaging Series Administrator, press the F1 key to access Help.

17. In the **User Settings Hardware Configuration** dialog, click **ImageXpress Ultra Filter Cube** and then click **Settings**.
18. Click **Load Filter Cubes**.

WARNING! Do not open the filter cube access door while the system is powered on.

19. Specify the label for the new filter cube (for example, DAPI;FITC;Cy3;Cy5). The position on the left is position 1. Separate each channel with a semicolon.

Note: The format for the filter names is Wavelength1;Wavelength2;Wavelength3;Wavelength4. Each entry must be separated by a semicolon. If a filter cube is missing from any filter cube assemblies, leave it blank between the semicolons.

20. Click **OK** in all open dialog and then close the **Meta Imaging Series Administrator** dialog.

21. Start the MetaXpress Software.
22. Click **Devices > Configure Illumination.**
23. Remove the old setting and create a new setting. Make sure that both the correct filter cube position as well as the correct main beam splitter have been selected. See To verify the filter cube settings on page 47.
24. Click **Add/Replace** to store the new setting.

Objectives

The objectives in the ImageXpress Ultra System are mounted in a four-position linear selector. Objectives are very delicate components and special care is required when handling them. Make sure you review the safety information at the beginning of this chapter and the information on the correct placement of the objective. See User Safety Instructions on page 135 and Correct Objective Placement on page 140.

If you add or replace any of the optical components in the factory-standard ImageXpress Ultra System:

- Change the component within the instrument.
- Update the software to reflect the new hardware configuration.

Correct Objective Placement

The 10X Plan Fluor Objective must remain in position 1. It should never be removed. This objective is used for system calibration.

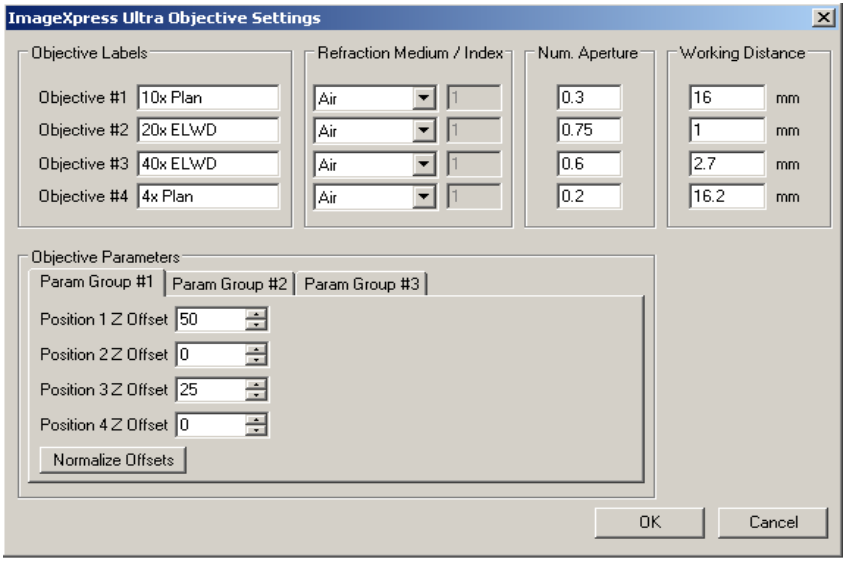
Changing Objectives

CAUTION! Wear powder-free gloves when you change the objectives to prevent skin oils from damaging the optical coatings.

Note: To configure objectives you must perform offset and parfocality procedures. See Configuring Objective Offsets and Parfocality on page 145.

To change objectives

1. Click **Start > Programs > MetaXpress > Meta Imaging Series Administrator**.
2. In the **Meta Imaging Series Administrator** dialog that appears, in the **List of Groups** field, select **MetaXpress** and then click **Configure Hardware**.
3. Select the desired hardware configuration (can be Default) and then click **Configure Devices**.
4. In the **User Settings for hardware configuration** dialog, click **ImageXpress Ultra Objectives** and then click **Settings**.
5. In the **ImageXpress Ultra Objective Settings** dialog, click the **Param Group #3** tab and then click **Open Door**.



6. Click the **Param Group #2** tab and then click **Move to Objective # eject position** to match the objective position of your choice.

   The objective holder and stage move so that you can access the objective (positions 1, 2 on one side of the stage; positions 3, 4 on the other side of the stage).

Note: The objectives are in numbered positions and must match the settings in the software.

7. Pull the objective up to engage the objective-holding device.

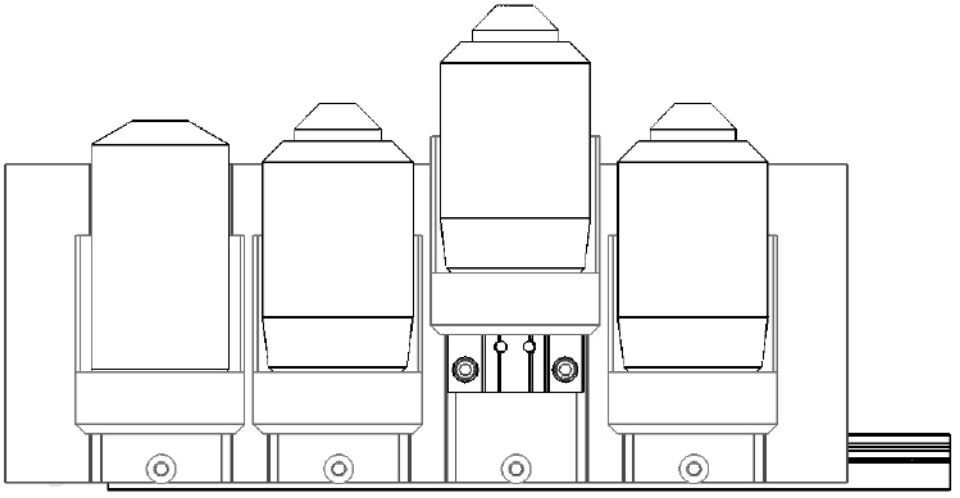


Figure 7-4 ImageXpress Ultra System objective changer with raised objective

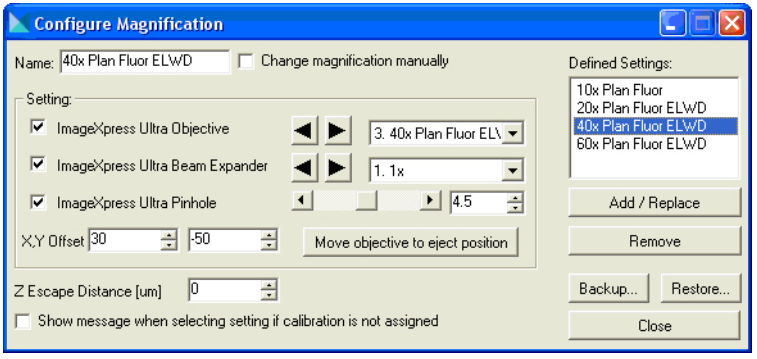
8. Loosen and remove the objective.

9. Replace the objective with the correction collar correctly set (if applicable).
10. In the **Meta Imaging Series Administrator** dialog, click **OK**.
11. In the **ImageXpress Ultra Objective Settings** dialog, click the **Param Group #3** tab and then click **Close Door**.
12. In the **Objective Labels** section, type the name of the new objective in the correct position.

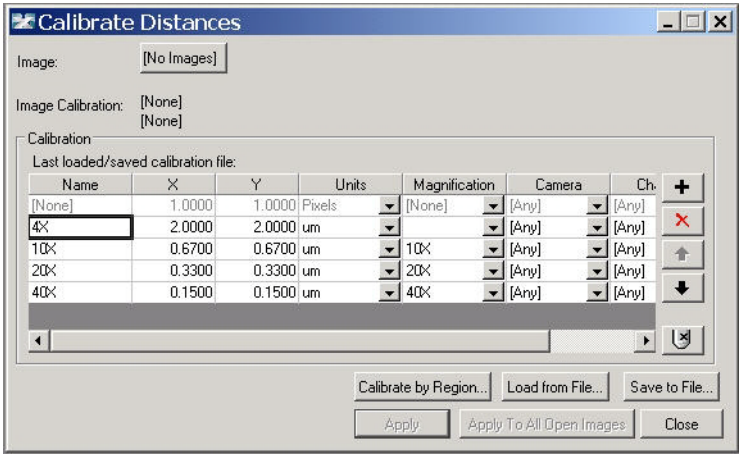
Note: The objective name must begin with a number, for example, 40X Plan Apo.

13. In the **Refraction Medium / Index** field, select the appropriate medium (for example, Air or Oil).
14. In **Num. Aperture** field, type the numerical aperture of the new objective, located on the barrel of the objective.
15. Click **OK** in all open dialogs and then click **OK** to close the **Meta Imaging Series Administrator** dialog.
16. Open the MetaXpress Software.
17. Click **Devices > Configure Magnification**.

18. In the **Configure Magnification** dialog, in the **Name** field, type the name of the new objective.
19. Select the **ImageXpress Ultra Objective**, **Beam Expander**, and **Pinhole** check boxes.
20. Select the default values. See Appendix A: Objectives Compatible with the ImageXpress Ultra System on page 173.



21. Remove the magnification setting that you replaced.
22. In the MetaXpress Software window, click **Measure > Calibrate Distances**.
23. In the **Calibrate Distances** dialog, click the **Setup** tab.



24. In the **Define Calibrations By** section, in the **Units / Pixel** fields, type the calibration for the new objective. For the calibration values, see Appendix A: Objectives Compatible with the ImageXpress Ultra System on page 173.
25. Set up laser autofocus for your plates using the information in Chapter 7.

Configuring Objective Offsets and Parfocality

The purpose of the following procedure is to center on and focus each objective on an asymmetrical feature provided by the GP-2 slide, noting the X, Y, and Z positions for each objective. These values are then entered into the software and used to compensate for XY and Z offsets inherent in the opto-mechanical positioning of the objectives.

Correctly setting objective XY offset and parfocality (Z offset) values enables you to switch objectives in the MetaXpress Software while maintaining the same region of interest and focal plane on the sample. Setting the XY offset between different objectives enables you to maintain the same point of interest on a sample when objectives are changed from one magnification to another. Setting parfocality (Z offsets) between different objectives enables you to maintain the same focal point on a sample when objectives are changed from one magnification to another.

To configure the objective offset and parfocality

1. Open the MetaXpress Software.

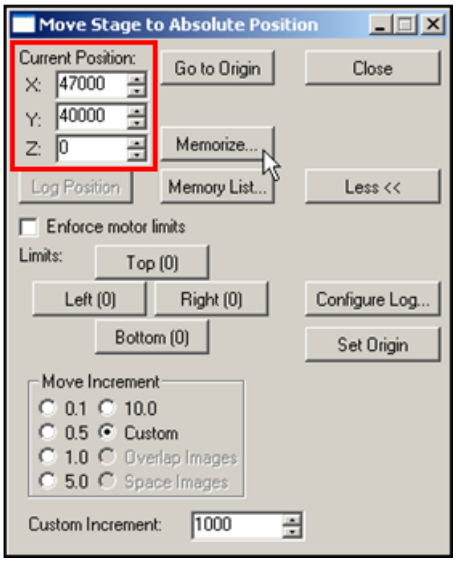
Note: The objectives must be installed and correctly configured in the Meta Imaging Series Administrator.

2. Open the top door.
3. Insert the GP-2 slide into the slide holder. Place the GP-2 target areas toward the calibration corner cut-out with the reflective surface facing down.

4. Insert the slide holder into the plate holder. Place the slide holder with the corner cut-out towards the plate clamp.

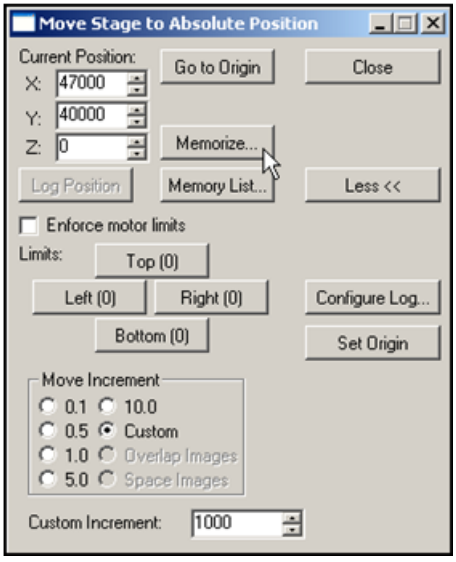
5. Click **Devices > Stage > Move Stage to Absolute Position**.

6. With the top door open, in the **Move Stage to Absolute Position** dialog, in the **Current Position** section, edit the values in the **X** and **Y** fields to move the stage so that a well-defined small feature is in the field of view of the 10X Plan Fluor objective.
   - The estimated value for the X field is 47000 and the Y field is 40000.

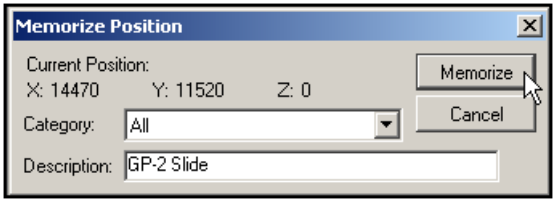


   - Use the up and down arrows to center the GP-2 target above the 10X objective. Increasing the X position moves the stage to the right and decreasing the X position moves the stage to the left.
   - Increasing the Y position moves the stage towards the front of the system (filter cube changer door), and decreasing the Y position moves the stage towards the back of the system.
   - In the Move Increment section, select Custom to vary the movement sizes and then in the Custom Increment field, type the desired number (1000 is the recommended starting value) of microns to move.

7. When the centre GP-2 target pattern is above the 10X objective, in the **Move Stage to Absolute Position** dialog, click **Memorize** to return to this exact XY stage position after you close the door. Multiple XY coordinates can be memorized and will remain available as long as the MetaXpress Software is not closed.

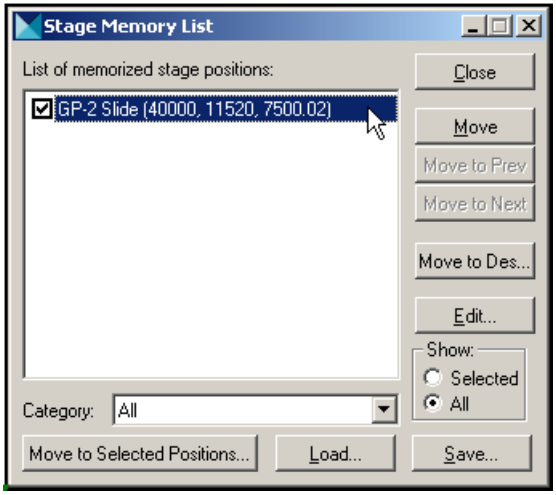


8. In the **Memorize Position** dialog, in the **Description** field, type **GP-2 Slide** and then click **Memorize**.



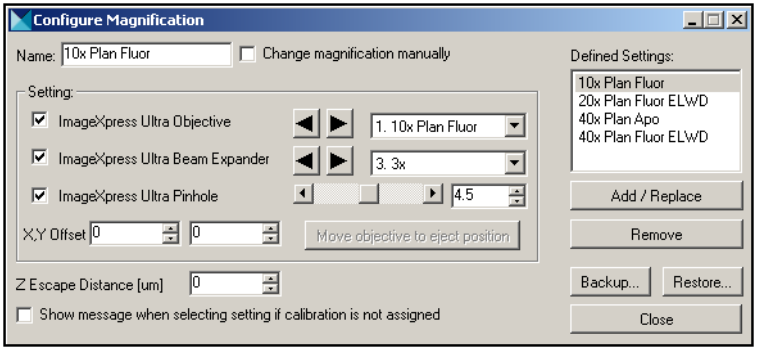
9. Close the top door.

10. In the **Move Stage to Absolute Position** dialog, click **Memory List** and highlight the position memorized (GP-2 Slide).



11. In the **Stage Memory List** dialog, click **Move** to move the stage to the saved location and then click **Save** to keep memory lists for multiple work sections.
12. In the MetaXpress Software, click **Devices > Configure Magnification**.
13. in the **Configure Magnification** dialog, click **Backup** to back up your current magnification settings.

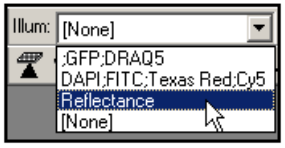
14. In the **Backup Magnification Settings** dialog, in the **File name** field, type a file name and then click **Save**. A useful nomenclature for the magnification setting is IXU-XXXXXXXX-YYYYMMDD format, where XXXXXXX is the serial number of the IXU system you are backing up, YYYY is the year, MM is the month, and DD is the day that you are backing up the magnification setting.



15. In the **Configure Magnification** dialog, you must confirm the name of each objective you wish to define XY and parfocality settings on.
    - Click **Remove** to delete any unwanted magnifications.
    - In the **Name** field, type any new magnifications settings and then click **Add and Replace** to add it to a defined settings.
    - Select the **ImageXpress Ultra Pinhole** check box. The default pinhole value is 4.5.
    - For the beam expander that is appropriate for each objective, see Selecting a Beam Expander for an Objective on page 176.
16. For this procedure, make sure that all objectives have XY offsets set to 0 before proceeding:
    - In the **Defined Settings** section, double click the objective.
    - In the **Setting** section, set the **X, Y Offset** field to **0** and **0** and then click **Add / Replace**.
    - In the **Configure Magnification** message that appears, click **Yes**.

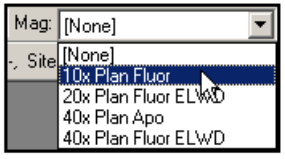
Note: The GP-2 slide is a chrome target layered on to glass and is reflective rather than fluorescent. Use the preconfigured Reflectance Illumination Setting (consisting of the 90-10 beamsplitter and empty emission filter set) with a single laser and PMT 4 to detect the image.

17. A preconfigured reflectance illumination setting should be configured on the instrument. On the **Device Control** toolbar, in the **Illum** field, click **Reflectance**.

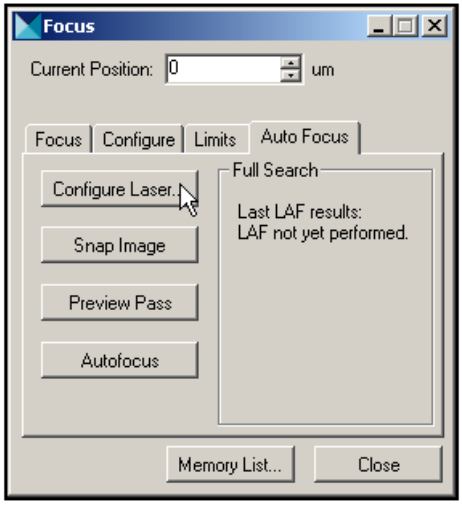


CAUTION! Reflective laser light can be very bright and damage your PMT. Make sure that the PMT is below 400 before proceeding.

18. On the **Device Control** toolbar, in the **Mag** field, select **10X Plan Fluor**.



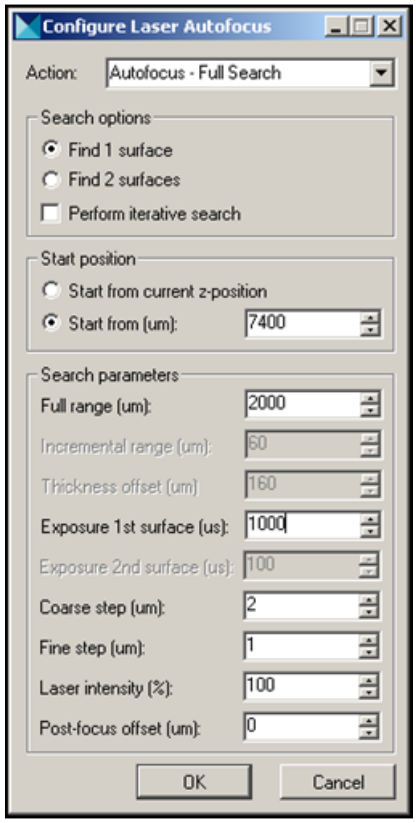
19. In the MetaXpress Software, click **Devices > Focus**.
20. In the **Focus** dialog, click **Configure Laser**.



21. In the **Configure Laser Autofocus** dialog, edit the fields:

Table 7-1 Configure Laser Autofocus Settings

Field / Section	Value
Action field	Select Autofocus - Full Search
Search options section	Select Find 1 Surface
Start position section	
Start from field	Type plate bottom reference position. This value is in the Plate Acquisition Setup - Autofocus dialog, in the Autofocus tab.
Search parameters section	
Full range field	2000
Exposure 1st surface	35
Coarse step	2
Fine step	1
Laser intensity	100

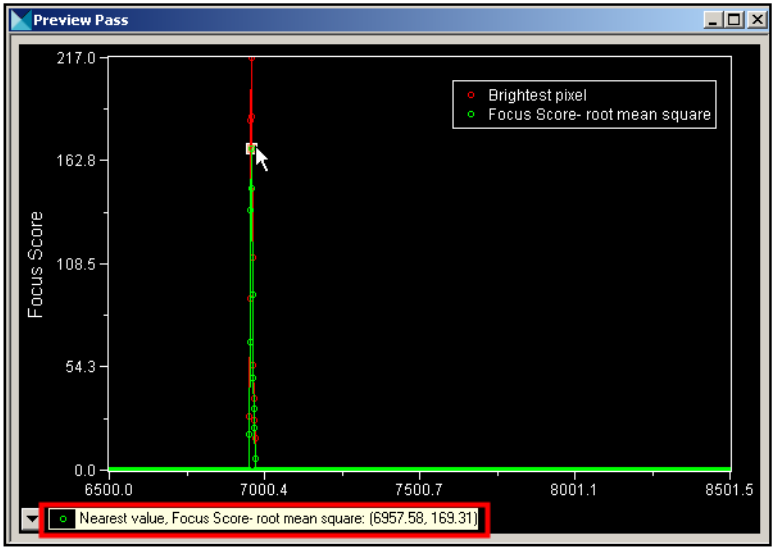


22. In the **Focus** dialog, click **Preview Pass**.

A graph window appears. If a surface is found and the exposure time is sufficient, a peak with overlaid red and green traces appears.

If no trace appears, in the **Configure Laser Autofocus** dialog, in the **Start from** field, increase the **Z** value by **500** and then in the **Focus** dialog, click **Preview Pass**. Repeat until a peak appears. If no peak appears and the **Start from** value exceeds **8500**, increase the exposure time by **50** and then restart the search starting at the value in the **Plate Bottom Reference Position** field.

Note: If two reflectance peaks appear, select the left one as it represents the bottom surface of the slide.



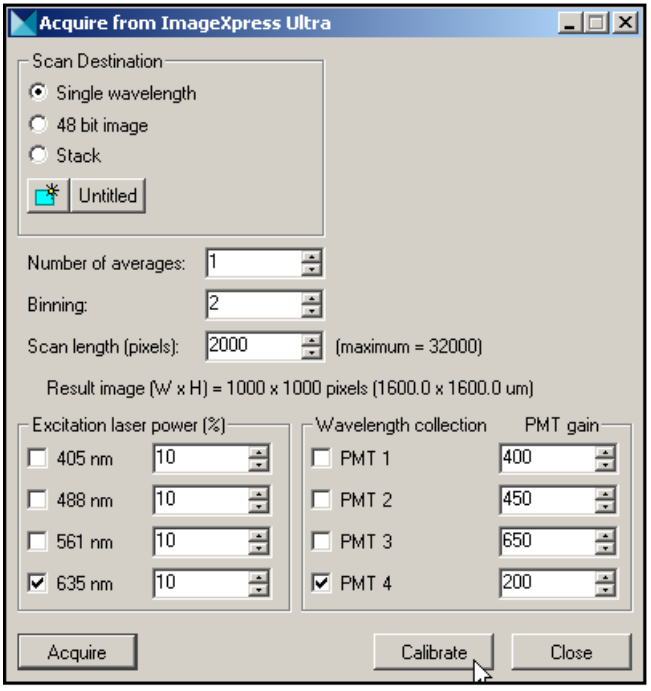
23. After a peak appears in the preview window, click the highest green point. The Z position of the focus position appears in the bottom left, as highlighted in the screen shown in step 21.
24. In the **Move Stage to Absolute Position** dialog, in the **Current Position** section, in the **Z** field, type the Z position found by selecting (clicking and holding the left mouse button down) the peak in the preview pass window. Text will appear in the bottom left indicating the nearest Z value.
25. Click **Acquire > Acquire from ImageXpress Ultra** to snap an image.
26. In the **Acquire from ImageXpress Ultra** dialog, edit the fields:

Table 7-2 Acquisition settings

Field	Value
Scan Destination	Single wavelength
Number of Averages	1
Binning	2
Scan Length	2000
Excitation laser power	Select the 635 check box only with 10% power
Wavelength collection	Select PMT 4 check box only with a PMT gain of 200

27. In the **Acquire from ImageXpress Ultra** dialog, depending on the MetaXpress Software version, click **Calibrate** or **Calibrate Galvo** and then wait until the instrument galvonometer completes calibration.

Note: Calibration takes about 15 to 30 seconds. The cursor becomes an hour glass icon while the instrument calibrates and then returns to a cursor upon completion of the successful calibration.



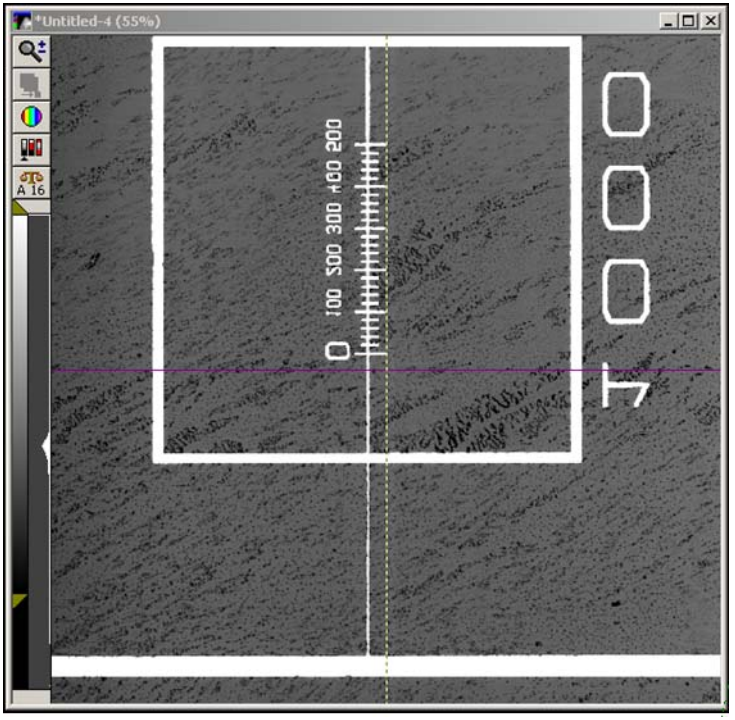
28. In the **Acquire from ImageXpress Ultra** dialog, click **Acquire**.

The system produces an image that is likely out of focus.

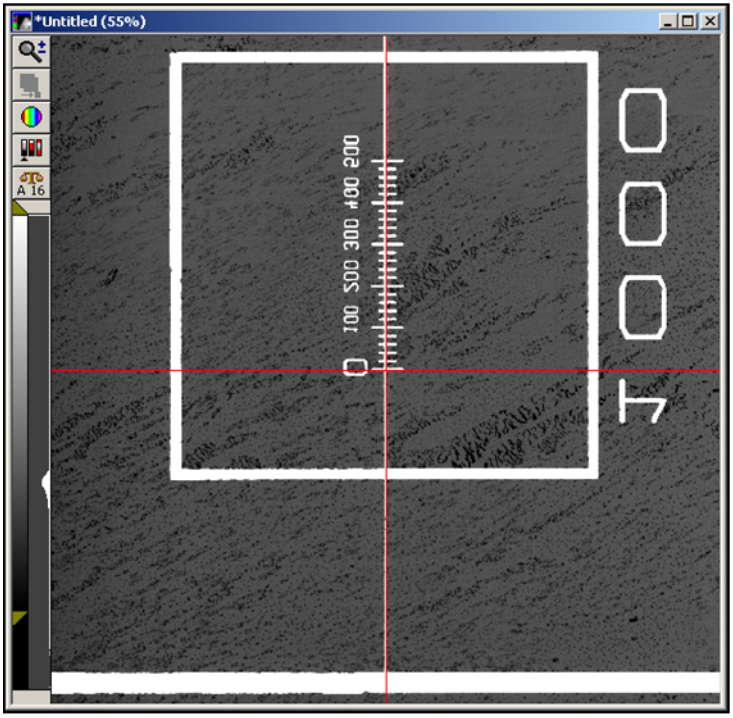
29. In the **Move Stage to Absolute Position** dialog, select **Custom** and then type a value of **100**. In the **Current Position** section, in the **Z** field, increase the value by **100**.

Tip! Obtain a sharp image by increasing or decreasing the current Z position (focus) value by using the up and down arrows next to the current position.

30. Repeat steps 27 and 28 until you obtain a sharp image.
31. To enable centering a feature with the field of view, using the region tool, draw a cross hairs in the image. A journal can be obtained from Technical Support if one is not already available on your instrument. The cross hairs and reference image are shown in the screen.

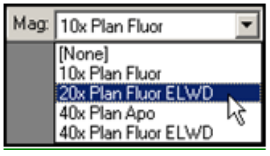


32. In the **Move Stage to Absolute Position** dialog, in the **Current Position** section, edit the **X** and **Y** fields to overlay the cross hairs with a well-defined feature as shown in the screen.



33. When the best focus image is displayed, record the X, Y, and Z positions for the 10X objective located in the **Device** toolbar.

34. On the **Device** toolbar, in the **Mag** field, select the next higher objective.



Note: The objectives in your ImageXpress Ultra System might differ from the ones described in this procedure.

35. Repeat steps 32 and 33 to find the XY and Z positions for each objective. With other objectives, you must modify certain parameters.

Table 7-3 Parameter Changes for Objectives

dialog	Change
Acquire from ImageXpress Ultra System	Increase or decrease the PMT gain to get an appropriate image.
Configure Laser Autofocus	
Exposure 1st surface field	Increase the value
Coarse step field	• 10X objective—3 to 5 • 20X objective—2 to 3 • 40X objective—1 to 2 • 60X objective and higher—1 to 2
Fine step field	• 10X objective—1 • 20X objective—0.5 • 40X objective—0.2 to 0.5 • 60X objective and higher—0.1 to 0.2
Start from field	Must be lower than the focal position of the bottom surface of the slide or plate. If you are unsure of an appropriate value, start very low and gradually step up until that surface is detected.
Full range field	Must never exceed about 1.5 times the working distance of the objective you are setting. This is critical for short working distance objectives to prevent the objective from bumping the plate.

36. Record the X, Y, and Z positions for all the objectives.

Table 7-4 Objective Positions

Objective	X position	Y position	Z position
10X Plan Fluor			
20X Plan Fluor ELWD			
40X Plan Apo			
40X Plan Fluor ELWD			

Note: The objectives listed in Table 7-4 are examples of objectives that can be installed in the instrument. Modify the list of objectives to match the ones installed in the instrument you are using.

Entering Objective Offset and Parfocality Values

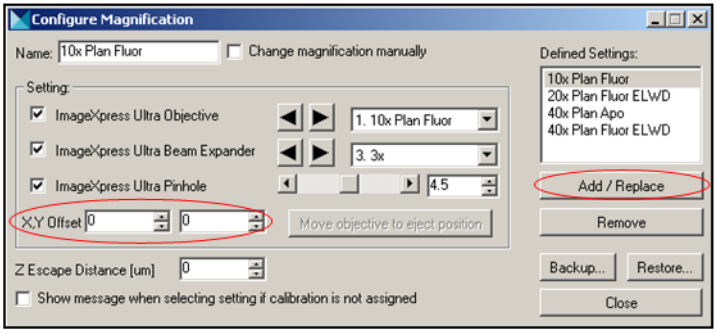
Use a spreadsheet to subtract the XY values of the 10X Plan Fluor objective from the values of each objective as shown in Table 7-5. The resulting values are entered in the X and Y offsets fields. The values in the table are examples only and the values you use to calculate the offsets might be different.

Table 7-5 Objective XY Offset Example Calculations

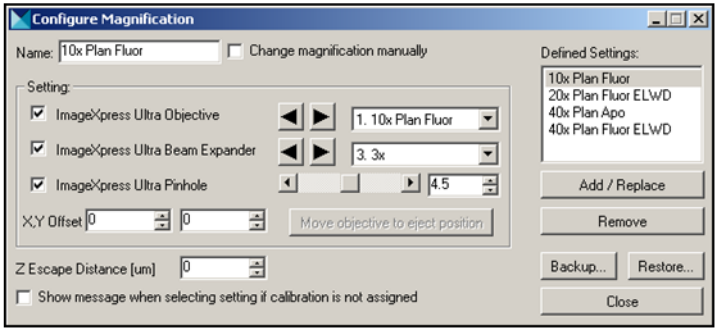
Objectives	X Position	Y Position	X Offset	Y Offset
10X Plan Fluor	48857	43259	48857 - 48857 = **0**	43259 - 43259 = **0**
20X Plan Fluor ELWD	48957	43239	48857 - 48957 = **100**	43259 - 43239 = **20**
40X Plan Apo	48980	43309	48857 - 48980 = **123**	43259 - 43309 = **50**
40X Plan Fluor ELWD	48757	43289	48857 - 48757 = -**100**	43259 - 43289 = **30**

To enter objective offset and parfocality values

1. In the MetaXpress Software, click **Device > Configure Magnification**.
2. In the **Configure Magnification** dialog that appears, in the **Defined Settings** section, in the **X** and **Y Offset** fields, type the value you calculated for each objective setting and then click **Add / Replace**.



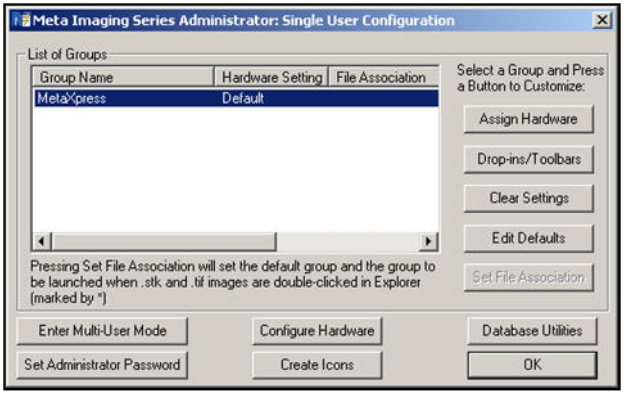
3. In the **Configure Magnification** message asking you if you want to replace the setting, click **Yes**.
4. In the **Configure Magnification** dialog, click **Backup**.



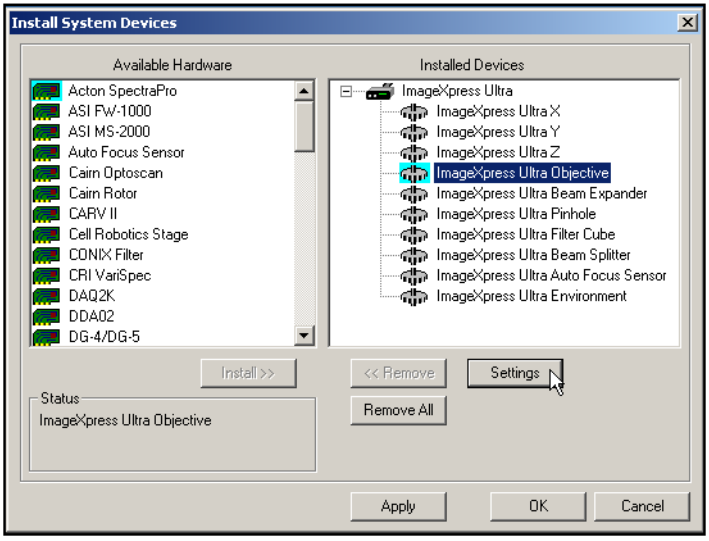
5. In the **Backup All Magnification Settings** dialog, in the **File name** field, edit the name using the IXUXXXXXXX-INSTALL format where XXXXXXX is the serial number for the IXU system being backed up and then click **Save**.
6. Close the MetaXpress Software.

   Type the parfocality (Z offset) values into the Objective Setting dialog in the Meta Imaging Series Administrator software.

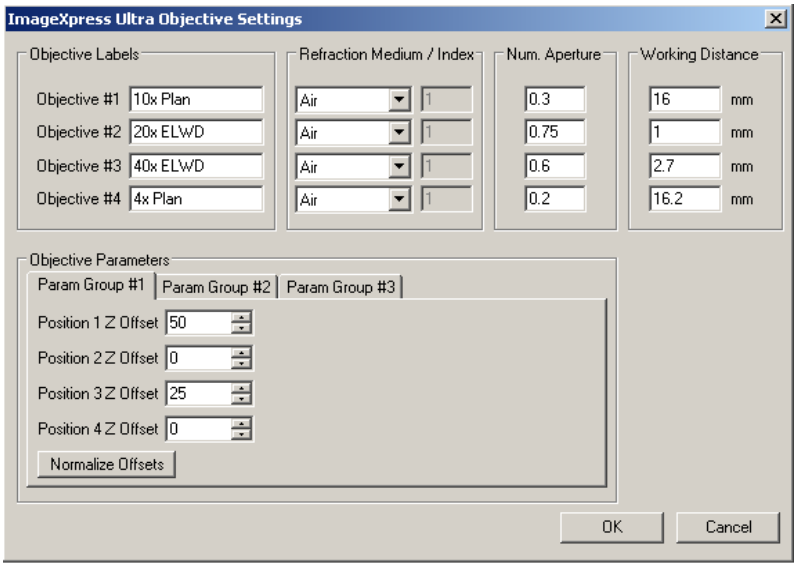
7. Open the **Meta Imaging Series Administrator** software and in the **Enter Administrator Password** dialog, in the **Enter Password** field, type the password and then click **OK**.
8. In the **Meta Imaging Series Administrator: Single User Configuration** dialog, click **Configure Hardware**.



9. In the **Configure Hardware** dialog, click **Install System Devices**.
10. In the **Install System Devices** dialog, in the **Installed Devices** section, click **ImageXpress Ultra Objective** and then click **Settings**.



11. In the **ImageXpress Ultra Objectives Settings** dialog, in the **Param Group #1** tab, in the **Position Z Offset** fields, type the values for each objective recorded in Step 36 on page 159.



Note: In a vacant position, use the lowest value recorded in step 35 to enter in that position.

12. Click **Normalize Offsets.**

Note: The Z offset positions adjust and one of the four position offset parameters is 0.

13. Click **OK** to exit the **ImageXpress Ultra Objectives Settings** dialog and then click **OK** to exit the **Install System Devices** dialog.

14. Repeat the process to add the parfocality value in the **Configure Devices** dialog.

Tip! For additional information about any of the dialogs in the Meta Imaging Series Administrator, press the F1 key to access the online Help for the active dialog. This includes accessing information on the single user and multi user modes.

15. In the **Configure Hardware** dialog, click **Configure Devices**.
16. In the **User Settings for X hardware configuration** dialog, in the **Claimed Devices** section, click **ImageXpress Ultra Objective** and then click **Settings**.
17. In the **ImageXpress Ultra Objectives Setting**s dialog, in the **Param Group #1** tab, type the Position Z Offset values recorded for each objective.

Note: If a position is not being used by an objective use the lowest value recorded in step 35 for that position.

18. Click **Normalize Offsets**.

Note: The Z Offset Positions change from the Z positions recorded to the Offset values from the position 1 reference position. Compare these values for each position with the values normalized in step 11. If the resulting offset values do not match, double check that the values have been correctly recorded.

19. Click **OK** to exit the **ImageXpress Ultra Objective Settings** dialog and then click **OK** to exit the **User Settings for X hardware configuration** dialog.
20. Click **OK** to exit the **Configure Hardware** dialog and then click **OK** to exit the **Meta Imaging Series Administrator** dialog.
21. After the parfocality and XY offsets have been set and saved in the software, load a GP-2 slide and then focus and center on a position with the 10X objective.
22. Switch objectives and without moving the stage or focus position, snap an image. The slide should remain relatively centered and in focus at different magnifications.

Cleaning Objectives

If debris or contaminants have collected on an objective, you must clean the objective lens. You must remove the objective before cleaning it.

To clean the objectives

1. In the MetaXpress Software, open the instrument door.
2. Close the MetaXpress Software and turn off the ImageXpress Ultra System at the main power switch located on the external power supply of the instrument.
3. Use compressed air to blow dust contaminants off objectives. Make sure you briefly purge your compressed air source before directing it at the objectives.

CAUTION! Do not use products that disperse aerosol propellants or fluid onto the lens surface. Do not invert compressed air cans as they disperse aerosol propellants.

4. Use lens paper and a solvent to wipe the objective free of contaminants.

CAUTION! Do not use a lint-free cloth to clean the objective lens. Contact the Nikon objective manufacturer for the preferred cleansing solvent and procedure.

Adjusting the Spherical-Aberration Correction Collar on Objectives

Certain Nikon objectives that are supplied with the ImageXpress Ultra System have adjustable correction collars, which are used to minimize spherical aberration in the image of the specimen. Changing this setting adjusts the distances between components inside the objective barrel. Image quality and resolution are largely dependent on properly setting these collars.

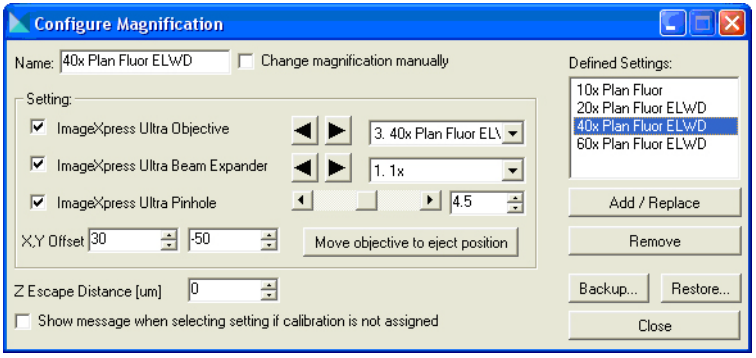
The settings depend on the thickness of the microplate well or slide on which the specimen is mounted. In general, the correction collar should be set for the physical thickness of the plate or slide that you are imaging. Determine the physical thickness in the following ways:

- Obtaining the plate specifications from the plate manufacturer.
- Breaking a spare plate and using calipers to measure the thickness.
- Measuring the optical thickness with the laser autofocus and multiplying it by the refractive index (1.59 for polystyrene; 1.52 for glass).

After you have determined the thickness of your plate or slide, adjust the correction collar.

To adjust the correction collar on the objectives

1. Turn on the ImageXpress Ultra System and open the MetaXpress Software.
2. Click **Devices > Configure Magnification** dialog.



3. In the **Defined Settings** field, double-click the magnification setting that you want to adjust.

4. Click **Move objective to eject position**.

   The top door opens and the stage and objective holder move so that you can access the objective.
5. Pull the objective up to engage the objective-holding device.
6. Locate the correction collar on the objective that you want to adjust.

Note: The graduated scale is on the barrel of the correction collar and shows the current setting of the objective. Use a flashlight to view the markings that are located on two sides of the objective.

7. Rotate the correction collar to its new setting.

Note: Some objectives are marked in mm, while others, such as the one shown, are marked in tens of microns.

8. Click **OK**.
9. Test the correction collar setting by examining the image quality of the acquired images. If the quality has degraded, re-adjust the correction collar. The correction collars are calibrated for glass, so the optimum setting for polystyrene bottom plates might differ slightly from the actual thickness of the plate.

Note: You might need to adjust the focus settings after adjusting the correction collar.

Using Oil-Immersion Objectives

You can use oil-immersion objectives with the ImageXpress Ultra System for research-mode imaging. Imaging with oil-immersion objectives is different from typical screening applications because you must keep oil between the objective and the plate. It is not possible to scan large areas (for example, entire slides or plates) in this configuration and the robustness of LAF cannot be guaranteed as the objective might move away from the sample during an LAF routine. Contact Molecular Devices Technical Support for ordering information for the objectives or assistance in completing this procedure.

To apply oil to the objective

1. In the MetaXpress Software, eject the plate to open the top door.
2. Remove any plates in the system.
3. Sparingly add oil to the top of the appropriate objective using a dropper bottle.
4. Insert the sample, either with a thin glass cover slip or in a microplate with a thin glass bottom (oil-immersion objectives are not compatible with most plastic microplates).
5. In the MetaXpress Software, slowly step up the objective until you are near to focus. Oil-immersion objectives are not recommended for scanning entire microplates.
6. After you have completed the process with the oil-immersion objective, eject the plate, remove the sample, and clean the top of the objective with lens paper.

Cleaning the ImageXpress Ultra System

Clean the plate-loading region and the outside of the instrument without damaging the internal components of the imaging system. Follow these guidelines carefully to prevent damaging the instrument:

- Do not remove the front panels of instrument during the cleaning procedure as the panels protect the ImageXpress Ultra System optics and electronics.
- Use disinfectant wipes (or lint-free cloth with 70% ethanol) to remove biological agents. Wipe the area with a damp wipe, followed by a second wipe with 100% ethanol to speed drying to remove non-viable biohazardous agents (carcinogens, toxins). With any aqueous cleaning agent, do not use too much as excess liquid will flow into the instrument.
- Do not use ultraviolet light for sterilization, as this can damage plastic components.
- Do not use any organic solvents without first consulting Technical Support.
- Do not pour or squirt water or alcohol directly on to the instrument, to prevent damaging internal components.

CAUTION! Wear appropriate personal protective equipment as detailed under your company's environmental and safety program when performing any cleaning procedure.

Robotic Plate Handling

8

If your ImageXpress® Ultra System has the robotic plate handler integrated with the base imaging system, there are additional hardware and user procedures to become familiar with. The robotic plate handler is integrated with the ImageXpress Ultra System in such a way that plates can be scanned for barcodes, loaded onto the imaging system where images are acquired and then returned to a home location.

If at any point you would like to upgrade your system with the robotic plate handler, contact your Molecular Devices Sales Representative.

CRS Catalyst Express Robot Hardware

The CRS robotic plate handler can be integrated with the ImageXpress Ultra System. The robot step-by-step protocol is as follows:

1. Get a microplate from a hotel shelf.
2. Scan the microplate barcode.
3. Load the microplate onto the ImageXpress Ultra System.
4. Acquire images.
5. Unload the microplate from the ImageXpress Ultra System.
6. Return the microplate to the original hotel shelf.

CRS Catalyst Express Robot User Procedures

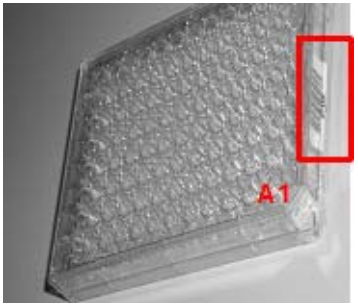
Loading Plates onto the CRS Catalyst Express Robot

You must properly load your plates onto the hotels of the robotic plate handler to use the CRS Catalyst Express robot to scan barcodes and to load and unload plates in the ImageXpress Ultra System. If the plates are not loaded in the correct orientation, two errors will occur:

1. The barcode will not be scanned.
2. Well position A1 will not be correctly located in the front left corner of the plate-loading region on the ImageXpress Ultra System.

To prevent improper loading of plates

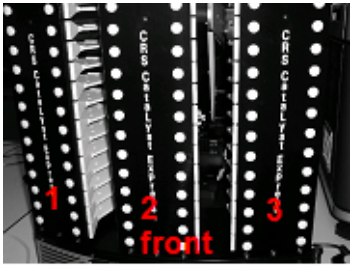
1. If you plan to scan barcodes, properly affix the barcode labels before loading plates onto the robotic plate handler. Labels are located on the front left side of the plate and they must be consistently located in the same position on all plates being scanned.



Note: Use labels with a minimum line height of no less than 7.5 mm, preferably 10 mm. For recommendations on compatible barcode vendors, contact Molecular Devices Technical Support.

2. The CRS Catalyst Express has three vertical racks (“hotels”) on to which you can load microplates.

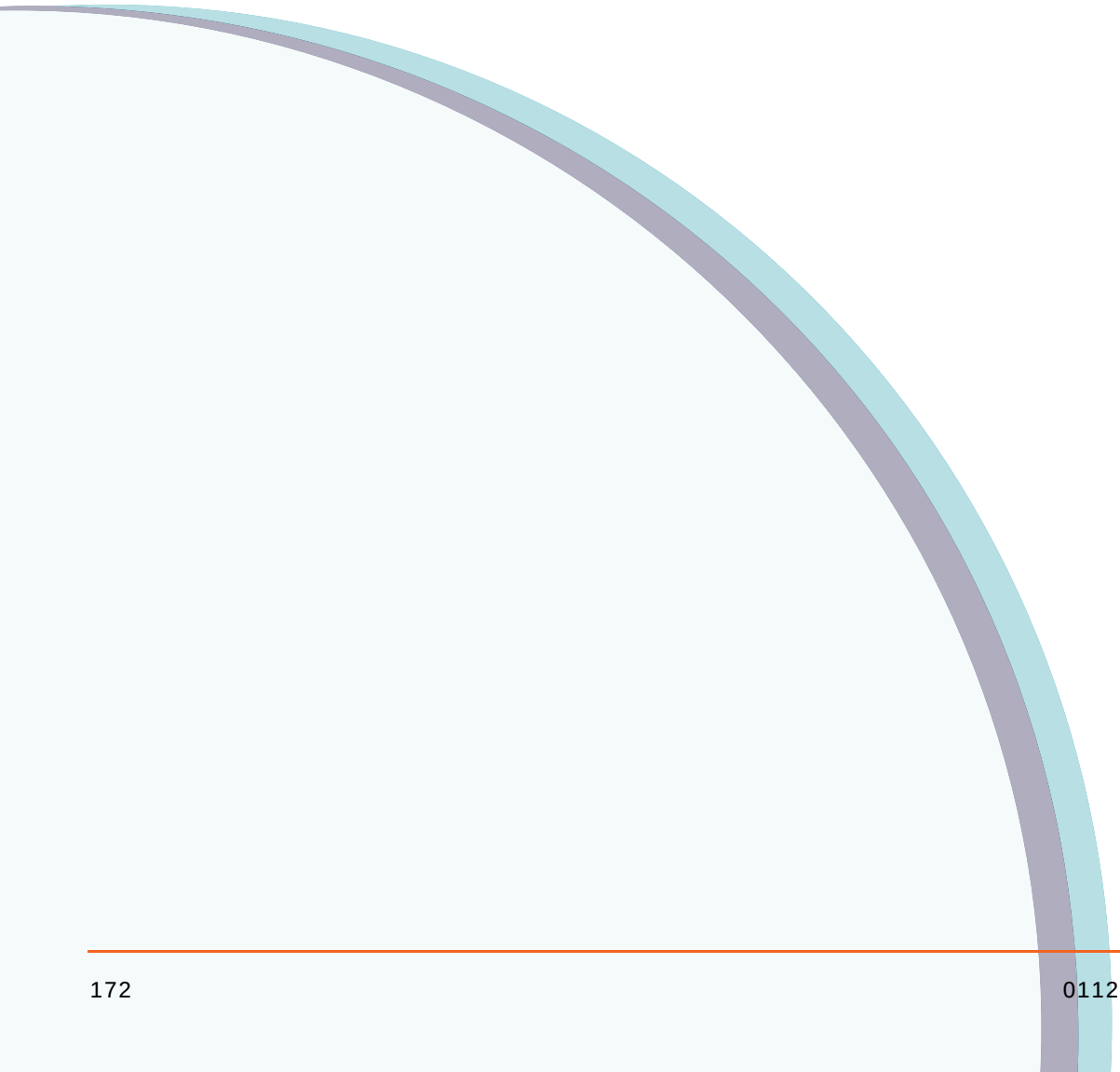
Note: When you are positioned in front of the robot, the hotel numbers (1–3) are at the front of the robot as shown in the photograph below.



3. Load the plates into the hotels so that each plate is set up tightly against the lip of the rear of the plate holder (toward the robot).

Tip! When the plates are correctly placed in the hotels, the well position A1 is located in the front left corner as viewed from the front of the robot.

4. If your plates are loaded with the barcode facing front and A1 in the front left location, then the plates will be scanned and correctly loaded on to the ImageXpress Ultra System plate-loading area.



Objectives Compatible with the ImageXpress Ultra System

A

Table A-1 details the Nikon objectives that are compatible with the ImageXpress® Ultra System, their properties, recommended settings for the beam expander, pinhole, and distance calibration. It also provides plate compatibility information.

Note: You can use oil objectives for research imaging but they are not suitable for screening applications.

Table A-1 Nikon Objectives Compatible with the ImageXpress Ultra System

		Objective Properties		Recommended Settings			Plate Compatibility		
Objective Magnification and Type	Part #	Numerical Aperture	Working Distance	IXU Beam Expander	IXU Pinhole Default	Distance Calibration	Cover Slips (0.17 mm)	Thin-bottom plates (0.17 mm)	Thick bottom plates (0.25 to 1 mm)
4X S Fluor	1-6300-0189	0.20	15.5 mm	3x	4.5 mm	2 µm/pixel	✓	✓	✓
4X Plan Apo	1-6300-0121	0.20	15.7 mm	3x	4.5 mm	2 µm/pixel	✓	✓	✓
10X Plan Fluor	1-6300-0190	0.30	16 mm	2x	4.5 mm	0.8 µm/pixel	✓	✓	✓
10X S Fluor	1-6300-0122	0.50	1.2 mm	3x	4.5 mm	0.8 µm/pixel	✓	✓	✓ (1)
20X Super Plan Fluor ELWD cc 0 to 2 mm	6500-0108	0.45	8.1 to 7.0 mm	2x			✓ (2)	✓ (2)	✓

Table A-1 Nikon Objectives Compatible with the ImageXpress Ultra System (cont'd)

		Objective Properties		Recommended Settings			Plate Compatibility		
Objective Magnification and Type	Part #	Numerical Aperture	Working Distance	IXU Beam Expander	IXU Pinhole Default	Distance Calibration	Cover Slips (0.17 mm)	Thin-bottom plates (0.17 mm)	Thick bottom plates (0.25 to 1 mm)
20X Plan Fluor ELWD cc 0 to 2 mm	1-6300-0123	0.45	8.1 to 7.0 mm	2x	4.5 mm	0.4 µm/pixel	✓ (2)	✓ (2)	✓
20X S Fluor	1-6300-0411	0.75	1 mm	2x	4.5 mm	0.4 µm/pixel	✓	✓	
20X Plan Apo	1-6300-0196	0.75	1 mm	2x	4.5 mm	0.4 µm/pixel	✓	✓	
40X Super Plan Fluor ELWD cc 0 to 2 mm	6500-0109	0.60	3.7 to 2.7 mm				✓ (2)	✓ (2)	✓
40X Plan Fluor ELWD cc 0 to 2 mm	1-6300-0124	0.60	3.7 to 2.7 mm	1x	4.5 mm	0.2 µm/pixel	✓ (2)	✓ (2)	✓
40X Plan Apo cc 0.11 to 0.23 mm	1-6300-0412	0.95	0.14 mm	2x	4.5 mm	0.2 µm/pixel	✓	✓	
40X S Fluor cc 0.11 to 0.23 mm	1-6300-0197	0.90	0.3 mm	2x	4.5 mm	0.2 µm/pixel	✓	✓	
40X Plan Fluor Oil	1-6300-0416	1.30	0.2 mm	2x	4.5 mm	0.2 µm/pixel	✓	✓	

Table A-1 Nikon Objectives Compatible with the ImageXpress Ultra System (cont'd)

		Objective Properties		Recommended Settings			Plate Compatibility		
Objective Magnification and Type	Part #	Numerical Aperture	Working Distance	IXU Beam Expander	IXU Pinhole Default	Distance Calibration	Cover Slips (0.17 mm)	Thin-bottom plates (0.17 mm)	Thick bottom plates (0.25 to 1 mm)
60X Super Plan Fluor ELWD cc 0 to 2 mm	6500-0110	0.70	1.8 to 2.62 mm	1x			✓ (2)	✓ (2)	✓
60X Plan Fluor ELWD cc 0.5 to 1.5 mm (Obsolete December 2008)	1-6300-0389	0.70	2.1 to 1.5 mm	1x	4.5 mm	0.133 µm/pixel			✓ (3)
60X Plan Fluor	1-6300-0414	0.85	0.3 mm	1x	4.5 mm	0.133 µm/pixel	✓	✓	
60X Plan Apo Oil	1-6300-0417	1.40	0.21 mm	1x	4.5 mm	0.133 µm/pixel	✓	✓	
100X Plan Fluor cc 0.14 to 0.20 mm	1-6300-0415	0.9	0.2 mm	1x	4.5 mm	0.08 µm/pixel	✓	✓	
100X Plan Fluor Oil	1-6300-0418	1.30	0.2 mm	1x	4.5 mm	0.08 µm/pixel	✓	✓	

1. Using microplates thicker than 0.3 mm will result in image degradation with the 10X S Fluor objective.
2. The 20X and 40X Plan Fluor ELWD objectives will image through cover slips and thin-bottom plates, but other objectives will give better resolution and higher light throughput.
3. The 60X Plan Fluor ELWD objective works well with plates of 0.5 to 1.5 mm but is not ideal for thick bottom plates of 0.25 mm.

Note: When used with thin-bottom plates, the short working distance of 20X S Fluor, 20X Plan Apo, 40X S Fluor, 40X Plan Apo, 40X Plan Fluor Oil, 60X Plan Fluor, 60X Plan Apo, 100X Plan Fluor, and 100X Plan Apo objectives can cause interference with microplate skirt when imaging edge wells. It is recommended to either omit the edge wells or use a plate with a low skirt.

Selecting a Beam Expander for an Objective

You can estimate the pupil diameter of the Nikon infinite conjugate objectives using the formula below.

Diameter = 2 *NA* objective focal length

2 *NA* (200 mm/objective magnification)

For the 10X Plan Fluor the NA= 0.3

Diameter = 2 *0.3* (200/10) = 12 mm

For the 40X Plan Apo NA = 0.95

Diameter = 2*0.95*5mm = 9.5 mm.

Use the guidelines in Table A-2 to select the appropriate beam expander for an objective. The default beam expander settings set the excitation laser beam diameter to be comparable to or slightly smaller than the objective pupil diameter. This represents a compromise between optical efficiency and axial resolution. Select a lower magnification beam expander to increase optical efficiency. Select a higher magnification beam expander to increase axial resolution.

Table A-2 Beam Expander and Objective

Beam Expander	Objective Pupil Diameter
1x	Up to 9 mm
2x	9 to 15 mm
3x	15 mm and above

ImageXpress Ultra System Specifications

The ImageXpress® Ultra System is designed to operate indoors in laboratory conditions. For optimal performance, site requirements must be met. As with any precision optical instrument, care should be taken to maintain a low-dust, low-vibration environment. Temperature and humidity extremes can compromise performance.

Note: Contact your Molecular Devices Sales Representative or Technical Support for the most current version of the site requirement document.

Table B-1 Site Requirements

Requirement	Description
Environmental Temperature	50° F to 86° F (10°C to 30°C)
Environmental Humidity	5% to 50% non-condensing
Altitude	Up to 1.25 miles (2000 m)
Power Requirements	The ImageXpress Ultra System can be directly connected to all international supply voltages. The input voltage range is from 100 to 240 V~ and input frequency range 50/60 Hz. No range switching is required. Fluctuations must not exceed ± 10% of the nominal voltage. Use the included IEC power cord to connect the external power supply to a grounded power receptacle that is rated for 15 A. The ImageXpress Ultra System requires one to three outlets. If the robot option is purchased, two additional power outlets are required. If using a power strip, do not connect the acquisition computer to the same power strip as the instrument. The acquisition computer and monitor are not necessarily auto switching between input voltages.
Power Consumption	ImageXpress Ultra System power consumption is 1100 watts for two to three seconds at initialization, 800 watts average operating RMS.

Table B-1 Site Requirements (cont'd)

Requirement	Description
Space Requirements	Table or bench top 36 inches (91 cm) deep. There needs to be space below the table for the power supply so that the power cable can easily reach the back of the instrument.
Rear Clearance	The rear of the instrument should be no closer than 6 inches (15 cm) to a wall.
Weight Requirements	The table must be sufficient to support 350 pounds (160 kg) with minimal vibration.

Electromagnetic Compatibility (EMC)

C

REGULATORY INFORMATION FOR CANADA (ICES/NMB-001:2006)

This ISM device complies with Canadian ICES-001.

Cet appareil ISM est confomre à la norme NMB-001 du Canada.

ISM EQUIPMENT CLASSIFICATION (Group 1, Class A)

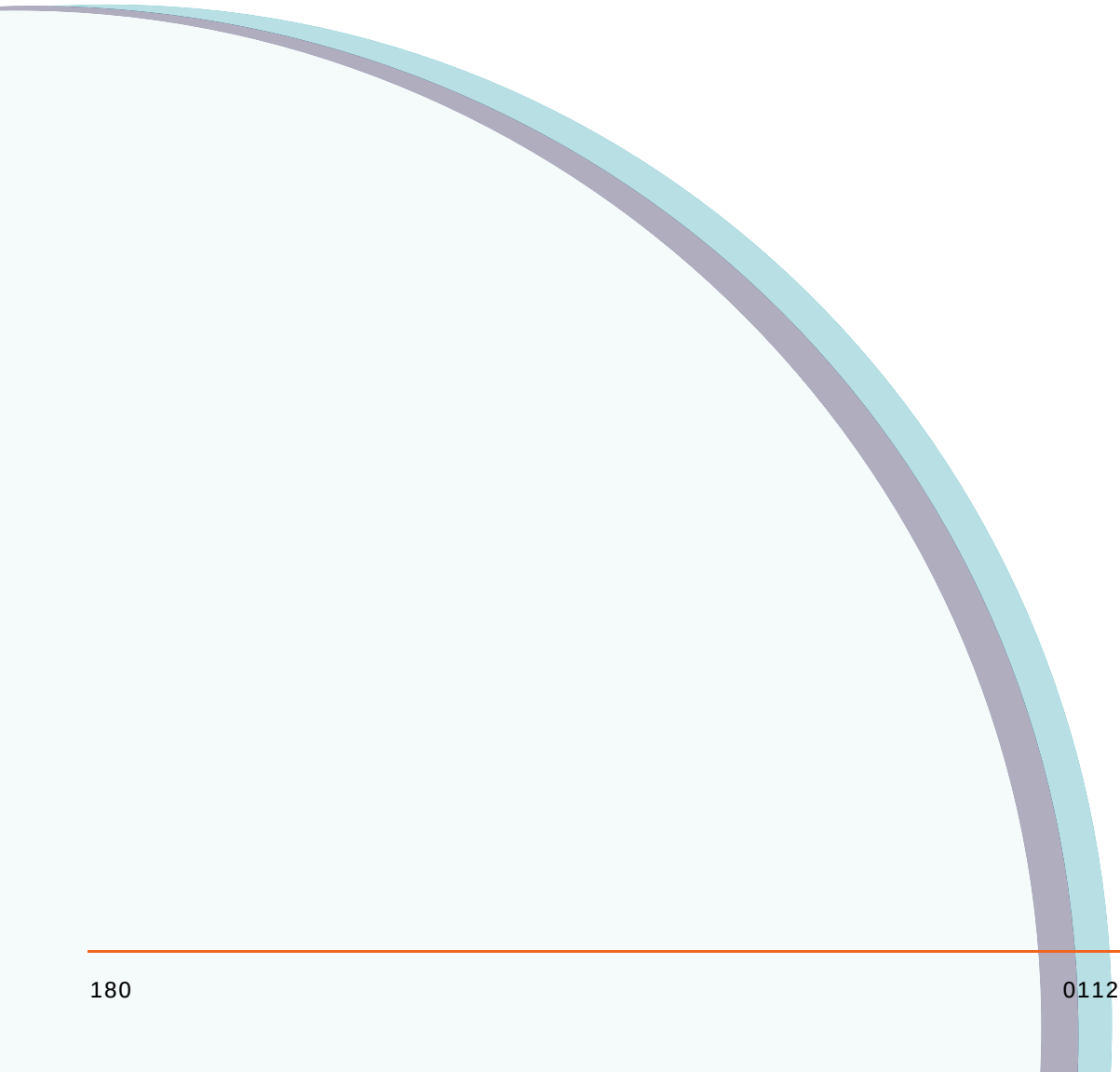
This equipment is designated as scientific equipment for laboratory use that intentionally generate and/or use conductively coupled radio-frequency energy for internal functioning, and are suitable for use in all establishments, other than domestic and those directly connected to a low voltage power supply network which supply buildings used for domestic purposes.

INFORMATION FOR THE USER (FCC NOTICE)

This equipment has been tested and found to comply with the limits for non-consumer ISM equipment, pursuant to part 18 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference in a non-residential installation. This equipment generates, uses, and can radiate radio frequency energy and if not installed and used in accordance with the instructions, may cause harmful interference to radio communications. However, there is no guarantee that interference will not occur in a particular installation. If this equipment does cause harmful interference to radio or television reception, which can be determined by turning the equipment off and on, the user is encouraged to try to correct the interference by one or more of the following measures:

- Reorient or relocate the receiving antenna.
- Increase the separation between the equipment and receiver.
- Connect the equipment into an outlet on a circuit different from that to which the receiver is connected.
- Consult the dealer or an experienced radio/TV technician for help.

In order to maintain compliance with FCC regulations, shielded cables must be used with this equipment. Operation with non-approved equipment or unshielded cables is likely to result in interference to radio and TV reception. The user is cautioned that changes and modifications made to the equipment without the approval of the manufacturer could void the user's authority to operate this equipment.



Index

Numerics

405 nm laser
  applications compatible with system 68
  compatible dyes 68
488 nm laser
  applications compatible with system 70
  dyes compatible with system 70
532 filter set configuration, filter cubes 35
532 nm laser
  applications compatible with system 72
  dyes compatible with system 72
561 filter set configuration, filter cubes 37
561 nm laser
  applications compatible with system 73
  dyes compatible with system 73
635 nm laser
  applications compatible with system 75
  dyes compatible with system 75

A

A1 center, verifying plate reference point 57
acquisition settings
  verifying, using Meta Imaging Series Administrator 50
adding
  experiment information 86
  pre-configured plate types 61
adjusting, correction collar on objectives 165
analysis methods, selecting 129
applications
  405 nm laser compatible dyes 68
  488 nm laser compatible dyes 70
  532 nm laser compatible dyes 72
  561 nm laser compatible dyes 73
  635 nm laser compatible dyes 75
  for experiments 68
applying, oil to objectives 167
autofocus laser, red diode 38
autofocus settings, configuring 118
averaging
  binning, combined 89
  plate acquisition 89
AxoTrace messages, logging 23

B

backing up
  calibration settings, MetaXpress Software 53
  magnification settings, MetaXpress Software 52
beam combiners
  imaging optics 30
beam expander
  purpose 30
  selecting for objective 176
  verifying settings 46
beam splitters
  standard configuration 31
  verifying settings 49

binning and averaging, combined 89
binning, configuring 87

C

calibration settings
  backing up using MetaXpress Software 53
  verifying in MetaXpress Software 53
cell counting, configuring on plates 100
changing
  binning settings 89
  filter cubes, maintenance 135
  objectives 141
  scan lines settings 89
cleaning
  instrument 168
  objectives 164
components
  imaging optics 29
  system 19
configuring
  autofocus settings 118
  binning settings 87
  display settings, plate acquisition 127
  laser autofocus 118
  lasers for scans 121
  number of scan lines 87
  objective offsets 145
  parfocality 145
  protocol settings 115
  sites to visit on plate 96
  wells to visit on plate 95
confirming
  laser autofocus response 54
  laser autofocus settings, plate files 62
confocal imaging 27
correction collar, adjusting on objectives 165
creating
  experiments 83
  experiments, using existing settings files 84
CRS Catalyst Express Robot
  loading plates 169
  preventing, improper loading of plates 170
  user procedures 169

D

display settings, configuring for plate acquisitions 127
dyes
  405 nm laser compatible with system 68
  488 nm laser compatible with system 70
  532 nm laser compatible with system 72
  561 nm laser compatible with system 73
  635 nm laser compatible with system 75
  for experiments 68

E

electromagnetic compatibility 179
electronics, instrument 29
EMC 179
emission filters, filter cubes 33
excitation lasers
  imaging optics 29
  optional 30
  standard 30

experiments
adding information 86
applications 68
creating 83
creating using existing settings files 84
dyes 68
fluorophores 68
laser power 65
objectives, choosing 67
planning 63
sample preparation 65

F

filter cubes
532 filter set configuration 35
561 filter set configuration 37
changing 135
comparison with eipfluorescent filter tubes 34
emission filters 33
standard configuration 35
verifying settings 47
fluorescence imaging 27
fluorophores 68
fuses, safety information 16

G

galvanometer 30
generating, shading correction files 105

H

hazardous material precautions 17
high voltage, safety information 16
high-content experiments, planning 63

I

images
setting multiple timepoints 102
troubleshooting poor quality 64
ImageXpress System installation 20
imaging optics
beam combiners 30
beam splitters 31
components 29
emission filters 33
galvanometer 30
motorized iris 32
objective lenses 32
photomultipler tubes 37
relay lens 31
tube lens 32
installing, ImageXpress System 20
instrument
cleaning 168
electronics 29
features 19
non-interlocked panels 14
overview 19
protective housing 13
safety information 10
safety label 11
schematic 28

J

journals, running for plate acquisitions 125

L

laser autofocus
  configuring 118
  confirming response 54
  confirming settings 62
laser focus, plate acquisition 94
laser power
  experiments 65
  samples, finding optimal 65
lasers
  configuring for scans 121
  incompatible probes 76
  safety information 14
lifting hazard, safety information 17
loading plates
  CRS Catalyst Express Robot 169
  preventing improper loading 170
logging, AxoTrace messages 23

M

magnification settings, backing up using MetaXpress Software 52
maintenance
  adjusting correction collar on the objectives 165
  applying oil to objectives 167
  changing objectives 141
  cleaning the instrument 168
  cleaning the objectives 164
  filter cubes, changing 135
  limitations for users 17
  user safety instructions 135
Meta Imaging Series Administrator
  device settings, verifying 39
  verifying, acquisition settings 50
MetaXpress Software
  backing up, calibration settings 53
  backing up, magnification settings 52
  starting 22
  verifying, calibration settings 53
motorized
  iris 32
  objective (Z) stage 37
  objective changer 37
motorized X-Y stage, sample X-Y stage 38
moving parts, safety information 16

N

Nikon objectives, compatible with system 173

O

objective (Z) stage
  motorized 37
  motorized objective changer 37
objective lenses 32
objective offsets
  configuring 145
  values 159

objectives
adjusting, correction collar 165
applying oil 167
changing 141
cleaning 164
correct placement 141
experiments, choosing 67
Nikon, compatible with system 173
numerical aperture 67
oil immersion 167
selecting beam expander 176
verifying settings 44
oil immersion objectives 167
optical thickness 94
optimal laser power, samples 65

P

panels, non-interlocked 14
parfocality
configuring 145
values 159
Photomultiplier Tubes (PMT) 37
pinhole settings
verifying 46
placing objectives correctly 141
planning, high-content experiments 63
plate acquisition
averaging 89
binning 87
combined binning and averaging 89
configuring binning settings 87
configuring laser autofocus 118
configuring lasers for scans 121
configuring plates 91
configuring scan lines 87
configuring, cell counting 100
creating experiment, using existing settings file 84
creating experiments 83
display settings, configuring 127
experiment information 86
journals, running 125
optical thickness 94
PMT gain overview 114
protocol settings 105
reviewing settings 131
running 25
scan lines 89
screening menu, MetaXpress Software 80
setting multiple timepoints 102
setting up 25
shading correction images, using 62
sites to visit, configuring 96
wells to visit, configuring 95
Plate Acquisition and Control dialog box, overview 81
Plate Acquisition Setup dialog box, overview 82
plate acquisitions
settings, saving 133
starting 132
plate clamp, sample X-Y stage 38
plate files, confirming laser autofocus settings 62
plate holder, sample X-Y stage 38
plate reference point, verifying A1 center 57

plate settings files, creating experiments 84
plate types
  adding, pre-configured 61
  guidelines for selecting 77
  verifying 61
plates, configuring 91
PMT gain, plate acquisition 114
PMTs, operational voltage range 114
power supply
  safety label 12
power supply, voltage range 16
pre-configured plate types, adding 61
probes
  compatible with lasers 68
  incompatible with lasers 76
protective housing, instrument 13
protocol settings
  configuring 115
  plate acquisition 105

R

red diode laser, autofocus 38
relay lens, imaging optics 31
reviewing, plate acquisition settings 131
robot plate handling, CRS Catalyst Express hardware 169

S

safety information
  fuses 16
  hazardous material precautions 17
  high voltage 16
  instrument 10
  lasers 14
  lifting hazard 17
  moving parts 16
  power supply 16
safety instructions, maintenance tasks 135
safety label
  instrument 11
  power supply 12
sample preparation, experiments 65
sample X-Y stage
  motorized X-Y stage 38
  plate clamp 38
  plate holder 38
  sample 38
samples
  optimal laser power, finding 65
saving, plate acquisition settings 133
scan lines
  configuring settings 87
  plate acquisition 89
scans, configuring lasers 121
schematic, instrument 28
screening menu, plate acquisition 80
selecting
  beam expander for objective 176
  data analysis methods 129
  plate types 77
setting up, plate acquisition 25
settings, plate acquisition 131
shading correction files
  generating 105
  verifying 62

site requirements, system specifications 177
sites to visit
  examples 98
  plate, configuring 96
standard configuration, filter cubes 35
starting
  MetaXpress Software 22
  plate acquisitions 132
system
  installing 20
  main connections 20
  turning off 25
  turning on 21
system specifications, site requirements 177

T

timepoints, setting to acquire images 102
troubleshooting
  poor quality images 64
tube lens, imaging optics 32
turning off, system 25
turning on, system 21

U

user safety instructions, maintenance tasks 135

V

verifying
  acquisition settings, Meta Imaging Series Administrator 50
  beam expander settings 46
  beam splitter settings 49
  calibration settings, MetaXpress Software 53
  device settings, Meta Imaging Series Administrator 39
  filter cube settings 47
  objective settings 44
  pinhole settings 46
  plate reference point, A1 center 57
  plate types 61
  shading correction files 62
  X Y Z settings 40
voltage range
  PMTs 114
  power supply 16

W

warning labels 13
wells to visit, configuring plates 95

X

X Y Z settings, verifying 40