Reflections of the key LASER lines on your confocal laser scanning microscope (CLSM) will be used to test the wavelength accuracy of your spectral detector. A mirror slide will be provided for this standard test.

1   Put a 10x magnification objective lens in place (or other low magnification lens).

2   Place the mirror slide on the stage with the coverslip facing the objective. Focus first on the edge of the mirror, either via the microscope or directly on the confocal.

3   Choose the **Lambda Mode** option found under the XY scan button.

**4**   Select the light path and dye button to view optical path.
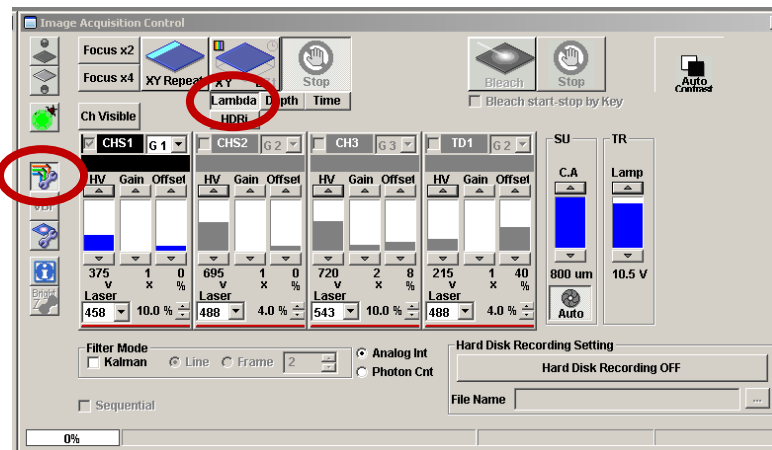
5   Select all of the laser lines available above 405 nm. In the **Light Path & Dyes** window choose the 20/80 mirror (**BS 20/80**).
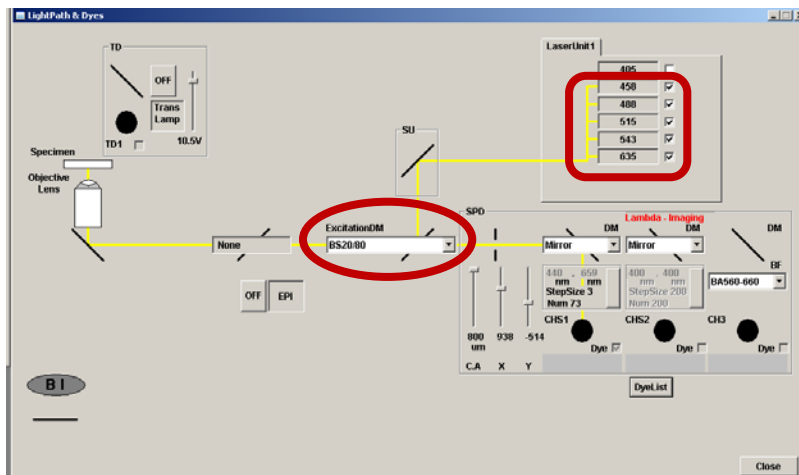
6 Under the **Acquisition Setting** window set the scan speed to 20 μs/pixel, the **size** of the image to 128 x 128, the **Zoom** setting to 2. The bit depth will set to 12-Bit by default.

Parameter	Setting
Frame Size	128x128
Scan Speed	20 μs/pix
Frame Averaging	1
Zoom Factor	2.0
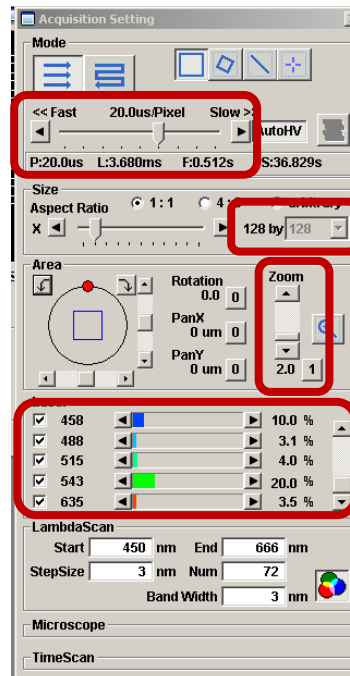
7 In the **Lambda Scan** window set the wavelengths to cover the range of lasers you will be using.

8 Set the spectral resolution to 3 nm.

9 Also in the acquisition setting window, under the "**Image Acquisition control**" menu set the parameters as follows (slight adjustments may need to be made):

Parameter	Setting
Pinhole	Optimal or 1 Airy Unit
HV Gain	300
Digital Offset	10
Digital Gain	0.0

10 Set the laser power for each laser line to give a signal of about 75% maximum (2000-3000 for 12 bit images).

11 Make sure using the range indicator LUT that you are not getting any saturated pixels (red) within any of the Lamda stack images.

12 Collect a Lamda stack of images by pressing the XY_L button. You may get interference patterns in the images from the laser reflections. This is normal. Notice how the periodicity of the interference pattern gets larger as you move to longer wavelength lasers.

2
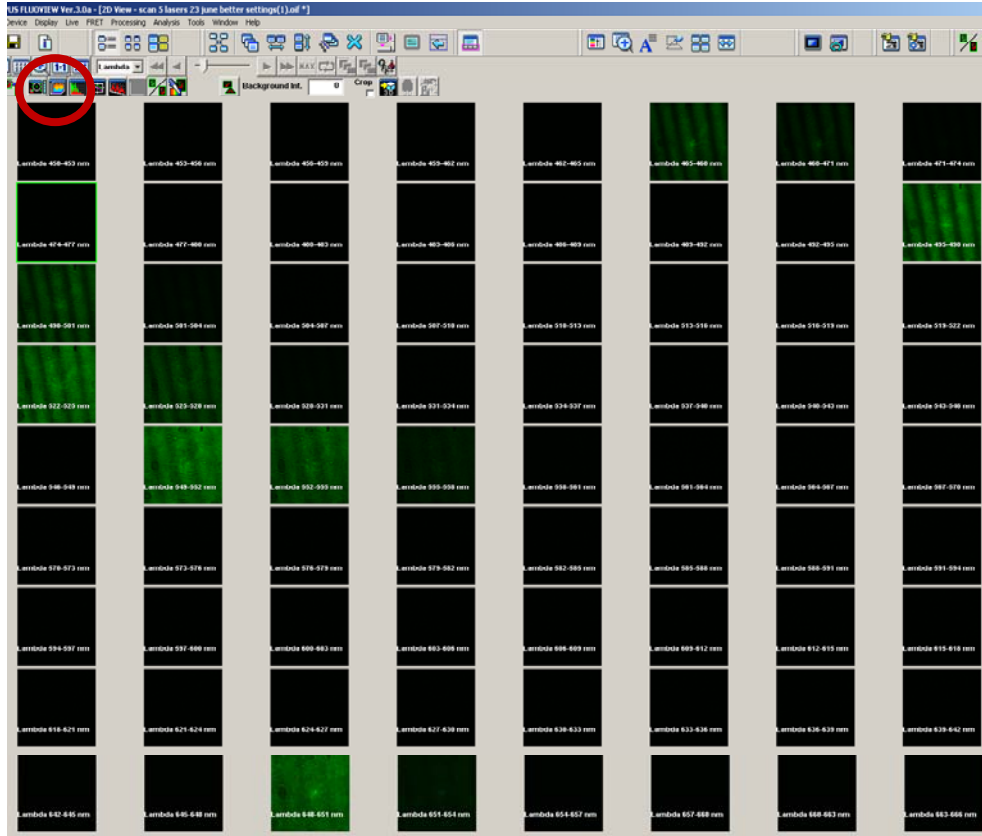
13 Select an ROI on the data set corresponding to the whole frame.

14 Select 'series analysis' button on top of the image. The spectra for the entire 128x128 images of the Lambda stack should appear. Verify that the laser peaks are falling within 2-3 nm of the expected values. In this example, there is a shift of 6-7 nm for each laser and a shift of 15 nm for the 633 line. In this case, the system needs to be recalibrated by a qualified technician.
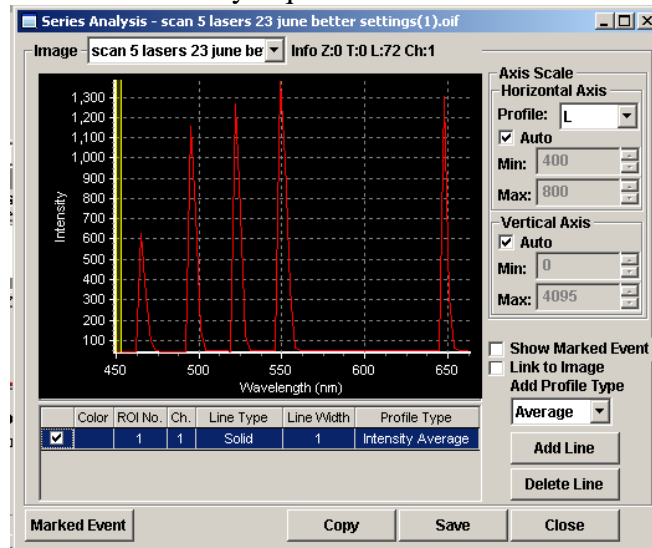
15 The text values for the spectra can be saved as an .xls or Excel format in order to determine more precisely the wavelengths for the peak values and the FWHM of the peaks in order to determine the spectral resolution of the system.

16 Name the file with your name and the name of the confocal platform you collected the data on. Send the text file (.txt or .xls) to the ABRF-LMRG at abrf.lmrg@gmail.com.