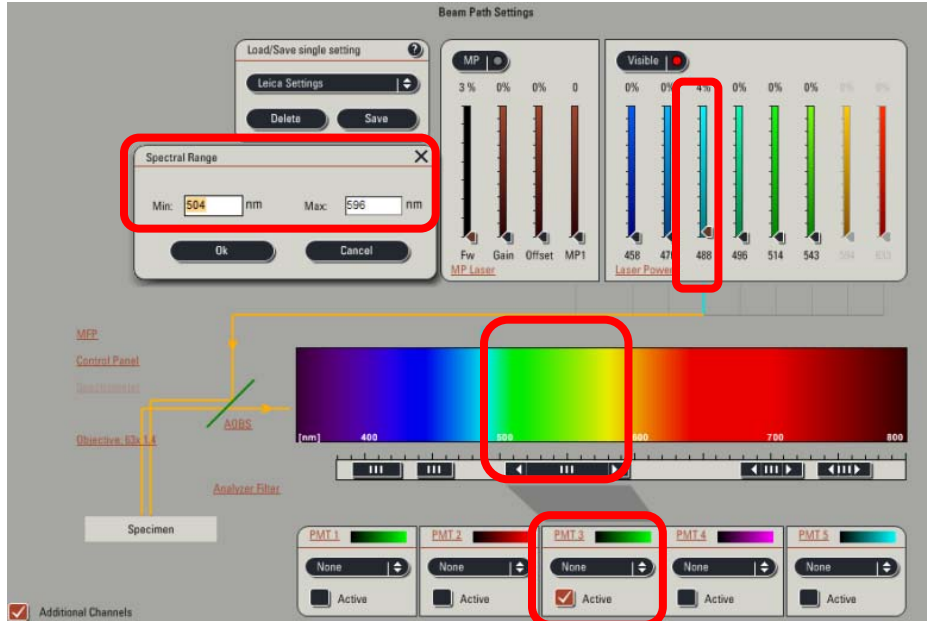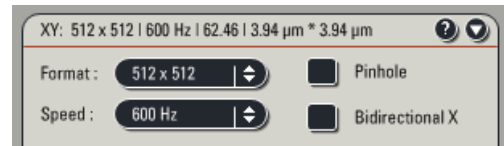**1** Under the **Beam Path Setting** window set up the confocal for imaging a green dye. For example, under **Visible** choose the 488 nm LASER, choose **PMT 3** and under **spectral range** collection of the emission light from **~504-596 nm**.

2 In the **Acquisition Mode** window set the **Format** to 512 x 512 pixels, **Speed** to 600 Hz, and unselect **bidirectional**. Set the **Line Average** to 4.

CRITICAL STEP: Ensure the bidirectional scanning is NOT selected because if it is not properly calibrated it can generate image artifacts.

3 Set the Channel detection PMT - **Gain** = 700-800.

4 CRITICAL STEP: The **Digital Offset** must be set above zero so that no pixels read zero intensity units (shown by green pixels) in order to avoid intensity data clipping. Saturated pixels will appear blue with this LUT.
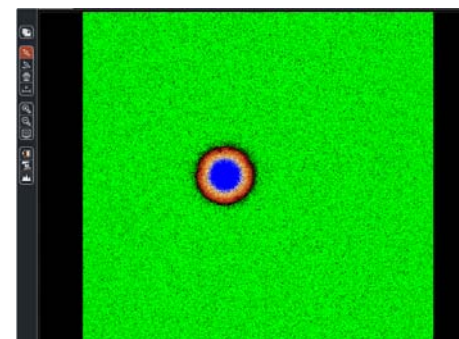
5   Choose the 488 nm LASER line and set the LASER power to ~0.5% (~8 µW). If the LASER power is set too high then pixels will saturate (shown as blue pixels above) and high intensity data clipping will result in non-quantitative data.

6   Set the **Pinhole** to 1 Airy unit and take an image of the microspheres.

7   Under the **Acquisition** tab check **zoom in,** then on the image pane draw a rectangle around one microsphere. This will result in image acquisition of only the region containing the microspheres. CRITICAL STEP: Verify that the **Pixel Size** is at Nyquist sampling, if not use the zoom factor to adjust. See details in the main paper.
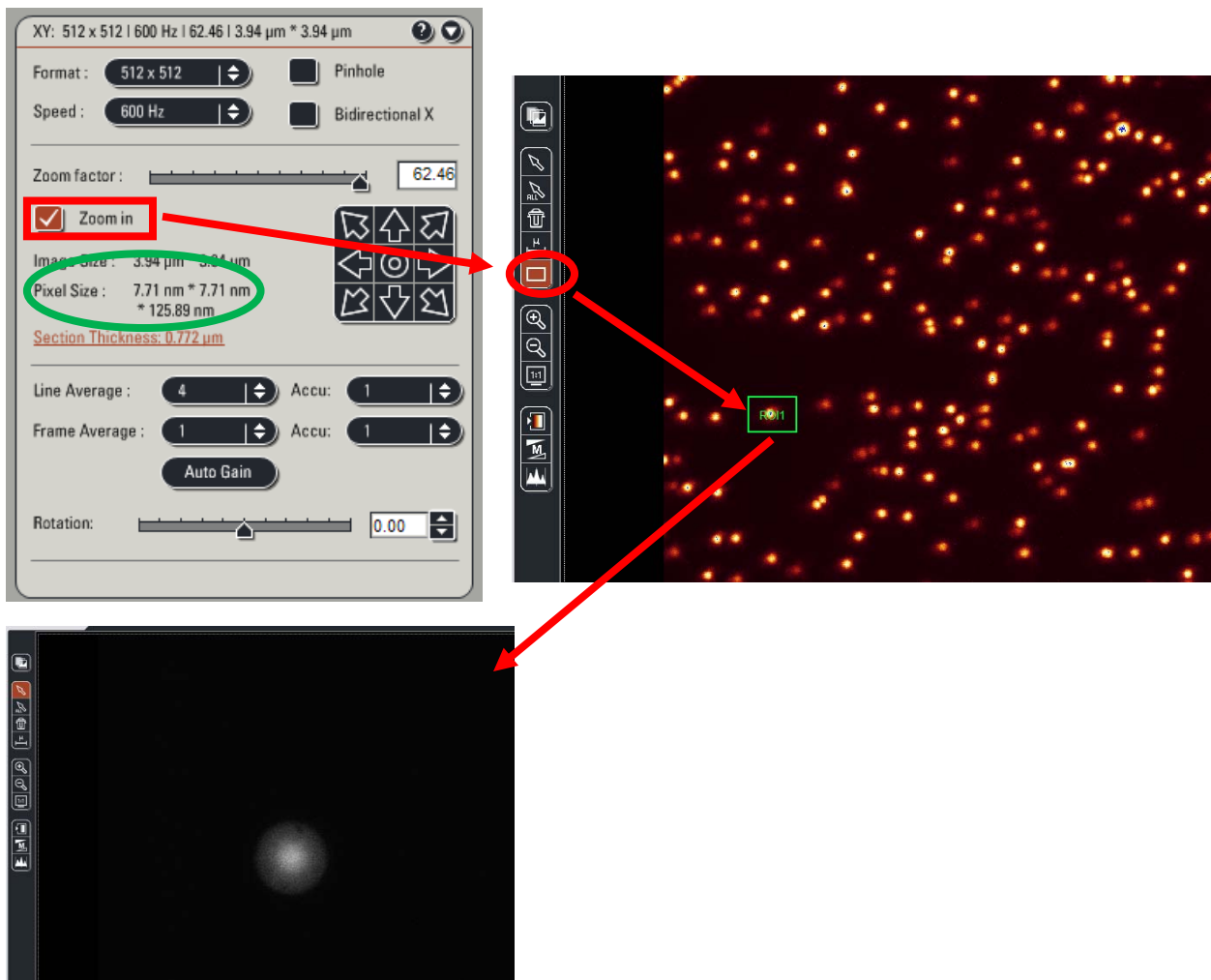
8 Verify the image acquisition settings using the **Range Indicator** Look Up Table (LUT). Zero intensity pixels are green and saturated pixels are blue. For ideal acquisition settings you should have no green or blue pixels. If there are green pixels increase the **Digital Offset**, if there are blue pixels reduce the **LASER power** or **PMT gain**.

9 See the main protocol paper for details on how to set the Z-image spacing.

10 Choose the **XZY Acquisition mode** and set up the y-axis scanning. Drag the white line to the center of the bead. Enter in the **Nr. of steps** and the **y-step size** to ensure you capture the entire PSF image at the appropriate resolution. The **Set Plane** button can also be used to set the first and last planes in the stack.

11 Collect the stack of images and save the file in both TIFF and Leica file formats.

12 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.