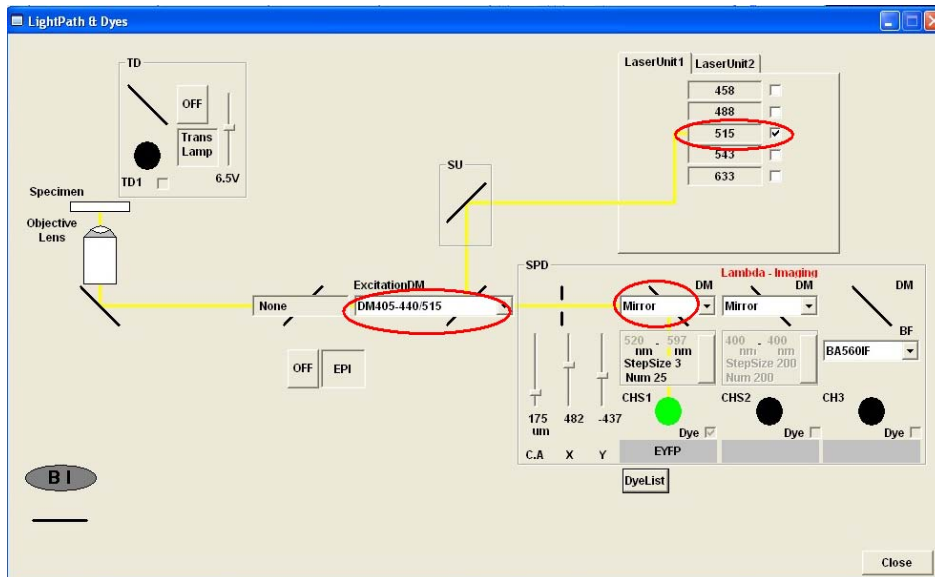
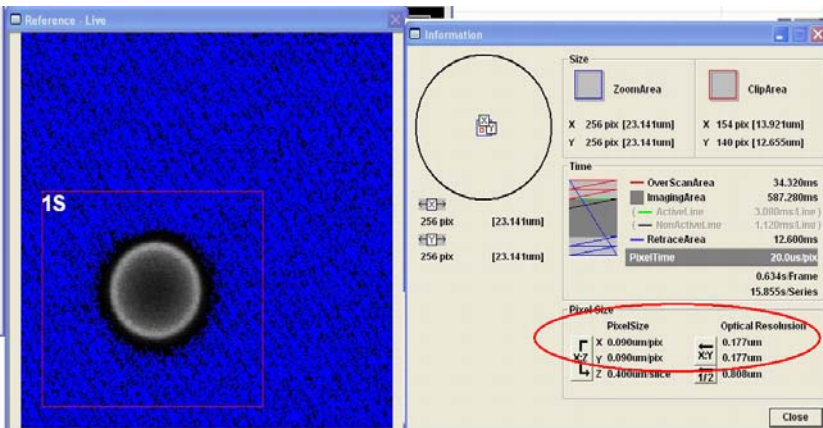


Spectral deconvolution (unmixing) protocol – Olympus FV1000

- 1) Start or restart the confocal software.
- 2) To ensure proper automatic calculation of the confocal aperture size corresponding to 1 Airy, select a dye such as EYFP from the dye list (the system will also pick the 515 nm laser for you, which is needed for this procedure). Check the instrument settings (click the LightPath button):

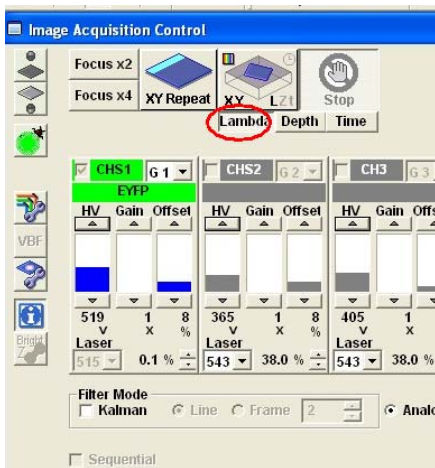


- 3) **Locate the fluorescent beads.** For uprights, make sure the DIC prism below the objective is out of the optical path. Locate the fluorescent beads as usual, using a low magnification objective, then switch to a high-NA objective, such as 60x/1.2 water/ 1.4 oil immersion or higher. Focus on



the center of the bead (the bead image will have maximum diameter) and set the zoom and Size (number of pixels) to optimum resolution (pixel size is approximately $\frac{1}{2}$ of the optical resolution). You can use the ClipScan feature to scan a

smaller rectangular area that contains the bead without affecting image sampling .



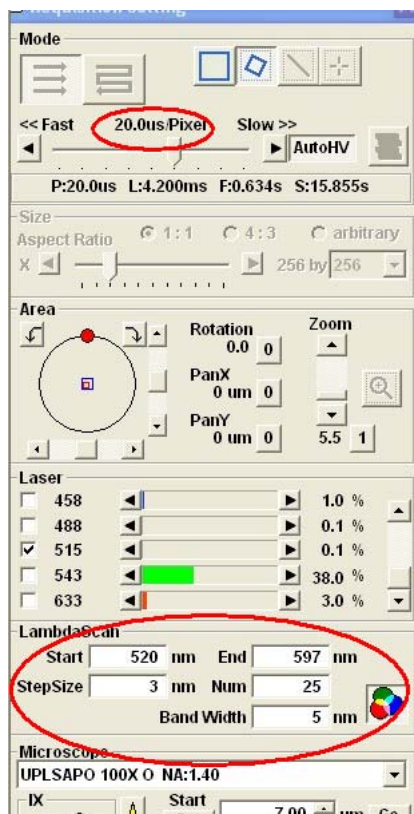
- 4) **Lambda settings:** Activate the “Lambda” button in the Acquisition control and adjust detection parameters:

Offset 8

Gain 1

HV about 500

515 nm laser power 0.5% or less



Set scanning speed to 20 μ s/pixel

Set the lambda scan: Start 520nm, End 597nm, Step Size 3 nm,

Band Width 5nm

- 5) **Intensity settings:** Start Live View (XY Repeat). Switch to HiLo Lookup table (Ctrl-H on keyboard) to highlight saturated pixels. Using the Spectral settings window (the rainbow-colored button in the bottom right of LambdaScan section) move the detection wavelength between 520 and 595, making sure the Band Width remains unchanged (5 nm) and adjust the HV and laser power so that there is no saturation (no red pixels) at any spectral position, especially at the peak. The maximum signal should be about 3000 in the brightest frame. Offset can be adjusted to reduce background outside the bead.



- 6) Stop live view, **acquire the lambda series.**

- 7) **Unmixing:**

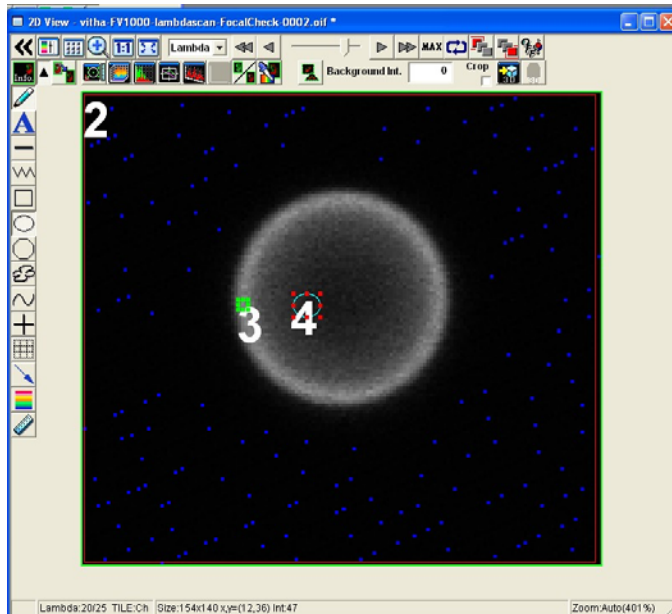
Menu commands “Processing” – “Spectral Deconvolution”

8A) Unmixing using Regions of Interest (ROIs) which is of interest here

For this method, some parts of the specimen have to contain only one or the other dye, but not both, which is the case here.

Draw a ROI in the spectral image series that only contains dye #1 (ROI 3 in the outer shell of the bead)

Draw another ROI that only contains dye #2 (ROI 4 in the core of the bead). ROI 2 in the whole image generated when using the clipping option; it is not important here and does not interfere.

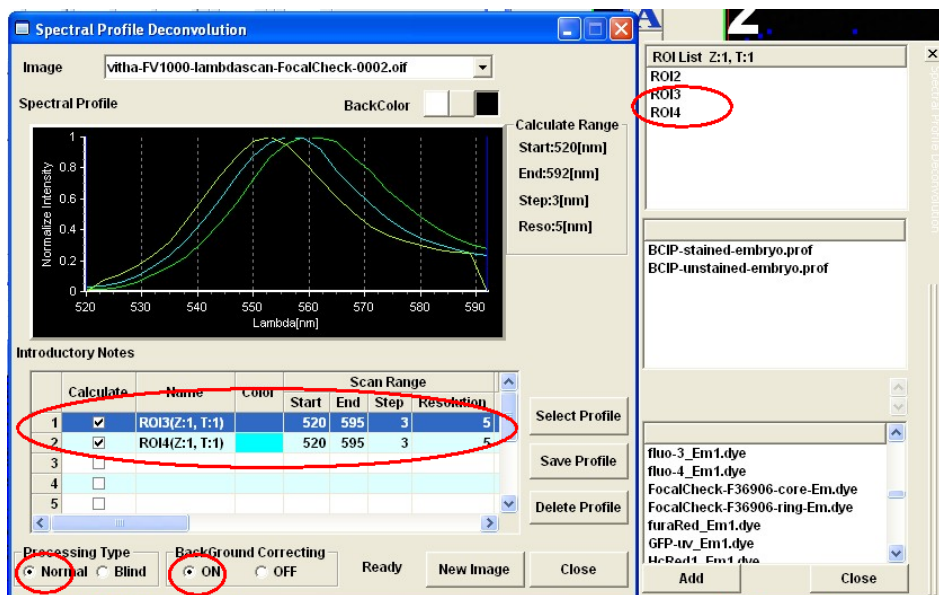


In the Spectral Profile Deconvolution window, select the ROI3 and ROI4 from the ROI list in the table on the right and click “Add” or double click on each.

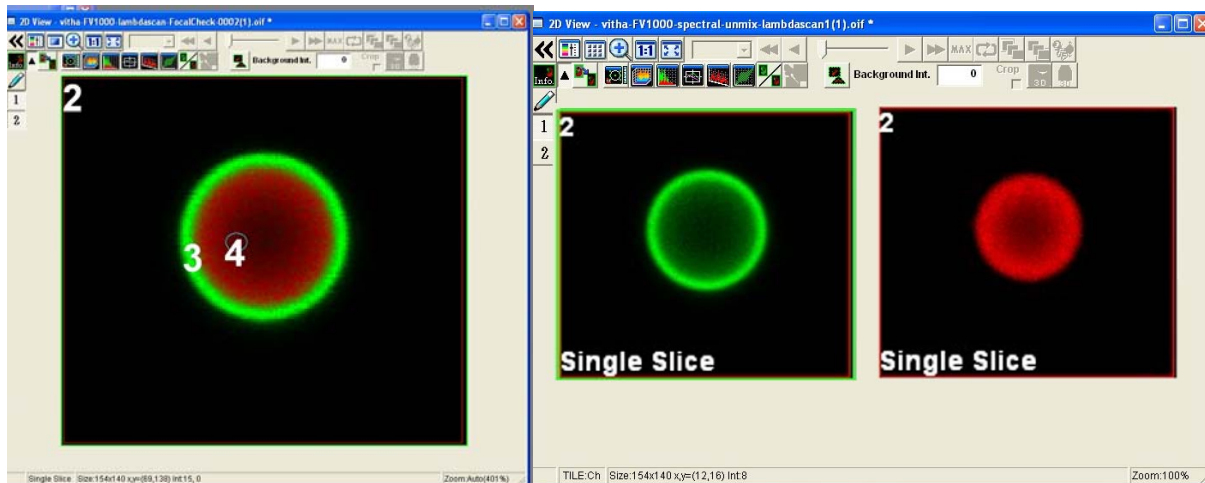
The two ROIs are now listed in the introductory notes below the graph, “Calculate” for both is checked.

Their spectral profiles, measured from the lambda series, are shown.

Select Processing Type Normal, as it is not purely blind, Background Correcting ON.



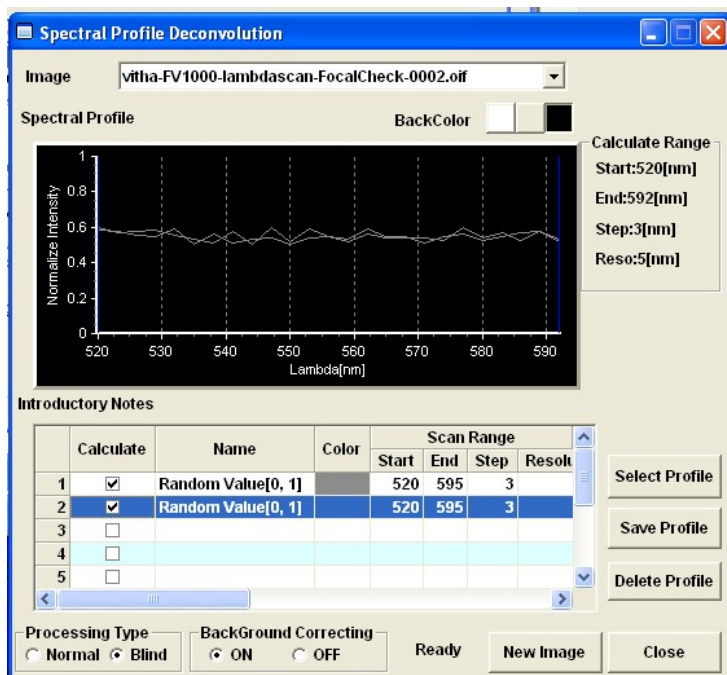
Click **New Image**. A spectrally unmixed image will be created:



8C) Blind Unmixing : this can be a little more difficult to obtain, and can vary from beads to beads.

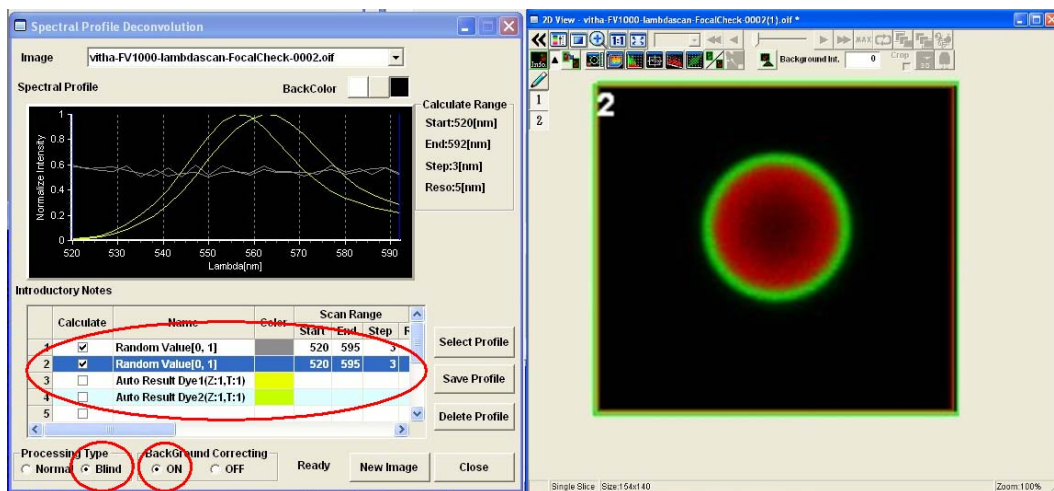
This method does not require reference spectra or ROIs containing the single dyes.

Check two “calculate” boxes in the “Introductory Notes” section. A random spectrum is calculated.



Select Processing Type: Blind, Background Subtraction: ON. Click “New Image”.

The new dye profiles are shown and a spectrally unmixed image is created:



Great thanks to Stanislav Vitha for this protocol.

9) Send the following information to the ABRF-LMRG at abrf.lmrg@gmail.com:

- Sample image as above of the separated data in 2D or 3D.
 - Image Z-stack of a single bead from the unmixed data stack
- Please indicate the following

- objective used
- pixel size
- laser power
- platform the images were collected on
- software and the method used for the un-mixing.