

### 5.2.18 Image Optimization

This section describes an example how to acquire an image, using an excitation wavelength of 488nm and a fluorescence emission range above 510 nm. We presume that no predefined configuration is available for this imaging task and that Smart Setup is not used to do this task.


The description applies to the use of any microscope.

#### 5.2.18.1 Requirements

- Click on the **Locate** tab on the **Left Tool Area** and set the microscope according to your needs.
- Position the specimen under the objective and focus onto the desired imaging plane.
- Click **Acquisition** tab in the **Left Tool Area** toolbar.

Click on **Show all tools** to display the tools in the tool group **Setup Manager**.

- Open the **Light Path** tool. Choose **Channel** mode for the image acquisition (Fig. 98).
- Click on the laser icon, select the 488 line, and set the transmission slider to about 2 %. Make sure other lasers are not checked (Fig. 99).

 Optimize the laser intensity subsequently via the transmission slider when imaging.

- Set the **VSD** to the position 500 nm and activate PMT 2 via the check box. You may want to assign a different color to the channel which will be taken over for the initial display of the image. Click the LUT part of the list to open the color table and select the desired color (Fig. 100).
- Click on the **Dye** pull down list and select the desired dye (e.g. FITC). This will display the emission spectrum of the desired dye in the upper part of the light path.
- If required, deactivate all the other channels (Ch1, transmission, R1-2) via the check boxes.

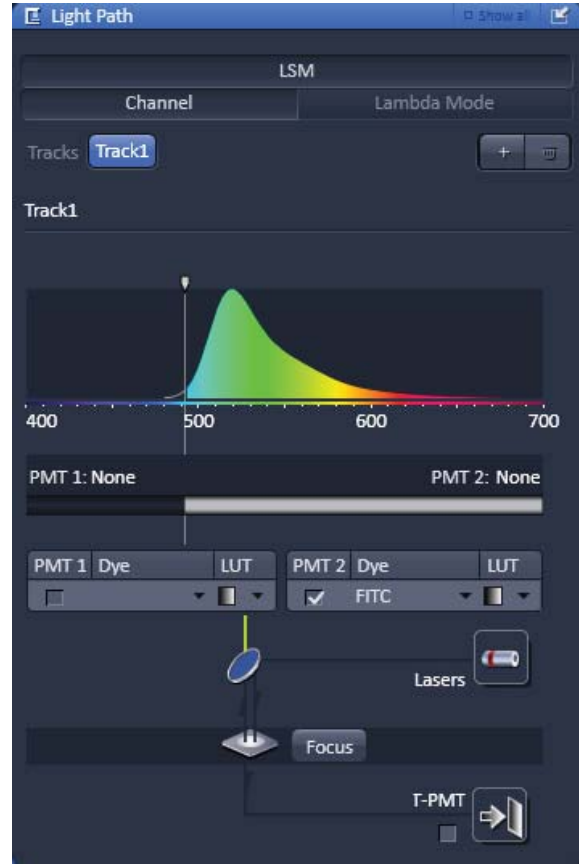


Fig. 98 Light Path tool

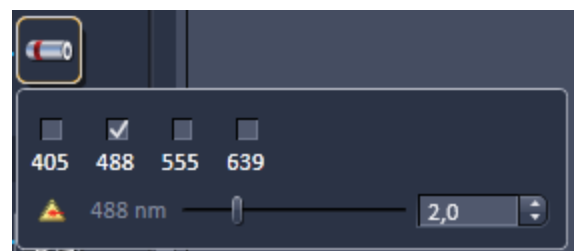


Fig. 99 Laser line and laser power selection

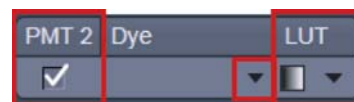
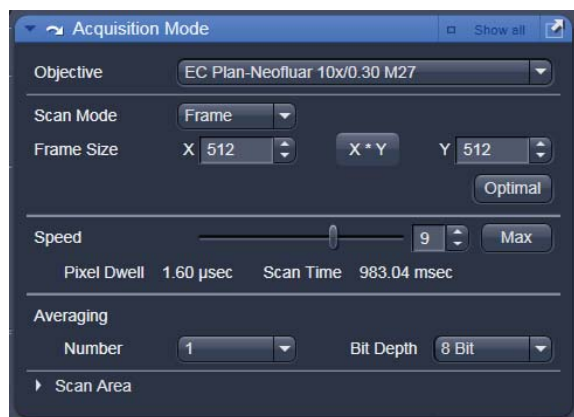



Fig. 100 Channel activation and color selection



**Fig. 101 Acquisition Mode tool**

- Start with the following settings on the **Pixel Depth, Scan Direction & Scan Average** panel:  
Data depth: 8 bits  
Average: Number: 1 (no averaging)

 The default settings for Acquisition Mode are suitable for good image quality and do not need to be changed for initial scanning.

### 5.2.18.2 Defining the Scan Parameters

- Open the **Acquisition Mode** tool (Fig. 101).
- Select **Frame** mode and a **Frame Size** of 512 x 512 to start with.
- The **Optimal** button automatically adjusts the frame size to match the magnification and resolution of the objective. Thus, images are acquired without collecting redundant image information.
- The Scan Speed is set to 9 per default. A lower speed produces better images as the light collection per pixel is higher. Use a speed from 7 to 9 to begin with.




**Fig. 102 Channels tool**

### 5.2.18.3 Defining the Detection Parameters

- Activate the **Channels** tool (Fig. 102).
- Set the **Pinhole** to two Airy Units using the slider. The value for the pinhole is displayed in the tooltip.
  - The pinhole diameter should be so small that there is still enough variation for the setting of the **Gain (Master)** and that sufficient image information is still available. 1 Airy is a good value to enable a confocal fluorescence XY-image to be obtained.
  - A small pinhole diameter will increase the depth of focus, but reduce the light intensity received by the PMT photomultiplier (for reflection mode confocal images start with a pinhole value of 0.3 Airy Units).
- Use the **Set Exposure** button.
  - The system determines the settings for the **Gain (Master)** and **Digital Offset** to match the scan speed, pinhole size and laser power.
  - Finally, the system builds up the image.

#### 5.2.18.4 How to Optimize Image Quality

As a rule, the first scanned image is not ideal, since the settings of the laser and therefore also the photomultiplier might not match the light output. More often than not, the screen image is dull and needs subsequent optimization.

- Use the lookup table **Range Indicator** (see section **Dimensions**). This display mode shows overexposed regions in red and underexposed regions in blue. Some red and some blue pixels should be visible to ensure the signal is spread over the dynamic range of the detector and no image information is lost.
  - Start a **Continuous** scan and quickly adjust the laser power, **Gain (Master)** and **Digital Offset** to set the image into the dynamic range.
  - Try to lower the impact of laser light onto the sample. Reduce the laser power and adjust the gain accordingly.
  - To adjust the black level (background), use the **Digital Offset** slider so that areas without picture content just show a trace of blue.
  - If necessary, re-amplify brightness with the **Digital Gain** slider.
-  Do not change the **Digital Gain** setting unless the settings made so far are insufficient for image optimization.
- Stop scanning.
  - Save the imaging configuration again as this time it will also save the correct settings for the detector, the laser, and the pinhole.

The signal-to-noise ratio can be substantially improved by reducing the scan speed to an acceptable level and averaging over several scans (i.e. with an average **Number** greater than 1 for the **Mean** average **Method** in the **Scan Control** window).


- Use the **Speed** slider in the **Acquisition Mode** tool set the slowest acceptable scan speed.
- In the **Number** text box of the **Averaging** panel enter the number of images to be averaged.



**Fig. 103** Image displaying bad adjustment of gain and offset (blue regions are underexposed, red regions are overexposed)



**Fig. 104** Image displaying good adjustment of gain and offset

-  Image optimization can be achieved faster if you select a smaller frame, since less data have to be processed.  
The greater the number of averages selected using **Mean** as average **Method**, the better the image quality will be; the scan time is prolonged accordingly.

A similar procedure applies for image optimization if more than one channel is used and simultaneous image acquisition is performed.

Make sure the suitable lasers are switched on and the channels are activated.

Pay attention to address the right channel when **Pinhole** size, **Gain (Master)**, and **Digital Offset** are controlled as these are set individually for each channel.

Use the split view (see section **Split View**) to see the effect of the controls onto each channel separately.

If the acquisition is performed sequentially in with multiple tracks it is faster to activate only one track at a time for image optimization.