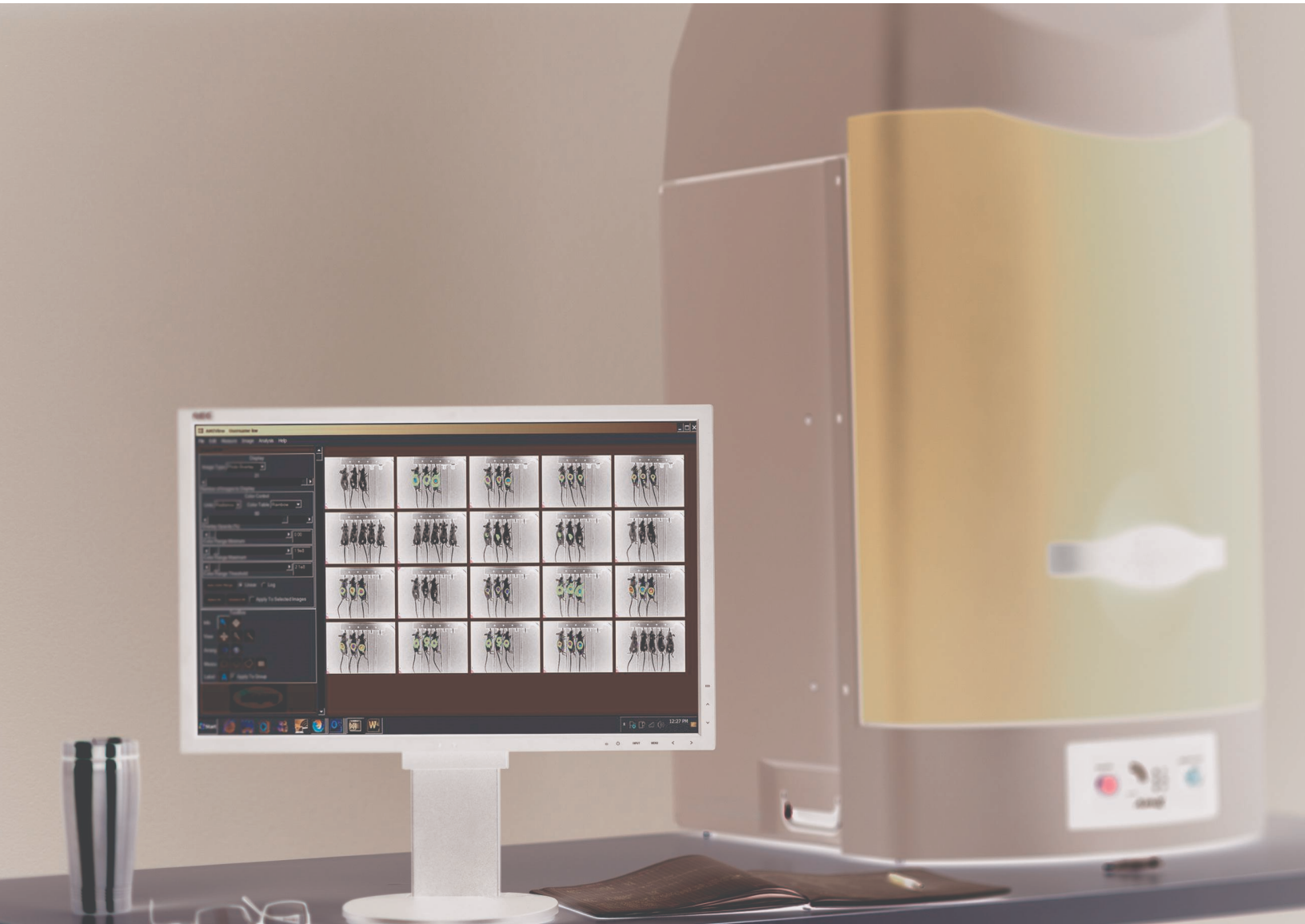
aura

imaging software by

spectral instruments imaging

USER MANUAL



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Patents
The SPECTRAL Ami and Ami X imaging systems are covered by U.S. Patents 8,901,516 and D648844. Other patents pending in the U.S. and other jurisdictions.

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Contents

Welcome

1.1 About This Manual

This manual is for use with the aura software and includes instructions which explain how to view, manage and analyze images.

Screenshots often supplement the detailed instructions in this manual. Sometimes the screenshots may not exactly match those on your screen.

Information may be presented in the following formats:

NOTE: Presents helpful information, for example, best practices for instrument operation or for using aura software.

IMPORTANT: Presents information that is essential to the correct operation of the software.

1.2 Technical Support

If you have questions about the aura software, please contact Spectral Instruments Imaging Technical Support:

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Getting Started

2.1 Starting aura Software

1. Double-click the aura icon on the desktop or single-click the aura icon in the taskbar.
2. Click **Click to Continue** in the IDL splash screen that appears (Figure 2.1). Alternatively, press **Enter** on the keyboard.

Figure 2.1 IDL Splash Screen

3. Enter a new or existing user name in the Spectral Instruments Imaging screen that appears (Figure 2.2) and press **Enter** on the keyboard.

NOTE: The aura user name is not password-protected and is not a security mechanism.

Figure 2.2 aura Login Screen

4. If you entered a new user name, click **Yes** in the prompt that appears (Figure 2.3). The software creates folders that are associated with the user name (Table 2.1).

Figure 2.3 New User Prompt

Table 2.1 Folders Associated With a User Name

Folder Location	Contents
Windows®: C:\Users**Windows Username**\aura Username Macintosh®: /Users/**Mac Username**/aura Username/	■ Image data (.ami) in the "data" folder. ■ Spectral unmixing library entries (.sli) in the "spectralLibs" folder. ■ ROIs (user-defined regions of interest which specify the area(s) to measure image signal) in the "savedROIs" folder.

* *"**Mac Username** and **Windows Username** are the user names used to log into that particular Mac or PC respectively. aura Username is the name chosen in Step 7 above.*

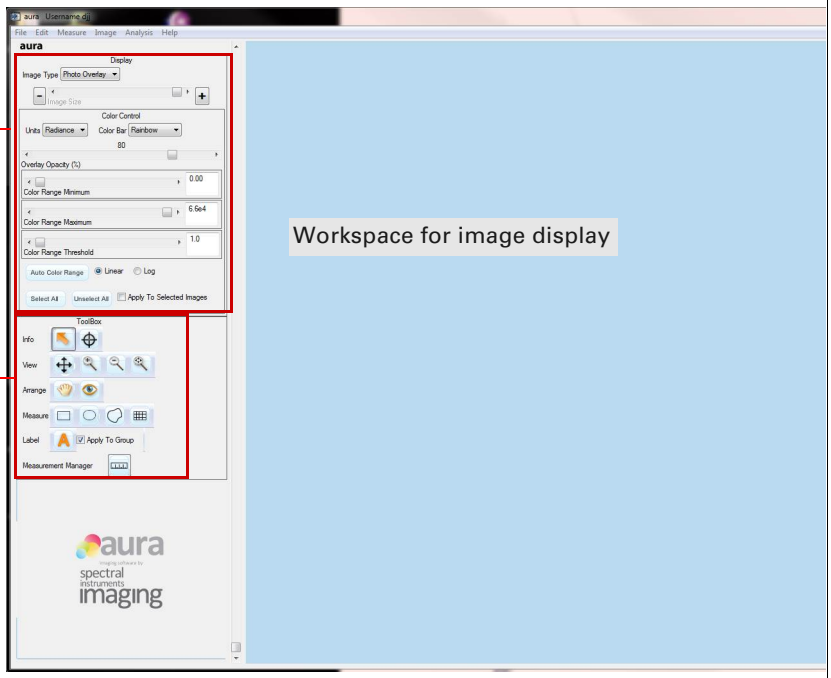
After entering a user name the main aura window appears (Figure 2.4).

Figure 2.4 aura Main Window

Image display and color controls. See Table 2.3 on page 9.

Tools for:
- Creating ROIs
- Displaying photometric data from ROIs
- Viewing coordinates (x and y) and light intensity (z) in an image
- Arranging images in the display space
- Zooming and panning
- Viewing images in an image set and image information
- Annotating images

See Table 2.3 on page 9.



2.2 Overview of How aura Images Are Acquired

See Appendix A on page 63 for details on the acquisition parameters.

In single image mode, multiple image types are usually acquired at the same time. For example, a photograph and a luminescence image can be taken together and saved as an image set (.ami file). Different combinations of the image types in an .ami file can be displayed together as an *overlay* (see Table 2.2 on page 5 for examples).

A series of multiple image sets can be acquired and saved as a *group*. All image sets in this group will be included in the same .ami file. Group acquisition is useful for time-dependent kinetic or longitudinal studies, fluorescence scans (acquisitions at different excitation/emission wavelengths), or acquisitions which vary an imaging parameter (for example, exposure time).

Imaging Modes

Table 2.2 shows the imaging modes available on the SPECTRAL line of imaging systems.

Table 2.2 Imaging Modes

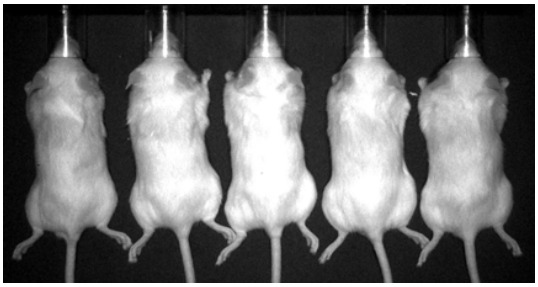
Imaging Mode	Description	Example
Photograph	An image of the white light reflected from a sample. Photographic images are displayed in grayscale (black and white). A photograph is usually acquired along with a luminescence or fluorescence image to provide a visual reference when viewing an overlay (see below).	

Table 2.2 Imaging Modes (continued)

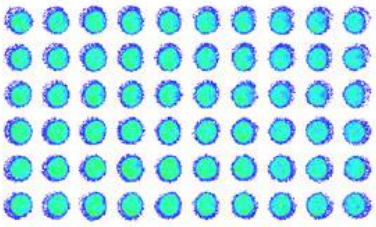
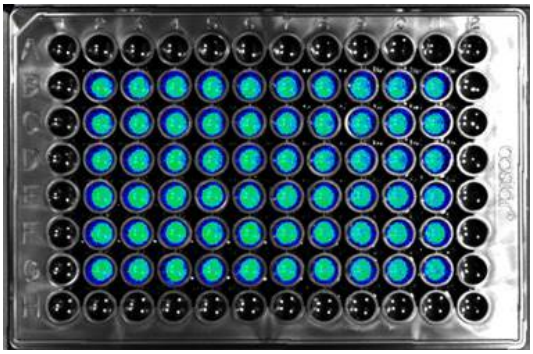
Imaging Mode	Description	Example
Luminescent	An image of light emitted from an unilluminated sample, for example, a bioluminescent source. No lights are on in the imaging chamber during acquisition. Luminescent sources are usually dim and may require exposure times of several minutes.	Luminescence image displayed using the rainbow color bar. See Appendix B on page 65 for more about image data display.  Overlay showing luminescence image on a photograph. 

Table 2.2 Imaging Modes (continued)

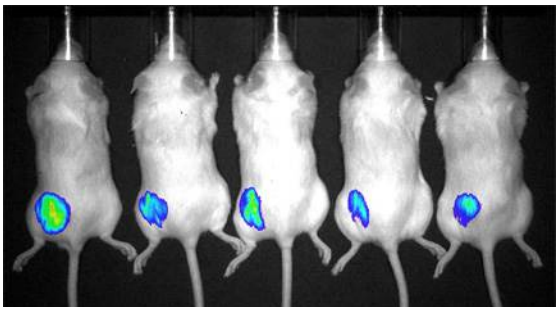
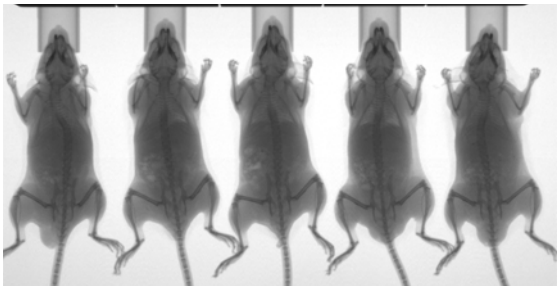
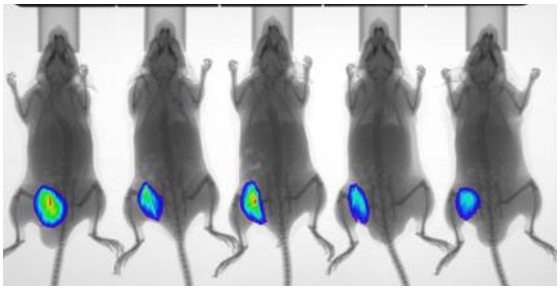
Imaging Mode	Description	Example
Fluorescent	An image of light emitted from a fluorescent reporter, at a specific wavelength when it is illuminated (excited) by a light source at a second, shorter wavelength.	Fluorescence image displayed using the rainbow color bar. See Appendix B on page 65 for more about image data display.  Overlay showing fluorescence image on photograph. 

Table 2.2 Imaging Modes (continued)

Imaging Mode	Description	Example
X-ray	An image of X-ray photons transmitted through an object. X-ray images provide information about internal sample structure and a visual reference when viewing an overlay.	This example image set includes a fluorescence image and an X-ray image. X-ray image  Overlay of fluorescence image on X-ray image. 

2.3 About aura Controls and Tools

Table 2.3 provides an overview of the image display controls, color controls, and tools and workspace preferences.

Table 2.3 Image Display and Color Controls

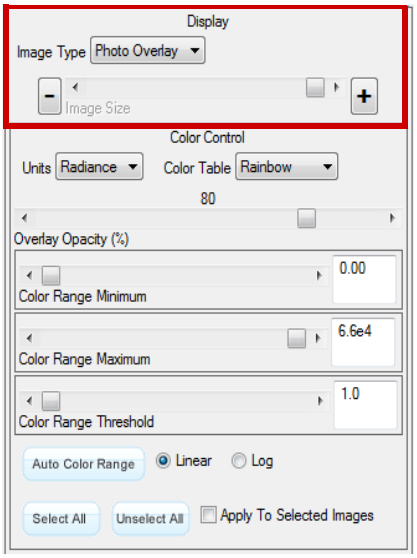
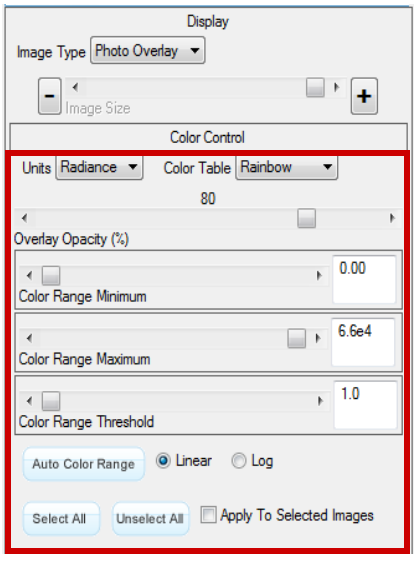
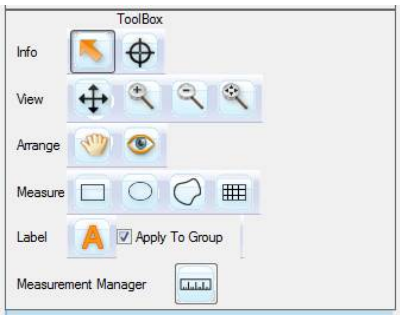
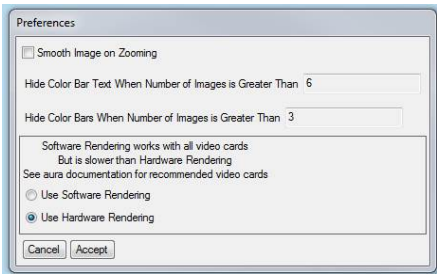
Item	Description	See Page
Display Controls 	Select the type of image or overlay to display from the Image Type drop-down list. If multiple images and/or groups are loaded, use the slider or arrows to select the number of images to display in the workspace incrementally. Use the +/- buttons to display only one image (**-** button) or all images (**+** button) in one click.	19 19
Color Controls 	Use these controls to... ■ choose the data units for image display: “Radiance” or “Counts” for luminescent images, “Efficiency” for fluorescent images. ■ select a color bar for luminescent or fluorescent images. ■ adjust how transparent the fluorescent or luminescent image is on top of the photo or x-ray image. ■ select the minimum pixel value assigned a color. ■ select the maximum pixel value assigned a color. ■ select the lowest pixel value displayed as a colored pixel, according to the color bar. ■ map the pixel values to colors on the color bar based on a linear or logarithmic scale. ■ select/de-select all images. ■ apply the current color bar settings to all selected images.	22

Table 2.3 Image Display and Color Controls (continued)

Item	Description	See Page
Toolbox 	Provides tools for... ■ organizing images in the workspace. ■ zooming and panning an image. ■ viewing image information. ■ measuring signal intensity using an ROI (region of interest). ■ annotating images. ■ displaying photometric data from ROIs.	 20 29 30 32 57 32
Preferences 	**File** → **Preferences** provides options for: ■ rounding out the square image pixels. ■ hiding the color bar text when the number of displayed images above a user-set number so that the images can be more clearly seen. ■ hiding the color bar when the number of displayed images above a user-set number so that the images can be more clearly seen. ■ selecting the rendering mode for images. **Use Hardware Rendering** is enabled by default and should remain selected unless your graphics processor driver is not compatible.	 28 28 28 29

Viewing Images

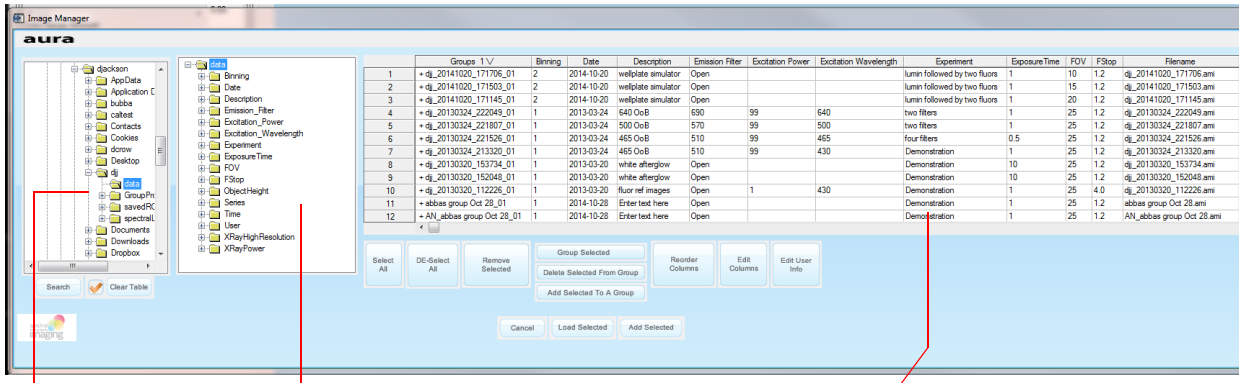
3.1 About the Image Manager

The Image Manager provides a convenient way to:

- Search for image data (.ami files)
- Load (open) image data
- Manage and create groups. (See *Creating a Group From Single Images* on page 59.)

Figure 3.1 and Table 3.1 provide an overview of the Image Manager.

Figure 3.1 Image Manager



Filesystem Tree

The software searches a user-selected folder, including subfolders, for images (.ami).

Tag Pane

Filters which images found in the user-selected folder get listed in the image table. Only the images which meet all the criteria selected in the tag pane will be listed in the image table (see Figure 3.2).

Displays the image tags and properties that are associated with the images found by the search as folders.

Expand a folder to view all tag values and image property values.

Image Table

Shows the images selected in the tag pane. The column headers are tags (as specified by a tag set or instrument setting tags which are automatically created).

Clicking any of the column headers toggles the sort order between ascending and descending.

A **+** sign indicates groups. Click the row to view the images in the group.

Use the image table to:

- Select and load images.
- Add or remove images from a group.
- Create a group from single images.

Figure 3.2 Selecting Images in the Tag Pane

Tag Pane

Tag pane shows the search criteria associated with the images found by search of a folder in the filesystem tree (Figure 3.1). In this example, the folder named "data" was searched. By default, no filters are selected in the tag pane and thus all images in the searched folder are displayed in the image

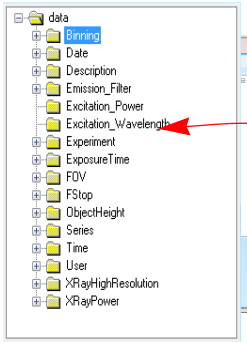
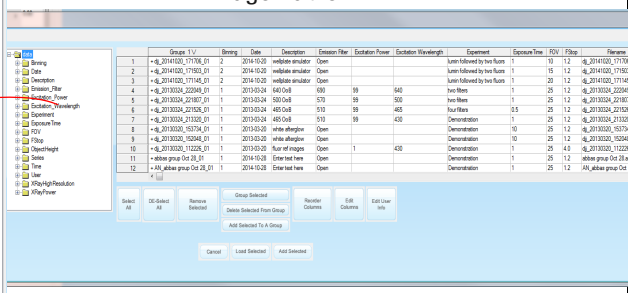
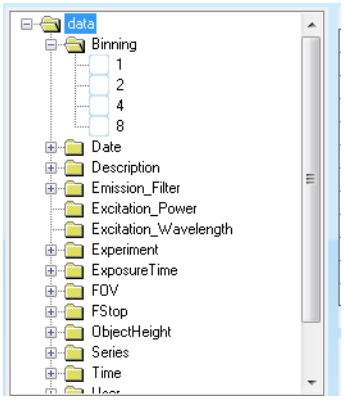


Image Table



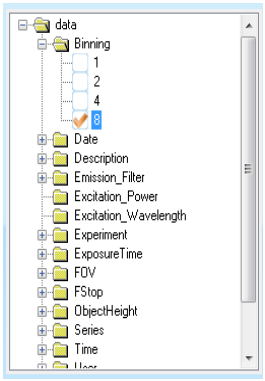
Exclude images from the image table by check marking the criteria of the images that should remain in that table. Then, select one or more of the remaining images in the image table.

Select a Criterion



In this example, the criterion "Binning" is chosen.

Select a Criterion Value



By selected a value of “8” for the binning criterion, the image table removes all images with binning levels other than 8.

Select Images

	Groups	Binning	Date
1	+ caltest_20170519_081808_01	8	2017-05-19
2	+ caltest_20170519_082057_01	8	2017-05-19
3	+ caltest_20170519_082427_01	8	2017-05-19
4	+ caltest_20170519_082952_01	8	2017-05-19
5	+ caltest_20170519_083310_01	8	2017-05-19
6	+ caltest_20170519_083649_01	8	2017-05-19
7	+ caltest_20170519_083939_01	8	2017-05-19
8			
9			
10			
11			
12			

Now, only the image files which met the search criteria remain in the image table.

Table 3.1 Image Manager

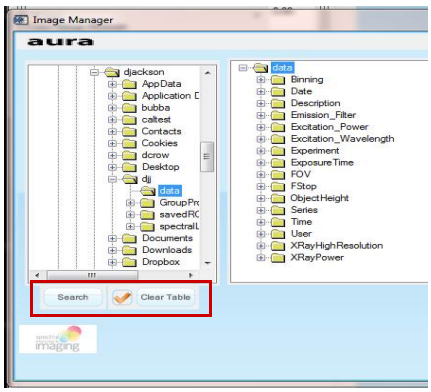
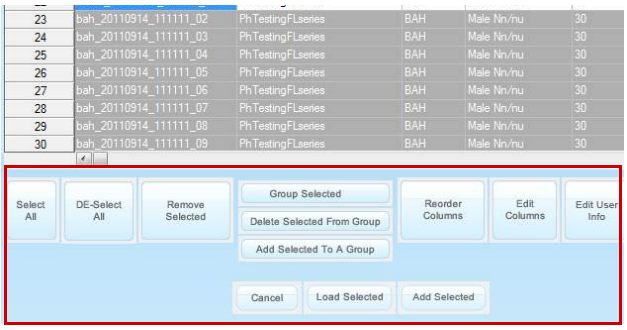
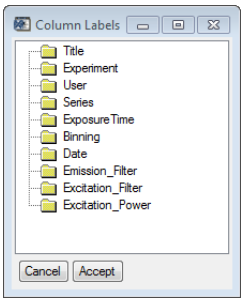
Item	Description
Filesystem Tree Tag Pane 	**Search** – Begins a search for images in the folder selected in the filesystem tree. **Clear Table** – If this option is selected (checked), the image table rows are cleared when a new search begins. The image table will include only the new search results. If this option is not selected, the image table is not cleared when a new search begins, and new results are added to the table.

Table 3.1 Image Manager

Item	Description
Image Table 	**Select All** – Selects all images (rows) in the image table. **De-Select All** – Clears the selection in the image table. **Remove Selected** – Removes selected images (rows) from the image table. This does not delete data from the system. It is the same as unchecking items in the tag pane. Check mark items in the tag pane to restore the data to the image table. **Reorder Columns** – Enables you to set the column order in the image table. Rearrange the column headers by dragging them in the dialog box that appears.  **Edit Columns** – Enables you to select the column headers (tags) to show or hide in the image table. **Edit User info**– Enables you to change the Experiment, Description, User and Series tags (depending on which of these are enable via "Edit Columns" above) of any image displayed. When this button is clicked, it changes into the **Update User Info** button and these four columns are highlighted green. You can then click any of these highlighted fields in the Image Table and type in the new information. When you are done making your changes, click the **Update User Info** button and click **Yes** in the small window which appears to save them. **Load Selected** –Displays only the selected images in the workspace. **Add Selected** –Displays the selected images in addition to the images already in the workspace. **Cancel** – Closes the Image Manager. **Group Selected** – Enables you to create a group from single images. See *Creating a Group From Single Images* on page 59 for more details. **Delete Selected From Group** – Enables you to delete an image from a group. **Add Selected To A Group** – Enables you to add an image to a group. See *Creating a Group From Single Images* on page 59 for more information on creating and managing groups using the Image Manager

3.2 Loading Images

Saved images can be loaded (opened) using the Image Manager, the **File → Load Images** menu commands, or by dragging and dropping an .ami file from an open file browser window into the aura program window.

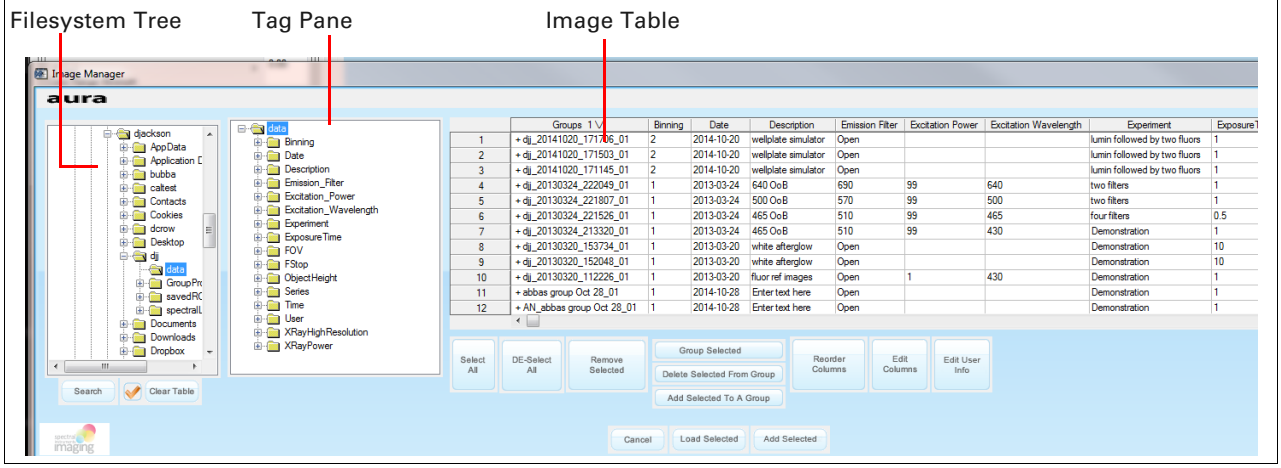
Loading Images Using the Image Manager

1. Select **File → Image Manager**. Alternatively, simultaneously press the **Ctrl** and **L** keys.

2. A small **Browse for Folder** window will appear. Browse through the filesystem tree to select the folder which contains the images you wish to load. The Image Manager will then appear (Figure 3.3), with all the saved images found in that folder displayed in the image table.
3. To locate specific images:
   a. Expand a node in the tag pane to view all criterion values.
   b. If you want to search through a different folder, browse for and click that folder in the filesystem tree pane on the left-hand side and click **Search**.

      If the folder contains images (.ami), the associated tags are listed in the tag pane (Figure 3.3).
4. Select the images to exclude from the image table by check marking a specific criterion value.

Figure 3.3 Image Manager Panes



5. Select images in the image table by clicking the row(s). To remove the selection, click the rows again.
6. Multiple images and groups can be selected:
   - To select all rows, click the **Select All** button.
   - To select contiguous rows, press and hold the Shift key while you click the first and last row in the selection.
   - To select non-contiguous rows, press and hold the Ctrl key while you click the table rows.
7. Click **Load Selected**.

   If **Load Selected** is clicked, any images already displayed in the workspace will be unloaded and only the images chosen in Image Manager will be displayed there. If **Add Selected** is clicked, then the images chosen in Image Manager will be displayed in addition to the images already in the workspace. Regardless of which of these is clicked, the Image Manager window will close, returning the user to the main aura window with the chosen images displayed (Figure 3.5 on page 15).

Loading Images From the File Menu

1. Select **File → Load Images** on the menu bar. Select **File → Load Images to Existing** in order add images to the ones already displayed in the workspace.
2. Browse for the image data (.ami file) in the dialog box that appears and click **Open** (Figure 3.4).

Multiple images or groups can be selected. To select contiguous files, press and hold the **Shift** key while you click the first and last file. To select non-contiguous files, press and hold the **Ctrl** key while you click the files.

NOTE: On a Macintosh, the dialog box shown in Figure 3.4 may appear behind other windows, including the main window.

The workspace displays the images (Figure 3.5 on page 15). If the workspace already contained images, they will be unloaded before the new images are displayed unless **File → Load Images** was chosen in step 1above.

Figure 3.4 Choose aura Files

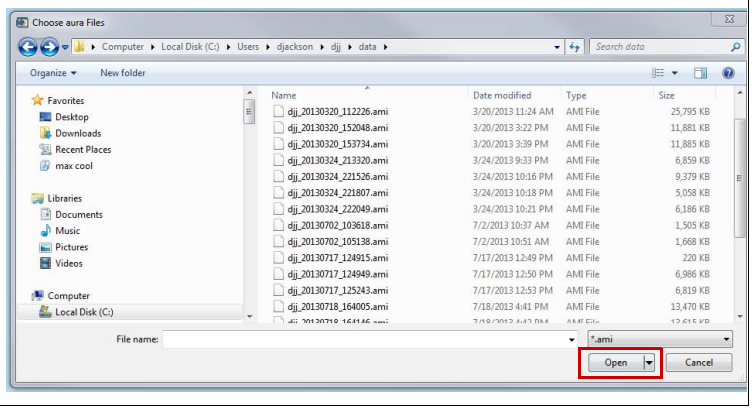


Figure 3.5 Images in the Workspace

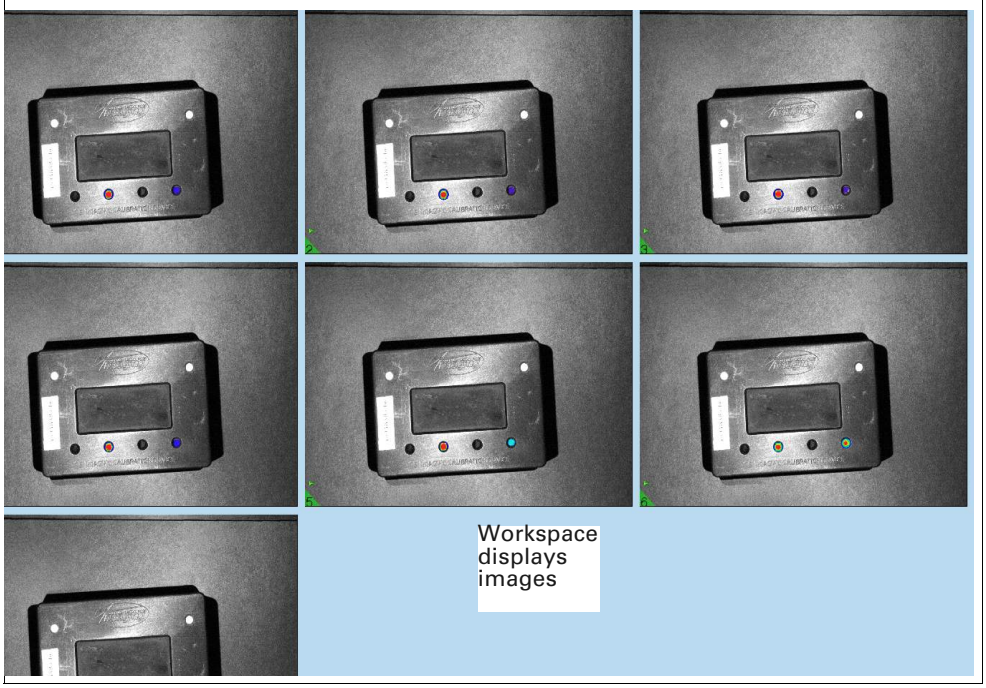
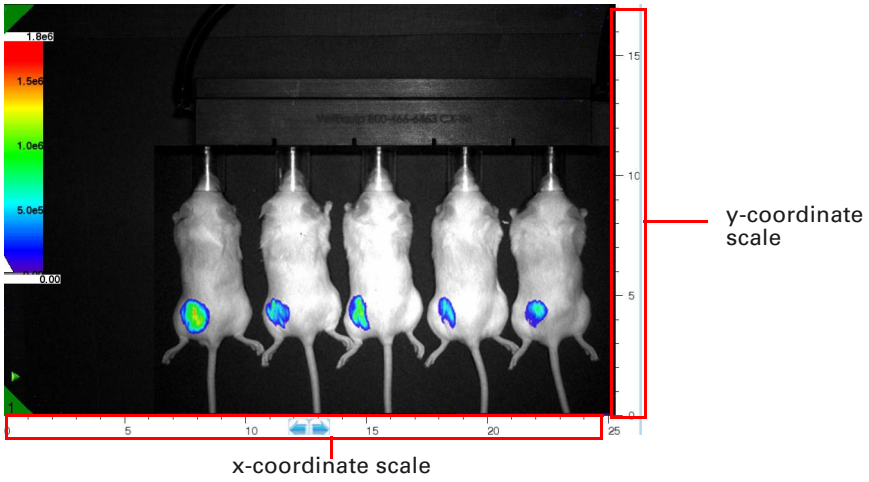


Table 3.2 Display and Color Controls

Item	Description
Image Type	Select the type of image or overlay to display: ■ **Photo Overlay** – Luminescence or fluorescence image on photograph. ■ **Fluor/Lumin** – Fluorescence or luminescence image only. ■ **Photograph** – Photograph only. ■ **X-Ray** – X-Ray image only. ■ **X-Ray Overlay** – Luminescence or fluorescence image on X-ray image. ■ **Info** – Information about the image (for example, file name, acquisition parameter settings, user-entered image label information). ■ **Saturated Image** – Shows only the pixels where signal is saturated, if there are any in the present image(s). If there are no saturated pixels, this image type option will not appear.
Number of Images to Display	If multiple images are loaded, use the slider to select how many to display. Incrementally select the number images to display by using the slider or arrows. Display all images at once by clicking the – button and display only one image at time by clicking the + button.
Units	Units for the color bar scale and signal measured by an ROI (region of interest). **Radiance** – A calibrated absolute measurement of photon emission from the subject (photons/second/cm^2/steradian). **Counts** – An uncalibrated relative measurement of the total number of photons incident on the imaging system CCD. **Efficiency** – Fluorescent emission normalized to the excitation intensity (radiance/ illumination intensity).
Color Bar	Specifies the color bar which represents signal intensity in the image. Several preset color bars are available in the drop-down list. Select: **Custom** to choose a different color or create a custom color for the color bar. **Wavelength** to choose a color bar by visible spectrum wavelength. See page 23 for more information.
Overlay Opacity	Use the slider to adjust the opacity of the luminescence or fluorescence image displayed in an overlay.
Color Range Minimum	Sets the lowest value in the color bar. The color range minimum is always ≤ color range threshold.
Color Range Maximum	Sets the highest value in the color bar.
Color Range Threshold	Sets a threshold value to display in the image. Pixels with signal values below this threshold are not assigned a color bar value and appear transparent (not visible) in the image. The color range threshold is always≥ color range minimum.
Auto Color Range	Linear – Applies a linear scale to the color bar and automatically sets the minimum, maximum, and threshold values. Log – Applies a logarithmic scale to the color bar.
Select All	Selects all images in the workspace. If this option is selected, image adjustments are applied to all of the images.
Unselect All	Clears the selection so that no images are selected.
Apply to Selected Images	If this option is selected, image adjustments are only applied to the selected images.

Table 3.3 Tool Box

Tool		Description
Info		Pointer tool that is used to select an image in the viewing area.
		Cursor measurement tool shows the x, y-coordinates using the scale around the image, and intensity (z-coordinate) at the cursor location in the image. 
View		Pan tool moves a magnified image within the viewing area. **Note:** The pan tool is only functional after an image has been magnified using the zoom tool.  .
		Magnifies an image (zooms in).
		Reduces magnification of an image (zooms out).
		Zooms out completely to original size (1:1).
Arrange		Use this tool to rearrange images in the viewing area when multiple images are loaded.
		Enables you to view the different images or image information for the .ami file. Select this tool and click an image to cycle through all of the available overlays.
Measure	   	Tools for creating an ROI on an image. aura software measures the signal intensity within the ROI. ROI shapes: rectangular, circle or oval, free-form, or grid (2x3, 3x4, 4x6, 8x12, or 16x24). See Chapter 4, *Analyzing Images* on page 31 for instructions on measuring signal using these tools.
Label		Use this tool to add notes to an image. Choose the "Apply to Group" option to add the note to all images in a group. See the instructions on page 57.
Measurement Manager		Opens the Measurement Manager when images are loaded in the workspace.

Unloading Images

Unloading images removes them from the workspace. The images remain available in the Image Manager.

To unload:

- An individual image – Right-click the image and select **Unload This Image** from the shortcut menu.
- Multiple images – Press and hold the Shift key (for contiguous images) or Ctrl key (for non-contiguous images) while clicking the images. Then select **File → Unload Selected Images** on the menu bar.
- A group – Right-click an image in the group and select **Unload This Image** from the shortcut menu. A message window will appear saying that images in a group can't be unloaded separately. Click **OK** on this window.

NOTE: Images which belong to a group can only be unloaded as a group, not individually.

- All images from the workspace – Click **Select All**, then select **File → Unload Selected Images** on the menu bar.

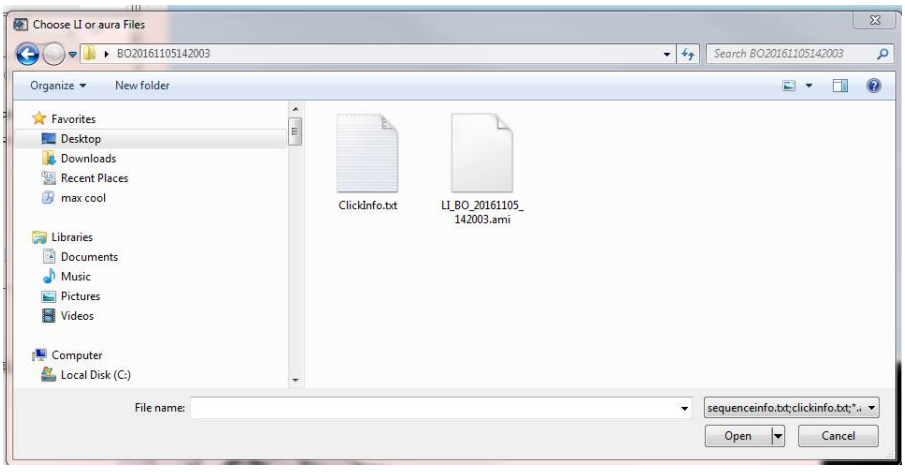
3.3 Loading Living Image® Files

In addition to being able to load image files acquired on a SPECTRAL imaging system, aura is also able to load and analyze Living Image® data.

To load a Living Image image folder into aura:

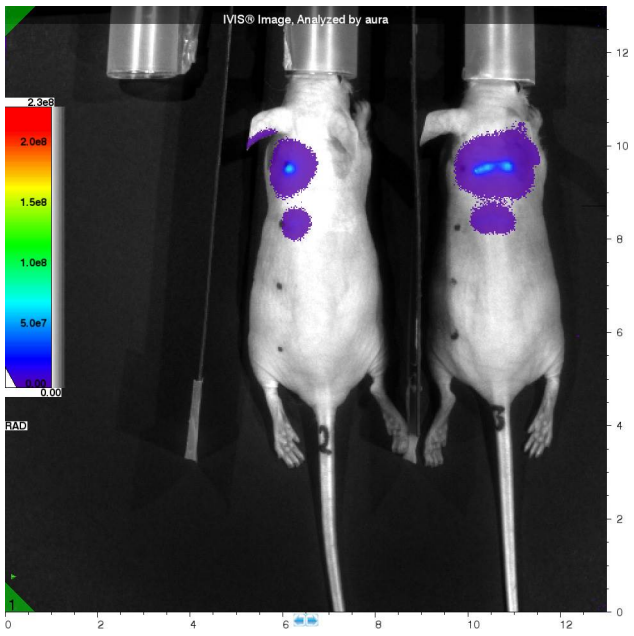
1. Click **File→Load Living Image Click Folders**.
2. In the window that appears (Figure 3.6), navigate to the folder of the Living Image® data you wish to analyze and click either the "clickinfo.txt" file (if it contains a single image) or the sequenceinfo.txt file (if it contains an image sequence) that folder.

Figure 3.6 Selecting Living Image® data to load



3. The image data loads into aura, allowing you to use all of aura's analysis tools, including ROIs, as seen in Figure 3.7 below.

Figure 3.7 Analysis of Living Image® data



3.4 Viewing Images

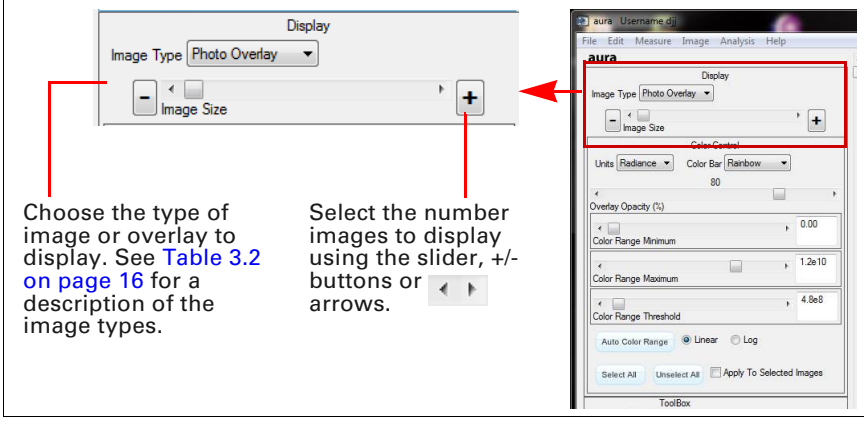
Image Type and Number of Images

Use the Display controls shown in Figure 3.8 to:

- Choose the type of image to view (Photo Overlay is the default display).
- Change the number of images displayed in the workspace when multiple images and/or groups are loaded.

NOTE: Select an image in the workspace, then use the mouse wheel to cycle through the available image types (for example, Photo Overlay, Fluor/Lumin, Photograph, X-Ray, X-Ray Overlay, or Info).

Figure 3.8 Select Type of Image and Number of Images to Display



Organizing Images in the Workspace

If multiple images and/or groups are loaded:

- The images are numbered according to their position in the workspace (Figure 3.9).
- Double-click an image to display only that image in the workspace.

To rearrange images:

1. Click the  button.
2. Click an image and hold the left mouse button while you move the image to a new position in the workspace, then release the button.
3. After the left mouse button is released, the multiple image view will change to a single image view with only the recently moved image visible in the workspace. To change back to multiple image mode, click on the minus (-) button of the **Number of Images to Display** slider (Figure 3.8).

Selecting Images

Some selections in the Display and Color Controls (for example, type of image or color bar) can be applied to multiple images at the same time. Use one of the following methods to select image(s). A green triangle indicates the active image(s) (Figure 3.9).

Figure 3.9 Multiple Images in the Workspace

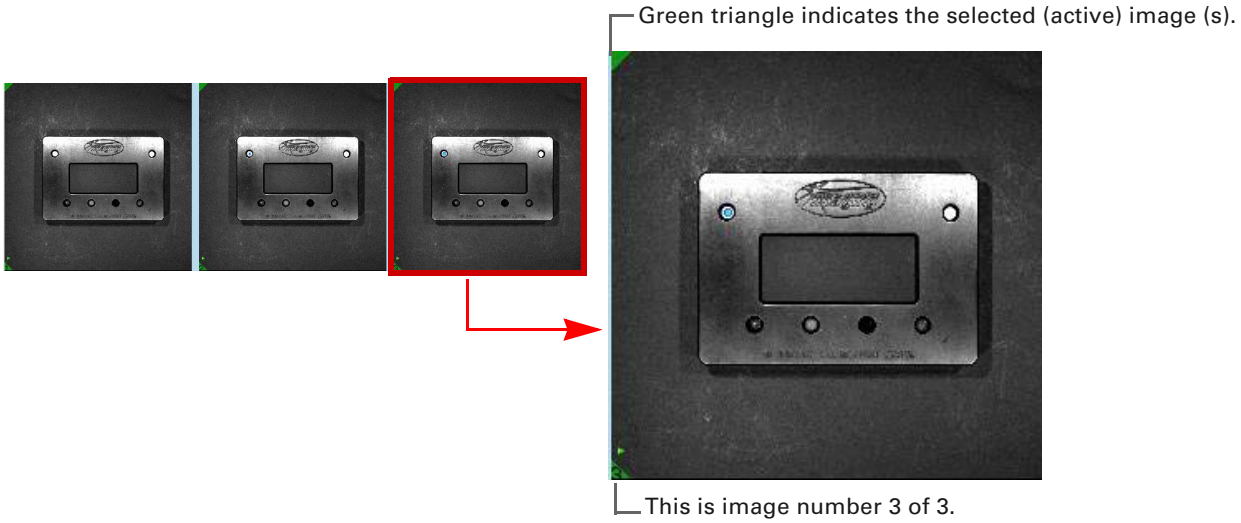
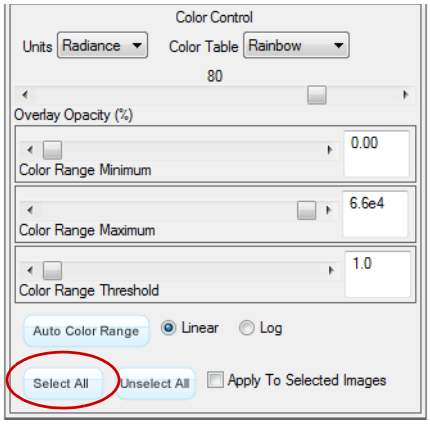


Table 3.4 Methods for Selecting Images

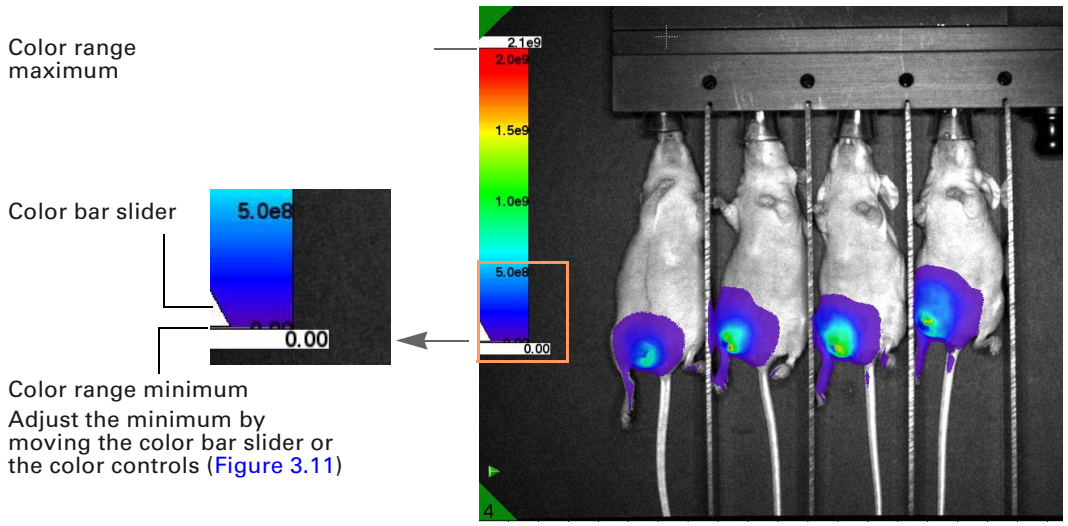
To Select:	Do This:
One image	Click the image in the workspace.
Specific images	Press and hold the Shift key (for contiguous images) or **Ctrl** key (for non-contiguous images) while you click the images in the workspace.
All images	Click **Select All**. 

3.5 Adjusting Image Appearance

Modifying the Color Bar

The default color bar uses the rainbow color scheme and units of radiance to render intensity data (Figure 3.10). The color bar scale defaults to displaying from 4% to 94% of the maximum intensity in the image data. See Appendix B, *Image Data* on page 65 for information about color bars.

Figure 3.10 Rainbow Color Bar



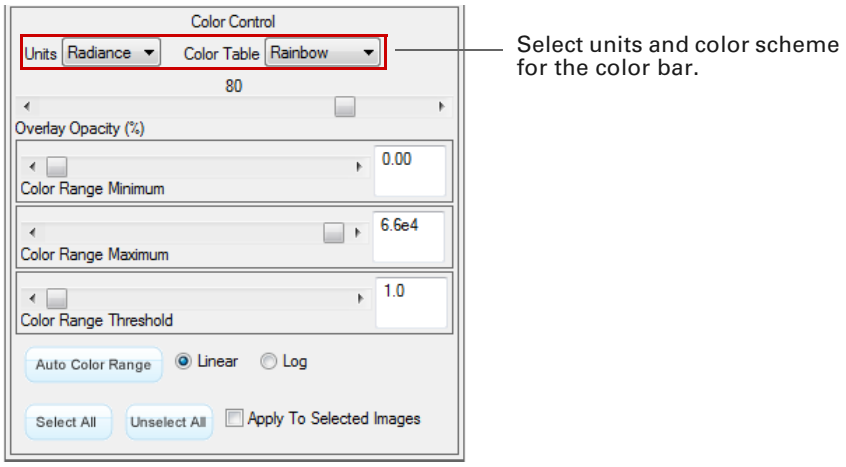
Sometimes it may be useful to adjust the intensity range displayed in an image. For example, increasing the color range threshold may help hide noise from the image display. Adjust the intensity data display using the color bar sliders or the Color Controls to modify the color bar (Figure 3.11). The intensity data display can be reset back to the original color gradient by clicking the **Auto Color Range** button in the color control menu.

NOTE: Modifying the color bar only affects the image display, it does not affect the image data.

Color Bar Units

The selection in the **Units** drop-down list (Figure 3.11) sets the units of the color bar scale.

Figure 3.11 Color Controls



The available units include:

- **Radiance** – A calibrated measurement of photon emission from the subject (photons/second/cm^2/steradian). This is the default unit for luminescent or fluorescent data.

NOTE: SPECTRAL imaging systems are absolutely calibrated instruments. Therefore, radiance image data acquired on SPECTRAL imaging systems are calibrated measurements which can be directly compared to radiance image data obtained on any other calibrated imaging system.

NOTE: Since the radiance of emitted light from a sample in fluorescent imaging mode is dependent on the intensity of the applied excitation light at that specific area, radiance data for fluorescent images may not be directly compared between instruments. Please use Efficiency (see below) values for directly comparing fluorescent images between imaging systems.

- **Counts** – An uncalibrated measurement of the total number of photons incident on the imaging system CCD.
- **Efficiency** – Fluorescence emission normalized to the excitation intensity (radiance/illumination intensity).

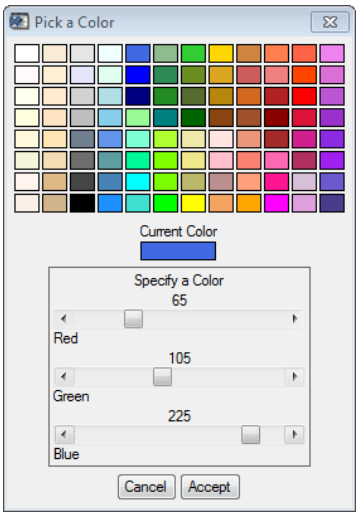
Color Scheme

Several preset color bar schemes are available in the Color Bar drop-down list (Figure 3.11). You can also create a custom color bar.

Create a Custom Color Bar – Select or Create a Color Using the Color Palette

1. Select **Custom** from the Color Bar drop-down list.
2. Do either of the following in the color palette that appears (Figure 3.12):
   - Click a color swatch.
   - Create a color using the Red, Green and Blue sliders.

Figure 3.12 Color Palette

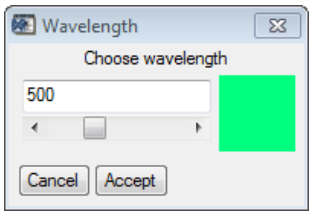


3. Click **Accept**.
   The color bar is applied to the selected image(s).

Create a Custom Color Bar – Select Color By Visible Spectrum Wavelength

1. Choose **Wavelength** from the Color Bar drop-down list.
2. Enter a wavelength or use the slider to select a wavelength in the dialog box that appears (Figure 3.13).

Figure 3.13 Choose Wavelength



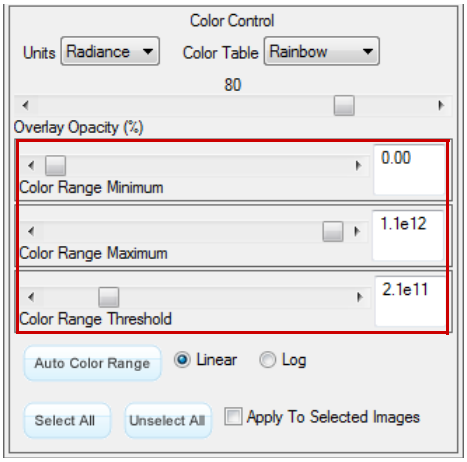
3. Click **Accept**.
   The color bar is applied to the selected image(s).

Color Range

The Color Range Minimum, Maximum, and Threshold settings determine the color bar range for image data display (Figure 3.14).

NOTE: Adjusting the color bar range only affects the data display; the data is unchanged.

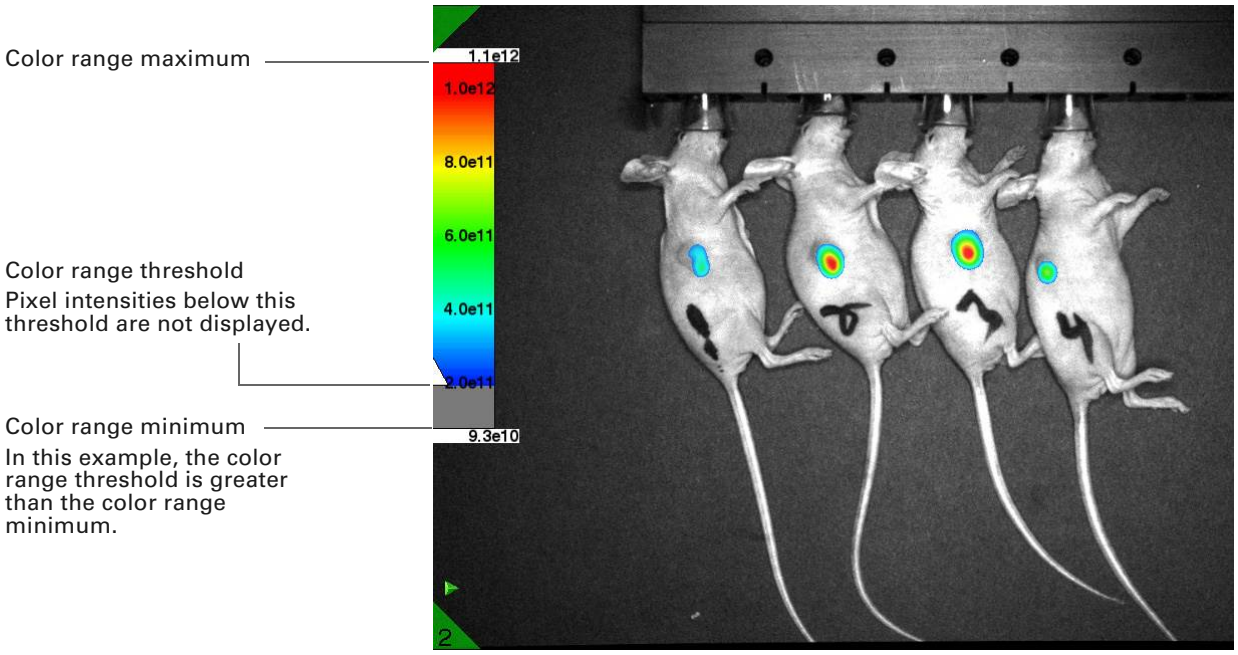
Figure 3.14 Color Bar Range Controls



Adjust the color range minimum and/or threshold levels to hide noise from the image display. Pixel intensities below the range minimum or threshold are not displayed.

NOTE: The color range minimum is the bottom of the color range on the color bar (Figure 3.15). The color range threshold is the lowest value displayed. Use these settings to match color ranges across multiple images and set individual thresholds for each image.

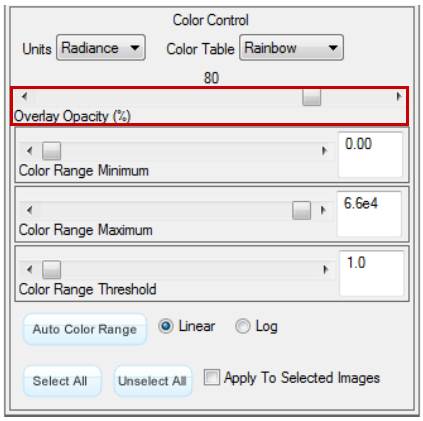
Figure 3.15 Example Color Bar



Overlay Opacity

Fluorescence or luminescence image data in a photo overlay are displayed at 80% opacity by default. Use the **Overlay Opacity** slider to adjust the fluorescence or luminescence data opacity. For example, reduce the opacity to view the photograph area under the image data (Figure 3.16).

Figure 3.16 Image Data Opacity Control



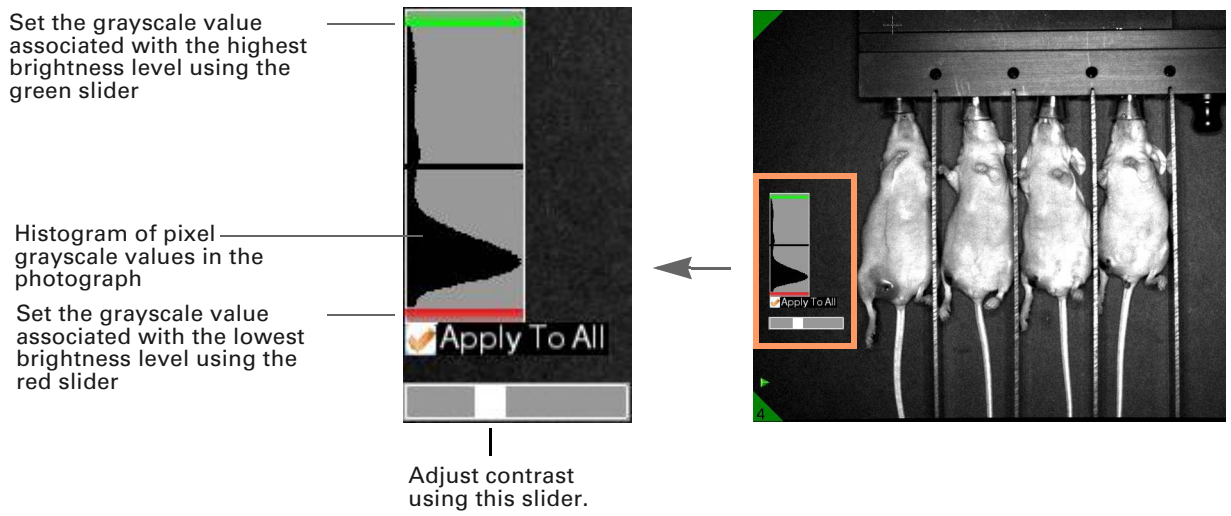
Adjusting Photograph Display

Adjust the brightness or contrast of photographs using the photo controls (Figure 3.17).

1. Select an image in the workspace and choose **Photograph** or **Overlay** from the Image Type drop-down list.
2. Select **Image** → **Show/Hide Photo Control** on the menu bar. Alternatively, right-click the image and select **Show Photo Control** on the shortcut menu.

   The photo control shows a histogram of pixel grayscale values in the photograph.
3. Adjust the photograph brightness by moving the green or red bars up or down. Adjust contrast by using the slider.
4. Select **Apply to All** to apply the display settings to all photographs in the workspace.

Figure 3.17 Photo Controls



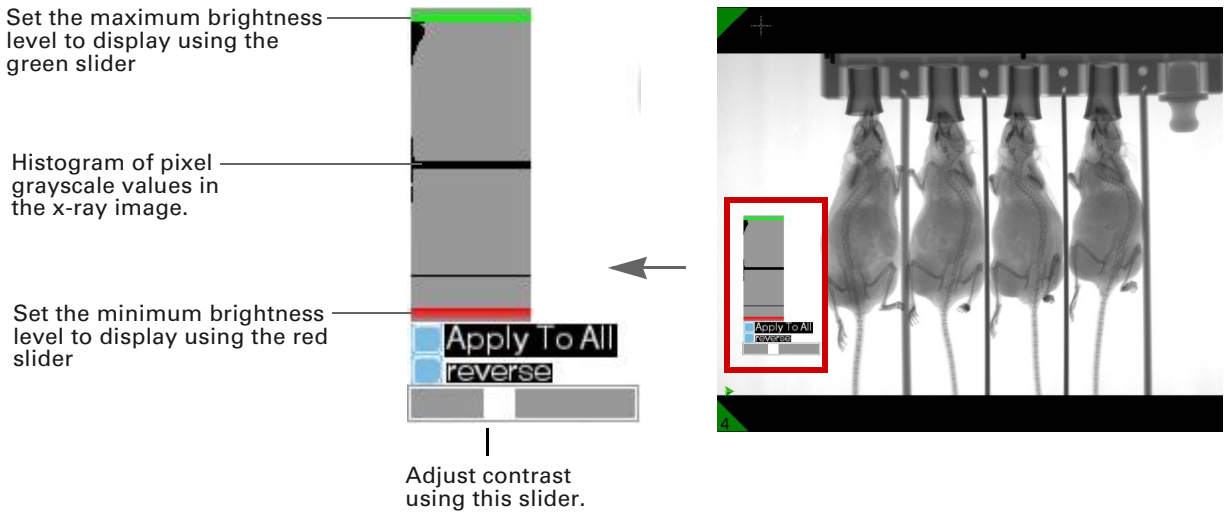
Adjusting X-Ray Image Display

Adjust the brightness or contrast of x-ray images using the x-ray controls (Figure 3.18).

1. Select an image in the workspace and choose **X-Ray** from the Image Type drop-down list.
2. Select **Image** → **Show/Hide Xray Control** on the menu bar. Alternatively, right-click the image and select **Show XRAY Control** on the shortcut menu.

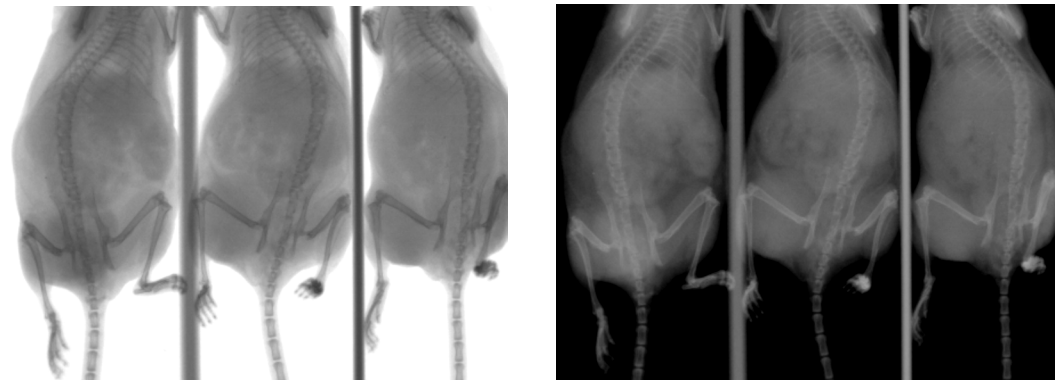
The x-ray control shows a histogram of pixel grayscale values in the x-ray image.

Figure 3.18 X-Ray Controls



3. Adjust the x-ray image brightness by changing the maximum and/or minimum brightness levels (Figure 3.18). Adjust contrast by using the slider.
4. Choose **reverse** to display the x-ray images using a reverse color-opacity map as shown in Figure 3.19.

Figure 3.19 Default and Reverse X-Ray Image Display



Default X-ray image display
Transparent = white, 100% opaque = black

Reverse X-ray image display
Transparent = black, 100% opaque = white

5. Select **Apply to All** to apply the display settings to all x-ray images in the workspace.

3.6 aura User Display Preferences

Each aura user has a set of default preferences which affect image appearance. These settings can be viewed and modified In the **File→ Preferences** window (see Figure 3.20).

Figure 3.20 aura User Display Preferences

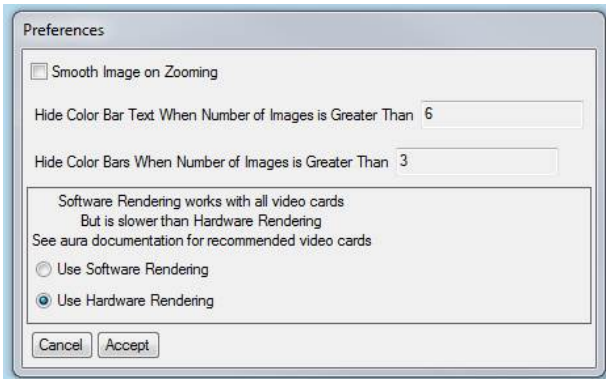


Image Smoothing

Since the individual pixels on the CCD sensor are square shaped, luminescent and fluorescent images can appear very "blocky" at high binning or high zoom levels. aura has a setting to round out these square pixels:

1. In the upper left corner of the aura window, click **File**.
2. Click **Preferences**.
3. Click the button for **Smooth Image on Zooming**.
4. Click **Accept.** The changes will take effect immediately.

Like all the other image display settings in aura, this pixel smoothing does not change the actual image data.

Color Bar Text Hiding

The numbered scales on the color bar displayed on top of images can be a useful gauge of the radiance, counts or efficiency values present in the image. While these numbers are easy to read when only a few images are displayed in the workspace, this text is too small to read when many images are displayed. You can specify the number of images above which aura hides this text:

1. In the upper left corner of the aura window, click **File**.
2. Click **Preferences**.
3. Enter the desired number in the **Hide Color Bar Text When Number of Images is Greater Than** field.
4. Click **Accept.** The changes will take effect immediately.

Color Bar Hiding

Like the numbers on the color bar, the color bar itself can be removed when more than a user-specified number of images is displayed in the workspace:

1. In the upper left corner of the aura window, click **File**.
2. Click **Preferences**.
3. Enter the desired number in the **Hide Color Bar When Number of Images is Greater Than** field.
4. Click **Accept.** The changes will take effect immediately.

Image Rendering Modes

The contrast sliders used in the pixel smoothing, photo control and x-ray tools use graphics hardware acceleration of image rendering by default in order to operate quickly and smoothly.This hardware rendering is enabled by default in aura. If this hardware acceleration does not work properly on the computer you use to do analysis, you can switch to the slower, but more compatible software rendering for these features:

1. In the upper left corner of the aura window, click **File**.
2. Click **Preferences**.
3. Click the button for **Use Software Rendering**.
4. Click **Accept**.

NOTE: Unlike the image smoothing, color bar and text hiding, the change in rendering mode requires a restart of aura before it takes effect.

3.7 Zooming or Panning

To zoom in (magnify the image:):

1. Click the  button, then click the image.
2. Continue to zoom in by clicking the image.
3. Click the  button when done zooming in.

To zoom out (reduce magnification):

1. Click the  button, then click the image.
2. Continue to zoom out by clicking the image.
3. Click the  button when done zooming out.

To zoom out completely:

1. Click the  button.
2. Click the  button.

To pan an image:

NOTE: Panning is only available if the image has been magnified.

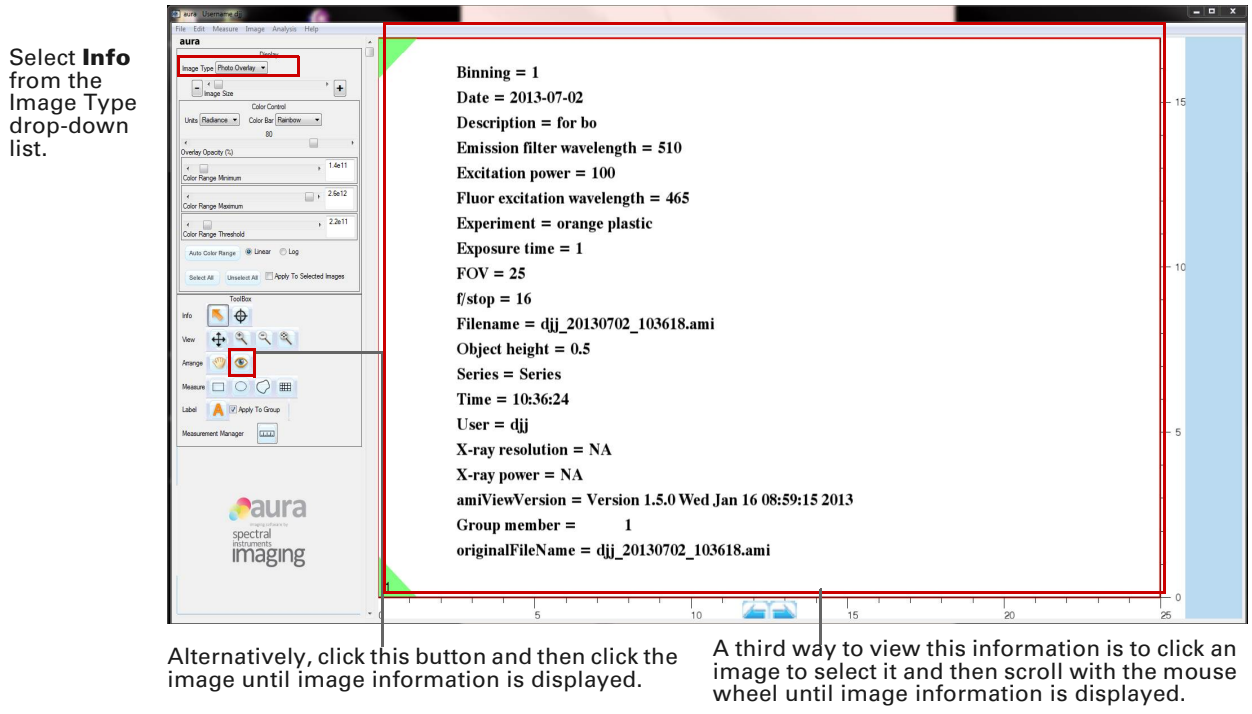
1. Click the  button.
2. Click and hold the left mouse button on the image, then move the mouse.

3.8 Viewing Image Information

Image information includes acquisition parameter values and tag information. Use either of the methods shown in Figure 3.21 to view image information.

NOTE: Select an image in the workspace, then use the mouse wheel to cycle through the available image types (i.e., Photo Overlay, Fluor/Lumin, Photograph, X-Ray, X-Ray Overlay, Saturated Image or Info).

Figure 3.21 Viewing Image Information



4

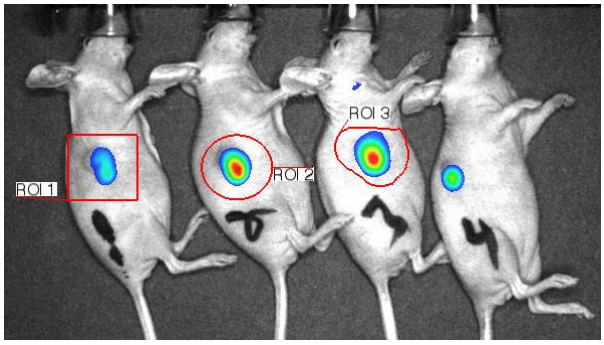
Analyzing Images

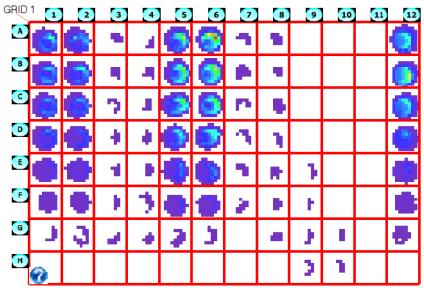
4.1 Overview of Quantitation

ROIs

A region of interest (ROI) defines the area of an image to quantify. Use the Measure tools to draw ROIs of different shapes on an image (Figure 4.1). aura software measures light emission within an ROI and displays the results in the Measurement Manager which provides a convenient way to view and export ROI measurements (Figure 4.2 on page 33).

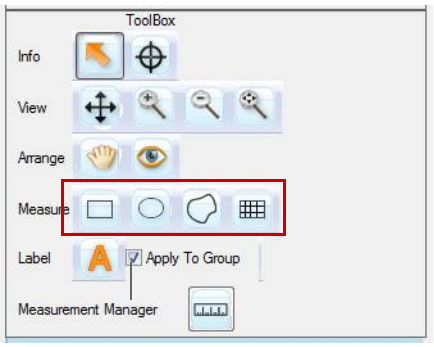
Figure 4.1 Example ROIs





(2x3, 3x4, 4x6, 8x12, or 16x24)

Draw ROIs using the Measure tools



If **Apply To Group** is selected, drawing an ROI on one image of a group will add the ROI to the same location in all images of the group.

About Background

Naturally occurring luminescence and fluorescence (*autofluorescence*) in animal and plant tissue produce background signal. Also, many objects and substances such as microplates, culture media, and imaging system components have autofluorescence.

For many imaging scenarios (especially bioluminescence), this background level is low enough relative to the reporter signal that it can be statistically ignored.

Often times, however, it is necessary to make background-corrected ROI measurements. This can be accomplished using one of the following methods:

- Correct an ROI measurement for background (see Section 4.3, page 40).
- Perform image subtraction to correct an image for background, then obtain ROI measurements (seeSection 4.4, page 42).
- Perform spectral unmixing (see Chapter 5 on page 45).

4.2 Making ROI Measurements

Drawing ROIs

1. Select an image in the workspace.
2. Select the measurement units in the **Units** drop-down list.
3. Click a Measure tool (Figure 4.1).

   NOTE: Select the **Apply to Group** option to make an ROI drawn on one image automatically appear in the same location on all other images in the same group.

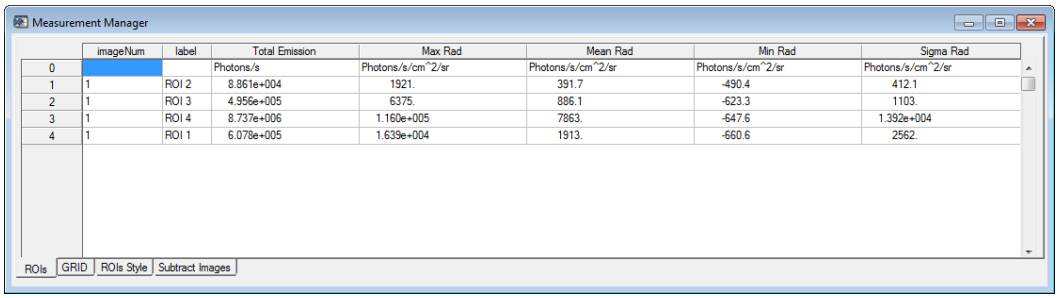
4. Click the image, then press and hold the mouse button while dragging the mouse to draw the ROI. Release the mouse button when finished.

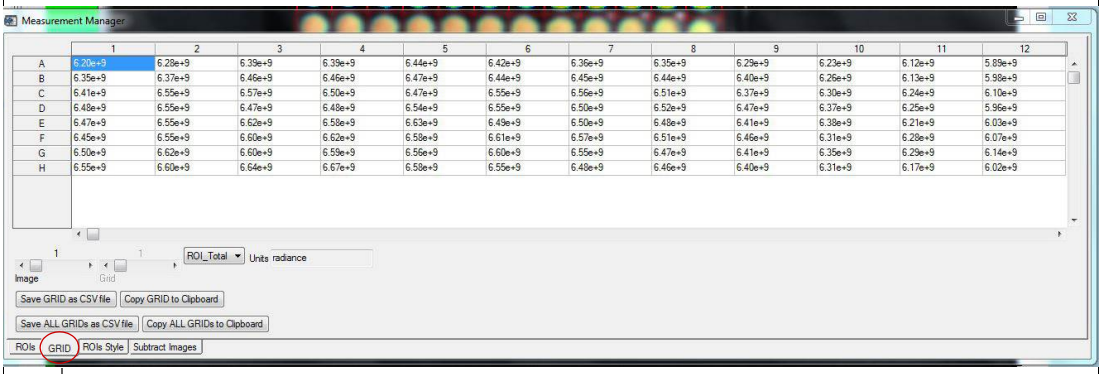
   If drawing a grid ROI ( ), select a grid option (2x3, 3x4, 4x6, 8x12, or 16x24) in the small window that appears. Click **Continue** and draw the ROI.

5. Right-click image and select **Measurement Manager** on the shortcut menu. Alternatively, click the button in the toolbox section, select **Measure → Measurement Manager** on the menu bar or press the Ctrl and M keys simultaneously.

   The Measurement Manager displays the computed ROI measurements (Figure 4.2). See page 35 for instructions on saving ROIs.

Figure 4.2 Measurement Manager – Example ROI Measurements





Click the **GRID** tab to view grid ROI measurements.

Table 4.1 Measurement Manager Column Labels

Item	Description
imageNum	The image number assigned in the workspace.
label	The ROI label name.
Total Emission	The total light emitted from the source within the ROI expressed as photons per second (when units are set to **Radiance**).
Total Efficiency	The combined efficiency of every pixel within the ROI expressed as cm^2(when units are set to **Efficiency**).
ROI Total	The total sum of all counts for the pixels inside an ROI (when units are set to **Counts**).
Sigma	When **Counts** units are selected, one sigma distribution of the count values inside the ROI.
Sigma Rad	When **Radiance** units are selected, one sigma distribution of the radiance values inside the ROI.
Sigma Efficiency	When **Efficiency** units are selected, one sigma distribution of the efficiency values inside the ROI.
X Centroid	X-coordinate value at the center of the ROI.
Y Centroid	Y-coordinate value at the center of the ROI.
Area	Area of the ROI ($pixels^2$).
BKGD ROI	Name of the background ROI linked to the measurement ROI (see Section 4.3, "Background ROIs," page 40).

Table 4.1 Measurement Manager Column Labels

Item	Description
Maximum Mean Minimum	When **Counts** units are selected: ■ The largest number of counts in a pixel within the ROI. ■ Total counts/number of pixels in the ROI. ■ The smallest number of counts in a pixel within the ROI. When **Radiance** units are selected: ■ The largest radiance measurement in a pixel within the ROI. ■ Total radiance/number of pixels in the ROI. ■ The smallest radiance measurement in a pixel within the ROI. When **Efficiency** units are selected: ■ The greatest efficiency measurement in a pixel within the ROI. ■ Total ROI efficiency divided by the number of pixels in the ROI. ■ The lowest efficiency measurement in a pixel within the ROI.
Stdev	The standard deviation of the pixel counts, radiance or efficiency values inside the ROI.

Copy and Paste ROIs

1. Double-click on an ROI to select it.
2. Right-click the ROI and select **Copy** on the shortcut menu.
3. Right-click the image and select **Paste ROI** on the shortcut menu.

   The Measurements Manager is automatically updated.

NOTE: You can also cut and paste an ROI by following step 1 to step 3 above, but select **Cut** instead of **Copy** on the shortcut menu.

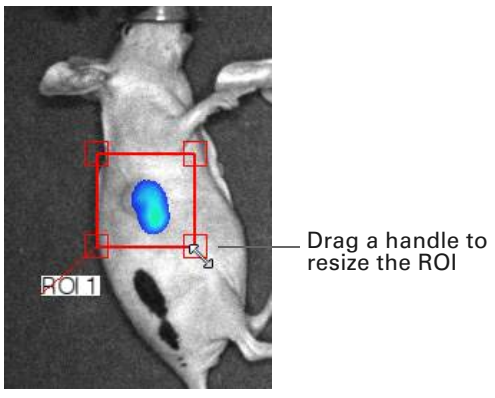
Resize ROIs

NOTE: ROIs drawn using the free-hand tool cannot be resized.

1. Right-click the ROI and select **Edit** on the shortcut menu. Alternatively, double-click inside the ROI.
2. Drag a handle that appears on the ROI.

   The Measurements Manager is automatically updated. The cells within a grid ROI will automatically resize as the height and width of whole grid are resized.

Figure 4.3 Resizing an ROI

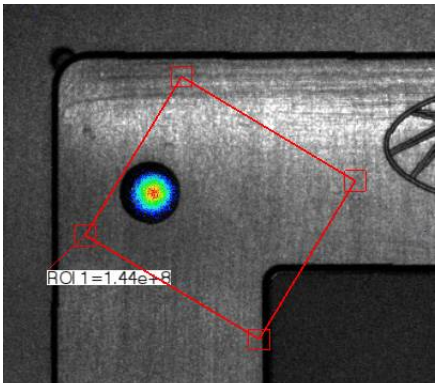


Rotating ROIs

1. Right-click the ROI and select **Edit** on the shortcut menu. Alternatively, double-click inside the ROI.
2. Hold down the **Shift** key and move the cursor to one of the square handles on the ROI (see Figure 4.4).
3. When the cursor becomes a circular arrow, click and drag that handle to rotate the ROI.
4. Release the left mouse button when finished rotating.

NOTE: This method also works on grid ROIs.

Figure 4.4 Rotating an ROI



Move ROIs

1. Double-click inside the ROI.
2. When the pointer becomes a ✥, press and hold the mouse button while you drag the ROI to a different location on the image. Release the mouse button when finished.

   The Measurement Manager is automatically updated.

Managing ROIs

Saved ROIs can be loaded onto one or more images in the workspace.

Saving ROIs

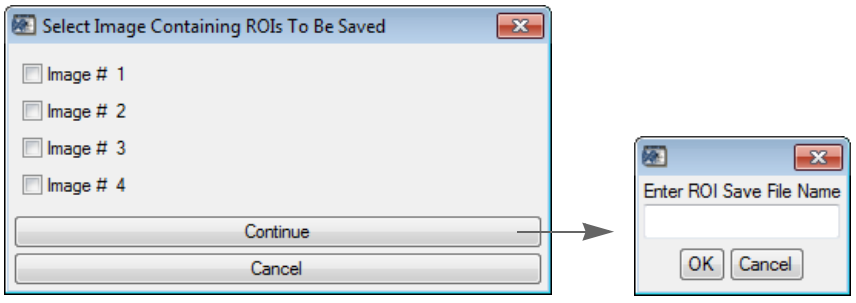
NOTE: If an image contains multiple ROIs, all of the ROIs will be saved in the same file.

1. Select **Measure → Save ROIs to File**.
2. Select an image in the dialog box that appears and click **Continue** (Figure 4.5).

   When all images loaded into the workspace have identical ROIs (e.g. a group image with all images selected), any image can be chosen to save its ROIs with the same end result.

   If there is more than one image loaded and they all don't have identical ROIs in size, shape and number, then the user has to decide which image has all the ROIs (and only the ROIs) to save using this dialog box.
3. Enter a name for the ROI file in the dialog box that appears and click **OK**.

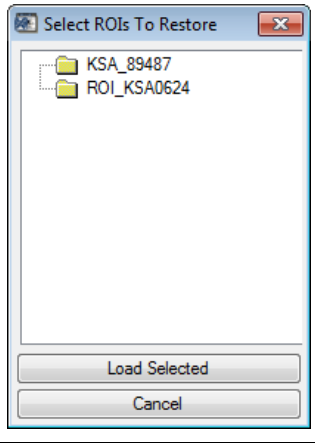
Figure 4.5 Select Image Containing ROIs to Save



Loading ROIs

1. Select **Measure** → **Restore ROIs from File** on the menu bar.
2. Select an ROI file or folder and click **Load Selected** in the dialog box that appears (Figure 4.6).

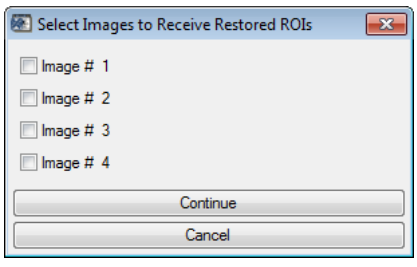
Figure 4.6 Select ROIs to Load



3. Select the image(s) on which to display the ROIs and click Continue in the next dialog box (Figure 4.7).

   The ROIs are added to the selected image(s).

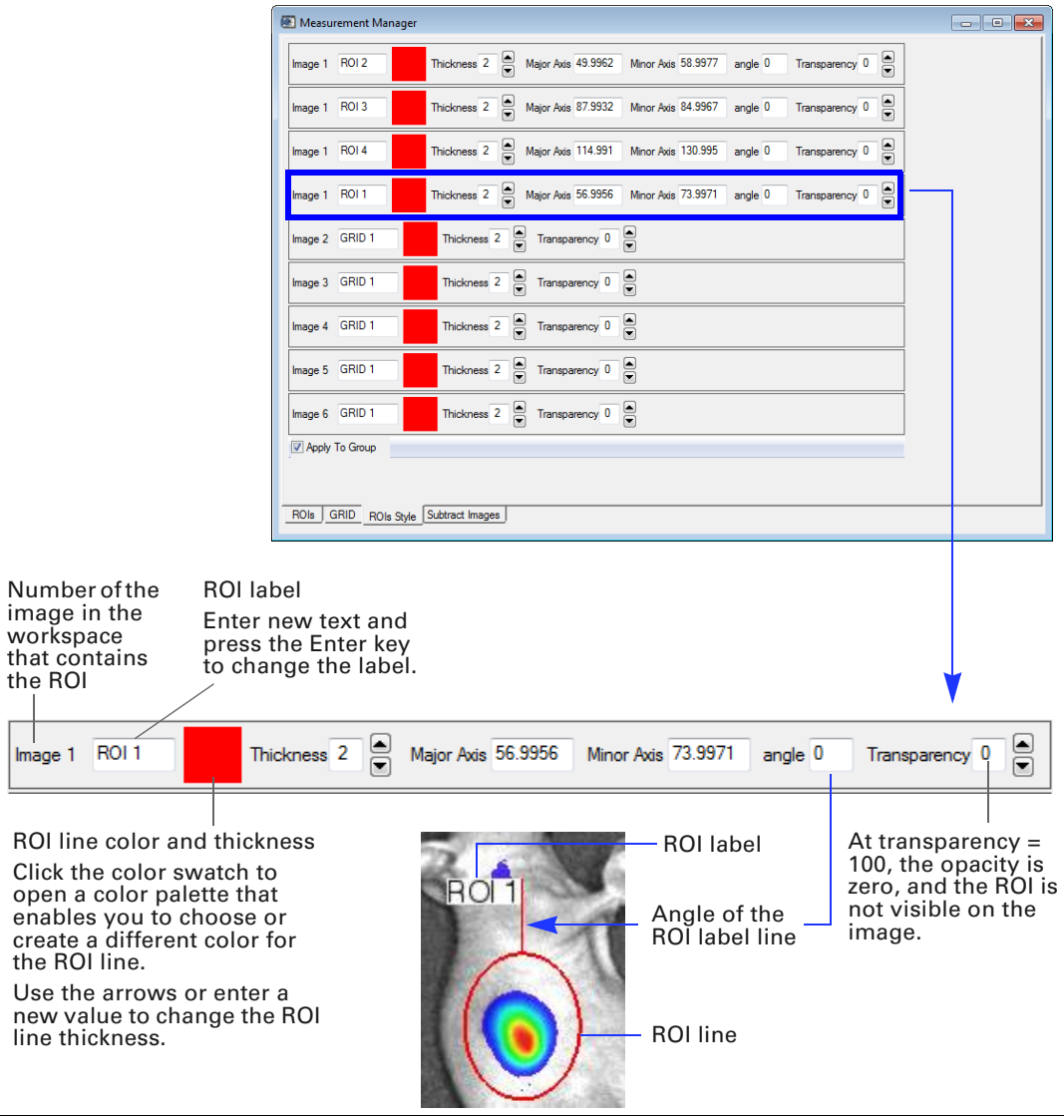
Figure 4.7 Select Image(s) for the ROIs



Editing ROI Style

Controls for ROI appearance and editing the ROI label are available in the ROIs Style tab of the Measurement Manager (Figure 4.8).

Figure 4.8 Measurement Manager – ROIs Style Tab



Deleting ROIs

NOTE: Deleting ROIs placed on an image does not permanently remove them from the system if those ROIs are saved.

To delete a □, ○, or ⬭ ROI:

1. Open the Measurements Manager (select **Measure → Measurement Manager** on the menu bar).
2. Select the ROIs (rows) to delete in the ROIs tab and click **Delete Selected ROIs**.

NOTE: Multiple rows can be selected.

- To select contiguous rows, press and hold the Shift key while you click the first and last row in the selection.
- To select non-contiguous rows, press and hold the Ctrl key while you click the table rows.

To delete a Grid ROI:

1. Right-click the ROI.
2. Select **Delete** from the shortcut menu or press the Delete key.

Exporting ROI Measurements

Although ROI values in Measurement Manager can be manually recorded in laboratory notebooks, there are a number of ways to easily export ROI data from Measurement Manager into a spreadsheet application like Microsoft Excel® or other software. ROI data from grid ROIs (Figure 4.9 for example) as well as from non-grid ROIs (like Figure 4.10 below) from one or many images can be easily exported from Measurement Manager. Please refer to these two figures below while following the instructions for exporting ROI data.

Figure 4.9 Example Grid ROI data

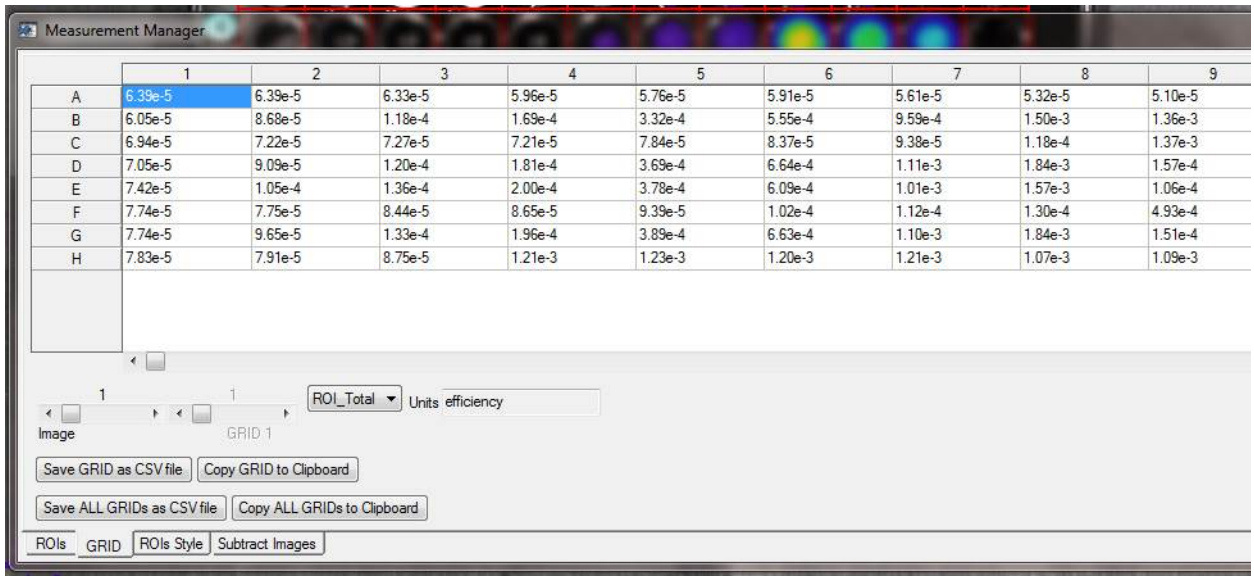
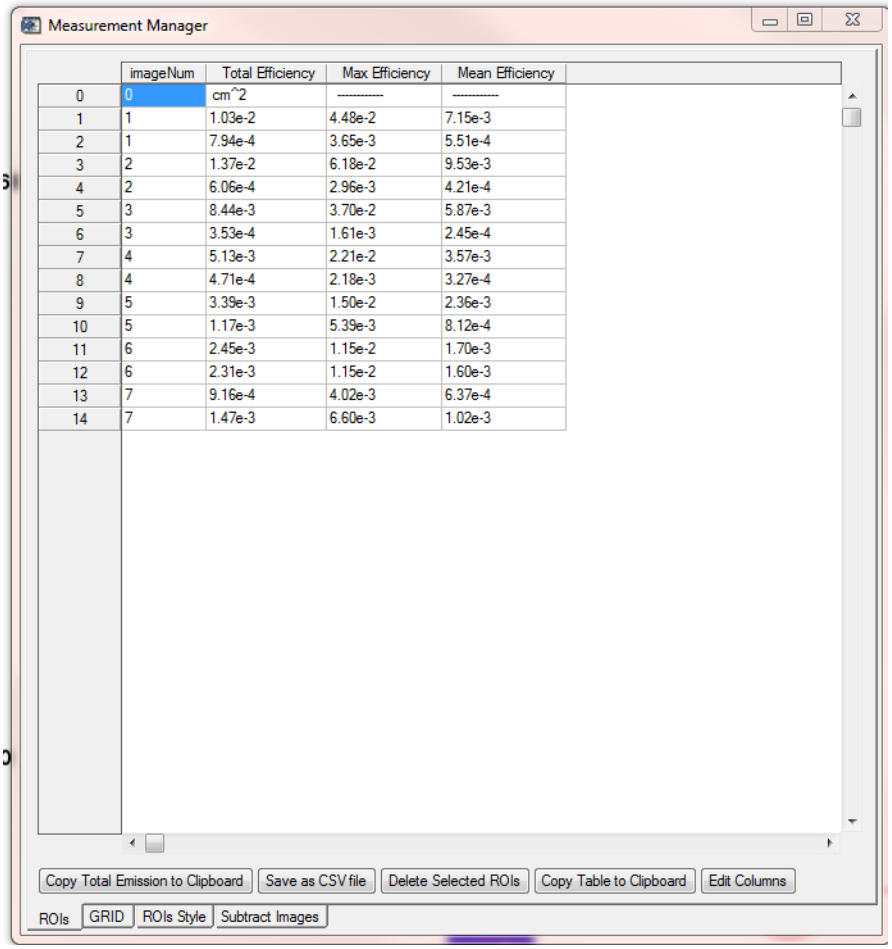


Figure 4.10 Example Non-Grid ROI data



Copying the Entire Measurement Manager Data Table

1. Draw either:
   a. at least one non-ROI on one or more images
   b. a single grid ROI on one image
2. Open Measurement Manager and click either the **ROIs** tab or the **GRID** tab on the bottom of the window depending on which type of ROI you created in Step 1.
3. If you created one or more non-grid ROIs, click the button labeled **Copy Table to Clipboard** in order to copy the data table as currently displayed in Measurement Manager.
4. If you created a single grid ROI on a single image, click the button labeled **Copy GRID to Clipboard** button.

NOTE: In order to copy all the Measurement Manager data tables from more than one image with a grid ROI, please see *, Copying and Pasting Grid ROI Data from All Images* below.

Copying and Pasting Only Total Emission Data

1. Place a non-grid ROI (e.g. rectangle, ellipsis or freehand) on one or more images.
2. Open Measurement Manager and click on the **ROIs** tab if that tab is not displayed already.
3. Click the **Copy Total Emission to Clipboard** button.
4. Paste this column of data (including the column title) into your software.

Copying and Pasting Grid ROI Data from All Images

1. Place a grid ROI on all images in the workspace you wish to gather data from.
2. Open Measurement Manager and click the **GRID** tab if it is not displayed already.
3. Click the button labeled **Copy ALL GRIDs to Clipboard.**
4. Paste the Clipboard contents into your application. For every image with a grid ROI, a separate table containing the user-specified data from the grid ROIs will appear.

Exporting Measurement Manager Data to a File

1. Place a single or grid ROI on all images in the workspace you wish to gather data from.
2. Open Measurement Manager Select either the **ROIs** or **GRID** tab depending on which type of ROI you placed on your image(s).
3. Click the appropriate button depending on the type and number of ROIs drawn and the number of images to be analyzed:
   a. For a single image with just one grid ROI, click the **Save GRID as CSV file** button.
   b. For more than one image containing a grid ROI or a single image containing more than one grid ROI, click the **Save ALL GRIDs as CSV file** button.
   c. To save data from one or more non-grid ROIs on one or more images, click the button labeled **Save as CSV file**.

4.3 Background ROIs

1. Correct the ROI measurement for background (optional).

   NOTE: This step is not necessary if image subtraction was performed to remove background.

   a. Draw an ROI on an area of the image which represents background (no probe signal present).
   b. Right-click the ROI and select **Make Background ROI** on the shortcut menu.

      A background ROI is denoted using a dashed line and **B_ROIXX** label name (Figure 4.11).
   c. Choose the measurement ROI(s) to link to the background ROI in the dialog box that appears and click **Accept** (Figure 4.11).

      NOTE: If the image is part of a group, the background ROI and links to measurement ROIs can be applied to all images in the group by selecting **Apply to Group**.

      The background-corrected measurements are computed and displayed in the Measurement Manager. (Right-click the image and select **Measurement Manager** on the shortcut menu to open the Measurement Manager.)

Figure 4.11 Linking a Background ROI to a Measurement ROI(s)

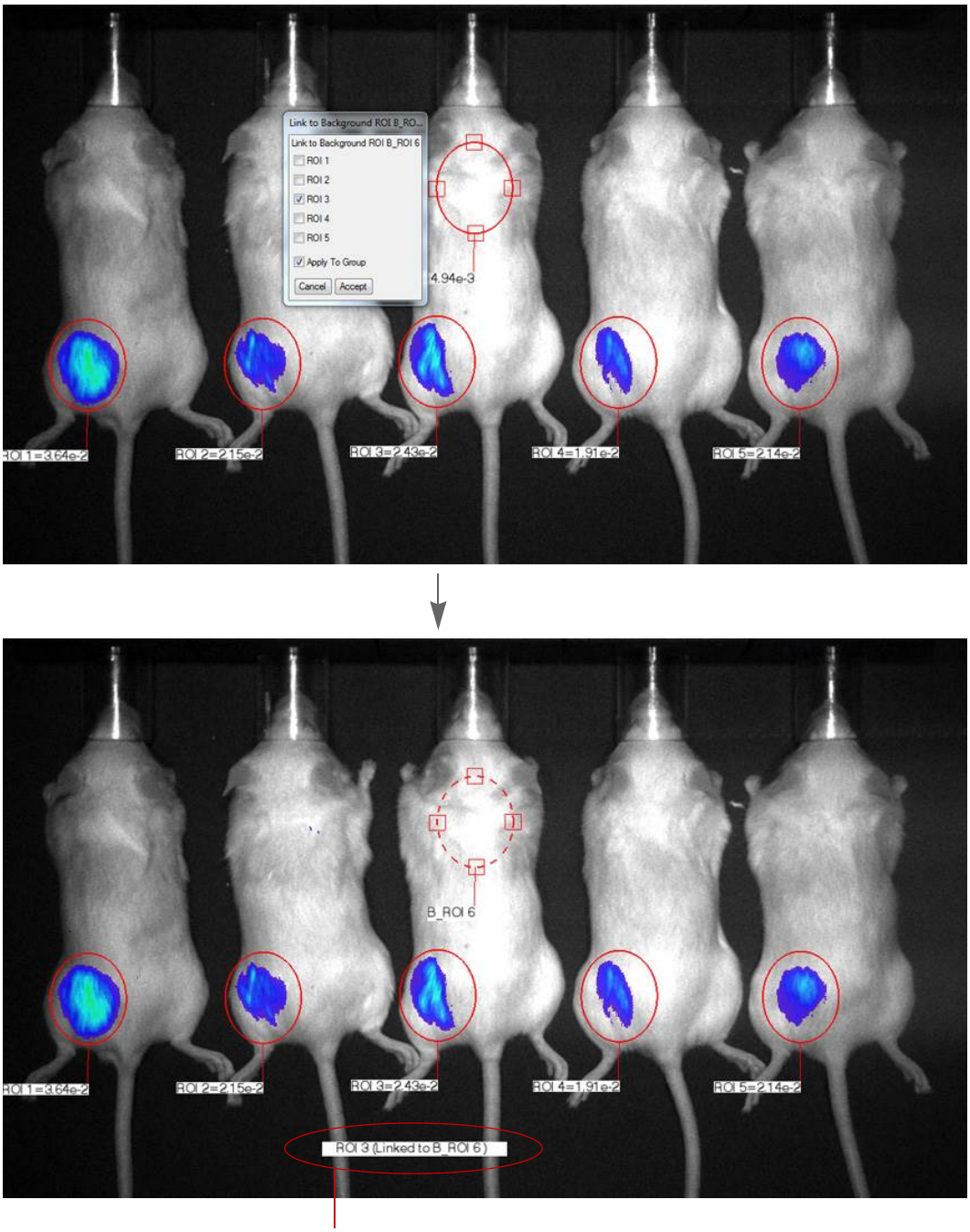


Image label indicates link between background and measurement ROIs.

2. To remove an ROI, right-click the ROI and select **Delete** on the shortcut menu.

NOTE: See page 35 for instructions on saving ROIs.

4.4 Image Subtraction

Image subtraction can be used to remove luminescence or autofluorescence background from an image before making ROI measurements. Image subtraction requires two images—a primary image and a background image. For example, an:

- Image of an object (primary image) and an image of the empty imaging system stage (background image).
- Image of a microplate containing cells and culture medium (primary image) and a microplate containing culture medium only (background image).

The both the primary and background fluorescent images will need to be taken with the same emission filter. Also, aura will not allow the emission and excitation wavelengths to be less than 35 nm apart.

To perform image subtraction:

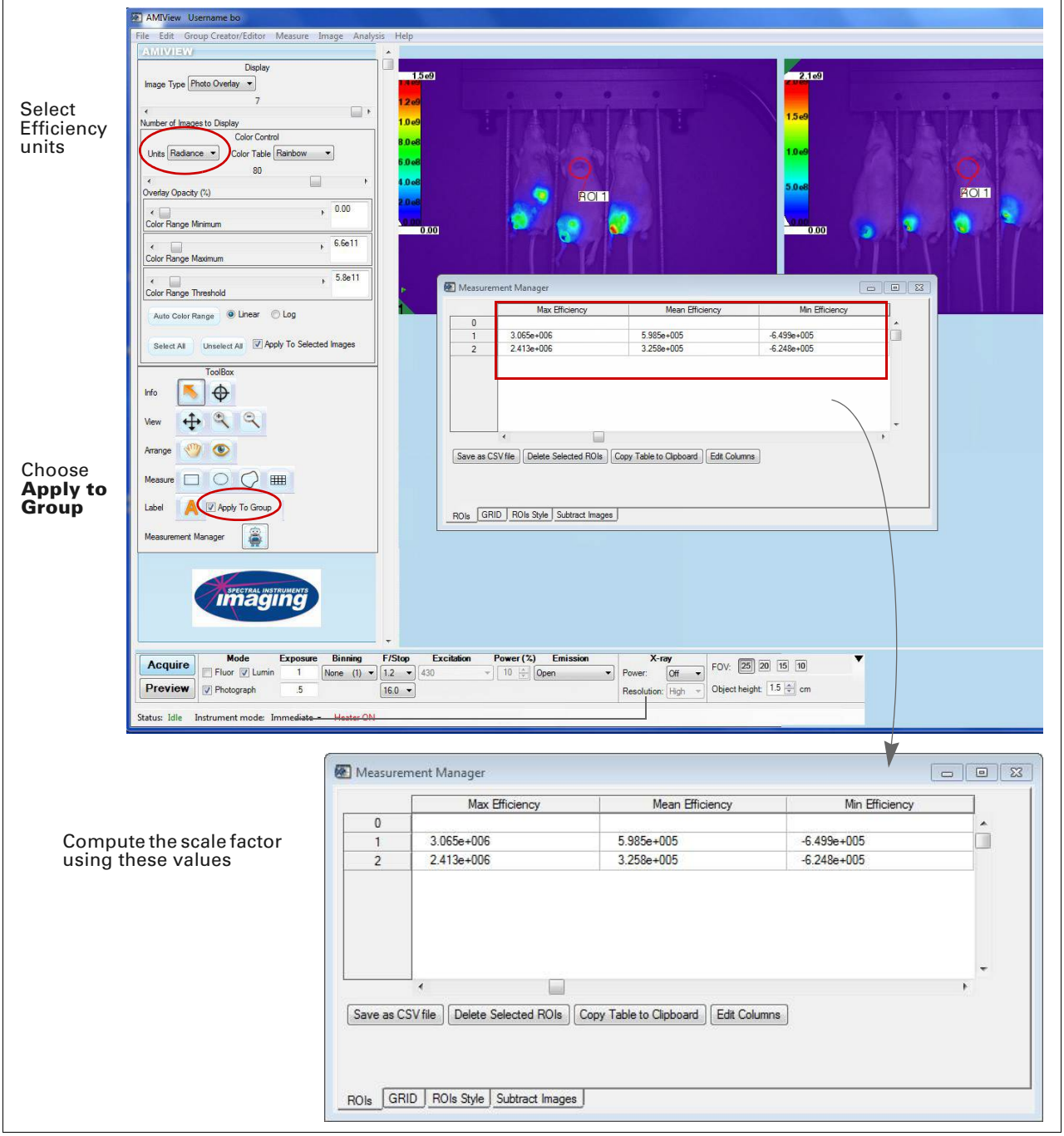
1. Obtain a primary and background image as single images.
2. Load both images into the workspace.
3. Create a group from the images and load the group. See *Creating a Group From Single Images* on page 59 for instructions.
4. Select **Apply to Group** in the ToolBox (Figure 4.12).
5. Place an ROI on one of the images in an area where there is no probe signal (Figure 4.12). (See *Making ROI Measurements* on page 32 for instructions.)

   The ROI appears on both images.
6. Select Efficiency in the Units drop-down list for fluorescent images. Select Radiance units for luminescent images.
7. Open the Measurement Manager (right-click an image and select **Measurement Manager** from the shortcut menu) (Figure 4.12).
8. Compute the scale factor (**Factor** in Figure 4.12) which will be used in image subtraction.

   For fluorescent images, Factor = ROI Mean Efficiency of the primary image/ROI Mean Efficiency of the background image.

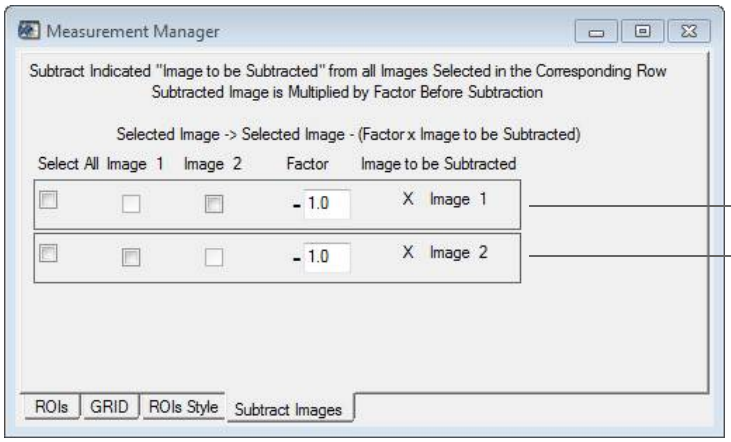
   For luminescent images, Factor = ROI Mean Radiance of the primary image/ROI Mean Radiance of the background image.

Figure 4.12 Measurement Manager – ROI Mean Efficiency



9. In the Subtract Images tab of the Measurement Manager (Figure 4.13):
   a. Check mark the primary image.
   b. Enter the scale factor computed in computed in step 8 and press the Enter key. The background image is immediately subtracted from the primary image.
10. To save the background-corrected image:
   a. Select **File** → **Save Image**.
   b. Enter a file name in the dialog box that appears.

Figure 4.13 Measurement Manager – Subtract Images Tab



If image 1 = background image and image 2 = primary image:

- Check mark **Image 2**.
- Enter the scale factor computed in step 8 on page 42.

If image 2 is the background image and image 1 = primary image:

- Check mark **Image 1**.
- Enter the scale factor computed in step 8 on page 42.

Spectral Unmixing

5.1 About Spectral Unmixing

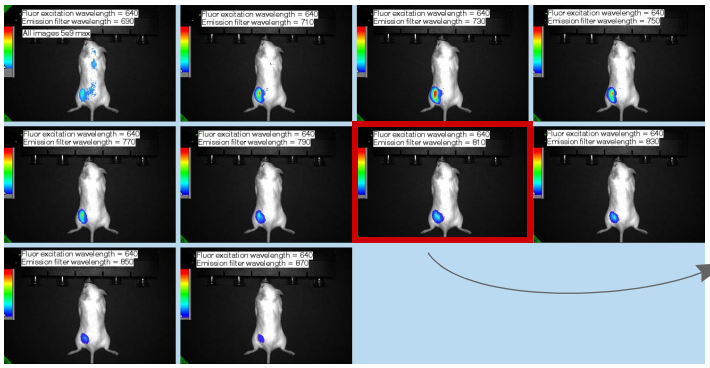
Spectral unmixing can be applied to image data containing multiple fluorescent or luminescent reporters (*components*) to create separate *component images*, each displaying a single component (Figure 5.1). Component images can be analyzed and recombined to create a *composite image* which shows component distribution. One common use for spectral unmixing is to remove autofluorescent or luminescent background from an image.

Spectral unmixing is best suited for images which have reporters that are spectrally too close together to be discriminated by an emission filter, but whose spectral peaks are far enough away so that they don't completely overlap.

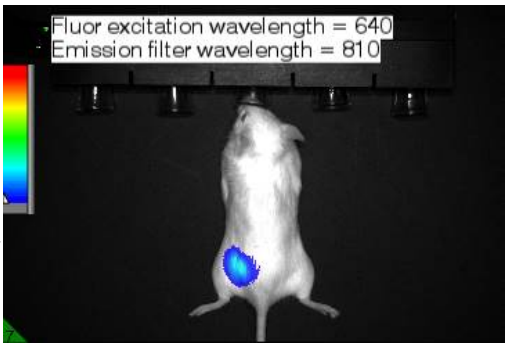
Spectral unmixing works best on images taken using spectrally contiguous emission filters (e.g. 490nm, 510nm, 530nm, 550nm instead of 490nm, 550nm, 570nm and 650nm) for the sample image and for measuring a component spectrum.

NOTE: See the SII technical note *Spectral Unmixing on SPECTRAL Imaging Systems* for additional information and examples of spectral unmixing.

Figure 5.1 Spectral Unmixing Overview
The image data in this example contains two components.

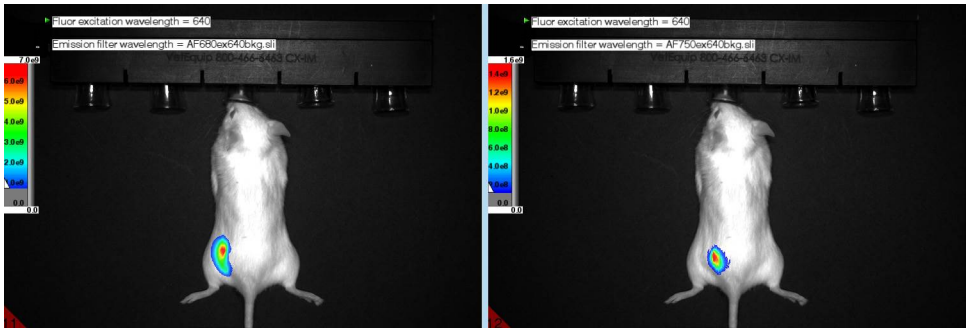


Acquire a group image for spectral unmixing



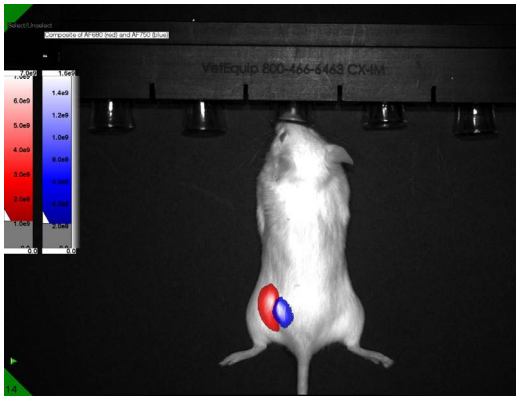
Two components are present, but are not distinguishable because they overlap

Perform spectral unmixing to determine the contribution from each component



Two component images produced by spectral unmixing

Create a composite image by recombining component images (optional)



Composite image displaying individual components using different color bars

Table 5.1 lists the steps to perform spectral unmixing. The spectral unmixing example in this chapter uses the Spectral Instruments Imaging Calibration Device (SIICD).

Table 5.1 Spectral Unmixing Workflow

Step	See Page
1. Define a component spectrum for each component to be unmixed and save for future use. Two methods are available: ■ Take a direct measurement. ■ Use published values.	 47 49
2. Load a group image data set for spectral unmixing.	53
3. Select component spectra from the library and choose unmixing options.	54
4. Create a composite image (optional).	54

5.2 Define Component Spectra

There are two ways to define component spectra:

- Direct measurement – Measure a control sample containing a single component using the Ami or Ami X Imaging System; for example, a specific dye in a particular animal model. See below for instructions.
- Use published values – Manually enter known values, for example, from a manufacturer's data sheet. See page page 49 for instructions.

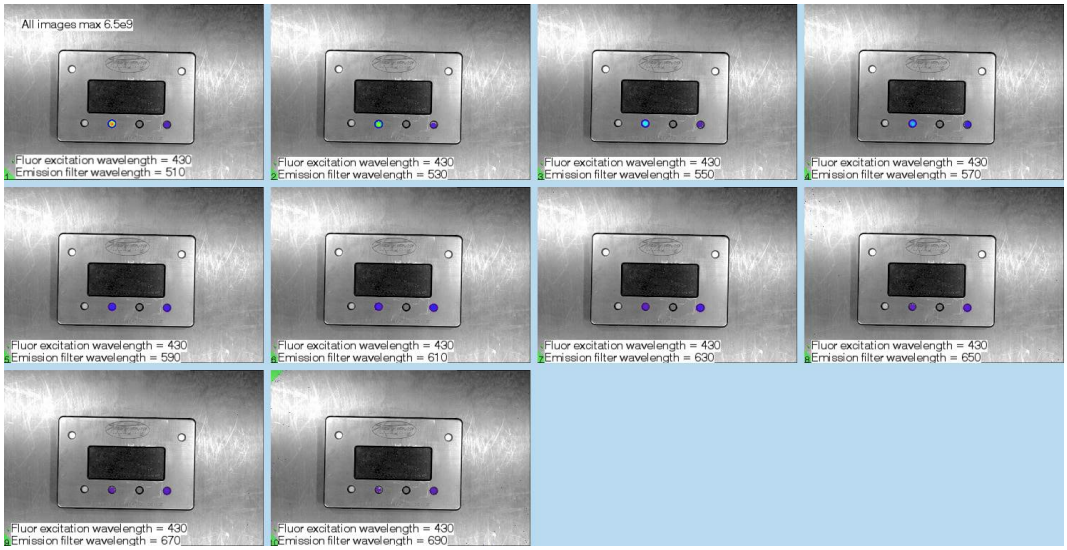
NOTE: Direct measurement is the best way to define component spectra because the sample environment may influence the spectrum. This is particularly true for deep tissue animal imaging where shorter wavelengths are preferentially absorbed and the observed spectrum is shifted toward the red.

A collection of component spectra is called a *library*. Library spectra are available for future use and can be shared with other users on the system or across systems.

Direct Measurement of a Component Spectrum

1. Acquire a group image following the image requirements for spectral unmixing.
2. Display the images using a color bar range which enables you to identify the location of the component (Figure 5.2). Usually, the automatic color scaling is sufficient.

Figure 5.2 SIICD Group Image
A 10 member group was acquired using a fluorescent excitation source at 430 nm for all images with emission filters ranging from 510 nm to 690 nm. The f-stop used was f/2.0 with 10% power and a one second exposure time for the fluorescent images. All images displayed using the same color bar range.

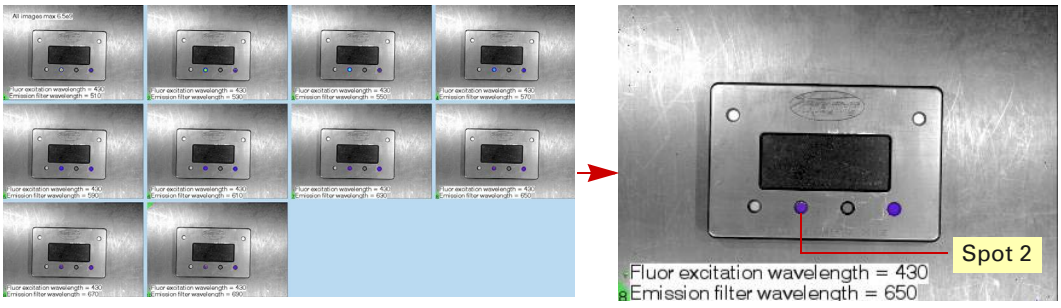


3. Verify that all the images are selected (i.e. there is a green triangle in the upper left hand corner). Place an ROI around the reporter signal in one of the images (Figure 5.3).

   The ROI is added to each image in the group.

> **NOTE:** If defining a component spectrum for autofluorescence or luminescence background, draw the ROI on an area of the subject where there is no reporter signal.

Figure 5.3 SCIID
A component spectrum will be determined for spot 2 of the SIICD.

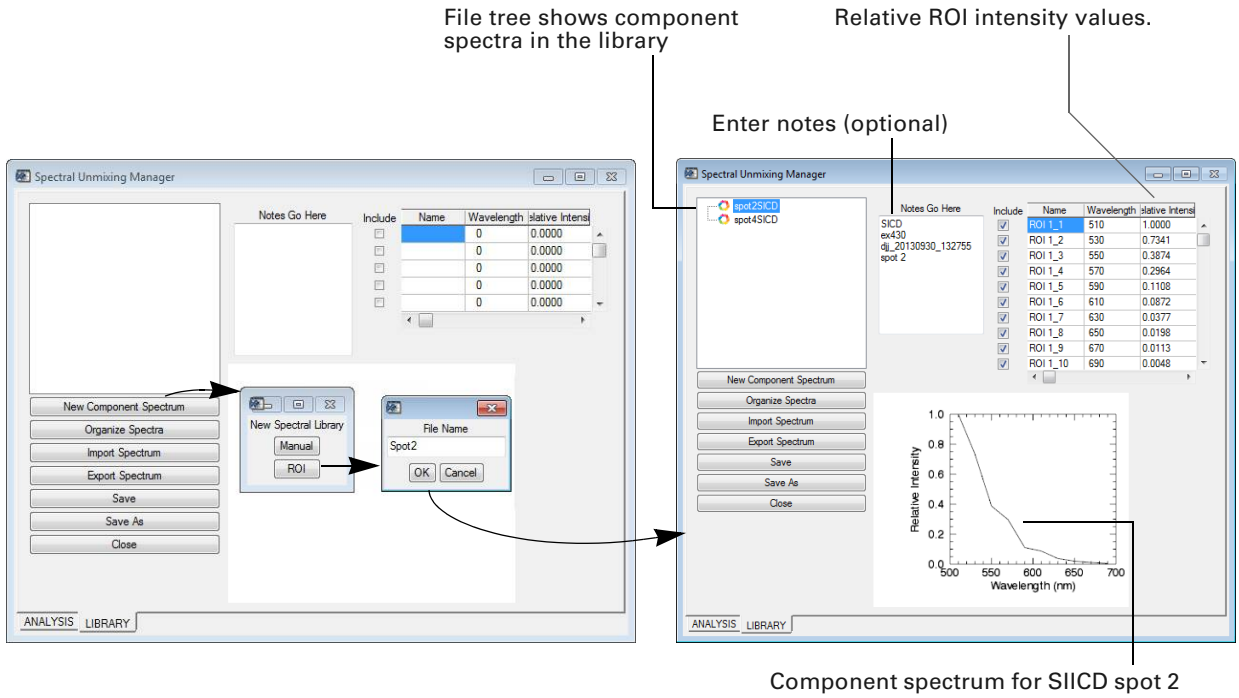


4. Open the Spectral Unmixing Manager by selecting **Analysis → Spectral Unmixing Manager** on the menu bar.
5. In the Library tab (Figure 5.4):
   - Click **New Component Spectrum**.
   - Choose **ROI** in the box that appears and enter a file name for the component spectrum in the next box.
   - Click **OK**.

The software computes the relative intensities of the ROI measurements, normalized to the brightest measurement, and displays the values in table and graph formats. The spectrum is added to the library.

NOTE: Removing the check mark next to an ROI excludes it from the analysis and automatically updates the relative intensity values in the table and graph. Click **Save** to save the updated spectrum.

Figure 5.4 Spectral Unmixing Manager – Library Tab
Library tab provides tools for creating, managing, and exporting/importing component spectra.



6. Enter notes about the spectrum (optional) and click **Save**.
   The information is saved with the spectrum.

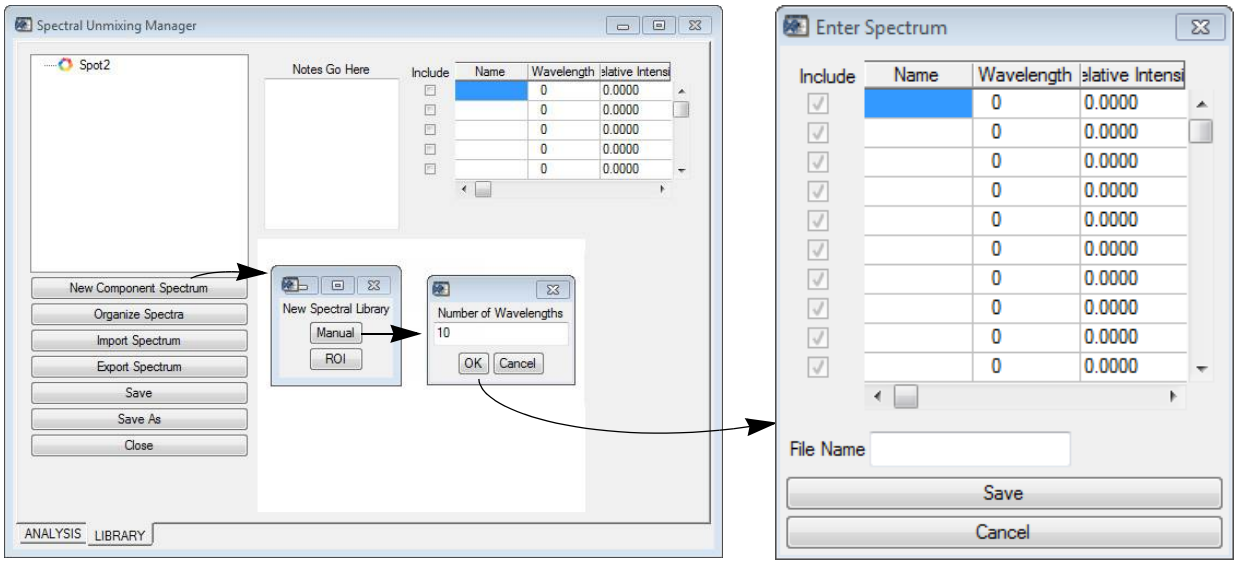
NOTE: Fluorescent component spectra are specific to the excitation wavelength as well as the solvent. Therefore, it is highly recommended to include the excitation wavelength in either the name of the saved component spectrum or in its notes.

Define a Component Spectrum Using Published Values

1. Open the Spectral Unmixing Manager by selecting **Analysis → Spectral Unmixing Manager** on the menu bar.
2. In the Library tab (Figure 5.5):
   - Click **New Component Spectrum**.
   - Choose **Manual** in the box that appears and enter the number of wavelengths in the next box.
   - Click **OK**.
3. Enter spectrum information in the next box (Figure 5.5). For each entry, enter a:
   - Name
   - Emission wavelength
   - Relative intensity normalized to the brightest emission value

- File name for the component spectrum.

Figure 5.5 Spectral Unmixing Manager – Library Tab



Enter spectrum information

4. Click **Save**.

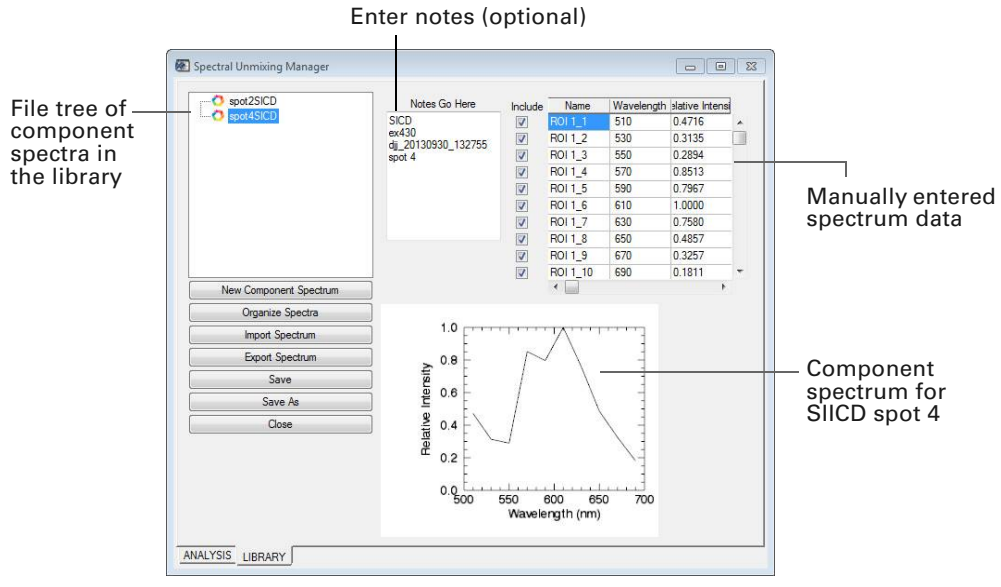
   The software creates plots the spectrum entries and adds the spectrum to the library (Figure 5.6).

> **NOTE:** Removing the check mark next to an entry excludes it from the spectrum and automatically updates the relative intensity values in the table and graph. Click **Save** to save the updated spectrum.

5. Enter notes about the spectrum (such as the excitation wavelength or solvent) and click **Save**.

   The information is saved with the spectrum.

Figure 5.6 Spectral Unmixing Manager – Library Tab

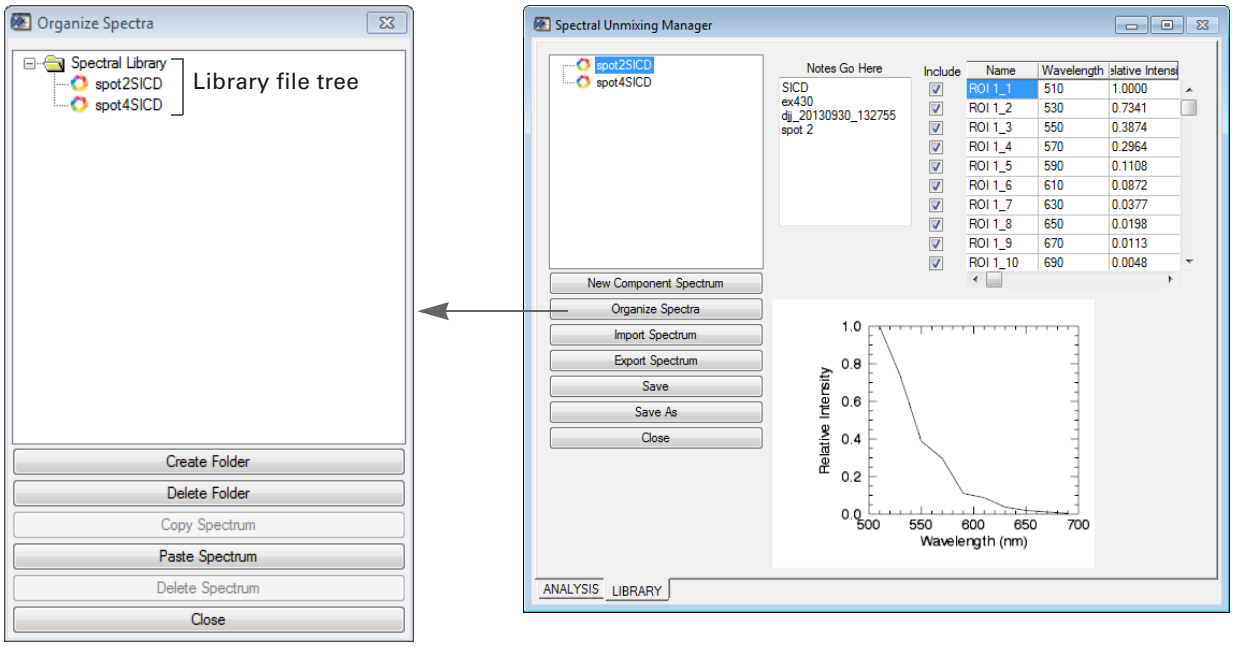


Managing Component Spectra

The file tree in the Spectral Unmixing Manager shows how library spectra are organized.

Click **Organize Spectra** in the Spectral Unmixing Manager to access tools for managing component spectra (Figure 5.7).

Figure 5.7 Organizing Component Spectra



Tools for organizing library spectra (see Table 5.2 for more information)

Table 5.2 Organizing Library Spectra

Item	Description
Create Folder	Adds a folder to the library file tree (Figure 5.7). To add folder: **1.** Select a folder in the Organize Spectra dialog box and click **Create Folder**. **2.** Enter a name for the folder in the box that appears and click **OK**.
Delete Folder	Deletes the selected folder in the Organize Spectra dialog box.
Copy Spectrum	Copies the selected library entry to the system clipboard.
Paste Spectrum	Pastes a library entry to the selected folder.
Delete Spectrum	Deletes a selected library entry.
Close	Closes the Organize Spectra dialog box.

Sharing Component Spectra

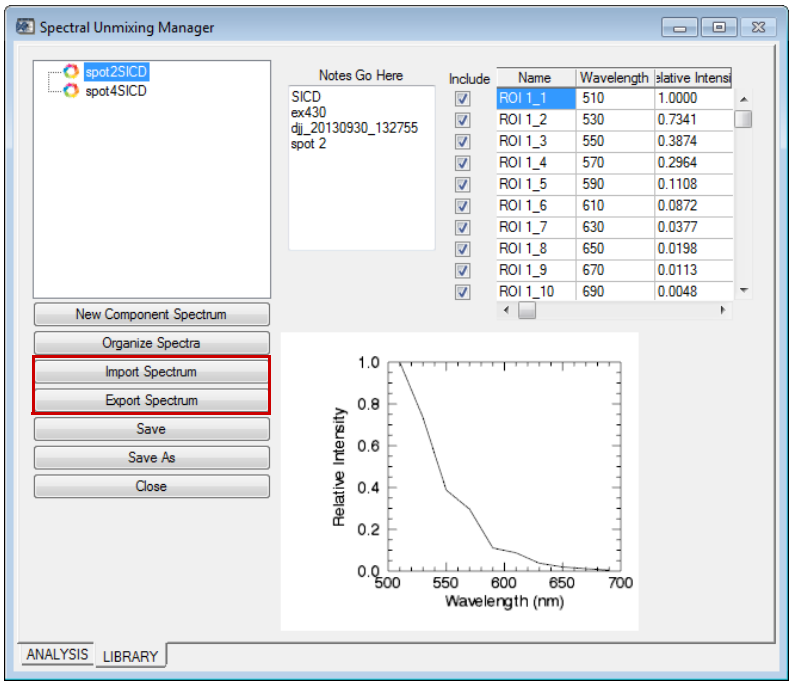
Library spectra (saved as plain text files with a ".sli" extension) can be shared with other aura users.

1. Open the Spectral Unmixing Manager (select **Analysis → Spectral Unmixing Manager** on the menu bar) (Figure 5.5).
2. To export a spectrum, click **Export Spectrum**, choose a directory in the box that appears, and click **OK**.
3. To import a spectrum, click **Import Spectrum**, choose a file (.sli) for import in the box that appears, and click **Open**.

   The spectrum is added to the library directory.

Figure 5.8 Spectral Unmixing Manager – Library Tab

Controls for importing or exporting a spectrum



5.3 Unmix Components

1. Load the group image to be unmixed.
2. Open the Spectral Unmixing Manager (select **Analysis → Spectral Unmixing Manager** on the menu bar) and click the Analysis tab (Figure 5.9).

Figure 5.9 Spectral Unmixing Manager – Analysis Tab

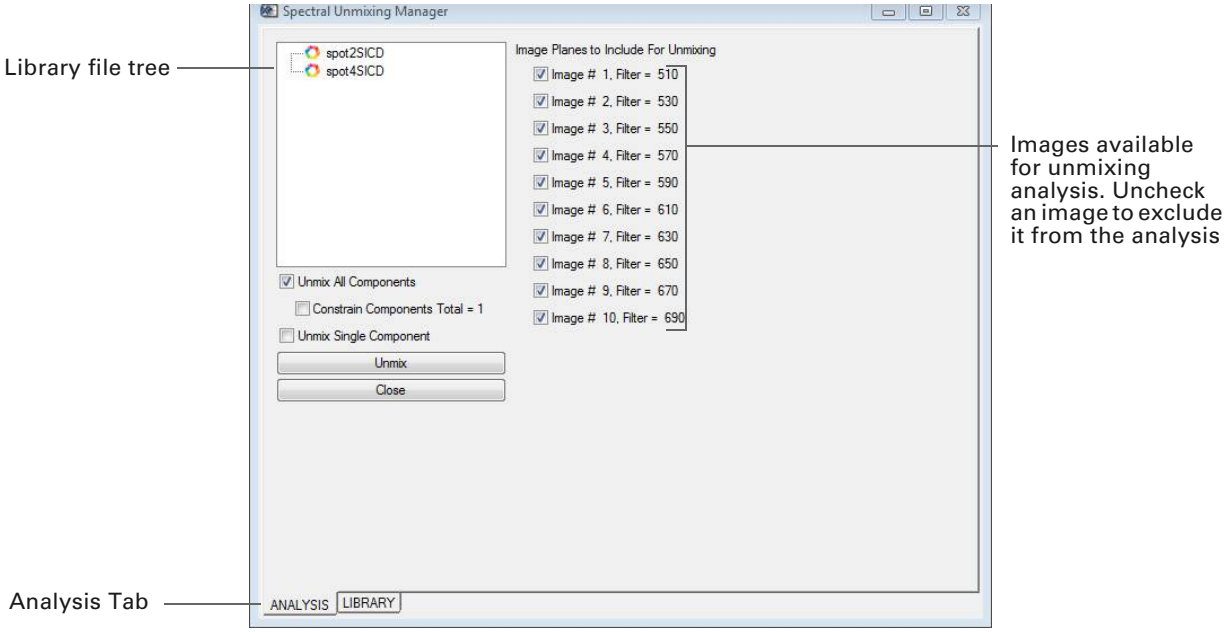


Table 5.3 Spectral Unmixing Manager – Analysis Tab

Item	Description
Library file tree	Choose component spectra to use for unmixing.
Unmix All Components	Choose this option to unmix all components using the selected component spectra. A component image is produced for each component.
Constrain Components Total = 1	Select this option to use a standard linear unmixing algorithm that constrains the component fractions in a pixel to sum to one. If this option is not selected, unmixing uses the MESMA algorithm, a very fast model that does not constrain component fractions in a pixel to sum to one.
Unmix Single Component	Choose this option to unmix one component using a selected component spectrum. Use single component unmixing if only one component is known, say autofluorescence, and other components are not known. One component image is produced.
Unmix	Click to begin the unmixing process.
Close	Closes the Spectral Unmixing Manager.

NOTE: For best (i.e. most accurate) unmixing results, select at least two component spectra known to be present in the sample and select the **Unmix All Components** option.

3. If you want to exclude an image from the analysis, remove the check mark next to the image.
4. Select component spectra in the library file tree.

   To select adjacent library entries, press and hold the Shift key while you click the first and last spectrum in the selection. To select non-adjacent entries, press and hold the Ctrl key while you click the spectra.
5. Choose unmixing options (see Table 5.3) and click **Unmix**.

   Component image(s) appear in the main workspace, with a blue triangle in the upper right corner labeling the image as an unmixed component image (see Figure 5.10).

NOTE: ROI measurements can be made on component images.

Figure 5.10 Component Images for SIICD Spot 2 and Spot 4

Only signal from spot 2 is visible in this image because emission is restricted to that which corresponds to the component spectrum determined for spot 2.

Only signal from spot 4 is visible in this image because emission is restricted to that which corresponds to the component spectrum determined for spot 4.

5.4 Using Component Images

Analyzing data from component images

ROIs can be placed on component images and their data analyzed. The data from component images, however is only validly compared against other unmixed images generated from group images using the same emission (and for fluorescent images, excitation) wavelengths and unmixed with the same component spectra.

5.5 Composite Images

Component images can be recombined to create a composite image that shows the location of each source (Figure 5.1 on page 46). Images in a composite are layered, one on top of the other. The composite can also include a photograph or an X-ray image.

NOTE: It is not possible to either save or place ROIs on composite images. ROIs can, however be placed on unmixed component images.

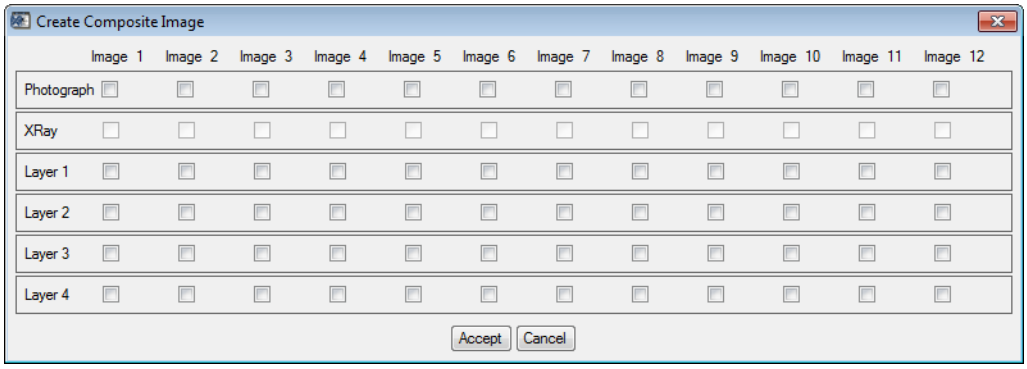
To create a composite image:

6. After component images are generated using spectral unmixing, select a different color bar for each component image. See Table 3.2 on page 16 for information on display color control.
7. Select **Analysis → Composite Image Manager** on the menu bar.

8. Put a check mark next to the images that will be used to create the composite (Figure 5.11).

NOTE: A composite image may include up to four layers, plus a photograph or an X-ray image. Layer 1 is at the bottom and layer 4 is displayed at the top of the composite image.

Figure 5.11 Composite Image Manager



9. Click **Accept**.

The workspace displays the composite image, labeled with a yellow triangle in the top right corner.

Figure 5.12 Composite image of the Spot 2 and Spot 4 components

Managing Images

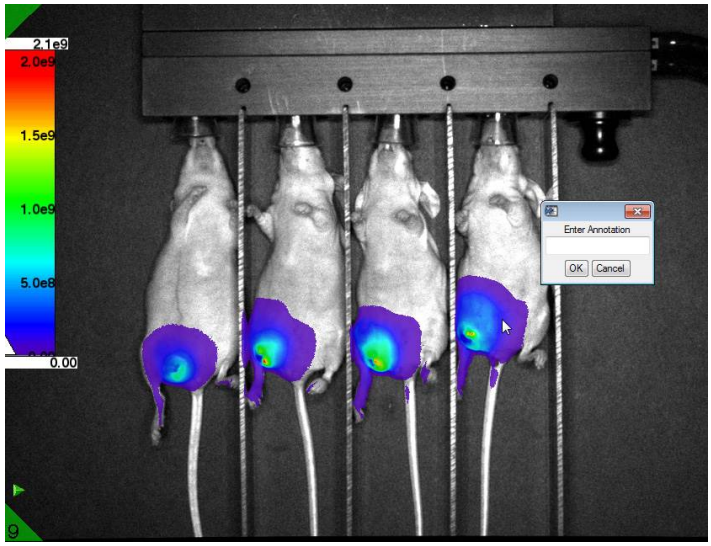
6.1 Annotating and Labeling Images

NOTE: Annotations can not be saved with the image nor exported separately. If you wish to save or share any annotations please take a screenshot by saving a graphics file (see Section 6.4, "Saving Graphic Files," page 60), but be aware that no image data is saved with the screenshot

Annotating an Image

1. Click the button in the ToolBox.
2. Click a location of interest in the image.
3. Enter notes in the dialog box that appears and click **OK** (Figure 6.1).

Figure 6.1 Adding an Annotation to an Image



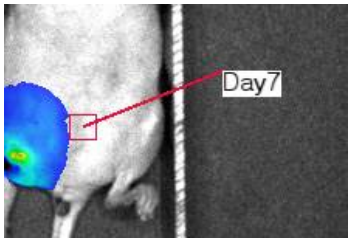
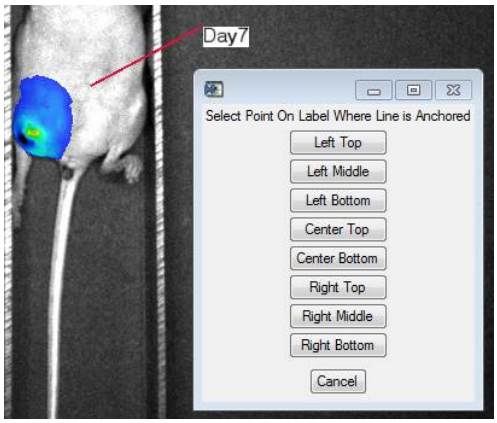
Managing Annotations

Right-click an annotation to see options for managing annotations (Table 6.1).

Table 6.1 Annotation Shortcut Menu

Item	Description
Delete	Removes the note from the image.
Color	Opens the color palette. Choose a preset color or create a custom color for the note line.

Table 6.1 Annotation Shortcut Menu (continued)

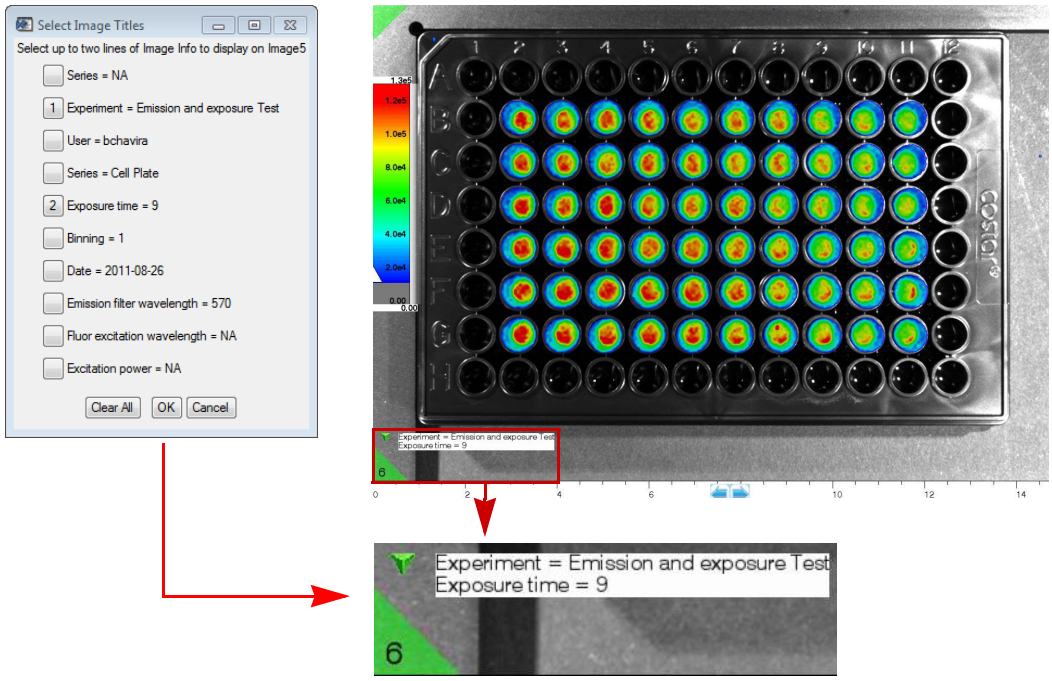
Item	Description
Rename	Choose this option to edit the note in the dialog box that appears.
Hide/Show Line	Hides or shows the line associated with the note.
Change Labeled Point	Enables you to move the line end point. Drag the line end point to a new location when the red box appears. 
Change Anchor Point	Provides options for changing the point on the label where the line is anchored. In this example, the line is anchored at the "Left Top" corner of the note. 
Cancel	Closes the shortcut menu.

Adding an Image Title

Images can be labeled with up to five lines of information.

1. Click the  icon on the image (located just above the image number, see Figure 6.1). Alternatively, select **Image** → **Configure Image Titles** on the menu bar.
2. Select up to two types of image information in the dialog box that appears and click **OK** (Figure 6.2).

Figure 6.2 Select Image Information for the Label



3. Click the icon to hide the image information.
4. To change or remove the information displayed on the image:
   a. Right-click the or icon.
   b. To change the information displayed, make new selections (up to five types of information) in the dialog box that appears. and click **OK**.
   c. To remove the information display, choose **Clear All** and click **OK**.

6.2 Creating a Group From Single Images

Single images can be organized into a group or added to a group. This may be helpful in some situations, for example, longitudinal time studies or when applying an ROI to a particular location that you want to examine in a series of images acquired using different imaging parameters.

NOTE: The group image which is created contains a copy of the single images. Therefore, those saved single image files will not disappear after the new group image file is created. This is also true of single images which are added to an already existing group.

To create a group from single images:

1. Select single images in the Image Manager and click **Group Selected**.
2. Enter a file name in the dialog box that appears and click **Save**.

To add images to an existing group:

1. Select images in the Image Manager and click **Add Selected To A Group**.
2. Choose a group in the dialog box that appears and click **Open**.

6.3 Exporting Image Data

Exporting image data creates numerical image data files that can be read by other analysis software (e.g. MATLAB®, Origin, ImageJ or Python). The exported data may include luminescent or fluorescent image data, depending on the image types in the .ami file. The units selected in the Units drop-down list determines the units of the exported data.

To export image data:

1. Select the images in the workspace that you want to export.
2. Select either of the following:
   - **File Export Data → Export Raw Images** – Images are not spatially adjusted.Output format is 32-bit floating point TIFF.
   - **File Export Data → Export Corrected Images** – Images are modified by removing lens related artifacts and co-registered so that all of the image layers have the same spatial scale. Output image format is also 32-bit floating point TIFF.
   - **File Export Data → Export Raw DICOM Images**– Images are not spatially adjusted. Output format is DICOM.

   or
   - **File Export Data → Export Corrected DICOM Images** – Images are modified by removing lens related artifacts and co-registered so that all of the image layers have the same spatial scale. Output image format is also DICOM.
3. Choose a folder for the data in the dialog box that appears and click **OK**.

NOTE: The exported data files are best opened in an image analysis program such as ImageJ. The exported files are also relatively large. For saving a simple picture of the image with a smaller file size, see 6.4, *Saving Graphic Files* below for instructions.

6.4 Saving Graphic Files

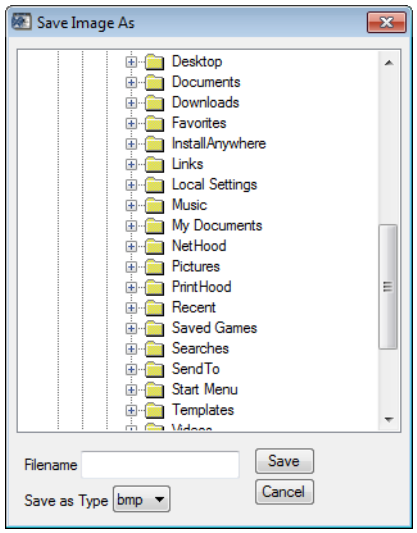
The workspace display can be saved as a graphic file (for example: jpeg, bmp, or png). The graphic file is an image (like a screenshot) that can be viewed in applications such as Microsoft Paint®, or PowerPoint®.

NOTE: A graphic file does not contain quantitative radiometric data and cannot be analyzed using aura software or other image processing applications.

To create a graphic file of the workspace display:

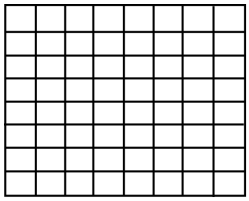
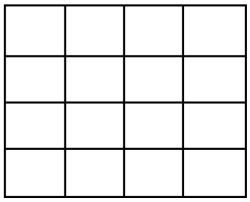
1. Select **File → Save Graphics** on the menu bar.
2. Select a folder and enter a file name in the dialog box that appears (Figure 6.3).
3. Make a selection from the **Save as** Type drop-down list and click **Save**.

Figure 6.3 Save Image as a Graphic File



Acquisition Parameters

Table A.1 Acquisition Parameters

Item	Description
Mode	The types of image acquisition: ▪ **Fluorescence** – Fluorescent mode acquires an image of light emitted from a fluorescent source at a specific wavelength when the subject is illuminated using a different (shorter) light source at a specific wavelength. ▪ **Luminescent** – Luminescent mode acquires an image of light emitted from an Unilluminated sample, for example, bioluminescent emission from plants, animals, or *in vitro* sources such a cells in microplate wells. There are no lights on inside the imaging chamber during acquisition. ▪ **Photograph** – Black and white image illuminated with white light.
Exposure (s)	The amount of time (seconds) the shutter is open during image acquisition. Longer exposure times result in higher sensitivity.
Binning	Sets the pixel size of the CCD camera. A higher level of binning increases sensitivity, but reduces spatial resolution. Higher sensitivity is due to a higher signal-to-noise ratio resulting from binning. A certain amount of read noise (the random number of electrons added during readout) is added to each pixel. If there is no binning, the read noise is added to each pixel. When individual pixels are binned to create a "super pixel", read noise is spread over the entire super pixel area, which reduces the read noise. For example, 8 x 8 binning results in read noise that is 1/64 the read noise with no binning. Thus, faint signals have a much better chance of rising above the noise. Also, binning increases signal. An 8 x 8 binning level produces a super pixel that is 64 times as large with 64 times the signal compared to a non-binned pixel.  No binning Pixels in a small area of the CCD shown with no binning.  Binning = 2 Combines four (2 x 2) pixels. Increases signal four times. Quadruples pixel size.  Binning = 4 Combines 16 (4 x 4) pixels. Increases signal 16 times. Increases pixel size by a factor of 16.  Binning = 8 Combines 64 (8 x 8) pixels. Increases signal 64 times. Increases pixel size by a factor of 64.
FStop	Setting that determines the size of the lens aperture. F-stop 1.2 is the largest aperture setting, and provides maximum sensitivity, but the smallest depth of field (the range over which the image is in focus). As the F-stop number increases, the aperture size decreases and the depth of field increases.
Excitation (nm)	Excitation light wavelength.
Power (%)	Power level of the excitation light source.
Emission (nm)	Emission filter center wavelength. Emission filters will allow light from 10nm shorter than this center wavelength to 10nm longer, thus a total transmission window of 20nm.
FOV	Field of view is the width of the imaged area.
Object height	Object height entered by user (the highest vertical point of the object).

B

Image Data

B.1 Numerical Images

The charge coupled device (CCD) camera in a SPECTRAL Imaging System is an integrated circuit etched onto a silicon surface and consists of light-sensitive elements called *pixels*. During image acquisition, photons incident on the CCD surface generate charge which is electronically read and converted into a two-dimensional array of numbers. Each number in the array represents the light intensity recorded by a pixel. The numerical value of a pixel is proportional to the intensity of the light it recorded. See Spectral Instruments online for more detailed information about CCDs and pixels.

aura software displays luminescent or fluorescent numerical images as a *pseudocolor image* (Figure B.1) which is generated by:

- Mapping pixel intensity values to colors defined by a color bar.
- Displaying each pixel filled with the color assigned to the pixel's intensity value (Figure B.1).

NOTE: The appearance of a pseudocolor image does not affect the numerical image data.

Image data is independent of the color bar, the minimum and maximum of the color bar range, or the color bar scale (linear or log) used to display the data. The color bar only affects data display, not the underlying pixel numerical values. This means that ROI measurements will give the same result, regardless of the color bar settings.

B.2 About aura Images

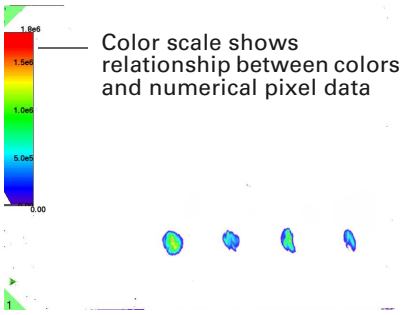
More than one type of image is usually acquired during acquisition, for example:

- A luminescent image and a photograph.
- A fluorescent image, x-ray image, and a photograph (if the imaging system has x-ray imaging capability).

aura software saves images and image information from an acquisition in one file (.ami). Load saved aura image files in the workspace to display and analyze images. The numerical image data in the file can also be exported (TIFF and DICOM) for use with other image processing software such as MATLAB®, Python, Origin, or ImageJ. For steps on how to export the numerical image data, see Exporting Image Data on page 60

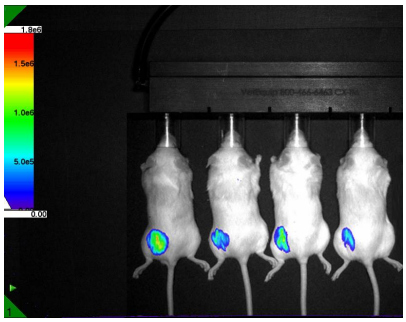
SPECTRAL Imaging Systems are absolutely calibrated. As a result, the numerical pixel values are related to a *standard* (a physical measurement of the photons per second emitted from a pixel area that is the same for the standard and image). Therefore, a measurement in radiance units has physical meaning and can be quantitatively compared to measurements made on any other absolutely calibrated instrument. In addition, the measurements are independent of instrument settings. If a subject emits twice as many photons compared to another, the radiance value will be twice as much, independent of the exposure time.

Figure B.1 Example Fluorescent Image, Photograph, and Overlay



Numerical image (fluorescent data) displayed as a pseudocolor image using a rainbow color bar

Photograph



Overlay of fluorescent image on photograph

B.3 Graphic Images

A photograph captured by a SPECTRAL Imaging System is an image of the light reflected from a subject when it is illuminated using the white lights near the top of the imaging system. aura software displays photographs using a grayscale color bar that assigns white to the largest pixel value and black to the smallest value, and shades of gray to the values in between (Figure B.1).

The reflected light in a photograph isn't calibrated against any standard; therefore, a photograph cannot be analyzed using ROI measurements. Refer to Saving Graphic Files on page 60 for how to export photographs.

Menu Commands

Table C.1 aura Menu Commands

Menu Bar Command	Description	See Page
File → Image Manager	Opens the Image Manager.	11
File → Load Images	Opens a dialog box that enables you to select .ami files to load in the workspace.	14
File → Load Images to Existing	Opens a dialog box that enables you to load more files in addition to the ones already loaded in the workspace	14
File → Unload Selected Images	Closes the images selected in the workspace.	18
File → Load Living Image Click Folders	Opens a dialog box that enables you to load image files originally taken with Living Image® software.	
File → Save Graphics	Enables you to save a screenshot of the workspace (for example, .jpg, .bmp, or .png). **Note:** This type of graphic image does not contain intensity data and cannot be analyzed using aura software or other image processing applications.	60
File → Save Image	Opens a dialog box which enables you to save an image (.ami). If you chose "Don't Save" during acquisition setup, use this menu command to save the image if you change your mind after acquisition is done.	
File → Print	Enables you to print the workspace contents.	
File → File Export Data → Export Raw Images	Enables you to export the selected luminescent, fluorescent, or x-ray image data (TIFF) without corrections for analysis by other image processing applications.	60
File → File Export Data → Export Corrected Images	Enables you to export the selected luminescent, fluorescent, or x-ray image data (TIFF) with corrections for analysis by other image processing applications.	60
File → File Export Data → Export Raw DICOM Images	Enables you to export the selected luminescent, fluorescent, or x-ray image data (DICOM) without corrections for analysis by other image processing applications.	60
File → File Export Data → Export Corrected DICOM Images	Enables you to export the selected luminescent, fluorescent, or x-ray image data (DICOM) with corrections for analysis by other image processing applications.	60
File → Preferences	Sets options for the appearance of the workspace.	10
File → Switch User	Enables you to change to another aura user name.	
File → Exit	Closes aura software.	
Edit → Edit Image Tags	Opens the Tag Editor that is used to create and manage tag sets.	27
Edit → Copy Graphics	Copies the workspace to the system clipboard.	
Image → Show/Hide Xray Control	Either display or remove the controls for adjusting the appearance of the x-ray image.	26
Image → Show/Hide Color Bar	Either display or removed the scale showing the color labeling of the pixel values.	28
Analysis → Spectral Unmixing Manager	Opens the Spectral Unmixing Manager, allowing you to measure or input component spectra, and generate unmixed images.	47

Table C.1 aura Menu Commands

Menu Bar Command	Description	See Page
Analysis → Composite Image Manager	Opens the Composite Image Manager window, allowing you to create a composite image from two or more component images.	54
Measure → Measurement Manager	Opens the Measurement Manager that enables you to view ROI measurements and manage ROIs.	32
Measure → Save ROIs to File	Saves the ROIs in the selected image to the system. The ROIs can be applied to other images.	35
Measure → Restore ROIs from File	Applies saved ROIs to an image.	36
Image → Configure Image Titles	Enables you to select image information to display on an image.	58
Image → Show/Hide Photo Control	Displays controls for adjusting photograph brightness and contrast.	26

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