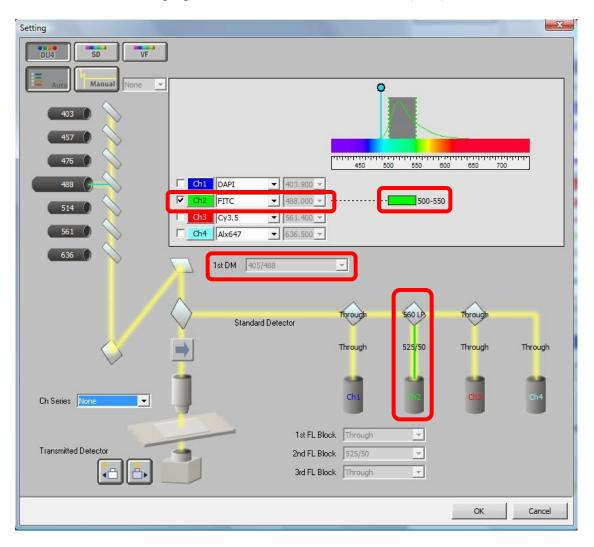
1 Set up the confocal light path for imaging a green dye (FITC) under the Setting window use the Auto mode. For example, the light path as shown here using the 488 nm LASER with appropriate beam splitter (405/488), a secondary 560 LP beam splitter, and a 525/50 emission filter detecting light from 500-550 nm in channel 2 (Ch2).



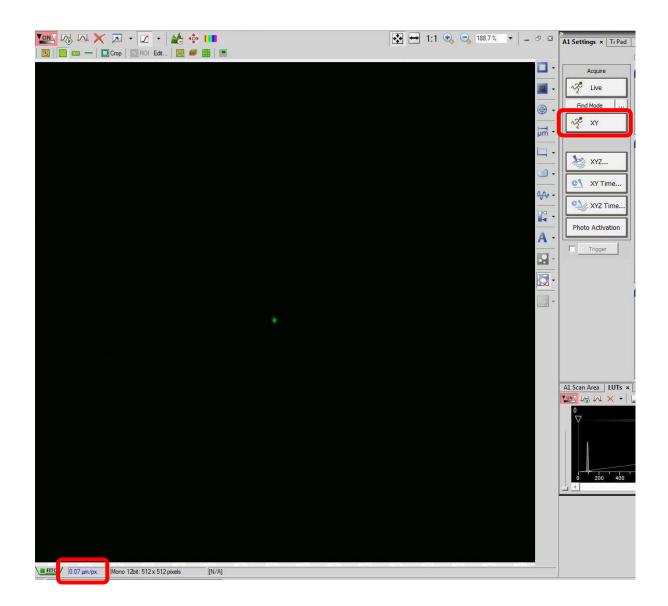
2 Under the Acquisition window set the detector to high sensitivity (HV=120). CRITICAL STEP: The Offset must be set below zero (-7) so that no pixel reads zero intensity units. Set the LASER Power to 0.6% and click the Home button to set the Pinhole to 1.0 Airy Units (A.U.).

Laser Power Monitor	AG B	HV Linear Correction
V Ch Z FITC HV 4 CONSISTENT Offset 4 CONSISTENT Laser 4 CONSISTENT	Laser 488.0 ► 120 ► -7 ► 0.6 0.0	
Pinhole 488.0 Home Home Home Home Dickness of optical section : 0.37 u Dictal Resolution : 0.10 um	⊃ ▶ 1.0 A.U. <- 29.4 um	TD HV ◀ Offset ◀

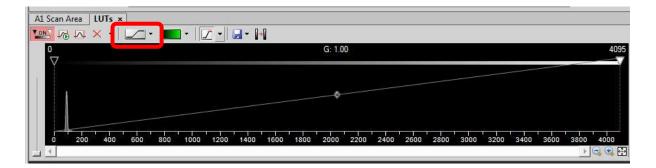
- 3 CRITICAL STEP: Under the Scan setting window set the Scan Direction to unidirectional scanning –indicated by two arrows pointing to the right. If not properly calibrated bidirectional scanning can generate image artifacts.
- 4 Set the Scan Size to 512x512, Scan Speed to 1/4 or so (Pixel Dwell of 5-25 μs), Line Average/Integrate to Average with a Count of 4 to reduce pixel noise, Line Skipping to None, and a Zoom factor of 6 (to get a pixel size of 70 nm with 60x NA 1.4 objective). The pixel size is indicated on the bottom left of each image.

Scan setting				9
Scan Direction	A		Zoom	5.988
Scan Size	512 🔹 🛨	512 512 recommend		
Scan Speed	1/4 •	Frame/sec(Pixel Dwell:9.9 u sec)		3.994x recommend
Line Average/Integrate	Average 💌	Count 4		
Line skipping	None			

5 Hit the **XY** button to take an image of the microspheres and verify the pixel size in X and Y is small enough to sample the PSF properly. Refer to Table 1 in the main article.

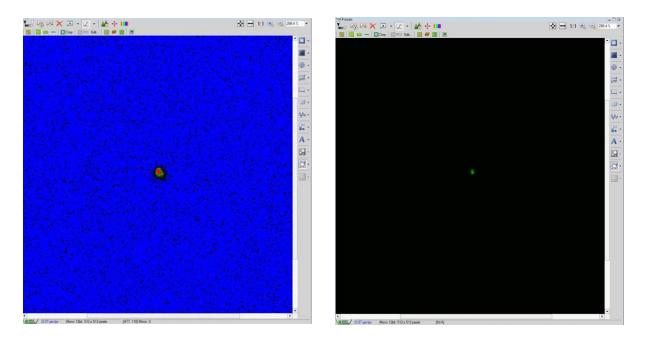


6 CRITICAL STEP: It is important when collecting confocal data that no image pixels read zero intensity units and no pixels show a saturated signal. If this is the case the intensity data will be clipped off and will not be accurate. Verify the image acquisition settings using the **oversaturated and undersaturated pixels** options. Under the **LUTs** tab click the button showing a sloped plot on a graph to activate the visual indication of saturated pixels.



You can choose the color for **Oversaturated pixels** and **undersaturated pixels** by clicking on the arrow on the right of the button. In this example, undersaturated pixels (zero intensity) are chosen to be **Blue** and oversaturated pixels are chosen to be **Red**. For ideal acquisition settings you should have no blue or red pixels. If there are blue pixels increase the **Offset**, if there are red pixels reduce the **LASER power**.

₩ xyz	Laser Power Monitor	AG 🖉	HV Linear Correction
	Ch 2 FITC	Oversaturated pixels	
C1 XY Time		None	
XT Time	HV I III	Complementary Color	
	Offset 🖪 🛲	Red	
SYZ Time		Green	
		Blue	
Photo Activation	Pinhole		ר □ □
	Home	Yellow	
Trigger	488.0 💌 🔤	Magenta	offset
	thickness of optical sectio	Cyan	
	Optical Resolution : 0.10	White	1
	A 100	Undersaturated pixels	
	Scan setting		•
		None	Zoom
	Scan Direction 📑 🛛	Complementary Color	< > 5.988
	Scan Size	Red	commend 🐹 🖸
		Green	Dwell:9.9 u sec) 3.994x recommend
	Scan Speed	Blue	Dwell:9.9 u sec) 3.994x recommend
	Line Average/Integrate	Yellow	
	Line skipping	Magenta	
		Cyan	
A1 Scan Area LUTs ×	ī	White	
	<u></u> • <u>_</u> +		
0		G: 1.00	409
Υ			
		¢.	
0 200 400 6	500 800 1000 1200 140	0 1600 1800 2000 22	00 2400 2600 2800 3000 3200 3400 3600 3800 4000
4		1000 110	

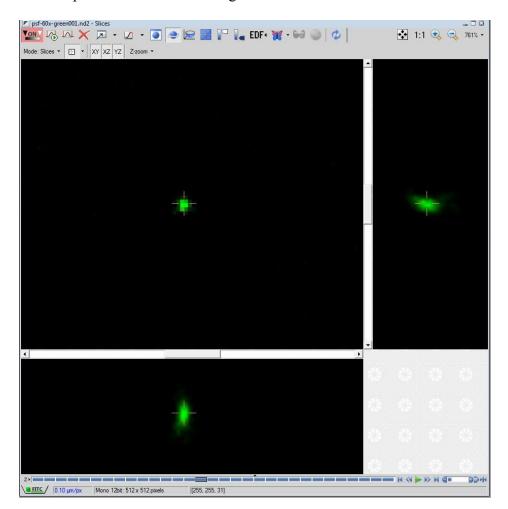


- 7 See the main protocol paper for details on how to properly set the Z-image spacing. Choose the ND Acquisition tab, choose Z-series and set up the Z-axis scanning. Start scanning by using the Live or Find mode buttons and set the Z-Series options in one of two ways:
 - A Bottom/Top: Focusing
 below the microsphere(s)
 of interest and marking the
 first plane when you see
 no intensity in the image
 by clicking the Bottom
 button. Then focus above
 the microsphere(s) of
 interest and mark the last
 plane when you see no
 intensity in the image by

U Juve	to File		
Path:	G:\users\fw\psf-09-05-11	Browse	
Filename	: psf-60x-green003.nd2	Record Data	
T Z	Bottom]]	
Step: 0. Range: 3.		295	

clicking the Top button.

- B Center: Focus on the centre of the microspheres and click on the Home button. Then click on the Relative button. Enter the total number of steps to image or the Step size in μm and the total Range. Ensure there are enough images to go well above and below the microspheres to image planes where essentially no signal is detected.
- 8 Check the **Save to File** option and choose the right **Path** and **Filename** to save your data as .nd2 files.
- 9 Perform the Z-Stack acquisition by clicking on the Run now button. Select the X-Y, X-Z and Y-Z image displays so you can verify with the orthogonal viewer that the sampling is sufficient to capture the entire PSF image.



- **10** Save all your files with your name and the name of the instrument you collected the data on. Send the following information to the ABRF-LMRG at <u>abrf.lmrg@gmail.com</u>:
 - a) Summary of the measured resolution in X,Y,Z for at least 5 microspheres measured with the pinhole set to 1 Airy Unit.
 - b) One representative MetroloJ report for data collected with the pinhole set to 1 Airy Unit.
 - c) Summary of the measured resolution in X,Y,Z for at least 5 microspheres measured with the pinhole set to 5 Airy Units.
 - d) One representative MetroloJ report for data collected with the pinhole set to 5 Airy Units.