M487E 10.10.NF.12 (3/3)

Nikon

NIS-Elements C

(For CONFOCAL MICROSCOPE C2/C2si)

Instructions

(Ver. 3.22)

Preface

Thank you for purchasing the Nikon products.

This instruction manual has been prepared for the users of the Camera Settings function of Nikon NIS-Elements.

To ensure correct usage, read this manual carefully before operating the instrument.

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- The contents of this manual are subject to change without notice.
- Although every effort has been made to ensure the accuracy of this manual, if you note any points that are unclear or incorrect, contact your nearest Nikon representative.
- Be sure to read the manuals for any other products that you are using with this product.
- Usage in a way not specified by the manufacturer may impair the product safety.
- Reference spectrum data of dyes on NIS-Elements are provided from Invitrogen Corporation / Molecular Probes Clontech Laboratories, Inc.

Invitrogen Corporation	http://www.invitrogen.com/
Clontech	http://www.clontech.com/

- The images of specimens as shown in this document are for reference only, and may appear somewhat different from those actually acquired.

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1 Before You Use

The C2 Settings window is used as a function of NIS-Elements. It cannot be used alone.
This section describes the starting/shutdown and structure of the C2 Settings window.

1.1 Starting and Shutting Down the C2 Settings Window

The C2 Settings window starts automatically when NIS-Elements starts.
Likewise, it automatically shuts down when NIS-Elements shuts down.

1.1.1 Starting the C2 Settings Window

Double-click the NIS-Elements icon.

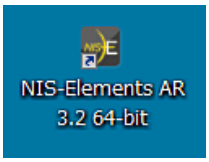


Figure 1.1-1 NIS-Elements icon

The NIS-Elements title window appears.
Then, the title window closes and NIS-Elements starts.
As NIS-Elements starts, the C2 Settings window starts automatically as well.

Figure 1.1-2 Title window

The [Driver selection] dialog box appears on the desktop before the C2 Settings window opens.

* If only one camera is installed, the camera is automatically selected and the [Driver selection] dialog box is not displayed.

To use the regular C2 Confocal system, select “Nikon Confocal.”

* To use the C2+TIRF system, check the [Enable Multi Camera] check box and select both Nikon Confocal and ANDOR. (see Chapter 12)

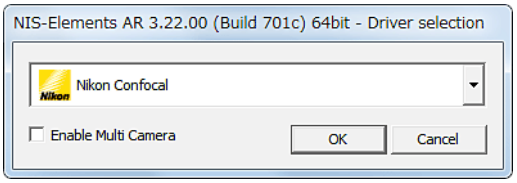


Figure 1.1-3 Driver selection dialog box

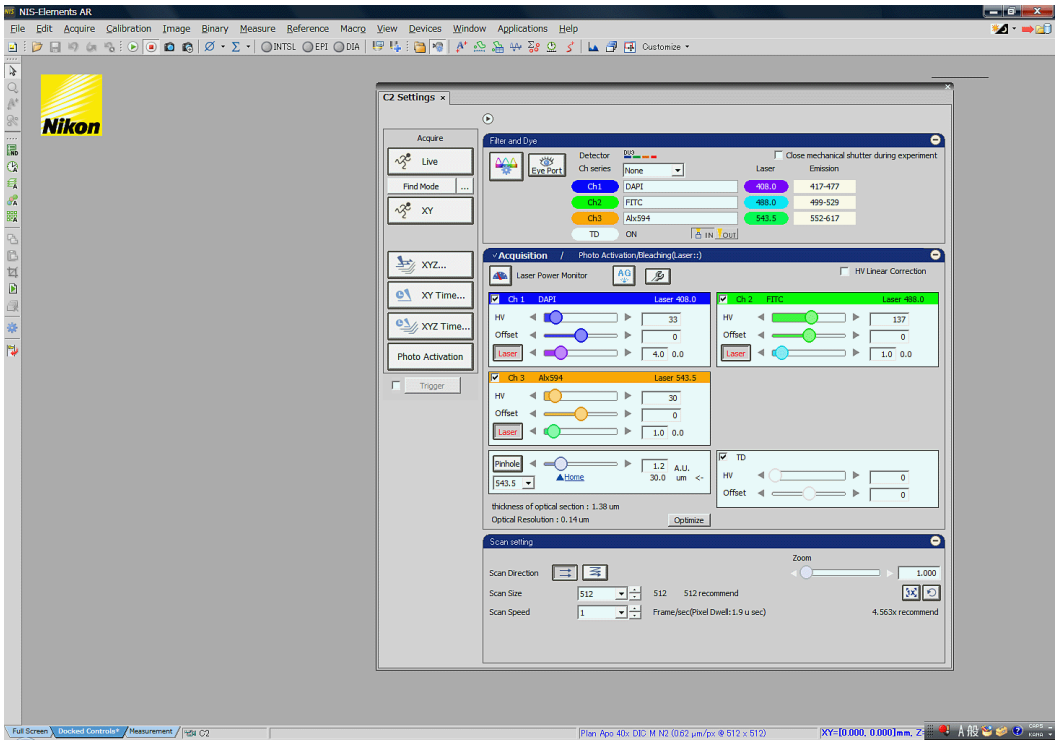


Figure 1.1-4 Initial NIS-Elements window and the C2 Settings window

1.1.2 Shutting Down the C2 Settings Window

The C2 Settings window automatically shuts down when NIS-Elements shuts down.

The layout of the C2 Settings window is memorized when it shuts down.

1.2 Structure of C2 Settings Window

The C2 Settings window enables to apply various settings, including the laser, adjusting the brightness of the image, the photo activation setting, and scan resolution/speed, to use the Confocal Microscope.

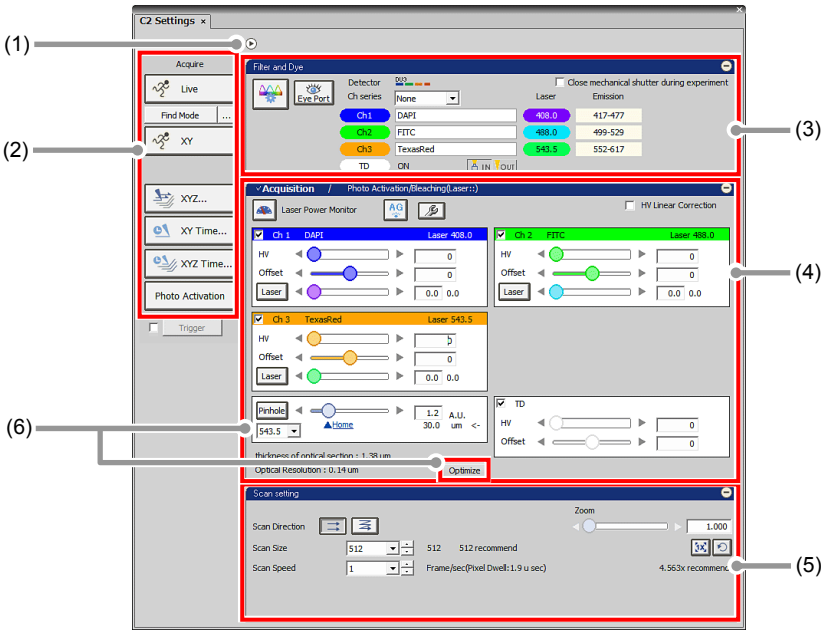


Figure 1.2-1 C2 Settings window

Table 1.2-1 Summary of C2 Settings window functions

Name		Function
(1)	Acquire window display/ nondisplay selection	Switches display/nondisplay of the Acquire window.
(2)	Acquire window	Enables to display live images, to acquire images (see Chapter 4) or to apply the photo activation settings (see Chapter 10). The functions available with NIS-Elements are arranged as buttons in this area.
(3)	Filter and Dye window	Enables to select the channel series to be used and set the optical path. (See “Filter and Dye” in the chapters concerning detector modes.)
(4)	Acquisition / Photo Activation window	The Acquisition window enables to set PMT brightness, laser power, and pinhole size. (See “Acquisition” in the chapters concerning detector modes.)
		The Photo Activation window enables to set the desired photo activation laser power. (see Chapter 10)
(5)	Scan setting window	Enables to set a scan method, resolution, scan speed, etc. (see Chapter 8)
(6)	Optimize button	Calculates the recommended value of resolution, zoom magnification, and Z stack step size based on the objective type and the selected excitation wavelength, and the indication/automatic application function can be set in detail.

2 Optical Path Changeover for C2 Scan Head

This chapter describes the optical path changeover for the C2 scan head.

2.1 Optical Path Changeover Lever for C2 Scan Head

Confocal Microscope C2 illuminates the specimen with the laser light (excitation light) transmitted from the laser unit, and detects the fluorescence from the specimen by the detector unit.
The detector unit to be used for the detection can be selected from the two types: the Standard Detector unit with a filter, or the Spectral Detector unit with a diffraction grating for the spectral function.
To select which detector the fluorescence from the specimen is transmitted to, use the optical path changeover lever on the C2 scan head.

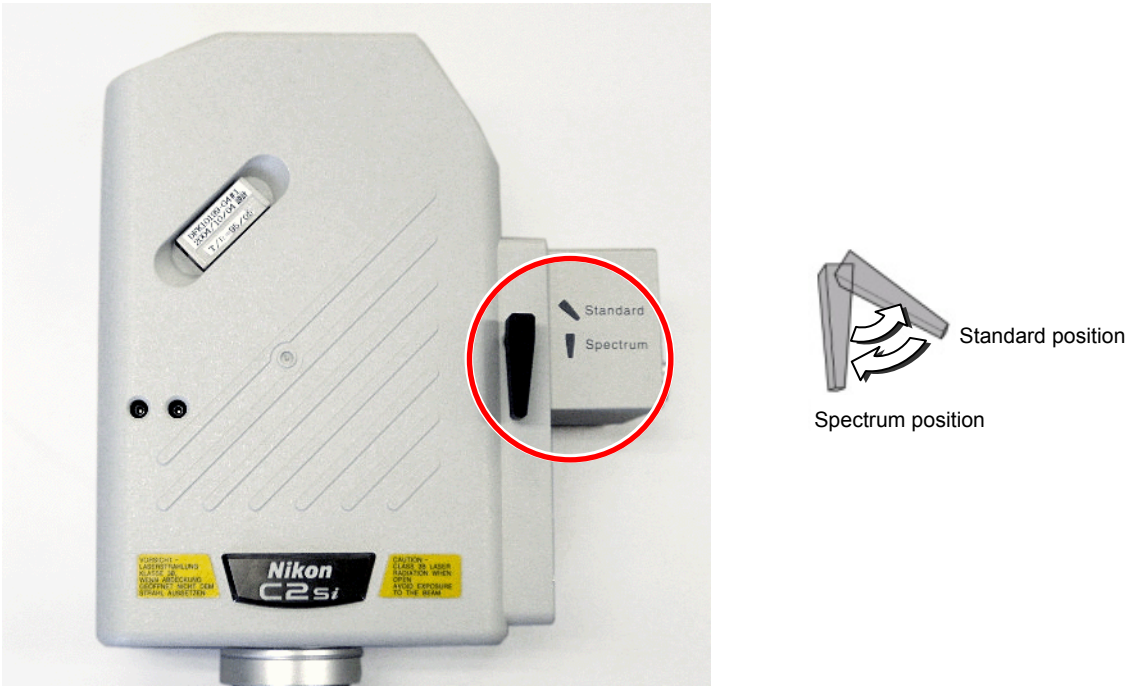


Figure 2.1-1 Optical path changeover lever for C2 scan head

Table 2.1-1 Overview of detector unit function

Lever position	Overview
Standard (Oblique)	Switches to Standard, and leads the fluorescence light from the pinhole to the Standard Detector unit, which has a filter. When this is selected, NIS-Elements C enters the “Standard Detector mode” in which the 3 channel images at maximum are acquired by using the filter.
Spectrum (Vertical)	Switches to Spectrum, and leads the fluorescence light from the pinhole to the Spectral Detector unit, which has a diffraction grating. When this is selected, either of the following two detection modes can be selected for NIS-Elements C: “Spectral Detector mode” in which the 32 channel images at maximum are acquired by the spectral function, or “Virtual Filter mode” in which 4 virtual channel images by four excitations at maximum can be acquired.

2.2 Detection Mode When Switching the Optical Path

This section describes the detection mode indication when the optical path is switched by the optical path changeover lever on the C2 scan head.

Table 2.2-1 Mode indication when the optical path is switched

Switching operation by the lever	Mode change
Standard ↓ **Spectrum**	The optical path is switched to the Spectral Detector unit side, and the Spectral Detector mode (SD) is used as the detection mode of the NIS-Elements C. For using the Virtual Filter mode (VF), select [VF] in the Optical path window to switch the detection mode. The last settings of the detection mode are recalled.
Spectrum ↓ **Standard**	The optical path is switched to the Standard Detector unit side, and the Standard Detector mode (DU3) is used as the detection mode of the NIS-Elements C. The Standard Detector mode (DU3) can be switched to directly from either the Spectral Detector mode (SD) or the Virtual Filter mode (VF). The last settings of the detection mode are recalled.

3 Basic Operations

This chapter describes the basic instructions for acquiring live images in the C2 Settings window.

Switch beforehand the optical path of the C2 scan head appropriately.
When selecting the Standard Detector mode [DU3] as the detection mode, set the optical path changeover lever on the C2 scan head to the [Standard] position.
When selecting the Spectral Detector mode [SD] or the Virtual Filter mode [VF], set the optical path changeover lever on the C2 scan head to the [Spectrum] position.

3.1 Acquiring the Live Image and Setting the Scan Area

1 Setting the Optical path

1. Display the Optical path window.
   Click the [Setting] button in the Filter and Dye window.
   For details of the Optical path settings, See “Filter and Dye” in the chapters concerning detection modes.

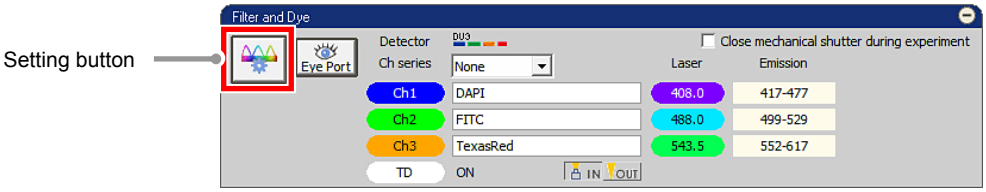


Figure 3.1-1 Filter and Dye window

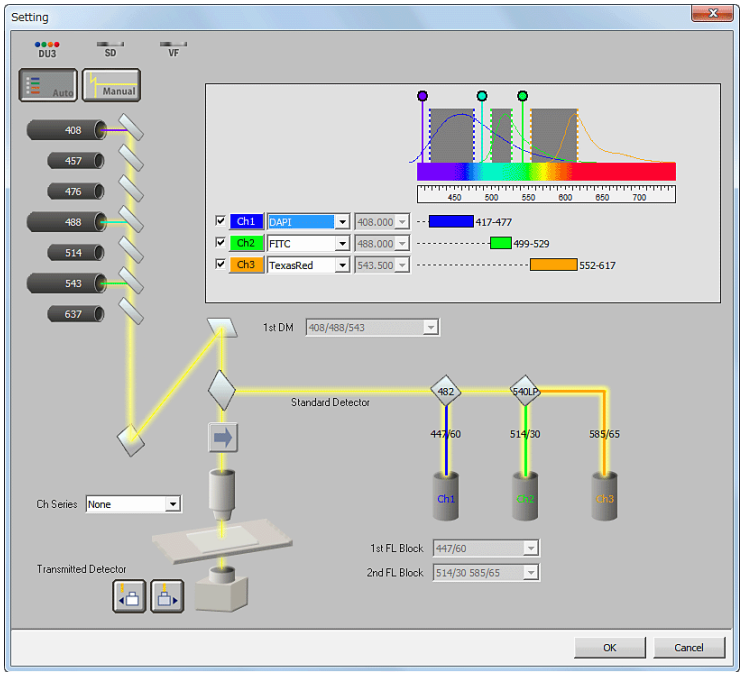


Figure 3.1-2 Optical path window

2. Select the detection mode (detector).
   (This step is not needed if the Standard Detector mode [DU3] or the Spectral Detector mode [SD] is used, as they are automatically selected as the detection mode when the optical path changeover lever on the C2 scan head is switched.)

   If the Virtual Filter mode [VF] is to be used, switch the optical path changeover lever on the C2 scan head to the [Spectrum] position, and then select the [VF] button in the Optical path window.

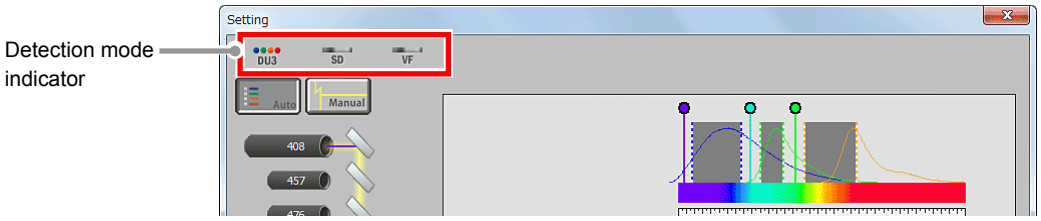


Figure 3.1-3 Displaying the Detection mode (when Standard is selected by the optical path changeover lever)

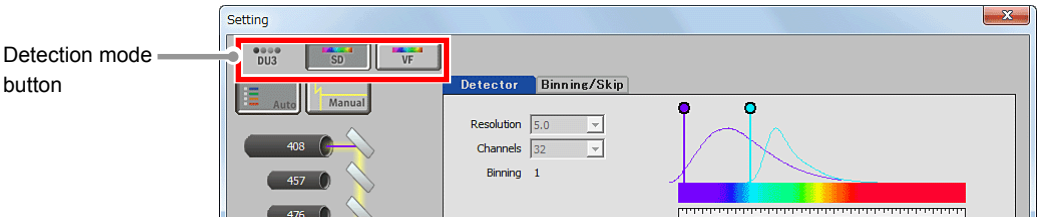


Figure 3.1-4 Selecting the Detection mode (when Spectrum is selected by the optical path changeover lever)

3. Activate the automatic mode of Optical path setting.
   Click the [Auto] button.

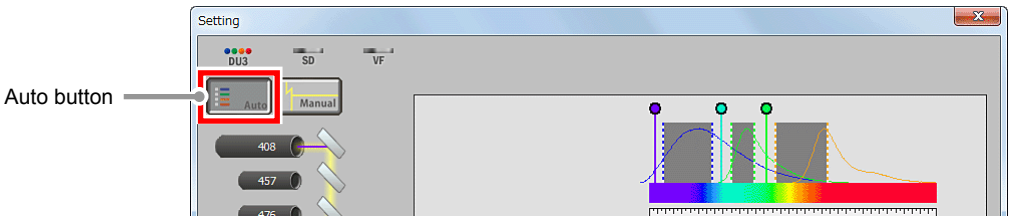


Figure 3.1-5 Selecting the auto mode

4. Select the fluorescence dyes for the channels to be used.
   For each channel to be used, select a fluorescence dye from the pull-down menu.
   Once a fluorescence dye is selected, appropriate laser and dichroic mirror are automatically selected.

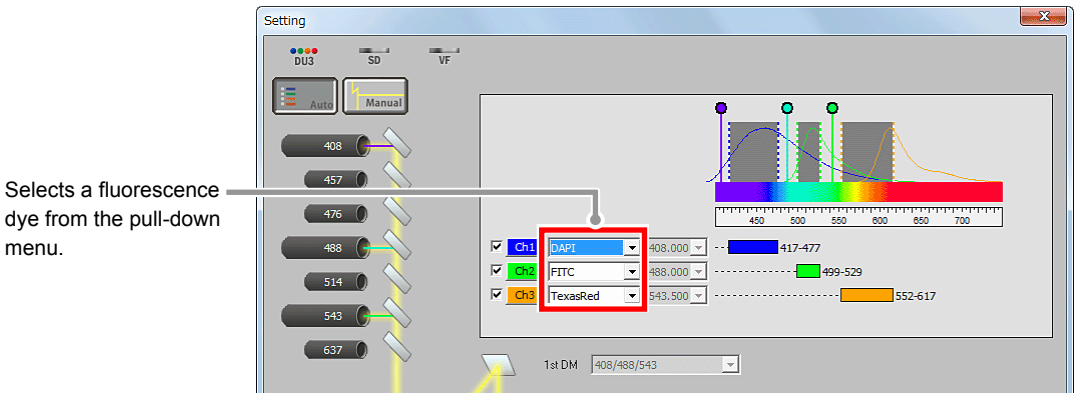


Figure 3.1-6 Selecting fluorescence dyes

5. Select the channels to be used.
   Check the check box for each channel to be used.

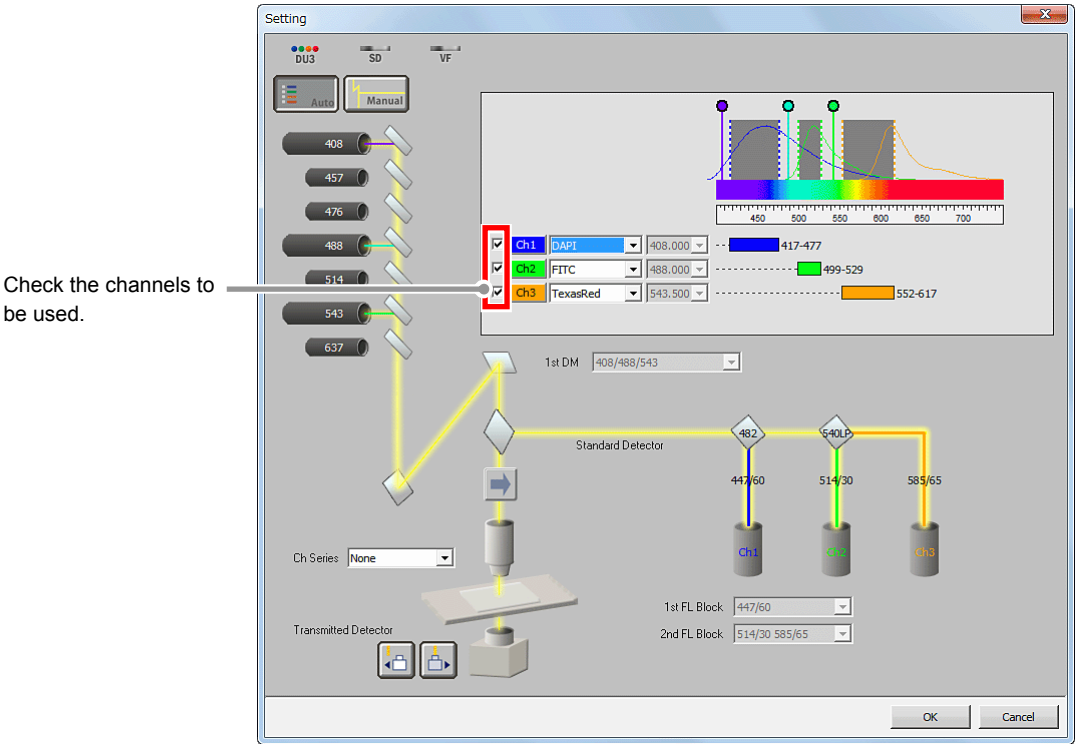


Figure 3.1-7 Selecting channels

6. Select the desired icon to use or disuse the transmitted detector.

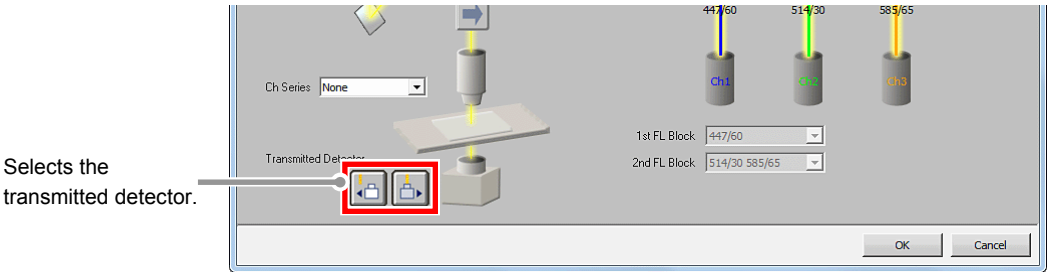


Figure 3.1-8 Selecting the transmitted detector

7. Determine the Optical path settings.
   Click the [OK] button.
   The Optical path settings are determined, and the Optical path window closes.

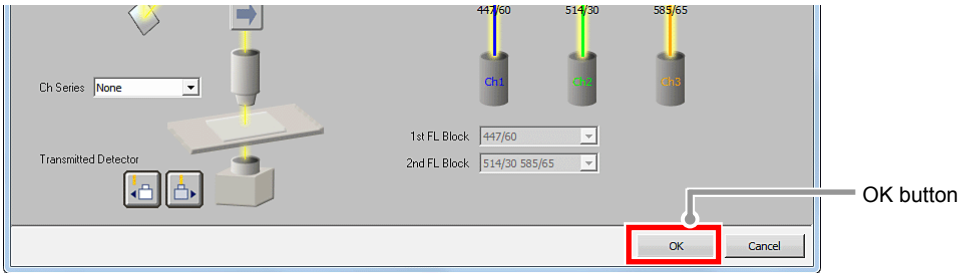


Figure 3.1-9 Determining the Optical path settings

2 Applying Scan settings

In the Scan setting window, apply various scan settings to acquire the live image.
For details of Scan settings, see Chapter 8, “Scan Setting Window.”

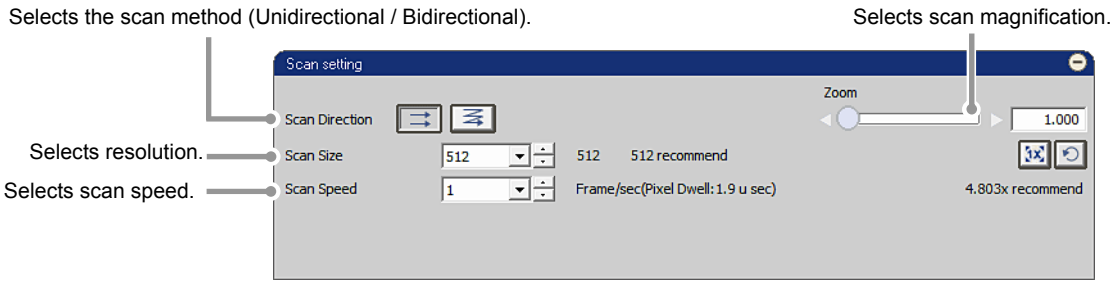


Figure 3.1-10 Scan setting window

3 Acquiring the live image

Click the [Live] button.
The live image is acquired and the Live window appears.

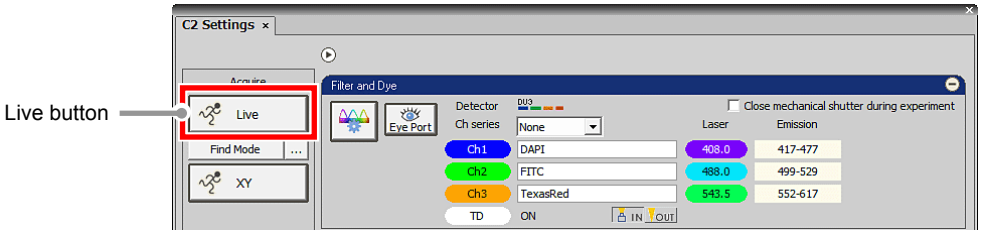


Figure 3.1-11 Acquiring the live image

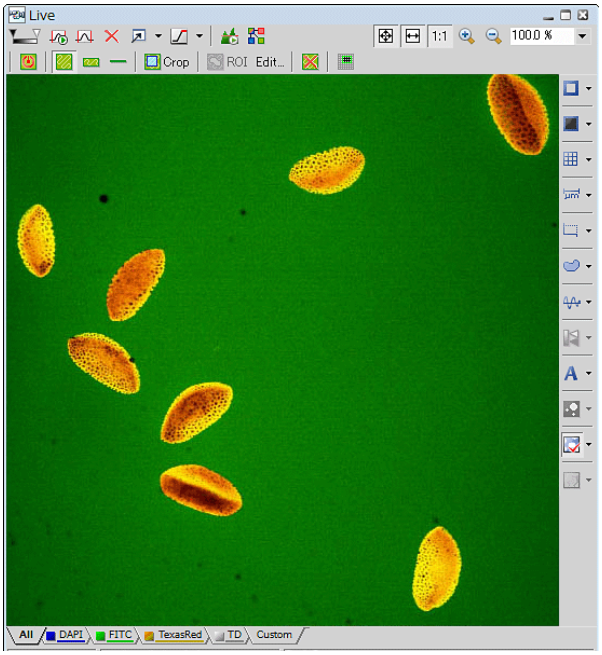


Figure 3.1-12 Live window

4 Adjusting the brightness of the live image

In the Acquisition window, adjust the brightness of the live image for each channel.
See “Acquisition window” in the chapters concerning detection modes.

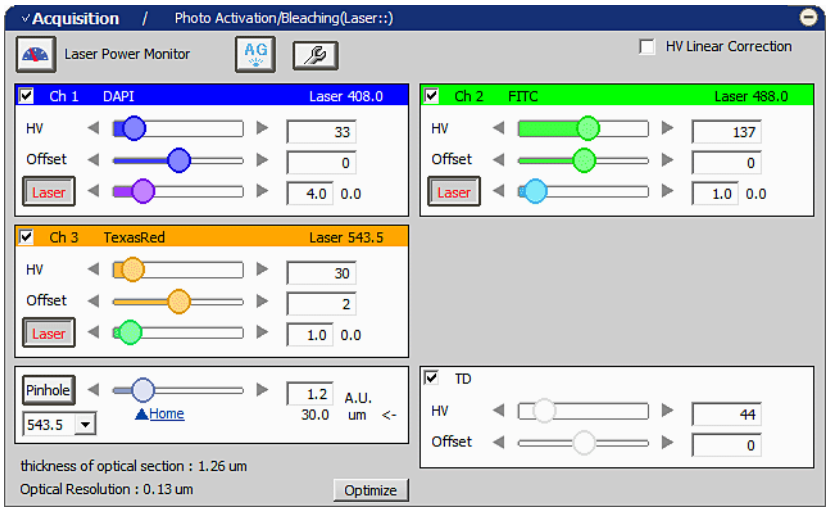


Figure 3.1-13 Acquisition window

5 Setting the scan area

Set the scan area for the acquired live image.
For details of the scan area, see Chapter 9, “Navigation Mode.”

1. Switch the Live window to the navigation mode.
   Click the [Show Scan Area] button in the Live window.

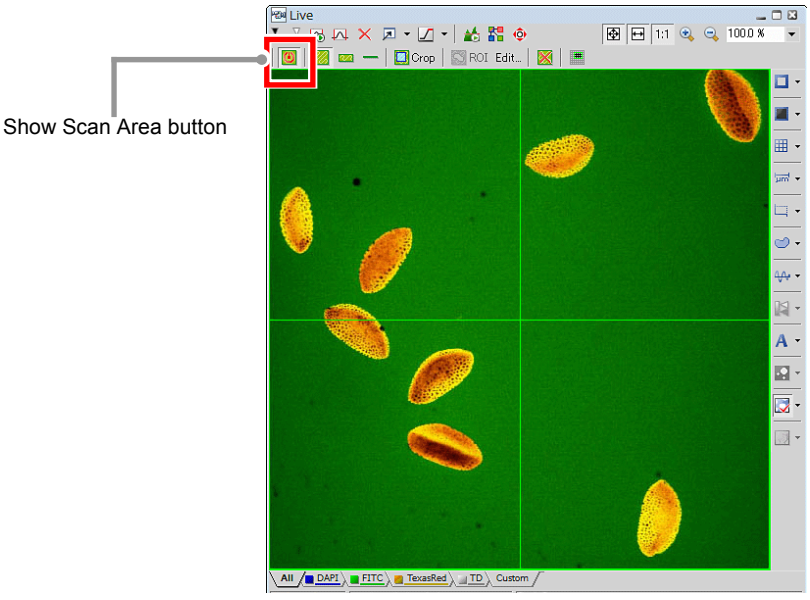


Figure 3.1-14 Switching to navigation mode

2. Select the scan area setting tool to be used.
   The scan area setting tools differ in their available shapes depending on the scan area selected.

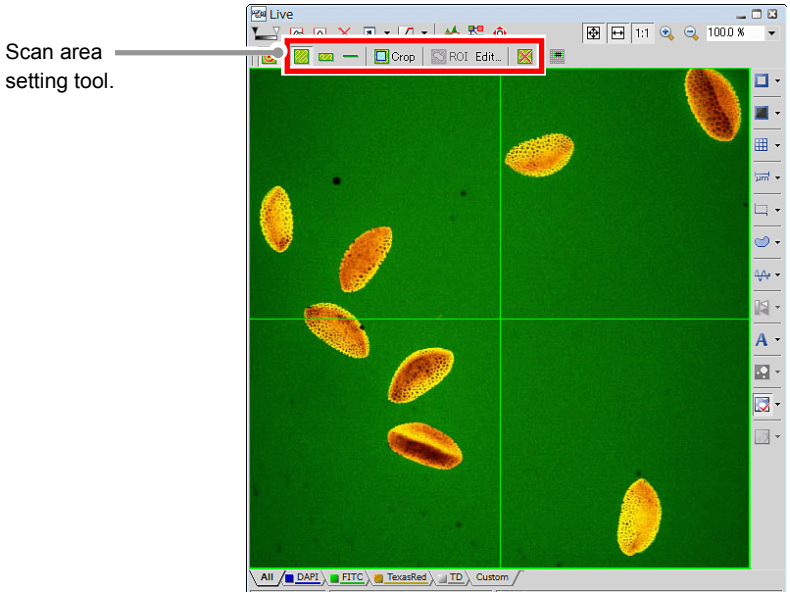


Figure 3.1-15 Selecting the scan area setting tool

3. Set the scan area with the tool selected.
   For instructions on selecting and using scan area setting tools, see Section 9.3.2, “Scan Area Setting Tools.”

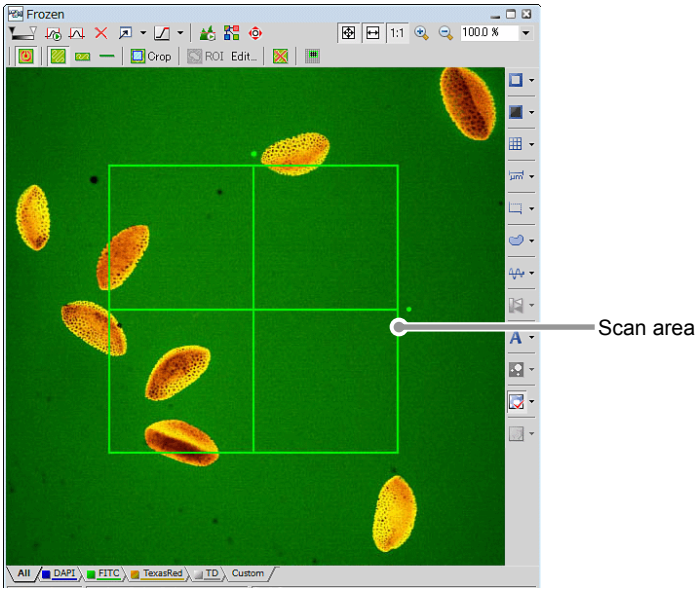


Figure 3.1-16 Setting the scan area

6 Acquiring the image of the set scan area

1. Right click on the drawn scan area.

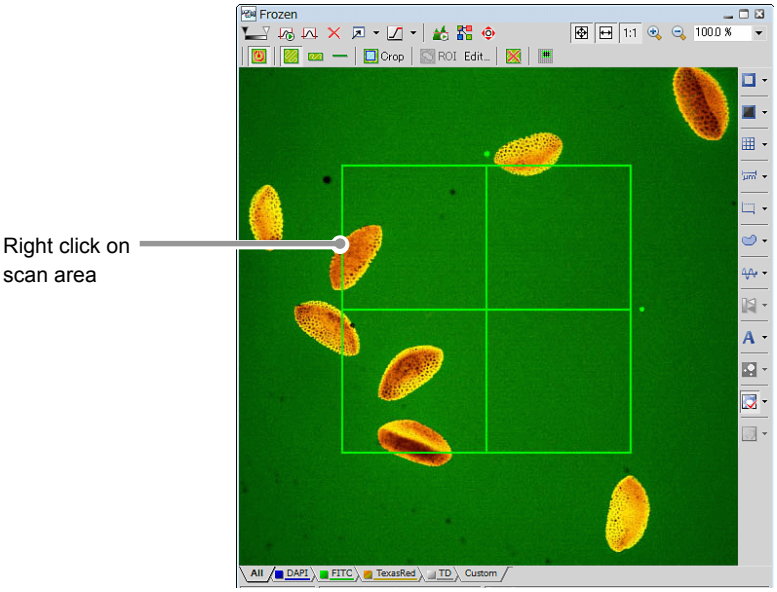


Figure 3.1-17 Acquiring the live image of scan area

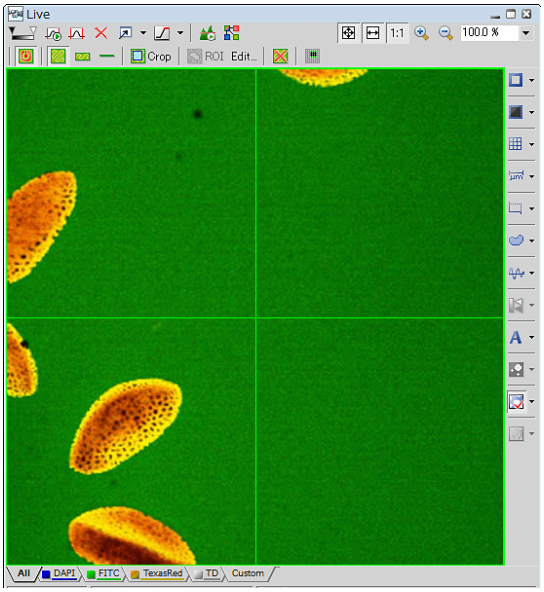


Figure 3.1-18 Live window after changing the scan area

* While working with Frozen image, the live image in the set scan area can also be acquired by clicking the [Live] button.

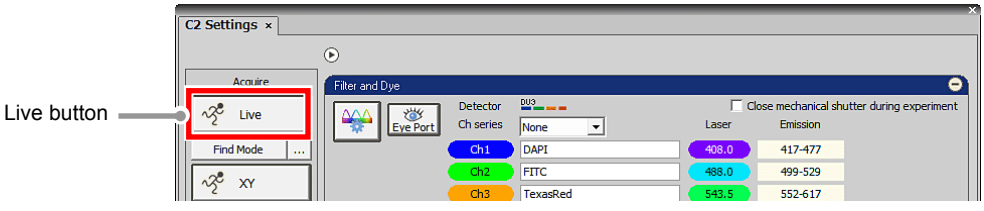


Figure 3.1-19 Acquiring the live image

- **Laser Interlocked**

  Indicates the interlock status of the microscope main unit.

  If the optical path of the microscope main unit is switched to the binocular system, all of the laser shutters close for safety purpose.
  At this time, the [Laser InterLocked] button blinks and the confocal image acquisition cannot be executed.

  When you execute the confocal image acquisition again, switch the optical path of the microscope main unit to "Confocal", and then click this [Laser InterLocked] button.

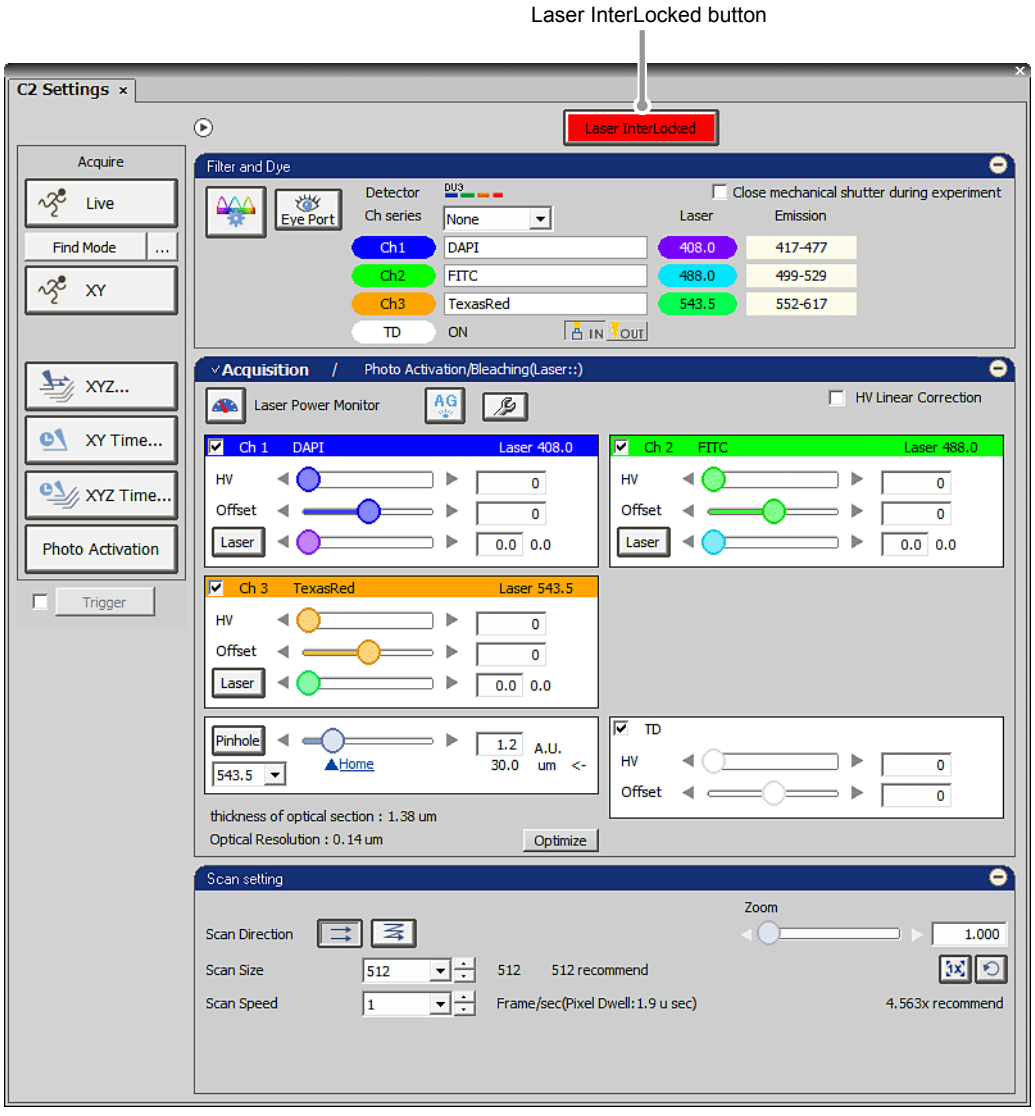


Figure 3.1-20 Laser InterLocked indicator

4 Acquire Window

The Acquire window enables to display the live image, acquire the image or apply the photo activation settings. Additionally, the functions available with NIS-Elements are arranged as buttons in this area.

4.1 Functions of Acquire Window

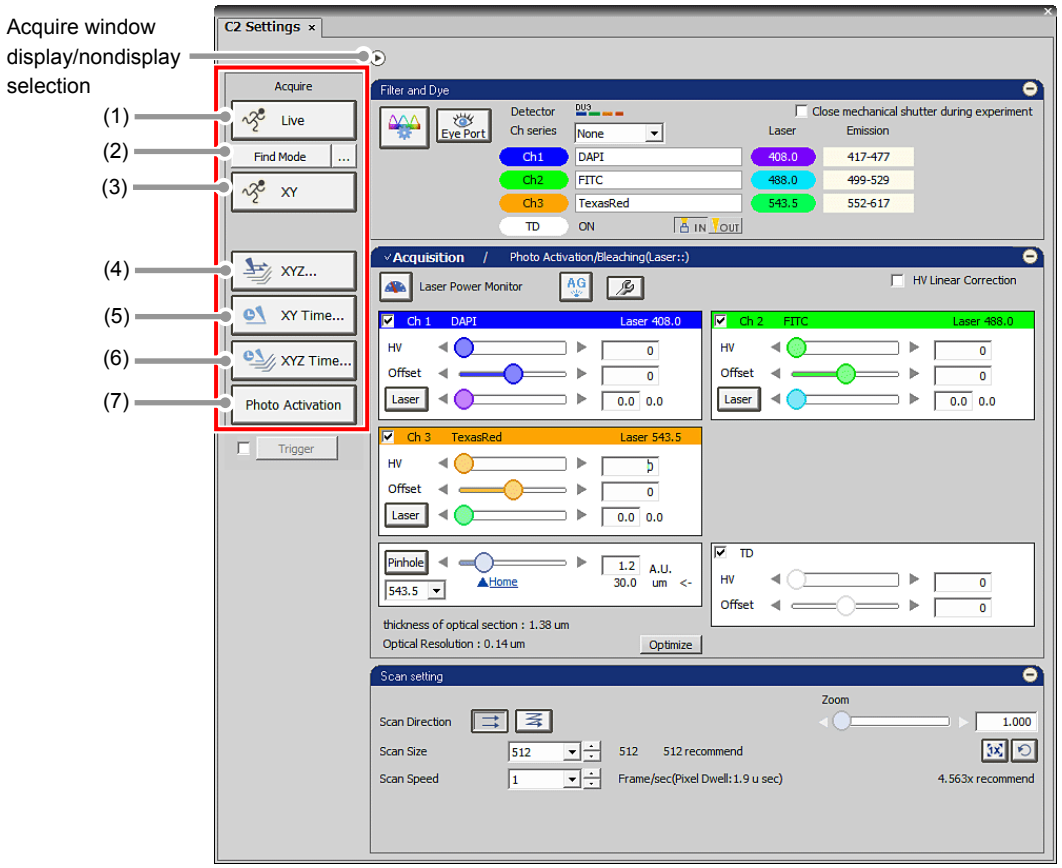


Figure 4.1-1 Acquire window

Table 4.1-1 Functions of Acquire window (sheet 1/3)

Name		Function	
(1)	Live button	Enables to display the live image. The Live window opens and displays the live image automatically. The live image is the real-time image that is currently observed with the microscope.	
(2)	Find Mode	Find Mode	Starts/stops live image acquisition in Find mode. Find mode is the mode where the live image acquisition is executed by temporarily switching to the high-frame-rate setting in order to ease the detection of the observation object such as a cell.
		...	Opens the [Find mode settings] dialog box. For details of the Find mode, See Section 4.1.2, “Find mode.”
(3)	XY button	Enables acquiring the captured image of the currently displayed live image. When the captured image is acquired, the Captured window appears separately from the Live window. The captured image is a still image that is acquired by re-scanning the scan area displayed in the current Live window.	

Table 4.1-1 Functions of Acquire window (sheet 2/3)

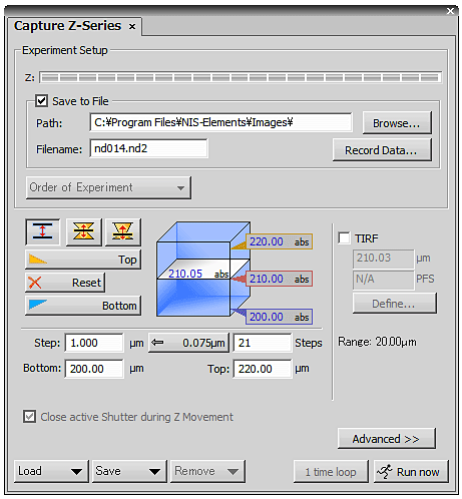
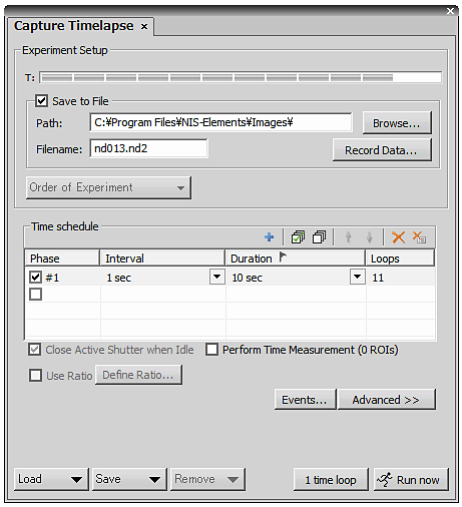
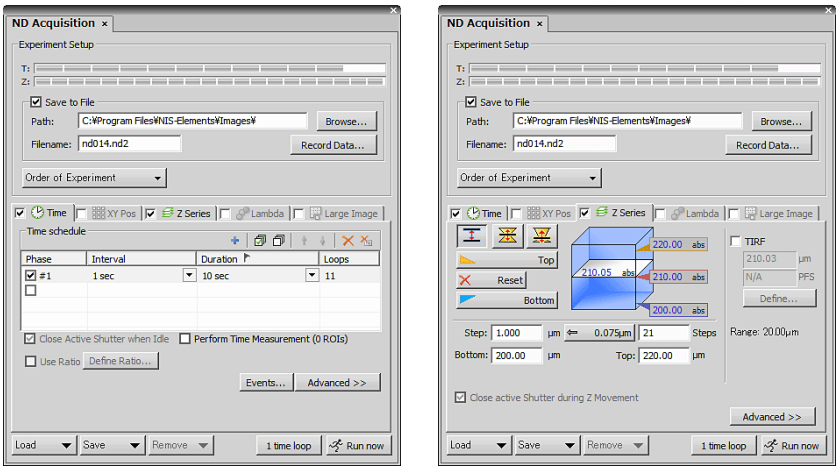
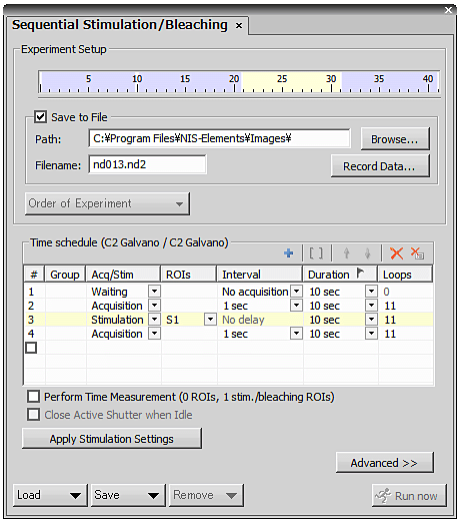
Name		Function
(4)	XYZ button	Opens the [Capture Z-Series] dialog box. Enables to acquire a three-dimensional (X-Y-Z) image. For operations of this dialog box, refer to “NIS-Elements Advanced Research User’s Guide.”  **Figure 4.1-2 Capture Z-Series dialog box** The same dialog box can also be opened with the following procedure: - Select [Acquire] on the menu bar and then select [Capture Z-Series] -> [Capture Automatically...] in this order.
(5)	XY Time button	Opens the [Capture Timelapse] dialog box. Enables to acquire two-dimensional (X-Y) images in time series. For operations of this dialog box, refer to “NIS-Elements Advanced Research User’s Guide.”  **Figure 4.1-3 Capture Timelapse dialog box** The same dialog box can also be opened with the following procedure: - Select [Acquire] on the menu bar and then select [Capture Timelapse] -> [Capture Automatically...] in this order.

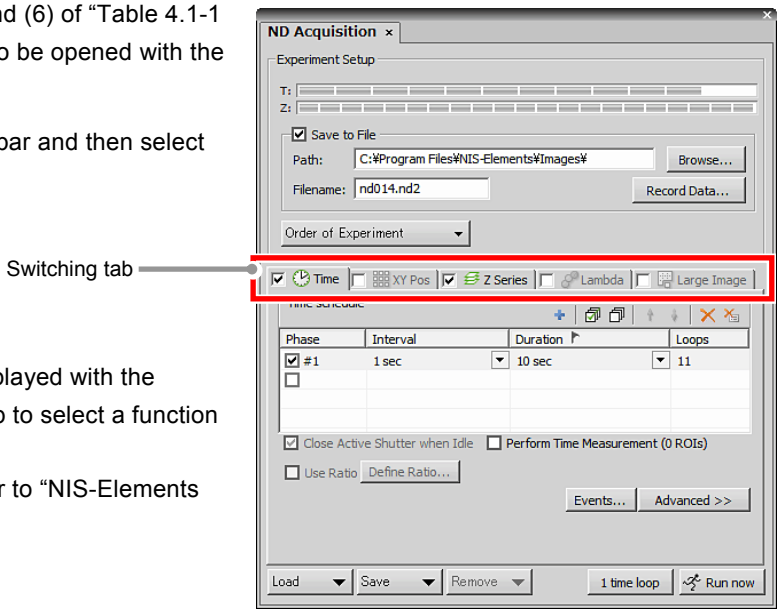
Table 4.1-1 Functions of Acquire window (sheet 3/3)

Name		Function
(6)	XYZ Time button	Opens the [ND Acquisition] dialog box. Enables to acquire three-dimensional (X-Y-Z) images in time series. For operations of this dialog box, refer to “NIS-Elements Advanced Research User’s Guide.”  **Figure 4.1-4 ND Acquisition dialog box**
(7)	Photo Activation button	Opens the [Photo Activation] dialog box. (This button is not displayed if a three-laser unit without AOM is connected.) The photo activate observation can be set. The dialog box to be opened depends on the conditions set in the C2 Settings window. For operations of this dialog box, see Chapter 10 in this instruction manual.  **Figure 4.1-5 Photo Activation dialog box** The same dialog box can also be opened with the following procedure: - Select [Applications] on the menu bar and then select [Define/Run Sequential Stimulation...] in this order.

4.1.1 ND Acquisition Dialog Box

The dialog boxes shown in (4), (5), and (6) of “Table 4.1-1 Functions of Acquire window” can also be opened with the following procedure:

- Select [Applications] on the menu bar and then select [Define/Run Experiment...].



In the [ND Acquisition] dialog box displayed with the above procedure, click a switching tab to select a function to use.
For operations of this dialog box, refer to “NIS-Elements Advanced Research User’s Guide.”

Figure 4.1-6 ND Acquisition dialog box

4.1.1.1 Notes on Use of ND Acquisition

There are some notes on the ND2 image acquisition with the Confocal Microscope C2 by using the [ND Acquisition] dialog box.

When using Perform Time Measurement

If the Time Measurement is executed with the [Perform Time Measurement] check box selected, a load on processing becomes so high that it may cause the following problems:

- When the [Loop] side is set, the time for transition to the next phase may be longer than the time supposed from the frame rate.
- When the [Duration] side is set, the number of the frames may be smaller than that supposed from the frame rate.

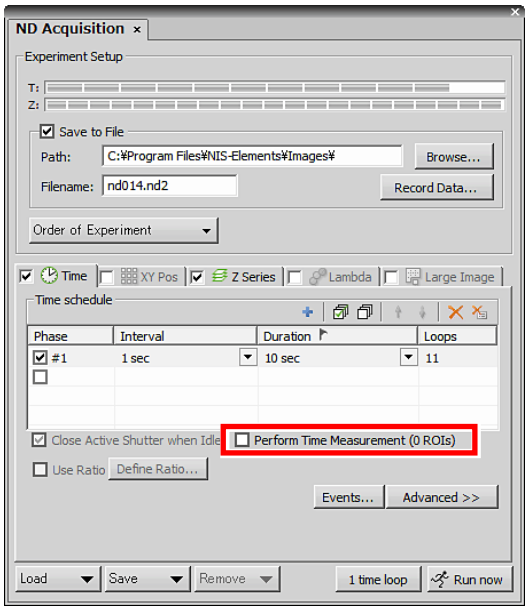


Figure 4.1-7 ND Acquisition dialog box

When Executing Large Image

The Large Image is a function to acquire a large image composed of multiple image frames and combine them to form a composite image by using the automatic algorithm, to be used when the target area is larger than the field of view (FOV) of the camera.

When this function is executed, the turning action is controlled by the stage. Therefore, it is necessary to execute the calibration before the Large Image function is executed.

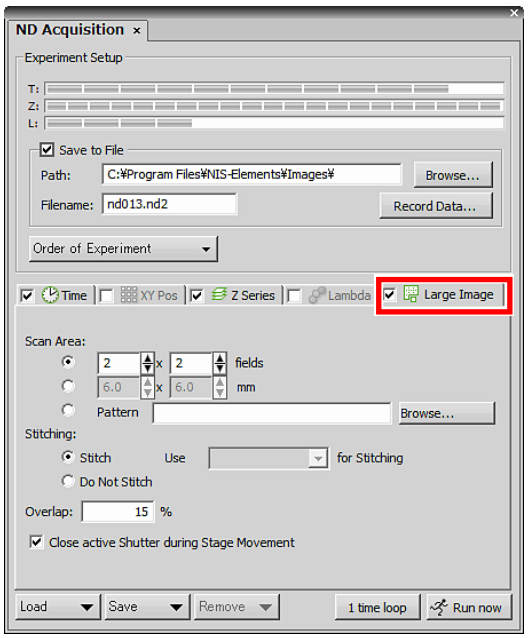


Figure 4.1-8 ND Acquisition dialog box

For operations of Auto calibration, refer to “NIS-Elements Advanced Research User’s Guide.”

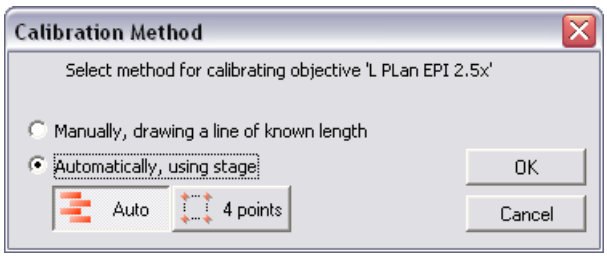


Figure 4.1-9 Auto calibration

4.1.2 Find Mode

By using the Find mode, you can acquire the live image by temporarily switching to the high-frame-rate setting in order to ease the detection of the observation object such as a cell.

4.1.2.1 Setting for Find Mode Settings Dialog Box

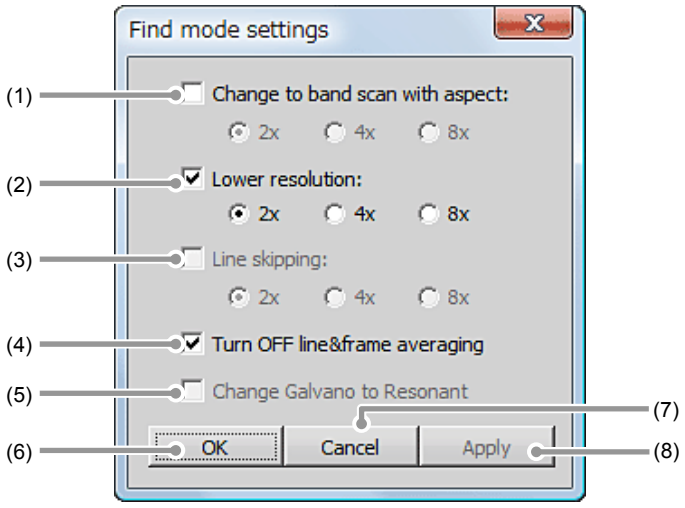


Figure 4.1-10 Find mode settings dialog box

Table 4.1-2 Summary of Find mode settings dialog box functions

Name		Function
(1)	Change to band scan with aspect:	Switches the band scan area by the specified ratio. E.g. If “2x” is selected in Scan Size 512 x 512, the band scan area is switched to 512 x 256 in the Find mode.
(2)	Lower resolution:	Changes the scan size. E.g. If “2x” is selected in Scan Size 512 x 512, the scan size is changed to 256 x 256 in the Find mode.
(3)	Line skipping:	Unusable in the C2.
(4)	Turn OFF line & frame averaging	Changes a setting for Frame Average. * C2 is not equipped with the line average function. E.g. Even if the Frame Average is set in the normal mode, the live image is acquired by changing the setting to “None” in the Find mode.
(5)	Change Galvano to Resonant	Unusable in the C2.
(6)	OK button	Determines the Find mode settings applied and closes the [Find mode settings] dialog box.
(7)	Cancel button	Discards the Find mode settings applied and closes the [Find mode settings] dialog box.
(8)	Apply button	Determines the Find mode settings.

4.2 Multi Position Acquisition

You can execute the experiment with multiple points within the same FOV by using the optical configuration (hereinafter referred to as O.C.) where different scan areas are respectively registered. (Photo activation experiment is not available.)

4.2.1 Procedure for Multi Position Acquisition Settings

1 Register the first scan area to O.C.

1. Specify a scan area on the acquired image.

∗ The scan areas usable in the multi position acquisition are the square scan area and the band scan area only. Also note that the rotated scan area cannot be used.

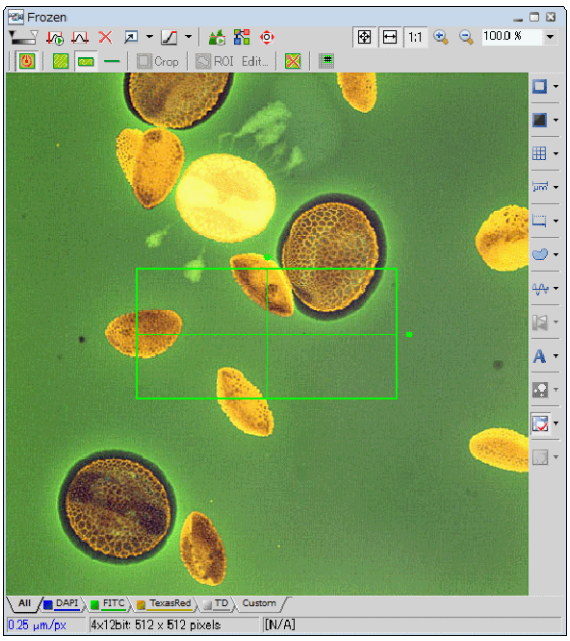


Figure 4.2-1 Specify a scan area

2. Register the specified scan area to O.C.
Select [Calibration] -> [New Optical Configuration…] from the menu bar to call the wizard.

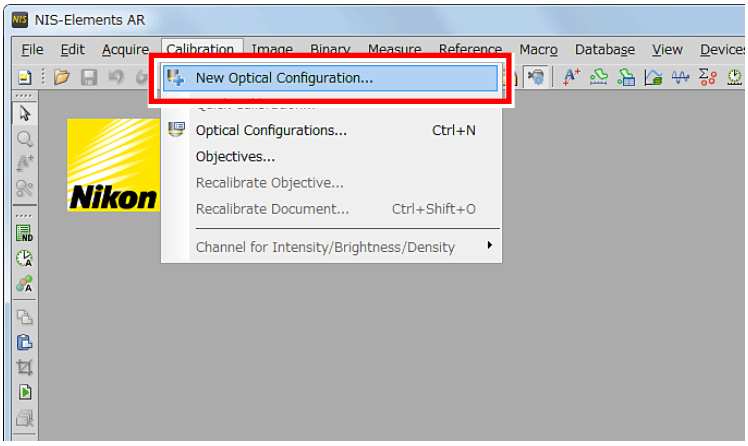


Figure 4.2-2 Call the Optical Configuration Wizard

3. Enter the name of O.C. to be registered.

4. Check the setting conditions, and then click the [Finish] button.

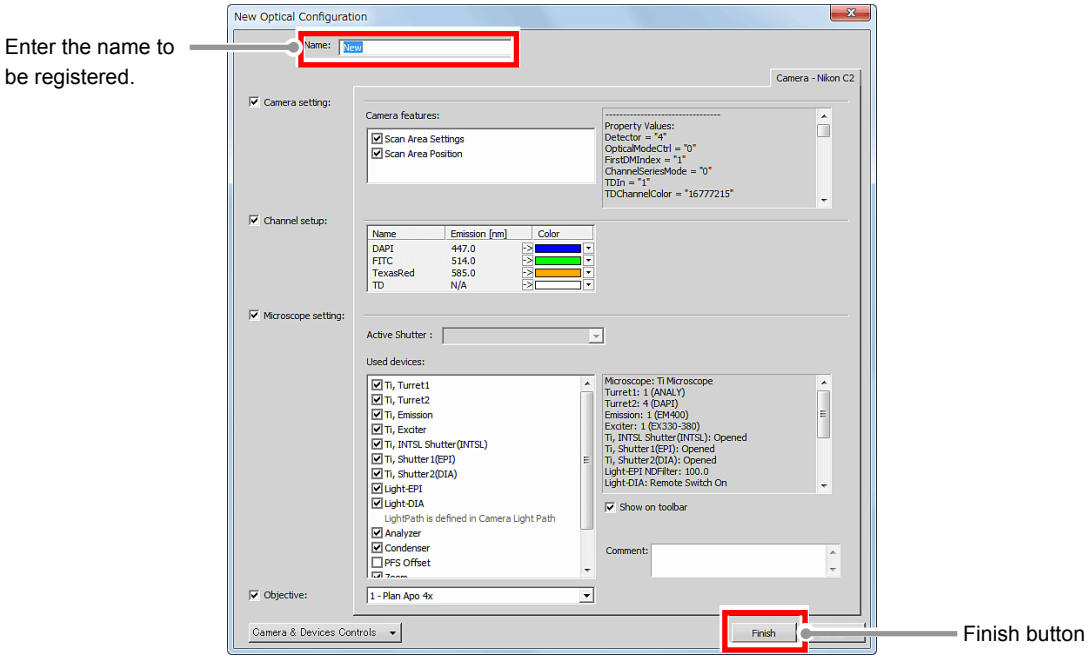


Figure 4.2-3 Register the optical configuration

2 Register the second and subsequent scan areas as separate O.C., respectively

After that, repeat “Step 1” to “Step 4” of **1** to register the O.C. of each scan area to be acquired in the multi position.

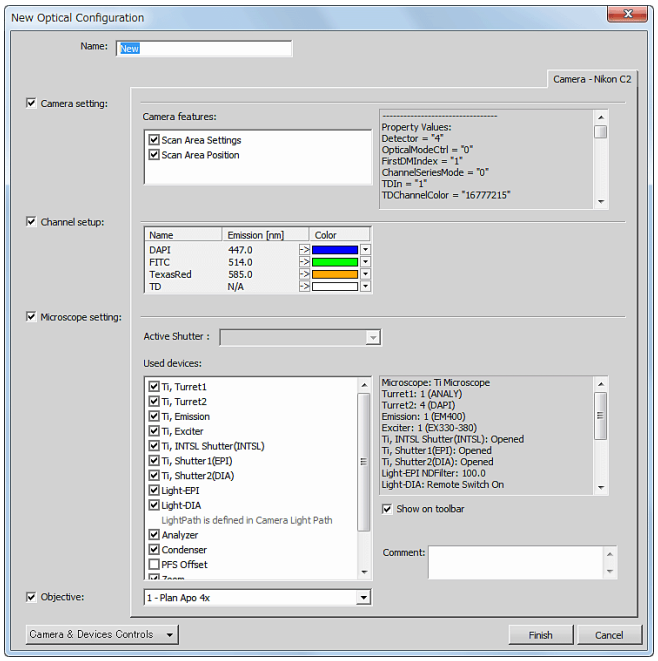


Figure 4.2-4 Register the optical configuration

3 Execute the multi position acquisition

Register the O.C. of each scan area for each action, and make the experiment setting, respectively.

1. Select [Applications] -> [Define/Run ND Sequence...] from the menu bar to open the [ND Sequence Acquisition] dialog box.

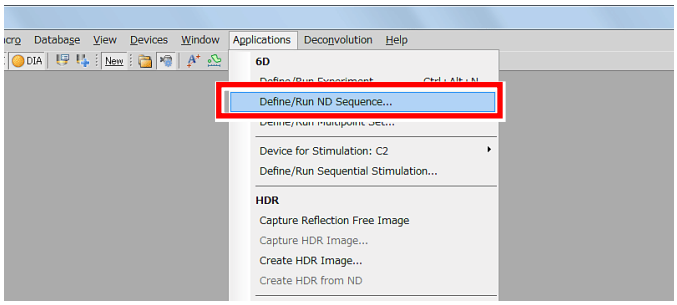


Figure 4.2-5 Call the ND Sequence Acquisition dialog box

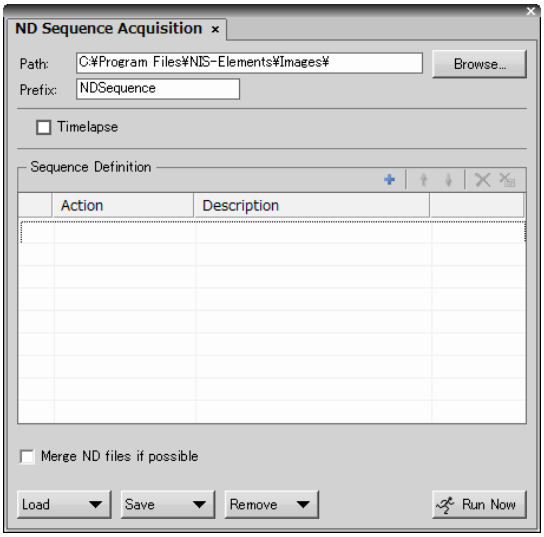


Figure 4.2-6 ND Sequence Acquisition dialog box

2. Click the first phase and select [ND Acquisition].

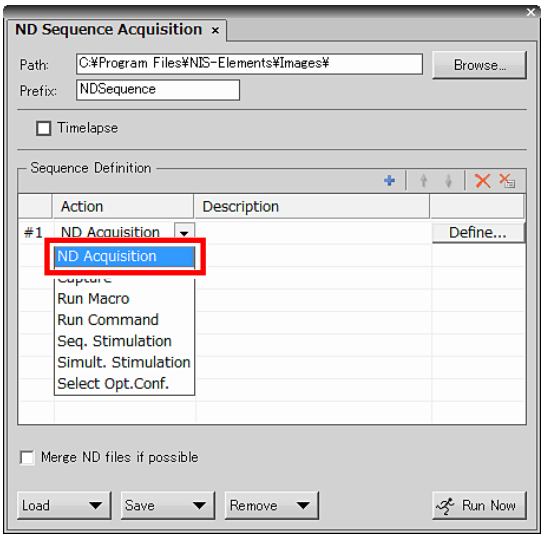


Figure 4.2-7 ND Sequence dialog box

3. Click the [Define…] button to open the experiment setting dialog box.

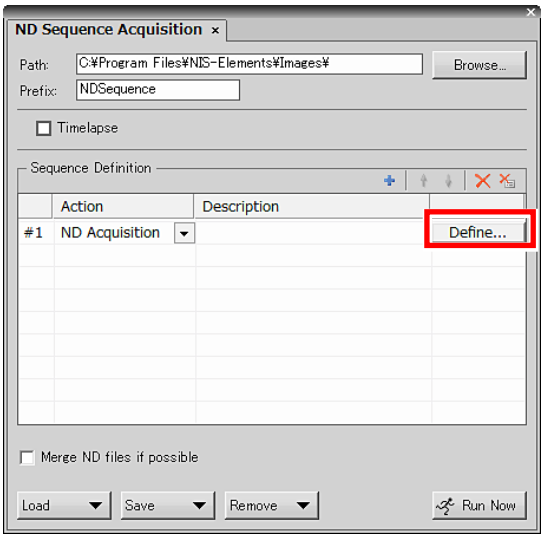


Figure 4.2-8 ND Sequence Acquisition dialog box

4. Select the Lambda series tab on experiment setting dialog box and specify the O.C. of the first scan area.

5. When the setting of the experiment sequence of the first scan area is completed, click the [OK] button to close the dialog box.
   The [ND Sequence Acquisition] dialog box is resumed.

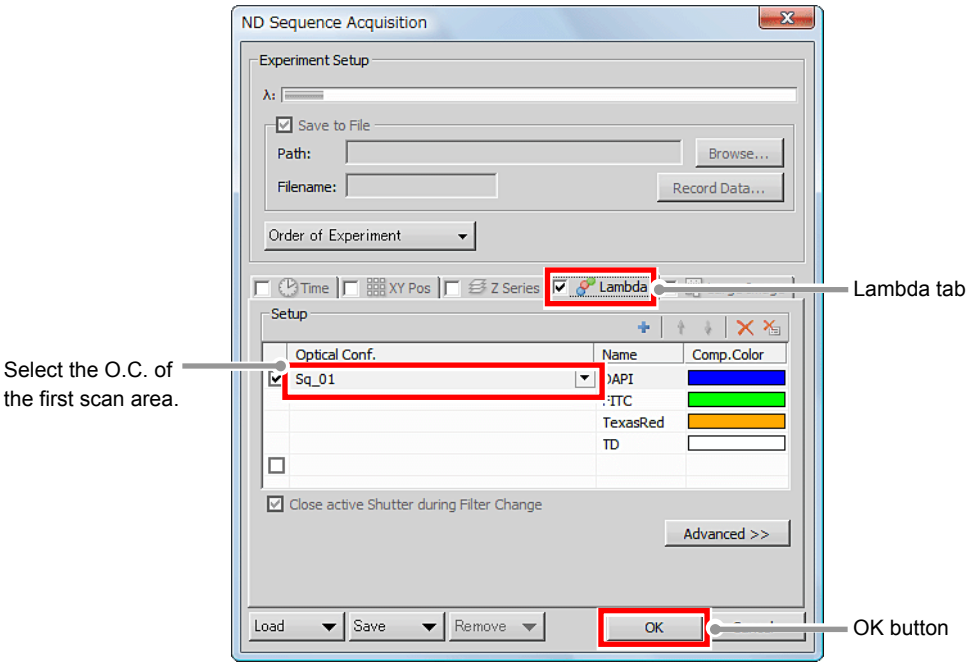


Figure 4.2-9 Experiment setting dialog box

* The Lambda series is used for the multi position acquisition. However, do not set multiple O.C.s in one Lambda series for the purpose of the multi position.
If O.C.s with different scan area types and/or sizes are set, displayed size will differ from the original image size, because the image size ratios must be matched within one ND image.

6. Click the next phase and select [ND Acquisition].
7. Click the [Define…] button to open the experiment setting dialog box.

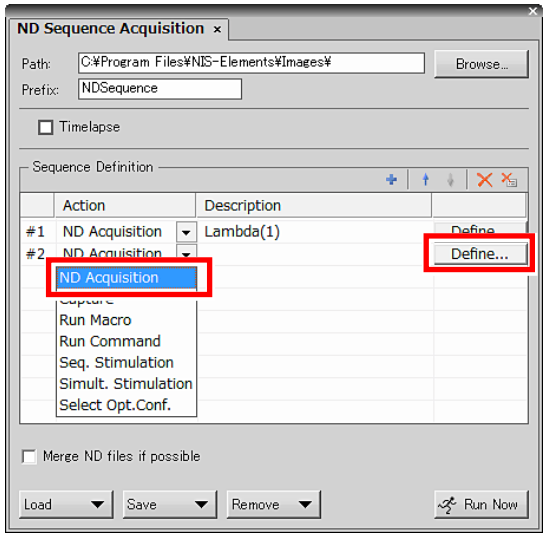


Figure 4.2-10 ND Sequence Acquisition dialog box

8. Specify the O.C. of the second scan area.
9. When the setting of the experiment sequence of the second scan area is completed, click the [OK] button to close the dialog box.
   The [ND Sequence Acquisition] dialog box is resumed.

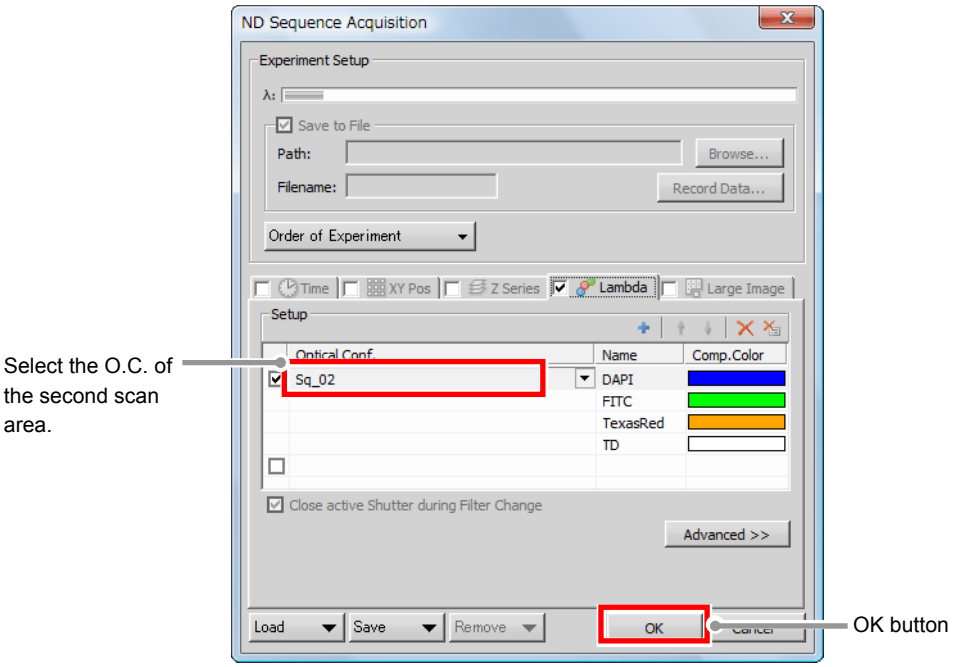


Figure 4.2-11 Experiment setting dialog box

10. After that, repeat “Step 6” to “Step 9” to make the experiment setting for the O.C. of the scan area registered for acquisition within the same FOV.

11. Click the [Run Now] button to execute the multi position acquisition.

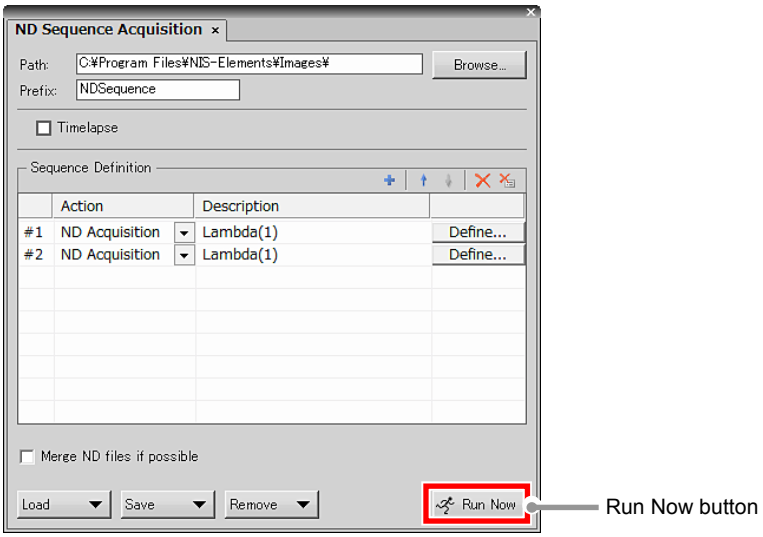


Figure 4.2-12 ND Sequence Acquisition dialog box

4.3 Functions of Z Intensity Control

When acquiring the images of a specimen at Z drive positions (tomographic images) by Z stack, acquiring images with identical conditions at all Z drive positions makes some of the images to be too bright or too dark depending on the Z drive position to acquire.
A solution to this problem is the Z Intensity Control function.

To use the Z Intensity Control function, first adjust the brightness on the live image by Z drive position you wish to acquire, and then register the optimum brightness setting values (laser power) for each Z drive position.

After that, acquire images by using the registered setting values. The brightness of the images for each Z drive position to acquire is automatically controlled, and images are acquired with the optimum brightness at all Z drive positions.

Minimum/recommended number of registrations for the Z Intensity Control function

To use the Z Intensity Control function, register at least two Z drive positions (Top and Bottom), and to acquire clearer images, it is recommended to register 4 or more positions (Top and Bottom plus two or more intermediate Z drive positions).

Z drive positions not registered to Z Intensity Control

For the setting values of Z drive positions not registered to Z Intensity Control are automatically complemented according to the setting values of the registered Z drive position, and the complemented setting values are used to acquire images.
To check the complemented setting values, open the Microsoft Excel file output by using the [Export...] button on the [Z Intensity Control] dialog box.

4.3.1 Usage of Functions of Z Intensity Control

1 Setting Z Stacks position for image acquisition and Z Device

Call the [Capture Z-Series] dialog box.
For setting Z stacks instructions, refer to “NIS-Elements Advanced Research User's Guide.”

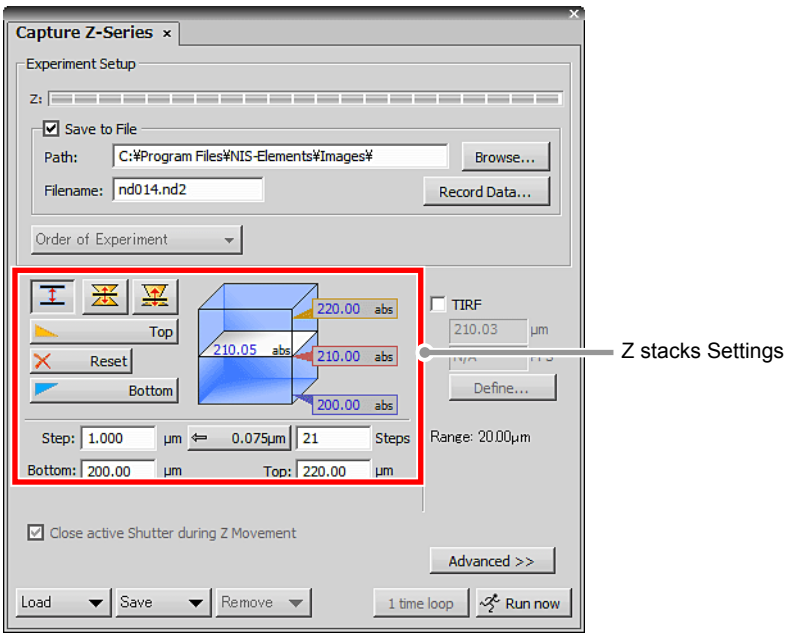


Figure 4.3-1 Z stacks Settings

2 Display the [Z Intensity Control] dialog box

1. Click the [Advanced] button to display the Advanced menu.

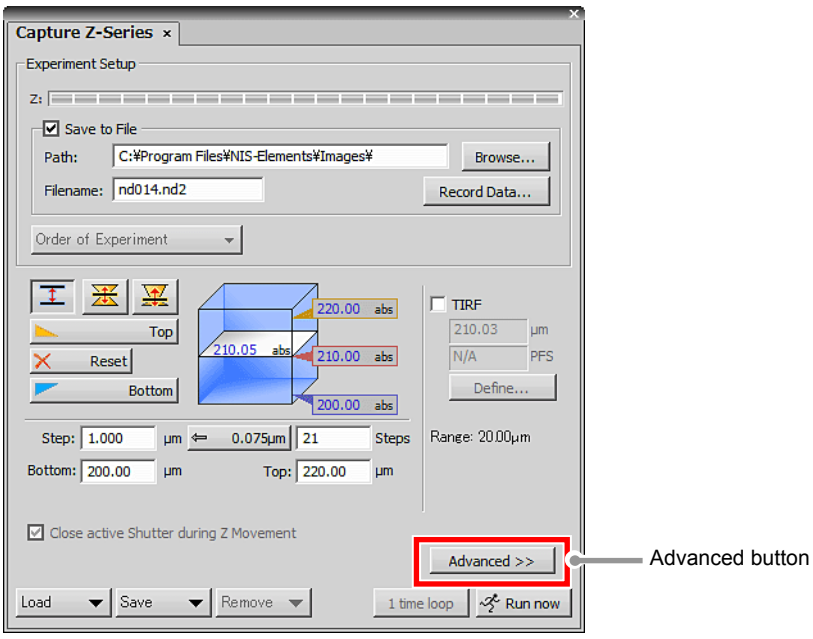


Figure 4.3-2 Displaying the Z intensity control button

2. Click the [Z intensity control] button to display the [Z Intensity Control] dialog box.

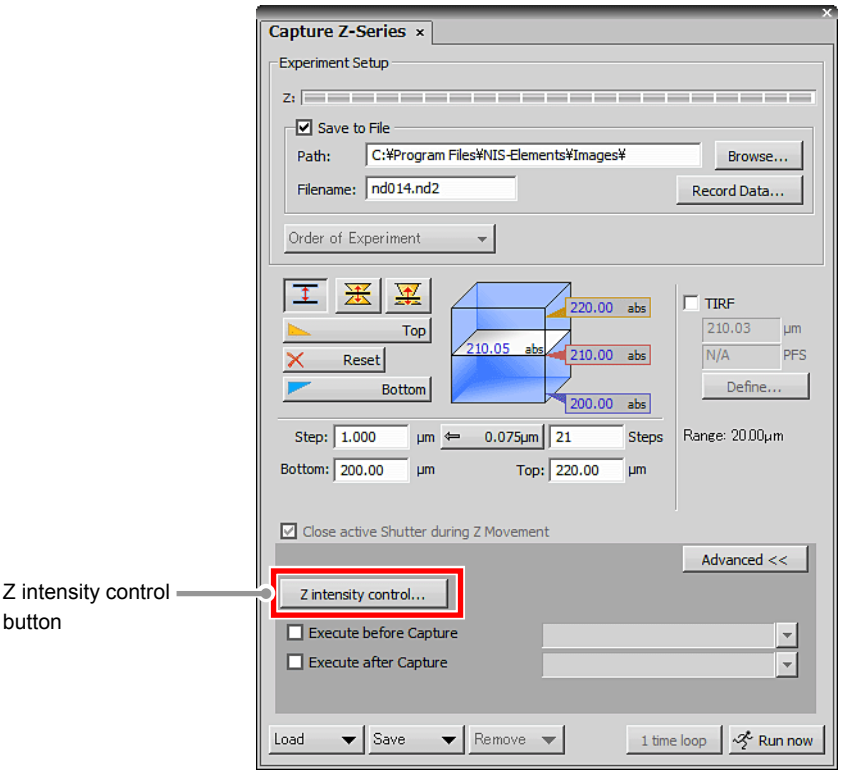


Figure 4.3-3 Displaying the Z Intensity Control dialog box

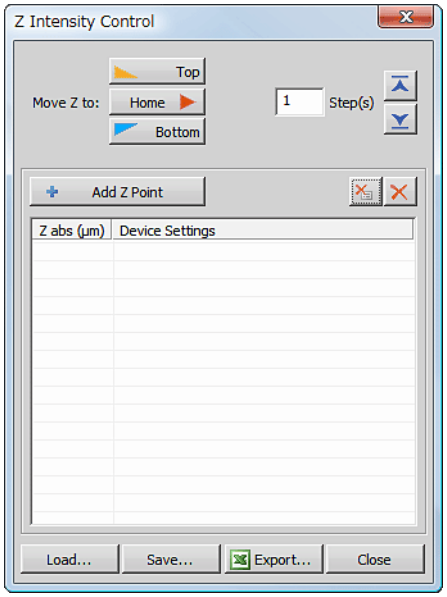


Figure 4.3-4 Z Intensity Control dialog box

3 Adjust brightness at the Z drive positions and register them to Z Intensity Control

1. Click the [Top] button in [Z Intensity Control] dialog box to move Z drive position to Top.

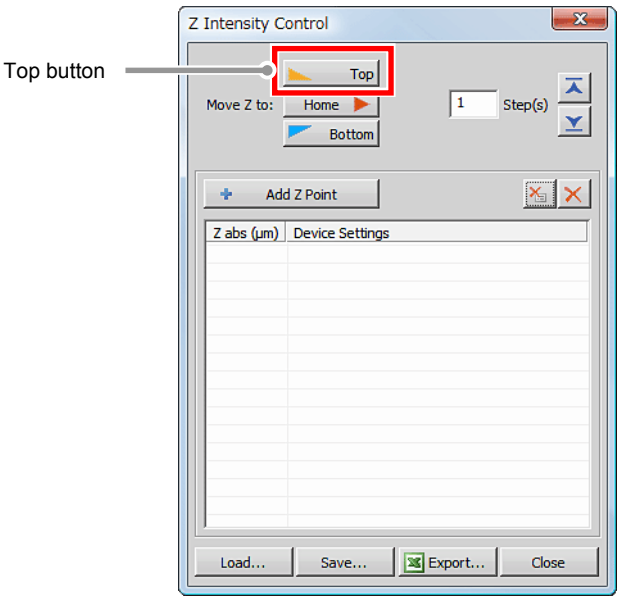


Figure 4.3-5 Z Intensity Control dialog box

2. Acquiring the live image of Top Z drive position.
   Click the [Live] button.
   The live image of Top Z drive position is acquired and the Live window appears.

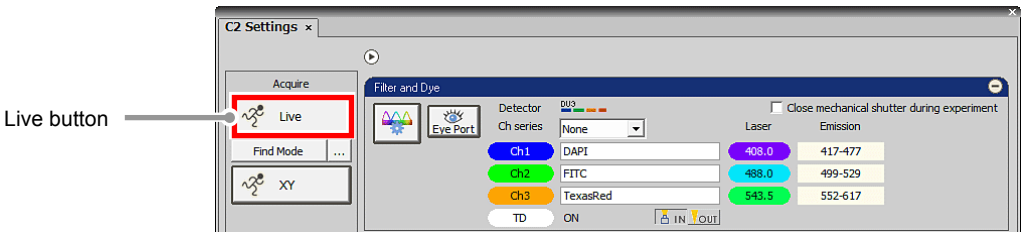


Figure 4.3-6 Live image acquisition

3. Adjusting the brightness of the live image.
   In the Acquisition window, adjust the brightness of the live image for each channel.

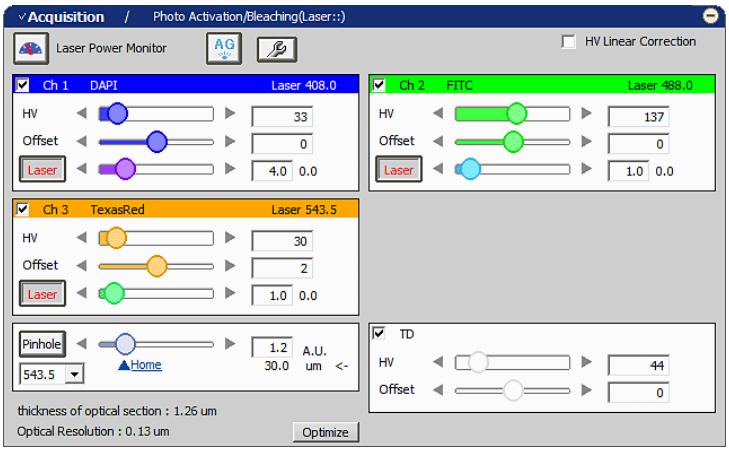


Figure 4.3-7 Acquisition window

4. Register the adjusted values.
   Click the [Add Z Point] button in the [Z Intensity Control] dialog box.
   The adjusted values are registered on the [Z Intensity Control] dialog box.

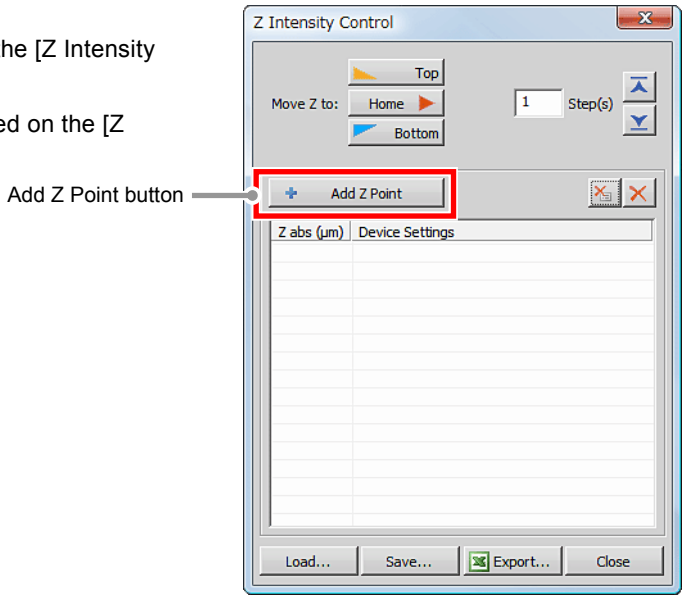


Figure 4.3-8 Z Intensity Control dialog box

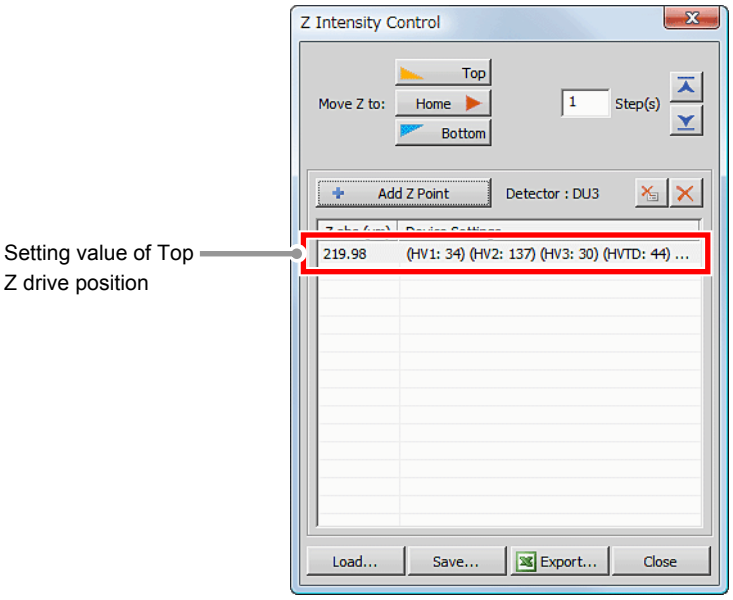


Figure 4.3-9 Z Intensity Control dialog box

5. Move to the Z drive position to be registered next.
   Click the button in the [Z Intensity Control] dialog box, to move the Z drive position to be registered next.
   After that, repeat “Step 2” to “Step 5” to register all the remaining Z drive positions.

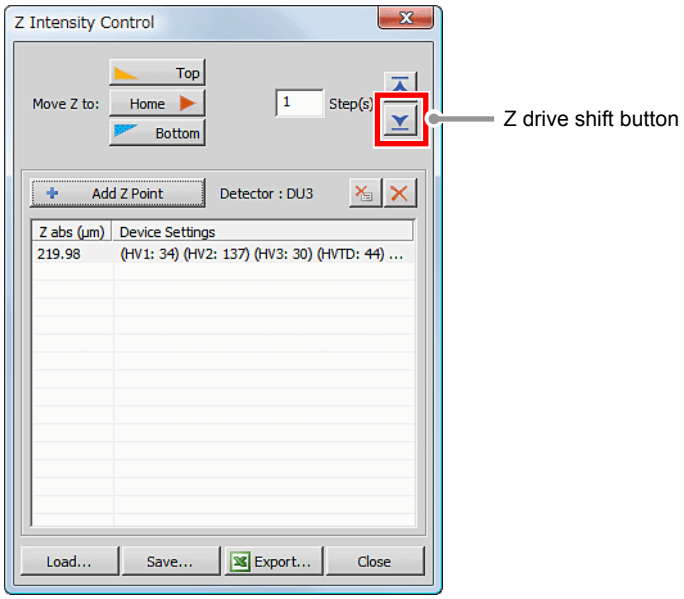


Figure 4.3-10 Z Intensity Control dialog box

6. After registering all of them, click the [Run now] button in the [Capture Z-Series] dialog box to execute the image acquisition.
   With the registered setting values, the brightness of the image for each Z drive position is automatically controlled and images are acquired with the optimum brightness at all the Z drive positions.

   The settings registered on the [Z Intensity Control] dialog box are exportable to an XML file by using the [Save...] button, and an exported XML file is loadable by using the [Load...] button.

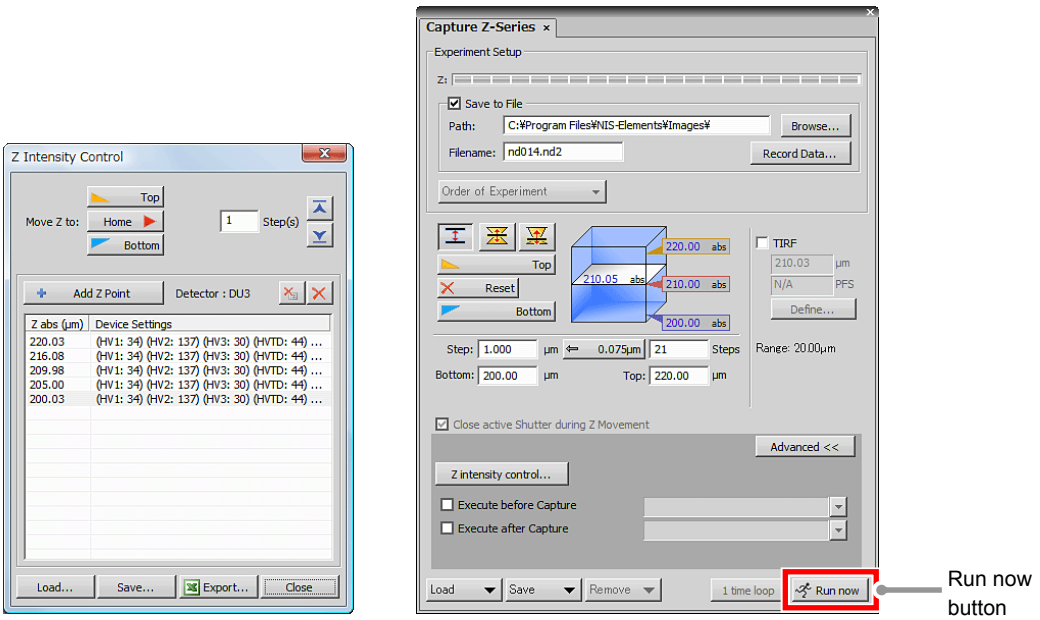


Figure 4.3-11 Image acquisition running

4.3.2 Z Intensity Control Dialog Box

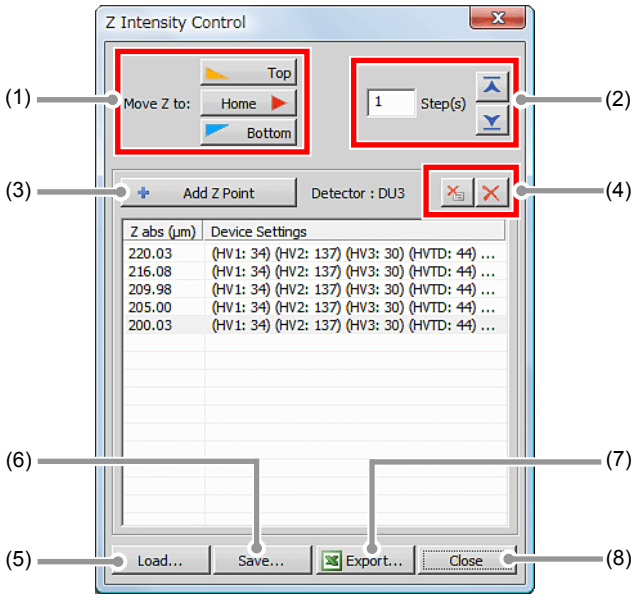


Figure 4.3-12 Z Intensity Control dialog box

Table 4.3-1 Functions of Z Intensity Control dialog box

Name		Function	
(1)	Move Z to:	Top button: Move the Z drive position to Top. Home button: Move the Z drive position to Home. Bottom button: Move the Z drive position to Bottom.	
(2)	Step (s)	Z drive shift button can move the Z drive position up and down by the value input to the field.	
(3)	Add Z Point button	Registers the values adjusted on the live image.	
(4)	Remove Registrations button		Removes all registrations.
			Removes the selected item.
(5)	Load... button	Retrieves the saved in an XML file.	
(6)	Save... button	Writes the registrations in an XML file and saves it.	
(7)	Export... button	Writes the registrations in a Microsoft Excel file. The exported file allows the user to check the complemented values of Z drive position with setting values unregistered.	
(8)	Close button	Closes the [Z Intensity Control] dialog box.	

5 Detection Mode (Standard Detector)

This chapter describes settings for the Standard Detector mode.

5.1 Filter and Dye window

This window enables to select the desired channel series and set the Optical path.

The Standard Detector mode can be used when the optical path changeover lever on the C2 scan head is set to the [Standard] position.

5.1.1 Structure of Filter and Dye Window

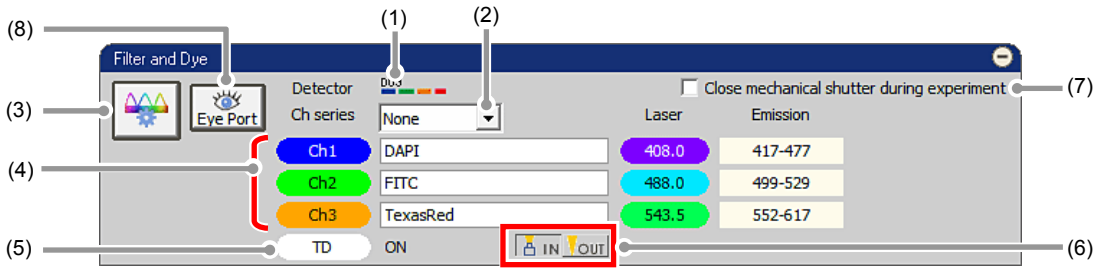


Figure 5.1-1 Filter and Dye window (Standard Detector-use)

Table 5.1-1 Functions of Filter and Dye window (Standard Detector-use)

Name		Function
(1)	Detector	Indicates that the Standard Detector mode [DU3] is used when the optical path changeover lever on the C2 scan head is set to the [Standard] position.
(2)	Ch series	Selects whether to perform scanning by simultaneously firing all lasers for the channels in use or by sequentially firing one laser after another. * Usable only when a four-laser unit is connected. * If the Bidirectional scan is selected, the Channel Series is fixed to “None” and cannot be used.
(3)	Setting button	Opens the Optical path window. To use, select the detector, the dichroic mirror, the channel, fluorescence dye for each channel, laser and others.
(4)	Status	Indicates for the settings for each channel (fluorescence dye name, laser wavelength, and wavelength band to be acquired).
(5)	TD	Indicates the status of the motorized transmitted detector.
(6)	TD IN/OUT button	Sets/removes the motorized transmitted detector in/from the optical path. (IN = Set in the optical path/ OUT = Remove from the optical path) As for the case where the TD IN/OUT button is not displayed, it will be displayed when the motorized transmitted detector is set in the optical path in the Optical path window.
(7)	Close mechanical shutter during experiment	If unchecked, the shutter remains open during the ND image acquisition. As the shutter is not opened/closed every image acquisition, the time for the image acquisition can be shortened. However, the laser remains emitted during the interval.
(8)	Eye Port button	Changes optical path to eye port.

- **Optical Configuration**

  Individual data items set in the Standard Detector mode can be managed collectively with the [Optical Configuration] dialog box.
  "NIS-Elements C" allows the user to store and retrieve the following settings: the laser power for image acquisition, offset of the transmission detector, PMT offset, channel selection, pinhole size, photo activation laser selection, the laser power for photo activation, averages, scan area and others.
  For storing and retrieving the [Optical Configuration] settings, see the sections concerning the optical configuration in the "NIS-Elements Advanced Research User's Guide."

5.1.2 Setting the Optical Path

Click the [Setting] button of "Filter and Dye" window to display the Optical path window.
The Standard Detector mode [DU3] setting screen is displayed when the optical path changeover lever on the C2 scan head is set to the [Standard] position.

There are two modes available for Optical path setting, [Auto] and [Manual].
Normally, the auto mode should be used.

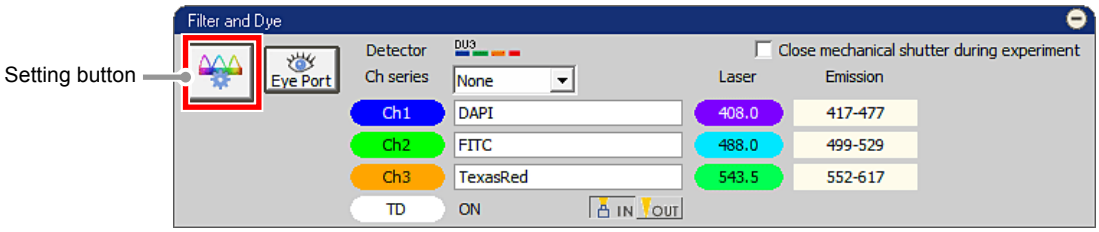


Figure 5.1-2 Filter and Dye window

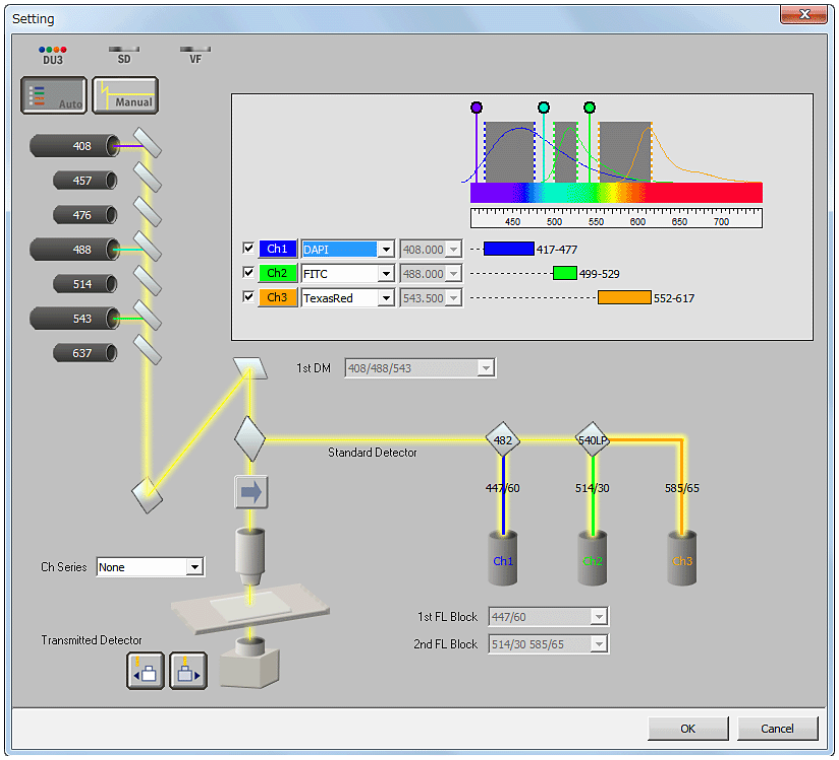


Figure 5.1-3 Optical path window (for auto mode)

5.1.3 Optical Path Window

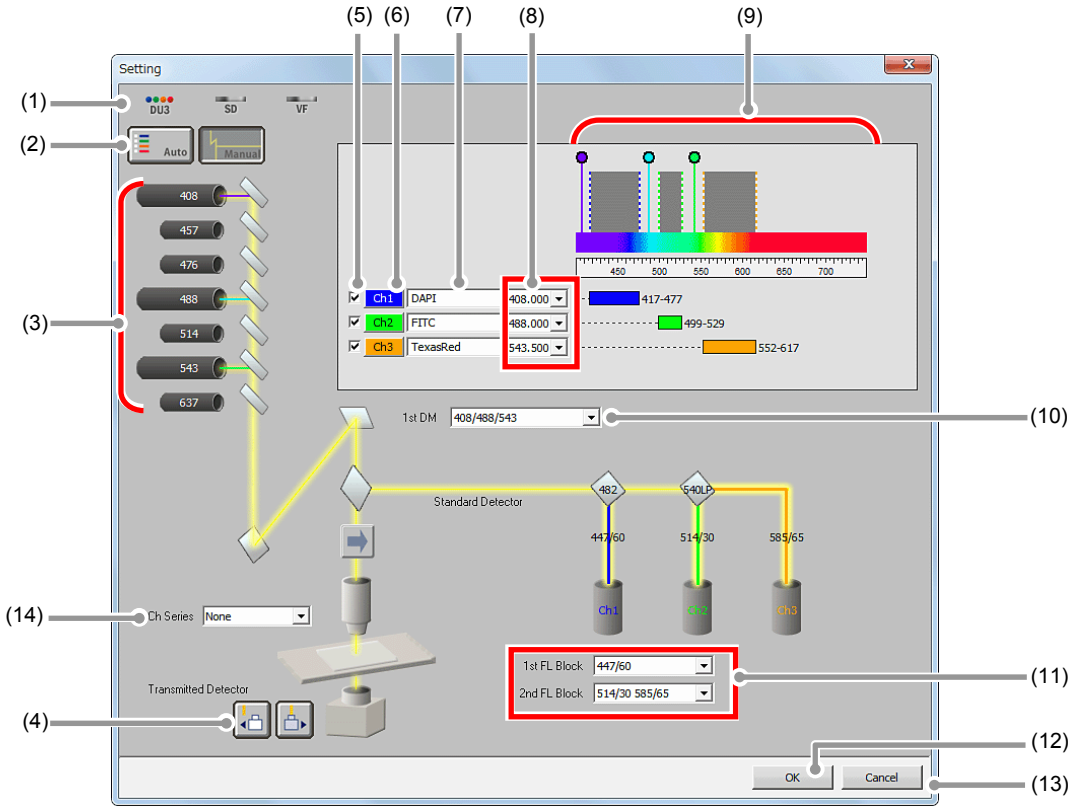


Figure 5.1-4 Optical path window (for Manual mode, Standard Detector-use)

Table 5.1-2 Functions of Optical path window (Standard Detector-use) (sheet 1/2)

Name		Function	
(1)	Detection mode selection Indication	DU3	Standard Detector mode. Enables to acquire the 3-channel + TD images.
(2)	Mode selector	Selects the desired mode for setting the Optical path.	
		Auto	Activates the auto mode. Once a fluorescence dye is selected, appropriate laser and the dichroic mirror are automatically selected.
		Manual	Activates the manual mode. Enables to set the lasers and the dichroic mirror to be used manually.
(3)	Excitation laser indicator	Displays the current setting for the laser. The currently set laser icon is displayed in a large size, and the optical path is indicated.	
(4)	Transmitted Detector selection button		Brings the transmitted detector into the Optical path, to enable the ability.
			Brings the transmitted detector out of the Optical path, to disable the ability.
(5)	Channel selection check box	Enables to select the channels to be used.	

Table 5.1-2 Functions of Optical path window (Standard Detector-use) (sheet 2/2)

Name		Function	
(6)	Channel color setting button	Displays the [Color Selection] dialog box, enables to set the desired color for each channel.	
(7)	Fluorescence dye selection/input:	In auto mode	Selects the fluorescence dye name to be used for each channel.
		In manual mode	Enters any desired fluorescence dye name for each channel.
(8)	Excitations laser select	These fields are only effective while in the manual mode. Enables to set the laser wavelength that is set with the software configuration, regardless of the setting of the Filter block display/select.	
(9)	Rainbow chart	Provides the following information: - Wavelength band for which to acquire images (shown in color and value for each channel) - Spectral profile of fluorescence dye - Excitation laser for fluorescence dye - A color band indicating the wavelengths in the entire band (400 to 750 nm) - Scale of the wavelengths in the entire band (400 to 750 nm)	
(10)	1st Dichroic mirror select	These fields are only effective while in the manual mode. Enables to manually select the 1st Dichroic mirror to be used.	
(11)	Filter block display/select	In manual mode only, the filter block to be mounted on the detector can be selected regardless of the excitation laser.	
(12)	OK button	Determines the Optical path settings applied and closes the Optical path window.	
(13)	Cancel button	Discards the Optical path settings applied and closes the Optical path window.	
(14)	Ch series selection	Selects whether to perform scanning by simultaneously firing all lasers for the channels in use or by sequentially firing one laser after another. For Ch series selection, see Section 5.1.4, “Selecting the Channel Series.”	

- **About the setting condition when the setting mode is switched**

  Auto mode → Manual mode:

  The entire settings in the Auto mode are retained.

  Manual mode → Auto mode:

  The fluorescence dye with the same channel name as set in the manual mode is automatically selected.

  If the same fluorescence dye name does not exist in the list, a fluorescence dye detectable by the laser wavelength is automatically selected from the list.

5.1.4 Selecting the Channel Series

The [Ch series] field enables to select whether to perform scanning by simultaneously firing all lasers for the channels to be used or by firing one laser after another.

There are two options for channel series, “None” and “Line”, either of which can be selected from the pull-down menu.

* Usable only when a four-laser unit is connected.

* If the Bidirectional scan is selected, the Channel Series is fixed to “None” and cannot be used.

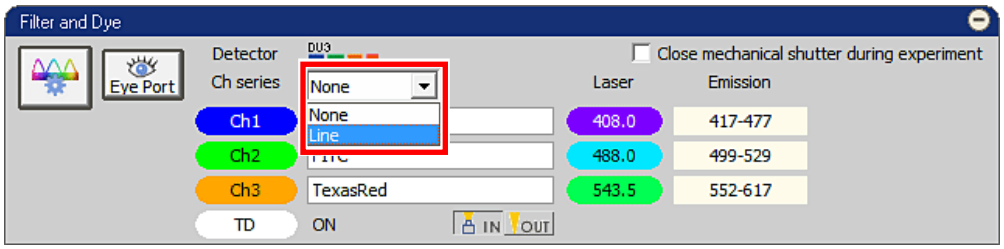


Figure 5.1-5 Selecting the channel series (Filter and Dye window)

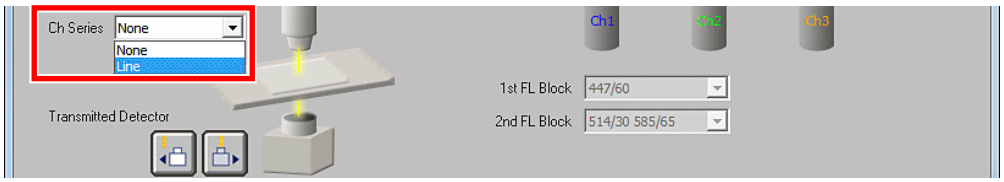


Figure 5.1-6 Selecting the channel series (Optical path window)

Table 5.1-3 Functions of channel series

Name	Function
None	Performs scanning by simultaneously firing all lasers for the channels to be used.
Line	Performs scanning by sequentially firing the lasers for the channels in use, one laser at a time. For each scan line, the lasers are fired in a sequence. This scan method is called the line sequence.

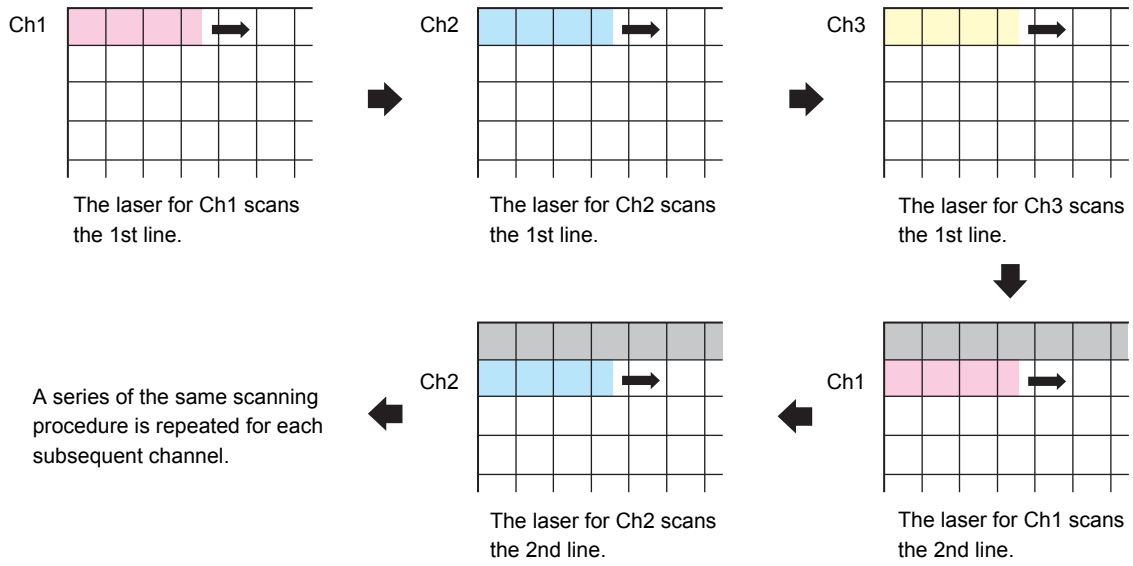


Figure 5.1-7 Scanning motion in line sequence

5.2 Acquisition Window

The Acquisition window enables to set PMT brightness (detection sensitivity), laser power, and pinhole size.

5.2.1 Structure of Acquisition Window

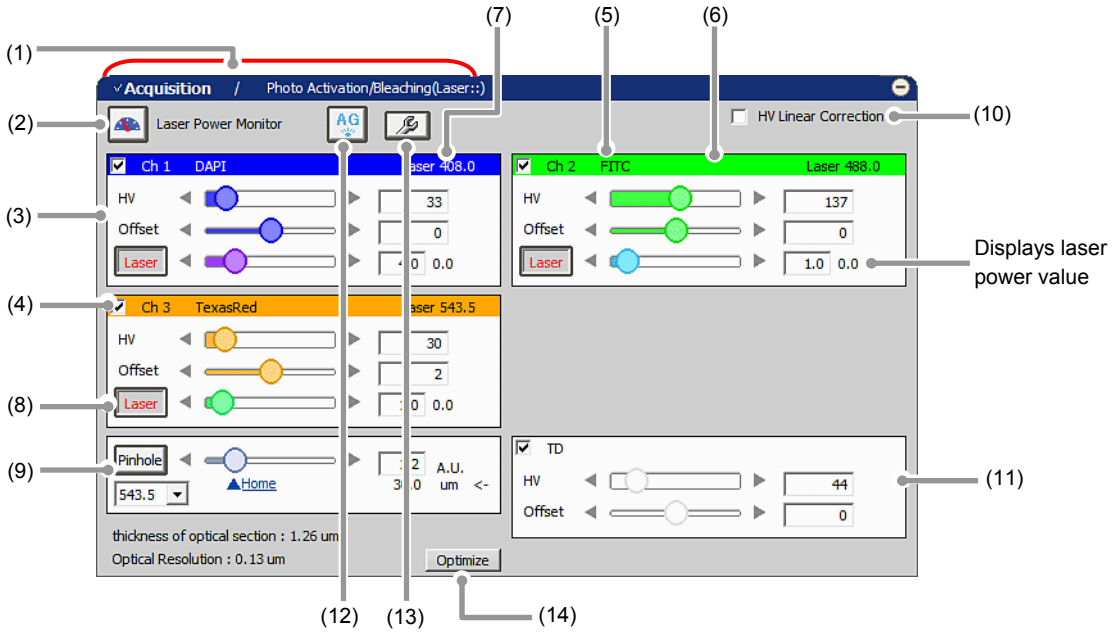


Figure 5.2-1 Acquisition window (Standard Detector-use)

Table 5.2-1 Functions of Acquisition window (Standard Detector-use) (sheet 1/2)

Name		Function
(1)	Acquisition/Photo Activation window switching	Switches between the Acquisition and Photo Activation windows. For the Photo Activation window, see Chapter 10.
(2)	Laser power monitor button	Displays the laser power value (integer obtained after A/D conversion divided by 10) of the current channel by clicking this button. During the image acquisition, the laser power cannot be measured and this button is grayed out.
(3)	Brightness adjustment for each channel	For each of the channels (Ch1 to Ch3), use the HV, Offset, and Laser controls to adjust the brightness of the live image.
(4)	Channel selection	Selects the channels (Ch1 to Ch3, and/or TD) to acquire the desired images. Do this by adding a check mark.
(5)	Fluorescence dye name indication	The fluorescence dye name specified in the Optical path window is indicated.
(6)	Channel color	Displays the channel color specified in the Optical path window.
(7)	Laser wavelength indication	Displays the currently selected laser wavelength.

Table 5.2-1 Functions of Acquisition window (Standard Detector-use) (sheet 2/2)

Name		Function	
(8)	Laser ON/OFF button	Selects whether the laser is emitted or not.	
		ON status	The laser is emitted.
		OFF status	The laser is not emitted. When switched from OFF to ON, the laser power value set in the previous ON status is applied.
(9)	Pinhole	Adjusts the pinhole size. (6 steps) For pinhole size, see Section 5.2.3, “Setting the Pinhole.”	
(10)	HV Linear Correction	Enables or disables HV Linear Correction. For HV Linear Correction, see Section 5.2.4, “HV Linear Correction.”	
(11)	Brightness adjustment for transmitted detector	For the transmitted detector, use the HV and Offset controls to adjust the brightness of the live image.	
(12)	AG button	Automatically adjusts the HV value (HV gain) of the currently selected channel to the optimum values. For Auto Gain, see Section 5.2.5, “Auto Gain.”	
(13)	Auto Gain setting button	Sets the ratio of saturation pixels used for automatic HV gain correction. The dialog box for range of the ratio of saturation pixels settings appears when this button is clicked. For Setting for Ratio of saturation pixels, see “Setting for Ratio of saturation pixels” in the Section 5.2.5, “Auto Gain.”	
(14)	Optimize button	Displays the [XYZ Size Setup] dialog box. In the [XYZ Size Setup] dialog box, the calculation method of the recommended values of the resolution, zoom magnification, and Z stack step size can be set. For [XYZ Size Setup] dialog box, see Section 5.2.1.1, “Recommended Value Indication/Automatic Application” in the next page.	

5.2.1.1 Recommended Value Indication/Automatic Application

By the function of the recommended value indication/automatic application, the recommended values of the appropriate resolution, zoom magnification, and Z stack step size are calculated based on the objective type and the selected excitation wavelength.
Using the calculated recommended values enables the image acquisition clearer and with less damage to the specimen.

Recommended Value Automatic Application

To automatically apply the recommended values to the parameters, set the [Nyquist XY] button of the Scan Area window to ON.

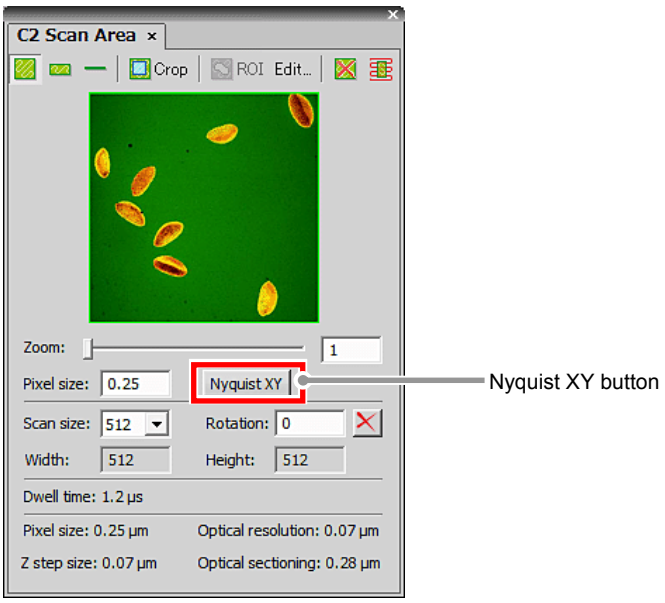


Figure 5.2-2 Scan Area window

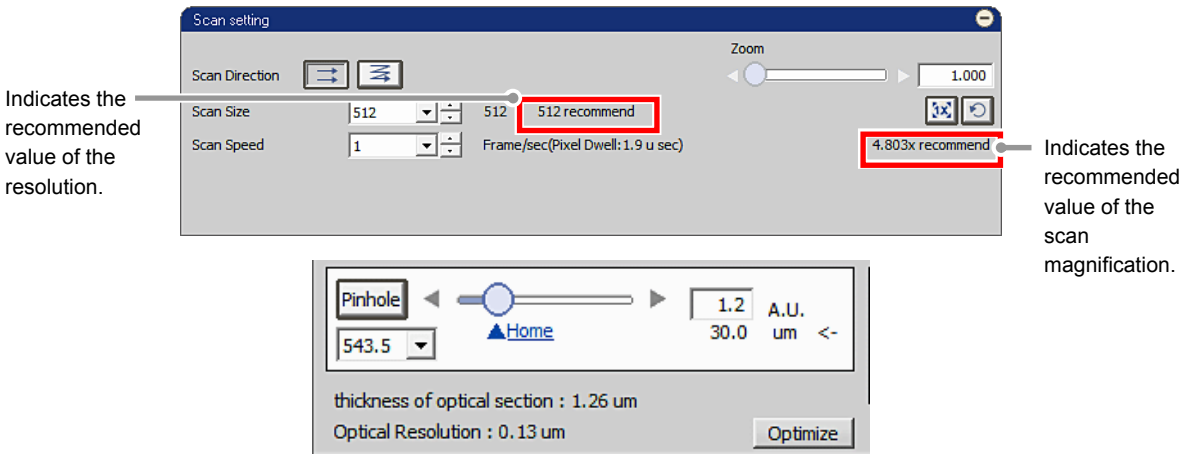


Figure 5.2-3 Location of Recommended Value Indication

* When the laser or objective in use is changed, the recommended values are recalculated, and newly indicated and automatically applied.

Recommended Value Settings

Detailed settings of the recommended values are made in the [XYZ Size Setup] dialog box that is displayed by clicking the [Optimize] button of the Acquisition window.
If the [Nyquist XY] button of the Scan Area window is ON, the recommended values are automatically applied to the parameters.
Or if the [Nyquist XY] button is OFF, the recommended values of the scan size and zoom are indicated in the Scan setting window.

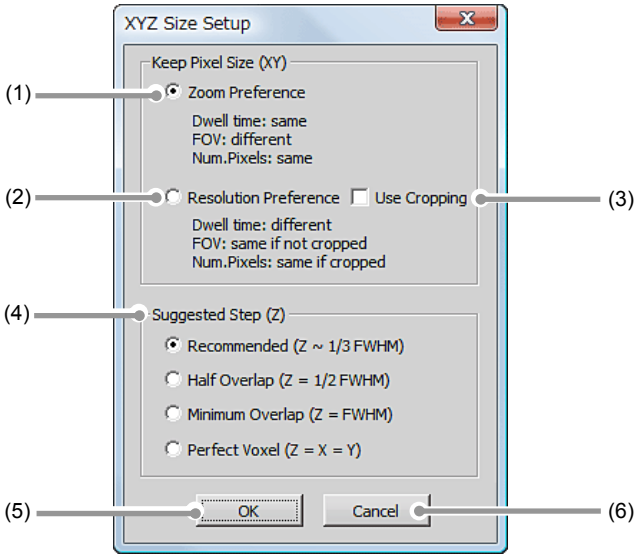


Figure 5.2-4 XYZ Size Setup dialog box

Table 5.2-2 Functions of XYZ Size Setup dialog box

Name		Function	
(1)	Zoom Preference	When the [Nyquist XY] button is ON, keeps the scan size and applied the recommended value of the zoom.	
(2)	Resolution Preference	When the [Nyquist XY] button is ON, keeps the zoom and applied the recommended value of the scan size.	
(3)	Use Cropping	Fits the scan size in detail by using Crop Scan.	
(4)	Suggested Step (Z)	Sets the Z step size calculation method.	
		Recommend (Z~1/3 FWHM)	Approximately one third of the thickness of optical section (FWHM value).
		Half Overlap (Z=1/2 FWHM)	One half of the thickness of optical section (FWHM value).
		Minimum Overlap (Z=FWHM)	The thickness of optical section (FWHM value).
		Perfect Voxel (Z=X=Y)	Value same as the pixel size.
(5)	OK button	Determines the XYZ Size Setup applied and closes the [XYZ Size Setup] dialog box.	
(6)	Cancel button	Discards the XYZ Size Setup applied and closes the [XYZ Size Setup] dialog box.	

5.2.2 Setting Image Brightness

For the live images of each channel, adjust HV, Offset, and Laser to obtain clear images.

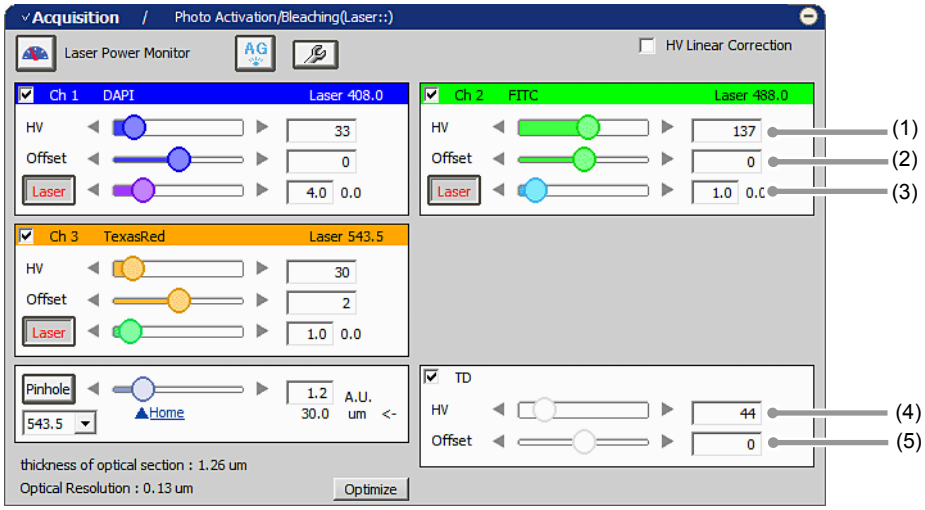


Figure 5.2-5 Setting the live image brightness (Standard Detector-use)

Table 5.2-3 Brightness adjustment functions for the live image (Standard Detector-use)

Name		Function
(1)	HV	Sets the voltage to be applied to the PMT. Slider bar: Slides to the right or left to set the HV value. Arrow buttons: Click either arrow button to increase or decrease the HV value stepwise. Direct entry in HV value display field: Type the desired setting value.
(2)	Offset	Sets the BL offset value of the PMT. Slider bar: Slides to the right or left to set the offset value. Arrow buttons: Click either arrow button to increase or decrease the offset value stepwise. Direct entry in offset value display field: Type the desired setting value.
(3)	Laser	Sets the laser power value. Slider bar: Slides to the right or left to set the laser power value. Arrow buttons: Click either arrow button to increase or decrease the laser power value stepwise. Direct entry in laser power value display field: Type the desired setting value.
(4)	HV (TD)	Sets the voltage to be applied to the transmitted detector. Slider bar: Slides to the right or left to set the HV value. Arrow buttons: Click either arrow button to increase or decrease the HV value stepwise. Direct entry in HV value display field: Type the desired setting value.
(5)	Offset (TD)	Sets the offset value of the transmitted detector. Slider bar: Slides to the right or left to set the offset value. Arrow buttons: Click either arrow button to increase or decrease the offset value stepwise. Direct entry in offset value display field: Type the desired setting value.

PMT Overload

If too much gain is applied to the illumination intensity, the gain is automatically shut down to protect PMT and/or transmitted detector (TD) and the following [PMT Overload] dialog box is displayed.
In this case, the PMT and/or TD HV value of the channel in which the overload occurs becomes "0". To continue the adjustment, set the PMT and/or TD HV value again.

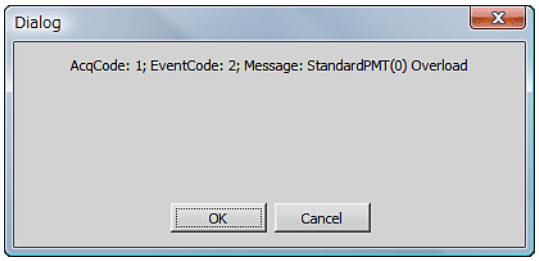


Figure 5.2-6 PMT Overload dialog box

5.2.3 Setting the Pinhole

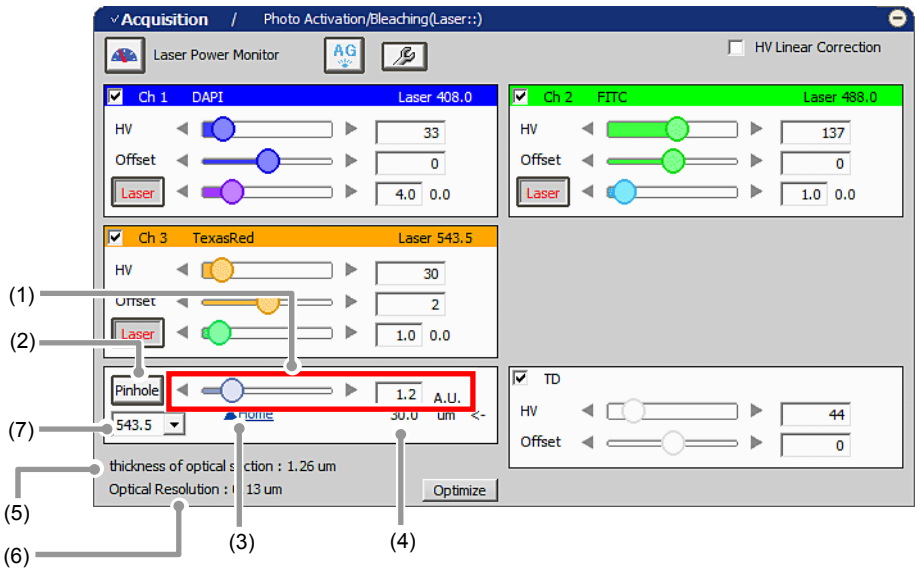


Figure 5.2-7 Setting the Pinhole (Standard Detector-use)

Table 5.2-4 Pinhole setting functions (Standard Detector-use)

Name		Function
(1)	Pinhole size setting	Sets a pinhole size for C2 system. Slider bar: Slides to the right or left to set the pinhole size. (Unit: A.U.) Arrow buttons: Click either arrow button to increase or decrease the pinhole size stepwise. Direct entry in pinhole size display field: Type the desired setting value.
(2)	Pinhole button	Displays the [A.U. Calculation Settings] dialog box to calculate the pinhole size. (For A.U. Calculation Settings, see Section 5.2.3.1, “Calculation Settings for Pinhole Size.”)
(3)	Home	Changes the pinhole to the predetermined home position. The value of the home position can be changed in the [A.U. Calculation Settings] dialog box. (For A.U. Calculation Settings, see Section 5.2.3.1, “Calculation Settings for Pinhole Size.”)
(4)	Pinhole size	Indicates pinhole size of C2 system. (Unit: um)
(5)	thickness of optical section	Indicates the FWHM (full width at half maximum) of z airy disk.
(6)	Optical Resolution	The actual size of 1 pixel square calculated from the optical information (for objectives and scan parameters) and the size acquired from an image.
(7)	Reference excitation wavelength for the pinhole size calculation	Selects the excitation wavelength as the reference of the automatic calculation of the pinhole size from the laser wavelengths, or enter it manually in the [A.U. Calculation Settings] dialog box. (For A.U. Calculation Settings, see Section 5.2.3.1, “Calculation Settings for Pinhole Size.”)

5.2.3.1 Calculation Settings for Pinhole Size

This section describes about the dialog box to calculate the pinhole size.

Click the [Pinhole] button in Acquisition window, the [A.U. Calculation Settings] dialog box appears. (Usually, the [Recommend] is selected to enable automatic calculation. [Recommend] calculates the A.U. value by using the Nikon-recommended EM and NA values.)

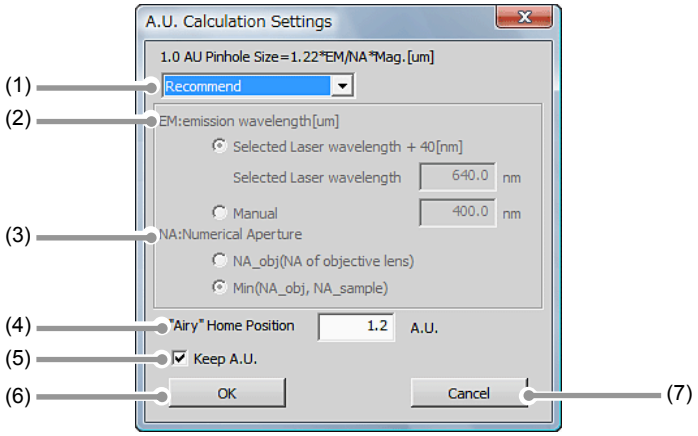


Figure 5.2-8 A.U. Calculation Settings dialog box

Table 5.2-5 A.U. Calculation Settings dialog box (sheet 1/2)

Name		Function	
(1)	Select calculation method	Recommend	Sets parameters automatically. (Recommended)
		User Setting	Allows the user to manually set parameters.
(2)	EM:emission wavelength[um]	Selected Laser wavelength	Calculates parameters by using the laser wavelength selected in the pinhole combo box of the Acquisition window as the emission wavelength (EM value). The wavelength displayed in the combo box is to be the laser wavelength set in the Optical Setting window.
		Manual	Allows the user to manually set parameters. (The parameter is calculated with the input value as the emission wavelength (EM value).) Enter the value directly from the keyboard.
(3)	NA: Numerical Aperture	Sets refractive index of the objective.	
		NA_obj(NA of objective lens)	Regardless of whether or not the objective NA value exceeds the refractive index of the sample (specimen), executes calculation by using the objective NA as the calculation parameter.
		Min(NA_obj, NA_sample)	When the objective NA value does not exceed the refractive index of the sample (specimen), executes calculation by using the objective NA as the calculation parameter. When the objective NA value exceeds the refractive index of the specimen, executes calculation by using the specimen refractive index.

Table 5.2-5 A.U. Calculation Settings dialog box (sheet 2/2)

Name		Function
(4)	“Airy” Home Position	Sets a home position of pinhole. Enter the value directly from the keyboard. * The pinhole size can be selected from six types in C2. Therefore, if the entered value does not match any of the types, the size that is larger than and the closest to the entered value is set as the home position.
(5)	Keep A.U. check box	When checked, the pinhole size is fixed by the A.U. when the selected wavelength or objective is changed. (However changes by the um.) When unchecked, the pinhole size is fixed by the um. (However changes by the A.U.) * The pinhole size can be selected from six types in C2. Therefore, if the to-be-fixed A.U. value does not match any of the types, the size that is larger than and the closest to the A.U. value is selected.
(6)	OK button	Determines the A.U. Calculation Settings applied and closes the [A.U. Calculation Settings] dialog box.
(7)	Cancel button	Discards the A.U. Calculation Settings applied and closes the [A.U. Calculation Settings] dialog box.

5.2.4 HV Linear Correction

When HV changes, Gain changes as shown in the graph captioned “Without HV Linear Correction” of Figure 5.2-9.
As HV increases, the gain variation (the variation of image brightness) is gradual initially, and it becomes steep beyond a certain point.
The gain variation can be automatically corrected to be linear with HV variation by the function called “HV Linear Correction.” With this correction, gain varies at the same rate as the HV adjustment.

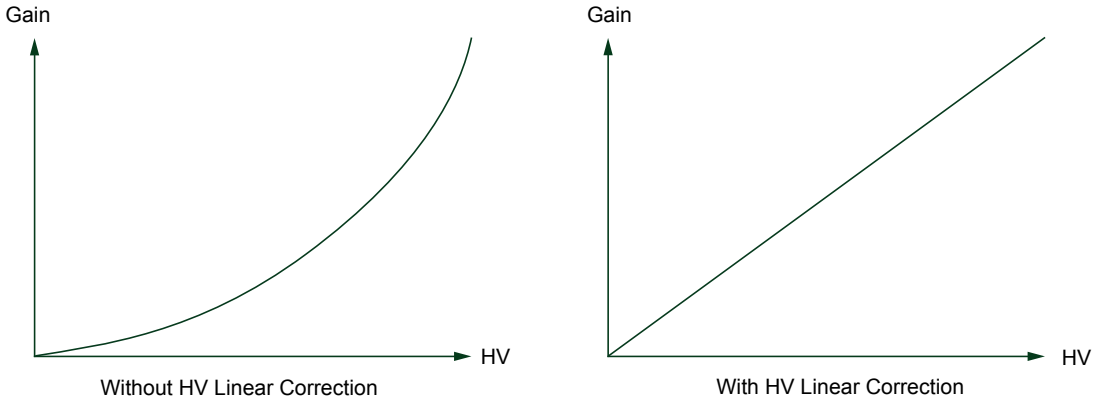


Figure 5.2-9 Gain vs. HV

To enable HV Linear Correction, check the HV Linear Correction check box.

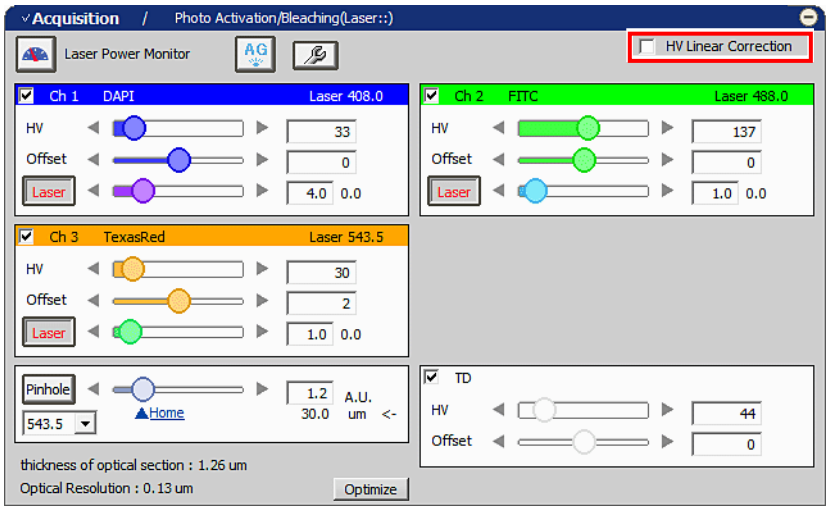


Figure 5.2-10 HV Linear Correction

- **When HV Linear Correction is enabled or disabled, HV is reset to 0 V once.**
- **If the Offset slider bar is moved, the accurate correction is not performed.**

5.2.5 Auto Gain

Auto Gain is a function to automatically correct the value of HV gain to set the optimum image brightness. Automatic HV gain correction is performed within the predetermined range of the ratio of saturation pixels.

Automatic HV gain correction is performed only when channels are selected.
For a TD, automatic adjustment is performed when it is selected.

After execution of Auto Gain, in the dialog box indicating the progress of Auto Gain, the correction values actually used (Ratio of saturation pixels) are displayed by channel.
For a channel on which Auto Gain failed, “x” is indicated and the HV value returns to its original value.

- **Auto Gain cannot be started during Scan.**
- **In line scan, Auto Gain is not executable.**
- **During execution of Auto Gain, do not execute manual adjustments in the Acquisition window.**

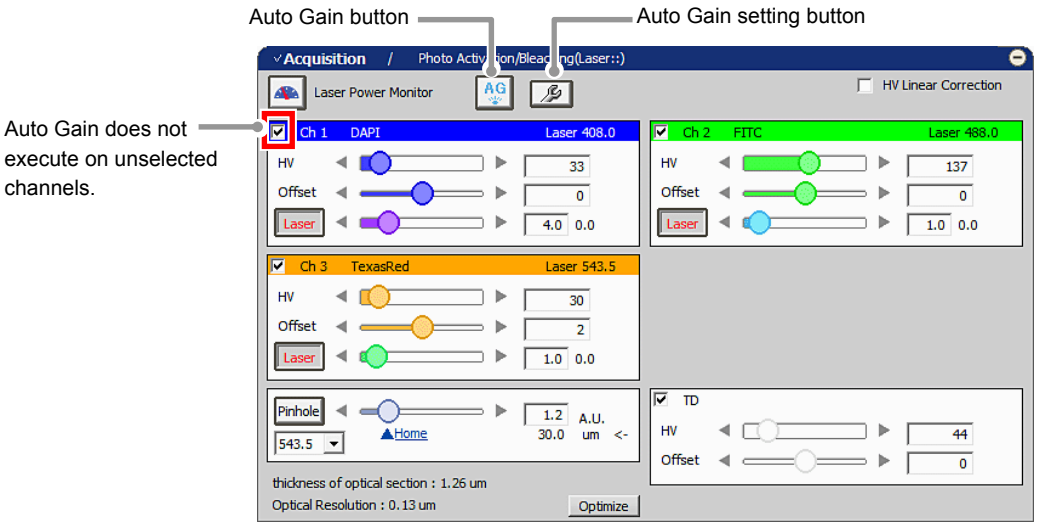


Figure 5.2-11 Execution of Auto Gain (Standard Detector-use)

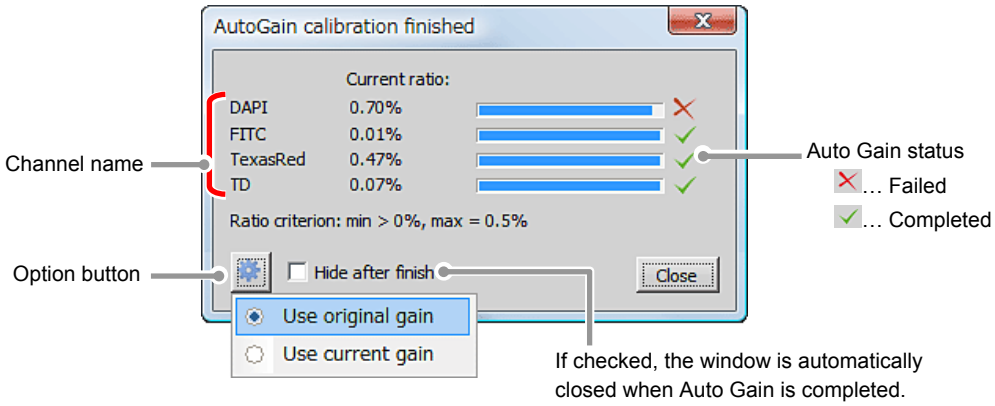


Figure 5.2-12 Auto Gain progress

Setting for Ratio of saturation pixels

Set the maximum and minimum value for the Ratio of saturation pixels used for automatic HV gain correction.

Click the [Auto Gain Setting] button to display the [Auto gain setup] dialog box.
Set the maximum and minimum value for the ratio of saturation pixels in [Auto gain setup] dialog box.

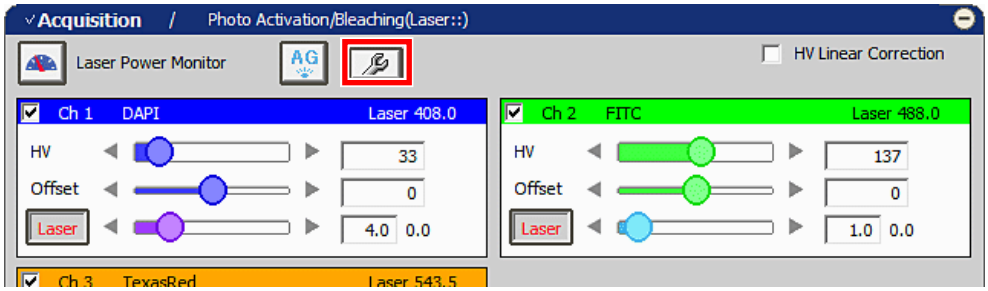


Figure 5.2-13 Displaying the Auto gain setup dialog box

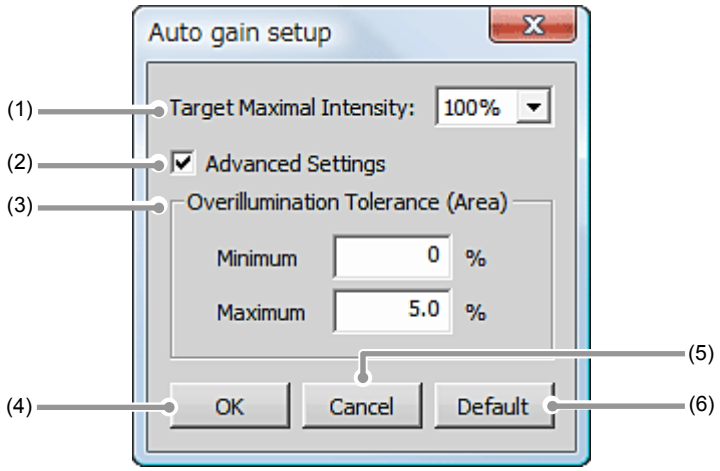


Figure 5.2-14 Setting for Ratio of saturation pixels

Table 5.2-6 Setting for Ratio of saturation pixels

Name		Function	
(1)	Target Maximal Intensity	Specifies the application ratio of the setting of the ratio of saturation pixels. Sets the percentage (%) of the maximum value to be applied.	
(2)	Advanced Settings	If checked, advanced settings of the ratio of saturation pixels are enabled.	
(3)	Overillumination Tolerance (Area)	Minimum	Sets the minimum value for Ratio of saturation pixels.
		Maximum	Sets the maximum value for Ratio of saturation pixels.
(4)	OK button	Determines the settings of Auto gain setup applied and closes the [Auto gain setup] dialog box.	
(5)	Cancel button	Discards the settings of Auto gain setup applied and closes the [Auto gain setup] dialog box.	
(6)	Default button	Resets the set values to the default values.	

5.3 Experiments by Using Lambda Series

By using the Lambda series as the function of the NIS-Elements AR, the multiwavelength excitation and emission experiments such as 2Ex 1Em (2 excitations 1 emission experiment) and 4Ex 4Em (4 excitations 4 emissions experiment) can be executed.

Multiwavelength excitation and emission experiment means an experiment made when using a fluorescence reagent whose fluorescence intensity varies with the wavelength of excitation lasers. The excitation lasers are changed, but the wavelength of acquired fluorescence is identical.

* About Lambda series:
When acquiring multiple excitation lights by emitting multiple lasers, the lasers are not emitted simultaneously but emitted in sequence.
By emitting lasers in sequence, the cross talk between channels can be avoided.

5.3.1 2Ex 1Em Acquisition

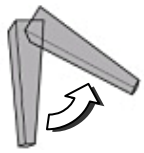
This section describes the settings to make the 2 excitations 1 emission experiment.

5.3.1.1 Procedure for 2Ex 1Em Settings

In the 2 excitations 1 emission experiment, the 2 excitation wavelengths are to be respectively registered to the optical configuration (hereinafter referred to as O.C.), and the O.C. is used by the Lambda series.

1 Switching the optical path of the C2 scan head

Switch the optical path changeover lever on the C2 scan head to the [Standard] position.



Standard position

Spectrum position

Figure 5.3-1 Switching the optical path of the C2 scan head

2 Setting the optical path of the 1st excitation wavelength to be registered to O.C.

1. Click the [Setting] button in the Filter and Dye window.
   For details of the Optical path settings, see Section 5.1, “Filter and Dye Window.”

Setting button

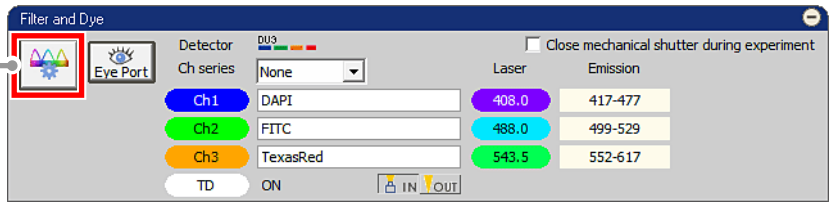


Figure 5.3-2 Filter and Dye window

2. Activate the Manual mode of Optical path setting.
   Click the [Manual] button.

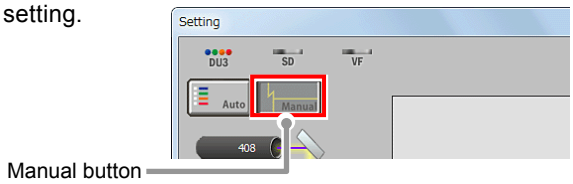


Figure 5.3-3 Selecting the Manual mode

3. Select a channel to be excited. (e.g. Ch1)

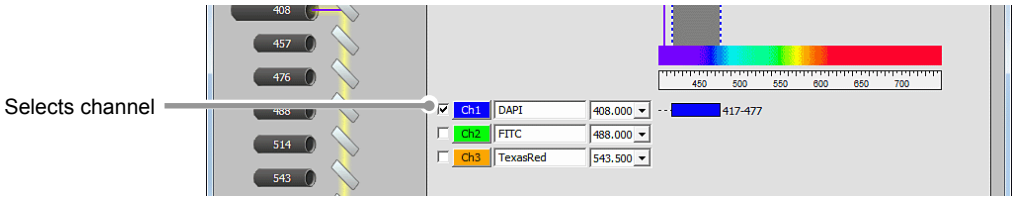


Figure 5.3-4 Selecting the channel

4. Select the 1st excitation wavelength. (e.g. 488nm)
   Select the wavelength for excitation lasers from the pull-down menu.

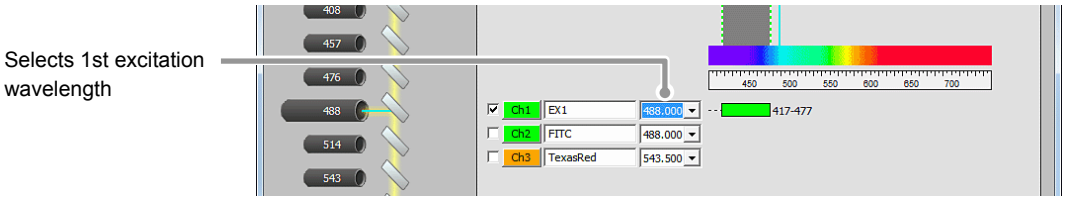


Figure 5.3-5 Selecting 1st excitation wavelength

5. Click [OK] button to determine the Optical path settings.

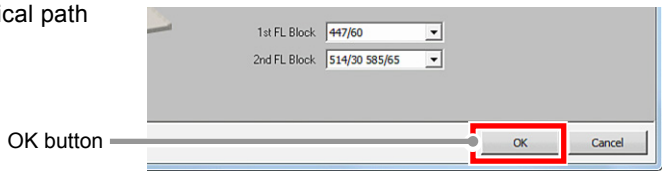


Figure 5.3-6 Optical path settings

6. In the Acquisition window, adjust a PMT for the excitation lasers.
   Set the HV value, the Offset value, and the Laser power value.
   For details of the acquisition settings, see Section 5.2, “Acquisition Window.”

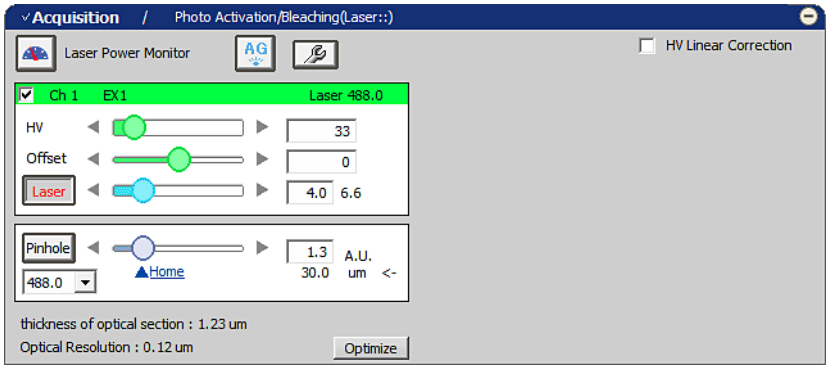


Figure 5.3-7 Acquisition window

3 Registering the set optical path as an O.C.

1. Select [Calibration] -> [New Optical Configuration] from the menu bar to open the Wizard.

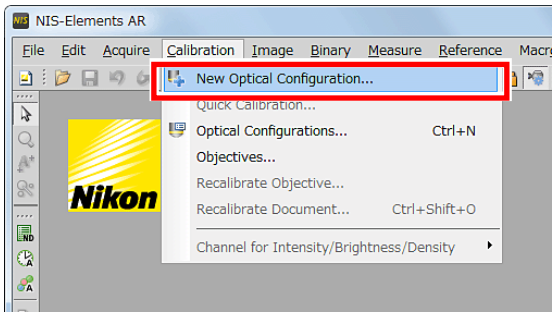


Figure 5.3-8 To display the Optical Configuration Wizard

2. Enter the name of O.C. to be registered, and then click the [Finish] button.

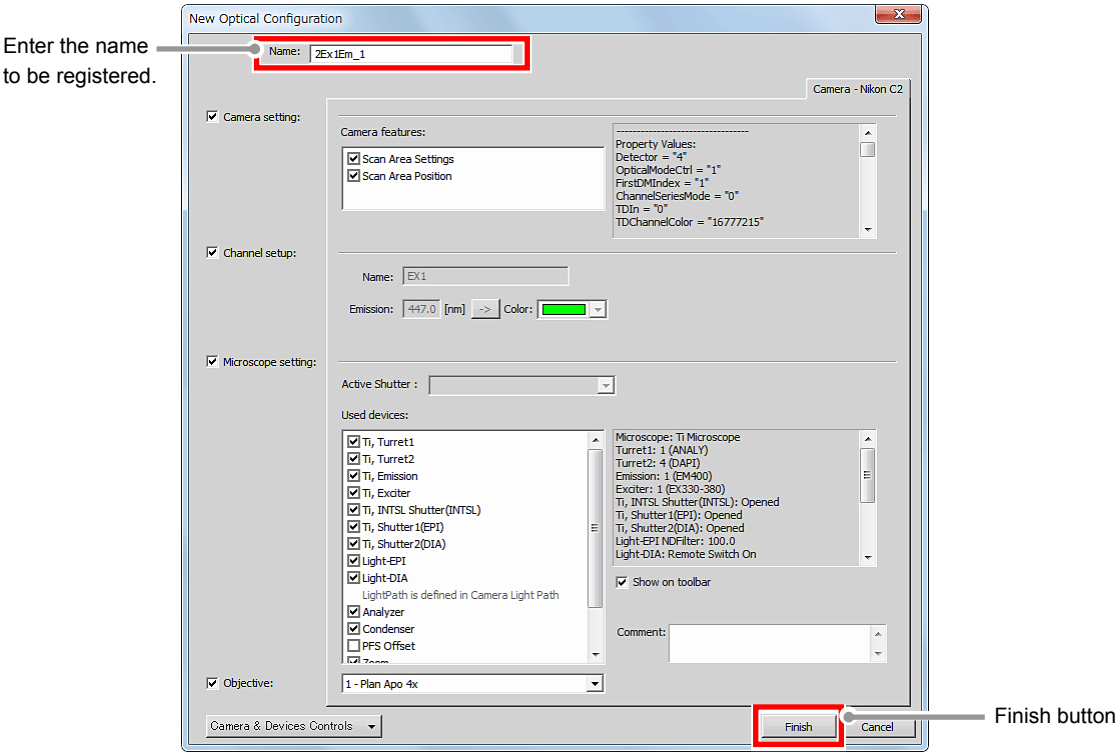


Figure 5.3-9 Registration of optical configuration

4 Setting the optical path of the 2nd excitation wavelength to be registered to O.C.

1. Click the [Setting] button in the Filter and Dye window.
   For details of the Optical path settings, see Section 5.1, “Filter and Dye Window.”

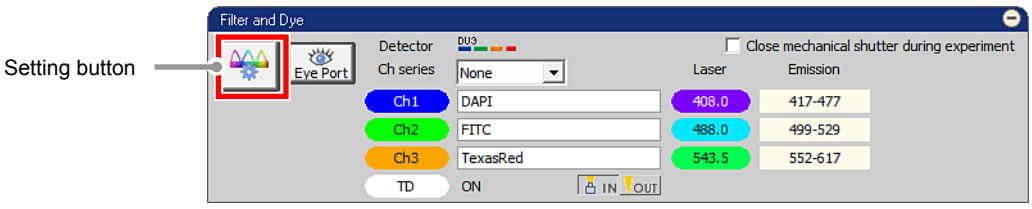


Figure 5.3-10 Filter and Dye window

2. Activate the Manual mode of Optical path setting.
   Click the [Manual] button.

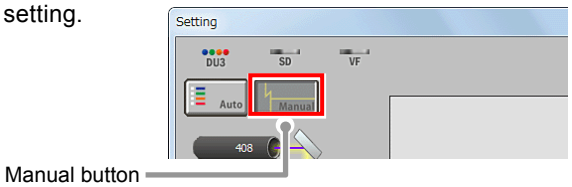


Figure 5.3-11 Selecting the Manual mode

3. Select the same channel as selected in the setting of the 1st excitation wavelength. (e.g. Ch1)

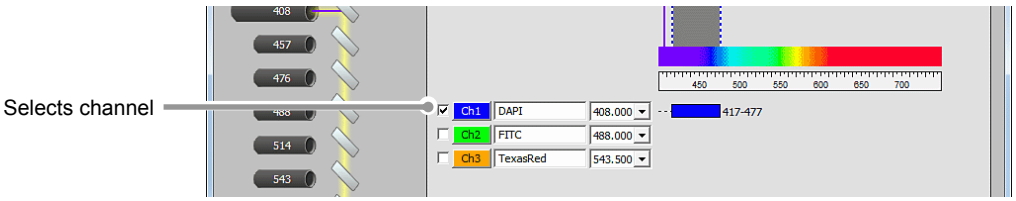


Figure 5.3-12 Selecting the channel

4. Select the 2nd excitation wavelength. (e.g. 543nm)
   Select the wavelength for excitation lasers from the pull-down menu.

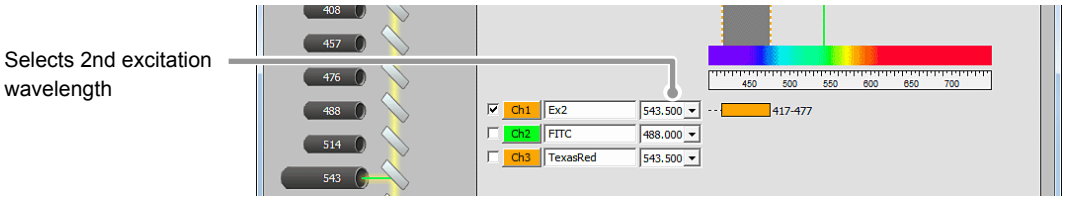


Figure 5.3-13 Selecting 2nd excitation wavelength

5. Click [OK] button to determine the Optical path settings.

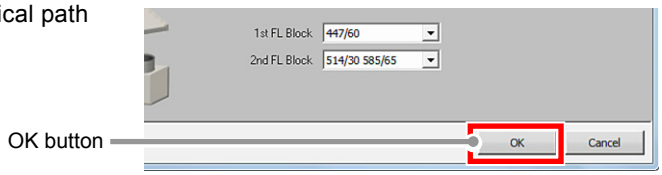


Figure 5.3-14 Optical path settings

6. In the Acquisition window, adjust a PMT for the excitation lasers.
   Set the HV value, the Offset value, and the Laser power value.
   For details of the acquisition settings, see Section 5.2, “Acquisition Window.”

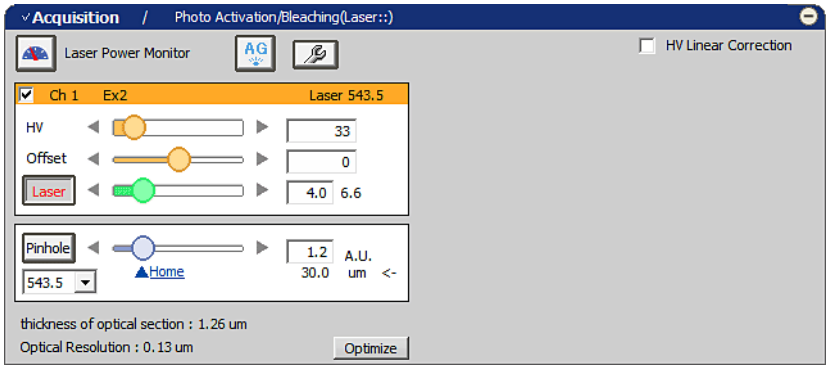


Figure 5.3-15 Acquisition window

5 Registering the optical path of the 2nd excitation wavelength as the second O.C.

1. Select [Calibration] -> [New Optical Configuration] from the menu bar to open the Wizard.

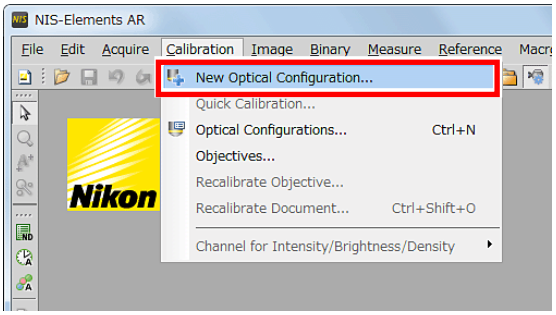


Figure 5.3-16 To display the Optical Configuration Wizard

2. Enter the name of O.C. to be registered, and then click the [Finish] button.

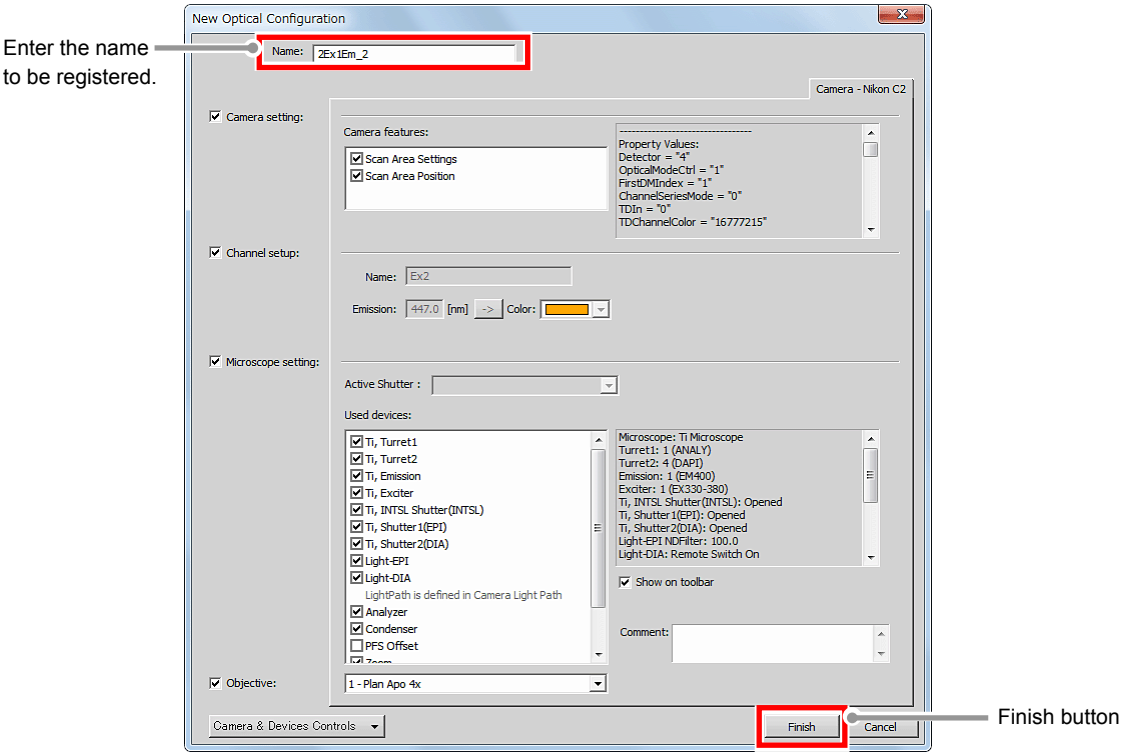


Figure 5.3-17 Registration of optical configuration

6 Execute the Lambda series

1. Select [Applications] -> [Define/Run Experiment...] from the menu bar to open the [ND Acquisition] dialog box.

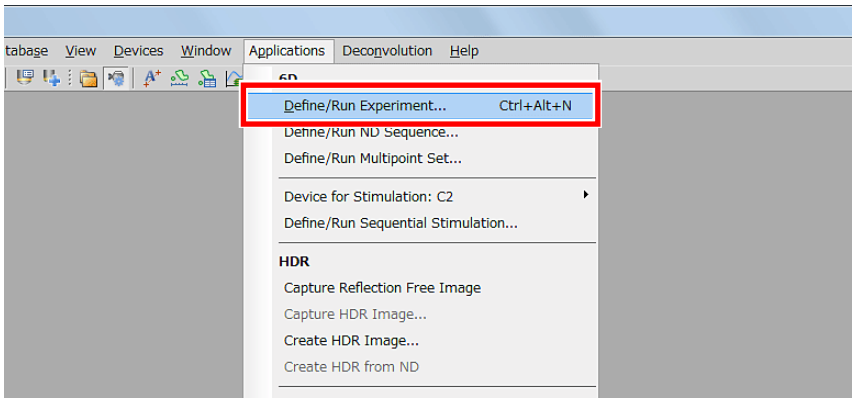


Figure 5.3-18 To display the ND Acquisition dialog box

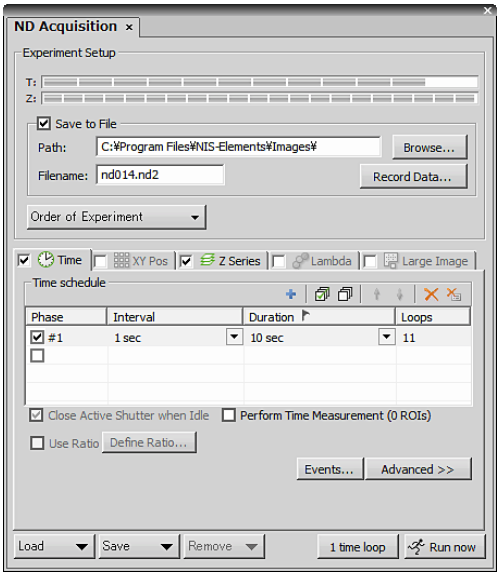


Figure 5.3-19 ND Acquisition dialog box

2. Select and check the [Lambda] tab among the tabs displayed in the [ND Acquisition] dialog box.
3. In the first column of the [Optical Conf.], select the O.C. to which the optical path of the 1st excitation wavelength has been registered.
4. In the second column of the [Optical Conf.], select the O.C. to which the optical path of the 2nd excitation wavelength has been registered.
5. Click [Run Now] button to acquire the image.

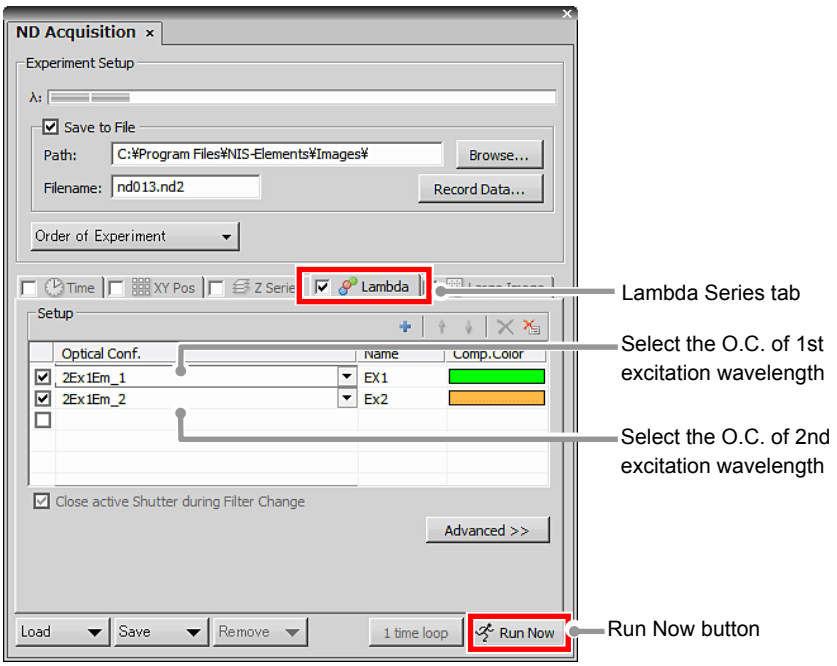


Figure 5.3-20 ND Acquisition dialog box

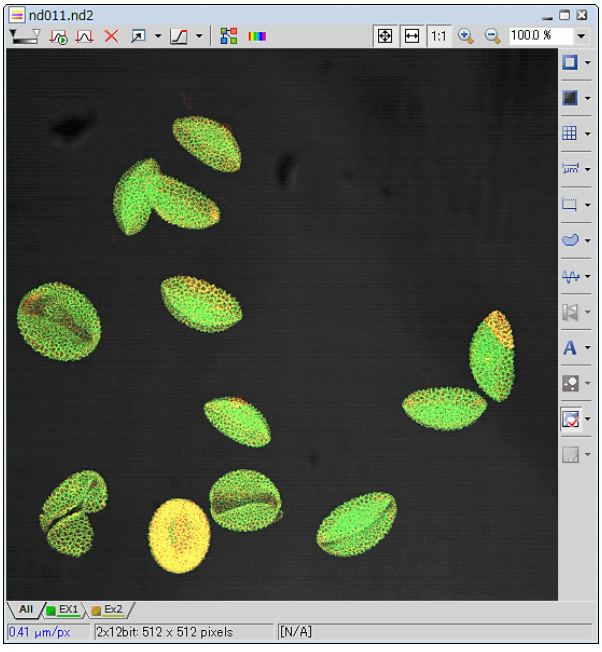


Figure 5.3-21 Acquired image

5.3.2 4Ex 4Em Acquisition

This section describes the settings to make the 4 excitations 4 emissions experiment.

To execute the 4 excitations 4 emissions experiment, a special filter block for “DAPI/CY5 Dual” needs to be used.

5.3.2.1 Procedure for 4Ex 4Em Settings

In the 4 excitations 4 emission experiment, the 4 excitation wavelengths are to be separately registered to the 2 optical configurations (hereinafter referred to as O.C.), and the O.C. is used by the Lambda series. (e.g., if the 4 channels of DAPI, FITC, TRITC, and Cy5 are to be acquired, the lasers of 405, 488, 543, and 640 are to be used.)

1 Switching the optical path of the C2 scan head

Switch the optical path changeover lever on the C2 scan head to the [Standard] position.

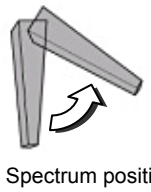


Figure 5.3-22 Switching the optical path of the C2 scan head

2 Setting the optical path of the 1st, 2nd, and 3rd excitation wavelength

1. Click the [Setting] button in the Filter and Dye window.
   For details of the Optical path settings, see Section 5.1, “Filter and Dye Window.”

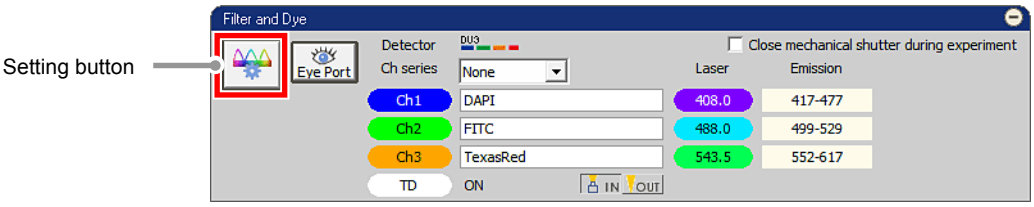


Figure 5.3-23 Filter and Dye window

2. Activate the Manual mode of Optical path setting.
   Click the [Manual] button.

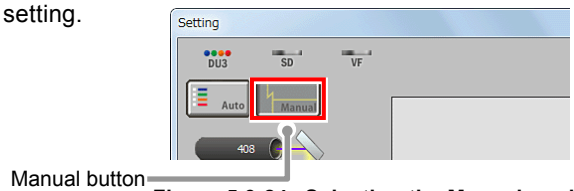


Figure 5.3-24 Selecting the Manual mode

3. Select all of the three channels to be excited.

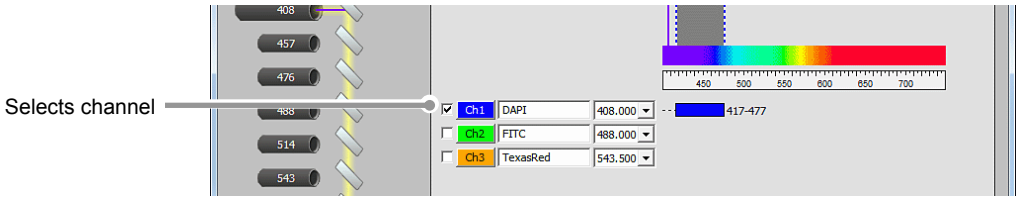


Figure 5.3-25 Selecting the channel

4. Assign an excitation wavelength to each of the selected channels. (e.g. 405nm, 488nm, and 543nm)
   Select the wavelength for excitation lasers from the pull-down menu.

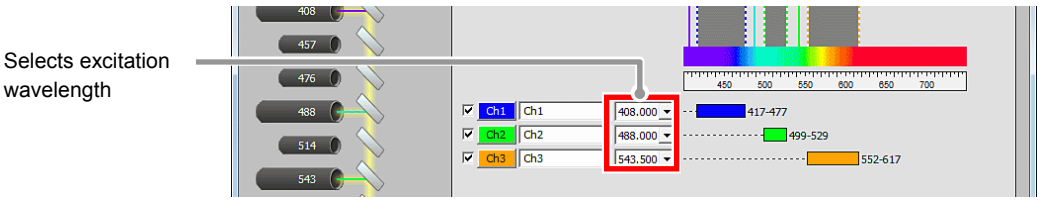


Figure 5.3-26 Selecting 1st, 2nd, and 3rd excitation wavelength

5. Click [OK] button to determine the Optical path settings.

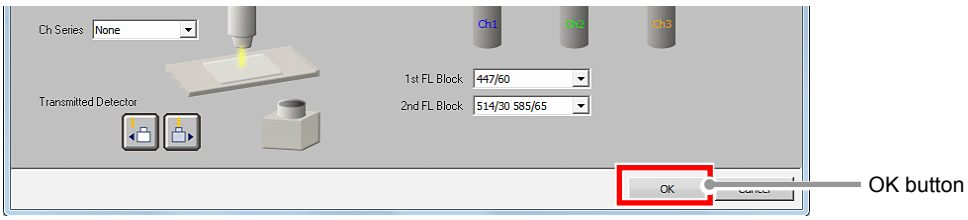


Figure 5.3-27 Optical path settings

6. In the Acquisition window, adjust a PMT for the excitation lasers.
   Set the HV value, the Offset value, and the Laser power value.
   For details of the acquisition settings, see Section 5.2, “Acquisition Window.”

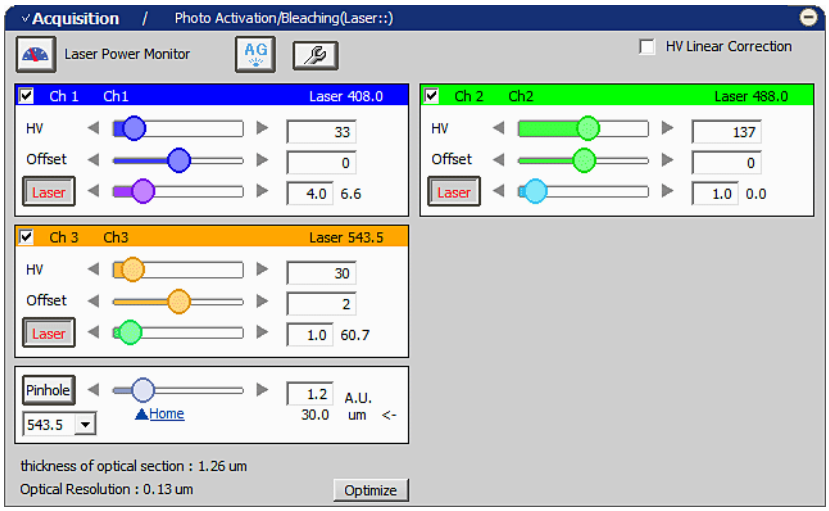


Figure 5.3-28 Acquisition window

3 Registering the set optical path as an O.C.

1. Select [Calibration] -> [New Optical Configuration] from the menu bar to open the Wizard.

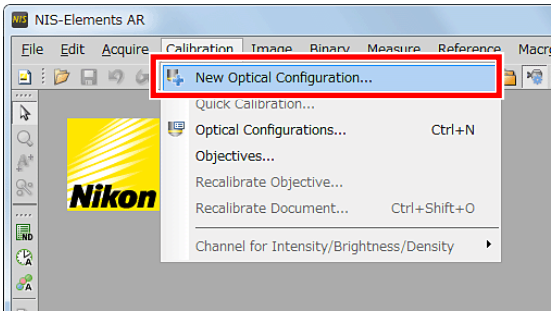


Figure 5.3-29 To display the Optical Configuration Wizard

2. Enter the name of O.C. to be registered, and then click the [Finish] button.

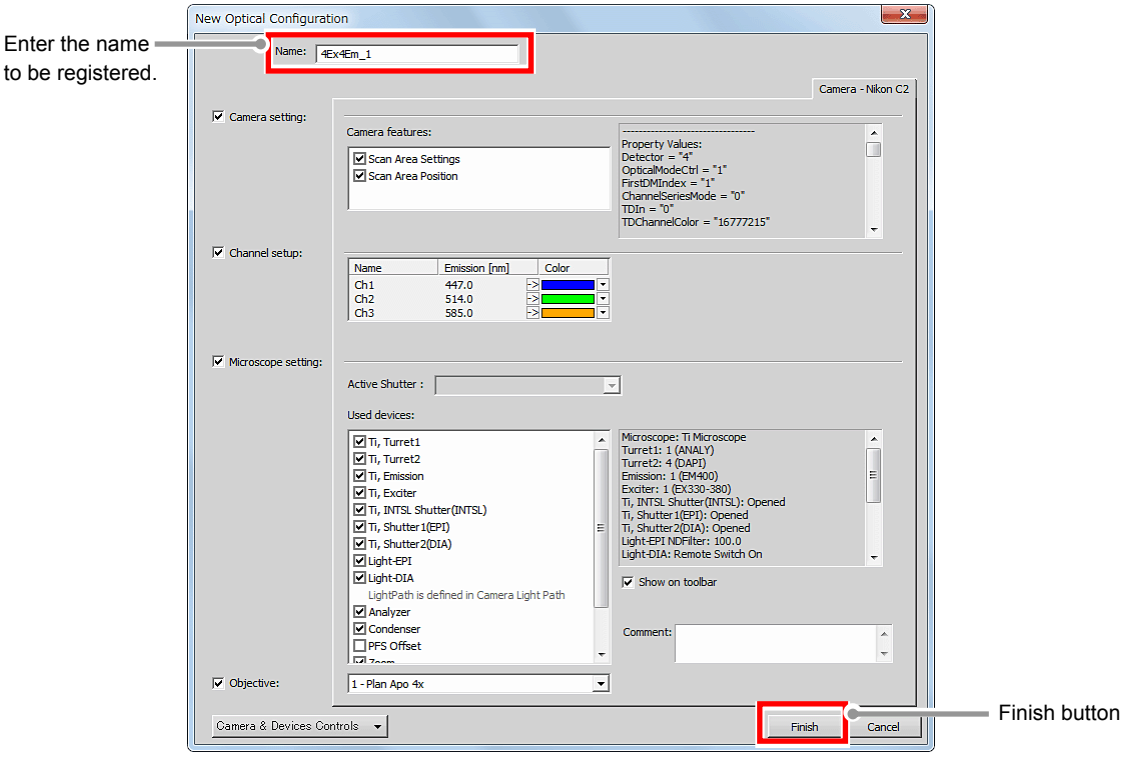


Figure 5.3-30 Registration of optical configuration

4 Setting the optical path of the 4th excitation wavelength to be registered to O.C.

1. Click the [Setting] button in the Filter and Dye window.
   For details of the Optical path settings, see Section 5.1, “Filter and Dye Window.”

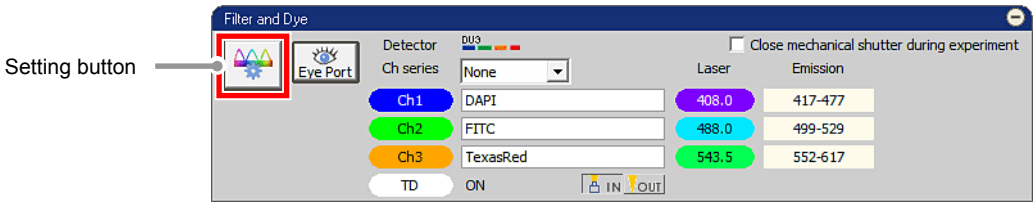


Figure 5.3-31 Filter and Dye window

2. Activate the Manual mode of Optical path setting.
   Click the [Manual] button.

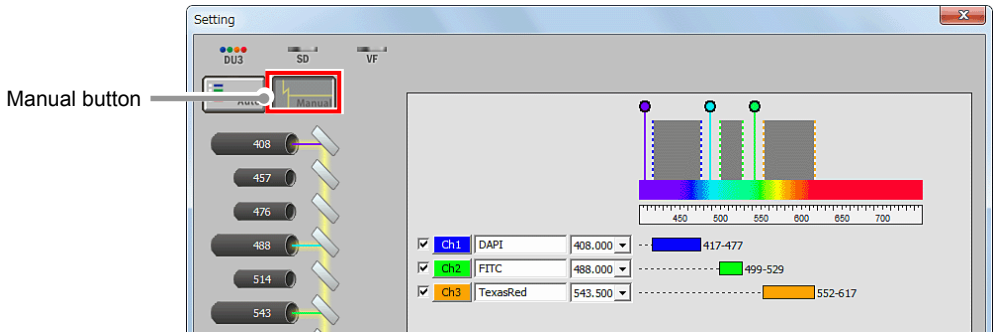


Figure 5.3-32 Selecting the Manual mode

3. Select a channel to be excited.

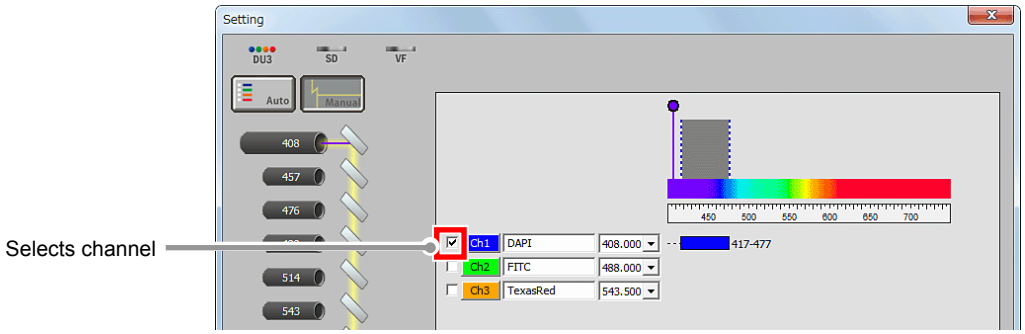


Figure 5.3-33 Selecting the channel

4. Select the excitation wavelength. (e.g. 640nm)
   Select the wavelength for excitation lasers from the pull-down menu.

* Set beforehand the special filter block for “DAPI/CY5 Dual” in the 1st FL Block.

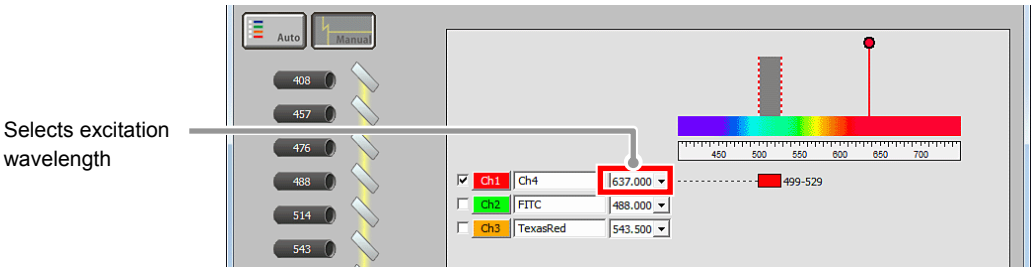


Figure 5.3-34 Selecting 4th excitation wavelength

5. Click [OK] button to determine the Optical path settings.

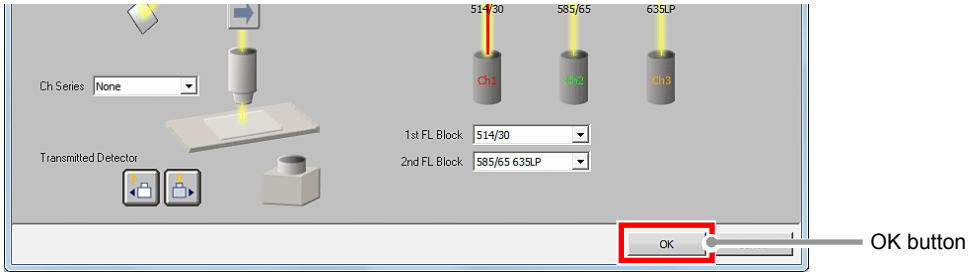


Figure 5.3-35 Optical path settings

6. In the Acquisition window, adjust a PMT for the excitation lasers.
   Set the HV value, the Offset value, and the Laser power value.
   For details of the acquisition settings, see Section 5.2, “Acquisition Window.”

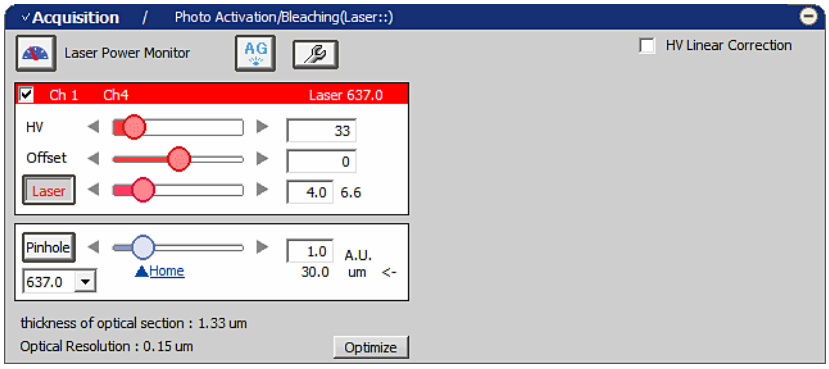


Figure 5.3-36 Acquisition window

5 Registering the optical path of the set 4th excitation wavelength as the 2nd O.C.

1. Select [Calibration] -> [New Optical Configuration] from the menu bar to open the Wizard.

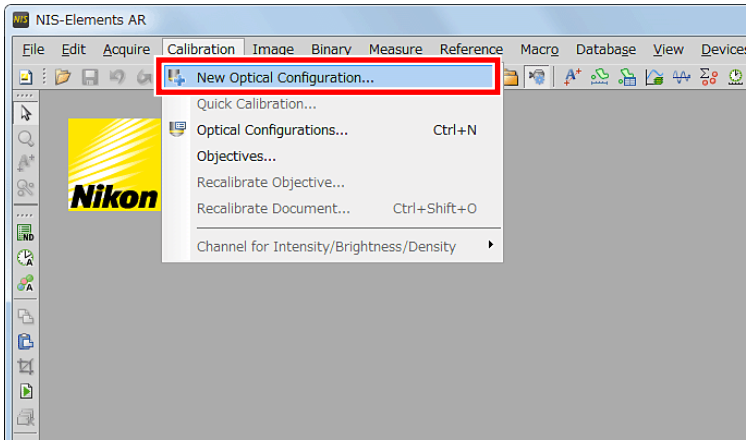


Figure 5.3-37 To display the Optical Configuration Wizard

2. Enter the name of O.C. to be registered, and then click the [Finish] button.

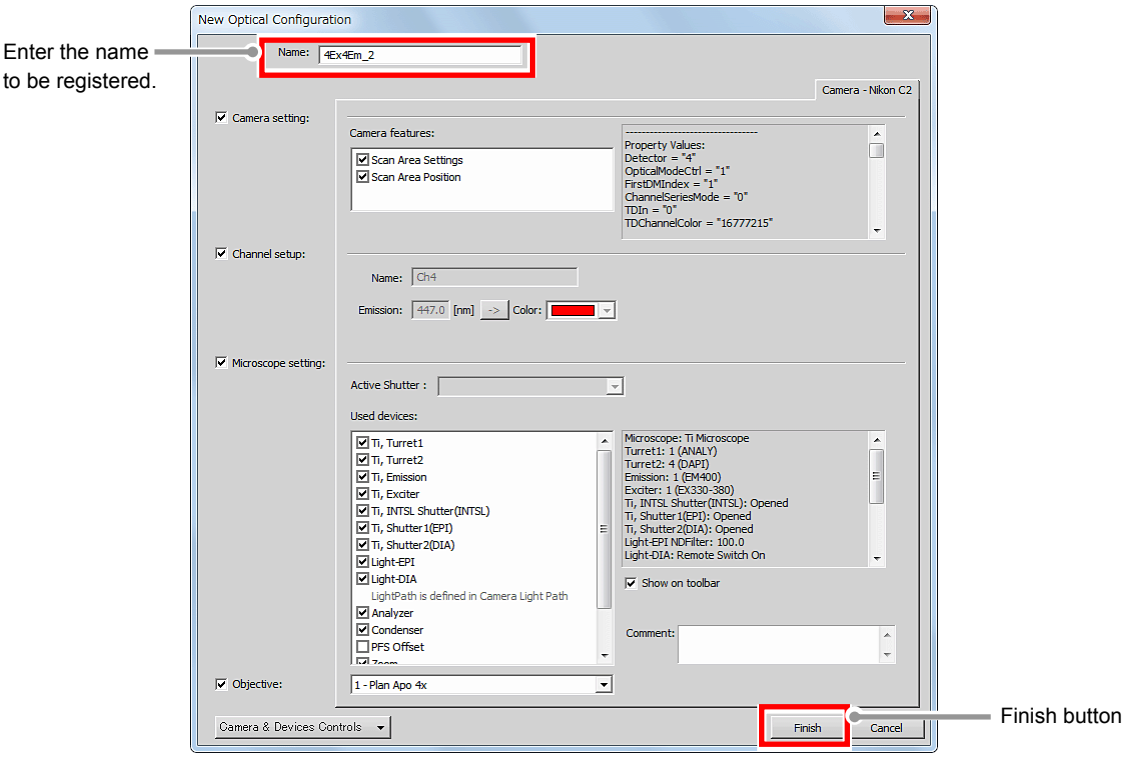


Figure 5.3-38 Registration of optical configuration

6 Execute the Lambda series

1. Select [Applications] -> [Define/Run Experiment...] from the menu bar to open the [ND Acquisition] dialog box.

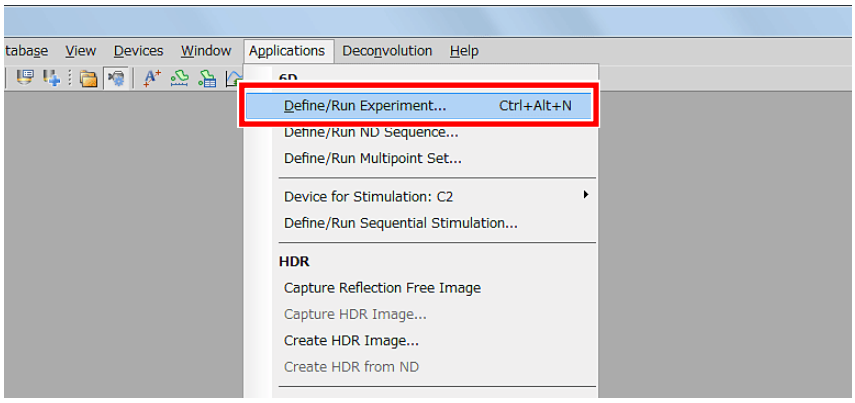


Figure 5.3-39 To display the ND Acquisition dialog box

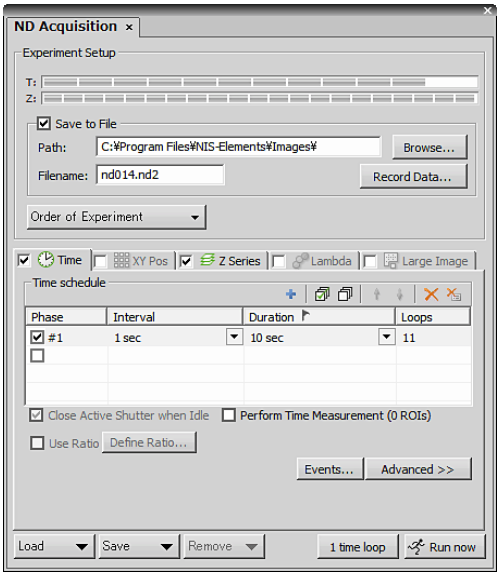


Figure 5.3-40 ND Acquisition dialog box

2. Select and check the [Lambda] tab among the tabs displayed in the [ND Acquisition] dialog box.
3. In the first column of the [Optical Conf.], select the O.C. to which the optical path of the 1st, 2nd, and 3rd excitation wavelengths have been registered.
4. In the second column of the [Optical Conf.], select the O.C. to which the optical path of the 4th excitation wavelength has been registered.
5. Click [Run now] button to acquire the image.

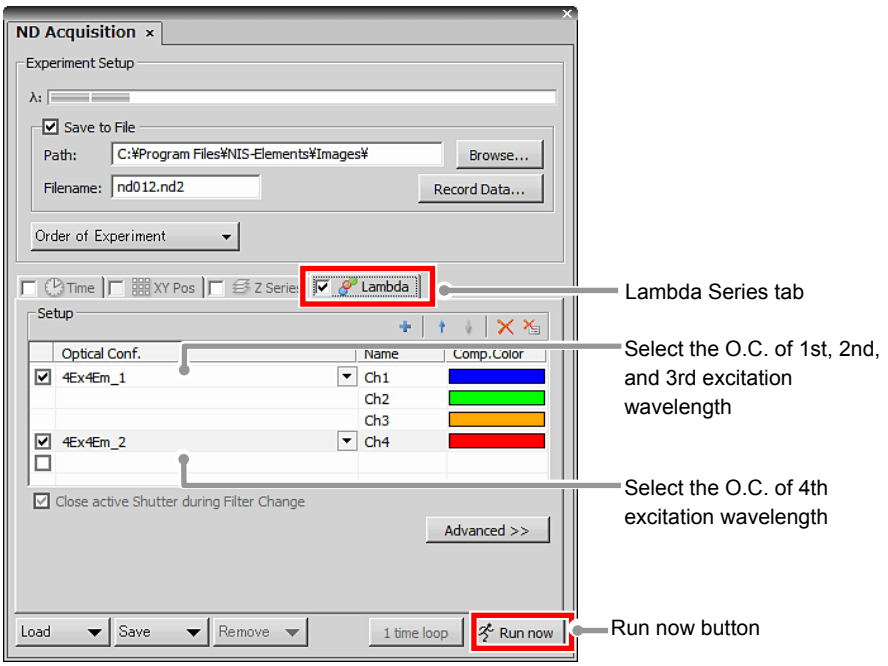


Figure 5.3-41 ND Acquisition dialog box

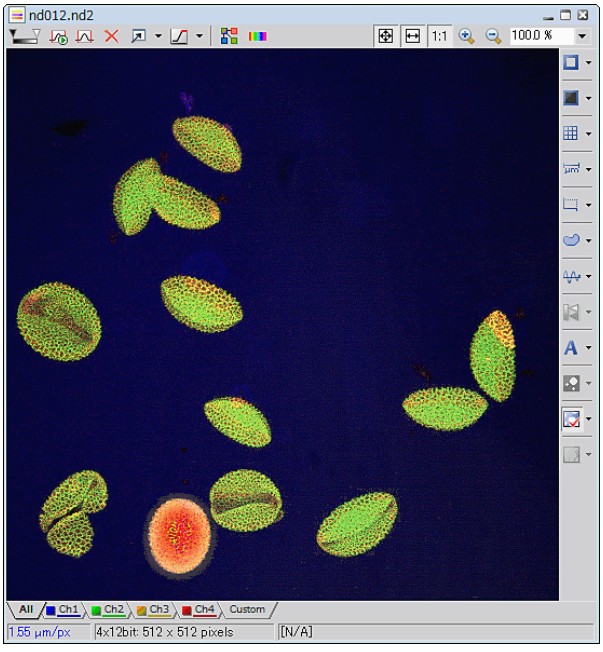


Figure 5.3-42 Acquired image

6 Detection Mode (Spectral Detector)

This chapter describes settings for the Spectral Detector mode.

6.1 Filter and Dye window

This window enables to set the Optical path.

The Spectral Detector mode can be used when the optical path changeover lever on the C2 scan head is set to the [Spectrum] position and the Spectral Detector (SD) is selected as the detection mode in the Optical path window.

6.1.1 Structure of Filter and Dye Window

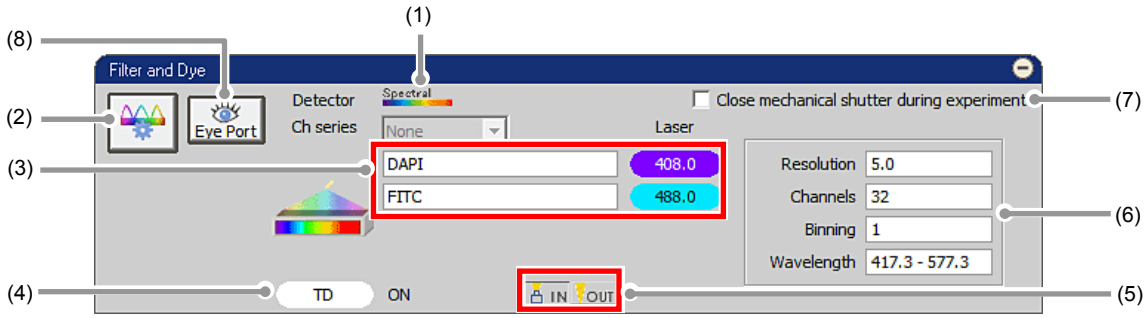


Figure 6.1-1 Filter and Dye window (Spectral Detector-use)

Table 6.1-1 Functions of Filter and Dye window (Spectral Detector-use)

Name		Function
(1)	Detector	Indicates that the Spectral Detector mode [SD] is used when the optical path changeover lever on the C2 scan head is set to the [Spectrum] position and the Spectral Detector (SD) is selected as the detection mode in the Optical path window.
(2)	Setting button	Opens the Optical path window. To use, select the detector, the dichroic mirror, the excitation laser, fluorescence dye for each excitation laser and others.
(3)	Status	Indicates for the settings for each excitation laser (fluorescence dye name, laser wavelength, and wavelength band to be acquired).
(4)	TD	Indicates the status of the motorized transmitted detector.
(5)	TD IN/OUT button	Sets/removes the motorized transmitted detector in/from the optical path. (IN = Set in the optical path/ OUT = Remove from the optical path) As for the case where the TD IN/OUT button is not displayed, it will be displayed when the motorized transmitted detector is set in the optical path in the Optical path window.
(6)	Spectral Detector setting information	Displays the information set on the Spectral Detector.
(7)	Close mechanical shutter during experiment	If unchecked, the shutter remains open during the ND image acquisition. As the shutter is not opened/closed every image acquisition, the time for the image acquisition can be shortened. However, the laser remains emitted during the interval.
(8)	Eye Port button	Changes optical path to eye port.

- **Optical Configuration**

  Individual data items set in the Spectral Detector mode can be managed collectively with the [Optical Configuration] dialog box.
  “NIS-Elements C” allows the user to store and retrieve the following settings: the laser power for image acquisition, offset of the transmission detector, PMT offset, excitation laser selection, pinhole size, photo activation laser selection, the laser power for photo activation, averages, scan area and others.
  For storing and retrieving the [Optical Configuration] settings, see the sections concerning the optical configuration in the “NIS-Elements Advanced Research User's Guide.”

6.1.2 Setting the Optical Path

Click the [Setting] button of “Filter and Dye” window to display the Optical path window.
The Spectral Detector mode [SD] setting screen is displayed when the optical path changeover lever on the C2 scan head is set to the [Spectrum] position and the Spectral Detector (SD) is selected as the detection mode in the Optical path window.

There are two modes available for Optical path setting, [Auto] and [Manual].
Normally, the auto mode should be used.

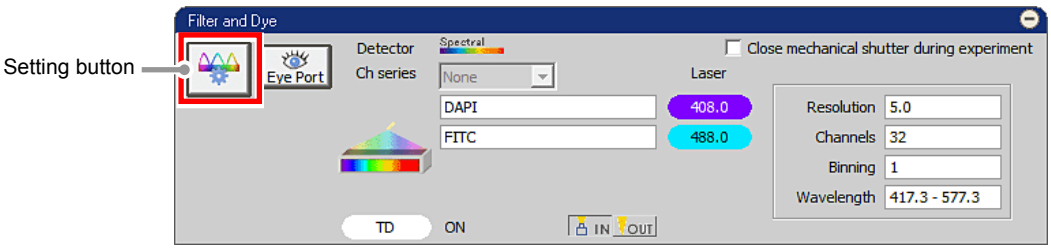


Figure 6.1-2 Filter and Dye window

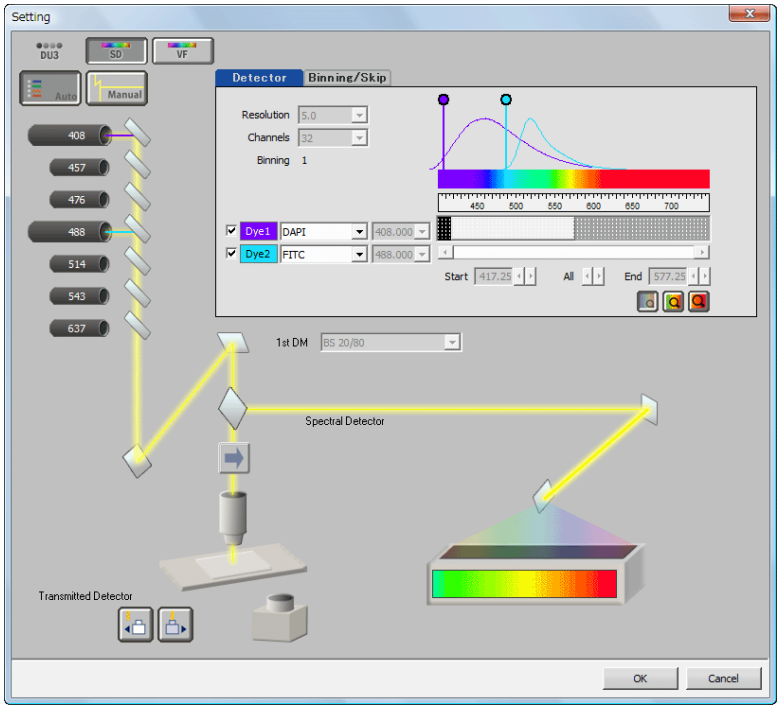


Figure 6.1-3 Optical path window (for auto mode)

6.1.3 Optical Path Window

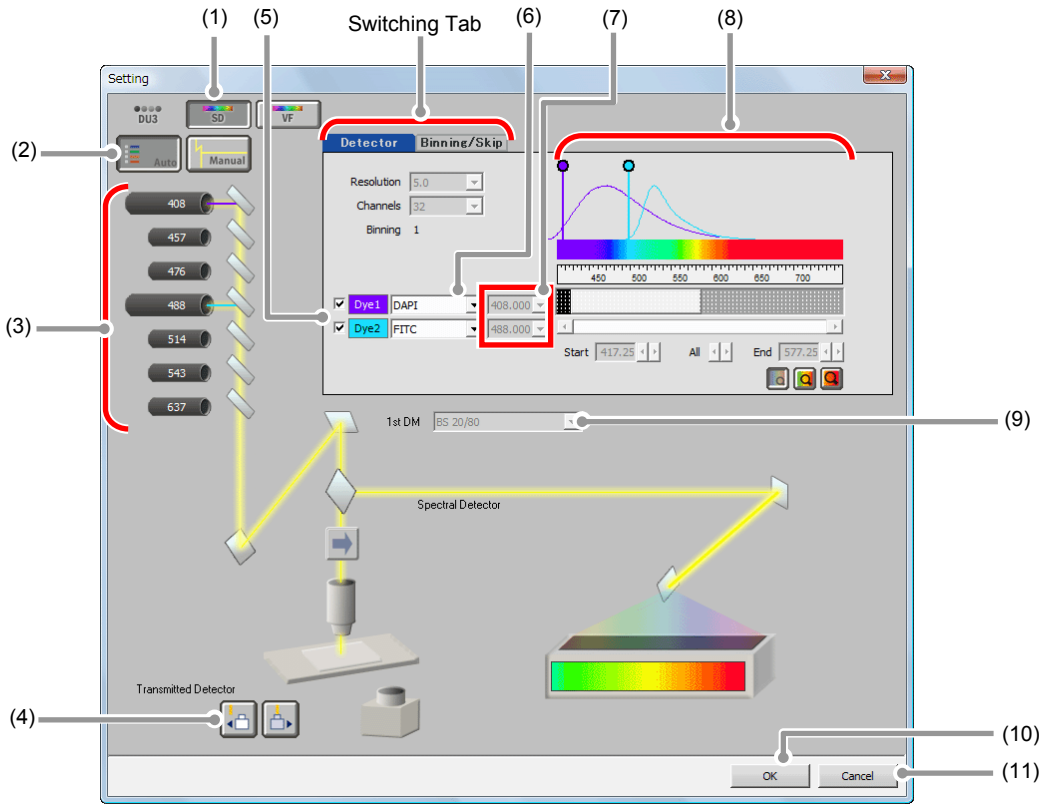


Figure 6.1-4 Optical path window (for auto mode, Spectral Detector-use)

Table 6.1-2 Functions of Optical path window (Spectral Detector-use) (sheet 1/2)

Name		Function	
(1)	Detection mode selection button		Enabled to select the Spectral Detector mode. Enables to acquire the 32-channel + TD spectral images simultaneously.
(2)	Mode selector	Selects the desired mode for setting the Optical path.	
			Activates the auto mode. Once the fluorescence dye to be used is selected, the appropriate laser and the dichroic mirror, and the acquired wavelength range and resolution are automatically selected. Up to 2 lasers can be selected.
			Activates the manual mode. Enables to set all of the laser, the dichroic mirror, acquired wavelength range, and resolution to be used manually. Up to 4 lasers can be selected.
(3)	Excitation laser indicator	Displays the current setting for the laser. The currently set laser icon is displayed in a large size, and the optical path is indicated.	
(4)	Transmitted detector selection button		Brings the transmitted detector into the Optical path, to enable the ability.
			Brings the transmitted detector out of the Optical path, to disable the ability.

Table 6.1-2 Functions of Optical path window (Spectral Detector-use) (sheet 2/2)

Name		Function
(5)	Excitation laser selection check box	Enables to select the excitation lasers to be used.
(6)	Fluorescence dye selection	These fields are only effective while in the auto mode. Selects the fluorescence dye name to be used for each excitation laser.
(7)	Excitation laser wavelength select	These fields are only effective while in the manual mode. Enables to set the laser wavelength that is set with the software configuration, regardless of the setting of the Filter block display/select.
(8)	Rainbow chart	Provides the following information: - Wavelength band for which to acquire images (shown in color and value for each excitation laser) - Spectral profile of fluorescence dye - Excitation laser for fluorescence dye - A color band indicating the wavelengths in the entire band (400 to 750 nm) - Scale of the wavelengths in the entire band (400 to 750 nm)
(9)	1st Dichroic mirror select	These fields are only effective while in the manual mode. Enables to manually select the 1st Dichroic mirror to be used.
(10)	OK button	Determines the Optical path settings applied and closes the Optical path window.
(11)	Cancel button	Discards the Optical path settings applied and closes the Optical path window.

- **About switching between SD and VF**

  SD → VF: The last settings in the Virtual Filter mode are recalled.

  VF → SD: The last settings in the Spectral Detector mode are recalled.

- **About the setting condition when the setting mode is switched**

  Auto mode → Manual mode:
  The entire settings in the Auto mode are retained.

  Manual mode → Auto mode:
  The last settings in the Auto mode are recalled.

6.1.4 Optical Path Window Switching Tab

The tab for switching between [Detector] and [Binning/Skip] is displayed on the right top of the Optical path window.

6.1.4.1 Detector Tab

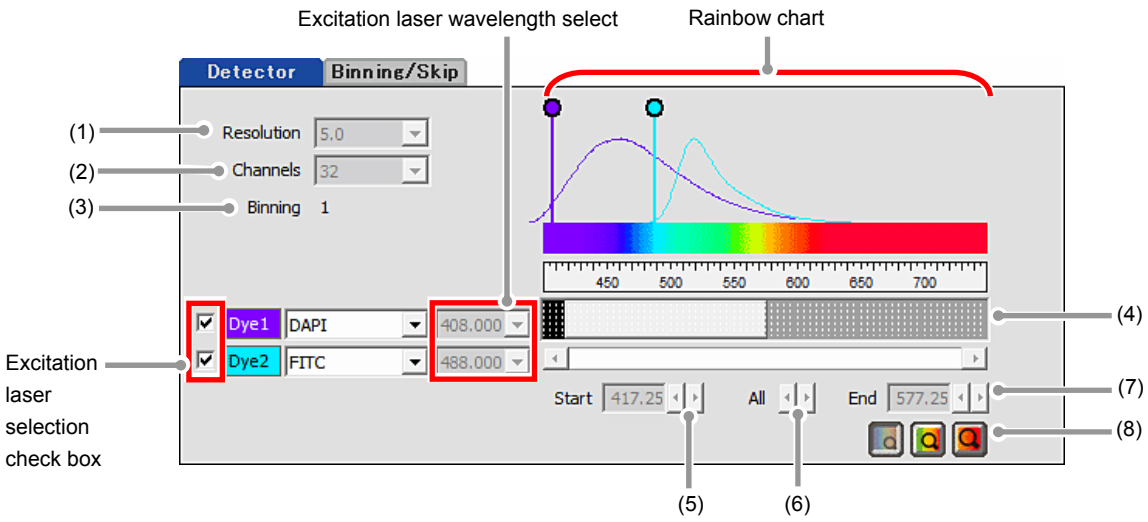


Figure 6.1-5 Optical path window (Detector tab)

Table 6.1-3 Functions of Detector tab

Name		Function
(1)	Resolution	Selects a wavelength resolution. (Enabled in the manual mode only.) Selectable from 2.5, 5, or 10nm.
(2)	Channels	Selects the number of channels (number of PMTs). (Enabled in the manual mode only.) Up to 32 channels can be selected in the wavelength range of 400nm to 750nm.
(3)	Binning	Displays the number of channel binning currently set.
(4)	Wavelength range setting bar	Sets a wavelength range in a wavelength range from 400nm to 750nm. (Enabled in the manual mode only.) Sets a range by shifting the wavelength range setting bar to the right or left or by enlarging or reducing it. (Linked with the above setting of the number of channels.) * A part of the wavelength range may be displayed in black depending on the setting conditions. In the wavelength range displayed in black, no wavelength range can be set.
(5)	Start	Displays the start wavelength of the wavelength range currently selected. Enabled to enlarge or reduce the range of the short wavelength in units of wavelength resolution with the right or left button in the manual mode.
(6)	All	In the currently selected wavelength range, enables shifting to the right or left in units of 0.25nm without changing the width of the wavelength. (Enabled in the manual mode only.)
(7)	End	Displays the end wavelength of the wavelength range currently selected. In the manual mode, the range of the long wavelength in units of wavelength resolution can be enlarged or reduced using the right and left buttons.
(8)	Enlarge button	Enlarges the rainbow chart. The display is switched in three levels.

- **Restriction on the detection wavelength range for the long wavelength**

  To prevent the incidence of the second-order light of excitation light to the detector, there are restrictions on the settings of the detection wavelength range for the long wavelength, as shown below.

  1 If there is a possibility of the incidence of the second-order light of excitation light to the detection wavelength range, the wavelength resolution of the diffraction grating is increased (the detection range is narrowed) to prevent the incidence of the second-order light of excitation light to the detection wavelength range.
  (The wavelength resolution of the diffraction grating automatically transits from 10.0nm to 5.00nm, and then to 2.50nm.)

  2 When the wavelength resolution is 2.50nm, the detection wavelength range is limited so as not to move to the wavelength longer than the wavelength of second-order light.

6.1.4.2 Binning/Skip Tab

With the inter-channel binning, the dark image can be brightened. (Enabled in the manual mode only.) Further, channels within the set wavelength range can be arbitrarily skipped. Since masked channel data is not acquired, the data volume can be reduced.
Set this tab after the setting of the [Detector] tab is determined.

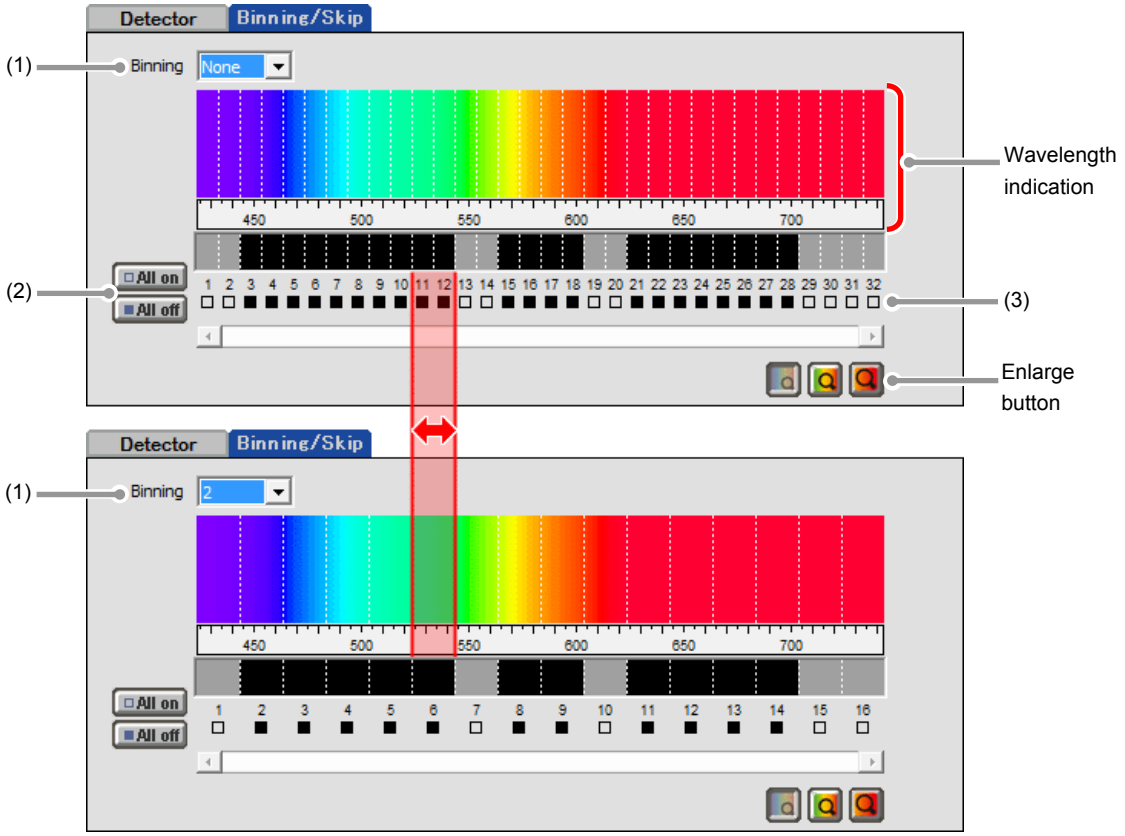


Figure 6.1-6 Optical Path window (Binning/Skip tab)

Table 6.1-4 Functions of Binning/Skip tab

Name		Function	
(1)	Binning	Sets the number of channels to be combined into one channel. Two to four channels can be set. When Binning is set, the number of channels set with the [Detector] tab is automatically re-set to the closest number of channels that can be divided by the binning value.	
(2)	PMT All on/off button	All on	Resets all PMT skips that have been set.
		All off	Leaves one channel and skips all of other PMTs.
(3)	PMT skip selection check box	Sets skip in each channel. If this box is clicked, ■ (black) is displayed and skip is set. Channel data with skip set is not acquired during scan.	

* If the setting of the [Detector] tab is changed, the setting with the [Binning/Skip] tab is cancelled.

6.2 Acquisition Window

The Acquisition window enables to set PMT brightness (detection sensitivity), laser power, and pinhole size.

6.2.1 Structure of Acquisition Window

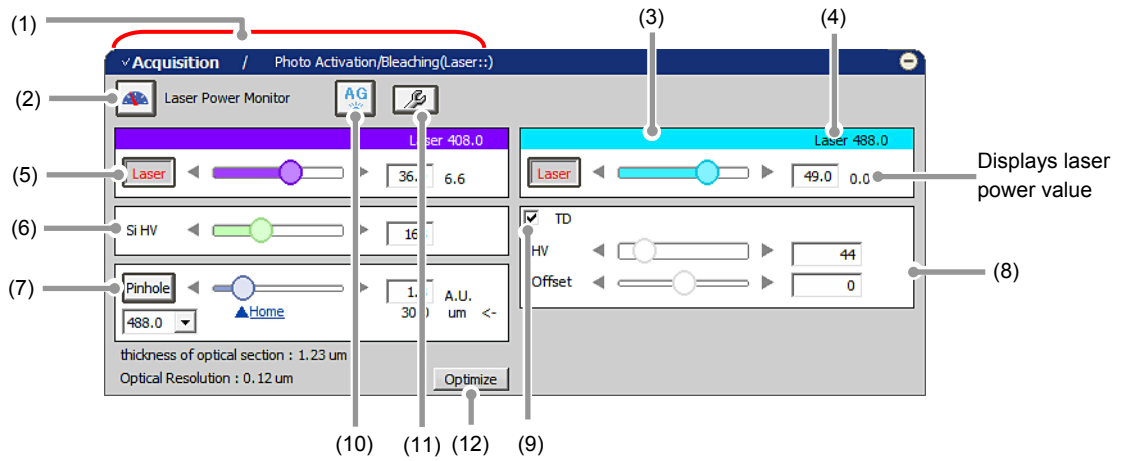


Figure 6.2-1 Acquisition window (Spectral Detector-use)

Table 6.2-1 Functions of Acquisition window (Spectral Detector-use) (sheet 1/2)

Name		Function	
(1)	Acquisition/Photo Activation window switching	Switches between the Acquisition and Photo Activation windows. For the Photo Activation window, see Chapter 10.	
(2)	Laser power monitor button	Displays the laser power value (integer obtained after A/D conversion divided by 10) of the current excitation laser by clicking this button. During the image acquisition, the laser power cannot be measured and this button is grayed out.	
(3)	Excitation laser color	Displays the excitation laser color specified in the Optical path window.	
(4)	Laser wavelength indication	The currently selected laser wavelength is indicated.	
(5)	Laser ON/OFF button	Selects whether the laser is emitted or not.	
		Laser ON status	The laser is emitted.
		Laser OFF status	The laser is not emitted. When switched from OFF to ON, the laser power value set in the previous ON status is applied.
(6)	Si HV	Adjusts HV of the Spectral Detector.	
(7)	Pinhole	Adjusts the pinhole size. For pinhole size, see Section 6.2.3, “Setting the Pinhole.”	
(8)	Brightness adjustment for transmitted detector	For the transmitted detector, use the HV and Offset controls to adjust the brightness of the live image.	
(9)	TD channel selection	Enables to acquire TD images by checking the check box.	

Table 6.2-1 Functions of Acquisition window (Spectral Detector-use) (sheet 2/2)

Name		Function
(10)	AG button	Automatically adjusts the Si HV value (Si HV gain) of the currently selected excitation laser to the optimum values. For Auto Gain, see Section 6.2.4, “Auto Gain.”
(11)	Auto Gain setting button	Sets the ratio of saturation pixels used for automatic Si HV gain correction. The dialog box for range of the ratio of saturation pixels settings appears when this button is clicked. For Setting for Ratio of saturation pixels, see “Setting for Ratio of saturation pixels” in the Section 6.2.4, “Auto Gain.”
(12)	Optimize button	Displays the [XYZ Size Setup] dialog box. In the [XYZ Size Setup] dialog box, the calculation method of the recommended values of the resolution, zoom magnification, and Z stack step size can be set. For [XYZ Size Setup] dialog box, see Section 6.2.1.1, “Recommended Value Indication/Automatic Application” in the next page.

6.2.1.1 Recommended Value Indication/Automatic Application

By the function of the recommended value indication/automatic application, the recommended values of the appropriate resolution, zoom magnification, and Z stack step size are calculated based on the objective type and the selected excitation wavelength.
Using the calculated recommended values enables the image acquisition clearer and with less damage to the specimen.

Recommended Value Automatic Application

To automatically apply the recommended values to the parameters, set the [Nyquist XY] button of the Scan Area window to ON.

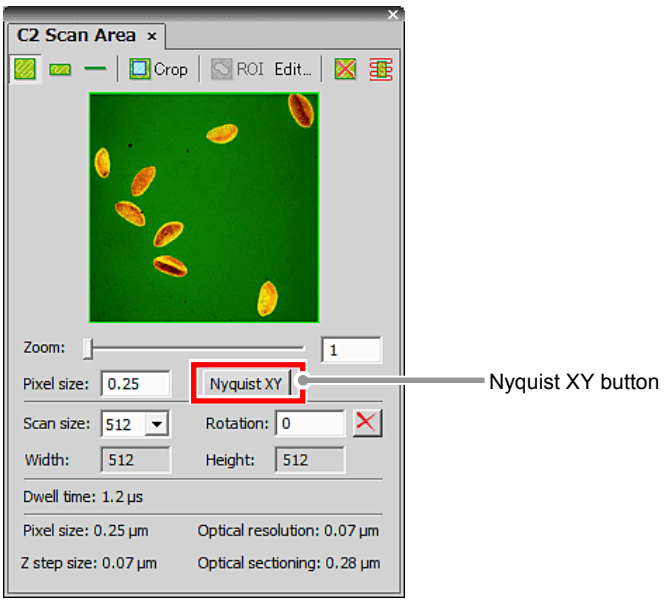


Figure 6.2-2 Scan Area window

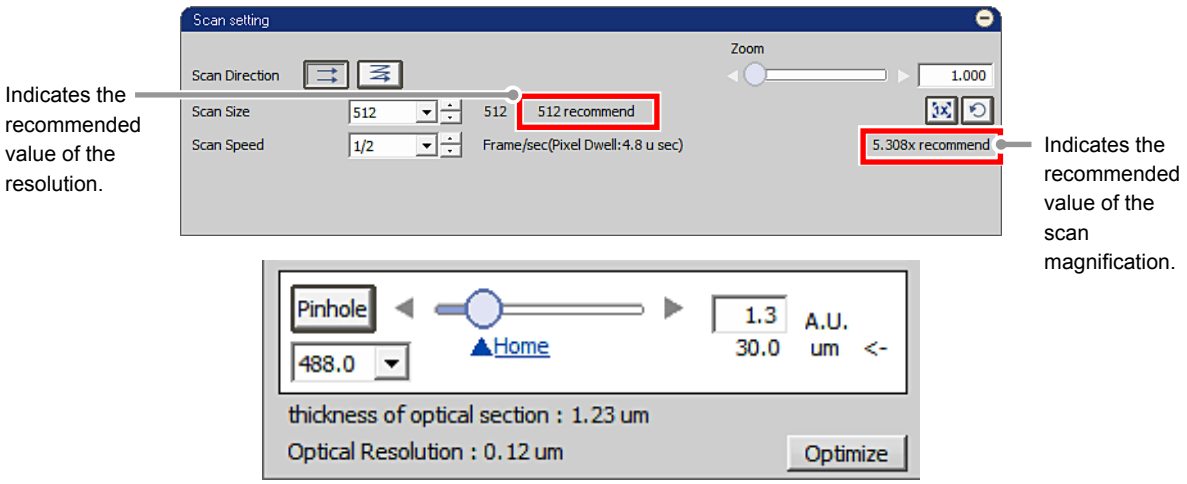


Figure 6.2-3 Location of Recommended Value Indication

* When the laser or objective in use is changed, the recommended values are recalculated, and newly indicated and automatically applied.

Recommended Value Settings

Detailed settings of the recommended values are made in the [XYZ Size Setup] dialog box that is displayed by clicking the [Optimize] button of the Acquisition window.
If the [Nyquist XY] button of the Scan Area window is ON, the recommended values are automatically applied to the parameters.
Or if the [Nyquist XY] button is OFF, the recommended values of the scan size and zoom are indicated in the Scan setting window.

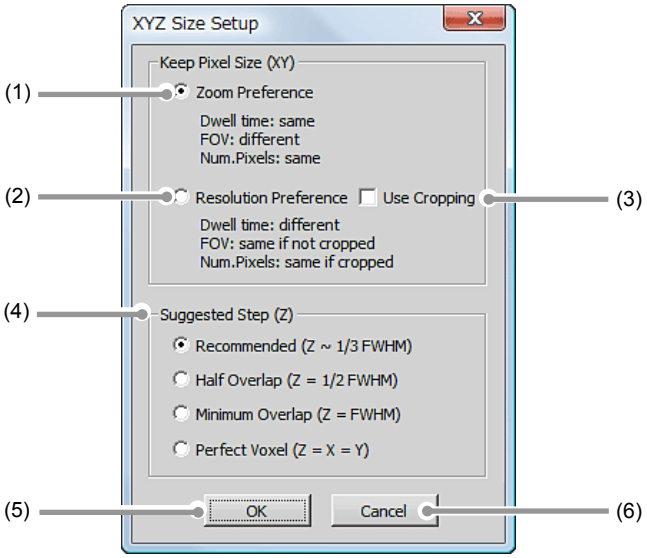


Figure 6.2-4 XYZ Size Setup dialog box

Table 6.2-2 Functions of XYZ Size Setup dialog box

Name		Function	
(1)	Zoom Preference	When the [Nyquist XY] button is ON, keeps the scan size and applied the recommended value of the zoom.	
(2)	Resolution Preference	When the [Nyquist XY] button is ON, keeps the zoom and applied the recommended value of the scan size.	
(3)	Use Cropping	Fits the scan size in detail by using Crop Scan.	
(4)	Suggested Step (Z)	Sets the Z step size calculation method.	
		Recommend (Z~1/3 FWHM)	Approximately one third of the thickness of optical section (FWHM value).
		Half Overlap (Z=1/2 FWHM)	One half of the thickness of optical section (FWHM value).
		Minimum Overlap (Z=FWHM)	The thickness of optical section (FWHM value).
		Perfect Voxel (Z=X=Y)	Value same as the pixel size.
(5)	OK button	Determines the XYZ Size Setup applied and closes the [XYZ Size Setup] dialog box.	
(6)	Cancel button	Discards the XYZ Size Setup applied and closes the [XYZ Size Setup] dialog box.	

6.2.2 Setting Image Brightness

For each excitation laser, adjust HV, Offset, and Laser to obtain clear images.

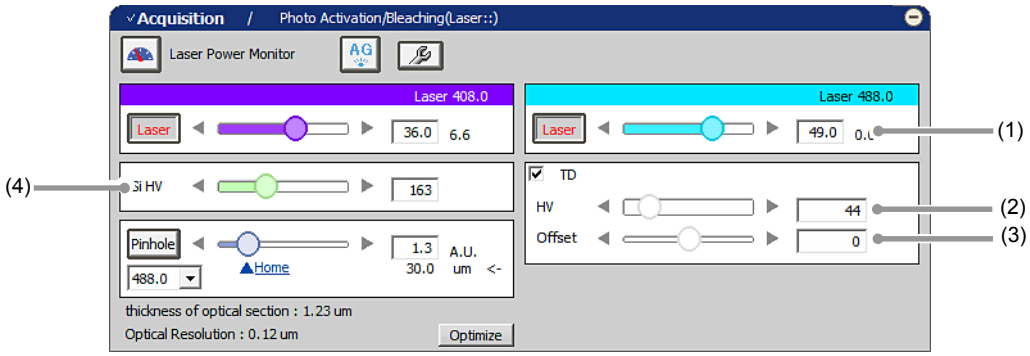


Figure 6.2-5 Setting the live image brightness (Spectral Detector-use)

Table 6.2-3 Brightness adjustment functions for the live image (Spectral Detector-use)

Name		Function
(1)	Laser	Sets the laser power value. Slider bar: Slides to the right or left to set the laser power value. Arrow buttons: Click either arrow button to increase or decrease the laser power value stepwise. Direct entry in laser power value display field: Type the desired setting value.
(2)	HV	Sets the voltage to be applied to the transmitted detector. Slider bar: Slides to the right or left to set the HV value. Arrow buttons: Click either arrow button to increase or decrease the HV value stepwise. Direct entry in HV value display field: Type the desired setting value.
(3)	Offset	Sets the offset value of the transmitted detector. Slider bar: Slides to the right or left to set the offset value. Arrow buttons: Click either arrow button to increase or decrease the offset value stepwise. Direct entry in offset value display field: Type the desired setting value.
(4)	Si HV	Adjusts HV of the Spectral Detector. (Applied to all excitation lasers.) Slider bar: Slides to the right or left to set the Si HV value. Arrow buttons: Click either arrow button to increase or decrease the Si HV value stepwise. Direct entry in Si HV value display field: Type the desired setting value.

PMT Overload

If too much gain is applied to the illumination intensity, the gain is automatically shut down to protect PMT and/or transmitted detector (TD), and then following [PMT Overload] dialog box is displayed.
In this case, the Si HV of Spectral Detector and/or TD HV value becomes "0".
To continue the adjustment, set the Si HV and/or TD HV value again.

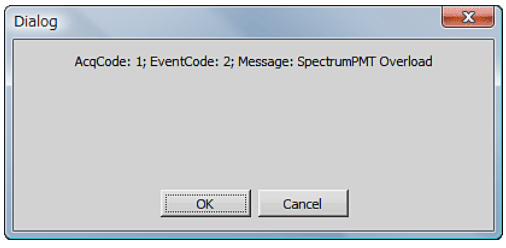


Figure 6.2-6 PMT Overload dialog box

6.2.3 Setting the Pinhole

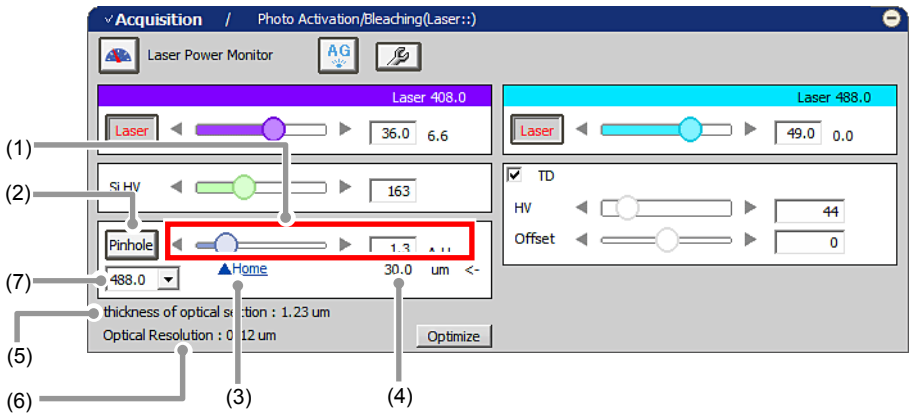


Figure 6.2-7 Setting the Pinhole (Spectral Detector-use)

Table 6.2-4 Pinhole setting functions (Spectral Detector-use)

Name		Function
(1)	Pinhole size setting	Sets a pinhole size for C2 system. Slider bar: Slides to the right or left to set the pinhole size. (Unit: A.U.) Arrow buttons: Click either arrow button to increase or decrease the pinhole size stepwise. Direct entry in pinhole size display field: Type the desired setting value.
(2)	Pinhole button	Displays the [A.U. Calculation Settings] dialog box to calculate the pinhole size. (For A.U. Calculation Settings, see Section 6.2.3.1, “Calculation Settings for Pinhole Size.”)
(3)	Home	Changes the pinhole to the predetermined home position. The value of the home position can be changed in the [A.U. Calculation Settings] dialog box. (For A.U. Calculation Settings, see Section 6.2.3.1, “Calculation Settings for Pinhole Size.”)
(4)	Pinhole size	Indicates pinhole size of C2 system. (Unit: um)
(5)	thickness of optical section	Indicates the FWHM (full width at half maximum) of z airy disk.
(6)	Optical Resolution	The actual size of 1 pixel square calculated from the optical information (for objectives and scan parameters) and the size acquired from an image.
(7)	Reference excitation wavelength for the pinhole size calculation	Selects the excitation wavelength as the reference of the automatic calculation of the pinhole size from the laser wavelengths, or enter it manually in the [A.U. Calculation Settings] dialog box. (For A.U. Calculation Settings, see Section 6.2.3.1, “Calculation Settings for Pinhole Size.”)

6.2.3.1 Calculation Settings for Pinhole Size

This section describes about the dialog box to calculate the pinhole size.

Click the [Pinhole] button in Acquisition window, the [A.U. Calculation Settings] dialog box appears. (Usually, the [Recommend] is selected to enable automatic calculation. [Recommend] calculates the A.U. value by using the Nikon-recommended EM and NA values.)

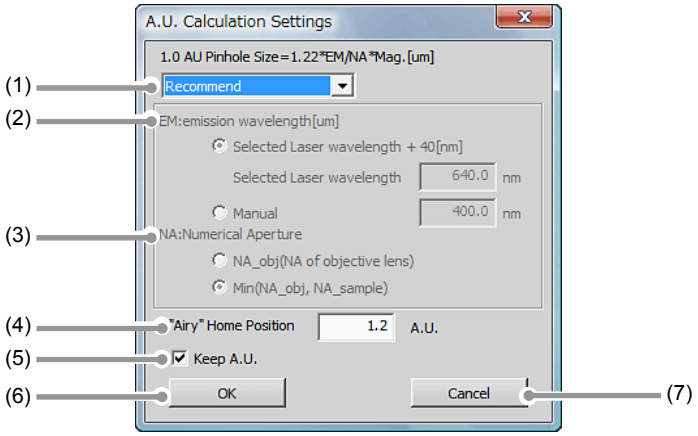


Figure 6.2-8 A.U. Calculation Settings dialog box

Table 6.2-5 A.U. Calculation Settings dialog box (sheet 1/2)

Name		Function	
(1)	Select calculation method	Recommend	Sets parameters automatically. (Recommended)
		User Setting	Allows the user to manually set parameters.
(2)	EM:emission wavelength[um]	Selected Laser wavelength	Calculates parameters by using the laser wavelength selected in the pinhole combo box of the Acquisition window as the emission wavelength (EM value). The wavelength displayed in the combo box is to be the laser wavelength set in the Optical Setting window.
		Manual	Allows the user to manually set parameters. (The parameter is calculated with the input value as the emission wavelength (EM value).) Enter the value directly from the keyboard.
(3)	NA: Numerical Aperture	Sets refractive index of the objective.	
		NA_obj(NA of objective lens)	Regardless of whether or not the objective NA value exceeds the refractive index of the sample (specimen), executes calculation by using the objective NA as the calculation parameter.
		Min(NA_obj, NA_sample)	When the objective NA value does not exceed the refractive index of the sample (specimen), executes calculation by using the objective NA as the calculation parameter. When the objective NA value exceeds the refractive index of the specimen, executes calculation by using the specimen refractive index.

Table 6.2-5 A.U. Calculation Settings dialog box (sheet 2/2)

Name		Function
(4)	“Airy” Home Position	Sets a home position of pinhole. Enter the value directly from the keyboard. * The pinhole size can be selected from six types in C2. Therefore, if the entered value does not match any of the types, the size that is larger than and the closest to the entered value is set as the home position.
(5)	Keep A.U. check box	When checked, the pinhole size is fixed by the A.U. when the selected wavelength or objective is changed. (However changes by the um.) When unchecked, the pinhole size is fixed by the um. (However changes by the A.U.) * The pinhole size can be selected from six types in C2. Therefore, if the to-be-fixed A.U. value does not match any of the types, the size that is larger than and the closest to the A.U. value is selected.
(6)	OK button	Determines the A.U. Calculation Settings applied and closes the [A.U. Calculation Settings] dialog box.
(7)	Cancel button	Discards the A.U. Calculation Settings applied and closes the [A.U. Calculation Settings] dialog box.

6.2.4 Auto Gain

Auto Gain is a function to automatically correct the value of Si HV gain to set the optimum image brightness. Automatic Si HV gain correction is performed within the predetermined range of the ratio of saturation pixels.

Automatic Si HV gain correction is performed only Si HV.
For a TD, automatic adjustment is performed when it is selected.

After execution of Auto Gain, in the dialog box indicating the progress of Auto Gain, the correction values actually used (Ratio of saturation pixels) are displayed.
If Auto Gain failed, “x” is indicated and the Si HV value returns to its original value.

- **Auto Gain cannot be started during Scan.**
- **In line scan, Auto Gain is not executable.**
- **During execution of Auto Gain, do not execute manual adjustments in the Acquisition window.**

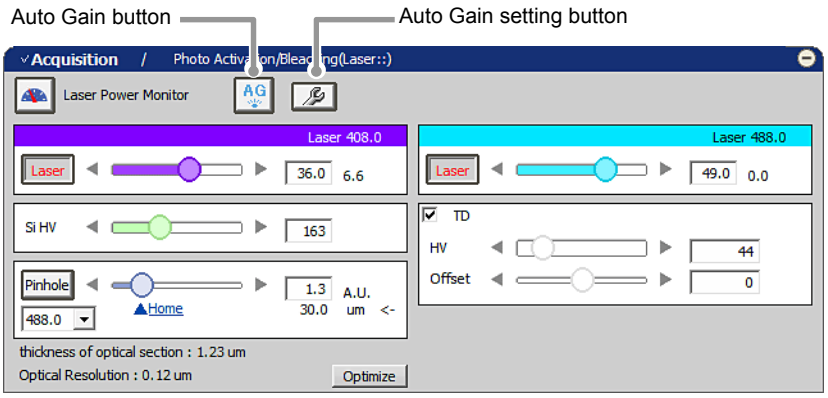


Figure 6.2-9 Execution of Auto Gain (Spectral Detector-use)

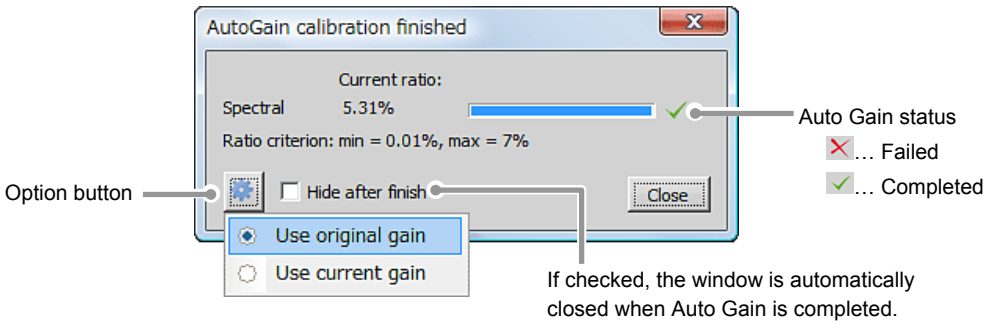


Figure 6.2-10 Auto Gain progress

Setting for Ratio of saturation pixels

Set the maximum and minimum value for the Ratio of saturation pixels used for automatic Si HV gain correction.

Click the [Auto Gain Setting] button to display the [Auto gain setup] dialog box.
Set the maximum and minimum value for the ratio of saturation pixels in [Auto gain setup] dialog box.

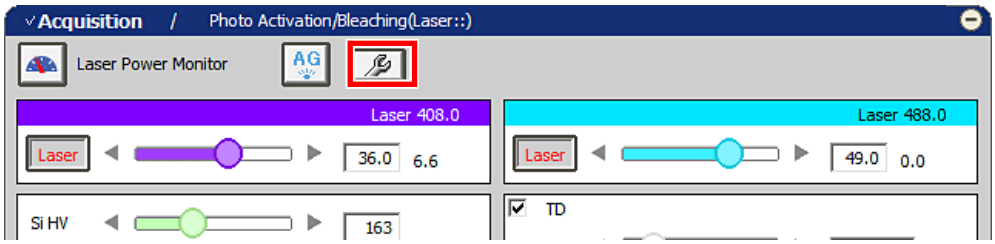


Figure 6.2-11 Displaying the Auto gain setup dialog box

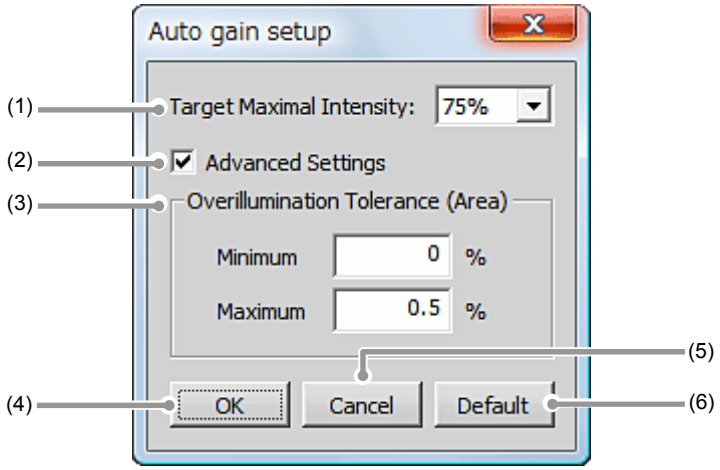


Figure 6.2-12 Setting for Ratio of saturation pixels

Table 6.2-6 Setting for Ratio of saturation pixels

Name		Function	
(1)	Target Maximal Intensity	Specifies the application ratio of the setting of the ratio of saturation pixels. Sets the percentage (%) of the maximum value to be applied.	
(2)	Advanced Settings	If checked, advanced settings of the ratio of saturation pixels are enabled.	
(3)	Overillumination Tolerance (Area)	Minimum	Sets the minimum value for Ratio of saturation pixels.
		Maximum	Sets the maximum value for Ratio of saturation pixels.
(4)	OK button	Determines the settings of Auto gain setup applied and closes the [Auto gain setup] dialog box.	
(5)	Cancel button	Discards the settings of Auto gain setup applied and closes the [Auto gain setup] dialog box.	
(6)	Default button	Resets the set values to the default values.	

6.3 Various Views (Spectral Detector-use)

This section describes various spectral views.

6.3.1 Channel View Setting

6.3.1.1 Channel Mixed View

From multiple channels acquired with the Spectral Detector, selected channels are mixed and displayed.

1. Open the Live window.

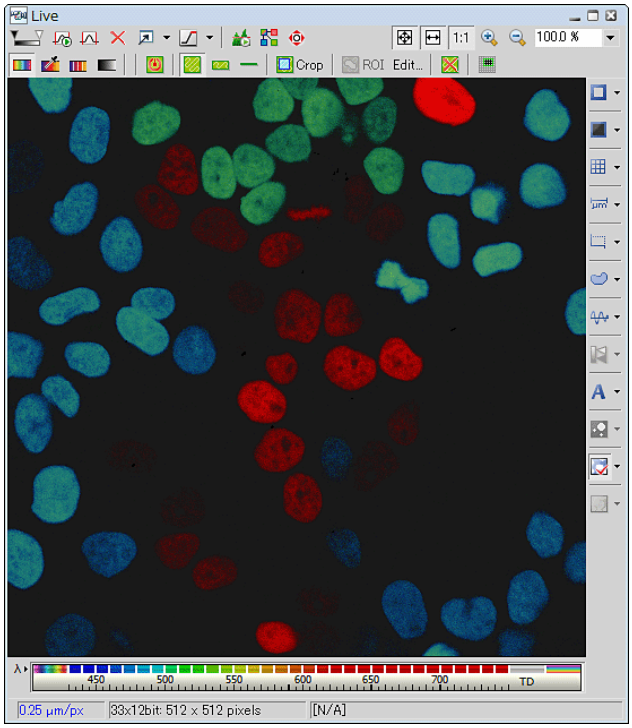


Figure 6.3-1 Live window

2. Select desired channels.
   While pressing the [Ctrl] key, click desired channels.
   To select a range, select the channel as the start point first, then while pressing the [Shift] key, click the channel as the end point.
   For selection of channels in multiple ranges, see Section 6.3.1.4 “Multi-Range Channel View.”

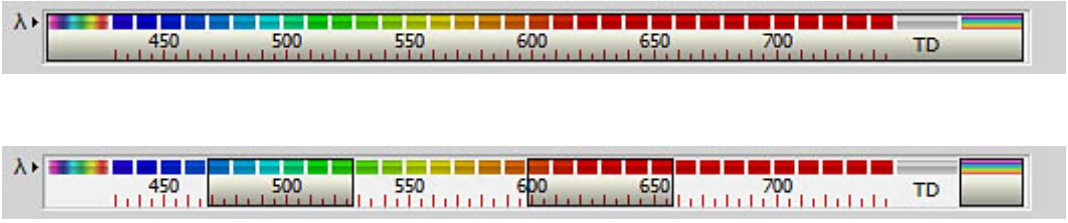


Figure 6.3-2 Channel view bar

6.3.1.2 Split Channel View

Selected channels are split into respective channels and displayed.

1. Click the [Split Components] button.
   “All image” mixing all channels, respective channel images, “TD image”, “Ratio image”, “Custom image” are displayed.

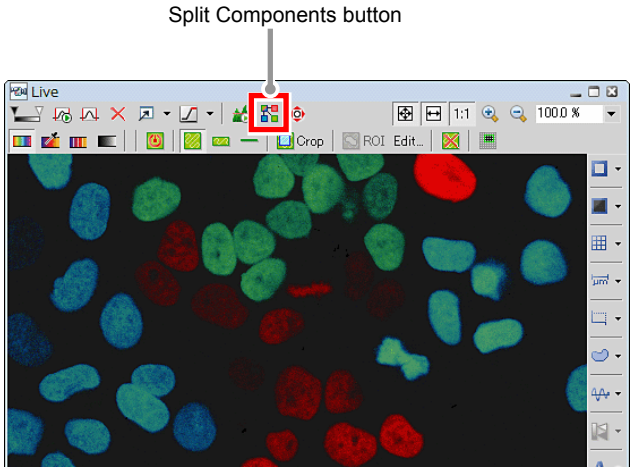


Figure 6.3-3 Live window

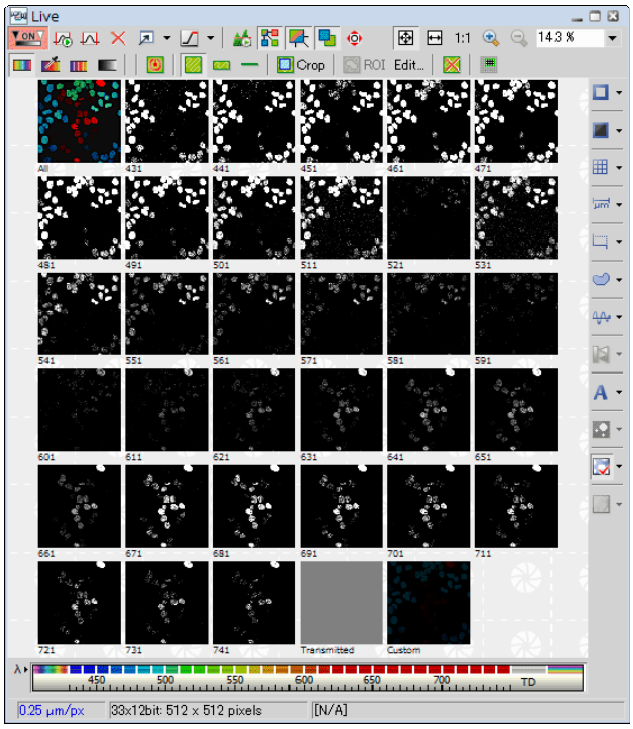
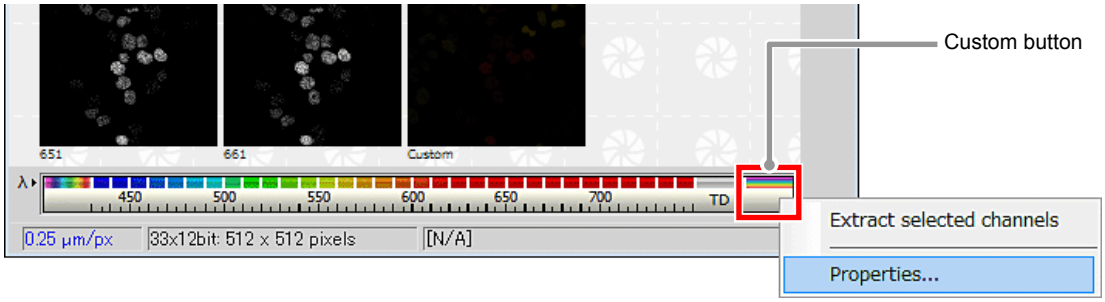
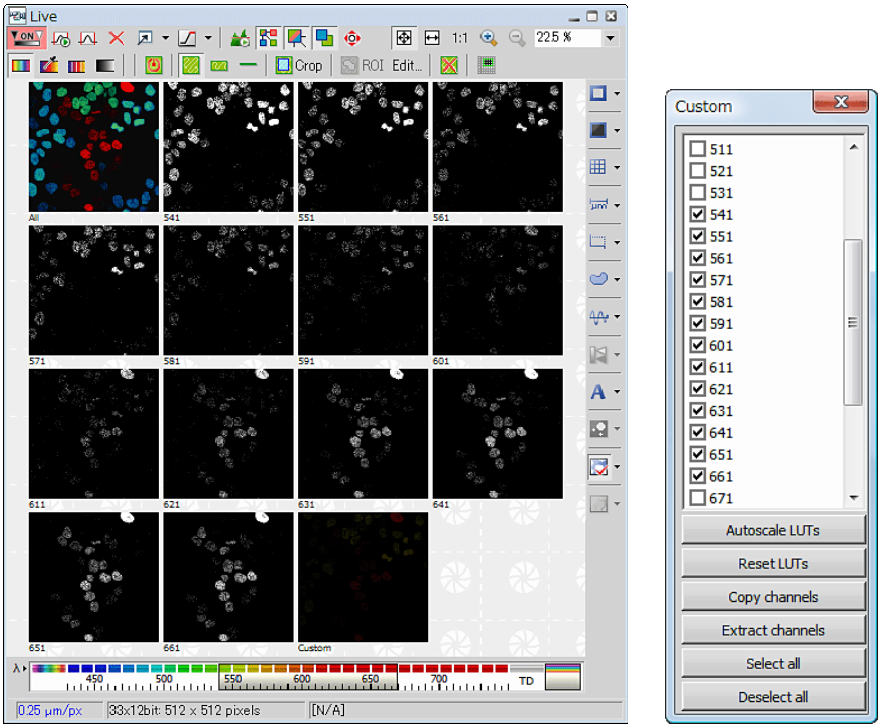


Figure 6.3-4 Split channel view

* For switching from Split channel view to Channel mixed view, click the [Split Components] button again.

2. Right-click on the [Custom] button and a menu appears. Select [Properties...] on the menu. The [Custom] dialog box appears to allow you to change the channels for the Custom View.





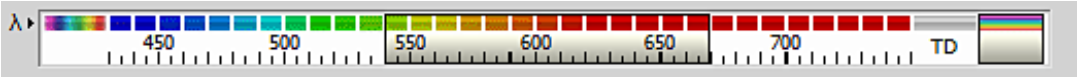


Figure 6.3-5 Split channel view (Custom image)

6.3.1.3 Ratio Image View

The Ratio image view is displayed.

Right-click on the window to display a menu.
Selecting [Ratio View] from the menu changes the window to the Ratio image.

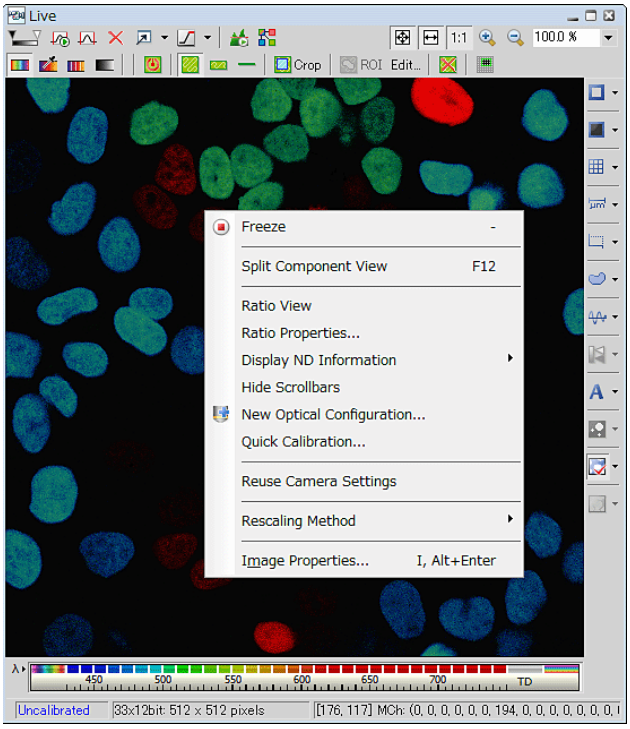


Figure 6.3-6 Displaying the Ratio image view

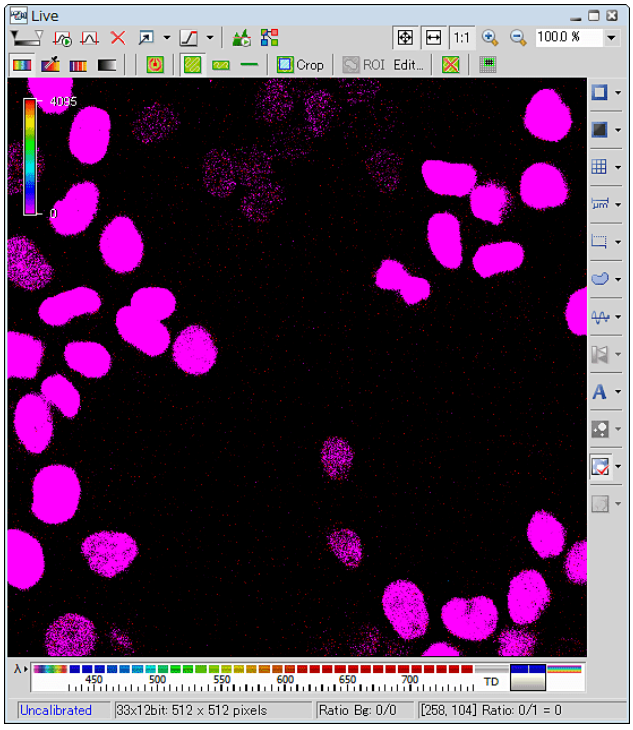


Figure 6.3-7 Ratio image view

6.3.1.4 Multi-Range Channel View

Mouse operation for displaying multi-range channels is as follows:

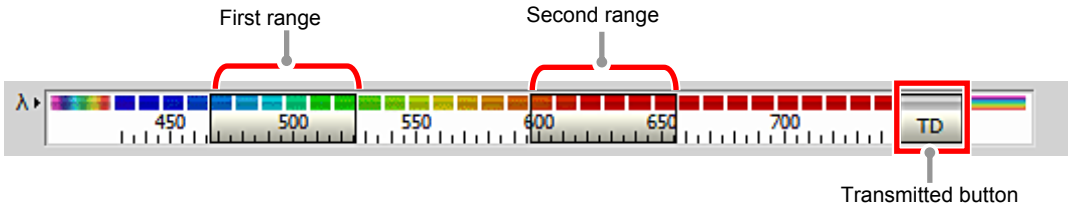


Figure 6.3-8 Multi-range channel view

1. Set and display the First range.
   Click the channel at the left end of the First range.
2. While pressing the [Shift] key, click the channel at the right end of the First range.
3. Select the Second range.
   While pressing the [Ctrl] key, click the channel at the left end of the Second range.
4. While pressing the [Ctrl] + [Shift] key, click the channel at the left end of the Second range.
5. Click the [Transmitted] button.
   While pressing the [Ctrl] key, click the [Transmitted] button. Then, the TD image and the images of the selected channels are mixed and displayed.

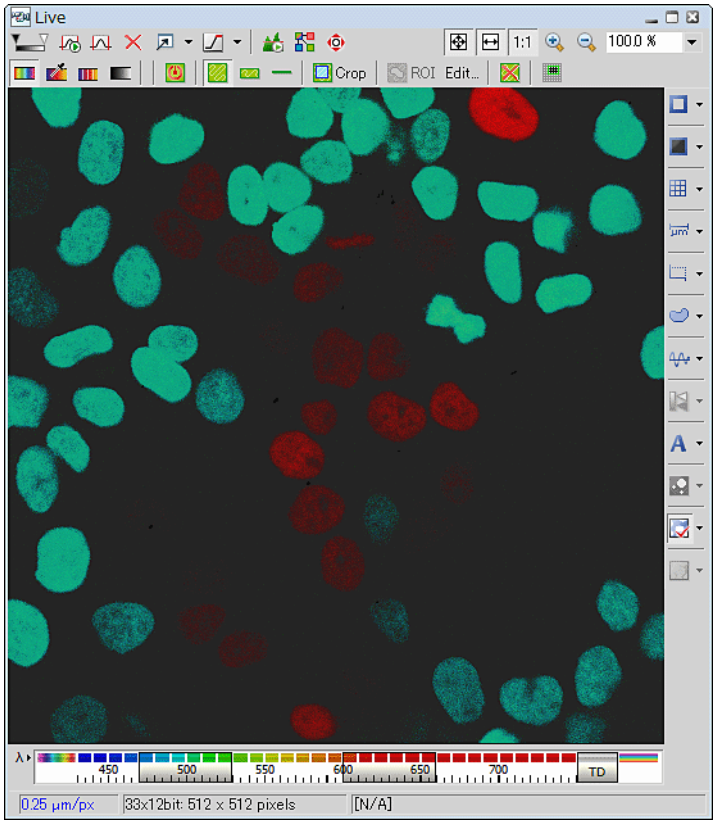


Figure 6.3-9 Channel mixed view

6.3.2 Color Mode Setting

6.3.2.1 Color Mode

The color mode switching method and channel color assignment are shown below.

Select the desired color mode from three modes; True Color, Custom Color, Grouped Color and Gray Scale and switch the display.
To set the color mode, be sure to turn “ON” the [Treat as Spectral] button. (If it is turned “OFF”, spectral information hidden.)

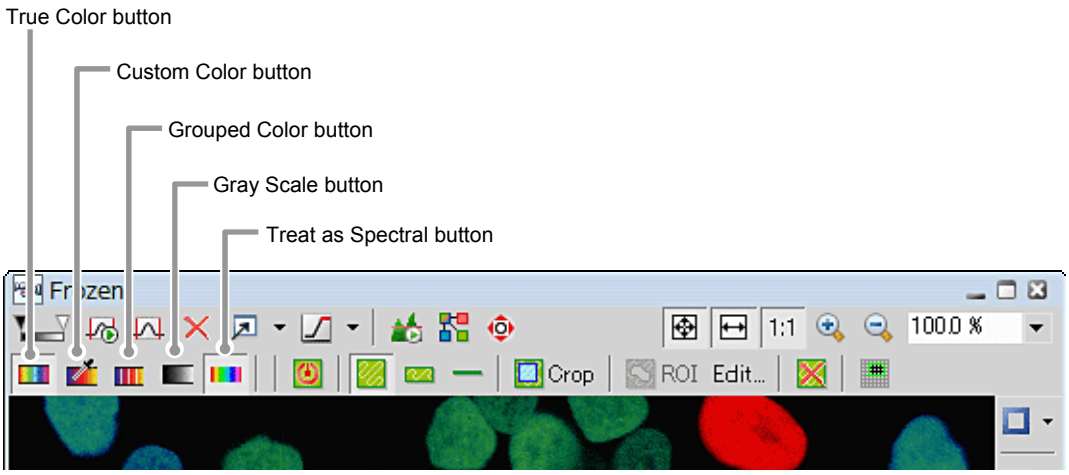


Figure 6.3-10 Frozen window

* **Settings of Custom Color, Grouped Color, Gray Scale**
To configure detailed settings, use the [LUTs] dialog box.
To Displaying the [LUTs] dialog box is shown below.

Click the [Show LUTs window] button or right-click on the gray area (without any dialog box and setting window displayed) to display a menu as shown below.
Select [Visualization Controls] -> [LUTs] in the menu to open the [LUTs] dialog box.

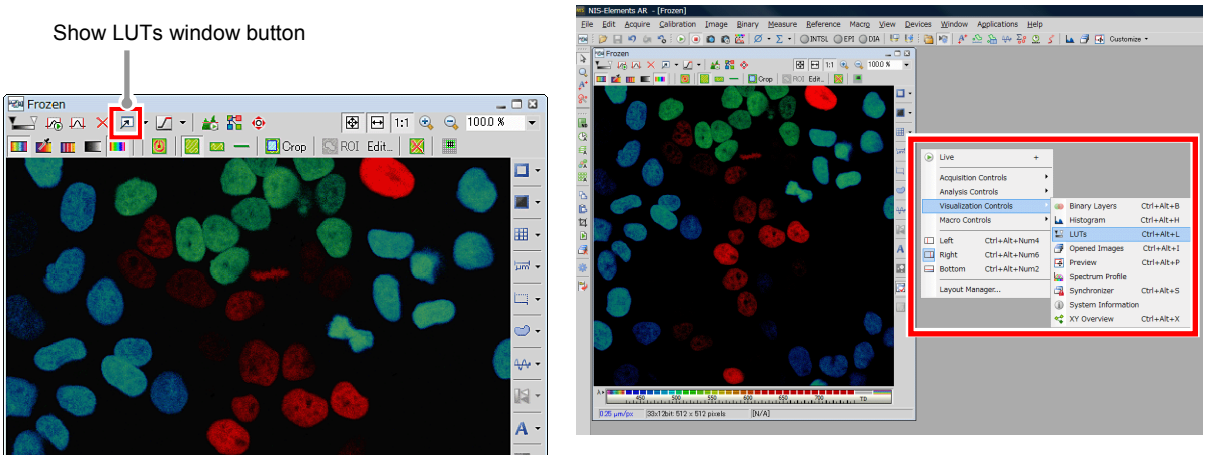


Figure 6.3-11 Displaying the LUTs dialog box

Displaying the True Color Image

Images of all channel data are displayed using the wavelength colors corresponding to the wavelength range provided during data acquisition.
Colors that are approximately same as those viewed by bare eyes are displayed.

Click the [True Color] button to display the True color image.

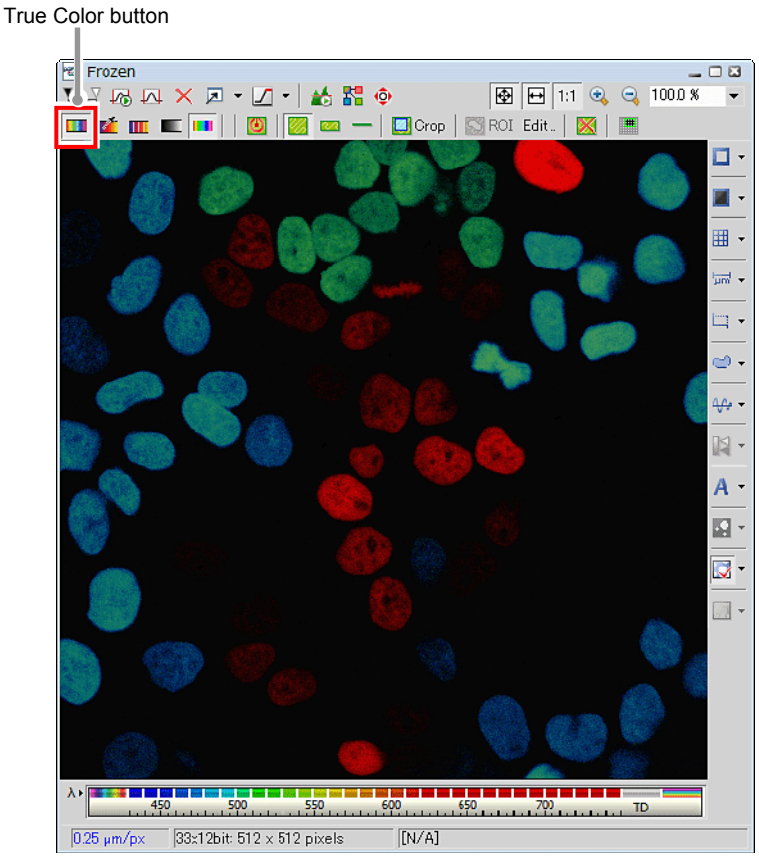


Figure 6.3-12 True Color image

Displaying the Custom Color Image

Custom Colors are assigned to respective channel data and images are displayed using multiple channel data. Custom Color assignment uses the [LUTs] dialog box.

Click the [Custom Color] button to display the Custom color image.

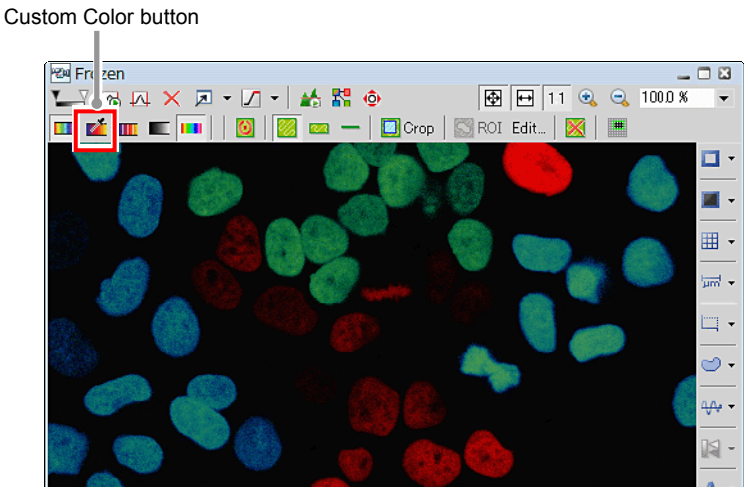


Figure 6.3-13 Custom Color button

Custom Color Setting

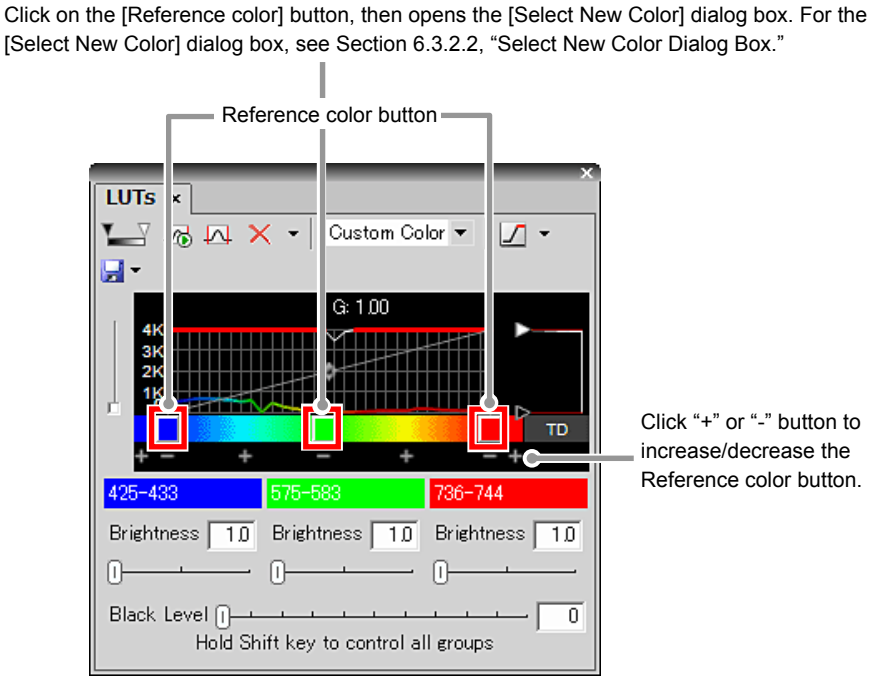


Figure 6.3-14 Custom Color setting dialog box

* In Custom Color mode, channels between [Reference color] buttons are color-interpolated and displayed.

Displaying the Grouped Color Image

With image acquired using the Spectral Detector, channels in a specified range can be grouped and colors can be assigned by group.
Grouped Color assignment uses the [LUTs] dialog box.

Click the [Grouped Color] button to display the Grouped color image.

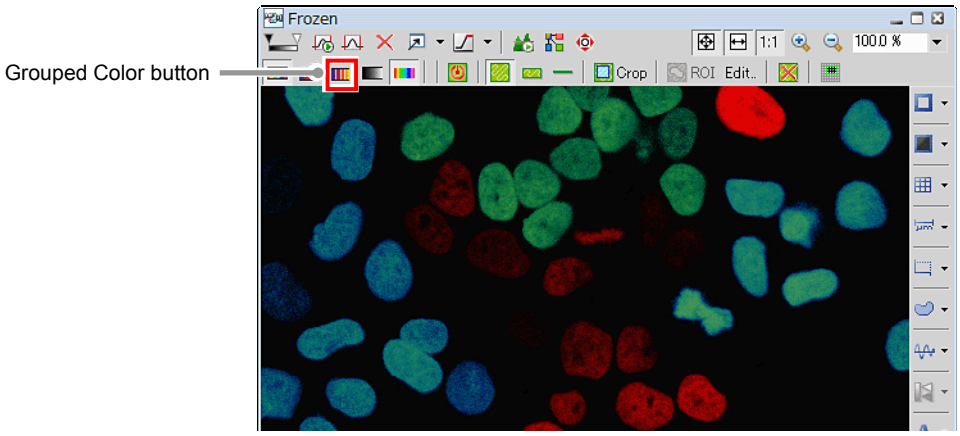


Figure 6.3-15 Grouped Color button

Grouped Color Setting

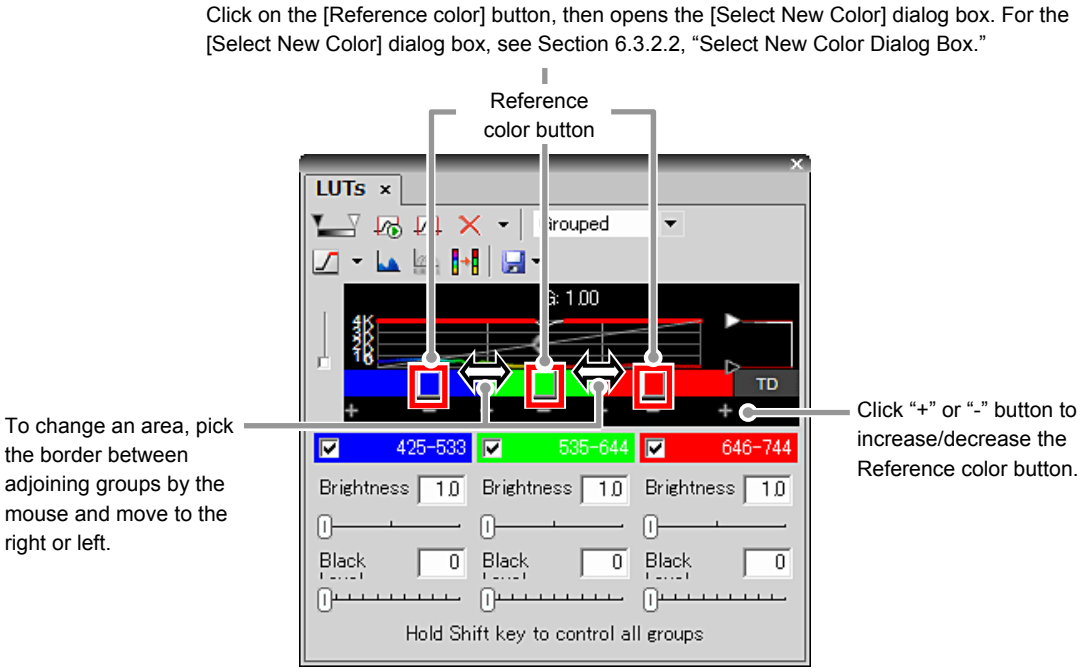


Figure 6.3-16 Grouped Color setting dialog box

* In Grouped Color mode, the area is split by the number set in [Reference color] button. Channels in each area are all displayed with the same color.

* In the channel bar of image window, can change an area too.
Click the group to change, then pick the border between adjoining groups by the mouse and move to the right or left.

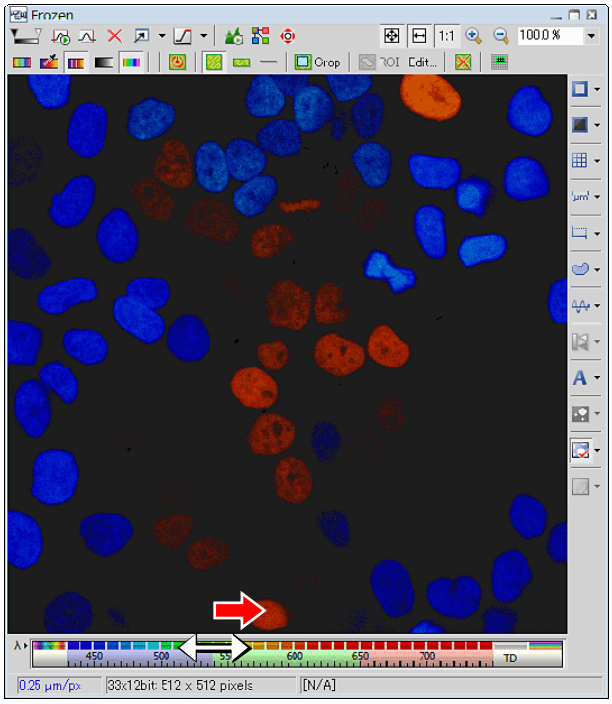


Figure 6.3-17 Grouped Color mode

Displaying the Gray Scale Image

Each channel is displayed with Gray Scale (Monochrome 256 gradations).
Gray Scale assignment uses the [LUTs] dialog box.

Click the [Gray Scale] button to display the Gray Scale image.

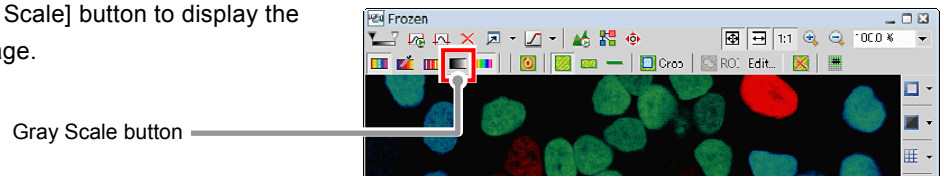


Figure 6.3-18 Gray Scale button

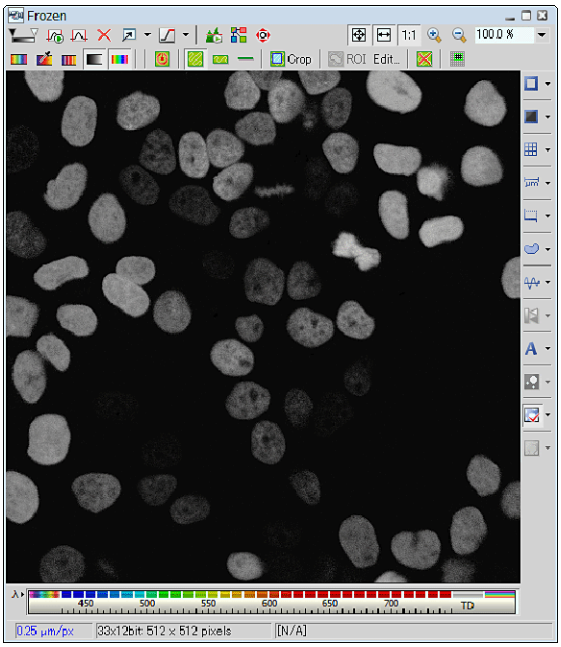


Figure 6.3-19 Gray Scale image

Gray Scale Setting

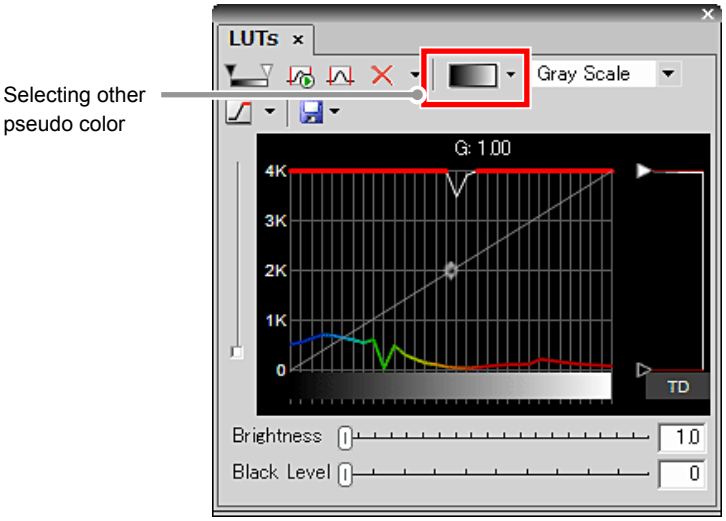


Figure 6.3-20 Gray Scale setting dialog box

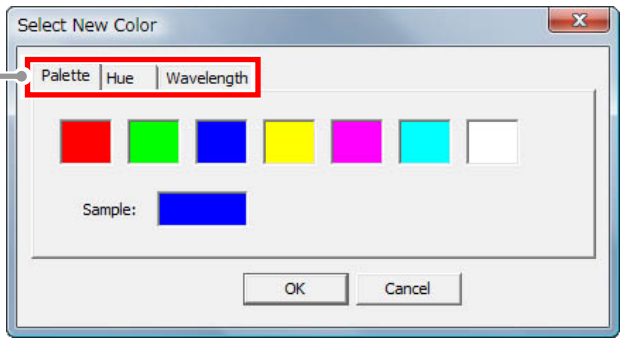
* The pseudo color menu also allows changing the displayed color settings.

6.3.2.2 Select New Color Dialog Box

In this dialog box, colors to be assigned to channels are selected.

Click the [Reference color] button on Custom Color or Grouped Color settings to display this dialog box.

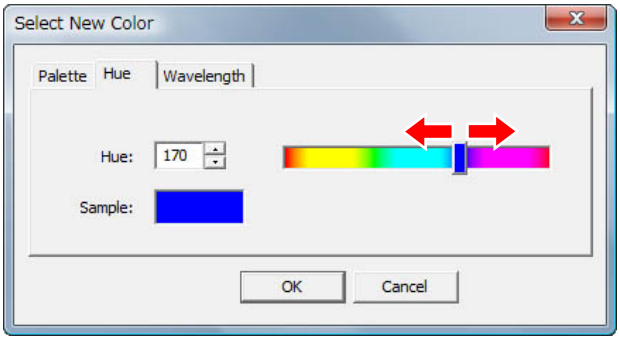
1. In the [Select New Color] dialog box, select the desired tab from three [Color palette] tabs.



- [Palette]
  Select a color from red, green, blue, yellow, purple, cyan, and white.
  Colors; yellow, purple, and cyan, support color weakness.

Figure 6.3-21 Select new color dialog box (Palette)

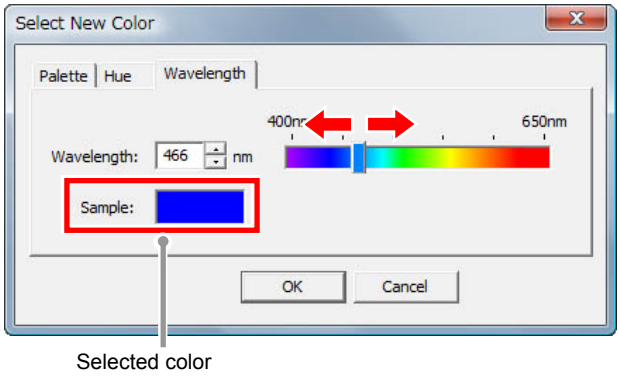
2. Select the color to be assigned.
   In the [Hue] and [Wavelength] tabs, a numeric value can be directly entered or the bar displayed in the color range can be moved to the right or left for selection.



- [Hue]
  A hue is set.
  A hue in a range of 0 to 240 can be set.
  A numeric value can be directly entered or the bar displayed in the color range can be moved to the right or left for selection.

Figure 6.3-22 Select new color dialog box (Hue)

3. The selected color is displayed in [Sample].



- [Wavelength]
  A color is set using a wavelength in the wavelength range.
  A wavelength is specified with a numeric value or bar to select a wavelength color.

Figure 6.3-23 Select new color dialog box (Wavelength)

6.3.3 Spectrum Profile

Brightness of the ROI area specified in the spectral image can be decomposed and displayed for each 32 channels.

6.3.3.1 Displaying the Spectrum Profile

1. Specify the ROI area in the spectral image. (If two or more ROI areas are selected, graphs are displayed for the colors of the ROI selected areas on the profile graph.)

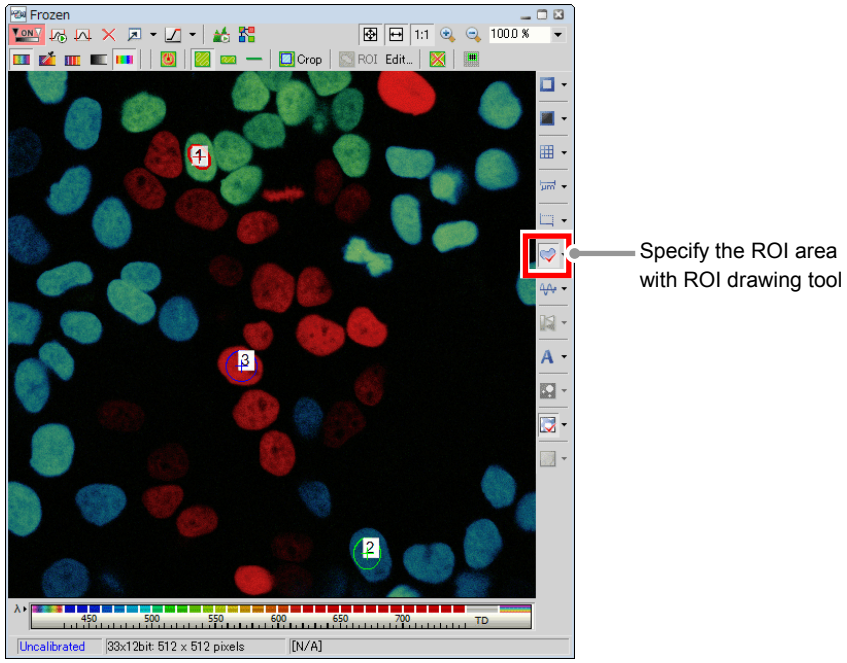


Figure 6.3-24 Specify the ROI area (Spectral image)

2. As shown below, right-click on the gray area (without any dialog box and setting window displayed) to display a menu.
   Select [Visualization Controls] -> [Spectrum Profile] in the menu to open Spectrum Profile.

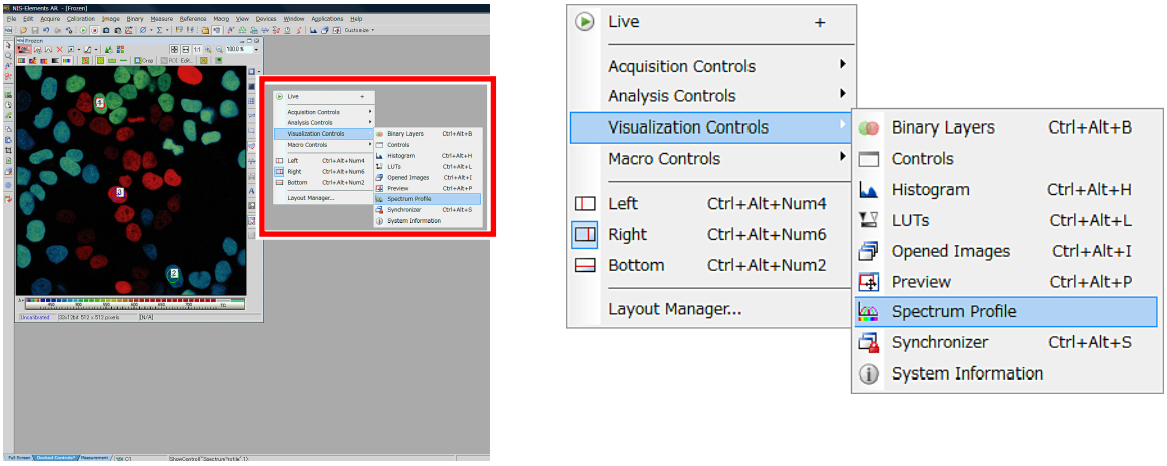


Figure 6.3-25 Displaying the Spectrum Profile

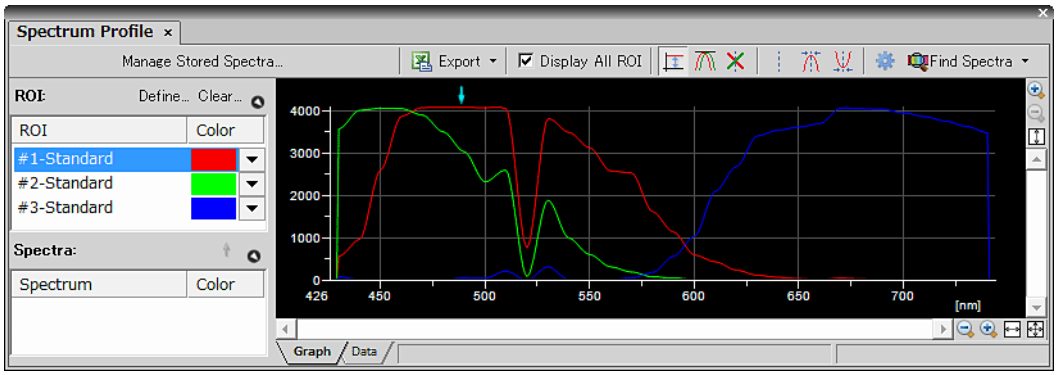


Figure 6.3-26　Spectrum Profile (all ROI areas are displayed)

3. To display the ROI on the graph, remove the check mark from [Display All ROI] and select the desired ROI from the ROI list.

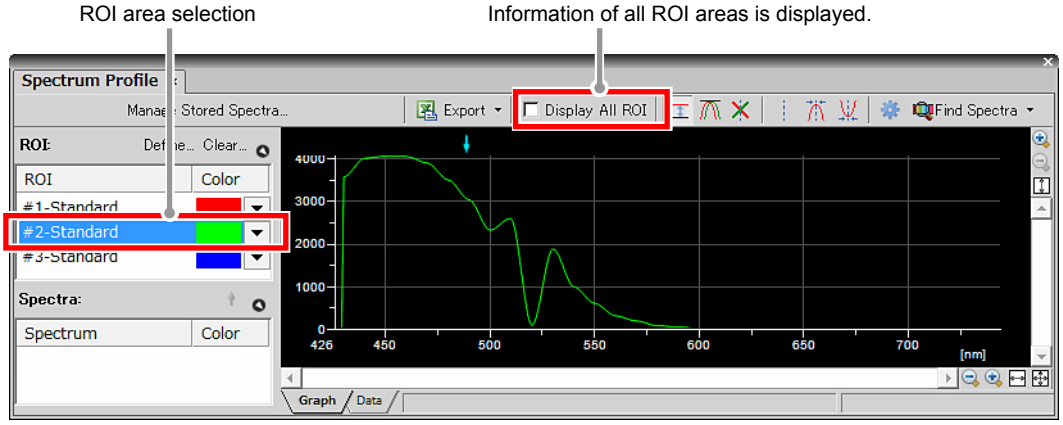


Figure 6.3-27　Spectrum Profile (displayed for each ROI area)

X-axis: 32-channel spectral colors displayed.
Y-axis: ROI brightness value or background brightness value displayed.

6.3.3.2 Spectrum Profile Setting

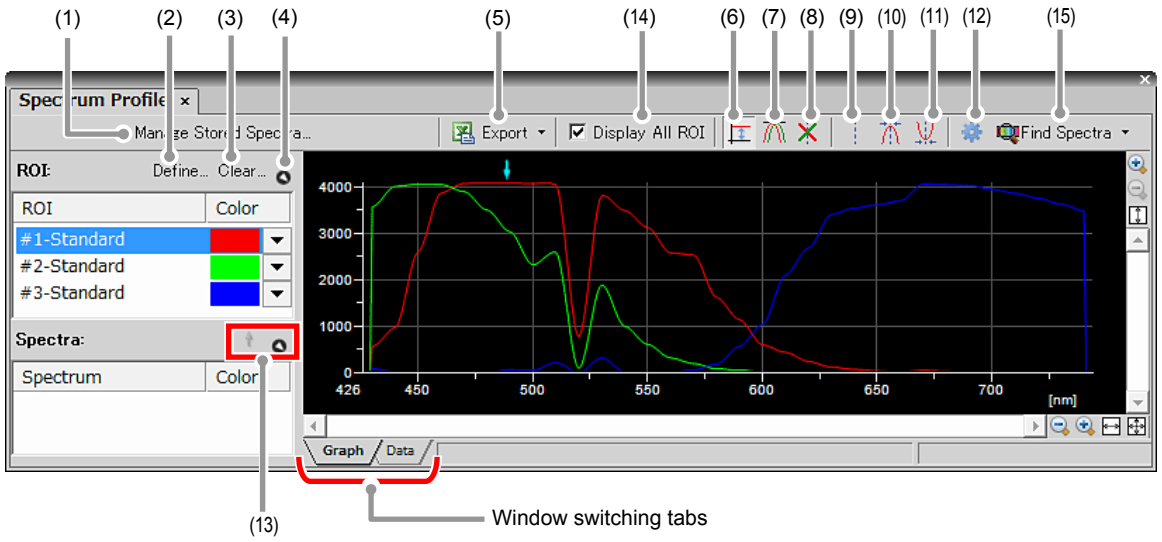


Figure 6.3-28 Spectrum Profile

* If any reagent is added in [Spectra:] the ideal line of reagent reaction is displayed on the graph and can be used as an indicator about whether the reagent is correctly reacting.

Table 6.3-1 Summary of Spectrum Profile graph functions (sheet 1/2)

Name		Function	
(1)	Manage Stored Spectra	Displays the item registered in Stored.	
(2)	Define	Opens the Simple ROI Editor tool.	
(3)	Clear	Clears the ROI area specified in the image. (Before clearing, a confirmation message is displayed.)	
(4)	Store Spectrum	Stores the user-defined spectrum (wavelength information).	
(5)	Export	Exports numeric data to Microsoft Excel.	
(6)	Vertical Scale Absolute		Enlarges the window assuming that the brightness minimum to maximum displayed in the graph as 100%.
(7)	Vertical Scale Normalized		Displays the brightness of each ROI in the Y-axis direction as a relative value to 100%. (Normalizing correction)
(8)	Scale to cursor		Calculates the aberration of the curve so that the cross point between bar graphs will be Y:1 and displays a relative graph.
(9)	Free cursor		Displays a cursor that can be moved to any position. When the cursor is picked with the mouse, brightness of the pixel at the cursor position can be checked as information.
(10)	Cursor to maximum		Moves the cursor to the maximum value of the specified ROI's brightness.
(11)	Cursor to minimum		Moves the cursor to the minimum value (0 or larger) of the specified ROI's brightness.

Table 6.3-1 Summary of Spectrum Profile graph functions (sheet 2/2)

Name		Function	
(12)	Options		Opens the [Options] dialog box for Spectrum Profile.
(13)	Move Up		Brings the selected spectra to one line above.
	Move down		Brings the selected spectra to one line below.
	Add spectra		Adds a spectrum as an indicator.
	Remove spectra		Removes a spectrum as an indicator.
(14)	Display All ROI	Displays all of the active ROIs.	
(15)	Find Spectra	Automatically detects spectra. Specifies the number of classifications (2 to 4) for spectra to automatically separate the wavelength or use “Auto Search” for separation without specifying the number of classifications.	

6.3.4 Spectral Unmixing Setting

Separate the wavelength information of a spectral image and display an Unmixing image.
If wavelengths overlap (because multiple reagents are in use) and differences are hard to identify, wavelength information can be separated and displayed.

6.3.4.1 Displaying the Spectral Unmixing Setting

1. Specify the wavelength to be separated in the spectral image or on the Frozen window using the ROI area.

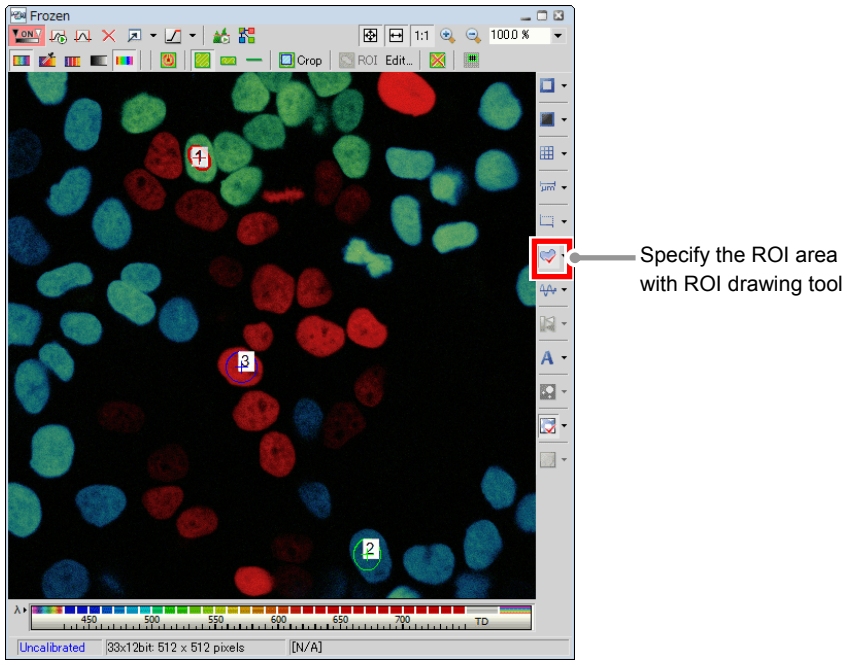


Figure 6.3-29 Specifying the ROI area (Spectral image)

2. Open the [Spectral Unmixing Setting] dialog box.
Select [Image] -> [Spectral Unmixing Setting…] on the menu bar.

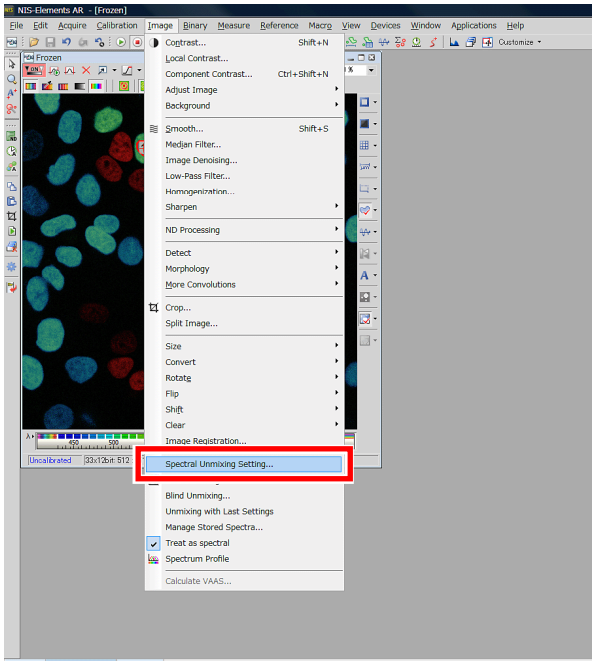


Figure 6.3-30 Displaying Spectral Unmixing Setting dialog box

3. If [ROIs] is selected from [Category:] in [Source Elements], [Elements:] displays the elements (ROIs) of the target to be separated.

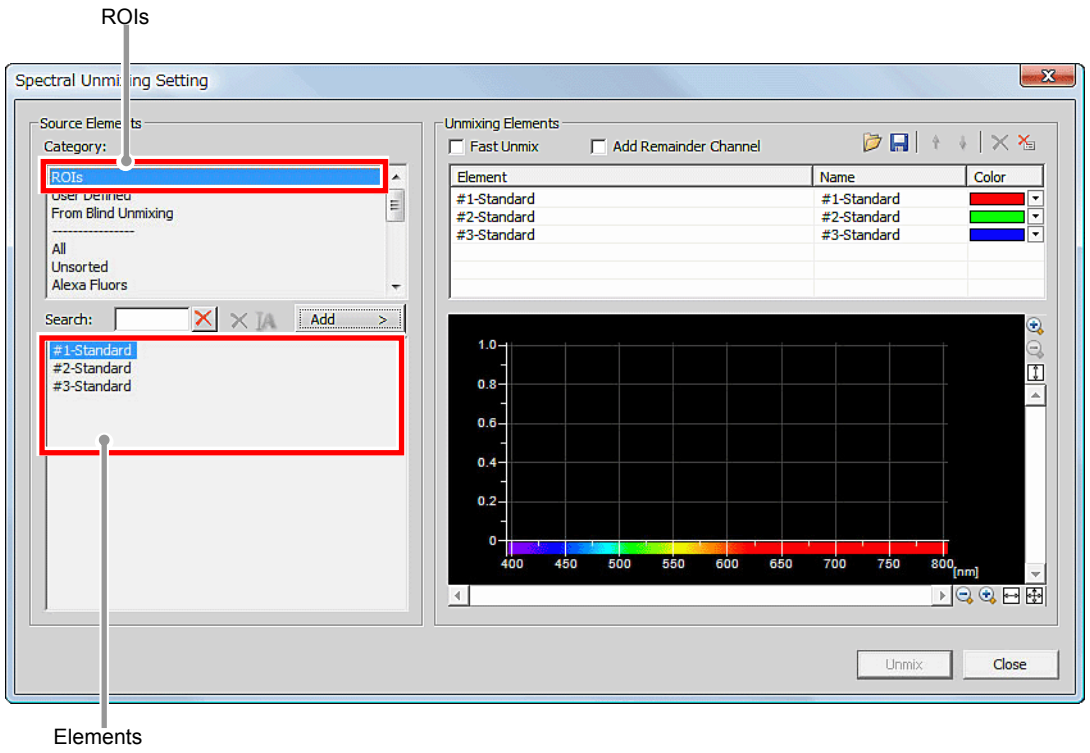


Figure 6.3-31 Spectral Unmixing Setting

4. Using the [Add] button, add the elements of the target to be separated from [Elements:] in [Source Elements] to [Unmixing Elements].

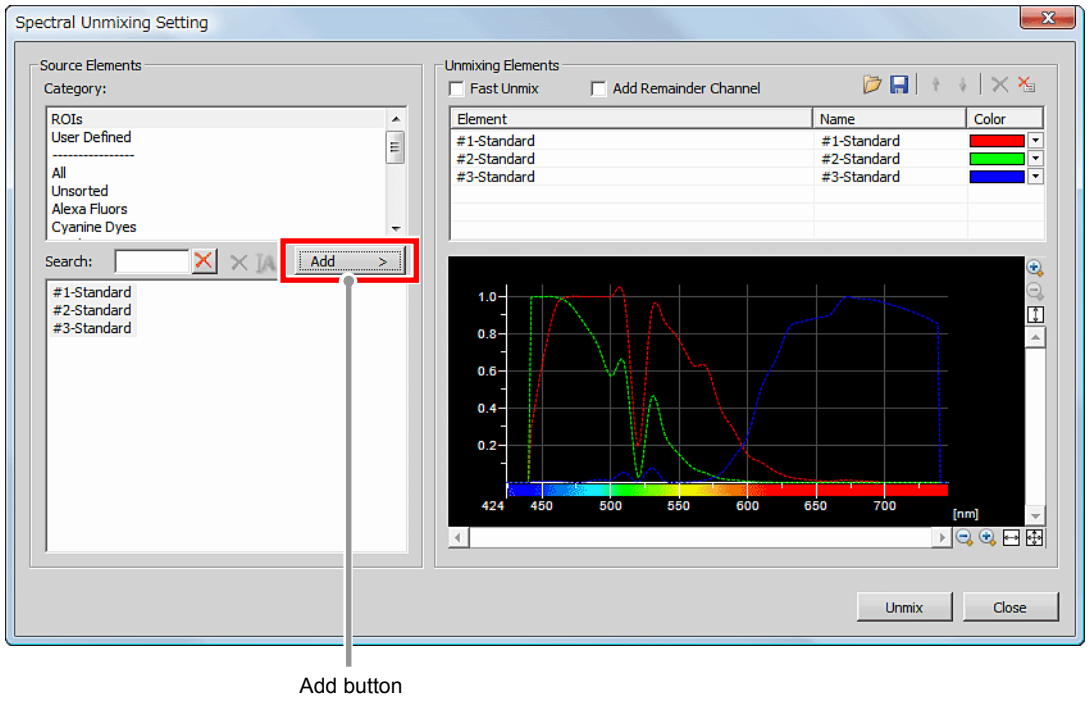


Figure 6.3-32 Spectral Unmixing Setting

5. Click the [Unmix] button to open the unmixed image window separately from the Frozen window.

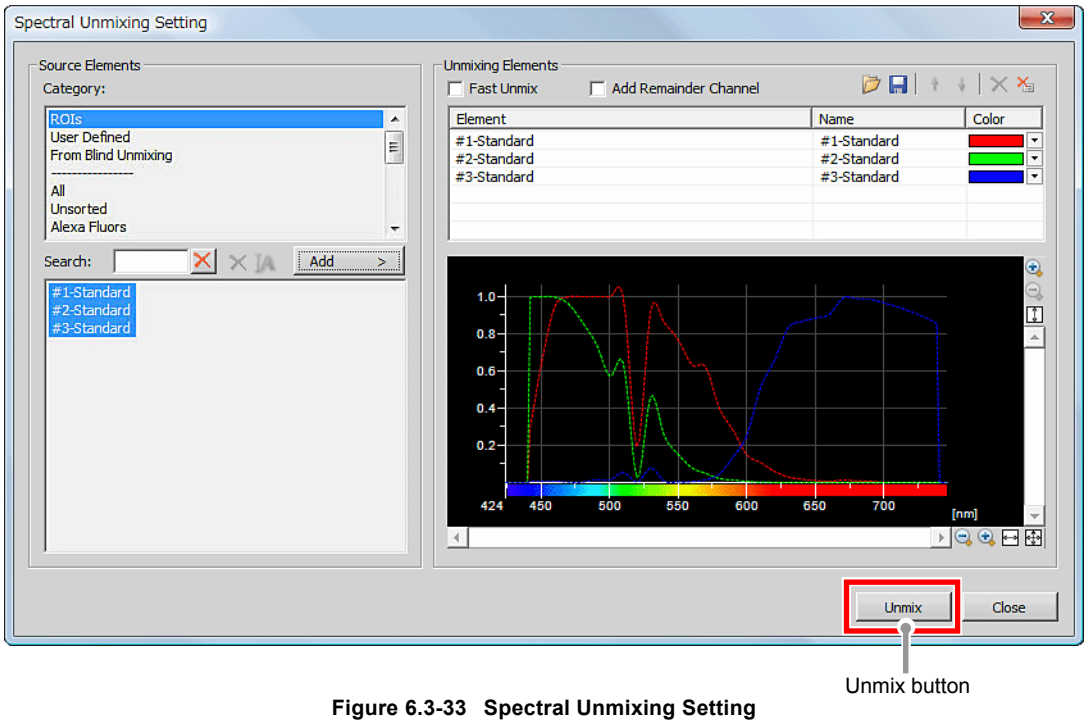


Figure 6.3-33 Spectral Unmixing Setting

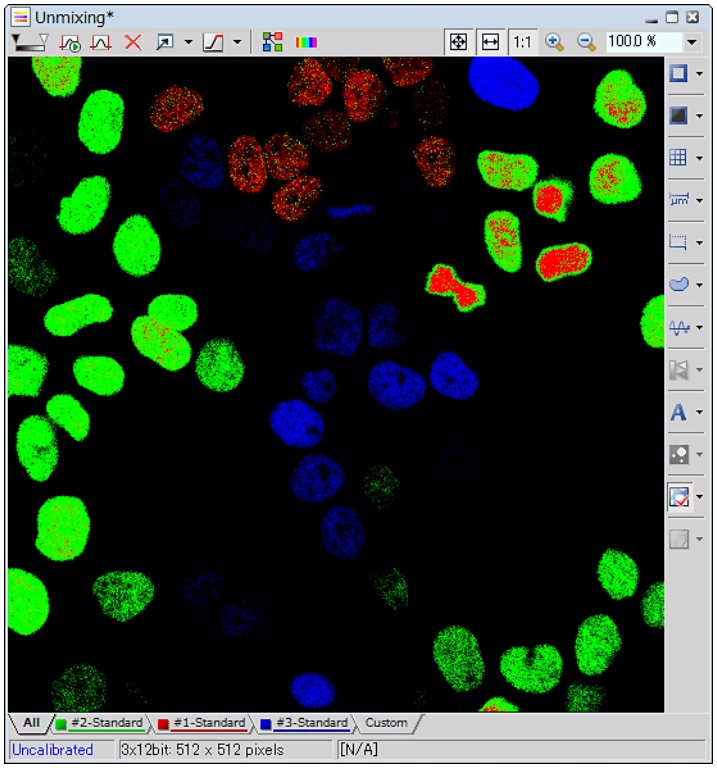


Figure 6.3-34 Spectral Unmixing view

* In addition to specifying using the ROI area, wavelength information can be separated by specifying the reagent in use.
However, noise provided upon image acquisition may appear.

* Specifying the background color of ROI.

As shown below, specify the ROI area in the part to be designated as the background color.

Right-click the mouse on the created ROI area to display a menu.

From the menu, select [Use as Background ROI].

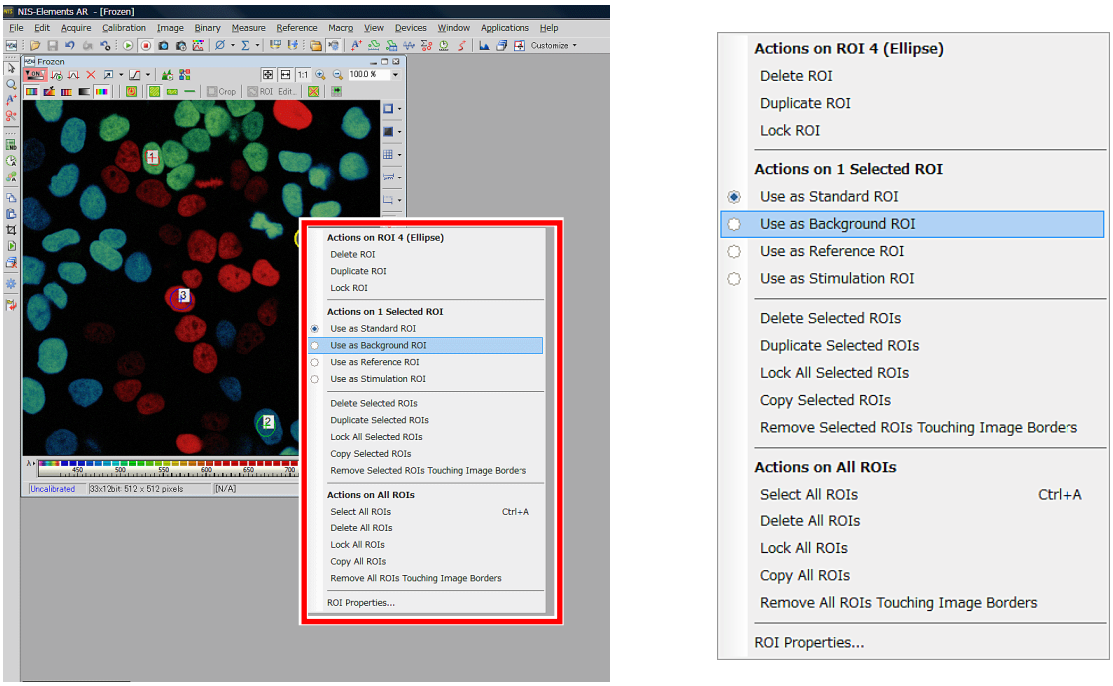


Figure 6.3-35 Changing the setting of the ROI area

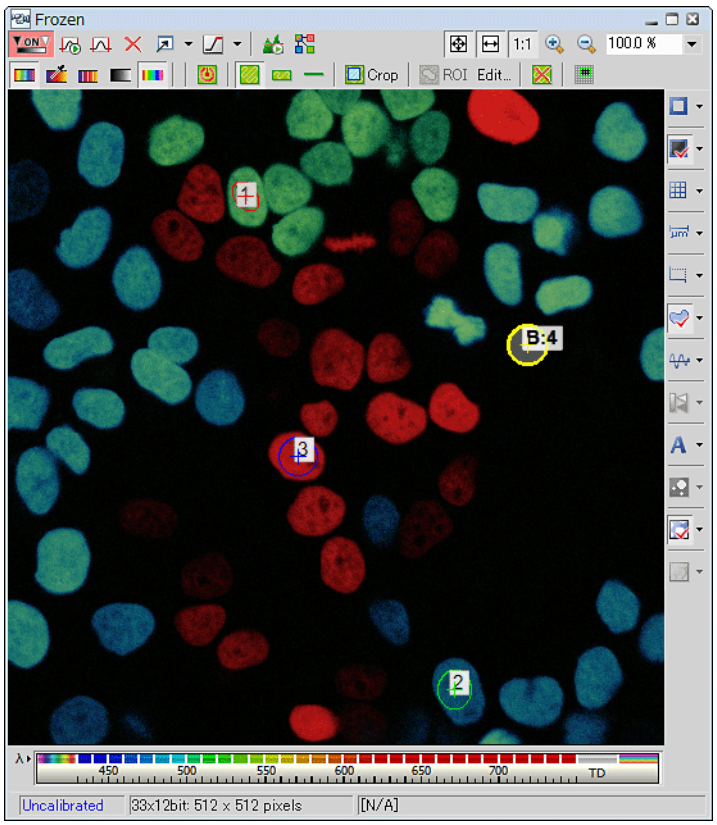


Figure 6.3-36 Spectral Unmixing view

6.3.4.2 Spectral Unmixing Setting

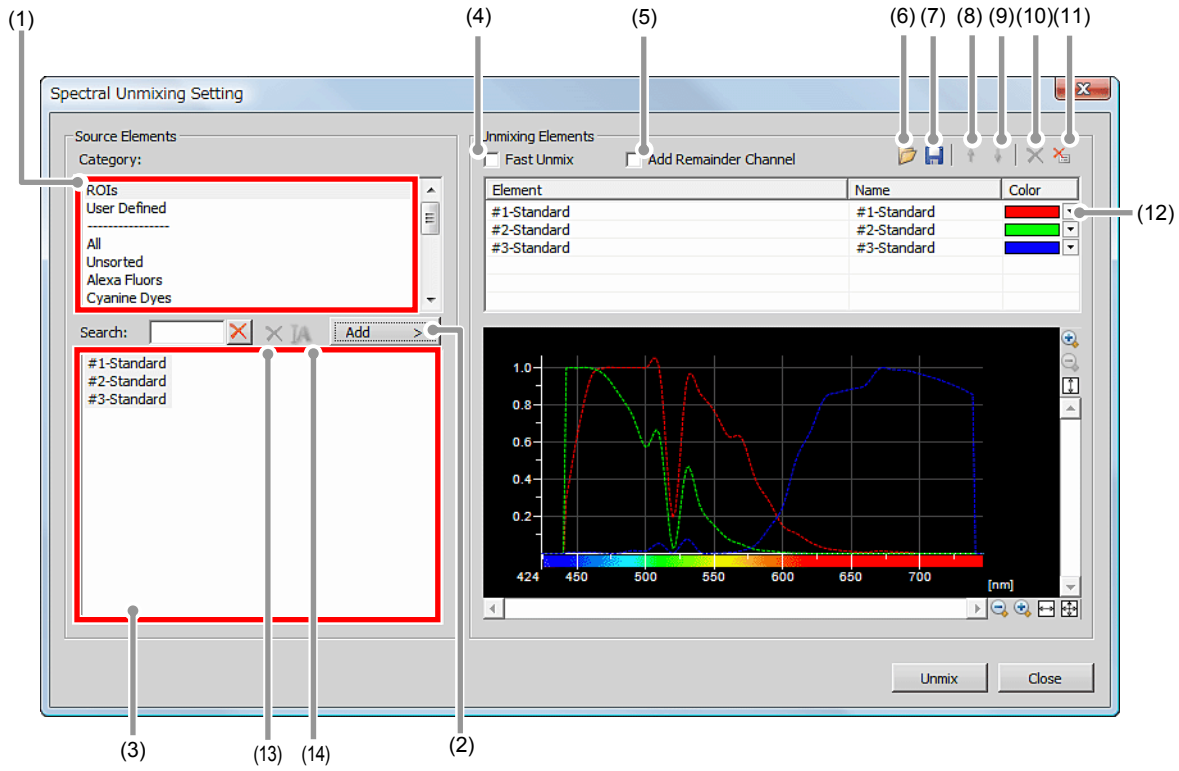


Figure 6.3-37 Spectral Unmixing Setting

Table 6.3-2 Summary of Spectral Unmixing Setting functions (sheet 1/2)

Name		Function	
(1)	Category:	Displays the category of the ROI, user-registered wavelength information, reagent, etc.	
(2)	Add>	Selects the elements of the target to be separated from [Elements:] and adds to [Unmixing Elements].	
(3)	Elements:	Selects the elements of the target to be separated.	
(4)	Fast Unmix	If check is turned “ON”, the calculation algorithm is simplified and higher-speed separation is performed compared with normal Unmix.	
(5)	Add Remainder Channel	This function enables calculation of remainder data in the Unmixing calculation. When selected, the remainder data is shown as an image in the Unmixing calculation result. When deselected, the remainder data is not shown.	
(6)	Open		Retrieves the setting information saved in an XML file.
(7)	Save		Writes the setting information in an XML file and saves it.
(8)	Move the Element one line Up		Brings the selected Element to one line above.
(9)	Move the Element one line down		Brings the selected Element to one line below.

Table 6.3-2 Summary of Spectral Unmixing Setting functions (sheet 2/2)

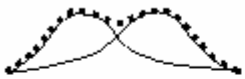
Name		Function	
(10)	Remove the Element		Removes the selected Element.
(11)	Remove all		Removes all Elements.
(12)	Color	The graph color and post-Unmix image can be set to any color.	
(13)	Remove Spectra	Enabled only when [User Defined] or [From Blind Unmixing] is selected for [Category:]. Removes the items selected in [Elements:].	
(14)	Rename Spectrum	Enabled only when [User Defined] or [From Blind Unmixing] is selected for [Category:]. Changes the names of items selected in [Elements:].	

Note

<Remainder data>

The Remainder data is used as a quality standard for the data produced by the Unmix calculation. The Remainder data is represented as an absolute value for the total of differences between measurement data (b) and the total of Unmixed data (a).

(a) Total of unmixed data: (b) Spectrum for measurement data: Remainder = | (b)-(a) |



$$S(\lambda) = \sum_n I_n \bullet R(\lambda)_n$$

$$\text{Remainder} = \sum_{\lambda} \left| S(\lambda) - E(\lambda) \right|$$

S(λ) = total unmixed data E(λ) = measurement data
In = unmixed data R(λ) = data used as calculation elements

This data is added as one channel data to Unmixed data.

6.3.5 Live Unmixing

Live observation is available in the state where spectral images are separated for each wavelength.

6.3.5.1 Displaying the Live Unmixing

1. Specify the wavelength to be separated in the spectral image using the ROI area.

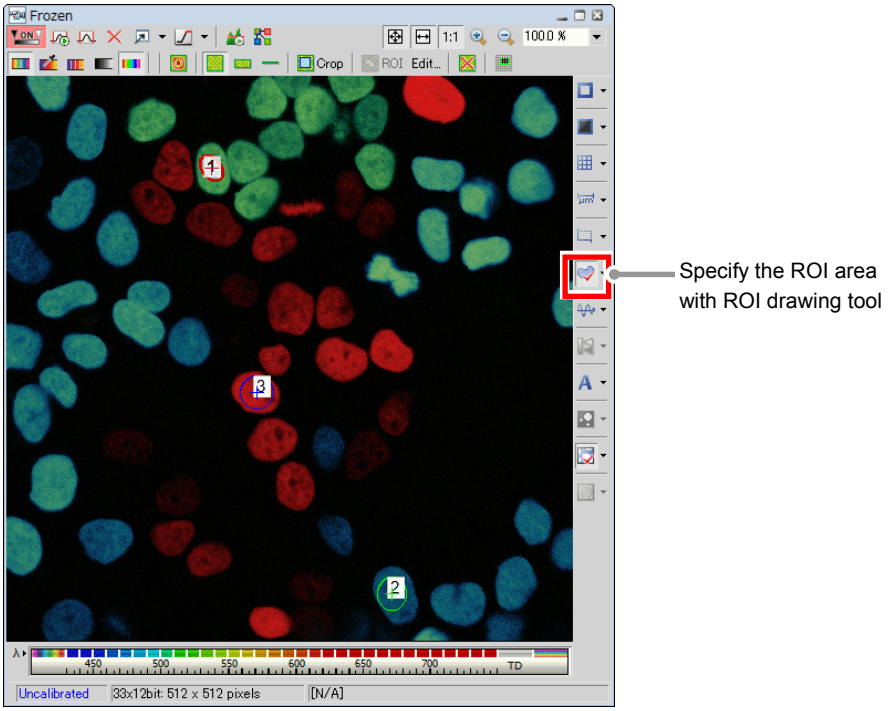


Figure 6.3-38 Specify the ROI area (Spectral image)

2. Open the [Spectral Unmixing Setting] dialog box.
   Select [Image] -> [Spectral Unmixing Setting…] on the menu bar.

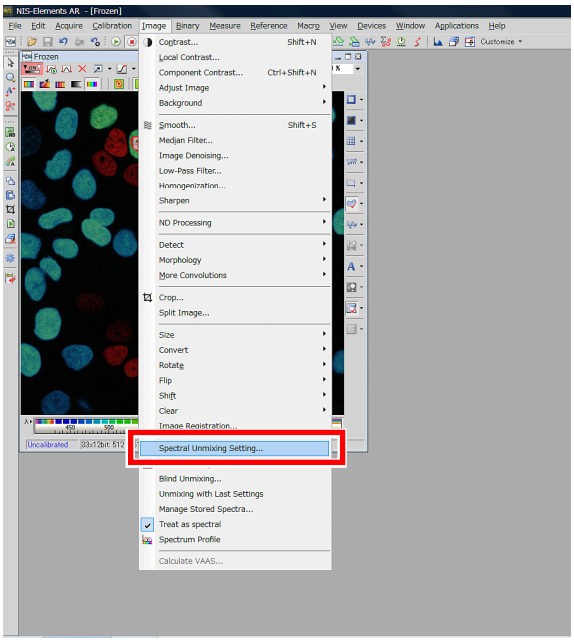


Figure 6.3-39 Displaying the Spectral Unmixing Setting dialog box

3. If [ROIs] is selected from [Category:] in [Source Elements], [Elements:] displays the elements (ROIs) of the target to be separated.

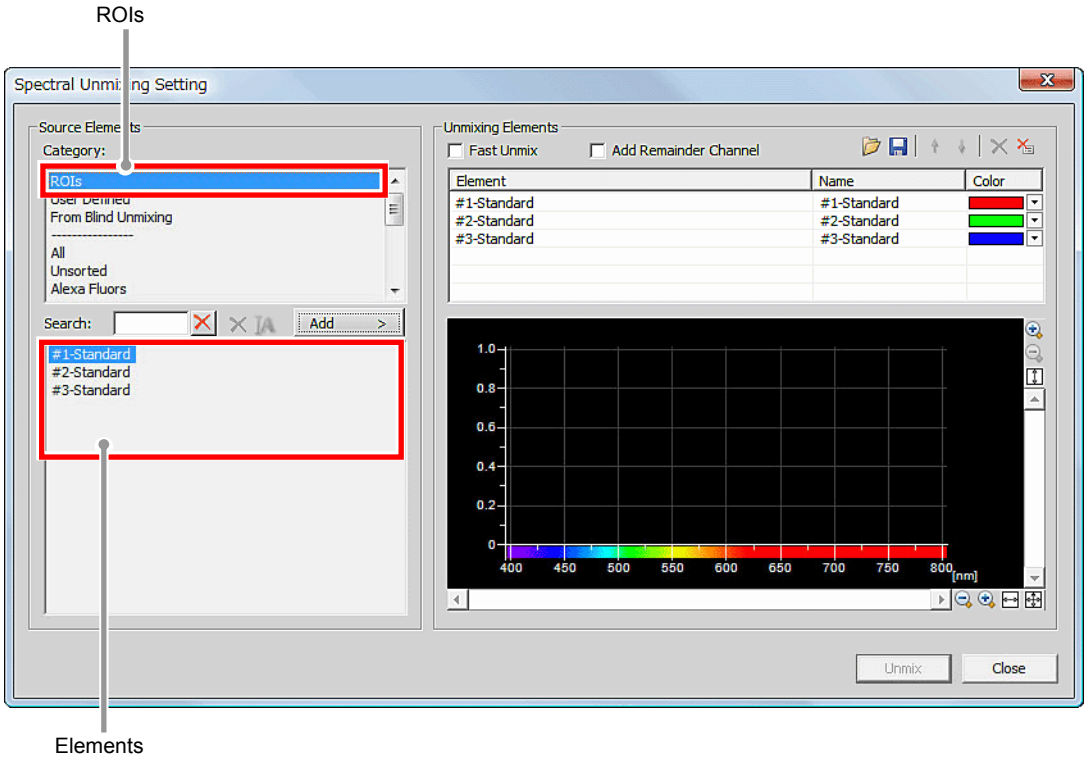


Figure 6.3-40 Spectral Unmixing Setting

4. Using the [Add] button, add the elements of the target to be separated from [Elements:] in [Source Elements] to [Unmixing Elements].

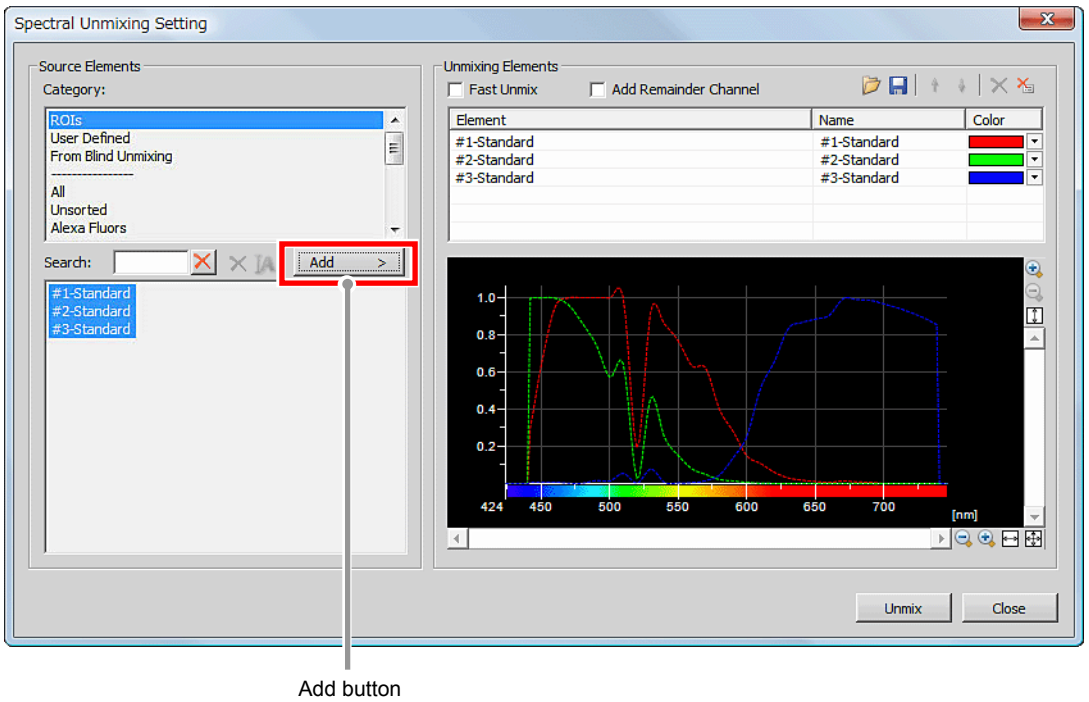


Figure 6.3-41 Spectral Unmixing Setting

5. Click the [Close] button to determine the wavelength you want to separate.
   (If you click the [Unmix] button instead, normal Unmix image starts to be captured.)

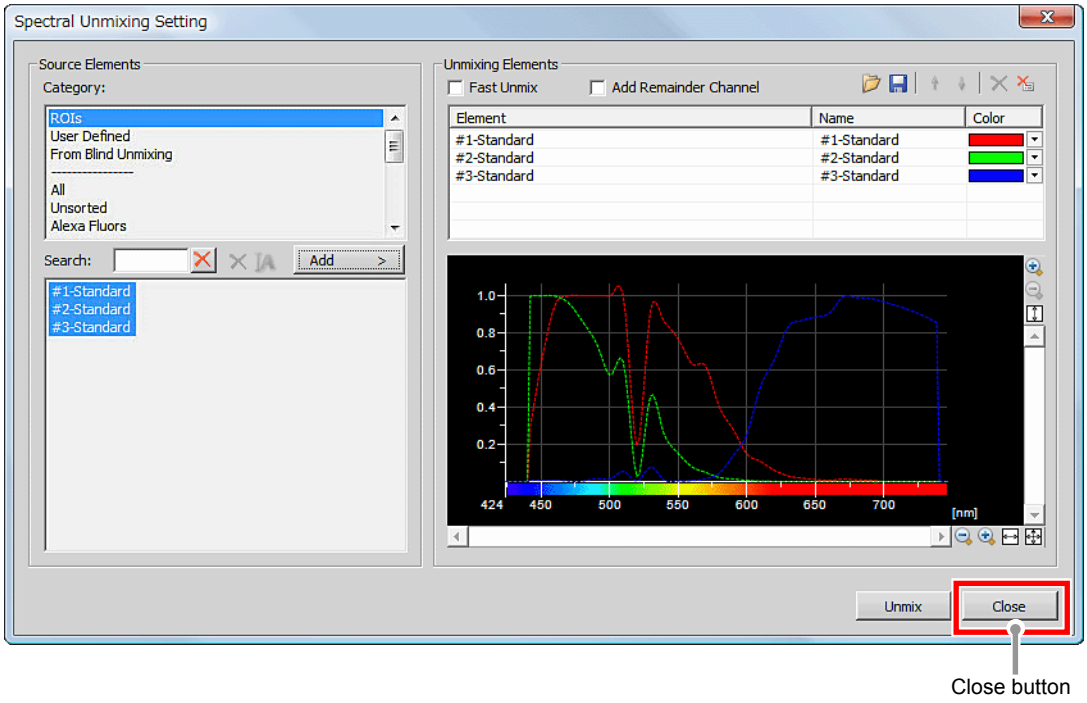


Figure 6.3-42 Spectral Unmixing Setting

* In addition to specifying using the ROI area, wavelength information can be separated by specifying the reagent in use.
However, noise provided upon image acquisition may appear.

6. Click the [Live Unmixing] button on the horizontal toolbar.
   If the wavelength to be separated is not specified, the message of “Invalid unmixing definition no unmixing elements defined.” appears.

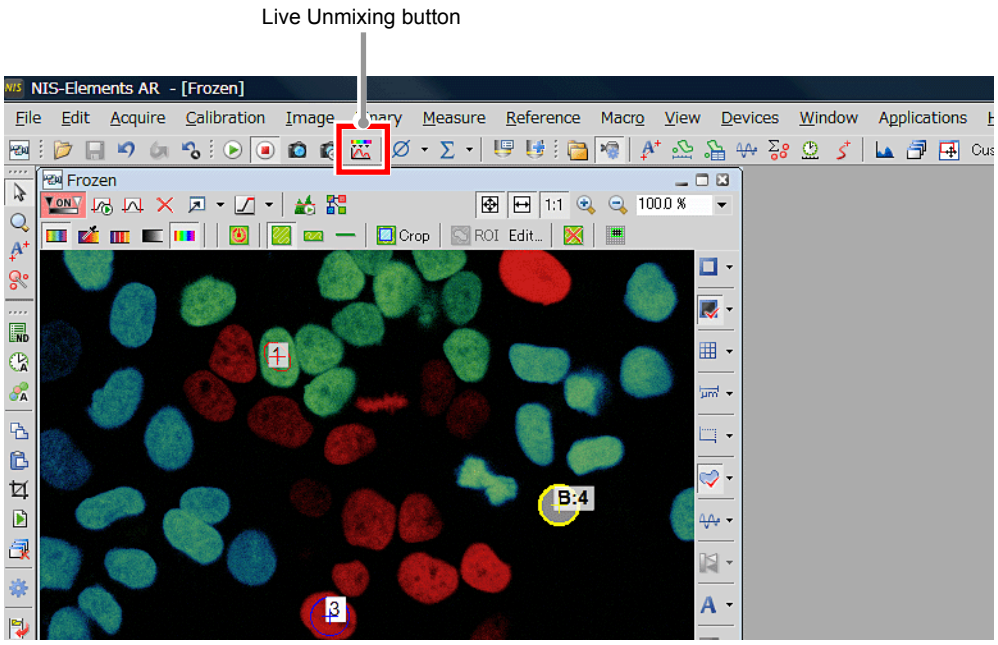


Figure 6.3-43 Live Unmixing

* Alternative method of switching to Live Unmixing
As shown in the figure on the right, select [Image] -> [Live Unmixing] on the menu bar.

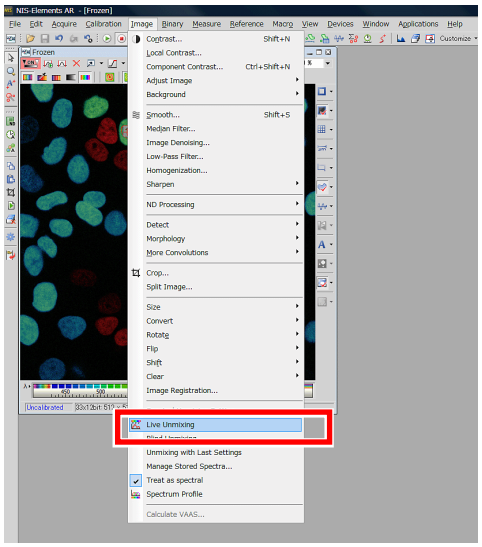


Figure 6.3-44 Switching to Live Unmixing

7. Click the [Live] button, the live image is switched to the Unmix live image.

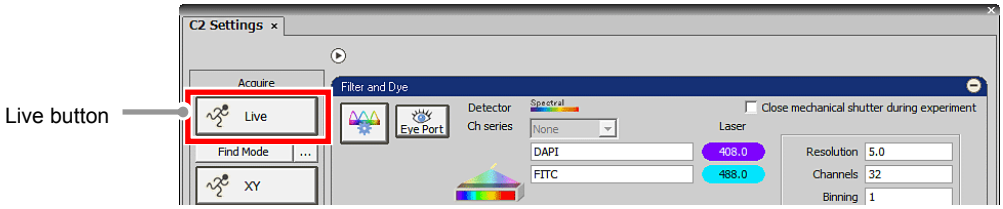


Figure 6.3-45 Acquiring the Unmix live image

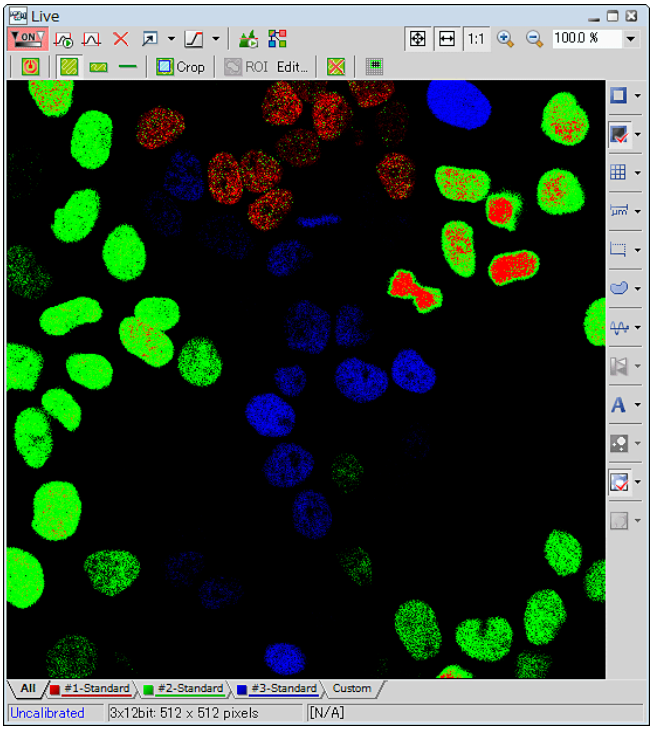


Figure 6.3-46 Live Unmixing

* Click the [Live Unmixing] button or select [Image] -> [Live Unmixing] on the menu bar again to return to the regular live image.

6.3.6 Blind Unmix

Automatically search for typical spectra and display an Unmix image separated by the spectral wavelength information.
If wavelengths overlap (because multiple reagents are in use) and differences are hard to identify, wavelength information can be separated and displayed.

Blind Unmix allows automatic separation by specifying the number of classifications or separation without specifying the number of classifications by using “Auto Search.”

6.3.6.1 Displaying the Blind Unmix Image

1. Open the [Unmix] dialog box while the acquired spectral image is displayed.
   Select [Image] -> [Blind Unmixing...] on the menu bar.
2. To specify the number of classifications, select one from “2” to “4” in the Number of Classifications pane.
   Select “Auto Search” when not specifying the number of classifications.
3. Click the [Find] button to execute the Blind Unmix.
   On completion of Blind Unmix, an image window opens for the image unmixed with the detected spectra.

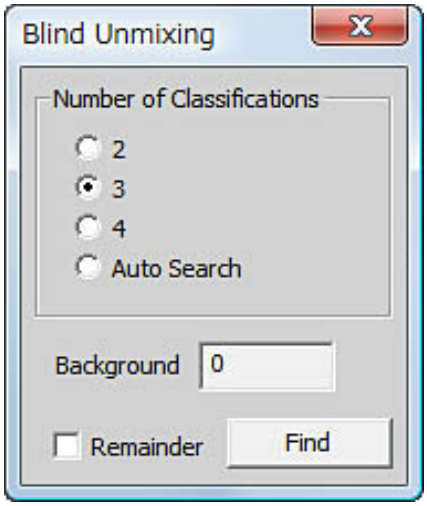


Figure 6.3-47 Blind Unmix dialog box

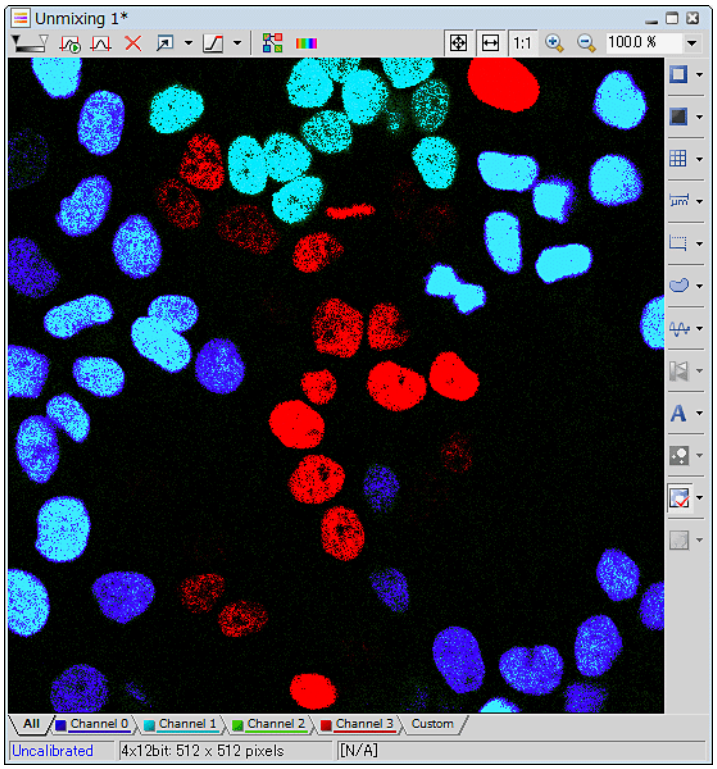


Figure 6.3-48 Spectral Unmixing view

6.3.6.2 Setting for Blind Unmix Dialog Box

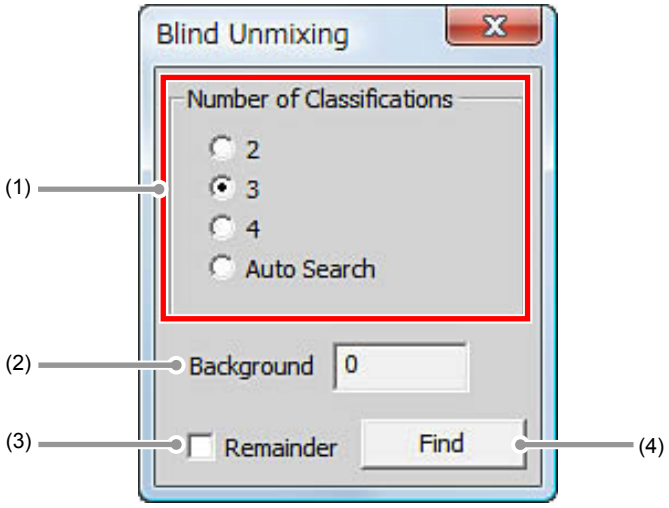


Figure 6.3-49 Blind Unmix dialog box

Table 6.3-3 Summary of Blind Unmix dialog box functions

Name		Function
(1)	Number of Classifications	Allows you to select the number of classifications for automatic separation of spectral wavelength information. Selects one from “2” to “4” to automatically separate the spectral wavelength information by the specified number of classifications. Selects “Auto Search” to automatically separate the spectral wavelength information without specifying the number of classifications.
(2)	Background	Allows you to set the threshold for elimination of the background offset noise. 0 to 4095 is specifiable. For a 16-bit spectral image, 0 to 65535 is specifiable. (Specifying the maximum value causes all to be regarded as background offset noise and no spectral wavelength information to be detected.)
(3)	Remainder	This function enables calculation of remainder data in the Unmixing calculation. When selected, the remainder data is shown as an image in the Unmixing calculation result. When deselected, the remainder data is not shown.
(4)	Find	Starts automatic detection of the spectral wavelength information.

7 Detection Mode (Virtual Filter)

This chapter describes settings for the Virtual Filter mode.

The Virtual Filter is a function that provides up to four binning groups for up to 32 channels spectral data and adjusts brightness of each group.

7.1 Filter and Dye window

This window enables to set the Optical path.

The Virtual Filter detection mode can be used when the optical path changeover lever on the C2 scan head is set to the [Spectrum] position and the Virtual Filter (VF) is selected as the detection mode in the Optical path window.

7.1.1 Structure of Filter and Dye Window

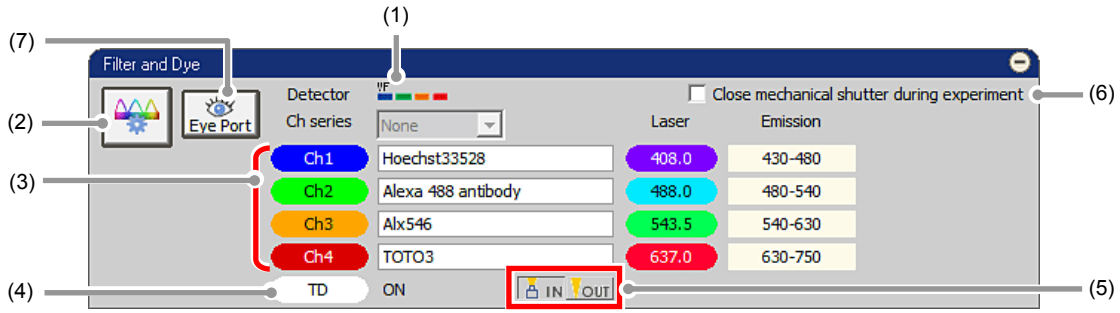


Figure 7.1-1 Filter and Dye window (Virtual Filter mode-use)

Table 7.1-1 Functions of Filter and Dye window (Virtual Filter mode-use)

Name		Function
(1)	Detector	Indicates that the Virtual Filter detection mode [VF] is used when the optical path changeover lever on the C2 scan head is set to the [Spectrum] position and the Virtual Filter (VF) is selected as the detection mode in the Optical path window.
(2)	Setting button	Opens the Optical path window. To use, select the detector, the dichroic mirror, the channel, fluorescence dye for each channel, laser and others.
(3)	Status	Indicates for the settings for each channel (fluorescence dye name, laser wavelength, and wavelength band to be acquired).
(4)	TD	Indicates the status of the motorized transmitted detector.
(5)	TD IN/OUT button	Sets/removes the motorized transmitted detector in/from the optical path. (IN = Set in the optical path/ OUT = Remove from the optical path) As for the case where the TD IN/OUT button is not displayed, it will be displayed when the motorized transmitted detector is set in the optical path in the Optical path window.
(6)	Close mechanical shutter during experiment	If unchecked, the shutter remains open during the ND image acquisition. As the shutter is not opened/closed every image acquisition, the time for the image acquisition can be shortened. However, the laser remains emitted during the interval.
(7)	Eye Port button	Changes optical path to eye port.

- **Optical Configuration**

  Individual data items set in the Virtual Filter mode can be managed collectively with the [Optical Configuration] dialog box.
  "NIS-Elements C" allows the user to store and retrieve the following settings: the laser power for image acquisition, offset of the transmission detector, PMT offset, channel selection, pinhole size, photo activation laser selection, the laser power for photo activation, averages, scan area and others.
  For storing and retrieving the [Optical Configuration] settings, see the sections concerning the optical configuration in the "NIS-Elements Advanced Research User's Guide."

7.1.2 Setting the Optical Path

Click the [Setting] button of "Filter and Dye" window to display the Optical path window.
The Virtual Filter detection mode [VF] setting screen is displayed when the optical path changeover lever on the C2 scan head is set to the [Spectrum] position and the Virtual Filter (VF) is selected as the detection mode in the Optical path window.

There are two modes available for Optical path setting, [Auto] and [Manual].
Normally, the auto mode should be used.

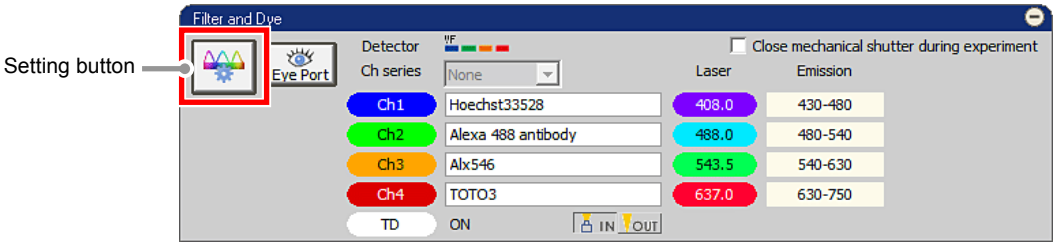


Figure 7.1-2 Filter and Dye window (Virtual Filter mode-use)

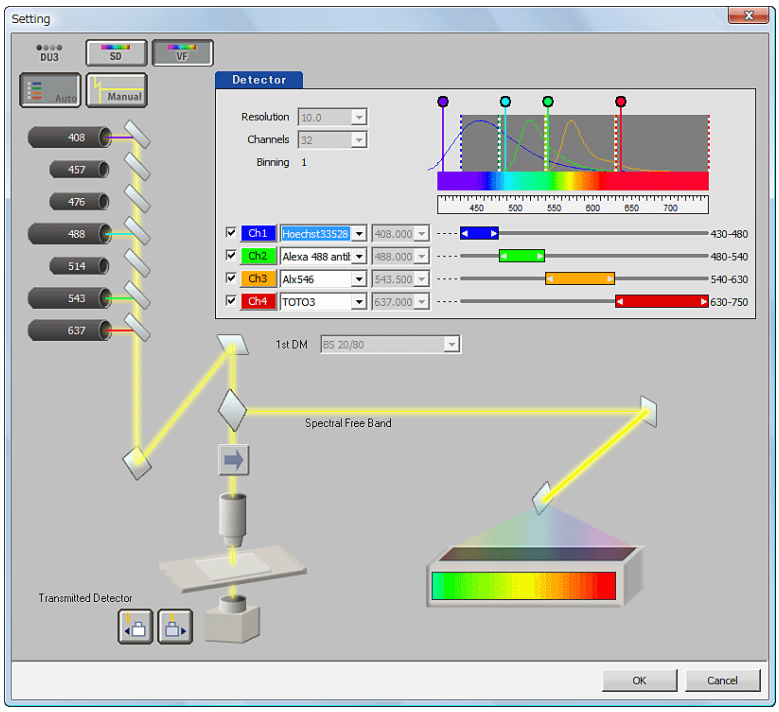


Figure 7.1-3 Optical path window (for auto mode, Virtual Filter mode-use)

7.1.3 Optical Path Window

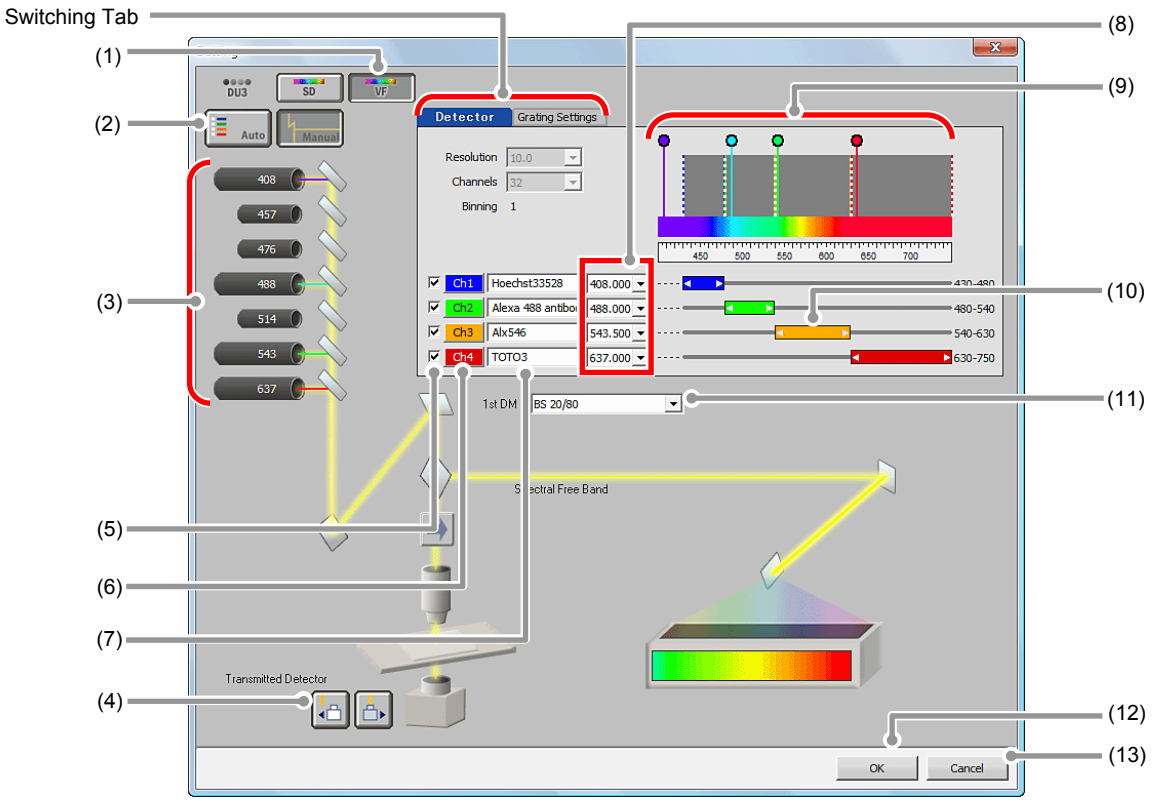


Figure 7.1-4 Optical path window (for manual mode, Virtual Filter mode-use)

Table 7.1-2 Functions of Optical path window (Virtual Filter mode-use) (sheet 1/2)

Name		Function	
(1)	Detection mode selection button		Enabled to select the Virtual Filter mode. Binning is performed for the spectral data of the concurrent 32 channels to group it into up to four groups, enabling acquisition of an image of light of a specified wavelength range.
(2)	Mode selector	Selects the desired mode for setting the Optical path.	
			Activates the auto mode. Once the fluorescence dye to be used is selected, the appropriate laser, the dichroic mirror, and the wavelength range acquired from the virtual channel are automatically selected.
			Activates the manual mode. Enables to set all of the laser, the dichroic mirror, and the wavelength range acquired from the virtual channel to be used manually.
(3)	Excitation laser indicator	Displays the current setting for the laser. The currently set laser icon is displayed in a large size, and the optical path is indicated.	
(4)	Transmitted detector selection button		Brings the transmitted detector into the Optical path, to enable the ability.
			Brings the transmitted detector out of the Optical path, to disable the ability.

Table 7.1-2 Functions of Optical path window (Virtual Filter mode-use) (sheet 2/2)

Name		Function	
(5)	Channel selection check box	Enables to select the channels to be used. (Up to 4 channel.)	
(6)	Channel color setting button	Displays the [Color Selection] dialog box, enables to set the desired color for each channel.	
(7)	Fluorescence dye selection/input:	In auto mode	Selects the fluorescence dye name to be used for each channel.
		In manual mode	Enters any desired fluorescence dye name for each channel.
(8)	Excitation laser select	These fields are only effective while in the manual mode. Enables to set the laser wavelength that is set with the software configuration, regardless of the setting of the Filter block display/select.	
(9)	Rainbow chart	Provides the following information: - Wavelength band for which to acquire images (shown in color and value for each channel) - Spectral profile of fluorescence dye - Excitation laser for fluorescence dye - A color band indicating the wavelengths in the entire band (400 to 750 nm) - Scale of the wavelengths in the entire band (400 to 750 nm)	
(10)	Acquisition range for each virtual channel slider bar	Specifies the laser wavelength range to be acquired for each virtual channel. * When shifting the slider bar in Auto mode, the Mode selector changes to manual mode.	
(11)	1st Dichroic mirror select	These fields are only effective while in the manual mode. Enables to manually select the 1st Dichroic mirror to be used.	
(12)	OK button	Determines the Optical path settings applied and closes the Optical path window.	
(13)	Cancel button	Discards the Optical path settings applied and closes the Optical path window.	

- **About switching between SD and VF**

  SD → VF: The last settings in the Virtual Filter mode are recalled.

  VF → SD: The last settings in the Spectral Detector mode are recalled.

- **About the setting condition when the setting mode is switched**

  Auto mode → Manual mode:
  The entire settings in the Auto mode are retained.

  Manual mode → Auto mode:
  The fluorescence dye with the same channel name as set in the manual mode is automatically selected.
  If the same fluorescence dye name does not exist in the list, a fluorescence dye detectable by the laser wavelength is automatically selected from the list.

  In the Auto mode, the resolution and the number of channels are automatically set so as to accommodate the wavelength range to detect all of the selected fluorescence dyes.

7.1.4 Optical Path Window Switching Tab

By selecting the manual mode at setting mode, the tab for switching between [Detector] and [Grating Settings] is displayed on the right top of the Optical path window.

7.1.4.1 Detector Tab

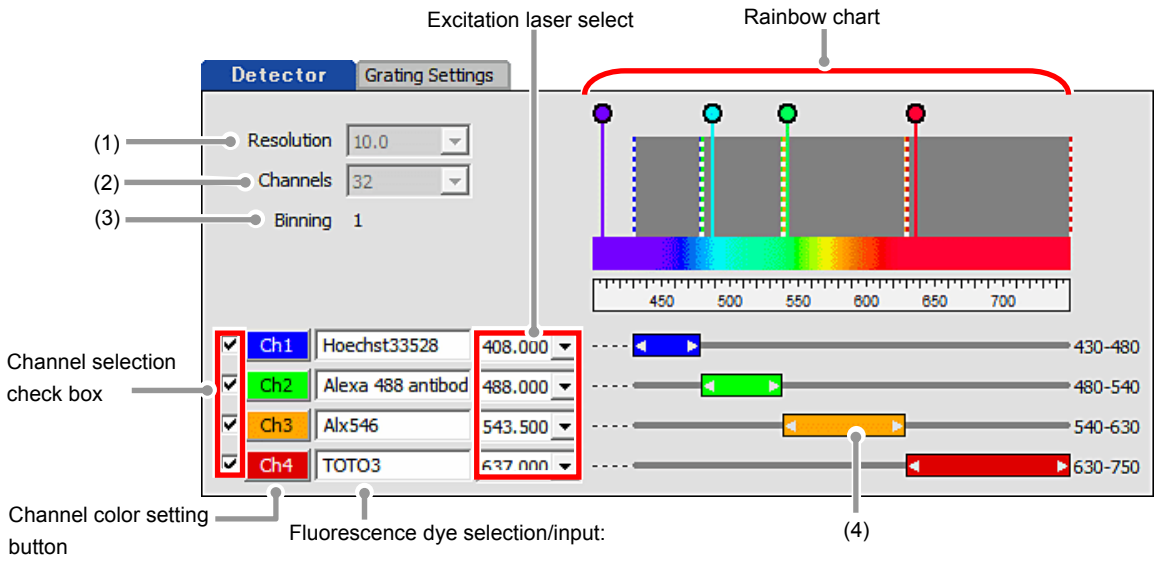


Figure 7.1-5 Optical path window (Detector tab)

Table 7.1-3 Functions of Detector tab

Name		Function
(1)	Resolution	Displays the wavelength resolution currently set.
(2)	Channels	Displays the number of channels (number of PMTs) currently set.
(3)	Binning	The number of channel binning is fixed to 1.
(4)	Acquisition range for each virtual channel slider bar	Specifies the laser wavelength range to be acquired for each virtual channel. The wavelength range can be overlapped between channels. The settable range is the grating range (the gray zone indicated in the rainbow chart).

7.1.4.2 Grating Settings Tab

The [Grating Settings] tab is displayed only when the manual mode is selected at setting mode.
Set the range for grating and set the wavelength range for the channels selected within the range.

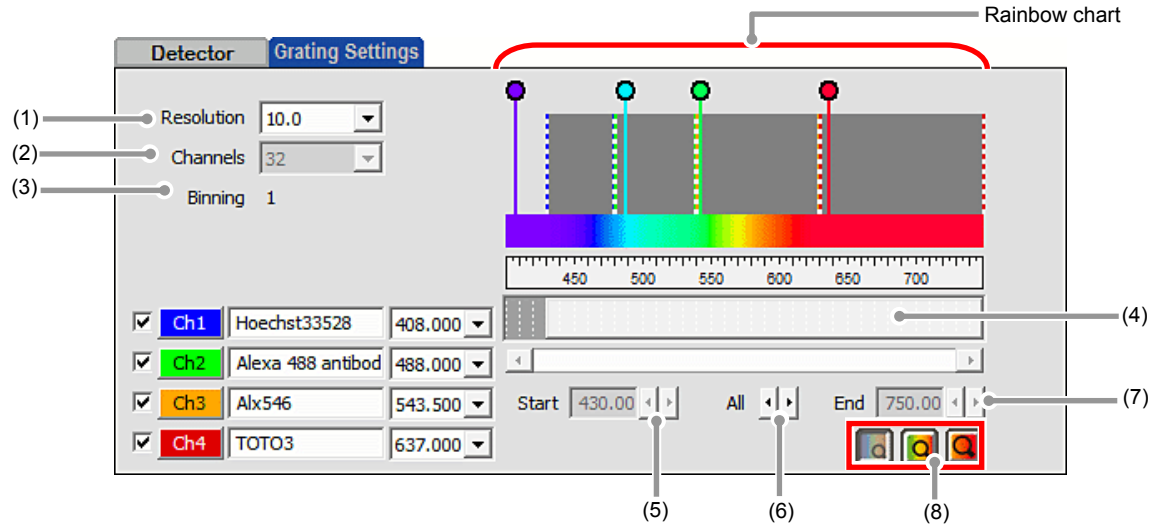


Figure 7.1-6 Optical path window (Grating Settings tab)

Table 7.1-4 Functions of Grating Settings tab

Name		Function
(1)	Resolution	Selects a wavelength resolution. Selectable from 2.5, 5, or 10nm.
(2)	Channels	The number of channel is fixed to 32.
(3)	Binning	The number of channel binning is fixed to 1.
(4)	Grating range setting bar	Sets a wavelength range in a wavelength range from 400nm to 750nm. The range depends on the grating resolution. It is shiftable horizontally but the width of the bar cannot be reduced.
(5)	Start	Displays the start wavelength of the Grating range currently selected. The right or left button cannot be use in Virtual Filter mode.
(6)	All	In the currently selected wavelength range, enables shifting to the right or left in units of 0.25nm without changing the width of the wavelength.
(7)	End	Displays the end wavelength of the Grating range currently selected. The right or left button cannot be use in Virtual Filter mode.
(8)	Enlarge button	Enlarges the rainbow chart. The display is switched in three levels.

* If the grating range is changed in the Grating Settings tab, return to the Detector tab and reset the acquisition range for each virtual channel.

7.2 Acquisition Window

The Acquisition window enables to set PMT brightness (detection sensitivity), laser power, and pinhole size.

7.2.1 Structure of Acquisition Window

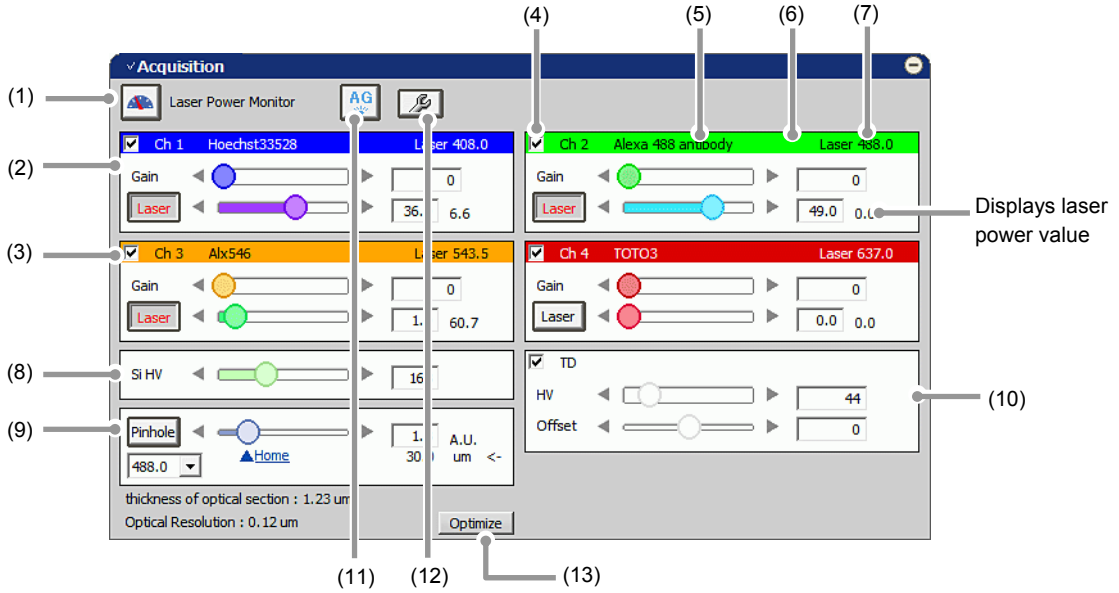


Figure 7.2-1 Acquisition window (Virtual Filter mode-use)

Table 7.2-1 Functions of Acquisition window (Virtual Filter mode-use) (sheet 1/2)

Name		Function	
(1)	Laser power monitor button	Displays the laser power value (integer obtained after A/D conversion divided by 10) of the current channel by clicking this button. During the image acquisition, the laser power cannot be measured and this button is grayed out.	
(2)	Brightness adjustment for each channel	For each of the virtual channels, use the Gain and Laser controls to adjust the brightness of the live image.	
(3)	Channel selection	Selects the virtual channels (Ch1 to Ch4, and/or TD) to acquire the desired images. Do this by adding a check mark.	
(4)	Laser ON/OFF button	Selects whether the laser is emitted or not.	
		ON status	The laser is emitted.
		OFF status	The laser is not emitted. When switched from OFF to ON, the laser power value set in the previous ON status is applied.
(5)	Fluorescence dye name indication	The fluorescence dye name specified in the Optical path window is indicated.	
(6)	Channel color	Displays the channel color specified in the Optical path window.	
(7)	Laser wavelength indication	The currently selected laser wavelength is indicated.	

Table 7.2-1 Functions of Acquisition window (Virtual Filter mode-use) (sheet 2/2)

Name		Function
(8)	Si HV	Adjusts HV of the Spectral detector.
(9)	Pinhole	Adjusts the pinhole size. For pinhole size, see Section 7.2.3, “Setting the Pinhole.”
(10)	Brightness adjustment for transmitted detector	For the transmitted detector, use the HV and Offset controls to adjust the brightness of the live image.
(11)	AG button	Automatically adjusts the Si HV value (Si HV gain) of the currently selected channel to the optimum values. For Auto Gain, see Section 7.2.4, “Auto Gain.”
(12)	Auto Gain setting button	Sets the ratio of saturation pixels used for automatic Si HV gain correction. The dialog box for range of the ratio of saturation pixels settings appears when this button is clicked. For Setting for Ratio of saturation pixels, see “Setting for Ratio of saturation pixels” in the Section 7.2.4, “Auto Gain.”
(13)	Optimize button	Displays the [XYZ Size Setup] dialog box. In the [XYZ Size Setup] dialog box, the calculation method of the recommended values of the resolution, zoom magnification, and Z stack step size can be set. For [XYZ Size Setup] dialog box, see Section 7.2.1.1, “Recommended Value Indication/Automatic Application” in the next page.

7.2.1.1 Recommended Value Indication/Automatic Application

By the function of the recommended value indication/automatic application, the recommended values of the appropriate resolution, zoom magnification, and Z stack step size are calculated based on the objective type and the selected excitation wavelength.
Using the calculated recommended values enables the image acquisition clearer and with less damage to the specimen.

Recommended Value Automatic Application

To automatically apply the recommended values to the parameters, set the [Nyquist XY] button of the Scan Area window to ON.

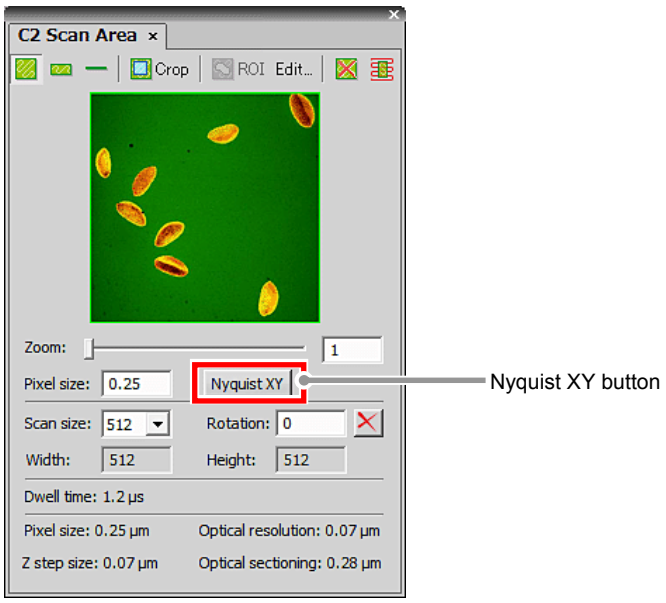


Figure 7.2-2 Scan Area window

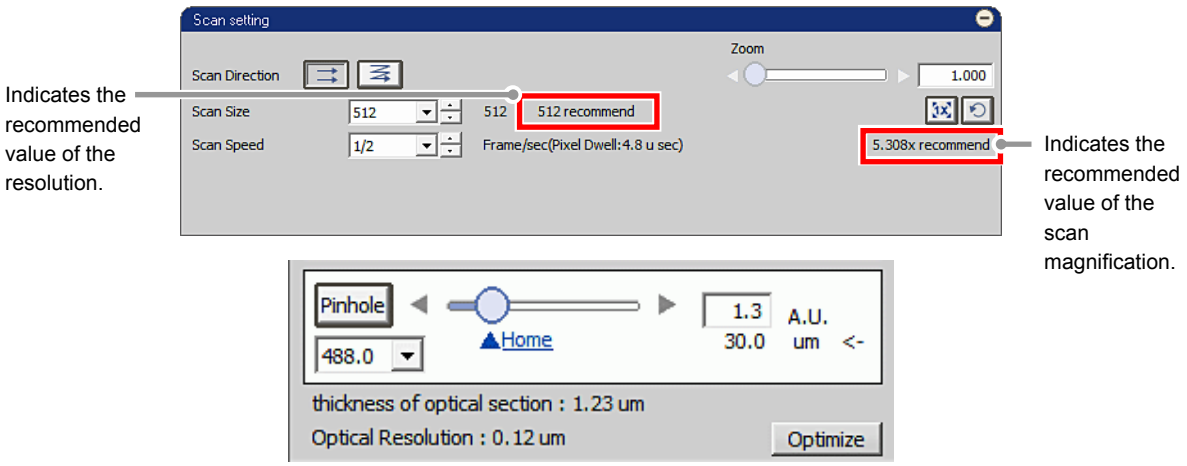


Figure 7.2-3 Location of Recommended Value Indication

* When the laser or objective in use is changed, the recommended values are recalculated, and newly indicated and automatically applied.

Recommended Value Settings

Detailed settings of the recommended values are made in the [XYZ Size Setup] dialog box that is displayed by clicking the [Optimize] button of the Acquisition window.
If the [Nyquist XY] button of the Scan Area window is ON, the recommended values are automatically applied to the parameters.
Or if the [Nyquist XY] button is OFF, the recommended values of the scan size and zoom are indicated in the Scan setting window.

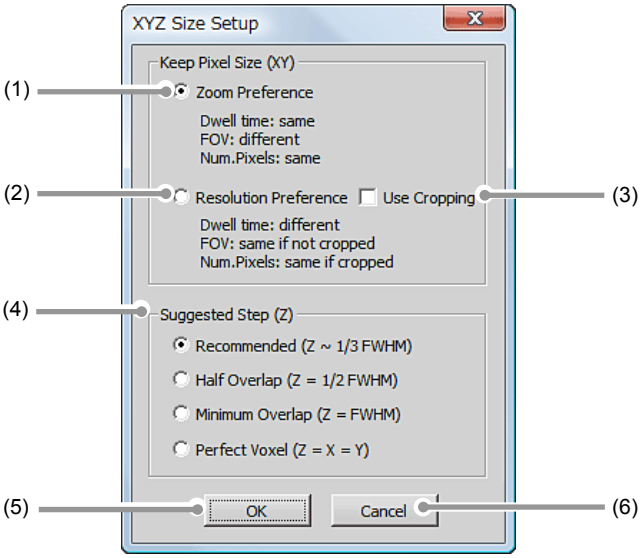


Figure 7.2-4 XYZ Size Setup dialog box

Table 7.2-2 Functions of XYZ Size Setup dialog box

Name		Function	
(1)	Zoom Preference	When the [Nyquist XY] button is ON, keeps the scan size and applied the recommended value of the zoom.	
(2)	Resolution Preference	When the [Nyquist XY] button is ON, keeps the zoom and applied the recommended value of the scan size.	
(3)	Use Cropping	Fits the scan size in detail by using Crop Scan.	
(4)	Suggested Step (Z)	Sets the Z step size calculation method.	
		Recommend (Z~1/3 FWHM)	Approximately one third of the thickness of optical section (FWHM value).
		Half Overlap (Z=1/2 FWHM)	One half of the thickness of optical section (FWHM value).
		Minimum Overlap (Z=FWHM)	The thickness of optical section (FWHM value).
		Perfect Voxel (Z=X=Y)	Value same as the pixel size.
(5)	OK button	Determines the XYZ Size Setup applied and closes the [XYZ Size Setup] dialog box.	
(6)	Cancel button	Discards the XYZ Size Setup applied and closes the [XYZ Size Setup] dialog box.	

7.2.2 Setting Image Brightness

For the live images of each Virtual channel, adjust Gain, Laser, Si HV, HV (TD), and Offset (TD) to obtain clear images.

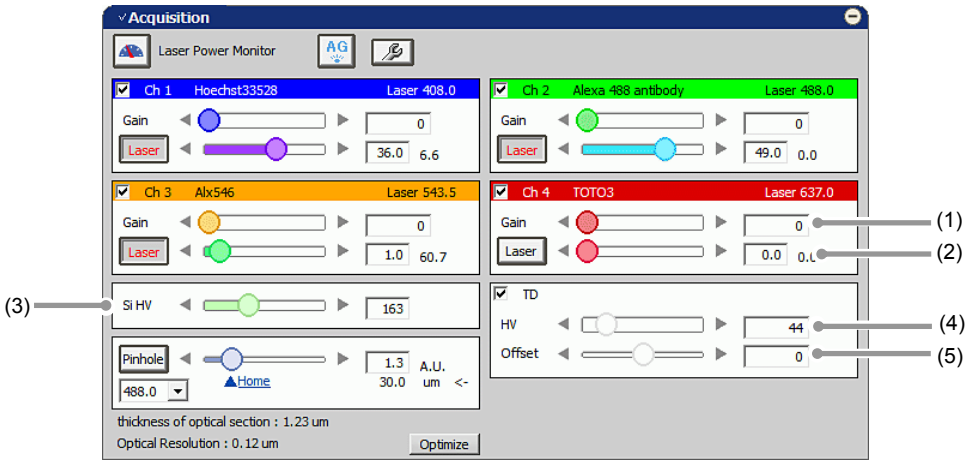


Figure 7.2-5 Setting the live image brightness (Virtual Filter mode-use)

Table 7.2-3 Brightness adjustment functions for the live image (Virtual Filter mode-use)

Name		Function
(1)	Gain	Sets the PMT Gain. Slider bar: Slides to the right or left to set the gain value. Arrow buttons: Click either arrow button to increase or decrease the gain value stepwise. Direct entry in gain value display field: Type the desired setting value.
(2)	Laser	Sets the laser power value. Slider bar: Slides to the right or left to set the laser power value. Arrow buttons: Click either arrow button to increase or decrease the laser power value stepwise. Direct entry in laser power value display field: Type the desired setting value.
(3)	Si HV	Adjusts HV of the Spectral detector. (Applied to all Virtual channel groups.) Slider bar: Slides to the right or left to set the Si HV value. Arrow buttons: Click either arrow button to increase or decrease the Si HV value stepwise. Direct entry in Si HV value display field: Type the desired setting value.
(4)	HV	Sets the voltage to be applied to the transmitted detector. Slider bar: Slides to the right or left to set the HV value. Arrow buttons: Click either arrow button to increase or decrease the HV value stepwise. Direct entry in HV value display field: Type the desired setting value.
(5)	Offset	Sets the offset value of the transmitted detector. Slider bar: Slides to the right or left to set the offset value. Arrow buttons: Click either arrow button to increase or decrease the offset value stepwise. Direct entry in offset value display field: Type the desired setting value.

PMT Overload

If too much gain is applied to the illumination intensity, the gain is automatically shut down to protect PMT and/or transmitted detector (TD), and then following [PMT Overload] dialog box is displayed.
In this case, the Si HV of Spectral Detector and/or TD HV value becomes “0”.
To continue the adjustment, set the Si HV and/or TD HV value again.

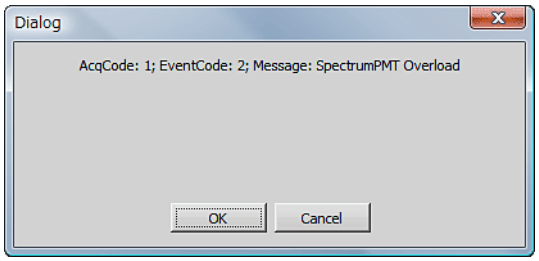


Figure 7.2-6 PMT Overload dialog box

7.2.3 Setting the Pinhole

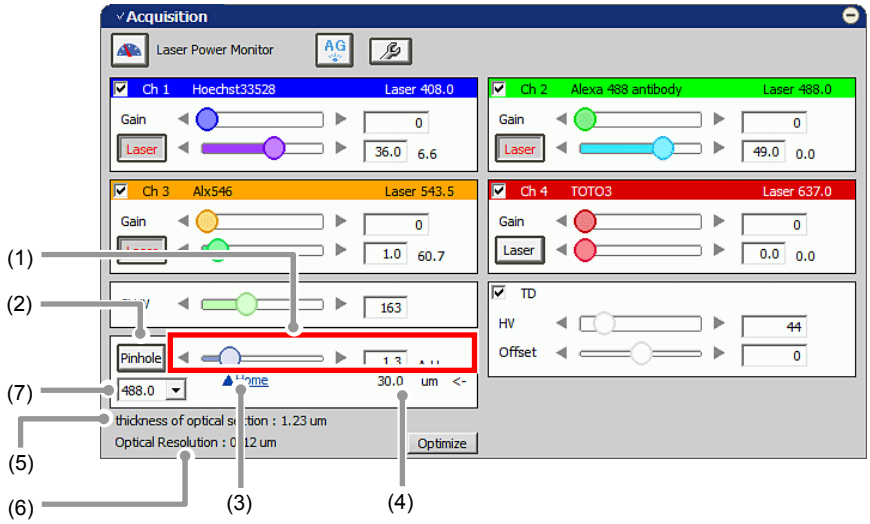


Figure 7.2-7 Setting the Pinhole (Virtual Filter mode-use)

Table 7.2-4 Pinhole setting functions (Virtual Filter mode-use)

Name		Function
(1)	Pinhole size setting	Sets a pinhole size for C2 system. Slider bar: Slides to the right or left to set the pinhole size. (Unit: A.U.) Arrow buttons: Click either arrow button to increase or decrease the pinhole size stepwise. Direct entry in pinhole size display field: Type the desired setting value.
(2)	Pinhole button	Displays the [A.U. Calculation Settings] dialog box to calculate the pinhole size. (For A.U. Calculation Settings, see Section 7.2.3.1, “Calculation Settings for Pinhole Size.”)
(3)	Home	Changes the pinhole to the predetermined home position. The value of the home position can be changed in the [A.U. Calculation Settings] dialog box. (For A.U. Calculation Settings, see Section 7.2.3.1, “Calculation Settings for Pinhole Size.”)
(4)	Pinhole size	Indicates pinhole size of C2 system. (Unit: um)
(5)	thickness of optical section	Indicates the FWHM (full width at half maximum) of z airy disk.
(6)	Optical Resolution	The actual size of 1 pixel square calculated from the optical information (for objectives and scan parameters) and the size acquired from an image.
(7)	Reference excitation wavelength for the pinhole size calculation	Selects the excitation wavelength as the reference of the automatic calculation of the pinhole size from the laser wavelengths, or enter it manually in the [A.U. Calculation Settings] dialog box. (For A.U. Calculation Settings, see Section 7.2.3.1, “Calculation Settings for Pinhole Size.”)

7.2.3.1 Calculation Settings for Pinhole Size

This section describes about the dialog box to calculate the pinhole size.

Click the [Pinhole] button in Acquisition window, the [A.U. Calculation Settings] dialog box appears. (Usually, the [Recommend] is selected to enable automatic calculation. [Recommend] calculates the A.U. value by using the Nikon-recommended EM and NA values.)

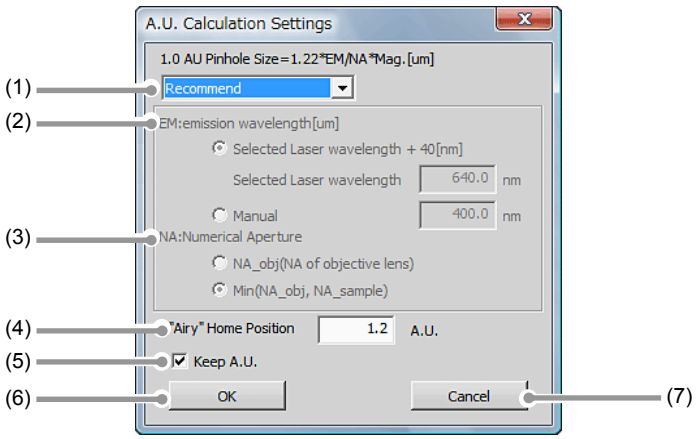


Figure 7.2-8 A.U. Calculation Settings dialog box

Table 7.2-5 A.U. Calculation Settings dialog box (sheet 1/2)

Name		Function	
(1)	Select calculation method	Recommend	Sets parameters automatically. (Recommended)
		User Setting	Allows the user to manually set parameters.
(2)	EM:emission wavelength[um]	Selected Laser wavelength	Calculates parameters by using the laser wavelength selected in the pinhole combo box of the Acquisition window as the emission wavelength (EM value). The wavelength displayed in the combo box is to be the laser wavelength set in the Optical Setting window.
		Manual	Allows the user to manually set parameters. (The parameter is calculated with the input value as the emission wavelength (EM value).) Enter the value directly from the keyboard.
(3)	NA: Numerical Aperture	Sets refractive index of the objective.	
		NA_obj(NA of objective lens)	Regardless of whether or not the objective NA value exceeds the refractive index of the sample (specimen), executes calculation by using the objective NA as the calculation parameter.
		Min(NA_obj, NA_sample)	When the objective NA value does not exceed the refractive index of the sample (specimen), executes calculation by using the objective NA as the calculation parameter. When the objective NA value exceeds the refractive index of the specimen, executes calculation by using the specimen refractive index.

Table 7.2-5 A.U. Calculation Settings dialog box (sheet 2/2)

Name		Function
(4)	“Airy” Home Position	Sets a home position of pinhole. Enter the value directly from the keyboard. * The pinhole size can be selected from six types in C2. Therefore, if the entered value does not match any of the types, the size that is larger than and the closest to the entered value is set as the home position.
(5)	Keep A.U. check box	When checked, the pinhole size is fixed by the A.U. when the selected wavelength or objective is changed. (However changes by the um.) When unchecked, the pinhole size is fixed by the um. (However changes by the A.U.) * The pinhole size can be selected from six types in C2. Therefore, if the to-be-fixed A.U. value does not match any of the types, the size that is larger than and the closest to the A.U. value is selected.
(6)	OK button	Determines the A.U. Calculation Settings applied and closes the [A.U. Calculation Settings] dialog box.
(7)	Cancel button	Discards the A.U. Calculation Settings applied and closes the [A.U. Calculation Settings] dialog box.

7.2.4 Auto Gain

Auto Gain is a function to automatically correct the value of Si HV gain to set the optimum image brightness. Automatic Si HV gain correction is performed within the predetermined range of the ratio of saturation pixels.

Automatic Si HV gain correction is performed only Si HV.
For a TD, automatic adjustment is performed when it is selected.

After execution of Auto Gain, in the dialog box indicating the progress of Auto Gain, the correction values actually used (Ratio of saturation pixels) are displayed.
If Auto Gain failed, “x” is indicated and the Si HV value returns to its original value.

- **Auto Gain cannot be started during Scan.**
- **In line scan, Auto Gain is not executable.**
- **During execution of Auto Gain, do not execute manual adjustments in the Acquisition window.**

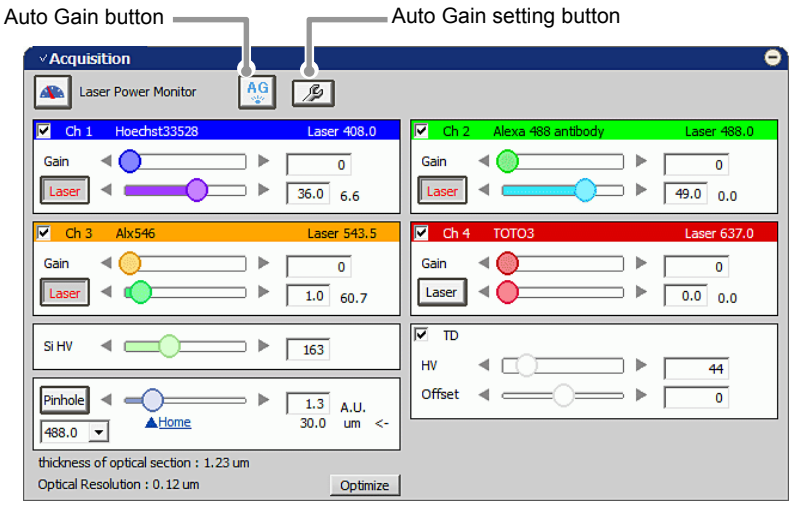


Figure 7.2-9 Execution of Auto Gain (Virtual Filter mode-use)

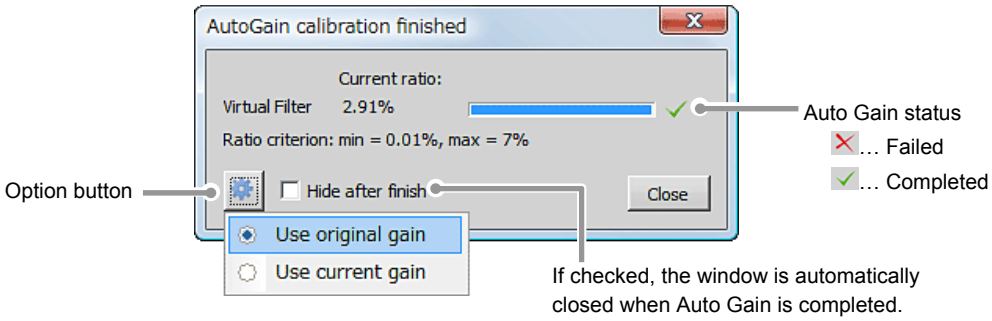


Figure 7.2-10 Auto Gain progress

Setting for Ratio of saturation pixels

Set the maximum and minimum value for the Ratio of saturation pixels used for automatic Si HV gain correction.

Click the [Auto Gain Setting] button to display the [Auto gain setup] dialog box.
Set the maximum and minimum value for the ratio of saturation pixels in [Auto gain setup] dialog box.

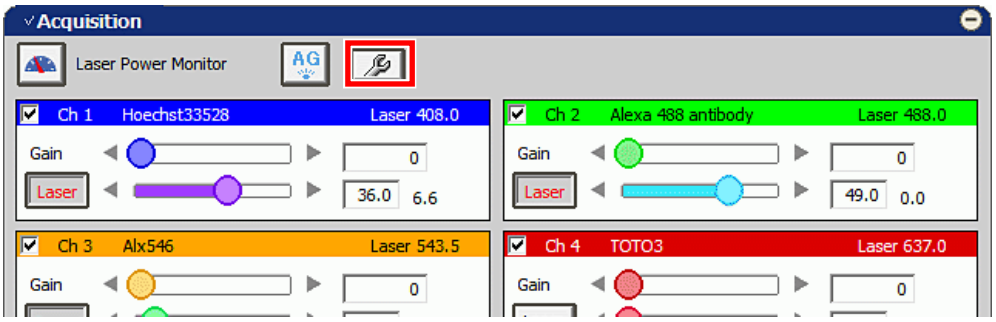


Figure 7.2-11 Displaying the Auto gain setup dialog box

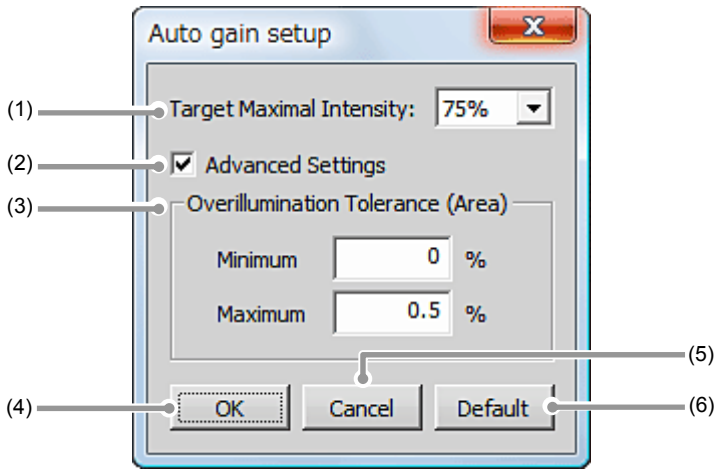


Figure 7.2-12 Setting for Ratio of saturation pixels

Table 7.2-6 Setting for Ratio of saturation pixels

Name		Function	
(1)	Target Maximal Intensity	Specifies the application ratio of the setting of the ratio of saturation pixels. Sets the percentage (%) of the maximum value to be applied.	
(2)	Advanced Settings	If checked, advanced settings of the ratio of saturation pixels are enabled.	
(3)	Overillumination Tolerance (Area)	Minimum	Sets the minimum value for Ratio of saturation pixels.
		Maximum	Sets the maximum value for Ratio of saturation pixels.
(4)	OK button	Determines the settings of Auto gain setup applied and closes the [Auto gain setup] dialog box.	
(5)	Cancel button	Discards the settings of Auto gain setup applied and closes the [Auto gain setup] dialog box.	
(6)	Default button	Resets the set values to the default values.	

7.3 Various Views (Virtual Filter mode-use)

This section describes various Virtual Filter mode views.

7.3.1 Channel View Setting

7.3.1.1 Channel Mixed View

Images acquired in the Virtual Filter mode are displayed in the method suitable to the purpose.

All image

The [All] tab is selected, all the virtual channels are mixed to display.

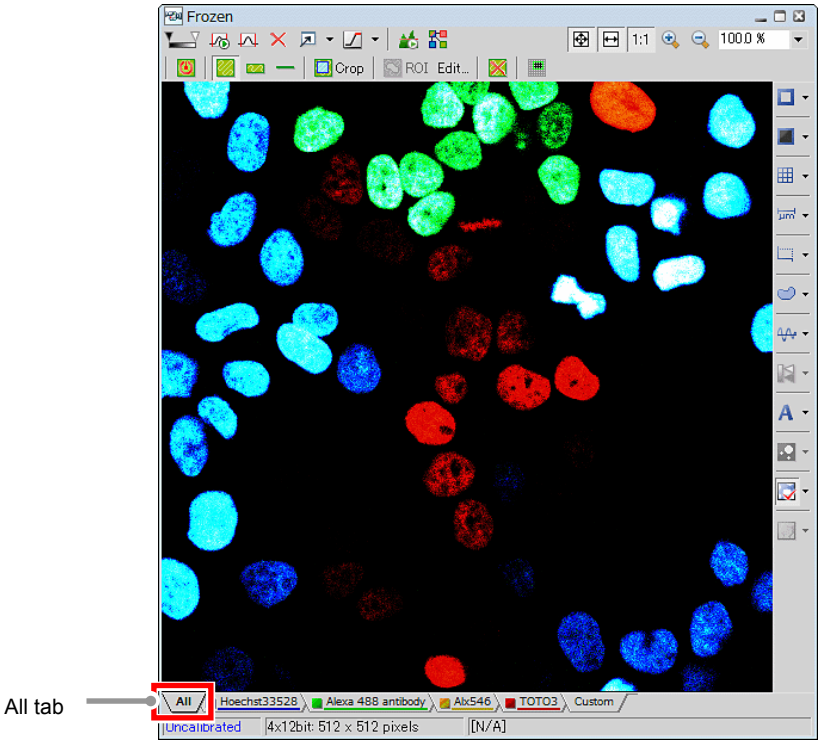


Figure 7.3-1 All image

Each channel image

To display the image of each virtual channel, select the tab corresponding to the channel.

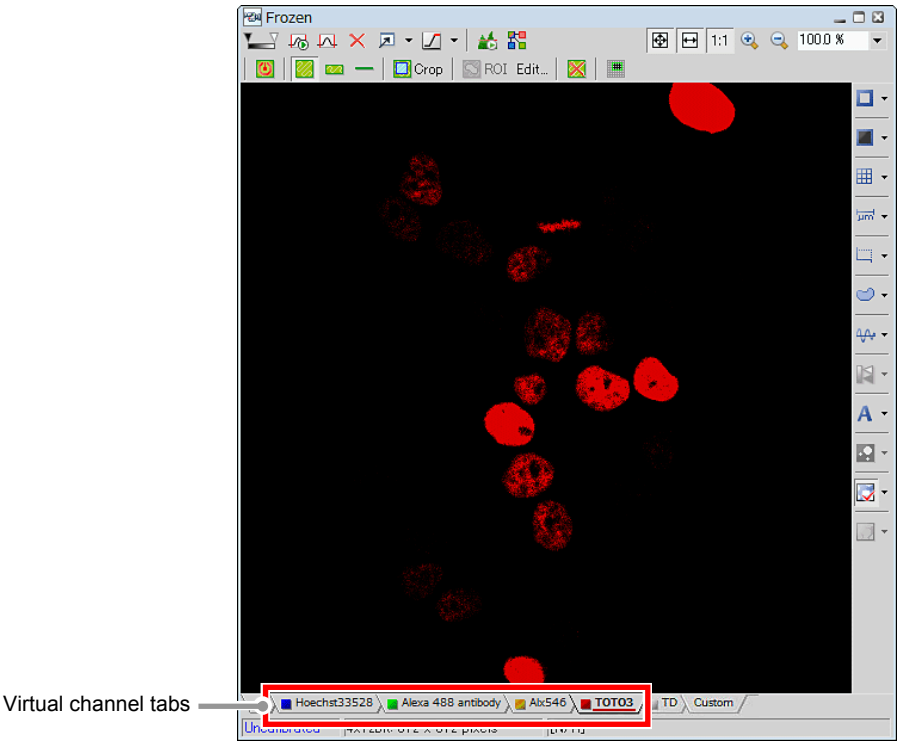


Figure 7.3-2 Each channel image

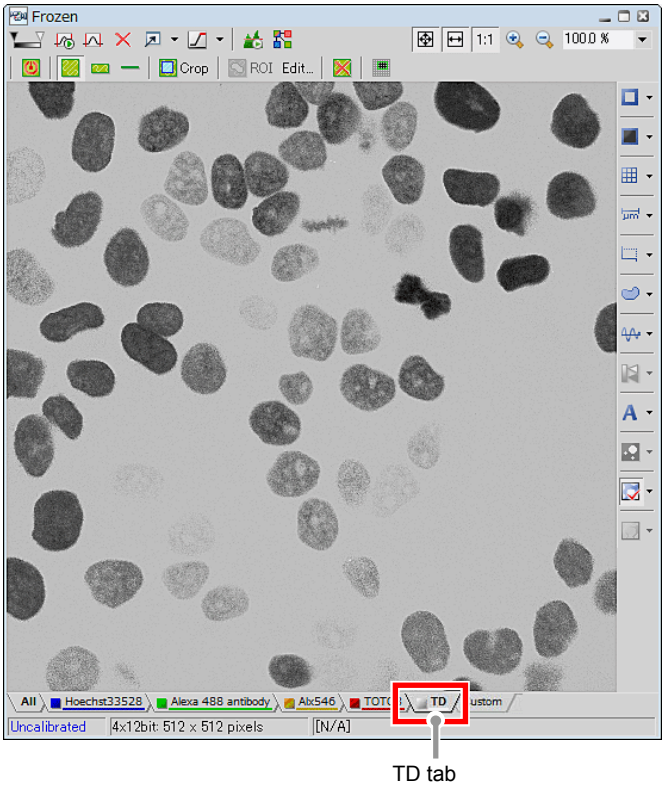


Figure 7.3-3 TD image

Custom image

Custom image displays a mixed image of selected multiple channels.
To change channels to be mixed, re-select channels.

Right-click on the [Custom] tab and a menu appears. Select [Properties...] on the menu.
The [Custom] dialog box appears to allow you to change the channels for the Custom View.

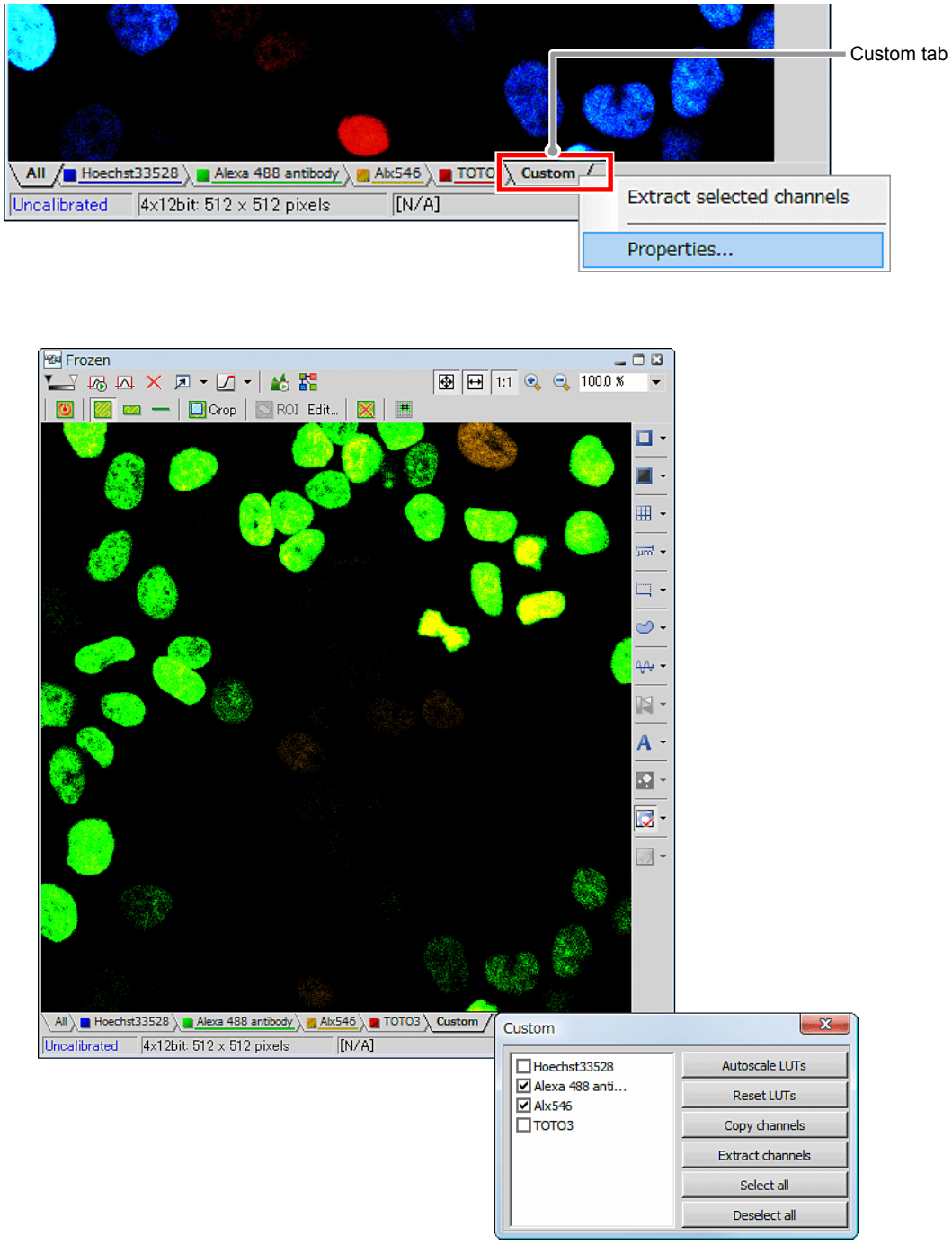


Figure 7.3-4 Selecting channels (Custom image)

Ratio image

The Ratio image view is displayed.

Right-click on the window to display a menu.
Selecting [Ratio View] from the menu changes the window to the Ratio image.

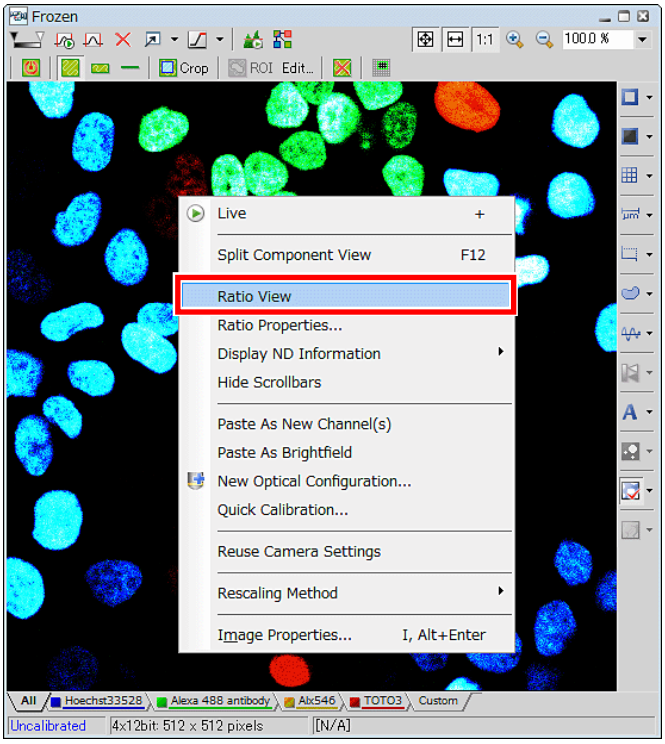


Figure 7.3-5 Displaying the Ratio image view

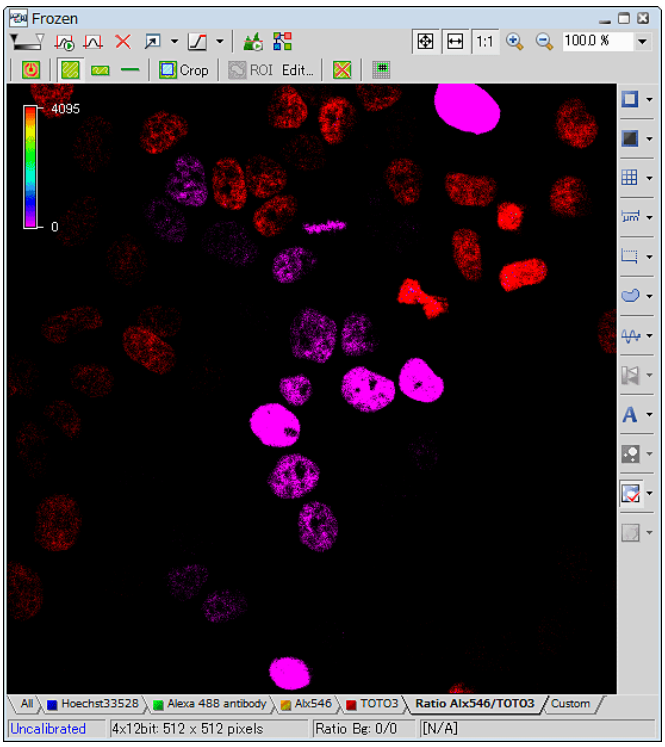


Figure 7.3-6 Ratio image

* You can change the combination of channels to be displayed in the Ratio View.
Right-click on the window and a menu appears. Select [Ratio Properties...] on the menu.
The [Ratio Properties] dialog box appears to allow you to change the channels for the Ratio View

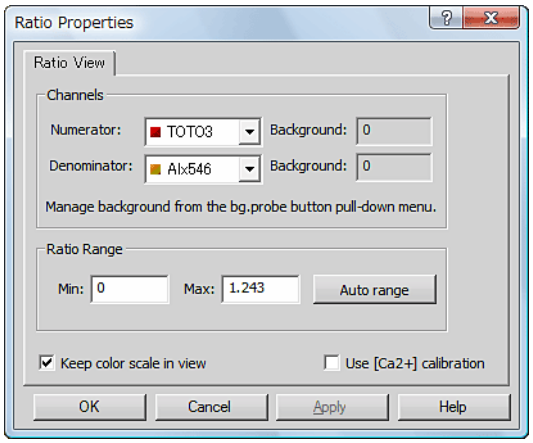


Figure 7.3-7　Ratio Properties dialog box

7.3.1.2 Split Channel View

Virtual channels are split into respective channels and displayed.

Click the [Split Components] button.
“All image” mixing all channels, respective channel images, “TD image”, “Ratio image”, “Custom image” are displayed.

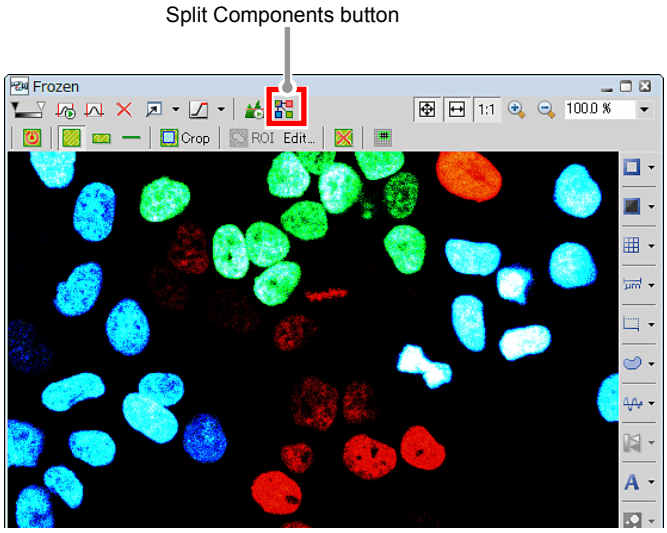


Figure 7.3-8 Frozen window

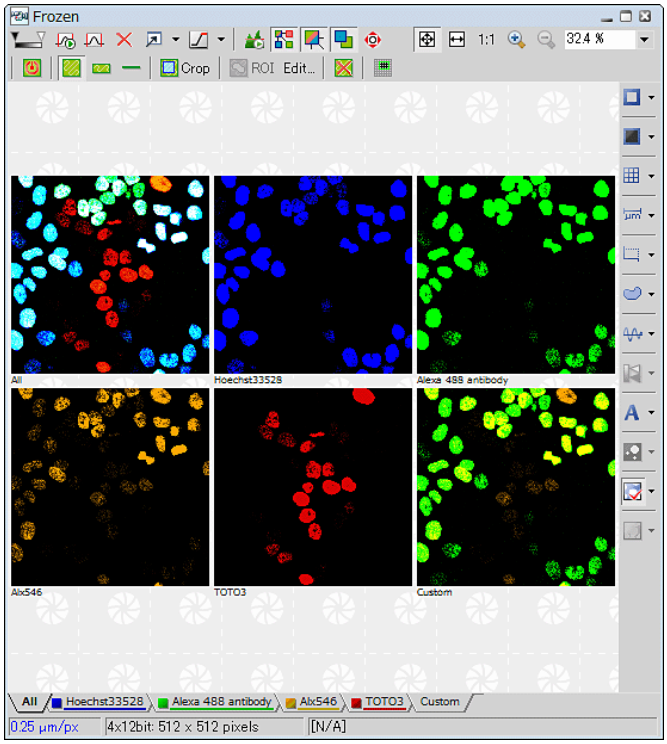


Figure 7.3-9 Split channel view

* For switching from Split channel view to Channel mixed view, click the [Split Components] button again.

8 Scan Setting Window

This window enables to set scanning conditions, such as resolution, scan speed, and magnification.
The setting items in the Scan setting window vary, for example depending on the scan area.

In the Standard Detector mode [DU3], imaging is possible with 2048 x 2048 pixels at maximum. In the Spectral Detector mode [SD] and Virtual Filter mode [VF], imaging is possible with 1024 x 1024 pixels at maximum.

8.1 Structure of Scan Setting Window

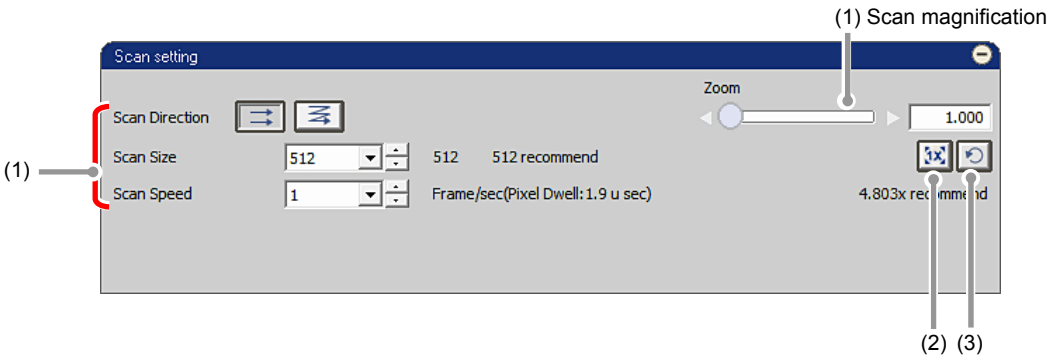


Figure 8.1-1 Scan setting window

Table 8.1-1 Summary of Scan setting window functions

Name		Function
(1)	Scan setting	Scan Direction: Selects Unidirectional or Bidirectional scan. Scan Size: Selects a resolution. (Unit=pixel) Scan Speed: Selects a scan speed. Scan magnification: Sets a scan magnification. For details of scanning setting parameters, see Section 8.3, “Scan Setting Parameters.”
(2)	Scan Zoom Reset button	Sets the scan magnification to 1.000.
(3)	Switch button for the previous setting	Returns to the previous setting.

8.2 Relationships among Scan Area Shape, Resolution, and Scan Speed

This section describes the relationship of the resolution and scan speed in the Scan setting window, and the relationship of the scan area shape set in the navigation mode versus the resolution and scan speed.

Relationship of Resolution and Scan Speed

Once a Scan Size (resolution) is set, the software automatically generates a list (see Table 8.2-1) of the scan speeds available with that resolution, making them selectable from the Scan Speed pull-down menu.

For example, suppose you set the resolution for the Square scan area to X = 512 and Y = 512 pixels, and select the Unidirectional scan. Then, values listed in the Scan Speed pull-down menu are: 1/16, 1/8, 1/4, 1/2, 1, or 2 (enable selecting at 4X or higher scan magnification.)

* The performance at the scan speed is not guaranteed. It varies depending on the environment.

Retention of Scan Setting Parameters for Different Scan Areas

For each scan area shape, the Scan setting parameters that have been previously set are retained.
Once a scan area is selected in the navigation mode, the navigation mode displays the scan area that has previously been set, and the Scan setting window displays the set values of Scan setting parameters.

Automatic Change of Scan Setting Parameters with Change in the Band Scan Area Shape

If the ratio of X and Y lengths of the Band scan area is changed:
Resolution: Does not change
Scan speed: Changes based on the new ratio of X and Y lengths, in a manner that gives the same pixel dwell.

Table 8.2-1 Combinations of resolution and scan speed (Square scan area)

Scan speed	Resolution					
	64 (not available in VF mode)	128 (not available in VF mode)	256	512	1024	2048 (not available in Spectral)
1/32					Uni-scan	Uni-scan
1/24					Uni-scan	Uni-scan
1/16				Uni-scan	Uni-scan	Uni-scan
1/8				Uni-scan	Uni-scan	Uni-scan
1/4			Uni-scan	Uni-scan	Uni-scan	Uni-scan
1/2			Uni-scan	Uni-scan	Uni-scan	
1		Uni-scan	Uni-scan	Uni-scan		
2		Uni-scan	Uni-scan	Uni-scan * Bi-scan		
3				Bi-scan *		
4	Uni-scan	Uni-scan	Uni-scan * Bi-scan			
6			Bi-scan *			
8	Uni-scan	Uni-scan *				
12	Uni-scan *					

Marks in the table

* mark indicates that selectable scan speed at 4X or higher scan magnification.

: Indicates that the combination is unavailable in the Spectral Detector mode.

For Band scan area

- The scan speed list is automatically changed depending on the Y resolution.

  Example 1.

  For resolution is 512 pixel and 1/2 band scan, "1/8, 1/4, 1/2, 1, 2, 4(*2)" are listed as the scan speed.

  Example 2.

  For resolution is 512 pixel and 1/4 band scan, "1/4, 1/2, 1, 2, 4, 8(*2)" are listed as the scan speed.

  Example 3.

  For resolution is 512 pixel and 1/16 band scan, "1, 2, 4, 8, 12(*1), 18(*2)" are listed as the scan speed.

  (*1) May not be proportional to the Y resolution.

  (*2) Selectable scan speed at 4X or higher scan magnification.

- **Scan in the Virtual Filter mode (VF)**

  When the Virtual Filter mode is selected, the scan time varies with the set number of channels. ("Time calculated by the displayed scan speed" x "number of channels")

Table 8.2-2 Combinations of resolution and scan speed (Line scan)

Scan speed \ Resolution	64 (not available in VF mode)	128 (not available in VF mode)	256	512	1024	2048 (not available in Spectral)
32				Uni-scan	Uni-scan	
128/3					Uni-scan	
64			Uni-scan	Uni-scan	Uni-scan	Uni-scan
256/3						Uni-scan
128		Uni-scan	Uni-scan	Uni-scan	Uni-scan	Uni-scan
256	Uni-scan	Uni-scan	Uni-scan	Uni-scan	Uni-scan	Uni-scan
512	Uni-scan	Uni-scan	Uni-scan	Uni-scan	Uni-scan	Uni-scan
768	Uni-scan *					
1024		Uni-scan *	Uni-scan * Bi-scan	Uni-scan * Bi-scan		
1536			Bi-scan *	Bi-scan *		

Marks in the table

* mark indicates that selectable scan speed at 4X or higher scan magnification.

: Indicates that the combination is unavailable in the Spectral Detector mode.

- **Scan in the Virtual Filter mode (VF)**

  When the Virtual Filter mode is selected, the scan time varies with the set number of channels. (“Time calculated by the displayed scan speed” x “number of channels”)

8.3 Scan Setting Parameters

This section describes the Scan setting parameters.
However, to change the Scan setting during scan, stop scan before making the change.

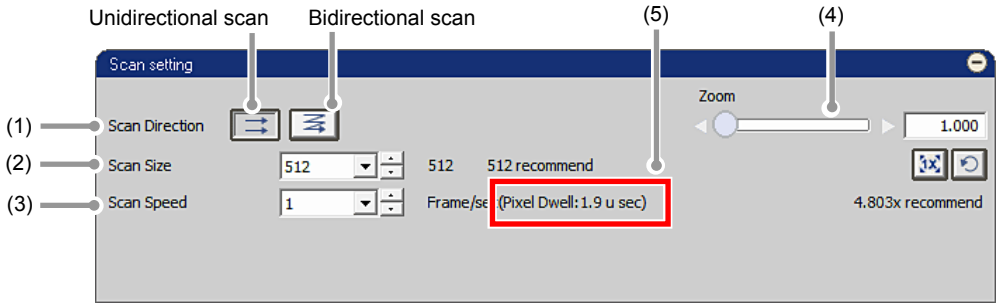


Figure 8.3-1 Scan setting parameters

Table 8.3-1 Functions of Scan setting parameters

Name		Function
(1)	Scan Direction	Toggles between Unidirectional and Bidirectional scan. Bidirectional scan is only selectable if the Square scan area or Band scan area is set. By default, Unidirectional scan is selected.
(2)	Scan Size	Sets the scan resolution in the X-direction. (Setting unit: Pixel) The resolution in the Y-direction is automatically calculated from the X to Y ratio of the scan area. Pull-down menu: Selects the desired resolution from this list. [▲] and [▼] buttons: Click these to select resolutions one after another.
(3)	Scan Speed	Sets scan speed. (Setting unit: Frame/Sec) Pull-down menu: Selects the desired scan speed from this list. [▲] and [▼] buttons: Click these to select scan speeds one after another.
(4)	Scan magnification	Sets scan magnification. Slider bar: Slides to the right or left to set the scan magnification. Arrow buttons: Click either arrow button to increase or decrease the scan magnification stepwise. Direct entry in scan magnification display field: Type the desired setting value.
(5)	Pixel Dwell	Indicates the laser irradiation time per pixel. This value is automatically determined from scan resolution and speed.

- **If the Spectral Detector mode or the Virtual Filter mode is selected as the detection mode, the bidirectional scan cannot be executed.**

Correcting the Image Shifting when Setting Bidirectional Scan

Image shifting correction when Bidirectional scan is selected is shown below.
When Bidirectional scan is selected from Scan Direction, the [Direction mismatch adjustment] button appears for shift correction. Click this button to display the slider bar and the correction value entry field.

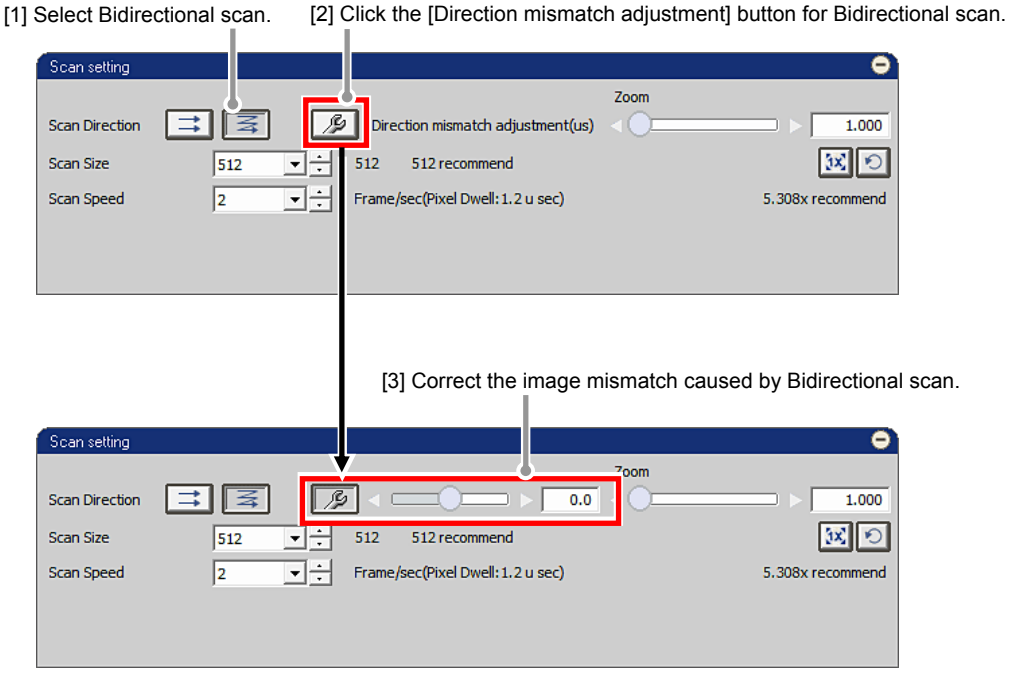


Figure 8.3-2 Correcting the image shifting for Bidirectional scan

Table 8.3-2 Correcting the image shifting for Bidirectional scan

Item	Description
Image shift correction range	-50 to 50
Image shift correction action	Slider bar: Slides to the right or left to set the correction value. Arrow buttons: Click either arrow button to increase or decrease the correction value in steps of 0.1. Direct entry in correction value display field: Type the correction value.

8.4 Unidirectional and Bidirectional Scan

8.4.1 Unidirectional and Bidirectional Scan Motion

Unidirectional scan consists of “forward paths” only, while Bidirectional scan uses both “forward and reverse paths.” Thus, Bidirectional scan takes less time to acquire a given image, but it causes shifting between the image scanned along the forward path and that scanned along the reverse path. It is therefore necessary to correct the image shifting when Bidirectional scan is selected.

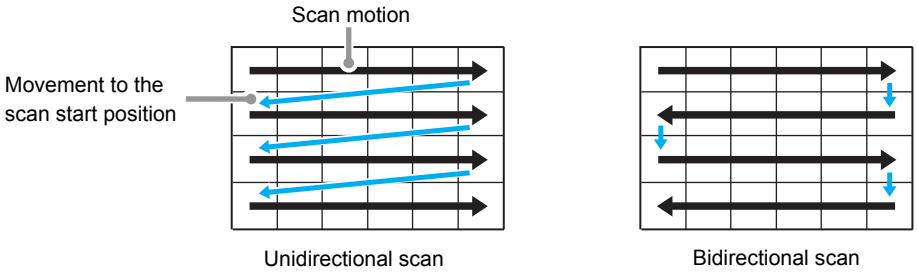


Figure 8.4-1 Unidirectional and Bidirectional scan motion

8.4.2 Scan Setting Parameters upon Toggling between Unidirectional and Bidirectional Scan

When you change from Bidirectional scan to Unidirectional scan, or vice versa, the new scan may not be executable with the current setting of scan parameters. In that case, the Scan setting parameters are automatically changed.

Table 8.4-1 Change of Scan setting parameters upon toggling between Unidirectional and Bidirectional scan

Scan direction toggling	Change of Scan setting parameters	
Bidirectional → Unidirectional	Resolution and scan speed remain unchanged.	
Unidirectional → Bidirectional	(A) If Bidirectional scan can be executed with the resolution and scan speed set for Unidirectional scan	Resolution and scan speed remain unchanged.
	(B) If Bidirectional scan cannot be executed with the resolution and scan speed set for Unidirectional scan	The resolution is changed to a value that can be used in the bidirectional scan and the closest to the set value.

9 Navigation Mode

The navigation mode enables to set the scan area in acquired images.
There are two types of navigation modes. If settings on either window are changed, display of the scan area, etc., on the other window changes in an interlocked manner.

9.1 How to Display Navigation Mode

The procedure of how to display each window is shown as follows.

Scan Area window

How to display the Scan Area window is shown below.

Click the button shown below to open the Scan Area window.

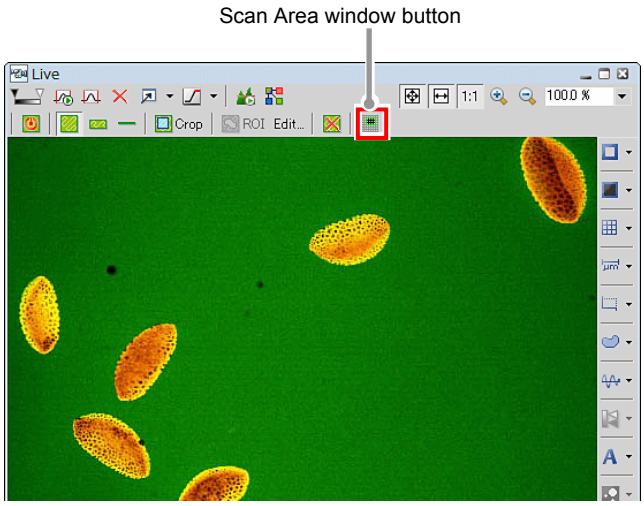


Figure 9.1-1 To display the Scan Area window

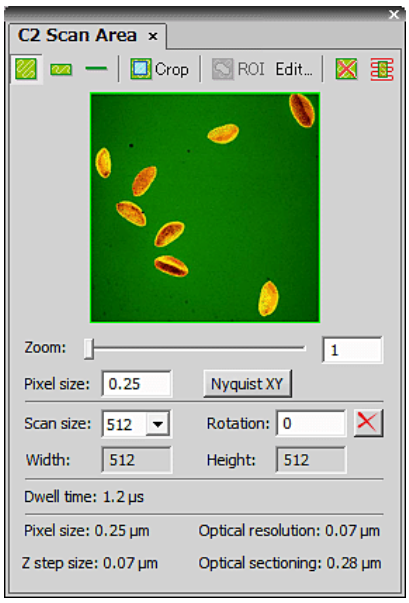


Figure 9.1-2 Scan Area window

* Other display methods
As shown below, right-click on the gray area (without any dialog box and setting window displayed) to display a menu. Then select [Acquisition Controls] -> [C2 Scan Area] in the menu to open the Scan Area window.

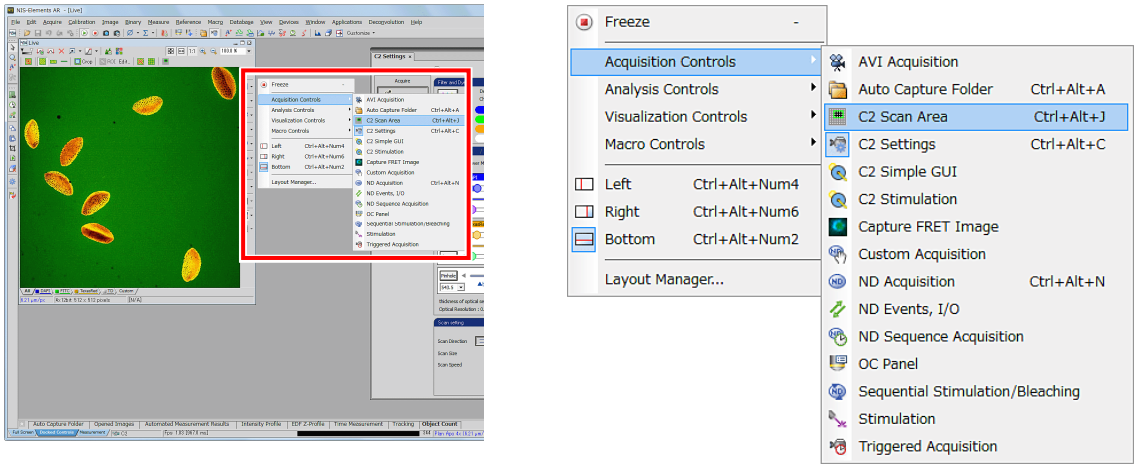


Figure 9.1-3 To display the Scan Area window

Navigation Mode

How to display the navigation mode is shown below.

The navigation mode is displayed by clicking [Show Scan Area] button in the Live window (which opens when the live image is acquired) or the Captured window (in which the live image was captured).

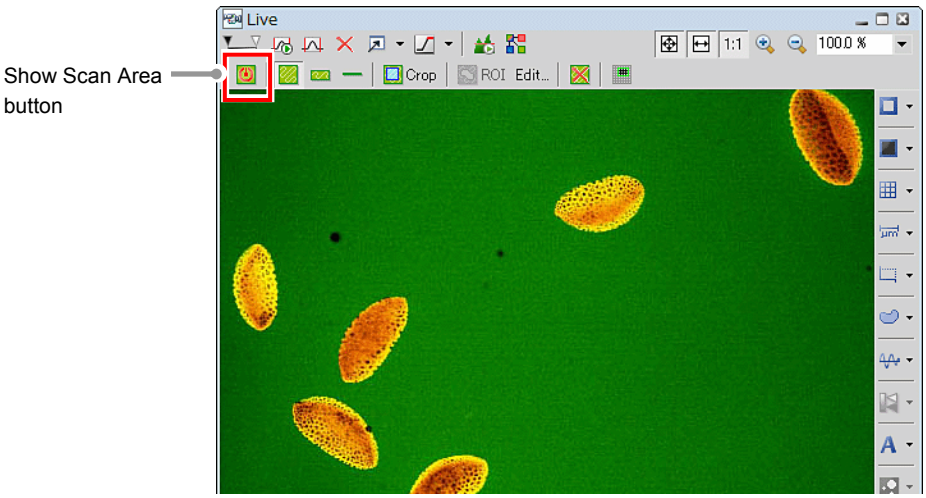


Figure 9.1-4 Live window

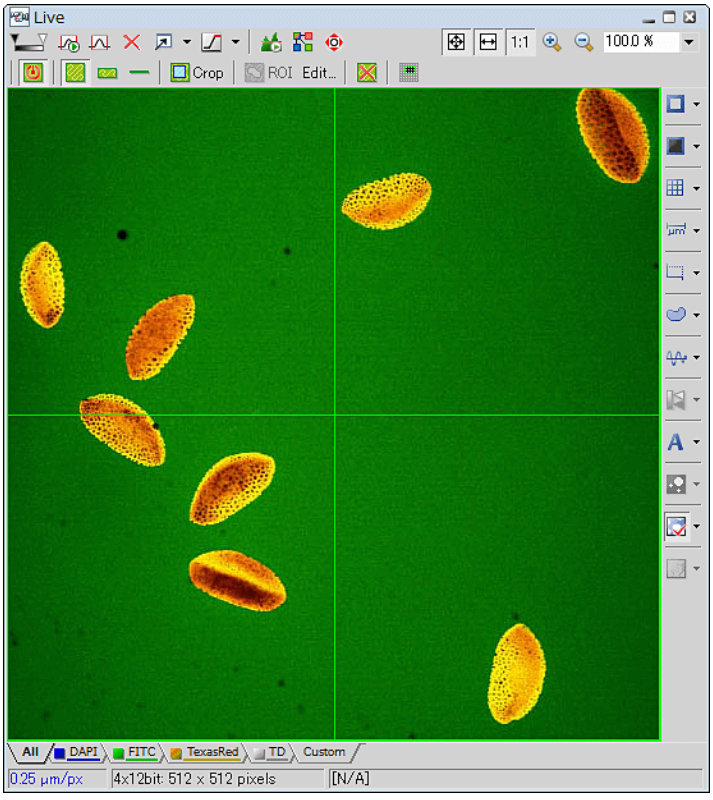


Figure 9.1-5 Navigation mode

9.2 Structure of Navigation Mode

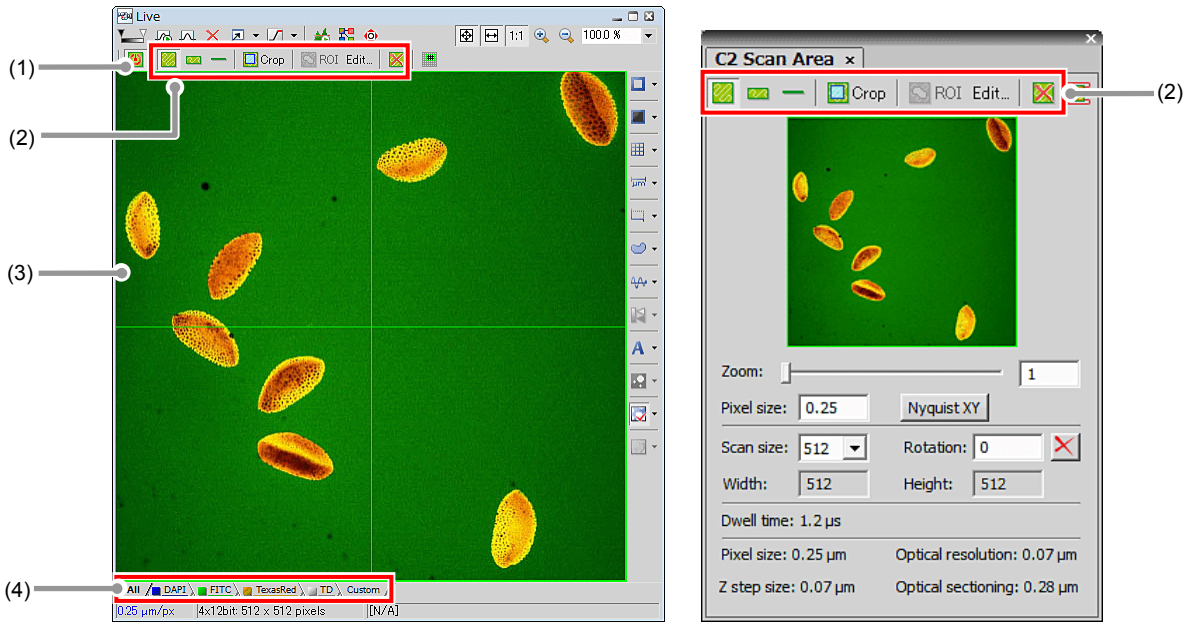


Figure 9.2-1 Navigation mode

Table 9.2-1 Summary of navigation mode functions

Name		Function	
(1)	Show Scan Area button	Switch the Live window to the navigation mode.	
(2)	Scan area setting tools	Provides tools for setting the scan area. A scan area of the selected shape can be set. Available tools vary depending on the scan mode and the type of scan area. For scan areas that can be set, see Section 9.3, “About Scan Areas.”	
(3)	Scan area view	A set scan area is displayed as green lines. If two or more ROI scan areas are set, colors of displayed lines are different. The lines are displayed in light blue for the Crop scan area.	
(4)	Channel selection tabs	All tab	Displays the overlaid images of all channels.
		Fluorescence dye name tabs	Displays the fluorescence dye names of each channel. Clicking each tab displays only the image of the corresponding channel.

9.3 About Scan Areas

There are three types of scan areas according to their shape. They are the Square scan area, the Band scan area and the Line scan.

Additionally, two other types are available. They are the ROI scan area and the Crop scan area, designed to serve particular purposes.

“NIS-Elements C” allows the user to store and retrieve the scan area settings (except for ROI scan area). For storing and retrieving the [Optical Configuration] settings, see the sections concerning the optical configuration in the “NIS-Elements Advanced Research User's Guide.”

9.3.1 Conditions for Setting Scan Areas

The following table shows conditions for setting scan areas.

Table 9.3-1 Conditions for setting scan areas

Scan area	Function	Parameter limits
Square	Rotation	-90 to 90°
	Magnification	As desired (1X to 1000X)
	Resolution	Both X and Y: 64 (*1), 128 (*1), 256, 512, 1024, or 2048 (*2) pixels
	X to Y ratio	X = Y
Band	Rotation	-90 to 90°
	Magnification	1X to 1000X
	Resolution	X: 64 (*1), 128 (*1), 256, 512, 1024, or 2048 (*2) pixels Y: 32, 64, 128, 256, 512, or 1024 (*2) pixels
	X to Y ratio	X > Y
Line	Line type	Straight line only
	Magnification	1X to 1000X
	Resolution	X: 64 (*1), 128 (*1), 256, 512, 1024, or 2048 (*2) pixels

(*1) Unsettable when the Virtual Filter [VF] is selected as the detection mode.

(*2) Usable only when the Standard Detector [DU3] is selected as the detection mode.

- **The rotation angle cannot be set when the Bidirectional scan is selected.**

- **The “ROI scan area” and the “Crop scan area” are effective for the Square scan area.**
- **Parameters of the “ROI scan area” and the “Crop scan area” depend on the selected scan area.**

9.3.2 Scan Area Setting Tools

The available scan area setting tools include the Square setting tool, Band setting tool, Line setting tool, ROI setting tool and Crop setting tool.

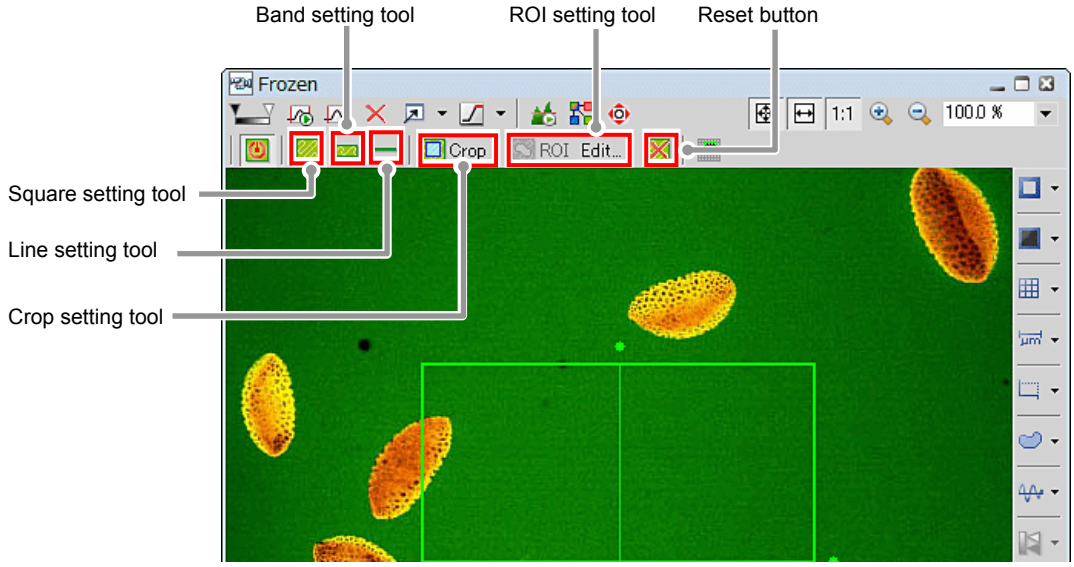


Figure 9.3-1 Scan area setting tools

Table 9.3-2 Functions of scan area setting tools

Name	Function	
Square setting tool	The Square scan area of a desired size can be set. The X and Y-directions are always of the same resolution.	
Band setting tool	The Band scan area of a desired size can be set. The Y-direction resolution is always lower than the X-direction resolution.	
Line setting tool	The Line scan (straight line scan) of a desired length and angle can be set. The line width is 1pixel. Images can be acquired in the X and T-directions.	
	X and T-directions	Timelapse observation is carried out only to the area specified by the Line.
ROI setting tool	Enables to set the scan area with any shape.	
Crop setting tool	Enables to set a smaller rectangular scan area within the Square scan area. The file size of image data can be decreased without changing the scan speed by cutting off unnecessary parts. The resolution in Y direction is the same or lower than the resolution in X direction.	
Reset button	Resets the current scan area settings.	

- **Z-direction setting when scanning a cross section**
  The Z-direction will be set by NIS-Elements. For setting instructions, refer to “NIS-Elements Advanced Research User’s Guide.”

Square Scan Area

The Square scan area appears when the square setting tool is selected.

- By default, the Square scan area occupies the whole of the image window.
- Only one Square scan area can be set in the single image.
- The Square scan area cannot be removed.
- The Square scan area that appears upon selecting the square setting tool is the one that was set previously.
- In the image window, the Square scan area can be set to any position and any size.

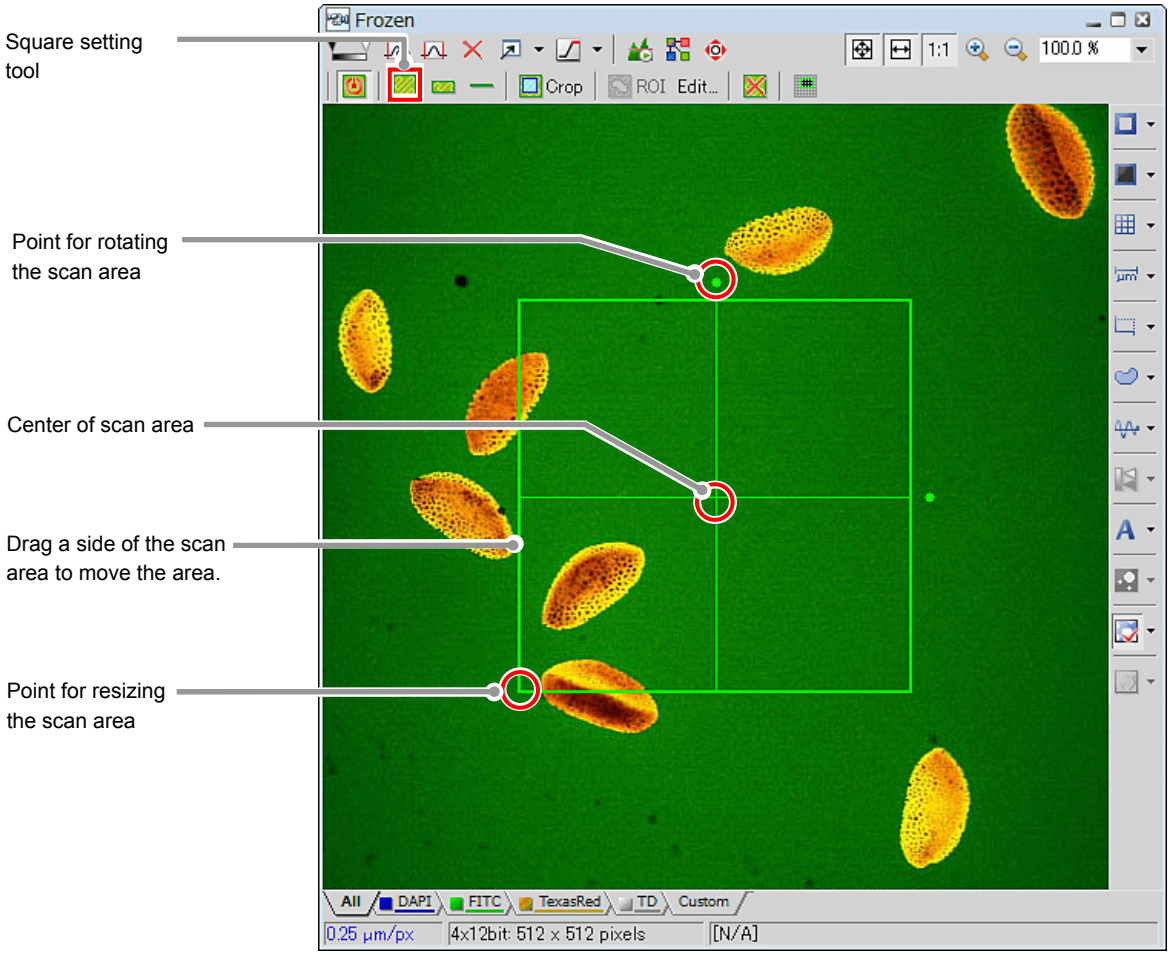


Figure 9.3-2 Square scan area

Table 9.3-3 Functions of the Square scan area and their operation

Function	Operation
Resize scan area	Drags the point placed at each corner, or at the center of each side, of the scan area. The scan area can be enlarged or reduced to a desired size, while retaining the square form.
Rotate scan area	Places the mouse pointer over the rotation point located outside the scan area. As the pointer changes to the rotation pointer, drag it to rotate the scan area. The rotation range is -90 to 90 degrees.
Move scan area	Places the mouse pointer on a side of the scan area. As the pointer changes to the move pointer, drag it to move the scan area. The scan area can be moved only within the display area of the image window. It cannot be moved outside the display area.

Band Scan Area

The Band scan area appears when the band setting tool is selected.

- By default, the Band scan area has the X-direction length equal to the width of the image window, and the Y-direction length equal to 1/2 of the X-direction length, with its center at the center of the image window.
- Only one Band scan area can be set in the single image.
- The Band scan area cannot be removed.
- The Band scan area that appears upon selecting the Band setting tool is the one that was set previously.
- The Band scan area can be set to any position in the image window, and to any size that meets the condition “X-direction length > Y-direction length.”

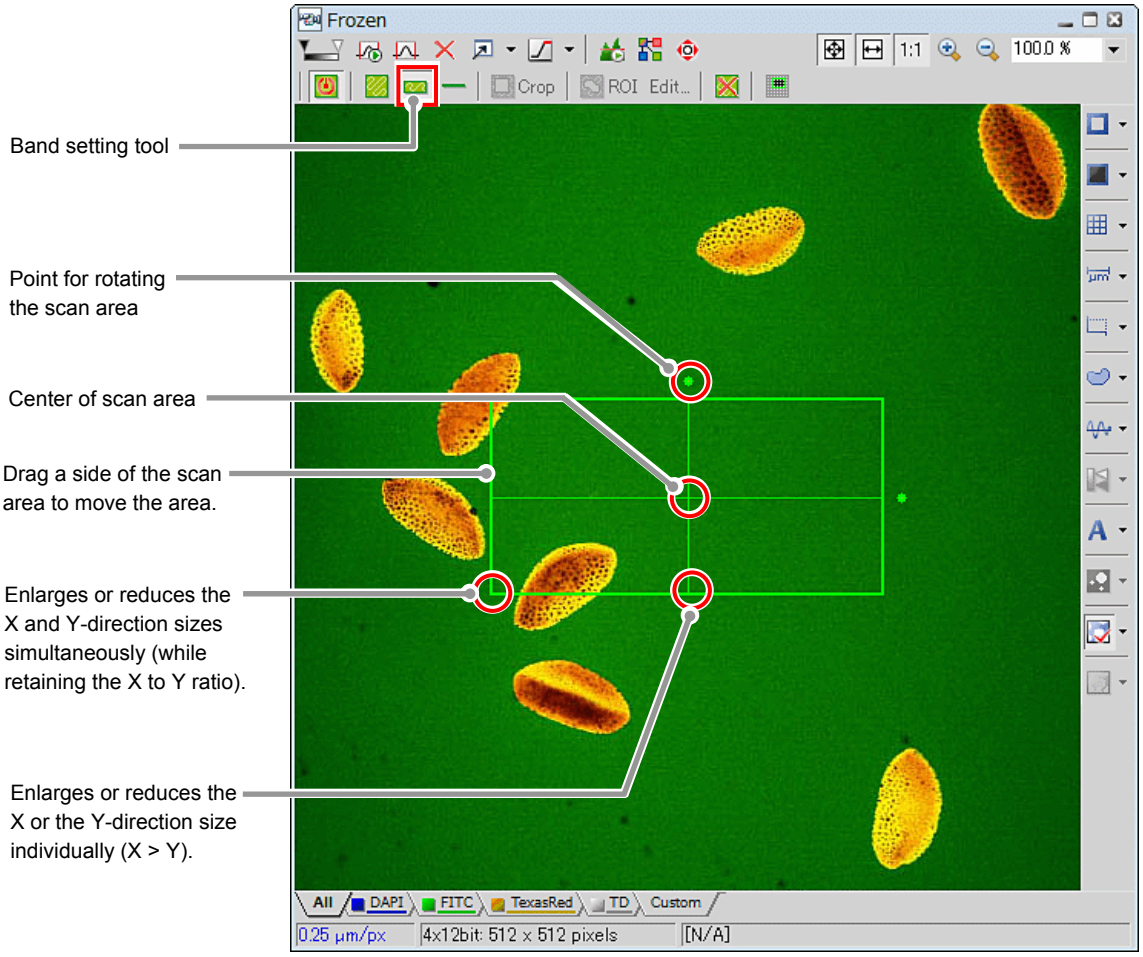


Figure 9.3-3 Band scan area

Table 9.3-4 Functions of the Band scan area and their operation (sheet 1/2)

Function	Operation	
Resize scan area	Drags the point placed at each corner, or at the center of each side, of the scan area to enlarge or reduce the size as desired.	
	Point at each corner	The size can be enlarged or reduced as desired while retaining the ratio of X to Y-direction lengths.
	Point at the center of each side	The X and Y sizes can be changed individually provided that “X-direction length > Y-direction length.”

Table 9.3-4 Functions of the Band scan area and their operation (sheet 2/2)

Function	Operation
Rotate scan area	Places the mouse pointer over the rotation point located outside the scan area. As the pointer changes to the rotation pointer, drag it to rotate the scan area. The rotation range is -90 to 90 degrees.
Move scan area	Places the mouse pointer on a side of the scan area. As the pointer changes to the move pointer, drag it to move the scan area. The scan area can be moved only within the display area of the image window. It cannot be moved outside the display area.

Resolution of the Band Scan Area

This section describes the X and Y-direction resolution of the Band scan area.

Table 9.3-5 Resolution of the Band scan area

X-direction resolution	A desired X-direction resolution is selected from 64 (*1), 128 (*1), 256, 512, 1024, and 2048 (*2) pixels
Y-direction resolution	The Y-direction resolution is automatically set as it is calculated from the ratio of X to Y-direction lengths. Example: If the X-direction resolution = 512, and the ratio of X-direction length to Y-direction length = 1:1/2, then the Y-direction resolution is set to “256.”

(*1) Unsettable when the Virtual Filter [VF] is selected as the detection mode.

(*2) Usable only when the Standard Detector [DU3] is selected as the detection mode.

• **If the Band scan area is resized:**

For the X-direction, the resolution does not vary even if the X-direction length is changed.
For the Y-direction, if the X-direction and/or the Y-direction length is changed, the ratio of X to Y-direction lengths varies. Based on the new ratio, the Y-direction resolution is automatically recalculated and set.

Example: Assume that the X-direction resolution = 512 pixels, the Y-direction resolution = 256 pixels, and the ratio of X to Y-direction length = 1:1/2.
If the Band scan area is changed and the resultant ratio of X to Y-direction lengths = 1:1/4, the Y-direction resolution is set to 128 pixels.

• **If the X-direction resolution is changed:**

The Band scan size does not vary either in the X or Y-direction.
The Y-direction resolution is automatically set as it is recalculated from the ratio of X and Y-direction lengths.

Example: Assume that the ratio of X-direction length to Y-direction length = 1:1/2, where the X-direction resolution = 512 pixels and the Y-direction resolution = 256 pixels.
If the X-direction resolution is changed to 256 pixels, the Y-direction resolution is set to 128 pixels.

Line Scan

The Line scan appears when the Line setting tool is selected.

- The Line scan has no default value.
- Line scan drawing can be set on the Live window, but is hidden after the live image has been acquired and is displayed only on the Scan Area window.
- Only one Line scan can be set in the single image.
- The Line scan cannot be removed.
- The Line scan that appears upon selecting the line setting tool is the one that was set previously.
- In the image window, the Line scan can be set to any position and any length and angle.
- The Line scan can be used in the live acquisition and in the timelapse acquisition with no delay.

Figure 9.3-4 Line scan

Table 9.3-6 Functions of the Line scan and their operation

Function	Operation
Change scan line	Drags the both ends of the line to change the line length or angle.
Rotate scan line	The Line scan allows a line to be drawn with a desired length and at a desired angle on the Scan Area window, thus it does not provide a function to rotate it.
Move scan line	Places the mouse pointer on a side of the scan line. As the pointer changes to the move pointer, drag it to move the scan line. The scan line can be moved only within the display area of the Scan Area window. It cannot be moved outside the display area.

ROI Scan Area

Clicking the [Edit ROIs inside Square Area] button of the ROI setting tool displays the ROI Editor. Use the ROI Editor to set the scan area with any shape.

- Unusable when the image resolution is 2048.
- Two or more ROI scan areas can be set on the image.

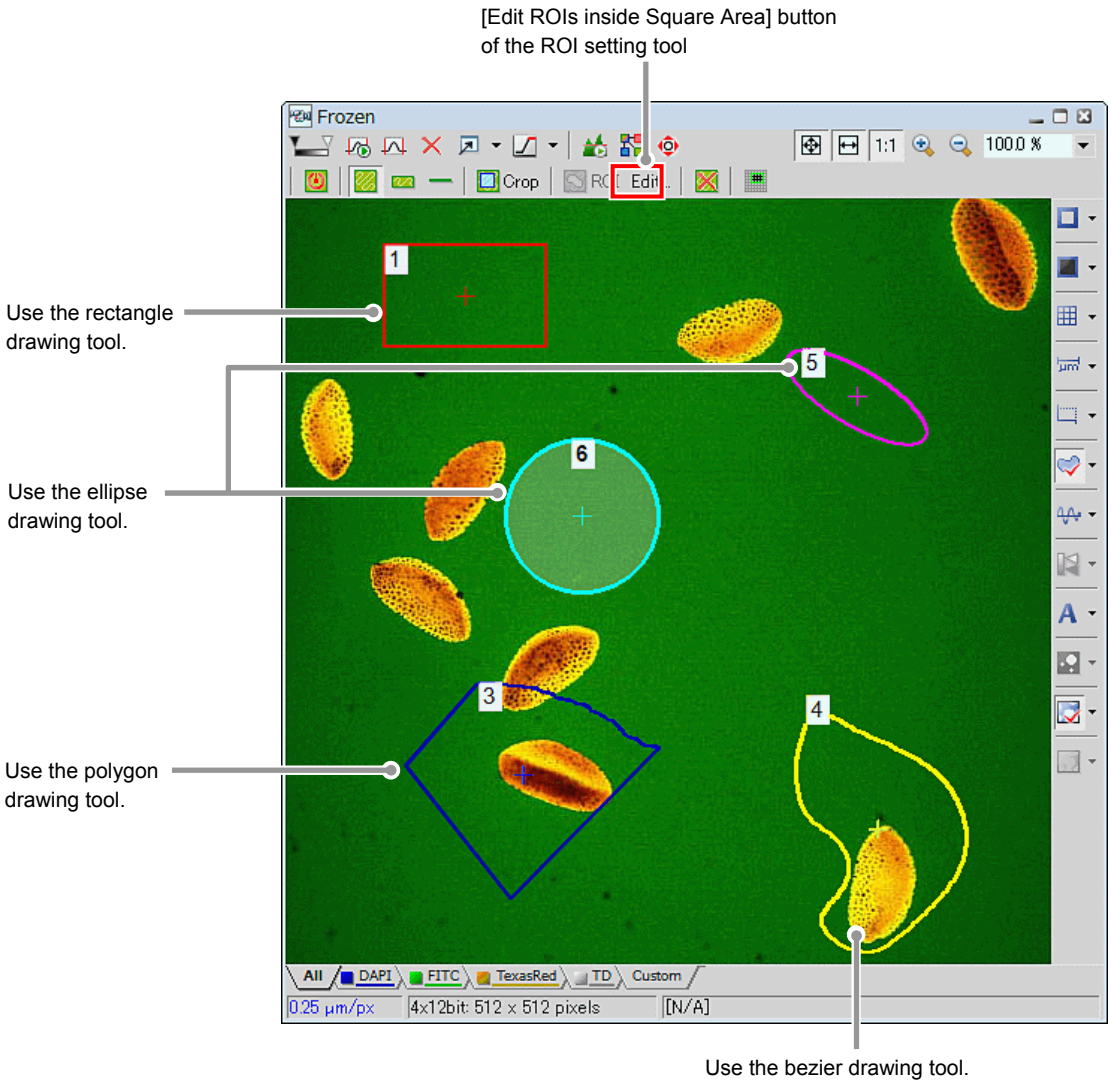


Figure 9.3-5 ROI scan area

* Drawn ROIs are hidden when the ROI Editor is closed, but you may check the scan areas on the Scan Area window.

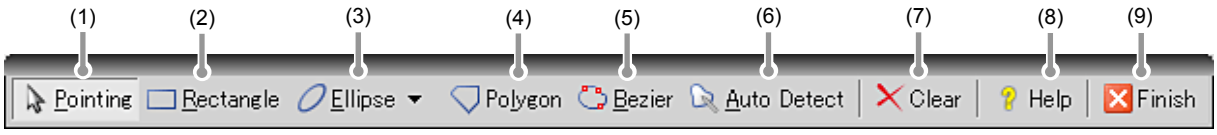


Figure 9.3-6 ROI Editor

Table 9.3-7 Functions of the ROI scan area drawing tool

Name		Functions and their operations
(1)	Pointing	Used to move a drawn ROI on the window.
(2)	Rectangle	Used to designate the scan area enclosed by a rectangle.
(3)	Ellipse	Used to designate the scan area enclosed by a circle. Clicks the center of the desired circle and drag to designate the size. When the drawn circle is picked and dragged with the mouse, the circle moves to another position. When □ on the top, bottom, right, or left of the circle is picked and dragged, the circle deforms. Right-clicking on the drawn circle designates the circle as the ROI scan area.
(4)	Polygon	Used to designate the scan area enclosed by straight lines. Designates the start point by clicking on the image and moving the pointer to the straight line ending position (end point) and clicking draws a straight line. Draw straight lines subsequently to draw a polygon. To close the selected area by connecting straight lines to each other, place the mouse pointer on the start point and double-click the mouse. Double-clicking the pointer at a position different from the start point can also close the selected area.
(5)	Bezier	Used for freehand drawing or for drawing a straight line or smooth curve by placing anchor points. For freehand drawing, click the mouse on the image then drag the mouse. To draw a curve using anchor points, drag the mouse in the curving direction. To close the selected area, right-click the mouse. (Double-clicking the mouse left button also closes the selected area.)
(6)	Auto Detect	Used to automatically detect and specify the similar color portion adjacent to the clicked position. By clicking the mouse on the image, the similar color portion adjacent to the clicked position is selected. To fix the selected area, right-click the mouse.
(7)	Clear	Clears the ROI scan area.
(8)	Help	Displays the help for ROI Editor.
(9)	Finish	Finishes drawing and editing of the ROI scan area and closes the ROI Editor.

Crop Scan Area

Enables to set a smaller rectangular scan area within the Square scan area.
The file size of image data can be decreased without changing the scan speed by cutting off unnecessary parts.

- For the Crop scan area, a rectangle only can be selected.
- Only one Crop scan area can be set on the image.
- Any position and any size can only be set within the Square scan area.
- If the Crop setting tool is selected, the previously set the Crop scan area is displayed.
- The resolution in Y direction is the same or lower than the resolution in X direction.
- Unusable in the Bidirectional scan.

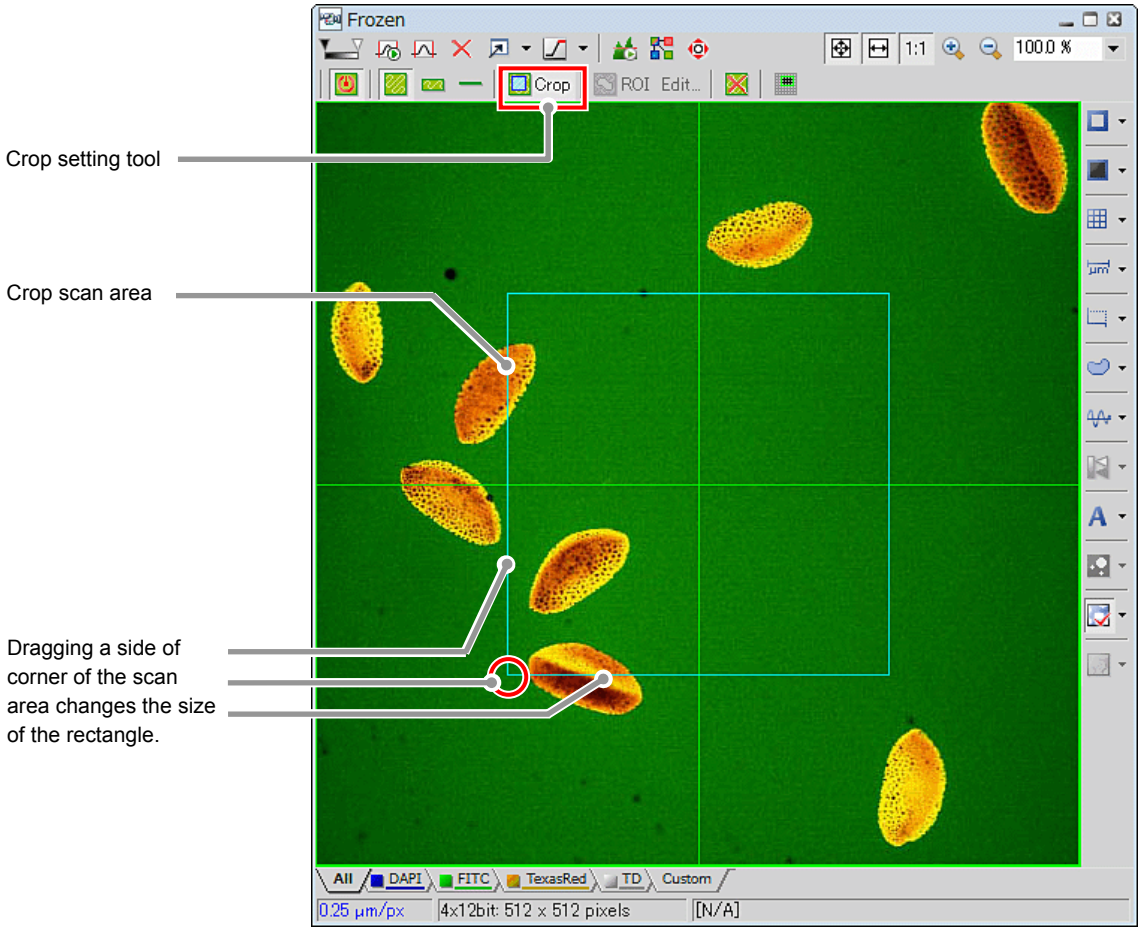


Figure 9.3-7 Crop scan area

Table 9.3-8 Functions of the Crop scan area and their operation

Function	Operation
Resize scan area	When the mouse pointer is placed on a corner or side of the Crop scan area, the arrow pointer is displayed. Clicking the mouse while the arrow is displayed and dragging in the arrow direction enables to enlarge or reduce to any size.

9.3.3 Switching Scan Area Setting Tools

The scan area setting tool can be switched by clicking the respective buttons.
Before using the Crop setting tool and the ROI setting tool, turn the [Show Scan Area] button to OFF.

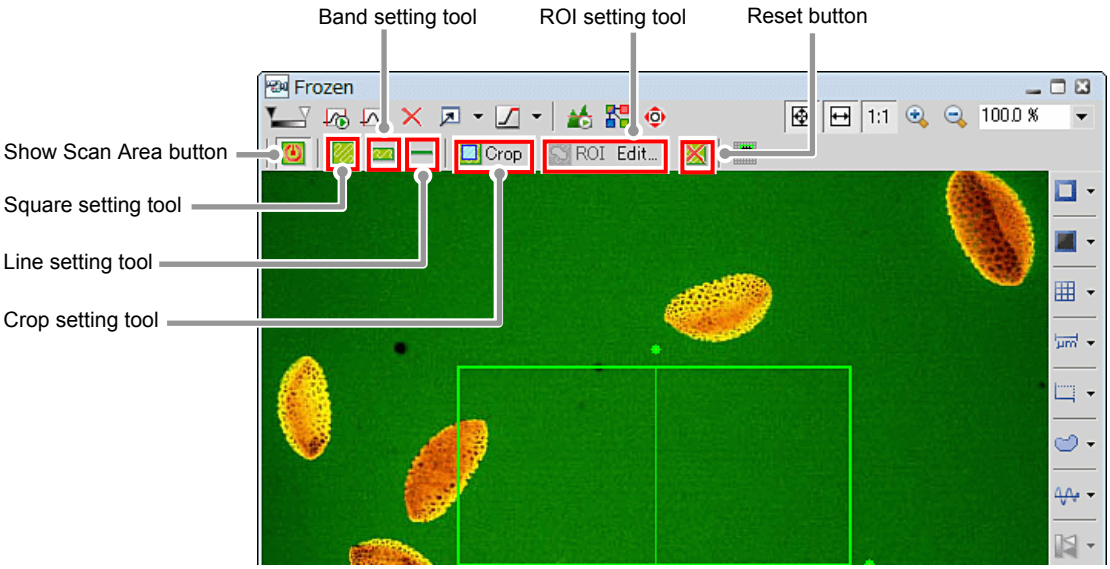
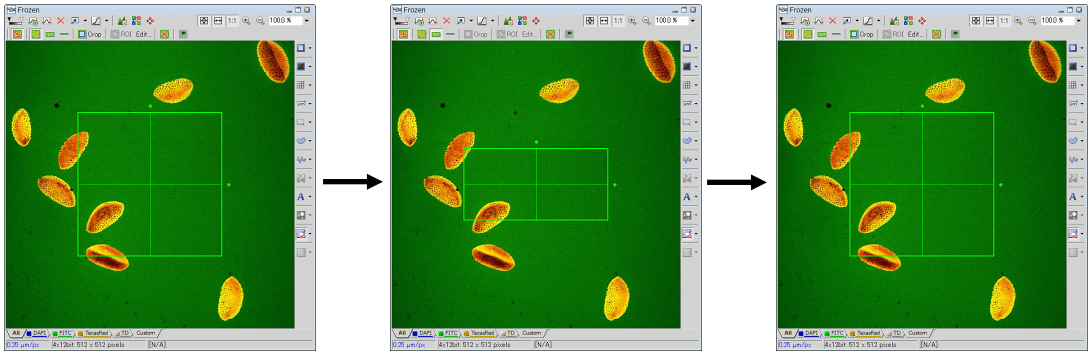


Figure 9.3-8 Scan area setting tools

When one scan area setting tool is switched to another setting tool, the information of the previous scan area is stored even after the display of the previous scan area disappears from the window.

For example, if the Square scan area is set using the square setting tool and then switches over to the band setting tool and set the Band scan area, when the square setting tool is switched back, the Square scan area appears. (As shown below)



[1] Set the Square scan area

[2] Switch over to the band setting tool and set the Band square area.

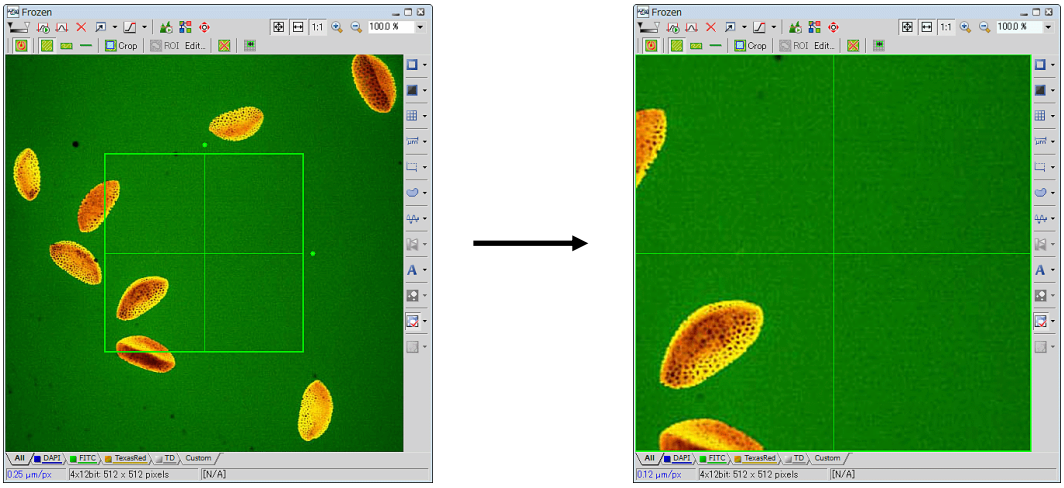
[3] Switch back to the square setting tool, and you see the Square scan area that you set before.

Figure 9.3-9 Storing scan area settings

9.3.4 Scan Area Zoom Function

The scan area zoom function is effective for the Square scan area and the Band scan area.

Set the Square or Band scan area around the portion of the live image to be enlarged. Then, acquire the live image, and the portion set with the scan area appears enlarged.



[1] Set a scan area on desired the portion to enlarge.

[2] Acquire the live image, and the set scan area appears enlarged across the image window.

Figure 9.3-10 Scan area zoom function

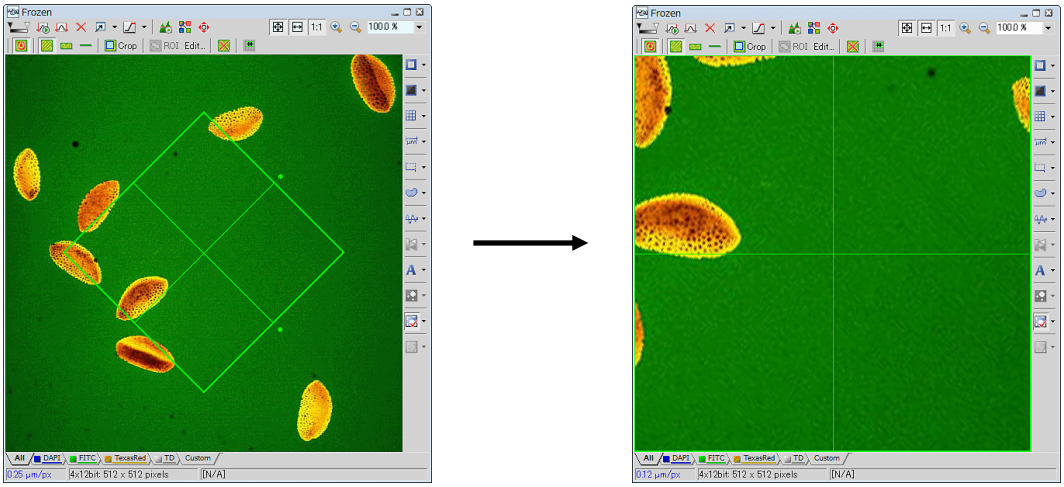
9.3.5 Scan Area Rotate Function

The scan area rotate function is effective for the Square scan area and the Band scan area.

Set the rotated Square or Band scan area around the portion of the live image to be rotated. Acquire the live image once again, and the rotated scan area appears in upright position.

This function rotates the set scan area, and at the same time, applies the scan area zoom function.

Unrotatable in the Bidirectional scan.



[1] Set a rotated scan area around the portion you want to rotate.

[2] Acquire the live image, and the set scan area appears in upright position with respect to the window and enlarged across the image window.

Figure 9.3-11 Scan area zoom and rotate function

10 Photo Activation Setting

This chapter describes the basic operation procedures to execute the photo activation experiment sequence that acquires images of target changes at a high speed while irradiating the photo activation laser beam and acquire the observed images.

* When a three-laser unit without AOM is connected, the photo activation experiment cannot be executed.

* When the Virtual Filter mode is selected, the photo activation experiment cannot be executed.

10.1 Photo Activation Setting Procedure

1. Acquires the specimen for photo activation.

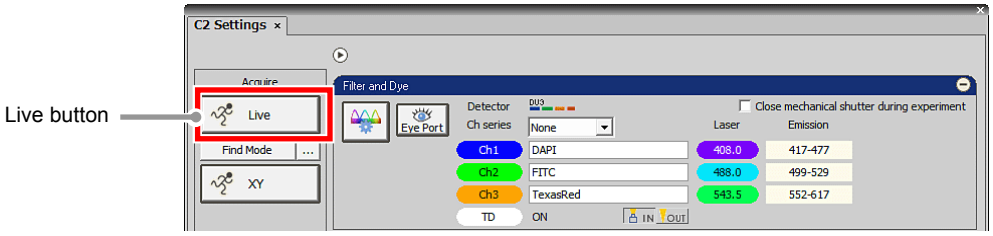


Figure 10.1-1 Acquire a live image

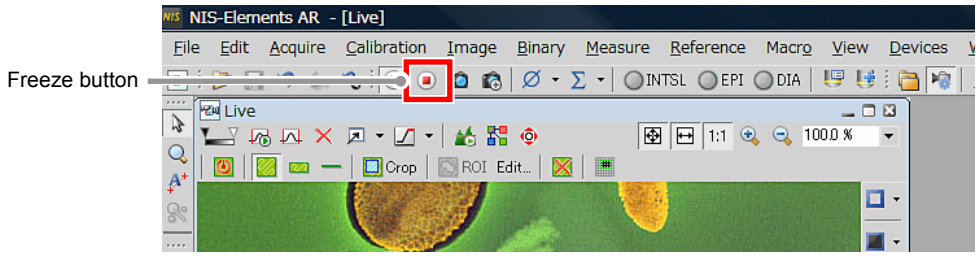


Figure 10.1-2 Acquire a Frozen image

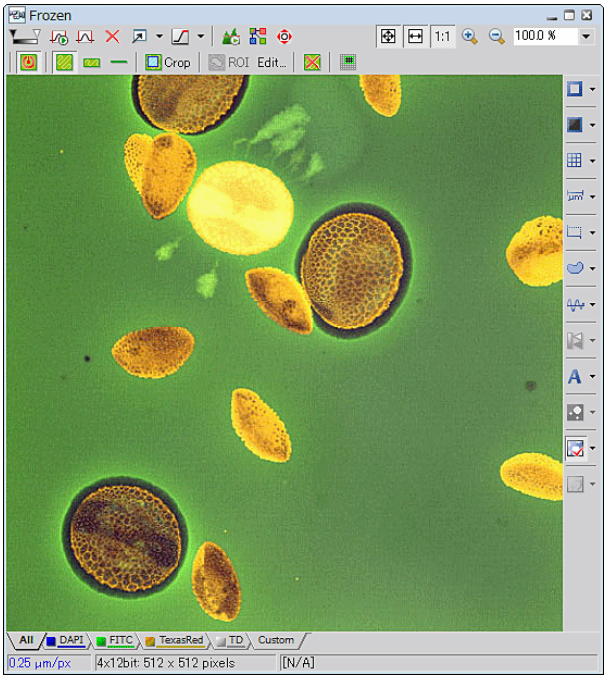


Figure 10.1-3 Frozen image

2. In the Acquisition window, set the image to be acquired through photo activation experiment observation.

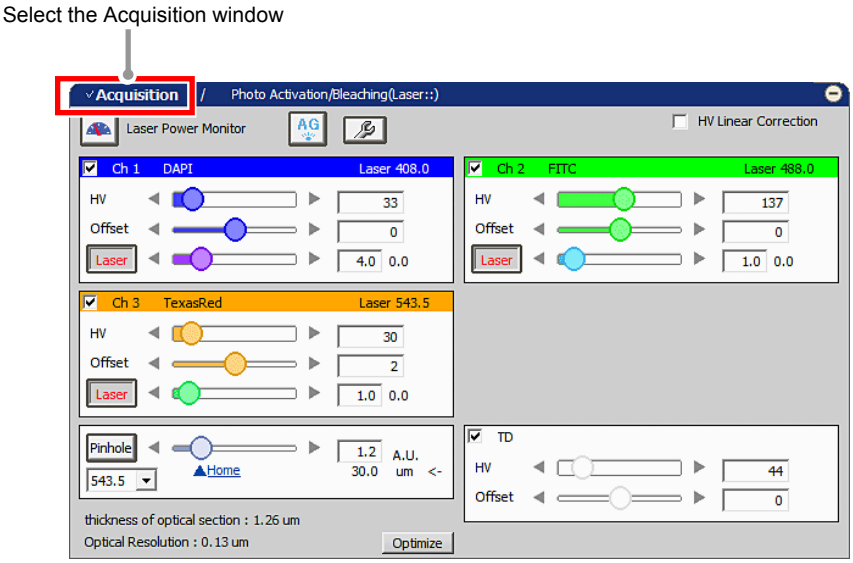


Figure 10.1-4 Acquisition window

3. Specify an area, point, or line to which the photo activation is to be applied with the Simple ROI Editor.
For details of the Simple ROI Editor, See Section 10.2.3, “Simple ROI Editor."

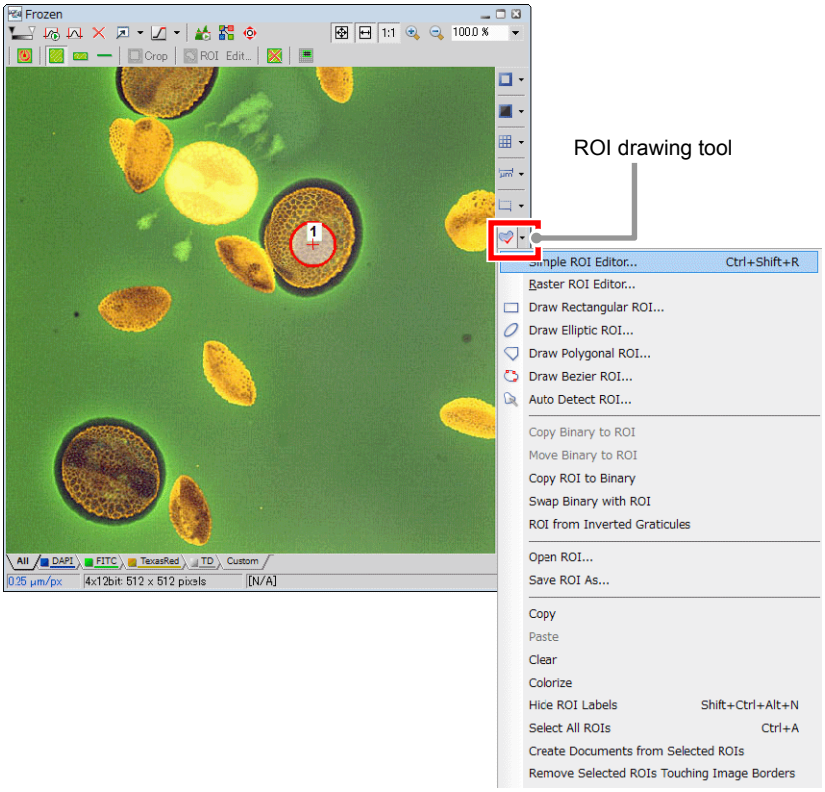


Figure 10.1-5 Setting of a ROI area

Figure 10.1-6 Simple ROI Editor

4. Specify a photo activation area.
   Draw a ROI area on the acquired image.
   Right-click on a designated ROI area to display a menu.
   Select [Use as Stimulation ROI] from the displayed menu and designate a ROI area as the photo activation area.

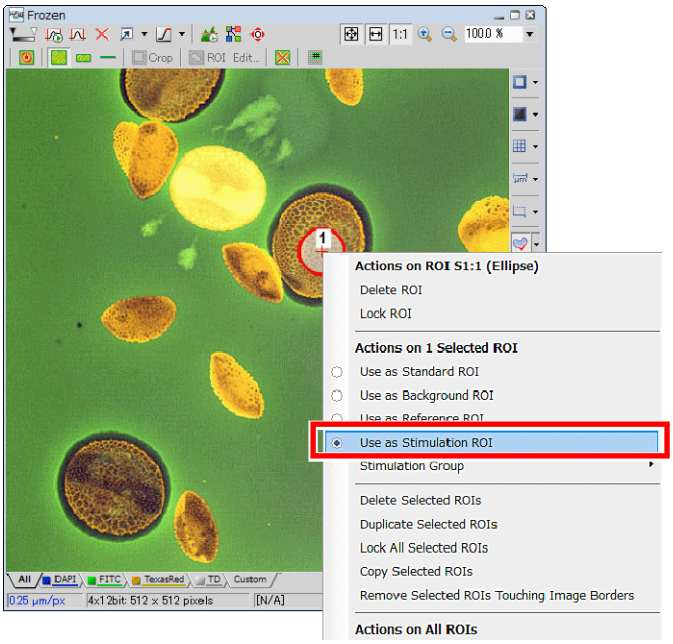


Figure 10.1-7　Setting of photo activation area

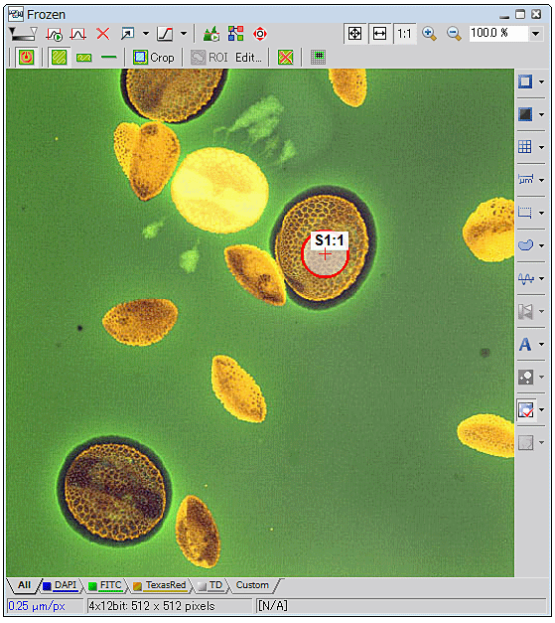


Figure 10.1-8　Photo activation area

* To specify a photo activation point, select [Stim. Point] of the Simple ROI Editor and specify a point on the image.

* To specify a photo activation line, select [Stim. Line] of the Simple ROI Editor and specify a line on the image.

* You can specify only one photo activation point/line and cannot specify multiple photo activation target areas.

5. If necessary, assign photo activation areas to 1 to 3 photo activation frames. (Only when a photo activation ROI area is selected)
   Up to three photo activation frames can be set.
   Right-click on the photo activation area and a menu appears. Select [Stimulation Group] on the menu and specify the photo activation frame 1 to 3.

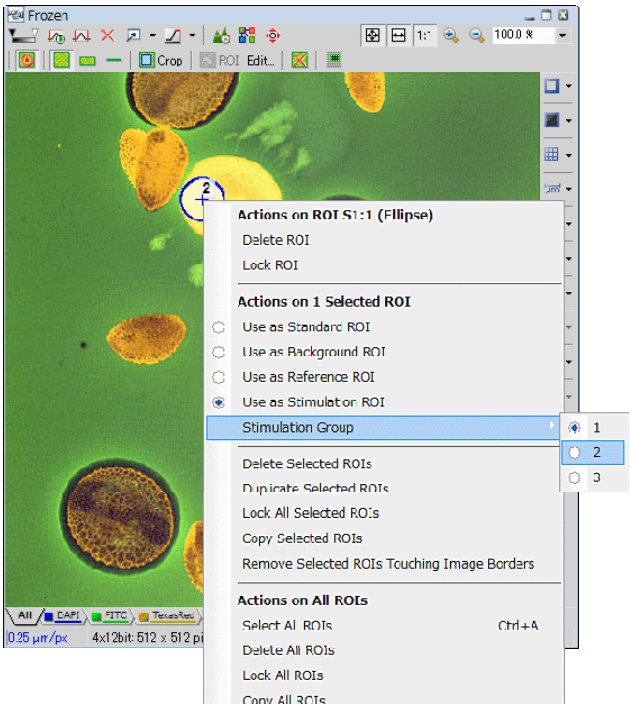


Figure 10.1-9　Selecting a photo activation frame

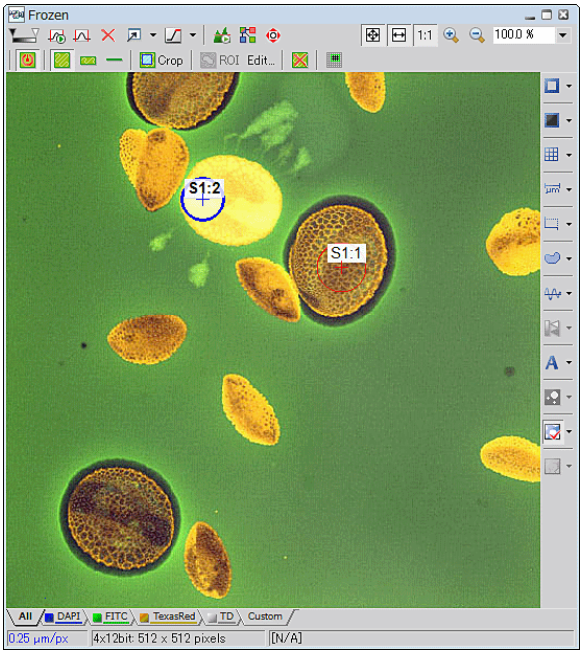


Figure 10.1-10　Photo activation area and photo activation frame

* You can crop the specified ROI area and set a non-photo activation area within the photo activation ROI area. Click the [Draw Holes] button of the Simple ROI Editor and select a drawing tool. Then draw an area to be cropped on the pre-selected ROI area. (Point drawing tools are unavailable for specifying a non-photo activation area.)

6. Switch to the Photo Activation window.
Select the photo activation laser beam and set the laser power and photo activation speed. (For photo activation laser setting, see Section 10.2.2, “Photo Activation Laser Setting.”)

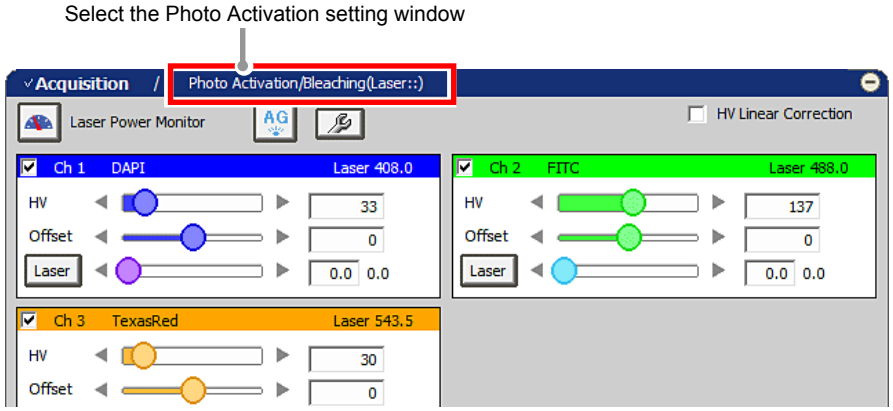


Figure 10.1-11 Switching to Photo Activation window

7. For each photo activation frame, set the photo activation laser beam, laser power, and photo activation speed. If you set multiple photo activation areas on the same frame, all of the specified ROIs are photo-activated at the same time.
(If two ROIs are the photo activation targets, a photo activation frame photo-activates the two ROIs. In this case, photo activation may take time even though each photo activation area (ROI) is small.)

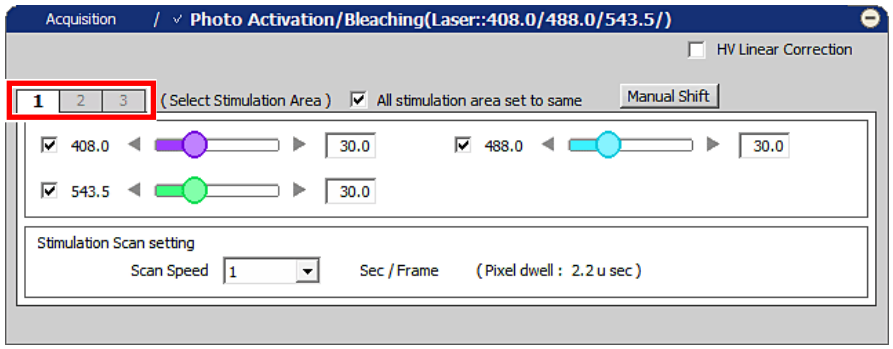


Figure 10.1-12 Photo activation laser setting (Specify the photo activation ROI area)

* When a photo activation point/line is specified, photo activation target area is limited to only one. Therefore photo activation frame cannot be set.
When a photo activation point is specified, the photo activation speed cannot be set.

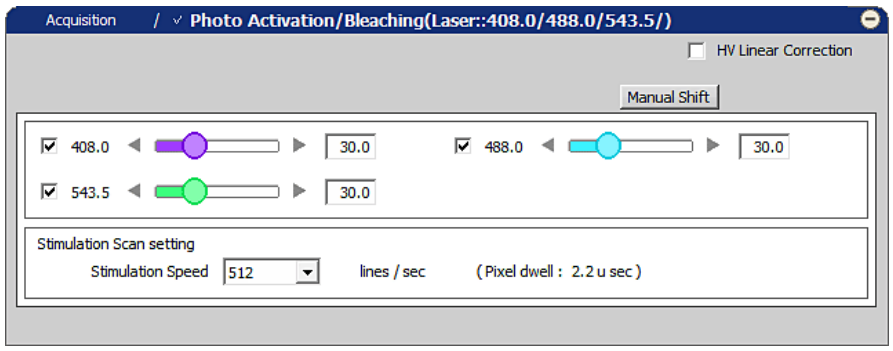


Figure 10.1-13 Photo activation laser setting (Specify the photo activation line)

8. Click the [Photo Activation] button to open the [Photo Activation] dialog box.

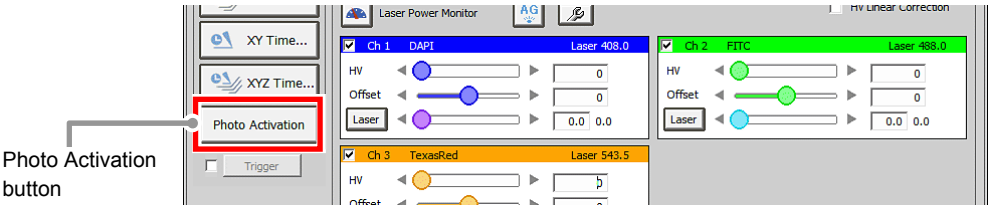


Figure 10.1-14 Photo Activation button

* Other display methods

As shown below, select [Applications] -> [Define/Run Sequential Stimulation…] from the menu bar to open the [Photo Activation] setting dialog box.

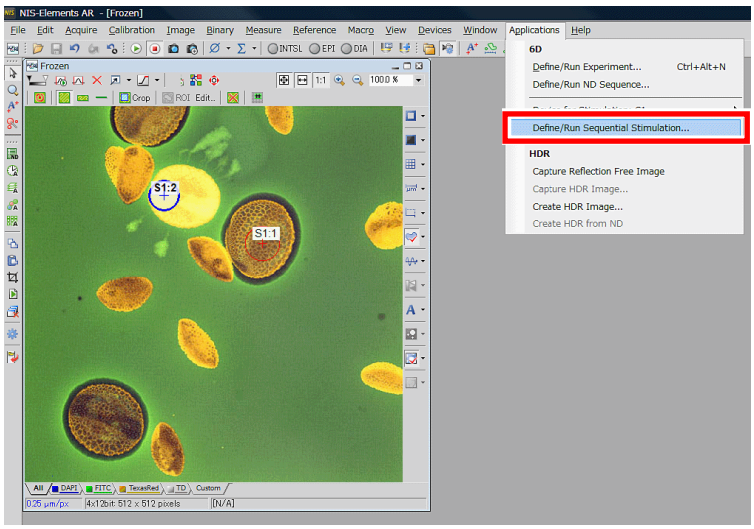


Figure 10.1-15 To display the Photo Activation setting dialog box

9. In the [Photo Activation] dialog box, set the photo activation experiment sequence.
(For the photo activation setting dialog box, see Section 10.2.1, “Photo Activation Experiment Sequence Setting.”)

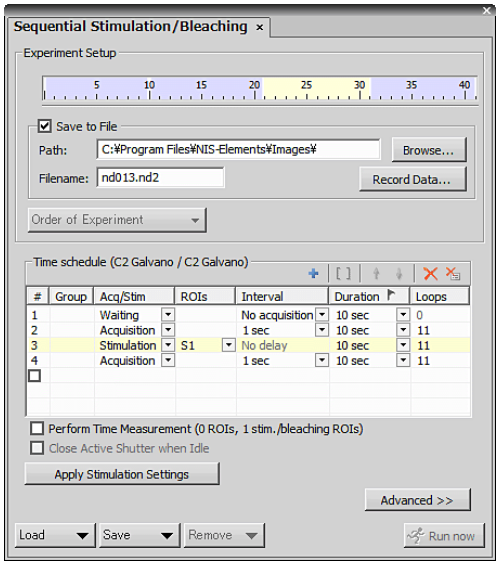


Figure 10.1-16 Experiment Sequence Setting

10. Click the [Apply Stimulation Settings] button to send the photo activation area information to the experiment sequence.

11. Click the [Run Now] button to execute the photo activation experiment sequence.

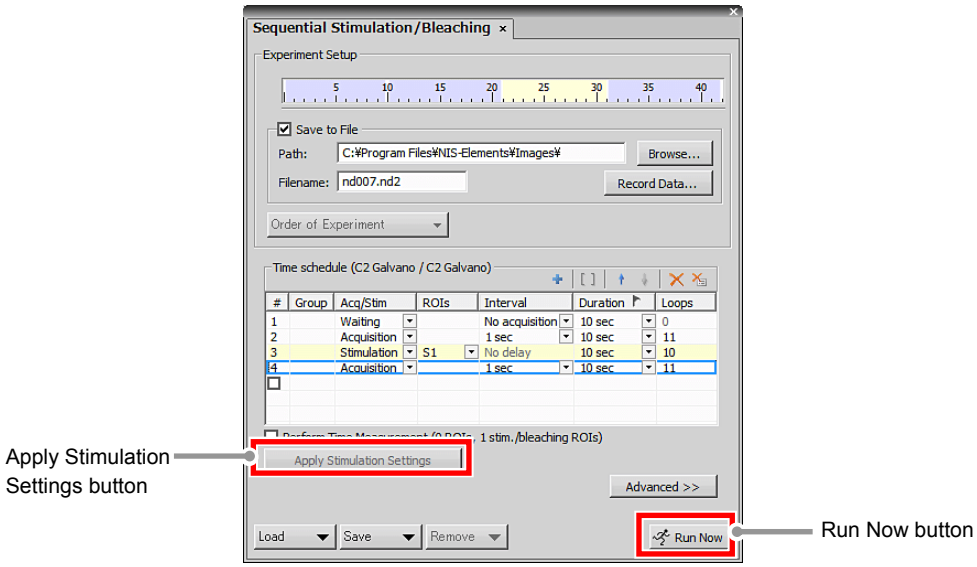


Figure 10.1-17 Experiment Sequence Setting

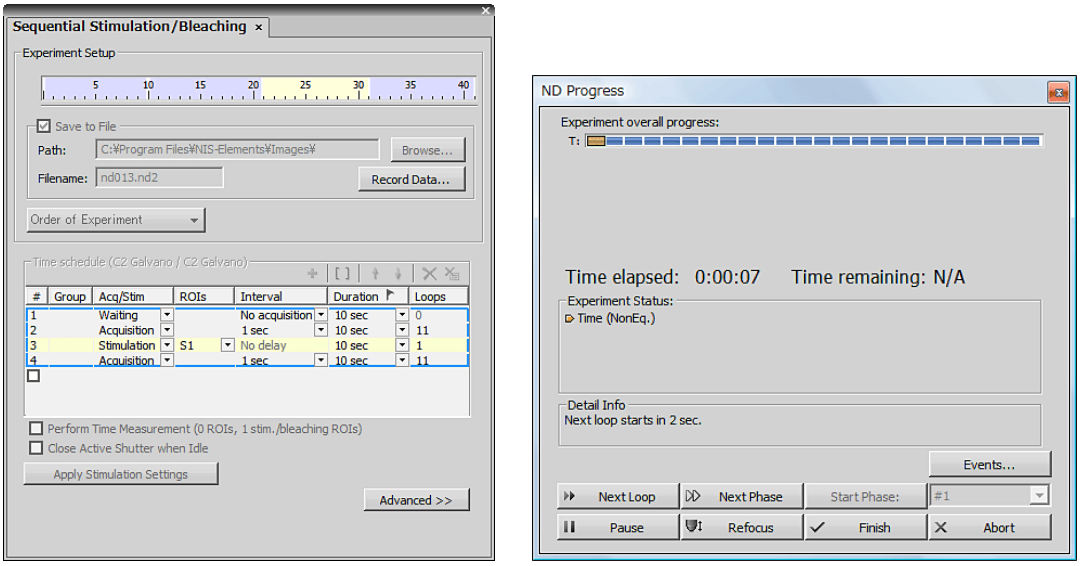


Figure 10.1-18 Experiment sequence running

* If the photo activation position is not correct, manually correct the photo activation position. For manual correction of the photo activation position, see “Correcting the Photo Activation Position Shift” on the next page.

Correcting the Photo Activation Position Shift

If the photo activation position is not correct, the photo activation position can be corrected manually.

1. Click the [Manual Shift] button on Photo Activation window to open [Manual Shift Alignment] dialog box.

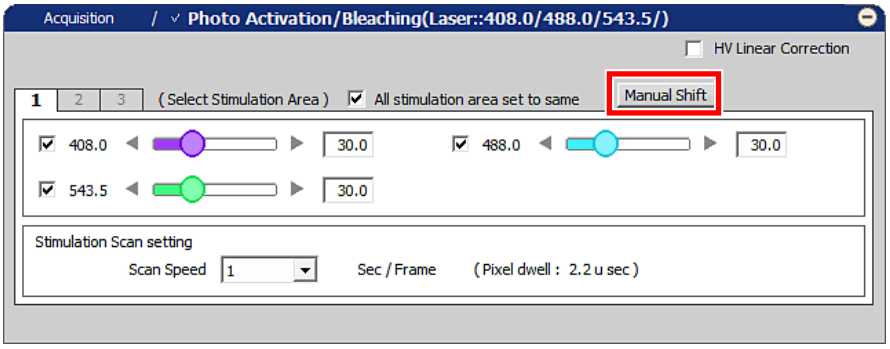


Figure 10.1-19 Check of the Photo activation position shift

2. Correct the shift by specifying the shift amount (pixel) for each photo activation group while checking the image acquired by the first photo activation sequence.

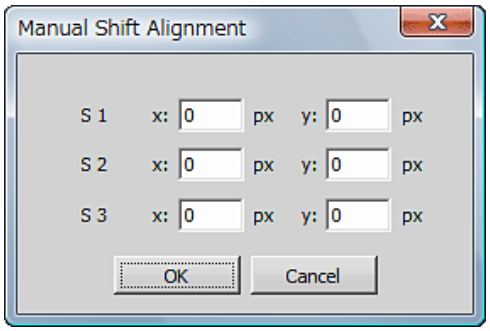


Figure 10.1-20 Manual Shift Alignment dialog box

3. After correcting the photo activation position shift, reexecute the photo activation experiment sequence.
   Click the [Photo Activation] button to open the [Photo Activation] dialog box.

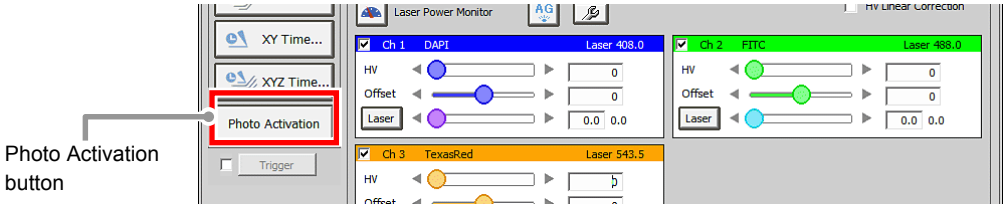


Figure 10.1-21 Photo Activation button

4. If the previous photo activation experiment sequence setting is to be maintained, click the [Apply Stimulation Settings] button to send the photo activation area information to the experiment sequence.

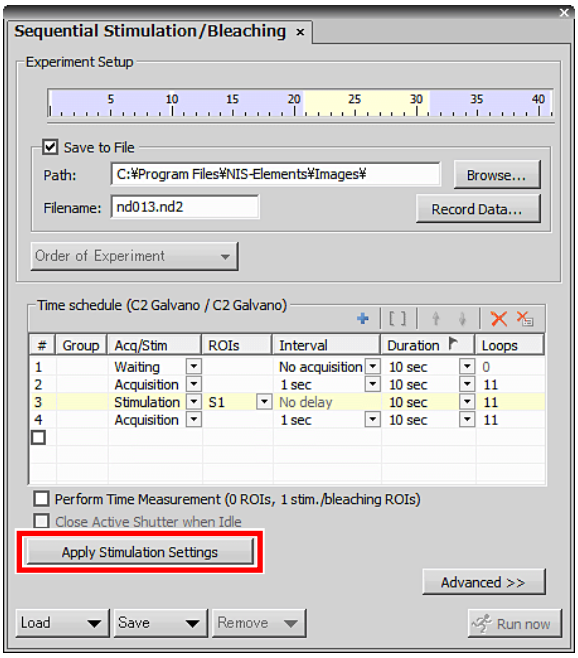


Figure 10.1-22 Experiment Sequence Setting

5. Click the [Run Now] button to execute the photo activation experiment sequence.

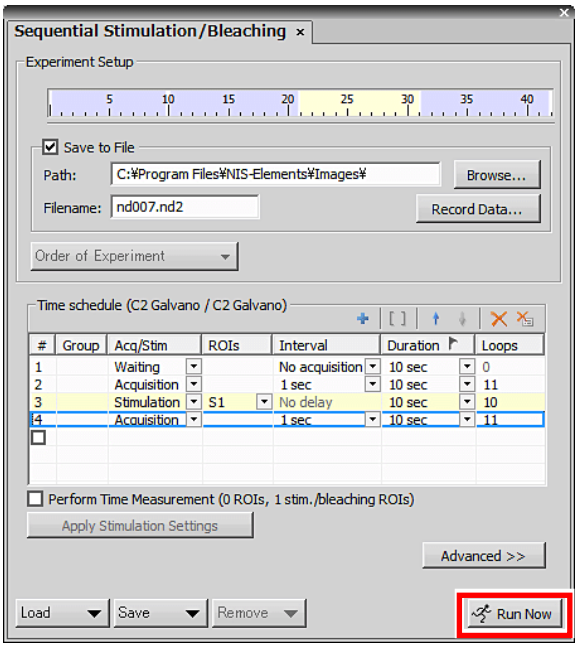


Figure 10.1-23 To execute the experiment sequence

10.2 Setting of Each Dialog Box

This section describes setting items in each dialog box on photo activation are explained.

10.2.1 Photo Activation Experiment Sequence Setting

Depending on the reaction speed, set the experiment sequence including observation before photo activation is given, during the photo activation period and after photo activation is given.
Photo activation and image acquisition are executed in time series for each phase set.

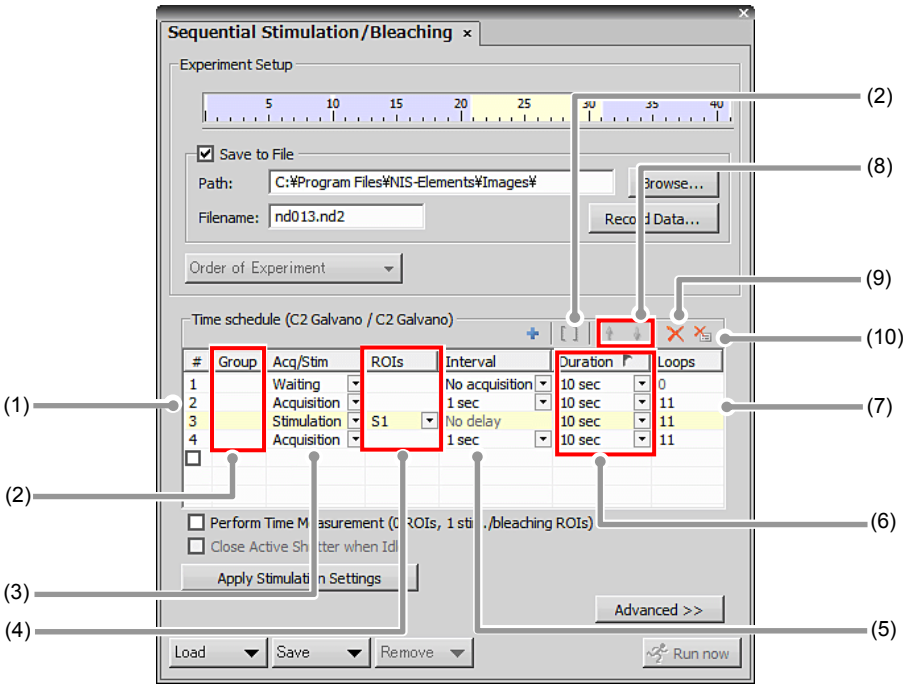


Figure 10.2-1 Photo activation sequence setting

Table 10.2-1 Functions of Sequential Stimulation window (sheet 1/2)

Name		Function	
(1)	Phase	Clicks □ to set phases of the experiment sequence.	
(2)	Group	[ ]	Group the phases. Selects the phases to be grouped while pressing the [Shift] key and click the Group button [ ] for grouping. Sets the number of repetitions in Group of [Time schedule].
(3)	Acq/Stim	Selects the items to be set from “Acquisition”, “Stimulation”, “Bleaching” and “Waiting.” The FRAP experiment can also be executed by making the phase settings as follows. #1 = Acquisition/#2 = Stimulation/#3 = Acquisition	
(4)	ROIs	Specifies the photo activation frame to run the set phase. - Frames 1 and 2: Select “S1” and “S2”. - Frames 1 and 3: Select “S1” and “S3”. - Frame 1 only: Select “S1”. (It is set as “S1” when a photo activation point/line is specified for a photo activation area.)	

Table 10.2-1 Functions of Sequential Stimulation window (sheet 2/2)

Name		Function	
(5)	Interval	Specifies the phase interval. - “No delay” No interval - “No acquisition” No interval and image acquisition If “Stimulation” or “Bleaching” is set in the [Acq/Stim] column, [Interval] is fixed to “No delay.”	
(6)	Duration	Specifies the continuation time of the selected phase. If the continuation time is designated, the number of execution times is automatically selected. (If [Interval] is set to “No delay” and [Loops] is changed, [Duration] is also changed in an interlocked manner.) * When photo activation point is designated, the setting of [Duration] can be set to less than 5 seconds.	
(7)	Loops	Specifies the number of execution times for the selected phase. (If [Interval] is set to “No delay” and [Duration] is changed, [Loops] is also changed in an interlocked manner.) * When the photo activation point or the photo activation line is designated, [Loops] cannot be set.	
(8)	Move the phase one line		Brings the selected phase to one line above.
			Brings the selected phase to one line below.
(9)	Remove the phase		Removes the selected phase.
(10)	Remove all		Removes all phases.

10.2.2 Photo Activation Laser Setting

The photo activation laser beam to be irradiated in the experiment sequence is set.

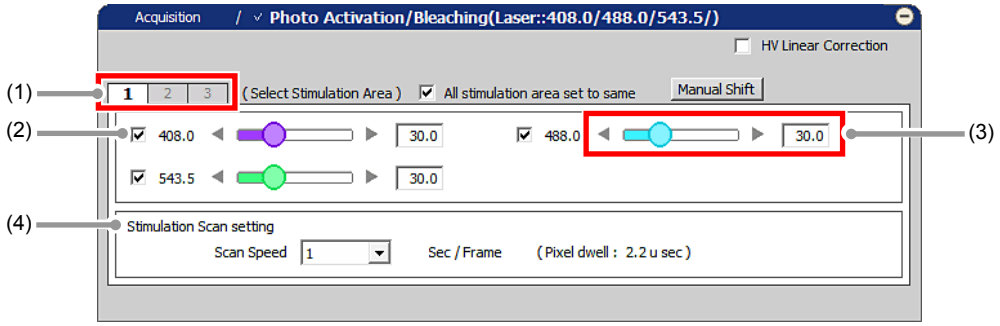


Figure 10.2-2 Photo activation laser setting (Specify the photo activation ROI area)

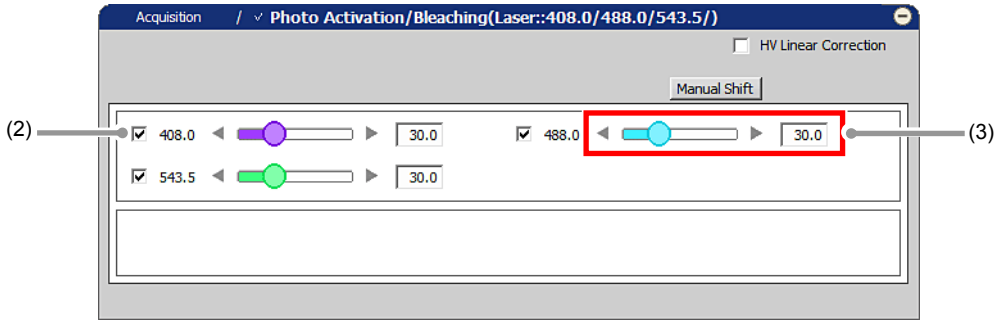


Figure 10.2-3 Photo activation laser setting (Specify the photo activation point)

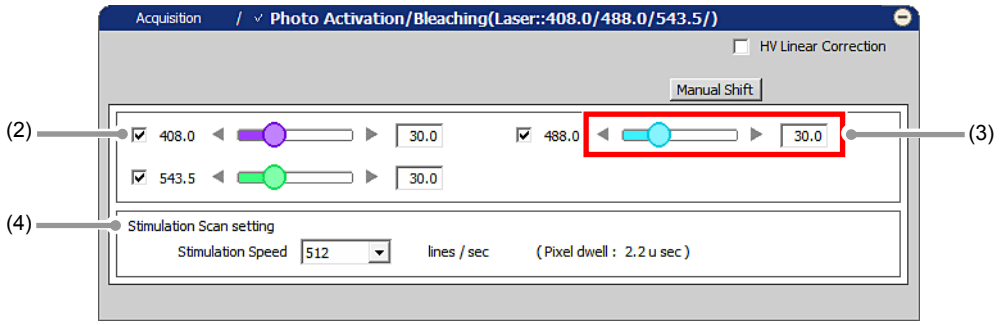


Figure 10.2-4 Photo activation laser setting (Specify the photo activation line)

Table 10.2-2 Functions of Photo Activation window

Name		Function
(1)	Photo activation frame tabs	Selects the photo activation frame to be set. (Only when a photo activation ROI area is selected)
(2)	Photo activation laser selection check box	Selects the photo activation laser beam to be irradiated on the specimen.
(3)	Photo activation laser power output adjustment	Adjusts the output power of the photo activation laser beam to be irradiated.
(4)	Photo activation scan speed setting	Sets the photo activation scan speed. The speed is expressed in Sec/Frame when a photo activation ROI area is selected, or in lines/sec when a photo activation line is selected.

10.2.3 Simple ROI Editor

This section describes the functions of the Simple ROI Editor drawing tool.

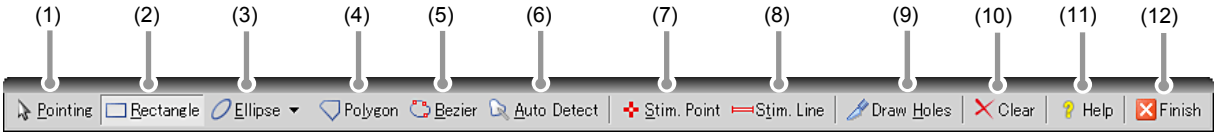


Figure 10.2-5 Simple ROI Editor

Table 10.2-3 Functions of the Simple ROI Editor drawing tool (sheet 1/2)

Name		Functions and their operations
(1)	Pointing	Used to move a drawn ROI on the window.
(2)	Rectangle	Used to designate the ROI area enclosed by a rectangle.
(3)	Ellipse	Used to designate the ROI area enclosed by a circle. Clicks the center of the desired circle and drag to designate the size.. When the drawn circle is picked and dragged with the mouse, the circle moves to another position. When □ on the top, bottom, right, or left of the circle is picked and dragged, the circle deforms. Right-clicking on the drawn circle designates the circle as the ROI area.
(4)	Polygon	Used to designate the ROI area enclosed by straight lines. Designates the start point by clicking on the image and moving the pointer to the straight line ending position (end point) and clicking draws a straight line. Draw straight lines subsequently to draw a polygon. To close the selected area by connecting straight lines to each other, place the mouse pointer on the start point and double-click the mouse. Double-clicking the pointer at a position different from the start point can also close the selected area.
(5)	Bezier	Used for freehand drawing or for drawing a straight line or smooth curve by placing anchor points. For freehand drawing, click the mouse on the image then drag the mouse. To draw a curve using anchor points, drag the mouse in the curving direction. To close the selected area, right-click the mouse. (Double-clicking the mouse left button also closes the selected area.)
(6)	Auto Detect	Used to automatically detect and specify the similar color portion adjacent to the clicked position. By clicking the mouse on the screen, the similar color portion adjacent to the clicked position is selected. To fix the selected area, right-click the mouse.
(7)	Stim. Point	Used to designate the photo activation point. You can specify only one photo activation point and cannot specify multiple photo activation target areas.
(8)	Stim. Line	Used to designate the photo activation straight line. You can specify only one photo activation line and cannot specify multiple photo activation target areas.

Table 10.2-3 Functions of the Simple ROI Editor drawing tool (sheet 2/2)

Name		Functions and their operations
(9)	Draw Holes	Used to draw a non-photo activation area in a ROI area drawn by using the various tools of Simple ROI Editor.
(10)	Clear	Clears the ROI area.
(11)	Help	Displays the help for Simple ROI Editor.
(12)	Finish	Finishes drawing and editing of the ROI area and closes the Simple ROI Editor.

11 Using Manual Microscope

This chapter describes how to make settings for operations from NIS-Elements with the Confocal Microscope C2 connected to Nikon manual microscope.

When using FN1 microscope, connect the C1-Y-TT Trinocular Tube (hereinafter referred to as trinocular tube). Combination of the trinocular tube and the “vertical movement device (Nikon “RFA” or Prior external Z Drive “Prior Z RFA”)” enables NIS-Elements to control the manually-operated ECLIPSE FN1.

11.1 Setting Manual Microscope Connection

Make settings using the following procedure to synchronize NIS-Elements with the manual microscope.

1 Call the [Manage devices] dialog box

Select [Devices] on the menu bar and then select [Manage devices...].
[Manage devices] dialog box appears.

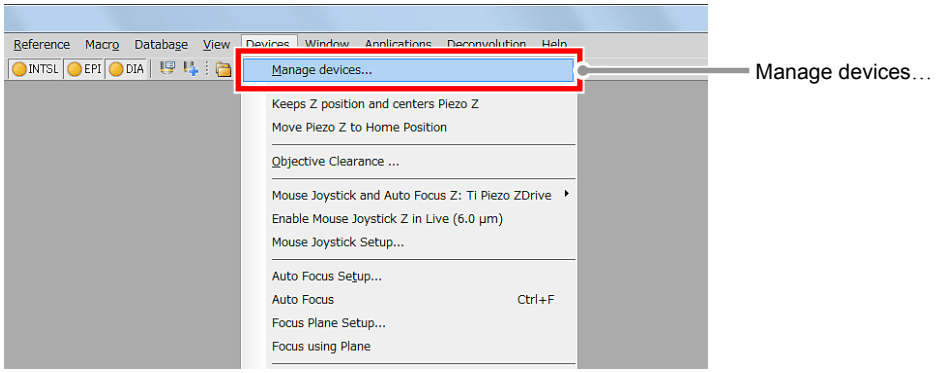


Figure 11.1-1 Devices menu

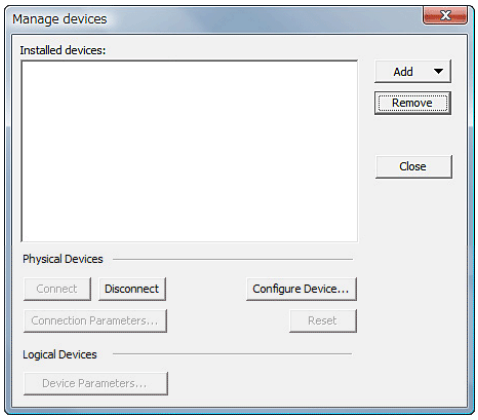


Figure 11.1-2 Manage devices dialog box

2 Add “Manual Microscope”

1. Click the [Add] button in the [Manage devices] dialog box to display the menu for devices to be added.

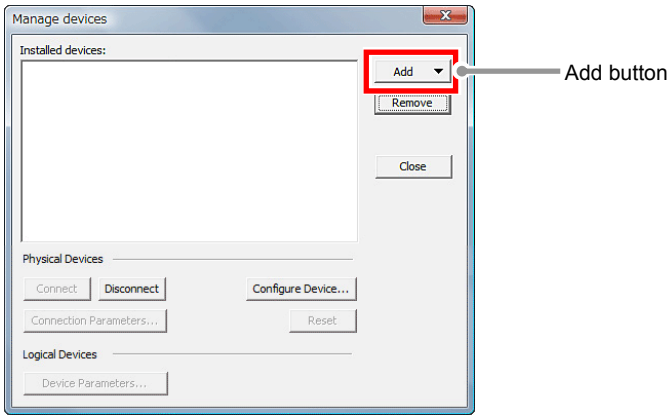


Figure 11.1-3 Manage devices dialog box

2. Select the “Manual Microscope” form pull-down menu.
“Manual Microscope” is added in the [Installed devices:] field.

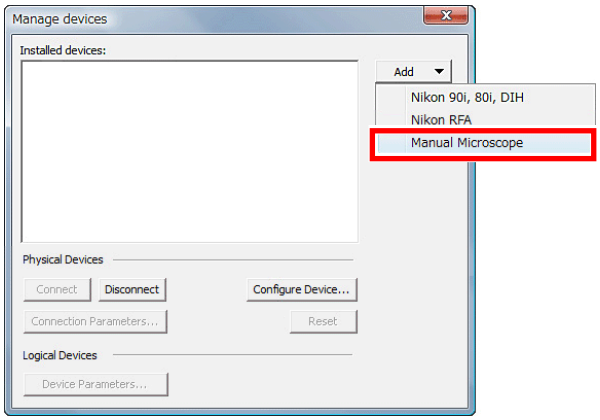


Figure 11.1-4 Manage devices dialog box

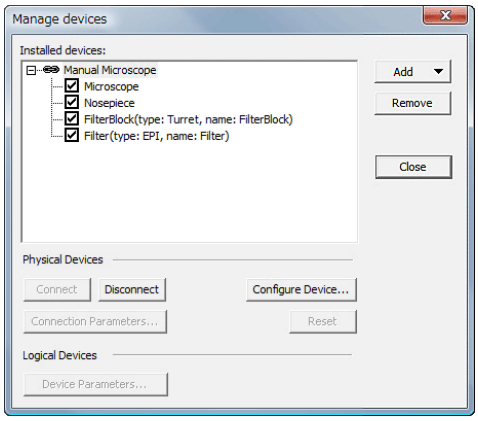


Figure 11.1-5 Manage devices dialog box

3 Add the vertical movement device (Nikon “RFA” or Prior external Z Drive “Prior Z RFA”)

1. Click the [Add] button to display the menu for devices to be added.
   Select the “Nikon RFA” or Prior external Z Drive “Prior Z RFA” form pull-down menu. (*)
   [Connection Parameters] dialog box appears.

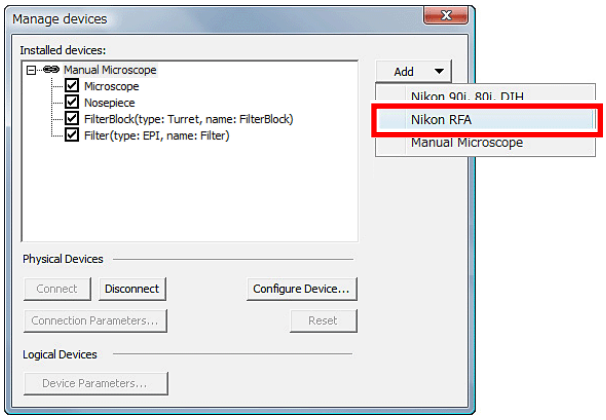


Figure 11.1-6 Manage devices dialog box

* Compatible Microscopes:
Nikon RFA : ECLIPSE AZ100, ECLIPSE 80i, ECLIPSE FN1
Prior Z RFA : ECLIPSE Ti-U, ECLIPSE FN1

2. Set the Serial port in the [Connection Parameters] dialog box.
   Select the serial port to which “Nikon RFA” or Prior external Z Drive “Prior Z RFA” is connected from the pull-down menu of [Serial port:].
   Click the [OK] button to finish the Serial port setting.

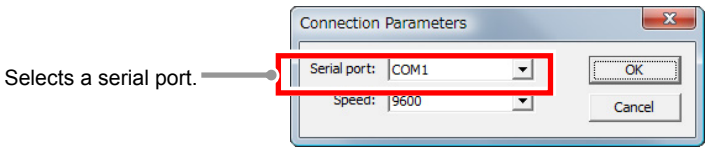


Figure 11.1-7 Connection Parameters dialog box

3. If you use “Nikon RFA” as the vertical movement device, set the model number.
   Select “Nikon RFA” in the [Installed devices:] field and click the [Configure Device…] button.
   [Model setting] dialog box appears.

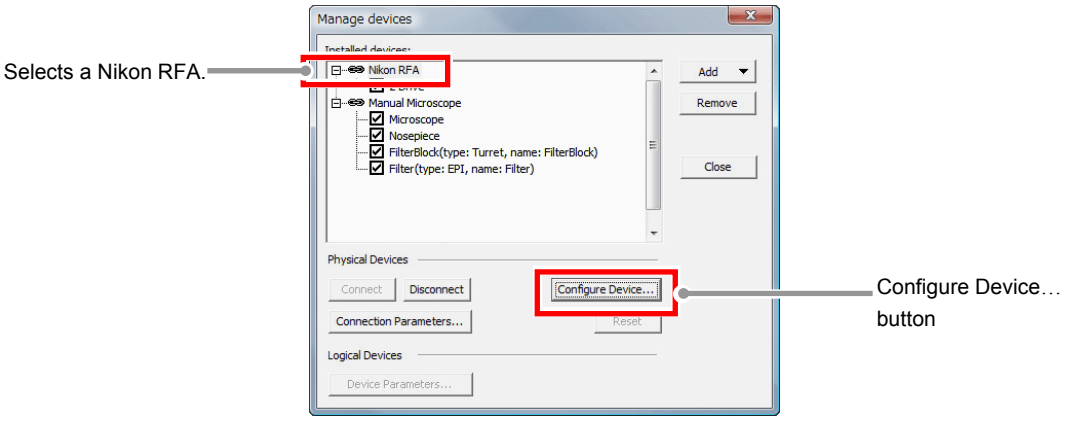


Figure 11.1-8 Manage devices dialog box

4. Set the model number in the [Model setting] dialog box.
   Select “99888” (*) from the pull-down menu of [Model:].
   Click the [OK] button to finish the Model number setting.

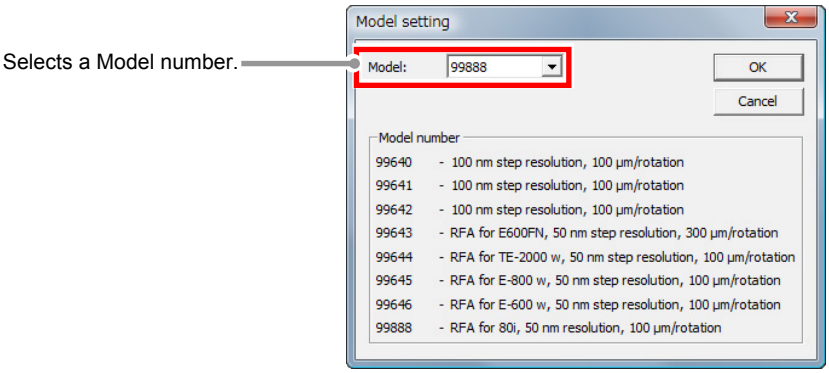


Figure 11.1-9 Model setting dialog box

* The model number differs depending on the microscope.
ECLIPSE AZ100, ECLIPSE 80i, ECLIPSE FN1 : 99888

5. Click the [Close] button to close the [Manage devices] dialog box.

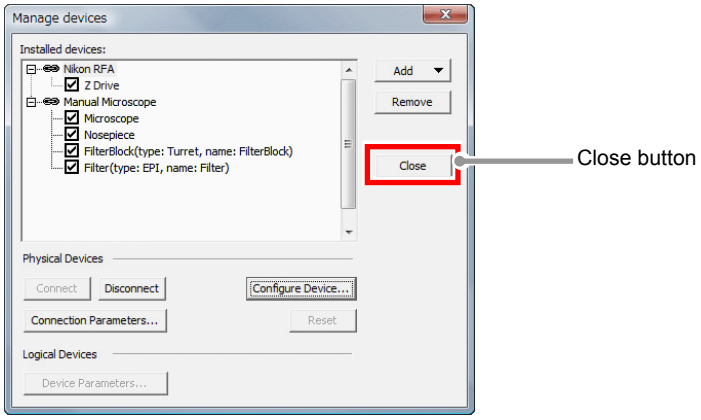


Figure 11.1-10 Manage devices dialog box

4 Setting the [Manual Microscope Pad]

1. Display the [Manual Microscope Pad].
   As shown below, right-click on the gray area (without any dialog box and setting window displayed) to display a menu. Then select [Acquisition Controls] -> [Manual Microscope Pad] in the menu.

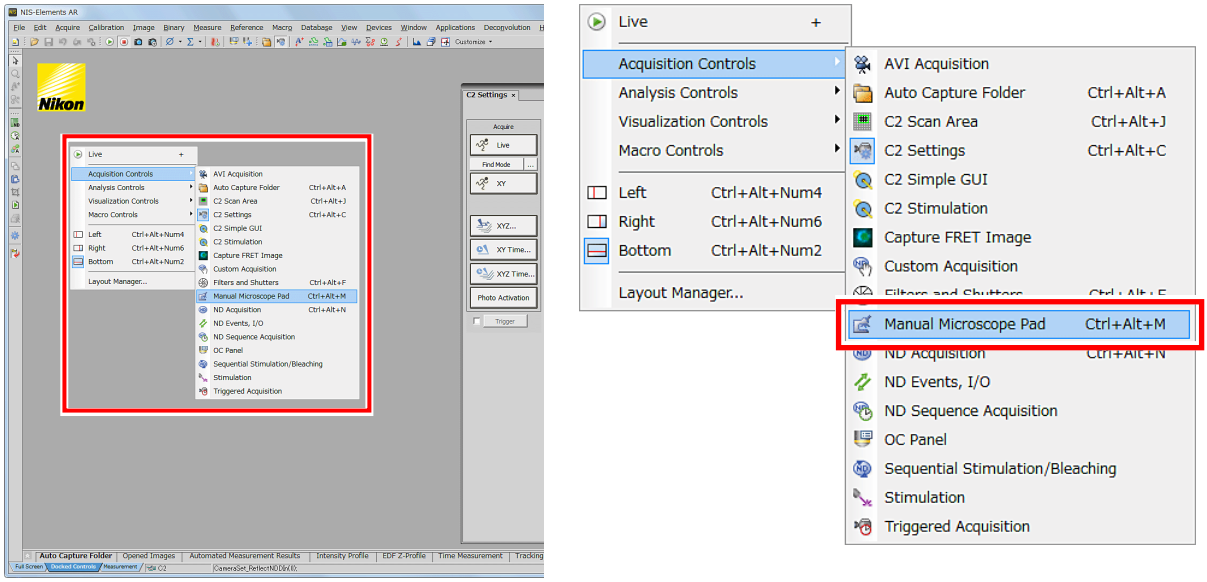


Figure 11.1-11 To display the Manual Microscope Pad

* Other display methods
And also, select [View] on the menu bar and then select [Acquisition Controls] -> [Manual Microscope Pad] to open the control pad.

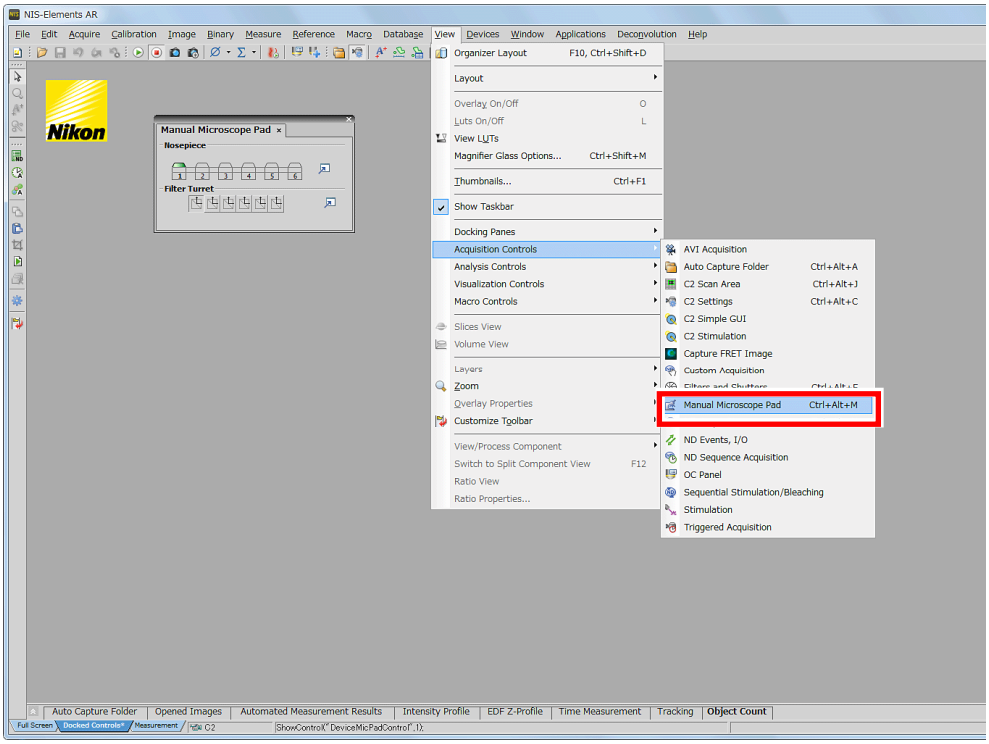


Figure 11.1-12 To display the Manual Microscope Pad

2. Set the Nosepiece information in the [Manual Microscope Pad].
   Click the button in the [Nosepiece] field.
   [Nosepiece & Objectives] dialog box appears.

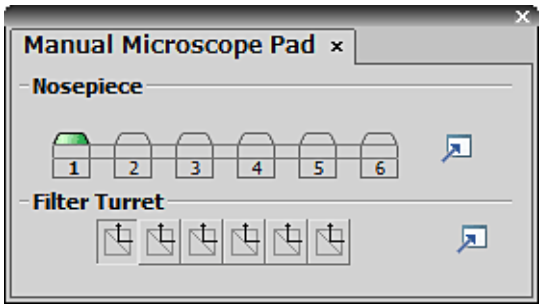


Figure 11.1-13 Manual Microscope Pad

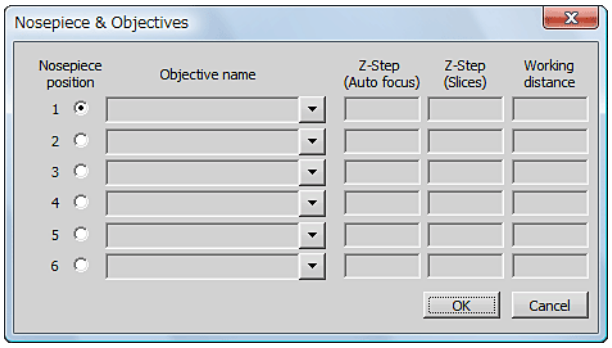


Figure 11.1-14 Nosepiece & Objectives dialog box

* When using manual microscope, information on the installed objectives cannot be read automatically by NIS-Elements. When using a manual microscope, be sure to select the objective to be used in the [Nosepiece & Objectives] dialog box.

3. Specify the Objective in the [Nosepiece & Objectives] dialog box.
   Select the Objective name to be used from the pull-down menu of [Objective name].

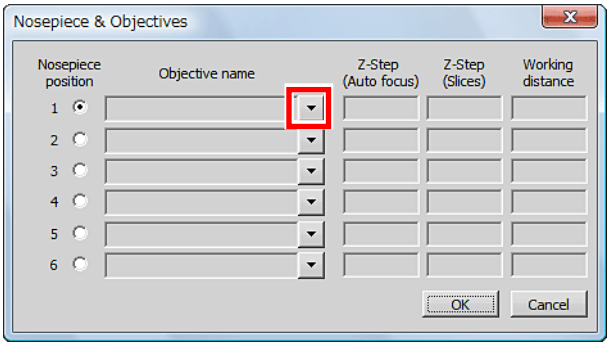


Figure 11.1-15 Nosepiece & Objectives dialog box

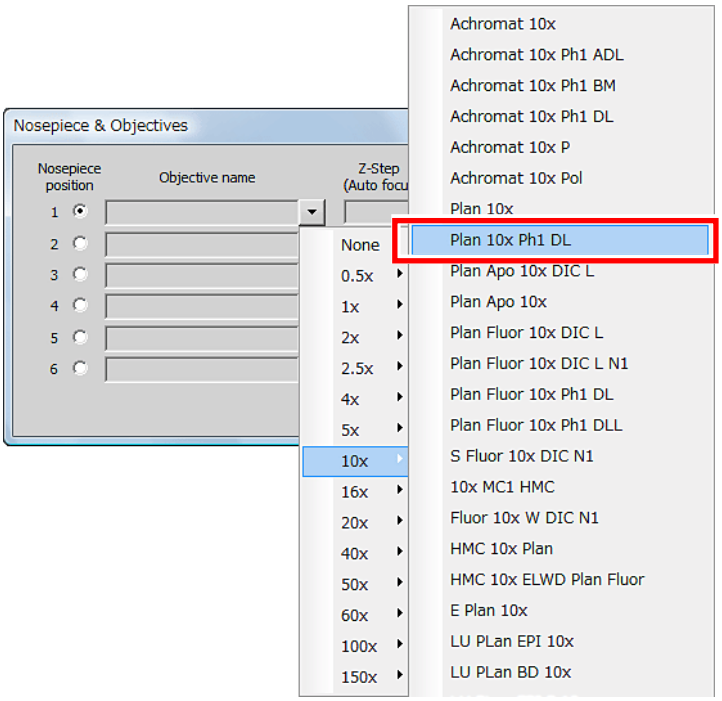


Figure 11.1-16 Selecting Objectives

4. Click the [OK] button to close the [Nosepiece & Objectives] dialog box.

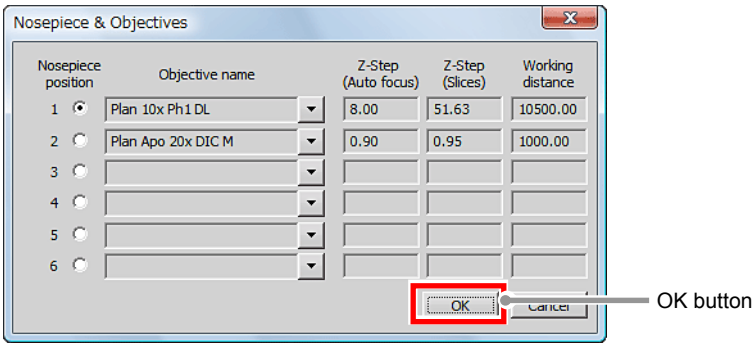


Figure 11.1-17 Nosepiece & Objectives dialog box

The connection setting procedure has been completed.

11.2 Manual Microscope Pad

The Microscope Control Pad for manual microscope consists of the following portions:

Nosepiece

Select the objective located in the light path.

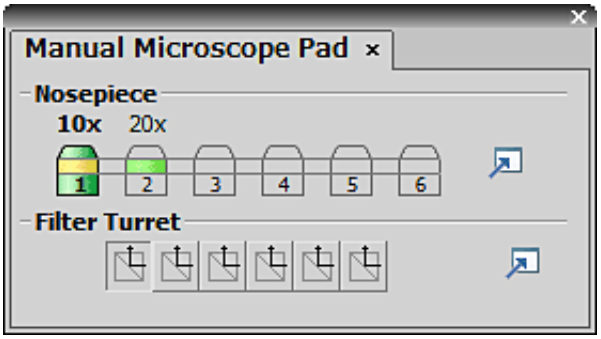


Figure 11.2-1 Manual Microscope Pad Settings

11.3 Operating the Z Drive

This section describes how to operate the Z drive of the manual microscope by NIS-Elements.

1. Display the [XYZ Navigation] dialog box.
   As shown below, right-click on the gray area (without any dialog box and setting window displayed) to display a menu. Then select [Acquisition Controls] -> [XYZ Navigation] in the menu.

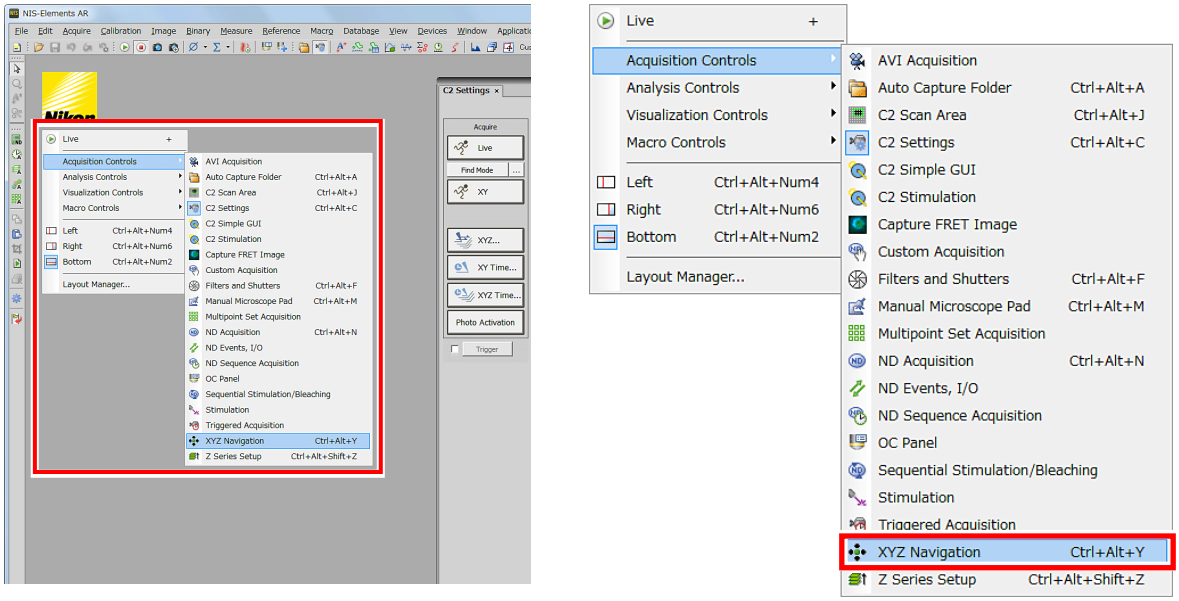


Figure 11.3-1 To display the XYZ Navigation dialog box

* Other display methods
And also, select [View] on the menu bar and then select [Acquisition Controls] -> [XYZ Navigation] to open the dialog box.

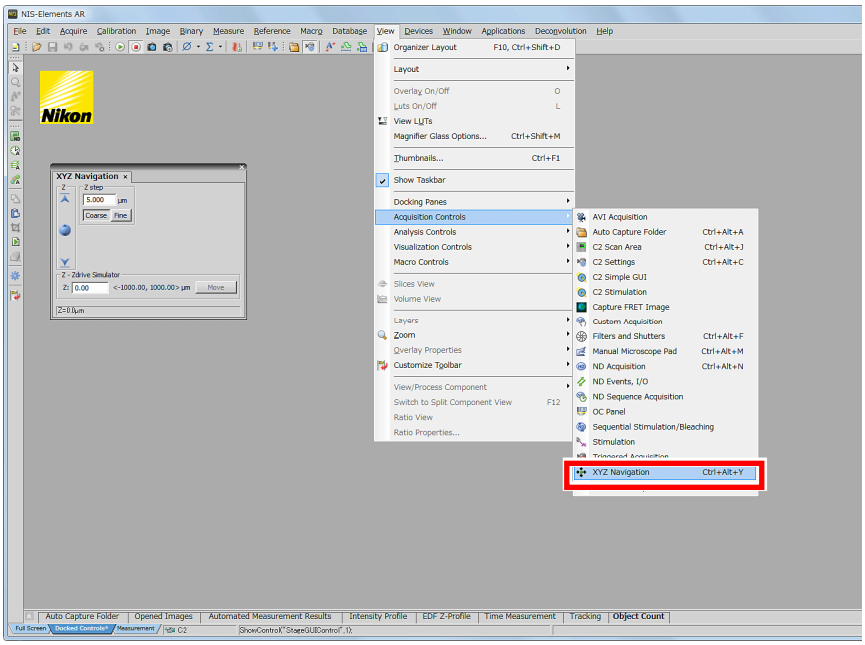


Figure 11.3-2 To display the XYZ Navigation dialog box

2. Control the Z drive position.
   Click the upper and lower blue arrow buttons to move the Z drive.
   Each clicking of the button can move the Z drive up and down by one step (predetermined length).

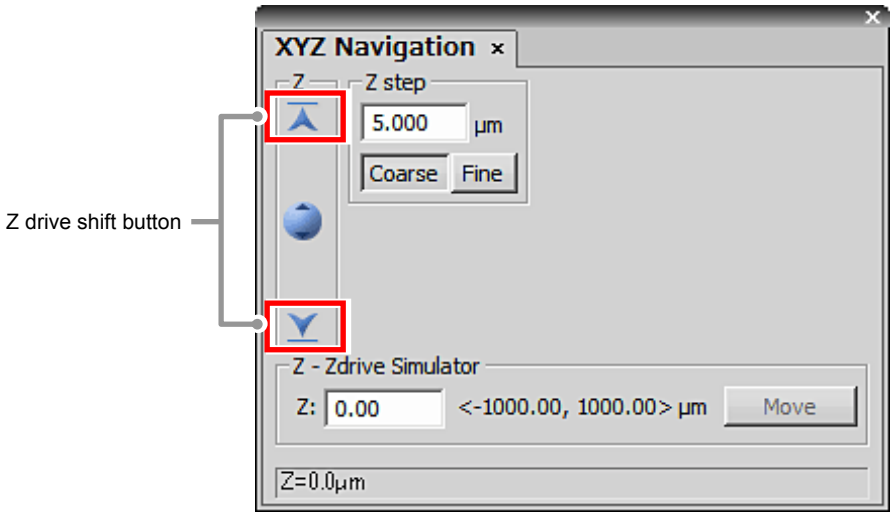


Figure 11.3-3 XYZ Navigation dialog box

* The step value can be set in the [Z step] field.
  Two accuracy settings of movement can be set - coarse and fine.

* The Z drive can also be moved directly to any position by entering its coordinate in the [Z-Nikon RFA ZDrive] edit box.

12 Using C2+TIRF System

By combining the observations of single molecules with laser TIRF and the sectioning capabilities of the C2, C2+TIRF System allows for multi-perspective cellular analysis.

Use the “digital imaging head for C2 for Nikon microscopes” attached to the left-side port of Nikon microscope [ECLIPSE Ti] and CCD camera attached to the back port, by switching between them.

With the C2, Z stack images (more than one) are acquired, and with the CCD camera for TIRF position (one image) is acquired.

12.1 Starting the C2+TIRF System

To use C2+TIRF System, you may select it at activation of NIS-Elements C or after the activation.

Steps to enable C2+TIRF System at activation of NIS-Elements C

1. On the [Driver selection] dialog box displayed at activation of NIS-Elements C, turn “ON” the [Enable Multi Camera] check box.
   On the [Driver selection] dialog box, you may select the second camera.

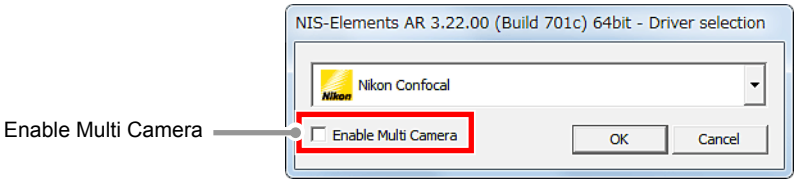


Figure 12.1-1 Driver selection dialog box

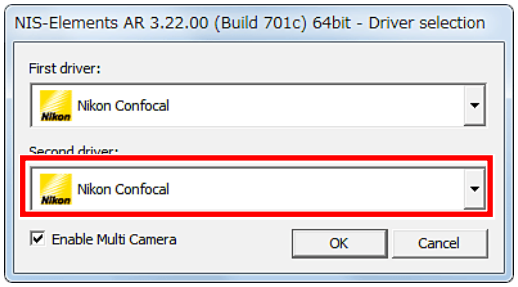


Figure 12.1-2 Driver selection dialog box

2. From the pull-down menu of the Second driver:, select the secondary camera.
   For the CCD camera, only [ANDOR] is selectable.

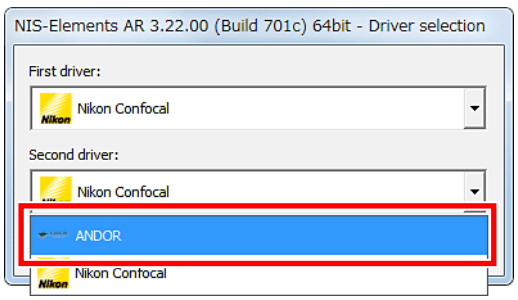


Figure 12.1-3 Selecting the second camera

3. Click [OK] button to starts the NIS-Elements C.
   After NIS-Elements C activates, the menu bar shows two camera operation icons.

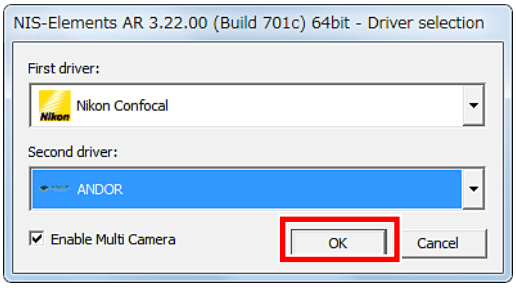


Figure 12.1-4 Starting the NIS-Elements C

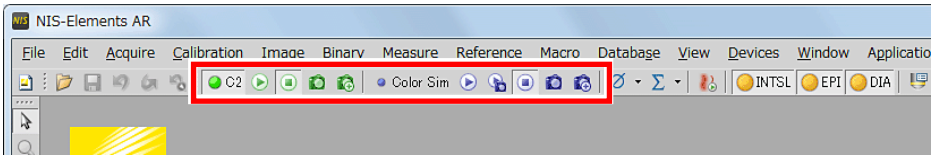


Figure 12.1-5 Menu bar for C2+TIRF System

Enabling C2+TIRF System after Activation of NIS-Elements C

1. Select [Acquire] on the menu bar and then select [Select Driver…].
   [Driver selection] dialog box appears.

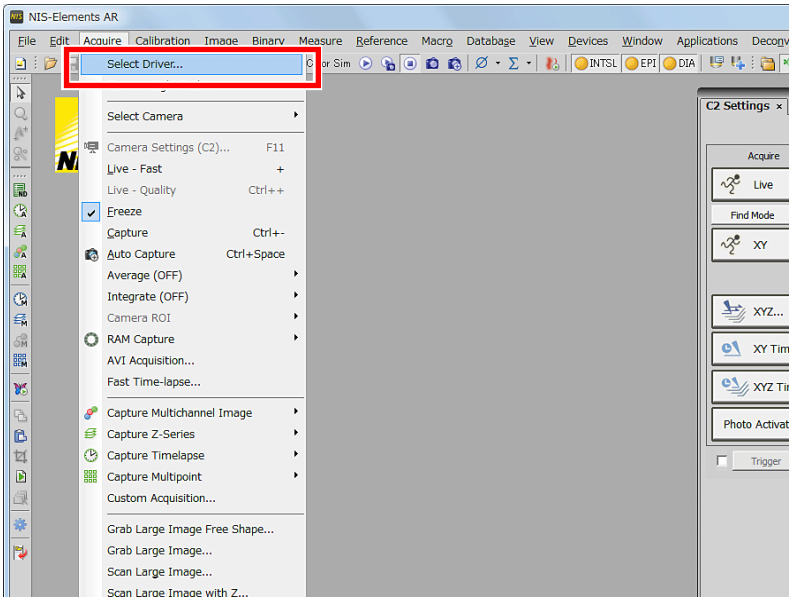


Figure 12.1-6 To display the Driver selection dialog box

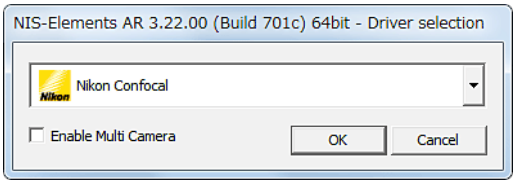


Figure 12.1-7 Driver selection dialog box

2. Turn “ON” the [Enable Multi Camera] check box.
   On the [Driver selection] dialog box, you may select the second camera.

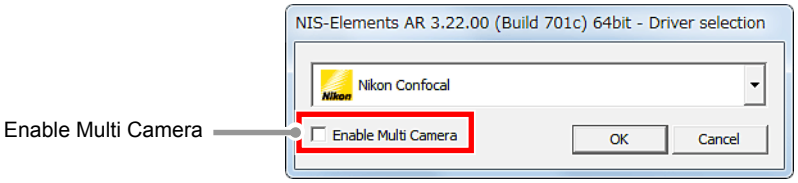


Figure 12.1-8 Driver selection dialog box

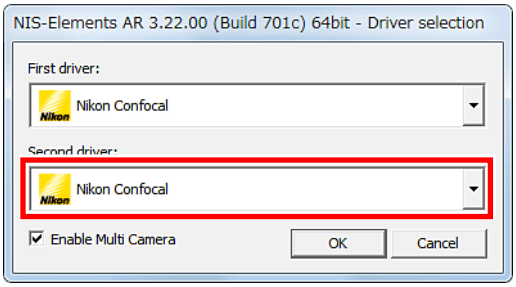


Figure 12.1-9 Driver selection dialog box

3. From the menu of the Second driver:, select the secondary camera.
   For the CCD camera, only [ANDOR] is selectable.

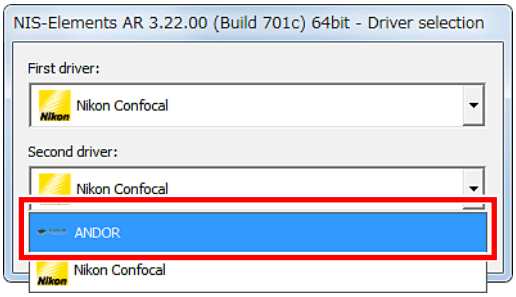


Figure 12.1-10 Selecting the second camera

4. Click the [OK] button to confirm it.
   The menu bar shows two camera operation icons.

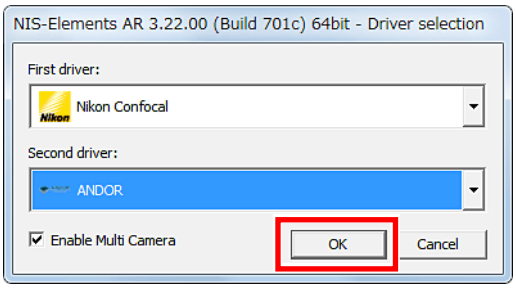


Figure 12.1-11 Switching to C2+TIRF System

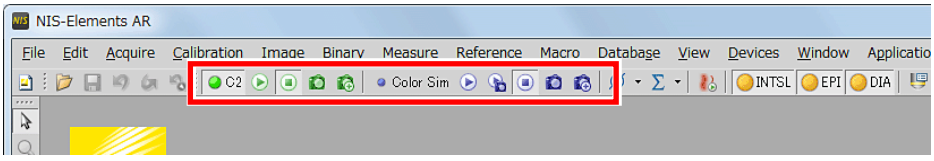


Figure 12.1-12 Menu bar for C2+TIRF System

12.2 Setting ECLIPSE Ti and Laser Connection

Make settings using the following procedure to synchronize NIS-Elements with the ECLIPSE Ti and LU4A (Laser).

1 Call the [Manage devices] dialog box

Select [Devices] on the menu bar and then select [Manage devices...].
[Manage devices] dialog box appears.

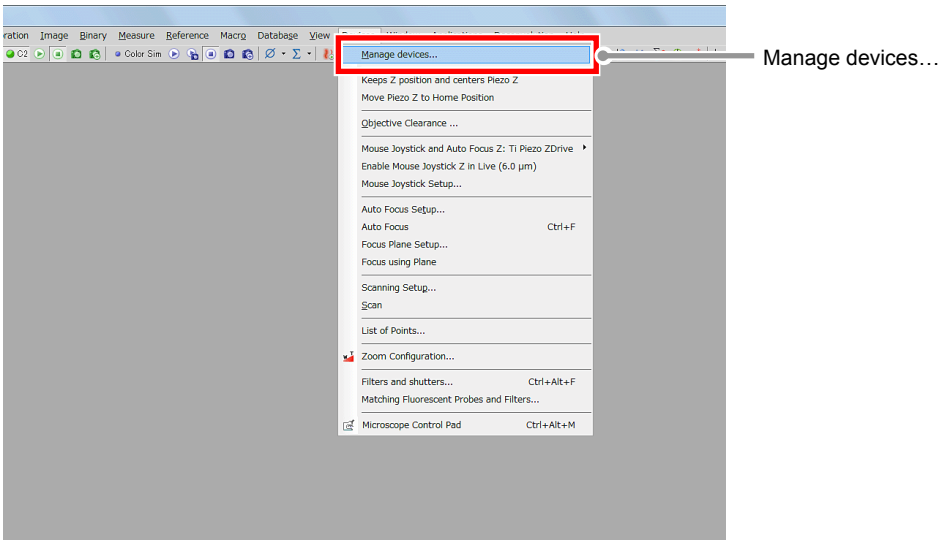


Figure 12.2-1 Devices menu

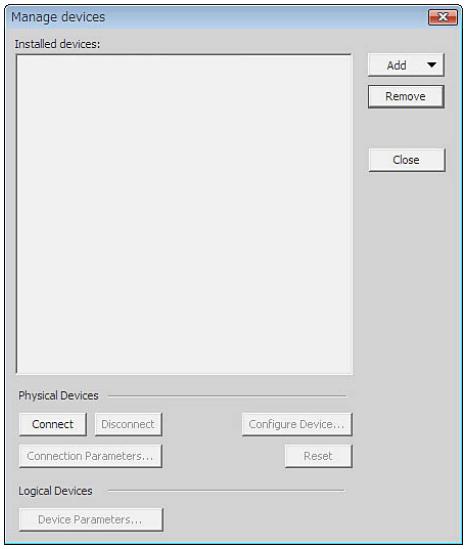


Figure 12.2-2 Manage devices dialog box

2 Add “Nikon Ti”

Click the [Add] button to display the menu for devices to be added.
Select the “Nikon Ti” form pull-down menu.
“Nikon Ti” is added in the [Installed devices:] field.

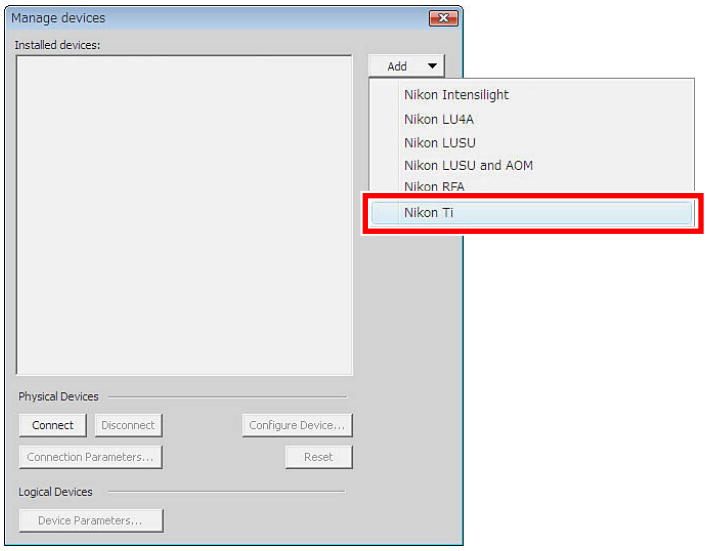


Figure 12.2-3　Manage devices dialog box

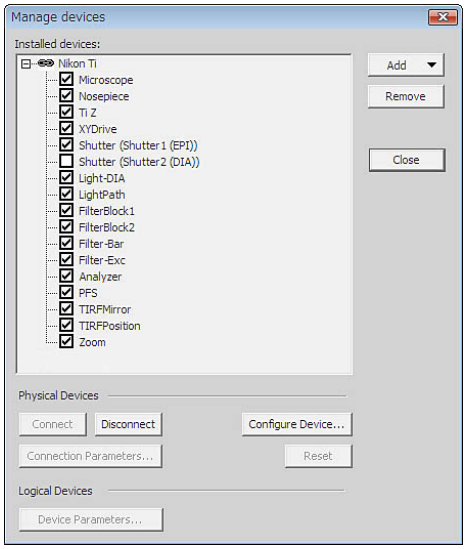


Figure 12.2-4　Manage devices dialog box

3 Add “Nikon LU4A”

Click the [Add] button to display the menu for devices to be added.
Select the “Nikon LU4A” form pull-down menu.
“Nikon LU4A” is added in the [Installed devices:] field.

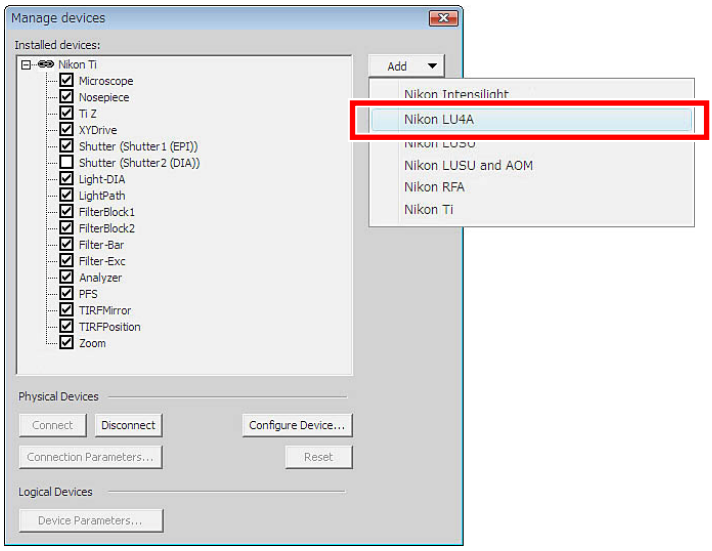


Figure 12.2-5 Manage devices dialog box

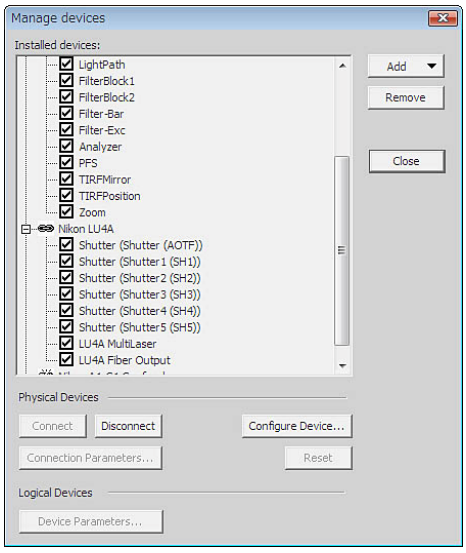


Figure 12.2-6 Manage devices dialog box

4 Setting the Lasers (Nikon LU4A)

1. Call the [LU4A Pad] dialog box.
   As shown below, right-click on the gray area (without any dialog box and setting window displayed) to display a menu. Then select [Acquisition Controls] -> [LU4A Pad] in the menu to open [LU4A Pad] dialog box.

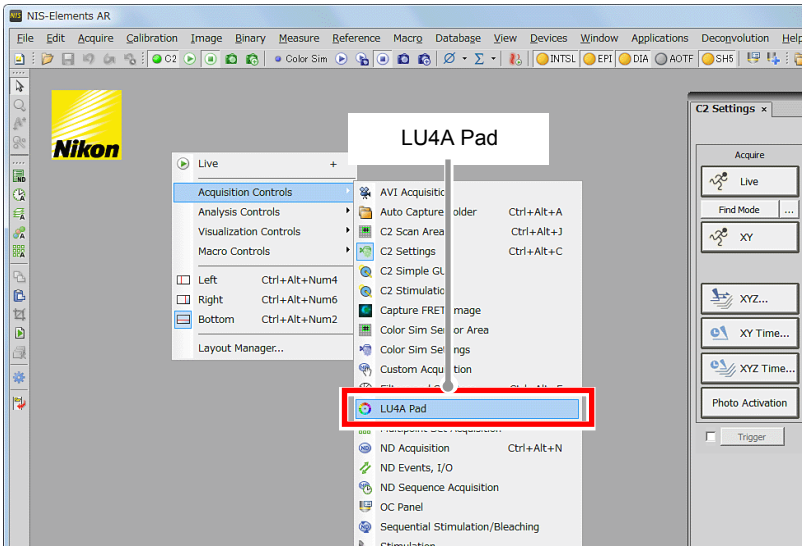


Figure 12.2-7 To display the LU4A Pad dialog box

2. Open the [LU4A Configuration] dialog box.
   Click the [Configure…] button in the [LU4A Pad] dialog box.
   [LU4A Configuration] dialog box appears.

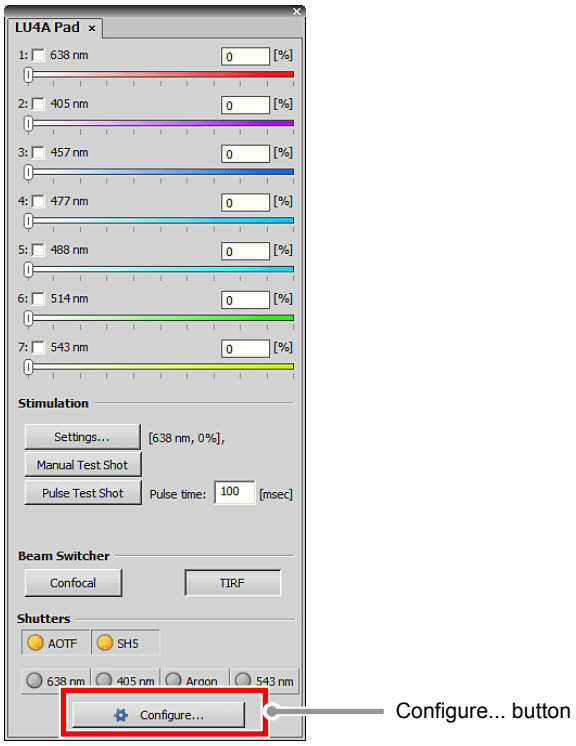


Figure 12.2-8 LU4A Pad dialog box

3. Select the laser to be used.
   Select the laser to be used from the pull-down menu of each laser.
   After selecting the laser, click the [OK] button to determine the settings.

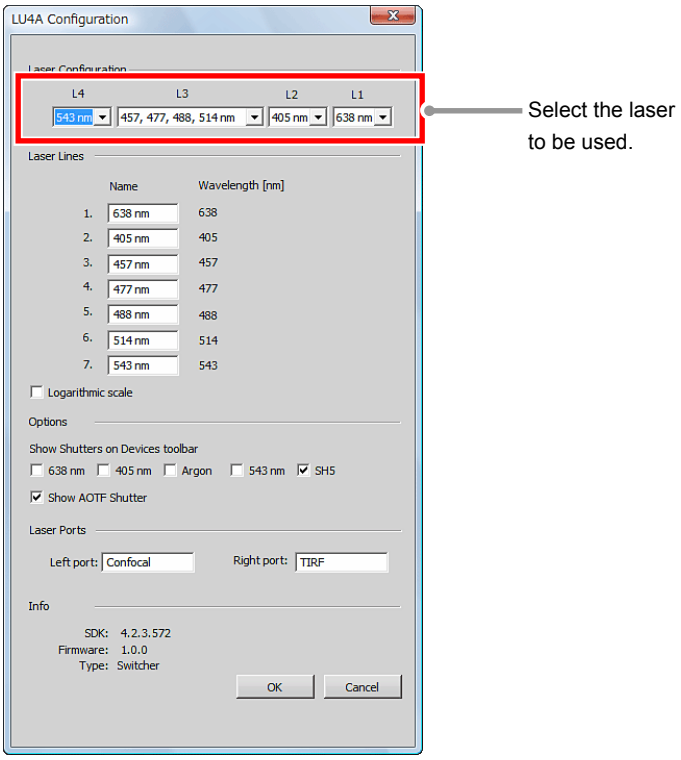


Figure 12.2-9 LU4A Configuration dialog box

12.3 Optical Configuration Setting

Register the settings for the confocal image acquisition with C2 and the TIRF image acquisition with CCD camera to the Optical Configuration files.

This section describes the setting of laser optical path switching (set on [LU4A Pad] dialog box) and setting of device selection (set on [Ti Pad]) for image acquisition that are to be configured only for C2+TIRF system.

12.3.1 Optical Configuration Setting for C2

1 Switch the laser optical path on the [LU4A Pad] dialog box

1. Call the [LU4A Pad] dialog box.
   As shown below, right-click on the gray area (without any dialog box and setting window displayed) to display a menu. Then select [Acquisition Controls] -> [LU4A Pad] in the menu to open the [LU4A Pad] dialog box.

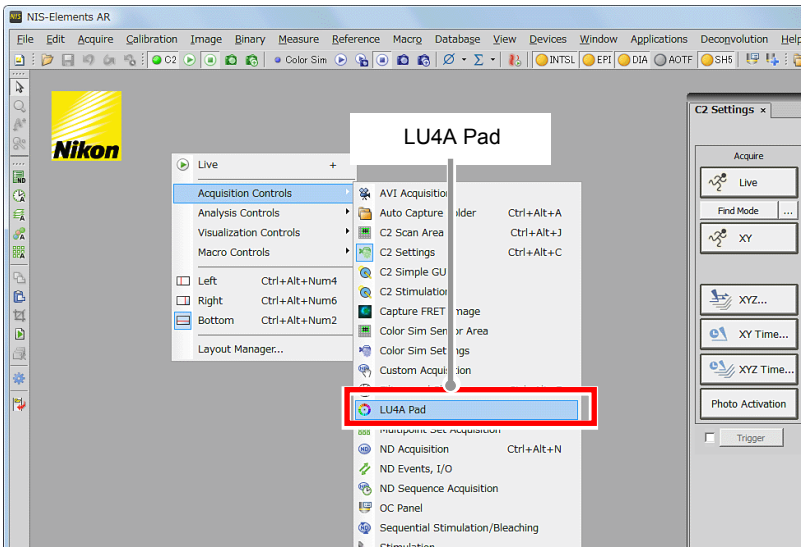


Figure 12.3-1 To display the LU4A Pad dialog box

2. With Beam Switcher in the [LU4A Pad] dialog box, select the laser optical path.
   For the optical configuration of the C2, select [Confocal].

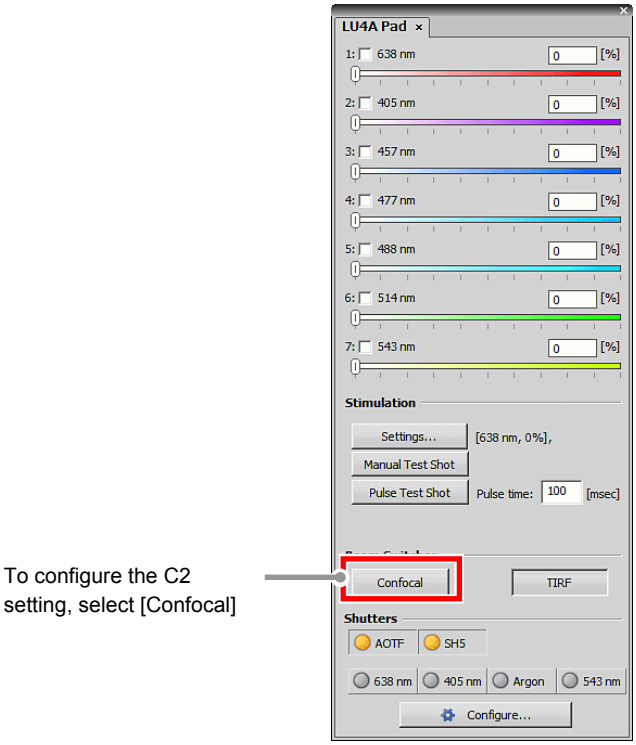


Figure 12.3-2 LU4A Pad dialog box

2 Select the device to be used for image acquisition with [Ti Pad]

1. Call the [Ti Pad].
   As shown below, right-click on the gray area (without any dialog box and setting window displayed) to display a menu. Then select [Acquisition Controls] -> [Ti Pad] in the menu to open the [Ti Pad].

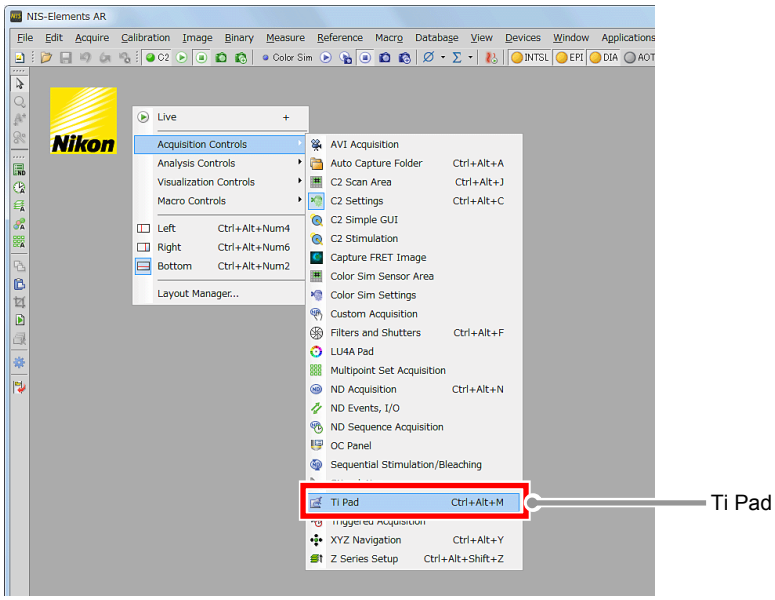


Figure 12.3-3 To display the Ti Pad

2. Select the device for [Ti Pad].
   For the optical configuration of the C2, select [EPI].

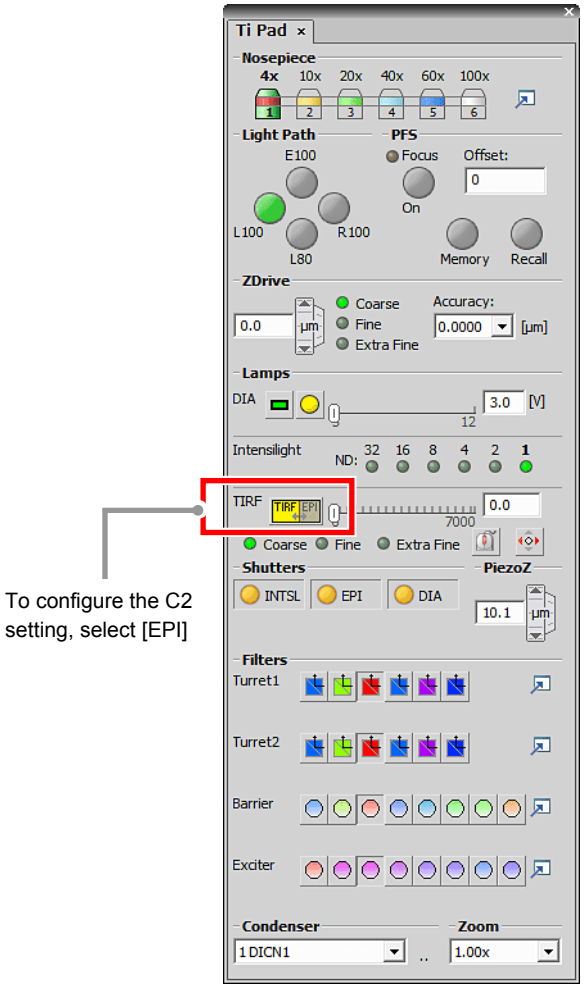


Figure 12.3-4 Ti Pad

3. Configure laser power and other settings on [C2 Settings], and register the configuration to the Optical Configuration file.
   For storing and retrieving the [Optical Configuration] settings, see the sections concerning the optical configuration in the “NIS-Elements Advanced Research User's Guide.”

12.3.2 Optical Configuration Setting for CCD Camera

1 Switch the laser optical path on the [LU4A Pad] dialog box

1. Call the [LU4A Pad] dialog box.

2. With Beam Switcher on the [LU4A Pad] dialog box, select the laser optical path.
   For the optical configuration of the CCD, select [TIRF].

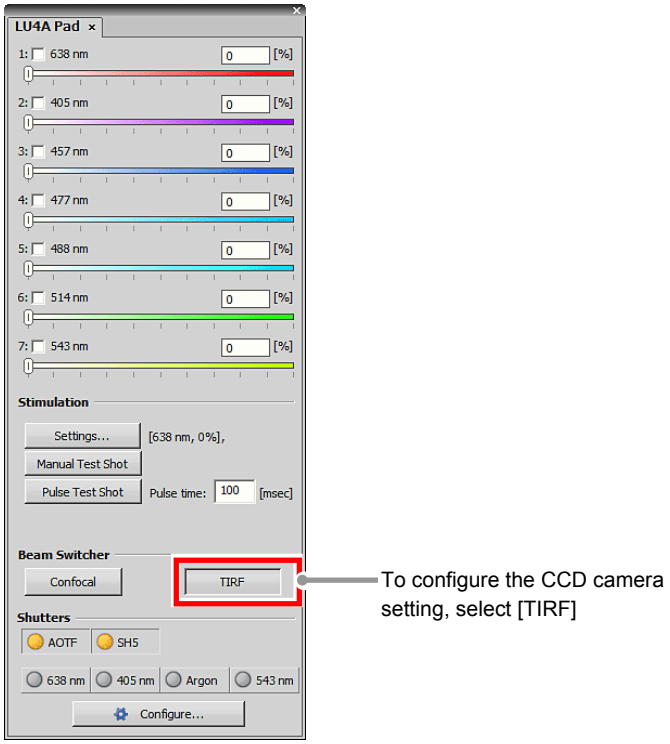


Figure 12.3-5 LU4A Pad dialog box

3. Select the lasers for CCD camera image acquisition and make power adjustments.

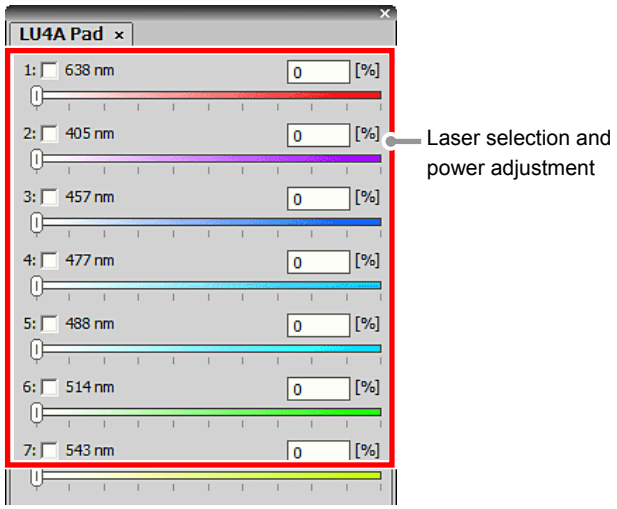


Figure 12.3-6 LU4A Pad dialog box

2 Select the device for image acquisition on [Ti Pad]

1. Call the [Ti Pad].

2. Select the device for [Ti Pad].
   For the optical configuration of the CCD, select [TIRF].

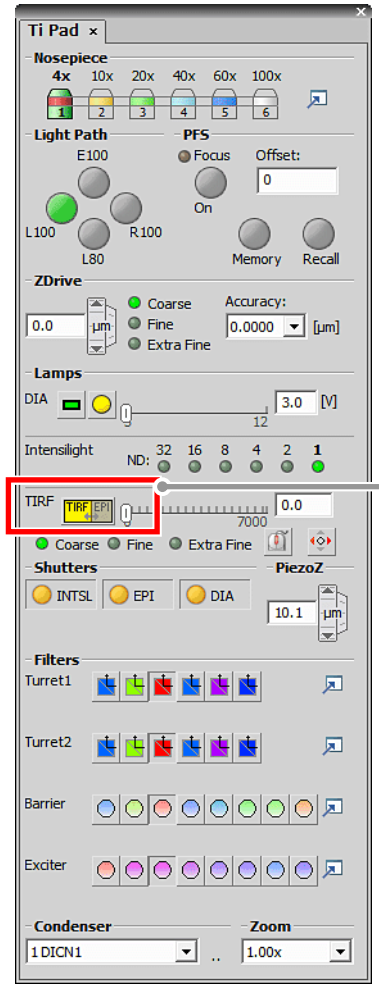


Figure 12.3-7 Ti Pad

3. Register the Optical Configuration file for the CCD camera.
   For storing and retrieving the [Optical Configuration] settings, see the sections concerning the optical configuration in the “NIS-Elements Advanced Research User's Guide.”

12.4 Procedure of Image Acquisition

Acquire the images by using the registered Optical Configuration files.

1 Call the [ND Acquisition] dialog box

Select [Applications] -> [Define/Run Experiment...] from the menu bar to open the [ND Acquisition] dialog box.

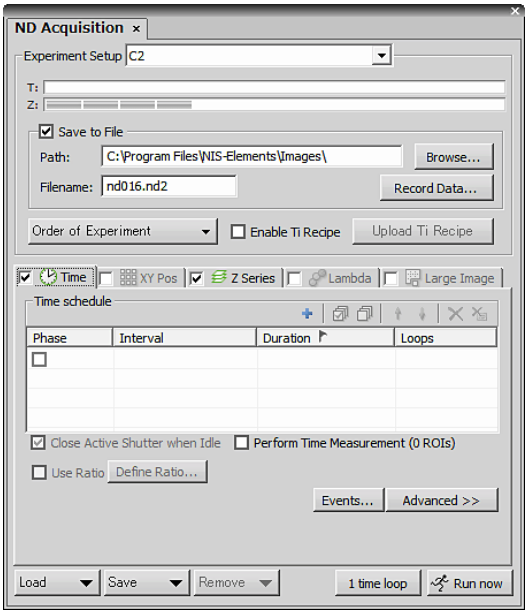


Figure 12.4-1 ND Acquisition dialog box

2 Set the Z stack for image acquisition with C2

1. Click the [Z Series] tab.

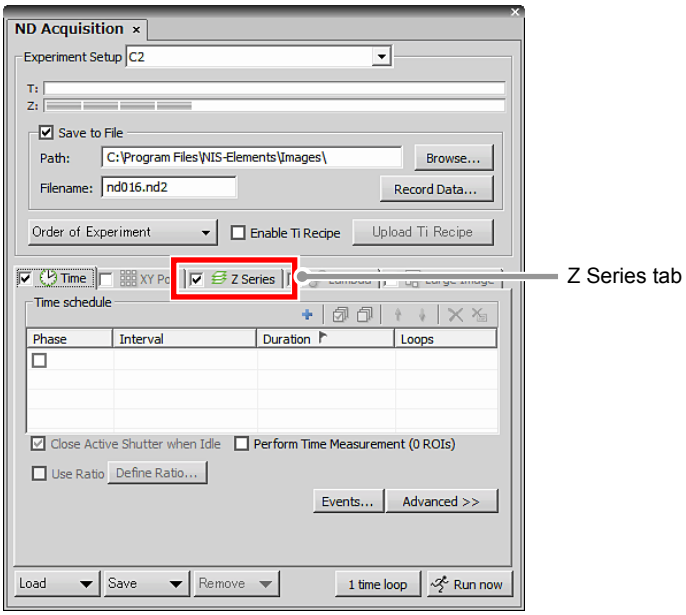


Figure 12.4-2 ND Acquisition dialog box

2. Set the Z stack for image acquisition with C2.
   For Z stacks settings, refer to “NIS-Elements Advanced Research User’s Guide.”

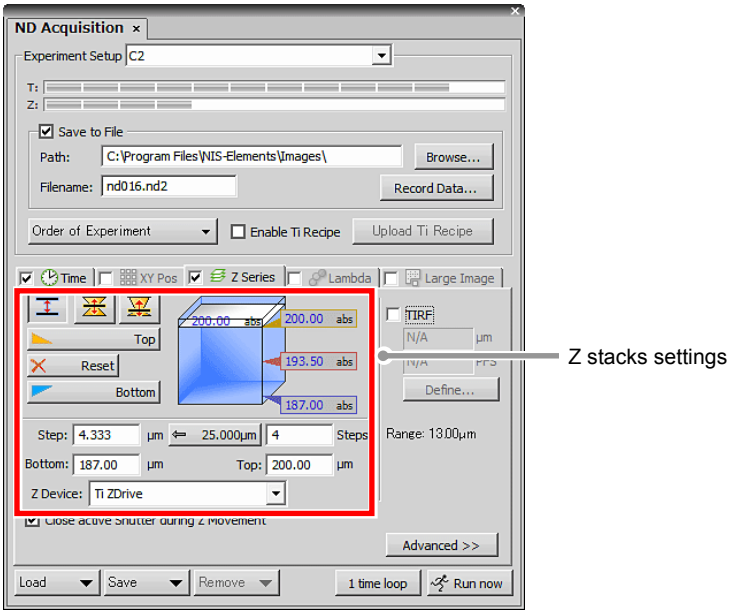


Figure 12.4-3 Z stacks settings

3 Set the TIRF position for image acquisition with CCD camera

1. Turn “ON” the [TIRF] check box.

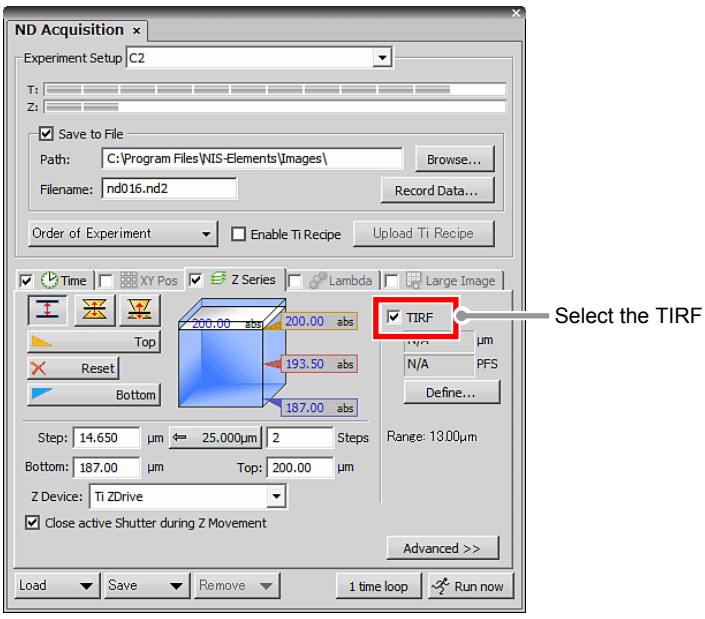


Figure 12.4-4 Select the TIRF

2. Set the TIRF position for image acquisition with CCD camera.
   Click the [Define] button.
   When the [Define TIRF Position] dialog box appears, adjust the Z stage to the TIRF position.

   You may adjust the Z stage to the TIRF position with either of the following methods:
   (1) Manually handle the Z stage.
   (2) Into [ZDrive] for Ti Pad, input the TIRF position to operate the Z stage.

   After adjusting the Z stage to the TIRF position, click [OK] on the [Define TIRF Position] dialog box.
   The Z stage is set to the TIRF position.

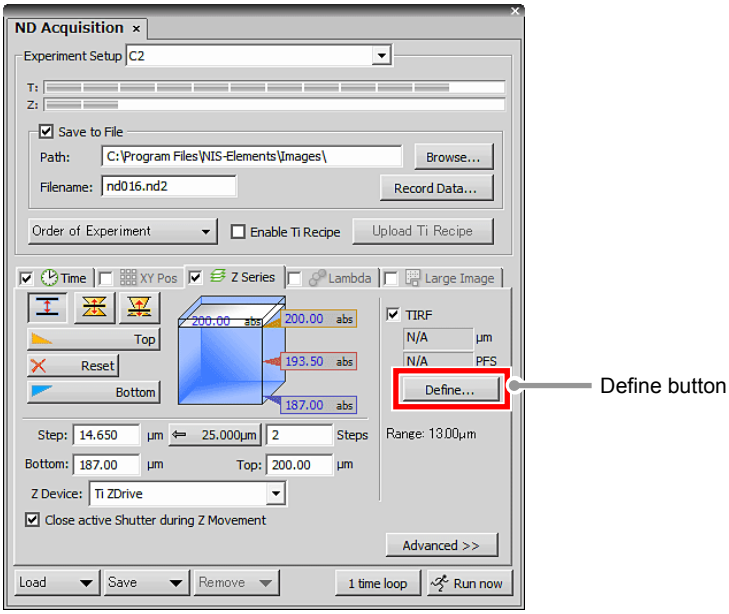


Figure 12.4-5 Define TIRF Position

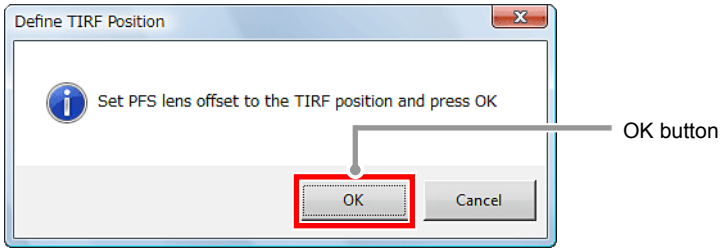


Figure 12.4-6 Define TIRF Position dialog box

4 Register the devices for image acquisition

1. Click the [Lambda] tab.

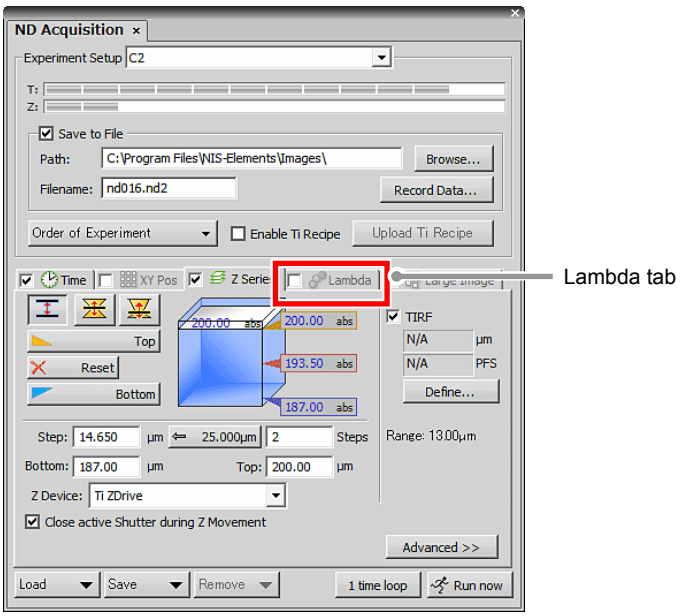


Figure 12.4-7 Select the Lambda tab

2. Register the C2 and CCD camera.
   Click below the [Camera] field, and then register the first device.
   Click on the second line, and then register the second device.

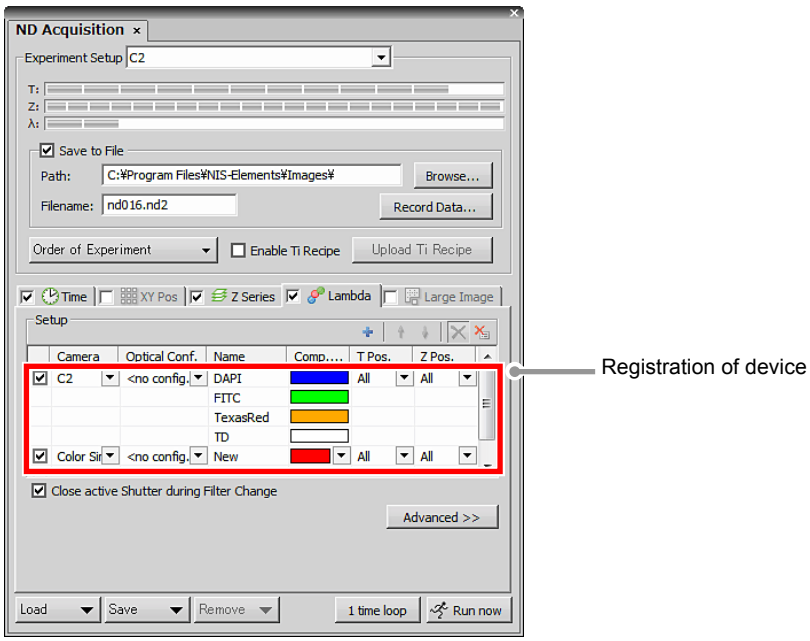


Figure 12.4-8 Registration of device

5 Select the Optical Configuration file

Select the Optical Configuration files for the C2 and CCD camera from the pull-down menu for Optical Conf.

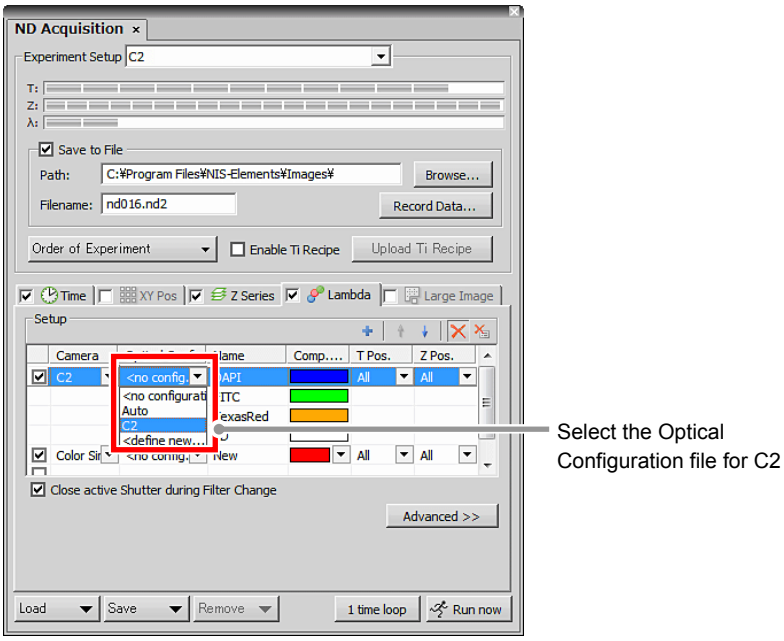


Figure 12.4-9 Select the Optical Configuration file for C2

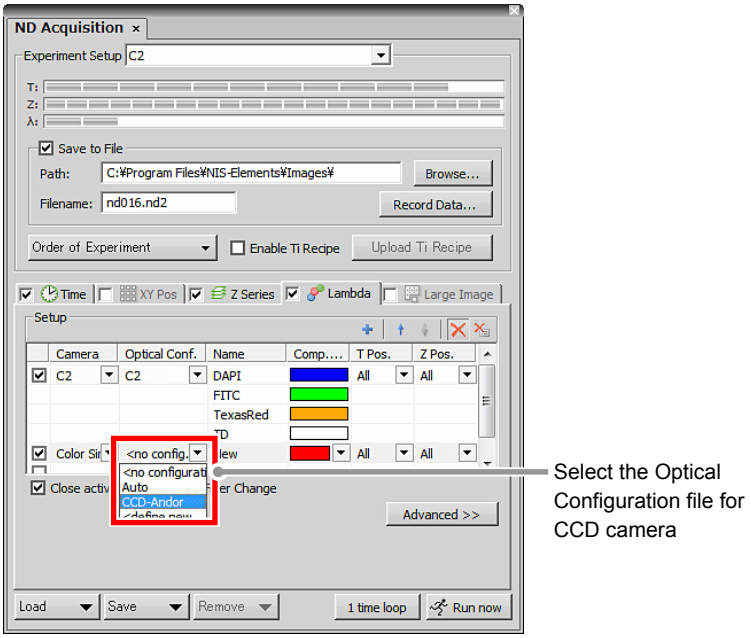


Figure 12.4-10 Select the Optical Configuration file for CCD camera

6 Setting the Z phase for TIRF

Select the [TIRF] from the pull-down menu of [Z phase] for CCD camera.
The C2 acquires images of all positions configured with Z stacks, thus the setting remains as [All] without change.

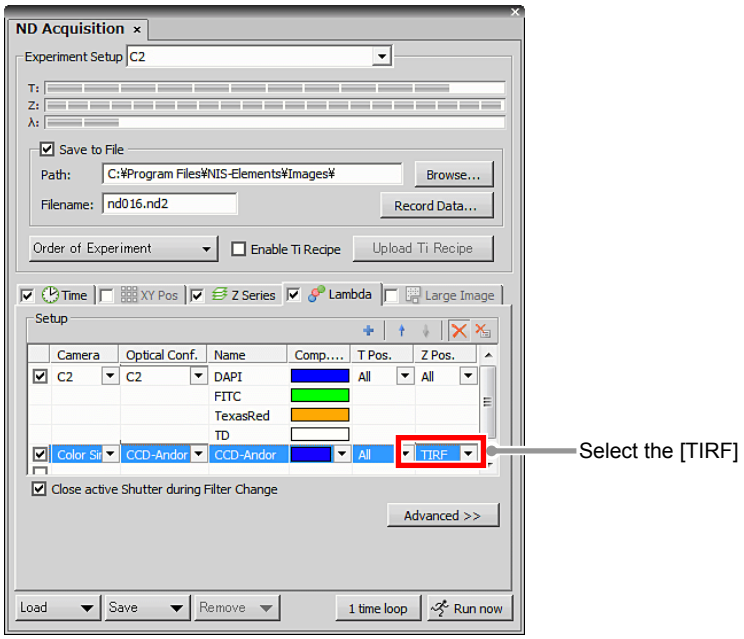


Figure 12.4-11 Z phase settings

7 Start the image acquisition

Click the [Run now] button to start the image acquisition.
After image acquisition, two image windows for C2 confocal image and CCD camera TIRF image are displayed.

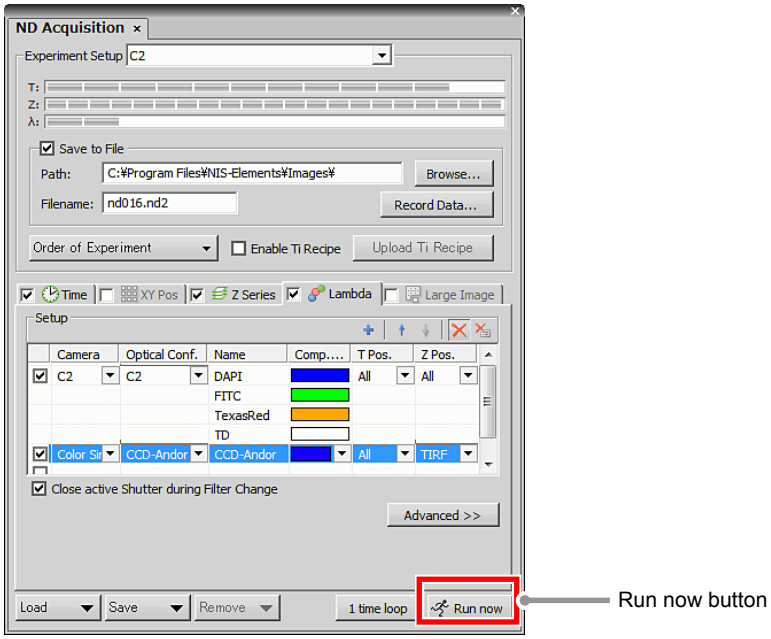


Figure 12.4-12 Run now

12.5 Merge the Confocal Image and TIRF Image

When the C2+TIRF System is in use, a confocal image acquired with C2 and a TIRF image acquired with CCD camera can be merged and acquired as one image.

Merge Camera

Turn “ON” the [Merge Camera] check box of the Advanced menu on the Lambda tab and click the [Run now] button to execute the image acquisition to get merged images.

Note that images are simply merged without any regard for the difference in the sizes of the C2 confocal image and the CCD camera TIRF image even if they are not in the same size, thus it is recommended to adjust the image sizes in advance.

Merge Camera + Stretch Camera Image to Same Size

To coordinate the confocal image and TIRF image sizes, turn “ON” both the [Merge Camera] check box and [Stretch Camera Image to Same Size] check box and then perform image acquisition.

This makes the smaller one expanded to the larger image size and a merged image is acquired with the same size.

However, even in that case, note that the image is simply expanded for size coordination regardless of what is represented in the image, thus it is recommended to adjust the image sizes in advance.

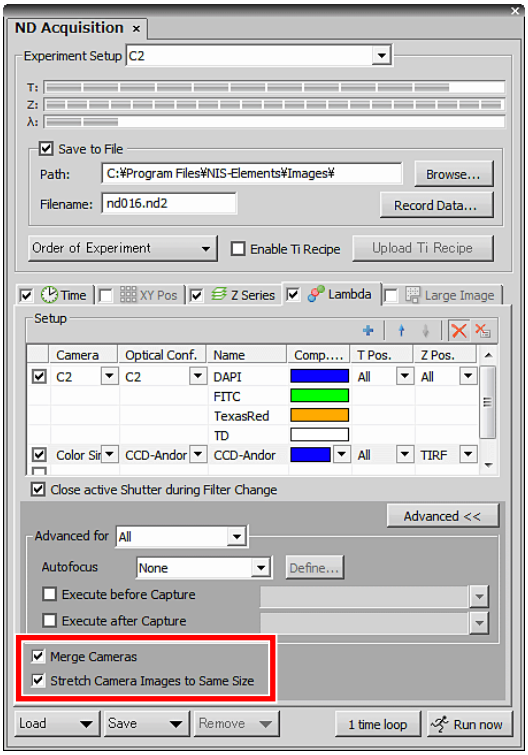


Figure 12.5-1 ND Acquisition dialog box

13 C2 Simple GUI

NIS-Elements allows you to use “C2 Simple GUI”, which is a simplified configuration screen supporting functions equivalent to those of “C2 Settings”, so that you can sufficiently use the window spaces.
This chapter describes how to show the screen and the functions available on it.

13.1 Displaying the C2 Simple GUI

As shown below, right-click on the gray area (without any dialog box and setting window displayed) to display a menu. Then select [Acquisition Controls] -> [C2 Simple GUI] in the menu to open [C2 Simple GUI] dialog box.

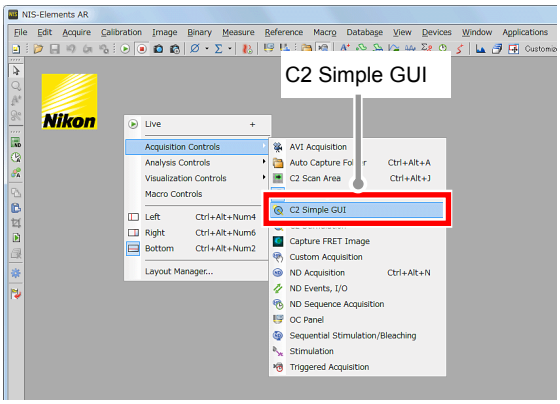


Figure 13.1-1 To display the C2 Simple GUI

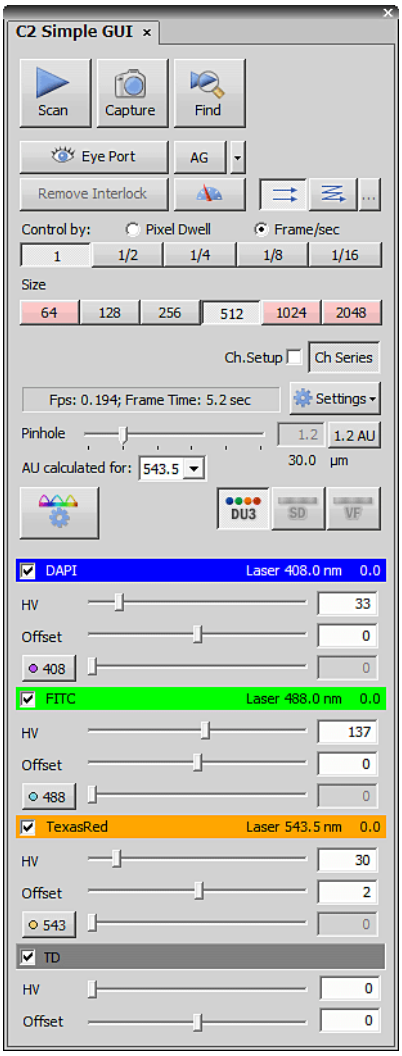


Figure 13.1-2 C2 Simple GUI (DU3 mode-use)

13.2 Functions of C2 Simple GUI

[C2 Simple GUI] allows you to configure settings for use of the Confocal Microscope C2 in the same manner as you configure with [C2 Settings].

The following shows an example of the screen where DU3 is selected for Detector mode.
For details of each item by detector, see the appropriate chapter.

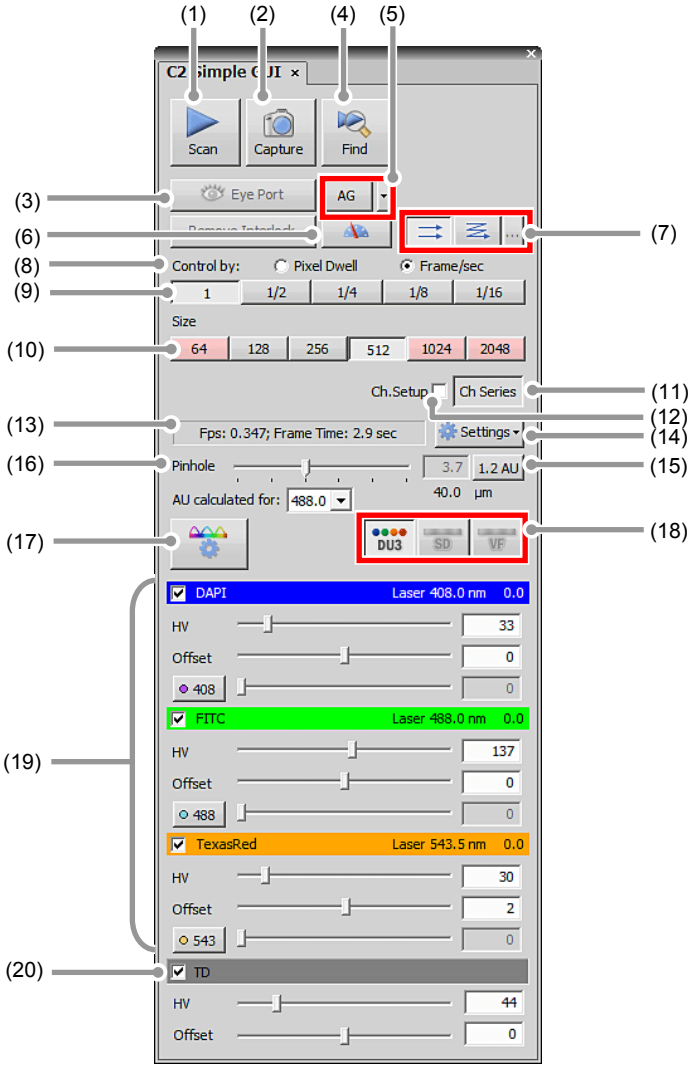


Figure 13.2-1 C2 Simple GUI (DU3 mode-use)

Table 13.2-1 Functions of C2 Simple GUI (sheet 1/3)

Name		Function
(1)	Scan button	Starts/stops live image acquisition.
(2)	Capture button	Captures the image.
(3)	Eye Port button	Changes optical path to eye port.
(4)	Find... button	Starts/stops live image acquisition in Find mode. Find mode is the mode where the live image acquisition is executed by temporarily switching to the high-frame-rate setting in order to ease the detection of the observation object such as a cell. For details, see Section 4.1.2, “Find Mode.”

Table 13.2-1 Functions of C2 Simple GUI (sheet 2/3)

Name		Function
(5)	Auto Gain	**Standard Detector-use** Automatically adjusts the HV value (HV gain) of the currently selected channel to the optimum values. * For details, see the following. Standard Detector: For Auto Gain, see Section 5.2.5, “Auto Gain.”
		Spectral Detector or Virtual Filter-use Automatically adjusts the Si HV value (Si HV gain) to the optimum values. * For details, see the following. Spectral Detector: For Auto Gain, see Section 6.2.4, “Auto Gain.” Virtual Filter: For Auto Gain, see Section 7.2.4, “Auto Gain.”
(6)	Laser power monitor button	Displays the laser power value (integer obtained after A/D conversion divided by 10) of the current channel by clicking this button.
(7)	Scan Direction	Toggles between Unidirectional and Bidirectional scan. Bidirectional scan is only selectable if the Square scan area or Band scan area is set. By default, Unidirectional scan is selected. * For details, see the following. Section 8.3, “Scan Setting Parameters.”
(8)	Control by:	Switches the Scan Speed selection form.
(9)	Scan Speed	Sets scan speed. (Setting unit: Frame/Sec) Pull-down menu: Selects the desired scan speed from this list. [▲] and [▼] buttons: Click these to select scan speeds one after another.
(10)	Scan Size	Sets the scan resolution in the X-direction. (Setting unit: Pixel) The resolution in the Y-direction is automatically calculated from the X to Y ratio of the scan area. Pull-down menu: Selects the desired resolution from this list. [▲] and [▼] buttons: Click these to select resolutions one after another.
(11)	Ch Series button	Settable only in Standard Detector-use. Selects whether to perform scanning by simultaneously firing all lasers for the channels in use or by sequentially firing one laser after another. * For details, see the following. See Section 5.1.4, “Selecting the Channel Series.”
(12)	Ch.Setup check box	Displayed when the [Ch Series] button is ON. When checked, the setting by the channel is facilitated. Automatically enters the state where only one channel is selectable.
(13)	Fps:	Indicates the current scan settings.
(14)	Settings button	Displays the menu to open dialog boxes for various settings such as HV Linear Correction.

Table 13.2-1 Functions of C2 Simple GUI (sheet 3/3)

Name		Function
(15)	AU button	Changes the pinhole to the predetermined home position. The value of the home position can be changed in the [A.U. Calculation Settings] dialog box. * For details, see the following. Standard Detector: See Section 5.2.3.1, “Calculation Settings for Pinhole Size.” Spectral Detector: See Section 6.2.3.1, “Calculation Settings for Pinhole Size.” Virtual Filter: See Section 7.2.3.1, “Calculation Settings for Pinhole Size.” The [A.U. Calculation Settings] dialog box is displayed by selecting [AU settings…] from the setting menu displayed by the [Settings] button.
(16)	Pinhole	Adjusts the pinhole size. Sets a pinhole size in Airy units (units of airy disk size). Slider bar: Slides to the right or left to set the pinhole size. (Unit: A.U.) Arrow buttons: Click either arrow button to increase or decrease the pinhole size stepwise. Direct entry in pinhole size display field: Type the desired setting value. * For details, see the following. Standard Detector: See Section 5.2.3, “Setting the Pinhole.” Spectral Detector: See Section 6.2.3, “Setting the Pinhole.” Virtual Filter: See Section 7.2.3, “Setting the Pinhole.”
(17)	Optical path Setting button	Opens the Optical path window. To use, select the detector and the dichroic mirror, the channels as well as the fluorescence dye, laser, and for each channel.
(18)	Detection mode indicator/selection button	Selects/displays the Detection mode for use. When the optical path changeover lever on the C2 scan head is set to the [Spectrum] position, [SD] or [VF] can be selected as the detector mode. When the optical path changeover lever on the C2 scan head is set to the [Standard] position, the detector mode is fixed to [DU3]. * For details of each item for each Detection mode, see the following. : See Chapter 5, “Detection Mode (Standard Detector).” : See Chapter 6, “Detection Mode (Spectral Detector).” : See Chapter 7, “Detection Mode (Virtual Filter).”
(19)	Brightness adjustment for each channel	For each of the channels (Ch1 to Ch3), use the HV, Offset, and Laser controls to adjust the brightness of the live image. Note that these items vary depending on the selected detector. * For details, see the following. Standard Detector: See Section 5.2.1, “Structure of Acquisition Window.” Spectral Detector: See Section 6.2.1, “Structure of Acquisition Window.” Virtual Filter: See Section 7.2.1, “Structure of Acquisition Window.”
(20)	Brightness adjustment for transmitted detector	For the transmitted detector, use the HV and Offset controls to adjust the brightness of the live image.

14 External Trigger Output

This chapter describes the external trigger output function of NIS-Elements.

This function allows trigger signals of (e.g., Acquisition or Photo Activation experiments) to be sent to an external device connected with the C2 controller.

Trigger signals are output frame by frame when an image is acquired.

One external trigger signal channel is available.
Note that the external trigger settings are unchangeable during Live or experiment.

14.1 Trigger Signal Output

14.1.1 Procedure for External Trigger Output Settings

1 Call the [External Trigger Output Settings] dialog box

1. [Trigger] check box to turn it “ON.”
   The external trigger output function is turned on, and [Trigger] button becomes effective.

   If trigger signals output is not to be executed, uncheck the [Trigger] check box.

2. Click the [Trigger] button to open the [External Trigger Output Settings] dialog box.

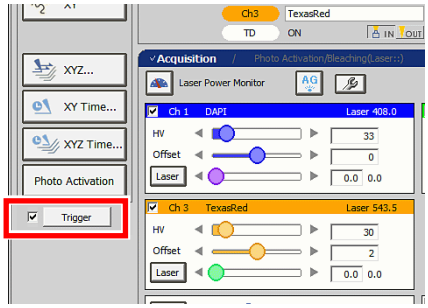


Figure 14.1-1 Trigger check box

2 Set the Polarity for the output trigger signal channels

1. Check the trigger signal output setting.

   [Acquisition] and [Photo Activation] are displayed only when the trigger signal output is ON.

2. Specify the level to output as the trigger signal.

   Sets the rising edge of the TTL level signal as the trigger signal.

   Sets the falling edge of the TTL level signal as the trigger signal.

3. Click [OK] button to finish the trigger signals polarity settings.

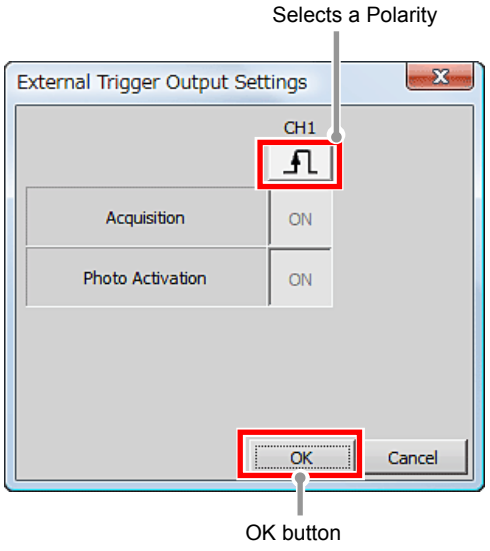
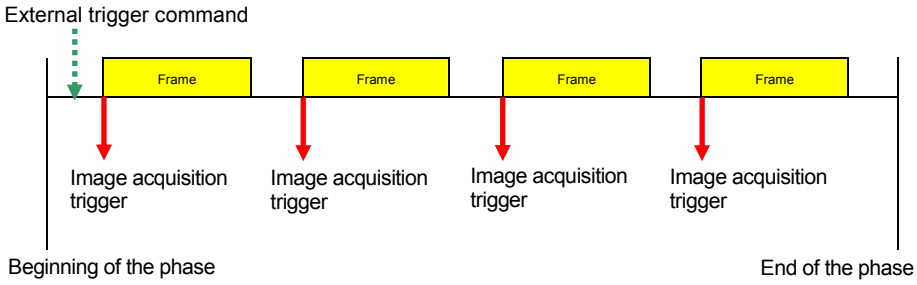
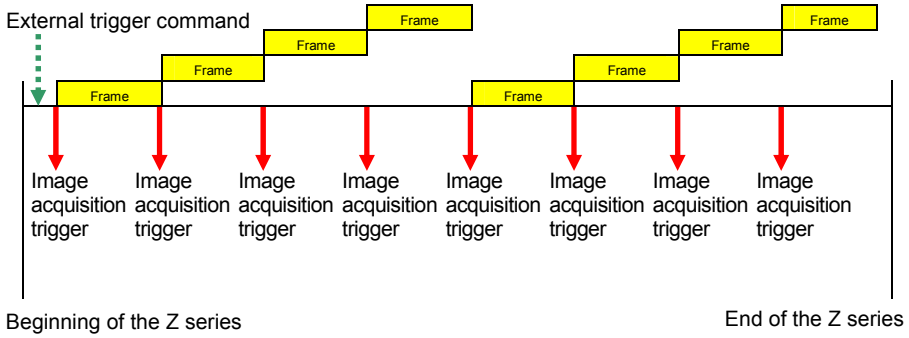
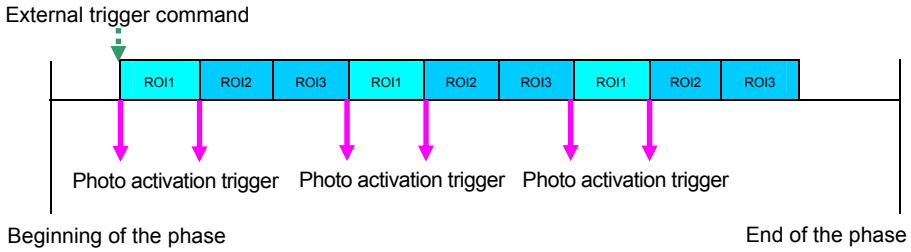
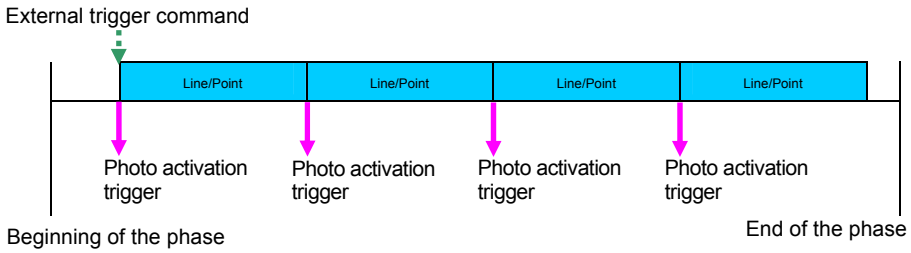
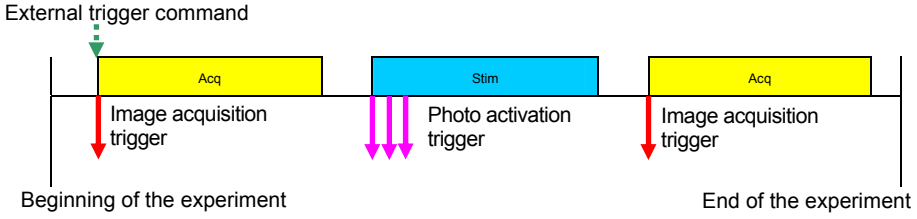


Figure 14.1-2 External Trigger Output Settings

14.1.2 External Trigger Output Operation List

The external trigger output operations are listed below.

Table 14.1-1 External trigger output operation list

Type	Operation
XYT	
XYZT	
ROI photo activation (for the case of ROI1: Group 1, and ROI2, ROI3: Group 2)	
Line/Point photo activation	
Photo activation	

Appendix Lists of Filter Block and Fluorescence Reagents

The Filter Block Information for the Optical Path

The Dichroic mirror or the Barrier filter to be registered on the Optical path window for the Confocal C2 is listed below.

1st Dichroic mirror

1st Dichroic mirror provided for the Optical C2 is shown below.

Table 1 1st DM List

	Name	Note:
1	BS 20/80	Excitation lights are unrestricted.
2	408/488/543	
3	405/488/561	
4	408/488/594	
5	440 (457)/514/594	
6	405/488/543/640	
7	405/488/561/640	
8	405 (408)/457 (440)/561/640 (633)	
9	440 (457)/514/561/640 (633)	
10	405 (408)/457 (440)/543/640 (633)	

Detector filter block

The Dichroic mirror and the Detector filter block provided for the Optical C2 is shown below.

Table 2 DM/BA filter List

	2nd DM	1st BA	3rd DM	3rd BA	2nd BA
1	455	None	None	None	None
2	482	438/24	540LP	585/65	494/41
3	511	447/60	560	561LP	510/84
4	540LP	482/35	593	594LP	514/30
5	560	494/41	648	635LP	525/50
6		510/84			537/26
7		514/30			550/49
8		525/50			585/65
9		537/26			593/40

Registered filter block name

The Detector filter block combined with the standard filter block is shown below.

1 – 8 (9 for 2nd Filter Block Set) indicates the preset setting, whereas 9 (10 for 2nd Filter Block Set) - 11 indicates the User registration, and 12 indicates no filter required.

The available Filter block is equivalent with the Filter block tab of the [Filter Block Setting] dialog box.

Table 3 Registration filter block name List 1 1st Filter Block Set

	Name	Registration number	2nd DM	BA1
1	438/24	1	455	438/24
2	447/60	2	482	447/60
3	482/35	3	511	482/35
4	494/41	4	540LP	494/41
5	510/84	5	560	510/84
6	514/30	6	540LP	514/30
7	525/50	7	560	525/50
8	537/26	8	560	537/26
9	User 3	9	None	None
10	User 4	10	None	None
11	User 5	11	None	None
12	Trough	12	Trough	Through

Table 4 Registration filter block name List 2 2nd Filter Block Set

	Name	Registration number	3rd DM	3rd BA	2nd BA
1	494/41,585/65	1	540LP	585/65	494/41
2	510/84,561LP	2	560	561LP	510/84
3	514/30,585/65	3	540LP	585/65	514/30
4	525/50,561LP	4	560	561LP	525/50
5	525/50,594LP	5	560	594LP	525/50
6	537/26,561LP	6	560	561LP	537/26
7	550/49,594LP	7	593	594LP	550/49
8	585/65,635LP	8	648	635LP	585/65
9	593/40,635LP	9	648	635LP	593/40
10	User 2	10	None	None	None
11	User 3	11	None	None	None
12	Trough	12	Trough	Trough	Trough

Fluorescence reagents

The Fluorescence dye selection available on C2 shows below.

Table 5 Fluorescence dye available for selection (sheet 1 of 2)

	Name	Excitation	Laser [nm]	Fluorescence	Category
1	Alexa Fluor 405	402	405/408	421	Blue
2	Cascade Blue	376	405/408	425	Blue
3	Hoechst33528	352	405/408	455	Blue
4	DAPI	345	405/408	461	Blue
5	ECFP	435	440/457	475	Cyan
6	ECFP (FRET Donor)	435	440/457	475	Cyan
7	Qdot 525	300	405	525	Green
8	Alexa 488 antibody	499	488	520	Green
9	Alexa 488 water	493	488	517	Green
10	Cy2	489	488	506	Green
11	AcridineOrange	502	488	526	Green
12	DiO	358.5	488	461	Green
13	FITC	495	488	519	Green
14	GFP-uv	395	405/408	509	Green
15	fluo-4	494	488	516	Green
16	Fluorescein	494	488	518	Green
17	YOYO1	490	488	510	Green
18	EGFP	488.5	488	509	Green
19	OregonGreen488	498	488	526	Green
20	BODIPY	503	488	512	Green
21	fluo-3	506	488	527	Green
22	Kaede (Before)	509.5	488	518.5	Green
23	Rhodamine Green	502	488	527	Green
24	Magnesium Green	506	488	532	Green
25	Calcium Green	507	488	529	Green
26	SNAFL-2	525	488	546	Green
27	EYFP	514	514	527	Yellow
28	EYFP (FRET Acceptor)	514	440/457	527	Yellow
29	Qdot 585	300	405	585	Orange
30	Qdot 605	300	405	605	Orange
31	m kusabira Orange	548	543	559	Orange

Table 5 Fluorescence dye available for selection (sheet 2 of 2)

	Name	Excitation	Laser [nm]	Fluorescence	Category
32	Alx546	561	543/561	572	Orange
33	DiI	551	543/561	569	Orange
34	Rhodamine Phalloidin	558	543/561	575	Orange
35	TRITC	543	543/561	580	Orange
36	Calcium Orange	549	543/561	576	Orange
37	rhod-2	556	543/561	576	Orange
38	DsRed2	563	543/561	581	Orange
39	Kaede (After)	573.5	543/561	581.5	Orange
40	Cy3.5	581	543/561	596	Orange
41	Rhodamine Red X	575	543/561	590	Orange
42	X-rhod-1	580	543/561	602	Orange
43	MitoTrackerRed	578	543/561	599	Orange
44	Calcium Crimson	588	543/561	611	Orange
45	Alx568	579	543/561	603	Orange
46	mCherry	580	594/561	610	Orange
47	HcRed1	588	543	614	Orange
48	Texas Red	595	543	613	Orange
49	Alx594	590	543	618	Orange
50	Alx633	632	633/640	647	Deep Red
51	Qdot 655	300	405	655	Deep Red
52	TO-PRO-3	642	640/638	661	Deep Red
53	TOTO3	642	633/640	661	Deep Red
54	Alx647	653	633/640	669	Deep Red
55	Cy5	650	633/640	670	Deep Red
56	Cy5.5	679	633/640	694	Deep Red