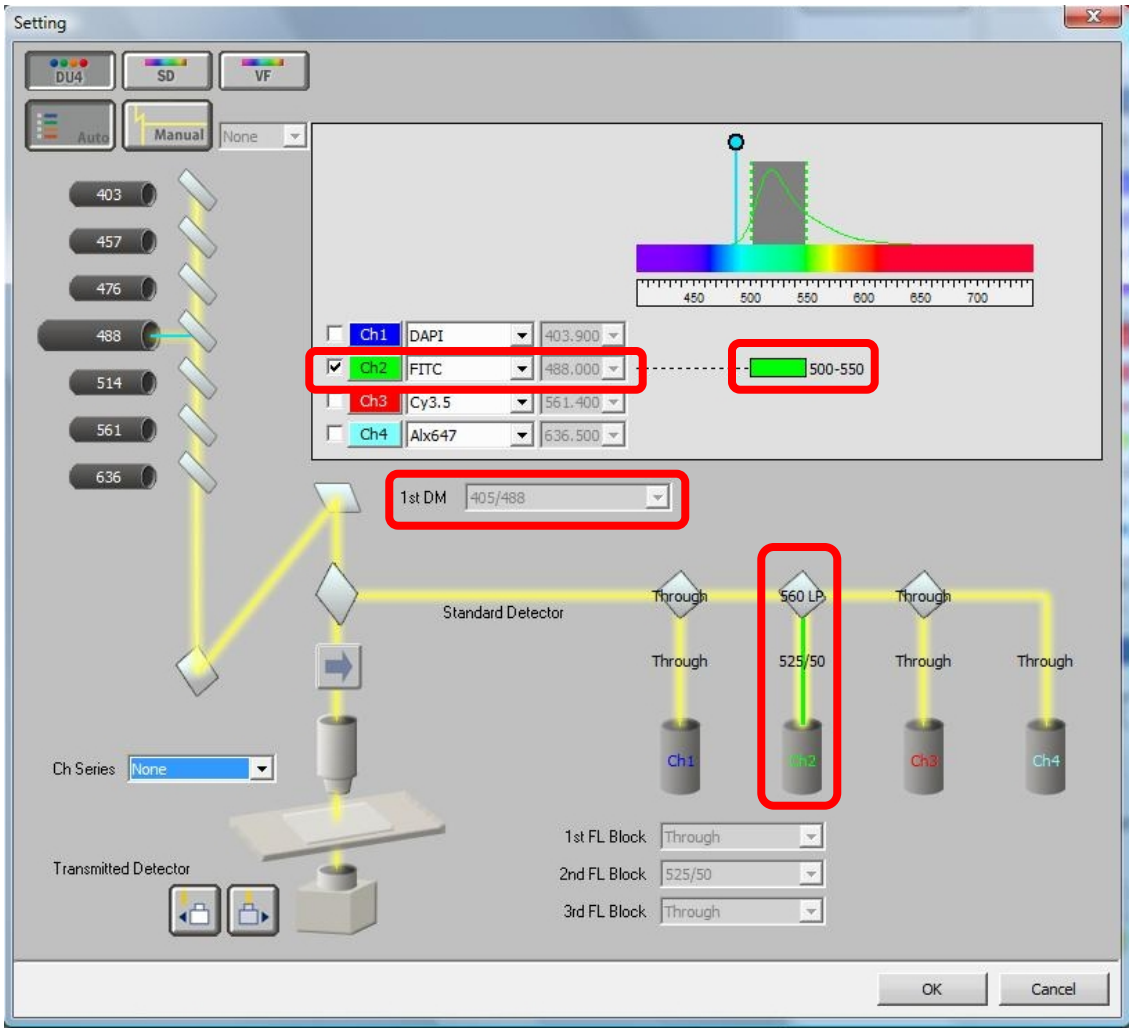
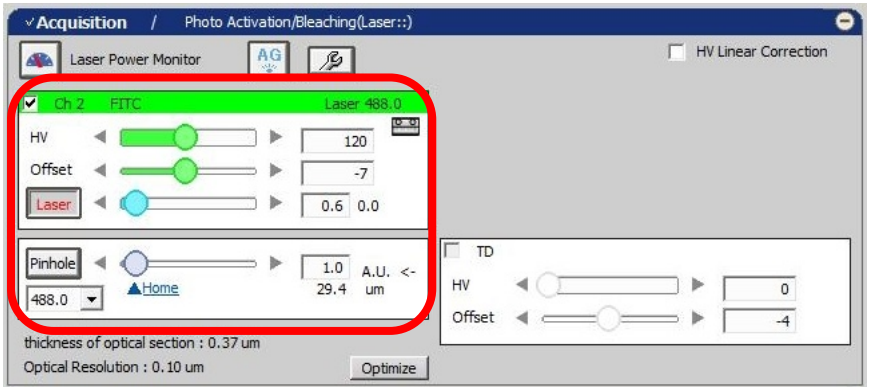
1 Set up the confocal light path for imaging a green dye (FITC) under the **Setting** window use the **Auto** mode. For example, the light path as shown here using the 488 nm LASER with appropriate beam splitter (**405/488**), a secondary **560 LP** beam splitter, and a **525/50** emission filter detecting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

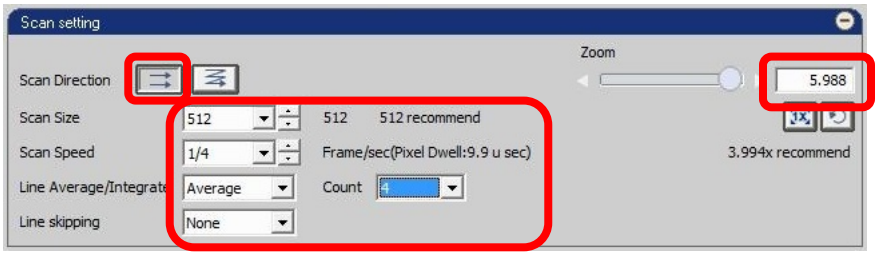


2 Under the **Acquisition** window set the detector to high sensitivity (**HV=120**). CRITICAL STEP: The **Offset** must be set below zero (**-7**) so that no pixel reads zero intensity units. Set the **LASER** Power to **0.6%** and click the **Home** button to set the **Pinhole** to 1.0 Airy Units (**A.U.**).

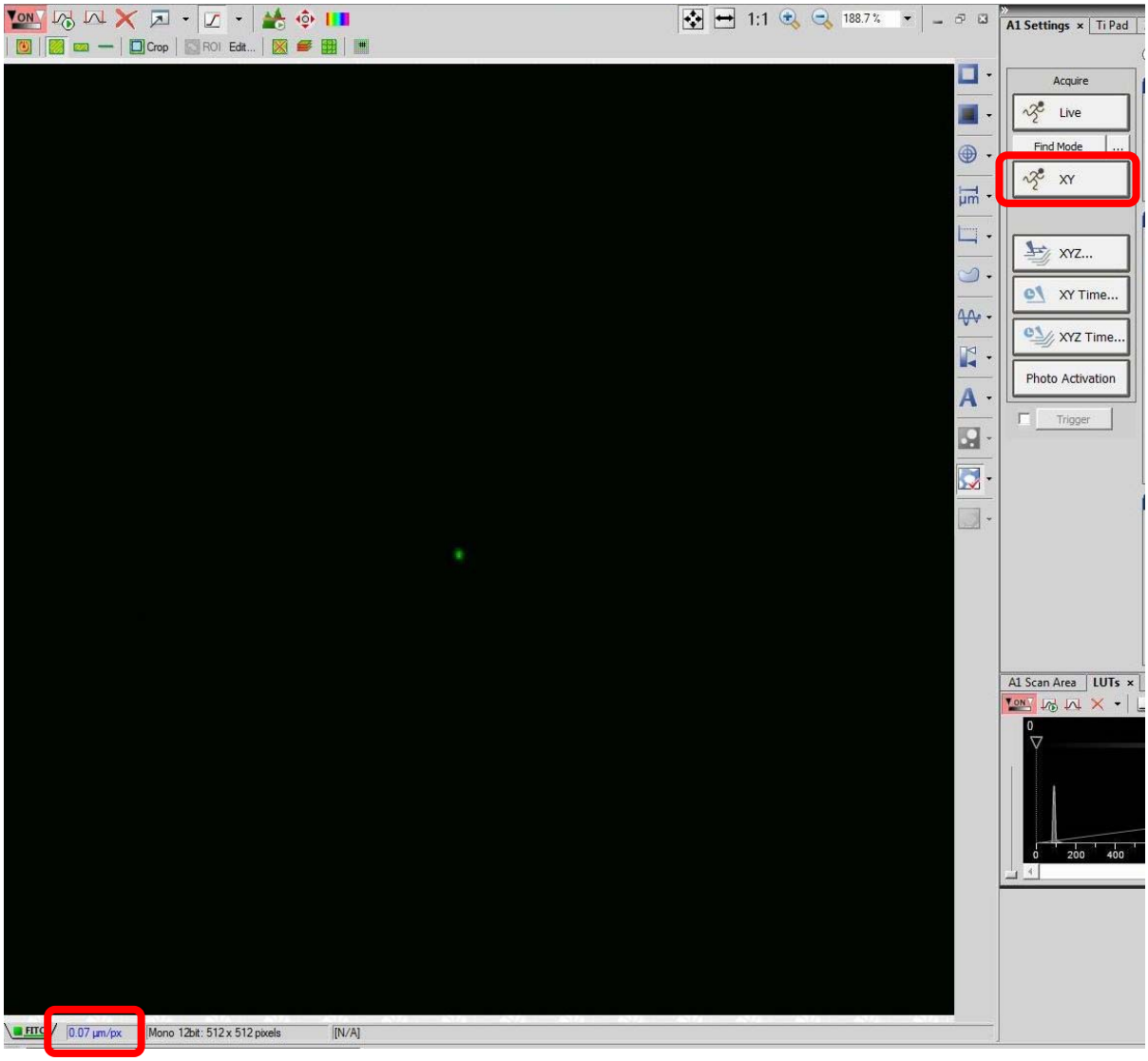


3 CRITICAL STEP: Under the **Scan setting** window set the **Scan Direction** to unidirectional scanning –indicated by two arrows pointing to the right. If not properly calibrated bidirectional scanning can generate image artifacts.

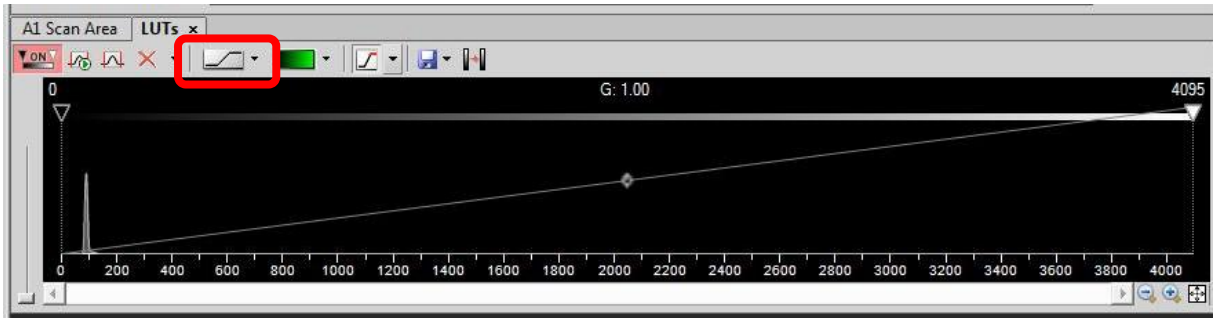
4 Set the **Scan Size** to 512x512, **Scan Speed** to 1/4 or so (**Pixel Dwell** of 5-25 µs), **Line Average/Integrate** to A**verage** with a **Count of** 4 to reduce pixel noise, **Line Skipping** to **None**, and a **Zoom** factor of 6 (to get a pixel size of 70 nm with 60x NA 1.4 objective). The pixel size is indicated on the bottom left of each image.



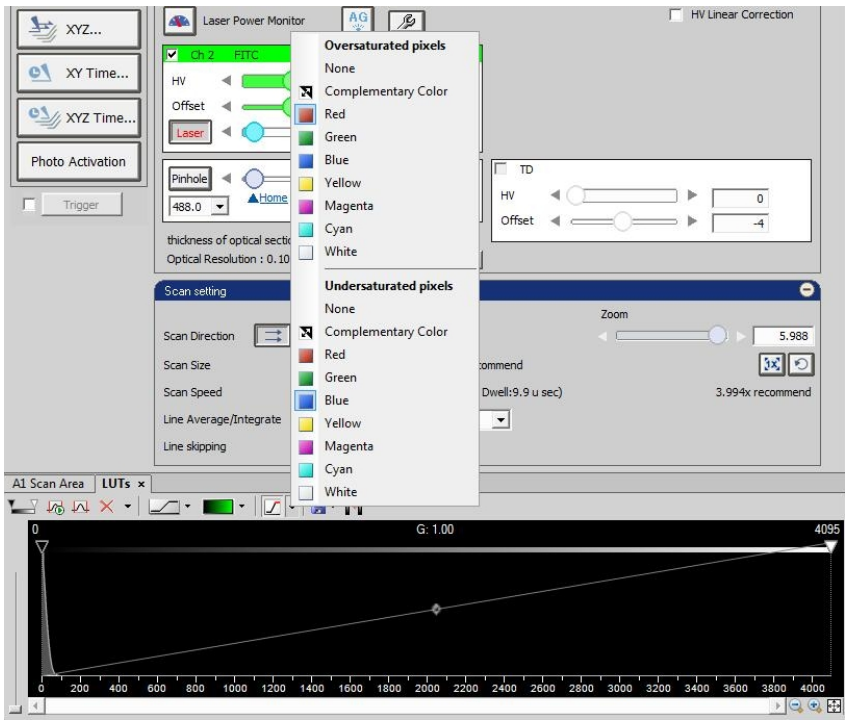
5 Hit the **XY** button to take an image of the microspheres and verify the pixel size in X and Y is small enough to sample the PSF properly. Refer to Table 1 in the main article.

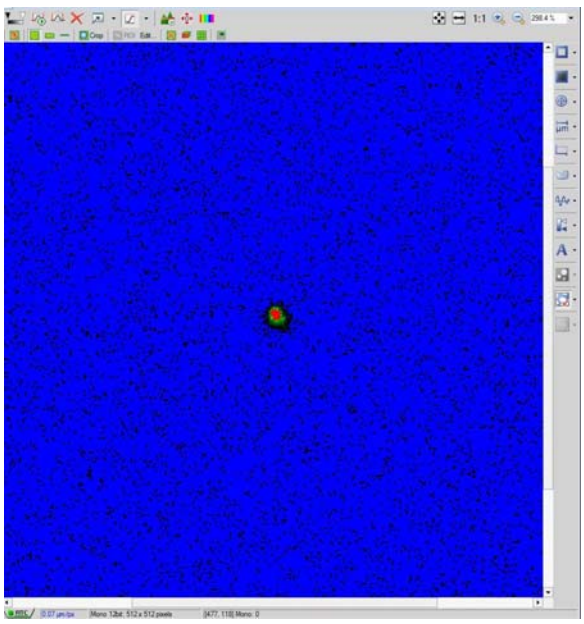


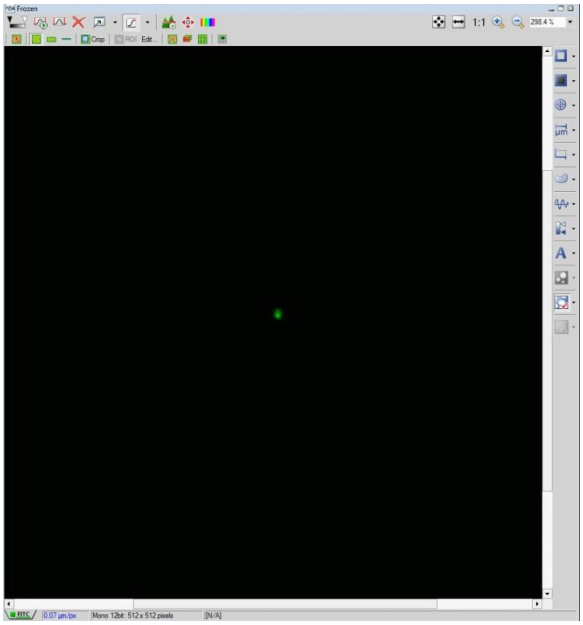
6 CRITICAL STEP: It is important when collecting confocal data that no image pixels read zero intensity units and no pixels show a saturated signal. If this is the case the intensity data will be clipped off and will not be accurate. Verify the image acquisition settings using the **oversaturated and 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**.

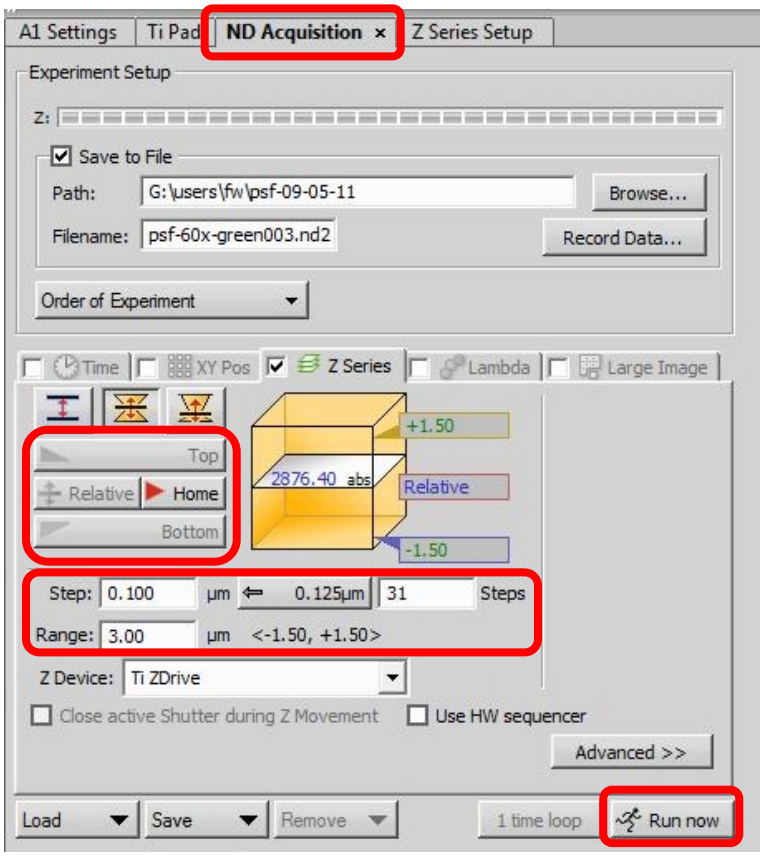






7 See the main protocol paper for details on how to properly set the Z-image spacing. Choose the **ND Acquisition** tab, choose **Z-series** and set up the Z-axis scanning. Start scanning by using the **Live** or **Find mode** buttons and set the **Z-Series** options in one of two ways:

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

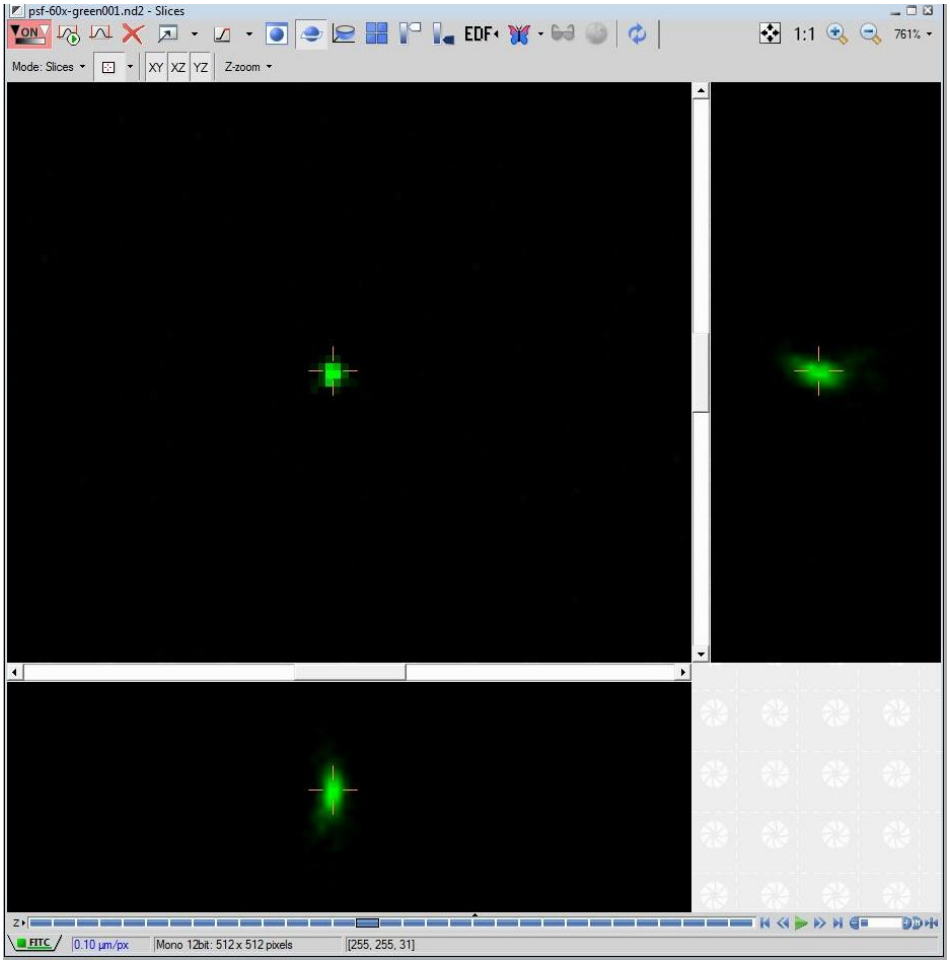


clicking the **Top** button.

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 abrf.lmrg@gmail.com:

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.