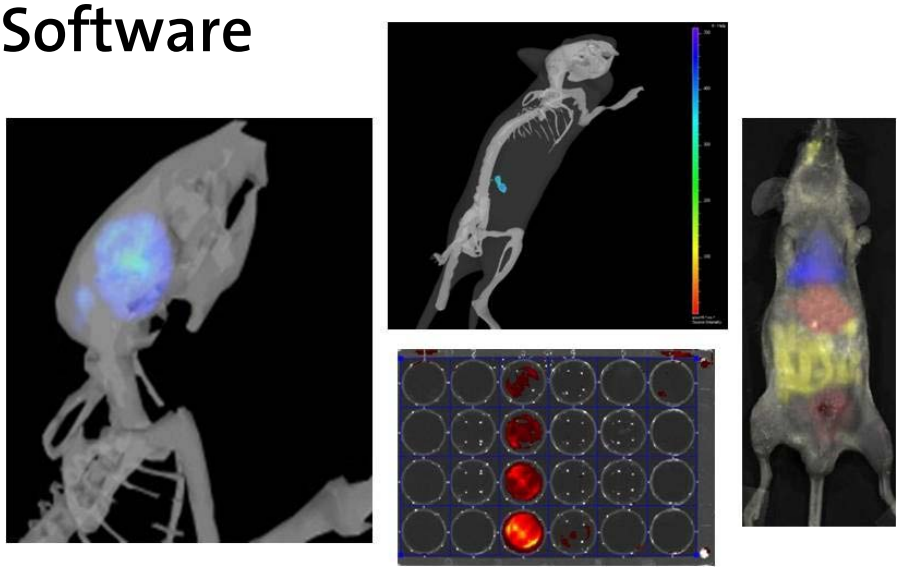
Caliper LifeSciences

Living Image® Software

User's Manual

Version 4.2



PN 133026 Rev 00

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1 Welcome

The Living Image® software controls optical image acquisition on IVIS® Imaging Systems, and provides tools for optimizing image display and analyzing images.

1.1 What's New In Living Image 4.2 Software

The following table provides a brief description of new or improved features in the Living Image software.

New or Improved Features	See Page
Image Acquisition and Analysis	
Living Image® acquisition and analysis sofware supports Windows® 7 operating system	
Batch mode for sequence acquisition	39
Expanded selection of fluorescent probes in the Imaging Wizard, including: BoneProbe680, Integrin750, RJ-2-DG-750	
Faster DLIT and FLIT algorithm speed. Dynamic voxel display during the DLIT or FLIT reconstruction process shows voxel size refinement during analysis.	
Working With Images	
Image Labels improvements: • Edit comments directly on image/sequence in the Info section • New toolbar button for quick access to the Image Label dialog box for editing image labels • New Options/Labels menu allows flexible selection of labels for display	 94 95 95
Add comments to images	95
Export well plate quantification results	190
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Voxel color scale with "transparent" as the minimum (or maximum in a reverse color table)	224
Measure voxels which have been copied from one surface and pasted to another using the 3D Source tools. Enables you to view all voxels ("original" and pasted) using one color scale.	221
Working With Volumetric Data	
Dedicated 3D Volumetric data browser with preview and playback capabilities. Enables loading of volumetric data without first loading an optical data set.	241
Save registration information for a particular volumetric and optical data set	246
Save a color-opacity map that can be applied to volumetric data	253

1.2 About This Manual

This user manual explains how to acquire optical image data on an IVIS® Imaging System and analyze images using the Living Image® software. The manual provides detailed instructions and screenshots that depict the system response.

NOTE

Sometimes the screenshots in the manual may not exactly match those displayed on your screen.

For more details on your IVIS Imaging System, please see the appropriate system manual.

Conventions Used In the Manual

Convention	Example
Menu commands are bolded.	To open image data, select **File** → **Open Dataset** on the main bar.
Toolbar button names are bolded.	To open image data, click the **Open Dataset** button  .
Numbered steps explain how to carry out a procedure.	1. To start the Living Image software, click the  icon on the desktop.
Document names are italicized.	*Living Image Software User's Guide*
Note information	**NOTE** A note presents pertinent details on a topic. or **Note:** Notes may also appear in this format.
Caution information	**CAUTION** **CAUTION!** A caution note warns you that your actions may have nonreversible consequences or may cause loss of data.
Important information	**IMPORTANT** **ALERT!** Important information advises you of actions that are essential to the correct performance of the instrument or software.

Living Image Help

There are several ways to obtain help on the software features:

- To view a tooltip about a button function, put the mouse cursor over the button.
- To view a brief description about an item in the user interface, click the  toolbar button, then click the item.
- Press F1 or select **Help** → **User Guide** on the menu bar to display the Living Image Software User's Manual (.pdf).

1.3 Contacting Caliper Technical Support

If you need technical support, please contact Caliper at:

Telephone:	1.877.522.2447 Toll Free in the United States
	1.508.435.9761
E-mail:	tech.support@caliperLS.com
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	Hopkinton, MA 01748
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2 Overview of Imaging & Image Analysis

This chapter provides a brief overview of imaging and image analysis. Images acquired on an IVIS® Imaging System are called *optical data*.

2.1 Workflow Overview

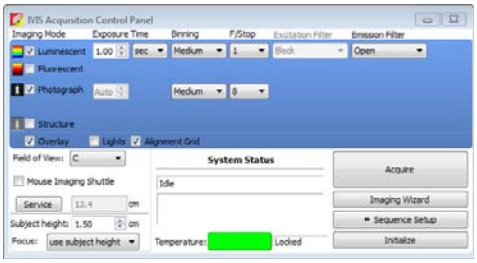
The Living Image® software provides image acquisition, viewing, and analysis functions for IVIS Imaging Systems. Figure 2.1 shows the steps to acquire an image. Figure 2.2 shows an example sequence acquisition workflow.

Figure 2.1 Steps to acquire a luminescent or fluorescent image

❶ Start the Living Image software and initialize the IVIS Imaging System.

❷ In the control panel:

- Select the imaging modes (for example, luminescent and photograph)
- Set the imaging parameters
- When you are ready to acquire the images, click **Acquire**



❸ Enter image label information (optional).

❹ The image window and Tool Palette appear when acquisition is finished.

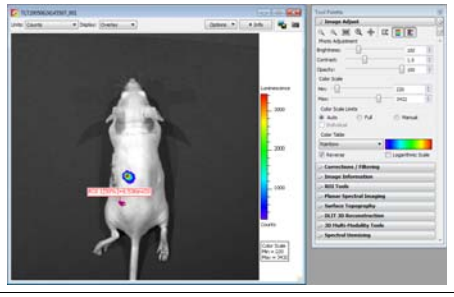
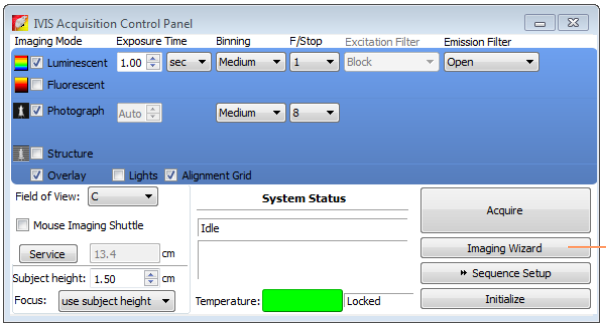


Figure 2.2 Steps to acquire an image sequence

❶ Start the Living Image software and initialize the IVIS® Imaging System

IVIS Acquisition Control Panel



Imaging Wizard

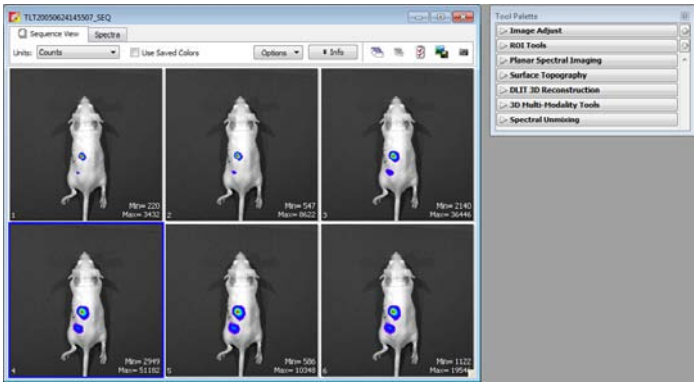
❷ Click **Imaging Wizard** in the control panel.

❸ In the Imaging Wizard, double-click Bioluminescence  or Fluorescence  and step through the wizard.

❹ In the control panel, click **Acquire Sequence**.

❺ Enter image label information (optional).

❻ The image window opens and displays the images as they are acquired. The Tool Palette is displayed.



2.2 Imaging Modes

Table 2.1 shows the imaging modes that are available on IVIS® Imaging Systems. Table 2.2 shows examples of the different types of images (*optical* data).

You can acquire:

- Single images, for example, a luminescent image and a photograph. After acquisition, the Living Image software automatically coregisters images to generate an *overlay* image.
- An image sequence - a collection of images that are grouped together in a single folder (Figure 2.3).

Table 2.1 IVIS Imaging Systems & imaging modes

Imaging Mode	IVIS Imaging System					
	Lumina II	Lumina XR	100 Series	200 Series	Spectrum	Kinetic
Photograph	✓	✓	✓	✓	✓	✓
Luminescent	✓	✓	✓	✓	✓	✓
Fluorescent	✓	✓	✓	✓	✓	✓
Structure				✓	✓	
X-ray		✓				
Kinetics						✓

NOTE

For details on your IVIS Imaging System, please see the imaging system hardware manual.

Figure 2.3 Example image sequence (overlay images: luminescent image on photograph
Double-click an image in the Sequence View to open it in a separate window.

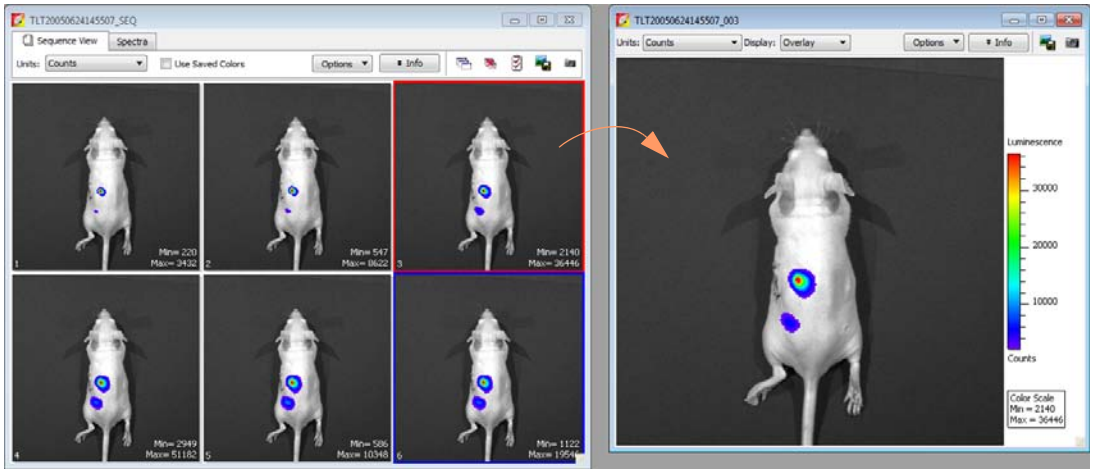


Table 2.2 Image types (optical data)

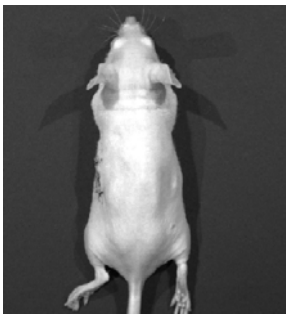
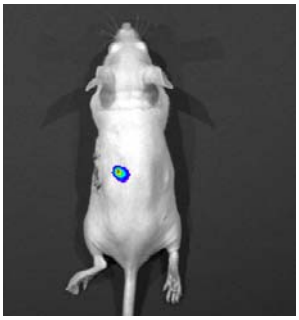
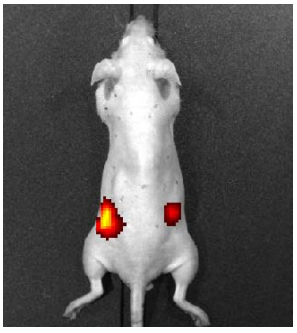
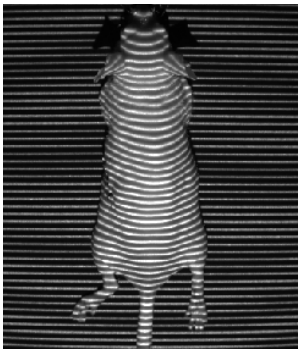
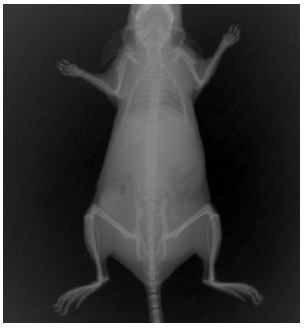
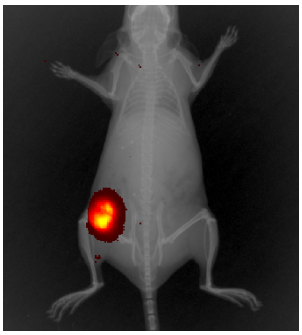
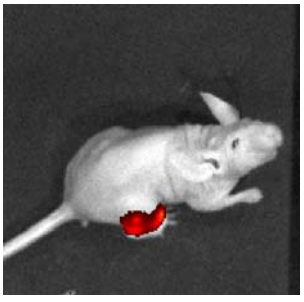
Imaging Mode	Description	Example
Photograph	A short exposure of the subject illuminated by the lights located in the ceiling of the imaging chamber. The photographic image is displayed as a grayscale image.	
Luminescent	A longer exposure of the subject taken in darkness to capture low level luminescence emission. The luminescent image is displayed in pseudocolor that represents intensity. For more details on luminescent image data, see Appendix D, page 279.	 Luminescent image  Overlay: Luminescent image on photograph
Fluorescent	An exposure of the subject illuminated by filtered light. The target fluorophore emission is captured and focused on the CCD camera. Fluorescent image data can be displayed in units of counts or photons (absolute, calibrated), or in terms of efficiency (calibrated, normalized). For more details on fluorescence image data, see Appendix F, page 291	 Fluorescent image  Overlay: Fluorescent image on photograph

Table 2.2 Image types (optical data) (continued)

Imaging Mode	Description	Example
Structure	A structured light image of parallel laser scanned across the subject. The surface topography of the subject is determined from the structured light image.	
X-ray	An exposure of the subject using the X-ray energy source on the Lumina XR. The X-ray image is displayed as a grayscale image.	 X-ray image  Overlay: Fluorescent image on X-ray image
Kinetic	A series of images captured on the IVIS Kinetic Imaging System that enables visualization of luminescent or fluorescent signals in real time.	 Play back kinetic data in real time or view a particular frame(s)

2.3 About Image Sequences

A sequence is a collection of images that are grouped together in a single folder. A sequence may include images that are acquired during the same session and are intended to be grouped together. For example, images taken at different time points or an image sequence for DLIT or FLIT 3-D tomographic analysis.

Images that were acquired during different sessions can also be grouped together to form a sequence (for more details, see page 117). For example, a time series could be constructed from images acquired on different days following an experimental treatment.

Some types of analyses are performed on an image sequence (see Table 2.3). The sequence requirements (number and type of images) depend on the type of analysis.

Table 2.4 shows the types of analyses that are possible on the different IVIS® Imaging Systems.

Table 2.3 Analyses that require an image sequence

Analysis	Description	Page
Planar spectral image	Computes the total flux and average depth of a luminescent source below the surface.	10
Display multiple fluorescent or luminescent reporters	Uses the Image Overlay function to display multiple luminescent or fluorescent images on one photographic image.	110
Subtract tissue autofluorescence using blue-shifted background filters	Uses the image math feature to subtract a background image from the primary image.	151
Spectral unmixing	Removes tissue autofluorescence from a fluorescence image.	165
DLIT	Reconstructs the brightness and 3D location of luminescent sources.	193
FLIT	Reconstructs the brightness and 3D location of fluorescent sources.	201

Table 2.4 IVIS® Imaging System capabilities

Acquire a Sequence for...	IVIS Imaging System					
	Lumina	**Lumina XR**	**100 Series**	**200 Series**	**Spectrum**	**Kinetic**
Planar spectral image analysis	optional*	optional*	optional*	yes	yes	optional*
Displaying multiple fluorescent or luminescent reporters	yes	yes	yes	yes	yes	yes
Subtracting tissue autofluorescence using blue-shifted background filters	yes	yes	yes	yes	yes	yes
Spectral unmixing	optional*	optional*	optional*	yes	yes	optional*
DLIT Analysis – 3D reconstruction of bioluminescent sources	no	no	no	yes	yes	no
FLIT Analysis – 3D reconstruction of fluorescent sources	no	no	no	no	yes	no

*Optional, requires premium filters

2.4 Image Display & Analysis

Most of the tools for adjusting image display and analyzing images are located in the Tool Palette. The Tool Palette automatically appears when acquisition is finished or when you open (*load*) image data. Its contents depend on the type of active image data. Figure 2.4 shows the tools that are available for an image or a kinetic sequence. Figure 2.5 shows the Tool Palette for an example image sequence.

Figure 2.4 Tools available for a single image (luminescent, fluorescent, X-ray) or a kinetic sequence

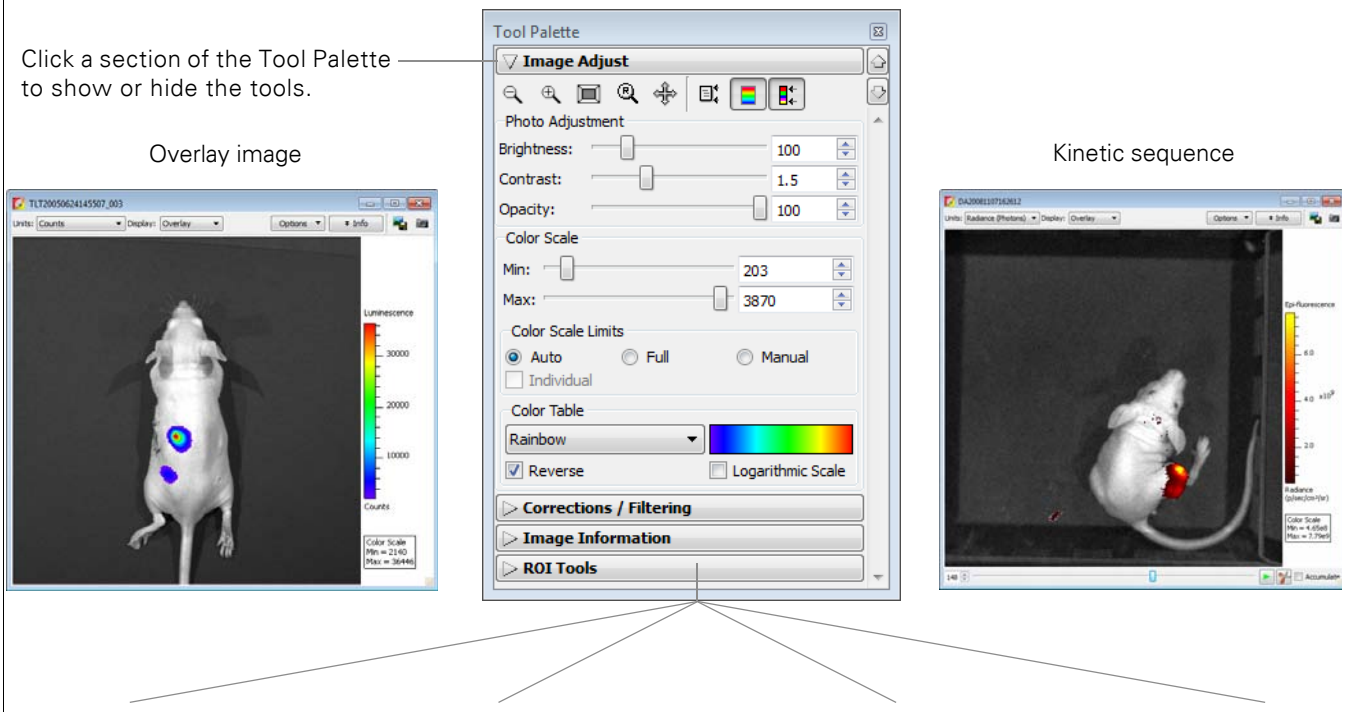


Image Adjust Tools
(page 97)

- Tune the photograph brightness, gamma (similar to contrast), or opacity
- Set the image display color scale minimum and maximum
- Select a color table for image display

Corrections/Filtering Tools
(page 99)

- Subtract dark background from the image data
- Apply flat field correction to the image data
- Specify pixel binning
- Smooth the pixel signal

Image Information Tools
(page 101)

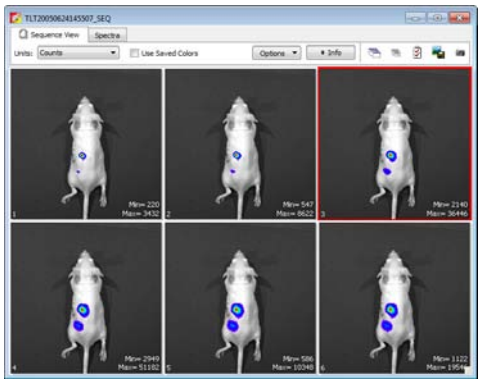
- Display x,y coordinates and intensity data at a user-selected location on the image
- Display a histogram of image pixel intensities
- Plot the intensity (y-axis) at each pixel (x-axis) along a user-specified line in the image
- 3D plot of intensity signals
- Measure distance in an image

ROI Tools
(page 119)

- Measure counts or photons in a user-specified region of interest (ROI) and compute measurement statistics (for example, average, min, max, standard deviation)
- Measure efficiency, radiant efficiency, or NTF efficiency in the ROI and compute measurement statistics (for fluorescent images only)

Figure 2.5 Tool palette for an example luminescent image sequence

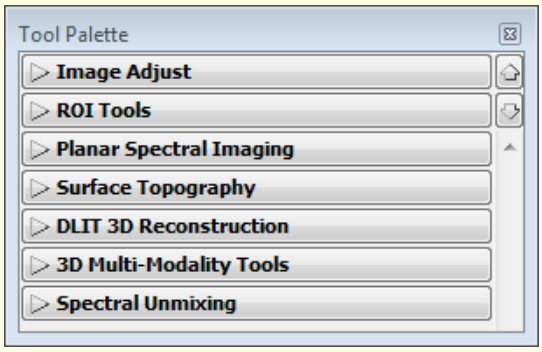
Image sequence (luminescent or fluorescent)



Tool Palette for an Image Sequence

Image Adjust Tools
(page 88)

- Tune the photograph brightness, gamma (similar to contrast), or opacity
- Set the image display color scale minimum and maximum
- Select a color table for image display



ROI Tools
(page 119)

- Measure counts or photons in a user-specified region of interest (ROI) and compute measurement statistics (for example, average, min, max, standard deviation)
- Measure efficiency in the ROI and compute measurement statistics (for fluorescent images only)

Analyses Requiring an Image Sequence

Planar Spectral Imaging
(page 157)

Determines the average depth and total photon flux of a luminescent point source in a user-specified region of interest. Analyzes a sequence of luminescent images acquired using different emission filters.

Surface Topography
(page 181)

Analyzes structured light images to reconstruct the animal surface. A surface is required for 3D reconstruction of luminescent or fluorescent sources inside a subject

DLIT Reconstruction
(page 193)

A 3-dimensional reconstruction of the subject that estimates the depth and intensity of a luminescent light-emitting source.

FLIT 3D Reconstruction
(page 201)

A 3-dimensional reconstruction of the subject that estimates the location and intensity of a fluorescent light-emitting source.

Spectral Unmixing
(page 165)

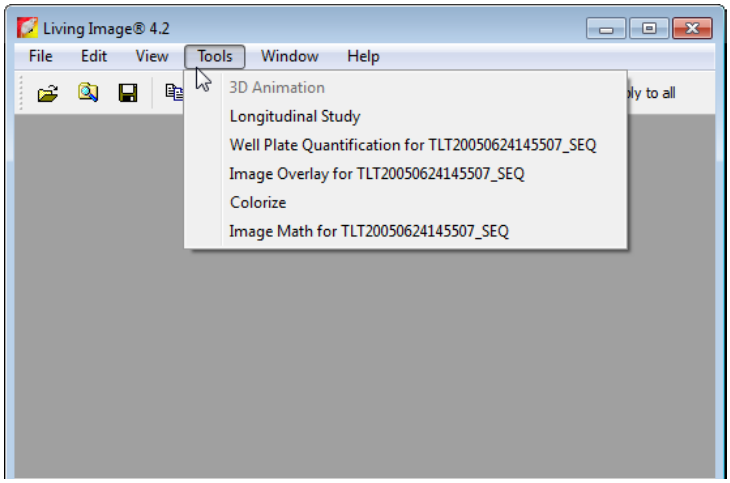
Extracts the signal of one or more fluorophores from the tissue autofluorescence. Distinguishes the spectral signatures of different fluorescent or luminescent reporters when more than one reporter is used in the same animal model.

NOTE

The 3D Multi-Modality tools require a separate license.

Additional tools are available in the menu bar after data are loaded.

Figure 2.6 Tools accessible from the menu bar



Tool	Description	See Page
3D Animation	Tools for creating an animation from an image sequence. For example, an animation can depict a rotating 3D scene. The animation can be recorded to a movie file (.mov, .mp4, or .avi)	231
Longitudinal Study	Enables you to view multiple DLIT and/or FLIT reconstruction results side-by-side; provides a convenient way to compare longitudinal study results. Voxel intensity within the entire surface or a user-selected area can be measured in all results.	213
Well Plate Quantification	Analyzes images of known serial dilutions of luminescent cells or fluorescent dye molecules and generates a quantification database. The software uses the quantification database to determine the number of cells in a DLIT source or the number of cells or dye molecules in a FLIT source.	185
Image Overlay	Displays multiple luminescent or fluorescent images on one photograph.	110
Colorize	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.	113
Transillumination Overview for <name>_SEQ	Available for FLIT image sequence. Generates an overview image for each filter pair that includes the data from all of the transillumination locations. The overview image can be analyzed using the tools in the Tool Palette.	109
Image Math	A method for mathematically combining two images (add, subtract, multiply, or divide). Use image math to remove autofluorescence from a fluorescent image.	151

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3 Getting Started

This chapter explains how to start the Living Image® software and initialize the IVIS® Imaging System. After it is initialized, the imaging system is ready to acquire images.

3.1 Starting the Living Image Software

The Living Image software on the PC workstation that controls the IVIS Imaging System includes both the acquisition and analysis features. The Living Image software on other workstations includes only the analysis features. For information on installing the software, see the Installation Guide included on the Living Image CD ROM. By default, the software is installed at:

PC (32-bit windows): C:Program Files:Caliper Life Sciences: Living Image

PC (64-bit windows): C:Program Files (x86):Caliper Life Sciences: Living Image

Macintosh: Applications:Caliper Life Sciences: Living Image

NOTE

All components of the IVIS® Imaging System 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.

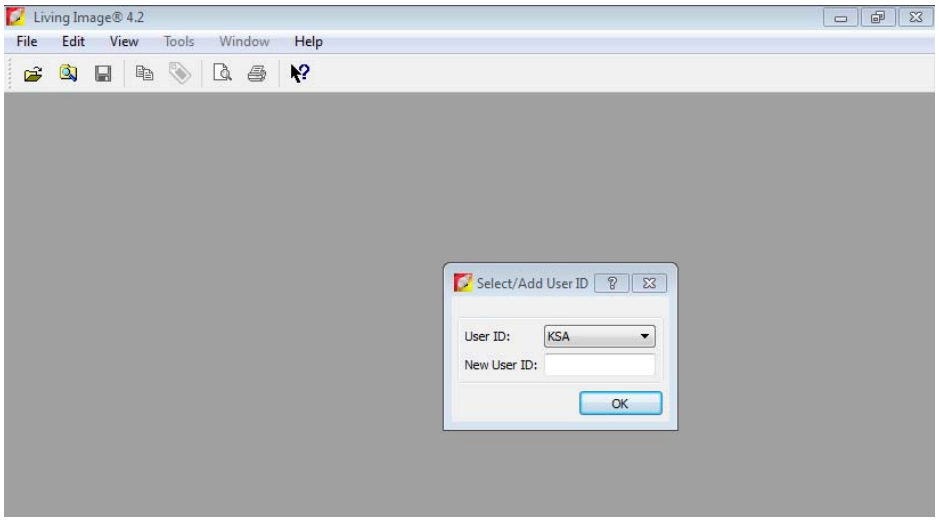
To start the software:

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

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

   — The main window appears (Figure 3.1).

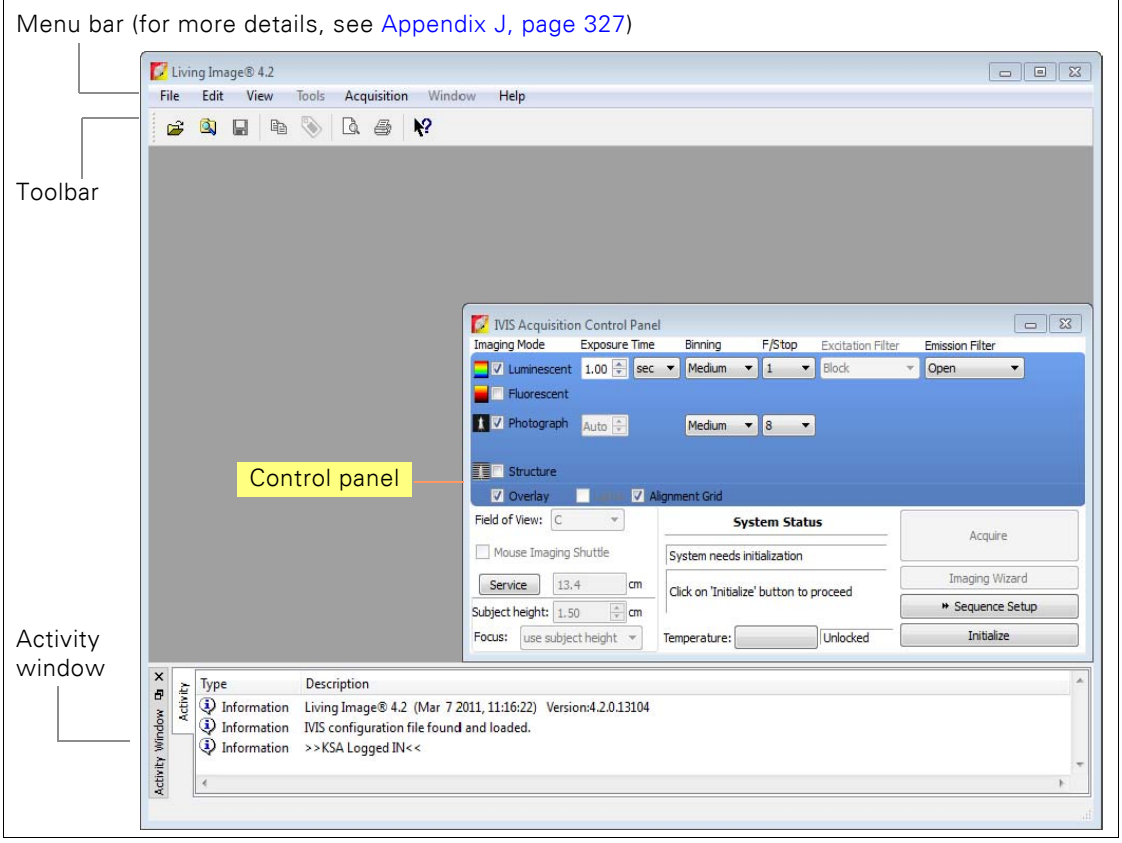
Figure 3.1 Living Image® main window



2. Select a user ID from the drop-down list or enter a new User ID (up to three letters), and click **OK**.

   — The control panel appears if the workstation controls the IVIS® Imaging System (Figure 3.2). For more details on the control panel, see Appendix A, page 259.

Figure 3.2 Living Image main window and IVIS Acquisition Control Panel



NOTE

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

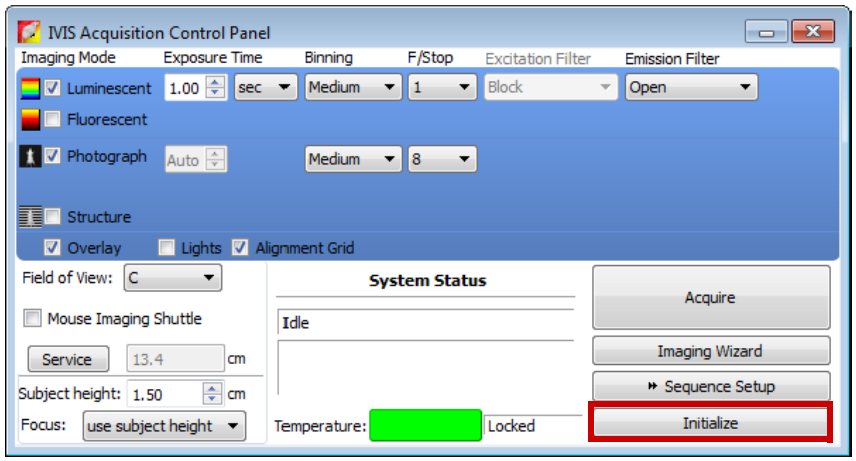
3.2 Initializing the IVIS Imaging System

The imaging system must be initialized each time the Living Image® software is started, or if the power has been cycled to the imaging chamber or the camera controller (a component of some IVIS® Imaging Systems). The initialization procedure 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. For further details on instrument operation, see your IVIS Imaging System hardware manual.

To initialize the IVIS Imaging System:

3. Start the Living Image software (double-click the  icon on the desktop).
4. In the control panel that appears, click **Initialize**.
   — You will hear the motors move.

Figure 3.3 IVIS® Acquisition Control Panel



NOTE

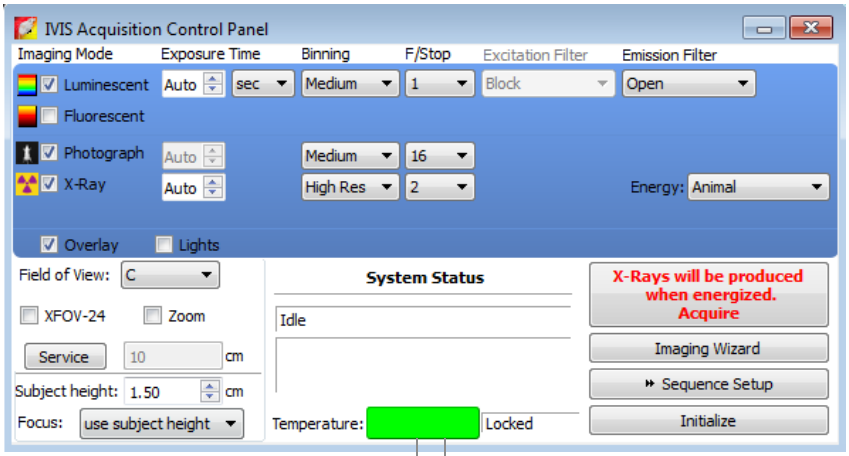
The control panel is only available on the workstation that controls the imaging system. The items available in the control panel depend on the particular IVIS Imaging System and the imaging mode selected (luminescent or fluorescent, Image Setup or Sequence Setup mode).

3.3 Checking the System Temperature

The IVIS acquisition control panel indicates the temperature status of the charge coupled device (CCD) camera (Figure 3.4). After the system is initialized, the temperature box turns green when the temperature is locked at the demand temperature (-90 C or -105 C for IVIS Systems cooled by a Cryotiger® unit), indicating the instrument is ready for operation and image acquisition.

The demand temperature for the CCD camera is fixed. Electronic feedback control maintains the CCD camera temperature to within a few degrees of the demand temperature. The default temperature of the stage in the imaging chamber is 37 C, but may be set to a temperature from 20-40 C.

Figure 3.4 IVIS® Acquisition Control Panel, IVIS Lumina XR



Click the temperature box to view the demand and measured temperatures of the CCD camera and stage

Temperature box color indicates:

- System not initialized
- System is initialized, but CCD camera temperature is out of range and not ready for imaging
- System is initialized and CCD camera is at or within acceptable range of the demand temperature and locked. The system is ready for imaging.

NOTE

The items in the control panel depend on the particular IVIS Imaging System and the imaging mode selected (luminescent or fluorescent, Image Setup or Sequence Setup mode). For more details on the control panel, see Appendix A, page 259.

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

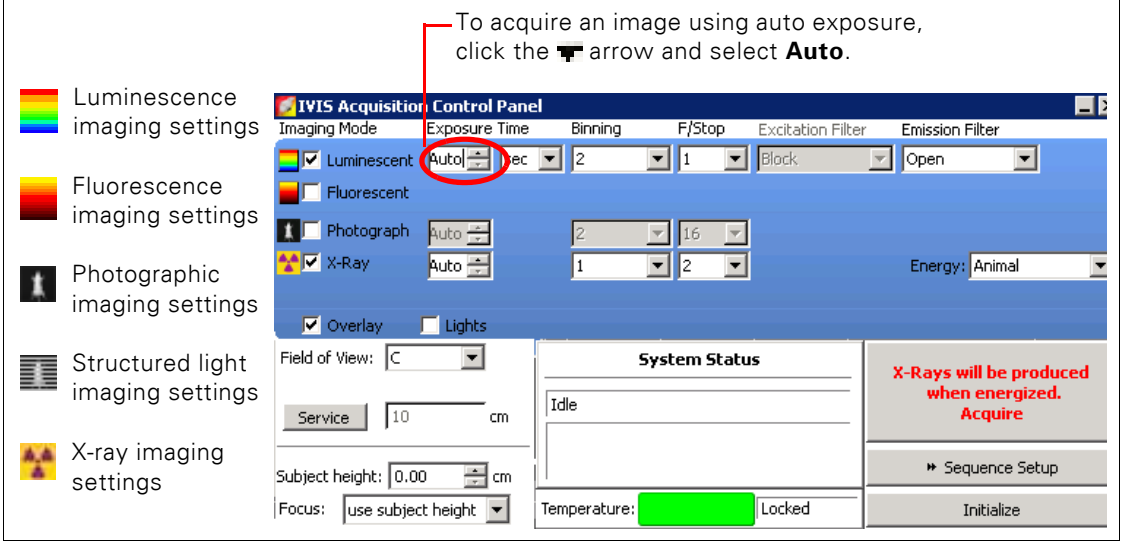
3.4 About the IVIS Acquisition Control Panel & Auto Exposure Feature

The control panel (Figure 3.5) provides the image acquisition functions. For details on the imaging parameters in the control panel, see Appendix A, page 259.

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 3.5), 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. For more details, see page 269.

Figure 3.5 IVIS Acquisition Control Panel



NOTE

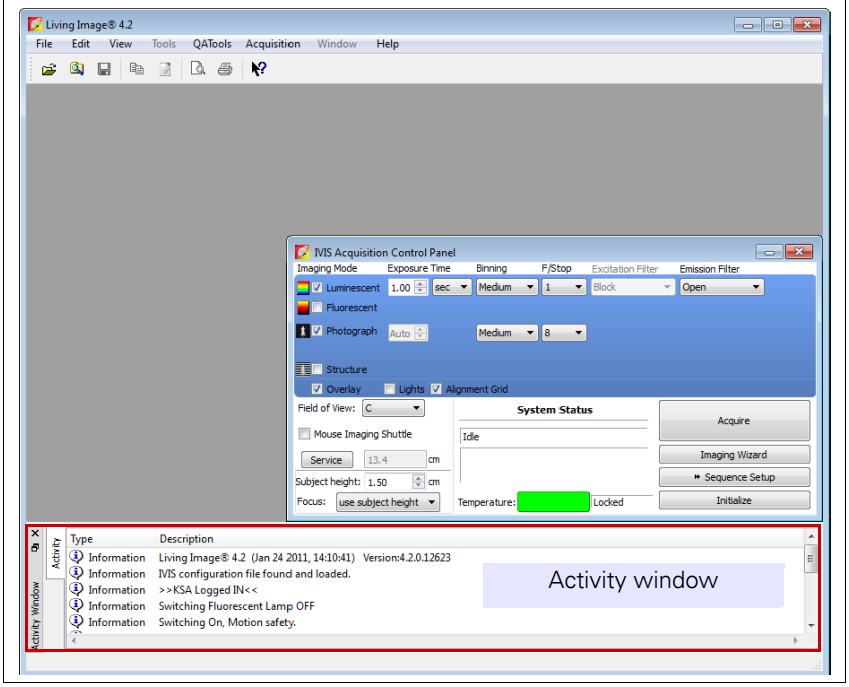
The options available in the control panel depend on the selected imaging mode, the imaging system, and the installed filter wheel or lens option.

3.5 Tracking System and User Activity

Activity Window

The Activity window shows the imaging system activities. The software creates and saves a log of the system activities related to data acquisition, for example, the type and number of acquired images, fluorescent lamp usage, X-ray tube accumulated usage, and kinetic camera usage. This information may be useful for Caliper 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.

Figure 3.6 Activity window



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>_2009.csv) located at C:Program Files\Caliper Life Sciences\Living Image\Usage).

Software Help

There are several ways to obtain help on the software features:

- To view a tooltip about a button function, put the mouse cursor over the button.
- To view a brief description about an item in the user interface, click the toolbar button, then click the item.

Press F1 or select **Help** → **User Guide** on the menu bar to display the Living Image Software User's Manual (.pdf).

4 Optical Imaging

Optical imaging measures the light emitted by bioluminescent or fluorescent light-producing reporters such as luciferase or fluorescent proteins.

The IVIS® Imaging System is ready to acquire optical images after the system temperature is initialized and the CCD camera reaches operating (*demand*) temperature (the system is then *locked*). This chapter explains how to acquire optical image data—a bioluminescent or fluorescent image or an image sequence.

4.1 Bioluminescent Optical Imaging

This section explains how to acquire a single bioluminescent optical image. For information on acquiring an image sequence, see *Acquire a Sequence Using the Imaging Wizard*, page 33.

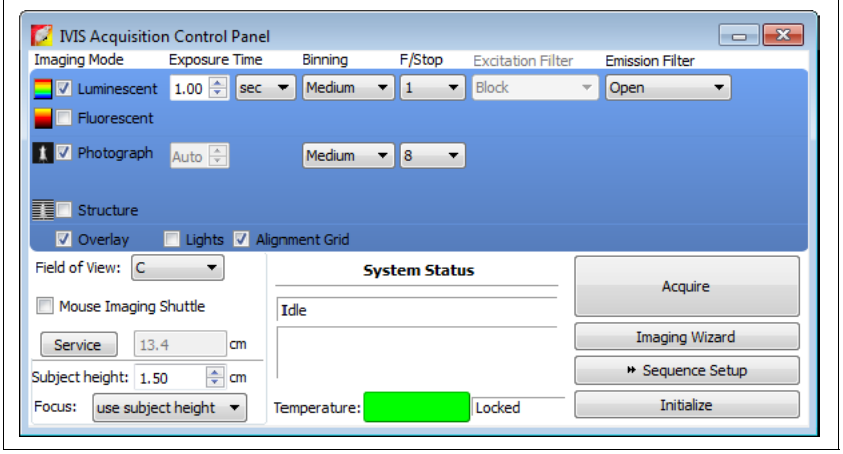
NOTE

Before setting the imaging parameters, the IVIS® Imaging System should be intialized and the temperature locked. For more details, see Chapter 3, page 15.

1. In the control panel, put a check mark next to **Luminescent** and select **Auto** exposure (click the arrow).

   When you select Auto exposure, the software automatically determines the binning and F/Stop settings. Alternatively, you can manually set the exposure, binning, and F/Stop. For more details on these control panel settings, see page 259.

Figure 4.1 Acquisition Control Panel



2. Put a check mark next to **Photograph** and select **Auto** exposure (click the arrow).
3. Make a selection from the Field of View drop-down list. For more details on the field of view, see page 261.
4. Set the proper **Focus** by choosing one of the methods below.

   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 will move down so that the top of the animal is at z = 0. You can either enter the height of the animal using the "use subject height" option or let the software determine the animal height by choosing the"scan mid range" option.

   - Select **use subject height** and use the arrows or the keyboard arrows to specify a subject height (cm).
   - Select **manual focus**. For more details on manual focusing see page 264.
   - Select **scan mid range** (available on the IVIS 200 or Spectrum Imaging System only). The imaging system determines the subject height using a laser scan. This value is automatically entered in the subject height box and is used to set the focal length.
5. To acquire an overlay image (coregistered images) for display after acquisition, put a check mark next to **Overlay**.

NOTE

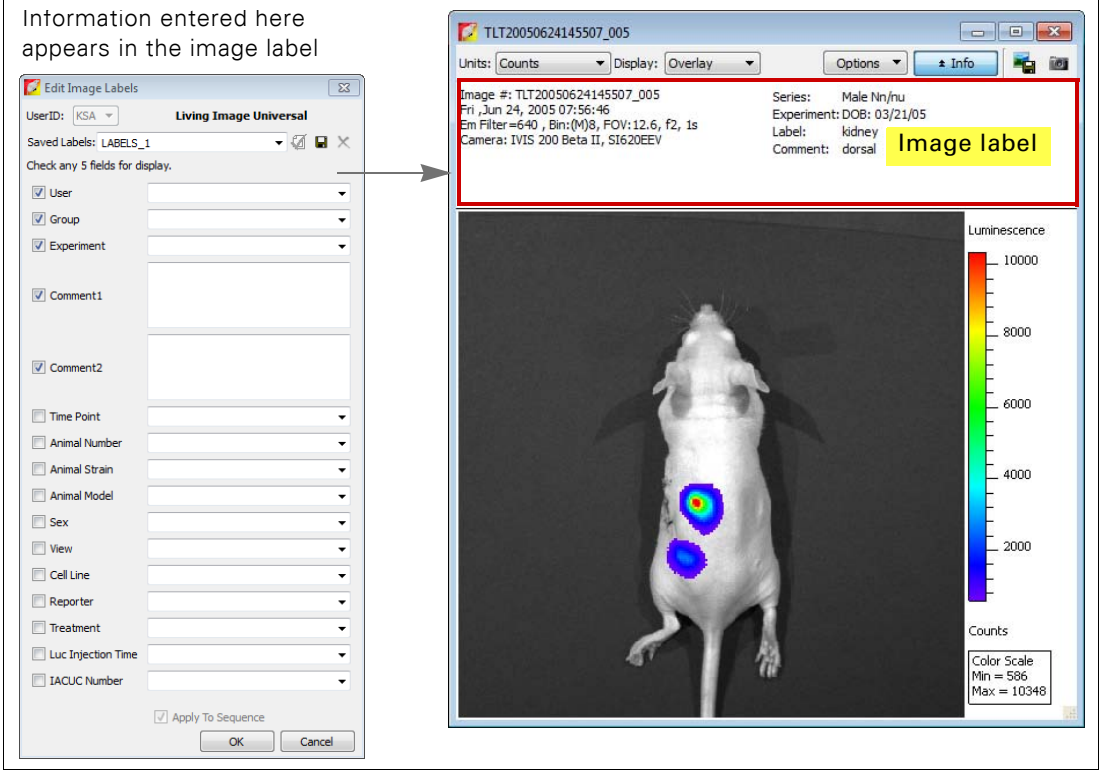
To view the subject(s) inside the chamber before image acquisition, take a photograph. Uncheck the Luminescent option, choose the Photograph and Auto options, and click **Acquire**.

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. Click this button to set up sequence acquisition. (For details on sequence setup, see page 33.)

Figure 4.2 Enter image label information (optional)



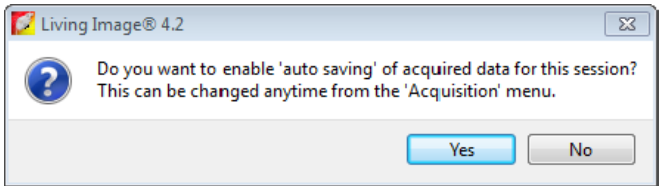
7. In the Edit Image Labels box that appears (Figure 4.2), enter information about the image and click **OK**.

NOTE

You can enter image label information at any time during or after acquisition. If you do not want to enter image information, click **Cancel**.

8. If this is the first image of the session, you are prompted to enable the autosave function.

Figure 4.3 Autosave prompt



9. To enable autosave, click **Yes** in the prompt and choose a folder in the dialog box that appears.

   If autosave is enabled, all images acquired during the session are automatically saved to the user-selected folder. You can choose a different folder at any time (select **Acquisition** → **Auto-Save** on the menu bar).

   Image acquisition proceeds. During acquisition, the control panel **Acquire** button becomes a **Stop** button. To cancel the acquisition, click **Stop** in the control panel. The image window appears when acquisition is completed (Figure 4.4).

Figure 4.4 Overlay (luminescent image on photograph) in the image window

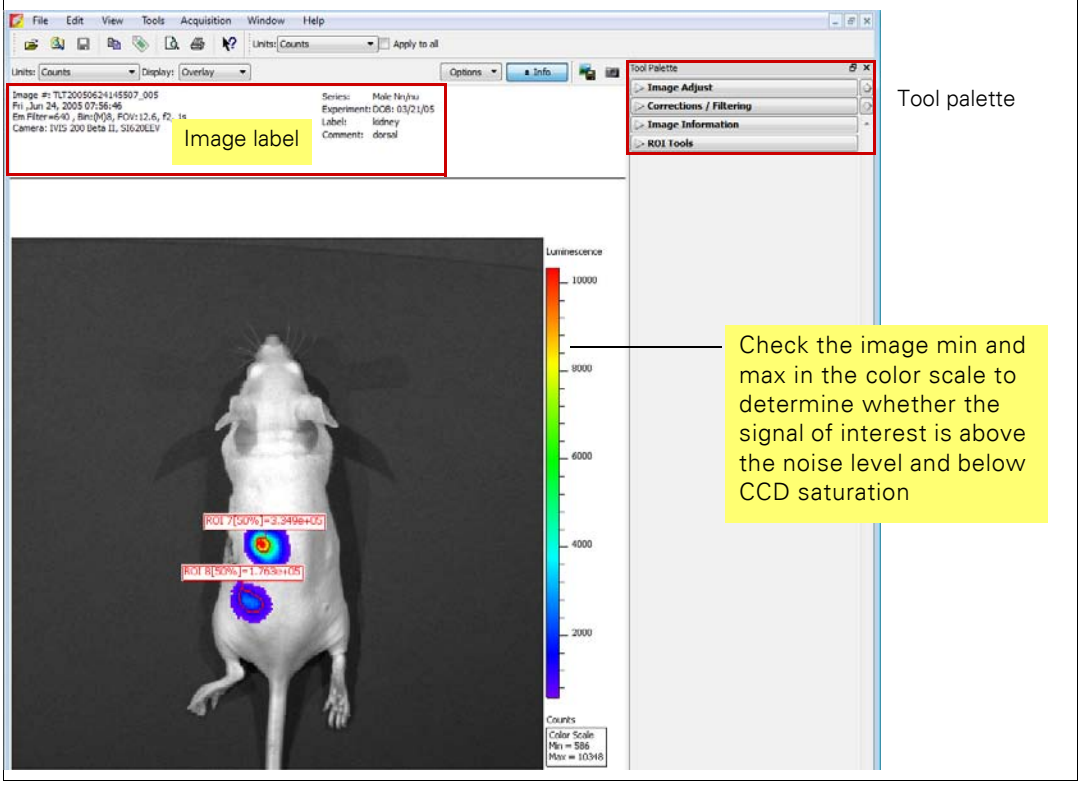
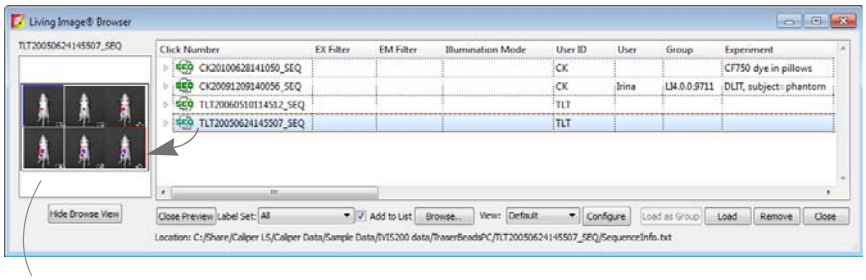


Table 4.1 Image window

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. For more details on measurement units, see page 281.
Display	A list of image types available for display, for example, overlay. For more details on the different types of image displays, see Table 2.2, page 8. **Note:** If the acquisition included more than two imaging modes (for example, luminescent, x-ray, and photograph), additional drop-down lists appear so you can conveniently choose any two images to overlay. Display: Overlay Luminescent on Photograph
Info	Click to display or hide the image label. The image label includes information you enter in the Edit Image Labels dialog box (Figure 4.2) 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 4.1 Image window (continued)

Item	Description
	Creates a preview picture (*snapshot*) of the image or thumbnails that the Living Image Browser displays when the data are selected in the browser. For more details on the browser, see page 83.  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 Optical Imaging With Epi-Illumination

This section explains how to acquire a single fluorescent optical image. Epi-illumination provides an excitation light source located above the stage. For more details about fluorescent imaging, see page 291. For information on acquiring an image sequence, see *Acquire a Sequence Using the Imaging Wizard*, page 33.

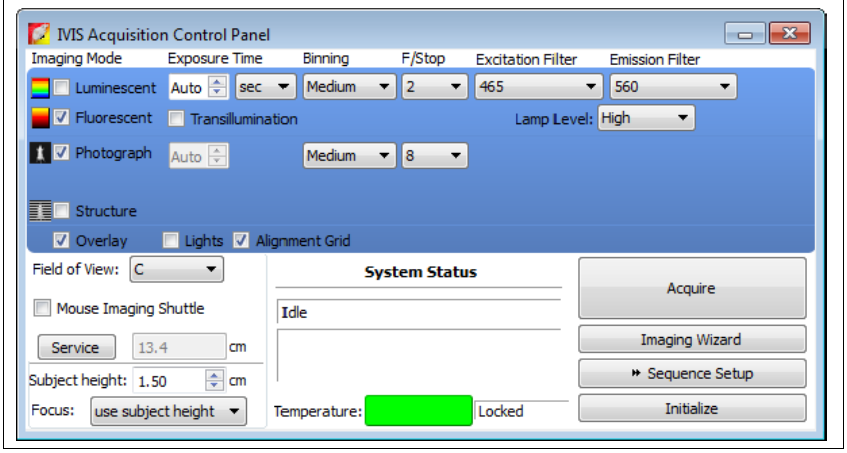
NOTE

Before setting the imaging parameters, the IVIS® Imaging System should be intialized and the temperature locked. For more details, see Chapter 3, page 15.

1. In the control panel, put a check mark next to **Fluorescent** and select **Auto** exposure (click the arrow).

   When you select Auto exposure, the software automatically determines the binning and F/Stop settings. Alternatively, you can manually set the exposure, binning, and F/Stop. For more details on these control panel settings, see page 259.

Figure 4.5 Acquisition control panel



2. Select an excitation and emission filter from the drop-down lists. For more information about the standard filter sets, see Table F.1, page 296.
3. Put a check mark next to **Photograph**.
4. Make a selection from the Field of View drop-down list. For more details on the field of view, see page 261.
5. Set the proper **Focus** by choosing one of the methods below.

   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 will move down so that the top of the animal is at z = 0. You can either enter the height of the animal using the "use subject height" option or let the software determine the animal height by choosing the"scan mid range" option.

   - Select **use subject height** and use the  arrows or the keyboard arrows to specify a subject height (cm).
   - Select **manual focus**. For more details on manual focusing see page 264.
   - Select **scan mid range** (available on the IVIS 200 or Spectrum Imaging System only). The imaging system determines the subject height using a laser scan. This value is automatically entered in the subject height box and is used to set the focal length.
6. To acquire an overlay image (coregistered images) for display after acquisition, put a check mark next to **Overlay**.

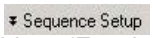
NOTE

To view the subject(s) inside the chamber before image acquisition, take a photograph. Uncheck the Fluorescent option, choose the Photograph and Auto options, and click **Acquire**.

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

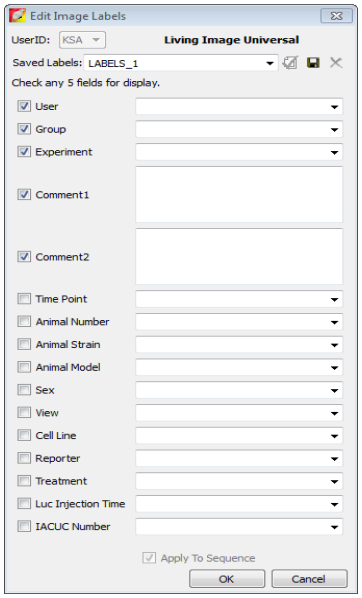
NOTE

If necessary, click 

in the control panel to operate in single image mode.In single image mode, the 

button appears in the control panel. Click this button to set up sequence acquisition. (For details on sequence setup, see page 33.)

Figure 4.6 Edit Image Labels box



The information entered here appears in the image label (Figure 4.8, page 28).

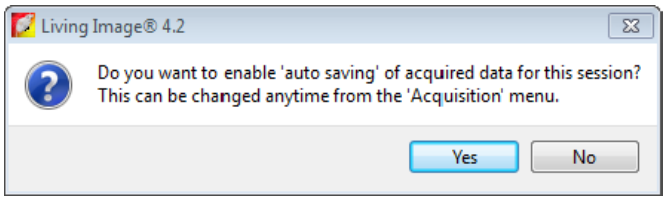
8. In the Edit Image Labels box that appears, enter information about the image and click **OK**.

NOTE

You can enter image label information at any time during or after acquisition. If you do not want to enter image information, click **Cancel**.

9. If this is the first image of the session, you are prompted to enable the autosave function.

Figure 4.7 Autosave prompt

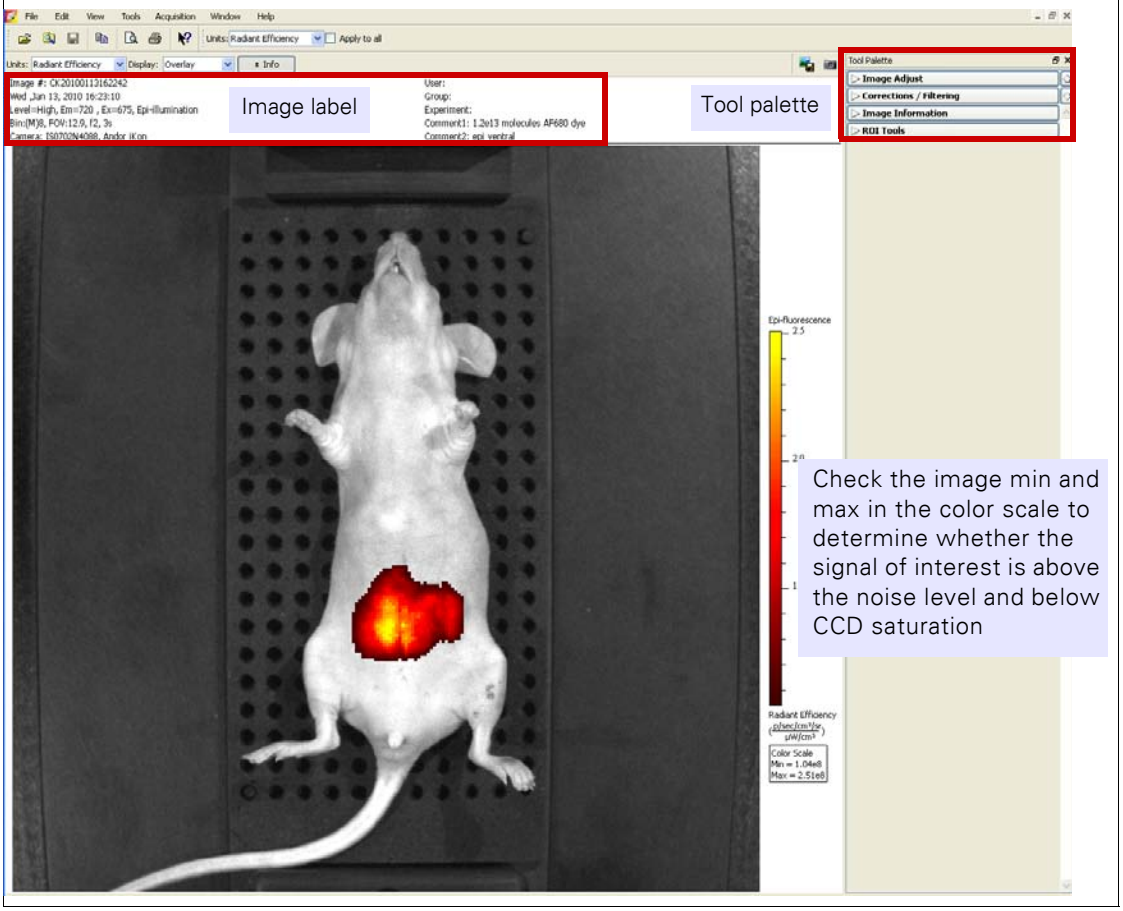


10. To enable autosave, click **Yes** in the prompt and choose a folder in the dialog box that appears.

    If autosave is enabled, all images acquired during the session are automatically saved to a user-selected folder. You can choose a different folder at any time (select **Acquisition** → **Auto-Save** on the menu bar).

    Image acquisition proceeds. During acquisition, the control panel **Acquire** button becomes a **Stop** button. To cancel the acquisition, click **Stop** in the control panel. The image window appears when acquisition is completed (Figure 4.8).

Figure 4.8 Overlay (fluorescent image on photograph) in the image window
For more details on the items in the image window, see Table 4.1, page 24.



4.3 Fluorescent Optical Imaging With Transillumination

This section explains how to acquire a fluorescent optical image with transillumination. Transillumination provides an excitation light source located below the stage. FLIT reconstruction of fluorescent sources analyzes a transilluminated image sequence. For more information about fluorescent imaging, see page 291.

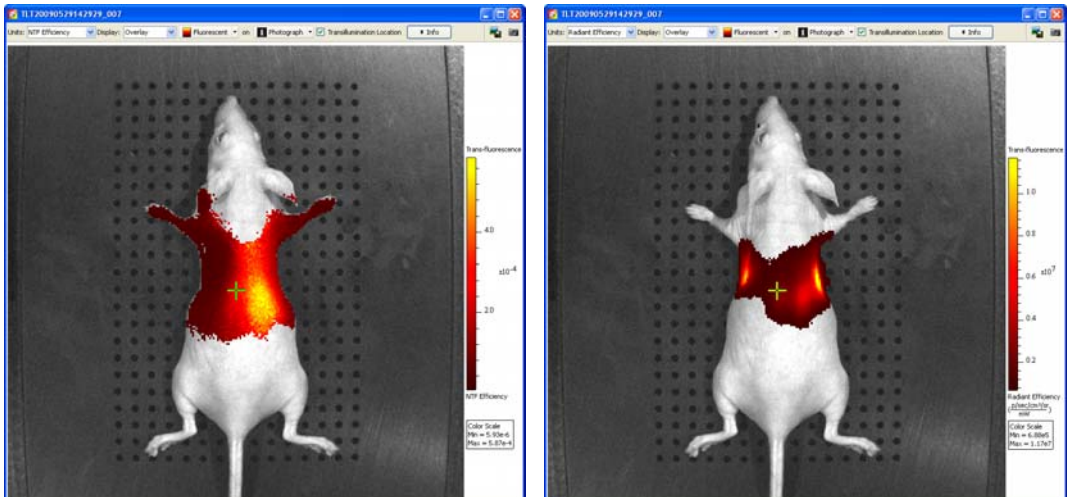
If the fluorescent source is deep relative to the imaged side of the animal, acquisition with transillumination is recommended. By default, acquisition with transillumination includes an Normalized Transmission Fluorescence (NTF) Efficiency image in which the fluorescent emission image is normalized by the transmission image measured with the same emission filter and open excitation filter (Figure 4.9).

NOTE

Transillumination is only available on the IVIS® Spectrum Imaging System.

Figure 4.9 Fluorescent images acquired with transillumination
In this example, the NTF Efficiency image highlights the presence of fluorescence in the animal, while the Radiant Efficiency image shows signal ambiguous with autofluorescence

NTF Efficiency Image | Radiant Efficiency Image



To acquire a fluorescent image with transillumination:

NOTE

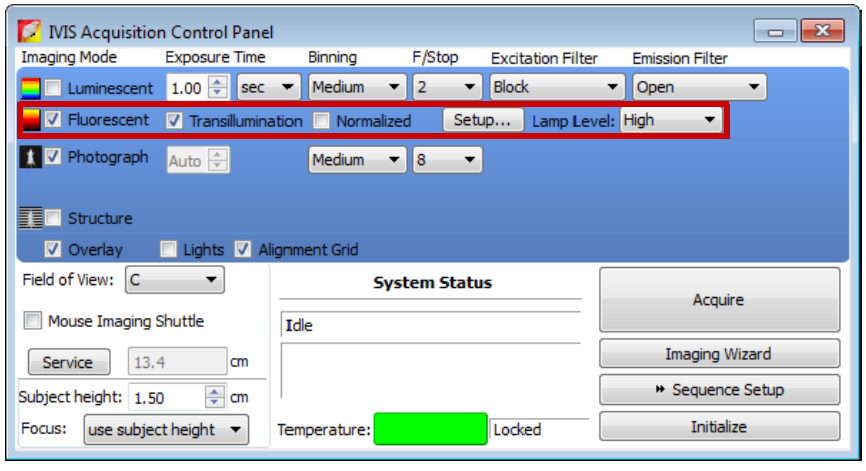
Before setting the imaging parameters, the IVIS® Imaging System should be intialized and the temperature locked. For more details, see Chapter 3, page 15.

1. In the control panel, put a check mark next to **Fluorescent** and **Transillumination** (Figure 4.10).

NOTE

The Normalization option is selected by default so that NTF Efficiency images can be produced.

Figure 4.10 Acquisition Control Panel



2. Select an excitation and emission filter from the drop-down lists. For more information about the standard filter sets, see Table F.1, page 296.
3. Click **Setup**.

   If you are prompted to acquire a subject photograph, click **Yes**.
4. In the Transillumination Setup box that appears (Figure 4.11), choose the location for transillumination and image acquisition (click a ■ square).

Figure 4.11 Opening the Transillumination Setup dialog box

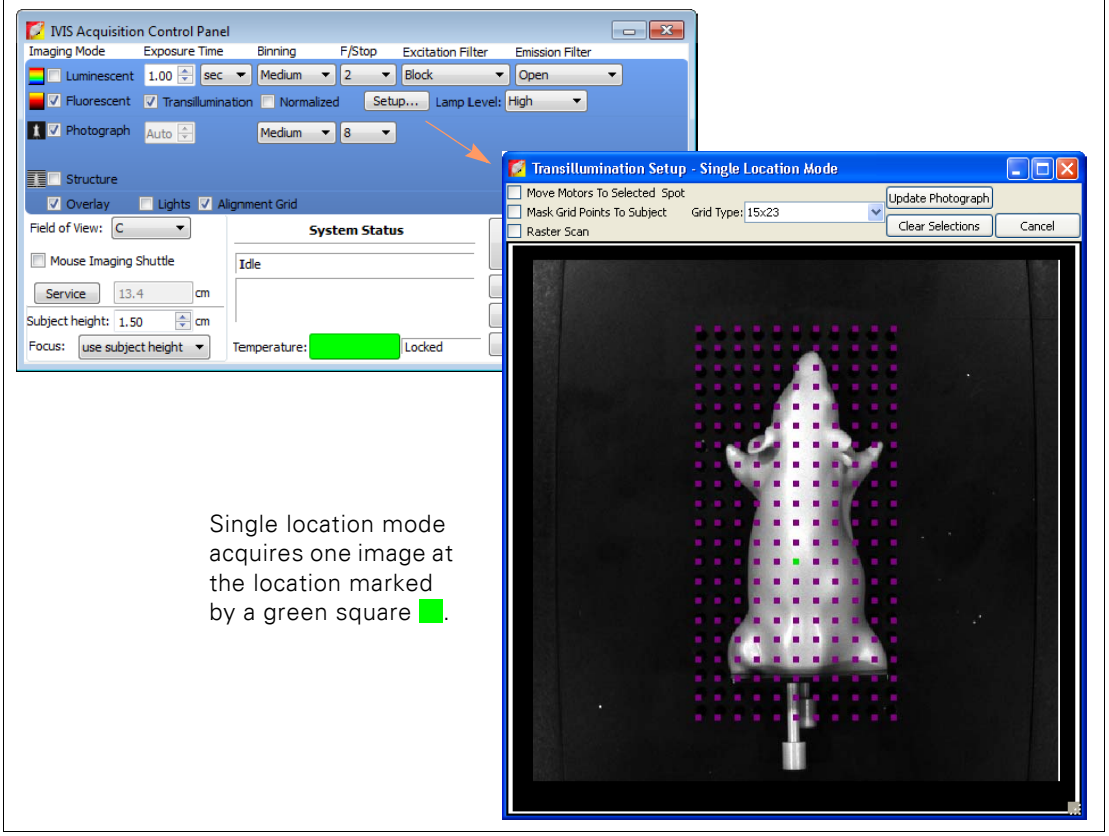


Table 4.2 Transillumination Setup box

Item	Description
Move Motors to Selected Spot	Transillumination motors will move the excitation light source to the grid location selected in the Transillumination Setup dialog box.
Mask Grid points To Subject	When setting up a transillumination sequence, choose this option to automatically select only the grid locations within the subject boundaries. Grid locations outside the subject are masked out. The mask prevents the transillumination excitation source from selecting an uncovered hole. Projecting light through an open hole would saturate the camera.

Table 4.2 Transillumination Setup box (continued)

Item	Description
Raster Scan	If this option is not selected, the software generates one image per transillumination location per filter pair. For example, a sequence setup that includes 20 locations using two filters will generate 20 images. If the raster scan option is selected, the software takes all of the images from the transillumination locations and adds them together into one image. The raster scan option may be helpful when trying to determine the optimal excitation and emission filters for a particular fluorescent probe.
Grid Type	Select a grid type from the drop-down list: 15x23, 11x23, 5x10, or 8x12 well plate, Xenogen Sparse Mask, 6x8x1cm.
Update Photograph	Click to acquire a new photographic image. If the chamber door is opened during transillumination setup, you are prompted to acquire a new photograph.
Clear Selections	Clears selected/ highlighted transillumination locations on the grid.

5. Confirm that the Lamp Level is set to High.

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

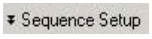
6. Make a selection from the Field of View drop-down list. For more details on the field of view, see page 261.

7. Set the Focus:
   - Select **use subject height** and use the  arrows or the keyboard arrows to specify a subject height (cm).
   - Select **manual focus**. For more details on manual focusing see page 264.
   - Select **scan mid range** (available on the IVIS 200 or Spectrum Imaging System only). The imaging system determines the subject height using a laser scan. This value is automatically entered in the subject height box and is used to set the focal length.

8. If necessary, click 

in the control panel to operate in single image mode.

NOTE

In single image mode, the 

button appears in the control panel. Click this button to set up sequence acquisition.

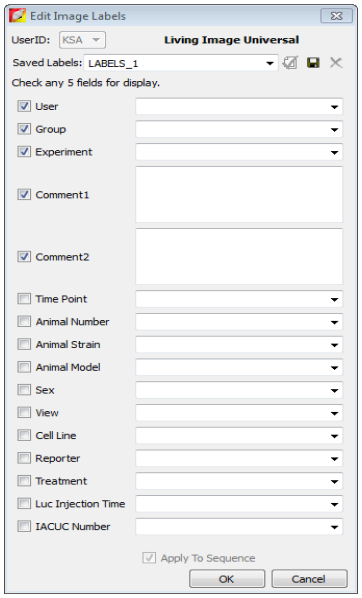
9. To acquire an overlay image (coregistered images) for display after acquisition, put a check mark next to **Overlay**.

NOTE

To view the subject(s) inside the chamber before image acquisition, take a photograph. Uncheck the Fluorescent option, choose the Photograph option, and click **Acquire**.

10. When you are ready to capture the image, click **Acquire**.

Figure 4.12 Edit Image Labels box



The information entered here appears in the image label (Figure 4.8, page 28).

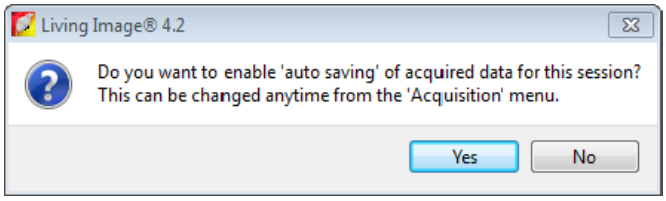
11. In the Edit Image Labels box, enter information about the image and click **OK**.

NOTE

You can enter image label information at any time during or after acquisition. If you do not want to enter image information, click **Cancel**.

12. If this is the first image of the session, you are prompted to enable the autosave function.

Figure 4.13 Autosave prompt



13. To enable autosave, click **Yes** in the prompt and choose a folder in the dialog box that appears.

    If autosave is enabled, all images acquired during the session are automatically saved to a user-selected folder. You can choose a different folder at any time (select **Acquisition** → **Auto-Save** on the menu bar).

    Image acquisition proceeds. During acquisition, the control panel **Acquire** button becomes a **Stop** button. To cancel the acquisition, click **Stop** in the control panel. The image window and Tool Palette appear when acquisition is completed (Figure 4.8, page 28).

4.4 Acquire a Sequence Using the Imaging Wizard

This section explains how to acquire a sequence of multiple images. To acquire an image sequence, first specify the acquisition parameters for each image in the sequence table (Figure 4.15). The Imaging Wizard provides a convenient way to do this for some imaging applications (Table 4.3). The wizard guides you through a series of steps, prompting you for the information that the software needs to set up the sequence. A sequence can also be set up manually (for more details, see page 41).

Figure 4.14 Imaging Wizard

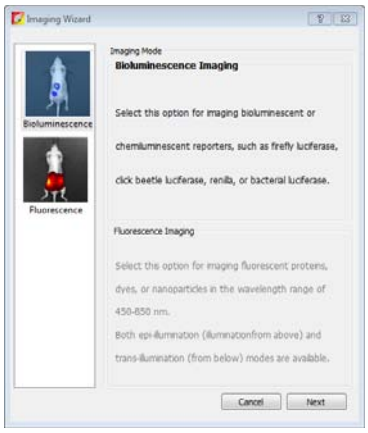


Table 4.3 Imaging Wizard options

Bioluminescence	Description	Page
Open Filter	Acquires an image at maximum sensitivity.	
Planar Spectral	Analyze the sequence to compute the average depth and total photon flux of a luminescent point source in a region of interest (ROI).	157
Spectral Unmixing	Analyze the sequence to determine spectral signature of different reporters in the same image and calculate the contribution of each reporter on each pixel in the image.	165
DLIT	Apply the DLIT algorithm to the sequence to reconstruct the 3D surface topography of the subject and the position, geometry, and strength of the luminescent sources.	193
Fluorescence	**Description**	**Page**
Filter Pair	Choose this option to acquire measurements of one or more fluorescent probes.	
Spectral Unmixing/ Filter Scan	Analyze a sequence to extract the signal of one or more fluorophores from the tissue autofluorescence. Helps you determine the optimum excitation and emission filter for a probe.	165
FLIT	Apply the FLIT™ algorithm to the sequence to reconstruct the 3D surface topography of the subject and the position, geometry, and strength of the fluorescent sources.	201

NOTE

For details on acquiring a sequence on the Lumina XR that includes X-ray images, see Chapter 5, page 49.

Sequence Setup Using the Imaging Wizard

1. Click **Imaging Wizard** in the control panel (Figure 4.15).

NOTE

If necessary, click **Restart** to show the first page of the wizard.

2. In the wizard that appears:
   a. Choose Bioluminescence or Fluorescence imaging.
   b. In the next wizard screen, choose the type of image sequence that you want to acquire (see Table 4.3, page 33).
   c. Step through the rest of the wizard.

      Each page of the wizard guides you with step-by-step instructions and descriptions. When you complete the wizard, the sequence information appears in the sequence table (Figure 4.15).
3. To clear the sequence, click the **Remove** button [Remove] and select **All**.

Figure 4.15 Opening the sequence table and Imaging Wizard

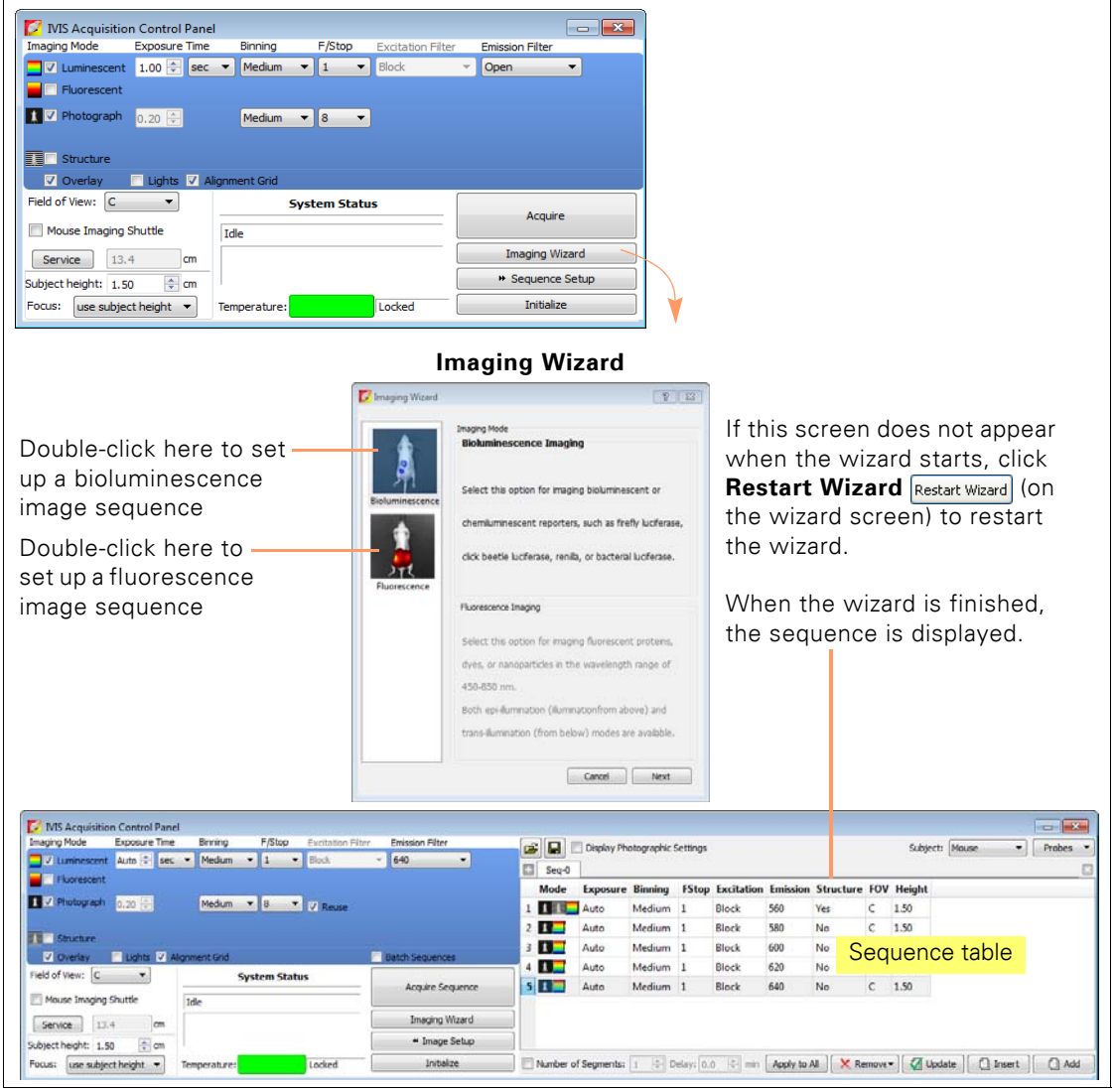
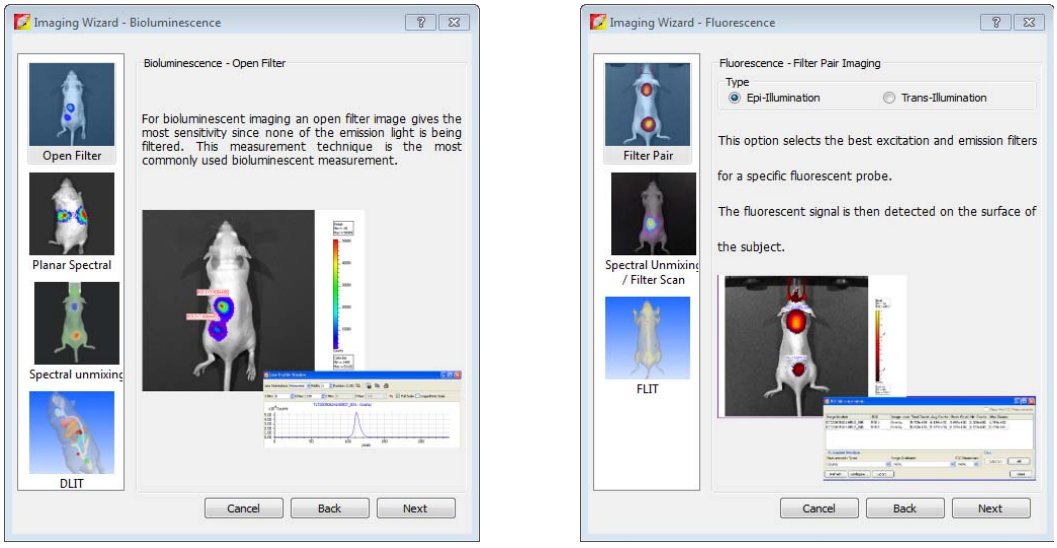


Figure 4.16 Choose the type of image sequence



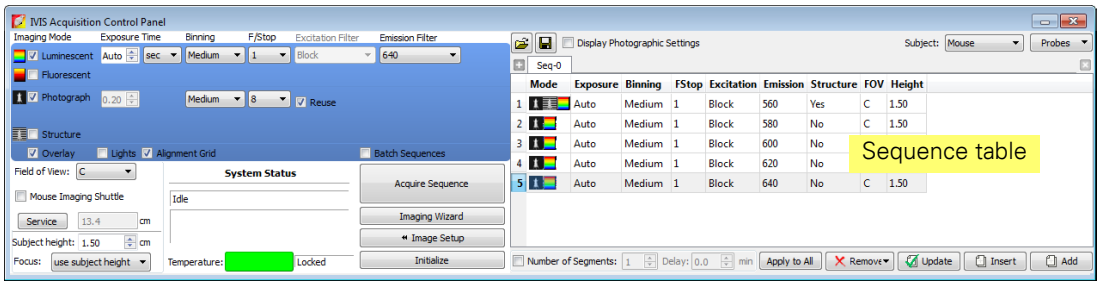
Imaging Wizard - Bioluminescence options

Imaging Wizard - Fluorescence options

NOTE

The imaging options available in the Imaging Wizard depend on the IVIS Imaging System and the installed filter set.

Figure 4.17 Control panel and sequence table during acquisition
Each row in the sequence table specifies the acquisition parameters for one image in the sequence. For more details on the sequence table, see Table 4.5, page 43.

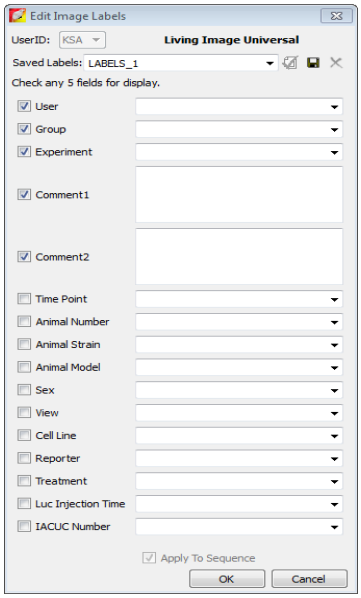


Acquire the Image Sequence

1. Confirm that the IVIS Imaging System is initialized and the CCD temperature is locked. (For more details, see page 17.)
2. When you are ready to acquire the images, click **Acquire Sequence** in the control panel.

   The Edit Image Labels box appears.

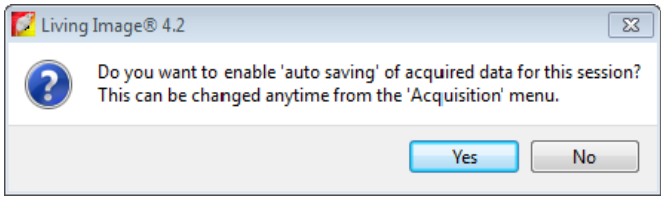
Figure 4.18 Edit Image Labels box



The information entered here appears in the image label (Figure 4.20).

3. In the Edit Image Labels box, enter information about the image and click **OK**. If you do not want to enter image information, click **Cancel**.
4. If this is the first image of the session, you are prompted to enable the autosave function.

Figure 4.19 Autosave prompt

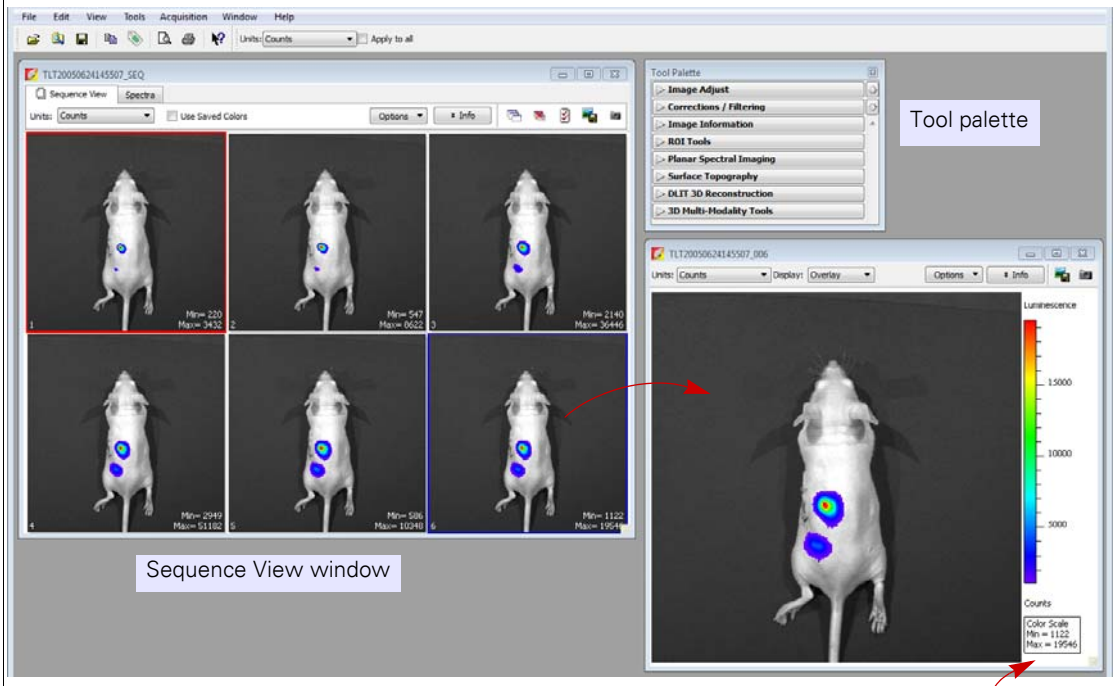


5. To enable autosave, click **Yes** in the prompt and choose a folder in the dialog box that appears.

   If autosave is enabled, all images acquired during the session are automatically saved to a user-selected folder. You can choose a different folder at any time (select **Acquisition** → **Auto-Save** on the menu bar).

   Image acquisition proceeds. The Sequence View window appears and displays the images as they are acquired. The Tool Palette appears when acquisition is completed (Figure 4.20).
6. To stop acquisition, click the **Stop** in the control panel. To pause acquisition, click **Pause** in the control panel.

Figure 4.20 Sequence View window & Tool Palette
Double-click an image in the sequence to open it in a separate image window.



NOTE

The Spectra window is available if the acquisition included multiple wavelengths. The Spectra window provides a convenient way to view probe spectra from the factory-installed library and ROIs. For more details, see page 171.

Table 4.4 Sequence View 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. For more details on measurement units, see page 281.
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 4.4 Sequence View window (continued)

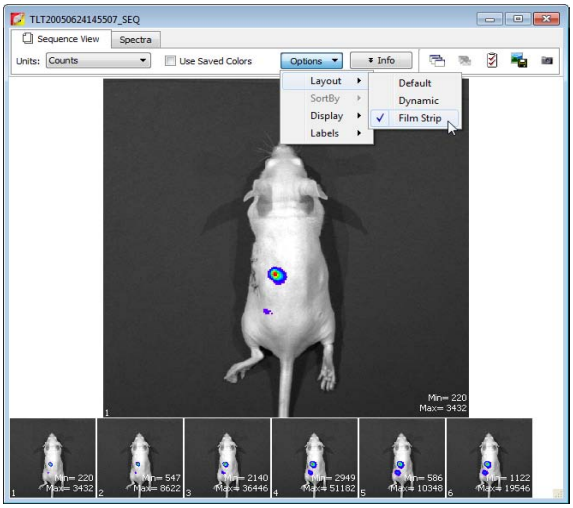
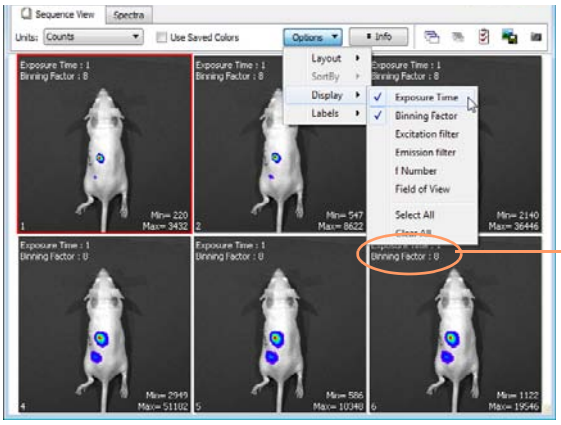
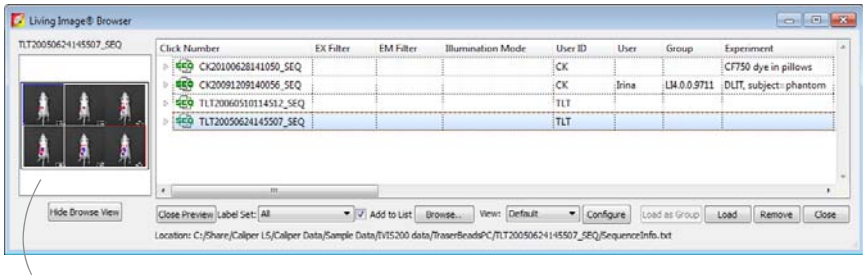
Item	Description
Options	Layout - Choose a display option for the images in a sequence (Default, Dynamic, or Film Strip). For example, here is Film Strip mode: 
	Sort by - Options for ordering images in the sequence window. This option only applies to images that were opened using the "Load as Group" function in the LIving Image browser. Default - Order in which the images are stored in the folder TimeStamp - Ascending order of the image acquisition time UserID - Ascending alphanumeric order of the user ID
	Display - Choose the types of information to display with each image.  In this example, exposure time and binning factor are displayed on each image
Info	Click to show or hide the image label information (Figure 4.20).
	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 4.4 Sequence View window (continued)

Item	Description
	Creates a preview picture (*snapshot*) of the image or thumbnails that the Living Image Browser displays when the data are selected.For more details on the browser, see page 83.



Preview picture of the selected data

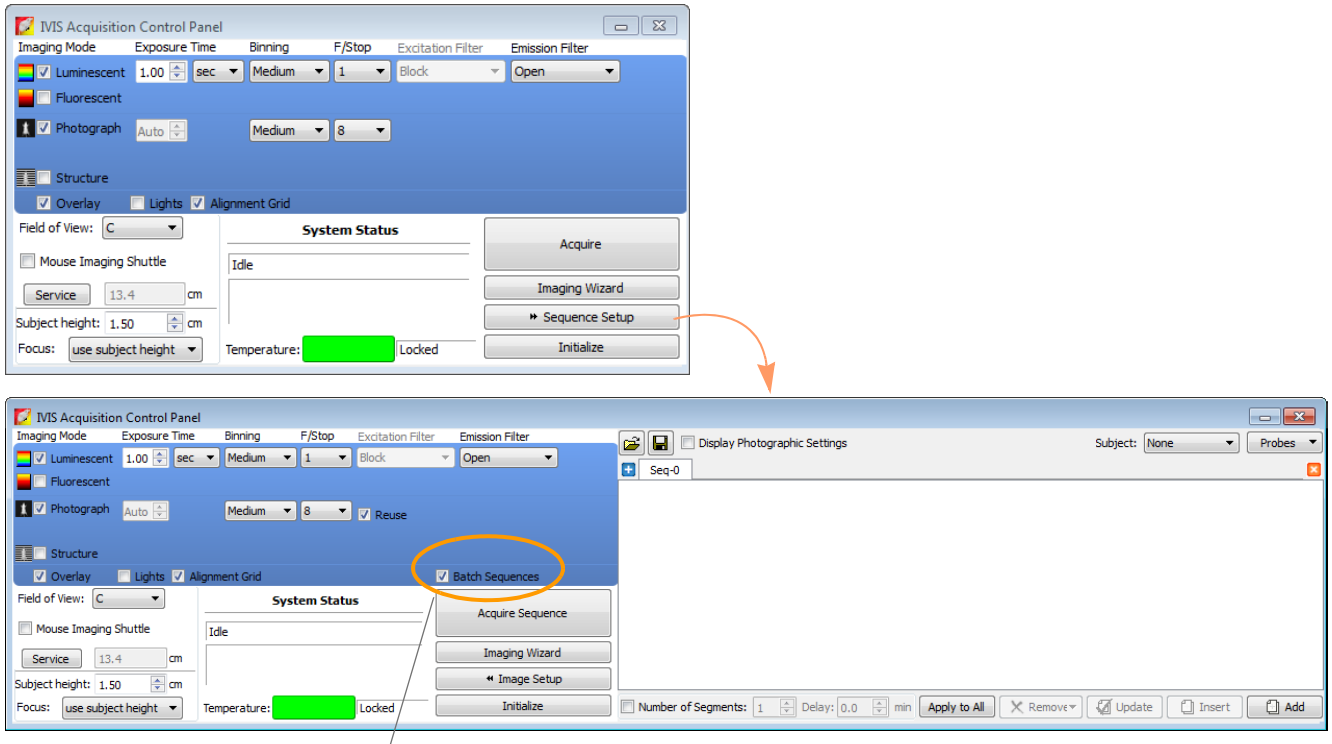
4.5 Acquire Multiple Sequences in Batch Mode

In batch mode, you can 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. In the Control Panel, click **Sequence Setup**.
2. Choose the Batch Sequences option.

Figure 4.21 Control Panel



Batch Sequences option

3. To set up the first sequence, do either of the following:
   - Click **Imaging Wizard** and step through the wizard (for more details on the Imaging Wizard, see page 33).

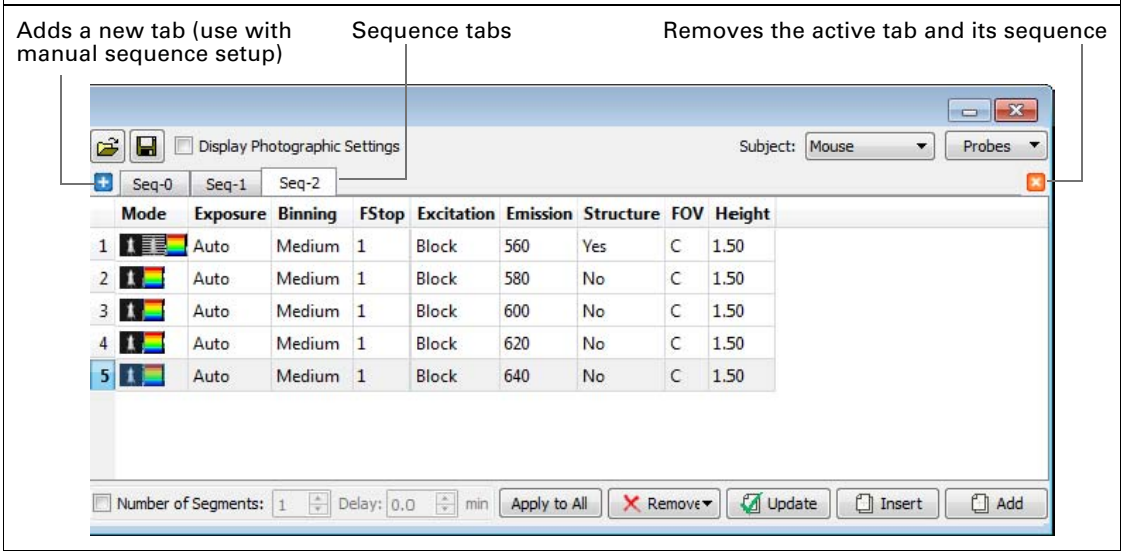
   OR
   - Set up the sequence manually (for more details, see page 41).
4. To set up the next sequence:
   - If using the Imaging Wizard, repeat step 3.

     Each sequence is dislayed 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 set up in the new tab.

NOTE

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

Figure 4.22 Multiple sequence tabs
In this example, three sequences are specified.



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

   Image acquisition proceeds with no intervening time delay between sequences.

NOTE

If the check mark is removed next to the Batch Sequences option (Figure 4.21), only the sequence in the active tab will be acquired.

4.6 Manually Set Up an Image Sequence

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

For details on image acquisition, see Acquire the Image Sequence, page 35.

NOTE

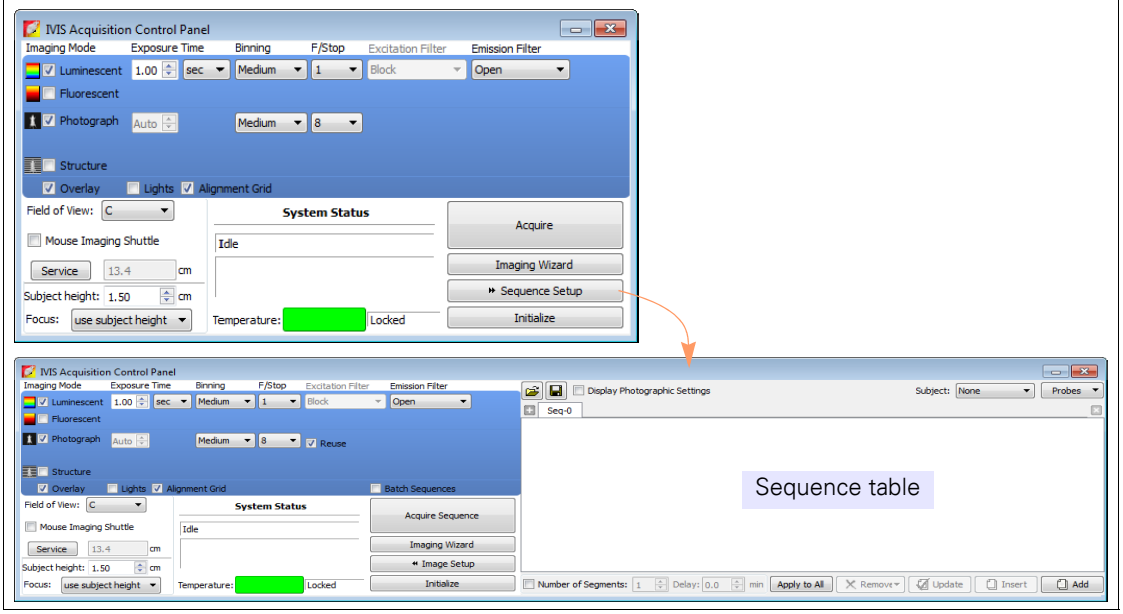
To create an image sequence, it may be convenient to edit a sequence setup generated by the Imaging Wizard or an existing sequence setup (.xsq). Save the revised sequence setup to a new name.

1. Click **Sequence Setup** in the control panel.

   The sequence table appears.

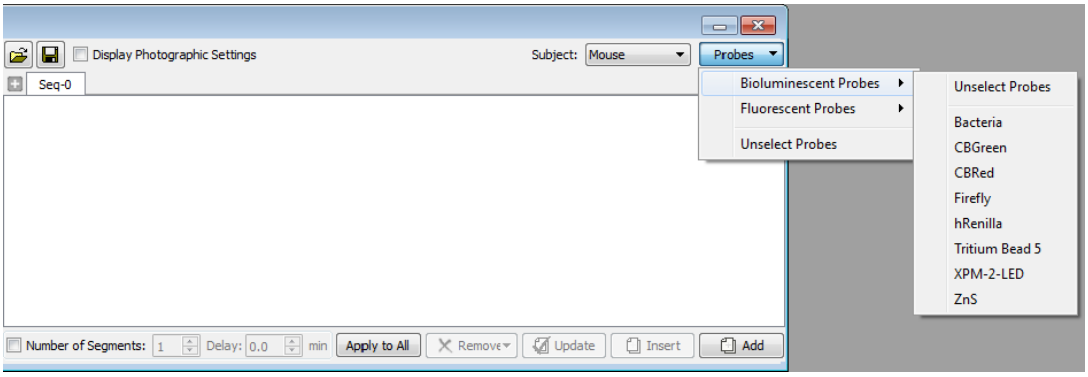
2. If necessary, click the **Remove** button Remove and select **All** to clear the sequence table.

Figure 4.23 Opening the sequence table



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

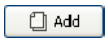
Figure 4.24 Choose a subject and probe



4. In the control panel, specify the imaging settings for the first luminescence or fluorescence image in the sequence and the photograph. (For details on the imaging parameters in the control panel, see page 259.)

NOTE

If you choose the photograph Reuse option in the control panel (Figure 4.25), the IVIS System acquires only one photograph for the entire sequence. If this option is not chosen, the system acquires a photograph for each image in the sequence.

5. Click the **Add** button 

.

   The acquisition parameters appear in the sequence table (Figure 4.25).

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

Figure 4.25 Control panel and sequence table with image settings
Each row in the sequence table specifies the acquisition parameters for one image in the sequence.

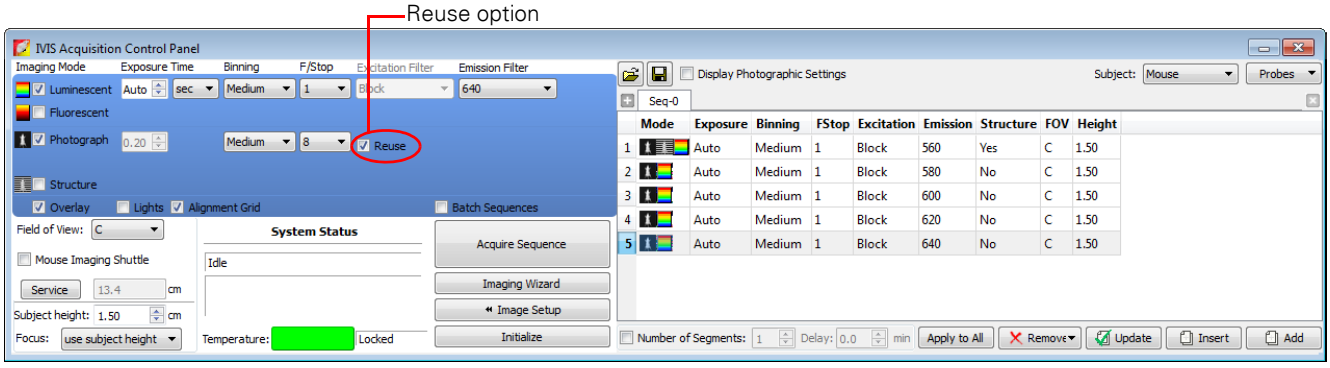


Table 4.5 Sequence table

Item	Description
Imaging Wizard	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 Surface Topography, DLIT, FLIT, Spectral Unmixing, and Planar Spectral Imaging tools. If a subject or probe is not selected here, the default parameters appear in the Tool Palette.
Number of Segments	The sequence specified in the sequence table is called a *segment*. Choose this option to set the number of segments to acquire and the time delay between segments. This is useful for acquiring data for kinetic analysis.
Delay	Specifies a time delay between each segment acquisition.
Apply to All	Applies the selected cell value to all cells in the same column.
Remove	Remove Selected - Deletes the selected row from the sequence table. Remove All - Removes all rows from the sequence table.
Update	Updates the selected row in the sequence table with the acquisition parameters in the control panel.
Insert	Inserts a row above the currently selected row using the information from the control panel.
Add	Adds a new row at the end of the sequence setup list.

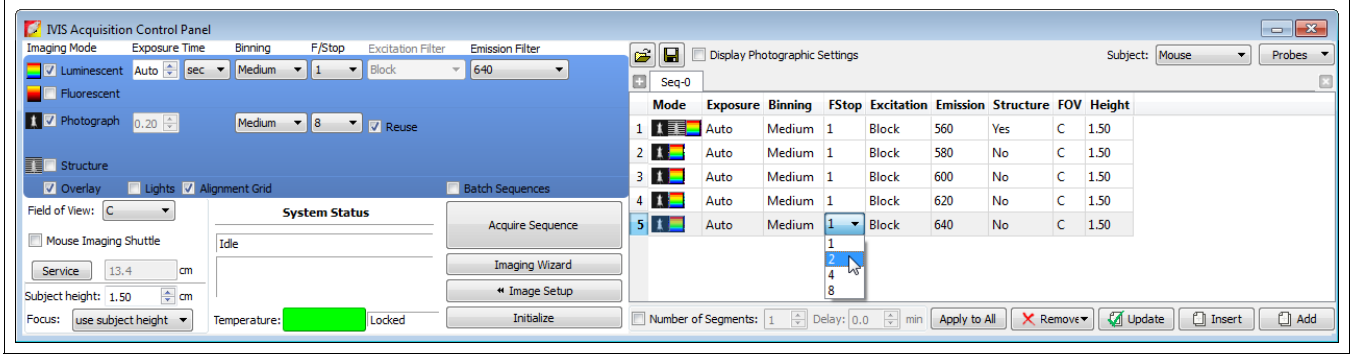
Editing Image Parameters

You can edit 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 Control panel and sequence table



2. Enter a new value in the cell or make a selection from the drop-down list.

To apply the new value to all of the cells in the same column, click Apply to All.

3. Click outside the cell to lose focus.

To edit a parameter in the control panel:

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

Inserting Images in a Sequence

Method 1:

1. In the sequence table, select the row next to where you want to insert the image.
2. Set the imaging mode and parameters in the control panel.
3. To insert the new image above the selected row, click Insert.

Method 2:

Select the row(s) of interest and right-click the sequence table to view a shortcut menu of edit commands.

Figure 4.27 Sequence table, edit commands in the shortcut menu

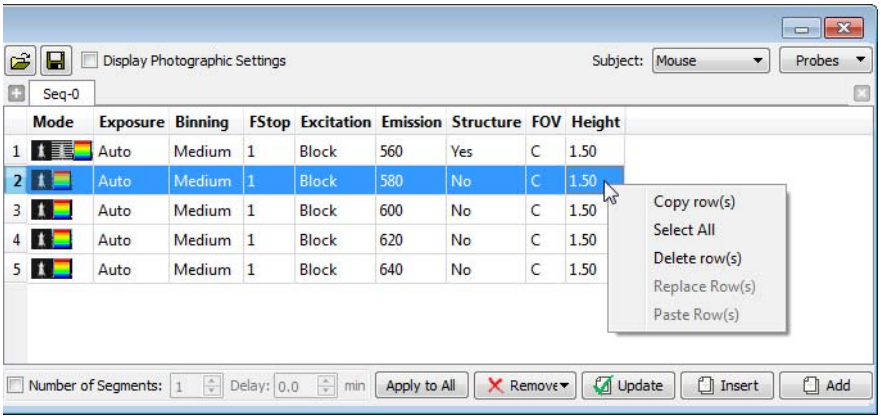


Table 4.6 Sequence table, shortcut menu edit commands

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

Method 1:

1. Select the row(s) that you want to delete.
2. Click Remove and choose **Selected** from the drop-down list.

Method 2:

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 High Resolution Imaging With the Optical Zoom Lens Attachment

The optional Optical Zoom Lens attachment enables close up and high resolution imaging on the IVIS Lumina, IVIS Lumina XR, and IVIS Kinetic Imaging Systems. When the Zoom lens attachment is installed, only the "Z" field of view setting (2.6 cm) is available for single-image or sequence acquisition.

The imaging system is set to the Z field of view position until the Zoom lens attachment is removed. If the Zoom lens attachment is installed when the Living Image software is closed, the stage will move to the Z position when the system is initialized.

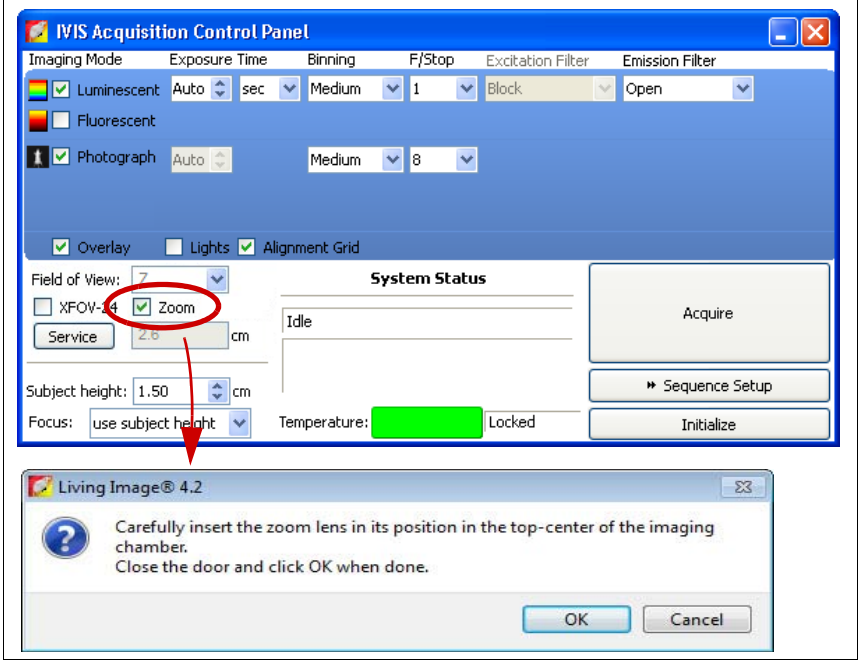
NOTE

When installing or removing the Optical Zoom Lens attachment, avoid touching the optical glass.

Installing the Zoom Lens Attachment

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

Figure 4.28 Control panel, Zoom option selected



2. After you install the Zoom Lens attachment in the imaging chamber, click **OK** in the prompt.

   The stage moves to the "Z" field of view position.

NOTE

When the Zoom Lens attachment is installed, single images or sequential images can only be acquired at the Z field of view setting. During sequence setup, if you attempt to change away from the Z setting, 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.

Removing the Zoom Len Attachment

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

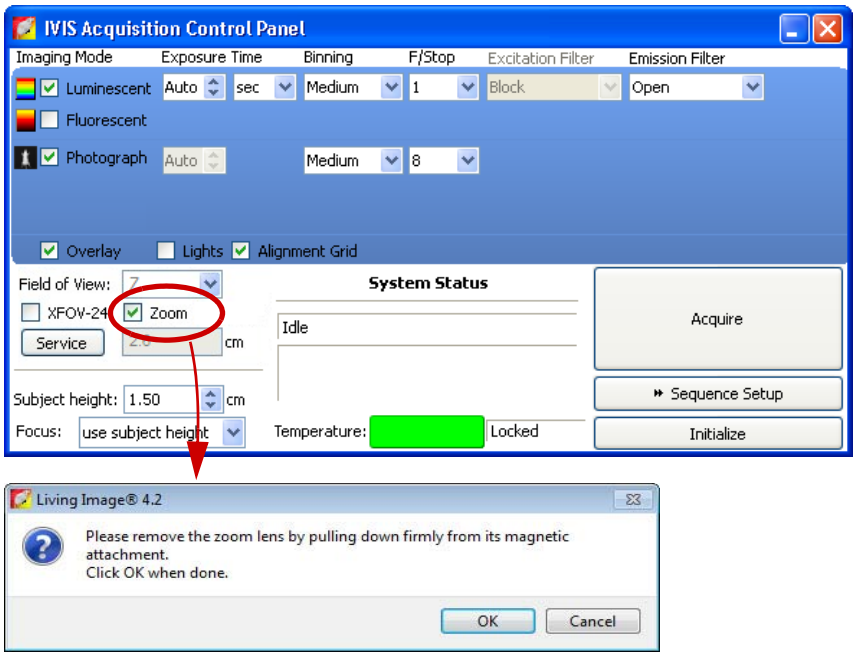
1. Remove the check mark next to Zoom in the control panel (Figure 4.29).

   The stage moves to position C, then you are prompted to remove the lens attachment.

2. After you remove the Zoom Lens attachment, click **OK** in the prompt.

   Always store the lens wrapped in its protective container.

Figure 4.29 Control panel, remove the check mark next to Zoom



4.8 Manually Saving Image Data

When you acquire the first image(s) of a session, you are prompted to enable the autosave feature. If autosave is enabled, all images acquired during the session are automatically saved to this folder. You can choose a different folder 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 you acquire an image or image sequence, click the **Save** button. Alternatively, select **File** → **Save** on the menu bar.
3. In the dialog box that appears, select a directory and click **OK**.

NOTE

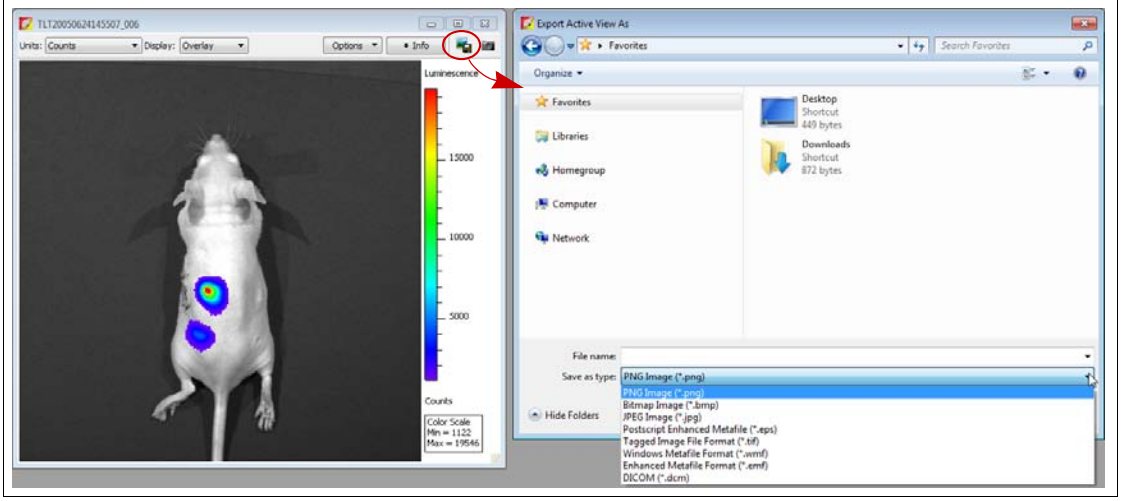
The software automatically includes the user ID, and a date and time stamp with the data.

4.9 Exporting Image Data

You can save the active image view in different file formats (for example, .bmp, .dcm).

1. Open an image or image sequence.
2. Click the **Export Graphics** button.

Figure 4.30 Exporting an image to a graphic file



3. In the dialog box that appears, select a directory, choose a file type, and enter a file name.
4. Click **Save**.

NOTE

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

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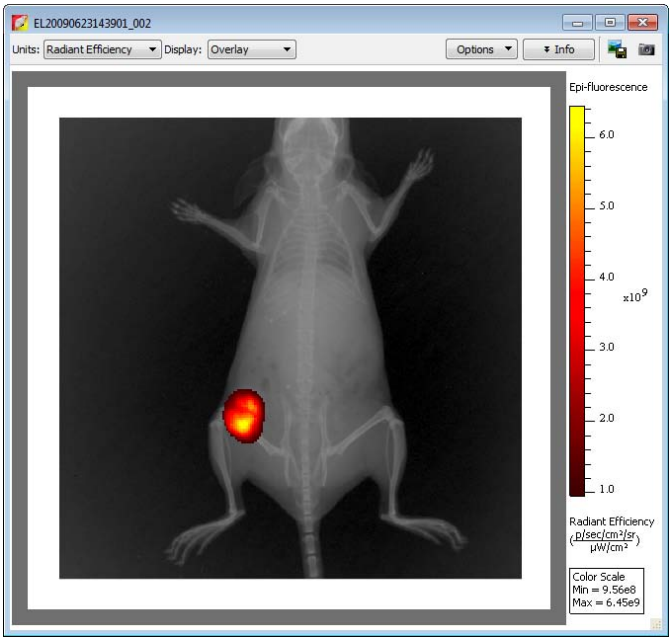
5 X-Ray Imaging

X-ray images can be acquired on the Lumina XR Imaging System. An X-ray image, luminescent or fluorescent image, and a photograph can be acquired at the same time. You can choose two images to create an overlay (Figure 5.1).

Figure 5.1 Example overlay: fluorescence image on X-ray image



5.1 Acquire an X-Ray Image

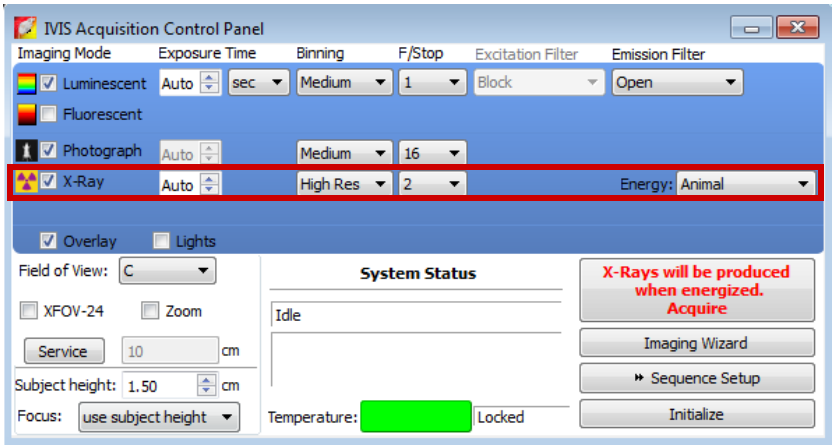
This section explains how to acquire an X-ray image on the Lumina XR Imaging System. For information on including a luminescent or fluorescent image in the acquisition, see Chapter 4, page 21.

1. Start the Living Image software (double-click the icon on the desktop).
2. Initialize the Lumina XR Imaging System and confirm or wait for the CCD temperature to lock. (For more details, see page 17.)
3. In the control panel, put a check mark next to **X-Ray** (Figure 5.2).

NOTE

To enable X-ray acquisition, verify that the X-ray enabling key on the front of the Lumina XR is set to ON, and the orange X-ray enable button has been depressed and is illuminated.

Figure 5.2 Lumina XR control panel



X-ray acquisition settings

4. Select the **Auto** exposure time (click the arrow). Alternatively, manually set the exposure, binning, and F/Stop. (For more details on the control panel settings, see page 259.)
5. Make a selection from the Energy drop-down list.

Table 5.1 Lumina XR energy options

Energy Option	Suitable For	X-Ray Energy Level
Animal	Living subject	35 Kv 100 A, filtered X-rays
Specimen	Non-living specimen	28 Kv 100 A, unfiltered X-rays

NOTE

A Caliper field service engineer can customize the default X-ray energy levels (Table 5.1) to settings from 5.0 - 40 Kv and from 1-100 A with or without the low energy X-ray absorbing (Al) filter.

6. Set the **Field of View:** Make a selection from the Field of View drop-down list. For more details on the field of view, see page 261.

NOTE

To view the subject(s) inside the chamber before image acquisition, take a photographic image (uncheck the luminescent or fluorescent option, choose the Photographic and Auto options, and click **Acquire**).

7. Set the **Focus:**
   - Select **use subject height** from the Focus drop-down list and use the arrows or the keyboard arrows to specify a subject height (cm).

NOTE

The subject height for X-ray images is restricted to 2.8 cm or less. The subject height is critical to ensure proper optical and X-ray overlay. The subject height is used to determine the X-ray resizing coefficient. Select a subject height which suits the region of interest.

or

- Select **Manual focus** from the Focus drop-down list. (For more details on manual focusing see page 264.)

8. If you want to acquire a photograph, set the **Photograph** image settings**:**
   a. Put a check mark next to **Photograph**.
   b. Enter an exposure time or choose the Auto option.
   c. Confirm the binning and f/stop defaults or enter new values.
9. If necessary, click Image Setup in the control panel to operate in single image mode.

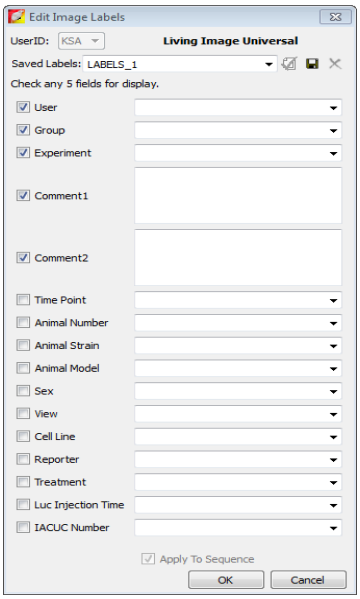
NOTE

In single image mode, the Sequence Setup button appears in the control panel. Click this button to set up sequence acquisition. (For more details on setting up a sequence, see page 54.)

10. When you are ready to acquire the image, click **Acquire**.

    The Edit Image Labels box appears.

Figure 5.3 Edit Image Labels box



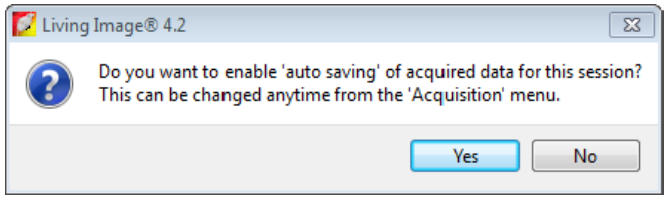
The information you enter here appears in the image label (Figure 5.6).

11. In the Edit Image Labels box, enter information about the image and click **OK** (Figure 5.3). If you do not want to enter image information, click **Cancel**.

    Image acquisition proceeds. During acquisition, the **Acquire** button becomes a **Stop** button. To cancel the acquisition, click **Stop**. When acquisition is complete, the image window appears (Figure 5.6).

12. If this is the first image of the session, you are prompted to enable the autosave function.

Figure 5.4 Autosave prompt



13. To enable autosave, click **Yes** in the prompt and choose a folder in the dialog box that appears.

    If autosave is enabled, all images acquired during the session are automatically saved to a user-selected folder. You can choose a different folder at any time (select **Acquisition** → **Auto-Save** on the menu bar).

    The acquisition proceeds and the control panel warns you that X-ray radiation is being produced (Figure 5.5). The image window and Tool Palette appear when acquisition is completed (Figure 5.6)

14. If you need to stop the acquisition, click **Stop** in the control panel 

.

Figure 5.5 Control panel during X-ray image acquisition

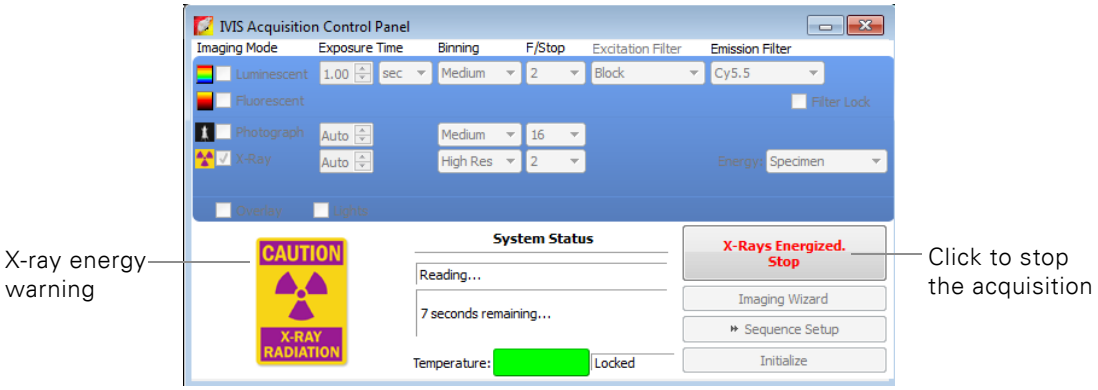
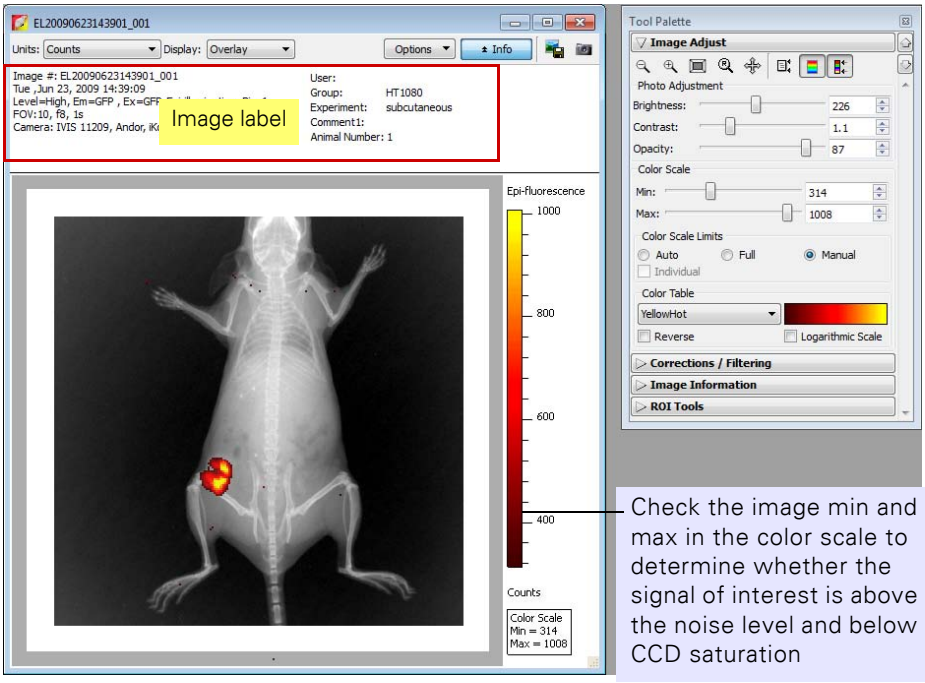


Figure 5.6 Overlay (fluorescent image on X-ray image) in the image window



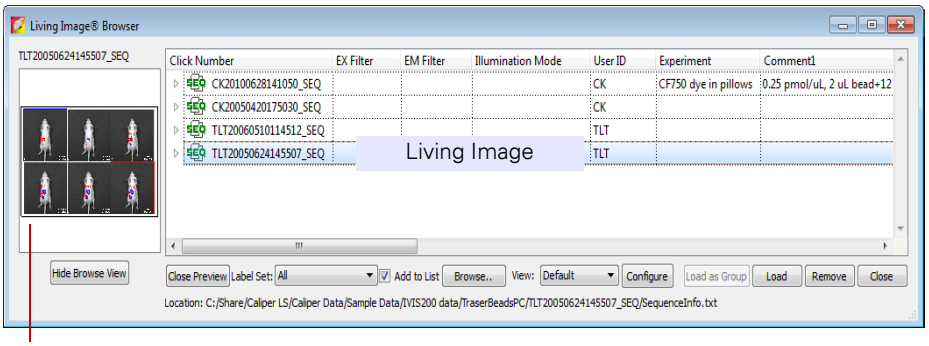
NOTE

It may be necessary to use the Image Adjust tools to optimize the overlay display. Use the Opacity control to adjust the appearance of the overlay. For more details on adjusting image appearance, see page 97.

Table 5.2 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. For more details on measurement units, see page 281.
Display	Select the image type (for example, X-ray) that you want to display from this drop-down list. For more details on the different types of image displays, see Table 2.2, page 8. **Note:** If the acquisition included more than two imaging modes (for example, luminescent, X-ray, and photograph), additional drop-down lists appear so you can conveniently choose any two images to overlay. 
Info	Click to display or hide the image label information.
	Opens a dialog box that enables you to export the active view as a graphic file.

Table 5.2 Image window (continued)

Item	Description
	Takes a "snapshot" that is displayed with the data in the Living Image Browser. 
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
Image label	Information about the image that the software automatically records and user-specified information entered in the Edit Image Label dialog box.

5.2 Acquire an Image Sequence That Includes X-Ray Images

To acquire an image sequence, first specify the acquisition parameters for each image in the sequence table (Figure 5.7). The Imaging Wizard provides a convenient way to do this for some imaging applications. The wizard guides you through a series of steps, prompting you for the information that the software needs to set up the sequence in the sequence table. If you don't use the wizard, you can set up a sequence manually (for more details, see page 41).

Sequence Setup Using the Imaging Wizard

1. Click **Sequence Setup** in the control panel (Figure 5.7).

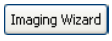
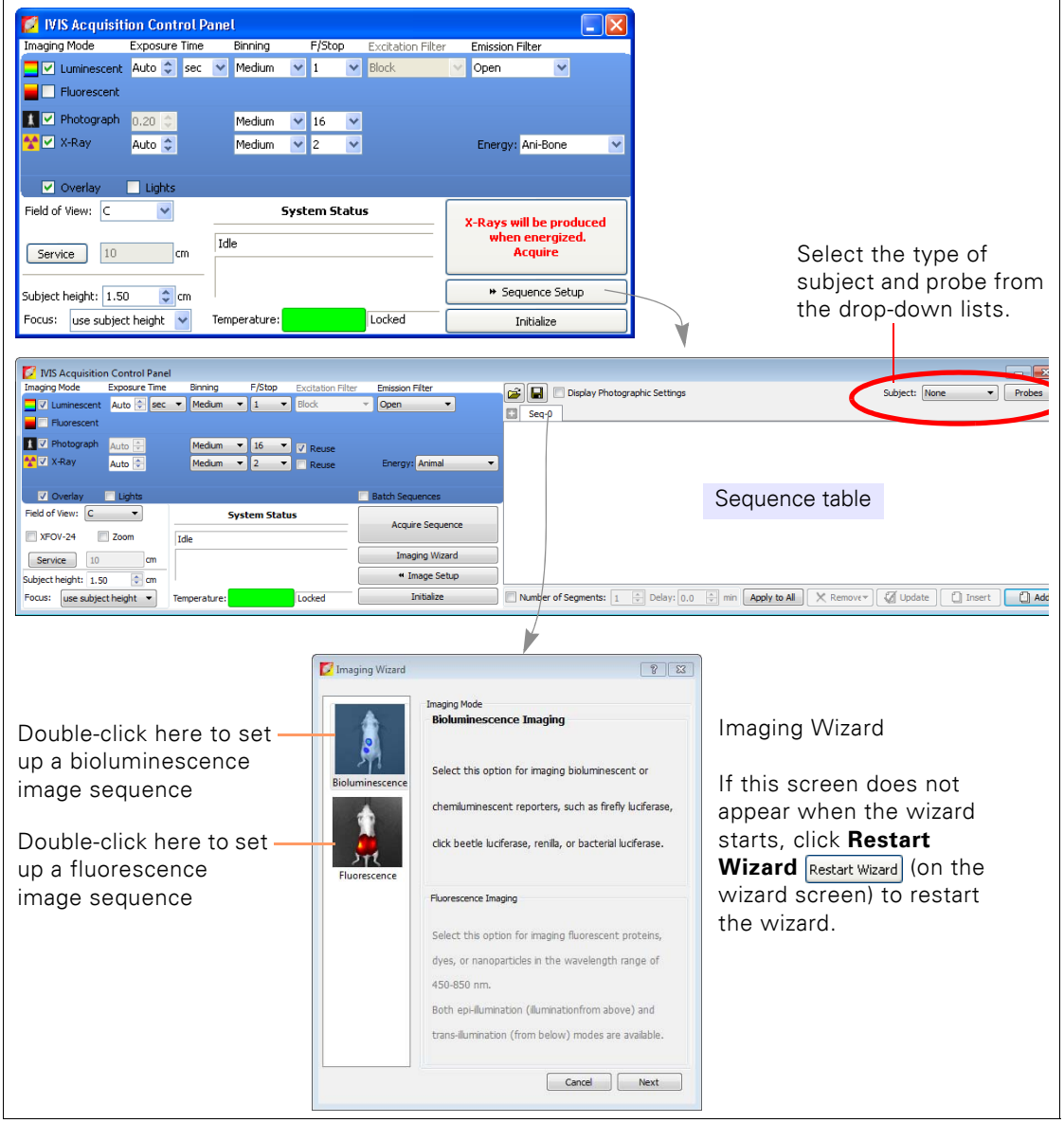
   The sequence table appears.
2. If necessary, click the **Remove** button  and select **All** to clear the sequence table.
3. Click the **Imaging Wizard** button  .

Figure 5.7 Opening the sequence table and starting the Imaging Wizard



4. In the wizard, choose bioluminescence or fluorescence imaging (Figure 5.7).
5. In the next wizard screen, choose the type of image sequence that you want to acquire. Step through the rest of the wizard
6. To include an X-ray image or photograph in the sequence, put a check mark next to the **X-ray** or **Photograph** option when you set the imaging parameters in the wizard (Figure 5.8).

Figure 5.8 Imaging Wizard, imaging parameters

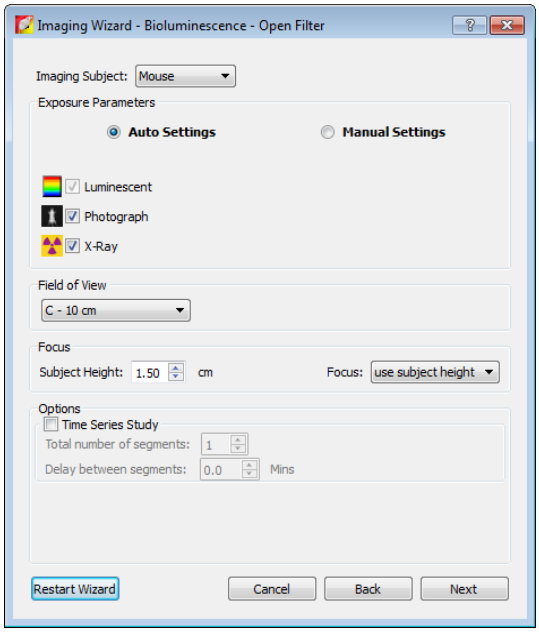


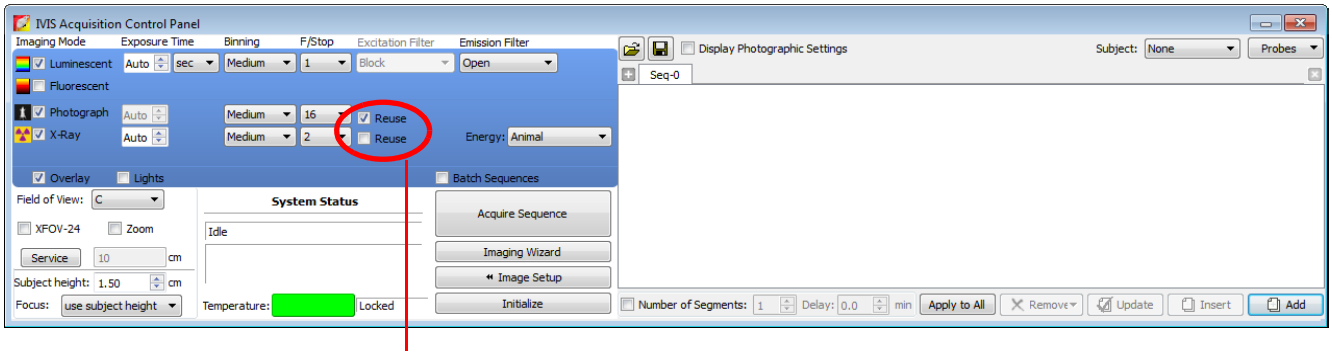
Table 5.3 Imaging Wizard

Item	Description
Imaging Subject	Choose the type of subject from this drop-down list.
Exposure Parameters	The Auto Settings exposure option is the default. To manually set the exposure parameters, select the Manual Settings option. For more details on the exposure parameters, see page 259.  To include a photograph or X-ray image in the acquisition, put a check mark next to the **Photograph** or **X-ray** option. **NOTE** In the control panel, the Photograph and X-ray Reuse option is selected by default (Figure 5.9). This means the same X-ray image and photograph will be used if camera conditions do not change (for example, binning or F/Stop). If you do not want to reuse the X-ray image or photograph, you can manually edit the image sequence in the sequence table (for more details, see page 44). Alternatively, remove the check mark next to **Reuse** in the control panel before you begin the Imaging Wizard.
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, but gives a higher resolution image. Select the FOV by choosing a setting (A, B, or C) from the drop-down list. For more details on the calibrated FOV positions, see Table A.3, page 263.

Table 5.3 Imaging Wizard

Item	Description
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 264.
Options	Time Series Study - Choose this option to specify the number of segments to acquire and a time delay between segments. This option is useful for acquiring data for kinetic analysis. **Note:** The sequence specified in the sequence table is called a *segment*.
Restart Wizard	Returns the wizard to the starting screen.

Figure 5.9 Control panel and sequence table

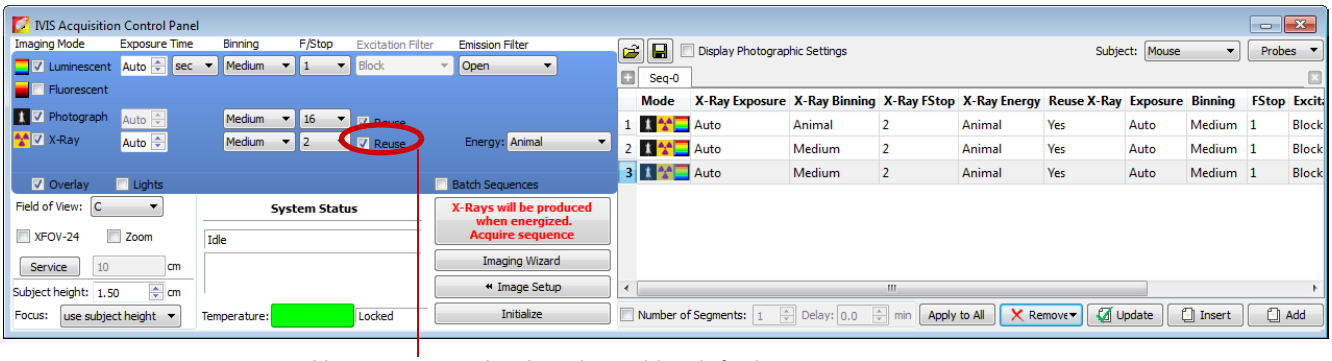


Reuse photograph & Reuse X-ray options

7. Complete the rest of the Imaging Wizard.

   When you complete the wizard, the sequence information appears in the sequence table (Figure 5.10).

Figure 5.10 Control panel and sequence table



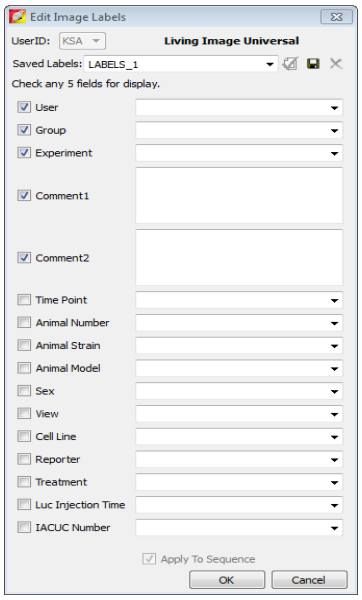
X-ray reuse option is selected by default.
One X-ray image is acquired for the sequence.

Acquire the Image Sequence

1. Confirm that the IVIS Imaging System is initialized and the CCD temperature is locked. (For more details, see page 17.)
2. When you are ready to acquire the images, click **Acquire Sequence** in the control panel.

   The Edit Image Labels box appears.

Figure 5.11 Edit Image Labels box



The information entered here appears in the image label (Figure 5.13).

3. In the Edit Image Labels box, enter information about the image and click **OK**. If you do not want to enter image information, click **Cancel**.
4. If this is the first image of the session, you are prompted for an autosave location. All images acquired during the session are automatically saved to this folder. You can choose a different folder at any time (select **Acquisition** → **Auto-Save** on the menu bar).

   To select a folder for autosaved data, click **Yes** in the prompt and choose a folder in the dialog box that appears.

Figure 5.12 Autosave prompt appears if this is the first acquisition of the session

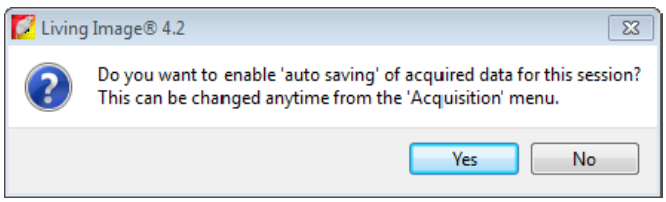


Image acquisition proceeds. The Sequence View window appears and displays the images as they are acquired. The Tool Palette appears when acquisition is completed (Figure 5.13).

5. To stop acquisition, click the **Stop** in the control panel. To pause acquisition, click **Pause** in the control panel.

Figure 5.13 Image sequence and Tool Palette
Double-click an image in the sequence to open it in a separate image window.

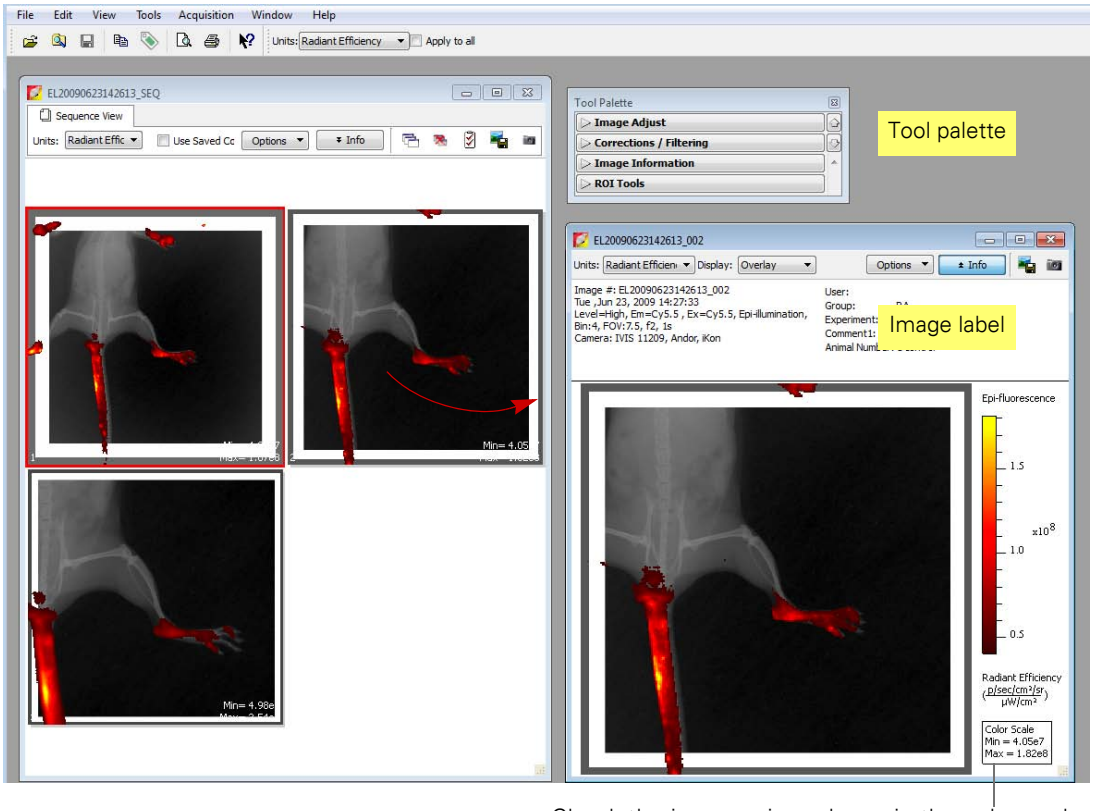


Table 5.4 Image window: Sequence view

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. For more details on measurement units, see page 281.
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 5.4 Image window: Sequence view (continued)

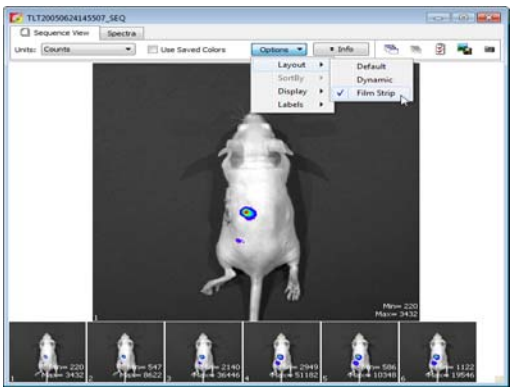
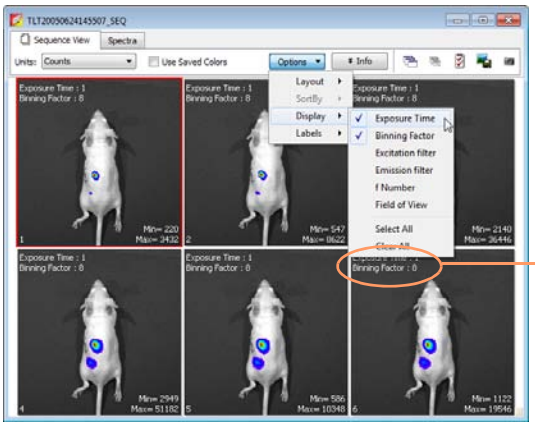
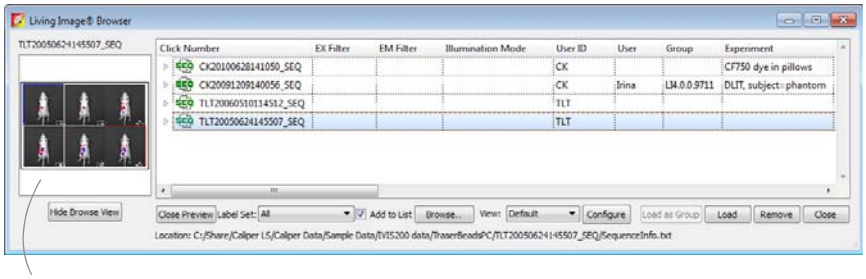
Item	Description
Options	Layout - Choose a display option for the images in a sequence. For example, here is Film Strip mode: 
	Sort by - Options for ordering images in the sequence window: Default - Order in which the images are stored in the folder TimeStamp - Ascending order of the image acquisition time UserID - Ascending alphanumeric order of the user ID
	Display - Choose the types of information to display with each image.  In this example, exposure time and binning factor are displayed on each image
Info	Click to show or hide the image label information (Figure 5.13).
	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 5.4 Image window: Sequence view (continued)

Item	Description
	Creates a preview picture (*snapshot*) of the image or thumbnails that the Living Image Browser displays when data are selected. For more details on the browser, see page 83.



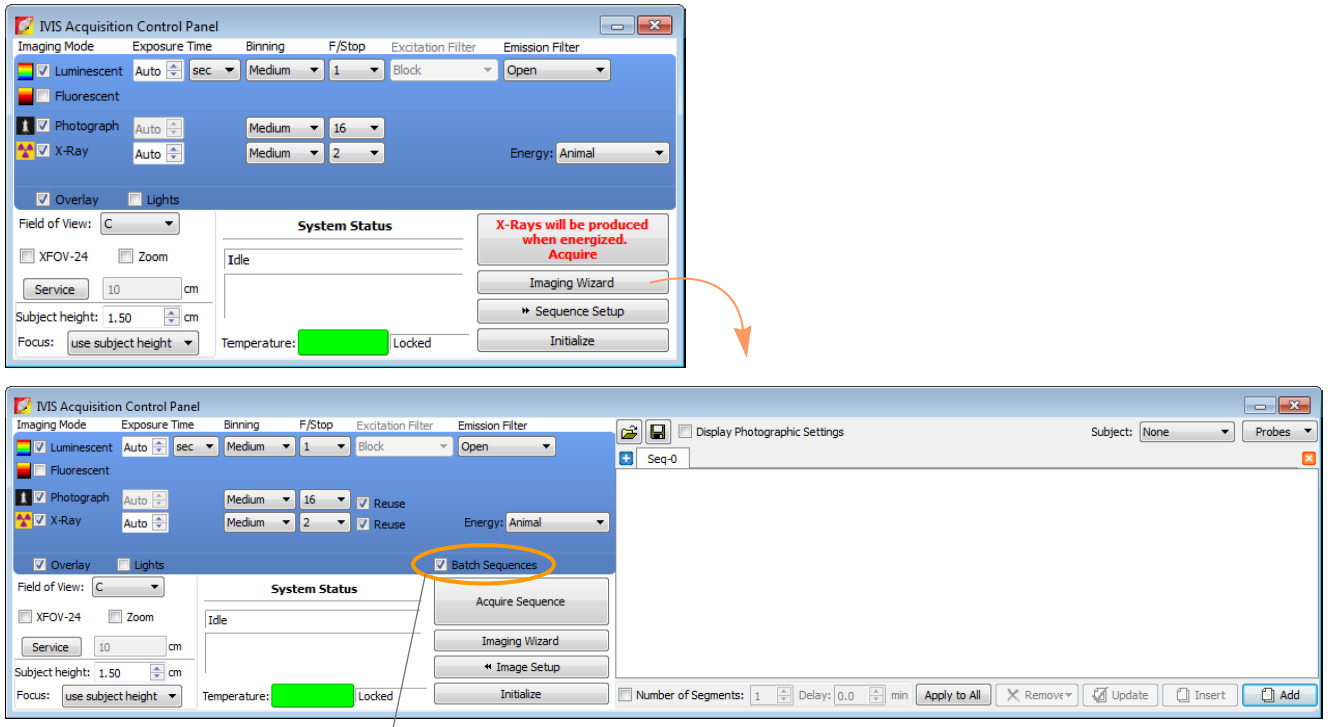
5.3 Acquire Multiple Sequences in Batch Mode

In batch mode, you can 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. In the Control Panel, click **Sequence Setup**.
2. Choose the Batch Sequences option.

Figure 5.14 Control Panel



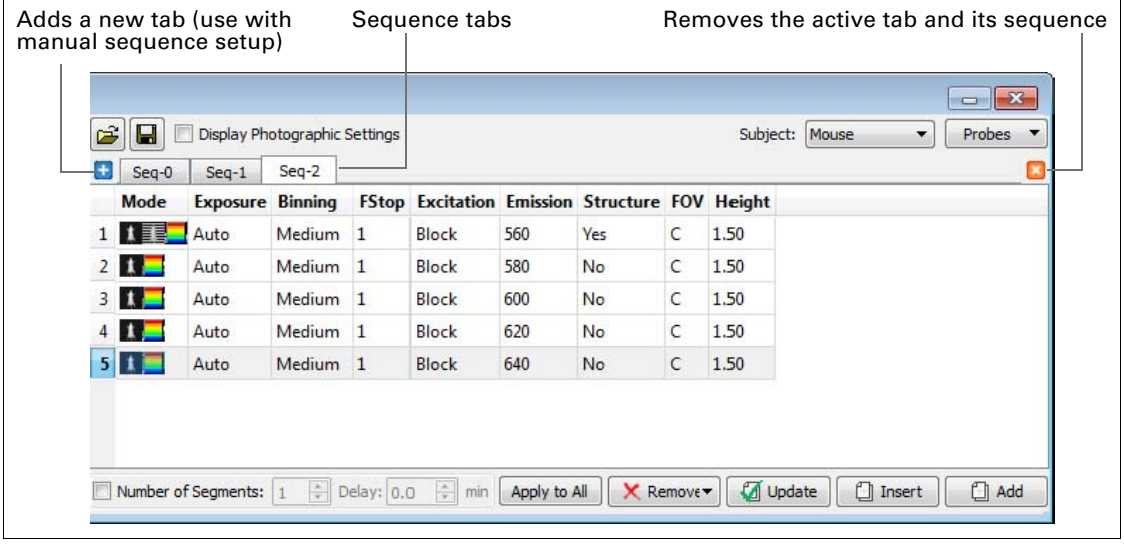
3. To set up the first sequence, do either of the following:
   - Click **Imaging Wizard** and step through the wizard (for more details on the Imaging Wizard, see page 54).

   OR
   - Set up the sequence manually (for more details, see page 41).
4. To set up the next sequence:
   - If using the Imaging Wizard, repeat step 3.
     Each sequence is dislayed 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 set up in the new tab.

NOTE

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

Figure 5.15 Multiple sequence tabs
In this example, three sequences are specified.



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

   Image acquisition proceeds with no intervening time delay between sequences.

NOTE

If the check mark is removed next to the Batch Sequences option (Figure 5.14), only the sequence in the active tab will be acquired.

5.4 High Resolution Imaging With the Optical Zoom Lens Attachment

The optional Optical Zoom Lens attachment enables close up and high resolution imaging on the IVIS Lumina, IVIS Lumina XR, and IVIS Kinetic Imaging Systems. When the Zoom lens attachment is installed, only the “Z” field of view setting (2.6 cm) is available for single-image or sequence acquisition.

The imaging system is set to the Z field of view position until the Zoom lens attachment is removed. If the Zoom lens attachment is installed when the Living Image software is closed, the stage will move to the Z position when the system is initialized.

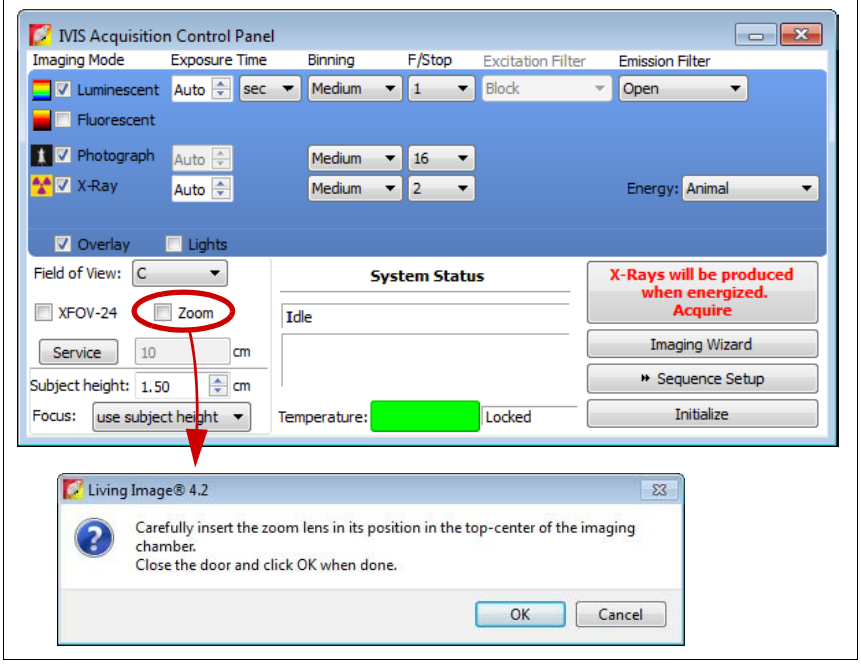
NOTE

When installing or removing the Optical Zoom Lens attachment, avoid touching the optical glass.

Installing the Zoom Lens Attachment

1. Choose the Zoom option in the control panel (Figure 5.16).
   You are prompted to insert the Zoom Lens attachment.

Figure 5.16 Control panel, Zoom option selected



2. After you install the Zoom Lens attachment in the imaging chamber, click **OK** in the prompt.
   The stage moves to the “Z” field of view position.

NOTE

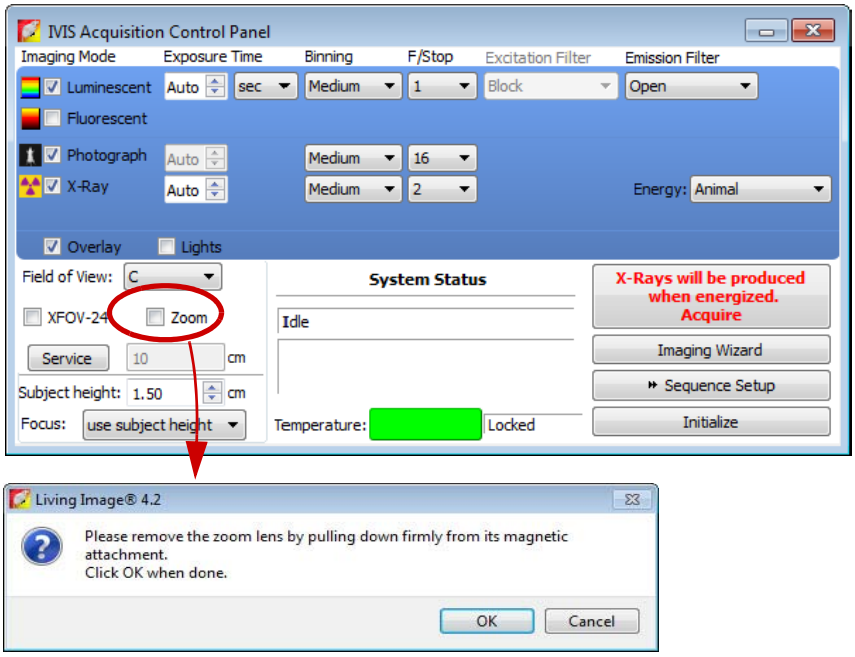
When the Zoom Lens attachment is installed, single images or sequential images can only be acquired at the Z field of view setting. During sequence setup, if you attempt to change away from the Z setting, the sequence table will be cleared. If you attempt to change from another field of view setting to the Z setting, the sequence table is cleared of all previous settings before the camera settings for the Z position are added.

Removing the Zoom Len Attachment

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

1. Remove the check mark next to Zoom in the control panel (Figure 5.17).
   The stage moves to position C, then you are prompted to remove the lens attachment.
2. After you remove the Zoom Lens attachment, click **OK** in the prompt.
   Always store the lens wrapped in its protective container.

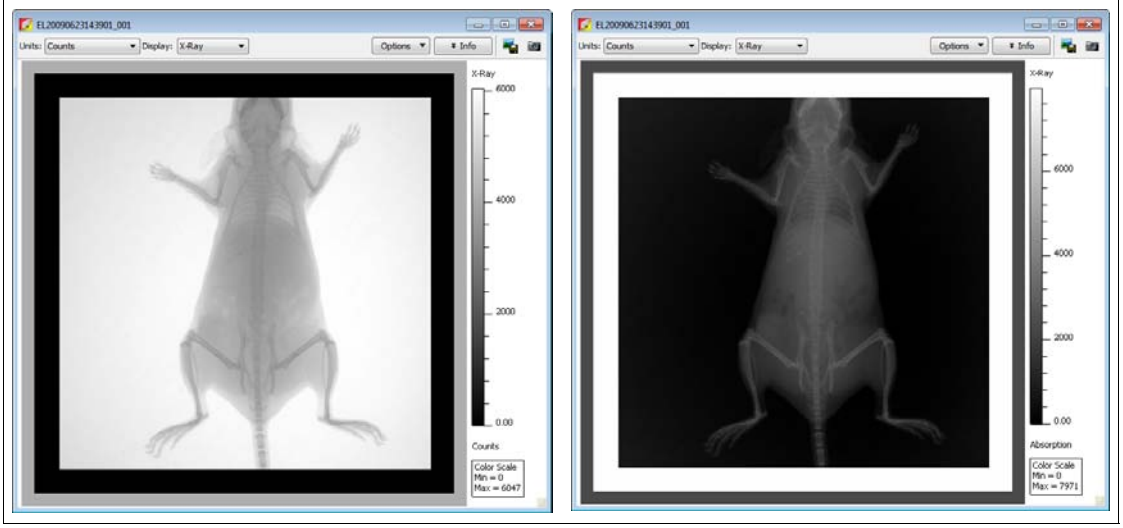
Figure 5.17 Control panel, remove the check mark next to Zoom



5.5 Measuring Relative Density

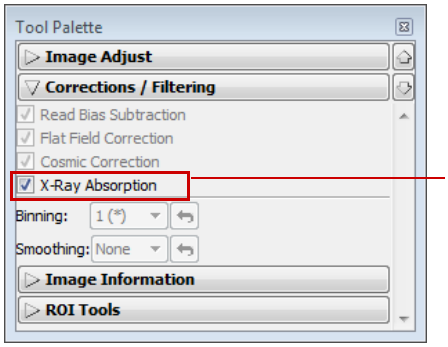
As density increases, tissue absorbs more X-ray energy and appears darker in a raw Lumina XR X-ray image (Figure 5.18). To enable measurements of this X-ray absorption, the raw X-ray image is mathematically transformed, resulting in an image where denser material appears lighter and intensity measurements are proportional to the tissue density (Figure 5.18).

Figure 5.18 X-ray images: raw (left) and in absorption units (right)



The Living Image software displays transformed X-ray images by default. To display raw X-ray images, remove the check mark next to **X-Ray Absorption** in the Corrections / Filtering tools (Figure 5.19).

Figure 5.19 Corrections/Filtering tools



If this option is not selected, no mathematical transformation is applied to the data and raw X-ray images are displayed (Figure 5.18).

When the X-ray data has been corrected for absorption, you can evaluate relative bone density by comparing the signal intensities of measurement ROIs. The ROI intensity increases with increasing tissue density.

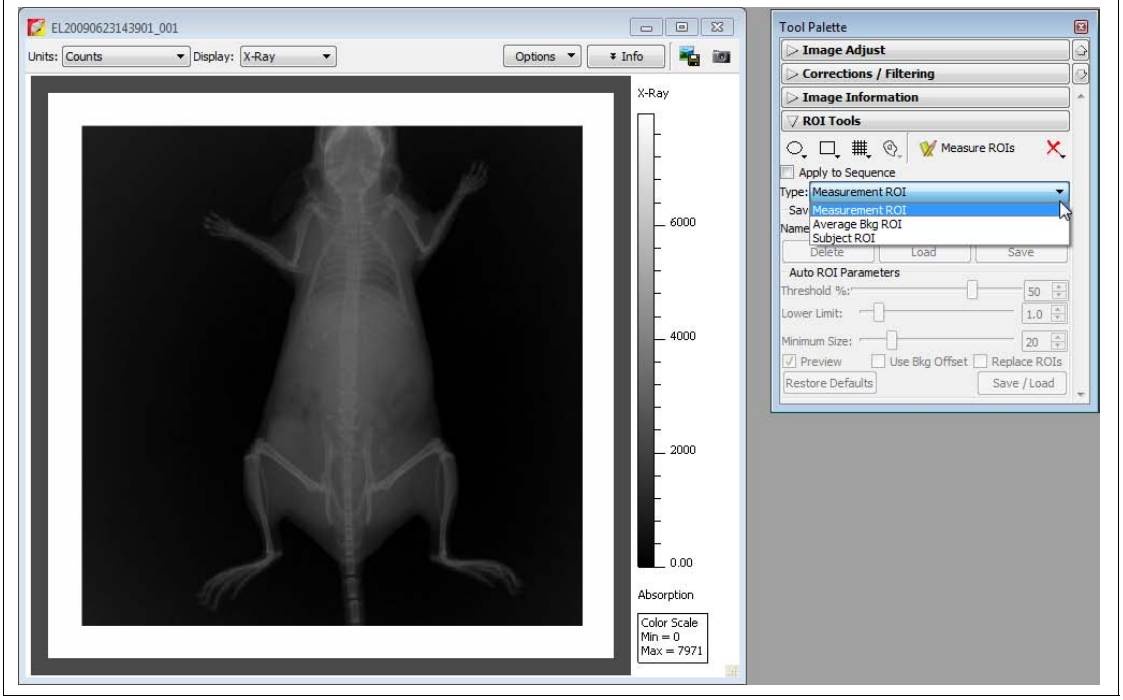
NOTE

When acquiring X-ray images for bone density evaluation, it is best if the subject does not fill the entire field of view since the X-ray absorption measurements are referenced to the exposed scintillator plate.

To determine relative bone density:

1. Load an X-ray image. (For more details on opening image data, see page 83.)
2. Confirm that the X-ray absorption correction (default) is applied (Figure 5.19).
3. In the ROI tools, select Measurement ROI from the Type drop-down list (Figure 5.20).

Figure 5.20 X-ray image with absorption correction



4. To select the ROI shape:
   a. Click the **Circle** or **Square** button.
   b. On the drop-down list that appears, select the number of ROIs that you want to add to the image.
      — The ROI(s) and intensity label(s) appear on the image. If you are working with a sequence, open an image to show the ROI intensity.
5. Adjust the ROI position:
   a. Place the mouse pointer over the ROI. When the pointer becomes a ✣, click the ROI.
   b. Drag the ROI.
6. Adjust the ROI dimensions:
   a. Place the mouse pointer over the ROI. When the pointer becomes a ✣, click the ROI.
   b. Place the mouse pointer over an ROI handle so that it becomes a ⤡. Drag the handle to resize the ROI.

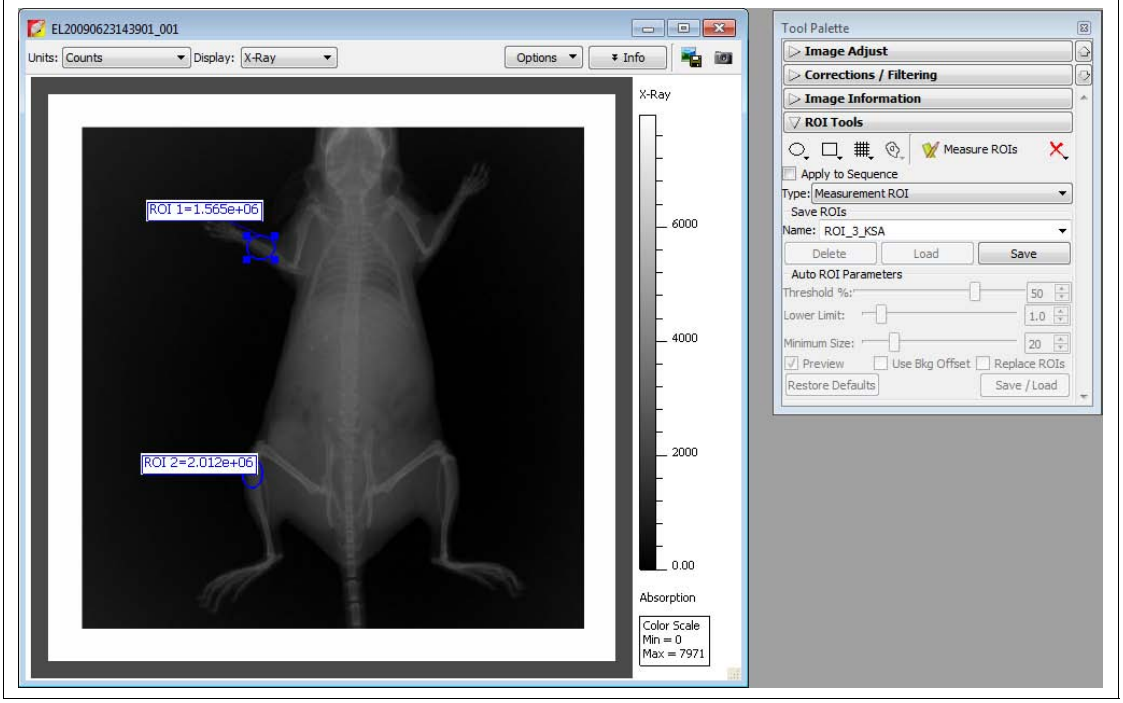
NOTE

You can also change the ROI position or size using the adjustment controls in the ROI Properties box (see "Moving an ROI," page 138 and "Editing ROI Dimensions," page 139).

7. Click the **Measure** button .

   The ROI intensity measurements appear in the X-ray image and the ROI measurements table appears. For more details on the table, see "Managing the ROI Measurements Table," page 145. For information on how to save ROIs, see page 142.

Figure 5.21 Measurement ROIs on X-ray image



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6 Kinetic Imaging

The IVIS Kinetic Imaging System is ready to acquire kinetic data after the system is initialized and the CCD camera reaches operating (*demand*) temperature (*locked*).

6.1 Kinetic Acquisition

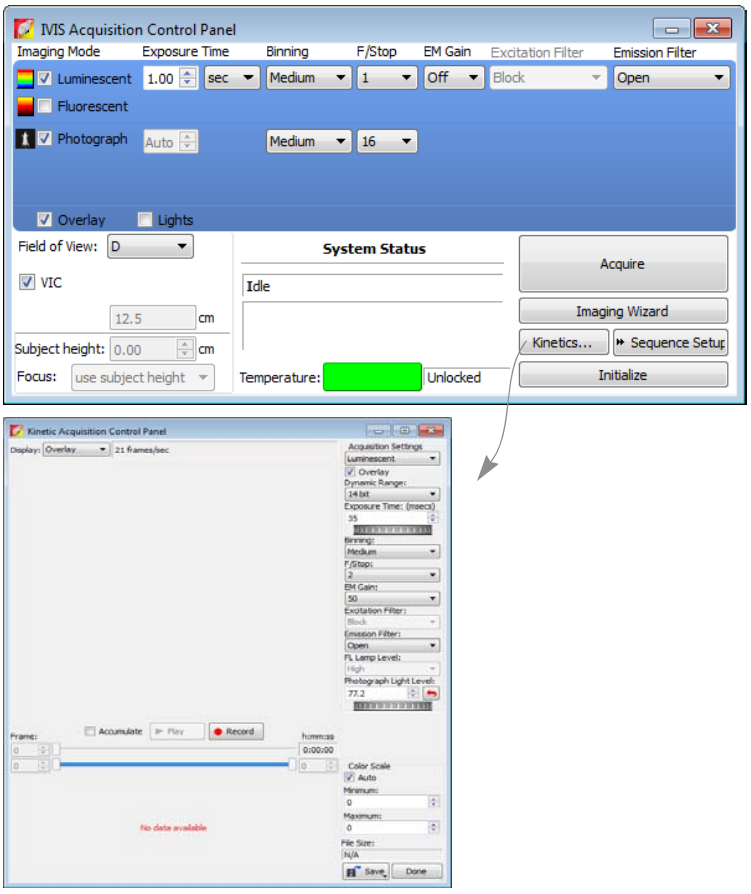
NOTE

Before setting the imaging parameters, the IVIS® Imaging System should be intialized and the temperature locked. For more details, see Chapter 3, page 15.

1. If you are acquiring ventral images (requires the subject be placed in the Ventral Imaging Chamber), choose the VIC option in the control panel.
2. When you are ready to begin imaging, click **Kinetics** in the control panel.

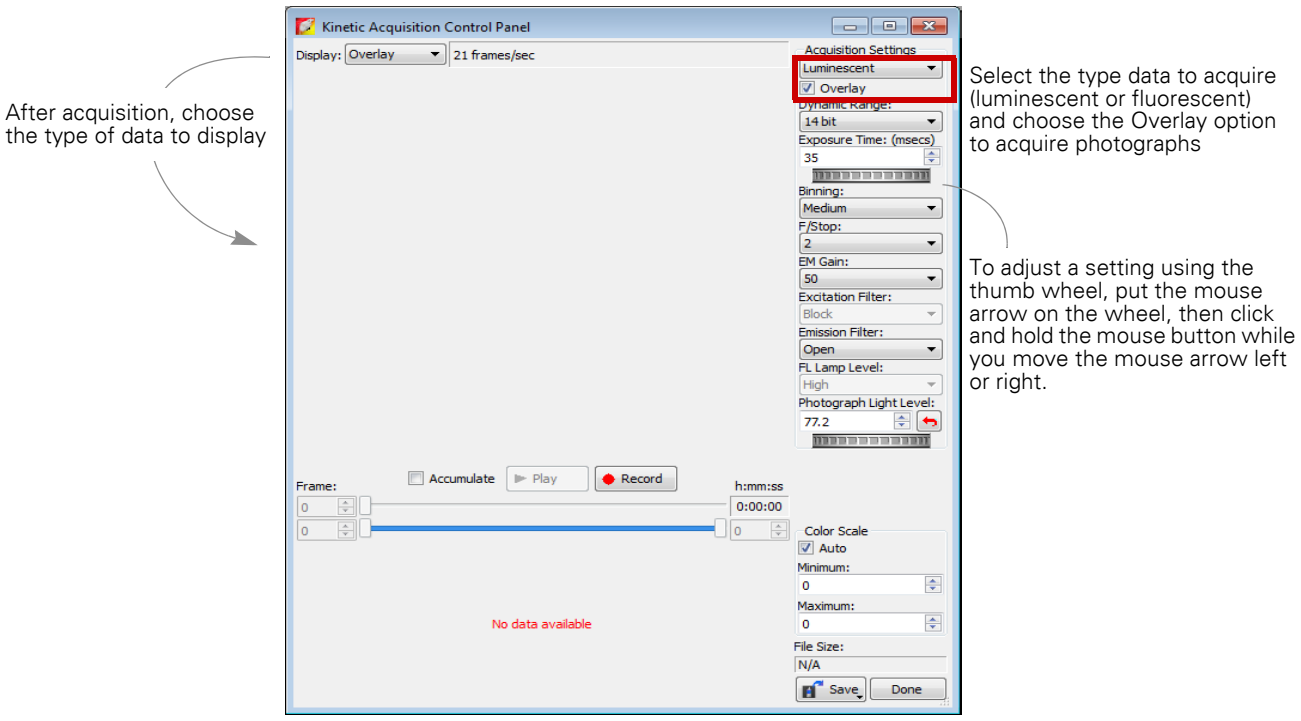
   The Kinetic Acquisition window appears.

Figure 6.1 Control panel (top) and Kinetic Acquisition window (bottom)



3. Select the type of data to acquire and set the acquisition parameters. (For more details on the acquisition parameters, see Table 6.1, page 71.)

Figure 6.2 Kinetic Acquisition window



4. Click the **Record** button Record to start acquisition. (After acquisition begins, the button changes to a **Stop** button Stop.) To stop acquisition, click the **Stop** button Stop.

   The maximum vs. time graph appears when kinetic acquisition begins and plots the maximum intensity signal in each frame. The graph provides a convenient way to look for signal trends or select particular frames for viewing.

Figure 6.3 Maximum vs. time graph

- Click a point in the graph to view the corresponding image (frame)
- Put the mouse pointer over the graph to view a tooltip that shows the frame number and time
- Right-click the graph to view the available shortcut menu of graph display options

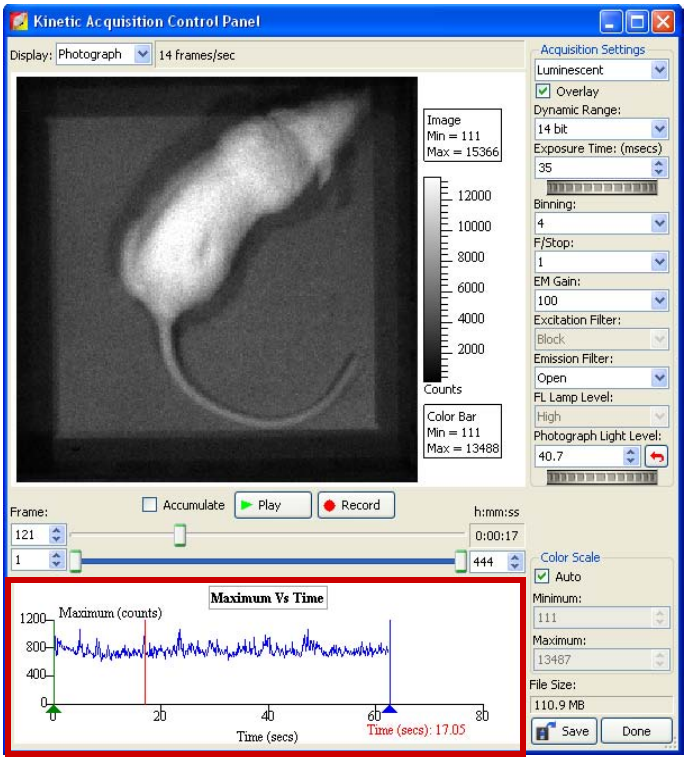


Table 6.1 Kinetic acquisition settings

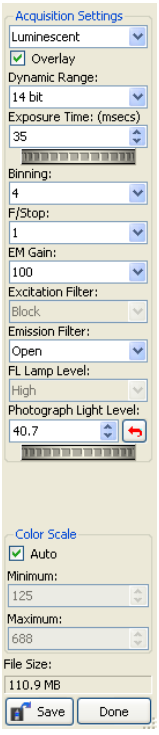
Item	Description
	Select the type of data to acquire (luminescent or fluorescent) from this drop-down list. Choose the Overlay option to also acquire photographs.
Overlay	Choose this option to acquire photographs.

Table 6.1 Kinetic acquisition settings (continued)

Item	Description
Dynamic Range	14 bit - If this option is chosen, the signal intensities range from 0 to 16383 counts per pixel. 16 bit - If this option is chosen, the signal intensities range from 0 to 65535 counts per pixel. **Note:** The14 bit dynamic range enables faster imaging.
Exposure Time (msecs)	The exposure time for the luminescent image. Shorter exposure times enable faster frame rates; longer exposure times provide greater sensitivity. The14 bit dynamic range enables faster imaging by attaining a higher frame rate at the cost of a smaller dynamic range.
Binning	Controls the pixel size on the CCD camera. Increasing the binning increases the pixel size, sensitivity, and frame rate, but reduces spatial resolution. Using larger binning for a luminescent or fluorescent image can significantly improve the signal-to-noise ratio. The loss of spatial resolution at high binning is often acceptable for *in vivo* images where light emission is diffuse. For more details on binning, see Appendix C, page 276. Recommended binning: 1-4 for imaging of cells or tissue sections, 4-8 for *in vivo* imaging of subjects, and 8-16 for *in vivo* imaging of subjects with very dim sources.
F/Stop	Sets the size of the camera lens aperture.The aperture size controls the amount of light detected and the depth of field. A larger f/stop number corresponds to a smaller aperture size and results in lower sensitivity because less light is collected for the image. However, a smaller aperture usually results in better image sharpness and depth of field. In kinetic mode, the photographic and luminescent (or fluorescent) image are acquired at the same F/Stop. For more details on f/stop, see Appendix C, page 275.
EM Gain	Multiplies the signal in real time. This option is useful for boosting low signals above the background noise. For kinetic imaging, the EM gain may be set to 50, 100, or 250. For conventional 16-bit still image acquisition, EM gain may be set to Off, 50, 100, or 250.
Excitation Filter	A drop-down list of fluorescence excitation filters. For fluorescent imaging, choose the appropriate filter for your application (GFP, DsRed, Cy5.5, or ICG). For luminescent imaging, **Block** is selected by default.
Emission Filter	A drop-down list of fluorescence emission filters. For fluorescent imaging, choose the appropriate filter for your application (GFP, DsRed, Cy5.5, or ICG). For luminescent imaging, **Open** is selected by default.
FL Lamp Level	Sets the illumination intensity level of the excitation lamp used in fluorescent imaging (Off, Low, or High). Low - This setting is approximately 18% of the High setting. **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.
Photograph Light Level	Controls the brightness of the lights at the top of the imaging chamber that are used to acquire photographic images.
Accumulate	Select this option to view the cumulative intensity signal in real time. When this option is chosen, the software computes and visualizes the cumulative signal in each frame.

Table 6.1 Kinetic acquisition settings (continued)

Item	Description
Color Scale	Auto - If this option is chosen, the software chooses the color scale minimum and maximum. **Note:** Do not choose this option if the Accumulate option is selected. Minimum - A user-specified threshold for the color scale minimum that is applied to the data if the Auto option is not selected. Intensity signals less than the minimum are not displayed. Maximum - A user-specified threshold for the color scale maximum that is applied to the data if the Auto option is not selected.
File Size	Displays the file size of the kinetic stream (.dcm) being acquired. The file size display is only available in the Kinetic Acquisition panel.
Save	Click to select an option for saving the data: Save Current Image - Saves the currently selected frame (single image, photograph, and read bias). Save Accumulated Image - Saves the accumulated signal for the selected frames (.tiff). Save Kinetic Data - Saves all selected photographic, luminescent or fluorescent images (frames) and the read bias image (.dcm). The signal is not accumulated.
Done	Closes the Kinetic Acquisition window

Accumulated Signal

The Accumulate option enables you to view increasing signals in real time. If you plan to accumulate signals, it is recommended that you perform a test acquisition to optimize settings so that the photographic image, luminescent, or fluorescent signal is not saturated.

To perform a test acquisition:

1. Confirm that the Accumulate option is selected. Do not select the Auto color scale option.
2. Start the acquisition (click the **Record** button 

).
3. If the photographic image is saturated, stop the acquisition (click the 

button) and reduce the photograph light level.

CAUTION

CAUTION! Extended acquisition of saturated images can shorten the life of the EMCCD and should be avoided.

4. Restart the acquisition. If necessary, repeat step 3 and step 4.

6.2 High Resolution Imaging With the Optical Zoom Lens Attachment

The optional Optical Zoom Lens attachment enables close up and high resolution imaging on the IVIS Lumina, IVIS Lumina XR, and IVIS Kinetic Imaging Systems. When the Zoom lens attachment is installed, only the "Z" field of view setting (2.6 cm) is available for single-image or sequence acquisition.

The imaging system is set to the Z field of view position until the Zoom lens attachment is removed. If the Zoom lens attachment is installed when the Living Image software is closed, the stage will move to the Z position when the system is initialized.

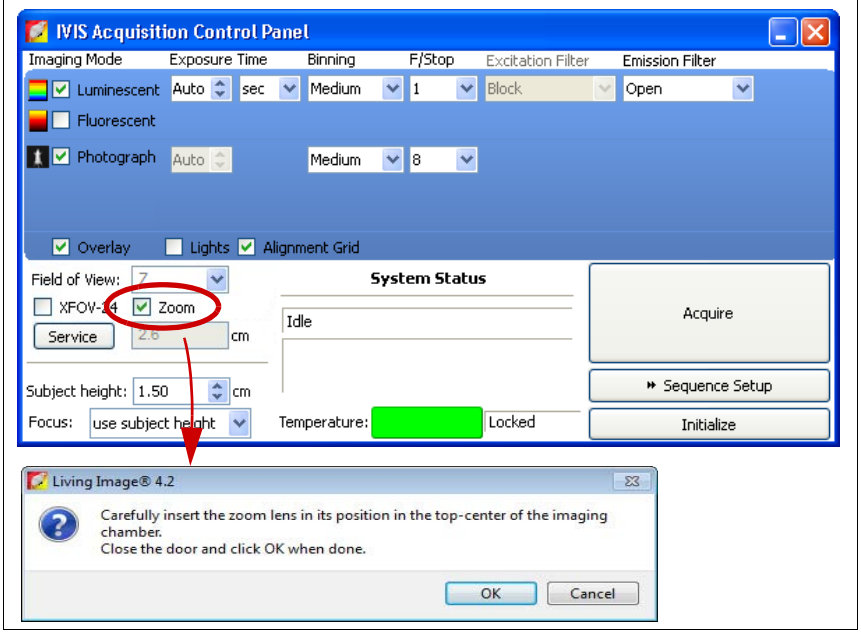
NOTE

When installing or removing the Optical Zoom Lens attachment, avoid touching the optical glass.

Installing the Zoom Lens Attachment

1. Choose the Zoom option in the control panel (Figure 6.4).
   You are prompted to insert the Zoom Lens attachment.

Figure 6.4 Control panel, Zoom option selected



2. After you install the Zoom Lens attachment in the imaging chamber, click **OK** in the prompt.
   The stage moves to the "Z" field of view position.

NOTE

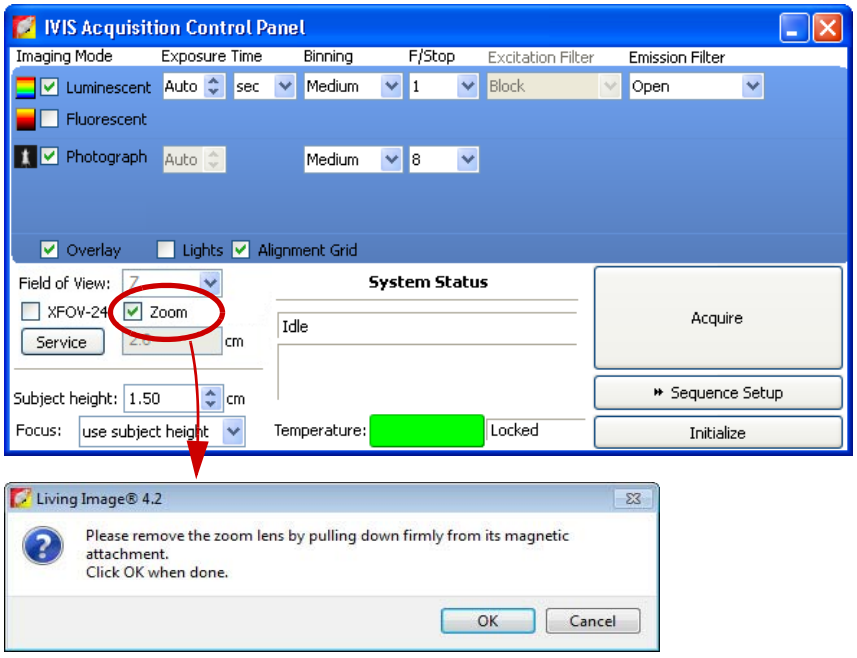
When the Zoom Lens attachment is installed, single images or sequential images can only be acquired at the Z field of view setting. During sequence setup, if you attempt to change away from the Z setting, the sequence table will be cleared. If you attempt to change from another field of view setting to the Z setting, the sequence table is cleared of all previous settings before the camera settings for the Z position are added.

Removing the Zoom Len Attachment

The imaging system is set to the “Z” field of view until the Zoom Lens attachment is removed.

1. Remove the check mark next to Zoom in the control panel (Figure 6.5).
   The stage moves to position C, then you are prompted to remove the lens attachment.
2. After you remove the Zoom Lens attachment, click **OK** in the prompt.
   Always store the lens wrapped in its protective container.

Figure 6.5 Control panel, remove the check mark next to Zoom



6.3 Viewing & Editing Data in the Kinetic Acquisition Window

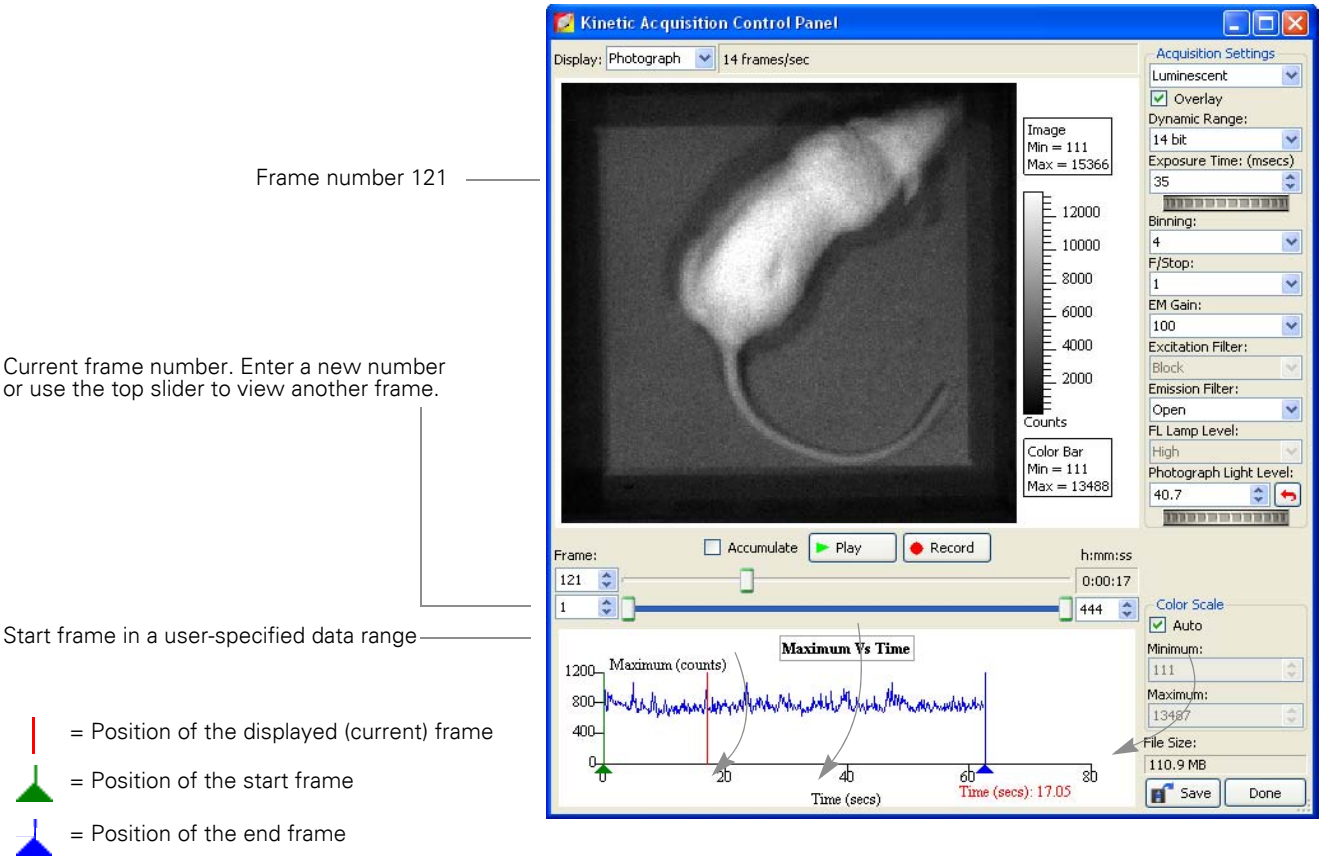
After stopping acquisition, you can view the data in the Kinetic Acquisition window.

1. To start the playback, click the **Play** button [Play]. (After playback starts, the button changes to a **Stop** button [Stop].).
2. To stop the playback, click the **Stop** button [Stop].
3. To view a particular frame, do either of the following:
   - Move the top frame slider or enter a frame number in the box next to the frame slider
   - Click a location in the Maximum vs. Time graph
4. To select a particular range of kinetic data, move the start and end frames selection handles. Alternatively, enter a frame number in the box next to each slider. Only the selected frames will be played back or saved.

NOTE

Kinetic data (.dcm) can also be edited in the Image window. For more details, see page 109.

Figure 6.6 Acquisition window



Viewing Options

After acquisition has been stopped, right-click the image to access a shortcut menu of viewing options.

Table 6.2 Kinetic view options

Item	Description
Zoom Area	To magnify a particular area, draw a box around the area that you want to zoom in on, right-click the area and select Zoom Area on the shortcut menu.
Zoom In	Incrementally magnifies the view.
Zoom Out	Incrementally reduces the magnification.
Reset Zoom	Returns the image to the default display magnification.
Pan View	Enables you to view a different area of a magnified image. To view another area of the image, choose this option, then click and hold the pointer while you move the mouse over the image.
Crop Area	To crop the image, draw a rectangle over the area of interest in the image, then right-click the area in the box and select Crop Area on the shortcut menu.
Draw Grid	Displays a grid over the frame.
Display Color Bar	Choose this option to display the color scale.
Display Color Min/Max	Choose this option to display the color scale minimum and maximum.
Display Image Min/Max	Choose this option to display the minimum and maximum signal.

6.4 Saving Kinetic Data

The IVIS Kinetic instrument enables you to acquire a real-time data stream which can generate very large files. The file size limit for DICOM data is 2GB. Kinetic data acquisition automatically stops when this file size limit is reached. Table 6.3 shows how binning conditions affect the total number of frames that can be collected in overlay or luminescent/fluorescent only mode.

Table 6.3 Frames collected per 1 GB DICOM file

Binning Level	Frame Size	Overlay Mode	Luminescent or Fluorescent Only	DICOM File Size
		Total Frames Collected		
Bin 1	2 MB	250	500	1 GB
Bin 2	512 KB	975	1950	
Bin 4	128 KB	3900	7800	
Bin 8	32 KB	15600	31250	
Bin 16	8 KB	62500	125000	

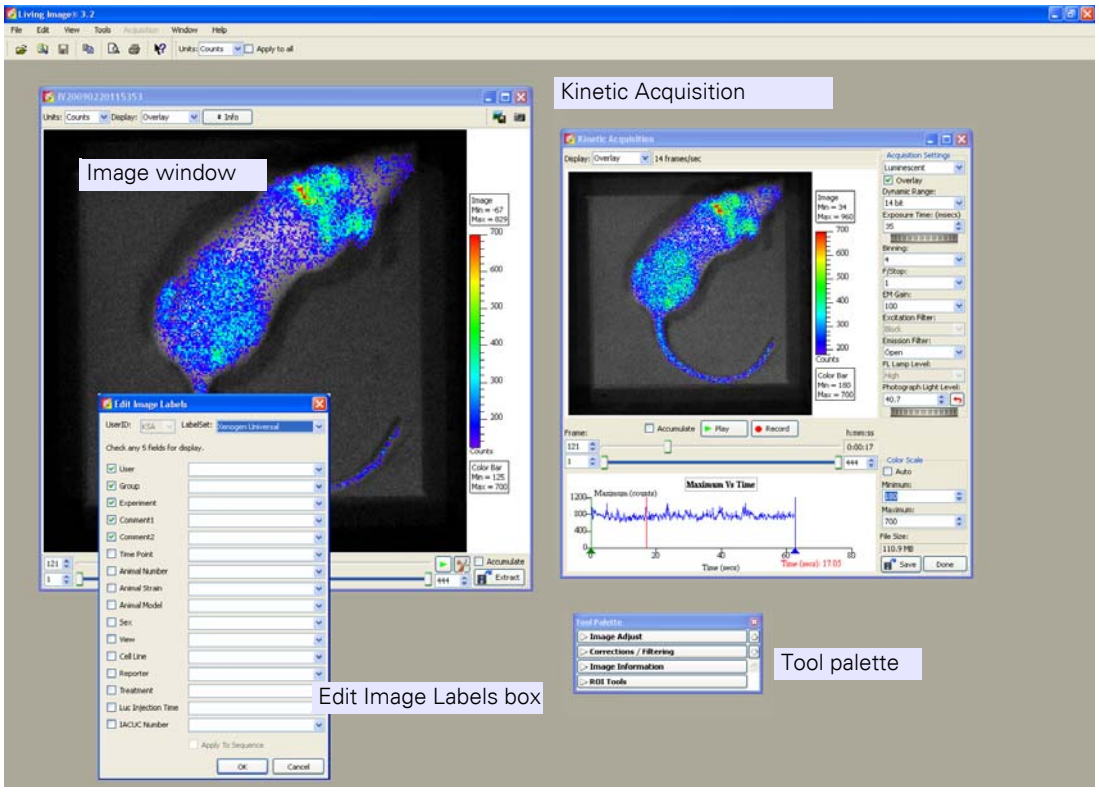
To save data:

1. In the Kinetic Acquisition window, click the **Save** button and select a save option.

Save Option	Description
Save → Current Image	Saves the currently displayed frame.
Save → Accumulated Image	Saves the accumulated signal for the selected frames (.tiff). **Note:** It is not necessary to select the Accumulate option to save an accumulated image.
Save → Kinetic Data	Saves the data (photographic frames, all luminescent or fluorescent frames, and read bias) in DICOM format (.dcm).

2. In the Edit Image Labels box that appears, enter information for the image label and click **OK**. If you do not want to enter label information, click **Cancel**.

Figure 6.7 The Edit Image Labels box, Image window, and Tool Palette appear when saving an image or kinetic stream



NOTE

You can edit and analyze kinetic data in the Image window.

6.5 Playing Kinetic Data

In the image window, you can:

- Play kinetic data
- Select and view a particular image
- Select a range of images and extract as a separate kinetic data set

Figure 6.8 Image window, kinetic data

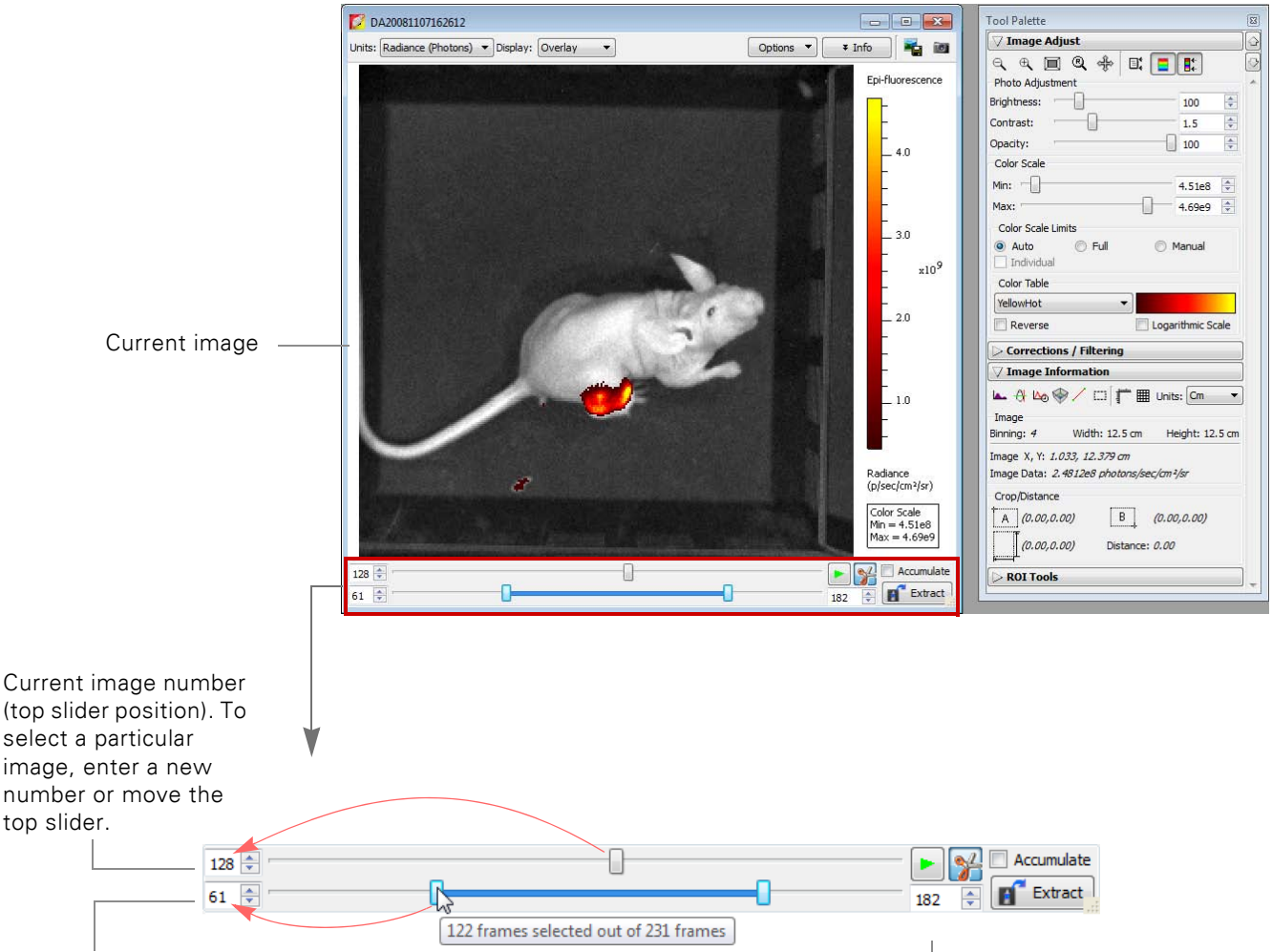


Figure 6.9 Image window, selecting kinetic data for export
Put the mouse arrow over a bottom slider to view a tooltip that shows the number of selected images (frames)

Table 6.4 Image window, kinetic data

Item	Description
Play	Starts playing kinetic data.
Stop	Stops playing kinetic data.
Edit and Save options	Shows or hides the bottom sliders that enable you to select a range of data and the Extract button that provides save options for the user-selected image or data.
Accumulate	If this option is chosen, the software computes and displays the cumulative intensity signal. Choose this option and playback the kinetic data to visualize accumulation as it happens.
Extract	Click to select a save option for the current image or selected data.
Extract Current Image	Displays the current image in a new image window.The software prompts you to save the image when you close the image window.
Extract Accumulated Image	The software computes the cumulative signal for each image (sum of the signal in all images up to and including the current image), then displays the cumulative signal of the current image in a new image window. The software prompts you to save the image when you close the image window.
Extract Kinetic Data	Choose this option if you want to save a particular range of images. Opens the Browse For Folder dialog box that enables you to select where to save the selected data.
Save As a Movie	Saves the kinetic data set as a movie (.mp4, .mov, .avi, .mpg)

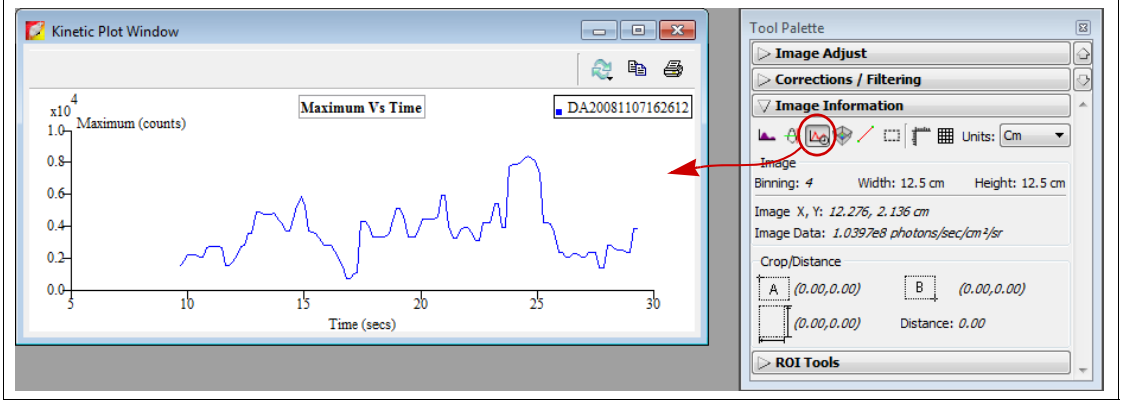
Viewing Kinetic Data

1. Open the kinetic data.
2. To start playing the kinetic data, click the **Play** button. If you want to start the playback at a particular image, first move the top slider to the starting image, then click the **Play** button.
3. To stop playing data, click the **Stop** button.
4. To view the cumulative signal during playback, choose the Accumulate option. If the accumulated image maximum exceeds the current color scale range, use the image adjust tools to adjust the color scale.

Kinetic Plot

The Kinetic Plot is a graph of the maximum signal versus time. To view the Kinetic Plot, click the button in the Image Information tools.

Figure 6.10 Kinetic Plot



Exporting Kinetic Data

You can select a range of images for export to DICOM format (includes photographs, intensity signal, and read bias) or to a movie.

1. In the image window, click the button (Figure 6.9, page 80).
2. If you want to select a particular range of data for export, use the frame range selection to select the data. Use the left slider to select the start image and the right slider to select the end image in the data range of interest.

   The top slider automatically moves to denote the location of the current image with respect to the selected data range.
3. To export the selected data to a movie:
   a. Click **Extract** and choose **Save as a Movie**.
   b. In the dialog box that appears, select a folder, enter a name for the movie, and choose the file format (for example, .mpg4).

Exporting an Image from a Kinetic Data Set

1. To select an image, move the frame slider or enter a frame number in the spin box.

Figure 6.11 Image window, selecting an image for export

2. Click **Extract** and choose **Extract Current Image**.

   A new image window appears and displays the selected image.

3. To save a snapshot of the current image, click the Export Graphics button in the image window. In the dialog box that appears, select a destination folder, enter a file name, select a file type, and click **Save**.

7 Working With Optical Image Data

This chapter explains how to open (*load*), view, and perform other operations on the optical image data obtained on an IVIS® Imaging System.

7.1 Opening Optical Image Data

You can load optical images from the:

- Living Image Browser (see below)
- Toolbar or menu bar (page 87)

Multiple data sets can be open at the same time.

NOTE

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

Loading Optical Images From the Living Image Browser

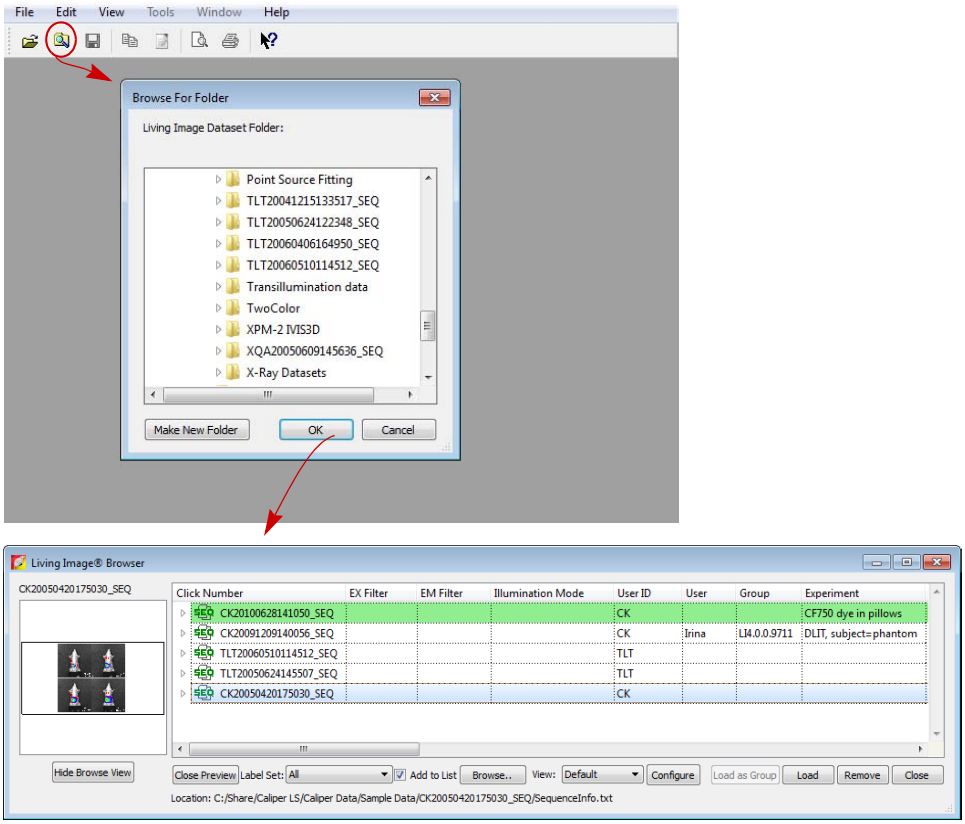
The Living Image Browser provides a convenient way to browse and preview optical data, view information about the data, and load the data.

To start the browser:

1. Click the **Browse** button  . Alternatively, select **File** → **Browse** on the menu bar.
2. In the dialog box that appears, select the folder of interest and click **OK**.

   The Living Image Browser appears (Figure 7.1).

Figure 7.1 Opening the Living Image Browser



Living Image Browser

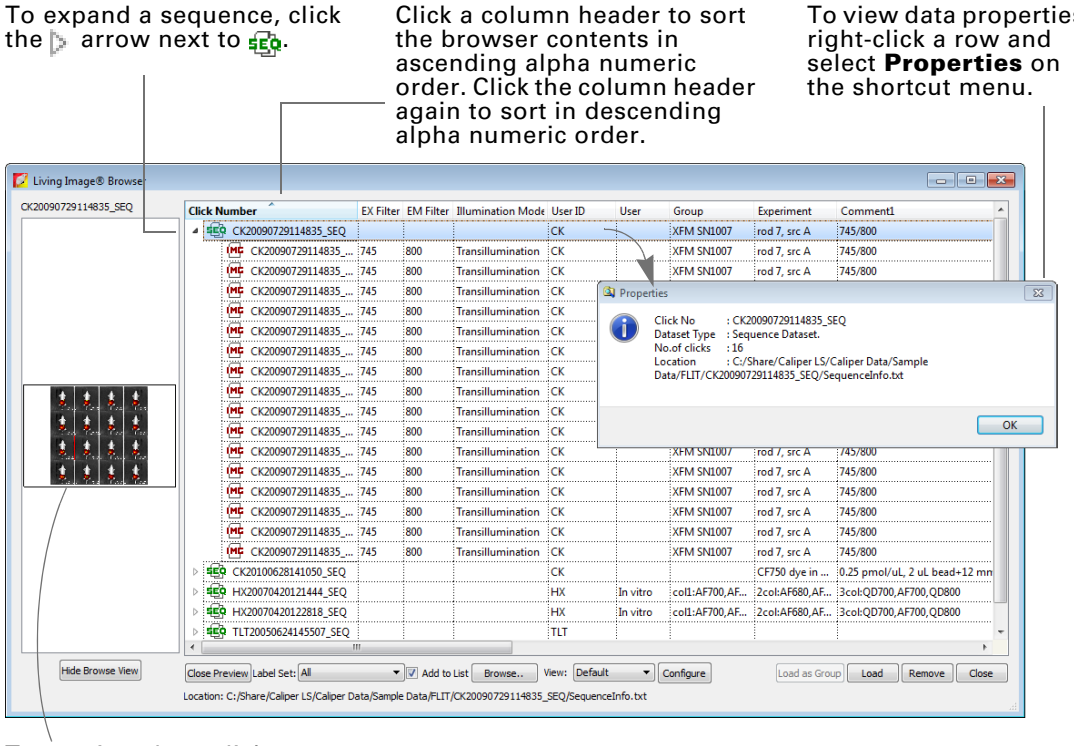
NOTE

The next time you start the Living Image software and open the Browse For Folder box, the software automatically returns to the last folder visited.

The Living Image Browser displays the selected data along with the user ID, label information, and camera configuration information.

- image
- image sequence
- kinetic data or image exported as DICOM file

Figure 7.2 Living Image Browser



Note: A preview is only available if a "snapshot" was taken of the image or sequence thumbnails in the image window. For more details on acquiring a snapshot, see Chapter 4, page 25.

3. To load data, do 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 thumbnail

   The image(s) and Tool Palette are displayed. Green rows in the browser indicate loaded data (Figure 7.3).

Figure 7.3 Image sequences opened ("loaded")
Multiple data sets can be loaded at the same time.

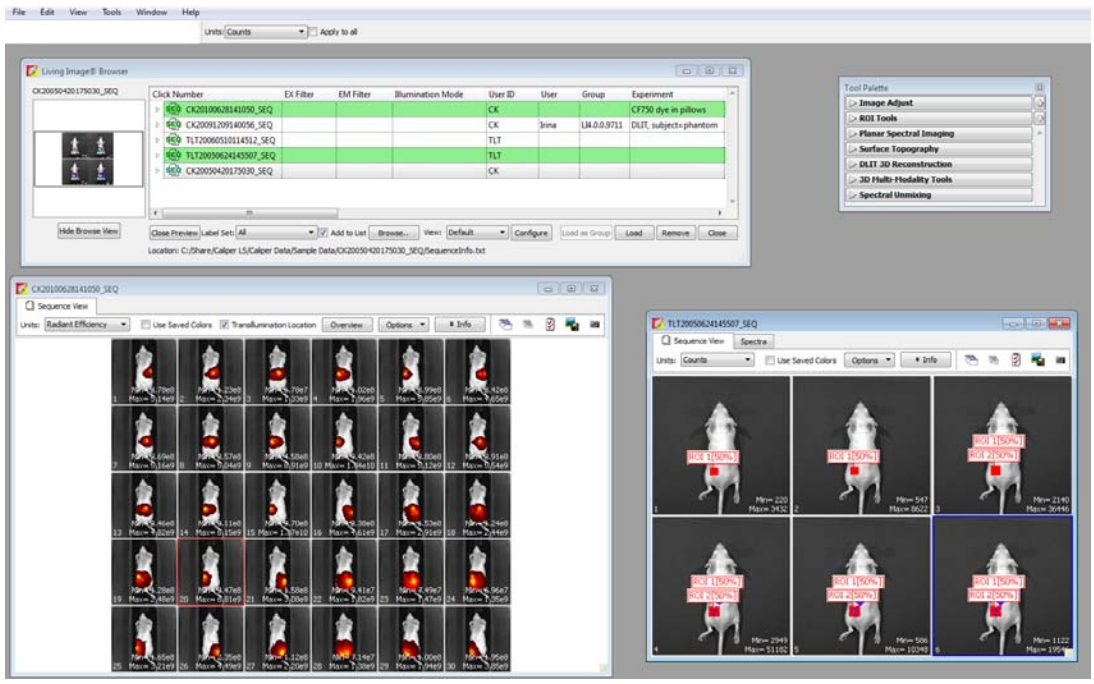


Table 7.1 Living Image Browser

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) that is displayed in the Living Image Browser.
Add to List	If this option is chosen, the selected in the Browse for Folder box is added to the Living Image Browser. If this option is not chosen, the data selected in the Browse for Folder box replaces the contents of the Living Image Browser, except for loaded data.
Browse	Opens the Browse For Folder box.
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 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 7.1 Living Image Browser (continued)

Item	Description
Load as Group	Enables you to select particular images that you want to view as a sequence. The images may be acquired during different sessions. To select adjacent images in the browser, press and hold the **Shift** key while you click the first and last file in the selection. To select non-adjacent images in the browser: PC users: Press and hold the **Ctrl** key while you click the images in the browser Macintosh users: Press and hold the **Cmd** key (apple key) while you click the images in the browser. **Note:** The **Load as Group** option is only available when two or more images (non-kinetic) are selected in the browser.
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.

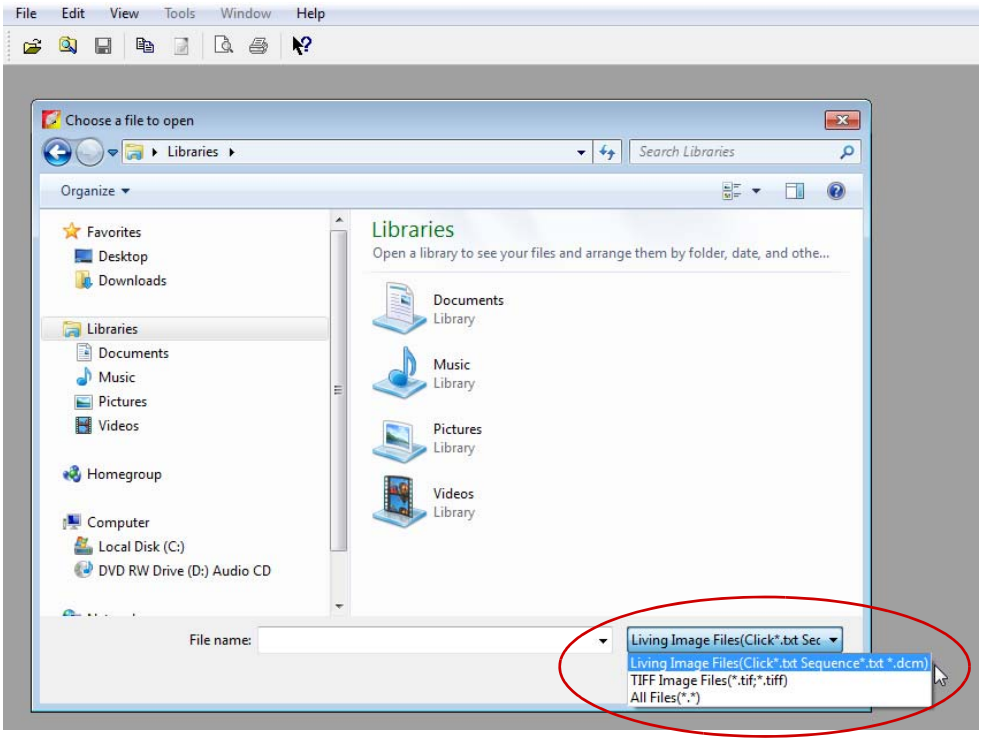
Opening Data from the Menu or Toolbar

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. In the box that appears, choose a file type filter from the drop-down list (Figure 7.4).

Figure 7.4 Opening data from the toolbar or menu bar



Select the file type(s)

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.

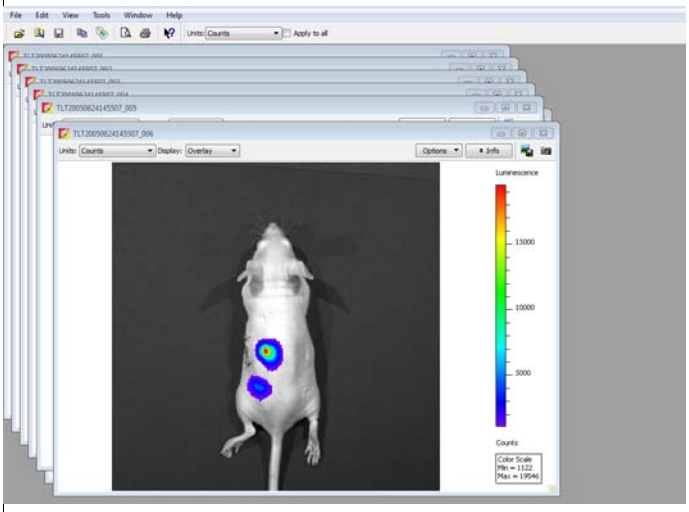
3. Navigate to the file and click double-click it. Alternatively, select the data and click **Open**.

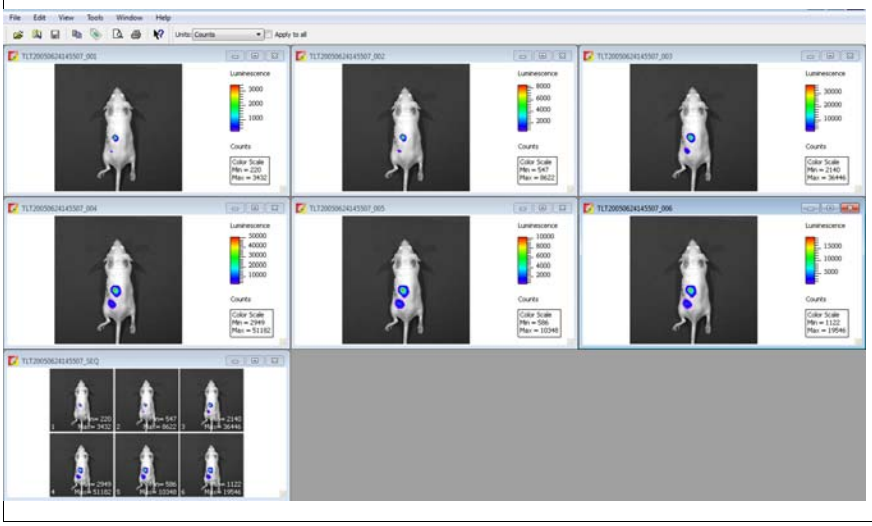
Organizing Images

When multiple image windows are open, you can organize them in a cascade or tile arrangement.

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

Figure 7.5 Image windows: cascade (top) or tiled (bottom)





7.2 About the Image Window & Tool Palette

Image Window

An image, image sequence, or kinetic data set is displayed in an image window. Multiple image windows can be open at the same time.

Figure 7.6 Image windows, sequence view and single image
The options available in the image window depend on the type of active image data.

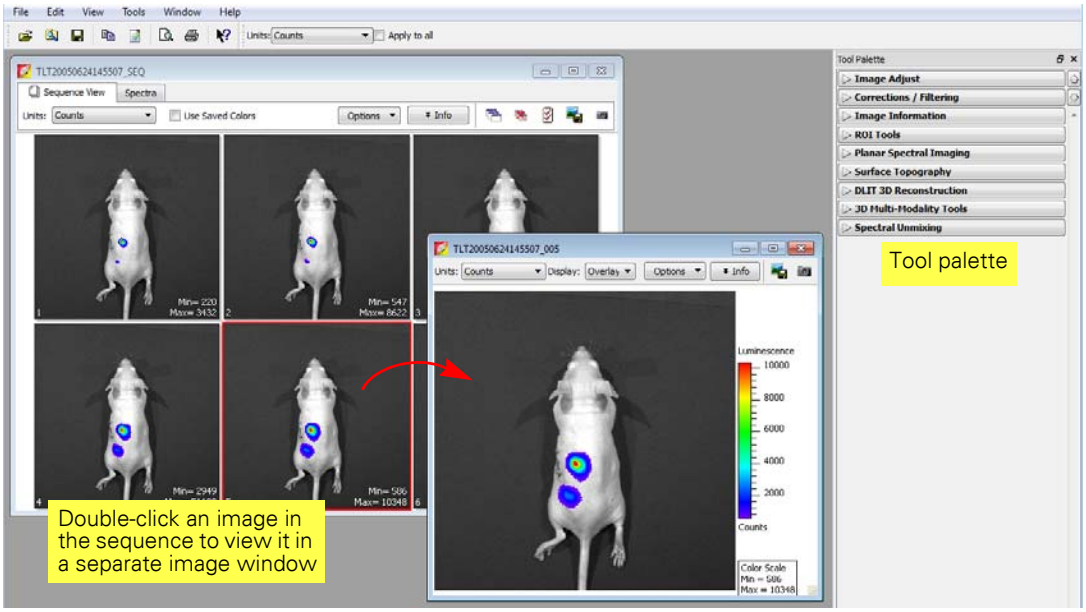


Table 7.2 Image window

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. For more details on measurement units, see page 281.
Use Saved Colors (image sequence)	Choose this option to display the image data using the color table that was specified in the Preferences at the time of acquisition. If this option is not selected, image data are displayed using the color table currently specified in the Preferences.

Table 7.2 Image window (continued)

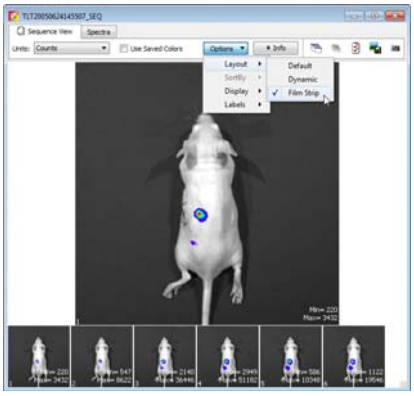
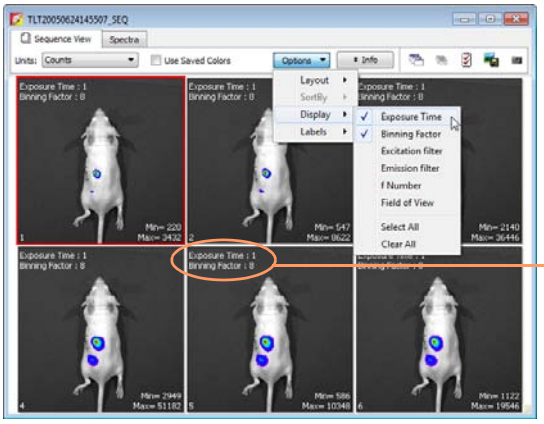
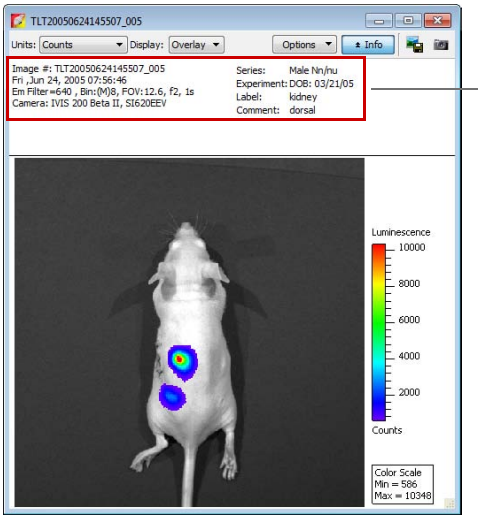
Item	Description
Options (image sequence)	Layout – Choose a display option for the images in a sequence (Default, Dynamic, or Film Strip). For example, here is Film Strip mode:  Sort by – Options for ordering images in the sequence window: Default – Order in which the images are stored in the folder. TimeStamp – Ascending order of the image acquisition time. UserID – Ascending alphanumeric order of the user ID. Display - Choose the types of information to display with each image.  In this example, exposure time and binning factor are displayed on each image Lablels – Enables you to select the information to include in the image label.  Image label

Table 7.2 Image window (continued)

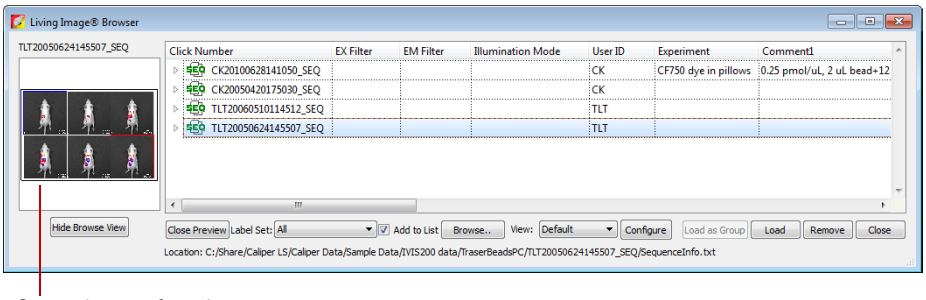
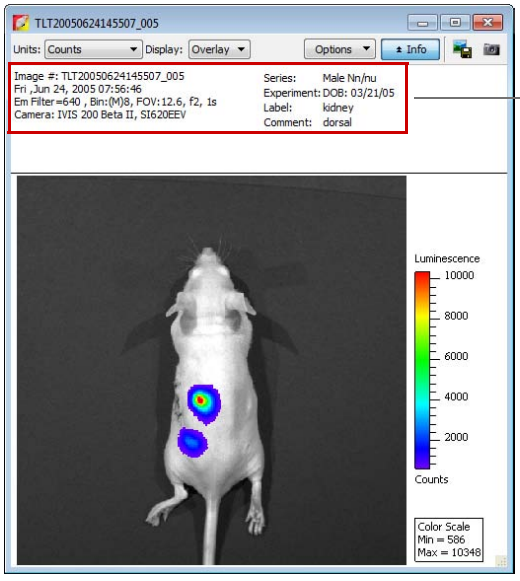
Item	Description
Info	Click to show or hide the image label. The image label includes information you enter in the Edit Image Labels dialog box (see page 23) and other information automatically recorded by the software.
	Opens all of the images in a sequence.
	Closes all open images of a sequence.
	Opens the Edit Sequence dialog box that enables you to add or remove images from a sequence.
	Opens a dialog box that enables you to export the active view as a graphic file.
	Takes a "snapshot" that is displayed with the data in the Living Image Browser. For more details on the browser, see page 83.  Snapshots of an image sequence
Display (single image)	A list of image types available for display, for example, overlay. For more details on the different types of image displays, see Table 2.2, page 8. **Note:** If the acquisition included more than two imaging modes (for example, luminescent, x-ray, and photograph), additional drop-down lists appear so you can choose any two images to overlay.  To change the foreground or background, click the button or select from the drop-down list.
Options (single image)	Enables you to select the type of information to include in the image label. 

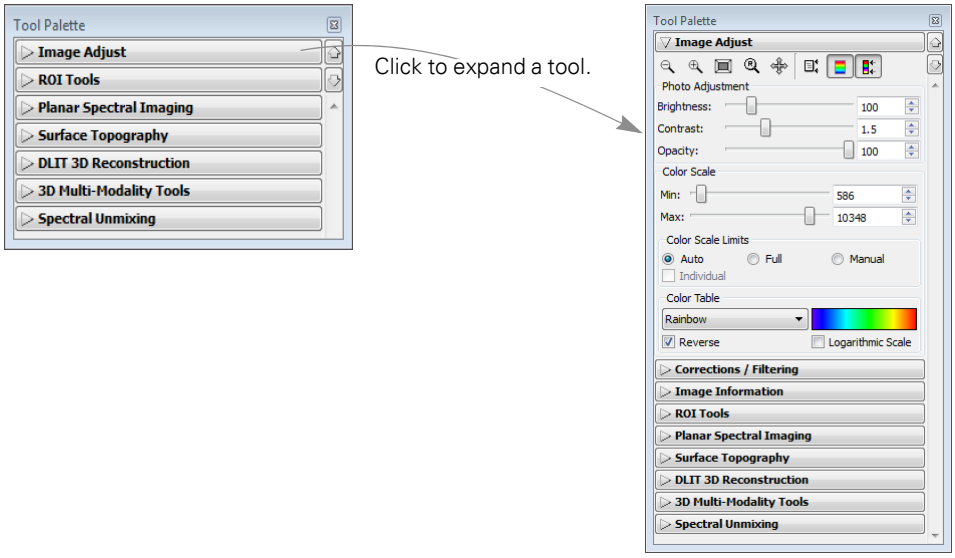
Table 7.2 Image window (continued)

Item	Description
Color Scale (single image)	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

Tool Palette

The Tool Palette appears when you open an image or sequence. The options available in the Tool Palette depend on the type of active image data.

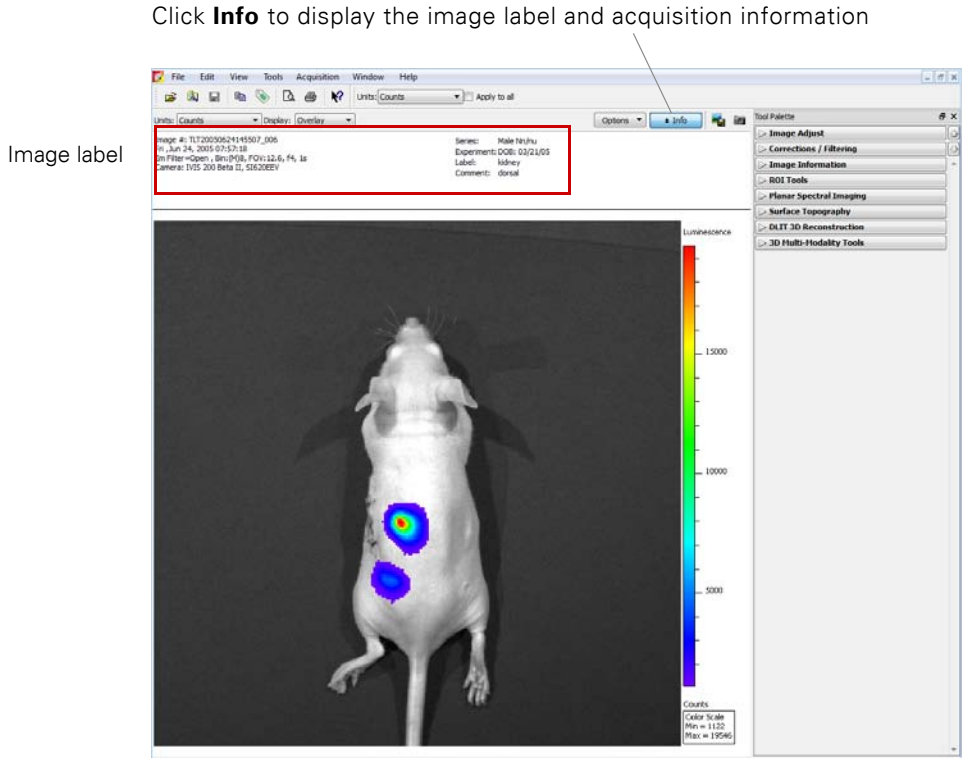
Figure 7.7 Tool palette
For an overview of the tools, see Figure 2.4 on page 11and Figure 2.5 on page 12.



7.3 Viewing Image Information

At acquisition, the software captures image information that includes all of the text information that is associated with an image, for example, camera parameters and any image label information entered at acquisition (Figure 7.8).

Figure 7.8 Image window displaying image information

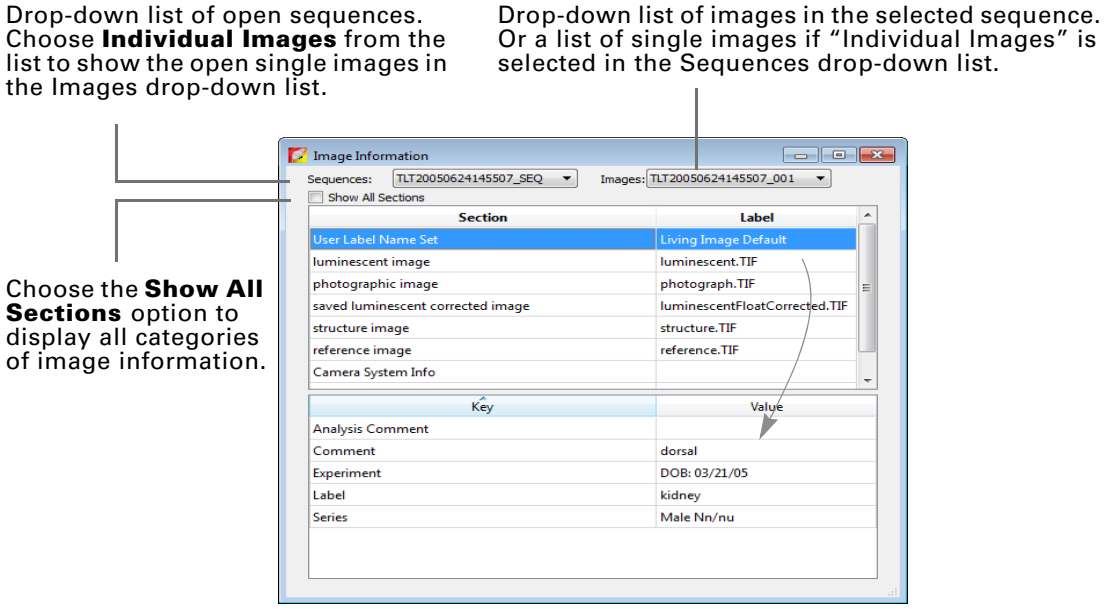


Another way to view information about images is available in the View menu.

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 7.9).

Figure 7.9 Viewing image information



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

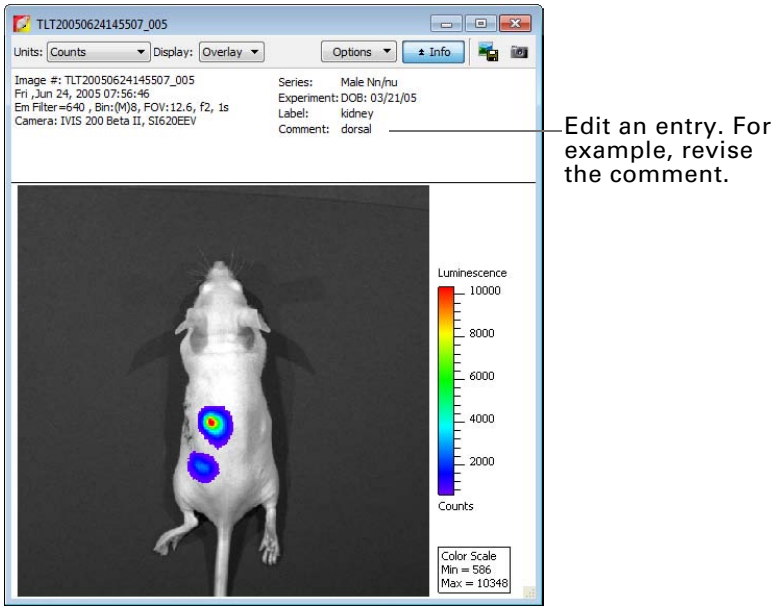
Editing the Image Label

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

To edit the image information:

1. Open an image or sequence.
2. Click **Info** to display the image label.

Figure 7.10 Image information



3. Edit the label information.

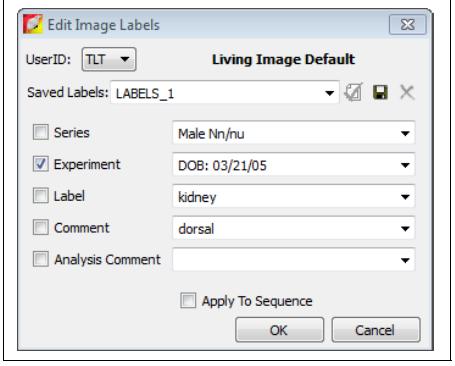
To add information to the image label:

1. Click the toolbar button. Alternatively, select **Edit** → **Image Labels** on the menu bar.
2. In the Edit Image Labels box that appears, select information and/or enter a comment (Figure 7.11).

NOTE

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

Figure 7.11 Edit Image Labels



3. When finished, click **OK**.

   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).

7.4 Adding Comments or Tags to an Image

Adding Comments

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

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 7.12).

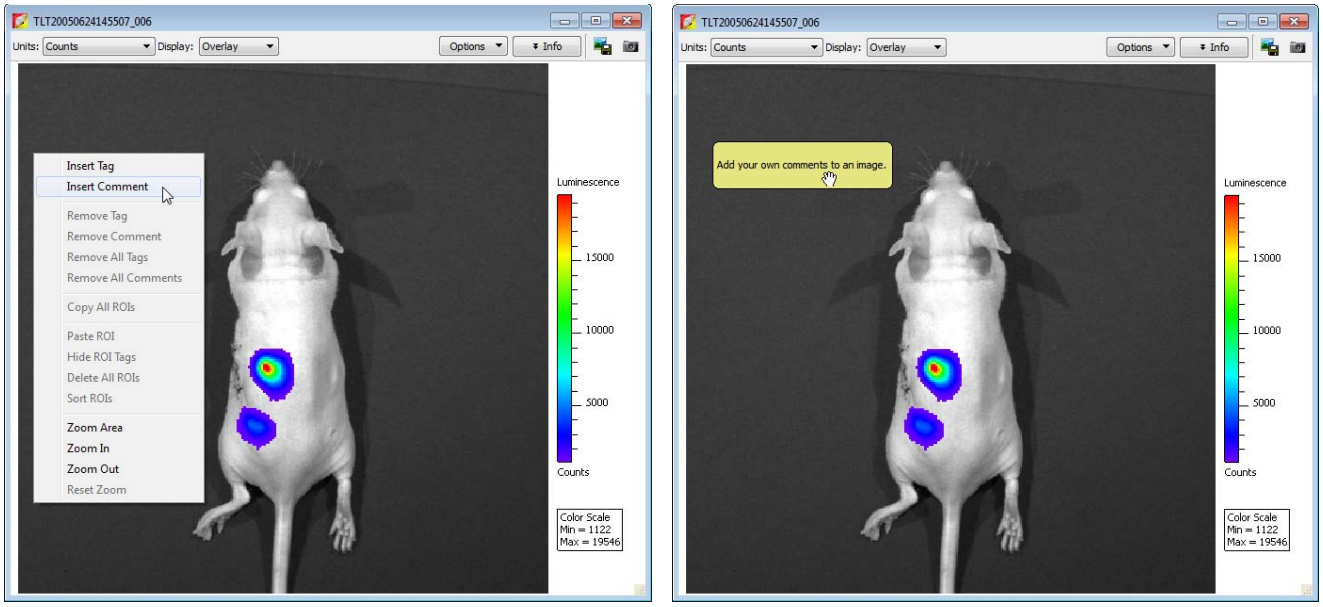
To reposition a comment:

1. Position the mouse pointer over the comment.
2. When the hand tool appears, use a click-and-drag operation to move the comment box, then click the mouse to set the location.

To remove a comment(s):

- To remove a comment, right-click the 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 7.12 Add a comment to an image



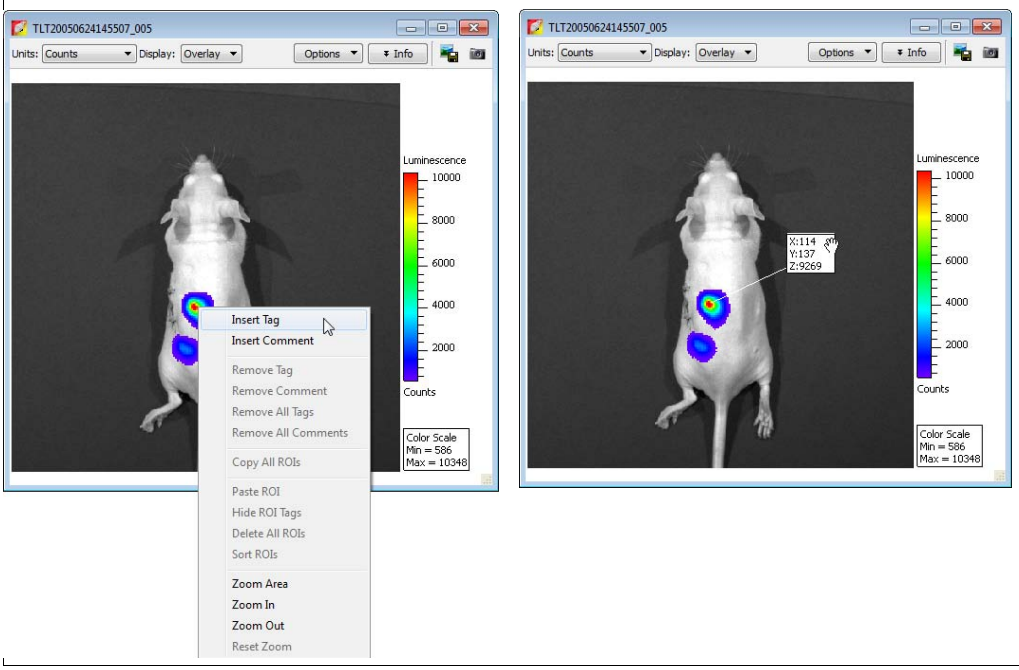
Tagging an Image

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

To apply a tag:

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

Figure 7.13 Insert a tag on an image (left), move the tag label (right)



To move a tag:

1. Position the mouse pointer over the tag.
2. When the hand tool appears, use a click-and-drag operation to move 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.

7.5 Adjusting Image Appearance

Use the image adjust tools to adjust the appearance of an image (Figure 7.14).

NOTE

Not all tools are available for all image display modes.

Figure 7.14 Tool palette, Image Adjust tools

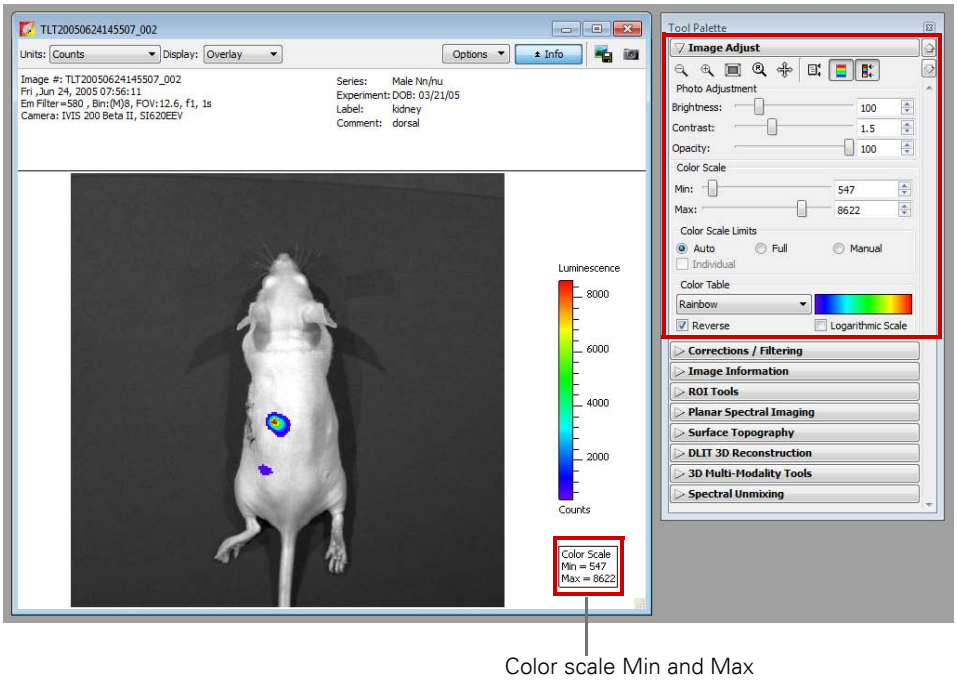


Table 7.3 Image Adjust tools

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 (**Ctrl**-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 to magnify the area inside a rectangle that you draw using a click-and-drag operation. (Sets the dimensions of the magnified area equal to image window dimensions.)
	Click this button to return the image to the default display magnification.

Table 7.3 Image Adjust tools (continued)

Item	Description
	Click this button to move a magnified image (*pan*) in the image window. For more details, see page 99.
	Click this button to hide or display the image min/max information in the image window
	Click this button to hide or display the color scale in the image window
	Click this button to hide or display the color scale min/max information in the image window
Photo Adjustment	Brightness – Click and move the slider left or right to adjust the brightness of an image displayed in overlay or photograph mode. Alternatively, enter a brightness value. Contrast – Click and move the slider left or right to adjust the *gamma* of an image displayed in overlay mode. Alternatively, enter a gamma value. (Gamma is related to image contrast.) Opacity – Click and move the slider left or right to adjust the opacity of the pseudocolor luminescent data of an image displayed in overlay mode. Alternatively, enter an opacity value.
Color Scale	Min – The minimum pixel intensity associated with the color scale for an image. Pixels less than the minimum value are not displayed. Max – The maximum pixel intensity associated with the color scale for an image. Pixels greater than the maximum value are displayed in the maximum color.
Color Scale Limits	Auto – If this option is chosen, the software sets the Min and Max values to optimize image display and suppress background noise. The Min and Max settings can be manually adjusted to further optimize the image display for your needs. Full – Choose this option to set the Max and Min values to the maximum and minimum data values in the image. Manual – Choose this option to enter Max and Min values for the image display. Individual – Applies a separate color table to each image in a sequence. **Note:** This option is only available when an image sequence is active.
Color Table	 Click the drop-down arrow to select a color table for the image data. (For more details on color tables, see *Pseudocolor Images*, page 279.) Reverse – Choose this option to reverse the selected color table. Logarithmic Scale – Choose this option to apply a log scale to the relationship between numerical data and the color range in the color table. A log scale increases the range of meaningful numerical data that can be displayed.

Magnifying or Panning in the Image Window

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

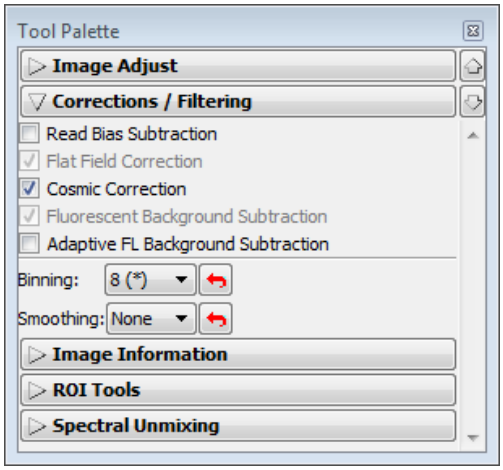
Panning helps you view different areas of a magnified image. If the image has not been magnified, you cannot pan the image.

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

7.6 Correcting Optical Image Data

Use the Corrections/Filtering tools to subtract background or apply corrections to the optical image data. (For more details on sources of background, see Appendix E, page 285.) You can also apply smoothing and soft binning to the image data. (For more information on binning and smoothing, see Appendix C, page 275.)

Figure 7.15 Tool palette, Corrections/Filtering tools



Read Bias Subtraction and Flat Field Correction are default mandatory corrections in photons mode. In counts mode, these corrections can be cleared.

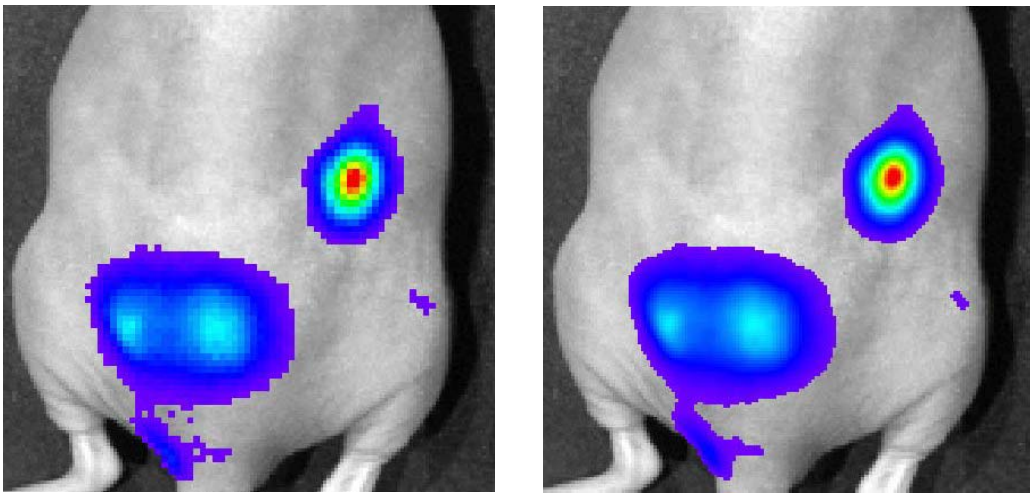
Table 7.4 Tool palette, Corrections/Filtering tools

Tool	Description
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. For more details on background, see Appendix E, page 285. **Note:** In Radiance (Photons) mode, dark background or read bias subtraction is a mandatory default. In counts mode, the check box can be cleared.

Table 7.4 Tool palette, Corrections/Filtering tools (continued)

Tool	Description
Flat Field Correction	Select this check box to apply a lens correction factor to the image data. For more details on flat field correction, see Appendix D, page 284. Note: In photons mode, flat field correction is a mandatory default. In counts mode, the check box can be cleared.
Cosmic Correction	Select this check box to correct image data for cosmic rays or other ionizing radiation that interact with the CCD. For more details on cosmic correction, see Appendix D, page 284.
Adaptive FL Background Subtraction	Opens the Photo Mask Setup box that enables you to set the photo mask for adaptive fluorescent background subtraction. For more details on adaptive fluorescent background subtraction, see Appendix F, page 305.
Binning	Specifies the number of pixels in the image data that are grouped together to form a larger pixel (called *soft* binning). Binning changes the pixel size in the image (Figure 7.16). For more details on binning, see Appendix C, page 276.
Smoothing	Computes the average signal of the specified number of pixels and replaces the original signal with the average signal (Figure 7.16). Smoothing removes signal noise without changing pixel size.
	Click this button to return the binning or smoothing to the previous setting and update the image.

Figure 7.16 Example of binning and smoothing image data



Binning at acquisition = 8, no smoothing

Binning = 2, smoothing = 5x5

7.7 Viewing Intensity Data & Making Measurements

The Image Information tools enable you to view intensity data and measure distance on an image. You can view pixel data in different formats:

Image Information	Description	See Page
x,y coordinates and associated intensity	The x,y pixel coordinates of the mouse pointer location in the image and the intensity (counts or photons) at that location.	102
Histogram	Histogram of pixel intensities in an image.	103
Line profile	Plots a line graph of intensity data at each pixel along a user-specified horizontal or vertical line in the image	104

Figure 7.17 Tool Palette, Image Information tools

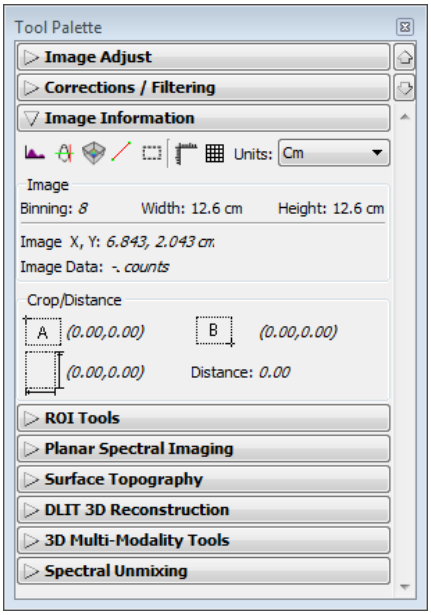


Table 7.5 Tool palette, Image Information tools

Tool	Description
	Click this button to display a histogram of pixel intensity. For more details, see page 103.
	Click this button to display a line profile. For more details, see page 104.
	Click this button to display a 3D representation of signal itensity. For mre details, see page 106.
	Click this button to display the distance measurement cursor in the image window. For more details, see page 107.
	Click this button to draw and measure a rectangle on an image. For more details, see page 108.
	Click this button to display/hide a scale on the x and y-axis of the image window.
	Click this button to display/hide a grid the image window.

Table 7.5 Tool palette, Image Information tools (continued) (continued)

Tool	Description
Units	Choose the units (cm or pixels) for distance measurements in the image window.
Image	Binning – The binning applied to the image. **Note:** If soft binning is applied to the image data, and the binning level is changed from 8 to 16, the new binning is indicated as 8x2. Width/Height – The FOV dimensions. **Note:** If "Pixels" is selected from the Units drop-down list, the dimensions are provided in terms of binned pixels. Image X,Y – The x,y pixel coordinates of the mouse pointer location in the image. Image Data – The intensity (counts or photons) at the pixel location of the mouse pointer.
Crop/Distance	 The x,y pixel coordinates at the upper left corner of the crop tool. OR The x,y pixel coordinates at the "A" end of the distance.  The x,y pixel coordinates at the lower right corner of the crop tool. OR The x,y pixel coordinates at the "B" end of the distance.  The width and height of the image crop tool. OR $^{\Delta}x$, $^{\Delta}y$ from the A to B end of the distance measurement cursor. For more details, see page 107 and 108.

Viewing X,Y Coordinates & Intensity Data

1. Open an image, and the Image Information tools, choose Cm or Pixels from the Units drop-down list.
2. Put the mouse pointer over a location of in the image.

   The x,y coordinates and intensity data are displayed in the Tool Palette.

Figure 7.18 x,y coordinates and intensity data at the mouse pointer location

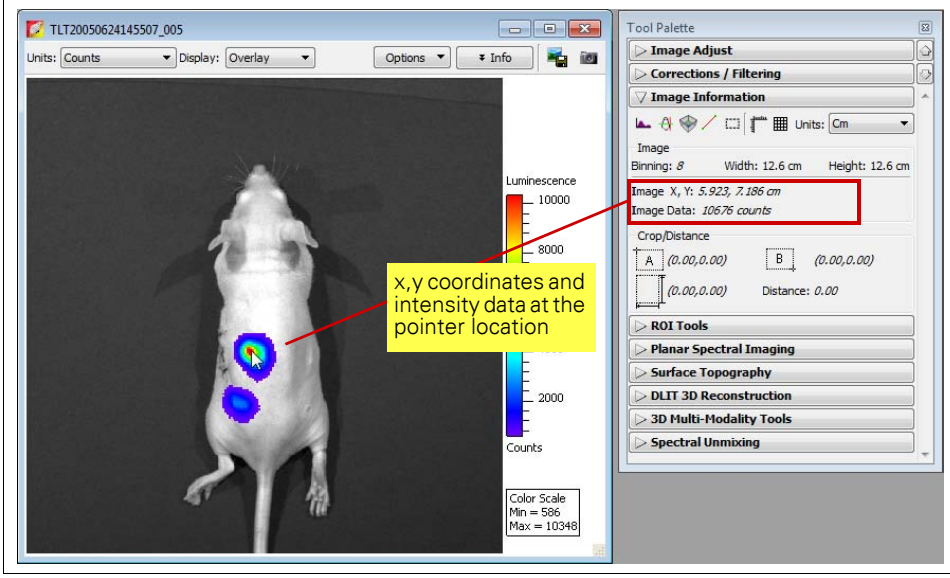


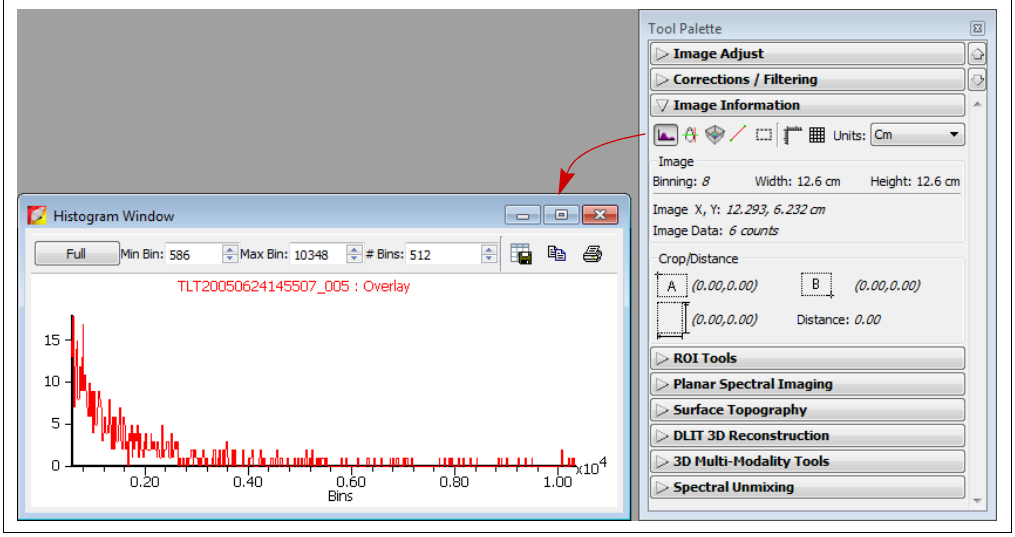
Image Histogram

The image histogram plots a frequency distribution of the pixel intensities in an image. The software sorts the intensities into groups or *bins* (x-axis) and plots the number of pixels per bin (y-axis).

To view the image histogram:

1. Open an image, and in the Image Information tools, click the **Image Histogram** button  .

Figure 7.19 View a histogram of pixel intensities



NOTE

By default the Auto min/max range of the image data determines the histogram range and bins (the software sets the min and max values to optimize image display and suppress background noise). To display the histogram using the full intensity range of the image, click **Full** in the Histogram window.

2. To edit the minimum or maximum bin intensity, enter a new value in the Min Bin or Max Bin box, or click the  arrows.
3. To edit the number of bins, enter a new value in the # Bins box or click the  arrows.

NOTE

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

Table 7.6 Histogram window

Item	Description
Full	Displays the histogram using the full intensity range of the image.
Min Bin	The lowest intensity bin.
Max Bin	The highest intensity bin.
# Bins	The total number of bins.
	Opens a dialog box that enables you to export the histogram (.csv).

Table 7.6 Histogram window (continued)

Item	Description
	Copies the histogram to the system clipboard.
	Opens the print dialog box.

Line Profile

The line profile plots intensity (y-axis) at each pixel (x-axis) along a user-specified line in the image. The line profile is automatically updated when you change the line position.

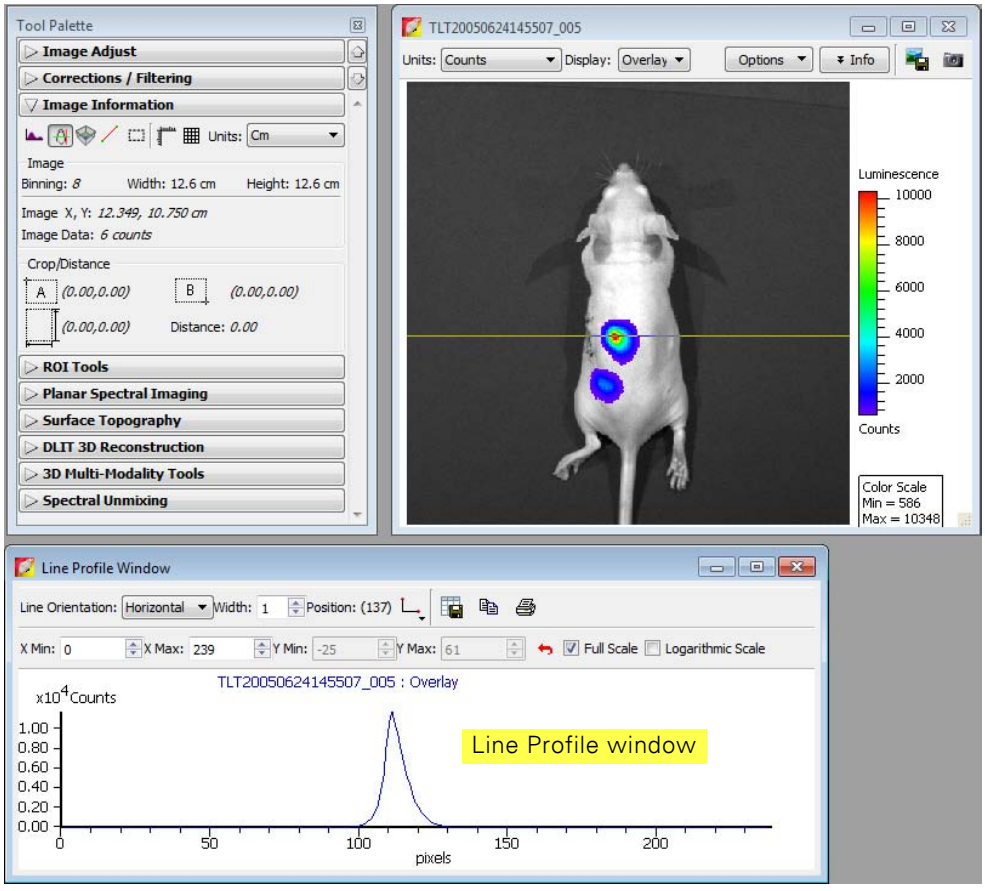
NOTE

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 the line profile:

1. Open an image, and in the Image Information tools, click the **Line Profile** button  .
   A line appears on the image and the Line Profile window appears.

Figure 7.20 Viewing a line profile of pixel intensities



2. To view the line profile at another location in the image, put the mouse pointer over the line. When the pointer becomes a ↕, drag the line over the image. The blue part of the line 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 7.7 Line Profile window

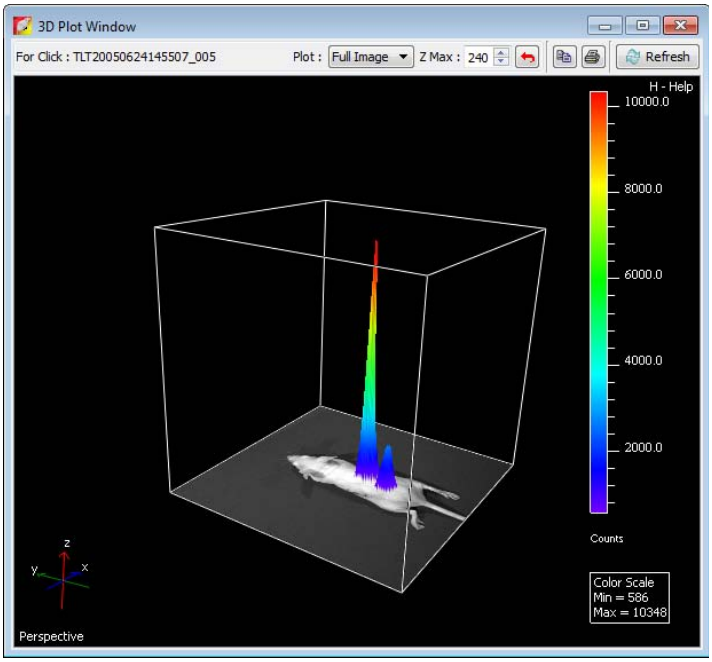
Item	Description
Line Orientation	Choose Vertical, Horizontal, or Free Hand from the drop-down list to set the orientation of the line in the image window. The Free Hand orientation enables you to drag each line segment endpoint to a user- selected position.
Width	Sets the line width.
Position	Line position (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.
	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 “photons” is selected in the image window, the x-axis units = pixels. If “counts” is selected in the image window, the x-axis units = cm. 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.

Viewing 3D Signal Intensity

1. Open an image and then click the Plot 3D button in the Image Information tools.

   A 3D representation of all signals in the image is displayed in the 3D Plot window (Figure 7.21).

Figure 7.21 3D intensity signal



2. To change the display, make a selection from the Plot drop-down list and click the **Refresh** button Refresh.

Table 7.8 3D Plot Window

Item	Description
Plot	Full Image – Displays all signals in the image. ROI <ROI number or name> – Displays the signal within the selected ROI. All ROIs – Displays the signal within all ROIs in the image.
Z Max	Height of the z-axis. Use the up/down arrows to change the height of the z-axis.
	Click to reset the z-axis to the default setting.
	Copies the 3D window to the system clipboard.
	Opens a Print dialog box that enables you to print the 3D window.

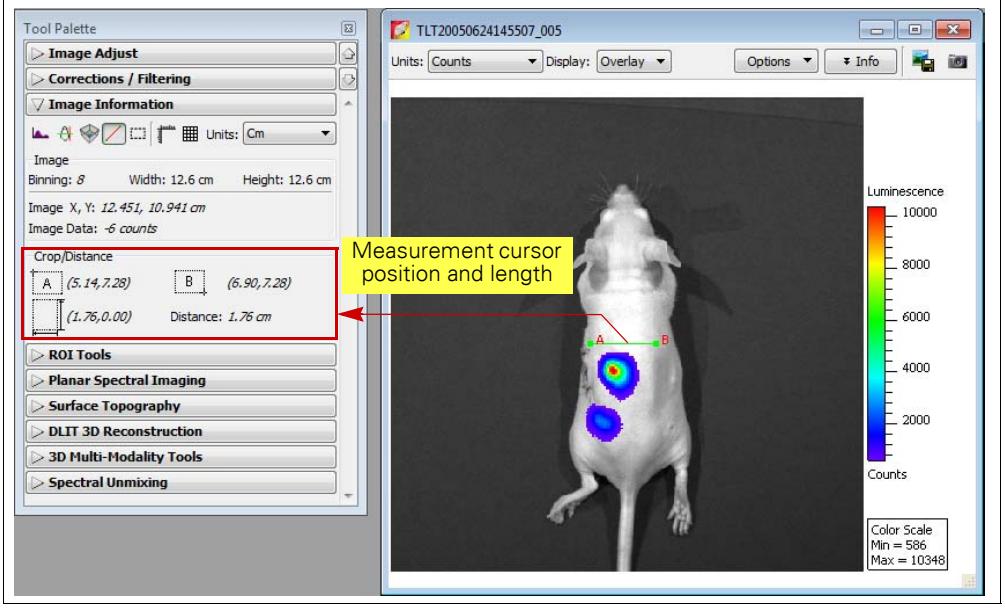
Making Measurements

To measure distance with the measurement cursor:

1. Open an image and click the **Distance Measurement Cursor** button in the Image Information tools.

   A measurement cursor (A——B) appears on the image (Figure 7.22). The Tool Palette shows the position and length of the cursor.

Figure 7.22 Measurement cursor
The Tool Palette displays the measurement cursor position and length.



2. To change the cursor position or size, drag 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. To hide the cursor, click the button.

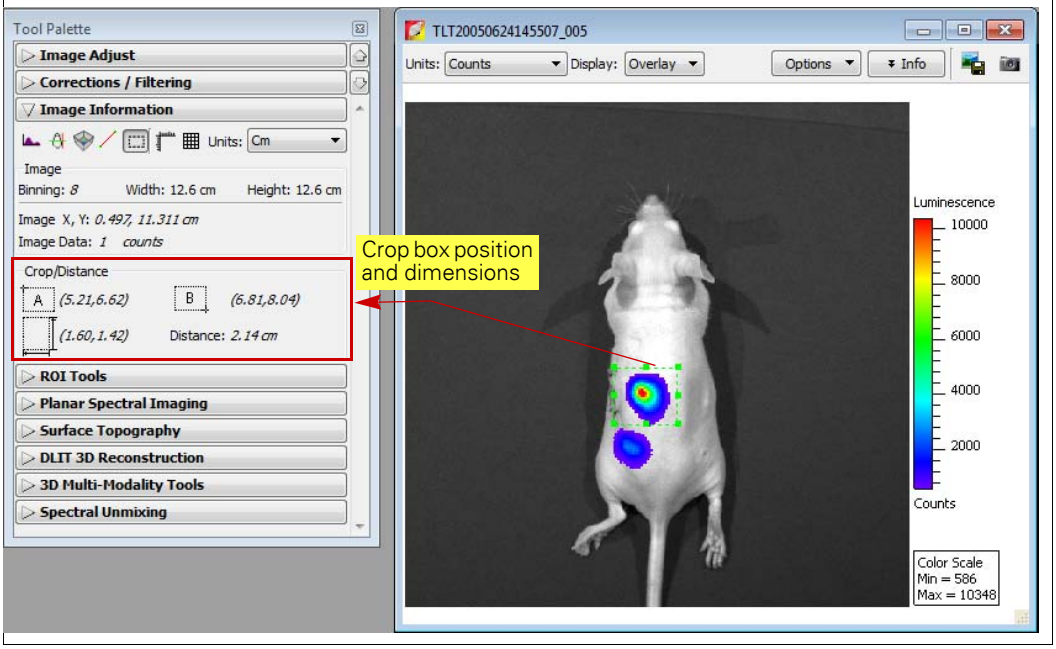
Table 7.9 Measurement cursor position & length

Item	Description
A	Pixel x,y coordinates of position A on the cursor.
B	Pixel x,y coordinates of position B on the cursor.
	Length of the cursor from A to B (number of pixels), vertical distance from A to B (number of pixels).
Distance	Length of the cursor from A to B (number of pixels).

To measure distance using the crop box:

1. Open an image, and in the Image Information tools, click the **Image Crop** button .

Figure 7.23 Using a crop box to make measurements



2. When the mouse pointer changes to a +, draw a rectangle on the area of interest.
3. To change the size or position of the crop box, drag a handle ■ at a corner or side of the box.
4. To delete the crop box from the image, click the button.

Table 7.10 Crop box position & dimensions

Item	Description
A	x,y coordinates at the upper left corner of the box.
B	x,y coordinates of lower right corner of the box.
	Box width and height.
Distance	Length of the diagonal from the upper left to lower right corner of the box.

7.8 Creating a Transillumination Overview

The transillumination overview tool combines the images of a FLIT sequence (a fluorescence sequence acquired in transillumination mode) into a single image. All of the individual fluorescent signals are stacked over one photograph and the intensity is summed. One overview is created per filter pair. If two filter pairs were used during acquisition, then two overview images will be created.

All transillumination locations are displayed simultaneously; a tool tip displays the transillumination position when you mouse over a transillumination point. An overview image is displayed in radiant efficiency and can be analyzed using the tools in the Tool Palette.

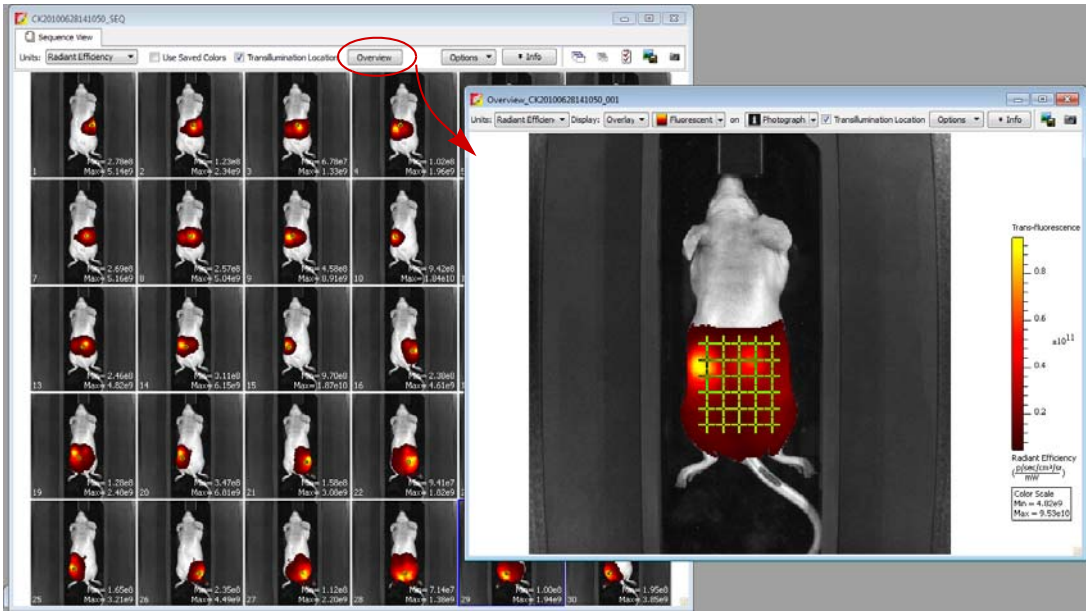
NOTE

If you choose the Raster Scan option in the Transillumination Setup box, the overview image is automatically generated. For more details, see page 30.

1. Load a sequence that was acquired in fluorescence transillumination mode.
2. Click the Overview button. Alternatively, select **Tools** → **Transillumination Overview for <name>_SEQ** on the menu bar.

   The overview appears.

Figure 7.24 Transillumination overview



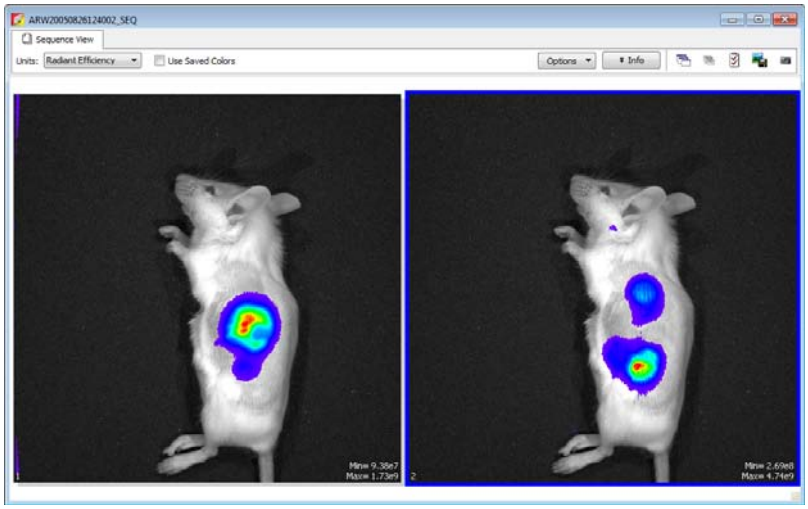
7.9 Overlaying Multiple Images

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.

To coregister 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 117.)
2. Load the image sequence.

Figure 7.25 Image sequence



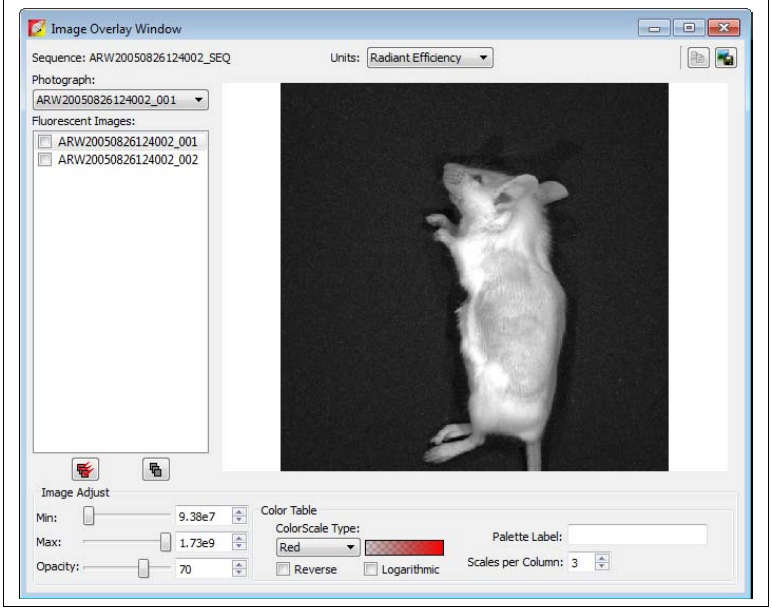
3. Open one of the images and optimize the image display using the color scale Min and Max sliders in the Image Adjust tools.

   To view all images in the sequence, click the **Display All** button to open each image (overlay mode) in a separate image window.

4. Select **Tools→ Image Overlay for <sequence name>_SEQ** on the menu bar.

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

Figure 7.26 Image Overlay window



5. To overlay all images, click the button.

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

Figure 7.27 Generated overlay

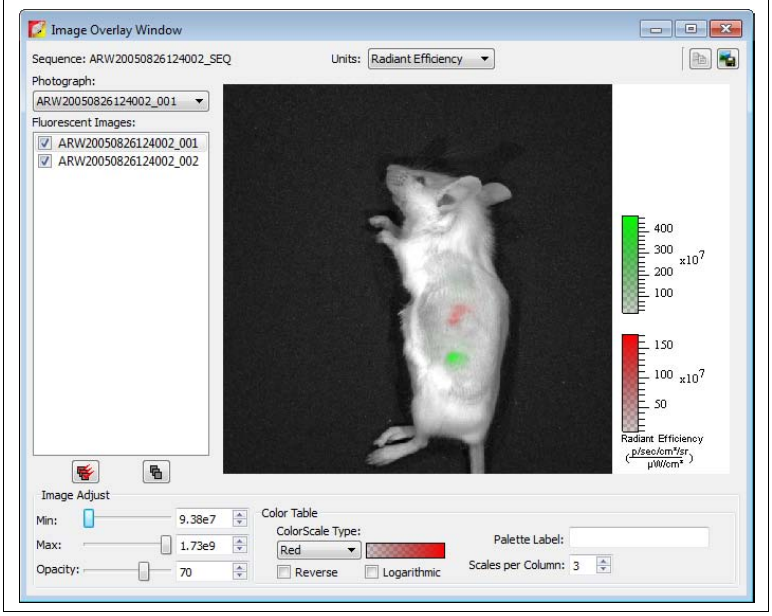


Table 7.11 Image Overlay window

Item	Description
Units	Choose the type of units for displaying the fluorescent or luminescent image. For more details on measurement units, see page 281.
Photograph	A drop-down list of the photographs in the image sequence.
Fluorescent or Luminescent Images	The sequence images.

Table 7.11 Image Overlay window

Item	Description
	Copies the overlay to the system clipboard.
	Click to export the overlay to a graphic file.
	Click to include all fluorescent or luminescent images in the overlay.
	Click to remove all fluorescent or luminescent images from the photograph.
Image Adjust	Tools for adjusting the appearance of the highlighted fluorescent or luminescent image. Adjustments can only be made on one image at a time. Min – The minimum pixel intensity associated with the color scale for an image. Pixels less than the minimum value are not displayed. Max – The maximum pixel intensity associated with the color scale for an image. Pixels greater than the maximum value are displayed in the maximum color. Opacity – Controls the opacity of the fluorescent or luminescent image.
Color Table	Tools selecting and modifying the color scale associated with an image. Color Scale Type – Choose BlackLevel to show black at the low end of the color scale. Choose WhiteLevel to show white at the low end of the color scale.  Click the drop-down arrow to select a color table for the image data. (For more details on color tables, see *Pseudocolor Images*, page 279.) Reverse – Choose this option to reverse the selected color table. Logarithmic – Choose this option to apply a log scale to the relationship between numerical data and the color range in the color table. A log scale increases the range of meaningful numerical data that can be displayed.
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.

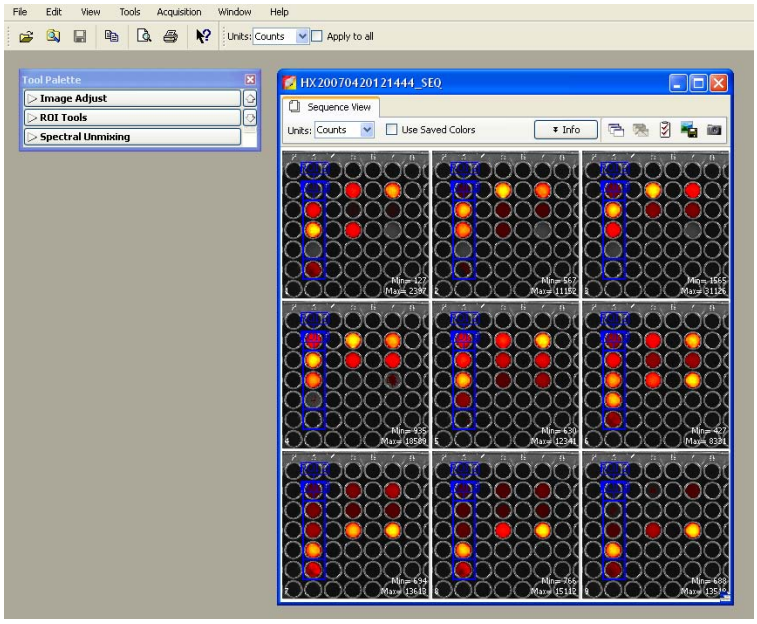
7.10 Rendering Intensity Data in Color

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

To view colorized intensity data:

1. Load an image sequence.

Figure 7.28 Microplate images
Images were acquired using different combinations of excitation and emission filters. The samples are quantum dot nanocrystals (700 or 800 nm).



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

   The software renders each luminescent or fluorescent image in color and combines them into a single image (Figure 7.29).

Figure 7.29 Colorize view

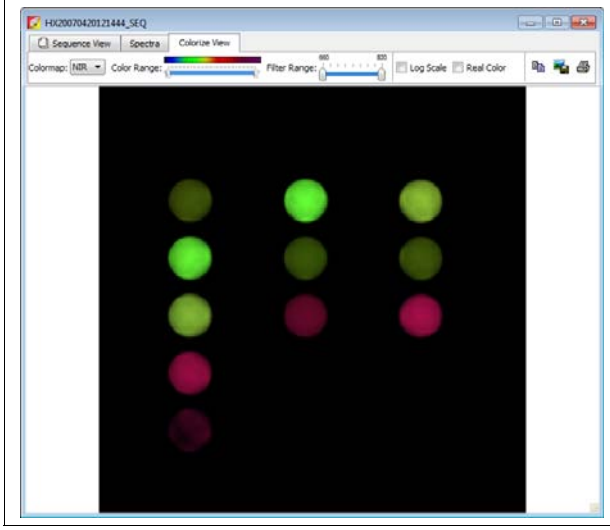


Table 7.12 Colorize tools

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

7.11 Exporting or Printing Images

The Image Layout window (Figure 7.30) provides an alternative way to:

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

1. To open the Image Layout window, select **View** → **Image Layout Window** on the menu bar.
2. To paste the active image into the Image Layout window, click the  button.
3. To resize the image, drag a handle ■ at a corner of the image.
4. To reposition the image in the window, drag the image.

Figure 7.30 Image Layout window

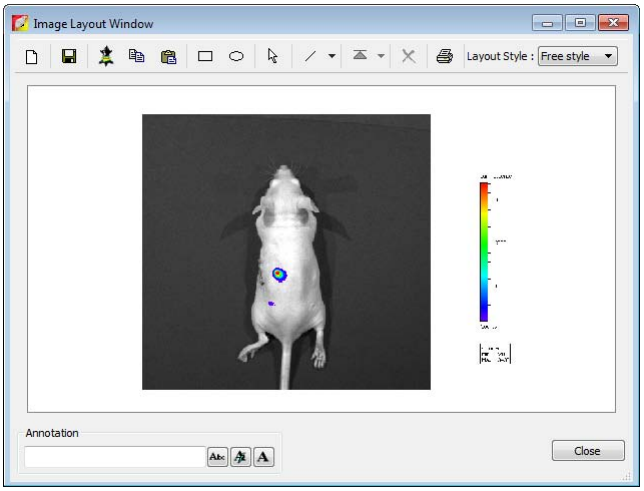


Table 7.13 Image Layout window

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

Table 7.13 Image Layout window (continued)

Item	Description
	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.

7.12 Editing an Image Sequence

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

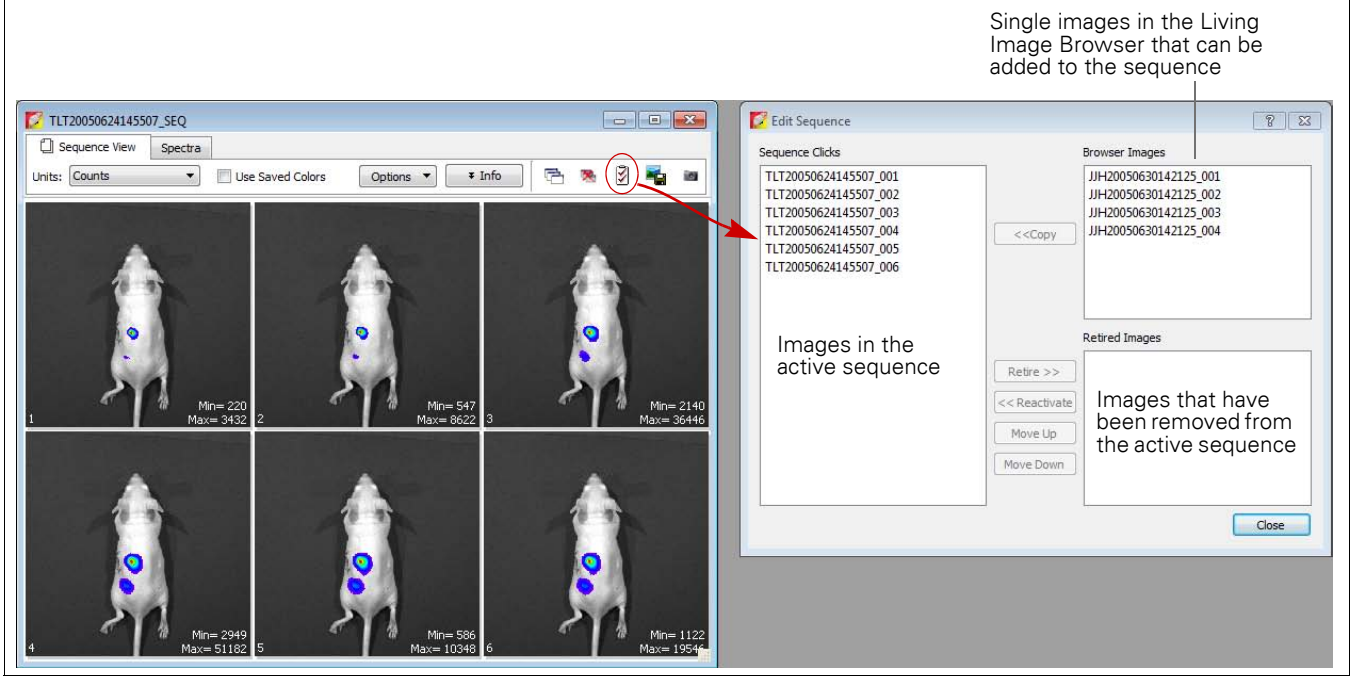
1. Open the image sequence that you want to edit.
2. If you plan to add images to the sequence, browse for the images that you want to add in the Living Image® browser. (For more details on browsing, see page 83.)

NOTE

If you plan to add images to the sequence, browse for the images that you want to add in the Living Image Browser. (For more details on the browser, see page 83.)

3. In the image window, click the **Edit** button  .

Figure 7.31 Opening the Edit Sequence dialog box



4. In the Edit Sequence box that appears, choose the image(s) to add or remove (*retire*) from the sequence (Figure 7.31).

   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. When you are finished editing the sequence, click **Close**.

   The updated image sequence is displayed.

7.13 Creating an Image Sequence from Individual Images

You can create a sequence from images acquired during different sessions.

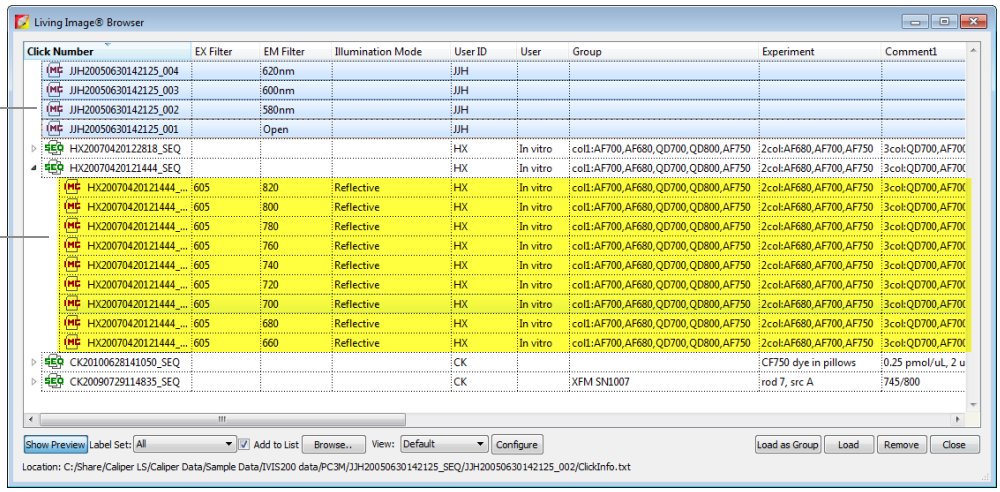
1. In the Living Image Browser, browse for the images of interest. (For more details on browsing, see page 83.)

NOTE

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

Figure 7.32 Living Image Browser

Individual images (highlighted blue in this example) that may or may not be part of a sequence can be selected for grouping into a new sequence.



Images loaded in the browser as part of a sequence (highlighted yellow in this example). These images can also be selected for grouping into another sequence.

2. In the browser, select the images that you want to group together.

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. To save the images as a sequence:

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

   b. In the dialog box that appears, select a folder and click **OK**.

8 ROI Tools

8.1 About ROIs

A *region of interest* (ROI) is a user-specified area in an optical image (Figure 8.1). The ROI tools enable you to create three types of ROIs: measurement, average background, or subject ROI (Table 8.1). During a session, the Living Image software records information about the ROIs you create and computes statistical data for the ROI measurements. The ROI Measurements table displays the data and provides a convenient way to review or export ROI information (Figure 8.1). (For more details, see *Managing the ROI Measurements Table*, page 145.)

Figure 8.1 Example measurement ROIs and ROI measurements table

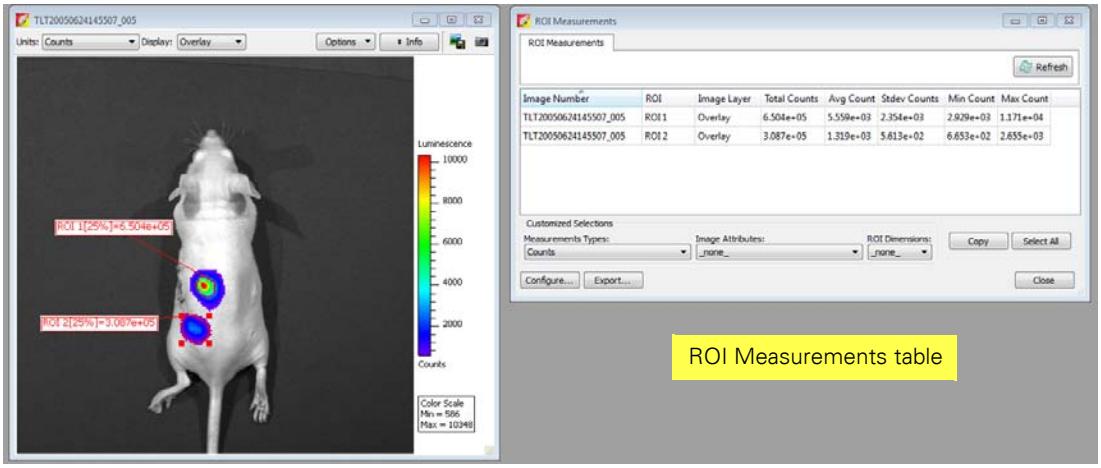


Table 8.1 Types of ROIs

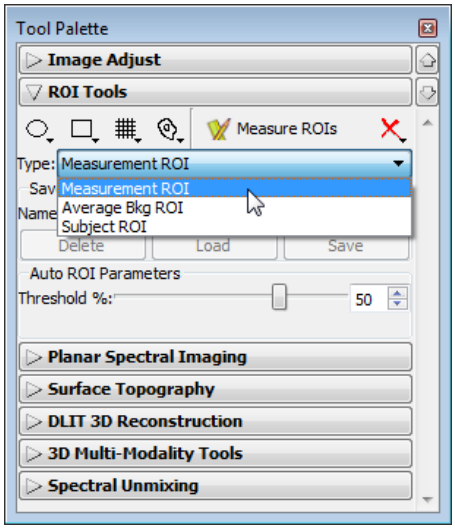
	Type of ROI		
	Measurement ROI	**Average Background ROI**	**Subject ROI**
Description	Measures the signal intensity in an area of an image.	Measures the average signal intensity in a user-specified area of the image that is considered background. **Note:** Using this type of ROI is optional. If the animal 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.	Identifies a subject animal in an image. **Note:** Using this type of ROI is optional. It provides a convenient way to automatically associate (link) a measurement and average background ROI for background-corrected ROI measurements when there is significant autoluminescence or autofluorescence.
Available ROI Drawing Methods	• Manual • Automatic • Free draw	• Manual • Free draw	• Manual • Automatic • Free draw
Available Shapes	Circle, square, grid, or contour	Circle or square	Square

8.2 Quick Guide: Drawing Measurement ROIs on an Image or Sequence

These steps provide a quick guide on how to apply a measurement ROI to an optical image or image sequence. For more details about measurement ROIs, see page 125.

1. Open an image or sequence and click ROI Tools in the Tool Palette.
2. In the ROI tools, select Measurement ROI from the Type drop-down list.

Figure 8.2 Select a type of ROI



3. Click the Contour button and make a selection from the drop-down list.

   Image or image or sequence – Select **Auto All** from the drop-down list.

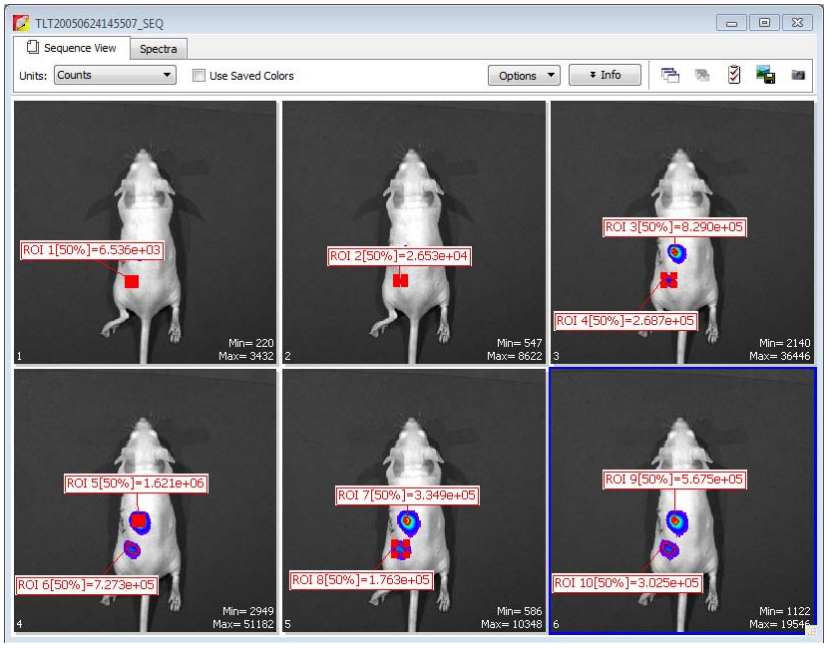
   Kinetic data – Select **Kinetic ROI**.

The software automatically draws measurement ROIs on all images. The ROI label shows the total intensity in the ROI and the Threshold % (Figure 8.3).

NOTE

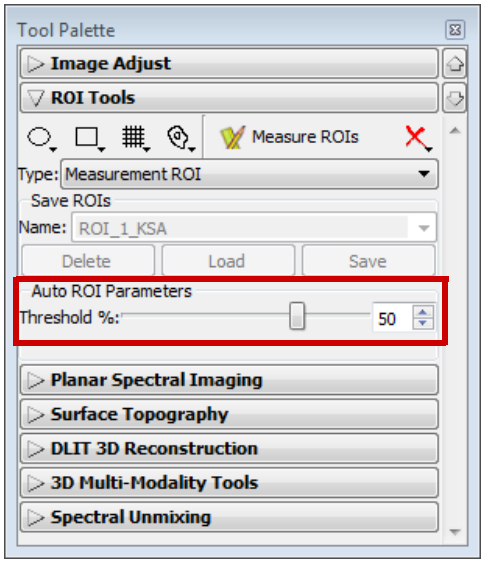
Auto ROIs are created and numbered in order from highest to lowest maximum signal within the ROI (ROI 1 contains the highest maximum signal). You may want to arrange the ROIs in a known order for easier comparison between images.To renumber the ROIs (ascending order from right to left), right-click the image and select Sort ROIs on 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 the sequence. The sort options are only available if the ROIs have not been sorted.

Figure 8.3 ROI intensity measurements



4. To adjust the ROI boundaries, change any of the auto ROI parameters using the slider or  arrows (Figure 8.4).

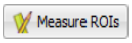
Figure 8.4 Auto ROI parameters



Threshold % - Specifies the minimum per cent of peak pixel intensity that a pixel must have to be included in an ROI identified by the software.

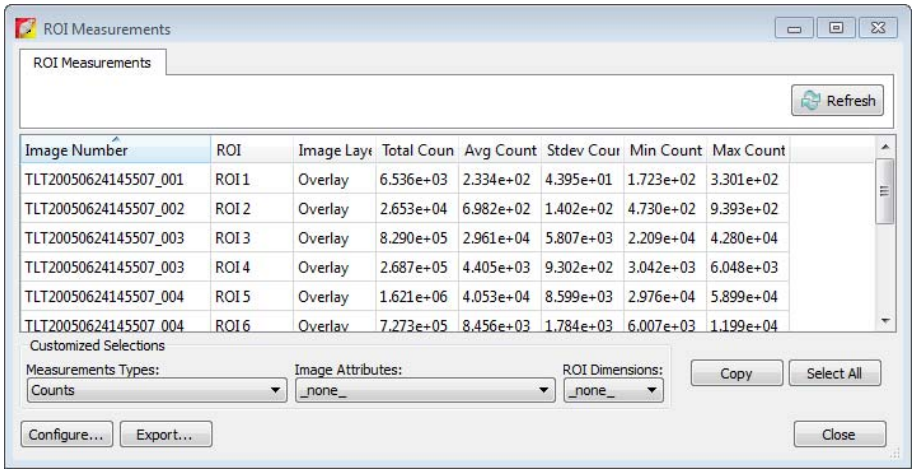
NOTE

After the ROIs have been created, 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.

5. Click the **Measure** button 

in the ROI tools to show the ROI Measurements table.

Figure 8.5 ROI Measurements table

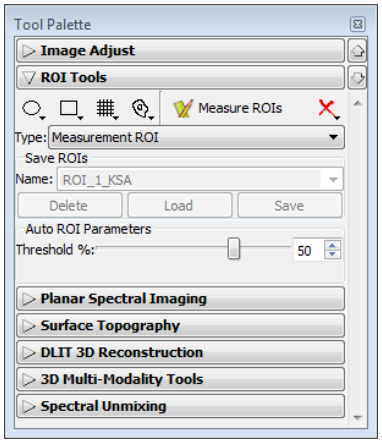


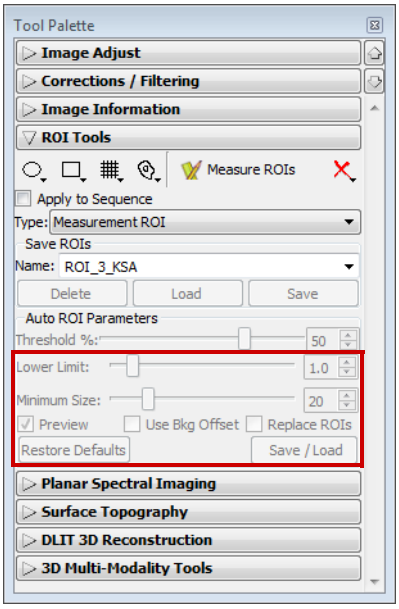
The ROI Measurements table displays data for all ROIs created in images or sequences during a session (one ROI per row). The table provides a convenient way to review and export ROI data. For more details on the table, see "Managing the ROI Measurements Table," page 145.

8.3 ROI Tools

Table 8.2 provides brief explanations for the ROI tools. The ROI tools that appear in the Tool Palette depend on the type of ROI selected from the ROI Type drop-down list, and whether an image or sequence is active. Some ROI parameters are only available if "Show Advanced Options" is selected in the General Preferences.

Figure 8.6 ROI tools





These Auto ROI parameters are available if "Show Advanced Options" is selected in the General Preferences. For more details on setting Preferences, see Appendix B, page 265.

Table 8.2 ROI tools

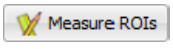
Item	Description
	Click to select the number of circle ROIs to add to the active image.
	Click to select the number of square ROIs to add to the active image.
	Click to specify the grid pattern for a measurement ROI that you want to add to the active image. This tool is useful for an image of a multi-well culture plate or microplate.
	Click and select **Auto All** to automatically draw ROIs in the image 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. For more details on using the auto ROI features, see page 127.
	Click to display the ROI Measurements table or compute intensity signal in an ROI.
	Click to display a drop-down list of options to delete an ROI(s) in the active image. For more details, see page 143. **Note:** These commands do not delete the ROIs that are saved to the system (listed in the Menu Name drop-down list).
Apply to Sequence	Choose this option to apply the selected ROI to all images in a sequence.

Table 8.2 ROI tools (continued)

Item	Description
Type	Choose the ROI type from the drop-down list: **Measurement** - Measures the signal intensity in an area of an image. **Average Bkg** - Measures the average signal intensity in a user-specified area of the image that is considered background. **Subject ROI** - Identifies a subject animal in an image. The software automatically associates a measurement and an average bkg ROI that are included in the same subject ROI. Using this type of ROI is optional.
Save ROIs	Creates a file that includes the ROI parameters (for example, the X,Y coordinates, type of ROI, color, shape, width/height). ROIs that have been saved to file can be recalled and applied for another image at any time.
Name	The name of the selected ROI set or the default name for a new ROI set.
Delete	Deletes the selected ROI set from the system. Note: This permanently removes the ROI from the system.
Load	Applies the ROI set selected from the Name drop-down list to the active image.
Save	Saves the ROI set in the active image. **Note:** This is a global save (the ROI is saved to the system) and the ROI set can be loaded onto any image. If you use the **File → Save** commands to save an image that includes an ROI, the ROI is saved with the image only (not a global save) and is not available for loading onto other images. For more details, see *Saving ROIs*, page 142.
Auto ROI Parameters	Parameters that specify how the auto ROI tool draws an ROI. **Threshold %** – If the Auto All or Auto 1 method is selected, the Threshold % specifies the minimum percent of peak pixel intensity that a pixel must have to be included in an ROI identified by the software. After ROIs are drawn on an image, if you modify the Threshold% (move the slider or enter a new value), the software automatically updates the ROIs.
	Note: The following Auto ROI parameters are only available if "Show Advanced Options" is selected in the general preferences. For more details on setting Preferences, see Appendix B, page 265.
	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 in the visible range.
	Minimum Size – Sets the minimum size of an ROI (measured in pixels). For example if the minimum size is set at 50, then ROIs created on the image must be greater than 50 pixels in size.
	Preview – If this option is chosen, the software draws the ROI each time a parameter is changed. ROI parameters can be saved without drawing the ROI.
	Use Bkg Offset – Choose this option to measure background-corrected signal. This is typically used to remove natural animal background luminescence, and should not be confused with the dark-charge and read-bias 'background' corrections that are applied (by default) to the raw CCD data to remove electronic noise before any measurements. For more details, see page 130.
	Replace ROIs – If this option is chosen, all auto ROIs are replaced when new ROI(s) are created.
	Restore Defaults – Restores the factory-set defaults for the auto ROI parameters.
Save/Load	Click to display or hide the tools that enable you to save, load, or delete auto ROIs in the active data. **Note:** The save function saves parameters, the not actual ROIs. This means that when you load saved auto ROI parameters, the software draws a new ROI using the saved values (Threshold%, Lower Limit, Minimum Size).

8.4 Measurement ROIs

To obtain the intensity signal in a user-specified area of an image, draw a measurement ROI on the image. This section explains in detail the three ways to draw measurement ROIs.

NOTE

For a quick guide to drawing measurement ROIs, see page 120.

Drawing Method	Description	See Page
Manual	Places one or more ROIs (circular, square, or grid shape) on the image.	120
Automatic	The software automatically locates and draws an ROI(s) on the image. To do this, the software locates the peak pixel intensities in the image and searches 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.	127
Free draw	Draw line segments that define the ROI.	129

Manually Drawing a Measurement ROI

1. Open an image or image sequence, and in the ROI tools, select Measurement ROI from the Type drop-down list.
2. Select the ROI shape:
   a. Click the **Circle**, **Square**, or **Grid** button.

      The grid shape is useful for drawing a grid of ROIs on an image of a well plate.

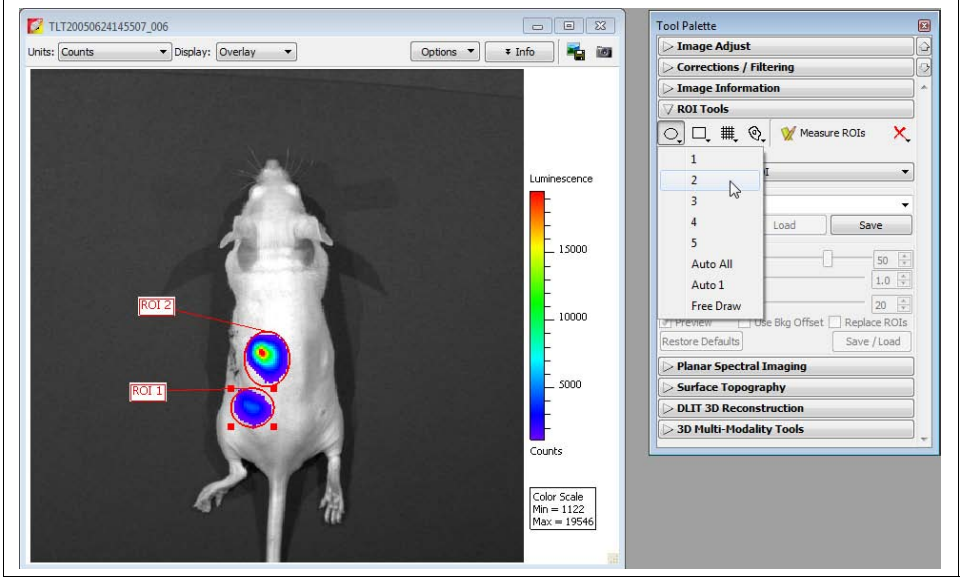
   b. On the drop-down list that appears, select the number of ROIs that you want to add to the image or the grid ROI dimensions.

      The ROIs and intensity measurements appear on the image.

NOTE

Manual ROIs are numbered in the order they are created. You may want to arrange the ROIs in a known order for easier comparison between images.To renumber the ROIs (ascending order from right to left), right-click the image and select Sort ROIs on 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 the sequence. The sort options are only available if the ROIs have not been sorted.

Figure 8.7 Placing two circular ROIs on the image



3. Adjust the ROI position:
   a. Place the mouse pointer over the ROI. When the pointer becomes a ✣, click the ROI.
   b. Drag ROI(s).

NOTE

To move multiple ROIs at the same time, press and hold the Shift key while you click the ROIs, and then drag them to a new location. Contour ROIs ( ) cannot be moved using this method.

4. Adjust the ROI dimensions:
   a. Place the mouse pointer over the ROI. When the pointer becomes a ✣, click the ROI.
   b. Place the mouse pointer over an ROI handle ■ so that it becomes a ⤡. Drag the handle to resize the ROI.

NOTE

You can also change the ROI position or size using the adjustment controls in the ROI Properties box (see "Moving an ROI," page 138 and "Editing ROI Dimensions," page 139).

5. Click the **Measure** button Measure ROIs.

   The ROI measurements and table appear. For more details on the table, see "Managing the ROI Measurements Table," page 145.

   For information on how to save ROIs, see page 116.

Automatically Drawing Measurement ROIs

The Living Image® software can automatically identify all of the ROIs in an image or image sequence that meet the auto ROI parameter thresholds or draw one ROI at a user-specified location.

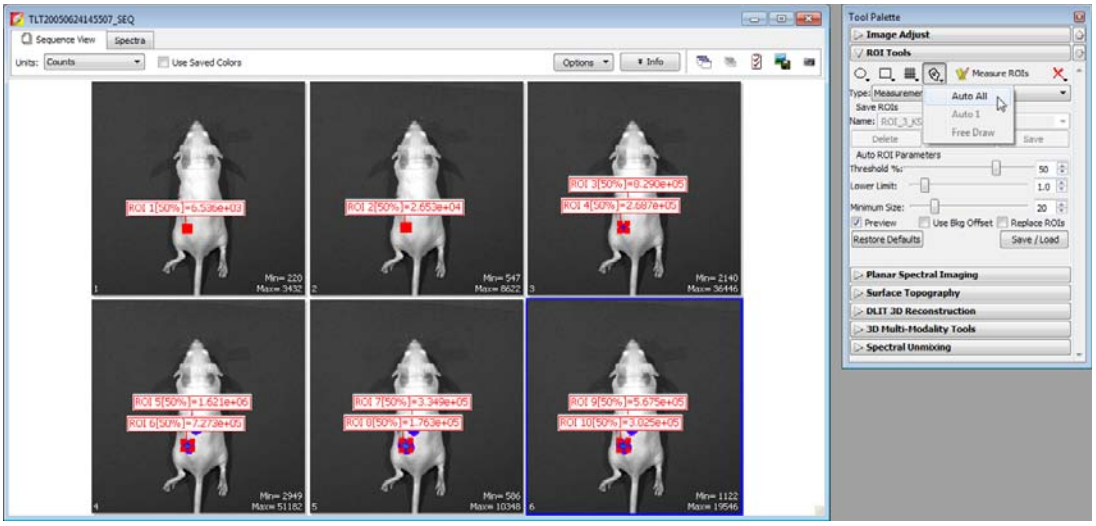
1. Open an image or image sequence, and in the ROI tools, select Measurement ROI from the Type drop-down list.
2. Click an ROI shape button (**Circle**, **Square**, or **Contour**) and select **Auto All** from the drop-down list.

   The ROIs appear on the image or sequence thumbnails. The ROI label includes the ROI intensity threshold (Threshold%) and intensity measurement.

NOTE

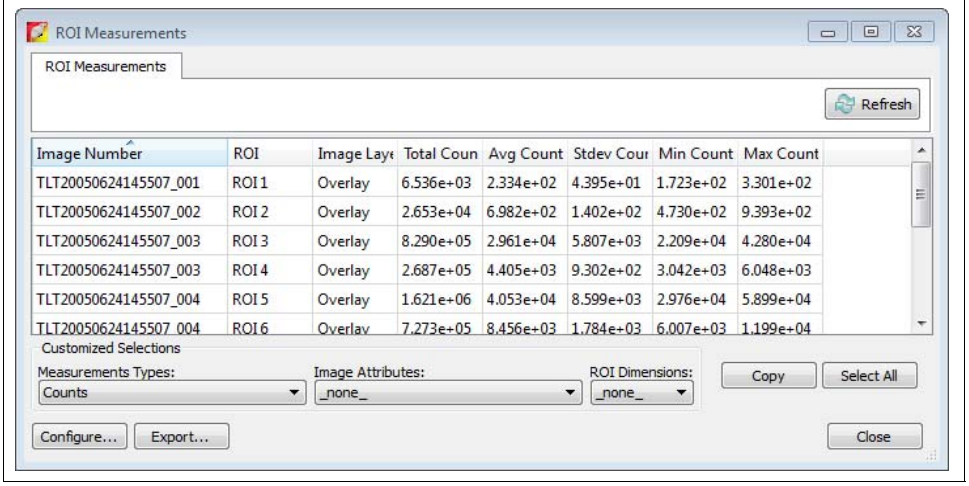
Auto ROIs are created and numbered in order from highest to lowest maximum signal within the ROI (ROI 1 contains the highest maximum signal). You may want to arrange the ROIs in a known order for easier comparison between images.To renumber the ROIs (ascending order from right to left), right-click the image and select Sort ROIs on 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 the sequence. The sort options are only available if the ROIs have not been sorted.

Figure 8.8 Automatically drawing measurement ROIs detected by the software



3. Click the **Measure** button Measure ROIs in the ROI tools to show the ROI Measurements table.

Figure 8.9 ROI Measurements table



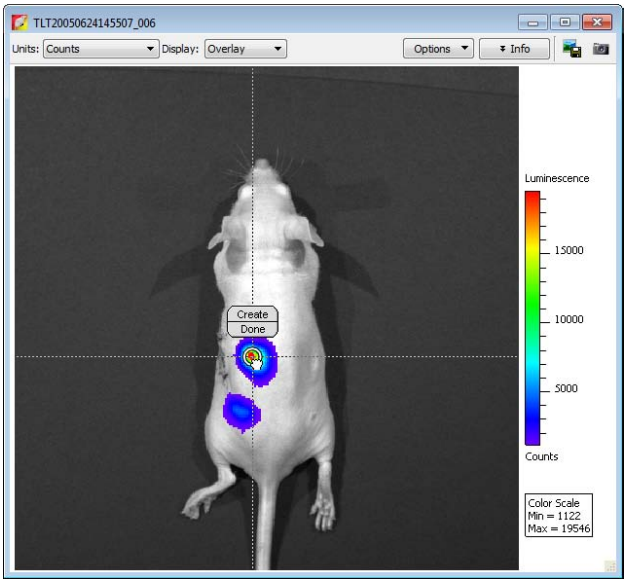
The ROI Measurements table displays data for all ROIs created in images or sequences during a session (one ROI per row). The table provides a convenient way to review and export ROI data. For more details on the table, see "Managing the ROI Measurements Table," page 145

To automatically draw an ROI at a user-specified location:

1. Open an image.
2. Click an ROI shape button (**Circle**, **Square**, or **Contour**) and select **Auto 1** from the drop-down list.

   The create tool appears on the image.

Figure 8.10 ROI create tool



3. Use the ring to move the create tool to the area where you want to draw the ROI, then click **Create**.

   The ROI appears on the image and the ROI label displays the intensity signal.

4. To draw another ROI on the image, repeat step 2. to step 3.

   For information on how to save ROIs, see page 142.

Drawing an ROI Using the Free Draw Method

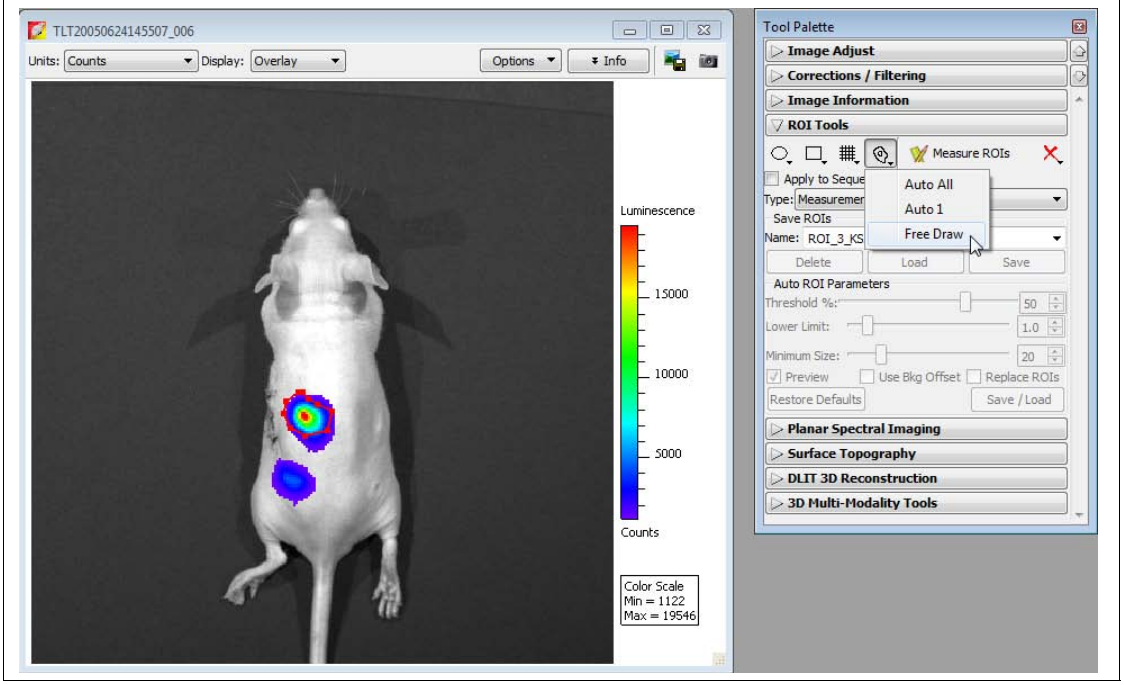
1. Open an image, and in the ROI tools, select the type of ROI that you want to draw from the Type drop-down list.
2. Click an ROI shape button (**Circle**, **Square**, or **Contour**) and select **Free Draw** from the drop-down list. In this example, the Contour shape was selected for the free draw method.

   The ROI shapes that are available depend on the type of ROI selected.
3. If you selected:

   or - Use the pointer (+) to draw the ROI.

   - Use the pointer (+) to click around the area of interest and draw line segments that define the ROI. Right-click when the last point is near the first point in the ROI.

Figure 8.11 Drawing an ROI using the free draw method



8.5 Subject ROIs

A subject ROI identifies a subject animal in an image. It provides a convenient way to automatically associate (link) a measurement and average background ROI for background-corrected ROI measurements when there is significant autoluminescence or autofluorescence. (For more details on background-corrected ROI measurements, see page 130.) Using a subject ROI is optional.

To draw a subject ROI using the auto ROI feature:

1. Select Subject ROI from the Type drop-down list.
2. Click the button.

3. Select **Auto All**.

To manually draw a subject ROI:

4. Select Subject ROI from the Type drop-down list.
5. Click the button, and select **1**.
6. Position the subject ROI so that it includes the measurement ROI(s) and the associated average background ROI.

8.6 Measuring Background-Corrected Signal

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

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

NOTE

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

The Image Adjust tools and zoom feature are helpful for selecting an appropriate area for an ROI. By setting the image minimum close to zero and zooming in on a background area in the image, you can determine where naturally occurring background luminescence or autofluorescence is present. For more details on the Image Adjust tools and the zoom feature, see *Adjusting Image Appearance*, page 97 and *Magnifying or Panning in the Image Window*, page 98.

To measure background-corrected signal:

1. Draw one or more measurement ROIs on the subject. (For more details, see page 129.)
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**.

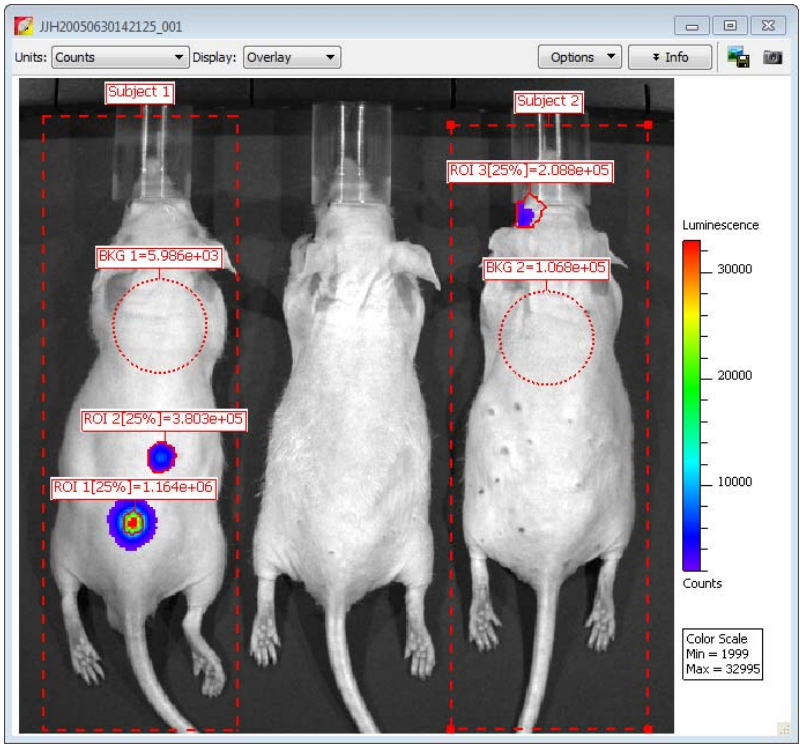
      The ROI is added to the image.For more details on adjusting the ROI position or dimensions, see page 138 and page 139.

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.

3. Use one of the following methods to associate the average background ROI with one or more measurement ROIs:
   - Method 1: Draw a subject ROI around the average background ROI and the measurement ROI(s) (Figure 8.12)
   - Method 2: Right-click the measurement ROI and select a background ROI from the shortcut menu that appears (Figure 8.13).
   - Method 3: Set the background ROI in the ROI properties dialog box (Figure 8.14)

Figure 8.12 Associate the average background ROI with measurement ROIs (Method 1)



Draw a subject ROI that includes the measurement ROI and the average background ROI. For details on how to draw a subject ROI, see page 129.

Figure 8.13 Associate an average background ROI with measurement ROIs (Method 2)

Right-click the measurement ROI and select an average background ROI from the shortcut menu.

Figure 8.14 Associate an average background ROI with measurement ROIs (Method 3)

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.

8.7 Kinetic ROIs

Kinetic ROIs help you track signal sources on an unanesthetized, mobile subject. The software automatically creates a separate ROI in each frame based on the user-specified auto ROI settings. As a result, kinetic ROIs are continuously displayed during kinetic data playback. You can draw a kinetic ROI using any of the methods or shapes in Table 8.1, page 120.

NOTE

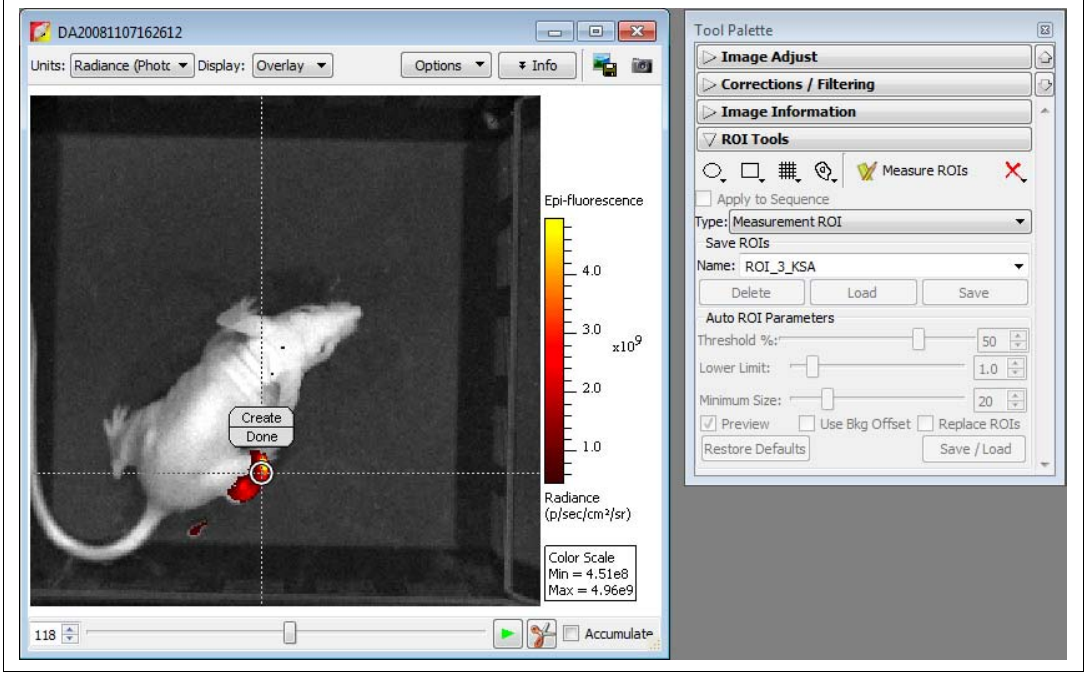
Large kinetic data sets may require more time to create, plot, and measure the ROIs because the software first applies corrections to a frame (specified in the Corrections/Filtering Tool Palette), then draws the ROIs in the frame. The process can be aborted at any time.

These steps provide a quick guide on how to apply a measurement ROI to kinetic data. For more details about measurement ROIs, see page 125.

1. Open the kinetic data and click ROI Tools in the Tool Palette.
2. In the ROI tools, select Measurement ROI from the Type drop-down list.
3. Click the Contour button and select **Kinetic ROI**.

   The create tool appears on the image.

Figure 8.15 ROI create tool



4. Use the ring to move the create tool to the area where you want to draw the ROI, then click **Create**.

   The ROI and label appear on the image.

NOTE

When drawing kinetic ROIs on kinetic data with multiple sources, it is recommended that you start with the brightest source, then the next brightest, and so on in order to create ROIs that can be distinguished based on the signal strength.

5. If it is necessary to adjust the ROI boundaries or change any of the auto ROI parameters (use the slider or arrows):

   Threshold % - Specifies the minimum per cent of peak pixel intensity that a pixel must have to be included in an ROI identified by the software

6. Click **Done** to hide the create tool.

   The kinetic data playback starts and shows the ROI in each image.

NOTE

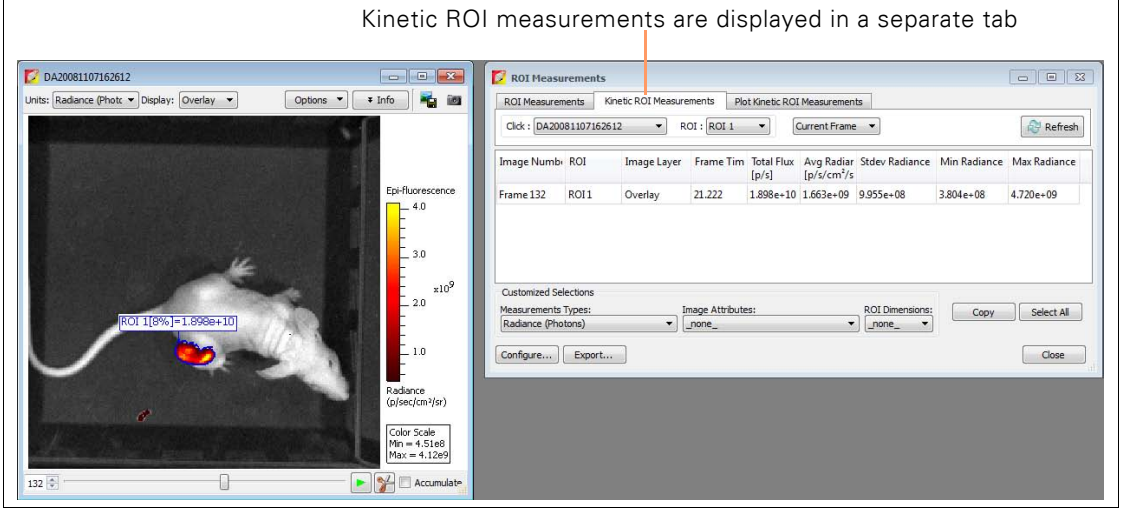
After the ROIs have been created, right-click an ROI to view a shortcut menu of ROI commands (Ctrl-click for Macintosh users). The shortcut menu provides easy access to many functions for managing ROIs and viewing ROI properties.

7. To measure intensity in the ROI, click the **Measure** button Measure ROIs in the Tool Palette.

   The Kinetic ROI Measurements table appears (Figure 8.16).

8. To view ROI measurements for all images, click the arrow next to Current Frame and select **All Frames**, then click the **Refresh** button.

Figure 8.16 ROI Measurements table



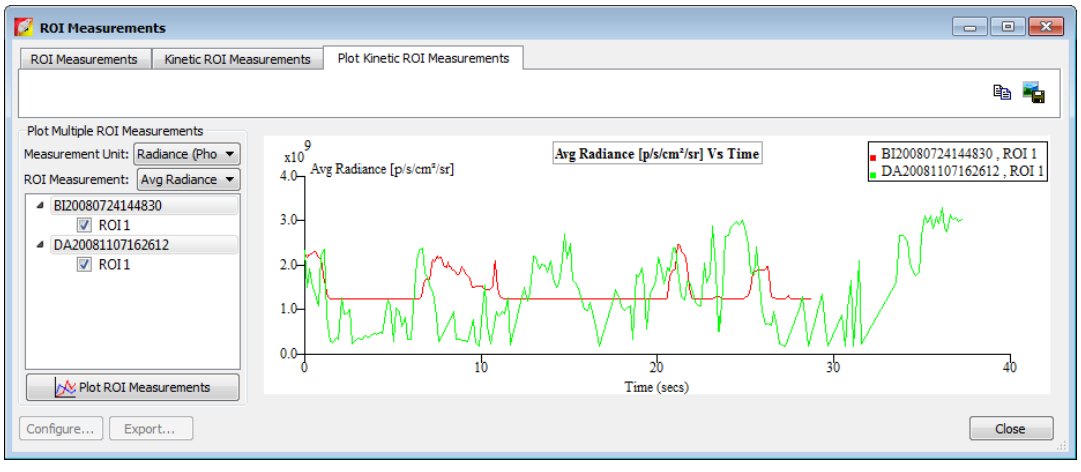
Plotting Kinetic ROI Measurements

The kinetic ROI plot provides a convenient way to view and compare kinetic ROI measurements across user-selected image frames from the same or different kinetic data sets.

1. Open one or more kinetic data sets.
2. Draw kinetic ROIs on the data sets in which you want to measure and compare ROIs.
3. In the ROI tools, click the ROI Measurements button  .

   The ROI measurements table appears.

Figure 8.17 Plot Kinetic ROI Measurements tab



4. Click the Plot Kinetic ROI Measurements tab.
5. Make a selection from the Measurement Unit and ROI Measurement drop-down lists.
6. Select a data set and an ROI.
7. Click **Plot ROI Measurements**.
8. To add other ROI data to the graph, repeat step 6 to step 7.

8.8 Managing ROIs

In the ROI Properties box, you can view information about an ROI, change the position of the ROI on the image, and edit the ROI label or line characteristics

Viewing ROI Properties

1. To view ROI properties, do one of the following:
   - 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 8.20).
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 8.18).

Figure 8.18 Opening the ROI Properties dialog box

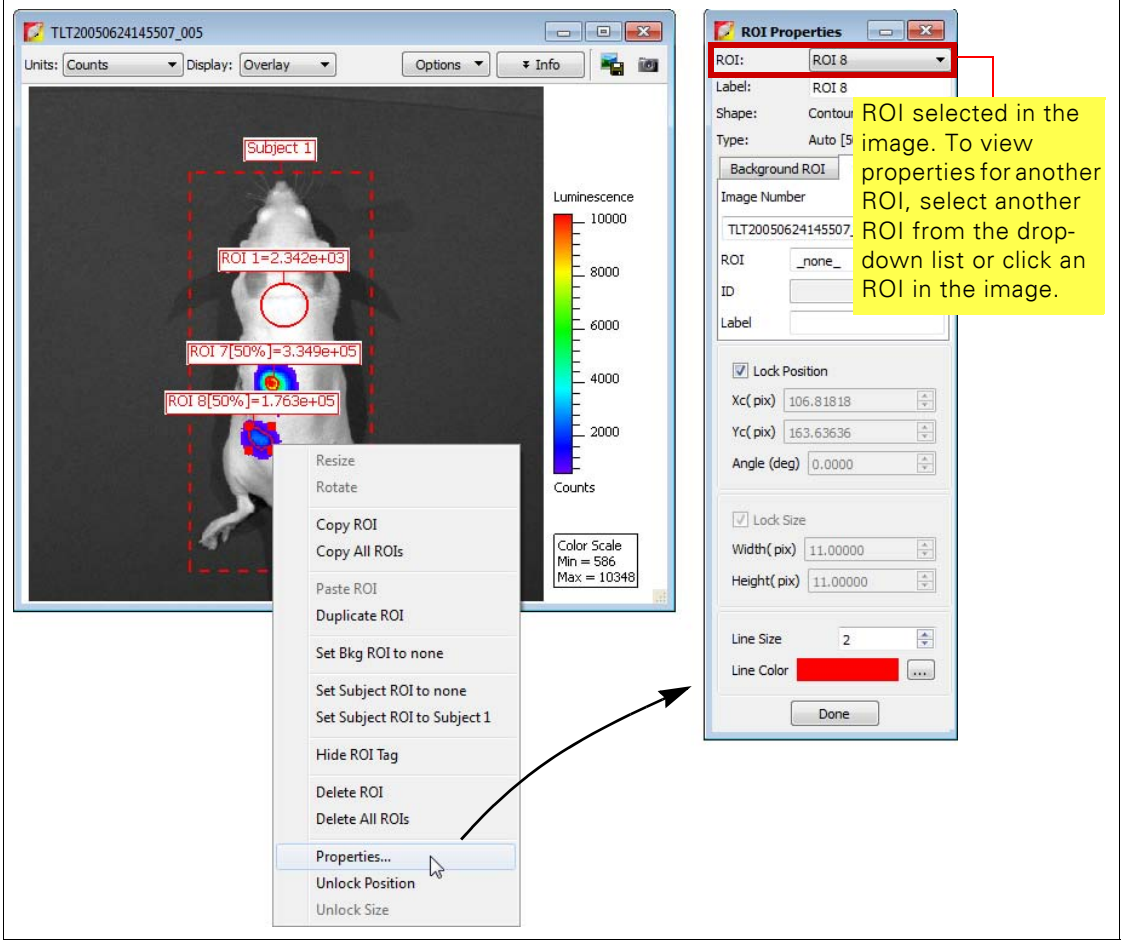


Figure 8.19 ROI Properties, Background ROI tab
The items in the ROI Properties box depend on the type of ROI selected in the image. For more details see Table 8.3, page 137.

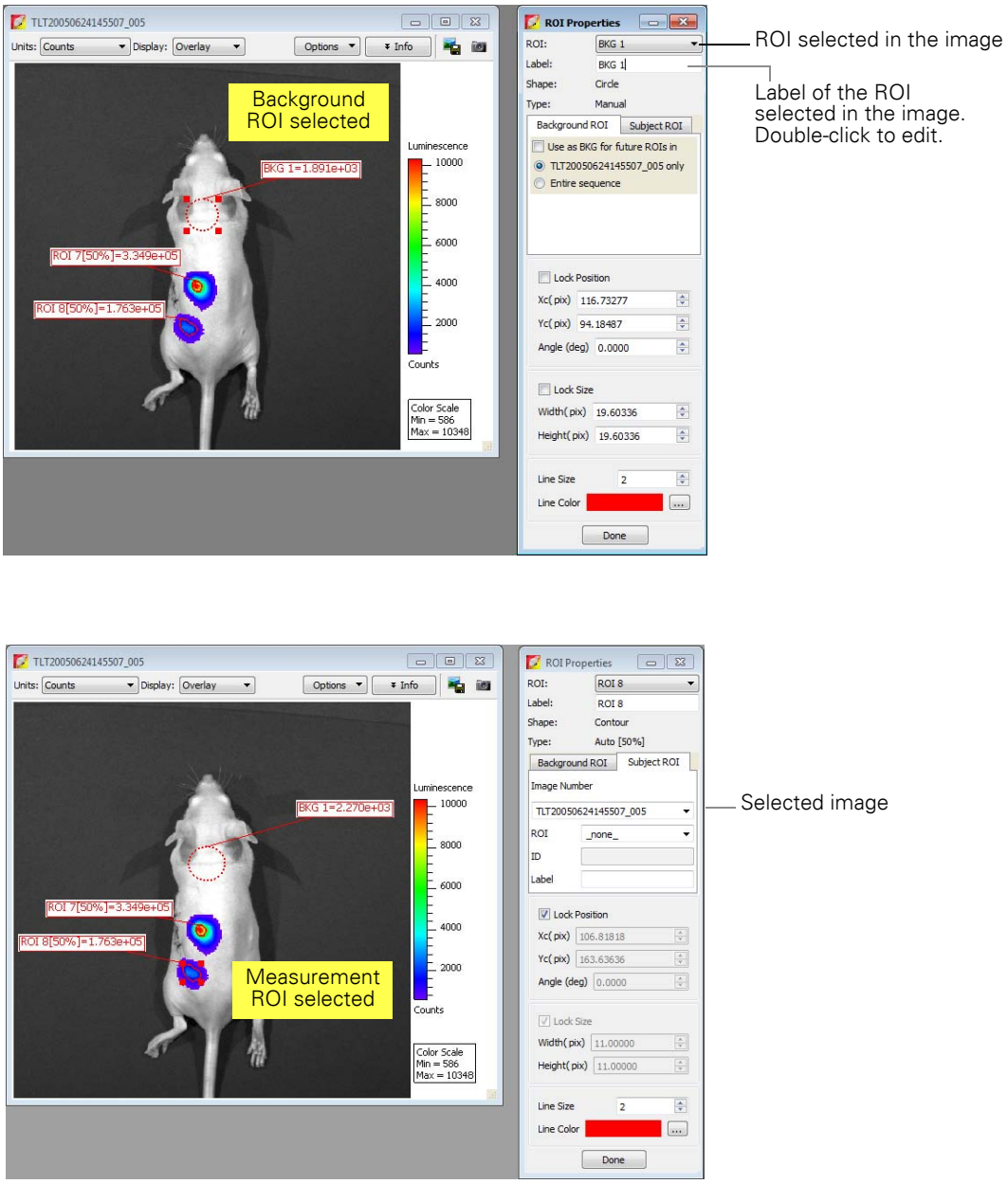


Figure 8.20 ROI properties, Subject tab
The items in the ROI Properties box depend on the type of ROI selected in the image. For more details see Table 8.3, page 137.

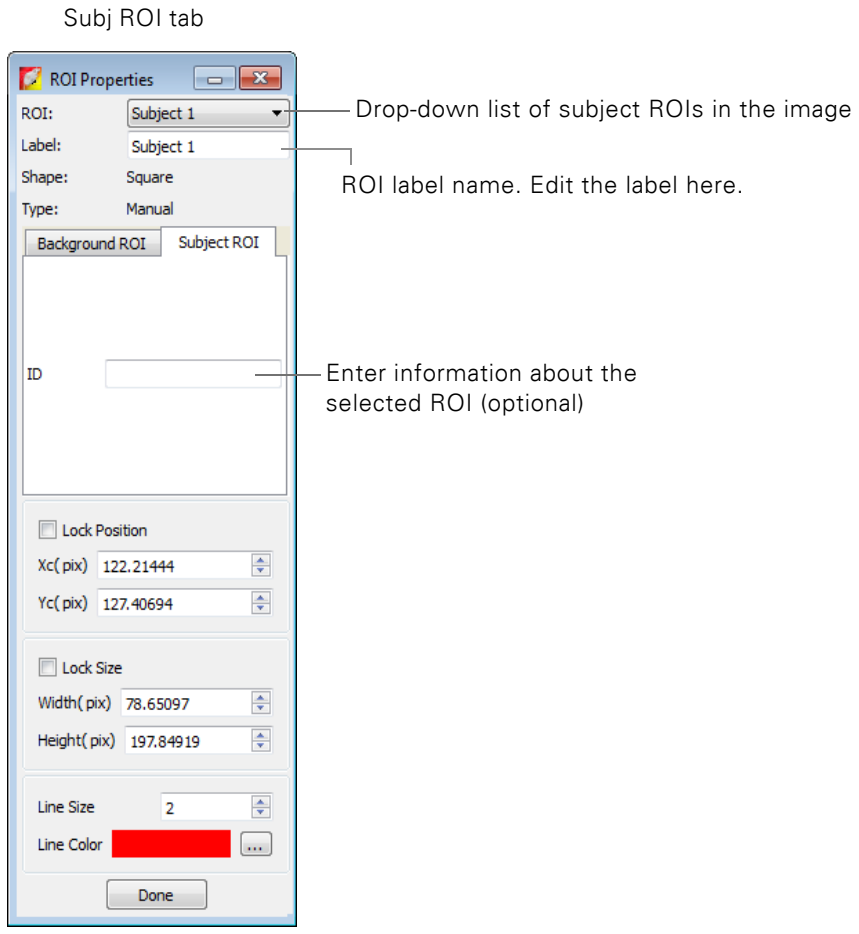


Table 8.3 ROI Properties

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).

Table 8.3 ROI Properties

Item	Description
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 shows 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.
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 (for more details on setting the units, see *ROI Dimensions*, page 147).
Height	Height (pixels or cm) of the ROI selected in the image.
Line Size	Specifies the ROI line thickness. To change the line thickness, enter a new value or click the up/down arrows.
Line Color	Specifies the color of the ROI line. To select a line color, click the **Browse** button ... .
Done	Click to close the ROI Properties box and apply any new settings, including: • Linkage between a measurement ROI and subject ROI (for more details, see *Drawing an ROI Using the Free Draw Method*, page 129). • ROI size dimensions or position • Subject ROI ID information

Moving an ROI

There are two ways to move an ROI on an image:

- Drag the ROI to a new location
- Edit the settings in the ROI Properties box

NOTE

An ROI cannot be moved if it was created using the auto ROI tool or if the ROI position is locked.

To drag an ROI:

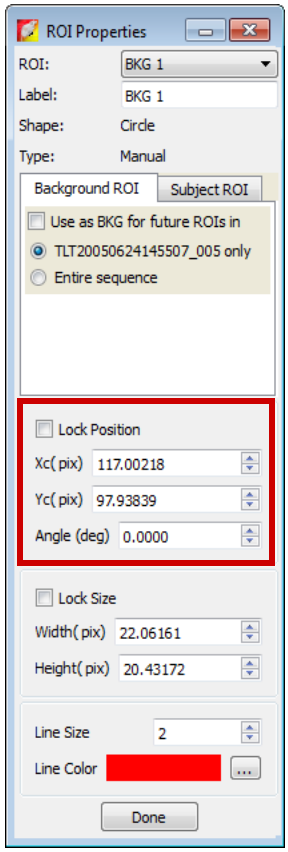
1. Put the mouse pointer over the ROI so that it becomes a ✥ arrow.
2. Drag the ROI.
3. Release the mouse button when the ROI is properly positioned.

To move an ROI using the ROI Properties dialog box:

1. Double-click the ROI in the image.

The ROI Properties box appears and displays the position and dimensions of the selected ROI.

Figure 8.21 ROI Properties dialog box



Position of the ROI selected in the image

2. To set ROI position, enter new coordinates for the center of the ROI (Xc (pix) and Yc (pix) values) in the ROI Properties box.
3. To rotate the ROI clockwise, enter the degrees in the Angle (deg) box and click outside the box.
4. To lock the current ROI position, choose the Lock Position option.

NOTE

The ROI position cannot be changed until the Lock Position option is cleared.

Editing ROI Dimensions

There are two ways to resize a circle or square ROI:

- Drag a handle on the ROI
- Edit the settings in the ROI Properties box

NOTE

You cannot change the size of an ROI that was created using the auto ROI or free draw tool.

To resize an ROI using a handle:

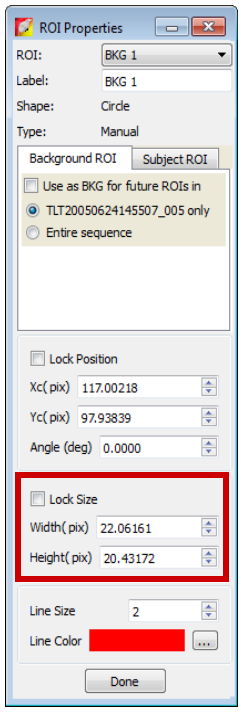
1. Select the ROI and put the mouse pointer over a handle (■) on the ROI.
2. When the pointer becomes a ↖↘ arrow, drag the handle.

To resize an ROI using the ROI Properties box:

1. Double-click the ROI in the image.

   The ROI Properties box appears and displays the positions and dimensions of the selected ROI.

Figure 8.22 ROI Properties dialog box



Dimensions of the ROI selected in the image

2. Enter a new width or height value in the ROI Properties box.
3. To lock the current ROI size, choose the Lock Size option.

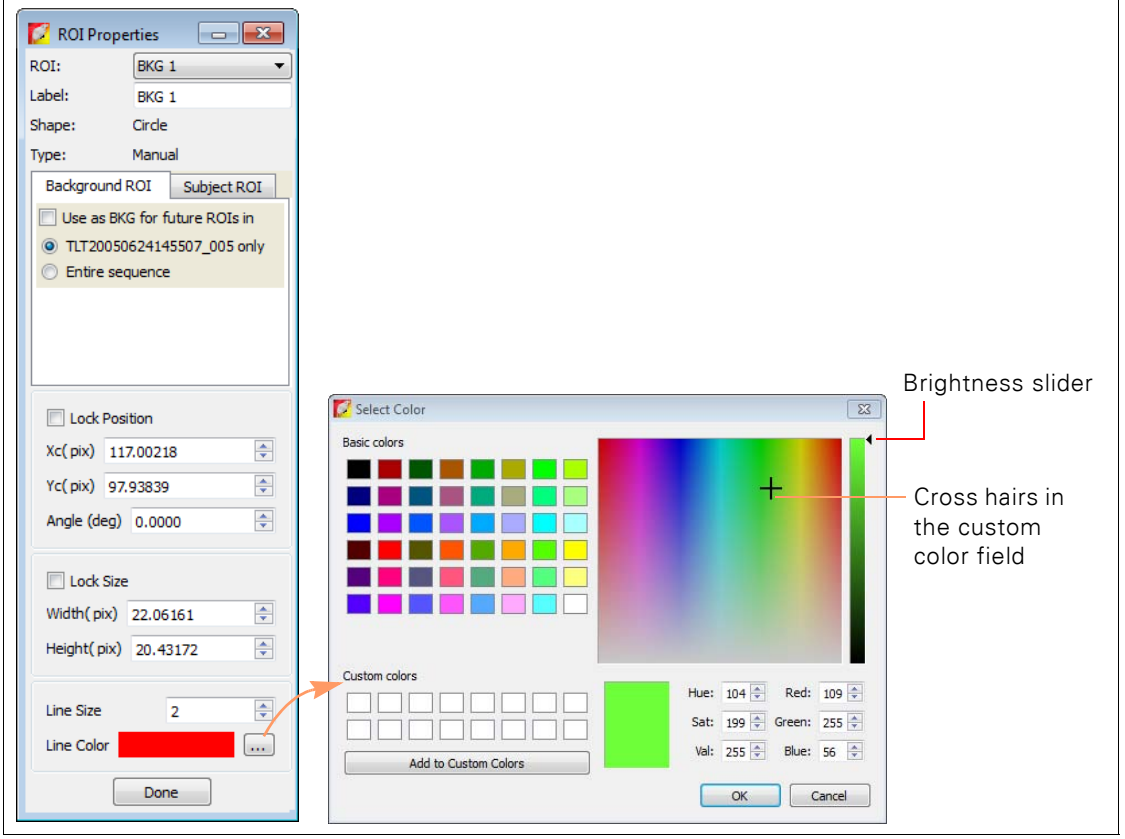
NOTE

The ROI size cannot be changed until the Lock Size option is cleared.

Editing the ROI Line

1. Double-click the ROI that you want to edit.
   The ROI Properties box appears (Figure 8.23).

Figure 8.23 Editing ROI properties



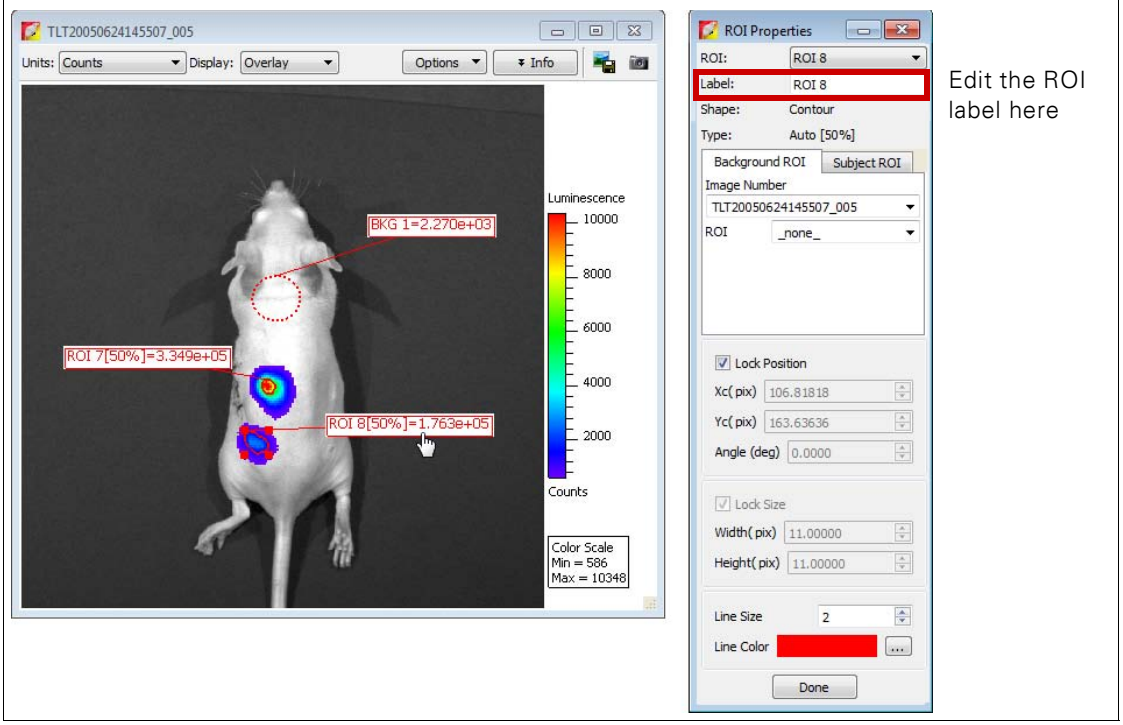
2. To edit the ROI line thickness, enter a new value in the Line Size box. Alternatively, click the ▲▼ arrows.
3. To change the ROI line color:
   a. Click the **Browse** button ....
   b. The Select Color box appears.
   c. To select a basic color for the ROI line, click a basic color swatch, and click **OK**.
   d. To define a custom color, drag the crosshairs in the custom color field, adjust the brightness slider, and click **Add to Custom Colors**.
   e. To select a custom color for the ROI line, click a custom color swatch, and click **OK**.

Move or Edit the 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, and then click to release the label at the new location (Figure 8.24).

Figure 8.24 Move or edit the ROI label



To edit the ROI label:

1. Double-click the ROI of interest. Alternatively, right-click the ROI (**Ctrl**-click for Macintosh users) and select **Properties** on the shortcut menu.
2. In the ROI Properties box that appears, edit the name in the ROI Label box and click **Done** (Figure 8.24).

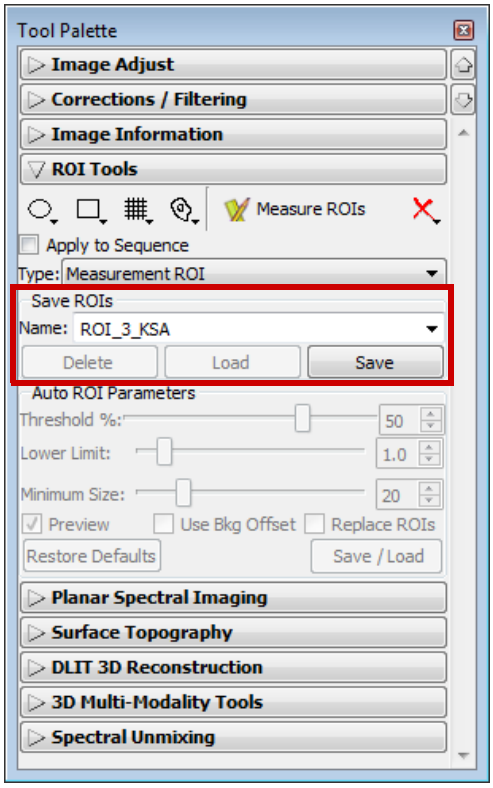
Saving ROIs

The software automatically saves ROIs with an image. The ROI measurements are saved in the AnalyzedClickInfo.txt file associated with the image. ROIs are saved per user and can be applied to other sequences. Additionally, ROI parameters can be saved per user and applied to other sequences.

To save ROIs to the system:

1. In the Name drop-down list, confirm the default name or enter a new name for the ROI(s).

Figure 8.25 Name and save the ROIs to the system



2. Click **Save**.

   The ROI(s) from the image are saved to the system and can be selected from the Name drop-down list.

To load ROIs on an image:

1. Open an image.
2. In the ROI tools, make a selection from the Name drop-down list and click **Load**.

NOTE

If you load ROI(s) onto an image, then draw additional ROIs, the **Save** button changes to **Overwrite**. If you want to save this collection of ROIs using the existing name, click **Overwrite**.

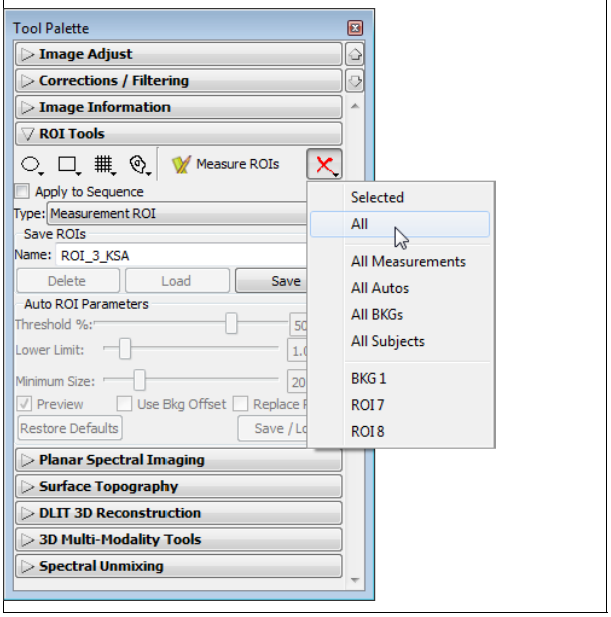
Deleting ROIs

You can delete ROIs from an image or permanently remove ROIs from the system.

To delete ROIs from an image:

1. In the ROI tools, click the button.

Figure 8.26 Removing ROIs from an image



2. In the drop-down list that appears, select a delete command.

   The specified ROIs are deleted from the image.

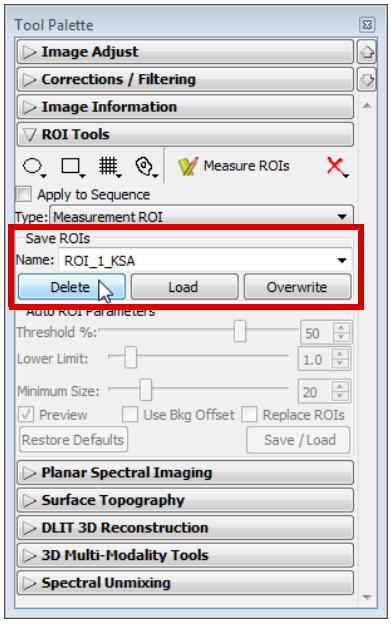
NOTE

This does not delete ROIs saved to the system (global save).

To permanently remove ROIs from the system:

1. Select the ROI(s) that you want to delete from the drop-down list of saved ROIs.
2. Click **Delete** (Figure 8.27).

Figure 8.27 Removing ROIs from the system



8.9 Managing the ROI Measurements Table

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 or photons, or in terms of *efficiency*. For more details, see *Quantifying Image Data*, page 281.

Viewing the ROI Measurements Table

1. Click the button. Alternatively, select **View** → **ROI Measurements** on the menu bar.

Figure 8.28 Opening the ROI Measurements table
Column headers in the table include ROI information, ROI measurements and dimensions, and information about the image recorded at acquisition.

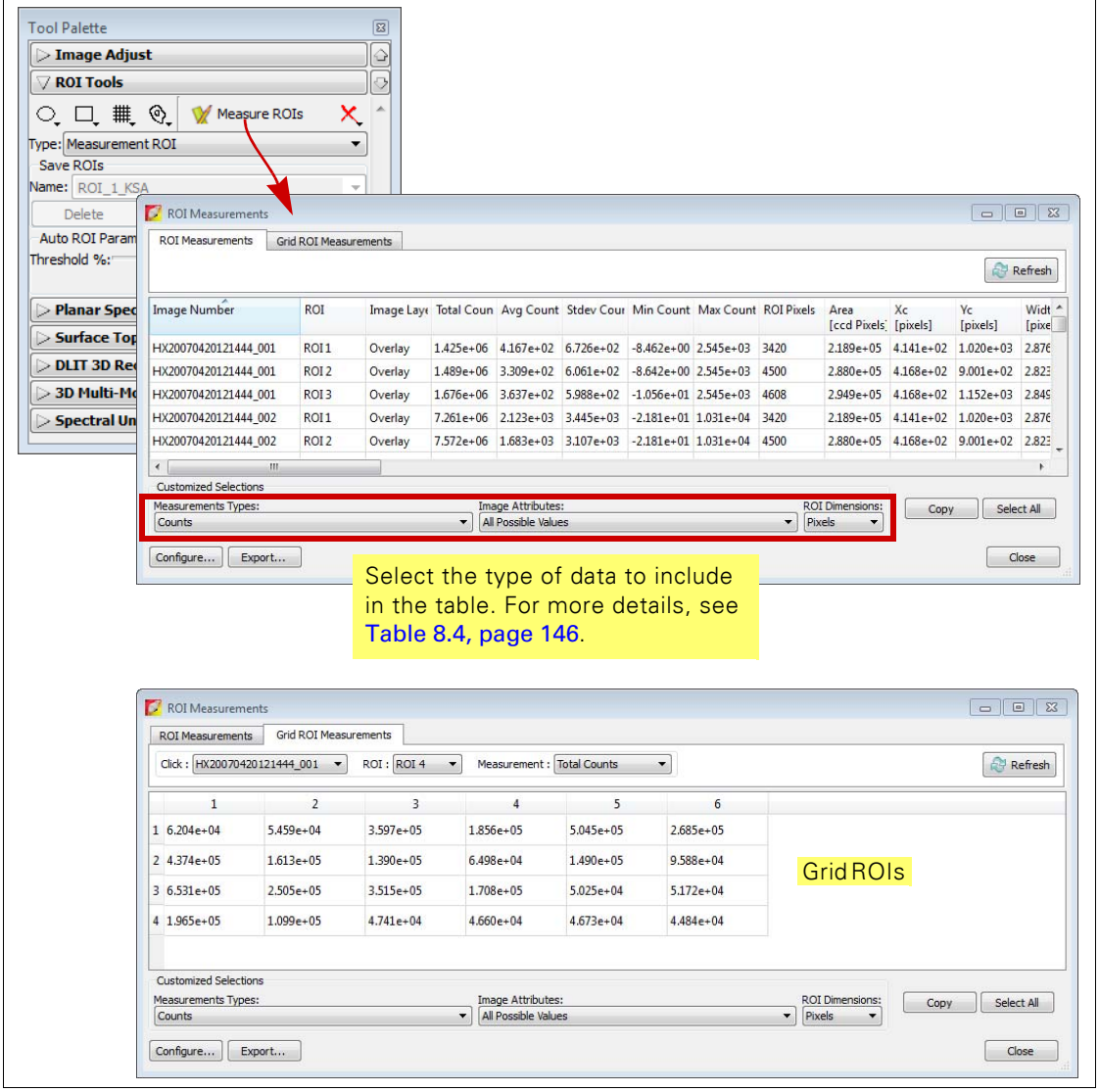


Figure 8.29 ROI Measurements table, kinetic ROIs

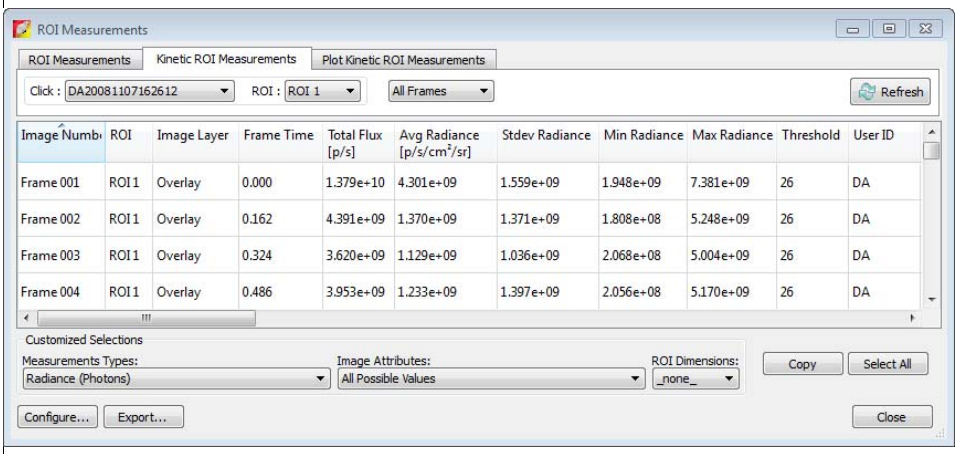


Table 8.4 ROI Measurements table

Item	Description
Measurement Types	Make a selection from the drop-down list to specify the type of ROI measurements to include in the table.
None	Excludes ROI measurements from the table.
Counts (luminescence)	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. For more details on count units, see page 281. **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.
Radiance (Photons) (fluorescence)	Total Flux = the radiance (photons/sec) in each pixel summed or integrated over the ROI area (cm^2) x 4π. Average Radiance = the sum of the radiance from each pixel inside the ROI/number of pixels or super pixels (photons/sec/cm^2/sr). Stdev Radiance = standard deviation of the pixel radiance inside the ROI Min Radiance = lowest radiance for a pixel inside the ROI. Max Radiance = highest radiance for a pixel inside the ROI. For more details on photon units, see page 282.
Radiant Efficiency (fluorescence)	Epi-fluorescence - Fluorescence emission radiance per incident excitation irradiance: p/sec/cm^2/sr/ W/cm^2 Transillumination fluorescence - Fluorescence emission radiance per incident excitation power: p/sec/cm^2/sr/mW
Efficiency (epi-fluorescence)	Fluorescent emission yield normalized to the incident excitation intensity (radiance of the subject/illumination intensity)
NTF Efficiency (transillumination fluorescence)	Fluorescent emission image normalized to the transmission image which is measured with the same emission filter and open excitation filter.

Table 8.4 ROI Measurements table (continued)

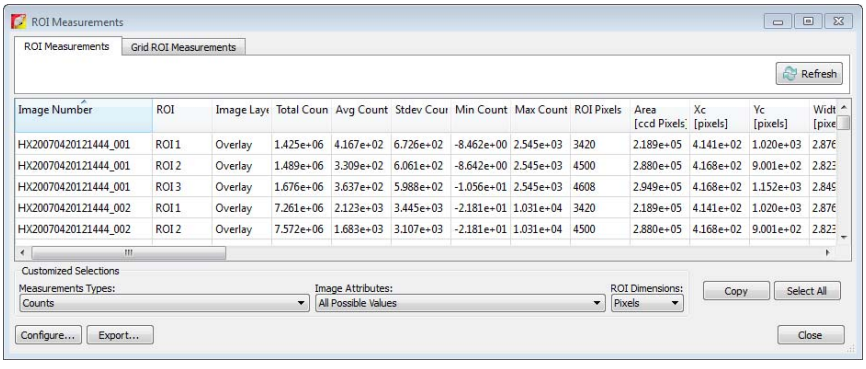
Item	Description
Image Attributes	Make a selection from the drop-down list to specify the click number (image file) information to include in the table. Click attributes include label name settings and camera settings.
None	Excludes image attributes from the table.
All Possible Values	Includes all of the image attributes (for example, label name settings and camera settings) in the table.
All Populated Values	Includes only the image attributes with values in the table.
Living Image Universal	Includes all Living Image Universal label name settings in the table.
ROI Dimensions	Make a selection from the drop-down list to specify the ROI dimensions to include in the table.
None	Excludes the ROI area, x,y-coordinates, and dimensions from the table.
Pixels	Includes ROI area, x,y-coordinates, and dimensions (in pixels) in the table.
cm	Includes ROI area, x,y-coordinates, and dimensions (in cm) in the table.
Copy	Copies the selected row(s) in the table to the system clipboard.
Select All	Copies all rows in the table to the system clipboard.
Refresh	Updates the ROI Measurements table (for example, after you draw new ROIs, move an ROI, and close or open image data).
Configure	Displays the Configure Measurements box that enables you to specify and organize the data categories (column headers) for the table.
Export	Displays the Save Measurements box so that the data can be saved to a .txt or .csv file. **Note:** Grid ROI measurements exported to a .csv file can be opened in a spreadsheet application like Microsoft® Excel®.
Close	Closes the ROI Measurements table.

Configuring the ROI Measurements Table

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

1. To reorder the columns, drag a column header (left or right) in the table.
2. To change the measurement units, make a selection from the Measurement Types drop-down list.

Figure 8.30 ROI Measurements table



3. To include image information in the ROI table, make a selection from the Image Attributes drop-down list.
4. To include ROI dimensions in the table, select units (Pixels or cm) from the ROI Dimensions drop-down list.

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 8.30). You can also create a custom table configuration.

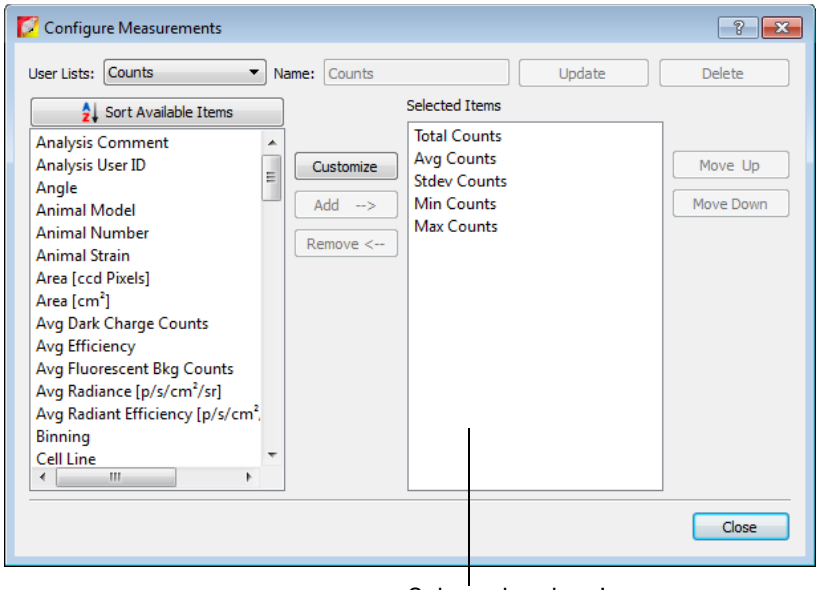
NOTE

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

1. In the ROI Measurements table, click **Configure**.

   The Configure Measurements box appears.

Figure 8.31 Configure Measurements dialog box



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

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

To delete a custom table configuration:

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

NOTE

Preset table configurations cannot be deleted.

Copying or Exporting the ROI Measurements Table

To export the table:

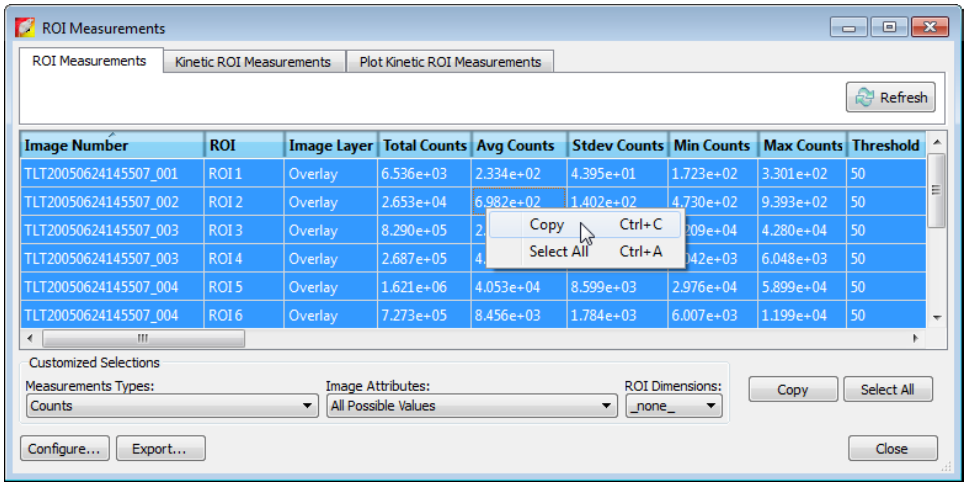
1. In the ROI Measurements table, click **Export**.
2. In the dialog box that appears, select a folder and enter a name for the file (.txt), then click **Save**.

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.

All rows - Click Select **All** and then click **Copy**. Alternatively, press **Ctrl+A**, then right click the table and choose **Copy** on the shortcut menu.

Figure 8.32 Copy all rows in the ROI Measurements table to the system clipboard



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9 Image Math

The Living Image software provides tools that enable you to mathematically combine two images to create a new image. The primary use of image math is to subtract tissue autofluorescence background from signal.

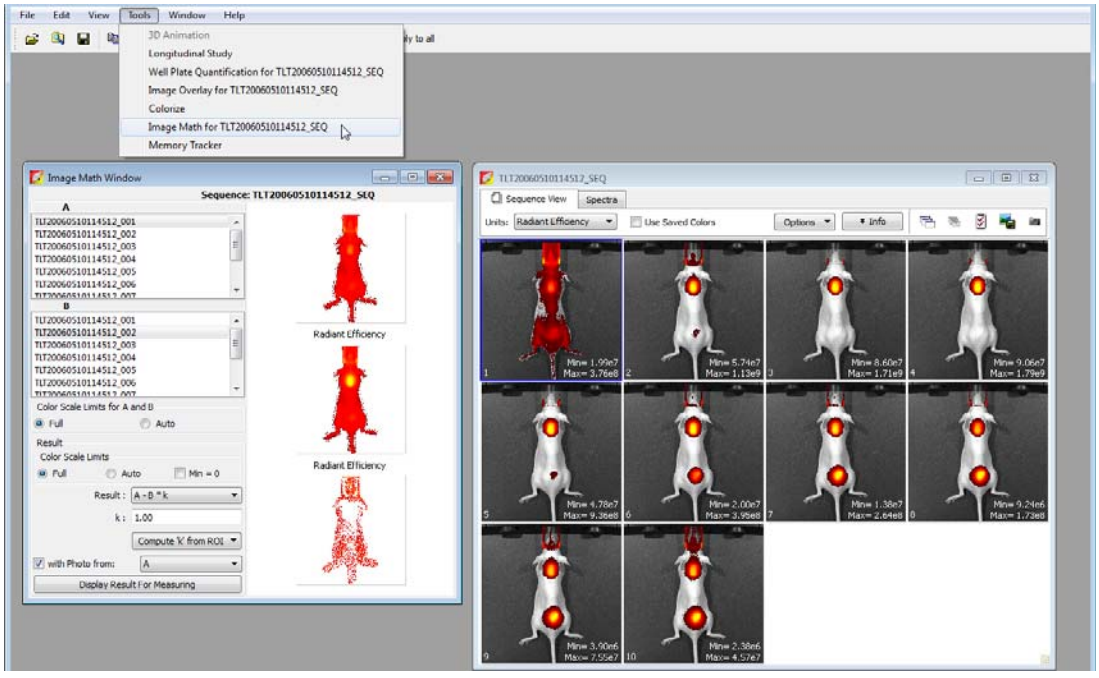
LIving Image Tool	Use This Tool To...	See Page
Image Math	Mathematically combine (add, multiply, subtract, or divide) two user-specified images.	151
Image Math	Remove autofluorescence from a fluorescent image.	153

To perform image math, open an image sequence or a group of images. For more details on creating a sequence from individual images, see page 117.

9.1 Creating a New Image Using Image Math

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

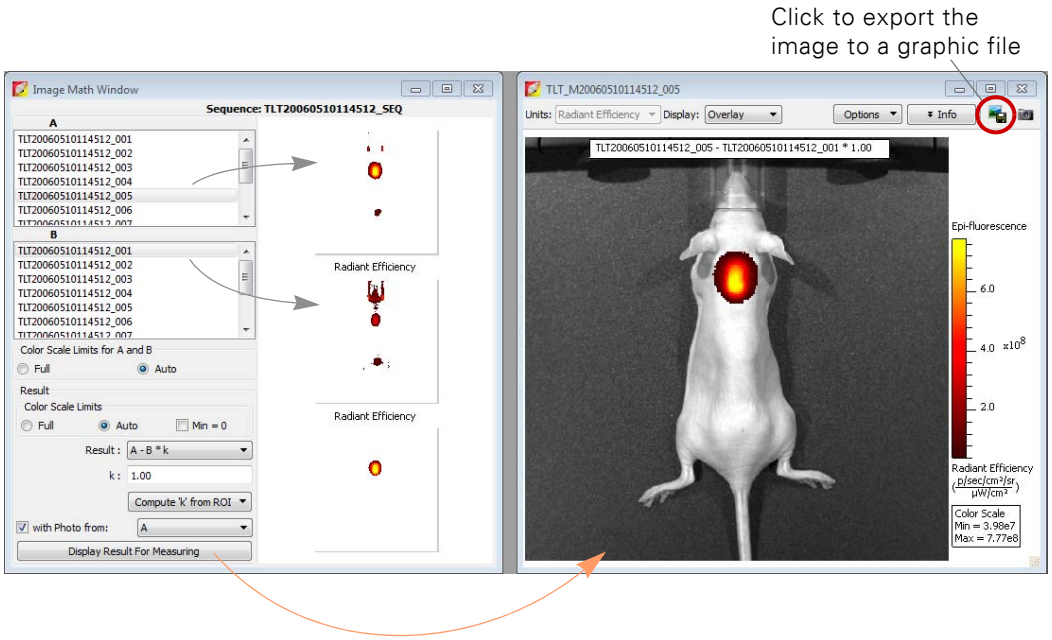
Figure 9.1 Opening the Image Math window



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 9.2 Image Math window and new image



NOTE

For more details on items in the Image Math window, see Table 9.1, page 153.

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. To view the new image, click **Display Result for Measuring**.

To save the new image:

1. Click the **Save** button . Alternatively, select **File** → **Save** on the menu bar.
2. In the dialog box that appears, select a directory, and click **Save**.

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

To export the image to a graphic file:

1. Click the **Export** button (Figure 9.2).
2. In the dialog box that appears, select a directory, enter a file name, and select the file type from the Save as type drop-down list.
3. Click **Save**.

Table 9.1 Image Math window

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

9.2 Subtracting Tissue Autofluorescence

To remove tissue autofluorescence from image data, the IVIS Imaging System implements a subtraction method using blue-shifted background filters that emit light at a shorter wavelength.

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. For more details on tissue autofluorescence, see Appendix F, page 305

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

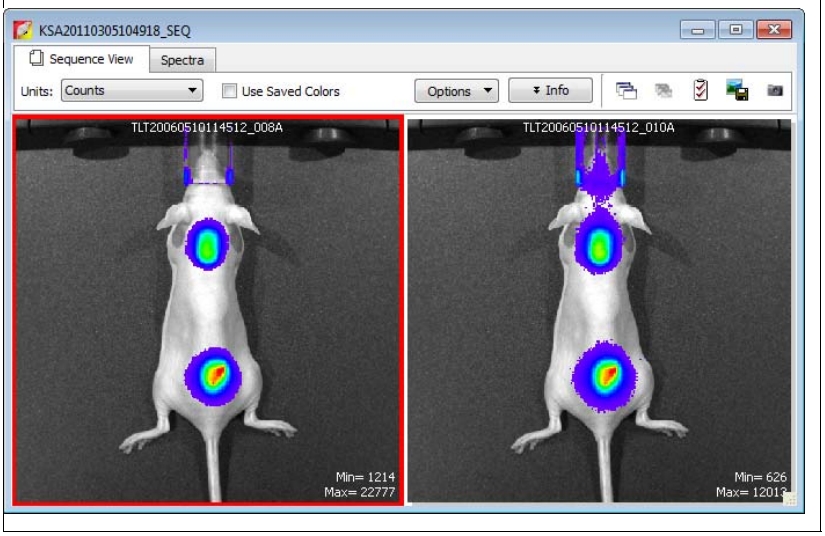
tissue autofluorescence due to different excitation wavelengths and filter transmission characteristics.

After you acquire an image sequence that includes a primary and background image, use the image math tool to subtract tissue autofluorescence. (For more details on acquiring an image sequence, see Chapter 4, page 33.)

To subtract tissue autofluorescence:

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

Figure 9.3 Image sequence

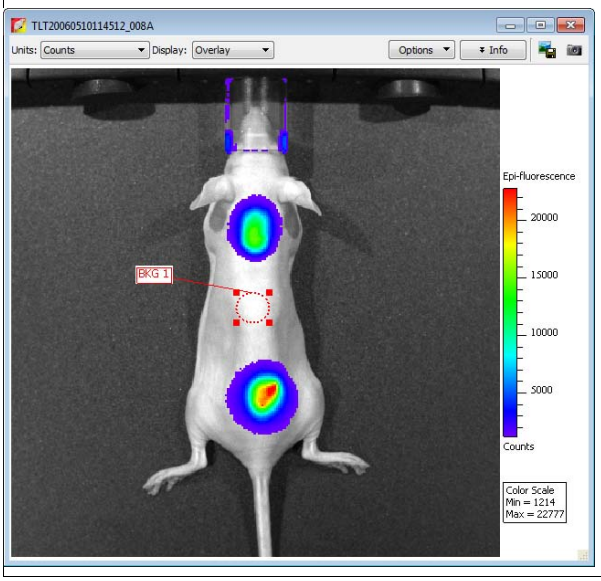


2. Open either the primary or background image and:
   a. Optimize the image display using the color scale Min and Max sliders in the Image Adjust tools.
   b. Draw a measurement ROI on an area of the animal that represents background signal (area where no fluorophore signal is present).

NOTE

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

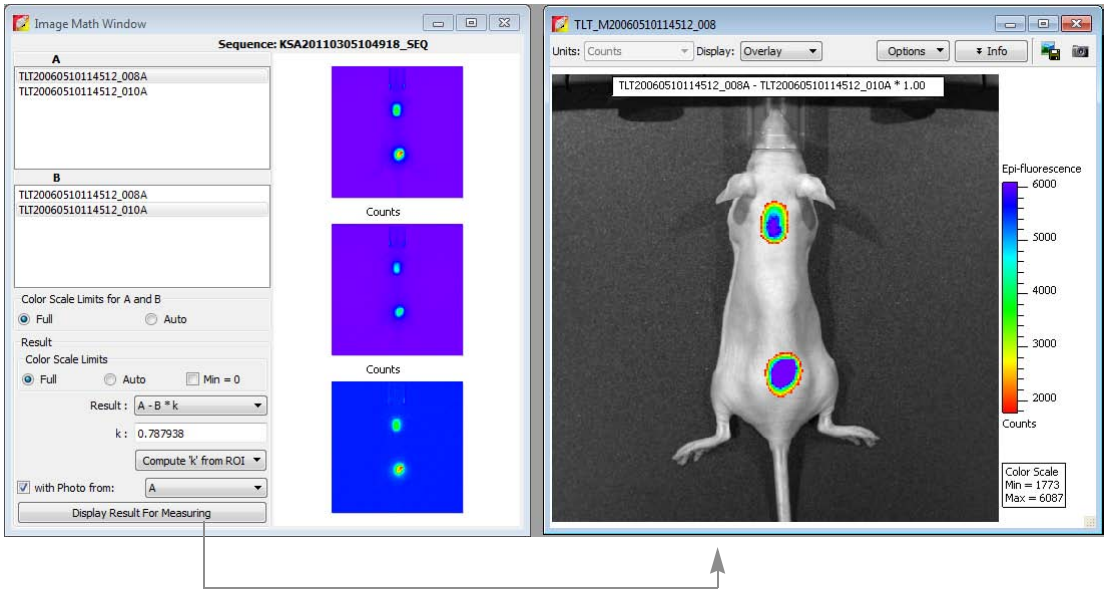
Figure 9.4 Draw measurement ROI on an area that represents background signal



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

   For more details on items in the Image Math window, see Table 9.1, page 153.
5. Select the math function 'A-B*k' in the Result drop-down list.

Figure 9.5 Select a math function and view the mathematical result



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

   The background-corrected signal is displayed.
7. To view the mathematical result (overlay mode) in a separate image window, click **Display Result For Measuring**.

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

To save the new image:

1. Click the **Save** button . Alternatively, select **File** → **Save** on the menu bar.
2. In the dialog box that appears, select a directory, and click **Save**.

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

To export the new image to a graphic file:

1. Click the **Export** button .
2. In the dialog box that appears, select a directory, enter a file name, and select the file type from the Save as type drop-down list.
3. Click **Save**.

10 Planar Spectral Image Analysis

The Living Image software applies planar spectral image analysis to a sequence to determine the average depth and total photon flux of a luminescent point source in a user-specified region of interest. For more information on planar spectral image analysis, see Appendix G, page 309.

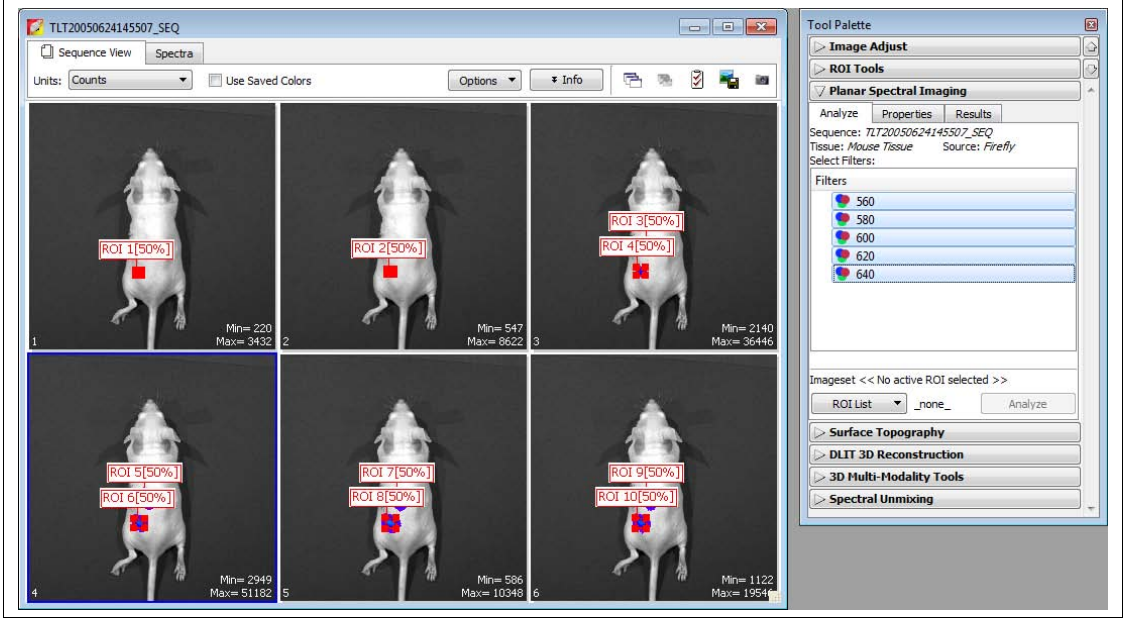
10.1 Image Sequence Requirements

Use the Imaging Wizard to setup the image sequence required for planar spectral image analysis. (For more details on the Imaging Wizard, see Chapter 4, page 33.) At a minimum, the sequence must include a photographic and luminescent image at the first wavelength and a luminescent image at a second wavelength (560-660).

10.2 Planar Spectral Image Analysis

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

Figure 10.1 Sequence for planar spectral image analysis

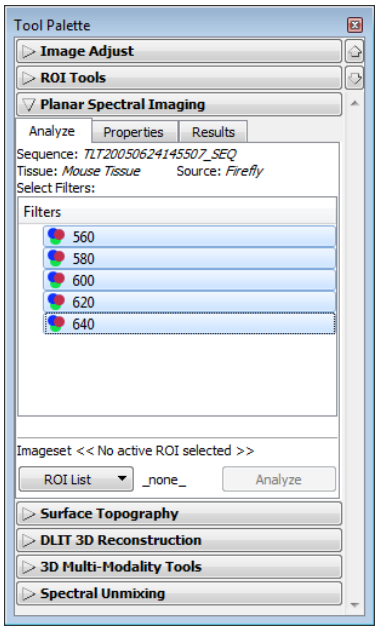


2. In the Tool Palette, click Planar Spectral Imaging.
3. In the Analyze tab, select the emission filter wavelengths for the analysis (Figure 10.2).

   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. (For more details, see *Measuring Background-Corrected Signal*, page 130.)

Figure 10.2 Planar spectral imaging tools, Analyze tab
For more details on the Analyze tab, see page 159.

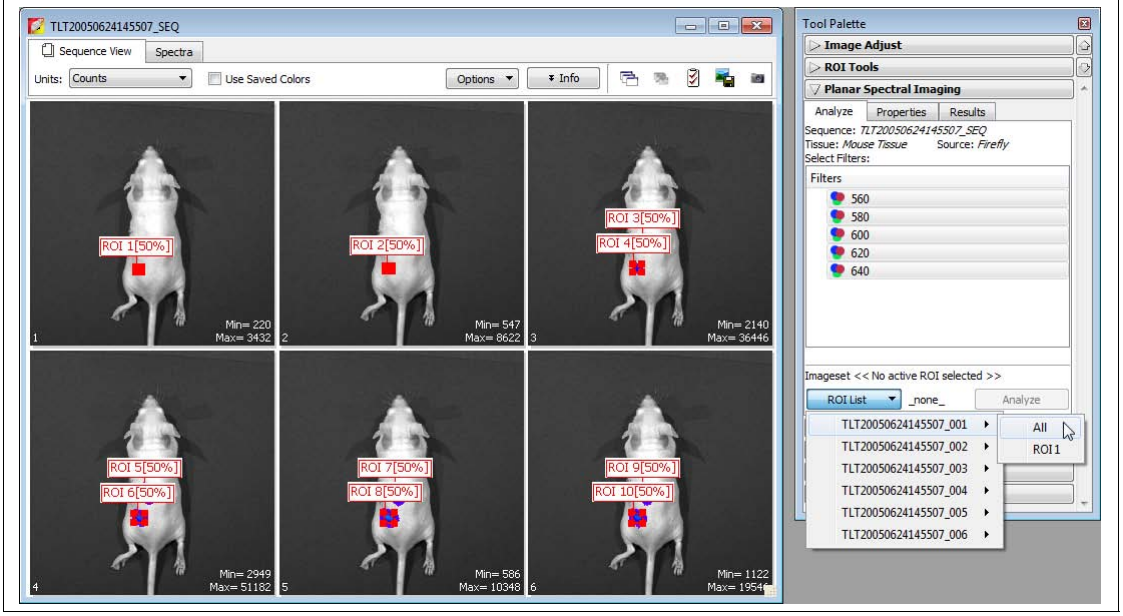


Emission filter wavelengths selected for the analysis

4. 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. (For more details on drawing ROIs, see page 120.)

   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.

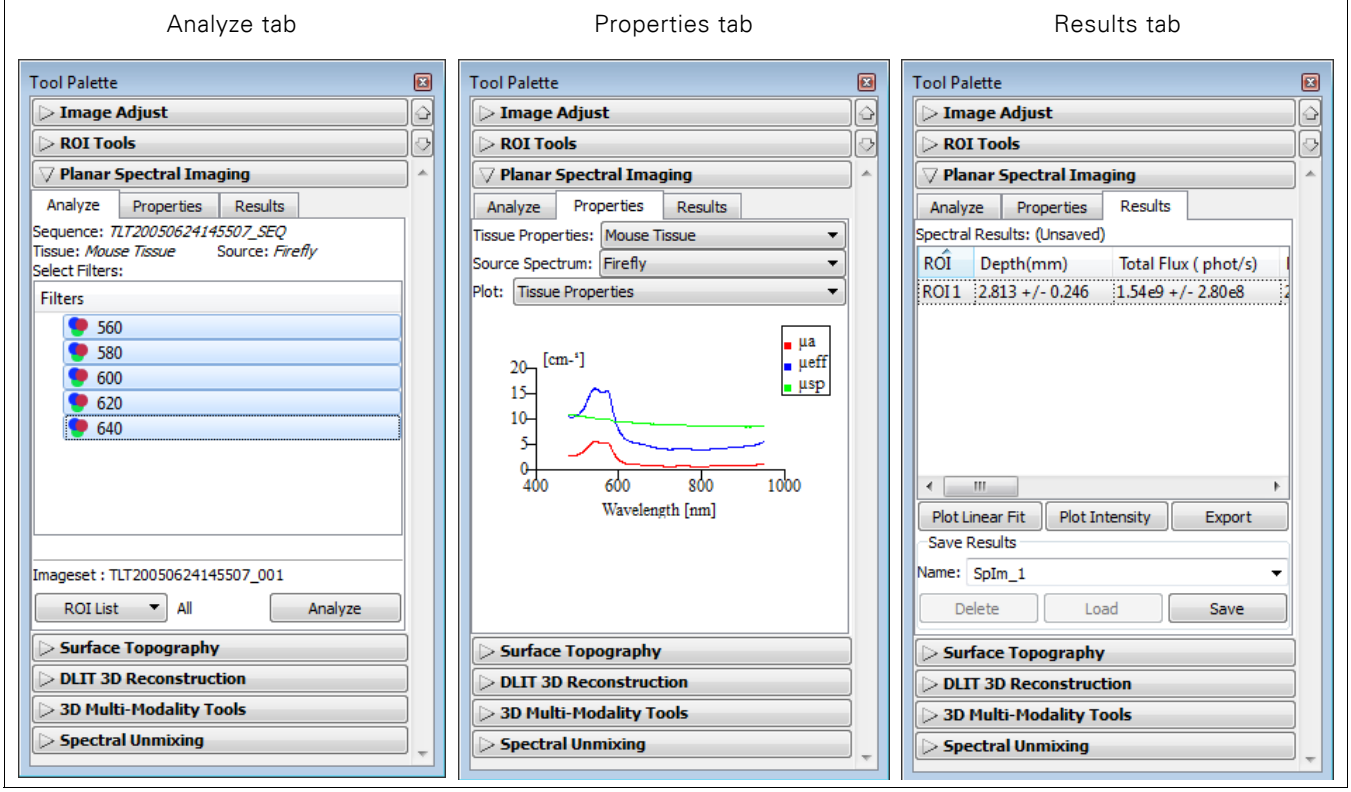
Figure 10.3 Selecting ROIs for planar spectral image analysis



5. Choose the tissue properties:
   a. 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

Figure 10.4 Planar spectral imaging tools



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 page 160.

Table 10.1 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.

Table 10.1 Planar spectral imaging tools (continued)

Item	Description
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 ($_a$), effective attenuation coefficient ($_{eff}$), and reduced scattering coefficient ($'_s$). 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 (phot/s) - Estimated total photon flux from the point source.
Plot Linear Fit	Displays a graph of normalized intensity versus the effective attenuation coefficient ($_{eff}$, 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 (SpIm_<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).

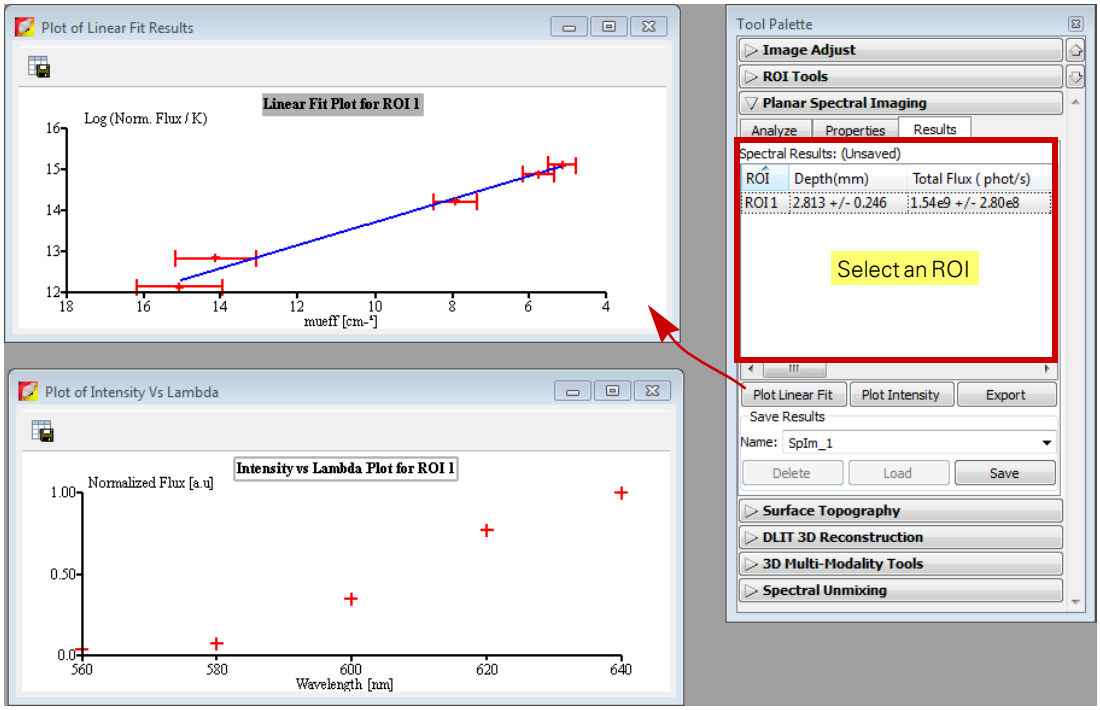
10.3 Viewing Graphical Results

1. In the Results tab, select an ROI.
2. Click **Plot Intensity** or **Plot Linear Fit** (Figure 10.5).

   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 ($_{eff}$). 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 ±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.

Figure 10.5 View the Linear Fit or Intensity graph



To export graph data:

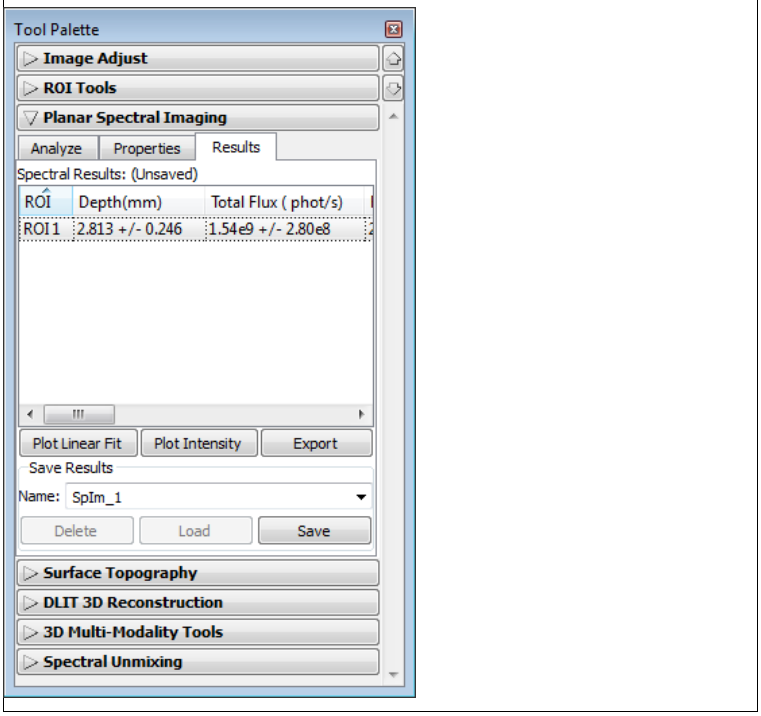
1. Click the **Export Data** button .
2. In the dialog box that appears, select a directory for the data and enter a file name (.csv).

   The data can be opened in a spreadsheet application such as Microsoft® Excel®.

10.4 Managing Planar Spectral Imaging Results

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

Figure 10.6 Planar spectral imaging tools, Results tab



To save results:

1. Select results (Splm_<name>) from the Name drop-down list.
2. Click **Save**.

   The planar spectral imaging results are saved with the image.

To view results:

1. Select results from the Name drop-down list.
2. Click **Load**.

To delete results:

1. Select the results that you want to delete from the Name drop-down list.
2. Click **Delete**.

To copy selected results:

1. Right-click the results (row) and select **Copy** from the shortcut menu that appears.

   The selected results are copied to the system clipboard.

To copy all results:

1. In the Results tab, 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.

To export results:

1. 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**.

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11 Spectral Unmixing

The Living Image software applies spectral unmixing to distinguish the spectral signatures of different fluorescent or luminescent reporters and calculate the respective contribution of each on every pixel of an image. Use spectral unmixing to:

- Extract the signal of one or more fluorophores from the tissue autofluorescence. Images can be acquired using epi-illumination (excitation light above the stage) or transillumination (excitation light below the stage)
- Analyze luminescent or fluorescent images when more than one reporter is used in the same animal model

11.1 Image Sequence Requirements

Use the Imaging Wizard to set up the image sequence that is required for spectral unmixing. For more details on the wizard, see Chapter 4, page 33.

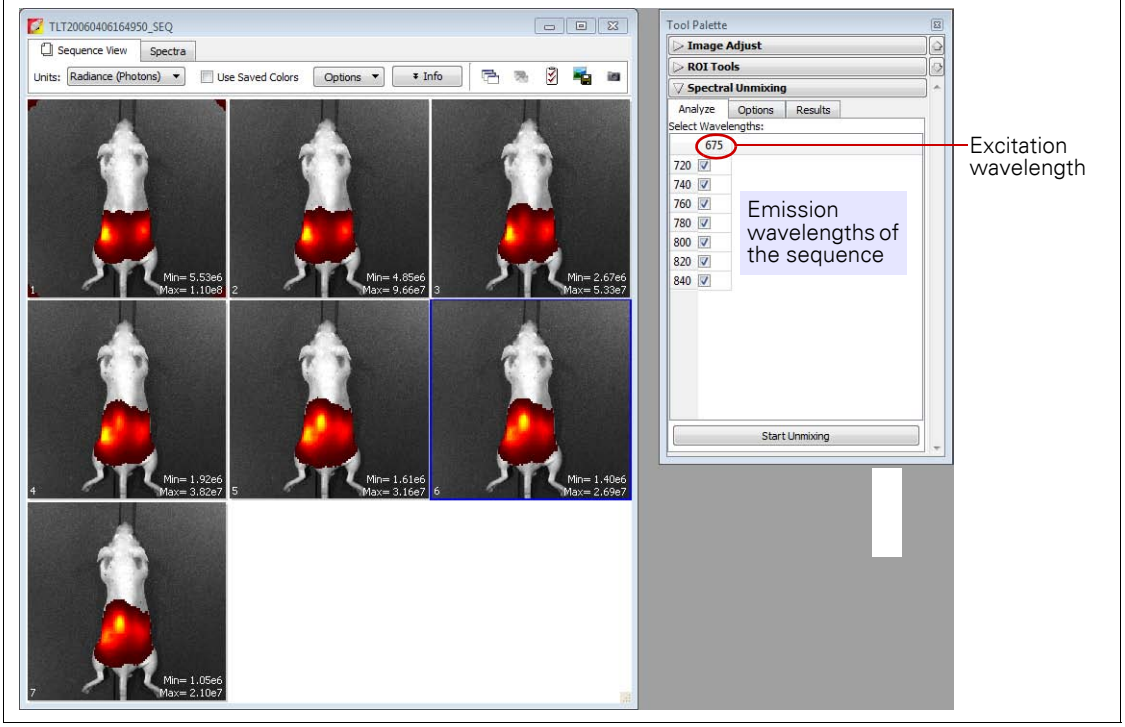
If you do not use the Imaging Wizard to set up the image sequence, it is recommended that the image sequence include images acquired using several filters that sample the emission or excitation spectra at multiple points across the entire range. Make sure that the band gap between the excitation and emission filters is sufficiently large (for example, >35 nm) so that the excitation light does not leak through the emission filter where it can be detected by the CCD.

11.2 Performing Spectral Unmixing

1. Load the image sequence.

   In the example in Figure 11.1, the fluorophore is Quantum Dots 800. Images were acquired using a 675 nm excitation filter and emission filters from 720 to 820 nm in 20 nm increments.

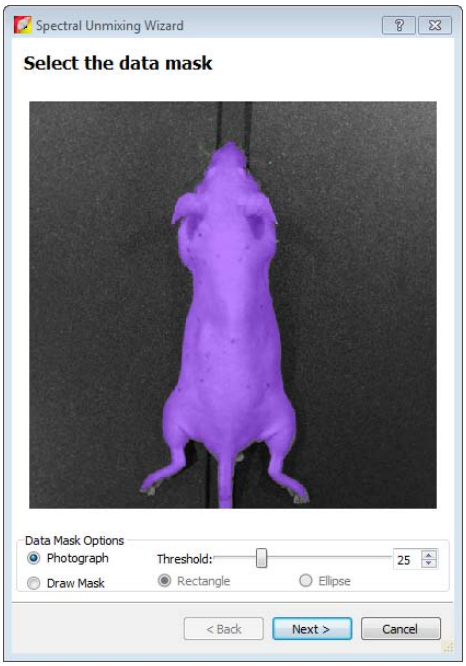
Figure 11.1 Sequence for spectral unmixing



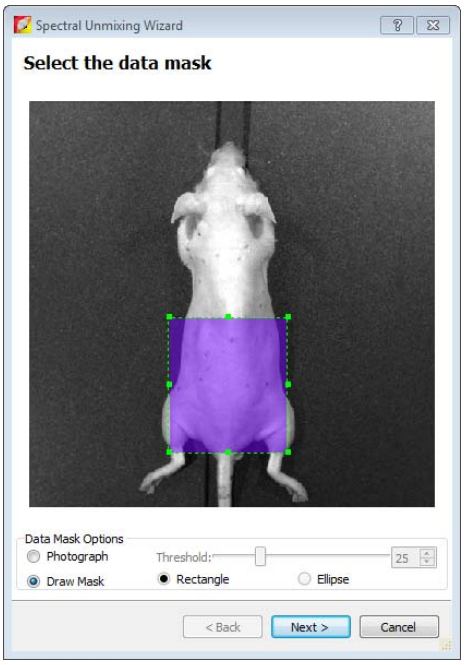
2. In spectral unmixing tools, click the Analyze tab, and put a check mark next to the emission wavelengths that you want to include in the analysis.
3. Click **Start Unmixing**.

   The Spectral Unmixing Wizard appears and shows the purple data mask that specifies the analysis area. By default, the data mask includes the entire subject.

Figure 11.2 Selecting the data mask
For more details on the data mask options, see Table 11.1.



Default data mask applied by the software



Data mask manually applied to a user-selected area

4. If you do not want to analyze the entire subject, draw a data mask on a user-selected area using the data mask options.

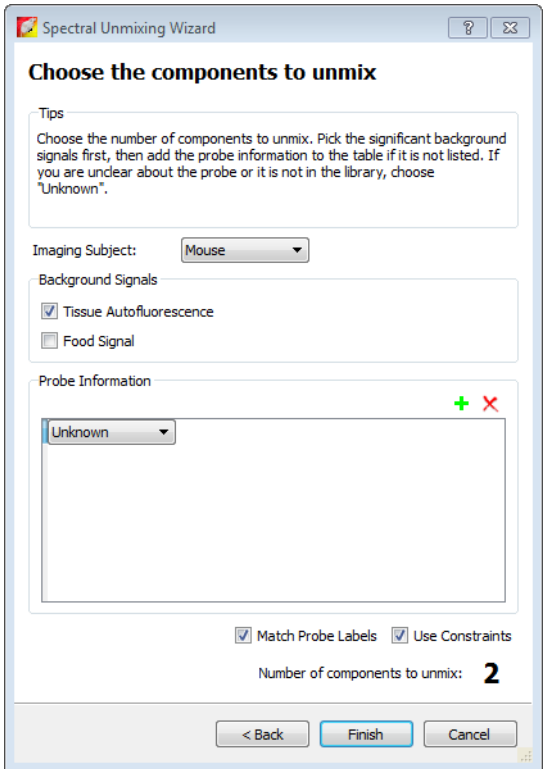
Table 11.1 Data mask options

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 unerlying 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. Click **Next** in the wizard.

   In this screen, you will select the subject type and signals (components) to unmix.

Figure 11.3 Choose the component to unmix



1. Choose a subject type from the drop-down list.
2. Choose one or both types of background signals.
3. If the probe does not automatically appear in this list, select the probe(s). If you are not sure about the probe that was used, choose "Unknown"

NOTE

Select at least two, but no more than four components to unmix.

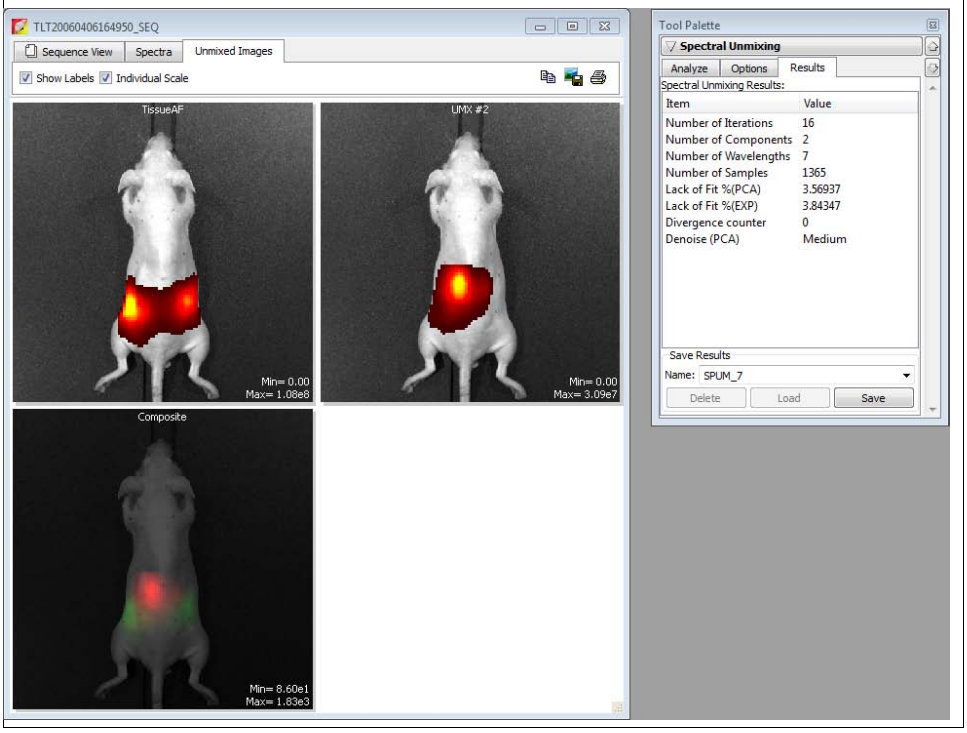
Table 11.2 Spectral unmixing wizard, choose the components to unmix

Item	Description
Imaging Subject	A drop-down list of subject types.
Background Signals	Any undesired fluorescence that the camera detects; for example, autofluorescence from the animal, food, or instrument background.
Probe Information	Specify the probes. - Click to add a probe. - Click to remove the selected probe.
Match Probe Labels	If the probe names are specified, the software attempts to automatically match the unmixed spectra with the specified probe names. **Note:** A correct match is not guaranteed due to the complexity of the *in vivo* spectra and filter sections, especially when only part of the emission/excitation spectrum is sampled.
Use Constraints	Choose this option to apply the recommended constraints when performing spectral unmixing. For more details on the constraints, see page 174. To disable the constraints, uncheck this option. Alternatively, in the Options tab of the Spectral Unmixing tools, click **Reset Values** and then click **Update**.
Number of components to unmix	The total number of components (background and probe signals) selected for unmixing.

6. Click **Finish** when you are done choosing the components to unmix.

   The unmixed images and results are displayed (Figure 11.4). The results include a signal distribution map of each unmixed result and a composite image that includes all of the fluorescent signals, each displayed in a different color.

Figure 11.4 Spectral unmixing results



7. To analyze an unmixed image, double-click the image.

   The image appears in a separate image window and the Tool Palette is available. This enables you to make ROI measurements and image adjustments that are saved with the image.

8. To adjust the appearance of the composite image, double-click the composite image.

   The composite image is displayed in a separate window.

Figure 11.5 Composite image window

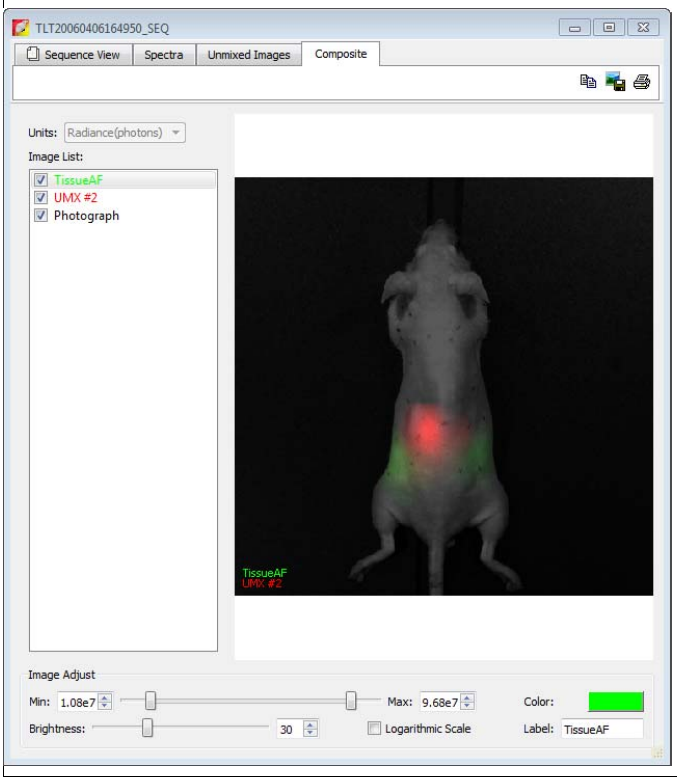


Table 11.3 Composite image window

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).
	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.

11.3 Spectra Window

The Spectra window plots the normalized spectra of the unmixed results.

Figure 11.6 Spectra window

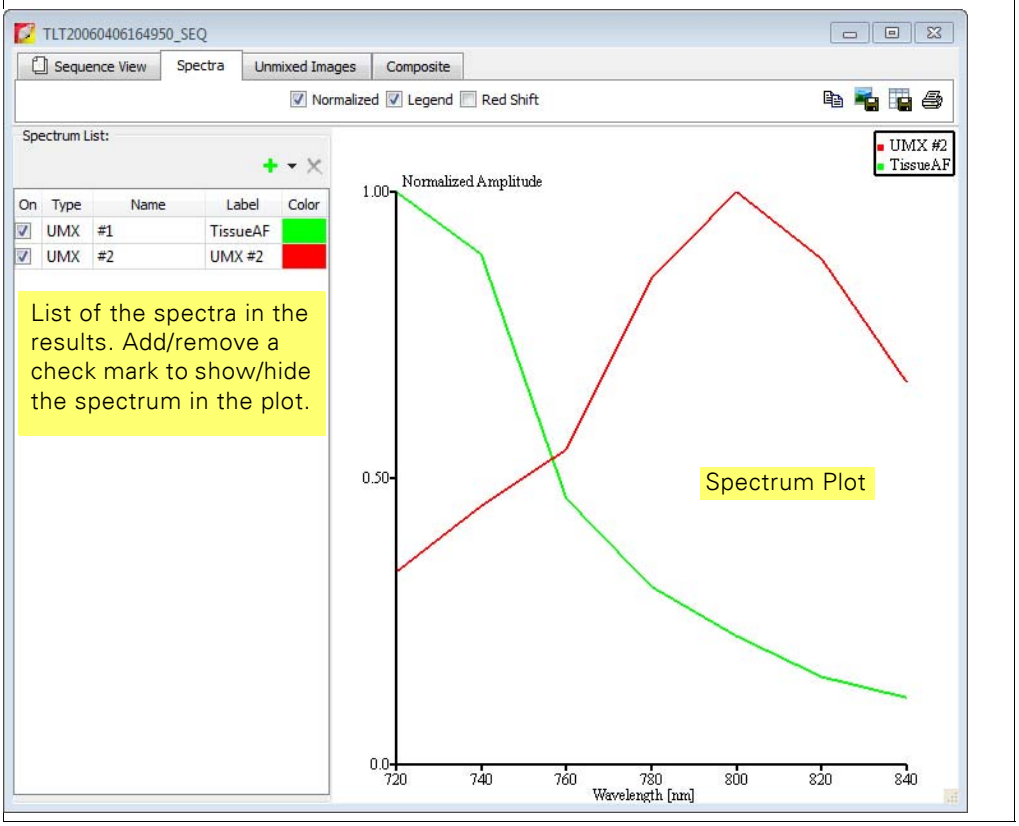


Table 11.4 Spectra window

Item	Description
	Enables you to select a spectrum to add to the graph. From Library - Choose this option to select a probe from the Living Image database. The library includes spectra of different sources obtained using excitation and emission filters. From ROI - Choose this option to display a spectrum calculated for a user-selected ROI.
	Deletes the spectrum selected in the spectrum list from the plot.
Type	The type of spectrum. UMX - A spectrum generated by the spectral unmixing algorithm. LIB - A user-selected library spectrum. The library includes spectra obtained of different sources obtained using excitation and emission filters. ROI - A spectrum calculated for a user-selected ROI.
Name	The spectrum identifier used by the unmixing algorithm. The name cannot be modified.
Label	The spectrum name in the spectrum plot key. Double-click the label to edit it.
	The plot line color for a particular spectrum. For the UMX type spectrum, it is also the color in the composite image. Double-click a color swatch to open the color palette that enables you to change the plot line color for the spectrum.

Table 11.4 Spectra window (continued)

Item	Description
Normalized	Normalizes ROI spectra to library spectra.
Legend	Click to show or hide the spectra plot legend.
Red Shift	Adjusts library and ROI spectra to compensate for tissue absorption (simulates the red spectral shift of a spectrum produced by a signal that is located at a depth of 5 mm in tissue).

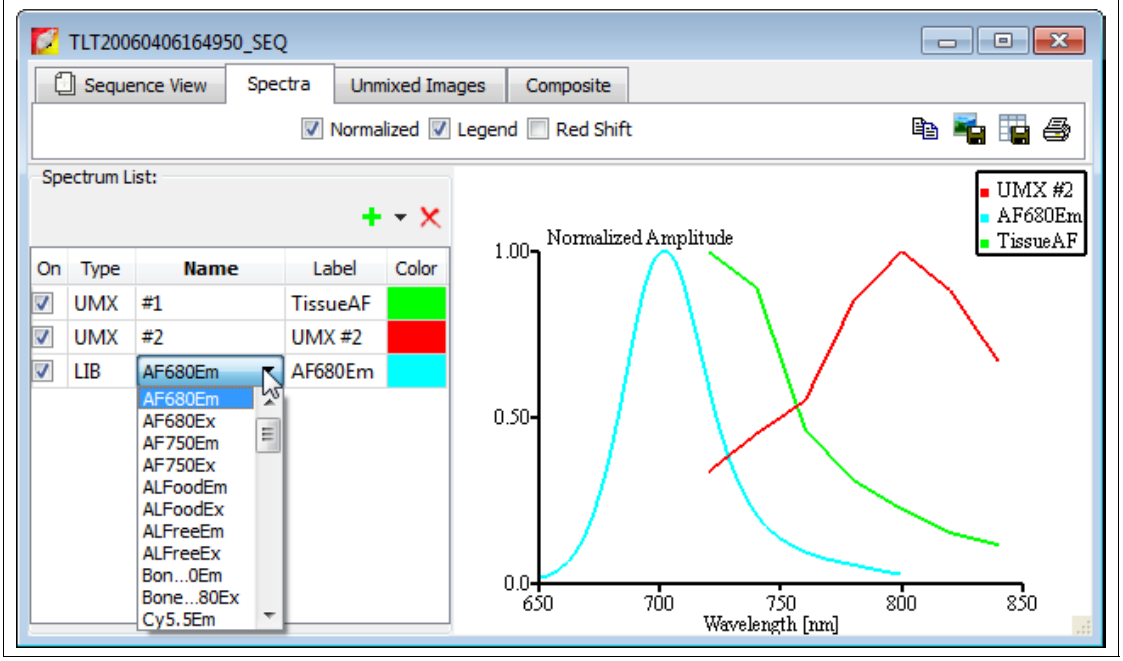
Adding Spectra to the Plot

You can add library spectra or a spectrum calculated for an ROI to the plot.

To display library spectra:

1. Click the + button arrow and select **From Library**.
   A new row appears in the spectrum list.
2. In the new row, select a probe name from the drop-down list.

Figure 11.7 Selecting a library spectrum to display



To add spectra from an ROI:

1. Create an ROI on an image and apply it to the sequence.
1. Click the + button arrow and select **From ROI**.
   A new row appears in the spectrum list.
2. In the new row, select an ROI name from the drop-down list.

To remove a spectrum:

1. In the spectrum list, select the spectrum (row) that you want to remove.
2. Click the ✕ button.

11.4 Spectral Unmixing Parameters

The Results tab in the Spectral Unmixing Tool Palette shows the optimized fit parameters used by the software to derive the spectral unmixing results (Figure 11.8).

Figure 11.8 Tool palette, Spectral unmixing tools, Results tab

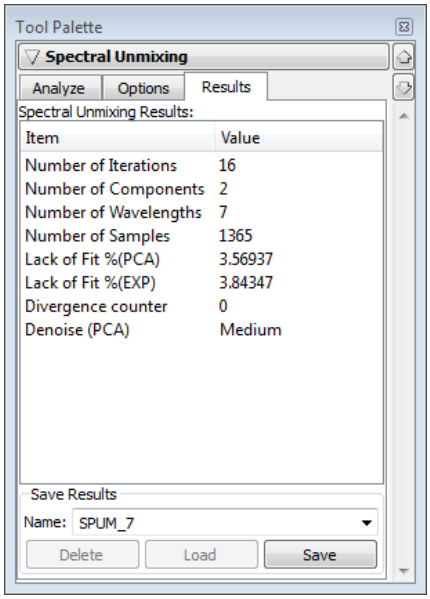


Table 11.5 Spectral unmixing tools, Results tab

Item	Description
Spectral Unmixing Results	
Number of Iterations	The number of iterations that the algorithm used.
Number of Components	The number of components unmixed.
Number of Wavelengths	The number of wavelength pairs used in the analysis.
Number of Samples	The number of pixel samples used in the analysis.
Lack of Fit (%PCA)	The fitting residue compared to the data filtered by principal component analysis.
Lack of Fit (% EXP)	The fitting residue compared to the experimental data.
Divergence Counter	The number of divergences that occurred.
Maximum Iterations	The maximum number of iterations allowed.
Denoise (PCA)	Indicates how much of the data was filtered by principal component analysis.
Normalization	The normalization method used in the analysis.
Non-negativity Method	The non-negativity method used in the analysis.
Weighting Mode	The weighting method applied to the data.
Column Weighting Mode	Indicates if column-wise weighting was used.
Row Weighting Mode	Indicates if row-wise weighting was used.
Save Results	

Table 11.5 Spectral unmixing tools, Results tab (continued)

Item	Description
Name	The name of the selected spectral unmixing results.
Delete	Removes the selected spectral unmixing results from the system.
Save	Saves the spectral unmixing results.

11.5 Spectral Unmixing Options

In the spectral unmixing tools, the Options tab shows the user-modifiable parameters in the spectral unmixing algorithm (Figure 11.9). It is recommended that you first perform spectral unmixing using the default settings. Then, if necessary, change the option settings and reanalyze the data.

Figure 11.9 Spectral unmixing options

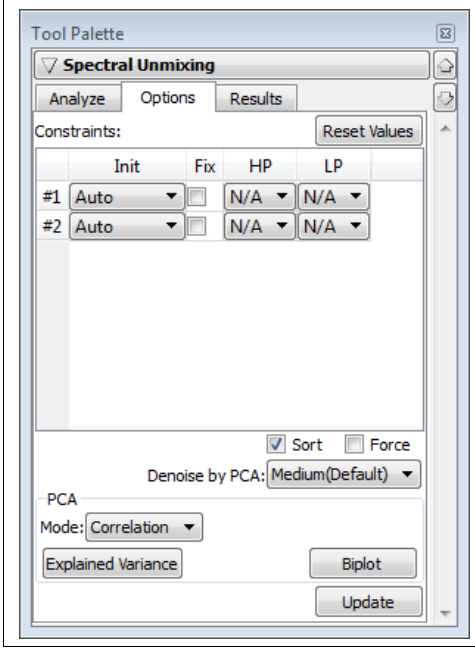


Table 11.6 Spectral unmixing options

Option	Description
Constraints	The constraints for unmixing the components.
Reset Values	Returns all constraint settings to the default values.
Init	The method for generating the initial guess of the spectrum for the selected component. "Auto" means this is automatically determined by the software. Alternatively, you can used a loaded spectrum as the initial guess.
Fix	This option determines whether the spectrum is allowed to change. If this option is chosen, the spectrum of that component is not updated during unmixing.
HP	Sets a high pass filter for the spectrum. Signal below the HP cut-off frequency is forced to zero. Choose N/A to turn off the high pass filter. Otherwise, the value represents the high pass cut-off frequency. This constraint can help isolate components that are physically mixed and difficult to distinguish.

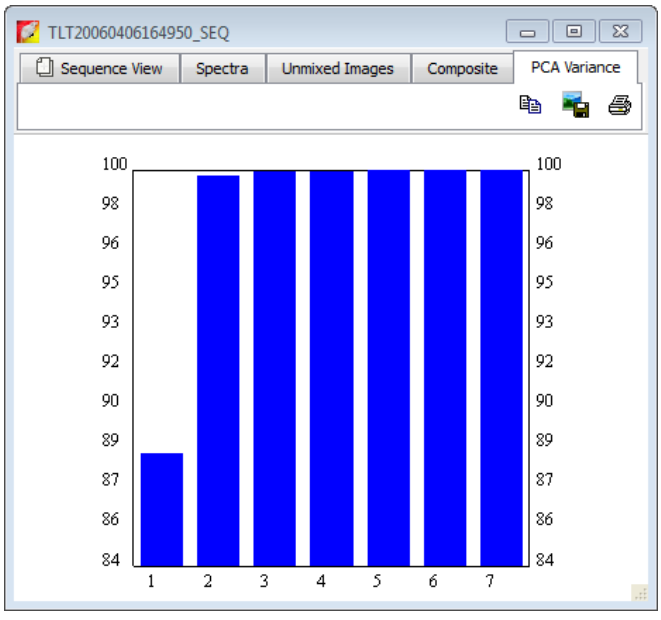
Table 11.6 Spectral unmixing options (continued)

Option	Description
LP	Sets a low pass filter for the spectrum Signal above the LP cut-off frequency is forced to zero. Choose N/A to turn off the low pass filter. Otherwise, the value represents the cut-off frequency of the low pass cut-off frequency. This constraint can help isolate components that are physically mixed and difficult to distinguish.
Sort	Choose this option to automatically sort the unmixed spectra in ascending order of their center wavelength.
Force	Choose this option to force the first component to non-zero throughout the image.
Denoise by PCA	Determines how much of the data will be filtered by principal component analysis. Stronger denoising means fewer principal components will be used in the data and more details will be lost. Stronger denoising also may slow down the unmixing.
PCA	
Mode	Standard - Principle component analysis performed on the original data. Correlation - Principle component analysis performed on the correlation matrix of the original data. Covariance - Principle component analysis performed on the covariance matrix of the original data. n
Explained Variance	Click to display the PCA variance plot (Figure 11.10).
Biplot	Click to display the biplot graph (Figure 11.11).
Update	Click to redo the spectral unmixing results with updated constraints.

PCA Explained Variance

The PCA Explained Variance histogram shows the part of variance (y-axis) that can be explained by a number of principal components (x-axis).

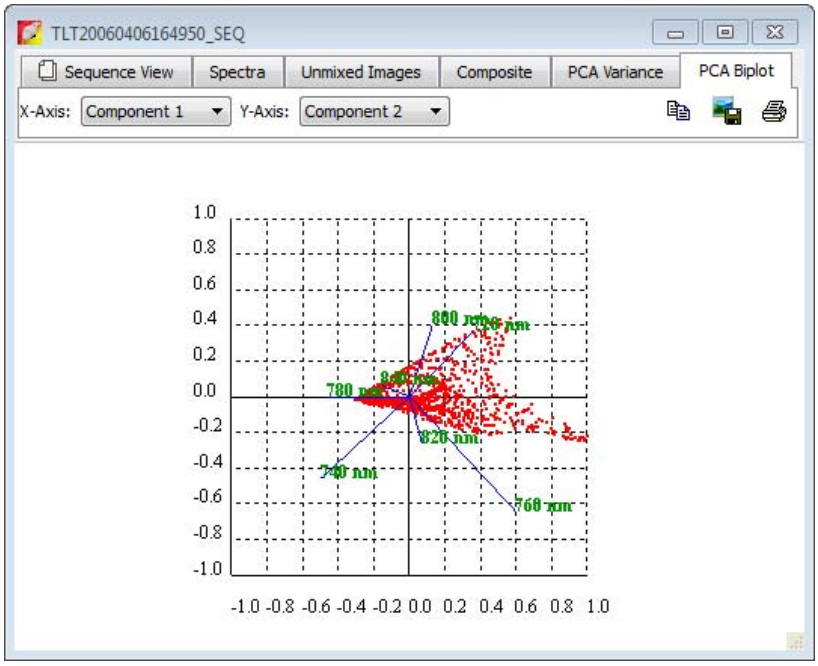
Figure 11.10 PCA explained variance histogram



PCA Biplot

The PCA biplot is a visualization tool for principal component analysis. It shows a simultaneous display of *n* observations (pixels) and *p* variables (wavelengths) on a two-dimensional diagram.

Figure 11.11 PCA biplot



12 Reconstructing a 3D Surface

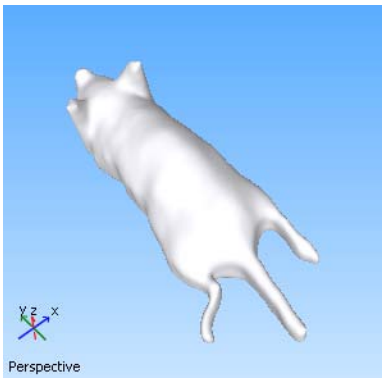
A *surface* is a 3D reconstruction of the animal surface (topography) derived from structured light images. The Living Image software requires a surface to perform some types of analyses (Figure 12.1).

You can:

- Save a surface and use it for any of the analyses shown below
- Export a surface for viewing in other 3D viewer applications
- Import a surface

For more details on how the software generates a surface, see Appendix H, page 317.

Figure 12.1 Example surface and analyses that require a surface



Generate a surface for:

DLIT Analysis
3D reconstruction of luminescent sources displayed as voxels (page 193)

FLIT Analysis
3D reconstruction of fluorescent sources displayed as voxels (page 201)

12.1 Generating a Surface

1. Load the image sequence for the reconstruction.

   For example, a sequence that was acquired for DLIT analysis.

2. In the surface topography tools, make a selection from the Object drop-down list (nude mouse, fur mouse, or phantom).

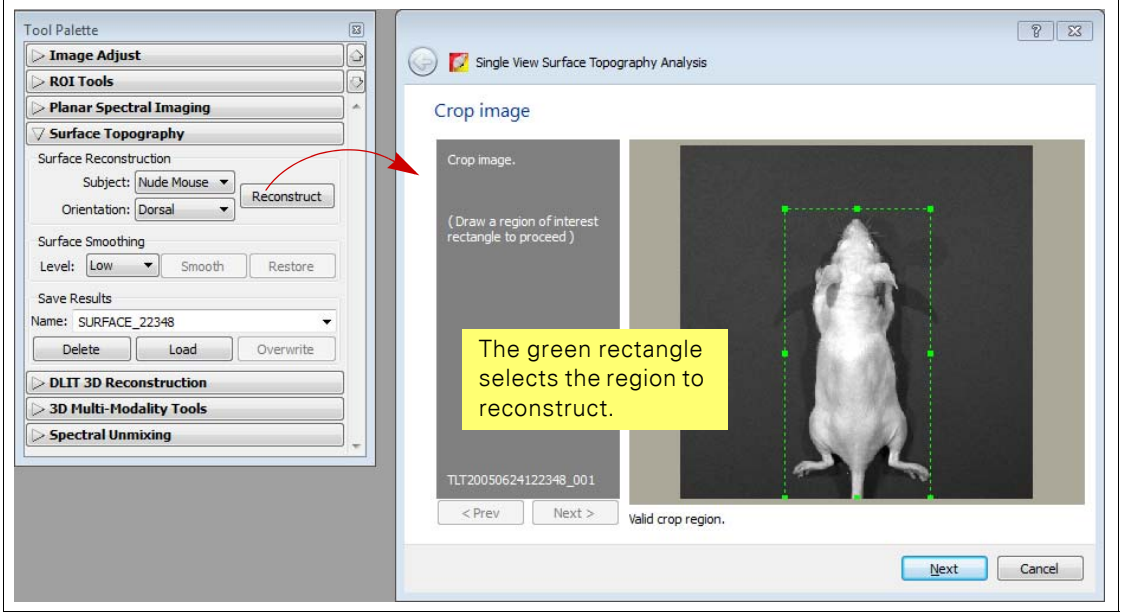
3. Select an orientation (dorsal or ventral).

4. Select a smoothing level.

5. Click **Reconstruct**.

   The Tomography Analysis box appears. By default, the entire subject is selected for the reconstruction.

Figure 12.2 Surface topography tools and tomography analysis box

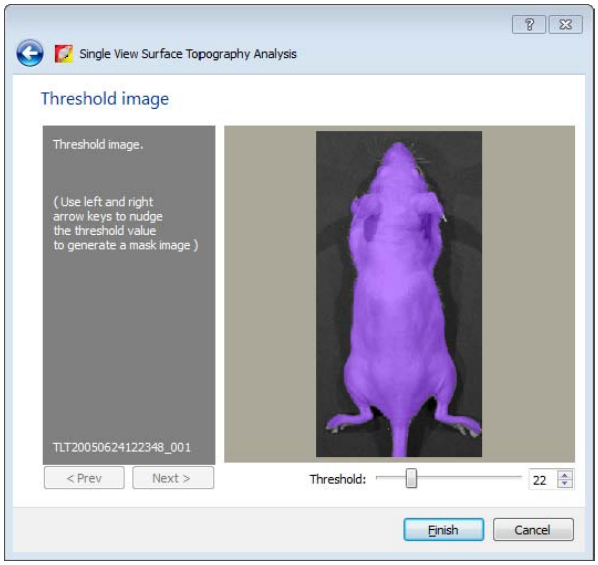


6. If you want to reconstruct only a particular region of the subject, resize the rectangle (drag a green handle ■) so that it includes only the area of interest.

7. Click **Next**.

   The purple data mask appears. The mask is an overlay on the subject image that defines the area of interest for the surface topography reconstruction. The mask should match the underlying photograph of the subject as closely as possible without including any area outside the subject image.

Figure 12.3 Data mask (purple)



8. If it is necessary, adjust the threshold value so that the mask fits the subject image as closely as possible. To change the threshold, do one of the following:
   - Press the left or right arrow keys on the keyboard.
   - Move the Threshold slider left or right.
   - Click the arrows or enter a new value in the box.
9. Click **Finish**.

   The surface and 3D tools appear in the Tool Palette. For more details on the Tool Palette, see page 220.

Figure 12.4 3D view and 3D tools in the toolbar and Tool Palette

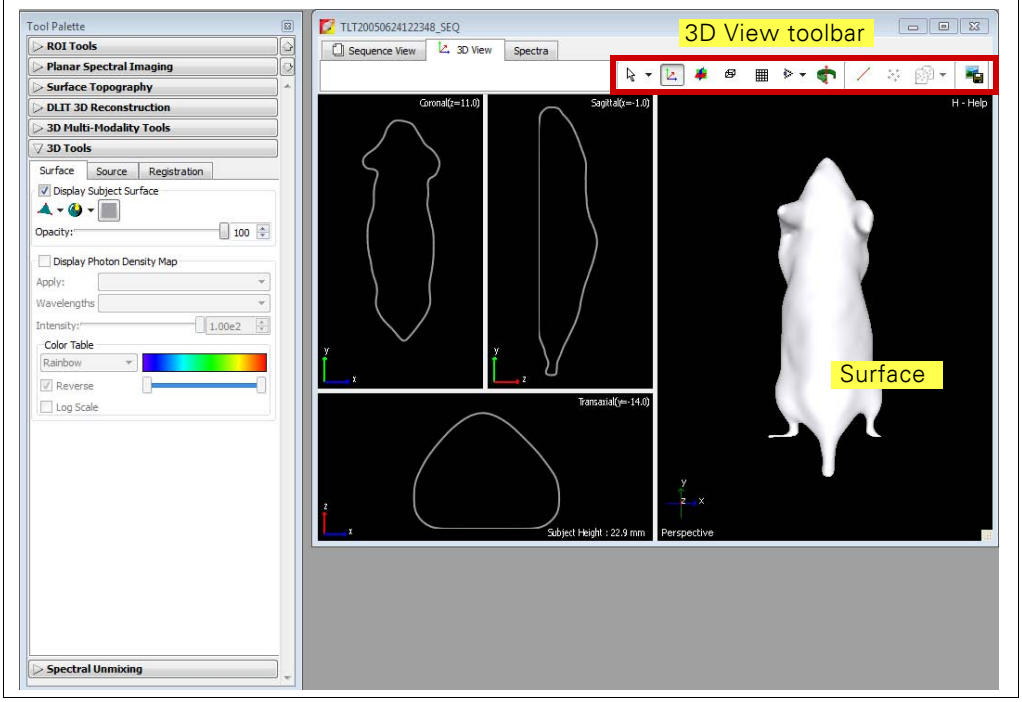


Figure 12.5 3D View toolbar

Table 12.1 3D view tools

Tool	Description
Image Tools 	A drop-down list of tools for viewing and working with the surface. Select to: • Click and display measurement dimensions in the coronal, sagittal, or transaxial view (in the 3D view window). • Drag a measurement cursor in the coronal, sagittal, or transaxial view and display measurement dimensions. (For details on measurement cursors, see page 107.) Select to zoom in or out on the image (use a click-and-drag operation). Select to move the subject in the window (use a click-and-drag operation). Select to rotate the subject around the x, y, or z axis (use a click-and-drag operation).
	Click to hide or show the x,y,z-axis display in the 3D view window.
	Click to hide or show coronal, sagittal, and transaxial planes through the surface in the 3D view window.
	Click to show or hide a bounding box around the surface.
	Click to show or hide a grid under the surface.
	Select this tool from the drop-down list to change the view perspective (top, bottom, left, right, front, back, or perspective view). For examples of the views, see Figure 12.7.
	Select this tool from the drop-down list to display the perspective view.
	Click to show or hide measurement cursors in the coronal, sagittal, or transaxial views.
	After you perform DLIT or FLIT analysis, click a voxel in the 3D reconstruction, then click this button to display measurements for the voxel in the 3D tools (source voxel measurements).
	Enables you to save the 3D view to a graphic file (for example, .jpg).

Changing the View Perspective

Figure 12.7 shows examples of the available views. You can view the surface from different perspectives by doing one of the following:

- Select to change the view (Figure 12.6)
- Alternatively, click the surface in the 3D View window, then press the **V** key to cycle through the different views of the surface

Figure 12.6 Surface, perspective view

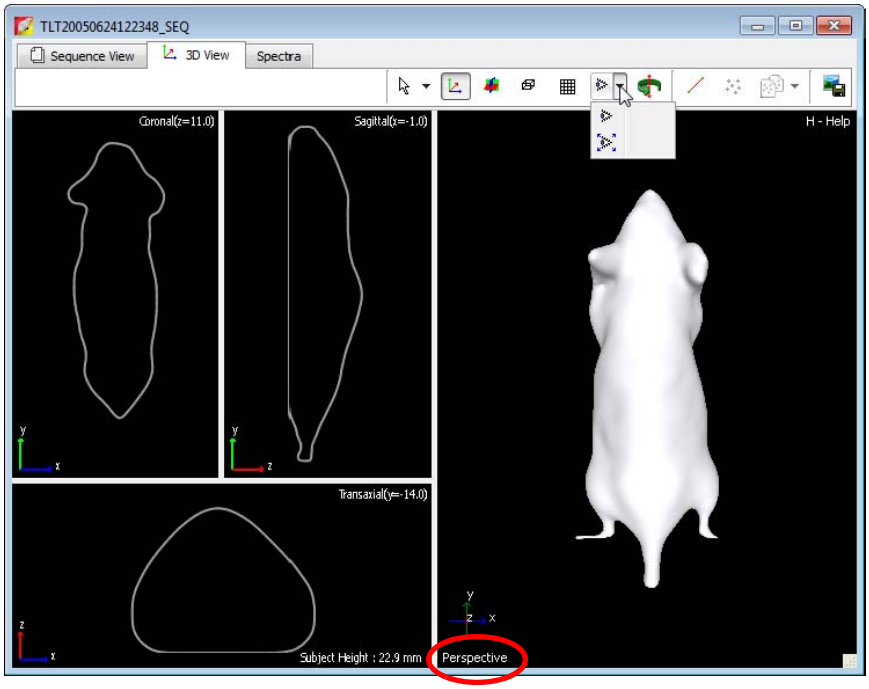
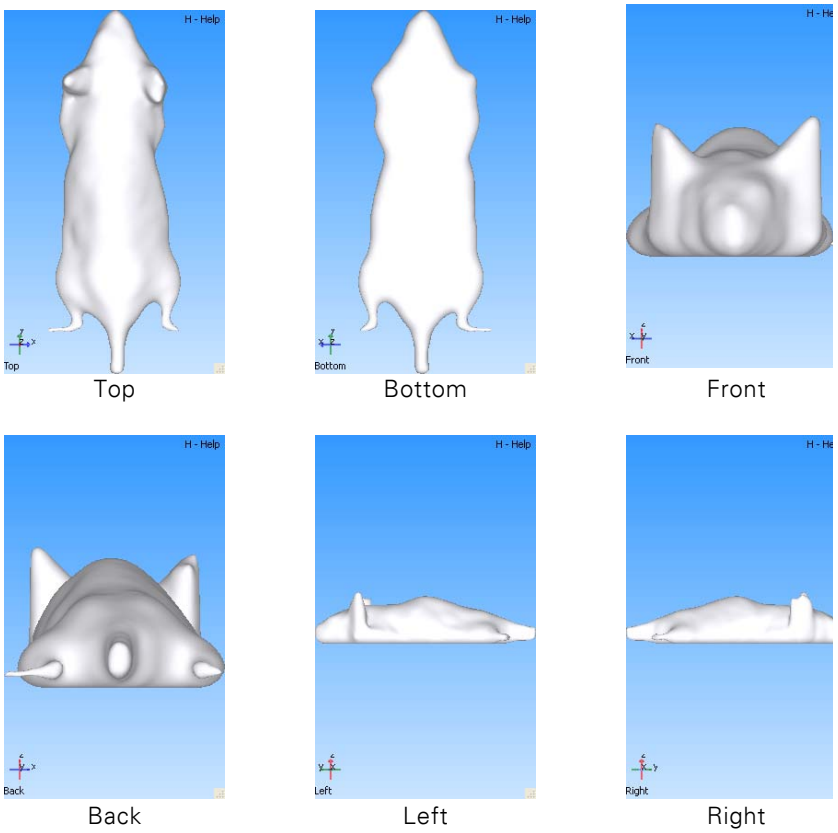


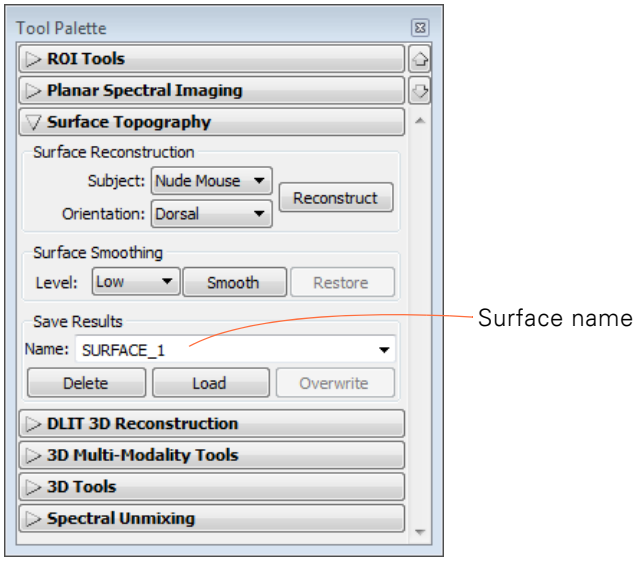
Figure 12.7 Alternate views of a surface



12.2 Managing Surfaces

After the surface is saved, it can be shared by the DLIT or FLIT tools.

Figure 12.8 Tool palette, Surface topography tools



Item in the Surface Topography Tools	Description
Name	Name of the selected surface.
Delete	Removes the selected surface from the system.
Load	Opens the selected surface.
Save	Saves a surface to the selected name.
Overwrite	Saves the surface and overwrites the previous surface results.

12.3 Export or Import a Surface

A surface can be shared with other users or viewed in other 3D viewer applications.

NOTE

Surface import capability is only available if Show Advanced Options is selected in the general preferences (see page 266).

1. Load a surface.
2. Select **File → Export (or Import) → 3D Surface** on the menu bar.
3. In the dialog box that appears, select a folder, enter a file name, and select a file type (see Table 12.2).

NOTE

Importing a surface by this method is for viewing purposes only, not for registration with optical reconstructions in Living Image software. To import a surface or other organs for registration purposes, import an organ atlas. For more details, see page 230.

Table 12.2 Surface file types

Export Option	Description	Export	Import
Surface mesh (.xmh)	A native file format of the Living Image software that is used to exchange 3D surface information between Living Image software and other third party analysis tools. It is based on a basic indexed face set format which stores all of the vertex information first, then stores the triangle information in terms of indexes into the vertex list.	yes	yes
AutoCAD DXF (.dxf)	Drawing exchange format that is compatible with most DXF file viewers.	yes	yes
VRML 1.0 (.wrl)	VRML 1.0 (.wrl) - Virtual reality modeling language format that is compatible with most VRML viewers.	yes	no
Open Inventor (.iv)	The ASCII version of the IV file format which is supported by all IV viewers.	yes	yes
STL (.stl or ASCII format)	Stereo lithography binary format compatible with most STL viewers.	yes (binary)	yes

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13 3D Quantification Database

It is possible to determine the number of cells in a DLIT source or the number of dye molecules or cells in a FLIT source if a quantification database is available. The database is derived from an analysis of images of known serial dilutions of luminescent cells or fluorescent cells or dye molecules.

13.1 Preparing & Imaging the Samples

1. Prepare a well plate (4 x 6, 6 x 4, 8 x 12, or 12 x 8 well format) that contains a dilution series of luminescent cells or fluorescent dye at four or more concentrations.
2. Include at least four background wells that contain diluent only.
3. Place the well plate on the IVIS stage, positioning it so that it is centered and squared in the field of view.

NOTE

All of the wells must be within view in the image. For wells containing fluorosphores, FOV D is recommended to reduce shadows from well walls and ensure more uniform excitation of the wells.

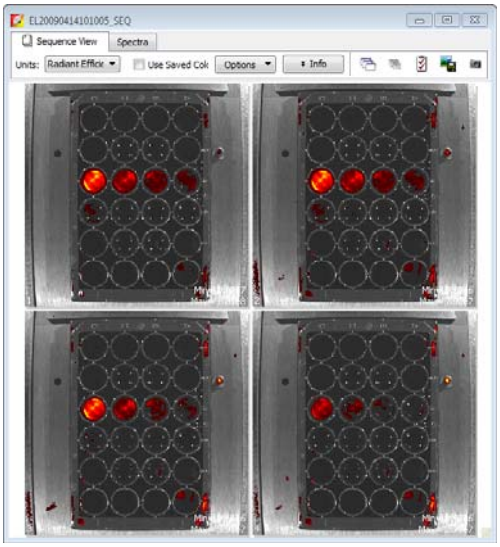
4. Acquire the images:

   Bioluminescent samples - Acquire one 'Open' filter image of the well plate

   Fluorescent samples - Acquire reflectance-illumination Filter Scan images using the appropriate excitation and emission bandpass filters.

   The well plate in Figure 14.1 contains a dilution series of a sample at four concentrations. The image sequence is a filter scan set of images with the excitation filter centered at 465 nm for all the images, and emission filter images centered at 520 nm, 540 nm, 560 nm, and 580 nm.

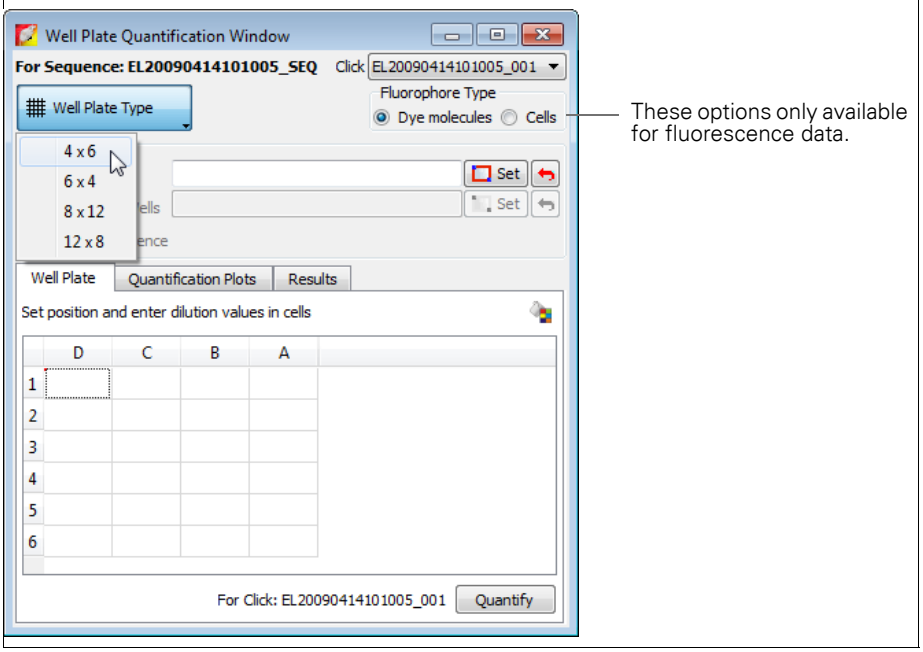
Figure 13.1 Well plate data



13.2 Creating a Quantification Database

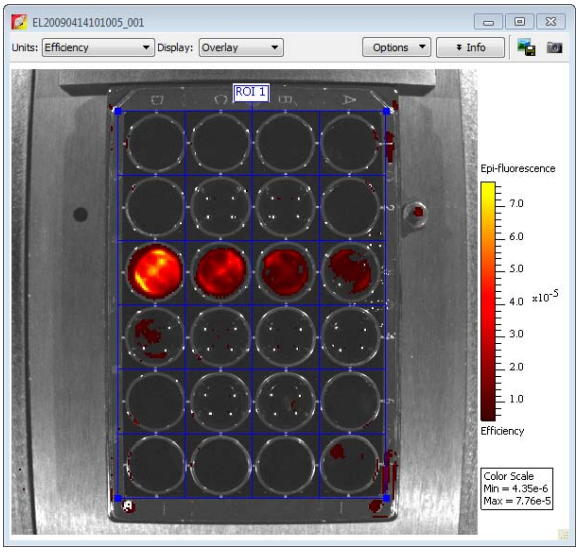
1. Load the well plate image sequence.
2. Select **Tools** → **Well Plate Quantification for "<name>_SEQ"** on the menu bar.
   The Well Plate Quantification window appears.
3. For fluorescent samples, choose the Dye molecules or Cells option.

Figure 13.2 Well Plate Quantification window



4. Select the well plate dimensions from the Well Plate Type drop-down list.
   The first image in the sequence opens and a grid ROI appears on the image.

Figure 13.3 Well plate image with grid ROI



5. Adjust the grid ROI to closely fit the plate wells.

6. In the well plate table, select the sample cells, and click **Set** (Figure 13.4).

   Clicking a row or column header selects the entire row or column.

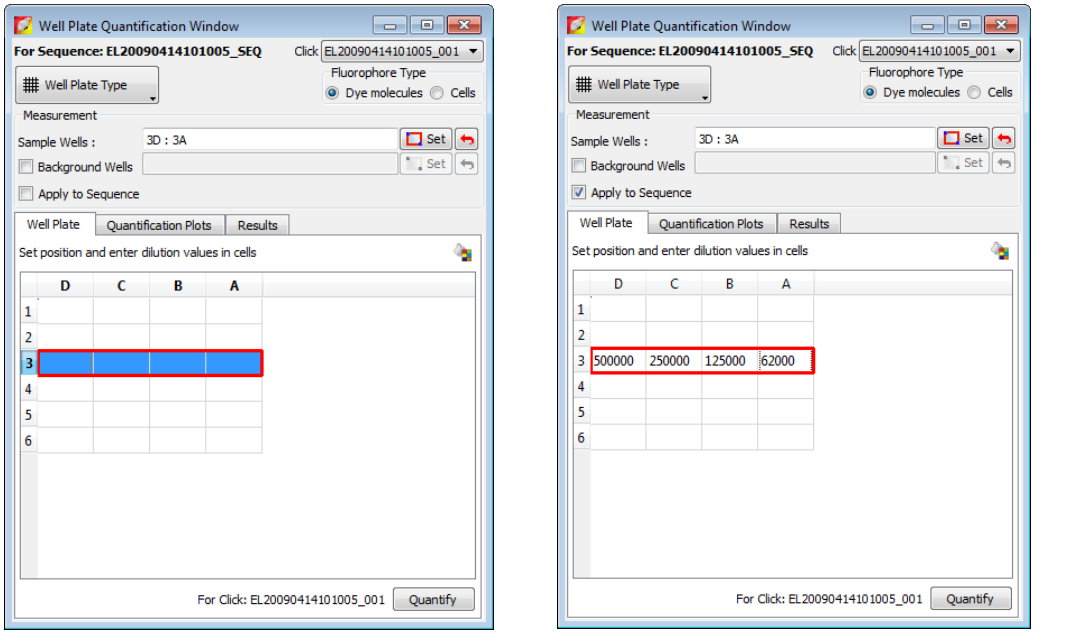
7. To remove the "sample" designations from table cells, select the cells and click the button.

8. To apply a color to table cells:

   a. Select the table cells and click the button. Alternatively, right-click the selected table cells and choose Background Color on the shortcut menu.

   b. Choose a color from the color palette that appears.

Figure 13.4 Select the sample wells and enter the number of cells or molecules



9. Enter the dilution values in the table cells.

NOTE

The values must be entered as dimensionless numbers. For example, for a picomole of dye molecules, enter "6.022e11".

10. To delete a dilution value, select the table cell and press the Delete key. Alterrnatively, right-click the number to view a shortcut menu of edit commands (for example, cut, copy, paste).

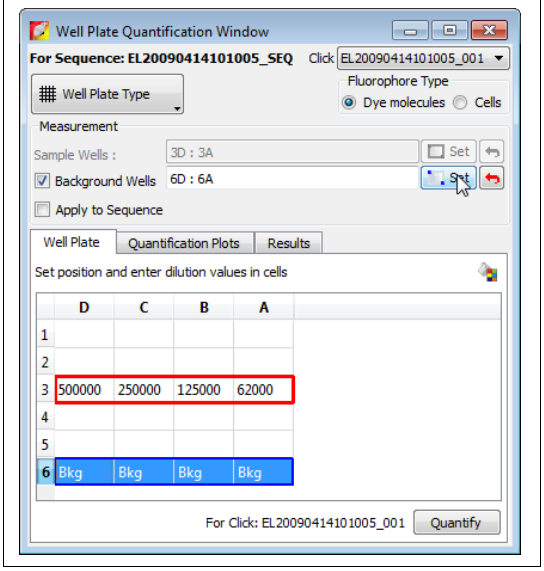
11. Choose the Apply to Sequence option.

12. Choose the Background Wells option.

13. In the well plate table, select the background wells and click **Set**.

    Clicking a row or column header selects the entire row or column. To remove the "background" well designations, click the button.

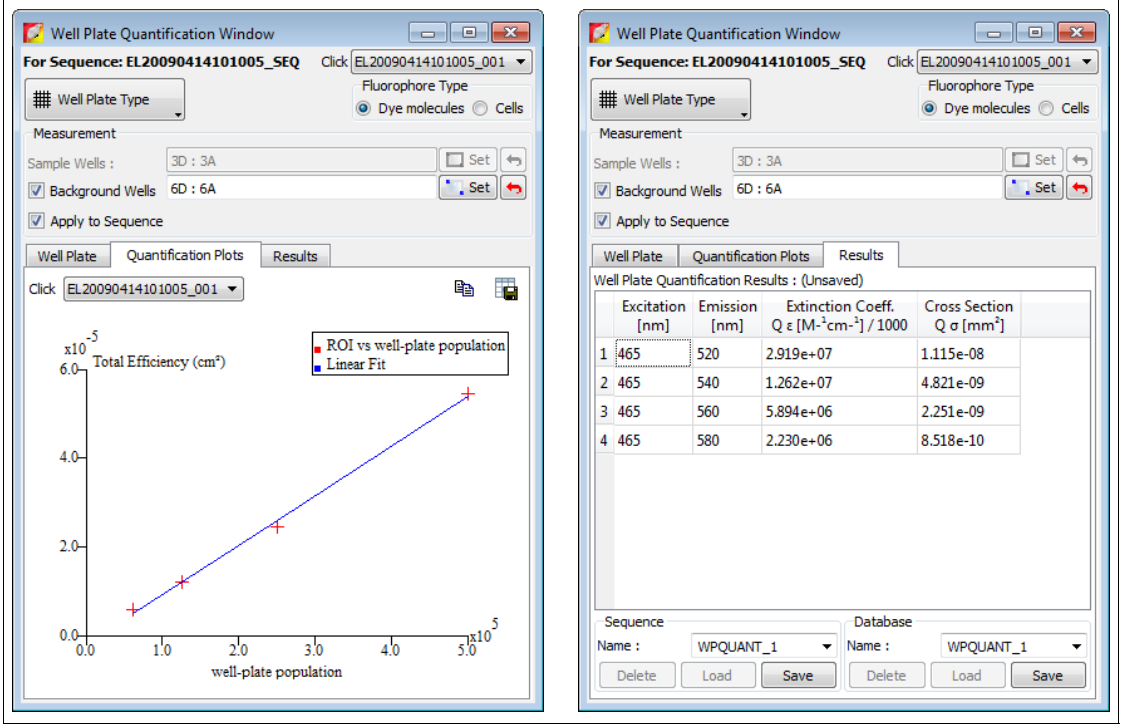
Figure 13.5 Set the background wells



14. Click **Quantify**.

    The results are displayed

Figure 13.6 Example fluorescence quantification plot and results



15. Check the linear fit of the data for each image in the quantification plot.

    A good straight line fit gives confidence to the results values. Large deviations from a straight line could indicate possible issues with the dilution series or errors when entering sample dilution values.

16. To export the quantification plot values:

a. Click the button.

b. In the dialog box that appears, select a folder for the file (.csv) and click **Save**.

17. To copy the quantification plot values to the system clipboard, click the button.

Table 13.1 Quantification results

Item	Description
Fluorescence	
Excitation (nm) Emission (nm)	The excitation and emission filter wavelengths for the image. 'Excitation' and 'Emission' filters will be specified for fluorescent images, and the 'Open' filter for 'Emission' will be specified for bioluminescent images.
Extinction Coeff	A measure of excitation photon absorption interaction with the well plate samples based on a base-10 logarithmic derivation. The quantum efficiency factor of the conversion of the absorbed photon to the emission wavelength is also included.
Cross Section	A measure of excitation photon absorption interaction with the well plate samples based on a natural logarithmic derivation. The quantum efficiency factor of the conversion of the absorbed photon to the emission wavelength is also included.
Bioluminescence	
Total Flux/cell	A measure of total flux (photon/sec) emitted from a single cell. This number can be used to estimate the number of cells from the total flux in the 3D quantification.

13.3 Managing Quantification Results

The quantification results can be saved with the image sequence and as a calibration database that is made available in the DLIT or FLIT 3D reconstruction tools (in the Properties tab). When you define the properties for performing a 3D reconstruction and a calibration database is specified, the 3D reconstruction results will be displayed in calibrated units for cell numbers or molecule quantities in picomole units.

Figure 13.7 Save the quantification results

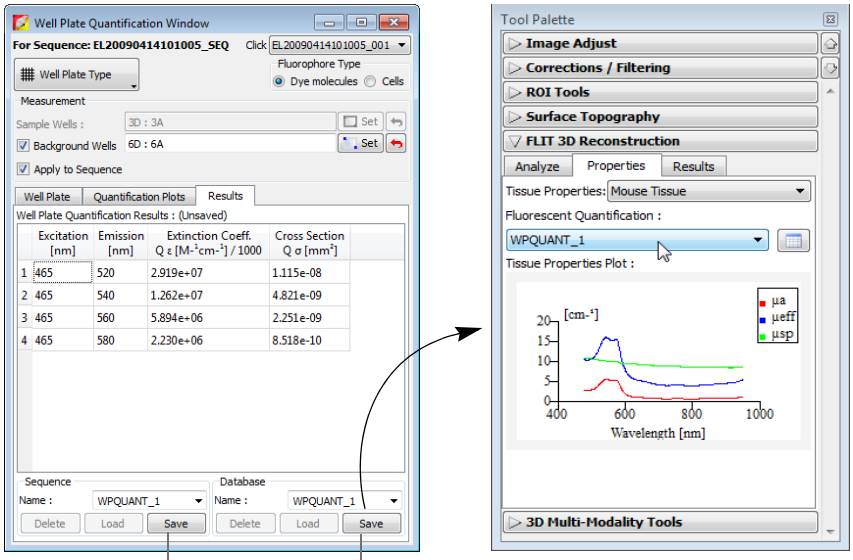
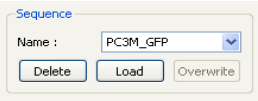
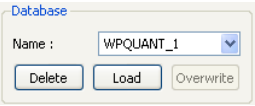


Table 13.2 Managing quantification results

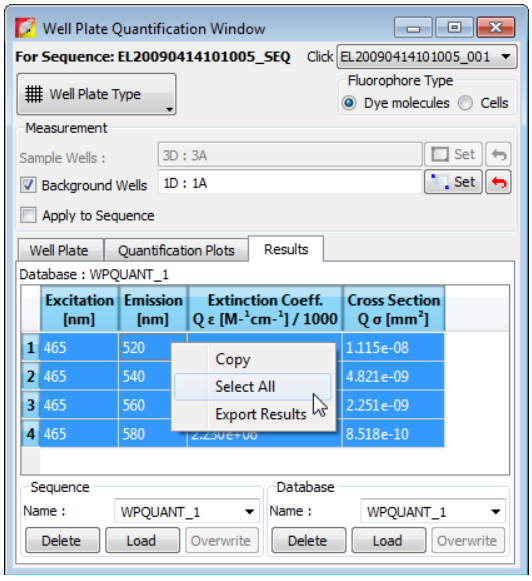
Item	Description
	Delete - Removes the active quantification results from the image sequence. Load - Opens quantification results from the sequence path. Save - Saves the quantification results with the selected image sequence. Overwrite - Saves the results with the selected image sequence and overwrites previous results.
	Delete - Deletes the database from the system. Load - Opens quantification results from the system path. Save - Saves the quantification results to a system database that is available for DLIT or FLIT reconstruction. Overwrite - Saves the results to the selected database name and overwrites previous results.

Exporting Quantification Results

Right-click the results table to view copy and export options.

- Copy – Copies the selected rows to the system clipboard
- Select All – Selects all rows in the results table
- Export Results – Opens a dialog box that enables you to export the selected results to a text file

Figure 13.8 Well plate quantification results



14 3D Reconstruction of Sources

The Living Image® software provides algorithms which analyze 2-dimensional image data to reconstruct 3-dimensional (3D) luminescent or fluorescent sources located inside an animal (*tomographic analysis*). For more details on the DLIT or FLIT algorithm, see Appendix H, page 317.

3D Reconstruction Algorithm	Description	Page
Diffuse Tomography (DLIT)	DLIT provides a complete 3D reconstruction of the luminescent source distribution within the subject. DLIT places no constraints on the geometry or spatial variation of the source strength throughout the volume. DLIT is well-suited for analyzing complex and spatially extended luminescent sources. The 3D reconstruction is presented as voxels. If a luminescent calibration database is available, the number of cells per source can be determined in addition to source intensity (photons/sec).	194
Fluorescent Tomography (FLIT)	FLIT provides a complete 3D reconstruction of the fluorescent source distribution within the subject.The 3D reconstruction is presented as voxels. If a fluorescent calibration database is available, the number of fluorophore molecules or cells per source can be determined in addition to the total fluorescence yield.	201

The input data to the DLIT algorithm for a 3D reconstruction of luminescent light sources includes:

- A structured light image that is analyzed to generate a surface for the imaging subject.
- A sequence of two or more images of the light emission from the surface of the subject acquired at different filter bandwidths (Table 14.1). Use the Imaging Wizard to acquire the images.

The input data to the FLIT algorithm for 3D reconstruction of fluorescent light sources includes:

- A surface that defines the surface of the subject.

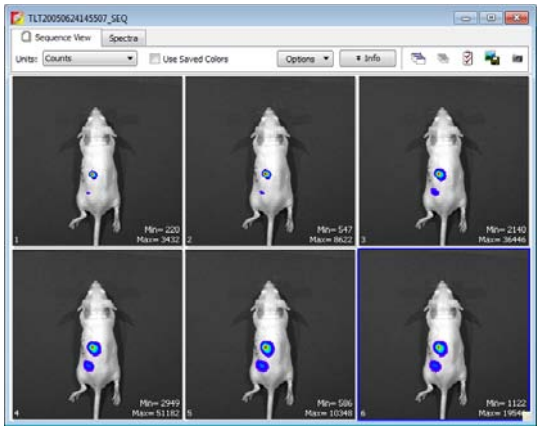
- A sequence of images acquired at different transillumination source positions using the same excitation and emission filter at each position. Use the Imaging Wizard to acquire the images.

Table 14.1 IVIS System filters for luminescence & fluorescence tomography

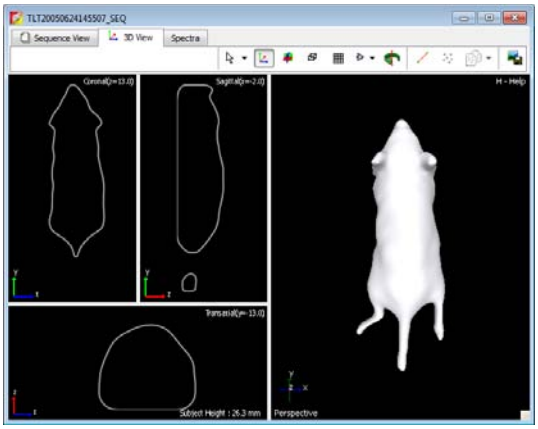
IVIS Imaging System	Filters	Bandwidth
200 Series	6 emission filters, 550-670 nm	20 nm
Spectrum	10 excitation filters, 415-760 nm	30 nm
	18 emission filters, 490-850 nm	20 nm

Figure 14.1 shows an example 3D reconstruction workflow.

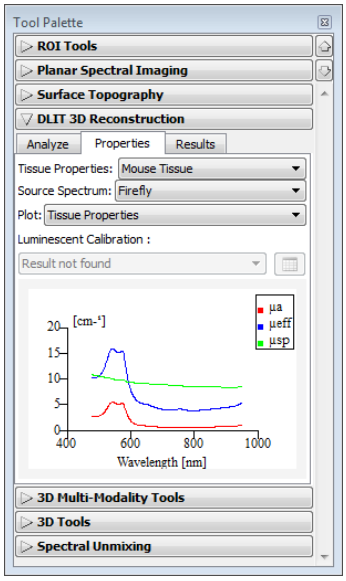
Figure 14.1 Basic workflow for 3D reconstruction of sources



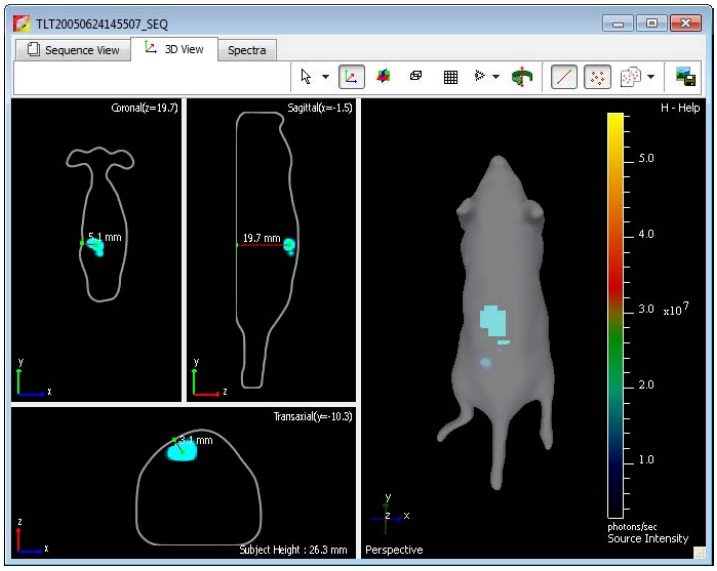
❶ Load an image sequence (see page 193 for image sequence requirements)



❷ Generate or load a surface



❸ Select tissue and source properties



❹ Reconstruct and view source measurements

14.1 Reconstructing Luminescent Sources

General Considerations

Animal Requirements

The best surface topography reconstruction is obtained from nude mice. It is possible to perform 3D imaging on white or light-colored furred mice if the fur is reasonably smooth over the mouse surface. Therefore it is recommended that you comb the fur before imaging to eliminate any "fluffy" areas that may trigger artifacts during the surface topography reconstruction. In this case, it is recommended that you shave the animals or apply a depilatory. 3D reconstructions are currently not possible on black or dark-colored furred mice.

Luminescent Exposure vs. Luciferin Kinetic Profile

It is important to consider the luciferin kinetic profile when you plan the image sequence acquisition. The DLIT algorithm currently assumes a flat luciferin kinetic profile. Therefore, to optimize the signal for DLIT 3D reconstruction, carefully plan the start and finish of image acquisition and ration the exposure time at each emission filter so that the sequence is acquired during the flattest region of the luciferin kinetic profile.

Image Sequence Requirements

Use the Imaging Wizard to setup the image sequence required for DLIT analysis. For more details on the Imaging Wizard, see page 33.

If you plan to manually set up the sequence, Table 14.2 shows the recommended image sequence. Analyzing more images usually produces more accurate results. At a minimum, the sequence must include data from at least two different emission filters (560-660 nm):

- Emission filter #1: Photographic, luminescent
- Emission filter #2: Luminescent image
- One structured light image

Table 14.2 Recommended image sequence for DLIT analysis

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

NOTE

It is recommended that the binning level be the same for all of the luminescent images.

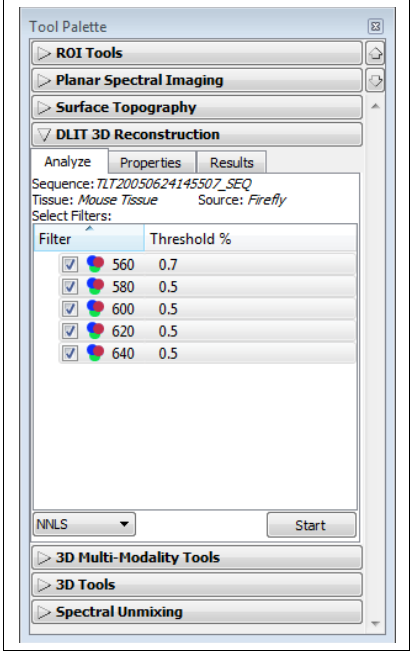
For more information on the DLIT algorithm and user-modifiable parameters, see Appendix H, page 317.

Steps to Reconstruct Luminescent Sources Using DLIT

1. Load an image sequence.
2. Generate or load a surface using the Surface Topography tools. For details on generating the surface, see page 181.
3. In the Tool Palette, choose **DLIT 3D Reconstruction**.

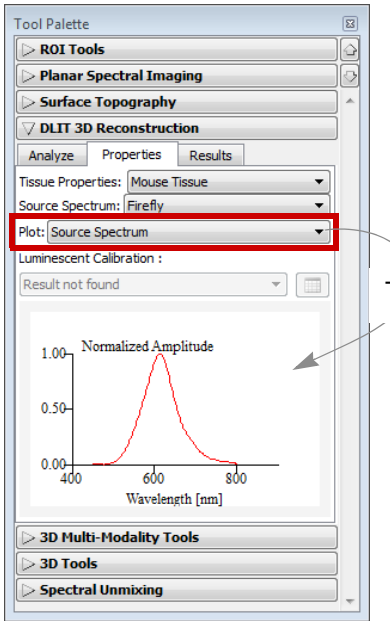
   The Analyze tab shows the data that the algorithm automatically selects for the reconstruction (Figure 14.2). For more details about the Threshold %, see page 198.

Figure 14.2 Analyze tab



4. In the Properties tab, make a selection from the "Tissue Properties" and "Source Spectrum" drop-down lists (Figure 14.3).

Figure 14.3 Properties tab

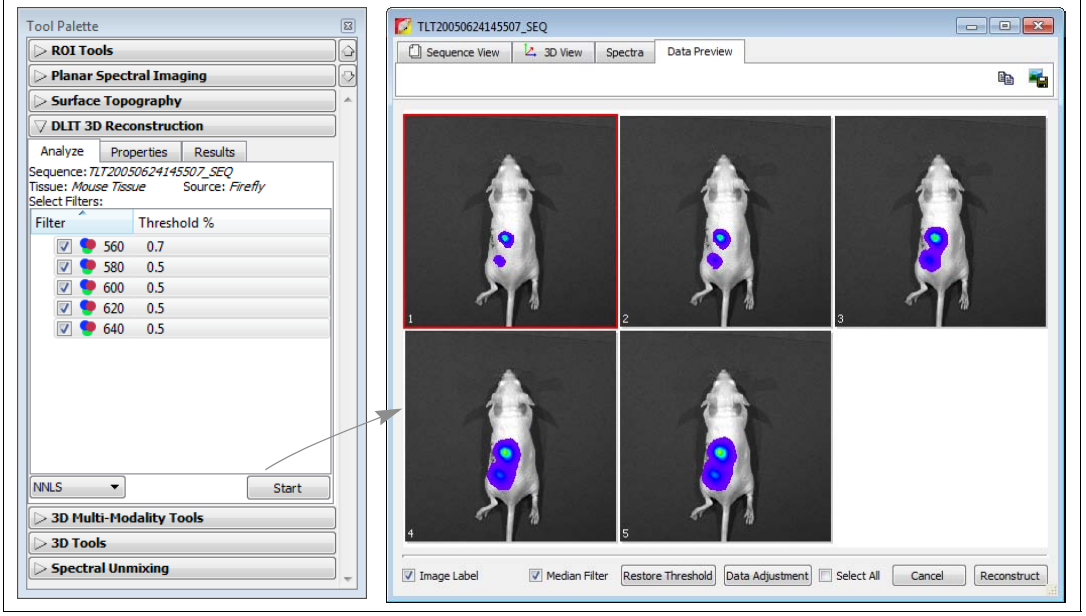


5. To view the tissue properties ($_a$, $_{eff}$, $'_s$) for the tissue and source you selected, make a selection from the Plot drop-down.
6. To compute the number of cells per source, select a luminescent calibration database.

   For details on generating a luminescent calibration database, see page 185.
7. In the Analyze tab, click **Start**.

   The Data Preview window appears and displays the image data that will be included in the reconstruction. Usually, no data adjustment is required. However, it is possible to exclude or include user-selected pixel data from the analysis. For more details, see page 198.

Figure 14.4 Data Preview window (right)



8. In the Data Preview window, click **Reconstruct**.

   The reconstruction normally requires less than one minute, depending on the reconstruction volume, parameter settings, and computer performance. When the analysis is finished:

   - The 3D View window displays the animal surface and the reconstructed sources
   - In the Tool Palette, the Results tab displays the results data and the algorithm parameter values
   - The 3D Tools appear after a reconstruction is generated or loaded. For more details on the 3D Tools, see page 220-231.

   For details on managing results (for example, save, load, or delete), see page 205.

Figure 14.5 DLIT reconstruction results

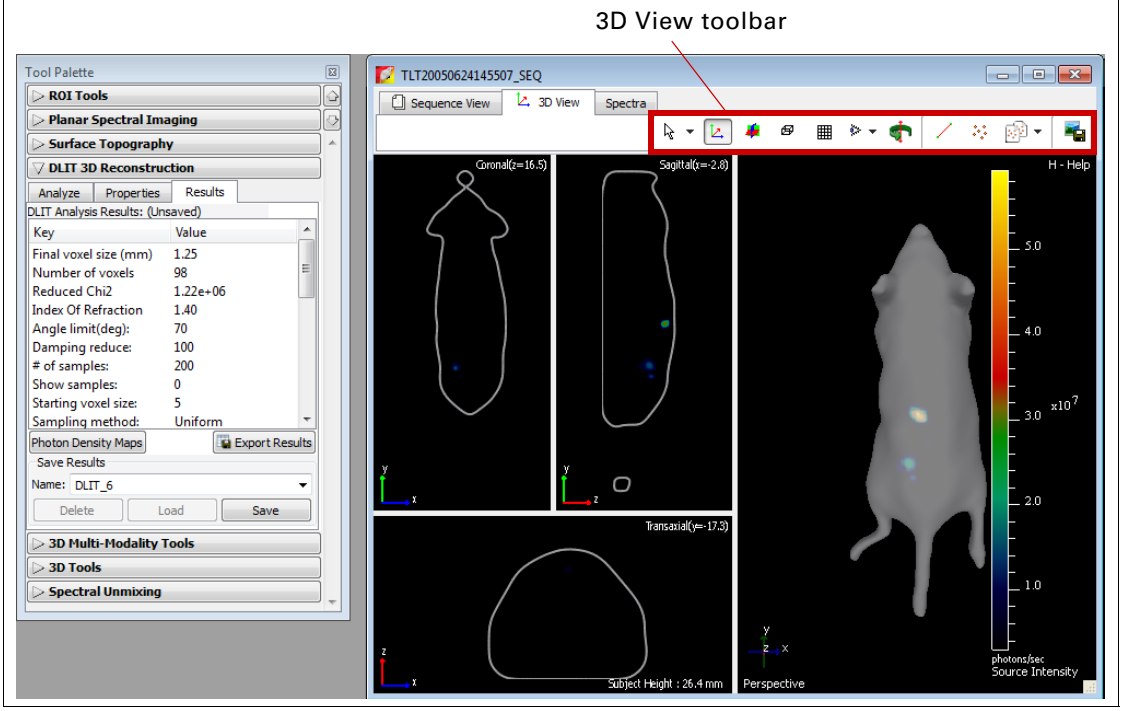


Figure 14.6 3D View toolbar

Table 14.3 3D View tools

Tool	Description
Image Tools 	A drop-down list of tools for viewing and working with the surface or DLIT results. or - Rotates or spins the surface in the x, y, or z-axis direction. - Moves the surface in the x or y-axis direction. - Zooms in or out on the image. To zoom in, right-click (Cmd key (apple key) +click for Macintosh users) and drag the toward the bottom of the window. To zoom out, right-click and drag the toward the top of the window.
	Displays the x,y,z-axis display in the 3D view window.
	Displays coronal, sagittal, and transaxial cross-sections through the subject in the 3D view window.
	Displays a bounding box around the subject.
	Displays a grid under the subject.
	Select this tool from the drop-down list to change the view perspective (top, bottom, left, right, front, back, or perspective view). For examples of the views, see Figure 14.35, page 229.
	Select this tool from the drop-down list to display the perspective view.
	Rotates the 3D reconstruction results in the 3D view window (*3D scene*). Click the + or - key to increase or decrease the rotation speed.To stop the rotation, click the 3D scene or the button.
	Displays measurement cursors in the coronal, sagittal, or transaxial views.
	Click this button, then select a source or a point in a source to obtain source measurements (total flux, volume, center of mass, host organ) in the 3D tools (Source tab). For more details, see page 209.
	Copies or pastes voxels or a source surface so that DLIT and FLIT reconstructions can be displayed on one surface. For more details, see page 213.
	Enables you to save the 3D view to a graphic file (for example, .jpg).

Data Preview Window

The Data Preview window shows the image data that the algorithm automatically selects for reconstruction. In special cases, you may want to include or exclude particular data from this default selection. There are two ways to do this:

- Change the Threshold % value - Applying a Threshold % value excludes or includes some pixels from the reconstruction. The software computes the minimum and maximum pixel values of an image based on an histogram of pixel intensities. If Threshold % = 0.5%, then pixels with intensity less than 0.5% of the maximum intensity value are excluded from the reconstruction. The Threshold % can be edited for individual images. The Data Preview window is updated when you change the Threshold % value.
- Region selection - Use the pencil tool to mark particular regions to include in the reconstruction. This may be useful for noisy images with high intensity pixels where changing the Threshold % value is not helpful. You can also use this method to focus on particular sources to reconstruct and ignore others.

To change the Threshold % for a selected image:

1. Click **Start** in the Analyze tab (Figure 14.7).

   The Data Preview window appears.
2. Click an image in the Data Preview window.

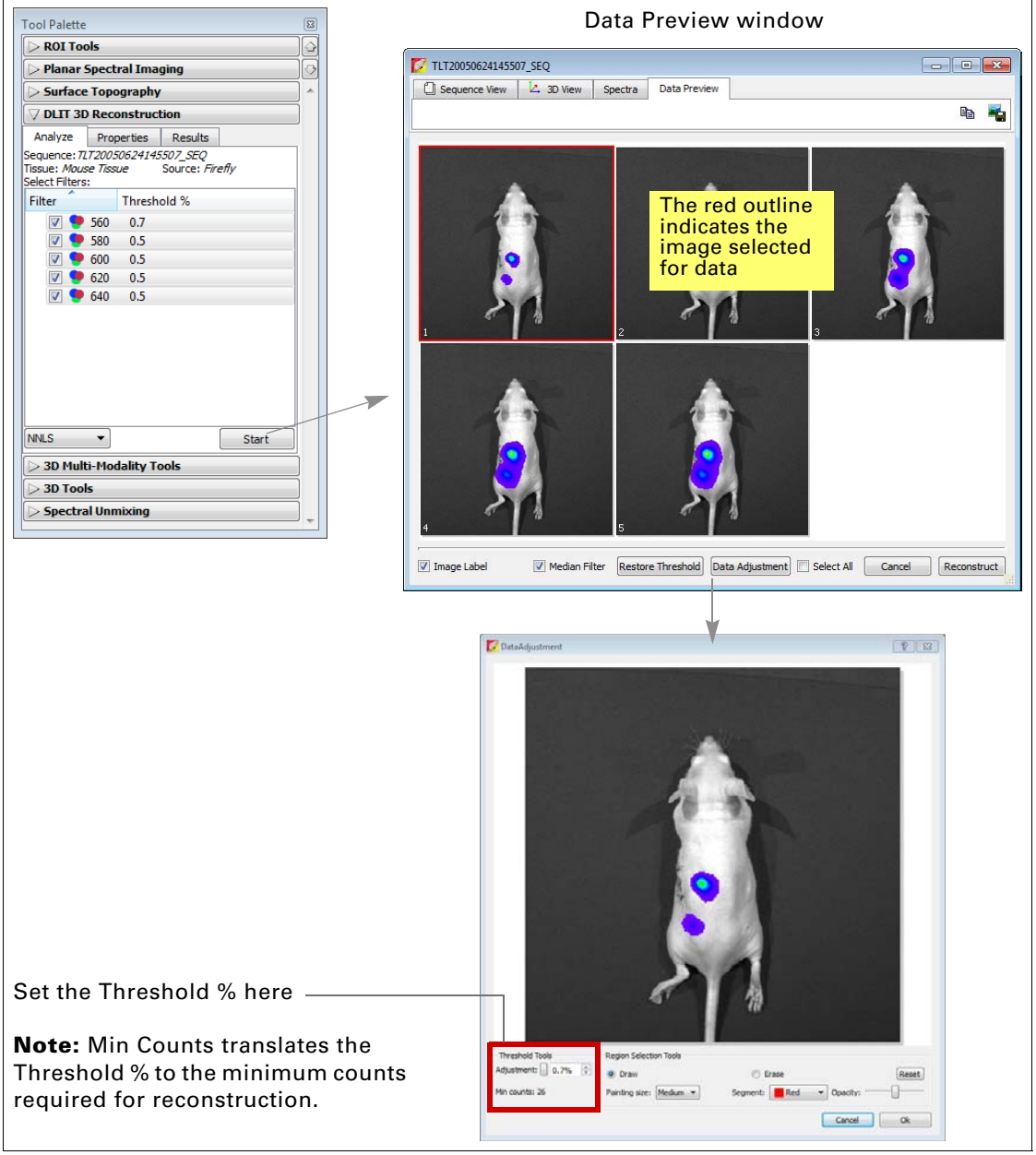
NOTE

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

3. Click **Data Adjustment**.
4. In the window that appears, enter a new Threshold % value.

   The new Threshold % appears in the Analyze tab.
5. To reset the Threshold % to the default value (for the selected images), click **Restore Threshold**.

Figure 14.7 Adjusting the Threshold %



Note: Min Counts translates the Threshold % to the minimum counts required for reconstruction.

To select particular regions for reconstruction:

1. Open the Data Preview window as shown in Figure 14.7.
2. Click **Data Adjustment**.
3. In the window that appears, choose the **Draw** option and put the mouse pointer over the image so that the pencil tool appears.
4. To automatically select all pixels in a source, right-click with the region with the pencil tool.

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

NOTE

If the pencil tool markings are applied to the image, only the marked pixels are included in the analysis.

Figure 14.8 Selecting regions to include in reconstruction

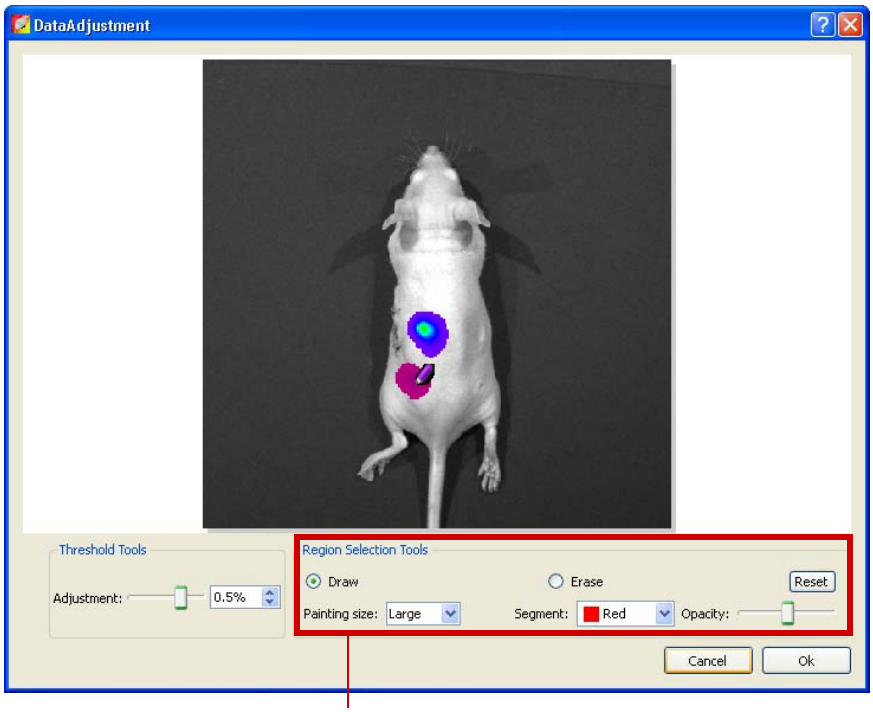


Table 14.4 Region Selection Tools

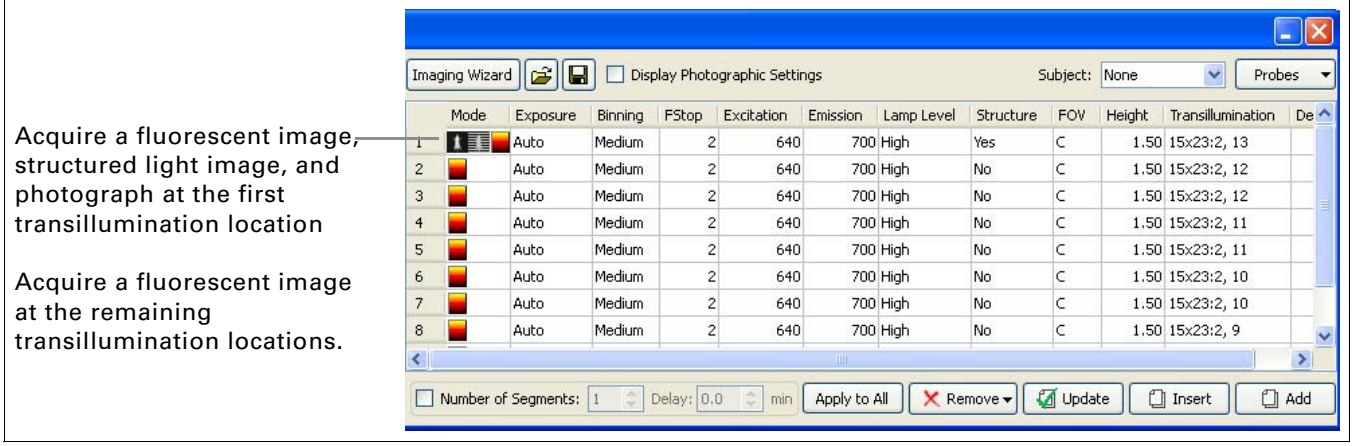
Item	Description
Draw	Choose this option to display the pencil tool. Use this tool to apply markings that select regions to include in the reconstruction.
Erase	Choose this option to display the eraser tool. Use the eraser to remove pencil tool markings (exclude pixels from the image).
Painting size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

14.2 Reconstructing Fluorescent Sources

Image Sequence Requirements

Use the Imaging Wizard to setup the image sequence required for FLIT analysis. (For more details on the Imaging Wizard, see page 33.) If you plan to manually set up the sequence, Figure 14.9 shows the an example image sequence. Acquire the images using transillumination on the IVIS® Spectrum Imaging System using the same excitation and emission filters from at least four source locations that form a rectangle.

Figure 14.9 Example sequence setup for FLIT

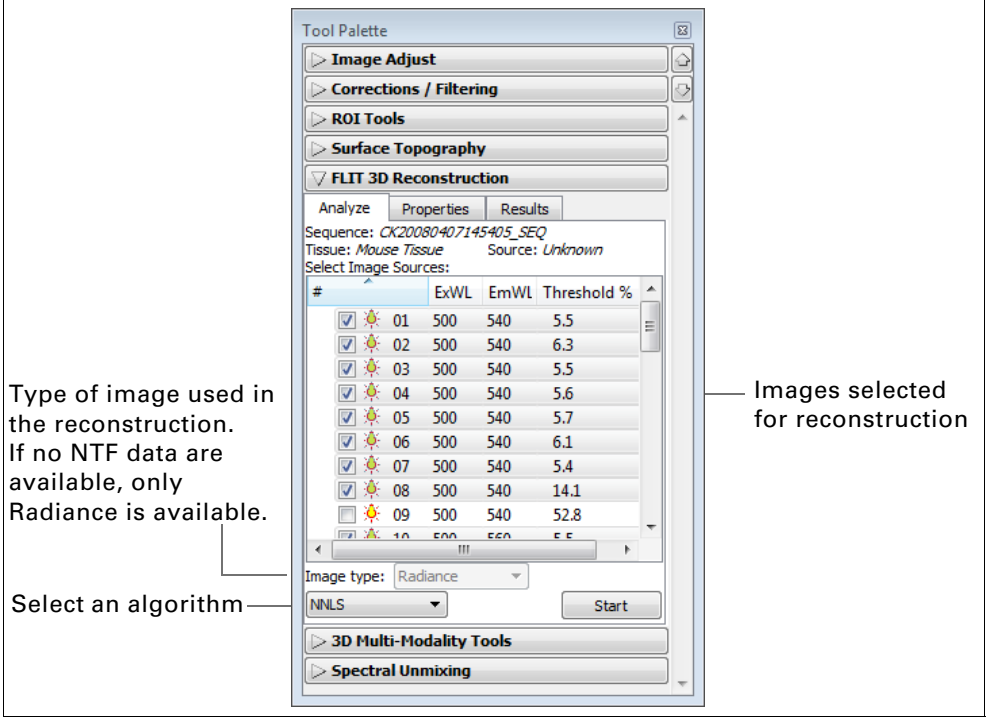


Steps to Reconstruct Fluorescent Sources

1. Load an image sequence.
2. Generate or load a surface. For details on generating the surface, see page 181.
3. In the Tool Palette, choose **FLIT 3D Reconstruction**.

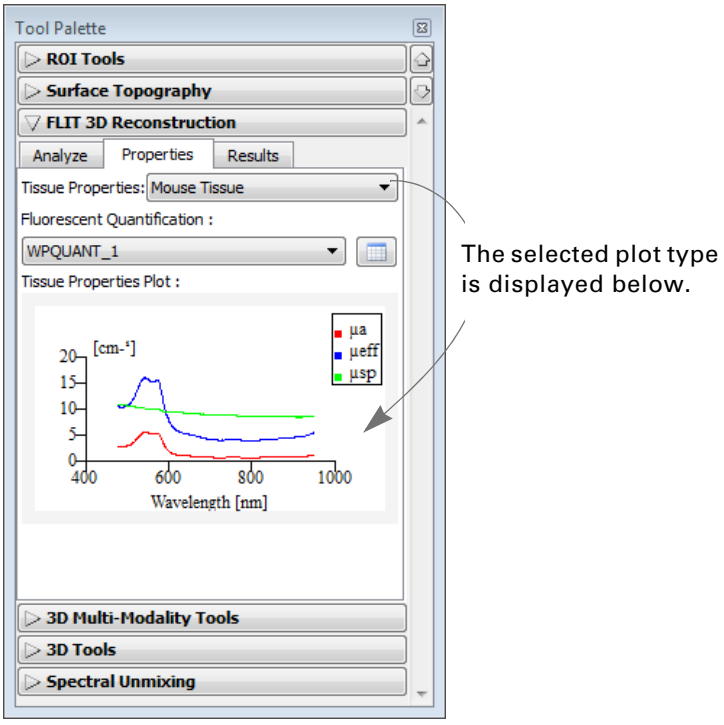
   The Analyze tab shows the images that the algorithm automatically selects for the reconstruction based on an appropriate signal level (Figure 14.2). For more details about the Threshold %, see page 198.

Figure 14.10 FLIT 3D Reconstruction tools, Analyze tab



4. Select the type of image used in the reconstruction: Radiance or NTF Efficiency (Figure 14.10).
5. In the Properties tab, make a selection from the "Tissue Properties" and "Source Spectrum" drop-down lists (Figure 14.3).

Figure 14.11 FLIT 3D Reconstruction tools, Properties tab



6. To view the tissue properties ($_a$, $_{eff}$, $'_s$) for the tissue you selected, make a selection from the Plot drop-down.
7. To include the number of fluorescent molecules/source in the results, select a fluorescent calibration database.

   For details on generating a luminescent calibration database, see page 185.
8. In the Analyze tab, click **Start**.
9. The Data Preview window appears and displays the image data that will be included in the reconstruction. Usually, no data adjustment is required. However, it is possible to exclude or include user-selected pixel data from the analysis. For more details, see page 198.

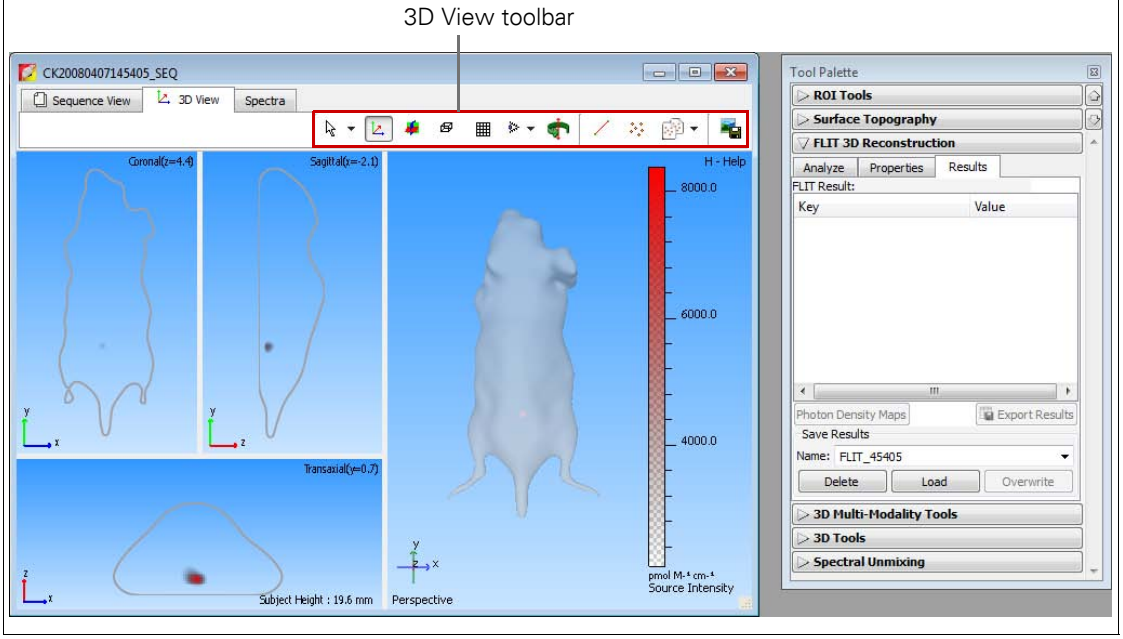
   You can also include or exclude image data by adding or removing the check mark next to the images listed in the Analyze tab (Figure 14.10).
10. Click **Reconstruct**.

    The reconstruction normally requires less than one minute, depending on the reconstruction volume, parameter settings, and computer performance. When the analysis is finished:

    - The 3D View window displays the surface and the reconstructed sources
    - In the Tool Palette, the Results tab displays the results data and the algorithm parameter values (Figure 14.13).
    - The 3D Tools appear in the Tool Palette. For more details on the 3D Tools, see page 220-231.

    For details on managing results (for example, save, load, or delete), see page 205.

Figure 14.12 FLIT results: 3D View window and Results tab
For details on the 3D View toolbar, see Table 14.3, page 197.



14.3 3D Reconstruction Results

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

DLIT or FLIT Results

NOTE

For more details on DLIT, see Appendix H, page 317. Sometimes adjusting the DLIT algorithm parameters improves the fit of the simulated photon density to the measured photon density data.

Figure 14.13 Example DLIT 3D reconstruction results

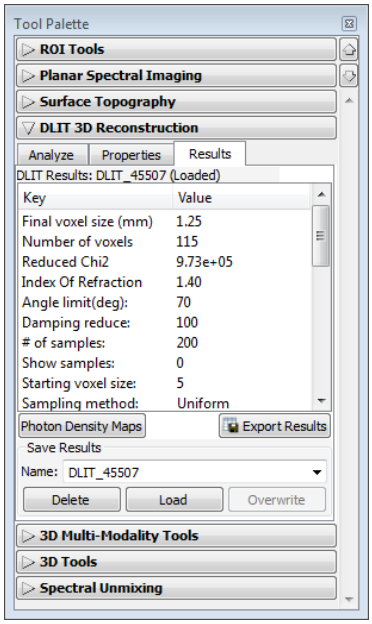


Table 14.5 DLIT or FLIT 3D reconstruction results

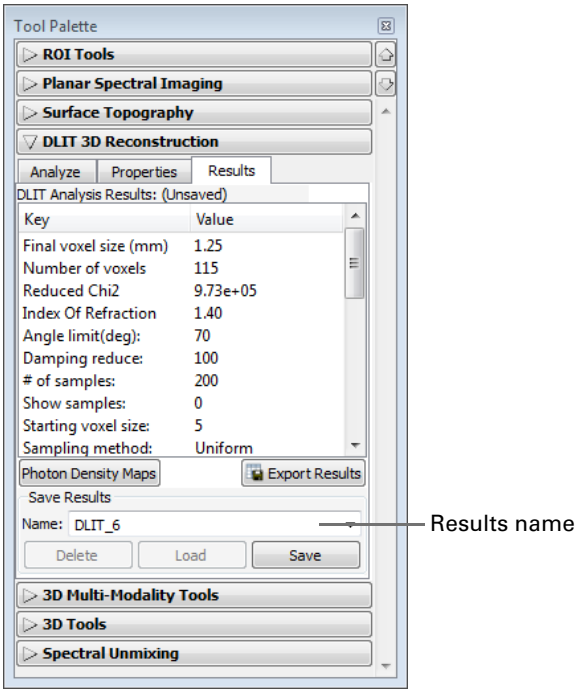
Item	Description
Final voxel size (mm)	The voxel size (length of a side, mm) that produces the optimum solution to the DLIT or FLIT analysis.
Number of voxels	The number of voxels that describe the light source(s).
Reduced Chi2	A measure of the difference between the computed and measured photon density maps at the optimum solution. A smaller χ^2 value indicates a better quality of fit.
Starting voxel size	The voxel size at the start of the analysis. The length of the side of the voxel cube in mm units for the coarsest initial grid size in the adaptive gridding scheme.
Nsurf (best)	The number of surface element data analyzed per wavelengths/ images.
Total surf samples	The total number of surface element data analyzed for all wavelengths/images.

Table 14.5 DLIT or FLIT 3D reconstruction results (continued)

Item	Description
Threshold angle	The angle that the object surface normal makes with the optical axis. The optical axis can be considered to be a line perpendicular to the stage. The default setting for this limit is 70 for IVIS Spectrum or IVIS 200 data.
Image Threshold	The percentage of the minimum radiance at each wavelength (DLIT) or source location (FLIT) is of the maximum radiance. This defines the minimum intensity included in the data.
Tissue Properties	The tissue properties for modeling the photon propagation.
Source Spectrum	The emission spectrum of the type of luminescent source.
Photon Density Maps	Click to view the photon density maps. For more details, see page 207.
Export Results	Opens a dialog box that enables you to save the results (.csv).
Save Results	
Name	The default name for the active DLIT or FLIT results. Select results from this drop-down list.
Delete	Click to delete the selected DLIT or FLIT results.
Load	Click to load the selected DLIT or FLIT results.
Save	Click to save the active DLIT or FLIT results.
Overwrite	If you reanalyze saved results, click to save the new results and overwrite the previous results.

Managing 3D Reconstruction Results

Figure 14.14 3D analysis results



To save 3D results:

1. In the Results tab of the DLIT/FLIT 3D reconstruction tools, confirm the default name or enter a new name.
2. Click **Save**.

   The results are saved to the sequence click number folder and are available in the Name drop-down list.

To open 3D results:

1. In the Results tab, make a selection from the Name drop-down list.
2. Click **Load**.

   The 3D results appear in the 3D View window.

To copy user-specified results:

1. In the Results tab, select the text of interest.
2. Right-click the results table and select **Copy** from the shortcut menu that appears.

   The selected results are copied to the system clipboard.

To copy all results:

1. In the Results tab, right-click the results table and choose **Select All** from the shortcut menu that appears.
2. Right-click the results table again and select **Copy** from the shortcut menu.

   All of the results are copied to the system clipboard.

To export results:

1. In the results tab, right-click the results table and select Export Results from the shortcut menu that appears.
2. In the dialog box that appears, choose a folder for the results, enter a file name, and click **Save**.

   The exported results are saved in .csv file format.

To delete results:

1. In the Results tab, make a selection from the Name drop-down list.
2. Click **Delete**.

   The results are deleted from the system.

14.4 Checking the Reconstruction Quality

To check the quality of a 3D reconstruction, it is useful to compare the measured and simulated photon density plots. The photon density is closely related to the measured radiance. Photon density is the steady state measure of the number of photons in a cubic millimeter. Light sources inside the tissue contribute to photon density in other portions of the tissue.

The reconstruction algorithms first convert the luminescent image of surface radiance to photon density just below the animal surface because this is what can be observed. Then the algorithm solves for point source locations inside the tissue which would produce the observed photon density near the surface.

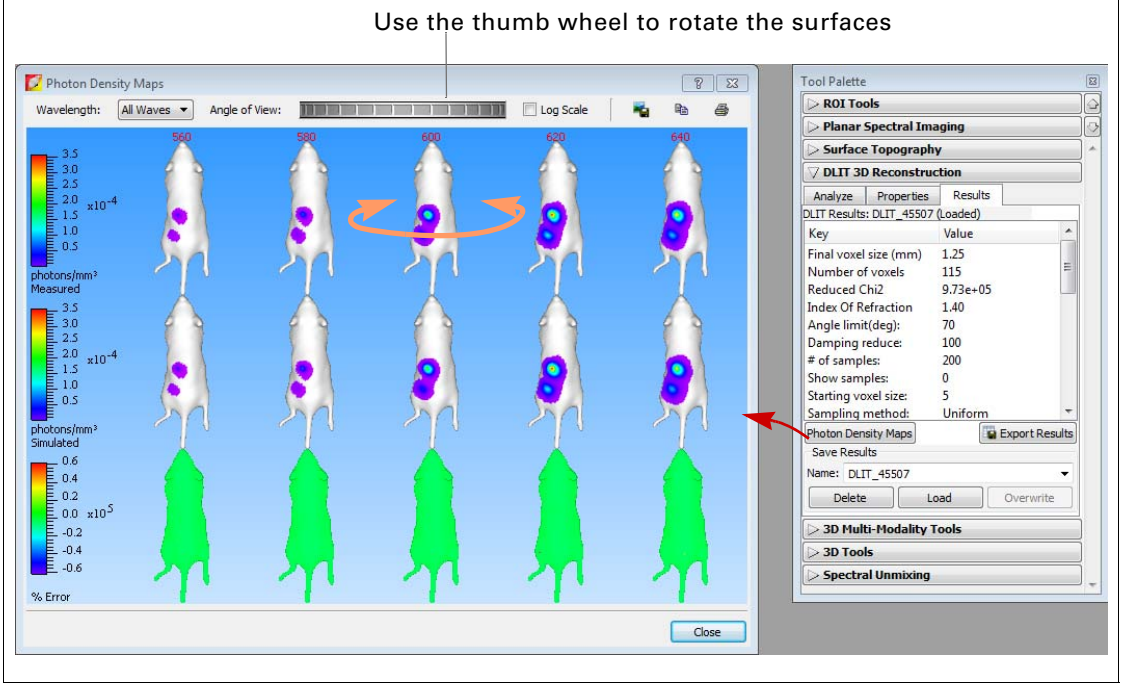
To view photon density maps:

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

   The photon density maps for all wavelengths are displayed (Figure 14.15).

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

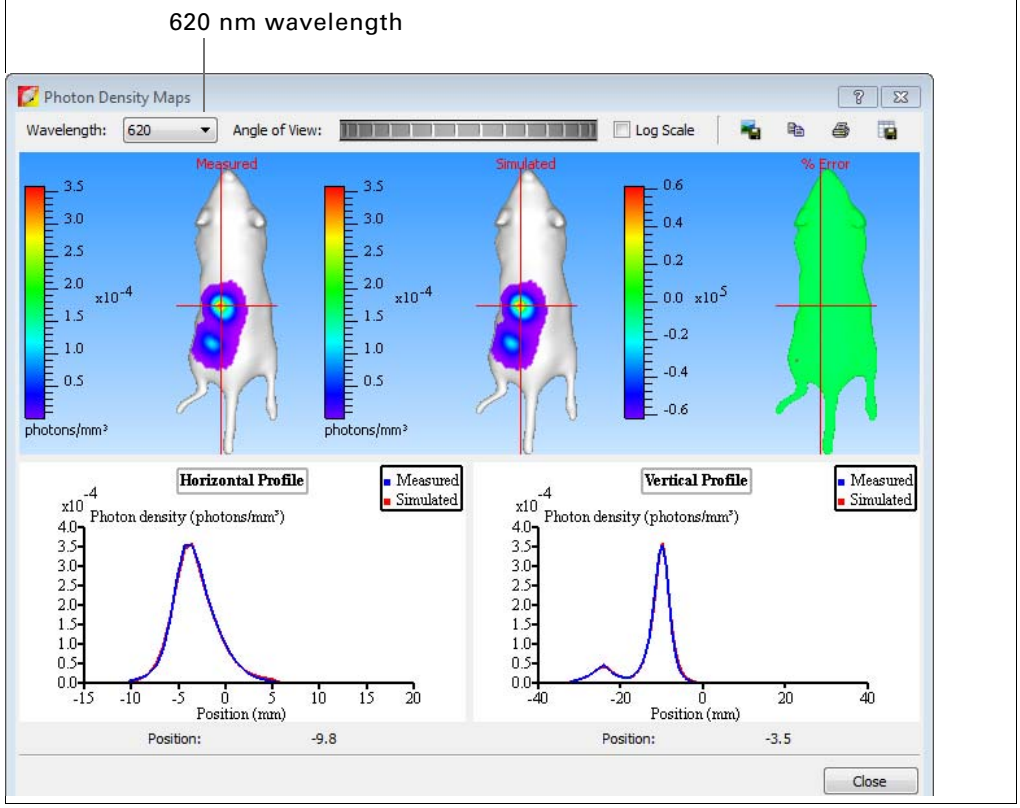
Figure 14.15 Photon density maps



3. Select a wavelength from the drop-down list

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

Figure 14.16 Simulated and measured photon density plots



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

Table 14.6 Photon Density Maps window

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

14.5 Measuring Sources

This section presents a convenient way to measure the source (total flux or total florescence yield, or if calibrated, the abundance in cells or picomoles. The volume, center of mass, and depth at the center of mass are also reported in the 3D Tools-Source tab.

NOTE

If the surface contains voxels pasted from other reconstruction results, choose a source in the 3D Source tools (Figure 14.17). For more details on pasting voxels, see page 213.

Determine Source Center of Mass

1. Click the  toolbar button and then drawing a box around the source.
2. Click **Center of mass** in the 3D tools Source tab (Figure 14.17) to obtain measured source information:

   Quantification – The integrated intensity within the selected sources.

   Volume – The total volume of the selected sources.

   Depth – The perpendicular distance from the source center of mass to dorsal surface.

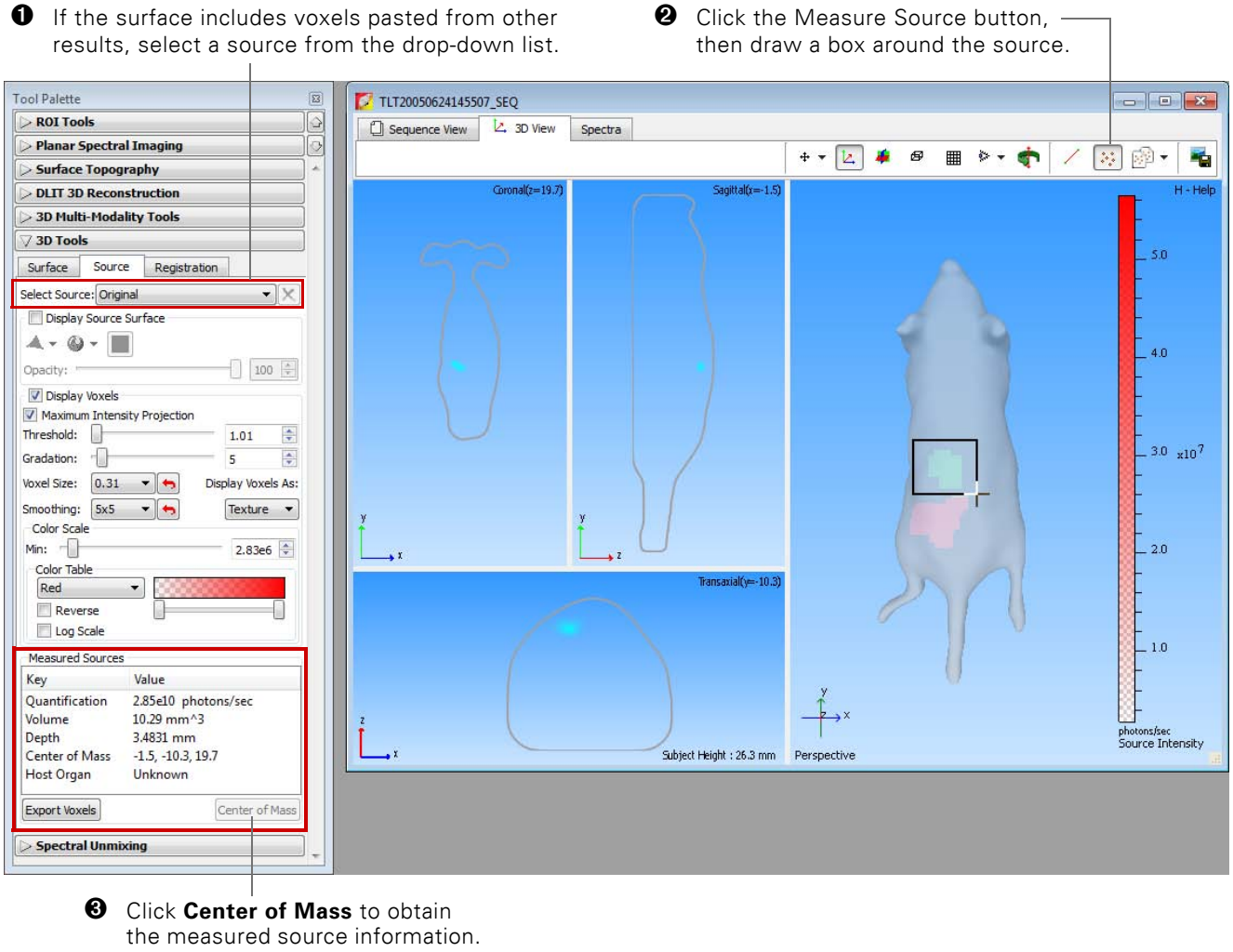
   Center of Mass – The weighted average x, y, and z-coordinates of the selected voxels, where the weights are the flux of each highlighted voxel.

   Host Organ – The organ in which the selected sources are located. This information is available if organs are displayed with the reconstruction. For more details on displaying organs, see 3D Tools - Registration, page 225

NOTE

The coronal, sagittal, and transaxial planes intersect at the center of mass of the selected source (Figure 14.18).

Figure 14.17 Select and measure source voxels in the 3D View window



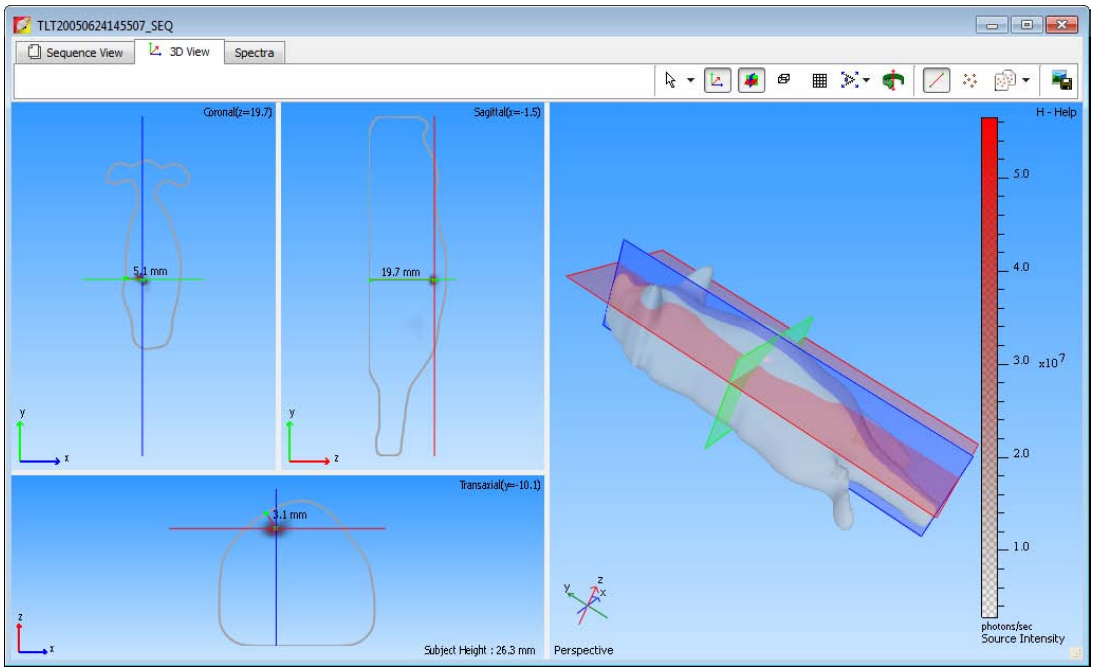
Measure Source Depth

1. Select the source by drawing a box around it (see Figure 14.17).
2. Click the  button.

   The distance from the center of mass to the surface is measured in the three planes (Figure 14.18).

   - Coronal and transaxial planes display the shortest distance from the center of mass to the surface
   - The sagittal plane displays the distance from the center of mass to the bottom of the subject.
3. To display slice planes through the center of mass, click the  button. For more details on planes, see page 212.

Figure 14.18 Slice planes
This example shows slice planes through a selected source center of mass and distance measurements from the source center of mass to the surface.



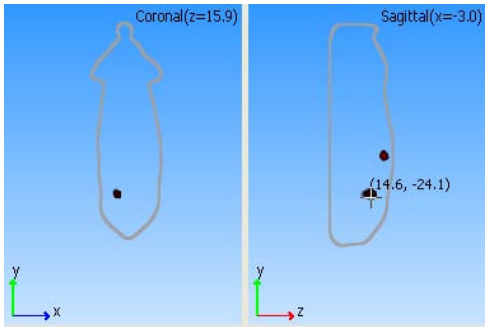
Viewing Coordinates

1. In the Coronal, Sagittal, or Transaxial windowpane, click a location in the reconstruction slice.

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

   - Coronal plane - Displays the x-y coordinates.
   - Sagittal plane - Displays the y-z coordinates.
   - Transaxial plane - Displays the x-z coordinates.

Figure 14.19 Viewing y,z coordinates in the sagittal plane

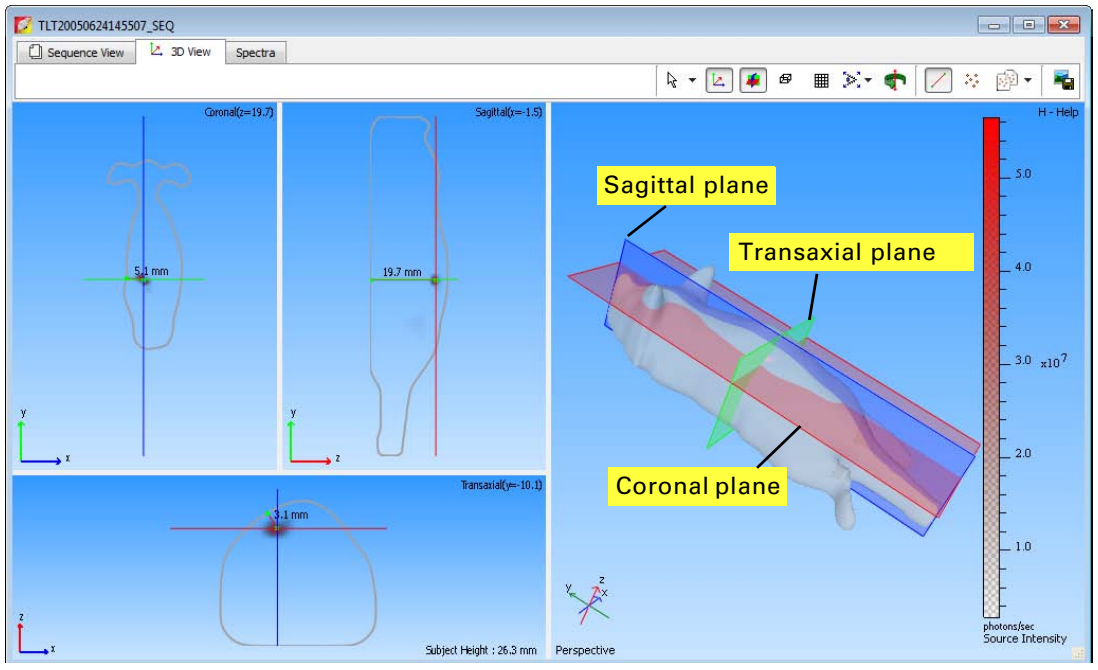


Displaying Slices Through a Reconstruction

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

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

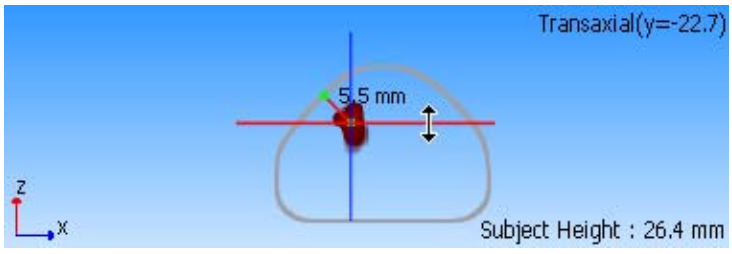
Figure 14.20 Planes cutting a reconstruction



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

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

Figure 14.21 Moving the coronal plane



14.6 Displaying Luminescent and Fluorescent Sources on One Surface

If an experiment includes luminescent and fluorescent reporters, DLIT and FLIT reconstructions can be displayed on one surface.

NOTE

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

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

NOTE

Pasted voxels can be measured. For more details on measuring sources, see page 209.

14.7 Comparing Luminescent and/or Fluorescent Sources

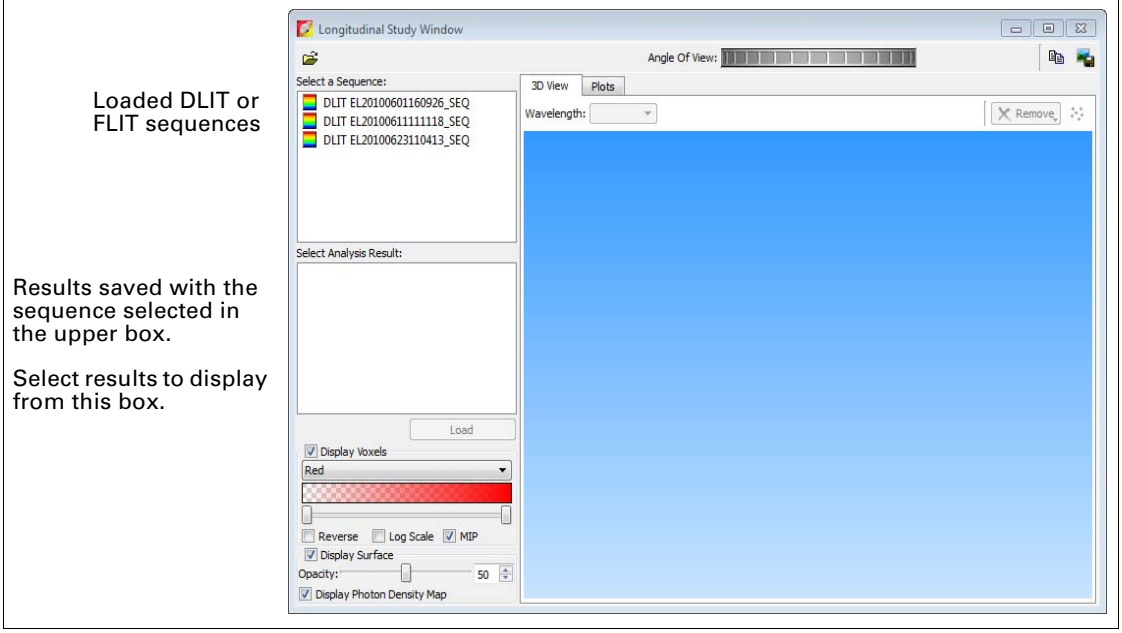
Multiple DLIT and/or FLIT reconstruction results can be viewed side-by-side in the Longitudinal Study window. Voxel intensity within the entire surface or a user-selected area can be measured in all results in the Longitudinal Study window. The Longitudinal Study window provides a convenient way to compare different results, for example, results obtained at different time points or results from different types of reporters.

Viewing Results in the Longitudinal Study Window

1. Load the DLIT and/or FLIT sequences with the results that you want to display. Select **Tools → Longitudinal Study** on the menu bar.

   The Longitudinal Study window appears.

Figure 14.22 Longitudinal Study window



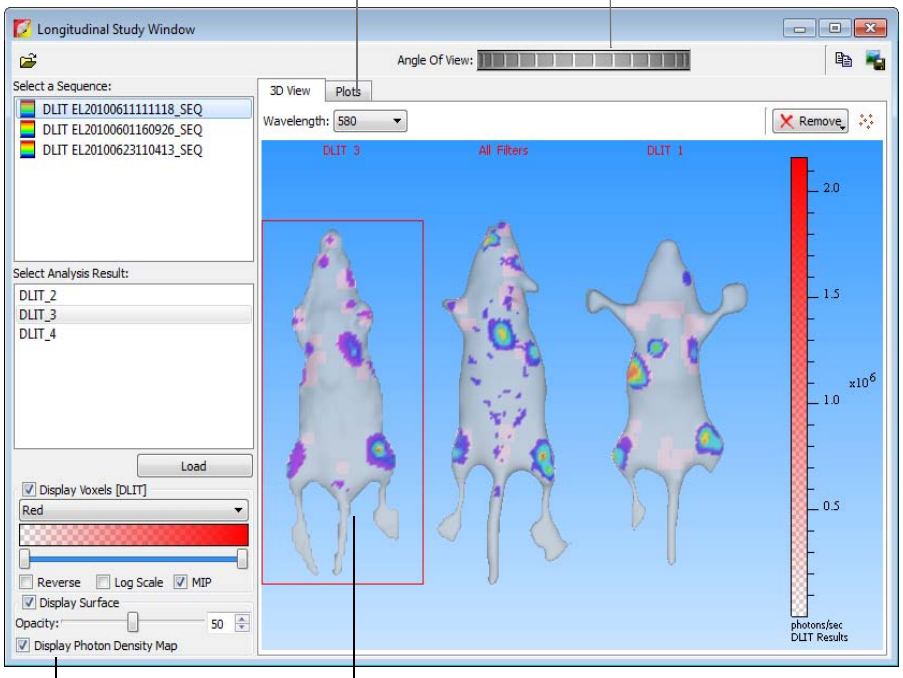
NOTE

After the Longitudinal Study window is open, more sequences can be added to the Longitudinal Study window by clicking the **Open** button and selecting sequenceinfo.txt files (found in the sequence data folder).

2. To show particular results:
   a. Select a sequence in the upper box.
   b. Select one or more analysis results in the lower box. To choose multiple adjacent results, press and hold the Shift key while you click the first and last result. To choose non-adjacent results, press and hold the Ctrl key while you click the results.
   c. Click **Load**.
3. To show more results, repeat step 2.
4. To remove results from the Longitudinal Study window, right-click a surface and select **Remove** on the shortcut menu. Alternatively, select a surface, click the Remove button Remove and choose **Selected Result**.

   To remove all results, click the Remove button Remove and choose **All Results**.
5. To view a particular image in a sequence:
   a. Click the surface.
   b. For DLIT results, make a selection from the Wavelength drop-down list. For FLIT results, make a selection from the Image drop-down list.

Figure 14.23 DLIT and FLIT results in the Longitudinal Study window



Measuring Intensity

1. After results are loaded, click the button.

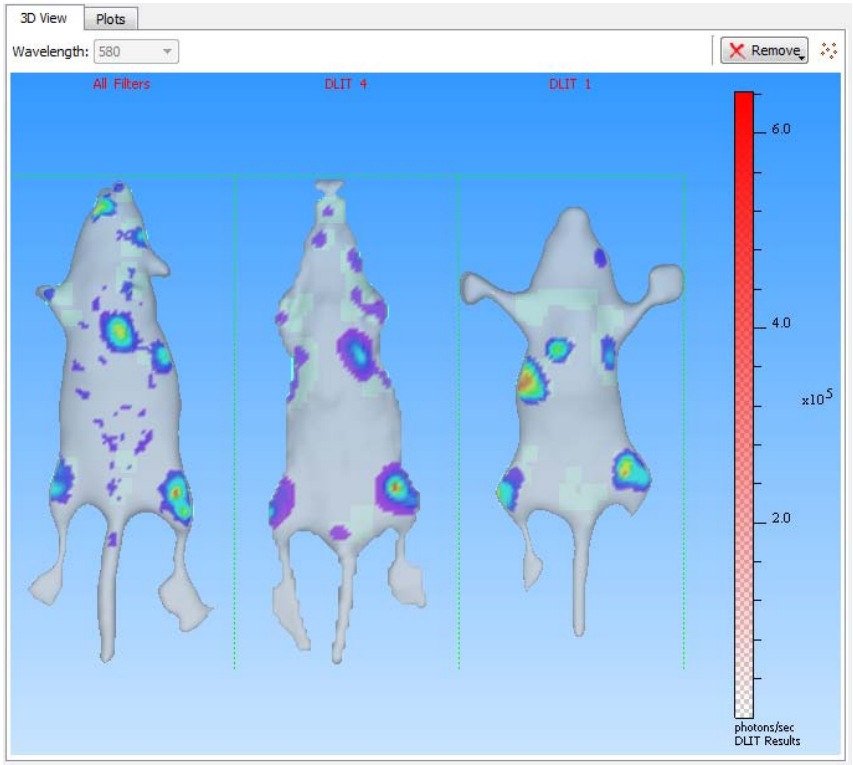
   By default, a selection box appears around each surface (Figure 14.24). This means that measurements for the entire surface will be computed.

2. To select a particular region of the surface for measurements, draw a box (by clicking and dragging the mouse) around the area.

   The same box is applied to the other surfaces in the Longitudinal Study window.

3. To clear boxes, click the button again.

Figure 14.24 Selection boxes around each surface



Viewing Plots

In the Plots tab, make a selection from the Analysis Type and Plot drop-down lists (Figure 14.25).

The following graphs are available in the Plots tab:

- Quantification Profile – Plots the measured intensity within the user-selected area on the surface. If no box was drawn on the surface, measures the total intensity for the entire surface.
- Reduced Chi-Squared Profile – A measure of the difference between the computed and measured photon density maps at the optimum solution. A smaller χ^2 value indicates a better quality of fit.
- Voxel Size – Plots the voxel size at the start of the 3D reconstruction and at the end of the 3D reconstruction.

Figure 14.25 Example Quantification plot

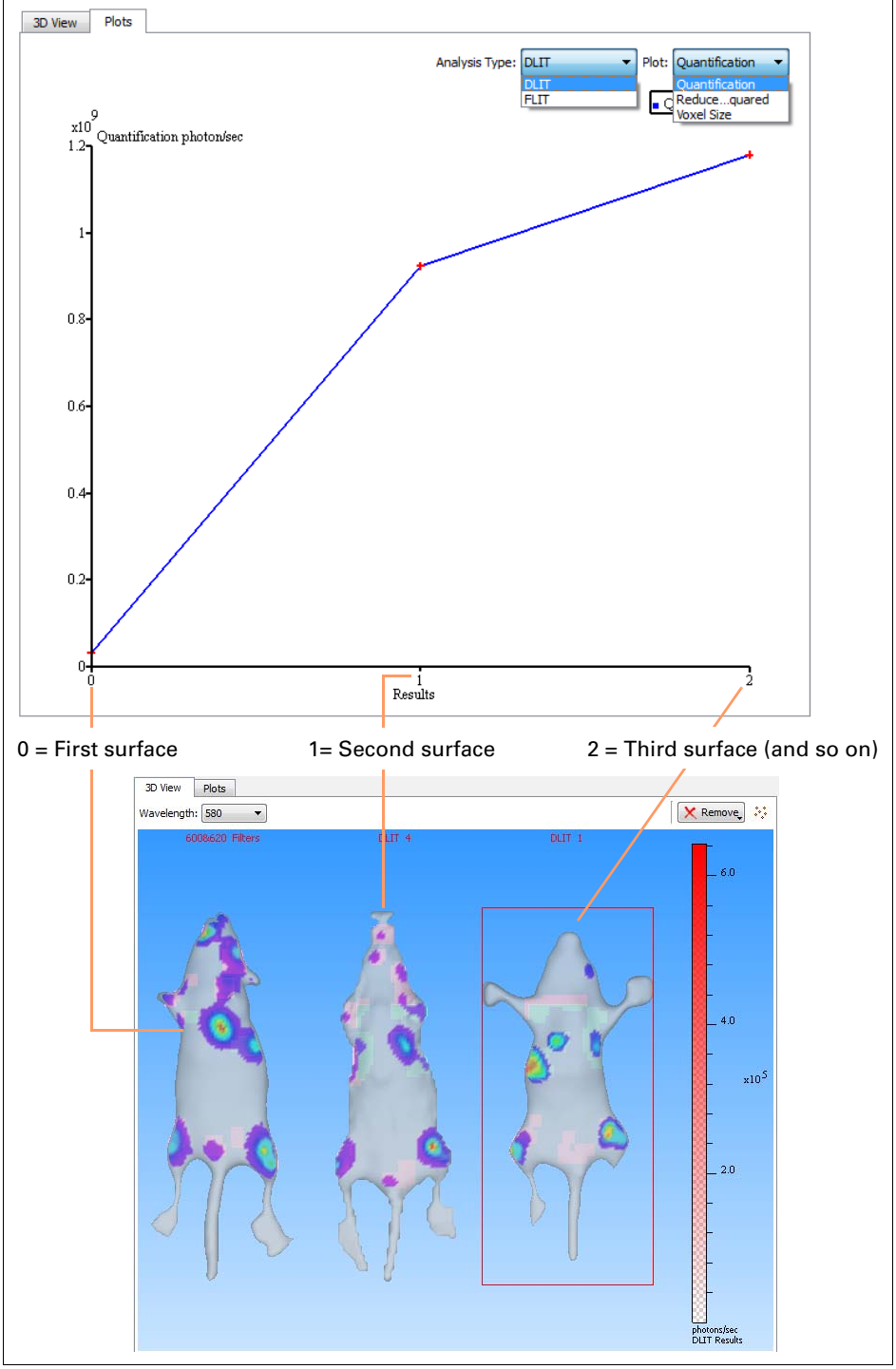
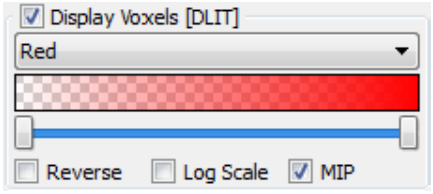


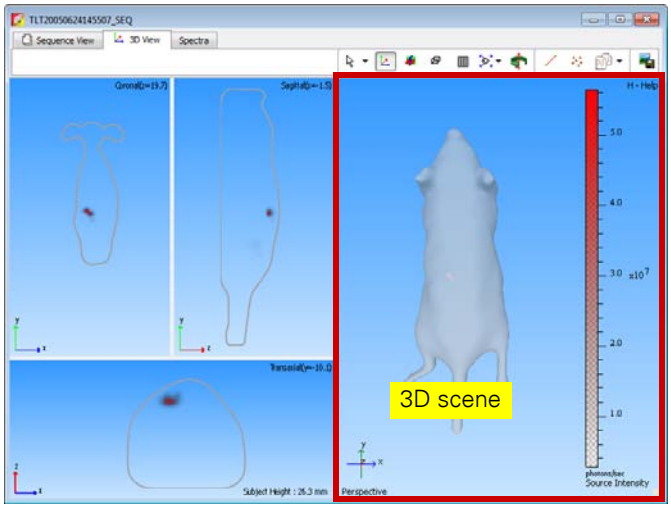
Table 14.7 Longitudinal Study window

Item	Description
	Voxel display controls: **Display Voxels** – Choose this option to show voxels with the surface. From the drop-down list, select a color scheme for the color scale. Move the sliders to adjust the color scale minimum and maximum values. **Reverse** – Choose this option to apply the colors of the selected color table in reverse order to the photon density scale. For example, the Red color table represents the source intensity (photons/sec) from low to high using a color scale from transparent to red. If **Reverse** is chosen, the source intensity (photons/sec) from low to high is represented using the color scale from red to transparent. **Log Scale** – Applies a log scale to the color scale. **MIP** – When this option is chosen, all maximum intensity voxels in the view are projected along the viewing direction into the viewing plane.
	Copies the 3D View tab in the Longitudinal Study window to the system clipboard.
	Opens a dialog box that enables you to export the 3D View tab to a graphic file (for example, .png).
	Enables you to select voxels for measurement. Measurements are displayed in the Plots tab.

14.8 Exporting a 3D Scene as DICOM

The items in the 3D View comprise a *3D scene*. For example, the 3D scene in Figure 14.26 includes a surface and voxels. The 3D scene can be exported to DICOM format and viewed in the Living Image DICOM Viewer or third party software.

Figure 14.26 3D scene

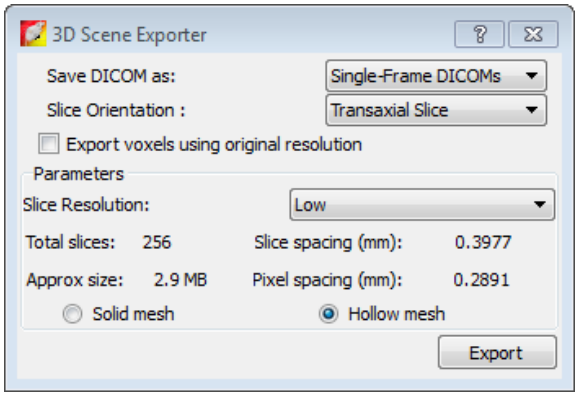


To export the 3D scene:

1. Load the results that you want to export.
2. Select **File** → **Export** → **3D Scene as DICOM** on the menu bar.
3. In the dialog box that appears, set the export options, and click **Export**.

   For more details on the 3D Scene Exporter, see Table 14.8.

Figure 14.27 3D Scene Exporter dialog box



4. In the Browse For Folder dialog box that appears, choose a folder for the DICOM files and click **OK**.

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

Table 14.8 3D Scene Exporter dialog box

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

Table 14.8 3D Scene Exporter dialog box (continued)

Item	Description
Hollow mesh	The intensity of pixels inside the surface is set to zero so that the exported surface appears as a hollow empty structure.

Viewing the DICOM Data

The 3D scenes exported to DICOM can be viewed in the Living Image® 3D Browser.

1. Select **File** → **Browse 3D Volumetric Data** on the menu bar.
2. In the dialog box that appears, select the DICOM data (.dcm or .dc3) and click **Open**. The 3D Browser window appears.
3. To

Figure 14.28 Living Image 3D Browser

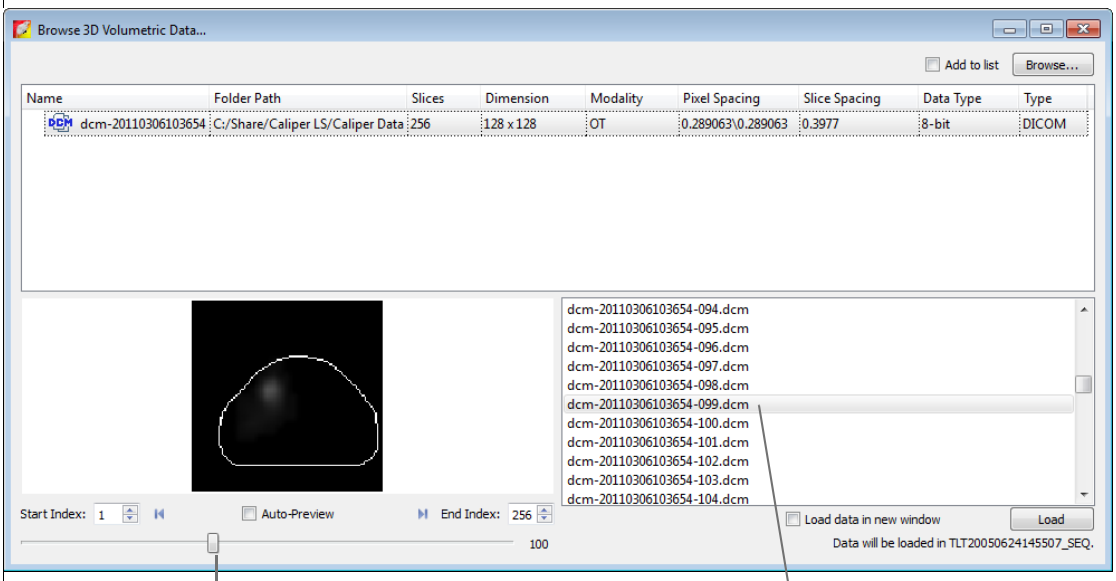


Table 14.9 Living Image 3D Browser DICOM viewing controls

Item	Description
Start Index	Specifies the first image (slice) for viewing.
Auto Preview	Select this option to automatically play back the images.
End Index	Specifies the last image (slice) for viewing.
Load	Opens the DICOM data in a 3D View window.
Load data in new window	If this option is selected, DICOM data are opened in a new 3D View window when you click **Load**. If this option is not selected, DICOM data are loaded in the active 3D View window.

14.9 3D Tools Overview

The Tool Palette includes the 3D Tools after you reconstruct or load a surface or 3D sources. The tools are organized by tabs:

3D Tools	Functions	Page
Surface Tools	Adjust the appearance of the reconstructed animal surface and photon density maps	See below
Source Tools	Adjust the appearance of reconstructed sources, make source measurements, export voxel measurements	222
Registration Tools	Display organs on the reconstructed surface, adjust the location or scale of organs on the surface, import an organ atlas	225
Animate Tools	Display preset animations of the 3D View scene. Enables you to create custom animations and record an animation to a movie file.	231

14.10 3D Tools - Surface

Use these tools to adjust the appearance of the reconstructed animal surface and photon density maps.

Figure 14.29 Surface tools and example DLIT reconstruction with photon density maps

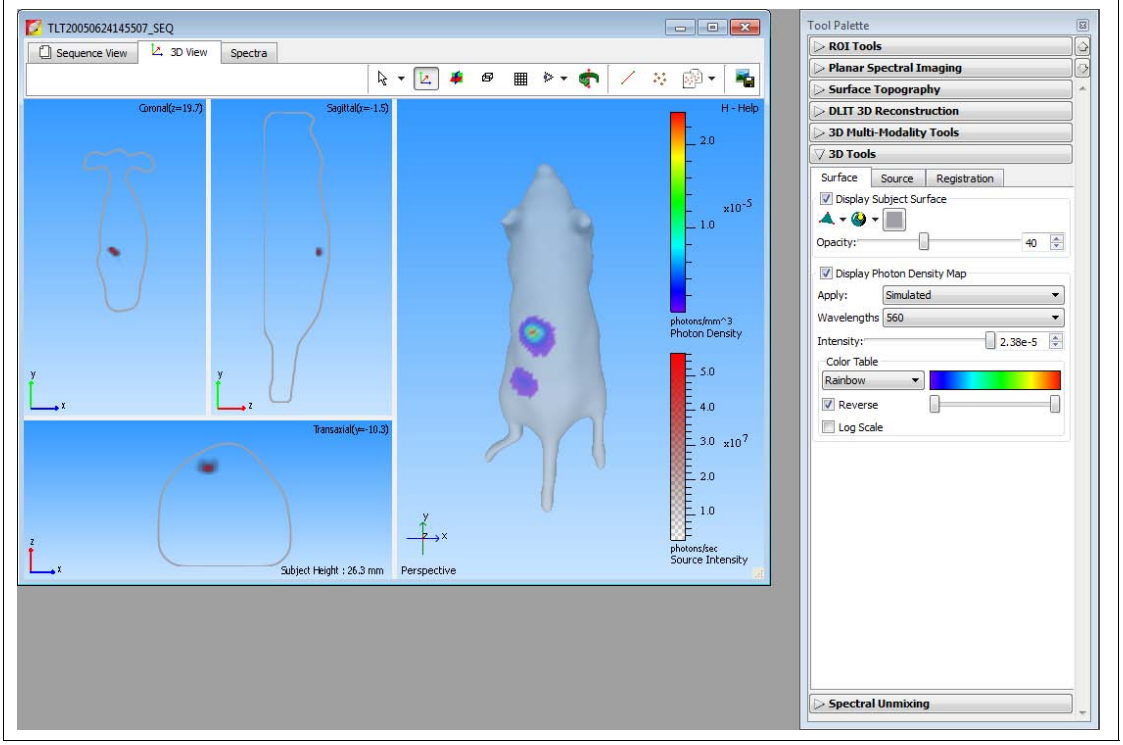


Table 14.10 Surface tools

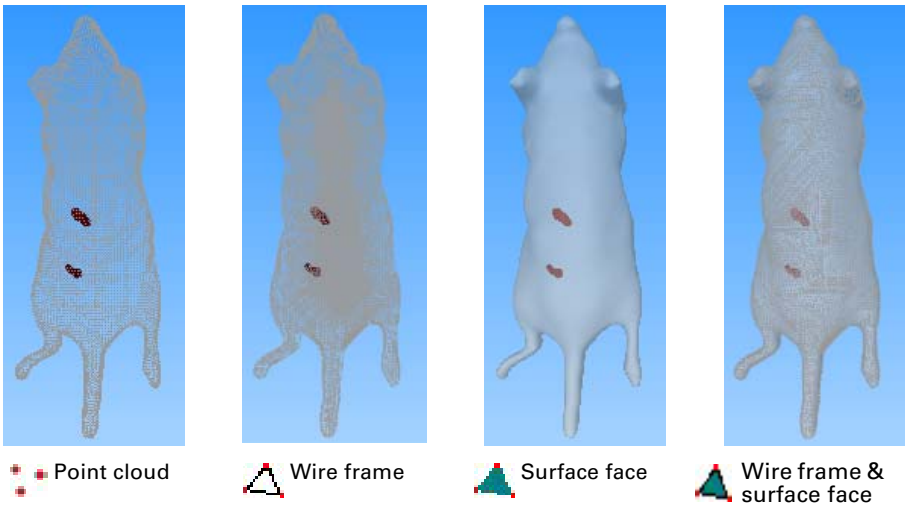
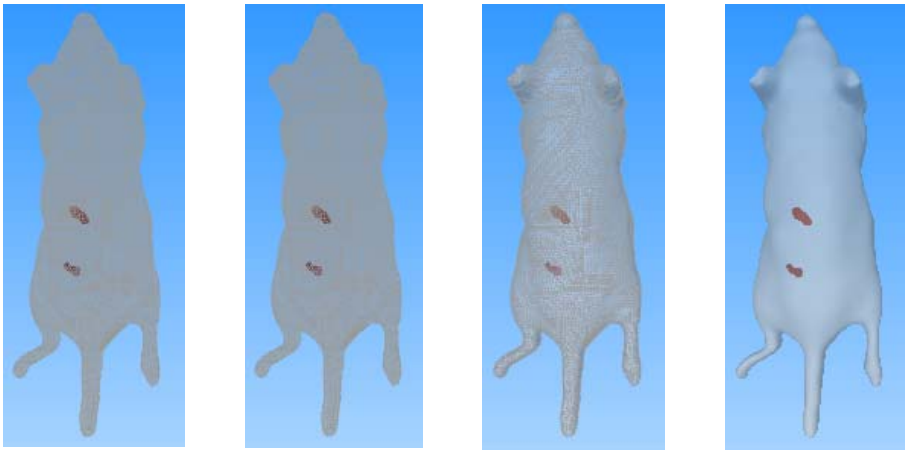
Item	Description
Display Subject Surface	Choose this option to display the surface in the 3D View window.
	Drawing styles for the surface.  Point cloud Wire frame Surface face Wire frame & surface face
	Shading styles for the surface.  Surface face Smooth surface face Reflect surface face Reflect smoot surface face
	Click to open the color palette from which you can select a display color for the surface and the cross section views.
Opacity	Adjusts the surface opacity.
Display Photon Density Map	Choose this option to display the photon density on the surface.
Apply	Choose measured or simulated photon density maps for display.
Wavelengths (DLIT) Images (FLIT)	Choose the data to display in the photon density map.
Intensity	Set the maximum intensity of the photon density map using the slider or by entering a value.
Color Table	Color scheme for the photon density map.

Table 14.10 Surface tools (continued)

Item	Description
Reverse	Choose this option to apply the colors of the selected color table in reverse order. For example, the Red color table represents the source intensity (photons/sec) from low to high using a color scale from transparent to red. If **Reverse** is chosen, the source intensity (photons/sec) from low to high is represented using the color scale from red to transparent.
Log Scale	Choose this option to apply a logarithmic scale to the photon density scale.

14.11 3D Tools - Source

Use the source tools to:

- Adjust the appearance of sources in DLIT or FLIT reconstructions
- Make source measurements (page 209)
- Export voxel measurements (.csv)

Figure 14.30 Source tools and example DLIT reconstruction

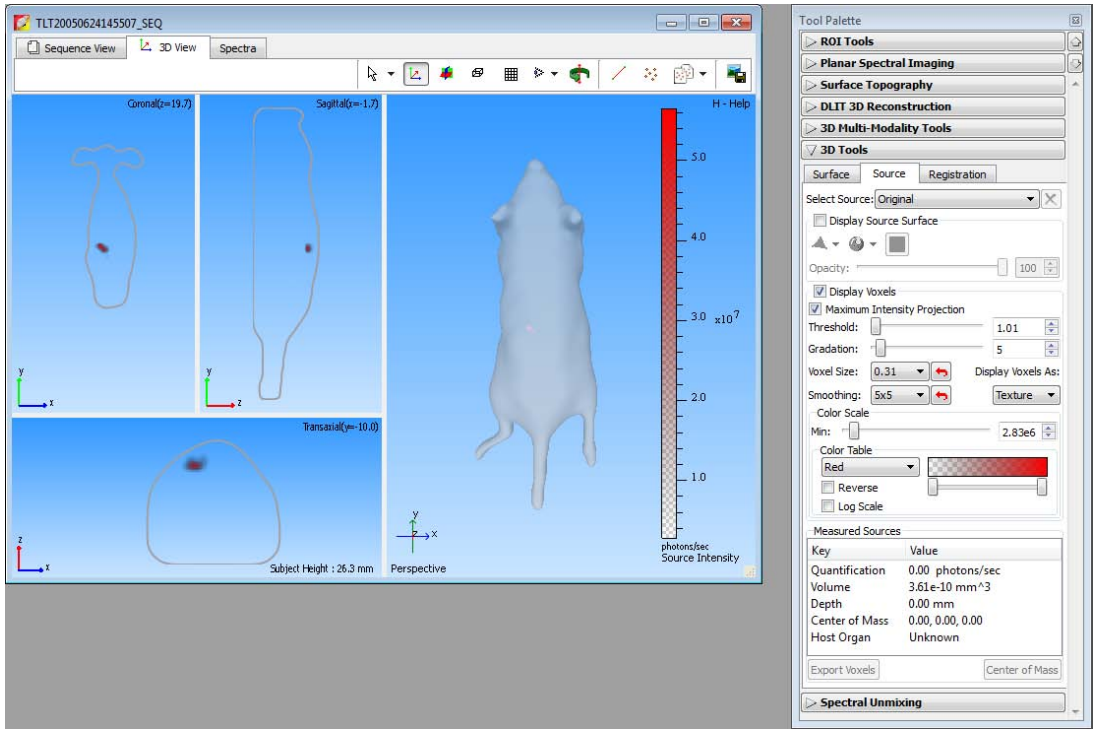


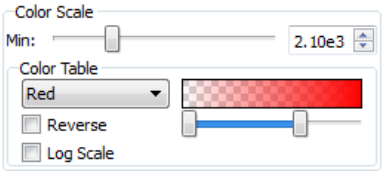
Table 14.11 Source tools

Item	Description
Select Source	A drop-down list of available sources. Original – Results saved with the data. <sequence name...SourceVoxels> – Pasted voxels. (Click the ✕ button to remove pasted voxels from the surface.)
Display Source Surface	Choose this option to display the source surfaces reconstructed using DLIT or FLIT. A surface will be wrapped around the currently displayed voxels. Adjust the voxel display by moving the Threshold slider.

Table 14.11 Source tools (continued)

Item	Description
	Drawing styles for the source surface (see "Display Source Surface").
	Shading styles for the source surface (see "Display Source Surface").
	Click to open the color palette from which you can select a display color for the source surface.
Opacity	Adjusts the source surface opacity.
Display Voxels	Choose this option to display the sources reconstructed using DLIT.
Maximum Intensity Projection	Choose this option to project all maximum intensity voxels in the view along the viewing direction into the viewing plane.
Threshold (DLIT/FLIT only)	Choose this option to apply a minimum threshold intensity to the voxel display.
Gradation (DLIT/FLIT only)	Use this slider to set a threshold for the percentage voxel intensity above which voxels are opaque and below which voxels will gradually face to transparent. The percentage voxel intensity is the percentage relative to the maximum intensity.
Voxel size	The 3D grid-spacing size for interpolation of the reconstructed source.
Smoothing	The smoothing box filter size.
Display voxels as	The voxel display mode (cubes, spheres, points, or texture).

Table 14.11 Source tools (continued)

Item	Description
Color Scale	 Min: Use the slider or up/down arrows to set the minimum value of the source color scale. Voxels with intensities less than the color scale minimum are not displayed in the reconstruction. Color Table – Color scheme for voxel display. Use the left and right sliders to set the minimum and maximum colors. Reverse – Choose this option to apply the colors of the selected color table in reverse order to the photon density scale. For example, the Red color table represents the source intensity (photons/sec) from low to high using a color scale from transparent to red. If **Reverse** is chosen, the source intensity (photons/sec) from low to high is represented using the color scale from red to transparent. Log scale – Choose this option to apply a logarithmic scale to the color table.
Measured Sources	Quantification (DLIT) – For uncalibrated sources, the total flux measured for the sources selected using the Measure Source tool. For calibrated sources, this unit will be in [cell] units. For details on using this tool, see page 209. Quantification (FLIT) – For uncalibrated sources, the fluorescence yield measured for the voxels selected using the Measure Source tool. Fluorescence yield is expressed in units of [pmol $M^{-1}cm^{-1}$] here for uncalibrated sources. For calibrated sources, this unit will be in either [cells] or [pmol]. For details using this tool, see page 209. Volume – Volume of the selected source (mm^3). Center of Mass (DLIT or FLIT) – The weighted average x, y, and z-coordinates of the selected voxels, where the weights are the flux of each highlighted voxel. Host Organ – The location of the selected source can be referenced to an organ atlas, and the organ from the atlas that is closest to the source will be reported here. This information is available if you select and register an organ atlas with the reconstruction. For more details, see page 230.
Export Voxels	Enables you to export the voxel measurements in their x-, y-, and z-coordinates and source intensities (.csv file).
Center of mass	Click to compute the center of mass for the source selected with the Measure Source tool. For details using this tool, see page 209.

14.12 3D Tools - Registration

Use the registration tools to:

- Display organs in the surface (page 226)
- Manually adjust the location or scale of organs in the surface (page 227)
- Check the organ fit (page 228)
- Import an organ atlas (page 230)

You can check the organ fit in the 3D View window (page 228)

Figure 14.31 3D registration tools and surface with fitted organs (“skin” not displayed)

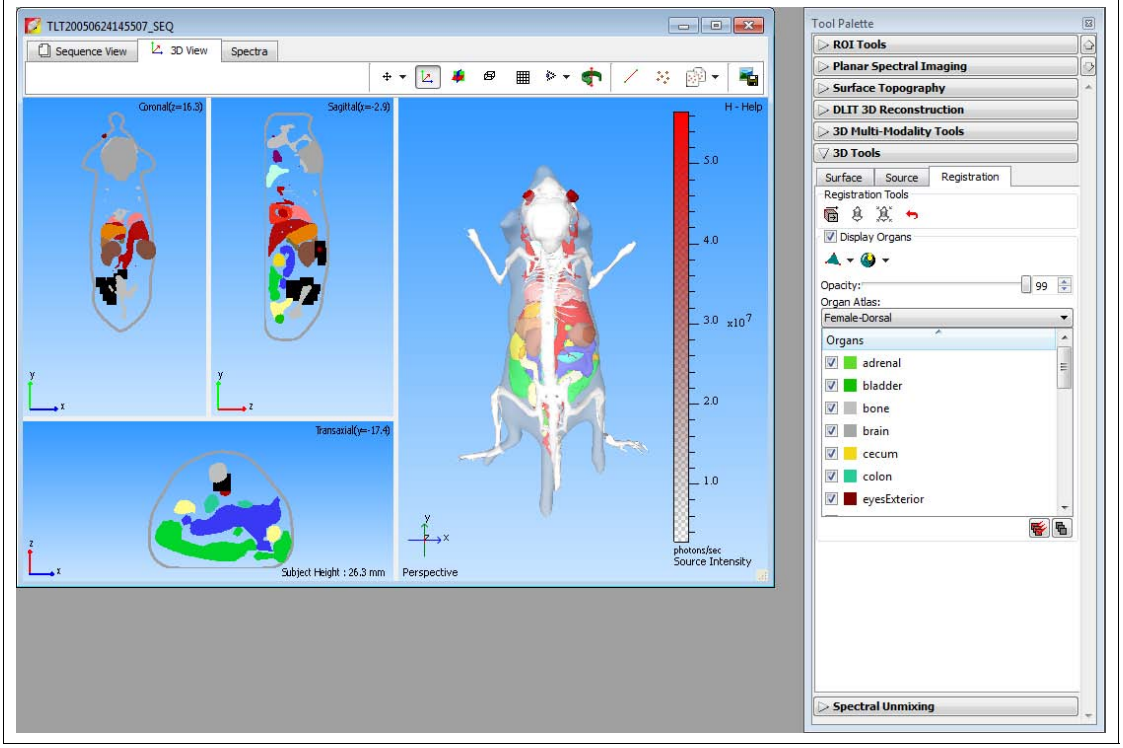


Table 14.12 Registration tools

Item	Description
	Use this tool to manually adjust the scale of location of organs. For more details, see page 227.
	Fits the organs to the surface using a linear transformation that keeps the shape of the atlas surface.
	Fits the organs to the surface using linear transformation and volume deformation.
	After fitting organs to the surface using the  or  tool, if necessary, you can click this button to restore the default fit.
Display Organs	Choose this option to display the organs on the surface. Organs that are check marked will be displayed. For more details, see page 226.

Table 14.12 Registration tools (continued)

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

Displaying Organs With the Reconstruction

1. Load reconstruction results and confirm that the surface is in the perspective view (click the  toolbar button in the 3D View window or press the **R** key).
2. In the 3D registration tools, choose the Display Organs option and select an organ atlas.

   The organs in the selected atlas appear on the surface.
3. To fit the organs to the surface, click a registration tool:

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

    Full registration - Performs linear transformation and volume deformation.

NOTE

For an optimum fit when there is a large difference between the orientation or size of the atlas organs and surface, first use the transformation tool to manually register the surface and atlas organs, then click a registration tool to automatically fit the organs. (For more details on manual registration, see below.)

4. If necessary, adjust the opacity of the organs using the slider or enter a number in the box.

   The organs are easier to view if you uncheck Skin in the Organs list.

5. To clear all organs from the surface, click the **Deselect All** button . To hide a particular organ, remove the check mark next to the organ name.
6. To display a specific organ(s), choose the organ name. To display all organs on the surface, click the **Select All** button .

NOTE

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

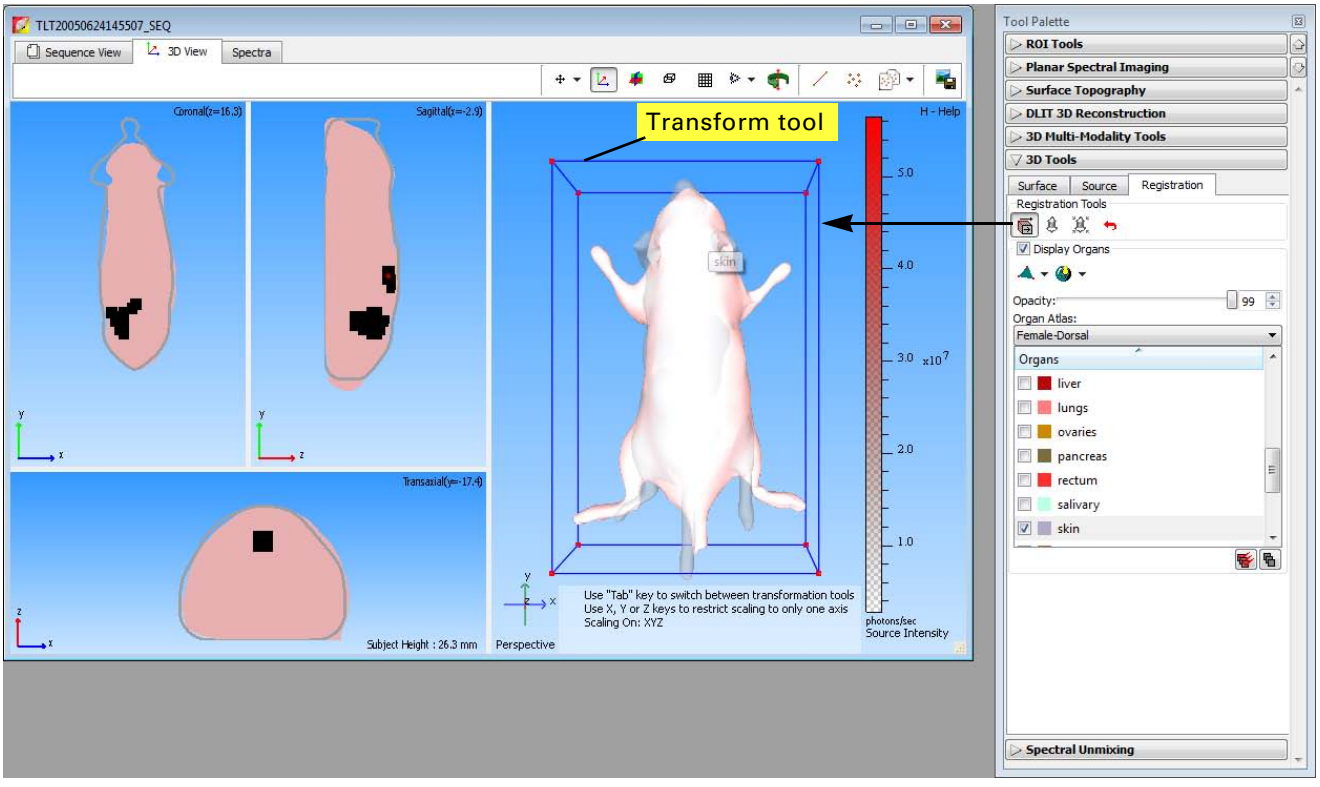
To manually adjust the scale or location of organs:

1. Load reconstruction results and confirm that the surface is in the perspective view (click the toolbar button in the 3D View window or press the **R** key).
2. In the 3D registration tools, choose the Display Organs option and select an organ atlas.

   The organs in the selected atlas appear on the surface. In Figure 14.32, only Skin is selected.
3. Click the **Transform tool** button .

   The transform tool appears.

Figure 14.32 Displaying the transform tool



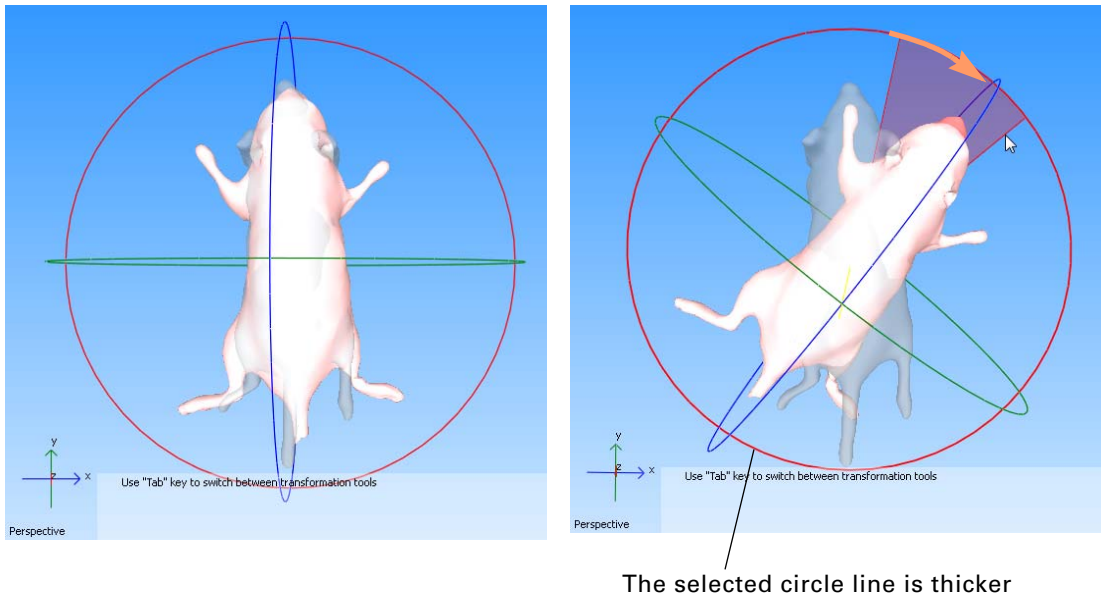
4. To adjust the x,y, or z-position of the organ, drag the transform tool.
5. Press the **Tab** key to put the transform tool in scale mode.

   A red cube appears at each corner of the transform tool.

6. To increase or decrease (*scale*) the size of the organ, drag a red cube at a corner of the transform tool. To restrict scaling to a particular axis, press the X, Y, or Z key, then drag a red cube.
7. Press the **Tab** key again to put the transform tool in rotate mode.

   A red, green, and blue circle appear around the surface.

Figure 14.33 Rotating the organ on the surface



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

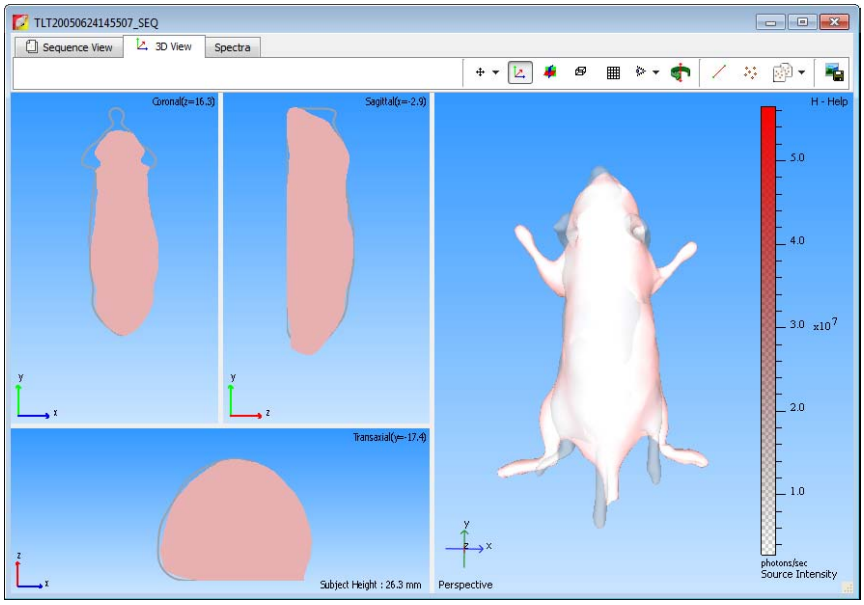
   To return the digital organ to the default position and size, click the **Reset** button, then button.
9. To turn off the transform tool, click the **Transform tool** button.

To check the organ fit:

1. Check the fit in the coronal, sagittal, and transaxial windowpanes.
2. Click the **Change view** toolbar button.

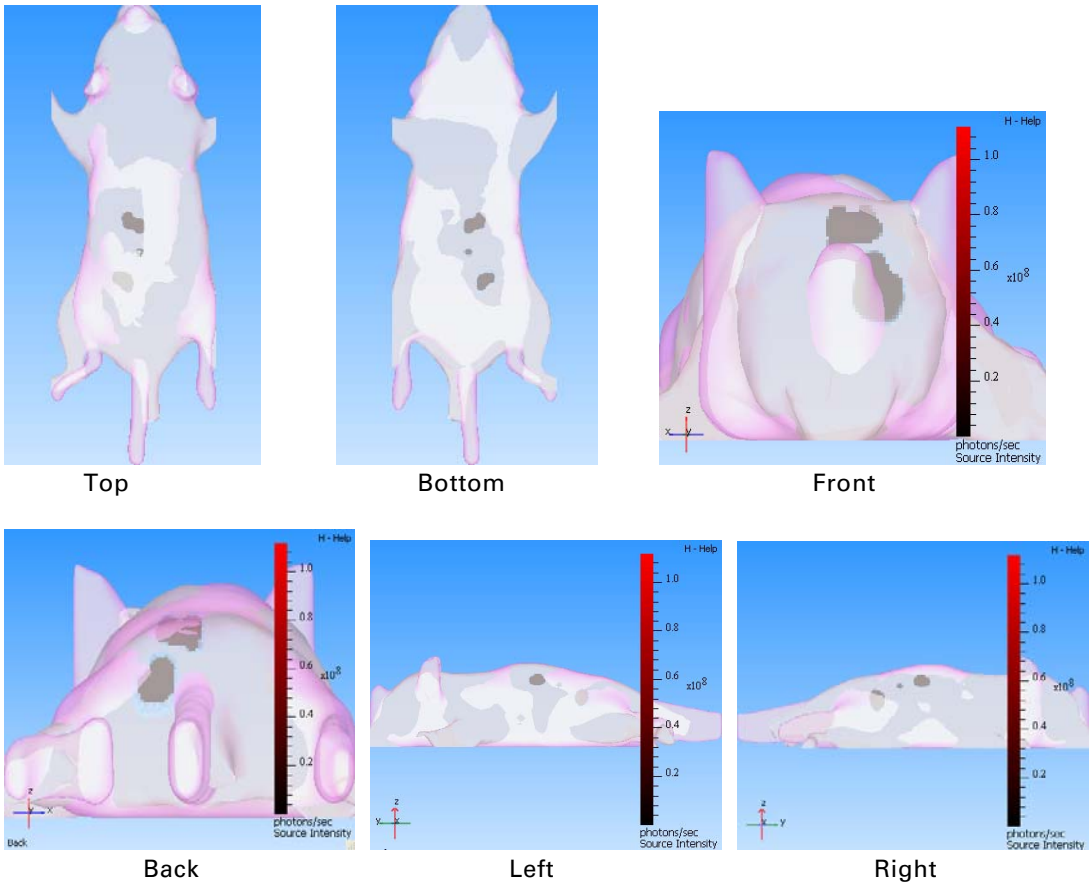
   The Top view is displayed.

Figure 14.34 Skin (pink) fitted to surface (gray)



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

Figure 14.35 Alternate views of the surface



Importing an Organ Atlas

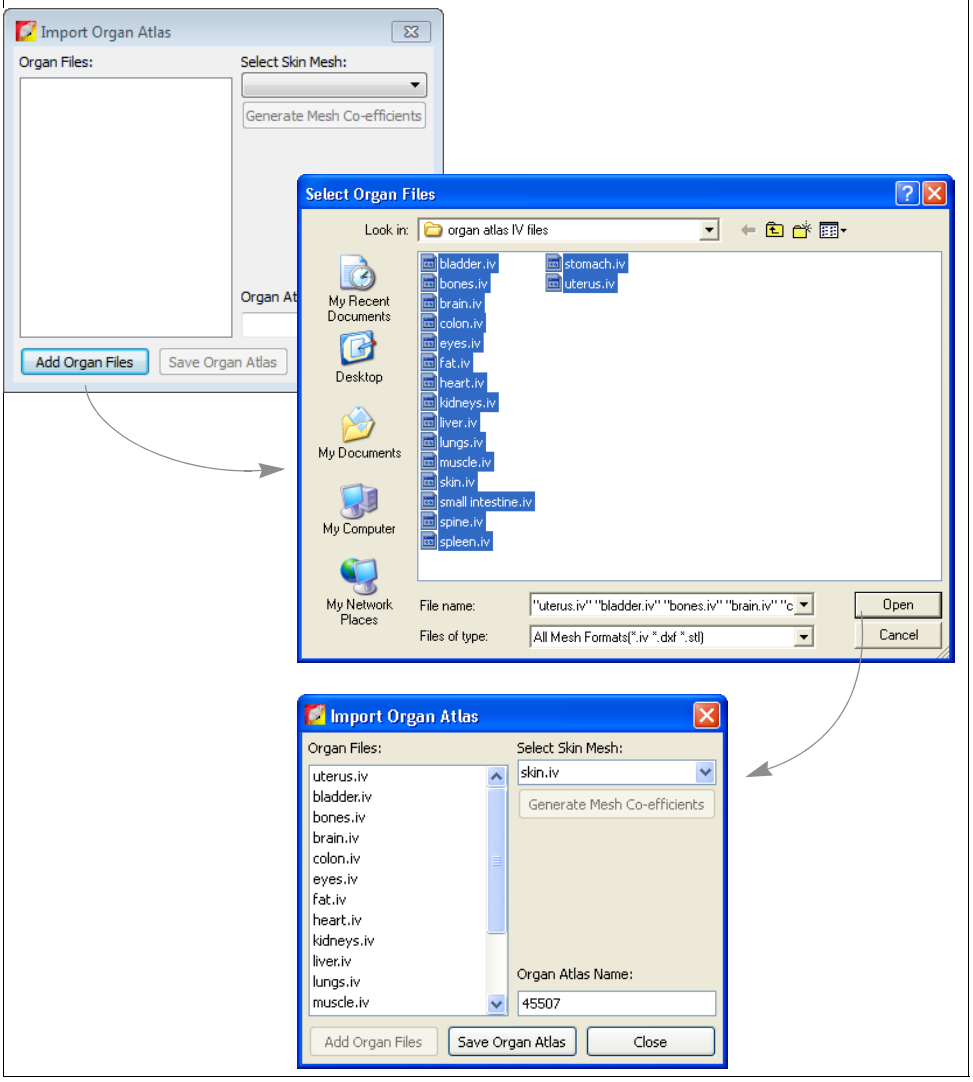
An organ atlas (.iv, .dxf, or .stl, one organ per file) consisting of segmented organ surfaces derived from an MRI or CT scan can be imported into the Living Image software for registration with the animal surfaces derived from IVIS data. Organ files must be segmented from MRI or CT 3D volumetric data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

NOTE

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

1. Load a DLIT or FLIT image sequence that is associated with the mouse comprising the organ files in *.iv, *.dxf or *.stl format.
2. Select **File** → **Import** → **Organ Atlas** on the menu bar.
3. In the dialog box that appears, click **Add Organ Files** (Figure 14.36).

Figure 14.36 Import Organ Atlas dialog box



4. In the next dialog box that appears, select all of the files (.iv, .dxf, .stl) that you want to include in the atlas (one file per organ) and click **Open**.
5. In the Select Skin Mesh drop-down list, select the skin organ file, which must include 'skin' in the file name.
6. Click **Generate Mesh Coefficients**.
7. Enter a name for the atlas and click **Save Organ Atlas**.

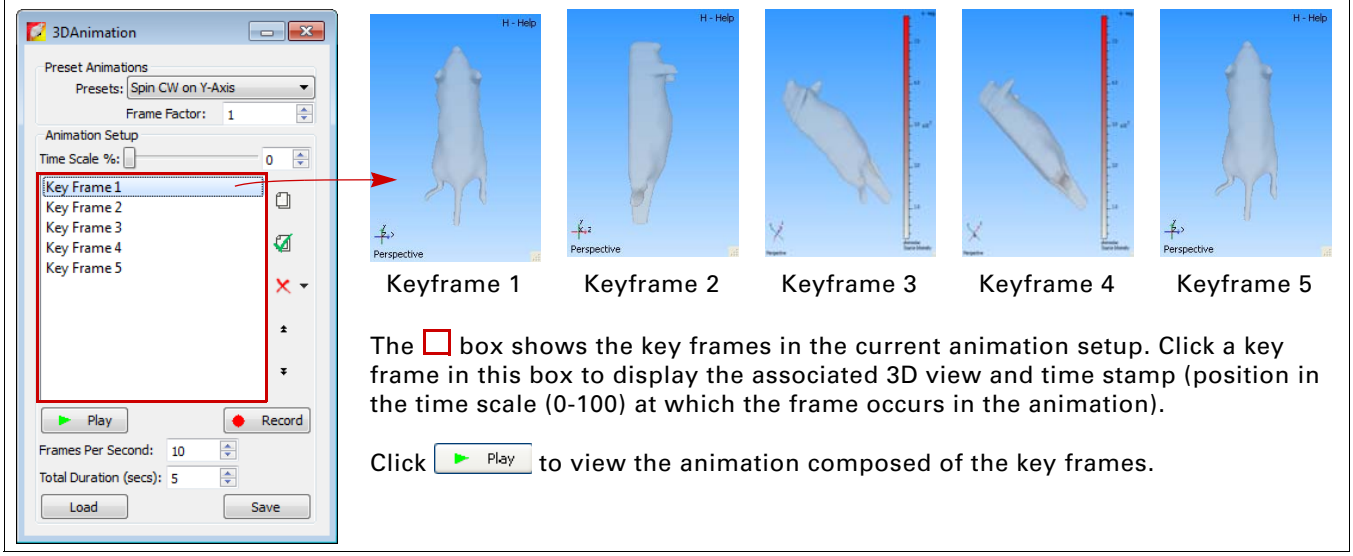
   The organ atlas (.atlas) is created and is added to the Organ Atlas drop-down list (in the 3D tools, Registration tab).

14.13 3D Animation

The Living Image software can create an animation from a sequence of 3D views (*key frames*). For example, an animation can depict a rotating 3D scene (Figure 14.37). The animation (series of key frames) can be recorded to a movie file (.mov, .mp4, or .avi). Use the animation tools to:

- View a preset animation (generated from a factory-loaded animation setup) (page 233)
- Create a custom animation (created from your custom animation setup) (page 234)
- Save an animation setup (page 235)
- Record an animation to a movie file (page 235)
- Edit an animation setup (page 236)

Figure 14.37 Individual 3D views (key frames) in the preset animation "Spin CW on Y-Axis"



The box shows the key frames in the current animation setup. Click a key frame in this box to display the associated 3D view and time stamp (position in the time scale (0-100) at which the frame occurs in the animation).

Click Play to view the animation composed of the key frames.

Table 14.13 3D animation tools

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

Viewing a Preset Animation

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

To view a preset animation:

1. Open an image sequence and load 3D reconstruction results.
2. Select properties to display in the 3D View window (for example, organs, sources, surface, or photon density maps).
3. Select **View** → **3D Animation** on the menu bar.
4. In the 3D Animation tools that appear:
   a. Clear the key frame box if necessary (click the button and select **Delete All**).
   b. Make a selection from the Presets drop-down list. See Table 14.13, page 232 for a description of the preset animations.

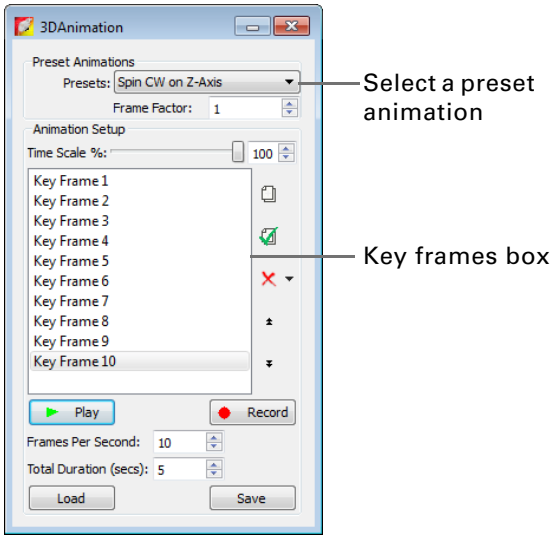
      After a preset animation is selected, a list of the key frames appears.

NOTE

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

5. To view the animation, click **Play**.

Figure 14.38 3D Animation tools
For more details on the animation tools, see Table 14.13, page 232.



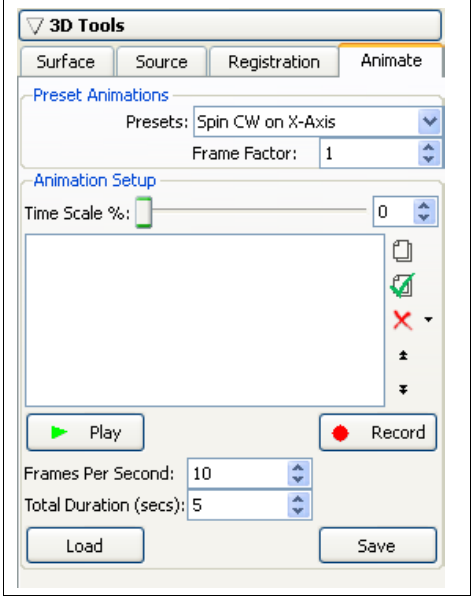
Creating a Custom Animation

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

1. Open an image sequence and load 3D reconstruction results.
2. Select properties to display in the 3D View window (for example, organs, sources, surface, or photon density maps).
3. Select **View** → **3D Animation** on the menu bar.

   The 3D Animation tools that appear:
4. Clear the key frame box if necessary (click the button and select **Delete All**).
5.

Figure 14.39 3D Animation tools

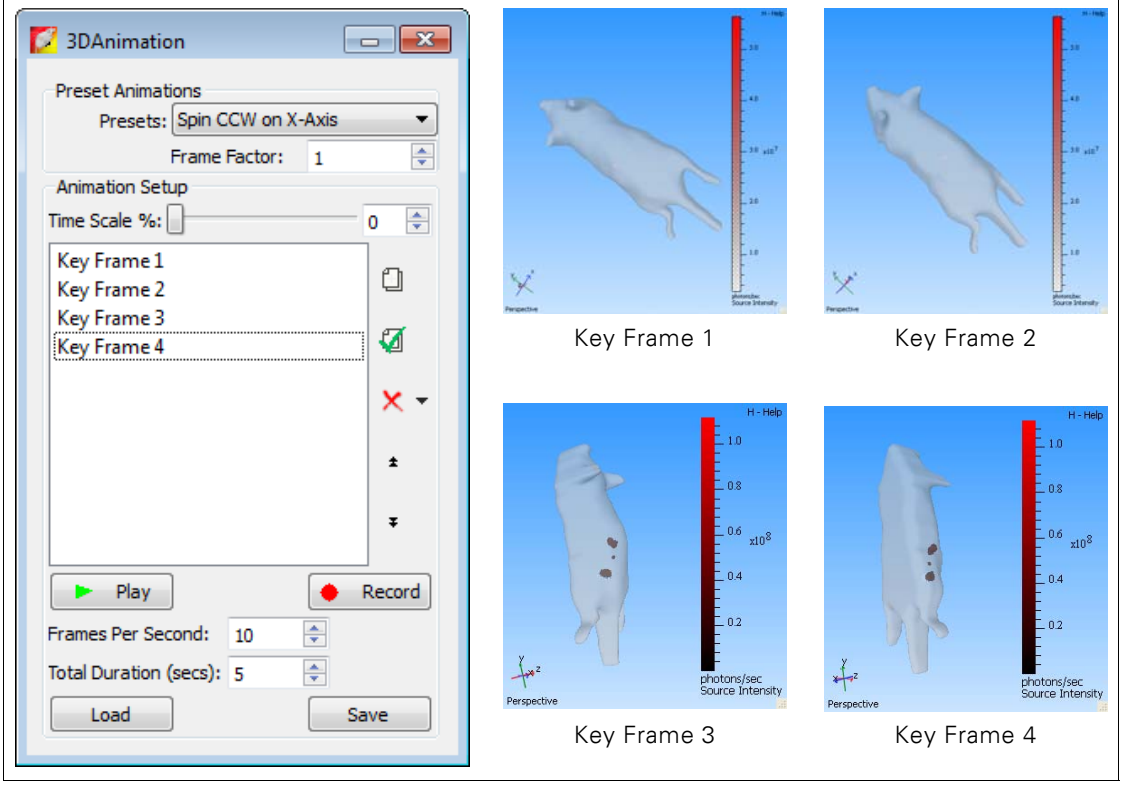


6. To capture the first key frame, click the button.

   The first key frame is added to the key frame box.
7. Adjust the position of the reconstruction in the 3D View using an image tool (for example, , or). For more details on the image tools, see page 197.
8. Click the button.

   The second key frame is added to the key frame box.

Figure 14.40 Example key frames for a custom animation



9. Repeat step 7 to step 8 until all of the key frames are captured. For details on how to edit the key frame sequence, see page 236.

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

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

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

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

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

To save an animation setup:

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

To record the animation to a movie:

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

To edit an animation setup:

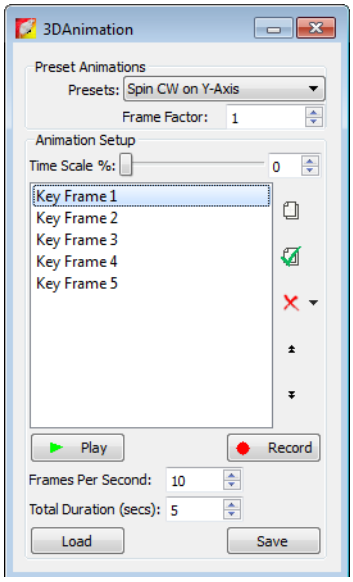
1. Open an image sequence and load a reconstruction.
2. Open an animation setup. To select a predefined setup, make a selection from the Preset drop-down list.

   To select a saved user-defined setup:

   a. Click **Load**.

   b. In the dialog box that appears, select an animation setup (.xkf).

Figure 14.41 List of key frames in the selected animation



3. To add a key frame:

   a. Adjust the position of the reconstruction in the 3D view using an image tool (for example, , or). For more details on the image tools, see page 197.

   b. Click the button.

4. To reorder a key frame in the sequence, select the key frame and click the or arrow.
5. To update a key frame:

   a. Select the key frame and adjust the 3D view.

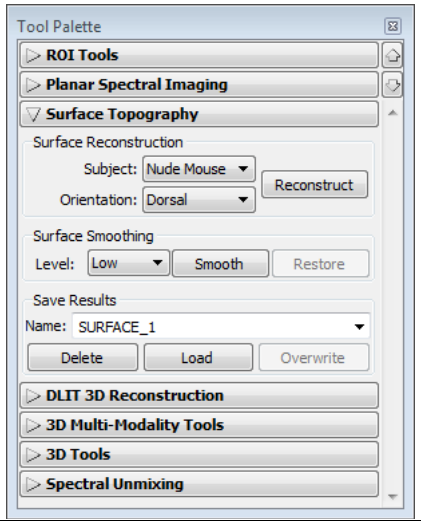
   b. Click the button.

6. To delete a key frame:

   a. Select the key frame that you want to remove.

   b. Click the button and select **Delete Current**.

14.14 DLIT/FLIT Troubleshooting

Issue	Solution
No sources in solution	• In DLIT or FLIT, this can occur if the surface is not correct. That is, if a surface is imported into the 3D View from another source other than from the Structured Light Analysis.
Surfaces are spiky	• The most common source of spiky surfaces are folds in the animal skin or fur, which corrupt the desired smooth lines projected on the animal from the laser galvanometer. – Choose the 'Fur Mouse' option for 'Subject' – Smoothing the surface by using the 'Smooth' feature in the Surface Topography tools can help improve the surface 
Bad Photon Density fit	• The optical properties or source spectrum may have been chosen erroneously. For example, for mice, 'Mouse Tissue' optical property is appropriate while 'XPM-2' is only appropriate for the Caliper phantom.

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15 3D Multi-Modality Tools

The Living Image® 3D Multi-Modality tools enable you to co-register 3D reconstructions of luminescent or fluorescent sources (optical imaging data) with CT or MRI images (3D volumetric data). Registering *multi-modal* data (optical and volumetric data) provides an anatomical context for interpreting biological (functional) information. Figure 15.1 shows the steps to register multi-modal data in the Living Image software.

15.1 3D Multi-Modality Tools Requirements

The Living Image 3D Multi-Modality tools require a separate license. Additionally, the graphics processing unit (GPU) must meet the minimum specifications shown in Table 15.1. If the appropriate license is not installed or the GPU does not meet these specifications, the 3D Multi-Modality tools will not appear in the Tool Palette.

NOTE

If you do not have the 3D Multi-Modality tools, you can still view DICOM data (see page 258); however, the 3D Multi-Modality tools are required to register optical and volumetric data.

Table 15.1 Minimum graphics card specifications

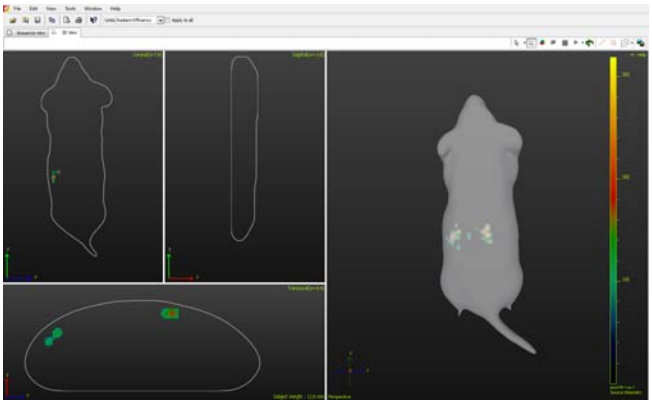
Specification	Description
OpenGL Version Requirement*	OpenGL 2.0 and above
OpenGL Extension Requirement*	GL-EXT-texture3D
Graphics Card Memory	Minimum: 256MB Dedicated + Shared) Recommended: 1GB (Dedicated)
Consumer Graphics Cards (Desktop/ Mobile, Windows/Mac)	Supported: – NVIDIA® GeForce® 8 Series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

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

Figure 15.1 Steps to register multi-modal data

❶ Load the optical data:

- Bioluminescence or fluorescence image sequence and structured light surface
- 3D source reconstruction (DLIT or FLIT results) (page 206)



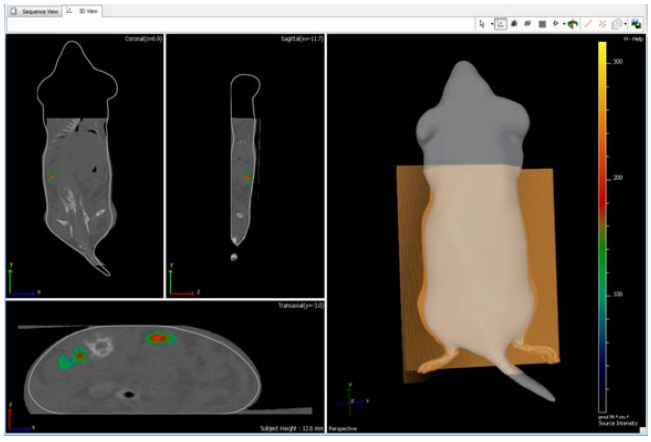
❷ Load 3D volumetric data (CT or MRI) (page 241).

❸ Register the 3D source reconstruction and the 3D volumetric data by performing either:

- Automatic fiducial registration–Available for data acquired on the Quantum FX µCT instrument using the Mouse Imaging Shuttle (page 245)

or

- Manual registration–Match animal surface representations using the Manual Registration tool (page 247)



❹ Classify the 3D volumetric data to help identify and separate objects (page 250). Save the color-opacity map (optional).

❺ Save the registered 3D multi-modality results (page 257).

15.2 Loading Data for Registration

1. Load a DLIT or FLIT image sequence and the 3D reconstruction results.

NOTE

The 3D Multi-Modality tools appear in the Tool Palette after you load optical image data. If the 3D Multi-Modality tools do not appear in the Tool Palette, confirm that the 3D Multi-Modality Tools license is installed and that the workstation graphics card meets the specifications in Table 15.1, page 239.

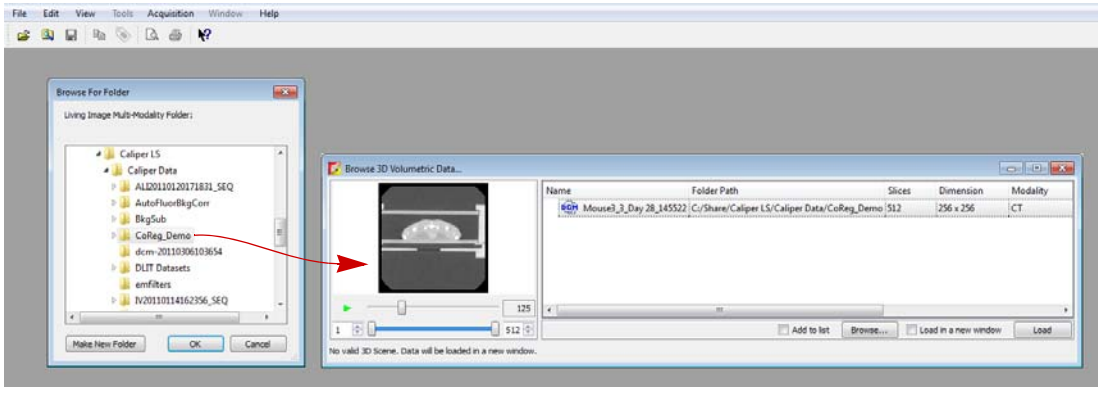
2. To browse for DICOM or TIFF volumetric data, select **File** → **Browse 3D Volumetric Data** on the menu bar.
3. In the Browse For Folder box that appears, select a data folder and click **OK**.

   The Living Image 3D Volumetric Browser appears (Figure 15.2).

NOTE

Only DICOM or TIFF data can be added to the 3D Volumetric browser. For details on loading other data types (.raw or .vox files) see page 243.

Figure 15.2 Opening the 3D Volumetric Browser



3D Volumetric Browser

NOTE

The next time you start the Living Image software and open the Browse For Folder box, the software automatically returns to the last folder visited.

The 3D Volumetric Browser automatically previews a playback of the data along with other information about the data (Figure 15.3).

- DICOM file
- TIFF file

4. To load volumetric data with the optical data:
   a. Confirm that the “Load in a new window” option is not selected. (If this option is selected, the volumetric data are loaded in a new window.)
   b. Double-click the data row in browser. Alternatively, select the data row and click **Load**.

The 3D volumetric data appears in the 3D View window of the optical data (Figure 15.4). The software converts loaded volumetric data into an 8-bit representation to reduce memory overhead and for easier color mapping. The 3D Multi-Modality tools provide an 8-bit color-opacity map for volume visualization which maps each voxel to an RGB color, or a color and opacity value.

A histogram of voxel intensities appears in the Multi-Modality tools and the software sets a default air/noise boundary.

Figure 15.3 3D Volumetric Data Browser

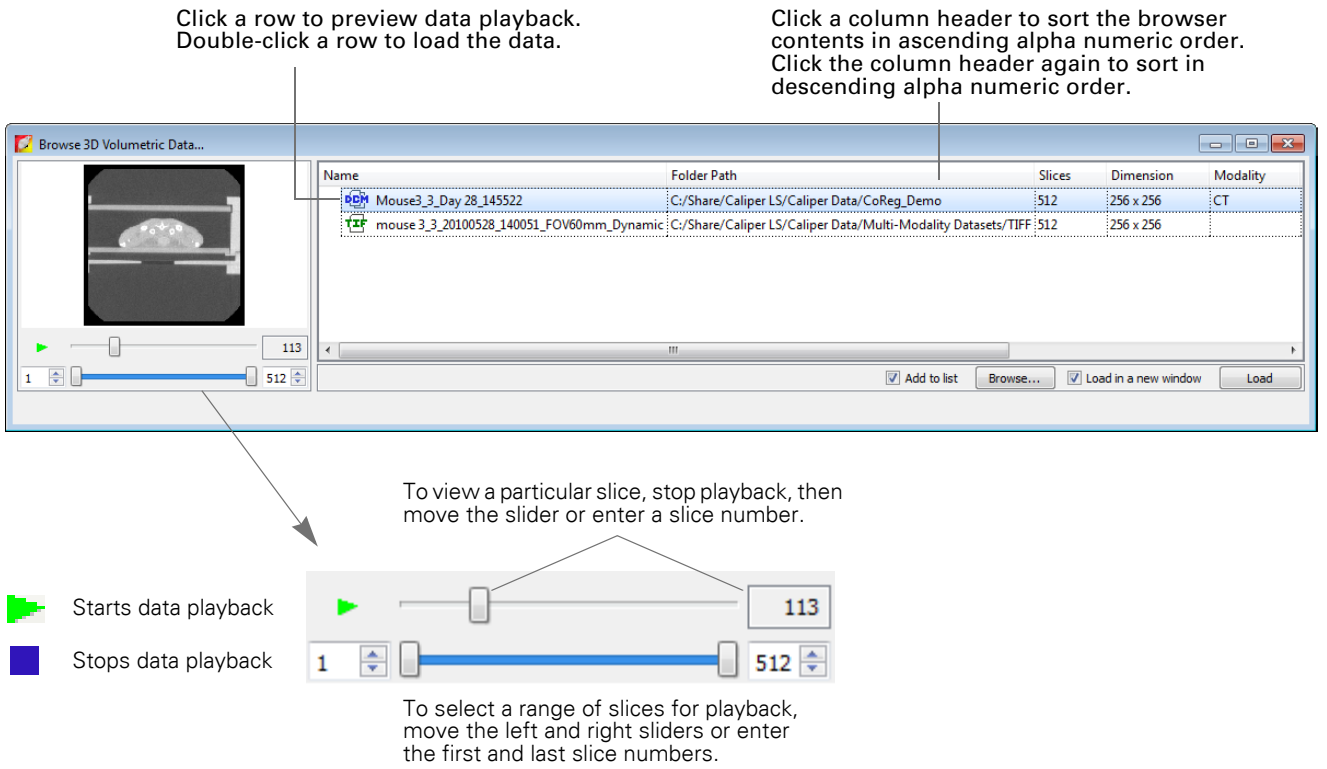
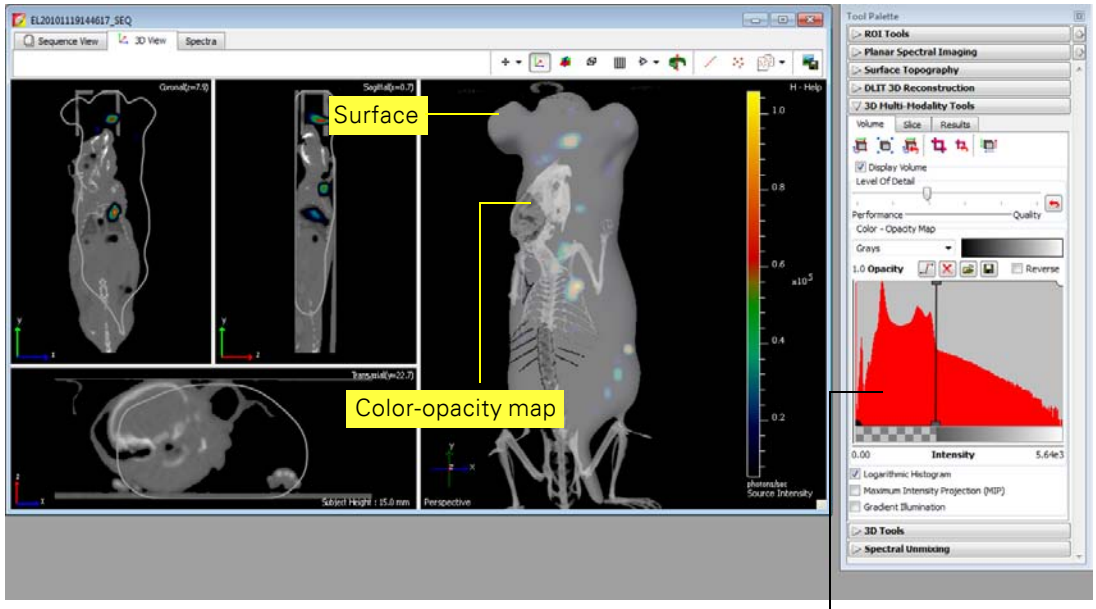


Table 15.2 3D Volumetric Data Browser

Item	Description
Add to List	If this option is chosen, the data selected in the Browse for Folder box is added to the 3D Volumetric Data Browser. If this option is not chosen, the data selected in the Browse for Folder box replaces the contents of the 3D Volumetric Data Browser, except for loaded data.
Browse	Opens the Browse For Folder box.
Load in a new window	If this option is chosen, multiple data sets can be loaded, each in a separate window. If this option is not chosen, only one data set can loaded at a time.
Load	Click to open the data selected in the 3D Volumetric Data Browser.

Figure 15.4 3D optical and 3D volumetric data loaded, but not registered



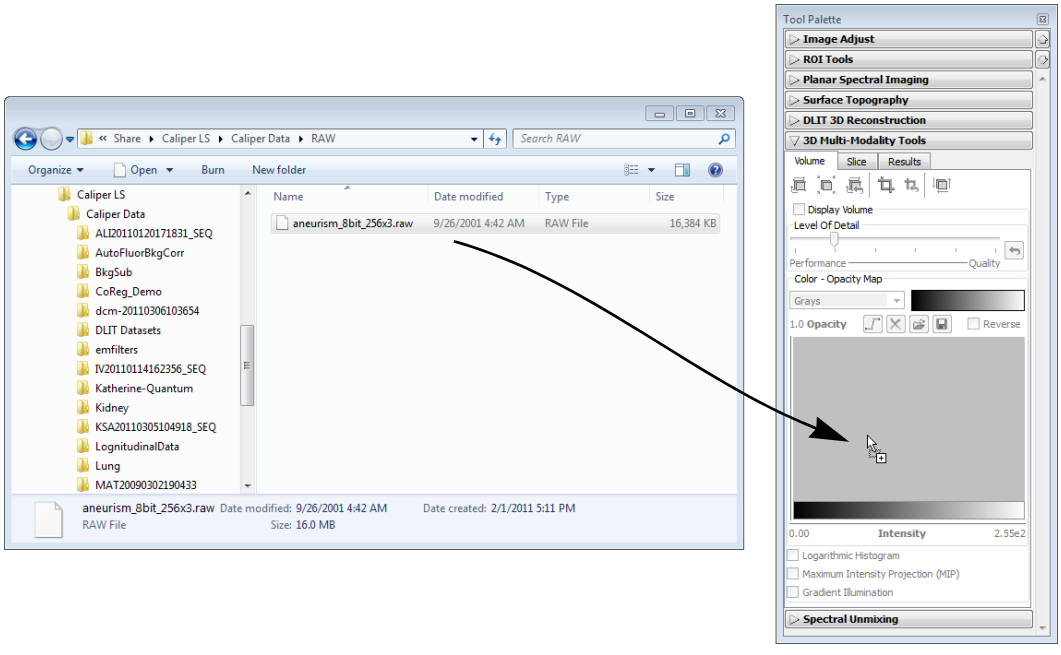
Loading RAW Volumetric Data

1. Drag a single RAW file (*.raw or *.vox) from Windows Explorer to the 3D Multi-Modality tools (Figure 15.5).

NOTE

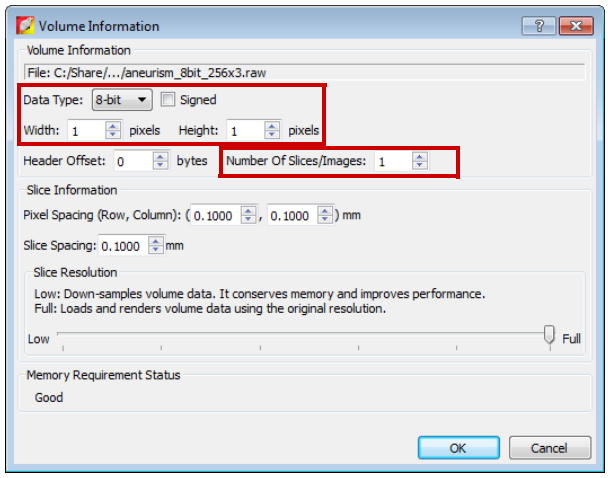
Only single *.raw or *.vox files consisting of multiple slices of a 3D volume can be loaded into Living Image.

Figure 15.5 Opening RAW volumetric data



2. In the Volume Information dialog box that appears (Figure 15.6), enter the:
   - Data width, height, and the number of slices.
   - Slice row, column pixel size, and the slice spacing in millimeters.

Figure 15.6 Volume information



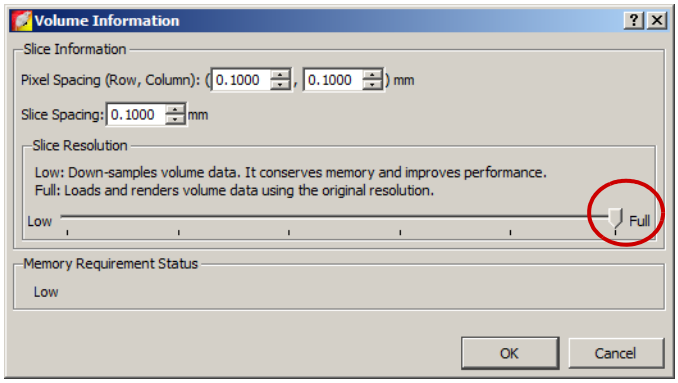
Select a data type.

Enter the:

- Width, height, and number of slices
- Slice row, column pixel spacing, and the slice spacing in millimeters

3. If loading the data will cause low memory, you are prompted to down-sample the data (Figure 15.7). Decrease the slice resolution by moving the Slice Resolution slider to the left until the Memory Requirement Status is "Good".

Figure 15.7 Down-sample 3D volumetric data to improve memory and performance



Changing the Orientation of RAW Volumetric Data

Occasionally, RAW files (*.raw or *.vox) may be loaded with the orientation "flipped" or reversed along the x, y, or z-axis. As a result, the slice views (transaxial, coronal, sagittal) may be flipped or rotated so that the actual view that is displayed does not match the 3D View windowpane name (for example, the Sagittal windowpane does not display a sagittal slice), or the data appears flipped with respect to the surface derived from the IVIS® Spectrum.

In such cases, you can:

- Invert the data along the x, y, or z-axis
- Manually rotate the data using the Transformation tool (for more details, see page 249).

To invert the subject orientation:

1. Click the Edit Spacing & Orientation button .
2. In the dialog box that appears, choose a "Subject Orientation" option and click **OK**.

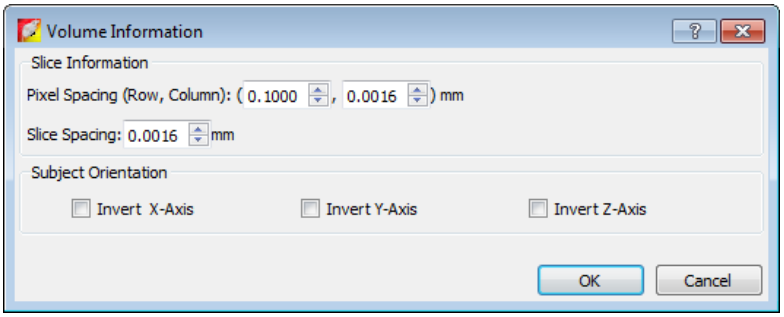


Figure 15.8 Volume Information dialog box

15.3 Registering Multi-Modal Data

Two multi-modal registration methods are available:

- Automatic fiducial registration – For experiments in which the Mouse Imaging Shuttle was used to transfer the animal between the IVIS® Spectrum and the Quantum FX µCT instrument. The Living Image® software automatically registers the optical surface reconstruction with 3D volumetric data acquired on the Quantum FX µCT instrument.
- Manual registration—Use the 3D Multi-Modality tools to register 3D volumetric data with a 3D surface reconstruction.

Automatic Fiducial Registration

Automatic fiducial registration is available for CT data acquired on the Quantum FX µCT instrument. The subject must be contained in the Mouse Imaging Shuttle during both optical and CT imaging, and the CT data must be exported to DICOM format.

After registration, classify the 3D volumetric data to help you identify and separate objects (see page 250).

About the Mouse Imaging Shuttle

The Mouse Imaging Shuttle (Caliper part no. 127744) contains the subject during imaging and enables the subject to be transferred between an IVIS® Imaging System and the Quantum FX µCT instrument without disrupting the subject's position.

The Mouse Imaging Shuttle must be correctly docked to the docking station in the IVIS Imaging System and the Quantum FX µCT instrument. The docking station in the Quantum FX µCT system is marked with a triangle-shaped fiducial pattern under the plane where the Mouse Imaging Shuttle docks. Automatic fiducial registration is available if both sides of the triangle fiducial pattern are included in the CT images. For more details on using the Mouse Imaging Shuttle, see the *Mouse Imaging Shuttle Instructions* (Caliper part no. 127820_RevA).

To perform automatic fiducial registration:

1. Load the data that you want to register (see page 241).
2. Click the Fiducial Registration button .

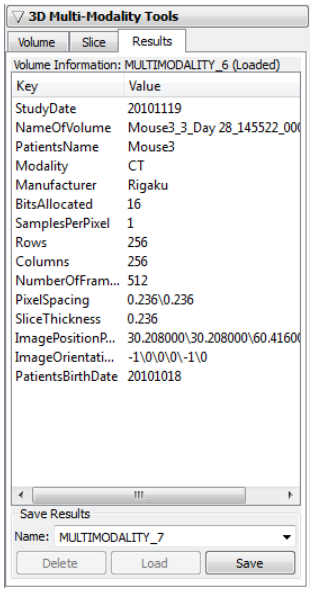
   The multi-modal data are automatically registered and cropped (Figure 15.9).
3. To undo the registration, click the Reset Registration button .

Figure 15.9 Registered 3D optical and 3D volumetric data

To save the registration information:

1. In the Results tab, confirm the default name or enter a name for the results.
2. Click **Save**.

Figure 15.10 3D Multi-Modality Tools, Results



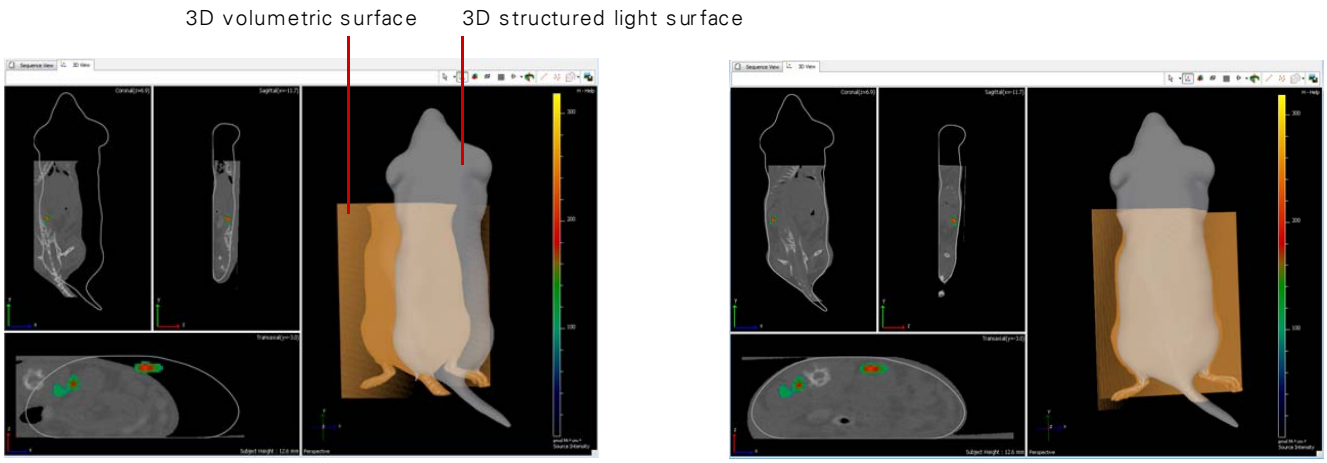
NOTE

Registration information is saved with the results for the volumetric data and is specific for a particular optical data set.

Manual Registration

To manually register data, use the 3D Multi-Modality tools to translate, scale, or rotate the 3D volumetric surface so that features common to both surfaces are matched and aligned in the x, y, and z planes. Examine the matched surfaces in the 3D slice views to help you fine tune the registration.

Figure 15.11 Example surfaces before and after registration



Surfaces before registration

Registered surfaces

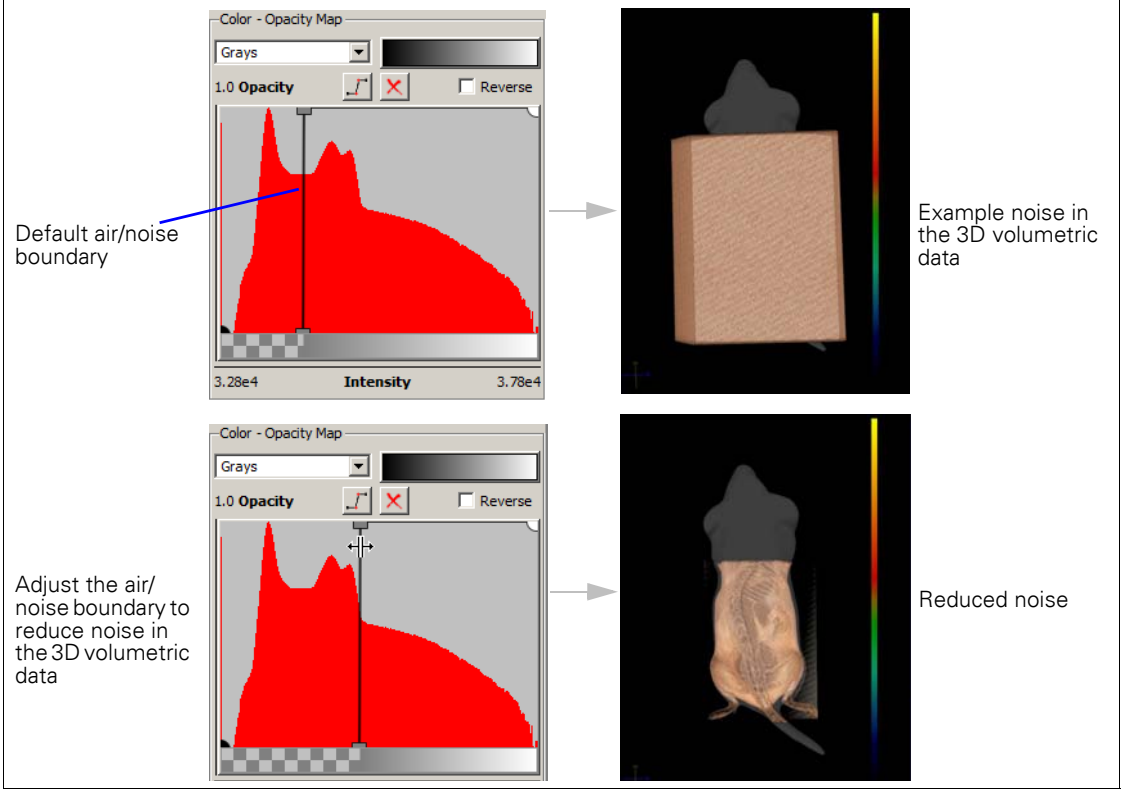
To manually register data:

1. Load the data that you want to register (for more details, see page 241).

   The software determines a default air/noise boundary for the 3D volumetric data (Figure 15.12).

2. If you need to remove noise from the 3D volumetric data, move the air/noise boundary to the right in the histogram tool.

Figure 15.12 Adjusting the air/noise boundary in the histogram tool



3. If the volumetric data needs cropping (for example, to remove structures such as the stage from the CT view), follow step a to step c below. If cropping is not needed, proceed to step 4.

   To crop the data:

   a. Click the crop tool button .

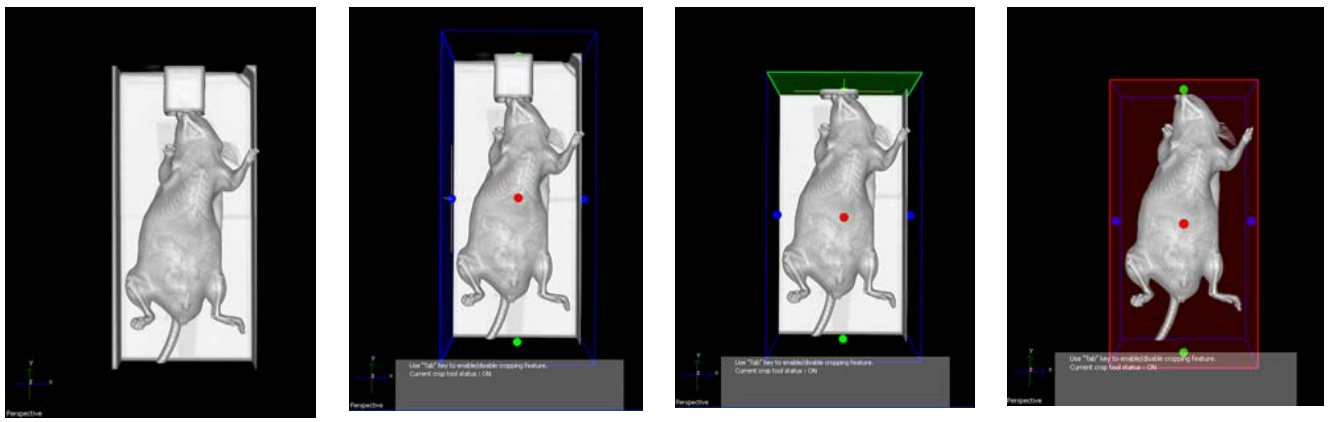
      The crop tool appears and has six control points:

      —Crops the data along the x-axis

      —Crops data along the y-axis

      —Crops data along the z-axis

Figure 15.13 Crop data along the x, y, or z-axis



No crop tool | X-axis crop tool | Y-axis crop tool | Z-axis crop tool

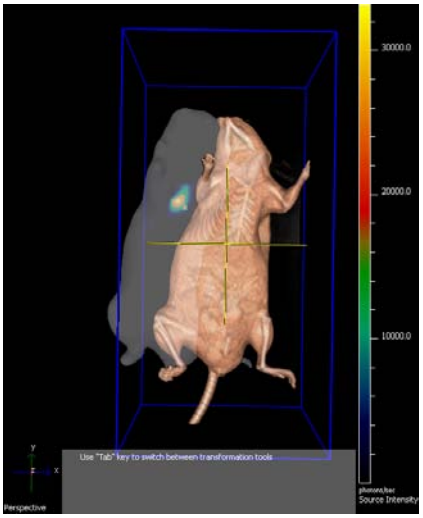
b. Click and hold a control point while you move the crop plane. As you move the crop plane, the slice views are updated. Release the mouse button to crop the data.

c. To reset the crop planes, click the button. When finished cropping, press the Tab key to turn off the crop tool.

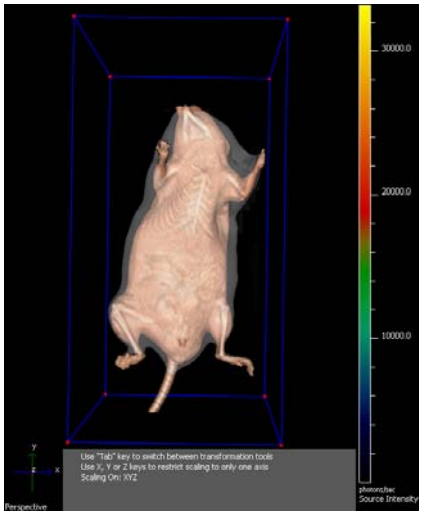
4. Click the Manual Registration button .

The transformation tool appears (Figure 15.14). The tool has three modes that enable you to translate, scale, or rotate the 3D volumetric data (press the Tab key to change the tool mode). The slice views are automatically updated when you use the tool.

Figure 15.14 Manual registration tool: transformation modes



Translate—Moves the volume in the x, y, or z-axis. Drag the tool to adjust the position of the volume.



Scale—Increases or decreases (scale the size of the volume, drag a red cube at a corner of the volume. To restrict scaling to a particular axis, press the X, Y, or Z key, then drag a red cube.

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

NOTE

Make sure that you click the transformation tool so that it is highlighted before you use it. Otherwise the dragging operation is applied to the optical data (structured light surface).

5. To return the 3D volumetric data to the default position and size, click the Reset Registration button .

6. For details on saving the registration information, see page 246.

NOTE

Registration information is saved with the results for the volumetric data and is specific for a particular optical data set.

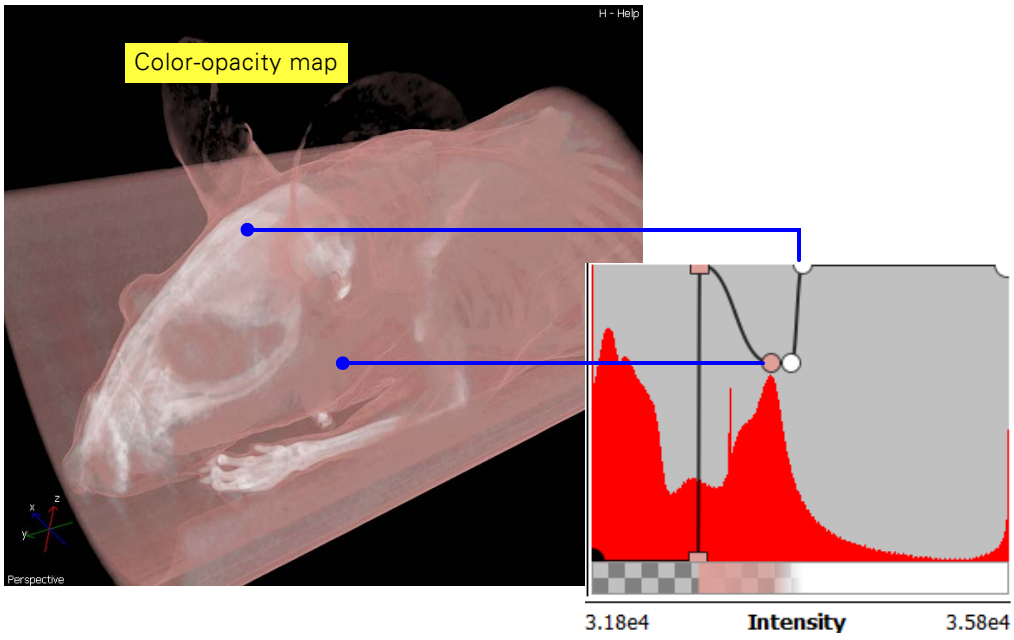
15.4 Classifying 3D Volumetric Data

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

For example, Figure 15.15 shows how the histogram tool designed a color-opacity map that shows both the skin and bone. The histogram tool enables you to easily re-design the color-opacity map to show just the skin or just the bone.

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

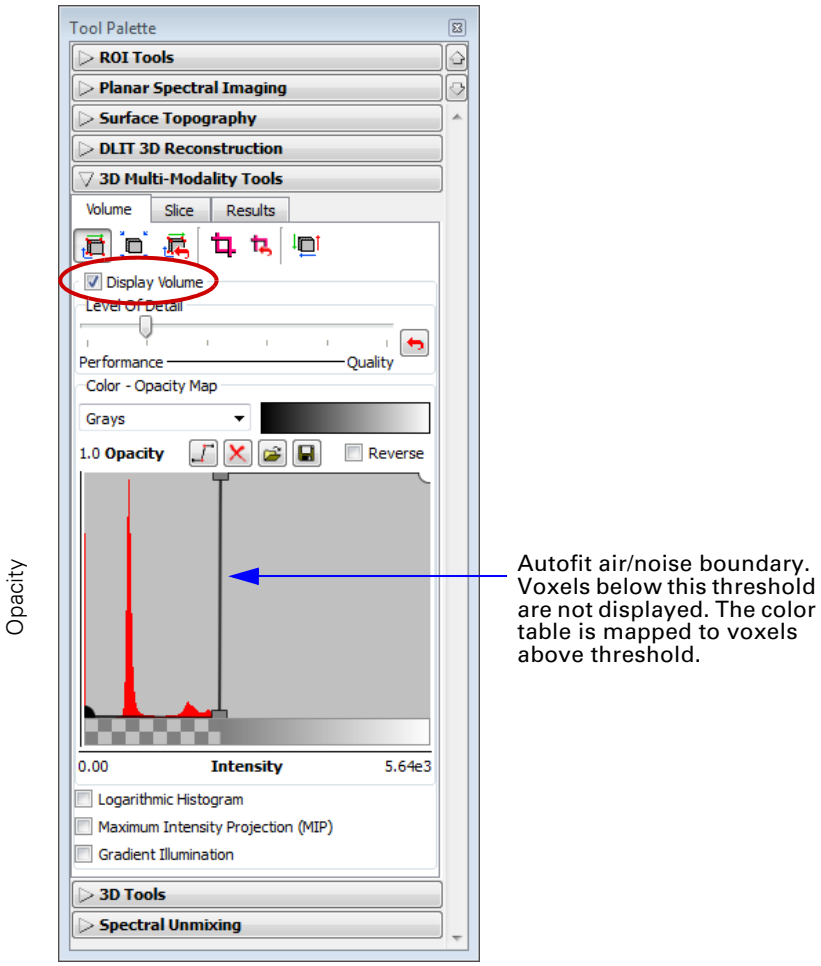
Figure 15.15 Histogram tool specifies the opacity for different voxel intensities



Specifying a Color-Opacity Map

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

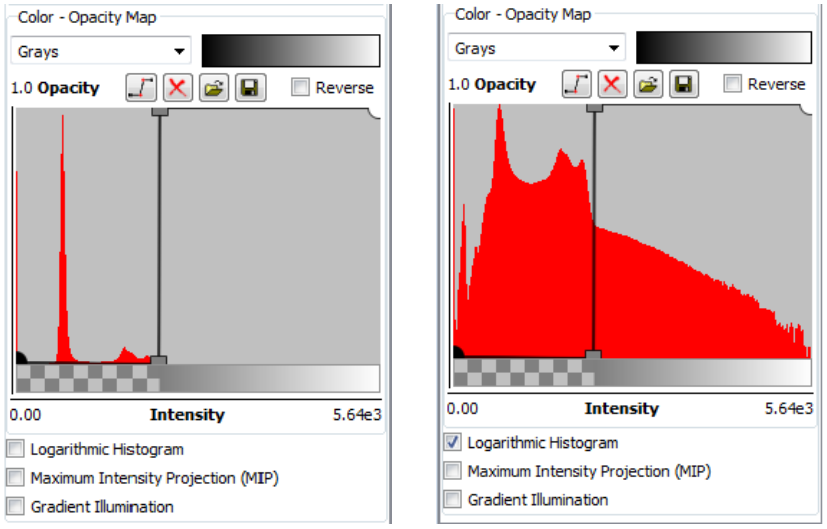
Figure 15.16 3D Multi-Modality tools



2. To change the color table for the color-opacity map, make a selection from the Color table-Opacity Map drop-down list. To apply the reverse color table, select the Reverse option.
3. If the histogram intensity range appears narrow or suppressed, choose the Logarithmic Histogram option.

   Choosing this option enhances the histogram display by magnifying the smaller regions of interest in the histogram while keeping noise and air-related intensity peaks high. It helps bring out hidden regions visible in the histogram for easier identification of interesting intensity ranges.

Figure 15.17 Example intensity histograms



Linear intensity scale

Logarithmic intensity scale

Managing Control Points

1. Place a control point on the histogram by clicking anywhere on the histogram between the ● point (represents the lowest intensity in the volume) and ○ point (represents the highest intensity in the volume).

   During volume rendering, the color-opacity maps are used to map color and opacity to the corresponding intensity value as well as interpolate color and opacity for all data between adjacent control points.

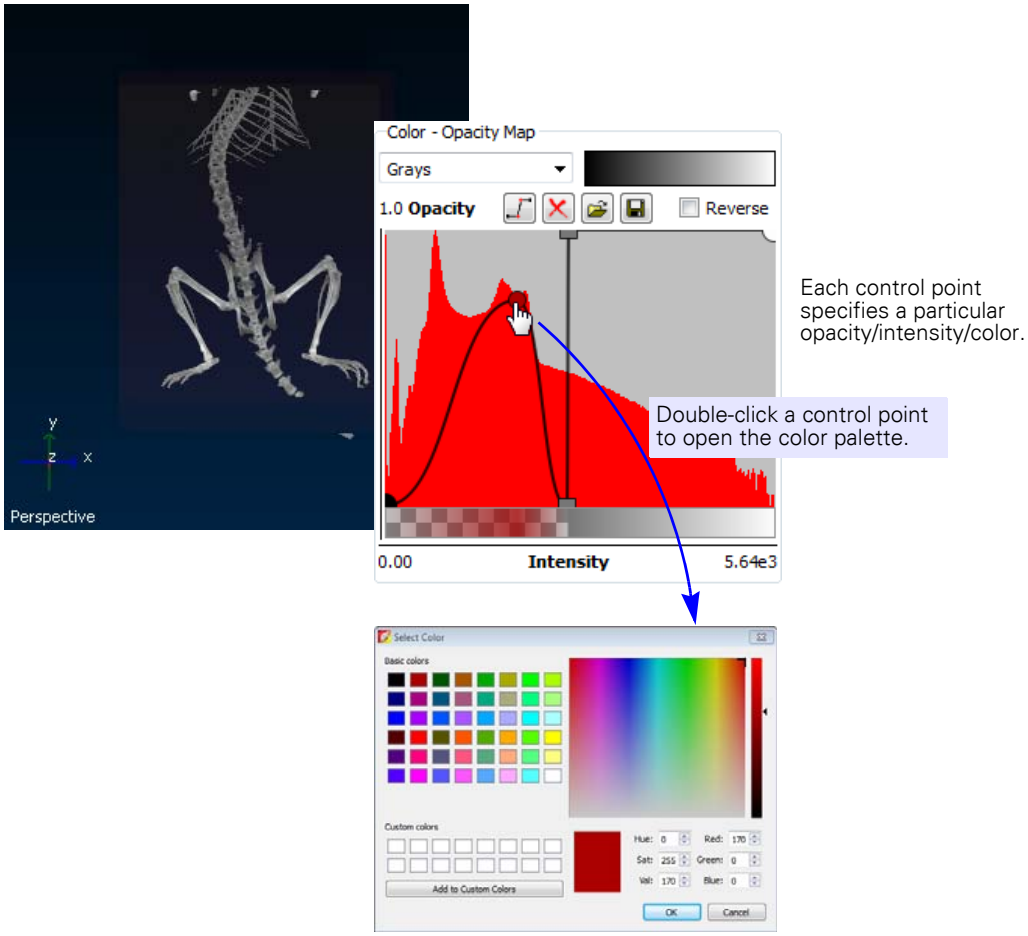
2. Drag any control point up or down to set the opacity level that is associated with the intensity value represented by the point. Drag a user-added control point left or right to change the intensity associated with the opacity specified by the point.

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

NOTE

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

Figure 15.18 Histogram tool



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

NOTE

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

Saving a Color-Opacity Map

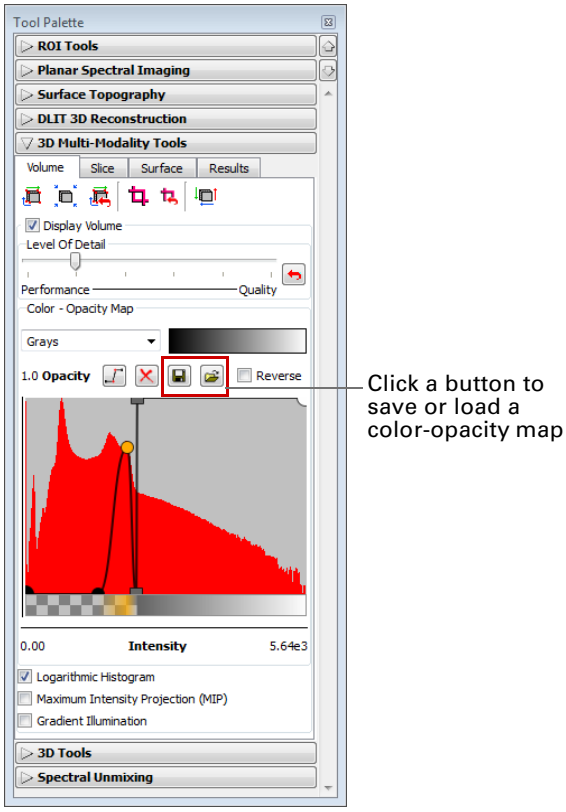
A color-opacity map can be saved and applied to any volumetric data set.

1. Click the Save button (Figure 15.19).
2. In the dialog box that appears, select a folder for the file (.tfn) and enter a file name.
3. Click **Save**.

Loading a Color-Opacity Map

1. Click the Open button (Figure 15.19).
2. In the dialog box that appears, navigate to the map file (.tfn), and click **Open**.

Figure 15.19 Save or load a color-opacity map



15.5 Volume Display Options

Adjusting the Image Quality

By default, the color-opacity map displays the volumetric data at original (1x) resolution. This means, for example, if the volume comprises 512 slices, then all of the 512 slices are displayed. You can increase or decrease the resolution of the data display from 0.5x to 3.0x resolution (for examples, see Table 15.3).

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

To adjust the image resolution:

1. Move the Level of Detail Slider to the left or right.

   The color-opacity map is updated.

2. To return the resolution to 1x, click the Reset button .

Figure 15.20 Level of Detail slider

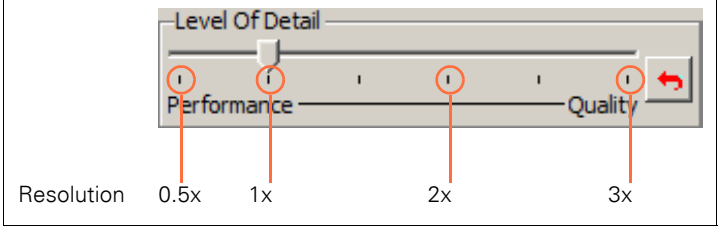


Table 15.3 Example volume with 512 slices at 1x resolution

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

Maximum Intensity Projection (MIP)

MIP projects all maximum intensity voxels in the view along the viewing direction into the viewing plane.

Gradient Illumination

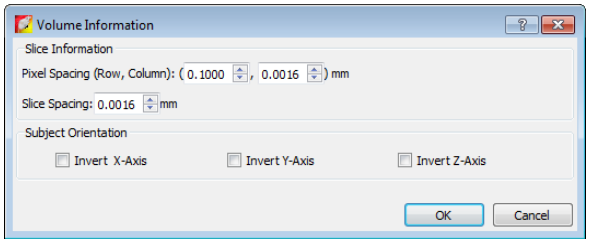
Gradient Illumination is based on the idea that light is reflected at boundaries between different voxel intensities, but is not affected when passing through homogeneous regions. Choosing this option illuminates the voxels at boundaries more than voxels within a homogeneous region. The boundaries are based on the gradient magnitude between heterogeneous regions or the change in intensities between neighboring voxels in heterogeneous regions. Choosing this option enhances the variation in tissue properties and may be helpful for visualizing the boundaries of different tissues.

Editing Volume Slices

You can modify the pixel and slice spacing of the volume. Changing the pixel or slice spacing modifies the volume resolution.

1. In the Volume tab, click the Edit Space and Orientation button .
2. In the dialog box that appears (Figure 15.21), edit the pixel or slice spacing.

Figure 15.21 Volume Information dialog box



15.6 Viewing a Slice

Slice Tab

In the 3D Multi-Modality tools, the Slice tab contains the color table options for the slice views.

Figure 15.22 Perspective view and slice views displayed using different color tables

1. To select a color table for the slice views, choose the Slice Color Table option, then make a selection from the Color Table drop-down list (Figure 15.22). To apply the reverse of the selected color table, choose the Reverse option.

NOTE

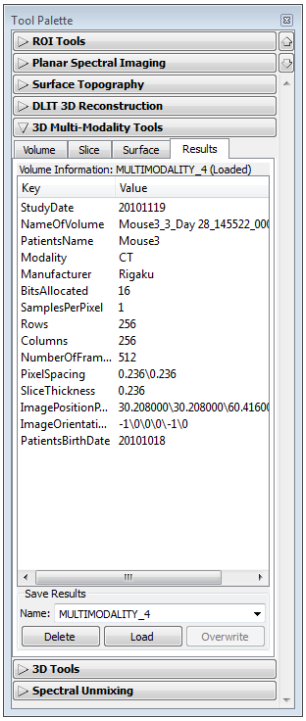
Black areas that appear around the optical sources in the overlay with the CT slices are due to the black color level at the low end of the color palette. To correct this, go to Sources tab in the 3D Tools, and move the colorbar low end slider up from the black level.

2. To apply the same color table as the color-opacity map, choose the Volume Color Table option. The volume color table is selected in the Volume tab.

15.7 Volume Information and Results

The Results tab displays information about the loaded data taken from the DICOM file header (Figure 15.23).

Figure 15.23 Volume information



You can save the registered and classified data. This provides a convenient way to share data. The software saves the following:

- Level of detail setting
- Color tables for the opacity map and slices
- Histogram tool control settings and the resulting color-opacity map
- Multi-modal registration settings
- Crop settings

Managing Results

To save registered results:

1. In the Results tab, confirm the default name in the Name drop-down list or enter a name.
2. Click **Save**.

   The registered 3D volumetric data, along with the color-opacity settings, appear in the 3D View window.

NOTE

The results are saved in XML format in the optical data set location. The results can only be accessed from the same optical data set.

To load results:

1. Select the results from the Name drop-down list.
2. Click **Load**.

To delete results:

1. Select the results from the Name drop-down list.
2. Click **Delete**.
3. Click **Yes** in the confirmation message that appears.

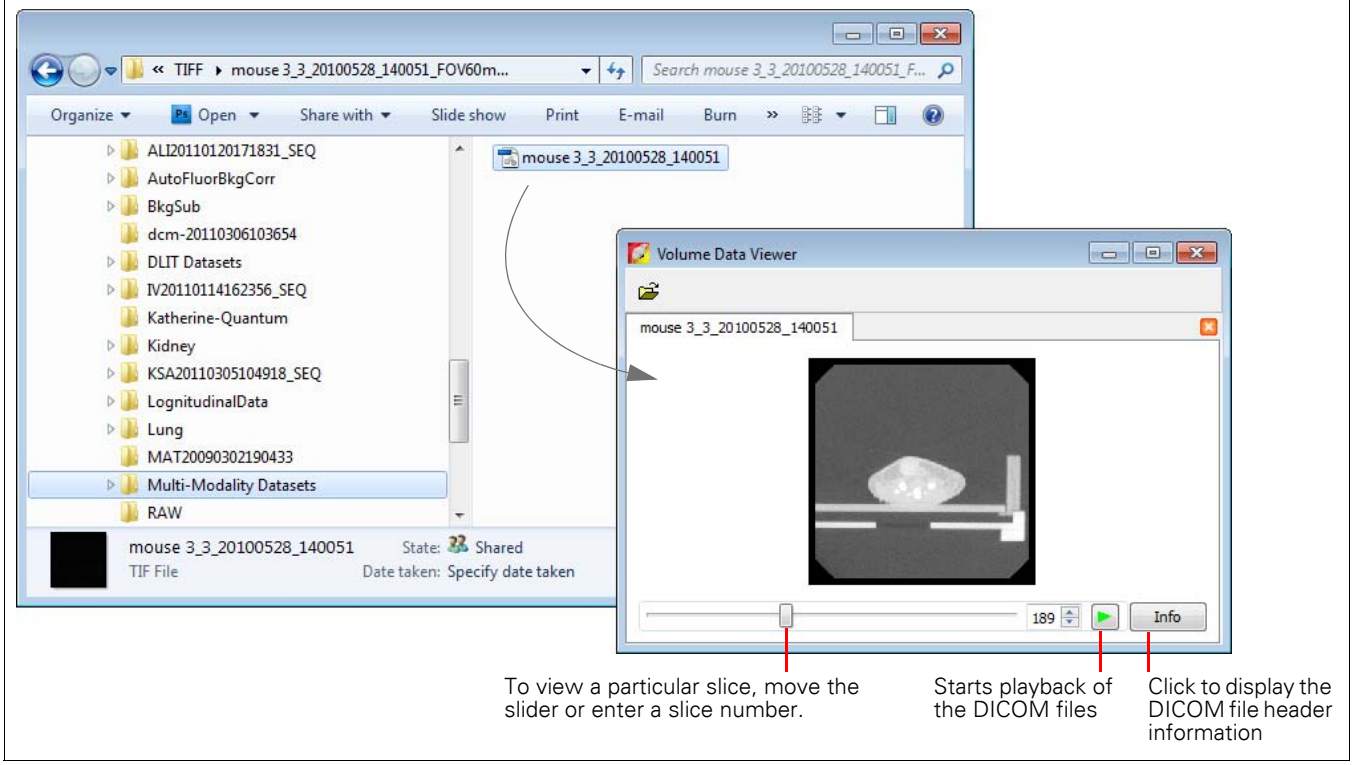
15.8 Volume Data Viewer

The Living Image software provides a viewer for volumetric data. The 3D Multi-Modality tools are not required to view DICOM or TIFF data.

1. Select **View** → **Volume Data Viewer** on the menu bar.
   The Volume Data Viewer appears.
2. Select volume data by doing either of the following:
   - Drag the data file (DICOM, TIFF) from Windows Explorer to the Volume Data Viewer window

     or
   - In the Volume Data Viewer, click the Open button, and in the dialog box that appears, select a DICOM or TIFF file, and click **Open**.
3. To clear the Volume Data Viewer, click the button.

Figure 15.24 Drag volume data from Windows Explorer to the Volume Data Viewer

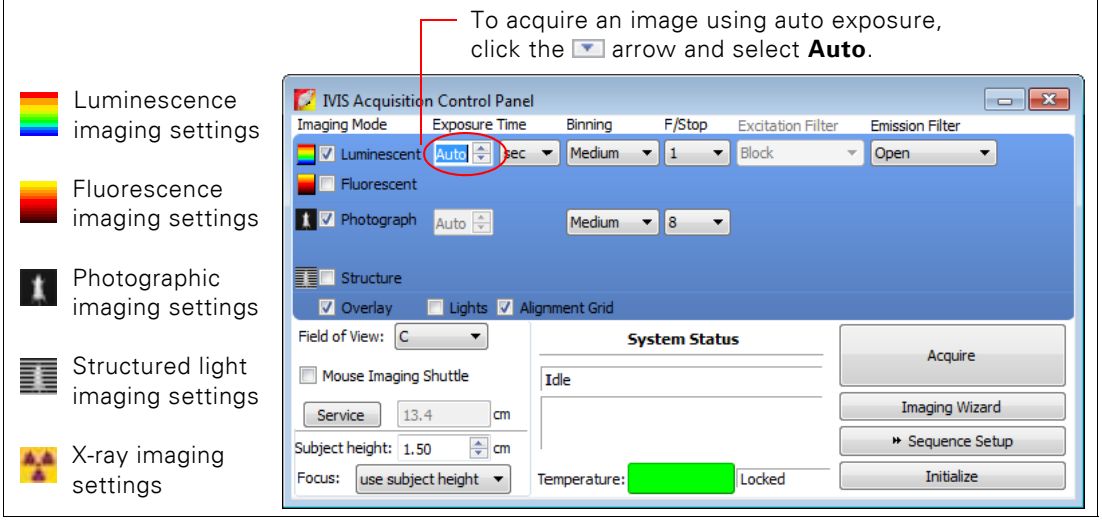


Appendix A IVIS Acquisition Control Panel

A.1 Control Panel

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

Figure A.1 IVIS acquisition control panel, auto exposure selected



NOTE

The options available in the IVIS acquisition control panel depend on the selected imaging mode, the imaging system, and the filter wheel or lens option that are installed.

Table A.1 IVIS acquisition control panel

Item	Description
Luminescent	Choose this option to acquire a luminescent image.
Fluorescent	Choose this option to acquire a fluorescent image. If the Fluorescent option is selected on the IVIS Spectrum Imaging System, the following options also appear in the control panel: Transillumination - Choose this option to acquire a fluorescent image using transillumination (excitation light located below the stage). Normalized - This option is selected by default when the Fluorescent and Transillumination options are chosen so that NTF Efficiency images can be produced.

Table A.1 IVIS acquisition control panel (continued)

Item	Description
Exposure time	The length of time that the shutter is open during acquisition of an image. The luminescent or fluorescent signal level is directly proportional to the exposure time. The goal is to adjust the exposure time to produce a signal that is well above the noise (>600 counts recommended), but less than the CCD camera saturation of ~60,000 counts. Luminescent exposure time is measured in seconds or minutes. The minimum calibrated exposure time is 0.5 seconds. The exposure time for fluorescent images is limited to 60 seconds to prevent saturation of the CCD. There is no limit on the maximum exposure time for luminescent images; however, there is little benefit to exposure times greater than five minutes.The signal is linear with respect to exposure time over the range from 0.5 to 10 minutes. Integration times less than 0.5 seconds are not recommended due to the finite time required to open and close the lens shutter.
Binning	Controls the pixel size on the CCD camera. Increasing the binning increases the pixel size and the sensitivity, but reduces spatial resolution. Binning a luminescent image can significantly improve the signal-to-noise ratio. The loss of spatial resolution at high binning is often acceptable for *in vivo* images where light emission is diffuse. For more details on binning, see Appendix C, page 276. Recommended binning: 1-4 for imaging of cells or tissue sections, 4-8 for *in vivo* imaging of subjects, and 8-16 for *in vivo* imaging of subjects with very dim sources.
F/stop	Sets the size of the camera lens aperture.The aperture size controls the amount of light detected and the depth of field. A larger f/stop number corresponds to a smaller aperture size and results in lower sensitivity because less light is collected for the image. However, a smaller aperture usually results in better image sharpness and depth of field. A photographic image is taken with a small aperture (f/8 or f/16) to produce the sharpest image and a luminescent image is taken with a large aperture (f/1) to maximize sensitivity. For more details on f/stop, see Appendix C, page 275.
Excitation Filter	A drop-down list of fluorescence excitation filters. For fluorescent imaging, choose the appropriate filter for your application. For luminescent imaging, **Block** is selected by default. If you select **Open**, no filter is present. For systems equipped with spectral imaging capability, choose the appropriate emission filter for your application. **Note:** On some models with standard filter sets, the excitation filter selection automatically sets the emission filter.
Emission Filter	A drop-down list of fluorescence emission filters located in front of the CCD lens. The emission filter wheel is equipped with filters for fluorescence or spectral imaging applications. The number of filter positions (6 to 24) depends on the system. For luminescent imaging, the **Open** position (no filter) is automatically selected by default.
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 *Adjusting Image Appearance*, page 97).
X-ray	Choose this option to acquire an X-ray image.

Table A.1 IVIS acquisition control panel (continued)

Item	Description
Structure	Choose this option to take a structured light image (an image of parallel laser lines scanned across the subject) when you click **Acquire**. The structured light image is used to reconstruct the surface topography of the subject which is an input to the Diffuse Luminescence Imaging Tomography (DLIT™) algorithm that computes the 3D location and brightness of luminescent sources. When this option is chosen, the f/stop and exposure time are automatically set to defaults for the structured light image (f/8 and 0.2 sec, respectively). The spatial resolution of the computed surface depends on the line spacing of the structured light lines. The line spacing and binning are automatically set to the optimal values determined by the FOV (stage position) and are not user-modifiable.
Overlay	If this option is chosen, the system automatically displays the overlay after acquisition is completed (for example, luminescent image on photograph).
Lights	Turns on the lights located at the top of the imaging chamber.
Fluor Lamp Level	Sets the illumination intensity level of the excitation lamp used in fluorescent imaging (Off, Low, High, and Inspect). The Low setting is approximately 18% of the High setting. Inspect turns on the illumination lamp so that you can manually inspect the excitation lamp. **Note:** Make sure that the filters of interest are selected in the filter drop-down lists before you select Inspect. The Inspect operation automatically positions the selected filters in the system before turning on the lamp. Subsequent changes to the filter popup menus will have no effect until another Inspect operation is performed.
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. For more details on the calibrated FOV positions A-E, see Table A.3, page 263.
Service	Moves the stage to a position for cleaning the imaging chamber below the stage. Only available on the IVIS Imaging System 200 Series and IVIS Spectrum.
Mouse Imaging Shuttle	Choose this option if the subject will be contained in the Mouse Imaging Shuttle during image acquisition. **Note:** The Mouse Imaging Shuttle or the Ventral Imaging Chamber can be used with the IVIS Kinetic, but not both at the same time.
Load	Moves the stage from the cleaning position back to the home position.
XFOV-24	**Note:** This check box is only available on an IVIS® System that includes the XFO-24 lens option. When the XFO-24 lens is installed, choose the XFOV-24 option. For more details on how to install the XFO-24 lens, see the *XFOV-24 Lens Instructions*. **IMPORTANT** **ALERT!** If you remove the XFO-24 lens from the system, be sure to remove the check mark from the XFOV-24 check box.
VIC	Select this option when using the Ventral Imaging Chamber to acquire ventral kinetic images on the IVIS Kinetic Imaging System. **Note:** The Mouse Imaging Shuttle or the Ventral Imaging Chamber can be used with the IVIS Kinetic, but not both at the same time.

Table A.1 IVIS acquisition control panel (continued)

Item	Description
Zoom	Select this option to install and acquire images using the Zoom lens on the IVIS Lumina, IVIS Lumina XR, or IVIS Kinetic Imaging System. After the Zoom lens is installed, the stage automatically moves to the Z position, adjusted by the specified subject height. The Zoom lens is focused to this 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. **IMPORTANT** **ALERT!** The IVIS® System has a protection system to prevent instrument damage, however always pay close attention to subject height, particularly on the IVIS Imaging System 200 Series. For example, it is possible for a large subject (10 cm ventral-dorsal height) to contact the top of the imaging chamber if you set the subject height = 0 and choose a small FOV.
Focus	Drop-down list of focusing methods available: **Use subject height** - Choose this option to set the focal plane at the specified subject height. **Manual** - Choose this option to open the Focus Image window so that you can manually adjust the stage position. For more details on manual focusing, see page 264.
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). For more details, see page 39.
Temperature	The temperature box color indicates the temperature and status of the system: White box – System not initialized. Red box – System initialized, but the CCD temperature is out of range. Green box System is initialized and the CCD temperature is at or within acceptable range of the demand temperature and locked. The system is ready for imaging. Click the temperature box to display the actual and demand temperature of the CCD and stage. For more details, see page 18.
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.
Sequence Setup	Click to display the sequence table so that you can specify and manage sequence acquisition parameters, or open sequence acquisition parameters (xsq). For more details on setting up an image sequence, see page 33.
Image Setup	Click to close the sequence table.
Initialize	Click to initialize the IVIS Imaging System. For more details on initializing the system, see page 17.

Table A.2 Additional controls for the IVIS Imaging System 200 Series or IVIS Spectrum

Item	Description
Alignment grid	Choose this option to activate a laser-generated alignment grid on the stage when the imaging chamber door is opened. The alignment grid is set to the size of the selected FOV. The grid automatically turns off after two minutes. If subject alignment is not completed in two minutes, place a check mark next to **Enable Alignment Grid** to turn on the grid. **Note:** The horizontal cross hair of the alignment grid is offset appropriately to take into account the height entered in the Subject height box.
Focus	**Scan Mid Image** - Choose this option in the Focus drop-down list to set the focal plane at the maximum dorso-ventral height of the subject at the middle of the animal. This focusing method uses the laser to scan horizontally across the middle of the subject to determine the maximum subject height along this line. This option is well suited for animal imaging because the peak height is clearly identified as the maximum height on the dorsal side along the mid-plane of the animal. **Note:** This focusing method is not recommended for microplates or when using a high magnification field of view (FOV A = 4.0 cm). In these situations, Manual or Subject Size focus methods are recommended.
Transillumination Setup (IVIS Spectrum only)	Choose this option to display the transillumination setup window that enables you to select the locations for image acquisition using bottom illumination that originates beneath the stage.

Table A.3 Typical field of view (FOV) settings

FOV Setting	IVIS Imaging System					
	Lumina	Lumina XR	100 Series	200 Series	Spectrum	Kinetic
	FOV (cm)					
A	5	5	10	4	4	4
B	7.5	7.5	15	6.5	6.5	7
C	10	10	20	13	13	10
D	12.5	12.5[a]	25	22.5 (19.5)[c]	22.5 (19.5)[c]	12
E	24[b]	N/A	N/A	22.5 (26)[c]	22.5 (26)[c]	24[b]
Z[d]	2.6	2.6	N/A	N/A	N/A	2.6

[a] Position D is not available for X-ray imaging on the Lumina XR.

[b] Available with removable lens option XFOV-24. Not available on the IVIS Lumina XR.

[c] Some imaging systems may have the FOV in parentheses. FOV 19.5 and 26 were replaced by FOV 22.5.

[d] Position Z is available when the Zoom lens is installed on the IVIS Lumina, IVIS Lumina XR, or IVIS Kinetic Imaging System.

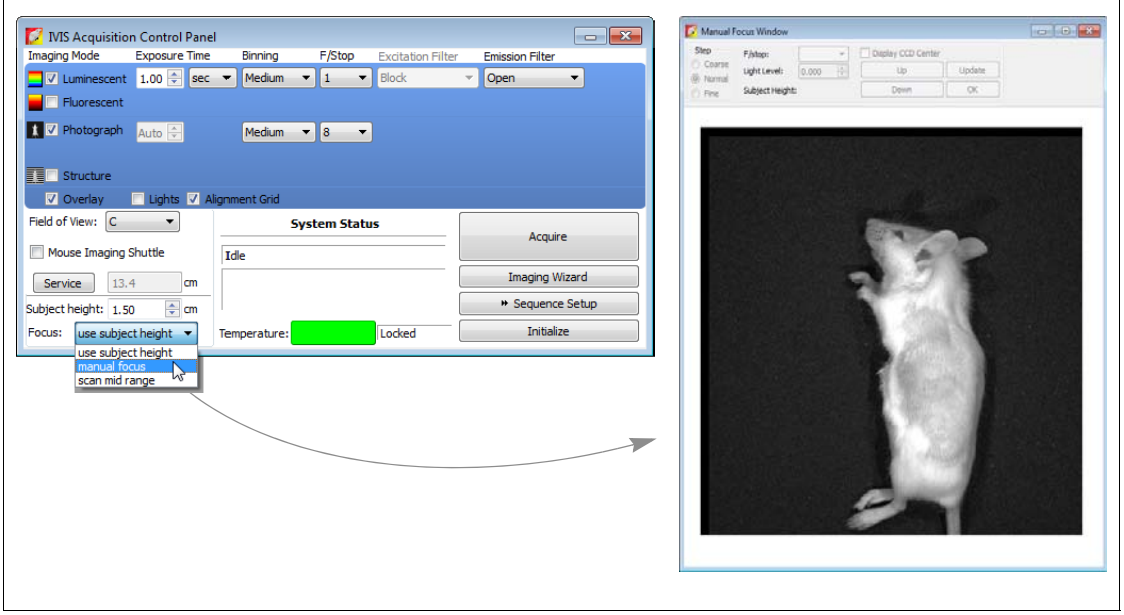
A.2 Manually Setting the Focus

The IVIS Imaging System automatically focuses the image based on subject height. If you do not want to use the automatic focus feature, you can manually set the focus.

1. In the control panel, choose **Manual Focus** in the Focus drop-down list.

   The Manual Focus window appears.

Figure A.2 Opening the Manual Focus window



2. To mark the center of the camera in the window, put a check mark next to Display CCD Center.
3. Select the size of the step increment that the stage moves: **Coarse**, **Normal**, or **Fine**.
4. Click **Up** or **Down** to move the stage and change the focus.
5. If necessary, select another F/stop setting from the drop-down list and adjust the light level using the arrows.
6. Click **Update** to apply the settings.

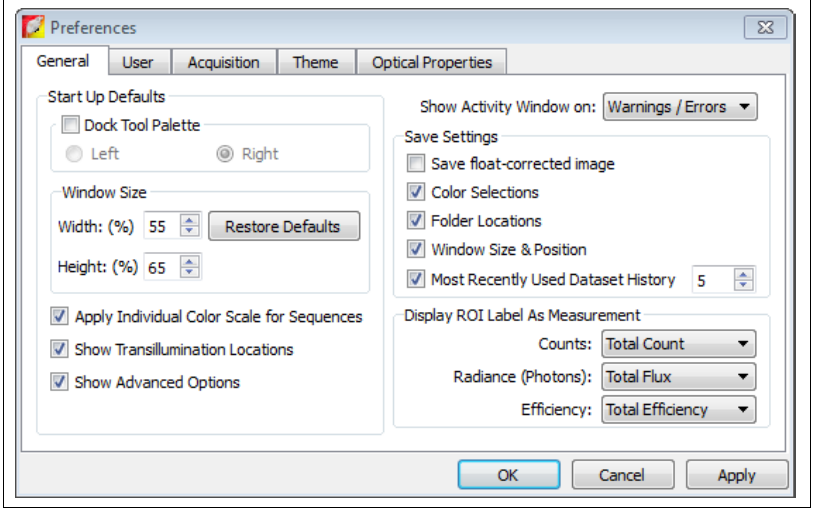
   The resulting focal plane (cm above the stage) is automatically entered in the Subject height box.
7. Click **OK** when the image is focused.

Appendix B Preferences

You can manage user IDs and specify defaults for some parameters that are associated with the user ID selected at the start of a new session.

To view the user-modifiable preferences after you log on, select **Edit** → **Preferences** on the menu bar.

Figure B.1 Preferences dialog box



NOTE

Any changes made to the Preferences are implemented at the start of the next session. The Acquisition tab is only available in the Living Image software that controls the IVIS Imaging System.

B.1 General Preferences

Figure B.2 General preferences

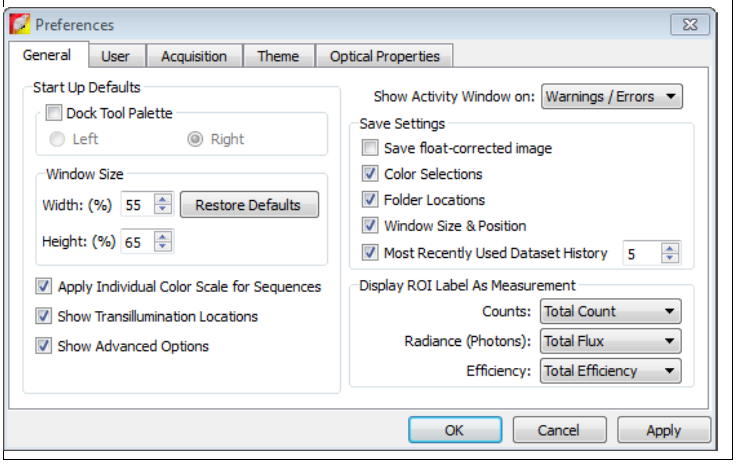


Table B.1 General preferences

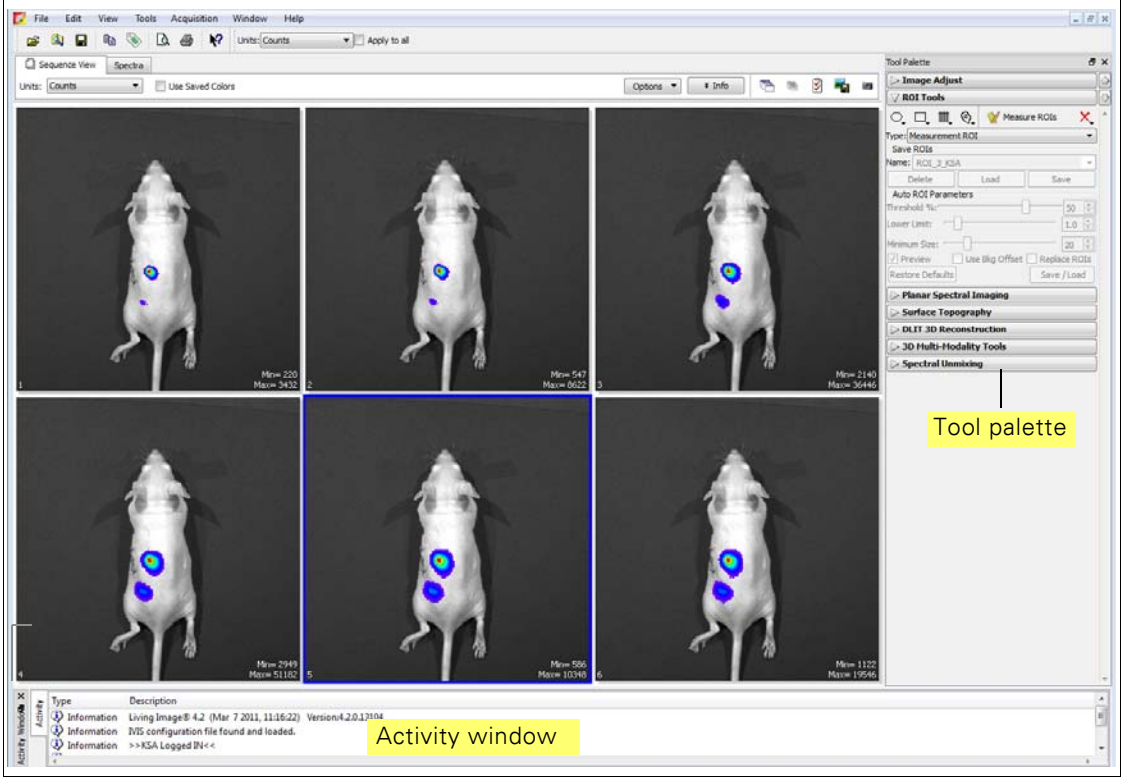
Item	Description
Start Up Defaults	Dock Tool Palette - Choose this option to set the position of the Tool Palette in the application window. Choose left or right. **Note:** To undock the Tool Palette, click on the palette title bar and drag it a distance greater than its width.
Window Size	Specifies the dimensions of the main application window. Width, Height - Sets the dimensions of the image window. Restore Defaults - Click to apply the default settings.
Apply Individual Color Scale for Sequences	Choose this option to apply a separate color scale to each thumbnail of a sequence. If this option is not chosen, all of the thumbnails are displayed using the same color scale.
Show Transillumination Locations	Choose this option to display a cross hair at each transillumination location when you load transillumination data. When you mouse over a cross hair, a tool tip displays the transillumination coordinates. If this option is not chosen, you can choose the Transillumination Location option in the sequence view window to display the transillumination locations.
Show Advanced Options	If this option is selected, advanced features are available in the menu bar and Tool Palette, including: • Additional ROI functionality for Auto ROI parameters • Additional export and import option for 3D surfaces and voxels
Show Activity Window on:	A drop-down list of options for when to display the activity log (Figure B.3).

Table B.1 General preferences (continued)

Item	Description
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 B.3). Window Size & Position - Applies the active image window size and position settings to subsequently opened image data. Most Recently Used Dataset History - Applies the active image window size and position settings to subsequently opened image data.
Display ROI Label As Measurement	Sets the type of measurement in counts, radiance (photons), or efficiency to show in the ROI label

Some of the general preferences specify how the main application window is organized. To undock the Tool Palette, click on the palette title bar and drag it a distance greater than its width. To dock the Tool Palette in the main window, drag the palette to the right or left side of the window and release.

Figure B.3 Main application window



B.2 User Preferences

Figure B.4 User preferences

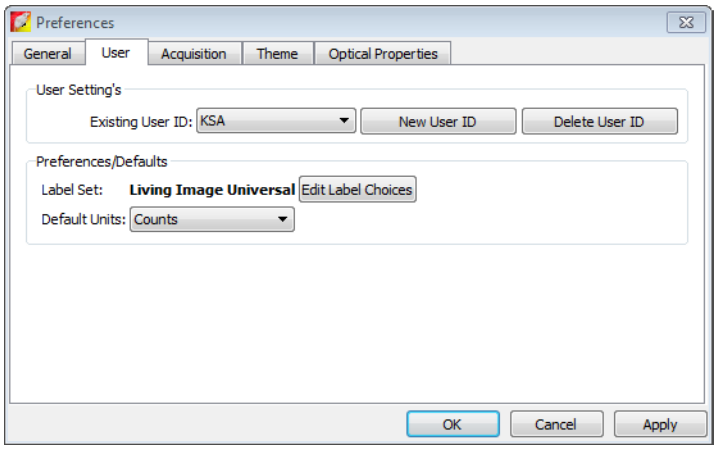


Table B.2 User preferences

Item	Description
User's Settings	Existing User ID - The user ID displayed in the log on dialog box at startup New User ID - Opens the Add New User box. A new user is added to the Existing User ID drop-down list. Delete User ID - Deletes the user selected from the Existing User ID drop-down list.
Preferences/Defaults	Edit User 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.

B.3 Acquisition

Figure B.5 Acquisition preferences, Auto Exposure

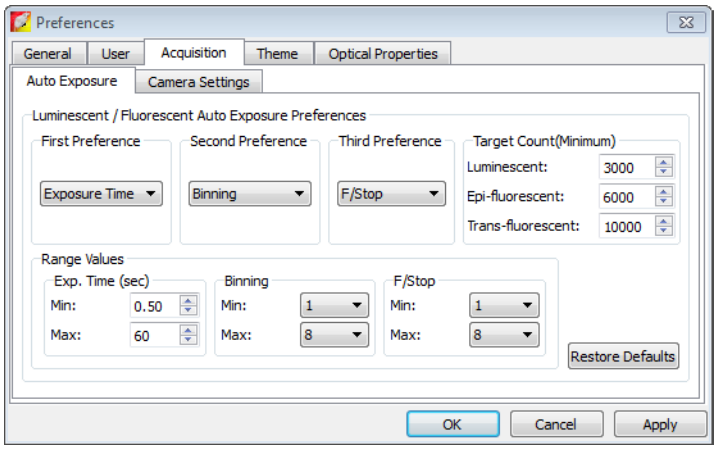


Table B.3 Auto exposure settings

Item	Description
Luminescent/Fluorescent Auto Exposure Preferences	
First Preference Second Preference Third Preference	During auto exposure, the software acquires a luminescent or fluorescent image so that the brightest pixel is approximately equal to the user-specified Target Count (Minimum). If the target minimum count cannot be closely approximated by adjusting the first preference (for example, exposure time), the software uses the first and second or first, second and third preferences to attempt to reach the target max count during image acquisition.
Target Count (Minimum)	A user-specified intensity.
Range Values Exp Time (sec) Binning F/Stop	The minimum and maximum values define the range of values for exposure time, F/Stop, or binning that the software can use to attempt to reach the target max count during image acquisition.
Restore Defaults	Click to apply default settings.

Figure B.6 Acquisition preferences, Camera Settings

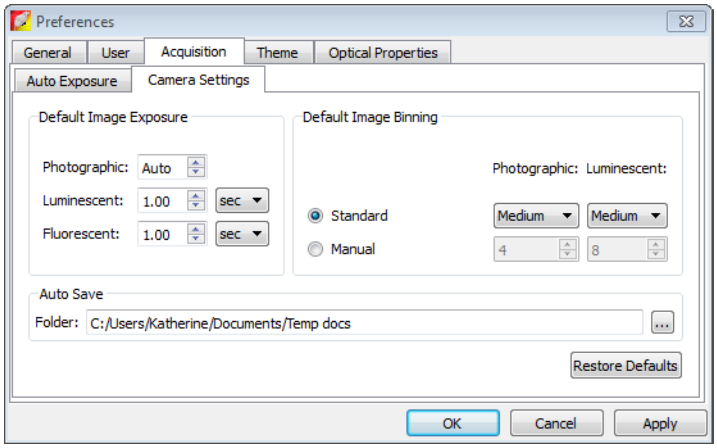


Table B.4 Camera settings

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,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.

B.4 Theme

Figure B.7 Image view preferences

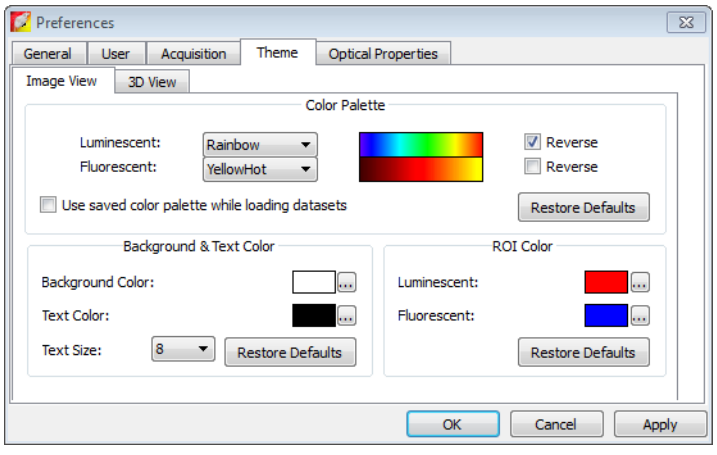


Table B.5 Image view preferences

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.

Table B.5 Image view preferences (continued)

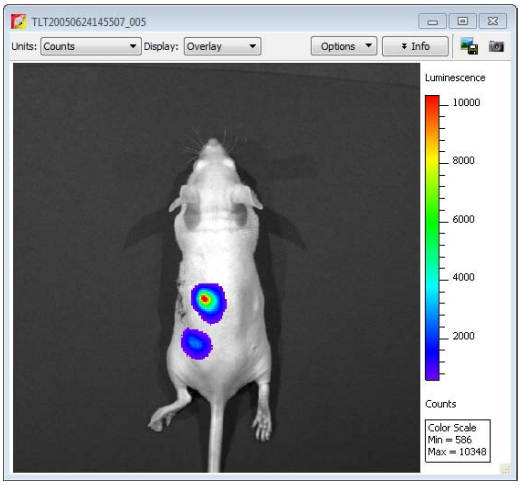
Item	Description
Use saved color palette while loading datasets	If this option is chosen, data are displayed using a user-specified color palette. For example, after you load data, specify a color table in the Image Adjust tools, and save the data. The user-specified color table is automatically applied whenever the data are loaded.
Background & Text Color	Sets the colors for the background and text in the image window shown here. To change a color, click the … button that opens the color palette. 
ROI Color	Sets the colors for the ROI outline. To change a color, click the … button that opens the color palette. Luminescent - Color of the ROI outline on a luminescent image. Fluorescent - Color of the ROI outline on a fluorescent image.
Restore Defaults	Click to apply the default settings.

Figure B.8 3D view preferences

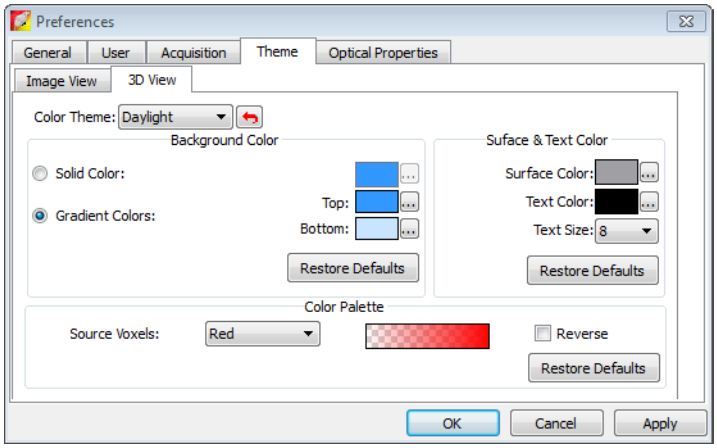
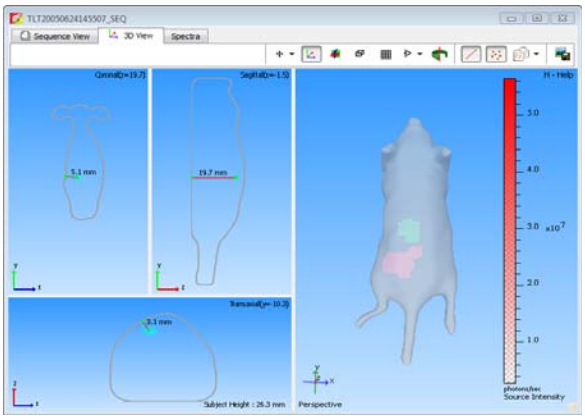


Table B.6 3D view preferences

Item	Description
Color Theme	Predefined color schemes available for the 3D View window shown here. Click the ↩ button to restore the defaults for the selected color theme. 
Background Color	Settings that modify the appearance of the background in the 3D View window. Solid Color - Choose this option to apply a non-gradient background color to the 3D view in the image window. Gradient Color - Choose this option to apply a gradient background color to the 3D view in the image window. Top = the color at the top of the window; Bottom = the color at the bottom of the window.
Surface & Text Color	Settings that modify the display of the surface and text in the 3D View window.
Color Palette	Source voxels - Choose a color table for voxel display. Reverse - Choose this option to reverse the min/max colors of the selected color table.
Restore Defaults	Click to apply the default settings.

B.5 Optical Properties

Figure B.9 Set the default optical properties preferences (left) for the Properties tab in the Planar Spectral Imaging, DLIT, or FLIT tools

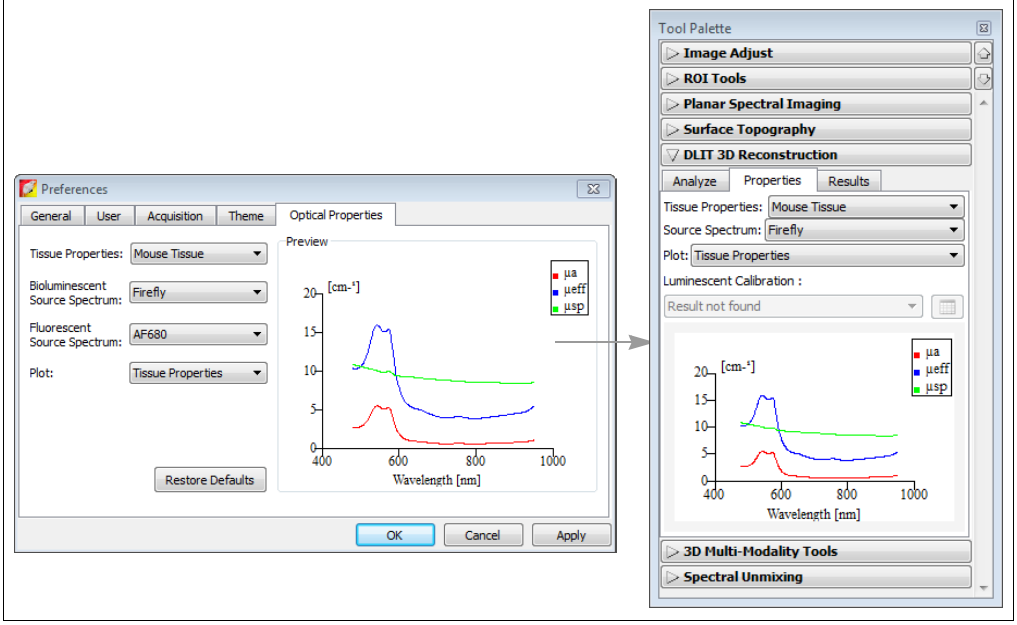


Table B.7 Tissue properties preferences

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.
Source Spectrum	Choose the default luminescent source. This Source Spectrum will be used if a Subject Type is not selected in the Imaging Wizard and saved during acquisition for DLIT sequences.
Plot	Tissue Properties - Choose this option to display a graph of the absorption coefficient ($_a$), effective attenuation coefficient ($_{eff}$), and reduced scattering coefficient ($'_s$ or sp). Source Spectrum - Choose this option to display the source spectrum for DLIT reconstructions. Bioluminescent Spectrum - Choose this option to display the spectrum of the bioluminescent source. Fluorescent Spectrum - Choose this option to display the spectrum of the fluorescent source.
Restore Defaults	Click to restore the defaults in the Optical Properties tab.

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Appendix C Detection Sensitivity

The parameters that control the number of photons collected (signal) and the image background (noise) determine the sensitivity of low light imaging. To maximize sensitivity, the goal is to increase signal and decrease background

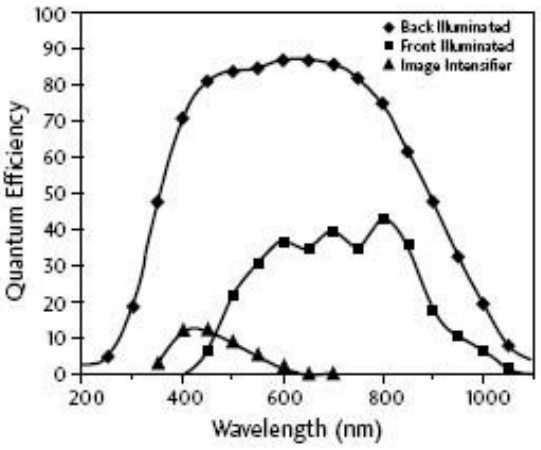
Several factors affect the number of photons collected, including the lens f/stop, image magnification, size and detection efficiency (quantum efficiency) of the CCD, transport efficiency of the imaging optics, and the image exposure time.

C.1 CCD Detection Efficiency

IVIS Imaging Systems use a back-thinned, back-illuminated CCD cooled to -90 to -105 C (depending on the system). This type of CCD provides high quantum efficiency of over 80% across the visible and near infrared part of the spectrum.

Figure C.1 shows detection efficiencies for several commonly used photon detectors. The back-illuminated CCD has the highest efficiency, particularly in the 600-800 nm region of the spectrum, the area of greatest interest for *in vivo* imaging.

Figure C.1 Quantum efficiencies



Bialkali photocathode (▲)
Back-illuminated CCD (◆)
Front-illuminated CCD (■).

IVIS systems use back-illuminated CCDs.

Lens Aperture

IVIS Imaging Systems are equipped with a high-light-collection f/1 lens. The sensitivity of the IVIS Imaging System can be adjusted by changing the f/stop setting that controls the lens aperture. The detected signal scales approximately as $1/(f/stop)^2$. For maximum sensitivity, select f/1, the largest aperture setting on the IVIS Imaging System (Figure C.2). This provides the greatest light collection efficiency, but results in the minimum depth of field for the image. The depth of field refers to the depth over which the image appears to be in focus and is determined by the f/stop and the field of view (FOV).

At f/1, the depth of field ranges from ~0.2 cm at FOV= 3.9 cm (IVIS Imaging System 200 Series only) to ~2 cm at FOV= 25 cm. You can use the manual focus option on the Control

panel to easily assess the depth of field at any f/stop and FOV setting. For more details on manual focusing, see page 264. Generally f/1 is recommended for low light luminescent images and f/2 or f/4 is recommended for brighter luminescent or fluorescent images.

Figure C.2 Lens f/stop positions.
Left: lens wide open at f/1; right: lens closed down at f/8.

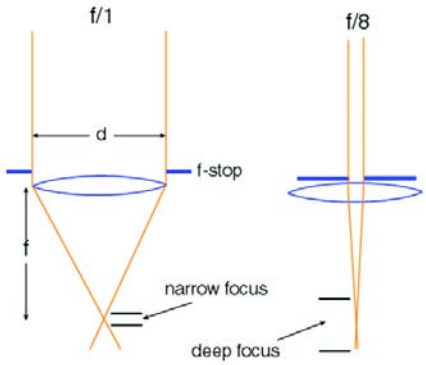


Image Exposure Time

The image exposure time also affects sensitivity. The number of photons collected is directly proportional to the image exposure time. For example, an image acquired over a two minute exposure contains twice as many detected photons as an image acquired over a one minute exposure. Longer exposure times are usually beneficial when imaging very dim samples. However, this may not always be true because some types of background, dark charge in particular, increase with exposure time. (For more details on backgrounds, see Appendix E, page 285.) An IVIS Imaging System has extremely low background that enables exposures of up to 30 minutes. However, animal anesthesia issues and luciferin kinetics limit practical exposure times for *in vivo* imaging to 5-10 minutes.

Field of View (FOV)

The FOV indirectly affects sensitivity. Changing the FOV without changing the binning or the f/stop does not significantly affect sensitivity. However, CCD pixels are effectively smaller at a smaller FOV (higher magnification) so that higher levels of binning can be applied without loss of spatial resolution.

For example, an image acquired at binning=4 and FOV=20 cm has the same spatial resolution as an image acquired at binning=8 and FOV=10 cm. Due to the increase in binning, the latter image has a four-fold increase in sensitivity compared to the former.

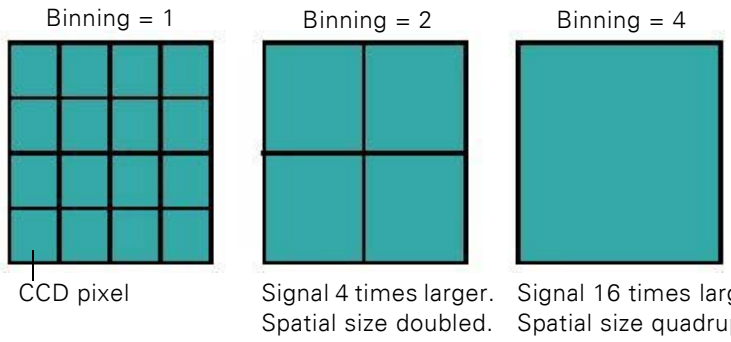
C.2 Binning

A charge coupled device (CCD) is a photosensitive detector constructed in a two-dimensional array of pixels. After an image is acquired, each pixel contains an electrical charge that is proportional to the amount of light that the pixel was exposed to. The software measures the electrical charge of each CCD pixel and assigns a numerical value (*counts*). (For more details on counts and other measurement units, see Appendix D, page 279.) The resulting image data comprise a two-dimensional array of numbers; each pixel contains the counts associated with the amount of light detected.

IVIS Imaging Systems are equipped with a CCD that ranges from 1024×1024 to 2048×2048 pixels in size, and thus have a high degree of spatial resolution. At binning=1,

each pixel is read and the image size (number of pixels) is equal to the physical number of CCD pixels (Figure C.3).

Figure C.3 A small segment of the CCD.
At binning = 2, 4 pixels are summed together; at binning = 4, 16 pixels are summed.



At binning=2, four pixels that comprise a 2×2 group on the CCD are summed prior to read out and the total number of counts for the group is recorded (Figure C.3). This produces a smaller image that contains one fourth the pixels compared to an image at binning=1. However, due to summing, the average signal in each pixel is four times higher than at binning=1. At binning=4, 16 pixels are summed prior to read out.

Binning significantly affects the sensitivity of the IVIS Imaging System. Binning at higher levels (for example, ≥ 4) improves the signal-to-noise ratio for read noise, an electronic noise introduced into the pixel measurement at readout. If four pixels are summed before readout, the average signal in the summed pixel (*super pixel*) is four times higher than at binning=1.

The read noise for the super pixel is about the same as it was for the individual pixels. Therefore, the signal-to-noise ratio for the read noise component of the image noise is improved by a factor of four. Read noise is often the dominant source of noise in *in vivo* images, so a high binning level is a very effective way to improve the signal-to-noise ratio.

Unfortunately, binning reduces the spatial resolution in an image. For example, at binning=2, a super pixel is twice as wide as a pixel at binning=1. This results in a factor of two loss in image spatial resolution. However, for *in vivo* imaging, the added sensitivity is usually more important than the spatial resolution. Further, since *in vivo* signals are often diffuse due to scattering in tissue, little is gained by increasing spatial resolution. (For more background on the propagation of light through tissue, see *Diffusion Model of Light Propagation Through Tissue*, page 310.) In such cases, high levels of binning may be appropriate (up to 10 or 16, depending on the CCD of the IVIS Imaging System). If signal levels are high enough that sensitivity is not an issue, then it is better to image at a lower binning level (two or four) in order to maintain a higher degree of spatial resolution.

NOTE

For application-specific questions regarding the appropriate binning level, please contact Caliper Corporation.

The IVIS System Control panel provides several binning options. The actual binning numbers associated with these settings depends on the CCD chip and type of image (Table C.1). These choices should satisfy most user needs. However, if you want to manually

control binning, you can specify **Manual Binning** in the Living Image Tools-Preference-Camera Settings box.

Table C.1 Binning settings

Binning	IVIS® System			
	100/200/Spectrum[1]	**Lumina**	**Lumina XR**	**Lumina Kinetic**
Small (high-resolution) Lumin	Bin 4	Bin 2	Bin 2	Bin 2
Medium Lumin[2]	Bin 8	Bin 4	Bin 4	Bin 4
Large (high-sensitivity) Lumin	Bin 16	Bin 8	Bin 8	Bin 8
Small (high-resolution) Photo	Bin 2	Bin 1	Bin 1	Bin 1
Medium Photo[2]	Bin 4	Bin 2	Bin 2	Bin 2

[1] Some early IVIS 100 Systems with Spectral Instruments SITe cameras, and all Roper and Princeton Instrument cameras, are not supported in Windows 7/Living Image 4.2 software.

[2] Default setting

You can also apply *soft binning* after an image is acquired. Conceptually, soft binning is the same as hardware binning— groups of pixels are summed and a smaller, lower resolution image is produced. However, in soft binning the summing is performed digitally on the stored image data, not on the electronic charge before readout as in hardware binning.

Although soft binning does not improve the signal-to-noise ratio for read noise, it may enhance the signal visibility because it reduces the statistical scatter of nearby pixel contents. Usually, hardware binning is preferred, but if it is not possible to take another image, applying soft binning to the data may provide a worthwhile solution.

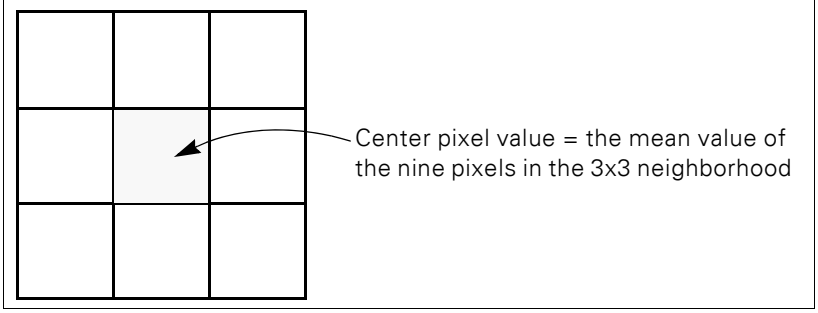
C.3 Smoothing

Smoothing is a filtering method that reduces noise in the image data. To apply smoothing, the software replaces the intensity of each pixel with the average intensity of a nearby pixel neighborhood that includes the pixel. Figure C.4 shows a 3x3 pixel neighborhood.

Smoothing does not change the pixel size and helps:

- Eliminate outlier pixel values that are extremely high or low.
- Reduce noise (fluctuations) in the image to help reveal small signals.

Figure C.4 3x3 pixel neighborhood



Appendix D Image Data Display & Measurement

D.1 Image Data

Scientific Image Data

Scientific image data is a two-dimensional array of numbers. Each element of the array (*pixel*) is associated with a number that is proportional to the light intensity on the element. A charge coupled device (CCD) camera used for scientific imaging is essentially an array of photo-sensitive pixels and each pixel collects photons during an image exposure.

The subsequent electronic readout provides a photon intensity number associated with each pixel. In a bright area of the image, more photons are detected and the photon intensity number is greater than the number in a dim area of the image.

The image data can be visualized in different ways, including pseudocolor images (generated by the Living Image software), contour plots, or isometric displays.

Graphic Image Data

A graphic image is a two-dimensional array of pixels with a color assigned to each pixel. There are several schemes for digitally storing the images. For example, a common scheme assigns a red-green-blue (RGB) color code to each pixel. The RGB code defines how much of each color to apply in order to create the final pixel color. Color photographs or color screenshots are examples of RGB images.

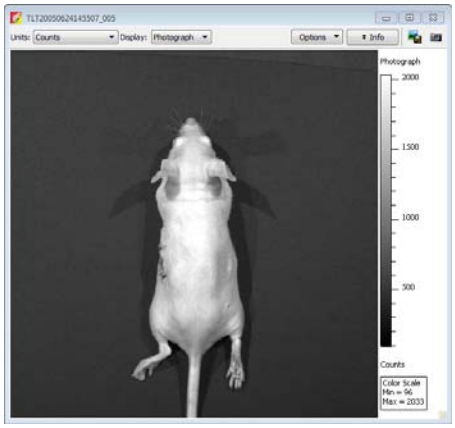
An RBG image is also a two-dimensional array of numbers, but unlike a scientific image, the numbers are only color codes and are not related to light intensity. A graphic image can be exported to a graphic display application.

Pseudocolor Images

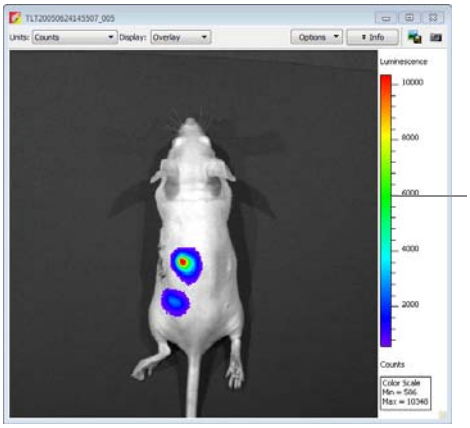
An image can be generated from scientific image data by assigning a color to each numerical value and plotting the array so that each pixel is filled with the color that corresponds to its numerical value. A *color table* defines the relationship between the numerical data and the displayed color. For example, a grayscale color table assigns black to the smallest number in the array, white to the largest number, and shades of gray to the values in between (Figure D.1). The resulting image is equivalent to a black and white photograph. An illuminated photographic image acquired on an IVIS Imaging System is an example of a grayscale pseudoimage.

The reverse rainbow color table is also commonly used and assigns violet to the smallest number on the array, red to the largest number, and all of the spectral colors of the rainbow to the values in between (Figure D.1).

Figure D.1 Example pseudoimages



A photograph is a gray scale pseudoimage



Overlay mode: pseudocolor image of the luminescent data is overlaid on a photograph

A pseudocolor scheme is typically used to display the numerical contents of scientific image data like the luminescent or fluorescent images acquired on an IVIS Imaging System. The pseudocolor scheme makes it easy to see areas of bright light emission. The amount of light emission can be quantified using measurement ROIs. (For more details, page 120.)

Measurement data is independent of the colors displayed in the pseudocolor image. You can change the appearance of the image data without affecting the underlying numeric pixel values. For example, you apply a different color table to the data or adjust the range of numeric values associated with the color table. Measurements that quantify pixel data produce the same results independent of the appearance of the pseudocolor display.

A pseudocolor image can be converted to an RGB color code and saved as an RGB image. The RGB image looks like a pseudocolor image, but does not include the numerical information derived from the light detected in each pixel. Therefore, the amount of light in an RGB image cannot be quantified.

Overlays

In the overlay display mode, the pseudocolor luminescent or fluorescent image is displayed over the associated grayscale photographic image (Figure D.1). Pixels in the luminescent or fluorescent image that are less than the minimum color table setting are not displayed. As a result, the lowest intensity color in the table is transparent and this enables you to view the underlying photographic image in regions where the luminescent light emission is low. While the pixels less than the minimum color table setting are not displayed, they still exist in the image data.

D.2 Quantifying Image Data

The Living Image software can quantify and display scientific image data for several types of measurements.

Table D.1 Data display units

Data Display	Description	Recommended For:
Counts	An uncalibrated measurement of the photons incident on the CCD camera.	Image acquisition to ensure that the camera settings are properly adjusted. Proper image parameter adjustment should avoid image saturation and ensure sufficient signal (greater than a few hundred counts at maximum).
Radiance (photons)	A calibrated measurement of the photon emission from the subject. Radiance is in units of "photons/second/cm²/steradian".	Luminescence measurements
Radiant Efficiency (fluorescence)	Epi-fluorescence - A fluorescence emission radiance per incident excitation power. Transillumination fluorescence - Fluorescence emission radiance per incident excitation power	Fluorescence measurements
Efficiency (epi-fluorescence)	Fluorescent emission normalized to the incident excitation intensity (radiance of the subject/illumination intensity)	Epi-fluorescence measurements
NTF Efficiency	Fluorescent emission image normalized to the transmission image which is measured with the same emission filter and open excitation filter.	Transillumination fluorescent measurements

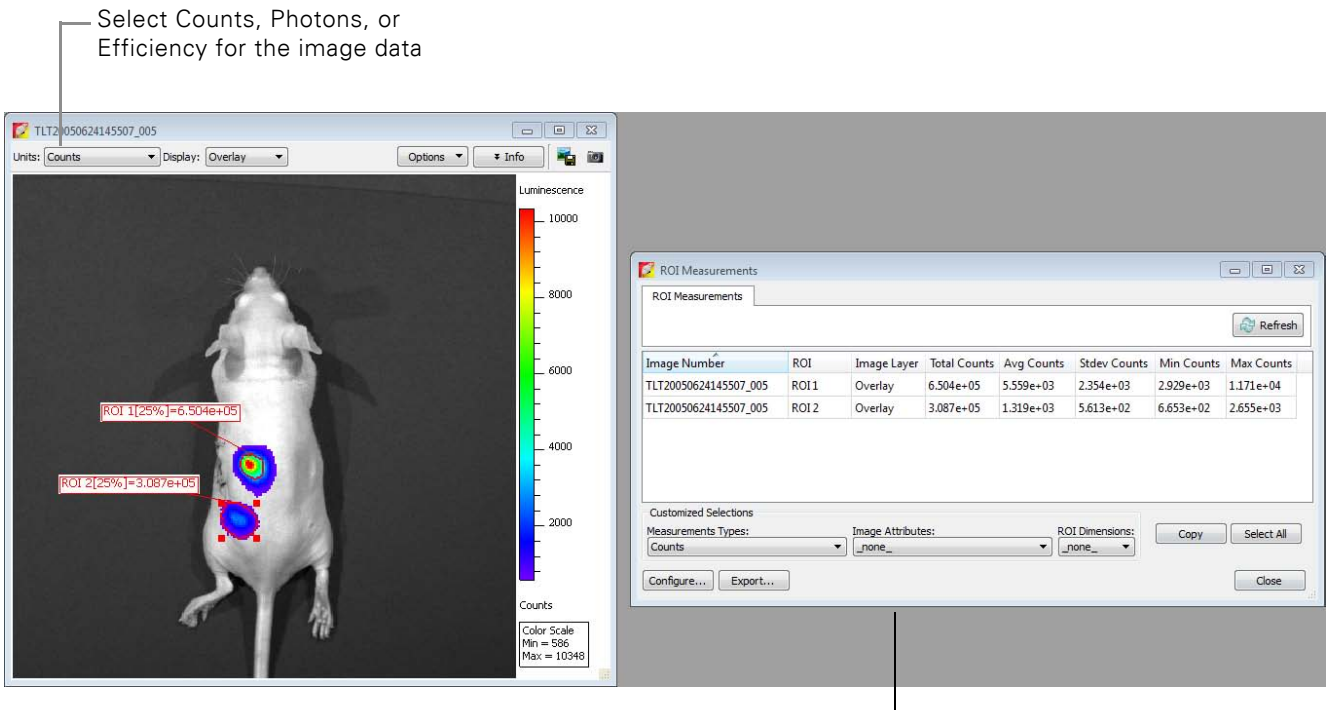
Counts

When image data is displayed in counts, the image pixel contents are displayed as the numerical output of the charge digitizer on the charge coupled device (CCD) (Figure D.2). The counts measurement (also known as *analog digitizer units* (ADU) or *relative luminescence units* (RLU)) is proportional to the number of photons detected in a pixel.

Counts are uncalibrated units that represent the raw amplitude of the signal detected by the CCD camera. A signal measured in counts is related to the photons incident on the CCD camera. The signal varies, depending on the camera settings (for example, integration time, binning, f/stop, or field of view setting).

All IVIS Imaging Systems include a CCD digitizer that is a 16-bit device, which means that the signal count range is from zero to 65,535. Sometimes the displayed signal count may appear outside of this range due to corrections applied to the image data (for example, background corrections).

Figure D.2 ROI measurements (counts mode)



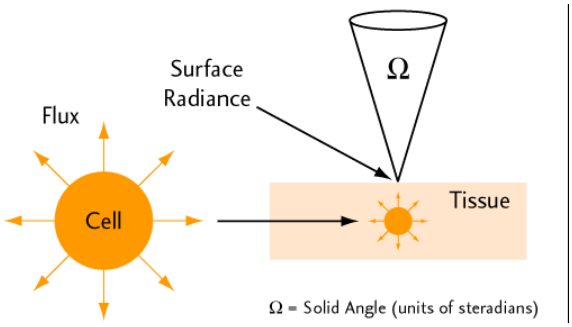
Radiance

When image data is displayed in radiance units ('photons' for short), the *photon emission from the subject* is displayed in photons/sec/cm²/sr. Counts are a relative measure of the photons incident on the CCD camera and radiance is in absolute physical units that measure the photon emission from the subject.

The radiance unit of photons/sec/cm²/sr is the number of photons per second that leave a square centimeter of tissue and radiate into a solid angle of one steradian (sr) (Figure D.3).

Figure D.3 Isotropic radiation

Isotropic radiation from a cell is called photon flux (photons/sec). When cells occur in tissue, photon emission from the tissue surface is called surface radiance (photons/sec/cm²/sr).



A steradian can be thought of as a three-dimensional cone of light emitted from the surface that has a unit solid angle. Much like a radian is a unit of arc length for a circle, a steradian is a unit of solid angle for a sphere. An entire sphere has 4π steradians. Lens systems typically collect light from only a small fraction of the total 4π steradians.

When image data is displayed in radiance mode, the units change to photons/sec/cm²/sr. These are units of photon radiance on the surface of the animal. A very important distinction between these absolute physical units and the relative units of counts is that the radiance units refer to photon emission *from the subject animal itself*, as opposed to counts that refers to a measurement of photons incident on the detector.

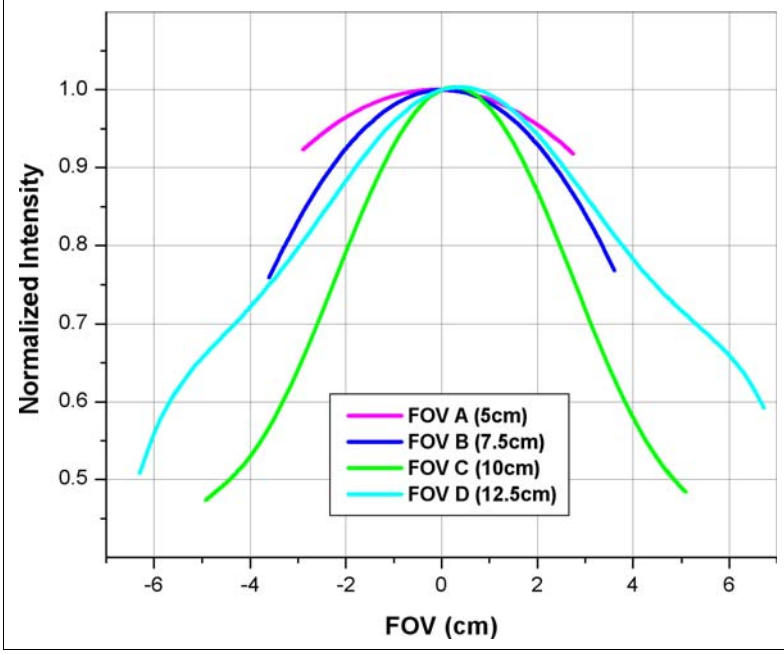
Measurements in units of radiance automatically take into account camera settings (for example, integration time, binning, f/stop, and field of view). As a result, images of the same subject acquired during the same session have the same signal amplitude regardless of the camera settings because the radiance on the animal surface does not change. The advantage of working with image data in radiance mode is that camera settings can be changed during an experiment without having to adjust the images or the measured ROI data. Images or ROI data can be quantitatively compared across different IVIS Imaging Systems.

Caliper Life Sciences calibrates the CCD response and lens of each IVIS® Imaging System for all the emission wavelengths. The response of the CCD is relatively flat (~10%) over the range from 500-700 nm which includes the spectral variation found in bacterial or firefly luciferase. Therefore, calibration is accurate over this range.

Efficiency

The fluorescent signal detected from a sample depends on the amount of fluorophore present in the sample and the intensity of the incident excitation light. The excitation light incident on the sample stage is not uniform over the field of view (FOV). The profiles for all stage locations peak near the center of the FOV. The illumination intensity profile varies by up to ±30% across the entire FOV (Figure D.4).

Figure D.4 Illumination profiles at different FOVs
Measurements were taken at the center of the FOV on the IVIS® Lumina Series.



Displaying fluorescent image data in terms of efficiency eliminates the variable excitation light from the measurement and enables a more quantitative comparison of fluorescent signals. When you select efficiency for the image data (Figure D.2), the software normalizes the fluorescent emission image to a reference image and computes:

Efficiency = Radiance of the subject/Illumination intensity

Prior to instrument delivery, Caliper Life Sciences generates a reference image of the excitation light intensity (no emission filter) incident on a highly reflective white plate for each excitation filter at every FOV and lamp power. The data are stored in the Living Image folder.

Image efficiency data does not have units. The efficiency number for each pixel represents the fraction of fluorescent photons relative to each incident excitation photon and is typically in the range of 10^{-2} to 10^{-9}. When ROI measurements are made, the total efficiency within the ROI is the efficiency per pixel integrated over the ROI area, so the resulting units of total efficiency is area or cm^2.

D.3 Flat Fielding

Flat fielding refers to the uniformity of light collected across the field of view (FOV). A lens usually collects more light from the center of the FOV than at the edges. The Living Image software provides a correction algorithm to compensate for the variation in the collection efficiency of the lens. This enables uniform quantitation of ROI measurements across the entire FOV.

To apply the correction algorithm, choose the Flat Field Correction option in the Corrections/Filtering tools. The algorithm multiplies each pixel by a predetermined scale factor. The scale factor for each pixel depends on its distance from the center of the image. The scale factor near the center of the field of view is one, but can be up to two or three near the corners on the IVIS® Imaging Systems and Spectrum.

You may notice an increase in noise near the edges and corners of the FOV when flat field correction is applied– this is normal.

D.4 Cosmic Ray Corrections

Cosmic rays are extraterrestrial high-energy particles that register a false signal on a CCD detector. Cosmic rays as well as other sources of ionizing radiation cause infrequent interactions (a few per minute) on the CCD. These interactions result in large signals that are usually isolated to a single pixel, making them easy to correct.

The Living Image software searches for isolated, high amplitude *hot pixels* and replaces them with a collective average of surrounding pixels. The Cosmic Correction option should always be selected for *in vivo* image data because hot pixels can significantly affect an ROI measurement.

Cosmic ray correction is not recommended when imaging very small objects such as individual cells. An individual cell may only light up one or two pixels and can sometimes be misinterpreted as a cosmic ray. In this case, clear the Cosmic Correction option in the Corrections/Filtering tools to avoid filtering out single-cell images.

Appendix E Luminescent Background Sources & Corrections

The background sources of light from luminescent images are inherently very low. This appendix discusses sources of background and how to manage them. Due to the extreme sensitivity of the IVIS Imaging System, residual electronic background (dark current) and luminescent emission from live animals (autoluminescence) are measurable and must be taken into account.

For information on fluorescent background, see Appendix F, page 299.

E.1 Electronic Background

The cooled CCD camera used in an IVIS Imaging System has electronic background that must be accurately measured and subtracted from the image data before the light intensity is quantified. Raw data that is not corrected for electronic background results in erroneous ROI measurements. Incorrect background subtraction may also result in serious errors. However, it is not necessary to subtract the electronic background when making a simple visual inspection of an image.

The types of electronic background include:

- *Read bias* - An electronic offset that exists on every pixel. This means that the zero photon level in the readout is not actually zero, but is typically a few hundred counts per pixel. The read bias offset is reproducible within errors defined by the *read noise*, another quantity that must be determined for quantitative image analysis.
- *Dark current* - Electronic background generated by the thermal production of charge in the CCD. To minimize dark current, the CCD is cooled during use.

Read Bias & Drift

Prior to a luminescent image exposure, the Living Image software initiates a series of zero-time exposures (*image readout*) to determine a read bias measurement.

If a dark charge background is available for the luminescent image, the average bias offset for the read bias image stored with the dark charge measurement is compared to the average bias offset determined with the read bias measurement made prior to the image. The difference, or *drift correction*, is stored with the luminescent image data, and is later used to correct minor drift (typically less than two counts/pixel) that may occur in the bias offset since measuring the dark charge background.

If a dark charge background is not available at the time of the luminescent image exposure, the software checks to see if the selected image parameters warrant a dark charge measurement (large binning and long exposure time). If a dark charge image is not required, the read bias will be used. If a dark charge is recommended, the software provides the option of using the read bias measurement instead. Since the read bias is by far the largest component of background, using a read bias measurement instead of a dark charge measurement is often acceptable. If read bias is used instead of a dark charge background, the read bias image is stored with the image data rather than the usual background information.

If the amount of dark charge associated with an image is negligible, read bias subtraction is an adequate substitute for dark charge background subtraction. Dark charge increases with exposure time and is more significant at higher levels of binning. A good rule of thumb is that dark charge is negligible if:

$$\tau B^2 < 1000$$

where τ is the exposure time (seconds) and B is the binning factor.

Under these conditions, dark charge contributes less than 0.1 counts/pixel and may be ignored.

Dark Charge

Dark charge refers to all types of electronic background, including dark current and read bias. Dark charge is a function of the exposure time, binning level, and camera temperature. A dark charge measurement should be taken within 48 hours of image acquisition and the system should remain stable between dark charge measurement and image acquisition. If the power to the system or camera controller (a component of some IVIS Imaging Systems) has been cycled or if the camera temperature has changed, a new dark charge measurement should be taken.

The dark charge is measured with the camera shutter closed and is usually performed automatically overnight by the Living Image software. The software acquires a series of zero-time exposures to determine the bias offset and read noise, followed by three dark exposures. The dark charge measurement usually takes more than three times as long to complete as the equivalent luminescent exposure.

E.2 Background Light On the Sample

An underlying assumption for *in vivo* imaging is that all of the light detected during a luminescent image exposure is emitted by the sample. This is not accurate if there is an external light source illuminating the sample. Any reflected light will be detected and is indistinguishable from emission from the sample.

The best way to deal with external light is to physically eliminate it. There are two potential sources of external light: a light leak through a crack or other mechanical imperfection in the imaging chamber or a source of external illumination.

IVIS Imaging Systems are designed to be extremely light tight and are thoroughly checked for light leaks before and after installation. Light leaks are unlikely unless mechanical damage has occurred. To ensure that there are no light leaks in the imaging chamber, conduct an imaging test using the High Reflectance Hemisphere (Figure E.1).

A more subtle source of external illumination is the possible presence of light emitting materials inside the imaging chamber. In addition to obvious sources such as the light emitting diodes (LEDs) of electronic equipment, some materials contain phosphorescent compounds.

NOTE

Do not place equipment that contains LEDs in the imaging chamber.

Phosphorescence is a physical process similar to fluorescence, but the light emission persists for a longer period. Phosphorescent materials absorb light from an external source (for example, room lights) and then re-emit it. Some phosphorescent materials may re-

emit light for many hours. If this type of material is introduced into the imaging chamber, it produces background light even after the chamber door is closed. If the light emitted from the phosphorescent material illuminates the sample from outside of the field of view during imaging, it may be extremely difficult to distinguish from the light emitted by the sample.

IVIS Imaging Systems are designed to eliminate background interference from these types of materials. Each system is put through a rigorous quality control process to ensure that background levels are acceptably low. However, if you introduce such materials inadvertently, problems may arise.

Problematic materials include plastics, paints, organic compounds, plastic tape, and plastic containers. Contaminants such as animal urine can be phosphorescent. To help maintain a clean imaging chamber, place animal subjects on black paper (for example, Artagain black paper, Strathmore cat. no. 445-109) and change the paper frequently. Cleaning the imaging chamber frequently is also helpful.

IMPORTANT

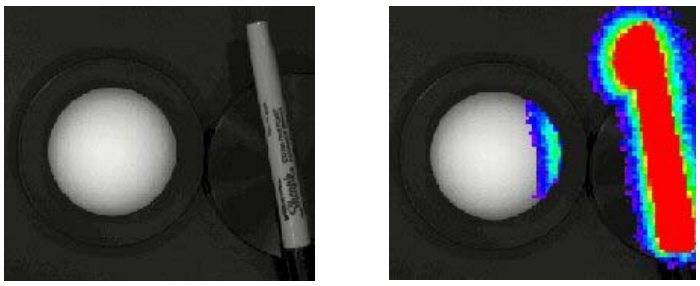
ALERT! Use only cleaning agents approved by Caliper. Many cleaning compounds phosphoresce! Contact Caliper technical support for a list of tested and approved cleaning compounds.

If it is necessary to introduce suspect materials into the imaging chamber, screen the materials by imaging them. Acquire an image of the material alone using the same settings (for example, FOV and exposure time) that will be used to image the sample to determine if the material is visible in the luminescent image.

Microplates (white, black, or clear plastic) can be screened this way. Screen all three types with a test image. White plates appear extremely bright by IVIS Imaging System standards and interfere with measurements. Black or clear plastic microplates do not phosphoresce, making them better choices.

The High Reflectance Hemisphere provides a more definitive way to determine the presence of an undesirable light source (Figure E.1). It is a small white hemisphere that is coated with a non-phosphorescent material. A long exposure image of the hemisphere should produce a luminescent image in which the hemisphere is not visible.

Figure E.1 High Reflectance Hemisphere and a plastic marker pen
Left: Photographic image. Right: Photograph with luminescent overlay. The hemisphere is illuminated by phosphorescence emitted from the pen.



If any part of the hemisphere exhibits what appears to be luminescent emission, it is actually the light reflected from a source illuminating the hemisphere. Observe the side of the hemisphere that is illuminated to help determine the source location.

In Figure E.1, the pen appears very bright due to phosphorescent emission that is also illuminating the portion of the hemisphere next to the pen. If the pen had been outside the field of view, it would not have been imaged, and the source of the phosphorescence would be less obvious. However, the illumination of the hemisphere would still be very apparent and indicative of a light pollution problem.

IMPORTANT

ALERT! Handle the High Reflectance Hemisphere by its black base plate while wearing cotton gloves provided by Caliper. Skin oils can phosphoresce and will contaminate the hemisphere. Latex gloves and the powder on them may also phosphoresce. If the hemisphere becomes contaminated, contact Caliper technical support for a replacement. There are no known agents that can clean the hemisphere. To check the hemisphere for contamination, take several images of the hemisphere, rotating it slightly between images. A glowing fingerprint, for example, will rotate with the hemisphere, while a glowing spot due to external illumination most likely will not.

E.3 Background Light From the Sample

Another source of background is the natural light emitted from a sample that is not due to emission from the source of interest in the sample. This type of background may be due to a material associated with the experimental setup. For example, the cell culture medium may phosphoresce. Materials should be screened so you can identify and eliminate problematic materials. If a background source is phosphorescent and the phosphorescent lifetime is relatively short, you can try keeping the sample in the dark for a long period before imaging to reduce background light emission.

Occasionally there is no way to eliminate the natural light emission of the sample. The natural light emission associated with living animals (*autoluminescence*) is a major area of interest in *in vivo* luminescent imaging. Most animals exhibit a low level of autoluminescence. Usually this is only a problem when looking for very low signals at the highest levels of sensitivity.

Caliper has conducted tests to try to minimize the source of the background light emission in mice.

Test Description	Observation
Test 1: Subject animals were housed in the dark 12 hours prior to imaging.	Background emission levels were not reduced. A phosphorescent component in mouse fur or skin is not the source of light emission.
Test 2: White-furred animals were shaved prior to imaging	No increase or decrease in background emission levels.
Test 3: Alfalfa (known to be phosphorescent) was eliminated from the animal diet.	An alfalfa-free diet reduced background emission slightly, but not significantly.

The sources of autoluminescence are not yet fully understood. No external sources have been proven to cause natural light emissions, so it is possible that a chemiluminescent process associated with metabolic activity in living animals is the source of animal background. This is supported by the observation that the level of background light drops significantly in euthanized animals.

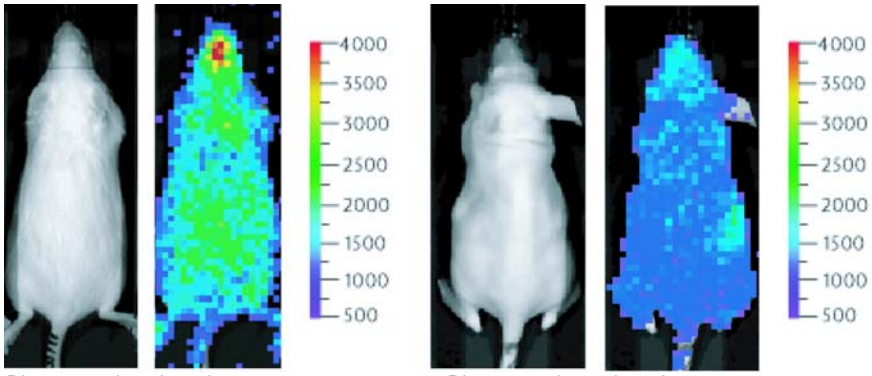
In Figure E.2, the background light emission is clearly visible in the images of a control white-furred mouse and a nude mouse. The images are five minute, high-sensitivity (high binning) exposures. The average emission from a white-furred mouse and a nude mouse is approximately 1600 photons/s/cm^2/sr and 1000 photons/s/cm^2/sr, respectively. Since

these values are well above the lower limit of detection of the IVIS Imaging System (~100 photons/s/cm²/sr), the background light emission from the mouse determines the limit of detection.

An approximation of this background (determined by making similar measurements on either control animals or regions of the subject animal that do not contain the primary signal) can be subtracted from ROI measurements. (For more information on ROI measurements, see Chapter 8, page 130.)

Note that the background light emission is not uniform over the entire animal. In Figure E.2, images of control animals (mice) show a somewhat higher background component originating from the abdominal and thoracic regions. Therefore, care must be taken when selecting a representative background area.

Figure E.2 Background light emission
Background light emission from a female white furred (Swiss Webster) (left) and a female nude (Nu/nu) mouse (right).



Usually only very low signals at the highest level of sensitivity require this type of background subtraction. For more information on how best to handle these types of measurements, please contact Caliper technical support.

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Appendix F Fluorescent Imaging

F.1 Description and Theory of Operation

System Components

The IVIS® Spectrum, IVIS 200 Series Imaging System, and IVIS Lumina offer built-in fluorescence imaging capability as standard equipment (Figure F.1, Figure F.2, Figure F.3). The IVIS Imaging System 100 and 50 Series use the XFO-6 or XFO-12 Fluorescence Option to perform fluorescence imaging. The fluorescence equipment enables you to conveniently change between luminescent and fluorescent imaging applications . For more details, see the appropriate hardware manual:

- *IVIS Spectrum System Manual*
- *IVIS Imaging System 200 Series System Manual*
- *IVIS Lumina System Manual*
- *XFO-6* or *XFO-12 Fluorescence Option Manual*

Figure F.1 IVIS Spectrum

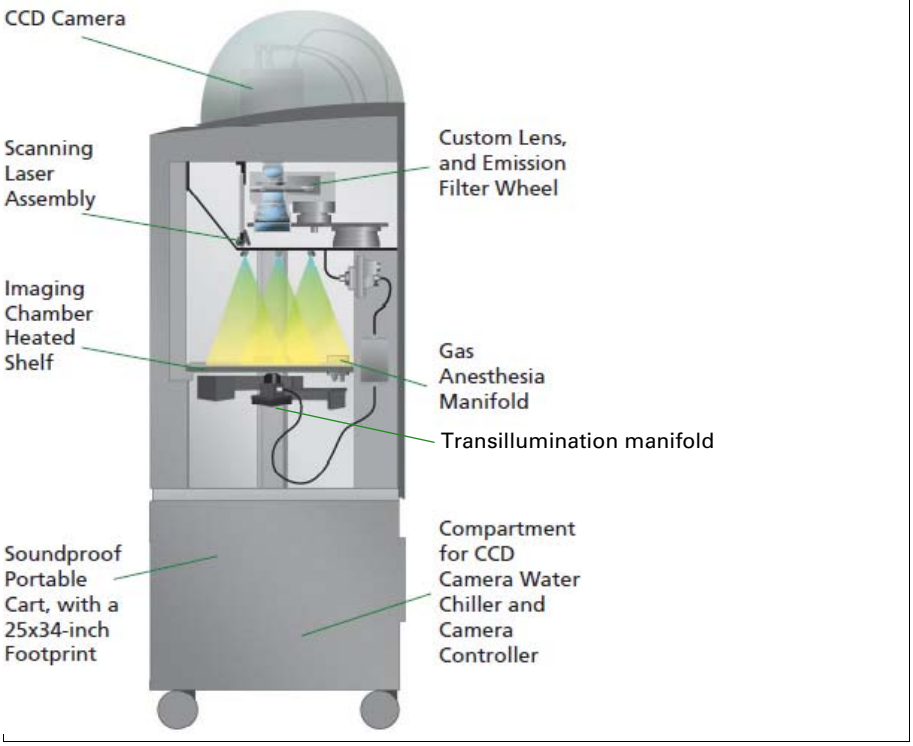


Figure F.2 IVIS Imaging System 200 Series

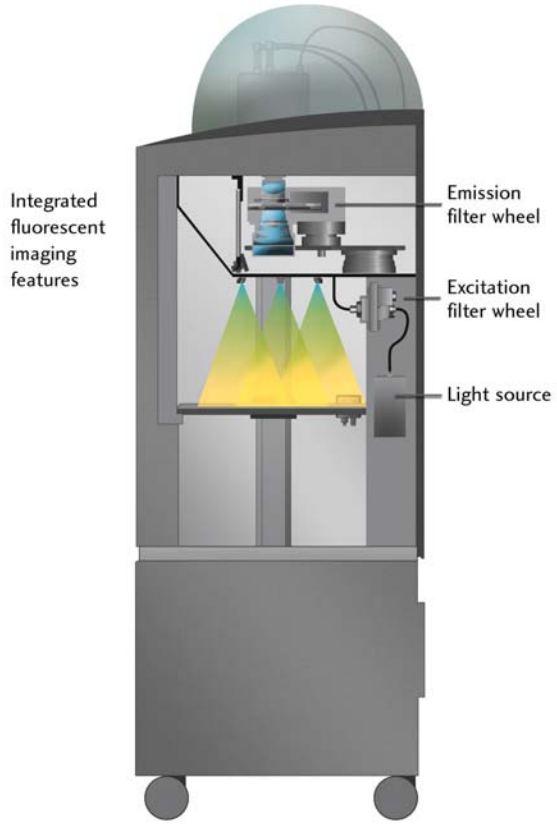
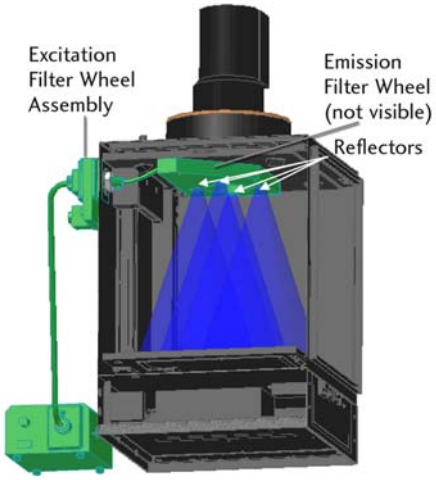


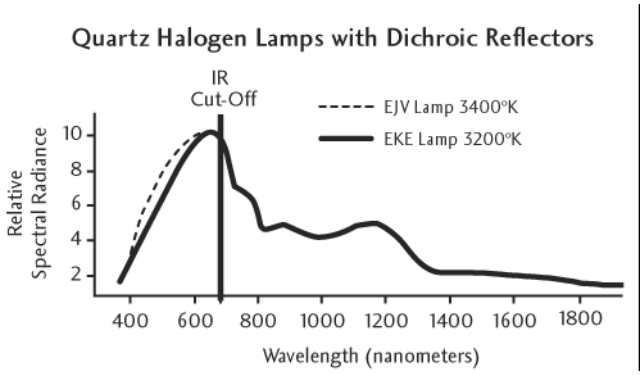
Figure F.3 IVIS Imaging System Lumina, Lumina 100 Series, and Lumina 50 Series



A 150-watt quartz tungsten halogen (QTH) lamp with a dichroic reflector provides light for fluorescence excitation. The relative spectral radiance output of the lamp/reflector combination provides high emission throughout the 400-950 nm wavelength range (Figure F.4). The dichroic reflector reduces infrared coupling (>700 nm) to prevent overheating of the fiber-optic bundles, but allows sufficient infrared light throughput to enable imaging at these wavelengths. The Living Image software controls the illumination

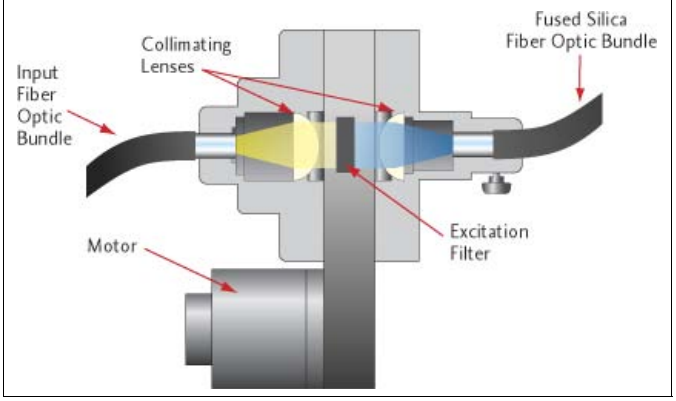
intensity level (off, low, or high). The illumination intensity at the low setting is approximately 18% that of the high setting.

Figure F.4 Relative spectral radiance output for the quartz halogen lamp with dichroic reflector.



The lamp output is delivered to the excitation filter wheel assembly located at the back of the IVIS Imaging System (Figure F.5). Light from the input fiber-optic bundle passes through a collimating lens followed by a 25 mm diameter excitation filter. The IVIS Imaging System provides a 12-position excitation filter wheel, allowing you to select from up to 11 fluorescent filters (five filters on older systems). A light block is provided in one filter slot for use during luminescent imaging to prevent external light from entering the imaging chamber. The Living Image software manages the motor control of the excitation filter wheel.

Figure F.5 Excitation filter wheel cross section



Following the excitation filter, a second lens focuses light into a 0.25 inch fused silica fiber-optic bundle inside the imaging chamber. Fused silica fibers (core and clad), unlike ordinary glass fibers, prevent the generation of autofluorescence.

The fused silica fiber bundle splits into four separate bundles that deliver filtered light to four reflectors in the ceiling of the imaging chamber (Figure F.1). The reflectors provide a diffuse and relatively uniform illumination of the sample stage. Analyzing image data in terms of efficiency corrects for nonuniformity in the illumination profile. When the efficiency mode is selected, the measured fluorescent image is normalized to a reference illumination image. (For more details on efficiency, see page 283.)

The IVIS Spectrum provides both transmission and epi-illumination. Emitted light from the excitation filter wheel feeds through a fiber optic bundle to illuminate the specimen

from either the top, in epi-illumination (reflectance) mode, or from underneath the stage, by means of an automated bundle switch. Transilluminating the subject from below at precise x,y-locations allows for transmission imaging, enabling more sensitive detection and accurate quantification of deep sources. Transmission fluorescence imaging also reduces the effects of autofluorescence. A computer-controlled imaging switch allows you to change between the two imaging modes (using IVIS Acquisition Control Panel or the Imaging Wizard).

The emission filter wheel at the top of the imaging chamber collects the fluorescent emission from the target fluorophore and focuses it into the CCD camera. All IVIS Imaging Systems require that one filter position on each wheel always be open for luminescent imaging.

IVIS Imaging System	Number of Emission Filter Wheel Positions	Number of Available Fluorescence Filters
Spectrum	24 (two levels, each with 12 positions)	22 (60 mm diameter)
Lumina	8	7 (4 sets of 7 high resolution filter wheels or a wheel with 4 standard filters)
100 or 50	6	5 (75 mm diameter)

F.2 Filter Spectra

High quality filters are essential for obtaining good signal-to-background levels (*contrast*) in fluorescence measurements, particularly in highly sensitive instruments such as the IVIS Imaging Systems. Figure F.6 shows typical excitation and emission fluorophore spectra, along with idealized excitation and emission filter transmission curves. The excitation and emission filters are called *bandpass filters*. Ideally, bandpass filters transmit all of the wavelengths within the bandpass region and block (absorb or reflect) all wavelengths outside the bandpass region. This spectral band is like a window, characterized by its central wavelength and its width at 50% peak transmission, or full width half maximum. Figure F.7 shows filter transmission curves of a more realistic nature.

Because the filters are not ideal, some leakage (undesirable light not blocked by the filter but detected by the camera) may occur outside the bandpass region. The materials used in filter construction may also cause the filters to autofluoresce.

Figure F.6 Typical excitation and emission spectra for a fluorescent compound. The graph shows two idealized bandpass filters that are appropriate for this fluorescent compound.

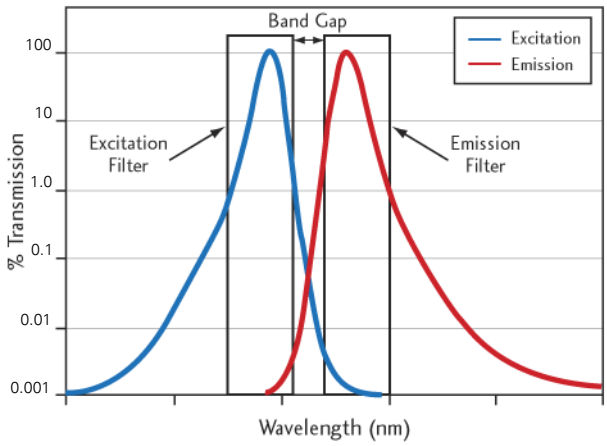
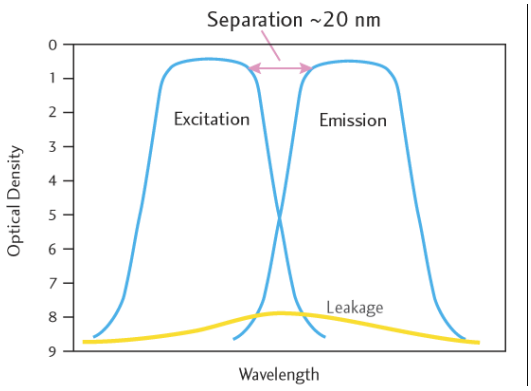


Figure F.7 Typical attenuation curves for excitation and emission filters



In Figure F.7, the vertical axis is optical density, defined as OD = -*log*(T), where T is the transmission. An OD=0 indicates 100% transmission and OD=7 indicates a reduction of the transmission to 10^{-7}.

For the high quality interference filters in the IVIS Imaging Systems, transmission in the bandpass region is about 0.7 (OD=0.15) and blocking outside of the bandpass region is typically in the OD=7 to OD=9 range. The band gap is defined as the gap between the 50% transmission points of the excitation and emission filters and is usually 25-50 nm.

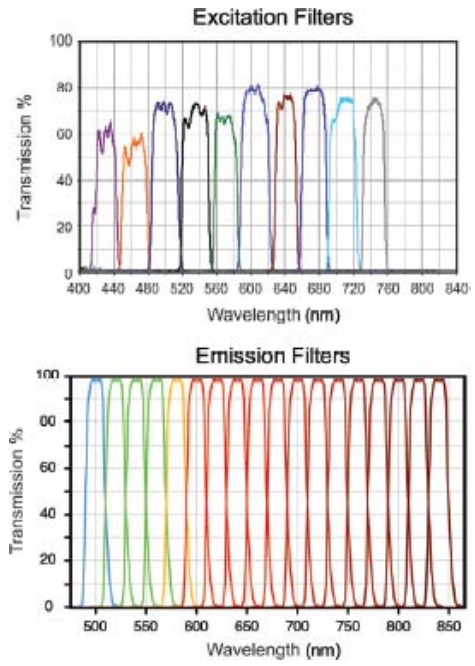
There is a slope in the transition region from bandpass to blocking (Figure F.7). A steep slope is required to avoid overlap between the two filters. Typically, the slope is steeper at shorter wavelengths (400-500 nm), allowing the use of narrow band gaps of 25 nm. The slope is less steep at infrared wavelengths (800 nm), so a wider gap of up to 50 nm is necessary to avoid cross talk.

Fluorescent Filters and Imaging Wavelengths

The IVIS® Spectrum excitation and emission filters enable spectral scanning over the blue to NIR wavelength region and include:

- 10 narrow band excitation filters: 415 nm – 760 nm (30 nm bandwidth)
- 18 narrow band emission filters: 490 nm – 850 nm (20 nm bandwidth)

Figure F.8 IVIS Spectrum excitation and emission filters



Eight excitation and four emission filters come standard with a fluorescence-equipped IVIS Imaging System (Table F.1). Custom filter sets are also available. Fluorescent imaging on the IVIS Imaging System uses a wavelength range from 400-950 nm, enabling a wide range of fluorescent dyes and proteins for fluorescent applications.

For *in vivo* applications, it is important to note that wavelengths greater than 600 nm are preferred. At wavelengths less than 600 nm, animal tissue absorbs significant amounts of light. This limits the depth to which light can penetrate. For example, fluorophores located deeper than a few millimeters are not excited. The autofluorescent signal of tissue also increases at wavelengths less than 600 nm.

Table F.1 Standard filter sets and fluorescent dyes and proteins used with IVIS Imaging Systems

Name	Excitation Passband (nm)	Emission Passband (nm)	Dyes & Passband
GFP	445-490	515-575	GFP, EGFP, FITC
DsRed	500-550	575-650	DsRed2-1, PKH26, CellTracker™ Orange
Cy5.5	615-665w	695-770	Cy5.5, Alexa Fluor® 660, Alexa Fluor® 680
ICG	710-760	810-875	Indocyanine green (ICG)
GFP Background	410-440	Uses same as GFP	GFP, EGFP, FITC
DsRed Background	460-490	Uses same as DsRed	DsRed2-1, PKH26, CellTracker™ Orange

Table F.1 Standard filter sets and fluorescent dyes and proteins used with IVIS Imaging Systems

Name	Excitation Passband (nm)	Emission Passband (nm)	Dyes & Passband
Cy5.5 Background	580-610	Uses same as Cy5.5	Cy5.5, Alexa Fluor® 660, Alexa Fluor® 680
ICG Background	665-695	Uses same as ICG	Indocyanine green (ICG)

F.3 Working with Fluorescent Samples

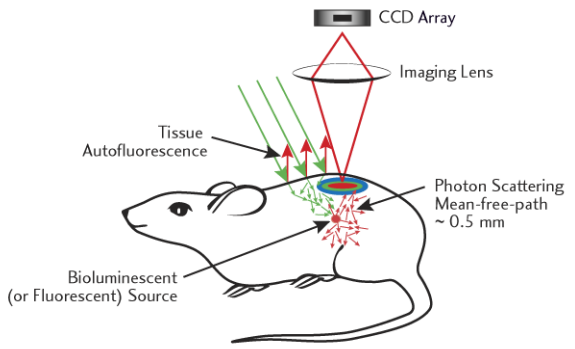
There are a number of issues to consider when working with fluorescent samples, including the position of the subject on the stage, leakage and autofluorescence, background signals, and appropriate signal levels and f/stop settings.

Tissue Optics Effects

In *in vivo* fluorescence imaging, the excitation light must be delivered to the fluorophore inside the animal for the fluorescent process to begin. Once the excitation light is absorbed by the fluorophore, the fluorescence is emitted. However, due to the optical characteristics of tissue, the excitation light is scattered and absorbed before it reaches the fluorophore as well as after it leaves the fluorophore and is detected at the animal surface (Figure F.9).

The excitation light also causes the tissue to autofluoresce. The amount of autofluorescence depends on the intensity and wavelength of the excitation source and the type of tissue. Autofluorescence can occur throughout the animal, but is strongest at the surface where the excitation light is strongest.

Figure F.9 Illustration of the *in vivo* fluorescence process



At 600-900 nm, light transmission through tissue is highest and the generation of autofluorescence is lower. Therefore it is important to select fluorophores that are active in the 600-900 nm range. Fluorophores such as GFP that are active in the 450-600 nm range will still work, but the depth of detection may be limited to within several millimeters of the surface.

Specifying Signal Levels and f/stop Settings

Fluorescent signals are usually brighter than luminescent signals, so imaging times are shorter, typically from one to 30 seconds. The bright signal enables a lower binning level that produces better spatial resolution. Further, the f/stop can often be set to higher values; f/2 or f/4 is

recommended for fluorescence imaging. A higher f/stop improves the depth of field, yielding a sharper image. For more details on the f/stop, see *Lens Aperture*, page 275.

F.4 Image Data Display

Fluorescent image data can be displayed in:

- Counts
- Radiance (photons)
- Radiant efficiency (Efficiency/Illumination Power)
- Efficiency (calibrated, normalized)
-

For more details, see *Quantifying Image Data*, page 281.

If the image is displayed in any units other than counts, you can compare images with different exposure times, f/stop setting, or binning level. When an image is displayed in terms of efficiency, the fluorescent image is normalized against a stored reference image of the excitation light intensity. Efficiency image data is without units and represents the ratio of emitted light to incident light. For more details on efficiency, see page 283.

Fluorescent Efficiency and Radiant Efficiency

The detected fluorescent signal depends on the amount of fluorophore present in the sample and the intensity of the incident excitation light. At the sample stage, the incident excitation light is not uniform over the FOV. It peaks at the center of the FOV and drops of slowly toward the edges (Figure F.10). To eliminate the excitation light as a variable from the measurement, the data can be displayed in terms of *efficiency* (Figure F.11).

Figure F.10 Illumination profiles for different FOVs on an IVIS® Lumina measured from the center of the FOV

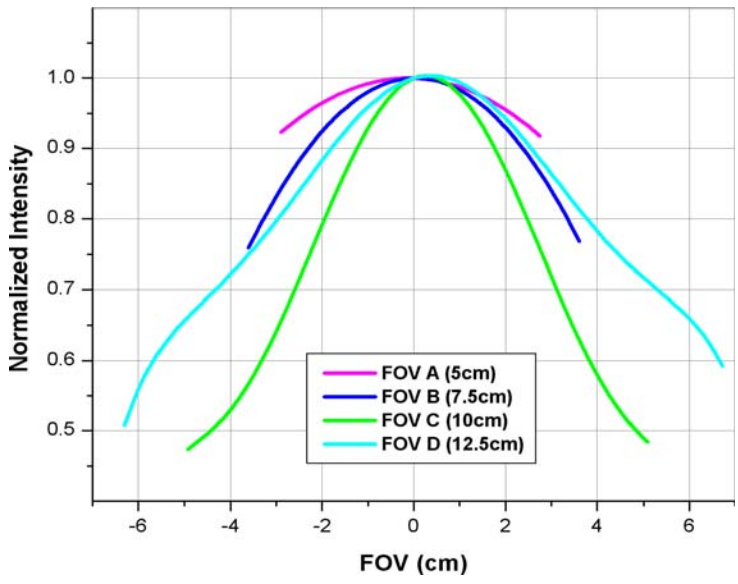
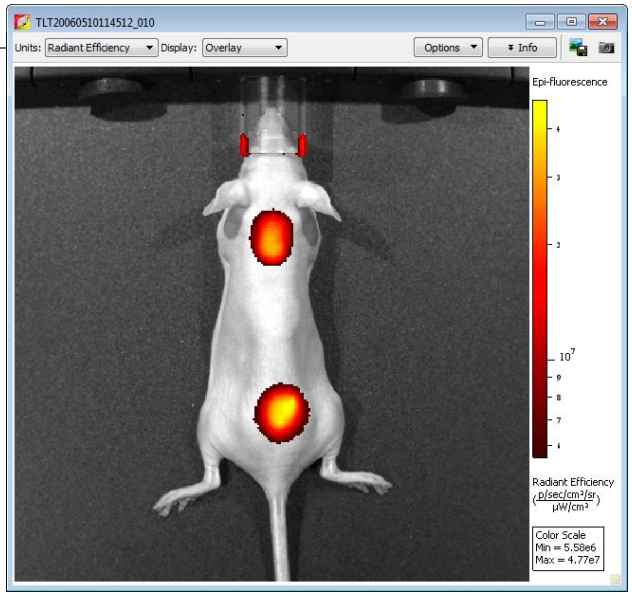


Figure F.11 Fluorescent image data displayed in terms of radiant efficiency

Choose Radiant Efficiency to enable a more quantitative comparison of fluorescent signals



When Radiant Efficiency is selected, the fluorescent image data is normalized (divided) by a stored, calibrated reference image of the excitation light intensity incident on a highly reflective white plate. The resulting image data is without units, typically in the range of 10^{-2} to 10^{-9}.

NOTE

On each IVIS Imaging System, a reference image of the excitation light intensity is measured for each excitation filter at every FOV and lamp power. The reference images are measured and stored in the Living Image folder prior to instrument delivery.

F.5 Fluorescent Background

Autofluorescence

Autofluorescence is a fluorescent signal that originates from substances other than the fluorophore of interest and is a source of background. Almost every substance emits some level of autofluorescence. Autofluorescence may be generated by the system optics, plastic materials such as microplates, and by animal tissue. Filter leakage, which may also occur, is another source of background light.

The optical components of the IVIS Imaging Systems are carefully chosen to minimize autofluorescence. Pure fused silica is used for all transmissive optics and fiber optics to reduce autofluorescence. However, trace background emissions exist and set a lower limit for fluorescence detection.

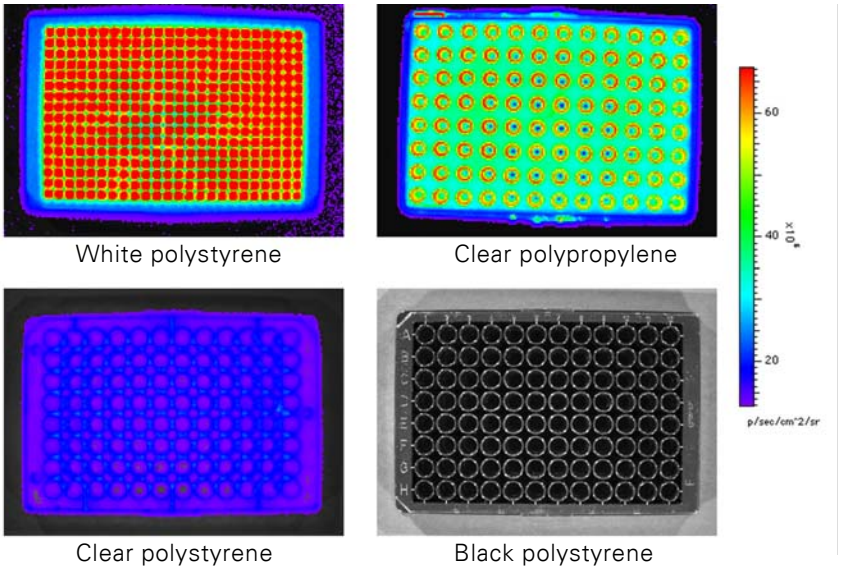
To distinguish real signals from background emission, it is important to recognize the different types of autofluorescence. The following examples illustrate sources of autofluorescence, including microplates, other materials, and animal tissue.

Microplate Autofluorescence

When imaging cultured cells marked with a fluorophore, be aware that there is autofluorescence from the microplate as well as native autofluorescence of the cell.

Figure F.12 shows autofluorescence originating from four different plastic microplates. The images were taken using a GFP filter set (excitation 445-490nm, emission 515-575nm).

Figure F.12 Examples of microplate autofluorescence emission
The black polystyrene plate emits the smallest signal while the white polystyrene plate emits the largest signal. (Imaging parameters: GFP filter set, Fluorescence level Low, Binning=8, FOV=15, f/1, Exp=4sec.)

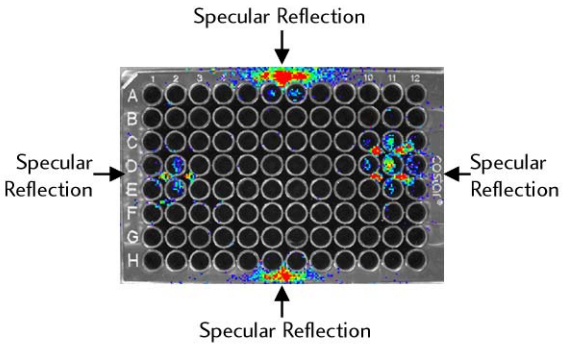


Two types of autofluorescent effects may occur:

Overall glow of the material - Usually indicates the presence of autofluorescence.

Hot spots - Indicates a specular reflection of the illumination source (Figure F.13). The specular reflection is an optical illumination autofluorescence signal reflecting from the microplate surface and is not dependent on the microplate material.

Figure F.13 Specular reflection.
The four symmetric hot spots on this black polystyrene well plate illustrate the specular reflection of the illumination source. (Imaging parameters: GFP filter set, Fluorescence level Low, Binning=8, FOV=15, f/1, Exp=4sec.)



Black polystyrene microplates are recommended for *in vitro* fluorescent measurements. Figure F.12 and Figure F.13 show that the black polystyrene microplate emits the smallest inherent fluorescent signal, while the white polystyrene microplate emits the largest signal. The clear polystyrene microplate has an autofluorescent signal that is slightly higher than that of the black microplate, but it is still low enough that this type of microplate may be used.

Control cells are always recommended in any experiment to assess the autofluorescence of the native cell.

Miscellaneous Material Autofluorescence

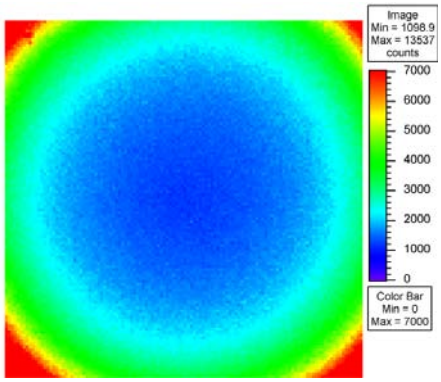
It is recommended that you place a black Lexan® sheet (Caliper part no. 60104) on the imaging stage to prevent illumination reflections and to help keep the stage clean. If you are working in transillumination mode, do not use the black Lexan sheet; it will block the signal.

NOTE

The black paper recommended for luminescent imaging (Swathmore, Artagain, Black, 9"x12", Caliper part no. 445-109) has a measurable autofluorescent signal, particularly with the Cy5.5 filter set.

Figure F.14 shows a fluorescent image of a sheet of black Lexan on the sample stage, as seen through a GFP filter set. The image includes optical autofluorescence, light leakage, and low level autofluorescence from inside the IVIS®System imaging chamber. The ring-like structure is a typical background autofluorescence/leakage pattern. The image represents the minimum background level that a fluorophore signal of interest must exceed in order to be detected.

Figure F.14 Light from black Lexan
This image shows the typical ring-like structure of light from a sheet of black Lexan, a low autofluorescent material that may be placed on the imaging stage to prevent illumination reflections. (Imaging parameters: GFP filter set, Fluorescence level High, Binning=16, FOV=18.6, f/2, Exp=5sec.)

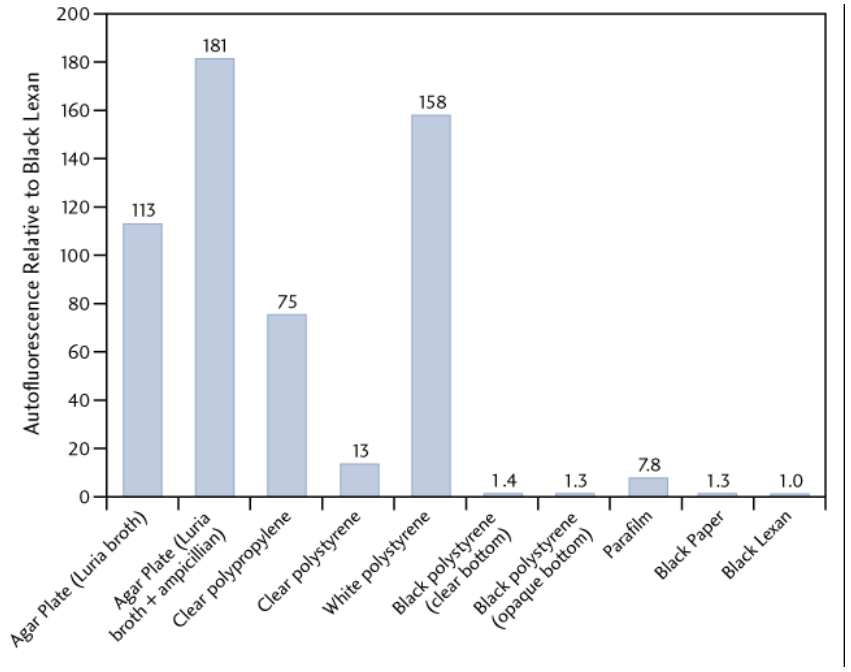


Other laboratory accessories may exhibit non-negligible autofluorescence. The chart in Figure F.15 compares the autofluorescence of miscellaneous laboratory materials to that of black Lexan. For example, the autofluorescence of the agar plate with ampicillin is more than 180 times that of black Lexan. Such a significant difference in autofluorescence levels further supports the recommended use of black polystyrene well plates.

NOTE

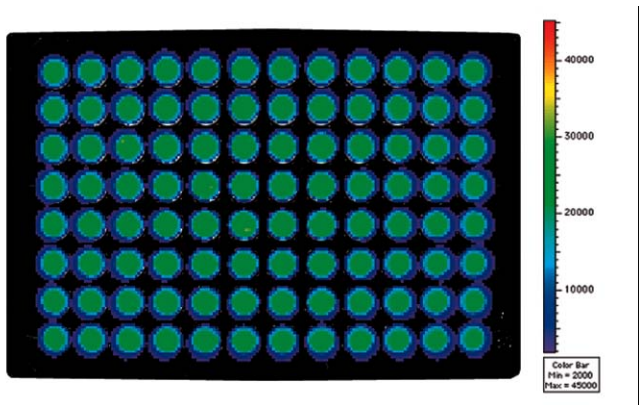
It is recommended that you take control measurements to characterize all materials used in the IVIS Imaging System.

Figure F.15 Comparison of autofluorescence of various laboratory materials to that of black Lexan



Despite the presence of various background sources, the signal from most fluorophores exceeds background emissions. Figure F.16 shows the fluorescent signal from a 96-well microplate fluorescent reference standard (TR 613 Red) obtained from Precision Dynamics Co. Because the fluorescent signal is significantly bright, the background autofluorescent sources are not apparent.

Figure F.16 96 well plate fluorescent reference standard (TR 613 Red)
The fluorescent signal is strong enough to exceed background emissions. (Imaging parameters: DsRed filter set, Fluorescence level Low, Binning=8, FOV=15, f/1, Exp=4sec.) Reference standard TR 613 Red is available through Precision Dynamics Co, http://www.pdcorp.com/healthcare/frs.html.



Animal Tissue Autofluorescence

Animal tissue autofluorescence is generally much higher than any other background source discussed so far and is likely to be the limiting factor in *in vivo* fluorescent imaging. Figure F.17 shows ventral images of animal tissue autofluorescence for the GFP, DsRed, Cy5.5, and ICG filter set in animals fed regular rodent food and alfalfa-free rodent food (Harlan Teklad, TD97184). Animals fed the regular rodent diet and imaged using the GFP and DsRed filter sets, show uniform autofluorescence, while images taken with the Cy5.5 and ICG filter sets show the autofluorescence is concentrated in the intestinal area.

The chlorophyll in the regular rodent food causes the autofluorescence in the intestinal area. When the animal diet is changed to the alfalfa-free rodent food, the autofluorescence in the intestinal area is reduced to the levels comparable to the rest of the body. In this situation, the best way to minimize autofluorescence is to change the animal diet to alfalfa-free rodent food when working with the Cy5.5 and ICG filter sets. Control animals should always be used to assess background autofluorescence.

Figure F.17 Images of animal tissue autofluorescence in control mice (Nu/nu females)
Animals were fed regular rodent food (top) or alfalfa-free rodent food (bottom). Images were taken using the GFP, DsRed, Cy5.5, or ICG filter set. The data is plotted in efficiency on the same log scale.

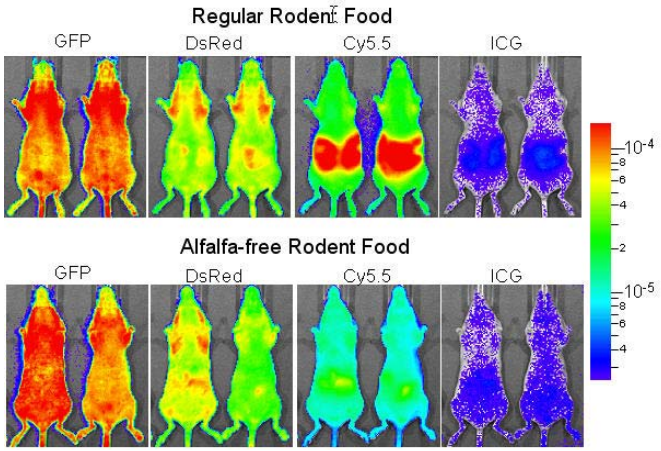
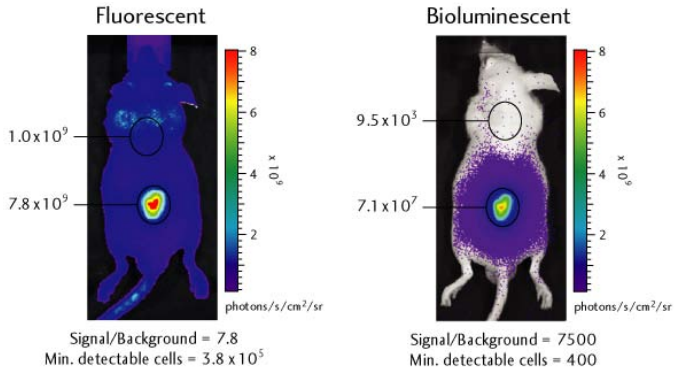


Figure F.18 shows a comparison of fluorescence and luminescence emission *in vivo*. In this example, 3×10^6 PC3M-luc/DsRed prostate tumor cells were injected subcutaneously into the lower back region of the animal. The cell line is stably transfected with the firefly luciferase gene and the DsRed2-1 protein, enabling luminescent and fluorescent expression. The fluorescence signal level is 110 times brighter than the luminescence signal. However, the autofluorescent tissue emission is five orders of magnitude higher. In this example, fluorescent imaging requires at least 3.8×10^5 cells to obtain a signal above tissue autofluorescence while luminescent imaging requires only 400 cells.

Figure F.18 Images of stably transfected, dual-tagged PC3M-luc DsRed cells. The images show the signal from a subcutaneous injection of $3x10^6$ cells in an 11-week old male Nu/nu mouse.



NOTE

When you make ROI measurements on fluorescent images, it is important to subtract the autofluorescence background. For more details, see *Subtracting Tissue Autofluorescence*, page 153.

F.6 Subtracting Instrument Fluorescent Background

The fluorescence instrumentation on an IVIS Imaging System is carefully designed to minimize autofluorescence and background caused by instrumentation. However a residual background may be detected by the highly sensitive CCD camera. Autofluorescence of the system optics or the experimental setup, or residual light leakage through the filters can contribute to autofluorescence background. The Living Image software can measure and subtract the background from a fluorescence image.

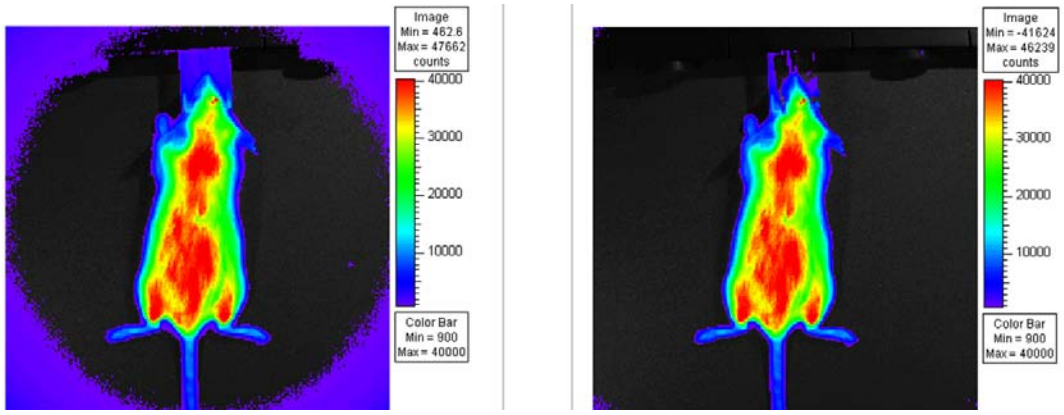
Fluorescent background subtraction is similar to the dark charge bias subtraction that is implemented in luminescent mode. However, fluorescent background changes day-to-day, depending on the experimental setup. Therefore, fluorescent background is not measured during the night, like dark charge background is.

After you acquire a fluorescent image, inspect the signal to determine if a fluorescent background should be subtracted (Figure F.19). If background subtraction is needed, remove the fluorescent subject from the imaging chamber and measure the fluorescent background (select **Acquisition** → **Fluorescent Background** → **Measure Fluorescent Background** on the menu bar). In the Living Image software, the **Sub Fluor Bkg** check box appears on the Control panel after a background has been acquired. You can toggle the background subtraction on and off using this check box.

NOTE

The fluorescence background also contains the read bias and dark charge. Dark charge subtraction is disabled if the Sub Fluor Bkg option is checked.

Figure F.19 Comparison of dark charge bias subtraction (left) and fluorescent background subtraction (right).
The autofluorescence from the nose cone and filter leakage have been minimized in the image on the right by using Sub Fluor Bkg option.



F.7 Adaptive Background Subtraction

Adaptive background subtraction is a simple way to reduce the "instrument fluorescent background" by fitting and removing the background using the existing image (for example, the left image in Figure F.19).

Unlike the method described in section F.6, Subtracting Instrument Fluorescent Background, where you acquire an actual instrument fluorescent background image by removing the fluorescent subject from the imaging chamber to correct the background, the new method uses software correction. To perform adaptive background subtraction:

- Identify the fluorescent subject in the original image using the photo mask
- The software automatically fits the instrument background to the whole image using the pixels outside of the subject
- The software subtracts the fitted instrument background from the original image

In most situations, such adaptive software correction works as effectively as the traditional method except the following cases:

- The subject is dark, making it is difficult to mask the subject using the photo (for example, experiments that use black well plates)
- The subject occupies most of the FOV (for example, high magnification or multiple mice in the FOV). As a result, there is not enough information outside the subject that can be used to help fit the background.

F.8 Subtracting Tissue Autofluorescence Using Background Filters

High levels of tissue autofluorescence can limit the sensitivity of detection of exogenous fluorophores, particularly in the visible wavelength range from 400 to 700 nm. Even in the near infrared range, there is still a low level of autofluorescence. Therefore, it is desirable to be able to subtract the tissue autofluorescence from a fluorescent measurement.

The IVIS Imaging Systems implement a subtraction method based on the use of blue-shifted background filters that emit light at a shorter wavelength. The objective of the background filters is to excite the tissue autofluorescence without exciting the

fluorophore. The background filter image is subtracted from the primary excitation filter image using the Image Math tool and the appropriate scale factor, thus reducing the autofluorescence signal in the primary image data. (For more details, see Chapter 9, page 153.) The assumption here is that the tissue excitation spectrum is much broader than the excitation spectrum of the fluorophore of interest and that the spatial distribution of autofluorescence does not vary much with small shifts in the excitation wavelength.

Figure F.20 shows an example of this technique using a fluorescent marker. In this example, 1×10^6 HeLa-luc/PKH26 cells were subcutaneously implanted into the left flank of a 6-8 week old female Nu/nu mouse. Figure F.21 shows the spectrum for HeLa-luc/PKH26 cells and the autofluorescent excitation spectrum of mouse tissue. It also shows the passbands for the background filter (DsRed Bkg), the primary excitation filter (DsRed), and the emission filter (DsRed). Figure F.20 shows the IVIS® images using the primary excitation filter, the background excitation filer, as well as the autofluorescent-corrected image.

The corrected image was obtained using a background scale factor of 1.4, determined by taking the ratio of the autofluorescent signals on the scruff of the animal. The numbers shown in the figures are the peak radiance of the animal background within the region of interest. In the corrected image, the RMS error is used to quantify the background. The signal-to-background ratio of the original fluorescent image (DsRed filter) is 6.5. The ratio increases to 150 in the corrected image, an improvement factor of 23. This improvement reduces the minimum number of cells necessary for detection from 1.5×10^5 to 6.7×10^3.

Figure F.20 Example of the autofluorescent subtraction technique using a background excitation filter.
a) primary excitation filter (DsRed), b) blue-shifted background excitation filter (DsRed Bkg), and c) corrected data. The corrected image was obtained by subtracting the scaled background filter image (multiplied by 0.47) from the primary filter image. The 6-week old female Nu/nu mouse was injected subcutaneously with 1×10^6 HeLa-luc/PKH26 cells in the left flank.

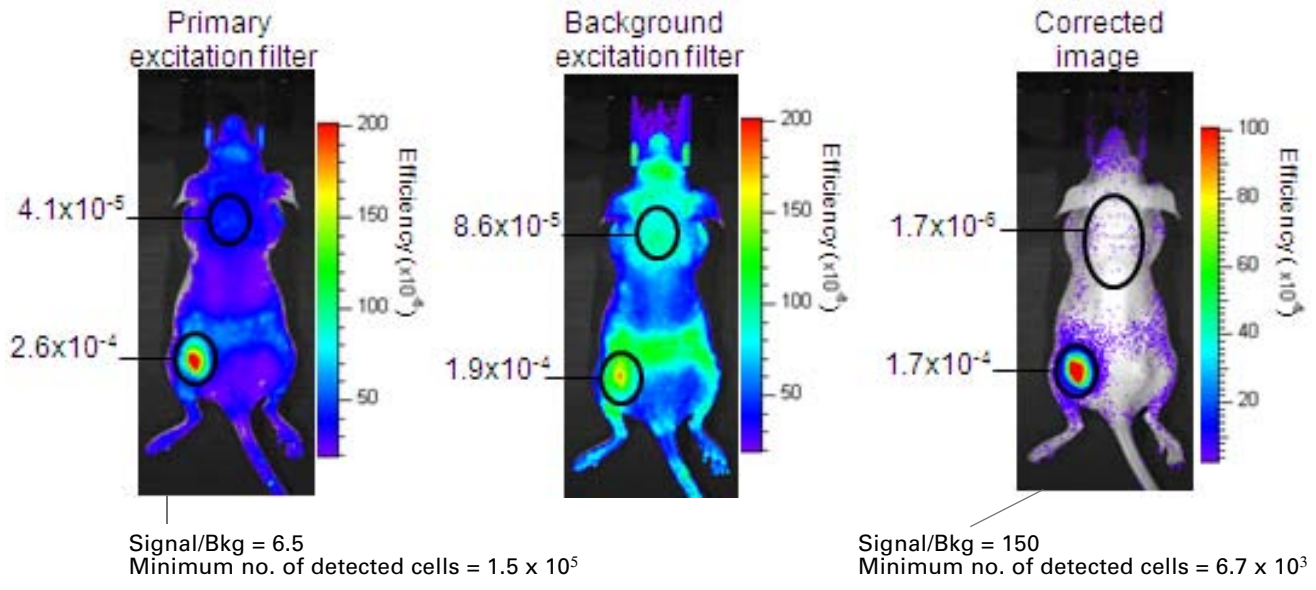
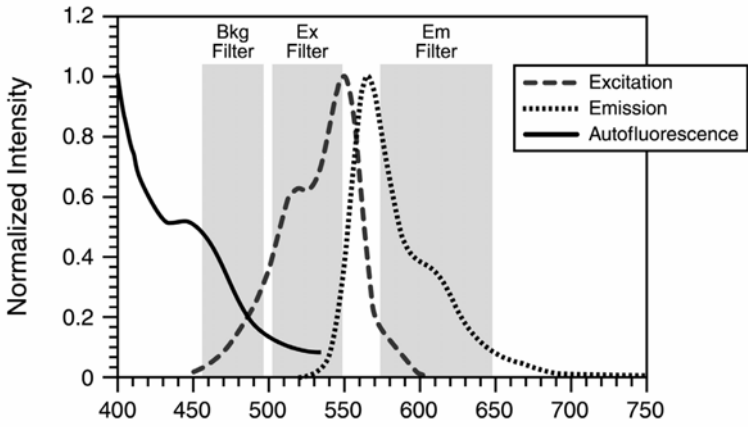


Figure F.21 Spectral data describing the autofluorescent subtraction technique using a background filter.
The graph shows the excitation and emission spectrum of PKH26 and the autofluorescent excitation spectrum of mouse tissue. Also included are the spectral passbands for the blue-shifted background filter (DsRed Bkg), the primary excitation filter (DsRed), and the emission filter used with this dye.



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Appendix G Planar Spectral Imaging

The unique spectral signatures of the luciferase emission spectrum and the optical properties of tissue enable the Living Image software to determine the depth and intensity of light sources inside a living animal. The planar spectral imaging algorithm relies on a diffusion model of light propagation in tissue and assumes a point source of light embedded in a flat surface approximation of the mouse. The algorithm is designed to provide a fast and robust method to approximate source location and brightness. The analysis requires two or more single-view images at wavelengths between 560 and 660 nm.

The Diffuse Tomography (DLIT) algorithm is a more complete and accurate model. It analyzes images of surface light emission to produce a three-dimensional (3D) reconstruction of the luminescent light sources in a subject. For more details on DLIT analysis, see Chapter 14, page 191 and Appendix H, page 317.

G.1 Planar Spectral Imaging Theory

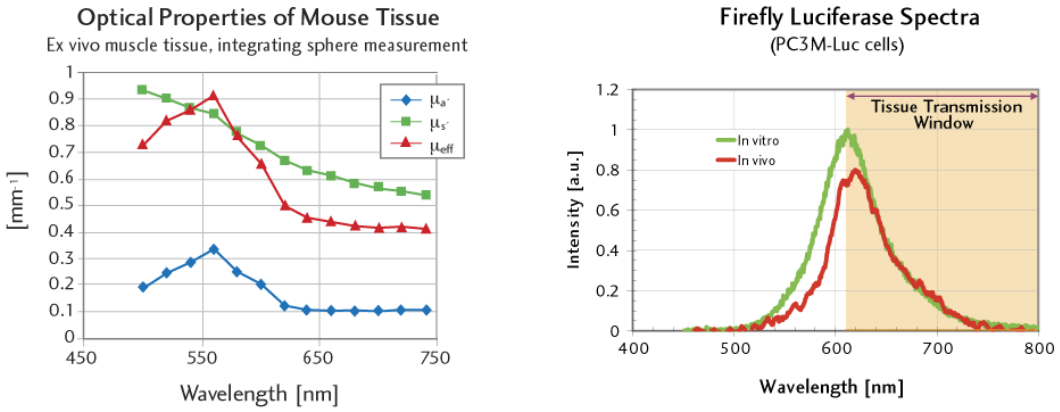
An image acquired on an IVIS Imaging System is a diffuse projection on the surface of the animal from the luminescent sources located deeper inside. Information about the depth of the luminescent cells can help quantify the source brightness and provide information on the location of the cells.

The Living Image software uses spectroscopic information from a single-view image to estimate the depth of the luminescent cells. The method takes advantage of the fact that firefly luciferase bioluminescence is emitted from 500 to 700 nm, a region of the spectrum where there are major contrasts in tissue optical properties (Figure G.1).

In this portion of the spectrum, tissue absorption drops off dramatically between 500-580 nm (green/yellow wavelengths) and 600-750 nm (red wavelengths), due mainly to the presence hemoglobin. As a result, the luminescent signal observed on the surface of the animal is dependent on both the wavelength and the thickness of the tissue through which it travels.

The depth and absolute photon flux of a single point source can be determined from two or more images acquired at different wavelengths using relatively simple analytical expressions derived from the diffusion model of the propagation of light through tissue.

Figure G.1 Optical Properties of Mouse Tissue and Firefly Luciferase Spectra
The luminescent signal from firefly luciferase (right) is emitted from wavelengths of 500-700 nm, which spans a region of the spectrum where there are major contrasts in the optical properties of mouse tissue (left). The firefly spectrum was measured at 37°C using PC3M cells.



Diffusion Model of Light Propagation Through Tissue

Light propagating through tissue undergoes scattering and absorption. The diffusion model assumes that scattering is the predominant phenomenon and the reduced scattering coefficient μ'_s >> absorption coefficient μ_a. This is valid mostly for wavelengths in the red and near infrared part of the spectrum. The model also assumes that the light is produced by a single point source and that the tissues are optically homogeneous.

Under these conditions, if we model the animal surface as flat and infinite in extent and integrate the light that is collected over the animal surface, the total integrated intensity I(λ) is reduced to a relatively simple expression:

$$I(\lambda) = SK(\lambda) \exp(-\ _{eff} d) \quad (1)$$

where S is the absolute total photon flux emitted by the luminescent source and d is the source depth.

The term μ_{eff} is the effective attenuation coefficient. It is determined by the tissue coefficient of absorption (μ_a) and reduced scattering (μ'_s) that quantify the two main phenomena light undergoes in tissue.

The function $K(\lambda)$ is a more complex expression that is derived from the model and includes terms that describe the effect of the tissue-air boundary on the light propagation. Both μ_{eff} and the function K are dependent on the wavelength, λ.

Equation 1 shows that if the total integrated intensity (ROI measurement) is measured at several wavelengths, it is proportional to an exponential function of the product of the depth and the optical property, μ_{eff}. Therefore, the steps to planar spectral image analysis include:

- Acquire two or more images at different wavelengths.
- Measure the total integrated intensity on each image.
- Fit the measured values to the exponential function of Equation 1.

The results of the fit are the total flux of the luminescence source S and the source depth d.

G.2 Optical Properties

Planar spectral image analysis requires prior knowledge of the tissue optical properties at the wavelength used at image acquisition. The two main optical parameters are the:

- *Absorption coefficient* (μ_a) that defines the inverse of the mean path before photons are absorbed by the tissue.
- *Reduced scattering coefficient* (μ'_s) that defines the inverse of the mean path before photons are scattered isotropically in the tissue.

The *effective attenuation coefficient* (μ_{eff}) is a function of the absorption and reduced scattering coefficients:

$$\mu_{eff} = (3\mu_a(\mu'_s + \mu_a))^{1/2} \quad (2)$$

Calculation of the function K in Equation 1 requires all three coefficients (μ_a, μ'_s, and μ_{eff}) as input. The function K includes a term called the *effective reflection coefficient* to account for the reflection of light at the air-tissue boundary due to a mismatch in the index of refraction. The tissue index of refraction is generally assumed to be close to 1.4.

The model assumes that the tissues are optically homogeneous and the Living Image software provides several factory set tissue optical property values to choose from.

G.3 Luciferase Spectrum

Analyzing spectrally filtered images requires knowledge of the spectral dependence of luminescent light emission. The luciferase luminescence spectrum was measured *in vitro* at 37° C and pH≈7 in various cell lines. This spectrum is used to normalize the photon flux values that the software measures at each wavelength.

Source spectra for several reporters are included in the database, including firefly, click beetle, renilla, and bacteria (Figure G.1).

NOTE

The firefly luciferase spectrum is temperature and pH dependent. The luciferase spectra included in the software are only valid for measurements performed at 37° C and pH 7.0-7.5. If you use other temperature or pH conditions for an experiment, the associated luciferase spectral curve is required for planar spectral image analysis. For more information on the pH and temperature dependence of the luciferase spectrum, please contact Caliper Corporation.

G.4 An Example of Planar Spectral Imaging

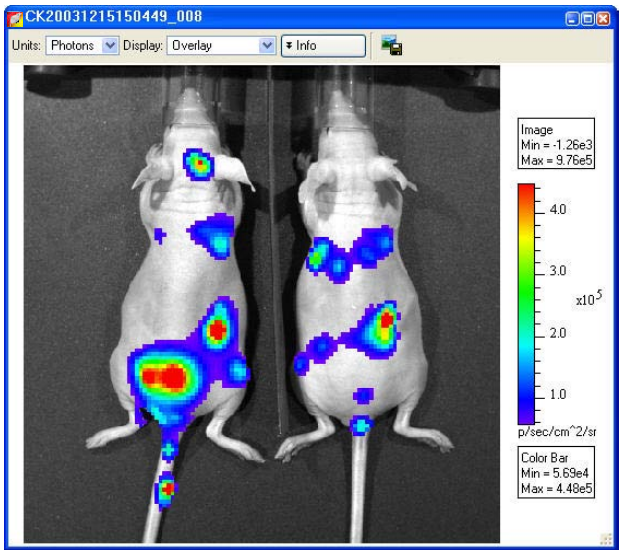
Melanoma cells were injected intravenously into the tail vein of nude mice. After 13 days, metastases developed in the lungs, kidney, and hind limb bone. An image sequence was acquired on the IVIS Imaging System 200 Series using filters at six wavelengths from 560 to 660 nm, in 20 nm intervals.

NOTE

When using the 560 nm and 580 nm band pass filters, tissue optics result in a larger attenuation of light (due mainly to hemoglobin absorption). A longer exposure time is recommended at these wavelengths.

Figure G.2 shows the metastasis sites. The signals from the lungs and right kidney are well defined in both animals. However, in the lower back area of the left mouse, the signals are in close proximity, causing an artifact in the planar spectral analysis.

Figure G.2 Metastatic sites in nude mice
Mice were imaged 13 days after a tail vein injection of $5x10^5$ B16F10 melanoma cells. Imaging parameters: high sensitivity binning, f/stop=1, FOV = C (13 cm), exposure time = 120 seconds at 560 and 580 nm, exposure time = 60 seconds at all other wavelengths. This resulted in signals of ~2000 counts on each image.



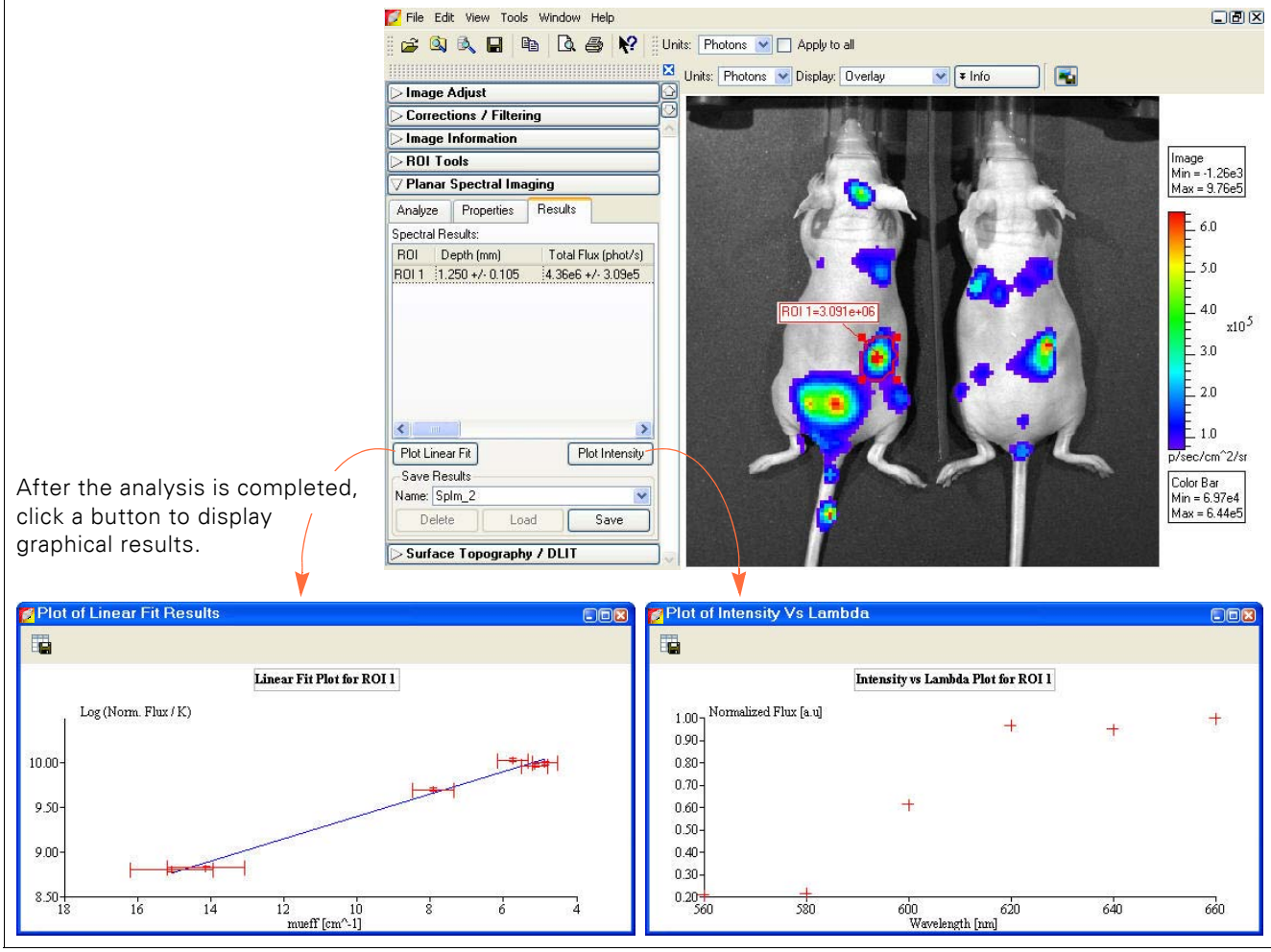
To perform the planar spectral analysis, draw a measurement ROI that captures the entire signal of each site of interest without including a neighboring metastasis (Figure G.3).

After the ROI is defined, start the planar spectral analysis (for more details, see page 157). The software:

- Measures the total flux inside the ROI on each filtered image.
- Normalizes the data to the luciferase spectrum (Plot of Intensity vs. Lambda, Figure G.4).
- Fits the normalized data to the analytical expression in Equation 1, page 310 where S = absolute total photon flux emitted by the luminescence source and d = source depth (Plot of Linear Fit Results, Figure G.4)

Figure G.3 Metastatic site
ROI includes the signal of the right kidney and separates it from other metastatic sites. The signal coming from the lower back area is spread out due to the presence of two bright spots. The dimmer signal in the lower bottom right of the image likely originates from the femoral bone of the animal.

Figure G.4 Planar spectral analysis results

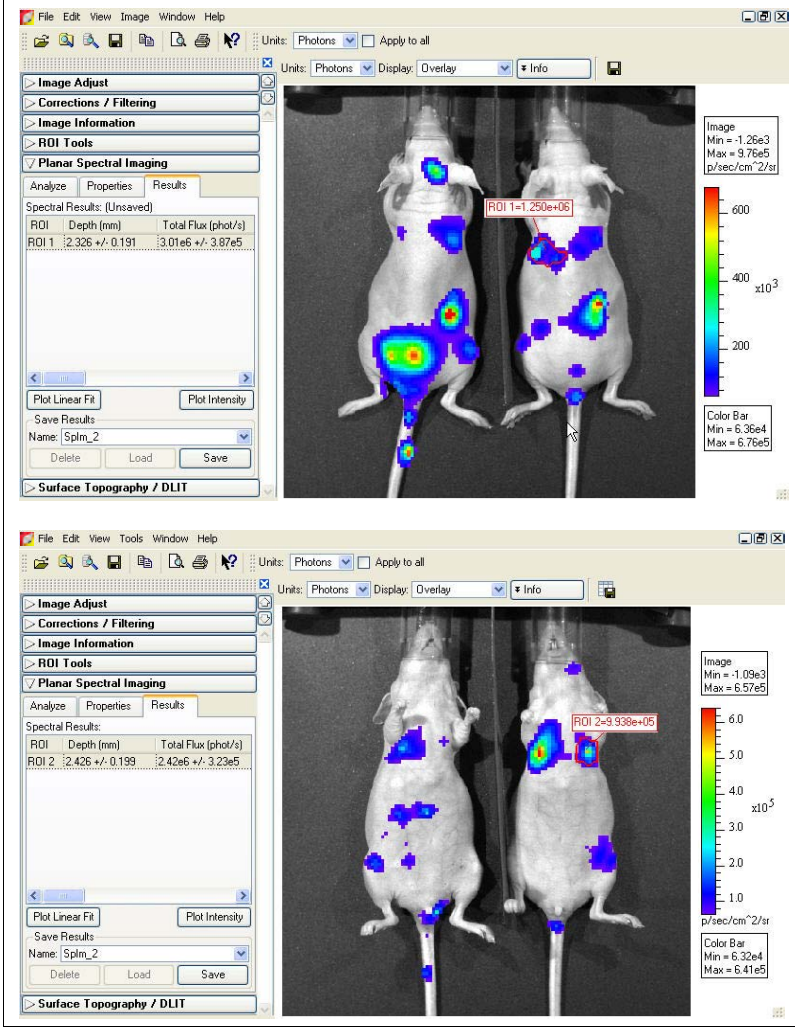


To estimate the cell count, divide the absolute photon flux by the flux per cell. This is best determined by making independent *in vitro* measurements of the cell line used in the experiment.

The Plot of Linear Fit Results is weighted by the uncertainty of the measured images and takes into account the uncertainty in the determination of the optical properties. The precision of the method is largely determined by the known precision of the optical properties. In most cases, the relative uncertainty in the depth determination is equal to the relative uncertainty in the optical properties.

An analysis of the dorsal and ventral views of the mouse left lung in **Figure G.5** results in total flux values that are very similar. The measured depth values are also close, indicating that the cells are distributed about the same distance from the front and back of the animal.

Figure G.5 Planar spectral analysis results
Top: Dorsal view of the left lung, bottom: ventral view of the left lung



G.5 Optimizing the Precision of Planar Spectral Analysis

The accuracy of the planar spectral analysis is highly dependent on the quality of the:

- Measured data for the firefly luciferase spectrum and the tissue optical properties.
- Fit of the experimentally measured total flux at each wavelength to $_{eff}$ (effective attentuation coefficient).

In general, more experimental values produce a better fit of the data. It is particularly important to be able to extract signals at all wavelengths to optimize the quality of the fit. If the software detects no signal above the animal background level at 560 nm and 580 nm (the wavelengths that absorb the most light), the dynamic range of the optical properties is reduced and with it, the precision of the fit.

If a luminescent signal is dim or buried deep in the tissue, it may barely exceed the tissue autoluminescence at the shorter, more absorbing wavelengths (560 and 580 nm). In this case, it is recommended that you subtract the tissue autoluminescence from the image data. (For more details on subtracting tissue autoluminescence, see Appendix E, page 288). It is also recommended that you inspect all images in the sequence to confirm that the luminescent signal is greater than the tissue autoluminescence. If the luminescent signal does not exceed the tissue autoluminescence at a particular wavelength, do not include that wavelength in the analysis.

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Appendix H DLIT & FLIT Reconstruction of Sources

Diffuse Luminescence Imaging Tomography (DLIT) is a technique that analyzes images of the surface light emission from a living subject to generate a three-dimensional (3D) reconstruction of luminescent light source distribution inside the subject.

Fluorescence Imaging Tomography (FLIT) analyzes images of surface light emission to generate a 3D reconstruction of fluorescent light source distribution inside the subject.

NOTE

To reconstruct luminescent sources, the Living Image software requires a photograph, a structured light image, and luminescent images obtained at two or more wavelength filters spanning the luciferase emission spectrum (for example, firefly luciferase 560-660 nm). To reconstruct fluorescent sources, the software requires a structured light and fluorescent images obtained using the same excitation and emission filters at different transillumination source positions on the IVIS Spectrum.

To localize and quantify the light sources in a subject, the software:

- Reconstructs the subject surface topography (*surface*) from structured light images. The surface is defined by a set of connected polygons or surface elements.
- Maps the surface radiance (photons/s/cm^2/steradian) to the photon density (photons/mm^3) just beneath the surface of each element of the surface. For NTF Efficiency data from normalized transmission fluorescence data, the NTF Efficiency 2D data is mapped to the 3D surface.
- Divides the interior of the subject into a solid surface of volume elements or *voxels*. Each voxel is considered to contain a point light source at its center that contributes to the photon density at each surface element.
- Defines equations that relate the source strength of each voxel to the measured data (photon density or NTF Efficiency) at each surface element.
- Determines the optimum approximate solution to the system of linear equations to reconstruct the source strength in each voxel.

H.1 Determining Surface Topography

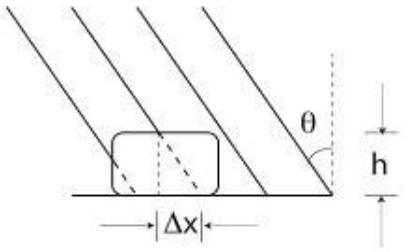
The software determines the surface topography from a structured light image. Parallel laser lines are projected onto the subject to produce a structured light image (Figure H.1).

NOTE

If the Structure option is chosen in the Control panel, a structured light image is automatically acquired.

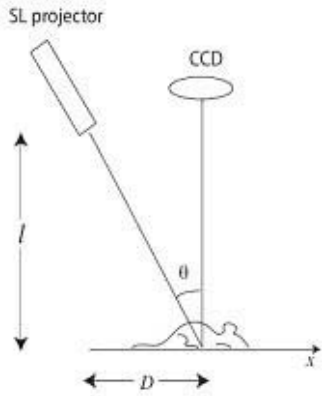
The surface topography of the subject is determined by analyzing the displacement (Δx) or bending of the laser lines as they pass over the subject. The displacement is defined as the difference between where the line should fall on the stage in the absence of the subject and where it appears in the image due to occlusion by the subject.

Figure H.1 Parallel laser lines projected onto a subject. Given knowledge of the angle θ, the height of the subject (h) can be determined by analyzing the displacement, Δx, of the laser lines as they pass over the object.



The parallel lines are projected onto the surface of the subject at an angle (θ). The angle is known by instrument calibrations of the distance between the structured light projector and the optical axis (D) and the distance between the stage and the structured light projector (l) (Figure H.2).

Figure H.2 Structured light projector and subject



D and l form two perpendicular sides of a triangle giving:

$$\tan \theta = D/l$$

Together Δx and h comprise a smaller version of this triangle. The height (h) can be determined from:

$$h = \Delta x/\tan \theta$$

by measuring the displacement Δx.

The software utilizes fast numerical methods to rapidly evaluate Δx over the entire image to determine the surface topography. The surface topography determination is limited to the topside of the object facing the lens.

H.2 Converting Light Emission to a Photon Density Map

The input data to the FLIT algorithm for 3D reconstruction of fluorescent light sources includes:

- A surface that defines the surface of the subject.
- A sequence of images acquired at different transillumination source positions using the same excitation and emission filter at each position.Use the Imaging Wizard to acquire the images.

The input data to the DLIT algorithm for a 3D reconstruction of luminescent light sources includes:

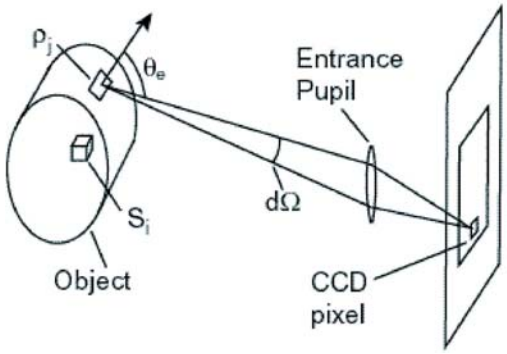
- A surface that defines the surface of the subject.
- A sequence of two or more images of the light emission from the surface of the subject acquired at different filter bandwidths (Table H.1). Use the Imaging Wizard to acquire the images.

Table H.1 IVIS System filters for luminescence and fluorescence tomography

IVIS Imaging System	Filters	Bandwidth
200 Series	6 emission filters, 550-670 nm	20 nm
Spectrum	10 excitation filters, 415-760 nm	30 nm
	18 emission filters, 490-850 nm	20 nm

The IVIS Imaging System 200 Series and the IVIS Spectrum are absolutely calibrated so that the electron counts on each CCD pixel can be mapped back to the surface of the object to produce an absolute value of the surface radiance (photon/s/cm^2/steradian) from each imaged surface element (Figure H.3).

Figure H.3 Light emission from a surface element passes through the lens entrance pupil and is recorded in the image



The imaging system collects the light emitted from the surface element at an angle (θ_e) (measured with respect to the normal to the surface element) into the solid angle $d\Omega$ subtended by the entrance pupil. The value of the surface radiance $L(\theta_e)$ is directly related to the photon density ρ (photons/mm^3) just inside the surface of the element. FLIT analysis uses NTF Efficiency data and takes into account the photon density of both the fluorescent image and transmission image.

H.3 Defining the Linear Relationship Between a Source and Photon Density or NTF Efficiency

The software divides the interior of the subject into a solid mesh of volume elements (*voxels)*. Each voxel is considered to contain a point light source at its center. The index i enumerates the set of voxels. S_i is the value of the strength of the point source inside the i^{th} voxel. The solid mesh defines a collection of point sources that approximate the actual source distribution. The accuracy of the approximation is improved by increasing the density of the solid mesh.

The reconstruction method is based on the principle that there is an approximately linear relationship between the source strength in each voxel (S_i) and the photon density or NTF Efficiency (ρ_j) at each surface element described by a Green's function G_{ij}. The photon density at the j^{th} surface element is the sum of the contributions from all the voxels:

$$\rho_j \cong \sum_i G_{ij} S_i \tag{1}$$

The Green's function contains information about the transport of photons through the tissue and the effects of the tissue-air boundary. By using a planar boundary approximation, the Green's function can be calculated analytically as a solution to the diffusion equation. Having an analytic expression for G allows Equation 1 to be computed very rapidly.

H.4 Determining the Best Approximate Solution to the Linear System

Once the Green's functions, G_{ij}, are known, the goal is to solve Equation 1 for the source strength S_i in each voxel. The DLIT and FLIT algorithms attempt to minimize χ^2 (Equation 2) while requiring that the source strength in each voxel is positive (Equation 3).

$$\chi^2 = \sum_j \frac{1}{\sigma_j^2} \left[\rho_j - \sum_i G_{ij} S_i \right]^2 \tag{2}$$

$$S_i \geq 0 \tag{3}$$

A Non-Negative Least Squares algorithm is used to find the approximate solution which minimizes χ^2. In order to reduce the number of variables in the problem, the code only uses surface elements with signal above a certain threshold and only keeps the voxels that contribute significantly to these surface elements.

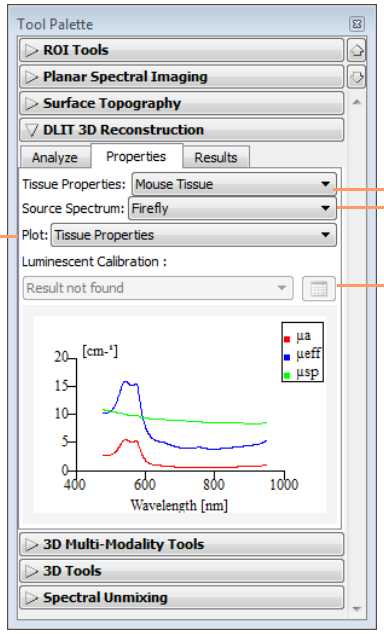
H.5 Source & Tissue Properties

DLIT analysis of spectrally filtered images requires knowledge of the spectral dependence of luminescent light emission. Table H.2 shows the factory set source spectra provided by the software.

NOTE

The source spectra is not an input to the 3D reconstruction of fluorescent sources.

Figure H.4 DLIT 3D reconstruction tools, Properties tab



Choose "Source Spectrum" from the Plot drop-down list to display the selected spectrum. This is required for DLIT.

Select Mouse Tissue or XPM-2 (mouse phantom) from the drop-down list.

Select a luminescent source spectrum

Browse for a 3D Quantification Database For more details on the database, see page 185.

Table H.2 Source spectra

Source Spectrum	Description
Bacteria	Bacterial luciferase
CB Green	Click beetle green luciferase
CB Red	Click beetle red luciferase
Firefly	Firefly luciferase
hRenilla	Sea pansy (*Renilla reniformis*) luciferase
Tritium Bead 5	Phosphor-coated glass bead containing tritium gas. Spectrum for bead #5.
XPM-2-LED	LED in the XPM-2 mouse phantom.

NOTE

The firefly luciferase spectrum is dependent on temperature and pH. The data provided are valid only for measurements performed at 37° C and at pH 7.0-7.5. Selecting other temperature and pH conditions for a specific experiment requires the use of the associated spectral curve for the spectral analysis. For more information about pH and temperature dependence of the luciferase spectrum, please contact Caliper Life Sciences technical support.

You can view tissue optical property values ($_{eff}$, $'_s$, $_a$) in the Tissue Properties drop-down list. The tissue properties are plotted as a function of wavelength. Select the optical property descriptor most representative of the imaged subject. "Mouse Tissue" is a good choice for general reconstructions *in vivo*.

NOTE

Default tissue optical properties and source spectra are specified in the Preferences box. For more details, see Appendix B, page 273.

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Appendix I IVIS Syringe Injection System

The IVIS Syringe Injection system is designed for use with the IVIS Kinetic Imaging System. You can control the infusion pump in the Living Image software or manually.

For more details on the setup and manual control of the infusion pump, see the *IVIS Syringe Injection System* instructions from Caliper or the *PHD 22/2000 Syringe Pump Series User's manual* from Harvard Apparatus. Both are included on the Living Image installation CDROM.

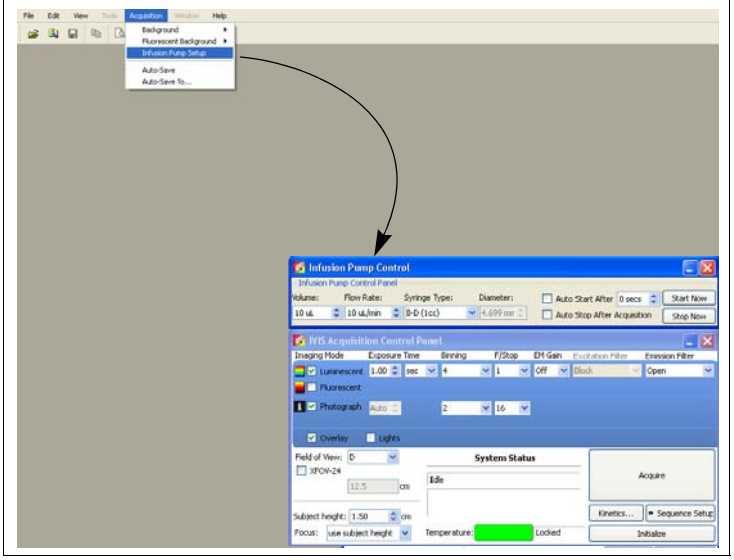
The IVIS Syringe Injection system can be used during kinetic or still image acquisition; however, subjects must remain immobile.

I.1 Controlling the Infusion Pump

After the IVIS Kinetic Imaging System is initialized and locked, you can access the infusion pump controls.

1. Select **Acquisition** → **Infusion Pump Setup** on the menu bar.
   — The Infusion Pump control panel appears above the IVIS acquisition control panel.

Figure I.1 Display the Infusion Pump control panel

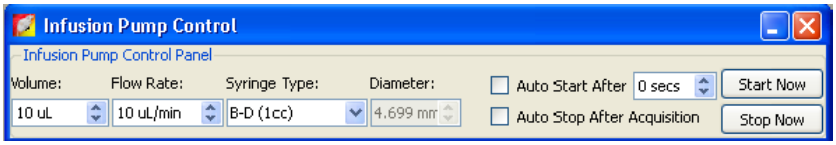


NOTE

If you are going to acquire kinetic data, open the infusion pump control panel before you open the kinetic acquisition control panel. When the kinetic control panel is open, the Acquisition menu is unavailable.

2. Set the volume and flow rate.

Figure I.2 Volume & flow rate settings



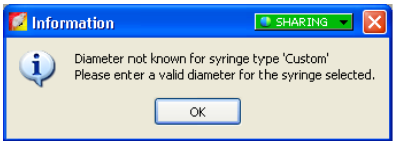
3. Make a selection from the Syringe Type drop-down list (the associated syringe diameter is automatically entered).

   To enter a custom syringe:

   a. Select **Custom** from the drop-down list.

   b. Click **OK** in the dialog box that appears.

Figure I.3 Confirmation message for a custom syringe



   c. Enter the syringe diameter in the infusion pump control panel.

NOTE

Custom syringe information that is entered in the infusion pump control panel is not saved to the system.

4. To automatically start the infusion pump after data acquisition begins, choose **Auto Start After** and enter the amount of seconds. For example, enter 10 to start infusion 10 seconds after acquisition begins.

   To manually start infusion, click **Start Now**.

5. To automatically stop infusion, choose **Auto Stop After Acquisition**. To manually stop infusion at any time, click **Stop Now**.

   If the auto stop option is not chosen and you do not manually stop the pump, the pump continues to run after acquisition ends until the specified infusion volume is reached.

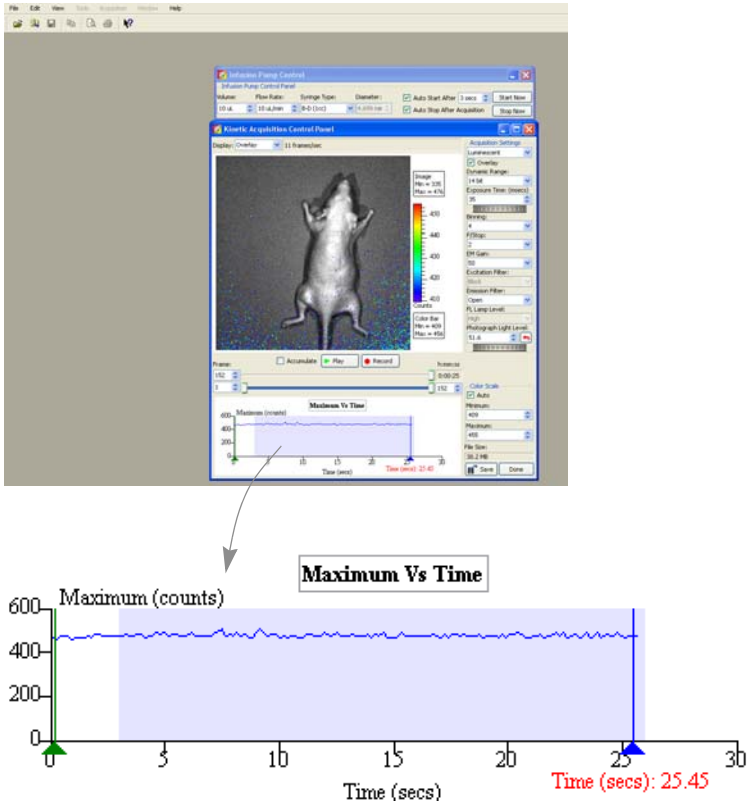
NOTE

The information in the infusion pump control panel is saved in the click info file. During acquisition, if you start infusion, then manually stop and restart the infusion, only the last actual start and stop is saved to the click info file, not the start/stop settings in the panel.

I.2 Tracking Infusion in the Maximum vs. Time Graph

During kinetic acquisition, the blue shaded region in the Max vs. Time graph indicates the infusion period. During acquisition, if you start infusion, then manually stop and restart infusion, only the last actual start and stop is recorded in the Maximum vs. Time graph. The graph stops recording infusion when acquisition stops (even though the pump may not be stopped).

Figure I.4 Tracking infusion



I.3 Closing the Infusion Pump Control Panel

1. Close the kinetic control panel.
2. Click **Acquisition** → **Infusion Pump Setup** on the menu bar.
   — The check mark is removed and the panel closes.

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Appendix J Menu Commands, Tool Bar, & Shortcuts

Figure J.1 Living Image toolbar

Table J.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.
File → Browse		Displays the Browse For Folder box so that you can select and an image data folder. The selected folder is displayed in the Living Image Browser.
File → Browse 3D Volumetric Data		Displays the Browse For Folder box so that you can select and a volumetric data folder (for example, DICOM format, TIF data). The selected folder is displayed in the 3D Browser.
File → Save		Saves (overwrites) the AnalyzedClickInfo text file to update the analysis parameters, but the original image data files are not altered.
File → Save As		Displays the Browse For Folder box so that you can specify a folder in which to save the image data. The original data is not overwritten.
File → Import → 3D Surface		Opens a dialog box that enables you to import a surface. **Note:** This menu item is only available if "Show Advanced Options" is selected in the Preferences (see page 266).
File → Import → 3D Voxels		Opens a dialog box that enables you to import a source volume. **Note:** This menu item is only available if "Show Advanced Options" is selected in the Preferences (see page 266).
File → Import →Organ Atlas		Opens a dialog box that enables you to import an organ atlas (.iv, .dxf, .stl).
File → Export → Image/ Sequence as DICOM		Opens the Browse for Folder dialog box that enables you to export the active image data to DICOM format (.dcm).
File → Export → 3D Surface		Opens a dialog box that enables you to save the 3D surface of the active data to a file such as Open Inventor format (.iv).
File → Export → 3D Voxels		Opens a dialog box that enables you to save the voxels from the active data.
File → Export → 3D Scene as DICOM		Opens a dialog box that enables you to save a 3D reconstruction and/or surface in DICOM format. The Multi-Frame DICOM option supports 3D CT reconstruction in third party software.
File → Print		Displays the Print box.
File → Print Preview		Displays the Print Preview box that shows what will be printed.
File → Recent Files		Shows recently opened data sets. **Note:** The number of files displayed can be set in the Preferences box (select **Edit →Preferences** and click the General tab).
File→ Exit		Closes the Living Image software.
Edit → Copy		Copies the active image window to the system clipboard.
Edit → Image Labels		Opens the Edit Image Labels dialog box that enables you to edit the label set information for the active data.
Edit → Preferences		Opens the Preferences box.

Table J.1 Menu bar commands and toolbar buttons (continued)

Menu Bar Command	Toolbar Button	Description
View → Tool Bar		Choose this option to display the toolbar.
View → Status Bar		Choose this option to display the status bar at the bottom of the main window.
View → Tool Palette		Choose this option to display the Tool Palette.
View → Activity Window		Displays the Activity window at the bottom of the main application window. The Activity window shows a log of the system activity.
View → Image Information		Displays the Image Information box that shows the label set and image acquisition information for the active data.
View → ROI Properties		Displays the ROI Properties dialog box.
View → ROI Measurements		Displays the ROI Measurements table.
View → Volume Data Viewer		Enables you to open and view DICOM data.
View → Image Layout Window		Opens the Image Layout window that enables you to paste an image of the active data in the window.
Tools → 3D Animation		Opens the 3D Animation window that enables you to view a preset animation or create an animation.
Tools → Longitudinal Study		Opens the Longitudinal Study window for side-by-side comparisons of DLIT or FLIT results.
Tools → Well Plate Quantification for ...		Opens the Well Plate Quantification window.
Tools → Image Overlay for...		Opens the Image Overlay window for the active data.
Tools → Colorize		Opens the Colorized View tab for the active sequence.
Tools → Image Math for...		Opens the Image Math window for the active data.
Acquisition → Background → Measure Dark Charge		Opens a dialog box that enables you to acquire a dark charge measurement.
Acquisition → Background → Add or Replace Dark Charge		Opens a dialog box that enables you to select an instrument luminescent background. This background measurement is subtracted from luminescent images.
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.

Table J.1 Menu bar commands and toolbar buttons (continued)

Menu Bar Command	Toolbar Button	Description
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 data set of the current image, replacing any existing background image that may be present in the data set.
Acquisition → Fluorescent Background → View Available Fluorescent Background		Opens a dialog box that displays the fluorescent background measurements for the system. If a fluorescent background is selected, the "Fluorescent Background Subtraction" option appears in the Corrections/ Filtering tool palette. Choose the "Fluorescent Background Subtraction" option to subtract the user-specified background measurement from the image data.
Acquisition → Fluorescent Background → Clear Available Fluorescent Background		Opens a dialog box that enables you to remove the fluorescent background measurements from the system.
Acquisition → Auto-Save		If Auto-Save is selected, all images are automatically saved to a user-selected folder.
Acquisition → Auto-Save To		Opens a dialog box that enables you to select a folder where images will be saved to automatically.
Window → Close		Closes the active image window.
Window → Close All		Closes all image windows.
Window → Cascade		Organizes the open image windows in a cascade arrangement (see page 88).
Window → Tile		Organizes the open image windows in a tiled arrangement (see page 88).
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 User Manual.
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 Caliper LS web page for In Vivo Imaging Reagents.
Help → About Living Image		Displays information about the Living Image software and Caliper technical support contact information.
		Click this button, then click an item in the user interface to display information about the item.

Table J.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.

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