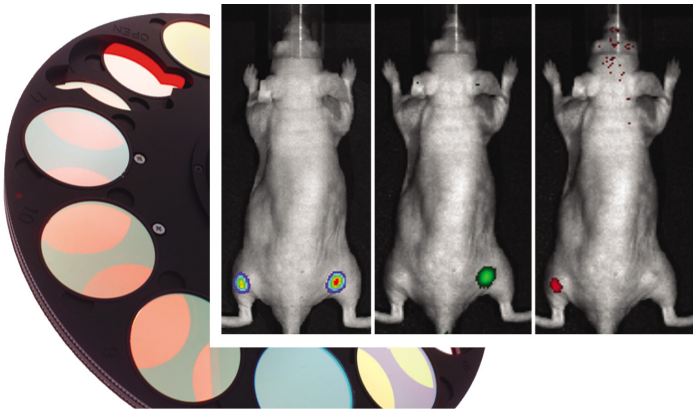
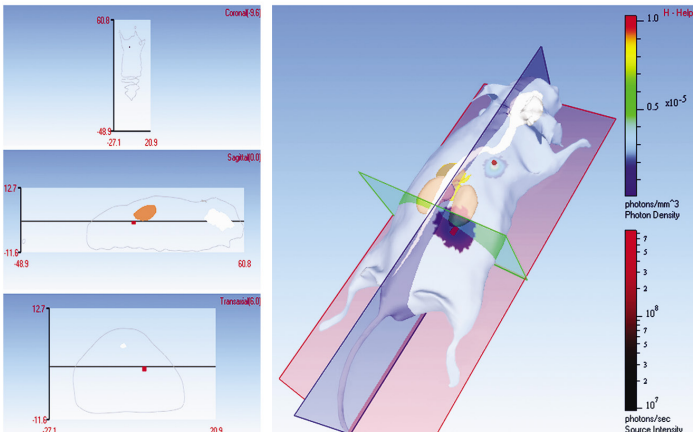
SOFTWARE MANUAL
FOR USE WITH
IVIS® SPECTRUM

Living Image® 4.5.4 Software

March 2017





PerkinElmer®

PerkinElmer Health Sciences

940 Winter Street
Waltham, Massachusetts 02451 USA
www.PerkinElmer.com

Technical Support
Telephone: +1 (800) 762-4000 (US) or +1 (203) 425-4602
Fax: +1 (203) 944-4904
E-mail: global.techsupport@perkinelmer.com

Sales: CustomerCareUS@perkinelmer.com

Trademarks

Copyright

Contents

1 Welcome

1.1 About This Manual

NOTE: This *Living Image 4.5.4 Software Manual* is only for use with the IVIS Spectrum Imaging System. If analyzing data acquired on a different type of IVIS Imaging System, say for example the IVIS SpectrumCT, please see the Living Image Software Manual specific for the IVIS SpectrumCT (see Table 1.1).

This manual explains how to acquire optical image data on the IVIS Spectrum and analyze the data using the Living Image software. The manual provides detailed instructions and screenshots for Living Image software tools that are available for data acquired on the IVIS Spectrum. Sometimes the screenshots in the manual may not exactly match those displayed on your screen.

Please see the *IVIS Spectrum Hardware Manual* (PN 121450) for information on the IVIS Spectrum instrument.

Table 1.1 Living Image Software Manuals

Living Image Software Manual for:
IVIS Lumina Series III (can also be used with the IVIS Lumina LT)
IVIS Lumina XRMS Series III (can also be used with the IVIS Lumina XR)
IVIS Lumina K Series III (can also be used with the IVIS Lumina Kinetic)
IVIS Spectrum
IVIS SpectrumBL (can also be used with the IVIS 200)
IVIS SpectrumCT

1.2 What's New in Living Image 4.5.4 Software

Software improvements specific to the IVIS Spectrum Imaging System are listed below.

Item	Description	See Page
Additional operating system support	Living Image now supports: ▪ Windows 10, 64-bit analysis. **Note:** Acquisition is only supported on Window 7 ▪ macOS 10.12 (Sierra). **Note:** OS X 10.11 (El Capitan) and 10.10 (Yosemite) are also supported. Older versions of OS X are no longer supported.	
New image export options in sequence view	In sequence view: ▪ Export all of the images in a sequence to one graphic file. ▪ Export each image of a user-selected subject to a separate graphic file.	89 89

1.3 Living Image Help

There are several ways to obtain help on the software features and related information.

To view:	Do this:
A tooltip about a button function	Put the mouse cursor over the button.
A brief description about an item in the Living Image user interface	Click the  toolbar button, then click the item.
The *Living Image Software User's Manual*	Press F1 or select **Help → User Guide** on the menu bar and select the manual specific for your imaging system.
Living Image technical notes (see Table 1.2 on page 2)	Select **Help → Tech Notes** on the menu bar. **Note:** Please see the In Vivo University download page for the most recent collection of technical notes.

Table 1.2 lists the tech notes that are available under the Help menu. There are three types of tech notes:

- Tech Notes – Quick guides for tasks using Living Image software tools.
- Biology Tech Notes – Protocols and procedures related to animal subjects.
- Concept Tech Notes – Background information on *in vivo* imaging topics.

Table 1.2 Technical Notes

Technical Notes
Adaptive Fluorescence Background Subtraction
Auto-Exposure
Subtracting Background ROI from a Sequence
Determine Saturation

Table 1.2 Technical Notes (continued)

Bioluminescence Tomography (DLIT) ▪ Setup and Sequence Acquisition ▪ Topography ▪ Source Reconstruction and Analysis
Drawing ROIs
Fluorescence Tomography (FLIT) ▪ Setup and Sequence Acquisition ▪ Topography ▪ Source Reconstruction and Analysis
High Resolution Images
Working With Image Math
Working With Image Overlay – 2D
Working With Image Overlay – 3D
Working With Imaging Wizard
Loading Groups of Images
Sending Large Files for Analysis
Spectral Unmixing
Subject ROIs
Transillumination ▪ Transillumination Fluorescence ▪ Transillumination – Raster Scan ▪ Transillumination – Normalized
Well Plate Quantification
Concept Technical Notes
Luminescent Background Sources and Corrections
Image Display and Measurement
Detection Sensitivity
Fluorescent Imaging
DLIT and FLIT Reconstruction of Sources
Planar Spectral Imaging
IVIS® Syringe Injection System

1.4 Contact Information

PerkinElmer Health Sciences
940 Winter Street
Waltham, Massachusetts 02451 USA
www.PerkinElmer.com

Technical Support
Telephone: +1 (800) 762-4000 (US) or +1 (203) 425-4602
Fax: +1 (203) 944-4904
E-mail: global.techsupport@perkinelmer.com

Sales: CustomerCareUS@perkinelmer.com

2 Imaging Overview

2.1 Example Imaging Workflow

Table 2.1 Example Imaging Workflow

Workflow Step	For More Details:

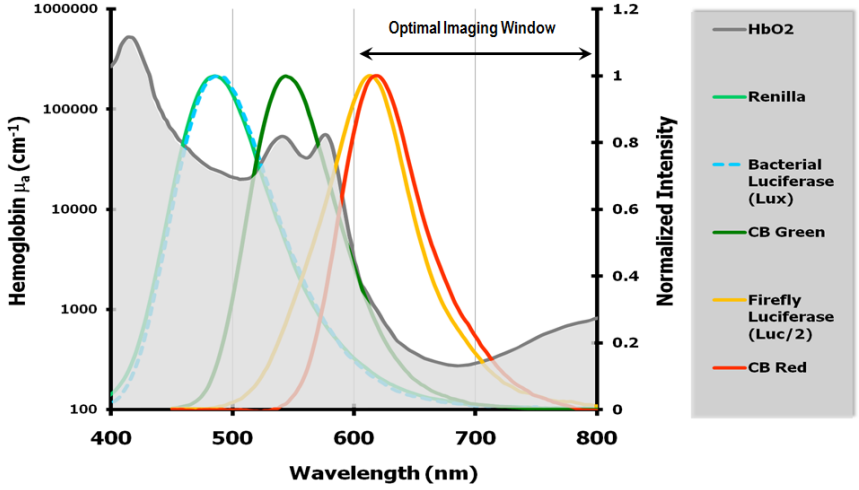
1. Plan the experiment.
▪ Best results are obtained using nude mice. Subjects with black or dark-colored fur and skin are not optimal and may give poor results.
▪ Determine the number of animals required:
  ▪ Always include control animals and replicates (for example, No disease + Probe, Disease + No Probe).
  ▪ Experimental animals (Disease + Probe and replicates).
▪ It may be necessary to change to low fluorescence mouse chow two weeks before the imaging study. Regular mouse chow contains chlorophyll which auto-fluoresces around 700 nm and can interfere with fluorophore signal.
▪ Select the type of imaging and probe:
  ▪ Luminescent signal is usually lower than fluorescent signal, but luminescent imaging has higher sensitivity due to low noise (instrument and animal autoluminescence). Optimal luminescence imaging is from 600 – 800 nm.
 	

Table 2.1 Example Imaging Workflow (continued)

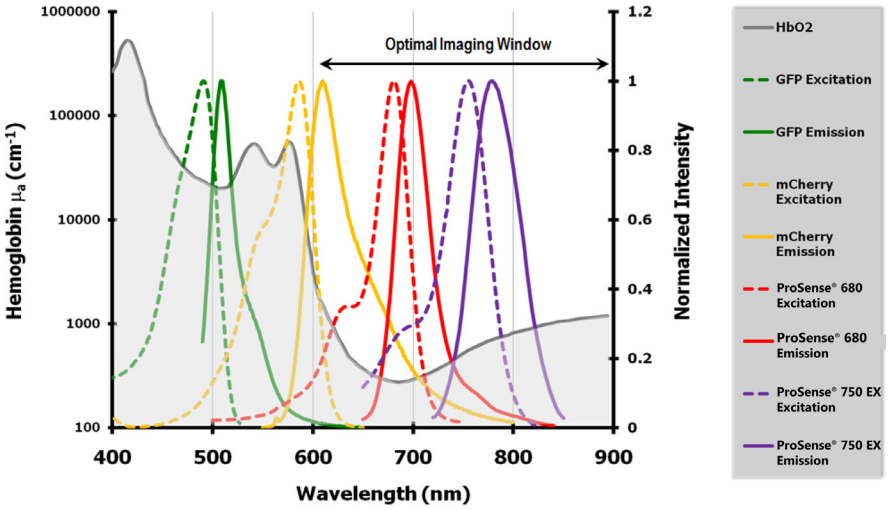
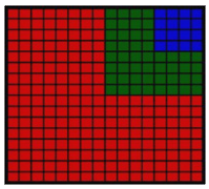
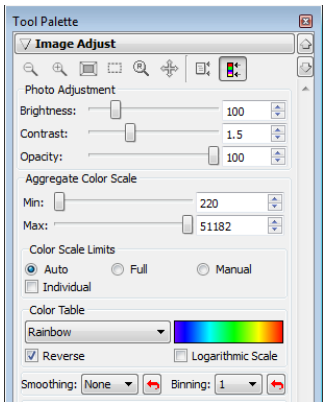
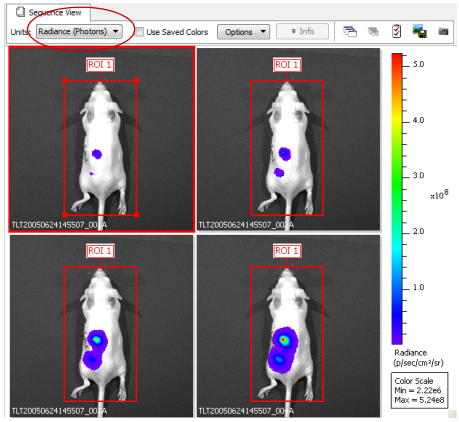
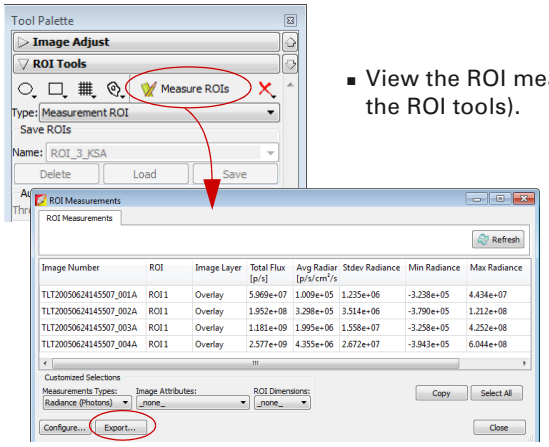
Workflow Step	For More Details:
1. Plan the experiment (continued). Fluorescent signal is usually higher than luminescent signal, but fluorescent imaging has lower sensitivity due to higher noise (instrument background and animal autofluorescence). Optimal fluorescence imaging is from 620 – 900 nm.  	
2. Determine the optimal imaging time post-injection. ▪ Luminescence imaging – Determine a probe kinetic curve for the animal model and cell line. Metabolic rates, which can differ among animal strains, and animal handling procedures affect probe kinetics. Always acquire images during the plateau of the kinetic curve for optimum quantitative results. ▪ Fluorescence imaging – Please see the Technical Data Sheet for the fluorescent imaging agent.	See the technical note *Kinetic Analysis of Bioluminescent Sources* for a protocol to determine a luciferin kinetic curve.
3. Prepare and image the subjects. ▪ If using white or light-colored furred mice, comb the fur before imaging to eliminate any "fluffy" areas that can alter the light emission pattern. It may be necessary to shave the animals or apply a depilatory. ▪ Acquire an image using autoexposure within the optimal time window. If necessary, manually adjust camera settings in the Control Panel (exposure time, binning, F/Stop) to obtain a signal between 600 and 60,000 counts. Signal within this range is above noise, but below saturation. ▪ Exposure time – Shorter exposure times increase throughput, longer exposure times increase signal intensity. If manually setting exposure, the time should be greater than 0.5 seconds and less than 5 minutes for luminescence imaging. ▪ Binning – Applies digital pixel binning to group pixels into one larger "super pixel".  ▪ Small binning (4 x 4 pixels/super pixel) – Lower sensitivity, higher resolution ▪ Medium binning (8x8 pixels/super pixel) ▪ Large binning (16x16 pixels/super pixel) – Higher sensitivity, lower resolution. ▪ F/Stop – Controls the amount of light the CCD detector receives. Changing the F-Stop, for example from F/1 to F/2, decreases counts by a factor of four. F1 – The lens aperture is wide open for maximum light collection (the default for luminescent imaging). F/8 – The smallest aperture opening. This setting provides the best resolution (default for photograph).	See imaging protocols for PerkinElmer *in vivo* imaging reagents such as ProSense® 680. Also see: Page 8 for an overview of image acquisition. Chapter 4, *Image Acquisition* on page 25. Appendix A, *IVIS Acquisition Control Panel* on page 253.

Table 2.1 Example Imaging Workflow (continued)

Workflow Step	For More Details:
4. Select images for viewing. ▪ Load the images as a group (select the images in the Living Image Browser and click Load as Group).	See page 57 for more about the Living Image Browser.
 ▪ If necessary, adjust the photo brightness, contrast, or opacity using the Image Adjust tools. ▪ Apply the same color scale to all images: ▪ Uncheck the "Individual" option. ▪ Adjust the color scale Min and Max. The changes are simultaneously applied to all images.	See page 66 for information on the Image Adjust tools.
5. Measure signals and analyze the data.  ▪ Choose the appropriate units: ▪ "Radiance (Photons)" for luminescence ▪ "Radiant Efficiency" or "NTF Efficiency" for fluorescence. Radiance, Radiance Efficiency, and NTF Efficiency are calibrated measurements (not dependent on camera settings) that enable quantitative comparison of signals across images. "Counts" is an uncalibrated measurement (dependent on camera settings) and cannot be used to compare signals in different images.	See *Concept Tech Note 2 – Image Display and Measurement* for more information on measurement units (select **Help → Tech Notes)**.
▪ Place ROIs on the images in sequence view. ▪ To make changes to related ROIs in all images while in sequence view, press and hold the Ctrl key while adjusting ROI size or position in an image. This ensures that the size and position of a particular ROI are the same in all of the images. ▪ If ROIs in an individual image need adjustment, for example to account for different animal positions in the images, adjust the ROI without using the Ctrl key.	See page 102 for instructions on measuring signal in optical images.
 ▪ View the ROI measurements (click Measure ROIs in the ROI tools). ▪ Click **Export** to save the ROI measurement data (.txt or .csv) for further analysis in a spreadsheet application.	See Table 6.8 on page 118 for information on ROI measurements.

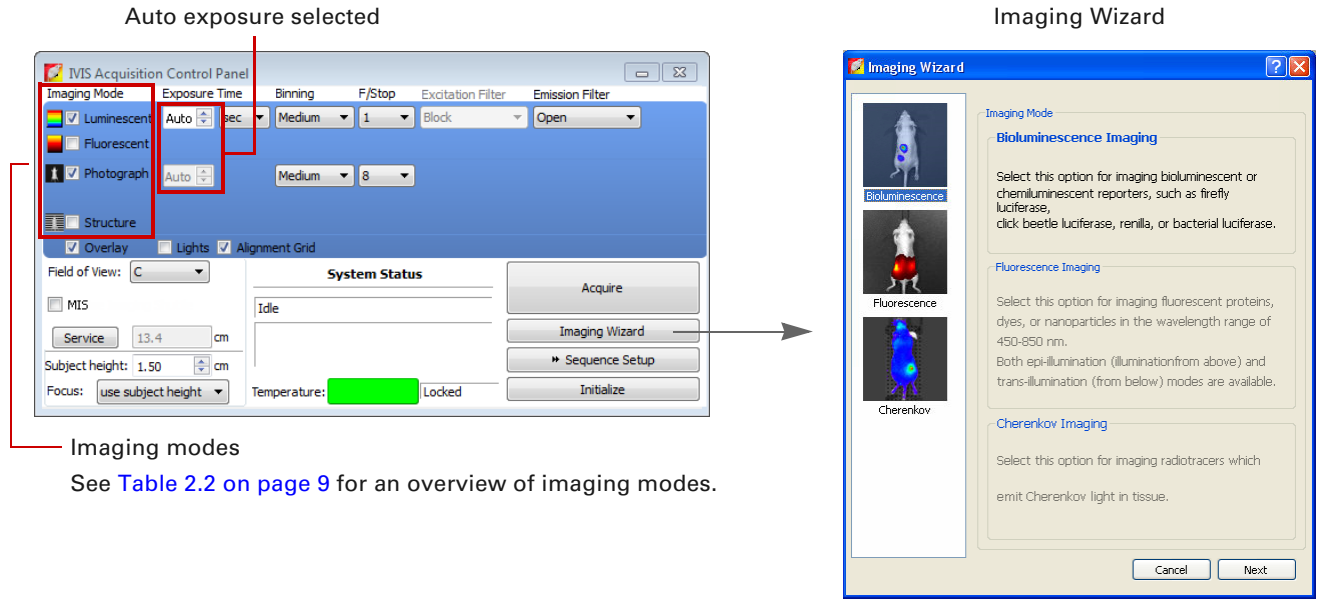
2.2 Overview of Image Acquisition

Control Panel

The control panel provides the image acquisition functions (Figure 2.1). See Appendix A on page 253 for details on the imaging parameters in the control panel.

NOTE: The control panel is only available on the PC workstation that controls the IVIS Imaging System. The items available in the control panel depend on the selected imaging mode (luminescent or fluorescent) and acquisition mode (Image Setup or Sequence Setup).

Figure 2.1 IVIS Acquisition Control Panel and Imaging Wizard



The auto exposure setting is useful in situations where the signal strength is unknown or varies widely, for example during a time course study. If Auto exposure is chosen (Figure 2.1), the system acquires an image at maximum sensitivity, then calculates the required settings to achieve, as closely as possible, an image with a user-specified target max count. If the resulting image has too little signal or saturated pixels, the software adjusts the parameters and takes another image.

In most cases, the default auto exposure settings provide a good luminescent or fluorescent image. However, you can modify the auto exposure preferences to meet your needs. See page 276 for more details.

Imaging Wizard

The Imaging Wizard provides a convenient option for image or sequence setup (see Figure 4.27 on page 44). The wizard guides you through a series of steps, prompting you for the information that the software needs to set up acquisition in the control panel. Table 4.4 on page 45 shows the types of images or sequences that the Imaging Wizard can set up.

Imaging Modes on IVIS Spectrum

Optical imaging detects photons in the visible range of the electromagnetic spectrum. Table 2.2 briefly explains the types of optical images that can be acquired on the IVIS Spectrum.

Table 2.2 Imaging Modes – IVIS SpectrumBL

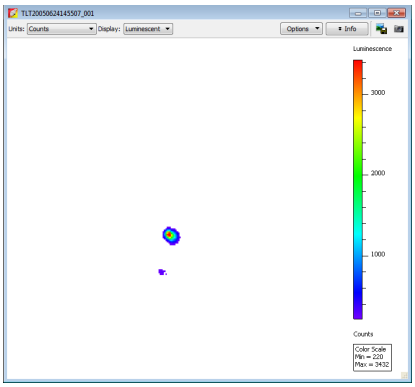
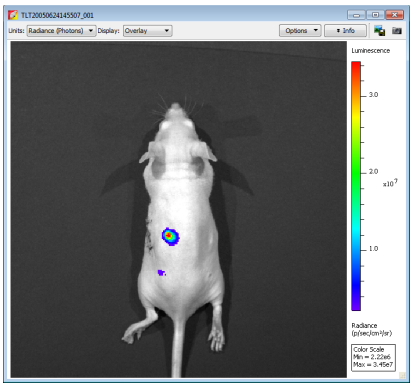
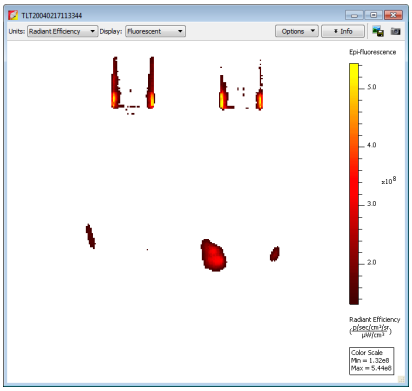
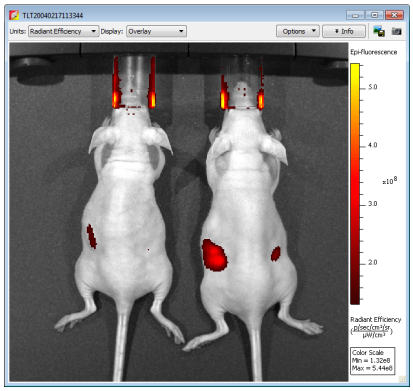
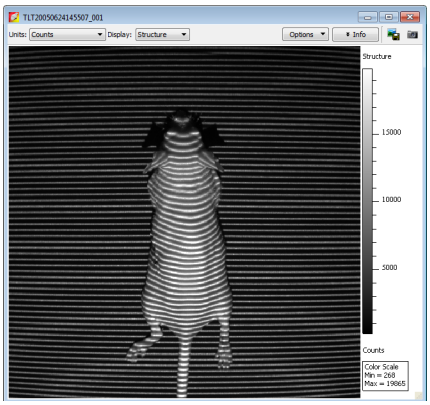
Imaging Mode	Description and Example	
 – Luminescent Luminescent optical imaging detects photons in the visible range of the electromagnetic spectrum	 Luminescent image – A longer exposure of the subject taken in darkness to capture low level luminescence emission from the surface of the subject. The optical luminescent image data is displayed in pseudocolor that represents intensity.	 Overlay: Luminescent image on photograph
 – Fluorescent Fluorescent optical imaging detects photons in the visible range of the electromagnetic spectrum	 Fluorescent image – An exposure of the subject illuminated by filtered light. The light source is located above the imaging stage (epi-illumination). The target fluorophore emission is captured and focused on the CCD camera. The optical fluorescent image data can be displayed in units of counts or photons (absolute, calibrated), or in terms of efficiency (calibrated, normalized). **Note:** See the concept tech note *Image Display and Measurement* for more on quantifying image data (select **Help → Tech Notes** on the menu bar).	 Overlay: Fluorescent image on photograph

Table 2.2 Imaging Modes – IVIS SpectrumBL (continued)

Imaging Mode	Description and Example
– Photograph	 A short exposure of the subject illuminated by the lights located in the ceiling of the imaging chamber. The photographic image is displayed as a grayscale image.
– Structure	 An image of parallel laser lines scanned across the subject which is used to reconstruct the surface topography of the subject. A surface topography is an input to the Diffuse Luminescence Imaging Tomography (DLIT™) algorithm that computes the 3D location and brightness of luminescent sources.

2.3 Overview of Living Image Tools and Functions

Living Image tools are organized in the Tool Palette or under "Tools" in the menu bar (Figure 2.2). Some tools are for use with a single image, others require an image sequence.

The Tool Palette can be docked in the main window (click the Tool Palette title bar, then drag and drop it at either side of the main window (Figure 2.2). Docking can also be set in the general preferences (see Table E.1 on page 273).

Figure 2.2 Living Image Tools are Located in the Menu Bar and Tool Palette

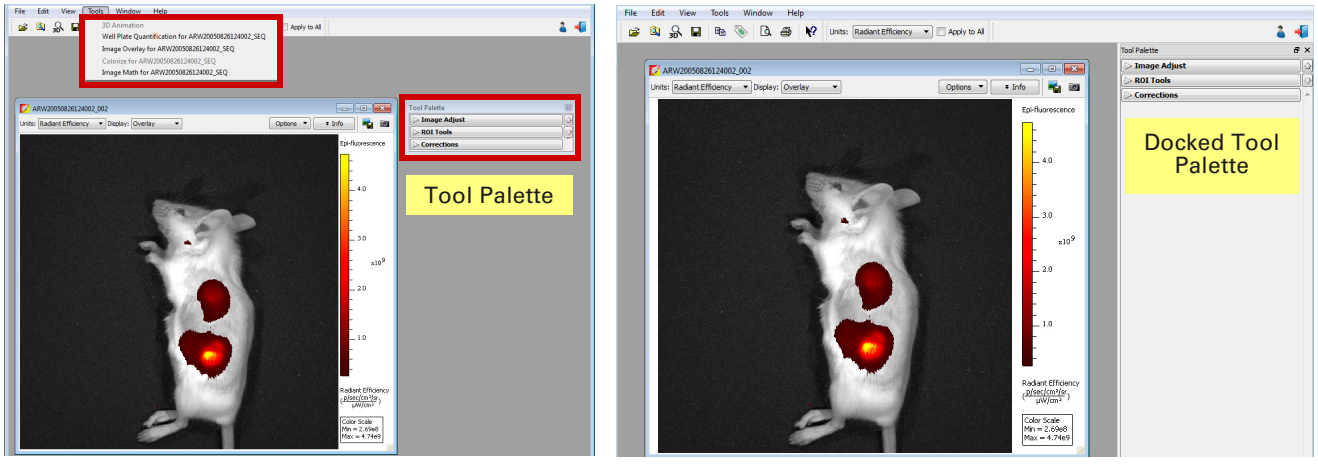


Table 2.3 provides an overview of the tools available for data acquired on the IVIS Spectrum. If analyzing data acquired on a different type of IVIS instrument, say for example the IVIS Spectrum CT, please see the Living Image Software Manual specific for that imaging system.

NOTE: The tools available in the Tool Palette or menu bar depend on the active image data.

Table 2.3 Living Image Tools Available for Data Acquired on the IVIS Spectrum

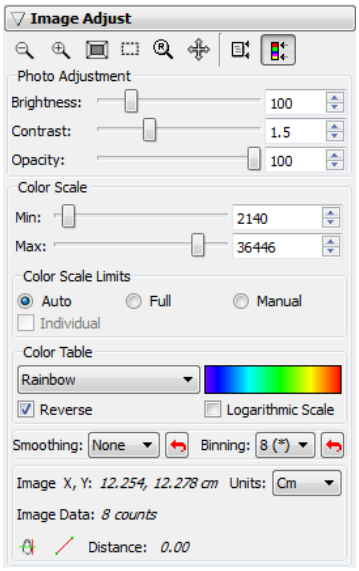
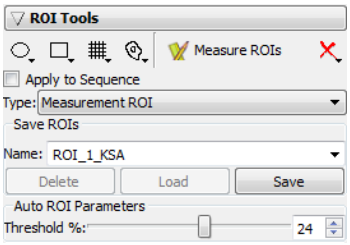
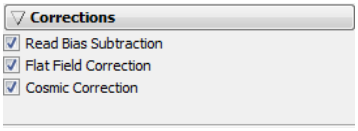
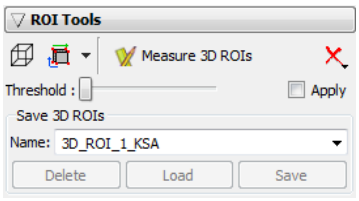
Living Image Tools and Functions	See Page
	66
Image Adjust ▪ Tools for image display (zoom, crop, or pan). ▪ Adjust photo display – Tune the photograph brightness, contrast, or opacity. ▪ Manage the color table for image display. ▪ Apply smoothing or binning to an image. ▪ View optical image data (counts or radiance) at an X,Y location. ▪ Measure distance in an image. ▪ View a line plot of pixel intensities.	
	100
ROI Tools for 2D Image Data Specify a region of interest (ROI) in an optical image and measure the signal intensity within the ROI.	
	258
Corrections (2D Image Data) Corrections for raw data.	
	174
3D ROI Tools (3D Reconstructions or 3D Volumetric Data) Specify a 3-dimensional region of interest (3D ROI). Measure within the 3D ROI: ▪ Signal intensity of source voxels. ▪ Tissue density in a CT scan or 3D DICOM data.	

Table 2.3 Living Image Tools Available for Data Acquired on the IVIS Spectrum (continued)

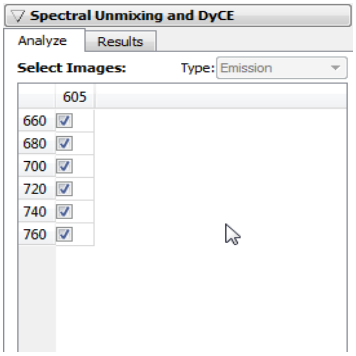
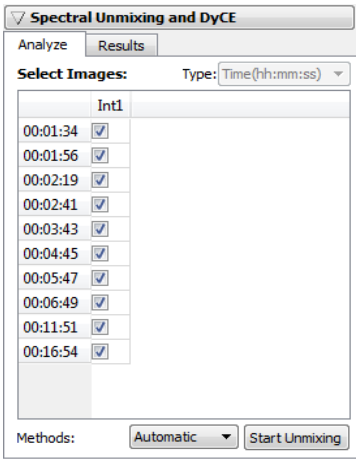
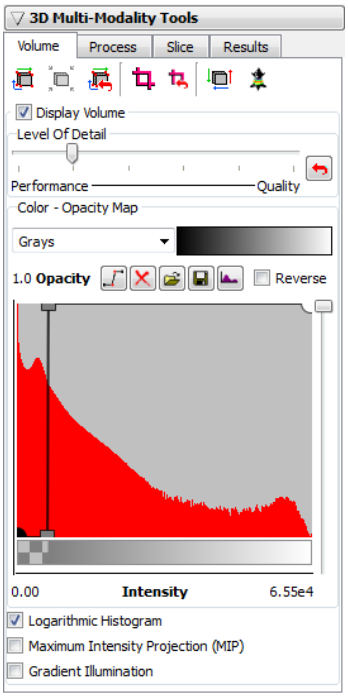
Living Image Tools and Functions	See Page
	**Spectral Unmixing** Use spectral unmixing to: ▪ Extract the signal of one or more fluorophores from the tissue autofluorescence. ▪ Analyze luminescent or fluorescent images when more than one reporter is used in the same animal model.	206
	**DyCE (Dynamic Contrast Enhancement)** Use DyCE to: ▪ Determine real-time pharmacokinetic (spatio-temporal biodistribution) of a probe or dye signal. ▪ Extract “temporal spectra” (signal intensity as a function of time) from particular anatomical regions. **Note:** DyCE acquisition and analysis tools require a separate license.	233
	**3D Multi-Modality Tools** **Note:** Spectrum Imaging System does not acquire volumetric data. However Living Image 3D Multi-Modality tools can analyze volumetric data (CT, PET, MRI) acquired on a different imaging system. The 3D Multi-Modality tools require a separate license. **Volume Tools** ▪ Set color and opacity values for different intensity ranges of a CT volume so that the color-opacity map shows the volume regions of interest (opaque in the map) and hides unimportant regions. ▪ Co-register 3D reconstructions of luminescent or fluorescent sources (biological information) with a CT volume to provide anatomical context for interpreting biological (functional) information. **Process Tools** – Apply smoothing to a CT volume. **Slice Tools** ▪ Options for rendering slices. ▪ View slice. ▪ Slice viewing options.	181

Table 2.3 Living Image Tools Available for Data Acquired on the IVIS Spectrum (continued)

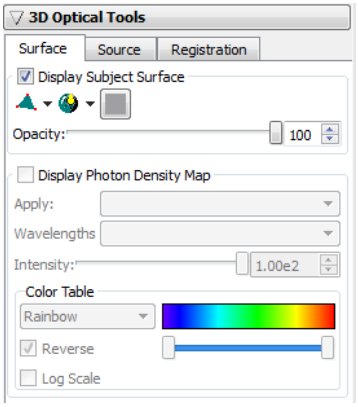
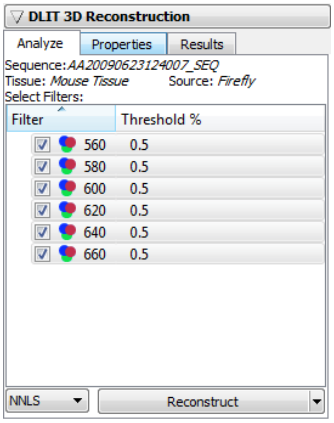
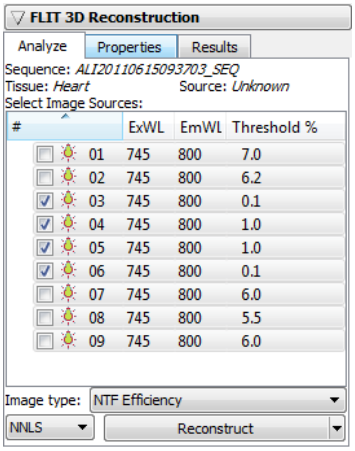
Living Image Tools and Functions	See Page
	**3D Optical Tools** Surface tools – Adjust the appearance of the reconstructed animal surface and the photon density maps. 144 Source tools – Adjust the appearance of reconstructed sources, make source measurements, export voxel measurements. 147 Registration tools – Display organs on the reconstructed surface, adjust the location or scale of organs on the surface, import an organ atlas. 157
	**DLIT 3D Reconstruction** 124 *Diffuse light tomography* (DLIT) analysis provides a complete 3D reconstruction of the luminescent source distribution within the subject. The 3D reconstruction is presented as volume elements called *voxels*. If a luminescent calibration database is available, the number of cells per source can be determined in addition to source intensity (photons/ sec).
	**FLIT 3D Reconstruction** 130 *Fluorescent imaging tomography* (FLIT) analysis provides a complete 3D reconstruction of the fluorescent source distribution within the subject. The 3D reconstruction is presented as volume elements called *voxels*. If a fluorescent calibration database is available, the number of fluorophore molecules or cells per source can be determined in addition to the total fluorescence yield.

Table 2.3 Living Image Tools Available for Data Acquired on the IVIS Spectrum (continued)

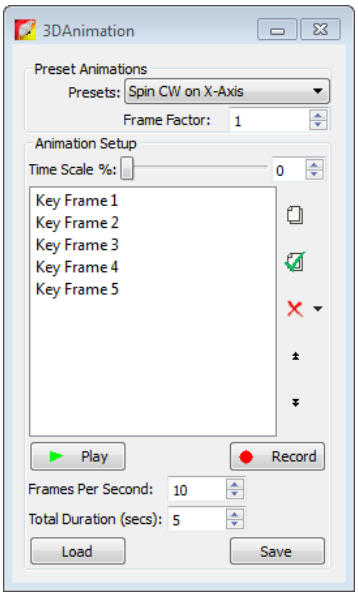
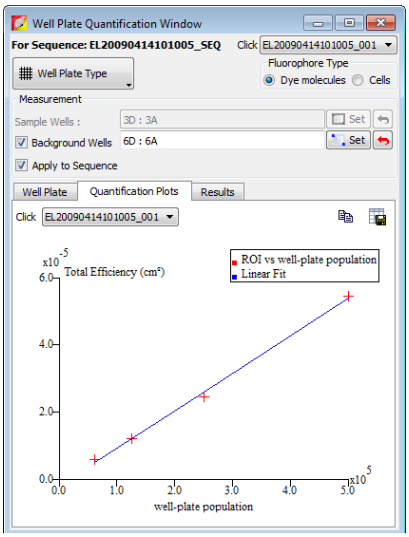
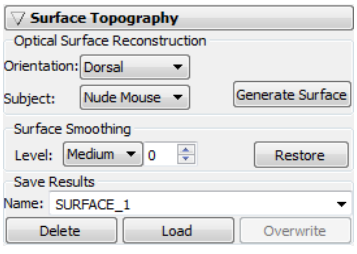
Living Image Tools and Functions	See Page
	**3D Animation Tools** 162 Select **Tools → 3D Animation** on the menu bar. Creates an animation from a sequence of 3D views (keyframes). For example, an animation can depict a rotating 3D scene. The animation (series of key frames) can be recorded to a movie file.
	**Well Plate Quantification** 260 Select **Tools → Well Plate Quantification for <sequence name>** on the menu bar. Generate a database of luminescence or fluorescence signal intensities by analyzing images of known serial dilutions of luminescent or fluorescent cells or dye molecules. Use the quantification database to extrapolate the number of cells in a DLIT source or the number of dye molecules or cells in a FLIT source.
	**Surface Topography** 267 Generate 3D reconstruction of the animal surface (*topography*) derived from a structured light image. A surface is a required input for: ▪ DLIT (*diffuse light tomography*) analysis which generates a 3D reconstruction of luminescent sources. ▪ FLIT (*fluorescence imaging tomography*) analysis which generate a 3D reconstruction of fluorescent sources.

Table 2.3 Living Image Tools Available for Data Acquired on the IVIS Spectrum (continued)

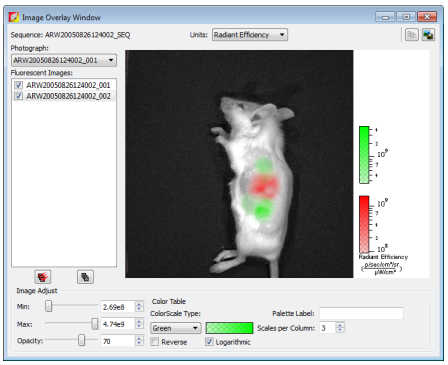
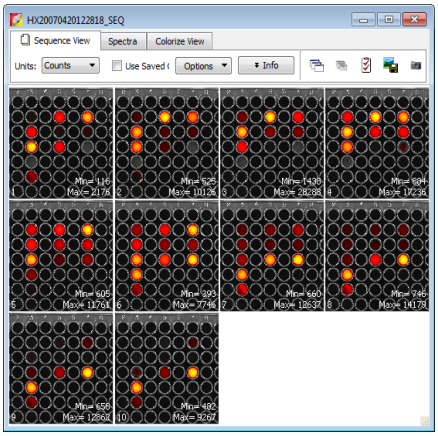
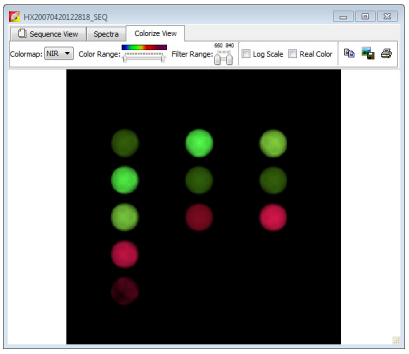
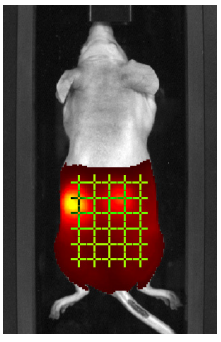
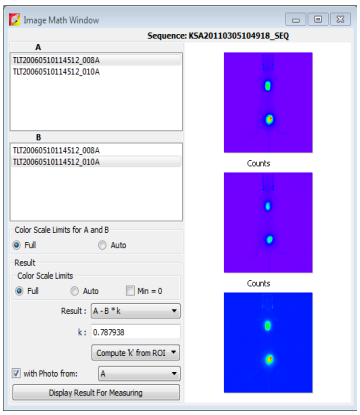
Living Image Tools and Functions	See Page
 **Image Overlay Window** Select **Tools → Image Overlay for <sequence name>** on the menu bar. View multiple fluorescent or luminescent signals in one 2-dimensional image in the Image Overlay window.	80
Colorize View Select **Tools → Colorize for <sequence name>** on the menu bar. The colorize tool renders each luminescence or fluorescence image of a sequence in color, and combines them into a single image. This enables you to see both intensity and spectral information in a single view. The tool provides a useful way to visualize multiple probes or scale probe signals that are not in the visible range.  Images of Quantum dot nanocrystals (700 or 800 nm) were acquired using different combinations of excitation and emission filters.  Colorize view of the combined images	83
 **Transillumination Overview** Select **Tools → Transillumination Overview for <sequence name>** on the menu bar. The transillumination overview tool combines the images of a FLIT sequence (a fluorescence sequence acquired in transillumination mode) into a single image. All of the individual fluorescent signals are stacked over one photograph and the intensity is summed. One overview is created per filter pair. If two filter pairs were used during acquisition, then two overview images will be created.	40 (Table 4.3)

Table 2.3 Living Image Tools Available for Data Acquired on the IVIS Spectrum (continued)

Living Image Tools and Functions	See Page
	**Image Math Window** 75 Select **Tools → Image Math for <sequence name>** on the menu bar. Mathematically combine (add, multiply, subtract, or divide) two user-selected images. For example, subtract a blue-shifted background filter image from the primary excitation filter image to remove tissue autofluorescence signal.

3 Getting Started

3.1 Starting Living Image Software

Living Image software on the PC workstation that controls the IVIS Spectrum includes both the acquisition and analysis features. Living Image software on other workstations includes only the analysis features.

See the Installation Guide on the Living Image CD ROM for software installation instructions. Table 3.1 shows the default software installation locations.

NOTE: If analyzing large datasets such as high resolution FLIT datasets or longitudinal studies with many subjects, a 64-bit analysis workstation with 16 GB memory capacity is recommended.-

Table 3.1 Living Image Software Installation Locations

Living Image Software	Operating System	Installation Location
32-bit version	32-bit Windows	C:\Program Files\Caliper Life Sciences\Living Image
	64-bit Windows	C:\Program Files(x86)\Caliper Life Sciences \Living Image
64-bit version	64-bit Windows	C:\Program Files\Caliper Life Sciences\Living Image

NOTE: All components of the IVIS Spectrum should be left on at all times due to the long cooling time required to reach operating (demand) temperature. It is also important to leave the system on to enable automatic overnight electronic background measurements. Periodically rebooting the computer is permissible and does not affect the camera operation.

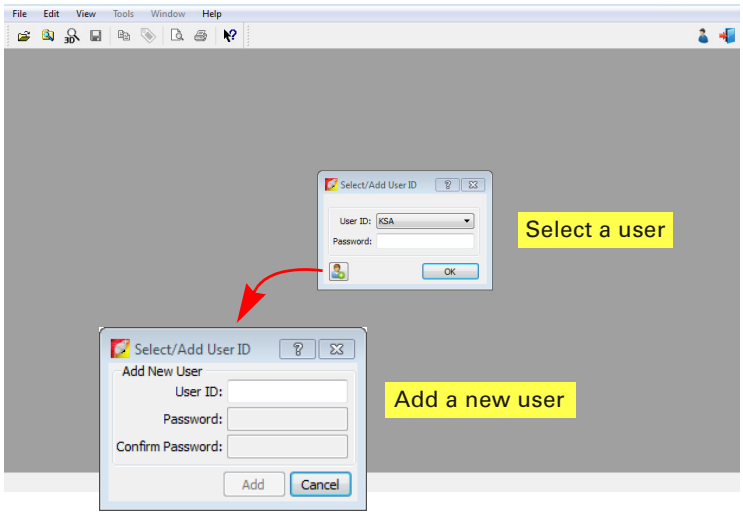
To start the software:

1. **PC Users:** Double-click the Living Image software icon on the desktop. Alternatively, click the Windows Start button and select **All Programs → Caliper Life Sciences → Living Image**.

   Macintosh Users: Double-click the Living Image icon on the desktop or run the software from the application folder.

   The main window appears (Figure 3.1).

Figure 3.1 Living Image Main Window at Startup



2. In the dialog box that appears, select a user ID from the drop-down list. If the user ID is password protected, enter the password and click **OK**.

   Alternatively, create a new user ID:

   a. In the Select/Add User ID box, click the button.

   b. Enter a user ID.

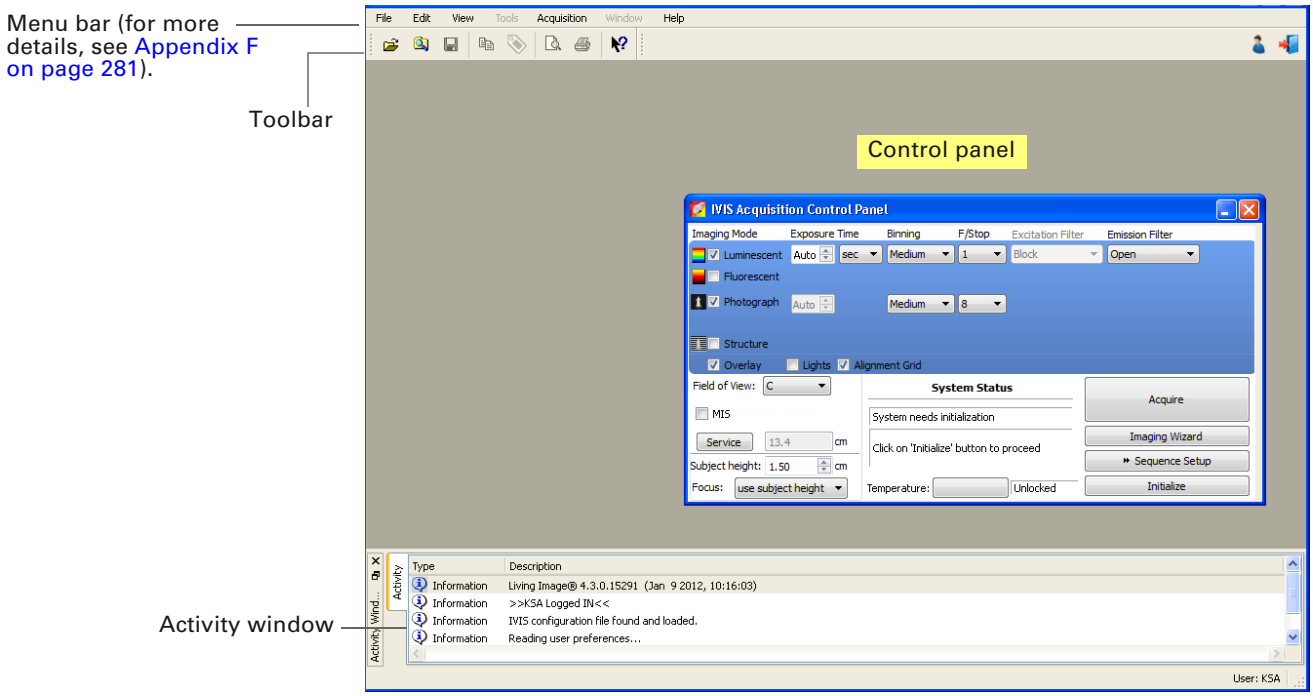
   c. Enter and confirm a password. This is optional.

   d. Click **Add** and **OK**.

   The control panel appears if the workstation controls the IVIS Spectrum (Figure 3.2). For more details on the control panel, see Appendix A on page 253.

NOTE: Living Image software has optional password protection for user accounts. See page 22 for more details.

Figure 3.2 Living Image Main Window and IVIS Acquisition Control Panel



NOTE: Living Image software on the PC workstation that controls the IVIS Imaging System includes both the acquisition and analysis features. Living Image software on other workstations includes only the analysis features. Macintosh users have access to the analysis features only.

3.2 Initializing the Imaging System and Checking Temperature

The IVIS Spectrum must be initialized each time Living Image software is started, or if the power has been cycled to the imaging chamber.

The initialization procedure is started from the control panel (Figure 3.3).

NOTE: The control panel is only available on the PC workstation that controls the IVIS Imaging System.

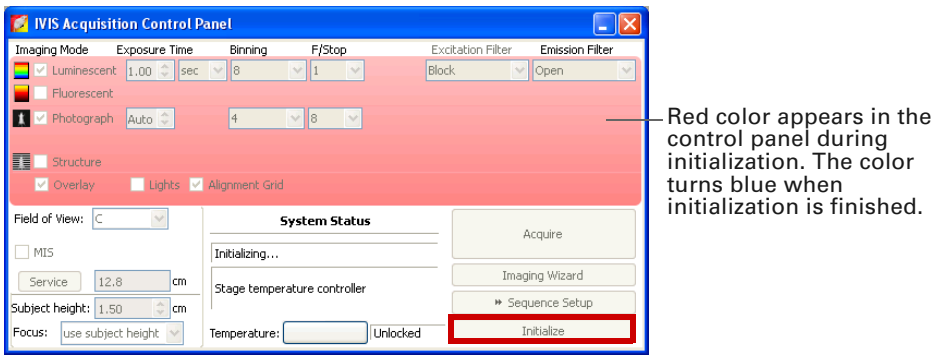
Initialization moves every motor-driven component in the system (for example, imaging stage and lens) to a home position, resets all electronics and controllers, and restores all software variables to the default settings. Initialization may be useful in error situations.

See the *IVIS® Spectrum Hardware Manual* (PN 133577) for further details on instrument operation.

Initializing the IVIS Spectrum

1. Start the Living Image software (double-click the icon on the desktop).
2. Click **Initialize** in the control panel that appears (Figure 3.3).
   After several seconds you will hear the instrument motors move.

Figure 3.3 IVIS® Acquisition Control Panel During Initialization



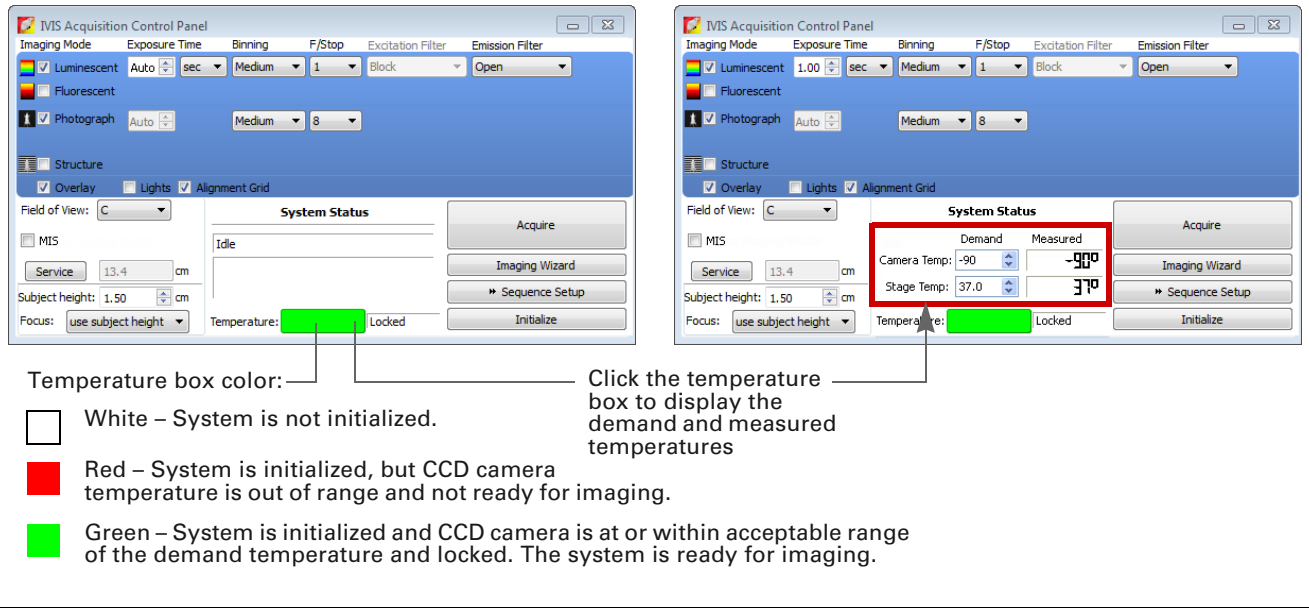
CCD Temperature

The IVIS Acquisition Control Panel indicates the temperature status of the charge coupled device (CCD) camera (see Figure 3.4 for a description of the temperature status colors). Immediately after initialization is completed, the temperature box is usually red and will turn green after several minutes. If this is not the case, contact PerkinElmer Technical Support (see page 4).

The demand temperature for the CCD camera is preset and generally should not be changed. Electronic feedback control maintains the CCD camera temperature to within a few degrees of the demand temperature.

The instrument is ready for imaging after the system is initialized and the operating (demand) temperature of the CCD camera is reached (locked).

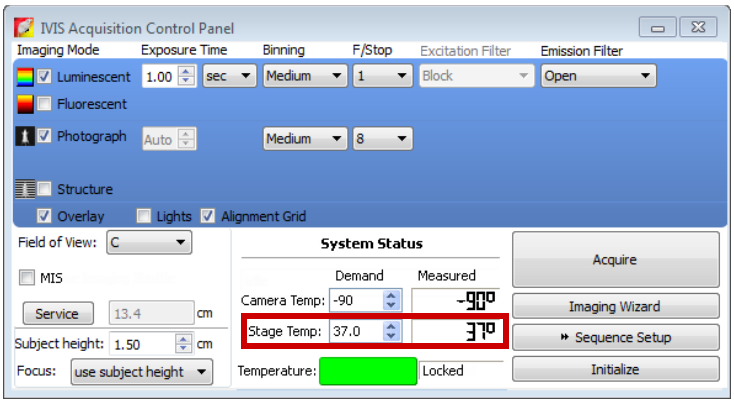
Figure 3.4 Control Panel – Instrument Temperature Status



Stage Temperature

The stage is temperature-controlled to keep subjects warm during imaging. The temperature control is enabled after the instrument is powered on and initialized from the Living Image software. The default temperature is 37 °C and is self-monitoring after the system is initialized. The imaging stage may be set to a temperature from 20 - 40 °C.

Figure 3.5 Set Imaging Stage Temperature in the Control Panel



3.3 Managing User Accounts

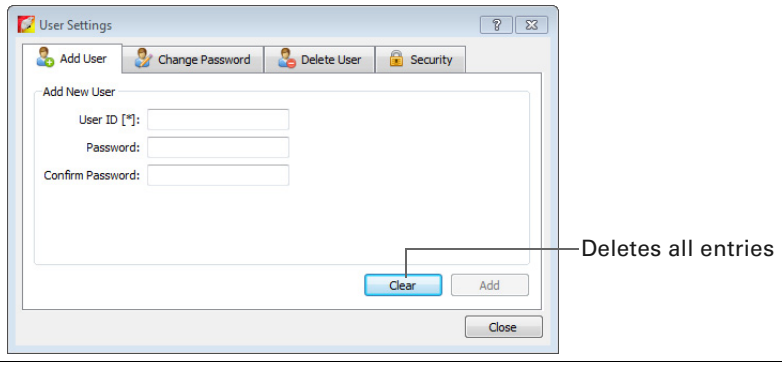
Adding Users

New users can be created in the:

- Main window at startup (see page 18).
- User Settings dialog box (Figure 3.6).

1. Select **Edit → User settings** on the menu bar.
2. Click the Add User tab in the dialog box that appears.

Figure 3.6 User Settings – Add User

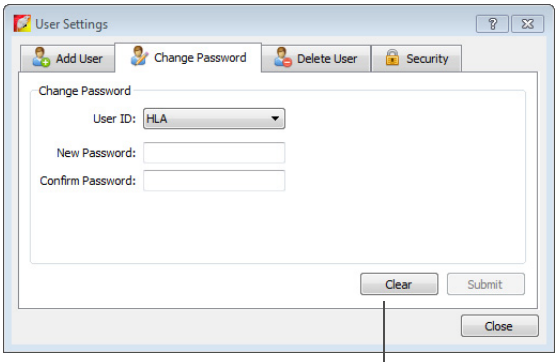


3. Enter a user ID.
4. Optional: enter and confirm a password.
5. Click **Add**.

Changing or Adding Passwords

1. Select **Edit → User settings** on the menu bar.
2. Click the Change Password tab in the dialog box that appears.

Figure 3.7 User Settings – Change Password



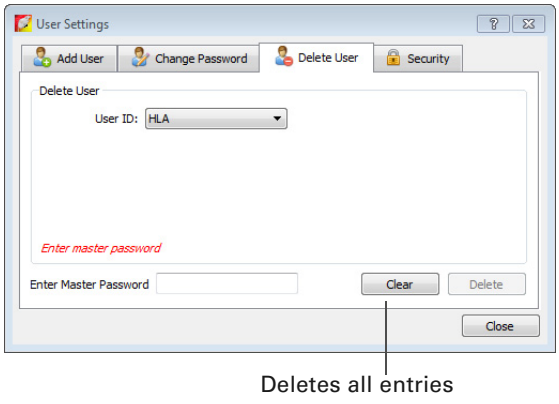
3. Select a User ID, enter and confirm a new password, and click **Submit**.

Deleting Users

NOTE: User accounts can be locked. If this security is applied, a master password is required to delete users from the system. See page 23 for more details on locking user accounts.

1. Select **Edit → User settings** on the menu bar.
2. Click the Delete User tab in the dialog box that appears.

Figure 3.8 User Settings – Delete User
In this example, user accounts are locked.



3. Select a User ID.
4. If the accounts are locked, enter the master password.
5. Click **Delete** and **Close**.

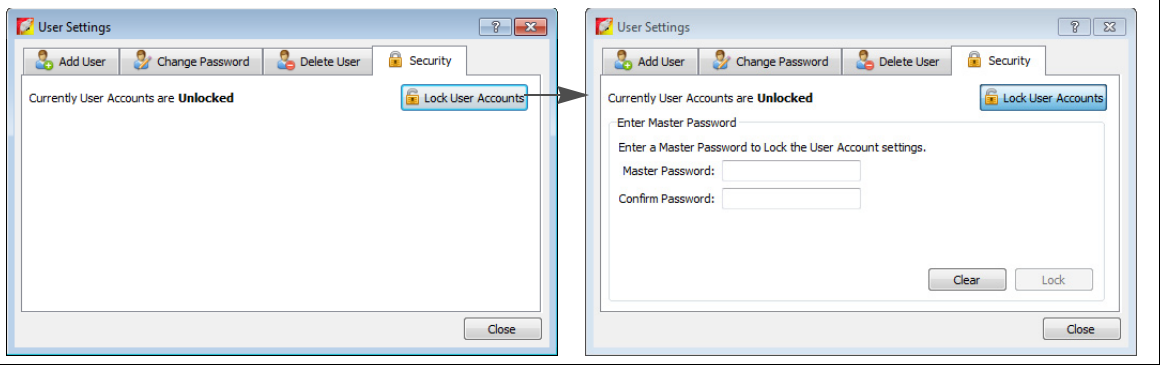
Locking User Accounts

If user accounts are locked, a master password is required to change user passwords, delete users, or unlock user accounts.

To lock user accounts:

1. Select **Edit → User settings** on the menu bar.
2. Click the Security tab in the dialog box that appears.
3. Click **Lock User Accounts**.

Figure 3.9 User Settings – Security

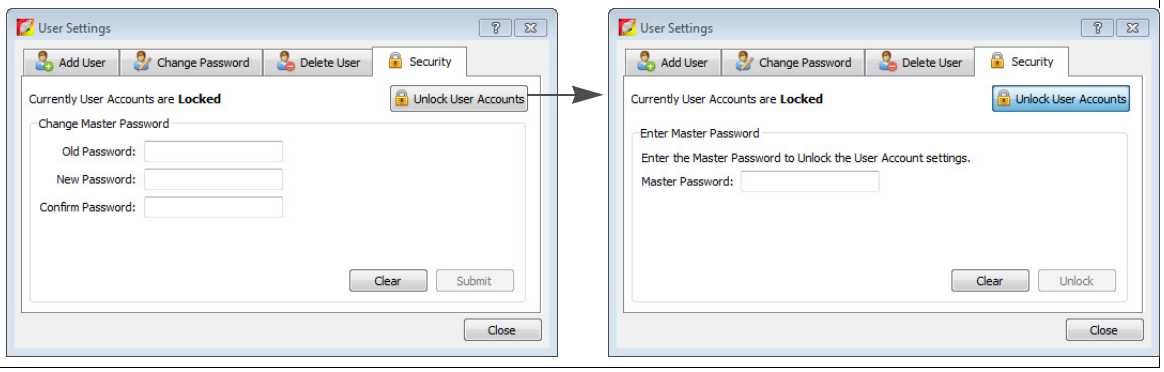


4. Enter and confirm a master password. Click **Close**.
   The master password will be required to delete users.

To unlock user accounts:

1. In the Security tab, click **Unlock User Accounts**.
2. Enter the master password and click **Unlock**. Click **Close**.

Figure 3.10 User Settings – Security



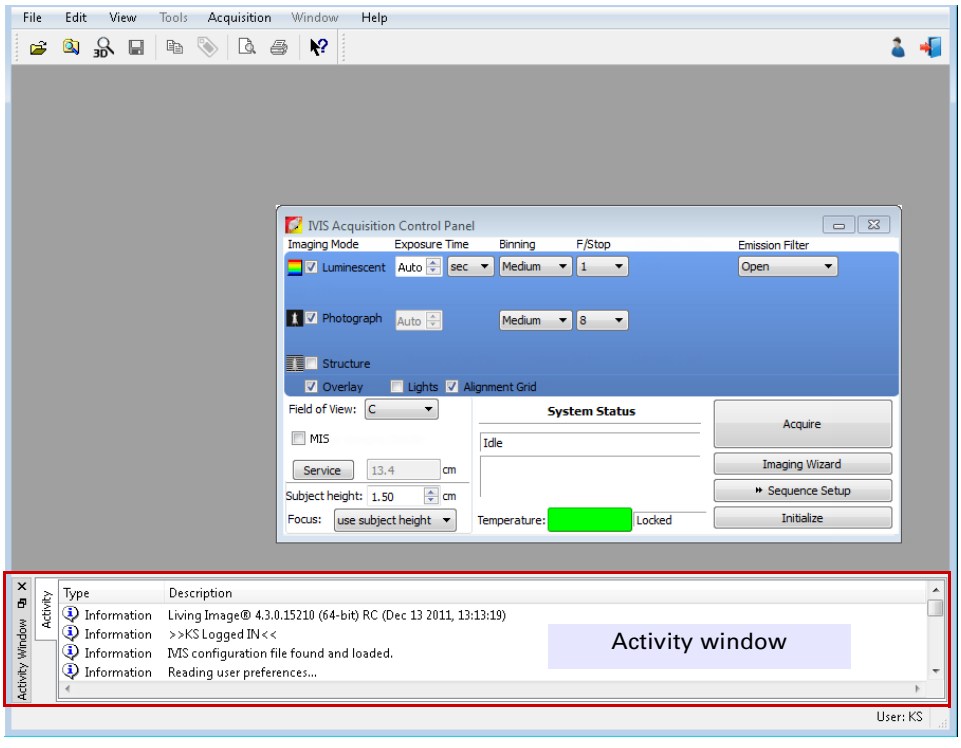
3.4 Tracking System and User Activity

Activity Window

The Activity window shows the imaging system activities (Figure 3.11). The software creates and saves a log of the system activities related to data acquisition.This information may be useful for PerkinElmer field service engineers to understand the imaging system behavior over time or for troubleshooting. The activity log is located at C:\Program Files\Caliper Life Sciences\Living Image.

The software tracks user time on the system (hr/min/sec per user ID) from logon until switching users or system shut down. The software creates a separate record for each month (for example, LI_USAGE_<MONTH>_2015.csv) located at C:\Program Files\Caliper Life Sciences\Living Image\Usage).

Figure 3.11 Activity Window



4 Image Acquisition

4.1 Luminescent Imaging

Luminescent imaging captures signals from luminescent molecular reporters. This section explains how to acquire a single luminescent optical image:

- Quick guide – See below.
- Detailed instructions – See page 26.

See page 44 for information on acquiring a luminescent sequence using the Imaging Wizard.

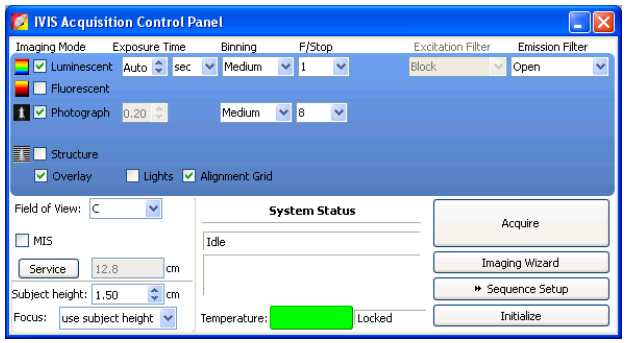
Quick Guide

1. Start Living Image software and initialize the IVIS Spectrum (see page 19 for details).

   NOTE: See the *IVIS Spectrum Hardware Manual* (PN 121450) for more information on the imaging system.

2. Place the anesthetized subjects in the imaging chamber and close the door.
3. In the control panel (Figure 4.1):
   a. Put a check mark next to **Luminescent** and select **Auto** exposure.
   b. Choose **Photograph** (optional, selecting **Photograph** automatically selects **Overlay**).
   c. Choose **Structure** (subject height will be automatically determined).
   d. Select a field of view (FOV) (see Table 4.1 on page 27).
   e. Click **Acquire**.

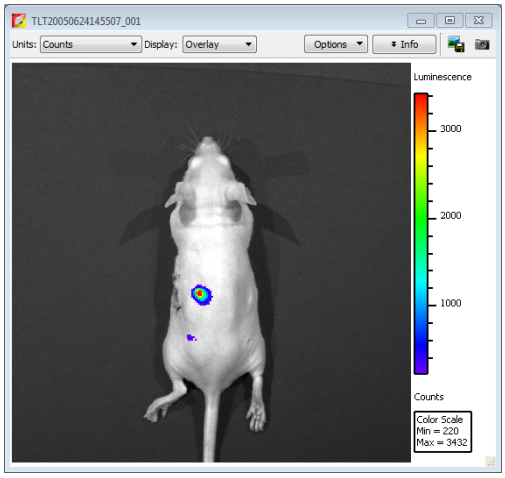
Figure 4.1 Control Panel



4. Select a location for the image data when prompted (optional, but strongly recommended).
   Image data acquired during the session will be automatically saved to this location.
5. Enter experiment and subject information in the dialog box that appears (optional, but strongly recommended).
   An image window and Tool Palette appear when acquisition is finished (Figure 4.2).

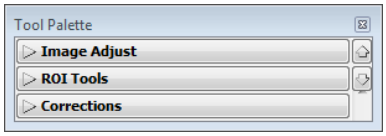
Figure 4.2 Image Window and Tool Palette

Image Window



See Table 4.2 on page 30 for more details on the image window.

Tool Palette



The Tool Palette includes the:

- Image Adjust Tools (page 66)
- ROI Tools (page 100)
- Corrections Tools (page 258)

Acquire a Luminescent Image

This section provides detailed instructions for luminescent imaging.

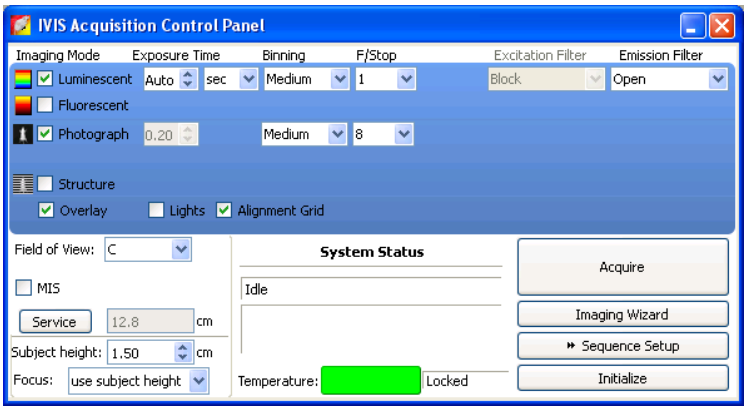
NOTE: The IVIS Spectrum should be initialized and the temperature locked before setting the imaging parameters in the control panel. See page 19 for more details.

1. Put a check mark next to **Luminescent** and select **Auto** exposure (click the ⇕ arrows) in the control panel.
   The software automatically determines the binning and F/Stop settings.

TIP: See the tech note *Auto-Exposure* for helpful information (select **Help → Tech Notes** on the menu bar).

Alternatively, manually set the exposure, binning, and F/Stop. See Appendix A on page 253 for details on these parameters.

Figure 4.3 Control Panel



2. Put a check mark next to **Photograph** (optional).

   Selecting **Photograph** automatically selects Overlay, so that an overlay image (registered photograph and luminescent image) is displayed after acquisition.

3. Select a field of view (FOV, size of the stage area to be imaged, see Table 4.1).

TIP: See the technical note *Detection Sensitivity* for more information about the field of view (select **Help → Tech Notes** on the menu bar).

Table 4.1 Field of View (FOV) Settings – IVIS Spectrum

FOV Setting	FOV (cm)
A[1]	4
B	6.5
C[2]	13
D	22.5 (19.5)[3]
E	22.5 (26)[2]

[1] Position A is not recommended for epi-fluorescent imaging because corrections for non-uniform excitation light pattern are not available.
[2] Position C is the default setting.
[3] Some IVIS Spectrum Imaging Systems may have the FOV in parentheses. FOV 19.5 and 26 were replaced with FOV 22.5.

4. Select a focus option in the control panel (Figure 4.4).

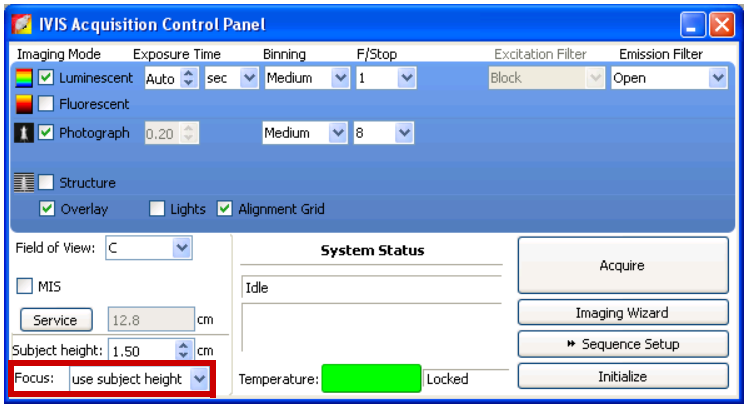
   The focal distance to the camera is set at stage z = 0 for each field of view. To focus at the top of the animal, the stage moves down so that the top of the animal is at z = 0. For example, if the subject height is 1.5 cm, the stage will move down 1.5 cm to set the plane of focus at the top of the subject.

   Do one of the following to set the focus for the area to be imaged:

   - If **Structure** is selected, choose the **use subject height** focus option.

     The subject height will be automatically determined from a structured light image.
   - If **Structure** is not selected, enter the subject height (cm) and choose the **use subject height** focus option.
   - Choose the **manual** focus option and follow the instructions in Appendix A on page 257.

Figure 4.4 Choose a Focus Option in the Control Panel

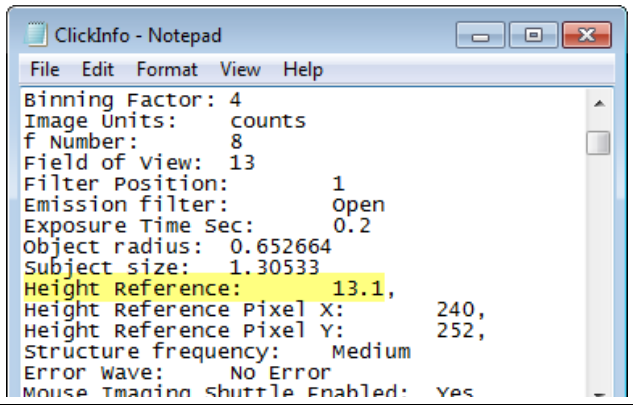


5. If you are using the Mouse Imaging Shuttle (MIS), select the "MIS" option (Figure 4.4). If not using the MIS, confirm that this option is cleared, otherwise the computed subject height will be inaccurate.

   When the MIS option is selected, Living Image software applies a correction factor to the subject height measurement to offset the MIS platform and sets binning at 4, the optimum binning level for computing height from structured light measurements when using the MIS.

NOTE: If image data was acquired without using the MIS, but the MIS option was selected, the subject height will be 2.9 mm less than actual due to the MIS correction factor. To correct this, manually edit the subject height in the ClickInfo.txt file by adding 2.9 mm to the Height Reference measurement (Figure 4.5).

Figure 4.5 ClickInfo Text File



6. If you want to check the subject inside the chamber before acquisition, take a photograph (uncheck **Luminescent**, choose **Photograph**, and click **Acquire)**. Be sure to select **Luminescent** after taking the photograph.
7. Click **Acquire** when you are ready to capture the image.

> **NOTE:** If necessary click Image Setup in the control panel to operate in single image mode. In single image mode, the Sequence Setup button appears in the control panel. Use this button to set up sequence acquisition (see page 44 for more details on sequence setup using the Imaging Wizard).

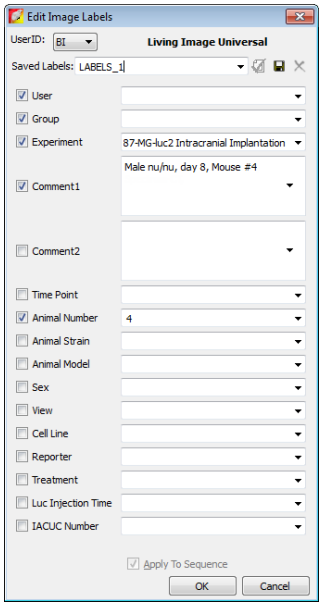
8. Enter information about the image in the dialog box that appears (optional, but strongly recommended) and click **OK**.

   You can enter image label information at any time during or after acquisition. If you do not want to enter image information, click **Cancel**. See page 93 for details on adding information to an image after acquisition.

Figure 4.6 Enter Information About the Image)

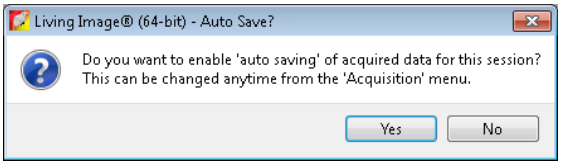
All information entered here will be saved with the image.

However, only the check marked values will appear in the image label (see Figure 4.8 on page 30).



If this is the first image of the session, you are prompted to enable the autosave function (Figure 4.7). When Autosave is enabled, all images acquired during the session are automatically saved to a user-selected location. A different location can be chosen at any time (select **Acquisition → Auto-Save** on the menu bar).

Figure 4.7 Autosave Prompt



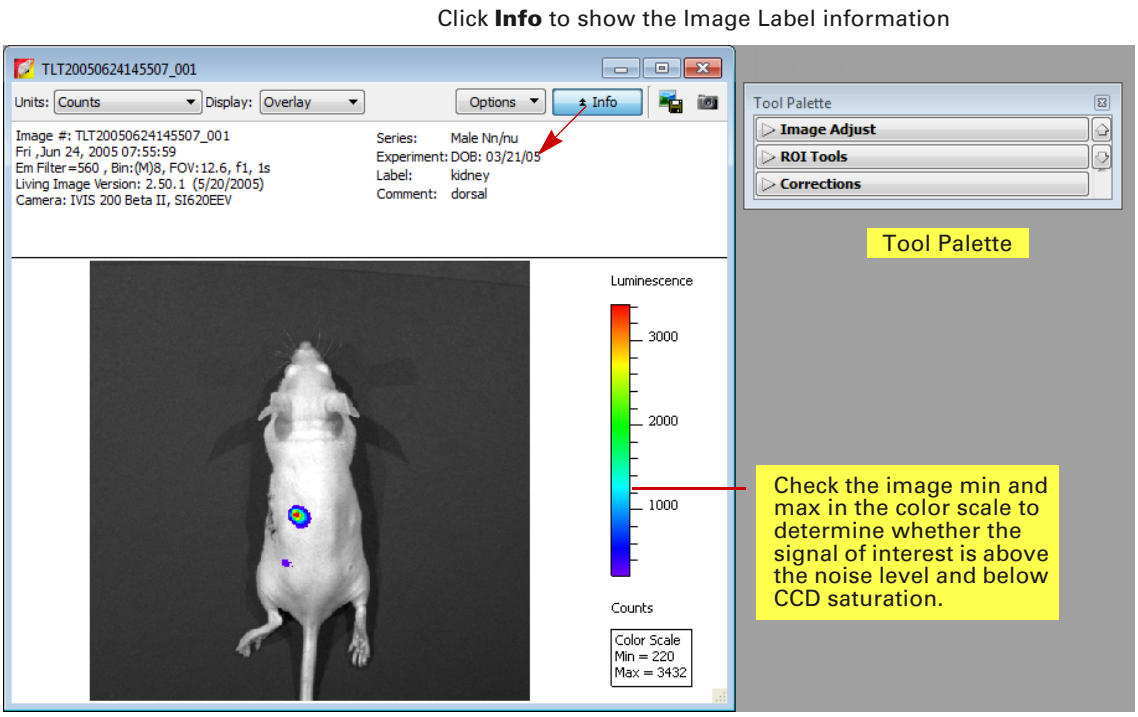
9. Click **Yes** in the prompt to enable autosave, then choose a location in the dialog box that appears. Alternatively, click **No** in the prompt and manually save the image data. See page 56 for details.

   Image acquisition begins and the upper area of the control panel changes to red color.

NOTE: During acquisition, the **Acquire** button in the control panel changes to **Stop**. Click **Stop** to cancel acquisition and reinitialize the imaging system.

The control panel returns to blue color when acquisition is finished and the image window appears (Figure 4.8).

Figure 4.8 Overlay (Luminescent Image on Photograph) in the Image Window

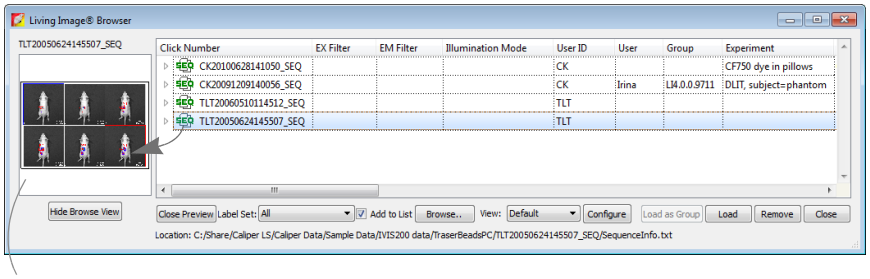


TIP: See the tech note *Determine Saturation* for information on pixel measurements (select **Help → Tech Notes** on the menu bar).

Table 4.2 Image Window

Item	Description
Units	Select the measurement units for the image display from this drop-down list. The available units depend on the type of image data. See the concept tech note *Image Display and Measurement* for more details (select **Help → Tech Notes** on the menu bar).
Display	A list of image types available for display, for example, overlay. For more details on the different types of image displays, see Table 2.2 on page 9.
Info	Click to display or hide the image label. The image label includes information you enter in the Edit Image Labels dialog box (Figure 4.8) and other image information automatically recorded by the software.
	Opens a dialog box that enables you to export the active view as a graphic file.

Table 4.2 Image Window

Item	Description
	Creates a preview picture (*snapshot*) of the image or thumbnails that the Living Image Browser displays when the data are selected in the browser. For more details on the browser, see page 57.  Preview picture of the data selected in the browser (blue row)
Color Scale	Provides a reference for the pixel intensities in a luminescent or fluorescent image. Pixels less than the color scale minimum do not appear in the image. Pixels greater than the color scale maximum are displayed in the maximum color.

4.2 Fluorescent Imaging With Epi-Illumination

Fluorescent imaging captures signals from fluorescent molecular reporters. This section explains how to acquire a single fluorescent optical image with epi-illumination (excitation light source located above the stage):

- Quick guide – See below.
- Detailed instructions – See page 33.

See page 38 for information on fluorescent imaging with transillumination (excitation light source located below the stage). See page 44 for information on acquiring a fluorescent sequence using the Imaging Wizard.

TIP: See the concept tech note *Fluorescent Imaging* for more about fluorescence imaging theory (select **Help → Tech Notes** on the menu bar).

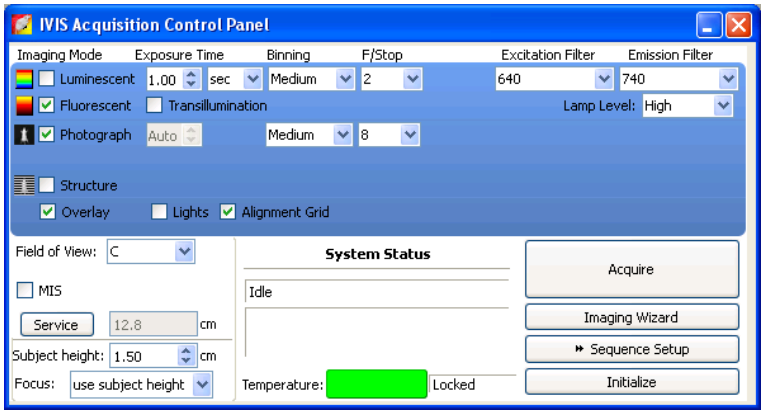
Quick Guide

1. Start Living Image software and initialize the IVIS Spectrum (page 19).

   NOTE: See the *IVIS Spectrum Hardware Manual* (PN 121450) for more information on the instrument

2. Place the anesthetized subjects in the imaging chamber and close the door.
3. In the control panel (Figure 4.9):
   a. Put a check mark next to **Fluorescent** and select **Auto** exposure in the control panel.
   b. Select an excitation and emission filter.
   c. Choose **Photograph** (optional, selecting **Photograph** automatically selects **Overlay**).
   d. Choose **Structure** (subject height will be automatically determined).
   e. Select a field of view (FOV) (see Table 4.1 on page 27).
   f. Click **Acquire**.

Figure 4.9 Control Panel



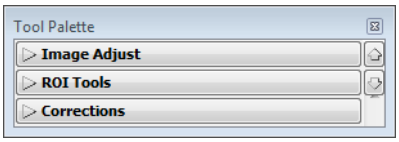
4. When prompted, select a location for the image data (optional, but strongly recommended).
   Image data acquired during the session will be automatically saved to this location.
5. Enter experiment and subject information in the dialog box that appears (optional, but strongly recommended).
   The image window and Tool Palette appear when acquisition is finished (Figure 4.10).

Figure 4.10 Image Window and Tool Palette

Image Window

See Table 4.2 on page 30 for more details on the image window.

Tool Palette



The Tool Palette includes the:

- Image Adjust tools (page 66)
- ROI Tools (page 100)
- Corrections tools (page 258)

Acquire a Fluorescent Image With Epi-Illumination

This section provides detailed instructions for image acquisition.

NOTE: The IVIS Spectrum should be initialized and the temperature locked before setting the imaging parameters in the control panel See page 19 for more details.

1. Put a check mark next to **Fluorescent** and select **Auto** exposure (click the arrows) in the control panel.

   The software automatically determines the binning and F/Stop settings.

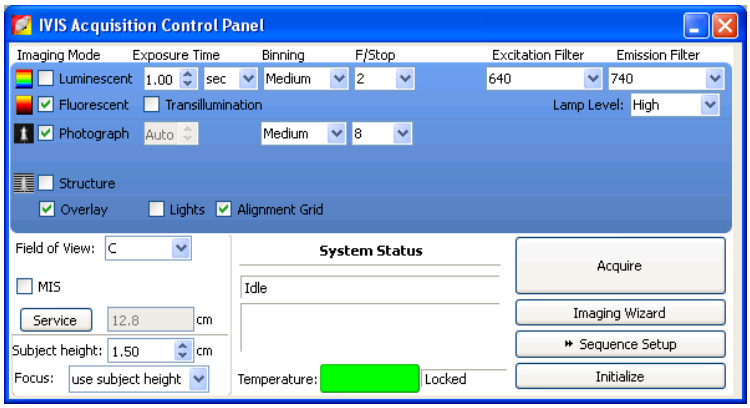
TIP: See the tech note *Auto-Exposure* for helpful information (select **Help → Tech Notes** on the menu bar).

   Alternatively, manually set the exposure, binning, and F/Stop. See Appendix A on page 253 for details on these parameters.

2. Put a check mark next to **Photograph**.

   Selecting **Photograph** automatically selects Overlay, so that an overlay image (registered photograph and luminescent image) is displayed after acquisition.

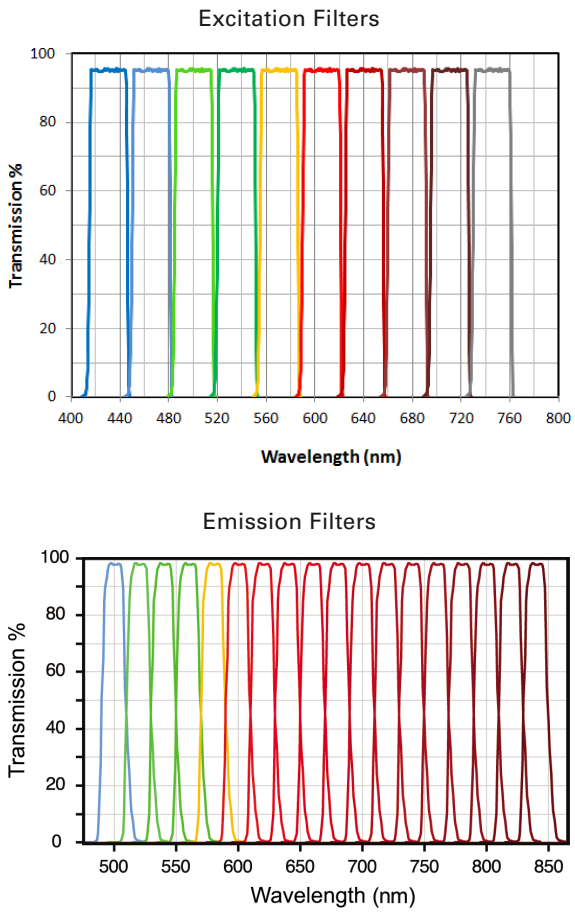
Figure 4.11 Control Panel



3. Select an excitation and emission filter from the drop-down lists.

The instrument has 18 narrow band emission filters (490-850nm, 20nm bandwidth) and 10 narrow band excitation filters (415-760nm, 30nm bandwidth), enabling spectral scanning over the blue to NIR wavelength region (Figure 4.12).

Figure 4.12 IVIS® Spectrum Excitation and Emission Filters



4. Select a field of view (FOV, size of the stage area to be imaged). See Table 4.1 on page 27 for a list of FOV settings.

TIP: See the concept technical note *Detection Sensitivity* for more information about the field of view (select **Help → Tech Notes** on the menu bar).

5. Select a focus option in the control panel (Figure 4.13).

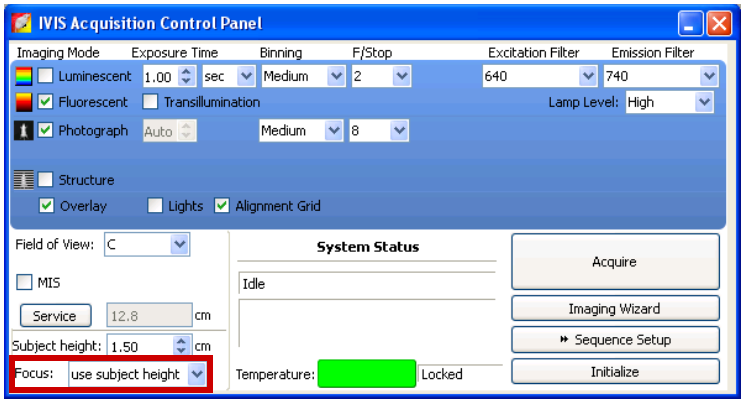
   The focal distance to the camera is set at stage z = 0 for each field of view. To focus at the top of the animal, the stage moves down so that the top of the animal is at z = 0. For example, if the subject height is 1.5 cm, the stage will move down 1.5 cm to set the plane of focus at the top of the subject.

   Do one of the following to set the focus for the area to be imaged:

   - If **Structure** is selected, choose the **use subject height** focus option.

     The subject height will be automatically determined from a structured light image.
   - If **Structure** is not selected, enter the subject height (cm), and choose the **use subject height** focus option.
   - Choose the manual focus option and follow the instructions in Appendix A on page 257.

Figure 4.13 Choose a Focus Option in the Control Panel

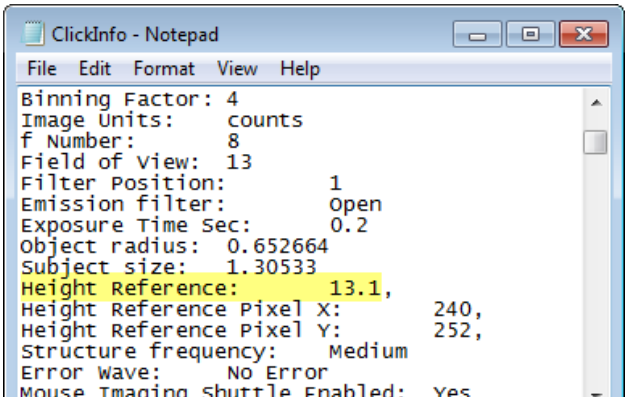


6. If you are using the Mouse Imaging Shuttle (MIS), select the "MIS" option (Figure 4.14). If not using the MIS, confirm that this option is cleared, otherwise the computed subject height will be inaccurate.

   When the MIS option is selected, Living Image software applies a correction factor to the subject height measurement to offset the MIS platform and sets binning at 4, the optimum binning level for computing height from structured light measurements when using the MIS.

NOTE: If image data was acquired without using the MIS, but the MIS option was selected, the subject height will be 2.9 mm less than actual due to the MIS correction factor. To correct this, manually edit the subject height in the ClickInfo.txt file by adding 2.9 mm to the Height Reference measurement (Figure 4.14).

Figure 4.14 ClickInfo Text File



7. If you want to check the subject inside the chamber before acquisition, take a photograph: uncheck **Fluorescent**, choose **Photograph**, and click **Acquire**. Be sure to select **Fluorescent** after taking the photograph.
8. Click **Acquire** when you are ready to capture the image.

> **NOTE:** If necessary click Image Setup in the control panel to operate in single image mode. In single image mode, the Sequence Setup button appears in the control panel. Use this button to set up sequence acquisition (see page page 44 for more details on sequence setup).

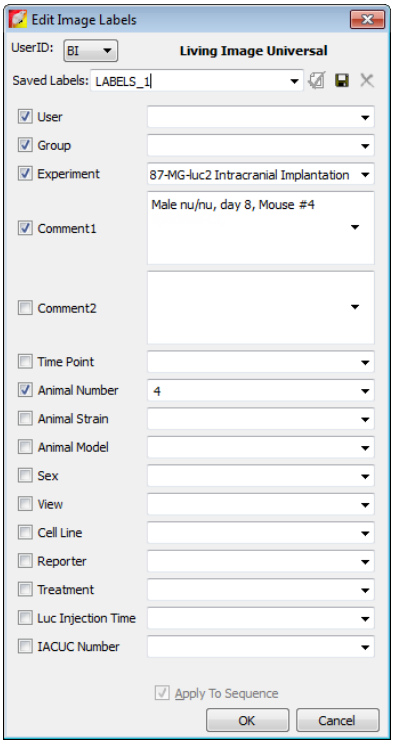
9. Enter information about the image in the Edit Image Labels box that appears (optional, but strongly recommended) and click **OK**.

   You can enter image label information at any time during or after acquisition. If you do not want to enter image information, click **Cancel**. See page 93 for details on adding information to an image after acquisition.

Figure 4.15 Enter Information About the Image

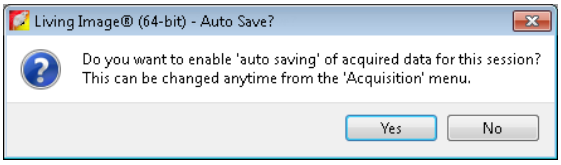
All information entered here will be saved with the image.

However, only the check marked values will appear in the image label (see Figure 4.17 on page 37).



If this is the first image of the session, you are prompted to enable the autosave function (Figure 4.16). When Autosave is enabled, all images acquired during the session are automatically saved to a user-selected location. A different location can be chosen at any time (select **Acquisition → Auto-Save** on the menu bar).

Figure 4.16 Autosave Prompt



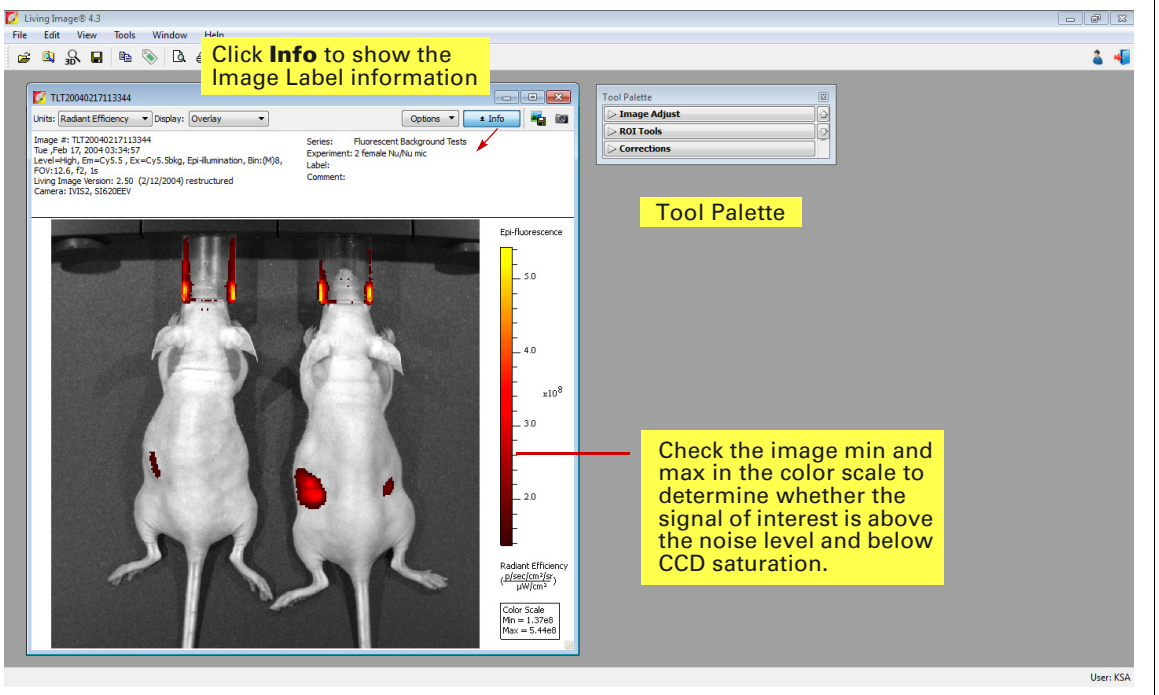
10. Click **Yes** in the prompt to enable autosave, then choose a location in the dialog box that appears. Alternatively, click **No** in the prompt and manually save the image data. See page 56 for details.

Image acquisition begins and the upper area of the control panel changes to red color.

NOTE: During acquisition, the **Acquire** button in the control panel changes to **Stop**. Click **Stop** to cancel acquisition and reinitialize the imaging system.

The control panel returns to blue color when acquisition is finished and the image window appears (Figure 4.17). See Table 4.2 on page 30 for details on the image window.

Figure 4.17 Overlay (Fluorescent Image on Photograph) in the Image Window



TIP: See the tech note *Determine Saturation* for information on pixel measurements (select **Help → Tech Notes** on the menu bar).

4.3 Fluorescent Imaging With Transillumination

Fluorescent imaging captures signals from fluorescent molecular reporters. Transillumination (excitation light source located below the stage) is recommended if the fluorescent source is deep relative to the imaged side of the animal.

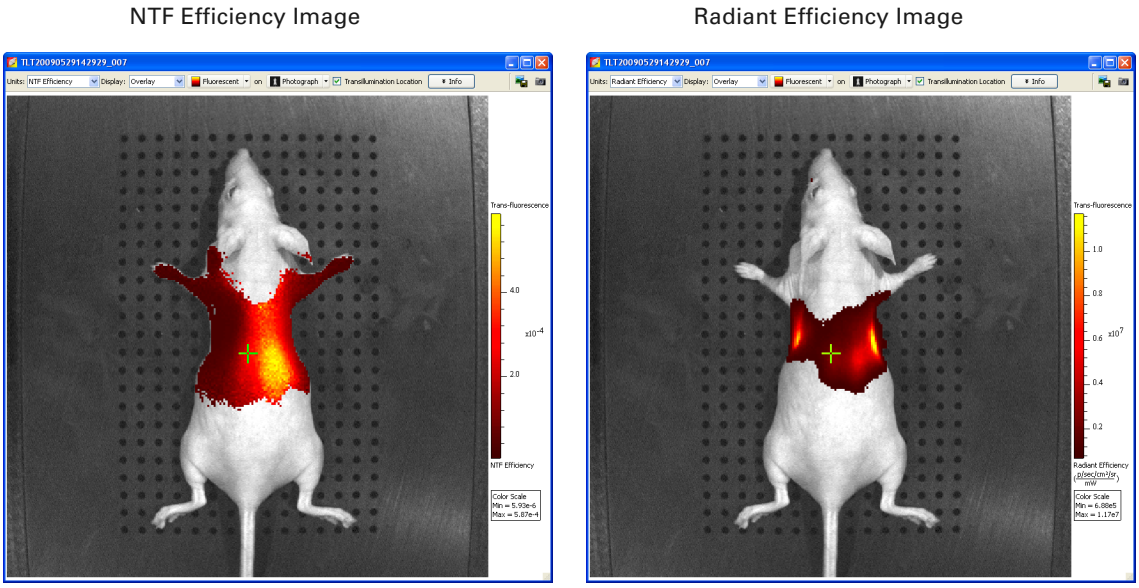
Acquisition with transillumination includes a Normalized Transmission Fluorescence (NTF) Efficiency image in which the fluorescent emission image is normalized by the transmission image measured with the same emission filter and open excitation filter (Figure 4.18).

TIP: See these tech notes for helpful information and quick guides (select **Help → Tech Notes** on the menu bar):

- *Transmission Fluorescence*
- *Transmission Fluorescence – Raster Scan*
- *Transmission Fluorescence – Normalized Transmission Fluorescence*
- *Transmission Fluorescence – Well Plates*

Figure 4.18 Fluorescent Images Acquired with Transillumination

The NTF Efficiency image in this example highlights the presence of fluorescence in the animal, while the Radiant Efficiency image shows signal ambiguous with autofluorescence.



This section explains how to acquire a single fluorescent optical image with transillumination. See page 44 for information on acquiring a fluorescent sequence.

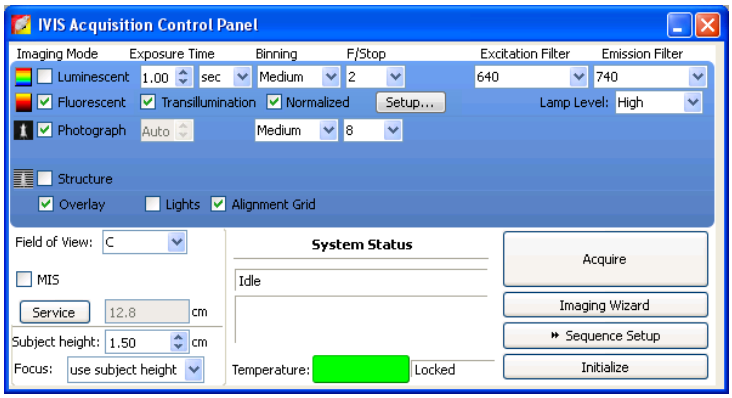
To acquire a fluorescent image with transillumination:

NOTE: Only one mouse can be imaged at a time using transillumination.

1. Put a check next to **Fluorescent** and **Transillumination** in the control panel.

NOTE: The Normalized option is selected by default so that NTF Efficiency images can be produced. See page 38 for more information.

Figure 4.19 Control Panel



2. Choose the **Auto** exposure option.
3. Put a check mark next to **Photograph**.

> **NOTE:** Selecting **Photograph** automatically selects **Overlay**, so that an overlay image (registered photograph and fluorescent image) is displayed after acquisition.

4. Select an excitation and emission filter from the drop-down lists.

   The instrument has 18 narrow band emission filters (490-850nm, 20nm bandwidth) and 10 narrow band excitation filters (415-760nm, 30nm bandwidth), enabling spectral scanning over the blue to NIR wavelength region (Figure 4.12 on page 34).
5. Click **Setup**. Click **Yes** if prompted to acquire a subject photograph.
6. Choose the locations (select ■ squares) for transillumination and image acquisition in the Transillumination Setup box that appears (Figure 4.20).

Figure 4.20 Open the Transillumination Setup Dialog Box

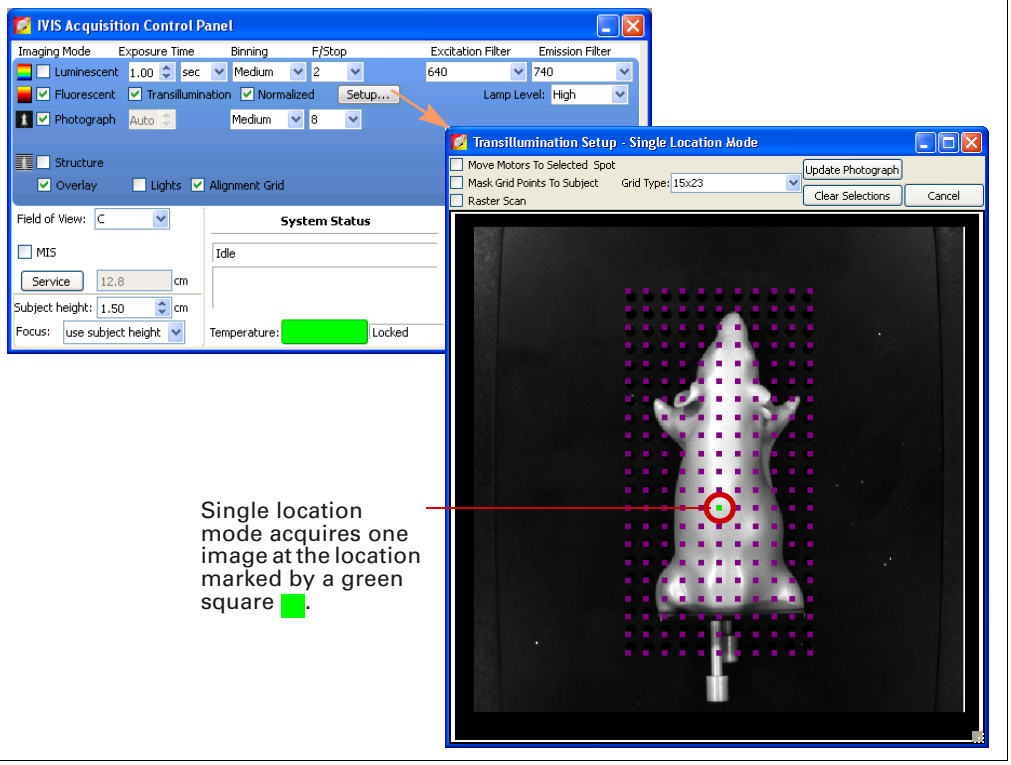


Table 4.3 Transillumination Setup Box

Item	Description
Move Motors To Selected Spot	Transillumination motors will move the excitation light source to the grid location selected in the Transillumination Setup dialog box.
Mask Grid Points To Subject	When setting up a transillumination sequence, choose this option to automatically select only the grid locations within the subject boundaries. Grid locations outside the subject are masked out. The mask prevents the transillumination excitation source from selecting an uncovered hole. Projecting light through an open hole would saturate the camera.
Raster Scan	If the raster scan option is selected, the software takes all of the images from the transillumination locations and adds them together into one image. All of the individual fluorescent signals are stacked over one photograph and the intensity is summed. One overview is created per filter pair. If two filter pairs were used during acquisition, then two overview images will be created. The raster scan option may be helpful when trying to determine the optimal excitation and emission filters for a particular fluorescent probe. All transillumination locations are displayed simultaneously; a tool tip displays the transillumination position when you mouse over a transillumination point. An overview image is displayed by default in radiant efficiency, and if transmission images are available, in normalized transmission fluorescence efficiency. Transillumination overview images can be analyzed using the tools in the Tool Palette. If this option is not selected, the software generates one image per transillumination location per filter pair. For example, a sequence setup that includes 20 locations using two filters will generate 20 images. In this case, a transillumination overview can be created manually by clicking the Overview button in the Sequence window. Alternatively, select **Tools** → **Transillumination Overview for <name>_SEQ** on the menu bar.
Grid Type	9x19 grid
Update Photograph	Click to acquire a new photographic image. If the chamber door is opened during transillumination setup, you are prompted to acquire a new photograph.
Clear Selections	Clears selected/ highlighted transillumination locations on the grid.

7. Confirm that the Lamp Level is set to **High** in the control panel.

NOTE: The lamp may be set to Low for certain applications, such as long wavelength data through thin tissue.

8. Select a field of view (FOV, size of the area to be imaged). See Table 4.1 on page 27 for a list of FOV settings.
9. Select a focus option in the control panel (Figure 4.21).

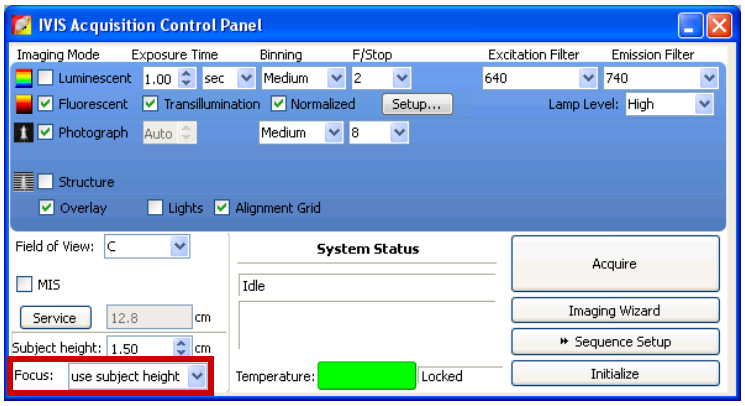
   The focal distance to the camera is set at stage z = 0 for each field of view. To focus at the top of the animal, the stage moves down so that the top of the animal is at z = 0. For example, if the subject height is 1.5 cm, the stage will move down 1.5 cm to set the plane of focus at the top of the subject.

   Do one of the following to set the focus for the area to be imaged:

   - If **Structure** is selected, choose the **use subject height** focus option.

     The subject height will be automatically determined from a structured light image.
   - If **Structure** is not selected, enter the subject height (cm), and choose the “use subject height” focus option.
   - Choose the **manual** focus option and follow the instructions in Appendix A on page 257.

Figure 4.21 Choose a Focus Option in the Control Panel

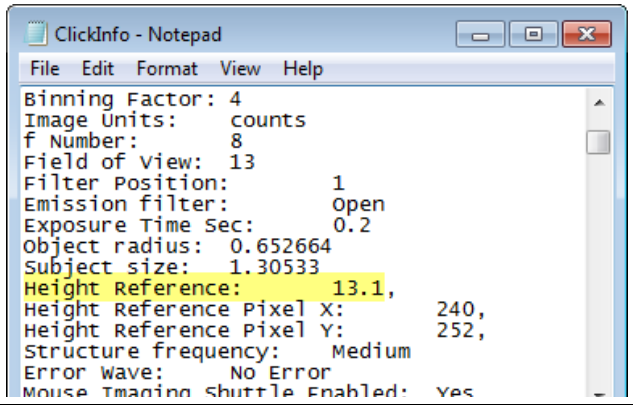


10. If you are using the Mouse Imaging Shuttle (MIS), select the "MIS" option (Figure 4.22). If not using the MIS, confirm that this option is cleared, otherwise the computed subject height will be inaccurate.

When the MIS option is selected, Living Image software applies a correction factor to the subject height measurement to offset the MIS platform and sets binning at 4, the optimum binning level for computing height from structured light measurements when using the MIS.

NOTE: If image data was acquired without using the MIS, but the MIS option was selected, the subject height will be 2.9 mm less than actual due to the MIS correction factor. To correct this, manually edit the subject height in the ClickInfo.txt file by adding 2.9 mm to the Height Reference measurement (Figure 4.22).

Figure 4.22 ClickInfo Text File



11. If you want to check the subject inside the chamber before acquisition, take a photograph: uncheck **Fluorescent**, choose **Photograph**, and click **Acquire**. Be sure to select **Fluorescent** after taking the photograph.

12. Click **Acquire** when you are ready to capture the image.

NOTE: If necessary click Image Setup in the control panel to operate in single image mode. In single image mode, the Sequence Setup button appears in the control panel. Use this button to set up sequence acquisition (see page 44 for more details on sequence setup).

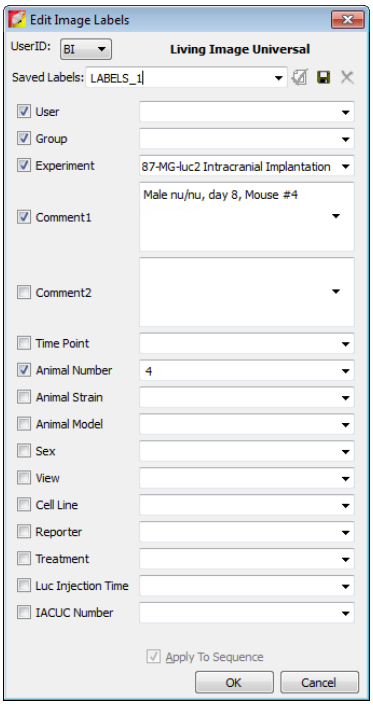
13. Enter information about the image in the dialog box that appears (optional, but strongly recommended) and click **OK**.

You can enter image label information at any time during or after acquisition. If you do not want to enter image information, click **Cancel**. See page 93 for details on adding information to an image after acquisition.

Figure 4.23 Enter Information About the Image

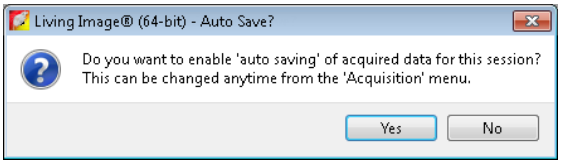
All information entered here will be saved with the image.

However, only the check marked values will appear in the image label (see Figure 4.25 on page 43).



If this is the first image of the session, you are prompted to enable the autosave function (Figure 4.24). When Autosave is enabled, all images acquired during the session are automatically saved to a user-selected location. A different location can be chosen at any time (select **Acquisition → Auto-Save** on the menu bar).

Figure 4.24 Autosave Prompt



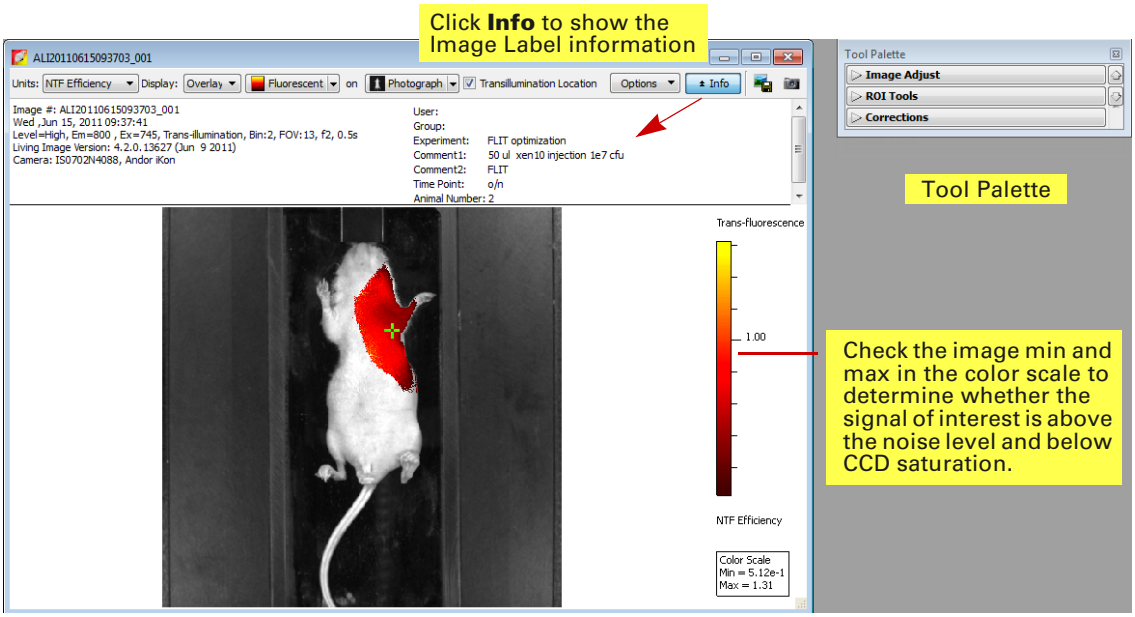
14. Click **Yes** in the prompt to enable autosave, then choose a location in the dialog box that appears. Alternatively, click **No** in the prompt and manually save the image data. See page 56 for details.

Image acquisition begins and the upper area of the control panel changes to red color.

NOTE: During acquisition, the **Acquire** button in the control panel changes to **Stop**. Click **Stop** to cancel acquisition and reinitialize the imaging system.

The control panel returns to blue color when acquisition is finished and the image window appears (Figure 4.25). See Table 4.2 on page 30 for details on the image window.

Figure 4.25 Overlay (Fluorescent Image on Photograph) in the Image Window



TIP: See the tech note *Identify Saturated Pixels in an Image* for information on pixel measurements (select **Help** → **Tech Notes** on the menu bar.

4.4 Cherenkov Imaging

Cherenkov luminescent imaging captures optical photons produced by Cherenkov radiation from radiotracer probes. Minutes of exposure time may be required because the Cherenkov signal can be very dim.

See page 44 for information on acquiring a Cherenkov sequence using the Imaging Wizard.

To acquire a Cherenkov image:

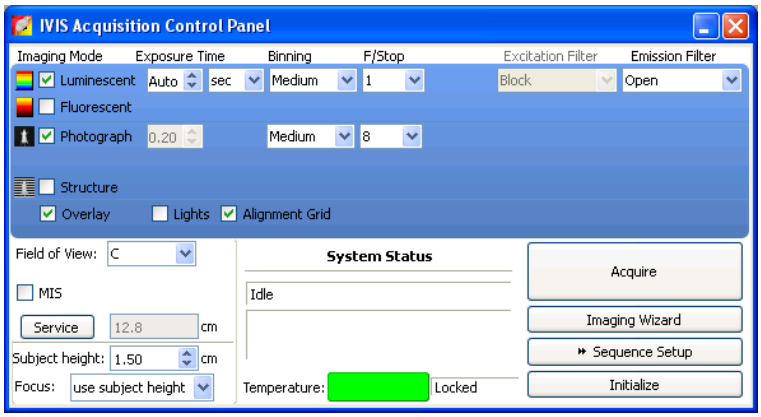
1. Put a check mark next to **Luminescent** and select **Auto** exposure in the control panel (Figure 4.26).

   The software automatically determines the binning and F/Stop settings.

   Alternatively, manually set the exposure time, binning, and F/Stop. See Appendix A on page 253 for details on these parameters. It may be helpful to increase the maximum time for Auto exposure in the user preferences to 5 minutes (see *Acquisition* on page 276).

TIP: See the tech note *Auto-Exposure* for helpful information (select **Help** → **Tech Notes** on the menu bar).

Figure 4.26 Control Panel



2. Perform step 2 on page 27 to step 9 on page 29.

4.5 Acquire a Sequence Using the Imaging Wizard

The acquisition parameters for each image in a sequence must be specified. The Imaging Wizard (Figure 4.27) provides a convenient way to set up a sequence for some imaging applications (see Table 4.4 on page 45). The wizard guides you through a series of steps, prompting you for the information that the software needs to set up the sequence.

This section explains how to start the Imaging Wizard and acquire a sequence of luminescent, fluorescent, or Cherenkov images. A sequence can also be set up manually (see page 52 for details).

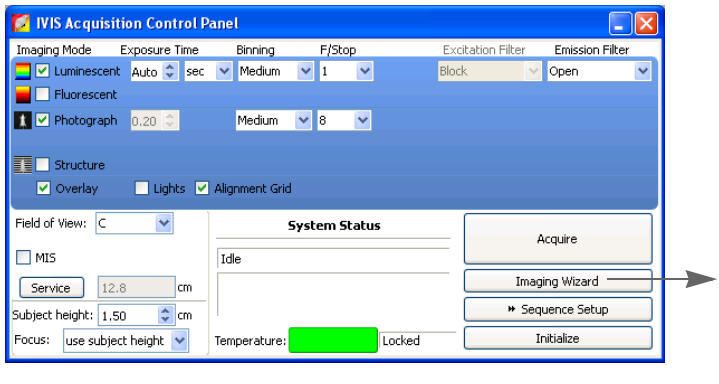
TIP: See the *Imaging Wizard* tech note for a quick guide (select **Help → Tech Notes** on the menu bar).

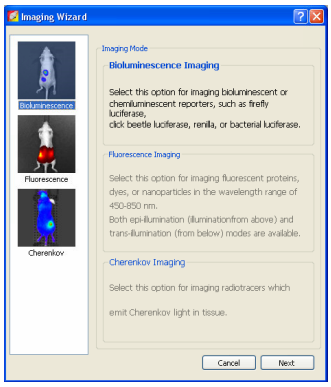
Start the Imaging Wizard and Setup a Sequence

NOTE: The IVIS Spectrum should be initialized and the temperature locked before setting imaging parameters. See page 19 for more details.

1. Click **Imaging Wizard** in the control panel (Figure 4.27). If necessary, click **Restart** in the Imaging Wizard to show the first screen of the wizard.

Figure 4.27 Open the Imaging Wizard





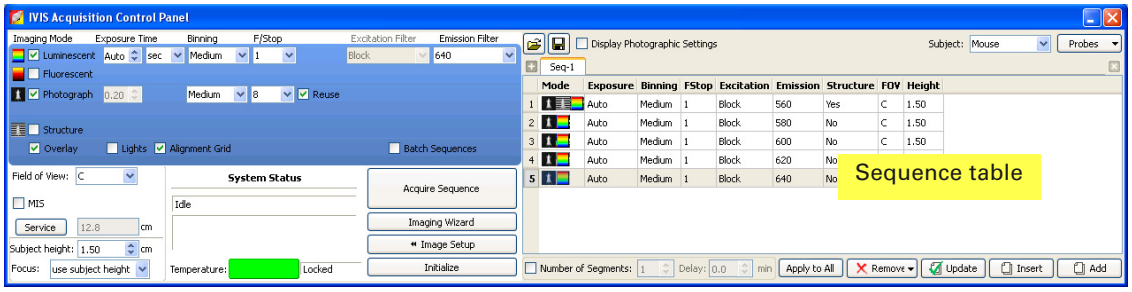
If this screen does not appear when the wizard starts, click **Restart Wizard** Restart Wizard (on the wizard screen) to restart the wizard.

2. Double-click an imaging mode: Bioluminescence  , Fluorescence  , or Cherenkov  .
3. Double-click an imaging option in the next screen (see Table 4.4 on page 45).
4. Step through the rest of the wizard.

   Each page of the wizard guides you with step-by-step instructions and descriptions. When you finish the wizard, it sets up the sequence to acquire (Figure 4.28).

Figure 4.28 Control Panel and Sequence Setup

Each row in the sequence table specifies the acquisition parameters for one image in the sequence. See page 53 for details on the sequence table.



5. To clear the sequence, click the **Remove** button  and select **All**.

   See additional information about:
   - Editing image parameters on page 54.
   - Inserting images in a sequence on page 55.
   - Removing images from a sequence on page 55.

Table 4.4 Imaging Wizard – Imaging Mode Options for IVIS Spectrum

Imaging Mode	Options	See Page
Bioluminescence	**Open Filter** – Acquires a luminescent image at maximum sensitivity.	
	Spectral Unmixing – Acquires an image sequence for analysis using the Spectral Unmixing tools which use a mathematical operation to separate the signals from multiple luminescent probes.	206
	DyCE[1] – Acquires a time series of optical images following a bolus injection of probe (radiotracer, bioluminescent, or fluorescent) to track probe biodistribution.	233
	DLIT (Diffuse Light Tomography) – Acquires an image sequence for analysis with the DLIT algorithm that reconstructs the position, geometry, and strength of 3D luminescent sources. **Note:** Hemoglobin (Hb and HbO_2) has an absorption peak near 540 nm. The Imaging Wizard automatically excludes the 540 nm emission filter from DLIT sequence setup to avoid long imaging times. This helps ensure that image acquisition occurs during the optimal time post-injection (determined by the probe kinetic curve for the animal model). If you want to include acquisition at 540 nm, manually add the image to the sequence table. See *Inserting Images in a Sequence* on page 55 for instructions.	124

Table 4.4 Imaging Wizard – Imaging Mode Options for IVIS Spectrum

Imaging Mode	Options	See Page
Fluorescence	**Filter Pair** – Selects the best excitation and emission filters for a specific fluorescent probe. Acquires a single image and detects the fluorescent signal on the surface of the subject. Epi-illumination or transillumination may be selected.	
	Spectral Unmixing/Filter Scan – Acquires an image sequence for analysis with the Spectral Unmixing tools to: ▪ Extract the signal of one or more fluorophores from the tissue autofluorescence. ▪ Determine the optimum excitation and emission filter for a probe.	206
	DyCE – Acquires a time series of optical images following a bolus injection of probe (radiotracer, bioluminescent, or fluorescent) to track probe biodistribution.	233
	FLIT (Fluorescence Imaging Tomography) – Acquires an image sequence for analysis with the FLIT algorithm that reconstructs the position, geometry, and strength of 3D fluorescent sources. This technique is only available in transillumination mode (light source below the stage).	130
Cherenkov	**Open Filter** – Acquires a Cherenkov image at maximum sensitivity.	
	Spectral Unmixing – Acquires an image sequence for analysis using the Spectral Unmixing tools which use a mathematical operation to separate the signals from multiple luminescent probes.	
	DyCE – Acquires a time series of optical images following a bolus injection of probe (radiotracer, bioluminescent, or fluorescent) to track probe biodistribution.	239

[1]DyCE analysis tools require a separate license.

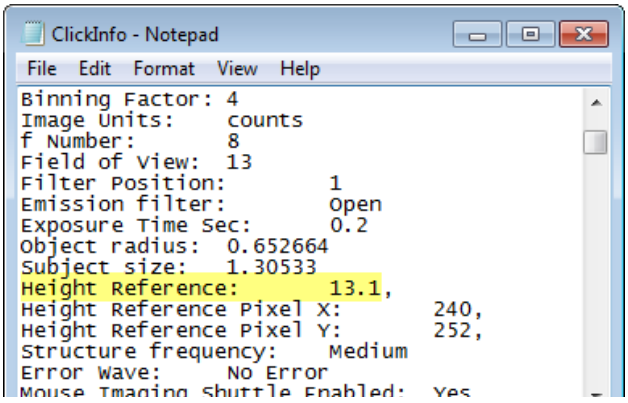
Acquire the Sequence

1. Confirm that the IVIS Spectrum is initialized and the CCD temperature is locked. (See page 19 for details.)
2. If you are using the Mouse Imaging Shuttle (MIS), select the "MIS" option (Figure 4.22). If not using the MIS, confirm that this option is cleared, otherwise the computed subject height will be inaccurate.

   When the MIS option is selected, Living Image software applies a correction factor to the subject height measurement to offset the MIS platform and sets binning at 4, the optimum binning level for computing height from structured light measurements when using the MIS.

NOTE: If image data was acquired without using the MIS, but the MIS option was selected, the subject height will be 2.9 mm less than actual due to the MIS correction factor. To correct this, manually edit the subject height in the ClickInfo.txt file by adding 2.9 mm to the Height Reference measurement (Figure 4.22).

Figure 4.29 ClickInfo Text File



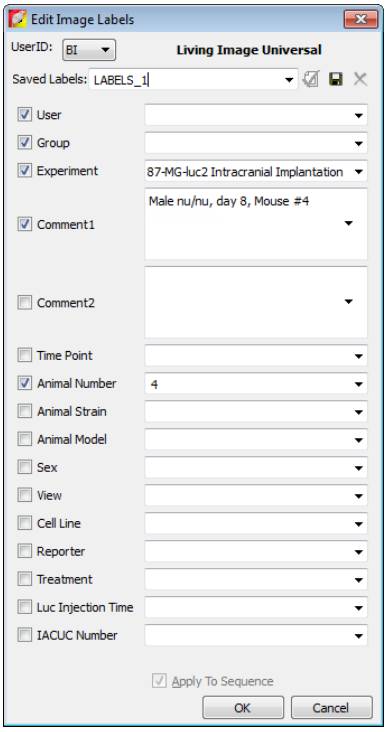
3. Click **Acquire Sequence** in the control panel when ready to begin acquisition.
4. Enter information about the image in the dialog box that appears (optional, but strongly recommended) (Figure 4.30). Click **OK.**

   You can enter image label information at any time during or after acquisition. If you do not want to enter image information, click **Cancel**. See page 93 for details on adding information to an image after acquisition.

Figure 4.30 Enter Information About the Image

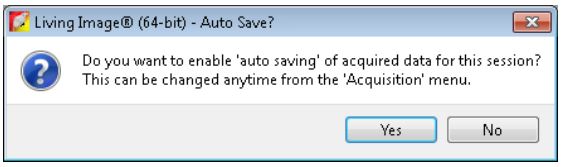
All information entered here will be saved with the image.

However, only the check marked values will appear in the image label (see Figure 4.32 on page 48).



If this is the first image of the session, you are prompted to enable the autosave function (Figure 4.31). When Autosave is enabled, all images acquired during the session are automatically saved to a user-selected location. A different location can be chosen at any time (select **Acquisition → Auto-Save** on the menu bar).

Figure 4.31 Autosave Prompt



5. Click **Yes** in the prompt to enable autosave, then choose a location in the dialog box that appears. Alternatively, click **No** in the prompt and manually save the image data. See page 56 for details.

   Image acquisition begins and the upper area of the control panel changes to red color.

> **NOTE:** During acquisition, the **Acquire** button in the control panel changes to **Stop**. Click **Stop** to cancel acquisition and reinitialize the imaging system.

The image window displays the images as they are acquired. The control panel returns to blue color when acquisition is finished and the Tool Palette appears (Figure 4.32).

The Image window may include multiple tabs, depending on the type of acquisition:

- Sequence View – Displays the image sequence.
- 3D View – Displays the 3D volume if the acquisition included DLIT or FLIT data.

> **TIP:** See the tech note *Saturated Pixels In an Image* for information on pixel measurements.

Figure 4.32 Image Window and Tool Palette

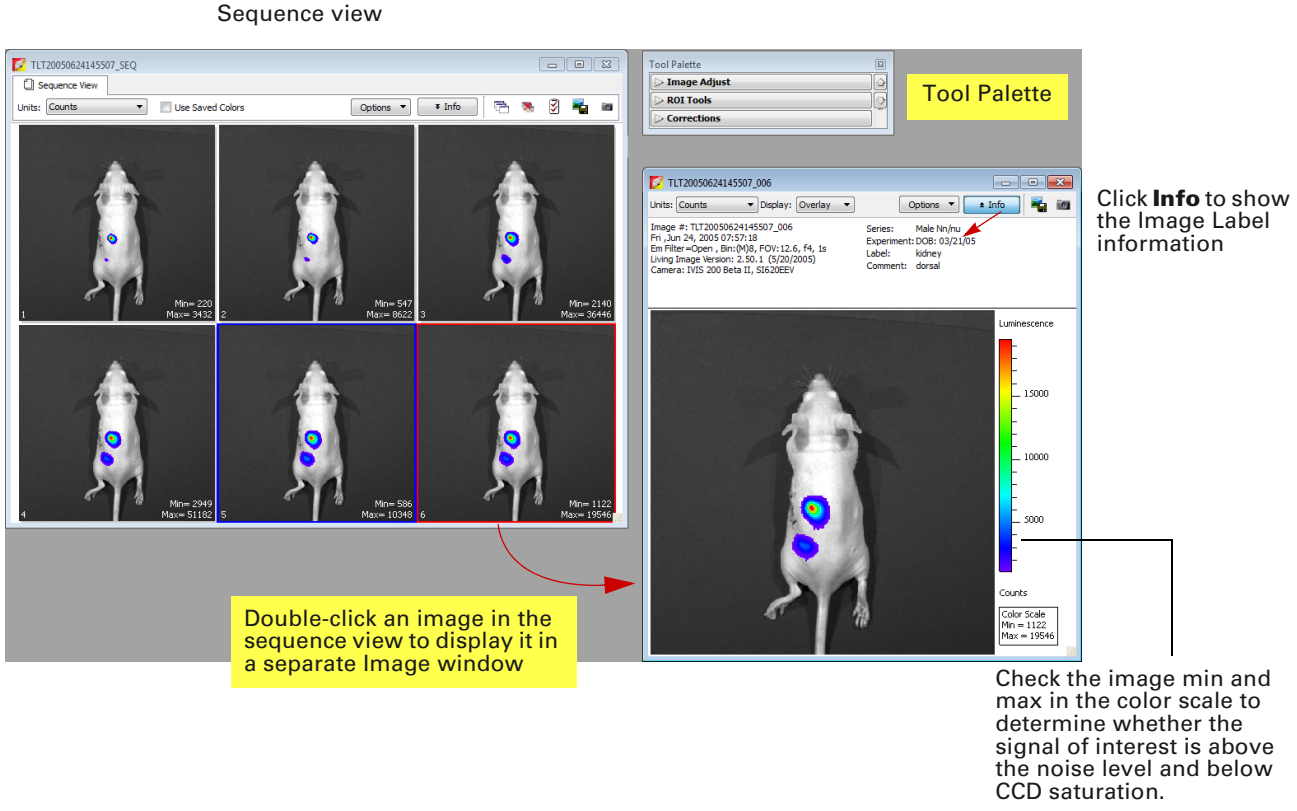


Table 4.5 Image Window – Sequence View

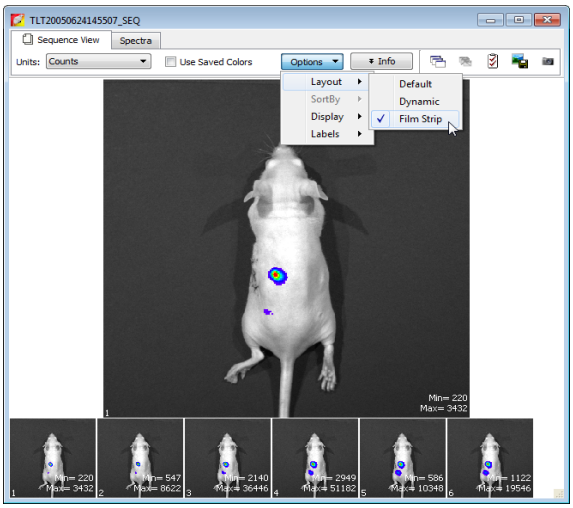
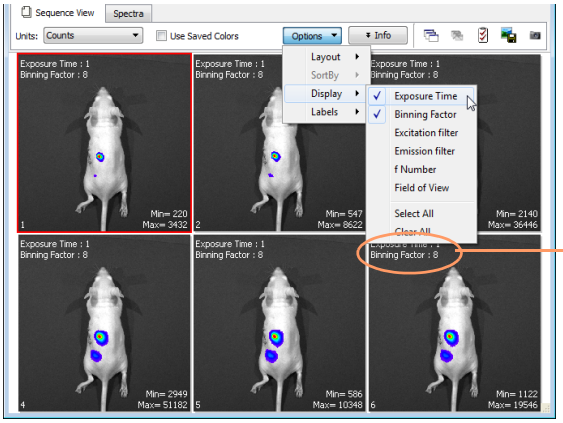
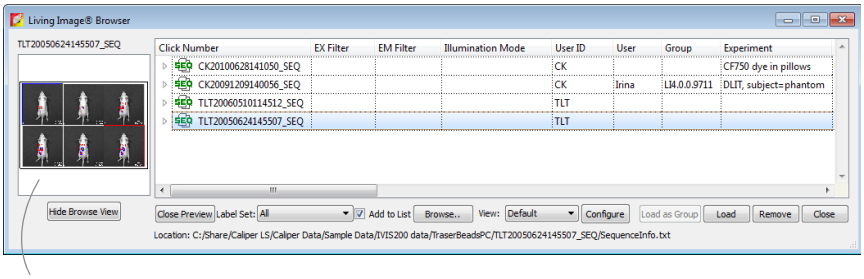
Item	Description
Units	Select the measurement units for the image display from this drop-down list. The available units depend on the type of image data. See the concept tech note *Image Display and Measurement* for more details (select **Help → Tech Notes** on the menu bar).
Use Saved Colors	Choose this option to display the image data using the color table that was specified in the Preferences at the time of acquisition. If this option is not selected, image data are displayed using the color table currently specified in the Preferences.
Options	Layout - Choose a display option for the images in a sequence (Default, Dynamic, or Film Strip). For example, here is Film Strip mode:  Sort by - Options for ordering images in the sequence window. This option only applies to images that were opened using the "Load as Group" function in the Living Image browser. Default - Order in which the images are stored in the folder. TimeStamp - Ascending order of the image acquisition time. UserID - Ascending alphanumeric order of the user ID Display - Choose the types of information to display with each image.  In this example, exposure time and binning factor are displayed on each image
Info	Click to show or hide the image label information (Figure 4.32).
	Opens all of the images in the sequence.
	Closes all open images.
	Opens the Edit Sequence dialog box that enables you to add or remove images from the sequence.

Table 4.5 Image Window – Sequence View (continued)

Item	Description
	Enables you to export the active image as a graphic file (for example, .png, .dcm).
	Creates a preview picture (*snapshot*) of the image or thumbnails that the Living Image Browser displays when the data are selected. See page 57 for more details on the browser.  Preview picture of the selected data

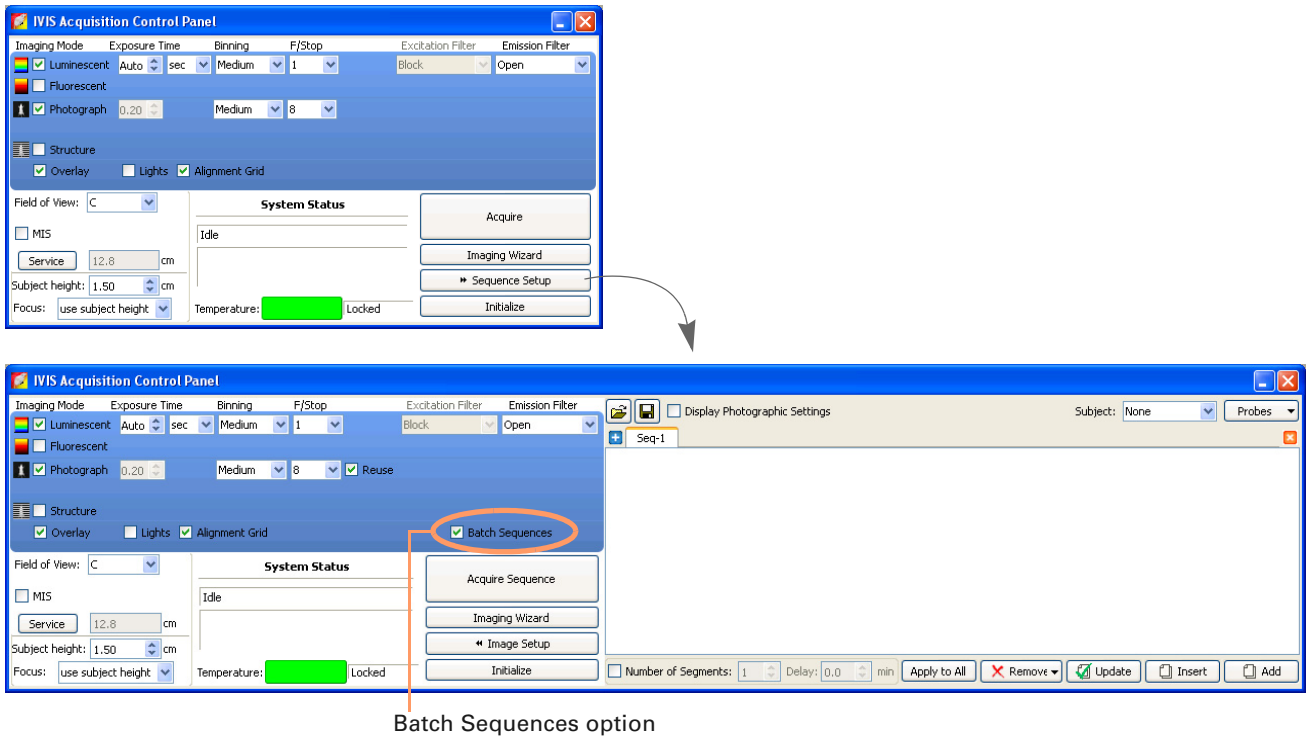
4.6 Acquire Multiple Sequences in Batch Mode

Use batch mode to set up multiple, separate sequences which will be automatically acquired, one after another, without manual intervention.

To setup and acquire sequences in batch mode:

1. Click **Sequence Setup** in the control panel.
2. Choose the **Batch Sequences** option (Figure 4.33).

Figure 4.33 Control Panel



3. To set up the first sequence, do either of the following:
   - Click **Imaging Wizard** and step through the wizard (see page 44 for details).

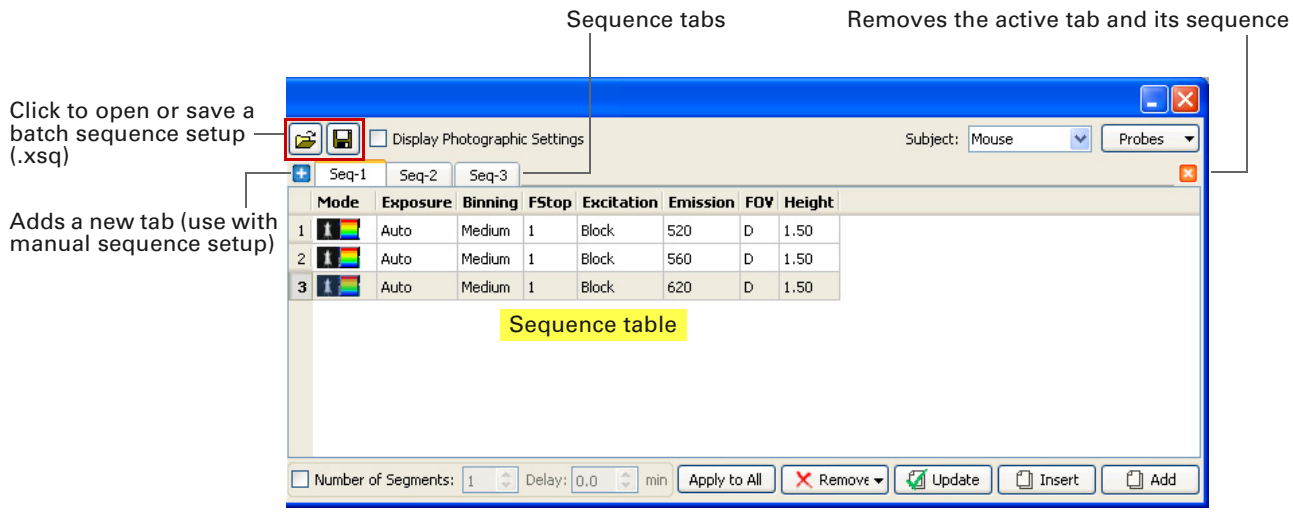
   OR
   - Set up the sequence manually (see page 52 for details).
4. To set up the next sequence:
   - If using the Imaging Wizard, repeat step step 3.

     Each sequence is displayed in a separate tab.
   - If setting up the sequence manually, click the button in the sequence table to add a new tab, then proceed with manual setup in the new tab.

NOTE: Sequence tabs can be renamed. Double-click a tab name to edit it. Alternatively, right-click the selected name to view a shortcut menu of edit commands (for example, Cut, Copy, Paste).

Figure 4.34 Multiple Sequence Tabs

Three sequences are specified in this example.



5. To remove a sequence, click the sequence tab and then click the button.
6. Click **Acquire Sequence** when you are ready to capture the sequences.

   Image acquisition proceeds with no intervening time delay between sequences. During acquisition, the **Acquire** button in the control panel changes to **Stop**. Clicking **Stop** cancels acquisition.

   The upper area of the control panel changes to red color during acquisition. The control panel returns to blue color when acquisition is finished.

NOTE: If the **Batch Sequences** option in the control panel is not selected (Figure 4.33), only the sequence in the active tab will be acquired.

7. To save the batch sequence setup:
   a. Click the **Save** button.
   b. Enter a file name (.xsq) and choose a location for the file in the dialog box that appears.

4.7 Manually Set Up a Sequence

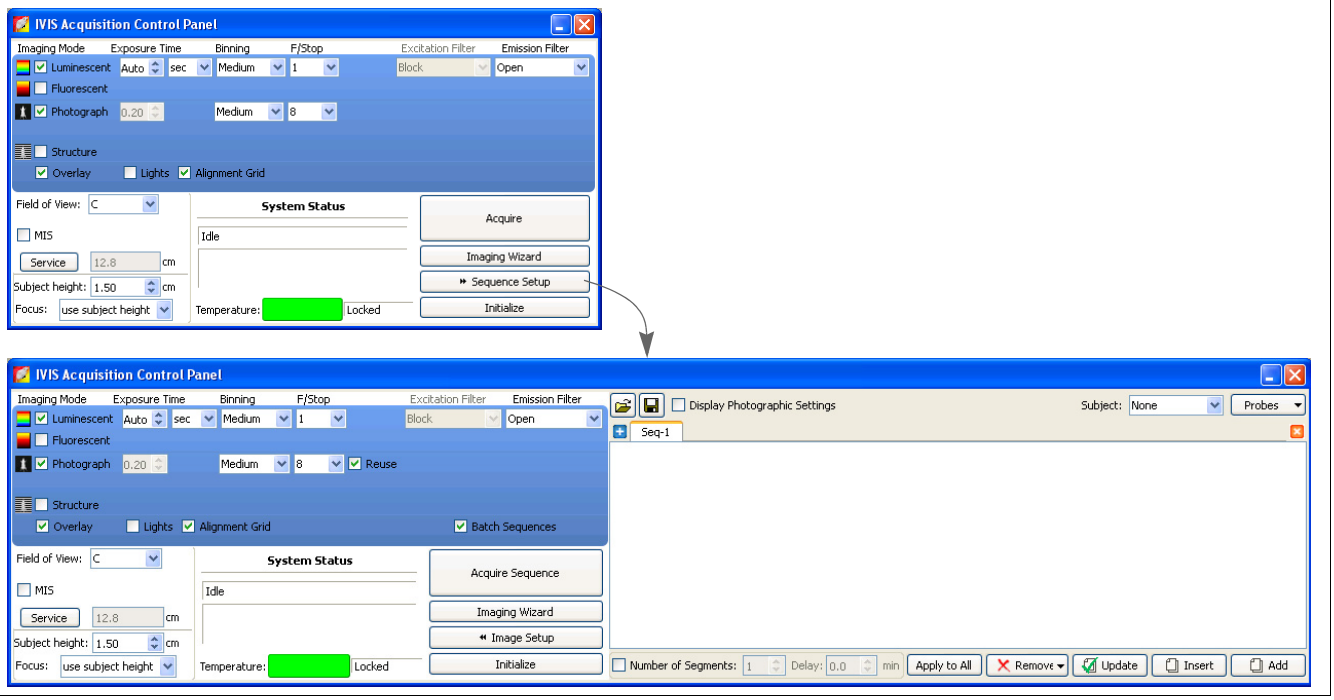
This section explains how to set up an image sequence if you do not use the Imaging Wizard. The sequence parameters in the sequence table can be saved as a Living Image Sequence Setup file (.xsq).

See *Acquire the Sequence* on page 46 for details on image acquisition.

TIP: It may be convenient to create an image sequence by editing a sequence setup generated with the Imaging Wizard or an existing sequence setup (.xsq). Save the modified sequence setup to a new name.

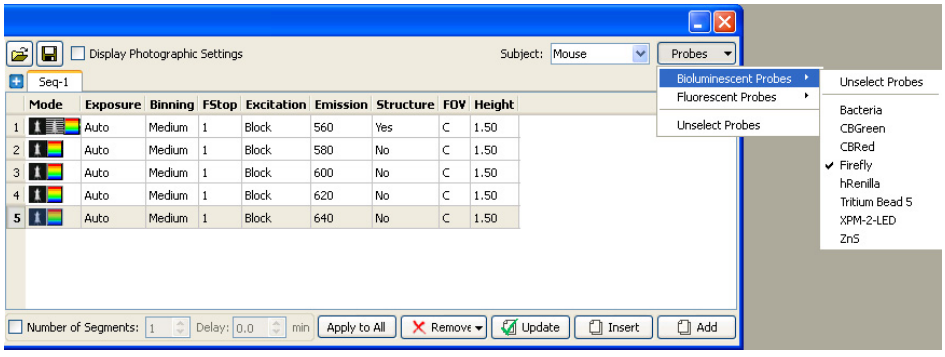
1. Click **Sequence Setup** in the control panel (Figure 4.35).
   The sequence table appears.
2. If necessary, click the **Remove** button Remove and select **All** to clear the sequence table.

Figure 4.35 Open the Sequence Table



3. Choose a subject and probe from the drop-down lists (Figure 4.36)

Figure 4.36 Choose Subject and Probe



4. Specify the imaging settings for the first image in the sequence. (See Appendix A on page 253 for details on the imaging parameters in the control panel.)

> **NOTE:** If you selected **Photograph** and the photograph **Reuse** option in the control panel (Figure 4.37), the IVIS Spectrum acquires only one photograph for the entire sequence. If **Reuse** is not chosen, the imaging system acquires a photograph for each image in the sequence.

5. Click the **Add** button 

.
The acquisition parameters appear in the sequence table (Figure 4.37).
6. Repeat step 4 to step 5 for each image in the sequence.
7. To set a time delay between each acquisition, enter a time (minutes) in the Delay box in the sequence table.
8. To save the sequence setup information (.xsq):
   a. Click the **Save** button in the sequence table.
   b. Select a directory, enter a file name, and click **Save** in the dialog box that appears.

Figure 4.37 Control Panel and Sequence Table with Image Settings

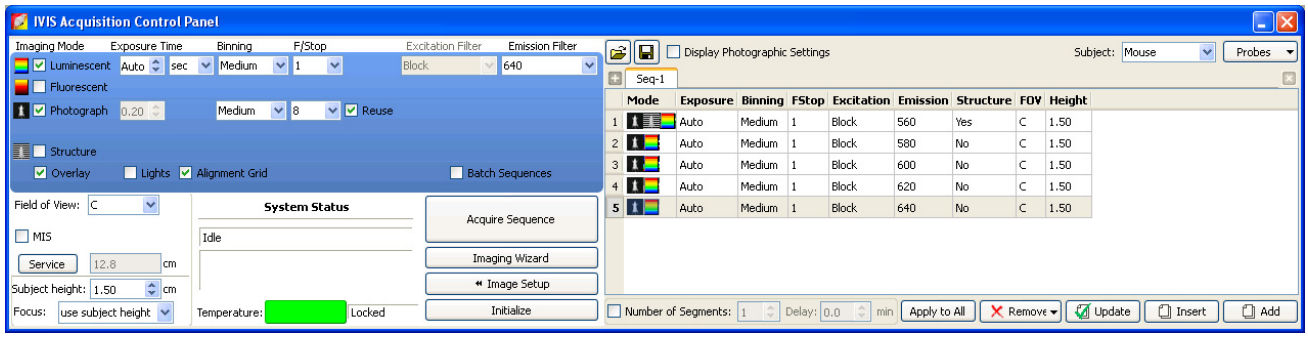


Table 4.6 Sequence Table

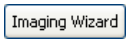
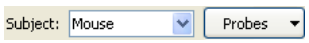
Item	Description
	Starts the Imaging Wizard.
	Displays a dialog box that enables you to select and open a sequence setup (.xsq), sequenceinfo.txt, or clickinfo.txt file.
	Displays a dialog box that enables you to save the information in the sequence table to a sequence setup file (.xsq).
Display Photographic Settings	Choose this option to include the photograph exposure time, binning, and F/Stop in the sequence table.
	If a subject and probe are specified (optional), the software uses the information to automatically set parameters in the Surface Topography, DLIT, FLIT, Spectral Unmixing, and Planar Spectral Imaging tools. If a subject or probe is not selected here, the default parameters appear in the Tool Palette.
Number of Segments	The sequence specified in the sequence table is called a *segment*. Choose this option to set the number of segments to acquire and the time delay between segments. This is useful for acquiring data for kinetic analysis.
Delay	Specifies a time delay between each segment acquisition.

Table 4.6 Sequence Table (continued)

Item	Description
Apply to All	Applies the selected cell value to all cells in the same column.
Remove	Remove Selected - Deletes the selected row from the sequence table. Remove All - Removes all rows from the sequence table.
Update	Updates the selected row in the sequence table with the acquisition parameters in the control panel.
Insert	Inserts a row above the currently selected row using the information from the control panel.
Add	Adds a new row at the end of the sequence setup list.

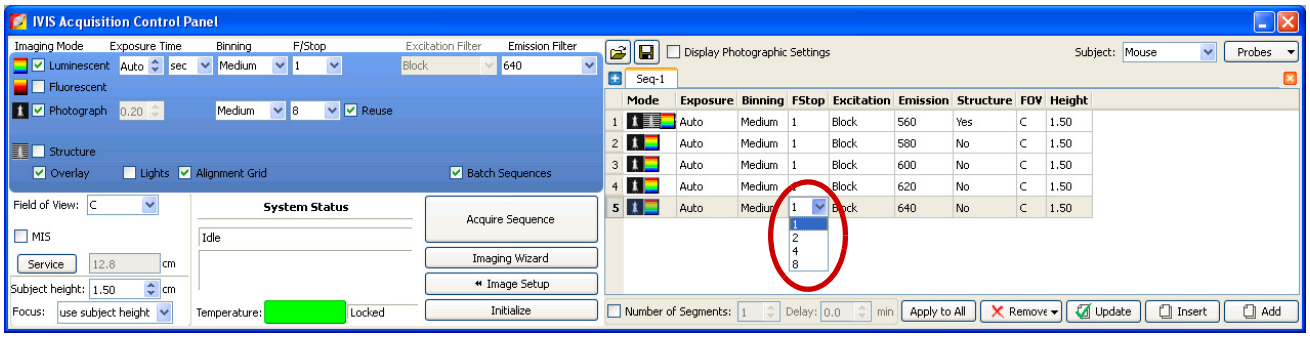
Editing Image Parameters

You can edit imaging parameters in the sequence table or in the control panel.

To edit a parameter in the sequence table:

1. Double-click the cell that you want to edit (Figure 4.38).

Figure 4.38 Edit Imaging Parameters



2. Enter a new value in the cell or make a selection from the drop-down list. To apply the new value to all of the cells in the same column, click Apply to All.
3. Click outside the cell to lose focus.

To edit a parameter in the control panel:

1. Select the row that you want to modify in the sequence table.
2. Set new parameter values and/or imaging mode in the control panel.
3. Click Update in the sequence table.

Inserting Images in a Sequence

Method 1:

1. Select the sequence table row that is below where you want to insert a new image (row).
2. Set the imaging mode and parameters in the control panel.
3. Click Insert to insert the new image above the selected row,

Method 2:

1. Select the row(s) of interest and right-click the sequence table to view a shortcut menu of edit commands (Figure 4.34 on page 57).

Figure 4.39 Sequence Table – Shortcut Menu Edit Commands

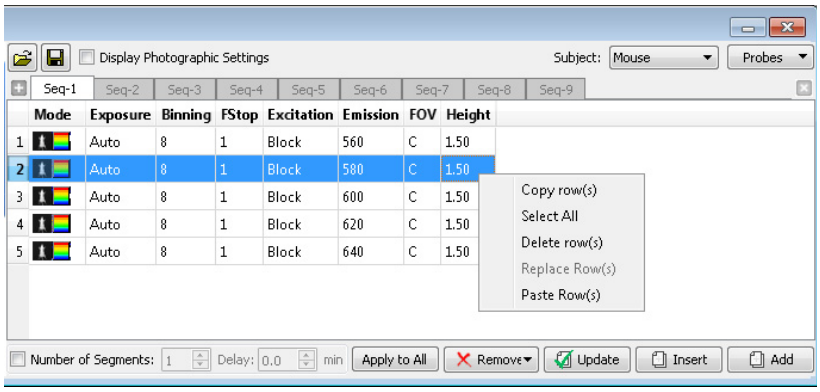


Table 4.7 Sequence Table – Shortcut Menu Edit Commands

Command	Description
Copy row(s)	Copies the selected row(s) to the system clipboard.
Select All	Selects all rows in the sequence table.
Delete row(s)	Deletes the selected row(s) from the sequence table.
Replace Row(s)	Replaces the row(s) selected in the sequence table with the rows in the system clipboard. **Note:** The Replace function is only available when the number of rows in the system clipboard is the same as the number of rows selected in the sequence table.
Paste Row(s)	Adds copied rows to end of the sequence.

Removing Images From a Sequence

Do either of the following:

- Select the row(s) that you want to delete. click Remove, and choose **Selected** from the drop-down list.
  or
- Select the row(s) of interest and right-click the sequence table to view a shortcut menu of edit commands (Figure 4.39).

4.8 Manually Save Image Data

Living Image software prompts you to enable the autosave feature during the first acquisition setup of an imaging session. If autosave is enabled, all images acquired during the session are automatically saved to a user-selected location. You can choose a different location at any time (select **Acquisition → Auto-Save** on the menu bar).

This section explains how to manually save data if you do not want to use the autosave feature.

1. Turn off the autosave feature (select **Acquisition** on the menu bar and remove the check mark next to **Auto Save**).
2. After image or sequence acquisition, click the **Save** button . Alternatively, select **File → Save** on the menu bar.
3. Select a directory in the dialog box that appears, and click **OK**.

   The data includes the user ID and a date/time stamp.

4.9 Exporting Images

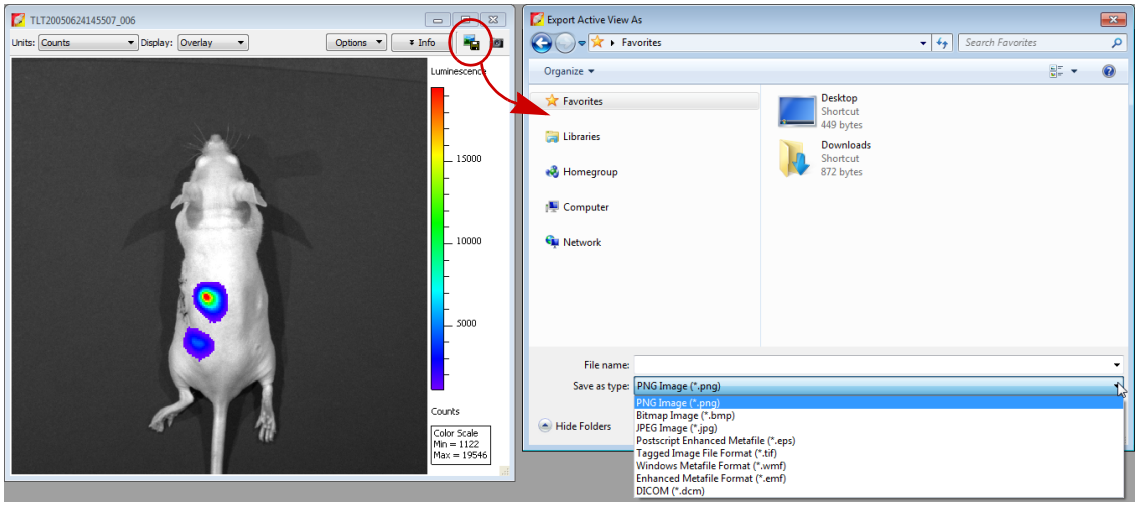
The active image view can be saved in different file formats (for example, .png, .bmp, .dcm).

NOTE: The Image Layout window provides an alternative way to export or print images. See page 86 for more information.

1. Open an image or sequence.
2. Click the **Export Graphics** button (Figure 4.40).
3. Select a directory in the dialog box that appears, enter a file name, and click **Save**.

NOTE: To export a sequence to DICOM (.dcm) format, select **File → Export → Image/Sequence As DICOM** on the menu bar. This creates a directory that contains the .dcm files and a SequenceInfo.txt.

Figure 4.40 Export Image to a Graphic File



5 Working With Images

5.1 Loading Image Data

Images can be loaded (opened):

- Using the Living Image Browser (see below).
- From the toolbar or menu bar (page 61).
- By dragging an image file or sequence folder to the Living Image main window.

Multiple datasets can be open at the same time.

NOTE: Select **File → Recent Files** on the menu bar to view recently opened files.

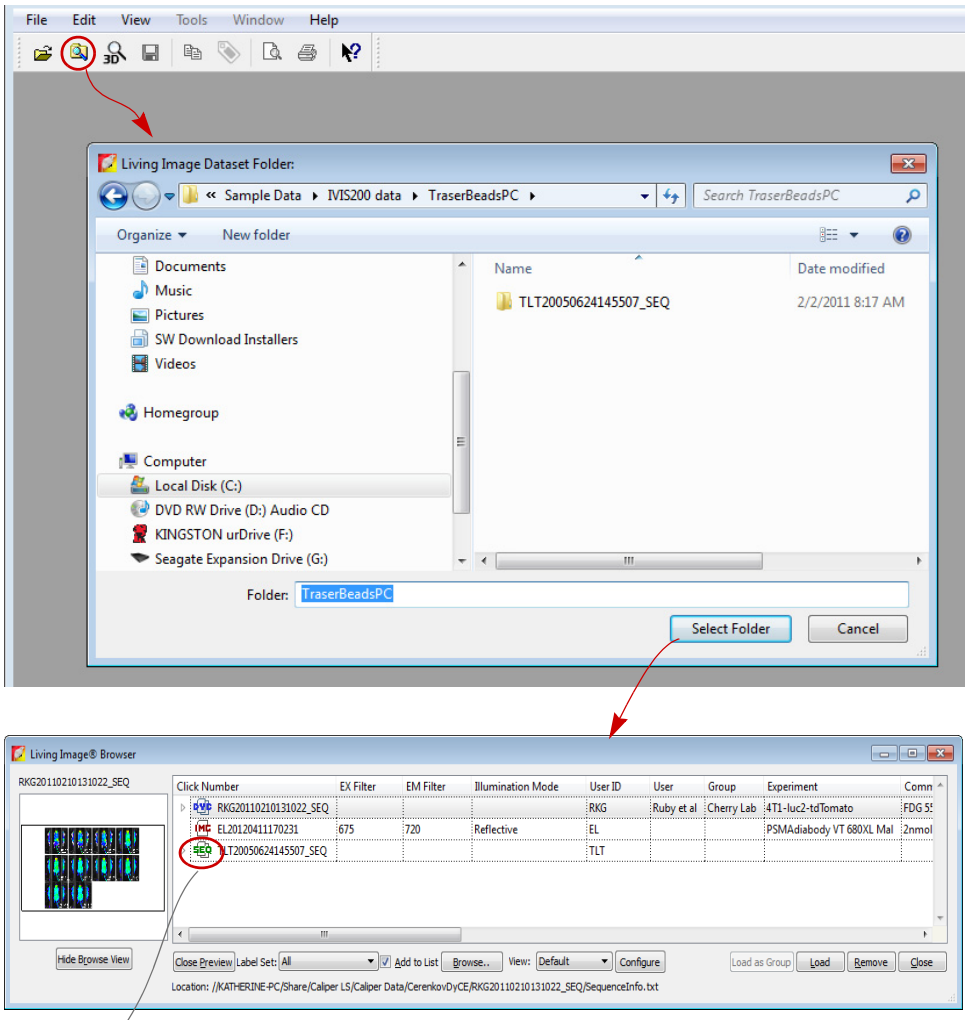
Preview and Load Data Using the Living Image Browser

The Living Image Browser provides a convenient way to browse and preview optical data, view information about the data, and load the data.

1. Start the Living Image Browser:
   a. Click the **Browse** button. Alternatively, select **File → Browse** on the menu bar.
   b. Select a folder in the dialog box that appears.
   The Living Image Browser appears (Figure 5.1). It displays all Living Image data located in the folder and its subfolders, along with the user ID, label information, and camera configuration information.

NOTE: The next time you start Living Image software and click the toolbar button, the software automatically returns to the last folder visited.

Figure 5.1 Opening the Living Image Browser



Living Image Browser

– Image

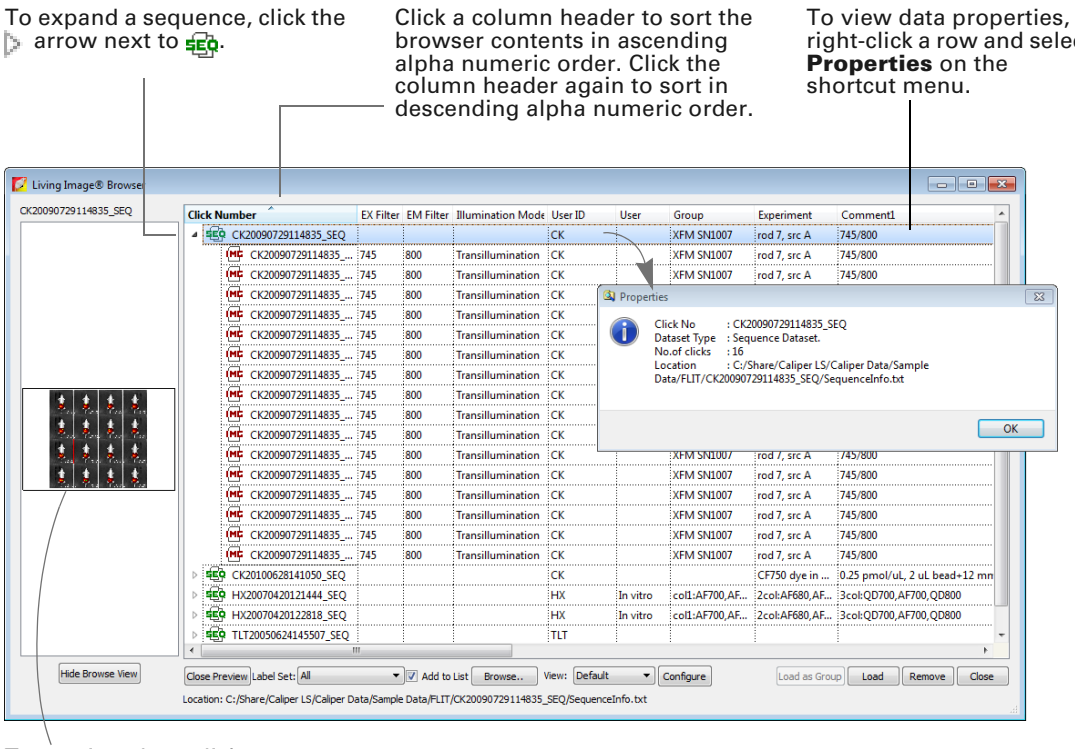
– Image with saturated pixels

– Image sequence

– Image exported as DICOM file

– DyCE or Cherenkov image sequence

Figure 5.2 Living Image Browser



Note: A preview snapshot is automatically taken at the time of image or sequence acquisition. A snapshot can also be captured manually (see page 65 for more details).

2. Load data by doing one of the following:
   - Double-click the data row.
   - Right-click the data name and select **Load** on the shortcut menu.
   - Select the data row and click **Load**.
   - Double-click the sequence thumbnail or, if available, image thumbnail.

   The image(s) and Tool Palette are displayed. Green rows in the browser indicate loaded data (Figure 5.3).

NOTE: Multiple datasets can be loaded.

Figure 5.3 Loaded Image Sequence

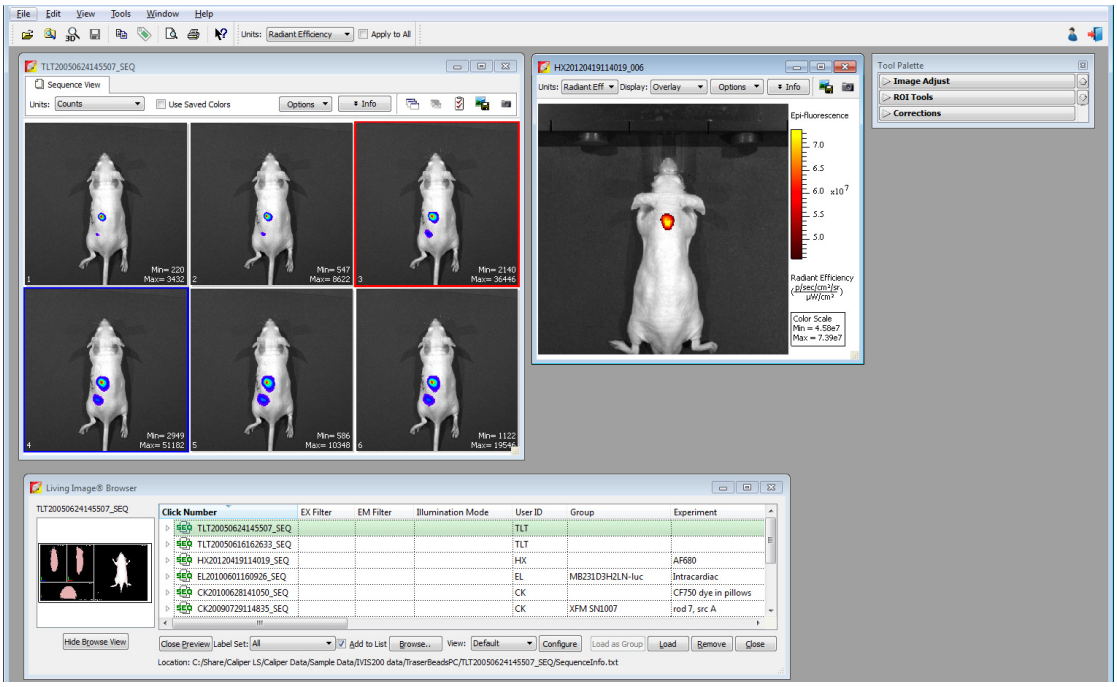


Table 5.1 Living Image Browser

Item	Description
Hide Browse View	Closes the browser table.
Close Preview	Closes the image preview box.
Label Set	A drop-down list of the available label sets which specify the image information (column headers) displayed in the Living Image Browser.
Add to List	If this option is chosen, the data selected in the "Living Image Dataset Folder" dialog box (Figure 5.1 on page 58) is added to the Living Image Browser. If this option is not chosen, the data selected in the dialog box replaces the contents of the Living Image Browser, except for loaded data.
Browse	Opens the "Living Image Dataset Folder" dialog box that enables you to choose data to add to the browser (Figure 5.1 on page 58).
View	The name of the Living Image Browser configuration (the column headers and their order in the browser).
Configure	Opens a dialog box that enables you to create and save custom Living Image Browser configurations. **Note:** To reorder a column in the browser, click the column header, then press the mouse key while you drag the header left or right. Release the mouse key to set the new position.

Table 5.1 Living Image Browser (continued)

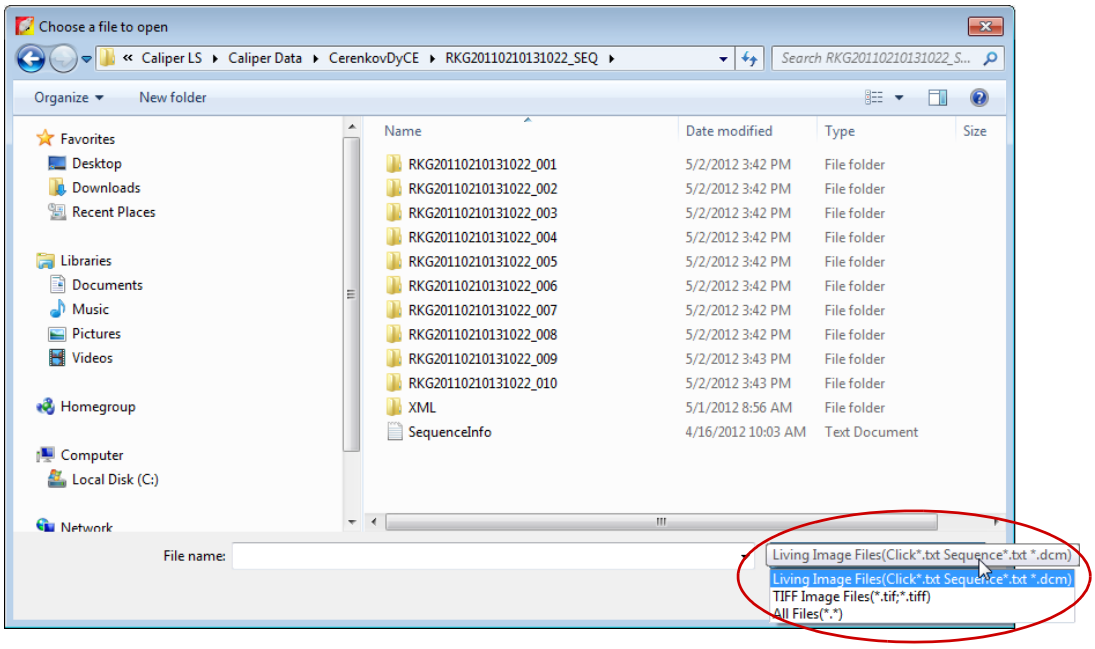
Item	Description
Load as Group	Enables you to select particular images that you want to view as a sequence. The images may be acquired during different sessions. To select adjacent images in the browser, press and hold the **Shift** key while you click the first and last file in the selection. To select non-adjacent images in the browser: PC users: Press and hold the **Ctrl** key while you click the images in the browser Macintosh users: Press and hold the **Cmd** key (apple key) while you click the images in the browser. **Note:** The **Load as Group** option is only available when two or more images (non-kinetic) are selected in the browser. **Tip:** See the tech note Loading Groups of Images for a quick guide (select **Help → Tech Notes** on the menu bar).
Load	Opens the selected image or image sequence.
Remove	Removes a user-selected image sequence(s) from the browser.
Close	Closes the Living Image Browser.

Load Data From the Menu Bar or Toolbar

NOTE: To open a recently viewed file, select **File → Recent Files** on the menu bar.

1. Click the **Open** button on the toolbar. Alternatively, select **File → Open** on the menu bar.
2. Choose a file type filter from the drop-down list in the box that appears (Figure 5.4).
   The default file type selection is "Click*.txt, Sequence*.txt, or *.dcm", which are the file types generally used to open a sequence or single image (see Table 5.2 on page 62).

Figure 5.4 Opening Data From the Toolbar or Menu Bar



Select the file type(s)

Table 5.2 File Filters

File Type Filter	Shows:
Living Image files	Click*.txt – an image (Living Image file format). Sequence*.txt – an image sequence (Living Image file format). *.dcm – kinetic data or an image that was exported to a DICOM file.
TIFF Image Files	Graphic files (*.tif, *.tiff).
All Files (*.*)	All file types.

3. Navigate to the file and double-click it. Alternatively, select the data and click **Open**.

About the Image Window and Tool Palette

An image or image sequence is displayed in an image window (Figure 5.5). Multiple image windows can be open at the same time.

Figure 5.5 Image Windows – Sequence View and Single Image

The options available in the image window depend on the type of active image data.

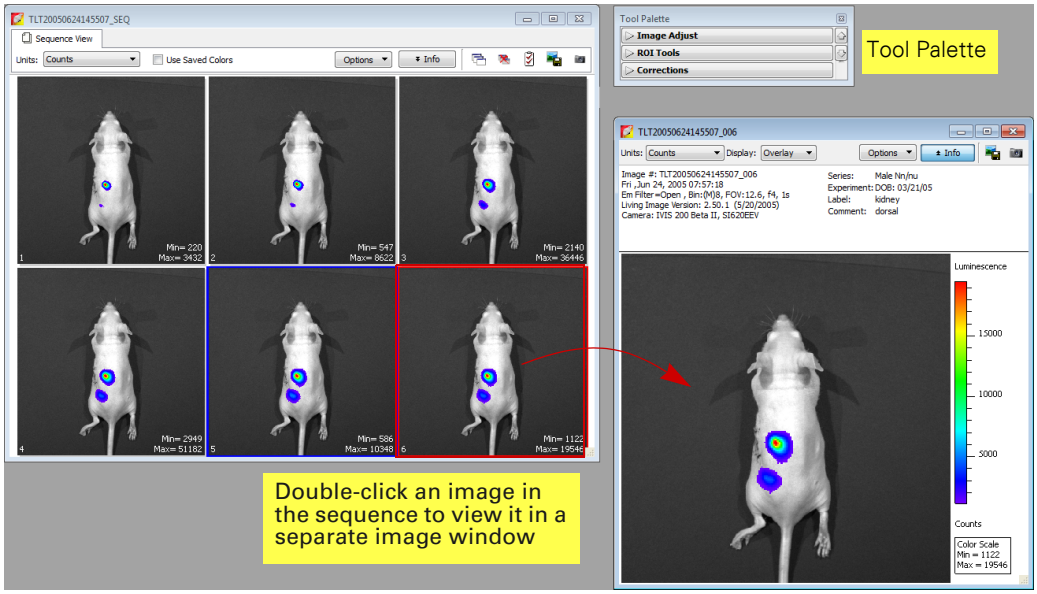


Table 5.3 Image Window

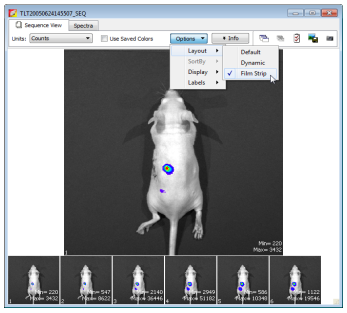
Item	Description
Units	Select the measurement units for the image display from this drop-down list. The available units depend on the type of image data. See the concept tech note *Image Display and Measurement* for more details on measurement units (select **Help → Tech Notes** on the menu bar). **Counts** – An uncalibrated measurement of the photons incident on the CCD camera. Recommended for image acquisition to ensure that the camera settings are properly adjusted. Proper image parameter adjustment should avoid image saturation and ensure sufficient signal (greater than a few hundred counts at maximum). **Radiance (photons)** – A calibrated measurement of the photon emission from the subject. Radiance is in units of "photons/second/cm^2/steradian". Recommended for luminescence measurements. **Radiant Efficiency (fluorescence)** – Recommended for fluorescence measurements. ▪ Epi-fluorescence - A fluorescence emission radiance per incident excitation power. ▪ Transillumination fluorescence - Fluorescence emission radiance per incident excitation power. **Efficiency** (epi-fluorescence) – Fluorescent emission normalized to the incident excitation intensity (radiance of the subject/illumination intensity). Recommended for epi-fluorescence measurements. **NTF Efficiency** – Fluorescent emission image normalized to the transmission image which is measured with the same emission filter and open excitation filter. Recommended for transillumination fluorescent measurements.
Use Saved Colors (image sequence)	Choose this option to display the image data using the color table that was specified in the Preferences at the time of acquisition. If this option is not selected, image data are displayed using the color table currently specified in the Preferences.
Options (image sequence)	Layout – Choose a display option for the images in a sequence (Default, Dynamic, or Film Strip). For example, here is Film Strip mode:  Sort by – Options for ordering images in the sequence window: ▪ Default – Order in which the images are stored in the folder. ▪ TimeStamp – Ascending order of the image acquisition time. ▪ UserID – Ascending alphanumeric order of the user ID.

Table 5.3 Image Window (continued)

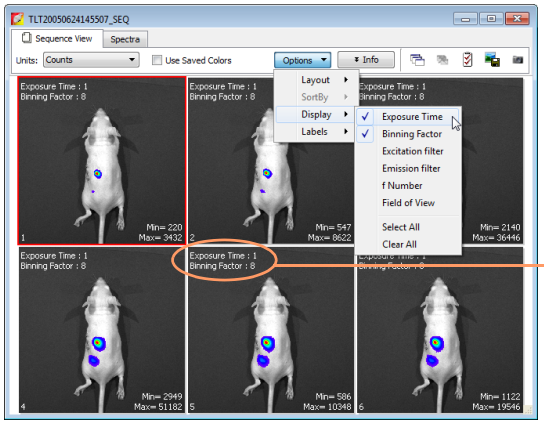
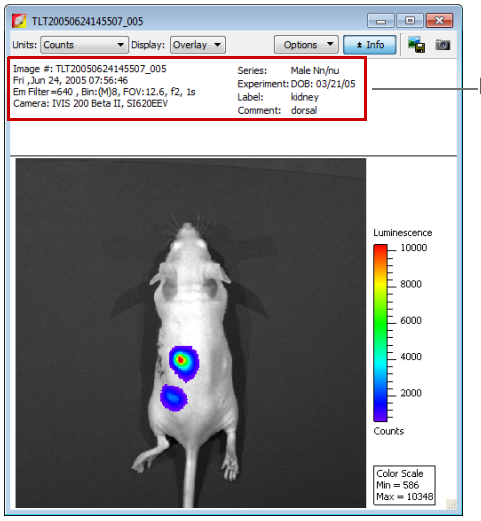
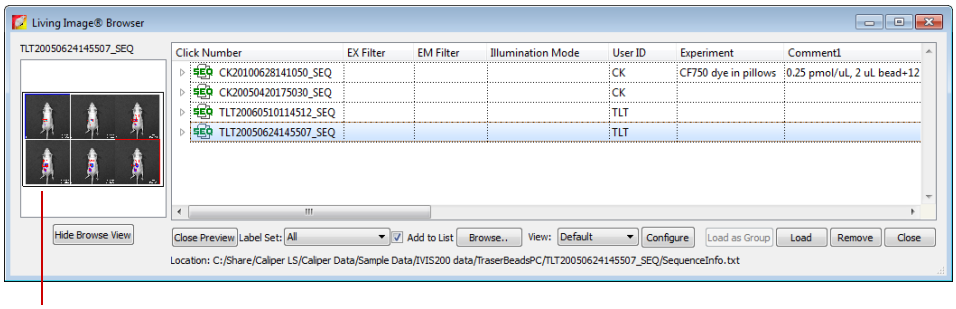
Item	Description
	Display - Choose the types of information to display with each image. 
	Labels – Enables you to select the information to include in the image label. 
Info	Click to show or hide the image label. The image label includes information you enter in the Edit Image Labels dialog box (see page 29) and other information automatically recorded by the software.
	Opens all of the images in a sequence.
	Closes all open images of a sequence.
	Opens the Edit Sequence dialog box that enables you to add or remove images from the sequence display.
	Opens a dialog box that enables you to export the active view as a graphic file.

Table 5.3 Image Window (continued)

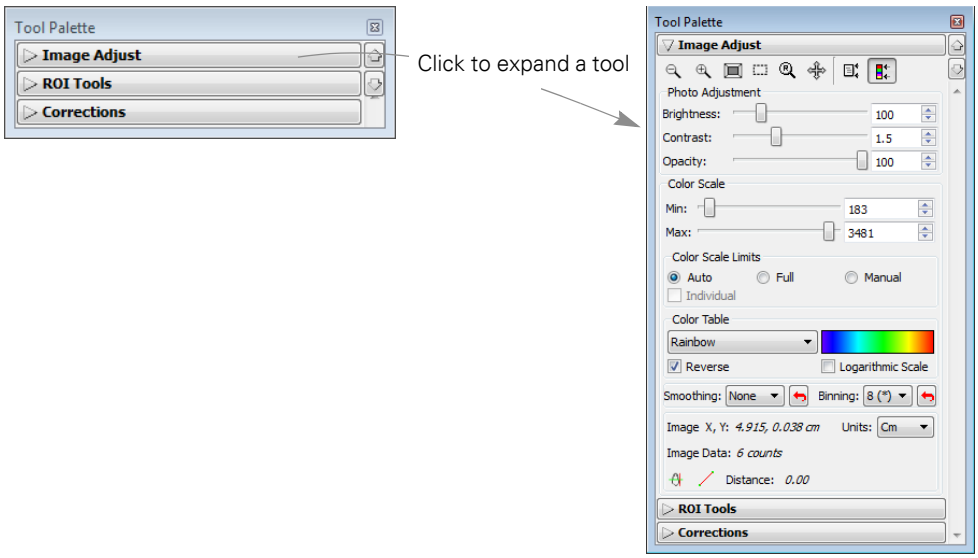
Item	Description
	Takes a "snapshot" that is displayed with the data in the Living Image Browser. See page 57 for more details on the browser.  Snapshots of an image sequence

The Tool Palette appears when an image or sequence is loaded (Figure 5.6). The options available in the Tool Palette depend on the type of active image data. A tool is only available if the dataset includes the components that the tool requires to perform the analysis.

NOTE: The 3D Multi-Modality tools and DyCE tools require a separate license.

Figure 5.6 Tool Palette

See page 11 for an overview of Living Image tools.

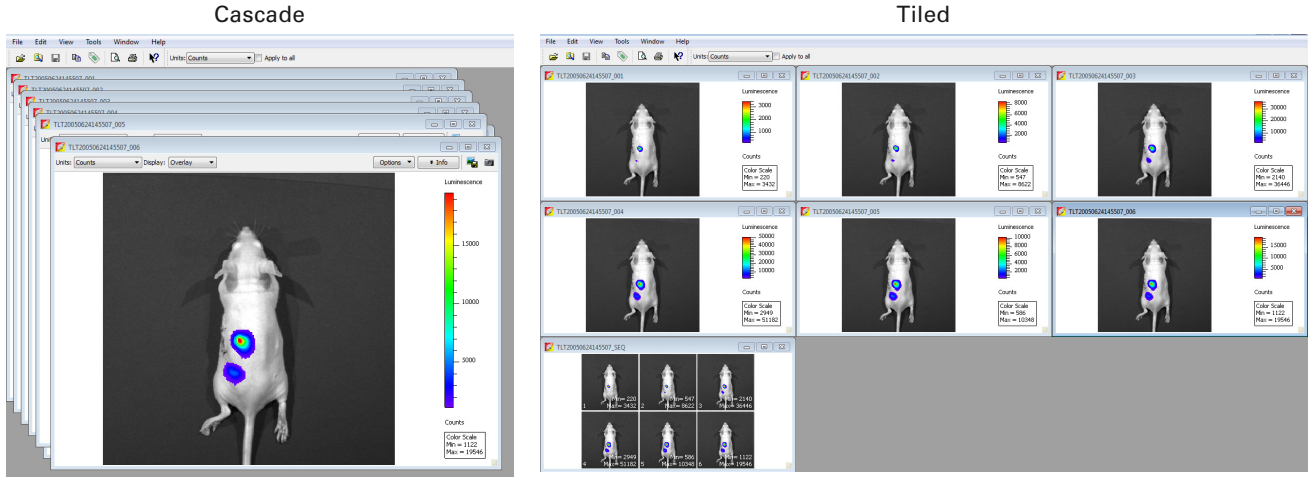


Organizing Images

If multiple image windows are open, they can be organized in a cascade or tile arrangement.

Choose **Window** → **Cascade** or **Window**→ **Tile** on the menu bar.

Figure 5.7 Image Windows



5.2 Adjusting Image Appearance

Use the Image Adjust tools to adjust image display (Figure 5.8). Most of the Image Adjust tools do not change the image data (for example, adjusting the color scale or color table). However, binning and smoothing may slightly change image data, and therefore should only be applied after image data have been analyzed.

NOTE: Not all tools are available for all image display modes. Some tools are available for single images, but not an image sequence and vice versa.

Figure 5.8 Tool Palette – Image Adjust Tools

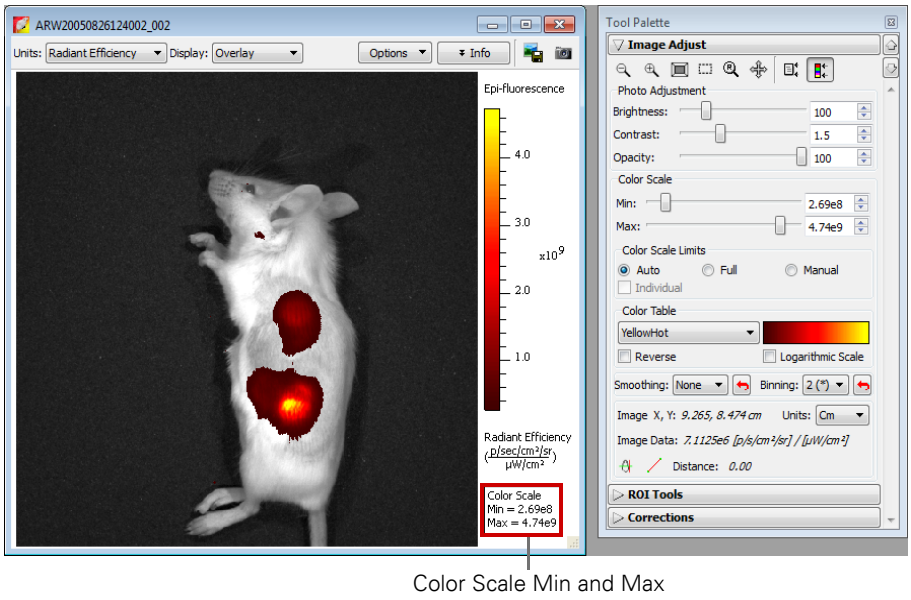


Table 5.4 Image Adjust Tools

Item	Description
	Click this button to incrementally zoom out on the image (reduces the image dimensions in the image window). **Note:** The zoom tools are also available in the shortcut menu when you right-click the image (**Cmd**-click for Macintosh users).
	Click this button to incrementally zoom in on the image (incrementally magnifies the image in the image window).
	Click this button, then draw a box on the image to magnify the area inside the box. (Sets the dimensions of the magnified area equal to image window dimensions.) See *Zooming or Panning* on page 68 for more information.
	Click this button to draw a box on an image that can be used to: ▪ Make measurements (see page 74) ▪ Select an area of the image to copy to the system clipboard.
	Click this button to return the image to the default display magnification.
	Click this button to move a magnified image (*pan*) in the image window. For more details, see page 68.
	Click this button to hide or display the image min/max information in the image window
	Click this button to hide or show the color scale in the image window.
Photo Adjustment	Brightness – Click and move the slider left or right to adjust the brightness of an image displayed in overlay or photograph mode. Alternatively, enter a brightness value. Contrast – Click and move the slider left or right to adjust the *gamma* of an image displayed in overlay mode. Alternatively, enter a gamma value. (Gamma is related to image contrast.) Opacity – Click and move the slider left or right to adjust the opacity of the pseudocolor luminescent data of an image displayed in overlay mode. Alternatively, enter an opacity value.
Color Scale	Min – The minimum pixel intensity associated with the color scale for an image. Pixels less than the minimum value are not displayed. Max – The maximum pixel intensity associated with the color scale for an image. Pixels greater than the maximum value are displayed in the maximum color.
Color Scale Limits	Auto – If this option is chosen, the software sets the Min and Max values to optimize image display and suppress background noise. The Min and Max settings can be manually adjusted to further optimize the image display for your needs. Full – Choose this option to set the Max and Min values to the maximum and minimum data values in the image. Manual – Choose this option to enter Max and Min values for the image display. Individual – Applies a separate color scale limit to each image in a sequence. **Note:** This option is only available when an image sequence is active.
Color Table	 Click the drop-down arrow to select a color table for the image data. (See the concept tech note *Image Display and Measurement* for more details on the color table (select **Help** → **Tech Notes** on the menu bar). Reverse – Choose this option to reverse the selected color table. Logarithmic Scale – Choose this option to apply a log scale to the relationship between numerical data and the color range in the color table. A log scale improves the visibility of dark areas in an image.

Table 5.4 Image Adjust Tools (continued)

Item	Description
Smoothing	Computes the average signal of a specified number of pixels (for example, 5 x 5) and replaces the original signal with the average signal (see Figure 5.9). Smoothing removes signal noise without changing pixel size. Smoothing can be applied to an image or a sequence.  Click this button to return smoothing to the previous setting and update the image.
Binning	Specifies the number of pixels in the image data that are grouped together to form a larger pixel (called *soft* binning). Binning changes the pixel size in the image (see Figure 5.9). Binning can be applied to an image or a sequence. See the tech note *Detection Sensitivity* for more details on binning (select **Help → Tech Notes** on the menu bar).  Click this button to return binning to the previous setting and update the image.
Image X,Y	The x,y pixel coordinates of the mouse pointer location in an image and the intensity (counts or photons) at that location. **Note:** This tool is only available when an image is active.
	Click this button to display a line profile (see page 71.) **Note:** This tool is only available when an image is active.
	Click this button to display the distance measurement tool in the image window (see page 73). **Note:** This tool is only available when an image is active.

Zooming or Panning

To incrementally zoom in or out on an image:

Click the  or  button. Alternatively, right-click the image and select **Zoom In** or **Zoom Out** on the shortcut menu.

To magnify a selected area in an image:

1. Click the  button. Alternatively, right-click the image and select **Area Zoom** on the shortcut menu.
2. When the pointer becomes a +, draw a rectangle around the area that you want to magnify.
   The selected area is magnified when you release the mouse button.

To reset the magnification (remove magnification):

Click the  button. Alternatively, right-click the image and select **Reset Zoom** on the shortcut menu.

To pan the image window:

NOTE: Panning helps you view different areas of a magnified image. Panning is only available if the image has been magnified.

1. Click the  button.
2. When the pointer becomes a  , click and hold the pointer while you move the mouse.

Smoothing and Binning

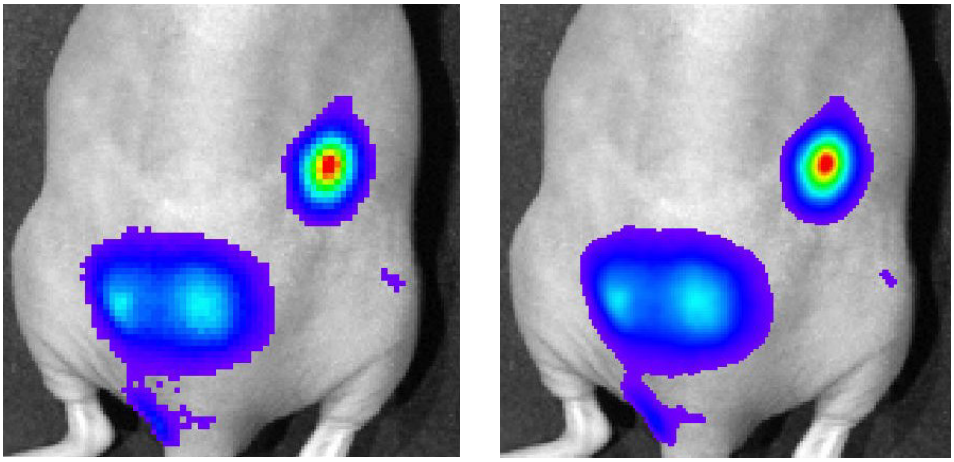
TIP: See the technical note *Detection Sensitivity* for helpful information about binning and smoothing (select **Help → Tech Notes** on the menu bar).

Smoothing computes the average signal of a specified number of pixels (for example, 5 x 5) and replaces the original signal with the average signal (Figure 5.9). Smoothing removes signal noise without changing pixel size

Binning specifies the number of pixels in the image data that are grouped together to form a larger pixel (called soft binning) (Figure 5.9). Binning changes the pixel size in the image.

Smoothing and binning can be applied to a single image or all of the images in a sequence.

Figure 5.9 Example of Binning and Smoothing Image Data



Binning at acquisition = 8, no smoothing

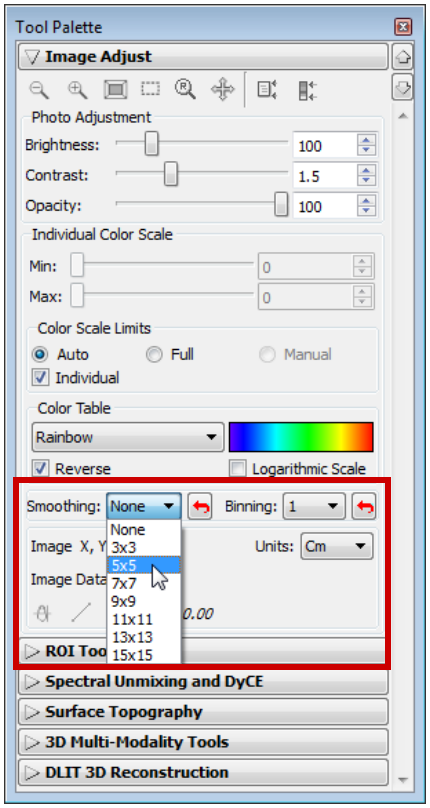
Binning = 2, smoothing = 5x5

To set smoothing and/or binning:

1. Load an image or a sequence.
2. Make a selection from the Smoothing and/or Binning drop-down lists in the Image Adjust tools (Figure 5.10).

   The image or all images in the sequence will be updated.
3. Click the button to return the smoothing or binning to the previous setting and update the image or sequence images.

Figure 5.10 Select Smoothing and/or Binning Levels



5.3 Viewing Intensity Data

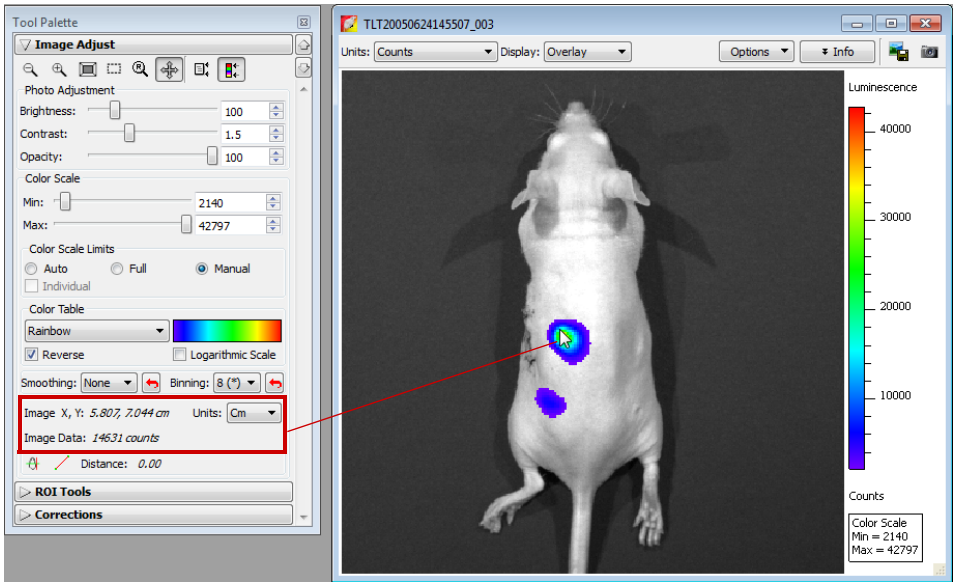
You can view intensity data:

- At a particular x,y location.
- Along a line drawn on the image.
- Within a user-selected *region of interest* (ROI). See Chapter 6 on page 98 for information on measuring signal in 2D images using an ROI.

X,Y Coordinates and Intensity Data

1. Open an image and choose Cm or Pixels from the Units drop-down list in the Image Adjust tools.
2. Put the mouse pointer over the image to view the:
   - x,y pixel coordinates of the mouse pointer location in the image (Figure 5.11).
   - Intensity at the pixel location of the mouse pointer. The intensity is represented in the units currently selected for the image.

Figure 5.11 X,Y Coordinates and Intensity Data at the Mouse Pointer Location



Line Profile

The line profile plots intensity (y-axis) at each pixel (x-axis) along a user-specified line on an image. It is particularly useful for inspecting the detailed character of the image data.

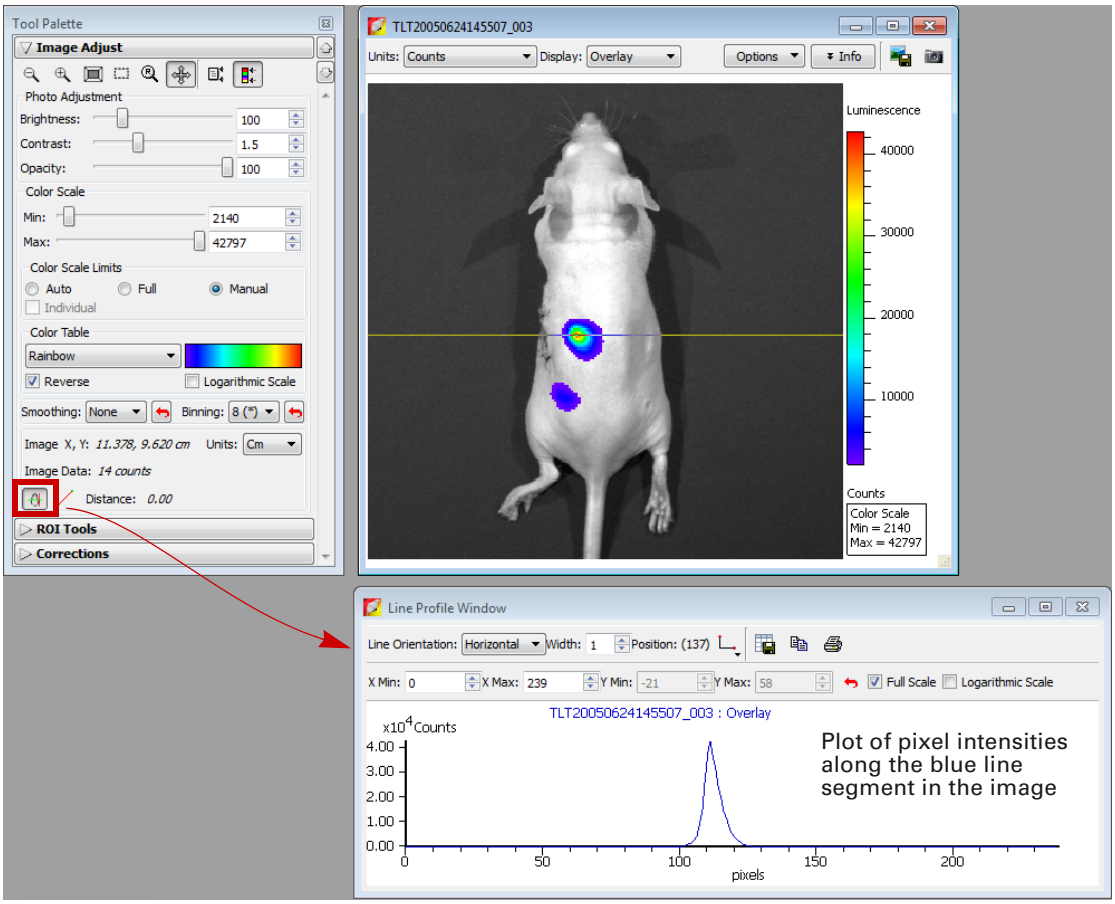
NOTE: In the Overlay display mode, the line profile plots the luminescent data. To obtain a histogram of the photograph, select Photograph from the Display drop-down list.

To display a line profile:

1. Open an image and click the **Line Profile** button in the Image Adjust tools (Figure 5.12).

   A line appears on the image and the Line Profile window opens. See Table 5.5 on page 72 for details on the Line Profile window.

Figure 5.12 View a Line Profile of Pixel Intensities



2. To view the line profile at another location in the image, put the mouse pointer over the line. When the pointer becomes a ⇳, drag the line over the image. The line segment colored blue indicates the pixel intensities that are plotted in the line profile graph.

   The line profile is updated as you move the line move over the image.

Table 5.5 Line Profile Window

Item	Description
Line Orientation	Choose Vertical, Horizontal, or Free Hand from the drop-down list to set the orientation of the line in the image window. The Free Hand orientation enables you to drag each line segment endpoint to a user- selected position.
Width	Sets the line width. The Line Profile window displays the average of the pixel values included in the line width.
Position	Line position (pixels).
	Enables you to choose the grid line pattern to display in the line profile window.
	Exports the line profile data to a .csv or .txt file.
	Copies the line profile graph to the system clipboard.

Table 5.5 Line Profile Window (continued)

Item	Description
	Opens the Print dialog box.
X Min X Max	Displays the minimum and maximum value of the x-axis. Use the  arrows to change the x-axis min or max. If a calibrated unit such as “radiance” is selected in the image window, the x-axis units = cm. If “counts” is selected in the image window, the x-axis units = pixels. To display the range available for the Min or Max, place the mouse pointer over the Min or Max edit box.
Y Min Y Max	Displays the minimum and maximum value of the y-axis. Use the  arrows to change the y-axis min or max. To display the range available for the Y Min or Y Max, place the mouse pointer over the Min or Max edit box.
	Click to reset the X and Y Min and Max values to the defaults.
Full Scale	Select this option to display the full X and Y-axis scales.
Logarithmic Scale	Select this option to apply a log scale to the y-axis.

5.4 Measuring Distance

Measure distance in an image using the distance measurement tool or image crop box.

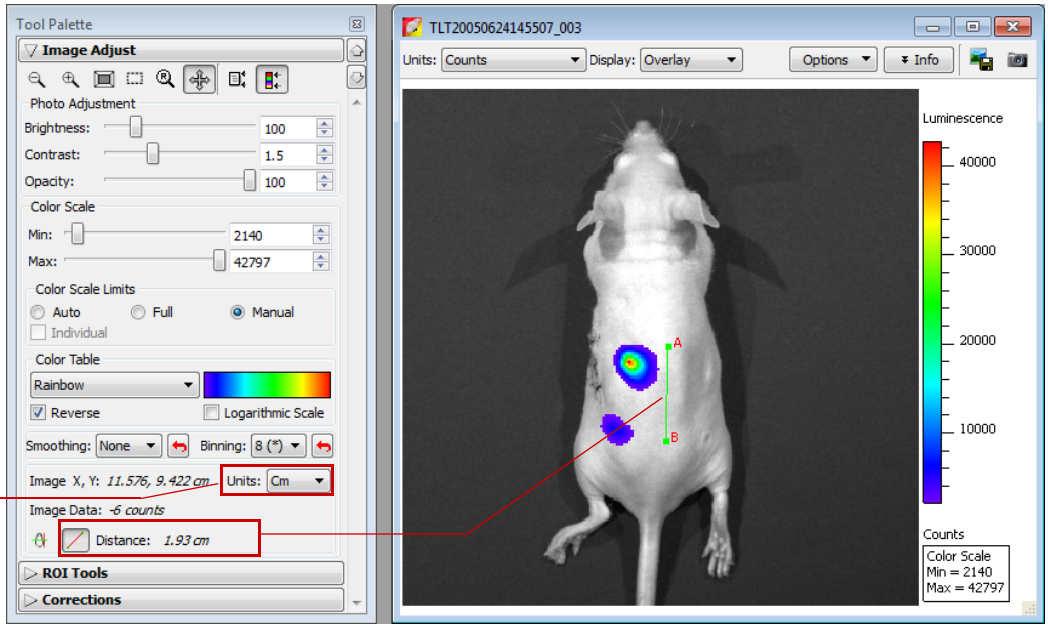
Distance Measurement Tool

1. Open an image, select Cm or Pixels from the Units drop-down list in the Image Adjust tools. and click the **Distance Measurement** button  .

   A measurement tool () appears on the image (Figure 5.13). The Tool Palette displays the length of the cursor.

Figure 5.13 Measuring Distance Using the Measurement Tool

The Tool Palette displays the measurement tool position and length.



2. Change the cursor position or size by dragging the A or B end of the cursor to a new location on the image.

   The measurement information in the Tool Palette is updated.

3. Click the button to hide the cursor.

Image Crop Box

1. Open an image and select Cm or Pixels from the Units drop-down list in the Image Adjust tools.
2. Click the **Image Crop** button in the Image Adjust tools (Figure 5.14).

   The mouse pointer changes a "+".

3. Draw a rectangle on the image using the mouse pointer.

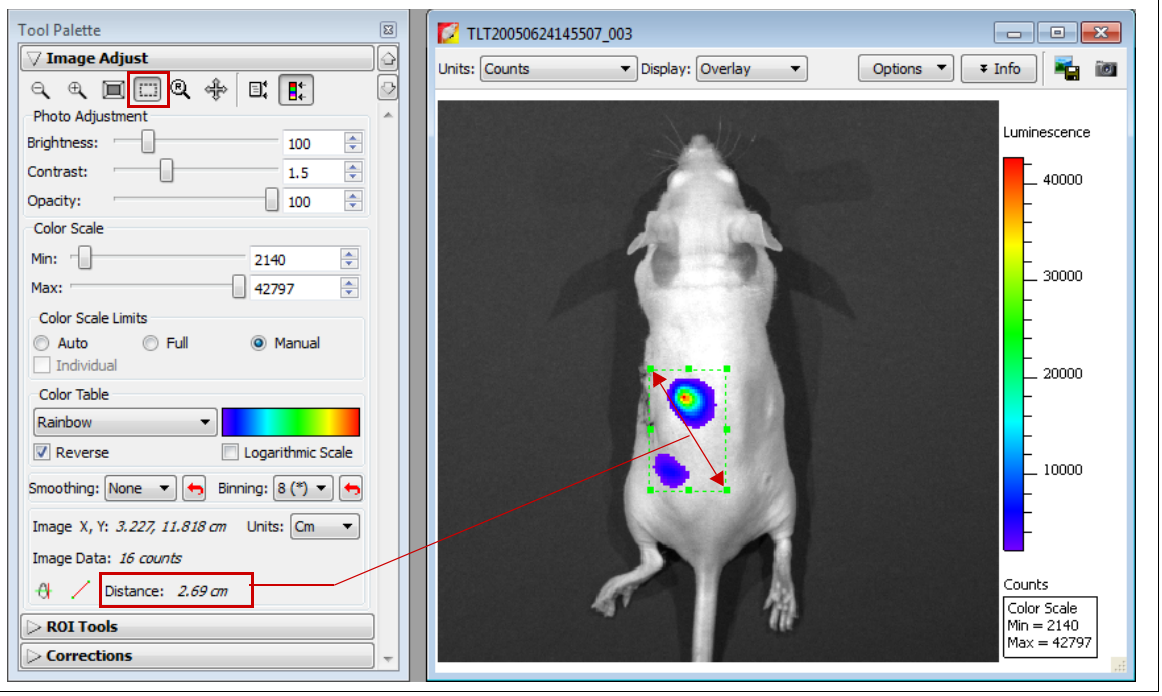
   The length of the crop box diagonal is displayed.

4. Change the size or position of the crop box by dragging a handle on the crop box.

   NOTE: Only the area within the crop box will be exported when you click the button.

5. Click the button to hide the crop box.

Figure 5.14 Measuring Distance Using a Crop Box



5.5 Combining Images Using Image Math

The Image Math tool mathematically combines two images to create a new image. Image math is primary used to subtract tissue autofluorescence background from signal. It provides an alternative to spectral unmixing for autofluorescence background subtraction.

To perform image math, open an image sequence or a group of images. See page 96 for more details on creating a sequence from individual images.

TIP: See the tech note *Image Math* for a quick guide (select **Help → Tech Notes** on the Help menu.

Subtracting Tissue Autofluorescence

To remove tissue autofluorescence from image data, you can use a subtraction method which uses a second excitation filter that is blue-shifted (a background filter) from the primary excitation filter.

The objective of using a background filter is to excite the tissue autofluorescence without exciting the fluorophore. To reduce autofluorescence signal in the primary image data, use the image math tool to subtract the background filter image from the primary excitation filter image.

The software computes the signal corrected for background: (A - B) × k, where:

A = primary image (acquired using the excitation filter)

B = background image (acquired using the background filter)

k = (primary signal/background signal)

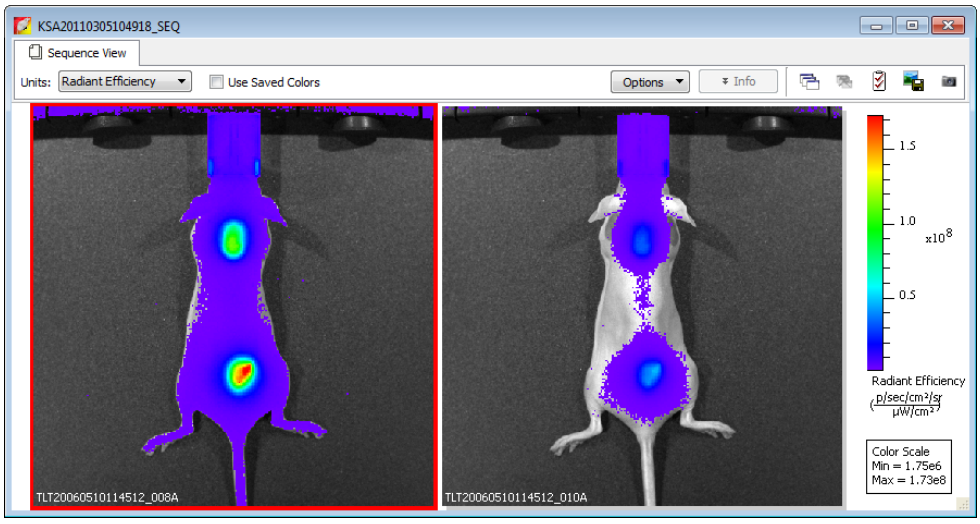
The background signal is obtained from a measurement ROI that is located in an area where no fluorophore signal is present. The scale factor k accounts for different levels of tissue autofluorescence due to different excitation wavelengths and filter transmission characteristics.

After you acquire an image sequence that includes a primary and background image, use the image math tool to subtract tissue autofluorescence. (See page 44 for more details on acquiring an image sequence.)

To subtract tissue autofluorescence:

1. Load the image sequence that includes the primary and background fluorescent images.

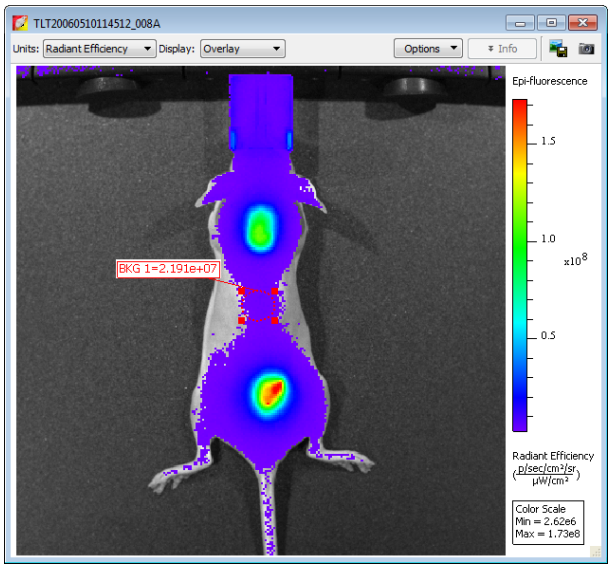
Figure 5.15 Image Sequence



2. Open either the primary or background image:
   a. Optimize the image display using the color scale Min and Max sliders in the Image Adjust tools.
   b. Draw a measurement ROI on an area of the animal that represents background signal (area where no fluorophore signal is present, Figure 5.16).

NOTE: You only need to draw the ROI on one of the images. The software copies the ROI to the other image.

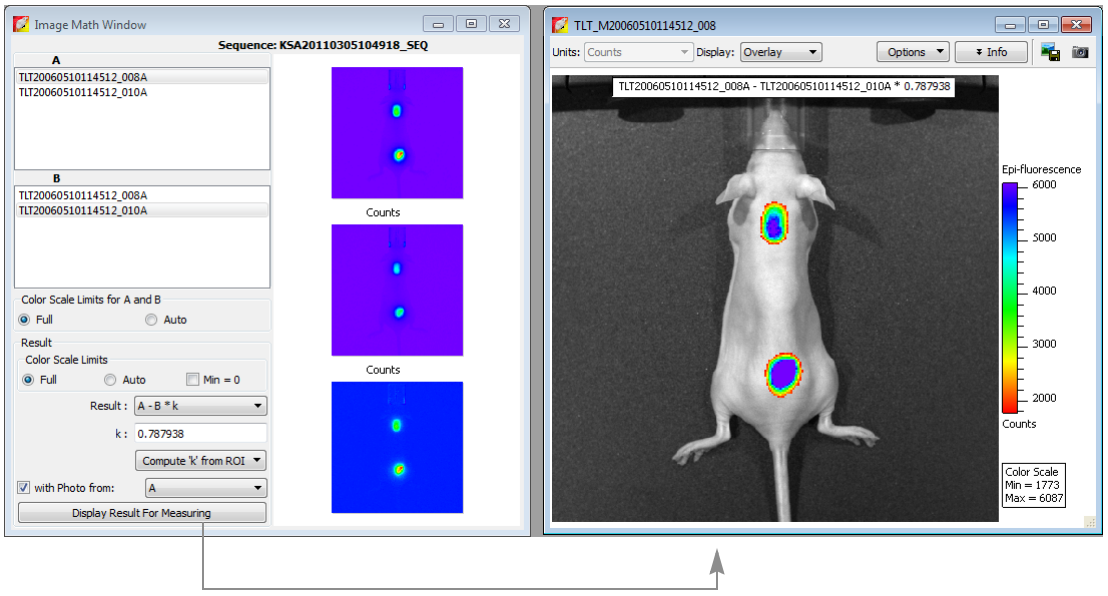
Figure 5.16 Draw Measurement ROI on an Area Representative of Background Signal



3. Select **Tools** → **Image Math for <name>_SEQ** on the menu bar.
4. In the Image Math window that appears, select the primary image in box A. Select the background image in box B (Figure 5.17).

   See Table 5.6 on page 79 more details on the Image Math window.
5. Select the math function 'A-B*k' in the Result drop-down list.

Figure 5.17 Select a Math Function and View the Mathematical Result



6. Click Compute 'k' from ROI and select the ROI (created in step 2) from the drop-down list.

   The background-corrected signal is displayed.

7. To view the mathematical result (overlay mode) in a separate image window, click **Display Result For Measuring**.

   If necessary, use the Color Scale Min and Max sliders in the Image Adjust tools to adjust the image display.

8. To save the new image:

   a. Click the **Save** button. Alternatively, select **File** → **Save** on the menu bar.

   b. Select a directory in the dialog box that appears and click **Save**.

   A folder of data is saved to the selected location (AnalyzedClickInfo.txt, ClickInfo.txt, luminescent and photographic TIF images).

9. To export the new image to a graphic file:

   a. Click the **Export** button.

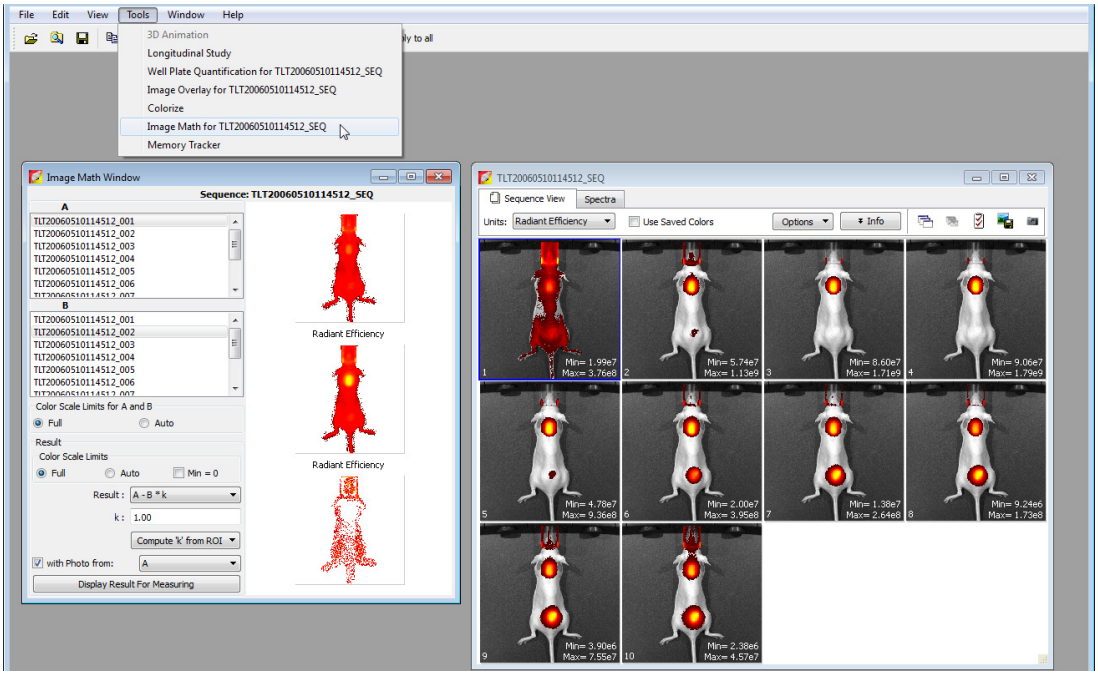
   b. Select a directory in the dialog box that appears, enter a file name, and select the file type from the "Save as type" drop-down list.

   c. Click **Save**.

Creating a New Image

1. Load an image sequence.
2. Select **Tools** → **Image Math for <name>_SEQ** on the menu bar.

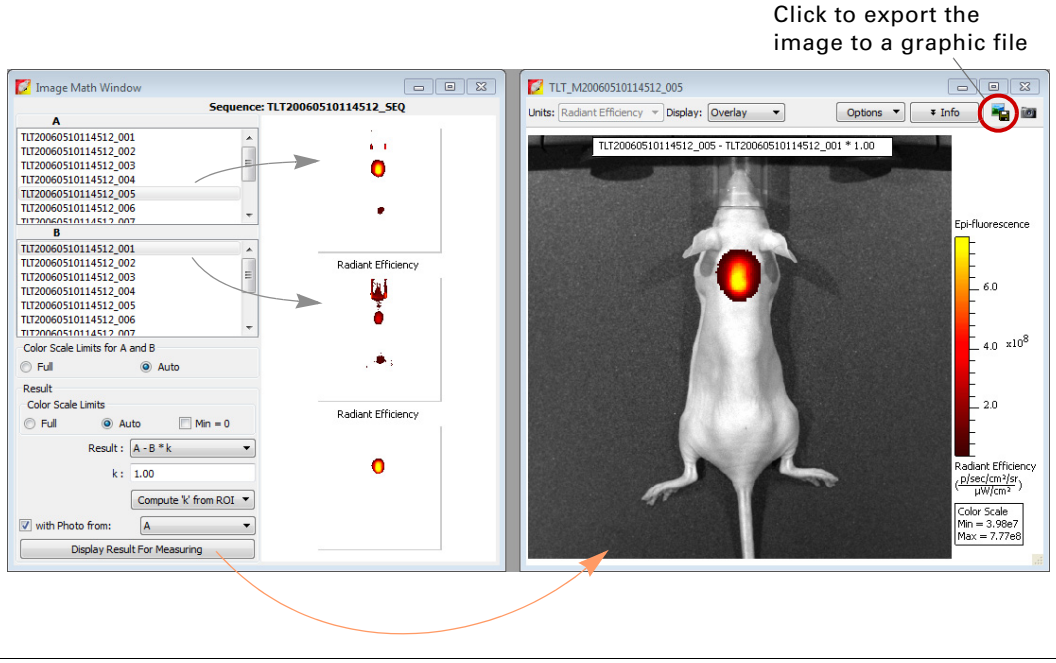
Figure 5.18 Opening the Image Math Window



3. In the Image Math window that appears, select an image from box A and from box B (Figure 5.19).

   The Image Math window shows a thumbnail of image A, image B, and the new image.

Figure 5.19 Image Math Window and New Image



NOTE: For more details on items in the Image Math window, see Table 5.6, page 79

4. Select a mathematical function from the Result drop-down list.
5. To include a scaling factor (k) in the function, enter a value for "k".
6. Click **Display Result for Measuring** to view the new image.
7. To save the new image:
   a. Click the **Save** button. Alternatively, select **File** → **Save** on the menu bar.
   b. Select a directory in the dialog box that appears and click **Save**.
      A folder of data is saved to the selected location (AnalyzedClickInfo.txt, ClickInfo.txt, luminescent and photographic TIF images).
8. To export the image to a graphic file:
   a. Click the **Export** button (Figure 5.19).
   b. Select a directory in the dialog box that appears, enter a file name, and select the file type from the "Save as type" drop-down list.
   c. Click **Save**.

Table 5.6 Image Math Window

Item	Description
Color Ranges for A and B	Full - Choose this option to set the Max and Min values to the maximum and minimum data values in the image. Auto - When this option is chosen, the software sets the Min and Max values to optimize image display and suppress background noise. The Min and Max settings can be manually adjusted to further optimize the image display for your needs. **Note:** The color scale does not affect the image math result.
Color Ranges for Result Image	Full - See above. Auto - See above. Min = 0 - Choose this option to set the minimum data value to zero.
Results	Drop-down list of mathematical functions that can be used to generate the new image, including: A - B*k A + B*k A * B*k A/B if Counts (B) > k (Useful for fluorescence tomography.) (A/B)*k
k, Image Math window	A user-specified scaling factor applied in the results function.
Compute ‘k’ from ROI	This option is useful for subtracting fluorescence background. Draw one ROI in an image on an area considered background. In the “Compute ‘k’ from ROI” drop-down list, select the this ROI.
with Photo from	Choose this option to display the new image in overlay mode using the selected photographic image. (This option is only available if one of the selected images is an overlay.
Display Result for Measuring	Opens the image generated by image math in an image window.

5.6 Overlaying Multiple Images

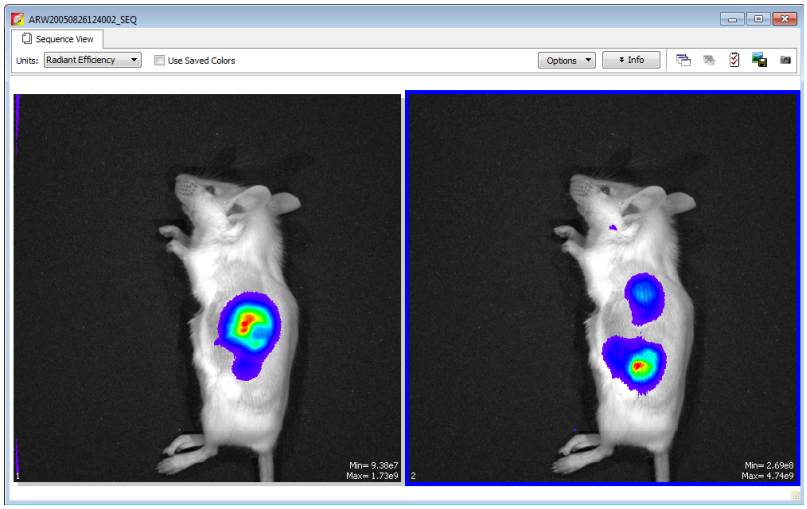
The image overlay tool provides a convenient way to view multiple reporters in one image. You can use the image overlay tool to display multiple luminescence or fluorescence images on one photographic image.

TIP: See the technical note *Image Overlay – 2D* for a quick guide (select **Help → Tech Notes** on the menu bar).

To overlay multiple images:

1. Acquire an image sequence using the appropriate filters for each reporter. Alternatively, create a sequence from images acquired during different sessions. (For more details, see page 96.)
2. Load the image sequence.

Figure 5.20 Image Sequence

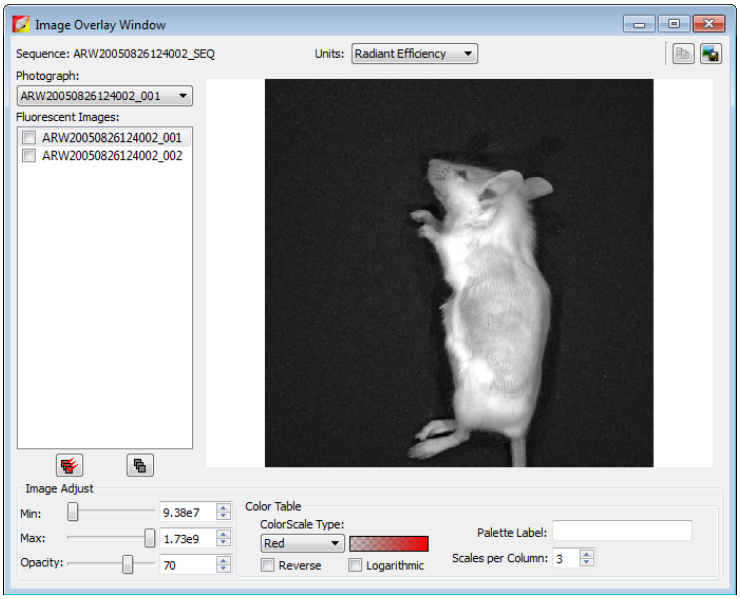


3. Open one of the images and optimize the image display using the color scale Min and Max sliders in the Image Adjust tools.

   To view all images in the sequence, click the **Display All** button to open each image (overlay mode) in a separate image window.
4. Select **Tools→ Image Overlay for <sequence name>_SEQ** on the menu bar.

   The image overlay window appears and shows the first photograph in the sequence.To view a different photograph, make a selection from the photograph drop-down list.

Figure 5.21 Image Overlay Window



5. To overlay all images, click the button.

The overlay appears. The photograph is at the bottom of the stack and the last fluorescent or luminescent image in the list is at the top of the stack.

Figure 5.22 Generated Overlay

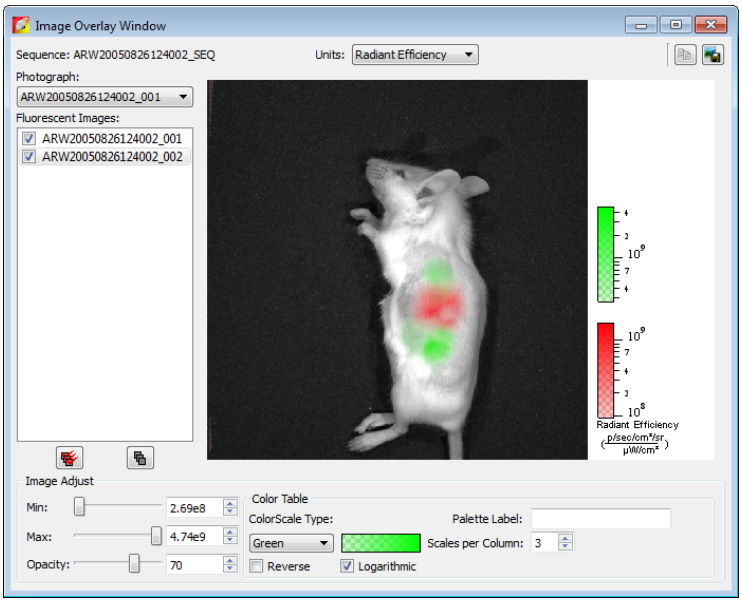


Table 5.7 Image Overlay Window

Item	Description
Units	Choose the type of units for displaying the fluorescent or luminescent image. See the concept tech note *Image Display and Measurement* for more details on measurement units.
Photograph	A drop-down list of the photographs in the image sequence.
Fluorescent or Luminescent Images	The sequence images.
	Copies the overlay to the system clipboard.
	Click to export the overlay to a graphic file.
	Click to include all fluorescent or luminescent images in the overlay.
	Click to remove all fluorescent or luminescent images from the photograph.
Image Adjust	Tools for adjusting the appearance of the highlighted fluorescent or luminescent image. Adjustments can only be made on one image at a time. Min – The minimum pixel intensity associated with the color scale for an image. Pixels less than the minimum value are not displayed. Max – The maximum pixel intensity associated with the color scale for an image. Pixels greater than the maximum value are displayed in the maximum color. Opacity – Controls the opacity of the fluorescent or luminescent image.
Color Table	Tools for selecting and modifying the color scale associated with an image. Color Scale Type – Choose BlackLevel to show black at the low end of the color scale. Choose WhiteLevel to show white at the low end of the color scale.  Click the drop-down arrow to select a color table for the image data. See the concept tech note *Image Display and Measurement* for more details on color tables (select **Help → Tech Notes** on the menu bar). Reverse – Choose this option to reverse the selected color table. Logarithmic – Choose this option to apply a log scale to the relationship between numerical data and the color range in the color table. A log scale improves the visibility of dark areas in an image.
Palette label	To include a brief line of text next to the color scale, enter text in the palette label box, then press the **Enter** key. To remove the text from the image window, delete the text in the palette label box and press **Enter**.
Scales per Column	Sets the number of color scales to display in a column.

5.7 Rendering Intensity Data in Color

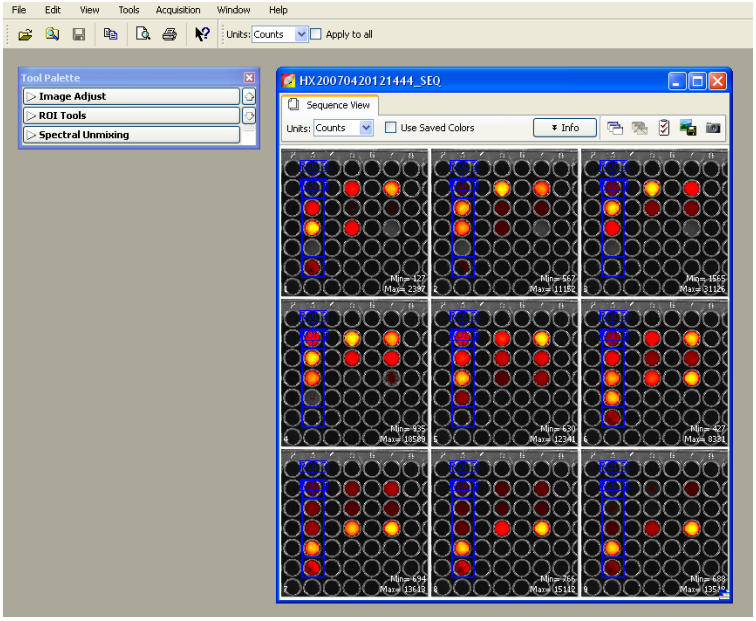
The colorize tool renders luminescence or fluorescence data in color, enabling you to see both intensity and spectral information in a single view. The tool provides a useful way to visualize multiple probes or scale probe signals that are not in the visible range.

To view colorized intensity data:

1. Load an image sequence.

Figure 5.23 Microplate Images

Images were acquired using different combinations of excitation and emission filters. The samples are quantum dot nanocrystals (700 or 800 nm).



2. Select **Tools** → **Colorize** on the menu bar.

   The software renders each luminescent or fluorescent image in color and combines them into a single image (Figure 5.24).

Figure 5.24 Colorize View

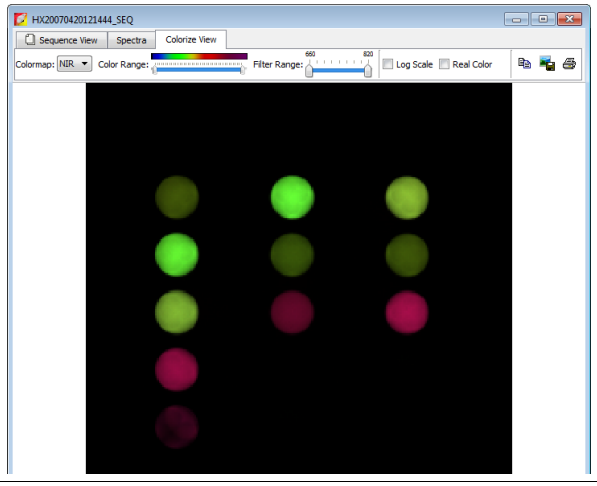


Table 5.8 Colorize Tools

Item	Description
Colorize View	
Color Map	NIR – A special camera setup that extends the color response into the near infrared range. Near infrared fluorophores appear red to purple using the NIR camera setup. VIS – Regular camera setup that mainly renders color in the visible range. It is similar to the color response of a commercial digital camera. NIR fluorophores appear dark red to invisible using the VIS camera setup.
Color Range	The color map indicates the color range of the selected camera setup from short to long wavelength. The two sliders determine the lower and upper limits of the color range that is used to render color. The parts of the color map outside the selected range are not used in the color rendering process. By default, the entire color range is selected.
Filter Range	The wavelength range of the luminescent images in the sequence. The two sliders determine the lower and upper end of the filter range. Only the parts of the image that are within the selected wavelength range are colorized. By default, the entire filter range is selected.
Log Scale	If this option is chosen, the dynamic range of the brightness in the image is compressed using a log scale. This improves the visibility of dark areas in the image.
Real Color	If this option is chosen, the colors are rendered using the wavelengths that directly correspond to the camera setup. For example, GFP appears green using real color rendering. If this option is not chosen, the original wavelength range of the image is modified to include the entire visible wavelength range of the camera setup. This helps improve the color contrast.
	Click this button to copy the colorize view to the system clipboard.
	Click this button to export the colorize view as a graphic file (for example, .jpg).
	Click this button to print the colorize view.

5.8 Annotating or Tagging Images

Adding Comments

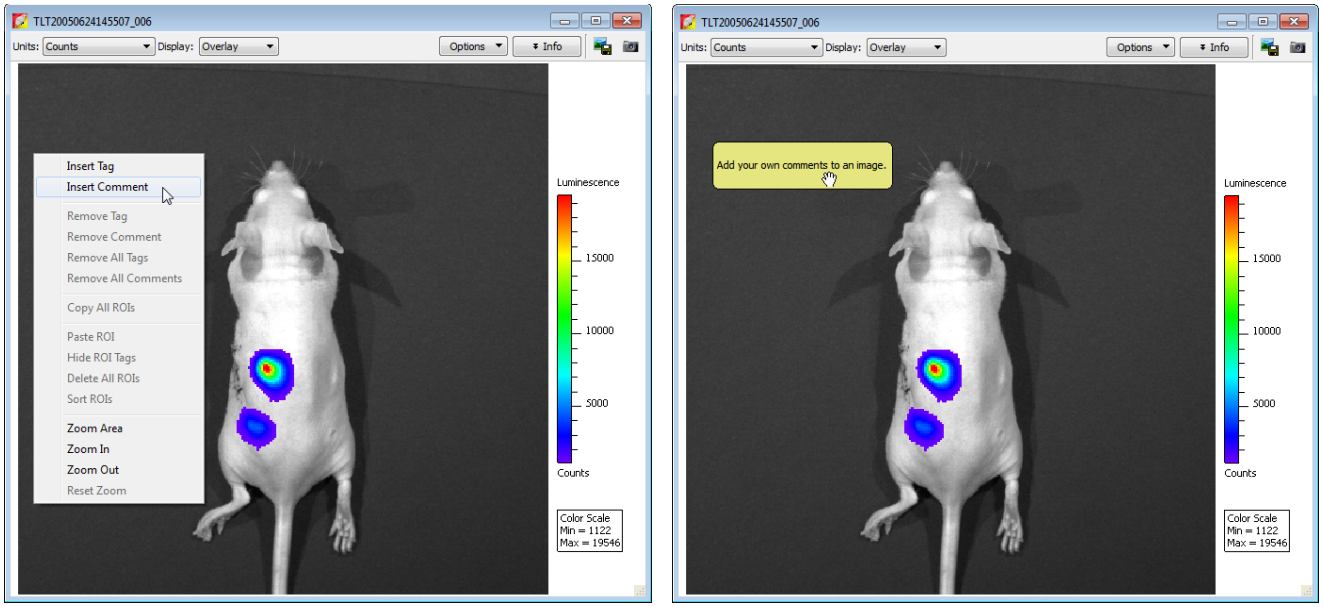
Comments can be added to an image and saved with the image.

To add comments:

1. Open an image.
2. Right-click the image and select **Insert Comment** on the shortcut menu. Enter comments in the yellow box that appears (Figure 5.25).
3. To move a comment in an image:
   a. Position the mouse pointer over the comment.
   b. When the hand tool appears, drag the comment box, then click the mouse to set the location.
4. Remove comments by doing either of the following:
   - Right-click a comment and select **Remove Comment** on the shortcut menu.

- To remove all comments, right-click the image and select **Remove All Comments** on the shortcut menu.

Figure 5.25 Adding Comments



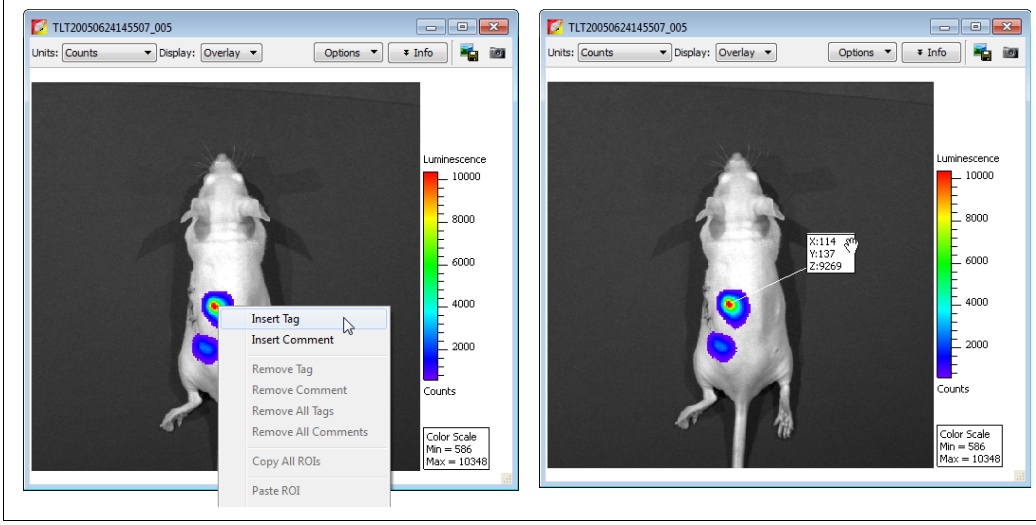
Applying Tags

An image tag displays the x,y pixel coordinates and the pixel intensity (z, counts or photons) at a user-selected location.

To apply a tag:

1. Right-click a location in the image.
2. Select **Insert Tag** on the short cut menu.

Figure 5.26 Tag an Image (left), Move the Tag Label (right)



3. To move a tag:
   a. Position the mouse pointer over the tag.
   b. When the hand tool appears, drag the tag, then click the mouse to set the tag location.
   A line between the pixel and the tag identifies the location associated with the tag.

5.9 Exporting an Image

The Image Layout window (Figure 5.27) provides an alternative way to:

- Annotate and export an image (for example, .bmp)
- Print an image
- Copy an image to the system clipboard

1. Load an image or image sequence and select **View → Image Layout Window** on the menu bar to open the Image Layout window.
2. Click the button to paste the active image into the Image Layout window.
3. Drag a handle ■ at a corner of the image to resize the image.
4. Drag the image to reposition it in the window.

Figure 5.27 Image Layout Window

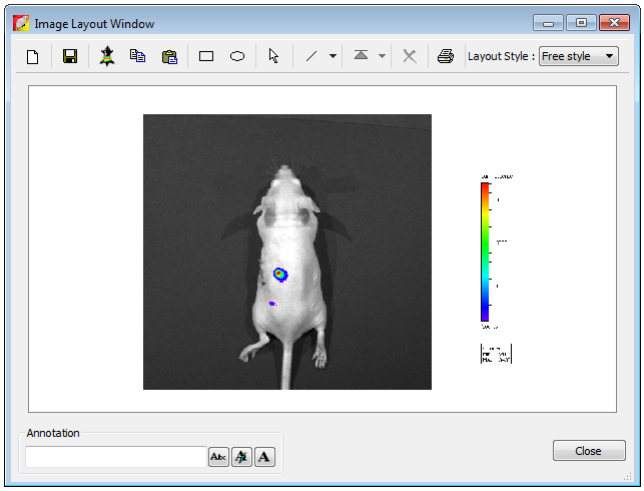
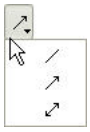
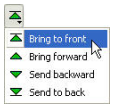
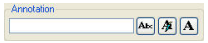


Table 5.9 Image Layout Window

Item	Description
	Clears the Image Layout window. **Note:** If you do not clear the layout (click the button) before you close the Image Layout window, the same window contents are displayed the next time the window is opened
	Opens a dialog box that enables you to save the Image Layout window contents to a graphic file.
	Pastes an image of the active data in the Image Layout window.
	Copies the contents of the Image Layout window to the system clipboard.

Table 5.9 Image Layout Window (continued)

Item	Description
	Pastes the contents of the system clipboard to the Image Layout window.
	Rectangle drawing tool
	Ellipse drawing tool
	Pointer tool
	Arrow and line drawing tool
	Select an the item in the Image Layout window. To move the item to the front or back in the window, choose an option from the  drop-down list.
	Deletes the selected image.
	A drop-down list of formatting options for the Image Layout window. For example, the 2x2 layout style provides 4 separate layout areas in the window. A different image can be pasted into each layout area.
	To apply notes to an image, enter text in the annotation box and press **Enter**. Drag the text to the location of interest in the image.
	Opens a dialog box that enables you to select a font or edit the font style and size.
	Opens a color palette that enables you to select a font color or specify a custom font color.
	Opens a text editor that enables you to edit the selected text.

5.10 Exporting an Image Sequence

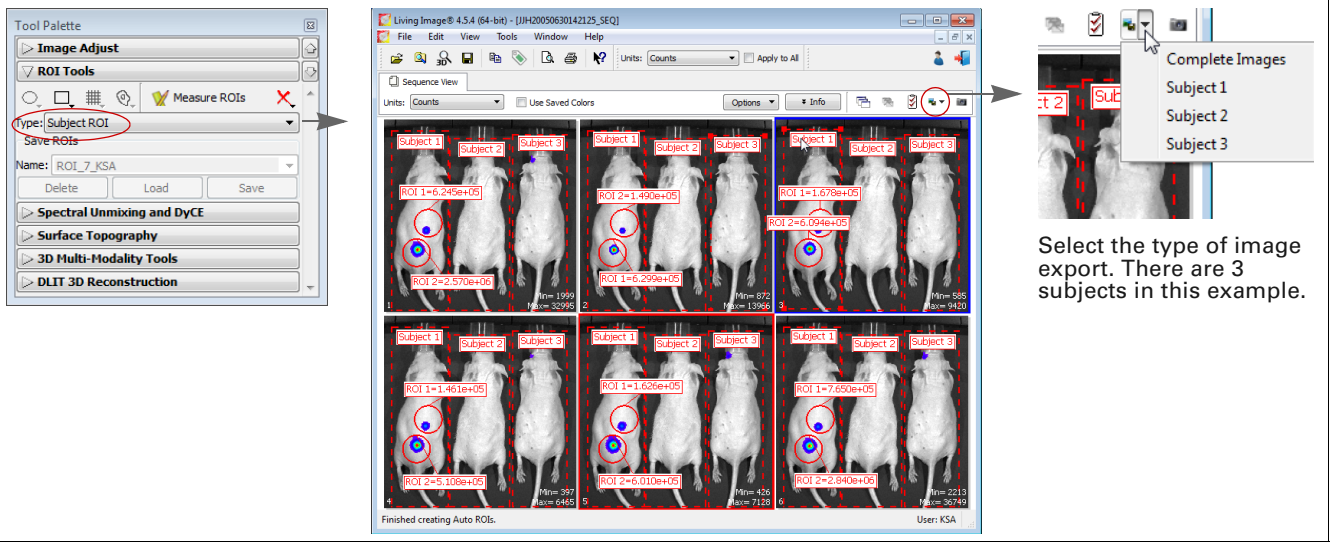
You can export sequence images or images of a user-selected subject from a sequence to a graphic file.

Preparing for Export

A subject ROI must be applied to each subject before exporting sequence images.

1. Load an image sequence.
2. Identify each subject with a subject ROI (Figure 5.28).
   a. Choose "Subject ROI" from the Type drop-down menu in the ROI tools
   b. Click the button and select **Auto All**.

Figure 5.28 Identify Each Subject With a Subject ROI



3. Click the Export Graphics button down arrow and choose an option from the drop-down list.
   - Export All Images – All images will be exported to one file (Figure 5.29 on page 89). See below for instructions.
   - Subject "N" – Images of the selected subject can be exported to either:
     - One file (Figure 5.32 on page 90). See page 89 for instructions.

       or
     - Separate files (Figure 5.35 on page 92). See page 91 for instructions.

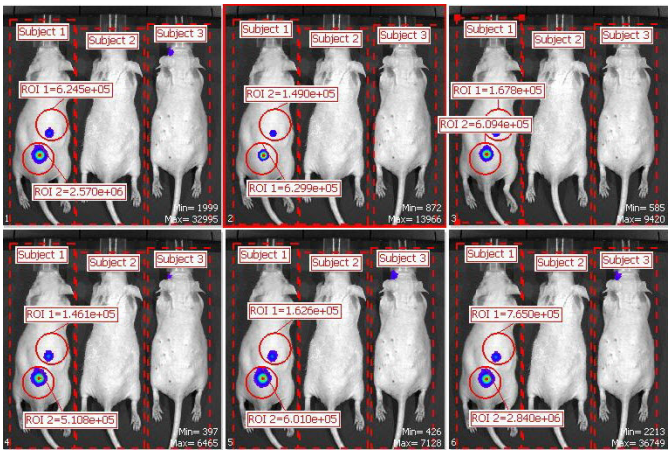
Export All Images

All images of the sequence will be exported to one file (Figure 5.29).

NOTE: Be sure to apply subject ROIs to the images before proceeding with the export. See *Preparing for Export* on page 88 for instructions.

1. Click the Export Graphics button down arrow and choose "Complete Images" from the drop-down list (Figure 5.28).
2. Select the file location, file type, and enter a file name in the dialog box that appears. Click **Save**. Figure 5.28 shows an example of the exported image.

Figure 5.29 Example "Complete Images" Export



Export Images of a Subject

NOTE: Be sure to apply subject ROIs to the images before proceeding with the export. See *Preparing for Export* on page 88 for instructions.

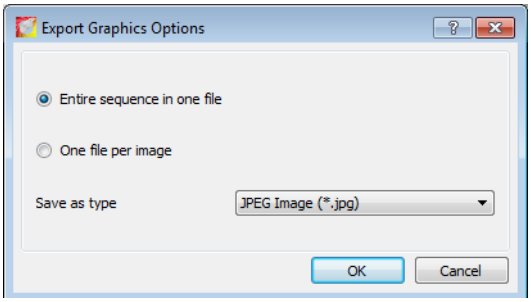
Each image of a subject can be exported to either:

- A single file – Images of the selected subject will be cropped to the subject ROI and placed side-by-side in a single row in the order in which they were acquired (Figure 5.32 on page 90).
- One image per file – Each image of the selected subject will be cropped to the subject ROI and exported to separate graphic file (Figure 5.35 on page 92).

Exporting the Images to a Single File

1. Click the Export Graphics button down arrow and select a subject from the drop-down list. The Export Graphics Options dialog box appears (Figure 5.30).

Figure 5.30 Export Graphics Option



2. Choose the "Entire sequence in one file" option, select a graphic file type, and click **OK** (Figure 5.30).
3. Select a location and enter a name for the file in the next dialog box that appears (Figure 5.31). Click **Select Folder**.

   The software will export the image to a subfolder (named with the sequence identifier "xxx_SEQ") in the selected folder.

Figure 5.31 Select a Folder for the Export

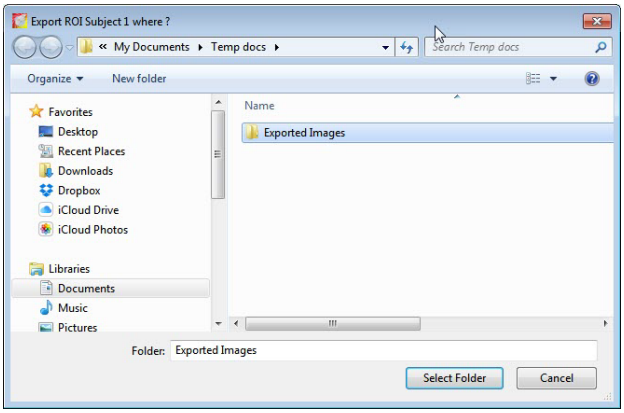
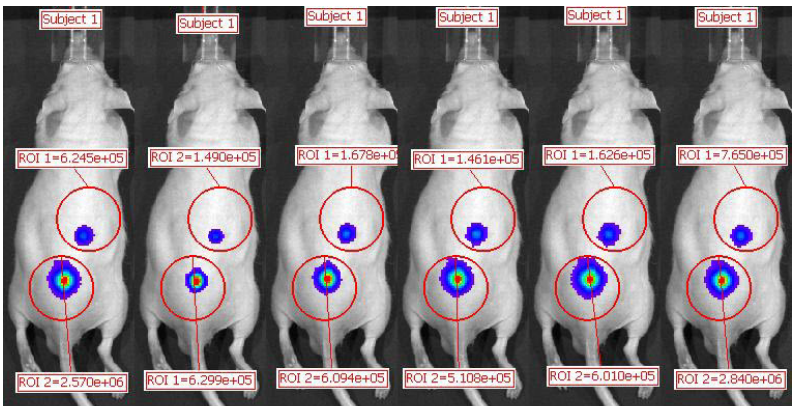


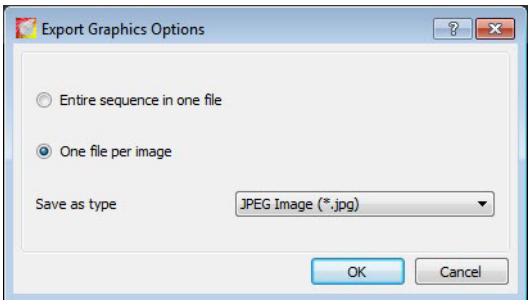
Figure 5.32 Subject Images Exported to One Graphic File



Exporting Each Image to a Separate File

1. Click the Export Graphics button down arrow and select a subject from the drop-down list. The Export Graphics Options dialog box appears (Figure 5.30).

Figure 5.33 Export Graphics Option



2. Choose the "One file per image" option, select a graphic file type, and click **OK** (Figure 5.33).
3. Select a folder location in the next dialog box that appears. Click **Select Folder** (Figure 5.34). The software will export each image to a subfolder (named with the sequence identifier "xxx_SEQ") in the selected folder (Figure 5.35).

Figure 5.34 Select a Folder for the Export

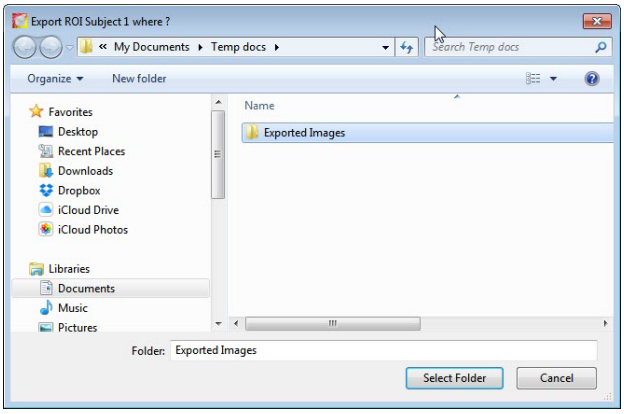
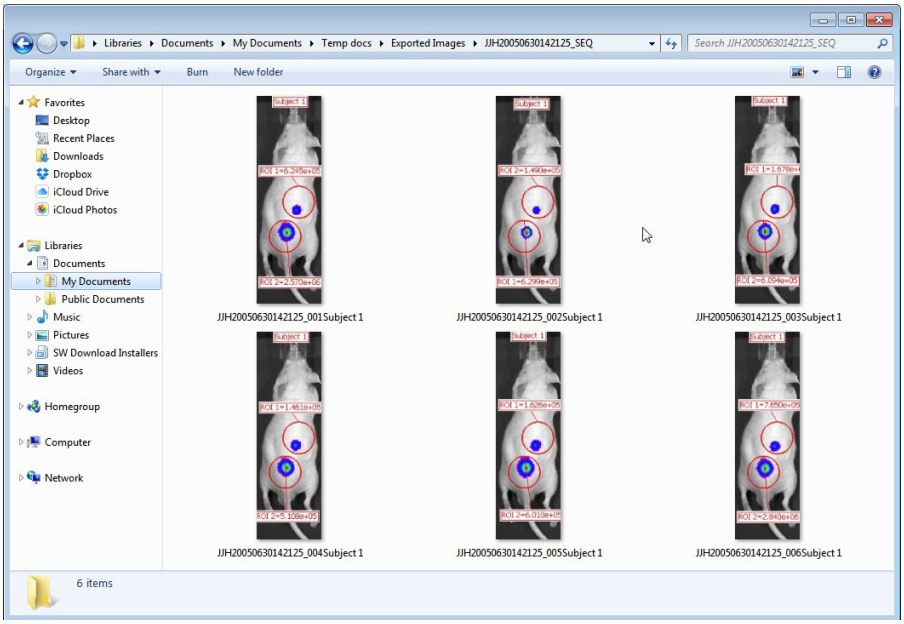


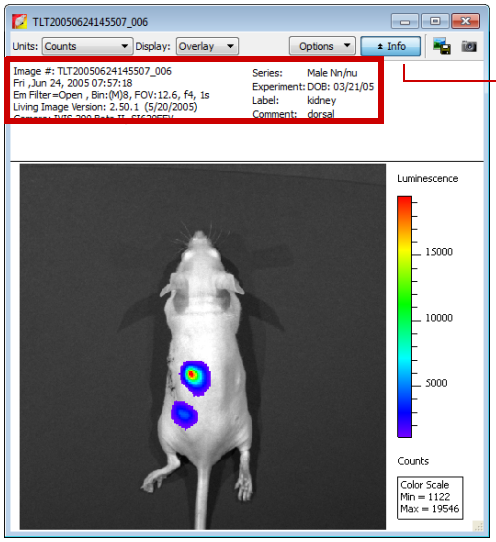
Figure 5.35 Subject Images Exported to Separate Graphic Files



5.11 Managing Image Information

At acquisition, the software captures image information such as camera parameters and any image label information you entered at acquisition time (Figure 5.36).

Figure 5.36 Image Label Information



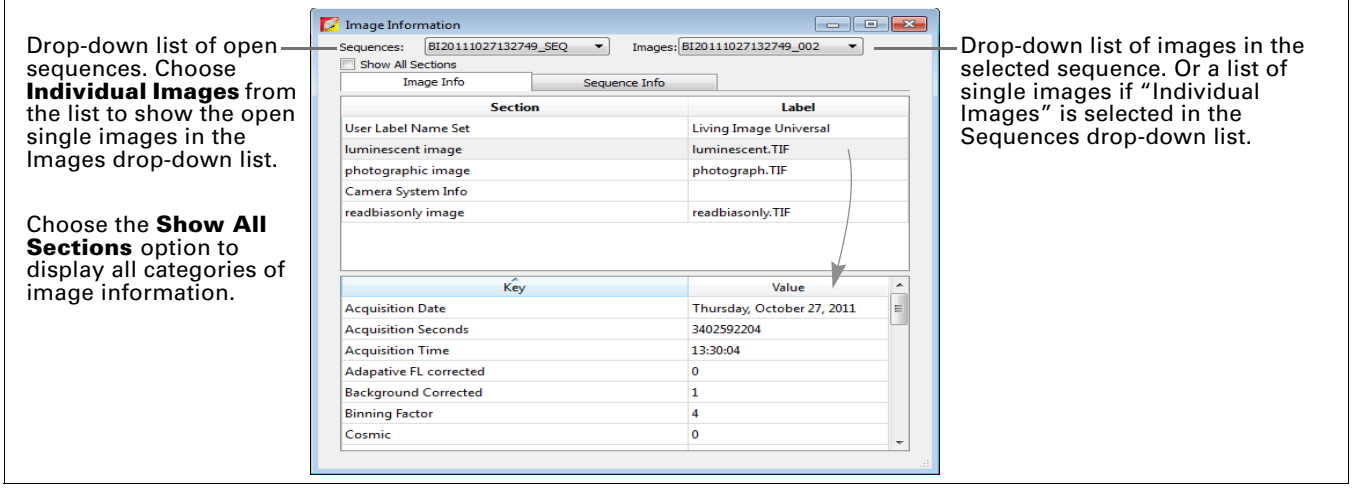
Viewing Image Information

Detailed information about images is available in the View menu.

1. Open an image or sequence.
2. Select **View** → **Image Information** on the menu bar.
   The Image Information window appears.

3. Choose an image by making a selection from the Sequences drop-down list and the Images drop-down list (Figure 5.37).

Figure 5.37 Viewing Image Information



4. To view particular information, select a category in the upper box to show the associated information in the lower box. For example, select luminescent image in the upper box to show the luminescent image acquisition parameters.

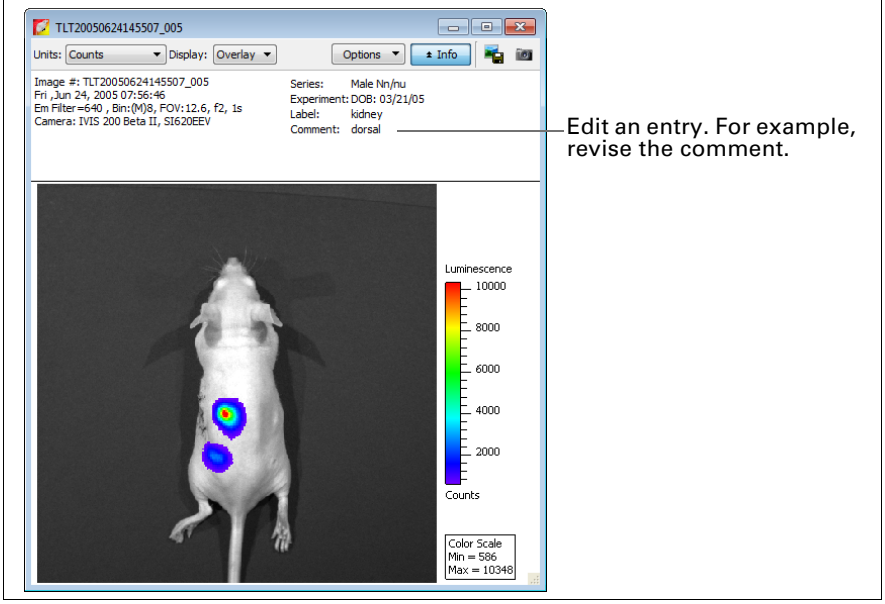
Editing the Image Label

You can edit image label information or add information to the label after acquisition.

To edit the image information:

1. Open an image or sequence.
2. Click **Info** to display the image label (Figure 5.38).

Figure 5.38 Image Information



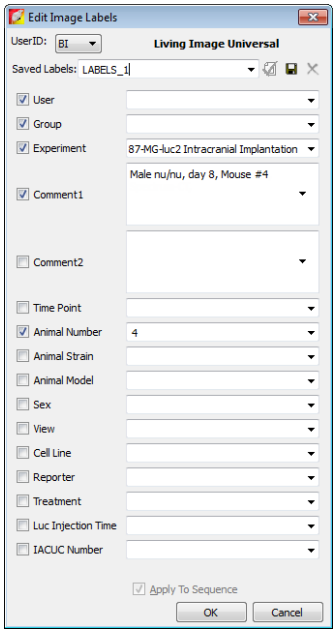
3. Edit the label information.

To add information to the image label:

1. Click the toolbar button. Alternatively, select **Edit** → **Image Labels** on the menu bar.
2. Select information and/or enter a comment in the Edit Image Labels box that appears (Figure 5.39).

NOTE: If a single image is active, changes are applied to that image only. If a sequence is active, changes are applied to each image of the sequence.

Figure 5.39 Edit Image Labels



3. Click **OK** when finished.

   The image information is updated.
4. Save the image to save the updated image label (select **File** → **Save** or **File** → **Save As** on the menu bar).

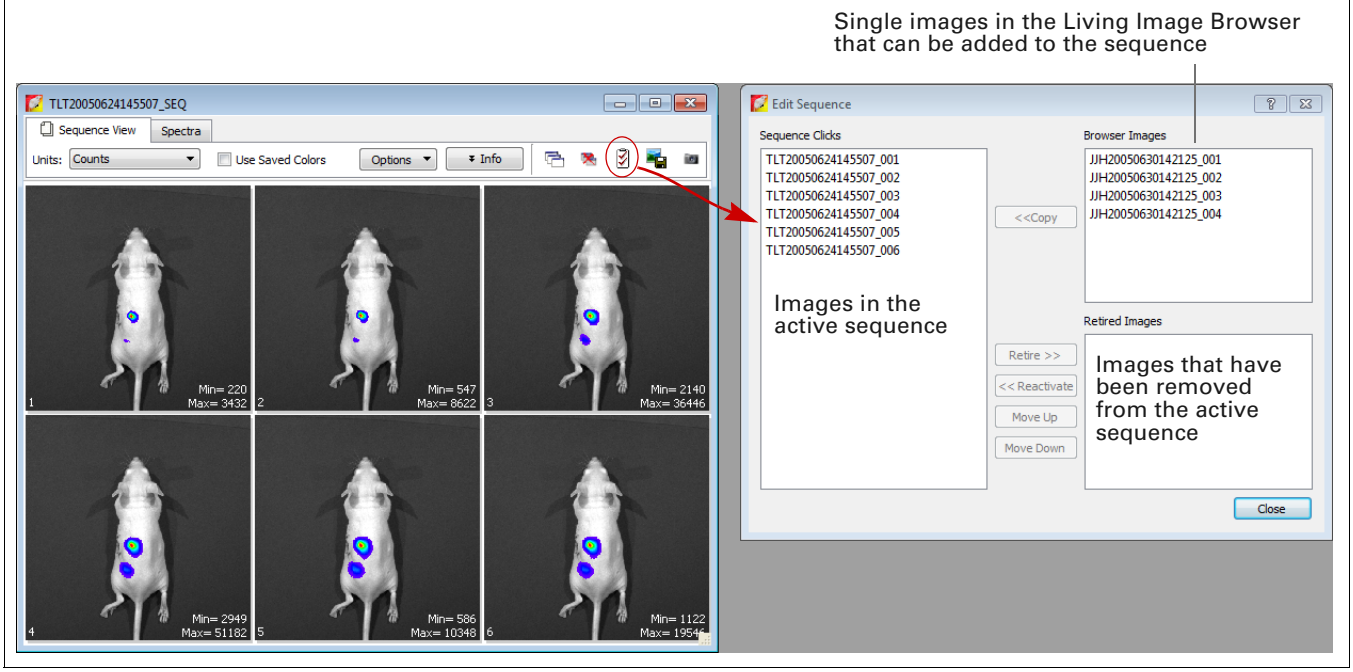
5.12 Managing Image Sequences

Editing a Sequence

You can add or remove individual images from a sequence. Only individual images, not an image sequence, can be added to a sequence.

1. Open the image sequence that you want to edit.
2. If you plan to add images to the sequence, browse for images in the Living Image browser. (See page 57 for more details on browsing.)
3. Click the **Edit** button in the image window(Figure 5.40).

Figure 5.40 Editing a Sequence



4. Choose the image(s) to add or remove (*retire*) from the sequence in the Edit Sequence box that appears (Figure 5.40).

   To add an image to the sequence, select an image from the "Browser Images" and click **Copy**. To remove an image from the sequence, choose an image from "Sequence Clicks" and click **Retire**.

5. To restore a retired image to the sequence, select the retired image and click **Reactivate**.

6. To reorder the sequence, select an image and click **Move Up** or **Move Down**.

> **NOTE:** The **Move Up** and **Move Down** buttons are only available when the sequence view window displays images in the default sort order. If the TimeStamp or UserID sort order is selected, the images cannot be reordered.

7. Click **Close** when you are finished editing the sequence.

   The updated image sequence is displayed.

Creating a Sequence From Individual Images

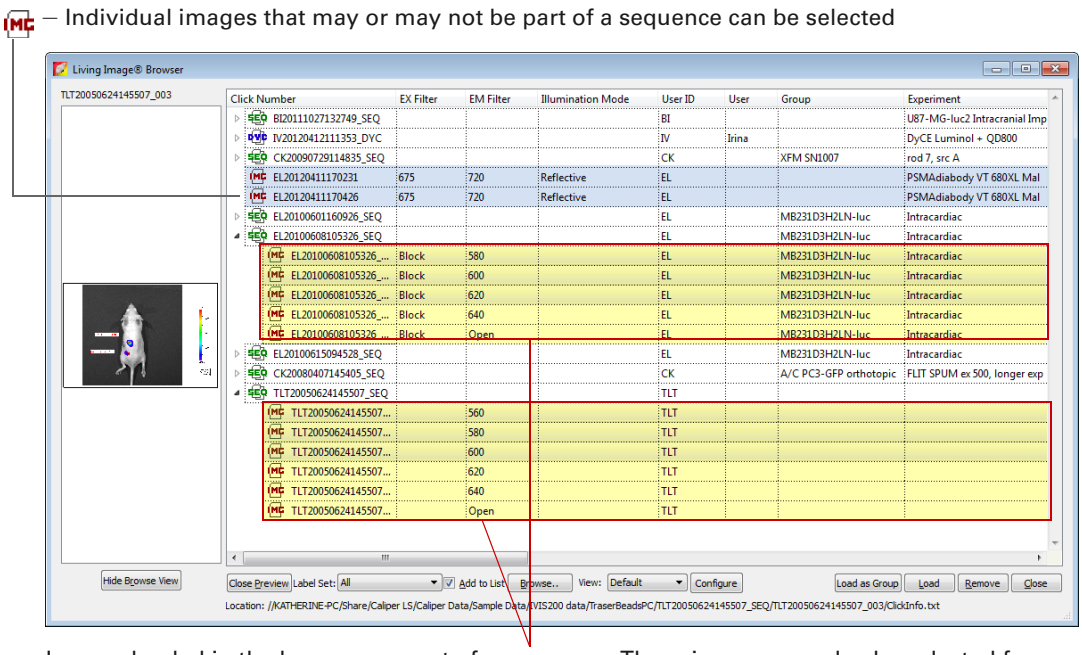
This section explains how to create a sequence from images acquired during different sessions.

TIP: Also see the tech note *Loading Groups of Images* for helpful information (select Help → Tech Notes on the menu bar).

1. Browse for the images of interest using the Living Image Browser. (See page 57 for more details on browsing.)

NOTE: Browse for individual images (which may or may not be part of a sequence), not image sequences.

Figure 5.41 Living Image Browser



2. In the browser, select the images that you want to group together (Figure 5.41).

   To select adjacent images in the browser, press and hold the **Shift** key while you click the first and last file in the selection.

   To select non-adjacent images in the browser:

   - PC users - Press and hold the **Ctrl** key while you click the images of interest in the browser.
   - Macintosh users - Press and hold the **Cmd** key (apple key) while you click the images of interest in the browser.

3. Click **Load as Group**.

   The image thumbnails are displayed together in an image window.

   NOTE: Images loaded as a group are displayed by default in Counts units with the Luminescent color table (set in the Preferences). If working with fluorescent images, be sure to change the units to Radiant Efficiency and, if desired, select a different color table.

4. Save the images as a sequence:

   a. Click the Save button . Alternatively, select **File** → **Save** on the menu bar.

   b. Select a folder in the dialog box that appears and click **OK**.

6 Measuring Signal in 2D Image Data

6.1 About ROIs

This chapter explains how to measure the signal (surface intensity) within a *region of interest* (ROI) in 2D image data. Four types of ROIs are available for images (Table 6.1).

Table 6.1 Types of ROIs

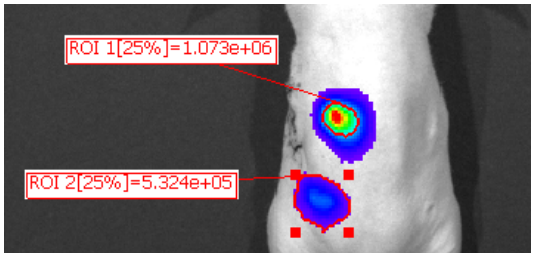
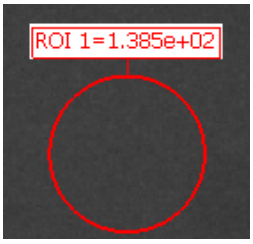
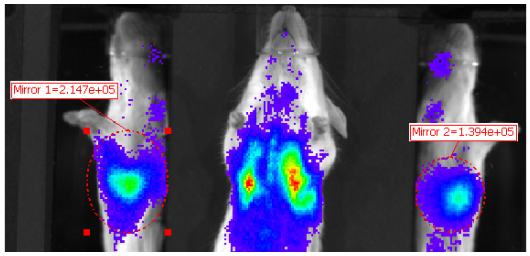
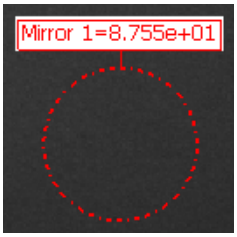
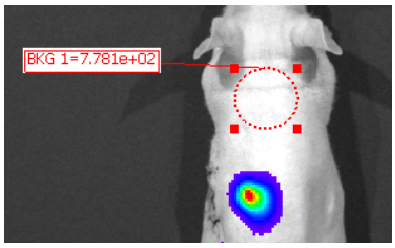
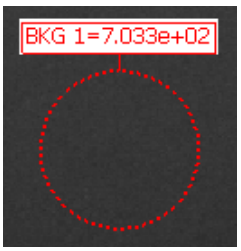
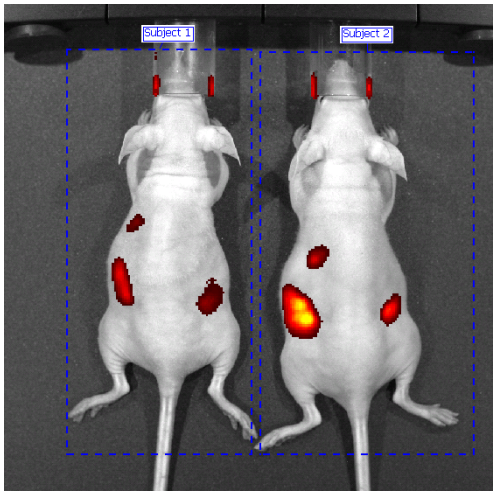
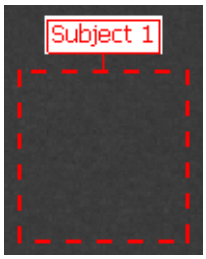
ROI Name	Description	ROI Line and Available Shapes	See Page
Measurement ROI	Measures the signal (surface intensity) in an area of an image. 	 Circle, square, grid, or contour	102
Mirror ROI	 Images acquired using the Side Imager have three views: left, right, and center. ▪ Left or right images – Measure signal using a mirror ROI. ▪ Center image (non-reflected view) – Measure signal using a measurement ROI.	 Circle or square	109

Table 6.1 Types of ROIs (continued)

ROI Name	Description	ROI Line and Available Shapes	See Page
Average Background ROI	Measures the average signal (surface intensity) in a user-specified area of an image that is considered background. Only available in image view. **Note:** Using this type of ROI is optional. If the animal has significant autoluminescence or autofluorescence, you can determine a background-corrected signal in a measurement ROI by subtracting an average background ROI from a measurement ROI. 	 Circle or square	106
Subject ROI	Identifies a subject in an image. **Note:** Using this type of ROI is optional. A subject ROI enables you to: ▪ Automatically associate (link) a measurement and average background ROI for background-corrected ROI measurements when there is significant autoluminescence or autofluorescence. ▪ Show the subject in which an ROI appears in the ROI table. This is helpful when one image includes multiple subjects and signals. 	 Square	108

6.2 Overview of ROI Tools

The ROI tools that appear in the Tool Palette depend on the type of ROI selected from the Type drop-down list, and whether an image or sequence is active. Table 6.2 provides a description of the ROI tools. Some ROI parameters are only available if "Show Advanced Options" is selected in the General Preferences.

ROI measurements and measurement statistics are available in the ROI Measurements table which provides a convenient way to review or export ROI information. See *ROI Measurements* on page 118 for more information.

Figure 6.1 ROI Tools

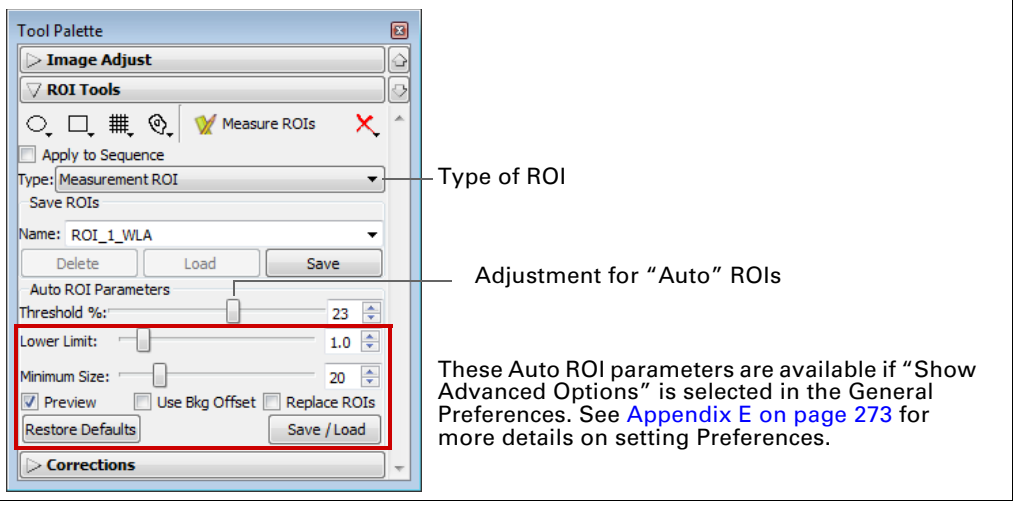


Table 6.2 ROI Tools

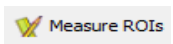
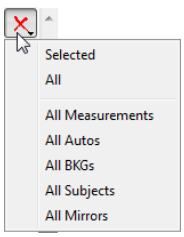
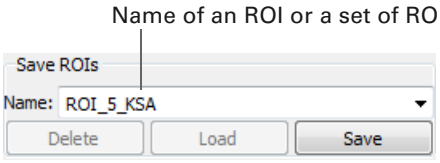
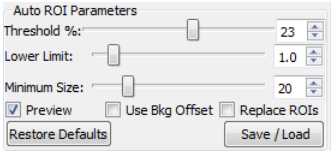
Item	Description
	Click to apply circle ROIs to an image or sequence.
	Click to apply square ROIs to an image or sequence.
	Click to specify the grid ROI to an image or sequence. This tool is useful for an image of a multi-well culture plate or microplate.
	Click to apply contour ROIs to an image or sequence. Select **Auto All** to automatically draw ROIs on an image or sequence using the auto ROI parameters. Click and select **Auto 1** to automatically draw one ROI at a user-selected location using the auto ROI parameters. See Table 6.3 on page 104 for more details on using the auto ROI features.
	Click to display the ROI Measurements table or compute intensity signal in an ROI. See *ROI Measurements* on page 118 for more information.
	Click  to display a drop-down list of ROI delete options for the active image data. These commands delete ROIs from image data. If an image is active and the "Apply to Sequence" option is selected, the delete operation is applied to all images in the sequence. **Note:** These commands do not delete named ROIs that are saved to the system (ROIs in the Name drop-down list).

Table 6.2 ROI Tools (continued)

Item	Description
Apply to Sequence	This option is available when an image of a sequence is open. If this option is selected: ▪ ROIs created on the active image will also be created on the other images of the sequence. ▪ Adjustments to an ROI in the image will be applied to related ROIs in the other images of the sequence. See Table 6.3 on page 104 for more information about related ROIs. ▪ Deleting an ROI in the image will delete related ROIs from the other images of the sequence.
Type	Choose the ROI type from the Type drop-down list: **Measurement** – Measures the signal intensity in an area of an image. **Average Bkg** – Measures the average signal intensity in a user-specified area of the image that is considered background. **Subject ROI** – Identifies a subject animal in an image. The software automatically associates a measurement and an Average Bkg ROI that are included in the same subject ROI. Using this type of ROI is optional. **Mirror ROI** – Measures the signal intensity in the left or right views of an image acquired using the Side Imager, taking mirror reflection effects into account.
Save ROIs to the system	 These ROI tools are only available when an image is active. ROIs (parameters only such as coordinates, type, shape, location) can be saved to the system (per user) and used to apply the ROIs to other images. These ROIs appear in the Name drop-down list. See page 112 for instructions. **Note:** ROIs can also be saved with the image data. The software prompts you to save ROIs before closing image data. ROIs saved with the image do not appear in the Name drop-down list.
Auto ROI Parameters	Parameters that specify how the auto ROI tool draws an ROI. **Threshold %** – If the Auto All or Auto 1 method is selected, the Threshold % specifies the minimum percent of peak pixel intensity that a pixel must have to be included in an ROI identified by the software. After ROIs are drawn on an image, if you modify the Threshold% (move the slider or enter a new value), the software automatically updates the ROIs.  **Note:** These Auto ROI parameters are only available if "Show Advanced Options" is selected in the general preferences. See Appendix E on page 273 for details on setting preferences. **Lower Limit** – Specifies a multiple (1 to 10) of the color scale minimum that sets the lower threshold for identifying an ROI. For example, if the lower limit = 2 and the color scale minimum = 1000 counts, then the auto ROI tool will only draw an ROI on areas of 2000 counts or greater. This helps create ROIs only within pixels visible on the image. **Minimum Size** – Sets the minimum size of an ROI (measured in pixels). For example if the minimum size is set at 50, then ROIs created on the image must be greater than 50 pixels in size. **Preview** – If this option is chosen, the software draws the ROI each time a parameter is changed. ROI parameters can be saved without drawing the ROI. **Use Bkg Offset** – Choose this option to measure background-corrected signal. This is typically used to remove natural animal background luminescence, and should not be confused with the dark-charge and read-bias 'background' corrections that are applied (by default) to the raw CCD data to remove electronic noise before any measurements. See page 106 for more details. **Replace ROIs** – If this option is chosen, all auto ROIs are replaced when new ROI(s) are created.
Auto ROI Parameters	**Restore Defaults** – Restores the factory-set defaults for the auto ROI parameters. **Save/Load** – Click to save auto ROI parameter settings to the system (per user) or load parameter settings selected from the Name drop-down list.

6.3 Measuring Signal

This section explains how to measure signal (surface intensity) within an ROI.

In sequence view, ROIs will be applied to all images of the sequence. If you want to apply ROIs to only one image of a sequence, open the image.

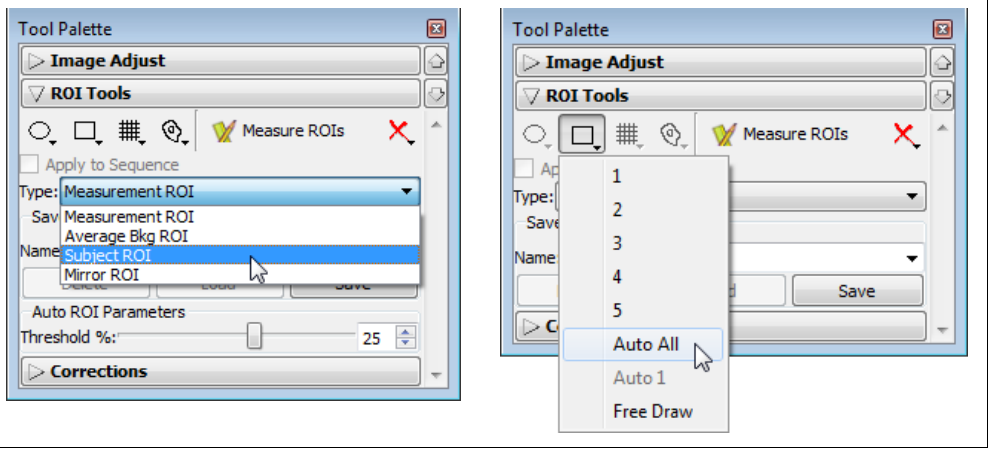
If an image of a sequence is active, selecting the “Apply to Sequence” option in the ROI tools will create related ROIs in the other images of the sequence as well.

ROIs that are applied to an image using the Auto All command are numbered from 1 to *n* (ROI 1 = brightest signal). If an image has multiple subjects and signals, it is helpful to first apply a subject ROI to each subject, then apply measurement ROIs. The ROI table will list the subject which contains each ROI.

NOTE: Ensure that the ROI table configuration includes "Subject" and/or "Subject Label" (if the subject ROI label was renamed). See *Creating a Custom ROI Table Configuration* on page 120 for instructions.

1. If there are multiple subjects in an image, apply subject ROIs. If not, skip to step 2.
   a. Select **Subject ROI** from the Type drop-down list (Figure 6.2).
   b. Click the square ROI shape and select **Auto All** from the drop-down list.

Figure 6.2 ROI Tools – Apply Subject ROIs

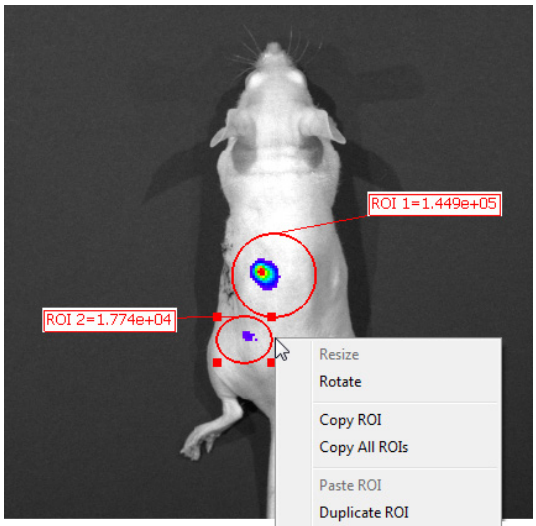


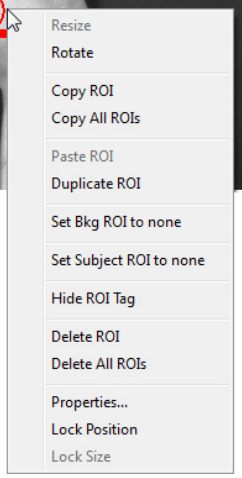
2. Select **Measurement ROI** from the Type drop-down list
3. Click an ROI shape (Circle, Square, Grid, or Contour) and make a selection from the drop-down list. If applying a grid ROI, choose the grid dimensions.

   The ROIs appear on the image(s) (Figure 6.3).

NOTE: If using subject ROIs, ensure that all of the measurement ROIs for a subject are completely within the subject ROI by resizing the subject ROI if necessary (see page 105 for instructions on resizing ROIs). Measurement ROIs not completely within a subject ROI will not be counted as part of the subject.

Figure 6.3 Example ROIs





ROI shortcut menu

Right-click an ROI or image (Ctrl-click for Macintosh users) to view a shortcut menu for easy access to many functions for managing ROIs and viewing ROI properties.

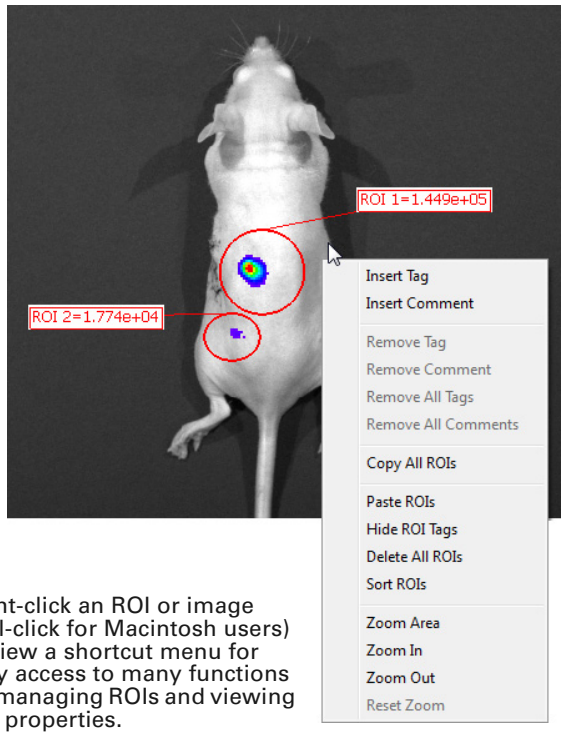
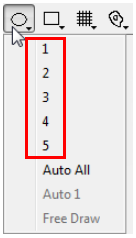
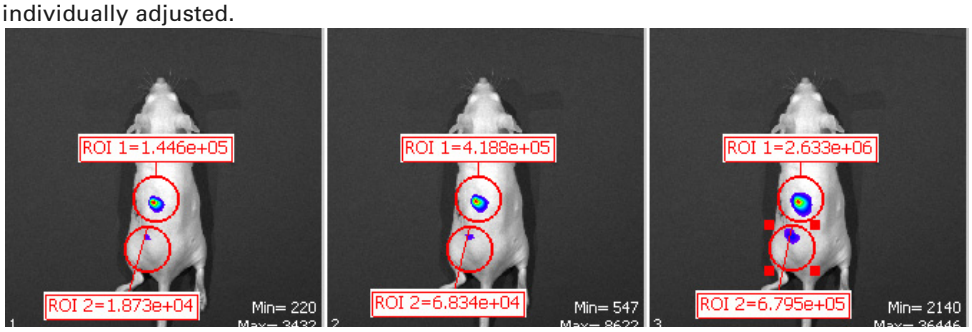
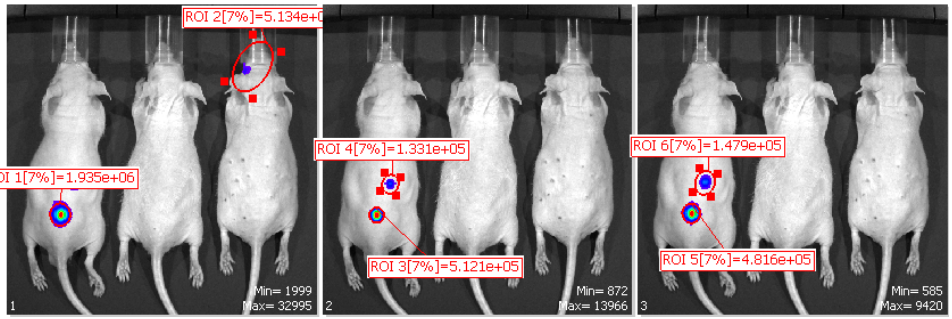
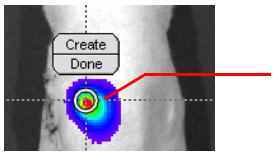


Image shortcut menu

NOTE: It may be helpful to arrange ROIs in a known order for easier comparison between images. To renumber ROIs (in ascending order from right to left), right-click the image and select **Sort ROIs** from the shortcut menu. If the "Apply to Sequence" option is selected in the ROI tools, choose **Sort ROIs in Sequence** to sort the ROIs in each image of the sequence. Sort options are only available if the ROIs have not been previously sorted.

Table 6.3 ROI Drawing Tools

Item	Description
	Select the number of ROIs to apply. The software places up to five ROIs on an image or each image of a sequence, whichever is the active view. ROI position and size will need manual adjustment. See Table 6.4 on page 105 for instructions on adjusting ROIs. Using this method in sequence view creates identical ROIs in each image of the sequence. The example below shows two ROIs that were added in sequence view. Each image of the sequence has ROI 1 and ROI 2. All ROIs named "ROI 1" in the sequence are "related" and can be moved or resized as a group in sequence view. Similarly, all of the ROIs named "ROI 2" are related. Moving or resizing related ROIs as a group is optional, an ROI can also be individually adjusted. 
Auto All	The software automatically applies ROIs by locating the peak pixel intensities in the image and searching the neighborhood around a peak pixel. A pixel is included in the ROI if the pixel intensity is greater than the Threshold%, a user-specified percentage of the peak pixel intensity.  ROIs created in sequence view using **Auto All** are numbered in ascending order starting in image 1. The numbering continues from left to right across the sequence images. ROIs created in image view using **Auto All** are numbered in ascending order where ROI 1 contains the highest maximum signal and the last ROI contains the lowest maximum signal. **Note:** Manually adding ROIs afterward may affect ROI numbering. If necessary, ROIs can be renumbered by editing the ROI labels. See *Managing ROIs* on page 112 for more information.
Auto 1	Only available in image view. Automatically identifies signal and applies an ROI using the auto ROI parameter thresholds at a user-selected location.  Drag the ring to the location for the ROI and click **Create**. Click **Done** when you finish drawing all ROIs.
Free Draw	Only available in image view. To draw a: ▪ Circle or square ROI – Drag the pointer (+) to draw and size the ROI around the signal. ▪ Contour ROI – Draw line segments around the signal by clicking the mouse pointer (+) at points that define the ROI perimeter. Right-click when the last point is near the first point of the ROI.

4. Adjust ROI size or position if necessary (see Table 6.4).

NOTE: The position and size of auto ROIs (ROIs created using Auto All or Auto 1) are locked by default. To unlock auto ROIs:

- Sequence view – Right-click the ROI and select **Properties** on the shortcut menu. Clear the lock options in the ROI Properties dialog box that appears (see Figure 6.12 on page 114).
- Image view – Right-click the ROI and select unlock options from the shortcut menu.

Table 6.4 Select and Adjust ROIs

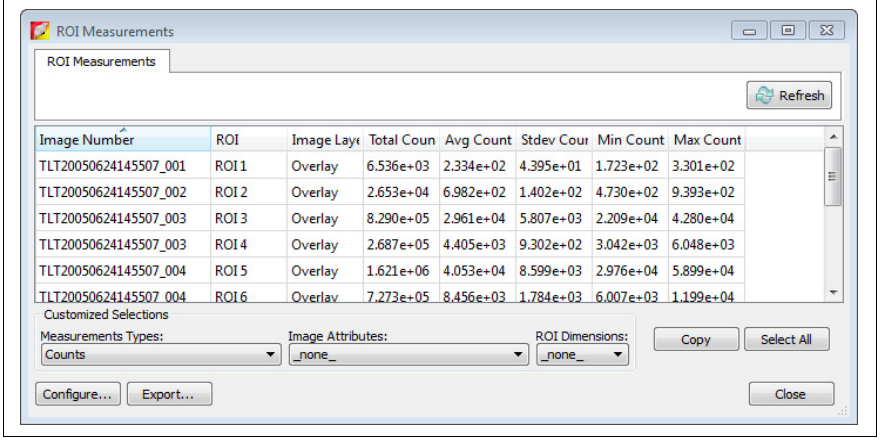
Operation	Single ROI	Multiple ROIs	Related ROIs Applied in Sequence View
Select	Click the ROI border. This will clear a previous ROI selection in the image.	Shift-click the border of each ROI. This does not clear a previous ROI selection in the image.	Press and hold the Control key while you click an ROI border.
Move	Put the mouse pointer over the ROI border. Drag the ROI when the pointer changes to ✥. **Note:** If an image of a sequence is active, choose the "Apply to Sequence" option in the ROI tools to move related ROIs in the other images of the sequence as well.	Select multiple ROIs and drag them when the pointer changes to ✥. **Note:** If an image of a sequence is active, choose the "Apply to Sequence" option in the ROI tools to move related ROIs in the other images of the sequence as well.	Press and hold the Control key while you move a selected ROI.
Resize	Circle, square, or contour ROI – Select the ROI. Put the mouse pointer over an ROI handle ■ and drag the handle when the pointer changes to ⤡. Grid ROI – Select the ROI. ▪ To move the entire grid, put the mouse pointer over the grid perimeter. Drag the grid when the pointer changes to ✥. ▪ To adjust width or height, put the mouse pointer over a grid handle ■ and drag the handle when the pointer changes to ⤡.		Put the mouse pointer over the ROI border and click the ROI when the pointer changes to ✥. Put the mouse pointer over an ROI handle ■. When the pointer changes to ⤡, press and hold the Control key while you drag the handle.

5. Click the **Measure** button Measure ROIs in the ROI tools to show the ROI Measurements table (Figure 6.4).

   The ROI Measurements table shows data for all ROIs created in images or sequences during a session (one ROI per row). The table display is automatically updated when new ROIs are created.

   The table provides a convenient way to review and export ROI data. See *ROI Measurements* on page 118 for more details.

Figure 6.4 ROI Measurements Table



6. Click **Yes** in the prompt when closing the data to save the ROIs with the image data. Alternatively, select **File → Save** on the menu bar.

   The ROIs will be displayed the next time the image data is loaded.

NOTE: ROIs can also be saved to the system (per user) and applied to other images. See page 117 for instructions.

6.4 Measuring Background-Corrected Signal

If a subject has significant autoluminescence or autofluorescence, a background-corrected measurement can be obtained by subtracting an average background ROI from a measurement ROI. The software computes:

Background-corrected intensity signal = Signal in the measurement ROI - Average signal in the average background ROI

NOTE: This is an optional "background" correction that is applied in addition to the electronic dark-charge and read-bias corrections that are applied to the raw CCD data.

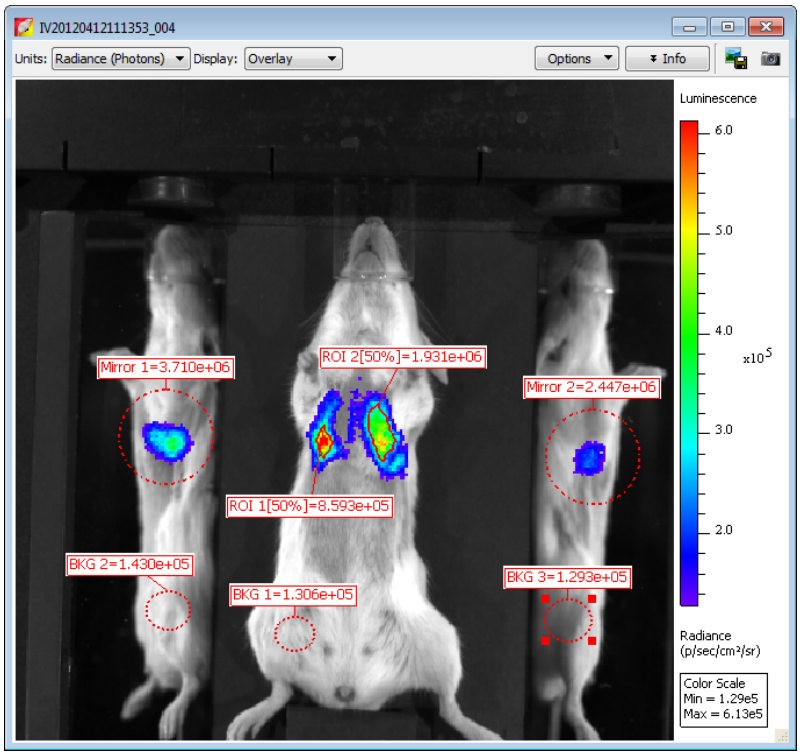
The Image Adjust tools and zoom feature are helpful for selecting an appropriate area for an ROI. By setting the image minimum close to zero and zooming in on a background area in the image, you can determine where naturally occurring background luminescence or autofluorescence is present. For more details on the Image Adjust tools and the zoom feature, see *Viewing Intensity Data* on page 70 and *Zooming or Panning* on page 68.

To measure background-corrected signal:

1. Open an image and draw one or more measurement ROIs on the subject (see page 102 for instructions).
2. Draw an average background ROI on the subject:
   a. Select Average Bkg ROI from the Type drop-down list.
   b. Click the **Square** or **Circle** button and select **1**.
      The ROI is added to the image.
   c. Adjust the ROI position or dimensions (see Table 6.4 on page 105 for instructions).

> **NOTE:** The average background ROI and measurement ROI do not need to be the same shape or size because the software computes the average intensity signal in each ROI. If the image was acquired using the Side Imager, draw a background ROI on each view (Figure 6.5).

Figure 6.5 Draw a Background ROI on Each Image View Acquired Using the Side Imager



3. Associate each background ROI with a measurement ROI(s) or mirror ROI(s) using one of the methods in Table 6.5.

Table 6.5 Methods for Associating Measurement or Mirror ROIs With a Background ROI

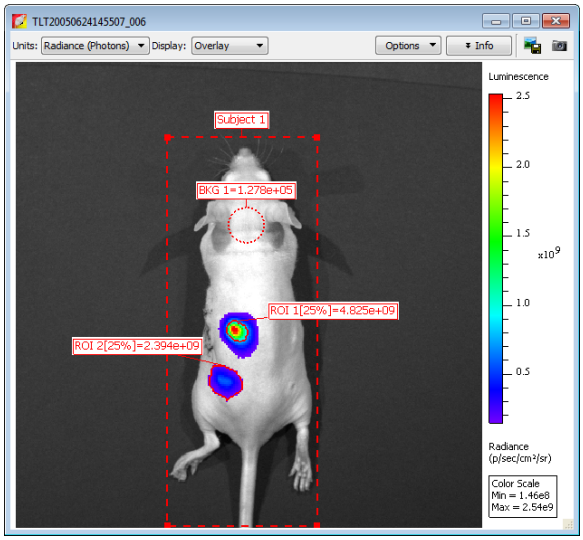
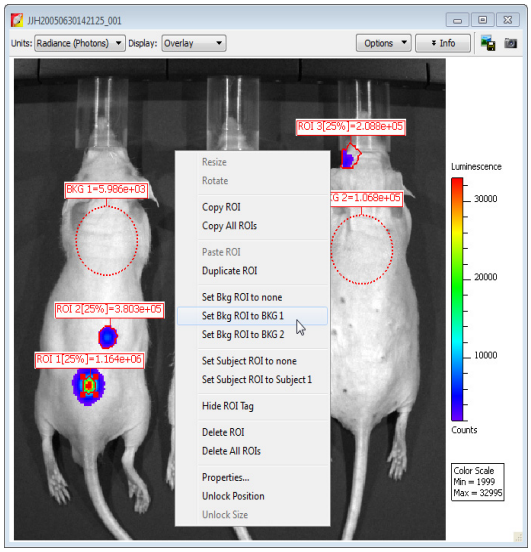
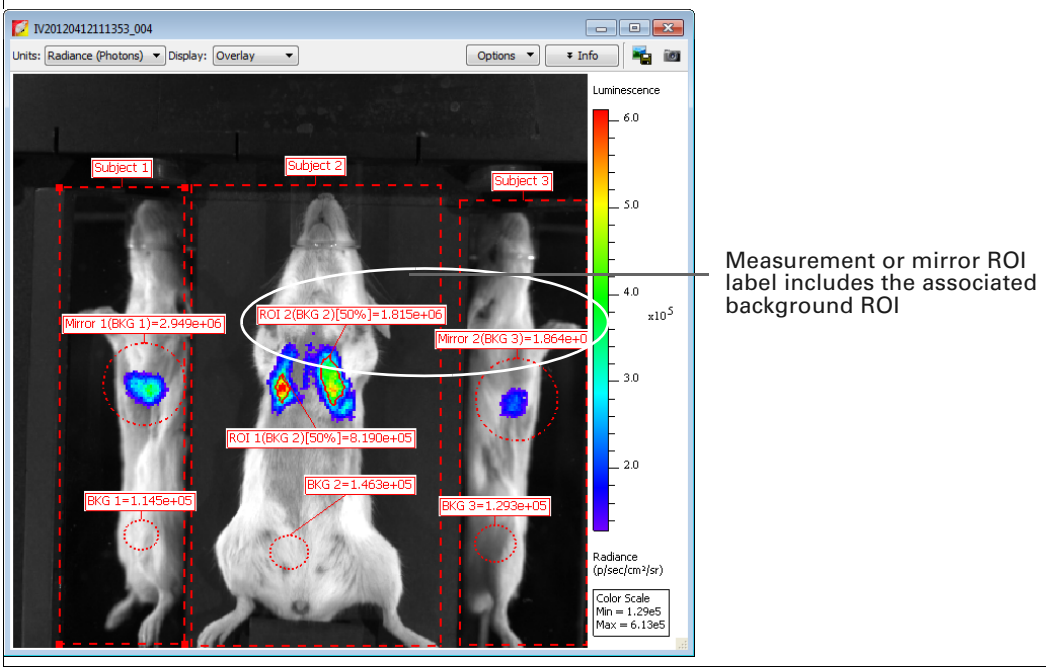
Methods for Associating ROIs	Example
Use a subject ROI to identify a subject in an image and automatically associate a measurement and average background ROI for background-corrected ROI measurements. To draw a subject ROI: **1.** Select "Subject ROI" from the Type drop-down list in the ROI tools. **2.** To apply ROIs: Automatic – Click the button and select **Auto All.** Manual – Click the button and select "1". Position the subject ROI so that it includes the measurement ROI(s) and the associated average background ROI.	
Right-click a measurement ROI and select an average background ROI from the shortcut menu.	
1. Right-click a background ROI and select **Properties** on the shortcut menu. **2.** In the ROI Properties box that appears, click the Background ROI tab and put a check mark next to **Use as BKG for future ROIs in**. **3.** Choose the image name or the **Entire sequence** option.	

Figure 6.6 Measurement ROIs Successfully Associated with a Background ROI



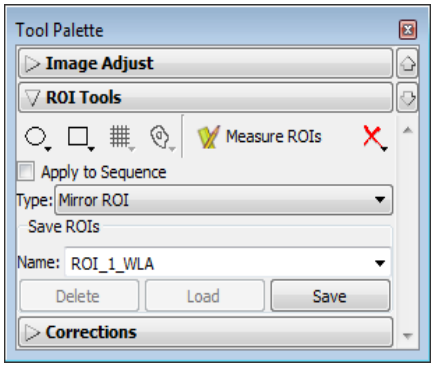
6.5 Measuring Signals Obtained Using the Side Imager

Use a mirror ROI to measure bioluminescence or fluorescence in the right or left mirror-reflected view of images acquired using the Side Imager. Measure signals in the center view using a measurement ROI. See page 102 for instructions on drawing a measurement ROI.

NOTE: Fluorescent image data acquired in reflectance/epi-illumination mode must include a photograph. Do not apply mirror ROIs on the center view or measurement ROIs on the left or right mirror-reflected views. This will result in incorrect ROI measurements.

1. Open an image or image sequence acquired with the Side Imager.
2. Select "Mirror ROI" from the Type drop-down list in the ROI tools (Figure 6.7). If analyzing a fluorescent image, choose the Photo Mask option.

Figure 6.7 ROI Tools



3. Select the ROI shape:
   a. Click the **Circle**, or **Square**, button.
   b. Select the number of ROIs to add to the image on the drop-down list that appears.

      If analyzing a reflectance/epi-illumination fluorescent image, go to step 4; otherwise, go to step 5.
4. For reflectance/epi-illumination fluorescent images only:
   a. Confirm the purple data mask in the dialog box that appears (Figure 6.8).

      The data mask includes the entire subject by default and defines the area of excitation light projection onto the animal. If you do not want to analyze the entire subject, select the Data Mask option and mask a particular area using the data mask options (Table 6.6).
   b. Click **OK**.

      The mirror ROIs and intensity measurements appear on the image (Figure 6.9 on page 111). Right-click an ROI to view a shortcut menu of ROI commands (Ctrl-click for Macintosh users). The shortcut menu provides easy access to many functions for managing ROIs and viewing ROI properties.

Figure 6.8 Excitation Projection Setup Dialog Box (For fluorescent images only.)

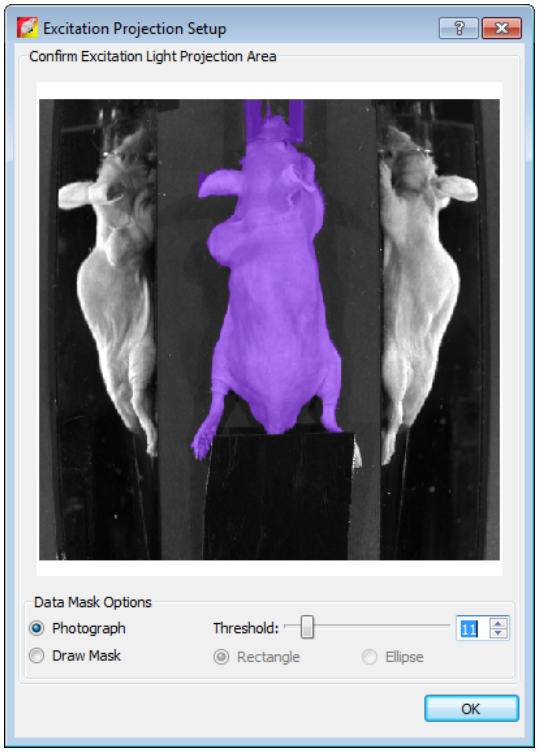


Table 6.6 Data Mask Options

Option	Description
Photograph	If this option is chosen, the software automatically draws the data mask by using higher intensities in the photograph. The mask selects high-valued photograph image pixels which are located continuously and centrally in the photograph image. The photograph mask works best with light-colored subjects.

Table 6.6 Data Mask Options (continued)

Option	Description
Threshold	If necessary use the threshold slider or arrows to adjust the mask so that it matches the underlying subject photograph as closely as possible without including any area outside the subject image.
Draw Mask	Choose this option to manually draw a data mask on an area of the photograph.
Rectangle	Specifies a rectangular shape for the manual data mask.
Ellipse	Specifies an elliptical shape for the manual data mask.

Figure 6.9 Mirror ROIs on Fluorescent Image Acquired with Side Imager

NOTE: Manual ROIs are numbered in the order they were created. You may want to arrange ROIs in a known order for easier comparison between images. To renumber ROIs, right-click the image and select **Sort ROIs** from the shortcut menu. If the "Apply to Sequence" option is selected in the ROI tools, choose **Sort ROIs in Sequence** to sort all of the ROIs in each image of the sequence. Sort options are only available if the ROIs have not been previously sorted.

5. Adjust ROI position or size following the instructions for a single ROI in Table 6.4 on page 105,
6. Click the **Measure** button Measure ROIs.

   The ROI measurements and table appear. See *ROI Measurements* on page 118 for more details.
7. Click **Yes** in the prompt when closing the dataset to save the ROIs with the data. Alternatively, select **File → Save** on the menu bar.

   The ROIs will be displayed the next time the image data is loaded.

NOTE: ROIs can also be saved to the system (per user) and applied to other images. See *Save ROIs to the system* on page 101 for instructions.

6.6 Managing ROIs

This section explains how to:

- View information about an ROI.
- Change the position of the ROI on the image.
- Edit the ROI label or line characteristics.

ROI Properties

1. Do one of the following view ROI properties:
   - Double-click an ROI in the image.
   - Right-click the ROI and select **Properties** from shortcut menu that appears.
   - Select the ROI, then select **View** → **Properties** on the menu bar.

   The ROI Properties box appears (for more details see Figure 6.12).
2. To view properties for another ROI, click the ROI in the image. Alternatively, select an ROI from the ROI drop-down list in the ROI Properties dialog box (Figure 6.10).

Figure 6.10 Viewing ROI Properties

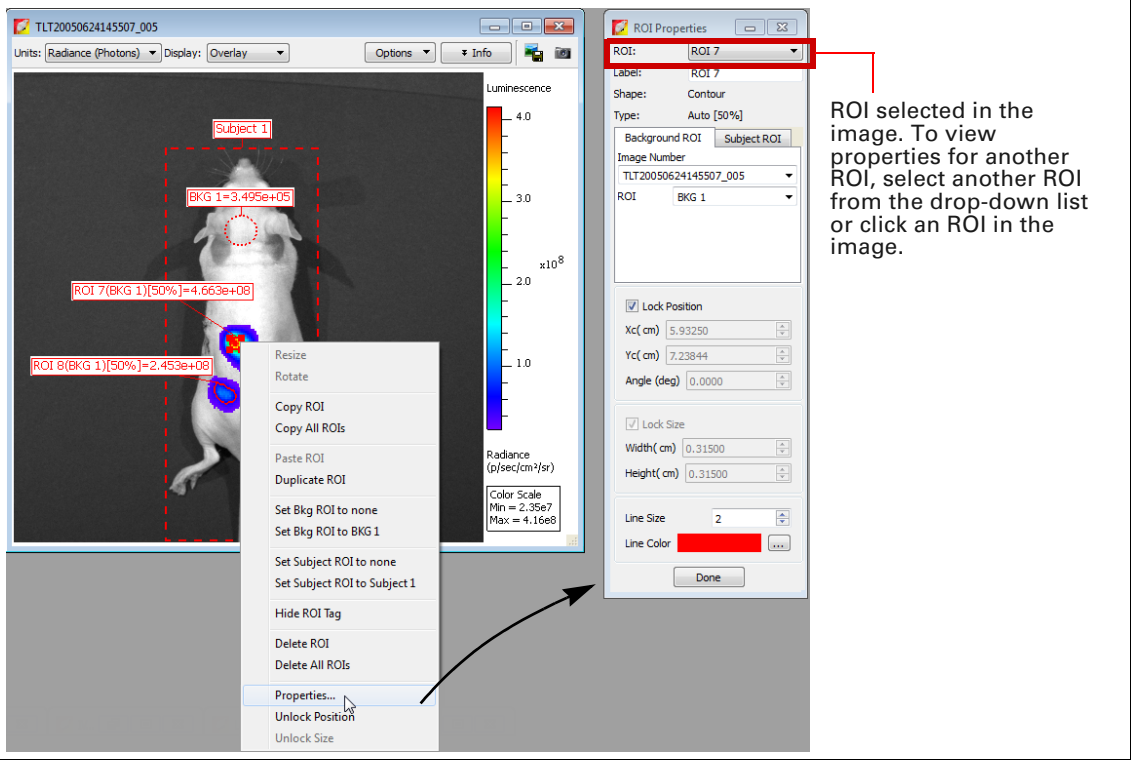


Figure 6.11 ROI Properties – Background ROI Tab

The items in the ROI Properties box depend on the type of ROI selected in the image. See Table 6.7, page 114 for more details.

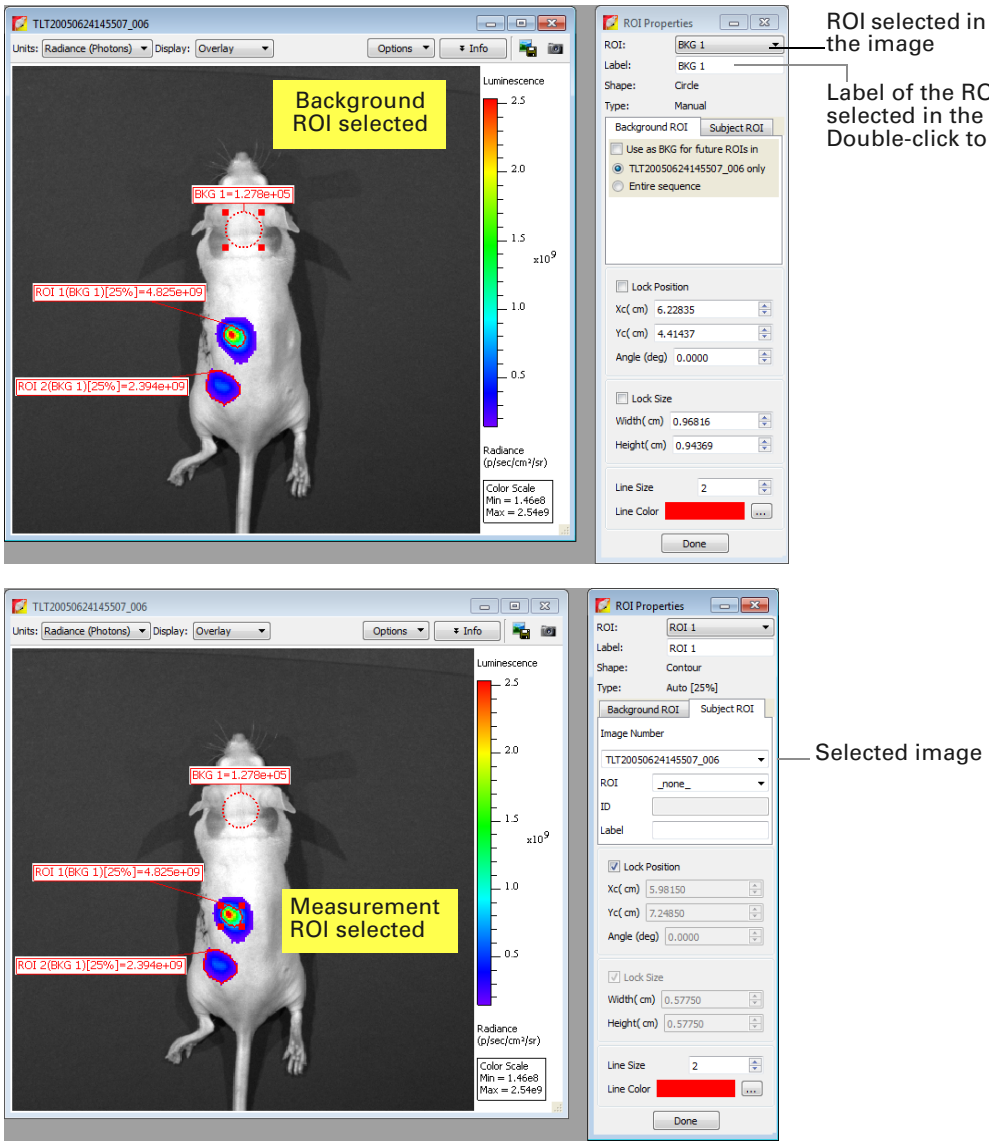


Figure 6.12 ROI Properties – Subject Tab

The items in the ROI Properties box depend on the type of ROI selected in the image. See Table 6.7 for more details.

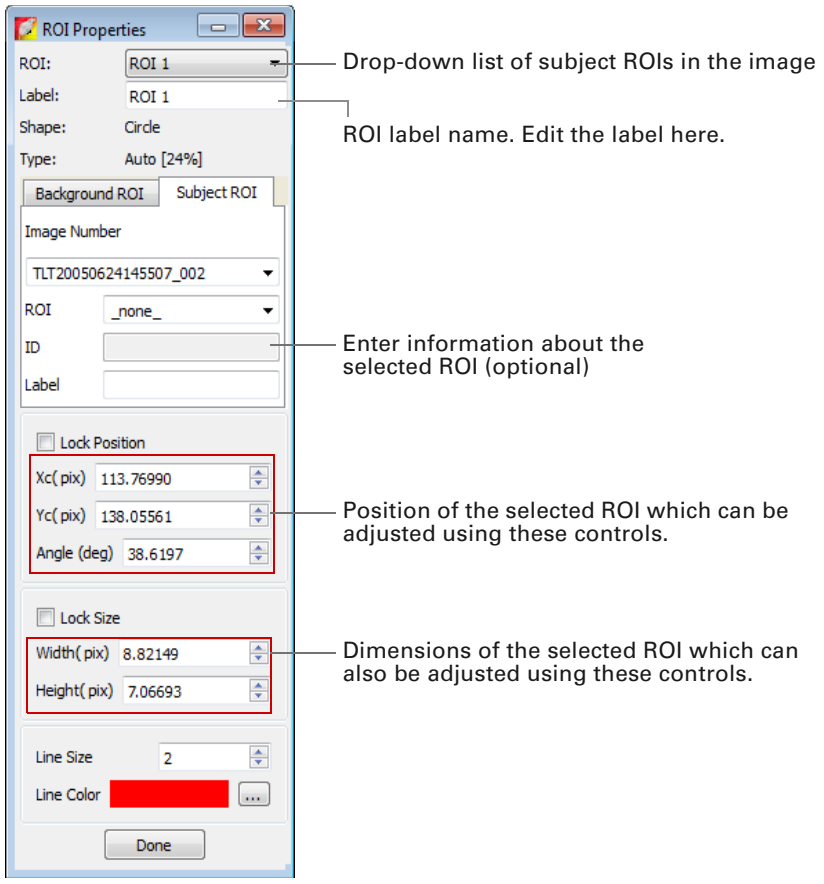


Table 6.7 ROI Properties

Item	Description
ROI	A drop-down list of ROIs in the active image or image sequence. To select an ROI, double-click the ROI in the image or make a selection from the drop-down list. Shape – The shape of the ROI (circle, square, grid, or contour) selected in the image. Type – Indicates the method that was used to draw the selected ROI (automatic, manual, or free draw).
ROI Label	Click to edit the selected ROI label name.
Image Number	A drop-down list of open images.
Background ROI tab	The Background ROI tab shows a drop-down list shows all average background ROIs in active image that can be linked to a user-specified measurement ROI or subject ROI (selected from the drop-down list at the top of the dialog box).
Subj ROI	The Subject ROI tab shows a drop-down list of all subject ROIs in the image number selected above that can be linked to a user-specified measurement ROI or average background ROI (selected from the drop-down list at the top of the dialog box). The Background ROI tab shows a drop-down list of all average background ROIs in the click number selected above that can be linked to a user-specified measurement ROI or subject ROI (selected from the drop-down list at the top of the dialog box).
ID	User-entered information about a subject ROI.

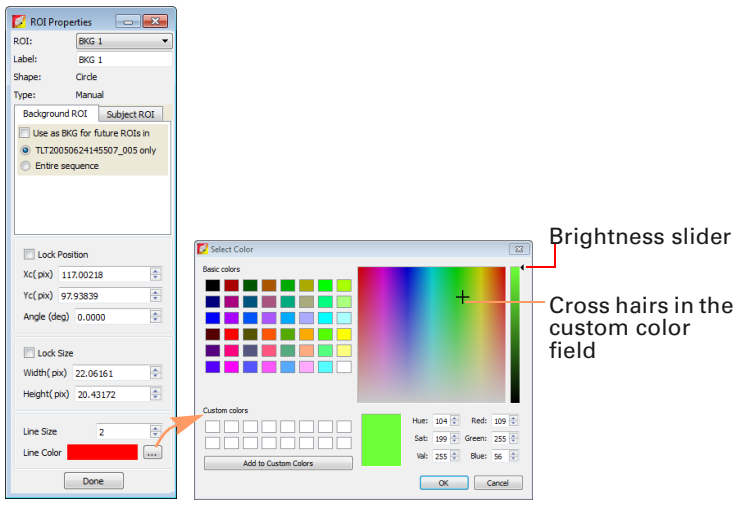
Table 6.7 ROI Properties (continued)

Item	Description
Label	Label name of the selected subject ROI.
Lock Position	Choose this option to lock the position of the ROI selected in the image.
Xc	x-axis coordinate at the center of the ROI selected in the image.
Yc	y-axis coordinate at the center of the ROI selected in the image.
Lock Size	Choose this option to lock the dimensions of the ROI selected in the image.
Width	Width (pixels or cm) of the ROI selected in the image (see *ROI Dimensions* on page 119 for more details on setting the units). Edit this value to resize an ROI, except for ROIs applied using the "Auto All" or "Auto 1" commands. Use the Threshold% slider to resize auto ROIs.
Height	Height (pixels or cm) of the ROI selected in the image. Edit this value to resize an ROI, except for ROIs applied using the "Auto All" or "Auto 1" commands. Use the Threshold% slider to resize auto ROIs.
Line Size	Specifies the ROI line thickness. To change the line thickness, enter a new value or click the up/down arrows .
Line Color	Specifies the color of the ROI line. To select a line color, click the **Browse** button .
Done	Click to close the ROI Properties box and apply any new settings, including: ▪ Linkage between a measurement ROI and subject ROI (See Table 6.5 on page 108 for more details). ▪ ROI size dimensions or position. ▪ Subject ROI ID information.

ROI Line

1. Double-click the ROI that you want to edit.
   The ROI Properties box appears (Figure 6.13).

Figure 6.13 Editing ROI Properties



2. To edit the ROI line thickness, enter a new value in the Line Size box. Alternatively, click the arrows.
3. To change the ROI line color:

a. Click the **Browse** button ....
The Select Color box appears.

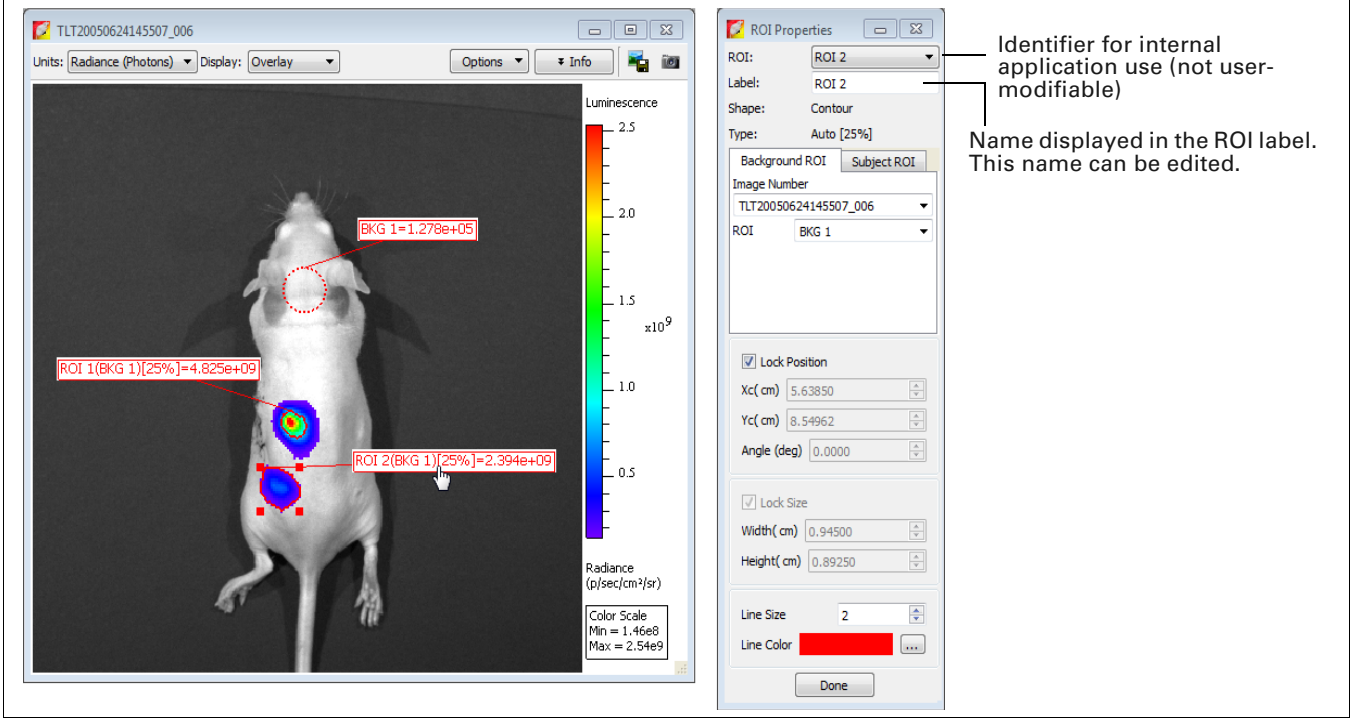
b. Select a basic color or create a custom color for the ROI line:
- Select a basic color – Click a basic color swatch and click **OK**.
- Define a custom color – Drag the crosshairs in the custom color field, adjust the brightness slider, and click **Add to Custom Colors**. Click a custom color swatch and click **OK**.

ROI Label

To move the ROI label:

1. Put the mouse pointer over the ROI label.
2. When the pointer becomes a , drag the label, then click to release the label at the new location (Figure 6.14).

Figure 6.14 Move or Edit the ROI Label



To edit the ROI label:

1. Double-click the ROI. Alternatively, right-click the ROI (**Ctrl**-click for Macintosh users) and select Properties on the shortcut menu.
2. Edit the name in the Label field in the ROI Properties box that appears (Figure 6.14).

Saving ROIs to the System

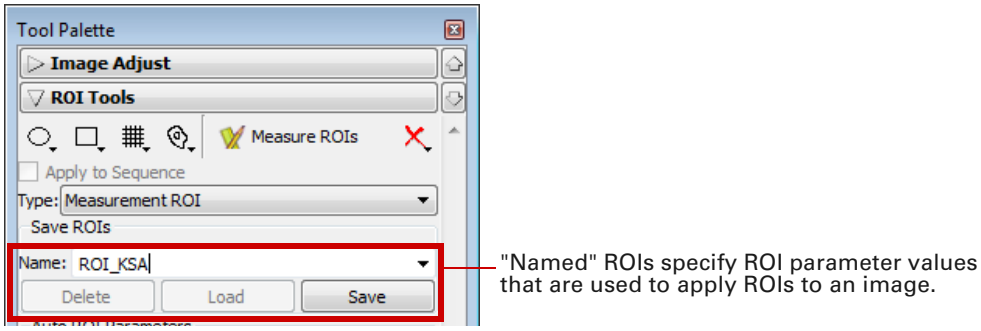
Living Image software saves ROIs with an image (the software prompts you to save before closing the image). ROI measurements are saved in the AnalyzedClickInfo.txt file associated with the image.

Additionally, ROI parameter values (for example, Threshold%, Lower Limit, Minimum Size) can be saved to the system (per user) as a "named" ROI and used to apply ROIs to other images (Figure 6.15). This section explains how to save ROIs to the system.

NOTE: Before closing a dataset, the software prompts you to save ROIs with the dataset. ROIs saved with an image do not appear in the "Name" drop-down list.

1. After one or more ROIs are applied to an image:
   a. Confirm the default name or enter a new name for the ROI in the Name drop-down list.
   b. Click **Save** (Figure 6.15).

Figure 6.15 Name and Save ROIs to the System

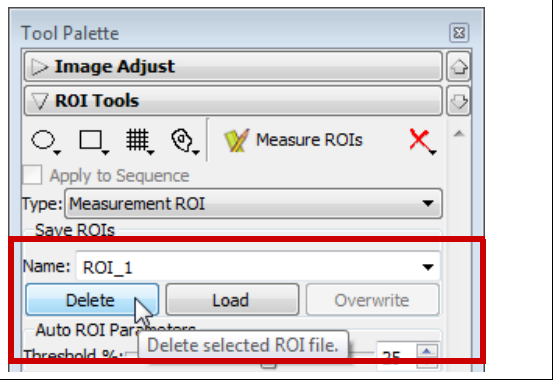


2. To apply a "named" ROI to an image, make a selection from the Name drop-down list and click **Load** (Figure 6.15).

NOTE: If you load an ROI, then apply or delete ROIs, the **Save** button changes to **Overwrite**. Click **Overwrite** to save the ROIs using the existing name. Alternatively, enter a new name and click **Save**.

3. To delete a "named" ROI from the system (per user), select the ROI from the Name drop-down list and click **Delete** (Figure 6.16).

Figure 6.16 Delete ROIs From the System



6.7 ROI Measurements

The ROI Measurements table shows information and data for the ROIs created during a session. The ROI measurements can be displayed in units of counts, radiance, radiant efficiency, efficiency, or NTF efficiency, depending on the type of image data.

TIP: See the technical note *Quantifying Image Data* for more details (select **Help → Tech Notes** on the menu bar).

Viewing ROI Measurements

1. Load an image or sequence that includes ROIs.
2. Click the Measure ROIs button in the ROI tools to display the ROI measurement table (Figure 6.17). Alternatively, select **View → ROI Measurements** on the menu bar.

Figure 6.17 ROI Measurements Table

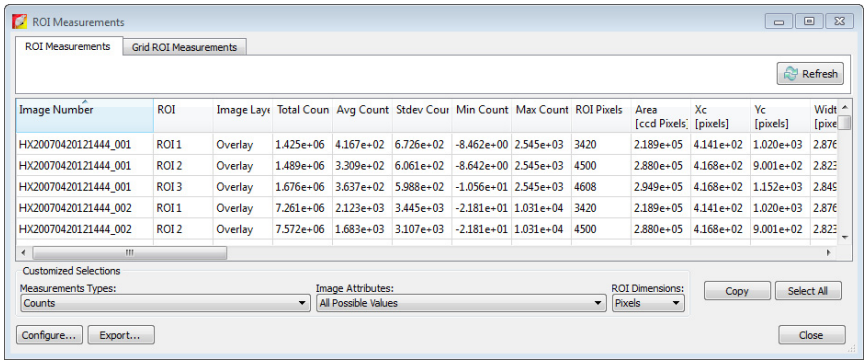


Table 6.8 ROI Measurements Table

Item	Description
Measurement Types	Make a selection from the this drop-down list to select the type of image unit for the ROI measurements in the table. Custom ROI table configurations also appear in this drop-down list. See *Configuring the ROI Measurements Table* on page 120 for instructions on creating a custom table.
None	Excludes ROI measurements from the table.
Counts (luminescence and fluorescence)	Includes Total Counts, Avg Counts, Stdev Counts, Min Counts, and Max Counts in the table. Total Counts = the sum of all counts for all pixels inside the ROI. Avg Counts = Total Counts/Number of pixels or super pixels. Stdev Counts = standard deviation of the pixel counts inside the ROI Min Counts = lowest number of counts in a pixel inside the ROI. Max Counts = highest number of counts in a pixel inside the ROI. **Note:** These numbers are displayed if the units selected in the ROI Measurements table and the image are the same. Otherwise, N/A appears in each column. **Tip:** See the tech note Image *Display and Measurement* for more details on count units (select **Help → Tech Notes** on the menu bar).

Table 6.8 ROI Measurements Table (continued)

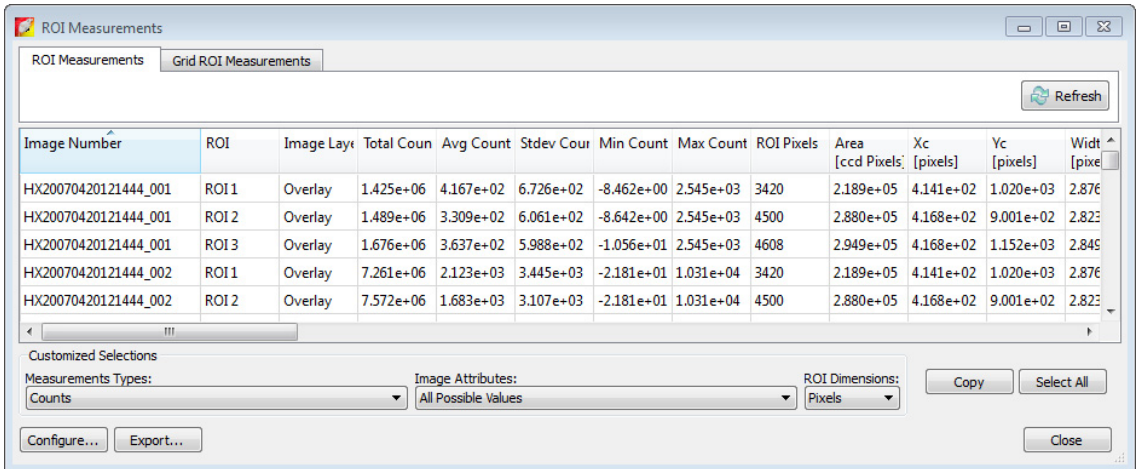
Item	Description
Radiance (Photons) (fluorescence and luminescence)	Total Flux (photons/sec) = the radiance (photons/sec/cm²/steradian) in each pixel summed or integrated over the ROI area (cm²) x 4π. Average Radiance = the sum of the radiance from each pixel inside the ROI/ number of pixels or super pixels (photons/sec/cm²/sr). Stdev Radiance = standard deviation of the pixel radiance inside the ROI Min Radiance = lowest radiance for a pixel inside the ROI. Max Radiance = highest radiance for a pixel inside the ROI. **Tip:** See the tech note *Image Display and Measurement* for more details on photon units (select **Help → Tech Notes** on the menu bar).
Radiant Efficiency (fluorescence)	Epi-fluorescence - Fluorescence emission radiance per incident excitation intensity: p/sec/cm²/sr/µW/cm²
Efficiency (epi-fluorescence)	Fluorescent emission yield normalized to the incident excitation intensity (radiance of the subject/illumination intensity)
Image Attributes	Make a selection from the drop-down list to specify the click number (image file) information to include in the table. Click attributes include label name settings and camera settings.
None	Excludes image attributes from the table.
All Possible Values	Includes all of the image attributes (for example, label name settings and camera settings) in the table.
All Populated Values	Includes only the image attributes with values in the table.
Living Image Universal	Includes all Living Image Universal label name settings in the table.
ROI Dimensions	Make a selection from the drop-down list to specify the ROI dimensions to include in the table.
None	Excludes the ROI area, x,y-coordinates, and dimensions from the table.
Pixels	Includes ROI area, x,y-coordinates, and dimensions (in pixels) in the table.
cm	Includes ROI area, x,y-coordinates, and dimensions (in cm) in the table.
Copy	Copies the selected row(s) in the table to the system clipboard.
Select All	Copies all rows in the table to the system clipboard.
Refresh	Updates the ROI Measurements table (for example, after you draw new ROIs, move an ROI, and close or open image data).
Configure	Displays the Configure Measurements box that enables you to specify and organize the data categories (column headers) for the table.
Export	Displays the Save Measurements box so that the data can be saved to a .txt or .csv file. **Note:** Grid ROI measurements exported to a .csv file can be opened in a spreadsheet application like Microsoft® Excel®.
Close	Closes the ROI Measurements table.

Configuring the ROI Measurements Table

You can customize the data and information (column headers) in the ROI Measurements table (Figure 6.18). Several preset categories are available in the Measurement Types, Click Attributes, and ROI Dimensions drop-down lists.

1. Drag a column header (left or right) in the table to reorder the columns.
2. Make a selection from the Measurement Types drop-down list to change the measurement units.

Figure 6.18 ROI Measurements Table



3. Make a selection from the Image Attributes drop-down list to include image information in the ROI table.
4. Select units (Pixels or cm) from the ROI Dimensions drop-down list to include ROI dimensions in the table.

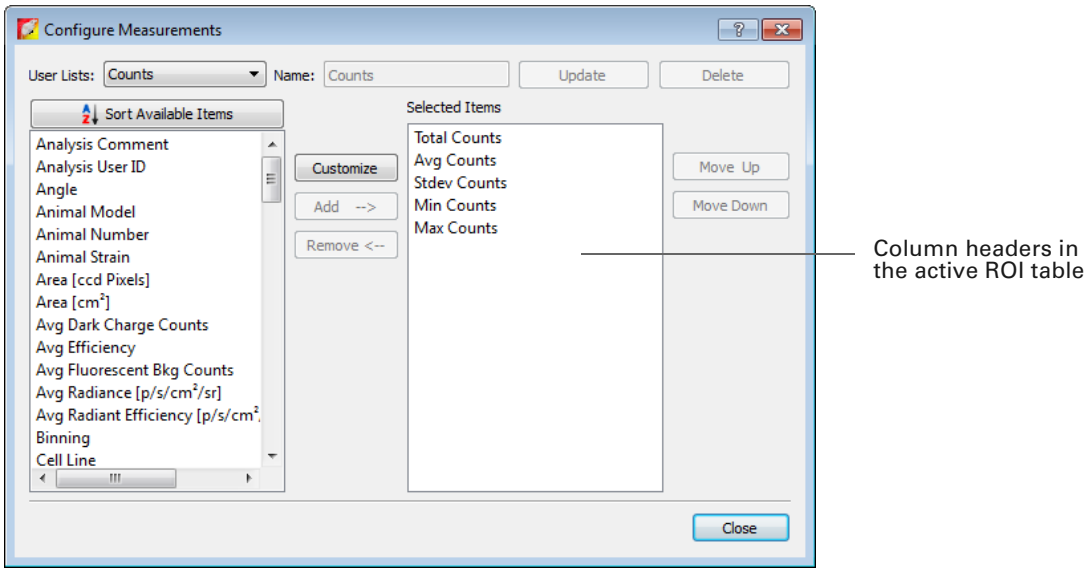
Creating a Custom ROI Table Configuration

A table configuration specifies the column headers in the ROI table. Several preset configurations are available (selected from the Measurements Types drop-down list in the ROI table, Figure 6.18). You can also create a custom table configuration.

NOTE: Preset table configurations cannot be edited. You can modify a preset configuration and save it to a new name.

1. Click **Configure** in the ROI Measurements table.
   The Configure Measurements box appears (Figure 6.19).

Figure 6.19 Configure Measurements Dialog Box



2. Select a configuration from the User Lists drop-down list and click **Customize**.
3. To add column header to the ROI table, make a selection from the "Available Item" list and click **Add**.
4. To remove column header from the ROI table, select the item that you want to remove in the Selected Items list, and click **Remove**.
5. To reorder an item in the Selected Items list, select the item and click **Move Up** or **Move Down**.

   The columns in the ROI Measurements table are updated.
6. Enter a name for the custom configuration in the Name box and click **Save**.

   The custom configuration is the added to the Measurements Types drop-down list in the ROI Measurements table (Figure 6.18). If a custom configuration is saved with the data, it becomes the default configuration.

To delete a custom table configuration:

Select the configuration from the User Lists drop-down list and click **Delete**.

NOTE: Preset table configurations cannot be deleted.

Copying or Exporting ROI Measurements

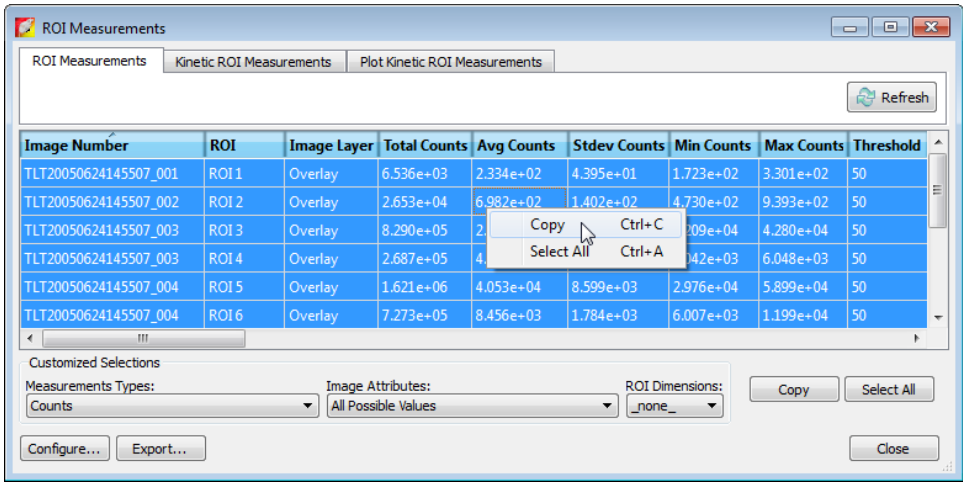
To export the table:

1. Click **Export** in the ROI Measurements table.
2. In the dialog box that appears:
   a. Select a folder and enter a name for the file.
   b. Select a file type (.txt or ,csv) and click **Save**.

To copy the table to the system clipboard:

- Copy selected rows – Select the rows of interest and click **Copy**. Alternatively, select the rows, then right-click the table and choose Copy on the shortcut menu (Figure 6.20).
- Copy all rows – Click Select **All** and click **Copy**. Alternatively, press **Ctrl+A**, then right-click the table and choose **Copy** on the shortcut menu.

Figure 6.20 Copy All Rows in the ROI Measurements Table to the System Clipboard



7 Reconstructing 3D Sources

7.1 About 3D Reconstruction

Living Image DLIT and FLIT algorithms analyze 2-dimensional optical image data to render 3-dimensional (3D) reconstructions of luminescent or fluorescent sources located inside an animal (*tomographic analysis*).

3D Reconstruction Algorithm	Description	See Page
Diffuse Tomography (DLIT)	DLIT provides a complete 3D reconstruction of the luminescent source distribution within a subject. DLIT places no constraints on the geometry or spatial variation of the source strength throughout the volume. DLIT is well-suited for analyzing complex and spatially extended luminescent sources. The 3D reconstruction is presented as voxels. If a luminescent quantification database is available, the number of cells per source can be determined in addition to source intensity (photons/sec).	127
Fluorescent Tomography (FLIT)	FLIT provides a complete 3D reconstruction of the fluorescent source distribution within a subject. The 3D reconstruction is presented as voxels. If a fluorescent quantification database is available, the number of fluorophore molecules or cells per source can be determined in addition to the total fluorescence yield.	132

Input data for 3D reconstruction of luminescent or fluorescent light sources include:

- An appropriate image sequence. Use the Imaging Wizard to set up and acquire a DLIT or FLIT sequence (acquisition instructions for DLIT on page 124, FLIT on page 130). If not using the Imaging Wizard, see *Manual Sequence Setup* on page 134 for instructions.
- A *surface* (a 3D reconstruction of the animal surface, also called a *topography*). Living Image software will automatically generate a surface during reconstruction or you can manually create and/or select a surface using the Surface Topography tools.
- A quantification database (optional). If a quantification database is available, it is possible to determine the number of cells in a luminescent source or the number of cells or dye molecules in a fluorescent source. A quantification database is derived from an analysis of images of known serial dilutions of luminescent cells, fluorescent cells, or dye molecules in a well plate. See Appendix C on page 260 for more details on generating a database.

TIP: See the technical note *DLIT and FLIT Reconstruction of Sources* for more details on the DLIT or FLIT algorithm (select **Help → Tech Notes** on the menu bar).

There are two reconstruction options:

- **"Single-Click"** – Uses the current settings in the Properties tab of the 3D Reconstruction tools (Figure 7.6). You do not need to manually create or select a surface (as in Living Image 4.4 and earlier versions).

NOTE: Living Image software uses the following rules to automatically generate or select a surface for "single-click" reconstruction:

- If the dataset has not yet been analyzed (no 3D reconstruction results or surface saved with the data), the software will automatically create a surface. When the analysis results are saved, the surface will be saved (available as "SURFACE_1" in the Surface Topography tools).
- If the dataset was previously analyzed, but no surface is loaded, the software loads and uses the surface most recently saved with the dataset.
- If the dataset was previously analyzed and a surface is loaded and displayed in the 3D View, the software uses that surface for the reconstruction.

- **Manual Reconstruction** – This is the method used in Living Image 4.4 software and earlier versions. Use manual reconstruction if you want to select particular pixel data to reconstruct or choose a surface. See *Manual 3D Reconstruction* on page 134 for instructions.

7.2 Reconstructing Luminescent Sources

NOTE: It is important to consider the luciferin kinetic profile when planning the image sequence acquisition. The DLIT algorithm assumes a stable luciferin kinetic profile. Therefore, to optimize the signal for DLIT 3D reconstruction, carefully plan the start and finish of image acquisition and ration the exposure time at each emission filter so that the sequence is acquired during the flattest region of the luciferin kinetic profile. See the technical note *Kinetic Analysis of Bioluminescent Sources* for a protocol to determine a kinetic curve for luciferin.

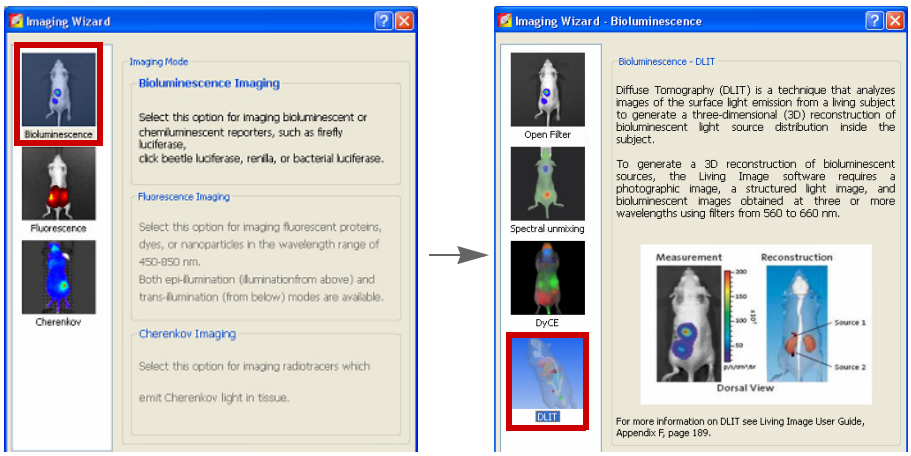
Acquire a Luminescent Sequence

NOTE: The IVIS Spectrum should be initialized and the temperature locked before setting the imaging parameters. See page 19 for more details.

1. Start the Imaging Wizard. See page 44 for instructions.
2. Double-click the Bioluminescence option. Double-click the DLIT option in the next screen (Figure 7.1).

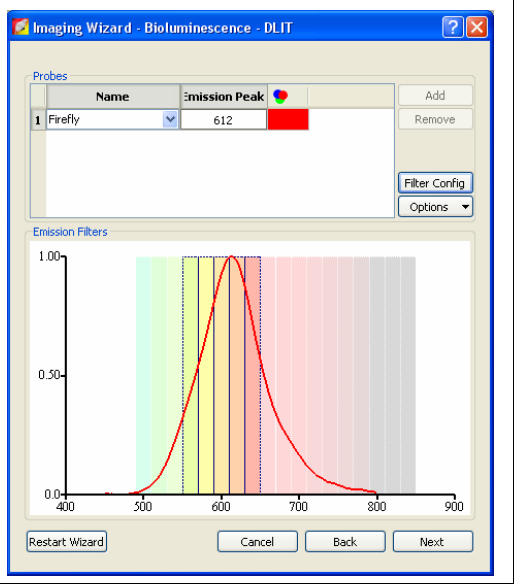
Figure 7.1 Imaging Wizard – Choose Bioluminescence and DLIT Options

If this screen does not appear when the wizard starts, click **Restart Wizard** Restart Wizard (on the wizard screen) to restart the wizard.



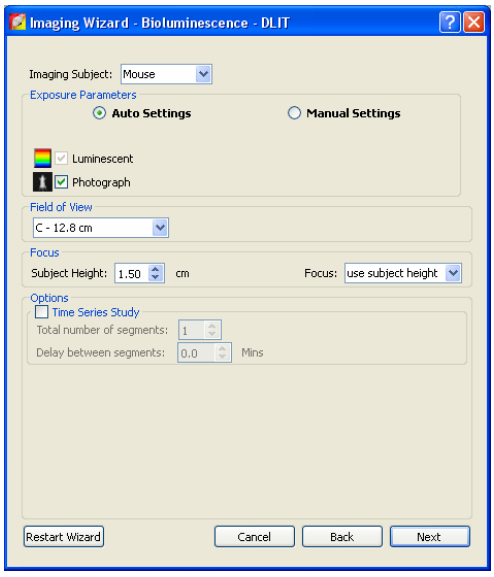
3. Select a probe from the Name drop-down list and click **Next** (Figure 7.2).

Figure 7.2 Select a Probe



4. In the next screen (Figure 7.3):
   a. Select the type of imaging subject.
   b. Choose the Auto Settings option for the exposure parameters.
   c. Select a field of view from the drop-down list.

Figure 7.3 Imaging Wizard – Bioluminescence DLIT



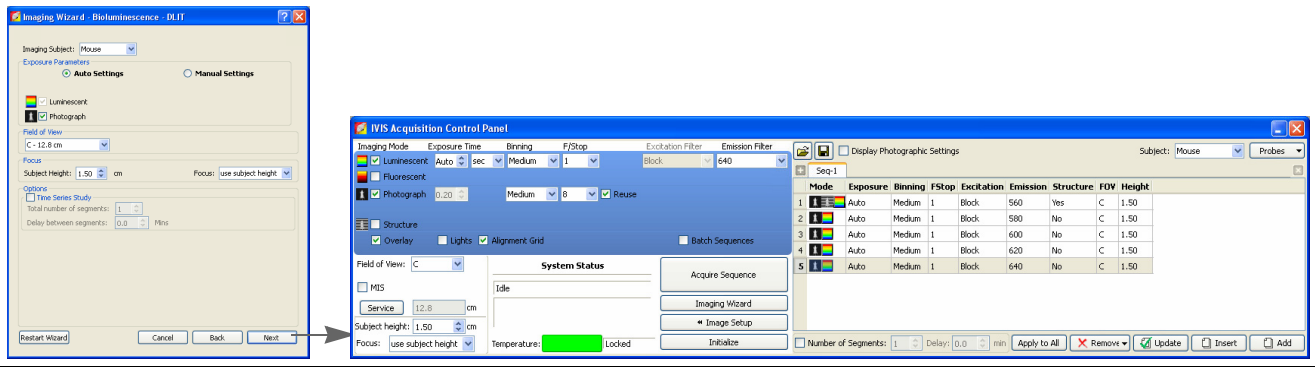
5. Set the focus by doing either of the following:
   - Enter a subject height and choose the “use subject height” focus option.

   OR
   - Choose the “manual focus” option from the Focus drop-down list and set the focus parameters in the Manual Focus Window that appears.

6. If performing a time series study, choose the Time Series Study option. Enter the number of segments to acquire and the delay between segment acquisition. A segment is an image sequence.
7. Click **Next**.

   The specified sequence appears in the sequence table (Figure 7.4).

Figure 7.4 Sequence Setup Complete

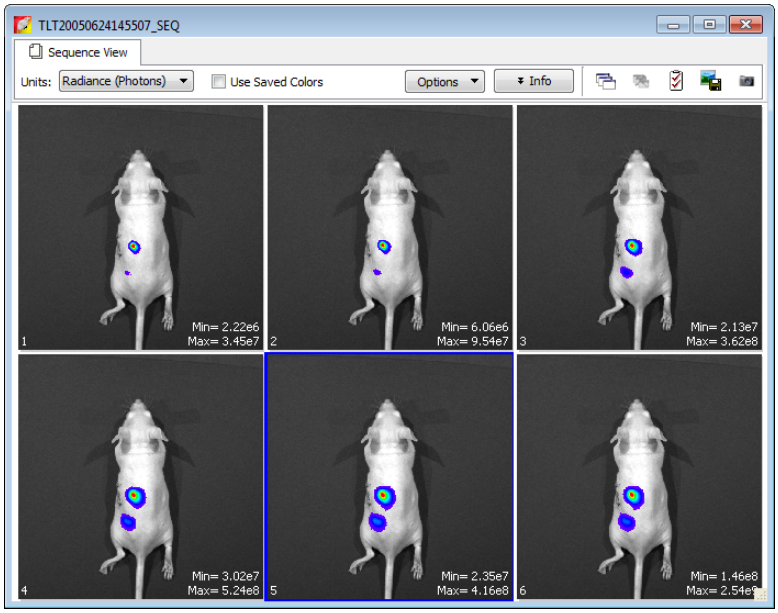


8. Acquire the sequence following the instructions on page 46.

   The image window appears when acquisition is completed (Figure 7.5). See Table 4.2 on page 30 for more details on the Image window.

   Next, proceed to *"Single-Click" DLIT 3D Reconstruction* on page 127.

Figure 7.5 Image Window – Sequence View

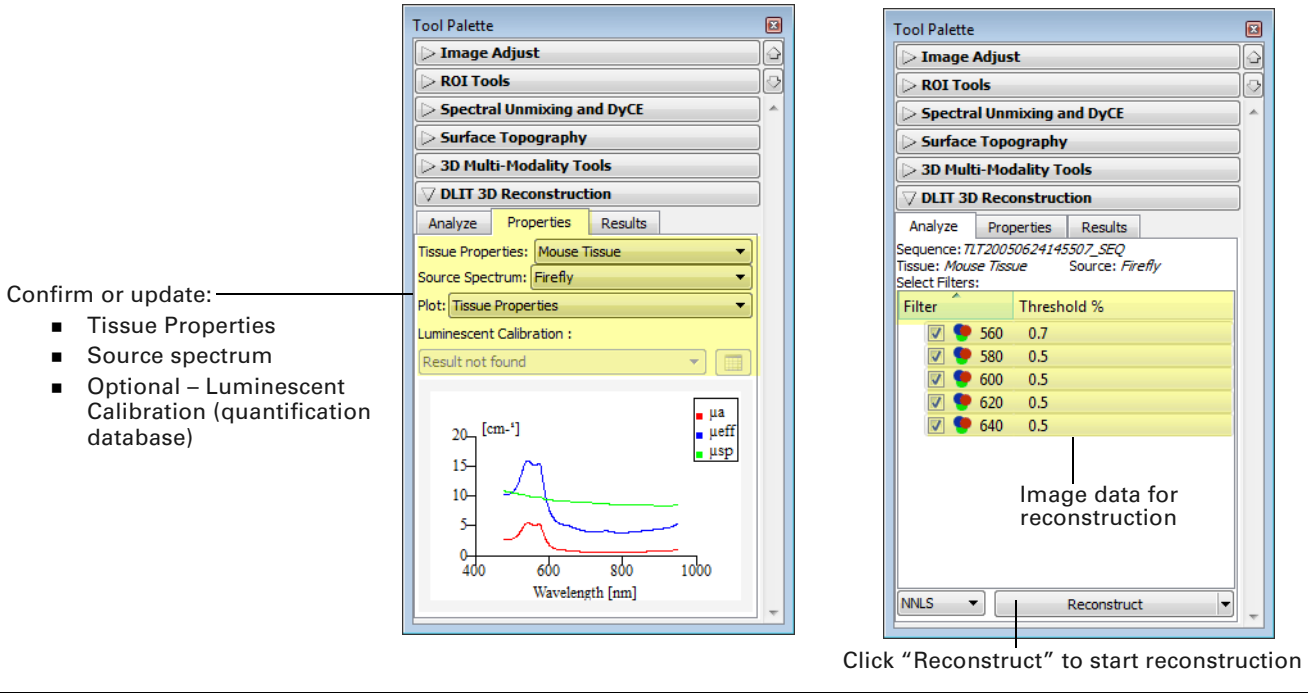


"Single-Click" DLIT 3D Reconstruction

NOTE: If the image sequence includes multiple subjects, for example two mice, perform manual 3D reconstruction for each subject separately (see page 135 for instructions). "Single-click" reconstruction is not available for this type of image data.

1. Load a luminescent image sequence.
2. Confirm that the correct tissue properties, source spectrum, and if using, quantification database are selected in the Properties tab (Figure 7.6). If necessary, change a property by making a selection from the drop-down lists.
3. Click **Reconstruct** (Figure 7.6).
4. Save reconstruction results (see *Managing 3D Reconstruction Results* on page 140).

Figure 7.6 DLIT 3D Reconstruction Tools



Reconstruction usually requires less than one minute, depending on the reconstruction volume, parameter settings, and computer performance. After the analysis is complete, the:

- 3D View window displays the animal surface and the reconstructed sources (Figure 7.7).
- 3D Optical Tools appear in the Tool Palette (see Table 7.1 for an overview of the tools).
- Results tab of the DLIT 3D Reconstruction tools displays the results data and the algorithm parameter values (see Table 7.6 on page 139 for more details).

Figure 7.7 DLIT Reconstruction Results

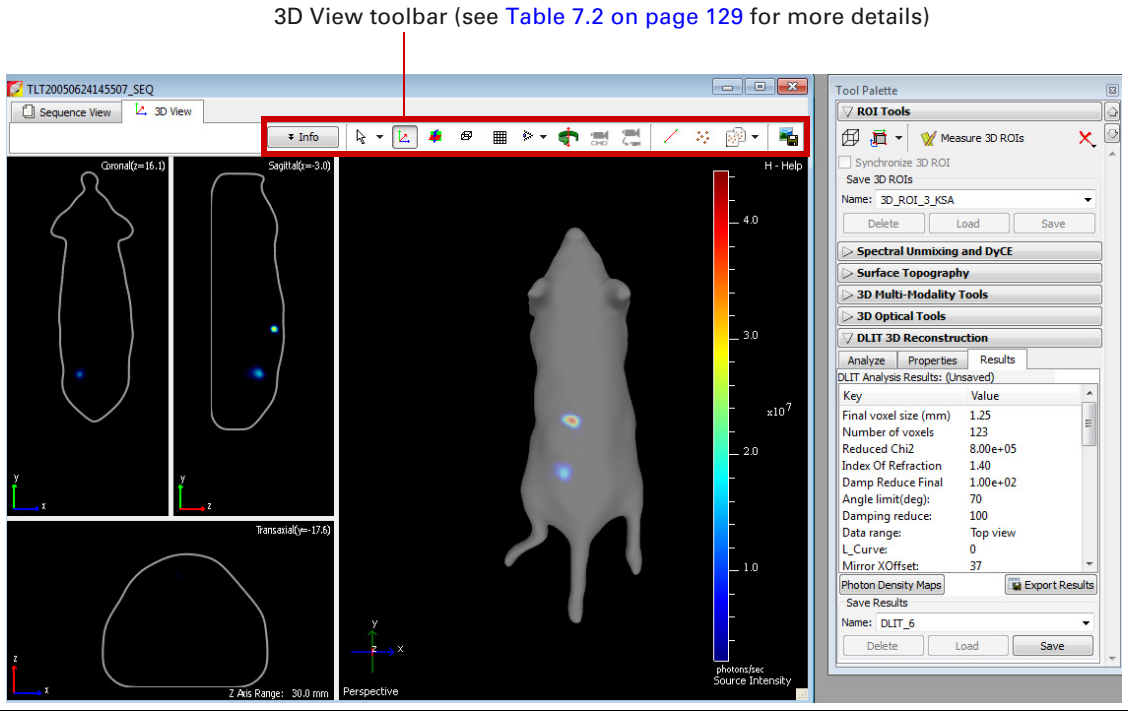


Table 7.1 3D Optical Tools

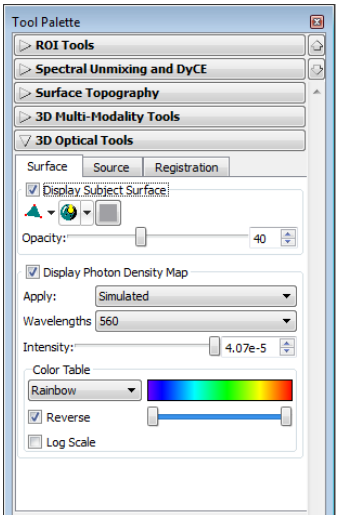
Tool Palette	3D Optical Tools	Functions	See Page
	Surface Tools	Adjust the appearance of the reconstructed animal surface and photon density maps.	143
	Source Tools	Make source measurements, adjust the appearance of reconstructed sources, export voxel measurements.	146
	Registration Tools	Display organs on the reconstructed surface, adjust the location or scale of organs on the surface, import an organ atlas.	156
	Animate Tools Select **Tools → 3D Animation** on the menu bar to view these tools.	Display preset animations of the 3D View scene. Enables you to create custom animations and record an animation to a movie file.	162

Figure 7.8 3D View Toolbar

Table 7.2 3D View Toolbar

Tool	Description
Info	Click to show or hide the image label. The image label includes information you enter in the Edit Image Labels dialog box (see Figure 4.6 on page 29) and other information automatically recorded by the software.
Image Tools 	A drop-down list of tools for viewing and working with the surface or 3D reconstruction. or - Rotates or spins the surface in the x, y, or z-axis direction. - Moves the surface in the x or y-axis direction. - Zooms in or out on the image. To zoom in, right-click (Cmd key (apple key) +click for Macintosh users) and drag the toward the bottom of the window. To zoom out, right-click and drag the toward the top of the window.
	Displays the x,y,z-axis display in the 3D view window.
	Displays coronal, sagittal, and transaxial cross-sections through the subject in the 3D view window.
	Displays a bounding box around the subject.
	Displays a grid under the subject.
	Select this tool from the drop-down list to change the view perspective (top, bottom, left, right, front, back, or perspective view). See Figure 8.14 on page 160 for examples.
	Select this tool from the drop-down list to display the perspective view.
	Rotates the 3D reconstruction in the 3D view window (*3D scene*). Click the + or - key to increase or decrease the rotation speed.To stop the rotation, click the 3D scene or the button.
	If multiple 3D reconstructions are loaded, click this button in one of the 3D views to turn on synchronization. All other 3D views will reset to display the same perspective as the active data. Click the button again to turn off synchronization. See page 154 for more details on synchronizing 3D views. **Note:** This button is only available if multiple 3D reconstructions are loaded.
	Applies the 3D view perspective of the active dataset to all loaded 3D reconstructions. **Note:** This button is only available if multiple 3D reconstructions are loaded and synchronization is turned off.
	Click to show or hide measurement tools in the coronal, sagittal, or transaxial views. Click and drag the green handle ( ) at either end of a measurement tool to resize and reposition it.
	Use this tool to obtain source measurements (for example, total flux, volume, center of mass, host organ). See *Source Quantitation* on page 149 for instructions.
	Copies or pastes voxels or a source surface so that DLIT and FLIT reconstructions can be displayed on one surface. See page 155 for more details.
	Enables you to save the 3D view to a graphic file (for example, .jpg).

7.3 Reconstructing Fluorescent Sources

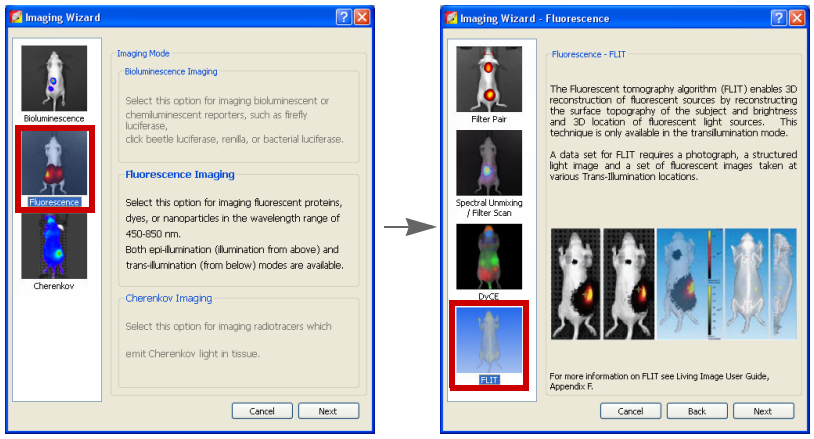
Acquire a Fluorescent Sequence

NOTE: The IVIS Spectrum should be initialized and the temperature locked before setting the imaging parameters. See page 19 for more details.

1. Start the Imaging Wizard. See page 44 for instructions.
2. Double-click the Fluorescence option. Double-click the FLIT option in the next screen (Figure 7.9).

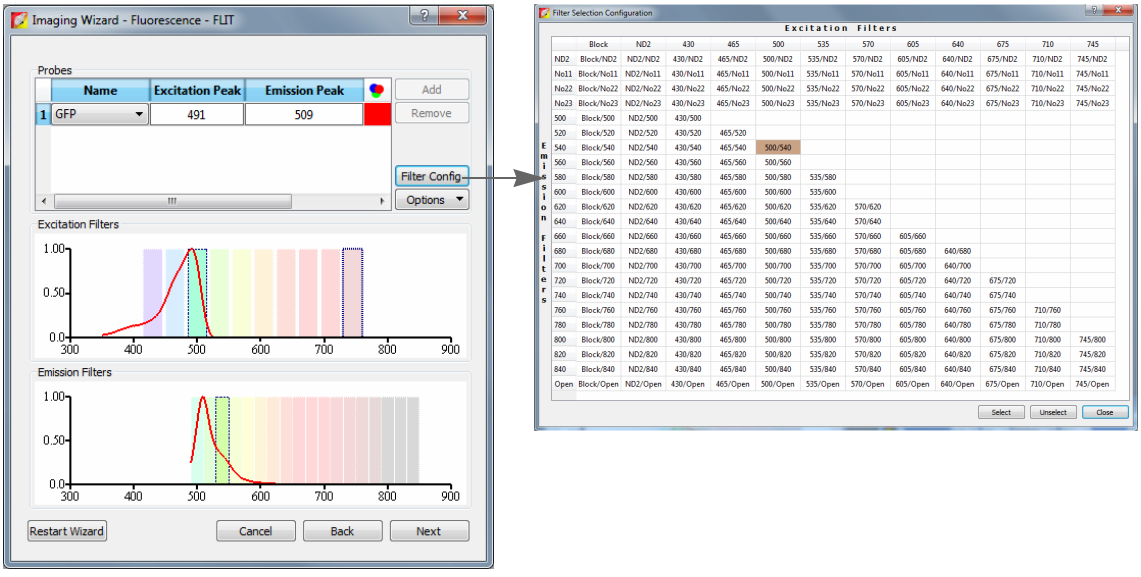
Figure 7.9 Imaging Wizard – Choose Fluorescence and FLIT Options

If this screen does not appear when the wizard starts, click **Restart Wizard** Restart Wizard (on the wizard screen) to restart the wizard.



3. Select a probe from the Name drop-down list and click **Next** (Figure 7.10).

Figure 7.10 Select a Probe

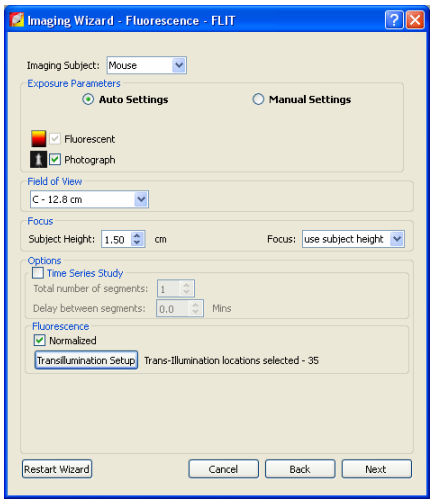


4. In the next screen (Figure 7.11):
   a. Select the type of imaging subject.

b. Choose the Auto Settings option for the exposure parameters.

c. Select a field of view from the drop-down list.

Figure 7.11 Imaging Wizard – Fluorescence FLIT



5. Set the focus by doing either of the following:

- Enter a subject height and choose the “use subject height” focus option.

OR

- Choose the “manual focus” option from the Focus drop-down list and set the focus parameters in the Manual Focus Window that appears.

6. If performing a time series study, choose the Time Series Study option. Enter the number of segments to acquire and the delay between segment acquisition. A segment is an image sequence.

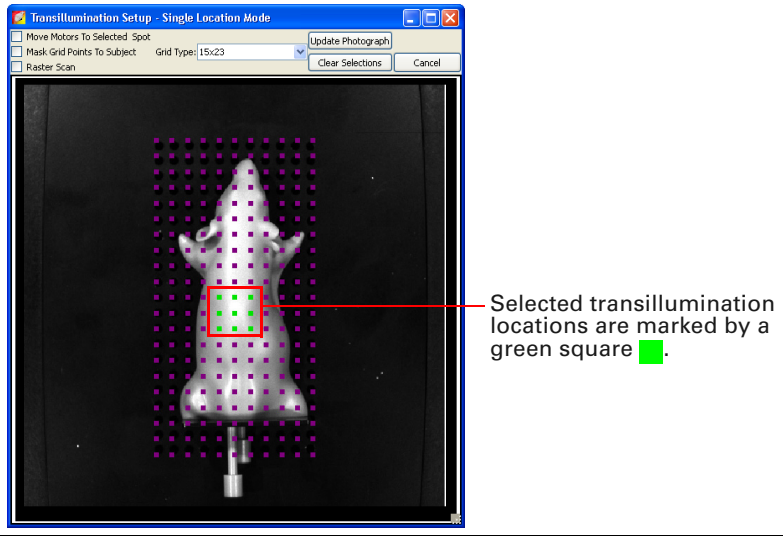
7. Select the transillumination locations.

a. Click **Transillumination Setup**.

b. Choose the transillumination locations in the Transillumination Setup box that appears (Figure 7.12).

See Table 4.3 on page 40 for more details on Transillumination Setup.

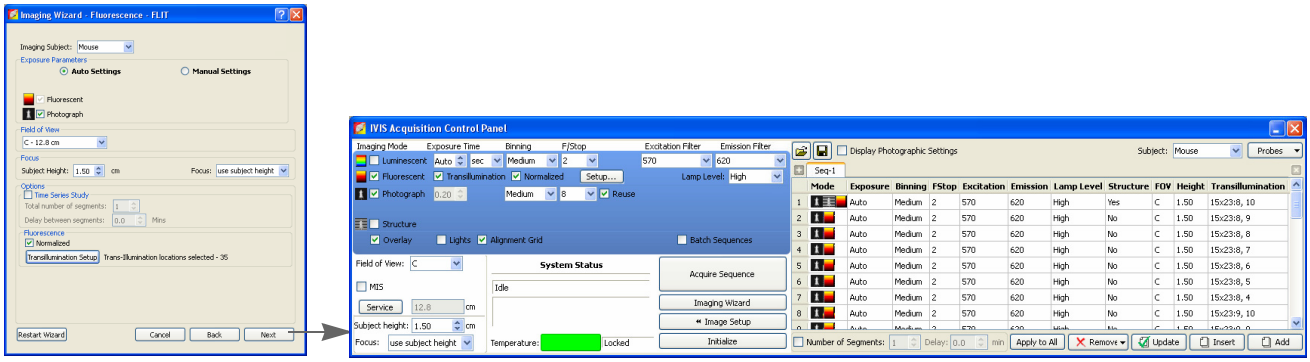
Figure 7.12 Transillumination Setup Box



8. Click **Next**.

   The specified sequence appears in the sequence table (Figure 7.13).

Figure 7.13 Sequence Setup Complete



9. Acquire the sequence following the instructions on page 46.

   The image window appears when acquisition is completed (Figure 7.5). See Table 4.2 on page 30 for more details on the Image window.

   Next proceed to *"Single-Click" FLIT 3D Reconstruction* on page 132.

"Single-Click" FLIT 3D Reconstruction

NOTE: If working with large datasets (e.g. high resolution FLIT datasets, longitudinal studies with many subjects), a 64-bit analysis workstation with 16 GB memory capacity is recommended.

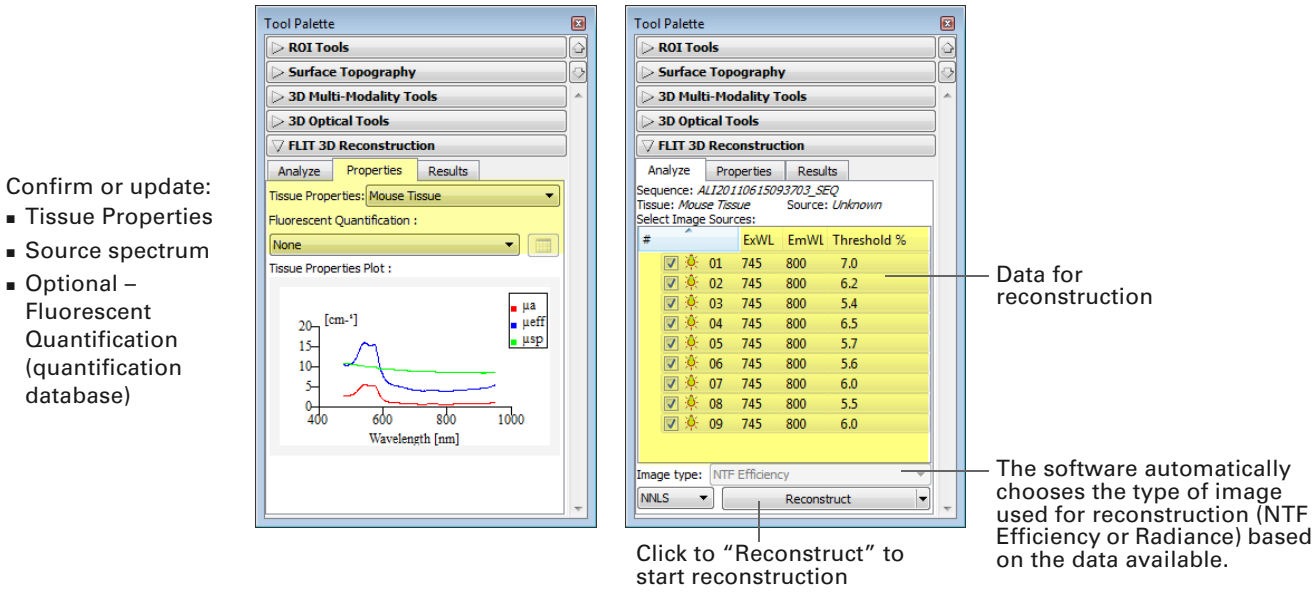
NOTE: If the image sequence includes multiple subjects, for example two mice, perform manual 3D reconstruction for each subject separately (see page 135 for instructions). "Single-click" reconstruction is not available for this type of image data.

1. Load a fluorescent image sequence.
2. Confirm that the default properties for reconstruction are correct (tissue properties and, if using, fluorescent quantification database) (Figure 7.6). If necessary, change a property by making a selection from the drop-down lists.

   NOTE: The software automatically chooses the type of image used for reconstruction (NTF Efficiency or Radiance) based on the available image data (Figure 7.14).
   - NTF Efficiency image is the default, if both types of image data are available.
   - Radiance image is selected if an NTF Efficiency image (transmission.tif) is not available in the click image folder.
   - If both types of image data are available and you want to use Radiance images for reconstruction, perform a manual reconstruction (see page 135 for instructions).

3. Click **Reconstruct** (Figure 7.6).
4. Save reconstruction results (see *Managing 3D Reconstruction Results* on page 140).

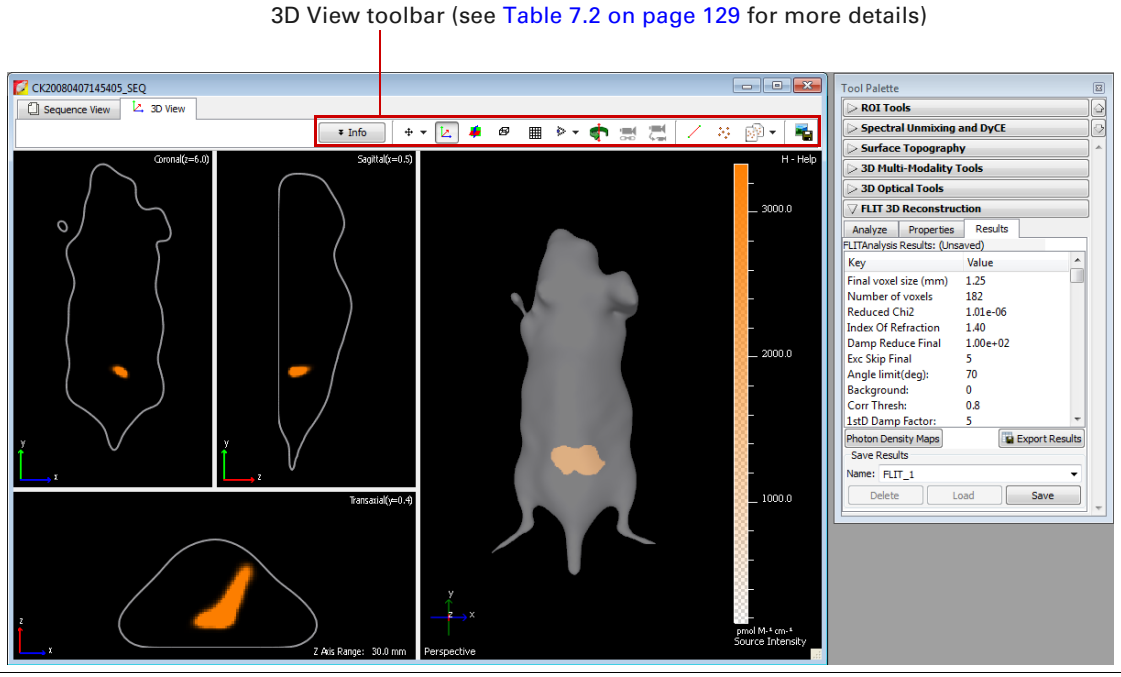
Figure 7.14 FLIT 3D Reconstruction Tools



Reconstruction time depends on the reconstruction volume, parameter settings, and computer performance. After the analysis is finished, the:

- 3D View window displays the animal surface and the reconstructed sources (Figure 7.15).
- 3D Optical Tools appear in the Tool Palette (see Table 7.1 on page 128 for an overview of the tools).
- Results tab displays the results data and the algorithm parameter values (see Table 7.6 on page 139 for more details).

Figure 7.15 FLIT results: 3D View Window and Results Tab



7.4 Manual 3D Reconstruction

This section explains how to:

- Manually set up a luminescence or fluorescence image sequence if you will not be using the Imaging Wizard.
- Perform DLIT or FLIT "manual reconstruction" which enables you to create or select a particular surface and include or exclude user-selected image data from the reconstruction.

Manual Sequence Setup

Table 7.3 lists the IVIS Spectrum filters available for DLIT or FLIT tomography.

Table 7.3 IVIS Spectrum Filters Available for Luminescence Tomography

Filters	Range	Bandwidth
10 excitation filters	415-760 nm	30 nm
18 emission filters	490-850 nm	20 nm

Luminescence Sequence for DLIT

Set up a sequence that includes:

- A structured light image.
- Optical data from at least two different emission filters (560 - 660 nm), at a minimum:
  - Emission filter #1: Photographic, luminescent.
  - Emission filter #2: Luminescent image.

Analyzing more optical images usually produces more accurate results. Table 7.4 shows the recommended optical image sequence.

Table 7.4 Manual Sequence Setup – Recommended DLIT Optical Image Sequence

Image Type	Emission Filter Options					
	560	580	600	620	640	660
Photograph	✓	Select the Reuse option in the control panel.				
Luminescent	✓	✓	✓	✓	✓	✓

NOTE: It is recommended to set the same binning level for all of the luminescent images.

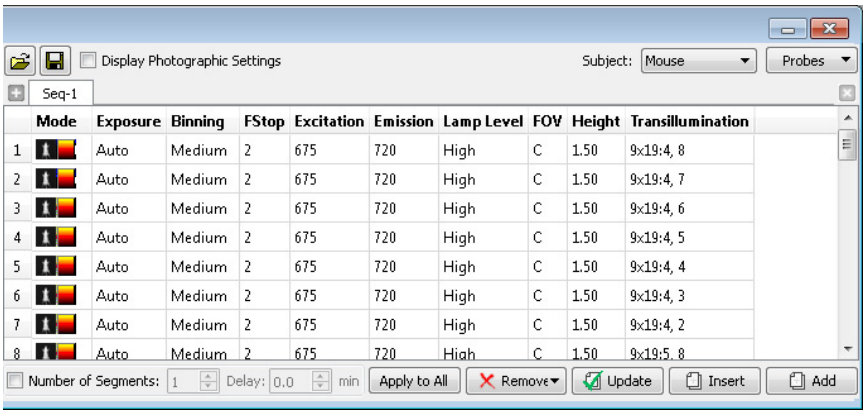
Fluorescence Image Sequence for FLIT

Use transillumination on the IVIS Spectrum and the same excitation and emission filters at a minimum of four source locations which form a rectangle. Acquire the following images:

- Fluorescent image and photograph at the first transillumination location.
- Fluorescent image at the remaining transillumination locations.
- A structured light image.

Figure 7.16 shows an example image sequence.

Figure 7.16 Example FLIT Sequence Setup



Manual 3D Reconstruction

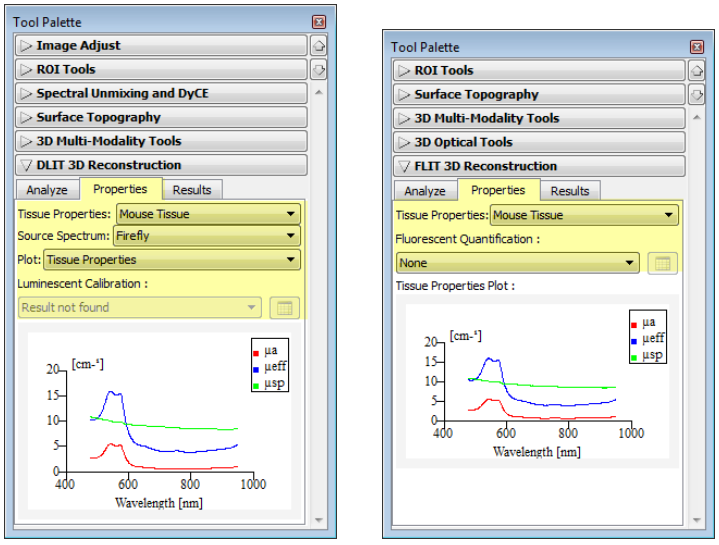
1. Load a DLIT or FLIT image sequence.
2. Generate or load a surface using the Surface Topography tools. See Appendix D on page 267 for instructions.

   NOTE: If the image sequence includes multiple subjects, you will need to reconstruct each subject separately. This requires a manually generated surface for each subject.

3. Select 3D reconstruction properties from the Properties drop-down lists (Figure 7.17):
   - DLIT – Select Tissue Properties, Source Spectrum, and if using, a quantification database ("Luminescent Calibration").
   - FLIT – Select Tissue Properties and, if using, a quantification database ("Fluorescent Quantification").

   NOTE: See Appendix C on page 260 for more details on quantification databases.

Figure 7.17 DLIT and FLIT 3D Reconstruction Properties



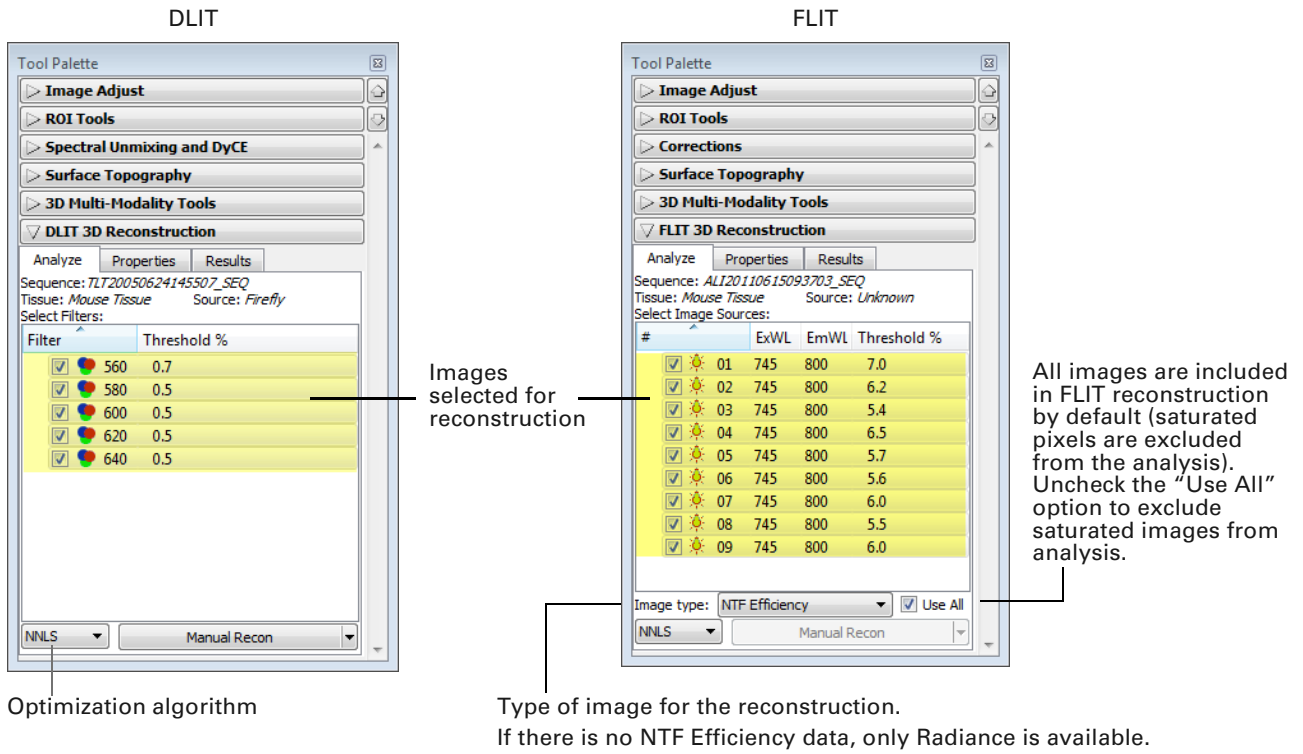
DLIT Properties FLIT Properties

4. Confirm the images selected for reconstruction (Figure 7.18).

   For FLIT, images with saturated pixels are included by default in the analysis. However, saturated pixels will be excluded from the analysis. Uncheck the "Use All" option to exclude saturated images from the analysis (Figure 7.18).

5. Choose the type of image to use in the 3D reconstruction from the "Image type" drop-down list (NTF Efficiency or Radiance) (Figure 7.18).

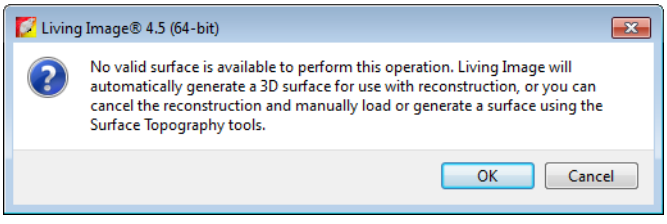
Figure 7.18 Images for Reconstruction



6. Click **Manual Recon** in the Analyze tab (Figure 7.18).
7. Select an option in the prompt that appears (Figure 7.19).
   - **OK** – Reconstruction will use the surface that was last generated by the software. If this surface is not appropriate for the active data, the software automatically generates a new surface.
   - **Cancel** – You will create (or select) and load a surface using the Surface Topography tools. See Appendix D on page 267 for instructions.

NOTE: If the image sequence includes multiple subjects, choose "Cancel" and select the surface (created in step 2) for the subject that you want to reconstruct.

Figure 7.19 Prompt to Select a Surface



8. Select pixels to include or exclude from the reconstruction in the Data Preview window that appears (Figure 7.20). There are two ways to do this:
   - Change the Threshold % value (see page 137) – Applying a Threshold % value excludes or includes some pixels from the reconstruction. The software computes the minimum and maximum pixel values of an image based on an histogram of pixel intensities. For example, if Threshold % = 0.5%, then pixels with intensity less than 0.5% of the maximum intensity value are excluded from the reconstruction. The Threshold % can be edited for individual images. The Data Preview window is updated when you change the Threshold % value. Min Counts translates the Threshold % to the minimum counts required for reconstruction. Keep the minimum counts > 200.
   - Select regions on an image (see page 138) – Use the pencil tool to mark particular regions to include in the reconstruction. This may be useful for noisy images with high intensity pixels where changing the Threshold % value is not helpful. This method also provides a useful way to focus on particular sources to reconstruct and ignore others.

To change Threshold %:

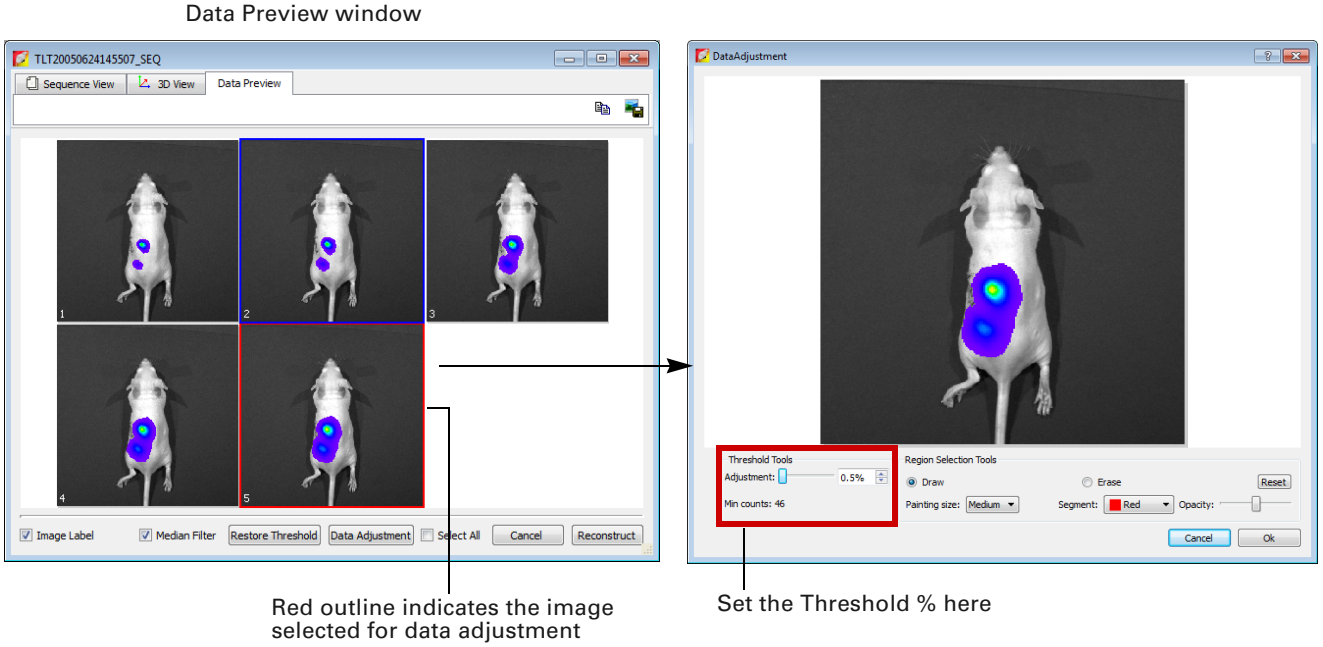
1. Click an image in the Data Preview window (Figure 7.20).

   NOTE: Changes to Threshold % are applied to the selected image only. To apply the change to all images, choose the **Select All** option.

2. Click **Data Adjustment**.
3. Enter a new Threshold % value in the window that appears.

   The new Threshold % appears in the Analyze tab. If necessary, click **Restore Threshold** to reset the Threshold % to the default value (for the selected images).
4. Click **Reconstruct** after you set the Threshold%.

Figure 7.20 Adjusting Threshold %



To select particular regions for reconstruction:

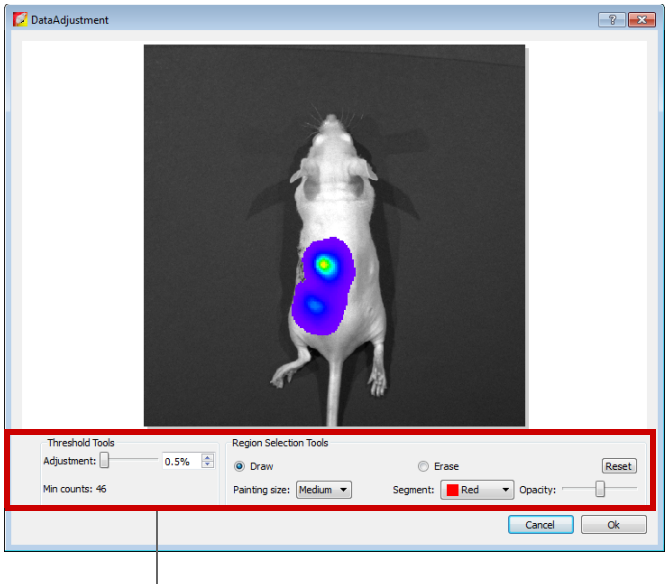
1. Open the Data Preview window as shown in Figure 7.20.
2. Click **Data Adjustment**.
3. Choose the **Draw** option In the window that appears, and put the mouse pointer over the image so that the pencil tool appears (Figure 7.21).
4. To automatically select all pixels in a source, right-click within the region with the pencil tool.

   Alternatively, put the pencil over the image and click the mouse key or press and hold the mouse button while moving the pencil over an area of the image.

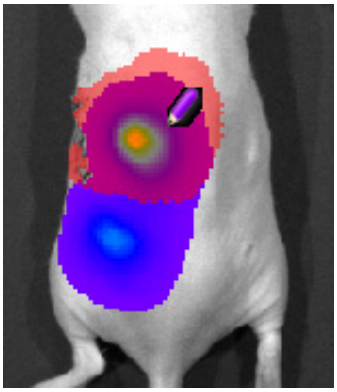
> **NOTE:** Pencil tool markings will be applied to all images of a sequence. If pencil tool markings are applied, only the marked pixels are included in the analysis.

5. Click **Reconstruct** after you are done marking the regions.

Figure 7.21 Selecting Regions to Include in Reconstruction



Use these tools to select particular image data to include in the analysis. See Table 7.5 for details on the tools.



Choose the "Draw" option then mark the area to include in the reconstruction using the pencil tool. In this example, the red area marked with the pencil tool will be reconstructed.

Table 7.5 Region Selection Tools

Item	Description
Draw	Choose this option to display the pencil tool when the mouse pointer is over the data adjustment image. Use this tool to apply markings that select regions to include in the reconstruction.
Erase	Choose this option to display the eraser tool. Use the eraser to remove pencil tool markings (exclude pixels from the image).
Painting size	Adjusts the width of the pencil tool mark or the eraser tool.
Segment	Colors available for the pencil tool.
Opacity	Adjusts the opacity of the pencil tool markings.
Reset	Removes all pencil tool markings.

7.5 3D Reconstruction Results

The Results tab displays information about the photon density, voxels, and algorithm parameters.

DLIT or FLIT Results

NOTE: For more details on DLIT, see the see the reference article *DLIT and FLIT Reconstruction of Sources* (select **Help → References** on the menu bar). Sometimes adjusting the DLIT algorithm parameters improves the fit of the simulated photon density to the measured photon density data.

Figure 7.22 Example DLIT 3D Reconstruction Results

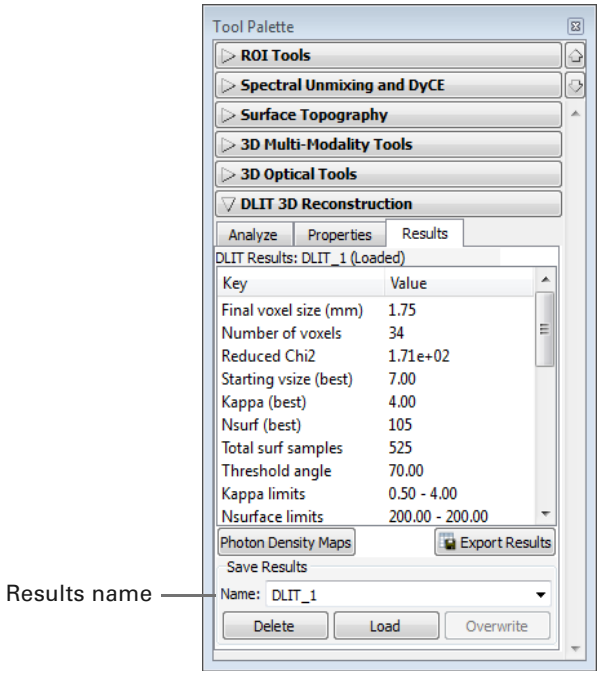


Table 7.6 DLIT or FLIT 3D Reconstruction Results

Item	Description
Final voxel size (mm)	The voxel size (length of a side, mm) that produces the optimum solution to the DLIT or FLIT analysis.
Number of voxels	The number of voxels that describe the light source(s).
Reduced Chi2	A measure of the difference between the computed and measured photon density maps at the optimum solution. A smaller χ^2 value indicates a better quality of fit.
Index of Refraction	Refractive index of light for the imaged subject.
Angle Limit(deg)	Angle limit of surface normal to optical axis, above which data will not be used in the reconstruction.
Damping reduce	The damping parameter is calculated from this reduction factor, relative to the maximum singular value of the system matrix.

Table 7.6 DLIT or FLIT 3D Reconstruction Results (continued)

Item	Description
Data range	For multi-view data, the image views used in the reconstruction.
Mirror XOffset	For multi-view data, the mirror location from the x center line.
Starting voxel size	The voxel size at the start of the analysis. The length of the side of the voxel cube in mm units for the coarsest initial grid size in the adaptive gridding scheme.
Total # of data pts	The total number of data points used in the reconstruction.
Median Filter	Indicates whether or not a median filter was applied to the data.
Image Threshold	The percentage of the minimum radiance at each wavelength (DLIT) or source location (FLIT) is of the maximum radiance. This defines the minimum intensity included in the data.
Samples of Image	The data in each image is sampled. This parameter shows the number of pixels sampled from each image.
Tissue Properties	The tissue properties for modeling the photon propagation.
Source Spectrum	The emission spectrum of the type of luminescent source.
Quantification Selection	A user-selected quantification database used in the reconstruction to convert reconstruction voxel units to 'cells' or 'picomoles' units.
Sequence name	Image data sequence name.
Version	Living Image® software version

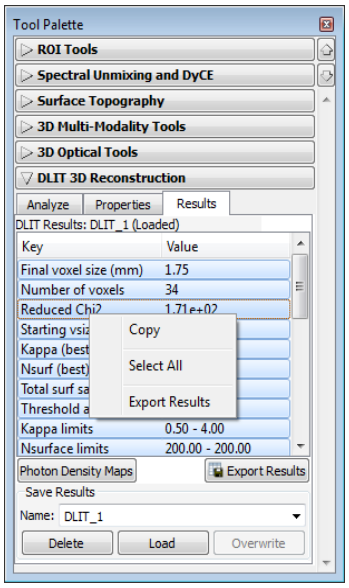
Managing 3D Reconstruction Results

Item in the DLIT/FLIT 3D Reconstruction Results Tab	Description
Name	The name for the active DLIT or FLIT results. Select results from this drop-down list.
Delete	Deletes the selected DLIT or FLIT results.
Load	Opens the selected reconstruction results in the 3D View.
Save	Saves the active DLIT or FLIT results to the selected name. The results are saved to the sequence click number folder and are available in the Name drop-down list.
Overwrite	If you reanalyze saved results, saves the new results and overwrites the previous results.
Export Results	Saves the results to a .csv file.

Copying Results to the System Clipboard

1. To copy all results:
   a. Right-click the results and chose **Select All** from the shortcut menu.
   b. Right-click the results again and select **Copy** from the shortcut menu.

Figure 7.23 Select and Copy Results



2. To copy user-selected results:
   a. Select the results.
   b. Right-click the selection and choose **Copy** from the shortcut menu.

7.6 Checking 3D Reconstruction Quality

Comparing the measured and simulated photon density plots is a useful way to check the reconstruction .

Photon density is closely related to the measured radiance. Photon density is the steady state measure of the number of photons in a cubic millimeter. Light sources inside the tissue contribute to photon density in other portions of the tissue.

The reconstruction algorithm first converts the luminescent or fluorescent image of surface radiance to photon density just inside the animal surface because this is what can be observed. The algorithm then solves for intensity values at locations inside the tissue which would produce the observed photon density near the surface.

For fluorescence reconstructions using NTF Efficiency data, the photon density of the fluorescence image is divided by the photon density of the transmission image, giving the NTF Efficiency. The NTF Efficiency values are the data just inside the animal surface for this type of dataset.

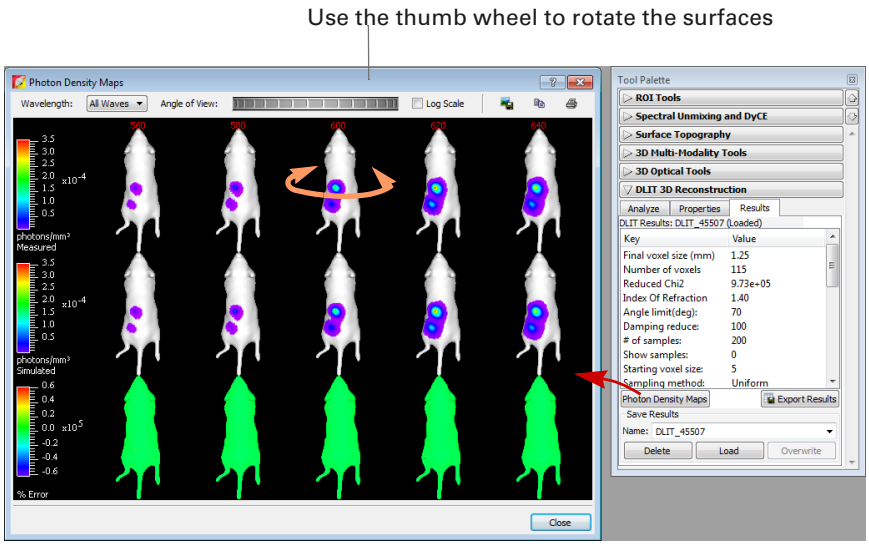
Viewing Photon Density or NTF Efficiency Maps

1. After the reconstruction is finished or results are loaded, click **Photon Density or NTF Efficiency Maps** in the Results tab.

   The photon density maps or NTF Efficiency maps for all image data are displayed (Figure 7.24).

2. To rotate the surface and view it from a different angle, move the thumb wheel to the left or right.

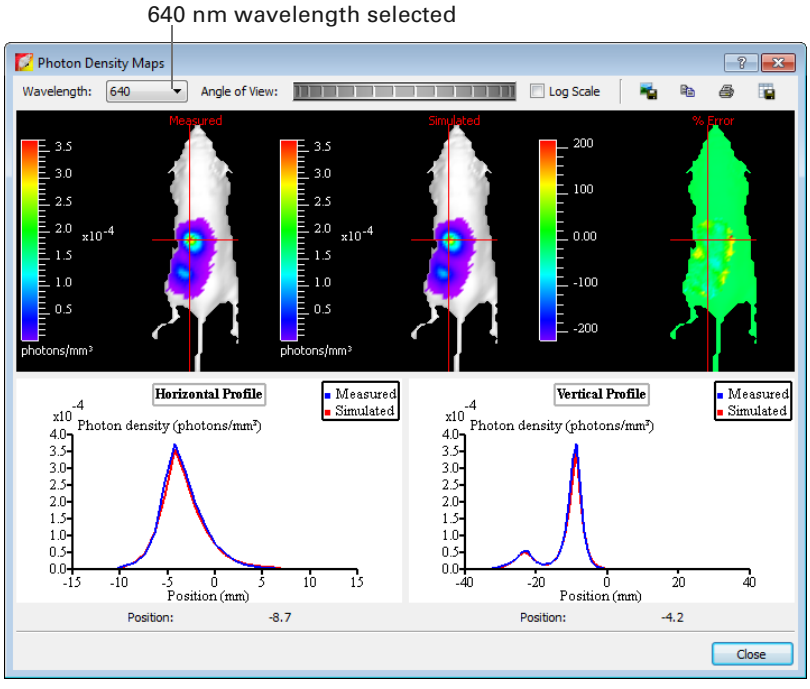
Figure 7.24 Photon Density Maps (or NTF Efficiency Maps for Fluorescence)



3. Select a wavelength from the drop-down list

   The photon density or NTF Efficiency profiles at the crosshairs location are displayed. In a good reconstruction, the simulated photon density or NTF Efficiency curves (red) closely resemble the measured photon density or NTF Efficiency curves (blue).

Figure 7.25 Simulated (Red) and Measured (Blue) Photon Density (or NTF Efficiency) Plots



4. To view the photon density or NTF Efficiency profile at another location on the animal surface, drag the cross hairs or click a point on the photon density or NTF Efficiency map.

Table 7.7 Photon Density Maps Window

Item	Description
Image sources	A list of images used in the reconstruction. Select all images or a particular image number to display.
Angle of View	The thumb wheel position. Turn the thumb wheel to rotate the surface on the vertical axis.
Log Scale	Choose this option to display the photon density or NTF Efficiency using a log scale.
Simulated	The photon density or NTF Efficiency computed from DLIT or FLIT source solutions which best fit the measured photon density or NTF Efficiency.
Measured	The photon density or NTF Efficiency determined from the image measurements of surface radiance.
Horizontal Profile	The photon density or NTF Efficiency line profile at the horizontal plane through the subject at the crosshairs location.
Vertical Profile	The photon density or NTF Efficiency line profile at the vertical plane through the subject at the crosshairs location.
Position (mm)	Horizontal Profile: The y-axis position of the crosshairs horizontal line. Vertical Profile: The x-axis position of the crosshairs vertical line.The x-y positions are relative to the center of the FOV (where x = 0 and y = 0).

Adjusting Surface and Photon Density Appearance

Use the 3D Optical Surface tools to adjust the appearance of the reconstructed animal surface and photon density maps.

Figure 7.26 Surface Tools and Example DLIT Reconstruction with Photon Density or NTF Efficiency Maps

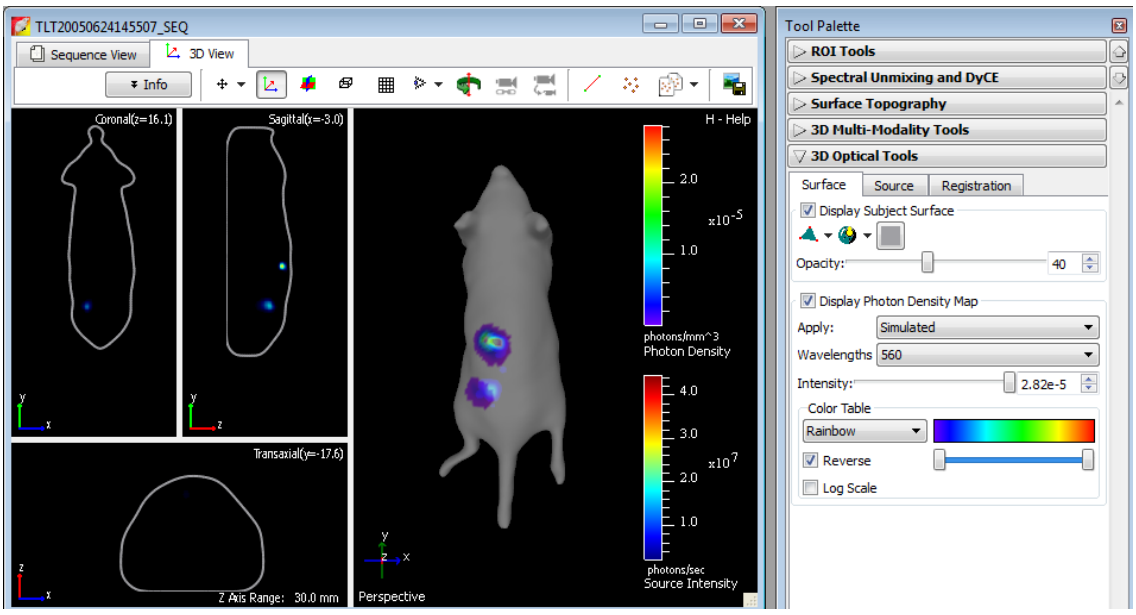
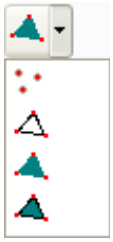
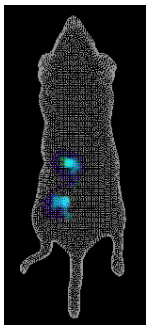
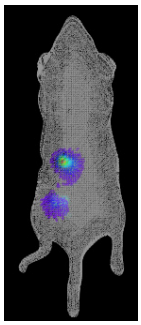
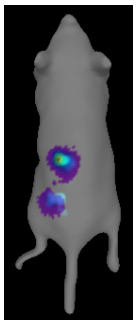
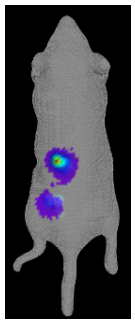
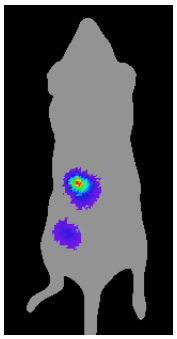
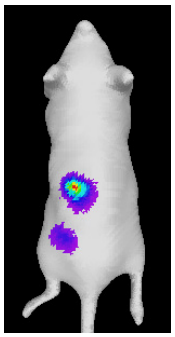
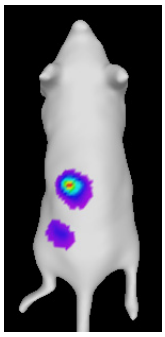
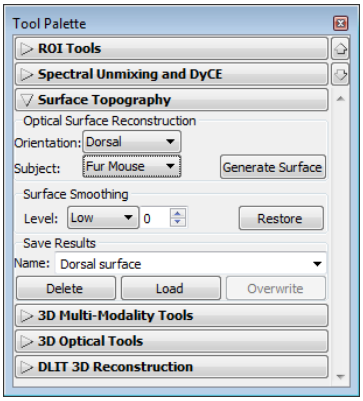


Table 7.8 3D Optical Tools, Surface Tab

Item	Description
Display Subject Surface	Choose this option to display the surface in the 3D View window.
	Drawing styles for the surface.  Point cloud  Wire frame  Surface face  Wire frame & surface
	Shading styles for the surface.  Surface face  Smooth surface face  Reflect surface face  Reflect smooth surface face
	Click to open the color palette from which you can select a display color for the surface and the cross section views.
Opacity	Adjusts the surface opacity.
Display Photon Density or NTF Efficiency Map	Choose this option to display the photon density or NTF Efficiency on the surface.
Apply	Choose measured or simulated photon density or NTF Efficiency maps for display.
Wavelengths (DLIT) Images (FLIT)	Choose the data to display in the photon density or NTF Efficiency map.
Intensity	Set the maximum intensity of the photon density or NTF Efficiency map using the slider or by entering a value.
Color Table	Color scheme for the photon density or NTF Efficiency map.
Reverse	Choose this option to apply the colors of the selected color table in reverse order. For example, the Red color table represents the mapped intensity from low to high using a color scale from transparent to red. If **Reverse** is chosen, the mapped intensity from low to high is represented using the color scale from red to transparent.
Log Scale	Choose this option to apply a logarithmic scale to the photon density or NTF Efficiency scale.

7.7 Troubleshooting

Issue	Solution
No sources in solution	This can occur in DLIT or FLIT if the surface is not correct. For example, if a surface is imported into the 3D View from another source other than a Surface Topography analysis.
Surface has spikes	The most common source of spiky surfaces are folds in the animal skin or fur which corrupt the desired smooth lines projected on the animal from the laser galvanometer. ▪ Choose the 'Fur Mouse' option for 'Subject'. ▪ Smoothing the surface by using the 'Smooth' feature in the Surface Topography tools can help improve the surface. 
Bad Photon Density or NTF Efficiency fit	The optical properties or source spectrum may have been incorrectly chosen. For example, 'Mouse Tissue' optical property is appropriate or mice, but 'XPM-2/XFM-2' is only appropriate for the mouse phantom.

8 Working With 3D Reconstructions

8.1 Adjusting Source Appearance and Making Measurements

Use the Source tools to:

- Adjust the appearance of sources in 3D reconstructions.
- Make source measurements.
- Export voxel measurements (.csv)

Figure 8.1 3D Optical Tools, Source Tab

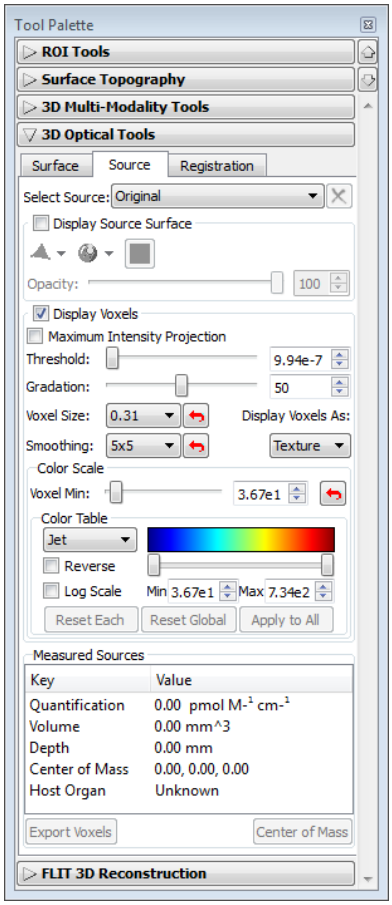


Table 8.1 3D Optical Tools, Source Tab

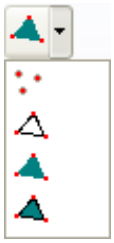
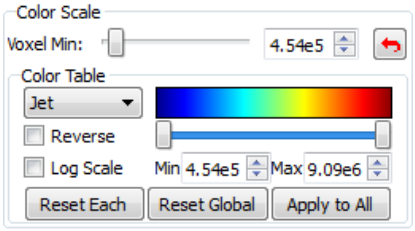
Item	Description
Select Source	A drop-down list of available sources. Original – Results saved with the data. <sequence name...SourceVoxels> – Pasted voxels. (Click the button to remove pasted voxels from the surface.) See *Viewing Luminescent and Fluorescent Sources on One Surface* on page 155 for more details on copying and pasting sources from one sequence to another.
Display Source Surface	Choose this option to display the source surfaces reconstructed using DLIT or FLIT. A surface will be wrapped around the currently displayed voxels. Adjust the voxel display by moving the Threshold slider. **Note:** Choosing "Display Source Surface" automatically turns off the "Display Voxels" option, and vice versa. If you uncheck "Display Source Surface", neither the surface nor voxels will be visible. Put a check mark next to "Display Voxels" to see voxels.
	Drawing styles for the source surface (see "*Display Source Surface*" above).
	Shading styles for the source surface (see "*Display Source Surface*" above).
	Click to open the color palette from which you can select a display color for the source surface.
Opacity	Adjusts the source surface opacity.
Display Voxels	Choose this option to display the sources reconstructed using DLIT or FLIT. **Note:** Choosing "Display Voxels" automatically turns off the "Display Source Surface" option, and vice versa. If you uncheck "Display Voxels", neither the voxels nor source surfaces will be visible. Put a check mark next to one of these display options to see voxels or source surfaces.
Maximum Intensity Projection	Choose this option to project all maximum intensity voxels in the view along the viewing direction into the viewing plane.
Threshold (DLIT/FLIT)	Choose this option to apply a minimum threshold intensity to the voxel display.
Gradation (DLIT/FLIT)	Use this slider to set a threshold for the percentage voxel intensity above which voxels are opaque and below which voxels will gradually face to transparent. The percentage voxel intensity is the percentage relative to the maximum intensity.
Voxel size	The 3D grid-spacing size for interpolation of the reconstructed source.
Smoothing	The smoothing box filter size.
Display voxels as	The voxel display mode (cubes, spheres, points, or texture).

Table 8.1 3D Optical Tools, Source Tab (continued)

Item	Description
Color Scale and Color Table	 Voxel Min – Use the slider, up/down arrows, or enter a value to set the minimum value of the source color scale. Voxels with intensities less than the color scale minimum are not displayed in the reconstruction. Color Table – Color scheme for voxel display. Use the left and right sliders, up/down arrows, or enter values to set the minimum and maximum colors. The Color Table Max is allowed up to 100 times the voxel maximum. Reverse – Choose this option to apply the colors of the selected color table in reverse order to the source voxel scale. For example, the Red color table represents the source intensity from low to high using a color scale from transparent to red. If **Reverse** is chosen, the source intensity from low to high is represented using the color scale from red to transparent. Log scale – Choose this option to apply a logarithmic scale to the color table. **Note:** The following items are available if multiple 3D reconstructions are loaded. Reset Each – Displays each 3D view using the default color table and scale for the dataset. Reset Global – Applies the color table of the active data and an aggregate scale to all 3D views. Apply to All – Applies the color table and scale of the active data to all 3D views. The software will alert you if the loaded results have different units, for example, a DLIT and FLIT reconstruction.
Measured Sources	Quantification (DLIT) – For uncalibrated sources, the total flux measured for the sources selected using the Measure Source tool. For calibrated sources, this unit will be in [cell] units. For details on using this tool, see page 149. Quantification (FLIT) – For uncalibrated sources, the fluorescence yield measured for the voxels selected using the Measure Source tool. Fluorescence yield is expressed in units of [pmol $M^{-1}cm^{-1}$] here for uncalibrated sources. For calibrated sources, this unit will be in either [cells] or [pmol]. For details using this tool, see page 149. Volume – Volume of the selected source (mm^3). Depth – Perpendicular distance from the source center of mass to dorsal surface. Center of Mass (DLIT or FLIT) – The weighted average x, y, and z-coordinates of the selected voxels, where the weights are the flux of each highlighted voxel. Host Organ – If reference atlas organs are displayed with the reconstruction, the organ that is closest to the source will be reported here. See page 156 for instructions on displaying organs on a reconstruction.
Export Voxels	Enables export of voxel measurements in their x-, y-, and z-coordinates and source intensities (.csv file).
Center of Mass	Click to compute the center of mass for the source selected with the Measure Source tool. For details using this tool, see page 149.

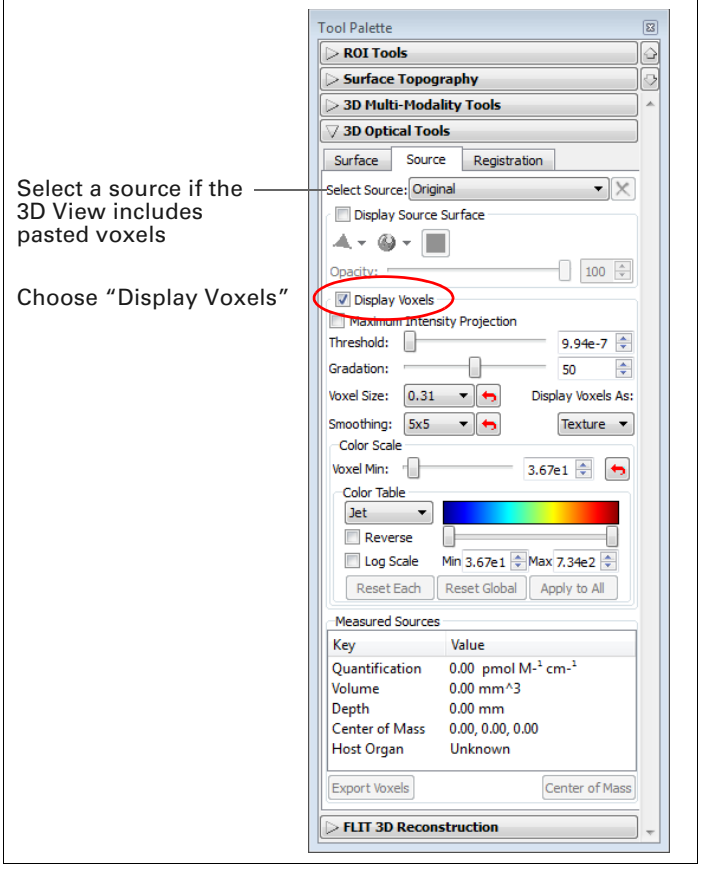
Source Quantitation

This section explains a convenient way to measure the source (voxels), total flux or total florescence yield, or if calibrated, the abundance in cells or picomoles. The volume, center of mass, and depth at the center of mass are also reported in the 3D Optical tools.

NOTE: Use 3D ROIs for more precise measurements. See page 174 for instructions.

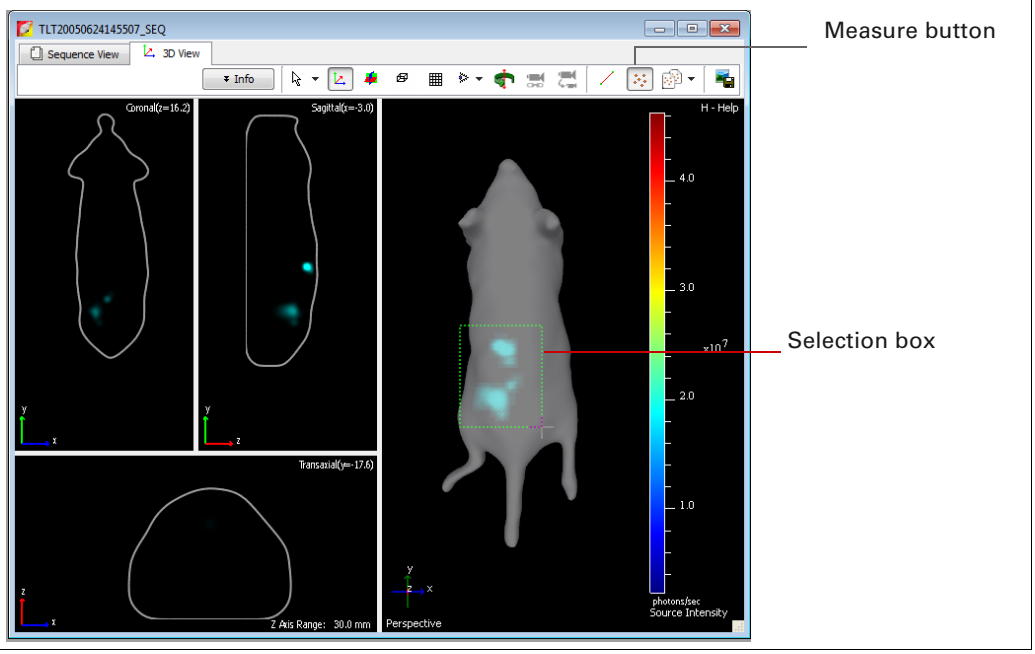
1. Click the Source tab in the 3D Optical tools.
2. If the surface includes voxels pasted from other results, select a source from the drop-down list (Figure 8.2).
3. Confirm that "Display Voxels" is selected, not "Display Source Surface".

Figure 8.2 Select and Measure Source Voxels



4. Click the Measure Source button, then draw a box around the source (Figure 8.3).

Figure 8.3 Select Source to Measure



5. Click **Center of Mass** to obtain the measurements (Figure 8.4). See Table 8.2 on page 151 for a description of the measurements.

   The coronal, sagittal, and transaxial planes intersect at the center of mass of the selected source (see Figure 8.5 on page 151).

Figure 8.4 Source Measurements

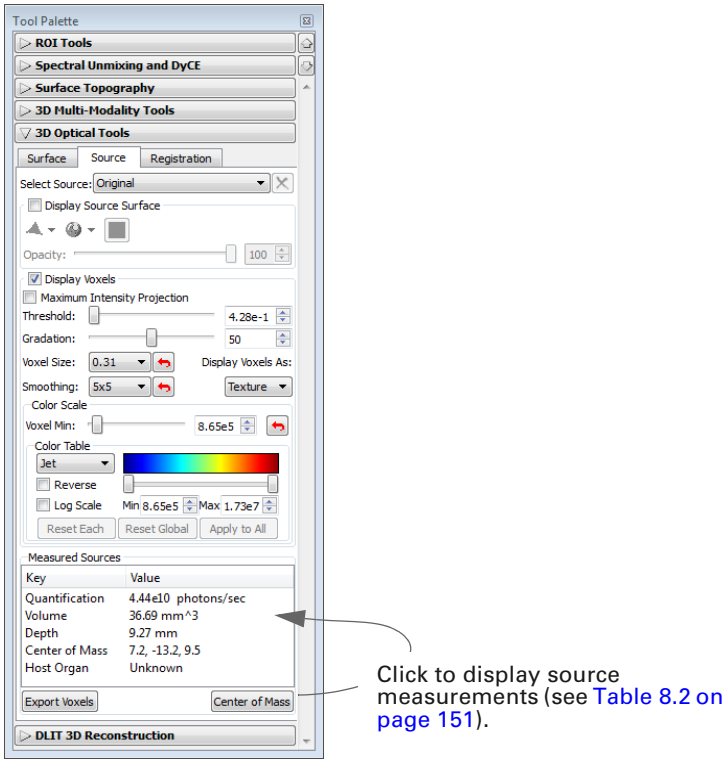


Table 8.2 Source Measurements

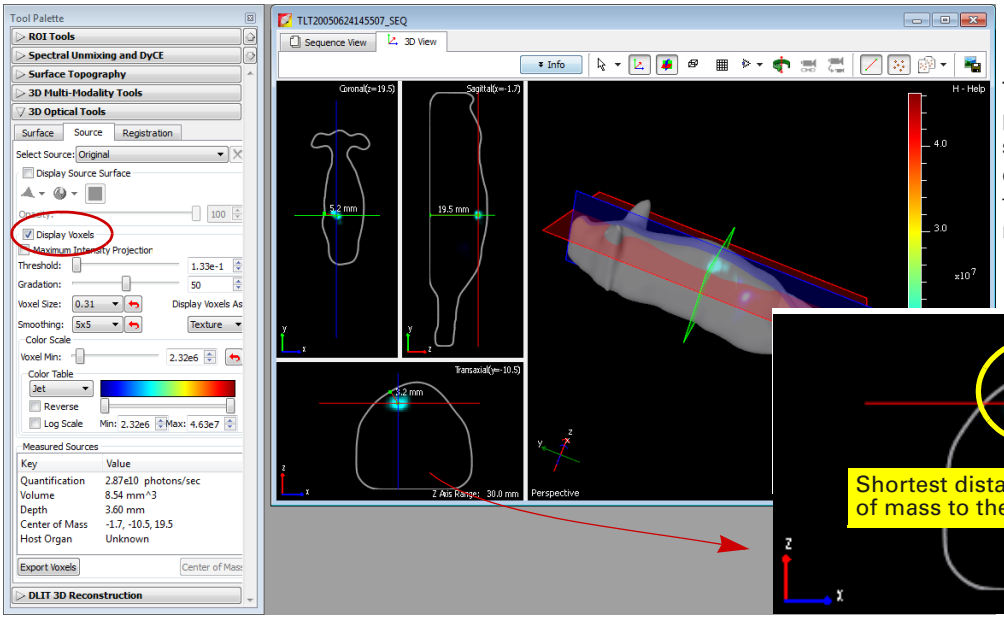
Source Measurement	Description
Quantification	The integrated intensity within the selected sources.
Volume	The total volume of the selected sources.
Depth	The perpendicular distance from the source center of mass to dorsal surface.
Center of Mass	The weighted average x, y, and z-coordinates of the selected voxels, where the weights are the flux of each highlighted voxel.
Host Organ	The reference atlas organ in which the selected sources are located. This information is available if organs are displayed with the reconstruction. See *Displaying Organs With a Reconstruction* on page 156 for more details.

Source Depth

This section explains another way to measure source depth. Follow the steps below after reconstruction is finished or results are loaded.

1. Click the Source tab in the 3D Optical tools.
2. If the surface includes voxels pasted from other results, select a source from the drop-down list.
3. Confirm that "Display Voxels" is selected, not "Display Source Surface".
4. Click the measurement tool button .
   The distance from the center of mass to the surface is measured in the three planes.
   - Coronal and transaxial planes display the shortest distance from the center of mass to the surface.
   - The sagittal plane displays the distance from the center of mass to the bottom of the subject.
5. Click the button to display slice planes through the center of mass. See page 152 for more information on planes.

Figure 8.5 Slice Planes



This example shows slice planes through a selected source center of mass and distance measurements from the source center of mass to the surface.

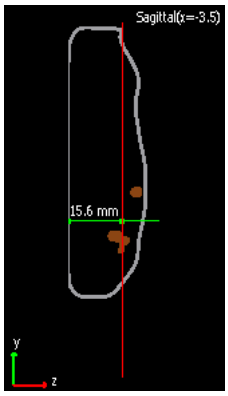
Viewing Location Coordinates

Click a location in the reconstruction slice in the Coronal, Sagittal, or Transaxial windowpane.

The coordinates (mm) of the position are displayed (Figure 8.6). The coordinates are updated when you press and hold the mouse button while you drag the cursor.

Slice Plane	Displays...
Coronal	The x-y coordinates of a position.
Sagittal	The y-z coordinates of a position.
Transaxial	The x-z coordinates of a position.

Figure 8.6 Viewing Y,Z Coordinates – Sagittal Plane

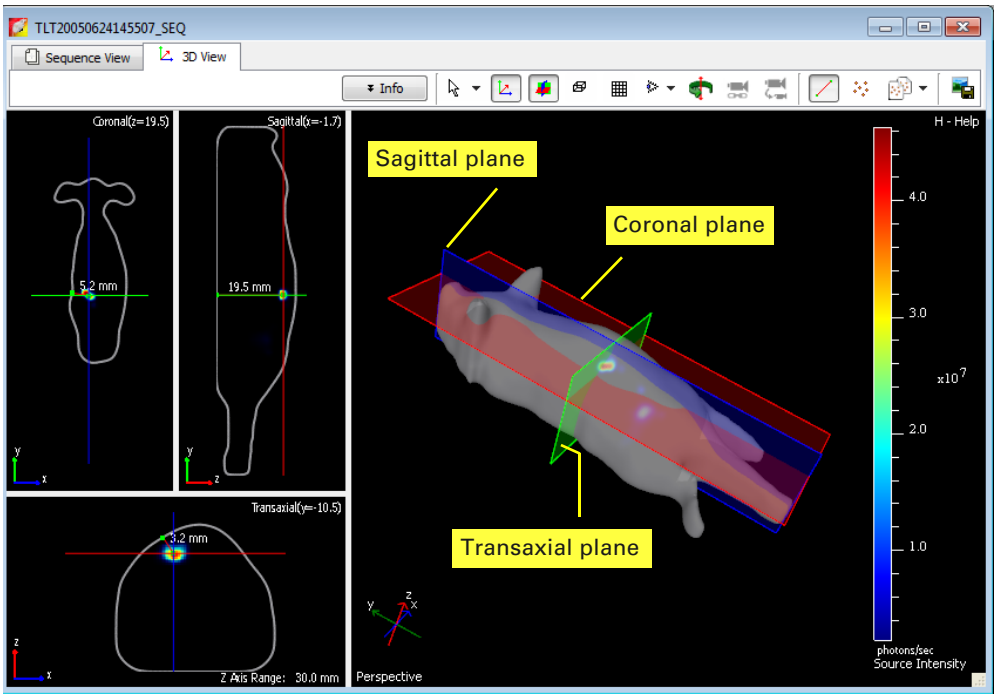


Displaying Slices Through a Reconstruction

1. Click a location on a source. Alternatively, click the toolbar button, draw a box around a source, then click **Center of mass** in the 3D Source tools.
2. Click the toolbar button.

   The Coronal, Sagittal, and Transaxial windowpanes show a slice through the surface taken by the associated plane (Figure 8.7).

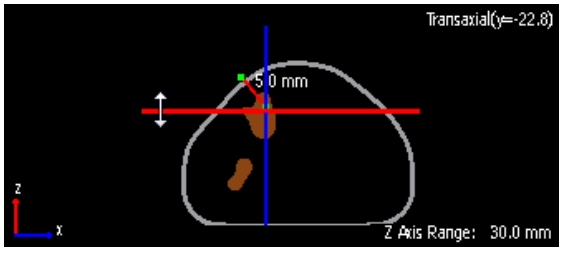
Figure 8.7 Planes Cutting a Reconstruction



3. To move a plane, put the mouse cursor over a line in the coronal, sagittal, or transaxial windowpane. When the cursor becomes a ↕ or ↔ arrow, drag the line.

   The view is updated in the windowpanes as you move the line.

Figure 8.8 Moving the Transaxial Plane



8.2 Synchronizing 3D Views

Synchronizing 3D views across multiple datasets provides a convenient way to compare results, for example in a longitudinal study.

Synchronized 3D ROIs can be also be applied across the datasets (see *Measuring Sources* on page 174 for more information).

NOTE: The 3D view synchronization feature replaces the Longitudinal Study window found in Living Image Software 4.4 and earlier versions.

1. Load multiple 3D reconstruction results and tile the windows (select **Windows → Tile** on the menu bar).
2. Choose from the 3D view synchronization options show in Table 8.3.

NOTE: 3D view synchronization options are only available if multiple 3D reconstructions are loaded. Synchronized views are only valid during a session and cannot be saved.

Table 8.3 3D View Synchronization Options

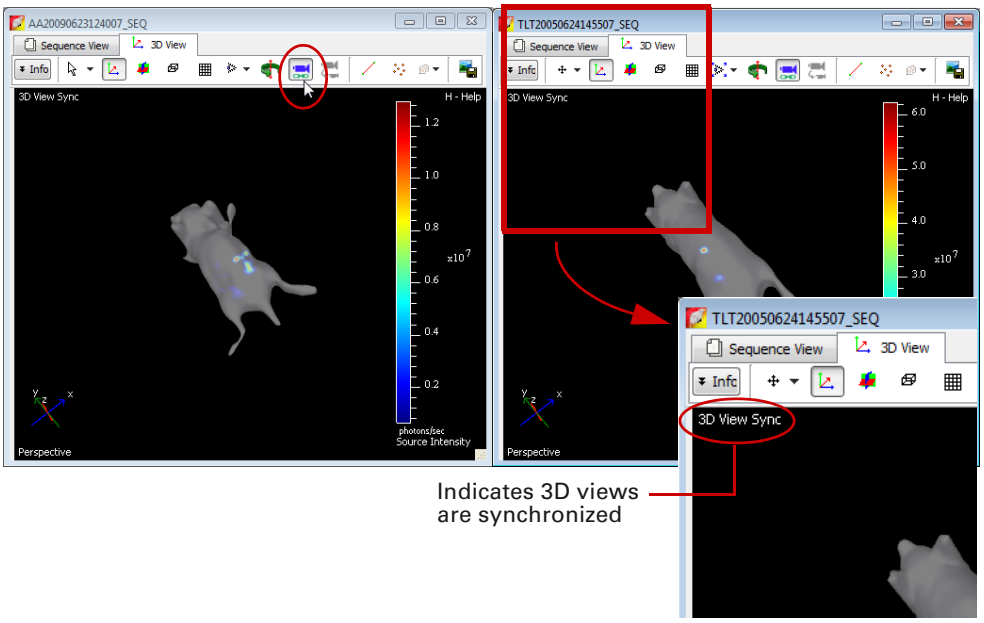
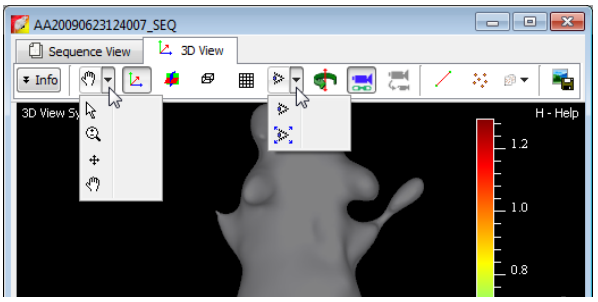
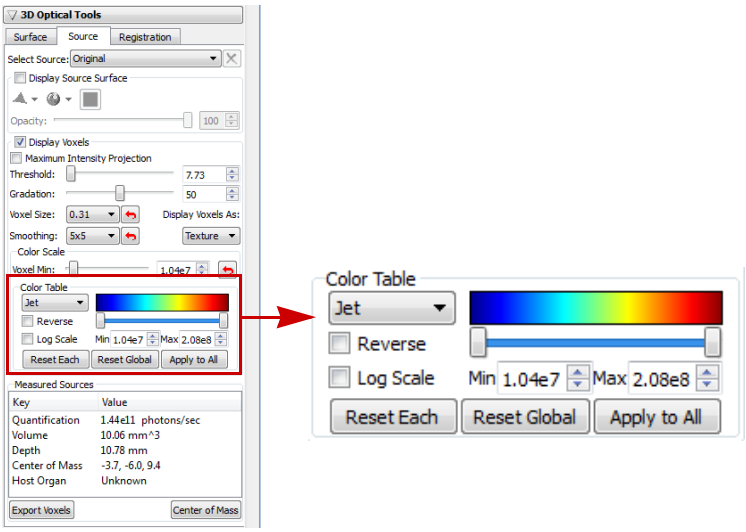
Item	Description
	When multiple 3D reconstructions are loaded, click this button in one of the 3D views to turn on synchronization. All other 3D views will reset to display the same perspective as the active data. Click the button again to turn off synchronization. 
	If 3D views are synchronized, these tools will be applied to all 3D views at the same time. 

Table 8.3 3D View Synchronization Options (continued)

Item	Description
	Applies the perspective of the active 3D view to all 3D views. **Note:** This button is only available if multiple DLIT or FLIT results are loaded and synchronization is turned off.
Color Table Options in 3D Optical Tools (Source tab)	 **Reset Each** – Displays each 3D view using the default color table and scale for the dataset. **Reset Global** – Applies the color table of the active data and an aggregate scale to all 3D views. See *Color Scale and Color Table* on page 148 for instructions on setting the min and max color table values using the 3D Optical Source tools. The software will alert you if the loaded results have different units, for example, a DLIT and FLIT reconstruction. **Apply to All** – Applies the color table and scale of the active data to all 3D views. **Note:** These options are only available if at least two 3D reconstruction results are loaded.

8.3 Viewing Luminescent and Fluorescent Sources on One Surface

When an experiment includes luminescent and fluorescent reporters, DLIT and FLIT reconstructions can be displayed on one surface if the luminescent and fluorescent imaging is done in the same imaging session, without moving the animal.

NOTE: If the DLIT and FLIT image sequences are acquired during the same session, the generated surfaces are nearly identical.

1. Load the DLIT and FLIT 3D reconstructions.
2. Choose one of the reconstructions, click the  button in the 3D View toolbar, and select **Copy source voxels**.
3. In the other reconstruction, click the  button and choose **Paste source voxels**.

NOTE: Pasted voxels can be measured. See page 149 for more details on measuring sources.

8.4 Displaying Organs With a Reconstruction

Mouse anatomy reference atlases are available for registration with 3D reconstructions. A mouse anatomy reference atlas is used when volumetric data from another imaging modality is not available. A reference atlas provides guidance for the luminescent or fluorescent source anatomical location.

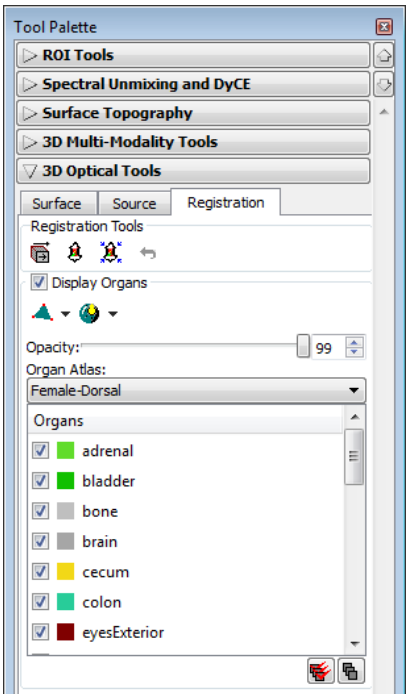
Use the 3D Optical Registration tools to:

- Display organs in the surface (see below)
- Manually adjust the location or scale of organs in the surface ((page 158))
- Check the organ fit (page 160)
- Import an organ atlas (page 161)

To display organs:

1. Load reconstruction results and confirm that the surface is in the perspective view (click the toolbar button in the 3D View window or press the **R** key).
2. Click the Source tab in the 3D registration tools and choose the "Display Organs" option (Figure 8.9).

Figure 8.9 3D Optical Registration Tools



3. Make a selection from the Organ Atlas drop-down list (for example, "Female–Dorsal").
4. The organs in the selected atlas appear on the surface (Figure 8.10).To fit the organs to the surface, click a registration tool:

 – Rigid registration: Performs linear transformation, but keeps the shape of the atlas surface.

 – Full registration: Performs linear transformation and volume deformation.

NOTE: For an optimum fit when there is a large difference between the orientation or size of the atlas organs and surface, first use the transformation tool to manually register the surface and atlas organs, then click a registration tool to automatically fit the organs. (See *Manually Adjusting Scale or Location of Organs* on page 158 for more details.)

5. If necessary, adjust the opacity of the organs using the slider or enter a number in the box. The organs are easier to view if you uncheck "Skin" in the Organs list. (Figure 8.10)
6. To clear all organs from the surface, click the **Deselect All** button. To hide a particular organ, remove the check mark next to the organ name.
7. To display a specific organ(s), choose the organ name. To display all organs on the surface, click the **Select All** button.

NOTE: After fitting organs to the surface using the or tool, if necessary, you can click Reset button to restore the default fit.

Figure 8.10 Surface with Fitted Organs ("Skin" not displayed) and 3D Optical Registration Tools

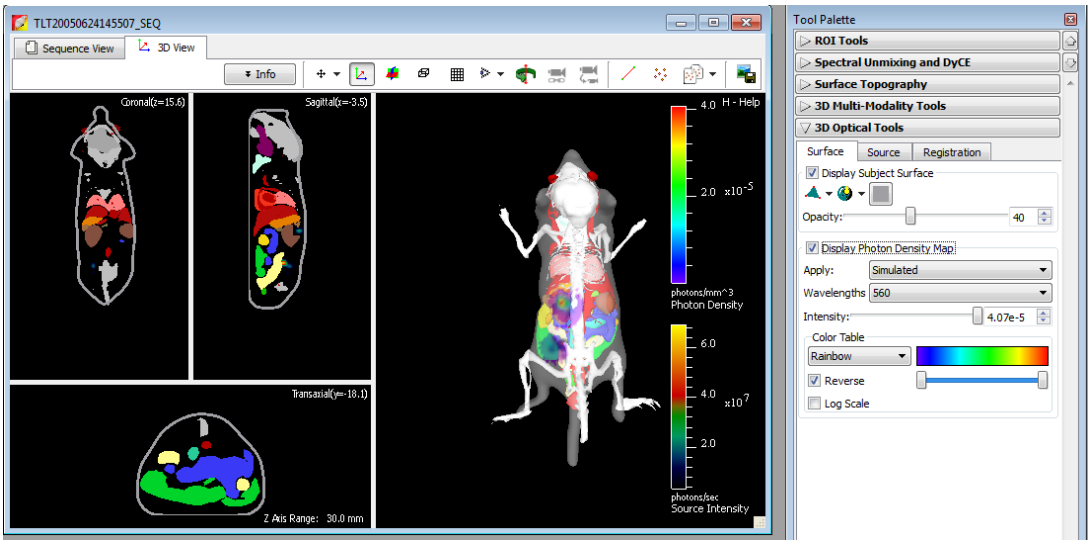
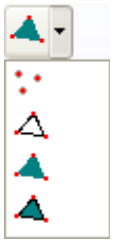


Table 8.4 3D Optical Registration Tools

Item	Description
	Use this tool to manually adjust the scale of location of organs. See page 158 for more details.
	Fits the organs to the surface using a linear transformation that keeps the shape of the atlas surface.
	Fits the organs to the surface using linear transformation and volume deformation.
	After fitting organs to the surface using the or tool, if necessary, click this button to restore the default fit.
Display Organs	Choose this option to display the organs on the surface. Organs that are check marked will be displayed. See for more details.

Table 8.4 3D Optical Registration Tools (continued)

Item	Description
	Drawing styles for the organs (see "*Display Organs*" above).
	Shading styles for the organs (see "*Display Organs*" above).
Opacity	Adjusts the opacity of the organ display.
Organ Atlas	Choose a type of organ atlas.
	Click to select all organs in the database and display them on the surface.
	Click to clear the selected organs and remove all organ diagrams from the surface.

Manually Adjusting Scale or Location of Organs

1. Load reconstruction results and display organs (see *Displaying Organs With a Reconstruction* on page 156 for instructions).

   The organs in the selected atlas appear on the surface. Only "Skin" is selected in Figure 8.11.
2. Click the **Transform tool** button  .

   The transform tool appears. Figure 8.12 explains the tool functions.

NOTE: It may be helpful to view the 3D image from different perspectives to check the organ position and size. To turn and rotate the 3D image:

- Click outside the transform tool box, then press and hold the left mouse key.
- Drag the mouse when the hand appears.

Figure 8.11 Displaying the Transform Tool

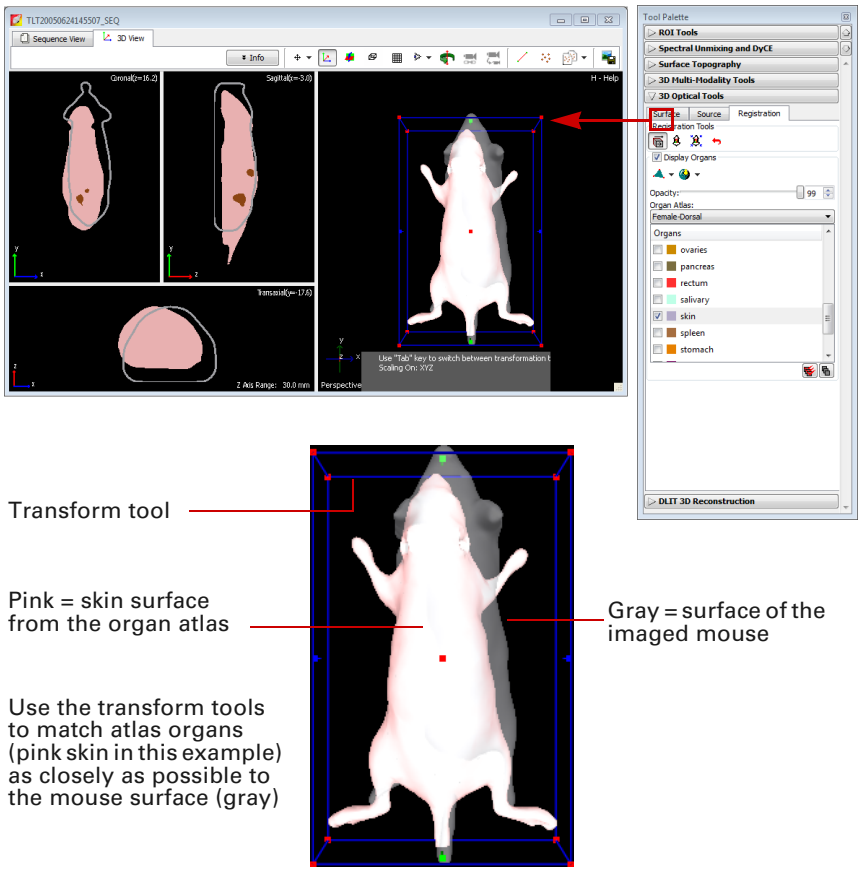
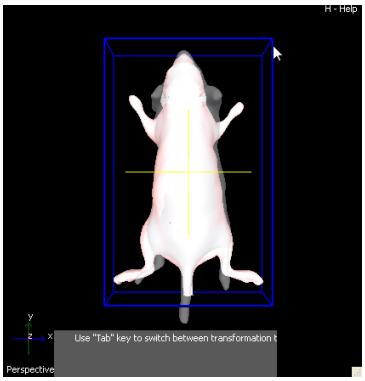
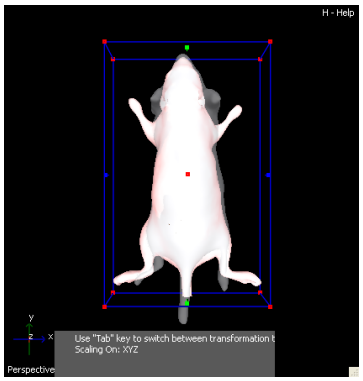


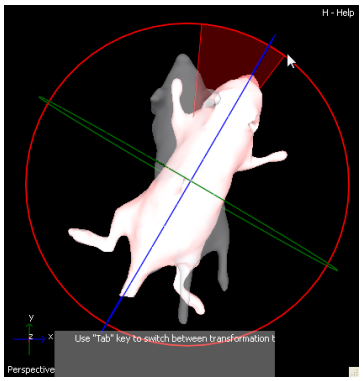
Figure 8.12 Transform Tools



Click and drag the organ(s) when the yellow "+" appears.



Click and drag a handle to scale (increase or decrease) the size of the organ(s).
Red – Scales on the z-axis.
Blue – Scales on the x-axis.
Green – Scales on the y-axis.



To rotate the organ(s) on the x,y, or z-axis, click the blue, green, or red circle and drag the mouse arrow in the direction of interest.

3. Press the Tab key to switch between the transform tools (Figure 8.12).

   The position of the organ(s) is updated in the slice windowpanes (coronal, sagittal, and transaxial views) after each adjustment.

4. Turn off the transform tool when you are done adjusting the position of the organ(s) (click the button).

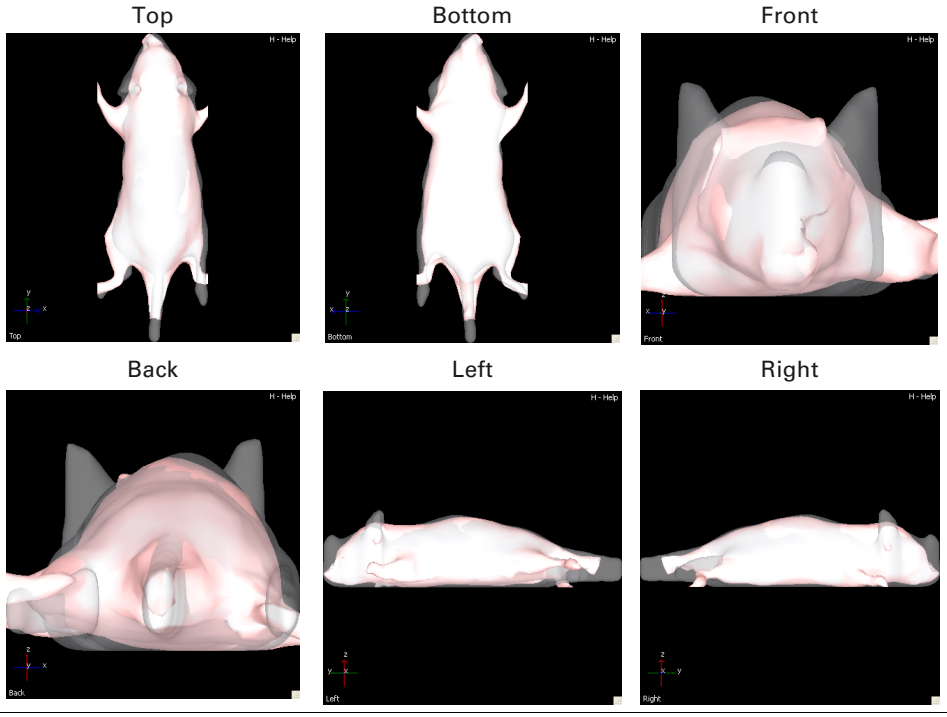
Checking the Organ Fit

1. Check the fit in the coronal, sagittal, and transaxial windowpanes.
2. Click the **Change view** toolbar button .
   The Top view is displayed (Figure 8.13).

Figure 8.13 Skin (pink) Fitted to Surface (gray)

3. Press the **V** key or the button to display alternative views of the surface.

Figure 8.14 Alternative Surface Views
In this example, "skin" is selected from the organ atlas (pink surface). The mouse surface is gray.



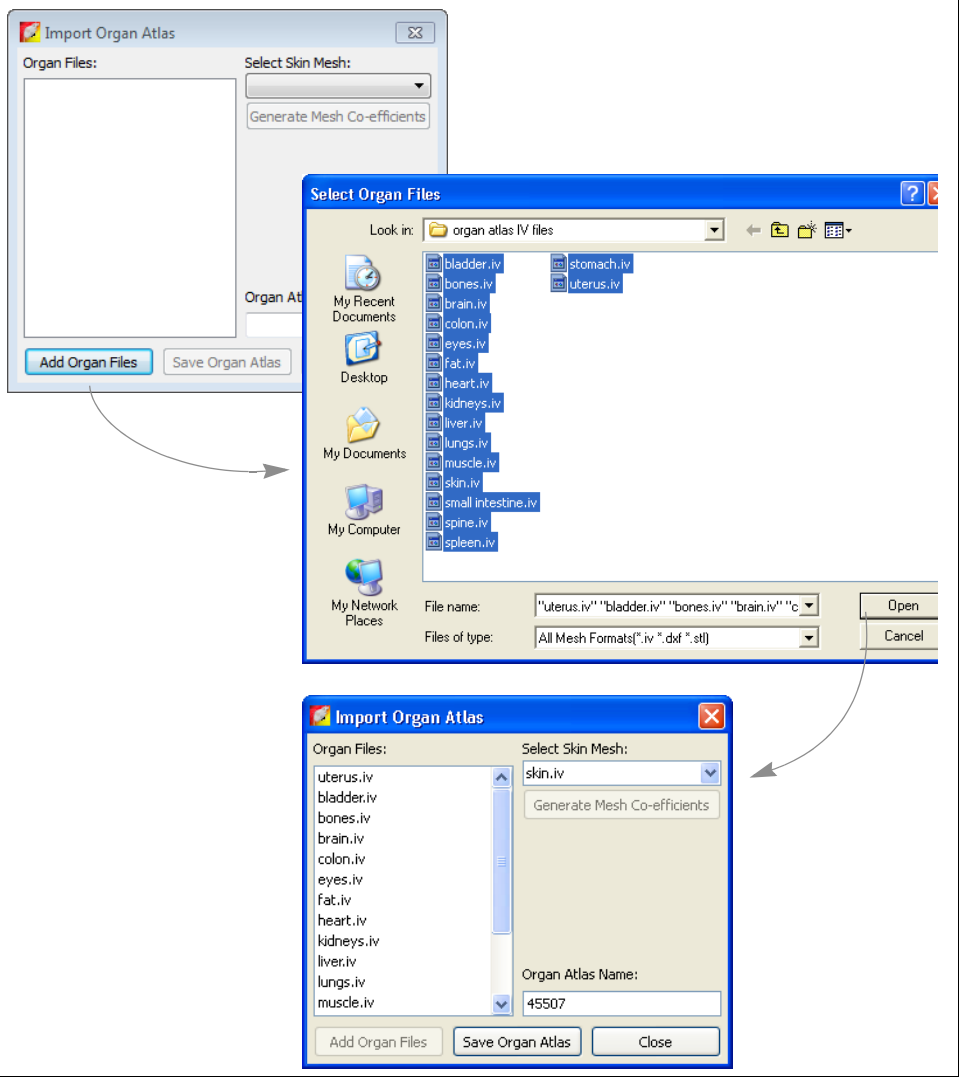
Importing an Organ Atlas

An organ atlas (.iv, .dxf, or .stl, one organ per file) consisting of segmented organ surfaces derived from an MRI or CT scan can be imported into the Living Image software for registration with the animal surfaces derived from IVIS data. Organ files must be segmented from MRI or CT 3D volumetric data in third party medical imaging analysis software.

NOTE: The imported atlas must include a surface (skin) file which delineates the animal surface. The file name must include the word "skin", for example *rat skin.iv*.

1. Load a DLIT or FLIT image sequence that is associated with the mouse comprising the organ files in *.iv, *.dxf or *.stl format.
2. Select **File** → **Import** → **Organ Atlas** on the menu bar.
3. In the dialog box that appears, click **Add Organ Files** (Figure 8.15).

Figure 8.15 Importing an Organ Atlas



4. In the next dialog box that appears, select all of the files (.iv, .dxf, .stl) that you want to include in the atlas (one file per organ) and click **Open**.

5. In the Select Skin Mesh drop-down list, select the skin organ file, which must include ‘skin’ in the file name.
6. Click **Generate Mesh Coefficients**.
7. Enter a name for the atlas and click **Save Organ Atlas**.

   The organ atlas (.atlas) is created and is added to the Organ Atlas drop-down list (in the 3D Optical Tools, Registration tab).

8.5 3D Animation

Living Image software can create an animation from a sequence of 3D views (*key frames*). For example, an animation can depict a rotating 3D scene (Figure 8.16). The animation (series of key frames) can be recorded to a movie file (.mov, .mp4, or .avi).

Use the animation tools to:

- View a preset animation (generated from a factory-loaded animation setup, page 164).
- Create a custom animation (created from your custom animation setup, page 165).
- Save an animation setup (page 166).
- Record an animation to a movie file (page 166).
- Edit an animation setup (page 166).

Figure 8.16 Individual 3D Views (key frames) in the Preset Animation “Spin CW on Y-Axis”

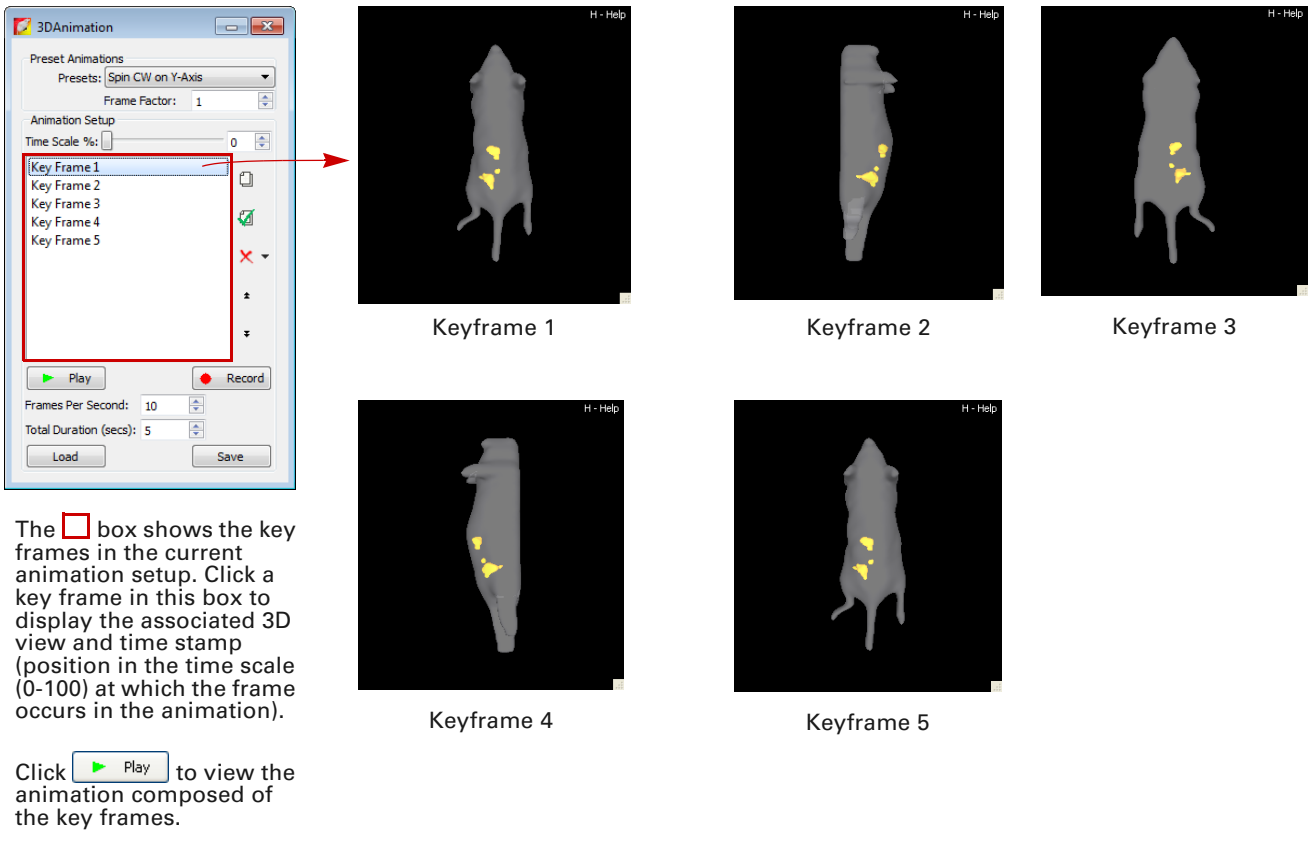


Table 8.5 3D Animation Tools

Item	Description
Time Scale%	The time stamp of a key frame in the animation on a time scale of 0-100. For example, if the animation is 10 sec long and includes five key frames: Key frame 1: Time stamp= 0; first frame of the animation. Key frame 2: Time stamp = 25%; frame occurs 2.5 seconds after the start of animation. Key frame 3: Time stamp = 50%; frame occurs 5.0 seconds after the start of animation. Key frame 4: Time stamp = 75%; frame occurs 7.5 seconds after the start of animation. Key frame 5: Time stamp = 100%; last frame of the animation.
Presets	A drop-down list of predefined animation setups.
Key frame	A 3D view. The software interpolates the key frames to create intermediate frames in real time, then generates an animated sequence from all of the frames. Each successive key frame in a sequence should differ slightly from the preceding one, so that motion is smoothly depicted when the frames are shown at a proper frame rate (frames/second). The Living Image software provides preset key frames or you can specify the 3D views for the key frames.
Preset Key Frame Factor	Determines how many key frames are used to generate one revolution in a spinning animation (No. of frames = (4 x Key Frame Factor) + 1). Increasing the key frame factor reduces the time period between key frames and creates the appearance of finer movement. Decreasing the key frame factor increases the time period between key frames and creates the appearance of coarser movement.
FPS	Frames displayed per second in the animation sequence.
	Creates a new key frame from the current 3D view.
	Updates the selected key frame to the current 3D view.
	Deletes a selected or all key frames from the key frame box.
	Moves a selected key frame up in the key frame box.
	Moves the selected key frame down in the key frame box.
Total Duration	The total time of the animation sequence.
Play	Click to view the animation sequence defined by the current key frames and animation parameters.
Record	Displays a dialog box that enables you to save the current animation to a movie (.mov, .mp4, or .avi, .mpg).
Animation Setup	
Load	Displays a dialog box that enables you to open an animation setup (.xml).
Save	Displays a dialog box that enables you to save the current key frames and animation parameters to an animation setup (.xkf).

Viewing a Preset Animation

Preset animations are factory-loaded animation setups. They include predefined key frames which are used to generate the animation.

To view a preset animation:

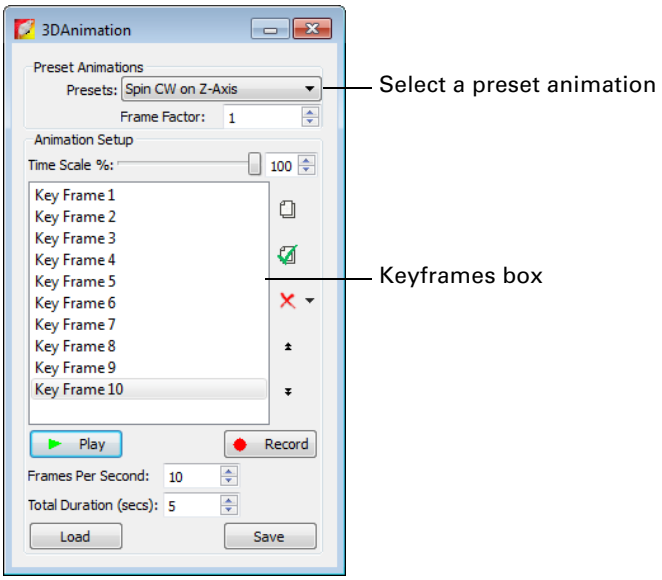
1. Open an image sequence and load 3D reconstruction results.
2. Select properties to display in the 3D View window (for example, organs, sources, surface, or photon density maps).
3. Select **Tools** → **3D Animation** on the menu bar.
4. In the 3D Animation tools that appear:
   a. Clear the key frame box if necessary (click the ✕ button and select **Delete All**).
   b. Make a selection from the Presets drop-down list. See Table 8.5 on page 163 for a description of the preset animations.

      After a preset animation is selected, a list of the key frames appears.

NOTE: You can view multiple animations sequentially. For example, if you select Spin CW on X-Axis and Spin CW on Y-axis from the Presets drop-down list, the animation shows the 3D reconstruction spinning clockwise on the x-axis, then spinning clockwise on the y-axis.

5. Click **Play** to view the animation.

Figure 8.17 3D Animation Tools\
See Table 8.5, page 163 for details on the animation tools.

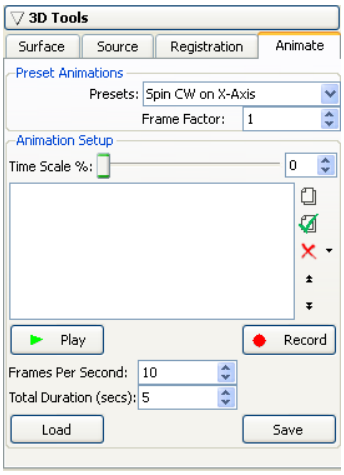


Creating a Custom Animation

To create an animation, specify a custom animation setup or edit an existing setup.

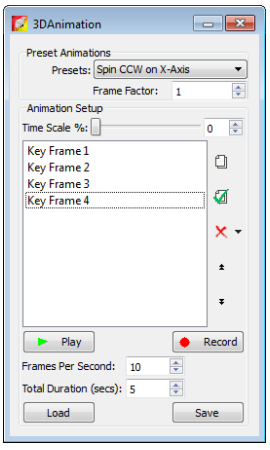
1. Open an image sequence and load 3D reconstruction results.
2. Select properties to display in the 3D View window (for example, organs, sources, surface, or photon density maps).
3. Select **View** → **3D Animation** on the menu bar.
   The 3D Animation tools appear (Figure 8.18).
4. Clear the key frame box if necessary (click the button and select **Delete All**).

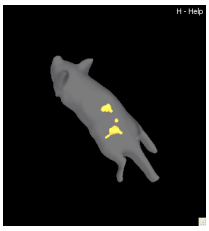
Figure 8.18 Key Frames Cleared

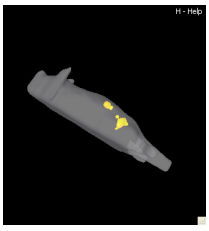


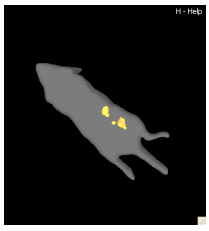
5. To capture the first key frame, click the button.
   The first key frame is added to the key frame box.
6. Adjust the position of the reconstruction in the 3D View using an image tool (for example, , or). See Table 7.2 on page 129 for more details on the image tools.
7. Click the button.
   The second key frame is added to the key frame box.

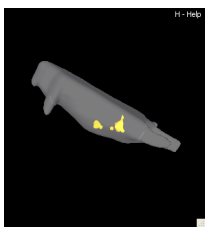
Figure 8.19 Example Key Frames for a Custom Animation




Key Frame 1


Key Frame 2


Key Frame 3


Key Frame 4

8. Repeat step 6 to step 7 until all of the key frames are captured. See *Edit an Animation Setup* below for details on how to edit the key frame sequence.

   Click a key frame to display the associated 3D view and the time stamp (position in the time scale (0-100) at which the frame occurs in the animated sequence).

9. Confirm the defaults for FPS (frames per second) and Total Duration (length of animation) or enter new values.

   FPS x Total Duration = No. of frames generated to create the animation. The number of generated frames should be ≥ to the number of key frames. Otherwise, the frames may not be properly animated.

10. To view the animation, click **Play**. To stop the animation, click **Stop**.

    An animation setup (series of key frames) can be saved (.xkf) or recorded to a movie (.mov, mp4, .avi, mpg).

Managing Animation Setups

Save an Animation Setup

1. Click **Save**.
2. Select a directory and enter a file name (.xkf) in the dialog box that appears.

Record an Animation to a Movie

1. Click **Record**.
2. Choose a directory, enter a file name (.mov, mp4, .avi), and click **Save** in the dialog box that appears.

Edit an Animation Setup

1. Open an image sequence and load a reconstruction.
2. Open an animation setup:

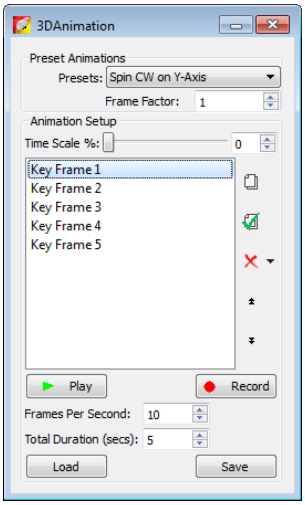
   To select a predefined setup, make a selection from the Preset drop-down list.

   To select a saved user-defined setup:

   a. Click **Load**.

   b. Select an animation setup (.xkf) in the dialog box that appears.

Figure 8.20 List of Key Frames in the Selected Animation

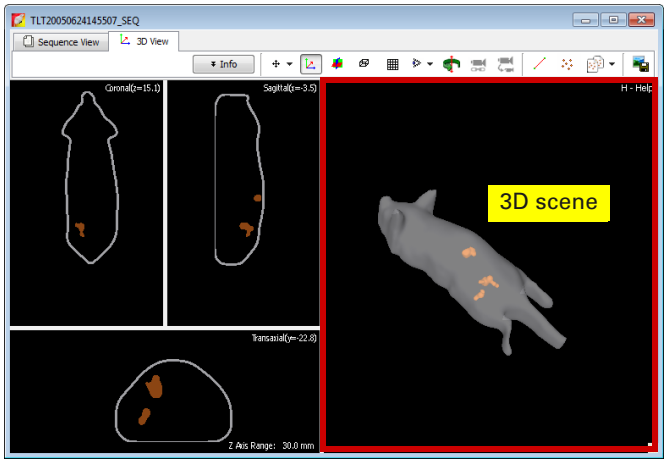


3. To add a key frame:
   a. Adjust the position of the reconstruction in the 3D view using an image tool (for example, , or). See Table 7.2 on page 129 for more details on the image tools.
   b. Click the button.
   c. To reorder a key frame in the sequence, select the key frame and click the or arrow.
4. To update a key frame:
   a. Select the key frame and adjust the 3D view.
   b. Click the button.
5. To delete a key frame:
   a. Select the key frame that you want to remove.
   b. Click the button and select **Delete Current**.

8.6 Exporting a 3D Scene as DICOM

The items in the perspective 3D View are called a *3D scene*. For example, the 3D scene in Figure 8.21 includes a surface and voxels. The 3D scene can be exported to DICOM format and viewed in the Living Image DICOM Viewer or third party software.

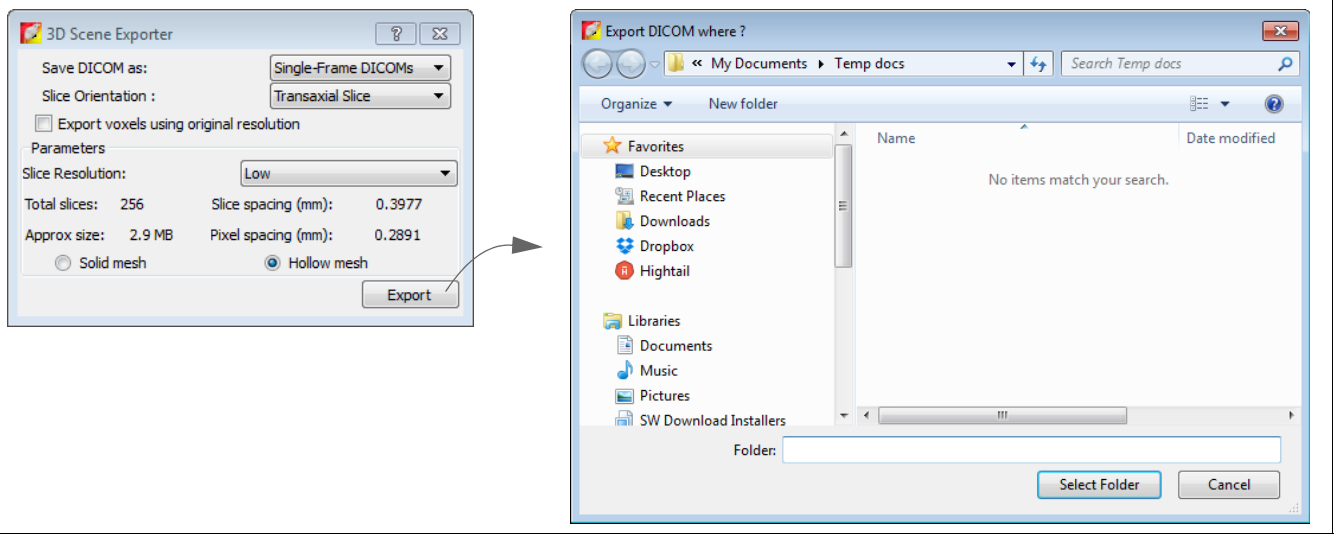
Figure 8.21 3DScene



To export the 3D scene:

1. Load the results that you want to export.
2. Select **File → Export → 3D Scene as DICOM** on the menu bar.
3. In the dialog box that appears, set the export options, and click **Export** (Figure 8.22).
   For more details on the 3D Scene Exporter, see Table 8.6 on page 168.

Figure 8.22 3D Scene Exporter Dialog Box



4. In the next dialog box that appears, choose a folder for the DICOM files and click **Select Folder**.

   During the export operation, the 3D View window displays the each slice in the export. For example, if Transaxial Slice is selected for export, then the transaxial windowpane cycles through a display of each exported slice.

Table 8.6 3D Scene Exporter Dialog Box

Item	Description
Save DICOM as:	Single-Frame DICOMs - Exports multiple files that contain a single frame each. Multi-Frame DICOM - Exports a single file that contains multiple frames. **Note:** Choose the Single-Frame or Multi-Frame DICOM option, depending on the third party software you will use to import and view the 3D scene. Some applications cannot reconstruct multi-frame DICOM files.
Slice Orientation	Choose transaxial, coronal, or sagittal slices for the export.
Export voxels using original resolution	Choose this option to export source voxels without any smoothing or binning. The original resolution of the source voxels is the resolution obtained after DLIT or FLIT reconstruction (approximately 1mm resolution).
Slice Resolution	Sets the number of slices required to accommodate the slice orientation with good slice sampling/spacing.
Total Slices Slice spacing Pixel spacing	Parameters that determine the number and resolution of the slices to export.
Solid mesh	If this option is chosen, voxels generated inside the hollow mesh are assigned an intensity so that they are displayed as “tissue” when loaded into visualization software. If no intensity is associated with the voxels, they are considered noise or air and appear hollow.
Hollow mesh	The intensity of pixels inside the surface is set to zero so that the exported surface appears as a hollow empty structure.

Viewing DICOM Data

3D scenes exported to DICOM can be viewed in the Living Image 3D Browser or a 3D View window.

1. Click the 3D toolbar button. Alternatively, select **File** → **Browse 3D Volumetric Data** on the menu bar.
2. If this is the first time browsing for volumetric data in the session, select a data folder in the dialog box that appears, and click **Select Folder**.

   The Living Image 3D Volumetric Browser appears (Figure 8.24).

NOTE: If the 3D Volumetric Browser was previously opened during the session, clicking the 3D button opens the browser. Click the Browse.. button in the browser, and in the dialog box that appears, select a DICOM data folder.

Figure 8.23 Opening the 3D Volumetric Browser

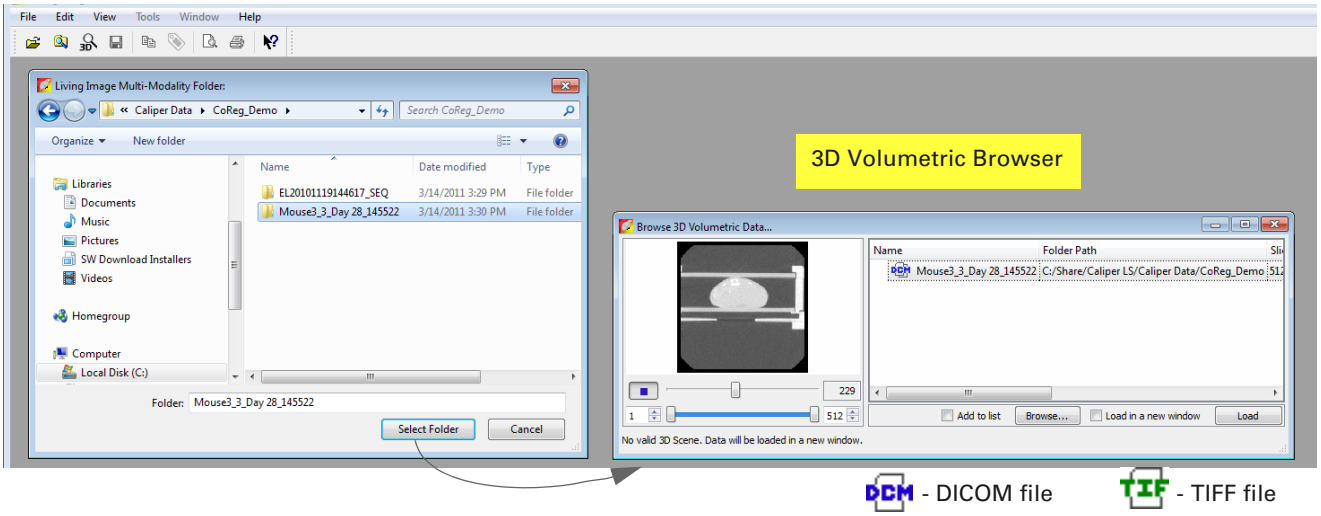


Figure 8.24 Living Image 3D Browser

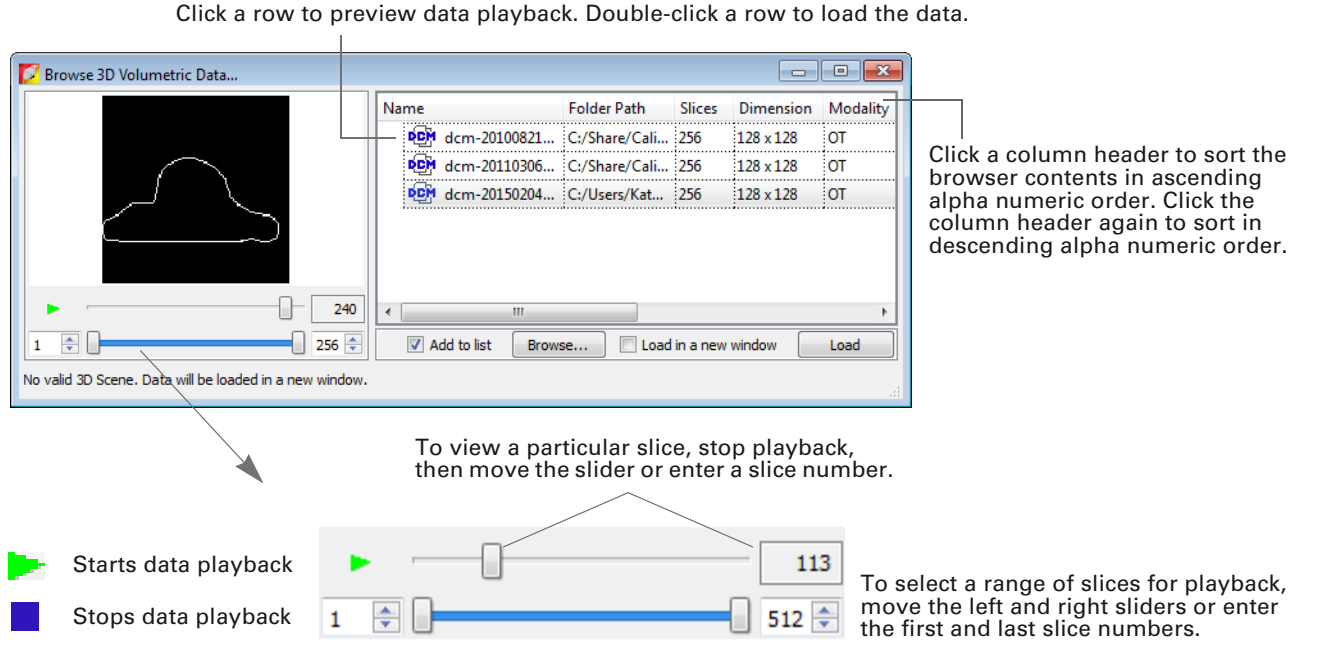


Table 8.7 Living Image 3D Browser DICOM Viewing Controls

Item	Description
Add to list	If this option is chosen, the selected data will be added to the browser. If this option is not chosen, the selected data replaces the contents of the browser, except for the loaded data.
Browse	Opens the dialog box that enables you to select data to display in the browser.
Load in a new window	If this option is selected, DICOM data will be opened in a new 3D View window when you click **Load**. If this option is not selected, DICOM data will be loaded in the active 3D View window.
Load	Opens the DICOM data in a 3D View window.

9 Measuring Signal in 3D Sources

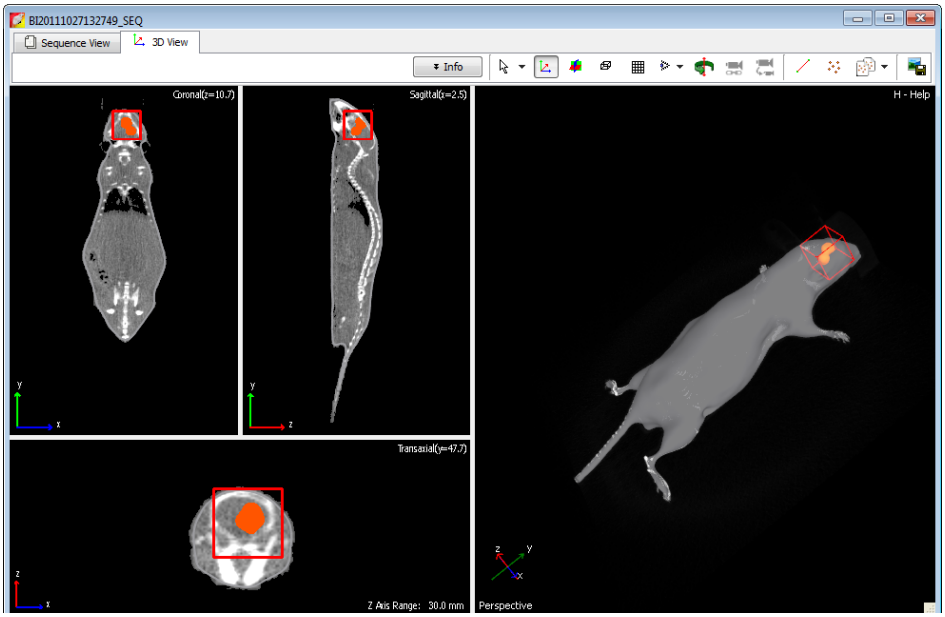
9.1 About 3D ROIs

A 3D *region of interest* (ROI) measures the signal intensity within a user-specified bounding box applied to a:

- 3D reconstruction of a luminescent source.
- 3D reconstruction of a fluorescent source.
- CT volume.

NOTE: CT imaging is not available on the IVIS Spectrum. Living Image software can analyze CT image data acquired on the IVIS Spectrum CT. 3D Multi-Modality tools (see page 181) are required to load IVIS Spectrum CT volumetric data or import volumetric data (PET, MRI, or CT data) from instruments other than the IVIS Spectrum CT).

Figure 9.1 Example 3D ROI



Living Image software records information about the ROIs you create during a session and computes statistical data for the ROI measurements. The ROI Measurements table displays the data and provides a convenient way to review or export ROI information (Figure 9.2).

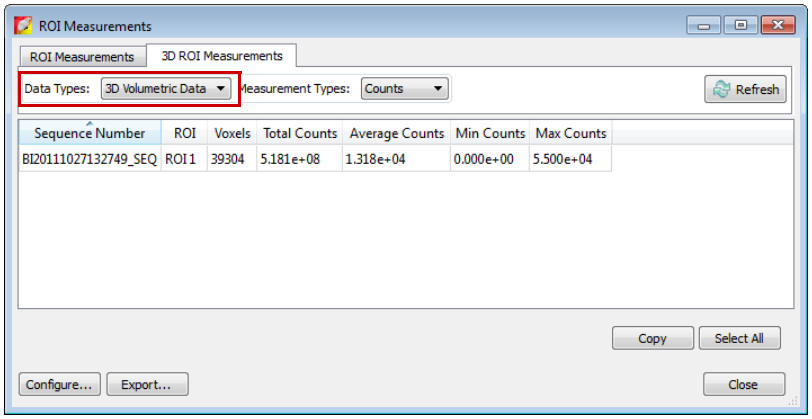
If a dataset includes ROIs on both 2D optical and 3D volumetric data, the measurements for the two types of ROIs are displayed in separate tabs of the ROI table (Figure 9.2):

- ROI Measurements tab shows ROI measurements for 2D optical data.
- 3D ROI Measurements tab shows measurements of structural data (for example, CT) or measurements of the 3D optical reconstruction.

Figure 9.2 3D ROI Measurements Table

Select "3D Volumetric Data" to view measurements of structural data, such as a CT volume

Select "Source Voxels" to view measurements of the 3D optical reconstruction.



9.2 Overview of 3D ROI Tools

Table 9.1 provides a description of the 3D ROI tools. ROI measurements and measurement statistics are available in the ROI Measurements table. The table provides a convenient way to review or export ROI information. See Table 9.2 on page 177 for more details on 3D ROI measurements.

Figure 9.3 3D ROI Tools

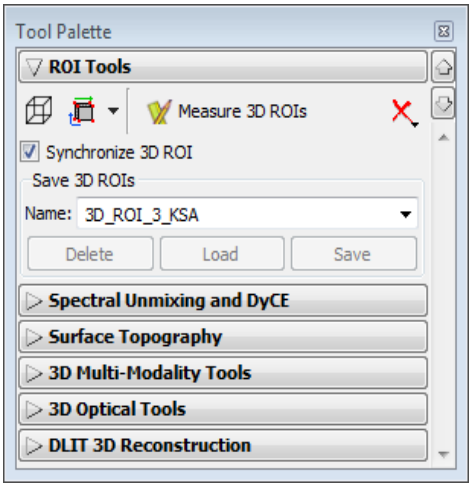
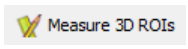
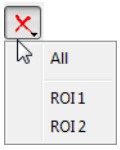
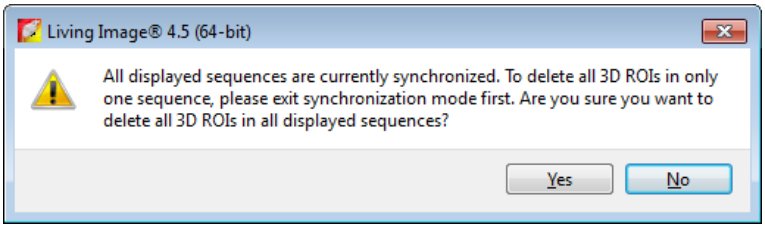
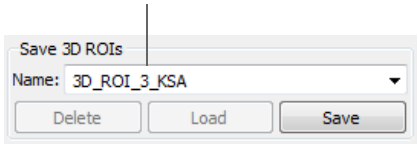


Table 9.1 3D ROI Tools

Item	Description
	Click to apply a 3D ROI. If multiple datasets are loaded and the "Synchronize 3D ROI" option is selected, a synchronized 3D ROI will be applied to all of the loaded datasets. Synchronized 3D ROIs can be moved and adjusted as a group using the 3D transform tool.
	Click to select a 3D ROI from the drop-down list and to turn on the 3D ROI transform tool. See page 175 for instructions on using the tool to move, scale, or rotate a 3D ROI.
	Click to display the 3D ROI measurements or compute intensity signal in a 3D ROI. See Table 9.2 on page 177 for more information on 3D ROI measurements.
	Click  for a drop-down list of delete options for 3D ROIs. If "All" is selected when multiple datasets with synchronized 3D ROIs are loaded, the software prompts you to confirm deleting the 3D ROIs from all of the datasets.  **Note:** These commands delete 3D ROIs from a dataset(s). They do not delete 3D ROIs that are saved to the system (3D ROIs which appear in the Name drop-down list, see below).
Synchronize 3D ROI	If multiple datasets are loaded and the "Synchronize 3D ROI" option is selected, clicking the  button applies a synchronized 3D ROI to all datasets. The transform tool and delete commands are applied simultaneously to synchronized 3D ROIs.
Save 3D ROIs to the system	Name of a 3D ROI or set of 3D ROIs  These ROI tools are only available when a 3D reconstruction or volume is loaded. 3D ROIs (parameters only such as coordinates and dimensions) can be saved to the system (per user) and used to apply the ROIs to other datasets. These 3D ROIs appear in the Name drop-down list. See page 180 for instructions. **Note:** 3D ROIs can also be saved with the dataset (the software prompts you to save before closing a dataset). 3D ROIs saved with a dataset do not appear in the Name drop-down list.

9.3 Measuring Sources

This section explains how to apply 3D ROIs on a 3D reconstruction or CT volume. If multiple datasets are loaded, identical 3D ROIs can be applied to all of the datasets at the same time. These related 3D ROIs are "synchronized" and can be moved or adjusted as a group. Synchronized ROIs provide a convenient way to compare results, for example, results obtained at different time points or from different reporters.

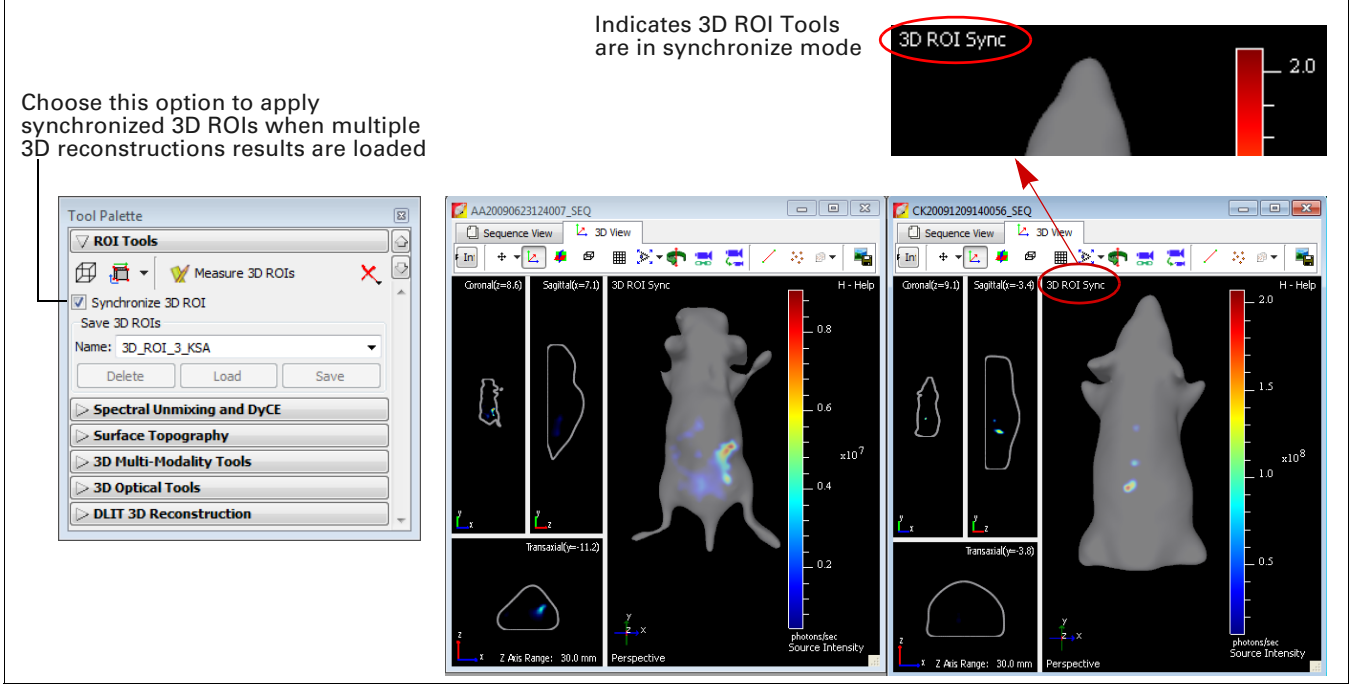
To apply 3D ROIs:

1. Load a 3D reconstruction or CT volume.

   If you want to apply synchronized 3D ROIs to multiple datasets:

   - Load multiple volumes or 3D reconstructions. Tile the windows (select **Window → Tile** on the menu bar).
   - Choose the "Synchronize 3D ROI" option in the Tool Palette (Figure 9.4).

Figure 9.4 3D ROIs – Synchronize Mode



2. Click the 3D ROI button in the ROI tools to apply the ROI (Figure 9.5).

NOTE: 3D ROIs applied before selecting the "Synchronize 3D ROI" option or after it is cleared are independent and unrelated to other 3D ROIs.

A red bounding box appears in the 3D View(s). If working with a CT volume and you do not see the red bounding box, do either of the following:

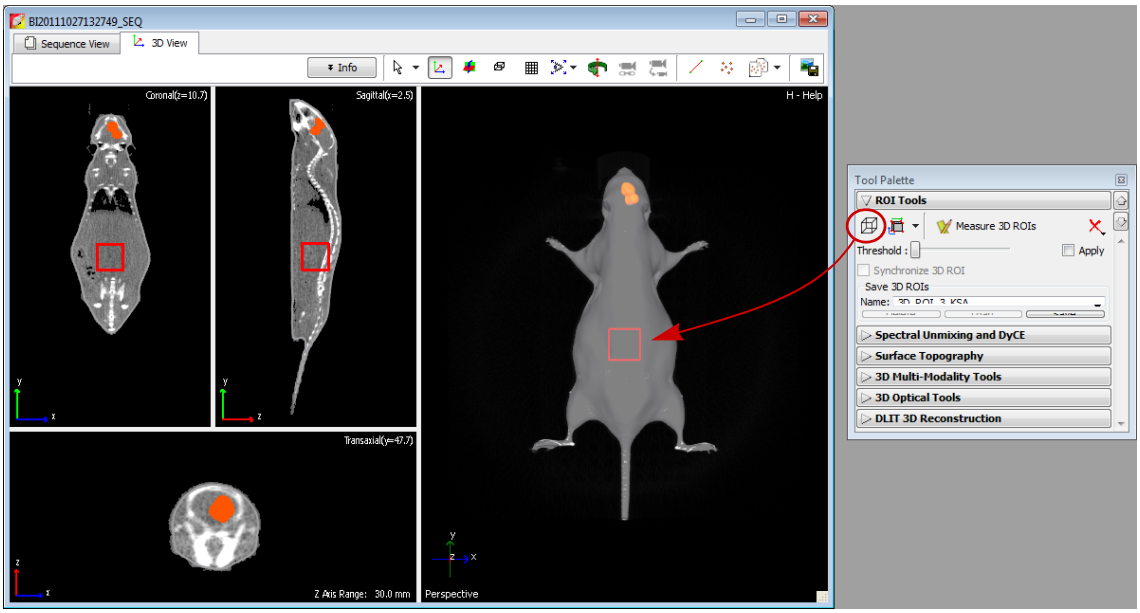
- Select the "Maximum Intensity Projection (MIP)" option in the 3D Multi-Modality tools.

  OR

- Reduce the volume opacity by adjusting the position of the Air/Noise Boundary in the 3D Multi-Modality tools.

NOTE: See Table 9.1 on page 173 for details on deleting 3D ROIs.

Figure 9.5 Applying a 3D ROI



3. It may be helpful to view the surface from different perspectives to check the 3D ROI position and size. To turn and rotate the surface, press and hold the left mouse key, then drag the mouse when the hand appears.

   If working with synchronized 3D ROIs, it may be helpful to synchronize the 3D views before using the tool. See *Synchronizing 3D Views* on page 154 for more details.

4. Adjust 3D ROI position using the transform tools. Adjustments will be applied simultaneously to all synchronized 3D ROIs.

   a. Click the 3D ROI Transform button and select the ROI from the drop-down list.

      The first 3D ROI created during a session is named "ROI 1" by default. A tooltip shows the ROI name when you put the mouse pointer over an ROI.

   b. Click a 3D ROI to begin using the transform tools.

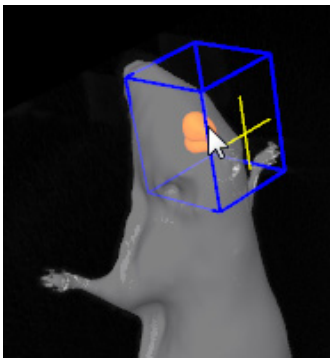
      Figure 9.6 explains the tool functions. The ROI position is updated in the slice windowpanes (coronal, sagittal, and transaxial views) after each adjustment.

      NOTE: If the 3D ROI disappears from view, right-click in the 3D view to redisplay the 3D ROI at the pointer location.

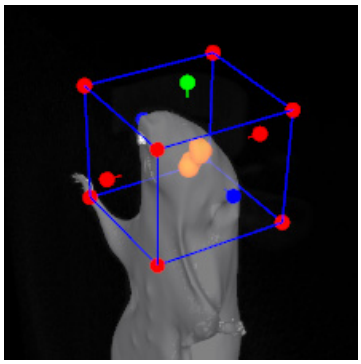
   c. Press the Tab key to switch between the transformations tools.

   d. Turn off the transform tool when you finish positioning the ROI (click the 3D ROI Transform button).

Figure 9.6 3D ROI Transform Tools



Click and drag the 3D ROI when the yellow "+" appears.

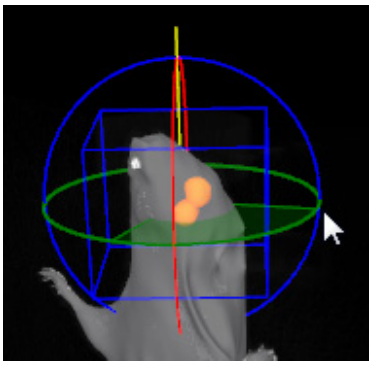


Click and drag a handle to scale (increase or decrease) the ROI size.

Red – Scales on the z-axis.

Blue – Scales on the x-axis.

Green – Scales on the y-axis.



To rotate the 3D ROI on the x,y, or z-axis, click the blue, green, or red circle and drag the mouse arrow in the direction of interest.

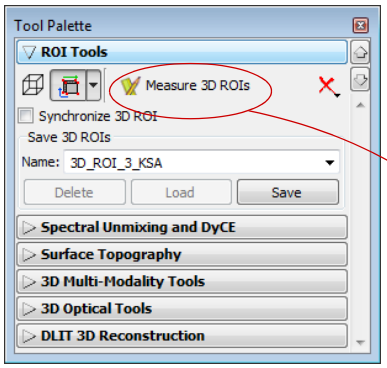
NOTE: The 3D ROI location (x, y, or z-coordinates) and dimensions (width, height, or depth) can be viewed and modified in the 3D ROI Properties dialog box. See page 178 for details.

5. Click the Measure 3D ROIs button in the Tool Palette to view the intensity measurements (Figure 9.7). See Table 9.2 for information about the measurements.
6. Click **Yes** in the prompt when closing the data to save the 3D ROIs with the data. Alternatively, select **File → Save** on the menu bar.

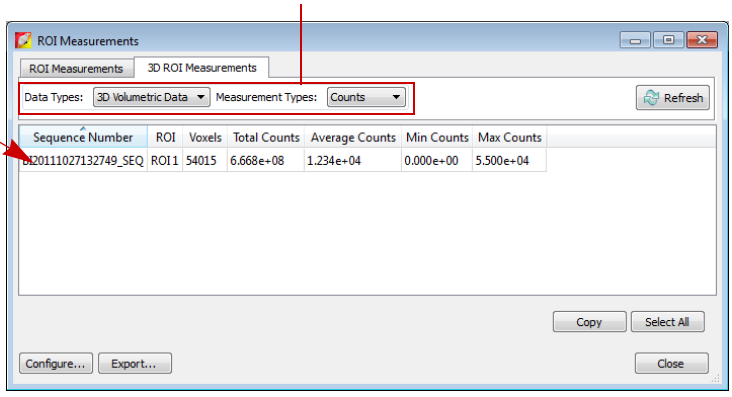
   The 3D ROIs will be displayed the next time the dataset is loaded.

NOTE: 3D ROIs from a dataset can be saved to the system (per user) and applied to other datasets. See *Saving 3D ROIs to the System* on page 180 for details.

Figure 9.7 3D ROI Measurements Table



Select the type of data and measurement (see Table 9.2)



See page 120 and page 122 for information about creating custom table configurations and exporting or printing the table.

Table 9.2 3D ROI Measurements Table

Item	Description
Data Types	3D Volumetric Data – Select this data type to measure the grayscale values of 3D volumetric data such as CT or MRI. Source Voxels – Choose this option to measure the source intensity of the voxels of a 3D optical image.
Measurement Types	3D Volumetric Data: Counts – A measurement of a voxel value. The scale is image specific and may not be consistent between images. Absorption – A measurement of the amount of X-rays absorbed by the voxels. Hounsfield – A measurement of voxel grayscale value in Hounsfield units. **Note:** Absorption and Hounsfield units are only available for IVIS Spectrum CT data. Source Voxels: photons/sec – The total flux of a luminescent source. cells – The number of cells for calibrated sources integrated over the 3D ROI. pmol M^{-1} cm^{-1} – Fluorescence yield for uncalibrated sources integrated over the 3D ROI. pmol – The number of picomoles for calibrated sources integrated over the 3D ROI.
Sequence Number	The identifier of the active image data.
ROI	Name of the 3D ROI.
Voxels	The number of voxels within the 3D ROI.
3D Volumetric Data: Counts measurements (16-bit scale with values that change from image to image)	Total Counts – the sum of all counts for all voxels inside the 3D ROI. Average Counts – Total Counts/Number of voxels in the 3D ROI Min Counts – The smallest number of counts in a voxel within the 3D ROI. Max Counts –The largest number of counts in a voxel within the 3D ROI.
3D Volumetric Data: Absorption Measurements (Fixed 32-bit scale with values that are consistent between images.) **Note:** These measurements are only available for IVIS Spectrum CT data.	Total Value – The sum of the absorption measurements of all voxels in the 3D ROI. Average Value – Total Value/Number of voxels in the 3D ROI. Stdev Value – Standard deviation of the absorption values for all voxels inside the ROI. Min Value – The smallest absorption value for any single voxel in the 3D ROI. Max Value – The largest absorption value for any single voxel in the 3D ROI.
3D Volumetric Data: Hounsfield measurements (Calibrated CT scale. Fixed from image to image.) **Note:** These measurements are only available for IVIS Spectrum CT data.	Total Hounsfield – The sum of the Hounsfield unit values for all of the voxels in the 3D ROI. Average Hounsfield – Total Hounsfield unit value/Number of voxels in the 3D ROI. Stdev Hounsfield – Standard deviation of the Hounsfield unit values for all voxels inside the ROI. Min Hounsfield – The minimum Hounsfield unit value for any single voxel in the 3D ROI. Max Hounsfield – The maximum Hounsfield unit value for any single voxel in the 3D ROI.

Table 9.2 3D ROI Measurements Table (continued)

Item	Description
Source Voxels: photons/sec measurements	Total Flux [ph/s] – The flux in each voxel summed or integrated over the 3D ROI. Average Flux [ph/sec] – Total flux/Number of voxels in the 3D ROI. Stdev Flux – Standard deviation of the flux of the voxels inside the ROI. Min Flux – The smallest flux value of a voxel. Max Flux – The largest flux value of a voxel.
Source Voxels: cells **Note:** This measurement type requires a quantification database. See Appendix C on page 260 for more details.	Total Cells – The number of cells in the 3D ROI. Average Cells – Total number of cells/Number of voxels in the 3D ROI. Stdev Cells – Standard deviation of the number of cells in the 3D ROI. Min Cell – The smallest number of cells in a voxel included in the 3D ROI. Max Cell – The largest number of cells in a voxel included in the 3D ROI.
Source Voxels: pmol M^{-1} cm^{-1} measurements	Total pmol M^{-1} cm^{-1} – The fluorescence yield summed or integrated over the 3D ROI. Average pmol M^{-1} cm^{-1} – Total fluorescence yield/Number of voxels in the 3D ROI. Stdev pmol M^{-1} cm^{-1} – Standard deviation of the fluorescence yield of the voxels in the 3D ROI. Min pmol M^{-1} cm^{-1} – The smallest fluorescence yield in the 3D ROI. Max pmol M^{-1} cm^{-1} – The largest fluorescence yield in the 3D ROI.
Source Voxels: pmol measurements **Note:** This measurement type requires a quantification database. See Appendix C on page 260 for more details.	Total pmol – Total picomoles of fluorescent probe within the 3D ROI. Average pmol – Total picomoles/Number of voxels. Stdev pmol – Standard deviation of the picomole values in the 3D ROI. Min pmol – Smallest picomole value in the 3D ROI. Max pmol – Largest picomole value in the 3D ROI.
Refresh	Updates the ROI Measurements table (for example, after you draw new ROIs, move an ROI, and close or open image data).
Copy	Copies the selected row(s) in the table to the system clipboard.
Select All	Selects all rows in the table .
Configure	Displays the Configure Measurements box that enables you to specify and organize the data categories (column headers) for the table. See for more details.
Export	Opens a dialog box that enables you to export the ROI measurements (.txt or .csv).
Close	Closes the ROI Measurements table.

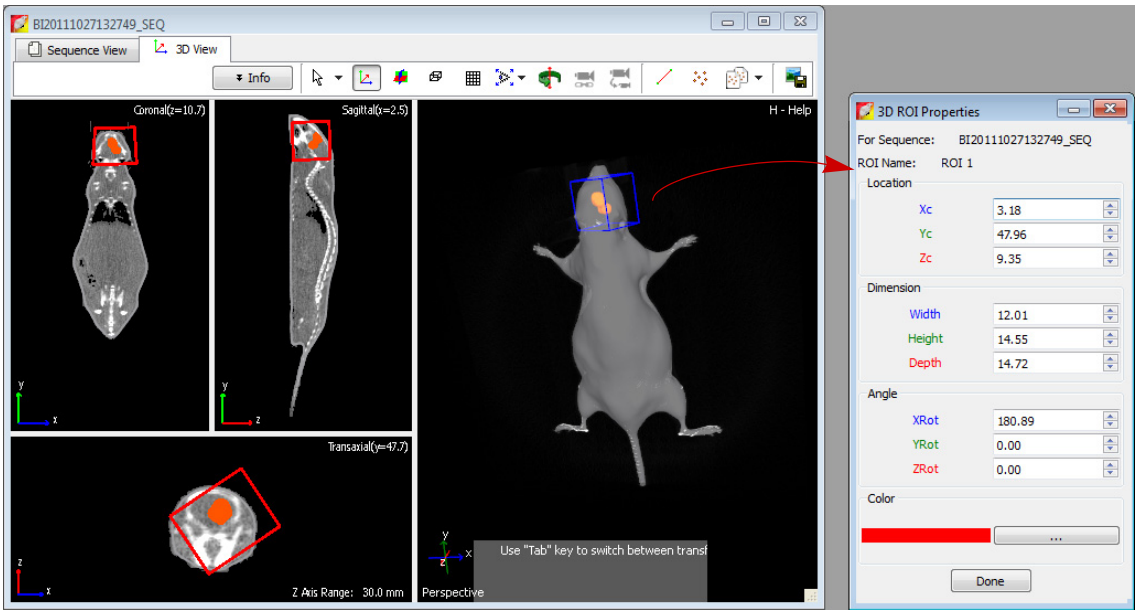
9.4 Managing 3D ROIs

ROI Properties

You can view information about the location and dimensions of a 3D ROI and edit these properties.

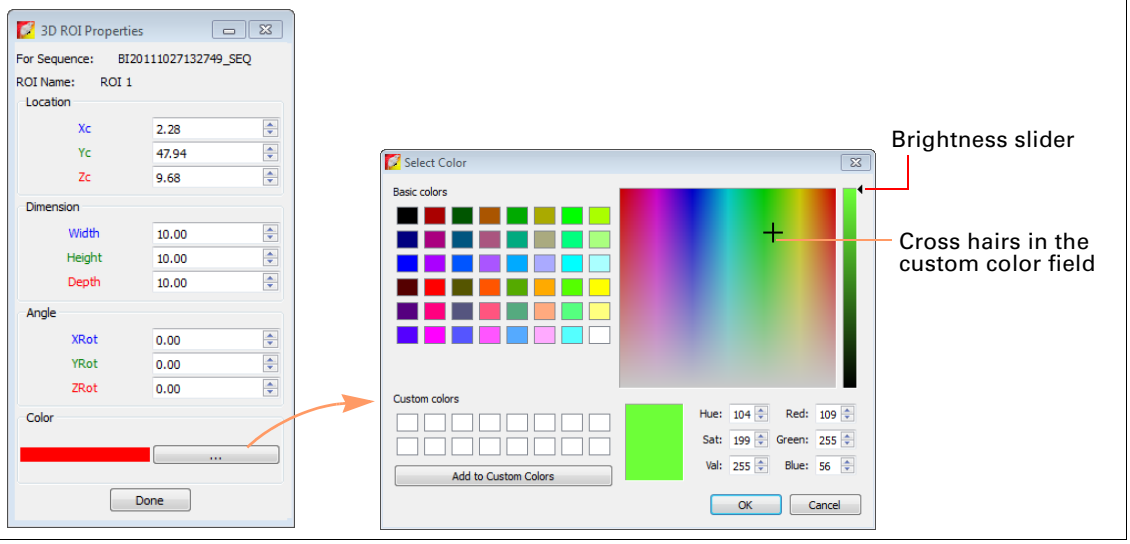
1. Click the 3D ROI Transform button and select an ROI from the drop-down list.
2. Double-click the 3D ROI.
   The 3D ROI Properties dialog box appears.

Figure 9.8 3D ROI Properties



3. Enter new values or use the arrows in the dialog box to modify the location or dimensions of the 3D ROI in the x, y, or z-planes.
4. Enter new values or use the arrows in the dialog box to rotate the 3D ROI in the x, y, or z-planes.
5. To change the color of the 3D ROI:
   a. Click the **Browse** button ... .
   The Select Color box appears (Figure 9.9).

Figure 9.9 Select 3D ROI Color



   b. To select a basic color for the ROI line, click a basic color swatch, and click **OK**.
   c. To define a custom color, drag the crosshairs in the custom color field, adjust the brightness slider, and click **Add to Custom Colors**.
   d. To select a custom color for the ROI line, click a custom color swatch, and click **OK**.

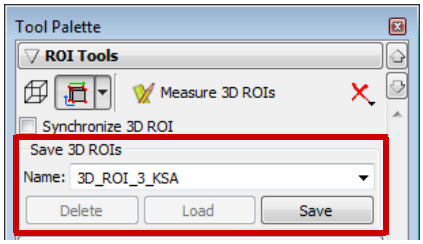
Saving 3D ROIs to the System

Living Image software saves 3D ROIs with a dataset (the software prompts you to save before closing the dataset). 3D ROIs (parameters only) can also be saved to the system (per user) and applied to other datasets. These 3D ROIs appear in the "Name" drop-down list (Figure 9.10). This section explains how to save 3D ROIs to the system.

NOTE: Before closing a dataset, the software prompts you to save 3D ROIs with the dataset. 3D ROIs saved with a dataset do not appear in the "Name" drop-down list.

1. After a 3D ROI(s) is applied to a dataset, save it to the system:
   a. Confirm the default name or enter a new name for the 3D ROI in the Name drop-down list.
   b. Click **Save** (Figure 9.10).

Figure 9.10 Name and Save 3D ROIs to the System

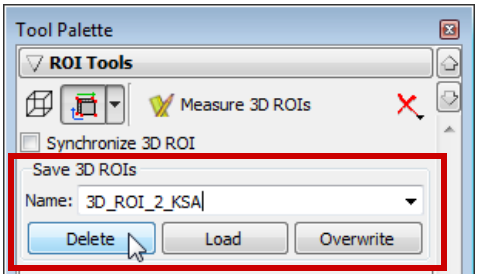


2. To apply a 3D ROI to a 3D reconstruction or volume, make a selection from the Name drop-down list and click **Load** (Figure 9.10).

NOTE: If you load a 3D ROI, then apply (or delete) 3D ROIs, the **Save** button changes to **Overwrite**. If you want to save the 3D ROIs using the existing name, click **Overwrite**. Otherwise, enter a new name and click **Save**.

3. To delete a 3D ROI from the system (per user), select a 3D ROI from the Name drop-down list and click **Delete** (Figure 9.11).

Figure 9.11 Delete 3D ROIs From the System



10 Working With Volumetric Data

10.1 About the 3D Multi-Modality Tools

Use the 3D Multi-Modality tools to:

- Classify volumetric data (reconstructed CT images representing 3D volumes).
- View volume slices.
- Refine the appearance of the volume (*volume processing*).
- Register optical and imported volumetric data (for example, CT, MRI, or PET data).

NOTE: IVIS Spectrum Imaging System does not acquire volumetric data. However, Living Image software can analyze volumetric data acquired on a different imaging system such as Spectrum CT Imaging System or Quantum microCT Imaging System.

Requirements

The Living Image 3D Multi-Modality tools require a separate license. Additionally, the graphics processing unit (GPU) must meet the minimum specifications shown in Table 10.1.

If the appropriate license is not installed or the GPU does not meet these specifications, the 3D Multi-Modality tools will not appear in the Tool Palette.

Table 10.1 Minimum Graphics Card Specifications

Specification	Description
OpenGL Version Requirement[1]	OpenGL 2.0 and above
OpenGL Extension Requirement[1]	GL-EXT-texture3D
Graphics Card Memory	Minimum: 256MB (Dedicated + Shared) Recommended: 1GB (Dedicated)
Consumer Graphics Cards (Desktop/ Mobile, Windows/Mac)	Supported: ▪ NVIDIA® GeForce® 8 Series and above (8, 9, 100, 200, 300 and 400 series) ▪ ATI Radeon™ HD 4000 Series and above (4000 and 5000 series) Recommended: ▪ Desktop - NVIDIA GeForce GT 240 and above ▪ Mobile - NVIDIA GeForce GT 230M and above
Workstation Graphics Cards (Desktop/ Mobile, Windows/Mac)	Supported: ▪ NVIDIA® Quadro® NVS Series and Above (NVS and FX series) ▪ ATI FireGL™ V5600 and Above (FireGL, FirePro and CrossFire series) Recommended: ▪ Desktop - Quadro FX 1800 and above ▪ Mobile - Quadro FX 880M and above

[1] If these specifications are not met, the 3D Multi-Modality tools do not appear in the Tool Palette.

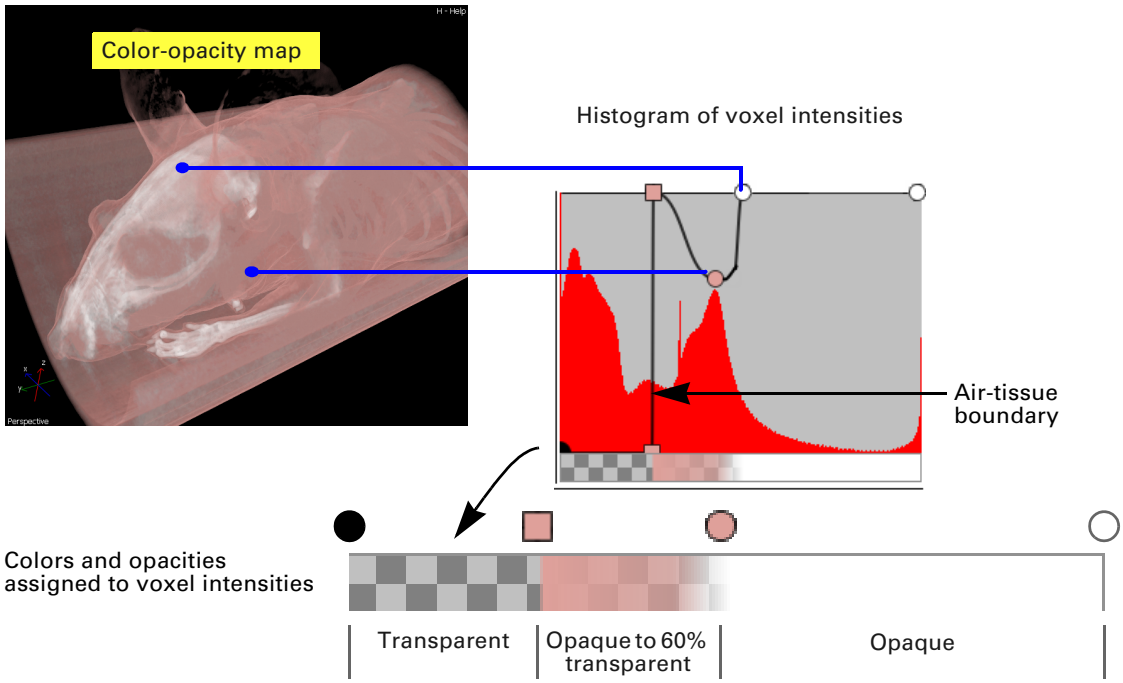
10.2 Classifying 3D Volumetric Data

3D Multi-Modality tools provide a histogram-based method for classifying 3D volumetric data. The histogram represents the distribution of voxel intensities in the 3D volumetric data and their color-opacity values. The goal of classification is to set color and opacity values for different intensity ranges so that the color-opacity map shows the volume regions that you are interested in (opaque in the map) and hides unimportant regions (transparent in the map).

For example, Figure 10.1 shows how the histogram tool designed a color-opacity map that shows both the skin and bone. The histogram tool enables you to easily re-design the color-opacity map to show only the skin or only bone.

3D Multi-Modality tools also enable you to classify the volumetric data by specifying color and opacity values for different intensity ranges so that you can view or hide certain parts of the data as needed. A color-opacity map can be saved.

Figure 10.1 Histogram Tool Specifies Opacity for Different Voxel Intensities



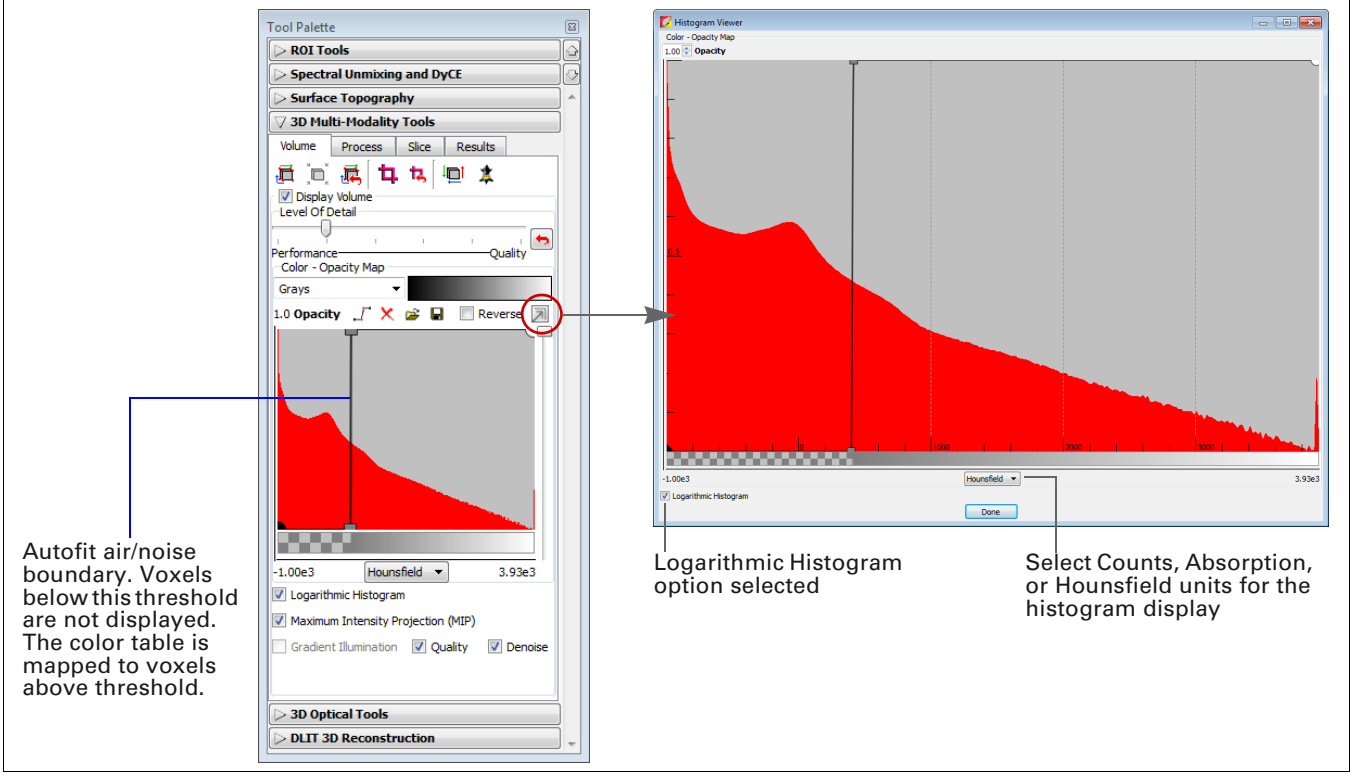
Specifying a Color-Opacity Map

After the surface and volume data are loaded, confirm that the Display Volume option is selected.

Histogram Display Options

- To change the color table for the color-opacity map, make a selection from the Color table-Opacity Map drop-down list. To apply the reverse color table, select the Reverse option.
- To view the histogram in a separate window, click the button (Figure 10.2).
- Select units for histogram display (Hounsfield, counts, or absorption). If the histogram intensity range appears narrow or suppressed, choose the Logarithmic Histogram option. This will enhance histogram display by magnifying the smaller regions of interest in the histogram while keeping noise and air-related intensity peaks high. It helps bring out hidden regions of the histogram for easier identification of interesting intensity ranges.

Figure 10.2 3D Multi-Modality Tools



Managing Control Points

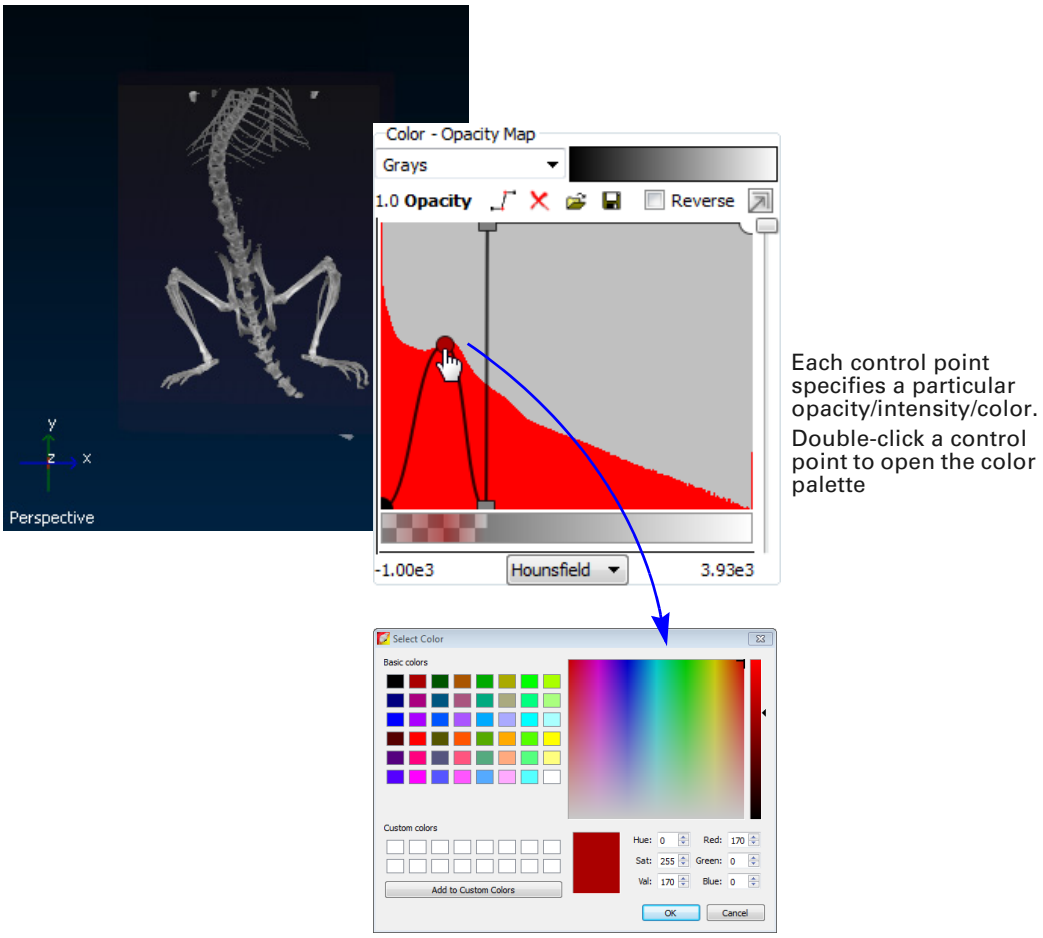
Edit the 3D volumetric data color-opacity map using "control points" (Figure 10.3). During volume rendering, the color-opacity map is used to map color and opacity to the corresponding intensity value as well as interpolate color and opacity for all data between adjacent control points.

1. Place a control point on the histogram by clicking anywhere on the histogram between the ● point (represents the lowest intensity in the volume) and ○ point (represents the highest intensity in the volume).
2. Drag any control point up or down to set the opacity level that is associated with the intensity value represented by the point. Drag a user-added control point left or right to change the intensity associated with the opacity specified by the point.

   When you add, delete, or modify a control point, the color-opacity map and the rendering of the volume data are updated in real-time.

NOTE: The minimum and maximum intensity levels associated with the ● and ○ control points cannot be changed. The opacity level associated with these points can be changed.

Figure 10.3 Histogram Tool



3. To select a color for particular data, double-click a control point. In the color palette that appears, choose a color and click **OK**. The software interpolates the color range between adjacent control points.
4. To delete a control point, right-click the point. To delete all control points, click the button.

NOTE: The ● and ○ control points cannot be deleted from the histogram.

Saving a Color-Opacity Map

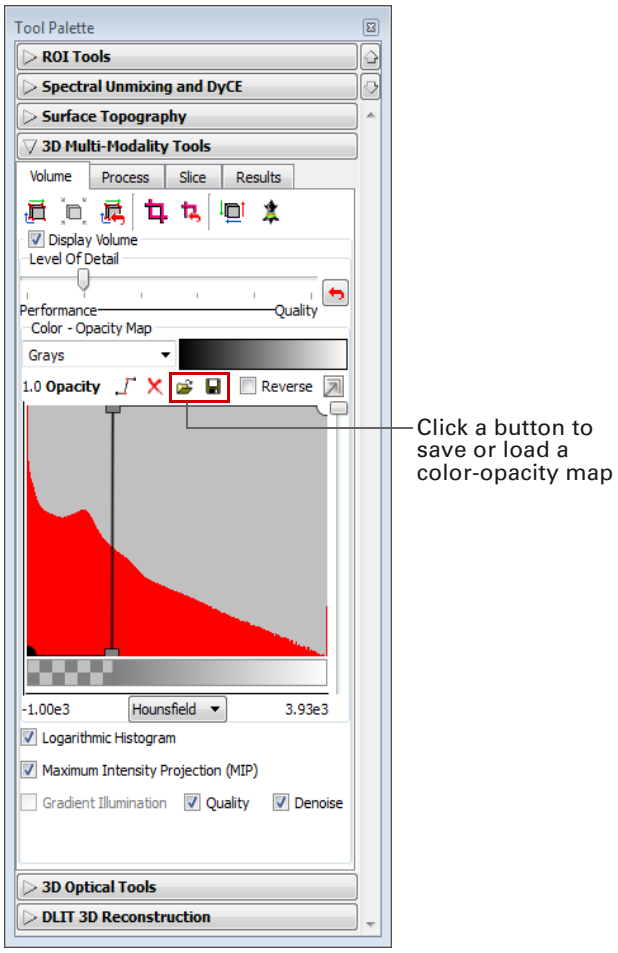
A color-opacity map can be saved and applied to any volumetric dataset.

1. Click the Save button (Figure 10.4).
2. In the dialog box that appears, select a folder for the file (.tfn) and enter a file name.
3. Click **Save**.

Loading a Color-Opacity Map

1. Click the Open button (Figure 10.4).
2. In the dialog box that appears, navigate to the map file (.tfn), and click **Open**.

Figure 10.4 Save or Load a Color-Opacity Map



10.3 Volume Display Options

Adjusting Image Quality

By default, the color-opacity map displays the volumetric data at original (1×) resolution. This means, for example, if the volume comprises 512 slices, then all of the 512 slices are displayed. You can increase or decrease the resolution of the data display from 0.5× to 3.0× resolution (see Table 10.2 for examples).

If the resolution is increased, the software interpolates the data and adds slices to the volume. If the processing performance is impacted at the original resolution, you may want to reduce the resolution to improve performance. Reducing the resolution down-samples the data and fewer slices are displayed.

To adjust the image resolution:

1. Move the "Level of Detail Slider" to the left or right (Figure 10.5).
   The color-opacity map is updated.
2. To return the resolution to 1×, click the Reset button .

Figure 10.5 Level of Detail Slider

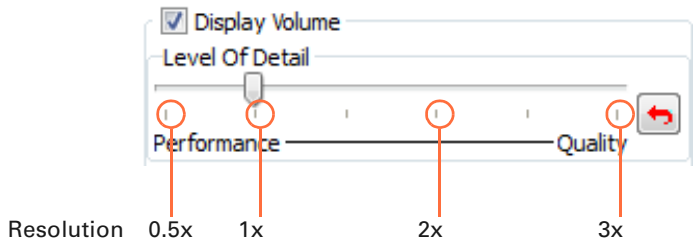


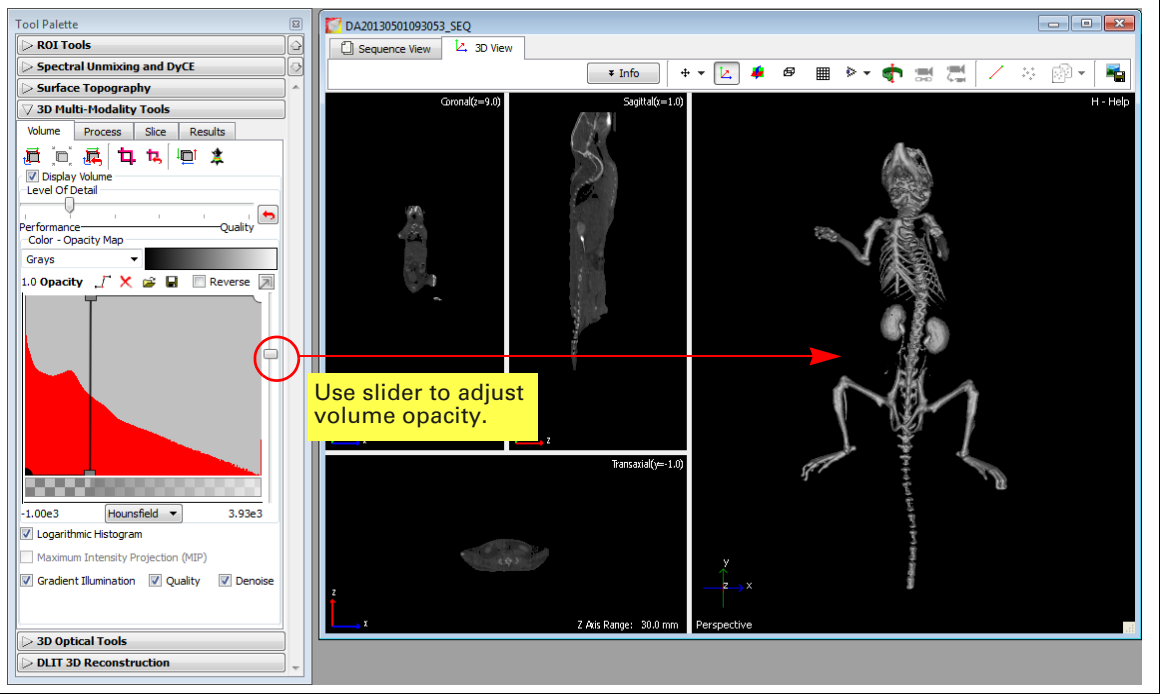
Table 10.2 Example Volume With 512 Slices at 1x Resolution

Volume Resolution	No. of Slices Displayed
0.5×	256
1× (original resolution)	512
1.5×	768
2×	1024
2.5×	1280
3×	1536

Adjusting Volume Opacity

Adjust the volume opacity using the slider in the 3D Multi-Modality tools.

Figure 10.6 Adjusting Volume Opacity



Maximum Intensity Projection

A maximum intensity projection (MIP) projects all maximum intensity voxels in the view along the viewing direction into the viewing plane (Figure 10.7). Living Image automatically extracts a 2D image from the MIP. The extracted image is similar in appearance to an X-ray image and is available in the image window. For example, Figure 10.8 shows an overlay of a luminescent image on an extracted 2D "x-ray" image.

NOTE: If you change the volume opacity (see Figure 10.6 on page 187) and want to extract new 2D images from the MIP, click the button (Figure 10.7).

Figure 10.7 Volume Display – Maximum Intensity Projection

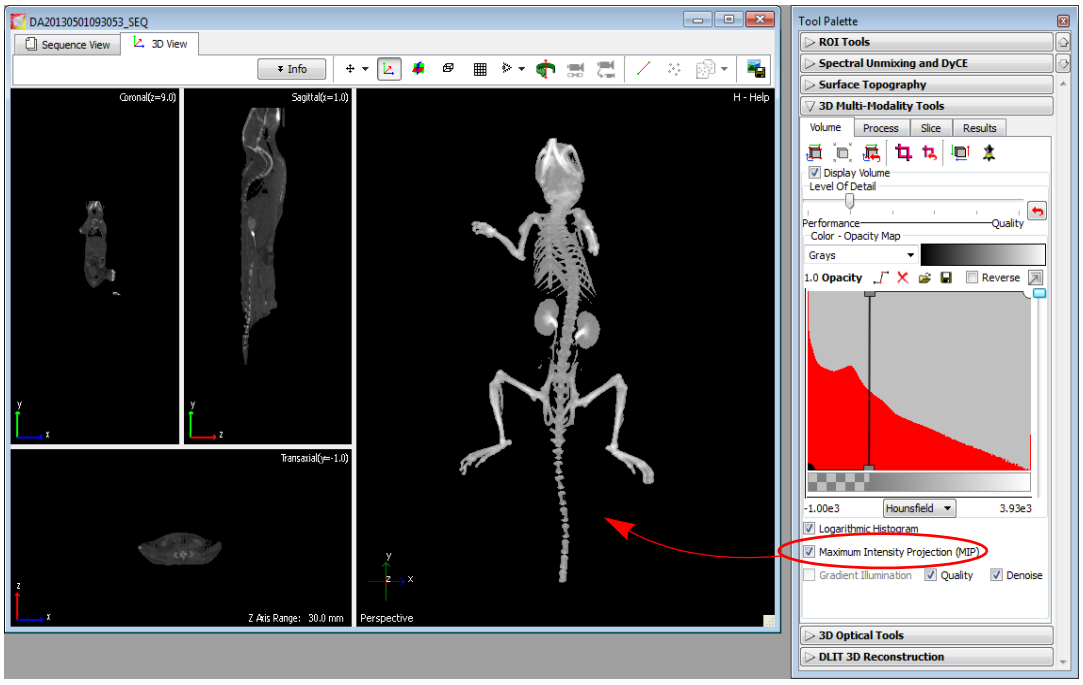
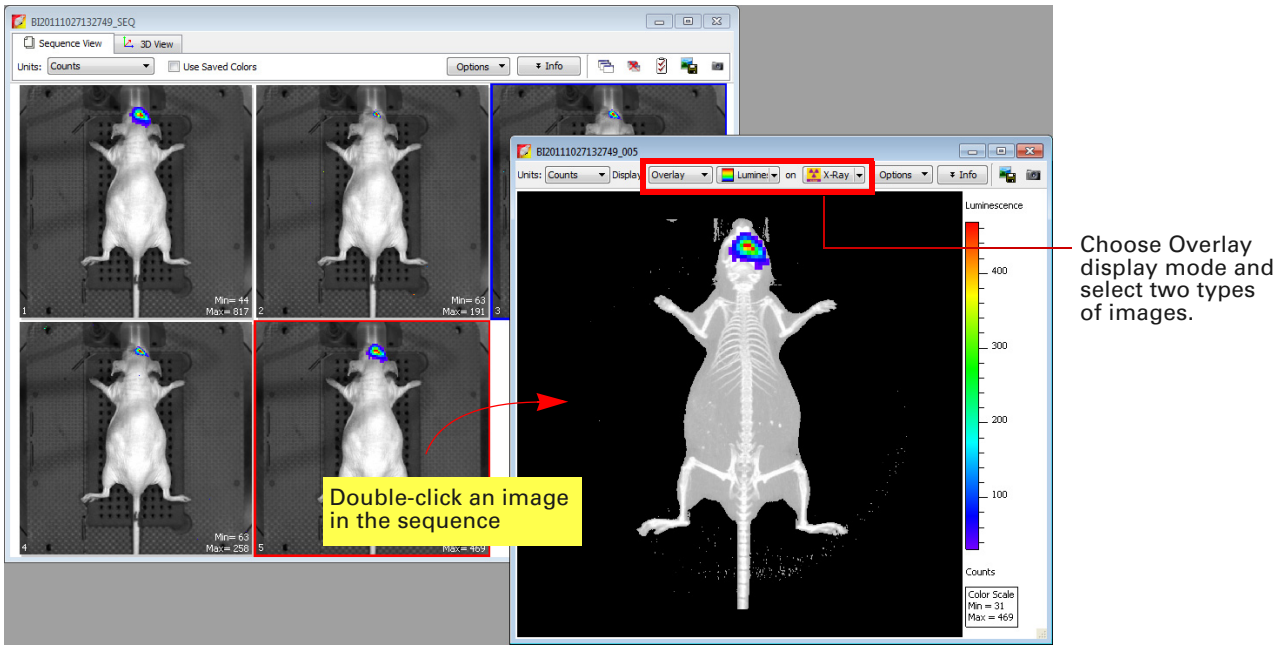


Figure 10.8 Image Window – Luminescent Image on X-ray Image



Gradient Illumination

Gradient illumination is based on the idea that light is reflected at boundaries between different voxel intensities, but is not affected when passing through homogeneous regions. Choosing this option illuminates the voxels at boundaries more than voxels within a homogeneous region. The boundaries are based on the gradient magnitude between heterogeneous regions or the change in intensities between neighboring voxels in heterogeneous regions. Using this option enhances the variation in tissue properties and may be helpful for visualizing the boundaries of different tissues.

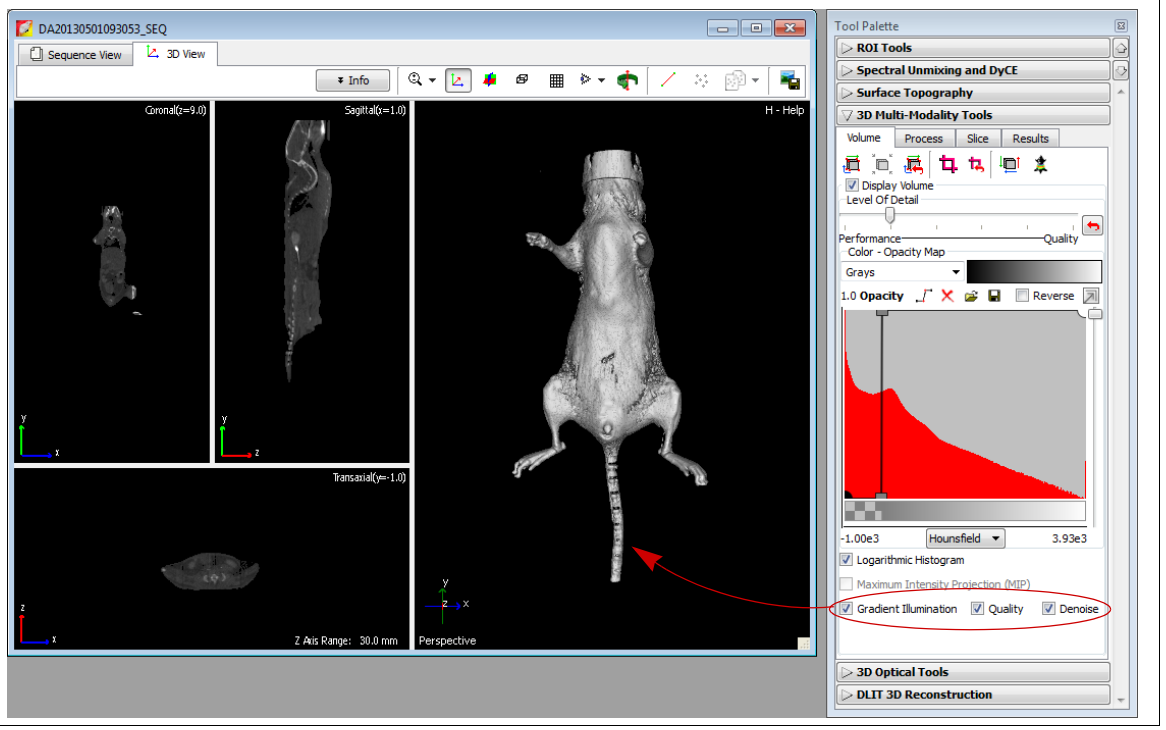
Two options are available for Gradient Illumination:

- Quality – This option will be automatically selected if your system has an appropriate graphics card. If this option is selected, the volume is displayed with more detail (Figure 10.9).

NOTE: If the system graphics card does not meet the recommended specifications (Table 10.1 on page 182), choosing the Quality option causes slow performance of actions such as rotating the volume.

- Denoise – Filters out image noise in the volume rendering. The raw data are not modified by this filter.

Figure 10.9 Volume Display – Gradient Illumination

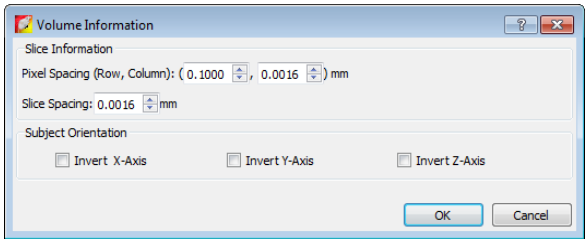


Modifying Volume Resolution

Changing the pixel or slice spacing modifies the volume resolution. Increasing the pixel or slice spacing reduces resolution, while reducing either increases resolution.

1. In the Volume tab, click the Edit Space and Orientation button .
2. In the dialog box that appears (Figure 10.10), edit the pixel or slice spacing.

Figure 10.10 Volume Information Dialog Box



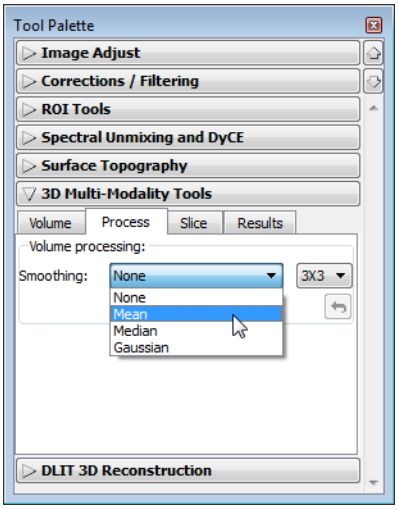
10.4 Smoothing a Volume

Smoothing a volume reduces noise in a CT, MRI, or PET image such as excessive variation in voxel grayscale values.

To apply smoothing:

1. Load the volumetric data.
2. Choose the type of smoothing and group size in the Process tab of the 3D Multi-Modality tools (Figure 10.11).
   - Mean – Applies the average grayscale value of a group of voxels (for example, a 3x3 group) to the central voxel of the group.
   - Median – Applies the median grayscale value of a group of voxels to the central voxel of the group.
   - Gaussian – Applies the weighted mean to the central voxel of the group. The weight distribution is similar to a normalized Gaussian shape with the highest value at the central voxel of the group.
3. Click the  button to remove the smoothing.

Figure 10.11 3D Multi-Modality Tools – Process Tab



10.5 Viewing and Rendering Slices

Viewing Slices

View volume slices by double-clicking the Coronal, Sagittal, or Transaxial windowpane (Figure 10.12).

Figure 10.12 Viewing Slices

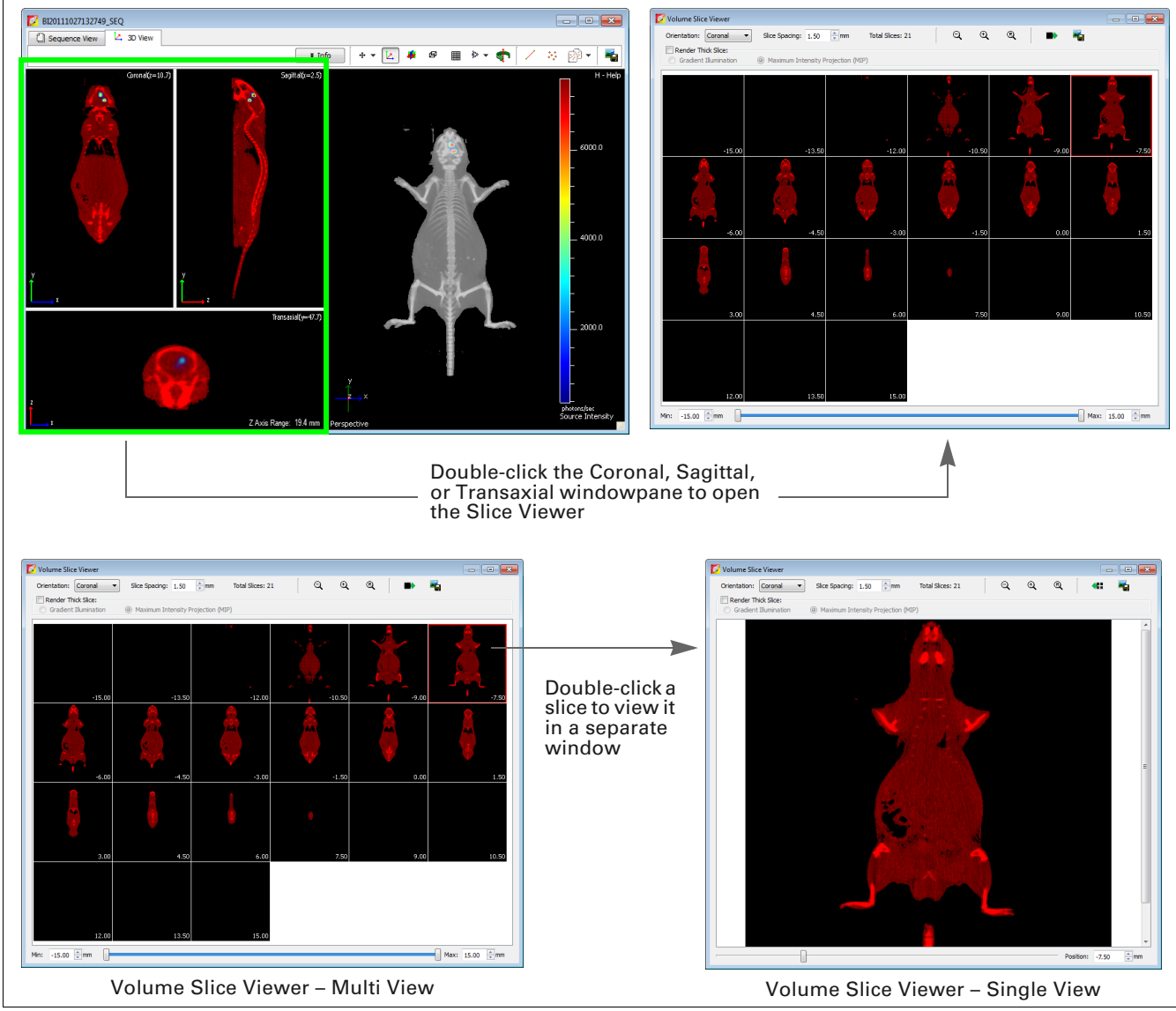
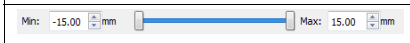
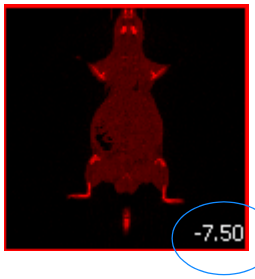


Table 10.3 Volume Slice Viewer

Item	Description
Orientation	Select a slice orientation from the drop-down list.
Slice Spacing	The distance between each slice in the Volume Slice Viewer. Enter a smaller value to increase the number of slices in the viewer or a larger value to decrease the number of slices in the viewer.
Total Slices	The number of slices shown in the viewer.
Render Thick Slice	This option is used to create a sequence of 3D or maximum intensity projection (MIP) renderings from the image stack. When this option is selected, "Slice Spacing" changes to "Slice Thickness". Increasing the slice thickness causes more slices to be extracted from the volume before creating the rendering.
Gradient Illumination	Gradient Illumination is based on the idea that light is reflected at boundaries between different voxel intensities, but is not affected when passing through homogeneous regions. Choosing this option illuminates the voxels at boundaries more than voxels within a homogeneous region. The boundaries are based on the gradient magnitude between heterogeneous regions or the change in intensities between neighboring voxels in heterogeneous regions. Using this option enhances the variation in tissue properties and may be helpful for visualizing the boundaries of different tissues.
Maximum Intensity Projection (MIP)	Projects all maximum intensity voxels in the view along the viewing direction into the viewing plane.
	Min – The slice coordinate of the first slice being viewed. Zero is defined as the center plane of the image. Max – The slice coordinate of the last slice being viewed. Specify the position range to include in the viewer using the Min and Max sliders or enter values.  Slice position
	Click to show the single view of the active slice in the multi view. Alternatively, double-click a slice in the multi view to show the single view.
	Click to show the multi view.
	If the single view has been magnified, click this button to zoom out incrementally.
	Magnifies the single view.
	Resets the single view to the default magnification.
	Click to export the slice view as a graphic file (for example, .bmp)

Rendering Slices

The Slice tab in the 3D MM tools contains rendering and viewing options for slices.

Figure 10.13 Perspective View and Slice Views Displayed Using Different Color Tables

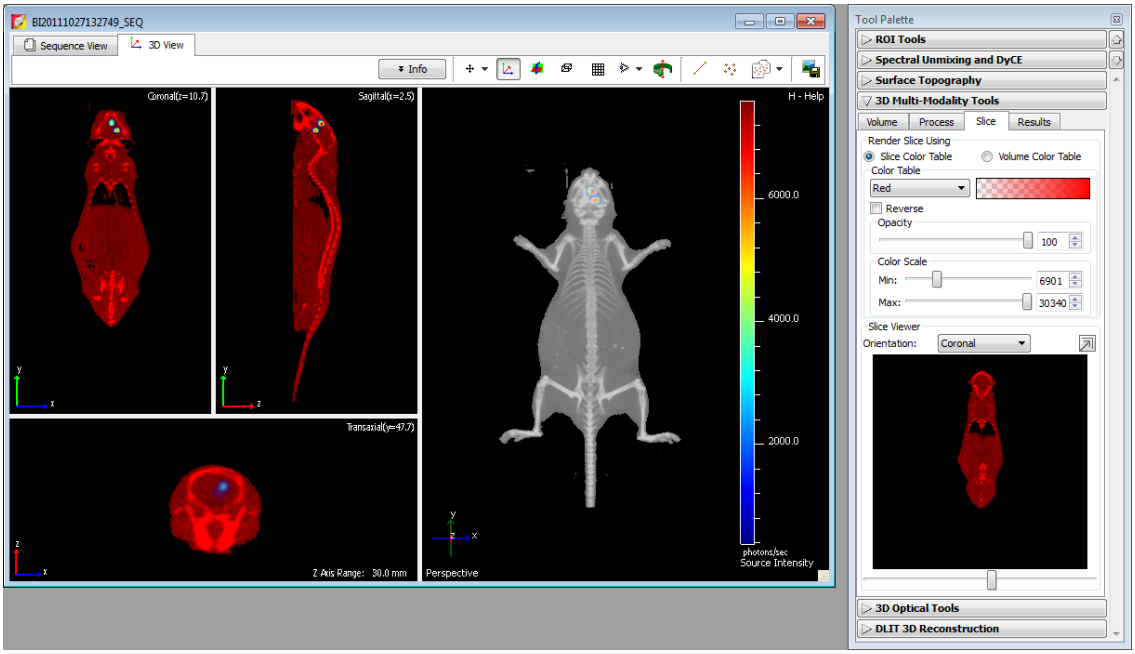
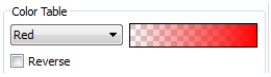
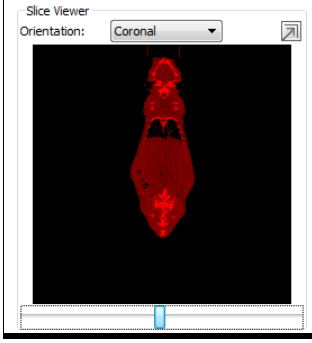


Table 10.4 3D Multi-Modality Tools for Rendering Slices

Item	Description
Slice Color Table	Choose this option to apply the color table selected from the Color Table drop-down list.
Volume Color Table	Choose this option to apply the volume color table of the volume color-opacity map that was selected in the Volume tab.
	Color table options. Choose the Reverse option to apply the inverse color table.
Opacity	Move the slider to adjust the color opacity.
Color Scale	Min – Sets the intensity level associated with the lowest color scale value. Max – Sets the intensity level associated with the maximum color scale value.
	The Tool Palette provides an alternative way to view slices and access the Slice Viewer. Choose a slice orientation from the drop-down list. Use the slider to move through the slices. Double-click the slice view or click the button to open the Slice Viewer. The selected slice is highlighted in the Slice Viewer.

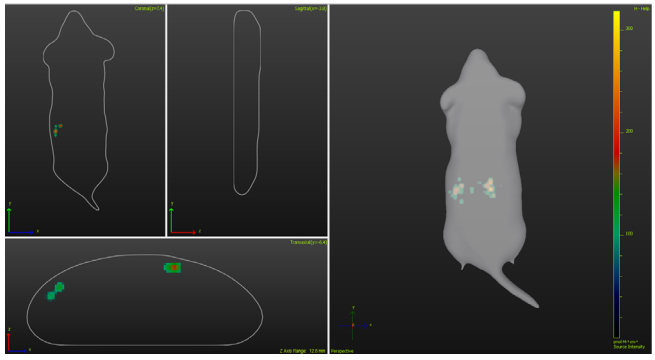
10.6 Registering Optical and Volumetric Data

Registering *multi-modal* data (optical and volumetric data) provides an anatomical context for interpreting biological (functional) information. Two registration methods are available:

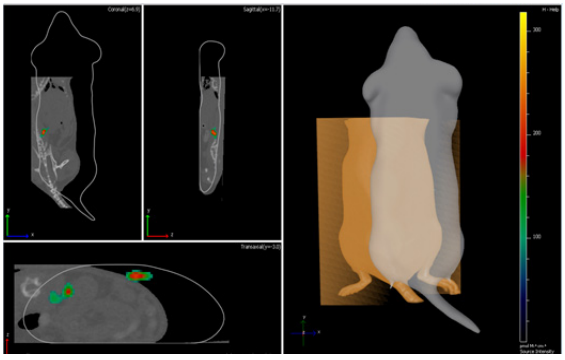
- Automatic fiducial registration – For experiments in which the optical data are acquired on the IVIS Spectrum and the CT data are acquired on the Quantum FX microCT pr Quantum GX microCT. The subject must be contained in the Mouse Imaging Shuttle during both optical and CT imaging, and the CT data must be exported to DICOM format. See page 197 for more details.
- Manual registration – Use the 3D Multi-Modality tools to register a 3D surface reconstruction with 3D volumetric data acquired on a third party instrument. See page 199 for more details.

Figure 10.14 shows an overview of the steps to register these types of multi-modal data. After registration, classify the 3D volumetric data to help identify and separate objects (see page 182).

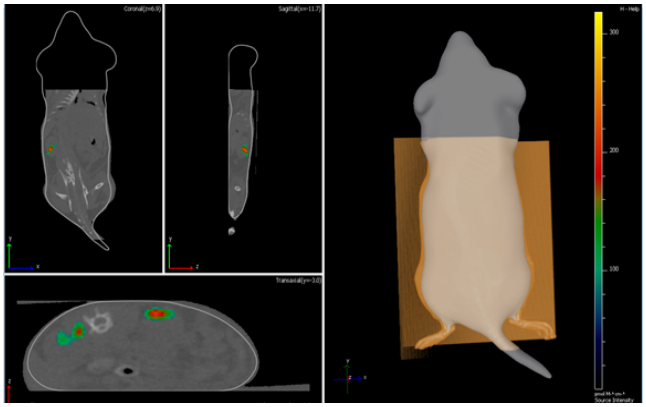
Figure 10.14 Registering Multi-Modal Data



1. Load the optical data:
   - Bioluminescence or fluorescence image sequence and structured light surface
   - 3D source reconstruction (DLIT or FLIT results)

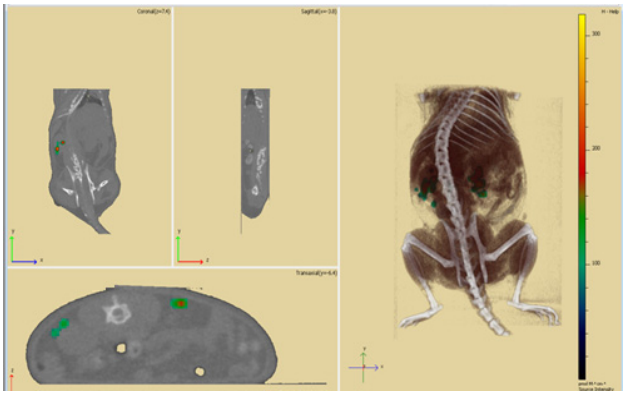


2. Load 3D volumetric data (CT or MRI) (page 196).



3. Register the 3D source reconstruction and the 3D volumetric data by performing either:
   - Automatic fiducial registration–Available for data acquired on the Quantum FX µCT instrument using the Mouse Imaging Shuttle (page 197)

   or
   - Manual registration–Match animal surface representations using the Manual Registration tool (page 199)



4. Classify the 3D volumetric data to help identify and separate objects (page 182). Save the color-opacity map (optional).
5. Save the registered 3D multi-modality results (page 202).

Loading Data for Registration

1. Load a DLIT or FLIT image sequence and the 3D reconstruction results.

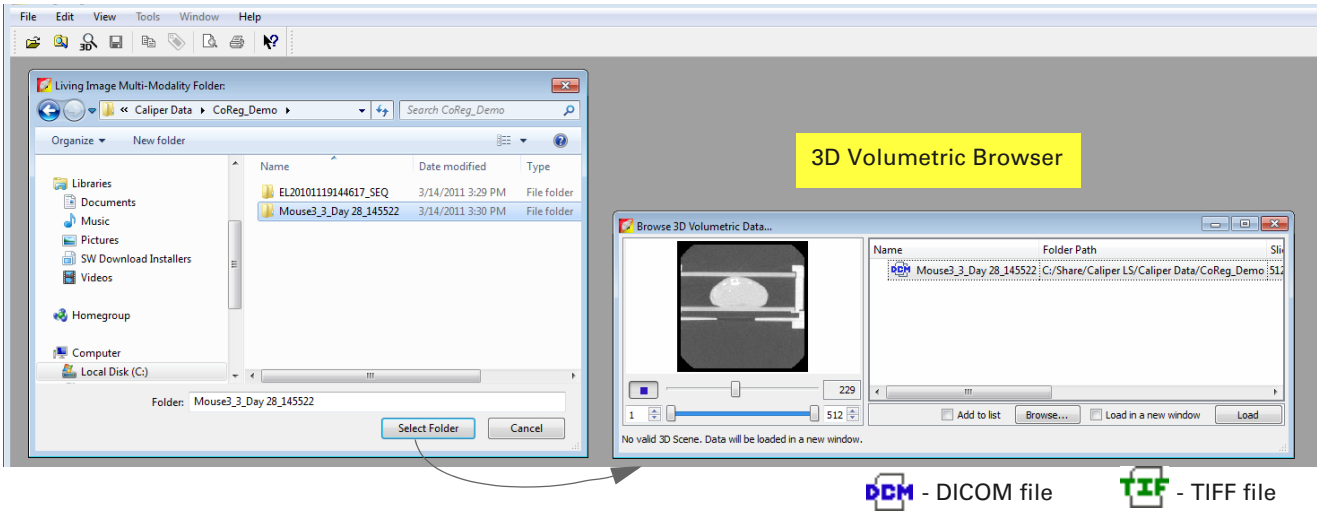
NOTE: The 3D Multi-Modality tools appear in the Tool Palette after you load optical image data. If the 3D Multi-Modality tools do not appear in the Tool Palette, confirm that the 3D Multi-Modality Tools license is installed and that the workstation graphics card meets the specifications in Table 10.1 on page 182.

2. Select the DICOM or TIFF volumetric data.
   a. Click the 3D toolbar button. Alternatively, select **File** → **Browse 3D Volumetric Data** on the menu bar.
   b. If this is the first time browsing for volumetric data in the session, select a data folder in the dialog box that appears, and click **Select Folder** (Figure 10.15).

      The Living Image 3D Volumetric Browser appears.

NOTE: If the 3D Volumetric Browser was previously opened during the session, clicking the 3D button opens the browser. Click the Browse.. button in the browser, and in the dialog box that appears, select a data folder. Only DICOM or TIFF data can be added to the 3D Volumetric browser. For details on loading other data types (.raw or .vox files) see page 202.

Figure 10.15 Opening the 3D Volumetric Browser



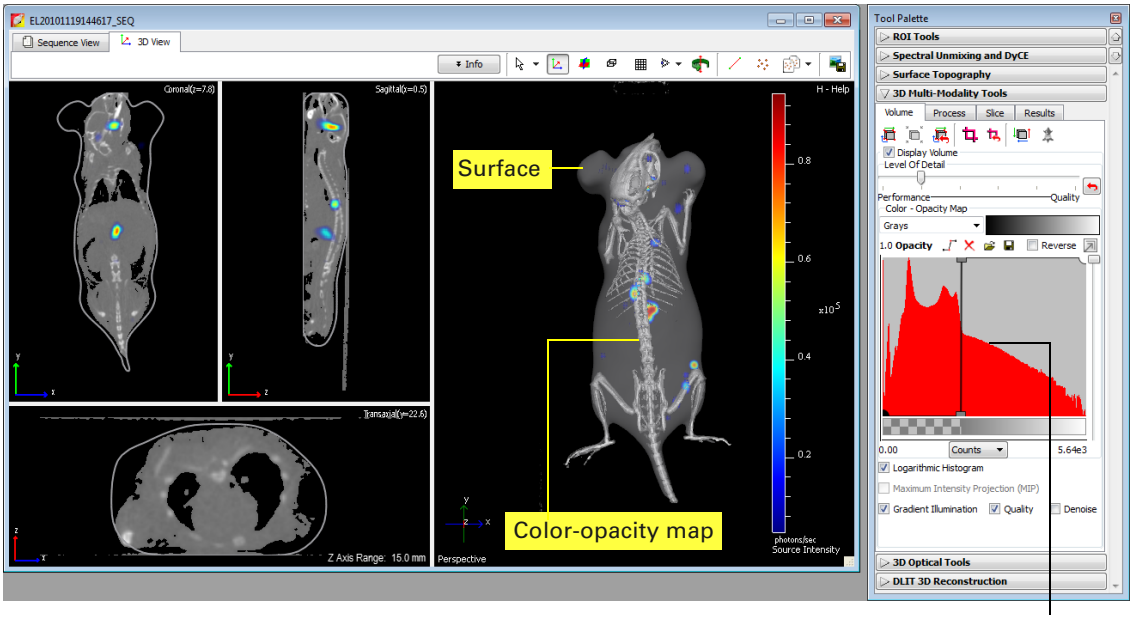
The 3D Volumetric Browser automatically previews a playback of the data along with other information about the data (Figure 10.15). See Figure 8.24 on page 169 for more details on the 3D Volumetric Browser.

3. Load the volumetric data with the optical data.
   a. Confirm that the “Load in a new window” option is not selected. (If this option is selected, the volumetric data are loaded in a new window.)
   b. Double-click the data row in the browser. Alternatively, select the data row and click **Load**.

The 3D volumetric data appears in the 3D View window of the optical data (Figure 10.16). The software converts loaded volumetric data into an 8-bit representation to reduce memory overhead and for easier color mapping. The 3D Multi-Modality tools provide an 8-bit color-opacity map for volume visualization which maps each voxel to an RGB color, or a color and opacity value.

A histogram of voxel intensities appears in the Multi-Modality tools and the software sets a default air/noise boundary.

Figure 10.16 3D Optical and 3D Volumetric Data



Registering Multi-Modal Data

Automatic Fiducial Registration

About the Mouse Imaging Shuttle

The Mouse Imaging Shuttle (PN 127744) contains the subject during imaging and enables the subject to be transferred between an IVIS® Imaging System and the Quantum FX µCT instrument without disrupting the subject's position.

The Mouse Imaging Shuttle must be correctly docked to the docking station in the IVIS Imaging System and the Quantum FX µCT instrument. The docking station in the Quantum FX µCT system is marked with a triangle-shaped fiducial pattern under the plane where the Mouse Imaging Shuttle docks. Automatic fiducial registration is available if both sides of the triangle fiducial pattern are included in the CT images. For more details on using the Mouse Imaging Shuttle, see the *Mouse Imaging Shuttle Instructions* (PN 127820).

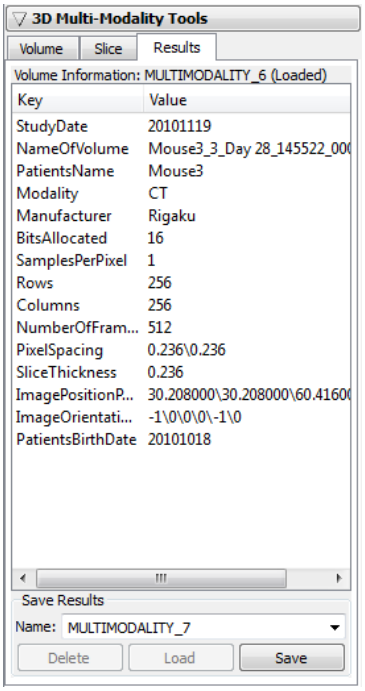
To perform automatic fiducial registration:

1. Load the data that you want to register (see page 196).
2. Click the Fiducial Registration button .
   The multi-modal data are automatically registered and cropped (Figure 10.17).
3. To undo the registration, click the Reset Registration button .

4. To save the registration information:
   a. Confirm the default name or enter a name for the results in the Results tab.
   b. Click **Save**.

Figure 10.17 Registered 3D Optical and 3D Volumetric Data

Figure 10.18 3D Multi-Modality Tools – Results

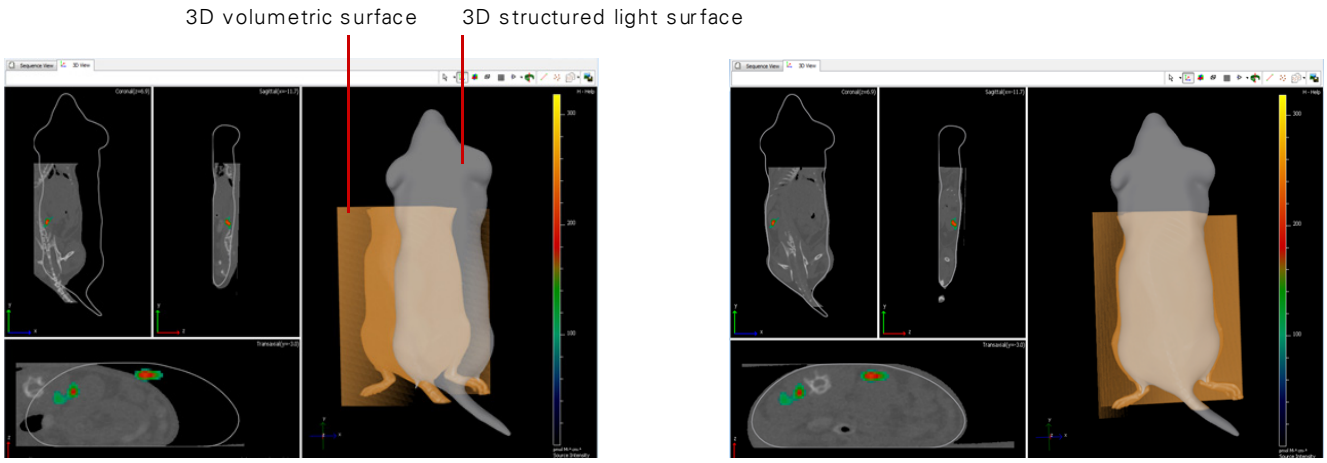


NOTE: Registration information is saved with the results for the volumetric data and is specific for a particular optical dataset.

Manual Registration

To manually register data, use the 3D Multi-Modality tools to translate, scale, or rotate the 3D volumetric surface so that features common to both surfaces are matched and aligned in the x, y, and z planes. Examine the matched surfaces in the 3D slice views to help you fine tune the registration.

Figure 10.19 Example Surfaces Before and After Registration



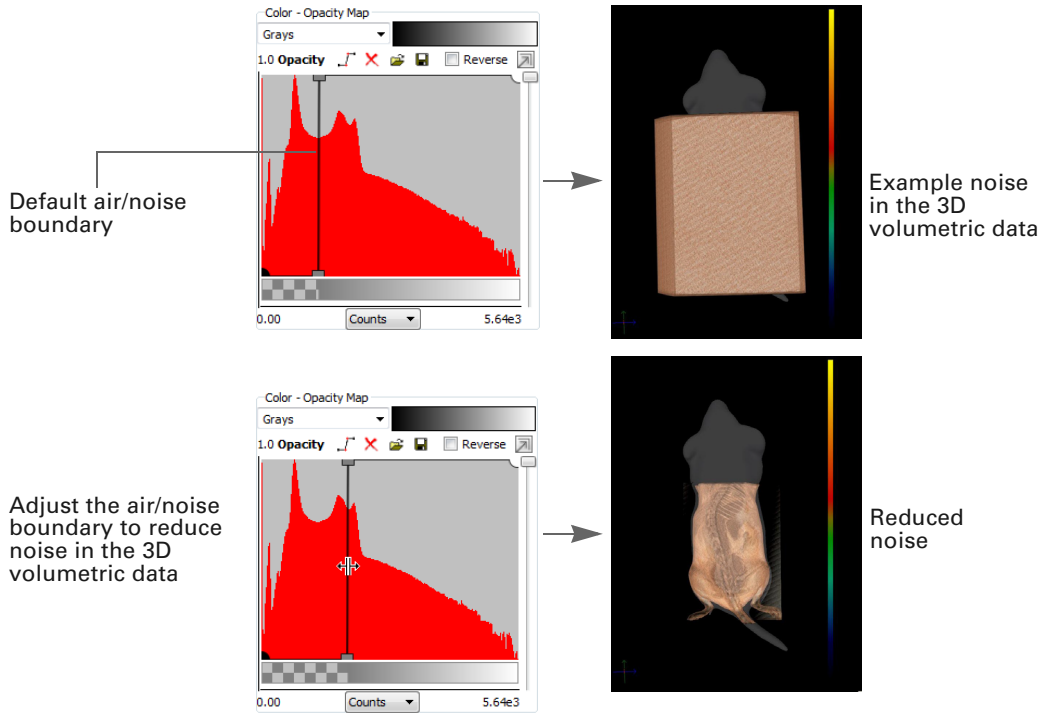
Surfaces before registration

Registered surfaces

To manually register data:

1. Load the data that you want to register (see page 196 for more details).
   The software determines a default air/noise boundary for the 3D volumetric data (Figure 10.20).
2. If you need to remove noise from the 3D volumetric data, move the air/noise boundary to the right in the histogram tool.

Figure 10.20 Adjusting Air/Noise Boundary Using Histogram Tool



3. If the volumetric data needs cropping (for example, to remove structures such as the stage from the CT view), follow step a to step c below. If cropping is not needed, proceed to step 4.

   To crop the data:

   a. Click the crop tool button .

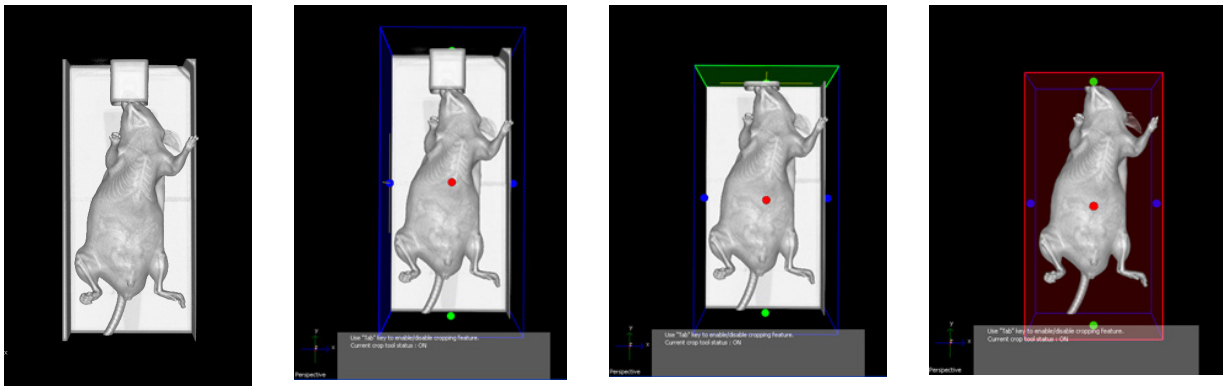
   The crop tool appears and has six control points:

   – Crops the data along the x-axis.

   – Crops data along the y-axis.

   – Crops data along the z-axis.

Figure 10.21 Crop Data Along X,Y, or Z-Axis



No crop tool | X-axis crop tool | Y-axis crop tool | Z-axis crop tool

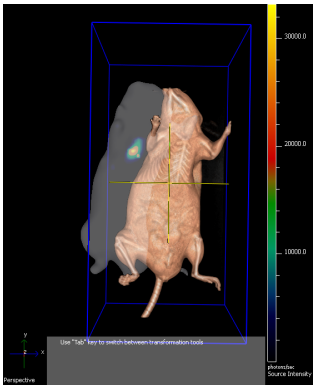
   b. Click and hold a control point while you move the crop plane. As you move the crop plane, the slice views are updated. Release the mouse button to crop the data.

c. To reset the crop planes, click the button. When finished cropping, press the Tab key to turn off the crop tool.

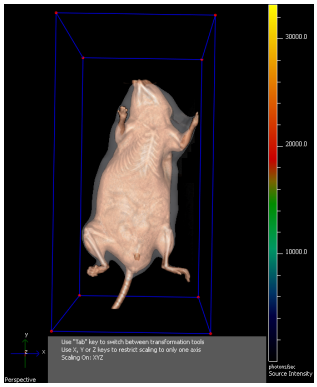
4. Click the Manual Registration button .

The transformation tool appears (Figure 10.22). The tool has three modes that enable you to translate, scale, or rotate the 3D volumetric data (press the Tab key to change the tool mode). The slice views are automatically updated when you use the tool.

Figure 10.22 Manual Registration Tool – Transformation Modes



Translate—Moves the volume in the x, y, or z-axis. Drag the tool to adjust the position of the volume.



Scale—Increases or decreases (scale the size of the volume, drag a red cube at a corner of the volume. To restrict scaling to a particular axis, press the X, Y, or Z key, then drag a red cube.

Rotate—To rotate the volum on the x, y, or z-axis, click th blue, green, or red circle and drag the mouse arrow in the direction of interest.

NOTE: Make sure that you click the transformation tool so that it is highlighted before you use it. Otherwise the dragging operation is applied to the optical data (structured light surface).

5. To return the 3D volumetric data to the default position and size, click the Reset Registration button .

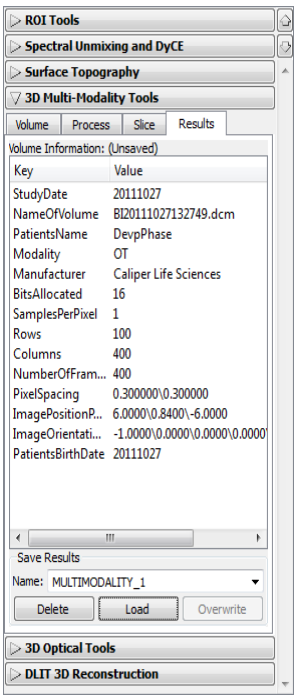
6. Save the registration information (see page 198).

NOTE: Registration information is saved with the results for the volumetric data and is specific for a particular optical dataset.

10.7 Volume Information and Results

The Results tab displays information about the loaded data taken from the DICOM file header (Figure 10.23).

Figure 10.23 Volume Information



Saving the registered and classified data provides a convenient way to share data. The software saves the following:

- Level of detail setting
- Color tables for the opacity map and slices
- Histogram tool control settings and the resulting color-opacity map
- Multi-modal registration settings
- Crop settings

Managing Results

Saving Registered Results

1. In the Results tab, confirm the default name in the Name drop-down list or enter a name.
2. Click **Save**.

   The registered 3D volumetric data, along with the color-opacity settings, appear in the 3D View window.

NOTE: The results are saved in XML format in the XML folder within the sequence folder. The results can only be accessed from the same optical dataset.

Loading Results

1. Select the results from the Name drop-down list.
2. Click **Load**.

Deleting Results

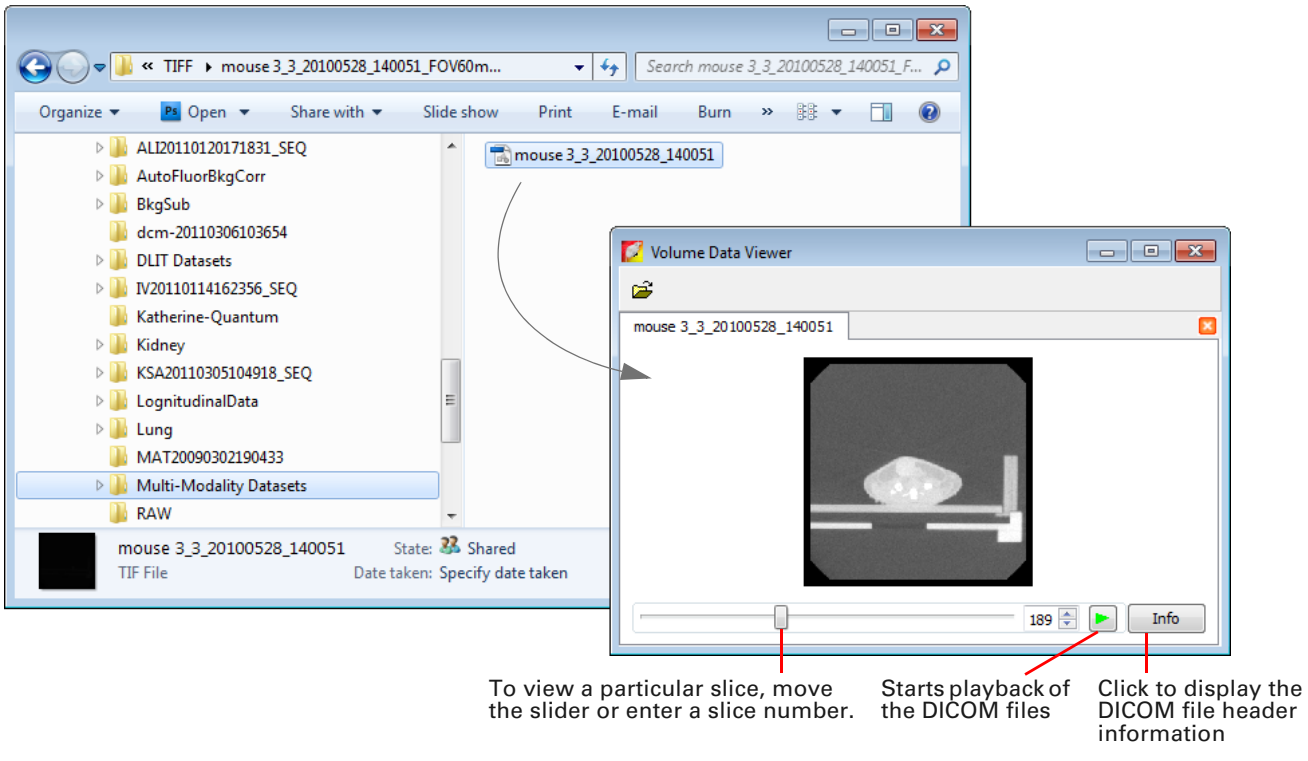
1. Select the results from the Name drop-down list.
2. Click **Delete**.
3. Click **Yes** in the confirmation message that appears.<$startrange>Tool Palette:3D Multi-Modality tools:Results

10.8 Volume Data Viewer

The Living Image software provides a viewer for volumetric data. The 3D Multi-Modality tools are not required to view DICOM or TIFF data.

1. Select **View** → **Volume Data Viewer** on the menu bar.
   The Volume Data Viewer appears.
2. Select volume data by doing either of the following:
   - Drag the data file (DICOM, TIFF) from Windows Explorer to the Volume Data Viewer window
     or
   - In the Volume Data Viewer, click the Open button, and in the dialog box that appears, select a DICOM or TIFF file, and click **Open**.
3. To clear the Volume Data Viewer, click the button.

Figure 10.24 Drag Volume Data from Windows Explorer to Volume Data Viewer

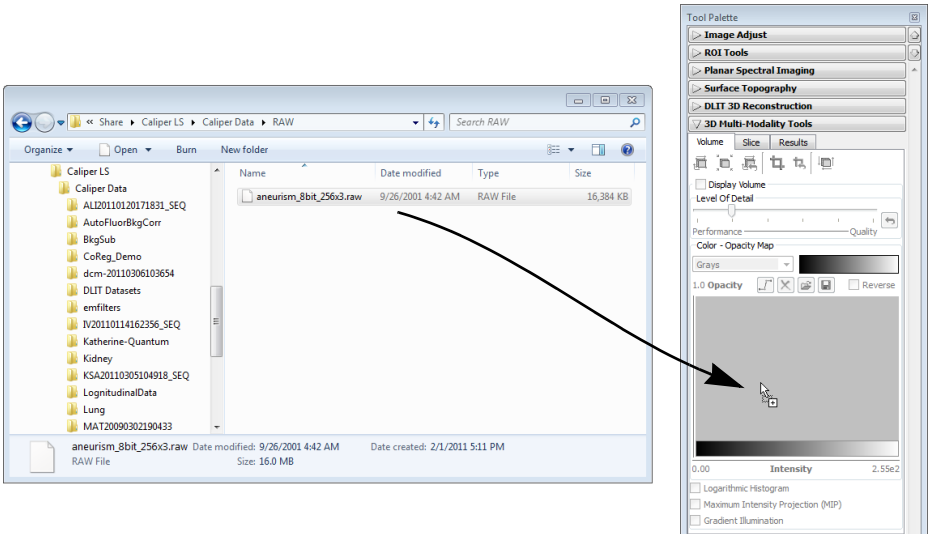


10.9 Viewing RAW Volumetric Data

1. Drag a single RAW file (*.raw or *.vox) from Windows Explorer to the 3D Multi-Modality tools (Figure 10.25).

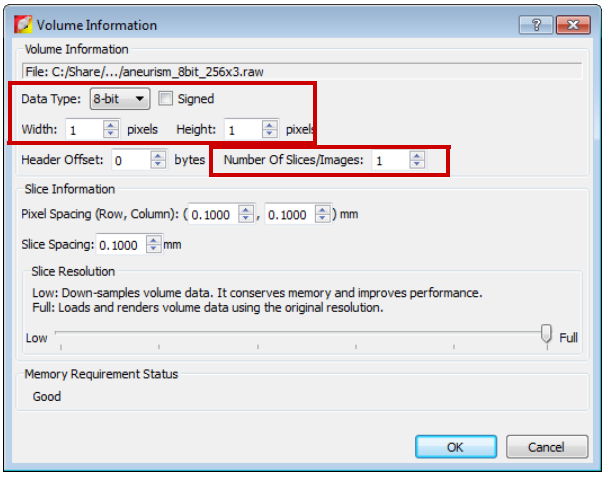
NOTE: Only single *.raw or *.vox files consisting of multiple slices of a 3D volume can be loaded into Living Image.

Figure 10.25 Opening RAW Volumetric Data



2. In the Volume Information dialog box that appears (Figure 10.26), enter the:
   - Data width, height, and the number of slices.
   - Slice row, column pixel size, and the slice spacing in millimeters.

Figure 10.26 Volume Information

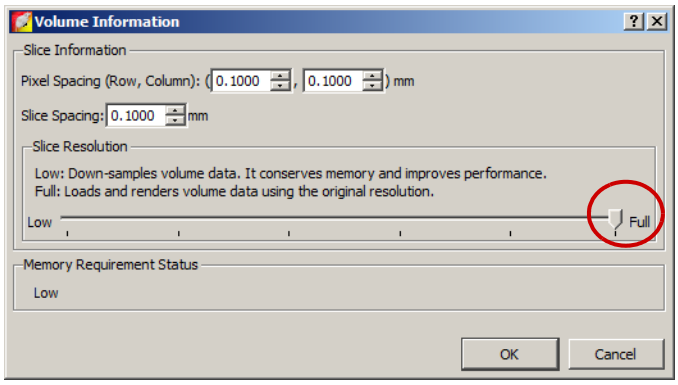


Select a data type.

Enter the:
- Width, height, and number of slices
- Slice row, column pixel spacing, and the slice spacing in millimeters

3. If loading the data will cause low memory, you are prompted to down-sample the data to improve memory and performance (Figure 10.27). Decrease the slice resolution by moving the Slice Resolution slider to the left until the Memory Requirement Status is “Good”.

Figure 10.27 Down-sample 3D Volumetric Data



Changing the Orientation of RAW Volumetric Data

Occasionally, RAW files (*.raw or *.vox) may be loaded with the orientation "flipped" or reversed along the x, y, or z-axis. As a result, the slice views (transaxial, coronal, sagittal) may be flipped or rotated so that the actual view that is displayed does not match the 3D View windowpane name (for example, the Sagittal windowpane does not display a sagittal slice), or the data appears flipped with respect to the surface derived from the IVIS® Spectrum.

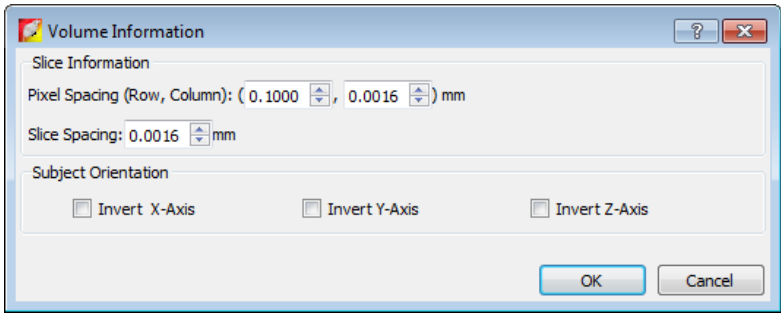
In such cases, you can:

- Invert the data along the x, y, or z-axis
- Manually rotate the data using the Transformation tool (see page 201 for more details).

To invert the subject orientation:

1. Click the Edit Spacing & Orientation button .
2. In the dialog box that appears, choose a "Subject Orientation" option and click **OK**.

Figure 10.28 Volume Information



11 Spectral Unmixing

11.1 About Spectral Unmixing

Living Image software applies spectral unmixing to distinguish the spectral signatures of different fluorescent or luminescent reporters and calculate the respective contribution of each on every pixel of an image. Use spectral unmixing to:

- Extract the signal of one or more fluorophores from the tissue autofluorescence. Images are acquired using epi-illumination (excitation light above the stage) or transillumination (excitation light below the stage).
- Analyze luminescent or fluorescent images when more than one reporter is used in the same animal model.

11.2 Acquire a Sequence for Spectral Unmixing

Set up an image sequence for spectral unmixing using the Imaging Wizard.

TIP: See the *Imaging Wizard* tech note for a quick guide on sequence acquisition (select **Help → Tech Notes** on the menu bar.

Choose an imaging mode in the wizard based on the type of probes.

Probe Type	Follow the Instructions for:
Luminescent	*Bioluminescence Imaging* on page 207
Fluorescent	*Fluorescence Imaging* on page 209
Radio-isotope	*Cherenkov Imaging* on page 212

If you are not using the Imaging Wizard to set up the image sequence, acquire a sequence using several filters which sample the emission or excitation spectra of all probes in the study at multiple points across the entire range. Include tissue autofluorescence for fluorescent spectral unmixing.

Make sure that the band gap between the excitation and emission filters is sufficiently large so that the excitation light does not leak through the emission filter where it can be detected by the CCD.

If a dataset includes multiple excitation and emission filter scans, the software automatically unmixes signal according to the filter type with the most entries. For example, a dataset acquired using three excitation filters and four emission filters will be unmixed by emission wavelength.

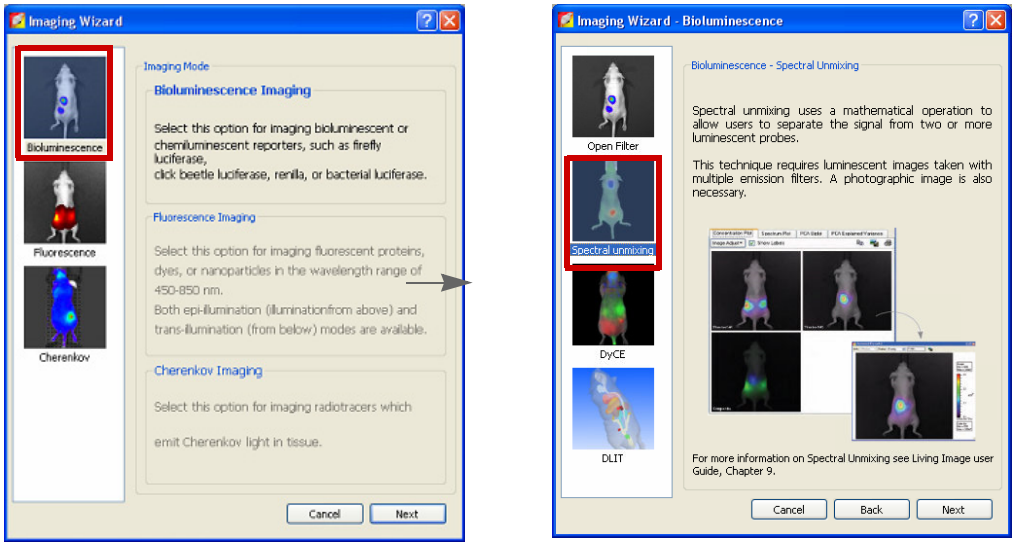
Bioluminescence Imaging

NOTE: The IVIS Spectrum should be initialized and the temperature locked before setting the imaging parameters. See page 19 for more details.

1. Start the Imaging Wizard. See page 44 for instructions.
2. Double-click the Bioluminescence option in the wizard. Double-click the Spectral Unmixing option in the next screen (Figure 11.1).

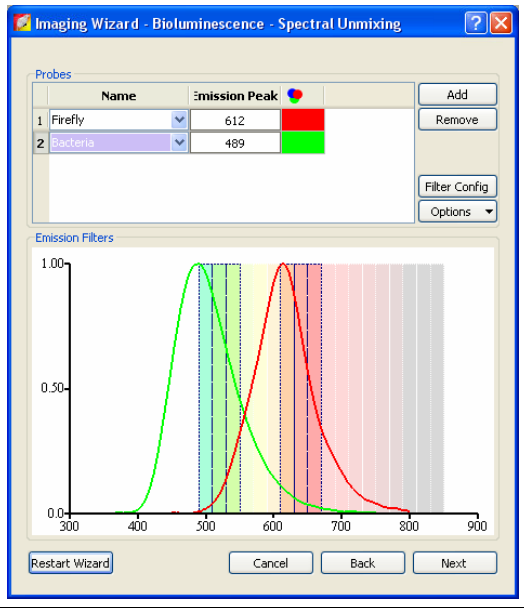
Figure 11.1 Imaging Wizard – Choose Bioluminescence and Spectral Unmixing Options

If this screen does not appear when the wizard starts, click **Restart Wizard** Restart Wizard (on the wizard screen) to restart the wizard.



3. Select a probe from the Name drop-down list (Figure 11.2).
4. Click **Add** and select another probe from the Name drop-down list. Repeat until all of the probes are added.

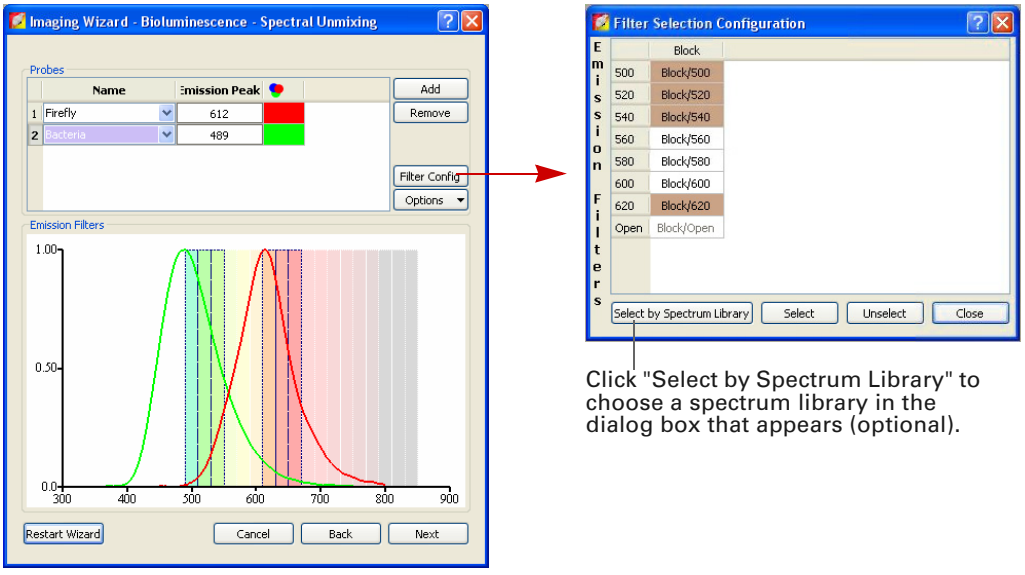
Figure 11.2 Select Probes



5. Optional: If you generated a spectrum library (a set of reference spectra), you can select it in the Imaging Wizard. Click **Filter Config**, then click **Select by Spectrum Library** in the dialog box that appears (Figure 11.3).

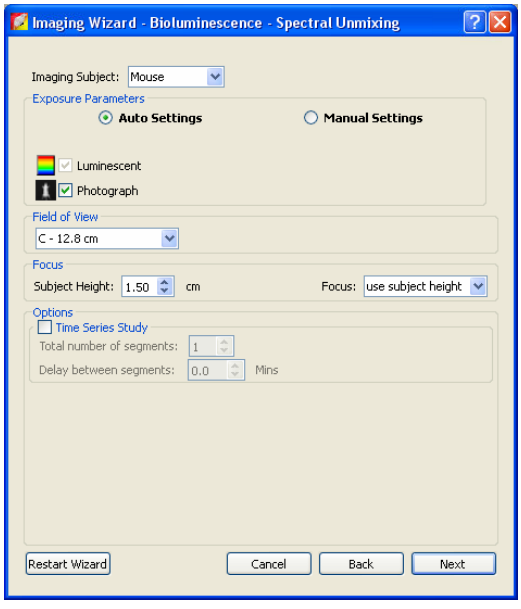
   See *Guided Method* on page 215 for instructions on creating a spectrum library.

Figure 11.3 Select a Spectrum Library (Optional)



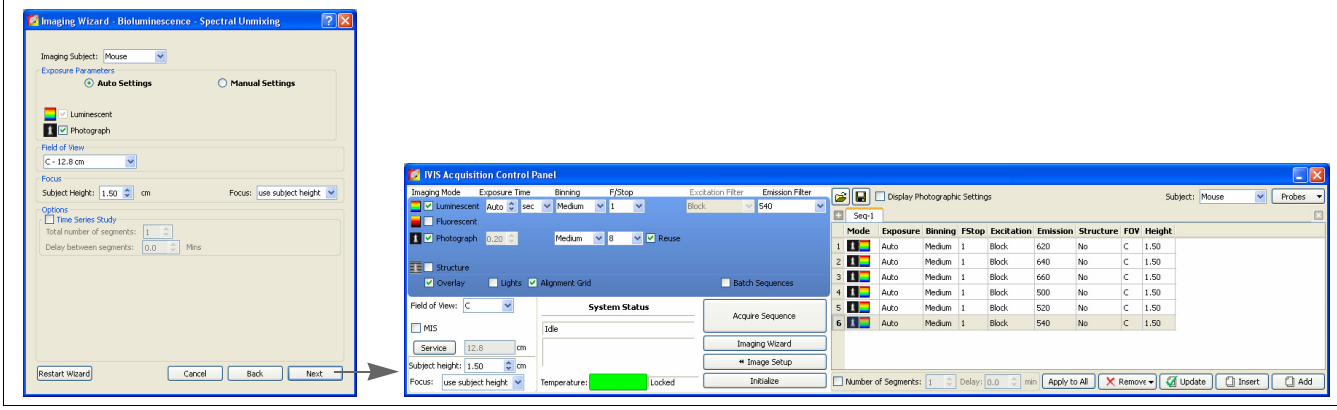
6. Click **Next** and in the screen that appears (Figure 11.4):
   - **a.** Select the type of subject.
   - **b.** Select a field of view.
   - **c.** Set the focus options.

Figure 11.4 Select Subject and Set Acquisition Parameters



7. To acquire a time series of images:
   a. Choose the Time Series Study option (Figure 11.4).
   b. Enter the number of segments and the delay between segments.
8. Click **Next**.
   The specified sequence appears in the sequence table (Figure 11.5).

Figure 11.5 Sequence Setup Complete



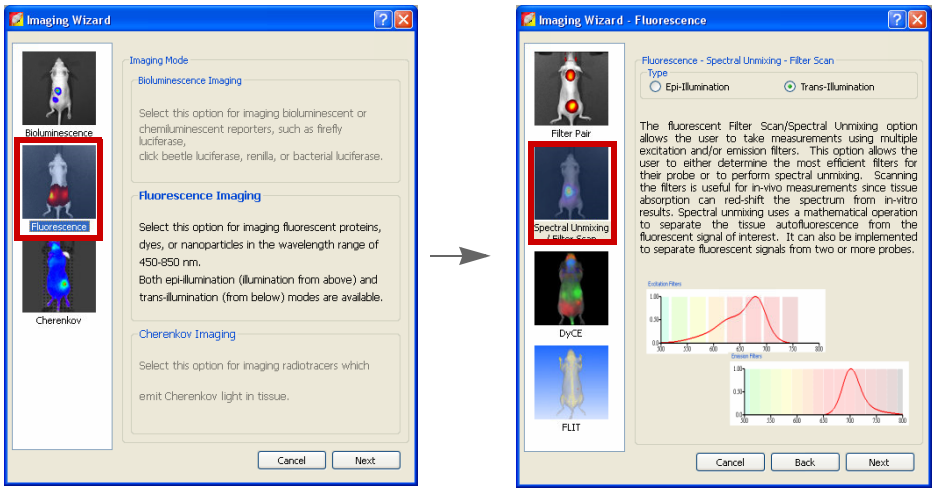
9. Acquire the sequence following the instructions on page 46.
   The image window appears when acquisition is completed (Figure 11.17). See Table 4.2 on page 30 for more details on the Image window.

Fluorescence Imaging

1. Start the Imaging Wizard. See page 44for instructions.
2. Double-click the Fluorescence option.
3. Select Spectral Unmixing in the next screen (Figure 11.6).
4. Select the type of illumination and click **Next**.
   - Epi-Illumination – Light source above the stage.
   - Trans-Illumination – Light source below the stage.

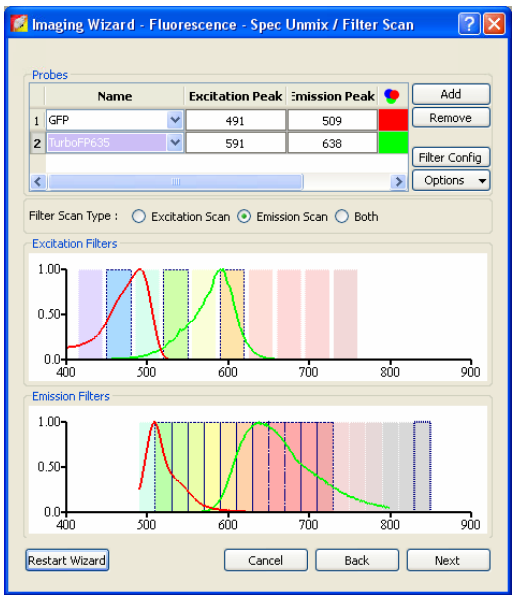
Figure 11.6 Imaging Wizard – Choose Fluorescence and Spectral Unmixing Options

If this screen does not appear when the wizard starts, click **Restart Wizard** Restart Wizard (on the wizard screen) to restart the wizard.



5. Select a probe from the Name drop-down list in the next screen (Figure 11.7).
6. Click **Add** and select another probe from the Name drop-down list. Repeat until all of the probes are added.

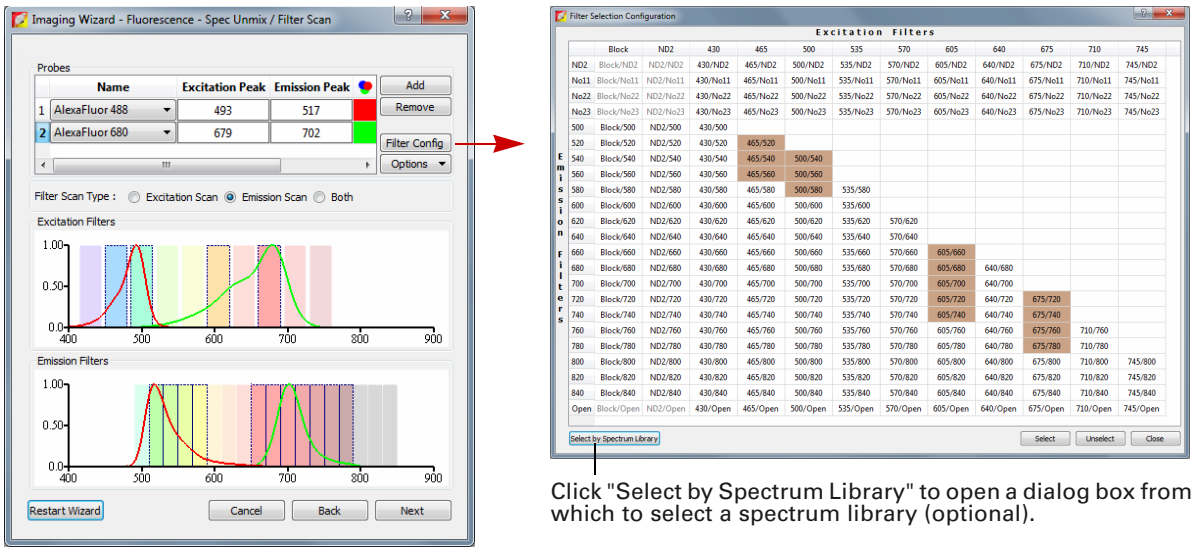
Figure 11.7 Select Probes



7. Optional: If a spectrum library (a set of reference spectra) is available, you can select it in the Imaging Wizard. Click **Filter Config**, then click **Select by Spectrum Library** in the dialog box that appears (Figure 11.8).

   See *Guided Method* on page 215 for instructions on creating a spectrum library.

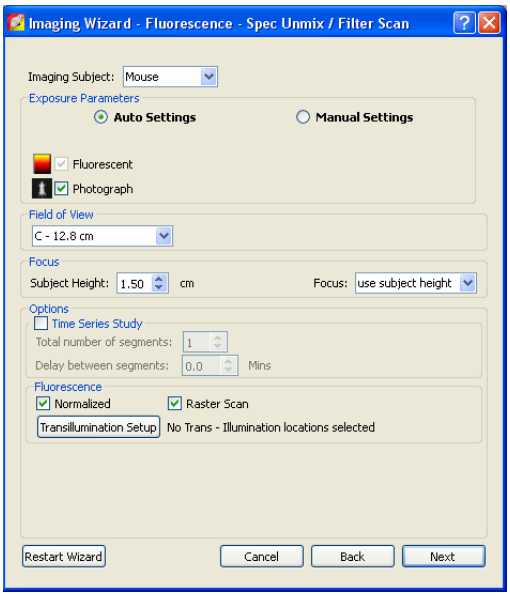
Figure 11.8 Select a Spectrum Library (optional)



Click "Select by Spectrum Library" to open a dialog box from which to select a spectrum library (optional).

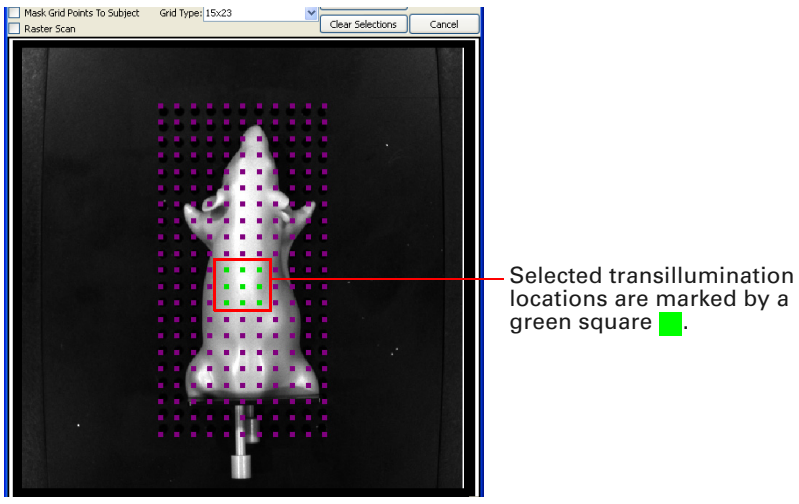
8. Click **Next** and in the screen that appears (Figure 11.9):
   a. Select the type of subject.
   b. Select a field of view.
   c. Set the focus options.

Figure 11.9 Select Subject and Set Acquisition Parameters



9. To acquire a time series of images:
   a. Choose the Time Series Study option (Figure 11.9).
   b. Enter the number of segments and the delay between segments.
10. If using transillumination, select the transillumination locations.
   a. Click **Transillumination Setup**.
   b. Choose the transillumination locations in the Transillumination Setup box that appears (Figure 11.10).
   See Table 4.3 on page 40 for more details on Transillumination Setup.

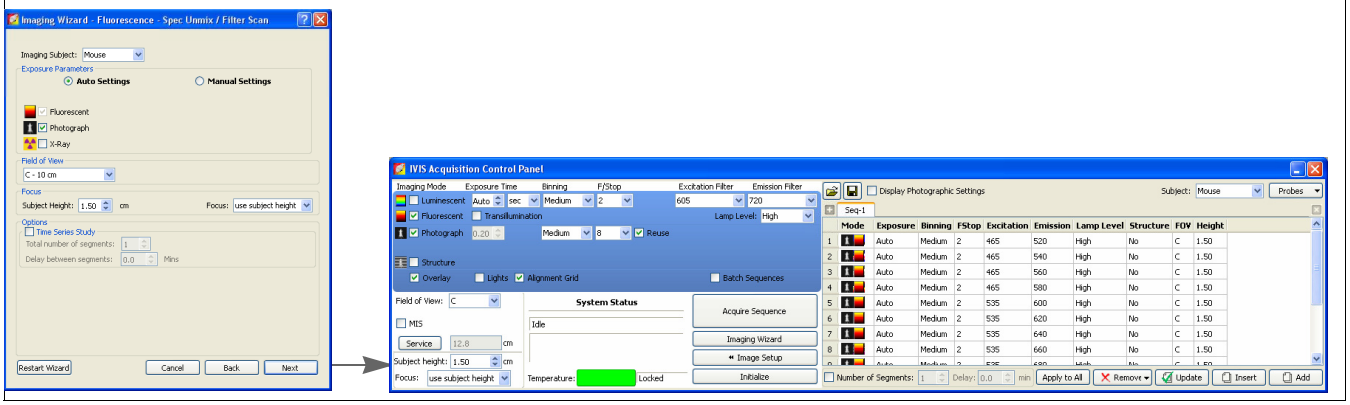
Figure 11.10 Transillumination Setup Box



11. Click **Next**.

The specified sequence appears in the sequence table (Figure 11.11).

Figure 11.11 Sequence Setup Complete



12. Acquire the sequence following the instructions on page 46.

The image window appears when acquisition is completed (Figure 11.17). See Table 4.2 on page 30 for more details on the Image window.

Cherenkov Imaging

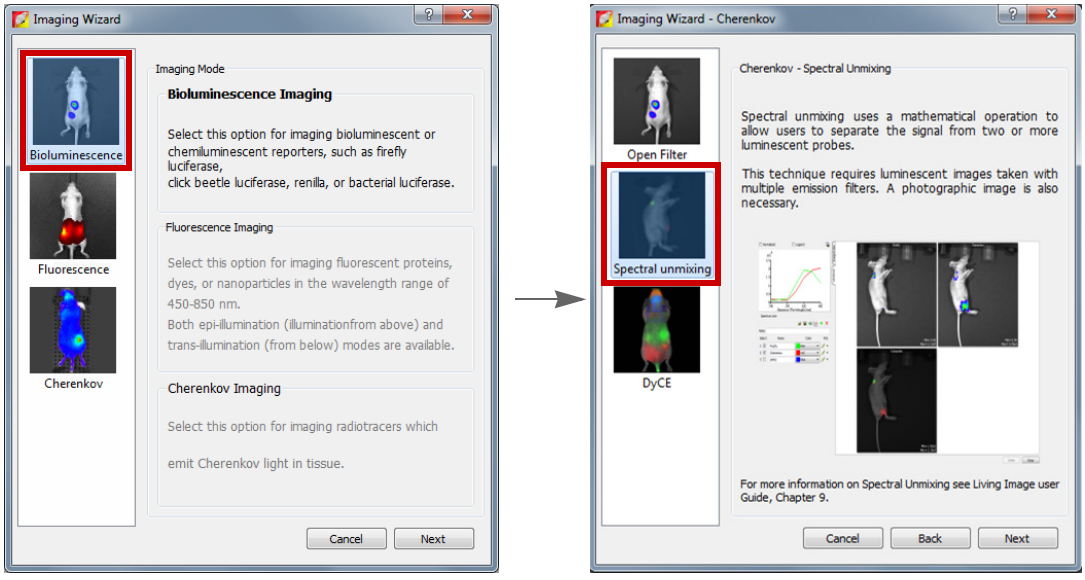
NOTE: The IVIS Spectrum should be initialized and the temperature locked before setting the imaging parameters. See page 19 for more details.

1. Start the Imaging Wizard. See page 44 for instructions.

2. Double-click the Cherenkov option. Double-click the Spectral Unmixing option in the next screen (Figure 11.12).

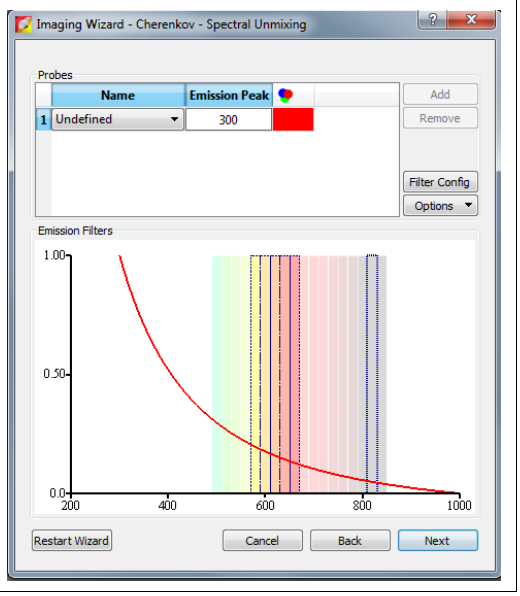
Figure 11.12 Imaging Wizard – Choose Cherenkov and Spectral Unmixing Options

If this screen does not appear when the wizard starts, click **Restart Wizard** Restart Wizard (on the wizard screen) to restart the wizard.



3. Select a probe from the Name drop-down list (Figure 11.13).
4. Click **Add** and select another probe from the Name drop-down list. Repeat until all of the probes are added.

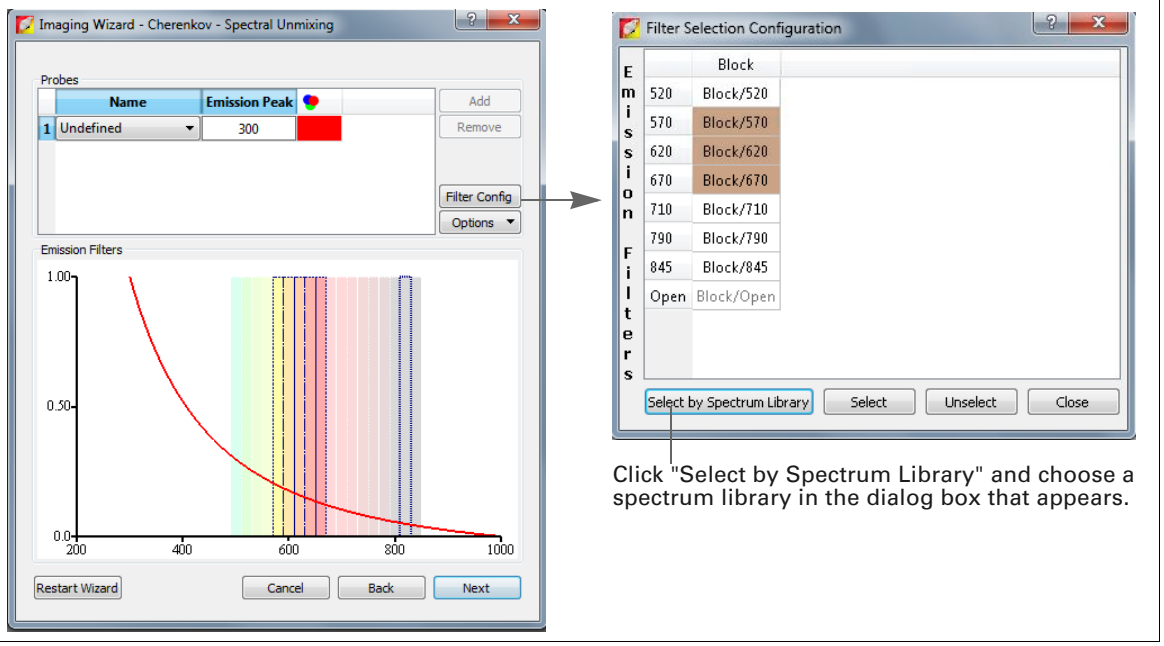
Figure 11.13 Select Probes



5. Optional: If you generated a spectrum library (a set of reference spectra), you can select it in the Imaging Wizard. Click **Filter Config**, then click **Select by Spectrum Library** in the dialog box that appears (Figure 11.14).

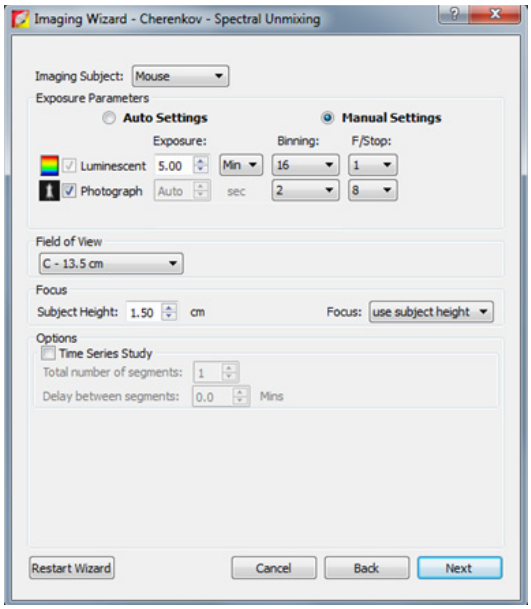
   See *Guided Method* on page 215 for instructions on creating a spectrum library.

Figure 11.14 Select a Spectrum Library (Optional)



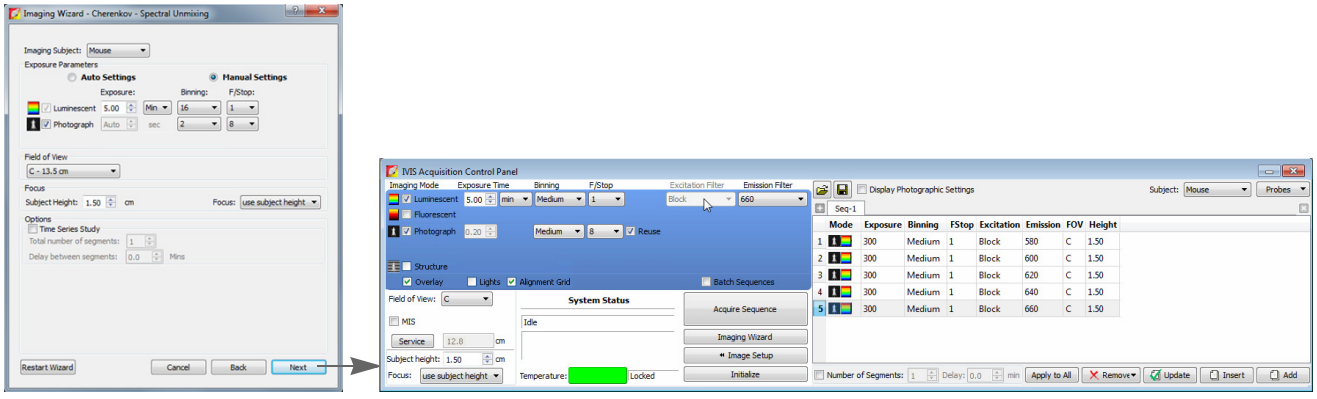
6. Click **Next** and in the screen that appears (Figure 11.15):
   c. Select the type of subject.
   d. Select a field of view.
   e. Set the focus options.

Figure 11.15 Select Subject and Set Acquisition Parameters



7. To acquire a time series of images:
   a. Choose the Time Series Study option (Figure 11.15).
   b. Enter the number of segments and the delay between segments.
8. Click **Next**.
   The specified sequence appears in the sequence table (Figure 11.16).

Figure 11.16 Sequence Setup Complete



9. Acquire the sequence following the instructions on page 46.
   The image window appears when acquisition is completed (Figure 11.17). See Table 4.2 on page 30 for more details on the Image window.

11.3 Spectral Unmixing Methods

Living Image software provides four spectral unmixing methods (Table 11.1).

Table 11.1 Spectral Unmixing Methods

Method	Description	See Page
Guided	Use this method when: ▪ Probe locations are known. ▪ Probe signals are mixed with background signal, but not other probe signals. **Note:** This method is not recommended if probe signals are overlapping. Use this method to generate a spectrum library (a set of reference spectra) for probes with known spectra and known locations.	215
Library	This method requires a user-generated spectrum library. The library method identifies pixels in the data with spectral characteristics that match the spectrum library. **Note:** The data being analyzed must be acquired using the same, or a subset of, the excitation/emission filter pairs of the spectrum library. The probe depth in the data being analyzed and the spectrum library dataset should be similar for optimum analysis results. For example, do not use a spectrum library generated from *in vivo* data to analyze *in vitro* data and vice versa.	218
Automatic	Use this method when: ▪ Probe locations are unknown. ▪ Probes are included in the spectrum library.	220
Manual	Use this method to: ▪ Unmix and create libraries for probe signals that overlap. ▪ Perform a manual analysis after an automatic analysis, if necessary, to identify additional probe locations. ▪ Unmix tissue autofluorescence. ▪ Generate a spectrum library.	223

Guided Method

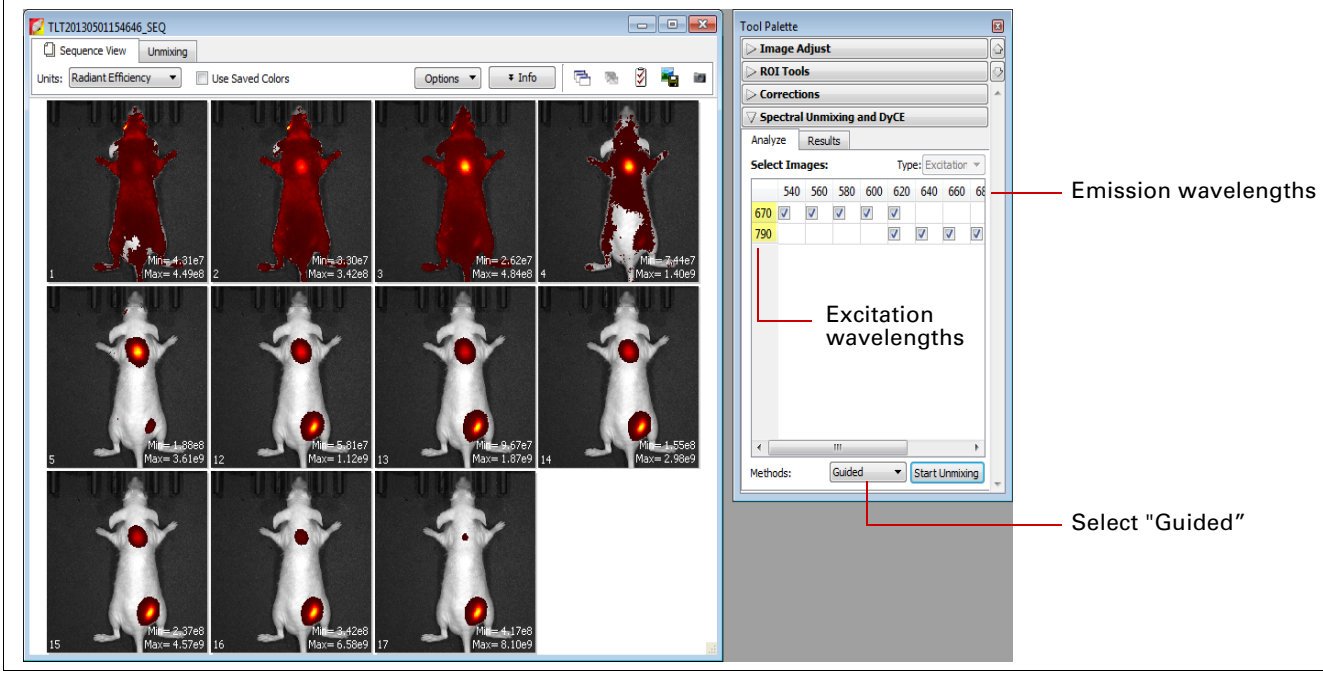
Use the guided method:

- When the probe locations are known and probe signals do not overlap.
- To generate a spectrum library for probes with known spectra and known locations

1. Load the image sequence.

   The fluorophores are Alexa Fluor 680 and Alexa Fluor 750 in Figure 11.17. Images were acquired using 680 and 790 nm excitation filters, and emission filters from 540 to 720 nm in 20 nm increments.

Figure 11.17 Sequence for Spectral Unmixing



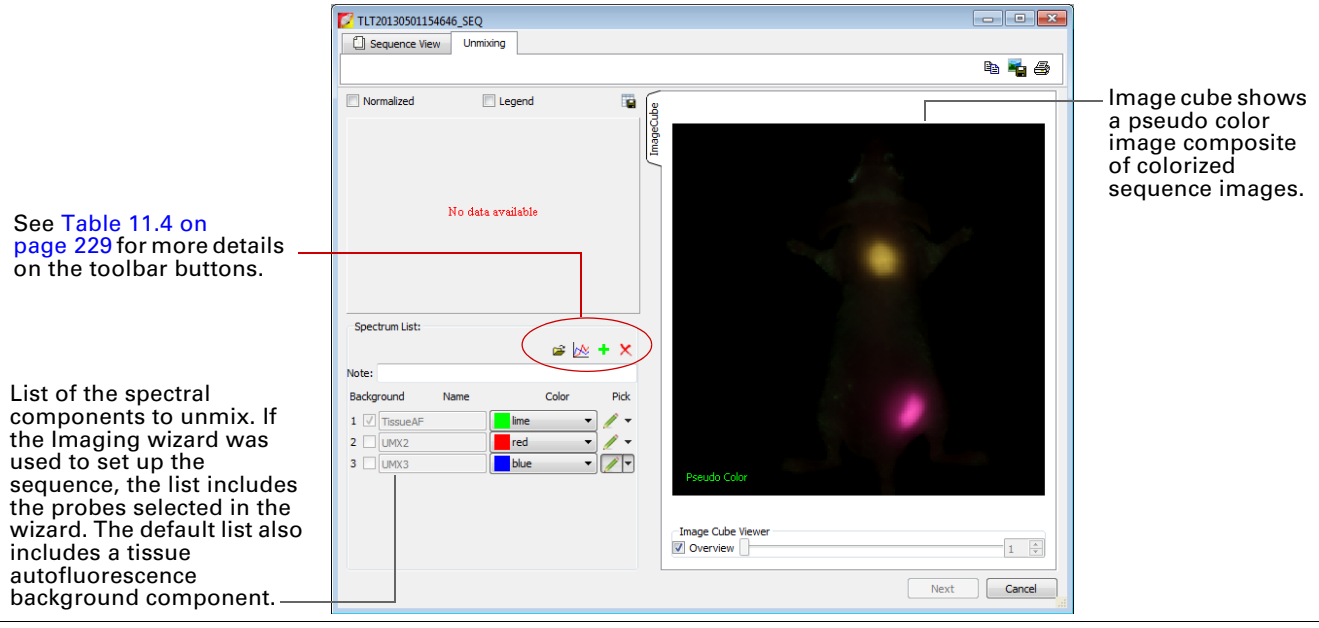
2. Click the Analyze tab of the Spectral Unmixing and DyCE tools.

   By default, all wavelengths are included in the analysis. Remove the check mark next to wavelengths that you want to exclude from the analysis.

3. Select "Guided" from the Methods drop-down list and click **Start Unmixing**.

   The Unmixing window appears (Figure 11.18).

Figure 11.18 Unmixing Window



The image cube represents a “stack” of the sequence images (sorted according to the spectral axis). When the Overview option is selected, the image cube shows a pseudo color image that is a composite of the stack images which have been colorized to encode spectral information.

The entire image cube is calibrated and visualized on the same scale. To view a particular image, remove the check mark next to the Overview option and move the slider or enter an image number.

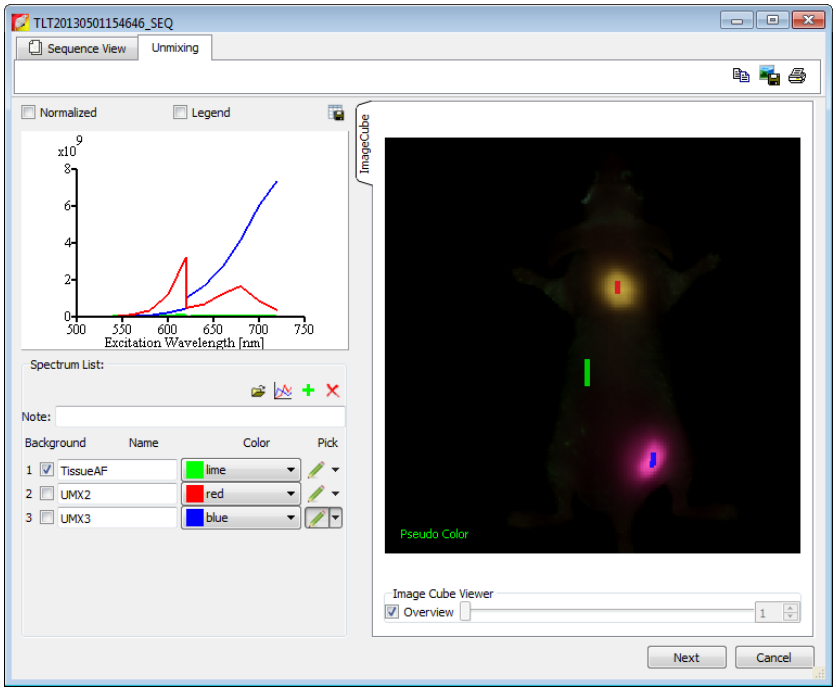
NOTE: In the Guided method, the Tissue AF component is preset as background. After you define the Tissue AF component (mark a region of tissue autofluorescence only on the image cube), the spectra of the other components that you mark on the image cube will be background-subtracted, not raw spectra from the data.

4. Move the mouse pointer over the image cube to see the spectrum at a particular location.
   The raw spectrum at the pointer location is updated as you move the pointer.
5. To specify a probe location for unmixing:
   a. Click the button for a spectrum.
   b. Using the mouse, draw a mark on an area of the image cube which represents the probe signal.
      The software plots a background-subtracted spectrum of the signal (Figure 11.19).

NOTE: For "Tissue AF", draw a mark on an area of the image cube where no probe signal is present.

   c. If necessary, right-click the image cube to erase the mark.
6. Repeat step step 5 to specify other probe locations.

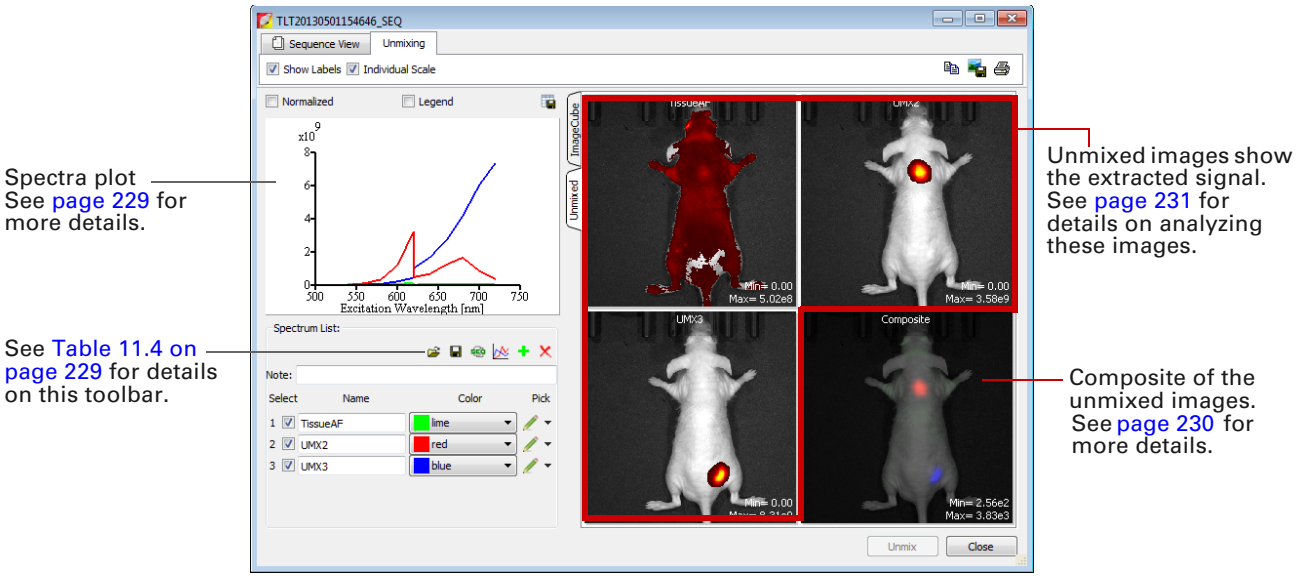
Figure 11.19 Mark Probe Locations for Unmixing on the Image Cube



7. Click **Next** after you finish marking the probe locations.
   The Unmixing window shows the analysis results which include unmixed spectra corrected for tissue autofluorescence, unmixed images, and a composite of the unmixed images (Figure 11.20).
   See *Spectral Unmixing Results* on page 228 for information about the results.

8. To save the results as a spectrum library:
   a. Click the button in the Spectrum List toolbar (Figure 11.20).
   b. Enter a file name in the dialog box that appears and click **Save**.

Figure 11.20 Spectral Unmixing Results



Library Method

The library method uses a user-generated spectrum library to analyze a dataset. If you plan to analyze data by this method, the data must be acquired using the same, or a subset of, the excitation/emission filter pairs of the spectrum library.

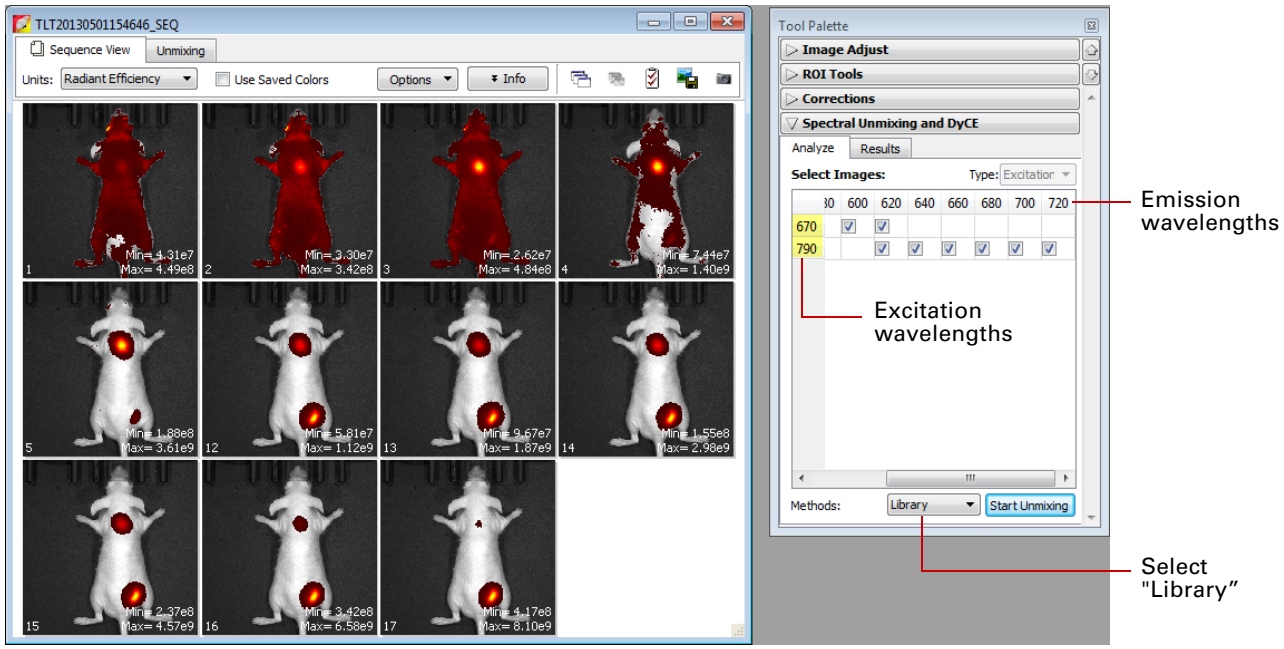
The probe depth in the dataset being analyzed and the spectrum library dataset should be similar for optimum analysis results. For example, do not use a spectrum library generated from in vivo data to analyze in vitro data.

NOTE: Use the guided or manual method to generate a spectrum library of known probes with known locations (see page 215 for guided method or page 223 for manual method).

1. Load the image sequence.

   In Figure 11.21, the fluorophores are Alexa Fluor 680 and Alexa Fluor 750. Images were acquired using 670 and 790 nm excitation filters, and emission filters from 540 to 720 nm in 20 nm increments.

Figure 11.21 Sequence for Spectral Unmixing



2. Click the Analyze tab of the Spectral Unmixing and DyCE tools.

   By default, all wavelengths are included in the analysis. Remove the check mark next to wavelengths that you want to exclude from the analysis.

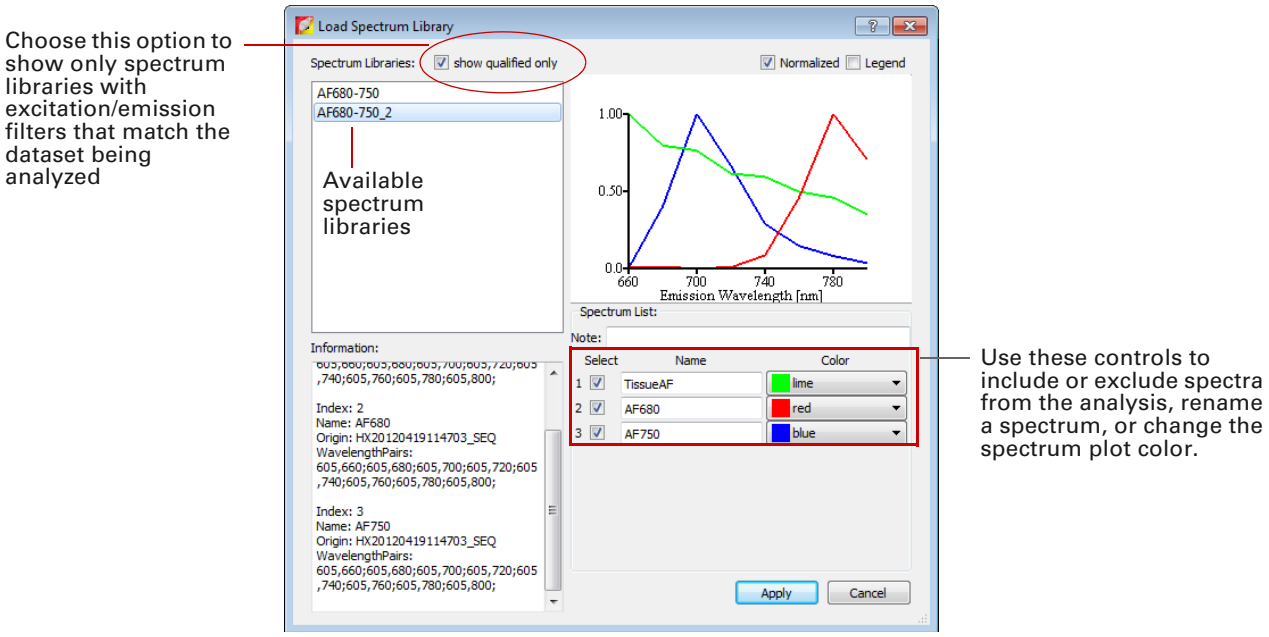
3. Select "Library" from the Methods drop-down list and click **Start Unmixing**.

4. Select a reference spectral library in the dialog box that appears and click **Apply** (Figure 11.22).

   The software identifies pixels with spectral characteristics that match the spectrum library. The Unmixing window shows the analysis results which include unmixed spectra, unmixed images, and a composite of the unmixed images (Figure 11.20 on page 218).

   See *Spectral Unmixing Results* on page 228 for information about the results.

Figure 11.22 Select a Reference Spectral Library



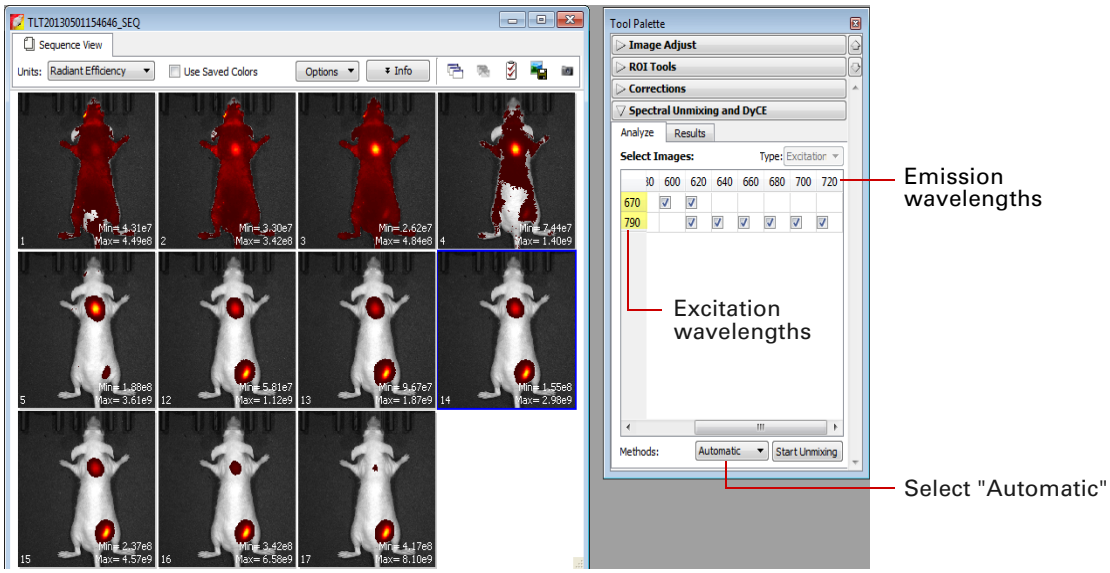
Automatic Method

Use the automatic method to analyze data when the probe locations are unknown and the probe is included in the spectrum library.

1. Load the image sequence.

   In Figure 11.23, the fluorophores are Alexa Fluor 680 and Alexa Fluor 750. Images were acquired using 670 and 790 nm excitation filters, and emission filters from 540 to 720 nm in 20 nm increments.

Figure 11.23 Sequence for Spectral Unmixing



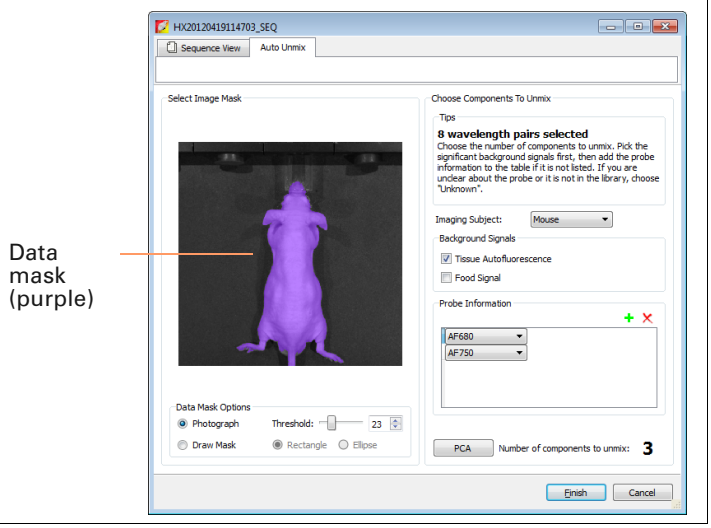
2. Click the Analyze tab of the Spectral Unmixing and DyCE tools.

   By default, all wavelengths are included in the analysis. Remove the check mark next to wavelengths that you want to exclude from the analysis.

3. Select "Automatic" from the Methods drop-down list and click **Start Unmixing**.

   The Auto Unmix window appears (Figure 11.24). The purple data mask shows the data that will be included in the analysis (the entire subject is included by default).

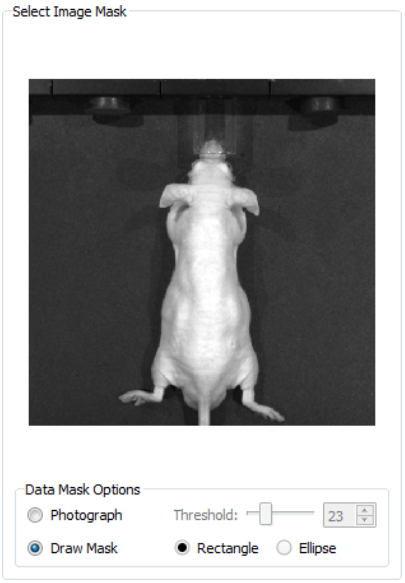
Figure 11.24 Auto Unmix Window



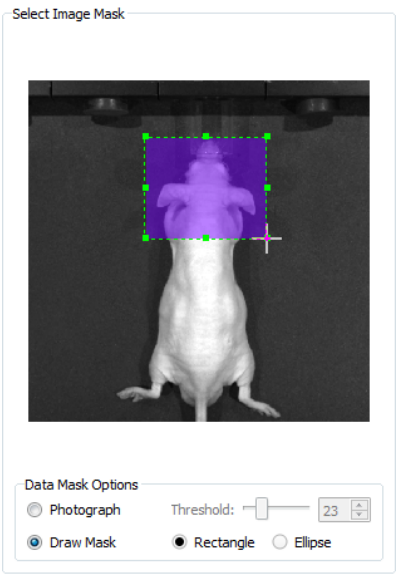
4. If you do not want to analyze the entire subject, draw a mask on a particular area (Figure 11.25). For example, it is useful to mask shaved or depilated areas.

Figure 11.25 Draw a Data Mask

See Table 11.2 on page 221 for more details on the data mask options.



Select the Data Mask option, and the Rectangle or Ellipse option.



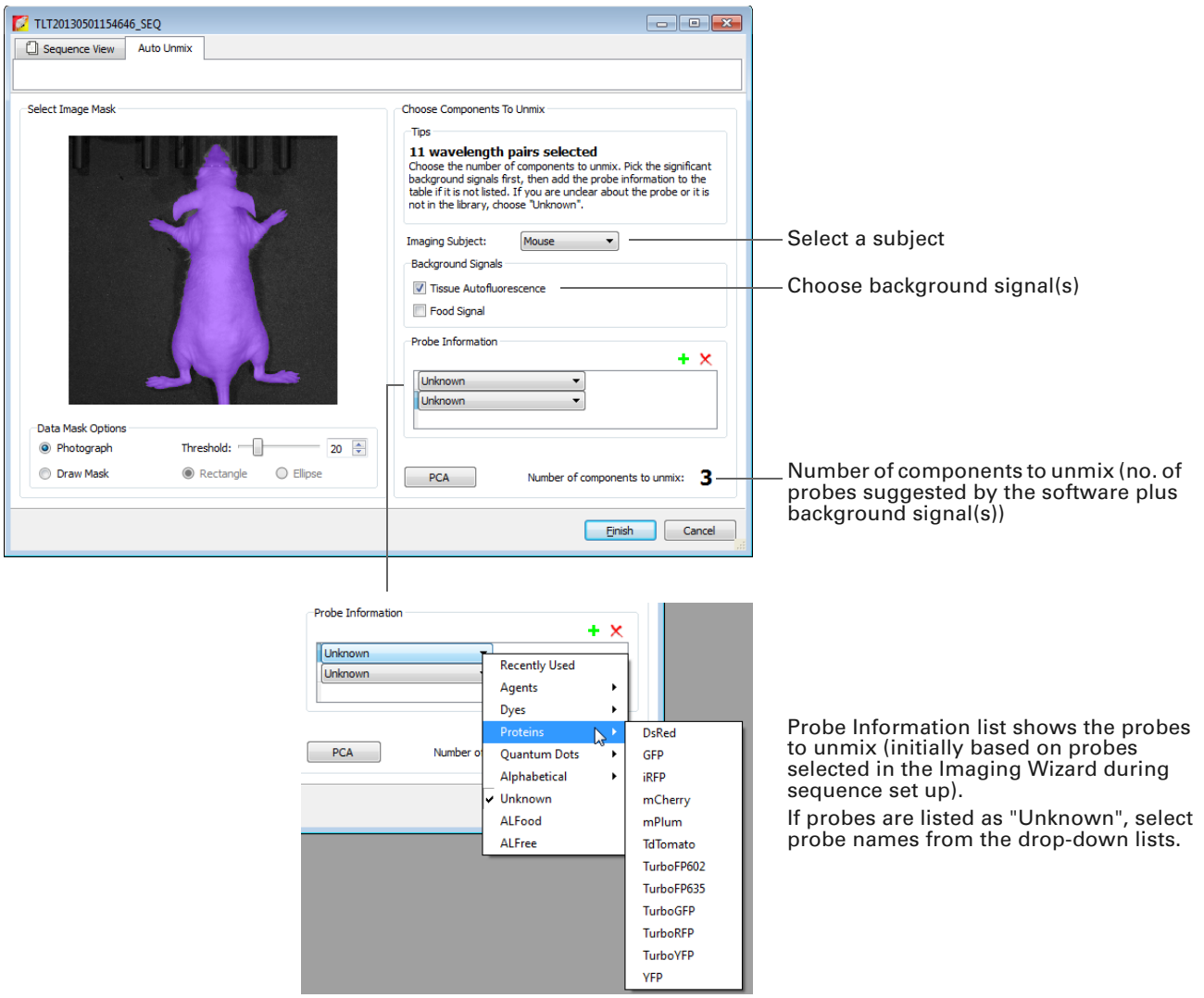
Draw a mask on a particular area using the mouse.

Table 11.2 Data Mask Options

Option	Description
Photograph	If this option is chosen, the software automatically draws the data mask so that it includes the entire photograph.
Threshold	If necessary use the threshold slider or arrows to adjust the mask so that it matches the underlying subject photograph as closely as possible without including any area outside the subject image.
Draw Mask	Choose this option to manually draw a data mask on an area of the photograph.
Rectangle	Specifies a rectangular shape for the manual data mask.
Ellipse	Specifies an elliptical shape for the manual data mask.

5. Choose an imaging subject and background signal(s) (Figure 11.26).

Figure 11.26 Auto Unmix Window

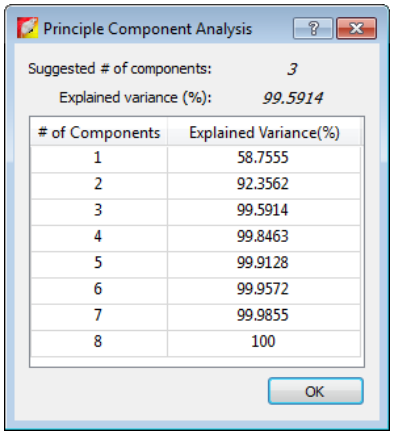


6. Click the **PCA** button.

   The Principle Component Analysis window shows the amount of signal explained by the suggested components (Figure 11.27). The three components in this example (tissue autofluorescence, probe AF680, and probe AF750) explain more than 99.5% of the signal. The small residual is due to noise.

   If the explained variance is low, add more components (probes) to unmix using the + button.

Figure 11.27 Principle Component Analysis



7. Click **Finish**.

   The Unmixing window shows the analysis results which include unmixed spectra, unmixed images, and a composite of the unmixed images (Figure 11.20 on page 218).

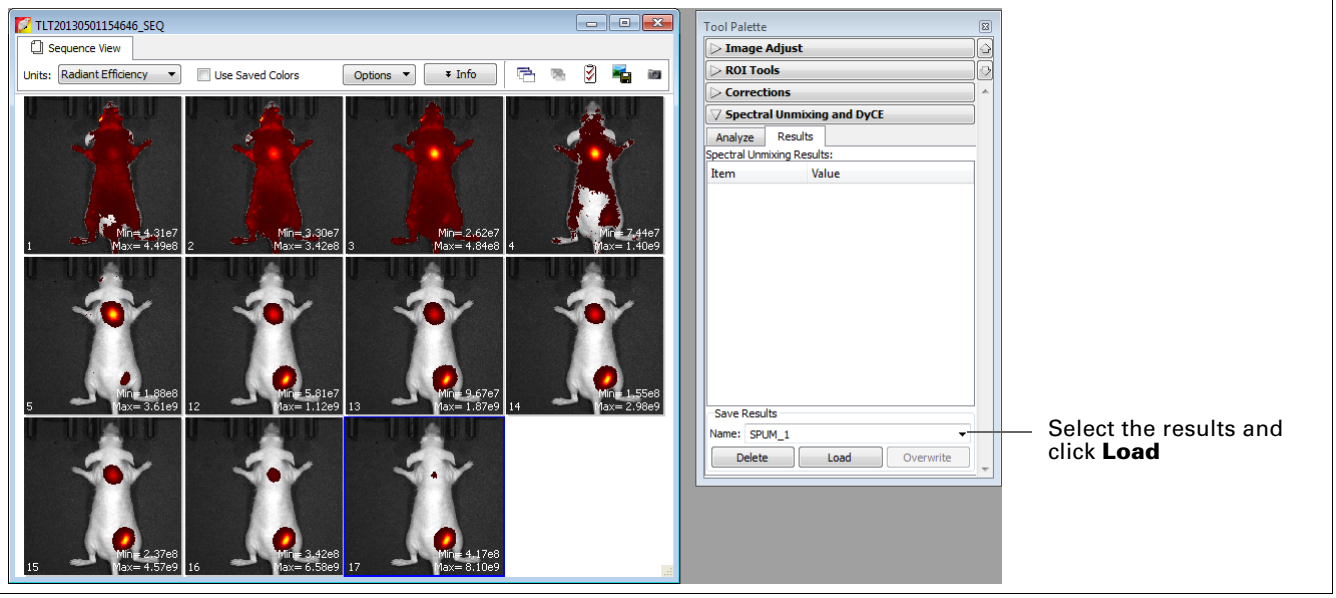
   See *Spectral Unmixing Results* on page 228 for information about the results.

Manual Method

Sometimes you may want to manually analyze results, for example, if the explained variance of the principle component analysis of an automatic analysis seems low or if the probe signals overlap. The example in this section shows how to manually analyze results from a previous analysis.

1. Open the image sequence.
2. Select the results and click **Load**.

Figure 11.28 Open a Sequence and Select Results to Load

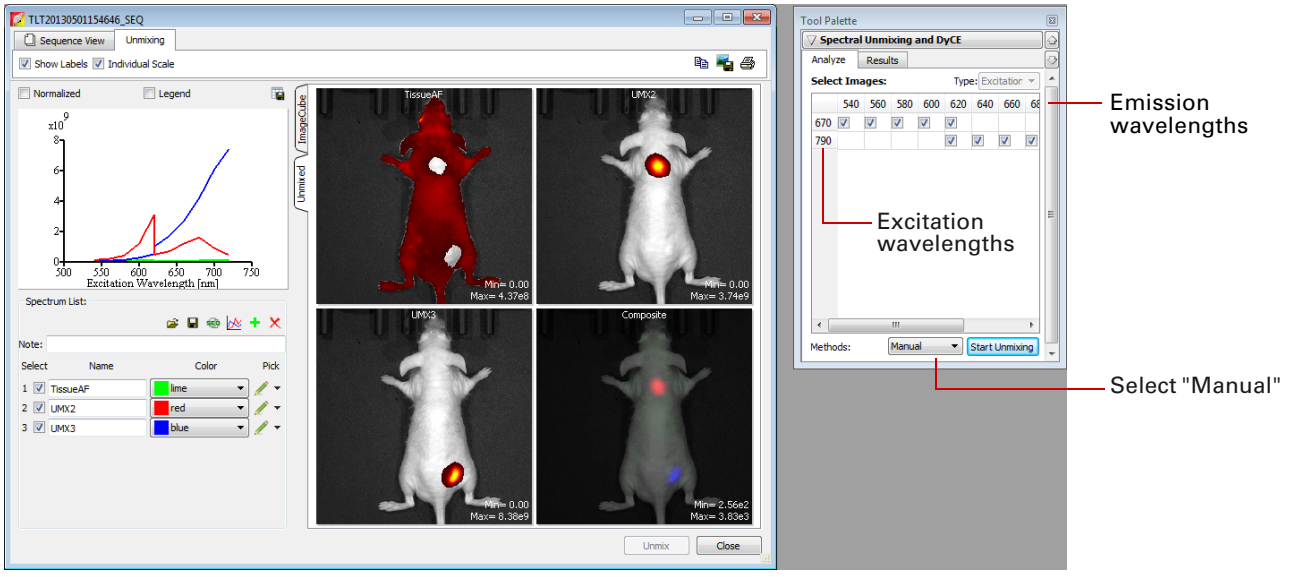


3. Click the Analyze tab of the Spectral Unmixing and DyCE tools.

All wavelengths are selected by default. Remove the check mark next to wavelengths that you want to exclude from the analysis.

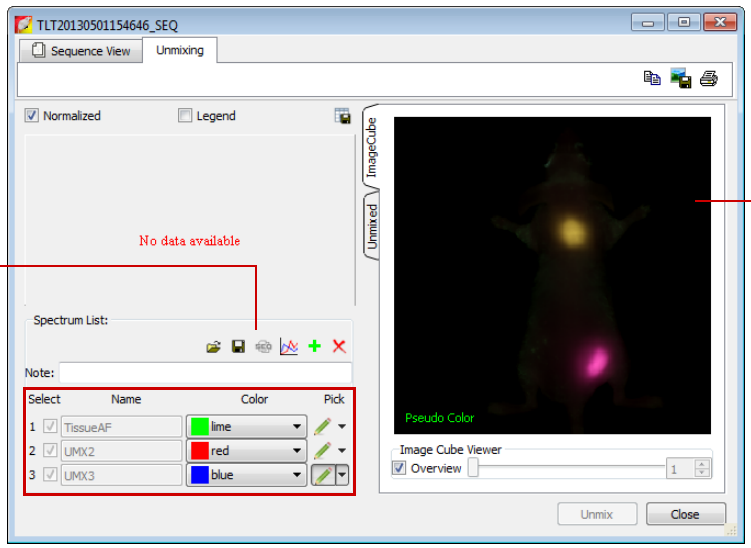
In Figure 11.29, the fluorophores are Alexa Fluor 680 and Alexa Fluor 750. Images were acquired using 670 and 790 nm excitation filters, and emission filters from 540 to 720 nm in 20 nm increments.

Figure 11.29 Unmixing Window – Results Loaded



4. Select "Manual" from the Methods drop-down list and click **Start Unmixing**.
   The Unmixing window appears (Figure 11.30).

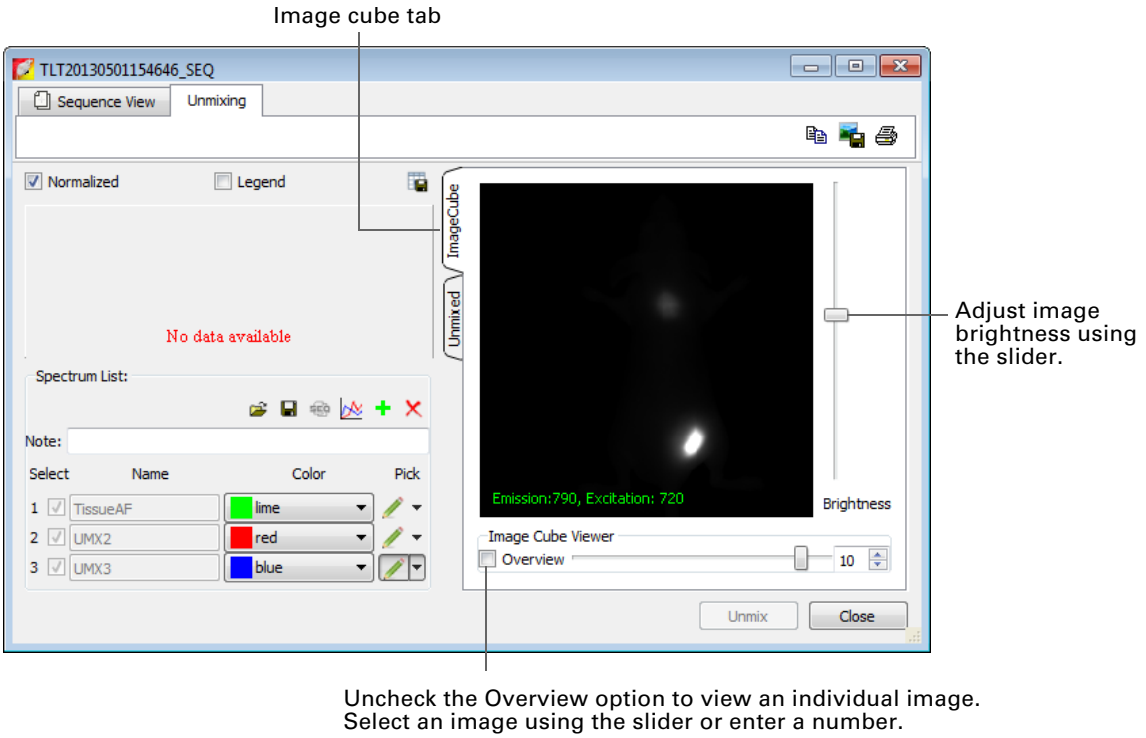
Figure 11.30 Unmixing Window



The image cube represents a “stack” of the sequence images (sorted according to the spectral axis). When the Overview option is selected, the image cube shows a pseudo color image that is a composite of the stack images which have been colorized to encode spectral information.

The entire image cube is calibrated and visualized on the same scale. To view a particular image, remove the check mark next to the Overview option and move the slider or enter an image number (Figure 11.31).

Figure 11.31 Image Cube – Single Image Mode



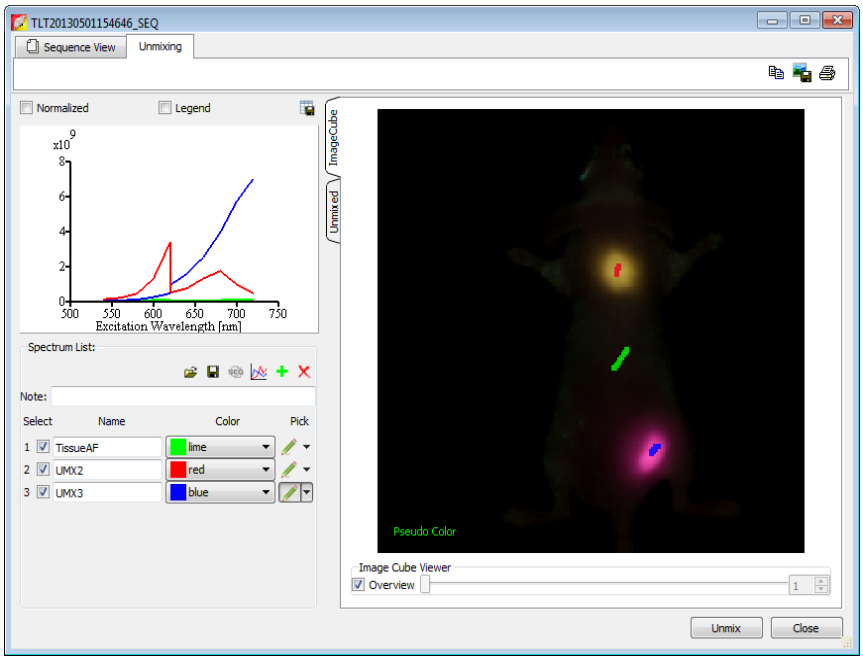
5. Move the mouse pointer over the image cube to see the spectrum at a particular location.
   The spectrum at the pointer location is updated as you move the pointer.
6. To specify a probe location for unmixing:
   a. Click the button for a spectrum.
   b. Using the mouse, draw a mark on an area of the image cube which represents the probe location.
      The software plots a normalized spectrum of the signal (Figure 11.32).

   NOTE: Mark a region of tissue autofluorescence only (where no probe signal is present) on the image cube for the Tissue AF component. The spectra of components that you mark on the image cube are raw spectra from the data when using the manual method.

   c. If necessary, right-click the image cube to erase the mark.
7. Repeat step step 6 to specify other probe locations.
8. Manually subtract autofluorescence background. See *Correcting Spectra* on page 226 for instructions.

Figure 11.32 Mark Probe Locations for Unmixing on the Image Cube

Raw spectra at the probe locations marked on the image cube



9. Click **Unmix** after you finish marking the probe locations and correct spectra for tissue autofluorescence.

   The Unmixing window shows the analysis results which include unmixed spectra, unmixed images, and a composite of the unmixed images (Figure 11.20 on page 218).

   See *Spectral Unmixing Results* on page 228 for information about the results.

11.4 Correcting Spectra

Spectra can be corrected for overlapping signal by subtracting one spectrum from another.

1. Click the  button in the Unmix window.
2. Choose the spectra to subtract in the dialog box that appears. (Figure 11.33).
3. Click **Apply** to add the computed spectrum to the spectrum plot and list in the Unmixing window. Alternatively, select an existing spectrum from the Name drop-down list and click **Apply** to overwrite the results.

Figure 11.33 Choose Spectra to Subtract: A - x*B = C

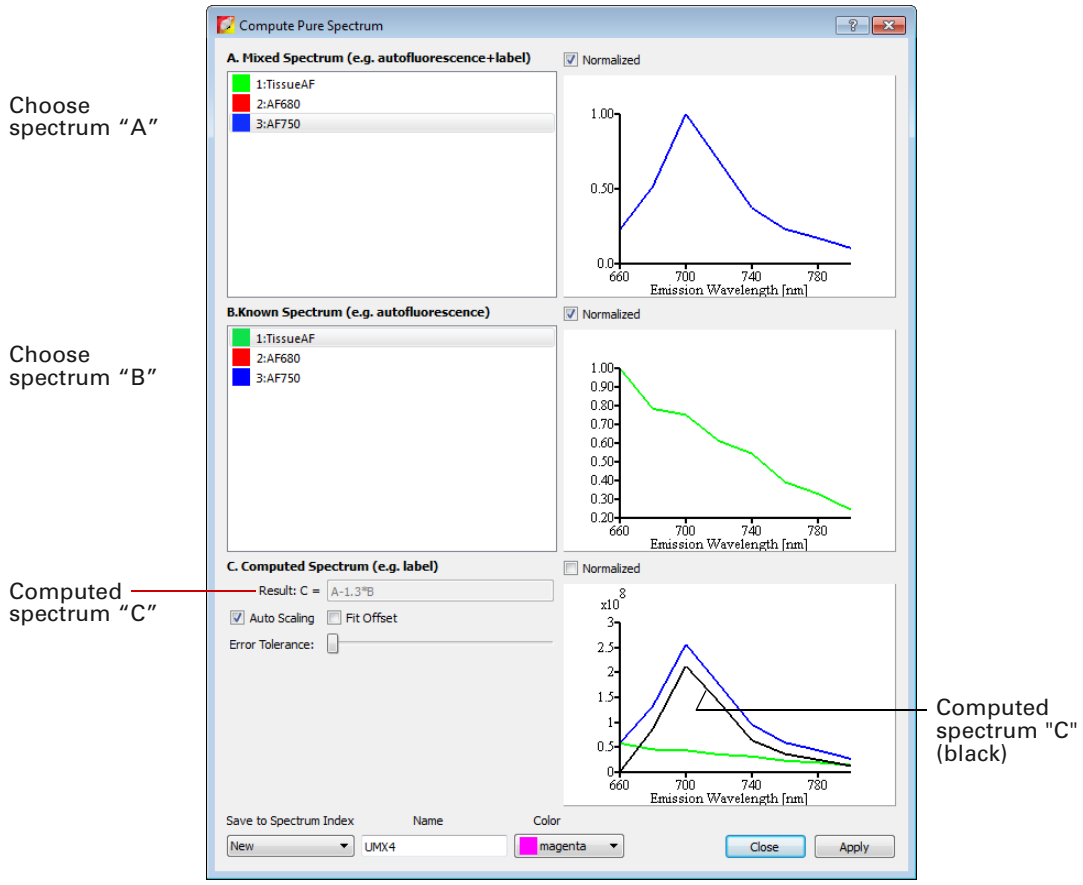
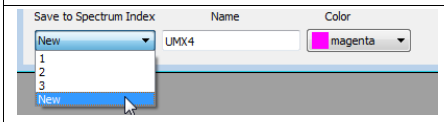


Table 11.3 Computed Spectrum

Item	Description
Normalized	Choose this option to display spectra normalized on a scale from zero to one.
Result: C = A - x*B	The subtraction performed by the software where "x" is a factor that ensures the residual signal is positive.
Autoscaling	Choose this option to display computed results on a normalized scale starting a zero.
Fit Offset	If this option is chosen, the software computes and removes an intensity baseline from the spectra.
Error Tolerance	The software computes a default error tolerance (the factor "x" for A - x*B) such that signal B is maximally removed from signal A with no negative result. Moving the slider adjusts the error tolerance and automatically updates the computed spectrum.
	Choose "New" to save computed spectrum with the specified name and color. Click **Apply** to add the computed spectrum to the spectrum plot and list in the Unmixing window. Choose a spectrum number from the drop-down list to overwrite that spectrum with the computed spectrum when you click **Apply**.

11.5 Spectral Unmixing Results

The results include a signal distribution map of each unmixed result and a composite image of all signals, each signal displayed in a different color. Remove the check mark next to "Individual Scale" to view a signal color scale (Figure 11.35).

Figure 11.34 Spectral Unmixing Results

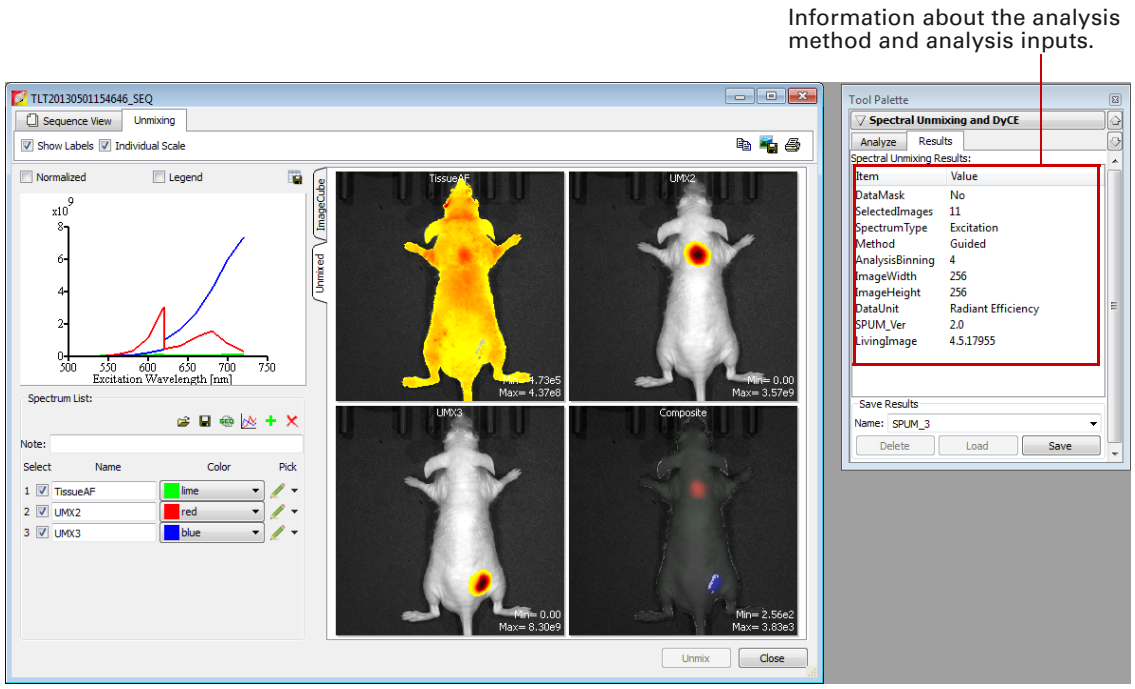
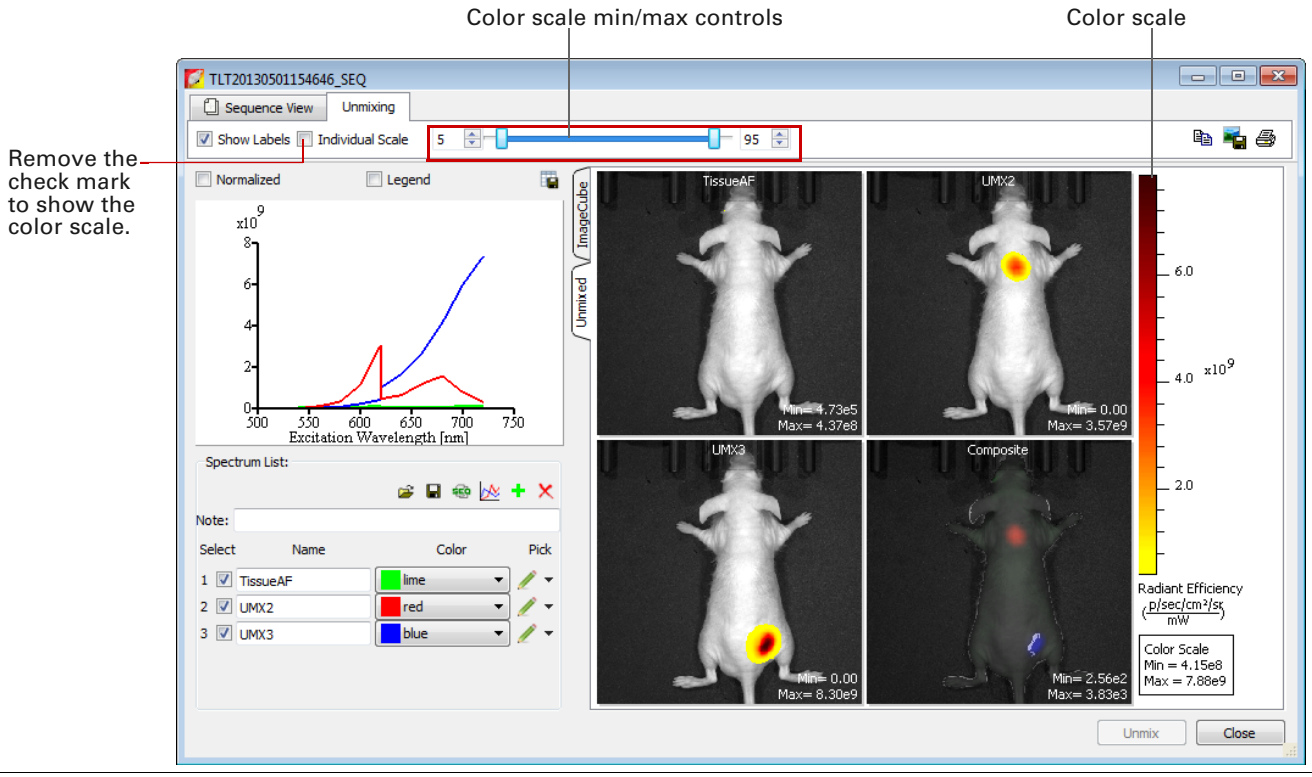


Figure 11.35 Spectral Unmixing Results With Color Scale



Spectra Plot

Spectra plots show the unmixed spectra.

Figure 11.36 Spectra Plots

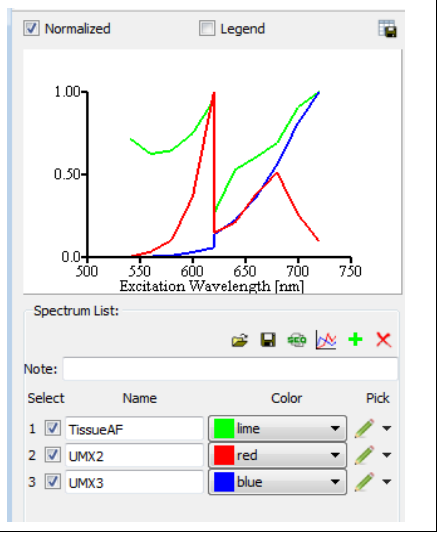


Table 11.4 Spectra Window

Item	Description
Normalized	Choose this option to display signals normalized on a scale from zero to one.
Legend	Choose this option to display a key for the spectra plot.
	Opens a dialog box that enables you to export the spectra plot data to a .csv file.
	Opens a dialog box that enables you to select and load a spectrum library.
	Opens a dialog box that enables you so save spectral unmixing results as a reference spectrum library for use with the "library" method of spectral unmixing. See page 218 for more details on the library method. **Note:** Do not save reference spectrum libraries at other locations. The software only looks for reference spectrum libraries in this specific folder.
	Enables you to view and save the unmixed images as a sequence dataset which can be analyzed using the Tool Palette.
	Opens a dialog box that enables you to correct a spectrum for overlapping signal by subtracting one spectrum from another (see *Correcting Spectra* on page 226).
	Adds a component to the spectrum list.
	Deletes the last spectrum in the spectrum list.

Adding Spectra to the Plot

To Add:	Do This:
A spectrum library	Click the  button and select a spectrum library in the dialog box that appears. **Note:** A spectrum library is a user-created set of reference spectra generated by analyzing probes with known spectra and known locations.
A spectrum from a user-defined region	Add a new spectrum to the list in the Unmix window and identify the region by drawing a mark on the image cube. See step 6 on page 225 for more details.

Composite Image

The composite image includes all of the signals, each displayed in a different color. Double-click the composite image to view it in a separate window (Figure 11.37).

Figure 11.37 Composite Window

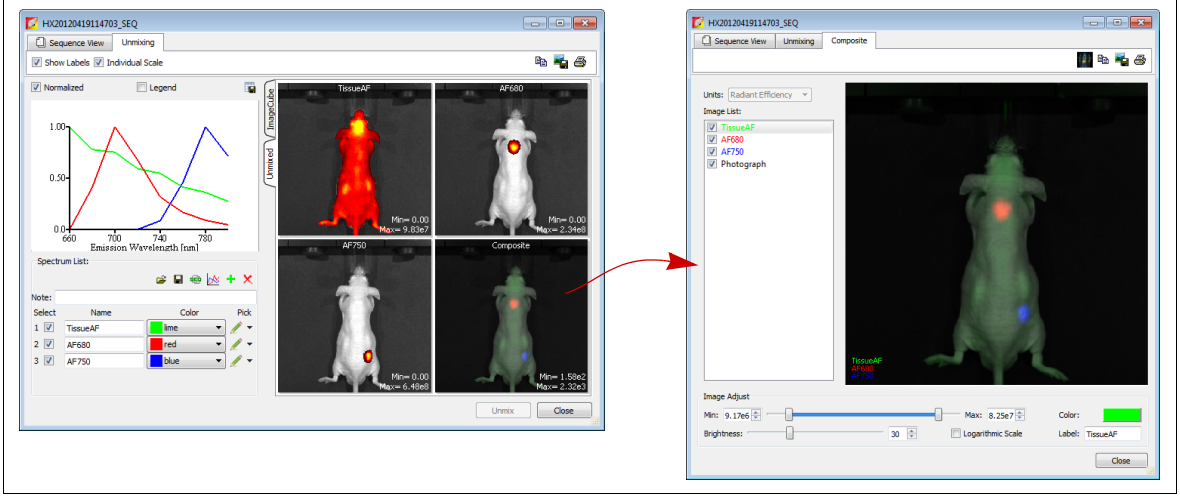


Table 11.5 Composite Window

Item	Description
Units	The type of data displayed in the composite image.
Image list	A list of the images that comprise the composite (background component(s), probe(s), and a photograph).
Min/Max	Sets the minimum and maximum count to display in the image.
Brightness	Adjusts the brightness of the component signals.
Logarithmic Scale	Choose this option to display signals using a logarithmic scale. This may be useful when probe signal strengths differ significantly, for example, a bright source and a dim source.
Color	Shows the color of the figure legend for the image selected in the image list. Click the color swatch to open a color palette that enables you to select a new color for the figure legend.
Label	The name of the image selected in the image list. To edit the name, double-click the name in this box. Right-click the label name to show a short-cut menu of edit commands (for example, Cut, Copy, Paste).

Table 11.5 Composite Window (continued)

Item	Description
	Sends the composite image to the "top" of the image cube. This helps improve the pseudo color visualization of the image cube.
	Copies the composite image to the system clipboard.
	Click to export the composite image to a graphic file (for example, .jpg).
	Opens the Print dialog box.

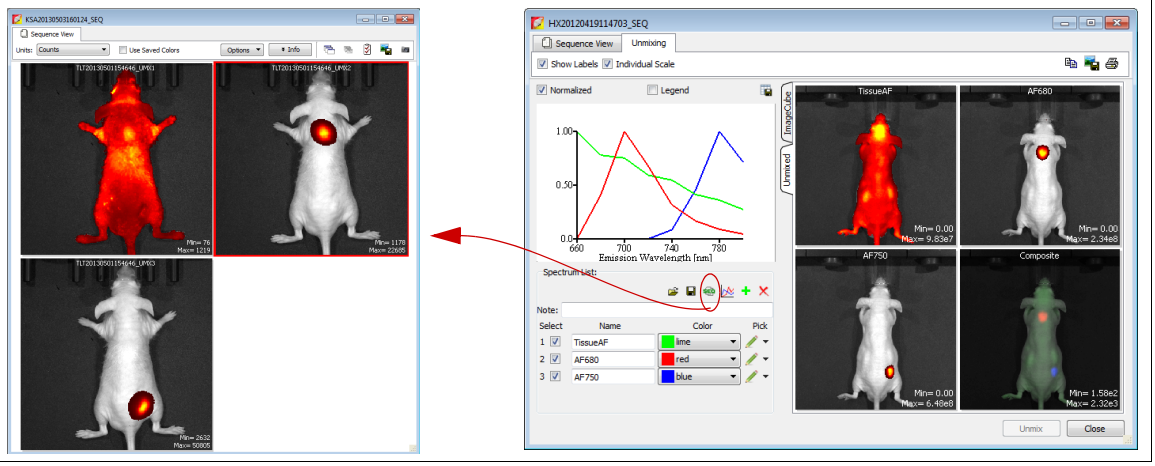
Analyzing Images

Do either of the following:

- Click the  button toolbar button to view all images as a sequence (Figure 11.38).
- Double-click a particular unmixed image.

  The image(s) appear in a separate window and the Tool Palette is available for image analysis. When closing the window, the software prompts you to save the sequence or image.

Figure 11.38 View Unmixed Images as a Sequence



Managing Spectral Unmixing Results

Figure 11.39 Spectral Unmixing Results

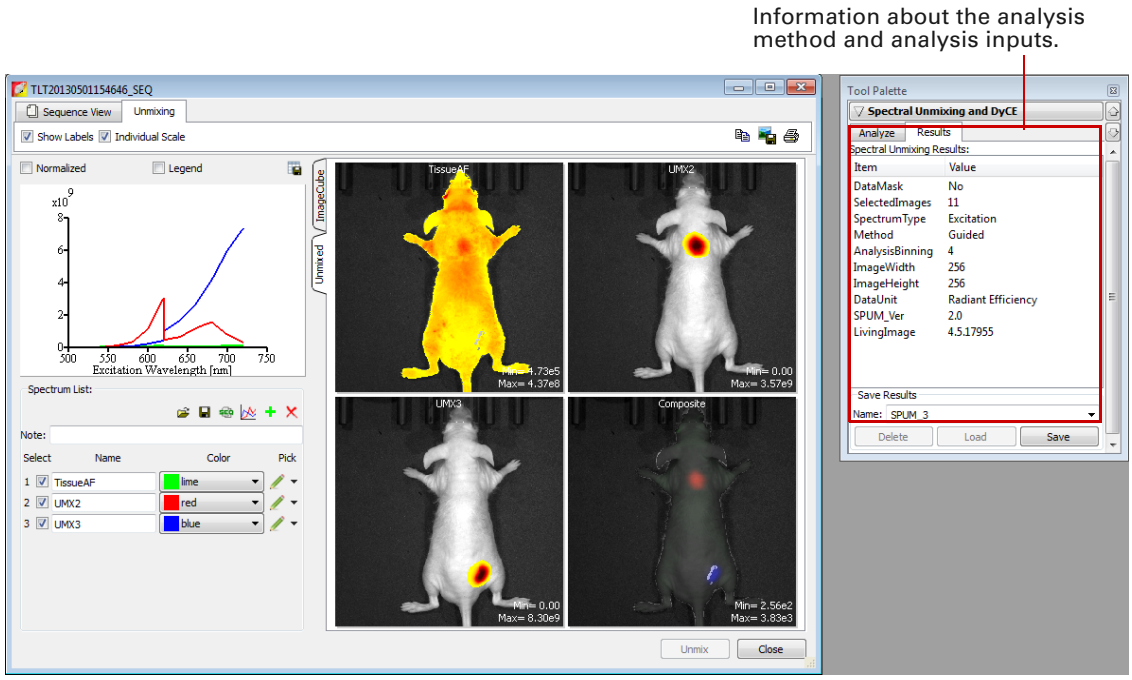


Table 11.6 Spectral Unmixing Results

Item	Description
Name	The name for the active spectral unmixing results. Select results from this drop-down list.
Delete	Deletes the selected results.
Load	Opens the selected results in the Unmixing window.
Save	Saves the active results using the selected name. The results are saved to the sequence click number folder and are available in the Name drop-down list.
Overwrite	If you reanalyze results, saves the new results and overwrites the previous results.

12 Biodistribution Studies Using DyCE Imaging

12.1 About DyCE (Dynamic Contrast Enhancement)

NOTE: The DyCE acquisition and analysis features of Living Image software require a separate license.

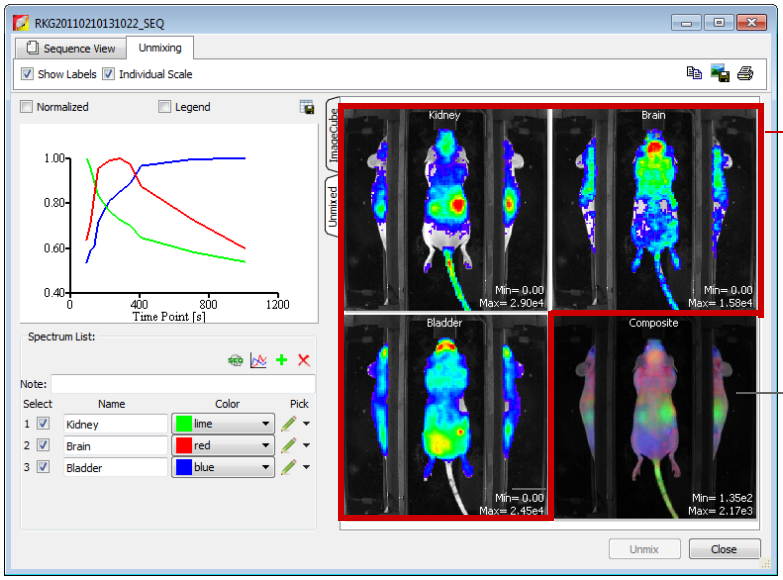
DyCE imaging and analysis is intended for biodistribution studies. DyCE imaging captures a time series of optical images immediately following a bolus injection of a probe or dye. Living Image software temporally unmixes the data on a pixel-by-pixel basis for each image of the time series and determines real-time spatio-temporal distribution of the probe or dye signal.

Living Image software presents the spatio-temporal information as:

- Temporal spectra – Line plots of signal intensity as a function of time. Each line plot represents the signal time course within a particular anatomical region.
- An unmixed image – An image representing the peak signal time point for a particular temporal spectrum.
- A composite image – An overlay of the unmixed images.

Figure 12.1 Example DyCE Results

Images were obtained using the Mouse Side Imaging Kit.



12.2 Acquire an Image Sequence for DyCE Analysis

A DyCE sequence is set up using the Imaging Wizard and includes a user-specified time delay between exposures. An acquisition can include up to three different time intervals where each interval is defined by duration and the delay between exposures.

NOTE: For optimum DyCE analysis results, acquire images using the Side Imager accessory (PN CLS135111).

Choose an imaging mode in the wizard based on your probe type.

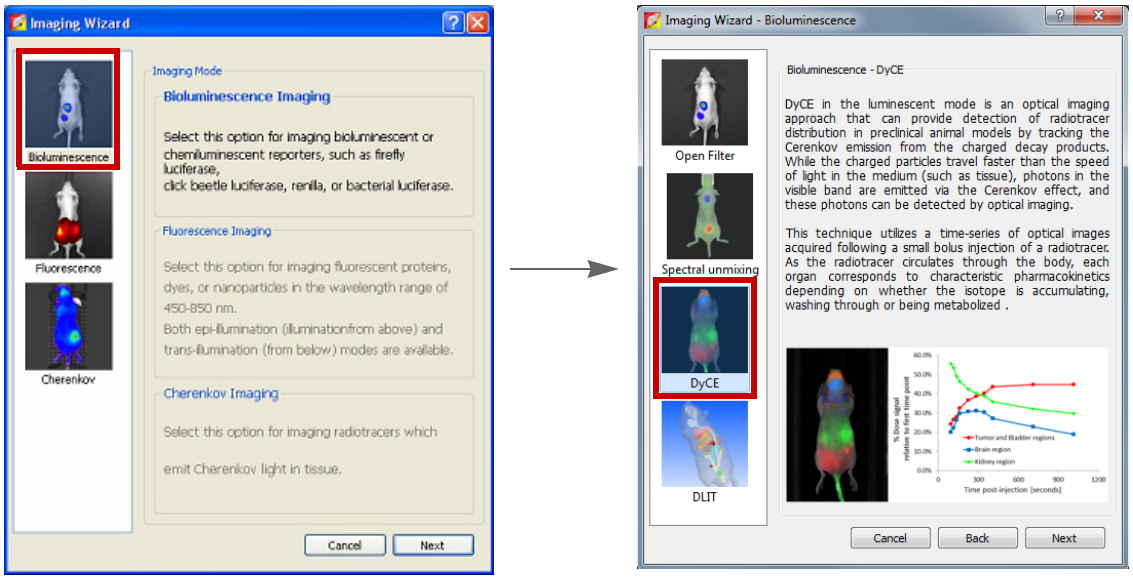
Probe Type	Follow the Instructions for:
Luminescent	*Bioluminescence Imaging* (below)
Fluorescent or near infrared	*Fluorescence Imaging* on page 236
Radiotracer	*Cherenkov Imaging* on page 239

Bioluminescence Imaging

NOTE: The IVIS Spectrum should be initialized and the temperature locked before setting the imaging parameters. See page 19 for more details.

1. Start the Imaging Wizard. See *Start the Imaging Wizard and Setup a Sequence* on page 44 for instructions.
2. Double-click the Bioluminescence option. Double-click the DyCE option in the next screen (Figure 12.2).

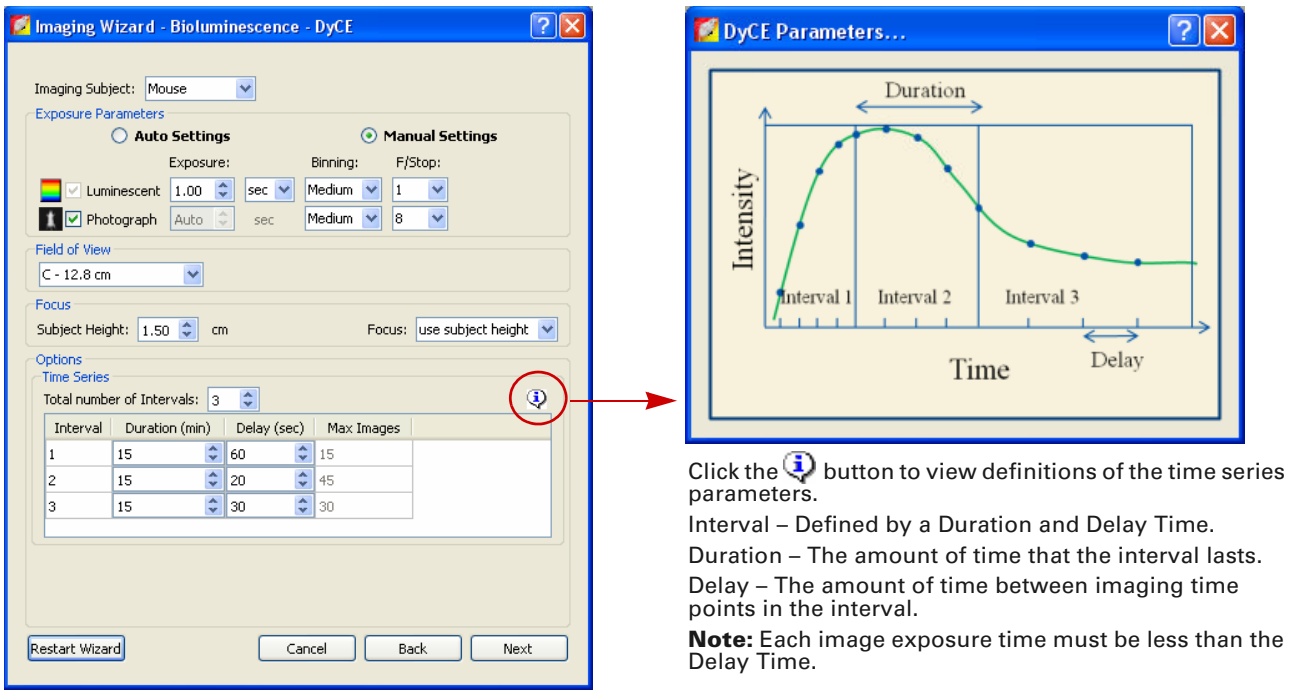
Figure 12.2 Imaging Wizard – Choose Bioluminescence and DyCE Options



If this screen does not appear when the wizard starts, click Restart Wizard (at the lower left wizard screen).

3. Select the type of imaging subject in the next screen (Figure 12.3).

Figure 12.3 Imaging Wizard – Bioluminescence DyCE



Click the button to view definitions of the time series parameters.

Interval – Defined by a Duration and Delay Time.

Duration – The amount of time that the interval lasts.

Delay – The amount of time between imaging time points in the interval.

Note: Each image exposure time must be less than the Delay Time.

4. Choose "Manual Settings" and set appropriate exposure parameter values for your probe.
5. Select a field of view from the drop-down list.
6. Set the focus by doing either of the following:
   - Enter a subject height and choose the "use subject height" focus option.

   OR

   - Choose the "manual focus" option from the Focus drop-down list and set the focus parameters in the Manual Focus Window that appears.

> **NOTE:** If using the Side Imaging accessory for bioluminescence DyCE, set the subject height = 0.0 cm and FStop = 2 (or larger). If using the Side Imaging accessory for fluorescence DyCE, choose the Manual Settings options and set the subject height = 0.0 cm and FStop = 4 (or larger).

7. Specify the time series.

> A time series can include up to three intervals. Each interval is defined by duration (minutes) and delay between images (seconds) (Figure 12.3).
> **Maximum number of images = Duration/Exposure if exposure is greater than delay.**
> **Maximum number of images = Duration/Delay if exposure is less than delay.**
> A time series can include a maximum of 200 images.

   a. Enter the number of intervals.

   b. Enter the duration and the delay between images for each interval.

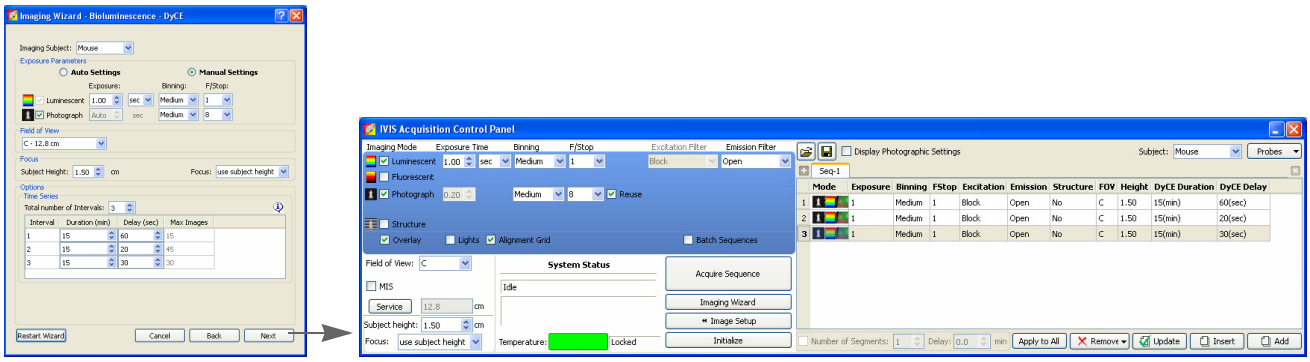
   The software computes the number of images to acquire during the interval.

> **NOTE:** The software alerts you if the number of images in the time series exceeds 200. If necessary, adjust the duration or delay between images of one or more intervals to reduce the number of images.

c. Click **Next**.

The specified sequence appears in the sequence table (Figure 12.4).

Figure 12.4 Sequence Setup Complete



8. Acquire the sequence following the instructions on page 46.

   The image window appears when acquisition is completed (Figure 12.13 on page 241). See Table 4.2 on page 30 for more details on the Image window.

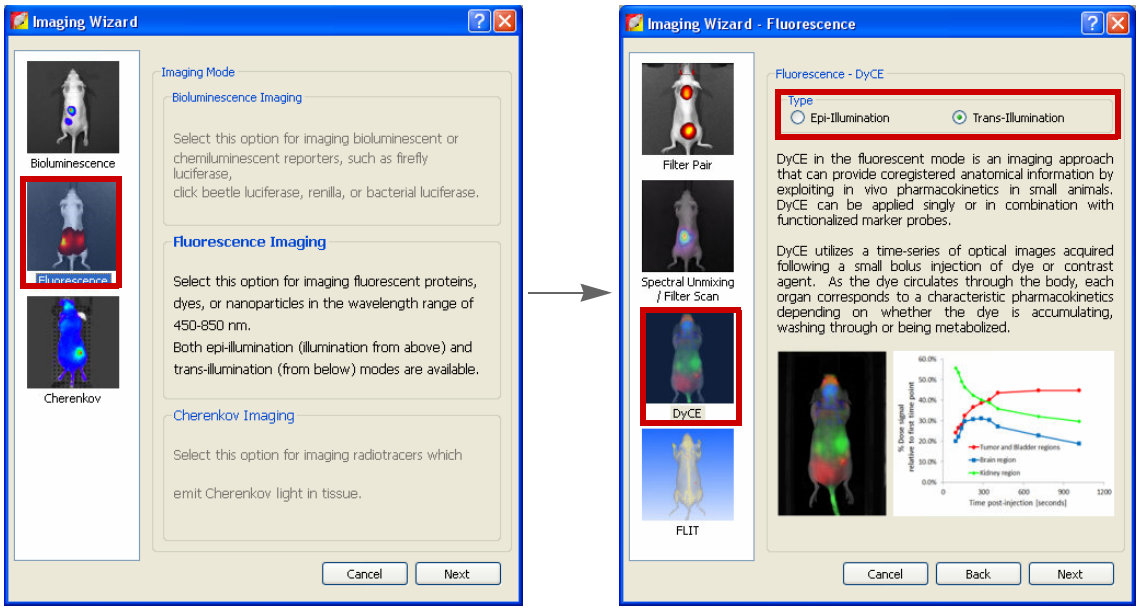
Fluorescence Imaging

NOTE: The IVIS Spectrum should be initialized and the temperature locked before setting the imaging parameters. See page 19 for more details.

1. Start the Imaging Wizard. See *Start the Imaging Wizard and Setup a Sequence* on page 44 for instructions.
2. Double-click the Fluorescence option (Figure 12.5).
3. Select DyCE and the type of illumination in the next screen (Figure 12.5):
   - Epi-Illumination – Excitation light source above the stage.
   - Trans-Illumination – Excitation light source below the stage. If this option is selected, NTF Efficiency images will be produced in which the fluorescent emission image is normalized by the transmission image measured with the same emission filter and open excitation filter.

TIP: See these tech notes for helpful information and quick guides (select **Help → Tech Notes** on the menu bar):
- *Transmission Fluorescence*
- *Transmission Fluorescence – Normalized Transmission Fluorescence*

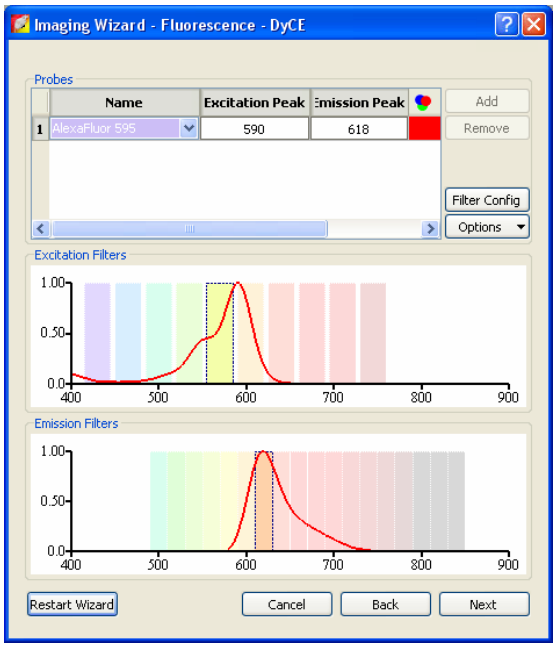
Figure 12.5 Choose the Fluorescence and DyCE Options



If this screen does not appear when the wizard starts, click Restart Wizard (at the lower left wizard screen)

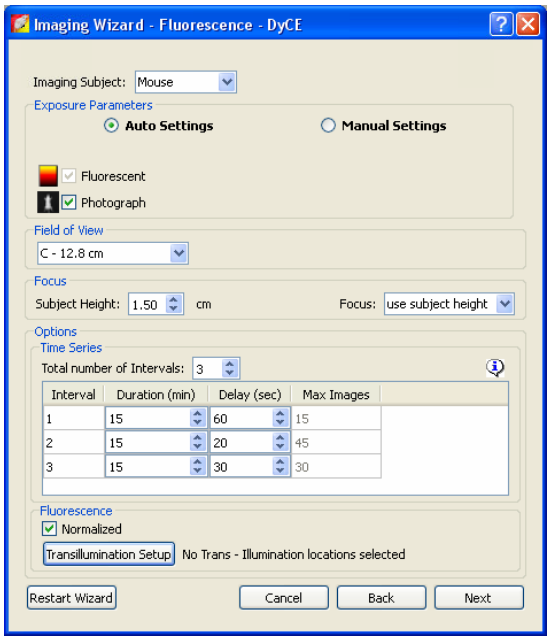
4. Click **Next**. Select a probe from the Name drop-down list in the next screen (Figure 12.6).
   If your fluorescent probe is not in the list, select "Input" and enter the fluorescence excitation and emission peak wavelengths. Click **Next**.

Figure 12.6 Select a Probe



5. Select the type of imaging subject in the next screen (Figure 12.7).
6. Choose the Auto Settings option.

Figure 12.7 Imaging Wizard



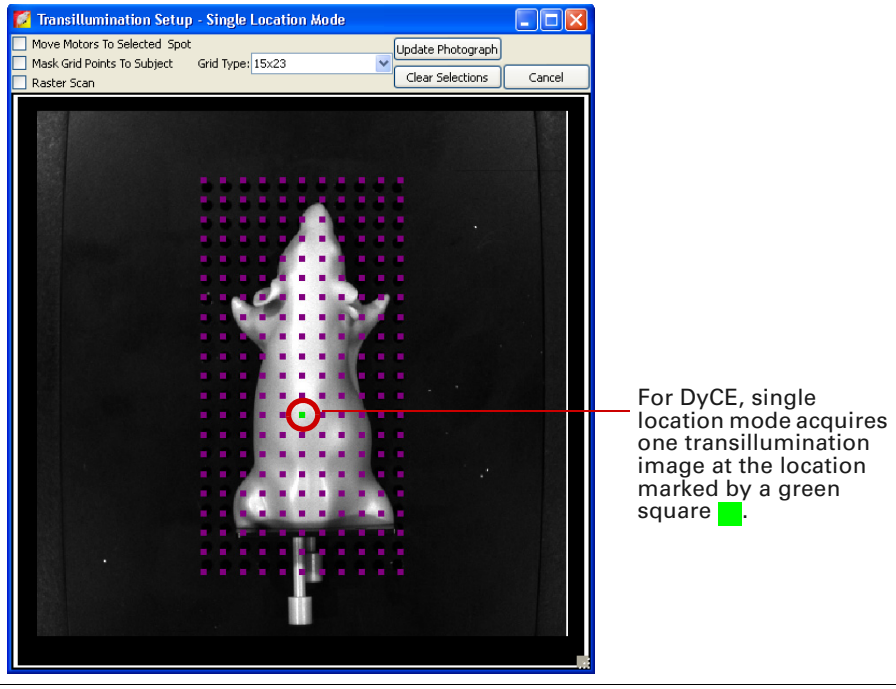
7. If using transillumination, select a transillumination location. Only one excitation point location is allowed.

a. Click **Transillumination Setup**.

b. Choose the location for transillumination by clicking a grid square in the Transillumination Setup box that appears (Figure 12.8).

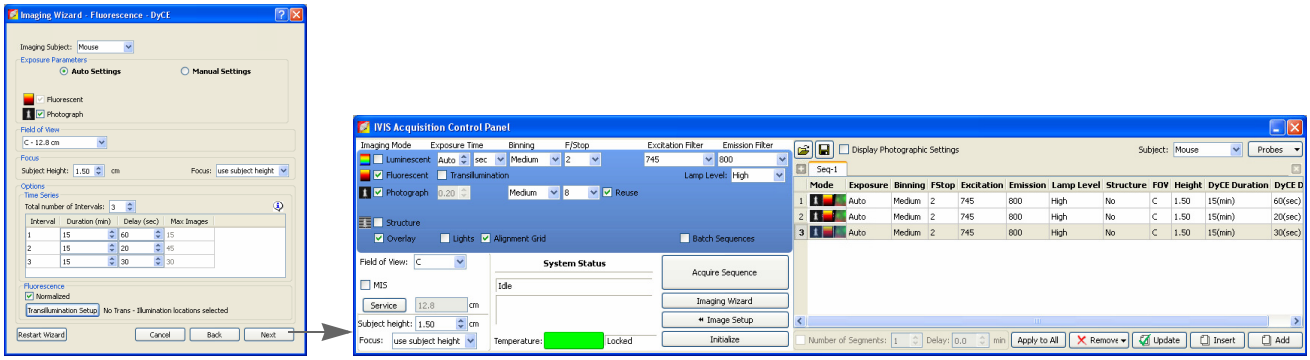
See Table 4.3 on page 40 for more details on the Transillumination Setup.

Figure 12.8 Transillumination Setup Box



8. Perform step 5 on page 235 to step 7 on page 235.
   The specified sequence appears in the sequence table (Figure 12.9).

Figure 12.9 Sequence Setup Complete



9. Acquire the sequence following the instructions on page 46.
   The image window appears when acquisition is completed (Figure 12.13 on page 241). See Table 4.2 on page 30 for more details on the image window.

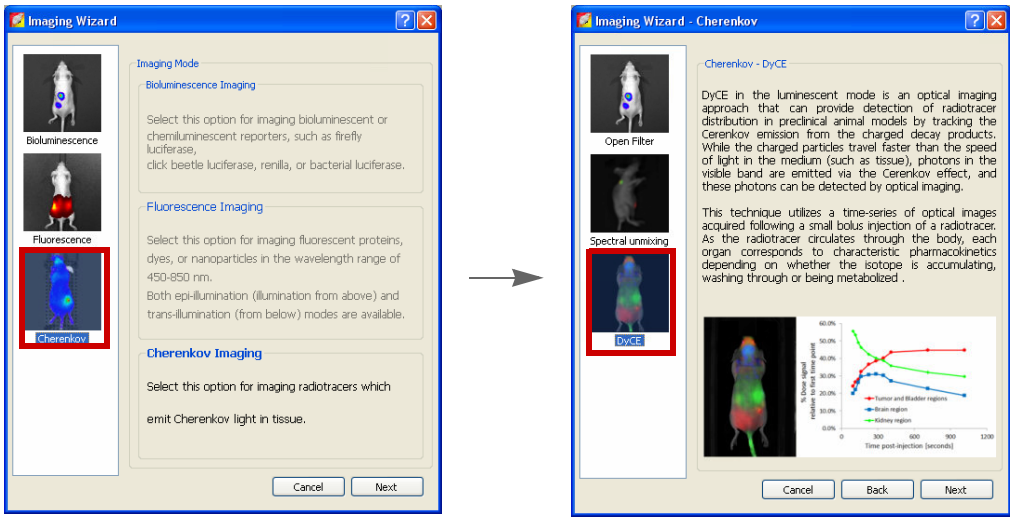
Cherenkov Imaging

NOTE: The IVIS Spectrum should be initialized and the temperature locked before setting the imaging parameters. See page 19 for more details.

1. Start the Imaging Wizard. See *Start the Imaging Wizard and Setup a Sequence* on page 44 for instructions.
2. Double-click the Cherenkov option. Double-click the DyCE option in the next screen (Figure 12.10).

Figure 12.10 Choose Cherenkov and DyCE Options

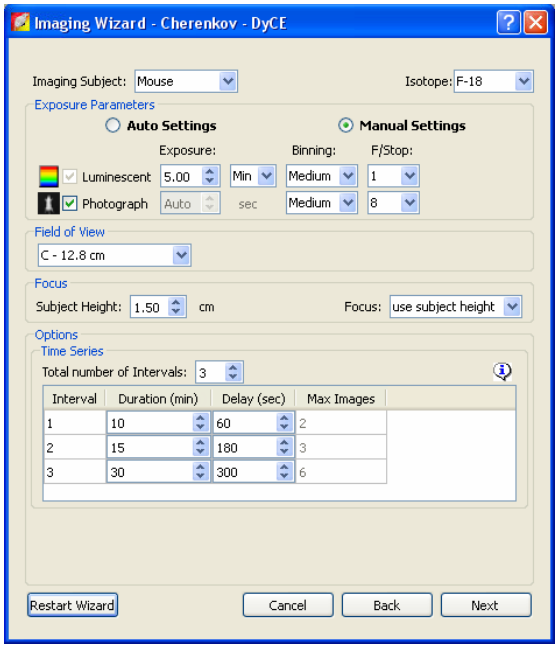
If this screen does not appear when the wizard starts, click Restart Wizard (at the lower left wizard screen).



3. Select the subject type and radio-isotope from the drop-down lists in the next screen (Figure 12.11). If your radio-isotope is not available in the list, choose "Undefined".
4. Choose the Manual Settings option and set exposure parameter values that are appropriate for your radiotracer probe.

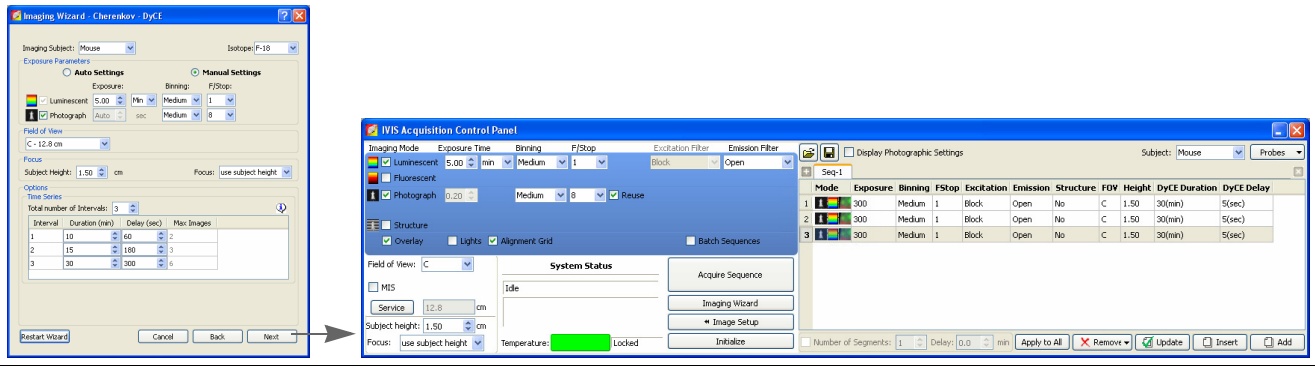
NOTE: Typical exposures are in the range of minutes because Cherenkov light emission is very low. Beta decays with higher energies allow shorter exposure times than lower energy beta decays.

Figure 12.11 Imaging Wizard – Cherenkov DyCE



5. Perform step 5 on page 235 to step 7 on page 235.
   The specified sequence appears in the sequence table (Figure 12.12).

Figure 12.12 Sequence Setup Complete



6. Acquire the sequence following the instructions on page 46.
   The image window appears when acquisition is completed (Figure 12.13 on page 241). See Table 4.2 on page 30 for more details on the image window.

12.3 DyCE Analysis

Automatic or manual DyCE analysis is available. PerkinElmer recommends performing an automatic analysis first, followed by manual analysis to identify possible additional temporal components.

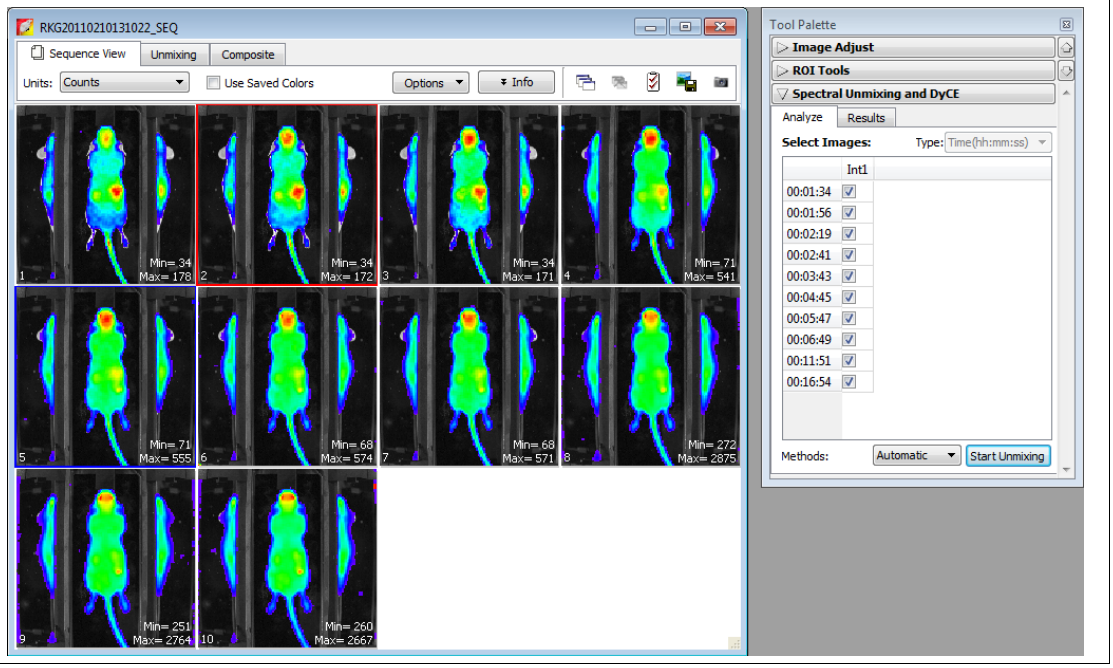
NOTE: If working with large datasets (e.g. studies with many subjects), a 64-bit analysis workstation with 16 GB memory capacity is recommended.

Automatic DyCE Analysis

1. Load a DyCE sequence. The DyCE icon in the Living Image browser indicates a DyCE sequence.

   NOTE: If the data is noisy, as is common for Cherenkov data, smooth all the images in the sequence using the Smoothing tools (under Image Adjust in the Tool Palette). This can be done in sequence view mode. See *Smoothing and Binning* on page 69 for details.

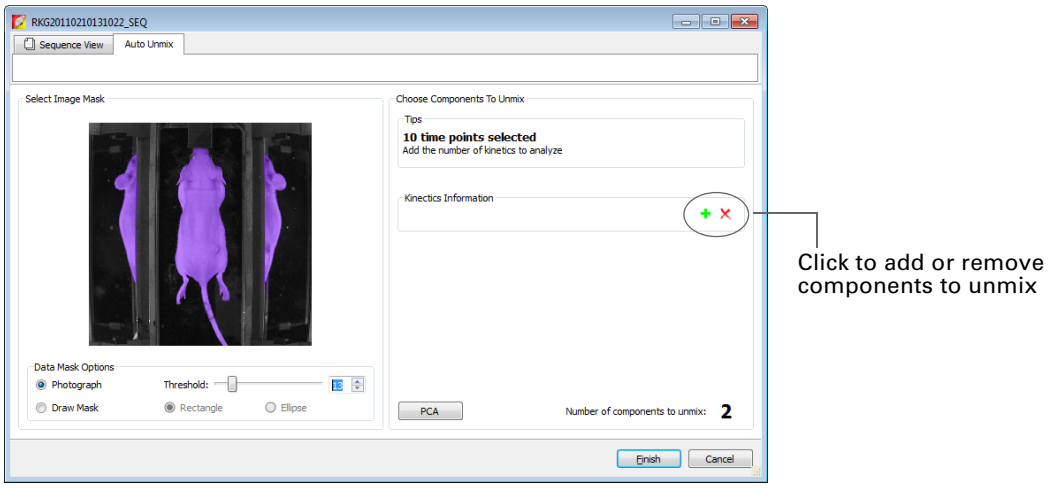
Figure 12.13 Load a DyCE Sequence



2. Click the Analyze tab in the Spectral Unmixing/DyCE tools.
3. Select **Automatic** from the Methods drop-down list and click **Start Unmixing**.

   The Auto Unmix Wizard appears and shows the purple data mask that specifies the analysis area (Figure 12.14). The data mask includes the entire subject by default.
4. If necessary, change the threshold level to adjust the purple mask so that it matches the underlying subject photograph as closely as possible without including any area outside the subject image.

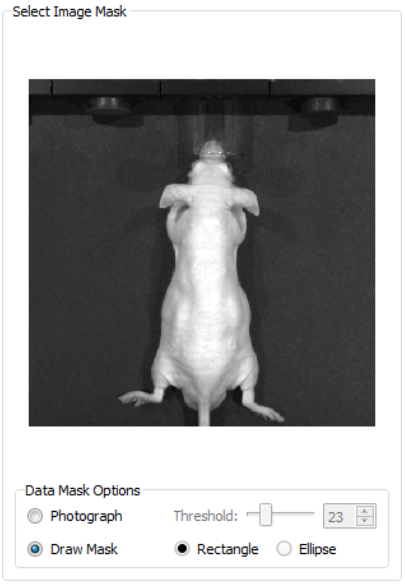
Figure 12.14 Auto Unmix Wizard



5. If you do not want to analyze the entire subject, draw a data mask on a particular area using the data mask options (Figure 12.15). See Table 12.1 for more details on the options.
   a. Select **Draw Mask** and choose the **Rectangle** or **Ellipse** option.
   b. Draw a mask over an area using the mouse. If necessary, click the mask to discard it, and redraw the mask.

Figure 12.15 Draw a Data Mask

See Table 12.1 for more details on the data mask options.



Select the Data Mask option, and the Rectangle or Ellipse option.

Draw a mask on a particular area using the mouse.

Table 12.1 Data Mask Options

Option	Description
Photograph	If this option is chosen, the software automatically draws the data mask so that it includes the entire photograph.
Threshold	If necessary use the threshold slider or arrows to adjust the mask so that it matches the underlying subject photograph as closely as possible without including any area outside the subject image.
Draw Mask	Choose this option to manually draw a data mask on an area of the photograph.
Rectangle	Specifies a rectangular shape for the manual data mask.
Ellipse	Specifies an elliptical shape for the manual data mask.

6. Click the ✚ button to add components to unmix.

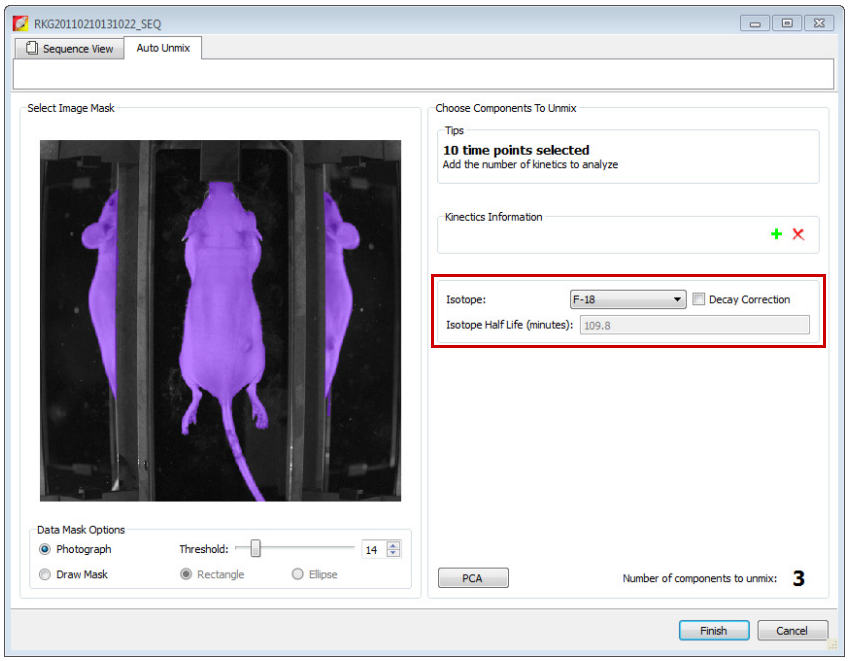
NOTE: Two or three components are recommended for the initial automatic analysis. The DyCE results obtained from the automatic analysis can be manually analyzed to identify possible additional components (see page 245 for details on manual analysis).

7. For Cherenkov data only:
   - Choose the Decay Correction option to apply decay correction to the image data before analysis.

NOTE: If using Decay Correction, Cherenkov decay correction will be applied to every pixel in image, including pixels where the Cherenkov isotope is not present. Therefore, ensure that the data mask covers only the image region of interest, for example, only the mouse.

   - If the radio-isotope used in the experiment was selected in the Imaging Wizard prior to acquisition, it will be displayed in the Isotope drop-down list. If the incorrect radio-isotope was selected at acquisition, choose a different radio-isotope from the drop-down list. If your radio-isotope is not available in the list, choose "Undefined" and enter the isotope half-life in minutes (Figure 12.16).

Figure 12.16 Auto Unmix Wizard – Cherenkov Data



Available for Cherenkov data only

8. Click **Finish**.

   The Unmixing window shows a plot of the temporal spectra, unmixed images, and a composite of the unmixed images (Figure 12.17).

Figure 12.17 DyCE Results – Three Temporal Components

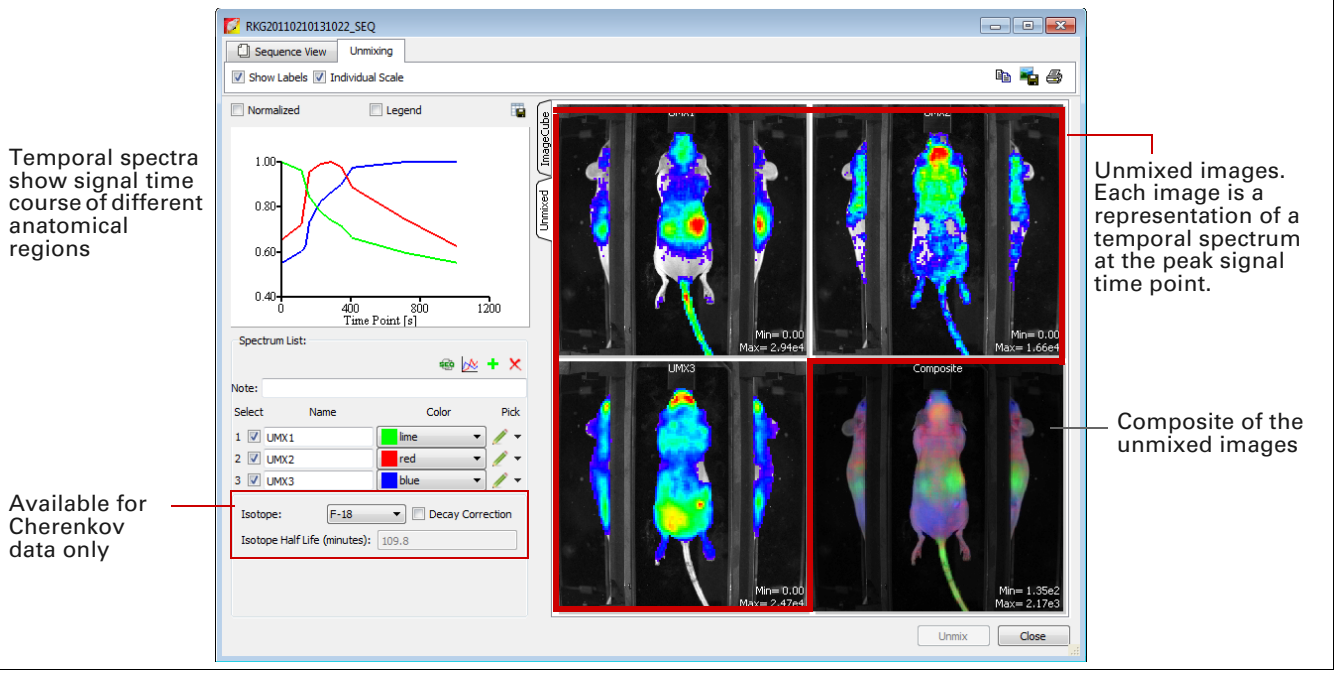


Table 12.2 Spectrum List Toolbar

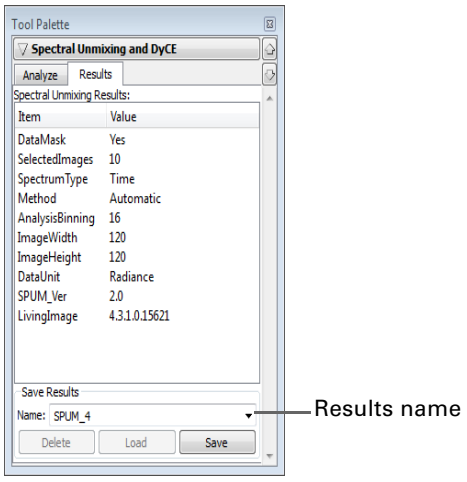
Item	Description
	Enables you to view and save the unmixed images as a sequence dataset. The image adjust, corrections/filtering, image information, or ROI tools are available for the images.
	Enables you to subtract one spectrum from another (see page 251).
	Adds a temporal component to the spectrum list when performing a manual analysis. See page 245 for more details on manual analysis.
	Deletes the last component in the spectrum list. Click **Unmix** after deleting a spectrum to view updated DyCE results.

9. To save the results:

   a. Enter a name in the Results tab of the Tool Palette (Figure 12.18).

   b. Click **Save**.

Figure 12.18 DyCE Results

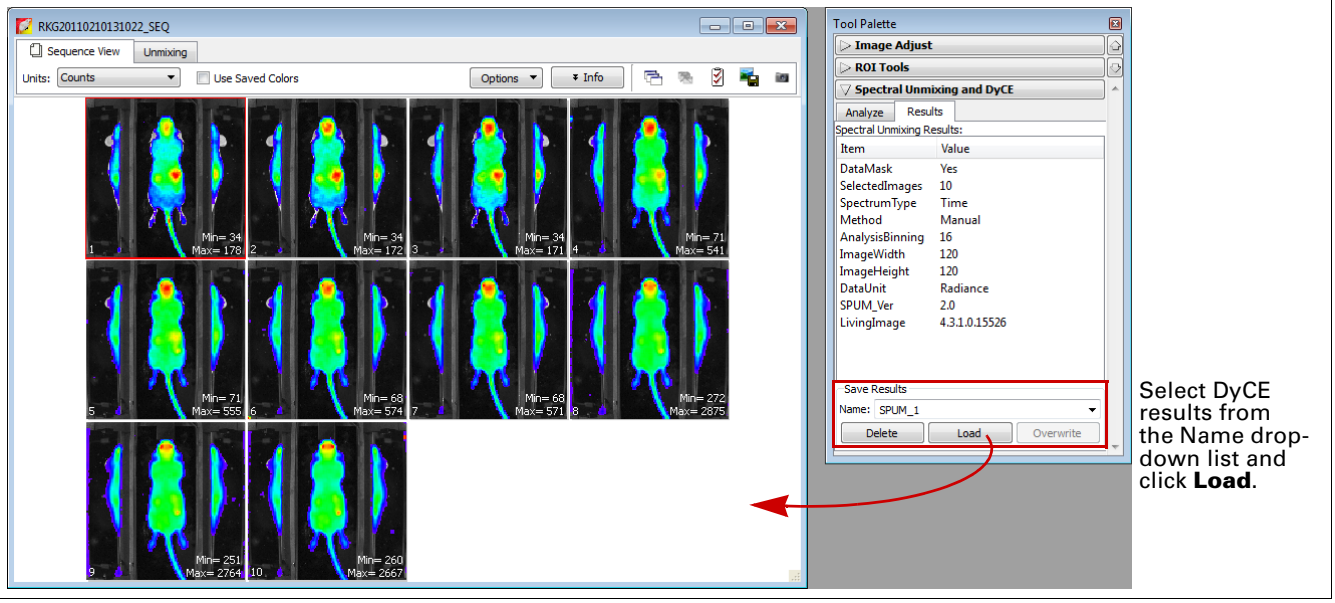


Manual DyCE Analysis

1. Load a DyCE image sequence. Alternatively, load DyCE results obtained from an automatic analysis (Figure 12.19).

NOTE: This section illustrates manual analysis of DyCE results obtained from an automatic analysis.

Figure 12.19 Load DyCE Results



2. Click the Image Cube tab (Figure 12.20).

   The image cube represents a “stack” of the DyCE sequence images. If the Overview option is selected, the image cube shows a composite of all images (Figure 12.20).

   To view a particular image, remove the check mark next to Overview and move the slider or enter an image number (Figure 12.21).

Figure 12.20 Image Cube – Overview Mode

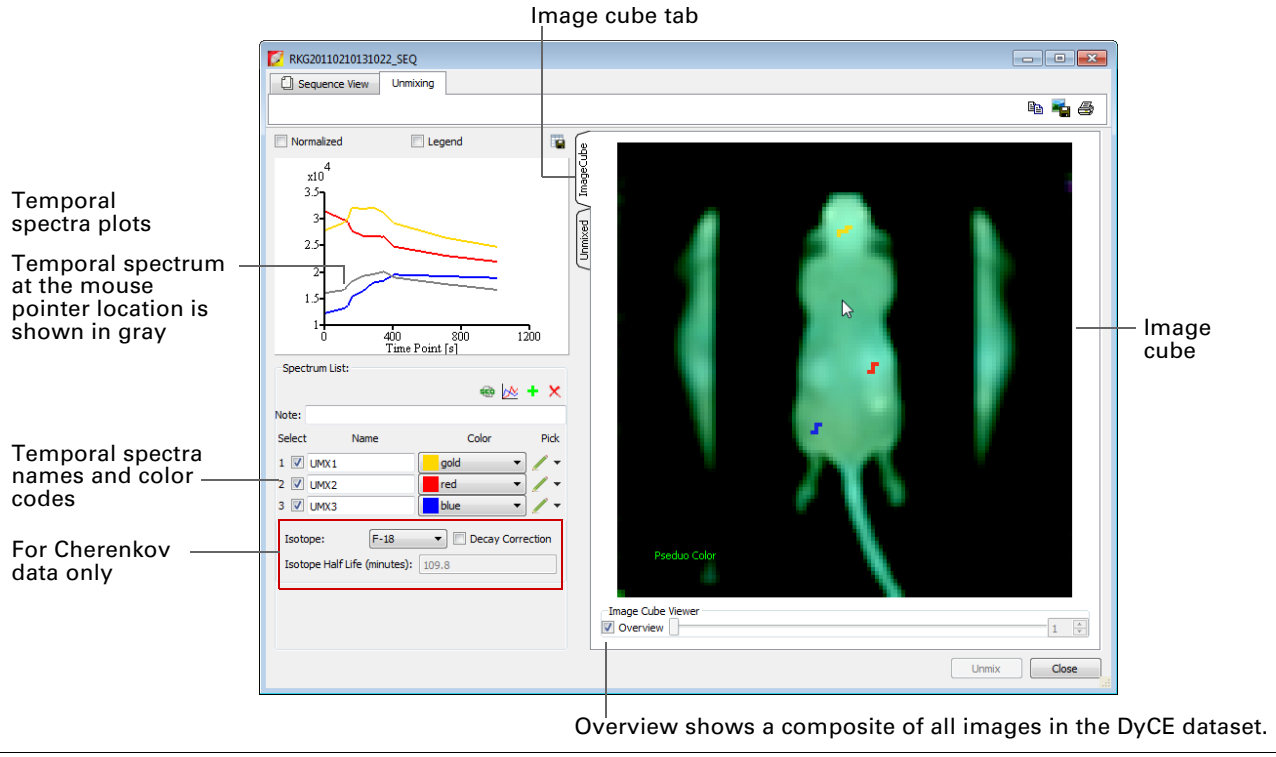
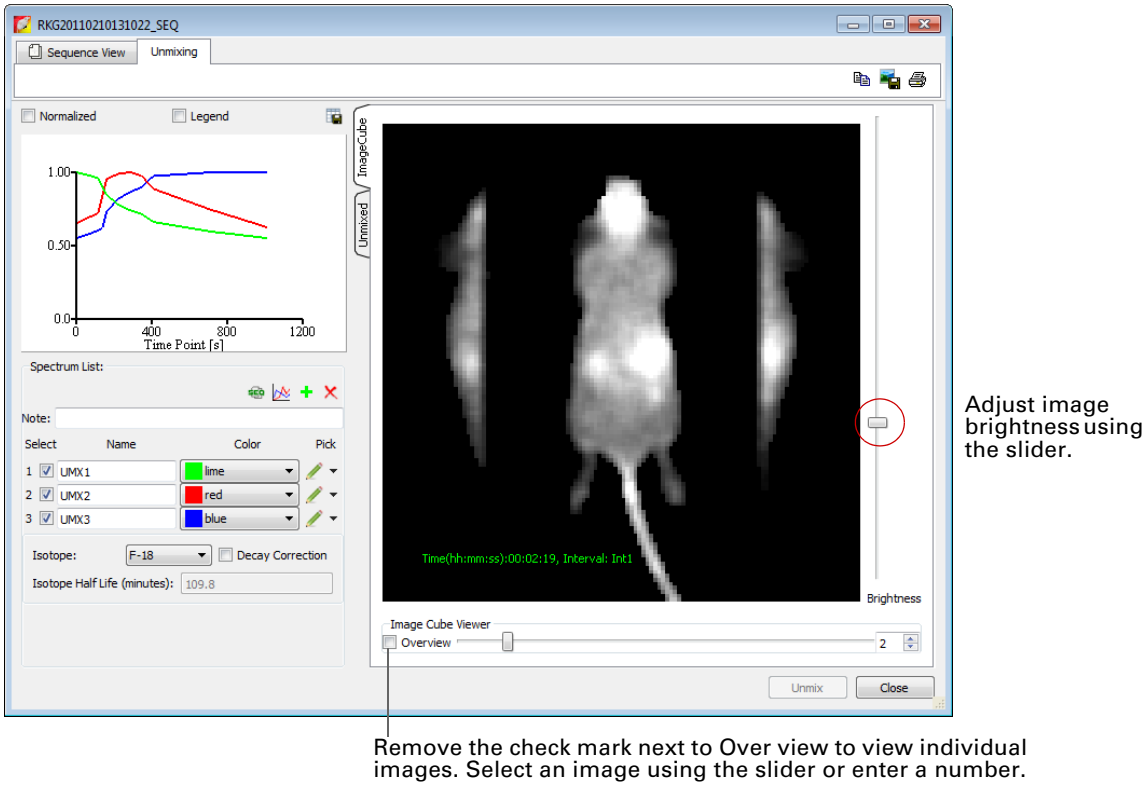


Figure 12.21 Image Cube – Single Image Mode



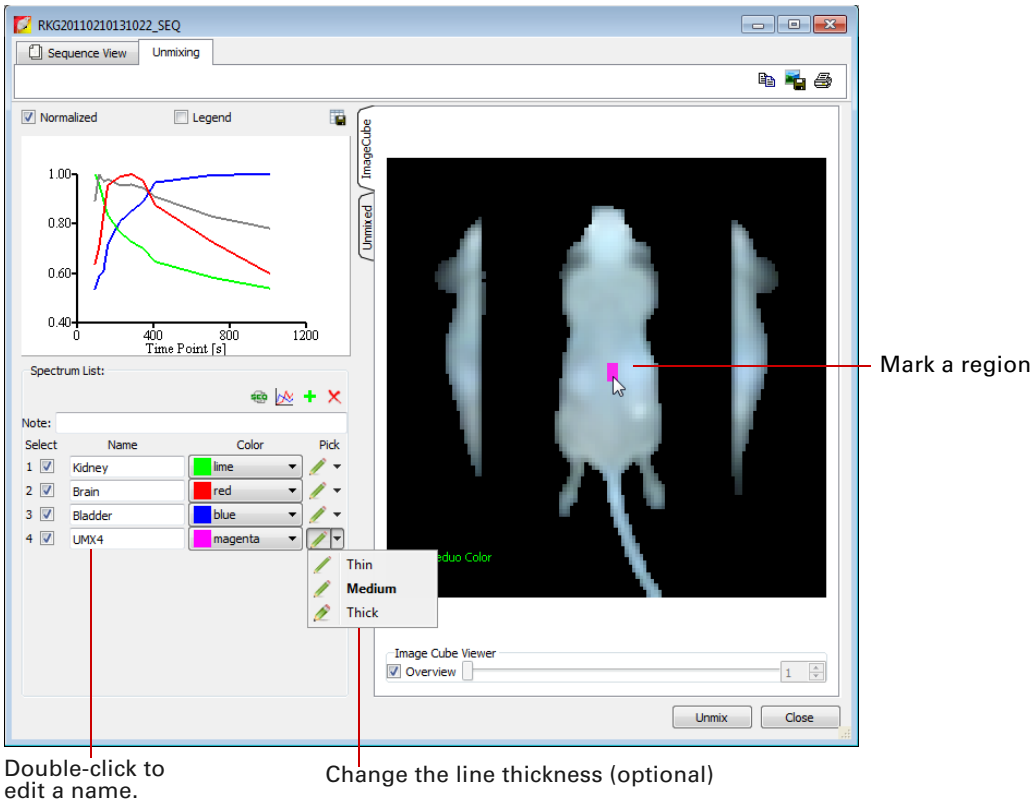
3. Move the mouse pointer over the image cube to see the temporal spectrum at a particular location.
   The temporal spectrum at the pointer location is updated as you move the pointer.

> **NOTE:** If analyzing DyCE results, the Normalized option for the spectrum plot must be checked to see all of the temporal spectra when the mouse pointer is over the image cube.

4. To add another component to unmix:
   a. Click the + button.
      A new name appears in the spectrum list (Figure 12.22)
   b. Specify the region by using the mouse to draw a mark on the image cube. If necessary, click the pencil button next to the spectrum name to select a different line thickness from the drop-down list.
   c. If necessary, right-click the image cube to erase the mark.
5. Repeat step 4 to specify additional temporal components.

> **NOTE:** A maximum of 10 components can be unmixed.

Figure 12.22 Mark the Area of a Temporal Component on the Image Cube



6. Click **Unmix** after you finish marking the regions.
   The software generates unmixed images for the new temporal spectra and updates the composite image with these components.

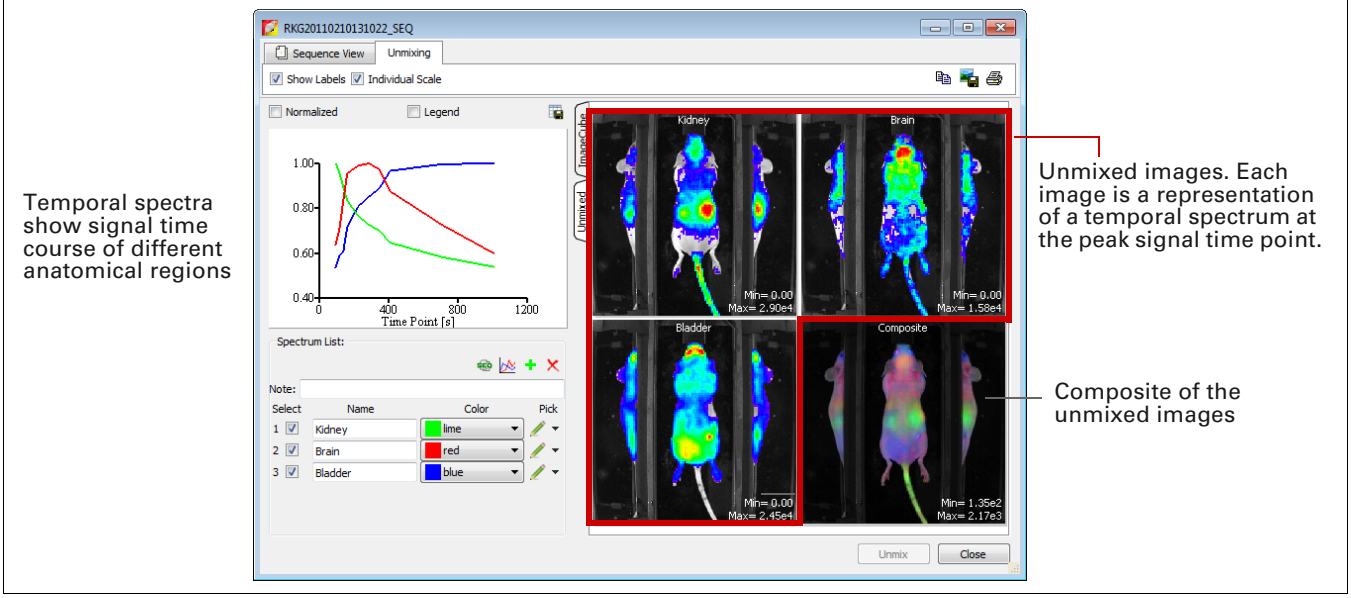
Table 12.3 Spectrum List Toolbar

Item	Description
	Enables you to view and save the unmixed images as a sequence dataset. The image adjust, corrections/filtering, image information, or ROI tools are available for the images.
	Enables you to subtract one spectrum from another (see page 251).
	Adds a component to the spectrum list.
	Deletes the last spectrum in the spectrum list.

12.4 DyCE Results

The Unmixing window shows the DyCE results. The example in Figure 12.23 shows three "temporal spectra" (signal as a function of time).

Figure 12.23 DyCE Results – Three Temporal Spectra

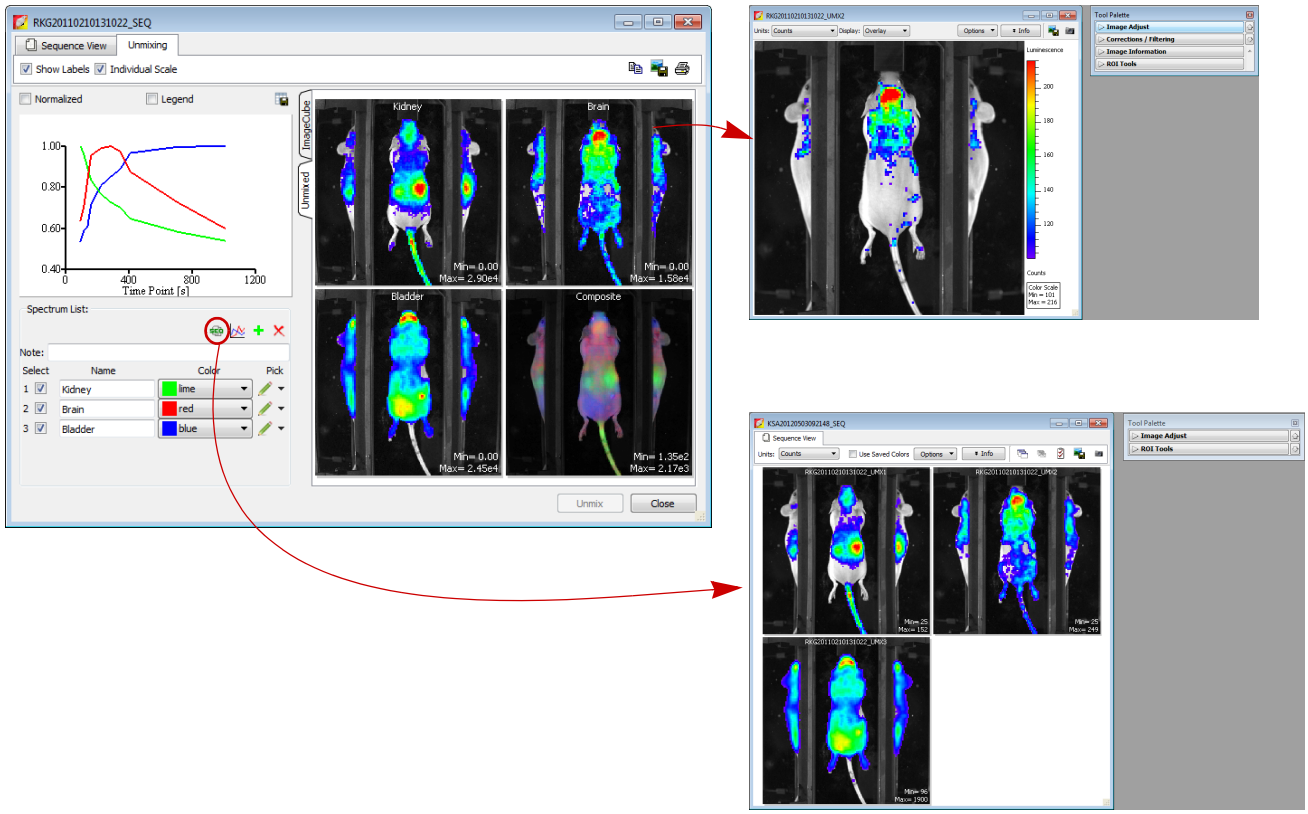


Viewing Unmixed Images

An unmixed image shows the maximum signal of a temporal spectrum.

- Double-click an unmixed image to view it in an image window (Figure 12.24). The Tool Palette is available for viewing and analyzing the image.
- Click the  button to view the unmixed images as a sequence(Figure 12.24). The Tool Palette is available for viewing and analyzing the sequence. The software prompts you to save the sequence when closing the Sequence View window.

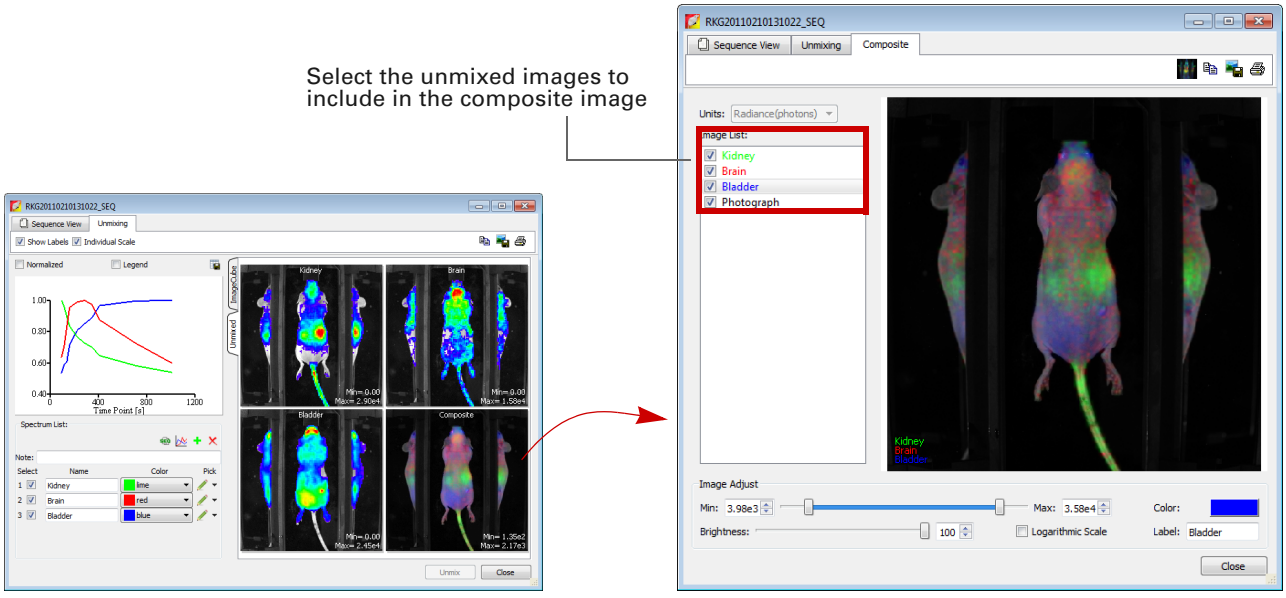
Figure 12.24 View an Unmixed image in an Image Window or View all Unmixed Images as a Sequence



Viewing the Composite Image

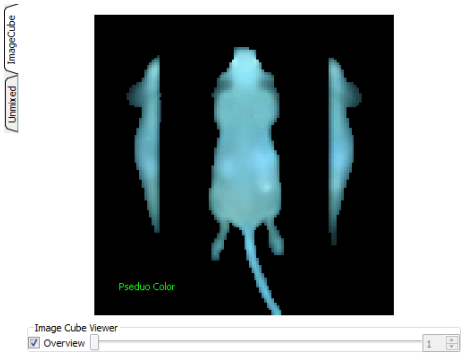
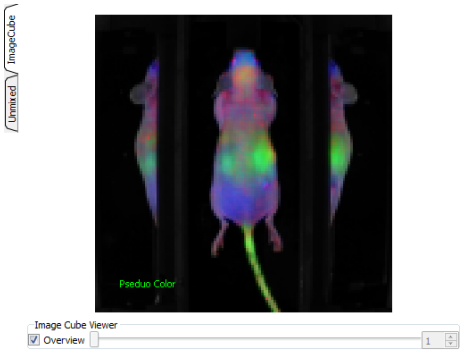
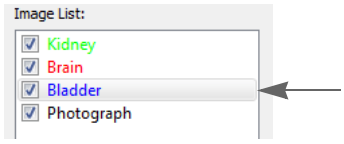
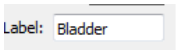
1. Double-click the composite thumbnail.
   The Composite window opens.

Figure 12.25 Open the Composite Window



2. Add or remove the check mark next to an image to include or exclude the data from the composite image.
3. Use the image adjust tools at the bottom of the Composite window to adjust the appearance of the composite image.

Table 12.4 Composite Window

Item	Description
	Sends the composite image to the "top" of the image cube.  Click the Image Cube tab in the Unmixing window to view the image cube. See Figure 12.20 on page 246 for more details on the image cube.  Composite image displayed on top of the image cube.
	Copies the Composite window to the system clipboard.
	Opens a dialog box that enables you to export the composite image to a graphic file (for example, .png).
	Opens the print dialog box.
 	Shows the color of the data for the highlighted image.  Click the color swatch to open the color palette which can be used choose a color for the selected image data.
 	Data name for the highlighted image. Double-click the name to edit it.

Correcting Temporal Spectra

Temporal spectra can be corrected for overlapping spectra; for example, correcting fluorescence temporal spectra for tissue autofluorescence.

NOTE: If correcting for tissue autofluorescence, one of the unmixed components of the dataset should be tissue autofluorescence signal only.

1. Click the button in the Unmixing window.
2. In the dialog box that appears, choose the spectra to subtract (Figure 12.26).

Figure 12.26 Choose Temporal Spectra to Subtract: A - x*B = C

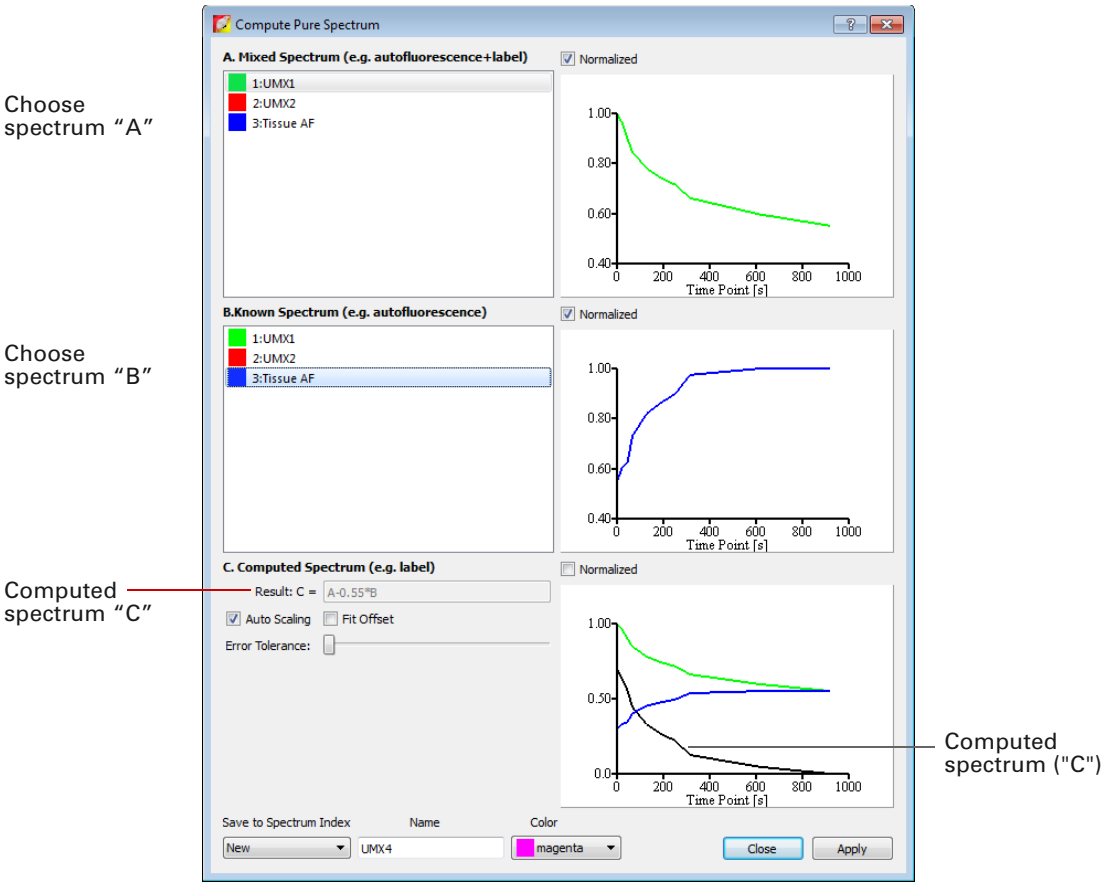
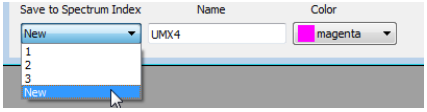


Table 12.5 Computed Spectrum

Item	Description
Normalized	Choose this option to normalize the spectra with respect to time zero.
Result: C = A - x*B	The subtraction performed by the software where "x" is a factor that ensures the residual signal is positive.
Autoscaling	Choose this option to normalize spectra signal on a scale of zero to one.
Fit Offset	If this option is chosen, the software computes and removes an intensity baseline from the spectra.

Table 12.5 Computed Spectrum (continued)

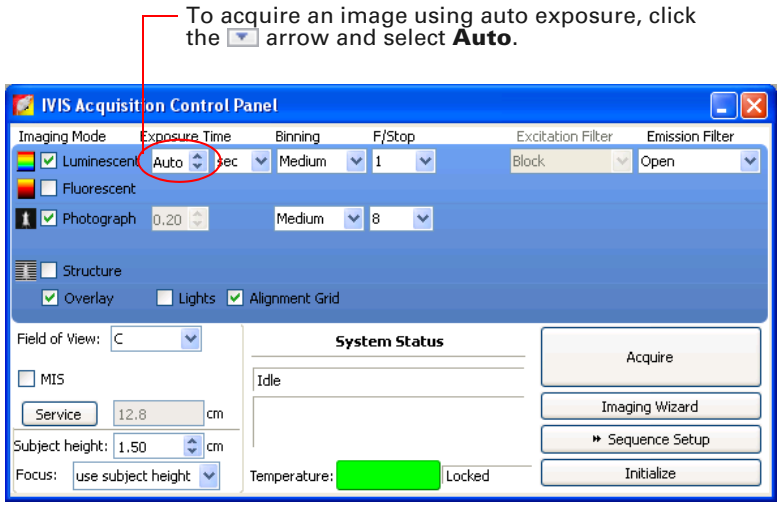
Item	Description
Error Tolerance	The software computes a default error tolerance (the factor "x" for A - x*B) such that signal B is maximally removed from signal A with no negative result. Moving the slider adjusts the error tolerance and automatically updates the computed spectrum.
	Choose "New" to save computed spectrum with the specified name and color. Click **Apply** to add the computed spectrum to the line plot and spectrum list in the Unmixing window. Choose a spectrum number from the drop-down list to overwrite that spectrum with the computed spectrum when you click **Apply**.

Appendix A IVIS Acquisition Control Panel

A.1 Control Panel Functions

The control panel provides the image acquisition functions (Figure A.1).

Figure A.1 IVIS Acquisition Control Panel – Auto Exposure Selected



NOTE: The control panel is only available on the PC workstation that controls the IVIS Imaging System. The options available in the IVIS acquisition control panel depend on the imaging system, selected imaging mode (Image Setup or Sequence Setup), and the filter wheel or lens option that are installed.

Table A.1 IVIS Acquisition Control Panel

Item	Description
Luminescent	Choose this option to acquire a luminescent image.
Fluorescent	Choose this option to acquire a fluorescent image. If the Fluorescent option is selected, the following options also appear in the control panel: Transillumination - Choose this option to acquire a fluorescent image using transillumination (excitation light located below the stage). Normalized - This option is selected by default when the Fluorescent and Transillumination options are chosen so that NTF Efficiency images can be produced.
Photograph	Choose this option to automatically acquire a photograph. The illumination lights at the top of the imaging chamber are on during a photographic image so that the system can acquire a black and white photograph of the sample(s). **Note**: You can adjust the appearance of the photographic image using the Bright and Contrast controls (see *Photo Adjustmento*n page 67.

Table A.1 IVIS Acquisition Control Panel (continued)

Item	Description
 Structure	Choose this option to take a structured light image (an image of parallel laser lines scanned across the subject) when you click Acquire. The structured light image is used to reconstruct the surface topography of the subject which is an input to the Diffuse Luminescence Imaging Tomography (DLIT™) algorithm that computes the 3D location and brightness of luminescent sources. When this option is chosen, the f/stop and exposure time are automatically set to defaults for the structured light image (f/8 and 0.2 sec, respectively). The spatial resolution of the computed surface depends on the line spacing of the structured light lines. The line spacing and binning are automatically set to the optimal values determined by the FOV (stage position) and are not user-modifiable.
Overlay	If this option is chosen, the system automatically displays the overlay after acquisition is completed (for example, luminescent image on photograph).
Exposure time	The length of time that the shutter is open during acquisition of an image. The luminescent or fluorescent signal level is directly proportional to the exposure time. The goal is to adjust the exposure time to produce a signal that is well above the noise (>600 counts recommended), but less than the CCD camera saturation of ~60,000 counts. Luminescent exposure time is measured in seconds or minutes. The minimum calibrated exposure time is 0.5 seconds. The exposure time for fluorescent images is limited to 60 seconds to prevent saturation of the CCD. There is no limit on the maximum exposure time for luminescent images; however, there is little benefit to exposure times greater than five minutes.The signal is linear with respect to exposure time over the range from 0.5 sec to 10 minutes. Integration times less than 0.5 seconds are not recommended due to the finite time required to open and close the lens shutter.
Binning	Controls the pixel size on the CCD camera. Increasing the binning increases the pixel size and the sensitivity, but reduces spatial resolution. Binning a luminescent image can significantly improve the signal-to-noise ratio. The loss of spatial resolution at high binning is often acceptable for *in vivo* images where light emission is diffuse. For more details on binning, see the reference article *Detection Sensitivity* (select **Help → References** on the menu bar). Recommended binning: Small (1-4) for imaging of cells or tissue sections, Medium (4-8) for *in vivo* imaging of subjects, or Large (8-16) for *in vivo* imaging of subjects with very dim sources.
F/stop	Sets the size of the camera lens aperture.The aperture size controls the amount of light detected and the depth of field. A larger f/stop number corresponds to a smaller aperture size and results in lower sensitivity because less light is collected for the image. However, a smaller aperture usually results in better image sharpness and depth of field. A photographic image is taken with a small aperture (f/8 or f/16) to produce the sharpest image and a luminescent image is taken with a large aperture (f/1) to maximize sensitivity. For more details on f/stop, see the reference article *Detection Sensitivity* (select **Help → References** on the menu bar).
Excitation Filter	A drop-down list of fluorescence excitation filters. For fluorescent imaging, choose the appropriate filter for your application. For luminescent imaging, **Block** is selected by default. If you select **Open**, no filter is present. For systems equipped with spectral imaging capability, choose the appropriate emission filter for your application. **Note:** On some models with standard filter sets, the excitation filter selection automatically sets the emission filter.
Emission Filter	A drop-down list of fluorescence emission filters located in front of the CCD lens. The emission filter wheel is equipped with filters for fluorescence or spectral imaging applications. The number of filter positions (6 to 24) depends on the system. For luminescent imaging, the **Open** position (no filter) is automatically selected by default.

Table A.1 IVIS Acquisition Control Panel (continued)

Item	Description
Lamp Level	Sets the illumination intensity level of the excitation lamp used in fluorescent imaging (Off, Low, High, and Inspect). The Low setting is approximately 18% of the High setting. Inspect turns on the illumination lamp so that you can manually inspect the excitation lamp. **Note:** Make sure that the filters of interest are selected in the filter drop-down lists before you select Inspect. The Inspect operation automatically positions the selected filters in the system before turning on the lamp. Subsequent changes to the filter popup menus will have no effect until another Inspect operation is performed.
Lights	Turns on the lights located at the top of the imaging chamber.
Alignment Grid	Choose this option to illuminate an alignment grid on the stage when the imaging chamber door is opened. The alignment grid shows the sizes and positions of the possible fields of view. If subject alignment is not completed in two minutes, place a check mark next to **Alignment Grid** to turn on the grid.
Field of View	Sets the size of the stage area to be imaged by adjusting the position of the stage and lens. The FOV is the width of the square area (cm) to be imaged. A smaller FOV gives a higher sensitivity measurement, so it is best to set the FOV no larger than necessary to accommodate the subject or area of interest. The FOV also affects the depth of field (range in which the subject is in focus). A smaller FOV results in a narrower depth of field. Select the FOV by choosing a setting from the drop-down list. See Table A.2 for more details on the calibrated FOV positions.
Service	Moves the stage to a position for cleaning the imaging chamber below the stage.
Load	Moves the stage from the cleaning position back to the home position.
MIS	Choose this option if the subject will be contained in the Mouse Imaging Shuttle during image acquisition.
Subject height (cm)	Sets the position of the focal plane of the lens/CCD system by adjusting the stage position. The subject height is the distance above the stage that you are interested in imaging. For example, to image a mouse leg joint, set the subject height to a few mm. To image the uppermost dorsal side of a mouse, set the subject height to the 1.5 - 2.0 cm. The default subject height is 1.5 cm. **IMPORTANT!** The IVIS Imaging System has a protection system to prevent instrument damage, however always pay close attention to subject height. For example, it is possible for a large subject (10 cm ventral-dorsal height) to contact the top of the imaging chamber if you set the subject height = 0 and choose a small FOV.
Focus	Drop-down list of focusing methods available: **Use subject height** – Choose this option to set the focal plane at the specified subject height. **Manual** – Choose this option to open the Focus Image window so that you can manually adjust the stage position. For more details on manual focusing, see page 257.
Batch Sequences	Choose this option if you want to specify multiple, separate image sequences for batch acquisition (multiple image sequences are automatically acquired, one after another, without user intervention). See page 50 for more details.
Temperature 	The temperature box color indicates the temperature and status of the system: ▪ White box – System not initialized. ▪ Red box – System initialized, but the CCD temperature is out of range. ▪ Green box – System is initialized and the CCD temperature is at or within acceptable range of the demand temperature and locked. The system is ready for imaging. Click the temperature box to display the actual and demand temperature of the CCD and stage. See page 20 for more details.
Acquire	Click to acquire an image using the settings and options selected in the control panel or to acquire an image sequence specified in the Sequential Setup table.

Table A.1 IVIS Acquisition Control Panel (continued)

Item	Description
Sequence Setup	Click to display the sequence table so that you can specify and manage sequence acquisition parameters, or open sequence acquisition parameters (xsq). See page 52 for more details on manually setting up an image sequence.
Imaging Wizard	Click to start the Imaging Wizard
Sequence Setup	Click to open the sequence table.
Image Setup	Click to close the sequence table.
Initialize	Click to initialize the IVIS Spectrum. See page 19 for more details on initializing the system.

Table A.2 Optical Field of View (FOV) Settings

FOV Setting	FOV (cm)
A[1]	4
B	6.5
C[2]	13
D	22.5 (19.5)[3]
E	22.5 (26)[2]

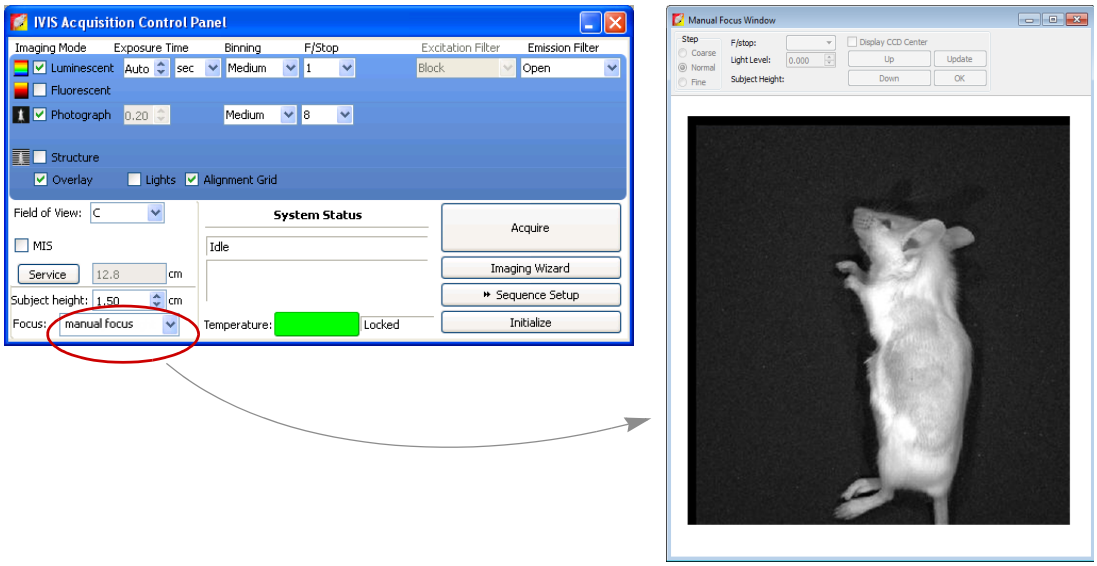
[1] Position A is not recommended for epi-fluorescent imaging because corrections for non-uniform excitation light pattern are not available.
[2] Position C is the default setting.
[3] Some IVIS Spectrum Imaging Systems may have the FOV in parentheses. FOV 19.5 and 26 were replaced with FOV 22.5.

A.2 Manually Setting the Focus

The IVIS Imaging System automatically focuses the image based on subject height. If you do not want to use the automatic focus feature, you can manually set the focus.

1. In the control panel, choose **Manual Focus** in the Focus drop-down list.
   The Manual Focus window appears.

Figure A.2 Opening the Manual Focus Window



2. To mark the center of the camera in the window, put a check mark next to Display CCD Center.
3. Select the size of the step increment that the stage moves: **Coarse**, **Normal**, or **Fine**.
4. Click **Up** or **Down** to move the stage and change the focus.
5. If necessary, select another F/stop setting from the drop-down list and adjust the light level using the arrows.
6. Click **Update** to apply the settings.
   The resulting focal plane (cm above the stage) is automatically entered in the Subject height box.
7. Click **OK** when the image is focused.

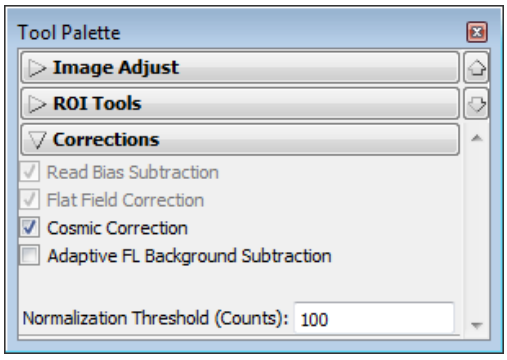
Appendix B Optical Image Data Corrections

The Corrections tools apply corrections or subtract adaptive fluorescence background from optical image data.

TIP: See these technical notes for helpful information (select **Help → Tech Notes** on the menu bar).

- *Luminescent Background Sources and Corrections.*
- *Fluorescent Imaging* for more about fluorescent background.
- Adaptive Fluorescence Background Subtraction.

Figure B.1 Tool Palette – Corrections Tools



NOTE: Read Bias Subtraction and Flat Field Correction are default mandatory corrections in Radiance units mode. These corrections can be cleared in counts mode.

Table B.1 Optical Image Data Corrections

Item	Description
Lens Distortion Correction	Select this option to correct for distortion at the perimeter of an image due to curvature of the CCD lens. Lens distortion correction is available for data acquired by Living Image® software version 4.3 and higher. The correction is particularly important for IVIS Spectrum CT data acquired for DLIT or FLIT.
Read Bias Subtraction/Dark Charge Subtraction	Select this check box to subtract dark background from the image data. If a dark charge image is available for the imaging conditions, the dark background image, including read bias noise, will be subtracted. Otherwise, only read bias noise will be subtracted. **Note:** In Radiance (Photons) mode, dark background or read bias subtraction is a mandatory default. In counts mode, the check box can be cleared. **Tip:** See the tech note *Luminescent Background Sources and Corrections* (select **Help → Tech Notes** on the menu bar).
Flat Field Correction	Select this check box to apply flat field correction to the image data. **Note:** In photons mode, flat field correction is a mandatory default. In counts mode, the check box can be cleared.

Table B.1 Optical Image Data Corrections (continued)

Item	Description
Cosmic Correction	Select this check box to correct image data for cosmic rays or other ionizing radiation that interact with the CCD. See the tech note *Image Data Display and Measurement* for more about cosmic correction (select **Help → Tech Notes** on the menu bar).
Adaptive FL Background Subtraction	Opens the Photo Mask Setup box that enables you to set the photo mask for adaptive fluorescent background subtraction. **Tip:** See the tech note *Adaptive Fluorescence Background Subtraction* (select **Help → Tech Notes** on the menu bar).
Normalization Threshold (Counts)	The minimum number of counts required to perform normalization.

Appendix C Quantification Database

If a quantification database is available, it is possible to determine the number of cells in a DLIT or FLIT source, or the number of dye molecules in a FLIT source. The database is derived from an analysis of images of known serial dilutions of luminescent cells or fluorescent cells or dye molecules. This chapter explains how to construct a database.

C.1 Preparing and Imaging Samples

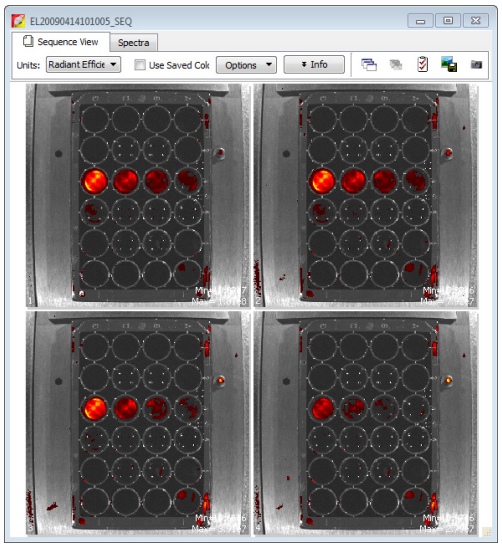
1. Prepare a well plate (4 x 6, 6 x 4, 8 x 12, or 12 x 8 well format) that contains a dilution series of luminescent cells or fluorescent dye at four or more concentrations.
2. Include at least four background wells that contain diluent only.
3. Place the well plate on the IVIS stage, positioning it so that it is centered and square in the field of view.

> **NOTE:** All of the wells must be within view in the image. For wells containing fluorophores, FOV D is recommended to reduce shadows from well walls and ensure more uniform excitation of the wells.

4. Acquire the images:
   - Bioluminescent samples – Acquire one 'Open' filter image of the well plate.
   - Fluorescent samples – Acquire reflectance-illumination Filter Scan images using the appropriate excitation and emission bandpass filters.

The well plate in Figure 14.1 contains a dilution series of a sample at four concentrations. The image sequence is a filter scan set of images with the excitation filter centered at 465 nm for all the images, and emission filter images centered at 520 nm, 540 nm, 560 nm, and 580 nm.

Figure C.1 Well Plate Data



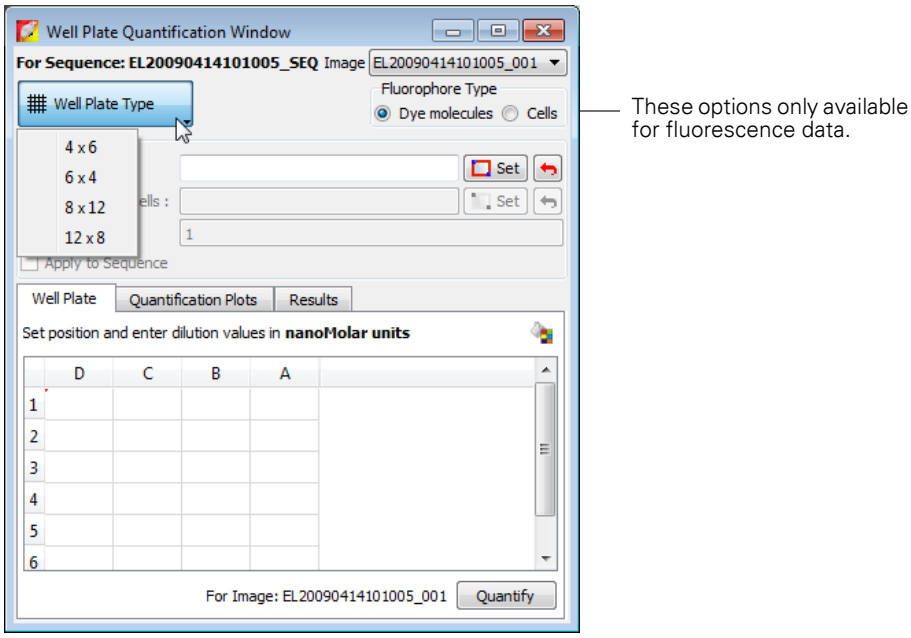
C.2 Creating a Database

1. Load the well plate image sequence.
2. Select **Tools → Well Plate Quantification for "<name>_SEQ"** on the menu bar.

The Well Plate Quantification window appears.

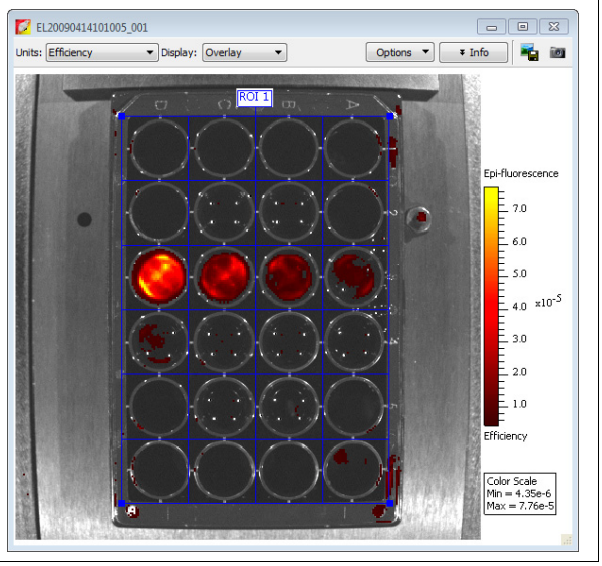
3. For fluorescent samples, choose the Dye molecules or Cells option.

Figure C.2 Well Plate Quantification Window



4. Select the well plate dimensions from the Well Plate Type drop-down list.
   The first image in the sequence opens and a grid ROI appears on the image.

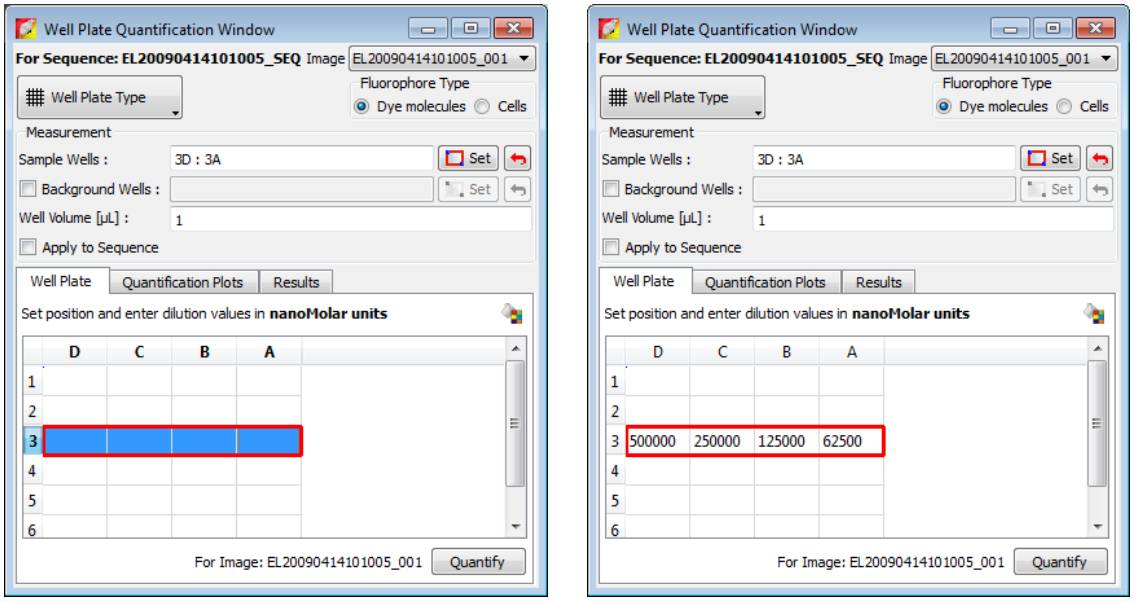
Figure C.3 Well Plate Image With Grid ROI



5. Adjust the grid ROI to closely fit the plate wells.

6. In the well plate table, select the sample cells, and click **Set** (Figure C.4). Clicking a row or column header selects the entire row or column.
7. To remove the "sample" designations from table cells, select the cells and click the button.
8. To apply a color to table cells:
   a. Select the table cells and click the button. Alternatively, right-click the selected table cells and choose Background Color on the shortcut menu.
   b. Choose a color from the color palette that appears.

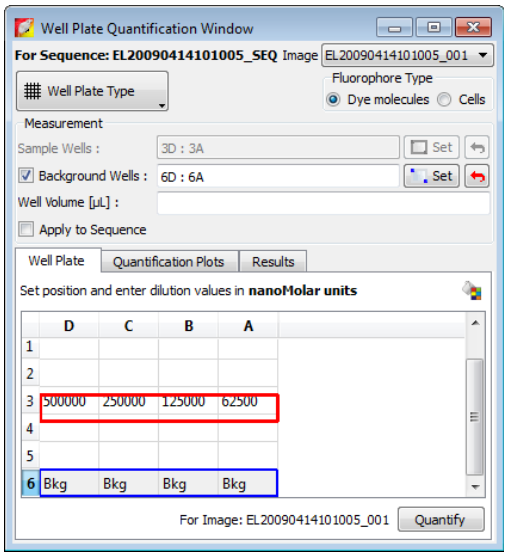
Figure C.4 Select Sample Wells and Enter Cell Numbers or Molecule Concentrations



9. Enter the concentration values in the table cells in nanomolar units, if calibrating fluorescent dyes. Enter the cell values in dimensionless units if calibrating cells.
10. To delete a concentration or cell value, select the table cell and press the Delete key. Alternatively, right-click a selected value to view a shortcut menu of edit commands (for example, cut, copy, paste).
11. If calibrating fluorescent molecules, enter the fluid volume (microliters) for the highlighted wells. The highlighted well volumes must be equal.
12. Choose the Apply to Sequence option.
13. Choose the Background Wells option.
14. In the well plate table, select the background wells and click **Set**.

    Clicking a row or column header selects the entire row or column. To remove the "background" well designations, click the button.

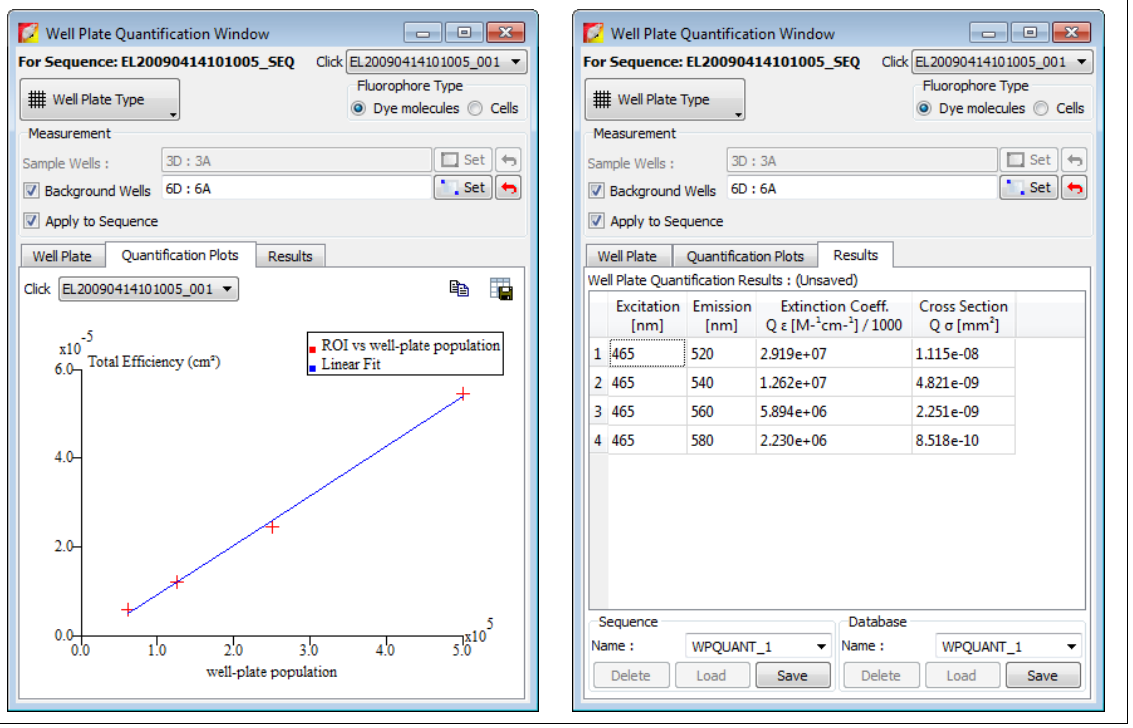
Figure C.5 Set Background Wells



15. Click **Quantify**.

The results are displayed

Figure C.6 Example Fluorescence Quantification Plot and Results



16. Check the linear fit of the data for each image in the quantification plot.

A good fit to the straight line gives confidence to the results values. Large deviations of individual points from a straight line could indicate possible issues with the dilution series or errors when entering sample dilution values.

17. To export the quantification plot values:

- **a.** Click the  button.
- **b.** In the dialog box that appears, select a folder for the file (.csv) and click **Save**.

18. To copy the quantification plot values to the system clipboard, click the  button.

Table C.1 Quantification Results

Item	Description
Fluorescence	
Excitation (nm) Emission (nm)	The excitation and emission filter wavelengths for the image. 'Excitation' and 'Emission' filters will be specified for fluorescent images, and the 'Open' filter for 'Emission' will be specified for bioluminescent images.
Extinction Coeff	A measure of excitation photon absorption interaction with the well plate samples based on a base-10 logarithmic derivation. The quantum efficiency factor of the conversion of the absorbed photon to the emission wavelength is also included.
Cross Section	A measure of excitation photon absorption interaction with the well plate samples based on a natural logarithmic derivation. The quantum efficiency factor of the conversion of the absorbed photon to the emission wavelength is also included.
Bioluminescence	
Total Flux/cell	A measure of total flux (photon/sec) emitted from a single cell. This number can be used to estimate the number of cells from the total flux in the 3D quantification.

C.3 Managing Quantification Results

Quantification results can be saved with the image sequence and as a calibration database that is available in the DLIT or FLIT 3D reconstruction tools (Properties tab). If a calibration database is selected when defining the properties for performing 3D reconstruction, the 3D reconstruction results will be displayed in calibrated units for cell numbers or molecule quantities in picomole units.

Save, Load, or Delete Results

Figure C.7 Saving Quantification Results

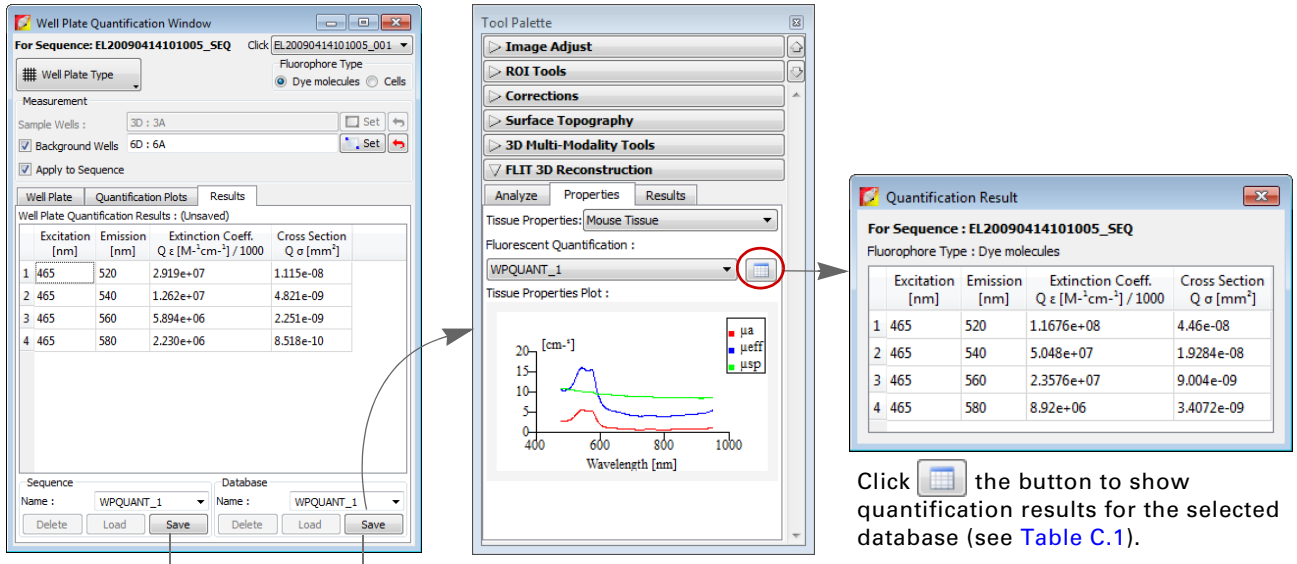
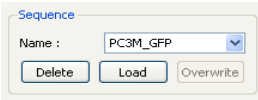
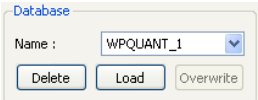


Table C.2 Managing Quantification Results

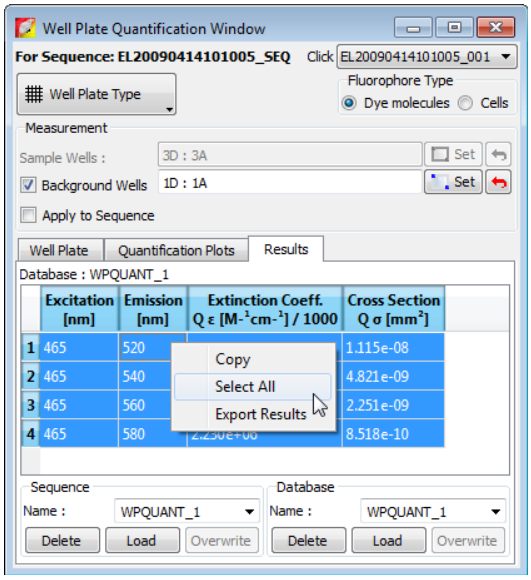
Item	Description
	Delete - Removes the active quantification results from the image sequence. Load - Opens quantification results from the sequence path. Save - Saves the quantification results with the selected image sequence. Overwrite - Saves the results with the selected image sequence and overwrites previous results.
	Delete - Deletes the database from the system. Load - Opens quantification results from the system path. Save - Saves the quantification results to a system database that is available for DLIT or FLIT reconstruction. Overwrite - Saves the results to the selected database name and overwrites previous results.

Exporting Quantification Results

Right-click the results table to view copy and export options.

- Copy – Copies the selected rows to the system clipboard
- Select All – Selects all rows in the results table
- Export Results – Opens a dialog box that enables you to export the selected results to a text file

Figure C.8 Well Plate Quantification Results

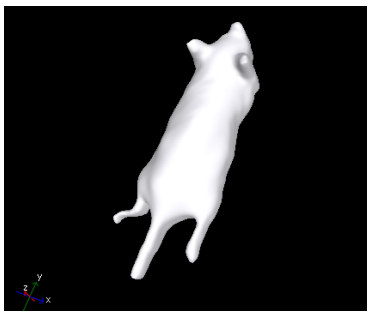


Appendix D Surface Topography

D.1 About Surfaces

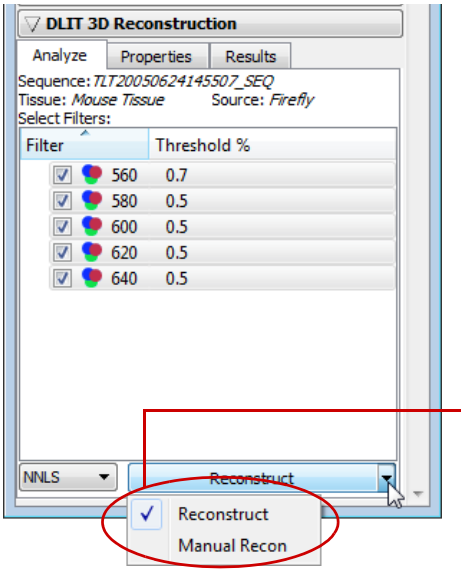
A *surface* is a 3D reconstruction of the animal surface (topography) derived from a structured light image (Figure D.1). A surface is a required input for 3D reconstruction of luminescent or fluorescent sources displayed as voxels. You can also import a surface or export a surface for viewing in other 3D viewer applications.

Figure D.1 Example Surface



Living Image software (version 4.5 and higher) automatically generates or selects a surface during reconstruction. You can also manually create or select a particular surface (Figure D.2). This appendix explains how to create and select a surface using the Surface Topography tools.

Figure D.2



Reconstruct – Choose this option for "Single-Click" reconstruction. Living Image software will automatically generate or select a surface during reconstruction.

Manual Recon – Choose this option to manually create and/or select a surface using the Surface Topography tools.

Animal Requirements

The best surface topography reconstruction is obtained from nude mice. Furred mice are not recommended for DLIT or FLIT. The Surface Topography tool can appropriately generate the surface of a furred mouse. However, the optical data pattern can be grossly shifted by the fur. 3D reconstructions using mice with black or dark-colored fur will give poor results.

It is possible to perform 3D imaging on white or light-colored furred mice if the fur is reasonably smooth over the mouse surface. Therefore it is recommended that you comb the fur before imaging to eliminate any "fluffy" areas that may alter the light emission pattern and/or trigger artifacts during the surface topography reconstruction. In this case, it is recommended that you shave the animals or apply a depilatory.

D.2 Generating a Surface

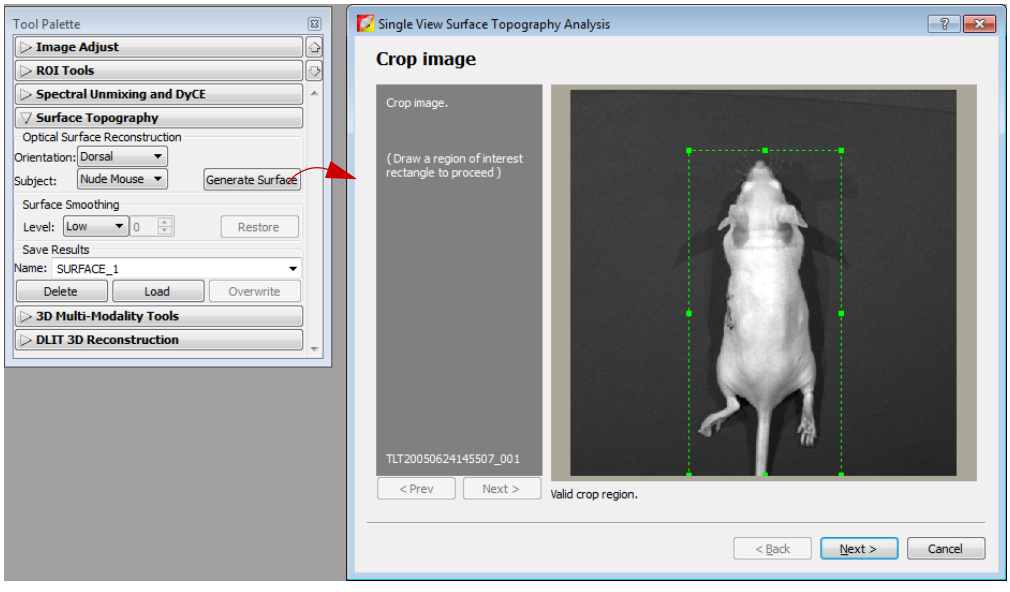
1. Load the image sequence for the reconstruction, for example, a sequence acquired for DLIT analysis or a sequence acquired for FLIT analysis.
2. Select an orientation (dorsal or ventral) and subject in the surface topography tools.
3. Select a smoothing level.

NOTE: The default "Low" smoothing level is sufficient in most cases, but it may be necessary to modify this if there are tufts of hair on the animal which disrupt the surface smoothness.

4. Click **Generate Surface**.

   The entire subject is selected for reconstruction by default in the Single View Surface Tomography window that appears (Figure D.3).

Figure D.3 Data Selected for Surface Reconstruction



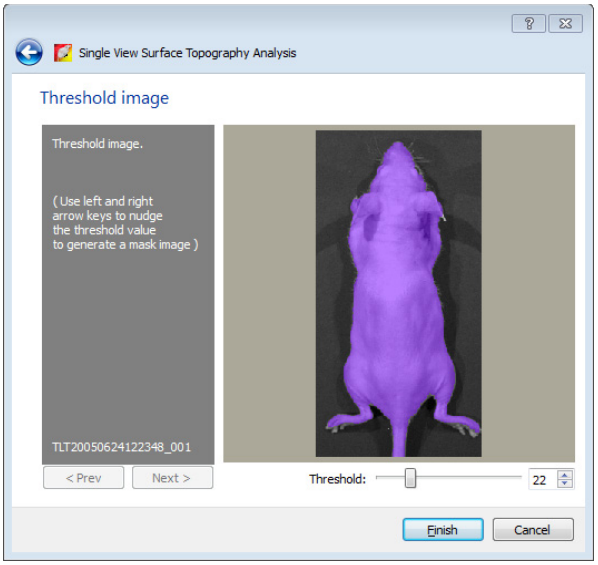
5. If you want to reconstruct only a particular region of the subject, resize the rectangle (drag a green handle ■) so that it includes only the area of interest.

NOTE: If the image sequence includes multiple subjects, manual 3D reconstruction will require a separate surface for each subject. Adjust the rectangle to select a particular subject for surface topography.

6. Click **Next**.

   The purple data mask appears. The mask is an overlay on the subject image that defines the area of interest for the surface topography reconstruction. The mask should match the underlying photograph of the subject as closely as possible without including any area outside the subject image.

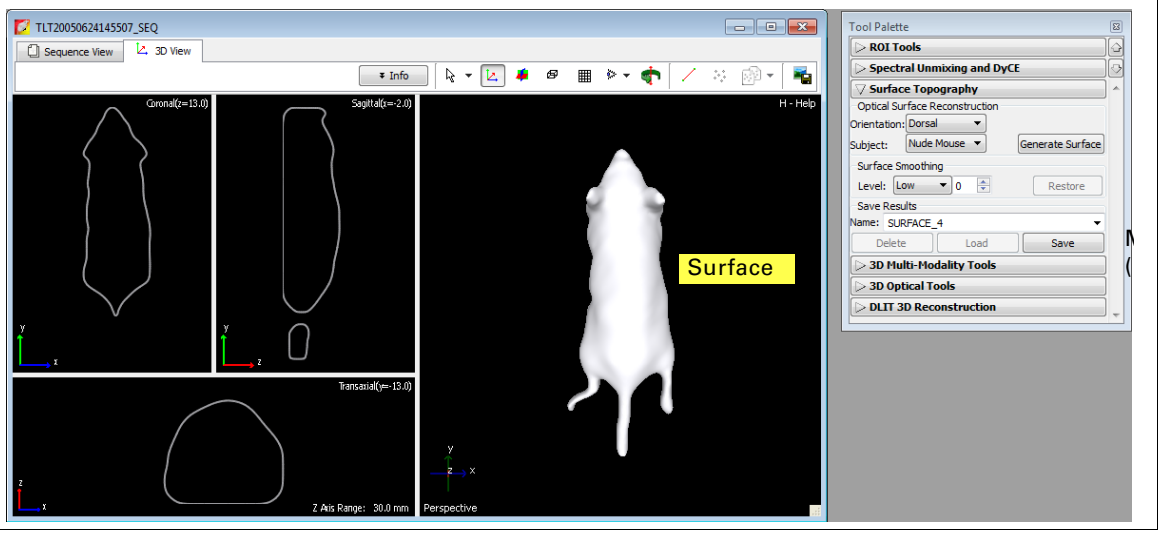
Figure D.4 Data Mask (purple)



7. If it is necessary, adjust the threshold value so that the mask fits the subject image as closely as possible. To change the threshold, do one of the following:
   - Press the left or right arrow keys on the keyboard.
   - Move the Threshold slider left or right.
   - Click the arrows or enter a new value in the box.
8. Click **Finish**.

   The surface appears in the 3D View and the 3D Optical Tools appear in the Tool Palette. See Table 7.1 on page 128 for more details on the 3D Optical Tools.

Figure D.5 3D View and 3D Tools in the Toolbar and Tool Palette



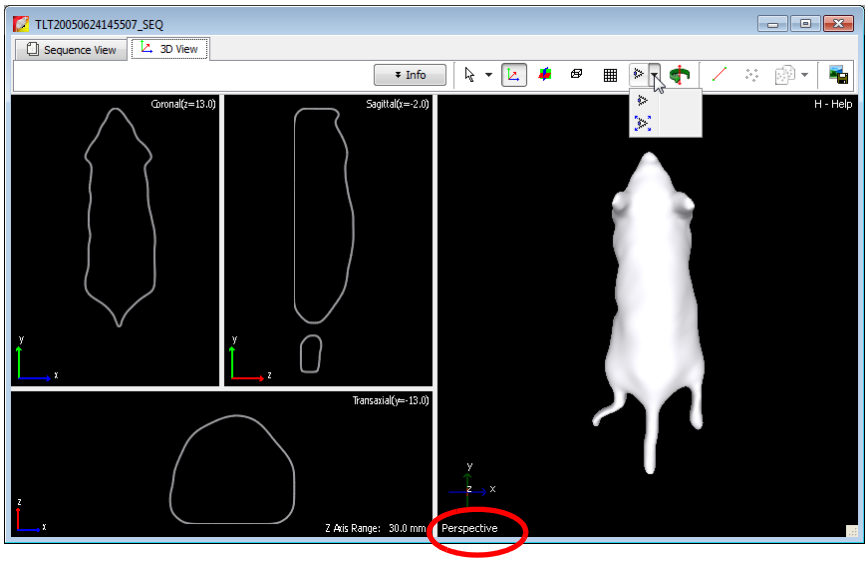
Changing the View Perspective

You can click and drag the surface to view it from different perspectives. Alternatively, do one of the following:

- Select to change the view (Figure D.6)
- Click the surface in the 3D View window, then press the **V** key to cycle through the different views of the surface.

Figure D.7 shows examples of the available views.

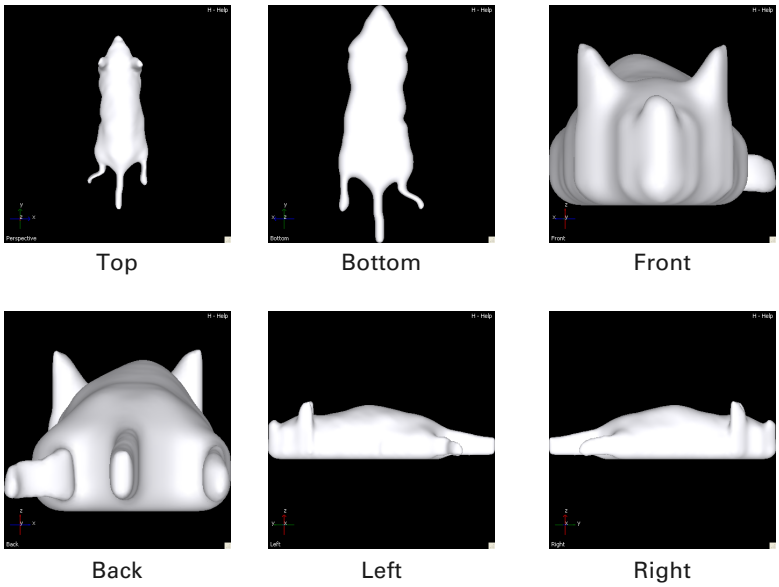
Figure D.6 Surface – Perspective View



View name

Figure D.7 Alternative Surface Views

Click the surface, then press the "V" key to change the view.



Top Bottom Front

Back Left Right

D.3 Managing Surfaces

After the surface is saved, it can be shared by the DLIT or FLIT tools.

Figure D.8 Tool Palette – Surface Topography Tools

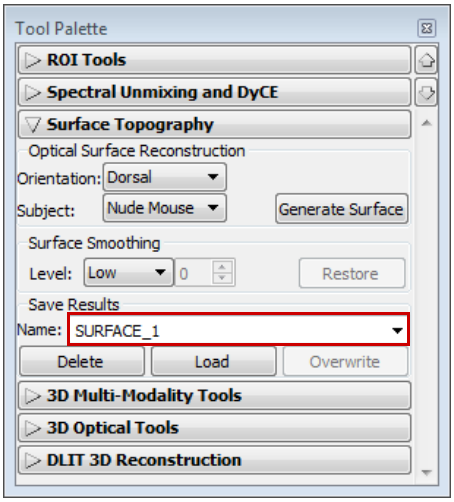


Table D.1 Surface Topography Tools – Managing Surfaces

Item	Description
Name	▪ If the loaded dataset has not been analyzed, the "Name" field shows the default name "SURFACE_1 (new)". ▪ If the dataset has been analyzed and the results saved, the "Name" field shows the name of the surface saved with the .sequence.
Delete	Removes the selected surface from the system.
Load	Opens the selected surface.
Save	Saves a surface to the default name (e.g. “SURFACE_2") or a user-specified name.
Overwrite	Saves the surface and overwrites the previous surface results.

Export or Import a Surface

A surface can be shared with other users or viewed in other 3D viewer applications.

NOTE: Surface import capability is only available if “Show Advanced Options” is selected in the general preferences (see page 274).

1. Load a surface.
2. Select **File → Export (or Import) → 3D Surface** on the menu bar.
3. In the dialog box that appears, select a folder, enter a file name, and select a file type (see Table D.2).

NOTE: Importing a surface by this method is for viewing purposes only, not for registration with optical reconstructions in Living Image software. To import a surface or other organs for registration purposes, import an organ atlas. See page 161 for more details.

Table D.2 Surface File Types

Export Option	Description	Export	Import
Surface mesh (.xmh)	A native file format of the Living Image software that is used to exchange 3D surface information between Living Image software and other third party analysis tools. It is based on a basic indexed face set format which stores all of the vertex information first, then stores the triangle information in terms of indexes into the vertex list.	yes	yes
AutoCAD DXF (.dxf)	Drawing exchange format that is compatible with most DXF file viewers.	yes	yes
VRML 1.0 (.wrl)	VRML 1.0 (.wrl) - Virtual reality modeling language format that is compatible with most VRML viewers.	yes	no
Open Inventor (.iv)	The ASCII version of the IV file format which is supported by all IV viewers.	yes	yes
STL (.stl or ASCII format)	Stereo lithography binary format compatible with most STL viewers.	yes (binary)	yes

Appendix E Preferences

You can manage user IDs and specify defaults for some parameters that are associated with the user ID selected at the start of a new session.

After you log on, select **Edit** → **Preferences** on the menu bar to view the user-modifiable preferences.

NOTE: Any changes made to the Preferences are implemented at the start of the next session. The Acquisition tab is only available in the Living Image software that controls the IVIS Imaging System.

E.1 General Preferences

Figure E.1 General Preferences

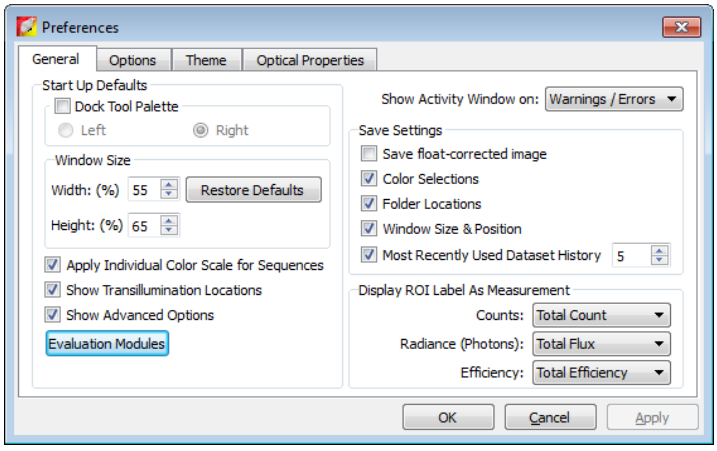


Table E.1 General Preferences

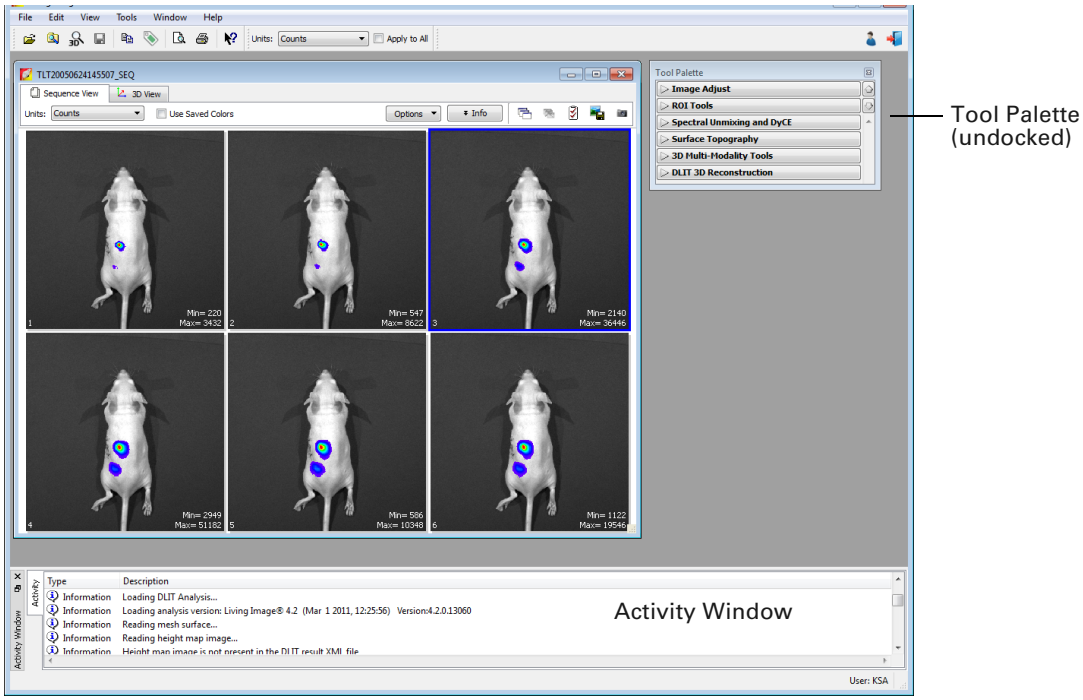
Item	Description
Start Up Defaults	Dock Tool Palette - Choose this option to set the position of the Tool Palette in the application window. Choose left or right. **Note:** To undock the Tool Palette, click on the palette title bar and drag it a distance greater than its width.
Window Size	Specifies the dimensions of the main application window. Width, Height - Sets the dimensions of the image window. Restore Defaults - Click to apply the default settings.

Table E.1 General Preferences (continued)

Item	Description
Apply Individual Color Scale for Sequences	Choose this option to apply a separate color scale to each thumbnail of a sequence. If this option is not chosen, all of the thumbnails are displayed using the same color scale.
Show Transillumination Locations	Choose this option to display a cross hair at each transillumination location when you load transillumination data. When you mouse over a cross hair, a tool tip displays the transillumination coordinates. If this option is not chosen, you can choose the Transillumination Location option in the sequence view window to display the transillumination locations.
Show Advanced Options	If this option is selected, advanced features are available in the menu bar and Tool Palette, including: ▪ Additional ROI functionality for Auto ROI parameters. ▪ Additional export and import option for 3D surfaces and voxels. ▪ Planar Spectral Imaging tools in the Tool Palette.
Show Activity Window on:	A drop-down list of options for when to display the activity log (Figure E.2).
Save Settings	Save float-corrected image - Saves an image after all corrections are applied (read bias subtraction, flat field correction, cosmic correction). Color Selections - Applies the color settings of the active image data to subsequently opened image data. Folder Locations - Sets the default folder path to the current folder path setting. Click the **Export** button in the image window to view the current folder path setting (Figure E.2). Window Size & Position - Applies the active image window size and position settings to subsequently opened image data. Most Recently Used Dataset History - Defines the number of recently opened datasets to remember and display when you select **File** → **Recent Files** → **Menu**.
Display ROI Label As Measurement	Sets the type of measurement in counts, radiance (photons), or efficiency to show in the ROI label

Some of the general preferences specify how the main application window is organized. To undock the Tool Palette, click on the palette title bar and drag it a distance greater than its width. To dock the Tool Palette in the main window, drag the palette to the right or left side of the window and release.

Figure E.2 Main Application Window



E.2 Options

Figure E.3 Preferences – Options

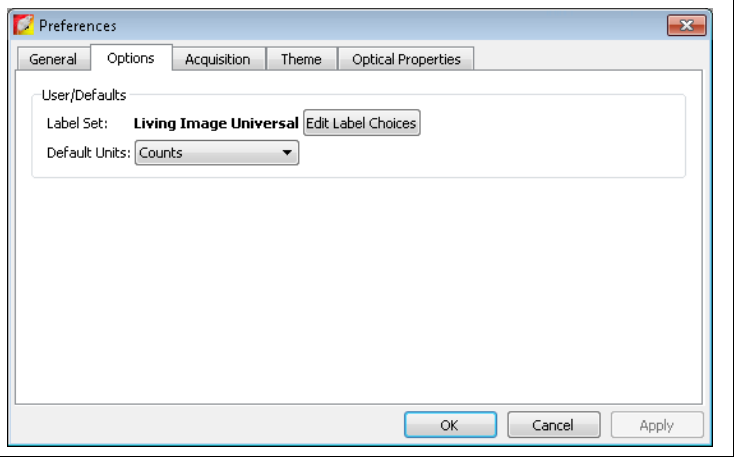


Table E.2 Preferences – Options

Item	Description
Edit label Choices	Opens a dialog box that enables you to edit the Living Image Universal label set.
Default Units	Choose counts or radiance (photons) for image display.

E.3 Acquisition

Figure E.4 Acquisition Preferences – Auto Exposure

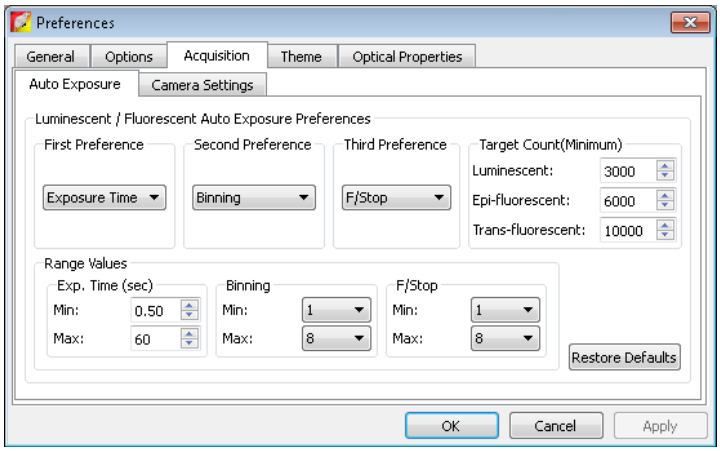


Table E.3 Auto Exposure Settings

Item	Description
Luminescent/Fluorescent Auto Exposure Preferences	
First Preference Second Preference Third Preference	During auto exposure, the software acquires a luminescent or fluorescent image so that the brightest pixel is approximately equal to the user-specified Target Count (Minimum). If the target minimum count cannot be closely approximated by adjusting the first preference (for example, exposure time), the software uses the first and second or first, second and third preferences to attempt to reach the target max count during image acquisition.
Target Count (Minimum)	A user-specified intensity.
Range Values Exp Time (sec) Binning F/Stop	The minimum and maximum values define the range of values for exposure time, F/Stop, or binning that the software can use to attempt to reach the target max count during image acquisition.
Restore Defaults	Click to apply default settings.

Figure E.5 Acquisition Preferences – Camera Settings

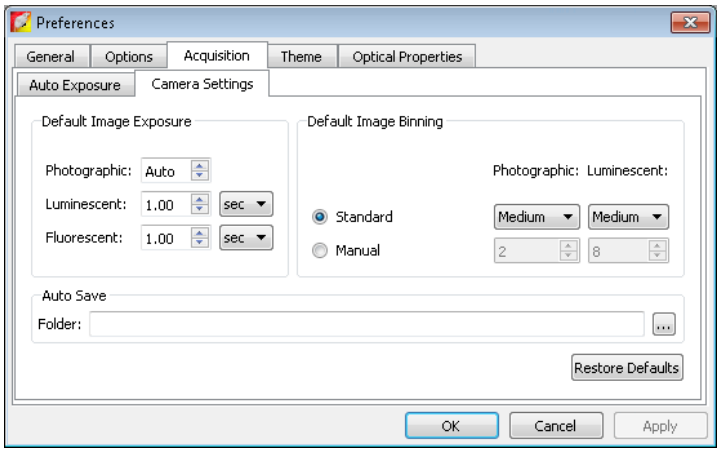


Table E.4 Camera Settings

Item	Description
Default Image Exposure	Sets the default exposure settings that appear in the IVIS acquisition control panel.
Default Image Binning	Standard - Binning choices include Small, Medium and Large. These are predetermined, factory-loaded binning values that depend on the imaging system camera. Manual - Allows the user to choose a binning value (1, 2, 4, 8 or 16)
Auto Save	Specifies the folder where images are automatically saved. Click the … button to select a folder.
Restore Defaults	Click to apply the default settings.

E.4 Theme

Figure E.6 Image View Preferences

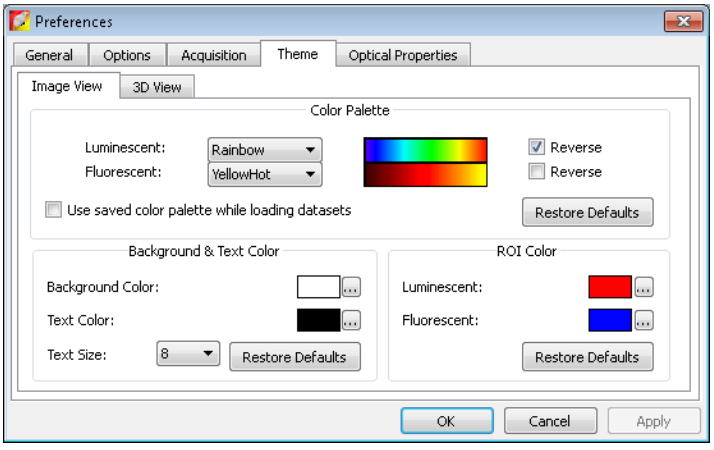


Table E.5 Image View Preferences

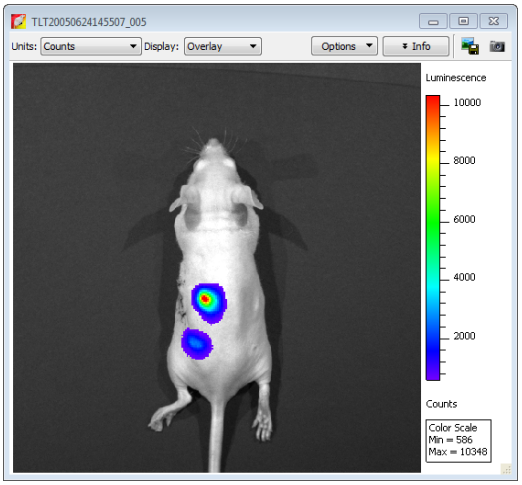
Item	Description
Color Palette	Use these controls to select a color table for luminescent and fluorescent image data. Choose the Reverse option to reverse the min/max colors of the selected color table.
Use saved color palette while loading datasets	If this option is chosen, data are displayed using a user-specified color palette. For example, after you load data, specify a color table in the Image Adjust tools, and save the data. The user-specified color table is automatically applied whenever the data are loaded.
Background & Text Color	Sets the color of the: ▪ Background in the image window (shown below) ▪ Text for the color bar To change a color, click the … button that opens the color palette. 
ROI Color	Sets the colors for the ROI outline. To change a color, click the … button that opens the color palette. Luminescent - Color of the ROI outline on a luminescent image. Fluorescent - Color of the ROI outline on a fluorescent image.
Restore Defaults	Click to apply the default settings.

Figure E.7 3D View Preferences

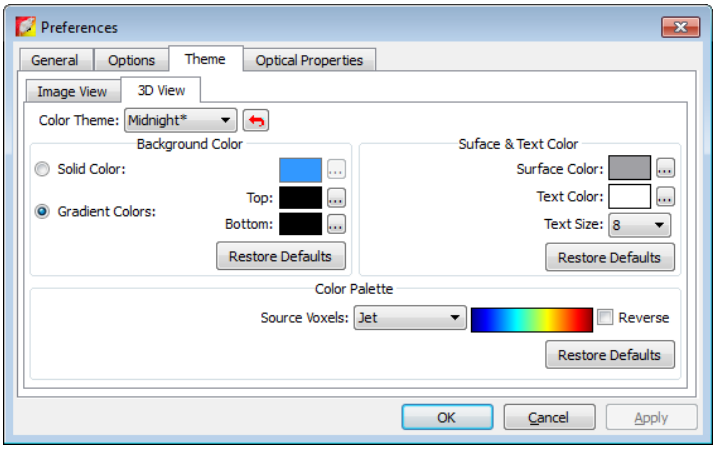
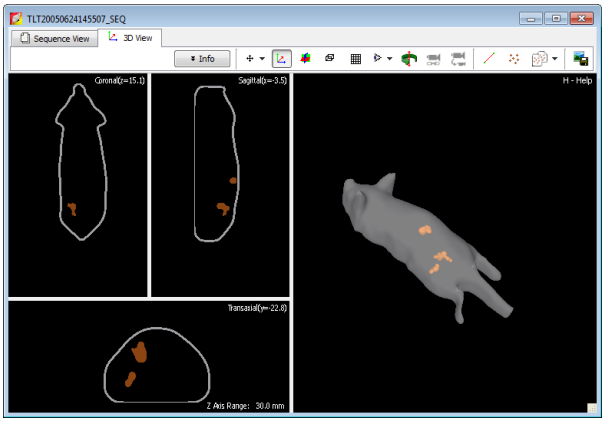


Table E.6 3D View Preferences

Item	Description
Color Theme	Predefined color schemes available for the 3D View window shown here. Click the ↩ button to restore the defaults for the selected color theme. 
Background Color	Settings that modify the appearance of the background in the 3D View window. Solid Color - Choose this option to apply a non-gradient background color to the 3D view in the image window. Gradient Color - Choose this option to apply a gradient background color to the 3D view in the image window. Top = the color at the top of the window; Bottom = the color at the bottom of the window.
Surface & Text Color	Settings that modify the display of the surface and text in the 3D View window.
Color Palette	Source voxels - Choose a color table for voxel display. Reverse - Choose this option to reverse the min/max colors of the selected color table.
Restore Defaults	Click to apply the default settings.

E.5 Optical Properties

Figure E.8 Set Default Optical Properties Preferences (left) for the Properties Tab in the Planar Spectral Imaging, DLIT, or FLIT Tools

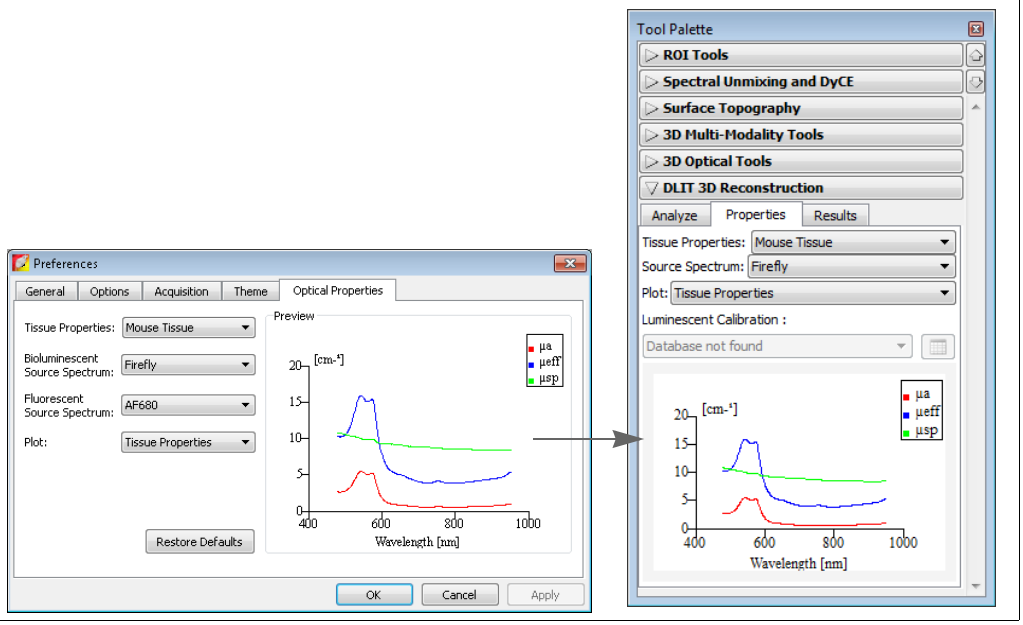


Table E.7 Preferences – Optical Properties

Item	Description
Tissue Properties	Choose a default tissue type that is most representative of the area of interest. This tissue type will be used if a Subject Type is not selected in the Imaging Wizard and saved during acquisition.
Source Spectrum	Choose the default luminescent source spectrum. This Source Spectrum will be used if a Subject Type is not selected in the Imaging Wizard and saved during acquisition for DLIT sequences.
Plot	Tissue Properties - Choose this option to display a graph of the absorption coefficient (μ_a), effective attenuation coefficient (μ_{eff}), and reduced scattering coefficient (μ'_s or μsp). Source Spectrum - Choose this option to display the source spectrum for DLIT reconstructions. Bioluminescent Spectrum - Choose this option to display the spectrum of the bioluminescent source (available for DLIT reconstructions only). Fluorescent Spectrum - Choose this option to display the spectrum of the fluorescent source (available for FLIT reconstructions only).
Restore Defaults	Click to restore the defaults in the Optical Properties tab.

Appendix F Menu Commands, Toolbars, and Shortcuts

Figure F.1 Living Image Toolbar

Table F.1 Menu bar commands and toolbar buttons

Menu Bar Command	Toolbar Button	Description
File → Open		Displays the Open box so that you can select and open an image data file. Double-click a SequenceInfo.txt file or ClickInfo.txt file to open the image data file (see page 61).
File → Browse		Displays the Browse For Folder box so that you can select and an image data folder. The selected folder is displayed in the Living Image Browser.
File → Browse 3D Volumetric Data		Displays the Browse For Folder box so that you can select a volumetric data folder (for example, DICOM format, TIF data). The selected folder is displayed in the 3D Browser.
File → Save		Saves (overwrites) the AnalyzedClickInfo text file to update the analysis parameters, but the original image data files are not altered.
File → Save As		Displays the Browse For Folder box so that you can specify a folder in which to save the image data. The original data is not overwritten.
File → Import → 3D Surface		Opens a dialog box that enables you to import a surface. **Note:** This menu item is only available if "Show Advanced Options" is selected in the Preferences (see page 273).
File → Import → 3D Voxels		Opens a dialog box that enables you to import a source volume. **Note:** This menu item is only available if "Show Advanced Options" is selected in the Preferences (see page 273).
File → Import → Atlas		Opens a dialog box that enables you to import an organ atlas (.iv, .dxf, .stl).
File → Export → Image/ Sequence as DICOM		Opens the Browse for Folder dialog box that enables you to export the active image data to DICOM format (.dcm).
File → Export → 3D Surface		Opens a dialog box that enables you to save the 3D surface of the active data to a file such as Open Inventor format (.iv).
File → Export → 3D Voxels		Opens a dialog box that enables you to save the voxel information from the active data.
File → Export → 3D Scene as DICOM		Opens a dialog box that enables you to save a 3D reconstruction and/or surface in DICOM format. The Multi-Frame DICOM option supports 3D CT reconstruction in third party software.
File → Print		Displays the Print box.
File → Print Preview		Displays the Print Preview box that shows what will be printed.

Table F.1 Menu bar commands and toolbar buttons (continued)

Menu Bar Command	Toolbar Button	Description
File → Recent Files		Shows recently opened datasets. **Note:** The number of files displayed can be set in the Preferences box (select **Edit → Preferences** and click the General tab).
File → Logout		Opens the Select/Add User ID dialog box so that another user can logon or a new user ID can be added to the system.
File → Exit		Closes the Living Image software.
Edit → Copy		Copies the active image window to the system clipboard.
Edit → Image Labels		Opens the Edit Image Labels dialog box that enables you to edit the label set information for the active data (see Figure 4.6 on page 29).
Edit → Preferences		Opens the Preferences box (see page 273).
View → Tool Bar		Choose this option to display the toolbar.
View → Status Bar		Choose this option to display the status bar at the bottom of the main window.
View → Tool Palette		Choose this option to display the Tool Palette.
View → Activity Window		Displays the Activity window at the bottom of the main application window. The Activity window shows a log of the system activity.
View → Image Information		Displays the Image Information box that shows the label set and image acquisition information for the active data.
View → ROI Properties		Displays the ROI Properties dialog box (see page 112).
View → 3D ROI Properties		Displays the 3D ROI Properties dialog box (see page 178).
View → ROI Measurements		Displays the ROI Measurements table.
View → Volume Data Viewer		Enables you to open and view DICOM data.
View → Image Layout Window		Opens the Image Layout window that enables you to paste an image of the active data in the window.
Tools → 3D Animation		Opens the 3D Animation window that enables you to view a preset animation or create an animation.
Tools → Longitudinal Study		Opens the Longitudinal Study window for side-by-side comparisons of DLIT or FLIT results.
Tools → Well Plate Quantification for ...		Opens the Well Plate Quantification window.
Tools → Image Overlay for...		Opens the Image Overlay window for the active data.
Tools → Colorize		Opens the Colorized View tab for the active sequence.
Tools → Image Math for...		Opens the Image Math window for the active data.
Acquisition → Background → Measure Dark Charge		Opens a dialog box that enables you to acquire a dark charge measurement.
Acquisition → Background → Add or Replace Dark Charge		Opens a dialog box that enables you to select an instrument luminescent background. This background measurement is subtracted from luminescent images.

Table F.1 Menu bar commands and toolbar buttons (continued)

Menu Bar Command	Toolbar Button	Description
Acquisition → Background → Measure and Replace Dark Charge		Measures the dark charge under the same conditions as the currently selected image. When the measurement is complete, the newly acquired dark charge image will be included in the dataset of the current image, replacing any existing dark charge image that may be present in the dataset.
Acquisition → Background → View Available Dark Charge		Opens a dialog box that enables you to view the dark charge measurements for the system.
Acquisition → Background → Clear Available Dark Charge		Clears all dark charge images from the system.
Acquisition → Background → Auto Background Setup		Opens a dialog box that enables you to acquire background images, or schedule or disable automatic background acquisition.
Acquisition → Fluorescent Background → Measure Fluorescent Background		Starts a measurement of the instrument fluorescent background.
Acquisition → Fluorescent Background → Add or Replace Fluorescent Background		Opens a dialog box that enables you to select an instrument fluorescent background measurement for the active image data. If the "Fluorescent Background" Subtraction option is chosen in the Corrections/Filtering Tool Palette, the background measurement is subtracted from the image data.
Acquisition → Fluorescent Background → Measure and Replace Fluorescent Background		Measures fluorescent background under the same conditions as the currently selected image. When the measurement is complete, the newly acquired background image will be included in the dataset of the current image, replacing any existing background image that may be present in the dataset.
Acquisition → Fluorescent Background → View Available Fluorescent Background		Opens a dialog box that displays the fluorescent background measurements for the system. If a fluorescent background is selected, the "Fluorescent Background Subtraction" option appears in the Corrections/Filtering Tool Palette. Choose the "Fluorescent Background Subtraction" option to subtract the user-specified background measurement from the image data.
Acquisition → Fluorescent Background → Clear Available Fluorescent Background		Opens a dialog box that enables you to remove the fluorescent background measurements from the system.
Acquisition → Auto-Save		If Auto-Save is selected, all images are automatically saved to a user-selected folder.
Acquisition → Auto-Save To		Opens a dialog box that enables you to select a folder where images will be saved to automatically.
Window → Close		Closes the active image window.
Window → Close All		Closes all image windows.
Window → Cascade		Organizes the open image windows in a cascade arrangement (see Figure 5.7 on page 66).
Window → Tile		Organizes the open image windows in a tiled arrangement (see Figure 5.7 on page 66).
Window → 1. <Image or Sequence name> **Window → 2. <Image or Sequence name>** **Window → etc.**		A list of the open image windows. Click a window in the list to make it the active window (indicated by a check mark).

Table F.1 Menu bar commands and toolbar buttons (continued)

Menu Bar Command	Toolbar Button	Description
Window → Other Windows → <window name>		Lists other windows that are open. For example, If the Living Image Browser is open, use these commands to make the browser the active window and display it on top of all other open windows.
Help → User Guide		Displays the Living Image Software Manual.
Help → Tech Notes		Displays a folder of technical notes. **Note:** For the most recent collection of technical notes, please see the IVIS University download page.
Help → License information		Displays the license information.
Help → Plug-in Information		Displays a list of tool plug-ins and Tool Palette plug-ins.
Help → IVIS Reagents		Opens the PerkinElmer web page for In Vivo Imaging Reagents.
Help → About Living Image		Displays information about the Living Image software and PerkinElmer technical support contact information.
		Click this button, then click an item in the user interface to display information about the item.

Table F.2 Keyboard shortcuts

Keys	Shortcut Description
Ctrl + B	Opens the Living Image Browser.
Ctrl + C	Copies the active image to the system clipboard.
Ctrl + D	Arranges open windows in a cascade.
Ctrl + O	Displays a dialog box that enables you to open data.
Ctrl + P	Open the Print dialog box.
Ctrl + S	Saves the active file or window.
Ctrl + T	Tiles the open windows.
Ctrl + W	Closes the active window.
Shift + F1	Changes the mouse pointer to the “What’s This” tool  . Click this button, then click an item in the user-interface to display information about the item.

NOTE: Macintosh users use the Cmd key (apple key) instead of the Ctrl key.

Index

Symbols

A

B

C

D

T

U

V

W

X

Z