

## Lab 6. Introduction to Confocal Microscopy

Objective: Learn about factors that affect resolution and signal discrimination.

Samples: RGB beads

Procedure: The sample will be placed on the microscope and ready for imaging. Use a 2-channel path configuration, i.e., separate path and channel for red and green detection.

**Use 585-615BP for the red channel and 505-535 for the green channel, zoom 2, 1024x1024. You will need only 0.1 – 1% laser power and low gain.**

**Simultaneous versus Sequential Imaging** (adjust the pinhole to achieve about 1 A.U.)

### **Start with a single track configuration**

Use a 10x/0.3 objective and find a field with a combination of red and green beads, preferably not touching each other. Focus near the center of 1 or more fluorescent beads (when the bead appears to have the largest diameter).

1. Optimize and save an image for the red channel using selective excitation and detection.
2. Optimize and save an image for the green channel using selective excitation and detection.
3. **Simultaneously (check both 488,543)** image and save both channels, using only the excitation for the green channel, but the optimized detection condition for both channels found in step 1 and 2.
4. **Switch to a Multi-track configuration for 2-channel (red, green)** fluorescence and repeat step 1, 2 then image both channels sequentially.

How do the images from simultaneous imaging compare to those from sequential imaging? Why?

**Pinhole size and resolution** (adjust the pinhole to achieve about 8 A.U.)

**Use Multi-track configurations for the remainder of the exercise.**

1. Repeat steps 1 and 2 from the previous exercise; you will need to reduce the laser excitation to account for the increased signal from a larger pinhole.  
*Why do you decrease the laser instead of the detector gain?*
2. Measure a line profile across an image of an isolated green bead.
3. Measure a line profile across an image of an isolated green bead from the previous exercise and compare the line profiles between the images acquired with 2 different pinholes.  
*How are they different? Why?*
4. Measure a line profile across an image of an isolated red bead.
5. Measure a line profile across an image of an isolated red bead from the previous exercise and compare the shapes of the line profiles between the images acquired with 2 different pinholes.  
*How are they different? How is the profile for the red channel different from the green channel at 8AU? Why?*
6. Collect a z-series for both channels.
7. Compare the 3-dimensional shape of the beads with those in the first exercise collected at a pinhole diameter of 1 A.U.  
*Are the shapes different? Why?*