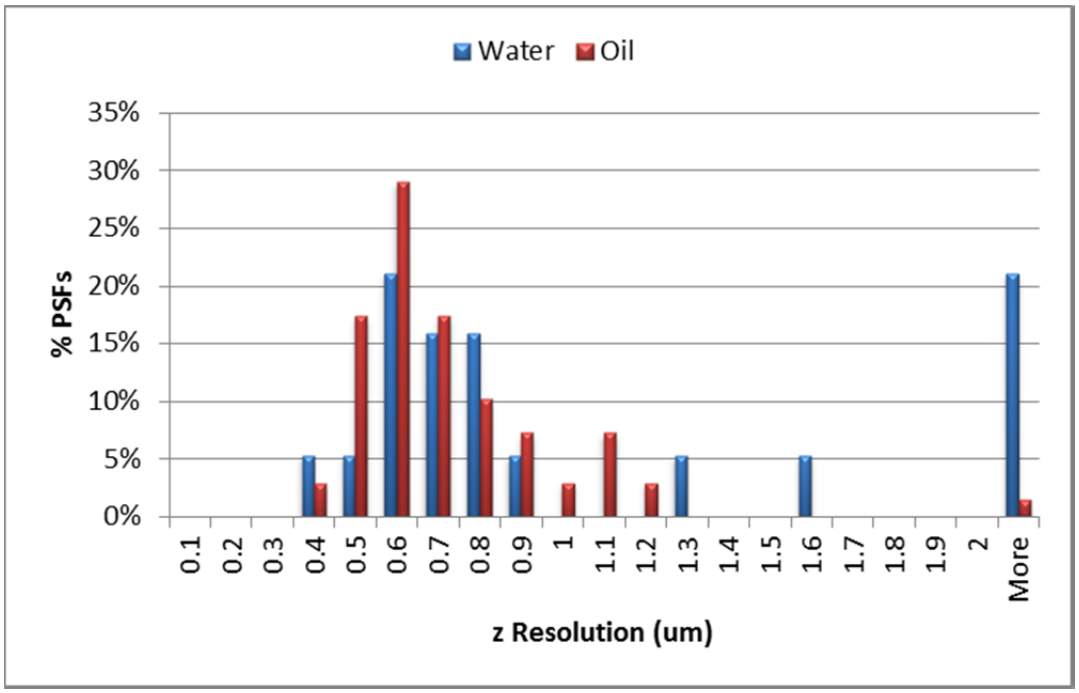
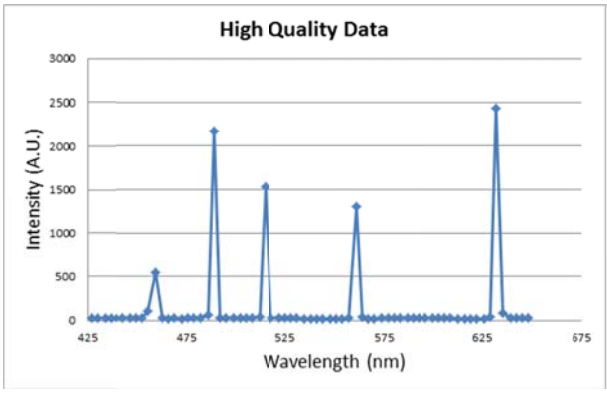
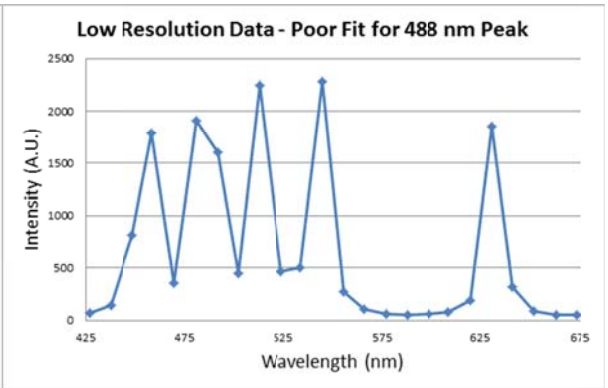


Supplemental Figure 1: Histogram of 63x Objective Lens z-axis Calculated Resolutions. Results from the MetroloJ z-axis fits for 5 beads from each lens with a 1 Airy unit pinhole setting. Many water lenses without correct correction collar adjustments show poor z-axis resolution relative to oil immersion lenses. This is evident from the large number of PSFs showing a z-axis resolution greater than 2 μm. The expected z-axis resolution was 460 nm (1.4 NA Oil Immersion) and 415 nm (1.3 NA Water Immersion) based on Equation 2 in the manuscript.

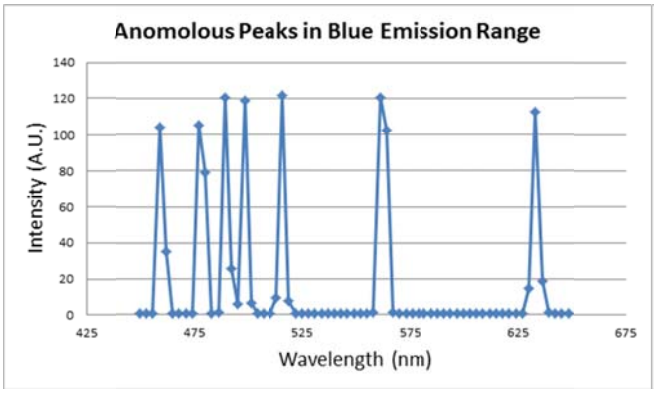
A



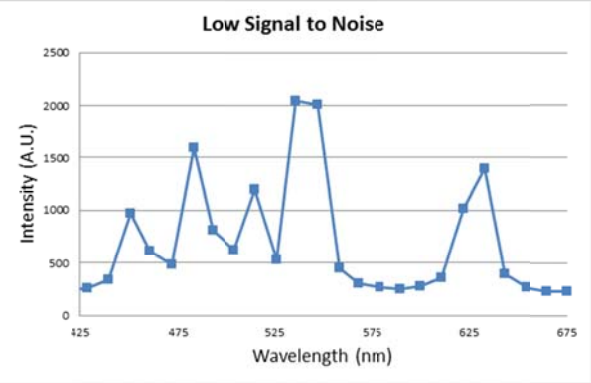
B



C

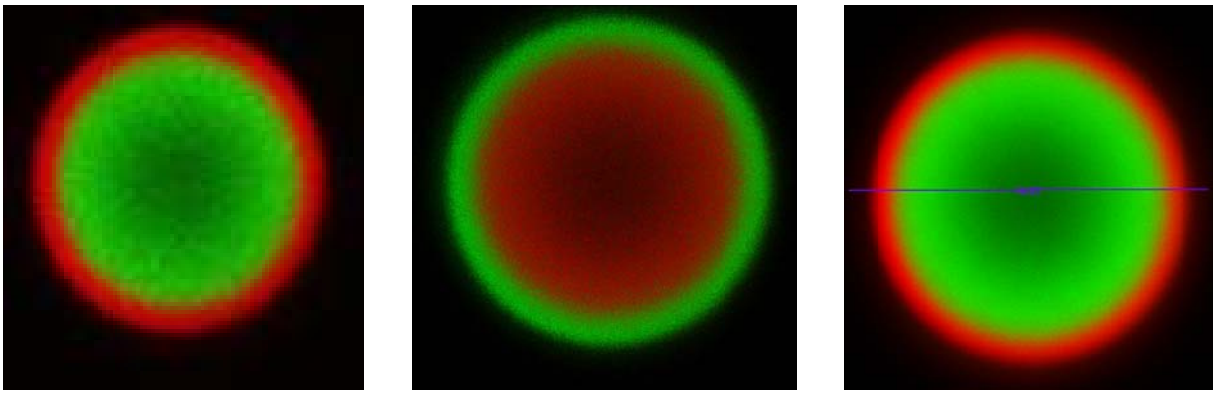


D

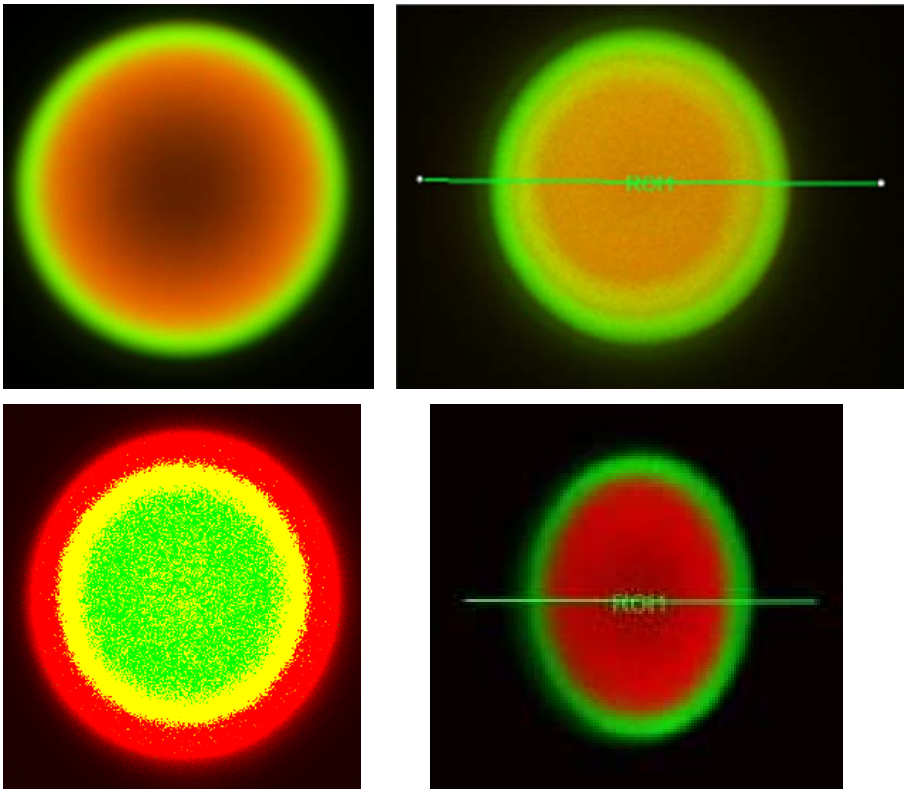


Supplemental Figure 2: High and low quality spectral accuracy data. Results from various instruments showing representative data of high quality spectral accuracy in A, low resolution data that made it difficult to fit the laser reflection peak at 488 nm in B, appearance of anomalous peaks in the emission spectra in C and low signal to noise data in D.

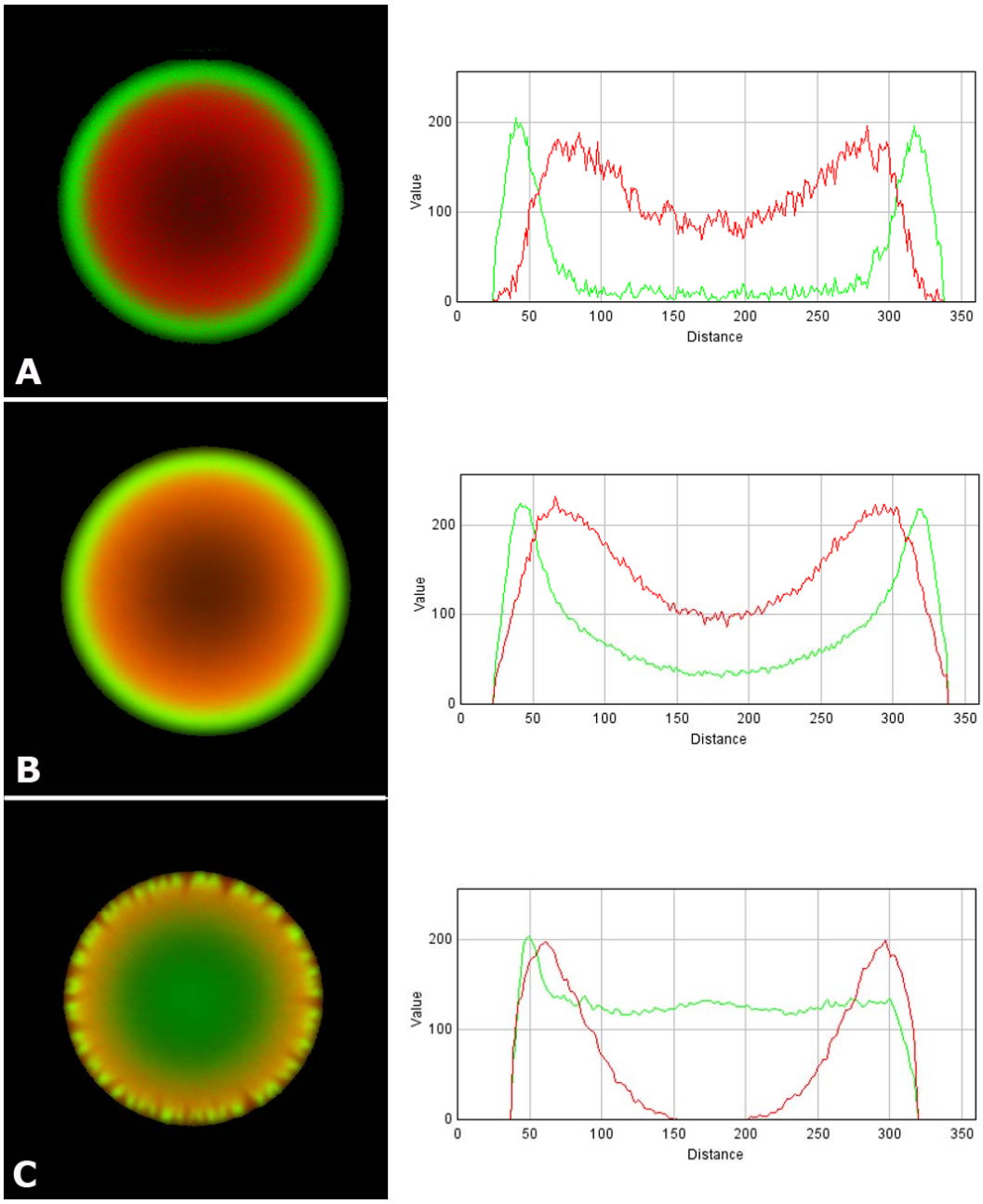
A



B



Supplemental Figure 3: High quality and Low quality spectral separation data. Results from various instruments showing high quality double orange bead spectral separation in A. In B, poor separation was seen due to the algorithm showing both dyes in the interior of the bead in the first two examples, fluorescence detector saturation, and distortion in the x-y image data collection. Beads are 6 μm in diameter.



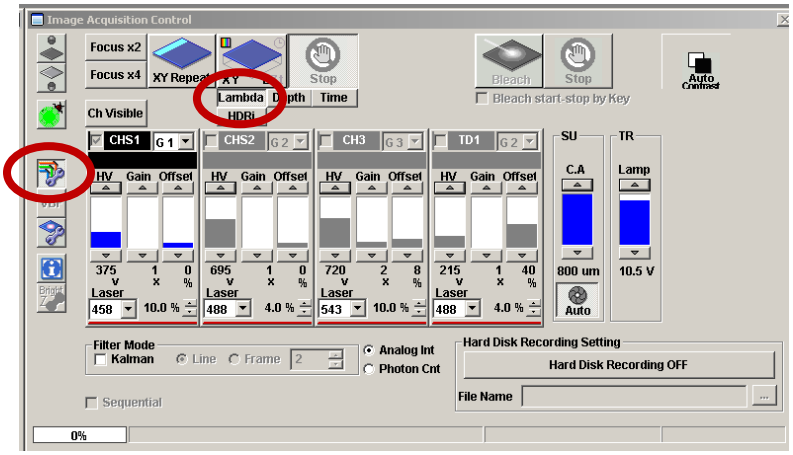
Supplemental Figure 4: Examples of data showing high, medium and low spectral separation quality scores. Representative spectrally separated bead images and corresponding line graph (center of the bead), demonstrating the broad range in separation quality submitted by respondents. The bead in panel A, demonstrates high quality separation, i.e. clear separation of the bead and outer ring and no saturated pixels. This "type of bead image" received a score of 5. The bead in panel B, demonstrates moderate quality separation, i.e. less separation of the bead and outer ring then in (A) and no saturated pixels, the "type of bead image" received a score of 3. The bead in panel C, demonstrates poor quality separation, i.e. improper separation of the bead and outer ring, the "type of bead image" received a score of 1.

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.

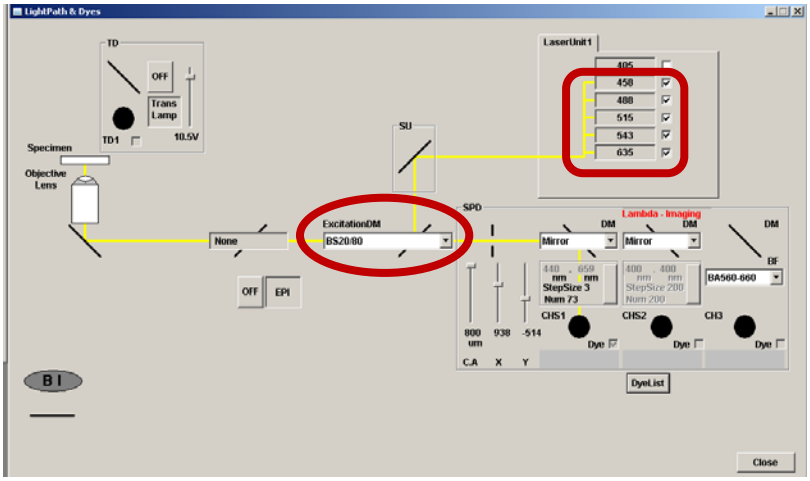
1 Put a 10x magnification objective lens in place (or other low magnification lens).

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.

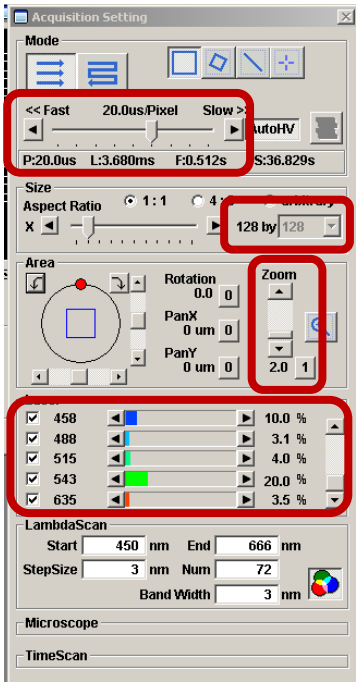
3 Choose the **Lambda Mode** option found under the XY scan button.
4 Select the light path and dye button to view optical path.



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.

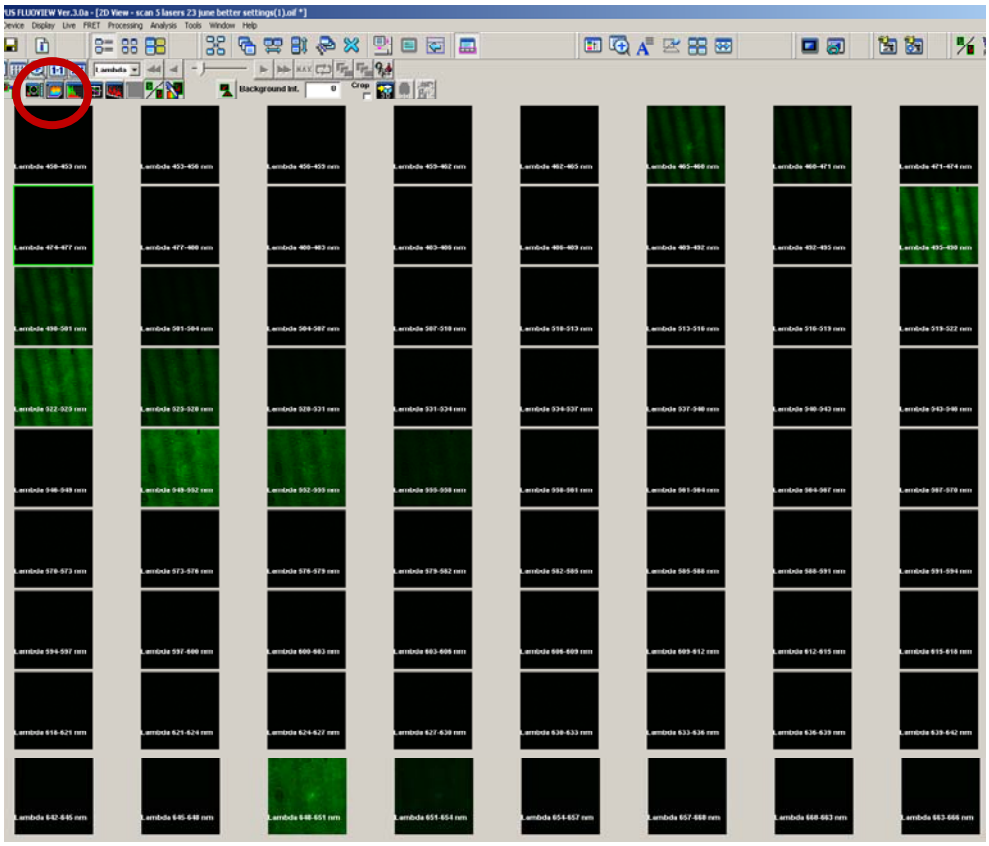
9 Also in the acquisition setting window, under the "**Image Acquisition control**" menu set the parameters as follows (slight adjustments may need to be made):

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).

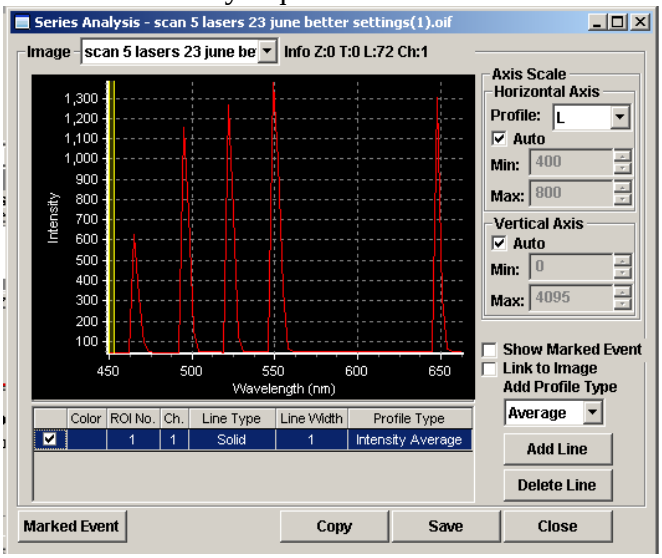
11 Make sure using the range indicator LUT that you are not getting any saturated pixels (red) within any of the Lamda stack images.

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.



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.

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. Under the **Microscope Control** window, 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 on the edge of the mirror via the microscope.

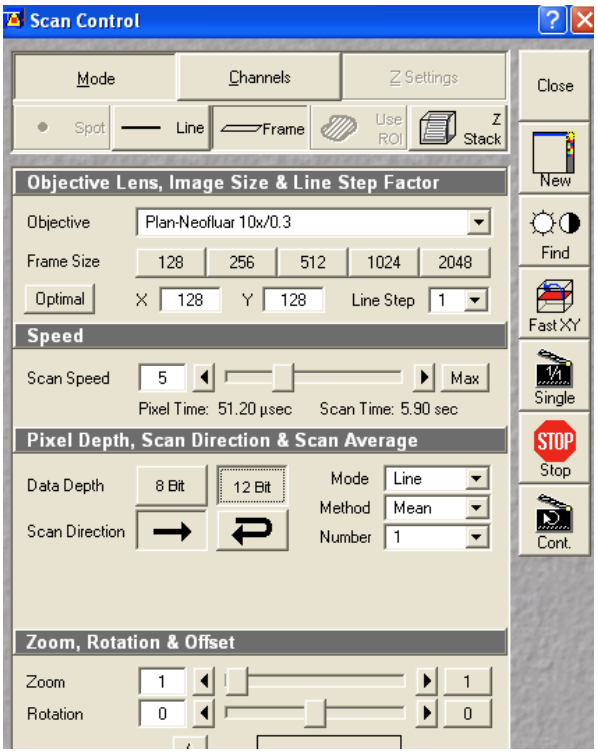
3. Choose the **Lambda Mode** under the **Configuration Control** window.

4. In the same window, put an 80/20 mirror (**NT80/20**) into the light path.

5. Adjust the range of wavelengths (under **lambda mode** menu) to cover the range of available lasers.

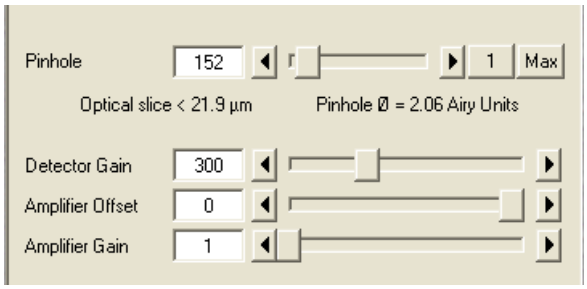
6. Do not select the 405 nm LASER because the detector does not measure wavelengths below about 420 nm.

7. Under the **Scan Control** menu in the **mode** tab, set the parameters to the following (see table below and image, right):



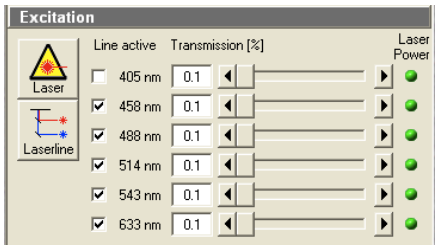
Parameter	Setting
Frame Size	128x128
Scan Speed	5-9
Frame Averaging	1
Bit Depth	12 Bit
Zoom Factor	1.0

8. Under the **Scan Control** within the **channels** tab set the parameters as follows (slight adjustments may need to be made):



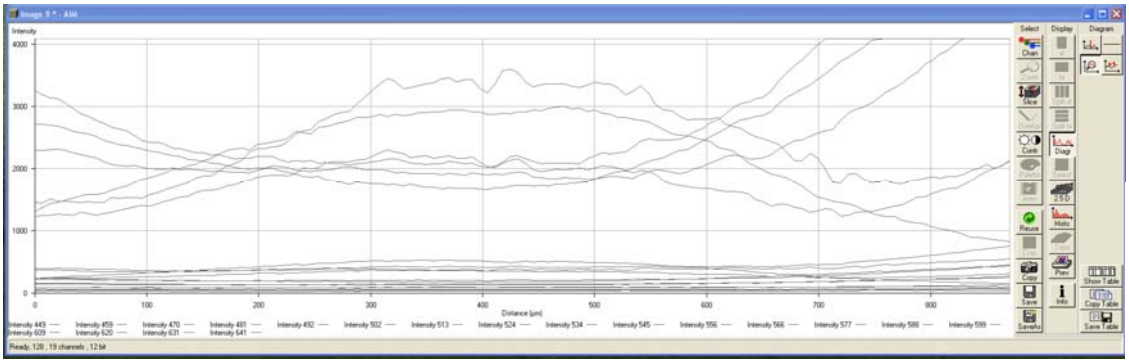
Parameter	Setting
Pinhole	1-2 Airy Units
Detector Gain	200-400
Amplifier Offset	>0
Amplifer Gain	1.0

9. Under the **Channels** tab in **Scan Control**, check off the lasers being tested (all lasers except the 405nm).

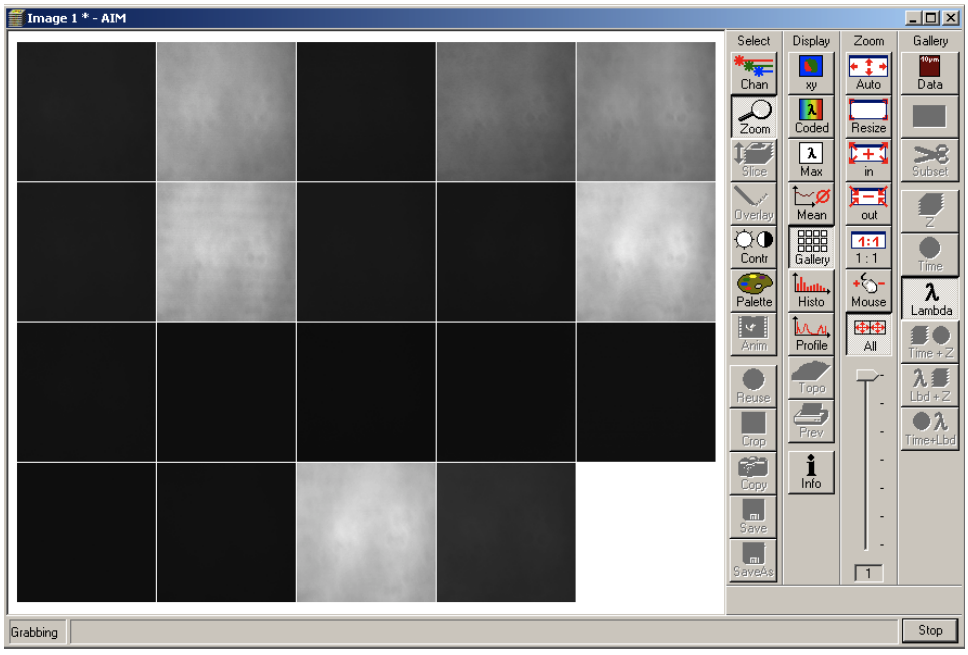


10. Set the laser power for each laser line to give an intensity signal of 2000-3000 for a 12-bit image (150-200 for an 8-bit image).

11. To aid in setting the intensity units, start a **continuous scan**, and in the **image** window under the **display menu**, select the **diagram** view. Change the percentage of power to the laser lines and/or the detector gain to adjust the intensities to within 2000-3000. If this view is not available use an ROI measurment.

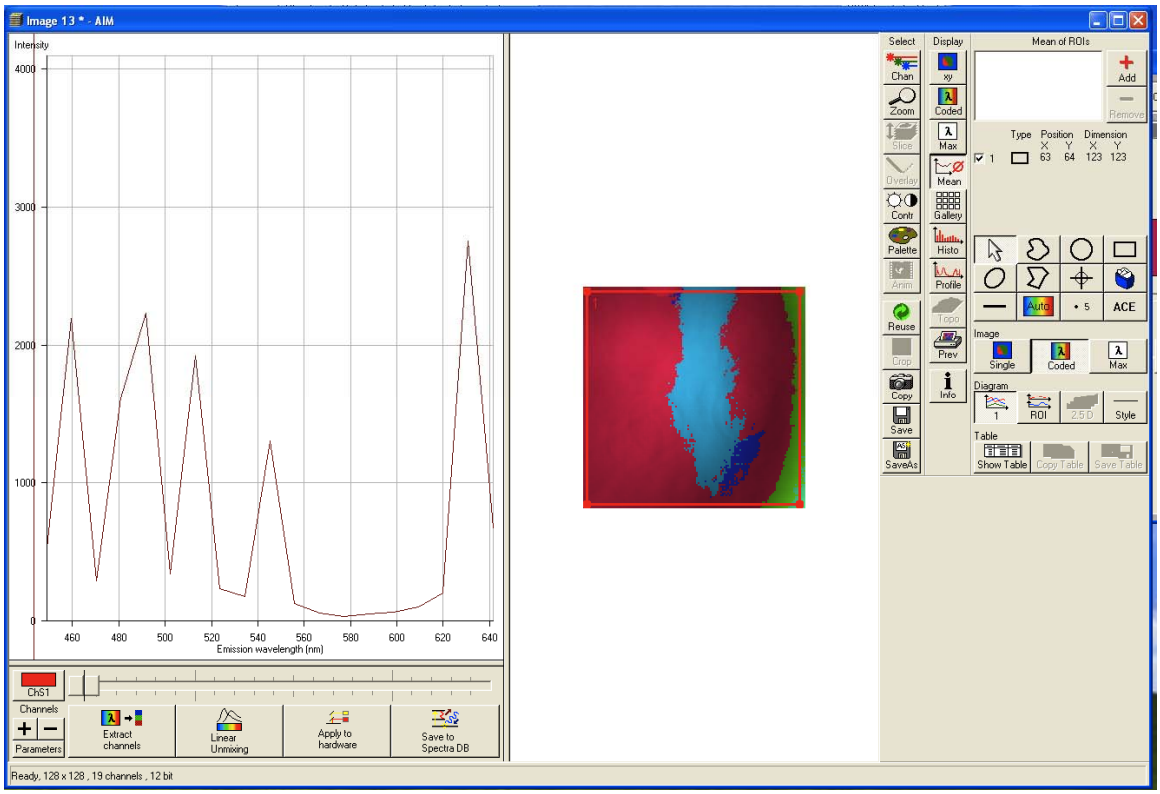


12. Use the **palette** and check off **range indicator** to ensure that there are no saturated pixels (red) within the Lambda stack. (See **gallery** view on **continuous** mode).



13. Collect a Lambda stack of images (single). 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.

14. After the picture has been taken, select **mean** from the **display** menu. Draw a region of interest over the entire image. Verify that the laser peaks are falling within 10-15 nm of the expected values. Notice the emission peaks at the wavelengths that correspond to the laser lines. The parameters should be set to only ChS1. Click on **show table** and then click on **save table** to log the data.



15. The text values for the spectra can be saved as a text file and imported into Excel.

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.

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.

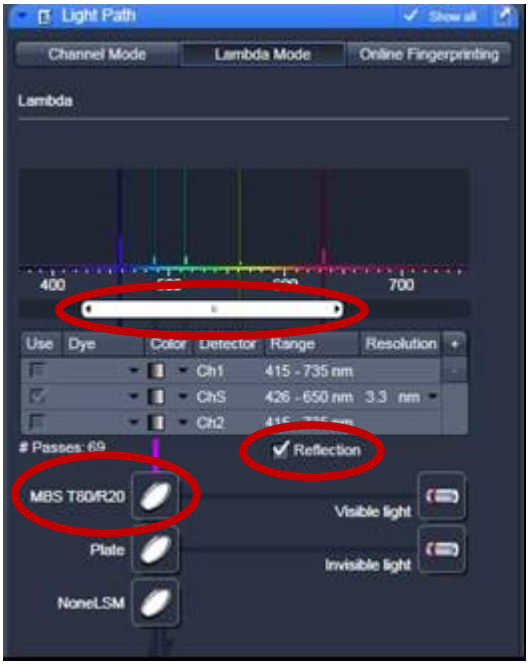
4. Put an 80/20 mirror (**T80/R20**) into the beam path.

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.

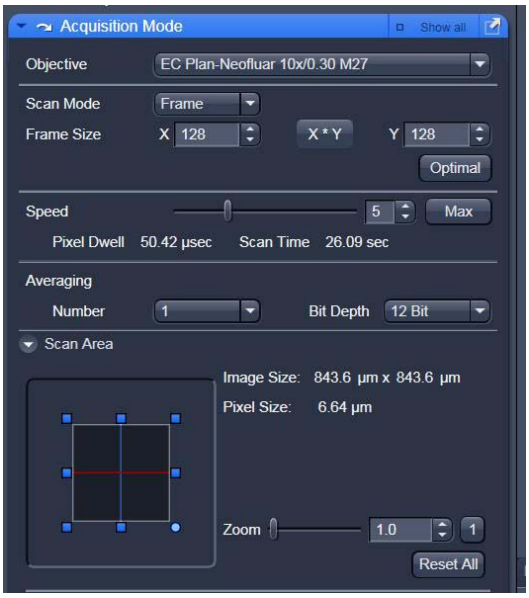
7. Set the spectral resolution to the smallest setting (3.3 nm on our system).

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



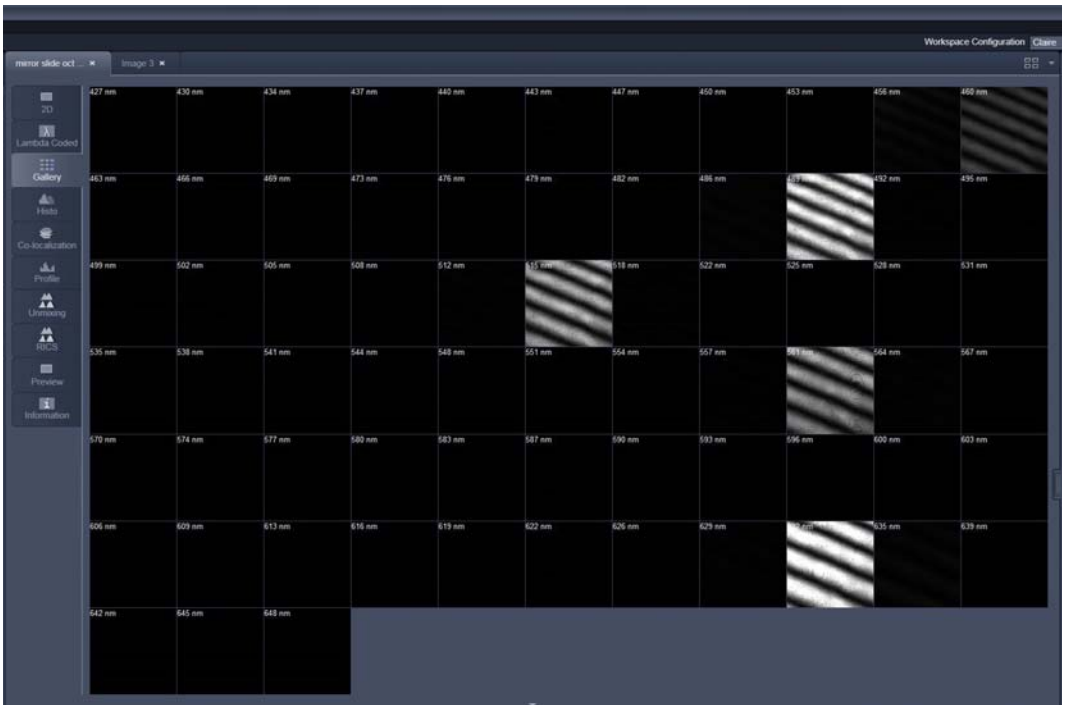
10. Under the **Channels** menu set the parameters as follows (slight adjustments may need to be made):

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).

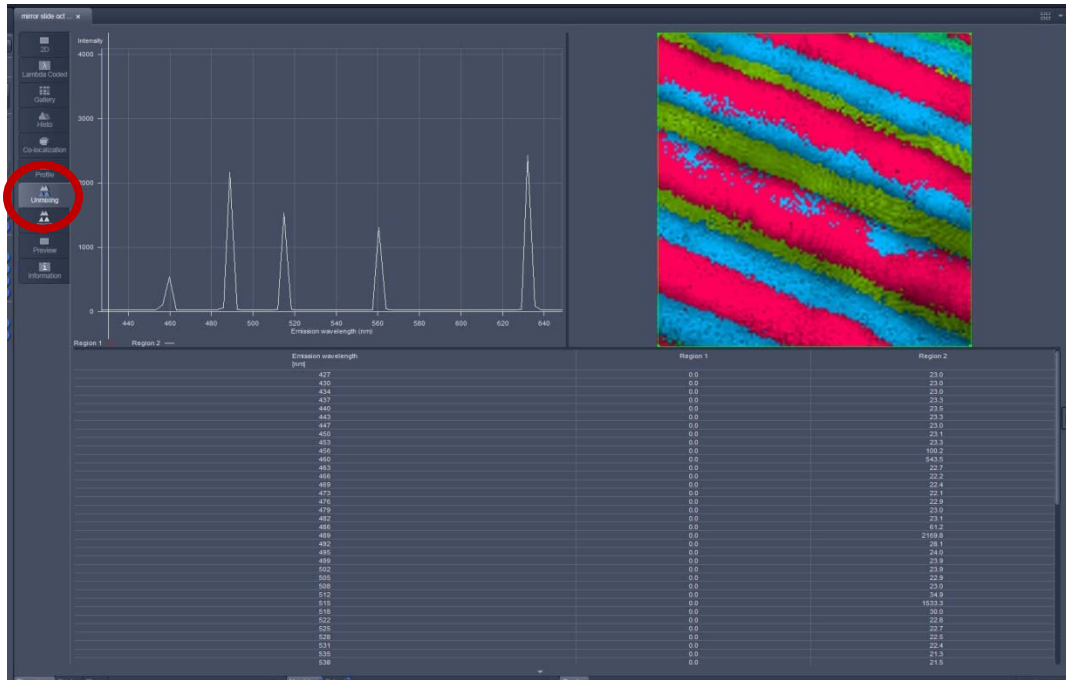
12. Make sure to use the range indicator LUT that you are not getting any saturated pixels (red) within any of the Lamda stack images.

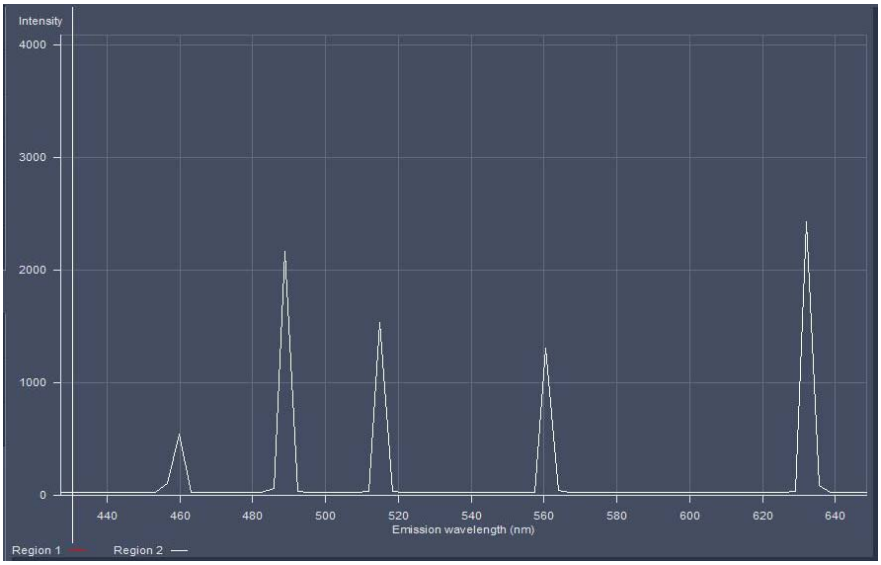
13. Collect a Lamda stack of images. You may or may not get stripped interference patterns in the images from laser reflections within the system. If you do this is normal and it will not affect the measurements. Notice how the periodicity of the interference pattern gets larger as you move to longer 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**.**

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.**

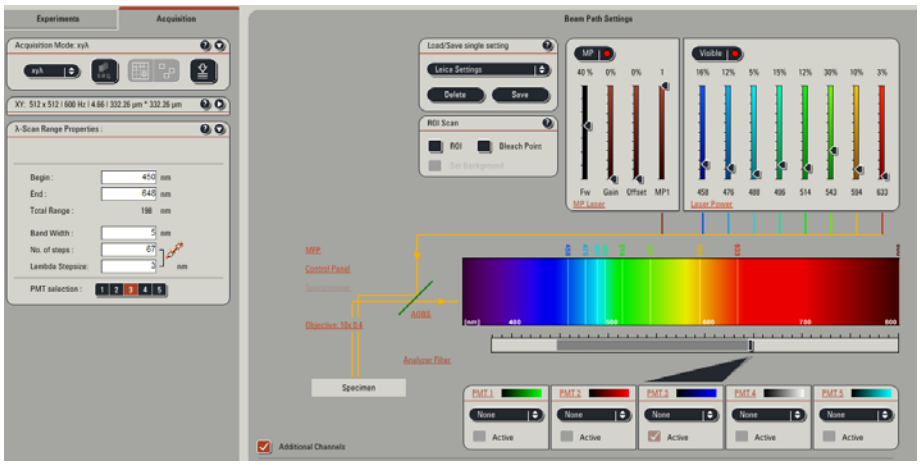




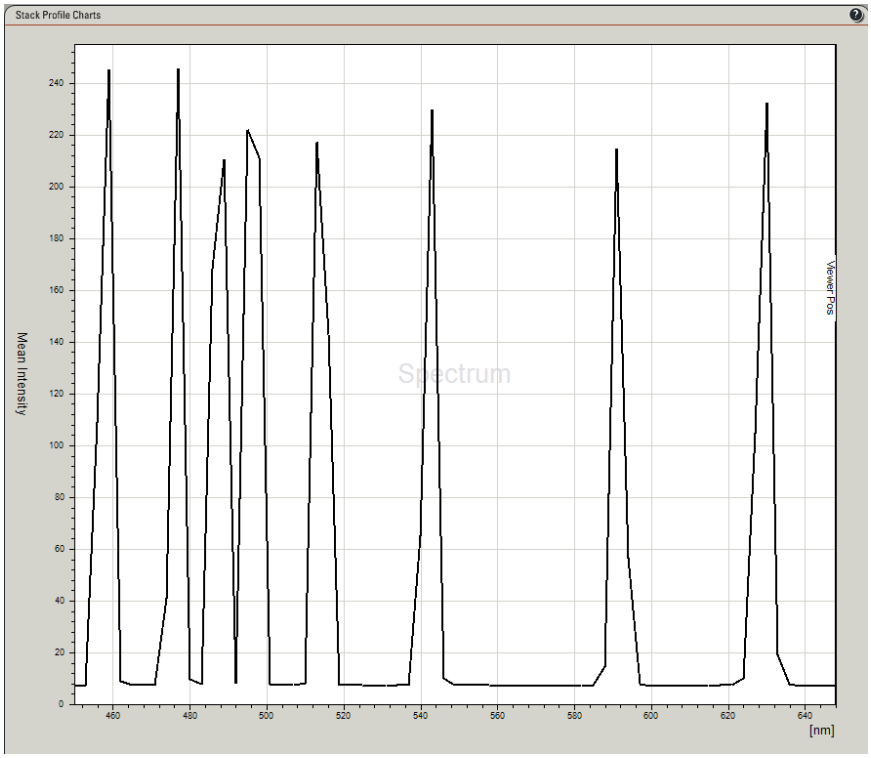
17. Name the file with your name and the name of the confocal platform you collected the data on. Send the text file (.txt or .xls) to the ABRF-LMRG at abrf.lmrg@gmail.com.

Using LASER as standards for spectral detection

Use the provided mirror slide w/lower magnification lens (i.e., 10x).
1.) Focus first on the edge of the mirror, either via the microscope or directly on the confocal. This will make it easier to find focus.
2.) Setup spectral detection; vary the LASER power to achieve similar output at a single gain setting.



On a Leica systems, as with most system, there is enough LASER light collected to over come any ND blocking.

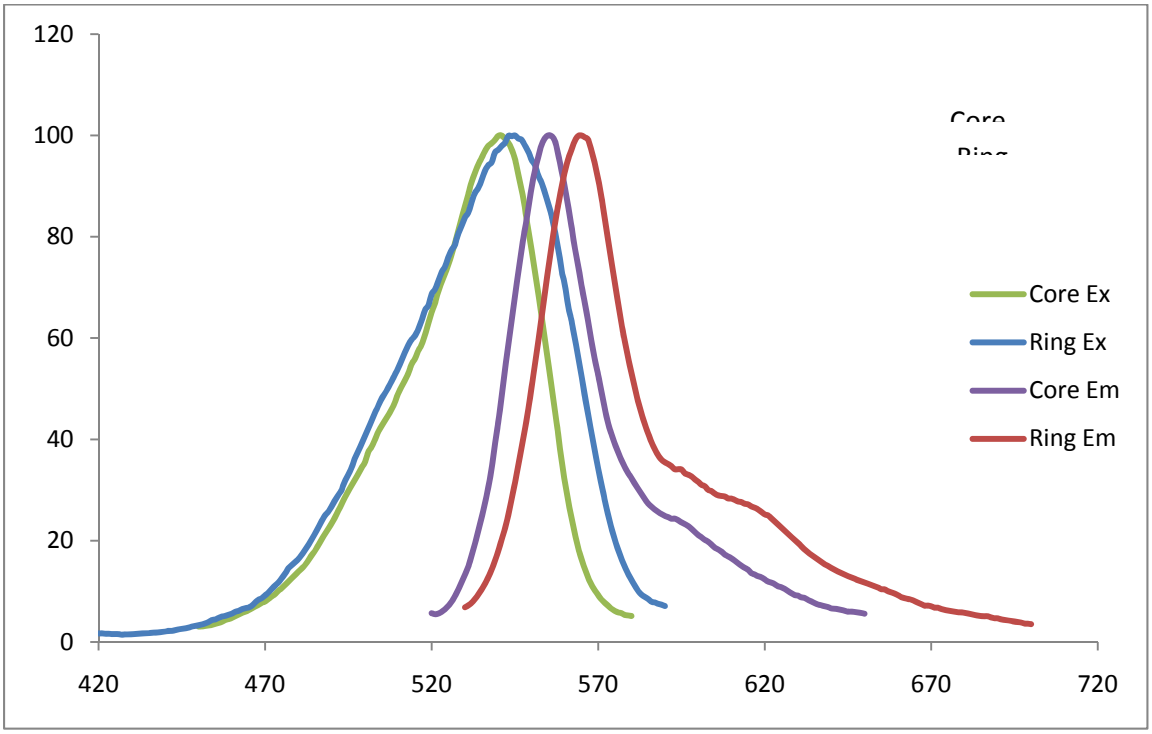


This is the plot of all 8 of our visible LASER lines. The absolute height of the peaks is irrelevant; the FWHM is what determines the resolution of the system

Protocol for spectral separation- *Mostly for Leica SP5 confocal microscopes*

Contents

Purpose: To check both spectral hardware and software. The d**ouble-orange fluorescent microspheres** are designed to test spectral separation on the Zeiss META system and other spectral imaging systems. These microspheres are stained with two different fluorescent dyes that appear similar in color by eye but are sufficiently different to be resolved by linear-unmixing techniques. When linear-unmixing data-processing algorithms are applied, the dyes are shown to be spectrally distinct and spatially separated – one appears only with in the outer ring and the other appears throughout the microsphere. Outer shell of the bead (532/552), core of the bead (545/565)

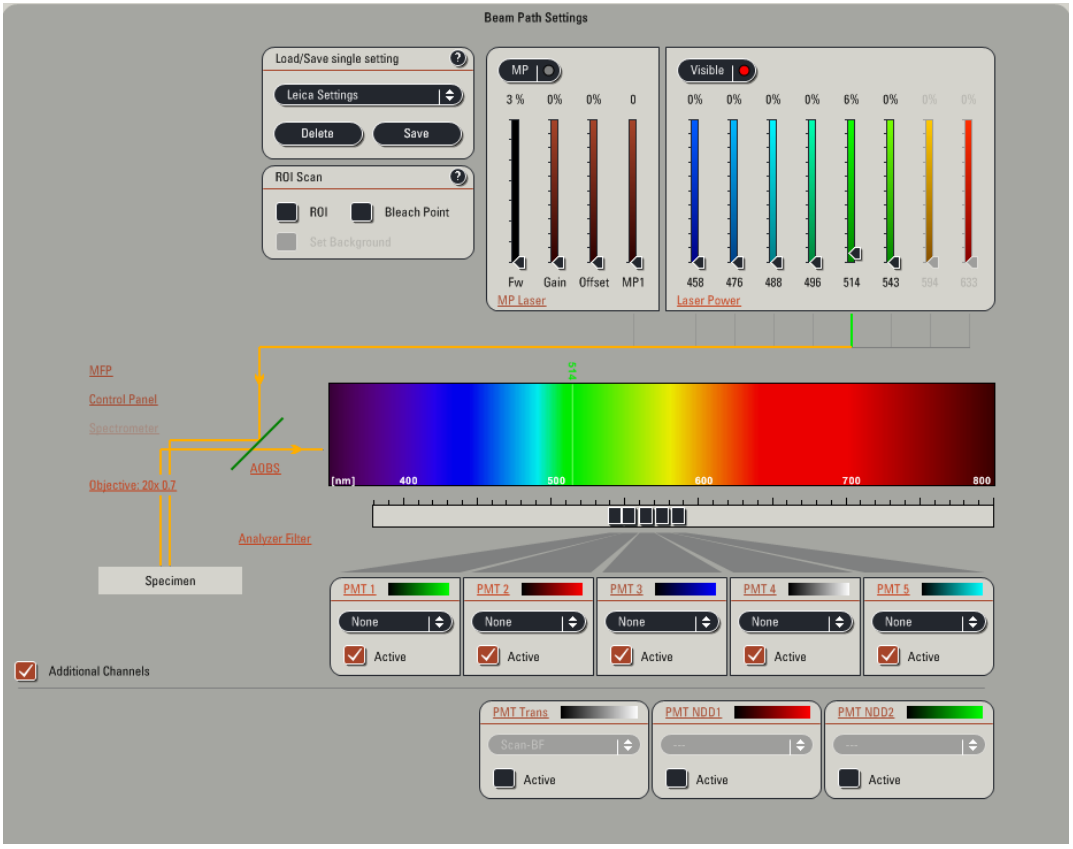


Materials: **Molecular Probes 6.0 µm FocalCheck™ beads mount with Prolong Gold™**

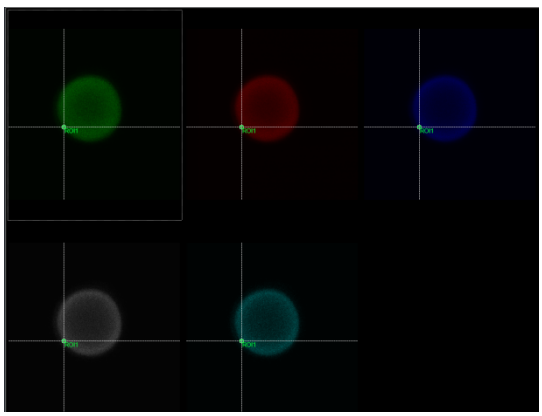
Multi-PMT detection method: This method use all available PMTs to simultaneously detect discreet portion of the emission spectra from both the core and shell (see above)

Procedure:

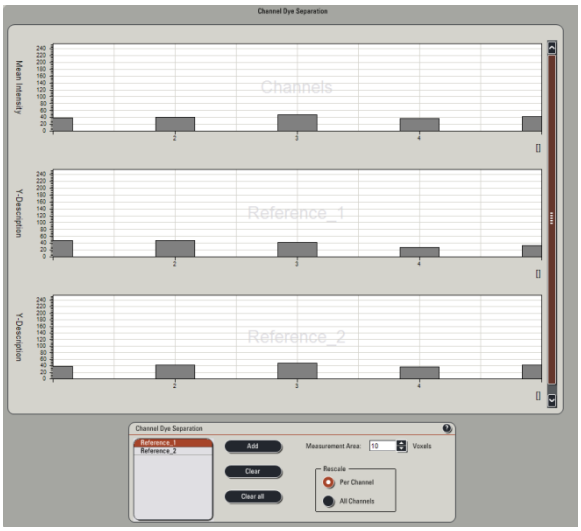
1) Set up as many PMT's as possible similar to below.
   a) *Note: Be certain to have PMT coverage from 545-570 in order to collect both flurophores*



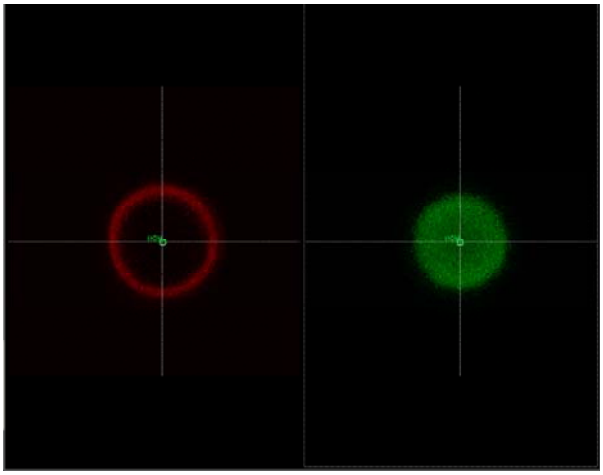
2) Find and image a bead use the 514 or equivalent LASER. The bead should be ~450 pixels ls in diameter or use ~13nm pixels and the intensity should be ~85% of saturation, i.e., for 8 bit image that would be 216 gray levels.
3) Focus to the ~ center of the bead.
4) Select: Process/Dye separation/channel
5) You can define a reference range for every fluorescent dye used to determine the current emission spectrum of the fluorescent dye. For this purpose, place the crosshairs on the edge of the bead, *careful not to select any of the core of the bead. (measurement area (Voxel) set to 10 & rescale to per channel).*



6) Click the Add radio button to add the values for the center of the bead
   a) Note: Select a position at which there are no or minimize the fluorescence signals in the remaining color channels. Otherwise, it may lead to errors during the separation of superimposed fluorescence signals.
7) Repeat for the center of the bead
   a) *Note: The clear button can be used to remove unwanted reference data.*

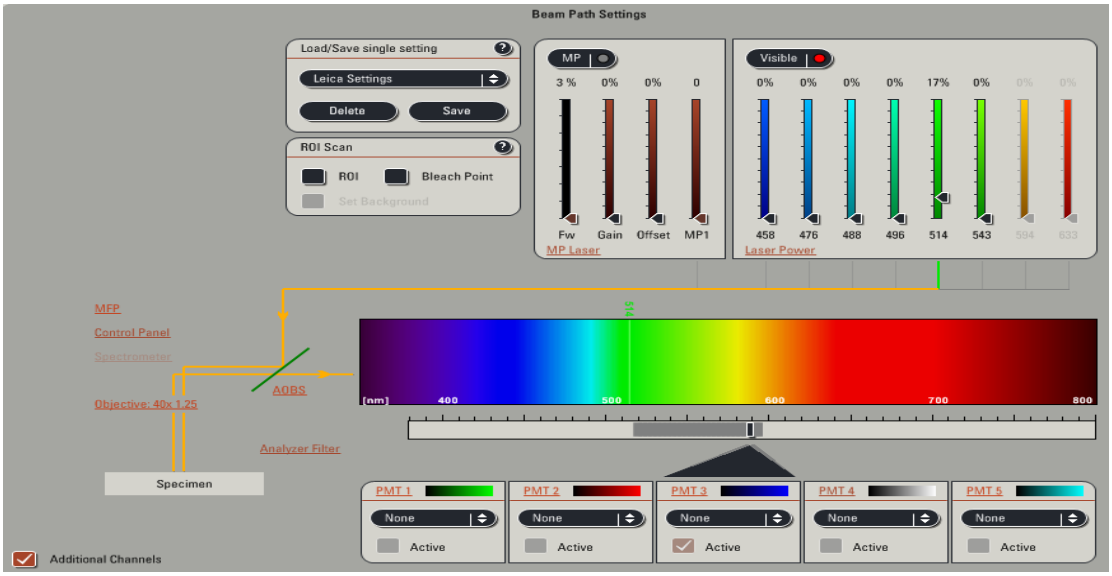


8) Once both spectra have been add, click apply radio button
   a) *Note: This will generate a new file based on spectra selected from the center and edge of the bead*.

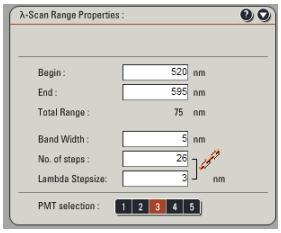


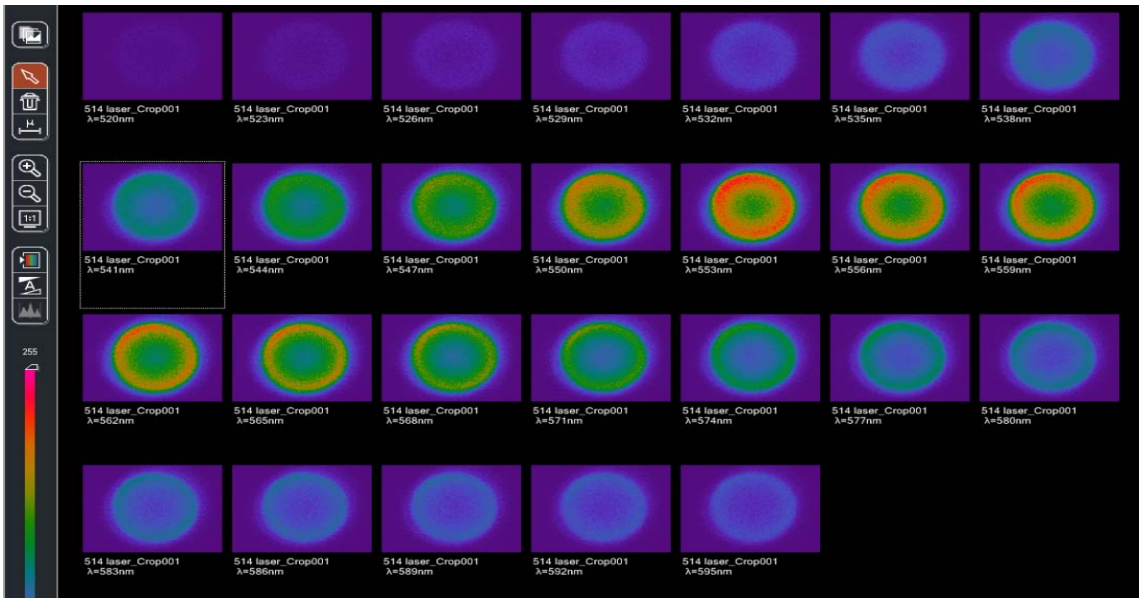
Spectral detection method: This method use one PMT, and scan the slit detection across the emission spectra from both the core and shell (see above)

1) **Procedure:** Set up the PMT that directly in-line with the optical axis (usually #3) as possible similar to below.



2) *Set the scan to 520-595 in order to collect both flurophores*
   a) *Set the Band width to 5*
   b) *No. of steps to 26*
   c) *Lamda Step size to 3*
3) *Collect series- should look similar to below*

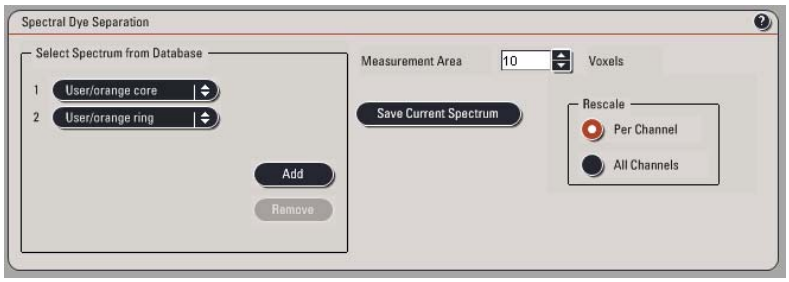




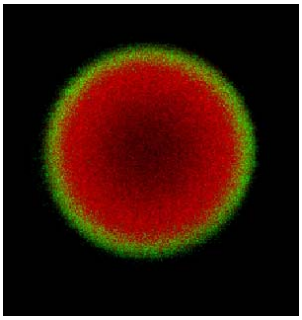
4) Select: Process/Dye separation/spectral

5) Copy the two files provied *.lsf* files into the database directory typically: *C:\documents and settings\username\leica microsystems\las af\database*

6) *Load the ring and core spectrum*



7) *Click apply and the software spectrally unmix the image using provided spectrum*



8) *The above image is pseudo-colored to visualize the two overlapping flurophores*

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

a) Sample image as above of the separated data in 2D or 3D.

b) Image Z-stack of a single bead from the unmixed data stack

Please indicate the following

i) objective used

ii) pixel size

iii) laser power

iv) platform the images were collected on

v) software used for the un-mixing.

Molecular probes bead information

Molecular Probes

Product Information

Revised: 16–July–2003

FocalCheck™ Fluorescent Microsphere Standards

Storage upon receipt:

- 2–6°C, for microsphere suspensions
- Room temperature, for microsphere slides
- Do not freeze
- Protect from light

Introduction

Molecular Probes' Focal Check™ fluorescent microspheres are specifically designed for examining the alignment, sensitivity and stability of confocal laser scanning microscopes.[1] They are particularly useful for confirming the optical sectioning thickness ("Z-resolution") in three-dimensional imaging applications. These polystyrene beads, available in either 15 µm or 6 µm diameters, have been prepared by a proprietary method in which fluorescent dye is used to stain only the outermost portion of each microsphere. The resulting beads have a well-defined dye layer, which, when viewed in cross section in the confocal laser-scanning microscope, appears as a fluorescent ring of varying dimensions depending on the focal plane (Figure 1). We refer to this proprietary staining procedure as ring staining, in order to differentiate it from routine staining throughout the bead.

FocalCheck products are currently available in several different multicolored configurations of fluorescent ring stains and/or contrasting fluorescent stain throughout the bead (Table 1). The excitation/emission maxima exhibited by the different stains in these microspheres — blue (365/430 nm), green (505/515 nm), orange (560/580 nm) and dark red (660/680 nm) — are well matched to the laser sources and optical filters commonly used in confocal laser scanning microscopes. Moreover, because the dyes are localized *within* the bead and therefore protected from environmental factors, the FocalCheck microspheres are brighter and more photostable than conventional surface-stained beads.

The sharp ring stains exhibited by the FocalCheck microspheres produce a striking visual representation of instrument misalignment or other aberrations, making them ideal as reference standards for confocal laser scanning microscopy. Correct image registration is indicated when the multiple ring images of the ring-stained FocalCheck beads (or the ring and disk images of the combination ring-stained and stained-throughout FocalCheck beads) are perfectly coincident in all dimensions (Figure 1). Furthermore, because the FocalCheck beads are available in a number of multicolor options, they are especially useful in testing and aligning confocal laser scanning microscopes that have multiple laser lines and detection channels.

Table 1. FocalCheck fluorescent microsphere suspensions and kits.

Cat #	Bead Size (µm)	Ring Stain *	General Stain * (Throughout)
F-7234	15	blue, orange	none
F-7240	15	green, dark red	none
F-7235 †	15	green, orange, dark red	none
F-14806 ‡	6	green, orange, dark red	none
F-7237 †	15	green	blue
F-14808 ‡	6	green	blue
F-7238	15	green	dark red
F-7236	15	orange	blue
F-7239 †	15	dark red	green
F-14807 ‡	6	dark red	green
F-36905 §	6	green-1 (500/512)	green-2 (512/525)
F-36906 §	6	orange-1 (532/552)	orange-2 (545/565)
F-36907 §	6	red-1 (580/610)	red-2 (569/574)
F-36908 §	6	far-red–1 (665/695)	far-red–2 (640/674)

* Ring and general stains are identified by fluorescence emission color.
† Also available mounted on slides in Kit F-24634.
‡ Also available mounted on slides in Kit F-24633.
§ Only available mounted on slides as a kit. The fluorescent stains are spectrally similar, but can be resolved by the technique of spectral unmixing. The numbers in parentheses refer to the absorption and emission maxima for the respective dyes, in nm.

Special FocalCheck microspheres are available for testing spectral separation. These microspheres are stained with two different fluorescent dyes, which appear similar in color by eye. However, when linear-unmixing data-processing algorithms are applied, the dyes can be spectrally resolved — one appearing only within the outer ring and the other throughout. These 6 µm, dual-stained microspheres are provided mounted on slides in the FocalCheck DoubleGreen, DoubleOrange, DoubleRed and DoubleFarRed Fluorescent Microspheres Kits (F-36905, F-36906, F-36907, F-36908). In addition, the kits contain mounted control microspheres that have been stained throughout with each of the two dyes separately.

Spectral data for beads

Core

wl	ex	Wl	em
400	3.890477	530	6.841007
401	4.301481	531	7.215827
402	4.127847	532	7.726619
403	4.107378	533	8.482014
404	3.881953	534	9.381295
405	3.712509	535	10.39568
406	3.556954	536	11.46043
407	3.401796	537	12.81295
408	3.161923	538	14.27338
409	3.001633	539	16.04317
410	2.838968	540	17.97842
411	2.639396	541	20.20863
412	2.619129	542	22.38129
413	2.399444	543	25.09353
414	2.257884	544	28.22302
415	2.22584	545	31.43165
416	2.103295	546	34.88489
417	1.999779	547	38.44604
418	1.863102	548	42.02158
419	1.811749	549	45.97842
420	1.706568	550	50.33094
421	1.716014	551	54.84173
422	1.640475	552	59.43885
423	1.642417	553	64.27338
424	1.577624	554	68.88489
425	1.587517	555	73.59712
426	1.566637	556	78.20144
427	1.455448	557	82.80576
428	1.522028	558	86.33094
429	1.50739	559	89.85612
430	1.521544	560	92.94964
431	1.621994	561	95.39568
432	1.677378	562	97.26619
433	1.685317	563	98.99281
434	1.711406	564	99.92806
435	1.740652	565	100
436	1.831822	566	99.64029

437	1.870536	567	99.13669
438	1.912665	568	96.83453
439	1.996873	569	94.53237
440	2.0631	570	91.22302
441	2.208906	571	87.69784
442	2.206568	572	83.52518
443	2.349559	573	79.20863
444	2.469951	574	74.89209
445	2.626569	575	70.82014
446	2.696235	576	66.90647
447	2.83131	577	62.7554
448	3.061524	578	59.38129
449	3.181503	579	56.09353
450	3.330171	580	52.8777
451	3.457084	581	50.07194
452	3.704365	582	47.54676
453	3.964489	583	45.1223
454	4.338381	584	42.8705
455	4.469847	585	41.17266
456	4.763881	586	39.43165
457	5.037619	587	38.04317
458	5.162105	588	36.82734
459	5.401014	589	35.98561
460	5.606294	590	35.47482
461	5.96514	591	35.1295
462	6.155754	592	34.66906
463	6.47504	593	34.09353
464	6.690617	594	34.10072
465	6.811498	595	34.05036
466	7.188324	596	33.30216
467	7.809784	597	33.02158
468	8.363384	598	32.69065
469	8.667058	599	32.11511
470	9.242364	600	31.6259
471	9.77939	601	31.03597
472	10.50785	602	30.73381
473	11.18187	603	30.03597
474	11.78317	604	29.78417
475	12.64806	605	29.28777
476	13.53136	606	28.92806
477	14.59242	607	28.82734
478	15.19009	608	28.72662
479	15.79907	609	28.33094
480	16.41786	610	28.30216
481	17.28922	611	28.03597

482	18.18031	612	27.72662
483	19.21251	613	27.61871
484	20.29502	614	27.28058
485	21.4416	615	27.15108
486	22.73784	616	26.7482
487	23.94326	617	26.5036
488	25.03949	618	26.15108
489	25.76013	619	25.66906
490	26.87383	620	25.18705
491	28.04711	621	24.97842
492	29.02735	622	24.40288
493	30.09283	623	23.82014
494	31.90607	624	23.20144
495	33.09334	625	22.57554
496	34.55146	626	21.92086
497	36.44422	627	21.33094
498	37.84082	628	20.78417
499	39.24909	629	20.08633
500	40.61238	630	19.57554
501	42.28775	631	18.8777
502	43.87936	632	18.30216
503	45.33834	633	17.69784
504	46.66482	634	17.1295
505	48.17059	635	16.71942
506	49.09944	636	16.2518
507	50.3001	637	15.84892
508	51.53836	638	15.33813
509	52.87043	639	15.00719
510	54.13295	640	14.58993
511	55.80024	641	14.25899
512	57.18971	642	13.8777
513	58.70215	643	13.58273
514	59.74477	644	13.30216
515	60.49501	645	13.03597
516	61.82087	646	12.70504
517	63.53349	647	12.48921
518	65.54087	648	12.15108
519	66.50893	649	11.97842
520	68.72831	650	11.70504
521	69.63339	651	11.51799
522	71.29749	652	11.20863
523	73.04858	653	11
524	74.1356	654	10.7554
525	76.00655	655	10.41007
526	77.43212	656	10.36691

527	78.45757	657	10.02158
528	80.72197	658	9.784173
529	82.18924	659	9.546763
530	83.84305	660	9.23741
531	84.78798	661	8.848921
532	86.93234	662	8.654676
533	88.67931	663	8.467626
534	89.65934	664	8.352518
535	91.25435	665	8.064748
536	93.05612	666	7.733813
537	94.14586	667	7.482014
538	94.7307	668	7.161871
539	96.67391	669	7.177698
540	97.18011	670	7.034532
541	97.92993	671	6.771223
542	98.56615	672	6.753957
543	99.91067	673	6.496403
544	99.86391	674	6.311511
545	100	675	6.228058
546	99.41013	676	6.064029
547	99.14238	677	5.983453
548	98.00266	678	5.859712
549	96.80797	679	5.857554
550	95.1765	680	5.766187
551	94.12777	681	5.617266
552	92.05662	682	5.456115
553	90.66251	683	5.334532
554	88.65729	684	5.193525
555	86.48811	685	5.127338
556	84.15008	686	5.063309
557	80.91924	687	5.066187
558	77.68977	688	4.831655
559	73.29311	689	4.67554
560	70.40483	690	4.651079
561	66.20309	691	4.436691
562	63.32262	692	4.358273
563	59.59101	693	4.26259
564	56.48409	694	4.153237
565	52.30759	695	3.98705
566	48.34763	696	3.921583
567	44.89988	697	3.858993
568	41.17215	698	3.633813
569	37.58221	699	3.593525
570	34.14511	700	3.507914
571	31.10624		

572	27.85016
573	24.99582
574	22.39846
575	20.08469
576	18.13883
577	16.40913
578	14.80357
579	13.42665
580	12.21157
581	11.12459
582	10.05398
583	9.353218
584	8.894608
585	8.498588
586	7.953624
587	7.811698
588	7.540247
589	7.371873
590	7.104142

RING STAIN

wl	ex	wl	em
Inc."	Orange-1		
450	3.066484	520	5.681617
451	3.067274	521	5.491604
452	3.165203	522	5.616438
453	3.267705	523	5.941228
454	3.372202	524	6.482545
455	3.583761	525	7.105612
456	3.766394	526	7.939682
457	3.97667	527	9.041096
458	4.332114	528	10.28833
459	4.516544	529	11.7985
460	4.723545	530	13.25674
461	5.108626	531	14.84755
462	5.395775	532	17.03491
463	5.74905	533	19.3217
464	5.946615	534	21.90676
465	6.271023	535	24.54706
466	6.609876	536	27.25365
467	7.010671	537	30.50155
468	7.330406	538	34.11401
469	7.738083	539	38.85329
470	7.999238	540	43.31639
471	8.480131	541	48.28767

472	9.004411	542	53.7229
473	9.439178	543	58.38489
474	10.12985	544	63.15731
475	10.63159	545	68.16173
476	11.28445	546	72.85683
477	11.91771	547	77.49669
478	12.57527	548	81.44057
479	13.22261	549	85.42863
480	13.88893	550	89.65974
481	14.5202	551	92.99602
482	15.16135	552	95.7247
483	16.13321	553	98.08882
484	17.17264	554	99.42554
485	17.99019	555	100
486	19.15101	556	99.86743
487	20.22556	557	98.96156
488	21.38725	558	96.3323
489	22.44187	559	93.0844
490	23.36968	560	89.86964
491	24.64606	561	86.00309
492	25.94896	562	82.09236
493	27.23949	563	77.77287
494	28.58957	564	74.30402
495	29.84569	565	70.45957
496	30.8981	566	66.88025
497	32.08309	567	63.25674
498	33.26927	568	59.40124
499	34.41848	569	55.76668
500	35.51701	570	52.77287
501	37.52164	571	49.35926
502	38.56718	572	46.19973
503	39.971	573	43.09545
504	41.52901	574	41.08484
505	42.68169	575	39.11843
506	43.74867	576	37.46133
507	44.72061	577	35.90367
508	46.00378	578	34.54485
509	47.33917	579	33.32965
510	49.1222	580	32.40168
511	50.44648	581	31.21962
512	51.68337	582	30.23641
513	53.02438	583	29.28635
514	54.86108	584	28.23685
515	55.92689	585	27.35307
516	57.51122	586	26.71233

517	58.69672	587	26.14892
518	60.6327	588	25.70703
519	62.83649	589	25.24304
520	65.09107	590	24.90057
521	66.85651	591	24.65753
522	69.15293	592	24.33716
523	71.09052	593	24.34821
524	72.65298	594	24.04993
525	74.46547	595	23.63014
526	76.48897	596	23.27662
527	78.73364	597	22.91206
528	81.10564	598	22.3376
529	83.685	599	21.7521
530	85.94105	600	21.1445
531	88.37197	601	20.68051
532	90.87639	602	20.18338
533	92.53937	603	19.80778
534	94.29398	604	19.25541
535	95.53104	605	18.63677
536	96.91003	606	18.28325
537	97.93995	607	17.86346
538	98.45802	608	17.27795
539	99.11784	609	16.90234
540	99.91909	610	16.52673
541	100	611	16.0517
542	99.37211	612	15.55457
543	98.665	613	15.05745
544	97.32022	614	14.65974
545	95.43414	615	14.26204
546	92.31964	616	13.61025
547	89.47731	617	13.32302
548	85.65515	618	12.9916
549	81.46805	619	12.78171
550	77.54657	620	12.36191
551	73.16071	621	11.93106
552	68.97528	622	11.73221
553	64.42641	623	11.41184
554	59.92033	624	11.00088
555	55.32888	625	10.81418
556	50.37329	626	10.50707
557	45.73629	627	10.02651
558	41.05845	628	9.681838
559	35.79909	629	9.275298
560	31.72838	630	9.150464
561	28.08866	631	8.813522

562	24.36168	632	8.696421
563	21.48103	633	8.309766
564	18.58652	634	8.011489
565	16.40817	635	7.690013
566	14.41043	636	7.388422
567	12.72121	637	7.261379
568	11.26811	638	7.048166
569	10.24453	639	6.913389
570	9.272878	640	6.630579
571	8.358532	641	6.553248
572	7.757345	642	6.486964
573	7.171688	643	6.353292
574	6.543628	644	6.168802
575	6.160867	645	5.982103
576	5.849847	646	6.001989
577	5.693052	647	5.941228
578	5.343721	648	5.823023
579	5.246725	649	5.75232
580	5.136218	650	5.579982

Confocal note on importing spectral data

Adding Fluorophores Spectrum to the Dyes Spectra Database

Prepared by Louise Bertrand
Applications and Technical Support Group, Leica Microsystems, Inc

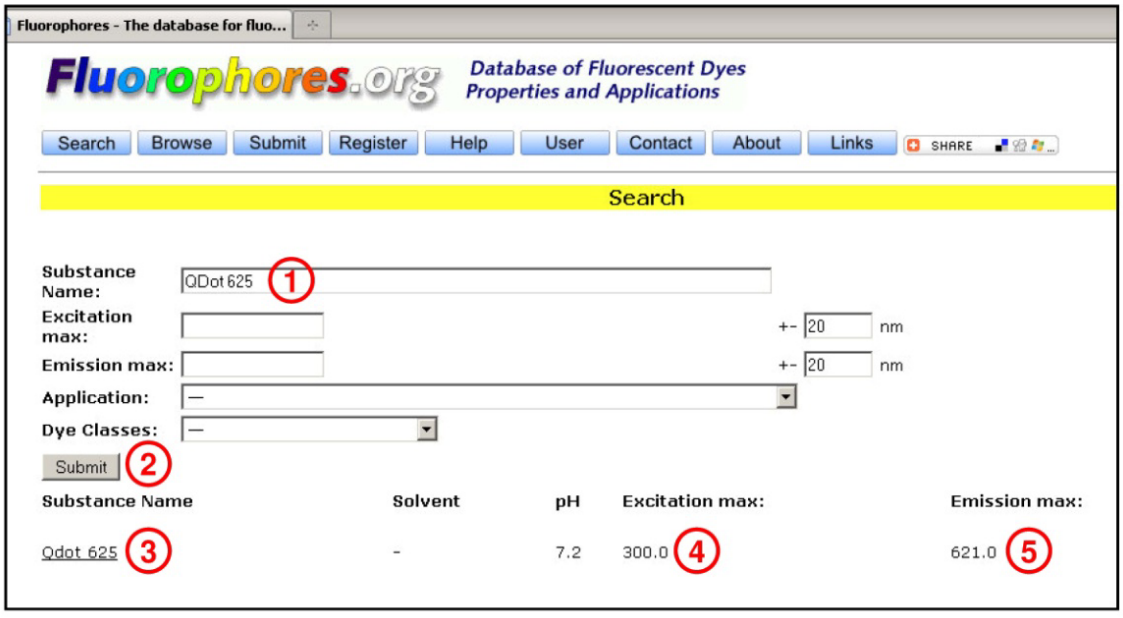
Many databases provide access to spectra of organic compounds, oxonol dyes, styryl-based dyes, fluorescent dyes and fluorescent proteins. These spectra are useful for rapid comparison and for setting up the range properties of your PMTs.

This application note will demonstrate how to add fluorophore spectra to the Dyes Spectra database. We chose for this purpose, Qdot 625.

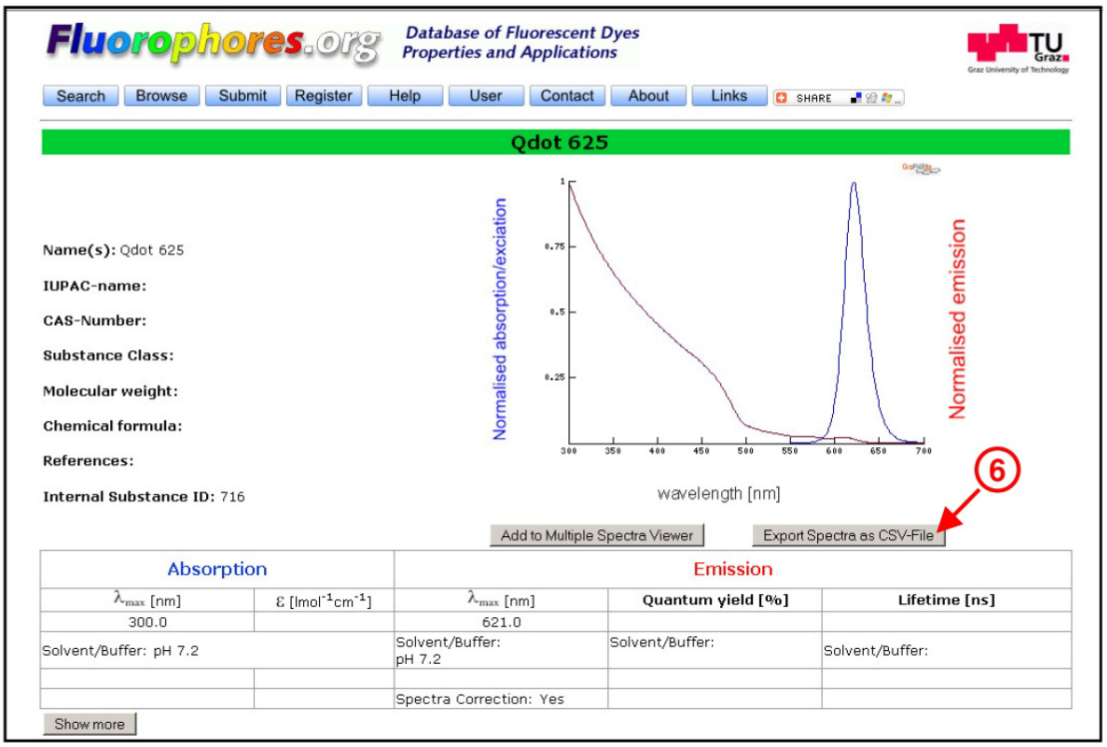
1. Go to your web browser and open your Spectra Database. In this case, *Fluorophores.org (http://www.fluorophores.tugraz.at/)*.

2. Click 'Search' and enter your fluorophore under 'Substance Name' (1) then, click Submit (2). Your fluorophore with appear under Substance Name (3) with the corresponding Excitation (Absorption) maximum peak (4) and Emission maximum peak (5).

Note: Please take note of the Excitation (Absorption) maximum peak value and Emission maximum peak value as you will need them when entering the new dye in LAS AF Dyes Spectra Database.



3. Click on the fluorophore **3** , a new window will appear.



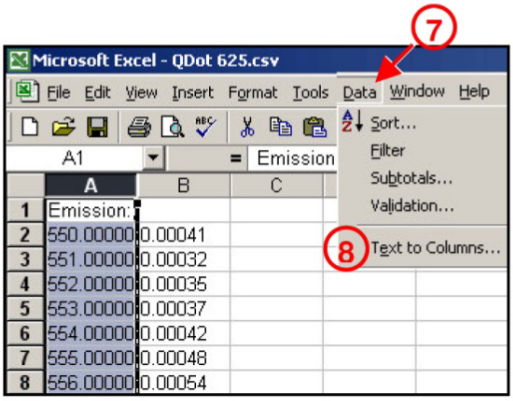
4. Click on 'Export Spectra as CSV-File' (6). A new window will appear asking if you want to download the file. Download the spectrum of this fluorophore to your computer.

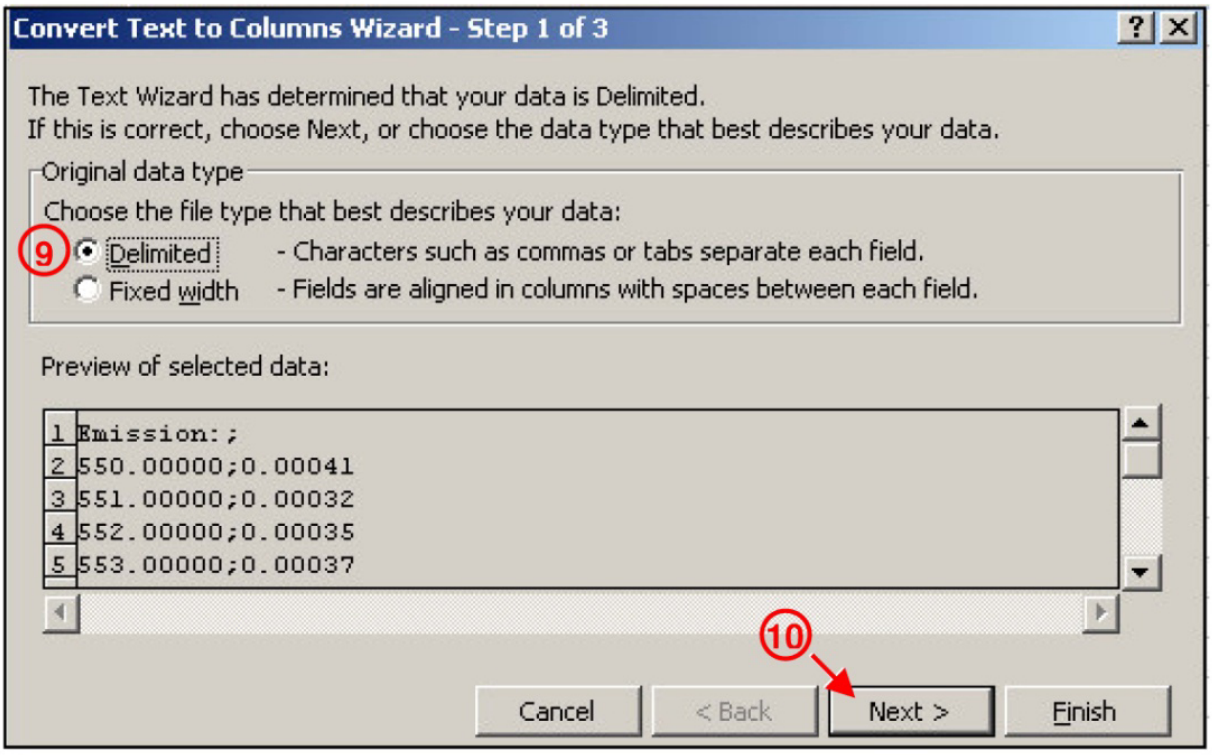
5. Open your Data Analysis tools - in this case, Microsoft Excel. Go to File > Open, select file type: *.cvs* and open your fluorophore 'spectra.cvs' document previously downloaded.

6. Select the first column (A), the column will highlight blue when selected.

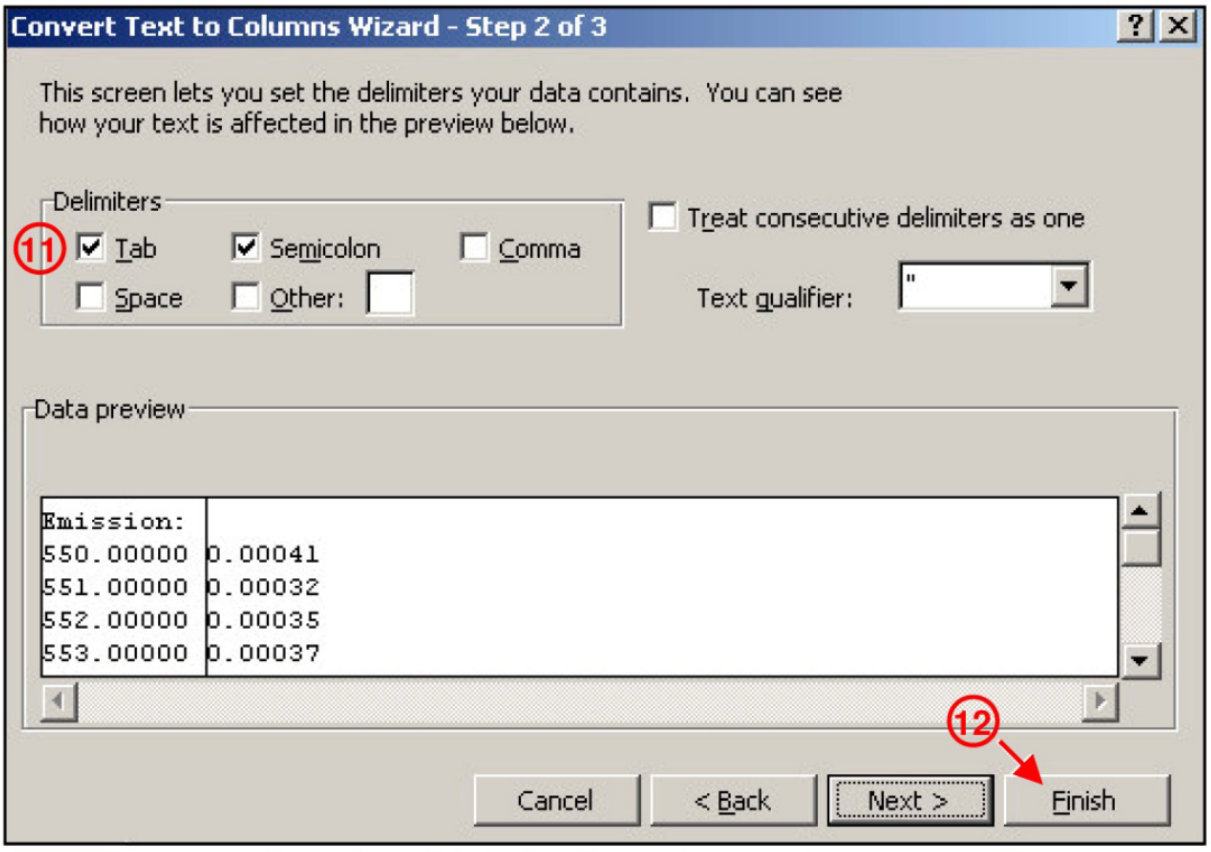
7. Go to Data (7), select Text to Columns.. (8).

8. Choose the file type that best describes your data. In this case, Delimited (9) and click Next (10).

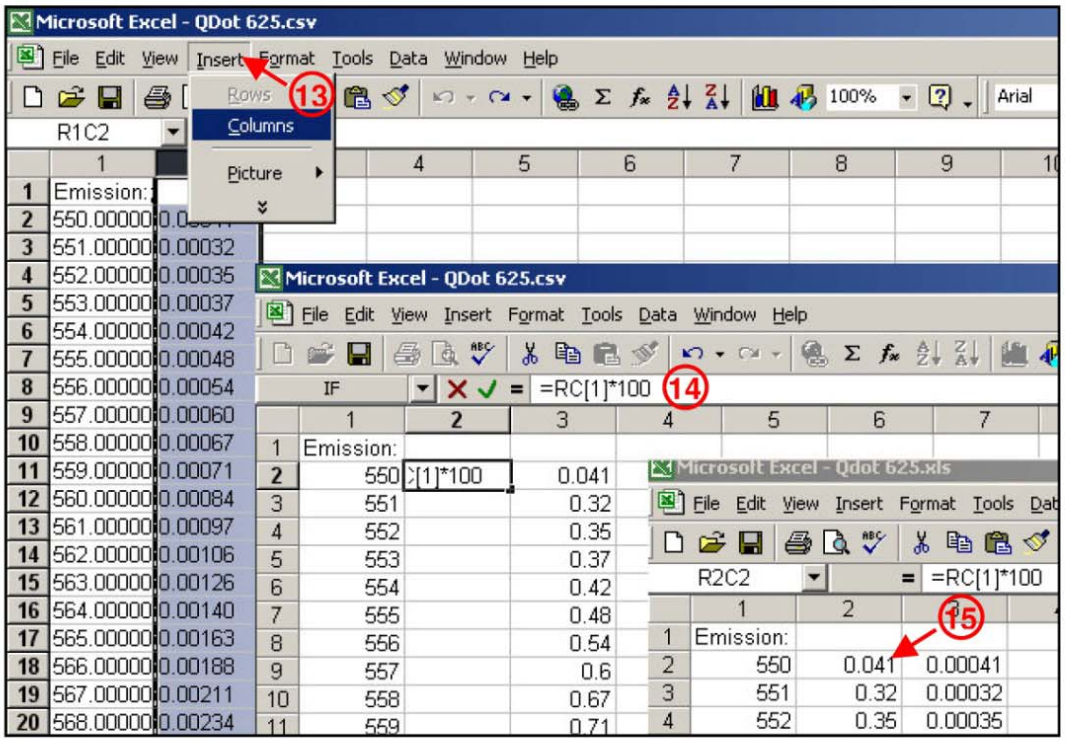




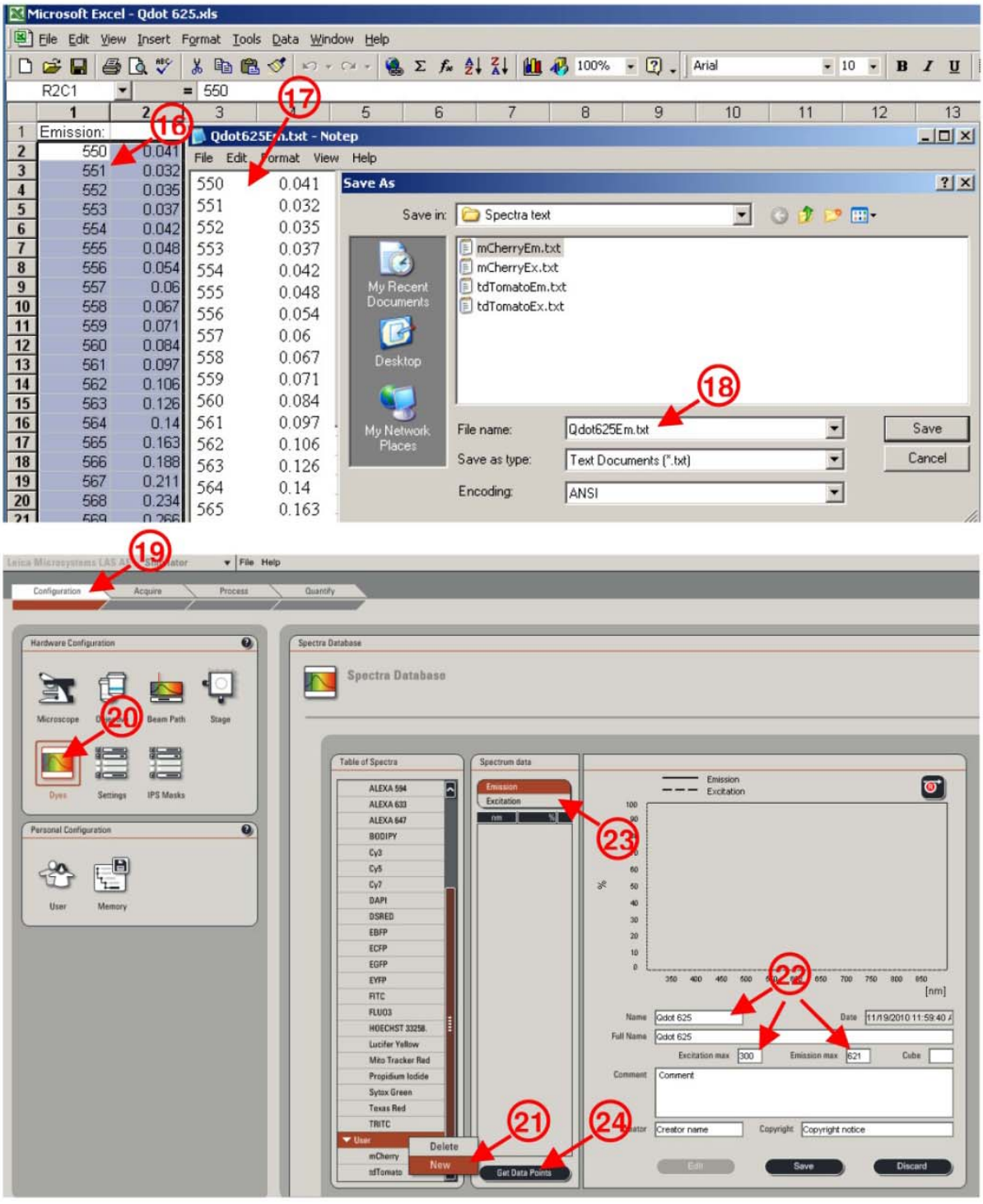
9. Under Delimiters (11), check the 'Tab' and 'Semicolon' boxes and then click 'Finish' (12).



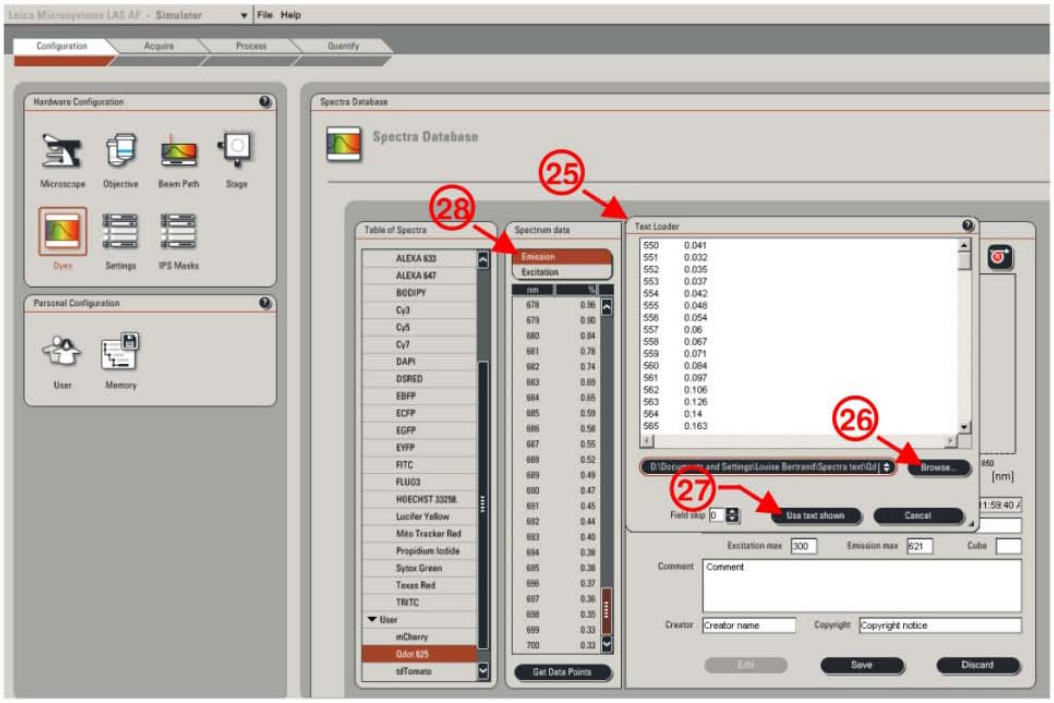
10. Select column 2 (column shows in blue when selected), go to Insert (13) and choose Columns. A new column will be inserted between column (wavelength) and column 2 (values).
    In this new column, multiply the emission-absorption values (column 3) by 100. Do so by writing in the tab (14) '=(select column 3, row 2)*100' and hit enter (15). Copy this first result (cell) and paste it into all the other cells below.



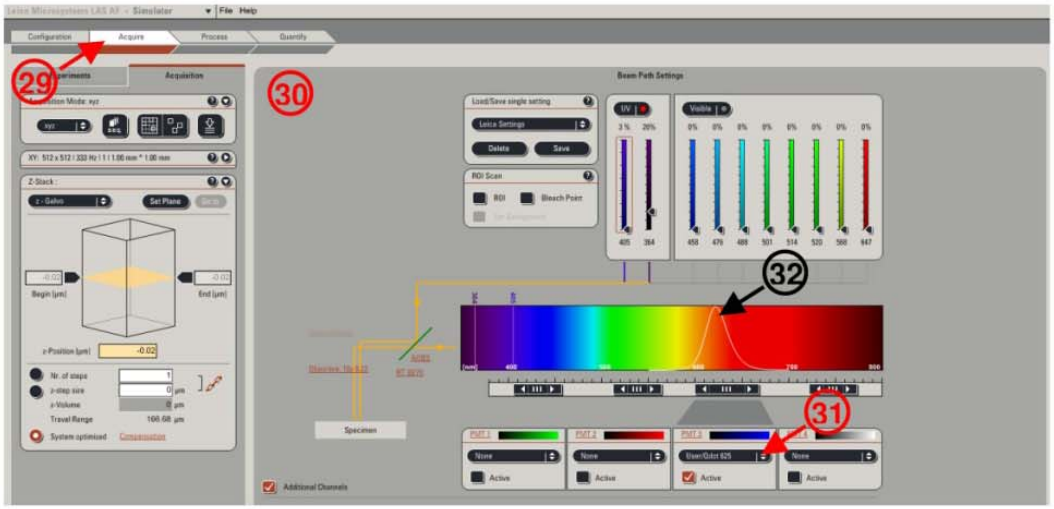
11. Save your document as the fluorophore.xls (In this example, Qdot 625.xls).
12. Open your Microsoft Notepad (Start > Accessories > Notepad).
13. From your Excel sheet, copy the Emission wavelength (column 1), and the values multiplied by 100 (column 2) (16) (both columns will be highlighted blue), and paste them onto your Notepad (17). Save your document as the FluorophoreEm (Qdot625Em.txt) (18).
14. Repeat for the Absorption (Excitation).
15. Open LAS AF.
16. Select the Configuration tab (19) > Dyes (20).
17. Under 'User' right-click, select 'New' (21), enter the name of the fluorophore (Qdot 625), the excitation peak value (300) and emission peak value (621) in the corresponding boxes (22).



18. Select 'Excitation'(23) and click 'Get Data Points'(24). The 'Text Loader' window will come up.
19. In the 'Text Loader' window(25), click Browse(26), select your fluorophoreEx.txt, click 'Open' and click 'Use text shown'(27). Click Save.



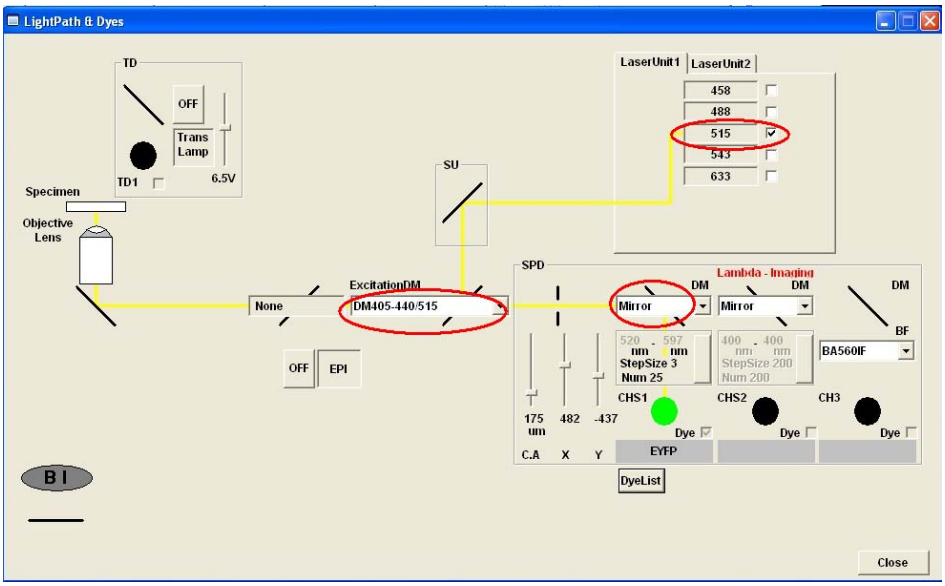
20. Select Emission (28), click Edit, click 'Get Data Points'. The 'Text Loader' window will come up. Repeat step 19 for loading the Emission data points and click 'Save'
21. Go to Acquire (29) > Beam Path Settings (30) > PMT and select your fluorophore from the drop down menu (31) 'User/Qdot 625'. The selected fluorophore emission curve will show up in the spectra window (32).



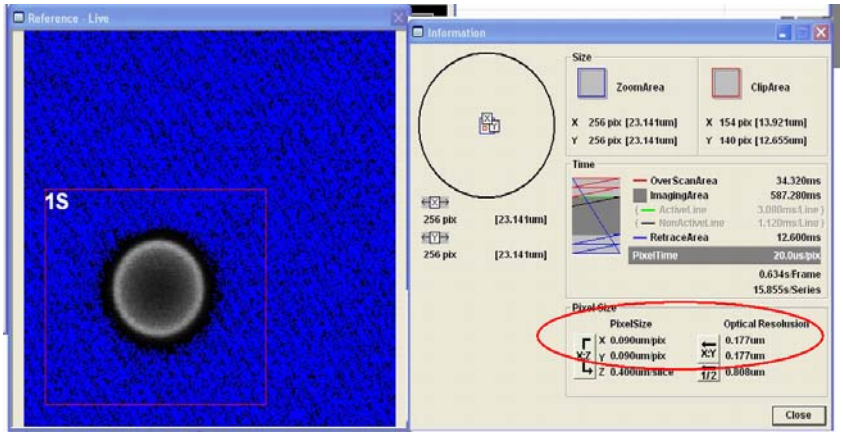
Spectral deconvolution (unnmixing) protocol – Olympus FV1000

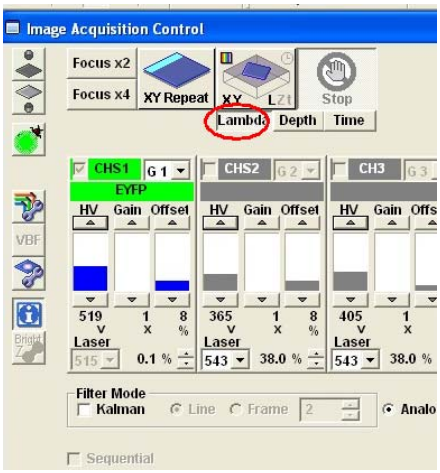
1) Start or restart the confocal software.

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 ½ of the optical resolution). You can use the ClipScan feature to scan a smaller rectangular area that contains the bead without affecting image sampling .





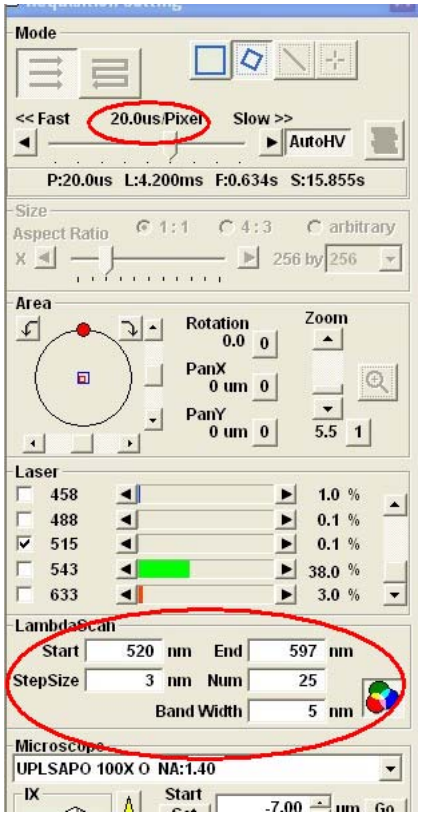
4) **Lambda settings:** Activate the "Lambda" button in the Acquisition control and adjust detection parameters:

Offset 8

Gain 1

HV about 500

515 nm laser power 0.5% or less



Set scanning speed to 20 µs/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 rights  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 nackground outside the bead.

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

7) Unmixing:

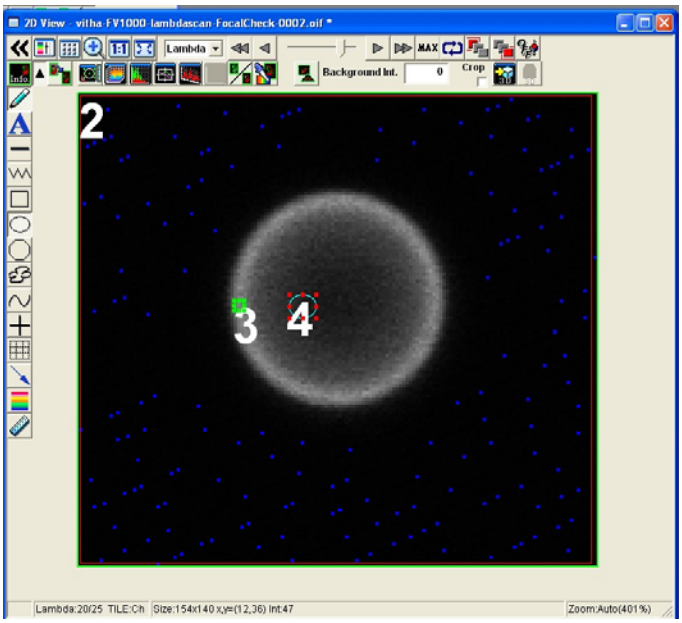
Menu commands "Processing" – "Spectral Deconvolution"

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.

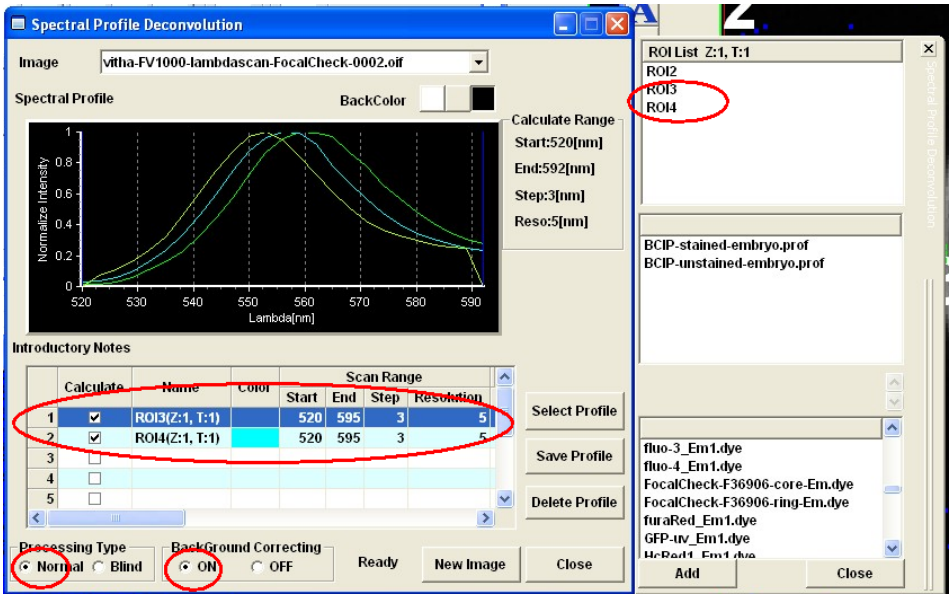


In the Spectral Profile Deconvolution window, select the ROI3 and ROI4 from the ROI list in the table on the right and click “Add” or double click on each.

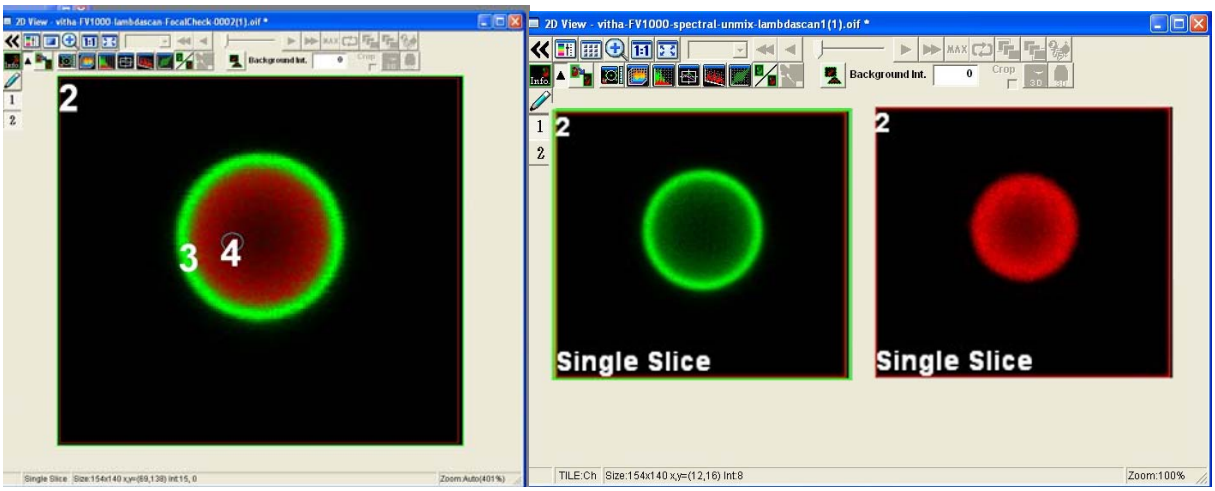
The two ROIs are now listed in the introductory notes below the graph, “Calculate” for both is checked.

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

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



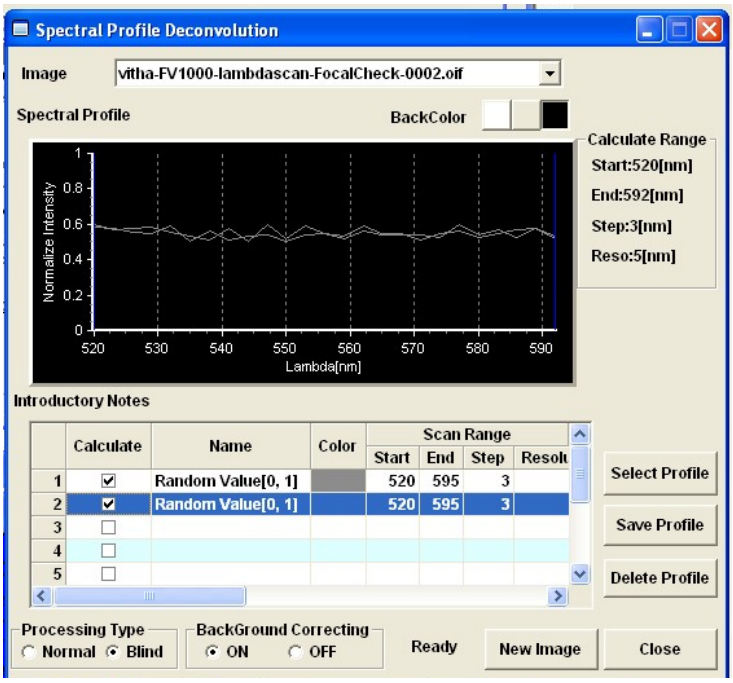
Click New Image. A spectrally unmixed image will be created:



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

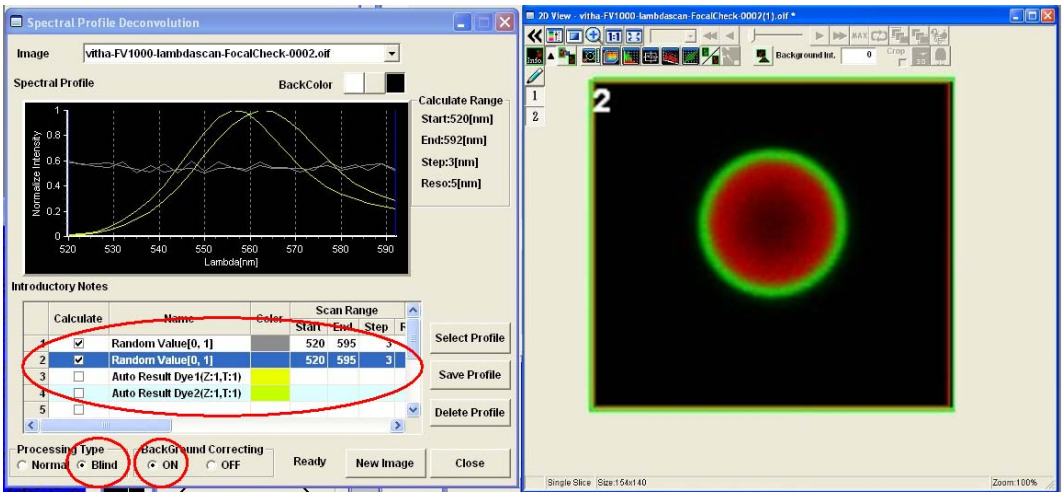
This method does not require reference spectra or ROIs containing the single dyes.

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:

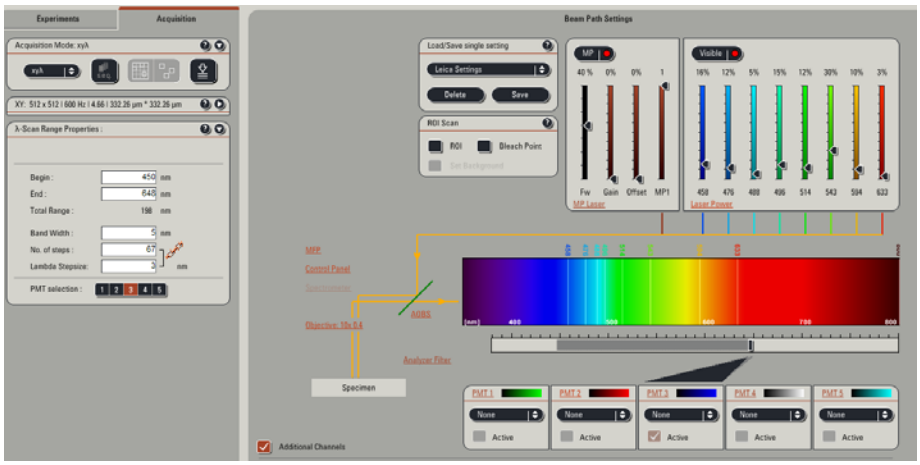


Great thanks to Stanislav Vitha for this protocol.

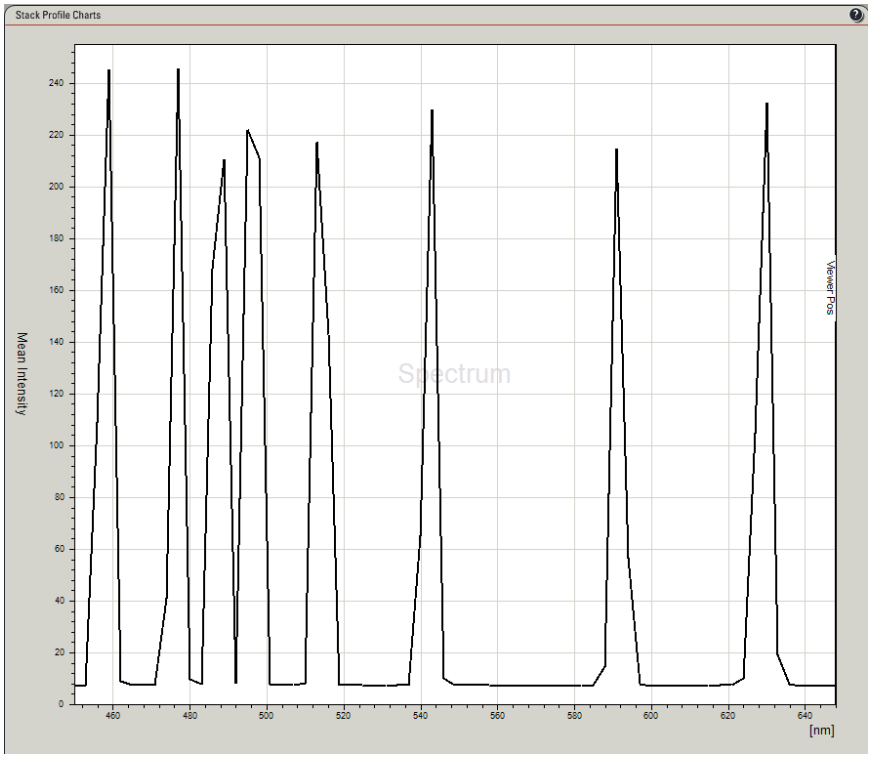
***9)* Send the following information to the ABRF-LMRG at abrf.lmrg@gmail.com:**
a) Sample image as above of the separated data in 2D or 3D.
b) Image Z-stack of a single bead from the unmixed data stack
Please indicate the following
i) objective used
ii) pixel size
iii) laser power
iv) platform the images were collected on
v) **software** *and the method used for the un-mixing.*

Using LASER as standards for spectral detection

Use the provided mirror slide w/lower magnification lens (i.e., 10x).
1.) Focus first on the edge of the mirror, either via the microscope or directly on the confocal. This will make it easier to find focus.
2.) Setup spectral detection; vary the LASER power to achieve similar output at a single gain setting.



On a Leica systems, as with most system, there is enough LASER light collected to over come any ND blocking.



This is the plot of all 8 of our visible LASER lines. The absolute height of the peaks is irrelevant; the FWHM is what determines the resolution of the system