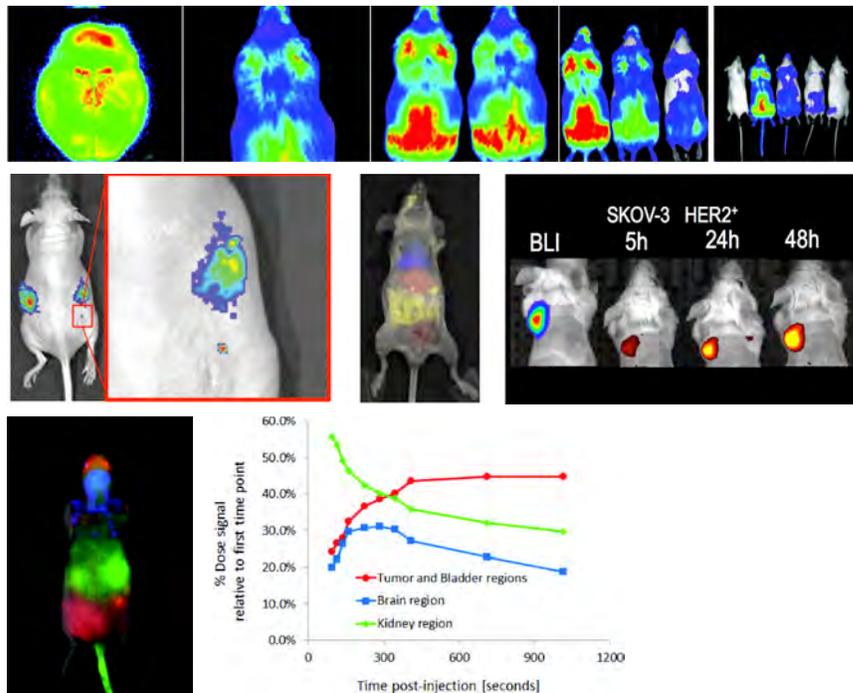




Living Image® 4.5.4 Software

March 2017



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1 Welcome

About This Manual

What's New in Living Image 4.5.4

Living Image Help on page 2

Contact Information on page 3

1.1 About This Manual



NOTE: This *Living Image Software 4.5.4 Manual* is only for use with the IVIS Lumina Series III or IVIS Lumina instrument. 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 Lumina Series III and analyze the data using Living Image software. The manual provides detailed instructions and screenshots for Living Image software tools that are available for data acquired on the IVIS Lumina Series III. Sometimes screenshots in the manual may not exactly match those displayed on your screen.

Please see the *IVIS Lumina Series III Hardware Manual* for information on the 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

Living Image software improvements specific to the IVIS Lumina Series III Imaging System are listed below.

Item	Description	See
Additional operating system support	Living Image now supports: <ul style="list-style-type: none"> ■ 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: <ul style="list-style-type: none"> ■ Export all of the images in a sequence to one graphic file. 	page 79
	<ul style="list-style-type: none"> ■ Export all images of a user-selected subject to one graphic file. 	page 79
	<ul style="list-style-type: none"> ■ Export each image of a user-selected subject to a separate graphic file. 	page 81

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.
<i>Living Image Software Manual</i>	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) <ul style="list-style-type: none"> ■ Setup and Sequence Acquisition ■ Topography ■ Source Reconstruction and Analysis
Drawing ROIs
Fluorescence Tomography (FLIT) <ul style="list-style-type: none"> ■ 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 <ul style="list-style-type: none"> ■ 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

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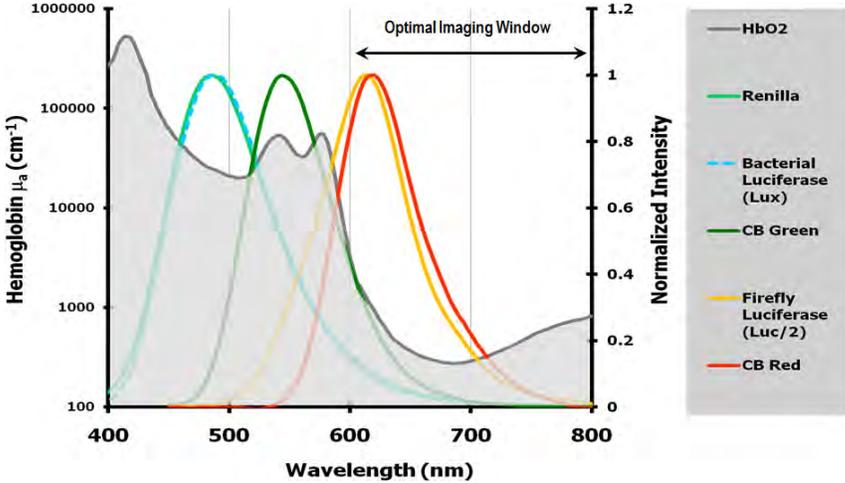
2 Imaging Overview

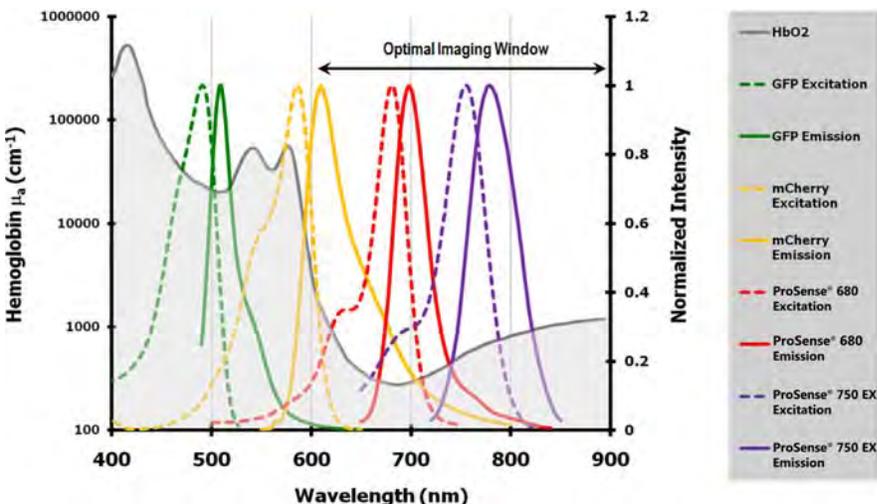
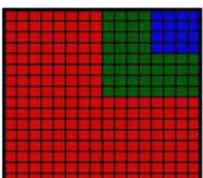
Example Imaging Workflow

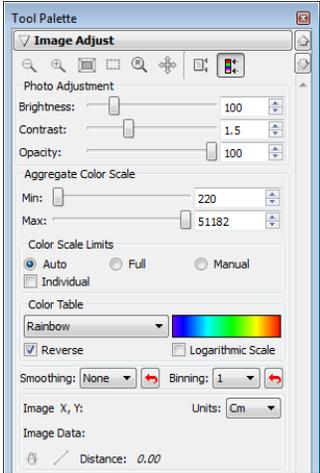
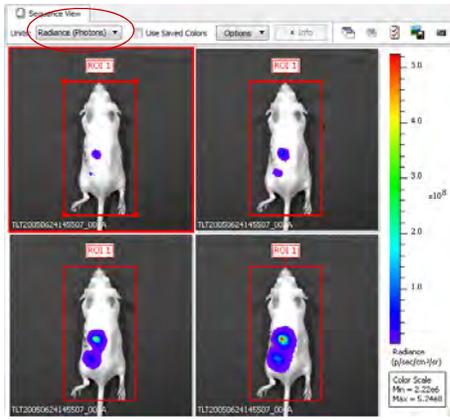
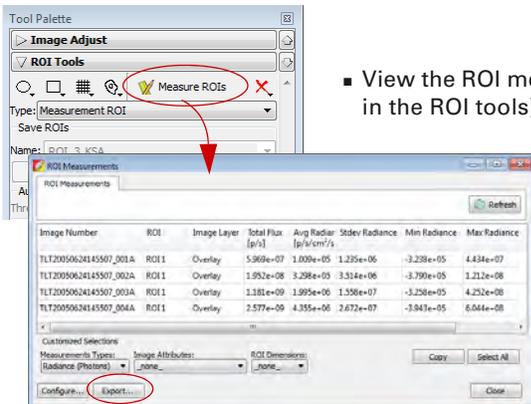
Overview of Image Acquisition on page 8

Overview of Living Image Tools and Functions on page 10

2.1 Example Imaging Workflow

Workflow Step	For More Details:
<p>1. Plan the experiment.</p> <ul style="list-style-type: none"> ■ 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: <ul style="list-style-type: none"> ■ 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: <ul style="list-style-type: none"> ■ 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. 	

Workflow Step	For More Details:
<p>1. Plan the experiment (continued).</p> <ul style="list-style-type: none"> 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. 	
<p>2. Determine the optimal imaging time post-injection.</p> <ul style="list-style-type: none"> 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. 	<p>See this technical note for a protocol to determine a kinetic curve for luciferin.</p>
<p>3. Prepare and image the subjects.</p> <ul style="list-style-type: none"> 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".  <ul style="list-style-type: none"> 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. <ul style="list-style-type: none"> 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. <ul style="list-style-type: none"> 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). 	<p>See imaging protocols for PerkinElmer <i>in vivo</i> imaging reagents such as ProSense® 680.</p> <p>Also see:</p> <ul style="list-style-type: none"> Page 8 for an overview of image acquisition. Chapter 4, Image Acquisition on page 22. Appendix A, IVIS Acquisition Control Panel on page 159.

Workflow Step	For More Details:
<p>4. Select images for viewing.</p> <ul style="list-style-type: none"> Load the images as a group (select the images in the Living Image Browser and click Load as Group).  <ul style="list-style-type: none"> If necessary, adjust the photo brightness, contrast, or opacity using the Image Adjust tools. Apply the same color scale to all images: <ul style="list-style-type: none"> Uncheck the “Individual” option. Adjust the color scale Min and Max. The changes are simultaneously applied to all images. 	<p>See page 49 for more about the Living Image Browser.</p> <p>See page 58 for information on the Image Adjust tools.</p>
<p>5. Measure signals and analyze the data.</p>  <ul style="list-style-type: none"> Choose the appropriate units: <ul style="list-style-type: none"> “Radiance (Photons)” for luminescence “Radiant Efficiency” for fluorescence. <p>Radiance and Radiance 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.</p> <ul style="list-style-type: none"> 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.  <ul style="list-style-type: none"> 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 	<p>See <i>Concept Tech Note 2 – Image Display and Measurement</i> for more information on measurement units (select Help → Tech Notes).</p> <p>See page 92 for instructions on measuring signal.</p> <p>See Table 6.8 on page 108 for information on ROI measurements.</p>

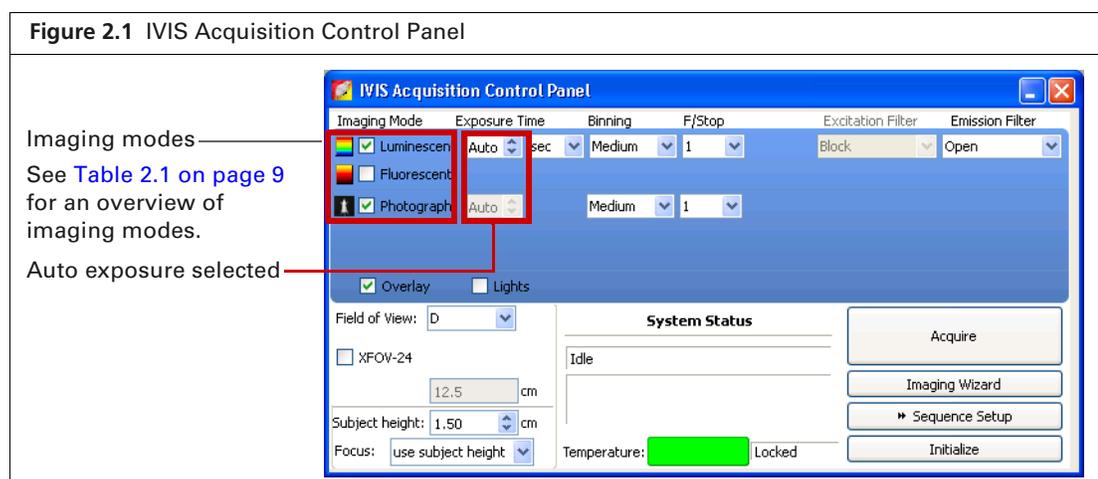
2.2 Overview of Image Acquisition

Control Panel

The control panel provides the image acquisition functions (Figure 2.1). See Appendix A on page 159 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), and the installed filter wheel or lens option.



Auto Exposure Feature

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 173 for more details.

Imaging Wizard

The Imaging Wizard provides a convenient option for image or sequence setup (see Figure 4.16 on page 35). 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 36 shows the types of images or sequences that the Imaging Wizard can set up.

Imaging Modes on IVIS Lumina Series III

Optical imaging detects photons in the visible range of the electromagnetic spectrum. [Table 2.1](#) briefly explains the types of optical images that can be acquired on the IVIS Lumina Series III.

Table 2.1 Imaging Modes – IVIS Lumina Series III

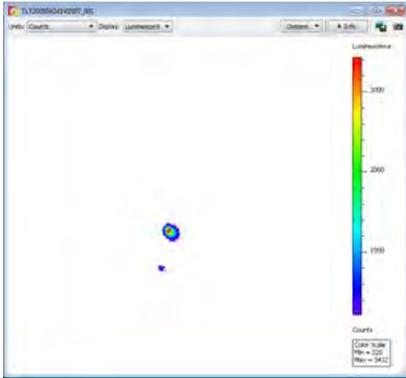
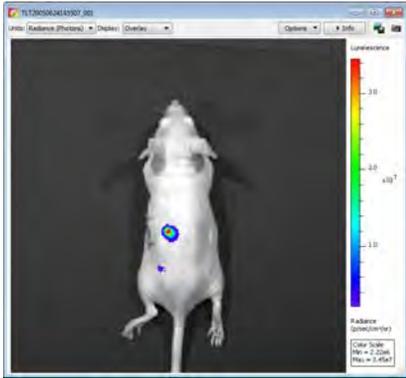
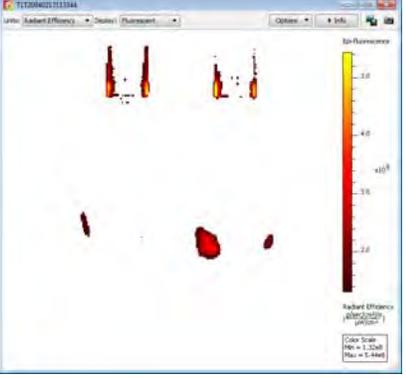
Imaging Mode	Description and Example
Luminescent optical imaging 	<div style="display: flex; justify-content: space-around;"> <div data-bbox="373 441 779 819">  </div> <div data-bbox="974 441 1380 819">  </div> </div> <p data-bbox="373 829 860 997">Luminescent image – A longer exposure of the subject 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.</p> <p data-bbox="982 829 1453 850">Overlay: Luminescent image on photograph</p>
Fluorescent optical imaging 	<div style="display: flex; justify-content: space-around;"> <div data-bbox="373 1018 779 1396">  </div> <div data-bbox="974 1018 1380 1396">  </div> </div> <p data-bbox="373 1407 909 1543">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.</p> <p data-bbox="373 1554 909 1659">The optical fluorescent image data can be displayed in units of counts or photons (absolute, calibrated), or in terms of efficiency (calibrated, normalized).</p> <p data-bbox="373 1669 909 1753">Note: See the concept tech note Image Display and Measurement for more on quantifying image data (select Help → Tech Notes on the menu bar).</p> <p data-bbox="982 1407 1453 1428">Overlay: Fluorescent image on photograph</p>

Table 2.1 Imaging Modes – IVIS Lumina Series III (continued)

Imaging Mode	Description and Example
Photograph 	 <p>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.</p>

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 D.1 on page 171).

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

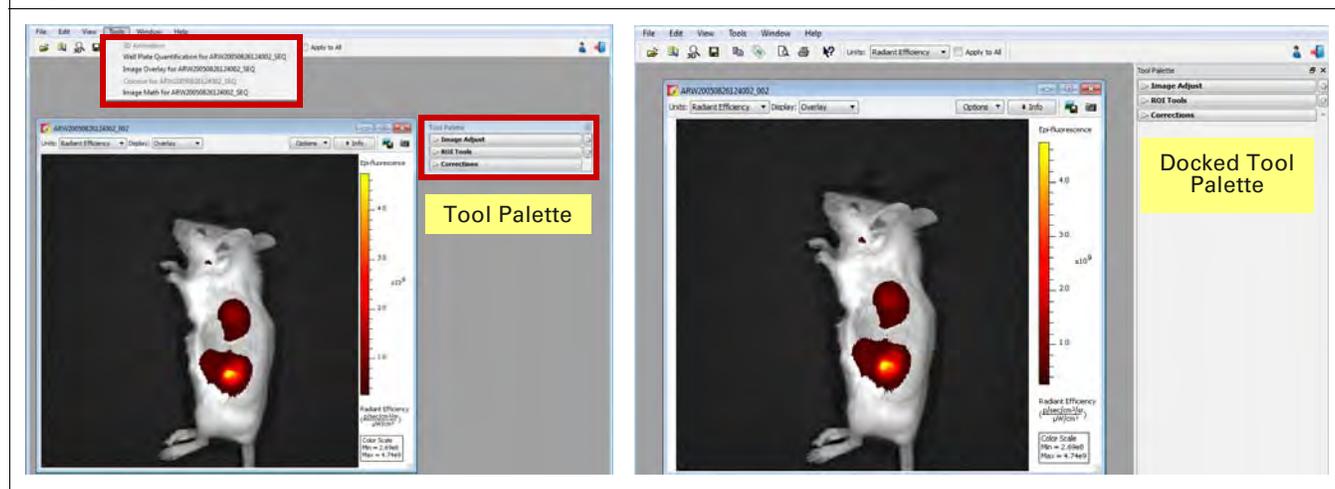


Table 2.2 provides an overview of the tools available for data acquired on the IVIS Lumina Series III. If analyzing data acquired on a different type of IVIS instrument, say for example the IVIS Spectrum CT, please see the Living Image Software Manual specific for that imaging system.



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

Table 2.2 Living Image Tools Available for Data Acquired on the IVIS Lumina Series III

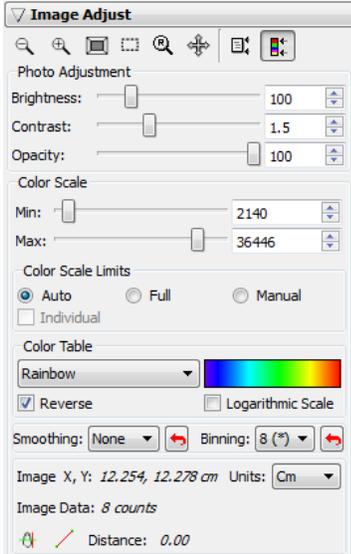
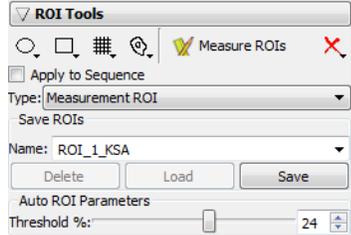
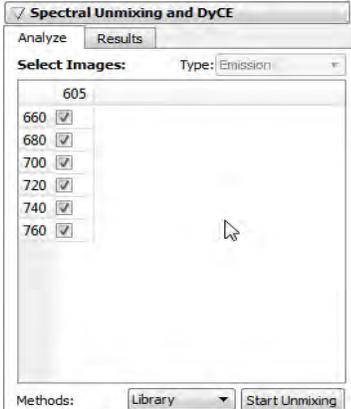
Living Image Tools and Functions	See Page
	<p>Image Adjust 62</p> <ul style="list-style-type: none"> ■ Tools for image display (zoom, crop, or pan). ■ Adjust photo display – Tune the photograph brightness, contrast, or opacity. ■ Manage the color table for image display. ■ Apply smoothing or binning to an image. ■ View image data (counts, radiance, or efficiency) at an X,Y location. ■ Measure distance in an image. ■ View a line plot of pixel intensities.
	<p>ROI Tools 88</p> <p>Specify a region of interest (ROI) in an image and measure the signal intensity within the ROI.</p>
	<p>Corrections 169</p> <p>Apply corrections to the raw data.</p>
	<p>Spectral Unmixing 113</p> <p>Use spectral unmixing to:</p> <ul style="list-style-type: none"> ■ 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

Table 2.2 Living Image Tools Available for Data Acquired on the IVIS Lumina Series III (continued)

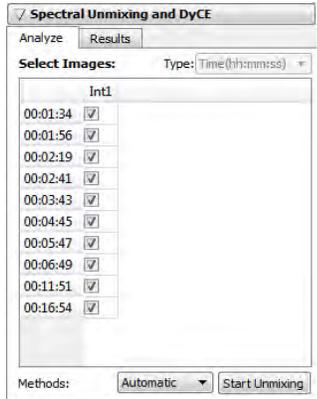
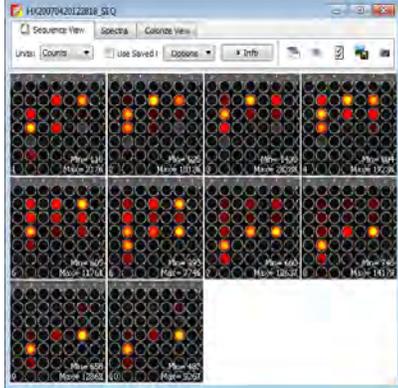
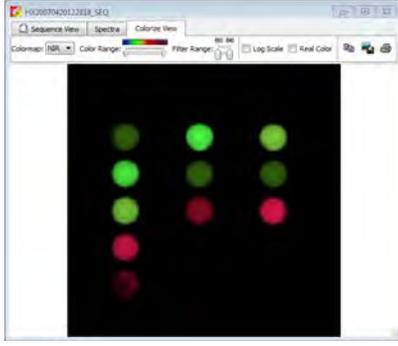
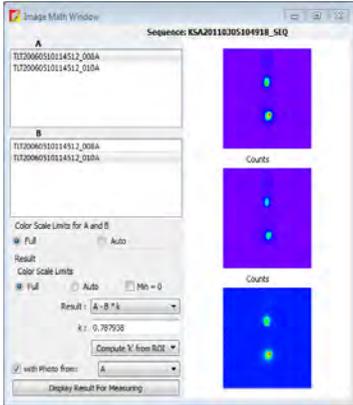
Living Image Tools and Functions	See Page
	<p>DyCE (Dynamic Contrast Enhancement) 140</p> <p>Use DyCE to:</p> <ul style="list-style-type: none"> ■ Determine real-time pharmacokinetic (spatio-temporal biodistribution) of a probe or dye signal ■ Extract “temporal spectra” (signal intensity as a function of time) from particular anatomical regions <p>Note: DyCE acquisition and analysis tools require a separate license.</p>
	<p>Image Overlay 70</p> <p>Select Tools → Image Overlay for <sequence name> on the menu bar.</p> <p>View multiple fluorescent or luminescent signals in one 2-dimensional image in the Image Overlay window.</p>
	<p>Colorize View 73</p> <p>Select Tools → Colorize for <sequence name> on the menu bar.</p> <p>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.</p> <p>The tool provides a useful way to visualize multiple probes or scale probe signals that are not in the visible range.</p>
<p>Images of Quantum dot nanocrystals (700 or 800 nm) were acquired using different combinations of excitation and emission filters.</p>  <p>Colorize view of the combined images</p>	

Table 2.2 Living Image Tools Available for Data Acquired on the IVIS Lumina Series III (continued)

Living Image Tools and Functions	See Page
	<p>Image Math Window 65</p> <p>Select Tools → Image Math for <sequence name> on the menu bar.</p> <p>Mathematically combine (add, multiply, subtract, or divide) two user-selected images.</p> <p>For example, subtract a blue-shifted background filter image from the primary excitation filter image to remove tissue autofluorescence signal.</p>

3 Getting Started

Starting Living Image Software

Initializing the Imaging System and Checking Temperature on page 16

Managing User Accounts on page 18

Tracking System and User Activity on page 21

3.1 Starting Living Image Software

Living Image software on the PC workstation that controls the IVIS Lumina Series III 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.

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



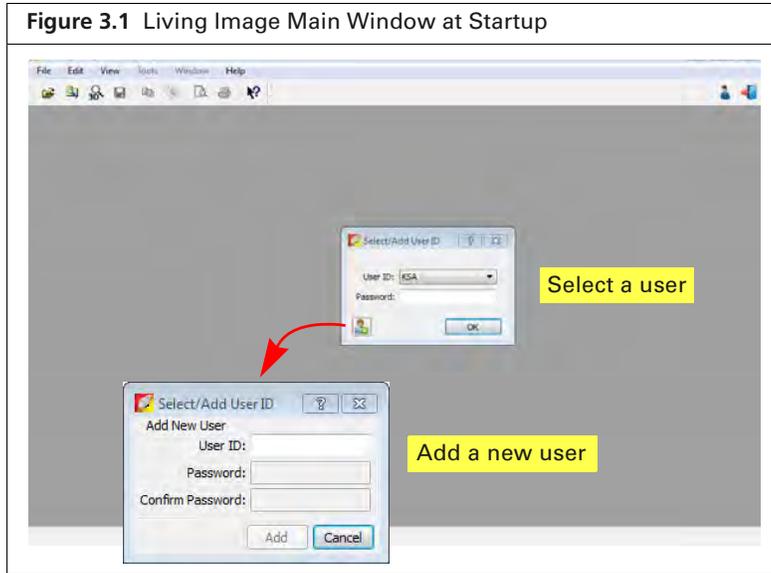
NOTE: All components of the IVIS Lumina Series III should be left on at all times due to the long cooling time required to reach operating (demand) temperature. It is also important to leave the system on to enable automatic overnight electronic background measurements. Periodically rebooting the computer 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](#)).



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**.

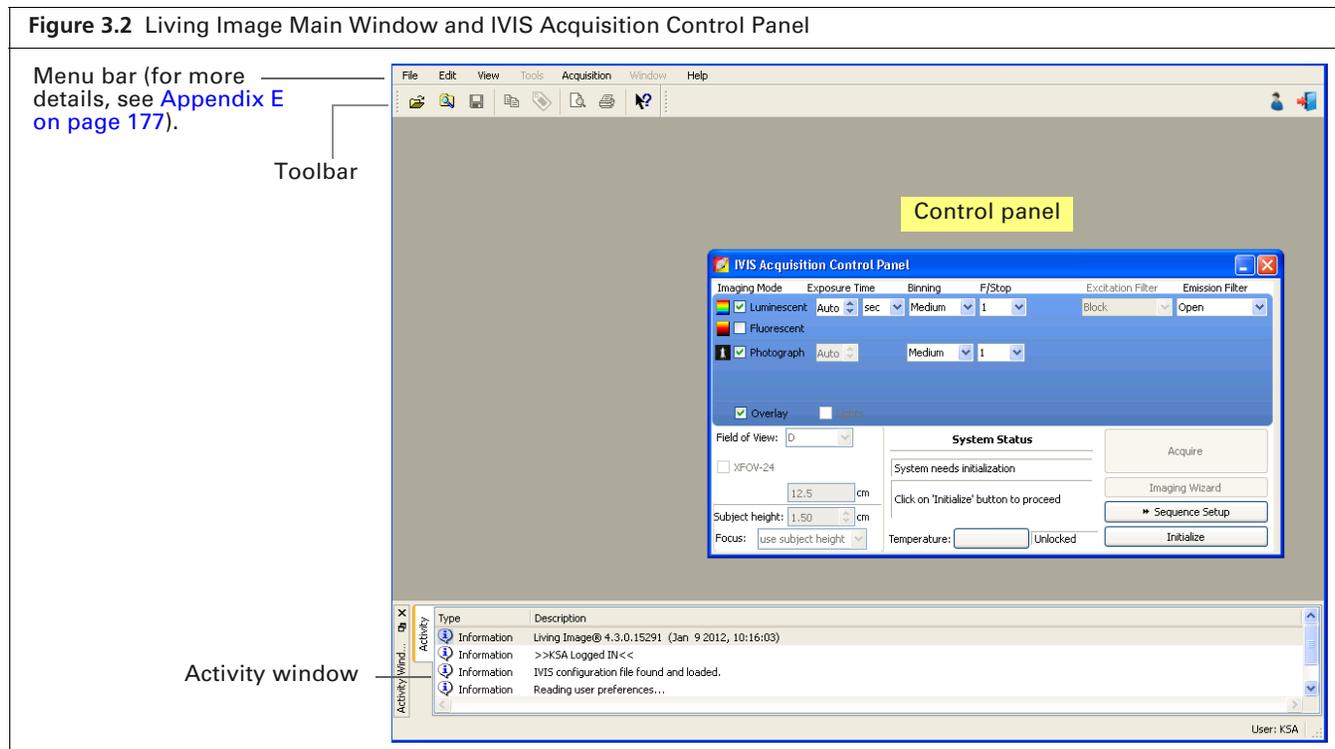
Alternatively, create a new user ID:

- 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 Lumina Series III (Figure 3.2). For more details on the control panel, see [Appendix A on page 159](#).



NOTE: Living Image software has optional password protection for user accounts. See [page 19](#) for more details.



3.2 Initializing the Imaging System and Checking Temperature

The IVIS Lumina Series III 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](#)).



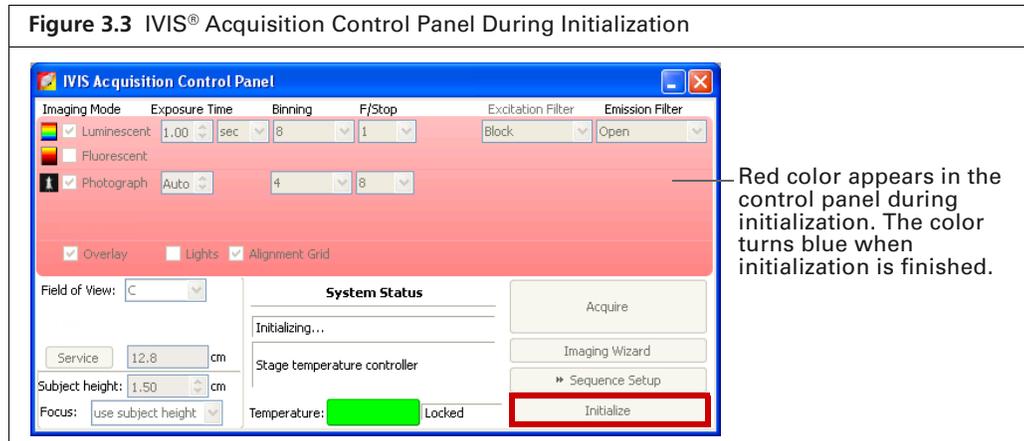
NOTE: The control panel is only available on the PC workstation that controls the IVIS Imaging System. The options available in the IVIS acquisition control panel depend on the imaging system, selected imaging mode (Image Setup or Sequence Setup), and the filter wheel or lens option that are installed.

Initialization moves every motor-driven component in the system (for example, 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 Lumina Series III Hardware Manual* for further details on instrument operation.

Initializing the IVIS Lumina Series III

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.

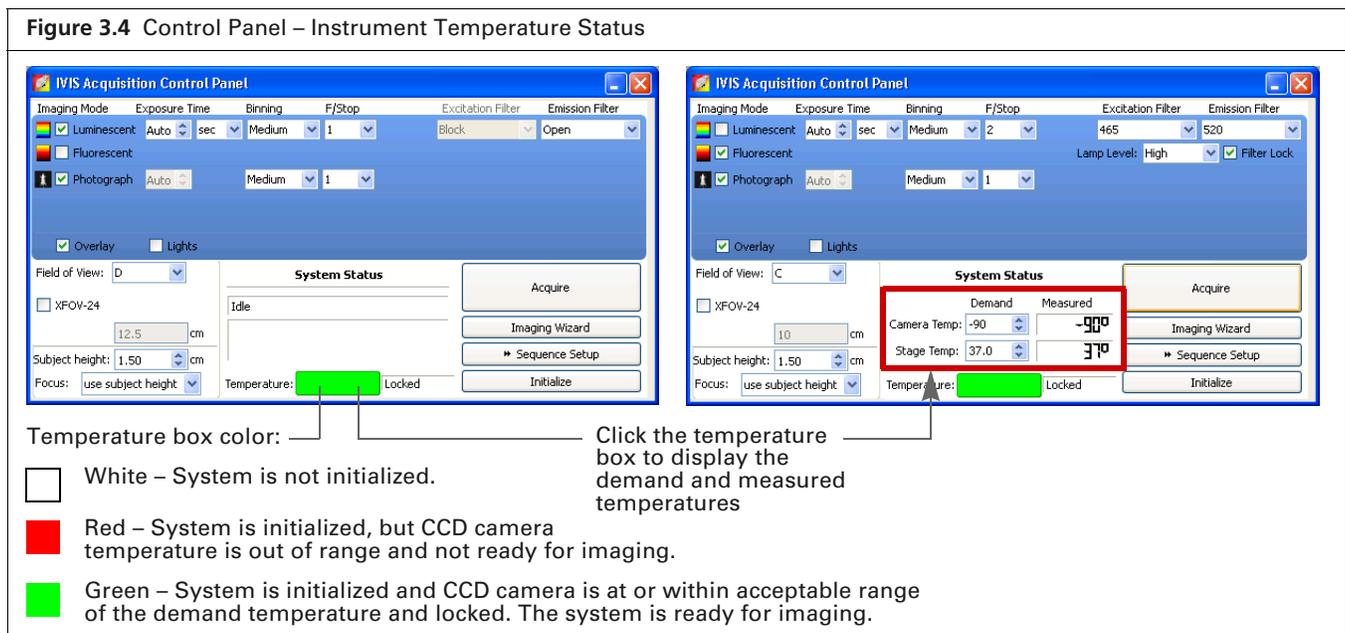


CCD Temperature

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 3).

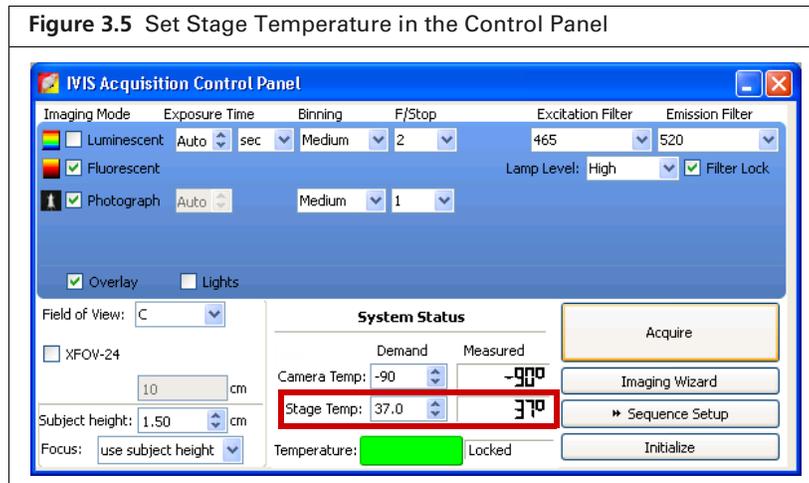
The demand temperature for the CCD camera is preset and generally should not be changed. Electronic feedback control maintains the CCD camera temperature to within a few degrees of the demand temperature.

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



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 stage may be set to a temperature from 20 - 40 °C.

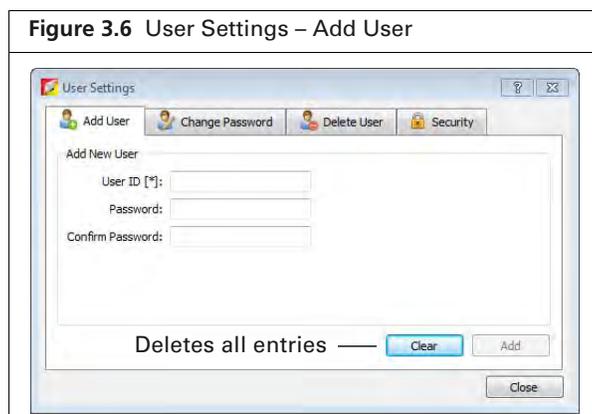


3.3 Managing User Accounts

Adding Users

New users can be created in the:

- Main window at startup (see [page 15](#)).
 - 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.



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.



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 20](#) 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.



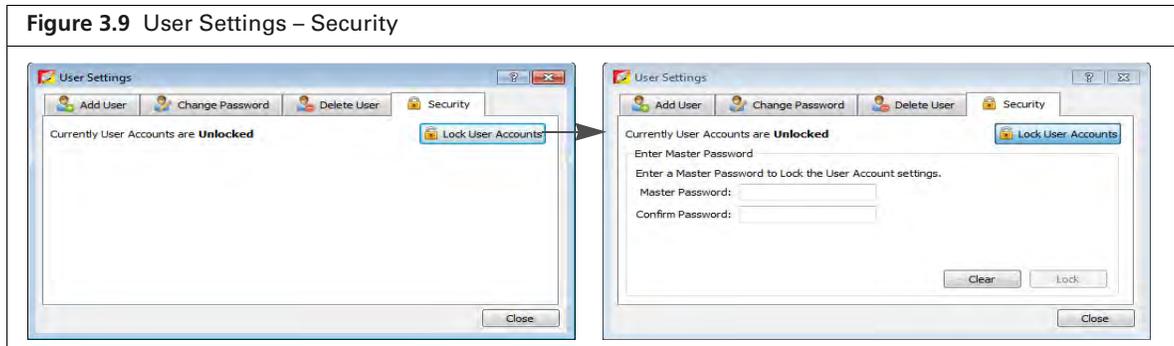
3. Select a User ID.
4. If the accounts are locked, enter the master password.
5. Click **Delete** and **Close**.

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:

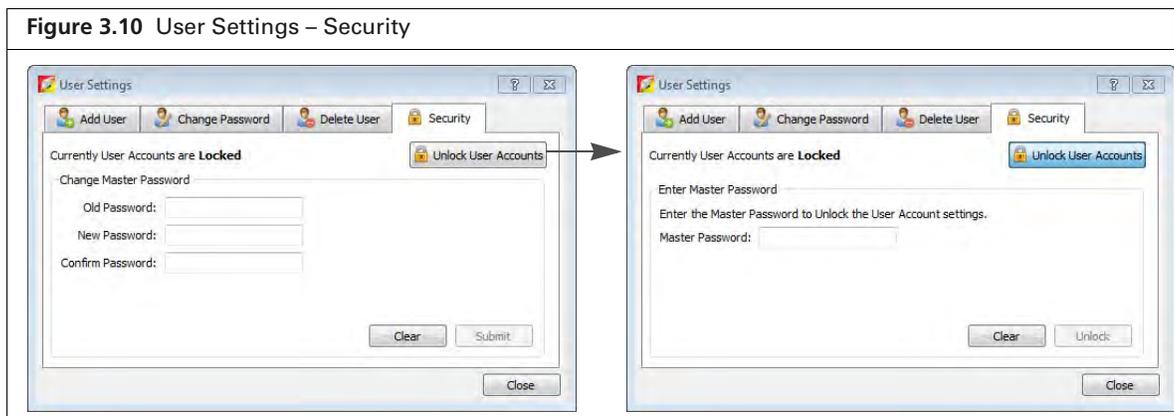
1. Select **Edit** → **User settings** on the menu bar.
2. Click the Security tab in the dialog box that appears.
3. Click **Lock User Accounts**.



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**.

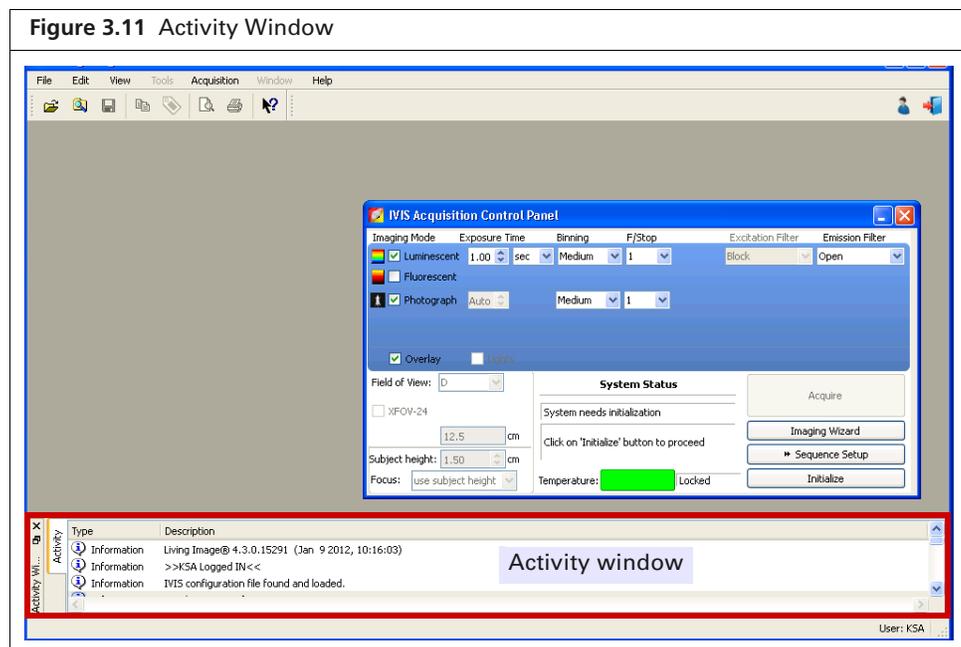


3.4 Tracking System and User Activity

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).



4 Image Acquisition

Luminescent Imaging

Fluorescent Imaging on page 28

Cherenkov Imaging on page 34

Acquire a Sequence Using the Imaging Wizard on page 34

Acquire Multiple Sequences in Batch Mode on page 40

Manually Set Up a Sequence on page 42

Imaging With the ZFOV-2.6 Zoom Lens on page 46

Manually Save Image Data on page 47

Exporting Images on page 48

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 24](#).

See [page 34](#) for information on acquiring a luminescent sequence using the Imaging Wizard.

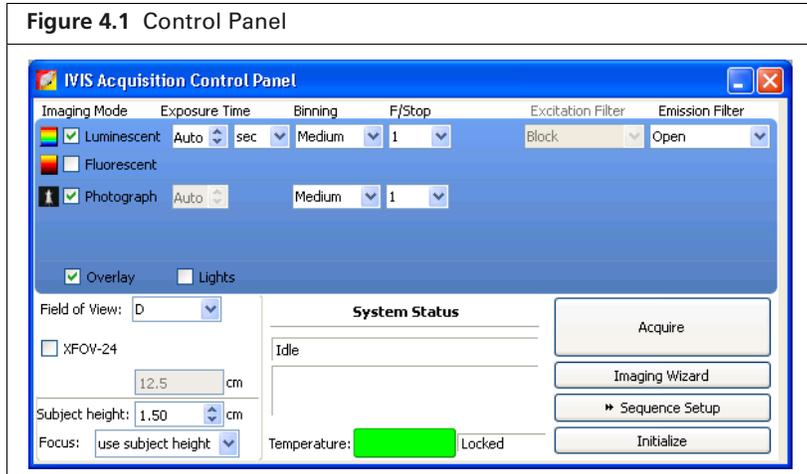
Quick Guide

1. Start Living Image software  and initialize the IVIS Lumina Series III (see [page 16](#) for details).

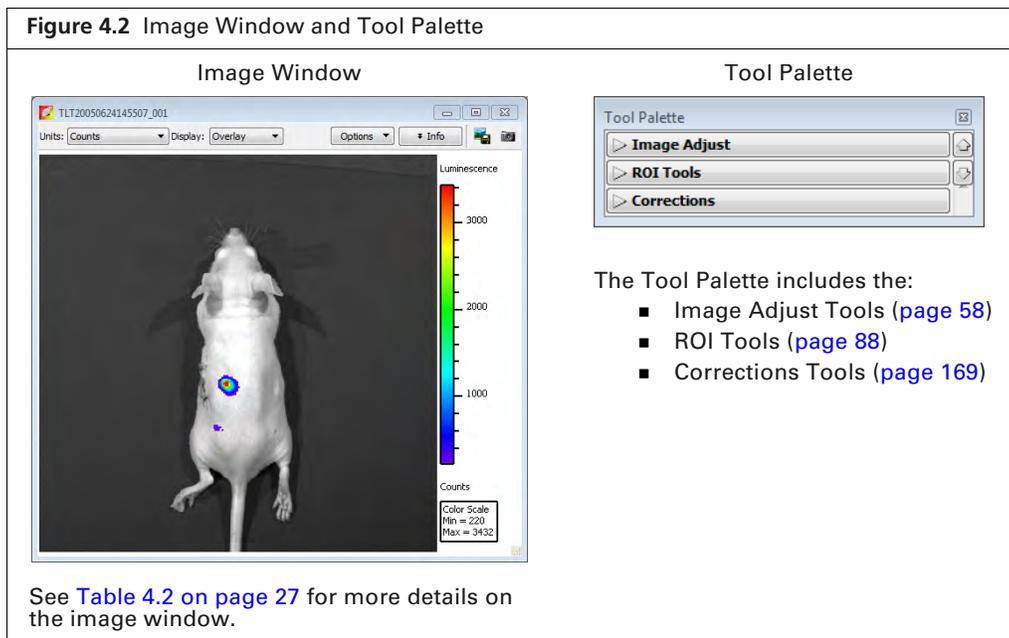


NOTE: See the *IVIS Lumina Series III Hardware Manual* for more information on the instrument

2. Place the anesthetized subjects in the imaging chamber and close the door.
3. In the control panel ([Figure 4.1](#)):
 - a. Put a check mark next to **Luminescent** and select **Auto** exposure.
 - b. Choose **Photograph** (optional, selecting **Photograph** automatically selects **Overlay**).
 - c. Select a field of view (FOV) (see [Table 4.1 on page 25](#)).
 - d. Enter the subject height (cm) and select the **use subject height** focus option.
 - e. Click **Acquire**.



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).



Acquire a Luminescent Image

This section provides detailed instructions for luminescent imaging.

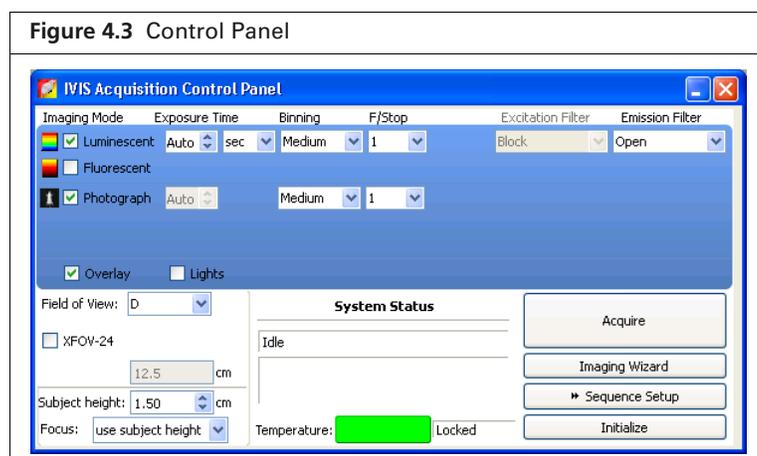
 **NOTE:** The IVIS Lumina Series III should be initialized and the temperature locked before setting the imaging parameters in the control panel. See [page 16](#) for more details.

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 159](#) for details on these parameters.



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 Lumina Series III

FOV Setting	FOV (cm)
A ¹	5
B	7.5
C ²	10
D	12.5
E ³	24
Z ⁴	2.6

¹Position A is not recommended for epi-fluorescent imaging because corrections for non-uniform excitation light pattern are not available.

²Position C is the default setting.

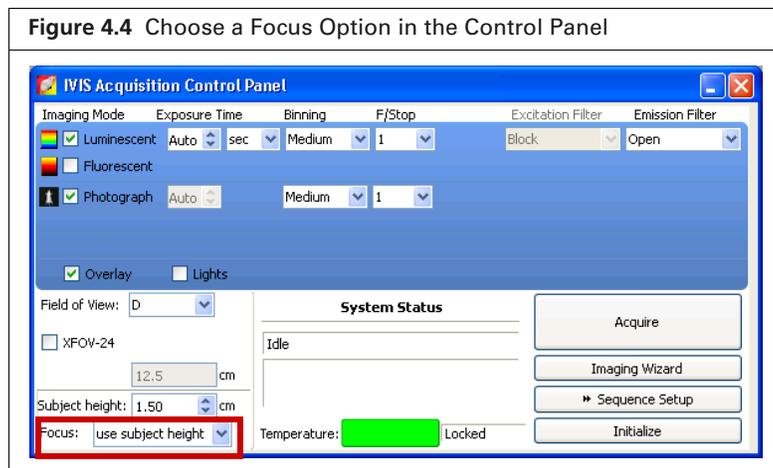
³Position E is available when the XFOV-24 Lens (PN 123177) is installed.

⁴Position Z is available when the ZFOV-2.6 Lens (PN 127285) is installed.

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.

- Enter the subject height (cm) and select the **use subject height** focus option.
- OR
- Choose the **manual** focus option and follow the instructions in [Appendix A on page 162](#).



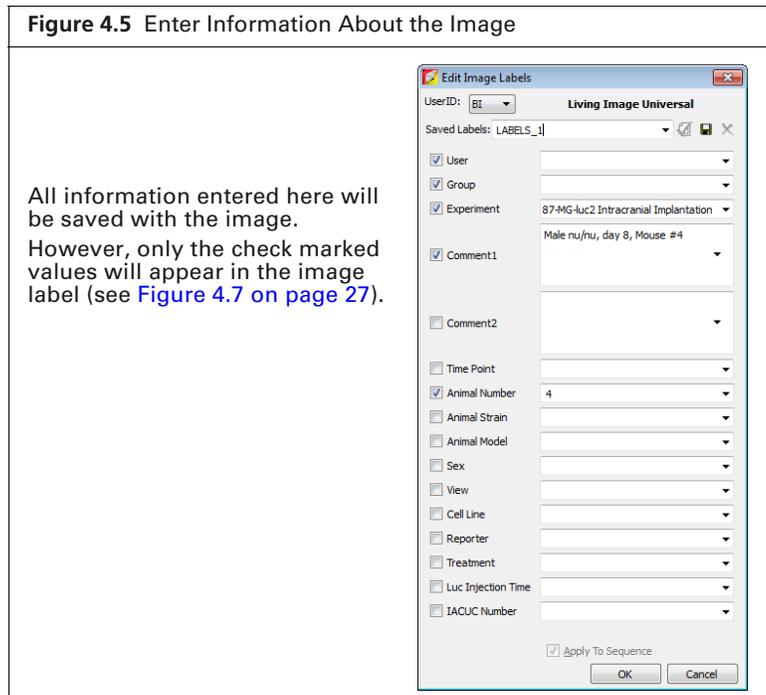
- 5.** If you want to check the subject inside the chamber before acquisition, take a photograph: uncheck **Luminescent**, choose **Photograph**, and click **Acquire**. Be sure to select **Luminescent** after taking the photograph.
- 6.** Click **Acquire** when you are ready to capture the image.



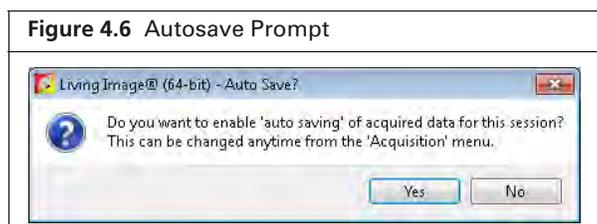
NOTE: If necessary click **Image Setup** in the control panel to operate in single image mode. In single image mode, the **Sequence Setup** button appears in the control panel. Use this button to set up sequence acquisition (see [page 42](#) for more details on sequence setup using the Imaging Wizard).

7. 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 84](#) for details on adding information to an image after acquisition.



If this is the first image of the session, you are prompted to enable the autosave function ([Figure 4.6](#)). 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).

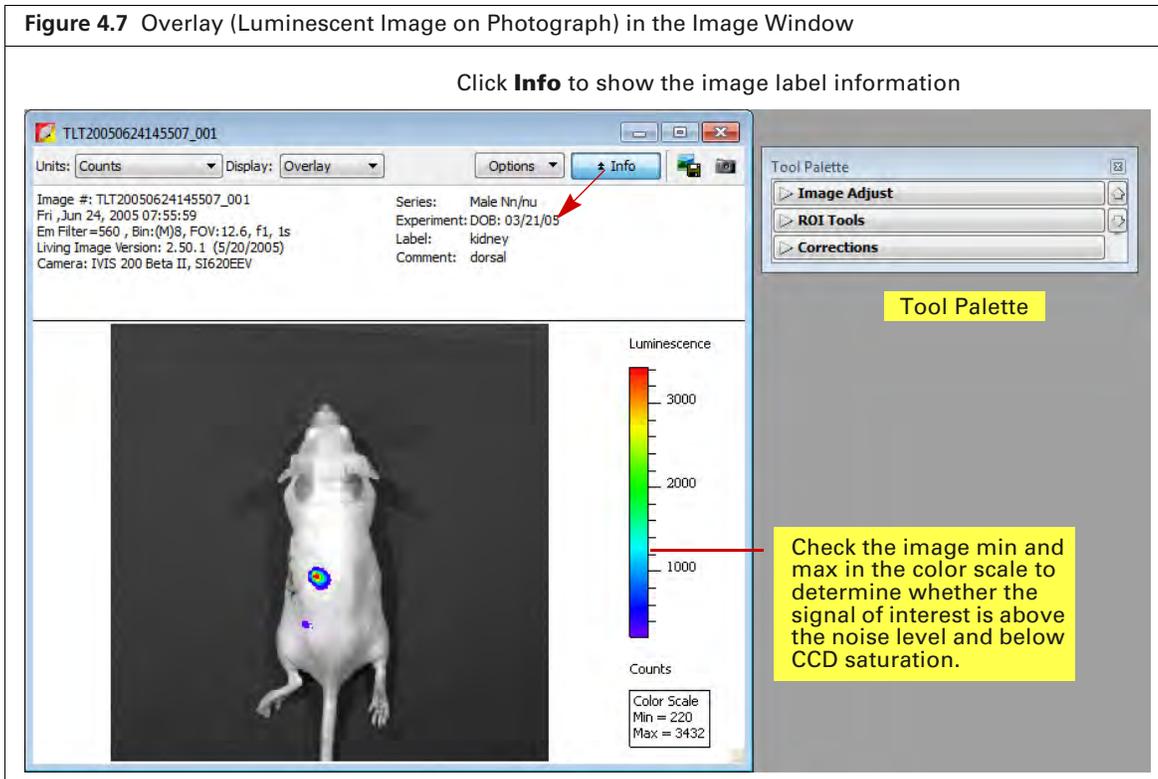


8. Click **Yes** in the prompt to enable autosave, then choose a location in the dialog box that appears. Alternatively, click **No** in the prompt and manually save the image data. See [page 47](#) for details. Image acquisition begins and the upper area of the control panel changes to red color.



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 4.7](#)).

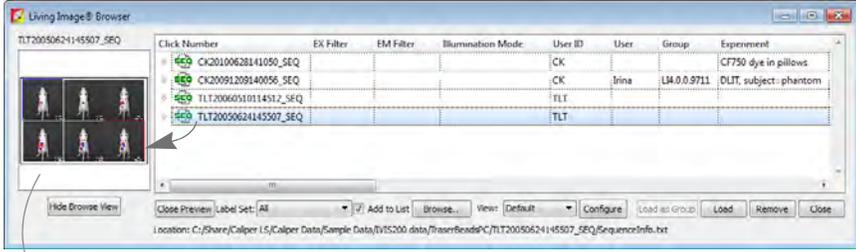


TIP: See the tech note *Determine Saturation* for information on pixel measurements (select **Help** → **Tech Notes** on the menu bar).

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 <i>Image Display and Measurement</i> 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 2.1 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 4.7) 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 (continued)

Item	Description
	Creates a preview picture (<i>snapshot</i>) of the image or thumbnails that the Living Image Browser displays when the data is selected in the browser. For more details on the browser, see page 49 .
	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

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

- Quick guide – See below.
- Detailed instructions – See [page 24](#).

See [page 34](#) 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).

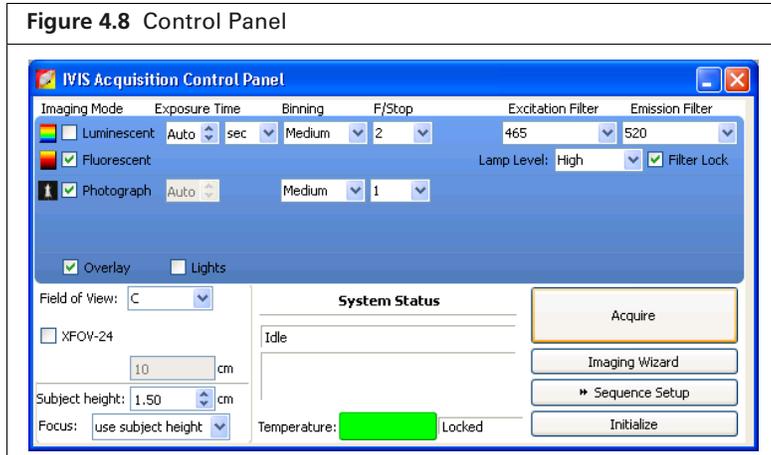
Quick Guide

1. Start Living Image software  and initialize the IVIS Lumina Series III (see [page 16](#) for details).

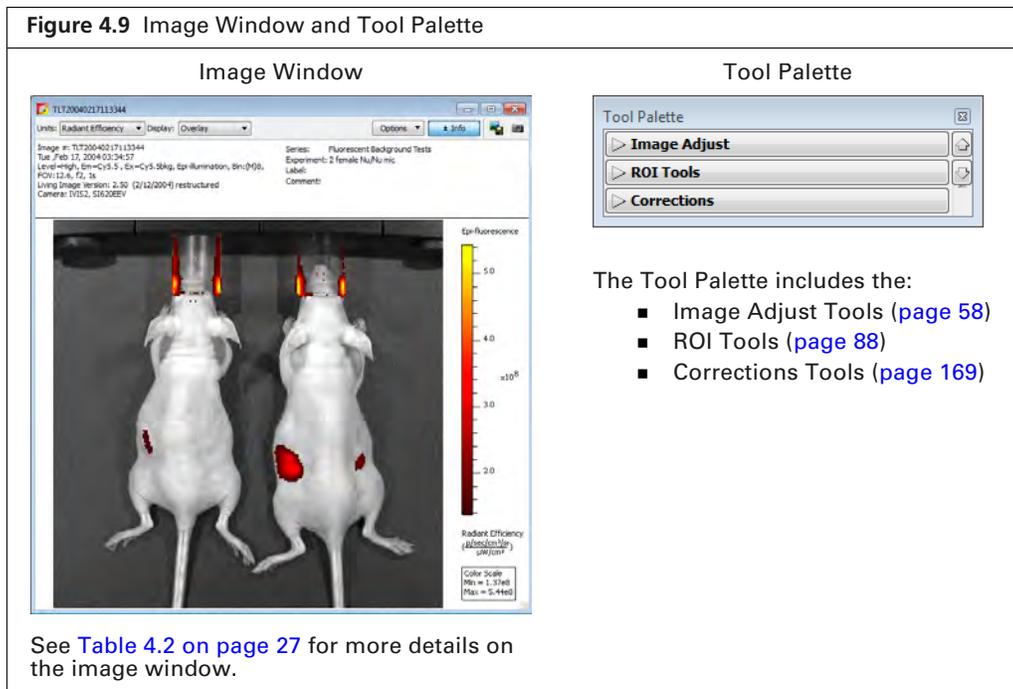


NOTE: See the *IVIS Lumina Series III Hardware Manual* for more information on the instrument

2. Place the anesthetized subjects in the imaging chamber and close the door.
3. In the control panel ([Figure 4.8](#)):
 - a. Put a check mark next to **Fluorescent** and select **Auto** exposure.
 - b. Select an excitation and emission filter.
 - c. Choose **Photograph** (optional, selecting **Photograph** automatically selects **Overlay**).
 - d. Select a field of view (FOV) (see [Table 4.1 on page 25](#)).
 - e. Enter the subject height (cm) and select the **use subject height** focus option
 - f. Click **Acquire**.



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 4.9).



- The Tool Palette includes the:
- Image Adjust Tools ([page 58](#))
 - ROI Tools ([page 88](#))
 - Corrections Tools ([page 169](#))

Acquire a Fluorescent Image

This section provides detailed instructions for fluorescent imaging with epi-illumination (excitation light source located above the stage).



NOTE: The IVIS Lumina Series III should be initialized and the temperature locked before setting the imaging parameters in the control panel. See [page 16](#) for more details.

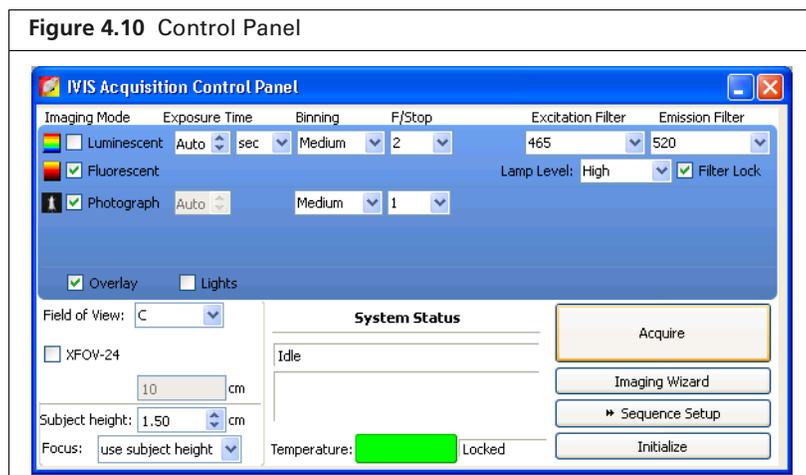
1. Put a check mark next to **Fluorescent** and select **Auto** exposure (click the arrows) in the control panel.

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 [Table A.1 on page 159](#) for details on these parameters.



2. Select an excitation and emission filter from the drop-down lists. [Table 4.3](#) shows the available excitation and emission filters.
3. 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.
4. Select a field of view (FOV, size of the stage area to be imaged). See [Table 4.1 on page 25](#) for a list of FOV settings.



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

Table 4.3 IVIS® Lumina Series III Filters

Excitation Filters		Emission Filters	
Center (nm)	Passband (nm)	Center (nm)	Passband (nm)
420	20	520	40
440	20	570	40
460	20	620	40
480	20	670	40
500	20	710	40
520	20	790	40
540	20	845	40
560	20		
580	20		
600	20		
620	20		
640	20		
660	20		
680	20		
700	20		
720	20		
740	20		
760	20		
780	20		

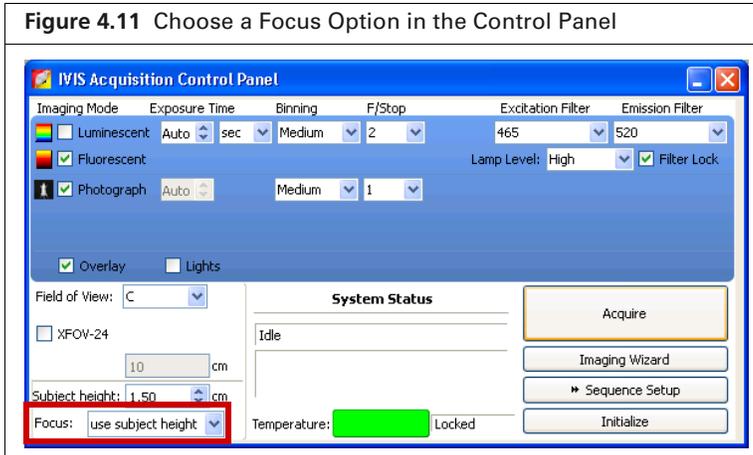
5. Select a focus option ([Figure 4.11](#)).

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.

- Enter the height of the animal (cm) and select the **use subject height** focus option.

OR

- Choose the **manual** focus option and follow the instructions in [Appendix A on page 162](#).

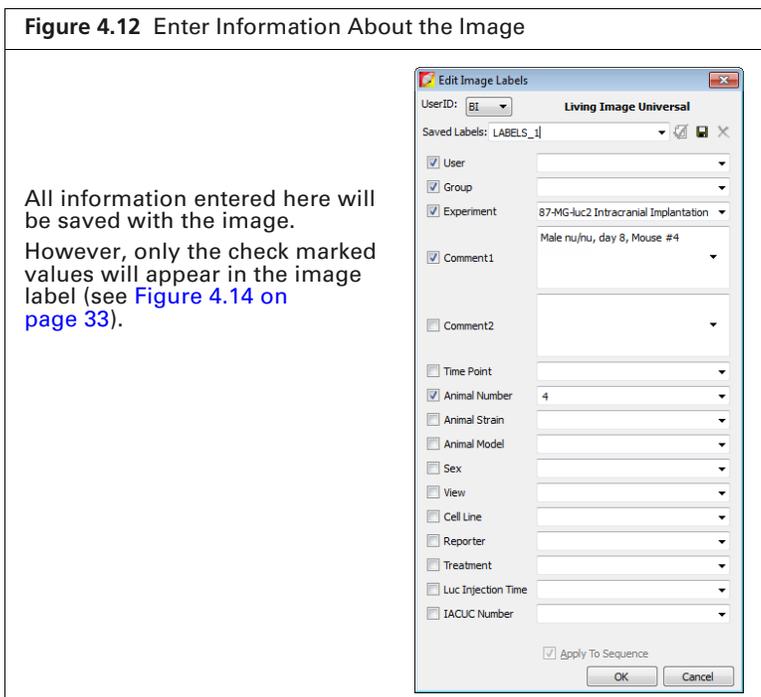


6. If you want to check the subject inside the chamber before acquisition, take a photograph—uncheck the Fluorescent option, choose the Photograph option, and click **Acquire**. Be sure to check the Fluorescent option after taking the photograph.
7. Click **Acquire** when you are ready to capture the 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 [page 34](#) for more details on sequence setup).

8. Enter information about the image in the dialog box that appears (optional, but strongly recommended). 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 84](#) for details on adding information to an image after acquisition.



If this is the first image of the session, you are prompted to enable the autosave function (Figure 4.13). 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).

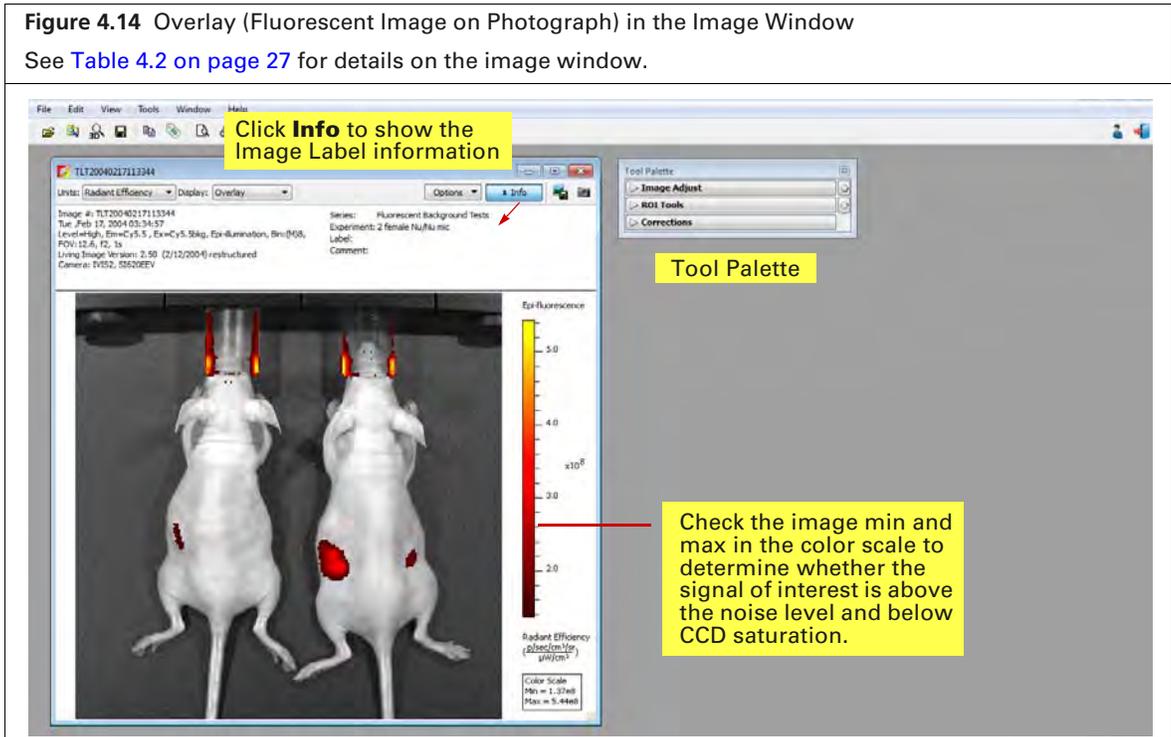


9. Click **Yes** in the prompt to enable autosave, then choose a location in the dialog box that appears. Alternatively, click **No** in the prompt and manually save the image data. See page 47 for details. Image acquisition begins and the upper area of the control panel changes to red color.



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 4.14).



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

4.3 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 36](#) for information on acquiring a Cherenkov sequence using the Imaging Wizard.

To acquire a Cherenkov image:

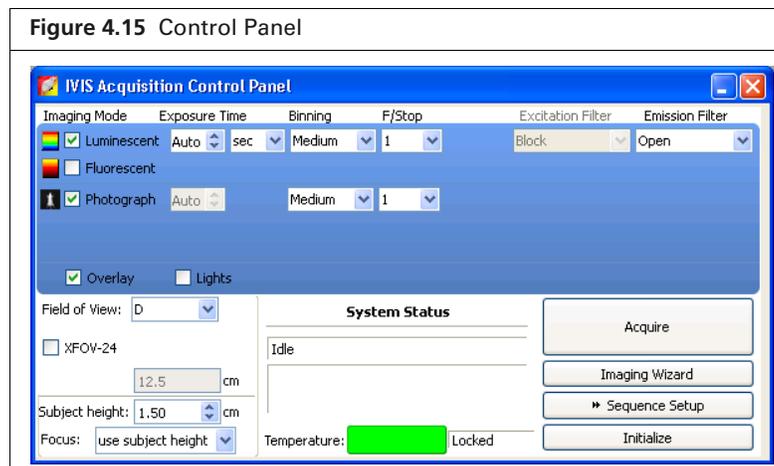
1. Put a check mark next to **Luminescent** and select **Auto** exposure in the control panel ([Figure 4.15](#)).

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 159](#) 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 173](#)).



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



2. Perform [step 2 on page 24](#) to [step 8 on page 26](#).

4.4 Acquire a Sequence Using the Imaging Wizard

The acquisition parameters for each image in a sequence must be specified. The Imaging Wizard ([Figure 4.16](#)) provides a convenient way to set up a sequence for some imaging applications (see [Table 4.4 on page 36](#)). 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 42](#) for details).



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

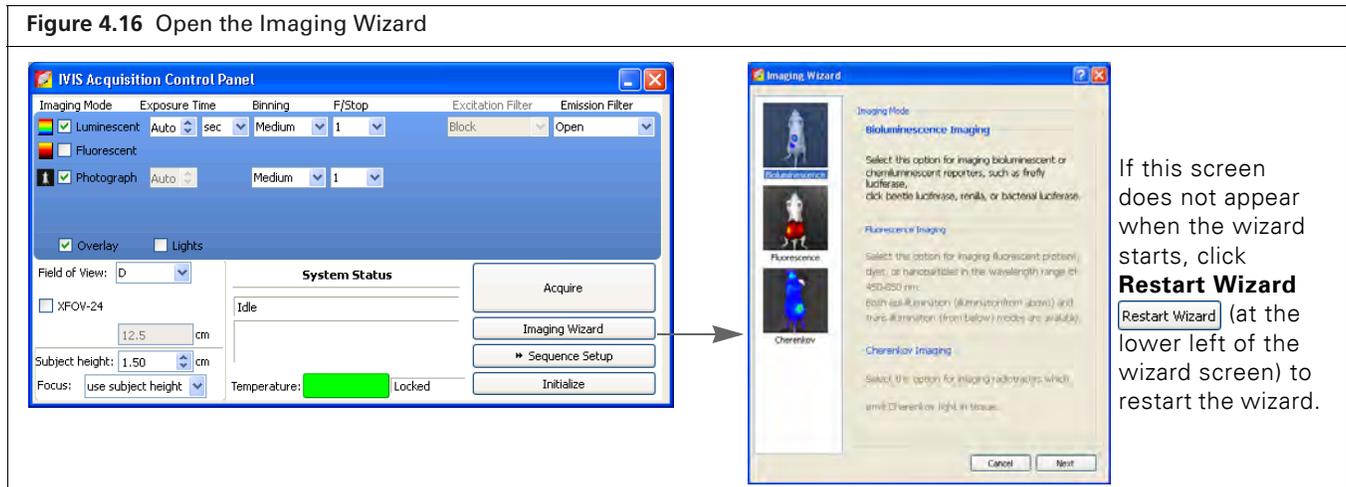
Start the Imaging Wizard and Setup a Sequence



NOTE: The IVIS Lumina Series III should be initialized and the temperature locked before setting imaging parameters. See [page 16](#) for more details.

1. Click **Imaging Wizard** in the control panel ([Figure 4.16](#)). If necessary, click **Restart** in the Imaging Wizard to show the first screen of the wizard.

Figure 4.16 Open the Imaging 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 36](#)).
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 4.17](#)).

Figure 4.17 Control Panel and Sequence Setup

Each row in the sequence table specifies the acquisition parameters for one image in the sequence. See [page 43](#) for details on the sequence table.



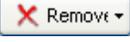
5. To clear the sequence, click the **Remove** button  and select **All**.
 See additional information about:
 - Editing image parameters on [page 44](#).
 - Inserting images in a sequence on [page 44](#).
 - Removing images from a sequence on [page 46](#).

Table 4.4 Imaging Wizard – Imaging Mode Options for IVIS Lumina Series III

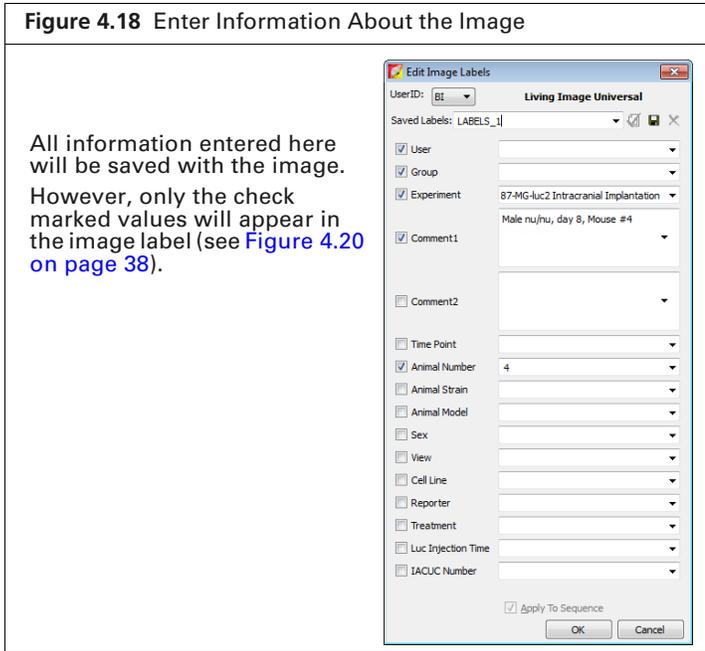
Imaging Mode	Options	See Page
Bioluminescence	Open Filter – Acquires a luminescent image at maximum sensitivity.	
	Spectral Unmixing – Acquires an image sequence for analysis using the Spectral Unmixing tools which use a mathematical operation to separate the signals from multiple luminescent probes.	114
	DyCE ¹ – Acquires a time series of optical images following a bolus injection of probe (radiotracer, bioluminescent, or fluorescent) to track probe biodistribution.	141
Fluorescence	Filter Pair – Selects the best excitation and emission filters for a specific fluorescent probe. Detects the fluorescent signal on the surface of the subject.	
	Spectral Unmixing/Filter Scan – Acquires an image sequence for analysis with the Spectral Unmixing tools to: <ul style="list-style-type: none"> ■ Extract the signal of one or more fluorophores from the tissue autofluorescence. ■ Determine the optimum excitation and emission filter for a probe. 	116
	DyCE – Acquires a time series of optical images following a bolus injection of probe (radiotracer, bioluminescent, or fluorescent) to track probe biodistribution.	143
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.	119
	DyCE – Acquires a time series of optical images following a bolus injection of probe (radiotracer, bioluminescent, or fluorescent) to track probe biodistribution.	145

¹DyCE analysis tools require a separate license.

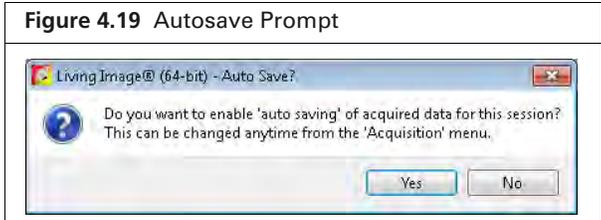
Acquire the Sequence

1. Confirm that the IVIS Lumina Series III is initialized and the CCD temperature is locked. (See [page 16](#) for details.)
2. Click **Acquire Sequence** in the control panel when ready to begin acquisition.
3. Enter information about the image in the dialog box that appears (optional, but strongly recommended) ([Figure 4.18](#)). 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 84](#) for details on adding information to an image after acquisition.



If this is the first image of the session, you are prompted to enable the autosave function ([Figure 4.19](#)). 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).



4. 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 47](#) 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 4.20).

Figure 4.20 Image Window and Tool Palette



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

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 <i>Image Display and Measurement</i> 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.

Table 4.5 Image Window – Sequence View (continued)

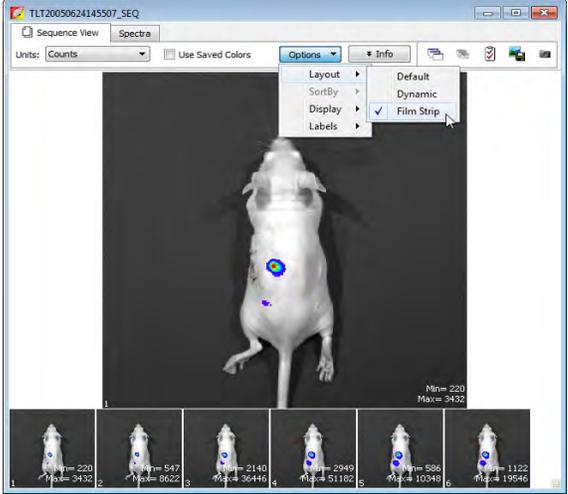
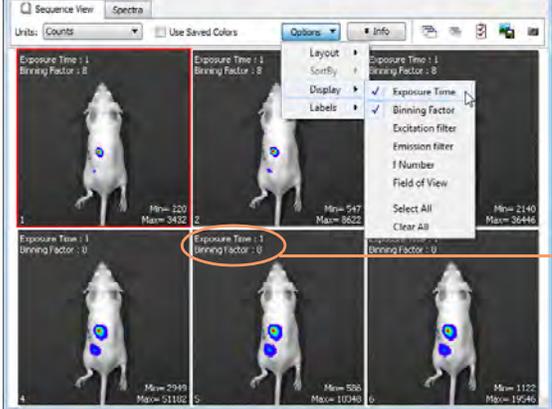
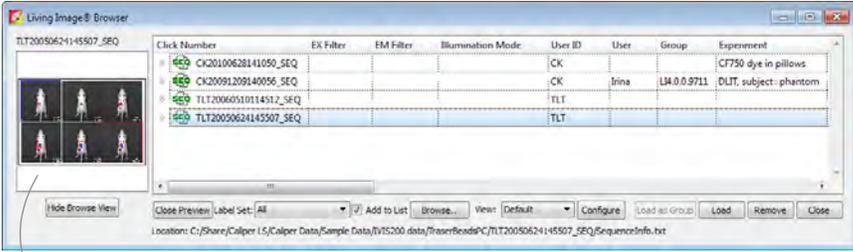
Item	Description
Options	<p>Layout - Choose a display option for the images in a sequence (Default, Dynamic, or Film Strip). For example, here is Film Strip mode:</p> 
	<p>Sort by - Options for ordering images in the sequence window. This option only applies to images that were opened using the “Load as Group” function in the Living Image browser.</p> <ul style="list-style-type: none"> ■ 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.
	<p>Display - Choose the types of information to display with each image.</p>  <p>In this example, exposure time and binning factor are displayed on each image</p>
Info	Click to show or hide the image label information (Figure 4.20).
	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.
	Enables you to export the active image as a graphic file (for example, .png, .dcm).

Table 4.5 Image Window – Sequence View (continued)

Item	Description
	Creates a preview picture (<i>snapshot</i>) of the image or thumbnails that the Living Image Browser displays when the data are selected. See page 49 for more details on the browser.



Preview picture of the selected data

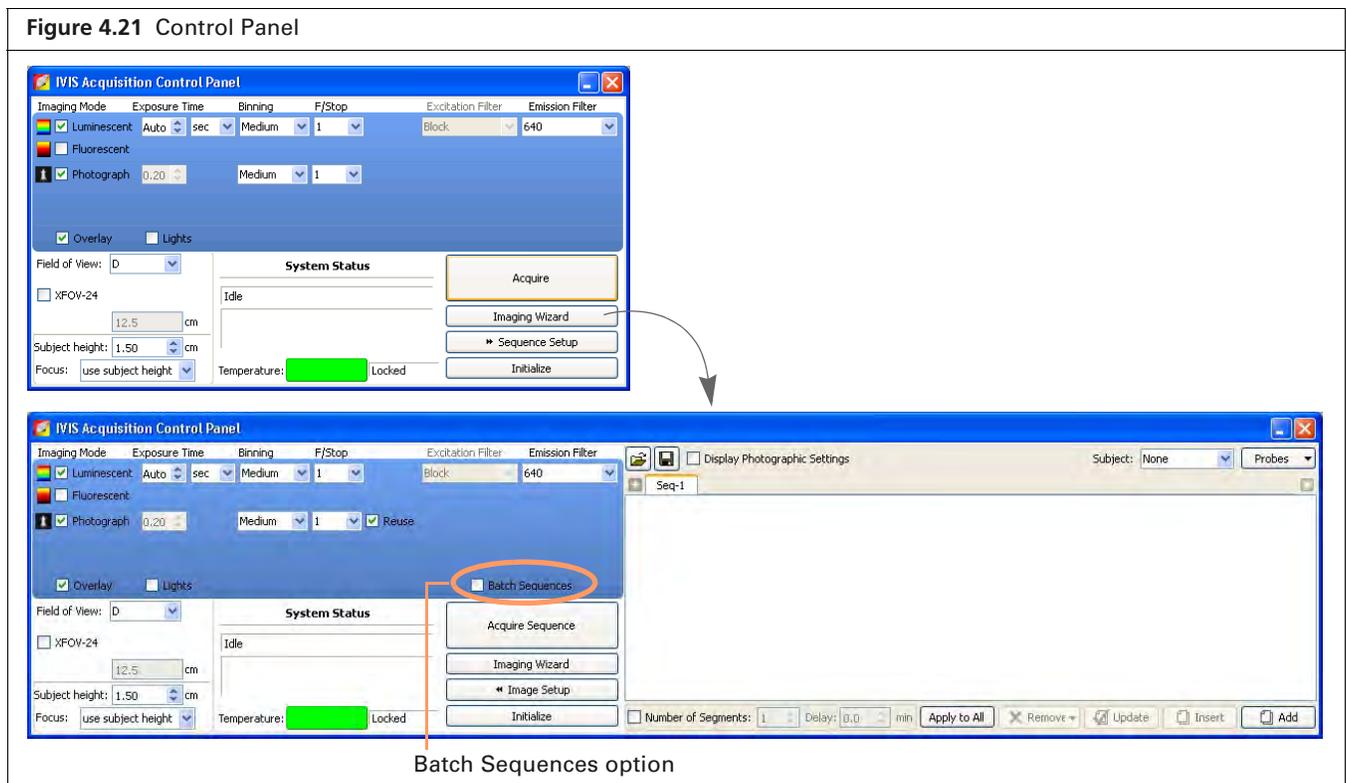
4.5 Acquire Multiple Sequences in Batch Mode

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:

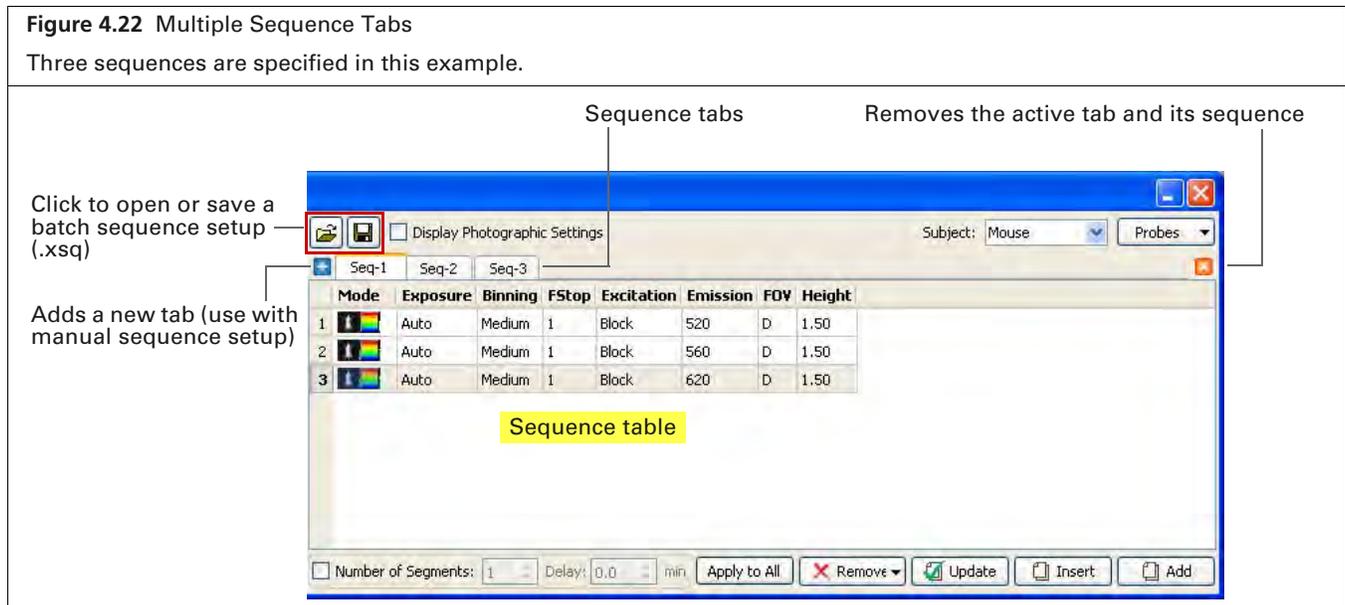
1. Click **Sequence Setup** in the control panel.
2. Choose the **Batch Sequences** option (Figure 4.21).

Figure 4.21 Control Panel



3. To set up the first sequence, do either of the following:
 - Click **Imaging Wizard** and step through the wizard (see page 34 for details).
 - OR
 - Set up the sequence manually (see page 42 for details).
4. To set up the next sequence:
 - If using the Imaging Wizard, repeat 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).



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 4.21), 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.

4.6 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 36 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.

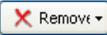
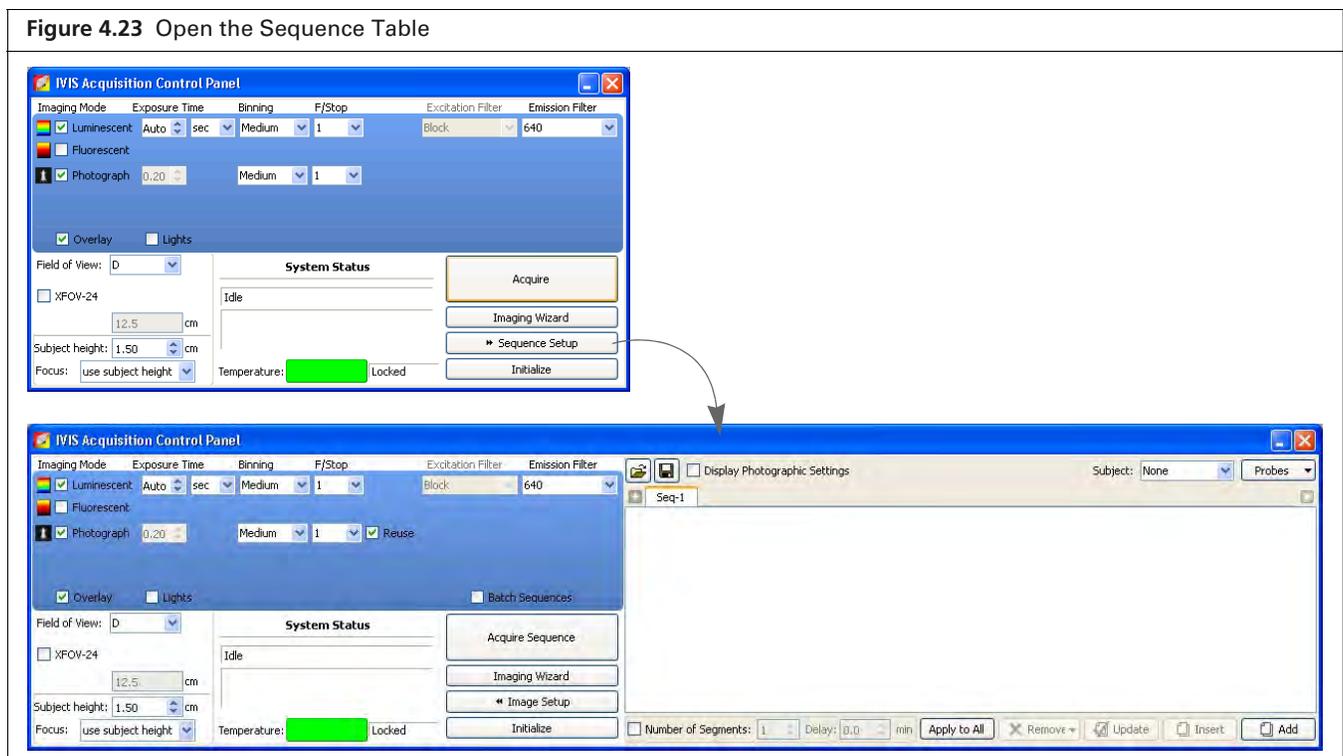
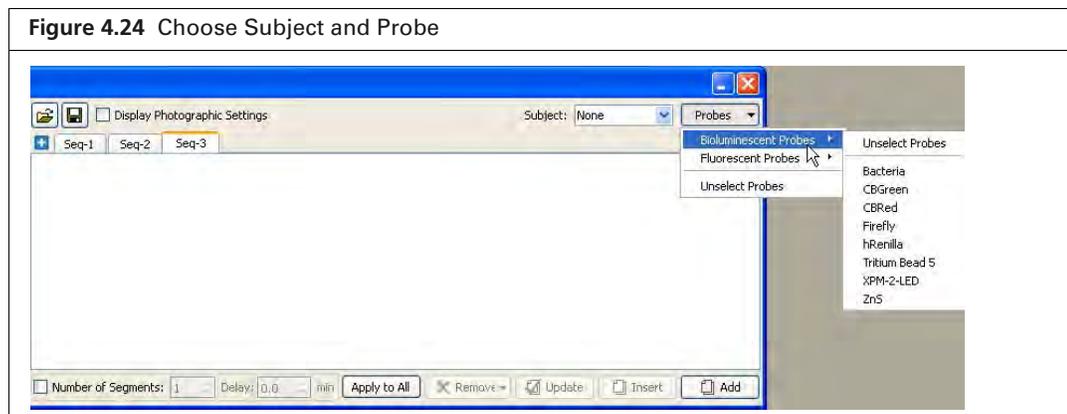
1. Click **Sequence Setup** in the control panel (Figure 4.23).
The sequence table appears.
2. If necessary, click the **Remove** button  and select **All** to clear the sequence table.

Figure 4.23 Open the Sequence Table



3. Choose a subject and probe from the drop-down lists (Figure 4.24)

Figure 4.24 Choose Subject and Probe



- Specify the imaging settings for the first image in the sequence. (See [Appendix A on page 159](#) 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 4.25](#)), the IVIS Lumina Series III 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.

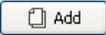
- Click the **Add** button . The acquisition parameters appear in the sequence table ([Figure 4.25](#)).
- Repeat [step 4](#) to [step 5](#) for each image in the sequence.
- To set a time delay between each acquisition, enter a time (minutes) in the Delay box in the sequence table.
- To save the sequence setup information (.xsq):
 - Click the **Save** button  in the sequence table.
 - Select a directory, enter a file name, and click **Save** in the dialog box that appears.

Figure 4.25 Control Panel and Sequence Table with Image Settings

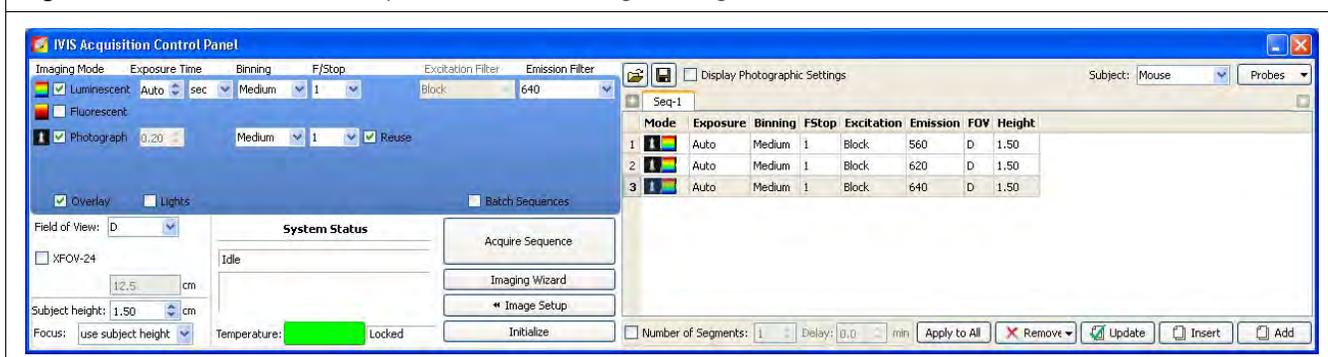


Table 4.6 Sequence Table

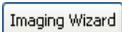
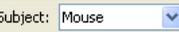
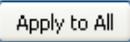
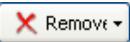
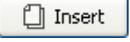
Item	Description
	Starts the Imaging Wizard.
	Displays a dialog box that enables you to select and open a sequence setup (.xsq), sequenceinfo.txt, or clickinfo.txt file.
	Displays a dialog box that enables you to save the information in the sequence table to a sequence setup file (.xsq).
Display Photographic Settings	Choose this option to include the photograph exposure time, binning, and F/Stop in the sequence table.
Subject:  Mouse  Probes	If a subject and probe are specified (optional), the software uses the information to automatically set parameters in the 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 <i>segment</i> . 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
	Applies the selected cell value to all cells in the same column.
	Remove Selected - Deletes the selected row from the sequence table. Remove All - Removes all rows from the sequence table.
	Updates the selected row in the sequence table with the acquisition parameters in the control panel.
	Inserts a row above the currently selected row using the information from the control panel.
	Adds a new row at the end of the sequence setup list.

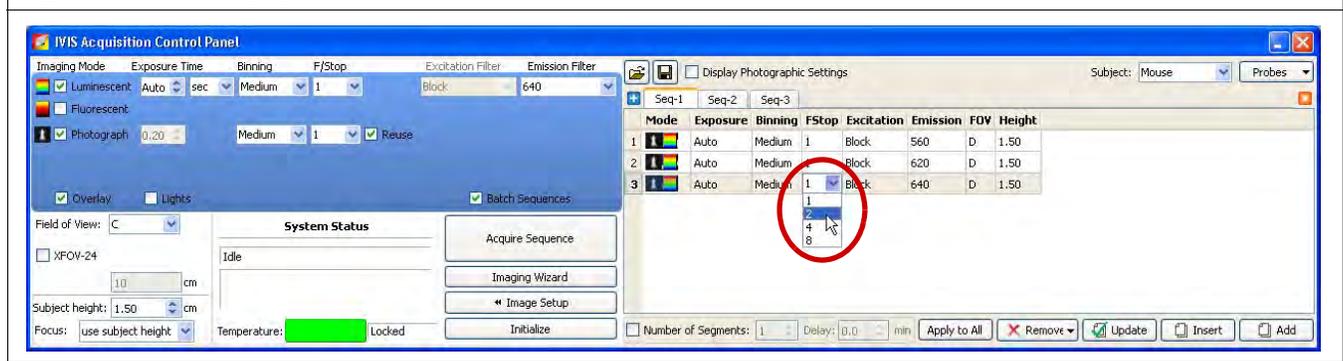
Editing Image Parameters

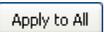
You can edit imaging parameters in the sequence table or in the control panel.

To edit a parameter in the sequence table:

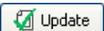
1. Double-click the cell that you want to edit (Figure 4.26).

Figure 4.26 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 .
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  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  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).

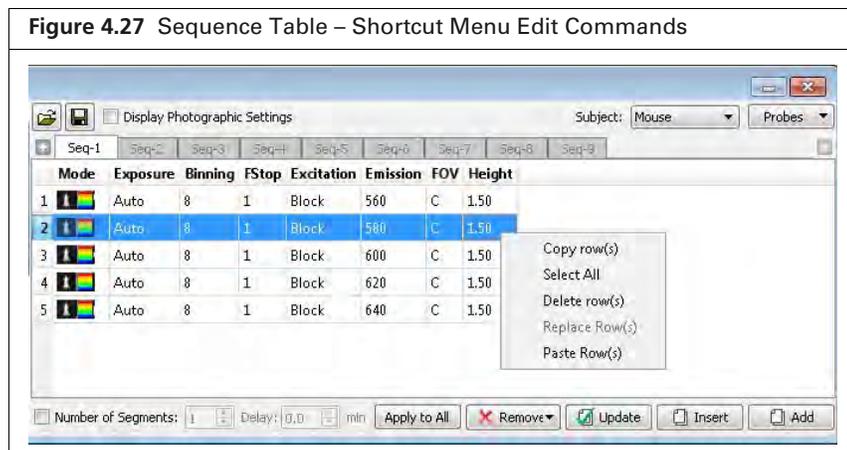


Table 4.7 Sequence Table – Shortcut Menu Edit Commands

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 , 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 4.27).

4.7 Imaging With the ZFOV-2.6 Zoom Lens

The optional ZFOV-2.6 Zoom Lens (PN 127285) enables close up imaging with a 2.6 cm field of view. If the ZFOV-2.6 Zoom Lens is installed, only the "Z" field of view is available for single-image or sequence acquisition.

If the ZFOV-2.6 Zoom Lens is installed when Living Image software is closed, the stage will move to the Z FOV position when the system is initialized. Keep the FOV set at "Z" until the ZFOV-2.6 Zoom Lens is removed.



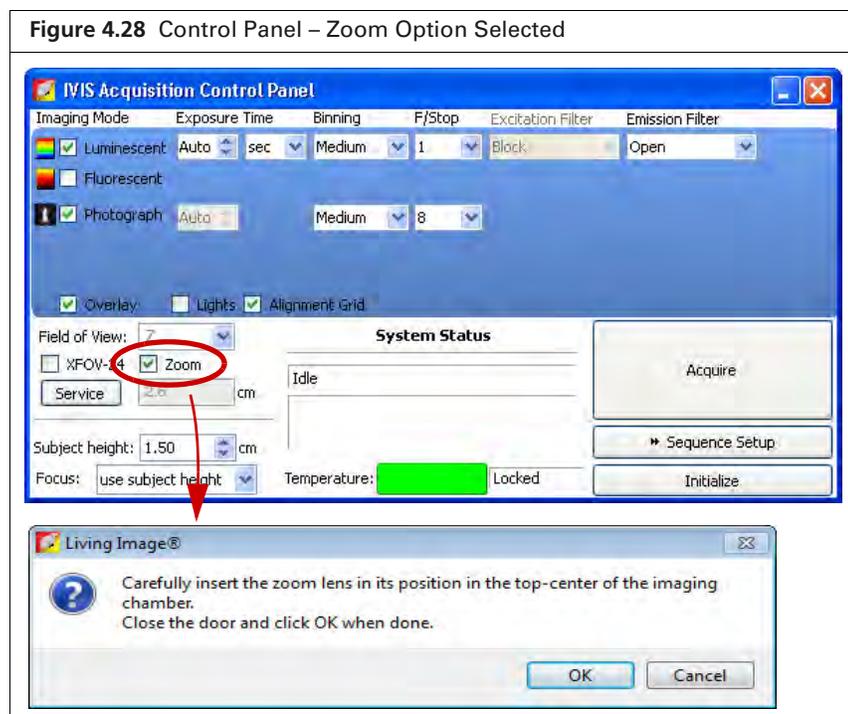
NOTE: Avoid touching the optical glass when installing or removing the ZFOV-2.6 Zoom Lens.

Installing the Zoom Lens



NOTE: Single images or sequential images can only be acquired at the Z field of view setting when the ZFOV-2.6 Zoom Lens is installed. If you attempt to change away from the Z setting during sequence setup, the sequence table will be cleared. If you attempt to change from another field of view setting to the Z setting, the sequence table is cleared of all previous settings before the camera settings for the Z position are added.

1. Choose the Zoom option in the control panel (Figure 4.28).
You are prompted to insert the ZFOV-2.6 Zoom Lens.



2. Click **OK** in the prompt after you install the ZFOV-2.6 Zoom Lens in the imaging chamber.
The stage moves to the "Z" field of view position.

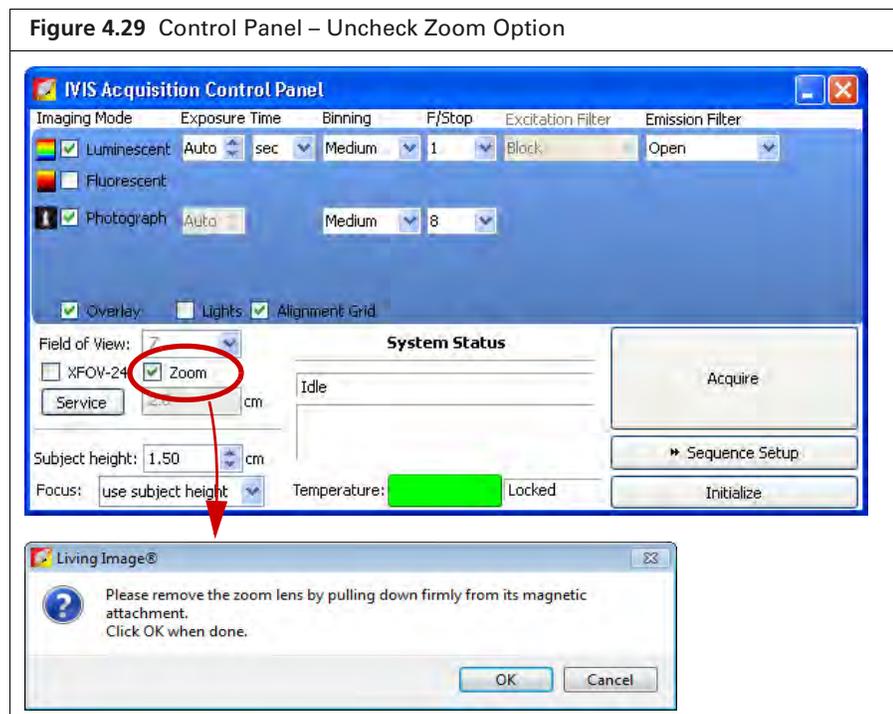
Removing the Zoom Lens

The imaging system is set to the "Z" field of view until the ZFOV-2.6 Zoom Lens is removed.

1. Remove the check mark next to "Zoom" in the control panel (Figure 4.29).
The stage moves to position C, then you are prompted to remove the lens.
2. Click **OK** in the prompt after you remove the ZFOV-2.6 Zoom Lens.



NOTE: Always store the ZFOV-2.6 Zoom Lens wrapped and in its protective container.



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.

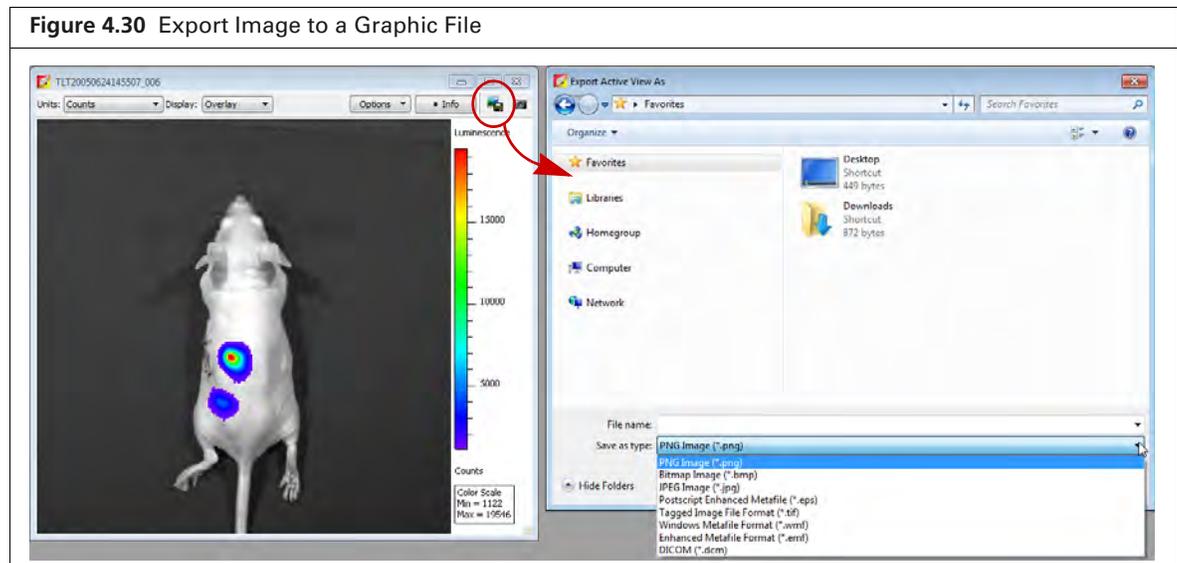
4.9 Exporting Images

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 76](#) for more information.

1. Open an image or sequence.
2. Click the **Export Graphics** button  (Figure 4.30).
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.



5 Working With Images

Loading Image Data

Adjusting Image Appearance on page 58

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Exporting an Image Sequence on page 78

Managing Image Information on page 83

Managing Image Sequences on page 85

5.1 Loading Image Data

Images can be loaded (opened):

- Using the Living Image Browser (see below).
- From the toolbar or menu bar ([page 53](#)).
- 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

The figure illustrates the process of opening the Living Image Browser. It shows two screenshots. The top screenshot is a 'Living Image Dataset Folder' dialog box. The path is 'Sample Data > IVIS200 data > TraserBeadsPC'. The folder 'TLT20050624145507_SEQ' is selected. The bottom screenshot is the 'Living Image Browser' window. It displays a table of image data. A red circle highlights the 'SEQ' icon in the table. Below the browser window is a legend for the icons used in the table.

Click Number	EX Filter	EM Filter	Illumination Mode	User ID	User	Group	Experiment	Comments
...
...
...
...

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

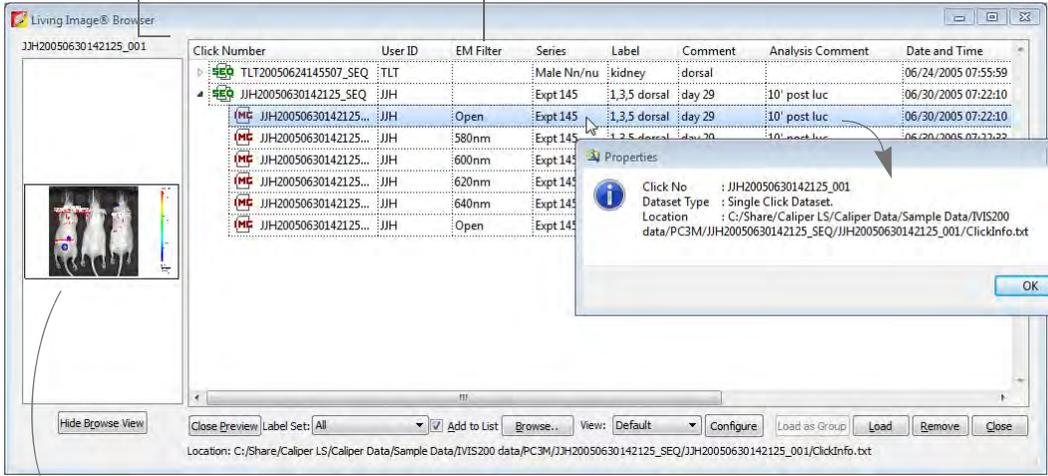
To expand a sequence, click the  arrow next to **SEQ**.

Click a column header to sort the browser contents in ascending alpha numeric order. Click the column header again to sort in descending alpha numeric order.

To view data properties, right-click a row and select **Properties** on the shortcut menu.

To preview data, click a row.

Note: A preview snapshot is automatically taken at the time of image or sequence acquisition. A snapshot can also be captured manually (see [page 28](#) for more details).



Click Number	User ID	EM Filter	Series	Label	Comment	Analysis Comment	Date and Time
TLT20050624145507_SEQ	TLT		Male Nn/nu	kidney	dorsal		06/24/2005 07:55:59
JJH20050630142125_SEQ	JJH			1,3,5 dorsal	day 29	10' post luc	06/30/2005 07:22:10
JJH20050630142125...	JJH	Open	Expt 145	1,3,5 dorsal	day 29	10' post luc	06/30/2005 07:22:10
JJH20050630142125...	JJH	580nm	Expt 145	1,3,5 dorsal	day 29	10' post luc	06/30/2005 07:22:10
JJH20050630142125...	JJH	600nm	Expt 145	1,3,5 dorsal	day 29	10' post luc	06/30/2005 07:22:10
JJH20050630142125...	JJH	620nm	Expt 145	1,3,5 dorsal	day 29	10' post luc	06/30/2005 07:22:10
JJH20050630142125...	JJH	640nm	Expt 145	1,3,5 dorsal	day 29	10' post luc	06/30/2005 07:22:10
JJH20050630142125...	JJH	Open	Expt 145	1,3,5 dorsal	day 29	10' post luc	06/30/2005 07:22:10

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.

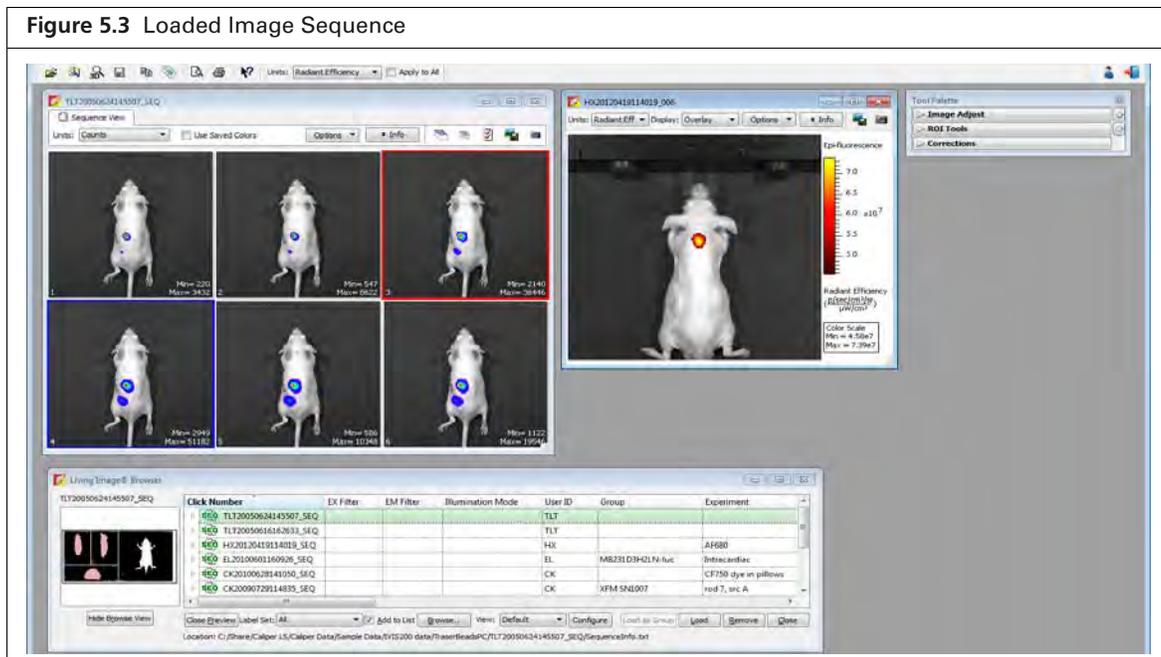


Table 5.1 Living Image Browser

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 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 50) 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 50).
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	<p>Enables you to select particular images that you want to view as a sequence. The images may be acquired during different sessions.</p> <p>To select adjacent images in the browser, press and hold the Shift key while you click the first and last file in the selection.</p> <p>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.</p> <p>Note: The Load as Group option is only available when two or more images (non-kinetic) are selected in the browser.</p> <p>Tip: See the tech note Loading Groups of Images for a quick guide (select Help → Tech Notes on the menu bar).</p>
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 54).

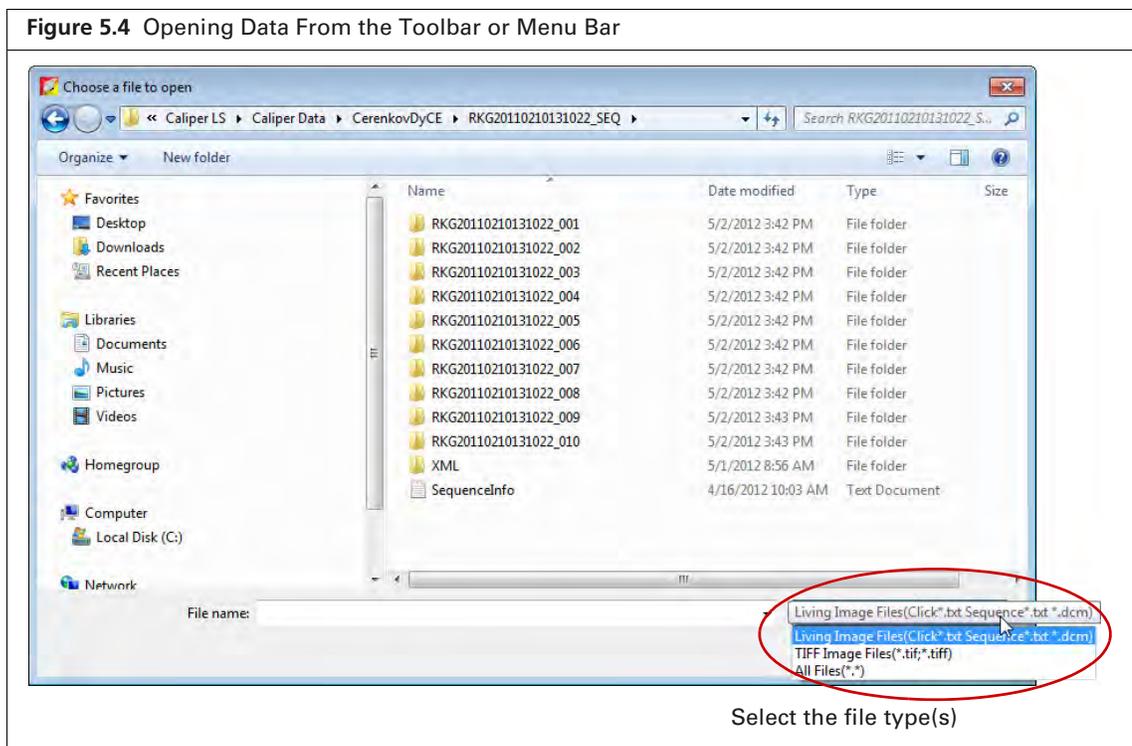


Table 5.2 File Filters

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.

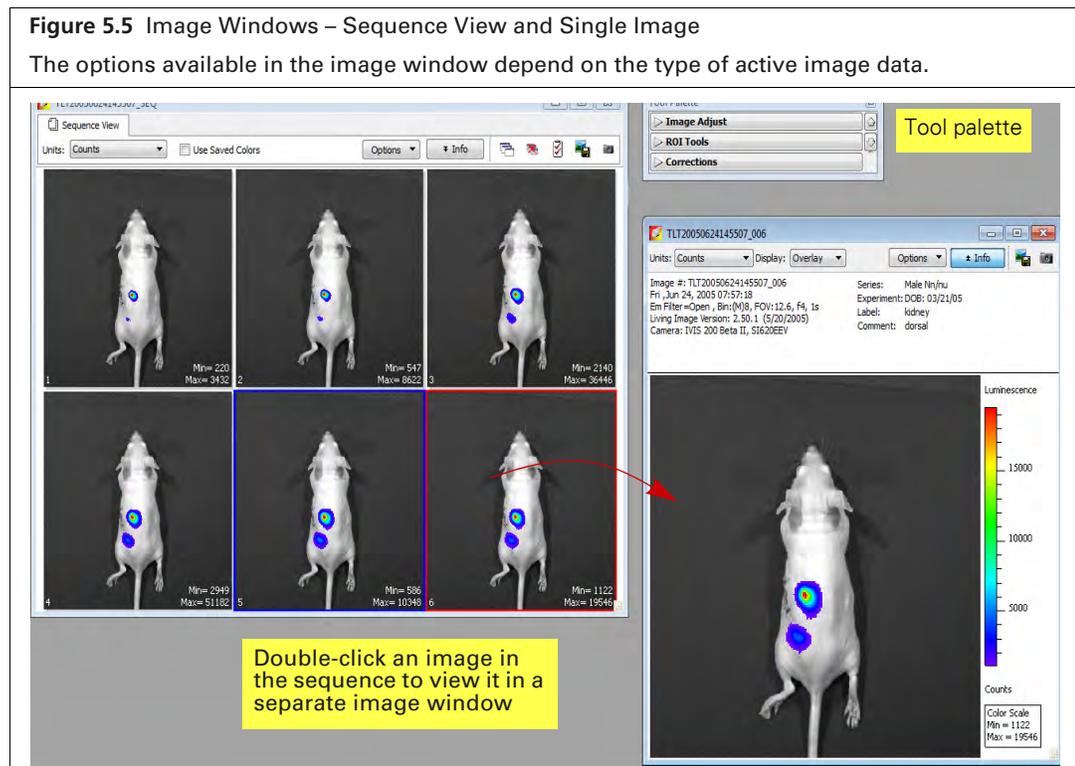


Table 5.3 Image Window

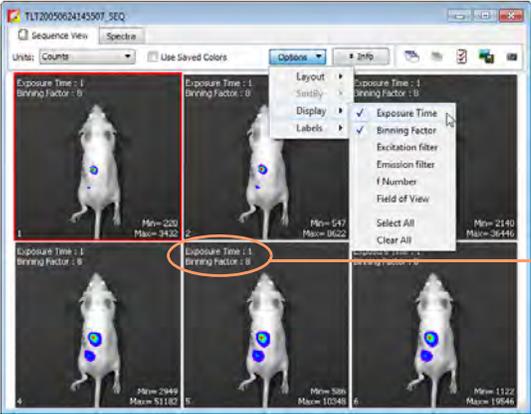
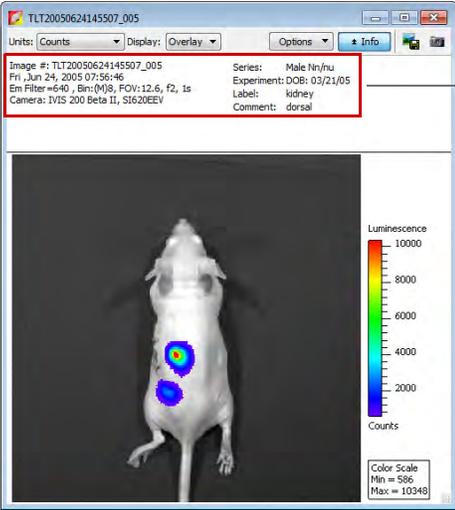
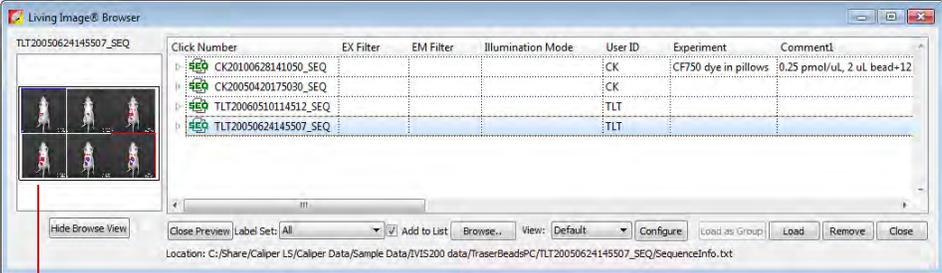
Item	Description
Units	<p>Select the measurement units for the image display from this drop-down list. The available units depend on the type of image data. See the concept tech note <i>Image Display and Measurement</i> for more details on measurement units (select Help → Tech Notes on the menu bar).</p> <p>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).</p> <p>Radiance (photons) – A calibrated measurement of the photon emission from the subject. Radiance is in units of "photons/second/cm²/steradian". Recommended for luminescence measurements.</p> <p>Radiant Efficiency – A fluorescence emission radiance per incident excitation power. Recommended for fluorescence measurements.</p> <p>Efficiency – Fluorescent emission normalized to the incident excitation intensity (radiance of the subject/illumination intensity). Recommended for epi-fluorescence measurements.</p>
Use Saved Colors (image sequence)	<p>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.</p>
Options (image sequence)	<p>Layout – Choose a display option for the images in a sequence (Default, Dynamic, or Film Strip). For example, here is Film Strip mode:</p> 
	<p>Sort by – Options for ordering images in the sequence window:</p> <ul style="list-style-type: none"> ■ 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.
	<p>Display - Choose the types of information to display with each image.</p>  <p>In this example, exposure time and binning factor are displayed on each image</p>

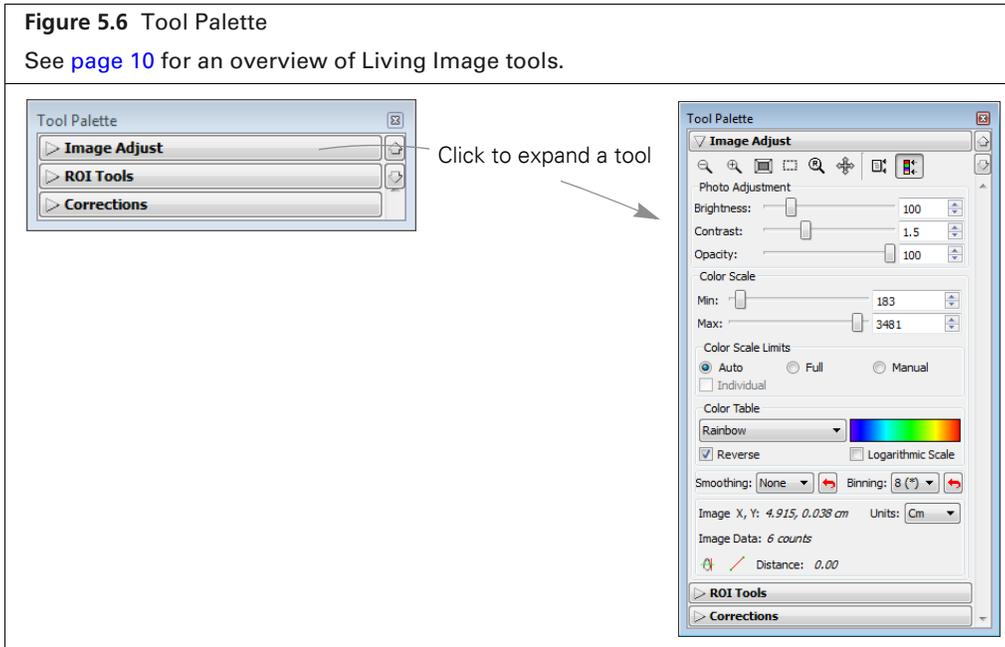
Table 5.3 Image Window (continued)

Item	Description
	<p>Labels – Enables you to select the information to include in the image label.</p>  <p style="text-align: right;">Image label</p>
Info	<p>Click to show or hide the image label. The image label includes information you enter in the Edit Image Labels dialog box (see page 27) and other information automatically recorded by the software.</p>
	<p>Opens all of the images in a sequence.</p>
	<p>Closes all open images of a sequence.</p>
	<p>Opens the Edit Sequence dialog box that enables you to add or remove images from the sequence display.</p>
	<p>Opens a dialog box that enables you to export the active view as a graphic file.</p>
	<p>Takes a “snapshot” that is displayed with the data in the Living Image Browser. See page 49 for more details on the browser.</p>  <p style="text-align: center;">Snapshots of an image sequence</p>

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.



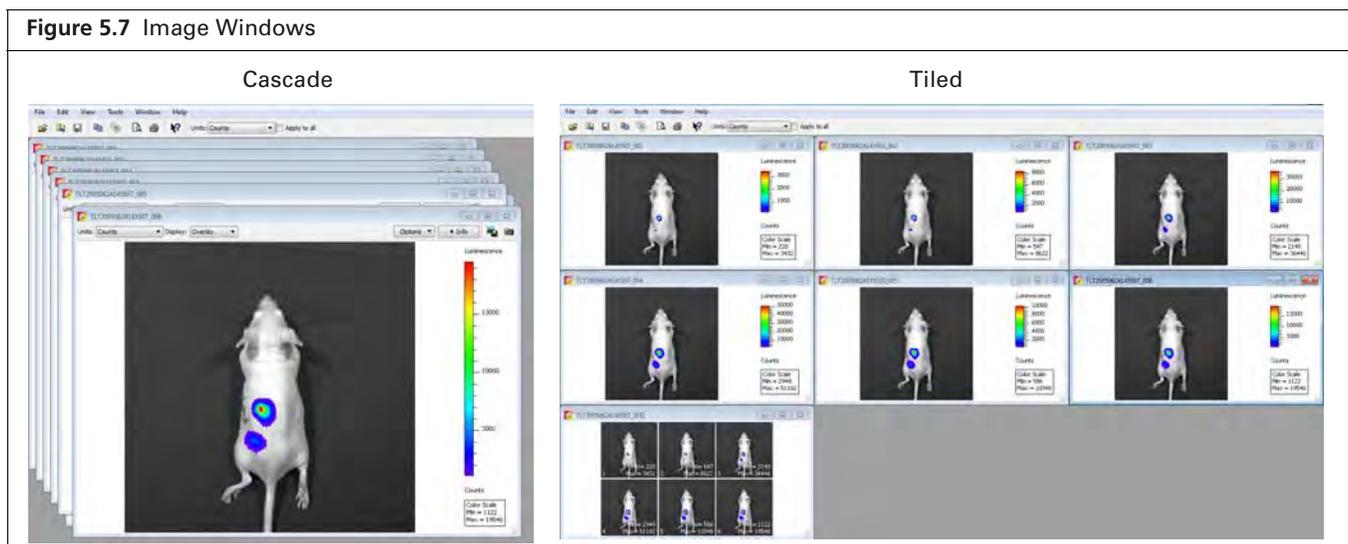
NOTE: The 3D Multi-Modality tools and DyCE tools require a separate license.



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.



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.

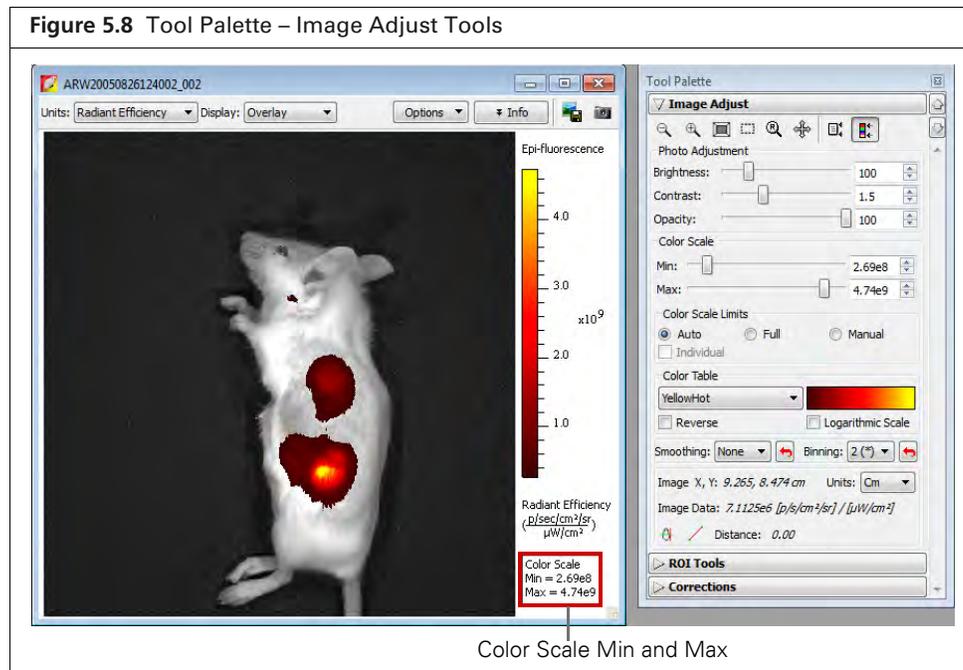


Table 5.4 Image Adjust Tools

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 60 for more information.
	Click this button to draw a box on an image that can be used to: <ul style="list-style-type: none"> ■ Make measurements (see page 65) ■ 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 (<i>pan</i>) in the image window. For more details, see page 60 .

Table 5.4 Image Adjust Tools (continued)

Item	Description
	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	<p>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.</p> <p>Contrast – Click and move the slider left or right to adjust the <i>gamma</i> of an image displayed in overlay mode. Alternatively, enter a gamma value. (Gamma is related to image contrast.)</p> <p>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.</p>
Color Scale	<p>Min – The minimum pixel intensity associated with the color scale for an image. Pixels less than the minimum value are not displayed.</p> <p>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.</p>
Color Scale Limits	<p>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.</p> <p>Full – Choose this option to set the Max and Min values to the maximum and minimum data values in the image.</p> <p>Manual – Choose this option to enter Max and Min values for the image display.</p> <p>Individual – Applies separate scale limit to each image in a sequence. Note: This option is only available when an image sequence is active.</p>
Color Table	<p></p> <p>Click the drop-down arrow to select a color table for the image data. See the concept tech note <i>Image Display and Measurement</i> for more details on the color table (select Help → Tech Notes on the menu bar).</p> <p>Reverse – Choose this option to reverse the selected color table.</p> <p>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.</p>
Smoothing	<p>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.</p> <p> Click this button to return smoothing to the previous setting and update the image.</p>
Binning	<p>Specifies the number of pixels in the image data that are grouped together to form a larger pixel (called <i>soft binning</i>). Binning changes the pixel size in the image (see Figure 5.9 on page 61). Binning can be applied to an image or a sequence. See the tech note <i>Detection Sensitivity</i> for more details on binning (select Help → Tech Notes on the menu bar).</p> <p> Click this button to return binning to the previous setting and update the image.</p>
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 62 .) 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 64). 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

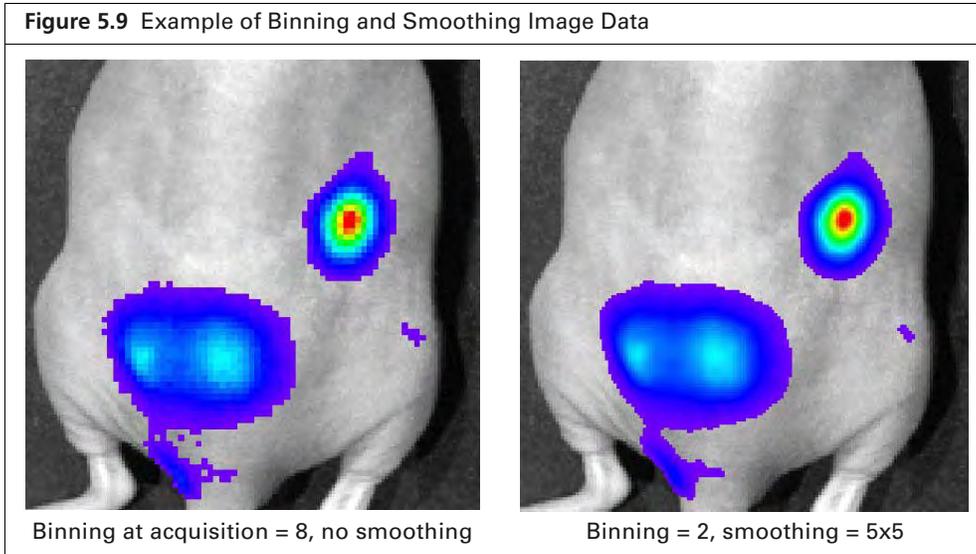


TIP: See the technical note *Detection Sensitivity* for helpful information about binning and smoothing (select **Help** → **Tech Notes** on the menu bar).

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

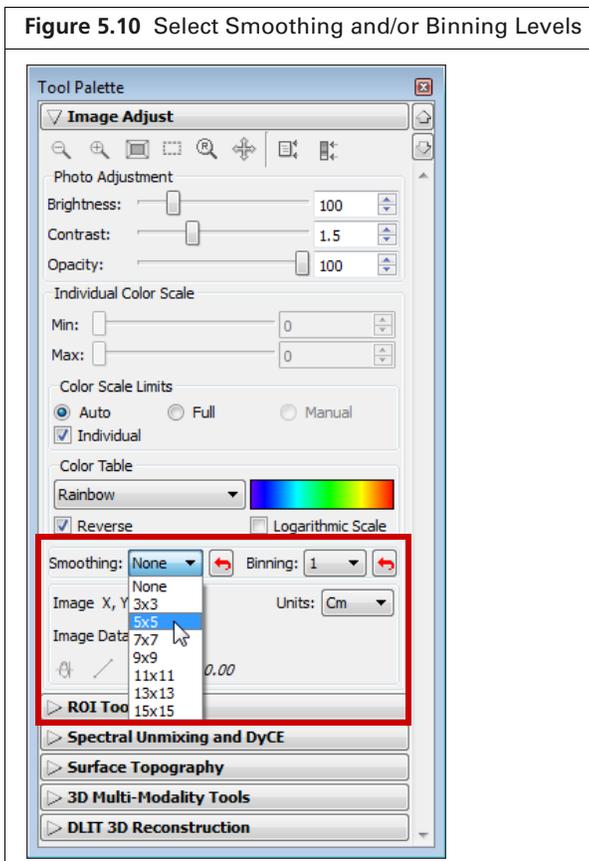
Binning specifies the number of pixels in the image data that are grouped together to form a larger pixel (called soft binning) (Figure 5.9). Binning changes the pixel size in the image.

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



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.



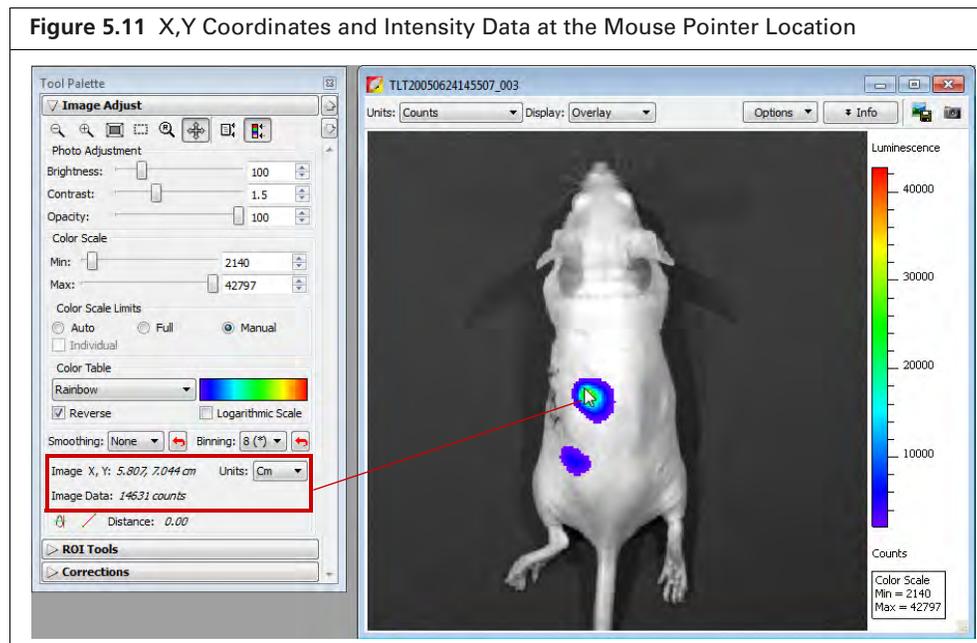
5.3 Viewing Intensity Data

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 88](#) 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.



Line Profile

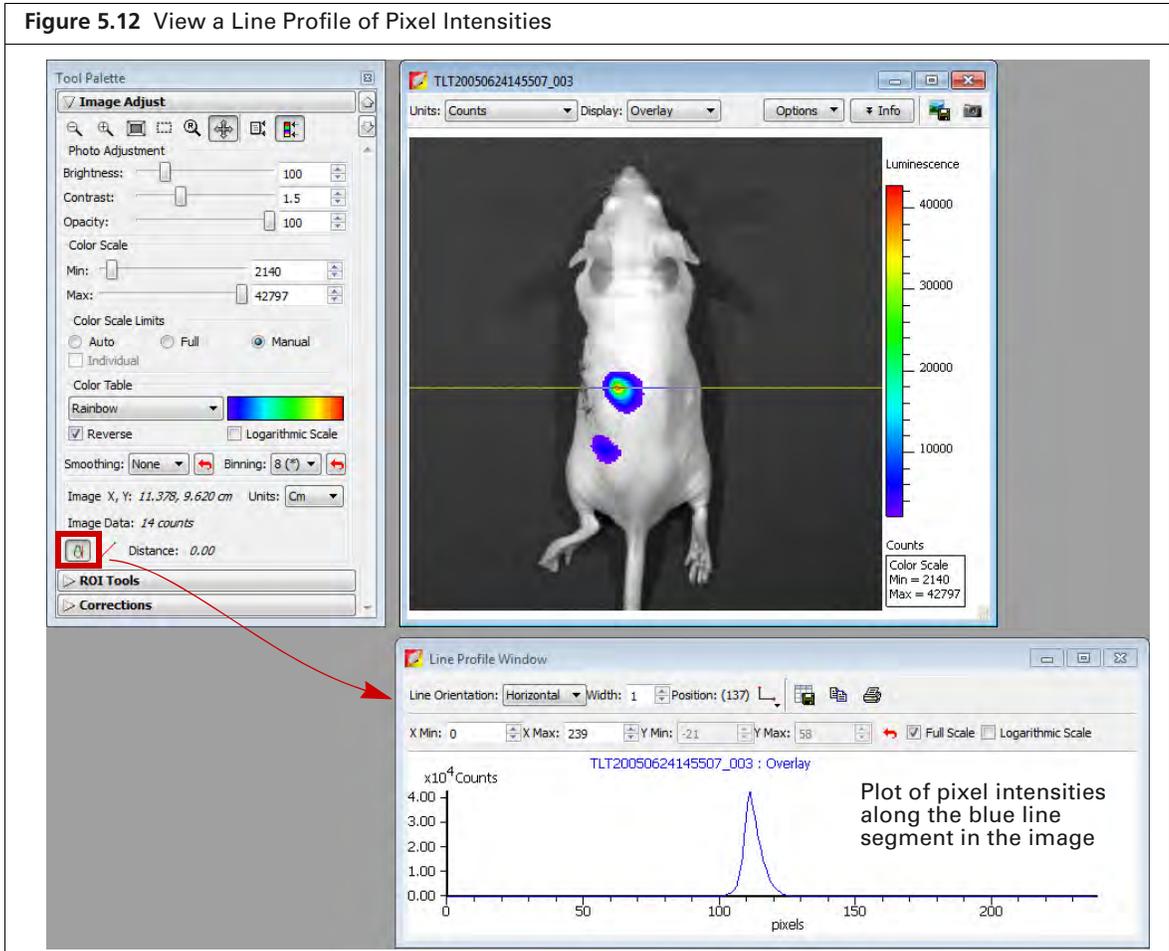
The line profile plots intensity (y-axis) at each pixel (x-axis) along a user-specified line on an image. It is particularly useful for inspecting the detailed character of the image data.



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 63](#) for details on the Line Profile window.



- 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.

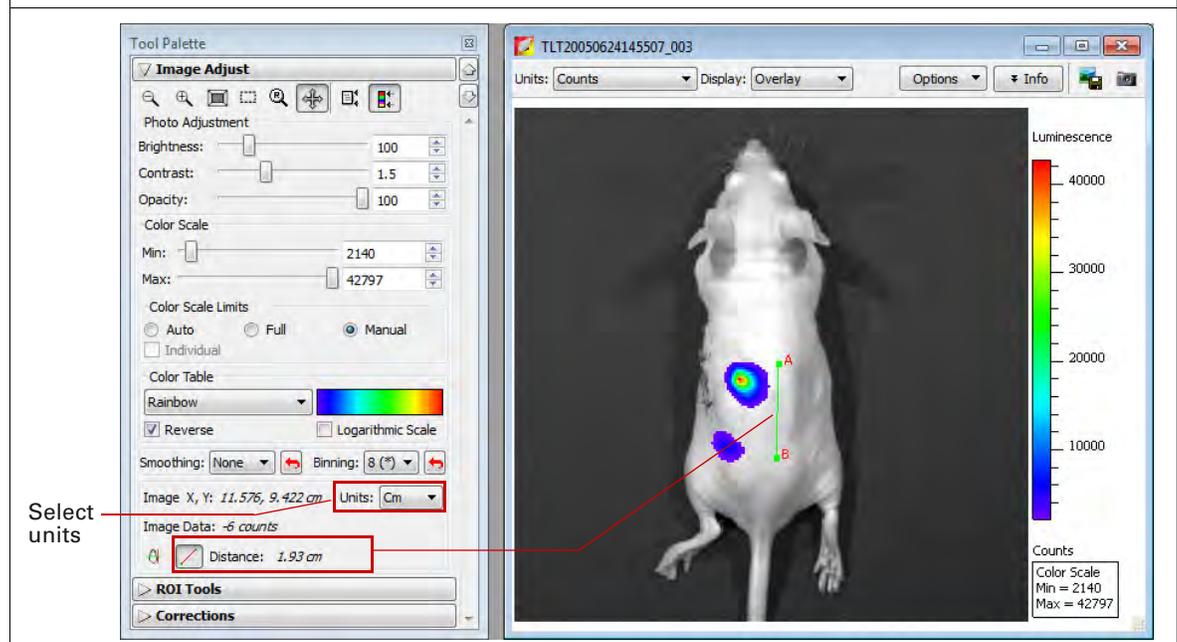
5.4 Measuring Distance

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.

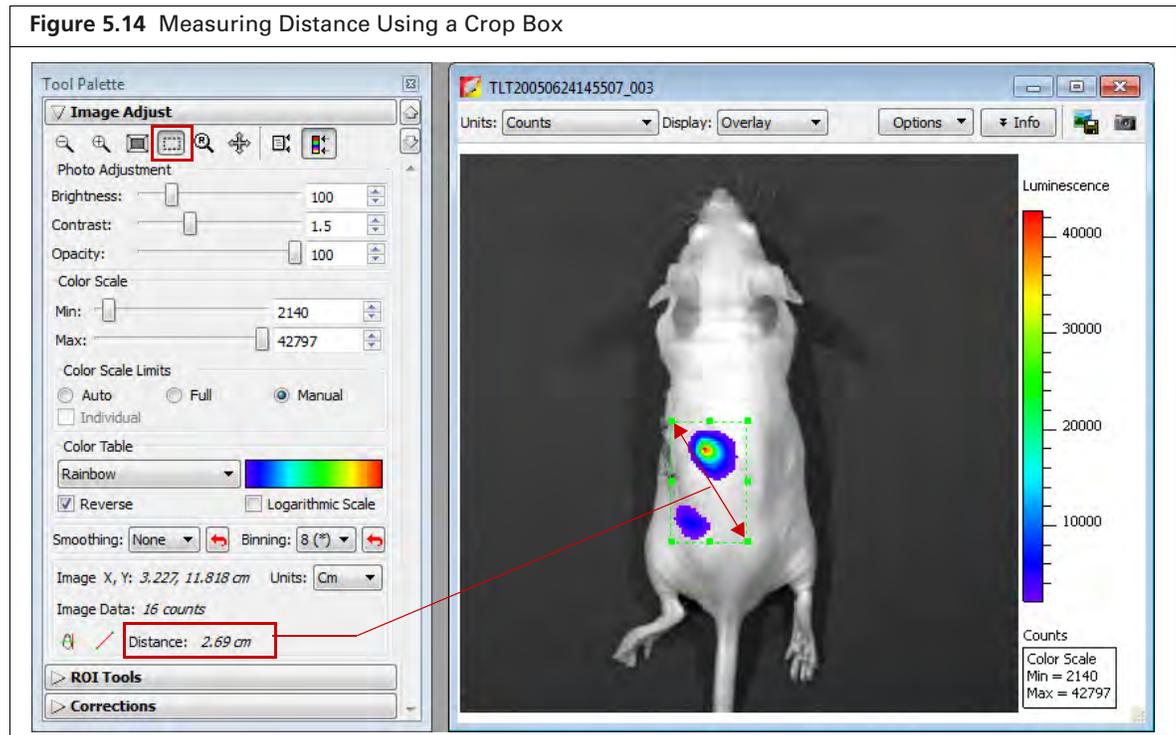
Figure 5.13 Measuring Distance Using the Measurement Tool
 The Tool Palette displays the measurement tool position and length.



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.
5. Click the  button to hide the 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 primarily 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 86](#) 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: $(A - B) \times k$, where:

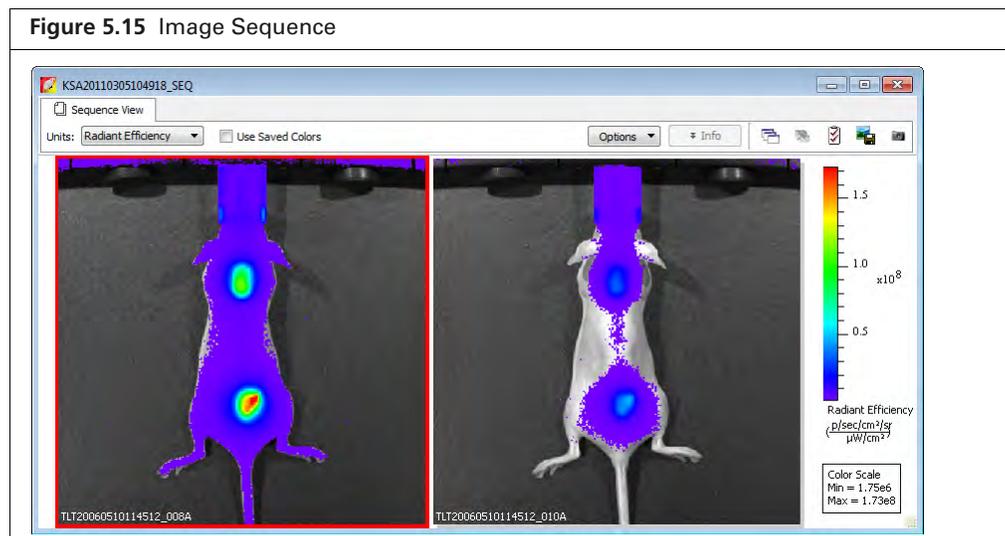
- A = primary image (acquired using the excitation filter)
- B = background image (acquired using the background filter)
- k = (primary signal/background signal)

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 34](#) for more details on acquiring an image sequence.)

To subtract tissue autofluorescence:

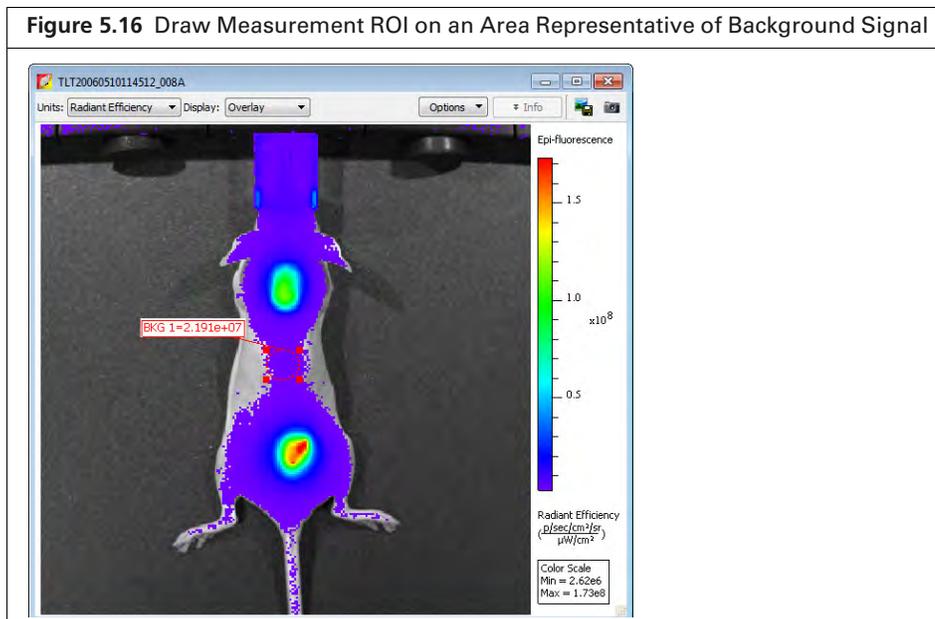
1. Load the image sequence that includes the primary and background fluorescent images.



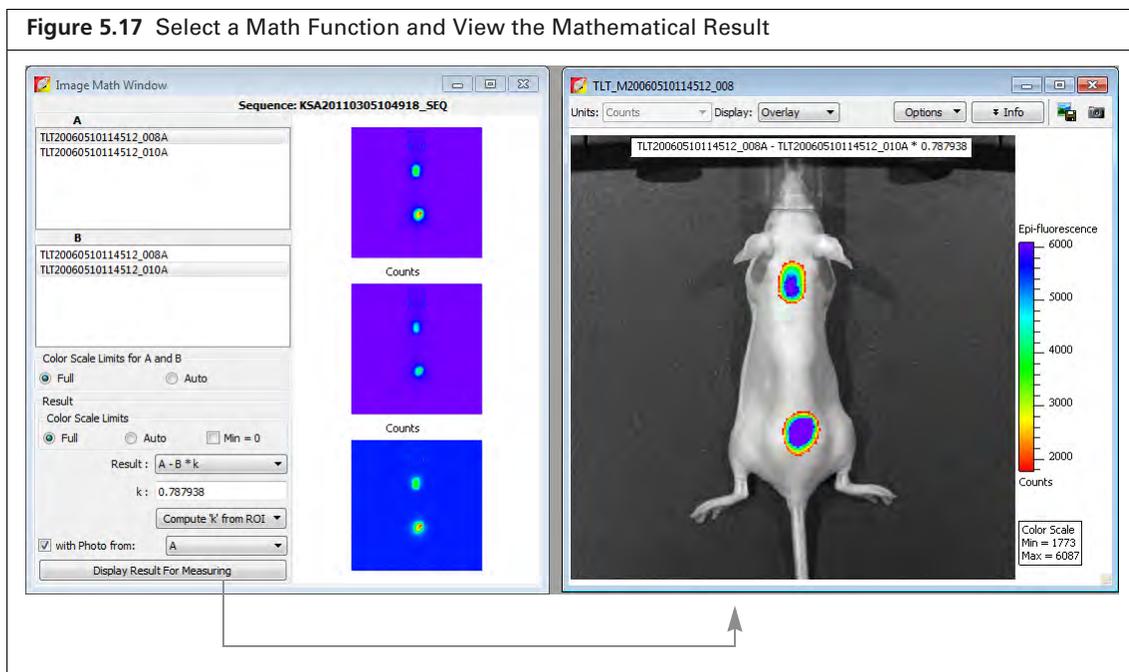
2. Open either the primary or background image:
 - a. Optimize the image display using the color scale Min and Max sliders in the Image Adjust tools.
 - b. Draw a measurement ROI on an area of the animal that represents background signal (area where no fluorophore signal is present, [Figure 5.16](#)).



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



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 69 for more details on the Image Math window.
5. Select the math function 'A-B*k' in the Result drop-down list.

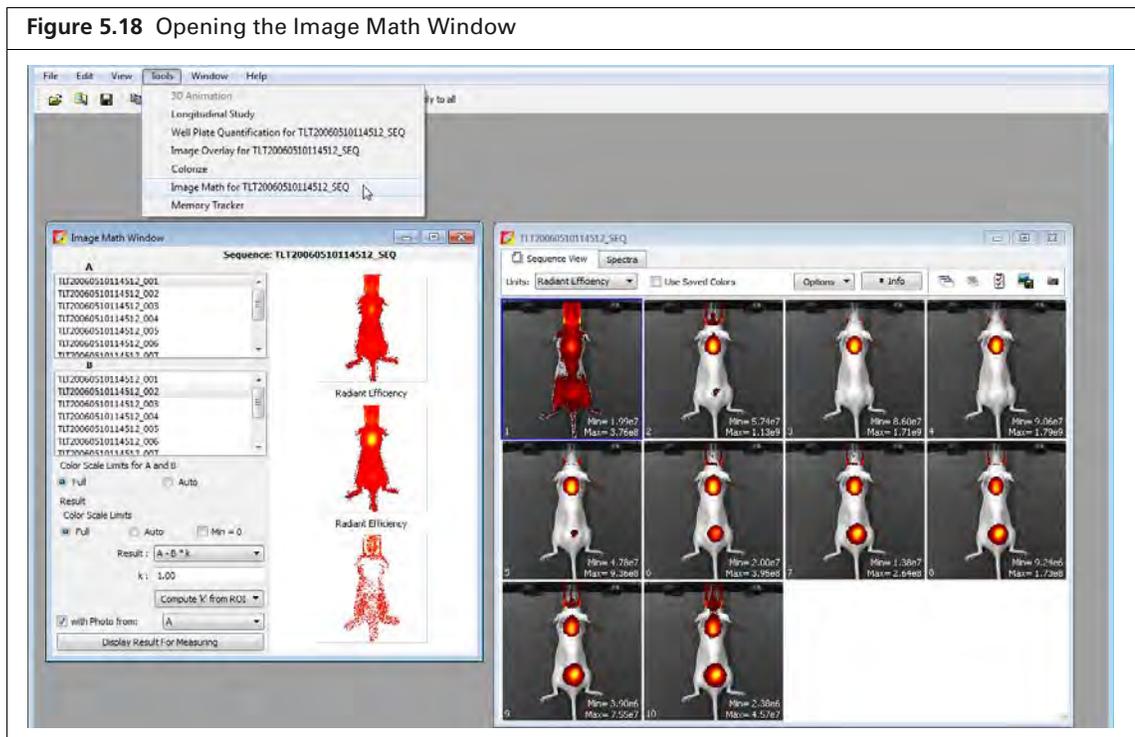


6. Click **Compute 'k' from ROI** and select the ROI (created in step 2) from the drop-down list.
 The background-corrected signal is displayed.
7. To view the mathematical result (overlay mode) in a separate image window, click **Display Result For Measuring**.
 If necessary, use the Color Scale Min and Max sliders in the Image Adjust tools to adjust the image display.

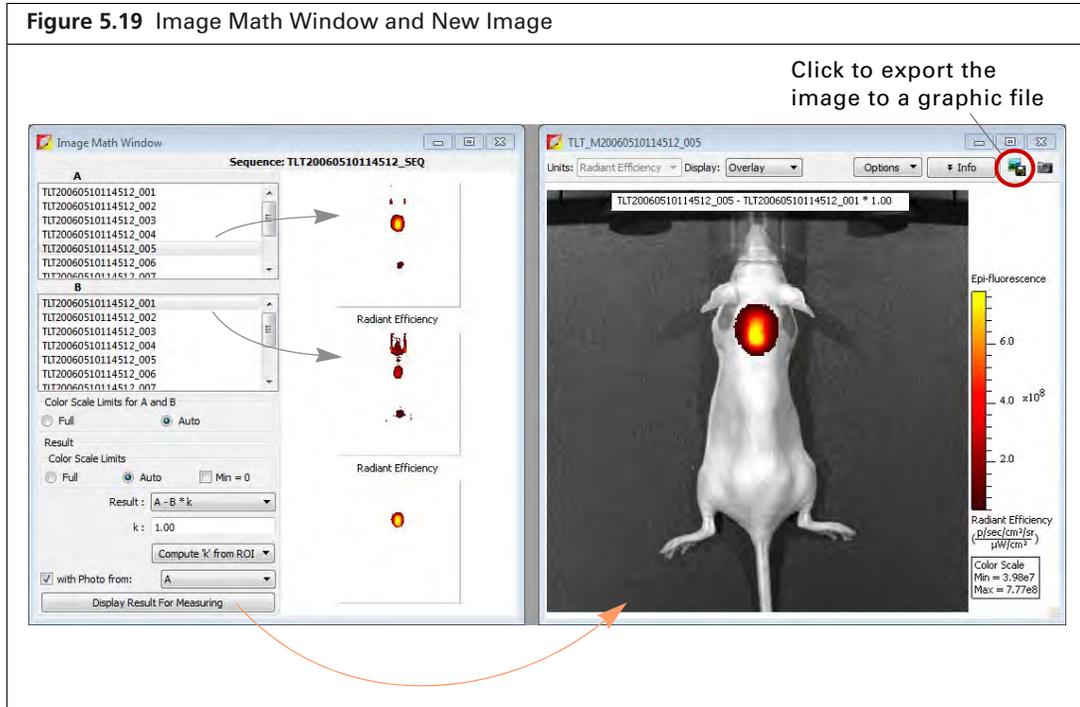
8. To save the new image:
 - a. Click the **Save** button . Alternatively, select **File** → **Save** on the menu bar.
 - b. Select a directory in the dialog box that appears and click **Save**.
 A folder of data is saved to the selected location (AnalyzedClickInfo.txt, ClickInfo.txt, luminescent and 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).



3. In the Image Math window that appears, select an image from box A and from box B. The Image Math window shows a thumbnail of image A, image B, and the new image (Figure 5.19).



NOTE: For more details on items in the Image Math window, see [Table 5.6, page 69](#)

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 c.
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	<p>Full - Choose this option to set the Max and Min values to the maximum and minimum data values in the image.</p> <p>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.</p> <p>Note: The color scale does not affect the image math result.</p>

Table 5.6 Image Math Window (continued)

Item	Description
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

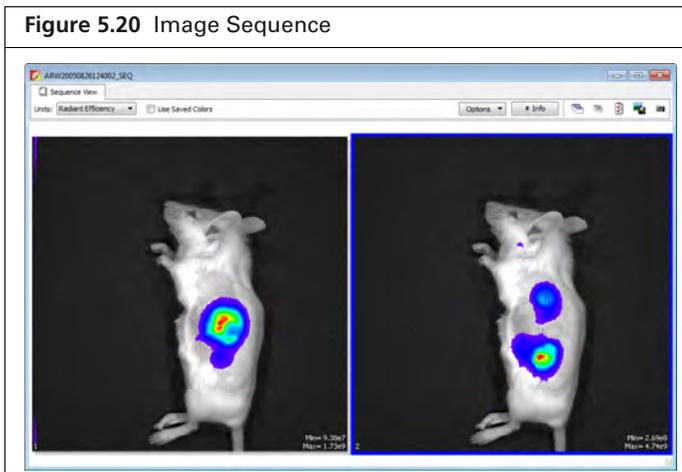
The image overlay tool provides a convenient way to view multiple reporters in one image. You can use the image overlay tool to display multiple luminescence or fluorescence images on one photographic image.



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 86](#).)
2. Load the image sequence.

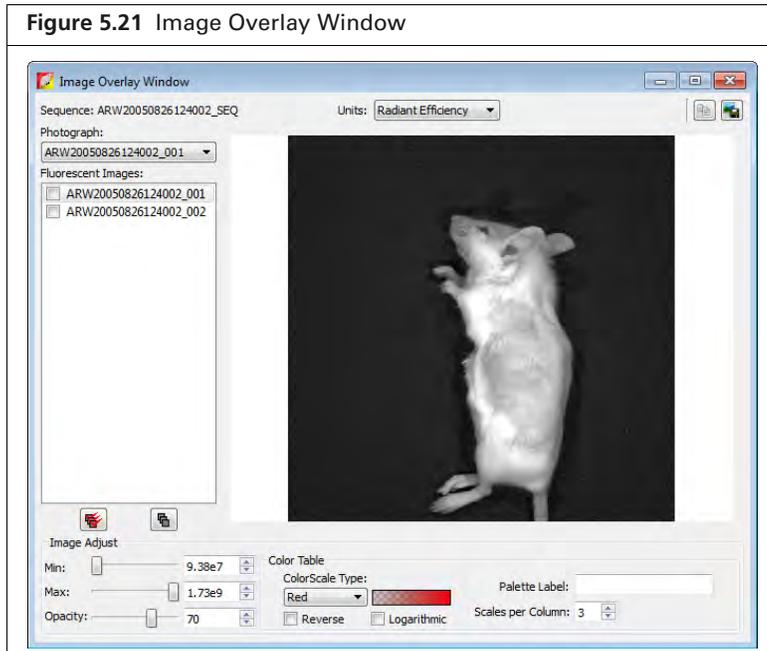


3. Open one of the images and optimize the image display using the color scale Min and Max sliders in the Image Adjust tools.

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.



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.

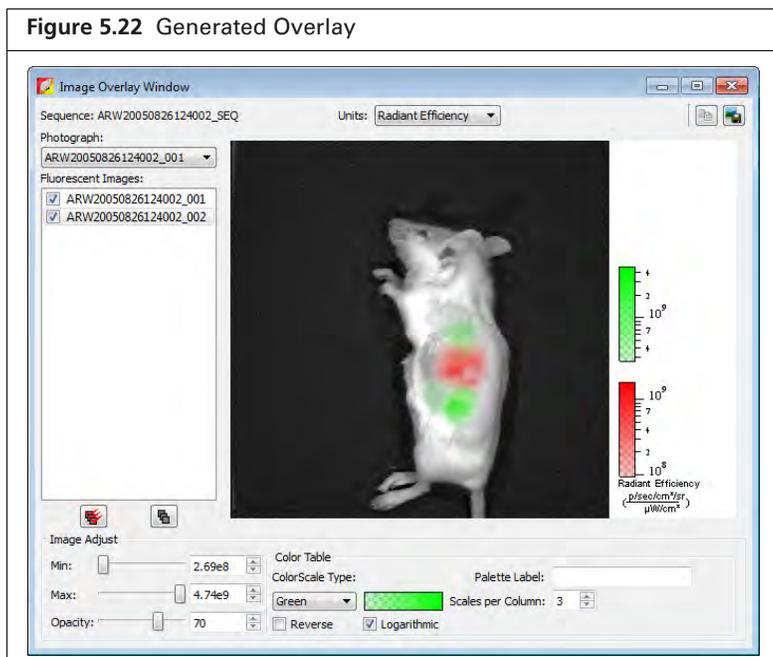


Table 5.7 Image Overlay Window

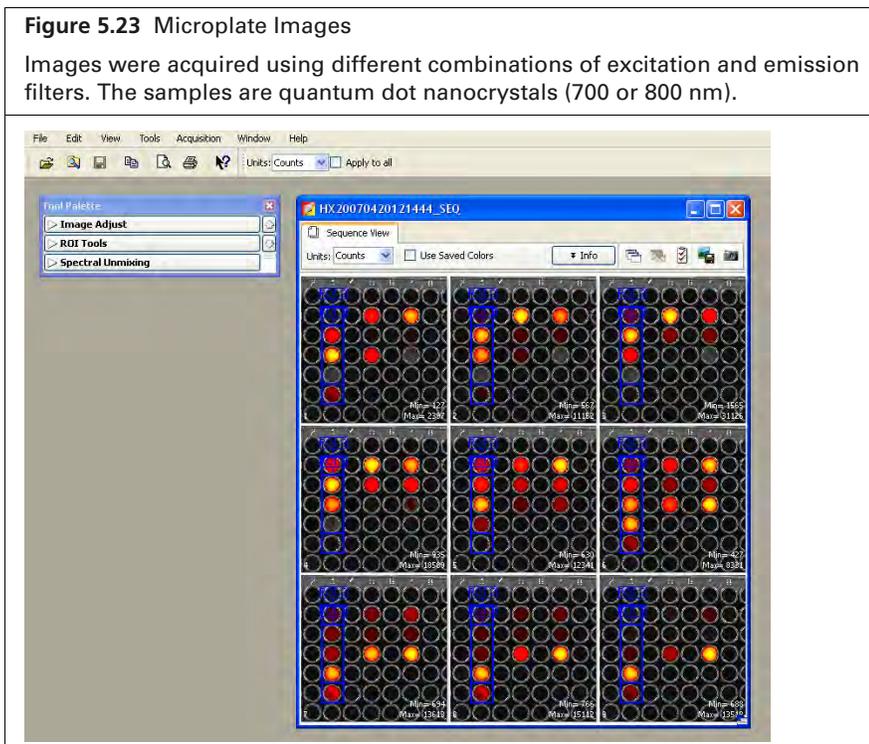
Item	Description
Units	Choose the type of units for displaying the fluorescent or luminescent image. See the concept tech note <i>Image Display and Measurement</i> for more details on measurement units.
Photograph	A drop-down list of the photographs in the image sequence.
Fluorescent or Luminescent Images	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	<p>Tools for adjusting the appearance of the highlighted fluorescent or luminescent image. Adjustments can only be made on one image at a time.</p> <p>Min – The minimum pixel intensity associated with the color scale for an image. Pixels less than the minimum value are not displayed.</p> <p>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.</p> <p>Opacity – Controls the opacity of the fluorescent or luminescent image.</p>
Color Table	<p>Tools for selecting and modifying the color scale associated with an image.</p> <p>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.</p> <p> Click the drop-down arrow to select a color table for the image data. See the concept tech note <i>Image Display and Measurement</i> for more details on color tables (select Help → Tech Notes on the menu bar).</p> <p>Reverse – Choose this option to reverse the selected color table.</p> <p>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.</p>
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.



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).

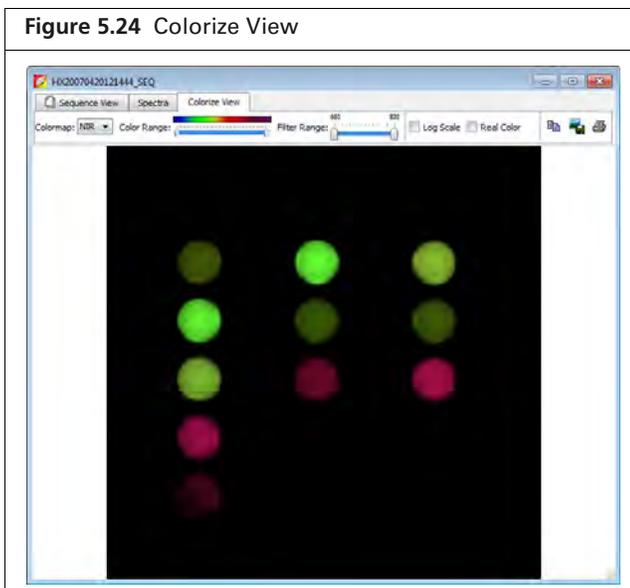


Table 5.8 Colorize Tools

Item	Description
Colorize View	
Color Map	<p>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.</p> <p>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.</p>
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	<p>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.</p> <p>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.</p>
	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

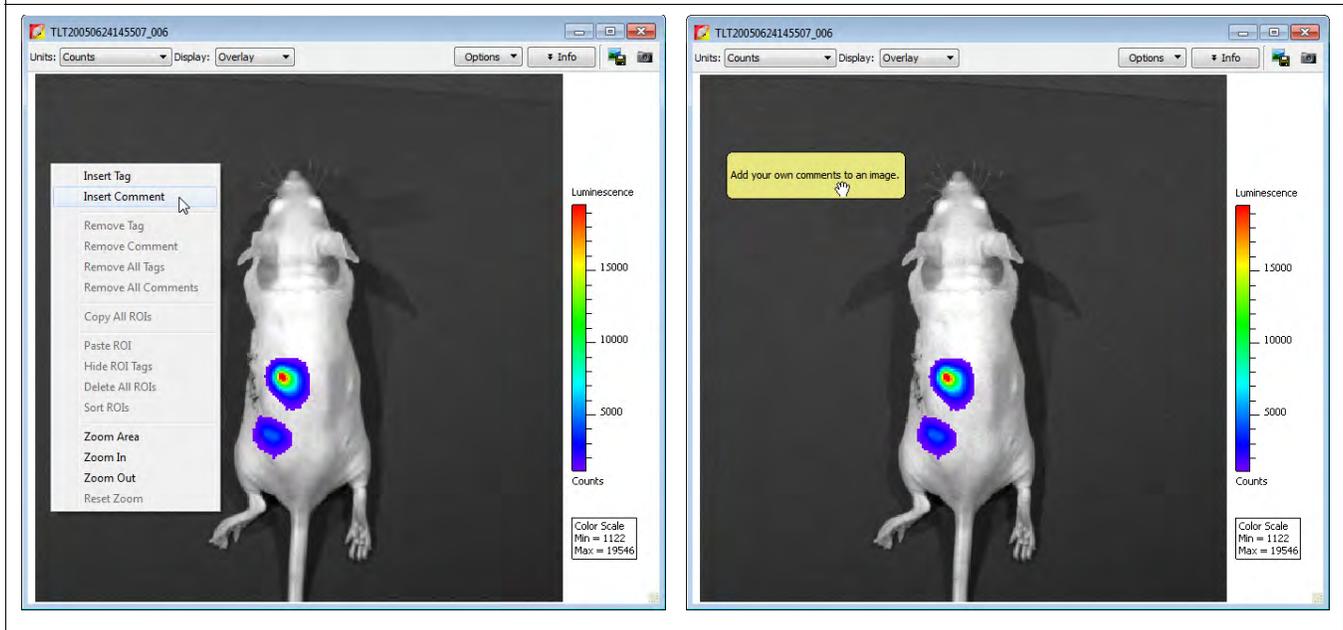
Comments can be added to an image and saved with the image.

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



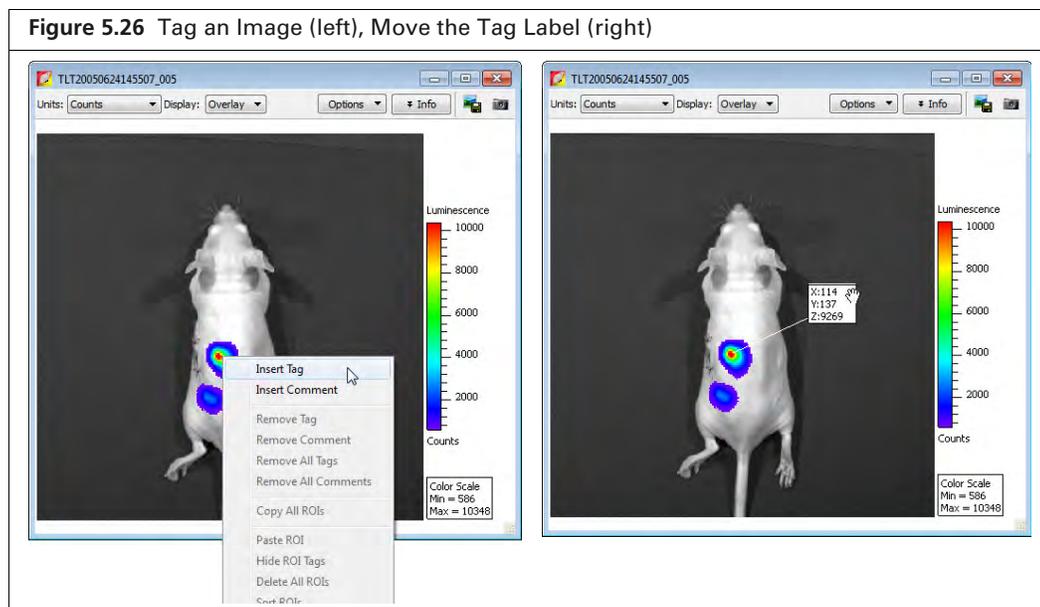
Applying Tags

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 a Single 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.

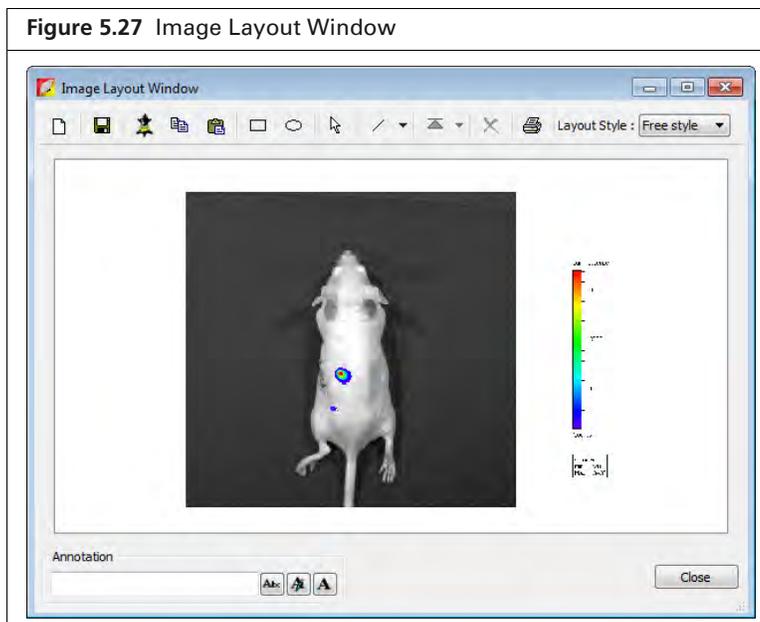
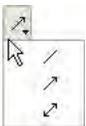


Table 5.9 Image Layout Window

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.

Table 5.9 Image Layout Window (continued)

Item	Description
	Copies the contents of the Image Layout window to the system clipboard.
	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

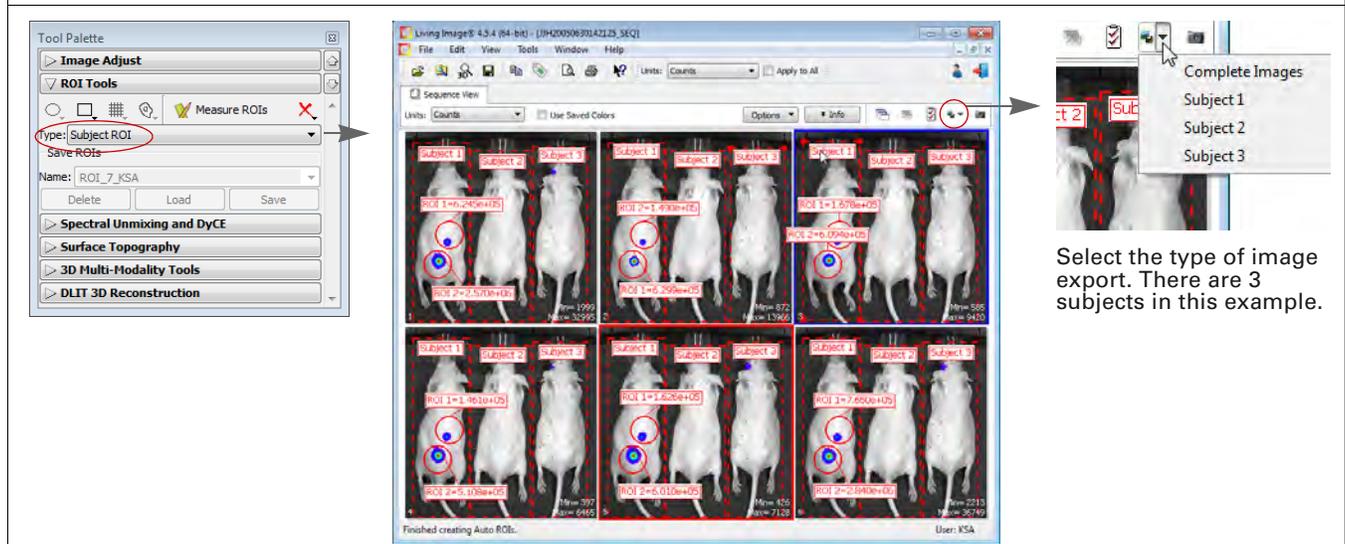
You can export sequence images or images of a user-selected subject from a sequence to a graphic file.

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



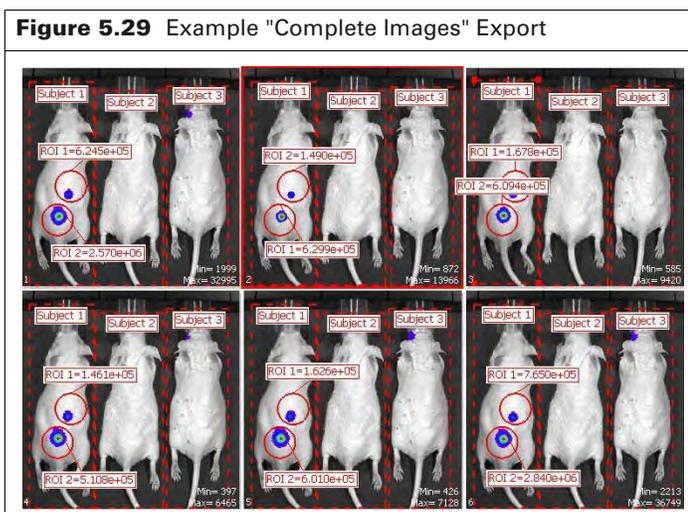
3. Click the Export Graphics button down arrow  and choose an option from the drop-down list.
 - Export All Images – All images will be exported to one file (Figure 5.29 on page 79). See below for instructions.
 - Subject "N" – Images of the selected subject can be exported to either:
 - One file (Figure 5.32 on page 80). See page 79 for instructions.
 - or
 - Separate files (Figure 5.35 on page 82). See page 81 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 78](#) 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.



Export Images of a Subject

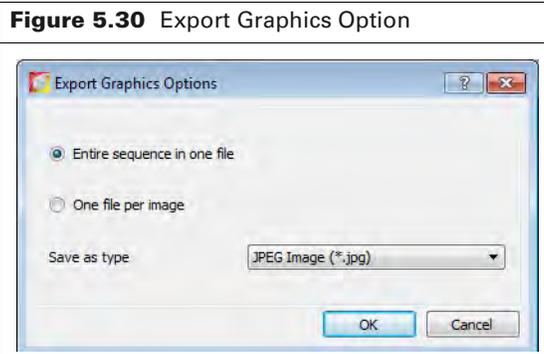
NOTE: Be sure to apply subject ROIs to the images before proceeding with the export. See [Preparing for Export on page 78](#) for instructions.

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 80).
- 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 82).

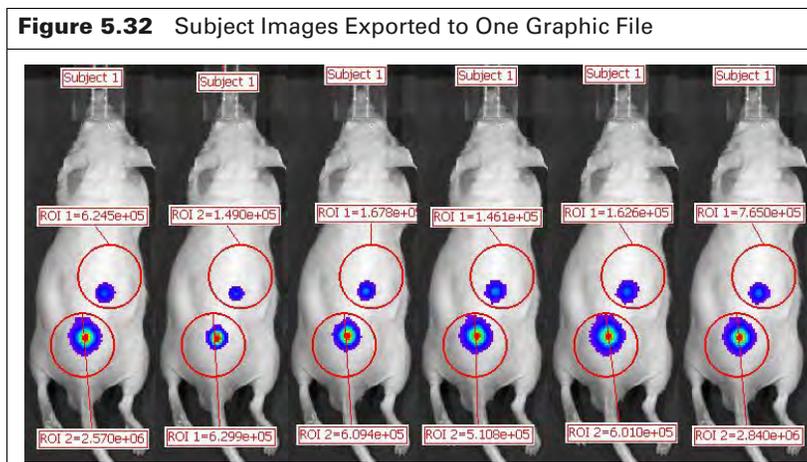
Exporting the Images to a Single File

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).



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**.

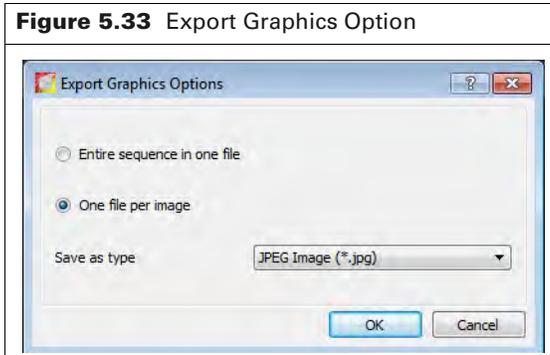
The software will export the image to a subfolder (named with the sequence identifier "xxx_SEQ") in the selected folder.



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

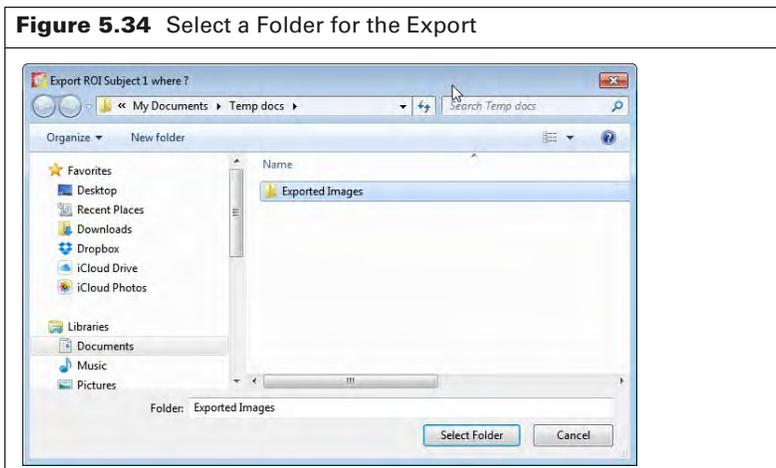
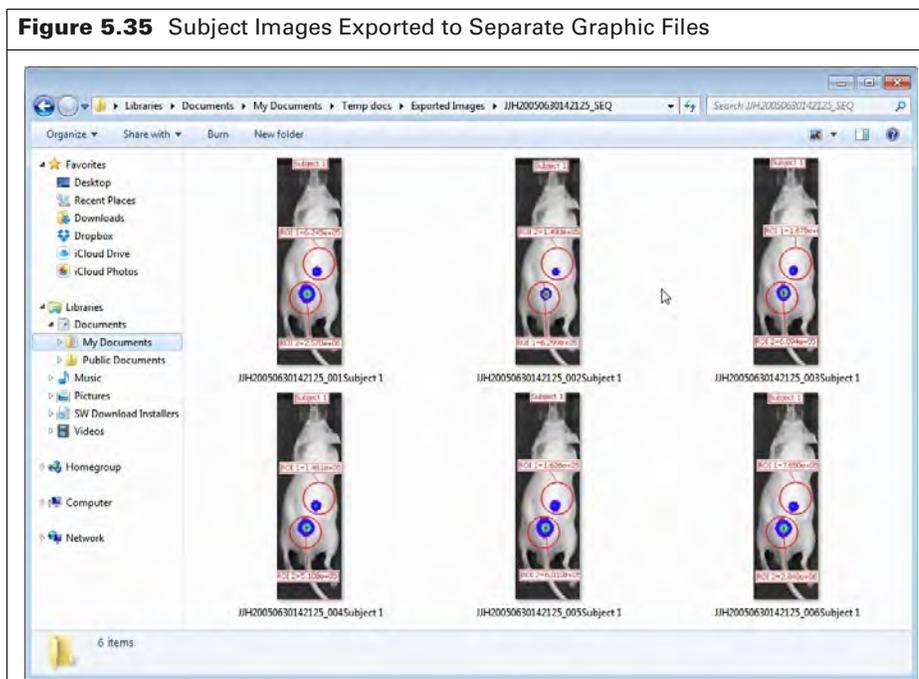
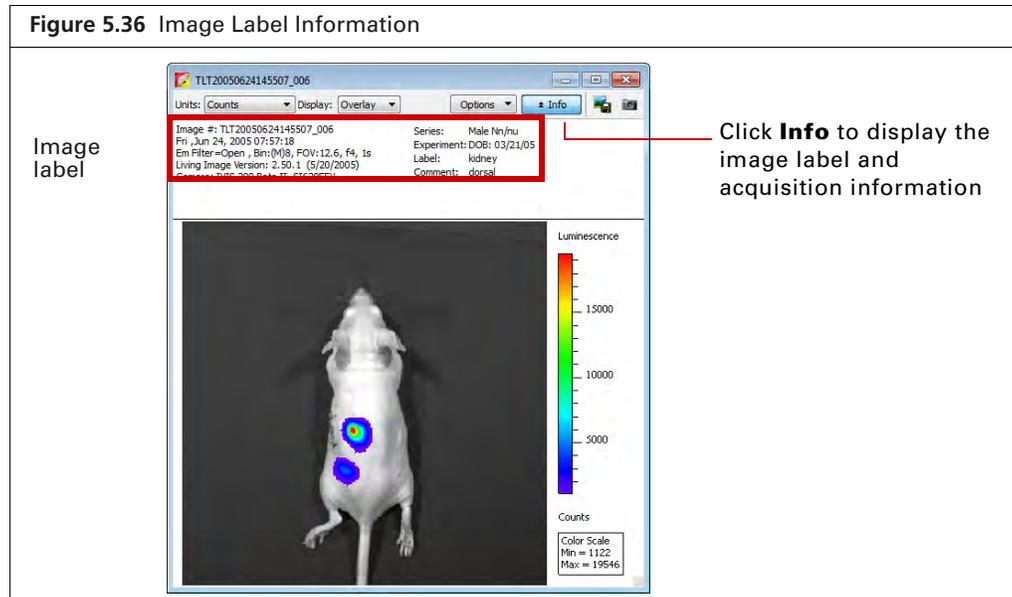


Figure 5.35 Subject Images Exported to Separate Graphic Files



5.11 Managing Image Information

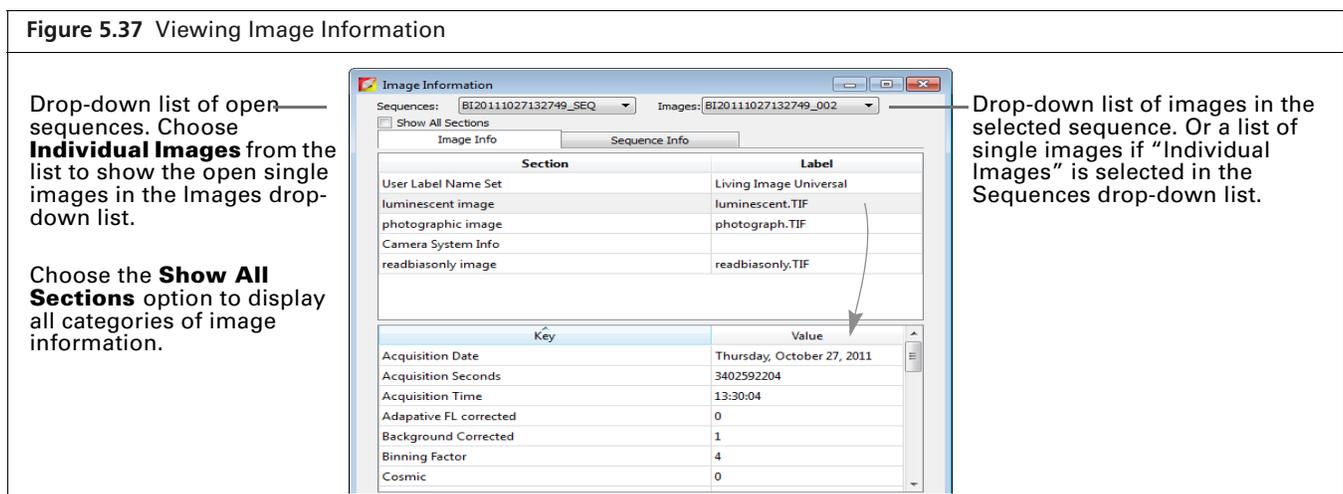
At acquisition the software captures image information such as camera parameters and any image label information you entered at acquisition time (Figure 5.36).



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).



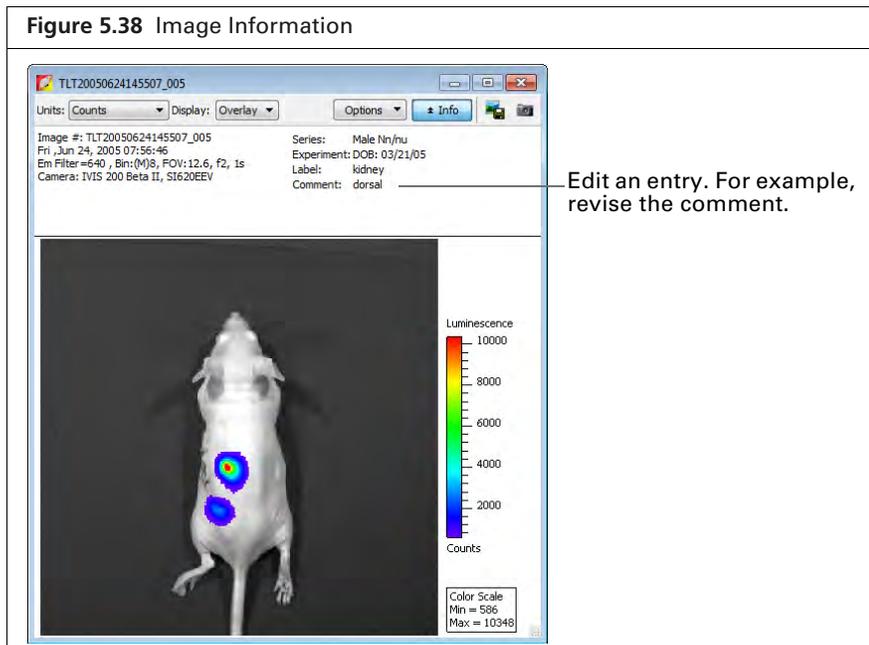
4. To view particular information, select a category in the upper box to show the associated information in the lower box. For example, select luminescent image in the upper box to show the luminescent image acquisition parameters.

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).



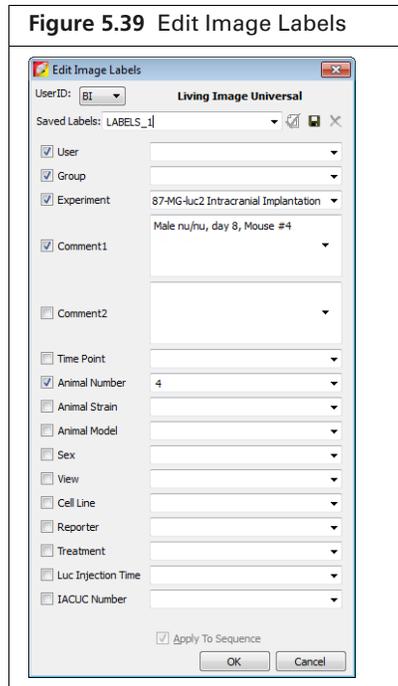
3. Edit the label information.

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.



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).

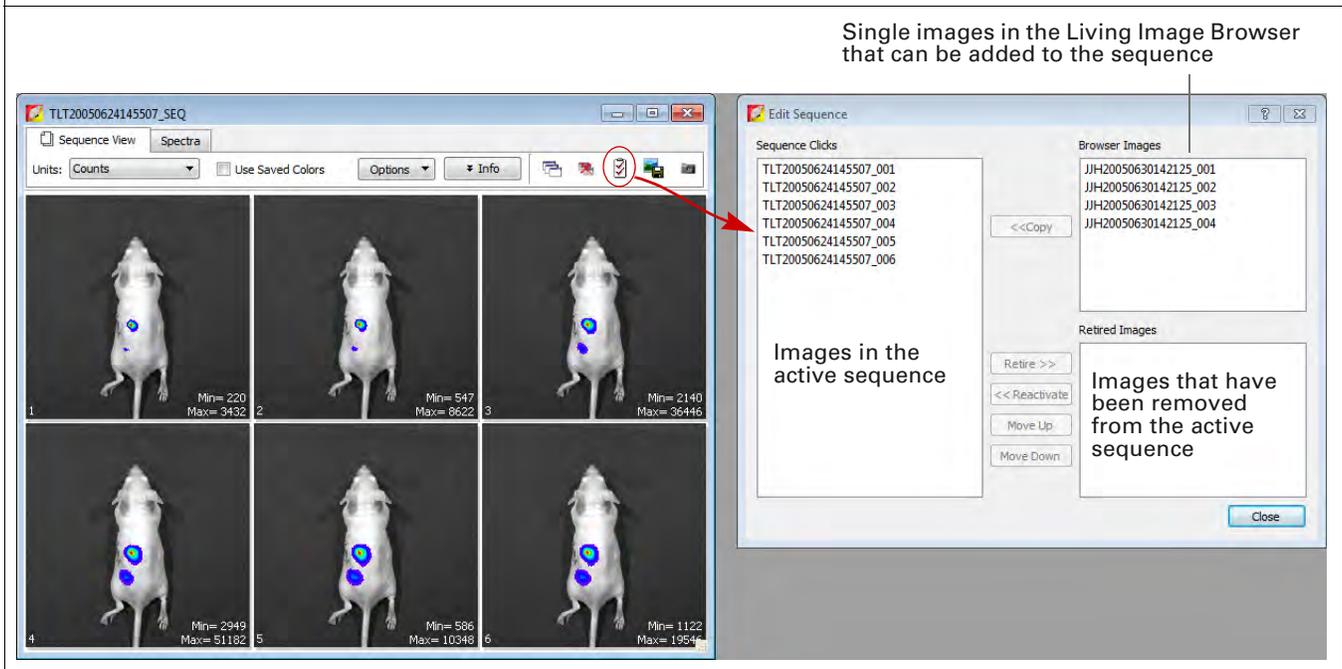
5.12 Managing Image Sequences

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 49](#) 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 49 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

Individual images that may or may not be part of a sequence can be selected

Images loaded in the browser as part of a sequence. These images can also be selected for grouping into another sequence.

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.
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

About ROIs

Overview of ROI Tools on page 90

Measuring Signal on page 92

Measuring Background-Corrected Signal on page 96

Measuring Signals Obtained Using the Side Imager on page 99

Managing ROIs on page 102

ROI Measurements on page 108

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

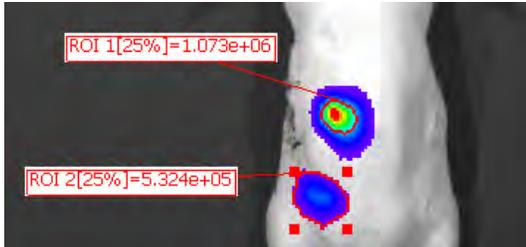
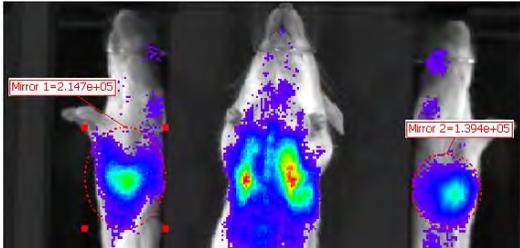
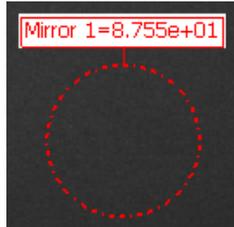
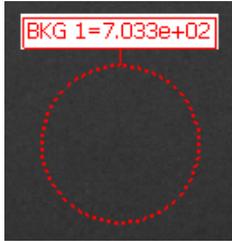
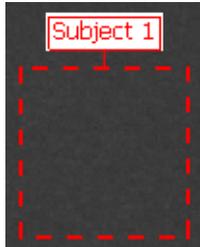
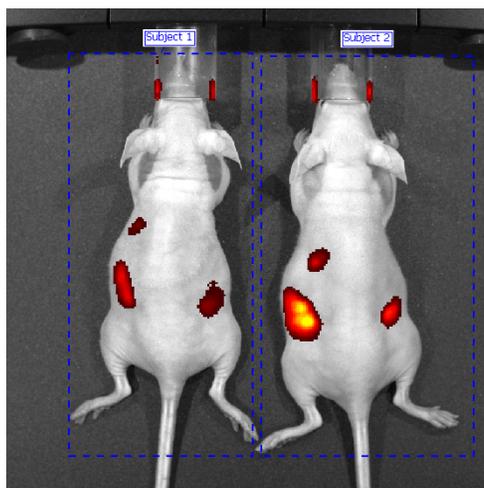
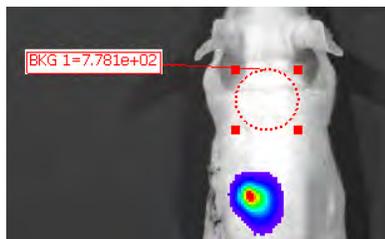
ROI Name	Description	ROI Line and Available Shapes	See Page
Measurement ROI	<p>Measures the signal (surface intensity) in an area of an image.</p> 	 <p>Circle, square, grid, or contour</p>	92
Mirror ROI	<p>Images acquired using the Side Imager have three views: left, right, and center.</p> <ul style="list-style-type: none"> ■ Left or right images – Measure signal using a mirror ROI. ■ Center image (non-reflected view) – Measure signal using a measurement ROI. 	 <p>Circle or square</p>	99

Table 6.1 Types of ROIs (continued)

ROI Name	Description	ROI Line and Available Shapes	See Page
<p>Average Background ROI</p>	<p>Measures the average signal (surface intensity) in a user-specified area of an image that is considered background. Only available in image view.</p> <p>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.</p>	 <p>Circle or square</p>	<p>96</p>
<p>Subject ROI</p>	<p>Identifies a subject in an image.</p> <p>Note: Using this type of ROI is optional. A subject ROI enables you to:</p> <ul style="list-style-type: none"> ■ 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. 	 <p>Square</p>	<p>98</p>



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 108](#) for more information.

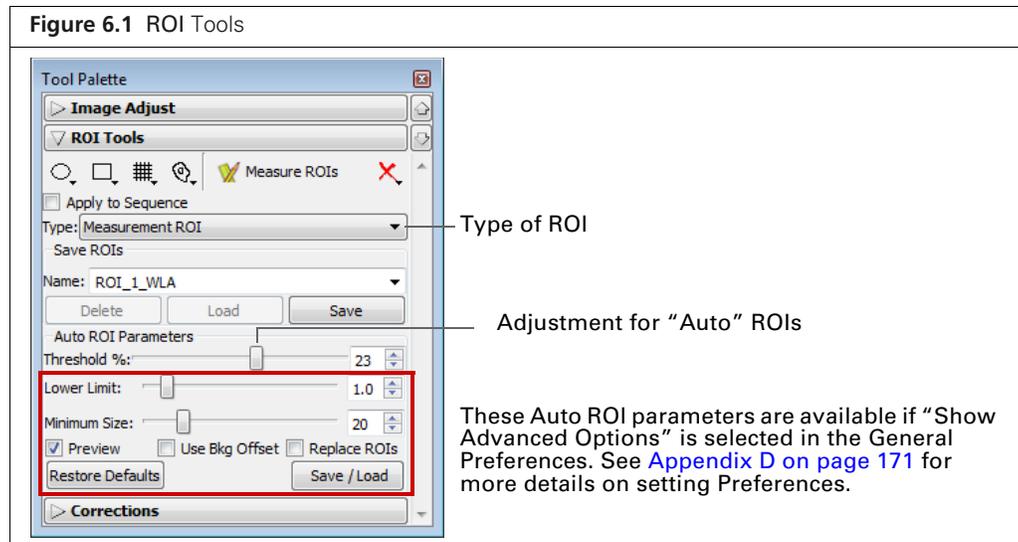


Table 6.2 ROI Tools

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 94 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 108 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)

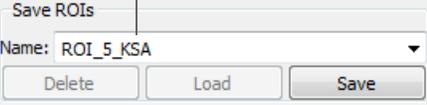
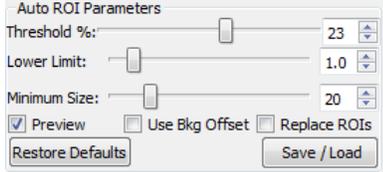
Item	Description
Apply to Sequence	<p>This option is available when an image of a sequence is open. If this option is selected:</p> <ul style="list-style-type: none"> ■ ROIs created on the active image will also be created on the other images of the sequence. ■ Adjustments to an ROI in the image will be applied to related ROIs in the other images of the sequence. See page 94 for more information about related ROIs. ■ Deleting an ROI in the image will delete related ROIs from the other images of the sequence.
Type	<p>Choose the ROI type from the Type drop-down list:</p> <p>Measurement – Measures the signal intensity in an area of an image.</p> <p>Average Bkg – Measures the average signal intensity in a user-specified area of the image that is considered background.</p> <p>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.</p> <p>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.</p>
Save ROIs to the system	<div style="display: flex; align-items: center;"> <div style="margin-right: 20px;"> <p style="text-align: center;">Name of an ROI or a set of ROIs</p>  </div> <div> <p>These ROI tools are only available when an image is active.</p> </div> </div> <p>ROIs (parameters only such as coordinates, type, shape, location) can be saved to the system (per user) and to apply the ROIs to other images. These ROIs appear in the Name drop-down list. See page 107 for instructions.</p> <p>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.</p>
Auto ROI Parameters	<p>Parameters that specify how the auto ROI tool draws an ROI.</p> <p>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.</p> <p>Note: The following Auto ROI parameters are only available if “Show Advanced Options” is selected in the general preferences. See Appendix D, page 171 for details on setting Preferences.</p> <div style="margin-bottom: 20px;">  </div> <p>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.</p> <p>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.</p> <p>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.</p> <p>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 96 for more details.</p> <p>Replace ROIs – If this option is chosen, all auto ROIs are replaced when new ROI(s) are created.</p>

Table 6.2 ROI Tools (continued)

Item	Description
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.

1. Open an image or image sequence.

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.

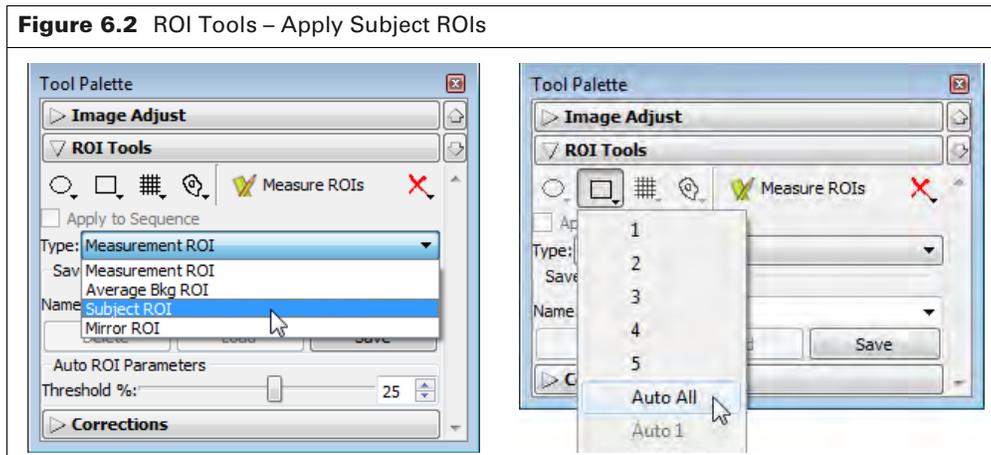
If an image of a sequence is active, selecting the “Apply to Sequence” option in the ROI tools will create related ROIs in the other images of the sequence as well.

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 110](#) for instructions.

2. If there are multiple subjects in an image, apply subject ROIs. If not, skip to [step 3](#).
 - 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.

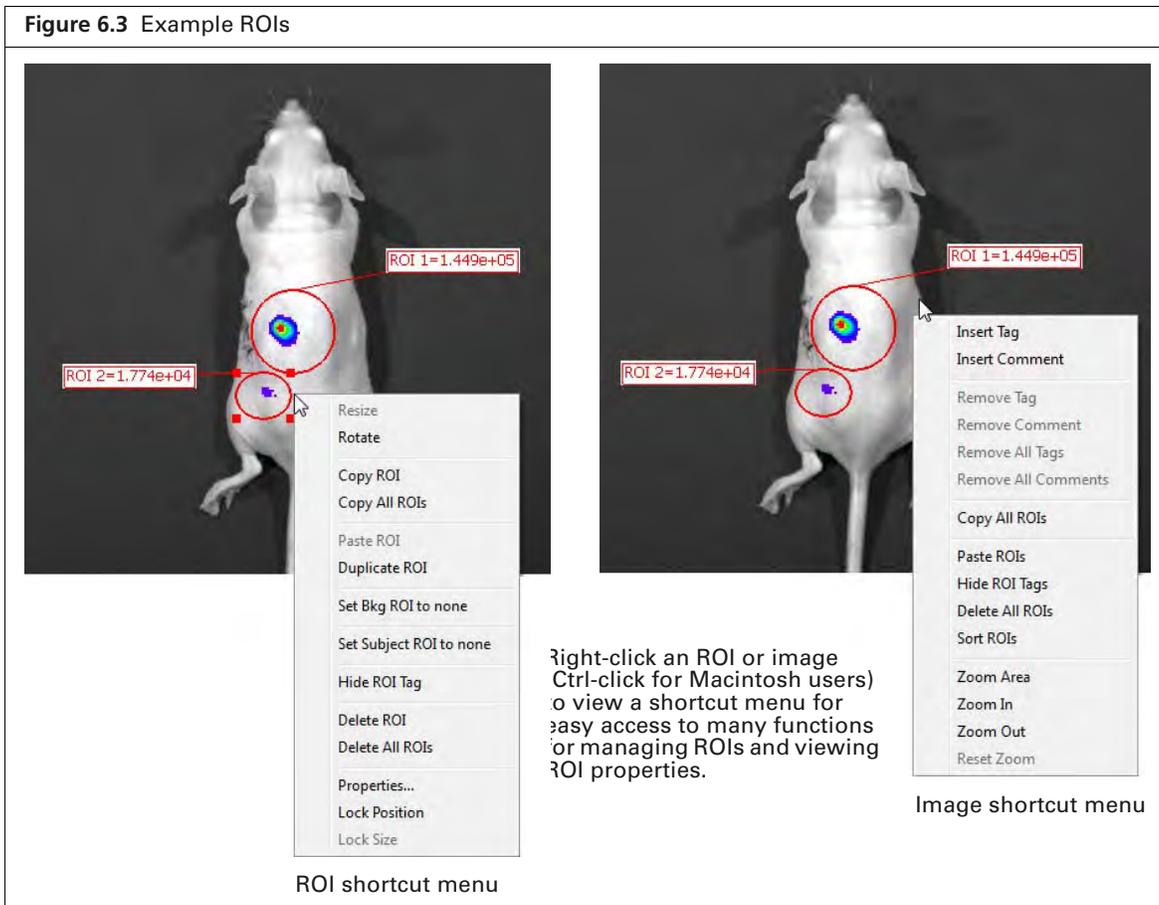


3. Select **Measurement ROI** from the Type drop-down list
4. Click an ROI shape (Circle , Square , Grid , or Contour ) and make a selection from the drop-down list. If applying a grid ROI, choose the grid dimensions.

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

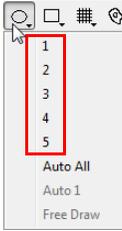
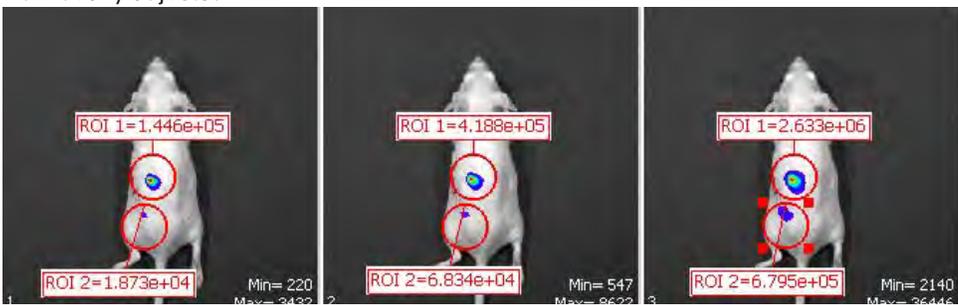
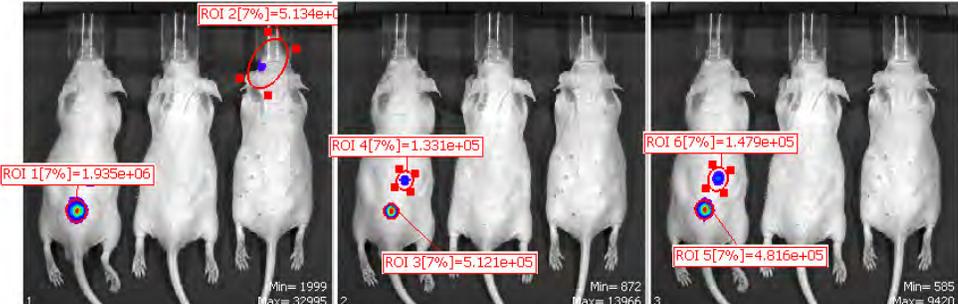
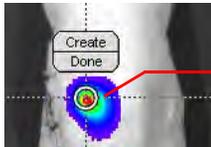


NOTE: If using subject ROIs, ensure that all of the measurement ROIs for a subject are completely within the subject ROI by resizing the subject ROI if necessary (see [page 95](#) for instructions on resizing ROIs). Measurement ROIs not completely within a subject ROI will not be counted as part of the subject.



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
	<p>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 95 for instructions on adjusting ROIs.</p> <p>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.</p> 
<p>Auto All</p>	<p>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.</p>  <p>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.</p> <p>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.</p> <p>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 102 for more information.</p>
<p>Auto 1</p>	<p>Only available in image view. Automatically identifies signal and applies an ROI using the auto ROI parameter thresholds at a user-selected location.</p>  <p>Drag the ring to the location for the ROI and click Create. Click Done when you finish drawing all ROIs.</p>
<p>Free Draw</p>	<p>Only available in image view. To draw a:</p> <ul style="list-style-type: none"> ■ 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.

5. Adjust ROI size or position if necessary (see [Table 6.4](#)).

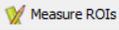


NOTE: The position and size of auto ROIs (ROIs created using Auto All or Auto 1) are locked by default. To unlock auto ROIs:

- 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 104](#)).
- Image view – Right-click the ROI and select unlock options from the shortcut menu.

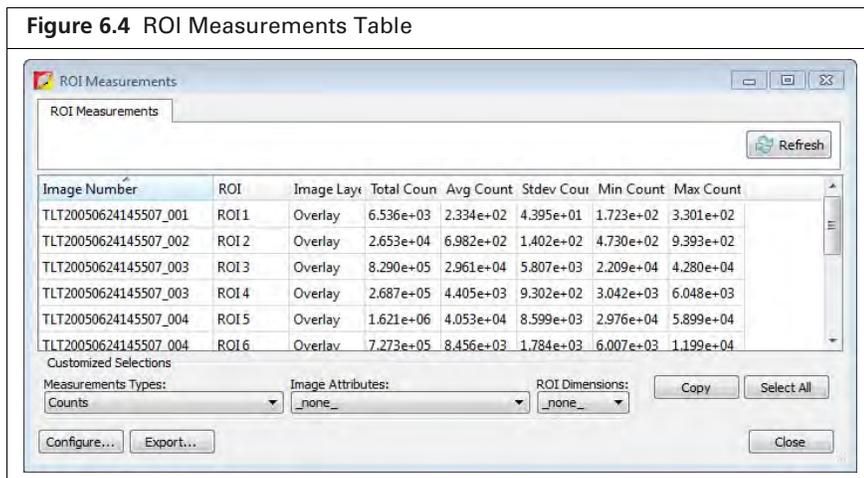
Table 6.4 Select and Adjust ROIs

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. <ul style="list-style-type: none"> ■ 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.

- Click the **Measure** button  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 108](#) for more details.



- 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 107](#) 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:

$$\text{Background-corrected intensity signal} = \text{Signal in the measurement ROI} - \text{Average signal in the average background ROI}$$

 **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.

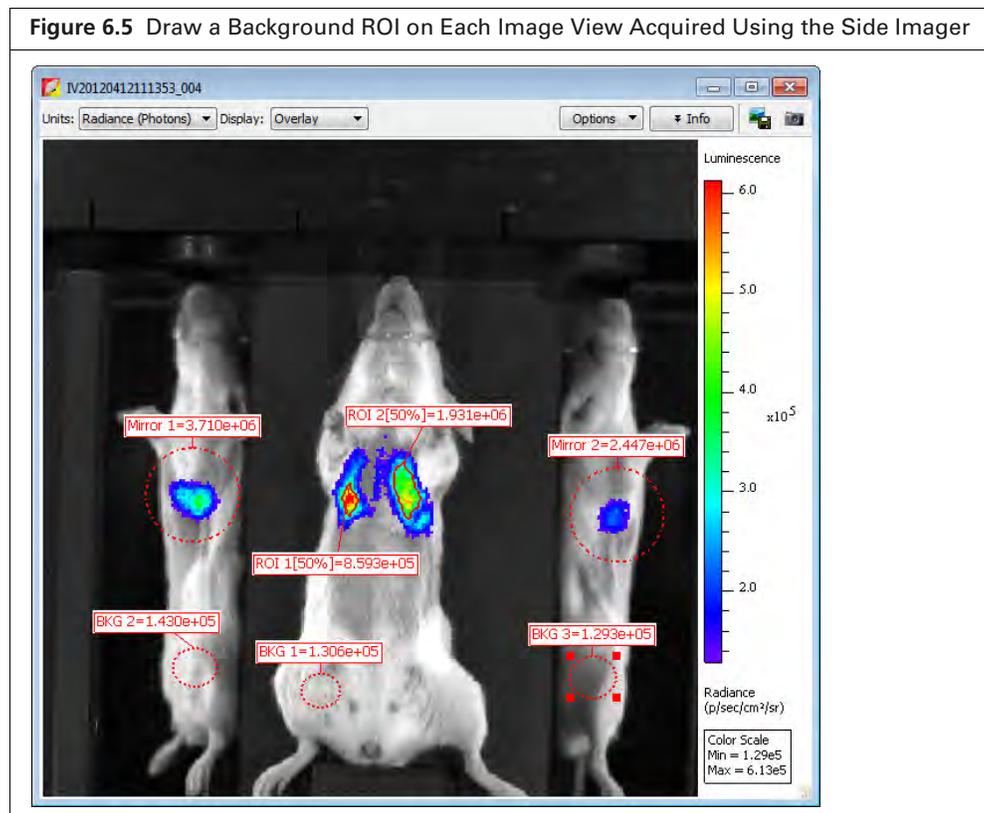
The Image Adjust tools and zoom feature are helpful for selecting an appropriate area for an ROI. By setting the image minimum close to zero and zooming in on a background area in the image, you can determine where naturally occurring background luminescence or autofluorescence is present. For more details on the Image Adjust tools and the zoom feature, see [Viewing Intensity Data on page 62](#) and [Zooming or Panning on page 60](#).

To measure background-corrected signal:

1. Open an image and draw one or more measurement ROIs on the subject (see [page 92](#) 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 95](#) for instructions).

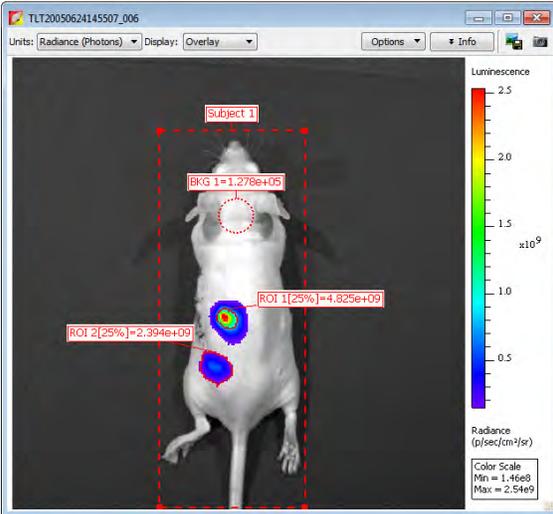


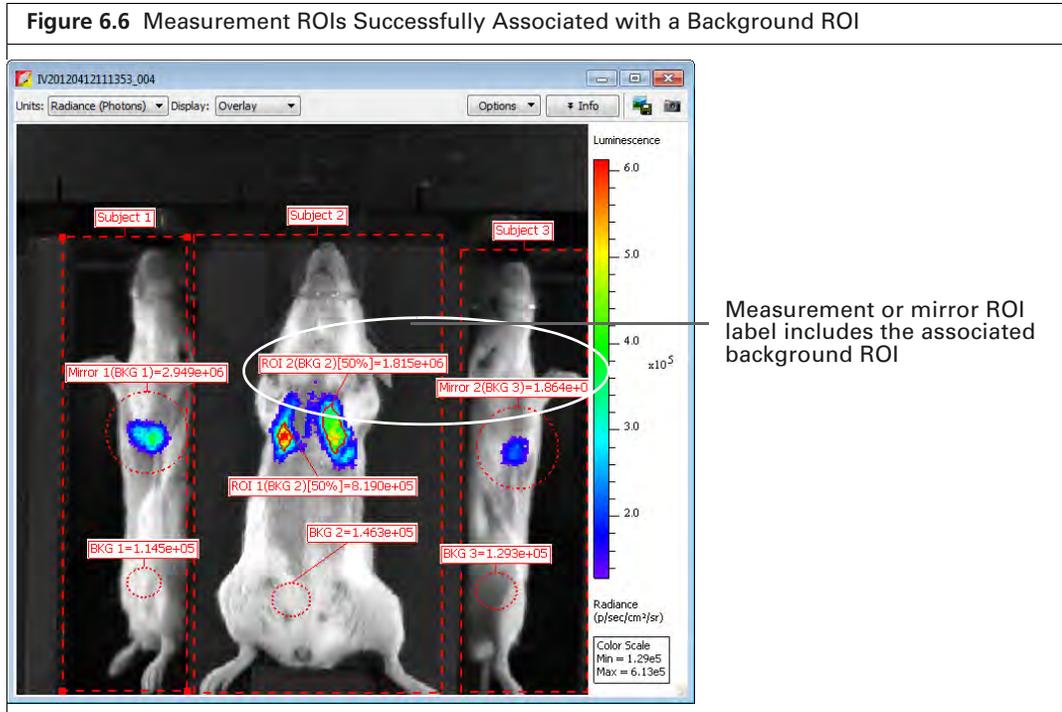
NOTE: The average background ROI and measurement ROI do not need to be the same shape or size because the software computes the average intensity signal in each ROI. If the image was acquired using the Side Imager, draw a background ROI on each view (Figure 6.5).



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
<p>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.</p> <p>To draw a subject ROI:</p> <ol style="list-style-type: none"> 1. Select "Subject ROI" from the Type drop-down list in the ROI tools. 2. To apply ROIs: <ul style="list-style-type: none"> 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. 	
<p>Right-click a measurement ROI and select an average background ROI from the shortcut menu.</p>	
<ol style="list-style-type: none"> 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. 	

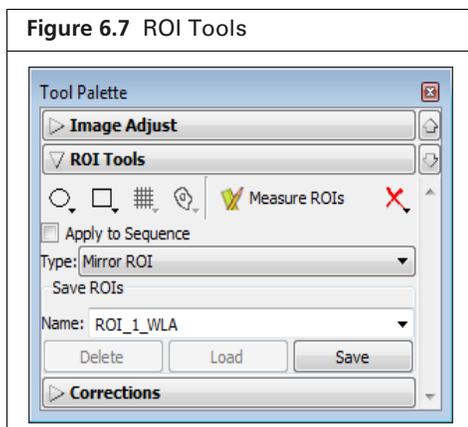


6.5 Measuring Signals Obtained Using the Side Imager

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 92](#) 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.



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 101](#)).
 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.)

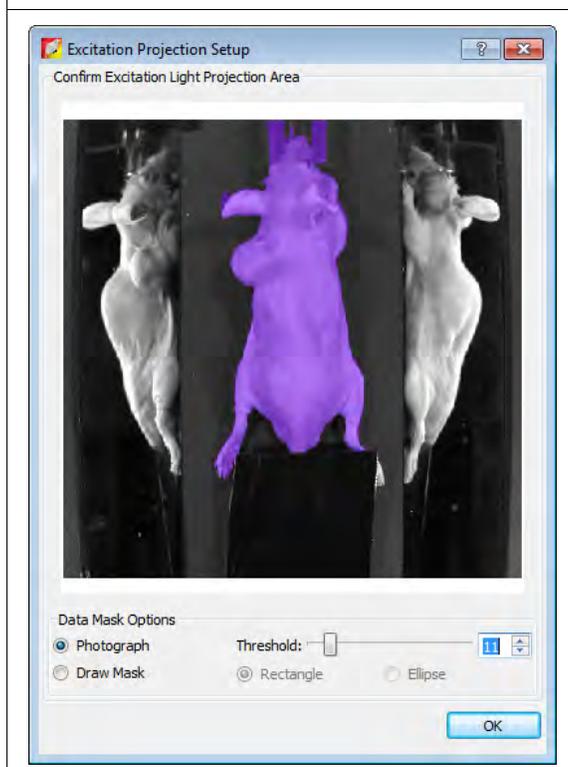


Table 6.6 Data Mask Options

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.

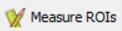
Table 6.6 Data Mask Options (continued)

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 95](#),
6. Click the **Measure** button  .
 The ROI measurements and table appear. See [ROI Measurements on page 108](#) 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 [page 107](#) 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](#)).

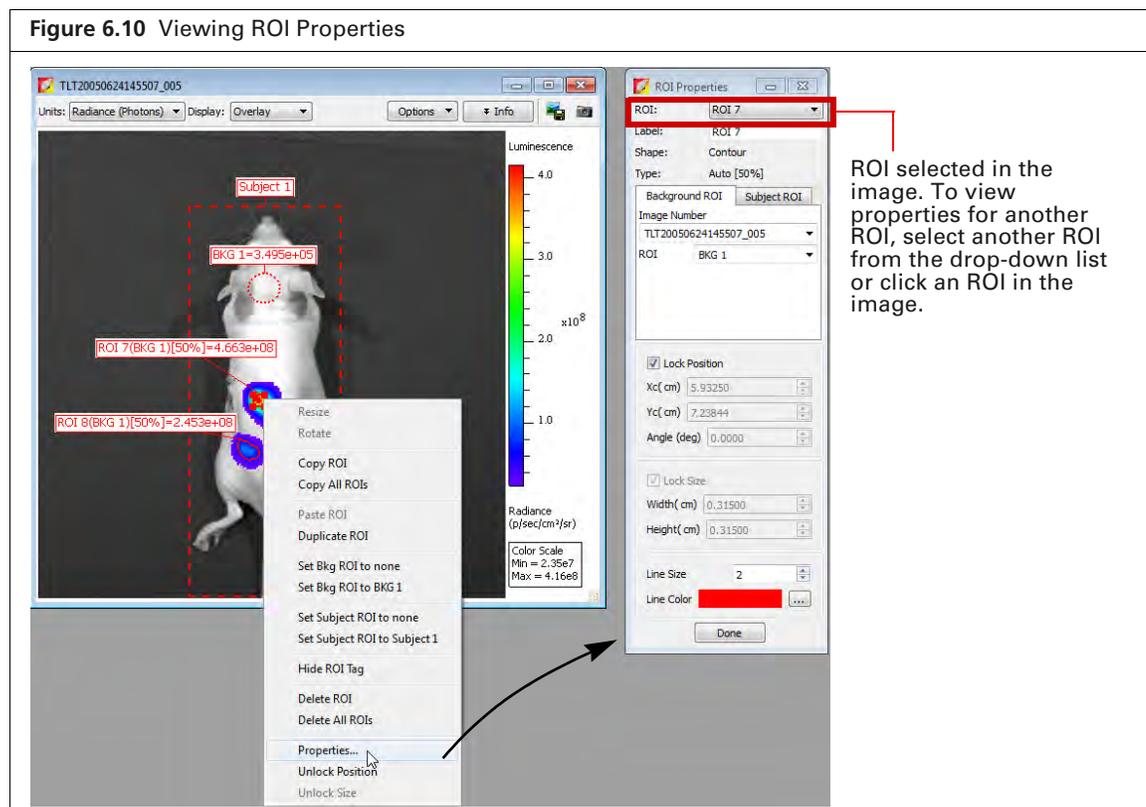


Figure 6.11 ROI Properties – Background ROI Tab

The items in the ROI Properties box depend on the type of ROI selected in the image. See [Table 6.7, page 104](#) for more details.

The figure displays two screenshots of the IVIS software interface, each showing a grayscale image of a mouse with overlaid ROIs and a corresponding ROI Properties dialog box.

Top Screenshot: Background ROI Selected

- The main image shows a mouse with three ROIs: a red circle on the head labeled "BKG 1=1.278e+05", a blue circle on the chest labeled "ROI 1(BKG 1)[25%]=4.825e+09", and a blue circle on the abdomen labeled "ROI 2(BKG 1)[25%]=2.394e+09". A yellow box with the text "Background ROI selected" is overlaid on the image.
- The ROI Properties dialog box shows:
 - ROI: BKG 1
 - Label: BKG 1
 - Shape: Circle
 - Type: Manual
 - Background ROI / Subject ROI tabs: "Background ROI" is selected.
 - Use as BKG for future ROIs in:
 - TLT20050624145507_006 only
 - Entire sequence
 - Lock Position:
 - Xc (cm): 6.22835
 - Yc (cm): 4.41437
 - Angle (deg): 0.0000
 - Lock Size:
 - Width (cm): 0.96816
 - Height (cm): 0.94369
 - Line Size: 2
 - Line Color: Red

Bottom Screenshot: Measurement ROI Selected

- The main image shows the same mouse with the same three ROIs. A yellow box with the text "Measurement ROI selected" is overlaid on the image.
- The ROI Properties dialog box shows:
 - ROI: ROI 1
 - Label: ROI 1
 - Shape: Contour
 - Type: Auto [25%]
 - Background ROI / Subject ROI tabs: "Subject ROI" is selected.
 - Image Number: TLT20050624145507_006
 - ROI: _none_
 - ID: [empty]
 - Label: [empty]
 - Lock Position:
 - Xc (cm): 5.98150
 - Yc (cm): 7.24850
 - Angle (deg): 0.0000
 - Lock Size:
 - Width (cm): 0.57750
 - Height (cm): 0.57750
 - Line Size: 2
 - Line Color: Red

Annotations with arrows point to the "ROI selected in the image" dropdown in the top dialog and the "Selected image" dropdown in the bottom dialog.

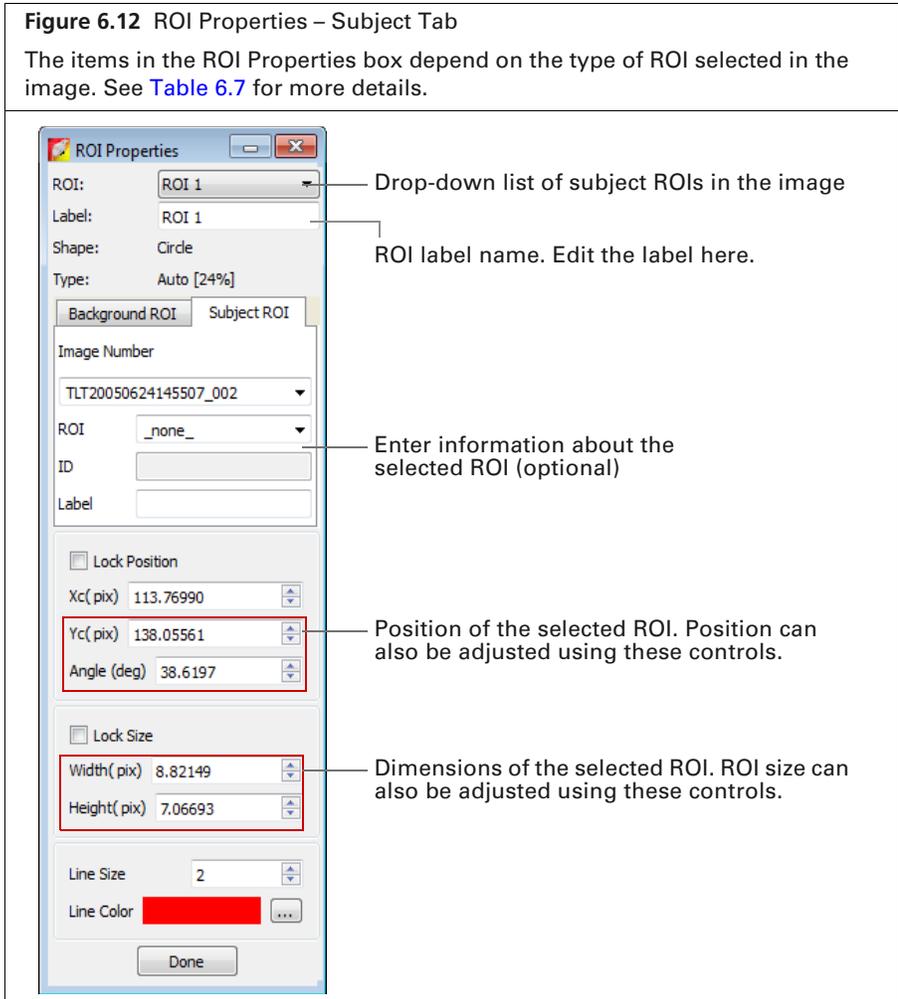


Table 6.7 ROI Properties

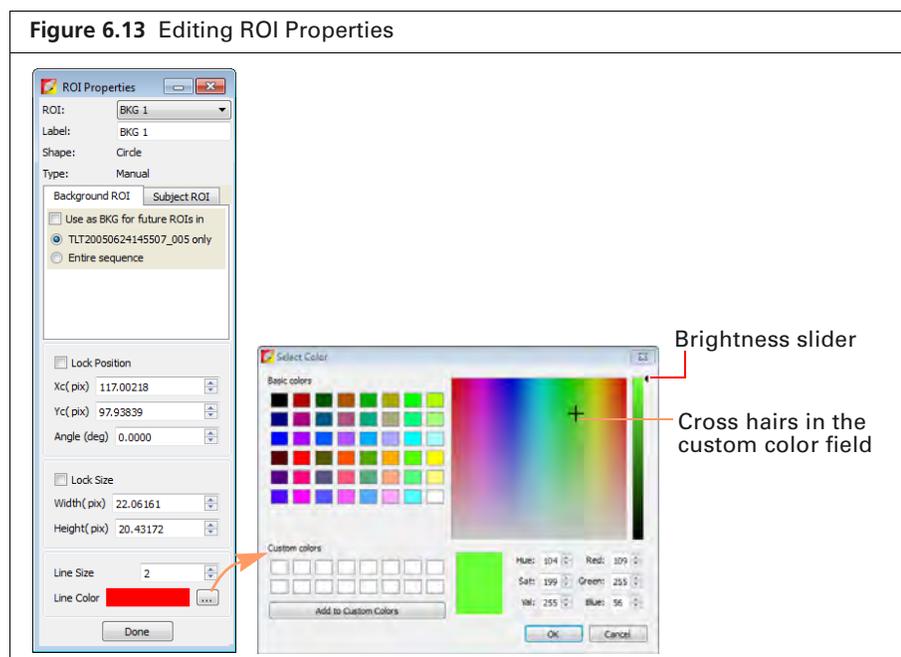
Item	Description
ROI	A drop-down list of ROIs in the active image or image sequence. To select an ROI, double-click the ROI in the image or make a selection from the drop-down list. Shape – The shape of the ROI (circle, square, grid, or contour) selected in the image. Type – Indicates the method that 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 <i>ROI Dimensions</i> on page 109 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: <ul style="list-style-type: none"> ■ Linkage between a measurement ROI and subject ROI (See Table 6.5 on page 98 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](#)).

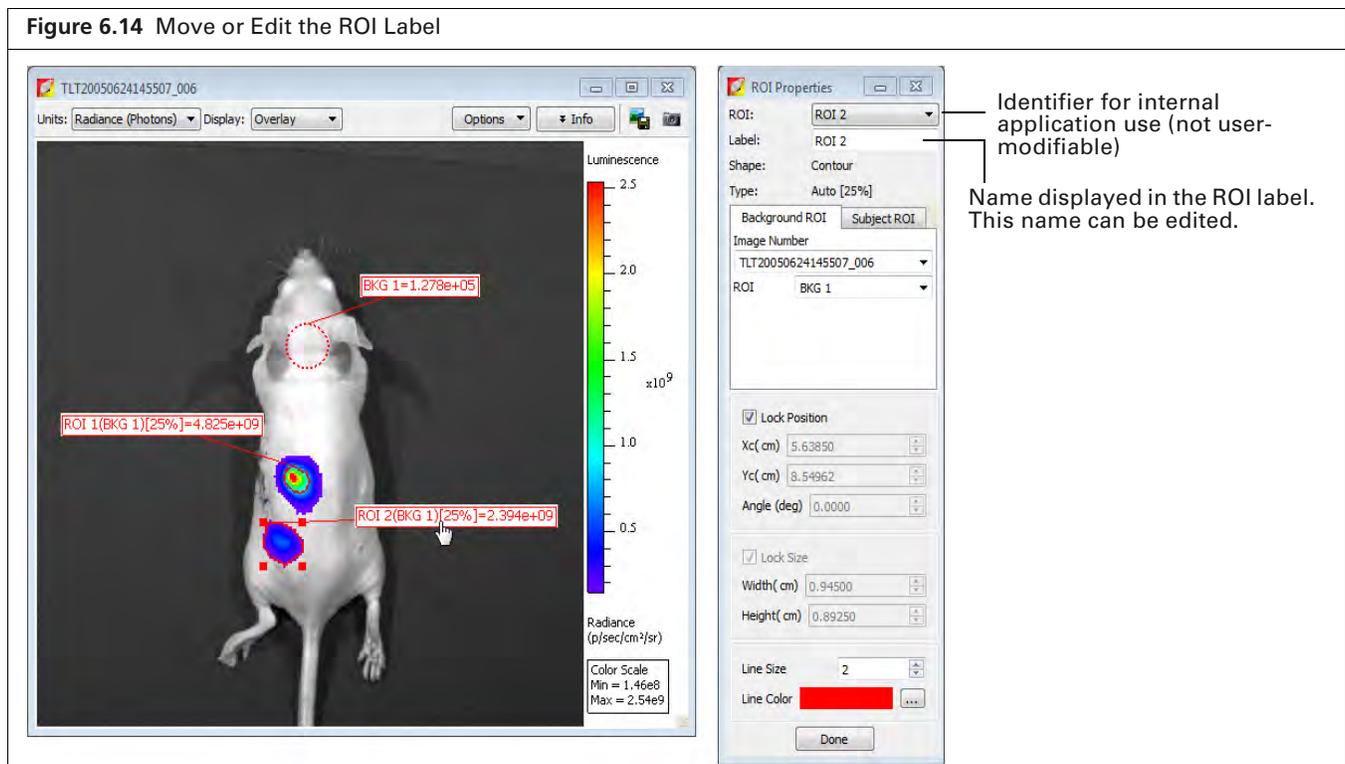


2. To edit the ROI line thickness, enter a new value in the Line Size box. Alternatively, click the  arrows.
3. To change the ROI line color:
 - a. Click the **Browse** button . The Select Color box appears.
 - b. Select a basic color or create a custom color for the ROI line:
 - Select a basic color – 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).



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).

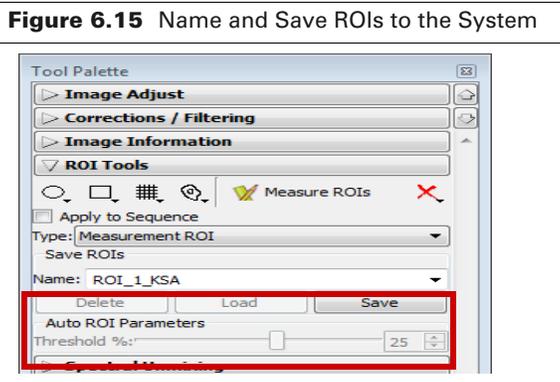
Saving ROIs to the System

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.

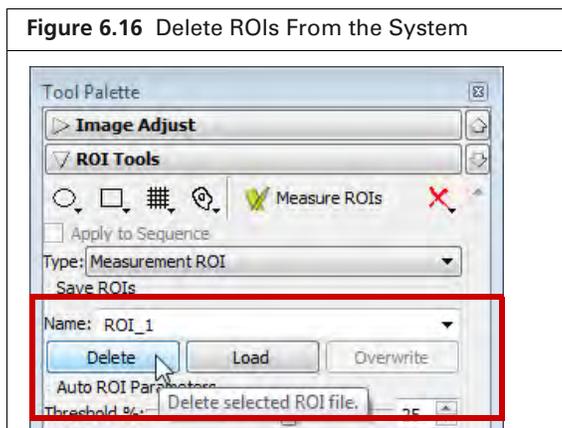
1. After one or more ROI(s) are applied to an image:
 - a. Confirm the default name or enter a new name for the ROI in the Name drop-down list.
 - b. Click **Save** (Figure 6.15).



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).

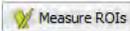


6.7 ROI Measurements

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  button in the ROI tools to display the ROI measurement table (Figure 6.17). Alternatively, select **View** → **ROI Measurements** on the menu bar.

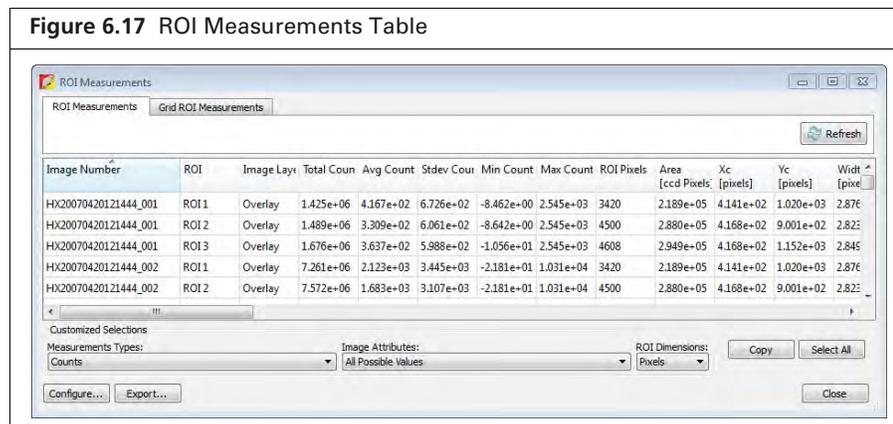


Table 6.8 ROI Measurements Table

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 110 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 <i>Image Display and Measurement</i> for more details on count units (select Help → Tech Notes on the menu bar).

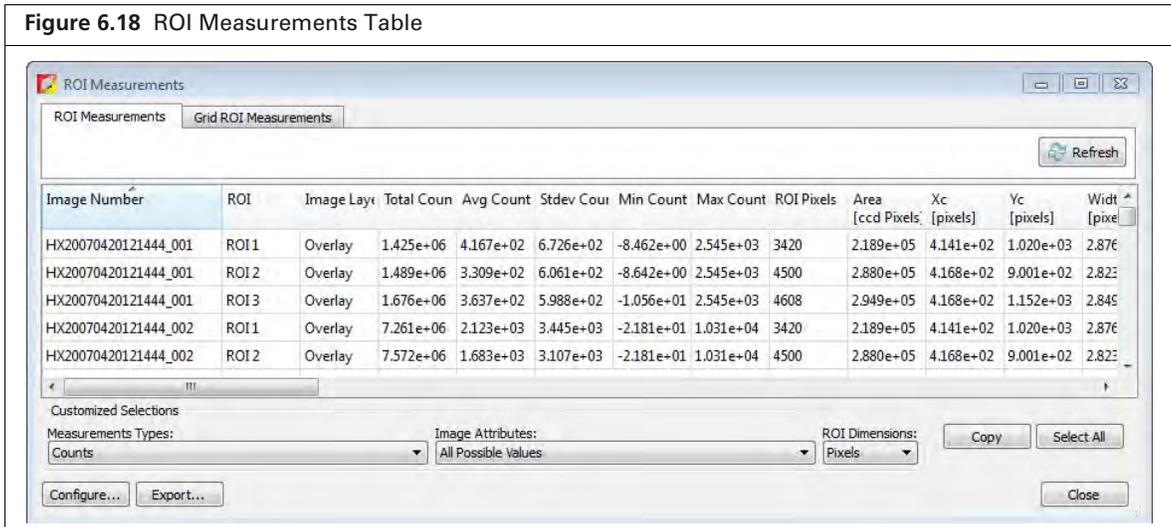
Table 6.8 ROI Measurements Table (continued)

Item	Description
Radiance (Photons) (fluorescence and luminescence)	<p>Total Flux (photons/sec) = the radiance (photons/sec/cm²/steradian) in each pixel summed or integrated over the ROI area (cm²) x 4π.</p> <p>Average Radiance = the sum of the radiance from each pixel inside the ROI/ number of pixels or super pixels (photons/sec/cm²/sr).</p> <p>Stdev Radiance = standard deviation of the pixel radiance inside the ROI</p> <p>Min Radiance = lowest radiance for a pixel inside the ROI.</p> <p>Max Radiance = highest radiance for a pixel inside the ROI.</p> <p>Tip: See the tech note <i>Image Display and Measurement</i> for more details on photon units (select Help → Tech Notes on the menu bar).</p>
Radiant Efficiency (fluorescence)	Epi-fluorescence - Fluorescence emission radiance per incident excitation intensity: p/sec/cm ² /sr/μW/cm ²
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	<p>Displays the Save Measurements box so that the data can be saved to a .txt or .csv file.</p> <p>Note: Grid ROI measurements exported to a .csv file can be opened in a spreadsheet application like Microsoft® Excel®.</p>
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.



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.

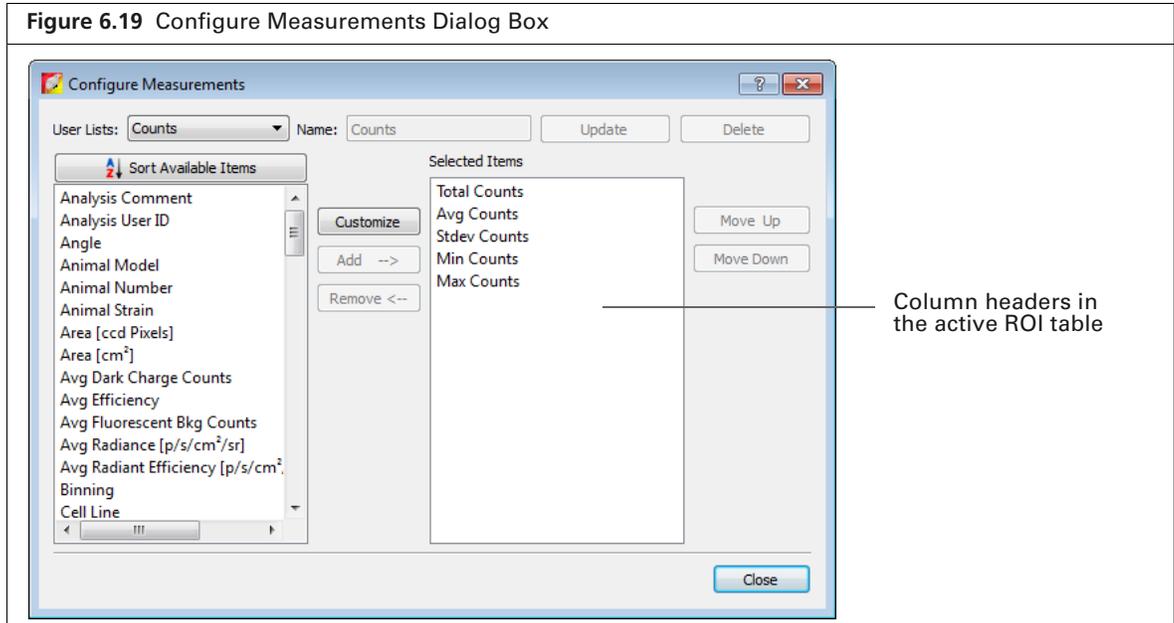
Creating a Custom ROI Table Configuration

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).



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 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.

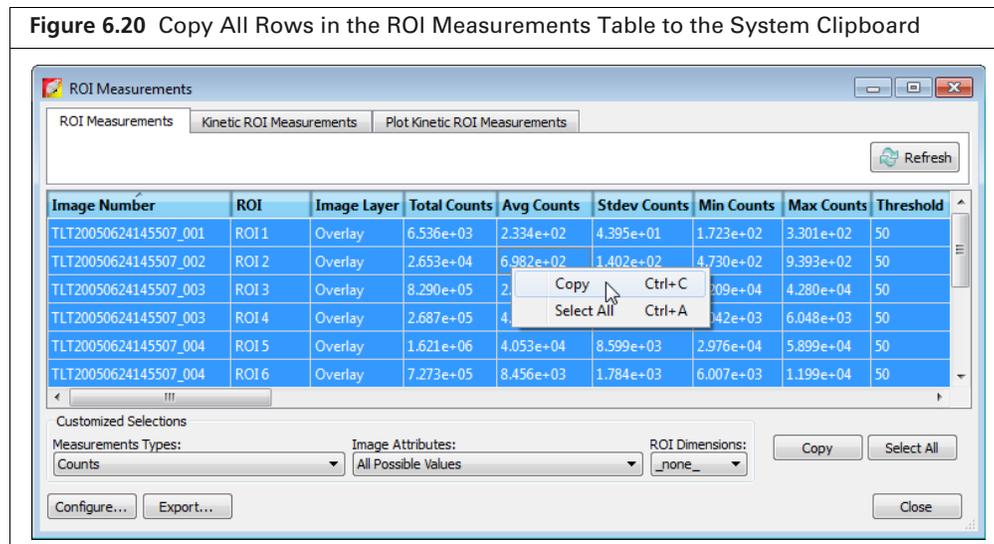
Copying or Exporting ROI Measurements

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.



7 Spectral Unmixing

About Spectral Unmixing

Acquire a Sequence for Spectral Unmixing

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Spectral Unmixing Results on page 134

7.1 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.
- Analyze luminescent or fluorescent images when more than one reporter is used in the same animal model.

7.2 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	<i>Bioluminescence Imaging</i> on page 114
Fluorescent	<i>Fluorescence Imaging</i> on page 116
Radio-isotope	<i>Cherenkov Imaging</i> on page 119

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.

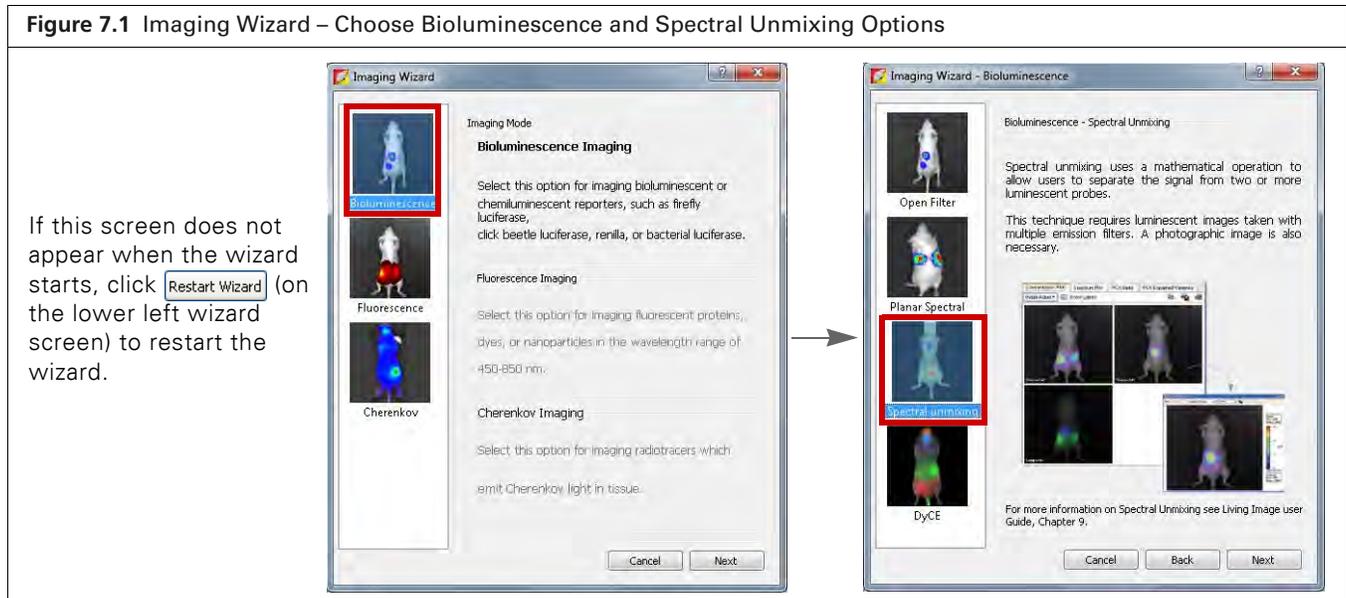
Bioluminescence Imaging



NOTE: The IVIS Lumina Series III should be initialized and the temperature locked before setting the imaging parameters. See [page 16](#) for more details.

1. Start the Imaging Wizard. See [page 35](#) for instructions.
2. Double-click the Bioluminescence option in the wizard. Double-click the Spectral Unmixing option in the next screen ([Figure 7.1](#)).

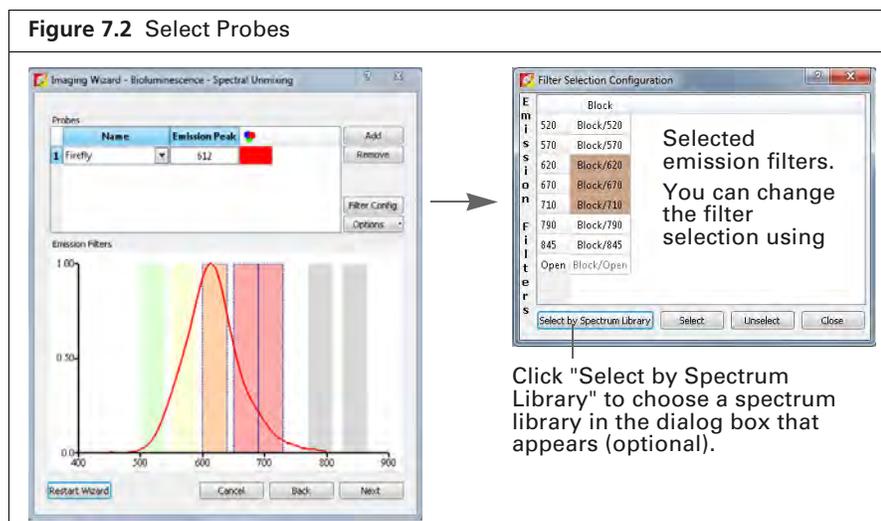
Figure 7.1 Imaging Wizard – Choose Bioluminescence and Spectral Unmixing Options



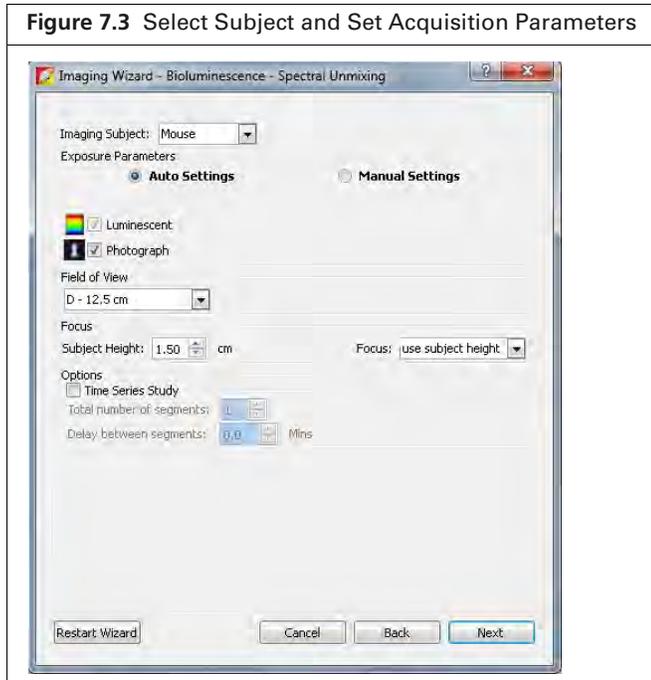
3. Select a probe from the Name drop-down list ([Figure 7.2](#)).
4. Click **Add** and select another probe from the Name drop-down list. Repeat until all of the probes are added.
5. Optional: If you generated a spectrum library (a set of reference spectra), you can select it in the Imaging Wizard. Click **Filter Config**, then click **Select by Spectrum Library** in the dialog box that appears ([Figure 7.2](#)).

See [Guided Method on page 121](#) for instructions on creating a spectrum library.

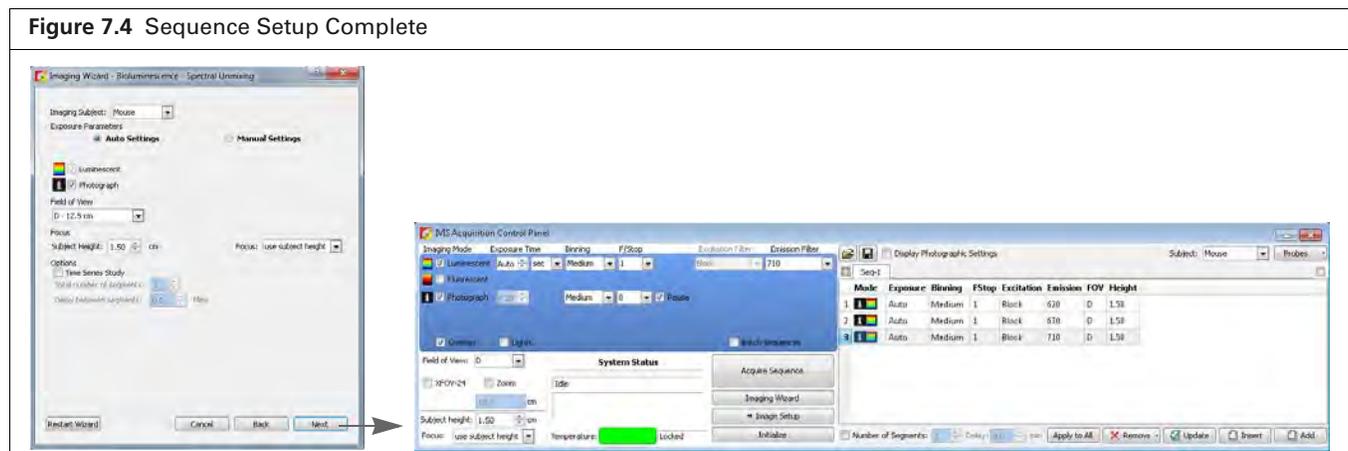
Figure 7.2 Select Probes



6. Click **Next** and in the screen that appears (Figure 7.3):
 - a. Select the type of subject.
 - b. Select a field of view.
 - c. Set the focus options.



7. To acquire a time series of images:
 - a. Choose the Time Series Study option (Figure 7.3).
 - b. Enter the number of segments and the delay between segments.
8. Click **Next**.
 The specified sequence appears in the sequence table (Figure 7.4).



9. Acquire the sequence following the instructions on [page 36](#).

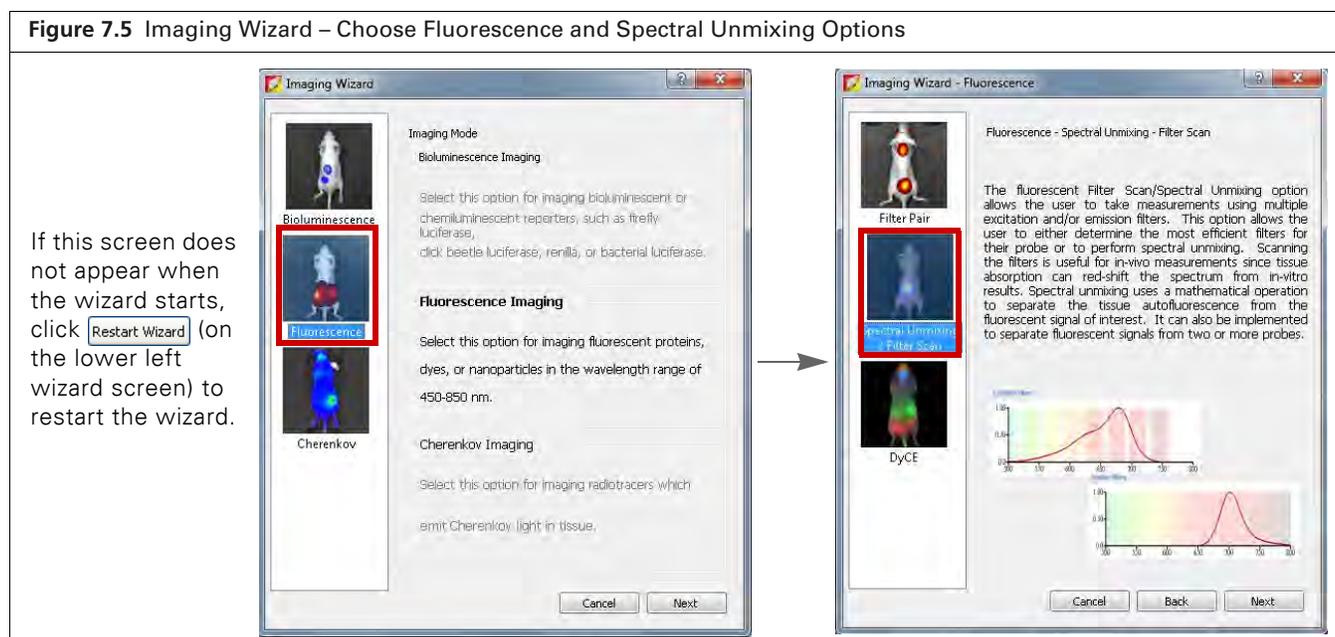
The image window appears when acquisition is completed ([Figure 7.14 on page 122](#)). See [Table 4.2 on page 27](#) for more details on the Image window.

Fluorescence Imaging

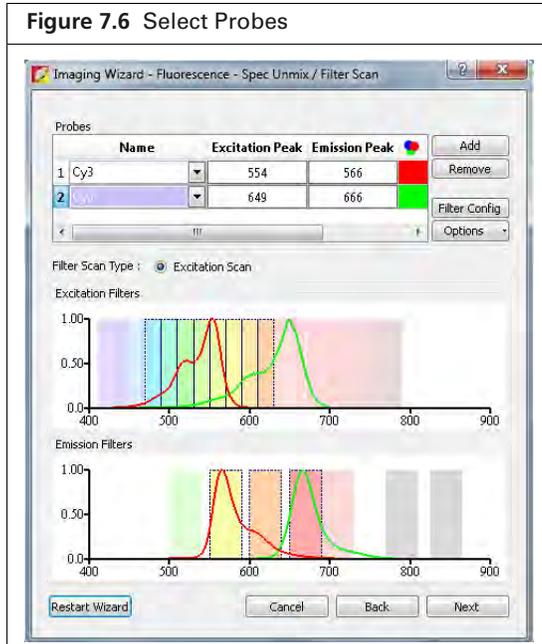


NOTE: The IVIS Lumina Series III should be initialized and the temperature locked before setting the imaging parameters. See [page 16](#) for more details.

1. Start the Imaging Wizard. See [page 35](#) for instructions.
2. Double-click the Fluorescence option. Double-click the Spectral Unmixing option in the next screen ([Figure 7.5](#)).

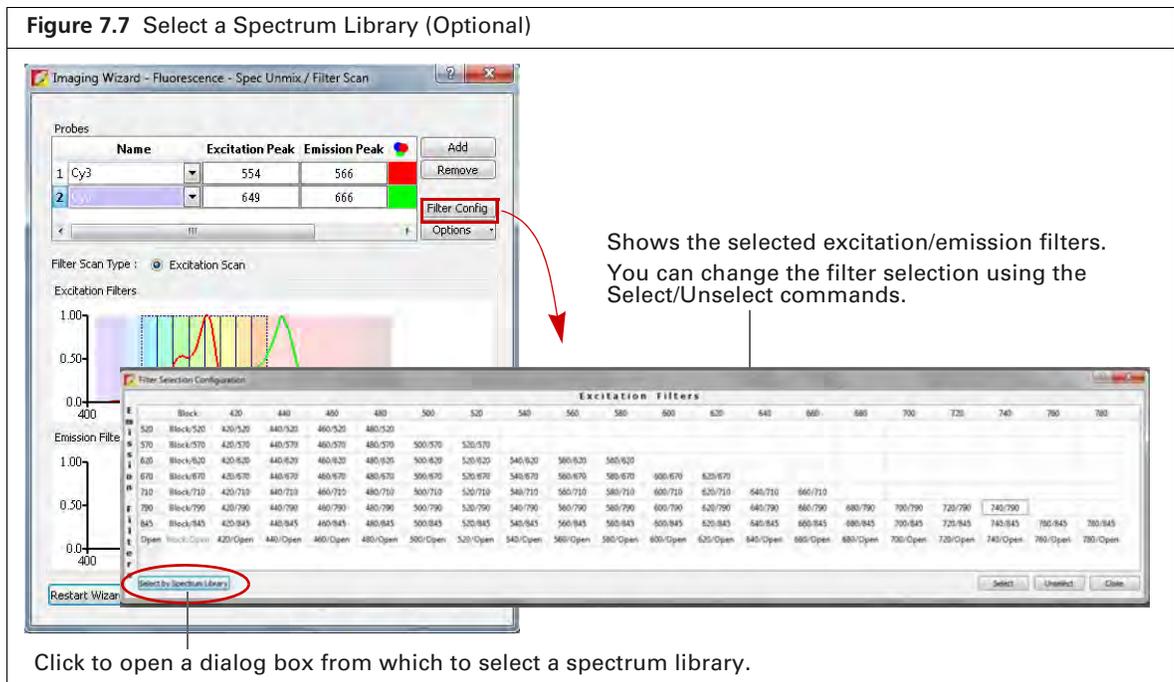


3. Select a probe from the Name drop-down list in the next screen ([Figure 7.2](#)).
4. Click **Add** and select another probe from the Name drop-down list. Repeat until all of the probes are added.

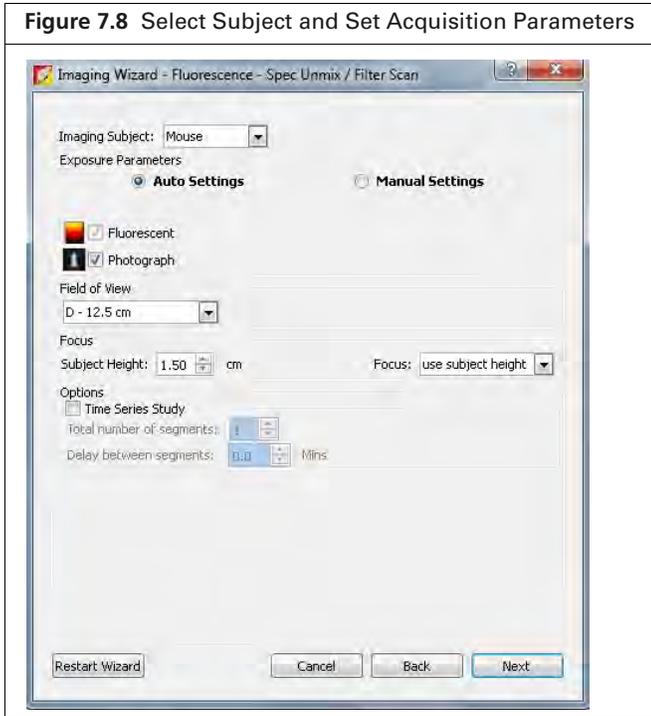


5. Optional: If a spectrum library (a set of reference spectra) is available, you can select it in the Imaging Wizard. Click **Filter Config**, then click **Select by Spectrum Library** in the dialog box that appears (Figure 7.7).

See *Guided Method* on page 121 for instructions on creating a spectrum library.



6. Click **Next** and in the screen that appears (Figure 7.3):
 - c. Select the type of subject.
 - d. Select a field of view.
 - e. Set the focus options.



7. To acquire a time series of images:
 - a. Choose the Time Series Study option (Figure 7.8).
 - b. Enter the number of segments and the delay between segments.
8. Click Next.

The specified sequence appears in the sequence table (Figure 7.9).



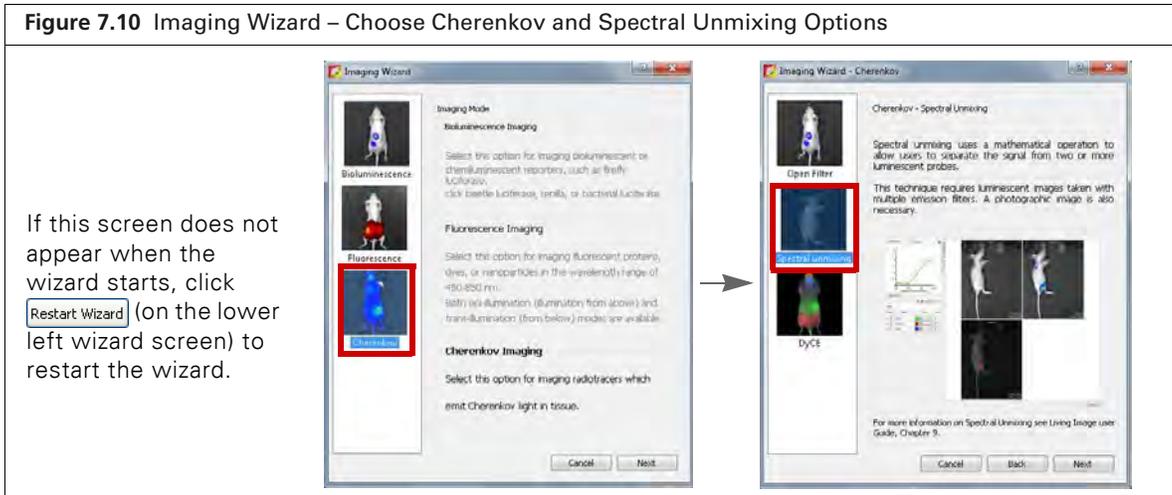
9. Acquire the sequence following the instructions on page 36.

The image window appears when acquisition is completed (Figure 7.14 on page 122). See Table 4.2 on page 27 for more details on the Image window.

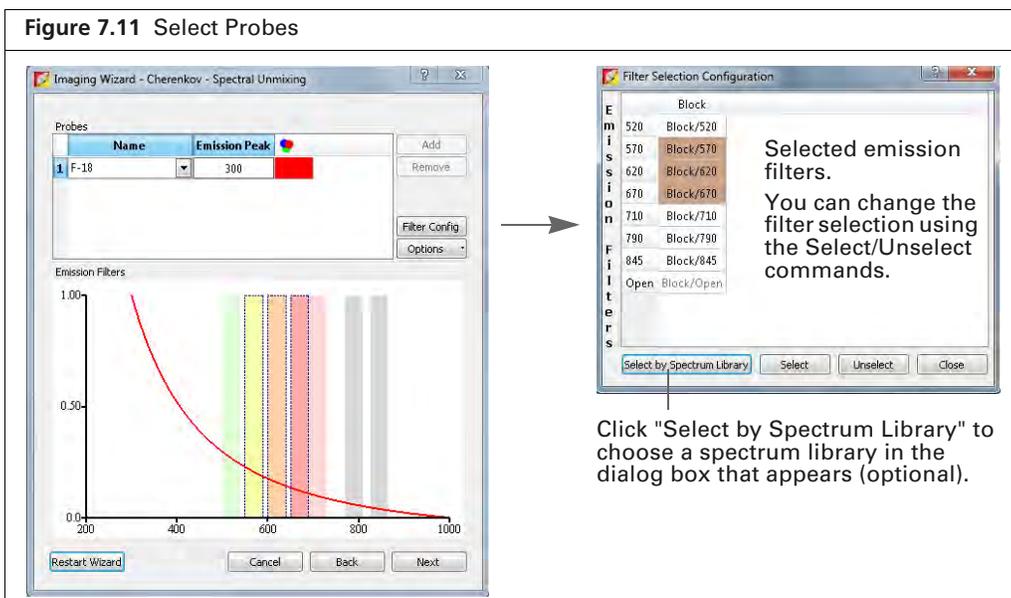
Cherenkov Imaging

NOTE: The IVIS Lumina Series III should be initialized and the temperature locked before setting the imaging parameters. See [page 16](#) for more details.

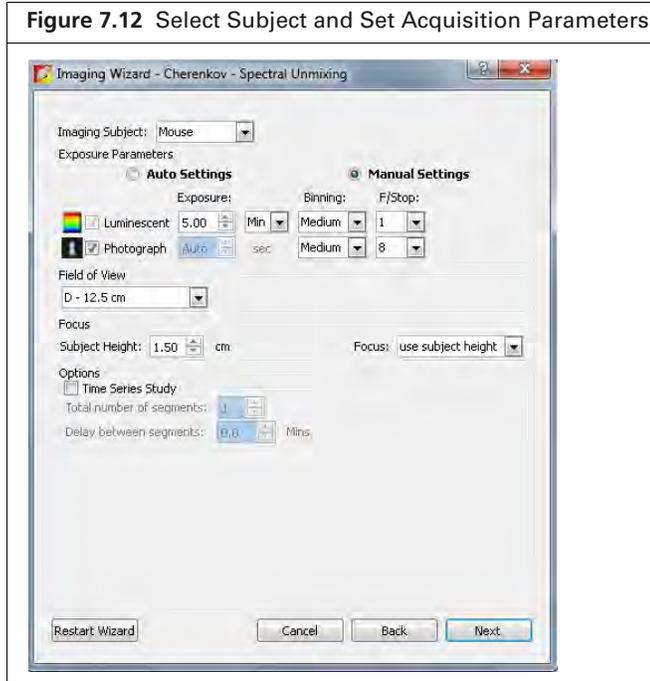
1. Start the Imaging Wizard. See [page 35](#) for instructions.
2. Double-click the Cherenkov option in the wizard. Double-click the Spectral Unmixing option in the next screen ([Figure 7.10](#)).



3. Select a probe from the Name drop-down list ([Figure 7.11](#)).
 4. Click **Add** and select another probe from the Name drop-down list. Repeat until all of the probes are added.
 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 7.11](#)).
- See [Guided Method on page 121](#) for instructions on creating a spectrum library.



6. Click **Next** and in the screen that appears (Figure 7.12):
 - c. Select the type of subject.
 - d. Select a field of view.
 - e. Set the focus options.



7. To acquire a time series of images:
 - a. Choose the Time Series Study option (Figure 7.12).
 - b. Enter the number of segments and the delay between segments.
8. Click **Next**.
 The specified sequence appears in the sequence table (Figure 7.13).



9. Acquire the sequence following the instructions on page 36.
 The image window appears when acquisition is completed (Figure 7.14 on page 122). See Table 4.2 on page 27 for more details on the Image window.

7.3 Spectral Unmixing Methods

Living Image software provides four spectral unmixing methods ([Table 7.1](#)).

Table 7.1 Spectral Unmixing Methods

Method	Description	See Page
Guided	<p>Use this method when:</p> <ul style="list-style-type: none"> ■ Probe locations are known. ■ Probe signals are mixed with background signal, but not other probe signals. <p>Note: This method is not recommended if probe signals are overlapping.</p> <p>Use this method to generate a spectrum library (a set of reference spectra) for probes with known spectra and known locations.</p>	121
Library	<p>This method requires a user-generated spectrum library. The library method identifies pixels in the data with spectral characteristics that match the spectrum library.</p> <p>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 <i>in vivo</i> data to analyze <i>in vitro</i> data and vice versa.</p>	124
Automatic	<p>Use this method when:</p> <ul style="list-style-type: none"> ■ Probe locations are unknown. ■ Probes are included in the spectrum library. 	126
Manual	<p>Use this method to:</p> <ul style="list-style-type: none"> ■ 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. 	129

Guided Method

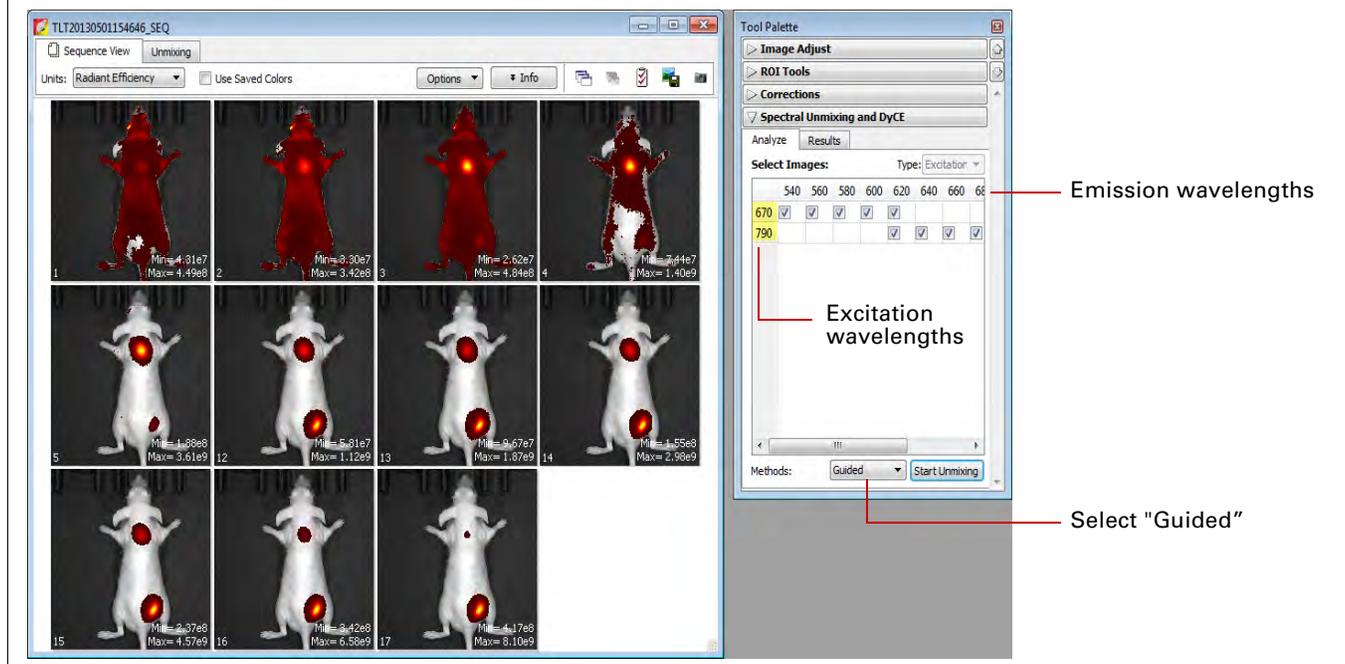
Use the guided method:

- 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.

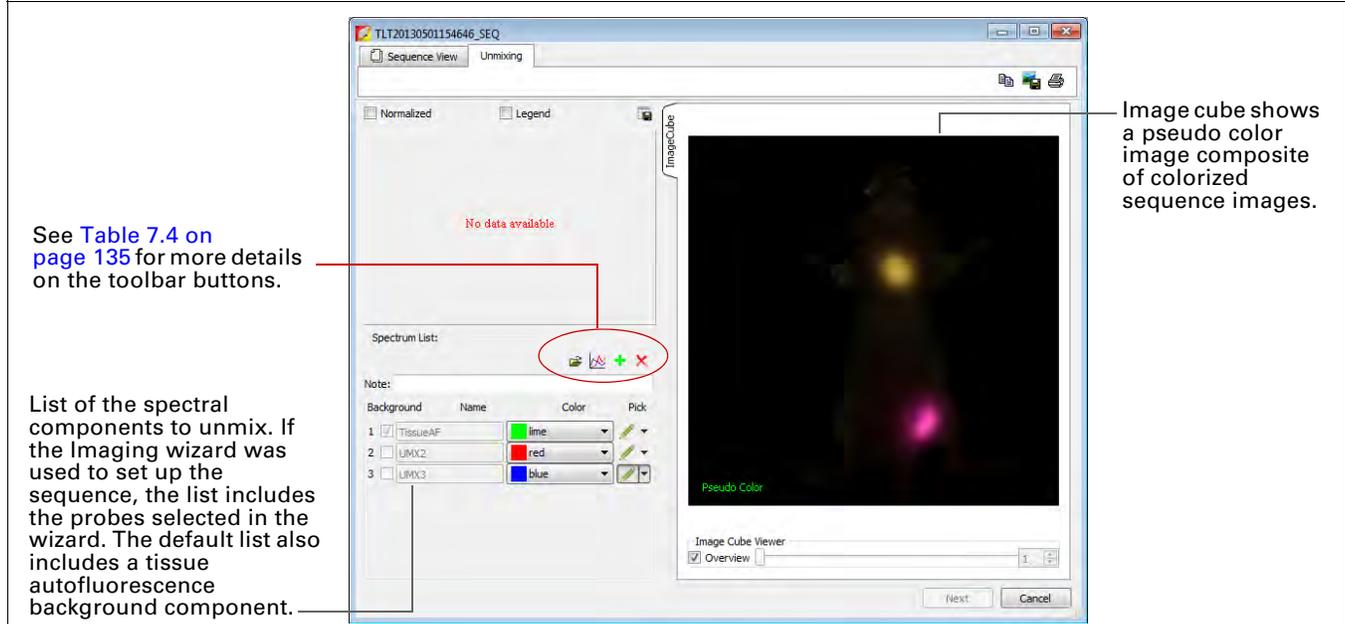
The fluorophores are Alexa Fluor 680 and Alexa Fluor 750 in [Figure 7.14](#). Images were acquired using 680 and 790 nm excitation filters, and emission filters from 540 to 720 nm in 20 nm increments.

Figure 7.14 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 7.15).

Figure 7.15 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.

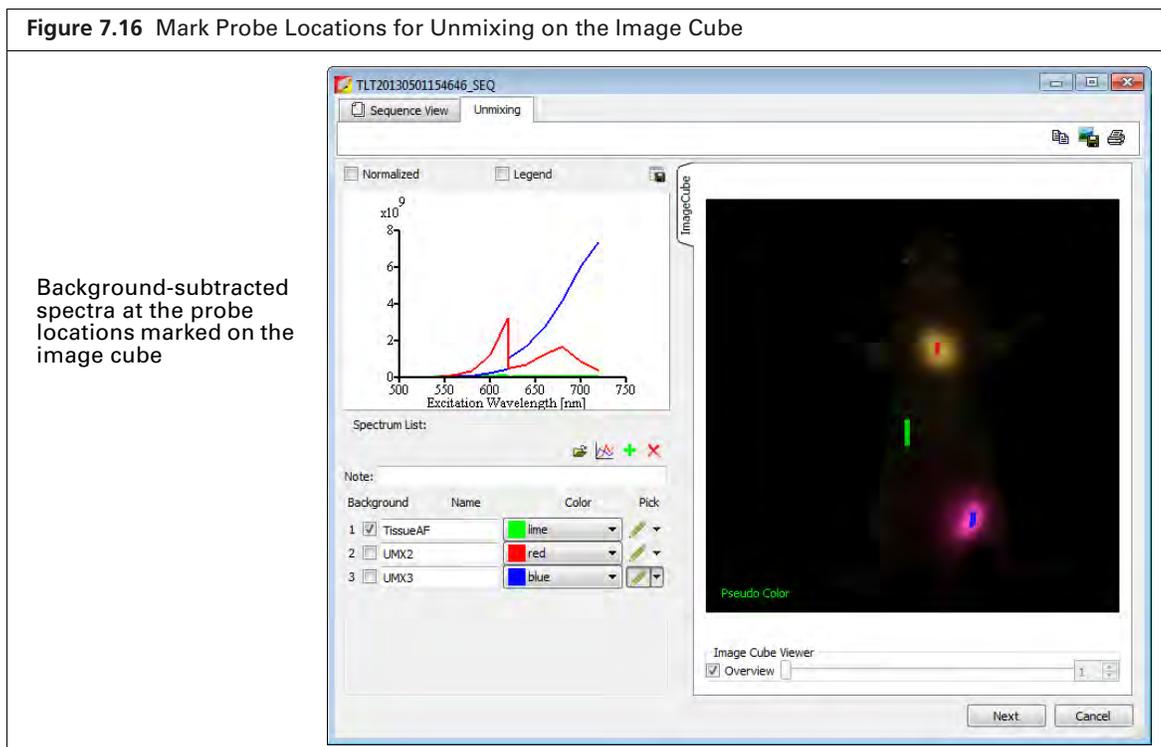
The entire image cube is calibrated and visualized on the same scale. To view a particular image, remove the check mark next to the Overview option and move the slider or enter an image number.

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 7.16](#)).

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.



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 7.17](#)). See [Spectral Unmixing Results](#) on [page 134](#) for information about the results.

8. To save the results as a spectrum library:
 - a. Click the  button in the Spectrum List toolbar (Figure 7.17).
 - b. Enter a file name in the dialog box that appears and click **Save**.



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.

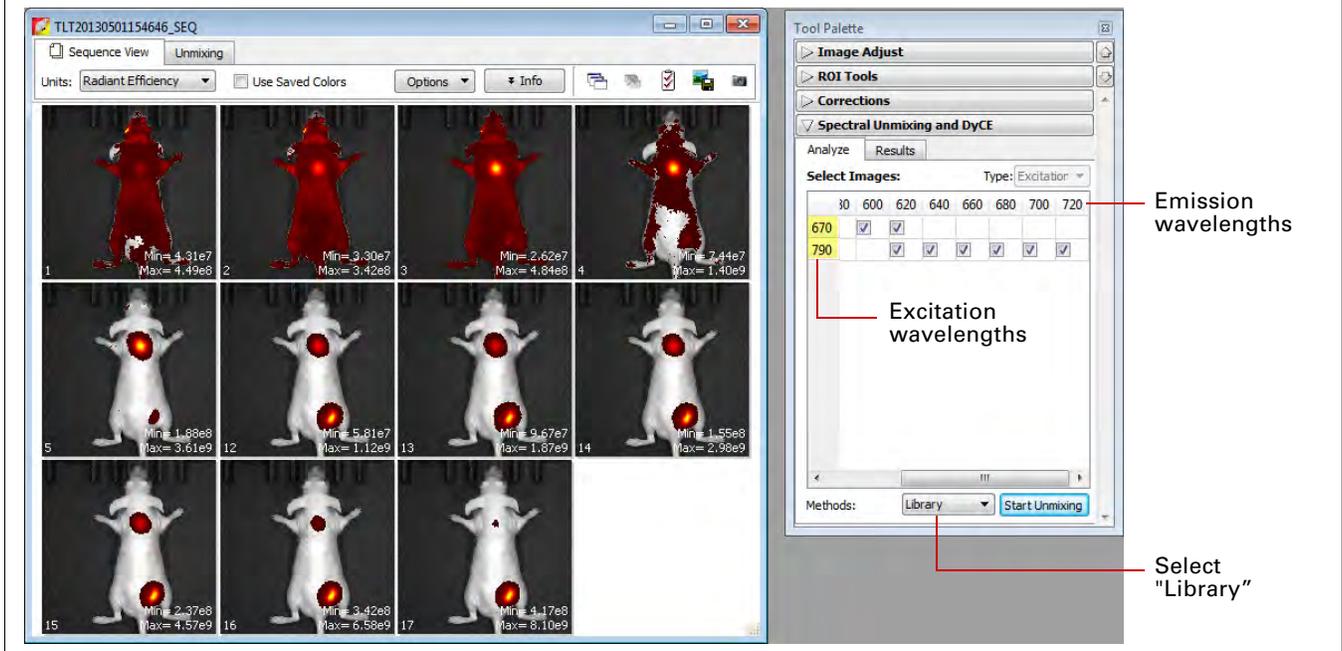


NOTE: Use the guided or manual method to generate a spectrum library of known probes with known locations (see page 121 for guided method or page 129 for manual method).

1. Load the image sequence.

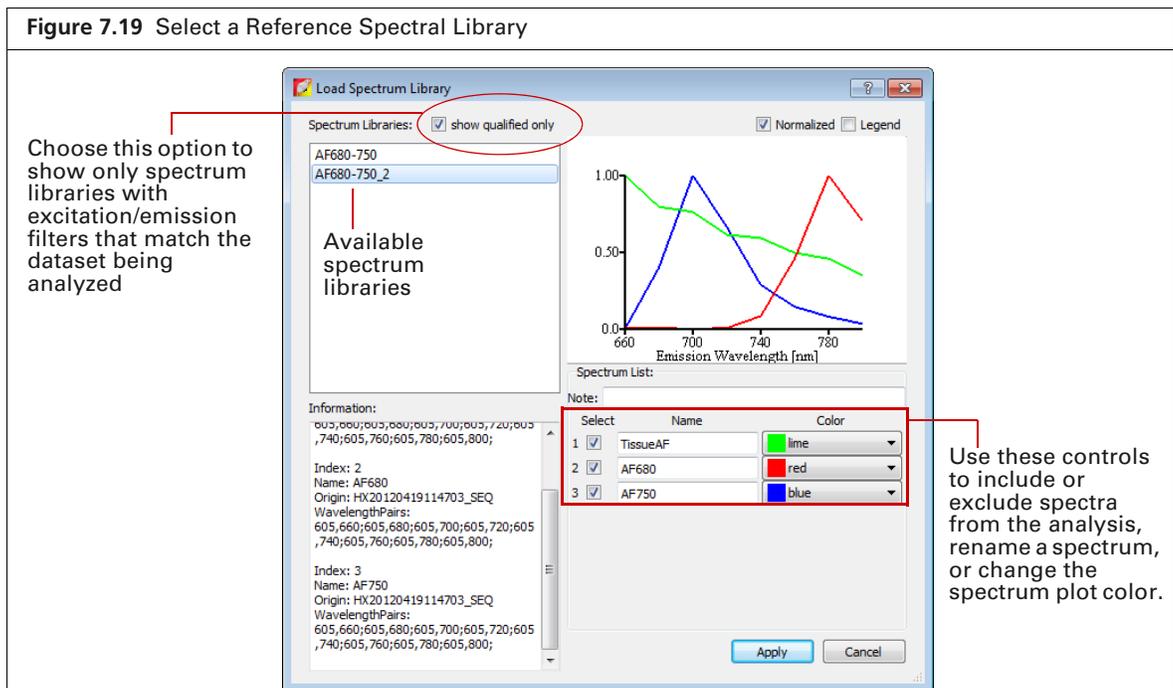
In Figure 7.18, the fluorophores are Alexa Fluor 680 and Alexa Fluor 750. Images were acquired using 670 and 790 nm excitation filters, and emission filters from 540 to 720 nm in 20 nm increments.

Figure 7.18 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 7.19).
 The software identifies pixels with spectral characteristics that match the spectral library. The Unmixing window shows the analysis results which include unmixed spectra, unmixed images, and a composite of the unmixed images (Figure 7.17 on page 124).
 See *Spectral Unmixing Results* on page 134 for information about the results.

Figure 7.19 Select a Reference Spectral Library

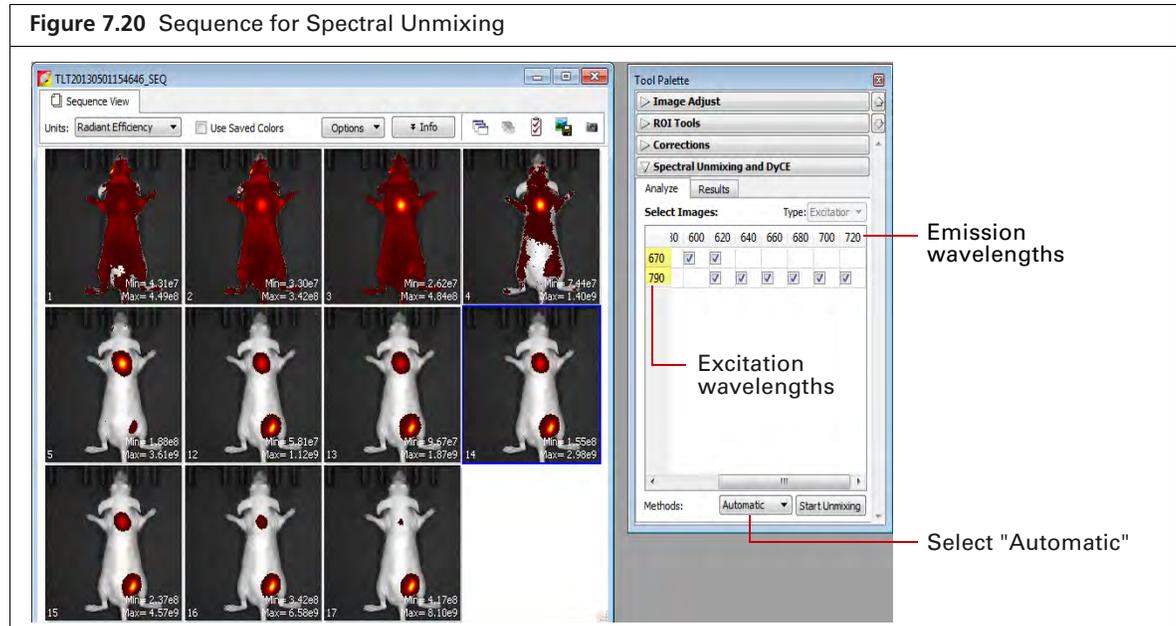


Automatic Method

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 7.20](#), 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.

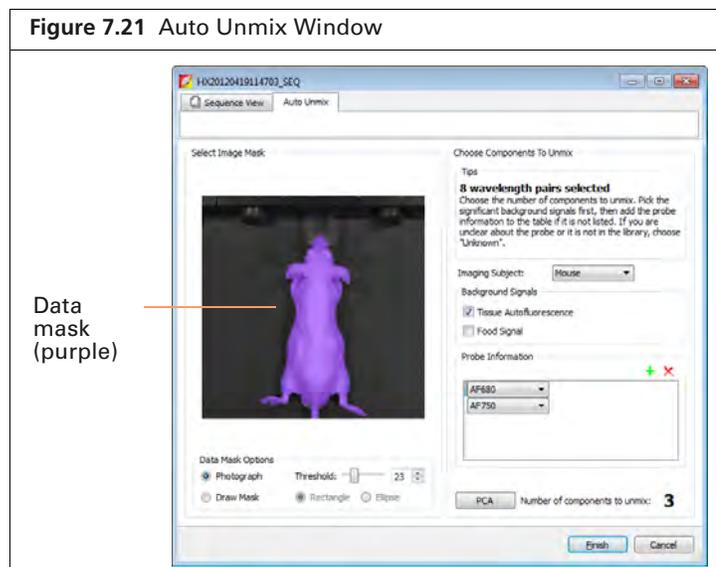


2. Click the Analyze tab of the Spectral Unmixing and DyCE tools.

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 7.21](#)). The purple data mask shows the data that will be included in the analysis (the entire subject is included by default).



- If you do not want to analyze the entire subject, draw a mask on a particular area (Figure 7.22). For example, it is useful to mask shaved or depilated areas.

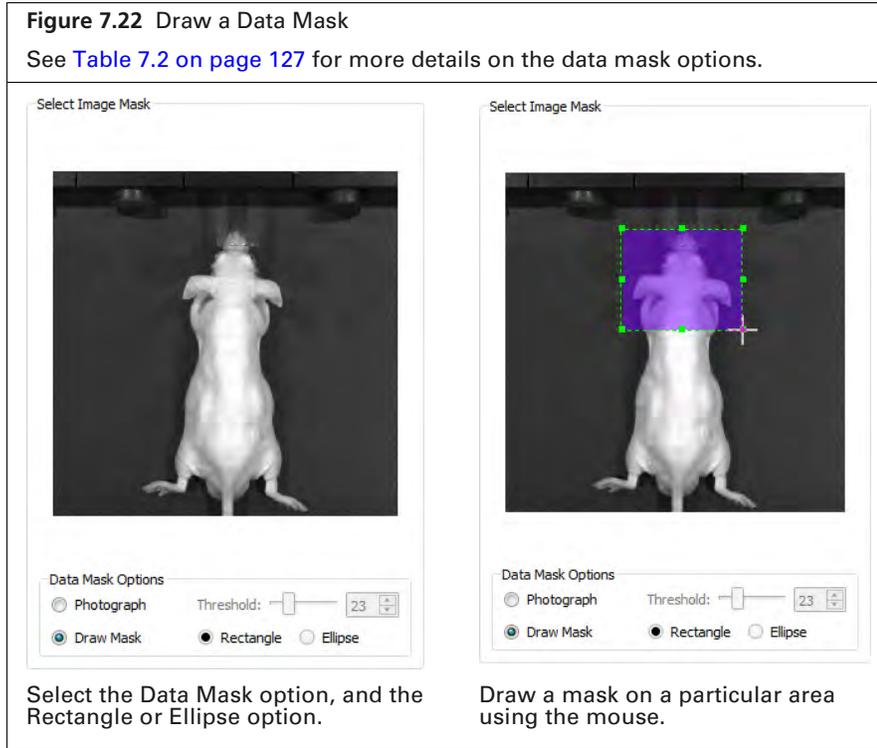


Table 7.2 Data Mask Options

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 7.23).

Figure 7.23 Auto Unmix Window

Select a subject

Choose background signal(s)

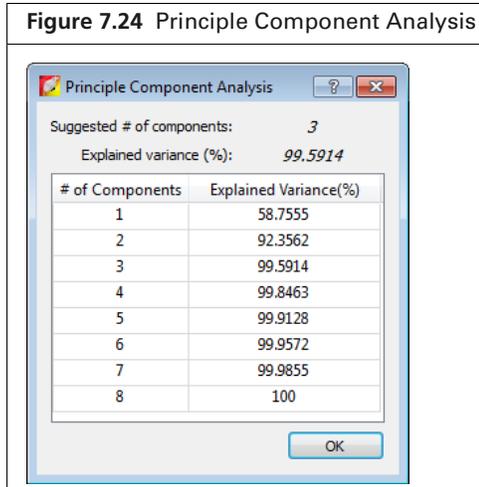
Number of components to unmix (no. of probes suggested by the software plus background signal(s))

Probe Information list shows the probes to unmix (initially based on probes selected in the Imaging Wizard during sequence set up).
 If probes are listed as "Unknown", select probe names from the drop-down lists.

6. Click the **PCA** button.

The Principle Component Analysis window shows the amount of signal explained by the suggested components (Figure 7.24). 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.



7. Click Finish.

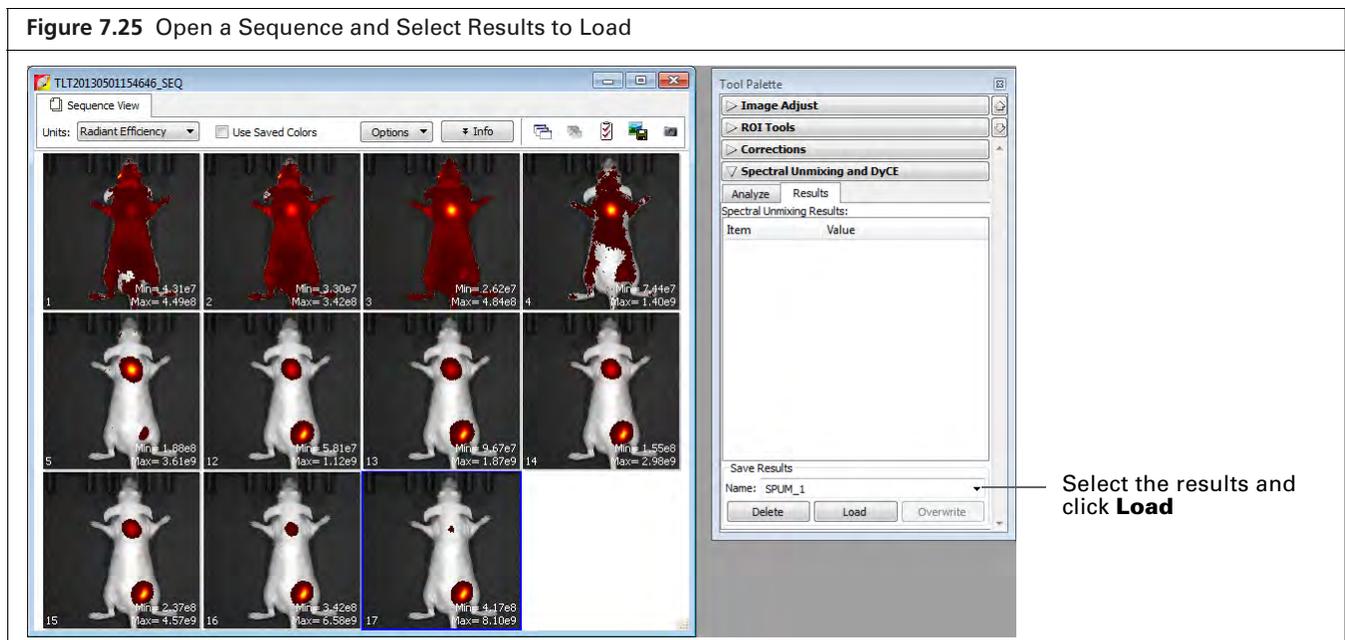
The Unmixing window shows the analysis results which include unmixed spectra, unmixed images, and a composite of the unmixed images (Figure 7.17 on page 124).

See *Spectral Unmixing Results* on page 134 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**.

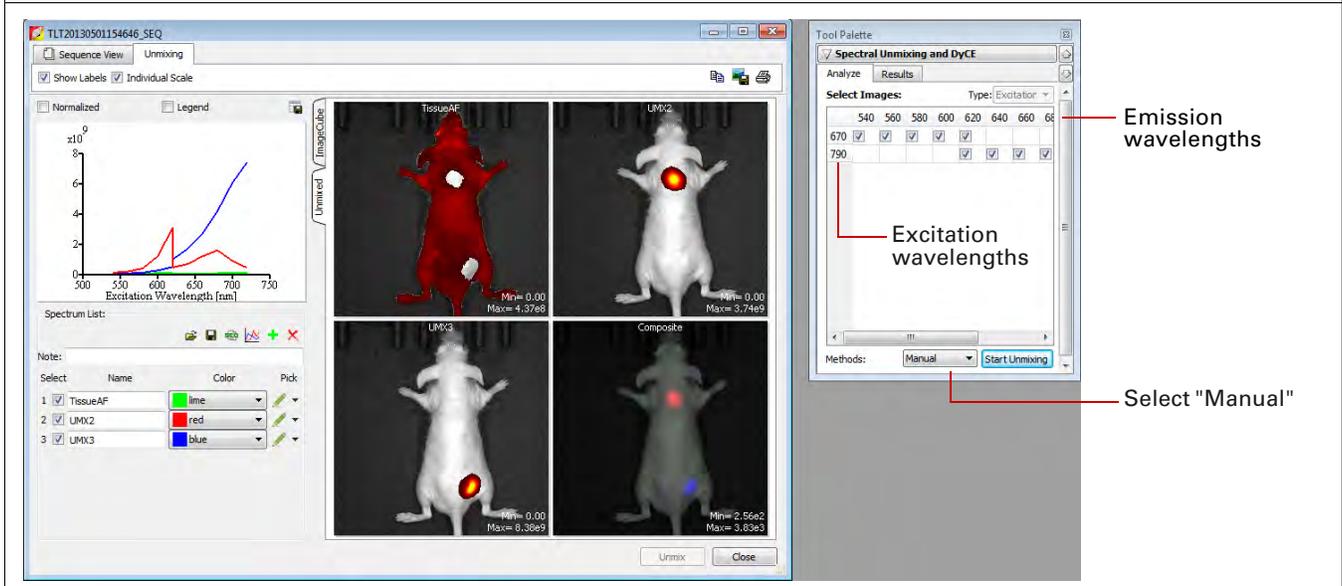


3. Click the Analyze tab of the Spectral Unmixing and DyCE tools.

All wavelengths are selected by default. Remove the check mark next to wavelengths that you want to exclude from the analysis.

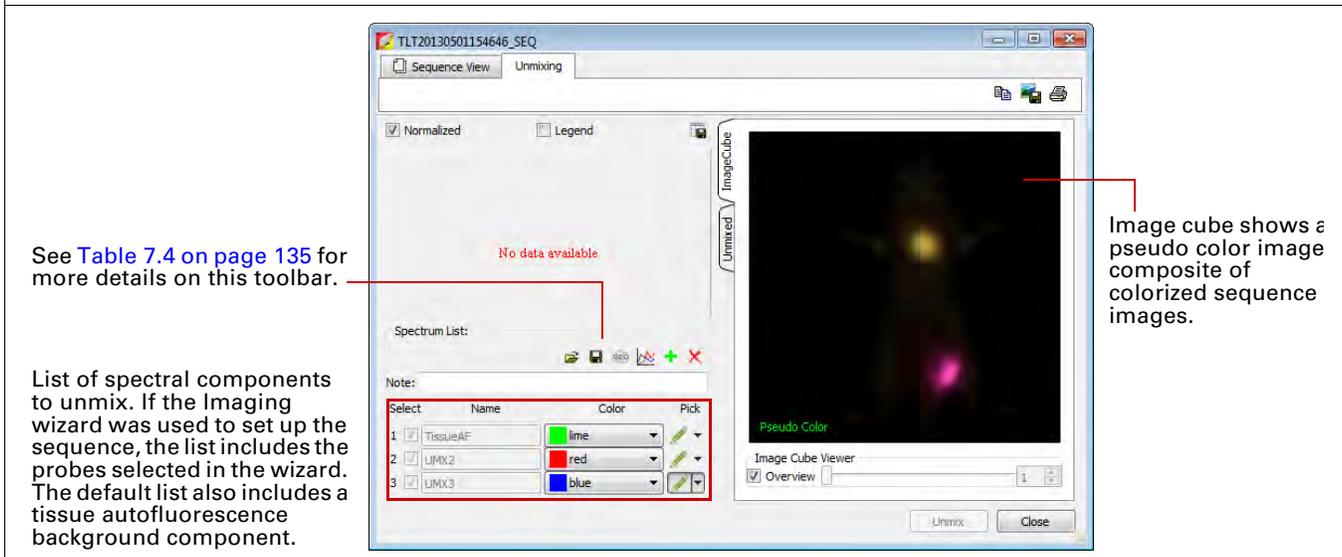
In [Figure 7.26](#), the fluorophores are Alexa Fluor 680 and Alexa Fluor 750. Images were acquired using 670 and 790 nm excitation filters, and emission filters from 540 to 720 nm in 20 nm increments.

Figure 7.26 Unmixing Window – Results Loaded



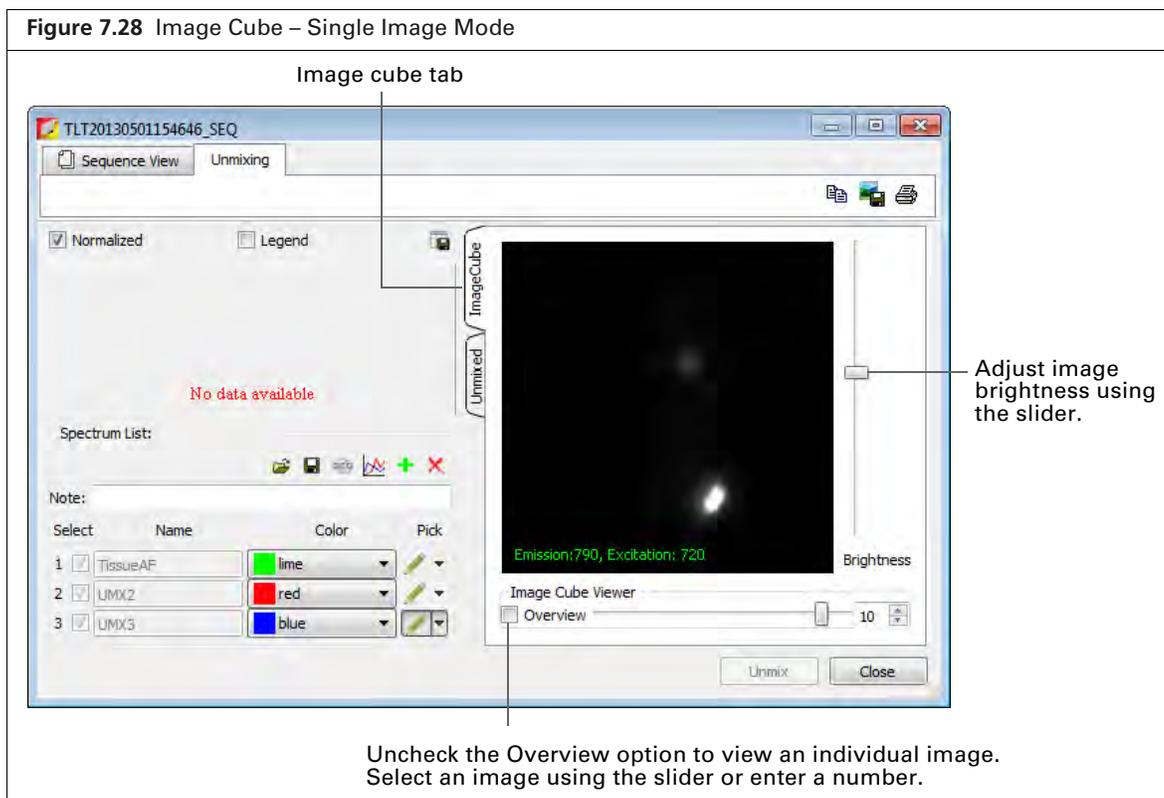
4. Select "Manual" from the Methods drop-down list and click **Start Unmixing**.
 The Unmixing window appears ([Figure 7.27](#)).

Figure 7.27 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 7.28).



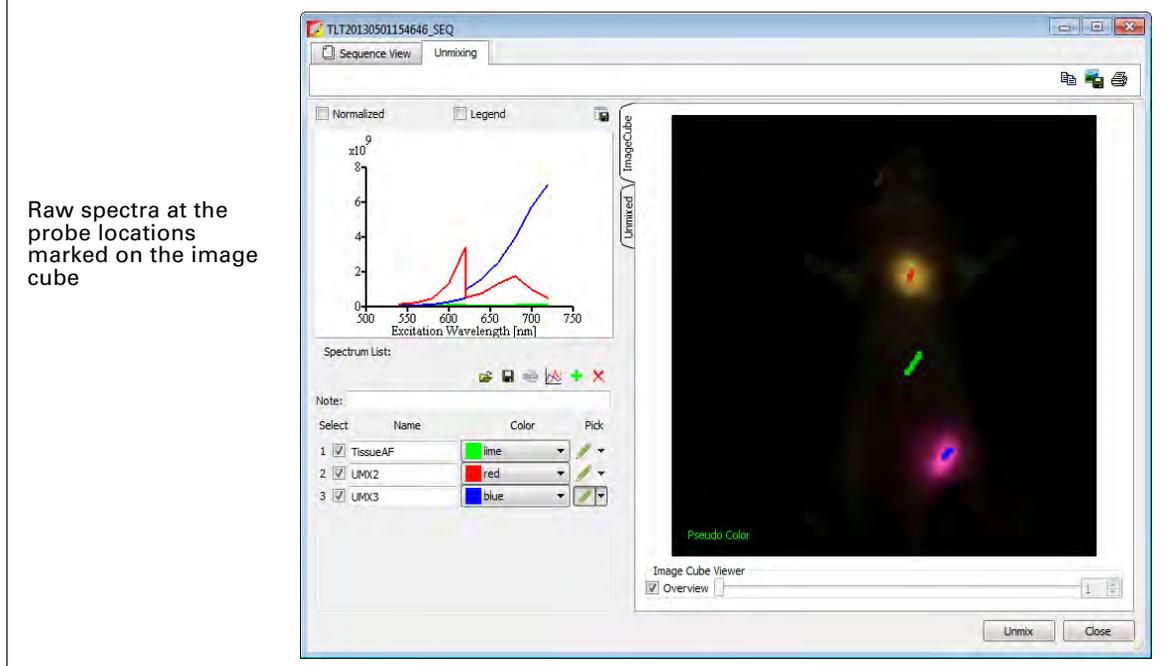
5. Move the mouse pointer over the image cube to see the spectrum at a particular location. The spectrum at the pointer location is updated as you move the pointer.
6. To specify a probe location for unmixing:
 - a. Click the  button for a spectrum.
 - b. Using the mouse, draw a mark on an area of the image cube which represents the probe location. The software plots a normalized spectrum of the signal (Figure 7.29).



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 132](#) for instructions.

Figure 7.29 Mark Probe Locations for Unmixing 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 7.17 on page 124).

See *Spectral Unmixing Results* on page 134 for information about the results.

7.4 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 7.30).
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.

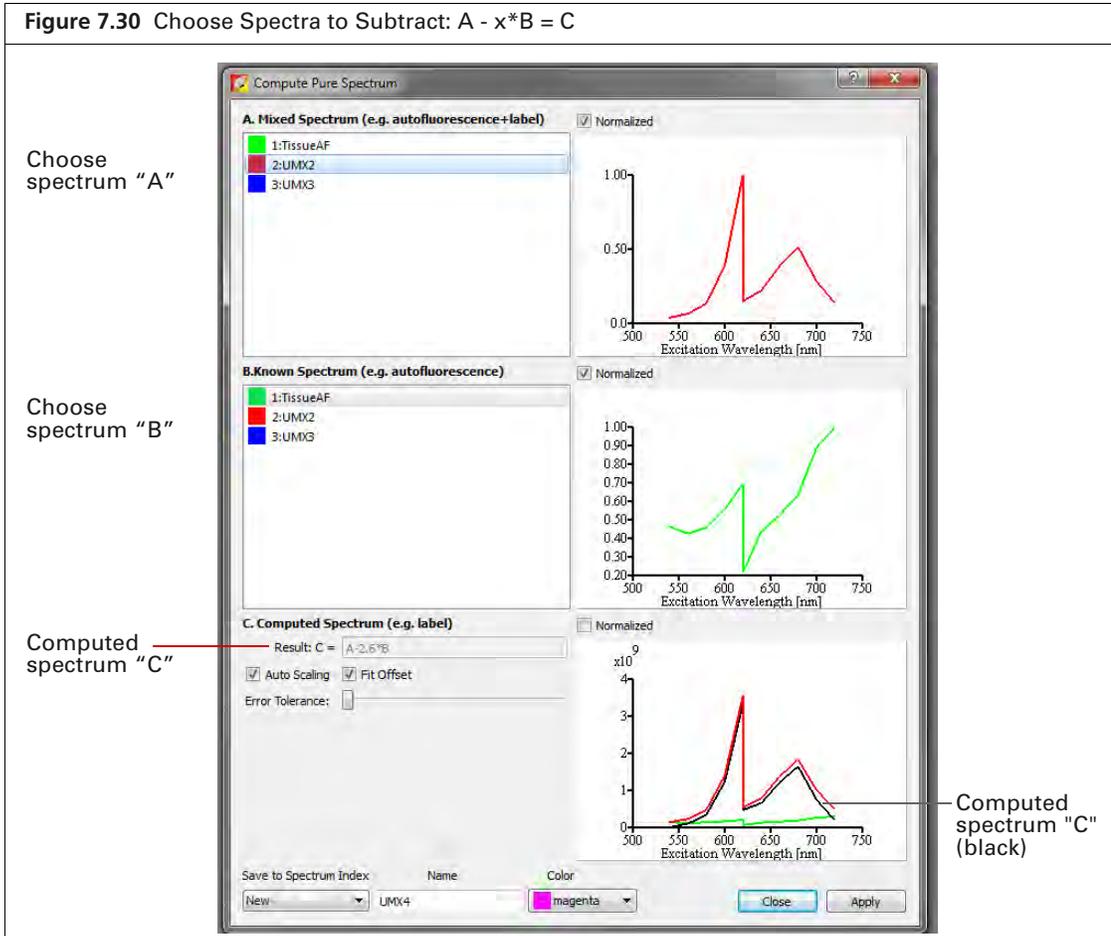
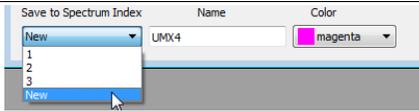
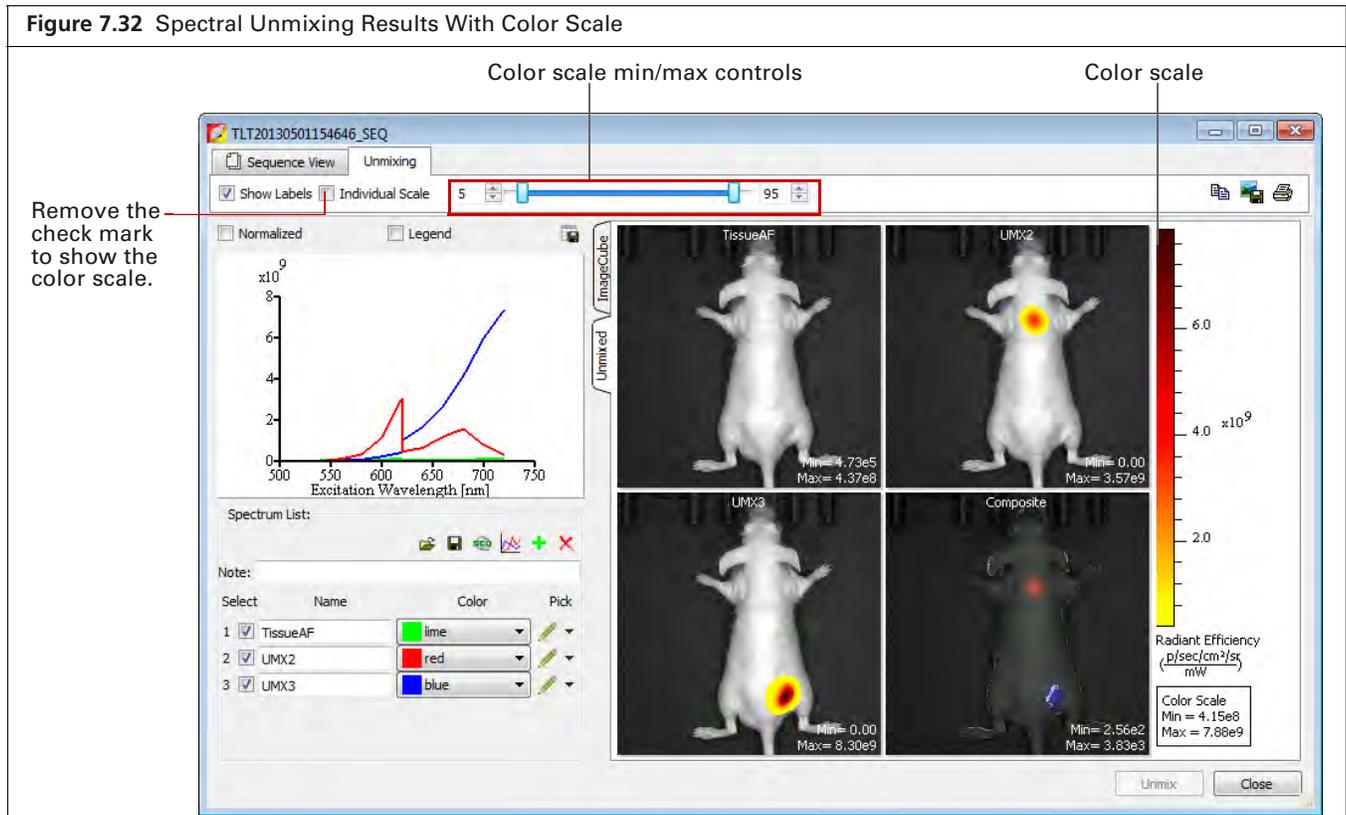
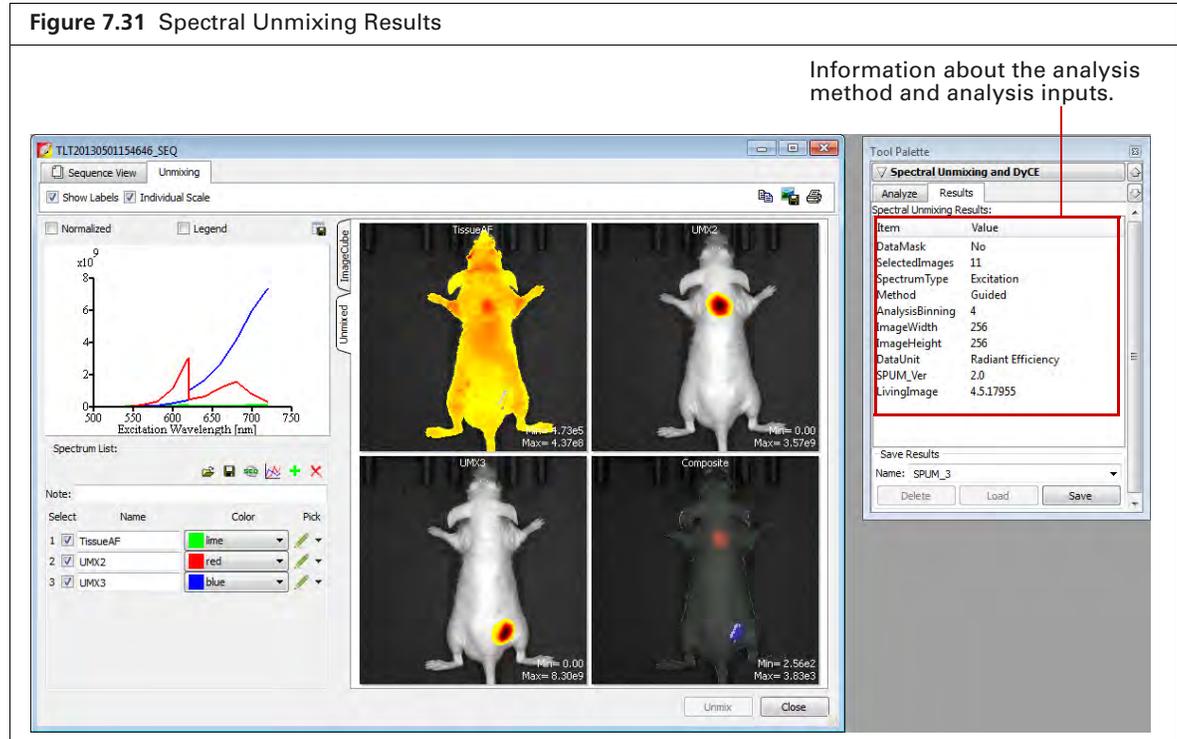


Table 7.3 Computed Spectrum

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 .

7.5 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 7.32).



Spectra Plot

Spectra plots show the unmixed spectra.

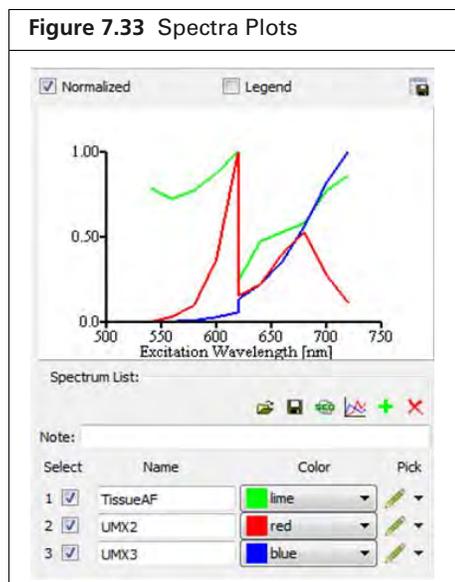


Table 7.4 Spectra Window

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 unmixed results as a reference spectrum library for use with the "library" method of spectral unmixed. See page 124 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 132).
	Adds a component to the spectrum list.
	Removes the check marked spectra from the "Select" list shown in Figure 7.33 .

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 131 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 7.34).

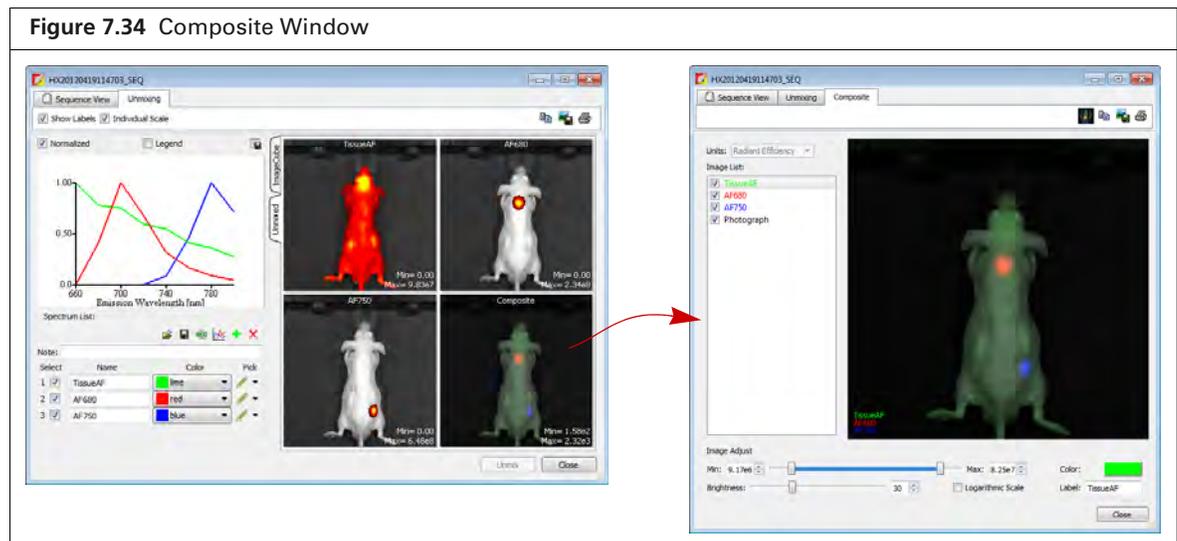


Table 7.5 Composite Window

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 7.5 Composite Window (continued)

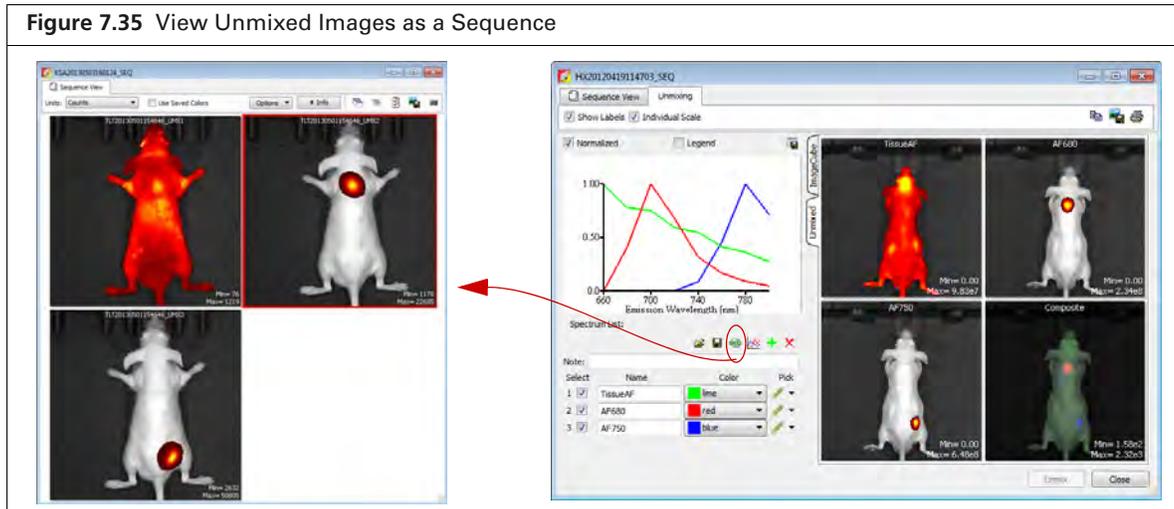
Item	Description
	Sends the composite image to the “top” of the image cube. This helps improve the pseudo color visualization of the image cube.
	Copies the composite image to the system clipboard.
	Click to export the composite image to a graphic file (for example, .jpg).
	Opens the Print dialog box.

Analyzing Images

Do either of the following:

- Click the  toolbar button to view all images as a sequence.
- 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 7.35).



Managing Spectral Unmixing Results

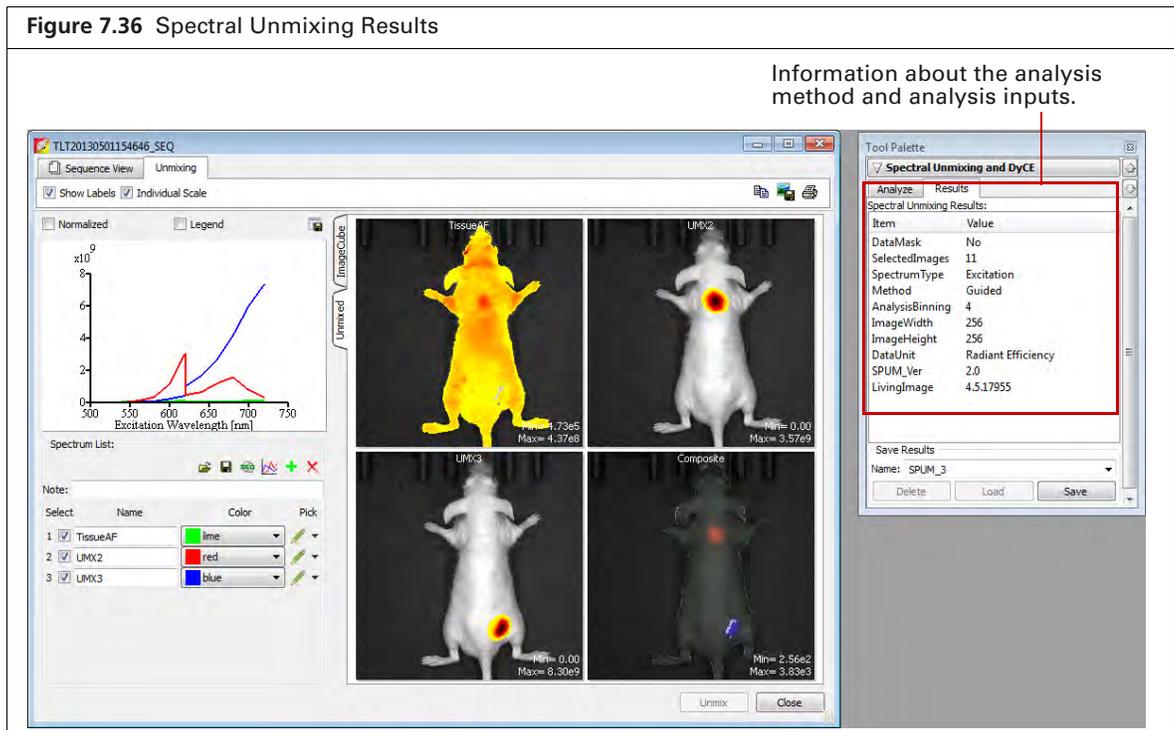


Table 7.6 Spectral Unmixing Results

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.

8 Biodistribution Studies Using DyCE Imaging

About DyCE (Dynamic Contrast Enhancement)

Acquire an Image Sequence for DyCE Analysis on page 141

DyCE Analysis on page 147

DyCE Results on page 154

8.1 About DyCE (Dynamic Contrast Enhancement)



NOTE: The DyCE acquisition and analysis features of Living Image software require a separate license.

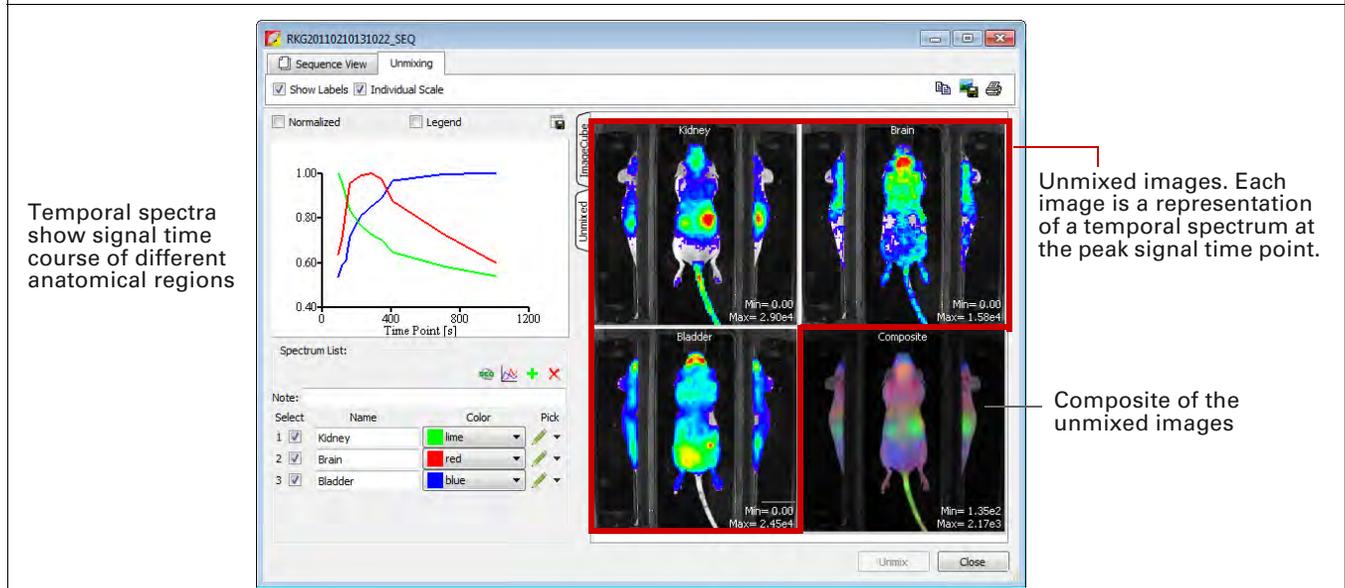
DyCE imaging and analysis is intended for biodistribution studies. DyCE imaging captures a time series of optical images immediately following a bolus injection of a probe or dye. Living Image software temporally unmixes the data on a pixel-by-pixel basis for each image of the time series and determines 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 8.1 Example DyCE Results

Images were obtained using the Mouse Side Imaging Kit.



8.2 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	<i>Bioluminescence Imaging</i> (below)
Fluorescent or near infrared	<i>Fluorescence Imaging</i>
Radiotracer	<i>Cherenkov Imaging on page 145</i>

Bioluminescence Imaging



NOTE: The IVIS Lumina Series III should be initialized and the temperature locked before setting the imaging parameters. See [page 16](#) for more details.

1. Start the Imaging Wizard. See [Start the Imaging Wizard and Setup a Sequence on page 35](#) for instructions.
2. Double-click the Bioluminescence option. Double-click the DyCE option in the next screen ([Figure 8.2](#)).

Figure 8.2 Imaging Wizard – Choose Bioluminescence and DyCE Options

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

3. Select the type of imaging subject in the next screen (Figure 8.3).

Figure 8.3 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.



NOTE: Typical exposures are in the range of minutes because Cherenkov light emission is very low. Beta decays with higher energies can afford shorter exposure times than lower energy beta decays.

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 8.3).

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.

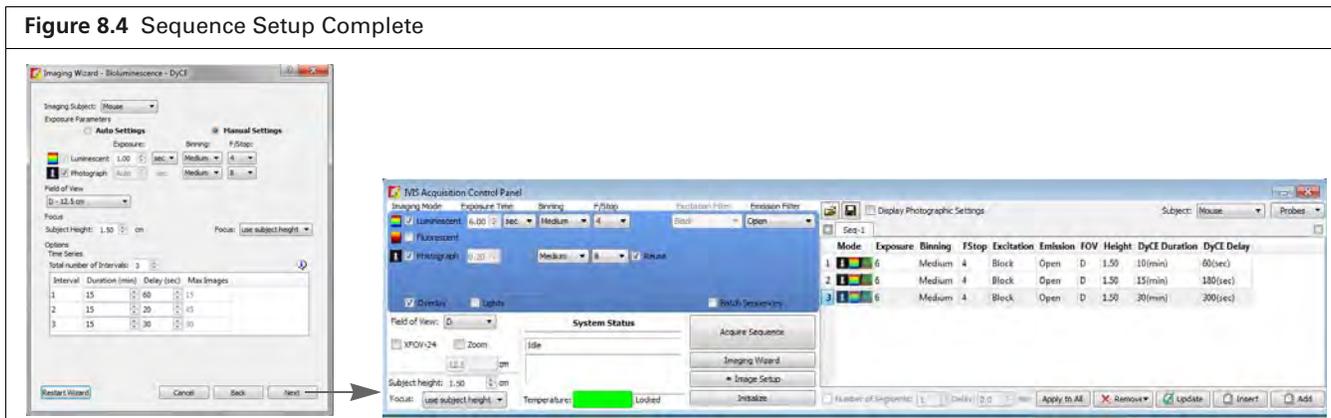


NOTE: The software alerts you if the number of images in the time series exceeds 200. If necessary, adjust the duration or delay between images of one or more intervals to reduce the number of images.

c. Click **Next**.

The specified sequence appears in the sequence table (Figure 8.4).

Figure 8.4 Sequence Setup Complete



8. Acquire the sequence following the instructions on page 36.

The image window appears when acquisition is completed (Figure 8.12 on page 147). See Table 4.2 on page 27 for more details on the Image window.

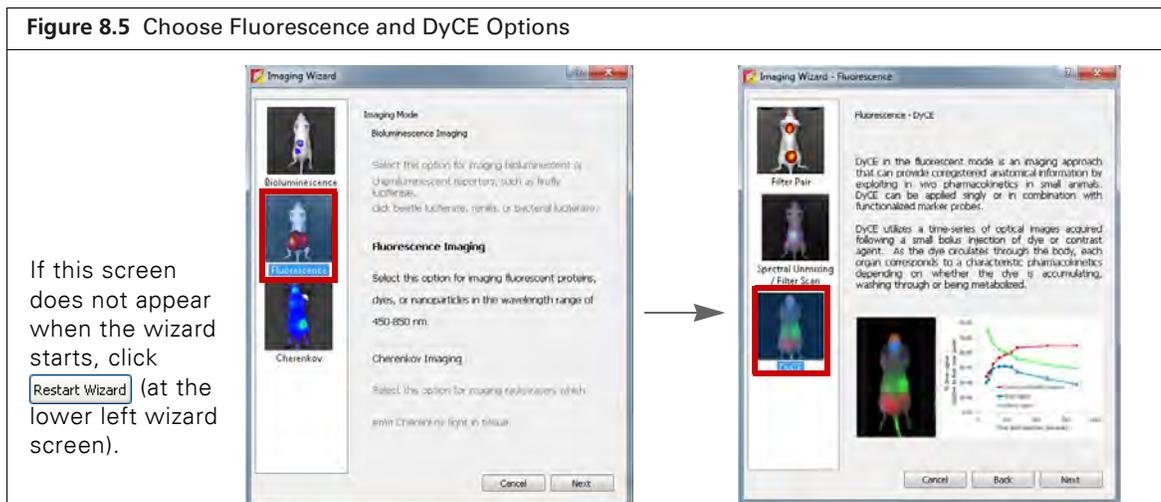
Fluorescence Imaging



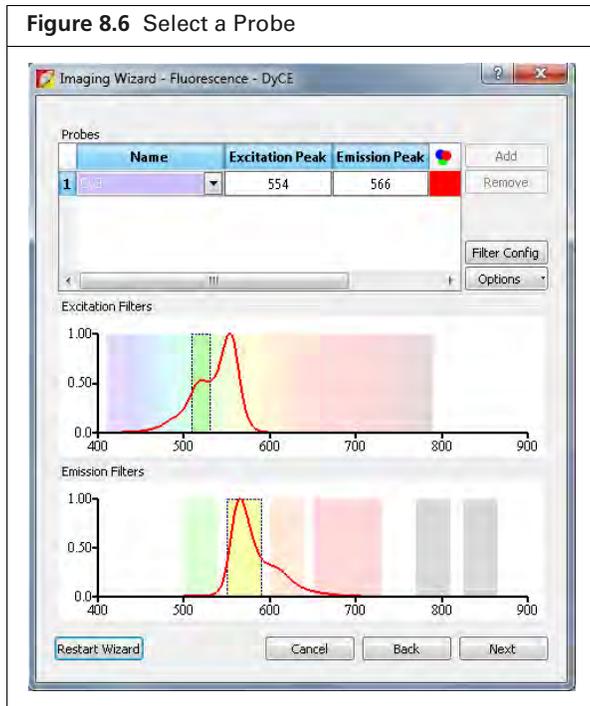
NOTE: The IVIS Lumina Series III should be initialized and the temperature locked before setting the imaging parameters. See page 16 for more details.

1. Start the Imaging Wizard. See *Start the Imaging Wizard and Setup a Sequence* on page 35 for instructions.
2. Double-click the Fluorescence option. Double-click the DyCE option in the next screen (Figure 8.5).

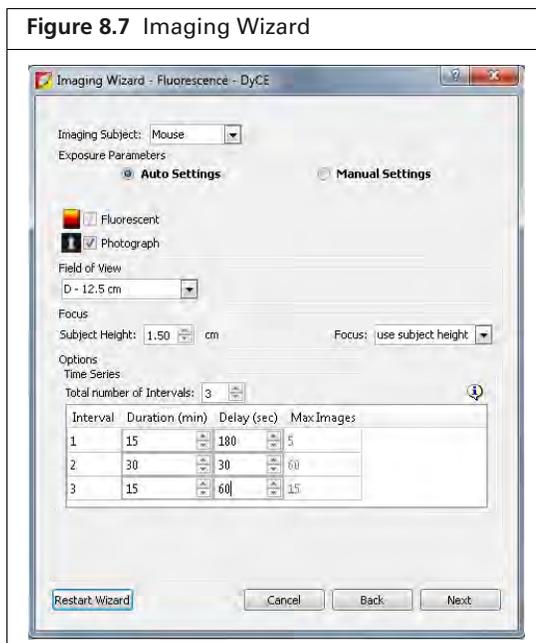
Figure 8.5 Choose Fluorescence and DyCE Options



3. Select a probe from the Name drop-down list in the next screen (Figure 8.6).
 If your fluorescent probe is not in the list, select “Input” and enter the fluorescence excitation and emission peak wavelengths. Click **Next**.

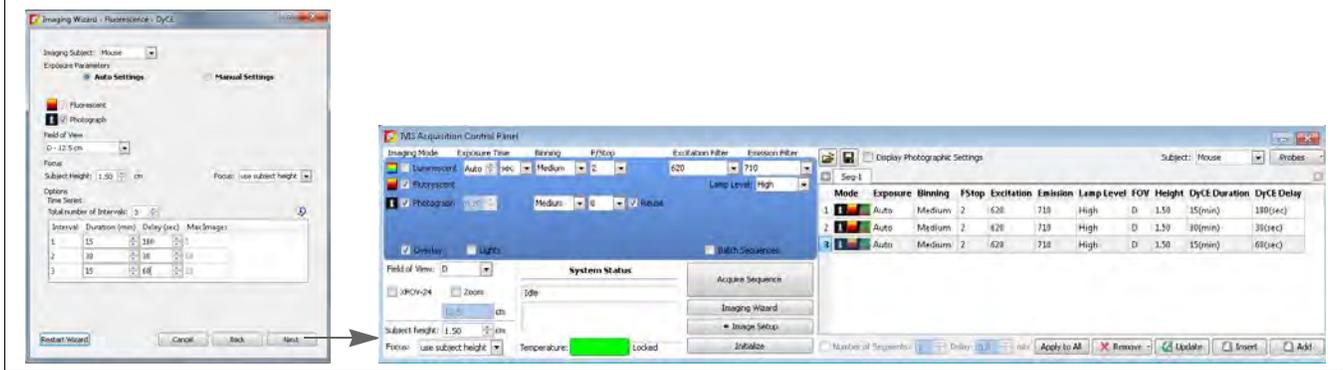


4. Select the type of imaging subject in the next screen (Figure 8.7).
5. Choose the Auto Settings option.



6. Perform step 5 to step 7 on page 142.
 The specified sequence appears in the sequence table (Figure 8.8).

Figure 8.8 Sequence Setup Complete



7. Acquire the sequence following the instructions on page 36.

The image window appears when acquisition is completed (Figure 8.12 on page 147). See Table 4.2 on page 27 for more details on the Image window.

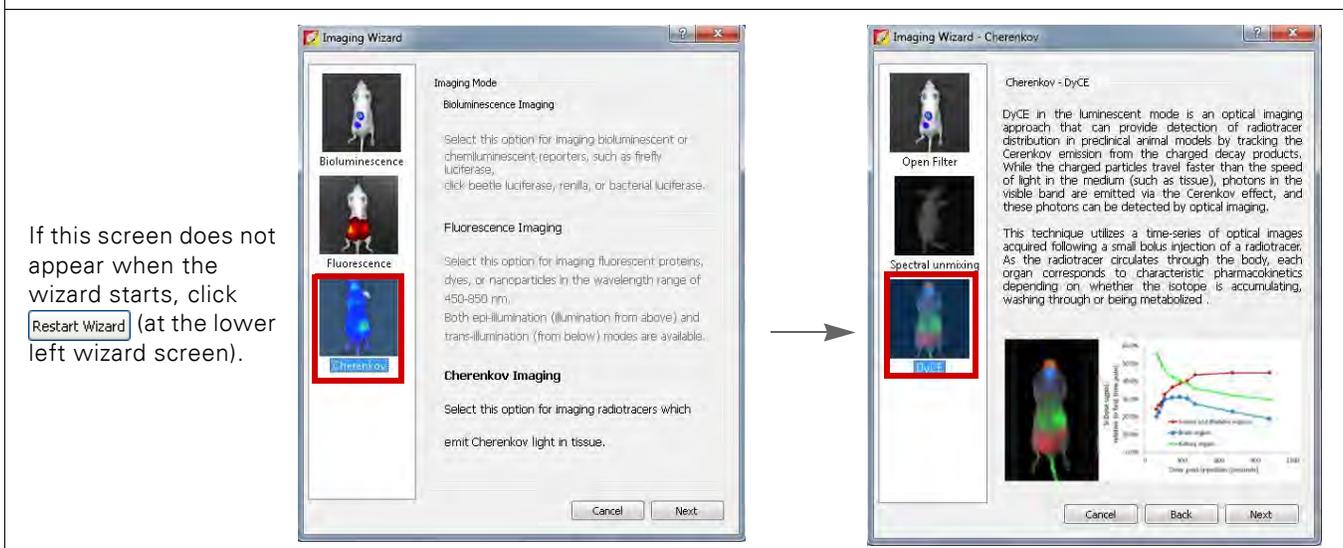
Cherenkov Imaging



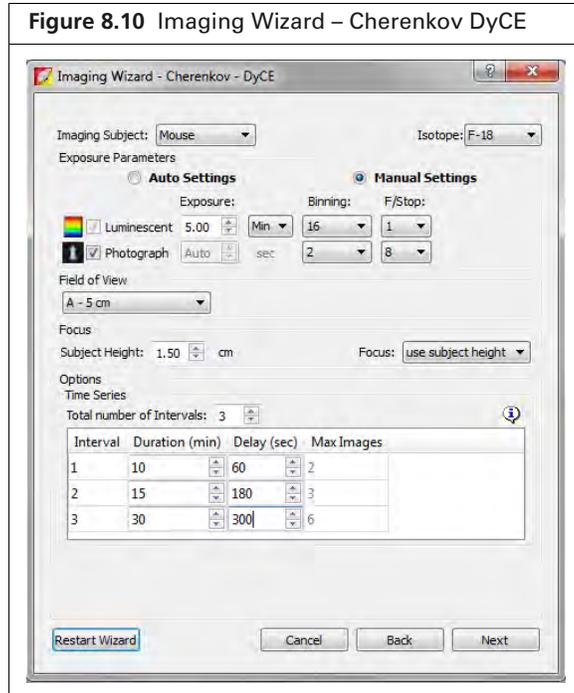
NOTE: The IVIS Lumina Series III should be initialized and the temperature locked before setting the imaging parameters. See page 16 for more details.

1. Start the Imaging Wizard. See *Start the Imaging Wizard and Setup a Sequence* on page 35 for instructions.
2. Double-click the Cherenkov option. Double-click the DyCE option in the next screen (Figure 8.9).

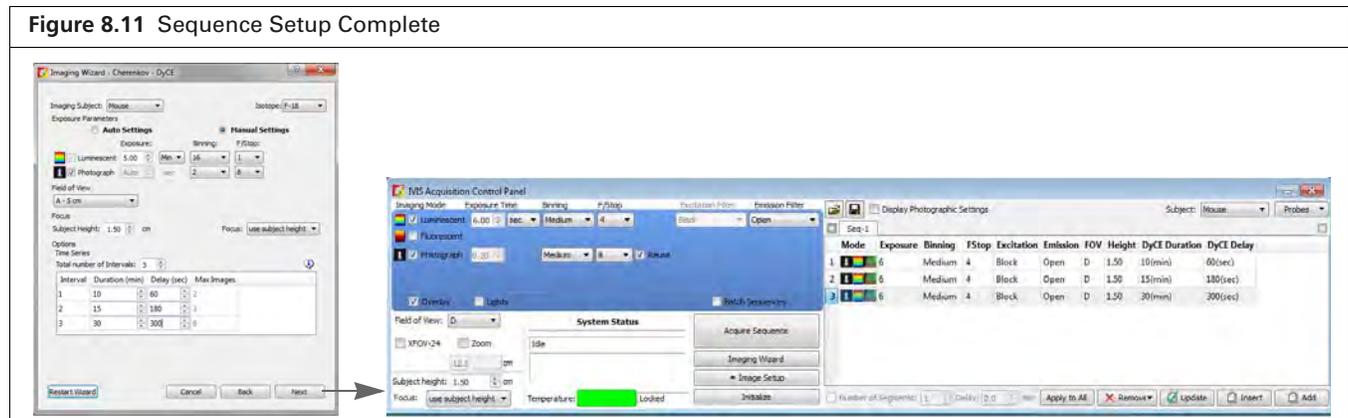
Figure 8.9 Choose Cherenkov and DyCE Options



3. Select the subject type and radio-isotope from the drop-down lists in the next screen (Figure 8.10). 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.



- Perform [step 5](#) to [step 7](#) on [page 142](#).
 The specified sequence appears in the sequence table ([Figure 8.11](#)).



- Acquire the sequence following the instructions on [page 36](#).
 The image window appears when acquisition is completed ([Figure 8.12](#) on [page 147](#)). See [Table 4.2](#) on [page 27](#) for more details on the Image window.

8.3 DyCE Analysis

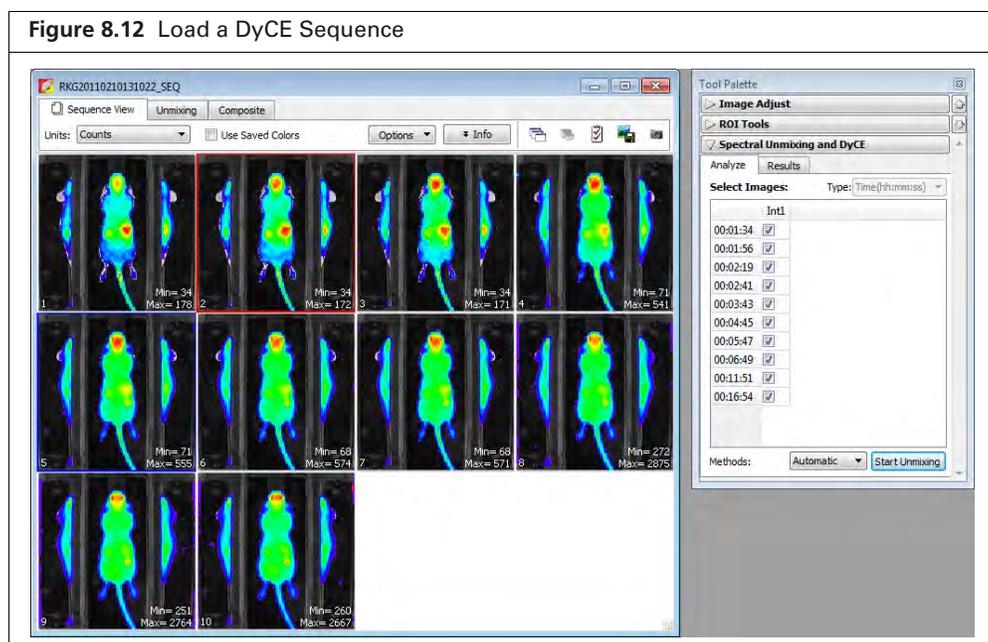
Automatic or manual DyCE analysis is available. PerkinElmer recommends performing an automatic analysis first, followed by manual analysis to identify possible additional temporal components.

Automatic DyCE Analysis

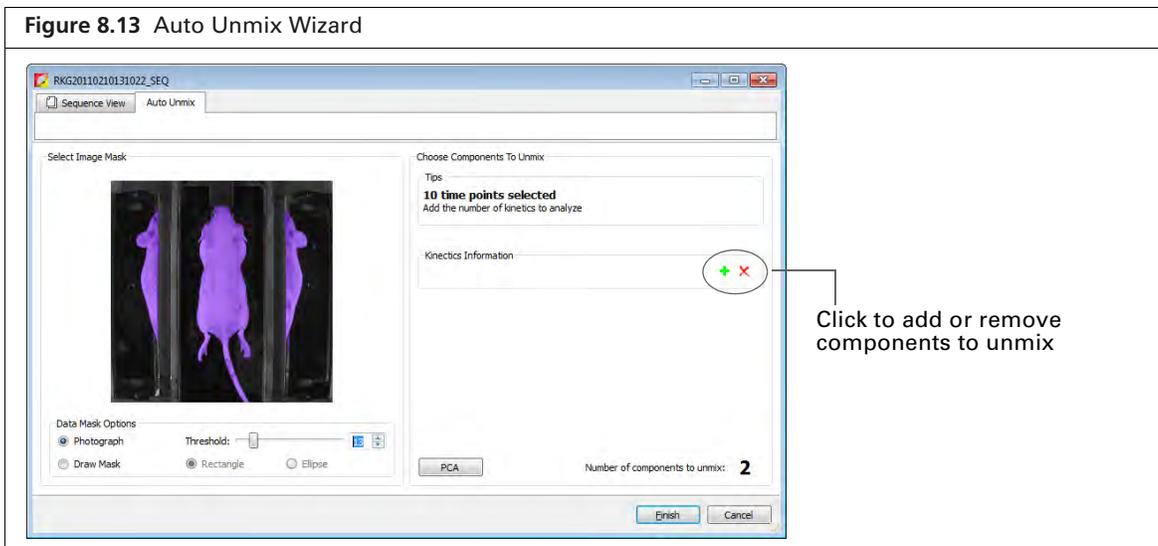
1. Load a DyCE sequence. The  icon in the Living Image browser indicates a DyCE sequence (Figure 8.12).



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 60](#) for details.



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 8.13). 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.



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 8.14). See Table 8.1 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.

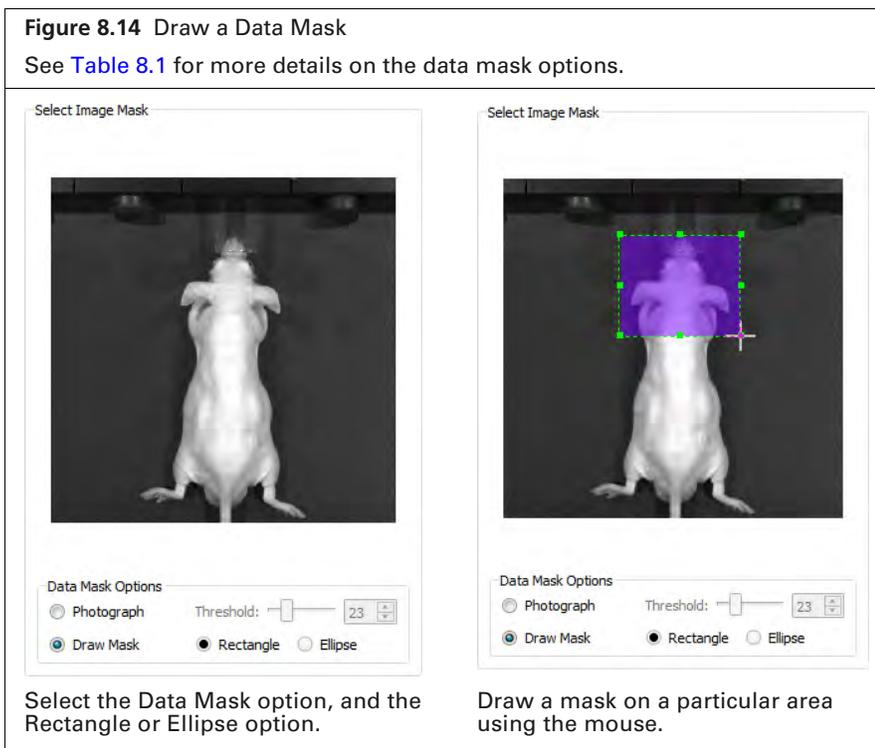


Table 8.1 Data Mask Options

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 151](#) for details on manual analysis).

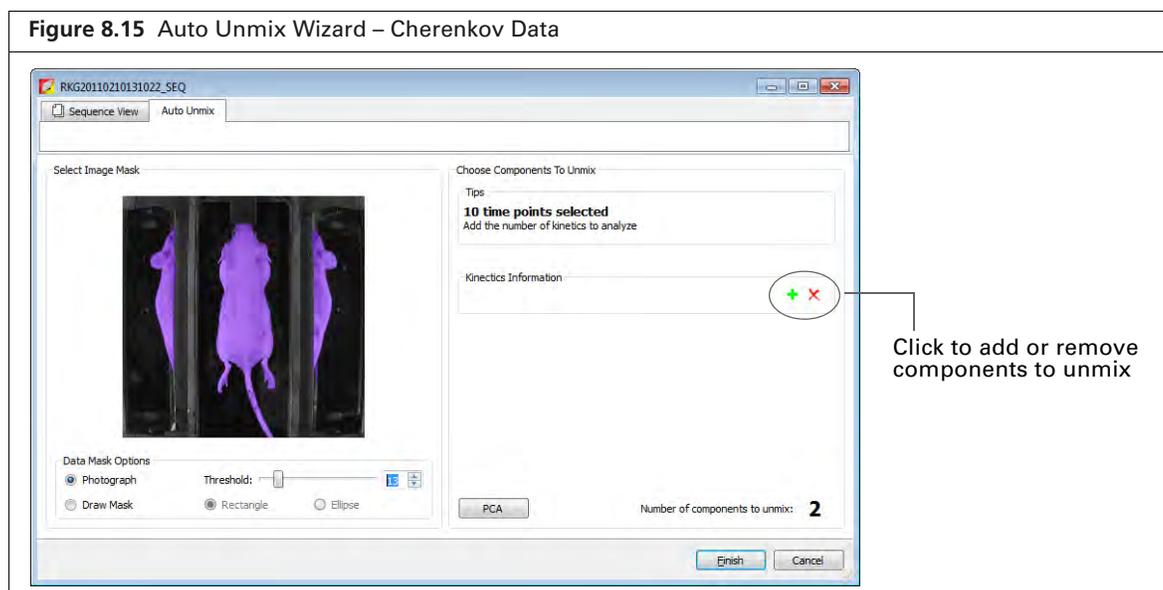
7. For Cherenkov data only:

- 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 8.15](#)).



8. Click Finish.

The Unmixing window shows a plot of the temporal spectra, unmixed images, and a composite of the unmixed images (Figure 8.16).

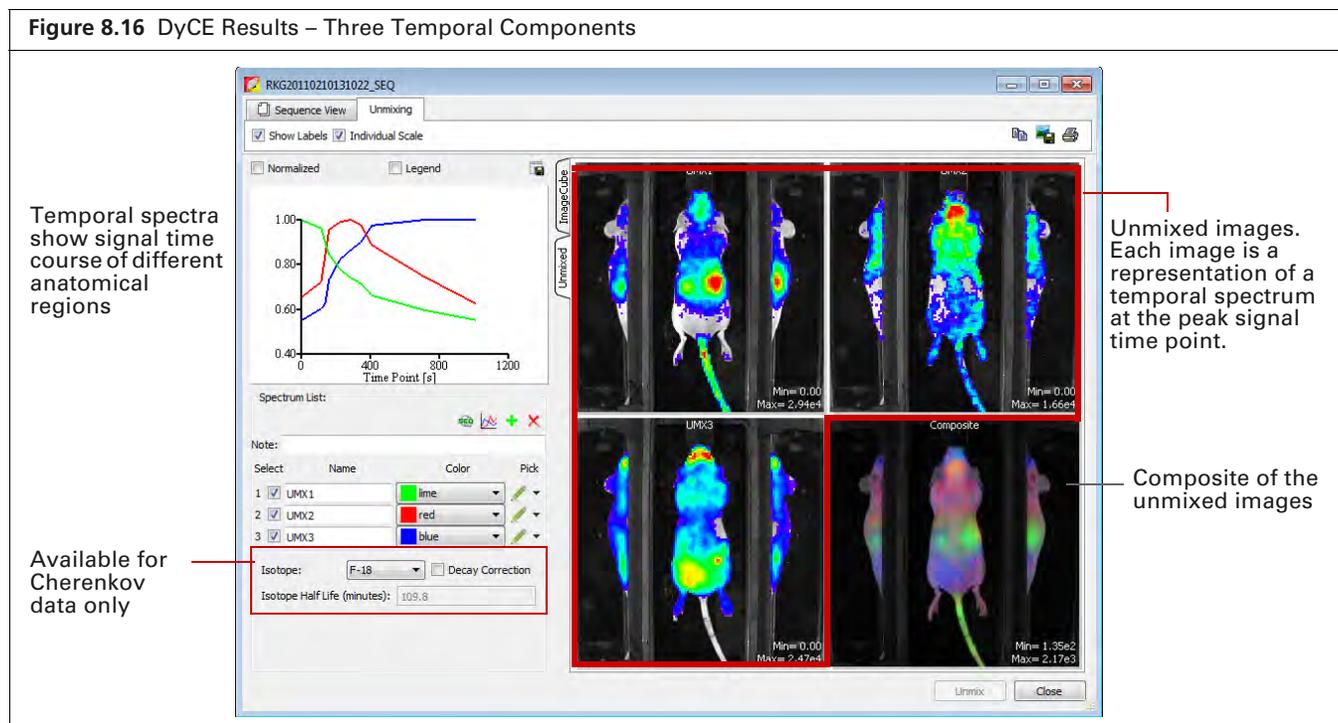
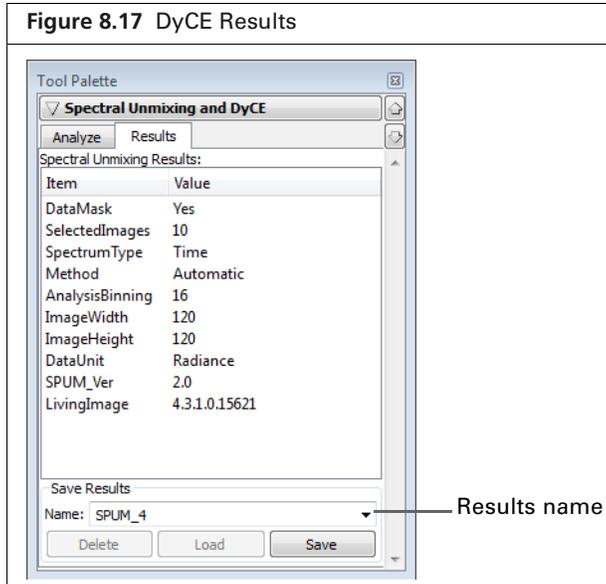


Table 8.2 Spectrum List Toolbar

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 157).
	Adds a temporal component to the spectrum list when performing a manual analysis. See page 151 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 8.17).
- b. Click **Save**.

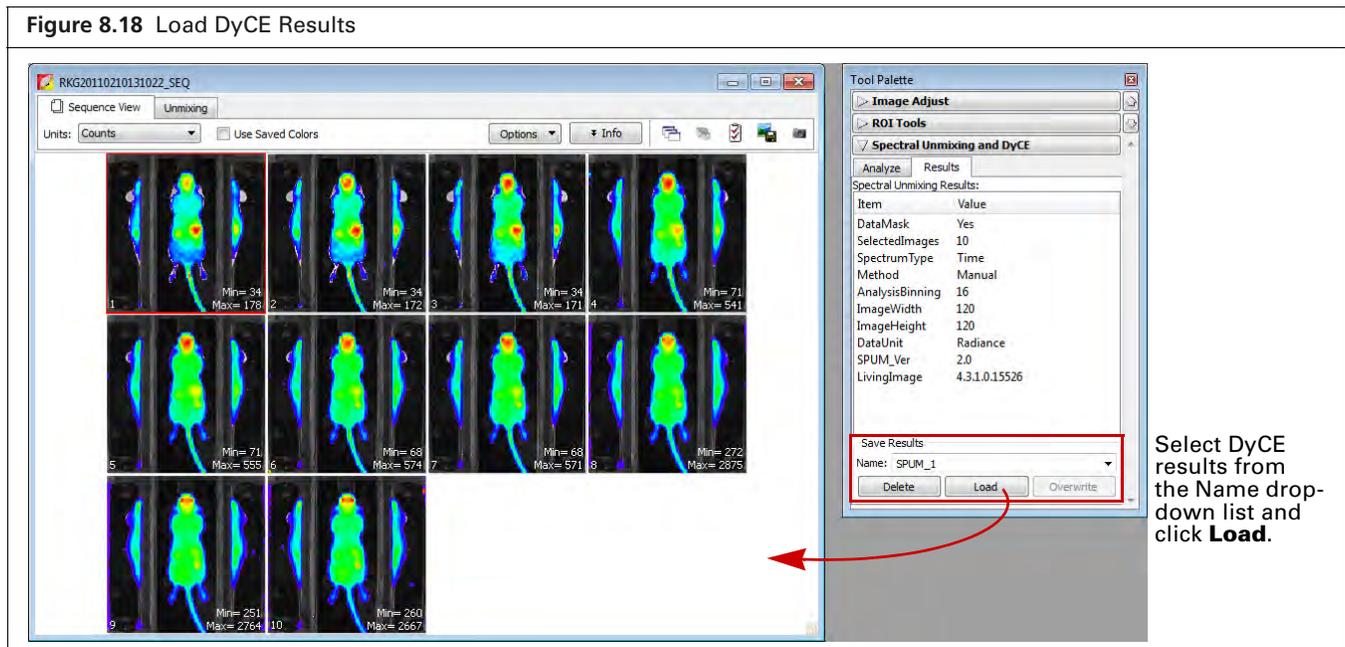


Manual DyCE Analysis

1. Load a DyCE image sequence. Alternatively, load DyCE results obtained from an automatic analysis (Figure 8.18).



NOTE: This section illustrates manual analysis of DyCE results obtained from an automatic analysis.



2. Click the Image Cube tab (Figure 8.19).

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 8.19).

To view a particular image, remove the check mark next to Overview and move the slider or enter an image number (Figure 8.20).

Figure 8.19 Image Cube – Overview Mode

Image cube tab

Temporal spectra plots

Temporal spectrum at the mouse pointer location is shown in gray

Temporal spectra names and color codes

For Cherenkov data only

Image cube

Overview shows a composite of all images in the DyCE dataset.

Figure 8.20 Image Cube – Single Image Mode

Adjust image brightness using the slider.

Remove the check mark next to Over view to view an individual image. Select an image using the slider or enter a number.

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.

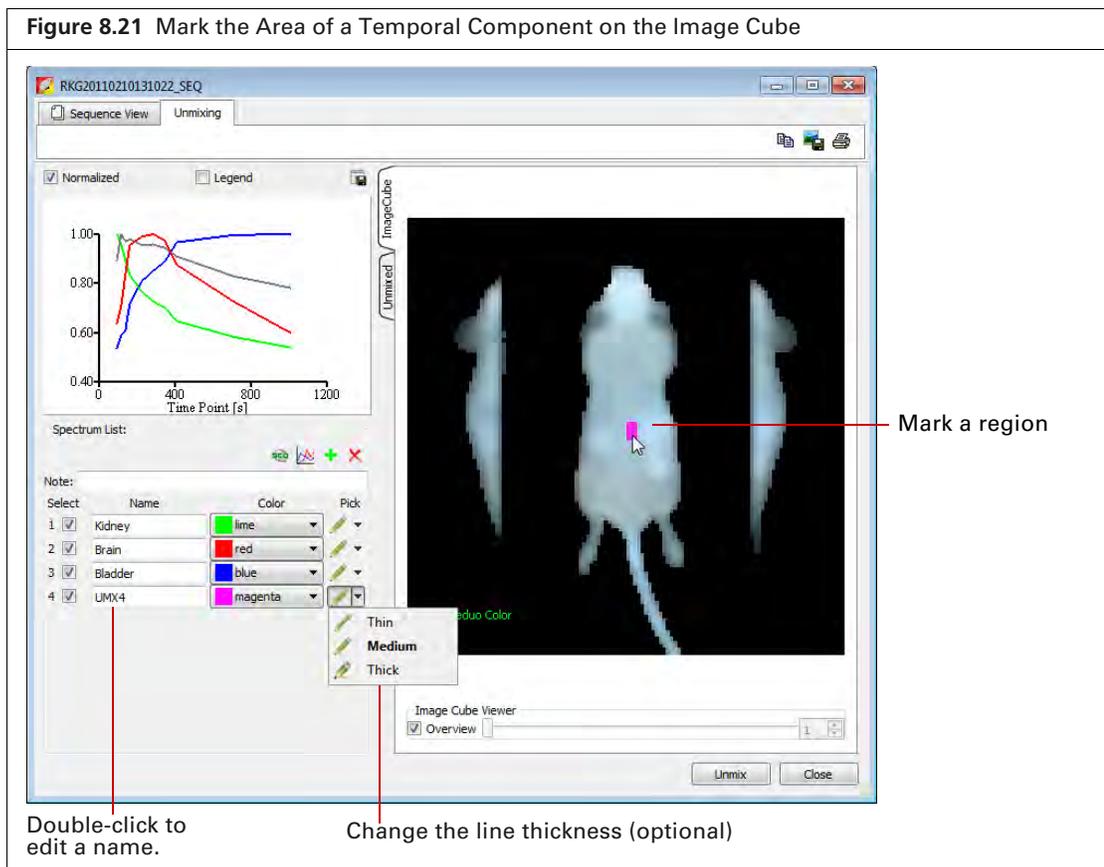


NOTE: If analyzing DyCE results, the Normalized option for the spectrum plot must be checked to see all of the temporal spectra when the mouse pointer is over the image cube.

4. To add another component to unmix:
 - a. Click the **+** button.
A new name appears in the spectrum list (Figure 8.21)
 - b. Specify the region by using the mouse to draw a mark on the image cube. If necessary, click the  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.



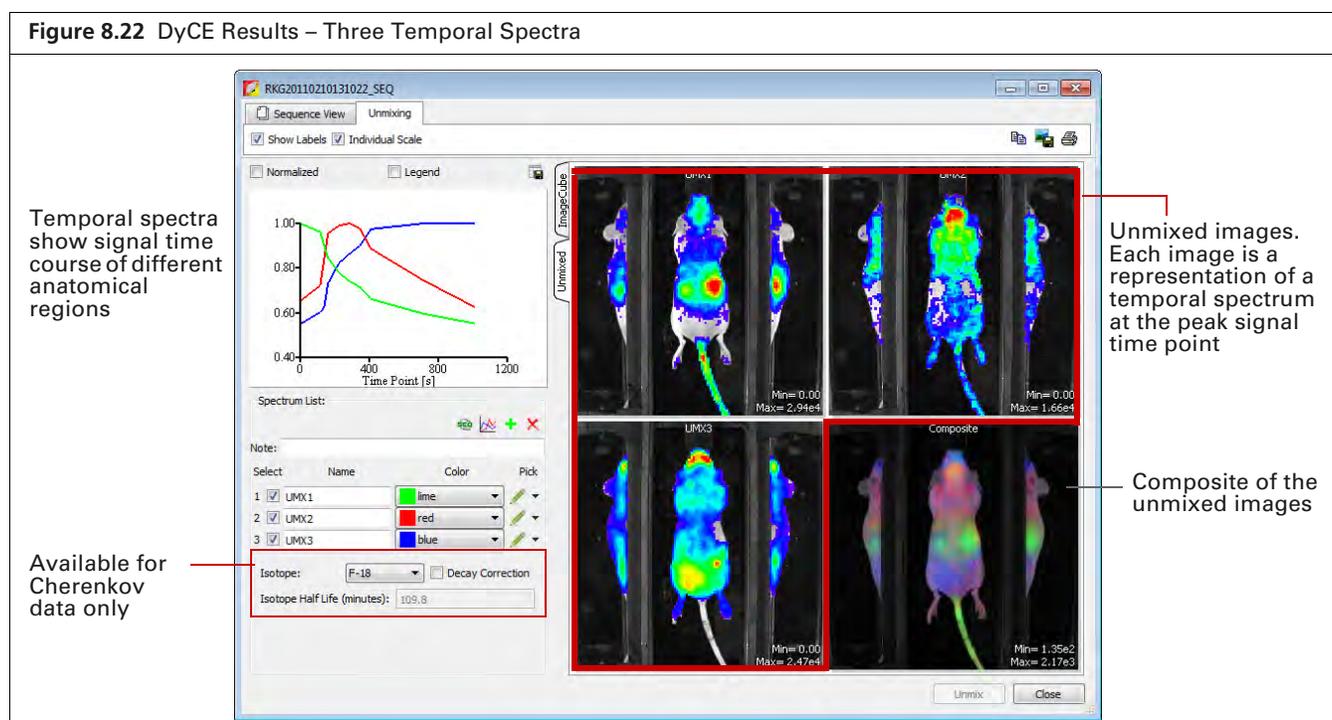
6. Click **Unmix** after you finish marking the regions.
The software generates unmixed images for the new temporal spectra and updates the composite image with these components.

Table 8.3 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 157).
	Adds a component to the spectrum list.
	Deletes the last spectrum in the spectrum list.

8.4 DyCE Results

The Unmixing window shows the DyCE results. The example in [Figure 8.22](#) shows three “temporal spectra” (signal as a function of time).

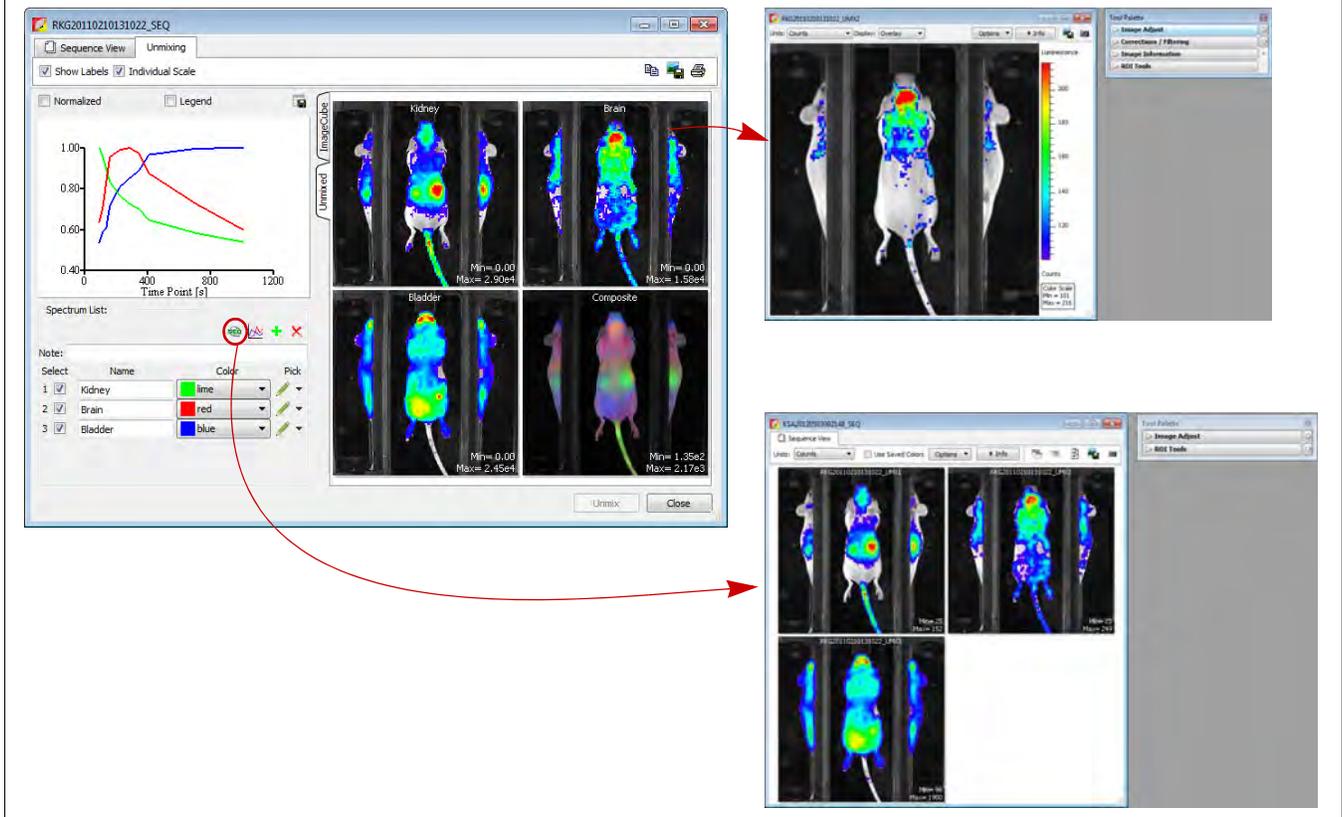


Viewing Unmixed Images

An unmixed image shows the maximum signal of a temporal spectrum.

- Double-click an unmixed image to view it in an image window ([Figure 8.23](#)). The Tool Palette is available for viewing and analyzing the image.
- Click the  button to view the unmixed images as a sequence ([Figure 8.23](#)). 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.

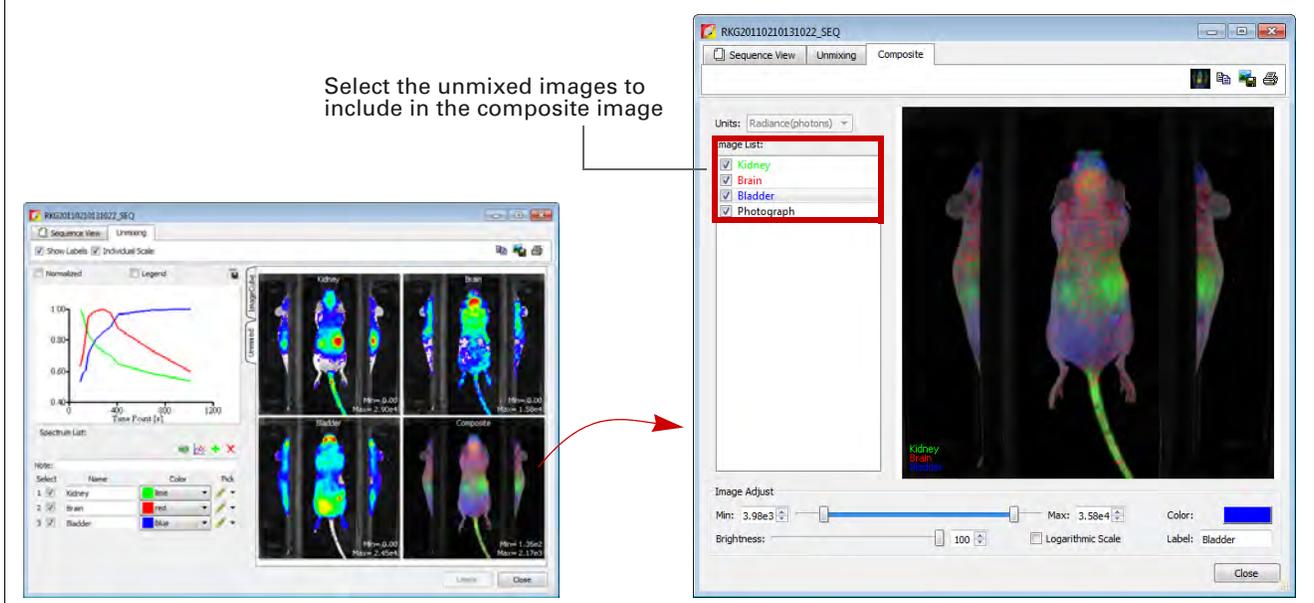
Figure 8.23 View an Unmixed image in an Image Window or View all Unmixed Images as a Sequence



Viewing the Composite Image

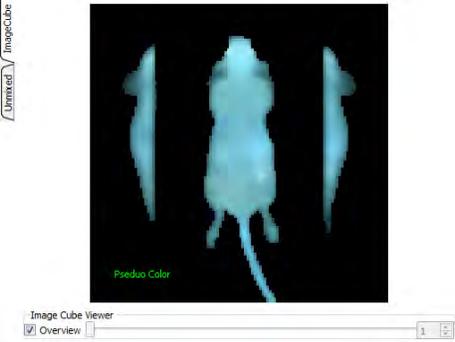
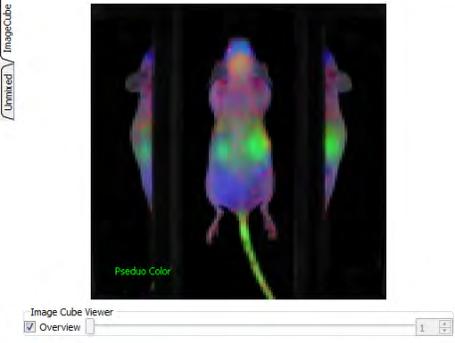
1. Double-click the composite thumbnail.
 The Composite window opens.

Figure 8.24 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 8.4 Composite Window

Item	Description
	<p>Sends the composite image to the “top” of the image cube.</p> <div style="display: flex; align-items: center;"> <div style="margin-right: 10px;">  </div> <div style="flex: 1;"> <p>Click the Image Cube tab in the Unmixing window to view the image cube. See Figure 8.19 on page 152 for more details on the image cube.</p> </div> </div> <div style="display: flex; align-items: center; margin-top: 10px;"> <div style="margin-right: 10px;">  </div> <div style="flex: 1;"> <p>Composite image displayed on top of the image cube.</p> </div> </div>
	<p>Copies the Composite window to the system clipboard.</p>
	<p>Opens a dialog box that enables you to export the composite image to a graphic file (for example, .png).</p>
	<p>Opens the print dialog box.</p>
<p>Color: </p>	<p>Shows the color of the data for the highlighted image.</p> <div style="border: 1px solid gray; padding: 5px; margin-bottom: 10px;"> <p>Image List:</p> <ul style="list-style-type: none"> <input checked="" type="checkbox"/> Kidney <input checked="" type="checkbox"/> Brain <input checked="" type="checkbox"/> Bladder <input checked="" type="checkbox"/> Photograph </div> <p>Click the color swatch to open the color palette which can be used choose a color for the selected image data.</p>
<p>Label: <input type="text" value="Bladder"/></p>	<p>Data name for the highlighted image. Double-click the name to edit it.</p>

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 8.25).

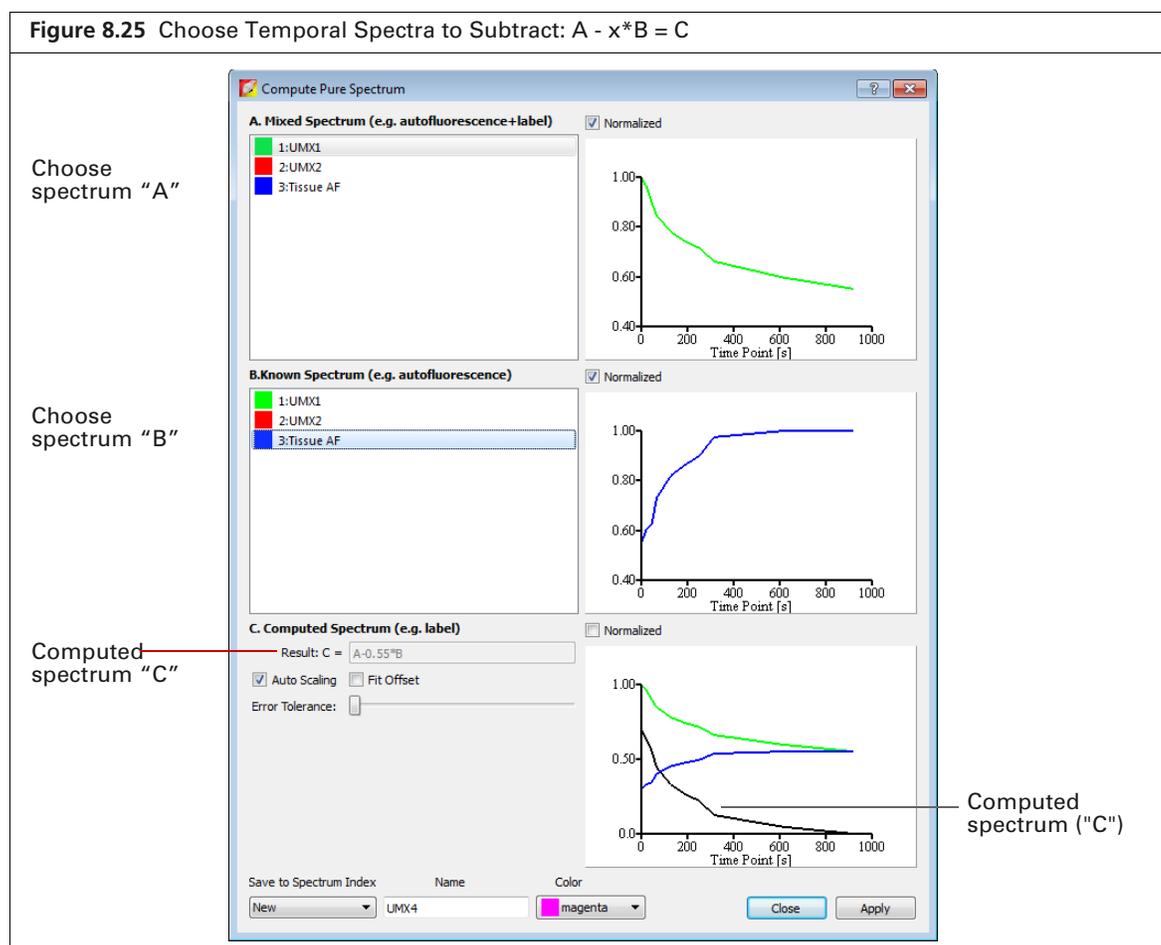
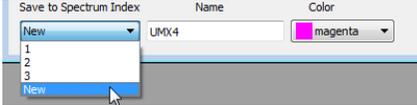


Table 8.5 Computed Spectrum

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 8.5 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.
	<p>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.</p> <p>Choose a spectrum number from the drop-down list to overwrite that spectrum with the computed spectrum when you click Apply.</p>

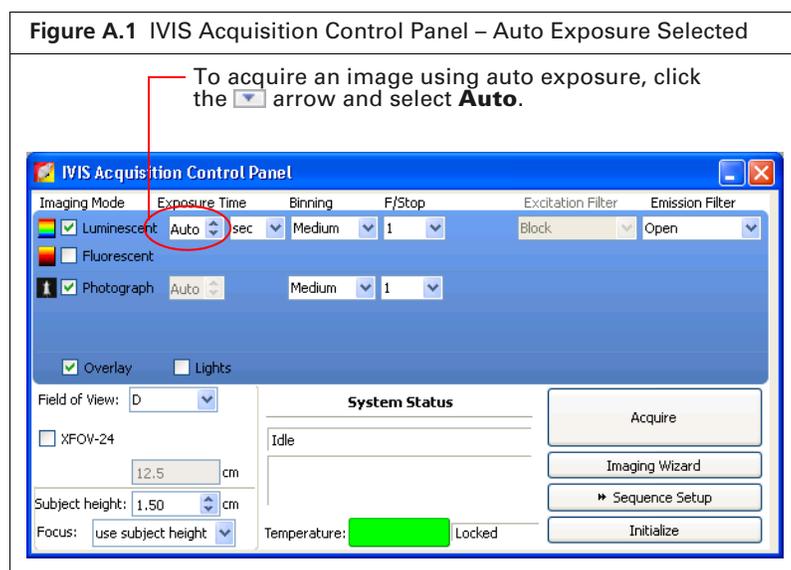
Appendix A IVIS Acquisition Control Panel

Control Panel Functions

Manually Setting the Focus on page 162

A.1 Control Panel Functions

The control panel provides the image acquisition functions (Figure A.1).



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.
 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 Viewing Intensity Data, page 62).

Table A.1 IVIS Acquisition Control Panel (continued)

Item	Description
Exposure time	<p>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.</p> <p>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.</p>
Binning	<p>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 <i>in vivo</i> images where light emission is diffuse. For more details on binning, see the reference article <i>Detection Sensitivity</i> (select Help → References on the menu bar).</p> <p>Recommended binning: Small (1-4) for imaging of cells or tissue sections, Medium (4-8) for <i>in vivo</i> imaging of subjects, or Large (8-16) for <i>in vivo</i> imaging of subjects with very dim sources.</p>
F/stop	<p>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.</p> <p>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 <i>Detection Sensitivity</i> (select Help → References on the menu bar).</p>
Excitation Filter	<p>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.</p> <p>Note: On some models with standard filter sets, the excitation filter selection automatically sets the emission filter.</p>
Emission Filter	<p>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.</p>
Lamp Level	<p>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.</p> <p>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.</p>
Overlay	<p>If this option is chosen, the system automatically displays the overlay after acquisition is completed (for example, luminescent image on photograph).</p>
Lights	<p>Turns on the lights located at the top of the imaging chamber.</p>

Table A.1 IVIS Acquisition Control Panel (continued)

Item	Description
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.
Mouse Imaging Shuttle	Choose this option if the subject will be contained in the Mouse Imaging Shuttle during image acquisition.
Load	Moves the stage from the cleaning position back to the home position.
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.
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 162 .
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 40 for more details.
Temperature 	The temperature box color indicates the temperature and status of the system: <ul style="list-style-type: none"> ■ 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 17 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.
Imaging Wizard	Click to start the Imaging Wizard
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 42 for more details on manually setting up an image sequence,.
Image Setup	Click to close the sequence table.
Initialize	Click to initialize the IVIS Lumina Series III. See page 16 for more details on initializing the system.

Table A.2 Field of View (FOV) Settings

FOV Setting	FOV (cm)
A	5
B	7.5
C	10
D	12.5
E ¹	24
Z ²	2.6

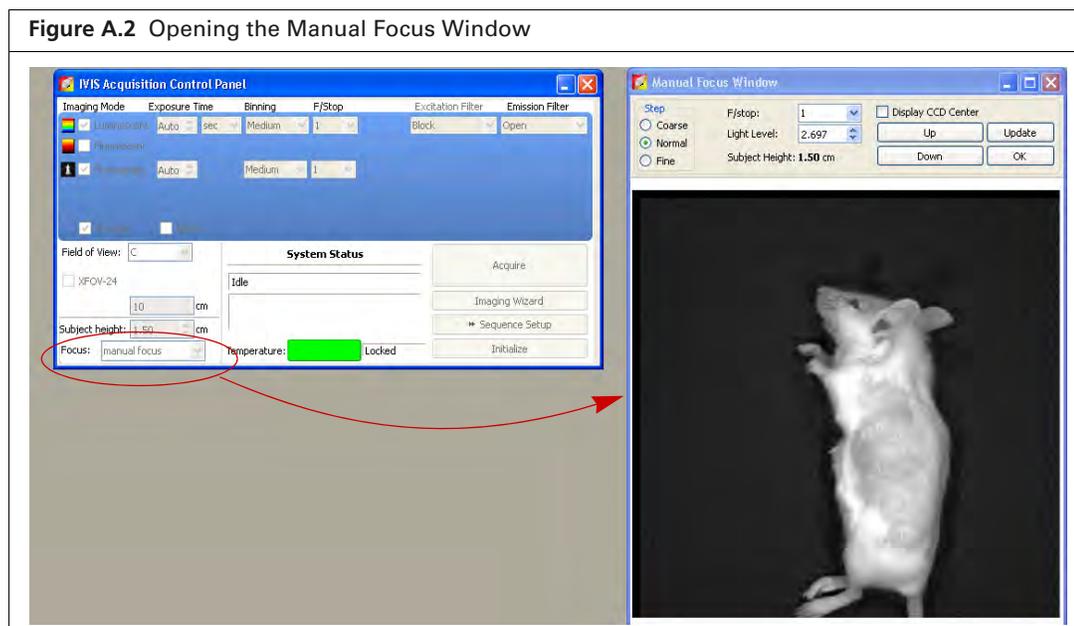
¹ Position E is available when the XFOV-24 lens (PN 123177) is installed.

² Position Z is available when the ZFOV-2.6 lens (PN 127285) is installed.

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.



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 Planar Spectral Image Analysis

About Planar Spectral Image Analysis

Image Sequence Requirements

Analyzing Data on page 164

Viewing Graphical Results on page 167

Managing Results on page 168

B.1 About Planar Spectral Image Analysis



NOTE: The Planar Spectral Imaging tools are only available if “Show Advanced Options” is selected in the user preferences. See [page 171](#) for details. Planar spectral imaging requires narrow band emission filters. The 40 nm wide emission filters that are standard on the IVIS Lumina Series III will not work. Planar spectral imaging requires a special set of 20 nm wide emission filters. Please contact PerkinElmer Technical Service (see [page 3](#)) for additional details.

Planar spectral image analysis can determine the average depth and total photon flux of a luminescent point source in a user-specified region of interest.



TIP: See the tech note *Planar Spectral Imaging* for theory and examples of planar spectral imaging. Select **Help** → **Tech Notes** on the menu bar.

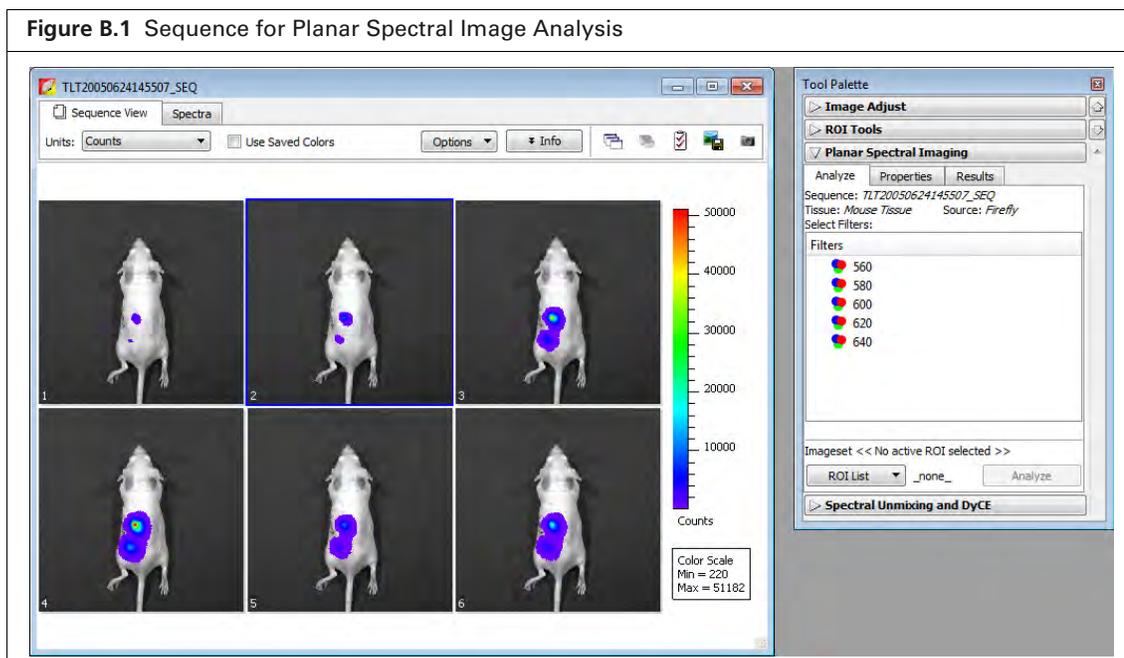
B.2 Image Sequence Requirements

Use the Imaging Wizard to set up the image sequence required for planar spectral image analysis. See [page 34](#) for more details on the Imaging Wizard.

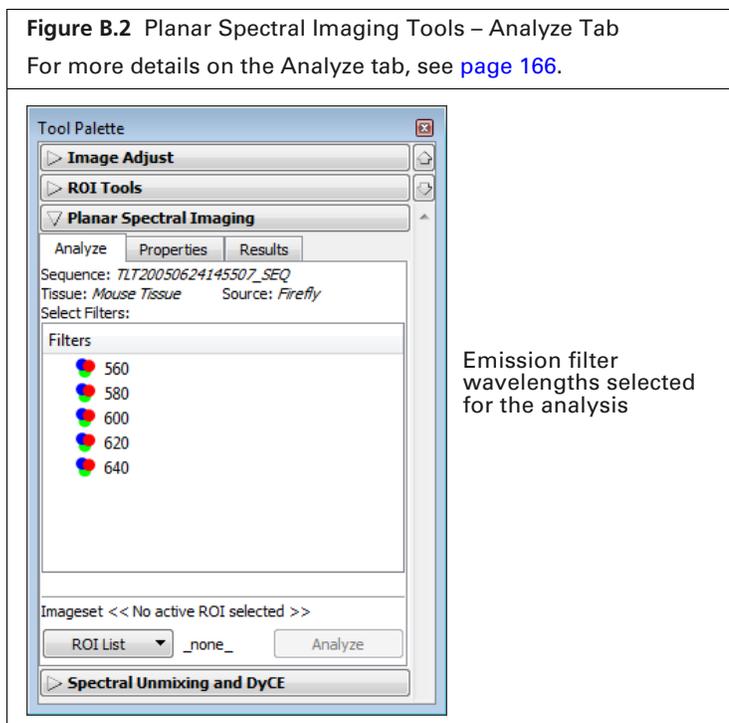
At a minimum, the sequence must include a photographic and luminescent image at the first wavelength and a luminescent image at a second wavelength. The data must be acquired in the wavelength range from 560-660 nm.

B.3 Analyzing Data

1. Load the image sequence that you want to analyze.

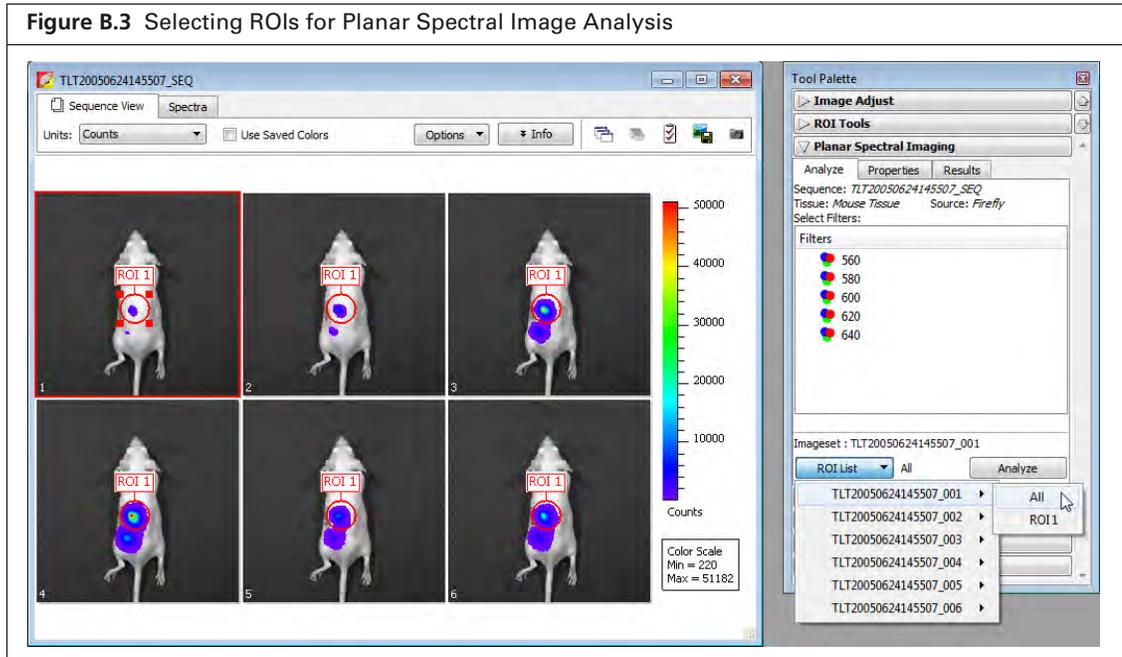


2. Click Planar Spectral Imaging in the Tool Palette.
3. Select the emission filter wavelengths for the analysis in the Analyze tab (Figure B.2).
 It is recommended that you do not include a wavelength in the analysis if the signal is less than or equal to the autoluminescent background. If autoluminescent background is a concern, you can create a background ROI and link it to the measurement ROI prior to planar spectral analysis. (See *Measuring Background-Corrected Signal* on page 96 for more details.)



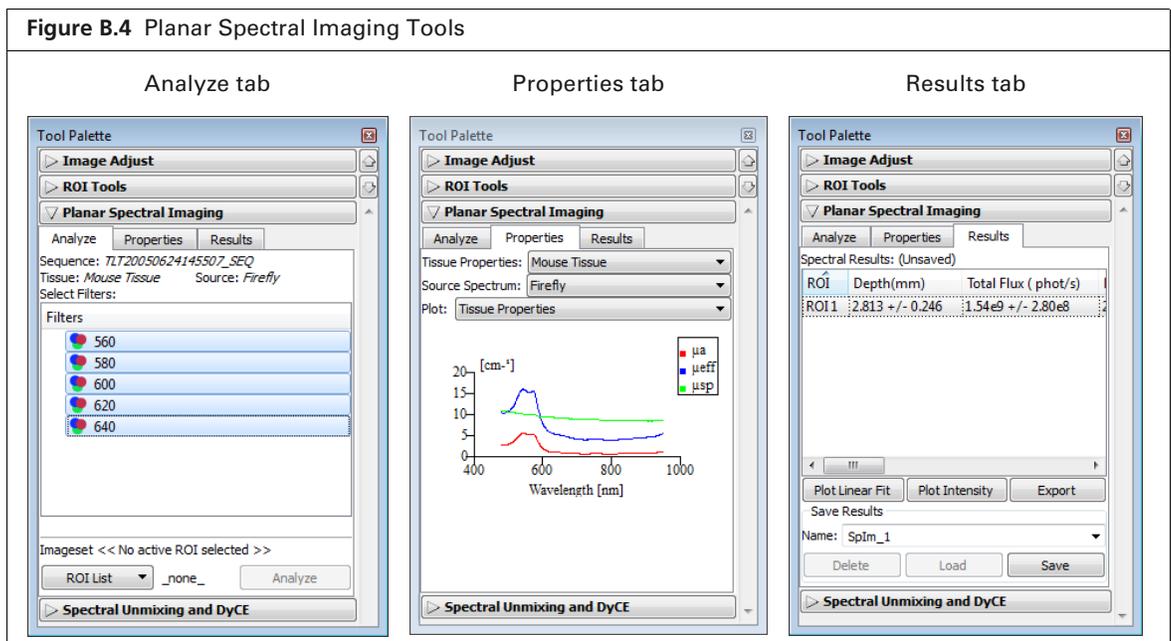
- In the ROI List drop-down, select **All** or a particular ROI in an image for the analysis. If there is no measurement ROI, draw an ROI that includes the area for analysis. (See [page 90](#) for more details on drawing ROIs.)

You only need to draw the ROI(s) on one image in the sequence. The software copies the ROI(s) to all other images of the sequence during the analysis. The ROI should include as much of the light emission from a single source as possible.



- Choose the tissue properties. In the Properties tab, choose Mouse Tissue or XPM-2/XFM-2 (mouse phantom) from the Tissue Properties drop-down list.

The software automatically sets the internal medium index of refraction based on the selection in the Tissue Properties list



6. Make a selection from the Source Spectrum drop-down list (Firefly in this example).
7. Click **Analyze** in the Analyze tab.

The Results tab displays the computed average depth (mm) and total flux (photon/sec) of the luminescent point source in the specified ROI(s). For more details on the results, see [Table B.1](#).

Table B.1 Planar Spectral Imaging Tools

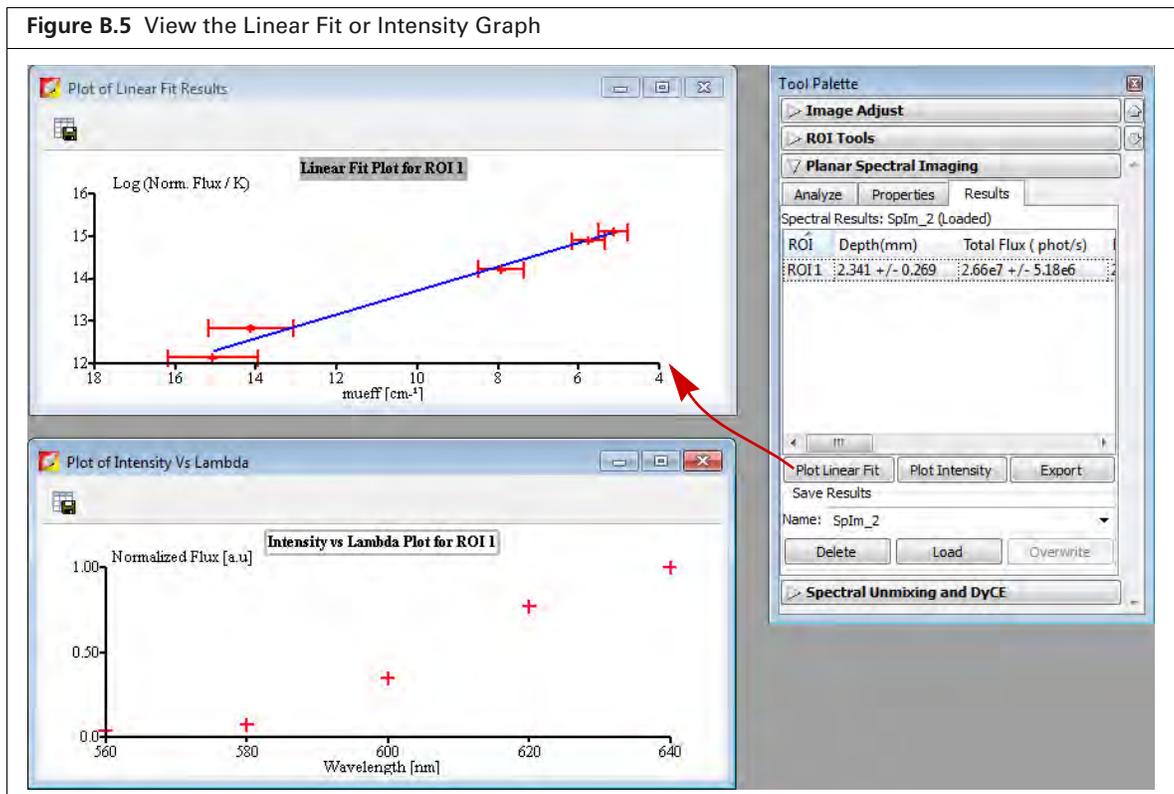
Item	Description
Analyze tab	
Sequence	Name of sequence used for the analysis.
Tissue, Source	The tissue properties and source spectrum selected in the Properties tab.
Select Filters	In the Filter box, select the acquisition wavelengths for the images in the selected sequence. To select non-adjacent wavelengths, press and hold the Ctrl key while you click the wavelengths. (Macintosh users, press and hold the Cmd key while you click the wavelengths.)
ROI List	A drop-down list of the ROIs in the active image.
Analyze	Click to perform the spectral analysis.
Properties tab	
Tissue Properties	Drop-down list of the absorption and scattering properties for Mouse Muscle or XPM-2/XFM-2 (mouse phantom). Note: If a result from an earlier version of Living Image software is loaded, the tissue type will be listed in this drop-down list.
Source Spectrum	Drop-down list of luminescent sources.
Plot	Tissue Properties - Click to display graphs (cm^{-1} vs. nm) of the absorption coefficient (μ_a), effective attenuation coefficient (μ_{eff}), and reduced scattering coefficient (μ'_s). Source Spectrum - Click to display the spectrum of the selected luminescent source (intensity versus wavelength, normalized to one).
Results tab	
Spectral Results	ROI - Name of the analyzed ROI. Depth (mm) - Estimated depth of the point source. Total Flux (photons/sec) - Estimated total photon flux from the point source.
Plot Linear Fit	Displays a graph of normalized intensity versus the effective attenuation coefficient (μ_{eff} the optical property of the tissue selected in the Tissue Properties drop-down list) along with the linear fit to these data determined by the spectral analysis code.
Plot Intensity	Displays a graph of normalized intensity versus wavelength. Intensity is normalized by the selected source spectrum and filter transmission properties.
Export	Opens a dialog box that enables you to save the results to a text file (.txt).
Save Results	Name - A drop-down list of saved results. Includes the default name for new unsaved analysis results (Splm_<name>). Delete - Deletes the selected results. Load - Opens the selected results. Save - Saves the analysis results. Results name appears in the Name drop-down list).

B.4 Viewing Graphical Results

1. In the Results tab, select an ROI.
2. Click **Plot Intensity** or **Plot Linear Fit** (Figure B.5).

Linear fit graph – Plots the logarithm of the intensity, normalized to the selected source spectrum and the filter transmission properties, against the optical property of the tissue (μ_{eff}). The slope of the line is the source depth. If any of the measured points (in red) deviate significantly from the straight line fit, then the analysis results may be suspect. The horizontal error bars represent the uncertainty in the optical properties (usually estimated at $\pm 10\%$). The vertical error bars represent noise in the image.

Intensity graph – Displays a graph of the measured intensity in the selected ROI at each wavelength in the analysis. The intensity is normalized to the selected source spectrum and the filter transmission properties.



To export graph data:

1. Click the **Export Data** button .
2. In the dialog box that appears, select a directory for the data and enter a file name (.csv).
The data can be opened in a spreadsheet application such as Microsoft® Excel®.

B.5 Managing Results

Go to the Results tab to select results that you want to view or manage.

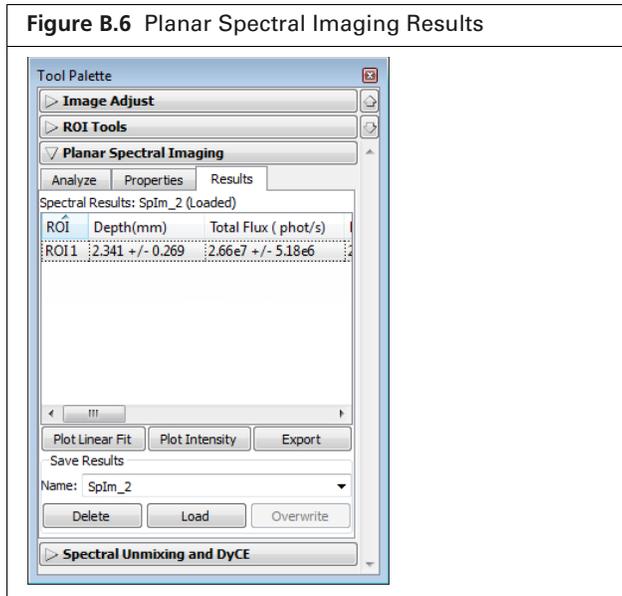


Table B.2 Managing Planar Spectral Imaging Results

To:	Do This:
Save Results	Select results (SpIm_<name>) from the Name drop-down list and click Save . The planar spectral imaging results are saved with the image.
View Results	Select results from the Name drop-down list and click Load .
Delete Results	Select the results the results to delete from the Name drop-down list and click Delete .
Copy Results	<ul style="list-style-type: none"> ■ To copy selected results, right-click the results (row in the Results tab) and select Copy from the shortcut menu that appears. The selected results are copied to the system clipboard. ■ To copy all results, right-click the results table and choose Select All from the shortcut menu that appears. All of the results are copied to the system clipboard.
Export Results	Right-click the results table and select Export Results from the shortcut menu that appears. In the dialog box that appears, choose a folder for the results, enter a file name (.txt), and click Save .

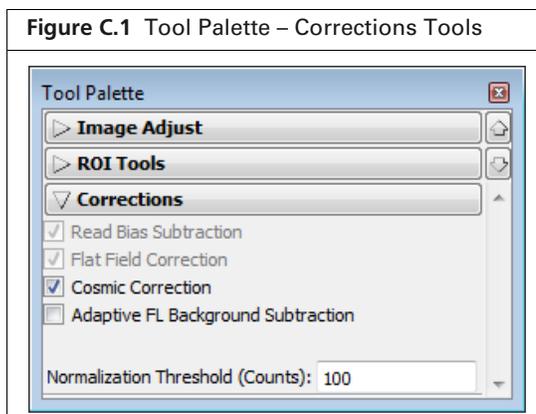
Appendix C Image Data Corrections

The Corrections tools apply corrections or subtract adaptive fluorescence background from 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.



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 C.1 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 <i>Luminescent Background Sources and Corrections</i> (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 C.1 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 <i>Image Data Display and Measurement</i> 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 <i>Adaptive Fluorescence Background Subtraction</i> (select Help → Tech Notes on the menu bar).
Normalization Threshold (Counts)	The minimum number of counts required to perform normalization.

Appendix D Preferences

General Preferences

Options on page 173

Acquisition on page 173

Theme on page 175

Optical Properties on page 176

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 Living Image software on the workstation that controls the IVIS Imaging System.

D.1 General Preferences

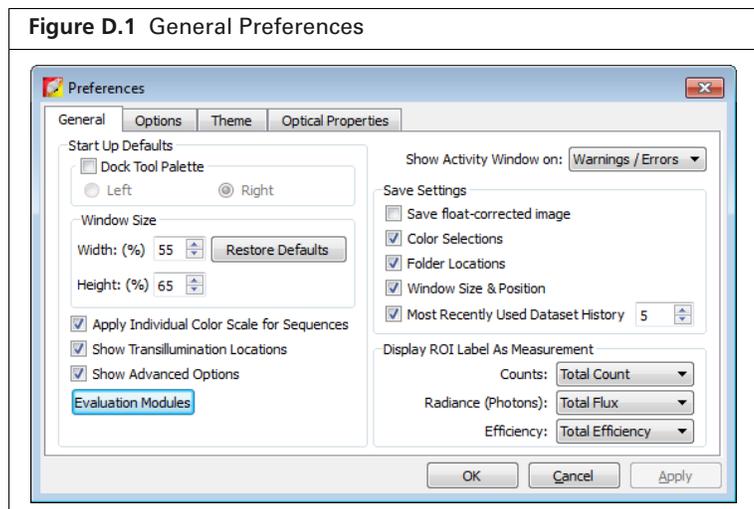


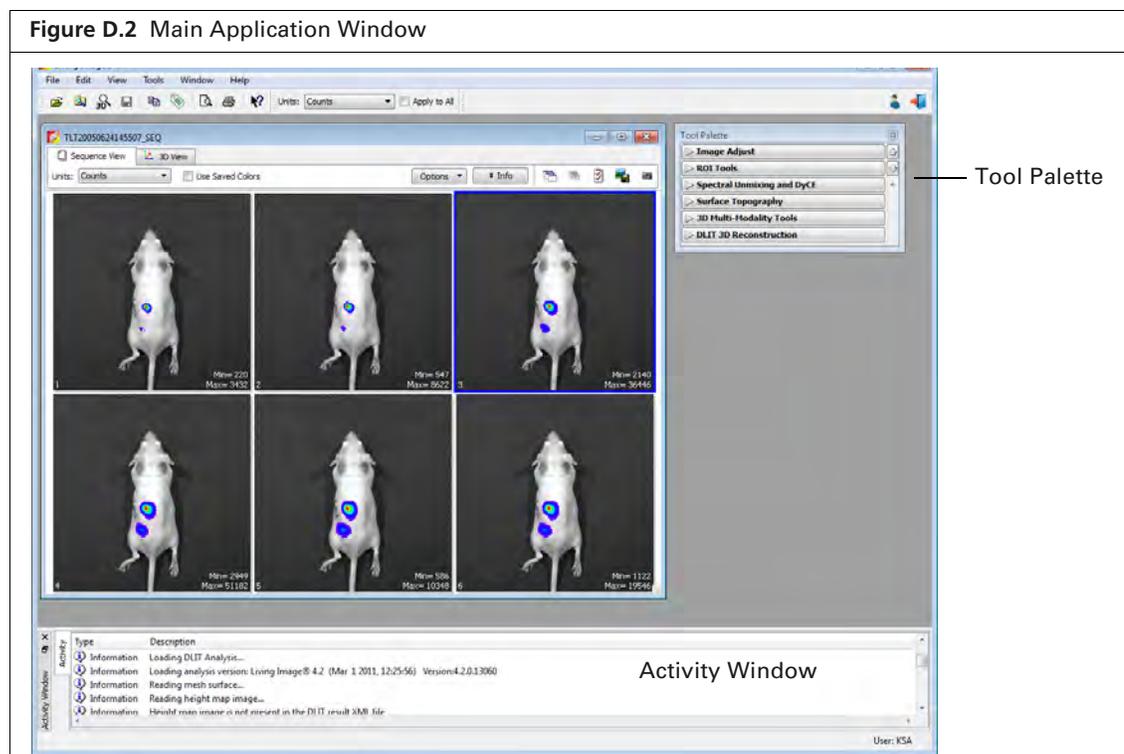
Table D.1 General Preferences

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 D.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 Advanced Options	If this option is selected, advanced features are available in the menu bar and Tool Palette, including: <ul style="list-style-type: none"> ■ Additional ROI functionality for Auto ROI parameters. ■ 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 D.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 D.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.



D.2 Options

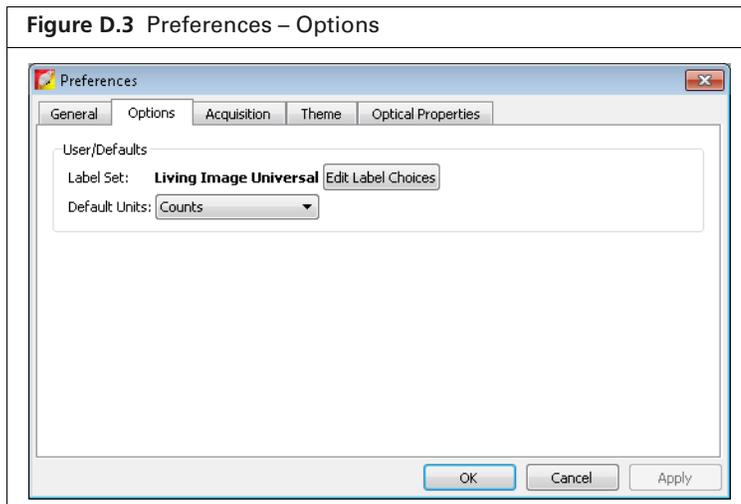


Table D.2 Preferences – Options

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.

D.3 Acquisition

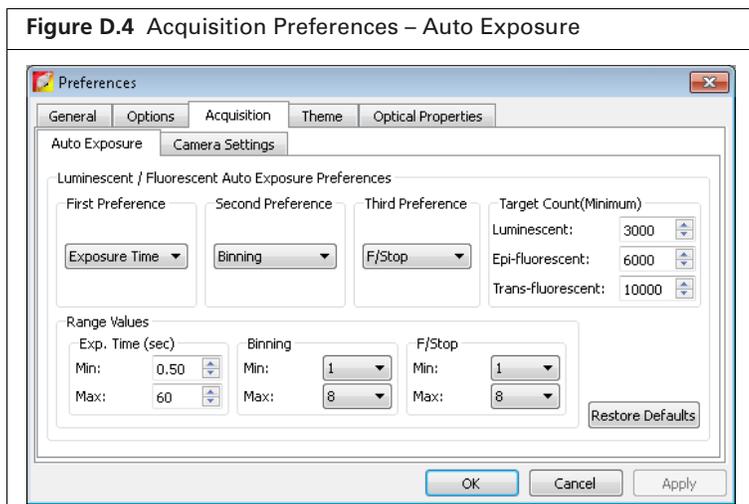


Table D.3 Auto Exposure Settings

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.

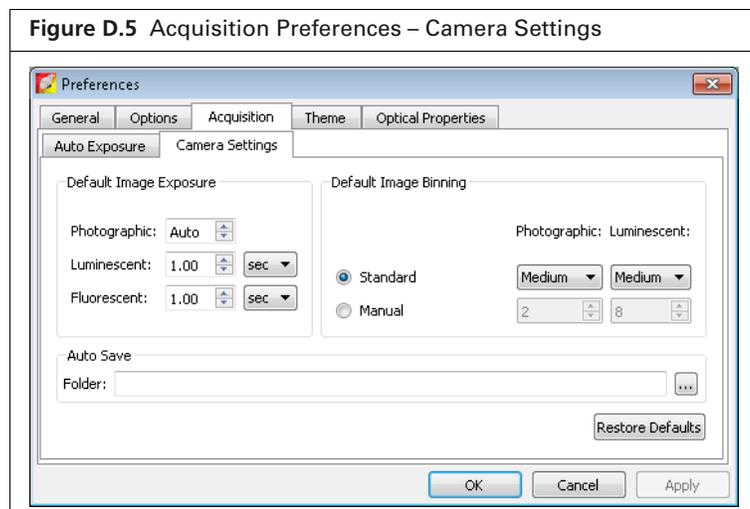


Table D.4 Camera Settings

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.

D.4 Theme

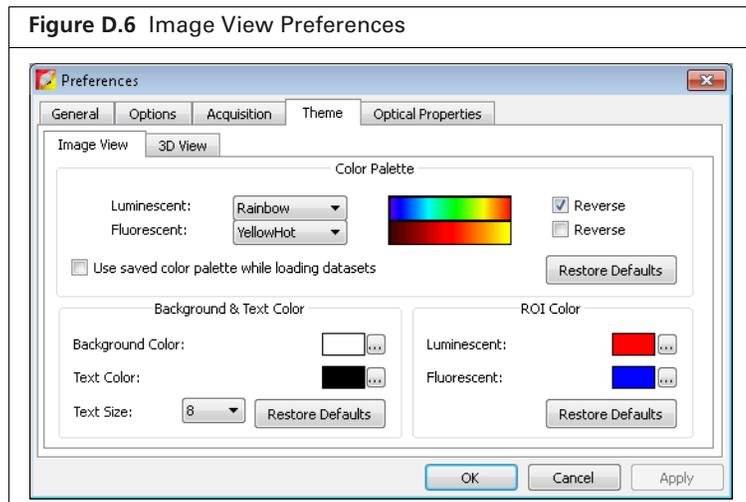
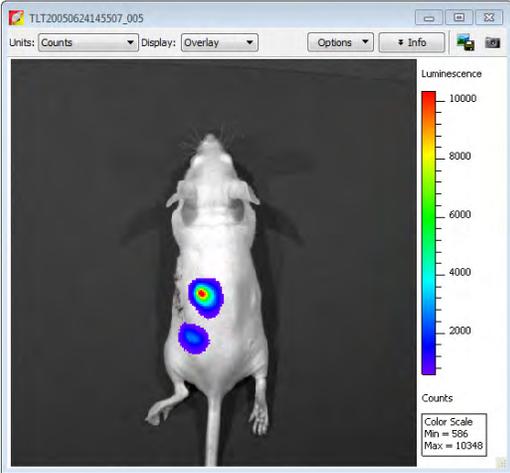


Table D.5 Image View Preferences

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	<p>Sets the color of the:</p> <ul style="list-style-type: none"> Background in the image window (shown below) Text for the color bar <p>To change a color, click the  button that opens the color palette.</p> 
ROI Color	<p>Sets the colors for the ROI outline. To change a color, click the  button that opens the color palette.</p> <p>Luminescent - Color of the ROI outline on a luminescent image. Fluorescent - Color of the ROI outline on a fluorescent image.</p>
Restore Defaults	Click to apply the default settings.

D.5 Optical Properties



NOTE: Some of the Optical Properties preferences (Figure D.7) are not applicable to data acquired on the IVIS Lumina Series III.

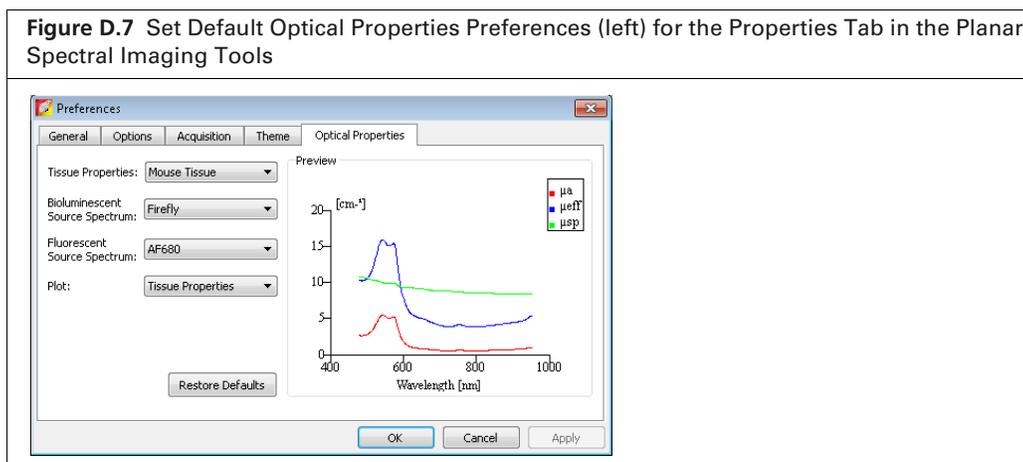


Table D.6 Tissue properties preferences

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.
Restore Defaults	Click to restore the defaults in the Optical Properties tab.

Appendix E Menu Commands, Toolbars, and Shortcuts

Figure E.1 Living Image Toolbar

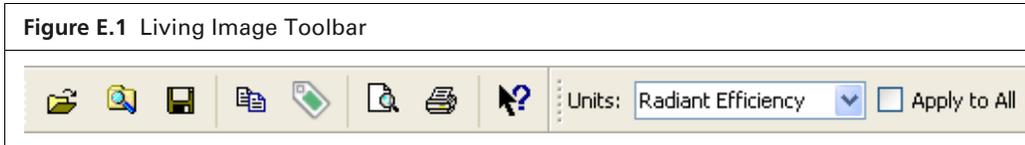


Table E.1 Menu Bar Commands and Toolbar Buttons

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 53).
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 → 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 → Atlas		Opens a dialog box that enables you to import an organ atlas (.iv, .dxf, .stl).
File → Print		Displays the Print box.
File → Print Preview		Displays the Print Preview box that shows what will be printed.
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 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 5.39 on page 85).
Edit → Preferences		Opens the Preferences box (see page 171).
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.

Table E.1 Menu Bar Commands and Toolbar Buttons (continued)

Menu Bar Command	Toolbar Button	Description
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 102).
View → ROI Measurements		Displays the ROI Measurements table.
View → Image Layout Window		Opens the Image Layout window that enables you to paste an image of the active data in the window.
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.
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.

Table E.1 Menu Bar Commands and Toolbar Buttons (continued)

Menu Bar Command	Toolbar Button	Description
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 page 54).
Window → Tile		Organizes the open image windows in a tiled arrangement (see page 54).
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).
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 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 E.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.