Living Image® Software

User's Manual

Version 3.2

PN 125112

Contents

1 Welcome

The Living Image® 3.2 software controls image acquisition on the IVIS® Kinetic. The application also provides tools for optimizing image display and analyzing images or kinetic data.

1.1 What's New In the Living Image 3.2 Software

The Living Image 3.2 software enables kinetic data acquisition on the IVIS® Kinetic and provides tools for visualizing and analyzing kinetic data.

New Feature or Updated Tool	See Page
Acquire and analyze luminescent or fluorescent signals in real-time	37
14-bit or 16-bit dynamic range	39
EM gain option multiplies the signal for fast imaging applications	40
Maximum signal vs. time graph plots the maximum signal in each frame of the kinetic data and provides a convenient way to see signal trends	41
View the cumulative signal to track signal changes in real time	40
Kinetic ROIs are applied to each frame in a kinetic data set and are displayed during kinetic data playback	94
Edit kinetic data	42
Save kinetic data in DICOM format (.dcm) or save kinetic images to a movie (for example, .mpg4)	43

1.2 About This Manual

This user manual explains how to acquire and analyze images or kinetic data on the IVIS® Kinetic. 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 the IVIS Kinetic, please see the appropriate *IVIS Kinetic 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.
A dash (—) precedes the description of the system response to a procedure.	— The main window appears.
Document names are italicized.	*Living Image Software User's Guide*
Note information	**NOTE** A note presents pertinent details on a topic. or **Note:** Notes 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 3.2 Software User's Manual (.pdf).

1.3 Contacting Caliper Technical Support

If you need technical support, please contact Caliper at:

Telephone:	1.877.LabChip (877.522.2447) Toll Free in the United States
	1.508.435.9761
E-mail:	tech.support@caliperls.com
Fax:	1.508.435.0950
Address:	Caliper Life Sciences
	68 Elm Street
	Hopkinton, MA 01748
	USA

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2 Getting Started

This chapter provides a brief overview of images and image analysis. It also explains how to start the Living Image® software and initialize the IVIS® imaging system.

2.1 Overview of Imaging & Image Analysis

For bioluminescence imaging, the imaging system acquires a photographic image and a bioluminescence image. The Living Image software automatically coregisters the images to generate an *overlay* image (Figure 2.1). For fluorescence imaging, the IVIS® imaging system acquires a photographic and fluorescence image that are used to generate an overlay image.

Table 2.1 Images used to generate an overlay image

Image Type	Description
Photographic	A short exposure of the subject illuminated by the lights located at the top of the imaging chamber (Figure 2.2). The photographic image is displayed as a grayscale image.
Bioluminescence	A longer exposure of the subject taken in darkness to capture low level bioluminescence emission. The bioluminescence image is displayed in pseudocolor that represents intensity (Figure 2.1). For more details on bioluminescence image data, see Appendix D, page 211.
Fluorescence	An exposure of the subject illuminated by filtered light. The target fluorophore emission is captured and focused on the CCD camera. Fluorescence 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 223

Figure 2.3 shows an example workflow on an IVIS® Imaging System.

| Photographic image | Bioluminescent image | Overlay image |
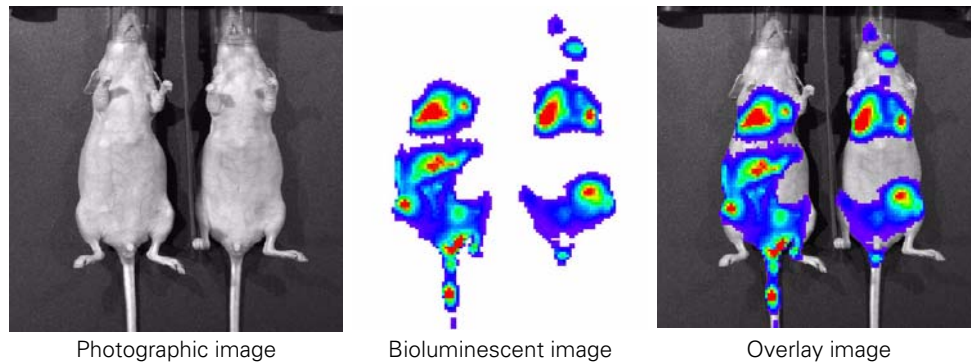
Figure 2.1 Example bioluminescence overlay image
The Living Image® software automatically coregisters the photographic and bioluminescence images to generate the overlay image.

Illumination LEDs

Camera lens opening

Sample stage
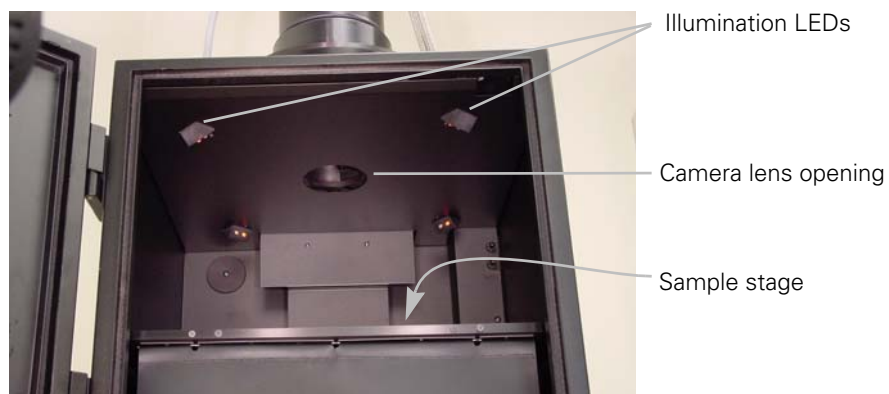
Figure 2.2 IVIS® Imaging System 100 Series, interior view.
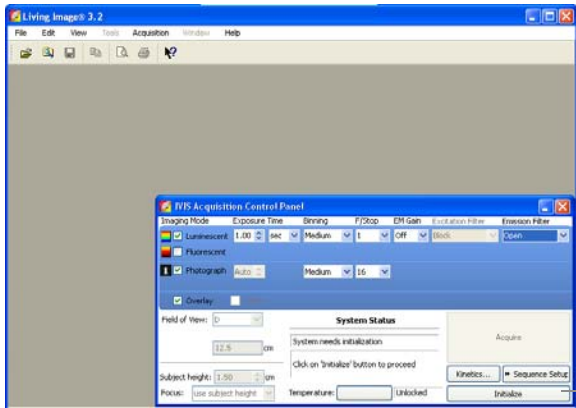
❶ Start the Living Image® software. In the control panel that appears, initialize the IVIS® imaging system.
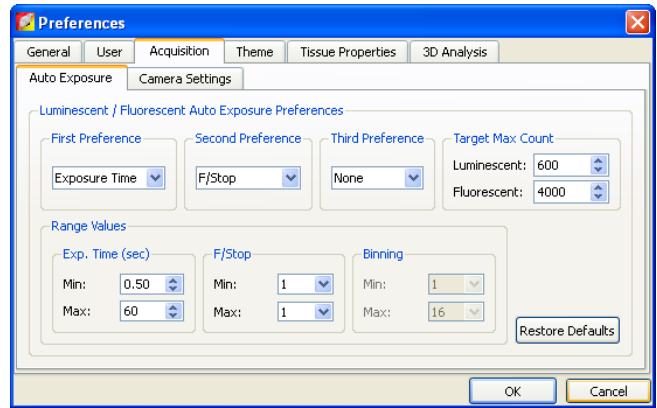
❷ Confirm the default user preferences or specify new preferences.
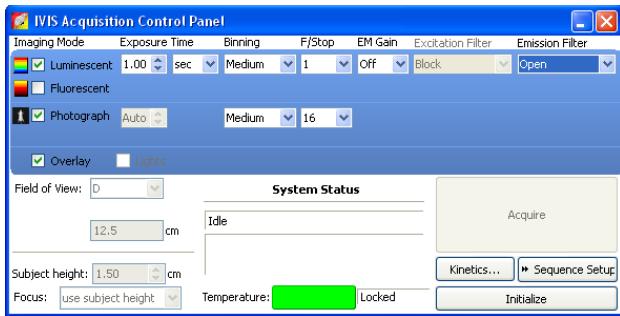
❸ Specify acquisition settings for a single image, image sequence, or kinetic sequence.

❹ Acquire the image(s) or kinetic sequence.
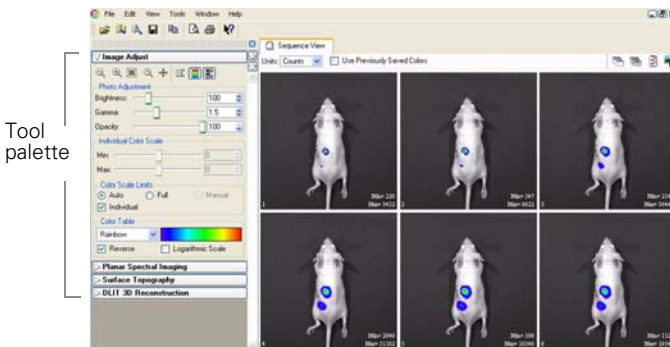
Tool palette

❻ View the image(s) or kinetic data. Analyze an image (Table 2.2), image sequence (Table 2.3), or kinetic data
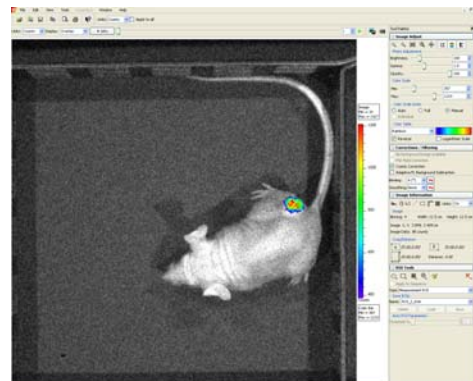
Figure 2.3 Example imaging and analysis workflow

Table 2.2 Image or kinetic sequence analysis tools

Tool Palette	Use The Tools to...	See Page
Image Adjust	• Tune the photograph brightness, gamma (similar to contrast), or opacity • Set the image display color scale min and max • Select a color table for image display	62
Corrections/Filtering	• Subtract dark background from the image data • Apply flat field correction to the image data • Specify pixel binning • Smooth the pixel signal	66
Image Information	• Display x,y coordinates and intensity data at a user-selected location on the image • Display a histogram of pixel intensities in an image • Plot the intensity (y-axis) at each pixel (x-axis) along a user-specified line in the image • Measure distance in an image	67
ROI	• 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)	81

Table 2.3 Analyzing image sequences

Analysis	Description	Wizard Available	See Page
Planar Spectral Image Analysis	Determines the average depth and total photon flux of a bioluminescent point source in a user-specified region of interest. Analyzes a sequence of bioluminescent images acquired using different emission filters.	Yes	121
Image Math	A method for mathematically combining two images (add, multiply, or subtract). Use image math to: • Remove autofluorescence from a fluorescent image • Display multiple bioluminescent or fluorescent images on the same photographic image so that you can view multiple reporters in one image	No	118
Spectral Unmixing	Removes tissue autofluorescence from a fluorescence image. Analyzes a sequence of fluorescence images acquired using the fluorophore excitation filter and several different emission filters. Spectral unmixing can be applied to images acquired using epi-illumination (excitation light above the stage) or transillumination (excitation light below the stage).	Yes	121
Point Source Fitting	Estimates the optical properties of tissue, the location and power of a point source, or the fluorescent yield of fluorophores.	Yes	143
3D Fluorescence Imaging Tomography (FLIT™)	A 3-dimensional reconstruction of the image (*tomographic analysis*) that estimates the location and intensity of a fluorescent light-emitting source. Analyzes a sequence of fluorescent images acquired on the IVIS Spectrum at different transillumination points (excitation light below the stage).	Yes	152
3D Diffuse Luminescence Tomography (DLIT™)	A 3-dimensional reconstruction of the subject (*tomographic analysis*) that estimates the depth and intensity of a bioluminescent light-emitting source.	Yes	156

> **NOTE**
>
> For more details on sequence setup using the imaging wizard, see page 24.

2.2 Starting the Living Image® Software

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: C:Programs:Xenogen:Living Image 3.2

Macintosh: Applications:Xenogen:LivingImage 3.2

To start the software:

1. **PC Users:** Click the Windows Start menu button ⊞ start and select **All Programs →
   Living Image**. Alternatively, click the Living Image® software desktop icon.

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

   - The main window appears.

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 (see next page).
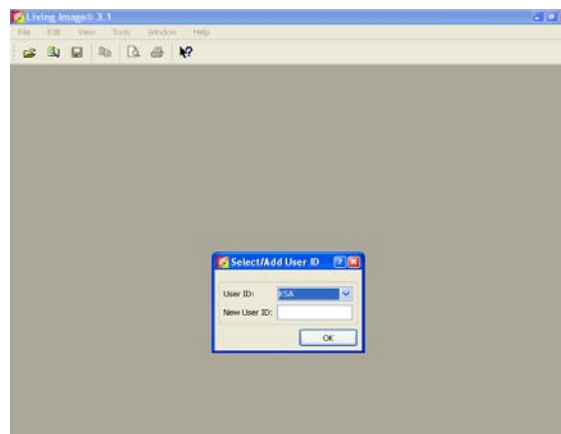
Menu bar (for more details, see Appendix J, page 261)

Toolbar
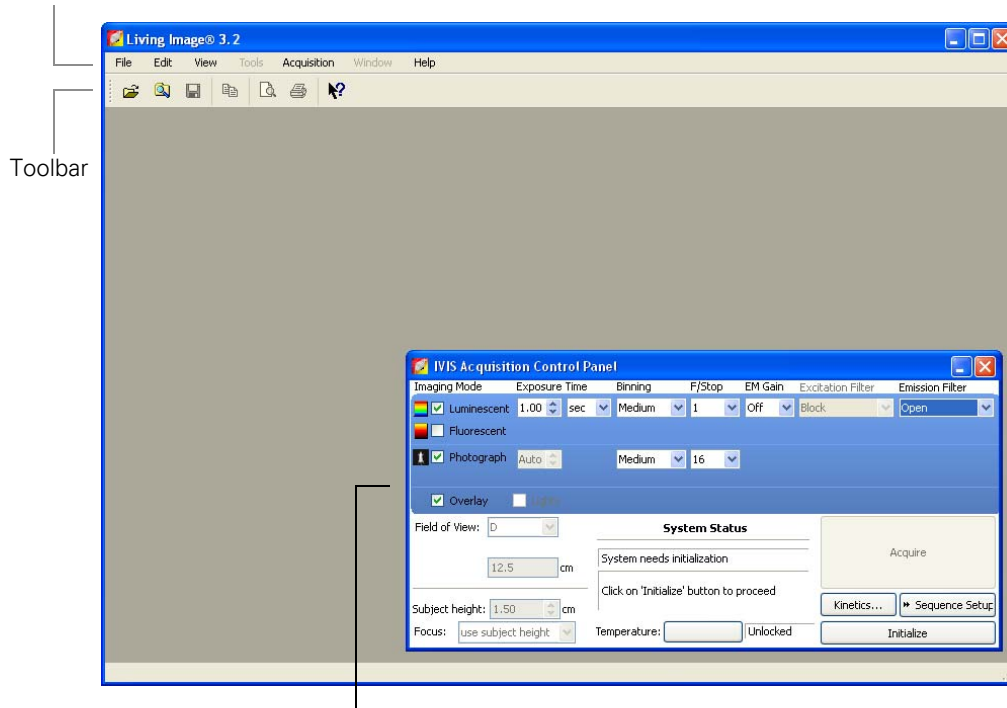
The acquisition control panel when the workstation controls the IVIS® Imaging System (for more details, see Appendix A, page 191).

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 only the analysis features of the Living Image software.

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 3.2 Software User's Manual (.pdf).

2.3 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 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 the hardware manual for your IVIS Imaging System.

To initialize the imaging system:

1. Start the Living Image software (double-click the 🖼 icon on the desktop).

2. In the control panel that appears, click **Initialize**.
   - You will hear the motors move.
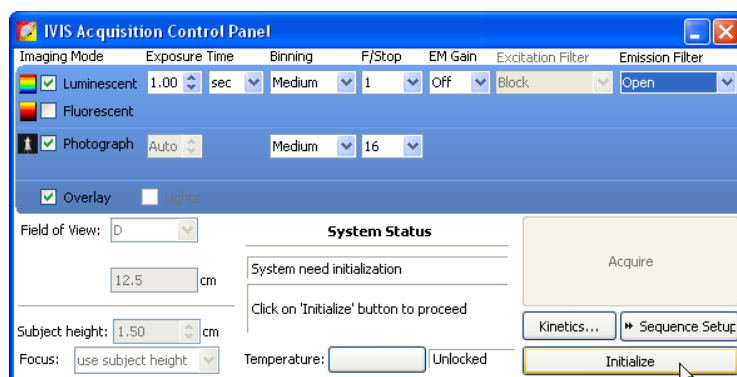
NOTE

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

2.4 Checking the System Temperature

The temperature box in the IVIS acquisition control panel indicates the temperature status of the charge coupled device (CCD) camera (Figure 2.4). After you initialize the system, 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 stage temperature on the IVIS® imaging system is 37° C, but may be set to a temperature from 25-40° C.
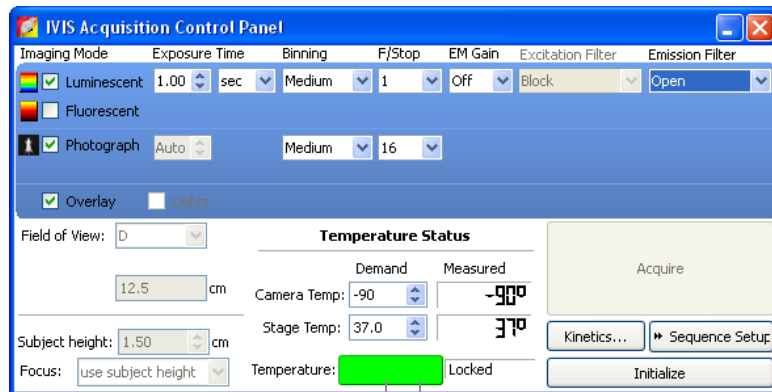
Click the temperature box to view the demand and measured temperatures of the CCD camera and stage.

Temperature box color indicates:

System not initialized.

System is initialized, but CCD camera temperature is out of range.

System is initialized and CCD camera is at or within acceptable range of the demand temperature and locked. The system is ready for imaging.

Figure 2.4 IVIS® acquisition control panel

NOTE

The items in the IVIS System 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 191.

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

2.5 About the IVIS Acquisition Control Panel & Auto Exposure Feature

The control panel (Figure 2.5) provides the image acquisition functions. For details on the imaging parameters in the control panel, see Appendix A, page 191.

The auto exposure setting is useful in situations where the signal strength is unknown or varies widely, for example during a time course study. When you choose auto exposure (Figure 2.5), 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 bioluminescence or fluorescence image. However, you can modify the auto exposure preferences to meet your needs. For more details, see page 199.

To acquire an image using auto exposure, click the arrow and select **Auto**.

Luminescence imaging settings

Fluorescence imaging settings

Photographic imaging settings

Structured light imaging settings
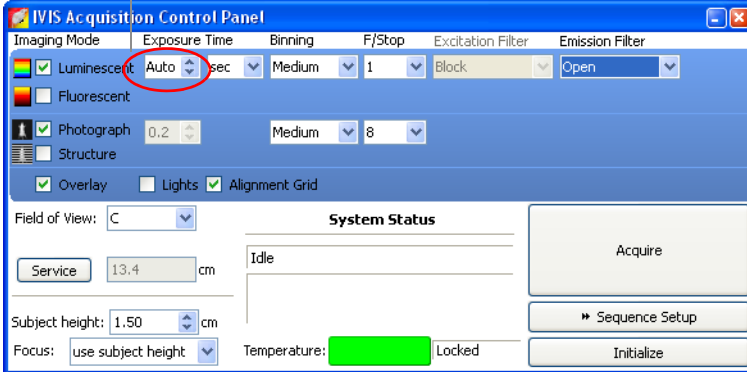
Figure 2.5 IVIS acquisition control panel, auto exposure selected

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.

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3 Acquire an Image or Image Sequence

The IVIS® Imaging System is ready for image acquisition after the system is initialized and the CCD camera reaches operating (*demand*) temperature (*locked*).

3.1 Acquire a Bioluminescence Image

1. Start the Living Image software (double-click the 🖼 icon on the desktop).

2. Initialize the IVIS System and confirm or wait for the CCD temperature to lock. (For more details, see page 11.)
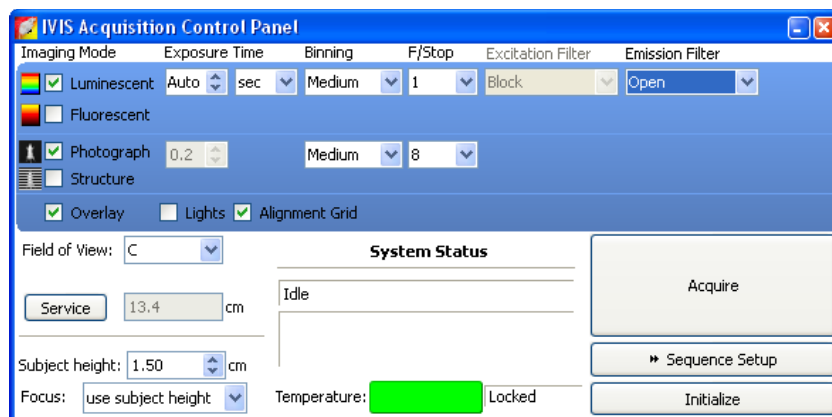
3. Put a check mark next to **Luminescent**.

4. Confirm that Excitation Filter setting is **Block** and the Emission Filter setting is **Open**.

5. Select the **Auto** exposure time (click the 🔽 arrow). Alternately, manually set the exposure, binning, and F/Stop. (For more details on the control panel settings, see page 191.)

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 160.

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).

   or

   • Select **Manual focus** from the Focus drop-down list. (For more details on manual focusing see page 34.)

8. **Set the photographic image settings:**

   a. Choose the Photographic option.

   b. Enter an exposure time or choose the Auto option.

   c. Confirm the binning and f/stop defaults or specify new settings for the photographic image.

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 22.)

10. When you are ready to acquire the image, click **Acquire**. During image acquisition, the **Acquire** button becomes a **Stop** button. To cancel the acquisition, click **Stop**.

    - The image window appears (Figure 3.1). If this is the first image of the session, you are prompted to choose an autosave location.

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

    - 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).

12. In the Edit Image Labels box that appears, enter information for the image label and click **OK** (Figure 3.1). If you do not want to enter label information, click **Cancel**.

Tool palette

Image label

Color bar

In the color bar, check the image min and max to determine whether the signal of interest is above the noise level and below CCD saturation. For 16 bit imaging, a signal ranging from ~600 - 60,000 counts is recommended; for 14-bit imaging, ~600-16,000 counts are recommended. If the signal level is unacceptable, adjust the exposure time or binning level. For more details on the image window, see Table 3.1, page 18.
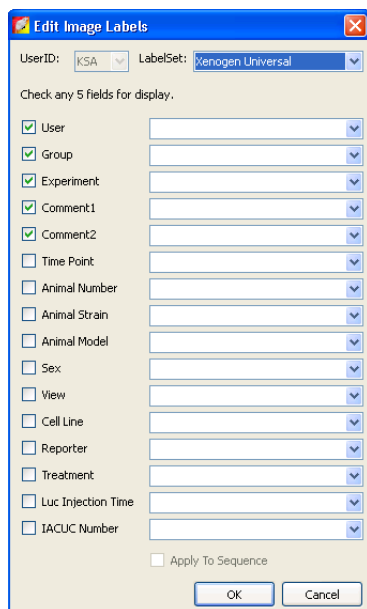
Edit the label information here.

Edit Image Labels box

Figure 3.1 Image window & Edit Image Labels box

Table 3.1 Image window

Item	Description
Units	Select the measurement units for the image display (counts, photons, or efficiency) from this drop-down list
Display	Select the image type (for example, overlay) that you want to display from this drop-down list. For more details on the different types of image displays, see Figure 5.6, page 57.
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.
Color bar	Shows the minimum and maximum pixel intensities in the image as well as the color bar. Pixels less than the color bar minimum or greater than the color bar maximum are not displayed in the image.
Image label	Information about the image that the software automatically records and user-specified information entered in the Edit Image Label dialog box.

3.2 Acquire a Fluorescence Image With Epi-Illumination

Epi-illumination uses an excitation light source located above the stage. For more details on fluorescence imaging, see page 169.

1. Start the Living Image software (double-click the 🖼 icon on the desktop).

2. Initialize the IVIS System and confirm or wait for the CCD temperature to lock. (For more details, see page 11.)
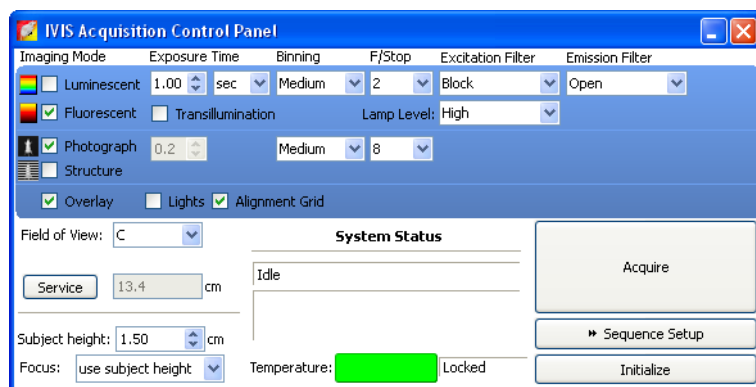
3. Put a check mark next to **Fluorescent**.

4. Confirm that the Fluorescent Lamp Level is set to the appropriate level (High or Low).

5. Select the **Auto** exposure time (click the ▼ arrow). Alternately, manually set the exposure, binning, and F/Stop. (For more details on the control panel settings, see page 191.)

6. **Set the FOV:** To adjust the field of view (FOV), make a selection from the Field of View drop-down list. For more details on FOV, see page 160.

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> **NOTE**
>
> To view the subject(s) inside the chamber before image acquisition, take a photographic image (clear 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).

     or

   - Select **Manual focus** from the Focus drop-down list. (For more details on manual focusing see page 34.)
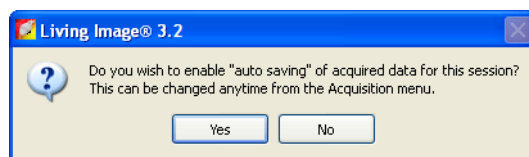
8. If necessary, click ⬍ Image Setup in the control panel to operate in single image mode.

> **NOTE**
>
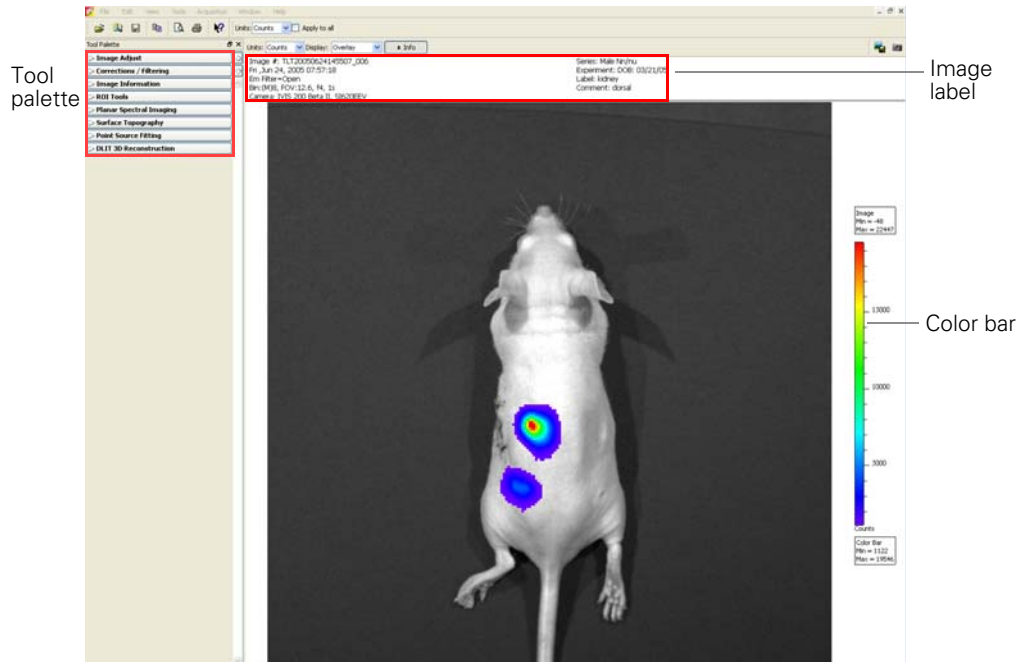> In single image mode, the ⬍ Sequence Setup button appears in the control panel. Click this button to set up sequence acquisition. (For more details on setting up a sequence, see page 22.)

9. When you are ready to acquire the image, click **Acquire**. During image acquisition, the **Acquire** button becomes a **Stop** button. To cancel the acquisition, click **Stop**.

   - The image window appears (Figure 3.1). If this is the first image of the session, you are prompted to choose an autosave location.
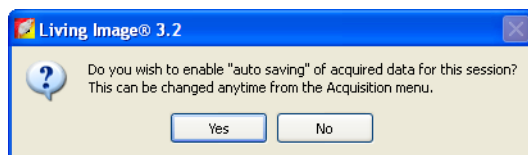
10. To specify a folder for autosaved data, click **Yes** in the prompt and choose a folder in the dialog box that appears.

    - 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).

11. In the Edit Image Labels box that appears, enter information for the image label and click **OK** (Figure 3.1). If you do not want to enter label information, click **Cancel**.

3.3 Acquire a Fluorescence Image With Transillumination

Transillumination uses an excitation light source located below the stage. For more details on fluorescence imaging, see page 169.

> **NOTE**
>
> Transillumination is only available on the IVIS® Spectrum imaging system.

1. Start the Living Image software (double-click the ![icon] icon on the desktop).

2. Initialize the IVIS System and confirm or wait for the CCD temperature to lock. (For more details, see page 11.)
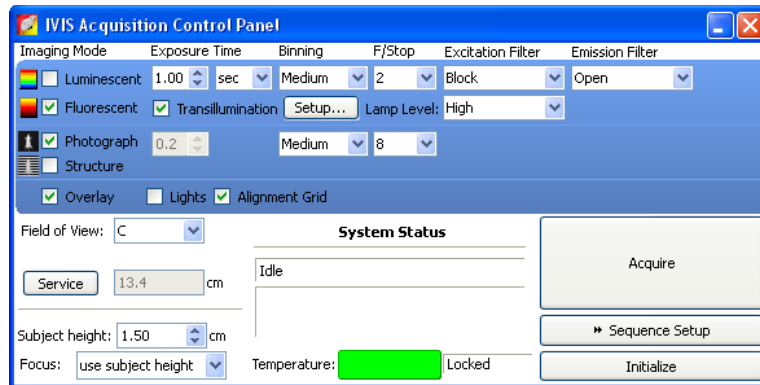
3. Put a check mark next to **Fluorescent** and **Transillumination**.

4. In the control panel, select an appropriate excitation filter and emission filter from the drop-down lists.

5. Click **Setup**.
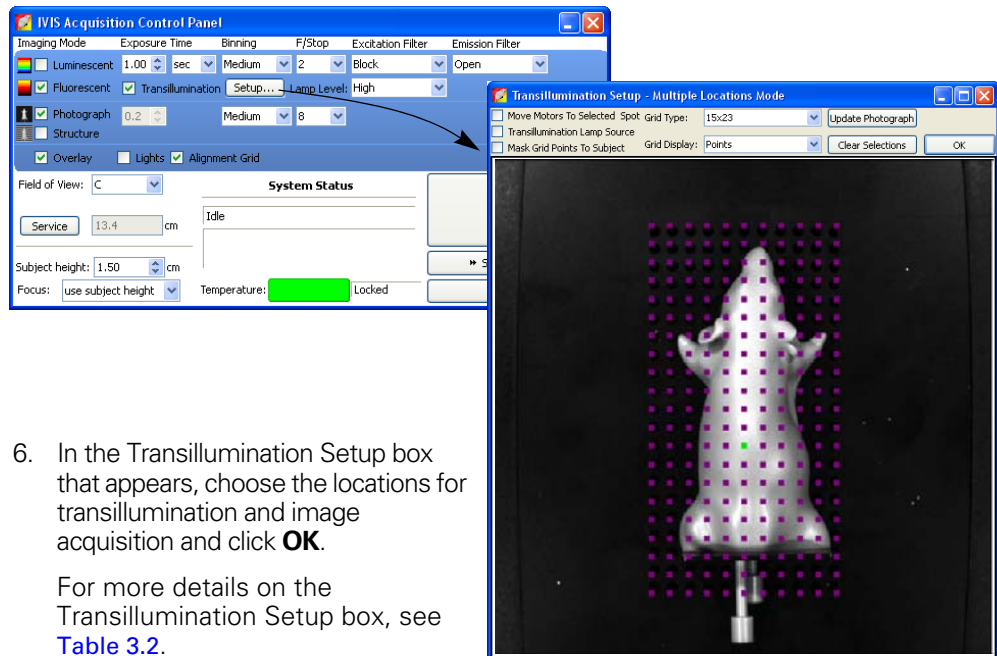
6. In the Transillumination Setup box that appears, choose the locations for transillumination and image acquisition and click **OK**.

   For more details on the Transillumination Setup box, see Table 3.2.

Table 3.2 Transillumination Setup box

Item	Description
Move Motors To Selected Spot	Transillumination motors will move to the grid location selected in the Transillumination Setup dialog box.
Transillumination Lamp Source	Choose this option to turn on the bottom illumination lamp.
Mask Grid points To Subject	Choose this option to select only the grid locations within the subject. Grid locations outside the subject are masked.
Grid Type	Select a grid type from the drop-down list: 15x23, 11x23, 5x10, or 8x12 well plate, Xenogen Sparse Mask, 6x8x1cm.
Grid Display	Select "Points" or "Cross Hair" to display the grid.
Update Photograph	Click to acquire a new photographic image. If the chamber door is opened during transillumination setup, you are prompted to acquire a new photograph.
Clear Selections	Clears selected/ highlighted transillumination locations on the grid.

7. **Set the FOV:** To adjust the field of view (FOV), make a selection from the Field of View drop-down list. For more details on FOV, see page 160.
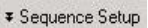
NOTE
To view the subject(s) inside the chamber before image acquisition, take a photographic image (clear the luminescent or fluorescent option, choose the Photographic and Auto options, and click **Acquire**).

8. **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).
     or
   - Select **Manual focus** from the Focus drop-down list. (For more details on manual focusing see page 34.)

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 22.)

10. When you are ready to acquire the image, click **Acquire**. During image acquisition, the **Acquire** button becomes a **Stop** button. To cancel the acquisition, click **Stop**.
   - The image window appears (Figure 3.1). If this is the first image of the session, you are prompted to choose an autosave location.

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

   - 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).

12. In the Edit Image Labels box that appears, enter information for the image label and click **OK** (Figure 3.1). If you do not want to enter label information, click **Cancel**.

3.4 Acquire an Image Sequence

A sequence is a collection of images that are grouped together in a single folder. A sequence may include images that were acquired during the same session and were intended to be grouped together (for example, images taken at different exposure times 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 example, a time series could be constructed from images acquired on different days following an experimental treatment. (For more details, see page 26.)

Some analyses are performed on an image sequence (see Table 3.3). The sequence requirements (number and type of images) depend on the analysis. The imaging wizard provides a convenient way to set up several types of image sequences (see page 24). You can also set up the sequence manually (see page 26).

Table 3.3 Application-specific image sequences

Analysis	IVIS Imaging System				
	Lumina	**100 Series**	**200 Series**	**Spectrum**	**Kinetic**
Planar spectral image analysis Computes the total flux and average depth of a bioluminescent source below the surface.	Optional*	Optional*	✓	✓	Optional*
Display multiple fluorescent or bioluminescent reporters. Uses the Image Overlay function to display multiple luminescent or fluorescent images on one photographic image.	✓	✓	✓	✓	✓
Subtract tissue autofluorescence using blue-shifted background filters. Uses the image math feature to subtract a background image from the primary image.	✓	✓	✓	✓	✓
Point Source Fitting Estimates the optical properties of tissue, the location and power of a point source, or the fluorescent yield of fluorophores.	✓	✓	✓	✓	✓
Spectral unmixing Removes tissue autofluorescence from a fluorescence image.	Optional*	Optional*	✓	✓	Optional*
DLIT™ Analysis Reconstructs the surface topography of the subject and the brightness and 3D location of bioluminescent sources.			✓	✓	✓
FLIT™ Analysis Reconstructs the surface topography of the subject and brightness and 3D location of fluorescent sources.				✓	✓

*Optional: with premium filters

Setting Up a Sequence Using the Imaging Wizard

The imaging wizard can automatically setup an image sequence for some applications that analyze an image sequence (Table 3.3). The wizard guides you through a series of steps, prompting you for the information that the software requires to set up the image sequence.

Table 3.4 Imaging wizard sequence options

Bioluminescence	Acquires...		Description	See Page
	Image	**Sequence**		
Open Filter	✓		Acquires an image at maximum sensitivity.	
Planar Spectral		✓	Analyze the sequence to compute the average depth and total photon flux of a bioluminescent point source in a region of interest (ROI).	121
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.	127
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.	151
Fluorescence			**Description**	**See Page**
2D	✓	✓	Use the data to make fluorescence measurements.	
Filter Scan		✓	Helps you determine the optimum excitation and emission filter for a probe.	
Spectral Unmixing		✓	Analyze the sequence to extract the signal of one or more fluorophores from the tissue autofluorescence.	127
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.	156

To set up a sequence using the imaging wizard:

1. Click **Sequence Setup** in the IVIS acquisition control panel.
   - The Sequence Editor appears.
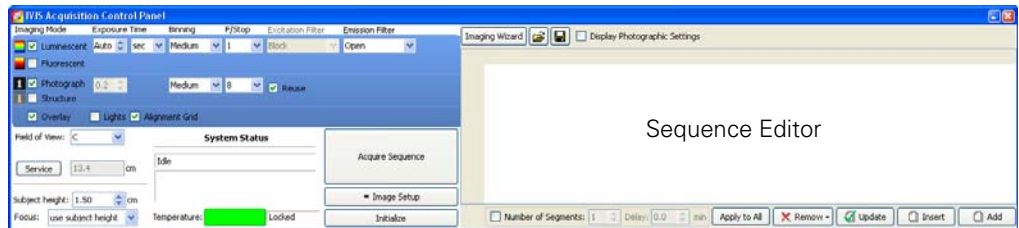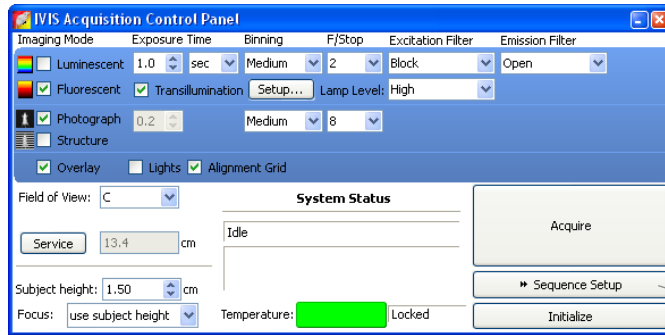
4. If necessary, click ❌ Remove ▾ and select **All** to clear the Sequence Editor.

5. Click **Imaging Wizard**.
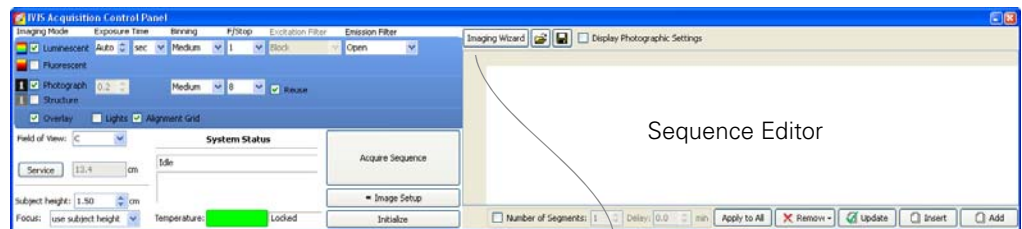
To begin setting up a bioluminescence image sequence, click here.

To begin setting up a fluorescence image sequence, click here.

6. Click **Next**.
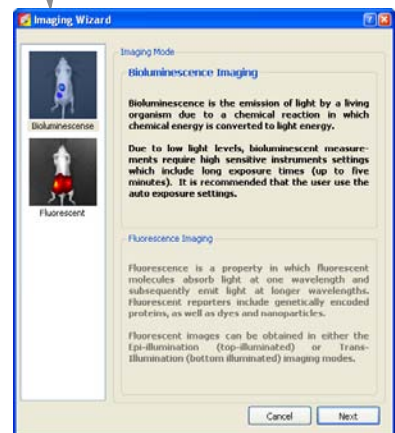
7. Double-click the type of bioluminescence or fluorescence sequence that you want to acquire and step through the rest of the imaging wizard.

   - The sequence setup appears in the Sequence Editor.
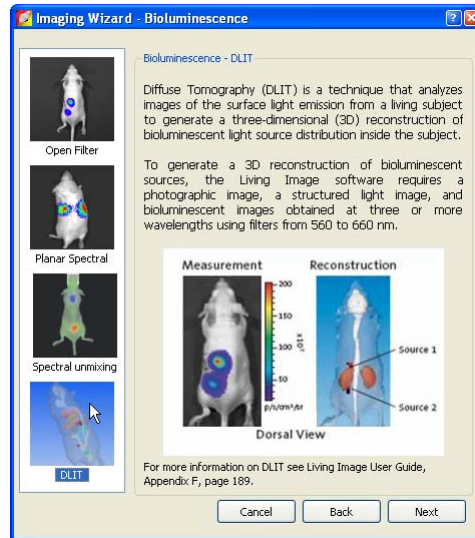
Imaging wizard - Bioluminescence
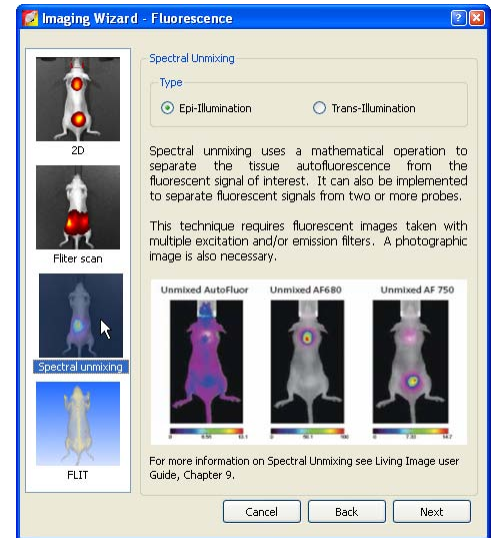
Imaging wizard - Fluorescence

NOTE

The imaging options available in the imaging wizard depend on the IVIS imaging system and the installed filter set.

Manually Setting Up a Sequence

You can manually set up an image sequence in the control panel and save the information to a Xenogen Sequence Setup file (.xsq).

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.
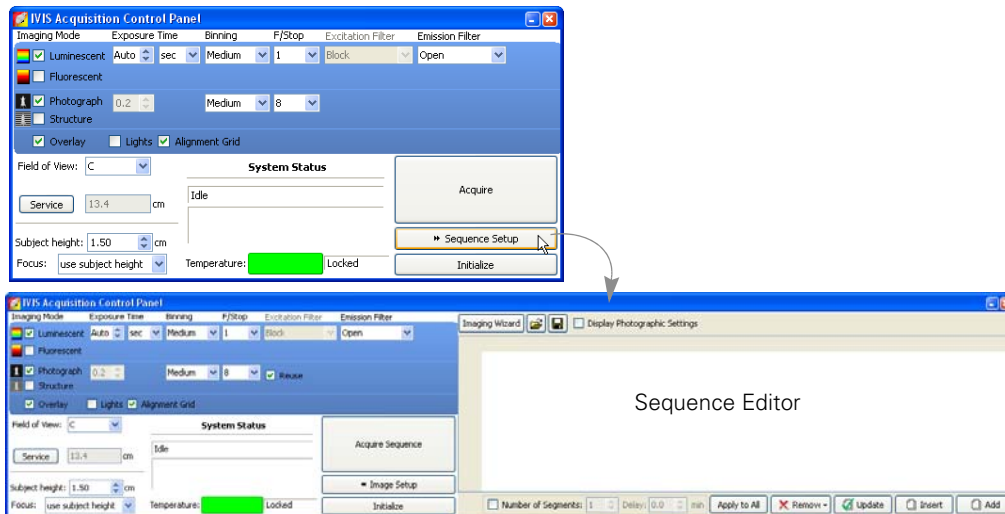
Sequence Editor

2. If it is necessary to clear the sequence editor that appears, click [✗ Remove ▾] and select **All**.

3. In the control panel, specify the settings for the first bioluminescence or fluorescence image in the sequence and the photographic image. (For details on the imaging parameters in the control panel, see .)
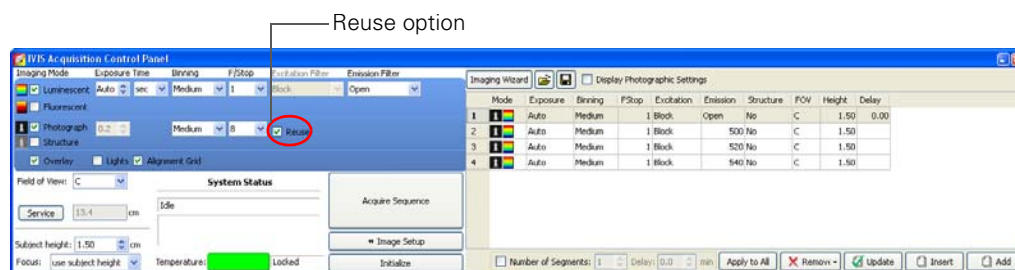
Reuse option

NOTE

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

4. Click [□ Add].
   - The acquisition parameters are added to the table.

Click to include the parameter settings
for the photographic image in the editor.

Each row in the editor specifies the acquisition
parameters for one image in the sequence

	Mode	Exposure	Binning	FStop	Excitation	Emission	Structure	FOV	Height	Delay
1		1	Medium	1	Block	Open	No	C	1.50	0.00
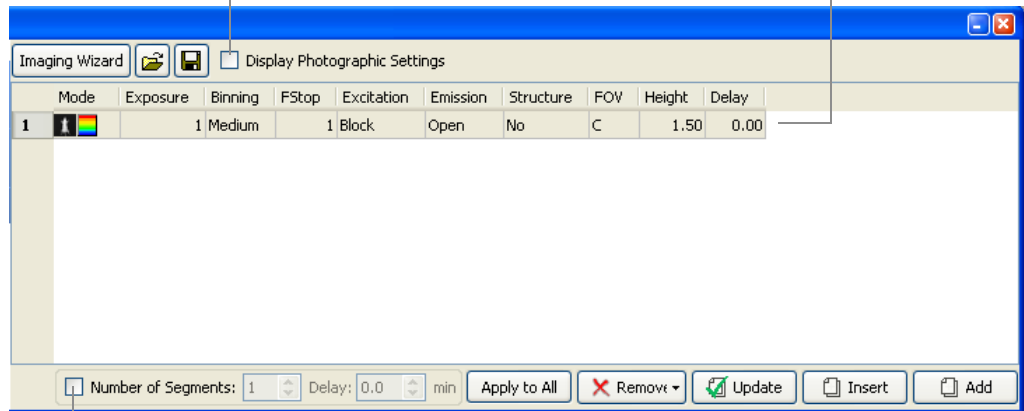
The image(s) in the sequence editor comprise one *segment*. You can acquire multiple
segments with a user-specified time delay between acquisitions. This is useful for
acquiring data for kinetic analysis.

5. Repeat step 3 to step 4 for each image in the sequence.

6. To specify a time delay between each acquisition, enter a time in the Delay (min)
    box in the Sequence Editor.

7. If you want to save the sequence setup information (.xsq):

    a. In the sequence editor, click the **Save** button 💾.

    b. In the dialog box that appears, select a directory for the file, enter a file name, and click
       Save.

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Editing an Image Sequence

You can edit a parameter value, as well as add or remove images from the sequence. A shortcut menu of edit commands is available when you right-click the Sequence Editor.

Changing an Imaging Parameter

1. In the sequence editor, double-click the cell that you want to edit.
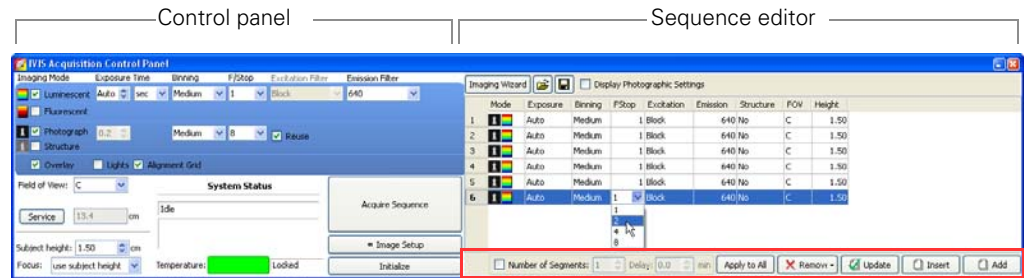
For details on these functions, see Table 3.5.

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

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

4. Click outside the cell to lose focus.
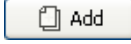
Editing a parameter using the control panel

1. In the sequence editor, select the row that you want to modify.

2. In the control panel, choose new parameter values and/or imaging mode.

3. Click [Update].

Table 3.5 Sequence Editor

Item	Description
Imaging Wizard	Starts the imaging wizard.
(open icon)	Displays a dialog box that enables you to select and open a sequence setup (.xsq), sequenceinfo.txt, or clickinfo.txt file.
(save icon)	Displays a dialog box that enables you to save a sequence setup (.xsq).
Display Photographic Settings	Choose this option to include the photograph exposure time, binning, and F/Stop in the sequence editor.
Number of Segments	Choose this option to set the number of segments to acquire and the time delay between segments. One segment = the sequence specified in the sequence editor.
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	Deletes the selected row from the sequence editor.
Update	Updates the selected row in the sequence editor with the acquisition parameters in the control panel.

Table 3.5 Sequence Editor

Item	Description
[🗐 Insert]	Inserts a row above the currently selected row using the information from the control panel.
[🗐 Add]	Adds a new row below the currently selected row using information from the control panel.

Adding or Deleting Images From a Sequence

1. Select the row(s) of interest and right-click the sequence editor to view a shortcut menu of edit commands (see Table 3.6).
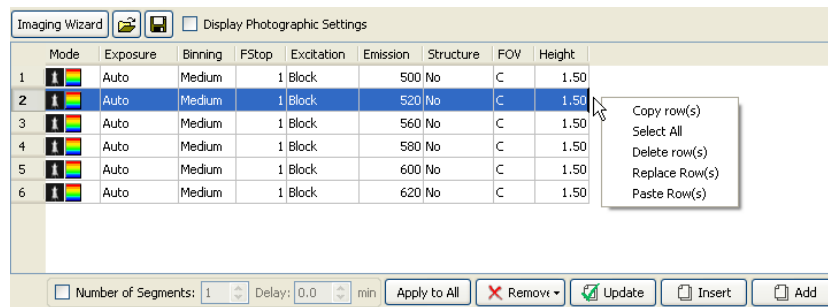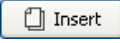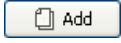
Table 3.6 Sequence editor, shortcut menu edit commands

Command	Description
Copy row(s)	Copies the selected row(s) to the system clipboard.
Select All	Selects all rows in the sequence editor.
Delete row(s)	Deletes the selected row(s) from the sequence editor.
Replace Row(s)	Replaces the row(s) selected in the sequence editor 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 editor.
Paste Row(s)	Adds copied rows to end of the sequence.

Alternate method to add a row:

1. In the sequence editor, 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]. To insert the new image below the selected row, click [🗐 Add].

Alternate method to delete one or more rows:

1. Select the row(s) that you want to delete.

2. Click [✕ Remove ▾] and choose **Selected** from the drop-down list.

To clear the sequence editor:

1. Click [✕ Remove ▾].

2. Choose **All** from the drop-down list.

Acquire the Image Sequence

The system is ready to acquire the image sequence after you set up the image sequence in the sequence editor.

1. Initialize the IVIS® System and confirm or wait for the CCD temperature to lock. (For more details, see page 11.)

2. When you are ready to acquire the images, click **Acquire Sequence** in the control panel.

 - Thumbnail images appear as the images are acquired. During acquisition, the **Acquire Sequence** button becomes a **Stop** button. To cancel the acquisition, click **Stop**.
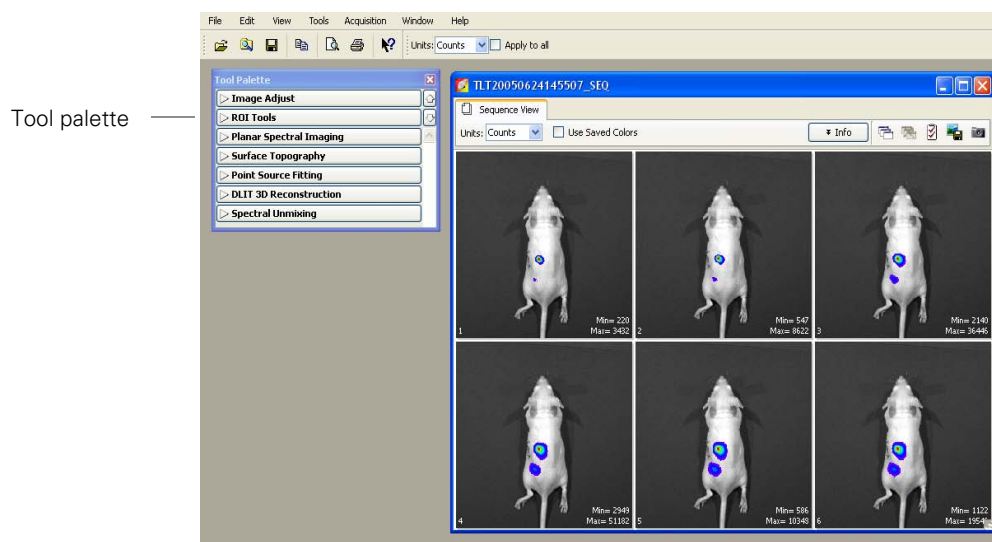
Tool palette

Thumbnail images
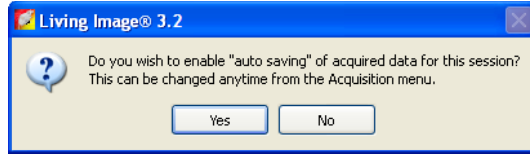
3. If this is the first acquisition of the session, you are prompted to choose an autosave location.

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

   - All sequences 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).

5. In the Edit Image Labels box that appears, enter information for the image label and click **OK** (Figure 3.1). If you do not want to enter label information, click **Cancel**.
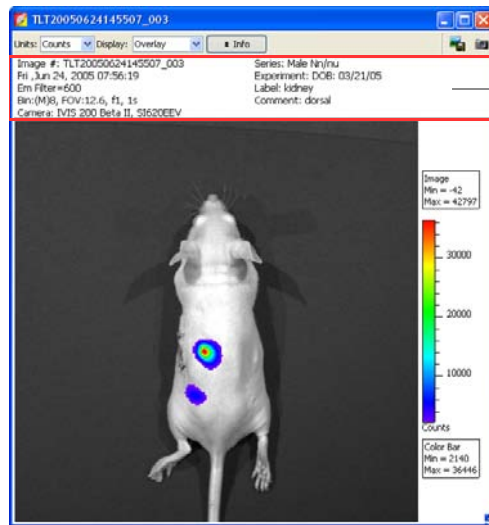
Image label

Open an image (double-click the thumbnail) to determine whether the signal of interest is above the noise level and below CCD saturation.

Check the image min and max in the color bar. A signal greater than 600 counts and less than ~60,000 counts is recommended. If the signal level is unacceptable, adjust the exposure time or binning level.

For more details on the image window, see Table 3.7
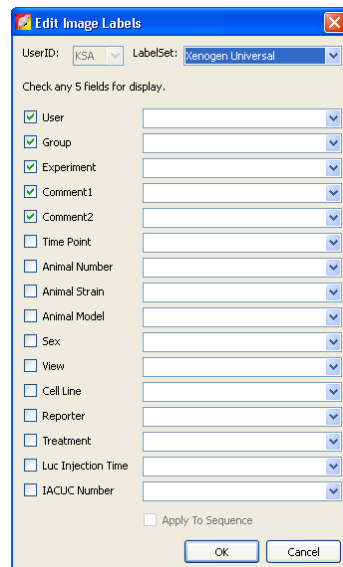
Edit the label information here.

Figure 3.2 Image window & Edit Image Label box

Table 3.7 Image window

Item	Description
Units	Select the measurement units for the image display (counts, photons, or efficiency) from this drop-down list
Display	Select the image type (for example, overlay) that you want to display from this drop-down list. For more details on the different types of image displays, see Figure 5.6, page 57.
Info	Click to display or hide the image label information.
	Opens a dialog box that enables you to export the image or thumbnails to a graphic file (for example, .bmp or DICOM format)
	Creates a preview picture of the image or thumbnails that the Living Image browser displays when the data is selected.
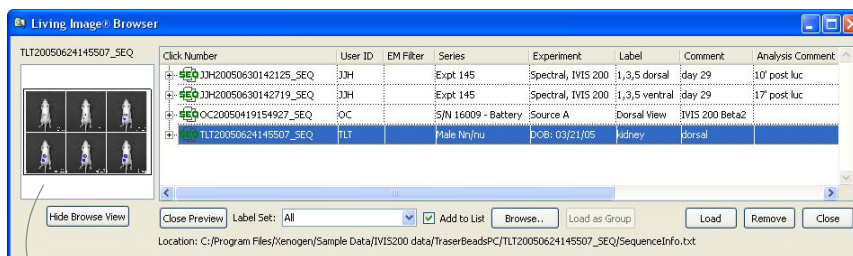
Preview picture of the selected data

Item	Description
Color bar	Shows the minimum and maximum pixel intensities in the image as well as the color bar. Pixels less than the color bar minimum are not displayed in the image.Pixels greater than the color bar 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.

3.5 Manually Setting the Focus

The IVIS Imaging System automatically focuses the image based on subject height (see page 193). If you do not want to use the automatic focus feature, you can manually set the focus.

1. In the control panel, choose **Manual Focus** in the Focus drop-down list.

   - The Manual Focus window appears.
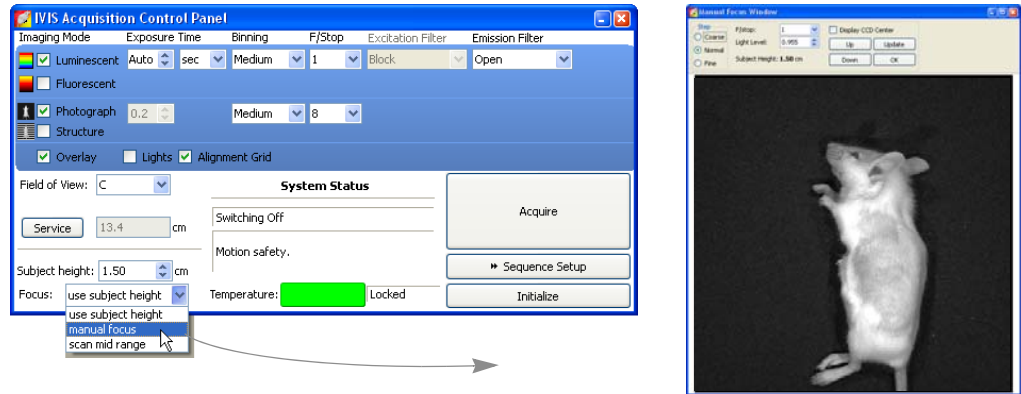
2. To mark the center of the camera in the window, put a check mark next to Display CCD Center.

3. Select the size of the step increment that the stage moves: **Coarse**, **Normal**, or **Fine**.

4. Click **Up** or **Down** to move the stage and change the focus.

5. If necessary, select another F/stop setting from the drop-down list and adjust the light level using the arrows.

6. Click **Update** to apply the settings.

   - The resulting focal plane (cm above the stage) is automatically entered in the Subject height box.

7. Click **OK** when the image is focused.

3.6 Manually Saving Image Data

When you acquire the first image(s) of a session, the autosave feature prompts you to choose the folder where you want to save image data. During the session, all images will be saved to this folder. You can change the autosave folder at any time (select **Acquisition →Auto-Save To** on the menu bar). If you do not want to use the autosave feature, you can manually save data.

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 of interest and click **OK**.

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

3.7 Exporting Image Data

You can export the image data in different file formats (for example, .bmp, .dcm).

1. Click the **Export Graphics** button 📥.
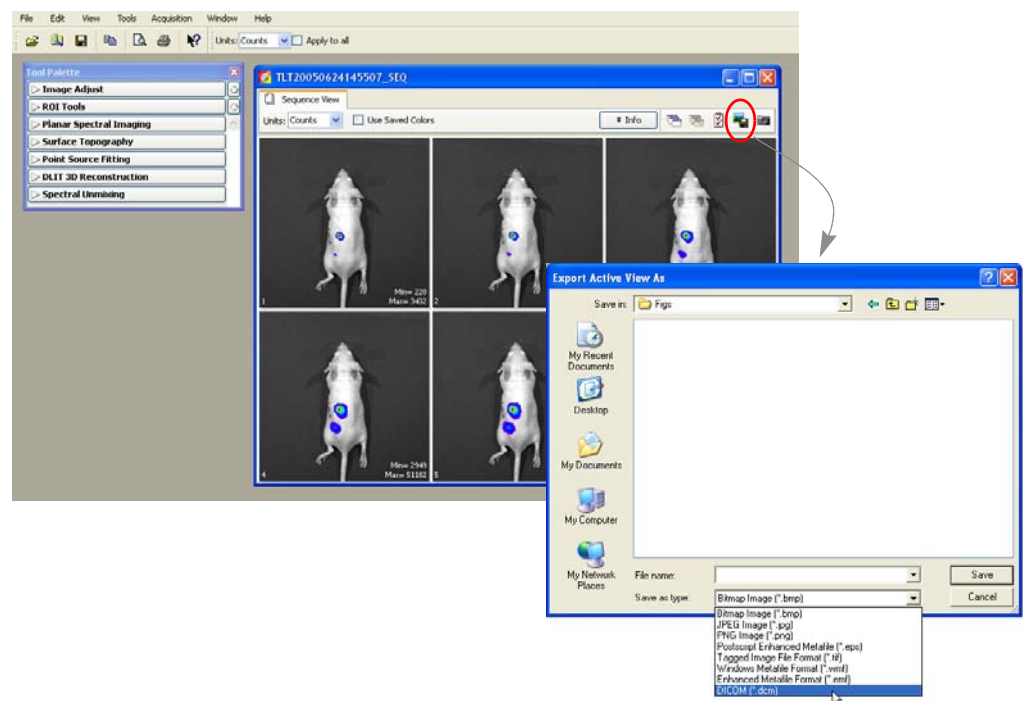
2. In the dialog box that appears, select a directory, choose a file type, and enter a file name.

3. Click **Save**.

> **NOTE**
>
> When you export to DICOM (.dcm) format, a directory that contains the .dcm files and a SequenceInfo.txt is created at the specified location.

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4 Acquire Kinetic Data

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

4.1 Kinetic Acquisition

1. Start the Living Image software (double-click the 🖼 icon on the desktop).

2. Initialize the IVIS System and confirm or wait for the CCD temperature to lock. For more details, see .)

3. When you are ready to begin imaging, click **Kinetics** in the control panel.

   - The Kinetic Acquisition window appears.
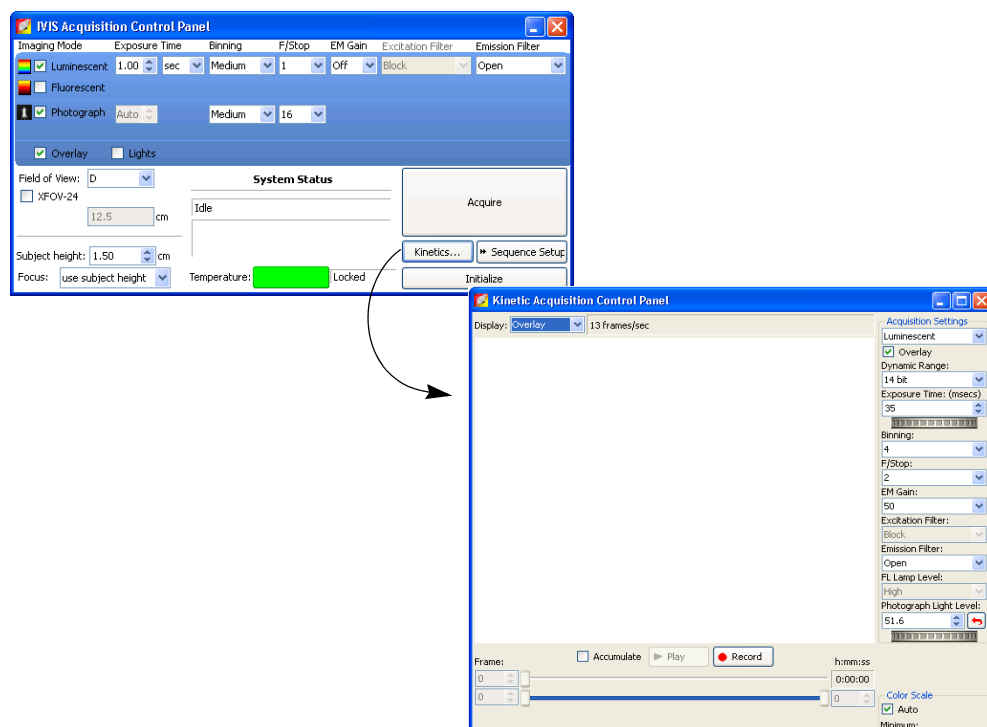
Figure 4.1 Control panel (top) and Kinetic Acquisition window (bottom)

4. Select the type of data to acquire and set the acquisition parameters. (For more details on the acquisition parameters, see .)

Select the type data to acquire
(luminescent or fluorescent)

After acquisition, choose
the type of data to display

To adjust a setting using thumb
wheel, put the mouse arrow on
the wheel, then click and hold the
mouse button while you move
the mouse arrow left or right.
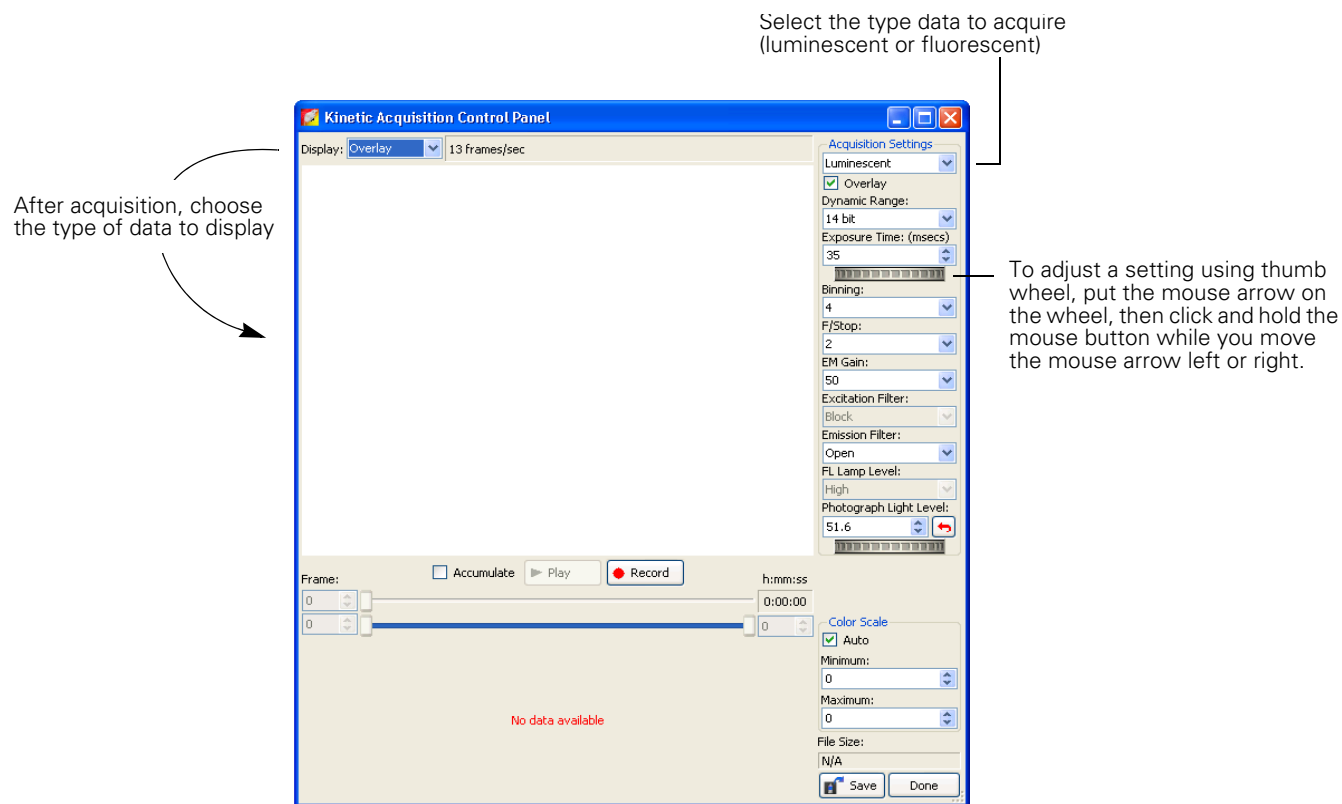
Figure 4.2 Kinetic Acquisition window

5. Click the [● Record] button to start acquisition. (After acquisition begins, the button changes to a [■ Stop].)

    - The Maximum vs. Time graph plots the maximum signal in each data frame. For more details on the graph, see page 41.

6. To stop acquisition, click the [■ Stop] button.

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 [■ Stop] button) and reduce the photograph light level.

⚠ CAUTION

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

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

Table 4.1 Kinetic acquisition settings
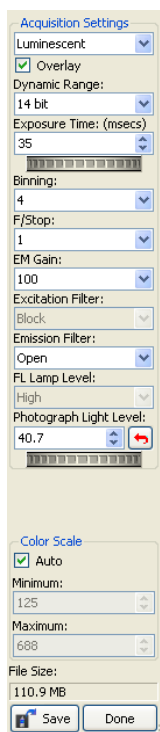
Item	Description
	Select the type of data to acquire (luminescent or fluorescent) from this drop-down list. Choose the Overlay option to acquire photographic images.
Dynamic Range	14 bit - If this option is chosen, the signal intensities range from 0 to 16384 counts per pixel. 16 bit - If this option is chosen, the signal intensities range from 0 to 65536 counts per pixel. **Note:** The 14 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. The 14 bit dynamic range enables faster imaging by attaining a higher frame rate at the cost of a smaller dynamic range.

Table 4.1 Kinetic acquisition settings (continued)

Item	Description
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 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 206.
	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 205**.
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 bioluminescent imaging, **Block** is selected by default. If you select **Open**, no filter is present.
Lamp Level	Sets the illumination intensity level of the excitation lamp used in fluorescent imaging (Off, Low, High, and Inspect).
	Low - This 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.
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.
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.

Table 4.1 Kinetic acquisition settings (continued)

Item	Description
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

Maximum vs. Time Graph

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.
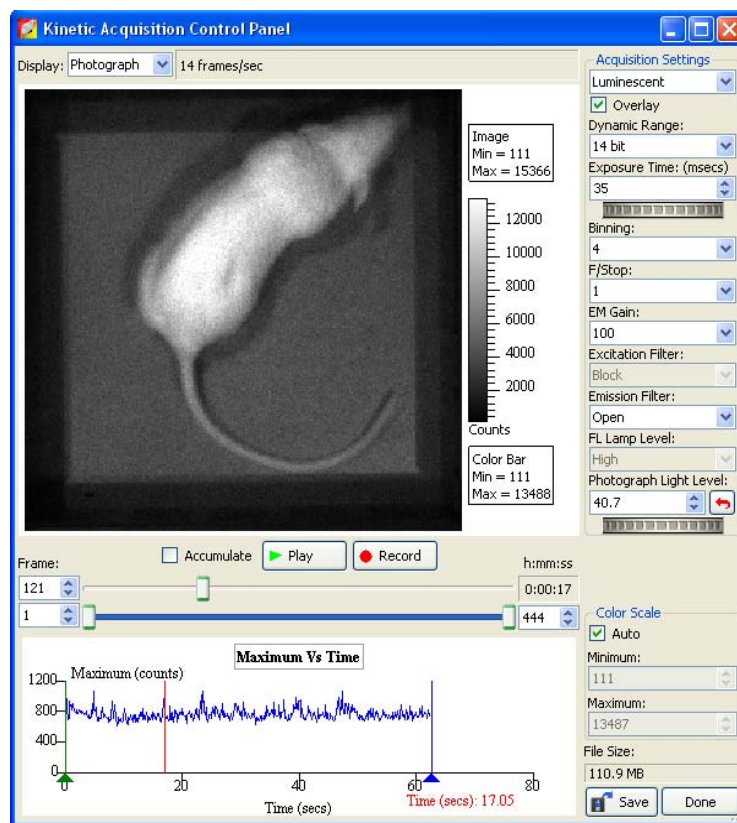
- Click a point in the graph to view the corresponding image (frame)

- 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

Figure 4.3 Maximum vs. time graph

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4.2 Viewing & Editing Data (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. (After playback starts, the button changes to [■ Stop].)

2. To stop the playback, click the [■ Stop] button.

3. To view a particular frame, do one 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. Alternately, 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 77.

Frame number 121 ⎯

Current frame number. Enter a new number or use the top slider to view another frame.

Start frame in a user-specified data range ⎯

| = Position of the displayed (current) frame

▲ = Position of the start frame

▲ = Position of the end frame
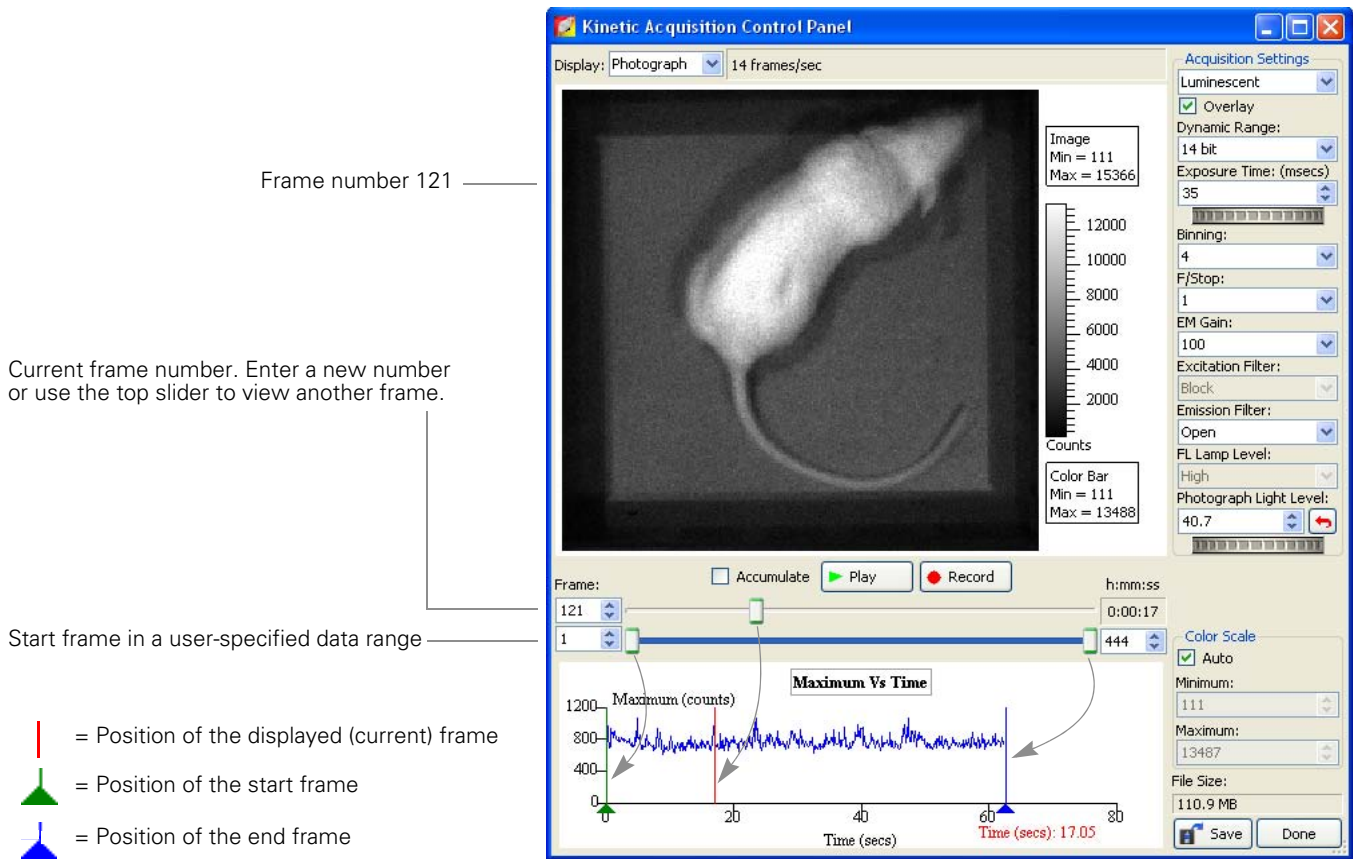
Figure 4.4 Acquisition window

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

Table 4.2 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.
Draw Scale	Displays a scale along the x- and y-axis of the frame.
Insert Tag	Displays a tag with x,y pixel information at a user-selected location of the image. To insert a tag, right-click a location in the image and choose Insert Tag on the shortcut menu.
Remove Tag	Removes a user-selected tag from the image.
Remove All Tags	Removes all tags from the frame.
Display Color Bar	Choose this option to display the color bar.
Display Color Min/Max	Choose this option to display the color bar minimum and maximum.
Display Image Min/Max	Choose this option to display the minimum and maximum signal.

4.3 Saving 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 4.3 shows how binning conditions affect the total number of frames that can be collected in overlay or luminescent/fluorescent only mode.

Table 4.3 Frames collected per 1 GB DICOM file

Binning Level	Frame Size	Overlay Mode	Luminescent or Fluorescent Only	DICOM File Size
		Total Frames Collected		
Bin1	2 MB	250	500	
Bin2	512 KB	975	1950	1 GB
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.
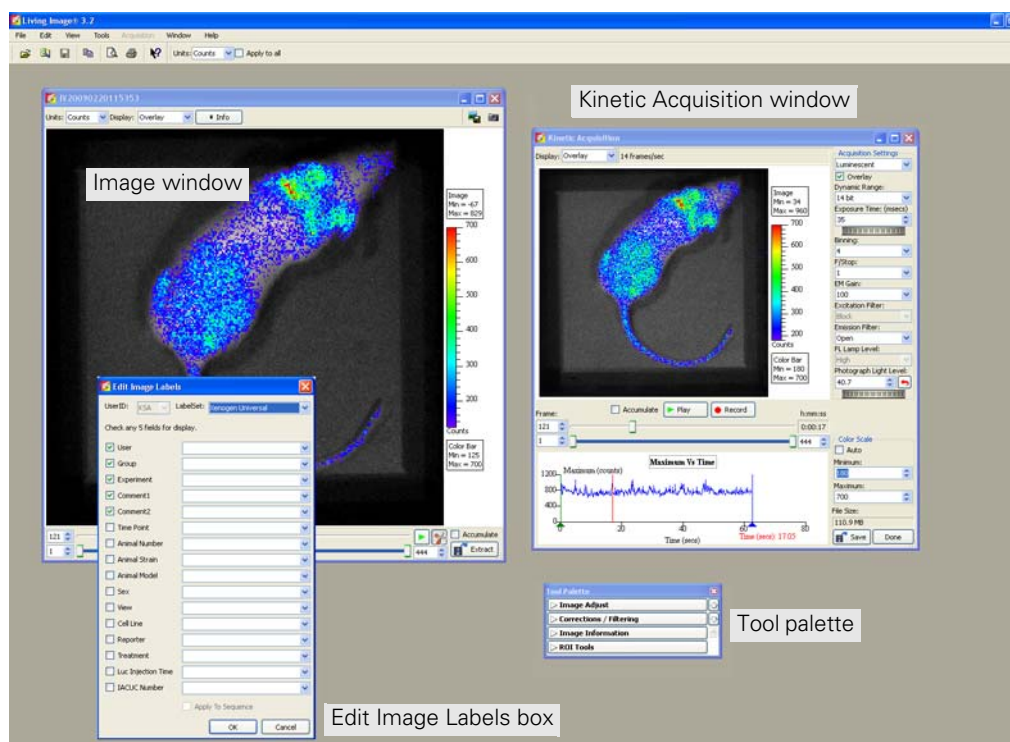
Figure 4.5 The Edit Image Labels box, Image window, and tool palette appear when you save an image or kinetic stream

NOTE

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

5 Working With Data

5.1 Browsing & Opening Data

The Living Image® Browser provides a convenient way to browse and preview data, view information about the data, and open a single image, image sequence or kinetic data. You can also navigate to data and open it without the browser (see).

Browsing Data

1. Click the **Browse** button 🔍. Alternatively, select **File →Browse** from the menu bar.

2. In the Browse for Folder box that appears, select the data of interest and click **OK**.
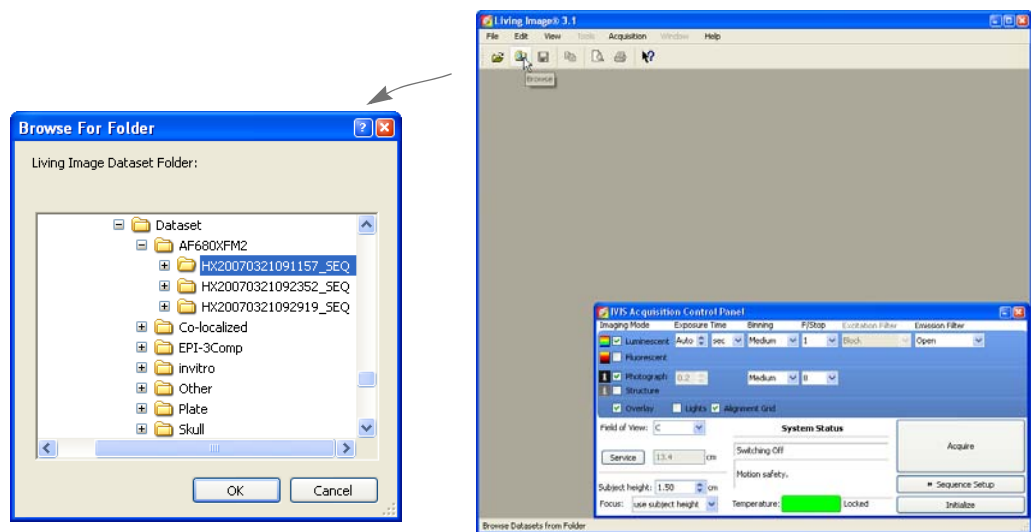
> **NOTE**
>
> The next time you start the Living Image software and open the Browse For Folder box, the software automatically returns to the last folder visited.

3. The selected data are displayed in the Living Image browser along with the user ID, label information, and camera configuration information.

IMC - an image

SEQ - an image sequence

DCM - a kinetic data

4. Click the + sign to show the images of a sequence. To view data properties, right-click the item and select **Properties** on the shortcut menu.
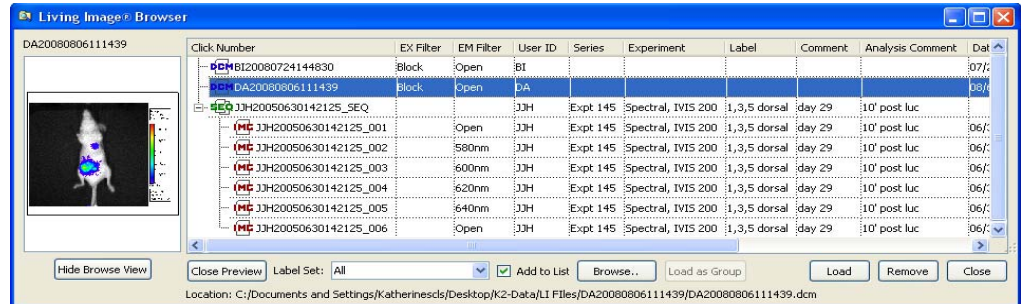
Table 5.1 Living Image® browser

Item	Description
Hide Browse View	Closes the browser table.
Close Preview	Closes the image preview box.
Label Set	A drop-down list of the available label sets which specify image information (column headers) that is displayed in the Living Image browser.
Add to List	If you choose this option, the data that you select in the Browse for Folder box is added to the Living Image browser. If this option is not chosen, the data that you select in the Browse for Folder box replaces the contents of the Living Image browser.
Browse	Opens the Browse For Folder box.
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 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

You can open data from the Living Image browser, the toolbar, or the menu bar. Multiple data sets can be open at the same time.

> **NOTE**
>
> To open recently viewed files, select **File** →**Recent Files** on the menu bar.

Opening Data from the Living Image Browser

1. To open data, do one of the following:

   • Double-click the data row:

   IMG image

   SEQ sequence

   DCM kinetic data

   • 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. Open data is highlighted in green in the browser.
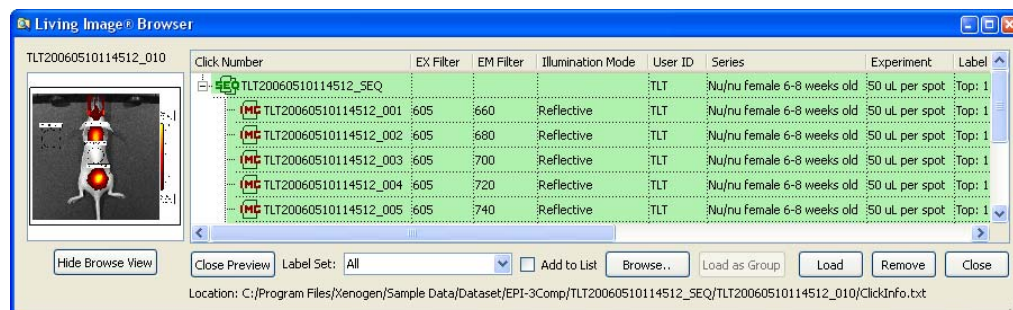
2. To show the images in a sequence, click the + sign next to a sequence (⊞ **SEQ**).

3. To open all images in a sequence, click the **Display All** button in the image window. To close all images, click the **Hide** button .
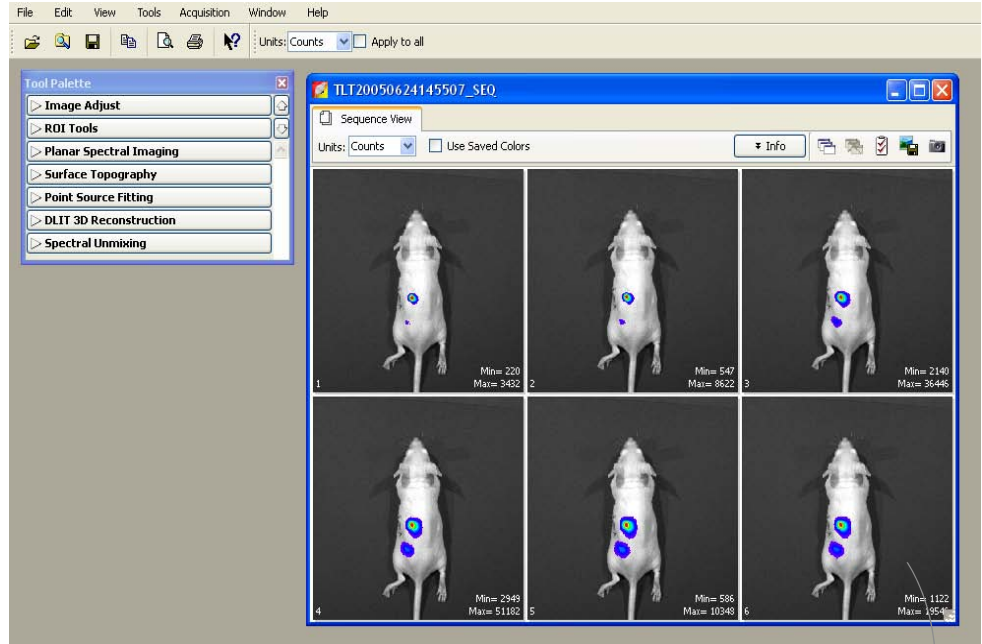
The image window displays thumbnails of sequence images using a single color table.

Double-click a thumbnail to open the image
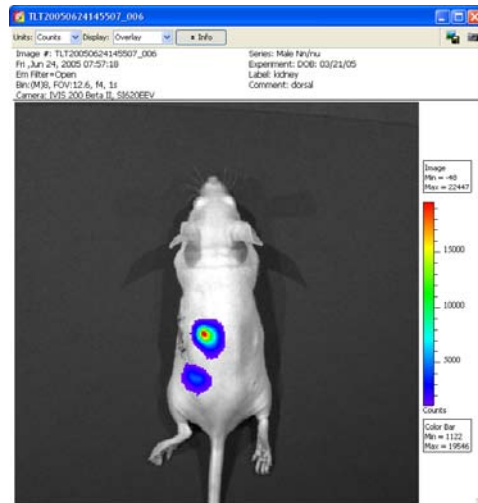
For more details on working with a sequence in the image window, see page 51.

Opening Data from the Menu or Toolbar

1. Click the **Open** button ⬀ on the toolbar. Alternately, select **File →Open** on the menu bar.

2. In the Open box that appears, double-click the file of interest. Alternately, select the data and click **Open**.

   File types:

   Click*.txt - an image (Living Image file format)

   Sequence*.txt - an image sequence (Living Image file format)

   *.dcm - kinetic data
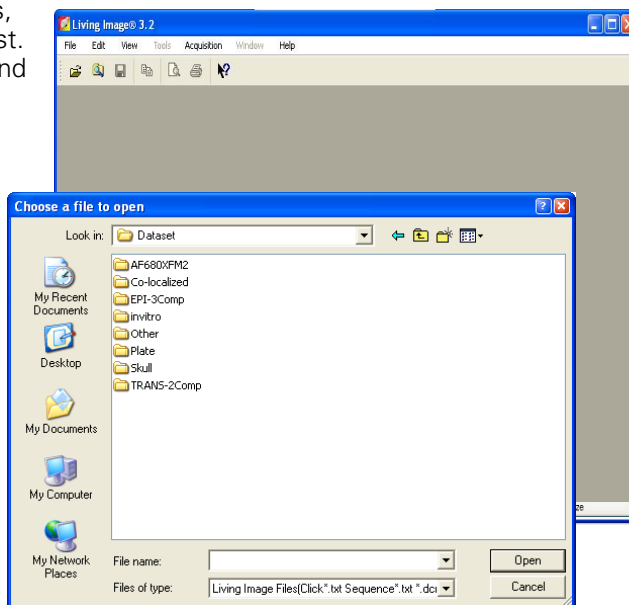
NOTE

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

5.2 The Tool Palette & Image Window

The tool palette contains information about the data and organizes the image analysis tools. The tools available in the tool palette depend on the type of data that is active

Click to expand a tool.
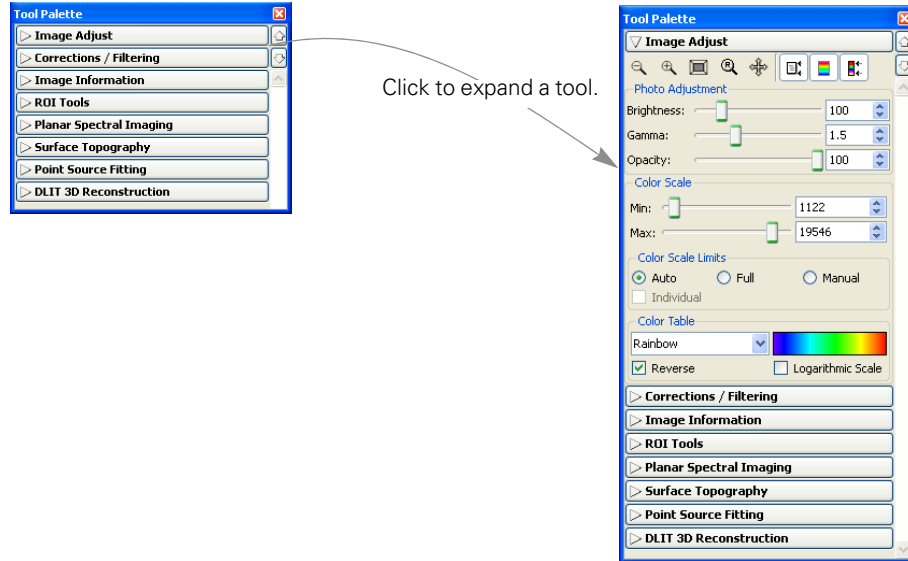
Figure 5.1 Tool palette

An image, image sequence, or kinetic data set is displayed in an image window. Multiple image windows can be open at the same time. The options available in the image window and tool palette depend on the type of image data.
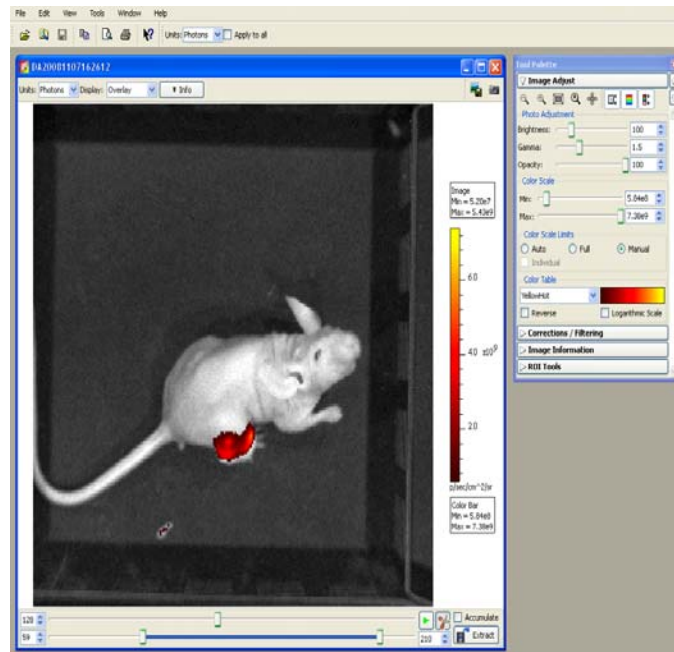
Figure 5.2 Image window, kinetic data

5.3 Working With an Image Sequence

When you open an image sequence, the image window displays thumbnails of the images in the collection. A single color table is applied to the images. (For details on how to open an image sequence using the Living Image browser, see page 47.)

For details on these items, see Table 5.2

Choose Individual to apply a separate color scale to each thumbnail in a sequence.
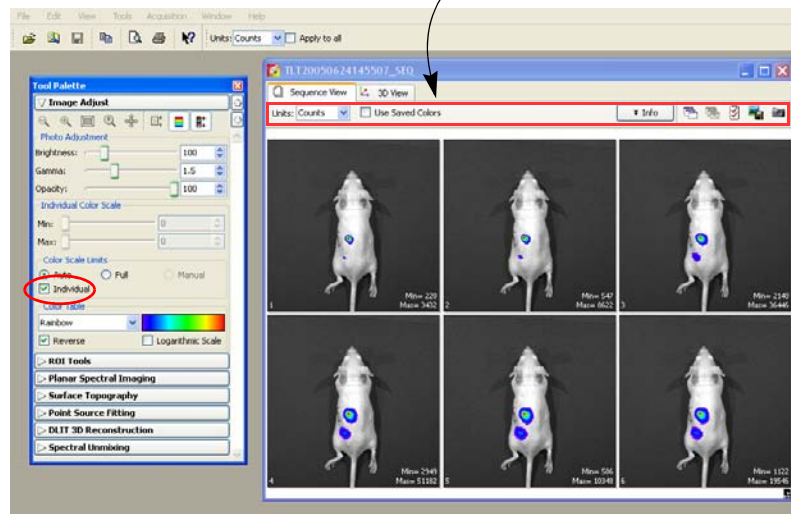
Figure 5.3 Image window, sequence view

If DLIT™ or FLIT™ analysis results are loaded, click the 3D View tab to display the 3D reconstruction of the luminescent sources (IVIS® Imaging System 200 or Spectrum only). For more details on 3D reconstruction, see page 151.
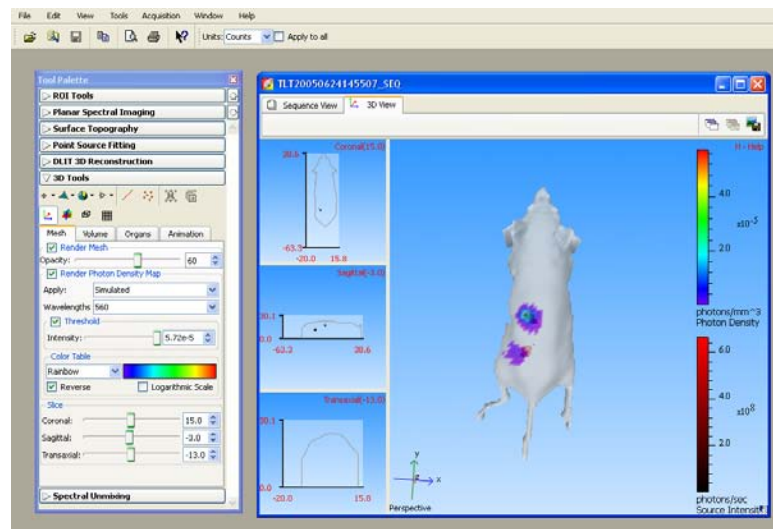
Figure 5.4 Image window, 3D view

Table 5.2 Image window, sequence view tab

Item	Description
Units	Choose counts, photons, or efficiency for the image data from the drop-down list. For more details on counts, photons, or efficiency see Appendix D, page 213.
Use Saved Colors	Choose this option to display an image using the color table that was last applied to the image data.
Info	Click to show or hide the sequence information.
	Click this button to open all images in a sequence.
	Click this button to close all open images in the active sequence.
	Click this button to open the Edit Sequence window that enables you to add or remove images from the active sequence. For more details on editing a sequence, see page 52.
	Click this button to acquire a preview of the sequence for display in the Living Image browser.

Editing an Image Sequence

You can add individual images to a sequence or remove user-specified images from a sequence.

1. Open the image sequence that you want to edit. (For details on how to open image data, see page 47).

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 45.)
    Note: Only individual images, not an image sequence, can be added to a sequence.

3. In the image window, click the **Edit** button .
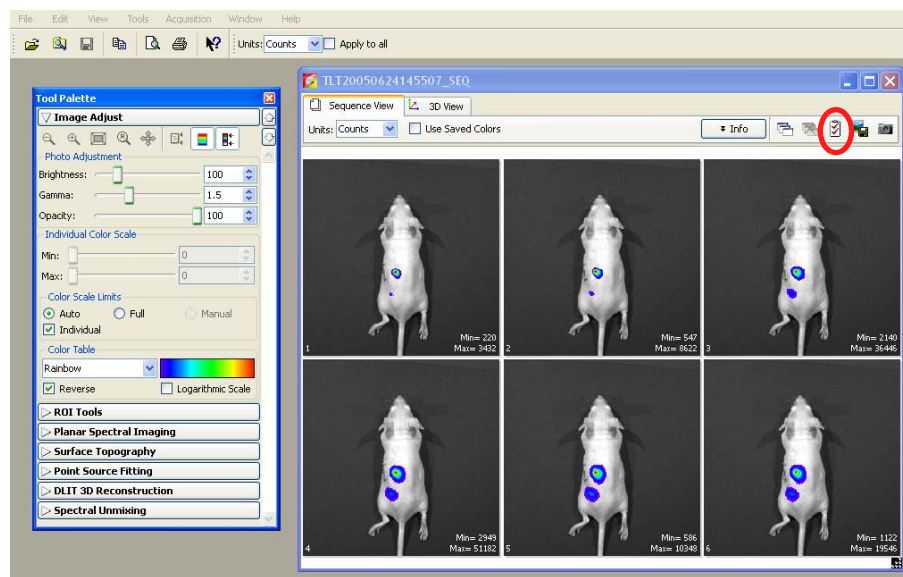
4. In the Edit Sequence box that appears, choose the image(s) that you want to add or remove from the sequence.

Images in the active sequence

Single images in the Living Image Browser

Images that have been removed from the active sequence
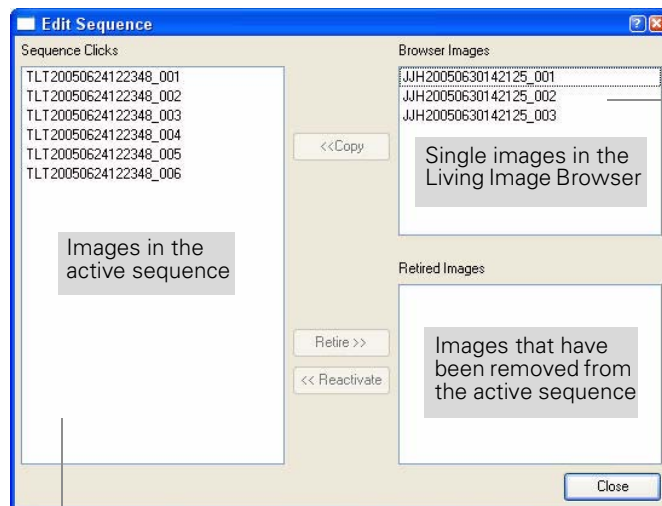
5. To add an image to the sequence, select an image and click **Copy**.

6. To remove an image from the sequence, select an image and click **Retire**.
   - The image is removed (retired) from the sequence.

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

8. When you are finished editing the sequence, click **Close**.
   - The updated image sequence is displayed.

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 45.)

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

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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.

Click Number	EX Filter	EM Filter	Illumination Mode	User ID	User	Group	Series	Experin
HX20070321091157_001	605	640	Reflective	HX	XFM-2 No.4 AF680 Testing	Scan Emission at Ex605		
HX20070321091157_002	605	660	Reflective	HX	XFM-2 No.4 AF680 Testing	Scan Emission at Ex605		
HX20070321091157_003	605	680	Reflective	HX	XFM-2 No.4 AF680 Testing	Scan Emission at Ex605		
HX20070321091157_SEQ				HX	XFM-2 No.4 AF680 Testing	Scan Emission at Ex605		
HX20070321091157_001	605	640	Reflective	HX	XFM-2 No.4 AF680 Testing	Scan Emission at Ex605		
HX20070321091157_002	605	660	Reflective	HX	XFM-2 No.4 AF680 Testing	Scan Emission at Ex605		
HX20070321091157_003	605	680	Reflective	HX	XFM-2 No.4 AF680 Testing	Scan Emission at Ex605		
HX20070321091157_004	605	700	Reflective	HX	XFM-2 No.4 AF680 Testing	Scan Emission at Ex605		
HX20070321091157_005	605	720	Reflective	HX	XFM-2 No.4 AF680 Testing	Scan Emission at Ex605		
HX20070321091157_006	605	740	Reflective	HX	XFM-2 No.4 AF680 Testing	Scan Emission at Ex605		
HX20070321091157_007	605	760	Reflective	HX	XFM-2 No.4 AF680 Testing	Scan Emission at Ex605		
HX20070321091157_008	605	780	Reflective	HX	XFM-2 No.4 AF680 Testing	Scan Emission at Ex605		

Show Preview   Label Set: All   ☑ Add to List   Browse..   Load as Group   Load   Remove   Close

Location: C:/Program Files/Xenogen/Sample Data/Dataset/Skull/HX20060809153031_SEQ/SequenceInfo.txt

Images loaded in the browser as part of a sequence (highlighted pink in this example). These images cannot 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.
   For details on how to save or export the image data, see Chapter 3, page 34.

5.4 Working With a Single Image

Table 5.3 explains the items in the image window. For details on how to browse data and open images, see page 45.

Choose the image display units.　　　　　　　　　　Select a display mode from the drop-down list.
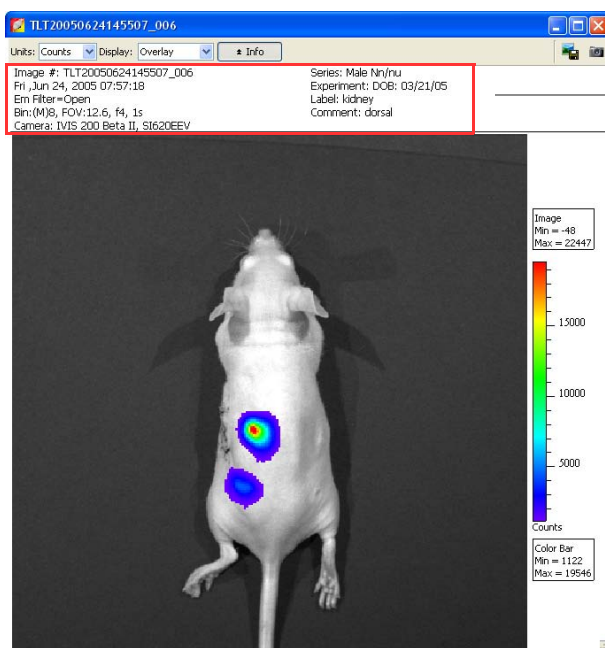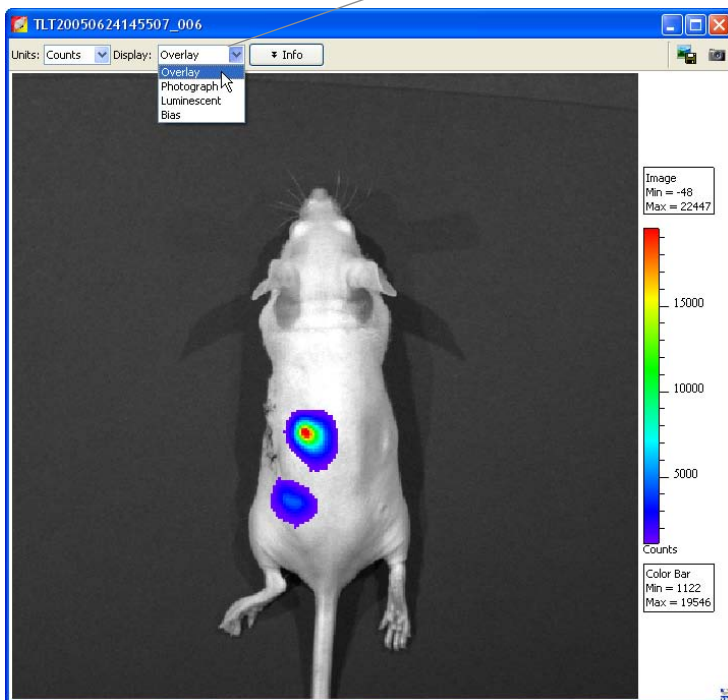
To display or hide the image information, click **Info**.

Figure 5.5 Image window, overlay display mode

Table 5.3 Image window

Item	Description
Units	Choose counts, photons, or efficiency from the drop-down list for the image data. For more details on counts, photons, or efficiency see Appendix D, page 213.
Display	To choose an image display mode in the image window, make a selection from the Display drop-down list. See Figure 5.6 for examples of the display modes.
Overlay	A pseudocolor image of luminescent or fluorescent image data displayed over a grayscale photographic image.
Photograph	A grayscale image that is captured when the IVIS® Imaging System illumination lights are activated.
Luminescent	A pseudocolor image of the luminescent data captured during an exposure when the IVIS Imaging System illumination lights are off.
Fluorescent	A pseudocolor image of the fluorescent data captured during an exposure when the IVIS Imaging System illumination lights are off.
Background	The CCD camera background acquired with the camera shutter closed. (See Appendix E, page 217.)
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.
Saturation Map	Displays image regions that saturated the CCD digitizer in red. ROI measurements should not be made on saturated regions. ROI measurements made on image regions that do not contain saturated pixels are accurate (unless the image is badly saturated).
Structure	A structured light image of parallel laser lines scanned across the subject. (Available in the IVIS® Imaging System 200 Series and IVIS Spectrum.) The surface topography of the subject is determined from the structured light image.
Reference	A structured light image of a white plate that is acquired and stored on disk prior to instrument installation.
3D View	A three-dimensional rendering of the subject. For more details see Appendix H, page 247.
Export	Opens the Export Active View As Image box so that the active image data can be exported (bmp, jpg, png, tiff, or postscript format).
Info	Click to display or hide information about the image in the image window.

Overlay

Photograph

Luminescent

Bias

Structure

Reference

Height Map

3D View
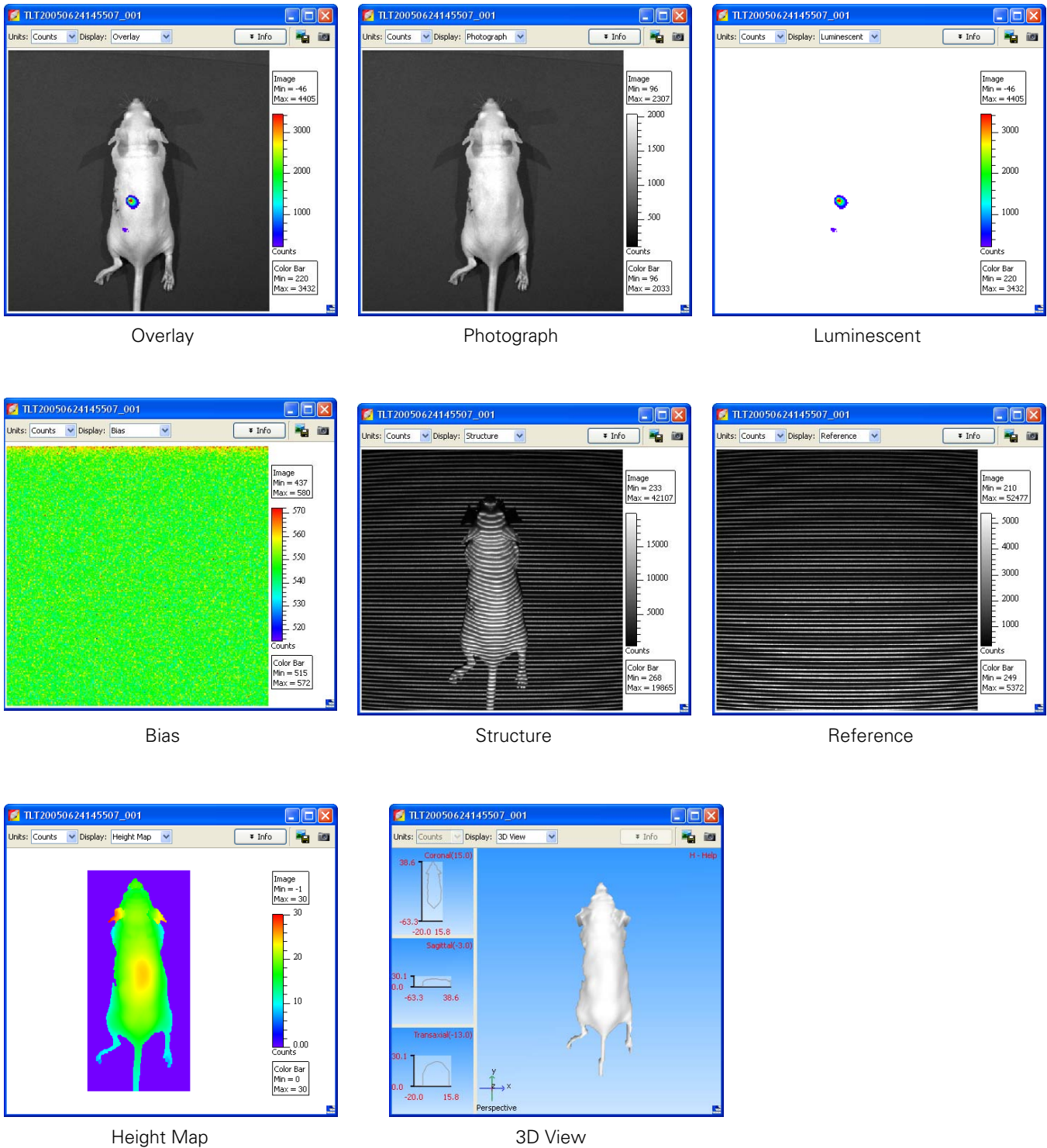
Figure 5.6 Display modes for a single image
The software coregisters the luminescent and photographic image to generate the overlay image.

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-specified 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.

To remove a tag:

1. Position the pointer over the tag.

2. Right-click the image and select **Remove Tag** on the shortcut menu.

3. To remove all tags, right-click the image and select **Remove All Tags** on the shortcut menu.
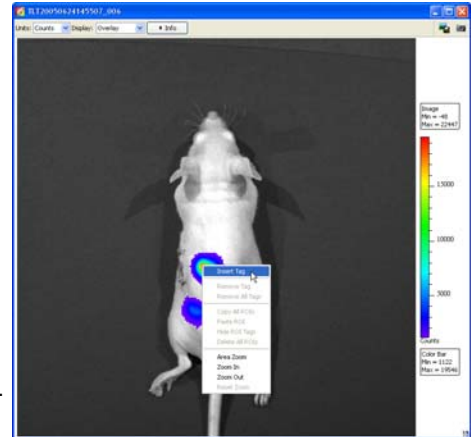
To move a tag:

1. Position the 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.
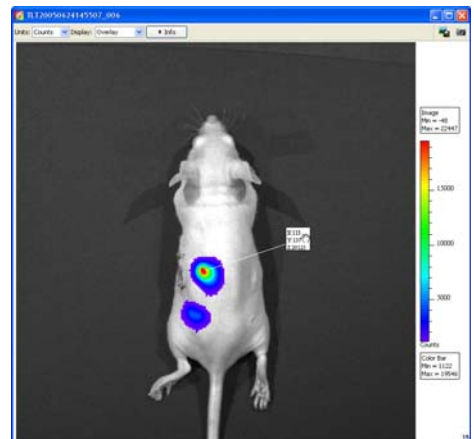
Organizing Images

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

To tile the open image windows, choose **Window → Tile** on the menu bar.
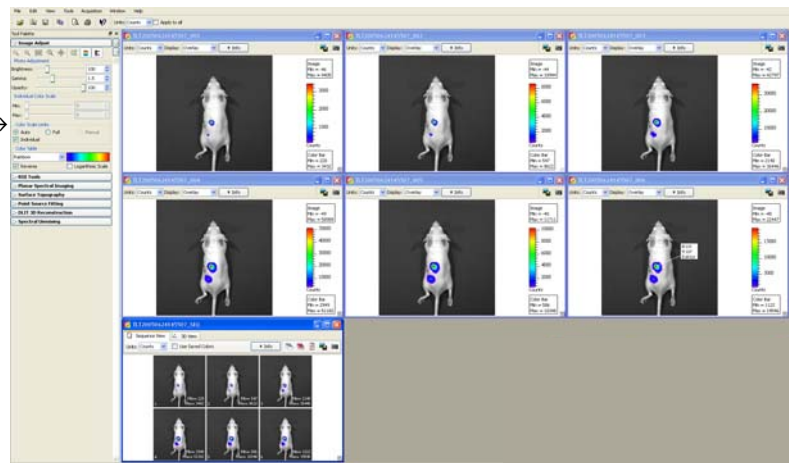
To organize the image windows in a cascade, choose **Window →Cascade** on the menu bar.
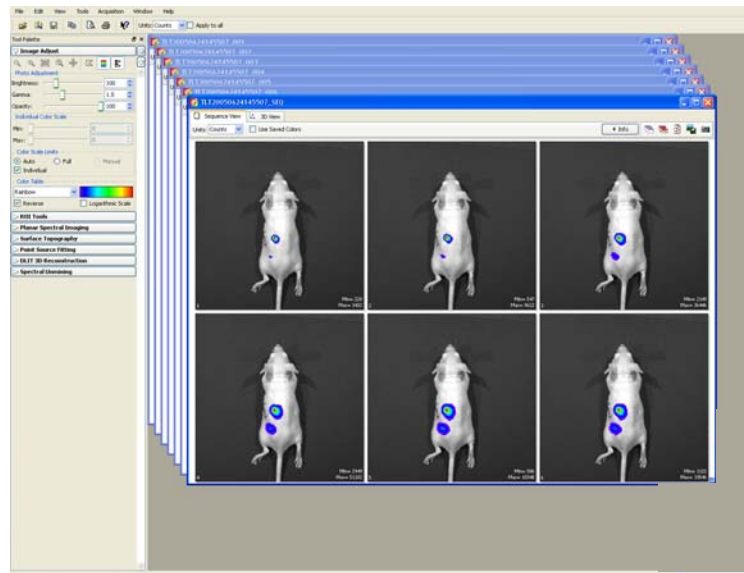
5.5 Viewing Image Information

At acquisition, the software captures image information that includes all of the text information that is associated with every image (for example, camera parameters and user labels).

Click **Info** to display the label set information and acquisition information for the image.
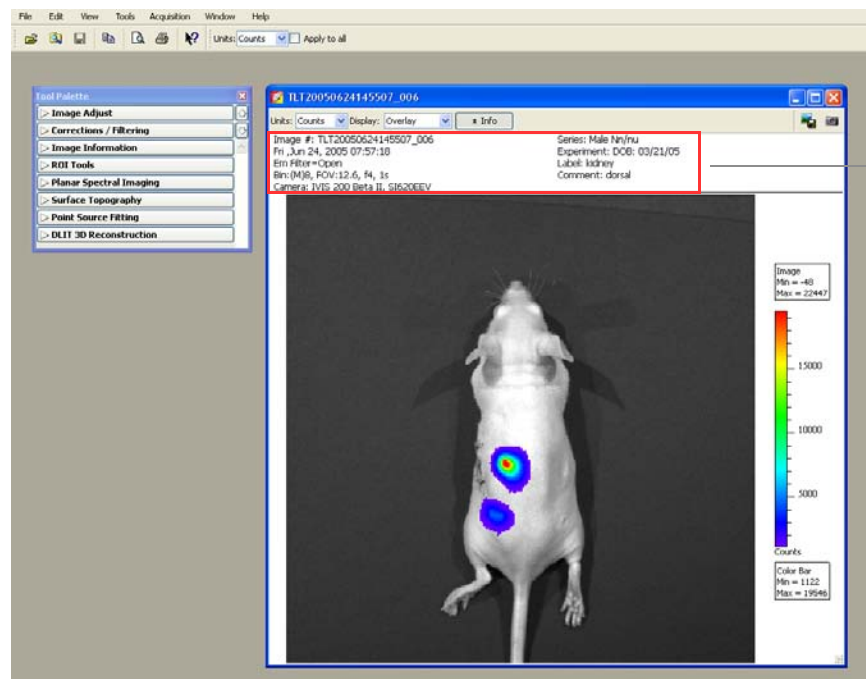
Label set information & acquisition parameters

To view information about an image:

1. Open the image or image sequence of interest. (For details on how to open data, see).

2. Select **View →Image Information** on the menu bar.

   - The Image Information window appears.

3. To choose an image, make a selection from the Sequences drop-down list and the Images drop-down list.

Drop-down list of open sequences. Choose **Individual Images** from the list to show the open single images in the Images drop-down list.

Drop-down list of images in the selected sequence. Or a list of single images if "Individual Images" is selected in the Sequences drop-down list.

Choose the **Show All Sections** option to display all categories of image information.
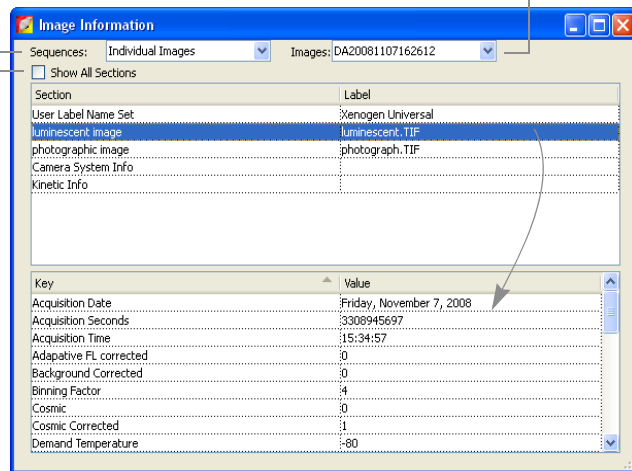
4. To view information of interest, select a category in the upper box to show the associated information in the lower box. For example, select luminescent image in the upper box to show the luminescent image acquisition parameters.

Editing the Image Label

You can edit the image label information after acquisition.

1. Open an image.

2. Select **Edit →Image Labels** on the menu bar.
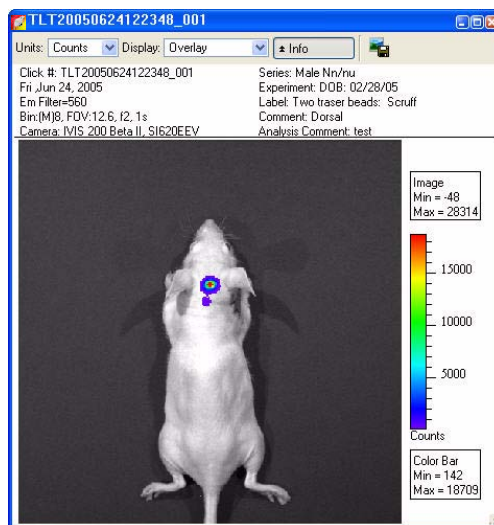
3. In the Edit Image Labels box that appears, edit the information of interest. You can also select a new label set to apply to the image or sequence.

4. When you are finished, click **OK**.

   - The image information is updated.

5. Save the image to save the updated image information.
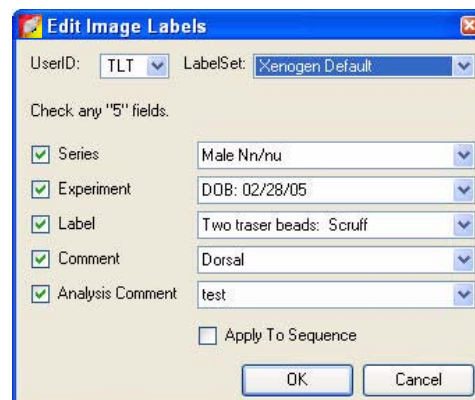
5.6 Image Layout Window

The Image Layout window provides a convenient way to annotate and save an image to a graphic file.

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.
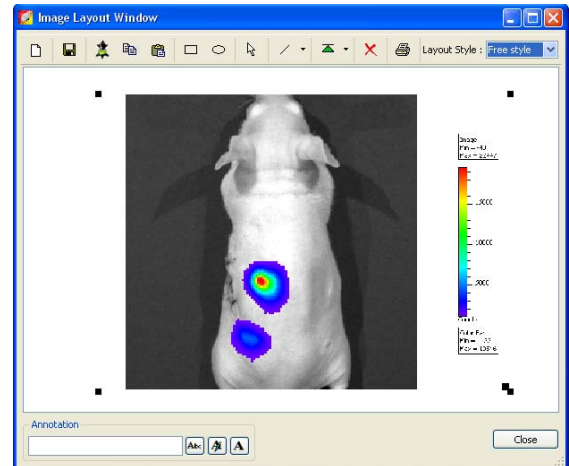
Table 5.4 Image layout window
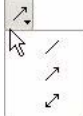
Item	Description
(clear icon)	Clears the Image Layout window. **Note:** If you do not clear the layout (click the button) before you close the Image Layout window, the same window contents are displayed the next time the window is opened
(save icon)	Opens a dialog box that enables you to save the Image Layout window contents to a graphic file.
(paste active image icon)	Pastes the active image in the Image Layout window.
(copy icon)	Copies the contents of the Image Layout window to the system clipboard.
(paste icon)	Pastes the contents of the system clipboard to the Image Layout window.
(rectangle icon)	Rectangle drawing tool
(ellipse icon)	Ellipse drawing tool
(pointer icon)	Pointer tool
(arrow/line icon)	Arrow and line drawing tool

Table 5.4 Image layout window (continued)

Item	Description
	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 ![icon] drop-down list.
	Deletes the selected image.
Layout Style: Layout 2x2	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.
Annotation	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.
Abc	Opens a dialog box that enables you to select a font or edit the font style and size.
A	Opens a color palette that enables you to select a font color or specify a custom font color.
A	Opens a text editor that enables you to edit the selected text.

5.7 Adjusting Image Appearance

Use the image adjust tools to adjust the appearance of an image (Figure 5.7).

> **NOTE**
>
> Not all tools are available for all image display modes.
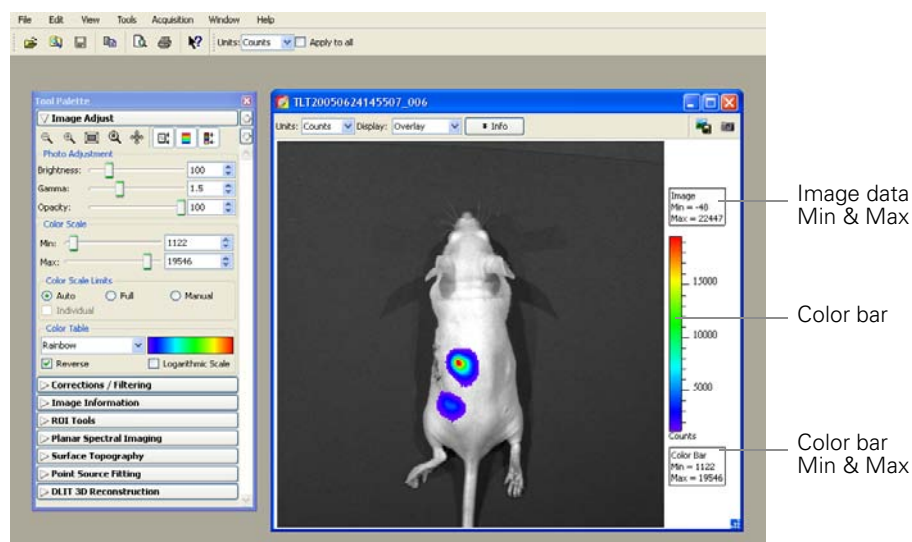
Figure 5.7 Tool palette, Image Adjust tools

Table 5.5 Image Adjust tools
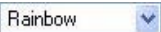
Item	Description
(zoom out icon)	Click this button to incrementally zoom out on the image (reduces the image dimensions in the image window). Note: The zoom tools are also available in the shortcut menu when you right-click the image (**Ctrl**-click for Macintosh users).
(zoom in icon)	Click this button to incrementally zoom in on the image (incrementally magnifies the image in the image window).
(magnify rectangle icon)	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.)
(reset zoom icon)	Click this button to return the image to the default display magnification.
(pan icon)	Click this button to move a magnified image (*pan*) in the image window. For more details, see page 65.
(min/max icon)	Click this button to hide or display the image min/max information in the image window
(color scale icon)	Click this button to hide or display the color scale in the image window
(color scale min/max icon)	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.
Gamma	Click and move the slider left or right to adjust the *gamma* of a 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 bar for an image. Pixels less than the minimum value are not displayed.
Max	The maximum pixel intensity associated with the color bar for an image. Pixels greater than the maximum value are displayed in the maximum color.
Limits	
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.
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.

Table 5.5 Image Adjust tools (continued)

Item	Description
Color Table Rainbow ⌄	Click the drop-down arrow to select a color table for the image data. (For more details on color tables, see *Pseudocolor Images*, page 211.)
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:

1. Click the ✣ button.

2. When the pointer becomes a ✥., click and hold the pointer while you move the mouse.

Note: Panning helps you view different areas of a magnified image. If the image has not been magnified, you cannot pan the image.
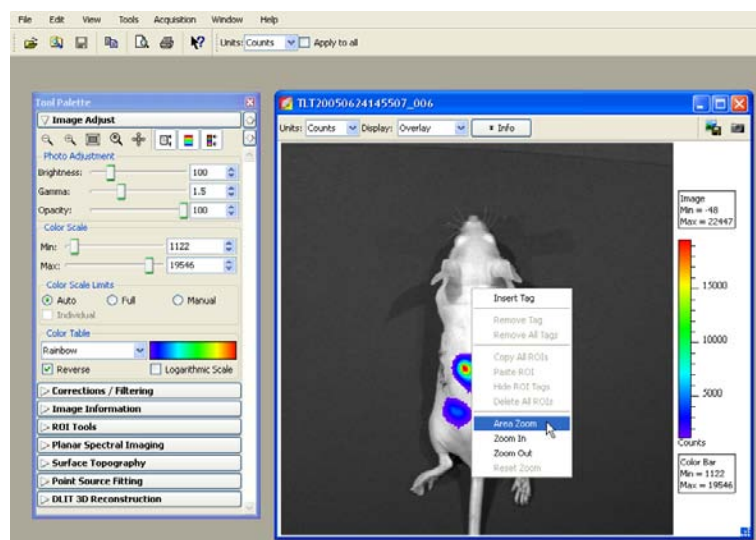
5.8 Correcting or Filtering Image Data

Use the Corrections/Filtering tools (Figure 5.8) to subtract background or apply corrections to the image data. (For more details on sources of background, see Appendix E, page 217.) You can also apply smoothing and soft binning to the image data. (For more information on binning and smoothing, see Appendix C, page 205.)
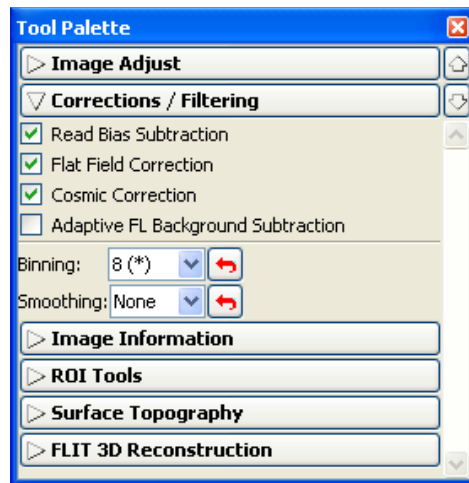
Read Bias Subtraction and Flat Field Correction are default mandatory corrections in photons mode. In counts mode, these corrections can be cleared.

Figure 5.8 Tool palette, Corrections/Filtering tools

Table 5.6 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 217.
	Note: In photons mode, dark background subtraction is a mandatory default. In counts mode, the check box can be cleared.
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 216. 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 216.
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 235.
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 5.9). For more details on binning, see Appendix C, page 206.

Table 5.6 Tool palette, Corrections/Filtering tools (continued)

Tool	Description
Smoothing	Computes the average signal of the specified number of pixels and replaces the original signal with the average signal (Figure 5.9). Smoothing removes signal noise without changing pixel size. **Note:** This type of smoothing is defined differently from the smoothing performed in the Living Image® software.
↩	Click this button to return the binning or smoothing to the previous setting and update the image.
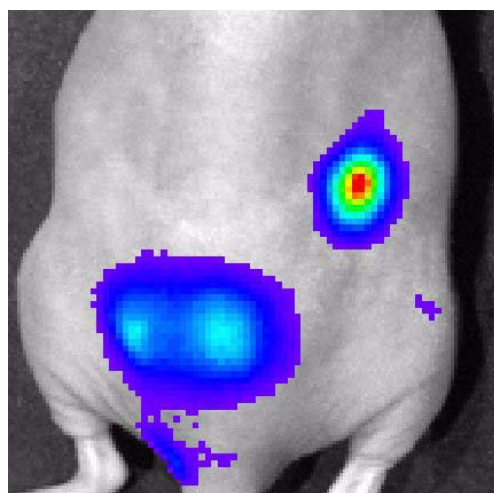
Binning at acquisition = 8, no smoothing   Binning = 2, smoothing = 5x5
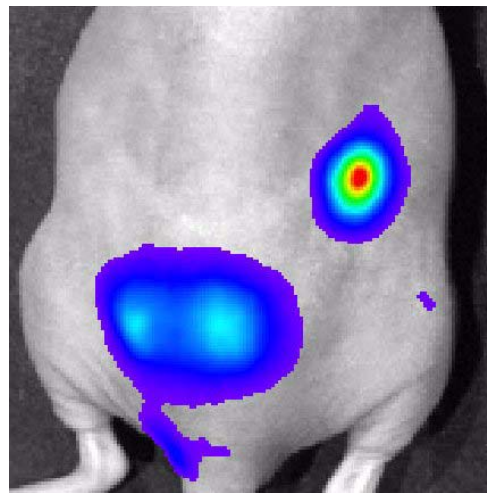
Figure 5.9 Example of binning and smoothing image data

5.9 Image Information Tools

You can view information about the active image using the Image Information tools (Figure 5.10).
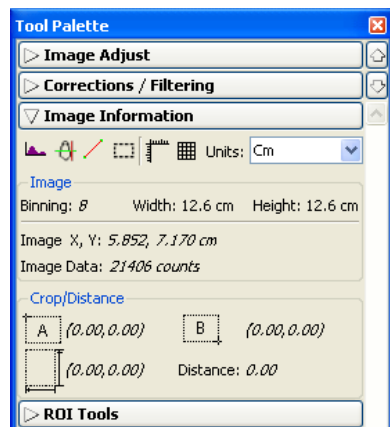
Figure 5.10 Tool palette, Image Information tools

Table 5.7 Tool palette, Image Information tools

Tool	Description	See Page
	Click this button to display a histogram of pixel intensity.	70
	Click this button to display a line profile.	71
	Click this button to display the distance measurement cursor in the image window.	72
	Click this button to draw and measure a rectangle on an image.	73
	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.	
Cm ▾	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.	69
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		
A	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 measurement cursor.	73
B	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 measurement cursor.	
	The width and height of the image crop tool. OR Δx, Δy from the A to B end of the distance measurement tool.	
Distance	The length of the diagonal from corner A to corner B in the image crop tool. OR The length of the distance measurement cursor.	

Use the tools to make measurements in an image and 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.	69
Histogram	Histogram of pixel intensities in an image.	70
Line profile	Plots a line graph of intensity data at each pixel along a user-specified horizontal or vertical line in the image	71

Viewing X,Y Coordinates & Intensity Data

1. In the Image Information tools, choose Cm or Pixels from the Units drop-down list.

2. Put the mouse pointer over the location of interest in the image.

   - The x,y coordinates and intensity data are displayed in the tool palette.

Note: The information is updated when you change the pointer position.
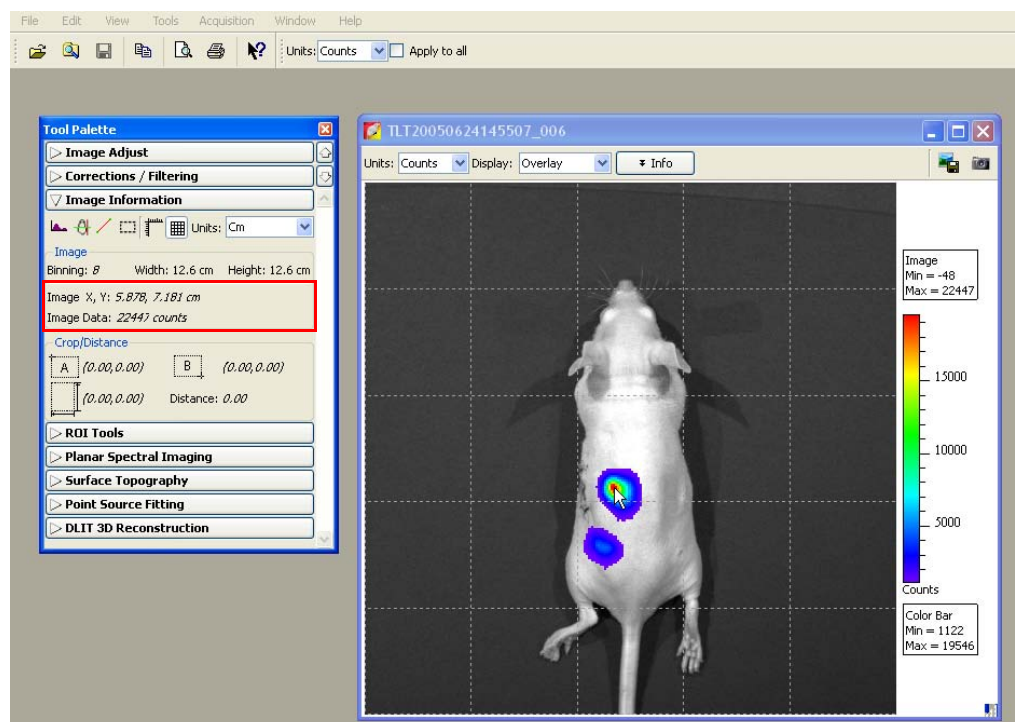
Viewing an Image Histogram

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 display the image histogram:

1. Open an image.

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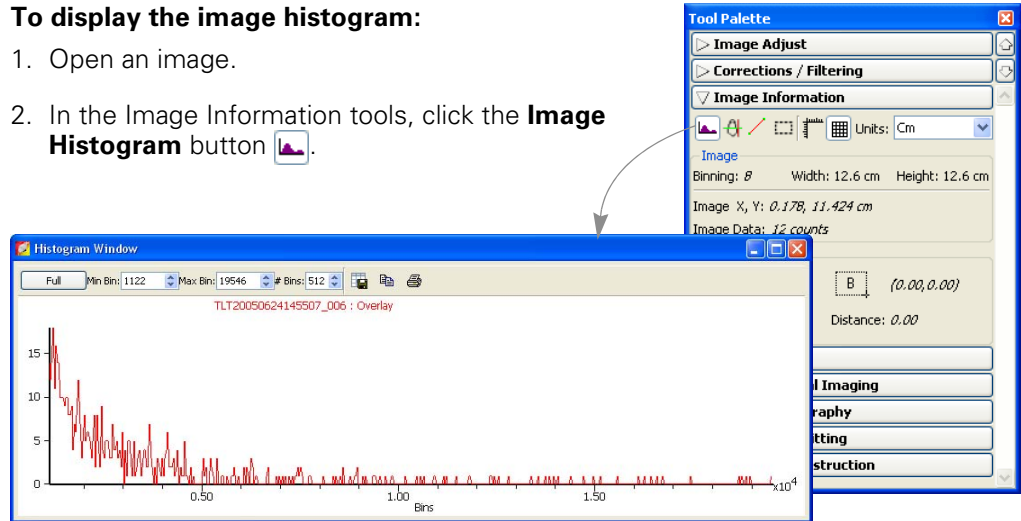
Note: By default the Auto min/max range of the image data determines the histogram range and bins (the software sets the min and max values to optimize image display and suppress background noise). To display the histogram using the full intensity range of the image, click **Full** in the Histogram window.

3. To edit the minimum or maximum bin intensity, enter a new value in the Min Bin or Max Bin box, or click the arrows.

4. 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 5.8 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).
	Copies the histogram to the system clipboard.
	Opens the print dialog box.

Viewing a 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 blue line appears on the image and the Line Profile window appears.
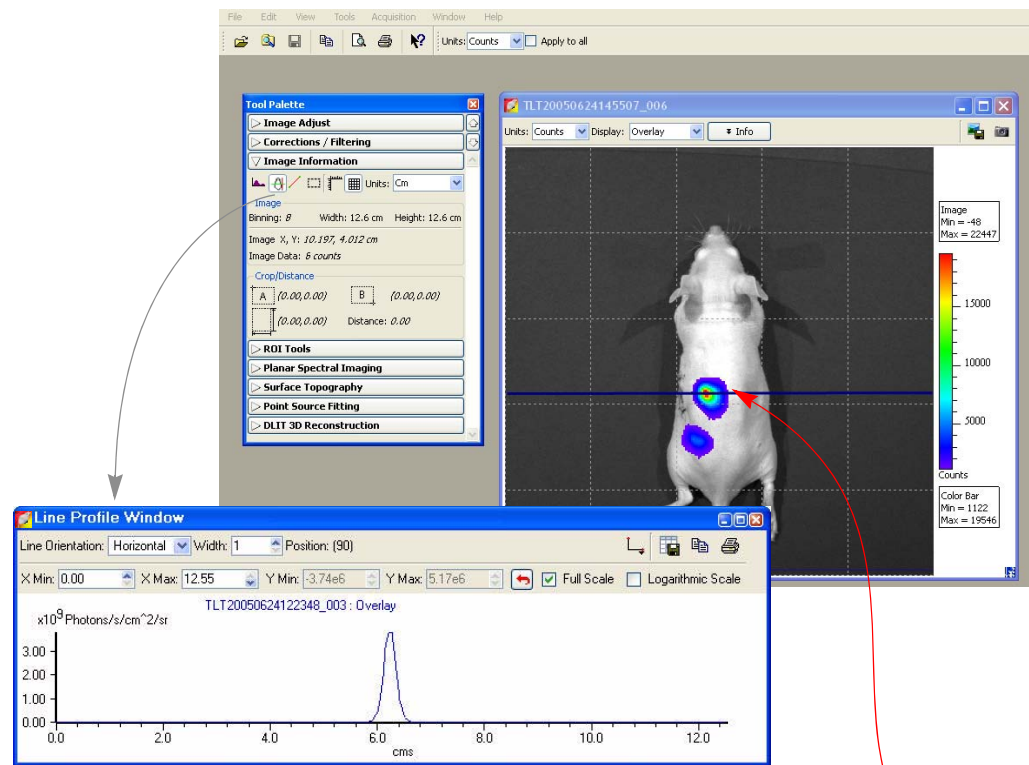
2. To view the line profile at another location in the image, put the mouse pointer over the line. When the pointer becomes a ⊥, drag the line over the image. The blue line determines the pixel intensities that are plotted in the line profile graph.

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

Table 5.9 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.

Table 5.9 Line Profile window (continued)

Item	Description
Position	Line position (pixels).
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.
⌐	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.

Making Image Measurements

To measure distance with the measurement cursor:

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

    - A measurement cursor (▪A——————▪B) appears on the image. The tool palette shows the position and length of the cursor.
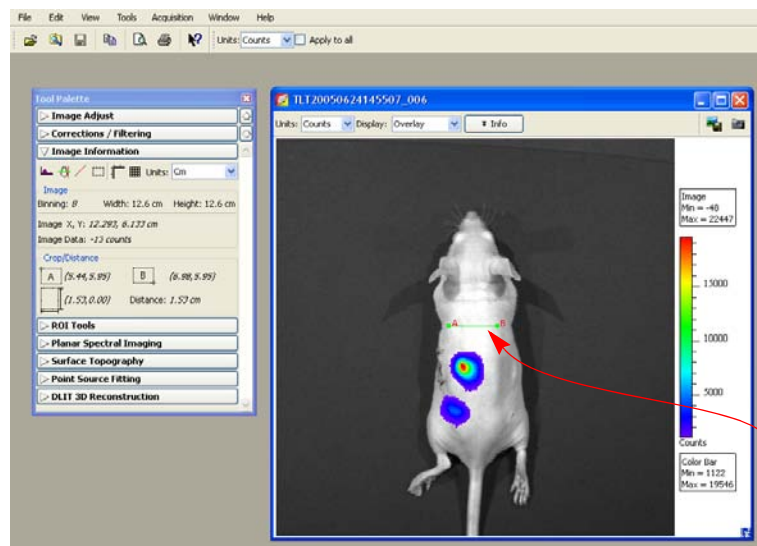
Measurement cursor

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 5.10 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 ⬚.

Crop box position & dimensions
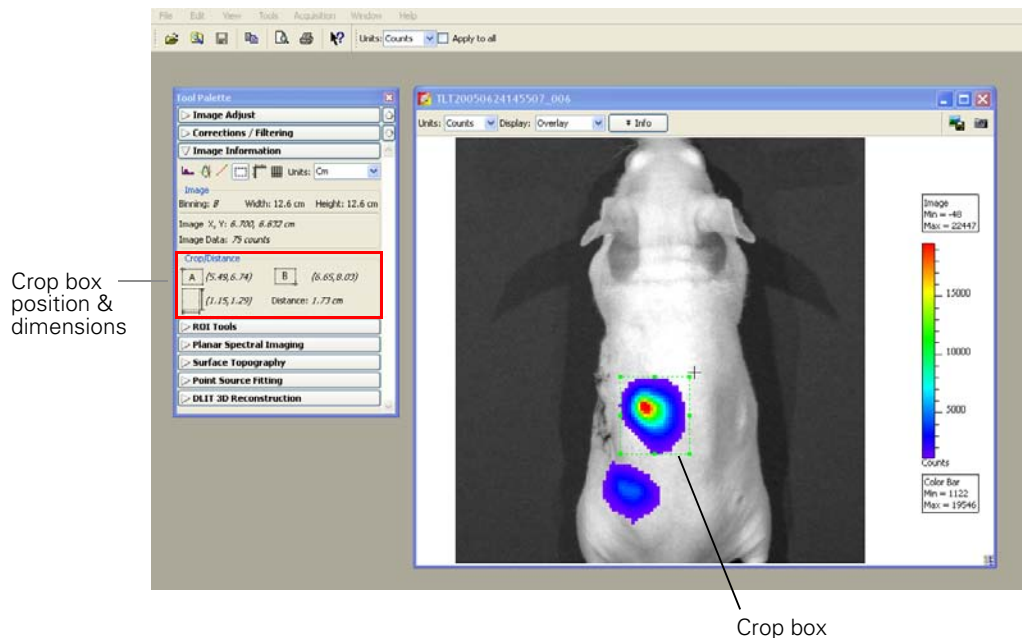
Crop box

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 5.11 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.
(width/height icon)	Box width and height.
Distance	Length of the diagonal from the upper left to lower right corner of the box.

5.10 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.

NOTE

The colorize tool is only available if Show Advanced Options is selected in the general preferences (see page 196).

To view colorized intensity data:

1. Open an image sequence.
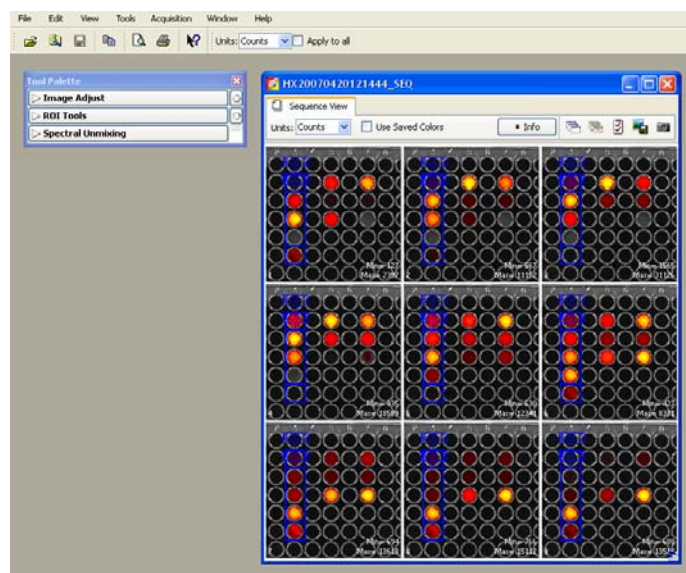
In this example, images were acquired using different combinations of excitation and emission filters. The samples are quantum dot nanocrystals (700 or 800 nm).

2. Select **Tools →Colorize "sequence name"_SEQ** on the menu bar.
   - The software renders each luminescent or fluorescent image in color and combines them into a single image.

3. To access controls for manually
   adjusting the color rendition, click
   Colorize.
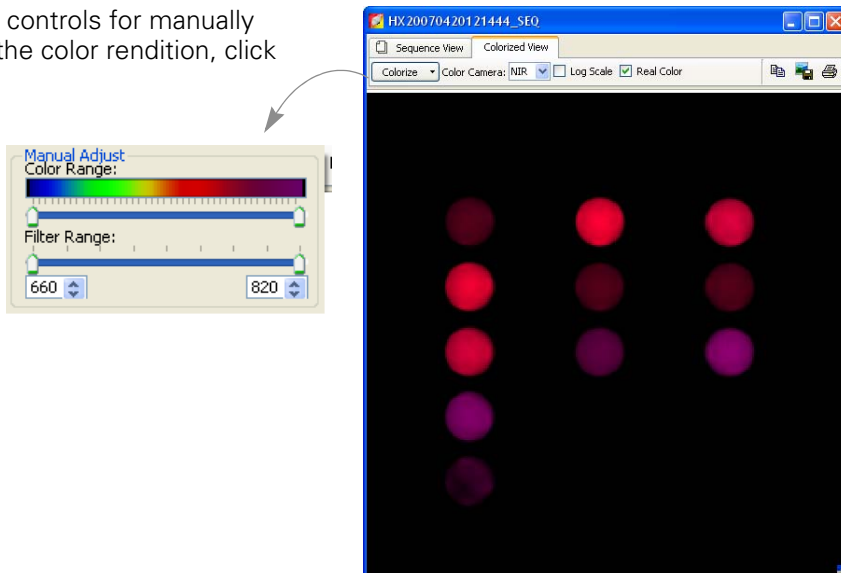
Table 5.12 Colorize tools

Item	Description
Colorize	
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.
Color Camera	
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.
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.
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.

5.11 Viewing Transillumination Data

The transillumination overview feature combines the images of a FLIT sequence (a fluorescence sequence acquired in transillumination mode) into a single image. All of the individual fluor 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 photon units and can be analyzed using the tools in the tool palette.

1. Open a sequence acquired in fluorescence transillumination mode.

2. Select **Tools →Transillumination Overview for xx_SEQ** on the menu bar.

   - The overview appears.
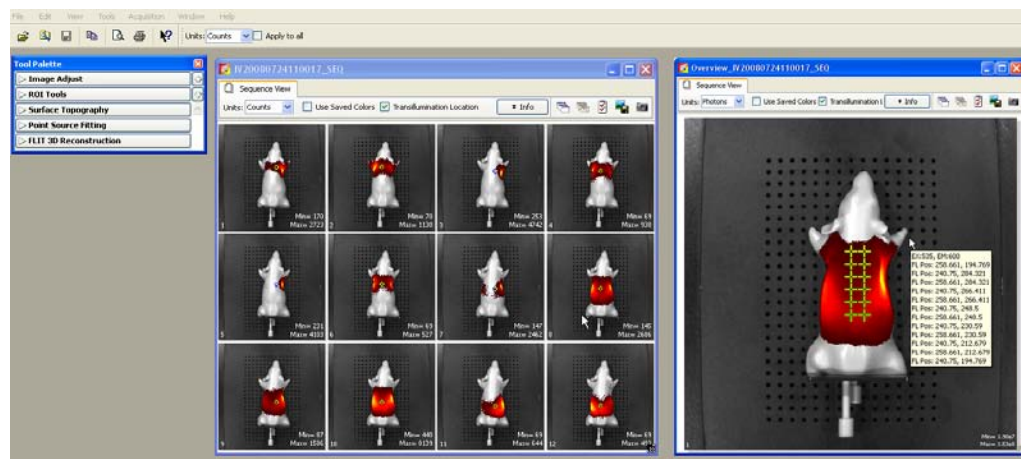
Sequence view   Transillumination overview

5.12 Viewing & Editing 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
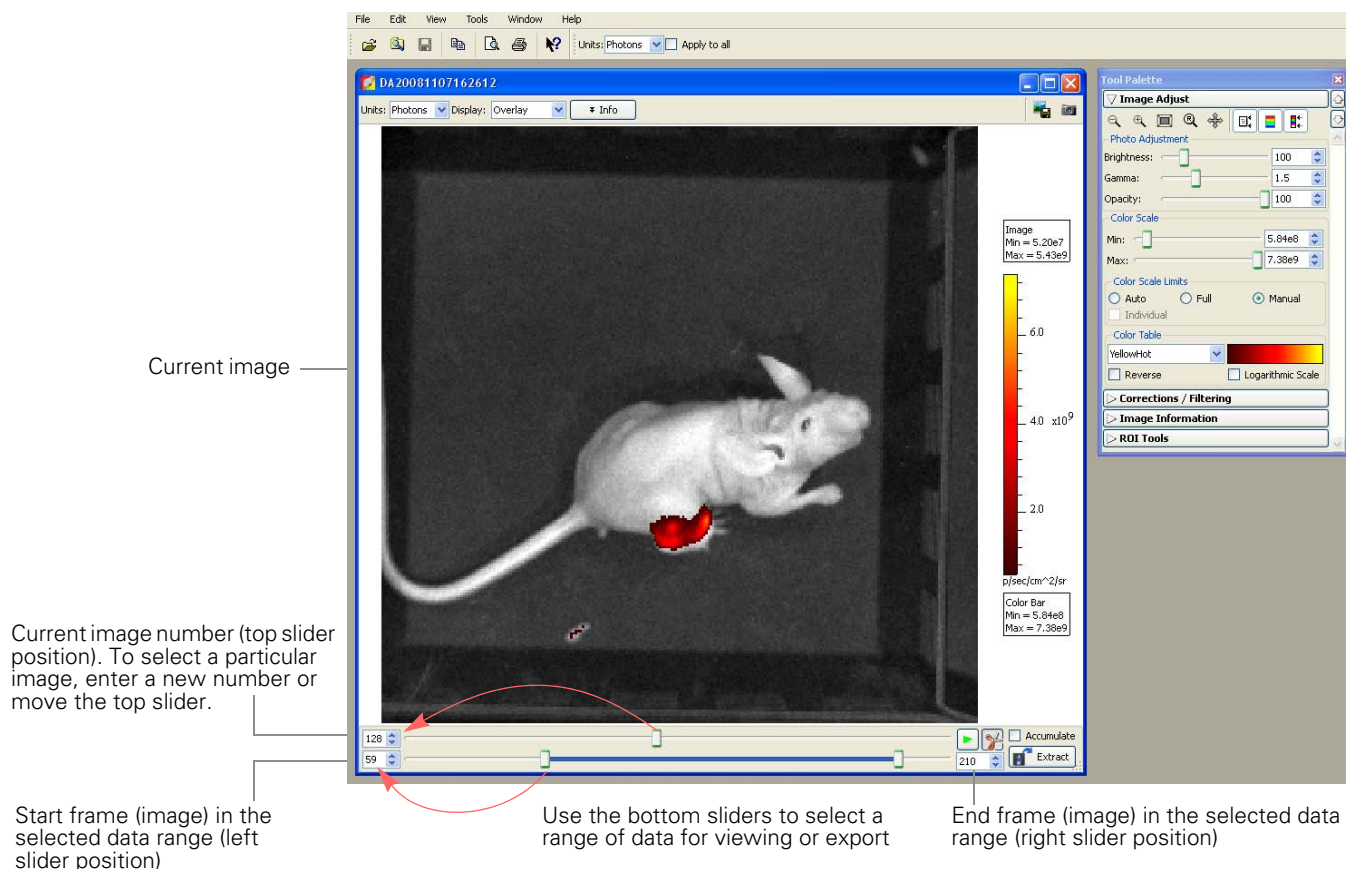
Figure 5.11 Image window, kinetic data
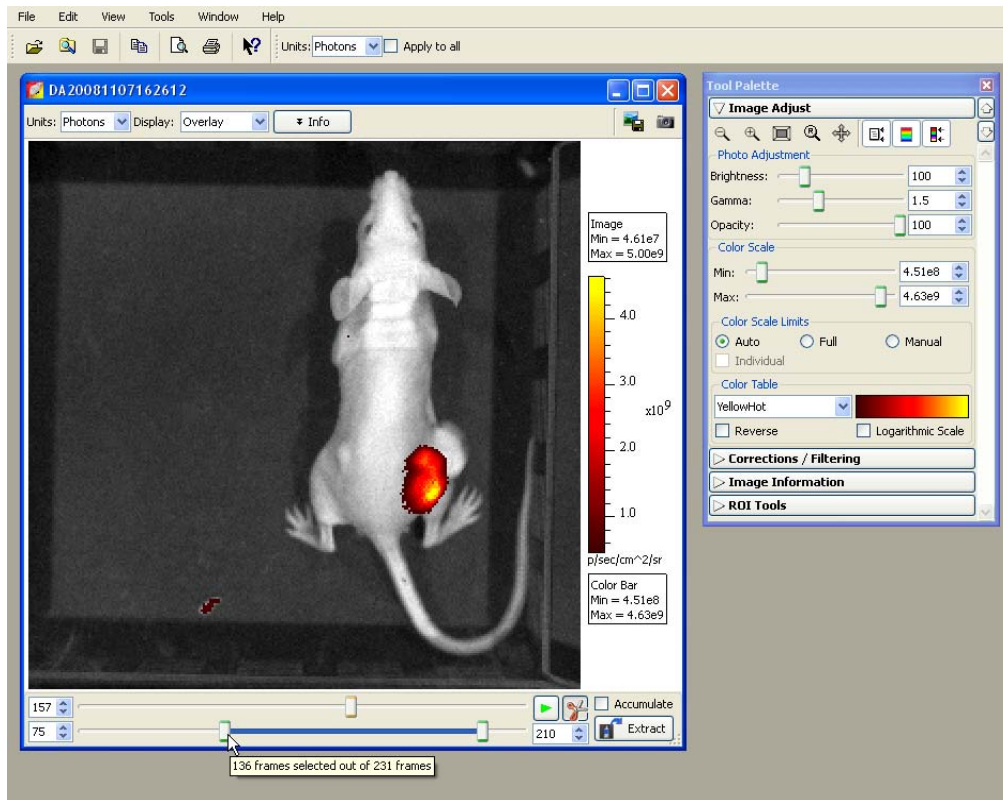
Figure 5.12 Image window, selecting kinetic data for export
Put the mouse arrow over a bottom slider to view a tooltip that shows the number of selected images (frames)

Table 5.13 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 accumulations 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.

Table 5.13 Image window, kinetic data (continued)

Item	Description
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.

Viewing Kinetic Data

1. Open the kinetic data.

2. To start playing the kinetic data, click the ▶ button. If you want to start the playback at a particular image, first move the top slider to the starting image, then click the ▶ button.

3. To stop playing data, click the ■ 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.

Editing & 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 5.12).

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.
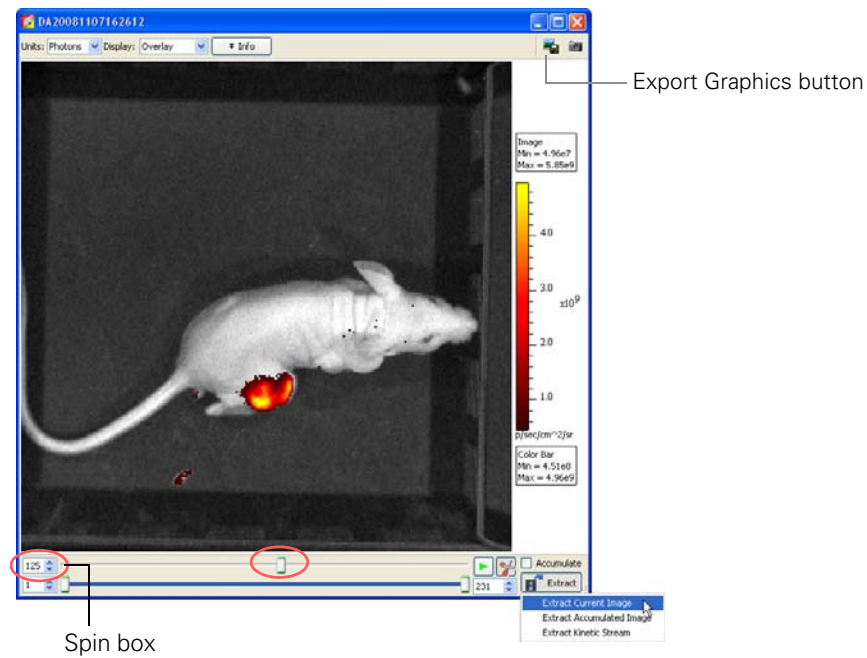
Figure 5.13 Image window, selecting an image for export

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 ![icon] 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**.

6 Working With ROI Tools

6.1 About ROIs

A *region of interest* (ROI) is a user-specified area in an image (Figure 6.1). The ROI tools enable you to create three types of ROIs: measurement, average background, or subject ROI (Table 6.1). 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 6.1). (For more details, see *Managing the ROI Measurements Table*, page 106.)
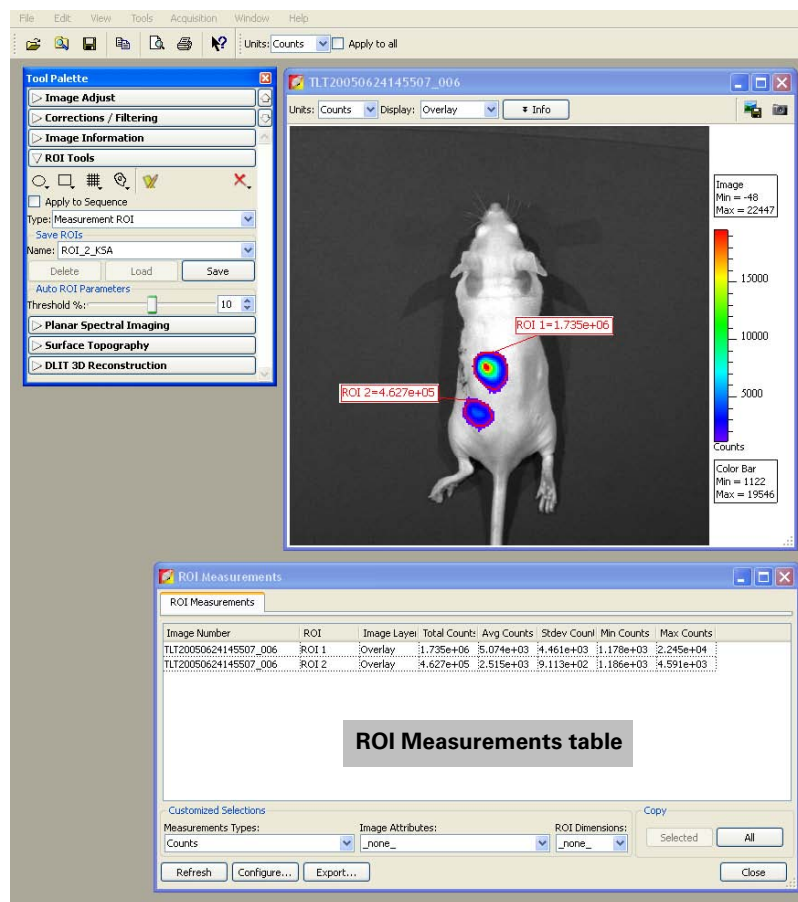
Figure 6.1 Example measurement ROIs and ROI measurements table

Table 6.1 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

---

NOTE

For a quick guide to drawing a measurement ROI, see page 84.

6.2 ROI Tools

To display the ROI tools:

1. Open an image or image sequence and click ROI Tools in the tool palette.
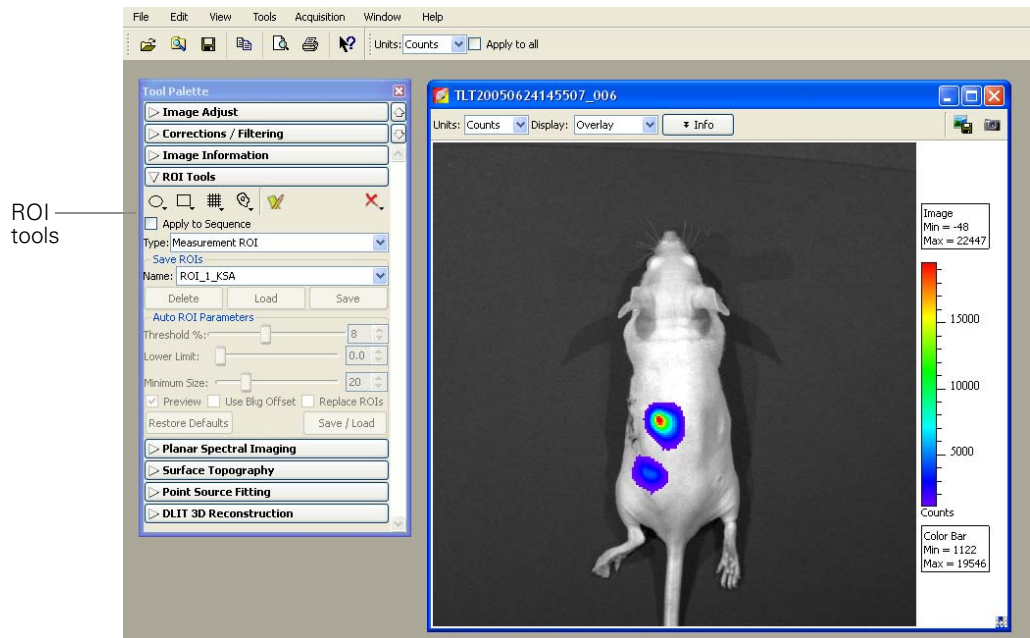
ROI tools

Table 6.2 Tool palette, 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 microtiter plate.
◔	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 88.
▼	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 105. **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.
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 associated a measurement and average bkg ROI included in the same subject ROI. Using this type of ROI is optional.
Save ROIs	
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 104.
Auto ROI Parameters	Parameters that specify how the auto ROI tool draws an ROI. **Note:** These are advanced options that are only available if "Show Advanced Options" is selected in the general preferences.
Threshold %	If the Auto All or Auto 1 method is selected, the 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 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.

Table 6.2 Tool palette, ROI tools (continued)

Item	Description
Lower Limit	Specifies a multiple (1 to 10) of the color bar minimum that sets the lower threshold for identifying an ROI. For example, if the lower limit = 2 and the color bar 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. For more details, see page 91.
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).

6.3 Measuring ROIs in an Image

To obtain the intensity signal in a user-specified area of an image, draw a measurement ROI on the image. There are three ways to draw measurement ROIs:

Drawing Method	Description	See Page
Manual	Places one or more ROIs (circular, square, or grid shape) on the image.	84
Automatic	The software automatically locates and draws a contour 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.	88
Free draw	Draw line segments that define the ROI.	90

Quick Guide: Drawing a Measurement ROI on an Image or Image Sequence

These steps provide a quick guide on how to apply a measurement ROI to an image or image sequence. For more details about measurement ROIs, see page 84.

1. Open an image or image sequence 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 ⚲ ⌄. For an image or image sequence, select Auto All from the drop-down list. For kinetic data, select Kinetic ROI.

    - The software automatically draws measurement ROIs on the image. The ROI label shows the total intensity in the ROI. If you are working with a sequence, open an image to view the intensity label. For more details, see .
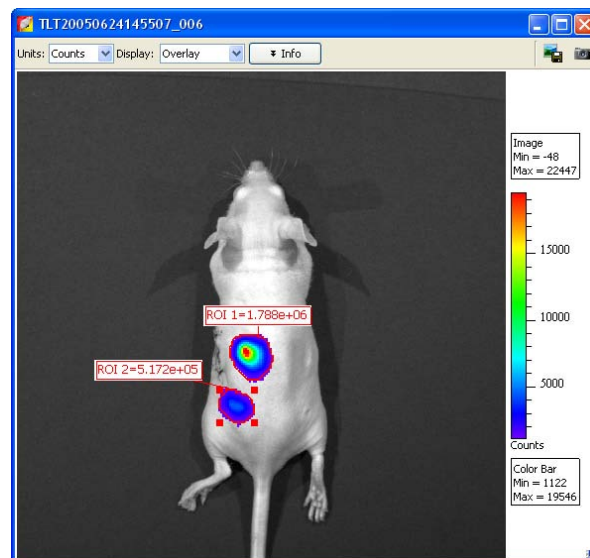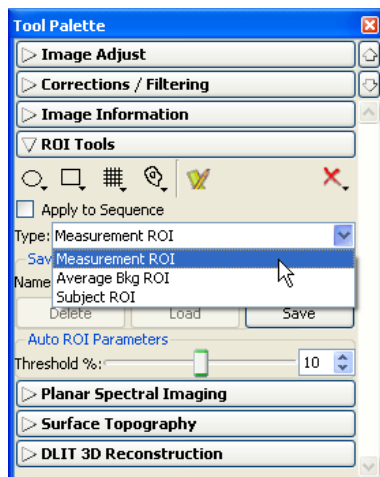
4. If it is necessary to adjust the ROI boundaries, change any of the auto ROI parameters (use the slider or ▲▼ arrows):

    • 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

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. To show the ROI Measurements table, click the Measure button 🟨 in the tool palette.
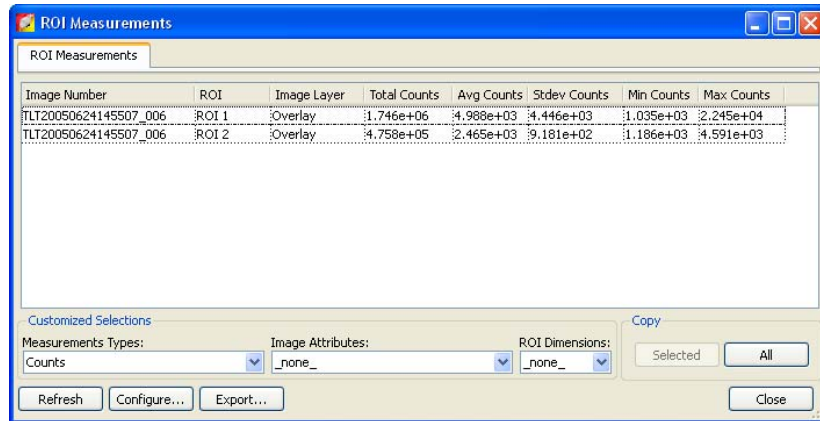
The ROI Measurements tab displays data for all ROIs created in images or sequences during a session (one ROI per row). The table provides a convenient way to review and export ROI data. For more details on the table, see *Managing the ROI Measurements Table*, page 106.

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. To specify 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 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.

3. Adjust the ROI position:

   a. Place the mouse pointer over the ROI. When the pointer becomes a ✥, click the ROI.

   b. Drag the ROI.

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.
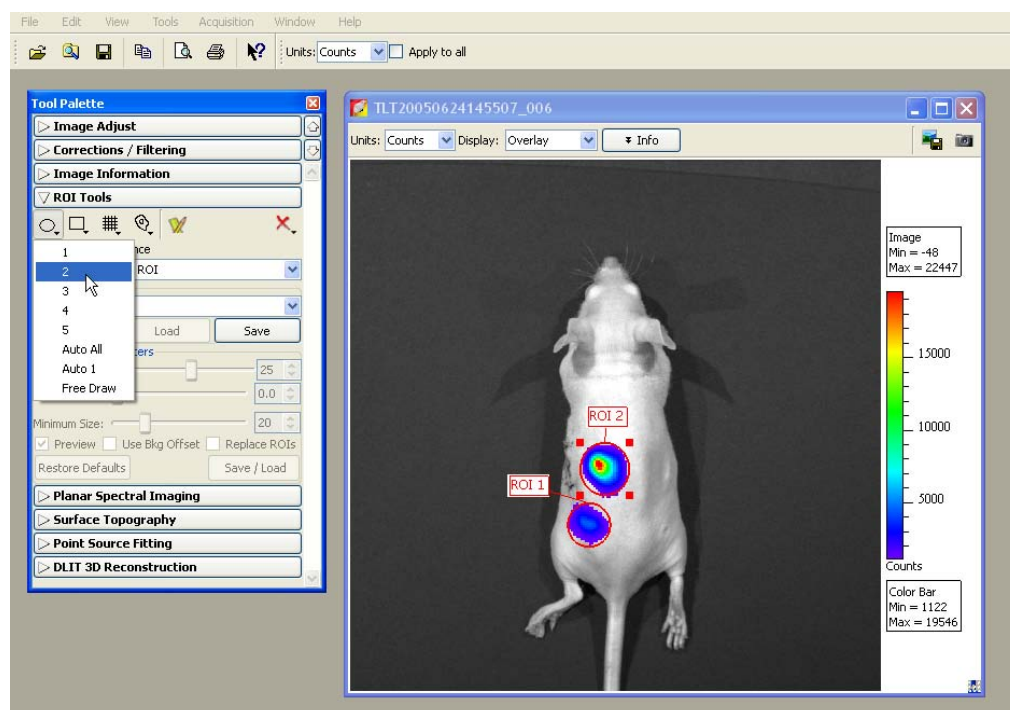
NOTE

You can also change the ROI position or size using the adjustment controls in the ROI Properties box (see *Moving an ROI*, page 100 and *Editing ROI Dimensions*, page 101).

5. Click the **Measure** button .

   - The ROI measurements and table appear. For more details on the table, see *Managing the ROI Measurements Table*, page 106.

Note: For information on how to save ROIs, see page 104.

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.

To automatically draw all ROIs detected by the software:

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 thumbnails.
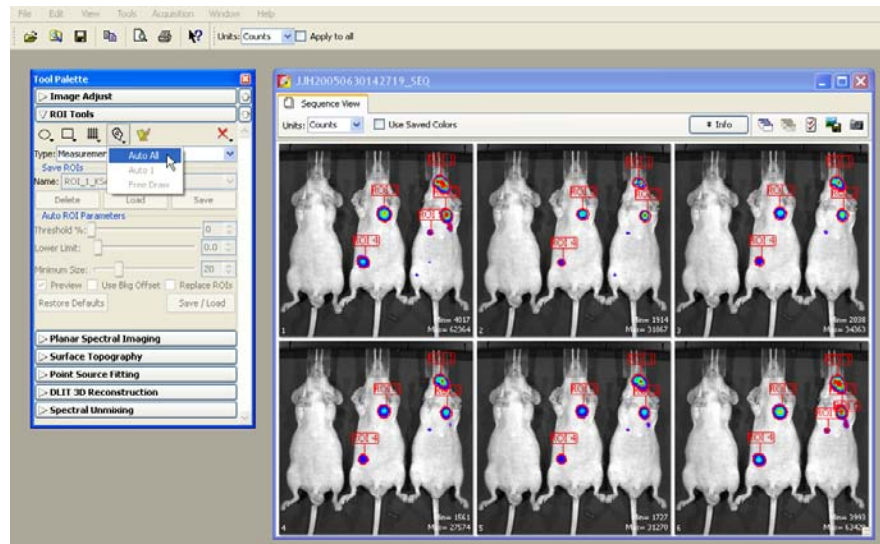
3. Click the **Measure** button ▥.

    - The ROI table appears. For more details on the table, see *Managing the ROI Measurements Table*, page 106.

4. Double-click a thumbnail to view the ROI measurements in the image.
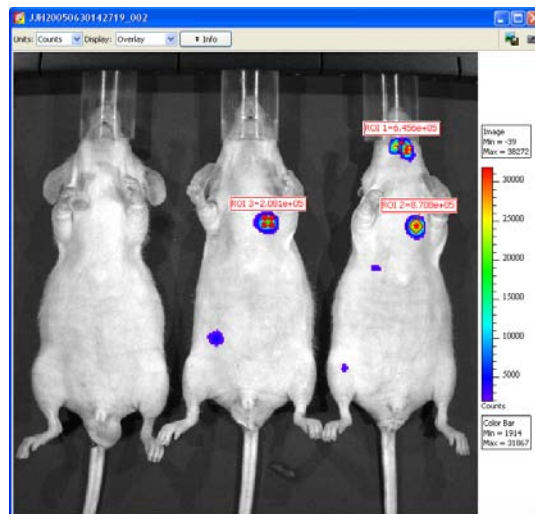
To automatically draw an ROI at a user-specified location:

1. Open an image.

2. Click an ROI shape button and select Auto 1 from the drop-down list.

   - The create tool appears on the image.

3. Use the ring ⊚ to move the create tool to the location for the ROI.

4. Click **Create** on the ring tool.

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

5. To draw another ROI, repeat step 3 to step 4.
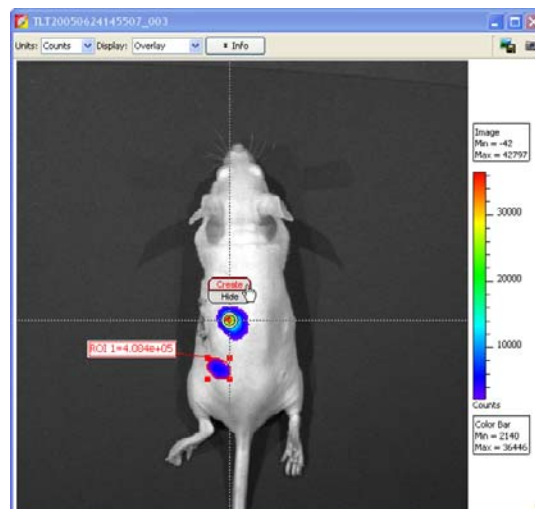
Note: For information on how to save ROIs, see page 104.

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.

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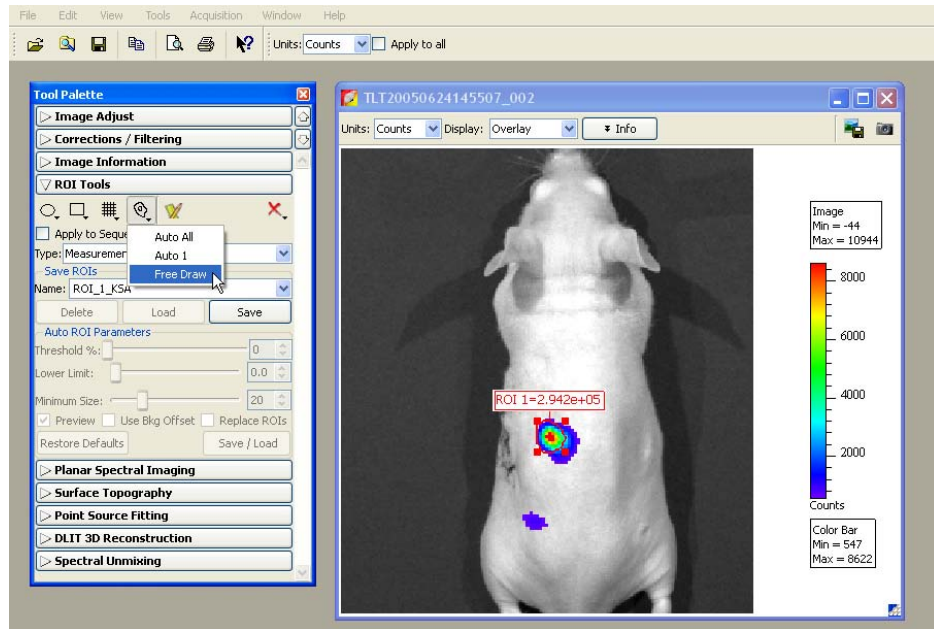
3. If you selected:

   ▢ 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.

Drawing a Subject ROI

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 91.) 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:

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

2. Click the ▣ button, and select **1**.

3. Position the subject ROI so that it includes the measurement ROI(s) and the associated average background ROI.

6.4 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

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 *Image Layout Window*, page 62 and *Magnifying or Panning in the Image Window*, page 65.

To measure background-corrected signal:

1. Draw one or more measurement ROIs on the subject. (For more details, see page 90.)

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 100 and page 101.

   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 three methods to associate the average background ROI with one or more 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 90.
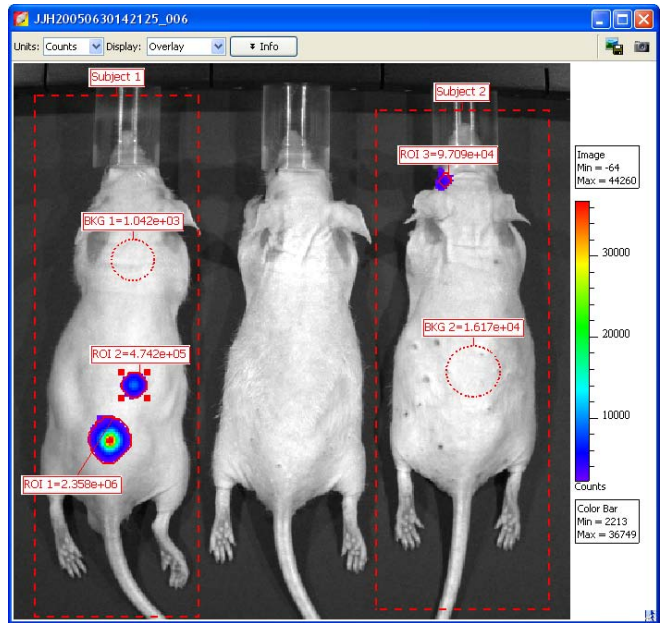
Method 2
Right-click the measurement ROI and select **Set BkG ROI to Bkg X** on the shortcut menu that appears.
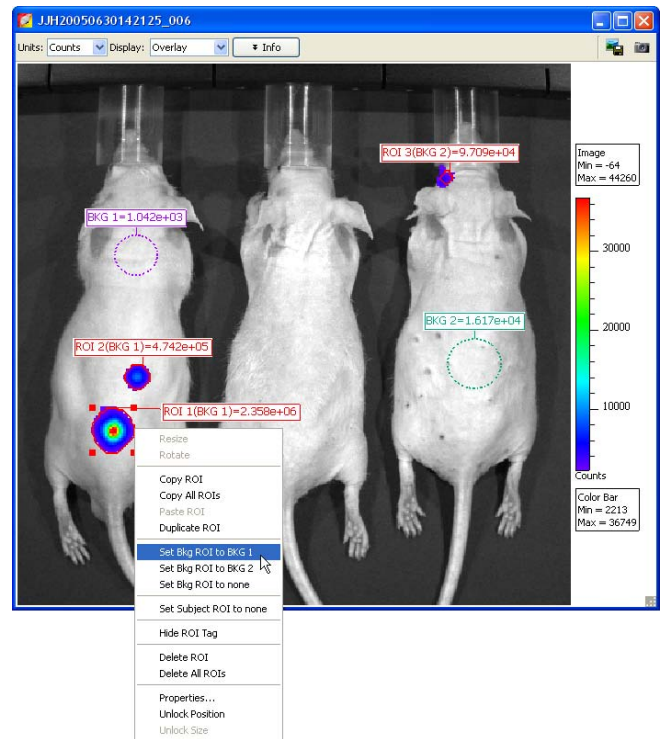
Method 3:

1. Right-click a background ROI and select **Properties** on the shortcut menu.

2. In the ROI Properties box that appears, click the Bkg ROI tab and put a check mark next to **Use as BKG for future ROIs in**.

3. Choose the image name or the **Entire sequence** option.
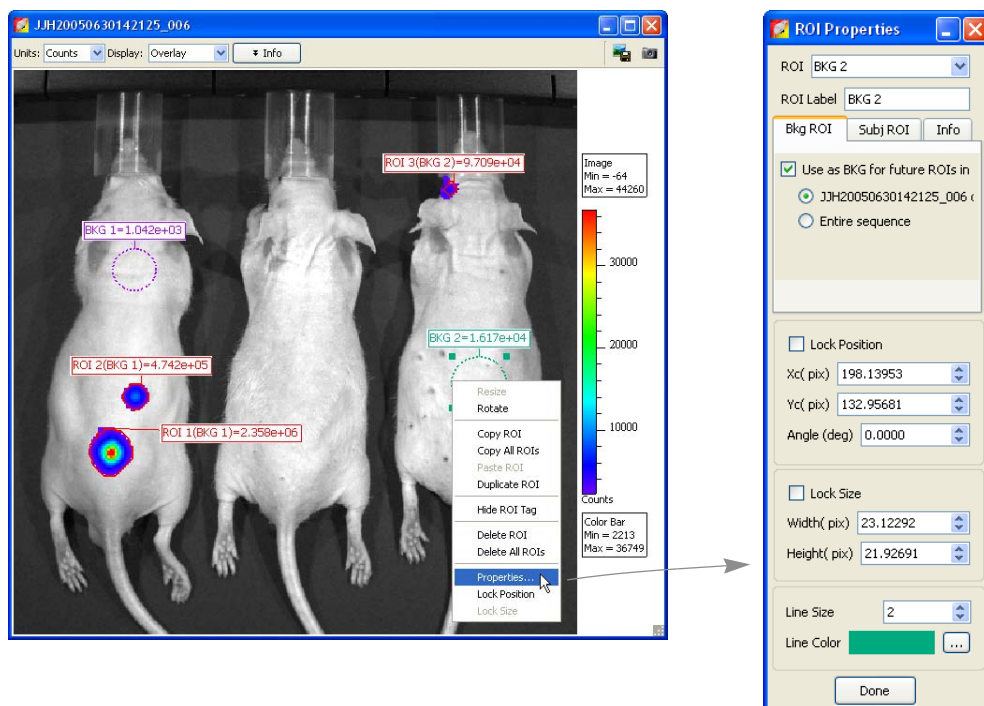
6.5 Measuring ROIs in Kinetic Data

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 6.1, page 82.

> **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.

Quick Guide: Drawing a Kinetic ROI

These steps provide a quick guide on how to apply a measurement ROI to kinetic data. For more details about measurement ROIs, see page 84.

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.

4. Use the ring ◎ to move the create tool to the area of the ROI.

> **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. Click **Create** on the tool.

   - The ROI and label appear on the image.

6. 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

7. 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.

---

8. To measure the ROIs, click the Measure button 🔳 in the tool palette.

   - The Kinetic ROI Measurements tab shows ROI information for the current image.

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

Kinetic ROI measurements are
displayed in a separate tab
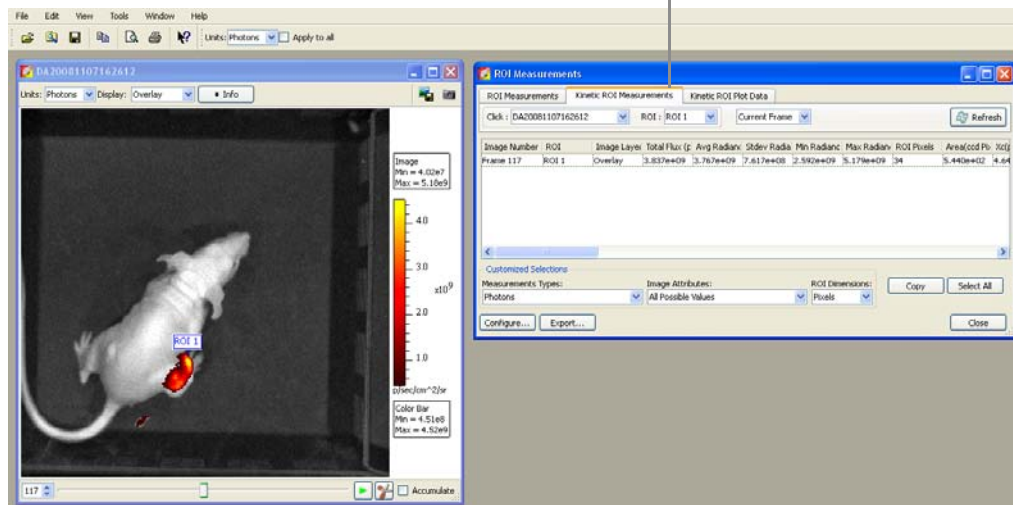
Plotting Kinetic ROI Measurements

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 dialog box appears.
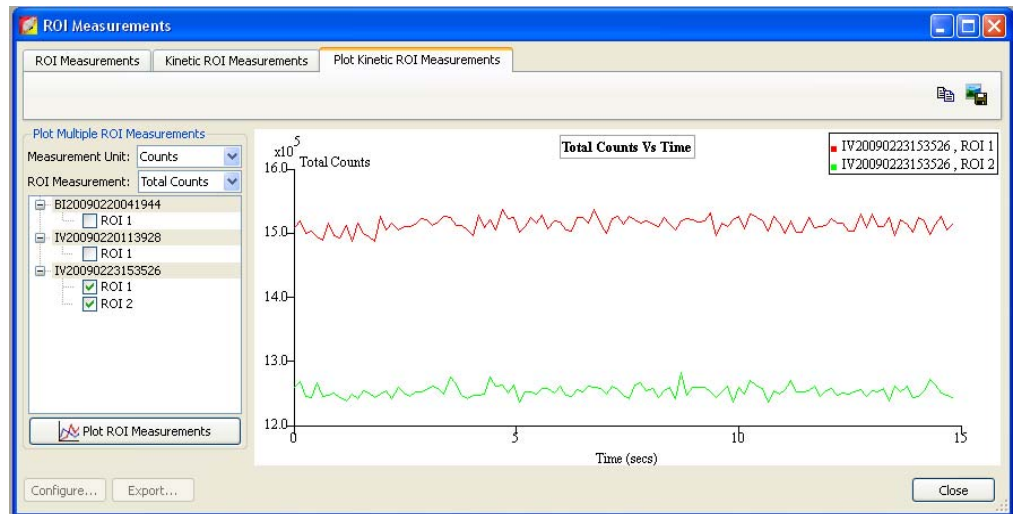
4. Click the Plot Kinetic ROI Measurements tab.

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.

6.6 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

To view ROI properties, do one of the following:

•Double-click the ROI of interest.

• Right-click the ROI and select Properties from shortcut menu that appears.

• Select the ROI, then select **View** →**Properties** on the menu bar.
The ROI Properties box appears (for more details see Figure 6.2).

• ROI selected in the image. To view properties for another ROI, select another ROI from the drop-down list.
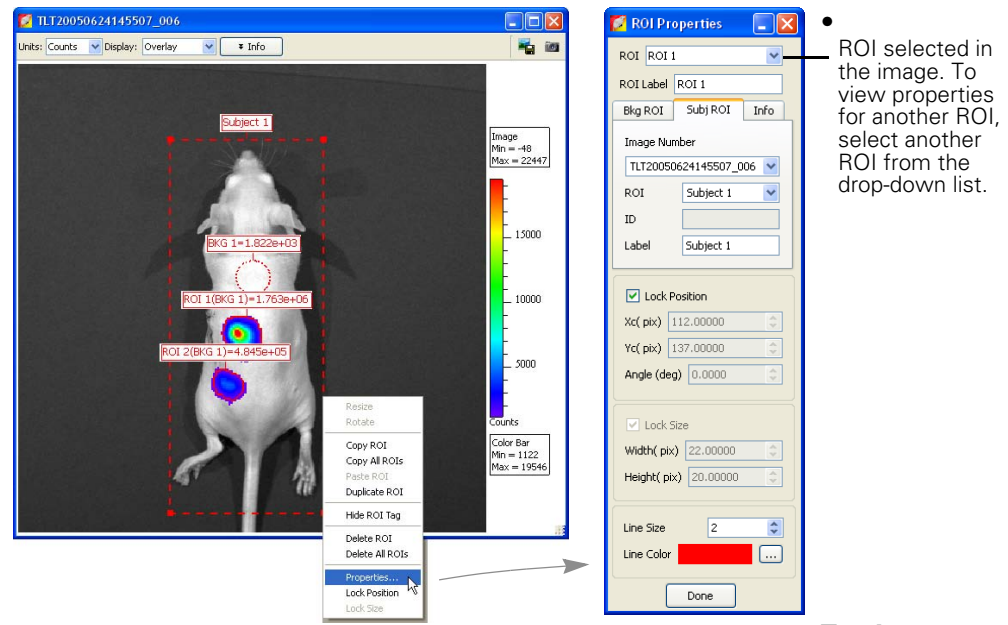
To view properties for another ROI:

• Click another ROI in the image. Alternately, select an ROI from the ROI drop-down list in the ROI Properties dialog box (Figure 6.2).

ROI selected
in the image

Label name of the ROI
selected above.
Double-click to edit.

Selected image

Drop-down list of
average background
ROIs in the image

Bkg ROI tab
(average background ROI
selected in the image)

Bkg ROI tab
(measurement ROI
selected in the image)
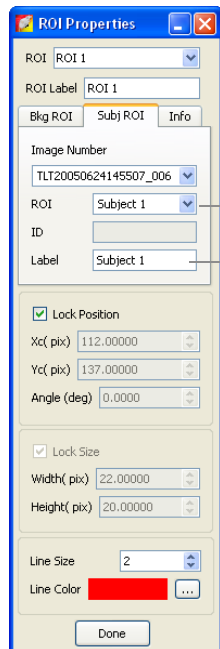
Drop-down list
of subject ROIs
in the image

Enter label information
here for the subject ROI
selected above
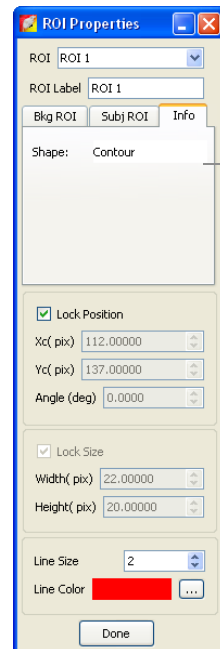
Information about the
ROI selected from the
drop-down list above

Subj ROI tab

Info tab

Figure 6.2 ROI properties
The items in the ROI Properties box depend on the type of ROI selected. For more details
see Table 6.3, page 99.

Table 6.3 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.
ROI Label	Click to edit the selected ROI label name.
Click Number	A drop-down list of open images.
Subj ROI BKG ROI Info tab	The Subject ROI tab shows a drop-down list of all subject ROIs in the click 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 Bkg 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-coordinate of the ROI selected in the image.
Yc	Y-coordinate 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 107).
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 90). • 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**
>
> You cannot move ROIs created using the auto ROI feature.

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.
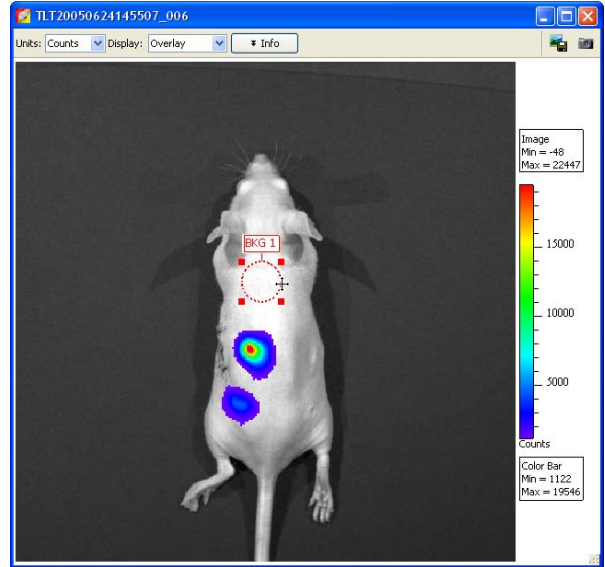
To move an ROI using the ROI Properties box:

1. Double-click the ROI in the image.

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

2. To set ROI position, enter new 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.
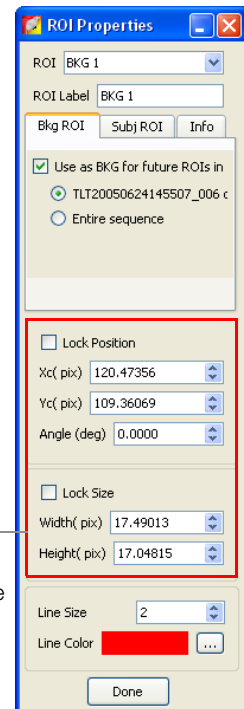
Position of the ROI selected in the image

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 place the pointer over a handle (■)on the ROI.

2. When the pointer becomes a ↖ arrow, drag the handle.
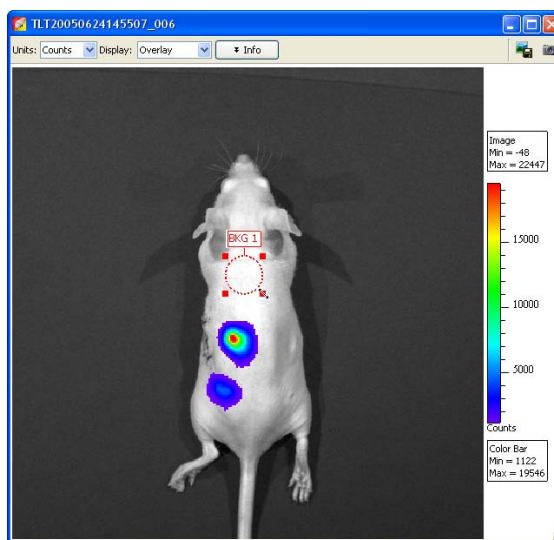
To resize an ROI using the ROI Properties box:

1. Double-click the ROI in the image.

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

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.
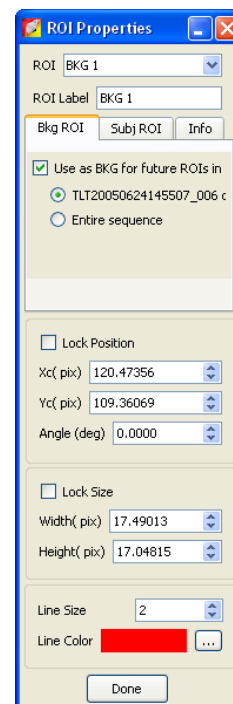
Editing the ROI Line

1. Double-click the ROI that you want to edit.

   - The ROI Properties box appears.

2. To edit the ROI line thickness, enter a new value in the Line Size box. Alternately, click the ⬍ arrows.

3. To change the ROI line color:

   a. Click the Browse button [...].

      - The Select Color box appears.

   b. To select a basic color for the ROI line, click a basic color swatch, and click **OK**.

   c. To define a custom color, drag the crosshair in the custom color field, adjust the brightness slider, and click **Add to Custom Colors**.

   d. To select a custom color for the ROI line, click a custom color swatch, and click **OK**.
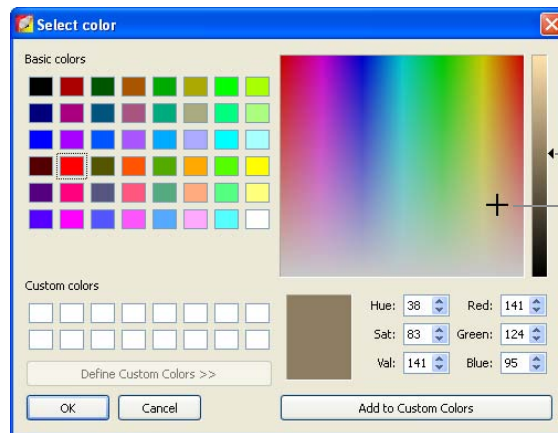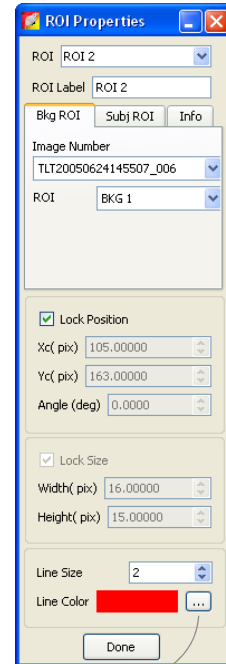
Brightness slider

Cross hairs in the custom color field

Moving or Editing an ROI Label

To move an ROI label:

1. Place the pointer over the ROI label.

2. When the pointer becomes a 🖐, drag the label.

3. Click to release the label at the new location.
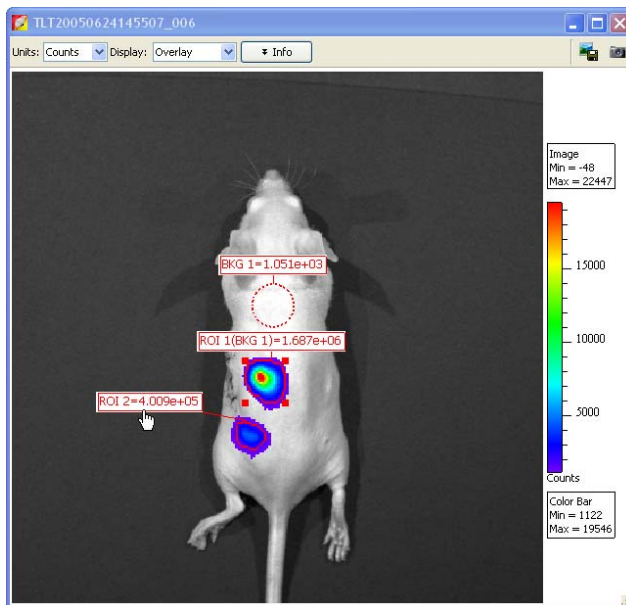
To edit an ROI label:

1. Double-click the ROI. Alternately, 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**.
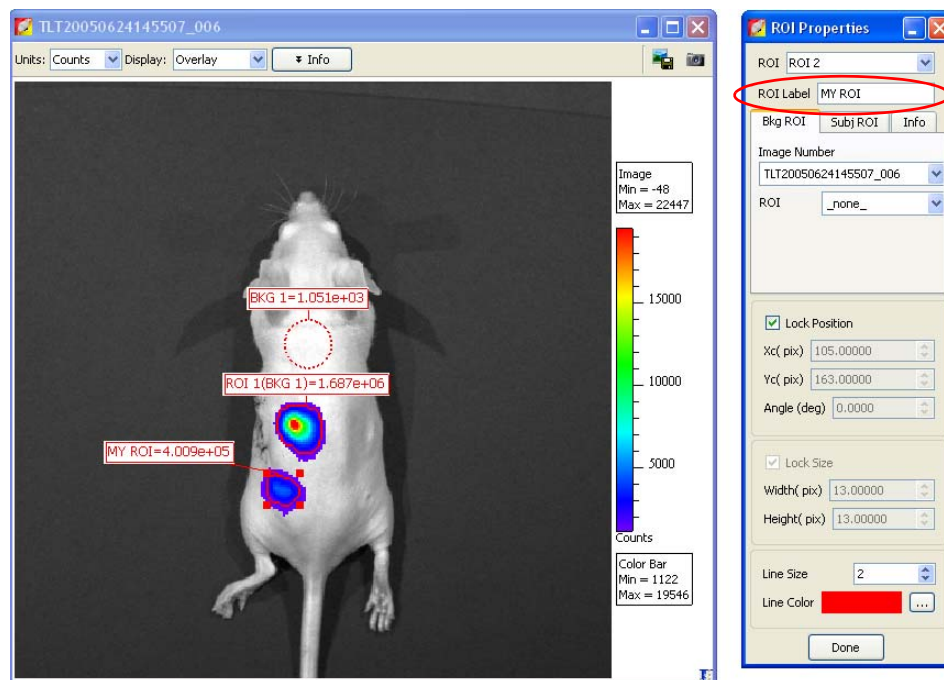
Saving ROIs

The software automatically saves ROIs with an image. The ROI measurements are saved to the AnalyzedClickInfo.txt file associated with the image. ROIs are saved per user and can be 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).
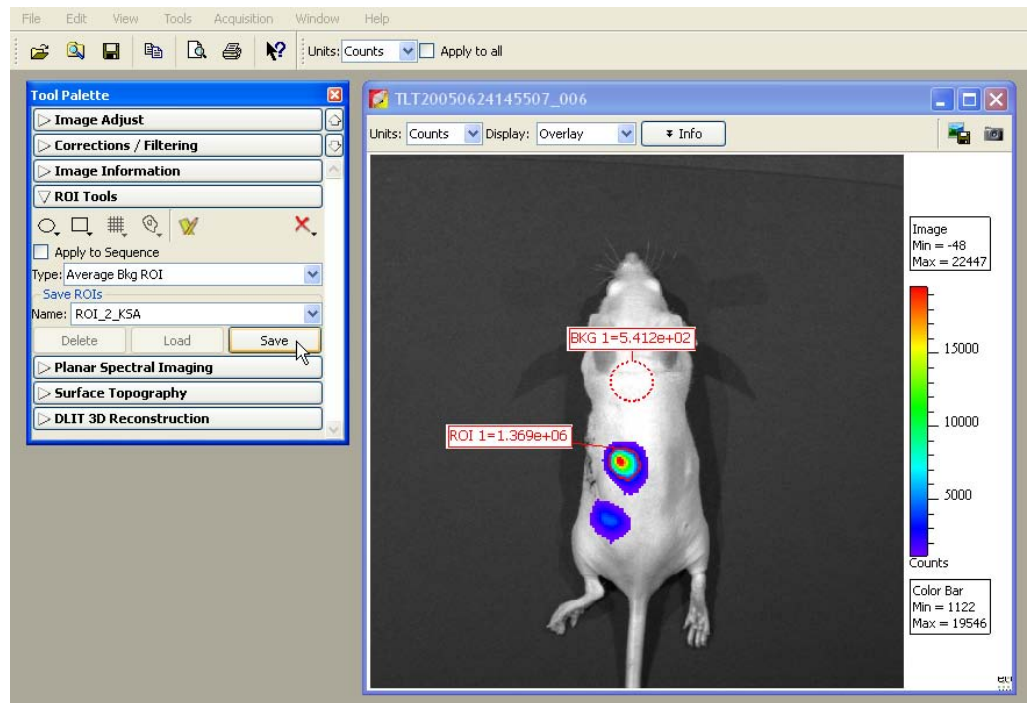
2. Click **Save**.

   - 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.
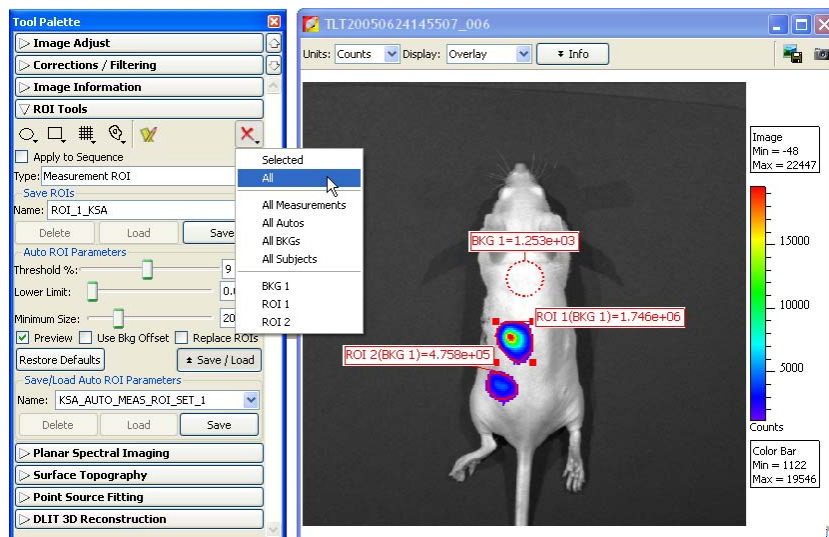
2. Make a selection from the drop-down list of delete commands.
   - The specified ROIs are deleted from the image.

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**.
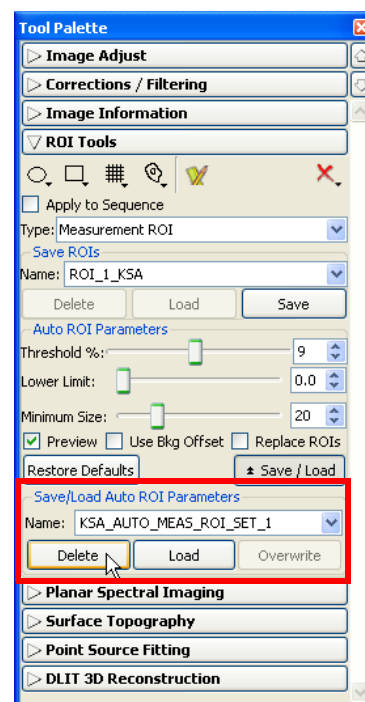
6.7 Managing the ROI Measurements Table

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 213.

To view the ROI Measurements table, click the ▨ button. Alternately, select **View →** **ROI Measurements** on the menu bar.
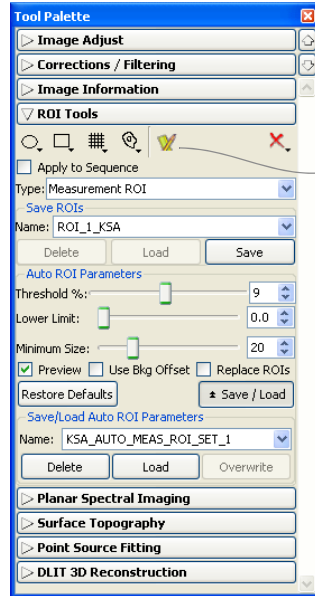
Column headers in the table include ROI information, ROI measurements and dimensions, and information about the image recorded at acquisition.
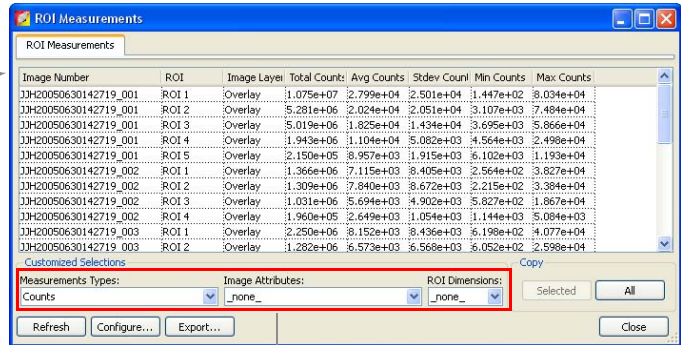
Select the type of ROI or image data to include in the table

Grid ROIs are displayed in a separate tab
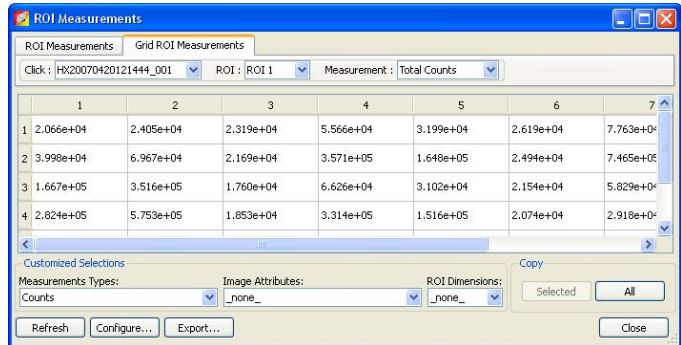
Kinetic ROIs are displayed in a separate tab
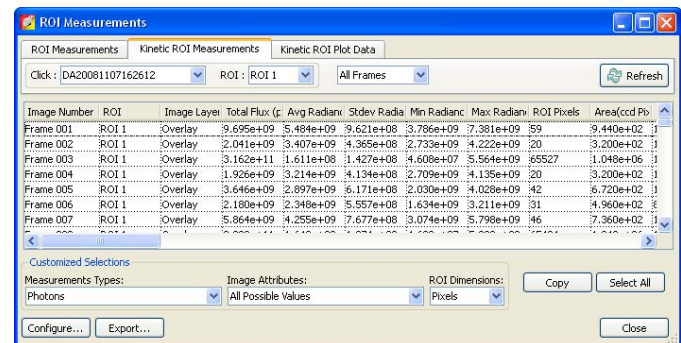
Table 6.4 ROI Measurements table

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	Exclude ROI measurements from the table.
Counts	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 213.)
	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.
Photons	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 214.)
Efficiency	Available for fluorescent images only. Includes Total Efficiency, Average Efficiency, Stdev Efficiency, Min Efficiency, and Max Efficiency in the table. (For more details on efficiency, see page 213.)
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.
Xenogen Universal	Includes all Xenogen 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	
Selected	Copies the selected row(s) in the table to the system clipboard.

Table 6.4 ROI Measurements table (continued)

Item	Description
All	Copies all rows in the table to the system clipboard.
Refresh	Updates the table.
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 predefined categories are available in the Measurement Types, Click Attributes, and ROI Dimensions drop-down lists.
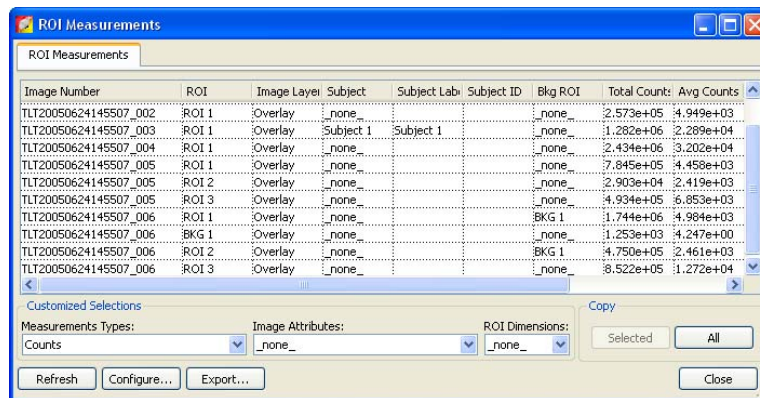
1. To reorder the columns, drag a column header (left or right) in the table.

2. To change the measurement units, make a selection from the Measurement Types drop-down list.

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.

To create & save a custom table configuration:

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

    - The Configure Measurements box appears.

Click to reorder the available items in ascending or descending alphabetical order

Available items (column headers) that can be added to the table

Items (column headers) currently in the table
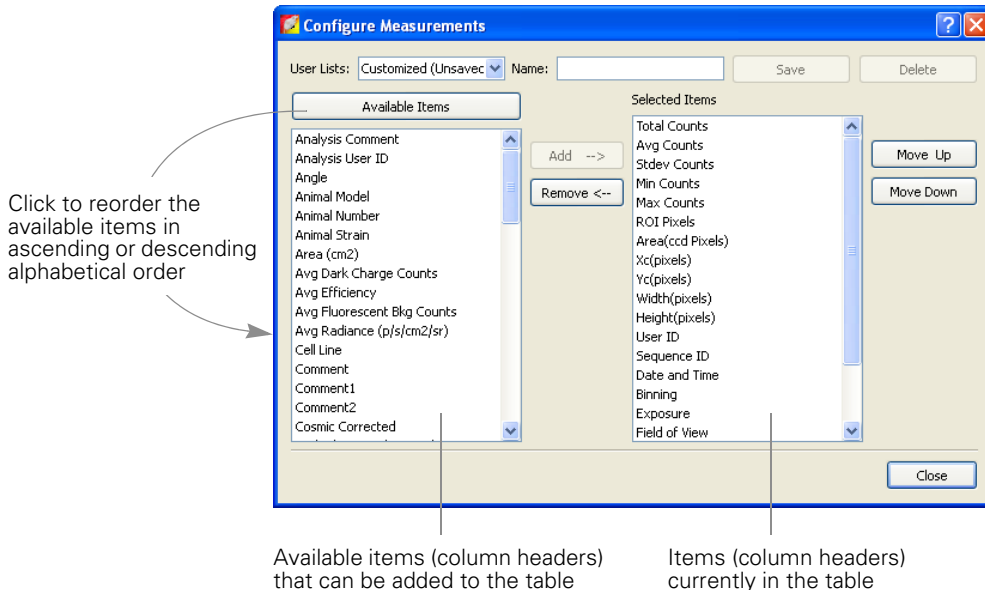
2. Do either of the following:

    •Select a configuration that you want to modify from the User Lists drop-down

    OR

    •Select Customized (Unsaved) from the User Lists drop-down to create a new configuration

3. To add an item to the table, click an item in the Available Item list and then click **Add**.

4. To remove an item from the table, select the item that you want to remove in the Selected Items list, and click **Remove**.
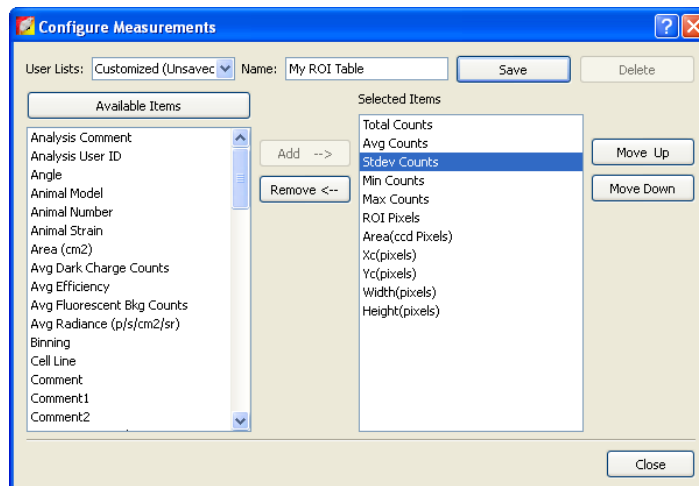
5. To reorder an item in the Selected Items list, select the item and click **Move Up** or **Move Down**.

    - The columns in the ROI Measurements table are updated.

6. To save the table configuration, enter a name in the Name box and click **Save**.
    Note: You cannot overwrite a factory loaded configuration. If you modify a factory loaded configuration, save it to a new name.

To delete a custom table configuration

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

2. **Note:** Factory loaded table configurations cannot be deleted.

Copying or Exporting the ROI Measurements Table

To export the table:

1. Click **Export**.

2. In the dialog box that appears, select a folder and enter a name for the file (.txt), then click **Save**.
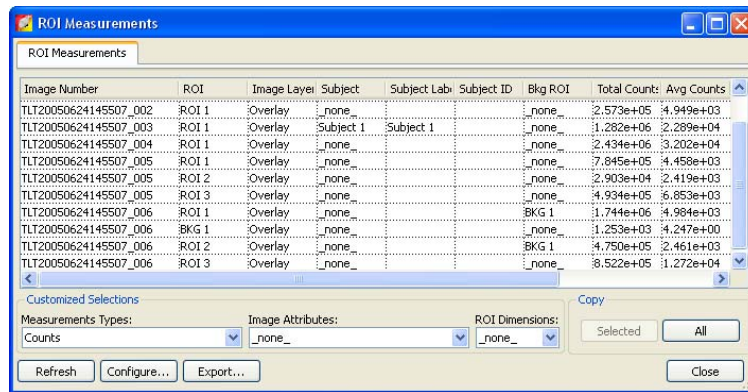
To copy the table to the system clipboard:

- Selected rows - Select the rows of interest and click **Selected**. Alternatively, select the rows then right-click the table and choose **Copy Selected** on the shortcut menu.

- All rows - Click **All**. Alternately, right-click the table and select **Copy All** on the shortcut menu.

7 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.	112
Image Math	Remove autofluorescence from a fluorescent image.	115
Image Overlay	Coregister multiple fluorescent or luminescent images on the same photographic image to view multiple reporters in a single image.	118

To perform image math, open an image sequence (see page 47) or a group of images (see *Creating an Image Sequence from Individual Images*, page 53).

7.1 Using Image Math to Create a New Image

To create a new image using image math:

1. Load an image sequence.
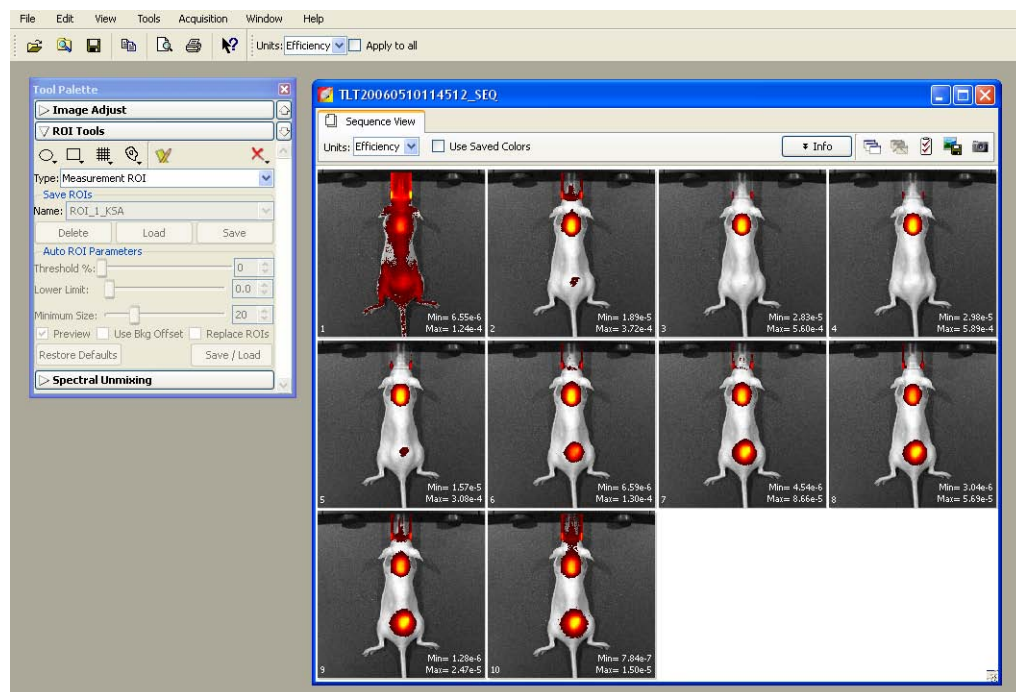
2. Select **Tools →Image Math for xx_SEQ** on the menu bar.

3. In the Image Math window that appears, select an image of interest from box A and box B.

   - The Image Math window shows a thumbnail of image A, image B, and the new image.
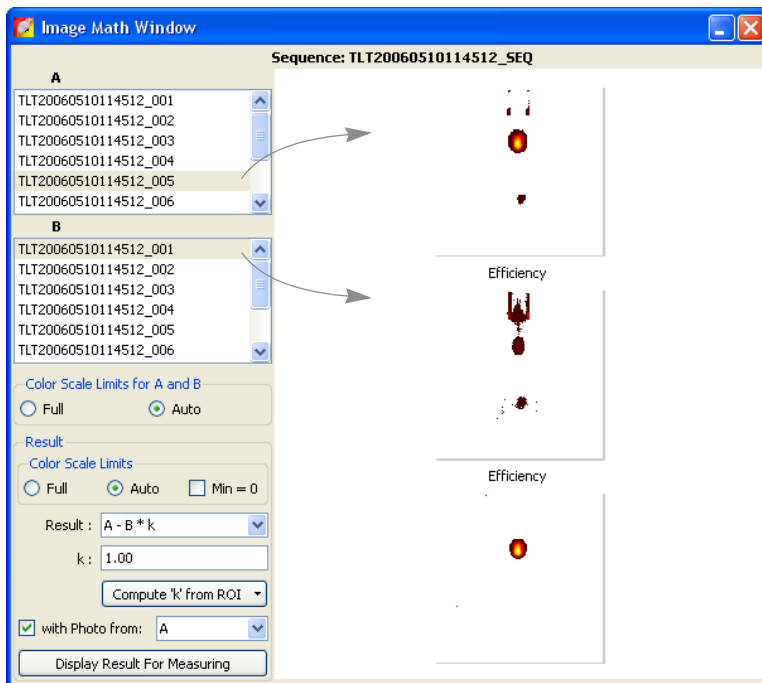
Note: For more details on items in the Image Math window, see Table 7.1, page 114.

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**.
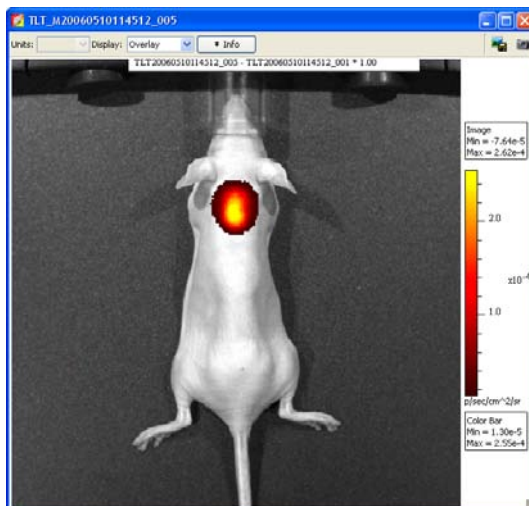
New image generated by the Result function

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 🖼️.

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 7.1 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 bar 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.)

k, Image Math window	A user-specified scaling factor applied in the results function.
k, Fluorescent Background Subtraction window	The software computes k = the ratio of the autofluorescent signal measured using the background filter to the autofluorescent signal measured using the excitation filter in a region on the animal where no fluorophore is present.
Compute 'k' from ROI	This option is useful for subtracting fluorescence background. Draw the same ROI in both images on an area considered background. In the "Compute 'k' from ROI" drop-down list, select the same ROI in each image.
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.

Table 7.1 Image Math window (continued)

Item	Description
Display Result for Measuring	Opens the image generated by image math in an image window.

7.2 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 (Table 7.2).

Table 7.2 Emission, excitation, and background filters used to acquire data that can be corrected for tissue autofluorescence

	Emission Filter	Excitation Filter (Primary Image)	Background Filter (Background Image)
Fluorophore	**Passband (nm)**		
GFP	515-575	445-490	410-440
DsRed	575-650	500-550	460-490
Cy5.5	695-770	615-665	580-610
ICG	810-875	710-760	665-695

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 236

The software computes:

Background-corrected signal = (A - B) × k, where:

A = primary image (acquired using the excitation filter)

B = background image (acquired using the background filter)

k = (primary signal/background signal)

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

After you acquire an image sequence that includes a primary and background image, use the image math tool to subtract tissue autofluorescence. (For more details on acquiring an image sequence, see Chapter 3, page 22.)

To subtract tissue autofluorescence:

1. Load the image sequence that includes the primary and background fluorescent images.
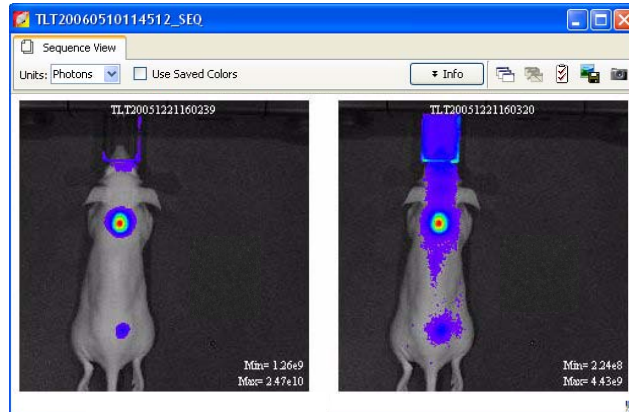
2. Open either the primary or background image and:

   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.
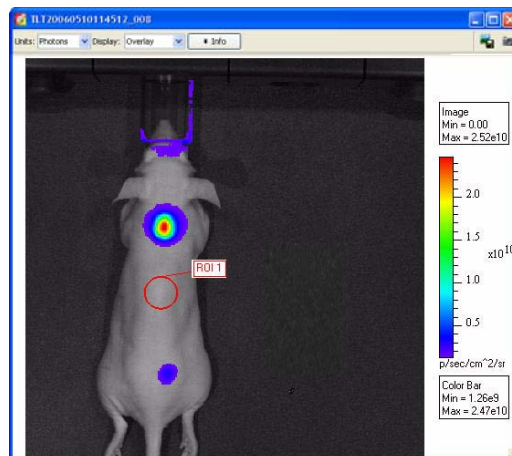
3. Select **Tools → Image Math for xx.SEQ** on the menu bar.

4. In the Image Math window that appears, select the primary image in box A. Select the background image in box B.

Note: For more details on items in the Image Math window, see Table 7.1, page 114.

5. Select the math function 'A-B*k' in the Result drop-down list.
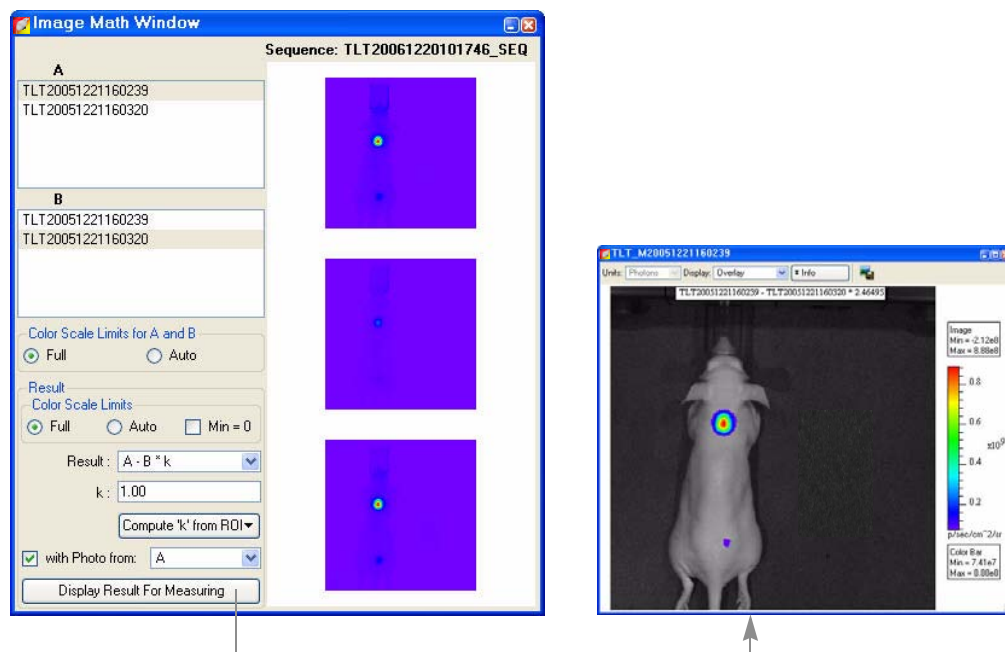
6. Click Compute 'k' from ROI and select the ROI (created in step 2) from the drop-down list.
   - The background-corrected signal is displayed.

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

   Note: 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 🖫. Alternately, 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**.

7.3 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 do this:

To coregister multiple images:

1. Acquire an image sequence using the appropriate filters for each reporter. Alternately, create a sequence from images acquired during different sessions. (For more details, see page 53.)

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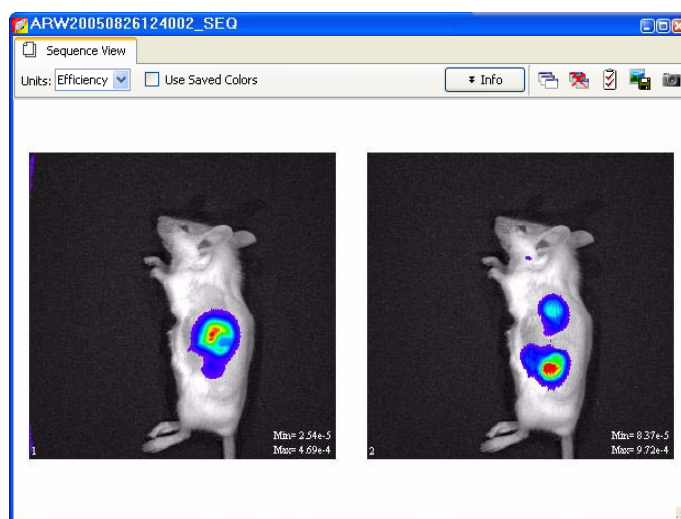
Note: To view all images in the sequence, click the **Display All** button   to open each image (overlay mode) in a separate image window.

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

4. Select **Tools→Image Overlay for xx_SEQ** on the menu bar.

   - The image overlay window appears and shows the first photographic image in the sequence.

5. Select a photographic
   image in the upper box.
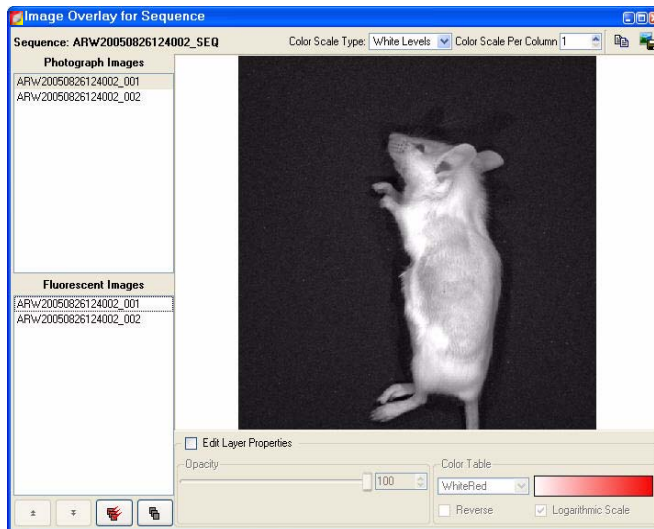
6. To select all of the fluorescent or luminescent images in the lower box, click the
   button.
   Alternately, to select particular images, do either of the following:

   • To select non-adjacent images in the list, press and hold the **Ctrl** key while you
     click the images.

   OR

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

   - In the overlay that is generated, the signal in each image is assigned a different
     color table. The photographic image is at the bottom of the stack and the last
     fluorescent or luminescent image selected from the list is at the top of the stack.
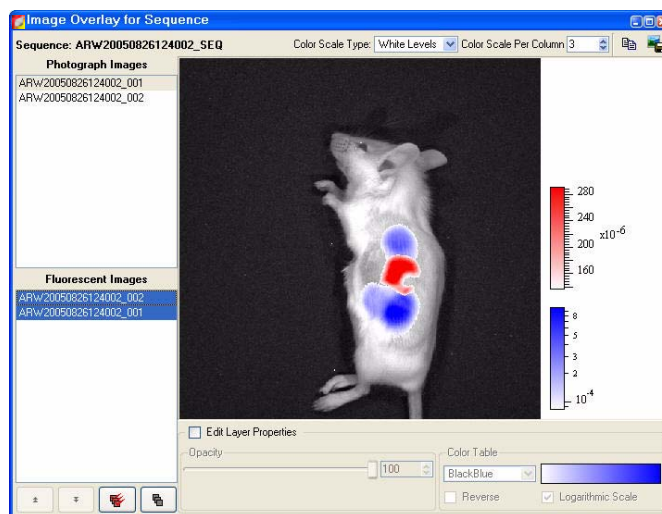
7. To remove all fluorescent or luminescent images from the overlay, click the ![icon] button.

8. To change the display of a fluorescent or luminescent image in the overlay:

    a. Select the image in the lower box.

    b. Choose the **Edit Layer Properties** option.

    c. Adjust the opacity, select a different color table, or edit the color table properties. When finished, clear the **Edit layer Properties** option.

Select white or black for the low end of the color scale

Set the number of color scales displayed per column

9. To reorder the images in the list:

    a. Choose the **Edit Layer Properties** option.

    b. Select an image.

    c. Click the ![up] or ![down] arrows.

10. To copy the overlay image to the system clipboard, click the **Copy** button ![icon].

11. To export the overlay image to a graphic file, click the **Export** button ![icon].

8 Planar Spectral Image Analysis

The Living Image® software enables you to apply planar spectral image analysis to a sequence to determine the average depth and total photon flux of a bioluminescent point source in a user-specified region of interest. For more information on planar spectral image analysis, see Appendix G, page 239.

Use the imaging wizard to setup the image sequence required for planar spectral image analysis. (For more details on the imaging wizard, see page 24.) 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).

8.1 Planar Spectral Image Analysis

1. Open the image sequence that you want to analyze.
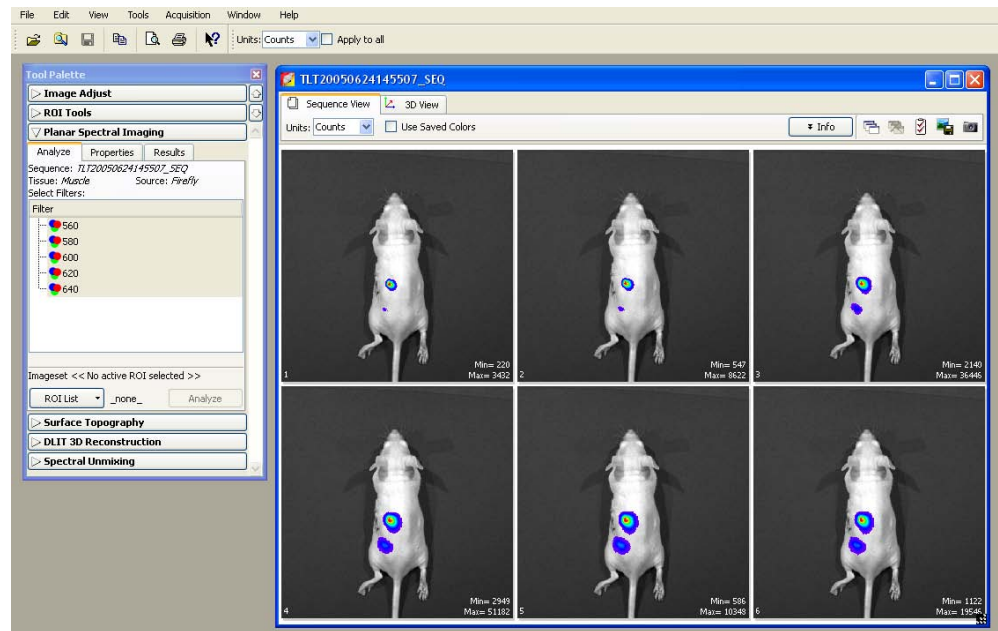
2. In the tool palette, click Planar Spectral Imaging.

3. In the Analyze tab, select the emission filter wavelengths for the analysis.

   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 91.)

4. In the ROI List drop-down, select **All** or a particular ROI 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 *Measuring ROIs in an Image*, page 84.)

   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.
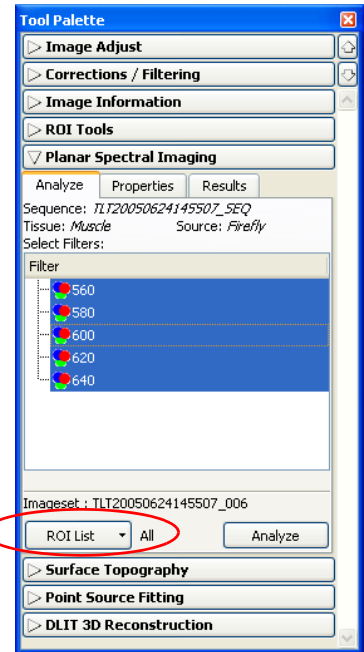
5. Choose the tissue properties:

   a. In the Properties tab, make a selection from the Tissue Properties drop-down list.

   b. Choose the tissue type most representative of the area of interest. Muscle is a good choice for a generic tissue type.

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

6. Make a selection from the Source Spectrum drop-down list.

7. Click **Analyze** in the Analyze tab.
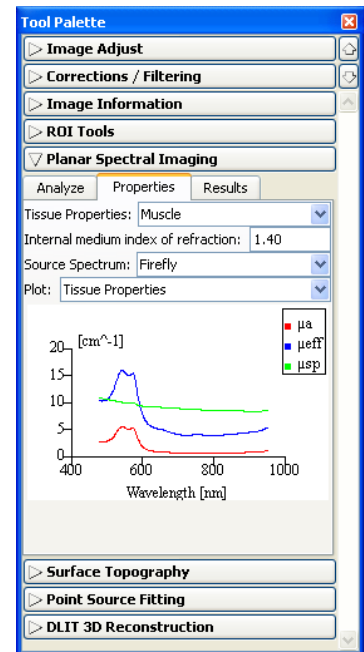
The Results tab displays the computed average depth (mm) and total flux (photon/sec) of the bioluminescent point source in the specified ROI(s).
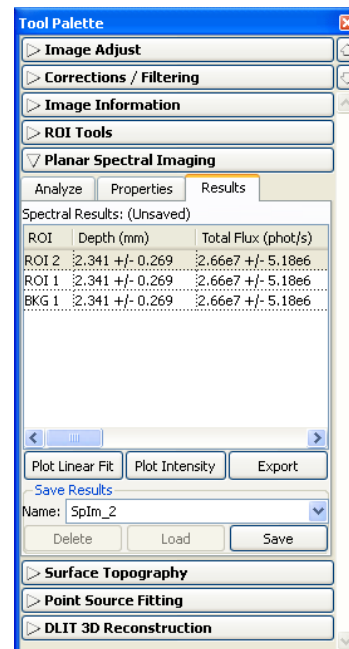
8.2 Planar Spectral Imaging Tools

The planar spectral imaging tools are displayed in three tabs.
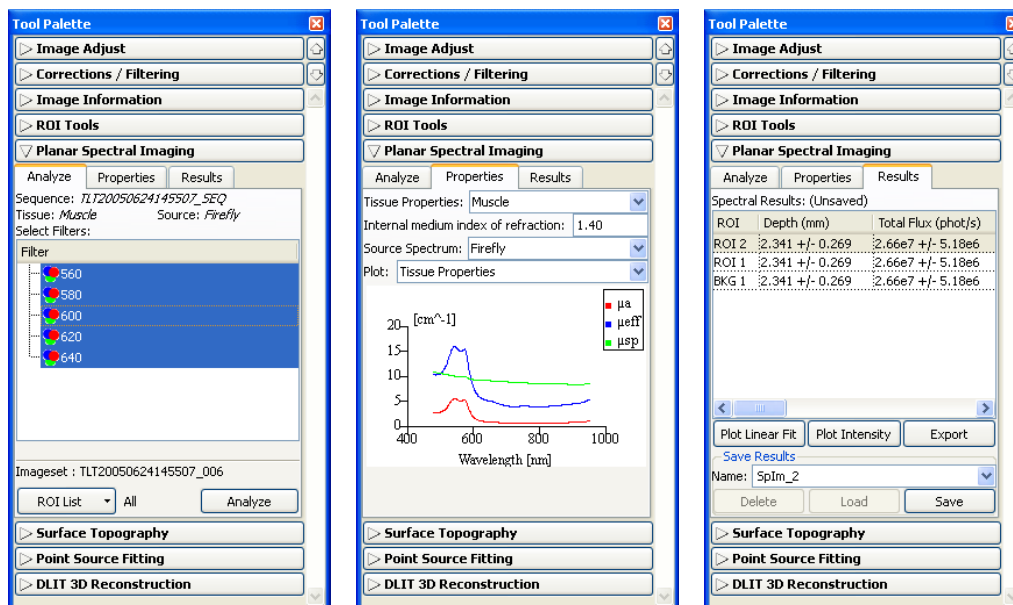
Figure 8.1 Tool palette, planar spectral imaging tools

Table 8.1 Planar spectral imaging tools & results

Item	Description
Analyze Tab	
Sequence	Name of sequence used for the analysis.
Tissue, Source	The tissue properties and source spectrum selected in the Properties tab.
Select Filters	In the Filter box, select the acquisition wavelengths for the images in the selected sequence. To select non-adjacent wavelengths, press and hold the **Ctrl** key while you click the wavelengths. (Macintosh users, press and hold the **Cmd** key while you click the wavelengths.)
ROI List	A drop-down list of the ROIs in the active image.
Analyze	Click to perform the spectral analysis.
Properties Tab	
Tissue Properties	Drop-down list of the absorption and scattering properties of various tissues.
Internal medium index of refraction	Tissue index of refraction that is automatically specified when you select a tissue property.
Source Spectrum	Drop-down list of bioluminescent sources.
Plot	
Tissue Properties	Click to display graphs (cm^{-1} vs nm) of the absorption coefficient (μ_a), effective attenuation coefficient (μ_{eff}), and reduced scattering coefficient (μ'_s).
Source Spectrum	Click to display the spectrum of the selected bioluminescent 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 (μ_{eff}, the optical property of the tissue selected in the Tissue Properties drop-down list) along with the linear fit to these data determined by the spectral analysis code.
Plot Intensity	Displays a graph of normalized intensity versus wavelength. Intensity is normalized by the selected source spectrum and filter transmission properties.
Export	Opens a dialog box that enables you to save the results to a text file (.txt).
Save Results	
Name	A drop-down list of saved results. Includes the default name for new unsaved analysis results (SpIm_xx).
Delete	Deletes the selected results.
Load	Opens the selected results.
Save	Saves the analysis results (results name appears in the Name drop-down list).

8.3 Viewing & Exporting Graphical Results

To view a graph of the results:

1. In the Results tab, select an ROI.

2. Click **Plot Intensity** or **Plot Linear Fit**.

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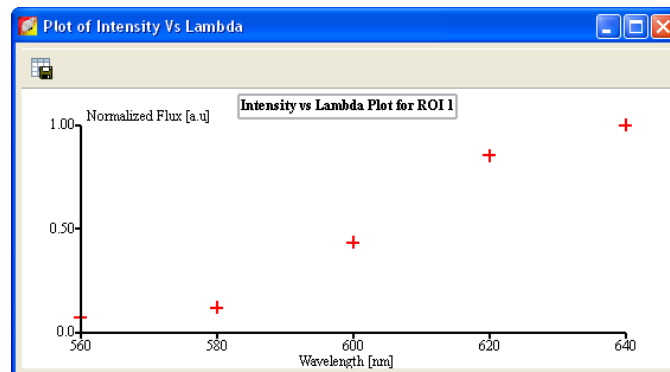
The intensity graph displays a graph of the measured intensity in the selected ROI at each wavelength in the analysis. The intensity is normalized to the selected source spectrum and the filter transmission properties.

To export graph data:

1. Click the **Export Data** button.

2. In the dialog box that appears, select a directory for the data and enter a file name (.csv).

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

8.4 Managing Planar Spectral Imaging Results

To save results:

1. Select the results of interest (SpIm_xx) from the Name drop-down list

2. Click **Save**.

   - The planar spectral imaging results are saved with the image.

To view results:

1. Select the results of interest 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) of interest and select **Copy Selected** 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 select **Copy All** from the shortcut menu that appears.

   - All of the results table is copied to the system clipboard.

To Export Results:

1. 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 (.txt), and click **Save**.
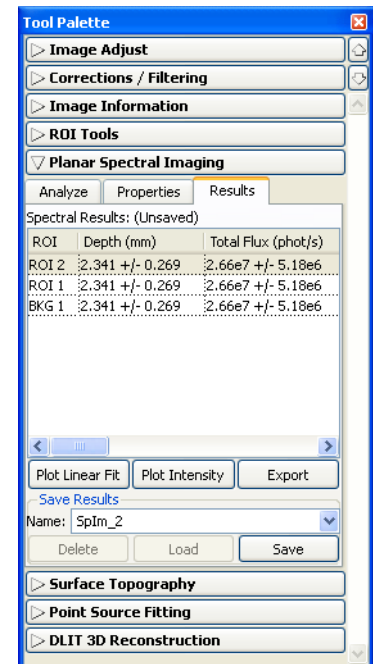
9 Spectral Unmixing

The Living Image® software uses spectral unmixing to distinguish the spectral signatures of different fluorescent or bioluminescent 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 bioluminescence images when more than one reporter is used in the same animal model

Use the imaging wizard to setup the image sequence required for spectral unmixing. (For more details on the imaging wizard, see .) 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, >40 nm) so that the excitation light does not leak through the emission filter where it can be detected by the CCD.

9.1 Spectral Unmixing

1. Load the image sequence.

    In this example, 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.

2. In the Analyze tab of the Spectral Unmixing tools, put a check mark next to the emission wavelengths that you want to include in the analysis.

3. In the Components drop-down list, select the number of spectral components to unmix in the images (the number of fluorophores +1 since tissue autofluorescence accounts for 1 component).

    For example, if the image data includes one fluorophore, then there are two components to unmix—the fluorophore signal and the tissue autofluorescence.
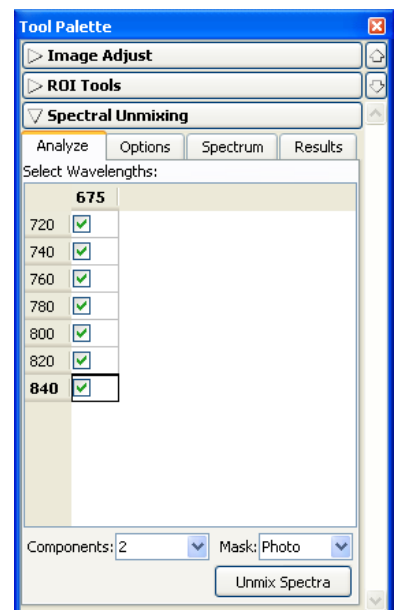
4. Make a selection from the Mask drop-down list:

- Photo - Opens the Photo Mask Setup window. The purple mask specifies the area for analysis.

  a. Use the Threshold slider or ⬍ arrows to adjust the mask so that it matches the underlying subject photograph as closely as possible without including any area outside the subject image.

  b. Click **Set**.

- All - The entire image is analyzed. This option is not recommended. It introduces many off-target pixels that might mislead and significantly slow down the analysis

- ROI - ROIs applied to the sequence are available in the drop-down list. Select an ROI to analyze just the area in the ROI. This option is recommended for well plates or dark objects where it is hard to achieve a good mask from the photograph.
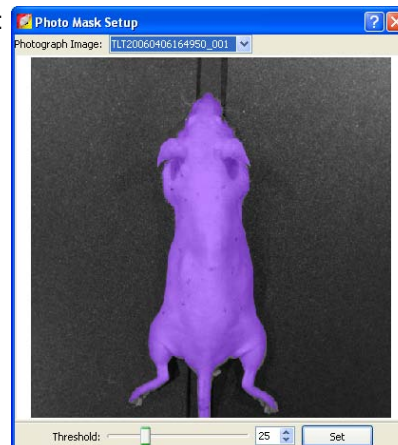
5. In the tool palette, click **Unmix Spectra**.

   - The spectral unmixing results appear. The Distribution tab shows a photon density map of each unmixed result and a composite image that includes all of the fluorescent signals, each displayed in a different color.

Unmixed 1 = Autofluorescence

Unmixed 2 = Fluorophore signal
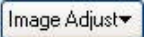
Composite = Unmixed 1 + Unmixed 2
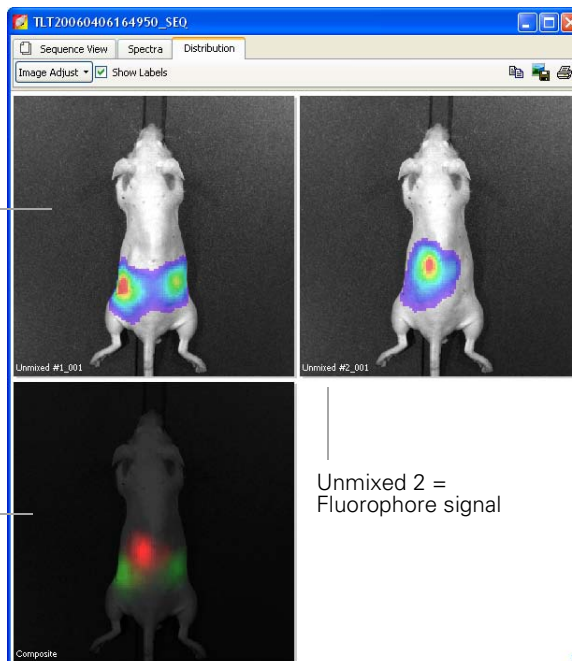
6. To analyze an unmixed image, double-click the image.

   - 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.
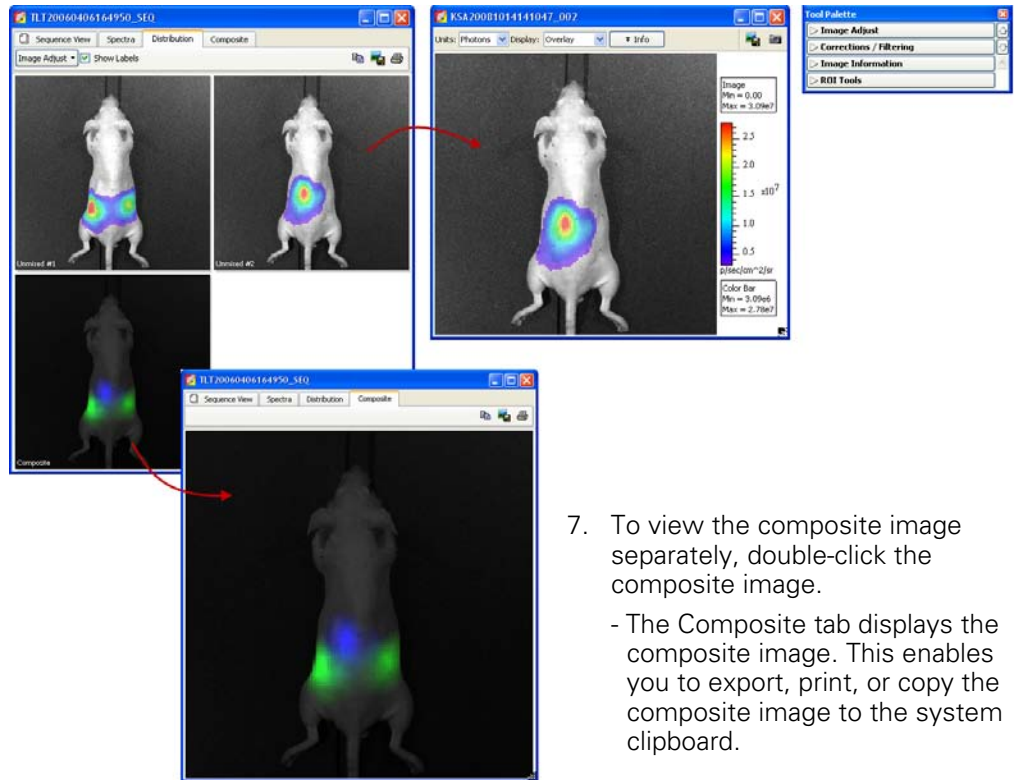
7. To view the composite image separately, double-click the composite image.

   - The Composite tab displays the composite image. This enables you to export, print, or copy the composite image to the system clipboard.

Item in the Concentration Plot	Description
Image Adjust▾	Click to show the controls to adjust the fluorescence opacity and the color table display in the concentration plot. Click anywhere in the window to hide the controls.
Show Labels	Choose this option to display image labels on the concentration plot and composite image.

9.2 Spectral Unmixing Results Window

The results are displayed in the three tabs of the Spectral Unmixing Results window.

Spectra Tab The spectrum plot shows the normalized spectra of the unmixed results. You can edit the appearance of the spectrum plot using the tools in the spectral unmixing tool palette (Spectrum tab) (Figure 9.1).

A list of the spectra in the results. Add/remove a check mark to show/hide the spectrum in the spectrum plot. Click a spectrum (row) to display in the preview pane.
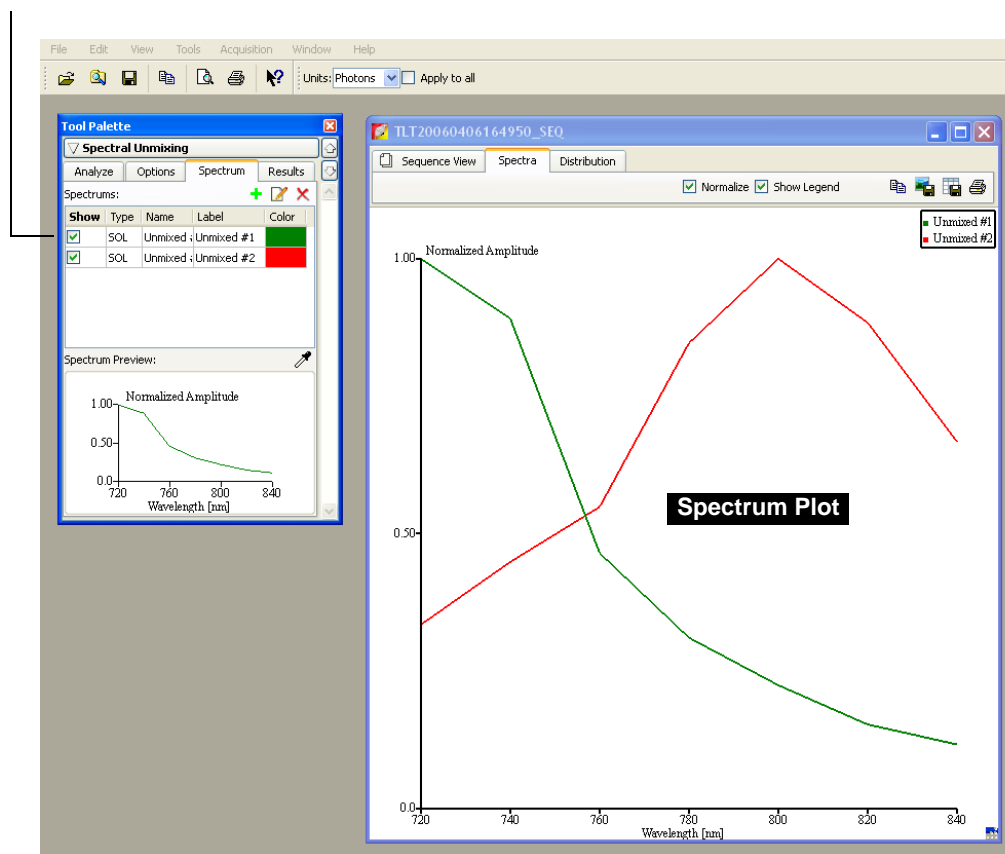
Figure 9.1 Spectral unmixing tools and spectrum plot

Table 9.1 Tool palette, spectral unmixing tools

Item	Description
✚	Opens a dialog box that enables you to select a spectrum to add to the spectrum plot.
📝	Opens a dialog box that enables you to edit a spectrum in the spectrum plot. **Note:** You can also double-click a spectrum row in the tool palette (Spectrum tab) to open the dialog box.
✗	Deletes the spectrum from the spectrum plot
Type	The type of spectrum.

Table 9.1 Tool palette, spectral unmixing tools (continued)

Item	Description
SOL	A spectrum generated by the spectral unmixing algorithm.
ROI	A spectrum calculated for a user-selected ROI.
LIB	A user-selected library spectrum. The library includes spectra obtained of different sources obtained using excitation and emission filters.
Name	The spectrum identifier used by the unmixing algorithm. The name cannot be modified.
Label	The spectrum name in the spectrum plot key. The label can be edited.
Color	The color of the spectrum in the spectrum plot. For the SOL type spectrum, it is also the color in the composite image.
Spectrum Preview	Shows the spectrum selected above (click a row above the preview pane).
	Use this tool to pick up a pixel in an opened image and plot the spectrum at this pixel in the spectrum preview.

9.3 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 9.2).
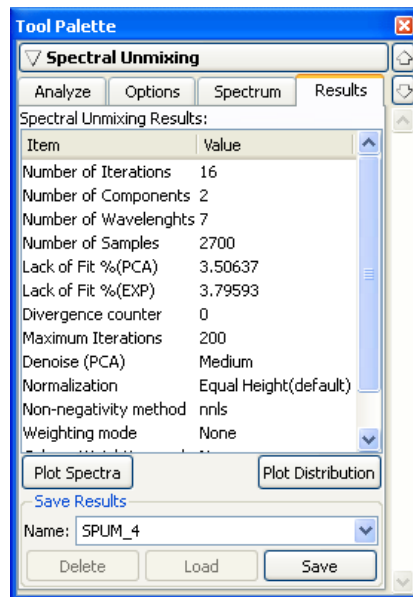
Figure 9.2 Tool palette, Spectral unmixing tools, Results tab

Table 9.2 Spectral unmixing tools, Results tab

Item	Description
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.
Plot Spectra	Click to display the spectrum plot tab.
Plot Distribution	Click to display the concentration plot tab.

9.4 Spectral Unmixing Options

In the spectral unmixing tools, the Options tab shows the user-modifiable parameters in the spectral unmixing algorithm (Figure 9.3). It is recommended that you first perform spectral unmixing using the default settings. Then, if necessary, change the option settings and reanalyze the data.
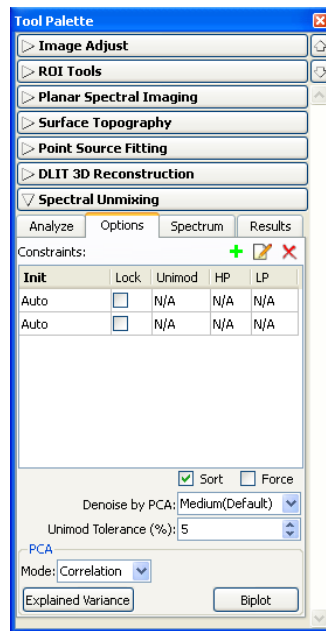
Figure 9.3 Tool palette, spectral unmixing options

Table 9.3 Spectral unmixing options

Option	Description
Constraints	The constraints for unmixing the components.
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.
Lock	The lock option determines whether the spectrum is allowed to change. If this option is chosen, the spectrum of that component is not updated during unmixing.
Unimod	Choose this option to apply the unimodality constraint. Unimodality forces the spectrum to have only one peak (one extremum). however, small magnitude extrema are allowed if they are less than the Unimod Tolerance value. This tolerance value limits the rising slope of the second spectral peak. For example, 5% tolerance means that the increase in magnitude of the neighboring nodes cannot exceed 5%.

Table 9.3 Spectral unmixing options (continued)

Option	Description
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.
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 less principal components will be used in the data and more details will be lost. Stronger denoising also may slow down the unmixing.
Unimod Tolerance (%)	The threshold for the unimodality constraint. It is the percentage of overshoot allowed for the second spectral peak.
PCA	
Mode	PCA can be performed on the original data, the correlation matrix of the original data, or the covariance matrix of the original data.
Explained Variance	Click to display the explained variance.
Biplot	Click to display the biplot graph.

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.
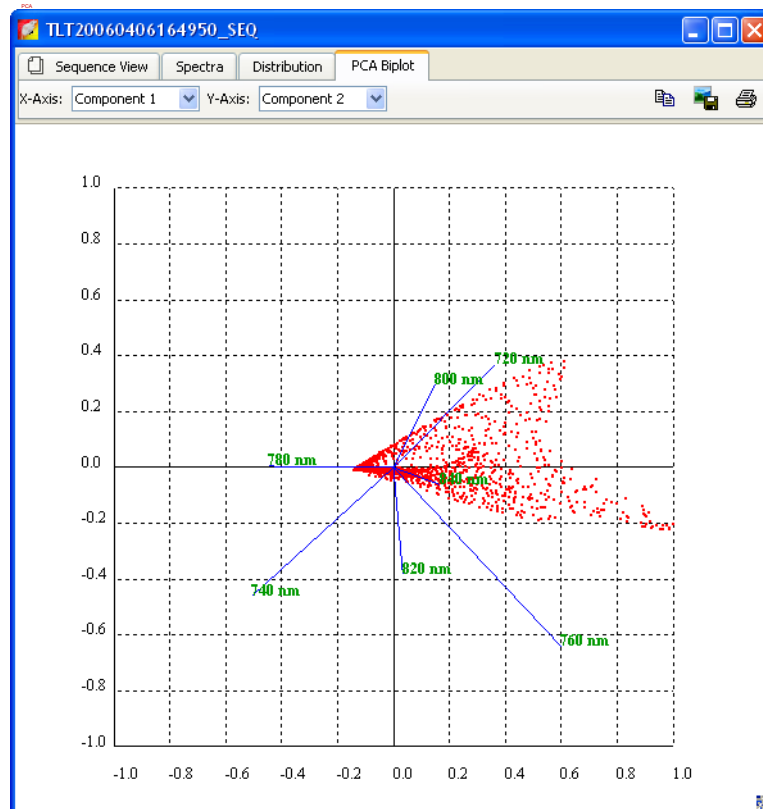
Figure 9.4 PCA biplot

**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).
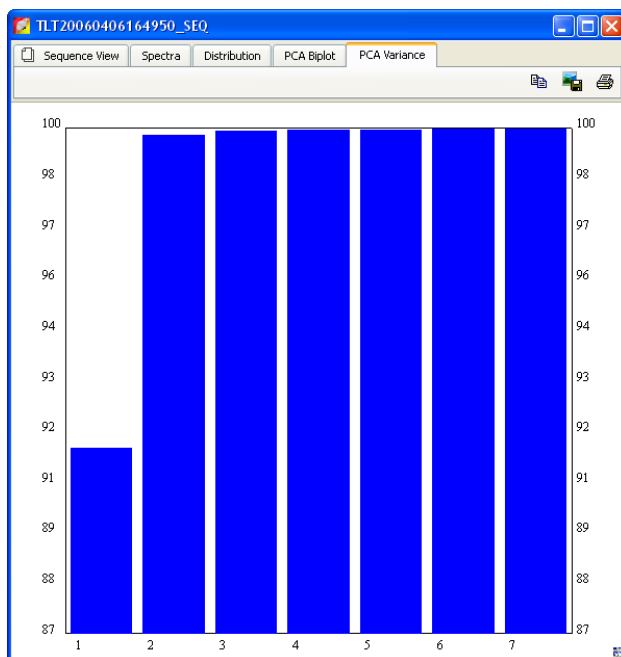
Figure 9.5 PCA explained variance

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10 Generating a Surface Topography

A surface topography (surface) is a reconstruction of the animal surface derived from structured light images. (For additional details on the surface topography, see page 247.) A surface is required for point source fitting and 3D reconstruction of bioluminescent sources (DLIT analysis) or fluorescent sources (FLIT analysis) inside a subject.

10.1 Generate the Surface Topography

1. Load the image sequence that you want to analyze. (For details on how to load a sequence, see page 45).

2. In the tool palette, click **Surface Topography**.

3. Click **Reconstruct**.

   - The tomography analysis box appears (Figure 10.1).
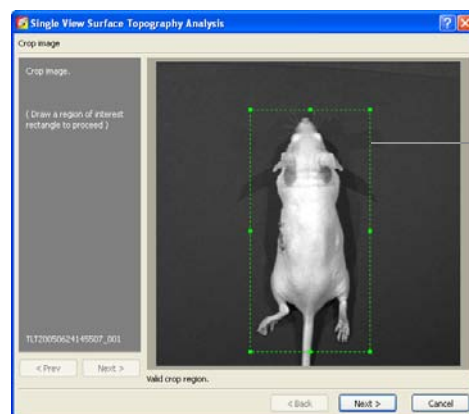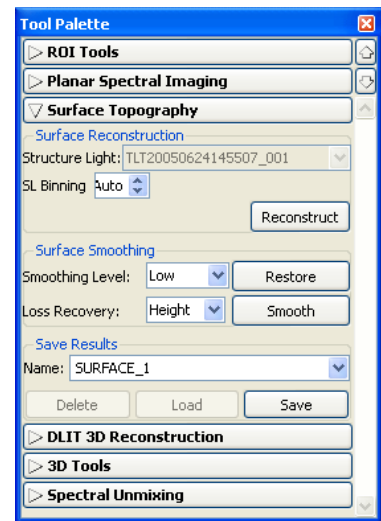
Crop box
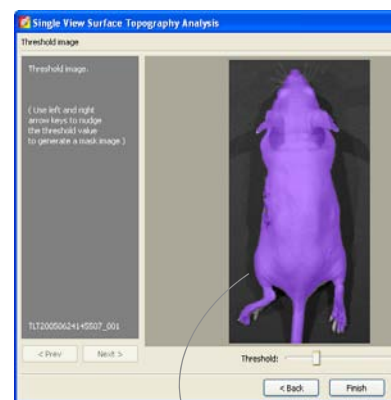
Mask (purple)

Figure 10.1 Tomography analysis box

4. Draw a crop box that includes a one cm margin around the subject, if possible.

5. Click **Next** to display the mask.

   The mask is a purple 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.

6. If you want to smooth the surface, confirm the default surface generation options and surface smoothing parameters or enter new values. (For more details on the parameters, see Table 10.1).

7. If you want to save the results, confirm the default name for the results or enter a new name.

8. If necessary, adjust the threshold value so that the mask fits the subject image as closely as possible without including any area outside of the subject. 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. For more details on the tools, see page 164.
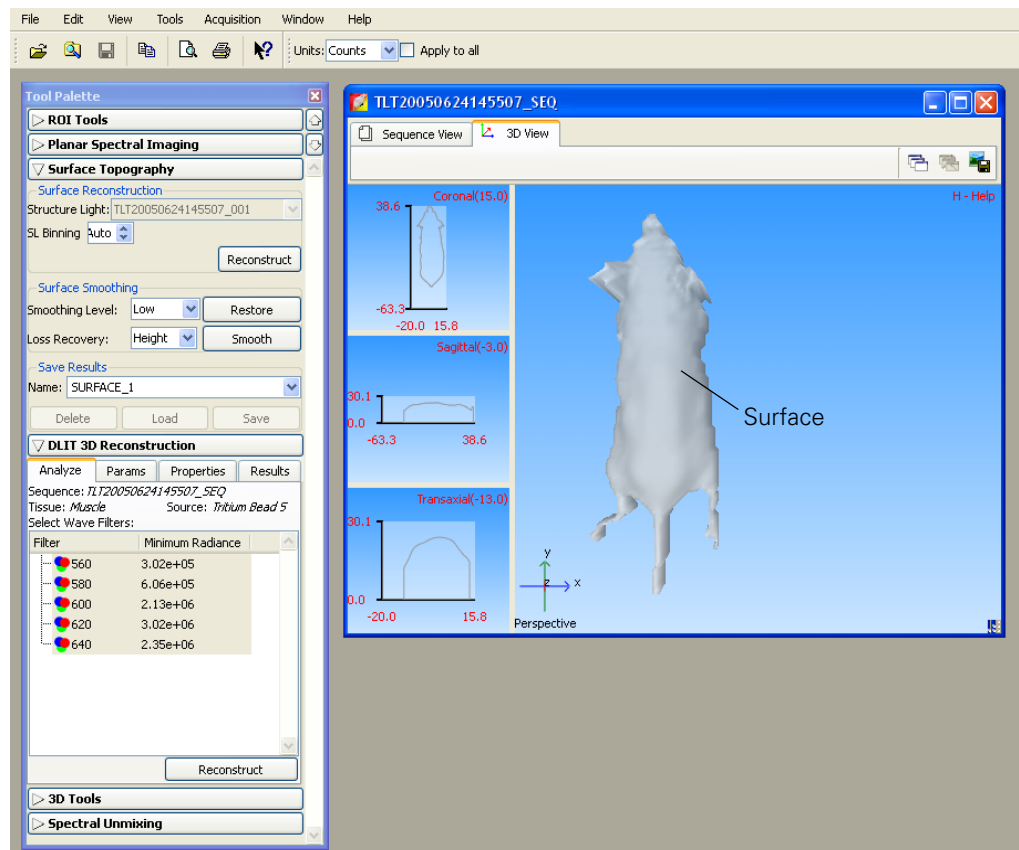
Table 10.1 Surface topography options & parameters

Item	Description
Surface Reconstruction	
Structured Light	A drop-down list of structured light images in the sequence. Select one for use in reconstructing the surface.
SL Binning	The binning level applied to the pixels that are used to construct the surface topography from the structured light image. If Auto is chosen, the software automatically determines the appropriate binning level. The higher the binning level, the lower the mesh resolution. For more details on binning, see page 206.
Reconstruct	Click to generate the surface.
Surface Smoothing	
Smoothing Level	The amount of smoothing to apply to a reconstructed surface.
Restore	Removes smoothing that was applied to a surface.
Loss Recovery	Smoothing can cause loss in the surface volume or height. Make a selection from the drop-down list to reduce losses. 'Height' is recommended for IVIS 200 or IVIS Spectrum surfaces.
Smooth	Initiates the smoothing specified.
Save Results	
Name	The name of the surface.
Delete	Click to delete the surface selected from the Name drop-down list.
Load	Click to load the surface selected from the Name drop-down list.
Save	Click to save the surface to the name entered in the Name drop-down list.

10.2 Managing Surfaces

The generated surface can be saved for shared use by the Point Source Fitting, DLIT, or FLIT tools.

Figure 10.2 Tool palette, Surface topography tools

Saving a Surface
1. Confirm the default name or enter a new name.

2. Click **Save.**

Loading a Surface
1. Select the surface that you want to use (for point source fitting, DLIT analysis, or FLIT analysis) from the Name drop-down list.

2. Click **Load**.

Deleting a Surface
1. Select the surface that you want to delete from the Name drop-down list.

2. Click **Delete**.

11 Point Source Fitting

The point source fitting algorithm is a tool for advanced users that can be used to estimate the optical properties of tissue, the location and power of a point source, or the fluorescent yield of fluorophores. The software analyzes the images in a sequence acquired in one of the following imaging modes:

- Bioluminescence
- Transillumination fluorescence (bottom-illuminated fluorescence)
- Epi-illumination fluorescence (top-illuminated fluorescence)
- Transmission

> **NOTE**
>
> The point source fitting algorithm requires an image sequence that includes one or more images and a structured light image.

11.1 Displaying the Point Source Fitting Tools

The default tool palette does not include the point source fitting tools.

To display the point source fitting tools in the tool palette:

1. Select **Edit →Preferences** on the menu bar.
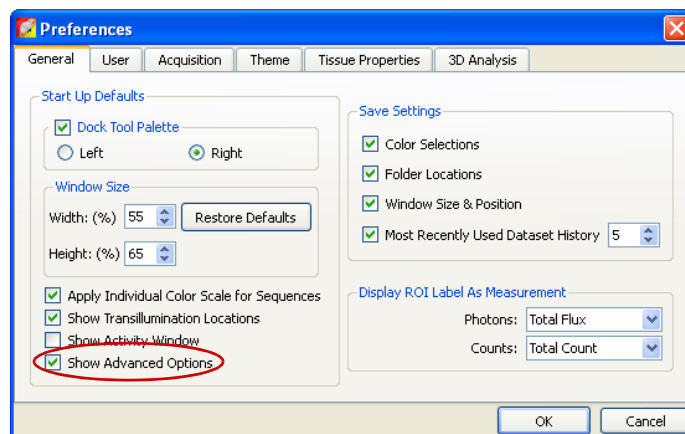
2. In the dialog box that appears, put a check mark next to Show Advanced Options, and click **OK**.

   - The point source fitting tools appear in the tool palette (Figure 11.1).

Analyze tab
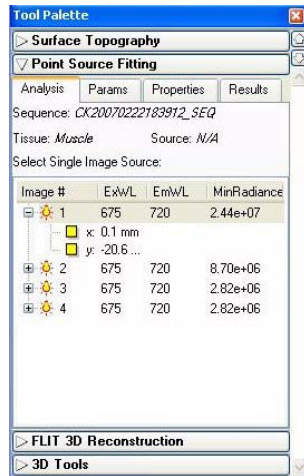
Analysis tab shows the active image sequence.

Click the + sign to display the position of the bottom illumination source read from the click info (x,y or x,y,z, depending on the image model).

Params tab
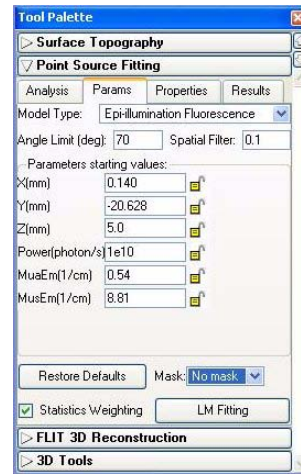
Starting parameter values.

Properties tab
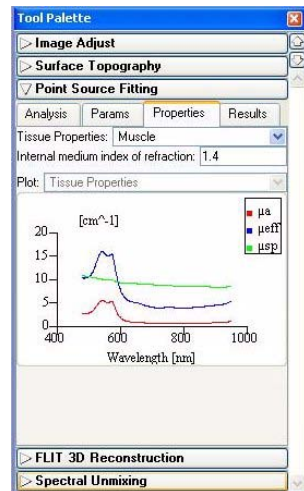
Select other starting values for the optical properties here.

Results tab
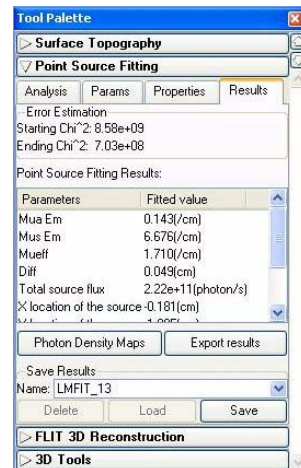
Point source fitting results.

Figure 11.1 Tool palette, point source fitting tools & results
If the image sequence does not include a structure light image, the point source fiting tools do not appear in the tool palette.

Table 11.1 Tool palette, point source fitting

Item	Description
Analysis Tab	
Image #	Image number in the active sequence.
ExWL	Excitation wavelength
EmWL	Emission wavelength
MinRadiance	Minimum surface radiance used for model fitting.
x,y	x- and y-coordinates of the bottom illumination source.
Params Tab	
Model Type	The image acquisition mode.
Angle Limit (deg)	The angle limit refers to the angle between the object surface normal and the optical axis. (For more details, see page 253.)
Spatial Filter	Filters out the noisy data at the mouse edges. A setting of 0.1 means that the analysis includes 90% of the data from the center of mass to the edges.
Parameter starting values	**Note:** Selecting a tissue Properties tab automatically updates MuaEm, MusEm/ MuaEx, and MusEx in the Params tab.
x, y, or z	Source coordinates.
F-yield/Power	Fluorescence yield/strength of illumination or bioluminescence source.
MuaEm	Absorption coefficient at the emission wavelength.
MusEm	Reduced scattering coefficient at the emission wavelength.
MuaEx	Absorption coefficient at the excitation wavelength.
MusEx	Reduced scattering coefficient at the excitation wavelength.
Restore Defaults	Resets the model type, algorithm starting parameters and algorithm options to the default values.
Mask	A drop-down list of ROIs in the selected image. Select an ROI to compute only the source in the ROI.
Statistics Weighting	Choose this option to apply a statistical weighting technique to help reduce the error associated with high radiance measurements.
LM Fitting	Click to begin the point source fitting.
Properties Tab	
Tissue Properties	Make a selection from this drop-down list to specify starting values for the parameters other than the defaults. **Note:** Selecting a tissue property automatically updates MuaEm, MusEm/ MuaEx, and MusEx in the Params tab.
Internal medium index of refraction	The internal medium index of the tissue selected from the Tissue Properties drop-down list. You can also enter a user-specified value.
Results Tab	
MuaEm	Absorption coefficient at the emission wavelength.
MusEx	Reduced scattering coefficient at the excitation wavelength.
Mueff	Effective attenuation coefficient $\text{Mueff} = \sqrt{3\mathbf{Mua}(\mathbf{Mua} + \mathbf{Mus})}$
Diff	Diffusion coefficient, Diff = (Mua +Mus)/3

Table 11.1 Tool palette, point source fitting (continued)

Item	Description
X location of the source	X-coordinate of the source location.
Y location of the source	Y-coordinate of the source location.
Z location of the source	Z-coordinate of the source location.
Starting ChiSqure	Error between the measured and simulated photon density at the start of the analysis.
Ending ChiSqure	Error between the measured and simulated photon density at the end of the analysis.

11.2 Point Source Fitting

Point source fitting is performed separately on each image in a sequence.

1. Open the image sequence that you want to analyze.
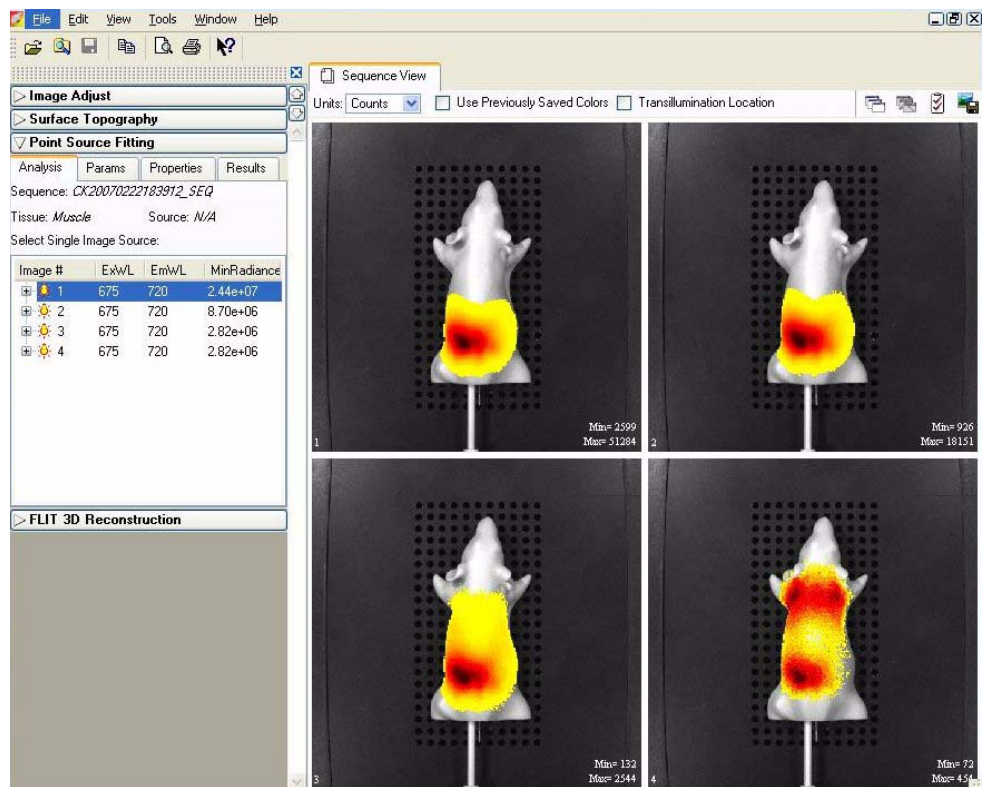
2. In the Analysis tab, select an image in the sequence.

3. In Surface Topography tools, generate or load a surface. For more details on generating the surface, see .
   Note: It is recommended that you use the smoothing tool to generate a good quality surface.

4. Click the Params tab.

 - The default starting values for the source location, power, and tissue optical properties are displayed.

 Note: The software automatically selects the correct model type for the image data.
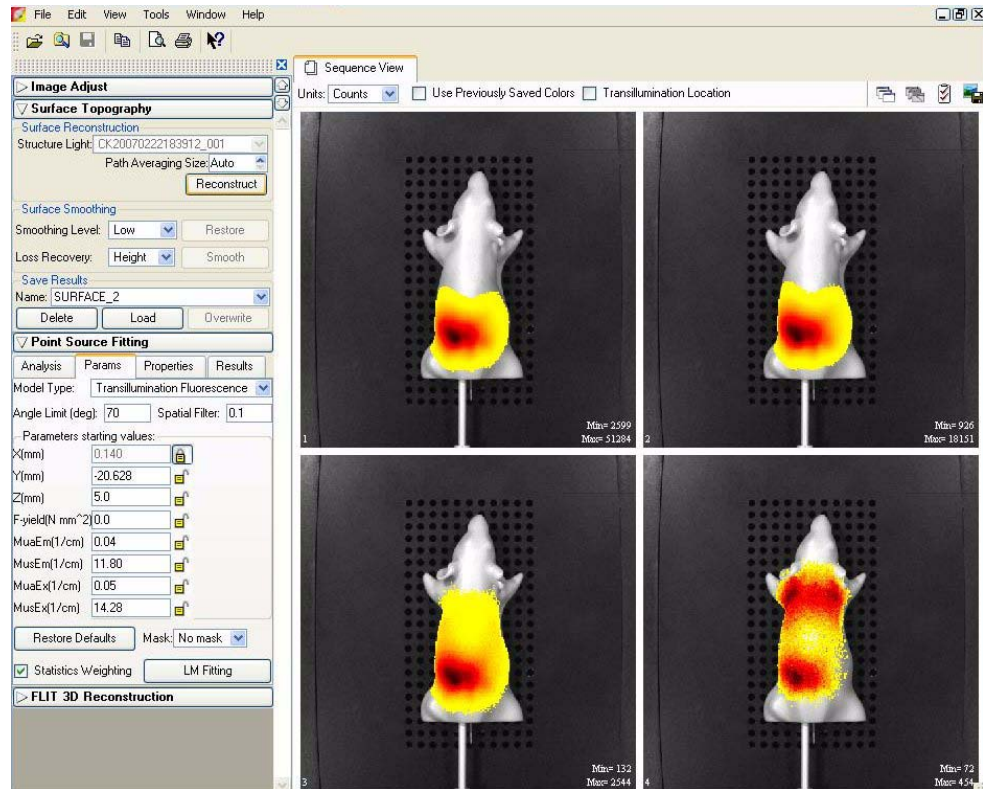
5. If you want to fix a parameter starting value, click the unlocked icon 🔓 so that it becomes a closed lock 🔒 .

6. If you want to construct the source only in a region of interest, make a selection from the Mask drop-down list.

7. Confirm the angle limit and spatial filter defaults or enter new values.

8. To specify different starting values for the optical properties:

 a. Click the Properties tab.

 b. Make a selection from the Tissue Properties drop-down list.

 c. Confirm the internal medium index of refraction or enter a new value.

9. In the Params tab, click **LM Fitting**.

 - The source appears on the mesh and the Results tab displays the point source fitting results.

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11.3 Checking the Point Source Fitting Results

1. In the Results tab, click Photon Density Maps.

    - The Photon Density Maps window appears.

2. Select the image from the Image sources drop-down list.

3. Compare the simulated and measured photon densities.
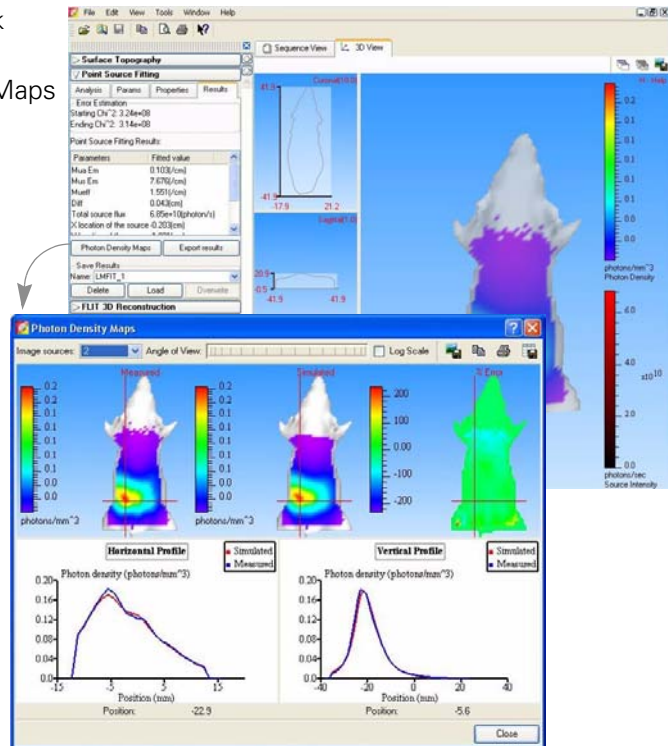
11.4 Exporting Results

To export all results:

1. In the Results tab, click **Export results**.

2. In the dialog box that appears, select the destination folder for the results and click OK.

    - The results include a .txt, .csv, .xsc (source information), and a .xmh (surface mesh) file.

To export user-selected results:

1. Right-click the item of interest in the results list, and select Export Results on the shortcut menu.

2. In the dialog box that appears, choose a folder for the results, enter a file name (.txt), and click **Save**.
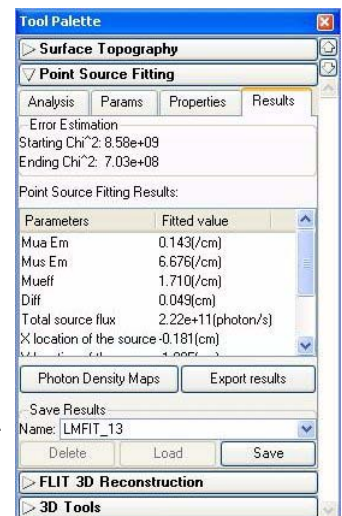
11.5 Managing Point Source Fitting Results

To save results:

1. Select the results of interest (LMFIT_xx) from the Name drop-down list

2. Click **Save**.

   - The point source fitting results are saved with the image.

To view results:

1. Select the results of interest 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) of interest and select **Copy Selected** 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 select **Copy All** from the shortcut menu that appears.

   - All of the results table is copied to the system clipboard.

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12 3D Reconstruction of Sources

The Living Image® software includes algorithms for 3D reconstruction of bioluminescent or fluorescent sources (*tomographic analysis*):

- Diffuse Tomography (DLIT™) algorithm - For tomographic analysis of bioluminescent sources. Analyzes a bioluminescent image sequence obtained on the IVIS® Imaging System 200 or IVIS Spectrum.

- Fluorescent Tomography (FLIT™) algorithm - For tomographic analysis of fluorescent sources. Analyzes a fluorescent image sequence obtained on the IVIS® Spectrum imaging system.

The steps to perform 3D reconstruction of bioluminescent/fluorescent sources include:

1. Acquire an image sequence.

2. Generate the surface topography (*surface*) of the subject.

3. Set the user-modifiable DLIT/FLIT algorithm parameters (for example, analysis wavelengths, source spectrum, and tissue properties) and reconstruct the position, geometry, and strength of the luminescent sources.

For more details on the DLIT/FLIT algorithm, see Appendix H, page 247.

12.1 3D Reconstruction of Bioluminescent 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. 3D reconstructions are currently not possible on black or dark-colored furred mice. In this case, it is recommended that you shave the animals or apply a depilatory.

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.

Generate a Surface Topography

For details on generating the surface, see

Set Up & Acquire an Image Sequence

Use the imaging wizard to setup the image sequence required for DLIT™ analysis. (For more details on the imaging wizard, see page 25.) If you plan to manually set up the sequence, Table 12.1 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, and structured light image.
- Emission filter #2: Luminescent image.

Table 12.1 Recommended image sequence for DLIT analysis

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

NOTE

The binning level must be the same for all of the luminescent images.

After the surface is generated, the 3D reconstruction of the light sources can proceed. Figure 12.1 shows example results. For more information on the DLIT algorithm and user-modifiable parameters, see Appendix H, page 247.

1. In the tool palette, click **DLIT 3D Reconstruction**.

2. In the Properties tab, make a selection from the "Tissue Properties" and "Source Spectrum" drop-down lists.

   "Muscle" is usually the best choice of tissue properties for general *in vivo* reconstructions.

   Note: The internal medium index of refraction is automatically entered when you select a tissue.

3. If you want to view the "Tissue Properties" (μ_a, μ_{eff}, μ'_s) or "Source Spectrum" for the tissue and light source selected above, make a selection from the Plot drop-down list in the Properties tab.
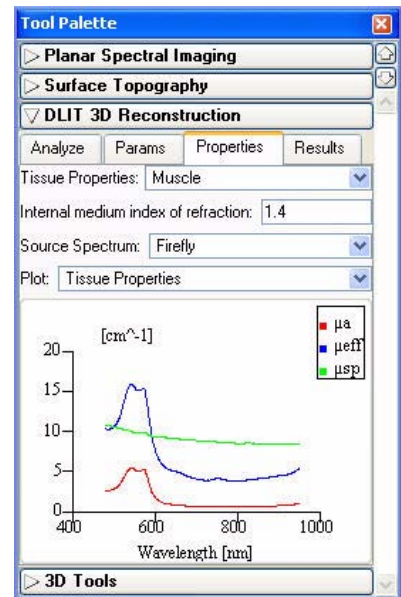
4. In the Analyze tab, select the acquisition wavelengths (560-660 nm).

5. If necessary, edit the minimum radiance associated with an acquisition wavelength or angle. For more details on the minimum radiance, see page 253.

   Note: It is recommended that you only analyze images that have signal well above the noise.

6. To edit the minimum radiance, double-click the entry and enter a new value.

   Note: The minimum radiance level can be observed by looking at individual images and adjusting the min level on the color bar, in radiance units (photons/sec/cm²).

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7. In the Params tab, confirm the parameter defaults or enter new values.

For more details on the parameters, see Appendix H, page 247.

Angle limit default is 70° for IVIS® Imaging System 200 Series or IVIS® Spectrum data.

DLIT algorithm user-modifiable parameters

Params tab
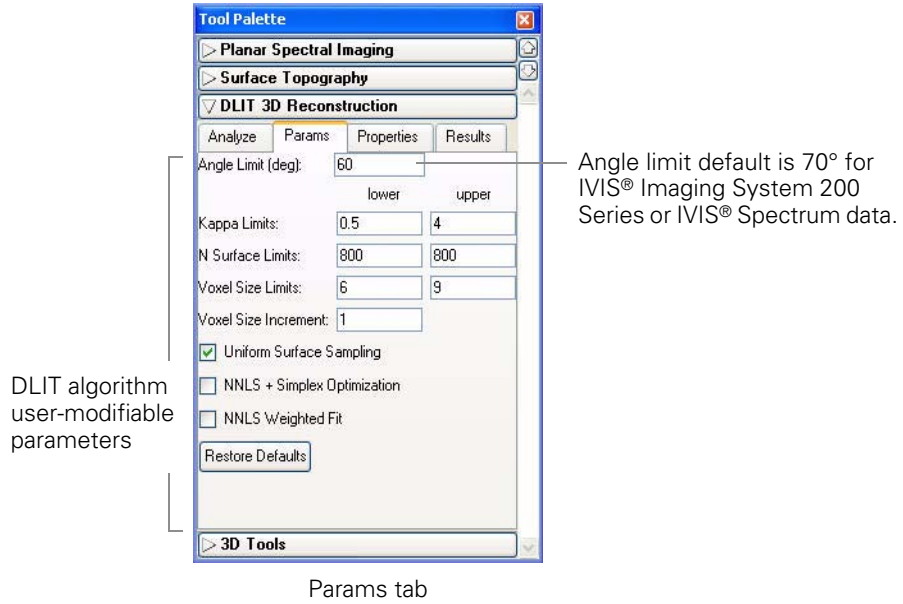
8. In the Analyze tab, click **Reconstruct**.

The reconstruction requires about 1-5 minutes, depending on the parameter settings and the processor speed.

Figure 12.1 shows example 3D reconstruction results.

The 3D View in the image window displays the surface, the 3D reconstruction of the bioluminescent light sources inside the subject (voxels), and the photon density map (photons/mm³ just below the surface).

Results tab displays the results data and the DLIT parameter values.
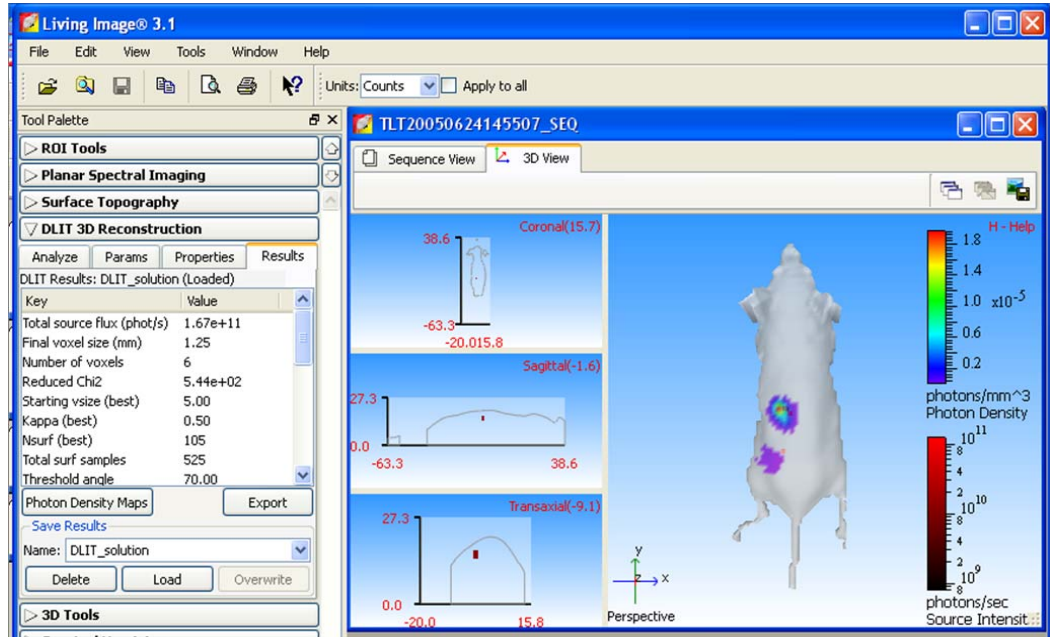
Image window, 3D view

To best view the light sources (voxels):

1. In the 3D tools, move the slider or enter an opacity value to adjust the surface opacity.

2. Clear the Render Photon Density Map option to display the surface without the photon density map.
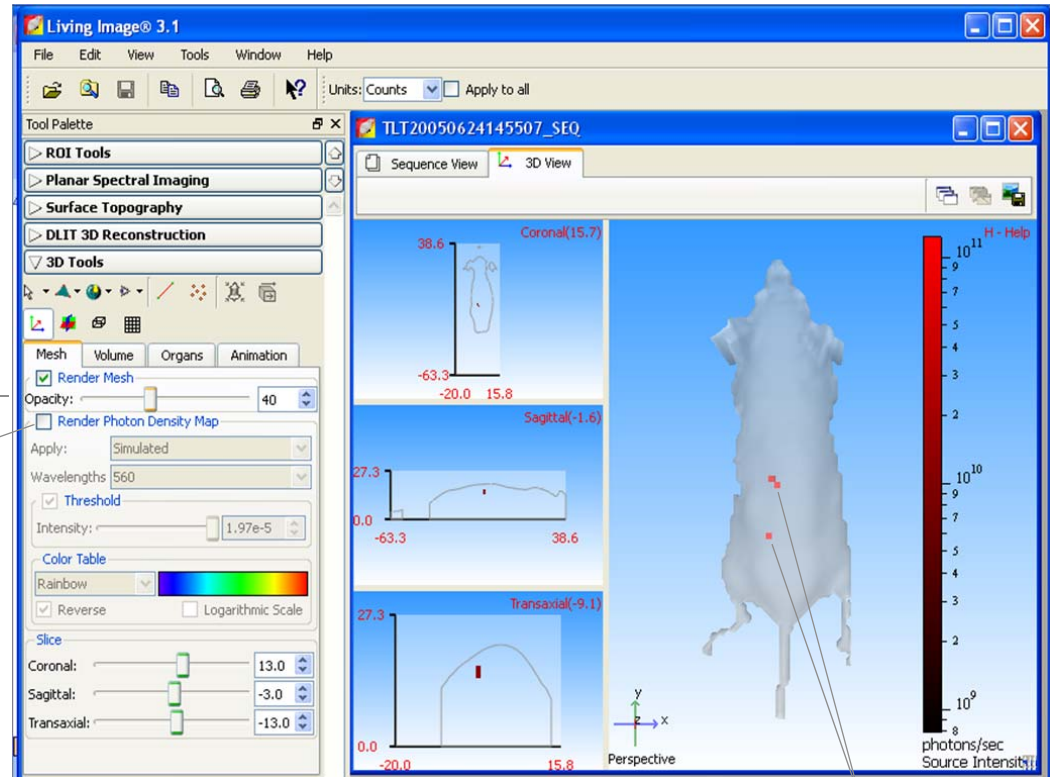
Light sources (voxels)

Figure 12.1 Viewing DLIT 3D reconstruction results, Results tab (top) and 3D tools (bottom)

12.2 3D Reconstruction of Fluorescent Sources

Set Up & Acquire an Image Sequence on the IVIS® Spectrum

Use the imaging wizard to setup the image sequence required for FLIT™ analysis. (For more details on the imaging wizard, see page 25.) If you plan to manually set up the sequence, Table 12.2 shows the recommended image sequence. Acquire the images using transillumination on the IVIS Spectrum using the same excitation and emission filters from at least four source locations.

Table 12.2 Example image sequence for FLIT analysis

Image Type	Source Location	
	First location	**Subsequent locations**
Photographic	✓	Use previous photo
Structured light	✓	
Fluorescent	✓	✓

Generate the Surface Topography

For instructions, see page 151.

Set the FLIT Parameters & Reconstruct the Sources

After the surface is generated, the 3D reconstruction of the light sources can proceed.

1. In the tool palette, click **FLIT 3D Reconstruction**.
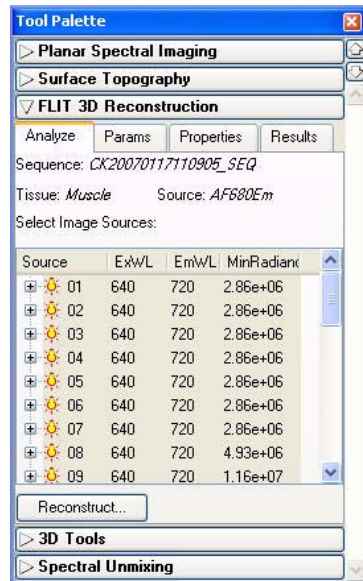
Analyze tab

Source 01 corresponds to the first image of the sequence, source 02 corresponds to the second image, and so on

2. In the Properties tab, make a selection from the Tissue Properties drop-down list. "Muscle" is usually the best choice of tissue properties for general *in vivo* reconstructions.

    Note: The internal medium index of refraction is automatically entered when you select a tissue.

Parameters tab
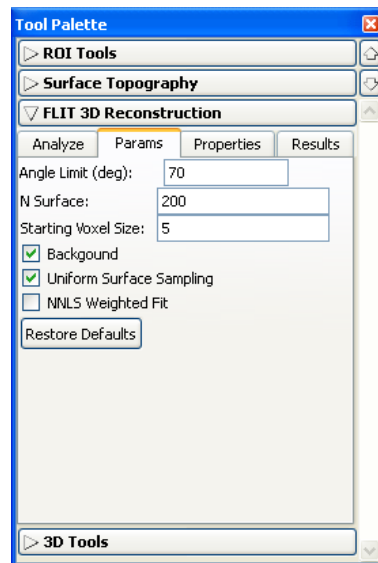
Properties tab
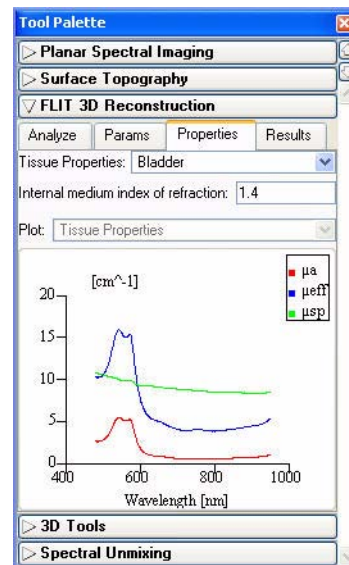
3. In the Params tab, confirm the parameter defaults or enter new values.

   For more details on the parameters, see Appendix H, page 247. It is recommended that you start with the default parameter settings. If necessary, fine tune the settings in the parameters tab.

4. In the Analyze tab, select the source locations to include in the analysis and click **Reconstruct**.

   - Figure 12.1, page 155 shows example 3D reconstruction results

12.3 DLIT & FLIT Results

The Results tab displays information about the photon density, voxels, and DLIT or FLIT algorithm parameters.
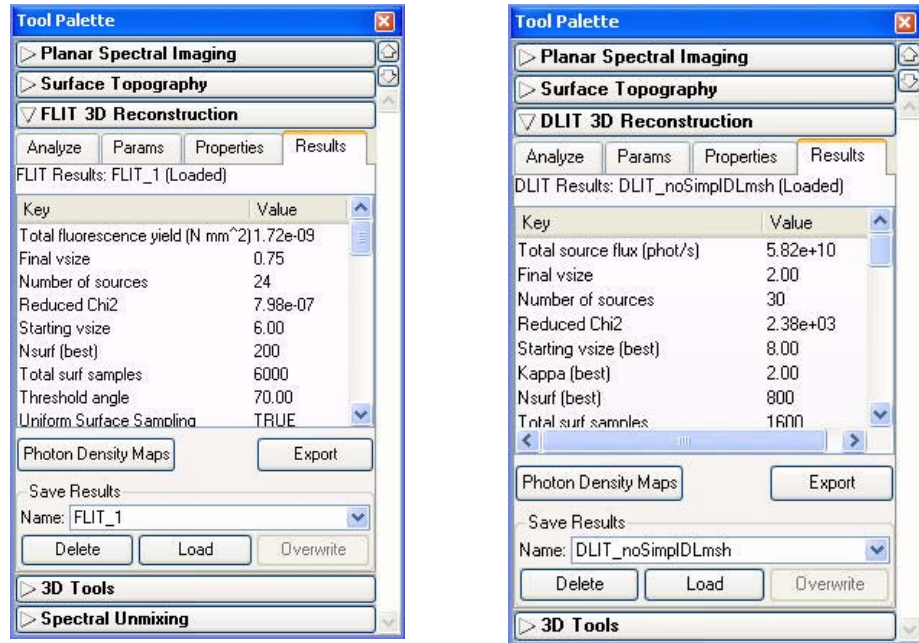
Figure 12.2 3D reconstruction results, FLIT (left) and DLIT (right)

NOTE

For more details on the DLIT and FLIT algorithm parameters, see Appendix H, page 247. Sometimes adjusting the DLIT algorithm parameters improves the fit of the simulated photon density to the measured photon density data.

Table 12.3 3D reconstruction results

Item	Description
Optimized fit parameters	
Total source flux (phot/s) (DLIT result)	The sum of the bioluminescent source intensities.
Total fluorescence yield (N mm^2) (FLIT result)	The total sum of the fluorescent yield. The quantity measured is:
	(Fluorescence quantum efficiency for the excitation wavelength to emission wavelength photons)*(Excitation wavelength photon absorption cross section)*(Fluorophore number density)*(Volume of voxel size). To convert the 'Fluorescence Yield' measurement to the number of fluorescent compounds, divide the 'Fluorescent Yield' values by measured quantities of [Fluorescence quantum efficiency of excitation wavelength conversion to emission wavelength]*[Excitation photon absorption cross section] for the fluorophore.

Table 12.3 3D reconstruction results (continued)

Item	Description
Final vsize	The voxel size (length of a side, mm) that produces the optimum solution to the DLIT or FLIT analysis.
Number of sources	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 vsize	The voxel size at the start of the analysis.
Kappa (best) (DLIT result)	The kappa value that produces the optimum solution.
N surf (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.
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. For more details, see *Angle Limit*, page 253.
Kappa limits (DLIT result)	The kappa parameter is a parameter that is searched during a reconstruction to determine the best fit to the image data. For more details, see *Kappa Limits (DLIT)*, page 254.
N surface limits (DLIT result)	The maximum number of surface intensity points to use in the reconstruction for each wavelength. The range is 200 to 800 and the default is 200. The reconstruction time is shorter for smaller values of N (for example, 200). However larger values of N may give a more accurate result because more data are included in the fit.
Starting Voxel Size (DLIT/FLIT)	The length of the side of the voxel cube in mm units for the coarsest initial grid size in the adaptive gridding scheme.
Voxel size limits (DLIT result)	The starting voxel size range evaluated by the algorithm to determine the optimum solution.
Voxel size increment (DLIT result)	The incremental change in voxel size evaluated at each iteration during the DLIT analysis.
Uniform Surface Sampling	TRUE = the option is chosen and the surface data for each wavelength is sampled spatially uniformly on the signal area. FALSE = the option is not chosen and the N brighter surface elements are used as data in the reconstruction.
NNLS + Simplex Optimization	TRUE = the option is chosen and a non-negative least squares optimization algorithm is used in addition to the SIMPLEX algorithm to seek the optimum solution. FALSE = only the NNLS algorithm is used to seek the optimum solution.
NNLS Weighted Fit	TRUE = the option is chosen and the DLIT or FLIT algorithm weights the wavelength data inversely proportional to its intensity in the NNLS reconstruction. FALSE = the option is not chosen
Min Radiance	The wavelength image data minimum radiance [photons/sec/cm²/sec] to use in the DLIT or FLIT analysis.
Index of Refraction	The internal medium index of refraction that is associated with the user-selected tissue.

Table 12.3 3D reconstruction results (continued)

Item	Description
Tissue Properties	The user-specified tissue in which the sources should be located.
Source Spectrum	The emission spectrum of the type of bioluminescent source.
Photon Density Maps	
Simulated	The photon density computed from DLIT/FLIT source solutions which best fit the measured photon density (see page 161).
Measured	The photon density determined from the image measurements of surface radiance.
Wavelength	The wavelength of the photon density map in the active image.
Source Image	The image number of the transillumination source image.
Photon Density Maps	Click to open the Photon Density Maps window.
Save Results	
Name	The default name for the active DLIT or FLIT results.
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.

Viewing Photon Density

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

To check the quality of the DLIT or FLIT construction, it is useful to compare the measured and simulated photon density plots. The photon density is closely related to the measured radiance.

To view the photon density maps:

1. In the Results tab, click **Photon Density Maps**.

   - The Photon Density Maps window displays the photon density maps for all wavelengths.

Select one or all wavelengths for display.

Move the wheel to the left or right to rotate the surface on the vertical axis.
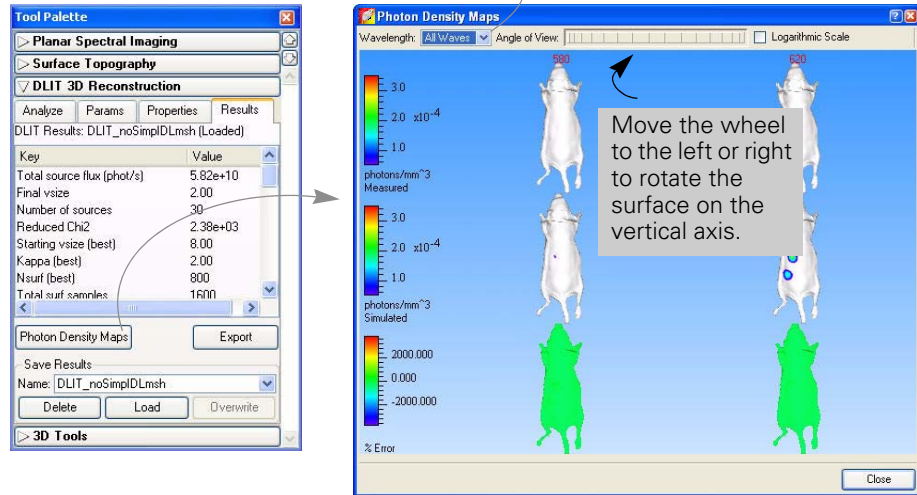
Note: The voxels are also automatically displayed when the 3D reconstruction is completed. For more details on measuring the voxels, see .
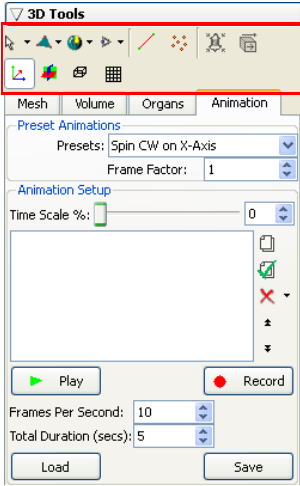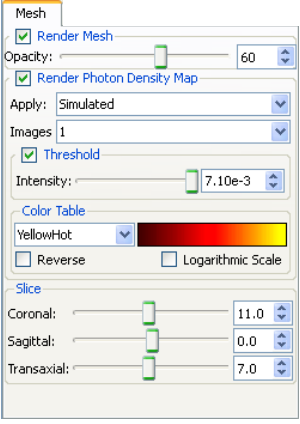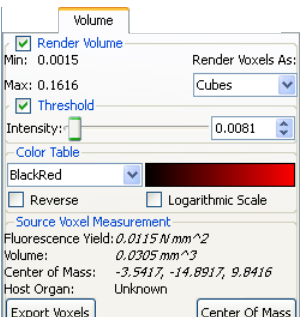
2. To display the measured and simulated photon density profiles:

   a. Select a wavelength.

   b. Drag the crosshair to the location of interest.

   - The horizontal and vertical photon density profiles are updated.
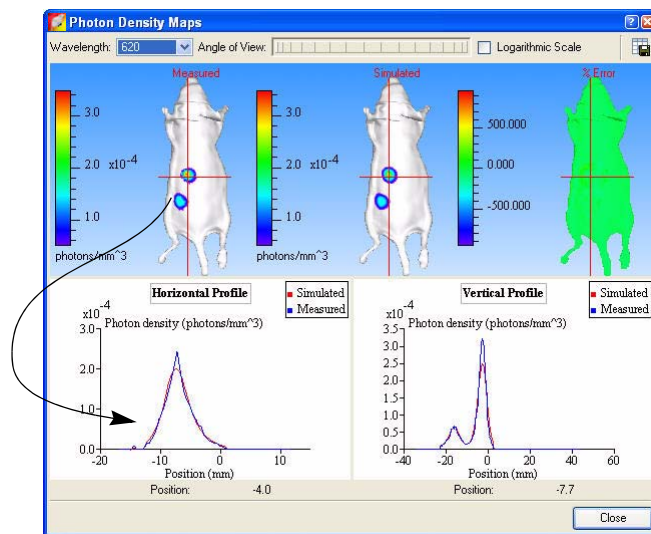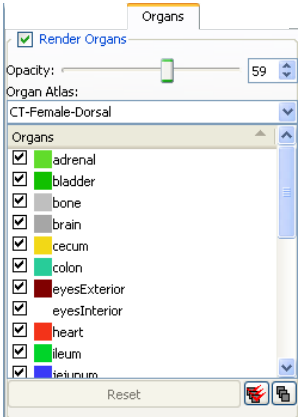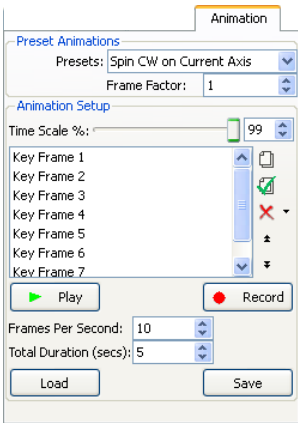
In a good reconstruction, the measured (blue) and simulated (red) photon density curves are close.

3D Tools

The 3D tools appear in the tool palette when a surface topography (surface) or 3D source is reconstructed, or when you open saved results.

For Details on...		See Page
3D tool buttons		**164**
Mesh tab		**170**
Volume tab		**172**

For Details on...		See Page
Organs tab		175
Animation tab		182

12.4 3D Tools

Click: 🔽 to select a tool to work with the surface or 3D results (perspective view only).

🔺 to select a surface drawing style.

🌐

to select a shading style for the surface.
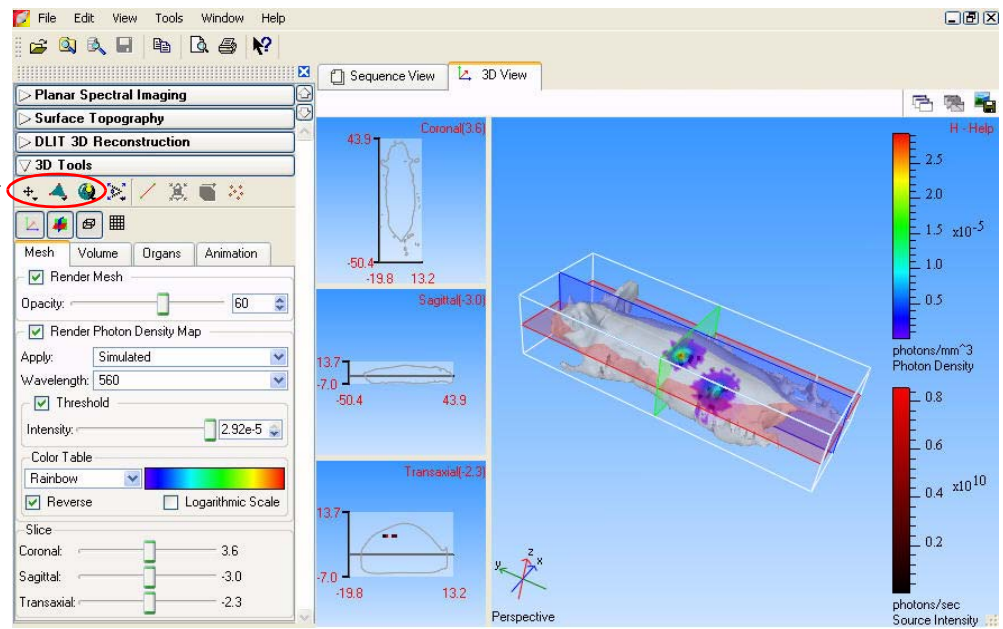
Figure 12.3 3D tools and DLIT results in the 3D view window

Table 12.4 3D Tools

Item	Description
Image Tools	A drop-down list of tools for viewing and working with the surface or DLIT results.
	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 173.)
	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).

Table 12.4 3D Tools

Item	Description
Drawing Styles	A drop-down list of drawing styles for the surface (for examples, see Figure 12.3, page 164). The Surface face drawing style ( ) is the default.
	Point cloud
	Wire frame
	Surface face
	Wire frame and surface face
Shading Styles	A drop-down list of shading styles for the surface (for examples, see , page 166). The Reflect smooth surface face shading style ( ) is the default.
	Smooth face
	Smooth surface face
	Reflect surface face
	Reflect smooth surface face
	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 12.6.
	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.
	Click to display the manual transform tool.
	Automatic atlas registration tool.
	Click a voxel in the 3D reconstruction, then click this button to display measurements for the voxel in the 3D tools (source voxel measurements).
	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 subject in the 3D view window.
	Click to show or hide a bounding box around the subject.
	Click to show or hide a grid under the subject.

**Rotate, Move, or Zoom
on a 3D Image**
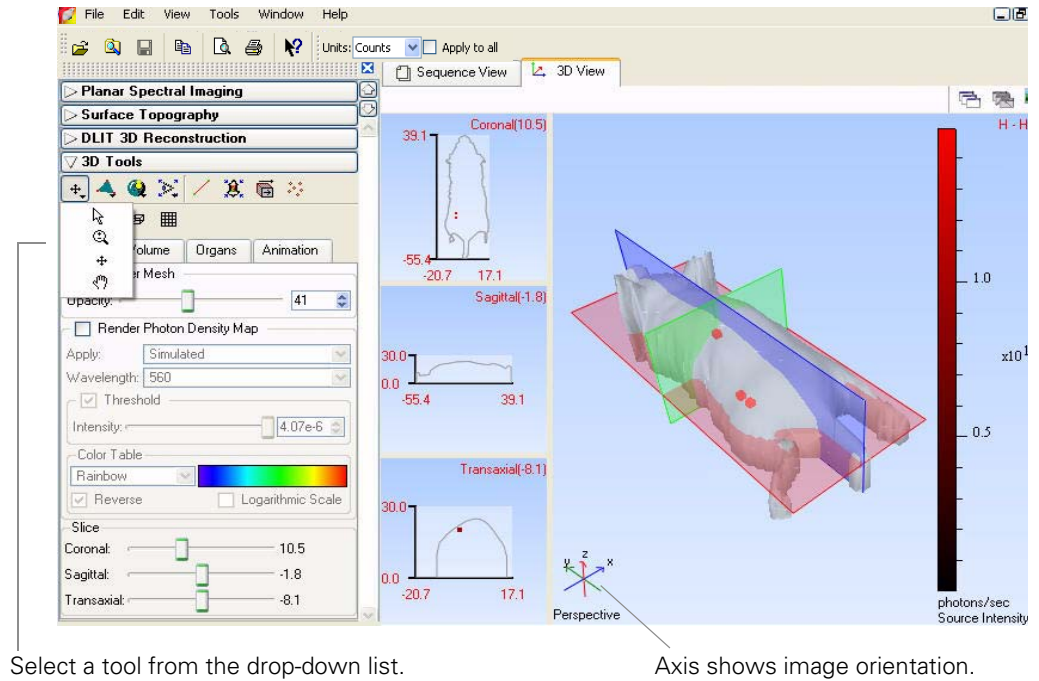
Select a tool from the drop-down list.   Axis shows image orientation.

To rotate the image:

1. Choose the ⌖ or ✋ tool.

2. Place the pointer in the 3D View window.

3. Click and drag the pointer in the x, y, or z-axis direction.
    - The x,y,z-axis shows the orientation of the image.

To move the image:

1. Select the ✥ arrow in the 3D tools and drag the image. Alternatively, press the
    Shift key while you drag the image.

To zoom in or out on the image:

1. Select the ✥ arrow in the 3D tools.

2. To zoom in on the image (magnify), right-click (Ctrl+click for Macintosh users) and
    drag the 🔍 toward the bottom of the window.
    To zoom out on the image, right-click and drag the 🔍 toward the top of the window.

Selecting a Drawing or Lighting Style for the Surface

You can choose a different drawing and lighting style to change the appearance of the surface.

To choose a drawing style, make a selection from the Drawing style drop-down list in the 3D tools.

Point cloud surface   Wire frame surface   Surface face surface   Wire frame & surface face
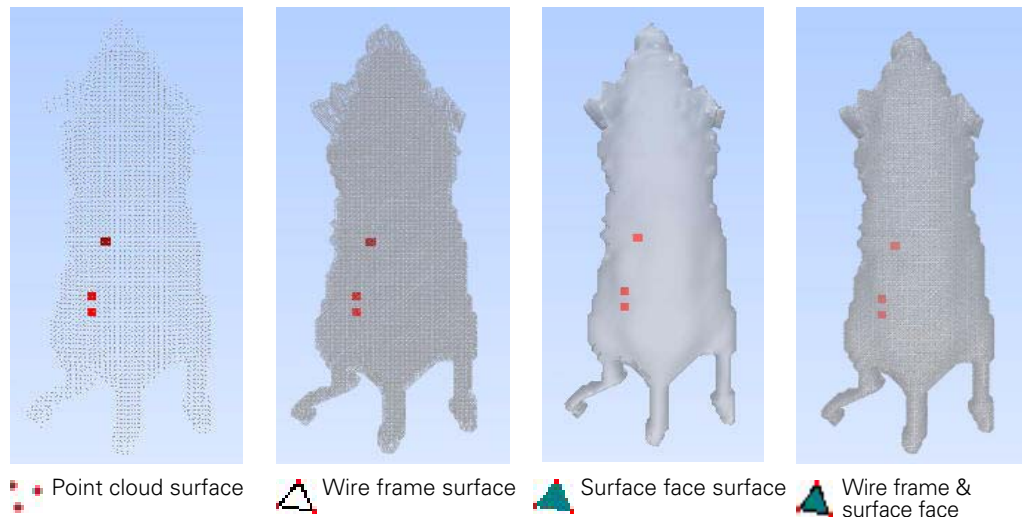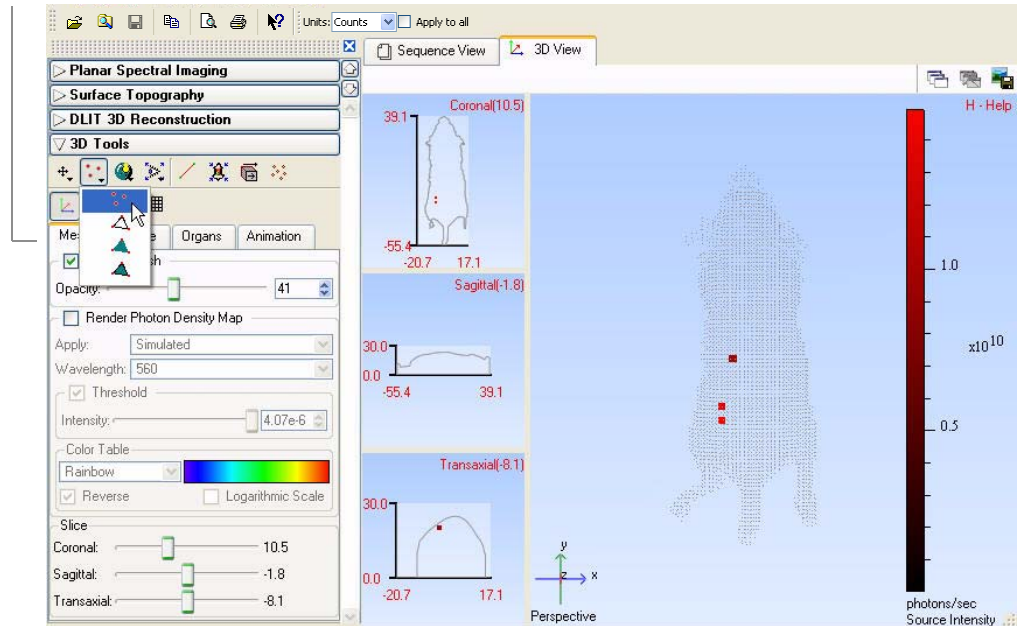
Figure 12.4 Surface drawing styles

To choose a shading style, make a selection from the Shading style drop-down list in the 3D tools.

 Surface face   Smooth surface face   Reflect surface face   Reflect smooth surface face
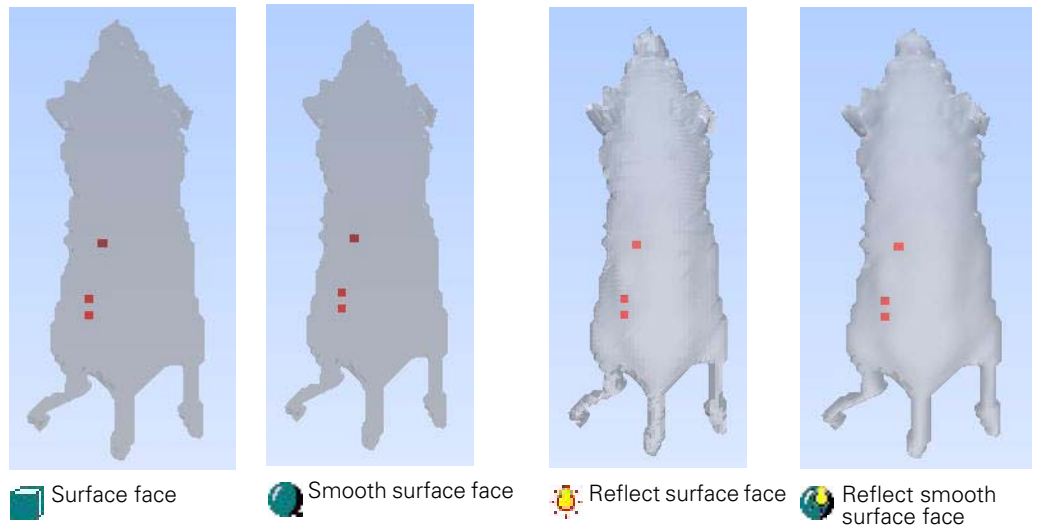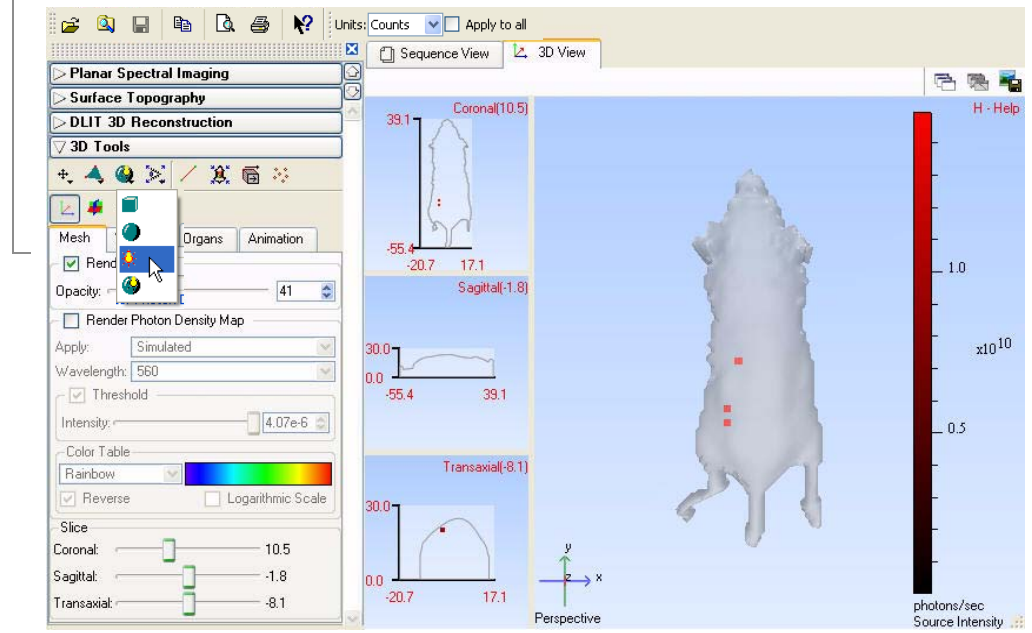
Figure 12.5 Surface shading styles

Changing the View Perspective

You can view a 3D image from different perspectives. Figure 12.6 shows examples of the other available views.

To change the view:

- Select ⬡ to change the view.

- Alternatively, click the surface, then press the **V** key to cycle through the different views of the surface (Figure 12.6).

- Select ⬡ to display the perspective view.

Note: Only the perspective view (the default view) can be rotated or moved in the 3D view window.
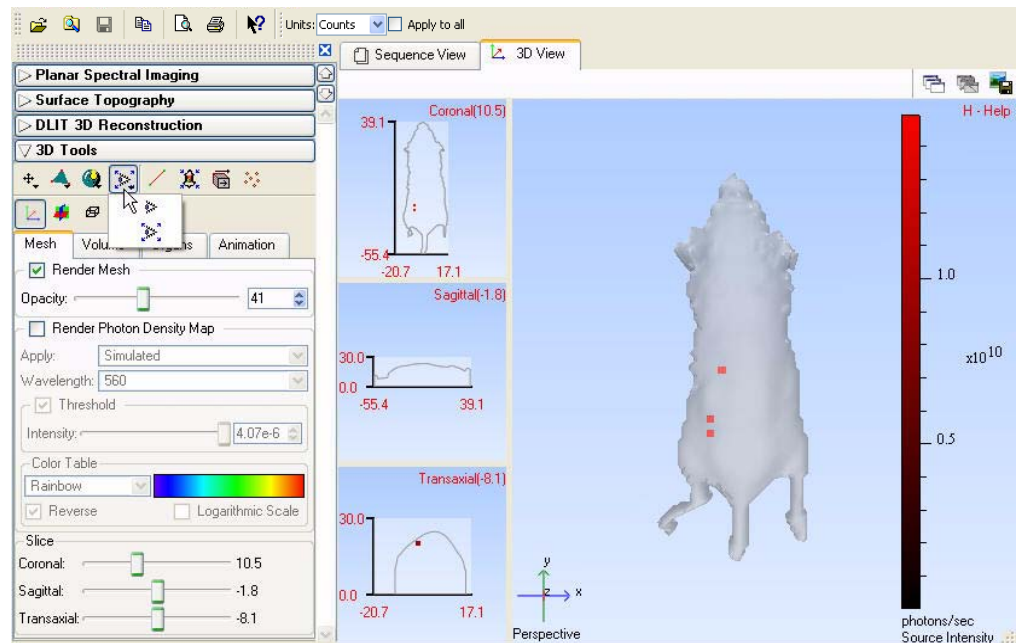
View name
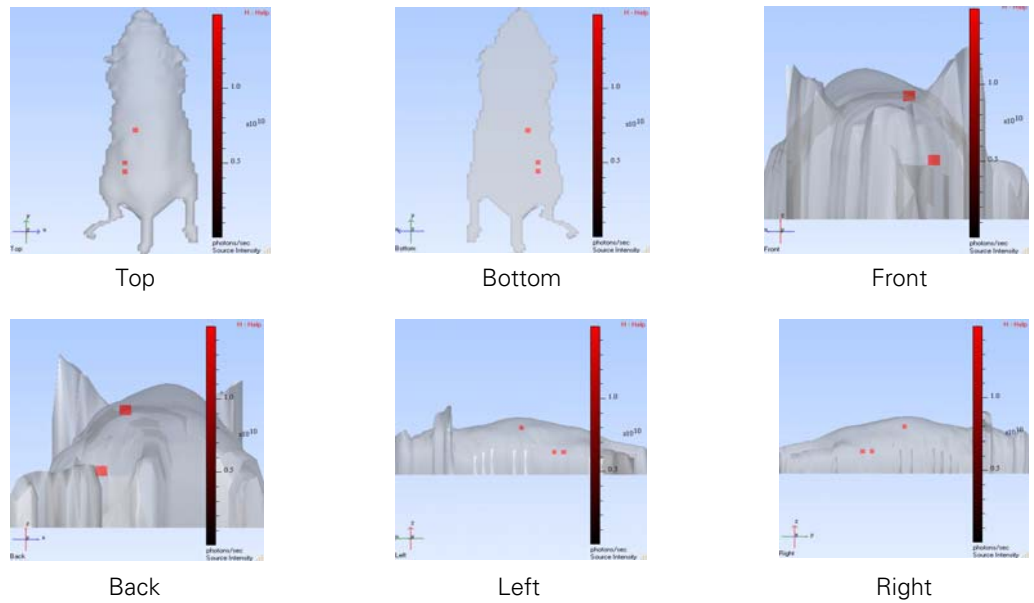
Figure 12.6 Alternative views of the surface

12.5 3D Tools - Mesh Tab

The Mesh tab includes tools for viewing the reconstructed surface, photon density maps, and planes through the 3D reconstruction.
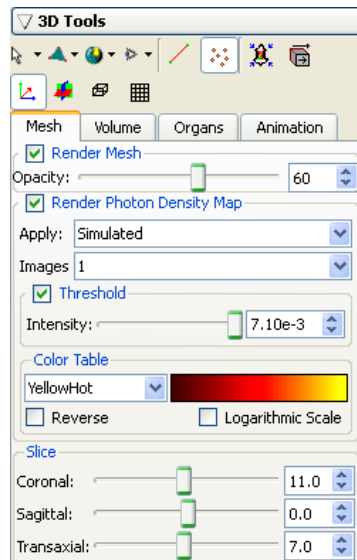
Figure 12.7 3D tools, mesh tab

Table 12.5 Mesh tab

Item	Description
Render Mesh	Choose this option to display the surface in the 3D view window. It may be helpful to turn off the surface to better view the photon source 3D reconstruction.
Opacity	Controls the surface opacity. A lower number makes the surface more transparent. This may be helpful for viewing the photon source 3D reconstruction.
Photon Density Map	Choose this option to display the photon density map. (If the DLIT reconstruction of the bioluminescent source has not been generated, this option is not available.)
Apply	Select the simulated (computed by the DLIT algorithm) or measured photon density for the photon density map.
Images	A drop-down list of the images used to reconstruct the surface. Make a selection from the list to view the DLIT/FLIT results associated with a particular image.
Threshold	Choose this option to apply a photon density threshold (photons/mm^3) to the photon density map.
Intensity	Use the Intensity slider, the ⬍ arrows or enter a value in the box to set the minimum intensity threshold.
Color Table	Specifies the color table for the source intensity scale.
Reverse	Choose this option to reverse the color table. For example, the BlackRed color table represents the source intensity (photons/sec) from low to high using a color scale from black to red. If **Reverse** is chosen, the source intensity (photons/sec) from low to high is represented using the color scale from red to black.
Logarithmic Scale	Choose this option to apply a logarithmic scale to the color table.
Slice	Move a slider to change the position of the coronal, sagittal, or transaxial plane through the surface. The intersection of the plane and subject (*slice*) is shown in the coronal, sagittal, and transaxial views in the 3D view window.

12.6 3D Tools - Volume Tab

The voxels are automatically displayed when the 3D reconstruction is complete. In the 3D tools, the Volume tab displays the voxel intensity and other data, and provides tools for voxel measurement and display.
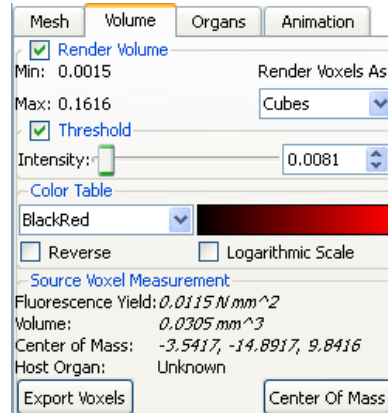
Figure 12.8 3D tools, Volume tab

Table 12.6 3D tools, Volume tab

Item	Description
Render Volume	Choose this option to display the voxels.
Min	The minimum voxel intensity (photons/sec for DLIT results; N mm² for FLIT results).
Max	The maximum voxel intensity (photons/sec).
Render voxels as	A drop-down list of shapes for voxel display.
Threshold	Choose this option to apply a minimum threshold intensity to the voxel display.
Intensity	Use the Intensity slider, the ⬍ arrows, or enter a value in the box to set the minimum threshold intensity.
Color Table	Specifies the color table for the voxel intensity scale.
Reverse	Choose this option to reverse the color table. For example, the BlackRed color table represents the source intensity (photons/sec) from low to high using a color scale from black to red. If **Reverse** is chosen, the source intensity (photons/sec) from low to high is represented using the color scale from red to black.
Logarithmic Scale	Choose this option to apply a logarithmic scale to the color table.
Source Voxel Measurement	Click the ⊠ button, then click a voxel in the 3D reconstruction to display measurements for the voxel.
Total Flux (DLIT results)	The total flux measured for the voxels selected using the voxel tool.
Total Fluorescence Yield (FLIT results)	N mm² measured for the voxels selected using the Measure Voxels tool ⊠.
Volume	Volume of the selected voxels (mm³).

Table 12.6 3D tools, Volume tab (continued)

Item	Description
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	Organ location of the selected voxel(s).
Center of Mass	Click to compute the center of mass for the selected voxel(s).
Export Voxels	Enables you to export the voxel measurements (.csv).

To display voxel measurements:

1. Click the **Measure Voxels** button.

2. On the surface, click a voxel. Alternately, draw a box around a group of voxels.
   - The Voxel tab displays the selected voxel data.

Voxel data

Coronal, sagittal, & transaxial views
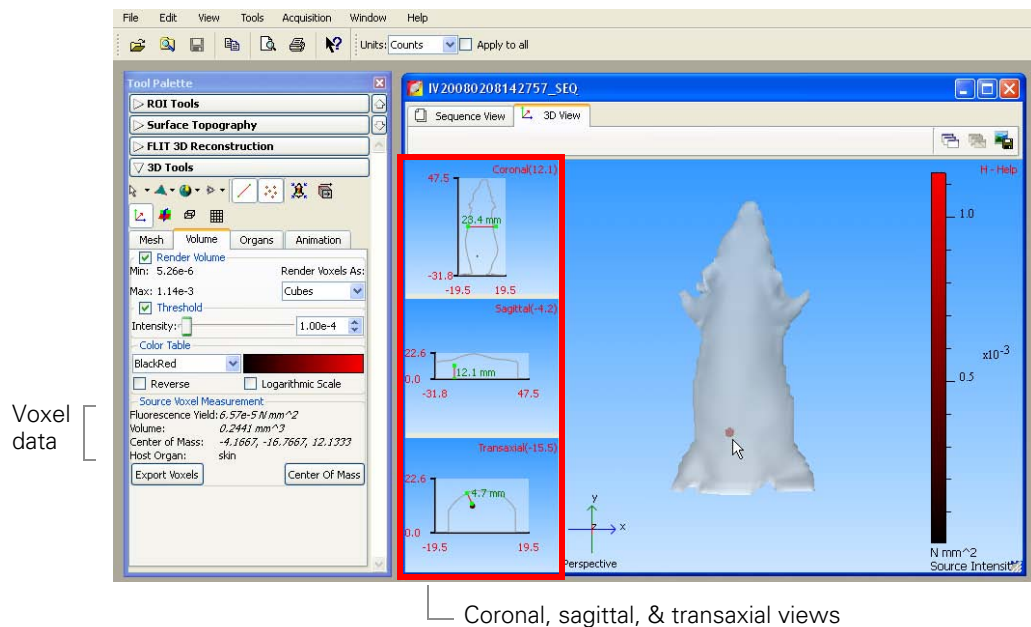
Figure 12.9 Example 3D reconstruction, FLIT results

3. Repeat step 2 to display data for other voxels.
   - The voxel data is updated.

4. To clear the voxel data, click any where in the 3D view window.

To measure the depth of a source(s):

1. Click the **Measure Voxels** button.

2. Click a voxel or draw a box around a group of voxels.

3. Click **Center of Mass**.

   The Coronal, Sagittal and Transaxial views display the intersection through the

voxels' center of mass.

4. Click the Measurement Cursor button ✎.

   - The Sagittal and Transaxial views show the distance between the voxel(s) and the dorsal or ventral surface (Figure 12.9).

5. To measure another distance, drag each end of the measurement cursor (●—●) to a new position.

   - The distance measurement is updated.

Viewing X,Y Coordinates

1. In the Coronal, Sagittal, or Transaxial windowpane, click the position of interest.

   - The x,y-coordinates (mm) of the position are displayed.

2. If you drag the mouse cursor, the coordinates are updated.
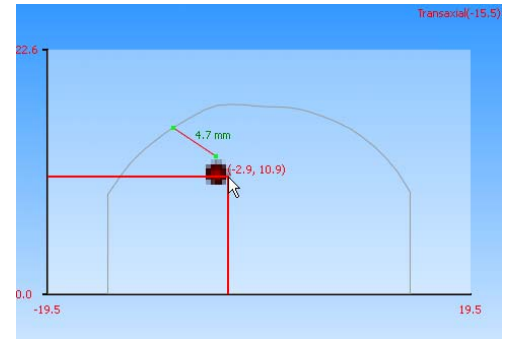
To display planes in the 3D image:

1. Click a voxel

2. Click the 🔳 button.

   - The Coronal, Sagittal, and Transaxial view shows the plane (*slice*) through the voxel.
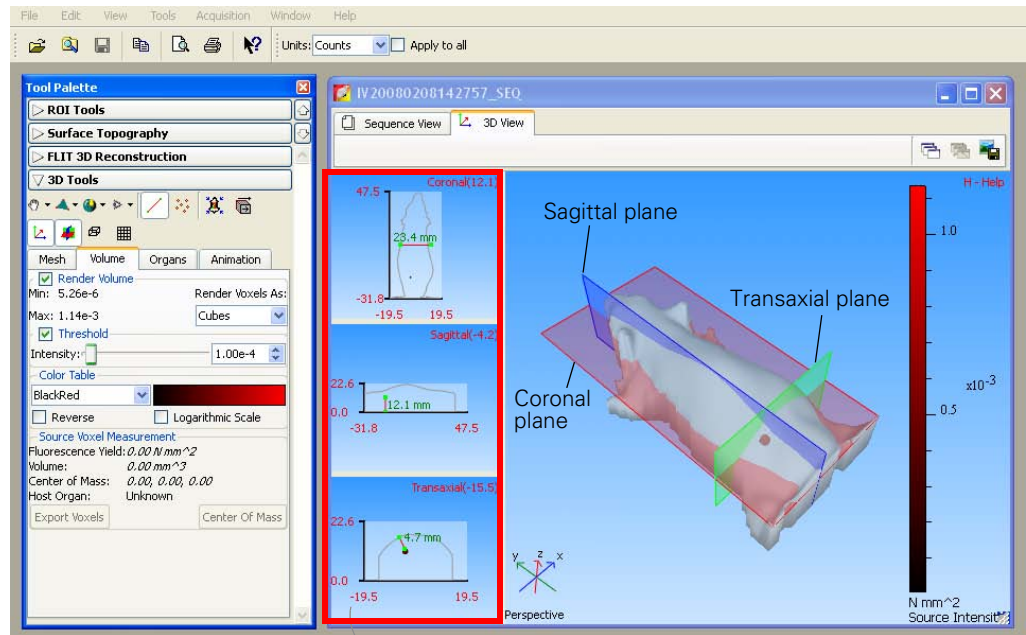
Coronal, sagittal, and transaxial views show the slice through the surface taken by the associated plane.

3. To change the location of a plane, move the coronal, sagittal, or transaxial slider left or right. Alternatively, click the slider, then press the ←or →keyboard arrow keys.

- The Coronal, Sagittal, or Transaxial windowpane is automatically updated.

12.7 3D Tools - Organs Tab

The Living Image® software provides digital mouse atlases that enable you to display a 3D skeleton and organs on the 3D reconstruction (Table 12.7). Select the atlas and organs that you want to display in the Organs tab. The software automatically aligns the organs on the surface. However, you can also manually adjust the scale or location of organs on the surface. You can also import a custom organ atlas created from Open Inventor files (.iv).
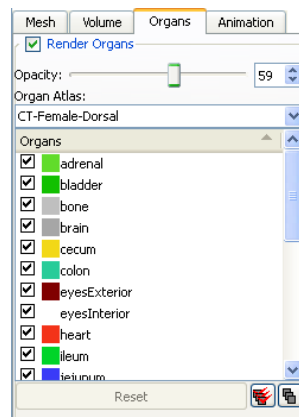
Figure 12.10 3D tools, Organs tab

Table 12.7 Organ atlases

Atlas	Description
Mouse Atlas-Female or Male	Male or female in a dorsal or ventral orientation. Atlas includes organs only, no skeleton.
CT-Female or Male	Male or female in dorsal, ventral, or lateral orientation. Atlas includes organs and skeleton.

Table 12.8 3D tools, Organs tab

Item	Description
Organs	Choose this option to enable the selection and display of organs on the surface.
Organ database	Choose the male or female organ database from the drop-down list.
Organs	A list of the organs in the selected organ database. Select the organ(s) that you want to display on the surface.
Reset	Click to display the selected organs in their default positions.
![select all]	Click to select all organs in the database and display them on the surface.
![clear]	Click to clear the selected organs and remove all organ diagrams from the surface.
Update scene	Click to display the selected organs on the surface.

Displaying Organs

1. In the 3D Tools, click the Organs tab.
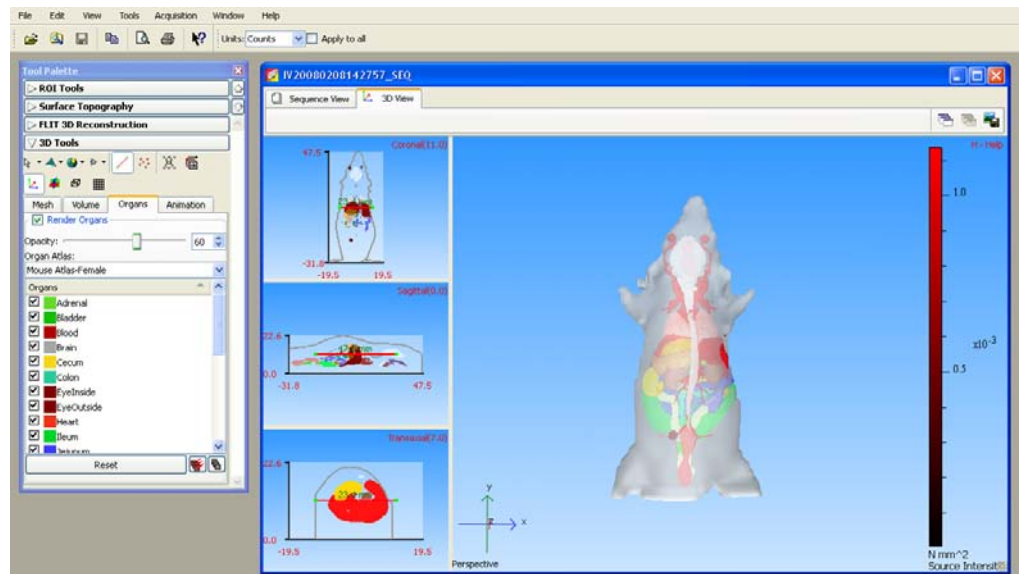
2. Confirm that the surface is in the perspective view (click the ![button] button or press the **R** key).

3. Choose the Render Organs option.

4. Select an organ atlas.

   - All of the organs in the selected atlas appear on the surface.

5. To co-register the digital organs and the surface:

   a. Click the **Fit organs to the mouse** button.

   b. On the drop-down list that appears, click a button to choose an option:

   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 the or tool to automatically fit the organs. (For more details on manual registration, see below.)

6. 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 do not select Skin in the Organs list.

7. To clear all organs from the surface, click the **Deselect All** button. To remove a specific organ, remove the check mark next to the organ name.

8. To display a specific organ(s), choose the organ name. To display all organs on the surface, click the **Select All** button.

> **NOTE**
>
> If you manually change the location, orientation, or scale of an organ(s), you can click **Reset** to restore the default size and position of the selected organs.

Manually Adjusting the Scale or Location of Organs

1. Follow step1 to step 4 above.

2. Click the **Transform tool** button.

   - The transform tool appears.
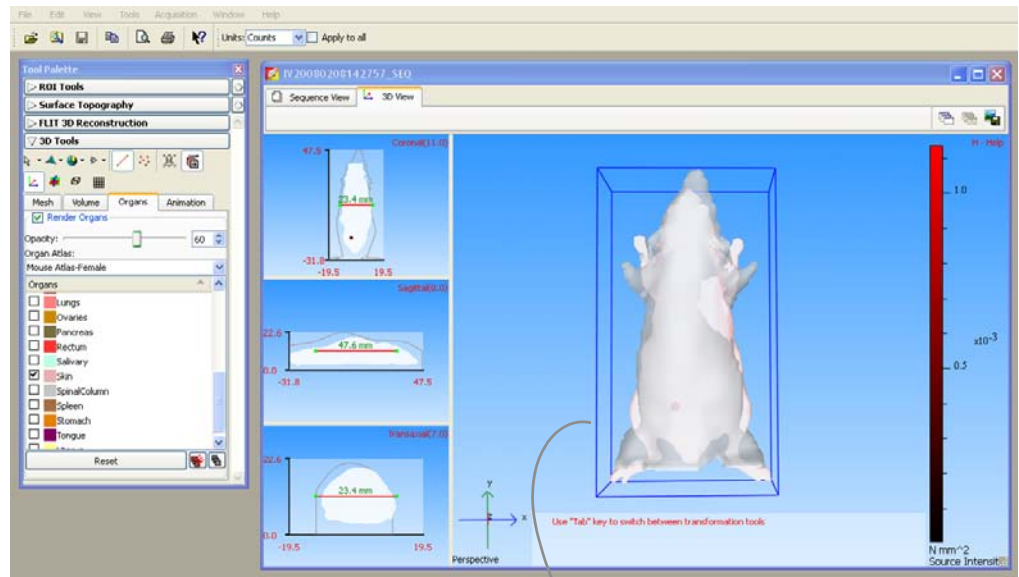
Transform tool
In this example, "Skin" is
selected from the organ list.

3. To adjust the x,y, or z-position of the organ, drag the transform tool.

4. To return the transform tool to the default location, click **Reset**.

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. **Note:** To restrict scaling to a particular axis, press the X, Y, or Z key, then drag a red cube.
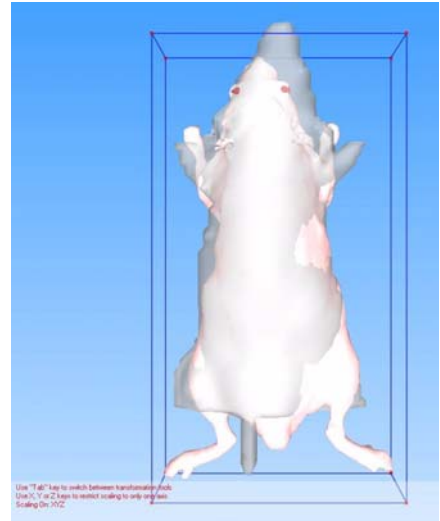
7. Press the **Tab** key again to put the transform tool in rotate mode.

   - A red, green, and blue circle appear around the surface.
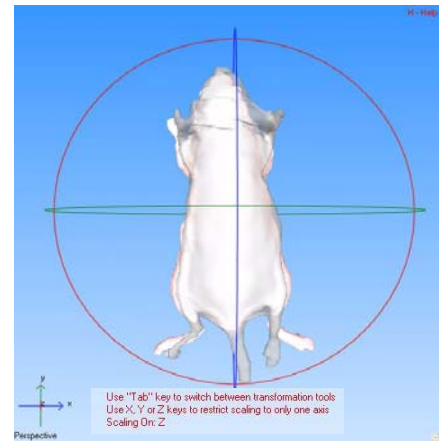
8. To rotate the organ on the x,y, or z-axis, click the blue, green, or red circle and drag the mouse arrow in the direction of interest.

   Note: To return the organ drawing to the default position and size, click **Reset** and the button.

9. To turn off the transform tool, click the button.

Circle line is thicker when selected
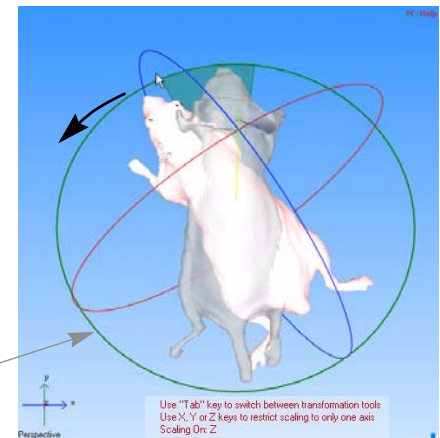
To check the organ and surface alignment:

1. Check the organ position in the Coronal, Sagittal, and Transaxial windowpanes.

2. In the 3D View tab, click in the windowpane with the surface.
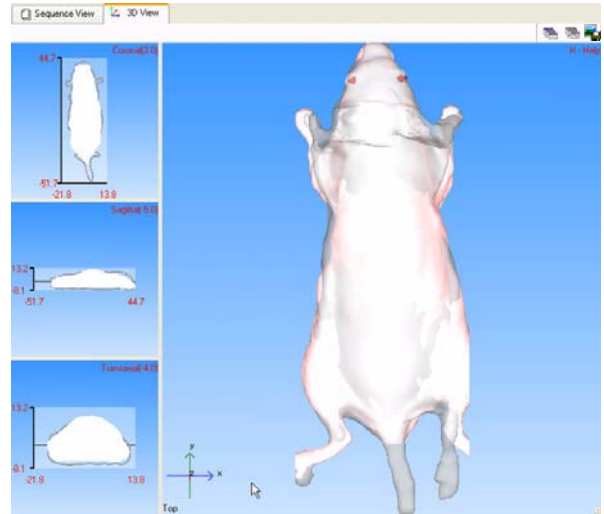
3. Press the **V** key or the 🔄 button to display alternative views of the surface.
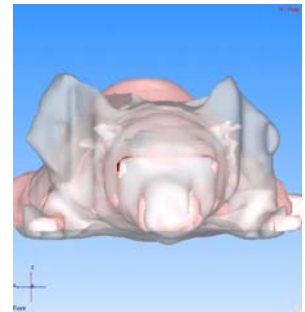
Top	Bottom	Front
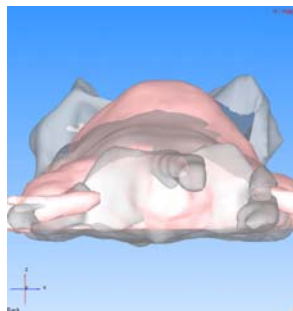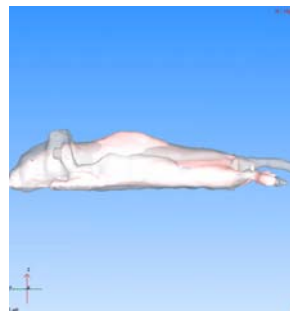
Back	Left	Right

Importing an Organ Atlas

You can import an organ atlas (Open Inventor format, one organ per .iv) derived from MRI or CT scans.

> **NOTE**
>
> The imported atlas must include a skin file named skin.iv.

To import an organ atlas:

1. Open the DLIT results that are associated with the organ atlas.

2. Select **File →Import →Organ Atlas** on the menu bar.
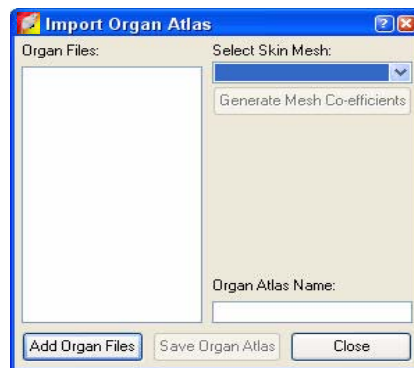
3. In the Import Organ Atlas box that appears, click **Add Organ Files**.

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

5. In the Select Skin Mesh drop-down list, select the skin organ file, which must have the file name 'skin.iv'.

6. Click **Generate Mesh Coefficients**.

7. Enter a name for the atlas and click **Save Organ Atlas**.

   - The organ atlas (.atlas) is created. The atlas name appears in the Organ Atlas drop-down list (in the 3D tools, Organs tab).

12.8 3D Tools - Animation Tab

The Living Image software can produce an animation from a sequence of 3D views or *key frames*. For example, an animation can depict:

- Magnifying (zooming in on) the 3D view.
- Spinning the 3D view on an axis.
- The surface or organs fading out (decreasing opacity) or fading in (increasing opacity).

The animation can be saved to a movie (.mov, .mp4, or .avi).

In the Animation Tab, You Can:	See Page
View a preset animation (generated from a predefined animation setup)	184
Create a custom animation (generated from your custom animation setup)	185
Edit & save an animation setup	186

Key frame box shows the key frames in the current animation setup

Click a key frame to display the associated 3D view and time stamp (position in the time scale (0-100) at which the frame occurs in the animation).
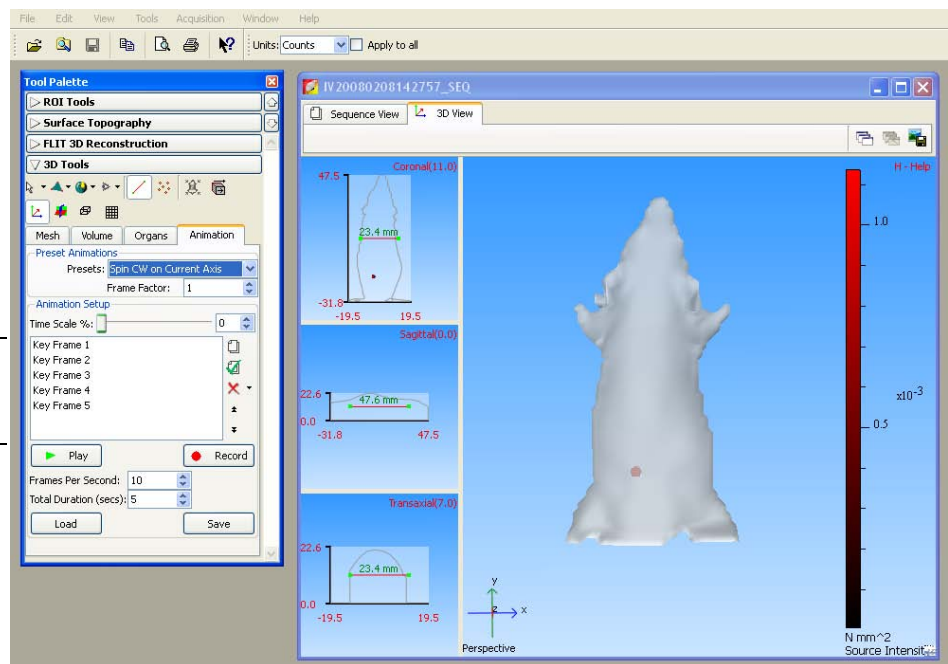
Figure 12.11 Animation tab

Table 12.9 3D tools, Animation tab

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.
	Click to create a new key frame from the current 3D view.
	Click to update the selected key frame to the current 3D view.
	Click to delete a selected or all key frames from the key frame box.
	Click to move a selected key frame up in the key frame box.
	Click to move 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).
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 (.xml).

Viewing a Preset Animation

To view a preset animation:

1. Open the DLIT/FLIT results.

2. Confirm that the 3D view shows the properties of interest (for example, organs, voxels, surface, or photon density maps).
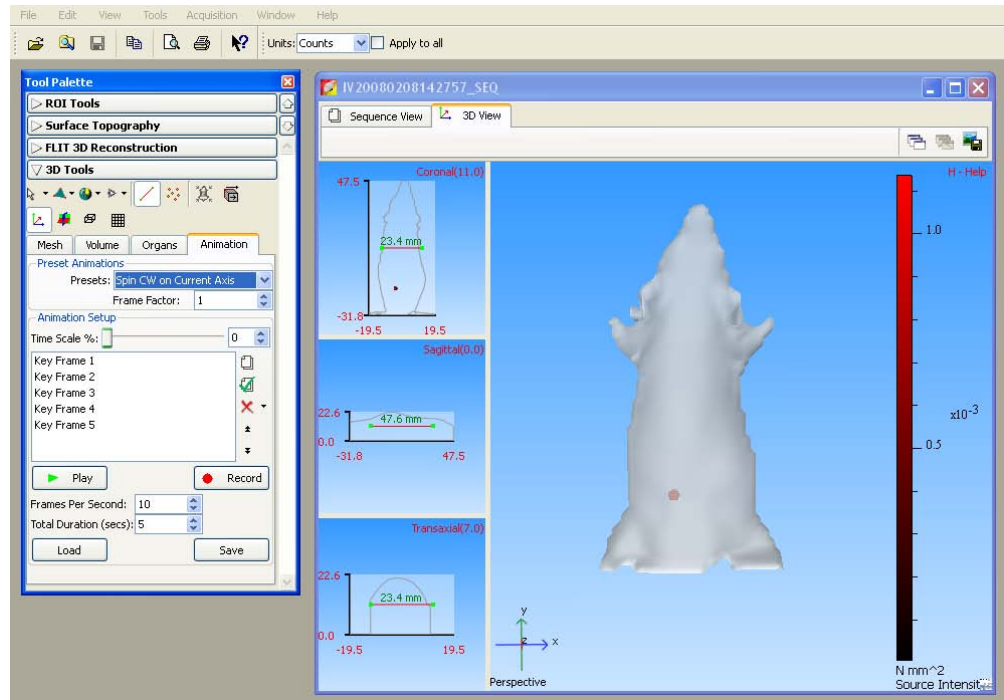
3. In the 3D Tools, click the Animation tab.

4. If necessary, clear the key frame box (click the ✖ button and select **Delete All**).

5. To view a preset animation, make a selection from the Presets drop-down list. (See Table 12.10 for a description of the preset animations.)

   - A list of the key frames appears.

6. To view the animation, click **Play**.

> **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.

To save 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**.

Table 12.10 Preset animations

Name	Choose This Animation Setup to...
Spin CW	Rotate the 3D reconstruction clockwise.
Spin CCW	Rotate the 3D reconstruction counterclockwise.
Zoom In	Magnify the 3D reconstruction.
Fade In	Increase opacity from 0-100%.
Fade Out	Decrease opacity from 100-0%.

Creating a Custom Animation

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

1. Open the 3D results of interest.

2. Confirm that the 3D view shows the properties of interest (for example, position or scale of the 3D reconstruction, organs, voxels, surface, or photon density maps).
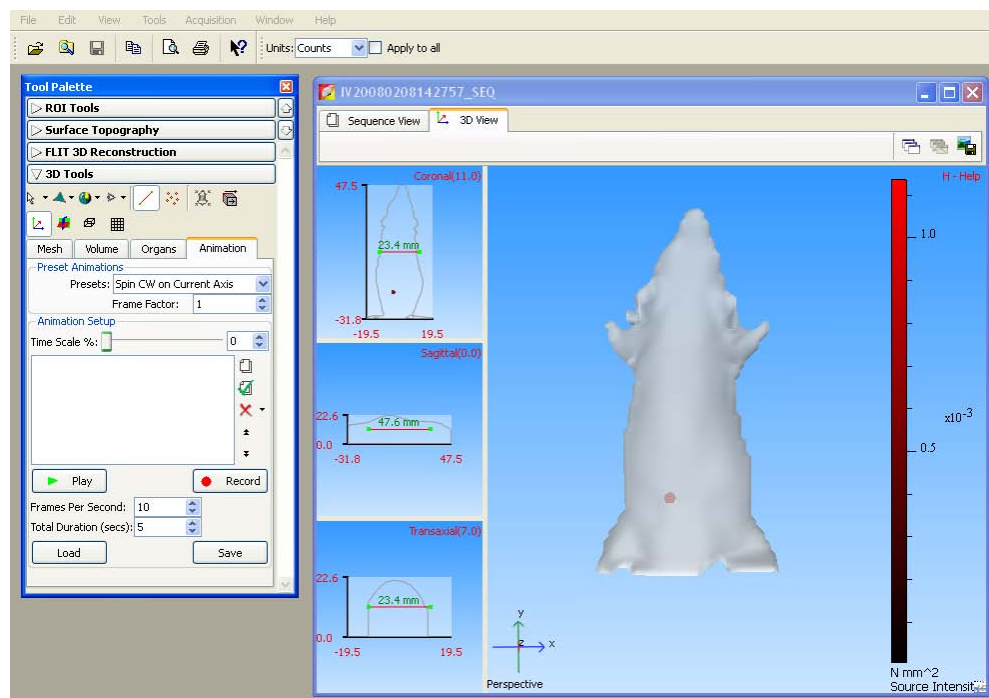
3. In the 3D tools, click the Animation tab.

4. If necessary, clear the key frame box (click the ✖ button and select **Delete All**.)

5. To capture the first key frame, click the ▯ button.
   - The first key frame is added to the key frame box.

6. To capture the next key frame, adjust the 3D view to show the properties of interest and click the ▯ button.
   - The second key frame is added to the key frame box.

7. Repeat Step 6 until all key frames of interest are captured. For details on how to edit

the key frame sequence, see page 186.

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).

8. 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.

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

To save the animation to a movie:

1. Click **Record**.

2. In the dialog box that appears, choose a directory and enter a file name (.mov, mp4, .avi), and click **Save**.

To save the animation setup:

1. Click **Save**.

2. In the dialog box that appears, select a directory and enter a file name (.xml).

Editing an Animation Setup

Open an animation setup:

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

   or

   To select a saved user-defined setup:

   a. Click **Load**.

   b. In the dialog box that appears, select the directory and animation setup (.xml) of interest.

To edit the key frame sequence:

1. Add a key frame:

   a. Adjust the 3D view to show the properties of interest.

   b. Click the ⬚ button.

2. To reorder a key frame in the sequence, select the key frame and click the ⬘ or ⬙ arrow.

3. Update a key frame:

   a. Select the key frame of interest.

   b. Adjust the 3D view.

   c. Click the ✅ button.
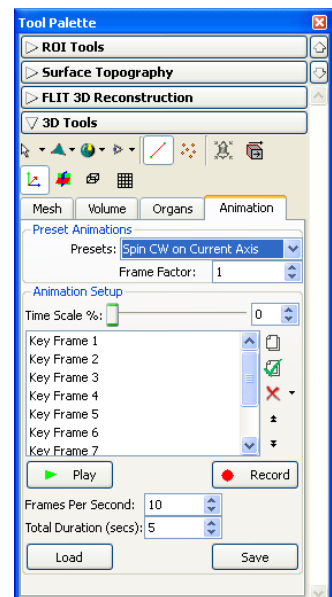
4. Delete a key frame:

   a. Select the key frame that you want to remove.

   b. Click the ✖ button and select **Delete Current**.

To save the animation setup:

1. Click **Save**.

2. In the dialog box that appears, select a directory and enter a file name (.xml).

12.9 Managing DLIT/FLIT Results

Default name for the results
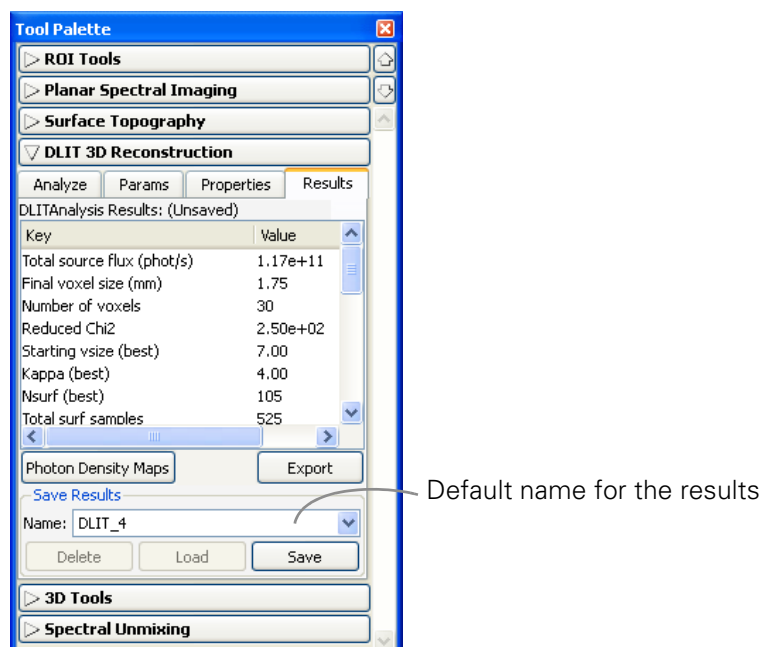
Figure 12.12 3D analysis results

To save results:

1. In the Results tab of the DLIT/FLIT 3D reconstruction tools, confirm the default file 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 results:

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

2. Click **Load**.

To copy user-specified results:

1. In the Results tab, select the results of interest.

2. Right-click the results table and select **Copy Selected** from the shortcut menu that

appears.

To copy all results:

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

    - All of the results table is 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**.

13 Troubleshooting Guide

13.1 DLIT/FLIT Analysis Troubleshooting

Table 13.1 DLIT/FLIT analysis

Issue	Solution
Large voxels (> 2 mm)	• The N_{data}>N_{voxels} is required for an overdetermined problem. It is possible that there are not enough data (N Surface). This can occur because it is desired that all wavelengths (DLIT) and all trans-illumination source positions (FLIT) are represented in the data equally. In the DLIT case, if the signal in a short wavelength is very low, then the sampled data can be too few for sufficient refinement of the voxel gridding.
	– To increase the data to be included, determine the [photons/sec/cm2/sec] level for ~30 counts on the low signal image (this is the level of noise) using Image Adjust in the Tool Palette. Enter in the [photons/sec/cm2/sec] value into the 'Minimum Radiance' column next to the wavelength/image number in the 'Analyze Tab' in the DLIT/FLIT 3D Reconstruction menu of the Tool Palette.
	• It is best to include the low signal data as it adds constraining information to the solution.
	Note: Using the Imaging Wizard should reduce the occurrence of low signal images in the data sequences.
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.
	• In FLIT, if you choose an emission Source Spectrum in the Properties Tab (an Advanced User feature) which is incorrect for the fluorophore of interest, this can also result in no sources.
	• In FLIT, if there is very little signal detected at the surface and the 'Background' box is checked in the Params tab, then it is possible that only the background fluorescence is simulated.
Surfaces are spiky	• The most common source of spiky surfaces are folds in the animal skin or animal fur, which corrupt the desired smooth lines projected on the animal from the laser galvanometer.
	– Adjusting the 'Path Averaging Size' in the 'Surface Topography' menu in the Tool Palette can help reconstruct an improved surface
	– Smoothing the surface by using the 'Smooth' feature in the tool palette Surface Topography menu can also help improve the surface.

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Appendix A   IVIS Acquisition Control Panel

The control panel provides the image acquisition functions (Figure A.1).
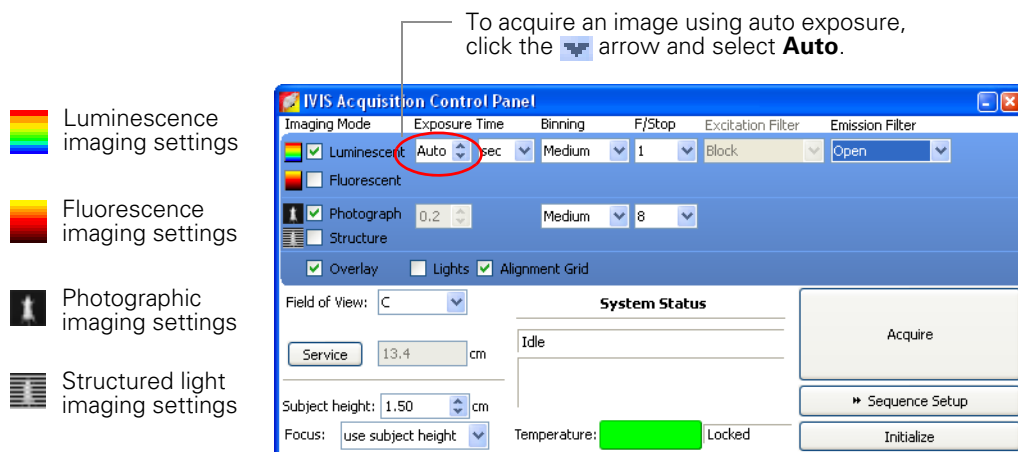
Figure A.1 IVIS acquisition control panel, auto exposure selected

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.
Exposure time	The length of time that the shutter is open during acquisition of a photographic or luminescent 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 206.
	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.

Table A.1 IVIS acquisition control panel (continued)

Item	Description
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 205.
Excitation Filter	A drop-down list of fluorescence excitation filters. For fluorescent imaging, choose the appropriate filter for your application. For bioluminescent 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:** The excitation filter selection automatically sets the emission filter position.
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 bioluminescent imaging, the **Open** position (no filter) is automatically selected by default.
Photograph	Choose this option to automatically acquire a photographic image. 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 Gamma controls (see *Image Layout Window*, page 62).
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 acquires a photographic image followed by a luminescent image, then coregisters the two images.
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 194.
Service	Click to move the stage to a position for cleaning the imaging chamber below the stage. Only available on the IVIS 200 and Spectrum imaging systems.
Load	Click to move the stage from the cleaning position back to the home position.

Table A.1 IVIS acquisition control panel (continued)

Item	Description
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.
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 34.
Temperature	The temperature box color indicates the temperature and status of the system: ☐ System not initialized. 🟥 System initialized, but the CCD temperature is out of range. 🟩 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 11.
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 Editor so that you can access the imaging wizard, specify and manage sequence acquisition parameters, or open sequence acquisition parameters (xsq). For more details on setting up an image sequence, see page 22.
Image Setup	Click to close the Sequence Editor.
Initialize	Click to initialize the IVIS Imaging System. For more details on initializing the system, see page 11.

Table A.2 Additional IVIS® System 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	100 Series	200 Series	Kinetic
	FOV (cm)			
A	4	10	3.9	4
B	7	15	6.5	7
C	10	20	13	10
D	12	25	19.5	12
E			26	

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.

1. After you log on, to view the user-modifiable preferences, select **Edit →Preferences** on the menu bar.

    - The Preferences box appears.
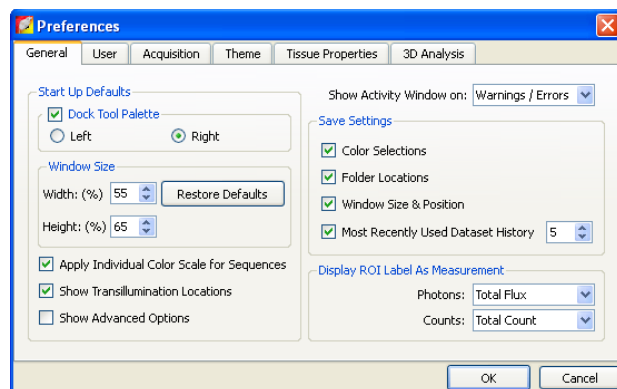
NOTE

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
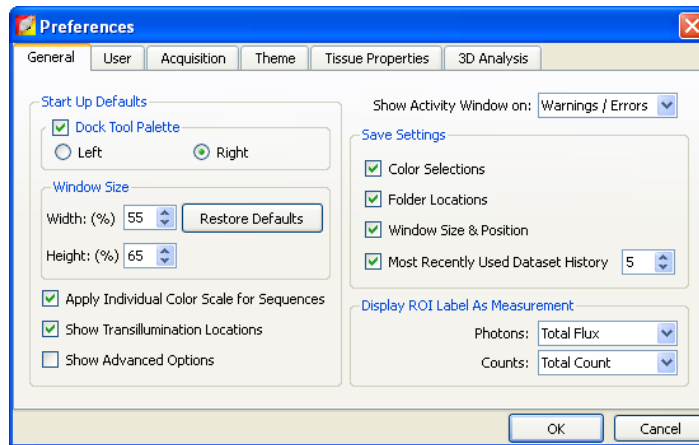
Figure B.1 General preferences

Table B1 General preferences

Item	Description
Start Up Defaults	
Dock Tool Palette	Sets 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 chosen, the tool palette includes the point source fitting tools and the image window includes the Colorized View tab.
Show Activity Window on:	A drop-down list of options for when to display the activity log (Figure B.2).
Save Settings	
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 ![export icon] in the image window to view the current folder path setting (Figure B.2).

Table B1 General preferences (continued)

Item	Description
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	
Photons	Select the type of measurement (in photons) to show in the ROI label.
Counts:	Select the type of measurement (in counts) to show in the ROI label.

Some of the general preferences specify how the main application window is organized.

Tool palette

Activity window (hidden by default)
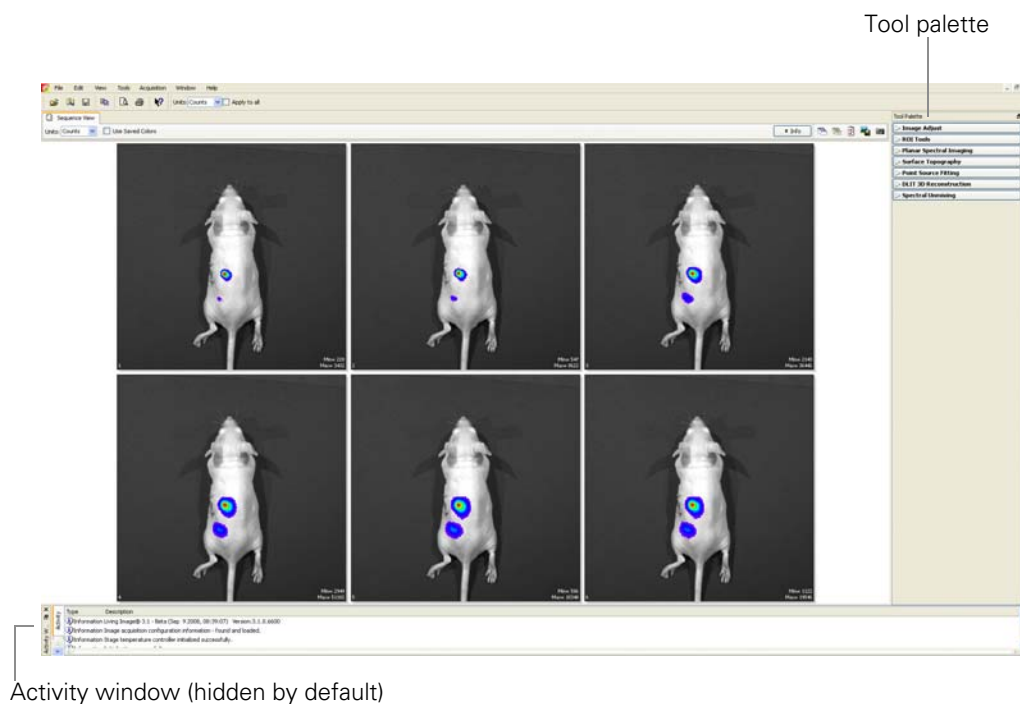
Figure B.2 Main application window
To undock the tool palette, click on the palette title bar and drag it a distance greater than its width. To dock the tool palette in the main window, drag the palette to the right or left side of the window and release.

B.2 User Preferences
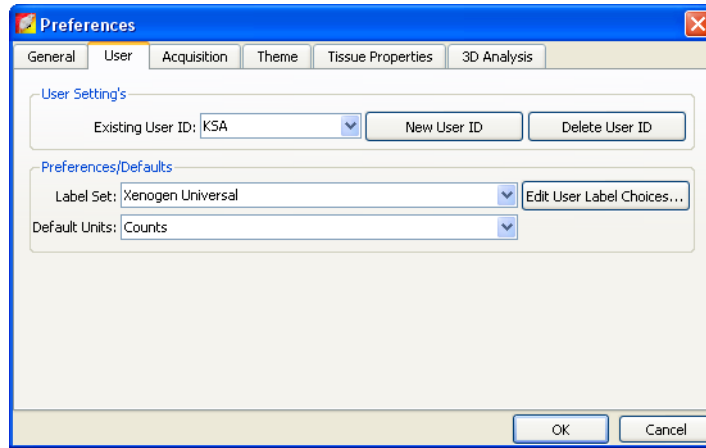
Figure B.3 User preferences

Table B2 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	
Label Name	Drop-down list of factory installed label name sets.
Edit User label Choices	Opens a dialog box that enables you to edit a label set.
Default Units	Specifies the units (photons or counts) for image display.

B.3 Acquisition
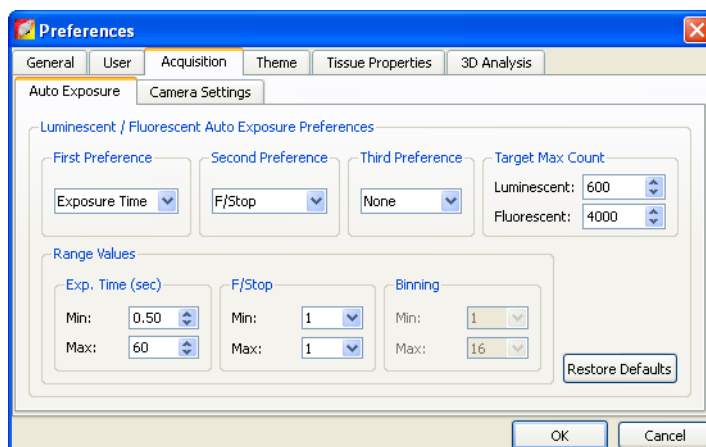
Figure B.4 Acquisition preferences, Auto Exposure

Table B3 Auto exposure settings

Item	Description
Luminescent/Fluorescent Auto Exposure Preferences	
First Preference Second Preference Third Preference	During auto exposure, the software acquires a bioluminescence or fluorescence image so that the brightest pixel is approximately equal to the user-specified target max count.
	If the target max 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 Max Count	A user-specified intensity.
Range Values Exp Time (sec) F/Stop Binning	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.

B.4 Theme

Click to apply the factory set defaults.

Click so select a different color or define a custom color.

Figure B.5 Theme preferences

Table B4 Theme preferences

Item	Description
3D View Background Color	
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 (Figure B.6). Top = the color at the top of the window; Bottom = the color at the bottom of the window.
Preferred Color Palette	
Luminescent	The color scale that is used to display luminescent images. Click **Reverse** to reverse the color scale.
Fluorescent	The color scale that is used to display fluorescent images. Click **Reverse** to reverse the color scale.
Use saved colors while loading data	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.
Preferred ROI Line Color	
Luminescent	Color of the ROI outline on a luminescent image.
Fluorescent	Color of the ROI outline on a fluorescent image.
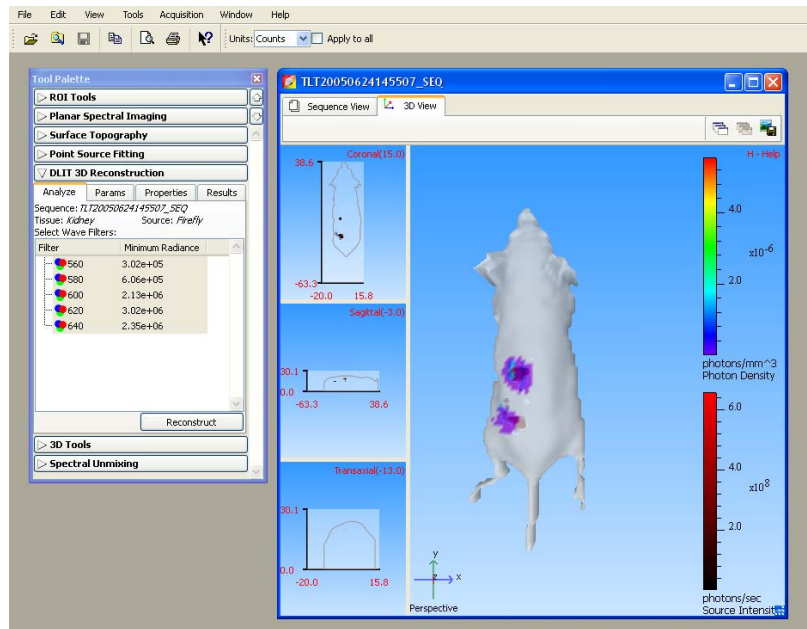
Figure B.6 Image window, 3D view with gradient background

B.5 Tissue Properties
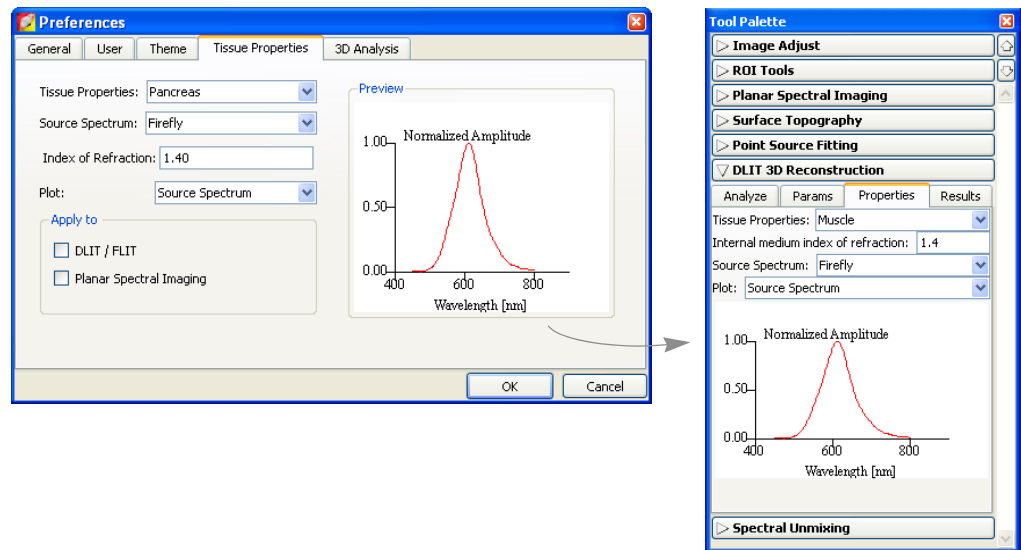
Figure B.7 Set tissue properties preferences (left) for the Properties tab in the Planar Spectral Imaging, DLIT, or FLIT tools.

Table B5 Tissue properties preferences

Item	Description
Tissue Properties	Choose a default tissue type that is most representative of the area of interest.
Source Spectrum	Choose the default luminescent source.

Table B5 Tissue properties preferences (continued)

Item	Description
Index of Refraction	The software automatically sets the internal medium index of refraction based on the selection in the Tissue Properties list
Plot	
Tissue Properties	Choose this option to display a graph of the absorption coefficient (μ_a), effective attenuation coefficient (μ_{eff}), and reduced scattering coefficient (μ'_s or μsp).
Source Spectrum	Choose this option to display the source spectrum.
Apply to	
DLIT/FLIT	Choose this option if the settings are for the Properties tab in the FLIT or DLIT tools.
Planar Spectral Imaging	Choose this option if the settings are for Properties tab in the Planar Spectral Imaging tools.

B.6 3D Analysis
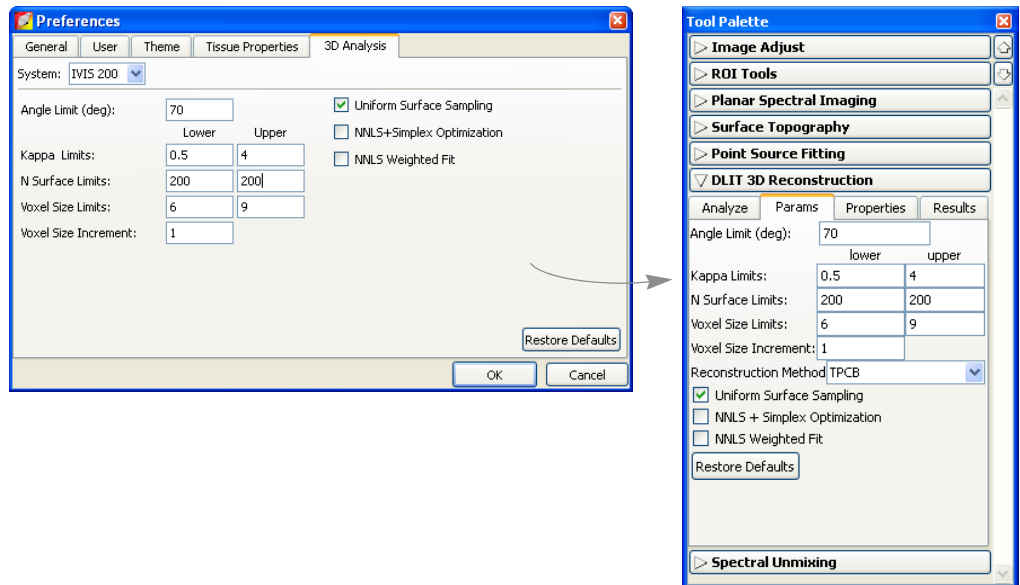
Figure B.8 Set 3D analysis preferences (left) for the DLIT reconstruction tools (right)

Table B6 3D analysis preferences

Item	Description
System	Select the IVIS Imaging System from the drop-down list.
Angle Limit (deg)	The angle between the object surface normal and the optical axis. For more details, see page 253.
Kappa Limits	Kappa (κ) is a parameter that is searched during a reconstruction to determine the best fit to the image data. Small values of kappa tend to favor deeper sources, while large values favor more shallow sources. For more details, see page 254.

Table B6 3D analysis preferences (continued)

Item	Description
N Surface Limits	The maximum number of surface intensity points to use in the reconstruction at a given wavelength. The range is 200 to 800 and the default is 200. The time required for reconstruction is shortest for smaller values of N (for example, 200). However, a large N value may give a more accurate result because more data are included in the fit.
Voxel Size Limits	Voxels are the small cubes of space inside a subject, each of which contains a light source (much like a pixel in a 2D image). The DLIT reconstruction begins with large voxels, specified by the voxel size limit (the length of a side of the voxel cube in mm). At each iteration, the algorithm reduces the size of the voxel by a factor of two until the optimum solution is found.
	The voxel size limits are a minimum of five and a maximum of 10. The default range is set to 6-9 mm. A larger range of voxel limits ensures a more reliable solution, but requires more computational time. The default range of 6-9 is usually adequate to determine the optimum solution.
Voxel Size Increment	This is the step increment in voxel size, stepping from the minimum voxel size limit to the maximum voxel size limit. For example, if the voxel size limit ranges from 6-9 mm, a voxel size increment = 1 gives four starting voxel sizes (6, 7, 8, and 9 mm).
	The default increment of 1 mm is usually adequate, however smaller increments can be used if you want to sample finer voxel sizes. Smaller increments will significantly increase the time required for reconstruction.
Uniform Surface Sampling	If this option is chosen, the surface data for each wavelength will be sampled spatially uniformly on the signal area. If this option is not chosen, the maximum 'N surface elements' will be sampled for the data. This means that the N brightest surface elements will be used as data in the reconstruction. Typically, non-uniform sampling is recommended if there is a single bright source, while uniform sampling is preferred if there are several scattered sources.
NNLS + Simplex Optimization	If this option is not chosen, the software uses a linear programming algorithm to seek the solution (Simplex solution). If this option is chosen, the software also applies a non-negative least squares optimization algorithm at each iteration to provide a better solution for source power. The Simplex solution is more robust, but tends to underestimate the source flux in each voxel. Therefore, the NNLS + Simplex option is recommended.
NNLS Weighted Fit	Choose this option to weight the wavelength data proportionally to its intensity in the NNLS reconstruction. This option is especially useful if the intensity of longer wavelength data is orders of magnitude greater than the intensity of shorter wavelength data.

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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.
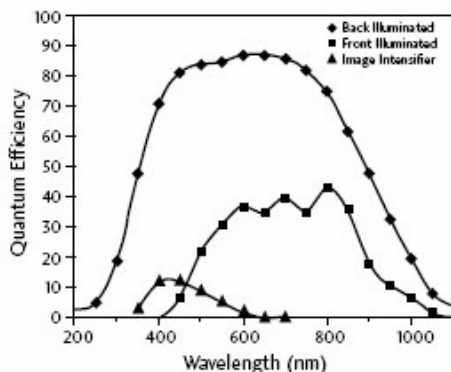
Figure C.1 Quantum efficiencies
Bialkali photocathode (▲), back-illuminated CCD (◆), and front-illuminated CCD (■). IVIS systems use back-illuminated CCDs.

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 34. Generally f/1 is recommended for low light luminescent images and f/2 or f/4 is recommended for brighter luminescent or fluorescent images.
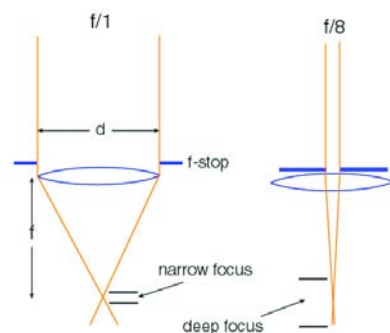
Figure C.2 Lens f/stop positions.
Left: lens wide open at f/1; right: lens closed down at f/8.

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 217.) 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 211.) The resulting image data comprise a two-dimensional array of numbers; each pixel contains the counts associated with the amount of light detected.

The 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).

Binning = 1	Binning = 2	Binning = 4

CCD pixel

Signal 4 times larger. Spatial size doubled.

Signal 16 times larger. Spatial size quadrupled.
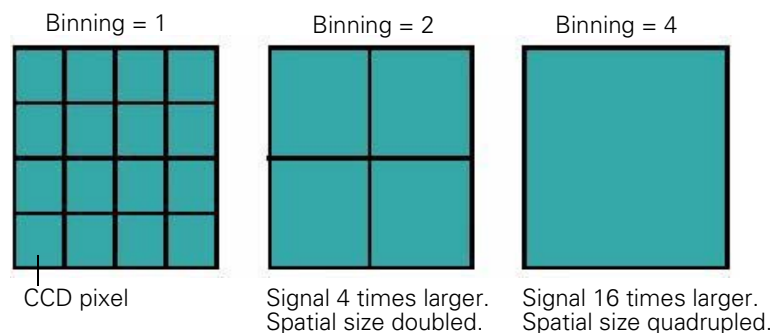
Figure C.3 A small segment of the CCD.
At binning = 2, 4 pixels are summed together; at binning = 4, 16 pixels are summed.

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 240.) 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 Xenogen 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	Camera			
	EEV	**ROPER**	**SITe**	**Andor**
Medium Lumin	Bin 8	Bin 5	Bin 4	Bin 4
Small (high-resolution) Lumin	Bin 4	Bin 2	Bin 2	Bin 2
Large (high-sensitivity) Lumin	Bin 16	Bin 10	Bin 8	Bin 8
Medium Photo	Bin 4	Bin 2	Bin 2	Bin 2
Small (high-resolution) Photo	Bin 2	Bin 1	Bin 1	Bin 1

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.

Center pixel value = the mean value of the nine pixels in the 3x3 neighborhood
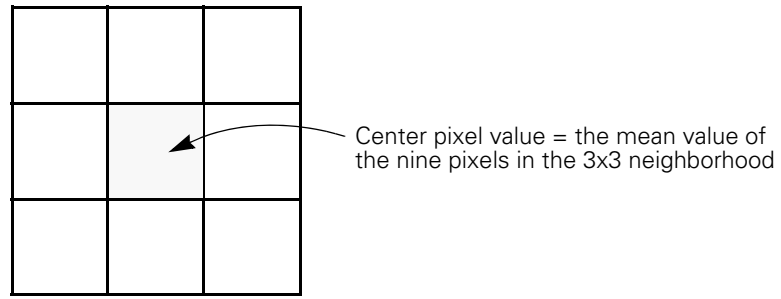
Figure C.4 3x3 pixel neighborhood

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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).
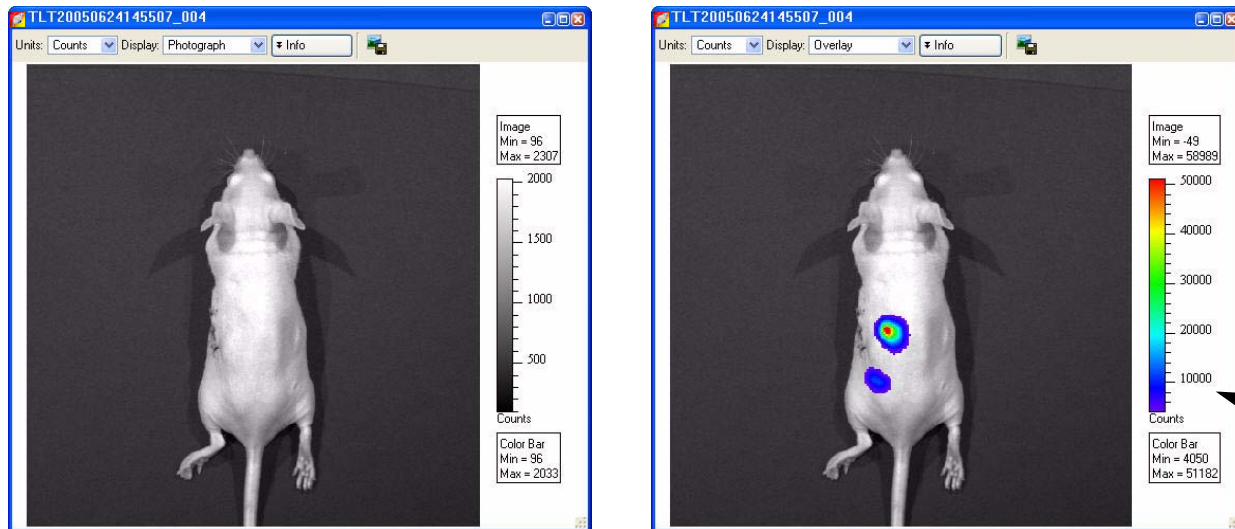
A photographic image is a gray scale pseudoimage.

In Overlay display mode, a pseudocolor image of the luminescent data is overlaid on a photographic image.

Color table associated with the data.

Figure D.1 Example pseudoimages

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, *Measuring ROIs in an Image*, page 84.)

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 on 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.

D.2 Quantifying Image Data

The Living Image software can quantify and display scientific image data using three types of measurements (Figure D.1):

- Counts
- Photons
- Efficiency (for fluorescent images only)

Data Display	Choose This to Display:	Recommended For:
Counts	An uncalibrated measurement of the photons incident on the CCD camera.	Image acquisition to ensure that the camera settings are property adjusted.
Photons	A calibrated measurement of the photon emission from the subject.	ROI measurements on bioluminescent images.
Efficiency	A fluorescence emission image normalized to the incident excitation intensity (radiance of the subject/ illumination intensity).	ROI measurements on fluorescent images.

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).

Select Counts, Photons, or Efficiency for the image data

In counts mode, the ROI measurements include:
Total Counts = Sum of all counts for all pixels inside the ROI

Average Counts = Total Counts/Number of pixels or superpixels

Quantity ROI Pixels = Number of binned pixels inside the ROI

Area (CCD pixels) = Number of unbinned CCD pixels inside the ROI

Figure D.2 Image window and ROI Measurements table (counts mode)

Photons

When image data is displayed in photons, the *photon emission from the subject* or *radiance* is displayed in photons/sec/cm²/sr. Counts are a relative measure of the photons incident on the CCD camera and photons are 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).
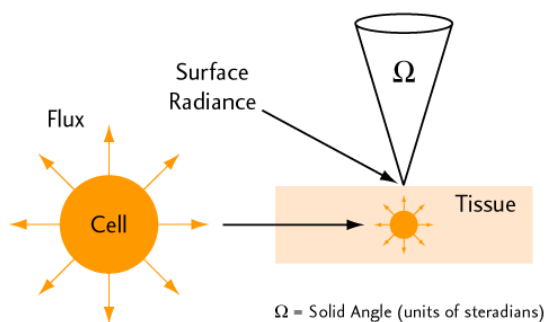
Figure D.3 Isotropic radiation
Isotropic radiation from a cell is called photon flux (photons/sec). When cells occur in tissue, photon emission from the tissue surface is called surface radiance (photons/sec/cm²/sr).

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 photons 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 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 photons 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.

Xenogen Corporation calibrates the camera settings of each IVIS Imaging System at 600 nm. 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). At FOV=10, there is a slightly dished illumination profile due to the close proximity of the stage to the illumination reflectors, while the profiles for the other stage locations are peaked near their center. The illumination intensity profile varies by up to ±30% across the entire FOV (Figure D.4).
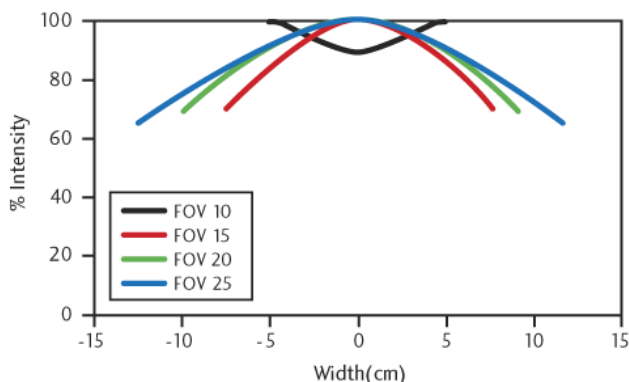
Figure D.4 Illumination profiles at different FOVs
Measurements were taken at the center of the FOV on the IVIS Imaging System 100 Series.

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, Xenogen Corporation 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® 3.0 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 System 100 Series. (The IVIS Imaging System 200 Series has a larger lens with a smaller flat field correction.)

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® 3.0 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 bioluminescent 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 229.

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 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 Xenogen 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 Xenogen approved cleaning agents. Many cleaning compounds phosphoresce! Contact Xenogen 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 Xenogen 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.
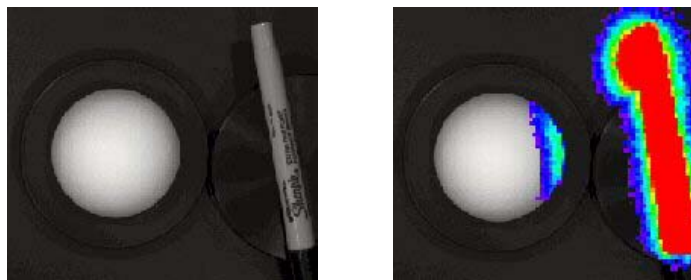
Figure E.1 Xenogen High Reflectance Hemisphere and a plastic marker pen
Left: Photographic image. Right: Photograph with luminescent overlay. The hemisphere is illuminated by phosphorescence emitted from the pen.

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 Xenogen High Reflectance Hemisphere by its black base plate while wearing cotton gloves provided by Xenogen. 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 Xenogen 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* bioluminescent 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.

Xenogen Corporation 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/cm²/sr and 1000 photons/s/cm²/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 6, page 91.)

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.

Photograph   Luminescent overlay   Photograph   Luminescent overlay
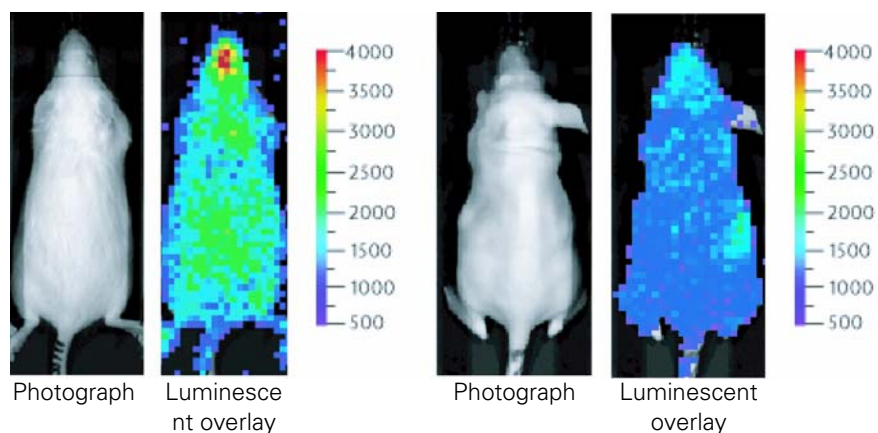
Figure E.2 Background light emission
Background light emission from a female white furred (Swiss Webster) (left) and a female nude (Nu/nu) mouse (right).

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 Xenogen technical support.

Appendix F   Fluorescent Imaging

F.1 Description and Theory of Operation

System Components The IVIS® Imaging System 200 Series and IVIS Lumina offer built-in fluorescence imaging capability as standard equipment. 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 bioluminescent and fluorescent imaging applications (Figure F.1). For more details, see the *IVIS Imaging System 200 Series System Manual,* the *IVIS Lumina System Manual,* or the *XFO-6 or XFO-12 Fluorescence Option Manual*.

IVIS Imaging System 200 Series

IVIS Lumina,
IVIS Imaging System 50 or 100 Series
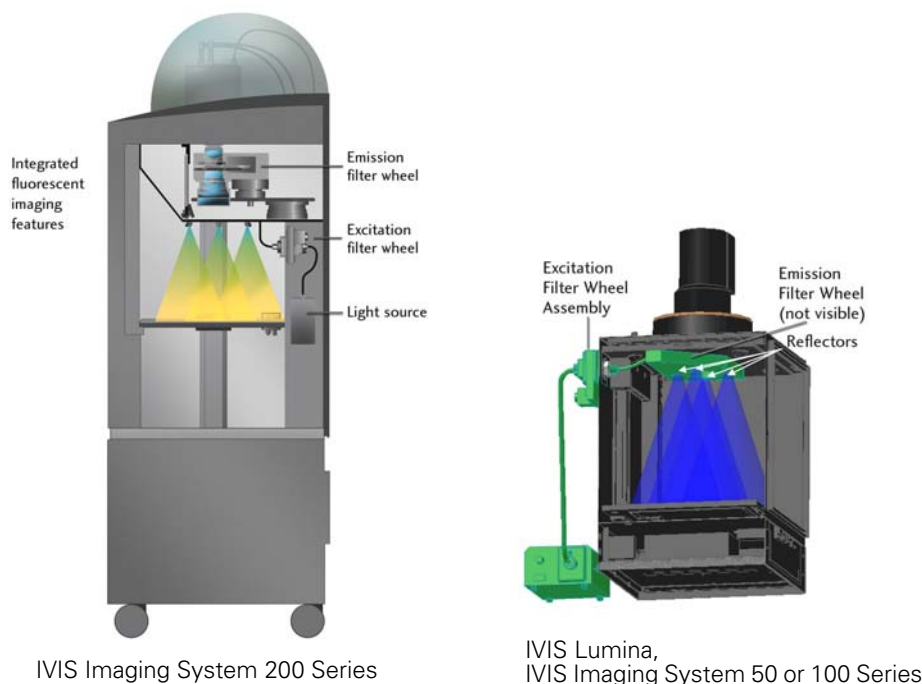
Figure F.1 Fluorescent imaging hardware

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.2). 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.
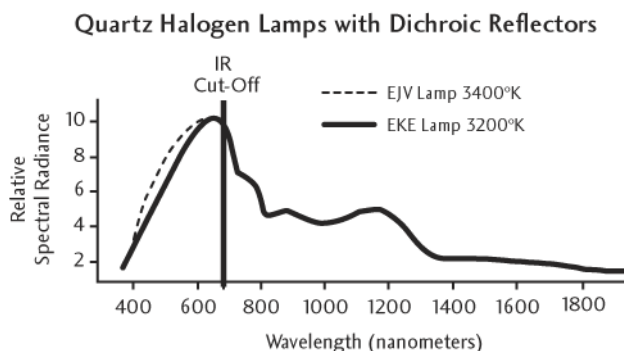
Figure F.2 Relative spectral radiance output for the quartz halogen lamp with dichroic reflector.

The lamp output is delivered to the excitation filter wheel assembly located at the back of the IVIS® Imaging System (Figure F.3). 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 bioluminescent imaging to prevent external light from entering the imaging chamber. The Living Image software manages the motor control of the excitation filter wheel.
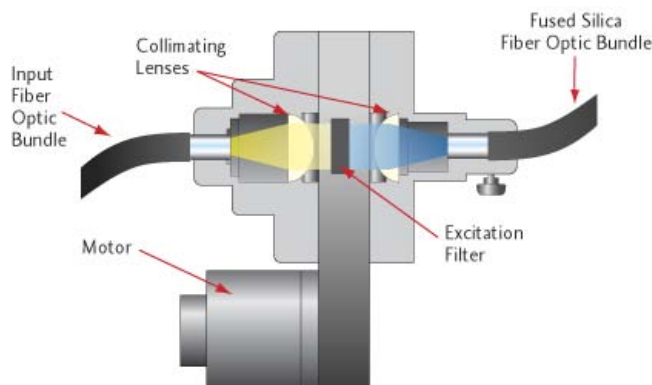
Figure F.3 Excitation filter wheel cross section.

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 215.)

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 bioluminescent imaging.

IVIS Imaging System	Number of Emission Filter Wheel Positions	Number of Available Fluorescence Filters
200 Series	24 (two levels, each with 12 positions)	22 (60 mm diameter)
Lumina	8	7
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.4 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.5 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.
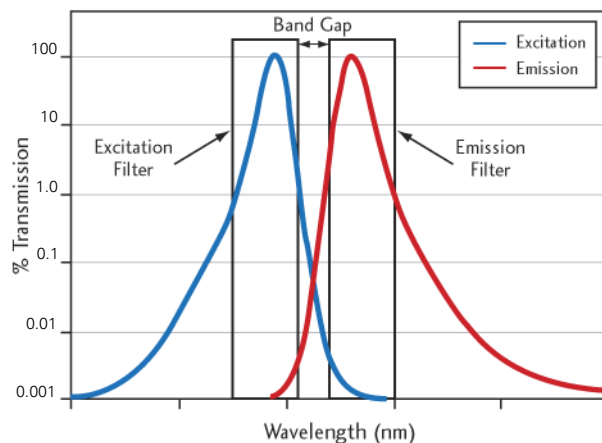
Figure F.4 Typical excitation and emission spectra for a fluorescent compound.
The graph shows two idealized bandpass filters that are appropriate for this fluorescent compound.
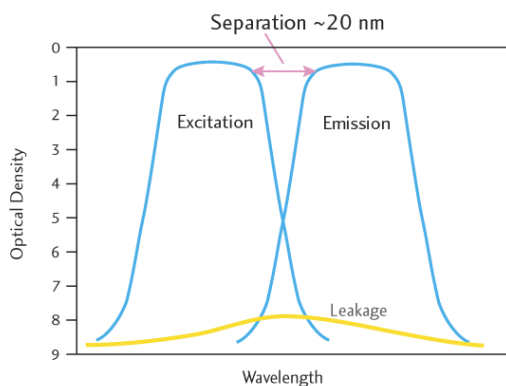
Figure F.5 Typical attenuation curves for excitation and emission filters.

In Figure F.5, 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.5). 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

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	615-665	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
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.6).

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.
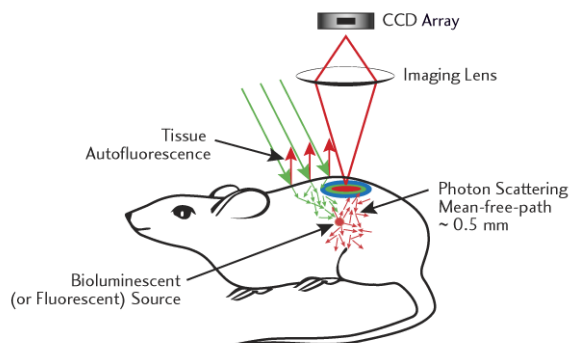
Figure F.6 Illustration of the *in vivo* fluorescence process.

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 bioluminescent 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 205*.

F.4 Image Data Display

Fluorescent image data can be displayed in units of counts or photons (absolute, calibrated), or in terms of efficiency (calibrated, normalized). For more details, see *Quantifying Image Data*, page 213.

If the image is displayed in photons, 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 215.

Fluorescent 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.7). To eliminate the excitation light as a variable from the measurement, the data can be displayed in terms of *efficiency* (Figure F.8).
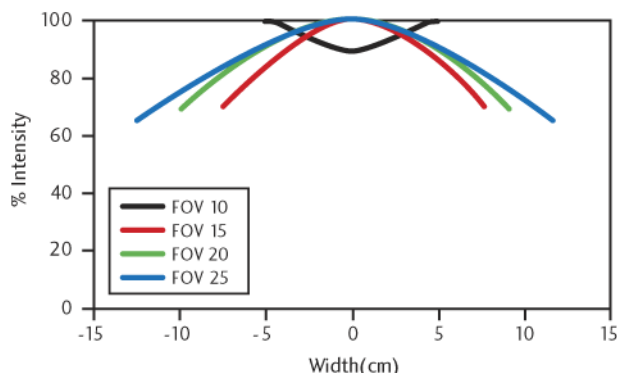
Figure F.7 Illumination profiles for different FOVs on an IVIS Imaging System 100 Series measured from the center of the FOV.

To enable a more quantitative comparison of fluorescent signals, choose Efficiency.

Figure F.8 Fluorescent image data displayed in terms of efficiency

When 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 every 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.9 shows autofluorescence originating from four different plastic microplates. The images were taken using a GFP filter set (excitation 445-490nm, emission 515-575nm).

White polystyrene   Clear polypropylene

Clear polystyrene   Black polystyrene
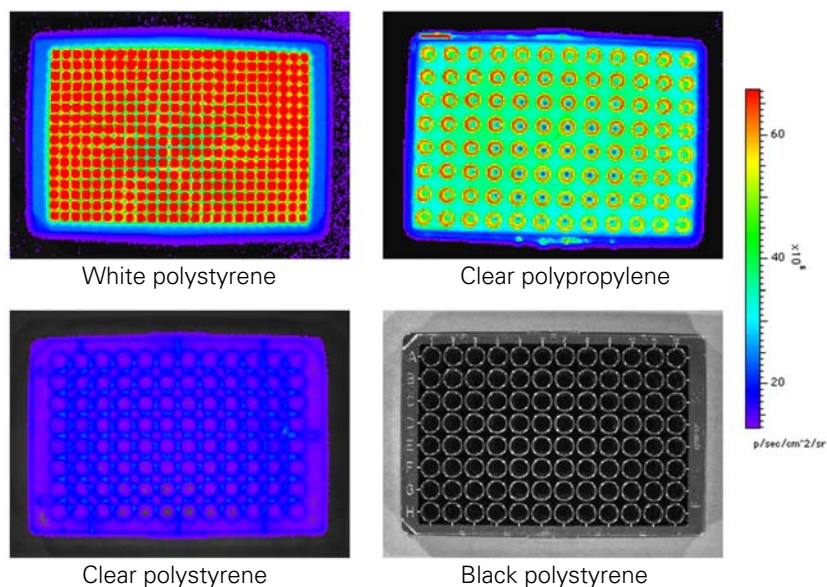
Figure F.9 Examples of microplate autofluorescence emission
The black polystyrene plate emits the smallest signal while the white polystyrene plate emits the largest signal. (Imaging parameters: GFP filter set, Fluorescence level Low, Binning=8, FOV=15, f/1, Exp=4sec.)

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.10). The specular reflection is an optical illumination autofluorescence signal reflecting from the microplate surface and is not dependent on the microplate material.

Figure F.10 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.)

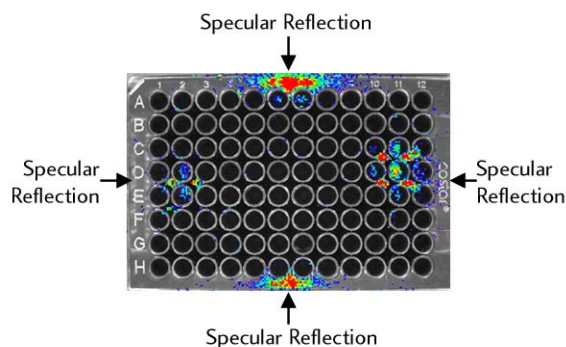Black polystyrene microplates are recommended for *in vitro* fluorescent measurements. Figure F.9 and Figure F.10 show that the black polystyrene microplate emits the smallest inherent fluorescent signal, while the white polystyrene microplate emits the largest signal. The clear polystyrene microplate 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 (Xenogen part no. 60104) on the imaging stage to prevent illumination reflections and to help keep the stage clean.

NOTE

The black paper recommended for bioluminescent imaging (Swathmore, Artagain, Black, 9"x12", Xenogen part no. 445-109) has a measurable autofluorescent signal, particularly with the Cy5.5 filter set.

Figure F.11 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.
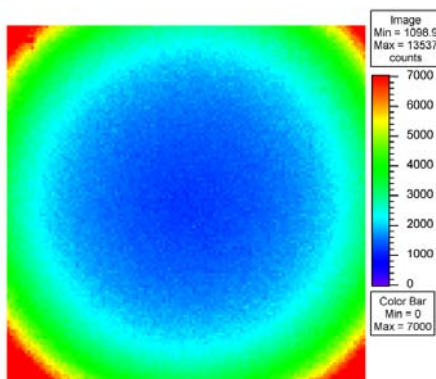
Figure F.11 Light from black Lexan
This image shows the typical ring-like structure of light from a sheet of black Lexan, a low autofluorescent material that may be placed on the imaging stage to prevent illumination reflections. (Imaging parameters: GFP filter set, Fluorescence level High, Binning=16, FOV=18.6, f/2, Exp=5sec.)

Other laboratory accessories may exhibit non-negligible autofluorescence. The chart in Figure F.12 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.12 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.13 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.
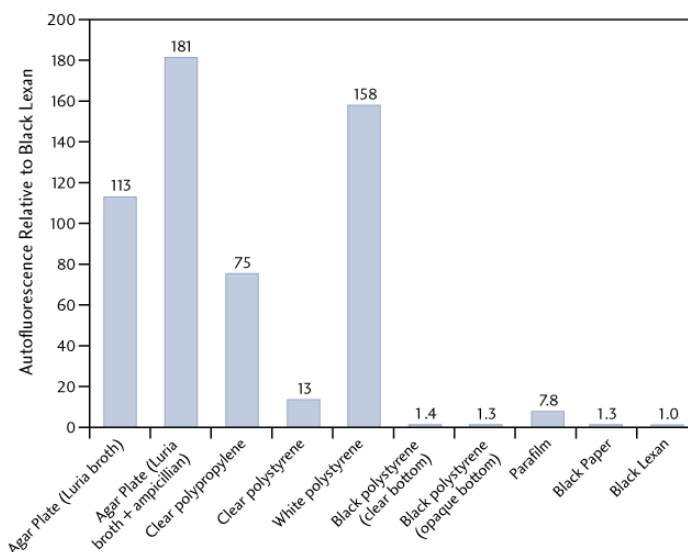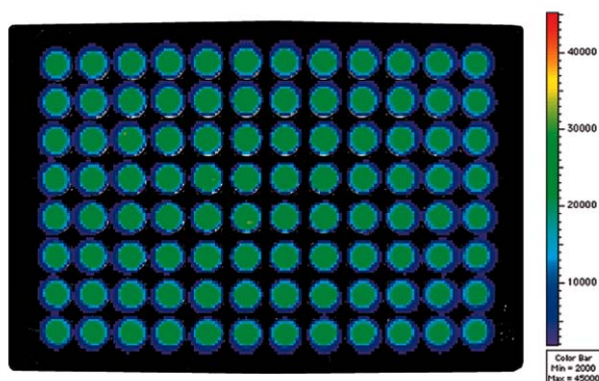

Figure F.13 96 well plate fluorescent reference standard (TR 613 Red)
The fluorescent signal is strong enough to exceed background emissions. (Imaging parameters: DsRed filter set, Fluorescence level Low, Binning=8, FOV=15, f/1, Exp=4sec.) Reference standard TR 613 Red is available through Precision 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 most limiting factor in *in vivo* fluorescent imaging. Figure F.14 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.
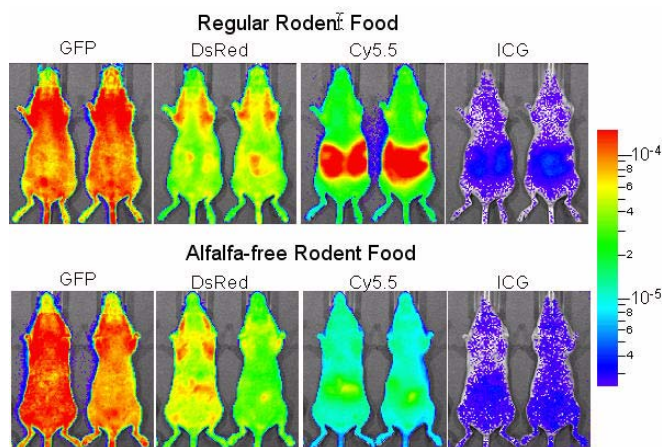
Figure F.14 Images of animal tissue autofluorescence in control mice (Nu/nu females)
Animals were fed regular rodent food (top) or alfalfa-free rodent food (bottom). Images were taken using the GFP, DsRed, Cy5.5, or ICG filter set. The data is plotted in efficiency on the same log scale.

Figure F.15 shows a comparison of fluorescence and bioluminescence 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 bioluminescent and fluorescent expression. The fluorescence signal level is 110 times brighter than the bioluminescence 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 bioluminescent imaging requires only 400 cells.
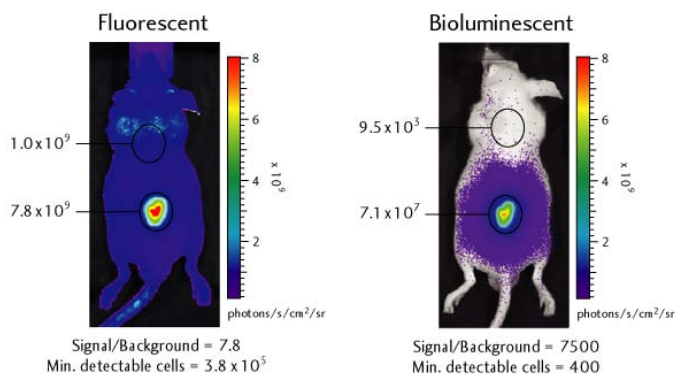
Figure F.15 Fluorescent (left) and bioluminescent (right) images of stably transfected, dual-tagged PC3M-luc DsRed cells.
The images show the signal from a subcutaneous injection of 3×10^6 cells in an 11-week old male Nu/nu mouse.

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 115.

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.16). 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 check box is checked.
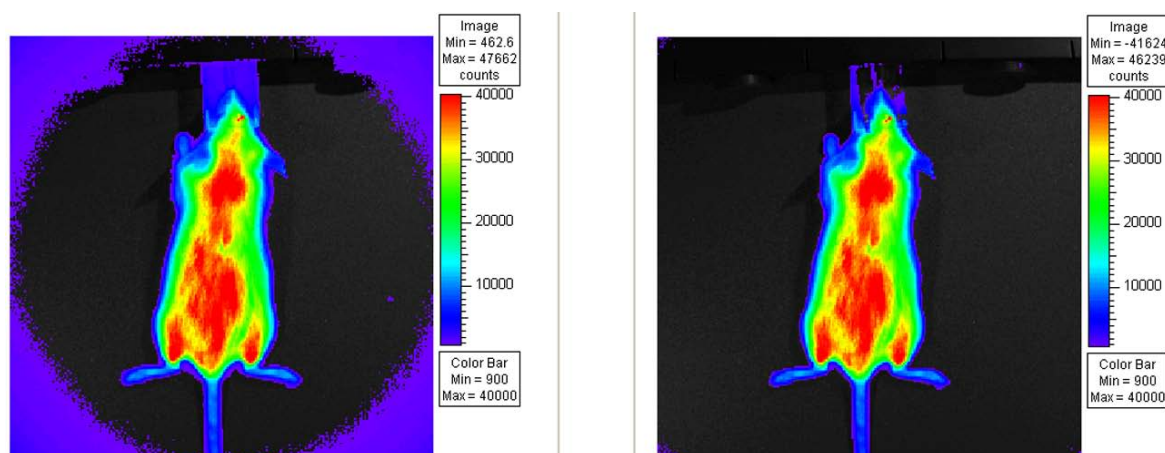
Figure F.16 Comparison of dark charge bias subtraction (left) and fluorescent background subtraction (right).
The autofluorescence from the nose cone and filter leakage have been minimized in the image on the right by using Sub Fluor Bkg option.

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.16).

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 (see Table 7.2, page 115). 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 7, page 115.) 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.17 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.18 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.17 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.
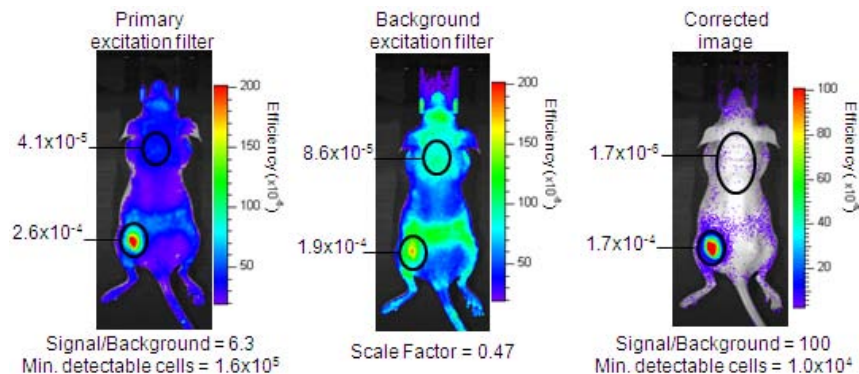

Figure F.17 Example of the autofluorescent subtraction technique using a background excitation filter.
a) primary excitation filter (DsRed), b) blue-shifted background excitation filter (DsRed Bkg), and c) corrected data. The corrected image was obtained by subtracting the scaled background filter image (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.
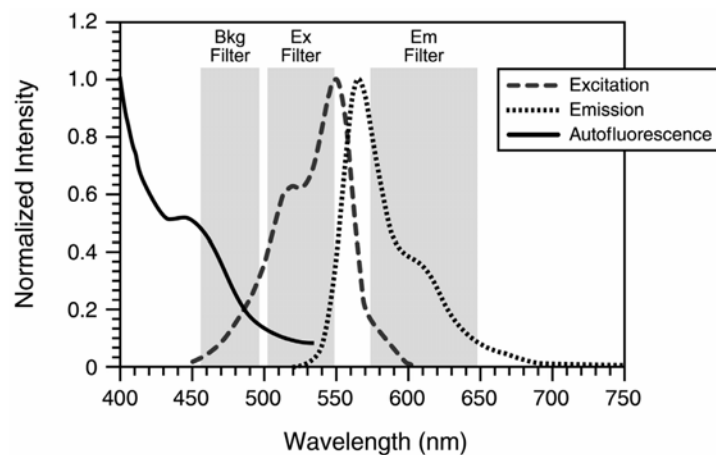

Figure F.18 Spectral data describing the autofluorescent subtraction technique using a background filter.
The graph shows the excitation and emission spectrum of PKH26 and the autofluorescent excitation spectrum of mouse tissue. Also included are the spectral passbands for the blue-shifted background 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 bioluminescent light sources in a subject. For more details on DLIT analysis, see Chapter 12, page 151 and Appendix H, page 247.

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 bioluminescent sources located deeper inside. Information about the depth of the bioluminescent 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 bioluminescent 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 bioluminescent 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.
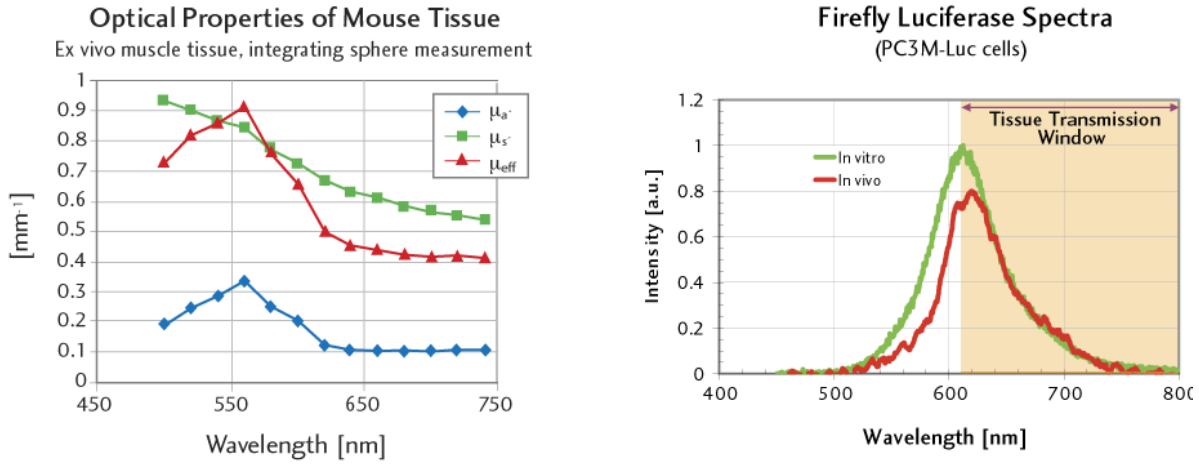
Figure G.1 Optical Properties of Mouse Tissue and Firefly Luciferase Spectra
The bioluminescent signal from firefly luciferase (right) is emitted from wavelengths of 500-700 nm, which spans a region of the spectrum where there are major contrasts in the optical properties of mouse tissue (left). The 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 $\mu'_s \gg$ 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 $I(\lambda)$ is reduced to a relatively simple expression:

$$I(\lambda) = SK(\lambda) \exp(-\mu_{eff}d) \tag{1}$$

where S is the absolute total photon flux emitted by the bioluminescent 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 bioluminescence 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:

$$\mu_{eff} = (3\mu_a\,(\mu'_s + \mu_a))^{1/2} \tag{2}$$

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 bioluminescent light emission. The luciferase bioluminescence 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 Xenogen 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.
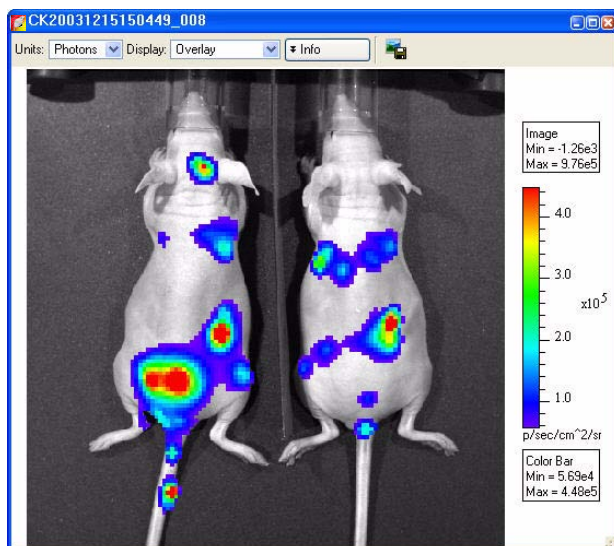

Figure G.2 Metastatic sites in nude mice.
Mice were imaged 13 days after a tail vein injection of 5×10^5 B16F10 melanoma cells. Imaging parameters: high sensitivity binning, f/stop=1, FOV = C (13 cm), exposure time = 120 seconds at 560 and 580 nm, exposure time = 60 seconds at all other wavelengths. 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 121). 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 240 where S = absolute total photon flux emitted by the bioluminescence 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.
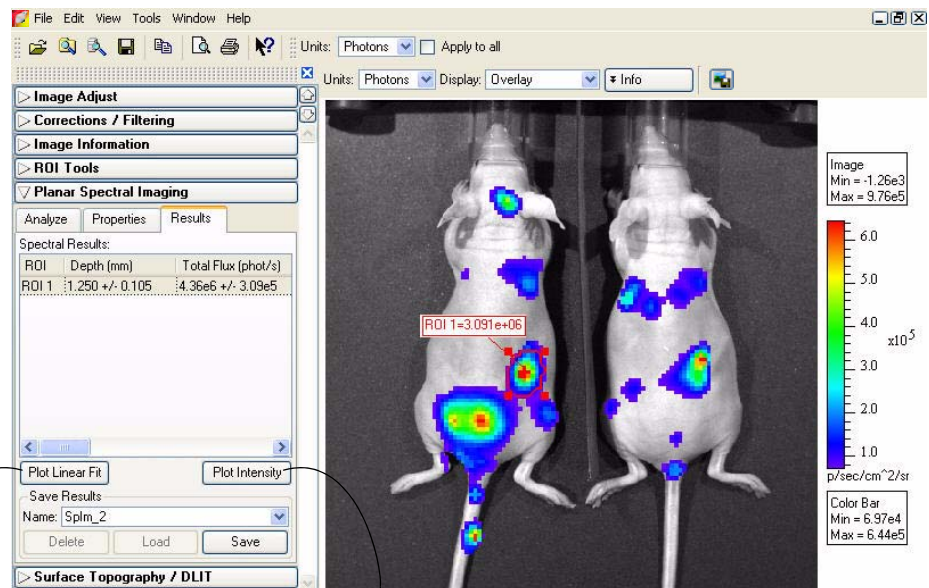
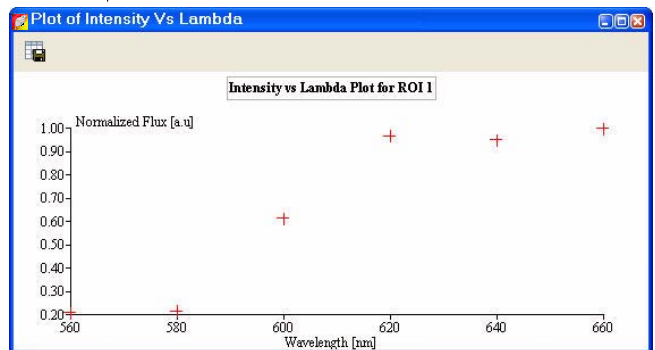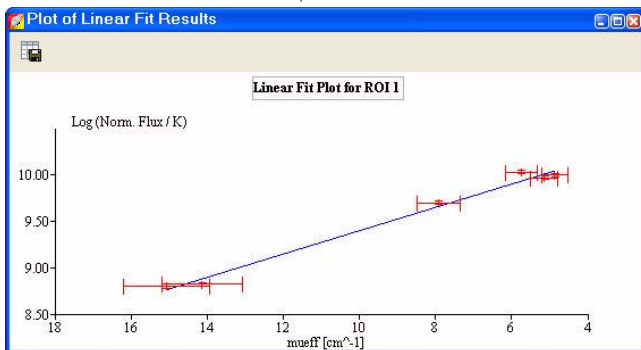After the analysis is completed, click a button to display graphical results.

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 μ_{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 bioluminescent 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 220). It is also recommended that you inspect all images in the sequence to confirm that the bioluminescent signal is greater than the tissue autoluminescence. If the bioluminescent 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   3D Reconstruction of Light Sources

Diffuse 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 bioluminescent light source distribution inside the subject.

Fluorescent Tomography (FLIT) analyzes images of surface light emission to generate a 3D reconstruction of fluorescent light source distribution inside the subject.

NOTE

To generate a 3D reconstruction of bioluminescent sources, the Living Image software requires a photographic image, a structured light image, and bioluminescent images obtained at two or more wavelength filters from 560-660 nm. To generate a 3D reconstruction of 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 (*mesh*) from structured light images. The mesh 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 mesh.

- Divides the interior of the subject into a solid mesh 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 photon density 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 or mesh 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.
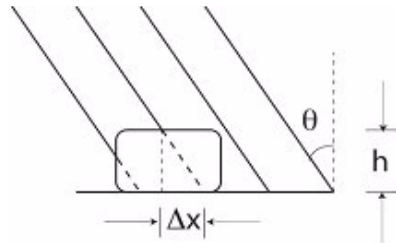

Figure H.1 Parallel laser lines projected onto a subject.
Given knowledge of the angle θ, the height of the subject (h) can be determined by analyzing the displacement, Δx, of the laser lines as they pass over the object.

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) .
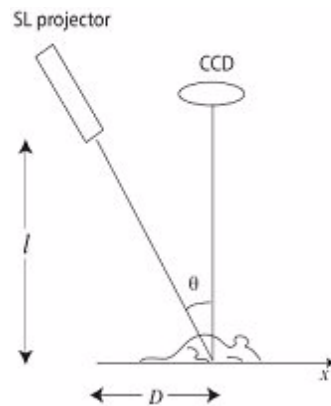
Figure H.2 Structured light projector and subject.

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.

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 mesh 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.

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

- A surface mesh 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 A.1)

Table A.1 IVIS System filters for bioluminescence & fluorescence tomography

IVIS® Imaging System	Filters	Bandwidth (nm)
200 Series	6 emission filters, 550-670 nm	20
Spectrum	10 excitation filters, 415-760 nm	20
	18 emission filters, 490-850 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).
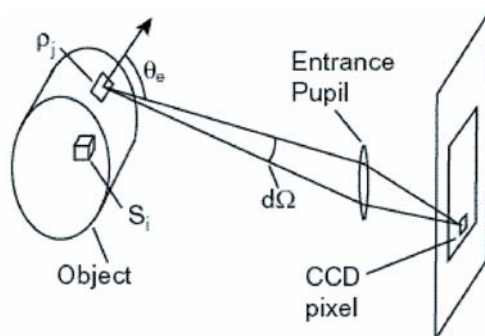
Figure H.3 Light emission from a surface element passes through the lens entrance pupil and is recorded in the image.

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.

Defining the Linear Relationship Between a Source and Photon Density

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 (or flux in photons/sec) of the point source inside the ith 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.

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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 (ρ_j) at each surface element described by a Green's function G_{ij}. The photon density at the jth 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.

Finding 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 algorithm attempts 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 combination of methods called Simplex and Non-Negative Least Squares are 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 (minimum radiance) and only keeps the voxels that contribute significantly to these surface elements.

Source & Tissue Properties

DLIT analysis of spectrally filtered images requires knowledge of the spectral dependence of bioluminescent light emission. Table H.1 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.

Select a bioluminescent source spectrum.

Select a tissue or organ from the drop-down list. The associated internal medium index of refraction is automatically entered.

Choose the Source Spectrum from the Plot drop-down list to display the selected spectrum.
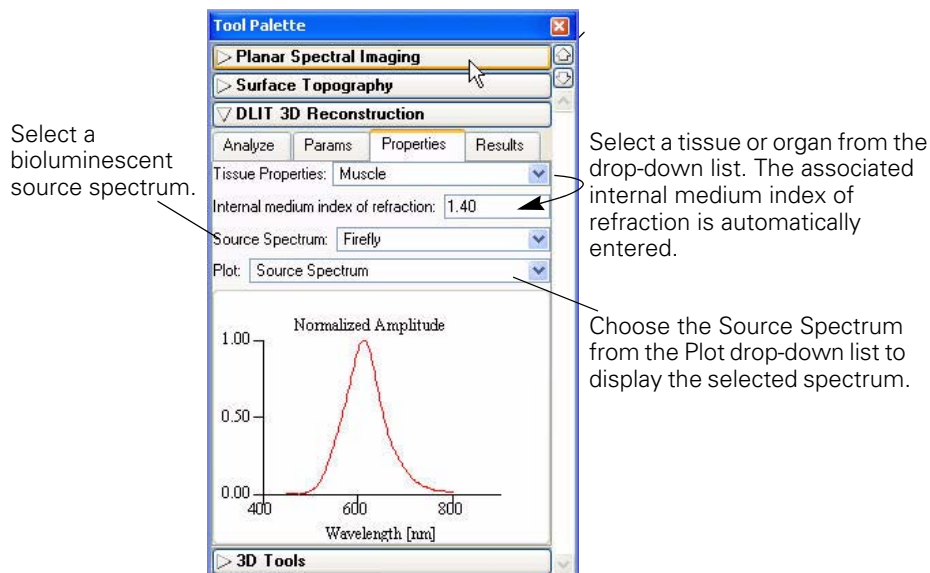
Figure H.4 DLIT 3D reconstruction tools, Properties tab

Table H.1 Source spectra

Source Spectrum	Description
Bacteria	Bacterial luciferase
CB Green	Click beetle green luciferase
CB Red	Click beetle red luciferase
Firefly	Firefly luciferase
XPM-2-LED	LED in the XPM-2 mouse phantom.
hRenilla	Sea pansy (*Renilla reniformis*) luciferase

> **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 (μ_{eff}) in the Tissue Properties drop-down list. The tissue properties are plotted as a function of wavelength. Select the tissue or organ most representative of the source location. Muscle is a good choice for general reconstructions *in vivo*.

NOTE

Default tissue optical properties and source spectrum are specified in the Preferences box. For more details, see Appendix B, page 201.

H.2 Algorithm Parameters & Options

This section explains the user-modifiable DLIT algorithm parameters and options.

Analyze Tab

Tissue and source are specified in the

Select the acquisition wavelengths for the DLIT analysis.

If DLIT analysis results are open, the 3D tools are available.

Active sequence
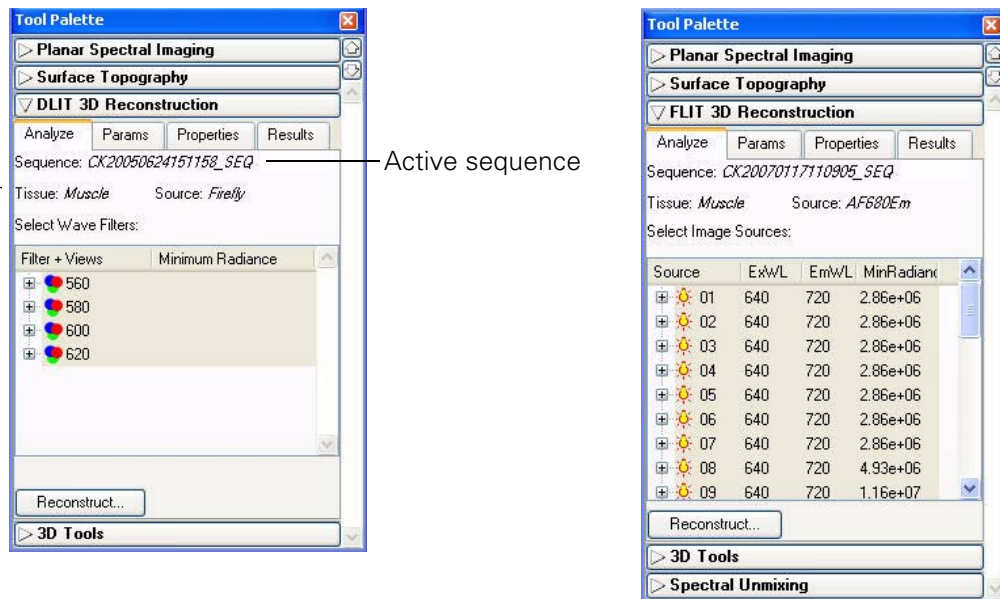
Figure H.5 3D reconstruction tools, Analyze tab, DLIT (left) and FLIT (right)

Wavelengths

For FLIT reconstruction of fluorescent sources, you must specify the transillumination source positions. It is recommended that you acquire images at a minimum of four source positions. (All images are acquired using the same excitation and emission filters.)

For DLIT reconstruction of luminescent sources, you must specify the acquisition wavelengths for the image sequence. It is generally recommended that you acquire image data using two to four wavelengths rather than a single wavelength so that more information is available for the analysis.

Ideally, chose wavelengths or source positions where the signal is well above zero (not buried in the CCD noise) and the optical property of the medium (μ_{eff}) exhibits a large change. The larger the difference in μ_{eff}, the higher the quality of information that the

wavelength data adds to the analysis. The recommended wavelengths are 580-640 nm for tissue and 560-620 nm for the Xenogen XPM-2 tissue phantom.

Minimum Radiance

The minimum radiance determines the lower radiance [photons/sec/cm²/sr] threshold of the data to be used in the DLIT/FLIT analysis.

The software automatically computes a default minimum radiance value and this is the recommended starting point for an analysis. If too much noise is included in the analysis, increase the minimum radiance value.

An optimum minimum radiance value can be evaluated by viewing the image data in photon units (photons/sec/cm²/sr) and adjusting the color bar Min to be above the level of noise in the image.

Parameters Tab
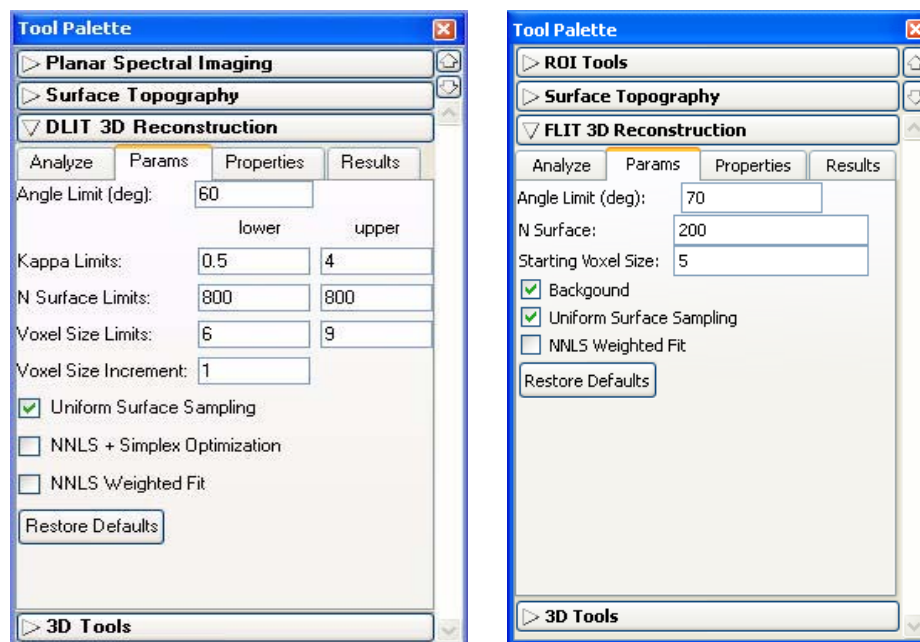
Figure H.6 3D reconstruction tools, Parameters tab, DLIT (left) and FLIT (right)

Angle Limit

The angle limit refers to the angle between the object surface normal and the optical axis. The optical axis can be considered to be a line perpendicular to the stage. The surface normal is a line perpendicular to a plane tangent to the surface point. For example, in a dorsal view of a mouse, the highest point on its back would have a normal line perpendicular to the stage. In this case the angle is zero. The side of a mouse abdomen would have a normal line parallel to the stage, so the angle here would be close to 90°.

The software uses luminescent image data for surface elements that are less than the angle limit. The default angle limit setting is 70° for the IVIS® Imaging System 200

Series or the IVIS Spectrum. For IVIS Spectrum or 200 Series data, if there is significant signal on the side of the subject, a larger angle of 70-85° can be used.

Kappa Limits (DLIT)

Kappa (κ) is a parameter that is searched during a reconstruction to determine the best fit to the image data. Small values of kappa tend to favor deeper sources, while large values favor more shallow sources.

The limits on kappa are minimum of 0.1 and a maximum of 10. The default range for kappa is 0.5-4. Kappa is doubled at each iteration, so for a selected range of 0.5-4, the kappa values for each iteration would be 0.5, 1, 2. and 4. Choosing a large range for kappa produces the most reliable solution, but requires more analysis time.

N Surface (FLIT)

The number of surface intensity points to use in the reconstruction at a given source position.

N Surface Limits (DLIT)

This is the maximum number of surface intensity points to use in the reconstruction at a given wavelength. The range is 200 to 800 and the default is 200. The time required for reconstruction is shortest for smaller values of N (for example, 200). However, a large N value may give a more accurate result because more data are included in the fit.

Starting Voxel Size (FLIT)

Voxels are the small cubes of space inside a subject, each of which contains a quantity of fluorescent yield. The FLIT reconstruction begins with large voxels, specified by the starting voxel size (the length of a voxel cube side in mm). At each iteration, the algorithm reduces the size of the voxels by a factor of 2 until the optimum solution is determined.

Starting Voxel Size Limits (DLIT)

Voxels are the small cubes of space inside a subject, each of which contains a light source (much like a pixel in a 2D image). The DLIT reconstruction begins with large voxels, specified by the voxel size limit (the length of a side of the voxel cube in mm). At each iteration, the algorithm reduces the size of the voxel by a factor of two until the optimum solution is found.

The voxel size limits are a minimum of five and a maximum of 10. The default range is set to 6-9 mm. A larger range of voxel limits ensures a more reliable solution, but requires more computational time.

Voxel Size Increment (DLIT)

This is the step increment in voxel size, stepping from the minimum voxel size limit to the maximum voxel size limit. For example, if the voxel size limit ranges from 6-9 mm, a voxel size increment = 1 gives four starting voxel sizes (6, 7, 8, and 9 mm).

The default increment of 1 mm is usually adequate, however smaller increments can be used if you want to sample finer voxel sizes. Smaller increments will significantly increase the time required for reconstruction.

Background (FLIT)

Choose this option to take the background fluorescence (for example, autofluorescence or non-specific probe in circulation) into account. Background fluorescence and fluorophore emission contribute to the photon density signal at the surface. The background fluorescence signal is modelled in order to isolate the signal due to the fluorophore only, where an average homogenous tissue background fluorescence yield is determined empirically.

Background fluorescence contribution to the photon density at the surface is forward-modelled. Simulated photon density data due to background at the surface is subtracted from the measured photon density so that the subsequent photon density used in the fit consists only of signal that is associated with the fluorophore.

The background option specifies whether or not the background fluorescence should be fit. This is not necessary for the XFM-2, long wavelength data. It is important when there is non-specific dye in circulation or a lot of autofluorescence.

Uniform Surface Sampling

If this option is chosen, the surface data for each wavelength will be sampled spatially uniformly on the signal area. If this option is not chosen, the maximum 'N surface elements' will be sampled for the data. This means that the N brightest surface elements will be used as data in the reconstruction. Typically, non-uniform sampling is recommended if there is a single bright source, while uniform sampling is preferred if there are several scattered sources.

NNLS Optimization + Simplex Optimization (DLIT)

If NNLS Optimization + Simplex option is chosen, the software uses a linear programming algorithm to seed the solution, followed by the NNLS optimization.

NNLS Weighted Fit

Choose this option to weight the data in the NNLS optimization.

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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 3.2 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.
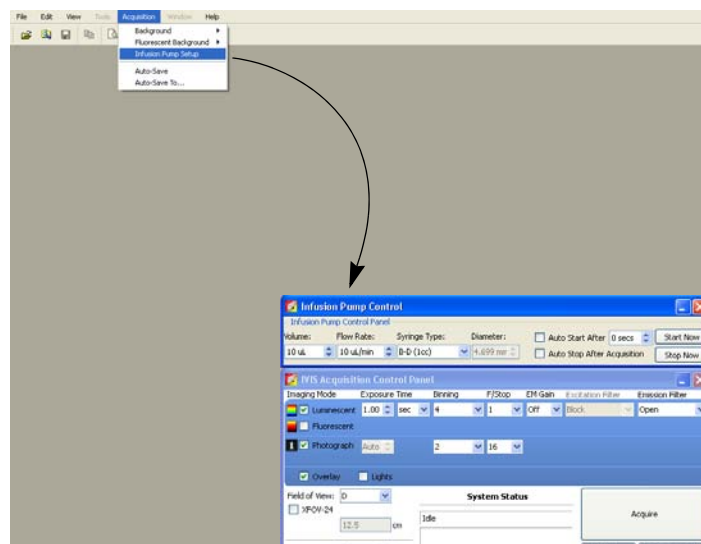
NOTE

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.
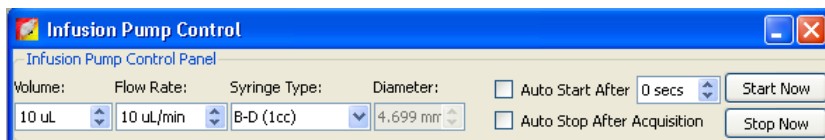
3. Make a selection from the Syringe Type drop-down list (the associated syringe diameter is automatically entered).

   To enter a custom syringe:

   a. Select **Custom** from the drop-down list.

   b. Click OK in the dialog box that appears.
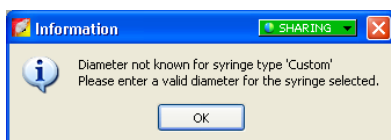
   c. Enter the syringe diameter in the infusion pump control panel.

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.

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).
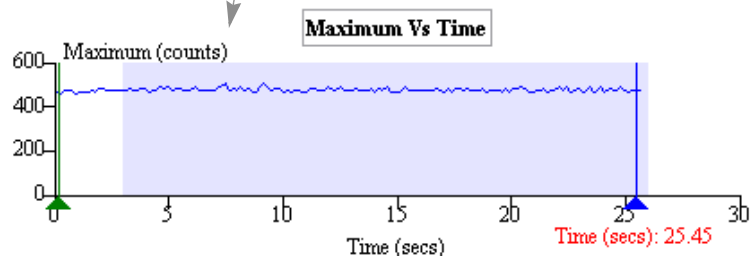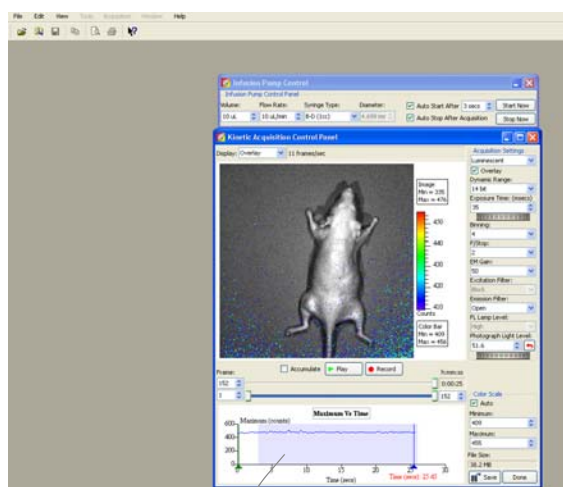
I.3 Closing the Infusion Pump Control Panel

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 Biotrue		Opens the Biotrue® CDMS Browser. **Note:** The browser is only available if the system includes the Biotrue CDMS option.
File → Save		Saves (overwrites) the active image data.
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 →Organ Atlas		Opens a dialog box that enables you to import an organ atlas (.atlas).
File → Import → DICOM		Opens a dialog box that enables you to import .dcm image data that can be viewed in the Living Image software.
File → Import → 3D Mesh		Opens a dialog box that enables you to import a mesh (.xmh). **Note:** This command is only available if an appropriate sequence is active (DLIT or planar spectral imaging sequence).
File → Import → 3D Volume		Opens a dialog box that enables you to import a source volume (voxels, .xsc). **Note:** This command is only available if an appropriate sequence is active (DLIT or planar spectral imaging sequence).
File → Export DICOM		Opens the Browse for Folder dialog box that enables you to export the active image data to DICOM format (.dcm).
File → Export → 3D Mesh		Opens a dialog box that enables you to save the 3D mesh of the active data in Open Inventor format (.iv).
File → Export → 3D Volume		Opens a dialog box that enables you to save the voxels from the active data in Open Inventor format (.iv).
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 Customize tab).
File→ Exit		Closes the Living Image software.

Table J.1 Menu bar commands and toolbar buttons (continued)

Menu Bar Command	Toolbar Button	Description
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.
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 → 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 → Tool Palette		Choose this option to display the tool palette.
View → Activity Window		Choose this option to display the activity bar at the bottom of the main window. The activity bar lists a history of the recent software activities.
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 → Image Layout Window		Opens the Image Layout window that enables you to paste an image of the active data in the window.
View → Acquisition Control Panel		Displays the control panel.
Tools → Image Math for...		Opens the Image Math window for the active data.
Tools → Image Overlay for...		Opens the Image Overlay window for the active data.
Tools → Colorize xx_SEQ		Opens the Colorized View tab for the active sequence. Note: This menu item is only available if "Show Advanced Options" is selected in the Preferences.
Tools → Transillumination Overview		Combines the images of a FLIT sequence into a single image (intensities are summed) that can be analyzed using tools in the tool palette.
Acquisition → Background → Replace		Opens a dialog box that enables you to select an instrument luminescent background. This background measurement is subtracted from luminescent images.
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		Opens a dialog box that enables you to remove the dark charge measurements from the system.
Acquisition → Background → Measure		Opens a dialog box that enables you to acquire a dark charge measurement.
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 Sub Fluor Bkg option is chosen in the control panel, the background measurement is subtracted from the image data.
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 a fluorescent background measurement.

Table J.1 Menu bar commands and toolbar buttons (continued)

Menu Bar Command	Toolbar Button	Description
Acquisition → Fluorescent Background → Clear Available Fluorescent Background		Opens a dialog box that enables you to remove the fluorescent background measurements from the system.
Acquisition → 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 Sub Fluor Bkg option appears in the control panel. Choose the Sub Fluor Bkg option to subtract the user-specified background measurement from the image data.
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 59).
Window → Tile		Organizes the open image windows in a tiled arrangement (see page 58).
Window → 1. xx **Window → 2. xx** **Window → etc.**		A list of the open data. Select the data of interest to it the active window and display it on top of all other open windows.
Window → Other Windows → Browser Window		If the Living Image browser is open, makes it the active window and displays it on top of all other open windows.
Help → User Guide		Displays the online help.
Help → About Living Image		Displays the online help index.
		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.

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