Drawing ROIs.

This tech note describes how to use the Living Image® software to draw measurement ROIs in 2D and 3D optical images taken on IVIS® instruments.

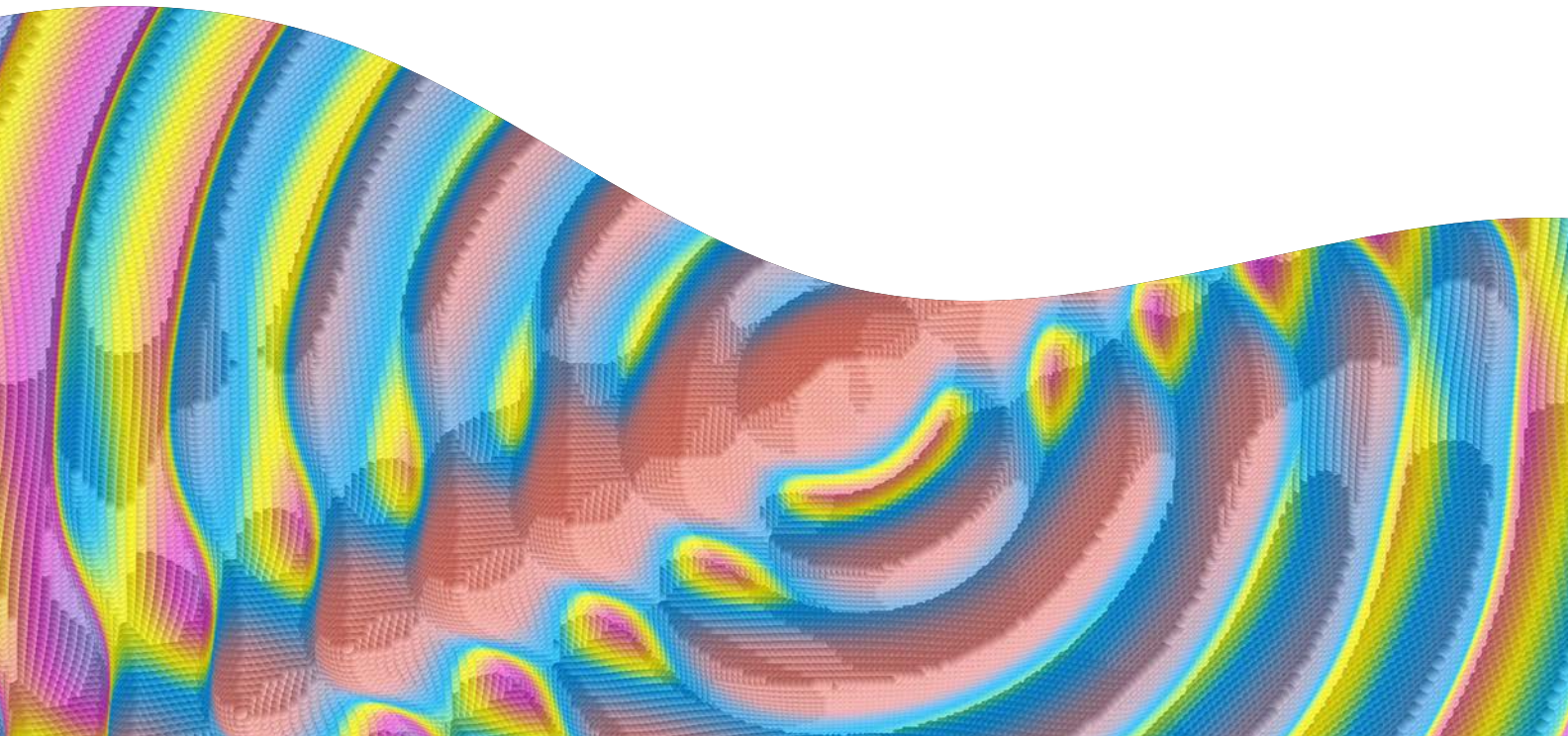
Regions of interest (ROIs) are user-defined areas within an image that have importance. This technical note discusses how to draw Measurement ROIs in images taken with IVIS® optical imaging systems. Measurement ROIs provide a quantitative value that can be monitored over time or compared to control subjects. There are separate technical notes for **Subject ROIs** and **Background ROIs.**

Drawing ROIs for 2D Bioluminescence Imaging (BLI)

Circle, square, grid (for well plates), or free draw options are available to draw your ROIs (Figure 1). ROI selections are userspecified and are dependent on the model being analyzed.

When you are finished drawing your ROIs, click on “Measure ROIs” in the Tool Palette to display the values in a table (Figure 2). The table can be customized and the customization settings saved. The data can be copied and pasted into Excel, or exported as an Excel-compatible .csv file.

For bioluminescence images, **larger ROIs are better.** Background bioluminescence levels are very low so it is advantageous to draw larger ROIs to include the entire area of signal diffusion. Do not focus only on the intense area generally represented by the red color. You must take into account the entire light diffusion pattern. The unit for 2D BLI is is labeled as “Radiance (Photons)” in the image window and “Total Flux” in the measurement table: photons/second = p/s.



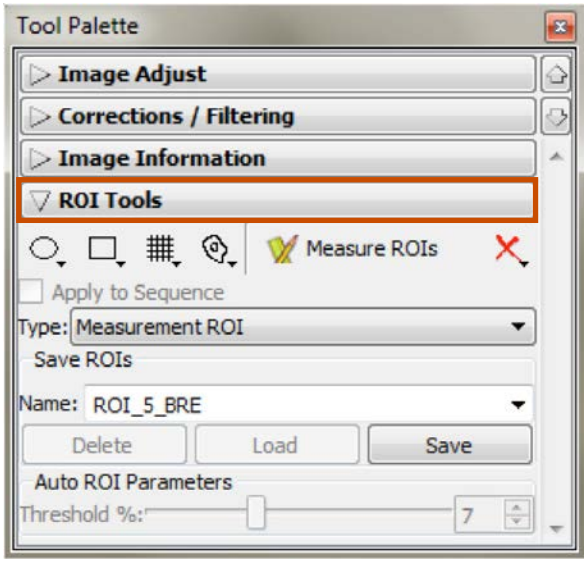


Figure 1: To draw ROIs on your image, select the ROI Tools tab in the Tool Palette. There, you can draw circles, squares, grids (for well plates), or contoured shapes around your area of interest.

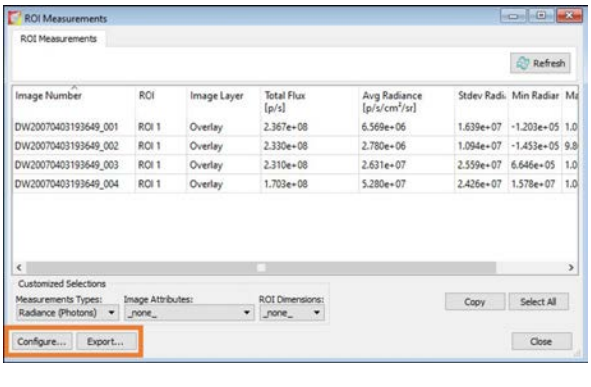


Figure 2: ROI measurements table. Click on the Configure button to customize the table. Click on the Export button to save the data as an Excel-compatible.csv file.

This tool is only for BLI (not FLI). Free draw gives you complete control over the shape of the ROI. This can be helpful for quantifying signal in resected organs. Draw by leftclicking points to create a spline around the area of interest until you have completed the desired shape. Close the shape by right-clicking once.

Drawing ROIs for 2D Fluorescence Imaging (FLI)

Analysis of fluorescence images can be more complicated than for bioluminescence images due to the many sources of background possible in fluorescence images (tissue and chow autofluorescence, instrument background, etc.).

1. Remove instrument background (Only if you used epi-illumination. If you used trans-illumination, skip to step#2). To remove the instrument background, see the tech note: **Adaptive Fluorescence Background Subtraction.**

2. Choose an area within your image or sequence of images to serve as background. See the tech note: **Background ROI.**

3. Draw your measurement ROIs. The background can automatically be subtracted from your measurements if selected in the ROI properties window (Figure 5). It is best to eep the size of the measurement ROIs consistent acrossimages that are to be compared. Alternatively, you an divide the total radiant efficiency by the ROI area to normalize for differences in size (e.g., for *ex vivo* organ analysis). The unit for 2D FLI (epi-illumination) takes the total flux and divides it by the power of the lamp. It's called "total radiant efficiency": [p/s] / [µW/cm^2]. The unit for 2D FLI (transillumination) is labeled as "NTF Efficiency" in the image window and "Efficiency" in the measurement table: cm^2.

For more information regarding sources of background and approaches to 2D FLI analysis, see the following tech note: **General and Technical Considerations for Background Subtraction in 2D Fluorescence using IVIS Imaging Systems.**

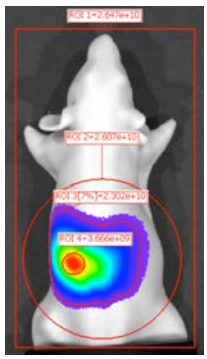


Figure 3: A 2D bioluminescence image with four ROIs of different shapes and sizes. It is better to draw the ROI bigger than the optical signal to capture the entire diffusion pattern.

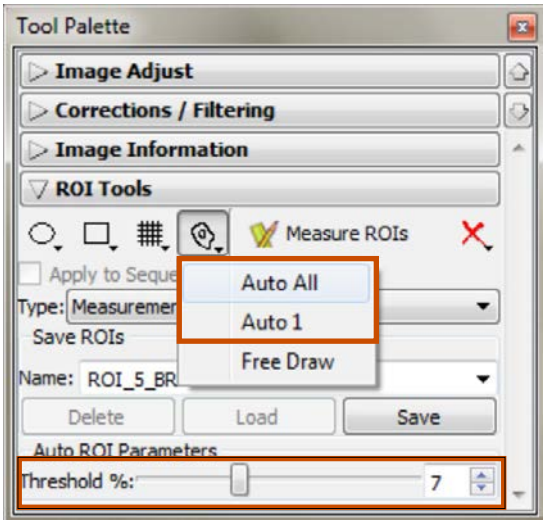


Figure 4: If desired, for 2D BLI, you can determine the extent of ROI selection based on a threshold (recommended thresholding is 5-10%).

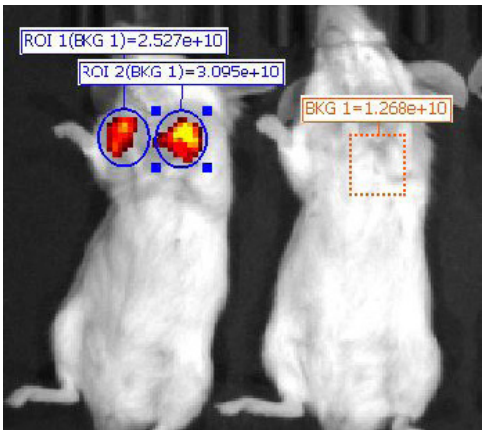


Figure 5: 2D epi-FLI showing the background ROI is being subtracted from the measurement ROIs.

Drawing ROIs for 3D BLI

Before you can begin analysis of 3D bioluminescence images, you must first reconstruct them from a series of 2D scans. See the technical notes in the **Bioluminescence Tomography** series - **Topography** and **Source Reconstruction and Analysis** - before proceeding.

The same rule of thumb applies to 3D BLI as for 2D BLI - the bigger the ROI, the better. You want to be sure to capture the whole diffusion pattern of light. Remember, there is little to no background signal in bioluminescence images.

Steps for drawing ROIs for 3D BLI:

1. Move all 2D views to your area of interest. Turn on the slice plane tool (Figure 6). Click and drag the planes in the 2D views (shown as red, green, and blue lines) so that the planes are centered on your area of interest.

2. Add your ROI. Go to the Tool Palette > ROI Tools and click on the cube (Figure 7).

3. The cube is red in the 3D view until you select it and it turns blue, indicating it can now be adjusted. Select the ROI you want to adjust by clicking on the pull-down menu to the right of the cube in the Tool Palette. To move the ROI, click and drag on the cube within the 3D view window.

4. To switch between the move/resize/rotate tools, press the Tab key (Figure 8A). Each time you press it, you will have a new adjustment tool option.

5. For more ROI options, double-click on the ROI cube in the 3D view and a Properties window will pop up (Figure 8B).

6. To access the ROIs in a table, click on “Measure 3D ROIs” in the Tool Palette. Then click on the “3D ROI Measurements” tab and choose the data type of “Source Voxels” (Figure 9). The measurement unit for 3D BLI is: photons/sec.

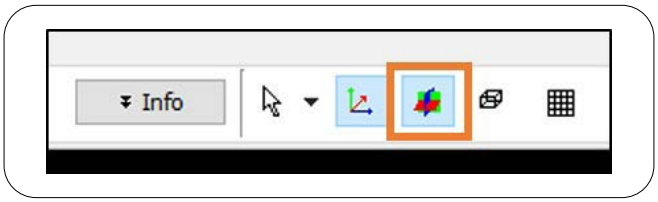


Figure 6: The slice plane tool allows you to center your 2D sagittal, coronal, and axial views to the region of interest.

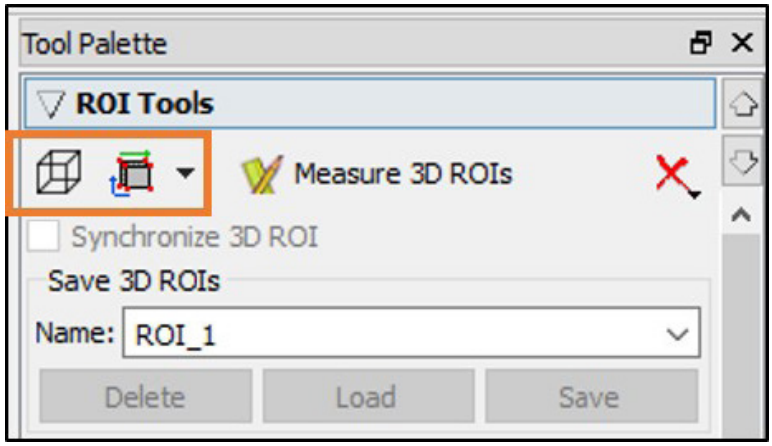


Figure 7: 3D ROIs are all cube-shaped. To add a new ROI, click the cube button in the Tool Palette. To adjust the position or size of an ROI, you must first select it by clicking on the pull-down menu to the right of the cube button.

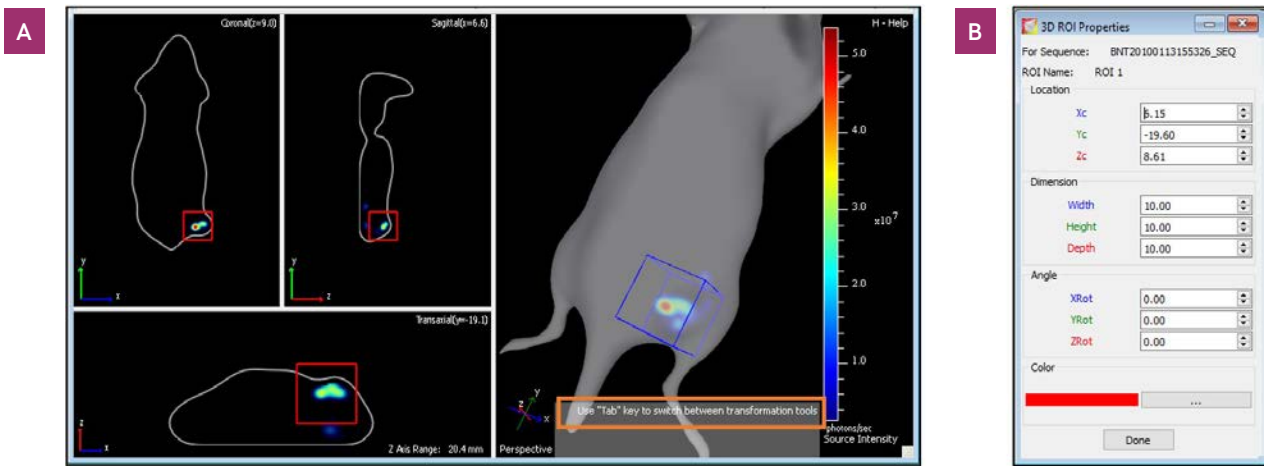


Figure 8: You can adjust the size and position of the 3D ROI using the (A) transformation tools and (B) 3D ROI Properties window.

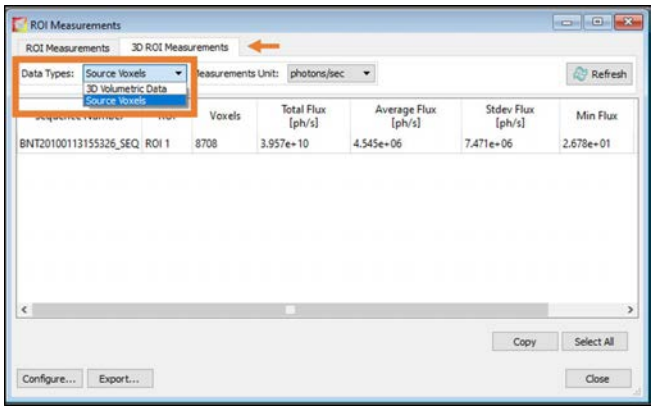


Figure 9: 3D ROI Measurements Table. To access the data, click on the "3D ROI Measurements" tab and select the data type of "Source Voxels." This table can be customized by clicking on "Configure." Clicking on "Export" allows you to save the data as an Excel-compatible .csv file.

Drawing ROIs for 3D FLI

Before you can begin analysis of 3D fluorescence images, you must first reconstruct them from a series of 2D scans. See the technical notes in the **Fluorescence Tomography** series - **Topography** and **Source Reconstruction and Analysis** - before proceeding.

The steps for drawing ROIs for 3D FLI are similar to those for 3D BLI in terms of adding the cube and moving/resizing it. To subtract background signal in a 3D fluorescence image, draw a second cube of equal size around an area considered background - this may be the contralateral side of the same animal or a separate control animal with no fluorescent reporter injected. You will have to manually subtract the background from the test ROI.

In the 3D ROI Measurements Table, click the 3D ROI Measurements tab and select the data type of “Source Voxels.” For quantification, 3D fluorescence measurements are reported as a fluorescence yield with units: pmol M^{-1} cm^{-1}.

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