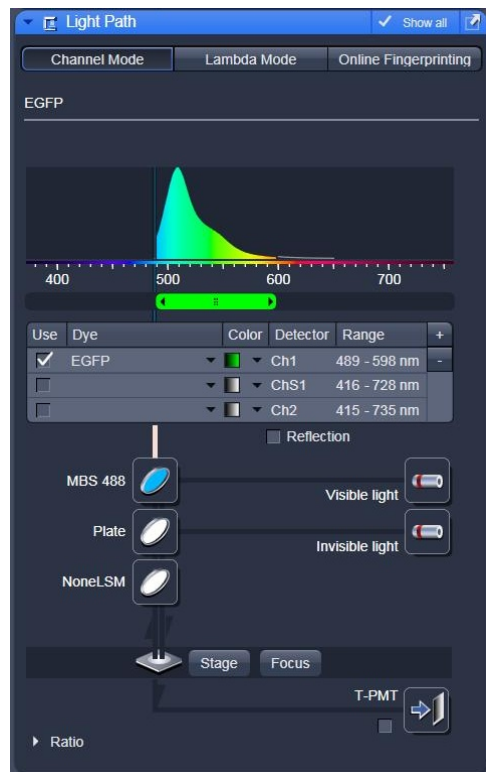


- Under the **Light Path** window set up the confocal for imaging a green dye (Alexa488-EGFP). For example, set up the light path as shown here using the 488 nm LASER (found under **Visible light**) reflecting off of the 488 nm Main Beam Splitter (**MBS 488**) and collection of the emission light from **~500-600 nm**.

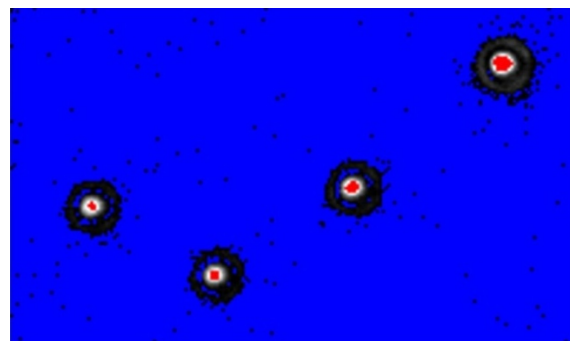
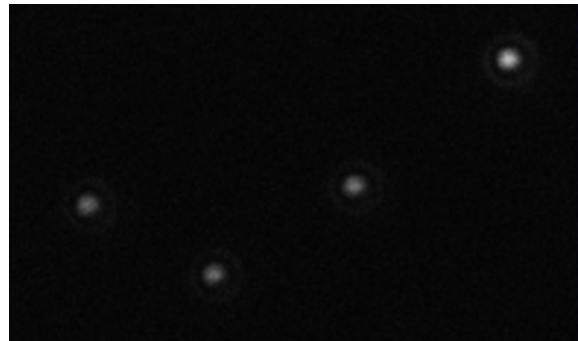


- In the **Acquisition Mode** window set the **Frame** scan mode, **Frame Size** of 1024x1024 pixels, **Line Step** of 1. **Scan Speed** of 5-9, mean line averaging of 4 to reduce pixel noise, **Bit Depth** of 12 Bit and a **Zoom** factor of 3. **CRITICAL STEP:** Set the instrument for unidirectional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



- Set the Channel detection PMT to - **Gain (Master)** = 700-800.

- 4 CRITICAL STEP: The **Digital Offset** must be set above zero (~12) so that no pixels read zero intensity units. If the offset is set too low pixels will read zero (shown by blue pixels below) clipping intensity data.



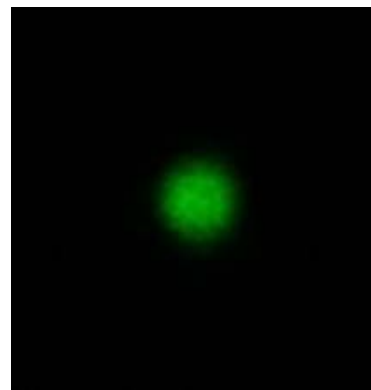
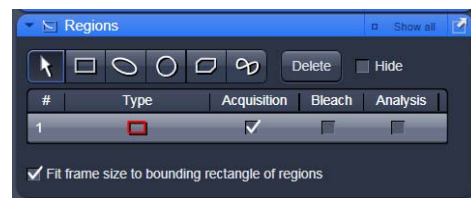
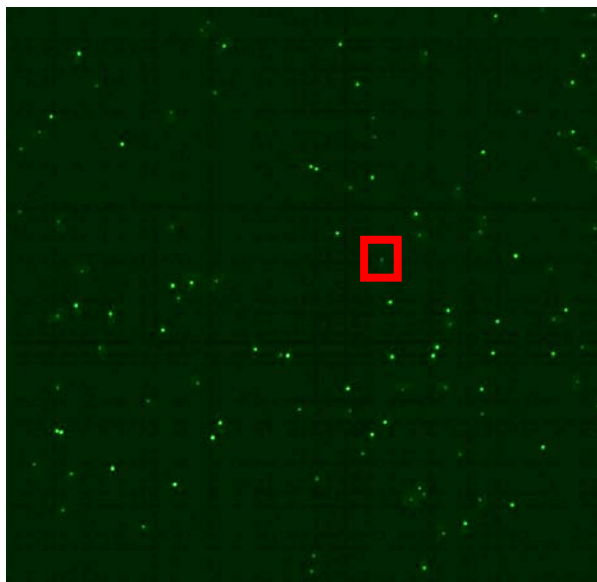
- 5 Set the **Digital Gain** to 1.
- 6 Choose the 488 nm LASER line and set the LASER power to ~0.5% (~8  $\mu$ W). If the LASER power is set too high (3.5%) then pixels will saturate (shown as red pixels above) and high intensity data clipping will result in non-quantitative data.

7 Set the **Pinhole** to 1 Airy unit (5 Airy units shown above).

8 Press the **Snap** button to take an image of the microspheres.



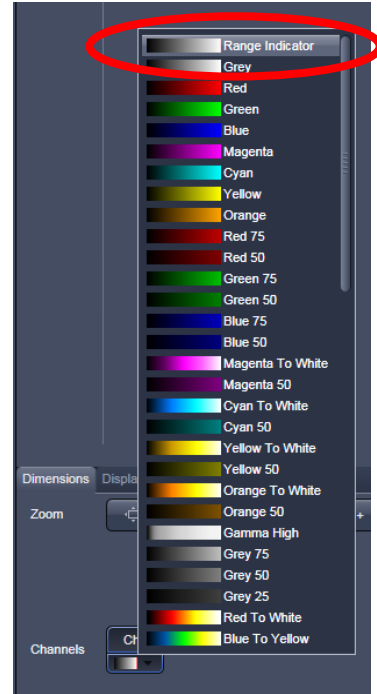
9 Under the **Regions** tab draw a rectangle around one microsphere. Unselect **Bleach** and **Analysis** and select **Acquisition**. Assure you check the box to select to **Fit frame size to bounding rectangle of regions** so that only the region containing the microspheres will be imaged during acquisition.



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

Click on the colour bar for each track in the image to change the colour. Under the **Dimensions** tab at the bottom of the image select the palette and choose the **Range**

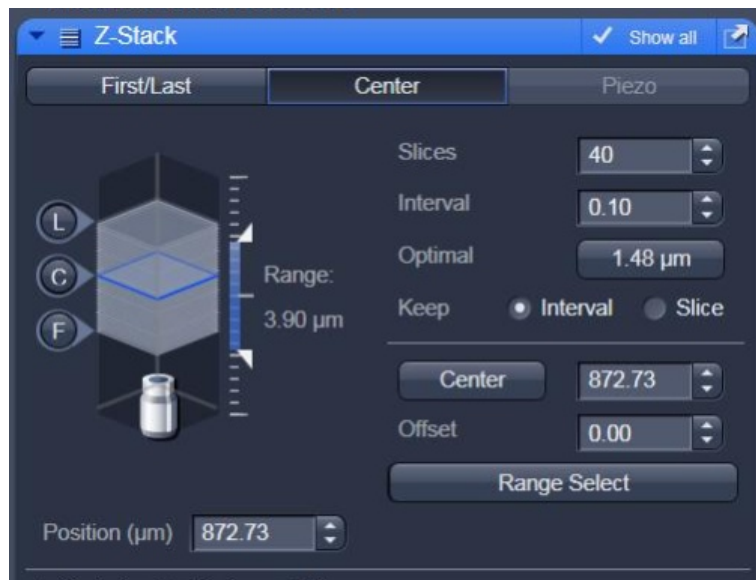
**Indicator** LUT (first in the list). Zero intensity pixels are blue and saturating are red. For ideal acquisition settings you should have no blue or red pixels. If there are blue pixels increase the **Digital Offset**, if there are red pixels reduce the **LASER power**.



- 11** See the main protocol paper for details on how to properly set the Z-image spacing. Choose the **Z-Stack** option and set up the Z-axis scanning. Use the **Live** or **Continuous** scanning mode and set the **Z-Stack** options in one of two ways:



- A First/Last:** Focusing below the microsphere(s) of interest and marking the first plane when you see no intensity in the image. Then focus above the microsphere(s) of interest and mark the last plane when you see no intensity in the image.
- B Center:** 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 Press the **Start Experiment** button to perform the **Z-Stack** acquisition.
- 13 Save the images as .lsm files and also as .tif files.
- 14 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](mailto: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.

