Fluorescence tomography: Setup and sequence acquisition.

This tech note will discuss how to set up an imaging experiment in Living Image® for fluorescence tomography using IVIS® imaging systems.

Fluorescence tomography - also referred to as fluorescence imaging tomography (FLIT) - utilizes the data obtained from a 2D transillumination fluorescence sequence in combination with a surface topography to reconstruct a fluorescent source in a 3D space. Utilizing FLIT, you can determine the depth of sources in your animal and calculate the absolute intensity of that source at depth. You can also convert measured fluorescence from a reconstructed 3D scan into pmol of dye or number of cells.

Note: The Fluorescence tomography technical notes are meant to be used as a series. Please be familiar with the **Fluorescence tomography: Topography technical note** and **Fluorescence tomography: Source reconstruction and analysis technical note** before continuing.

Note about topography: Nude mice result in best FLIT reconstructions. Furred mice, especially symptomatically stressed furred mice, do not create superior topography maps due to the unevenness of the structured light images required to reconstruct the surface topography. Without the reconstructed animal surface, FLIT analysis cannot be performed. It may be necessary to remove the fur from the mouse body, either by shaving or applying a depilatory substance. Furthermore for FLIT, we will be utilizing transillumination points below and recording signal on the surface of the animal. For best results, please shave both sides of the animals that you will be using for these procedures. Additionally, it is very difficult to perform FLIT analysis on black furred animals even after shaving. The pigmentation in the skin does not allow the algorithms to distinguish between the subject and the stage.

Notes about setup: This guide will walk you through the steps of manually entering your sequences for the FLIT sequence. The Living Image software versions include an Autoexposure setting and an Imaging Wizard. It is highly recommended that the Imaging Wizard be used for Fluorescence Tomography setup. For questions on how to use these two features, please see the **Autoexposure Technical Note** and the **Imaging Wizard Technical Note.** These features are specifically designed for ease of use and to streamline sequence setup.

Automated setup (Recommended)

1. Confirm you have the proper hardware in the instrument to perform transillumination scans (the perforated plate, Figure 1). The rectangular plate for the IVIS Spectrum has "front" and "back" written on the bottom of the plate. Make sure it's oriented properly.
2. To begin, click on the Imaging Wizard in the acquisition control panel.
3. Select "Fluorescence" then "FLIT."
4. Select your reporter. The software automatically chooses the filters to use. Click "Next."
5. Select your imaging subject, acquisition parameters (Auto settings is recommended), and field of view. The "Normalized" box should be checked by default. Do not uncheck the box.
6. Click on "Transillumination Setup." The Transillumination Setup window will appear, where you can select transillumination points by clicking the purple dots corresponding to the location of interest (Figure 2). To select multiple points, left click and drag the mouse to select a grid. You can also hold down the CTRL key and click on each dot. For 3D images, we recommend selecting 8-15 points in a continuous region for a good reconstruction. Click "Next."

Note: The technical note: **Optical Imaging on the IVIS SpectrumCT System: General and Technical Considerations for 2D and 3D Imaging** provides additional information on area selection for 3D fluorescence tomography.

7. The acquisition control panel will populate with a sequence of images to be taken. Click "Acquire Sequence."

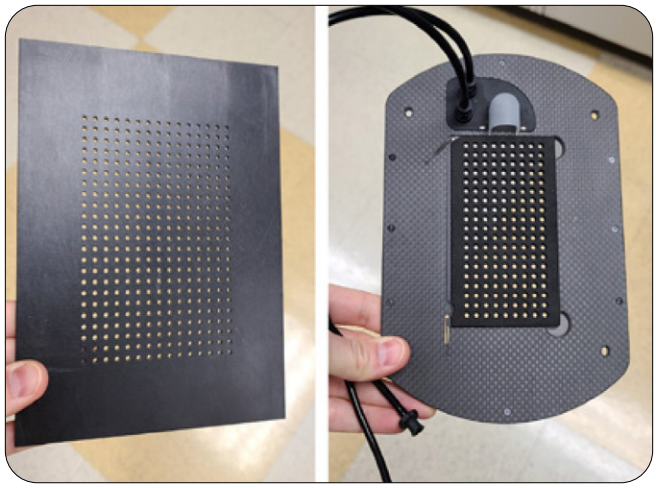

Figure 1: Transillumination plates for the IVIS Spectum (left) and SpectrumCT (right) instruments.

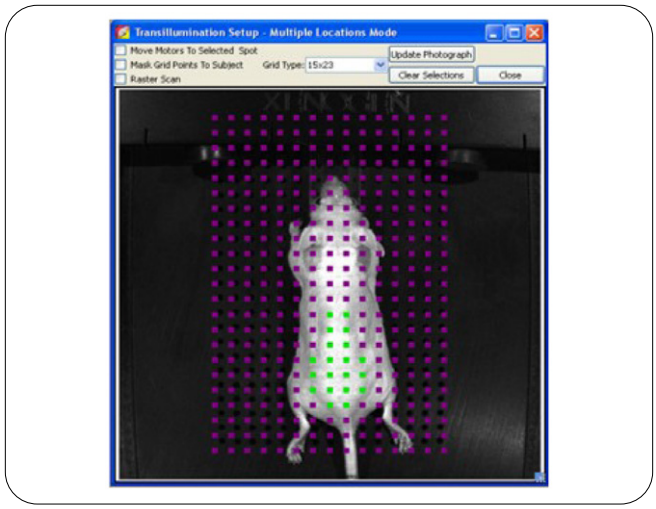

Figure 2: Transillumination setup. Click on the squares where you want to illuminate. The selected squares turn green.

Manual setup

*Confirm you have the proper hardware installed (Figure 1) before beginning.

1. Click "Sequence Setup" in the control panel to operate in sequence acquisition mode and the sequence editor window will open (Figure 3).
2. If necessary, click Remove and then select All to clear the table.
3. In the control panel, check "Fluorescent" then check "Transillumination" and specify the settings for the fluorescence image (exposure time, binning, F/stop, excitation filter, emission filter).

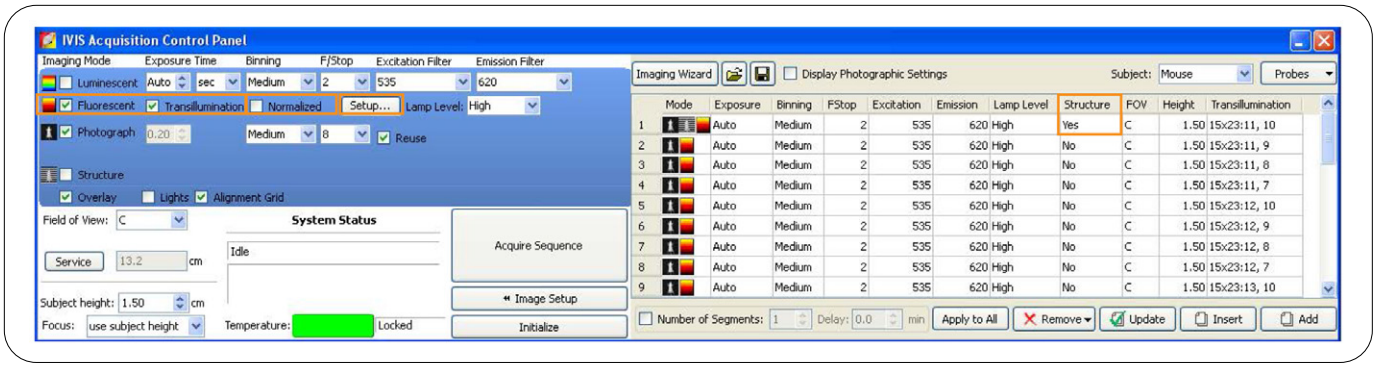

Figure 3: Manual Sequence Setup. Click on the "Sequence Setup" button and the control panel will expand.

Notes about setup: F/Stop should remain consistent for all fluorescent images for optimal 3D results. There is an option to unselect **Normalized** if the Normalized Transmission Fluorescence (NTF) option is not desired; however it is selected by default and is recommended. NTF is a method for subtracting nonspecific light leakage and allows imaging deeper into the animal with fewer artifacts. Please see the supplemental information in **Transillumination fluorescence: Normalized technical note** for more information.

Note: Auto exposure settings are available for Living Image 4 users. For information on this feature, please see the **Autoexposure Technical Note.** It is recommended to use this feature.

4. Specify the settings for the photographic image and make sure the **Reuse** box is checked.

5. In the Control Panel, click **Setup** and the **Transillumination Setup** window will appear.

6. The software will prompt for an update the photographic image, click **Yes** to acquire a new photograph.

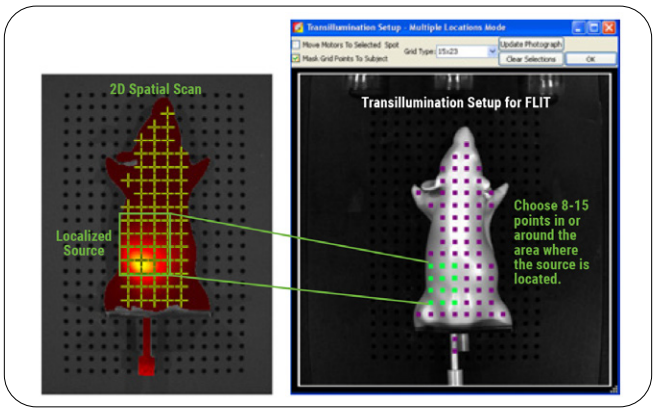

Figure 4: Taking a 2D raster transillumination scan of the whole animal (left) first can be helpful for selecting the proper points when setting up a 3D transillumination scan (right).

7. In the **Transillumination Setup** window, select 8-15 points immediately surrounding the source of interest (for more information on Transillumination Sequence Setup see the **Transillumination fluorescence: Setup technical note)**. To choose these points with the most precision, the first step is to determine where your sources are located in the subject. This is accomplished with a 2D transillumination spatial scan of the animal (Figure 4). **Raster scanning cannot be used in conjunction with FLIT as it is required spatial information that is only provided when images are acquired using multiple excitation points underneath the animal.** Therefore, scan times will increase dramatically as an image will be acquired at each point selected. If this were not the case, we could simply image using every point in the animal as with a 2D transillumination spatial scan but this would be very time consuming. Use the acquired spatial scan to select points in or around the immediate area where the source is located.

8. In the sequence editor, click **Add** while the Transillumination Setup window is open and the acquisition parameters will be added to the table. Each row will represent one transillumination point selected in the setup window. An image will be acquired for each point selected.

9. If using the IVIS Spectrum, one structured light image is required to reconstruct the surface of the animal. In the sequence editor window under the column labeled as **Structure**, click in the cell corresponding to row one and a Yes/No box will appear. Check this box to acquire your structured light image with image one in the sequence (Figure 5).

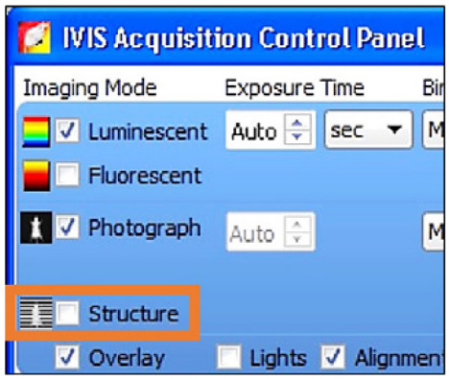

Figure 5: Check the structure button to ensure a structured light image is taken. This image is required to perform 3D reconstructions.

Note: The structured light image will automatically be assigned when using the **Imaging Wizard.**

10. To acquire the images, click **Acquire Sequence** in the control panel.

Note: During image acquisition, the **Acquire Sequence** button becomes a **Stop** button. To cancel the acquisition, click **Stop**.

11. Once image acquisition is complete, confirm that the signal is within proper limits – between 600 - 60,000 counts (Figure 6).

12. The transillumination locations can be shown/hidden on the images in the sequence by checking the tab at the top of the image.

13. Switch to calibrated units – **NTF Efficiency** – in the **Units** drop down at the top of the image.

14. The **Overview** button can be used to show a cumulative view of the diffusion pattern acquired during the sequence (Figure 7).

15. At this point, the data can be saved until a later point in time or the procedure can be continued by following the steps in the **Fluorescence tomography: topography technical note.**

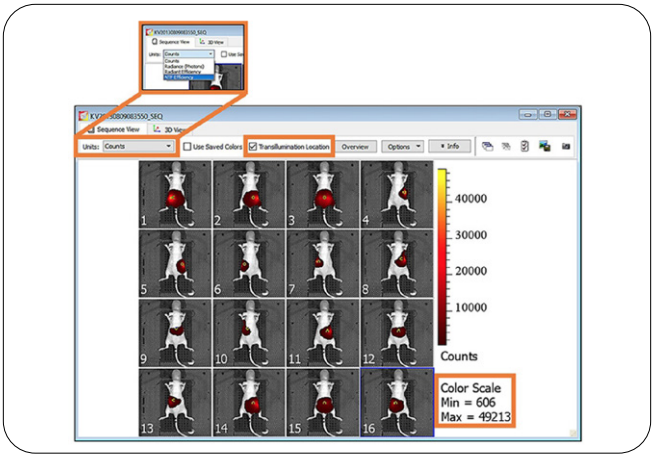

Figure 6: Acquisition results from a fluorescence tomography scan (before reconstruction). Click the "Transillumination Location" button to show where the transilluminator was for each image. Check counts to ensure they are within the linear range of the camera (600-60,000 counts). If they are out of range, you need to reacquire the image with different sensitivity settings. To quantify ROIs in the 2D images, change the units to NTF Efficiency.

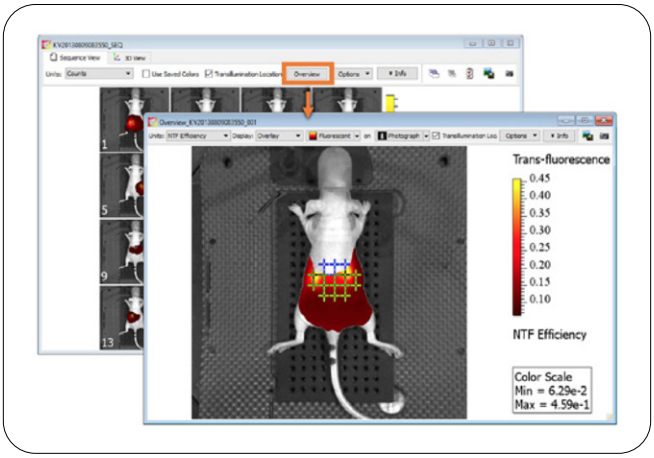

Figure 7: Clicking the "Overview" button opens a window that shows the cumulative diffusion pattern and transillumination points utilized.

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