**BP210: Biological Light Microscopy**
**Lab 5: Introduction to confocal, TIRF microscopy and Multi-Photon microscopy**

**Goal:** Use laser-scanning confocal, spinning disk confocal, TIRF, and multi-photon microscopes, and understand their light paths.

For this lab we'll rotate between microscopes so you can get experience with a Laser-Scanning Confocal Microscope (LSCM), Spinning Disk Confocal Microscope (SDCM), and a Total Internal Reflection Fluorescence (TIRF) microscope. The Multi-Photon demo will be in the CVRI.

**TIRF:**

1. Go over the TIRF light path.
    a. What lasers do you have?
    b. What optics are between the lasers and the objective?
    c. How do you align the TIRF light path and adjust the critical angle?
2. Prepare TIRF sample of diluted beads
    a. A great test sample for TIRF is a dish filled with diluted beads of the kind you use for measuring point spread functions. When you're out of TIRF (in epi-fluorescence) you can see beads freely diffusing in solution. When you're in TIRF, the beads in solution disappear, and you'll only see those beads stuck to the coverslip, or brief flashes of light as a bead in solution diffuses into the TIRF zone. To make such a sample, take a coverslip-bottom 35 mm dish, add a few mL of distilled water. Then add diluted FocalCheck beads to a final dilution of 1:5000 - 1:50000.
3. Examine the sample in TIRF and epi-fluorescence
    a. Set up your microscope for TIRF, and adjust the incident angle to see how the image changes
    b. What does the image look like if the incident angle is less than the critical angle?
    c. What does the image look like if the incident angle is greater than the TIRF angle?
    d. What is the critical angle, and how could you calculate it?
    e. If you can safely view the back focal plane, look there while you repeat these adjustments to the TIRF angle.

**Spinning Disk Confocal:**

1. Go over the spinning disk light path.

    a. What lasers are used for illumination?
    b. What dichroics, excitation, and emission filters are in the spinning disk light path?
    c. Where is the pinhole disk?
    d. How do you set up the microscope for spinning disk imaging?
2. Acquire some images with the spinning disk confocal. How do they compare to widefield images of the same sample?
3. Stop the disk so you can examine the pinholes
    a. How are they arranged?
    b. What is the size of the pinholes?
    c. How does this compare to the width of the objective's point spread function?

**Laser Scanning Confocal:**

1. Go over the light path
    a. What lasers are used for illumination?
    b. What dichroics, excitation, and emission filters are in the light path?
    c. Where is the pinhole?
    d. Is it fixed or adjustable?
    e. Where are the detectors?
    f. How do you set up the microscope for confocal imaging?
2. Acquire some images with the confocal
    a. How do they compare to widefield images of the same sample?
    b. What is the effect of adjusting the laser power and detector gain?
3. Learn how to change the scan area
    a. What scan area settings do you need to acquire a Nyquist-sampled image?
    b. What is physically changing in the microscope when you adjust this setting?
    c. What other scan parameters are adjustable?
    d. What are their effects?

Multi Photon:

Visit Anna Celli for a demonstration!