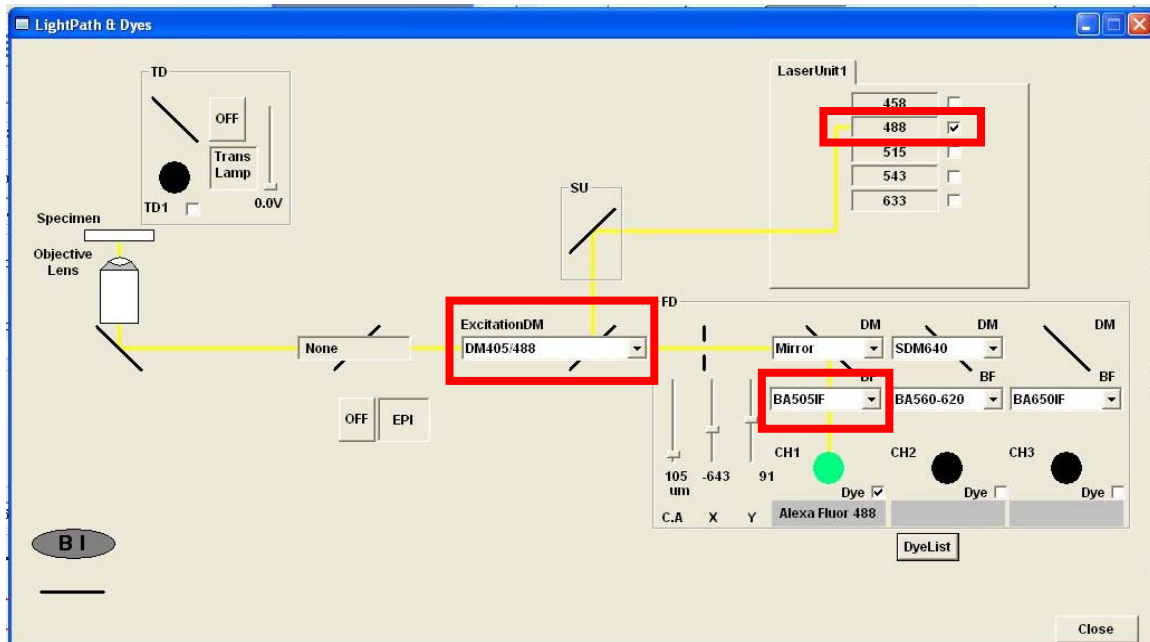
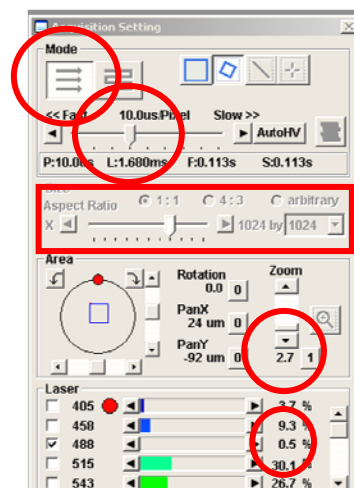


- 1 Set up the confocal light path for imaging a green dye (Alexa488-EGFP). For example, the light path as shown here using the 488 nm LASER (**Laser Unit 1**) reflecting off of the 405/488 nm Dichroic mirror (**ExcitationDM – DM405/488**) and collection of the emission >510 nm with a long pass interference filter (**BA505IF**).



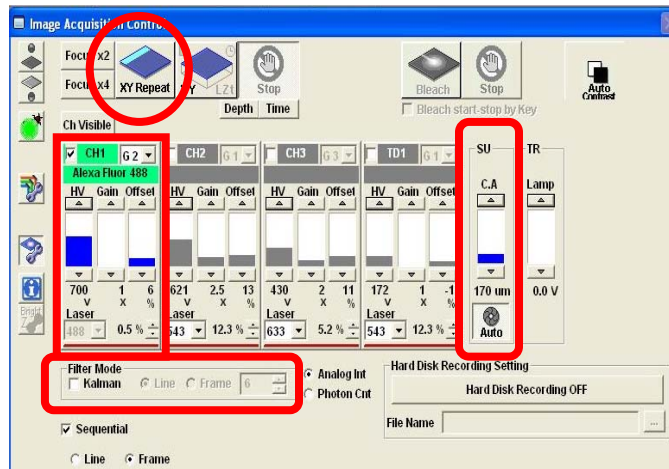
- 2 Open the **Acquisition Settings** window and set the image acquisition with the **mode** as unidirectional scanning - indicated by the arrows pointing to the right. **CRITICAL STEP:** Do not use bidirectional or raster scanning because if it is not properly calibrated it can generate image artifacts.



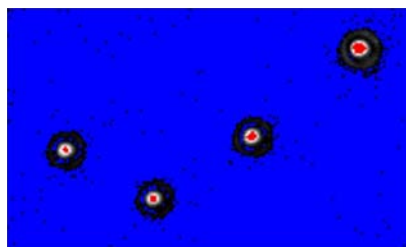
- 3 Set the scan speed to medium or 10-20 $\mu\text{s}/\text{pixel}$.
- 4 Keep the **Size** at an **Aspect Ratio** of 1 and select 1024 x 1024.
- 5 Under **Area** select a **Zoom** of ~3 (2.7 shown here).
- 6 Set the **Laser** power to 0.5%.

7 Open the **Image Acquisition**

Control window. Set up the Channel detection (**CH1**) so that it is at high detector sensitivity (**HV ~700**), low **Gain (1)** and low **offset (~6%)**. Use the **XY Repeat** function and scan the bead sample. The image should



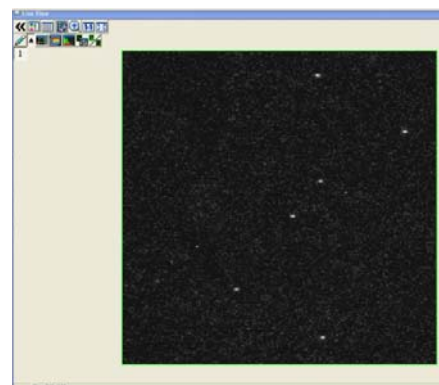
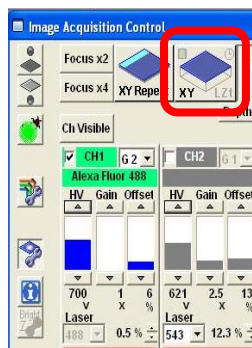
default to a 12-Bit format. Set the confocal aperture (**C.A.**) or pinhole to 1 Airy Unit by clicking the **Auto** button. **CRITICAL STEP:** Under **Filter Mode** make sure the Kalman filtering is not selected as any image filtering can cause image artifacts. Use the Hi-Lo look up table (LUT) setting and make sure there are no blue pixels (measuring zero intensity), and no red pixels (measuring saturation). You can press **Ctrl-H** to select the Hi-Lo LUT. If there are blue pixels put down the offset, if there are red pixels reduce the LASER power. If you reach minimum LASER power you can reduce the **HV voltage** setting.



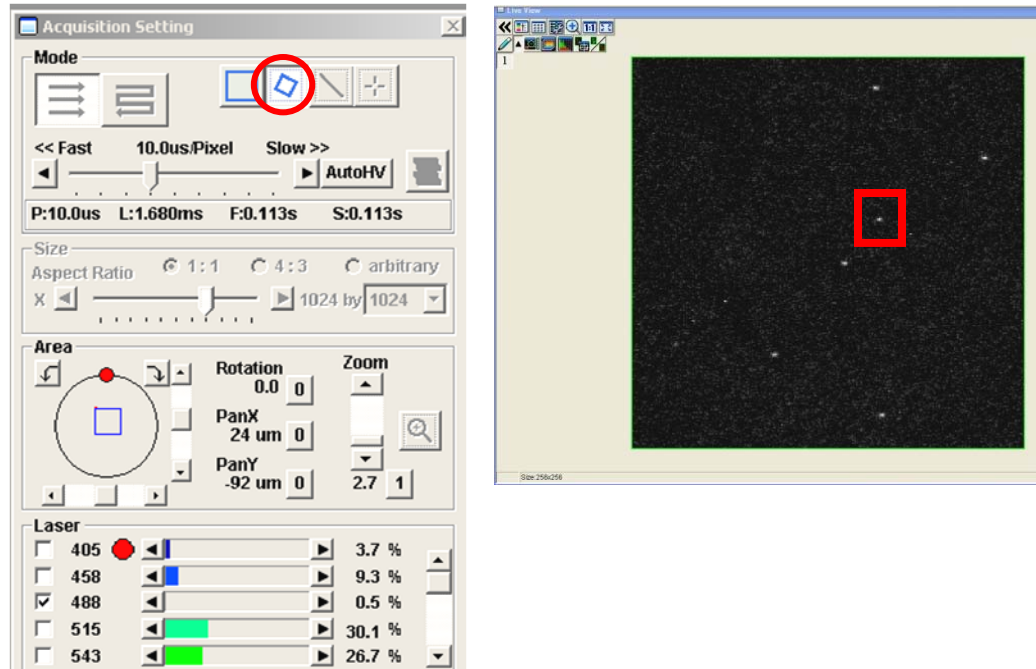
8 Press the **XY** button on the

Image Acquisition Control

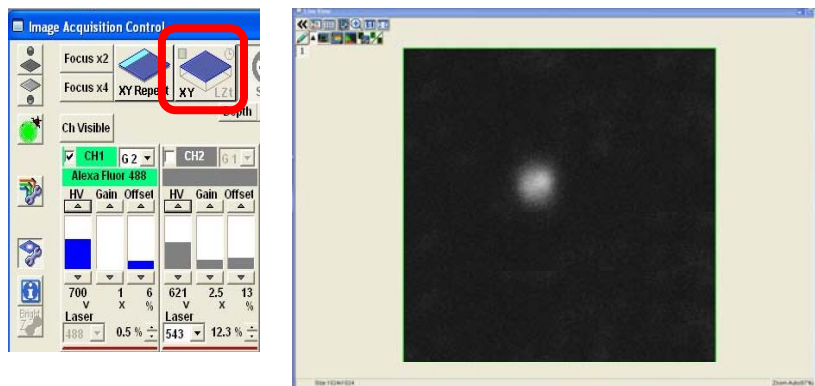
window to generate an image of the beads.



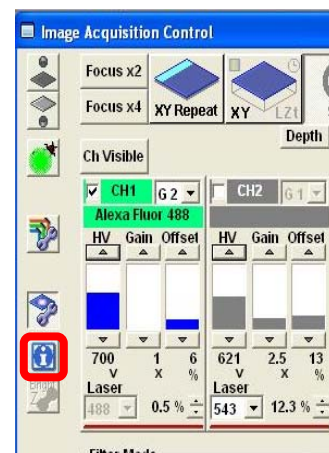
- 9 Under the **Acquisition Settings** use the **clip** tool to select a single bead for imaging.



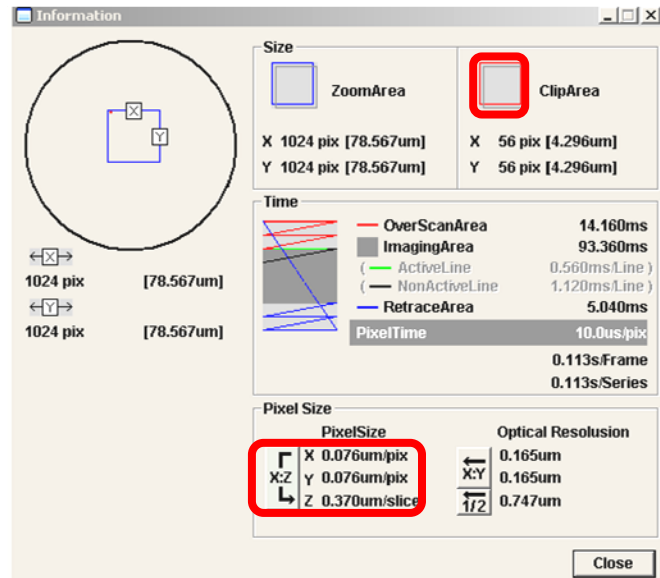
- 10 Press the **XY** button on the **Image Acquisition Control** window and take an image of the single bead.



- 11 Select the **Image Information** button on the **Image Acquisition Control** window. This will open up the **Information** window where the image pixel size and the clip region information will appear.

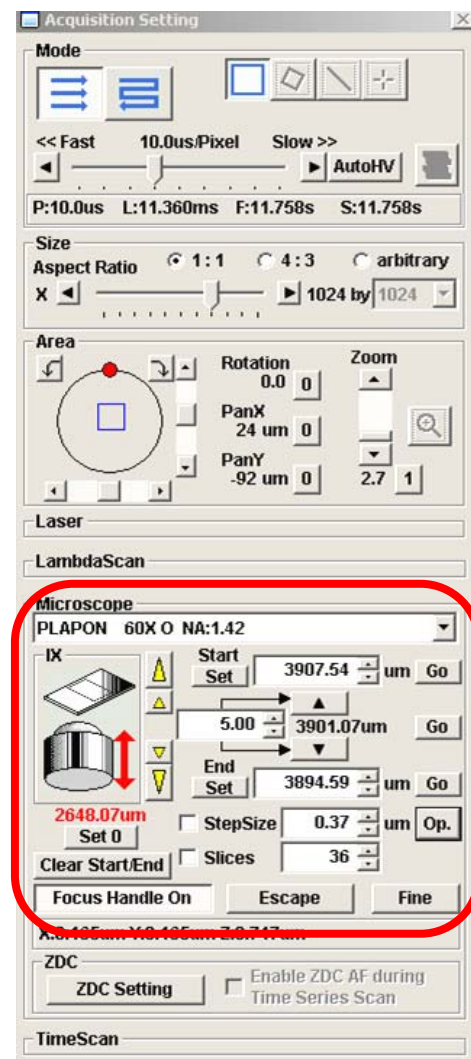
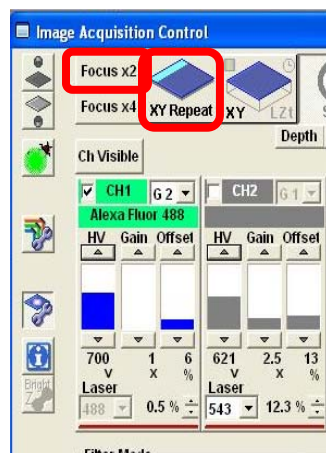


- 12 Verify that the image pixel size is within the recommended settings in Table 1 of the main protocol ($0.076\ \mu\text{m}$ or $76\ \text{nm}$ for a $60\times/1.4\ \text{NA}$ oil immersion objective lens). If it is not adjust the zoom factor to obtain ideal sampling.



- 13 Verify the image acquisition settings using the **Hi-Lo** LUT.

- 14 See the main protocol paper for details on how to properly set the Z-image spacing. Under the **Acquisition Setting** window activate the **Microscope** window. Use the **XY repeat** or **Focusx2** scanning mode and set the **Z-Stack** options. Manually focus or click on the yellow arrow buttons to focus below the beads. Click the **Set** button **Start**. Focus above the microsphere and click the **Set** button below **End**. Press the **Stop** button to stop scanning.



- 15** Adjust the **StepSize** or the **Slices** to achieve ideal sampling in Z (see the main protocol).
- 16** Perform the **Z-Stack** acquisition by selecting **Depth** and pressing on the **XY** button.
- 17** Right click on the image to save the image stack as an OIF file and also a .tif file.
- 18** 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.lmr@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.