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** under the Light Path menu.

4. Put an 80/20 mirror (**T80/R20**) into the beam path.

5. If using the Zeiss 710 check the **Reflection** box. This will remove the laser blocking filters from the light path.

6. Set the wavelengths on Channel S (ChS) to cover the range of lasers you will be using.

7. Set the spectral resolution to the smallest setting (3.3 nm on our system).

8. Do not select the 405 nm LASER because the detector does not measure wavelengths below about 420 nm.
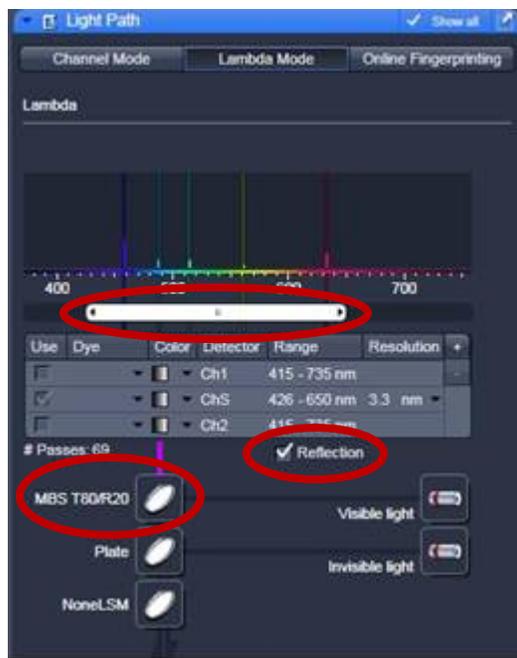
9. Under the **Acquisition Mode** menu set the parameters to the following:

| Parameter	Setting
Frame Size	128x128
Scan Speed	7
Frame Averaging	1
Bit Depth	12 Bit
Zoom Factor	1.0
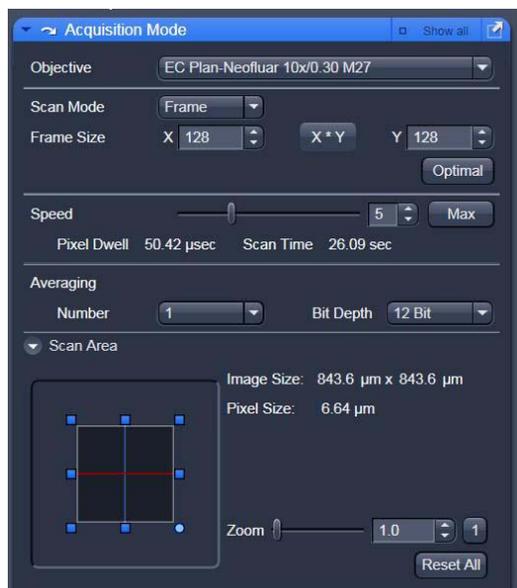
10. Under the **Channels** menu set the parameters as follows (slight adjustments may need to be made):

Parameter	Setting
Pinhole	1-2 Airy Units
PMT Gain	200-400
Digital Offset	20
Digital Gain	1.0

11. Set the laser power for each laser line to give an intensity signal of 2000-3000 for a 12-bit image (150-200 for an 8-bit image).

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

13. Collect a Lamda stack of images. You may or may not get stripped interference patterns in the images from laser reflections within the system. If you do this is normal and it will not affect the measurements. Notice how the periodicity of the interference pattern gets larger as you move to longer wavelength lasers.
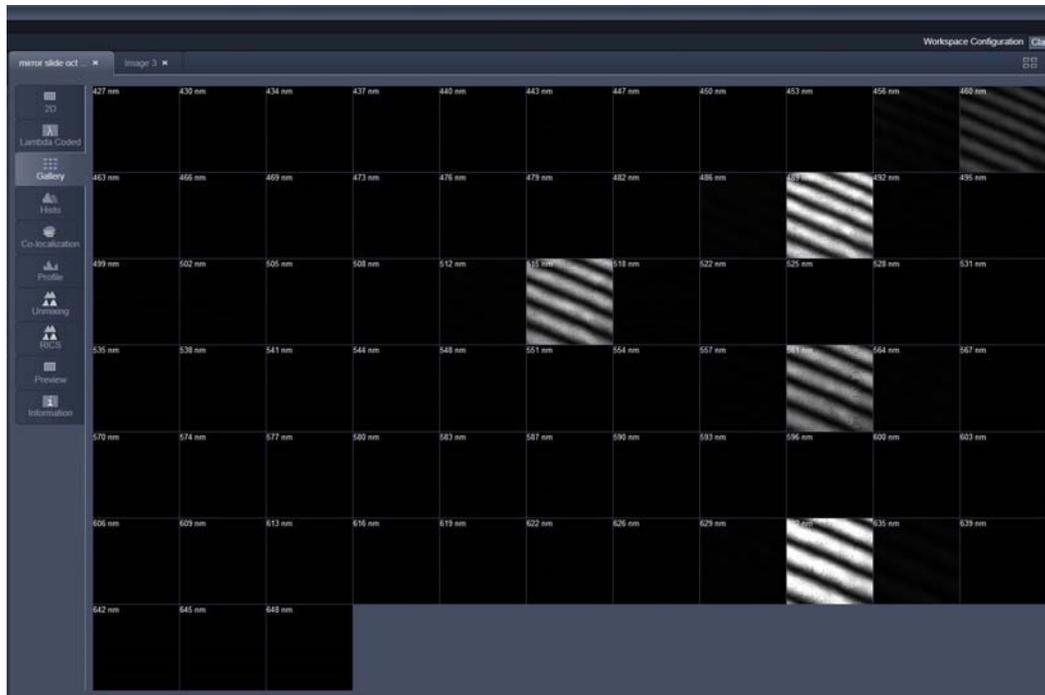
14. Note: if the wavelengths are not displayed on the images in the gallery view: Go to the **Gallery** tab and check **Show Text** in the **Transparent** drop-down menu**.**

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15. Go to the **Unmixing** tab on the data set. 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.

16. To save the text data, right click over the table and click **save data.** The text values for the spectra can be saved as a text file and imported into Excel 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. **NOTE: Depending on your instrument settings you may or may not see these interference stripes.**
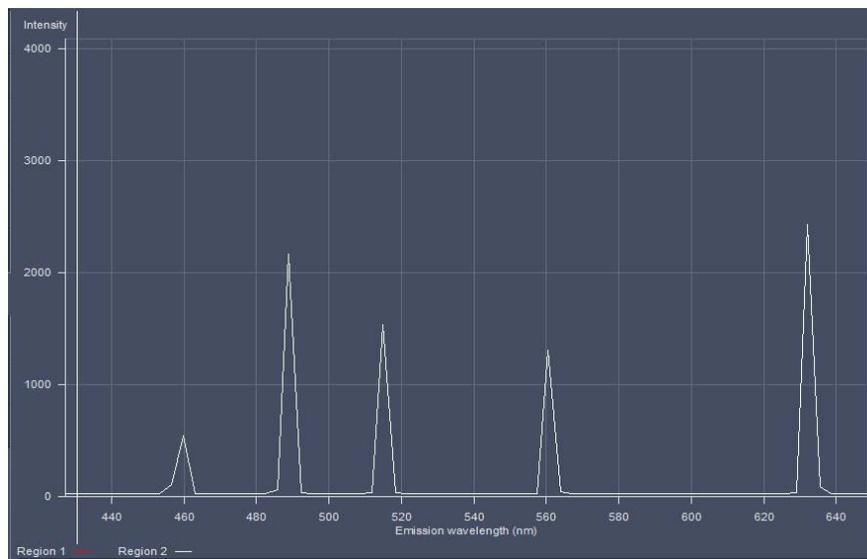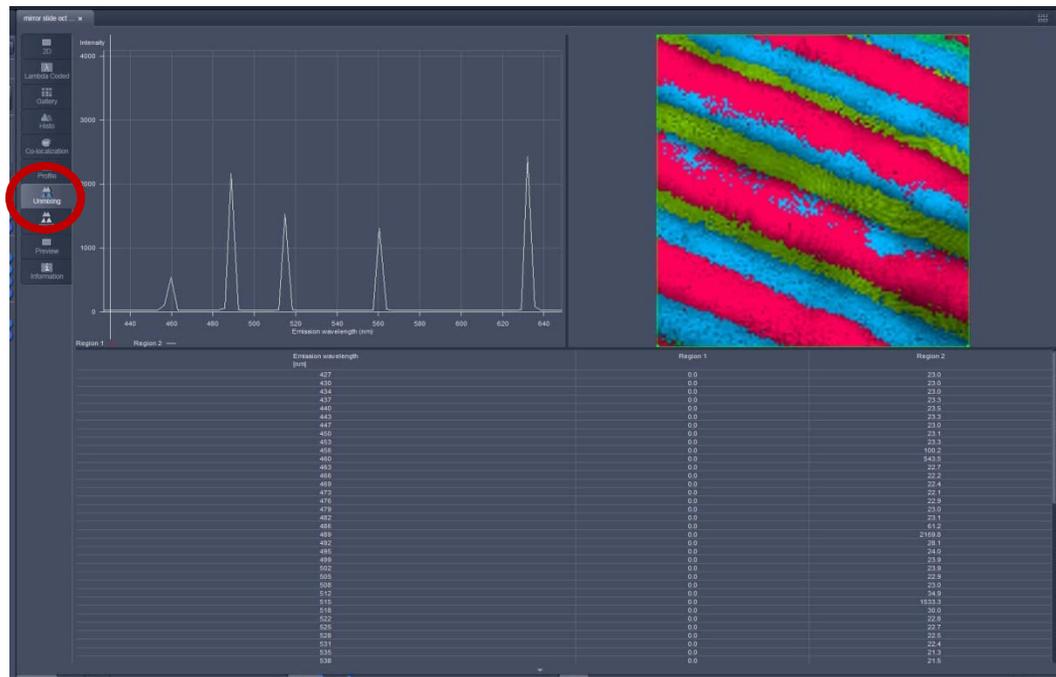
17. Name the file with your name and the name of the confocal platform you collected the data on. Send the text file (.txt or .xls) to the ABRF-LMRG at abrf.lmrg@gmail.com.