Contents

1 Welcome .................................................. 1
   1.1 IVIS Imaging Systems ................................. 1
   1.2 Living Image® Software .............................. 4
   1.3 Xenogen Technical Support ......................... 6

2 Quick Start Guide to Acquiring Images .......................... 7

3 Control Panel for IVIS® Imaging Systems ...................... 9
   3.1 Basic Controls ........................................ 9
   3.2 Fluorescence Option .................................. 16
   3.3 IVIS Imaging System 200 Series Options .......... 17
   3.4 IVIS Imaging System 3D Series Options .......... 18

4 Image Display and Analysis Window ............................ 21
   4.1 Image Display Tools .................................. 22
   4.2 Region of Interest Tools .............................. 22
   4.3 Physical Units ......................................... 26
   4.4 Display Mode .......................................... 27
   4.5 Image Correction Check Boxes ...................... 31
   4.6 Print & Zoom ........................................... 32
   4.7 Image Window Contextual Menus .................... 34

5 Sequence Window ........................................... 39
   5.1 Display Tools ........................................... 40
   5.2 Sequence Editing ....................................... 41

6 Main Menu Bar ............................................... 43
   6.1 File .................................................... 43
   6.2 Edit ..................................................... 44
   6.3 Living Image ............................................. 44
   6.4 LI Tools ................................................ 53

7 Image Display Modes .......................................... 69
   7.1 Understanding the Differences Between Scientific Image Data and Graphic Images 69
   7.2 Pseudocolor Images .................................... 70
   7.3 Overlays ............................................... 71
   7.4 Blends .................................................. 71

8 Sensitivity and Binning ........................................ 73
   8.1 Binning ................................................ 73
   8.2 Sensitivity .............................................. 75
   8.3 Image Saturation ...................................... 77
9 Measurements, Units, and Calibrations ........................................ 79
  9.1 Regions of Interest (ROI) .................................................. 80
  9.2 Units ............................................................................. 83
  9.3 Flat Fielding ................................................................. 85
  9.4 Cosmic Ray Corrections ..................................................... 86

10 Auto ROIs ........................................................................... 87
  10.1 Measurement ROIs .......................................................... 87
  10.2 Subject ROIs ................................................................... 90

11 Background Sources ............................................................. 93
  11.1 Electronic Background ...................................................... 93
  11.2 Background Light On the Sample ...................................... 94
  11.3 Background Light From the Sample ................................... 96

12 Dark Charge Management .................................................... 99
  12.1 Dark Charge Measurement ................................................ 99
  12.2 Dark Charge Subtraction .................................................. 100
  12.3 Read Bias and Drift .......................................................... 101
  12.4 Automatic Background Measurements ............................... 101

13 Data Management ................................................................. 103
  13.1 Storing Living Image® Data ................................................ 103
  13.2 Retrieving Living Image Data .............................................. 104
  13.3 Importing Data into Living Image Software ........................... 106

14 Fluorescent Imaging ............................................................... 107
  14.1 Description and Theory of Operation ................................. 107
  14.2 Understanding Filter Spectra .............................................. 110
  14.3 Acquiring Fluorescent Images ............................................ 112
  14.4 Image Units .................................................................... 114
  14.5 Working with Fluorescent Samples .................................... 114

15 Spectral Imaging (IVIS® Imaging System 200 & 3D Series) ...... 127
  15.1 Spectral Imaging Procedure .............................................. 127
  15.2 Spectral Imaging Theory .................................................... 130

16 Structured Light (IVIS® Imaging System 200 & 3D Series Only) .. 139
  16.1 Theory of Operation .......................................................... 139
Living Image® Software Manual

1 Welcome

The Xenogen IVIS® Imaging System is a highly sensitive, low light-level system optimized for in vivo (whole, living animals) imaging. Living Image® software is a custom software package developed by Xenogen that runs the IVIS System and provides tools for image display and analysis. Additionally, the Living Image software automates many aspects of important functions such as dark charge management, data storage and retrieval, and data quality assurance. Living Image software also serves as a guide to help inexperienced users through the complex steps associated with quantitative in vivo imaging.

1.1 IVIS Imaging Systems

The IVIS Imaging System is a modular software and hardware system. The Living Image software supports hardware for IVIS Imaging System 50, 100, 200, 3D Series and Lumina (see Table 1.1).

<table>
<thead>
<tr>
<th>Imaging System</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IVIS Imaging System 50 Series</strong></td>
<td>A 0.5 square inch CCD camera that is cooled to -90° C.</td>
</tr>
<tr>
<td>(Figure 1.1)</td>
<td>Field-of-view: 4-12.5 cm.</td>
</tr>
<tr>
<td><strong>IVIS Lumina</strong> (Figure 1.2)</td>
<td>A 0.5 square inch CCD camera that is cooled to -90° C.</td>
</tr>
<tr>
<td></td>
<td>Field of view: 5-12.5 cm.</td>
</tr>
<tr>
<td><strong>IVIS Imaging System 100 Series</strong></td>
<td>A 1.0 square inch CCD camera that is cooled to -105° C (cryogenic cooling) or -90° C (thermoelectric cooling).</td>
</tr>
<tr>
<td>(Figure 1.3)</td>
<td>Field of view: 10-25 cm.</td>
</tr>
<tr>
<td><strong>IVIS Imaging System 200 Series</strong></td>
<td>A 1.0 square inch CCD camera that is cooled to -105° C (cryogenic cooling) or -90° C (thermoelectric cooling). Includes integrated fluorescence and single-view 3D capabilities.</td>
</tr>
<tr>
<td>(Figure 1.4)</td>
<td>Field of view: 4-25 cm.</td>
</tr>
<tr>
<td><strong>IVIS Imaging System 3D Series</strong></td>
<td>A 1.0 square inch CCD camera that is cooled to -90° C. The system takes images of a single sample at multiple views (angles) around the sample.</td>
</tr>
<tr>
<td>(Figure 1.5)</td>
<td></td>
</tr>
</tbody>
</table>
1. Welcome

Figure 1.1  IVIS® Imaging System 50 Series

Figure 1.2  IVIS Lumina
Figure 1.3 IVIS® Imaging System 100 Series

Figure 1.4 IVIS Imaging System 200 Series
1. Welcome

The system takes very low-light level images, stores them, and displays them for subsequent analysis. This manual describes the use of the IVIS Imaging System and Living Image software for a typical in vivo imaging application. Some aspects of general purpose, low light-level imaging such as sensitivity and binning, measurements and calibrations, background sources of light, and dark charge management are also discussed.

1.2 Living Image® Software

The software is developed within a powerful data analysis and programming environment called WaveMetrics IGOR Pro\(^1\). The Living Image software creates a custom environment that is used for both data acquisition and analysis as described in this manual. Living Image software and Igor Pro run on both Macintosh\(^\circledast\) and Windows\(^\circledast\) computers. To start Living Image software under Windows, look for the Living Image software icon in the Start-Programs-Living Image menu or on the desktop. For Macintosh computers, look for an alias in the Apple\(^\circledast\) menu or on the desktop.

An overview of the Living Image software interface is shown in Figure 1.6. The standard panels include an image acquisition control panel, image display and analysis window, system status and dialog window, and a lab book window. Located in the top menu bar are tools for both IGOR Pro and Living Image software. Menu items that apply to Igor

---

1. WaveMetrics, Inc. retains copyright of Igor Pro 1992-2004
Pro only, and are not supported by Living Image software, remain inactive (disabled), helping to avoid interface clutter and confusion. See Chapter 6, page 43, for information on enabling these features. Refer to the Igor Pro manual (available in the Help menu) for information on commands not supported by Living Image software.

Sections of the Living Image manual may be viewed by selecting Living Image-Living Image Help. A PDF format version of the manual is also available in Start-Programs-Living Image (Windows) and in the Help menu (Mac). Help for specific controls can also be viewed as shown below.

<table>
<thead>
<tr>
<th>Operating System</th>
<th>Procedure for Viewing Help</th>
</tr>
</thead>
<tbody>
<tr>
<td>Windows</td>
<td>Hold the cursor over the item of interest and look for the help message in the system status line.</td>
</tr>
<tr>
<td>Macintosh OS X</td>
<td>Same as above but the user must first activate Show IGOR Tips from the top level Help menu.</td>
</tr>
</tbody>
</table>

This Living Image software manual is organized according to the panels and menu items shown in Figure 1.6. Commands and controls associated with the system control panel, the image display and analysis window, and the main menu bar items are described in separate sections. Later sections provide more details on a number of topics related to in vivo imaging.

The nomenclature in this manual uses bold text to indicate buttons, checkboxes, and menu items, e.g. Help. Bold text separated by hyphens
is used to indicate pull-down menus, e.g., Living Image Tools-Show Cursors. Names of windows and panels are capitalized but not bold, e.g., Measurements Table.

1.3 Xenogen Technical Support

Technical Support: 1.888.810.8055 (US)
                  1.510.291.6275 (International)
Main Telephone:  1.510.291.6100
E-mail:          IVISTechSupport@xenogen.com
Fax:             1.510.291.6196
Address:         Xenogen Corporation
                  860 Atlantic Avenue
                  Alameda, CA 94501
2 Quick Start Guide to Acquiring Images

This section consists of a "Quick Start" procedure to acquire an illuminated image of a sample (photographic image) followed by a non-illuminated image of light emission from the sample (luminescent image). No analysis is described. This procedure is intended as a brief guide that can be followed step by step. For more detailed information, see the sections that immediately follow. This procedure assumes that the system power is on. If power to the system is not on, see the IVIS® Imaging System Hardware Manual.

1. If it is not already on, start up the acquisition computer and start the Living Image® 2.6 software program from the Windows Start Menu. Log in by selecting your initials from the drop down menu, or if you are a new user, enter your initials and click DONE. A system control panel will appear in the lower right corner of the monitor (Figure 2.1).

2. Click the Initialize IVIS system button in the camera control panel. After initialization, the Temperature Status box in the

Figure 2.1 IVIS System Control Panel.
2. Quick Start Guide to Acquiring Images

center of the panel should be green, indicating that the CCD camera is adequately cooled. (If you are using the IVIS® Imaging System 50 Series or the IVIS Lumina, allow 10 to 15 minutes for the camera to reach the proper temperature.) The Temperature Status box changes from red to green when the CCD camera has reached the proper operating temperature.

3. Place the sample to be imaged in the center of the stage in the imaging chamber. Close the door.

4. Select the desired Field of View from the pull down menu on the left side of the control panel.

5. Enter the approximate (1 cm) Subject Height in the lower left entry box.

6. Click the Acquire continuous photos button to check the subject position. (This step is optional.) Close this window and reposition the subject if necessary.

7. Check the Overlay box in the control panel and set the Exposure Time, binning, and f/stop for the luminescent image. If unsure of what exposure time to use, it is best to start with low sensitivity settings (10 sec, Medium, f/1) and increase as necessary.

8. Click the Acquire button on the control panel.

9. After the exposure is complete, the overlaid image is displayed. Edit the information in the Change Information window and click Done. Always click Done in the Image Information window before proceeding.

10. Confirm that the signal of interest is above the noise level (recommend >600 counts) and below CCD saturation (~60,000 counts). If the signal level is unacceptable, adjust Exposure Time or Binning and repeat the image acquisition.

11. Upon acquisition, an image is not automatically saved, but it is automatically named using the operators initials, and a date and time stamp. To save the image, choose Save Living Image Data (under the Living Image menu item), select the Format "Save all Living Image Data Files", check the box “Save Lab Book”, then click Save.

This completes the data acquisition. If desirable, adjust the display with the Max Bar or Min Bar slider. To obtain additional images, begin with Step 3 above.

Subsequent analysis is described in Chapter 4, page 21, and can be conducted immediately or deferred until later, after additional images of the sample have been acquired.
3 Control Panel for IVIS® Imaging Systems

3.1 Basic Controls

Typically, image acquisition on all IVIS Imaging Systems involves placing a sample on the stage, adjusting camera settings such as field-of-view (FOV), sample height, and exposure time, binning, and acquiring the image. Most sample images are actually a composite of two images. The first image is a short exposure of the sample illuminated by lights located in the top of the imaging chamber. See Figure 3.1. This image is referred to as a "photographic image" and is displayed as a grayscale (black and white) image. The second image is a more sensitive exposure of the sample taken in darkness to record low-level luminescent emissions. This portion of the image is referred to as the "luminescent image". The luminescent information, displayed in pseudo-color representing intensity, is overlaid on top of the photographic image to form an "Overlay Image" to create what's referred to as an "Overlay image". See Figure 3.2. The acquisition and overlay of the two images is done automatically by Living Image software. For IVIS Imaging Systems with fluorescent imaging capabilities, a fluorescent image may be acquired and displayed in the same manner as the luminescent image. See Section 3.2, page 16.
3. Control Panel for IVIS® Imaging Systems

The IVIS System Control panel controls acquisition functions for all IVIS Imaging Systems. See Figure 3.3. Optional controls for the IVIS Imaging System 200 Series and systems with fluorescence capabilities will be discussed later in this section. Each control contains a built-in help message that can be displayed by moving the cursor over the control. The help message will appear in the system status line (typically located in the lower left corner of the screen on an IVIS Imaging System acquisition computer). A detailed description of each control is given below. The commands are listed in the general order in which they should be used when acquiring an image. See Chapter 2, page 7 for step-by-step procedures.

**Initialize IVIS System** initializes the IVIS Imaging System. It must be used each time Living Image software is started or if the power has been cycled on either the camera controller or the imaging chamber. It can also be used to reset to startup conditions, which is sometimes useful in error situations. The initializing procedure moves every motor-driven component (stage, lens, etc.) in the system to a "home" position, resets
all electronics and controllers, and restores all software variables to default settings.

**System Status** is the region of the camera control panel that displays CCD temperature information and status messages describing current operation of the system. The temperature settings should be checked after initialization. The top line, labeled **Temperature**, monitors the current temperature of the CCD. If the measured CCD temperature is within the acceptable range, a green box is displayed. If the temperature is out of range, a red box is displayed; and if the system has not been initialized, a blank box is displayed. Click once on the colored box to view the actual temperature. Current and demand temperatures will be visible for five seconds. The CCD demand temperature is fixed and should not be changed by the user. Electronic feedback control maintains the CCD temperature to within a few degrees of the demand temperature. If the temperature status control box is green, this indicates that it is "locked," and the IVIS is ready for imaging. If the status box indicates "unlocked" for a long period, contact IVIS Technical Support.

On the IVIS Imaging System 200 Series, the sample stage temperature is also displayed and controlled from the System Status panel. The sample stage temperature is set to 37° C by default, but may be changed by the user to any temperature within the range of 25°- 40° C.

**Field of View** (FOV) sets the size of the imaging region on the sample stage by adjusting the height of the sample stage and adjusting the position of the lens. The FOV is the width, in centimeters, of the square area on the stage that is to be imaged. Standard calibrated FOV positions are indicated by A, B, C, D, E, and correspond approximately to the widths shown in the table below.

<table>
<thead>
<tr>
<th>Imaging System</th>
<th>FOV values for positions A, B, C, and D respectively (and E for IVIS 200)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVIS® Imaging System 50 Series and IVIS Lumina</td>
<td>5, 7.5, 10 or 12.5 cm</td>
</tr>
<tr>
<td>IVIS Imaging System 100 Series</td>
<td>10, 15, 20, or 25 cm</td>
</tr>
<tr>
<td>IVIS Imaging System 200 Series</td>
<td>4, 6.5, 13, 20, 25 cm</td>
</tr>
</tbody>
</table>

In addition to selecting the viewing area, the FOV control indirectly affects the depth-of-field. The depth-of-field refers to the range over which the image is in focus. The depth-of-field is affected by both the FOV and the f-stop (see f-stop discussion below). Similar to a microscope, the depth-of-field decreases as the magnification increases. A smaller FOV (higher magnification) will have a narrower depth-of-field.

**Focus and Subject Height** is a group of controls that sets the focal plane at a particular location above the stage. Focusing is done by one of two methods (a third method is available with the IVIS Imaging System 200 Series). One method is to enter the estimated height of the subject, in centimeters (accuracy of 0.5 cm is usually adequate), in the **Subject Height** box. This adjusts the stage position so that the focal plane of the lens/CCD system is positioned at the estimated height.
above the stage. The estimated height does not have to be the maximum height of the subject. For example, if the goal is to monitor luciferase expression in the joint of a mouse leg, then the subject height should be the height of the leg above the platform, typically a few millimeters. However, if the user is interested in imaging a tumor on the uppermost dorsal side of a mouse, the full height of the animal (about 1.5-2.0 cm) should be used. The default setting at startup for subject height is 1.5 cm.

In addition to getting a sharp image, there are safety implications related to the subject height, particularly on IVIS Imaging System 200 Series. Placing a large subject, e.g., 10 cm high, on the stage and entering a height of zero and choosing a small FOV will result in the stage being positioned such that the subject may make contact with the top of the imaging chamber. Although built-in protections prevent damage to the instrument, users should always pay close attention to the subject height.

A second method for focusing, called **Manual** focus, is available in the pull down menu. In this method, a photographic image is acquired and displayed in the Manual Focus Control panel, using the f/stop setting specified for the Luminescent image. See Figure 3.4. The **Up** and **Down** buttons refer to the incremental movement of the stage, while **Fine**, **Normal**, and **Coarse** refer to the size of the increment. Clicking the **Up** and **Down** buttons changes the focus. When optimum focus is achieved, close the panel. The resulting focal plane height will be entered automatically into the **Subject Height** box.

![Figure 3.4 Manual Focus Control Panel. Illustration of manual focusing at f/1. Use the Up and Down controls to fine tune the focus position.](image)

**Imaging Mode** presents three checkboxes that are used to select between the photographic and luminescent (or fluorescent) imaging modes described above. The default is **Overlay**, which automatically takes a photographic image followed by a luminescent image, then overlays them. **Overlay** is the mode recommended for most imaging applications.

**Exposure Time** controls the length of time that the shutter is open for photographic and luminescent images. In the photographic settings, when "Auto" is checked, the exposure time will automatically be
adjusted to produce a good photograph. Luminescent images typically have longer exposure times that need to be adjusted, depending on the brightness of the subject. The luminescent or fluorescent signal level is directly proportional to exposure time. Luminescent exposure time is measured in units of seconds or minutes. The minimum calibrated exposure time is 0.5 seconds. There is no limit on the maximum exposure time, but usually there is little benefit to exceeding 5 minutes for animal imaging. The goal is to adjust the exposure time to produce a signal that is well above the noise (recommend >600 counts) but less than the CCD camera saturation of ~60,000 counts.

**Binning** increases the pixel size on the CCD, which delivers higher sensitivity at the expense of spatial resolution. The high resolution CCDs used in IVIS Imaging Systems contain between 1024x1024 and 2048x2048 elements. Depending on the subject being imaged, this resolution is usually much higher than necessary. The CCD hardware can be binned so that NxN areas of the CCD are summed before readout, where N is the binning level. This concept is illustrated in Figure 3.5. Binning a luminescent image results in a significant improvement in the signal-to-noise ratio. Although spatial resolution is degraded at high binning (pixels become large), this is often acceptable for in vivo images where light emission is rather diffuse. **Binning** pull-down menu choices for luminescent images are small, medium, and large, with the default set to medium. Each change in binning changes the sensitivity by a factor of four. For example, Medium binning is four times more sensitive than small binning. The user can opt to see the actual binning numbers by selecting the Living Image Tools-Preferences option from the pull-down menu and using the Camera Settings button to select Manual Binning Values. Recommended binning numbers for luminescent images are 1-4 for high-resolution imaging of cells or tissue sections, 4-8 for standard in vivo images, and 8-16 for very dim in vivo images requiring the highest sensitivity. See Chapter 8, page 73.

![Binning Example](image)

**Figure 3.5** A small segment of the CCD at binning = 1, binning = 2 (4 pixel summed together), and binning = 4 (16 pixels together)

**f/stop** controls the aperture of the lens on the camera. A larger number indicates a smaller aperture setting and lower sensitivity. The smallest f/stop on all IVIS Imaging Systems is f/1, which corresponds to the widest lens opening and the most sensitive setting. The detected signal scales inversely with the square of the f-number. The aperture size, or
f/stop, controls both the amount of light detected and the depth-of-field. See Fig. 3.6. A smaller aperture results in reduced sensitivity but improved depth of field, which produces a sharper image of an object with varying height. Typically, the photographic image is taken with a small aperture (f/8 or f/16) to give the sharpest image. The luminescent image is taken with a large aperture (f/1) to maximize sensitivity. All f/stop values are calibrated and the user is free to change these settings. For cases in which spatial resolution is known to be important, the user should select the Focus-manual option to see how sharp the image will be at the selected f/stop. This is especially important at the high magnification settings available on the IVIS Imaging System 200 Series because the depth of field is very narrow, on the order of 1-3 mm.

![f/stop diagram](image)

**Figure 3.6** f/stop. Left: lens wide open at f/1; right: lens closed down at f/8.

**Emission Filter** controls the filter wheel in front of the CCD lens. This wheel may have different types of filters installed for either fluorescence or spectral imaging applications. The number of filter slots ranges from 6 - 24, depending on the system. The desired filter is chosen from the pull down menu. For bioluminescent imaging, the filter wheel is typically set to "Open," which is a position with no filter.

**Acquire** initiates the image acquisition using the conditions chosen by the photographic and/or luminescent controls prior to the start of acquisition. During image acquisition, the Acquire button becomes a Stop button that can be used to terminate the exposure. At the end of acquisition, a Change Info panel is presented. This panel allows the user to enter descriptive information about the image. The descriptors appear as labels in the image window and can be used to help identify the image data later. Always click **Done** on the Change Info panel before proceeding, even if you don't wish to label the image. See Chapter 13, page 103.

**Acquire Continuous Photos** sets the camera in "live" photographic imaging mode. In this mode, a series of illuminated photographic images (frames) are taken rapidly and displayed sequentially to give a video-like effect that is useful for checking subject position and
watching for animal movement. The exposure time per frame, f/stop, and filter setting used in this mode are the same as those set for photographic mode. The system will take ten photos, then automatically stop in order to lengthen the service life of the shutter. **Lights On** manually turns the illumination lights in the top of the imaging chamber on and off for inspection. This is not needed for imaging, since the lights turn on and off automatically.

**Select Sequential Mode** opens up a new panel that allows the user to define an entire sequence of images to be taken with a single click of the **Acquire Sequence** button. See Figure 3.7. This option can be used to take a kinetic series of images (a sequence in time), a sequence of images with different filters, or a sequence varying any other parameter available in the IVIS System Control panel. Select the camera settings for the first image using the IVIS System Control panel. Then click the **Set** button in the Sequential Setup panel. This will load the parameters into the Sequential Setup table. Enter a delay time if desired, which is the time from the start of one image to the next, change the acquisition parameters if desired, then click **Set** again to load the next image. If the

---

**Figure 3.7** Sequential Imaging Panel used to define a sequence of images for acquisition.
3. Control Panel for IVIS® Imaging Systems

Delay Time is set to a number smaller than the acquisition time (for example, 0.1 minutes), then the acquisition of the subsequent image will start immediately. Up to 99 entries can be made in the sequence table. The Clear One and Clear All buttons allow the user to clear a single entry or all entries from the table, respectively. The Save and Recall buttons allow the user to save the setup on disk and recall it later. To edit an existing Sequence, click Edit Sequence, highlight the sequence number you want to edit, change the acquisition settings to the new parameters, then click Change. When finished editing, click Done. After setup of the sequential table has been completed, click Acquire Sequence to start the sequence. Thumbnail views of the images will appear as the image acquisition proceeds. To return to single-image acquisition, click on the Select non-sequential mode button. See Chapter 5, page 39 for more information on analysis and display of sequences.

3.2 Fluorescence Option

For IVIS Imaging Systems configured with the fluorescence option, there are additional controls that appear in the IVIS System Control panel. See Figure 3.8. For more details on fluorescent imaging and operation of the fluorescence acquisition controls, see Chapter 14, page 107.

The Excitation Filter allows the user to select from up to 11 fluorescence excitation filters (plus one blocked position) from the pull-down menu. By default, the filter is set to “Block” for bioluminescent images. It is important to always match filters (GFP excitation with GFP emission), as a mismatch, e.g., GFP excitation with Open emission, may result in an image that is over-exposed or saturated.

Filter Lock locks the excitation and emission filters together (for example, GFP excitation with GFP emission) to avoid mismatched filters and the possibility of over-exposed images. This option should be checked during normal fluorescence imaging.
**Fluor Lamp Level** allows the user to set the fluorescence lamp emission level to **Off**, **Low**, **High**, or **Inspect**. Low emission is approximately 18% of **High**. It is recommended to always use **High** if possible. If a fluorescent image is too bright, then use other controls such as **f/stop**, **Binning**, or **Exposure Time** to reduce the signal.

The **f/stop** setting defaults to **f/2** for fluorescence imaging. While this is the recommended starting point, the **f/stop** may be adjusted if necessary.

**Inspect** turns the fluorescent lamp on manually, allowing the user to inspect the illumination inside the IVIS Imaging chamber. Before selecting **Inspect**, verify that the emission and excitation filters are selected as desired. When **Inspect** is selected, the software will first move the excitation and emission filters to the positions currently set in the respective drop-down menus before turning on the fluorescent lamp. This control is only used for inspection. The software controls on/off cycling of the lamp automatically during imaging and will return the **Fluor Lamp Level** to its previous **High** or **Low** setting for imaging.

### 3.3 IVIS Imaging System 200 Series Options

In addition to the fluorescence control panel settings described above, the IVIS Imaging System 200 Series offers control options relating to the scanning galvanometer. These options are shown in Figure 3.9 and described below.

#### Enable Alignment Grid

Activates a laser-generated alignment grid inside the IVIS System 200 Series imaging chamber when the door is opened. The alignment grid is set to the size of the FOV selected in the **Field of View** pull-down menu. The grid will turn off automatically after 2 minutes to avoid excessive wear on the laser. If alignment of the subject is not complete after 2 minutes, check the **Enable alignment**
grid box again to turn on the grid. The horizontal cross hair on the alignment grid is offset appropriately to take into account the height of the object entered in the Subject Height box.

Focus-Scan Mid Image is another option for determining subject height and is selected from the Focus drop down list. When this option is selected, the laser beam is scanned horizontally across the middle of the image to determine the maximum subject height along this line. This option works reasonably well 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. This method of focusing is not recommended for objects with a great deal of structure such as well plates, or for the high magnification (FOVA = 4.0 cm) field of view. In these or similar situations, Manual focus or Subject Size focus methods are recommended.

Structure enables the structured light system for determining surface topography. Selecting this option will acquire an image of parallel laser lines scanned across the subject. This is called the structured light image. From this image, the surface topography of the subject can be determined. This information serves as input for the Diffuse Luminescence Imaging Tomography (DLIT) code for reconstructing the location and brightness of luminescent sources. When Structure is selected, the structured light image will be acquired automatically along with the photographic and luminescent (if selected) images when the Acquire button is clicked. The f/stop is set to a default setting of f/8 and the exposure time is set to 0.2 seconds for the structured light image. The spatial resolution of the computed surface depends on the line spacing of the structured light lines. Line spacing and image binning are automatically set to the optimal values determined by the stage position (field of view) and cannot be changed by the user.

3.4 IVIS Imaging System 3D Series Options

The IVIS Imaging System 3D Series offers control options relating to the position of the CCD camera during image acquisition. These options are shown in Figure 3.10.
Angle indicates the starting position of the CCD camera relative to the imaging platform. The first image in a sequence is acquired at this angle.

Inc is the number of degrees between each successive position of the CCD camera during the acquisition of an image sequence.

Structure enables the structured light system for determining surface topography (for more details see page 18).

NOTE

There is no FOV option for the IVIS Imaging System 3D Series since images are acquired at the same FOV, but at different angles.
4  Image Display and Analysis Window

Image Display Tools ............................................. 22
Region of Interest Tools ........................................ 22
Physical Units ..................................................... 26
Display Mode ...................................................... 27
Image Correction Check Boxes ............................... 31
Print & Zoom ....................................................... 32
Image Window Contextual Menus ............................ 34

Figure 4.1 represents a typical image window display that appears when a Living Image data set is opened or acquired. The main part of the window is most often a pseudocolor image referred to as the luminescent or fluorescent image overlaid on a photographic image. Generally, anything that is described in this manual as being applicable to the luminescent image will also be applicable to fluorescent images unless otherwise noted. At the top of the image window are analysis tools used for controlling the image display and taking measurements. Individual controls in the analysis panel are described below. On the right side of the image window is a color bar that shows the relationship between the pseudocolors in the image and the numerical values of the image data. At the bottom of the window display is a section that contains labeling information generated by both the user and the imaging system. This section describes use of the individual controls found in the image window.

Figure 4.1  Living Image data set in the Image Window.
4. Image Display and Analysis Window

4.1 Image Display Tools

**Max Bar** sets the maximum value for the range of data associated with the color bar for the image.

**Min Bar** and **Min Bar Slider** sets the minimum value for the range of data associated with the color bar for the image. The minimum value also serves as a threshold below which data in luminescent or fluorescent images are not displayed. When creating overlays, this allows the underlying photographic image to be viewed in regions where top-layer luminescent data is below the threshold.

**Note**

While **Max Bar** and **Min Bar** can dramatically affect the appearance of the displayed image, the numerical values associated with the image contents DO NOT change and the resulting measurement is independent of display conditions. For more details on color bars and pseudocolor images, see Chapter 7, page 69.

**Log** can be used to change the pseudocolor display from a linear relationship between numerical data and displayed colors to a logarithmic relationship. In doing so, the range of the meaningful numerical data that can be displayed is significantly increased.

**Full** sets the **Max Bar** and **Min Bar** values to correspond to the maximum and minimum data values in the image, respectively.

**Auto** uses an algorithm to set **Max Bar** and **Min Bar** to levels that provide a visually pleasing display, while suppressing the background noise. However, the Auto settings may not always give desirable results, in which case further adjustments can be made manually using the **Max Bar** and **Min Bar** controls.

**Bright** adjusts the brightness of the photographic image.

**Gamma** adjusts the "gamma" of the photographic image, which is related to image contrast. This often improves the quality of photographic images, particularly for dark samples.

4.2 Region of Interest Tools

**Create** may be used to create one or more new Regions of Interest (ROIs), which are measurement tools used to quantify the amount of light emission in specific areas of the image. If the **Apply to Sequence** check box is present, new ROIs are created in all the images within the sequence. See Chapter 5, page 39. An example of a Circle ROI is shown in Figure 4.2. For more on measurements and ROIs, see Chapter 9, page 79.
The **Number** pull-down menu below **Create** determines the number of ROIs to be created. This control may also contain **Auto** choices that are used to automatically create one or more ROIs. See Chapter 10, page 87, as well as the discussion below.

**Shape** determines the shape of the ROI to be created. The shape of the ROI—Circle, Square, Contour, Grid—is determined by the **Shape ROI** pull-down menu. ROIs can be sized and positioned by dragging, or by using the ROI Properties panel. (To display this panel, double-click on the ROI or select **Living Image Tools - Show ROI Properties**.) Circles and squares can be stretched and rotated to make ellipses and rectangles. The most common way to handle rotation is to drag the ROI handle while holding down the **Ctrl** key (Windows) or option key (Mac). Alternatively, the **Angle** control in the ROI Properties panel may be used. Grid ROIs are particularly useful for microtiter well plate measurements. When **Grid** is selected in the **ROI Shapes** menu, the **Number** pull-down control changes to a pull-down listing standard sizes of well plates, e.g. 12x8, 24x16, etc.

**Type** determines the type of ROI to be created—**Measurement ROI, Average Bkg ROI, Subject ROI, Crop**. User-created custom ROI choices may also be added to the menu. See Section 6.4, page 53. For use of various types of ROIs, see Chapter 9, page 79, and Chapter 10, page 87. For details on **Crop ROI**, see Section 6.1, page 43.
Settings is located to the right of the type pull-down and is only displayed when Auto 1 or Auto All is selected in the Number pull-down menu. See Figure 4.4. This button is displayed during auto creation of both Measurement ROIs and Subject ROIs. Selecting this button opens up the settings panels described below. Also see Chapter 10, page 87.

The Auto ROI Settings panel controls the auto ROI settings for Measurement ROIs in the top image window. See Figure 4.5. The ROI Edge Value controls the fraction (displayed as a percent) of the peak value at which the ROI contour is drawn. If the Use Background Offset check box is checked and an appropriate Background ROI is set to be used as background for future ROIs (see the ROI Properties Panel and Contextual Menus) then the average background level in the background ROI will be removed before computing the ROI Edge Value. A background offset is also used when this box is checked and the new Measurement ROIs created are inside a Subject ROI that has a Background ROI linked to it. The Lower Limit controls the minimum peak height of an auto ROI created in an Auto All search. This limit is expressed as a multiple of the image window Min Bar setting. Min ROI size controls the minimum required ROI size (expressed in pixels) for auto creation of an ROI. Reset to Default Values restores the check box to "checked" and sets the three numerical values described above to the default values of 5, 2, and 20 respectively. Recall Preferred Values can be used to recall stored values for the three numerical controls previously saved using the Store as Preferred Values button. Once selected, these preferred values act as default values for all image windows. A set of values unique to each individual user can be stored. The user ID of the current user is displayed in these buttons. In Figure 4.5, the user ID is MDC. The Create Auto ROIs button allows the creation of auto ROIs in the top image window and is equivalent to creating ROIs with the Create button in the image window. This button
allows faster testing of different Auto ROI settings. The **Remove existing Auto ROIs first** check box controls whether existing ROIs in the top image window are removed before the creation of new Auto ROIs. This function is equivalent to the **Remove** button in the image window and exists merely to allow faster testing. **Done** closes the Auto ROI Settings panel. The selected settings are stored for later use with the top image window.

The Auto Subject ROI Settings panel controls the settings for drawing Subject ROIs in the top image window. See Figure 4.6. Subject ROIs boxes are used to enclose a particular subject, usually a single animal. Auto creation of Subject ROIs will attempt to locate individual animals in the photographic image and enclose each one with a Subject ROI. The default settings in this panel are optimized to locate animals, particularly light-colored mice on a dark background, but may also give good results with other types of subjects. **Cut-off parameter** controls a numerical value that may be used to "trim" distant parts of the animal, such as the tail, from the desired Subject ROI. The smaller the value, the larger the trim. **Scale Factor** is a multiplier applied to the size of the Subject ROI prior to display. A larger **Scale Factor** value may be used to offset the effect smaller values of the **Cut-off parameter** have on a Subject ROI. **Small ROI Limit A** sets a lower limit for the smallest Subject ROI created. This limit is expressed as a percentage of the largest Subject ROI to be created. By default, no Subject ROI will be created if it is smaller than 10% of the size of the largest Subject ROI. **Small ROI Limit B** sets a second lower limit relative to the size of the total image area. Both of these limits are useful in preventing the creation of Subject ROIs around very small parts of an image, such as reflections or dust. The **Thresholding Method** may be used to select one of several algorithms designed to locate the edge of individual animals within the image. The default is a **Bimodal Method** that works well for light colored animals on a dark background, but if problems are encountered the user can try other algorithms for, possibly, better results. Other controls, including **Reset to Default Values**, **Recall Preferred Values**, **Store as Preferred Values**, **Create Auto ROIs**,
4. Image Display and Analysis Window

Remove existing Auto ROIs, and Done—function as described above in the Auto ROI Settings panel.

![Auto Subject ROI Settings](image)

Figure 4.6 Auto Subject ROI Settings for auto creation of subject ROIs.

Measure initiates a measurement of any or all of the ROIs present in the Image Window. Measurement results appear in both the Lab Book and the Measurements Table. See "Lab Book," page 58 and "Measurements Table," page 59. A brief summary of the measurement results appears in the tools section of the image window above the image display. The pull-down located directly below the Measure button controls which ROIs are to be measured. The default for all measures is All, however, individual ROIs may be selected. If the Apply to Sequence check box is present, the specified measurement applies to all images in the sequence. For more details, see Chapter 5, page 39.

Remove may be used to remove the ROIs selected using the pull-down located directly below the Remove button.

Apply To Sequence is present in all images that are part of a sequence. See Figure 4.7. If this box is checked, many of the image window controls and their actions are set to modify all the images contained in the sequence. See Chapter 5, page 39.

![Apply to Sequence](image)

Figure 4.7 Apply to Sequence option is available for images that are part of a sequence.

4.3 Physical Units

The Units pull-down menu is located immediately to the right of the Remove button. It can be seen in Figure 4.8, where it is set to Photons. This pull-down allows users to control absolute calibration of image measurement data, one of the most powerful features of the Living Image software and IVIS Imaging Systems. For more details, see Chapter 9, page 79. Individual settings for this control—Counts, Photons, Efficiency—are described below.
Counts allows the user to select the basic uncalibrated, raw data mode of image display. In this mode, image pixel contents are displayed as the numerical output of the charge digitizer on the CCD. This numerical output is often referred to as counts, analog digitizer units (ADU), or relative luminescence units (RLU). It is a number that is proportional to the number of photons detected in the pixel. CCD digitizers on all models of IVIS Imaging Systems are 16-bit devices, which means that the number can vary from 0 to ~60,000. When a region of interest is integrated, the units become photons/second and represent the number of photons per second that radiate omnidirectionally from the region specified. The value displayed in an image may sometimes be slightly outside this range due to corrections made on the image data. See Section 4.5, page 31.

Photons displays image data in terms of absolutely calibrated photon emission from the source. It is important to understand that while raw data (Counts) is a measurement of photons reaching the CCD detector, the data displayed in Photons mode is calibrated for all instrument settings and parameters, and thus represents the actual physical emission from the sample being imaged. Therefore, it is possible to compare measurements made with different instrument settings (e.g., differing exposure times, binning, FOV, etc.) as well as to compare measurements made on different instruments. In Photons mode, the quantity displayed is termed "radiance" and the units are photons/s/cm²/sr. In order for the displayed image to be calibrated, some of the image corrections described in Section 4.5, page 31 must be applied. When Photons mode is selected, these corrections are applied and the corresponding controls are deactivated. See Chapter 9, page 79.

Efficiency is a choice present for fluorescent images only (available only on IVIS Imaging Systems equipped with fluorescent capabilities). If Efficiency is selected, the fluorescent emission image is normalized (divided) by a stored reference image of the excitation light intensity, which is measured for each excitation filter at every FOV and lamp power. These reference images are measured and stored in the Living Image folder prior to instrument delivery. Normalizing fluorescent images by the incident excitation intensity not only converts the display to useful units, but also reduces the effect of spatial variations of the incident light. See Chapter 14, page 107.

4.4 Display Mode

The Display Mode pull-down menu is located immediately to the right of the Units pull down. It is used to control the type of image being displayed. The choices are described below. See Chapter 7, page 69.
Photograph is a display mode that shows only an underlying grayscale image, which is captured when the IVIS imaging system illumination lights are activated. See Figure 4.10.

Luminescent is a display mode that shows only the pseudocolor luminescent image obtained in an exposure with the IVIS Imaging System illumination lights deactivated. An example is shown in Figure 4.11. For image data taken in fluorescent mode on IVIS imaging systems with fluorescent capability, the equivalent display mode is termed fluorescent, rather than luminescent. See Chapter 7, page 69.

Overlay is the default display mode where either a luminescent or fluorescent pseudocolor image is overlaid on a photographic image. See Figure 4.12. The lower limit of the pseudocolor image is controlled with the Min Bar control, which sets a threshold below which data in the luminescent or fluorescent image is not displayed. This allows the underlying photographic image to be viewed in regions of the image.
where the luminescent or fluorescent data is below the established threshold.

![Figure 4.12](image.png)

**Figure 4.12** Section of an image window in Overlay display mode. The image comprises a luminescent image overlaid on a photographic image.

**Blend** displays a blended image created from a combination of photographic and luminescent (or fluorescent) images where the luminescent image is partially transparent. When **Blend** is selected, a slider control appears and may be used to control the degree of opacity of the luminescent image. See **Figure 4.13**. A value of one is totally opaque and is effectively an overlay. A value of zero is totally transparent and is effectively a photographic image. This mode is useful when you want to see the surface of the sample underlying the pseudo color.

![Figure 4.13](image.png)

**Figure 4.13** Blend slider controls the opacity of a blended image.
4. Image Display and Analysis Window

**Dark Charge** displays the dark charge image stored in the image data set. When viewing this image, it should be understood that dark charge images are multiplied by a factor (typically 10) that is stored along with the image data. Hence, the displayed numerical values are not necessarily the values being subtracted from the image data. See Chapter 12, page 99.

**Saturation Map** displays the region of the image, if any, for which photon emission is too intense to be quantitatively recorded when detected by the CCD camera. ROI measurements should not be made on these parts of the image. Measurements in other parts of the image not containing the saturated pixels will still be accurate (unless the image is badly saturated).

**Fluorescent Bkg** displays the instrument fluorescence background (optional) that may have been taken with a fluorescent image. The
background is an instrument background and is similar in concept to a "dark charge image" taken in luminescent mode. See "Fluorescent Background," page 52.

### 4.5 Image Correction Check Boxes

Image Correction checkboxes are located directly under the Display mode pull-down. They may be used to make limited corrections and modifications to a luminescent image, depending on how the image was acquired. Individual controls are discussed below.

**Sub Dark Charge** determines if a dark charge correction has been applied to the image data. This box will be present only if a dark charge measurement has been made within 48 hours of the image being acquired. Dark charge measurements can be taken manually or automatically during the systems nightly calibration and diagnostic routine. See Chapter 12, page 99. This same box may be labeled **Sub Bias Only** if a somewhat less accurate measurement, or "bias image", of the dark charge was used at the time the image was acquired. Although slightly less accurate than Sub Dark Charge, Sub Bias Only is perfectly acceptable for measuring bright subjects using low sensitivity settings, e.g. Integration Time=30 seconds, Binning =Small. When this box is checked, and a dark charge image or bias measurement is present in the image data set, it is subtracted from the luminescent image. If available, dark charge is automatically subtracted when the image is acquired or loaded. This is usually the desired situation and, typically, the user has no reason to uncheck the box. In certain instances when this correction must be made, e.g. when using calibrated photons mode, the control will be deactivated to prevent user modification.

**Flat Field** determines if a lens correction related to lens distortion has been applied. All lenses are more efficient at collecting light in the center of the image than near the edges. This efficiency curve is very reproducible and is measured for all IVIS Imaging Systems. The **Flat Field** correction is a multiplier that varies with distance from the center of the image and is applied to the values in the luminescent or fluorescent image. This normalizes the data values throughout the luminescent image to that of the value in the center of the image. If flat fielding calibration data is present in the image data set, a flat field correction is applied at the time the image is acquired or loaded. Use of the Flat Field is usually the desired situation and the user typically has no reason to uncheck this box. See Chapter 8, page 73.

**Cosmic** determines if a correction has been made that removes small bright spots from the luminescent or fluorescent image. These bright
spots appear in longer exposures and are due to the interaction of cosmic rays or other ionizing radiation in the CCD. Such an interaction typically results in a large signal in one pixel, or possibly several, pixels. The “artifact” signal is easy to identify and the affected pixel contents are replaced with a signal average of neighboring pixels. By default, the cosmic correction is applied at the time the image is acquired or loaded. The user should uncheck Cosmic only when viewing images that have isolated hot spots that are only a few pixels wide, such as images of individual cells.

**Sub Fluor Bkg** is a check box that is present if a fluorescent background has been applied to the fluorescent image. See Section 14.3, page 112. A fluorescent background incorporates a dark charge measurement, so either the Sub Dark Charge or the Sub Fluor Bkg check box can be selected, but not both.

![Print & Zoom settings](image)

**Figure 4.17** Sub Fluor Bkg option is available to fluorescent images for which a fluorescent background was acquired.

### 4.6 Print & Zoom

The **Print** button is located under the Units pull-down and is used to print the entire image window along with the color bar and the label section below the image. To print parts of the image rather than the entire window, see "Save Graphics," page 43, or "Export Graphics," page 44. Both Save Graphics and Export Graphics are used in conjunction with the Crop ROI tool.

The **Zoom** button is located directly beneath the **Print** button and is displayed only when a region of the image has been selected for zooming. To select a region, click-drag the mouse over the area of interest. Click the **Zoom** button to create an enlarged display of the area. The button will automatically change to **No Zoom** status. Click **No Zoom** to return to the original view. A zoomed image can be zoomed further using the same click-drag process.
Figure 4.18  Zoom button is available after an image region is selected by click-dragging the mouse.
4.7 Image Window Contextual Menus

Contextual menus are pull-down menus that vary based on the context of the operation the user is attempting. Several contextual menus are available in the image window and can be used to perform a variety of useful functions. Some contextual menus are redundant with other controls or menu items within Living Image software but provide a simpler way to achieve the desired action. In other instances, the contextual menu may be the only means by which to achieve the desired action. Contextual menus provide the user with a number of added conveniences and should be thoroughly explored.

The Image Contextual Menu is displayed by right-clicking (PC) or command-clicking (Mac) inside the image display area of the image window. See Figure 4.19. The menu appears near the click point and contains some or all of the items shown. The top section of the menu has functions for editing ROIs. When a particular ROI is selected, the menu will vary. These variations are described below. The remainder of the menu contains items used to create various types of ROIs. Unlike the Create tools found at the top of the image window, these menu items act relative to the click point used to activate the contextual menu, thus providing a way to make an ROI at a particular location within the image. There is no major advantage provided here over the Create tools. However, in some cases, ROIs may be created more rapidly, and with a little less clicking and dragging. Create Manual creates one ROI at a time, while Create Auto is equivalent to the Auto 1 function in Create tools. The Cancel command deactivates the contextual menu and is equivalent to simply releasing the mouse button.

![Image Contextual Menu](image)

Figure 4.19 To view the Image Contextual menu, right-click (PC) or command-click (Mac) in the image display region of the image window.

The Image Contextual Menu as displayed when the Apply to Sequence box is checked is shown in Figure 4.20. The functionality of this menu is identical to that of the previous Image Contextual Menu when the Apply to Sequence box is either not present or unchecked. However, when the Apply to Sequence box is checked, any action taken will be applied to all the images within the sequence. For example, creating an ROI at a particular location within the displayed image will create an ROI at that same location in all the images within the sequence.
Subsequent dragging and resizing of the ROI will also be reflected in all the images within the sequence as long as the Apply to Sequence box is checked.

![Image Contextual Menu](image)

**Figure 4.20** To view the Image Contextual menu, right-click (PC) or command-click (Mac) in the image display region of the image window. This menu indicates that the Apply to Sequence option has been checked.

The Measurement ROI Contextual Menu is activated by right-clicking (PC) or command-clicking (Mac) on a Measurement ROI. See Figure 4.21. The top section of this menu contains items similar to those found in the Image Contextual Menu plus some additions specific to the selected ROI. The second section of this menu may be used to activate the ROI Properties panel (To display this panel, double-click on the ROI or select **Living Image Tools-Show ROI Properties**.) This panel may be used to display and modify a number of ROI properties, including **Lock Position** and **Lock Size**, which can also be modified from the contextual menu. More often, these commands will be used to **Unlock Position** and/or **Unlock Size** for ROIs that are automatically created throughout a sequence but require subsequent manual position adjustments. The **Set Bkg ROI** items provide the ability to link Background ROIs to Measurement ROIs. See Chapter 9, page 79. When the user selects the appropriate **Set Bkg ROI** menu item, the chosen Measurement ROI can be linked to that Background ROI. Similarly, users can link Measurement ROIs to Subject ROIs using the appropriate **Set Subject ROI** menu items.
4. Image Display and Analysis Window

The Measurement ROI Contextual Menu as displayed when the **Apply to Sequence** box is checked is shown in Figure 4.22. The functionality of this menu is identical to that of the previous Measurement ROI Contextual Menu when the **Apply to Sequence** is either not present or unchecked. However, when the **Apply to Sequence** box is checked, any action taken will be applied to all the images within the sequence.

The Background ROI Contextual Menu is activated by right-clicking (PC) or command-clicking (Mac) on a Background ROI. See Figure 4.23. This menu contains items similar to those found in the Measurement ROI Contextual Menu plus others that are appropriate for using the Background ROI for Measurement and Subject ROIs. Similarly, the user may establish a link from either direction. Use the **Set Bkg ROI** to link a single Measurement ROI and a single Subject ROI.
Background ROI, or establish the link in reverse (starting with the Background ROI) with the Use As Bkg ROI. Where there are multiple ROIs, however, one menu may be preferable to the other in order to save time. For example, if several Measurement ROIs exist and all are to be linked to a single Background ROI, it may be best to select the Use as Bkg ROI for All Measurement ROIs item found in the Background Contextual menu. Use as Bkg for future ROIs sets the selected Background ROI so that it will be linked to any new Measurement or Subject ROIs created. Only one Background ROI per image (or sequence) can be set for future use. This item is particularly useful in conjunction with the Use Background Offset check box in the Auto ROI Settings Panel (see Chapter 10, page 87).

![Figure 4.23 To view the Background ROI Contextual menu, right-click (PC) or command-click (Mac) on a Background ROI.](image)

The Background ROI Contextual Menu as displayed when the Apply to Sequence check box is checked is shown in Figure 4.24. The functionality is identical to that of the previous Background ROI Contextual Menu when this box is either not present or unchecked. However, when the Apply to Sequence box is checked, any action taken will be applied to all the images within the sequence. (This also is true for Use as Bkg ROI for all Measurement ROIs and other similar menu items that do not necessarily indicate their applicability to all images within the sequence.) Checking the Apply to Sequence box provides a convenient way to assign a single background ROI, such as one positioned on a control animal, to multiple Measurement ROIs in different images. If the different images are not in a sequence, see “Load as Group”, page 45.
The Subject ROI Contextual Menus are not shown in any figures, but are quite similar in content and logic to the Background ROI contextual menus. They can be used to link a subject ROI to one or more Measurement or Background ROIs. And there are appropriate changes to the menu items and actions when the Apply to Sequence check box is checked.

Zoom/Crop is a small contextual menu that is activated by clicking inside the selected region of the image window. See Figure 4.25. To Zoom in, click-drag the mouse over the desired area inside the image region of the image window. Using the Zoom menu item is equivalent to clicking the Zoom button. If the Crop menu item is selected, a crop ROI is created around the selected region. See "Save Graphics," page 43, or "Export Graphics," page 44.
5 Sequence Window

Display Tools .......................................................... 40
Sequence Editing ....................................................... 41

Figure 5.1 shows a typical Sequence Window display of a Living Image data set that has been saved as a sequence. Sequences may be created from images acquired simultaneously at acquisition or grouped together from a collection of individual images. The sequence shown in Figure 5.1 was created from a group of six individual images taken on different days. See Chapter 3, page 9, and “Load as Group,” page 45.

Figure 5.1 Sequence data set in a Living Image Sequence Window.
5. Sequence Window

5.1 Display Tools

**Aggregate Color Range** controls the setting of a common color scale for all the images in the sequence. A color bar applicable to all the images is created, as well. **Autoscale** attempts to select a color range in which all the images will be displayed appropriately. See Figure 5.2. Note that the thresholds for the last two images in the sequence are somewhat lower than desirable. This is a common result for an Aggregate Color Range as it may be difficult to find a single color range appropriate for all images. **Full** may be used to display the entire data range. **Manual** allows the user to set the color range bar manually using the **Min Bar** and **Max Bar** controls. See Section 4.1, page 22.

![MDC20040219115115_SEQ](image)

**Figure 5.2** Sequence window displaying an autoscaled aggregate color range. The color bar (right) applies to all images in the sequence.

**Individual Color Ranges** controls the setting of a color range for each image in the sequence individually. See Figure 5.1. No color bar appears since the color bar is different for each image. The **Autoscale** and **Full**
controls serve the same function as described for **Aggregate Color Ranges** except the ranges are set for each image individually.

**Bright** adjusts the brightness of all photographic images in the Sequence Window.

**Gamma** adjusts the "gamma" of all photographic images in the Sequence Window. Gamma is related to image contrast and often improves the quality of photographic images, particularly for dark samples.

**Log** can be used to change the pseudocolor display from a linear relationship between the numerical data and the displayed colors to a logarithmic relationship. This can greatly increase the range of the numerical data that can be meaningfully displayed.

**Units**, shown set to Photons in Figure 5.1, allows users to change the units displayed in the Sequence Window.

**Display** and the pull-down directly to the right control the display of individual image windows. The pull-down may be set to **All** or used to select any individual image. See Figure 5.1. Subsequent use of the **Display** button will open individual image windows. Because the Sequence Window does not have measurement tools, individual image windows must be opened in order to do image analysis. Double-clicking on the desired image in the Sequence Window will also open an individual image window.

**Use Current Colors** sets the color bars for individual image windows that are opened from the Sequence Window to be the equivalent of the color bar of each image in the Sequence Window.

**Prev Saved Colors** sets the color bars in the individual image window to the settings that were saved in the image data file.

### 5.2 Sequence Editing

**Edit**, shown in Figure 5.1 can be used to open the Sequence Editing Window. See Fig. 5.3. Any changes made to the contents of the sequence may be saved using **Living Image-Save Living Image Data**. See "Save Living Image Data," page 48.
Sequence Clicks are the click numbers for the images currently contained in the sequence.

Browser Clicks are the click numbers for any other images contained in the Living Image Browser Window but not in the sequence.

Copy may be used to make a copy of a selected image in the Browser Clicks section and add it to the current sequence.

Retire may be used to move a selected image from the sequence into the Retired Clicks section.

Reactivate moves Retired Clicks back into the sequence.

Done closes the window and updates the contents of the edited sequence.
6 Main Menu Bar

The Main Menu Bar in Living Image software contains active items, including File, Edit, Living Image, Living Image Tools, and Help, as well as a number of deactivated Igor Pro menu items. Although Igor Pro menu items are not directly supported by Living Image software, advanced users may choose to activate them by selecting Igor Menu Enable from the Living Image Tools menu. See "Igor Menus", page 67. Care should be taken that users have studied the Igor Pro manual and are knowledgeable in the use of these menu items before activating them.

6.1 File

File menu functions supported directly by Living Image software are limited to Save Graphics, Page Setup, Print, and Print Image. They are described below.

Save Graphics is active when an appropriate graphics window, such as the Image Display window, is the top window. It allows the user to save the image window as a graphics file, suitable for export to another application, e.g., Microsoft PowerPoint. The dialog presented allows the user to choose from a variety of file formats. If QuickTime is installed, several additional file format choices will be available. For a free download of QuickTime (Windows and Macintosh), visit www.apple.com/quicktime. When installing QuickTime, select the Recommended (vs. Minimum) configuration to implement the additional file formats successfully.

It is important to remember that Save Graphics is saving a graphics file and not the actual image data set, which is saved using Save Living Image Data in the Living Image menu. See Section 7.1, page 69.

Page Setup performs the usual function of specifying the paper size, orientation, and other printing functions. Page Setup specifications are window specific and will vary, depending on which window is active.

Print, in various forms, e.g., Print Layout, Print Notebook, is used to print the active window, as long as it is supported by the Print
command. For Living Image software, this includes windows such as Lab Book and Layout. It does not include the Living Image image window. To print an image, use the Print button in the image window or select Print Image from the File menu.

Print Image is active when an image window is the top window. It will print this window. The Copy Image to Layout command located in the Living Image menu is also useful for printing image windows, especially if the user wants to print more than one image window per page.

6.2 Edit

Edit menu functions supported directly by Living Image software are limited. Which Edit menu functions are active depends on which window is active, e.g., many more text functions are active when a text window such as Lab Book is active. Among the active functions in the Edit menu that have uses customized to Living Image are Copy and Export Graphics. These are described below.

Copy is active when an active window contains text or images that may be copied to the clipboard. If the active window is an image window, the entire window is copied unless a Crop ROI is present. If a Crop ROI is present, only the cropped region is copied to the clipboard. For details about creating a Crop ROI, see Section 4.2, page 22.

Export Graphics is active when the top window is an image that can be exported. Export Graphics functions in nearly the same way as Save Graphics, described in the File menu, Section 6.1, page 43, except that the resulting graphic is pasted into the clipboard instead of being written to a file. This is useful for transferring images to other applications, e.g., Microsoft PowerPoint. The dialog presented allows the user to choose from several file formats. If QuickTime is installed, several additional file format choices will be available.

6.3 Living Image

The Living Image menu contains a variety of selections, which may be used to control several basic functions. They are described below.

Browse for LI Data opens the Living Image Browser window. Most saved Living Image data will be located and opened using this tool. When using this tool, select the folder containing the image(s) of interest, not the specific image you want to open. The results of a directory search for Living Image data using Browse for LI Data are displayed in Figure 6.1. If the Living Image Browser window is not empty, using the menu item Browse for LI Data opens the window, or brings it forward where it can be modified using the controls described below. If the Living Image Browser window is empty, Browse for LI Data opens a dialog window that is used to locate a directory, which may then be searched for Living Image data and the results displayed in the browser window. Displayed data is composed of either individual images or sequences of images. Contents of sequences can be displayed by using the +/- control in the leftmost column of the browser window.
Each entry (also known as a "click") represents a collection of image data stored in a directory named with the identifier shown in the Click Number column. See Section 13.1, page 103.

<table>
<thead>
<tr>
<th>Click Number</th>
<th>User ID</th>
<th>Series</th>
<th>Experiment</th>
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**Figure 6.1** Living Image Browser Window is used to locate and open most Living Image data.

The **Load** button may be used to open one or more selected image sets. If the resulting images are individual images, they will be displayed in image windows of the type described in Chapter 4, page 21. If the resulting images are sequences, they will be displayed in a sequence window. See Chapter 5, page 39. Double-clicking on an entry will also load the image.

**Load As Group** enables you to select one or more images and open them in a new sequence window (Figure 6.2). Saving this data creates a stored sequential data set, which future browses will display as a sequence. This is the basic method for creating sequences from data that is captured originally as individual images, e.g., data acquired on different days.

**NOTE**

The original individual data files are not altered in any way when creating and storing the new sequence, they are simply copied.
Sort allows the displayed data to be organized by a prioritized sort with up to three levels controlled by the Browser Sort panel. See Figure 6.3. Alternatively, clicking on any individual column heading will perform a single level sort relative to that column.

Sorting is useful for locating a particular image set when a large number of entries are present in the window. Locating a particular image is greatly aided by proper use of the data labeling tools provided by Living Image. These tools are discussed in more detail in Chapter 13, page 103, and Living Image Tools "Change Top Image's Info," page 57. The Columns pull-down menu controls which columns are displayed in the browser window. A number of selections may be present in this pull-down menu, with All Populated Values being the default. This selection brings up a window that displays every column within the browsed data set for which an entry has been located. All Possible Values shows every possible column as defined by the entries.
set up currently in all Label Name Sets. For more information on Label Name Sets, see Chapter 13, page 103, and "Preferences," on page 53. Additional entries include the Label Name Sets currently defined, which will produce a display containing columns of results specific to a particular Label Name Set. Custom configurations as defined by Customize in the Columns pull-down menu will appear as separate entries, e.g., Fig. 6.3 shows the three Label Name Sets that come standard with Living Image software, plus a single custom configuration called "Mike's Demo."

![Figure 6.4 Columns pull-down menu items in the Living Image Browser Window.](image)

The Add button opens a dialog with which a directory may be located, searched, and the Living Image data in the directory added to the current contents of the browser window. Remove removes all selected entries from the browser window except those displayed in an open image window. Remove All removes all entries from the browser window except for those currently displayed in an open image window. Export allows the contents of the browser window to be exported to a text file that can be opened from another application. The browser window contents may also be copied and pasted into another application. Measurements opens and displays the Measurements Table, and is equivalent to using Measurements Table in the Living Image Tools menu.

Load LI Data allows the user to find and open a single Living Image data set. A Living Image data set consists of a folder containing several files. See Chapter 13, page 103. The user may load the entire data set by selecting and opening any single file contained in the data set. Living Image data must be opened with this menu item or by using the browser.

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**NOTE**

Double-clicking on the folder containing the data set or on the files within the folder will not allow access to the data.

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The user can create an alias (Macintosh) or shortcut (Windows) of the Igor Pro application that can be used to open a Living Image data set. The alias or shortcut can reside on the desktop or other easily accessible
location. Any file inside a Living Image data set that is dragged onto the Igor icon when Living Image is running, will initiate a load of the entire Living Image data set.

**Load TIFF Data** allows general TIFF data to be loaded into Living Image for subsequent display and analysis. In this way, data may be imported from other imaging hardware or software. **Load TIFF Data** is used infrequently, if ever, by most Living Image users. This is the only way to import numerical scientific TIFF data. See Section 7.1, page 69.

**Save Living Image Data** saves the data displayed in the active image window to a Living Image data set. This menu item opens the Save Data Window if the active image window is an individual image window. See Figure 6.5. The Save Sequential Data window opens if the active data window is a sequence window. See Figure 6.6. The default for both, **All Living Image Data Files** - saves a full Living Image data set to a folder. The name of the directory will be the click number of the displayed images. See Chapter 13, page 103. The alternative to this may vary, depending on the display mode chosen in the image window or the type of image window. For an individual image window, most display modes, such as overlay, luminescent, etc., allow users to select **Luminescent Image TIFF File**, which saves a TIFF file containing the numerical (rather than graphical) content of the image. See Figure 6.5.

![Figure 6.5 Save Data Window.](image)

![Figure 6.6 Save Sequential Data Window.](image)
For a photographic display mode, the selection, **Photographic Image TIFF File**, saves the numerical contents of the photographic image. For a blended image display mode users can elect to save the blended image as a Windows or Macintosh graphics file. These options, which may be used to export Living Image data to other applications only, will appeal only to a select group of users. Most will find that using **File** menu item **Save Graphics** or **Edit** menu item **Export Graphics** is preferable, unless the image analysis is to be done on the exported image data that will require numerical TIFF data. For a sequence window, the selection **PNG Bitmap File...** saves the displayed sequence window as a PNG graphics file. The **Save Lab Book** check box controls whether the current Lab Book will be saved together with the Living Image data files. (Subsequent openings of the Lab Book file are controlled from a setting in **Living Image Tools - Preferences** menu item.) **Save** will save the data as a new set of files if the data have never been previously saved. If the data have been previously saved, **Save** will update the descriptor file “AnalyzedClickInfo.txt” to record the latest image analysis and display conditions. **Save** will never overwrite the original image data files. Use **Save Copy** to save a complete data set to a different directory. **Save Copy** creates a new copy of the existing data only. **Cancel** closes the window and cancels the save.

**Copy Image to Layout** provides a page layout capability for displaying and annotating one or more images. This option produces the Auto Layout Control panel (Figure 6.7). This panel may be used to copy open images and paste them into a "layout" window. The layout window is a simple graphics manipulation window in which one or more images may be arranged and text and drawings added using a standard set of tools. The resulting layout may be printed using either the button in the Layout Control window or the **File - Print Layout** menu item, which is available whenever a layout is the top window.

![Auto Layout Control Panel](image)

**Figure 6.7** Auto Layout control panel for laying out and printing multiple images.
Using the **Select Layout** and **Page Orientation** pop-up menus in the Auto Layout Control panel, select an existing or new layout for the desired number of images - 2-up, 8-up, and so on. Click **Copy Image to Layout** to copy the topmost image to the selected layout. The image will be sized automatically and placed in the first open position, or slot, in the layout. The upper left corner of the image will "snap to" the defined position for that slot. Each additional image will be placed in the next open position. If necessary, any existing images will be repositioned automatically. An image may be repositioned at any time by dragging it to another slot in the layout. To use the manual method for positioning and sizing images in a layout, click the **Use Manual Layout** button.

**Analysis Option** contains the submenus **Histogram Measurement ROIs**, **Structured Light Analysis**, and **Spectral Analysis**, which control various analysis functions.

- **Histogram Measurement ROIs** toggles to/from a mode where a histogram plot of the contents of the measured ROI(s) is created when a measurement is made. This option is seldom used for in vivo imaging.

- **Structured Light Analysis** is available only on the IVIS Imaging System 200 Series and 3D Series. This option launches a procedure that analyzes structured light images to obtain the 3D surface topography of the animal. See Chapter 16, page 139 for details of the analysis procedure and theory of operation.

- **Spectral Analysis** is only available only on the IVIS Imaging System 200 Series or IVIS Imaging System 50 and 100 Series fitted with an optional spectral imaging filter set. This option launches a procedure for determining the depth of a source in tissue based on spectral information. See Section 15 for details of the analysis procedure and the theory behind this algorithm.

**Dark Charge** submenus - **Measure**, **Replace**, **View Available**, and **Clear Available** - allow the user to manipulate the dark charge image associated with the image data set. Because the submenus are available only in the acquisition version of Living Image software (installed on the IVIS® Imaging System acquisition computer), all dark charge image manipulation must occur at the time the image is acquired. Typically, the manipulation tools provided in the Dark Charge menu are used only if a problem arises, as the automatic dark charge functions within Living Image software usually result in an appropriate dark charge image stored with the image data. See Chapter 12, page 99.

- **Measure** initiates a detailed measurement of electronic dark charge sources, which are dependent upon the luminescent exposure time, luminescent binning level, and the camera temperature. Before starting a measurement it is important to ensure that the appropriate values are entered in the Camera Control panel. The temperature should be locked at the appropriate Demand Temperature.

Upon starting the dark charge measurement, a series of messages will ask the user if the imaging parameters selected are those desired, and inform them of the time it takes to complete the measurement. The dark charge measurement is actually done with the camera shutter closed; so low-level luminescent samples may be left in the chamber. However, the door must remain closed.
during the measurement as the camera shutter is not light tight and, therefore, cannot prevent interference from higher levels of light, such as room lights. A series of zero-time exposures to determine the bias offset and read noise will then start followed by three dark exposures. Typically, the dark charge measurement takes more than three times as long to complete as the equivalent luminescent exposure. Upon completion of the dark charge measurement, a folder containing the data is created and stored in a dark charge folder in the Living Image Files folder. An entry corresponding to this measurement will also be placed in the View Available dark charge summary. See below.

**Replace** replaces the dark charge image associated with an image with a different dark charge image. This may be useful if it is determined that there is a problem with the dark charge measurement or if an appropriate dark charge image does not exist at the time of the image acquisition and there is a need to be remeasure it. The **Replace** command will offer to use an appropriate (correct exposure time, binning, temperature) available dark charge, if one exists. (This is typical if the dark charge measurement is made within a short time after image acquisition.) The user can accept this dark charge or refuse it, in which case a dialog is offered, allowing the user to manually select the desired dark charge parameters. If there is no appropriate dark charge image available, the **Replace** command automatically offers a manual selection dialog.

**View Available** displays a summary of the most recent dark charge image taken for each unique set of luminescent exposure time, luminescent binning, and camera temperature. Dark Charge images on the list that are more than 48 hours old will not be used by Living Image software automatically, but may be added to the image data set manually, using **Replace**.

**Clear Available** clears the Available Dark Charge summary that is displayed with **View Available**. No dark charge data is erased - the data are still contained in the dark charge folder as described above. Only the summaries are removed.

![Figure 6.8 Fluorescent Background Menu and Submenu Items.](image-url)
Fluorescent Background submenus – Add or Replace, Measure, Measure and Replace, Clear Available, and View Available Fluorescent Backgrounds – allow the user to subtract unwanted instrument fluorescence background from the fluorescent image, including autofluorescence due to anesthesia equipment, optics, or the chamber and any residual light leakage though the filter.

The submenu items are only available in the acquisition version of Living Image software when fluorescence capability has been installed on the IVIS Imaging System acquisition computer. Therefore, all fluorescent background manipulation must be done at the time the image is acquired.

Unlike Dark Charge measurement, Fluorescent Instrument measurement is done with the shutter open, lamp on, and excitation and emission filter in place. The chamber must not contain the sample during this process. Once complete, the fluorescence background can be subtracted from the appropriate fluorescent image in which the sample is present in the imaging chamber.

The **Sub Fluor Bkg** check box located in the upper part of the image window controls subtraction of this fluorescent background image. The check box appears if a fluorescent background was measured at the time a fluorescent image was acquired. See Figure 6.9. If a fluorescent background is present it may be subtracted from the image rather than the dark charge only (or bias image if no dark charge images are present). See Section 4.5, page 31. Both boxes cannot be checked at the same time.

The Fluorescent Background submenus are described below.

**Add or Replace Fluorescent Background** allows the user to either add a fluorescent background to an image or replace the existing fluorescent background with that of a previously acquired fluorescent background that has the desired imaging parameters (binning, time, FOV and f/stop). If two or more backgrounds are available with identical imaging parameters, the Living Image software chooses the one measured most recently.
Measure Fluorescent Background initiates a measurement of the fluorescent background and, upon completion, enters and stores the fluorescent background measurement in the Available Fluorescent Background table, which summarizes all fluorescent backgrounds taken during the current imaging session. See Figure 6.10. If an equivalent entry is already present in the Available Fluorescent Background table, the table will be updated and the more recent entry will be used for subtraction. All backgrounds in the table will be available for use in subsequent images taken during the session only. Fluorescent backgrounds are lost when Living Image software is closed because (unlike Dark Charge measurements) no data file is created.

Measure and Replace Fluorescent Background initiates a measurement of a fluorescent background as described above. Upon completion, the entry replaces the fluorescent background measurement of the active (topmost) fluorescent image displayed.

Clear Available Fluorescent Backgrounds clears all of the available fluorescent backgrounds from the Available Fluorescent Backgrounds table. Cleared entries are no longer available for use in subsequent fluorescent images.

View Available Fluorescent Backgrounds displays a summary of the fluorescent backgrounds that have been measured, entered, and stored in the Available Fluorescent Backgrounds table during the current Living Image session. See Figure 6.10

Living Image Help displays any one of a number of available Living Image Help documents.

Quit (Macintosh®) or Exit (Windows®) quits Igor Pro and therefore Living Image. This is equivalent to the File menu item Quit/Exit.

6.4 LI Tools

The LI Tools (Living Image Tools) menu and submenus contain a range of functions that may be used to control image analysis.

Preferences allows an individual user to set a variety of user-specific options using the Preferences submenus described below. These settings are saved for each user and are identified by the user ID at log-in.

Existing User ID selects an existing user profile (a two- or three-letter ID) from the Existing User ID pull-down menu, e.g., MDC.
Selection of an ID sets all the customizable values to those last saved by this user.

**Figure 6.11** Preferences settings for customizable options.

- **New User ID** adds a profile to the **Existing User ID** list.
- **Delete This User ID** deletes the profile selected in the **Existing User ID** pull-down menu.
- **Label Name Set** selects the default **Label Name Set** for the user, i.e., the names associated with the user-supplied labels stored with each image. See Chapter 13, page 103. These labels provide a system for identifying and locating image data. Several standard **Label Name Sets** are provided with Living Image software and customized versions can be created.
Edit User Label Choices displays a panel that allows a set of standard values to be defined for each label. See Figure 6.12. For example, the values in the right hand column will be saved as standard values for the selected label name in the left hand column. These values are then available as pull-down menu choices when image labels are being created at the end of image acquisition or when image information is changed. See “Living Image Tools - Change Top Image’s Info,” page 57.

By selecting a label in the left hand column, the buttons on the right side of the panel may be used to create and edit the standard values associated with the label selected. The up and down arrows on the labels column allow users to scroll through the labels listed (as do the up and down arrows on the keyboard when the labels column is active). Simply clicking on the label value to be edited works as well. Upon closing this panel, the current values will be saved and available whenever the current user’s ID is active.
6. Main Menu Bar

Camera Settings is present only on the acquisition version of Living Image software (installed on the IVIS Imaging System acquisition computer). Clicking this button opens the Camera Settings panel. See Figure 6.13. This panel sets values for the indicated camera parameters. Whenever the IVIS Imaging System is initialized, these parameters will be reset to the indicated values.

Select Default Units pull-down menu determines the units to be used when acquiring or opening an image. The default value is photons. See Chapter 9, page 79.

Lab Book Setting specifies whether to replace the Lab Book when loading analyzed data, retain the Lab Book, or display a prompt before replacing the Lab Book.

Autosave Directory allows the user to specify a directory (folder) into which all images will be saved automatically. This control is only present in the acquisition version of Living Image software (installed on the IVIS Imaging System acquisition computer). The directory path can be specified either by typing in the reference or by using the Browse button to locate an existing directory. When the Autosave On check box is checked, all newly acquired images will automatically be saved into this directory.

The Save ROIs button allows the user to save one or more ROIs for use in subsequent analyses. See, Section 4.2, page 22. A Save button on the Preferences panel is present only when the top image window contains one or more ROIs. Clicking the Save button will save the size, shape, and position of all of the ROIs in the image window to a file, and the file reference will be stored in one of the five files located directly below the button. The numerical control to the right of the button determines which of the five files will be used to store the ROI information.

For all items in the file list that have the check box at the far right checked, a menu item will be added to the ROI Types pull-down menu in the image window. See, Section 4.2, page 22. Menu item names are specified in the Menu Names boxes directly next to the

Figure 6.13 Set values for some camera settings in this panel after system initialization.
checkboxes. For example, in Figure 6.11, page 54, the menu items that will be added to the image window ROI Types pull-down menu will be My ROI, Fred’s ROI, and May 11 ROI.

The file references in the file list can be manually linked to an existing file either by typing in the file path or by using the Browse button to locate the desired file reference. The Clear button deletes an existing file reference and clears the entry. The file will not be erased and may be relinked later.

Done saves the current information in the Preferences panel and closes the panel.

Cancel closes the Preferences panel without saving any changes.

Change Top Image's Info opens the Change Info panel, which allows the user to modify the label values for the top image window. See Fig. 6.13. (This panel is identical to the panel opened at the end of image acquisition, when the image was first created.) Label values may be typed in manually, or, if standard label values have been defined using Edit User Label Choice, page 55, the user may select a value using the pull-down menu directly to the right of the label. See Fig. 6.14. The Label Name Set and User ID may also be changed in this panel. Because Label Name Sets may have more than five entries, the user may use the checkboxes on the left to determine which of the entries will be displayed in the five available label lines.

Figure 6.14 Edit user-entered labels in this panel.

NOTE Once the changes are made, the image window will show the new labels. However, if any image was loaded from a stored data set, the original data will still have the old label. To update an old label, the image must be saved and used to overwrite the original data. See “Save Living Image Data”, page 48.
6. Main Menu Bar

Lab Book is a word processor window that allows the user to write and save notes about an experiment. Submenu choices Display, Save As, Clear, and Replace are used to control the Living Image Lab Book. Information such as when an image is acquired or loaded is logged automatically. The user may edit any of the information or add comments and documentation associated with one or more images.

Several important features of the Lab Book include the ability to record ROI measurement results directly to the Lab Book. See Figure 6.16. The Lab Book also allows the user to copy and paste the measurement results to another application or save the Lab Book to another application as a separate file.
**Display** opens the Lab Book window or makes it the top window if already open.

**Save As** saves a copy of the Lab Book in one of a variety of text formats. Plain text is the most universally readable format, but formatting information is lost. Formatted text saves the Lab Book as an Igor Pro notebook (opens with Igor Pro only) and preserves all formatting. This is the preferred format if the Lab Book will be opened using only Living Image software. Rich text will preserve most, if not all, of the formatting and can be opened by many applications, especially word processors. Finally, HTML is a useful option if the data in the Lab Book is to be used for web publishing.

**Clear** deletes all information in the current Lab Book.

**Replace** deletes all information in the current Lab Book and opens an existing, saved Lab Book. The user may modify the new Lab Book, e.g., add information, etc., before saving it. This proves useful when there is a need to stop a set of experiments or an analysis and resume them later.

**Measurements** opens or brings forward the Measurements Table window. See Figure 6.17. This window is a user-customizable summary of measurement results that is useful for collecting and exporting measurement data to other applications. The user may select which columns will be displayed using the **Configure** button. Export allows the user to save the contents as a text file for export to another application. The user may also copy and paste the contents of the window to other applications. Whenever the user makes a new measurement, the results are updated and displayed as either **All Loaded Clicks** (images displayed in open image windows) or **All Browsed Clicks** (all entries present in the browser), either of which may be selected using the **Display Measurements For** pull-down menu.

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**NOTE**

The contents of the Lab Book are saved together with the individual image data if the check box “Save Lab Book” is selected when saving the Image. The Lab Book is a summary document that contains information from an imaging or analysis session, much as a paper lab book would if the user were making entries as data were acquired or analyzed.
Click Info opens a window containing a summary of information associated with the top image window. (Living Image software data sets are sometimes referred to as clicks, hence the name.)

Smoothing increases the number of pixels in the luminescent image in the top image window. The number of pixels in the original luminescent image is determined at the time it is acquired but may be smoothed later using software smoothing. See Chapter 8, page 73. Smoothing increases the number of pixels by a factor that is specified by the user. The resulting image is filled with data interpolated from the original image. See Figure 6.18. While the data looks smoother and has more pixels it is not higher resolution data. Software smoothing does not recover spatial information that is not present in the original image. Therefore, it cannot transform a lower resolution (blurry) image into a higher resolution (sharper) image.
Smoothing changes to Undo Smoothing when software Smoothing is active. Undo Smoothing restores the image to its original number of pixels.

Binning sums groups of pixels in the luminescent image in the top image window and displays them in an image with a smaller number of pixels. This is the software equivalent of hardware binning. See Chapter 8, page 73, for a discussion as to why it is often better to use hardware binning during image acquisition. However, if, for example, the image is stored data, in which case hardware binning is not an option, then software binning can help the user to better observe a low intensity signal. See Figure 6.18.

Binning changes to Undo Binning when software Binning is active. Undo Binning restores the image to its original number of pixels.

Show ROI Properties opens the ROI Properties panel, which controls the ROI label, links, shape, position, width, and line size and color in the top image window when an ROI(s) is present. See Fig. 6.18. The user also may open the ROI Properties Panel by double-clicking on any ROI.

The top control on the ROI Properties panel allows the user to define the ROI Label to be displayed in the image window rather than the default, e.g., ROI 1, ROI 2, ROI 3, etc. The tabbed section of the panel directly below the ROI Label shows the relationship of active links between measurement, background, and subject ROIs. One Background ROI in an image or sequence can have the Use as bkg for future ROIs check box shown in Figure 6.19b checked, in which case this Background ROI will be linked to any new Measurement or Subject ROIs created. This feature is particularly useful when used in conjunction with the creation of Auto ROIs as described in the discussion of the Auto ROI Properties Panel. The Info tab shown in Figure 6.19c displays information describing the type of ROI.

Figure 6.19 shows the properties of a Measurement ROI and possible links to background ROIs (Bkg ROI) and subject ROIs (Subject ROI). Links may be changed or implemented here, but are more likely to be links set up as described in Section 4.7, page 34. Generally, the user will use the ROI Properties panel only to monitor the effects of these changes.

In the middle of the panel, the user may manually enter values to control the position and size of the selected ROI. This is sometimes convenient, such as when trying to duplicate an ROI used in another measurement. The user can also rotate the ROI using the Angle control. The line size and color for the ROI may also be modified using the controls at the bottom of the panel.

NOTE

ROIs are more commonly positioned and sized by dragging. The most common way to handle rotation is to drag the ROI handle while holding down the Ctrl key (Windows) or option key (Mac).
Image Math allows the user to add, subtract, multiply, or divide one image from another. This is typically used in conjunction with "background excitation fluorescent filters", for purposes of subtracting tissue auto fluorescence. For this option to be activated, the desired images must be loaded together as a group by clicking on the Load As Group button on the Living Image Browser window. See Figure 6.1, page 45. If the images were taken in sequential mode, or saved together as a group, the user may simply load the sequence.

Once the images are loaded, the top most (or active sequence) click number is shown as Image Math for click number_SEQ. See Figure 6.20. Once the user selects Image Math from the menu, an Image Math panel is displayed. See Figure 6.21.
In the Image Math panel, the left column shows the individual click numbers of the images that were loaded in the sequence. See Figure 6.21. The images corresponding to the highlighted click numbers are displayed in the right column. When the user selects a new click number, the image on the right is updated immediately. The images may be viewed in units of counts photons, or efficiency. Changing the units in the sequence panel (left column) will change the image (right column) to the same type of units automatically.

NOTE

The image must be flat field and dark charge corrected for the image math to be viewed in units of photons or efficiency.

Figure 6.20 Image Math Menus for a single active image (left) and with the click number of an available active sequence (right).
The Result = pull-down menu on the Image Math panel) allows the user to choose the desired image math function. See Figure 6.21. The user begins by inputting a value for the constant "k" in the variable control box. The user may then choose which photograph to include with the newly calculated data by choosing A or B from the pull-down menu. If no photograph is desired, the user should uncheck the with Photo from: box. To display the new data, click on the Display Results for Measuring button. The first image displayed will appear with a unique click number containing "_M" after the first three letters in the click number; subsequent images will be appended with "_N," "_O," etc. The new image may be analyzed and saved like any typical IVIS Imaging System image data.

A text box appearing at the top the new image describes the mathematical operation used to create it. See Figure 6.22.
Show Cursors creates a set of cursors in the top image window. See Figure 6.23. The user may drag or move the cursors using the arrows on the computer keyboard. A message line in the controls section of the image window shows the position of the active cursor (the last one moved) as well as the distance between the cursors. The value labeled Z is the numerical value of the pixel contents at the location of the active cursor. When cursors are active, Show Cursors changes to Hide Cursors, which removes the cursors from the image.

Place Tag creates a "tag" at the location of cursor A. See Figure 6.23. The user may position the tag by dragging. Double-clicking on the tag opens a window that allows editing of the tag contents (when IGOR menus are enabled). Tags remain in place after the cursors are removed. To remove the tag, the user should double-click on the tag and use the Delete button in the editing window. Tags are not stored as part of a Living Image data set.
6. Main Menu Bar

Hide ROI Tags controls the display of the tags that label the ROIs in the image window. The user may use Hide ROI Tags to hide the tags from view. Hide ROI Tags changes to Show ROI Tags when tags are active. Using Show ROI Tags redisplays the ROI tags. ROI tags are not stored as part of a Living Image data set. ROI labels, however, are saved as part of the data set.

Color Table provides a selection of color tables for the luminescent image that the user may substitute for the default color table, Reverse Rainbow. See Chapter 7, page 69.
**Manual Overlay** allows the user to take a photographic image from one image window and a luminescent image from another image window and create an overlay of the two images in a third window. The two images may then be stored as a single image data set, which, when reopened, will be an overlay of the two images. This may be useful for combining photographic and luminescent images that were previously saved individually. The windows containing the photographic and luminescent images must be opened and displayed prior to using **Manual Overlay**.

**Igor Menus** allows users to **Enable** or **Disable** the Igor Pro menus, which provide access to a wide variety of tools not directly supported by Living Image software.

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**NOTE**

Use of Igor Pro tools is recommended for advanced users only. Users who are not familiar with Igor Pro should NOT enable Igor Pro menus. Incorrect use of these tools can corrupt Living Image data. Advanced users who enable Igor Pro menus should use the Igor Pro **Help** menu to access the Igor Pro manual. As a precaution, always make a backup copy of all data before attempting to use Igor Pro menu items.
6. Main Menu Bar

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7 Image Display Modes

Understanding the Differences Between Scientific Image Data and Graphic Images

Pseudocolor Images

Overlays

Blends

The main types of images obtained using an IVIS® Imaging System are the reference photographic images and the luminescent (or fluorescent) images, which contain the quantitative information of interest. Both types of images are essentially arrays of numbers that can be displayed in a variety of ways. The display options offered by Living Image® software are described in this section.

7.1 Understanding the Differences Between Scientific Image Data and Graphic Images

Before discussing display options, it is important to understand the two major categories of image types commonly encountered by scientific users. The first category is referred to as scientific image data. These images are two-dimensional arrays of numbers, where each element of the array (a pixel) contains a number that is proportional to the light intensity on that element. CCD (Charge Coupled Device) cameras used for scientific imaging are essentially an array of photo-sensitive pixels, each of which collects photons during an image exposure.

A subsequent electronic readout provides a photon intensity number associated with each pixel. Photon intensity numbers in the brighter areas of the image (more photons detected) are larger than those in dimmer areas. Scientific image data then needs to be visualized effectively. Typically, this is done by displaying the image in a variety of ways, including contour plots, isometric displays, and, more importantly as it applies to Living Image software, or pseudocolor images. Pseudocolor images are discussed in more detail below.

The other type of image data typically encountered is a graphic image, or a two-dimensional array of pixels with a color assigned to each pixel. There are several schemes for storing such images digitally, with one of the most common being to assign an RGB (Red-Green-Blue) color code to each pixel. The RGB code defines how much of each of these colors to mix in order to create the final color for each pixel displayed. These RGB images differ from scientific images in that even though they are stored arrays of numbers, the numbers are not related to the intensity of light - they are just color codes. RGB images are used to store images such as color photographs or color screenshots, or picture information void of numerical information. Some of the graphic export options in Living Image software can be used to save RGB images of...
7. Image Display Modes

Living Image software displays. See File - Save Graphics, page 43, and Edit - Export Graphic As, page 44. This type of image is then appropriate for transfer to a graphics display program such as Microsoft® PowerPoint® or Adobe® Illustrator®.

7.2 Pseudocolor Images

Images may be created from scientific image data by assigning a color to each numerical value and plotting the array such that each pixel is filled with the color corresponding to its numerical value. The relationship between the numerical data and the displayed colors is referred to as a color table. A commonly used color table is a grayscale color table where black is assigned to the lowest number in the array and white to the highest number. Shades of gray are assigned to values in between. The resulting image, e.g., an illuminated photographic image taken with an IVIS Imaging System, is then equivalent to a black and white photograph. See Figure 7.1.

Another widely used color table is a reverse rainbow color table where violet is assigned to the lowest number in the array, red to the highest, and all the spectral colors of the rainbow to values in between. See Figure 7.1. This and other pseudocolor or false color schemes are typically used when the quantity of interest is the numerical contents of the image, e.g., the luminescent images obtained with an IVIS Imaging System. The pseudocolor scheme makes it easy to spot regions of bright light emission. The amount of light emission can then be quantified using measurement techniques such as those described in Chapter 9, page 79. It is important to understand that the measurements are not in any way dependent upon the colors displayed in the pseudocolor image. The user can adjust these colors by changing color tables or varying the range of numeric values associated with the colors. See Max Bar and Min Bar controls, page 22. The underlying numeric values are unchanged - measurements to quantify these numbers will produce the same result independent of the appearance of the pseudocolor display.

To add to the general confusion usually associated with image types, a pseudocolor image display can be converted to its corresponding RGB color codes and saved as an RGB image. When displayed, the RGB image will appear as it did previously, but without the numerical

Figure 7.1 Pseudocolor image overlaid on a grayscale photograph.
information associated with the light detected in each pixel. This means that the amount of light in the image can no longer be quantified, because all that remains is a pretty picture.

7.3 Overlays

Overlays in Living Image software are defined as a pseudocolor luminescent image displayed over a grayscale photographic image. It is also worth noting that after the upper (Max Bar) and lower (Min Bar) limits for the color table display range have been set, all pixels within the luminescent image that are below the Min Bar setting are not displayed. Effectively, this makes the bottom color in the color table a "transparent color." The purpose of this is to allow the underlying photographic image to be viewed in regions of the image where the luminescent light emission is low.

7.4 Blends

Image blends are very similar to Overlays, except that the final image is created using a special processing technique known as image blending, or alpha blending. In this technique, the color associated with the top luminescent image has been mixed with the color in the underlying photographic image to produce the final displayed color. This has the visual effect of making the top image look partially transparent. The degree of transparency is determined by the parameter alpha, which can vary from zero to one, where one is totally opaque and is effectively an overlay. A value of zero is fully transparent and is effectively a photographic image. In Living Image software, the alpha value is changed with a slider control that appears when Blend is selected from the Image Display Mode.
In vivo imaging of bioluminescent or fluorescent reporters typically involves the detection of extremely low light levels. The IVIS® Imaging System is designed specifically to have the high sensitivity and low backgrounds required for this type of work. This section describes some of the sensitivity related issues to consider when determining optimum imaging conditions.

8.1 Binning

Before discussing specific sensitivity issues, it is important to understand the concept of binning, which affects significantly the sensitivity associated with an IVIS Imaging System image. CCDs are photosensitive detectors constructed in a two dimensional array of pixels as shown in Figure 8.1. Here, the left image represents Binning=1, which corresponds to the actual physical number of pixels contained in a small segment of the CCD. After an image is acquired, each pixel contains an electrical charge proportional to the amount of light to which the pixel has been exposed. The CCD is then read out, i.e., the electrical charge is measured and assigned a numerical value, referred to as counts. See Chapter 9, page 79. The resulting image data is comprised of a two-dimensional array of numbers, with each pixel containing the counts associated with the amount of light detected.

Figure 8.1 A small segment of the CCD at binning = 1, binning = 2 (4 pixel summed together), and binning = 4 (16 pixels together)
Looking at the image on the left in Figure 8.1, Binning=1, means that every pixel is read out and the image size (number of pixels) is equal to the physical number of pixels on the CCD. IVIS Imaging System CCDs range from 1024x1024 to 2048x2048 pixels in size, and thus have a high degree of spatial resolution. In the center image, where Binning=2, 4 pixels comprising a 2x2 group on the CCD are summed prior to read out and the total number of counts for the group is recorded. This results in a smaller image containing four times fewer pixels than Binning=1. However, due to summing, the average signal in each pixel is now four times higher than Binning=1. The right hand image shows Binning=4, meaning 16 pixels are summed prior to read out.

The advantage of binning is the improvement in signal to noise for higher levels of binning. Binning helps improve the signal relative to an electronic noise called read noise that is introduced to the measurement of each pixel as it is read out (converted from an analog voltage, to a digital number). If four pixels are summed before read out, the average signal in the summed pixel, or super pixel, is four times higher. Because the read noise for this super pixel is about the same as it was for the individual pixels, the signal-to-noise ratio for the read noise component of the image noise is improved by a factor of four. For many in vivo images, the read noise is the dominant noise source, making high levels of binning very effective in improving signal to noise.

Binning, unfortunately, does bring about a reduction in spatial resolution in the image. For a binning factor of two, as seen in Figure 8.1, the super pixels are twice as wide as binning=1. This results in a factor of two loss in image spatial resolution. With in vivo imaging, however, the added sensitivity is usually more important than the improved spatial resolution. Since in vivo signals are often diffuse due to scattering in tissue, little is gained by increasing spatial resolution. See Chapter 9, page 79. In such cases, high levels of binning (up to 8 or 16 depending on the CCD installed in the IVIS Imaging System) may be appropriate. If signal levels are high enough that sensitivity is not an issue, such as in vitro imaging, then it is better to image at lower binning levels (2 or 4) in order to maintain a higher degree of spatial resolution.

For application specific questions as to the appropriate degree of binning, please contact Xenogen technical support.

The Binning pull-down menu on the IVIS Imaging System Control panel provides users with three binning options – Large (High Sensitivity), Medium, and Small (High Resolution). The actual binning numbers corresponding to these settings depends on the CCD chip and type of image as summarized in the table below. These choices should satisfy the needs of most users. However, if manual control of binning is desired, the user will need to specify Manual Binning in the Living Image Tools-Preferences-Camera Settings dialog.

It is also possible to do "soft binning" after the image is acquired. The concept is the same as hardware binning – groups of pixels are summed and a smaller, lower resolution image is displayed. However, the summing is done digitally on the stored image data as opposed to
hardware binning where the summing is done on the electronic charge before read out. Soft binning also does not offer any benefit related to read noise. However, soft binning may enhance the visibility of a signal because of its ability to reduce the statistical scatter in nearby pixel contents. Typically, it is better to do hardware binning, but when taking another image is no longer a viable option, performing soft binning on stored data may prove a worthwhile solution. See Section 8.1, page 73.

<table>
<thead>
<tr>
<th>Luminescent or Fluorescent</th>
<th>IVIS 50</th>
<th>IVIS Lumina</th>
<th>IVIS 100 EEV*</th>
<th>IVIS 100 SiTe</th>
<th>IVIS 100 Roper</th>
<th>IVIS 3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
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<td>2</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Medium</td>
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<td>4</td>
<td>5</td>
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</tr>
<tr>
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<td>8</td>
<td>16</td>
<td>8</td>
<td>10</td>
<td>16</td>
</tr>
</tbody>
</table>

* If you need help determining the type of IVIS® Imaging System 100 Series you have, contact Xenogen technical support.

## 8.2 Sensitivity

Sensitivity of low-light imaging is dependent upon a number of parameters that control two major quantities - the number of photons collected (signal) and the background or noise of the image. When conducting low light imaging, it is advantageous to increase the signal and decrease the background for maximum sensitivity. See Chapter 11, page 93 and Chapter 12, page 99.

The number of photons collected is dependent upon several things - the f-number of the lens, the image magnification, the size, and detection efficiency (quantum efficiency) of the CCD, the transport efficiency of the imaging optics, and the image exposure time. In general, high-
quality imaging systems use state of the art imaging optics with anti-
reflection coatings to achieve high transport efficiency. Also, many
imaging systems will use a fast f/1 lens, which is near the practical limit
for a general-purpose optical system. Ultimately, the main differences
among imaging systems are related to the CCD. IVIS Imaging Systems
use CCDs from several sources, but all are large format back-
iluminated CCDs with detection efficiencies of more than 80% across
the visible region of the spectrum. Figure 8.2 shows detection
efficiencies for several commonly used photon detectors; the back
illuminated CCD being the highest efficiency choice, particularly in the
600-800 nm region of the spectrum, the area of greatest interest for in
vivo imaging.

In addition to binning, which is discussed in the previous section, the
sensitivity of IVIS Imaging Systems may be adjusted by varying the f-
number, which controls the lens aperture. The detected signal will scale
approximately as $1/(\text{f-number})^2$. For maximum sensitivity, select f/1,
which corresponds to the largest aperture for IVIS Imaging Systems.
See Figure 8.3. This provides the greatest light collection efficiency, but
will have the minimum depth of field of the image. The depth of field
refers to the depth over which the image appears to be in focus. This
depends on the f-number and the field of view. At f/1, the depth of field
varies from about 0.2 cm at FOV = 3.9 cm (IVIS Imaging System 200
Series only) to about 2 cm at FOV=25 cm. The depth of field can easily
be assessed at any given f-number and FOV by using the Manual
Focus option on the IVIS Imaging System Control panel. Generally, f/
1 is recommended for low-light luminescent images and f/2 or f/4 for
brighter luminescent or fluorescent images.

The FOV affects sensitivity indirectly. Changing the FOV without
changing binning or the f-number will not affect sensitivity
significantly. However, CCD pixels are effectively smaller at a smaller
FOV (higher magnification) so that higher levels of binning can be used
without sacrificing spatial resolution. In other words, a Binning=4
image at FOV=20 cm will have the same spatial resolution as
Binning=8 at FOV=10 cm. This means that the Binning=8, FOV=10
cm image will have four times better sensitivity than the FOV=20 cm
image because of the change in binning.
Finally, sensitivity is also affected by image exposure time. The number of photons collected is directly proportional to the image exposure time - a two-minute image contains twice as many detected photons as a one-minute image. When imaging very dim samples, longer exposure times are beneficial. This is not necessarily true for all imaging systems in that there are some backgrounds that also increase with exposure times, most notably dark charge, which can decrease the efficacy of increasing exposure time. IVIS Imaging Systems have extremely low dark charge backgrounds, allowing effective use of imaging times up to 30 minutes. Typically, for in vivo imaging, animal anesthesia issues and luciferin kinetics limit practical exposure times to 5-10 minutes.

8.3 Image Saturation

Another concern related to sensitivity and binning is handling bright images. Because IVIS Imaging Systems are extremely sensitive, a relatively dim sample can produce a saturated or overexposed image. Image saturation occurs if a CCD pixel is exposed to more photons than it is capable of recording. The saturation limit of a CCD is a function of the pixel electron integration capacity and the analog-to-digital electronics. The CCD camera's used in IVIS imaging systems have saturation limits varying from 60,000 to 65,534 counts.

To tell if an image has saturated pixels, or is close to saturation, view the image in units of Counts (located in the Units pull-down menu on the image window), making sure that all corrections - flat field, cosmic, dark charge- are first turned off. The maximum pixel values in the image may then be viewed. Alternatively, the Saturation Map display mode may be used to find the location of saturated pixels. For more details, see “Saturation Map,” page 30. Generally, saturated images should not be a problem. Living Image software will warn the user if a saturated pixel is detected during image acquisition. If this warning appears, the user should adjust the image settings, e.g., set a shorter exposure time, and reacquire the image.

If saturated data is present when a stored image is loaded, Living Image software will issue a warning and the image will be labeled as saturated. The Saturation Map can be used to determine where the image is saturated. If the saturation is in an area where measurements need to be made, then the measurements will not be accurate. ROI measurements made outside of a slightly saturated region should still be accurate. Badly saturated images should not be analyzed; instead, adjust the camera parameters and reacquire the image. If you need to measure a dim source, but have a bright source in the same sample that you are not interested in, it is good practice the cover the bright source with black paper, to prevent that region from saturating the CCD.
8. Sensitivity and Binning

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P0-Para0
Quantitative analysis of bioluminescent (or fluorescent) images is generally achieved by drawing regions of interest (ROIs) on the image and summing, or integrating, the signal within the regions. Achieving the best results with ROI measurements requires some understanding of the limitations of CCD-based imaging systems, issues related to calibration, and the possibility that background emission may be present. These issues are reviewed in this section, along with a discussion of the physical units available for measurements when using Living Image® software.

It is important to understand some of the basic physical concepts related to the propagation of light through tissue. Tissue is a highly scattering turbid medium. As a result, photons emitted from cells buried deep within the animal scatter many times before reaching the surface. The photons are attenuated and diffused (spread out) by the time they reach the surface. See Figure 9.1. The IVIS® Imaging System measures the light intensity emitted from the tissue surface, usually referred to as the surface radiance. The surface radiance depends on the brightness and depth of the source below the surface. To the extent that a given source remains at a fixed depth, the radiance on the surface is directly proportional to the brightness of the internal source. This is generally
assumed to be the case. However, be aware that the surface radiance could change if the position of the source within the animal varies.

### 9.1 Regions of Interest (ROI)

In this section, we provide an overview of ROI measurements and discuss techniques and strategies that will help improve the consistency of ROI measurements. For a full listing and description of the various commands, see Chapter 4, page 21.

In Fig. 9.2, a typical image is shown with three basic types of ROIs - a measurement ROI, an average background ROI, and a subject ROI. The measurement ROI identifies the signal of interest to the user; there can be one or more measurement ROIs on each image.

*NOTE* The measurement ROI will contain both signal of interest from, for example, a tumor, plus possible background emission that is not of interest.

In Figure 9.2, the signal from the lungs is relatively low so that the background autoluminescence from the entire animal becomes significant. See Section 11.3, page 96. Using a background ROI can reduce the effect of the background emission on ROI measurements, e.g., BKG 1 in Figure 9.2. The background ROI identifies a region that the user recognizes as having a background similar to the measurement region - typically either a non-signal emitting region on the animal or a similar location on a control animal. When this background ROI is linked to the measurement ROI using the contextual pull-down menus, clicking the Measure button will subtract the average signal in the background ROI from the average signal in the measurement ROI.

*NOTE* The background ROI and measurement ROI do not need to be the same size, as all subtractions are based on averages.

The third ROI shown in Figure 9.2 is the subject ROI, which allows the user to identify one subject animal from another. Here, the mouse on the left has been identified as Mouse 1. By drawing a bounding box around Mouse 1 and linking it to the measurement ROI using the contextual pull-down menu, the measurement will be linked to Mouse 1 in the Measurements Table. This information is useful when sorting data in spreadsheets or other types of database programs.
The Measure button sends ROI measurements output to both the Lab Book window and the Measurements panel. The Lab Book output for the ROIs in Figure 9.2 is shown in Figure 9.3. The Lab Book serves as a useful location to store measurement results and notes about the experiment. The user also has the option of saving the Lab Book along with image data.

The Lab Book shows the ROI measurement data from both the background ROI and measurement ROI. The measurement ROI has been corrected for average background as indicated by the BKG 1 label in the Avg Bkg column. The output data for this measurement, made in units of photons (more on units below), includes Total Flux (integrated signal in ROI), Avg Radiance (average signal is the sum divided by the area), Stdev (standard deviation of signal in ROI), and the Min and Max signal within the ROI. In most cases, it is recommended that the total ROI signal be used for
quantification rather than the average, as the average signal within an ROI is very much dependent upon the size of the ROI whereas the total is not. Average is more appropriate for characterizing signals that are uniform over a large area, such as backgrounds.

The Measurements table is a flexible and convenient tool for exporting After the ROIs have been drawn and measurements have been made, the user may save the results upon closing the image. A pull-down menu will appear and ask the user if the changes to the image should be saved. Clicking Yes will save the ROI data in the AnalyzedClickInfo.txt file along with the image. The actual image (TIFF) data are unaffected by this operation.

Although all three types of measurement ROIs were demonstrated in Figure 9.2, it is not necessary to use all three for all images. Often, when there is only one subject animal and the background is small compared to the signal, extracting the data of interest with a single measurement ROI will be adequate.

Using a consistent technique for drawing the ROIs improves the consistency and quality of ROI data. Diffuse luminescent spots on the surface often extend over a wide spatial region, so the user must decide
how large to make an ROI. Generally, the objective is to select as much signal as possible while minimizing background. The size of the ROI to best accomplish this depends on the specific image. Drawing an ROI at 1-5% of the peak signal is a reasonable place to start. Setting Min Bar equal to 5% of Max Bar is a quick and easy way for the user to determine where to draw a 5% ROI. Auto ROIs also provide a convenient way for users to draw an ROI at a specific level.

At times, the signal may be too low to draw an ROI at the 5% level and a higher level must be used. This is the case in Figure 9.2. Here, the ROI was drawn at 30% of peak value. A reasonably accurate determination of the ROI signal was still possible because an effort was made to subtract the background.

Although only circular ROIs were demonstrated here, other shapes such as elliptical, rectangular, and contours can be utilized. See Section 4.2, page 22.

9.2 Units

Images in Living Image may be displayed and quantified in units of counts or photons. For fluorescent images, an additional set of units called efficiency is available. Typically, counts are recommended for use during image acquisition to ensure camera settings are adjusted properly. Photons are recommended for ROI measurements on bioluminescent images and efficiency is recommended for analysis of fluorescent images. These selections are available from the Units pull-down menu on the image window. See Section 4.3, page 26.

Counts are uncalibrated units that refer to the raw amplitude of the signal detected by the CCD camera. The 16-bit CCD digitizer limits the signal level (in counts for each pixel) to a value between 0 and ~60000. Because a signal measured in counts is related to the number of photons incident on the CCD, the signal level varies, depending upon camera settings such as integration time, binning, f/stop and field-of-view. Thus, if the user changes these settings in the middle of an experiment to keep the signal below the digitizer saturation level of ~60000 counts, ROI measurements must then be corrected for the change in camera settings in order to compare two images taken with different settings. In the case of changing the integration time, the scaling of signal is straightforward because the signal is directly proportional to integration time. However, changes in f/stop or field-of-view result in changes to the signal level for which scaling is more difficult. In such cases, physical units of photons provide the user with a simpler, more direct comparison. See below.

When performing an ROI measurement in the counts mode, the quantity, Total Counts, (reported in the Lab Book or Measurements table) refers to the sum of all counts for all pixels inside the ROI. The Average Counts (Avg) is Total Counts divided by the number of pixels, or superpixels. The quantity ROI Pixels is the number of binned pixels inside the ROI, while Area (CCD pixels) is the number of unbinned CCD pixels inside the ROI.

When the Units menu is switched to Photons, the units on the displayed image change to photons/sec/cm$^2$/sr where sr=steradian (more on this below). 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 discussed above is that the radiance units refer to photon emission from the subject animal itself, as opposed to counts that refer to photons incident on the detector. As a result, the measurements in units of radiance have already taken into account settings such as integration time, binning, f/stop, and field-of-view. If the user were to take several images of an animal during a single session, with different integration times or fields-of-view, the displayed images would all have the same signal amplitude because the radiance on the surface of the animal is not changing – only the camera settings are changing. (Xenogen Corporation calibrates the camera settings of each IVIS Imaging System prior to shipment.) One advantage for the user is that camera settings can now be changed during an experiment without having to adjust the images or the measured ROI data. Another advantage of using absolute physical units is that images or ROI data may now be compared quantitatively between multiple IVIS Imaging Systems and, potentially, systems located at different facilities.

Presently, IVIS Imaging Systems are calibrated at 635 nm only. The response of the CCD is relatively flat (~10%) over the range 500-700 nm, which covers the spectral variation typically found in bacterial or firefly luciferase, so the calibration is accurate over this range.

The radiance units of photons/sec/cm$^2$/sr refers to the number of photons per second that are leaving a square centimeter of tissue and radiating into a solid angle of one steradian (sr). See Figure 9.5. The user does not need a detailed understanding of steradians to make use of these units. To summarize, 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 4$\pi$ steradians. Typically, lens systems collect light from only a small fraction of the total 4$\pi$ steradians.

ROI measurements made using photon units differ somewhat from those made with the counts mode described above. The total signal within the ROI is now called Total Flux (photons/sec) in the ROI Measurements table. This refers to the radiance in each pixel summed, or integrated, over the ROI area (cm$^2$). The result is also multiplied by 4$\pi$ to indicate the number of photons that are radiating omni-directionally, even though only a small fraction of these photons are collected. The rate of photons leaving a sphere surrounding a light source is typically called flux, hence the name Total Flux. See Figure 9.5. The average signal in photon mode is called Average Radiance, which is the sum of the radiance from each pixel inside the ROI divided by the number of pixels. The quantity Area in the Measurements table is now the ROI area (cm$^2$).
Measuring the brightness of a luciferase-tagged cell line is one example of how these units may be used, e.g., if the user images N cells in a well plate, draws an ROI around the well of interest, and performs the measurement, the total flux (photons/sec) emitted from that particular well will be known. By dividing the Total Flux by the number of cells (N), the user can determine the number of photons/sec radiating isotropically from each cell, which represents the absolute brightness of the cell line.

Efficiency is the final unit of measure available in the image window. Efficiency is only available for fluorescent images and represents the radiance of the subject animal divided by the illumination intensity. Since the illumination profile is not uniform over the field of view, it is desirable to normalize the image to this illumination profile. See Chapter 14, page 107. To achieve this, images of a white plate illuminated with excitation light (no emission filter) are acquired and the radiance of the white plate is calculated. These images are taken prior to shipping the IVIS Imaging System and are stored in the Living Image folder for subsequent use in making the efficiency calculation. The final image in units of efficiency is the original image divided by the white plate illumination image. The efficiency number for each pixel represents the fraction of fluorescent photons relative to each incident excitation photon. Typically, the efficiency is small, on the order of $10^{-3} - 10^{-7}$. Measurements made in efficiency units will result in a Total Efficiency that is the average efficiency multiplied by the area of the ROI. Total Efficiency is thus expressed in units of cm$^2$.

### 9.3 Flat Fielding

Flat Fielding refers to the uniformity of light collected across the field of view (FOV). Typically, lenses collect more light from the center of the FOV than from the edges. Living Image software allows the user to achieve uniform quantification of ROI measurements across the entire FOV by using a correction algorithm to compensate for the collection efficiency variation of the lens.
The **Flat field** check box, located on the top right of the Image Window, activates the correction algorithm. See Section 4.5, page 31. 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. Typically, 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.) The user will notice an increase in noise near the edges and corners of the FOV upon activating the flat field correction – this is normal. The accuracy of the flat field correction is best in the center of the image, tapering off toward the corners; therefore users should avoid placing animals near the outer edges or corners of the FOV. Using the flat field correction is strongly recommended for ROI measurements.

### 9.4 Cosmic Ray Corrections

Cosmic rays are extraterrestrial high-energy particles that register a false signal on CCD detectors. These cosmic rays as well as other sources of ionizing radiation cause infrequent interactions (few per minute on the CCD) that result in large signals that are usually isolated to a single pixel, making them easy to correct.

The Cosmic Ray algorithm in Living Image software searches for isolated, high amplitude "hot pixels" and replaces them with a collective average of surrounding pixels. See Section 4.5, page 35. Cosmic ray correction should always be used for in vivo imaging, as hot pixels can significantly affect an ROI measurement. The only time the cosmic ray correction is not recommended is when imaging very small objects such as individual cells. An individual cell will only light up one or two pixels and can sometimes be misinterpreted as a cosmic ray. The user should turn off the cosmic ray correction to avoid filtering out single-cell images.
This section provides a general description of how to generate and use automatic ROIs, which can be either Measurement ROIs or Subject ROIs. Specific controls and their use for generating automatic ROIs are described in Chapter 4, page 21.

Auto ROIs are regions of interest that are generated automatically based on a set of commands and algorithms in Living Image software. The user should view auto ROIs as convenient, timesaving devices that often give the desired result, but will occasionally give results that require editing. ROI tools are designed to make subsequent editing of the Auto ROI results quick and easy. To get the best results in as many cases as possible, the user should experiment with the Auto ROI Settings for typical examples of his/her data.

10.1 Measurement ROIs

Measurement ROIs are regions of interest that surround a portion of a luminescent (or fluorescent) image for which the user wants to quantify the numerical contents. Luminescent and fluorescent images are two-dimensional arrays where each entry, or pixel, contains a numerical value. Typically, these arrays are displayed as pseudocolor images. See Section 4.4, page 27. However, in discussing automatic generation of ROIs, it may be helpful to view the data as a surface plot. See Figure 10.1.

![Figure 10.1 Surface plot of the same luminescent image data displayed as a pseudocolor image in Fig. 10.2.](image)

In this type of display, the user should think of the data as being composed of triplets (X, Y, Z) where the X and Y values are the
position of the pixel in the 2-dimensional image and the Z value corresponds to the numerical content of the pixel. In a surface plot, such as seen in Figure 10.1, the Z value is shown as a height above the XY plane. Figure 10.1 is also labeled with the same pseudocolor scheme as used in Fig. 10.2. Displaying the data as a surface plot makes it easier for the user to think of the bright areas in the image as peaks in the luminescent display and auto ROIs as a means of finding those peaks and defining their boundaries in terms of a fractional peak height.

Use of the automatic ROI generation tools initiates a search for one or more peaks in the luminescent (or fluorescent) image. In Figure 10.2, Auto 1, seen in the pull-down menu directly below the Create button in the image window, initiates an automatic search for a single peak in the area surrounding a user-defined point, seen here as cursor A. The same action may be initiated using contextual menus. See Figure 4.19, page 34. In this case, the click point used to bring up the contextual menu is the search starting point. Upon finding a peak, a contour is generated at the fraction, or edge value, of the peak height as defined in the Auto ROI Settings panel. See Figure 4.5, page 25. (A contour is a line traced around the edges of the peaks and connects each pixel containing a value equal to the prescribed edge value of the peak height.) In Figure 10.2, ROI 1 is defined by an edge value of 20% of peak height.

![Image 1](image1.png)

**Figure 10.2** Image window showing an auto ROI generated using the Auto 1 option.

In Figure 10.3, Auto All, seen in the pull-down menu directly below the Create button, initiates an automatic search for every well-defined peak in a luminescent (or fluorescent) image. The search first locates
the highest peak before searching for peaks down to the lowest limit as defined in the Auto ROI Settings panel. Peaks lower than the value indicated in the Auto ROI Settings panel will not be identified. Peaks that have been identified are used to define a contour from which an ROI is created. However, if the new ROI overlaps an existing ROI created at a higher peak, the new ROI will not be assigned.

An example of this is shown in Figure 10.3, where Auto All searched for peaks having an edge value of 25% and a 10-pixel minimum ROI. The peak to the left of ROI 1 was not assigned an ROI because a 25% contour around this peak encompasses the region already defined as ROI 1. The overlap can be seen in Figure 10.4 where Auto 1 was used to create an ROI of the smaller peak. Use of Auto 1 is not restricted by any requirements to not overlap existing ROIs.

Figure 10.3 Auto All (in the pull-down menu below the Create button) initiates an automatic search for every well-defined peak in a luminescent (or fluorescent) image. The search first locates the highest peak before searching for peaks down to the lowest limit as defined in the Auto ROI Settings panel. Peaks lower than the value set in the Auto ROI Settings panel are not identified. Identified peaks define a contour from which a ROI is created. However, if the new ROI overlaps an existing ROI created at a higher peak, the new ROI will not be assigned.
In addition to being able to create auto ROIs defined as Contour, users may create auto Circle or Square ROIs. A Circle ROI is an ellipse that best fits the shape of the contour and is of equal area. A Square ROI is a rectangle created using the major and minor axes of the ellipse corresponding to a Circle ROI.

Auto ROIs allow the user to define an ROI in a consistent, non-subjective manner. For example, an ROI with a 10% contour around the maximum peak edge value is completely defined and easily reproducible. Consistent use of auto ROIs with a defined edge value will result in better quantification of most experimental data than other methods.

### 10.2 Subject ROIs

Subject ROIs are rectangular-shaped boxes that enclose a particular, often single, subject animal. Auto creation of Subject ROIs attempts to locate individual animals in a photographic image and enclose the animal in a Subject ROI. Default Auto Subject ROI settings are optimized to locate light-colored animals on a dark background. However, the settings may be varied to give good results for subject animals of different types. Settings values are discussed in more detail in Chapter 4, page 21.

Another use of Subject ROIs is that of creating a group box in which all ROIs contained or placed in the box are assigned the properties associated with the Subject ROI, e.g., if a background ROI is associated with a subject ROI, all measurement ROIs in the subject ROI will be associated with this background. See Section 9.1, page 80.

Xenogen is working to extend the usefulness of Subject ROIs, specifically, giving Living Image software the ability to locate, within a number of stored images, the image data associated with one particular animal. These potential Subject ROIs would be assigned a particular Subject ID, or identifier, that could be used to find, e.g., Mouse 123456, in a set of multiple images. See ROI Properties panel, page 35. Presently, Living Image software allows users to create subject animal IDs, but does not yet provide the ability to locate them across all images.
Figure 10.5 Auto creation of three Subject ROIs, one for each of animals in the photographic image.
11 Background Sources

Electronic Background ........................................ 93
Background Light On the Sample .......................... 94
Background Light From the Sample ...................... 96

Background sources of light from bioluminescent images are inherently very low. However, because of the extreme sensitivity of IVIS Imaging System instrumentation, residual electronic backgrounds (dark current) and luminescent emissions from live animals (autoluminescence) are measurable and must be taken into account. In addition, there could be sources of background light from leaks that develop in the imaging chamber or phosphorescent materials that are inadvertently introduced into the chamber. These issues are discussed in this section. Backgrounds sources that occur during fluorescence imaging are discussed in Chapter 14, page 107.

11.1 Electronic Background

Cooled CCD cameras such as those used in IVIS Imaging Systems have electronic background sources that must be accurately measured and subtracted from an image before light intensity may be measured quantitatively. However, it is not necessary to subtract these backgrounds when making a simple visual inspection of an image.

One such electronic background source is called the read bias, which is an electronic offset that exists on every pixel. This means that the zero photon level in the readout is not actually zero but, typically, a few hundred counts per pixel. The read bias offset is reproducible within errors defined by the read noise, which is another quantity that must be determined for quantitative image analysis. Another electronic background source is dark current, which is generated by the thermal production of charge in the CCD. To minimize dark current, the CCD is cooled during use. The dark current must be accurately characterized so that it can be subtracted from images prior to quantification.

The cameras used in IVIS Imaging Systems are high-quality, scientific research-grade devices that deliver the lowest values currently achievable for electronic background sources. This low background, together with imaging chamber integrity and high-efficiency light collection capabilities, make IVIS Imaging Systems the most sensitive low-level imaging systems currently available. But to fully exploit this high sensitivity, careful measurements of these electronic backgrounds must be made and used appropriately in image analysis. For more details on this "dark charge management", see Chapter 12, page 99.
11.2 Background Light On the Sample

An underlying assumption for in vivo imaging is that all the light detected during a luminescent image exposure is being emitted from the sample. This is not accurate if there is an external light source illuminating the sample. Any reflected light will be detected and will be 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. One is a light leak, e.g., light that leaks through a crack or other mechanical imperfection in the imaging chamber. IVIS Imaging Systems are designed to be extremely light tight and are thoroughly checked for light leaks before and after installation. Unless mechanical damage has occurred, it is unlikely that light leaks will be a problem. To ensure that there are no light leaks in IVIS Imaging System imaging chamber, conduct an imaging test using the Xenogen High Reflectance Hemisphere (Xenogen part no. XRH-2). See Figure 11.1 and Figure 11.2.

A subtler source of external illumination is the possible presence of light emitting materials inside the imaging chamber. In addition to the obvious sources, such as light emitting electronic equipment (do not place anything containing LEDs in the imaging chamber), there are materials that contain compounds that phosphoresce. Phosphorescence is a physical process similar to fluorescence, but applies to light emission that persists for a longer period. Phosphorescent materials absorb light from an external source, e.g., room lights, and then re-emit it. For some phosphorescent materials this re-emission may go on for many hours. Hence, if such a material is introduced into the imaging chamber, it produces background light even after the door to the imaging chamber 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 from 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. Still, if the user introduces such materials inadvertently, problems may arise. Materials that users should be particularly aware of include plastics, paints, and organic compounds. Plastic tape and plastic containers have also been found to be problematic. Contaminants such as animal urine can be phosphorescent. Performing imaging on black paper that is changed frequently will help to maintain a clean imaging chamber. Cleaning the imaging chamber frequently is also helpful, but only if using Xenogen-approved cleaners - many cleaning compounds phosphoresce! Xenogen can supply black paper for imaging as well as a list of tested and approved cleaning compounds.

If it is necessary to introduce suspect materials into the imaging chamber, users can screen such materials simply by imaging them. Position the suspect material in the desired field of view and take an exposure equivalent to that which will be used during image sampling, e.g., if a five minute exposure time will be used in the sample imaging in which the suspect material is present, take a five minute exposure of the suspect material to determine if it is visible in the luminescent
image. Screening of this nature may be done with common microtiter well plates, which are typically made of different types of plastic - white, black, clear. Screen all three types with a test image. The white plate will appear extremely bright by IVIS Imaging System standards and therefore interfere with measurements. The black and clear well plates will not phosphoresce, making them better choices.

A more definitive method for determining the presence of undesirable light sources is the Xenogen High Reflectance Hemisphere (Figure 11.1) – a small white hemisphere, coated with a material that is known not to phosphoresce. A long exposure image of the hemisphere should produce a luminescent image in which the hemisphere is not visible. If any part of it does exhibit what appears to be luminescent emission, it is really the light reflected from a source illuminating the hemisphere. Observing which side of the hemisphere is illuminated is often useful in determining the location of the source.

An example of the Xenogen High Reflectance Hemisphere at work is shown in Figure 11.2. The pen appears very bright due to phosphorescent emission, which also is illuminating the portion of the hemisphere closest to the pen. Had the pen been outside the field of view, it would not have been imaged directly, thus making the source of the phosphorescence less obvious. However, the illumination of the hemisphere still would still be very apparent and indicative of a light pollution problem.
11. Background Sources

11.3 Background Light From the Sample

Another source of background is the natural light emitted from the sample, and not due to emissions from the source of interest within the sample. This background light may be due to material associated with setting up the experiment, in which case, a less problematic material may be substituted. An example of natural background light is the growth medium used in an in vitro image of a well plate. Many of these materials phosphoresce and, therefore, should be screened, with those proving problematic eliminated. In addition, if the source of the background is phosphorescent and the phosphorescent lifetime is relatively short, users may want to keep the sample in the dark for a long period prior to imaging. This may help to reduce background light emission.

Occasionally, there is no way to eliminate the natural light emission of the sample. An area of major interest for in vivo bioluminescence imaging is the natural light emission associated with living animals, or autoluminescence. Most, if not all, animals exhibit a low level of autoluminescence. Fortunately, this is a problem only for users working at the highest levels of sensitivity and looking for very low signals. The sources of autoluminescence are not yet fully understood, but several experiments attempting to characterize this emission have been performed.

Xenogen has conducted tests to try to minimize the source of the background light emission in mice. Test #1: The phosphorescent component in mouse fur or skin was ruled out as a source, as housing the animals in the dark 12 hours prior to imaging proved unsuccessful in reducing background emission levels. Test #2: A shaving study was conducted to see how fur affected the signal. Shaved white-furred animals showed no increase or decrease in background emissions. Test #3: The alfalfa in mouse food, which is known to be phosphorescent, was eliminated from the animals' diet to try to reduce the signal. Although alfalfa-free food reduced background emission slightly, it was not significant.

IMPORTANT
The Xenogen High Reflectance Hemisphere (Xenogen part no. XRH-2) must be handled by its black base plate only (skin oils, which can phosphoresce, will contaminate the hemisphere) while wearing cotton gloves provided by Xenogen (latex gloves and the powder on them may also phosphoresce). If the hemisphere becomes contaminated, contact Xenogen Corporation for a replacement, as there are no known agents with which to clean the hemisphere. To check 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.
Because no external sources have been proven to cause natural light emissions, it is believed that a chemiluminescent process associated with a yet-to-be-determined metabolic activity in living animals is the source of animal background. This is corroborated by the fact that, in euthanized animals, the level of background light drops significantly.

Figure 11.3 shows images of a control white-furred mouse and a nude mouse where the background light emission is clearly visible. 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. These values are considerably above the lower limit of detectability for the IVIS Imaging System (~100 photons/s/cm²/sr), therefore, the background light emission from the mouse determines the limit of detectability when using these types of animals. 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. See Chapter 9, page 79. It should be noted that background light emission is not uniform over the entire animal. Images of control animals (mice) show a somewhat higher background component originating from the abdominal and thoracic regions. Therefore, care must be taken when choosing a representative area to subtract out.

Typically, this type of background should be considered only by users working at the highest levels of sensitivity and with very low signals. For more information on how best to handle these measurements, users should contact Xenogen Corporation.
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12 Dark Charge Management

Among the possible background sources described in the previous section, the electronic sources are the easiest to characterize and subtract from an image prior to any quantitative measurement of signal intensity. Electronic sources are also the most important source to subtract because, while they are reproducible and can be accurately subtracted, they also are relatively large. Raw data with no electronic background subtraction produces erroneous ROI measurement results. Incorrect background subtraction may also result in serious errors.

Living Image software is designed to measure and subtract electronic background sources from images in a way that is virtually transparent to the user. The term "dark charge" refers to all electronic backgrounds, including dark charge and read bias. This section describes how to measure dark charge for various imaging conditions, organize and keep track of the measurements, and subtract them appropriately. More detailed information on menu items associated with dark charge and background sources is contained in "Dark Charge," page 50, and "Fluorescent Background," page 52.

NOTE

Background measurements are appropriate for luminescent or fluorescent images only. Photographic images contain no quantitative information on signal intensity, so only the appearance is significant. It is not necessary to subtract a background from the image if no measurements are to be made.

12.1 Dark Charge Measurement

Dark charge depends upon the exposure time, binning level, and camera temperature. Before starting a manual dark charge measurement the user must first set up the Camera Control panel for image acquisition. The temperature should be locked at the appropriate demand temperature. See Chapter 3, page 9.

Dark charge measurements can be started manually using the Living Image - Dark Charge - Measure menu item. Alternatively, dark charge measurements may be set up to start at a given time, automatically. See Section 12.4, page 101. Upon starting the dark charge measurement, a series of messages will ask the user if the imaging parameters are correct, and inform them of the time it will take to...
complete the dark charge measurement. The dark charge measurement is actually done with the camera shutter closed; so low-level luminescent samples may be present. In addition, the door must remain closed during the measurement, as the camera shutter is not light tight and, therefore, cannot prevent interference from higher levels of light, such as room lights. A series of zero-time exposures to determine the bias offset and read noise will then start followed by three dark exposures. Typically, the dark charge measurement takes more than three times as long to complete as the equivalent luminescent exposure.

Upon completion of the dark charge measurement, a folder containing the data is created and stored in a dark charge folder in the Living Image Files folder. A summary of the available dark charge images can be viewed by selecting *Living Image - Dark Charge - View Available*.

Typically, a dark charge measurement should be taken in close proximity to the image acquisition and the system should remain stable between the dark charge measurement and the image acquisition. If power to the system or camera controller has been cycled and/or the camera temperature has been changed, the user should take a new dark charge measurement. *Living Image - Dark Charge - Clear Available* may be used to remove outdated dark charge measurements from the summary.

### 12.2 Dark Charge Subtraction

When starting a luminescent (or fluorescent) image acquisition, Living Image software automatically checks a summary of dark charge measurements stored in the dark charge folder to determine if there is an existing dark charge measurement that matches the current exposure time and binning. If a matching dark charge measurement is present, and the measurement was taken within 48 hours, Living Image software will automatically use this dark charge background. No prior notification is given.

**IMPORTANT**

If something occurred within 48 hours that invalidates this background, e.g., system power has been cycled or the camera temperature changed, and the *Living Image - Dark Charge - Clear Available* menu item was not used, you will receive no warning that an inappropriate background was used. If, after an image is acquired, the user becomes aware of such a situation, *Living Image - Dark Charge - Replace* may be used to substitute a new background measurement.

When a valid dark charge measurement is associated with an image, it is stored together with the image data when the image is saved. When the image data is displayed later in a Living Image image window, the associated dark charge is automatically subtracted if the *Sub Dark Charge* check box appears in the image window.
12.3 Read Bias and Drift

Prior to every luminescent image exposure, Living Image software initiates a series of zero-time exposures, or image readout, to determine a read bias measurement. See Chapter 11, page 93.

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 then compared to the average bias offset determined with the read bias measurement made prior to the image. The difference, or drift correction, is stored, along with the luminescent image data, and used later in correcting for minor drifting (typically less than two counts/pixel) that may have occurred 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 code will first check to see if the selected image parameters warrant a dark charge measurement (large binning and long image times). If a dark charge image is not required, the read bias will be used automatically. If a dark charge is recommended, the user will be offered the option of using the read bias measurement instead. Because 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.

Read bias subtraction is an adequate substitute for a dark charge background subtraction when the amount of dark charge associated with the image is negligible. Because dark charge increases with exposure time and is more significant for higher levels of binning, a good rule of thumb is that it is negligible if $tB^2 < 1000$, where $t$ is the exposure time in seconds and $B$ is the binning factor. For these conditions, dark charge contributes less than 0.1 counts/pixel and may be neglected.

If read bias is used instead of a dark charge background, the read bias image will be stored along with the rest of the Living Image data set rather than the usual background information. The read bias will be subtracted when the data is subsequently displayed in a Living Image image window, and the image window background subtraction check box, which, typically, is labeled Sub Dark Charge, will be labeled Sub Bias.

12.4 Automatic Background Measurements

Living Image software's automatic dark charge measurement tool allows users to make a predetermined set of dark charge measurements every day at a specified time, usually during periods of system inactivity, e.g., early morning hours, and have them available for use later the same day. The backgrounds to be measured are contained in a list called Living Image Auto Dark Charge List, which may be accessed via the Living Image item in the Start menu on an IVIS Imaging System acquisition computer. This list is a Microsoft® Excel spreadsheet that can be modified and saved by the user. Changes to the list will be implemented during the next automatic dark charge measurement set.
The automatic dark charge background list is comprised of exposure times and binning conditions that the user expects to use. If a particular exposure time and bin factor is entered on the list, and the corresponding dark charge is measured automatically, images subsequently taken with these settings will automatically use the correct dark charge background, store it with the image data, and subtract it from the displayed image. In this way, dark charge background subtraction is virtually transparent to the user.

A default list containing a number of commonly used exposure times and bin factors is installed on each IVIS Imaging System. Users may modify existing entries or add new entries, keeping in mind that each measurement requires considerable time and trying to predetermine every conceivable set of imaging conditions may prove burdensome to the system. The total time required to make all the measurements for the entire list is calculated and appears in the spreadsheet. The default list provided with the IVIS Imaging System requires about 3-4 hours per day to perform automatic background measurements. This means the system is unavailable to users during that period. The default starting time for automatic dark charge background measurements is 1:00 a.m. While changing the default starting time is relatively easy, interested users should contact IVIS Imaging technical support for more information.
13 Data Management

- Storing Living Image® Data ........................................ 103
- Retrieving Living Image Data ..................................... 104
- Importing Data into Living Image Software .................. 106

Multiple users compiling large amounts of image data can generate a database of large proportions in little time. The task of managing the data is an important one. Living Image® software provides a basic data management system for organizing and referencing image data, and allows users fast and easy access to their data.

13.1 Storing Living Image® Data

Living Image software provides several options for saving image data. The most common is for the user to select Living Image - Save Living Image Data, followed by All Living Image Files from the resulting pull-down menu. See "Save Living Image Data," page 48. This creates a folder that contains the image data in a generic format (TIFF). Additional information, such as camera settings and ROI positions and measurements, is also saved in a text file called ClickInfo.txt.

The folder name, sometimes called a "click number," is created automatically from the user's name, date, and time of creation. A click number is unique to the data set it represents. See Figure 13.1 for an example of stored Living Image data sets.
The Living Image data set format is easy to maintain and uses only the basic file manipulation tools provided by the user's computer operating system. Folders may be named, dragged, and reorganized in a manner familiar to virtually all computer users.

**WARNING!** While the user can manipulate entire folders, the individual files contained therein should not be moved outside the folder or modified in any way.

The file formats (text and TIFF) are non-proprietary, cross-platform, stable formats. Living Image software provides users with a basic set of tools for searching and sorting image data. See, "Retrieving Living Image Data," below. However, if a user group requires faster and more sophisticated file search capabilities, contact Xenogen Corporation for information on converting Living Image data sets to the desired database structure.

In addition to saving image data manually, as described above, Living Image software provides an Autosave feature that allows users to save image data automatically as each image is acquired. See Section 6.4, page 53.

### 13.2 Retrieving Living Image Data

**Living Image - Load LI Data** allows users to locate and access a specific image data set. See "Load LI Data" on page 47. More commonly, users may select **Living Image - Browse for LI Data** to
Living Image data sets can be stored with multiple types of data, making it easy and convenient for the user to keep imaging-related information, e.g., write-ups, summaries, Living Image Lab Books, etc., together with their actual imaging work.

Using the Living Image browser, users can perform a directory-specific search and locate all relevant Living Image data, while ignoring all other types of data and files. A summary of data locations is compiled and displayed in the Living Image Browser table. See Figure 13.2. The summary table lists the click number and all text labels and camera configurations for each image. The user can sort the information in the table by column or by any combination of columns using the Sort button. The Columns pull-down menu may be used to customize any displayed column.

Living Image data sets are labeled as each image acquisition is completed. See Section 3.1, page 9. Living Image software provides a simple, generic labeling scheme (default) and several, more detailed group-specific labeling schemes, e.g., Xenogen Oncology and Xenogen Infectious Disease. Companies that desire user- or group-specific labeling schemes should contact Xenogen Corporation. Xenogen will create a custom file containing the desired labeling schemes that can be installed on the company's IVIS Imaging System and the specified user's analysis computers. The customized labeling schemes may then be selected at the end of the image acquisition.

It is highly recommended that users consider establishing a labeling system that provides adequate information for locating the image data sets. Implementing Edit User Label Choices, as described in Section 6.4, page 53, can significantly speed the labeling of data, and, therefore, increase the probability that users will do detailed data labeling. It is also recommended that users develop a simple data structure that limits the number of data sets organized in any one folder, e.g., Create a master folder for each major experiment and use subfolders to house...
image data sets acquired during a specified time period – weekly, bi-weekly, monthly or bi-monthly, etc.

Users may, at any time, elect to search larger numbers of data sets, but need to remember that the speed limitations of the standard Living Image browser may extend search times significantly.

13.3 Importing Data into Living Image Software

**Living Image - Load TIFF Data** allows the loading of general TIFF data and provides the means by which to import image data produced using other software. **Load TIFF Data** should not to be confused with the Load Image Data option, which is used to load Living Image Data Sets.

One possible point of confusion associated with the Load TIFF command is related to scientific image data versus graphic image. See Chapter 7, page 69. The TIFF format supports storage of either numerical scientific image data or graphic RGB images. **Load TIFF Data** assumes the TIFF being loaded is a numerical scientific image data. If the TIFF is actually a graphic image, errors will result.
14 Fluorescent Imaging

14.1 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, while the IVIS® Imaging System 3D, 100, or 50 Series requires the use of the XFO-6 or XFO-12 Fluorescence Option to perform fluorescence imaging. The fluorescence equipment enables the user to conveniently switch between bioluminescent and fluorescent imaging applications. See Figure 14.1, or for more details, refer to the IVIS Imaging System 200 Series System Manual, the IVIS Lumina System Manual, or the XFO-6 or XFO-12 Fluorescence Option Manual.

Figure 14.1 Fluorescent imaging hardware. IVIS® Imaging System 200 Series (left). IVIS Imaging System 50 and 100 Series, and IVIS Lumina (right).
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. See Figure 14.2. The dichroic reflector reduces infrared coupling (>700 nm) to prevent overheating of fiber-optic bundles, while allowing sufficient infrared light throughput to permit imaging at these wavelengths. Illumination intensity levels – Off, Low, and High – are computer-controlled through Living Image software, with the Low setting being approximately 18% of the High setting.

![Quartz Halogen Lamps with Dichroic Reflectors](image)

**Figure 14.2** Relative spectral radiance output for the quartz halogen lamp with dichroic reflector.

The lamp output is delivered to the excitation filter wheel assembly located on the back of the IVIS Imaging System. See Figure 14.3. 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 the user 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 IVIS Imaging System imaging chamber. Motor-control of the excitation filter wheel is managed through Living Image software.
Following the excitation filter, a second lens focuses light into a 0.25-inch fused silica fiber-optic bundle inside the IVIS Imaging System imaging chamber. Fused silica (core and clad) fibers, 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 located on the ceiling of the IVIS Imaging System imaging chamber. See Figure 14.1. The reflectors provide a diffuse and relatively uniform illumination of the sample stage. Figure 14.4 shows the illumination profiles for fields of view of 10 to 25 cm on an IVIS Imaging System 100 Series.

**NOTE** At a FOV of 10 cm 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 14.3 Excitation filter wheel cross section.](image)

![Figure 14.4 Illumination profiles for different FOVs on an IVIS Imaging System 100 Series measured from the center of the FOV.](image)
14. Fluorescent Imaging

Nonuniformity in the illumination profile is corrected for by analyzing images in units of Efficiency. When Efficiency is selected, the measured fluorescent image is divided by a reference illumination image, thus normalizing the image. See Section 4.3, page 26.

Fluorescent emission from the target fluorophore is collected through an emission filter wheel located at the top of the IVIS System imaging chamber and focused into the CCD Camera. The IVIS Imaging System 200 Series provide a 24-position emission filter wheel (two levels, each with 12 positions), allowing the user to select from up to 22 fluorescence filters measuring 60 mm in diameter. The IVIS Lumina provides an 8-position emission filter wheel allowing the user to select from up to seven fluorescence filters. The IVIS Imaging System 100 Series and 50 Series provide a 6-position emission filter wheel, allowing the user to select from up to five fluorescence filters measuring 75 mm in diameter. All IVIS Imaging Systems require that one filter position on each wheel always be open for bioluminescent imaging.

14.2 Understanding Filter Spectra

The use of high-quality filters is essential for obtaining good signal-to-background levels, or contrast, in fluorescence measurements, particularly in highly sensitive instruments such as the IVIS Imaging Systems. Typical excitation and emission fluorophore spectra, along with idealized excitation and emission filter transmission curves are shown in Figure 14.5. The excitation and emission filters are called bandpass filters; ideally they transmit all the wavelengths within the bandpass region and block, i.e., absorb or reflect, all wavelengths outside the bandpass. This spectral band is like a window, characterized by its central wavelength and its width at 50% peak transmission, or full width half maximum (FWHM). Filter transmission curves of a more realistic nature are shown in Figure 14.6.

![Figure 14.5 Typical excitation and emission spectra for a fluorescent compound. Included in the graph are two idealized bandpass filters that would be used with this fluorescent compound.](image-url)
Because the filters are not ideal, some leakage, or 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.

![Typical attenuation curves for excitation and emission filters.](image)

**Figure 14.6** Typical attenuation curves for excitation and emission filters.

In Figure 14.6, the vertical axis is optical density, defined as $\text{OD} = -\log(T)$, where $T$ is the transmission. An optical density of 0 indicates 100% transmission, whereas $\text{OD}=7$ indicates a reduction of the transmission to $10^{-7}$. In high-quality interference filters, such as those used in the IVIS Imaging Systems, transmission of a filter in the bandpass region is about 0.7 ($\text{OD}=0.15$), with blocking outside of the bandpass region, typically, in the $\text{OD}=7$ to $\text{OD}=9$ range. The band gap is defined as the gap between the 50% transmission points of the excitation and emission filters. The band gap is typically from 25 to 50 nm.

There is a slope in the transition region from bandpass to blocking. See Figure 14.6. 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. At infrared wavelengths (800 nm), the slope is less steep so a wider gap of up to 50 nm is necessary to avoid cross talk.

Eight excitation and four emission filters come standard with a fluorescence equipped IVIS Imaging System. (see Table 14.1). Custom filter sets are also available. Fluorescent imaging conducted on the IVIS Imaging System uses a wavelength range of 400-950 nm. Therefore, a wide range of fluorescent dyes and proteins are available for fluorescent applications. It is important to keep in mind that for in vivo applications, wavelengths greater than 600 nm are preferred. At wavelengths below 600 nm animal tissue absorbs significant amounts of light, limiting the depth to which light can penetrate, e.g., fluorophores located deeper than a few millimeters will not be excited. The autofluorescent signal of tissue also increases at wavelengths below 600 nm.
### 14.3 Acquiring Fluorescent Images

**IVIS System Control Panel Overview**

Acquiring fluorescent images using Living Image software is controlled through the IVIS System Control Panel. See Figure 14.7. To acquire a fluorescent image, the user must check the **Fluorescent** box on the left side of the panel. Once selected, controls for the illumination lamp – **Fluor Lamp Level**, and **Filter Lock** – will appear in the top half of the panel. Checking the **Filter Lock** box ensures that the excitation and emission filters are properly paired. During image acquisition, the QTH lamp is computer-controlled through Living Image software. The **Fluorescent level** pull-down menu controls the illumination intensity level of the lamp with options – **Off**, **Low**, **High**, and **Inspect**. The **Low** setting is approximately 18% of the **High** setting. **Inspect** turns on the QTH illumination lamp, allowing the user to manually inspect the excitation lamp.

**NOTE**

Make sure the desired filters are currently selected in the filter popup menus before selecting Inspect. The Inspect operation will automatically position the filters currently selected in the filter popup menus before turning on the lamp. Subsequent changes to the filter popup menus will have no effect until another Inspect operation is done.
Procedure for Acquiring Fluorescent Images

1. If it is not already on, start up the acquisition computer and start the Living Image® software program from the Windows Start Menu. A system control panel will appear in the lower right corner of the monitor. See Figure 14.7, page 113.

2. Click the Initialize IVIS system button in the camera control panel. After initialization, the Temperature Status box in the center of the panel should be green, indicating that the CCD camera temperature is locked. (IVIS® Imaging System 50 Series and IVIS Lumina users should allow 10 to 15 minutes for the camera to reach the proper temperature.) The Temperature Status box changes from red to green when the CCD camera has reached the proper operating temperature.

3. Place the sample to be imaged in the center of the stage in the imaging chamber. Close the door.

4. Select the fluorescence check box.

5. Select the desired Field of View from the pull down menu on the left side of the control panel.

6. Enter the approximate (1.5 cm) Subject Height (height) in the lower left entry box (or focus manually).

7. Select the Emission Filter and Excitation Filter. If the Filter Lock box is checked, the software automatically selects the second filter. Select only one, as the other filter will be selected automatically.

8. Select High or Low from the Fluorescent level pull-down menu. High is the recommended setting.

9. Set the desired Exposure Time, Binning, and f/stop. Fluorescence is generally brighter than bioluminescence, so the exposure is shorter and f/stop higher (smaller lens opening). Typical fluorescent image camera settings might be 10 sec exposure time, Binning=small, and f/2.

10. Click the Acquire button in the control panel.
14. Fluorescent Imaging

11. After the exposure is complete, the overlaid image is displayed. From the Main Menu Bar, select **Living Image-Save Living Image Data** to save the displayed data.

This completes the data acquisition. To obtain additional images, repeat the process, beginning with Step 3.

The Living Image image window in which the fluorescent image is subsequently displayed displays annotations specific to fluorescence, including emission filter, excitation filter, and fluorescence level. These annotations are in addition to standard annotations such as exposure time, f/stop, FOV, and date/time of exposure.

### 14.4 Image Units

Data obtained from fluorescent images can be displayed in units of **Counts**, **Photons** (absolute, calibrated), or **Efficiency** (calibrated, normalized). See Chapter 9, page 79. If the user opts for units of **Photons**, images with different exposure times, f/stops, and binning may be compared. If **Efficiency** is selected, the fluorescent image is normalized (divided) by a stored reference image of the excitation light intensity. Efficiency images are unitless and represent the ratio of light emitted to light incident.

### 14.5 Working with Fluorescent Samples

There are a number of issues to consider when working with fluorescent samples, including the position of the sample on the stage, leakage and autofluorescence, background signals, and appropriate signal levels and f/stop settings. These issues are discussed below.

![Illustration of in vivo fluorescence process.](image)

**Figure 14.8 Illustration of in vivo fluorescence process.**
Tissue Optics Effects

When conducting in vivo fluorescence imaging, the excitation light must be delivered to the fluorophore inside the animal for the fluorescent process to begin. See Figure 14.8. Once the excitation light is absorbed by the fluorophore, the fluorescence is emitted. However, due to the optical characteristics of tissue, the excitation light undergoes scattering and absorption both before it reaches the fluorophore and after it leaves the fluorophore, and is detected on the surface of the animal. 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 may occur throughout the animal, but will be strongest on the surface of the animal, where the excitation light is strongest.

The transmission of light through tissue is highest at wavelengths of 600-900 nm, while the generation of autofluorescence is lower. Therefore, selecting fluorophores in the 600-900 nm range is highly desirable. Fluorophores active in the 450-600 nm range, such as GFP, 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

Because fluorescent signals are generally brighter than bioluminescent signals, imaging times are shorter, typically, from one to 30 seconds. The bright signal allows the user to select a lower binning to achieve better spatial resolution. In addition, the f/stop can often be set to higher values; f/2 or f/4 is recommended for fluorescence imaging. A higher f/stop will improve the depth of field, yielding a sharper image. See Section 3.1, page 9.

Instrument Background Subtraction

The fluorescence instrumentation on IVIS Imaging Systems has been carefully designed to minimize autofluorescence and background caused by instrumentation. However, because of the highly sensitive CCD camera utilized in these instruments, a residual background may be detected. This background is due to autofluorescence of the system optics or experimental setup, or residual light leakage through the filters. This background may be measured and subtracted from a fluorescence image using the Living Image® software.

Fluorescent background subtraction is similar to dark charge bias subtraction, which is implemented in luminescent mode. However, fluorescent backgrounds will change day-to-day, depending on the experimental setup. Therefore, fluorescent backgrounds are not automatically measured during the night, as are dark charge backgrounds.

Typically, the user takes a fluorescent image, inspects the signal, and determines if the fluorescent background should be subtracted. See Figure 14.9. If a subtraction is needed, the fluorescent subject should be removed from the imaging chamber and the fluorescent background measured, by clicking Living Image - Fluorescent Background - Measure Fluorescent Background. See Section 6.3, page 52. After a fluorescent background has been measured, it must be applied to the sample image by clicking Living Image - Fluorescent Background - Add or Replace Fluorescent Background. The Sub Fluor Bkg check box appears on the image analysis panel. See Figure 14.7. The user can toggle the background subtraction on and off using this check box.
The detected fluorescent signal from a sample depends on the amount of fluorophores present in the sample and the intensity of the incident excitation light. The incident excitation light hitting the sample stage is not uniform over the FOV; the illumination peaks at the center of the FOV and drops off slowly toward the edges. See Figure 14.4. To eliminate the excitation light as a variable from the measurement, data may be displayed in calibrated units of Efficiency, which provide a more quantitative comparison of the fluorescent signals. If Efficiency is selected, the fluorescent emission image is normalized (divided) by a stored, calibrated reference image of the excitation light intensity incident on a highly reflective white plate. The resulting image is a unitless number, typically in the range of $10^{-2}$ to $10^{-9}$.

A reference image of the excitation light intensity is measured for each excitation filter at every FOV and lamp power. These reference images are measured and stored in the Living Image folder prior to instrument delivery.
Understanding Autofluorescence

Autofluorescence is a fluorescent signal originating 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 well plates, and by animal tissue. Filter leakage, which also may occur, is another source of background light.

Optical components used within the IVIS Imaging System are carefully chosen to minimize the level of 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.

In distinguishing real signals from background emission, it is important for the user to recognize the signatures of different types of autofluorescence. The images that follow illustrate the different sources of autofluorescence.

Well Plate Autofluorescence

When making well plate measurements of cells marked with a fluorophore, the user must be aware of both well plate and native cell autofluorescence. Figure 14.10 shows autofluorescence originating from four different plastic well plates. The images were taken using a GFP filter set (excitation 445-490nm, emission 515-575nm).

Two types of autofluorescent effects may appear. One is the overall glow of the material, which, typically, indicates the presence of autofluorescence. The other is "hot spots", which indicates a specular reflection of the illumination source. See Figure 14.11. The specular reflection is an optical illumination autofluorescence signal reflecting
from the surface of the well plate, and is not dependent upon the plate material.

![Specular Reflection](image)

**Figure 14.11** 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.)

The images demonstrate that black polystyrene well plates emit the smallest inherent fluorescent signal, while white polystyrene plates emit the largest signal. The clear polystyrene well plate, which has a slightly higher autofluorescence than the black well plate, is low enough to still be useful. Black polystyrene well plates are recommended for *in vitro* fluorescent (and bioluminescent) measurements.

Control cells are always recommended in any experiment to assess the autofluorescence of the native cell.

**Miscellaneous Material**

**Autofluorescence**

Xenogen Corporation recommends that a sheet of black Lexan (Xenogen Corporation Part 60104) be placed on the imaging stage to prevent illumination reflections and to assist in keeping the stage clean. The black paper (Swathmore, Artagain, Black, 9”x 12”, Part #445-109) often recommended for bioluminescent imaging has a measurable autofluorescent signal, particularly with the Cy5.5 filter sets, and should not be used for fluorescent imaging.
Figure 14.12 is a fluorescent image of Black Lexan on a sample stage, as seen through a GFP filter set. The image includes optical autofluorescence, light leakage, and low level autofluorescence from inside the IVIS Imaging 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.

Other laboratory accessories may also exhibit non-negligible autofluorescence. See Figure 14.13. This chart compares the autofluorescence of miscellaneous laboratory materials to that of black Lexan, e.g., the agar plate with ampicillin is over 180 times more fluorescent than black Lexan. Such a significant difference in autofluorescence levels further supports the recommended use of black polystyrene well plates.

NOTE

It is also recommended that all materials used inside the IVIS Imaging System be characterized by taking control measurements.
Despite the presence of various background sources, the signals from most fluorophores will exceed background emissions. Figure 14.14 shows the fluorescent signal from a 96 well plate 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.

The autofluorescence of animal tissue is generally much higher than any other background source discussed so far, and is likely to be the
most limiting factor in vivo fluorescent imaging. Figure 14.15 shows ventral images of animal tissue autofluorescence for the GFP, DsRed, Cy5.5, and ICG filter sets for animals fed regular rodent food and alfalfa-free rodent food (Harlan Teklad TD97185 and TD97184, non-irradiated and irradiated, respectively). Animals fed the regular rodent diet and imaged using he GFP and DsRed filter sets, show uniform autofluorescence, while images taken with the Cy5.5 and ICG filter sets show the autofluorescence to be concentrated in the intestinal area. The chlorophyll in the regular rodent food is causing the autofluorescence in the intestinal area. When the animals’ diet is changed to the alfalfa-free rodent food, the autofluorescence in the intestinal area is relegated to levels seen in the rest of the body. In this situation, the best way to minimize autofluorescence is to switch the animals to a diet of alfalfa-free rodent food when working with the Cy5.5 and ICG filter sets. Control animals should always be used to assess background autofluorescence.

A comparison of fluorescence versus bioluminescence emission in vivo is shown in Figure 14.16. In this example, $3 \times 10^6$ PC3M-luc/DsRed prostate tumors are 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, allowing for bioluminescent and fluorescent expression. The signal level for fluorescence is 110 times brighter than that for bioluminescence. However, the autofluorescent tissue emission is five orders of magnitude higher.

![Figure 14.15](image)

**Figure 14.15** Images of animal tissue autofluorescence in control mice (Nu/nu males) fed regular rodent food (top) or alfalfa-free rodent food (bottom). Images were taken using GFP, DsRed, Cy5.5, or ICG filter sets.
this example, fluorescent imaging requires at least $3.8 \times 10^5$ cells to obtain a signal above tissue autofluorescence while bioluminescent imaging requires only 400 cells.

Figure 14.16 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 $3 \times 10^6$ cells in an 11-week old male Nu/nu mouse.

Subtracting Tissue Autofluorescence Using Excitation 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 out 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 14.1). The objective of these 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 on image math, see page 62.) 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.

Table 14.1 Summary of the background, excitation, and emission filters

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Background Filter</th>
<th>Excitation Filter</th>
<th>Emission Filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>410-440</td>
<td>445-490</td>
<td>515-575</td>
</tr>
<tr>
<td>DsRed</td>
<td>460-490</td>
<td>500-550</td>
<td>575-650</td>
</tr>
<tr>
<td>Cy5.5</td>
<td>580-610</td>
<td>615-665</td>
<td>695-770</td>
</tr>
<tr>
<td>ICG</td>
<td>665-695</td>
<td>710-760</td>
<td>810-875</td>
</tr>
</tbody>
</table>
Correction Procedure for Tissue Autofluorescence

1. Determine if a fluorescent instrument background needs to be taken (for more details, see page 115).

---

**NOTE**

If a fluorescent instrument background is necessary, it must be measured for both the primary and the background excitation filter image.

---

2. Load the primary (excitation filter) image and the background filter image as a sequence.

The Image Math tool is only available if an image sequence is open. You can create an image sequence by grouping individual images together or by acquiring multiple images using the Sequential mode.

**To load separate images as a group in the Living Image® 3D Analysis Package software:**

Select the two images from the Browser window and click **Load As Group**.

— The images are loaded together in a new sequence window.

**To acquire an image sequence in the Living Image software:**

a. Click **Select as Sequential Mode**.

— The sequential imaging panel appears.

b. Configure the imaging parameters for the primary fluorescent image and click **Set**.

c. Change the excitation filter to the corresponding background filter and click **Set**.

d. Click **Acquire Sequence**.

— The instrument automatically acquires the two images as a sequence.

3. After the image sequence(s) is loaded, click **Image Math**.

— The Image Math window appears (Figure 14.17).
14. Fluorescent Imaging

4. Choose the subtraction mode, A-B×k, from the Result drop-down list.

5. Enter a value for the scale factor, k.

The scale factor is determined by taking 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 with no fluorophore present. The scale factor accounts for different levels of tissue autofluorescence due to different excitation wavelengths and filter transmission characteristics.

6. To display the corrected image, click **Display Result for Measuring**.

   — In the corrected image, autofluorescence is significantly reduced, while leaving most of the fluorophore signal.

Figure 14.18 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 14.19 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 14.18 shows the IVIS® images using the primary excitation filter, the background excitation filter, 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 1.5×10^3 to 6.7×10^3.

**Figure 14.18 Example of the autofluorescent subtraction technique using a background excitation filter.**

The images show: 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 1.4) 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 14.19 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.
The unique spectral signatures of both the luciferase emission spectrum and the optical properties of tissue allow quantitative information on the depth of bioluminescent reporters to be extracted by acquiring images at various wavelengths. The spectral imaging technique incorporated in Living Image software uses a diffusion model for light propagation in tissue in order to extract the depth and intensity of sources inside a living animal. The spectral model requires the acquisition of two or more single-view images acquired at wavelengths between 560 and 660 nm.

Spectral imaging is standard on the IVIS Imaging System 200 and 3D Series, and is optional on the IVIS Imaging System 50 or 100 Series. Spectral imaging is not available on the IVIS Lumina.

15.1 Spectral Imaging Procedure

Image Acquisition

The sequence of operation assumes that the Living Image software is running and the imaging system has been initialized.

1. Click the Select Sequential Mode button on the System Control panel. A Sequence Control panel and setup table will open.

2. Select the desired filter from the Emission Filter pull-down menu on the System Control panel. This sets the image acquisition parameters for a specific wavelength.

   The IVIS Imaging System 200 Series includes six 20 nm band pass filters, ranging from 560-660 nm in 20 nm intervals, as standard equipment. An extended set of filters (Xenogen part no. XFS-60SI) adds 11 additional filters that together with the standard equipment provide a range from 420-740 nm.

3. Once all image acquisition parameters have been defined, click the Set button. The set parameters will appear in a column of the Sequential Setup table.

4. Repeat Steps 2 and 3 for all desired wavelengths. A minimum of two wavelengths is required to perform spectral imaging analysis. Typically, more wavelengths will yield a more accurate result. Imaging with each of the six filters is recommended.
5. The **Sequential Setup** table can be edited prior to image acquisition by using the buttons, **Clear One**, **Clear All**, and **Set**.

6. Click the **Acquire Sequence** button on the Camera Control panel to launch the image sequence acquisition.

### Spectral Analysis

1. Load the desired image sequence for spectral analysis.

2. From the **Main Menu Bar**, select **Living Image-Analysis Options-Spectral Analysis**. See Figure 15.1. This will open all images in the sequence. The images are displayed in units of photons. A Spectral Imaging Control panel will appear at the top right section of the screen. See Figure 15.2.

### Figure 15.1  Spectral Analysis Option selected from the Living Image menu.

3. Define an ROI for spectral analysis. If no ROI exists, one must be created. If an ROI is present and is not the desired ROI, it must be removed or modified until it is suitable for analysis.

### IMPORTANT

The spectral analysis will be performed only on the ROI labeled "ROI1." If more than one ROI is present, the additional ROIs will not be analyzed.

4. Click the **Spectral Analysis** button. See Figure 15.2. This copies the ROI (ROI1) defined on the upper-most image into all images in the sequence. The ROI is then measured in all the images and the measurement results fitted to the spectral analysis model.
5. The spectral analysis results shown in Figure 15.3, are displayed in a four-part format comprised of 1) the last image in the sequence, including the analyzed ROI; 2) the values extracted from the fit to determine the total flux of the source and its average depth below the surface; 3) a plot showing normalized intensity vs. wavelength (intensity is normalized by the luciferase spectrum and filter transmission properties); 4) a plot of the normalized intensity vs. effective attenuation coefficient $\mu_{\text{eff}}$ (optical property of mouse tissue). The analytic function used in this fit is an exponential. When displayed on a logarithmic scale, it becomes a straight line, the slope of which is the depth of the source.

![Spectral Imaging Controls Panel.

Figure 15.2 Spectral Imaging Controls Panel.

Figure 15.3 Spectral Analysis Results. This image shows the four-part spectral analysis results of a recently defined ROI.
15.2 Spectral Imaging Theory

Images obtained using the IVIS Imaging System are a diffuse projection on the surface of the animal from the bioluminescent sources located deeper inside. There is a strong interest in obtaining information about the depth of bioluminescent cells, both to improve quantification of the source brightness and to provide information on the location of the cells, e.g., in what organ did a metastatic lesion develop.

One rigorous approach used to derive depth localization of bioluminescent sources inside living tissue is known as diffuse tomographic analysis. This approach requires the acquisition of multiple images at various viewing angles and a 3-dimensional reconstruction algorithm to retrieve information on the precise location of the tumor. Instrumentation and software related to 3-D diffuse tomography is in development at Xenogen. In the mean time, a simpler algorithm that can provide depth estimates from a single-view image may be used.

This simpler form of spectral imaging analysis uses spectroscopic information to estimate the depth of the bioluminescent cells. This method takes advantage of the fact that the bioluminescent signal from firefly luciferase is emitted from 500 to 700 nm, a region of the spectrum where major contrasts in tissue optical properties exist. See Figure 15.4. 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 of hemoglobin. As a result, the bioluminescent signal observed on the surface of the animal is dependent on both the wavelength and the thickness of tissue through which it travels. Relatively simple analytical expressions using the diffusion approximation to model the propagation of light through tissue allow the extraction of the depth and absolute photon flux of a single point source from two or more images acquired at different wavelengths.
Light propagating through tissue undergoes scattering and absorption. The diffusion model used assumes that scattering is the predominant phenomenon occurring in biological tissues, as compared to absorption, i.e., reduced scattering coefficient $\mu'_s$ \(\gg\) absorption coefficient $\mu_a$. This statement 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.

Given these conditions, if one integrates the light collected over the animal surface, modeled as flat and infinite in extent, the total integrated intensity $I(\lambda)$ is reduced to a relatively simple expression:

$$I(\lambda) = S K(\lambda) \exp(-\mu_{\text{eff}}d)$$

where $S$ is the absolute total photon flux emitted by the bioluminescence source and $d$ is the source depth. The term $\mu_{\text{eff}}$ is called an effective attenuation coefficient. It depends on the tissue coefficient of absorption $\mu_a$ and reduced scattering $\mu'_s$, which quantify the two main phenomena occurring to light in tissue. The function $K(\lambda)$ is a more complex expression that is derived from the model and includes terms describing the effect of the tissue-air boundary to the light propagation. Both $\mu_{\text{eff}}$ and the function $K$ are dependent on the wavelength $\lambda$.

Equation 1 shows that if the total integrated intensity is measured at several wavelengths, it is proportional to an exponential function of the product of depth and the optical property $\mu_{\text{eff}}$. Therefore, the strategy employed in the spectral analysis technique is to analyze two or more images recorded at different wavelengths, measure the total integrated intensity on each image, and then fit the measured values to the exponential function appearing in Equation 1. The results of the fit are the total flux of the bioluminescence source $S$ and the source depth $d$. 

Figure 15.4 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 through 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 on PC3M cells.
Optical Properties

Prior knowledge of the optical properties at all wavelengths used is necessary to perform the spectral analysis. The two main optical parameters are the absorption coefficient $\mu_a$, which defines the inverse of the mean path before photons are absorbed by the tissue, and the reduced scattering coefficient $\mu'_s$, which defines the inverse of the mean path before photons are scattered isotropically in the tissue. The effective attenuation coefficient $\mu_{\text{eff}}$ is a function of the absorption and reduced scattering coefficients $\mu_{\text{eff}} = (3\mu_a (\mu'_s + \mu_a))^{1/2}$.

Calculation of the function $K$ appearing in Equation 1 requires as input all three coefficients ($\mu_a$, $\mu'_s$, and $\mu_{\text{eff}}$). In addition, the function $K$ accounts for the reflection of light at the air-tissue boundary, due to a mismatch in the index of refraction, by including a term called the effective reflection coefficient. The value for the tissue index of refraction is generally assumed to be close to 1.4.

As stated above, the model assumes that the tissues are optically homogeneous. The Living Image software currently uses one set of values for mouse optical properties. For more details, see the file "OptProp-Mouse.txt" located in the Spectral Imaging sub-directory of the Living Image software.

Luciferase Spectrum

The analysis of spectrally filtered images requires knowledge of the spectral dependence of bioluminescent light emission. Therefore, the luciferase bioluminescence spectrum was measured in vitro on various cell lines, at 37° C and pH≈ 7. This spectrum is used to normalize the photon flux values measured at each wavelength with Living Image software analysis tools.

Currently, all models developed at Xenogen Corp use luciferase obtained from the firefly to tag genes or tumor cell lines. The spectrum used by the Living Image spectral analysis is therefore the firefly luciferase spectrum measured in vitro. See Figure 15.4.

NOTE

The firefly luciferase spectrum is dependent on temperature and pH. The data provided is valid only for measurements performed at 37° C and for pH values in the 7.0-7.5 range. Selecting other temperature and pH conditions for a specific experiment would require the use of the corresponding spectral curve for the spectral analysis. For more information about pH and temperature dependence of luciferase spectrum, please contact Xenogen Corporation.

Spectral Imaging: Example

The spectral imaging technique was used to analyze images obtained on a metastasis model. Melanoma cells were injected intravenously into the tail vein of nude mice and began colonizing the lungs. After 13 days, metastasis had developed in several areas of the mouse body, including lungs, kidney and rear limb bone. Two animals were imaged in an IVIS Imaging System 200 Series. A sequence was acquired to record images filtered at six wavelengths, ranging from 560 to 660 nm, at intervals of 20 nm. Because tissue optics are responsible for a larger attenuation of light when using 560 nm and 580 nm band pass filters, due mainly to hemoglobin absorption, it is best to use longer imaging
times for these wavelengths when defining the acquisition sequence. In the example shown here, the conditions selected were high-sensitivity binning, f/stop=1, FOV C (13 cm). Exposure times of 120 seconds were used for 560 nm and 580 nm wavelengths, while 60 seconds exposure times were used on all other wavelengths. This resulted in signals of approximately 2000 counts on each image.

The dorsal view image shown in Figure 15.5 illustrates the various metastasis sites. On first observation, the image shows that the signals coming from the lungs and right kidney to be rather well defined in both animals. However, the signal in the lower back area of the mouse on the left features two bright spots in close proximity, causing an artifact in the spectral analysis.

To proceed with the spectral analysis, an ROI targeting each site needs to be defined, with the goal of each ROI being the capture of the entire signal of its intended target only and not that of the neighboring metastases. At this point, the user draws an ROI on the image in the sequence that appears most likely to locate the signal coming from the site of interest.

Figure 15.5 This image shows metastatic sites in nude mice imaged 13 days after a tail vein injection of 5x10^5 B16F10 melanoma cells.
Once the ROI is drawn, the user launches the spectral analysis. The ROI total flux is measured on each filtered image and normalized to the luciferase spectrum. The analytical expression in Equation 1 is used to fit the normalized data with two floating parameters, the total flux of the bioluminescence source $S$ and the depth $d$. See Figure 15.7. To obtain an estimate of the cell count, the absolute photon flux measured must be divided by the flux per cell. This is best determined by performing another independent measurement on the cell line used in the experiment.

Figure 15.6 Enlarged View of Metastatic Site. An ROI of the left mouse in Figure 15.5 was defined to include the signal of the right kidney and separate it from other 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 is likely originating from the femoral bone of the animal.
The fit is weighed 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 governed mainly 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. The experiment result shows a relative uncertainty of approximately 23% in depth, and 28% in total flux measured. See Figure 15.7.

Using the same image set, other sites have been analyzed. See Figure 15.8. On first observation, it can be surmised that, even though the total flux measured from the dorsal view of the lung of mouse #4 (far right) is dimmer at the surface than the flux coming from the kidney of mouse #3 (Figure 15.7), it is a brighter source ($2.76 \times 10^{8}$ ph/s vs. $1.81 \times 10^{8}$ ph/s), yet buried deeper inside the tissue (7.0 mm for lung vs. 4.8 mm for kidney). This is a typical example highlighting the usefulness of the spectral imaging technique. Another interesting observation is made...
15. Spectral Imaging (IVIS® Imaging System 200 & 3D Series)

when analyzing the left lung of mouse #4 from the dorsal and ventral views; the values for source total flux are very close. The measured depth also is similar, which would indicate that the cells are distributed at almost the same distance from the front and back of the animal. Adding the two depths obtained from the dorsal and the ventral views should yield an estimate of the animal height at this location. These results would mean that the mouse is approximately 14.7 mm tall in this region, a very reasonable estimate.

Optimizing the Precision of Spectral Analysis

Spectral analysis performance is highly dependent upon the quality of the \( a \text{ priori} \) measured data for the luciferase spectrum and the tissue optical properties. In addition, the precision of the analysis is dependent on the quality of the fit to the experimentally measured total fluxes at each wavelength. As a general observation, the more experimental values, the better the fit. In particular, it is important to be able to extract signals at all wavelengths to optimize the quality of the fit. If no signal above the animal background level is recorded at the wavelengths that absorb the most light, 560 nm and 580 nm, the dynamic range of the optical properties will be reduced and, with it, the precision of the fit.

Figure 15.8 Spectral Analysis Results: This image displays the results of a spectral analysis of the dorsal view (left) and the ventral view (right) of the left lung of mouse #4.
The spectral analysis includes a criterion that uses a minimum threshold to accept data from an image in order to avoid confusion between a bioluminescent source and the autoluminescence of the animal. However, this selection is not perfect as autoluminescence can vary from animal to animal, particularly for different strains. Therefore it is highly recommended that the user inspects the images in order to make sure a signal is present in the site of interest at all wavelengths used in the analysis. Of course, it is not always possible to extract a signal at all wavelengths, as is the case for sources deeply buried in tissue. If such image sequences are analyzed, the user has the option to discard one or more images from the analysis. By closing the undesired image before clicking on the **Spectral Analysis** button, the image will not be included in the measurements used in the fit.
Structured Light (IVIS® Imaging System 200 & 3D Series Only)

Theory of Operation

Structured Light refers to the method of determining the 3D surface topography of an animal. This information is required input for the Diffuse Luminescence Imaging Tomography (DLIT) algorithm, which uses models of photon transport in tissue to localize and quantify photon sources. Surface topography information is necessary to sufficiently model the boundary conditions of photon transport at the air-tissue interface.

16.1 Theory of Operation

Surface topography can be determined from structured light images. Parallel lines of light are drawn across the imaging subject to produce a structured light image. See Figure 16.1. The surface topography of the object can be determined from analyzing the displacement or bending of these lines as they pass over the object. The displacement, denoted by $\Delta x$, is defined to be the difference between where the line should fall on the stage in the absence of the object, and where it appears in the image due to occlusion by the object. The parallel lines are projected onto the surface of the object at an angle. This angle, $\theta$, 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, $l$. See Figure 16.2. $D$ and $l$ form two perpendicular sides of a triangle giving

$$\tan \theta = \frac{D}{l}$$

$\Delta x$ and $h$ make up a downscaled version of this triangle. The height, $h$, can be determined using

$$h = \frac{\Delta x}{\tan \theta}$$

by measuring the displacement $\Delta x$.

Living Image software utilizes fast numerical methods to rapidly evaluate $\Delta x$ over the entire image to determine the surface topography. The surface topography determination is limited to the topside of the object facing the lens.
16. Structured Light (IVIS® Imaging System 200 & 3D Series Only)

Structured Light Acquisition

To determine the surface topography of an animal, the user must acquire and store structured light images and a photographic image. The structured light image is acquired automatically if the Structure box is checked in the IVIS Imaging System 200 or 3D Series Camera Control panel. See Section 3.3, page 17.

Structured Light Analysis

The surface topography can be determined from structured light images at any time after the user acquires the images. The following steps describe a typical structured light analysis procedure, and are graphically represented in Figure 16.3 to Figure 16.6.

1. Browse for the data and load the desired Living Image data set.

2. Select Living Image-Analysis Options-Structured Light Analysis.

3. Crop the image. See Figure 16.3.
a. The “Crop - Step 1” window opens, a photographic image is displayed.

b. Make a rectangular crop around the desired animal. Starting in upper left hand corner of the crop, press and hold the left button of the mouse, and drag it down to the lower right hand corner of the crop.

c. Click on the Crop button of the Crop window when satisfied with the crop.

d. The Cancel button will cause the Structured Light Analysis program to exit.

4. Threshold. See Figure 16.4.

— The “Threshold – Step 2 window” opens.

a. A green line traces regions of image pixel intensities above a designated threshold. It is possible to adjust the threshold up or down until the green line traces around the animal, separating it from the stage. This is done either by entering values into the Threshold field, or by clicking on the up/down arrows of the keyboard. Ideally, the goal is to select the entire animal and none of the stage.

b. Click the OK button when satisfied with the trace.
5. Furry Analysis Option. See Figure 16.5.
   a. A dialog box opens and asks the user if this option should be applied. Click Yes if the animal is furry. Click No for nude subjects. An additional step may be necessary if the subject is furry.

6. Computed Height Map. See Figure 16.6.
   a. Computed Height Map window appears after the computation is completed. A computed height map of the animal is displayed.
   b. The displayed image contains a photographic image with contour line overlays indicating specified heights.
c. The **Background** pull-down menu allows the user to change the image displayed in the background (The default background is the photographic image).

d. The **Colors** pull-down menu controls the color table used to display the background image.

![Computed Height Map](image)

**Figure 16.6** Computed Height Map. This window displays an example of a photographic image with a contour overlay.
Index

A
acquire
  continuous photos 14
acquire image 14
  quick start 7–8
alignment grid 17
animal tissue autofluorescence 120–126
auto ROI
  measurement ROI 87
auto ROIs 87–91
autofluorescence 117
  animal tissue 120–126
  correction procedure 123–125
  miscellaneous 118–120
  subtract using background filters 122–126
  well plate 117–118
autosave 56
autosave directory 56
autoscale 40
average counts 83
average radiance 81, 84

B
background
  automatic measurements 101–102
  electronic 93
    dark current 93
    read bias 93
fluorescent 52
  add/replace 52
  clear 53
  measure 53
  measure and replace 53
  view available 53
light from sample 96–97
light on sample 94–95
system optics autofluorescence 115–116
tissue autofluorescence 122–126
background ROI 80
background ROI contextual menu 36–38
band gap 111
bandpass filter 110
binning 13, 61
blends 71

C
CCD camera 69
  saturation 13
  temperature 11
click number 45, 103
color table 66
colors
  current 41
  pre-saved 41
contextual menu
  background ROI 36–38
  image 34–35
  measurement ROI 35–36
  subject ROI 38
  zoom/crop 38
continuous photos 14
correcting for tissue autofluorescence 123–125
cosmic ray correction 86
counts
  average 83
  definition 83
  total 83
current colors 41

dark charge 50, 99
  clear available 51
  management 99–102
  measurement 50, 99–100
  replace 51
  subtraction 100
  view available 51
dark current 93
data
  sequence 39–42
diffusion model 131
drift 101

efficiency 85, 110, 114, 116
electronic background 93
dark current 93
read bias 93
emission filter 14, 110
excitation filter
    selecting 16
export ROI data 82
exposure time 12

F
f/stop 13
    fluorescent imaging 115
field of view 11
filter
    bandpass 110
    emission 14
    excitation 16
    fluorescent 111
    lock 16
filter lock 112
filter spectra 110
filter wheel
    emission 110
flat fielding 85
fluor lamp level 17
fluorescence efficiency 116
fluorescent background 52
    add/replace 52
    clear 53
    measure 53
    measure and replace 53
    view available 53
fluorescent filters 111
fluorescent images
    acquiring 112–114
    units 114
fluorescent imaging 16–17
    efficiency 85, 110
    excitation filter 16
    f/stop 115
    normalization 110
fluorescent samples
    tissue optics effects 115
focus 11
    focus-scan mid 18
    manual 12

G
gamma 41

H
Help 5, 10
High Reflectance Hemisphere 95
histogram 50

I
IGOR Pro 4
    menus 67
image
    acquire 14
    acquire (quick start) 7–8
    blends 71
    display modes 69–71
    fluorescent 16–17
    overlays 71
    photographic 9
    pseudocolor 70–71
    RBG 69
    sequential 15, 39
    sorting 46
image contextual menu 34–35
image data
    importing 106
    retrieving 104–106
    saving 103–104
    sequence 39–42
image math 62–65, 124
    result = 64
image window 21
    contextual menus 34–38
images
    add, subtract, multiply, divide 62–65
imaging mode
    overlay 12
importing image data 106
individual color range 40
initialize system 10
IVIS Imaging System 200 Series
    alignment grid 17
    components 107–110
    focus-scan mid 18
    structure 18, 19
IVIS Imaging System components
100 Series 1
200 Series 1, 107–110
50 Series 1

L
Lab Book 58–59, 81
label name set 54
labeling scheme 105
lights on 15
Living Image Browser window 44
Living Image software
   starting 4
log 41
luciferase spectrum 132

M
manual focus 12
manual overlay 67
measurement ROI 80, 87
   contextual menu 35–36
measurements table 82
miscellaneous autofluorescence 118–120

N
normalization 110

O
optical density 111
optical properties for spectral imaging 132
overlay imaging mode 12
   manual 67
overlays 71

P
photographic image 9
photon 69, 79, 83, 114
photon radiance 83
pixel 69
   quantity ROI 83
   RBG color code 69
   scale factor 86
place tag 65
preferences 53–57
pre-saved colors 41
pseudocolor image 70–71

Q
quantity ROI pixels 83

R
radiance
   average 81
   photon 83
radiance units 84
RBG color code 69
read bias 93, 101
result = 64
retrieving image data 104–106
ROI 80–83
   background contextual menu 36–38
   cosmic ray correction 86
drawing 82
exporting data 82
flat field correction 85
hide ROI tags 66
histogram 50
measurement contextual menu 35–36
min and max signal 81
properties 61–62
quantity ROI pixels 83
save 56
standard deviation 81
subject contextual menu 38
total flux 84
ROI tags
   hide 66
ROI types
   background ROI 80
   measurement ROI 80
   subject ROI 80

S
saturation 13
saving image data 103–104
scale factor 86
sequence image data 39–42
sequence window 39–42
sequential
   images 15, 39
   mode 15
show cursors 65
smoothing 60
Index

sorting image sets 46
spectral analysis 50, 128–129
  optimizing precision 136
spectral imaging 127, 130–137
  diffusion model 131
  example metastasis model 132–136
  luciferase spectrum 132
  optical properties 132
  procedure 127–128
start
  Living Image software 4
steradian 84
structure 18, 19
structured light 139
  theory of operation 139–140
structured light analysis 50
structured light images
  acquiring 140
  analysis 140–143
subject height 11
subject ROI 80
  contextual menu 38
surface radiance 79
surface topography 50, 140–143
  See structured light.
system optics autofluorescence 115–116
system status 11

T
  technical support 6
  temperature 11
tissue autofluorescence 122–126
tissue optics effects 115
total counts 83
total flux 81, 84

U
  units 41
  user ID 53

W
  WaveMetrics IGOR Pro 4
  well plate autofluorescence 117–118
windows
  image display 21
  Lab Book 58–59

Xenogen technical support 6

Z
  zoom/crop contextual menu 38