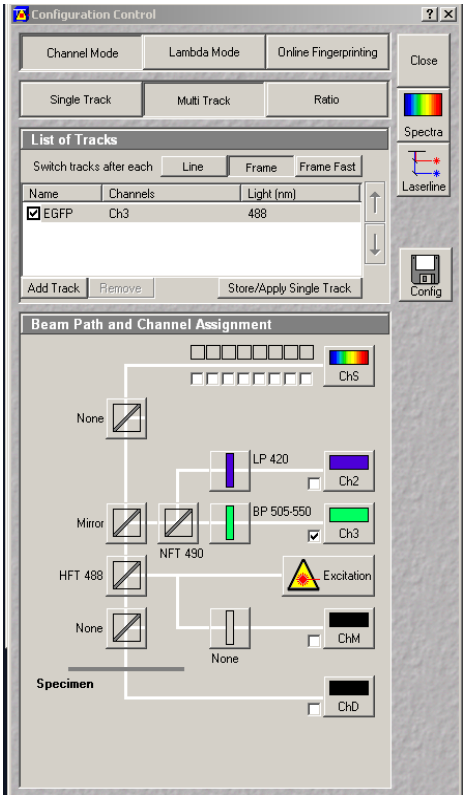
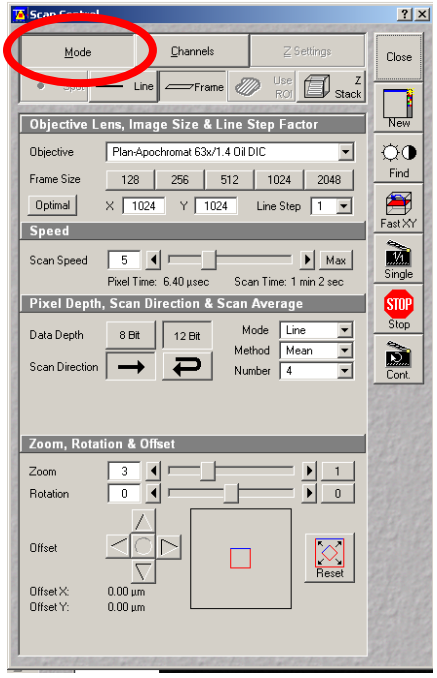
1 Set up the confocal light path for imaging a green dye (e.g. Alexa488-EGFP). For example, under the **Configuration Control** window the light path could be set up as shown here using the 488 nm LASER (found under **Excitation**) reflecting off of the 488 nm Main Beam Splitter (**HFT 488**) and collection of the emission light from **~505-550 nm** using a band-pass filter (**BP 505-550**).



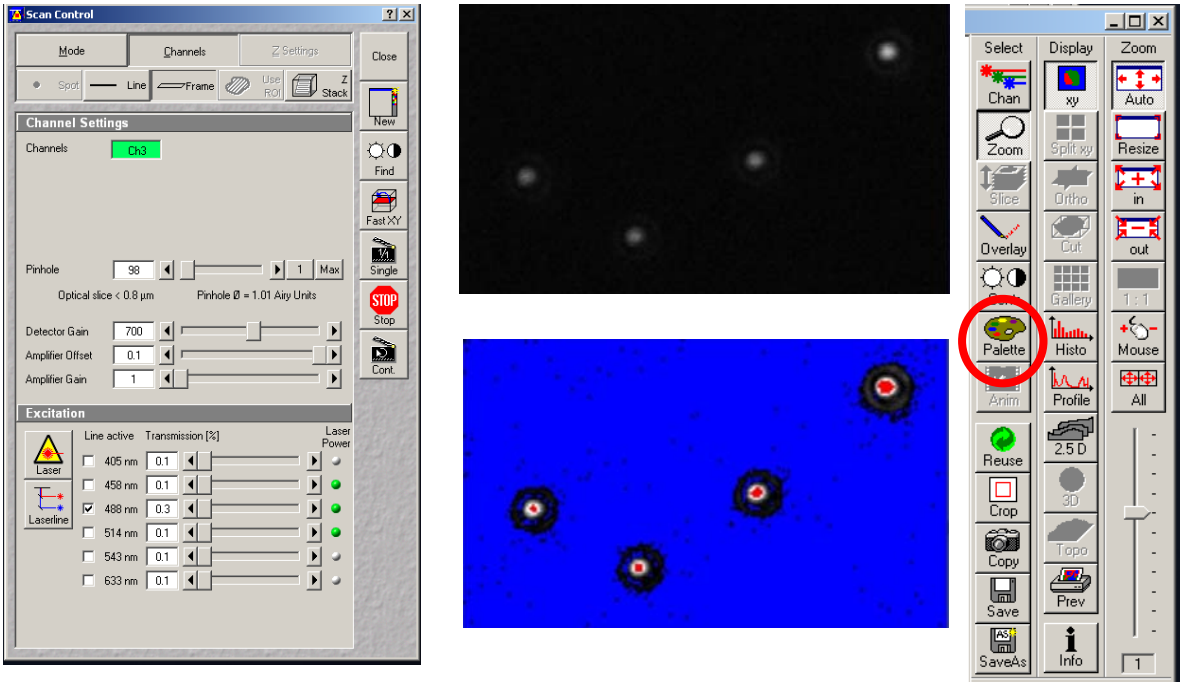
2 Under the **Scan Control** window choose the **Mode** button. Set the image acquisition in **Frame** scan mode, **Frame Size** of 1024x1024 pixels, **Line Step** of 1. Scan **Speed** of 5-9, mean line averaging of 4 to reduce pixel noise, **Bit Depth** of 12 Bit and a **Zoom** factor of 3. CRITICAL STEP: Set the instrument for unidirectional scanning – **Direction** indicated by an arrow pointing to the right. If not properly calibrated bidirectional scanning can generate image artifacts.



3 Choose the **Channels** button and set the **Channel Settings**. Set the **Pinhole** to 1 Airy unit.

4 Set the PMT detector for high sensitivity, **Detector Gain** = 700-800.

5 CRITICAL STEP: The **Amplifier Offset** must be set above zero (~0.1) so that no pixels read zero intensity units. Select the **Palette** button on the side of the image and choose the **range indicator** LUT. Adjust the offset until no pixels read zero (shown by blue pixels).

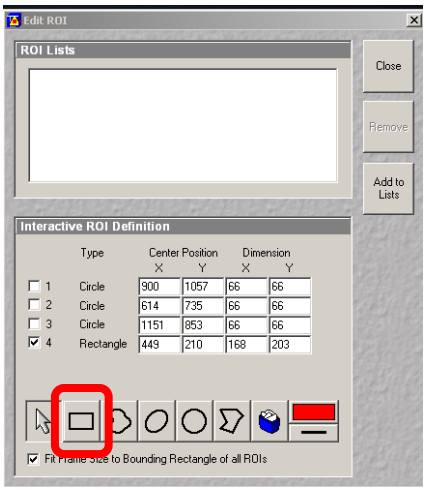


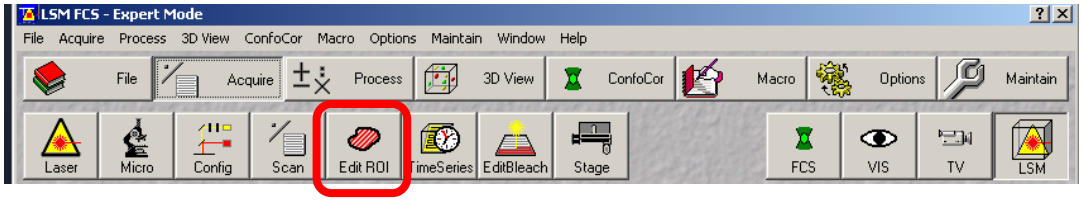
6 Set the **Amplifier Gain** to 1.

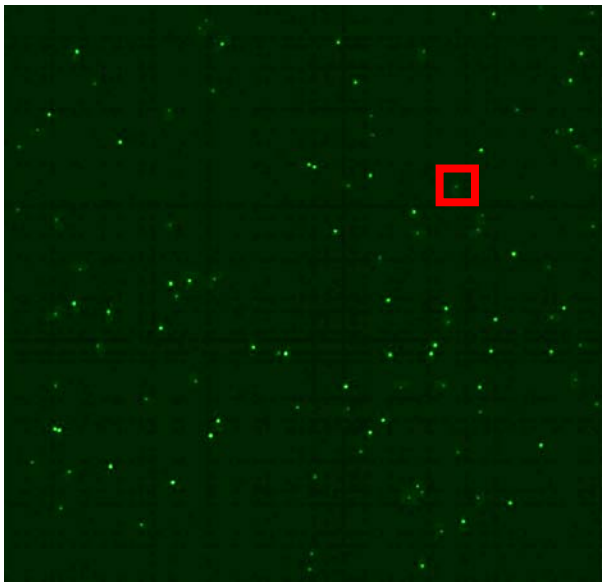
7 Choose the 488 nm LASER line and set the LASER power to ~0.3% (~6 µW). If the LASER power is set too high then pixels will saturate (shown as red pixels with the **range indicator** LUT) and high intensity data clipping will result in non-quantitative data. Adjust the LASER power so no pixels are measuring saturated intensity. If minimum LASER power is reached adjust the **Detector Gain** voltage to a lower value until no red pixels are observed.

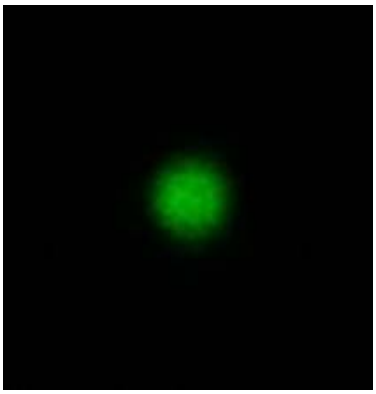
8 Press the **Single** button on the side of the **Scan Control** window and take an image of the microspheres.

9 Choose the **Edit ROI** button and then choose the **square drawing tool** in the window that pops up. Draw a rectangle around one microsphere. Assure you check the box to select to **Fit Frame Size to Bounding Rectangle of all ROIs** so that only the region containing the microspheres will be imaged during acquisition.







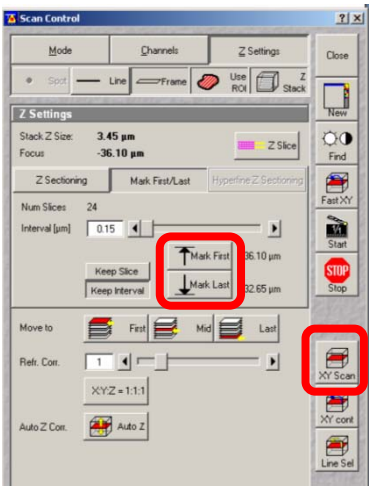


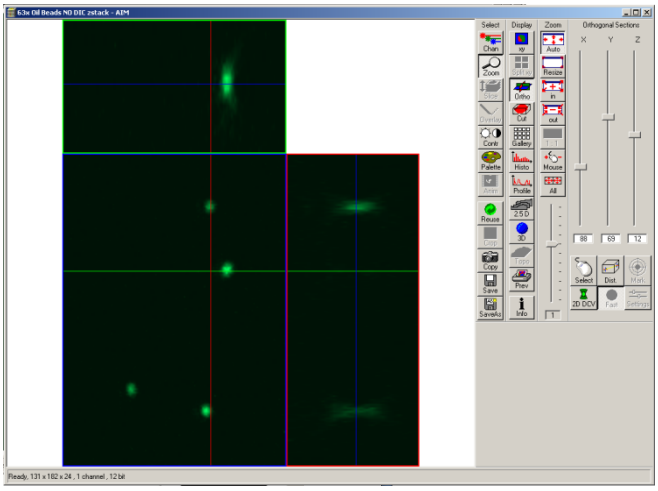
10 Verify the image acquisition settings using the **Range Indicator** Look Up Table (LUT).

11 See the main protocol paper for details on how to properly set the Z-image spacing. Under the **Scan Control** window choose the **Z-Settings** option and press the **Z Stack** button. Set up the Z-axis scanning parameters. Use the **Fast XY** or **Cont (Continuous)** scanning mode and set the **Z-Stack** options in one of two ways**:**

A **Mark First/Last:** Focusing below the microsphere(s) of interest and press the **Mark First** button marking the first plane when you see no intensity in the image. Then focus above the microsphere(s) of interest and press the **Mark Last** button and mark the last plane when you see no intensity in the image.

B **Z Sectioning:** Focus on the centre of the microspheres and click on the **Center** button. Enter the total number of **Slices** to be imaged. Ensure there are enough images to go well above and below the microspheres to image planes where essentially no signal is detected.





12 Perform the **Z-Stack** acquisition by pressing the **XY Scan** button.

13 Press the **Ortho** button to view the x-y, x-z and y-z image planes.

14 Save the data as .lsm files and also as .tif files.

15 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.