**BI227 ASSIGNMENT #4** **FALL 2007**

## Goals for this week:
Single channel 3D imaging
Measuring Point spread functions

## Samples
1) Carolina Prepared slides (pollen, rotifers, stems). **Clean off oil with OptiPad when done**.
2) Green (505/515 nm) and orange (540/560 nm) fluorescent microspheres (0.175 $\mu$m) mounted on cover slides

## Assignments
**1) Single Channel 3D Imaging Carolina Pollen and/or Rotifer Slides. (40x Air, 40x oil, or 63x oil objective)**
**1a) Find Settings for 3D Imaging**
Collect *Z*-stacks by exciting with either the 488nm or the 543nm laser. Compare stacks collected with different intervals, optimal (~50% overlap) versus larger intervals. Get comfortable setting up Z-stack collection on these samples before you go on to try the prepared thick samples. What happens as you image deeper into the specimen? Are the optimal brightness and contrast settings the same for the entire stack? Can you get all of the way through the specimen?

**1b) Compare Effect of Pinhole Aperture**
Acquire two Z-stacks of the same sample (Carolina slides) with different pinhole size settings (wide open,and smaller), adjusting the intensity, exposure time, brightness, and contrast such as tto fill the whole dynamic range.

Make sure to record your *X*-scale, *Y*-scale, and *Z*-interval values; when visualizing your samples on Imaris, update the image properties with these values. What is the effect of changing intervals on the 3D renderings?

**2) Point Spread Function Measurement**
Collect stacks containing images from at least one fluorescent microsphere for different pinhole size settings. In particular, acquire one with the pinhole completely open and one with the pinhole set to about 1 Airy unit. Adjust intensity, exposure time, brightness, and contrast such that the image values fill the whole dynamic range.

Acquire two z-stacks for the same pinholes sizes as for the two stacks in 1b). Make sure to choose the bead color to match the color imaged in 1b).

## Considerations for Z-sectioning

**PLEASE BE CAREFUL:** ***Watch out for clearance of the objectives! It is very important that you do not plow the objective into the coverslips. Adjust the focus slowly and watch for and avoid contact with the coverslip. Oil objectives with high NA are notorious for short working distances. Good objectives cost thousands of dollars and hitting the slide may ruin them.***

The best Z-stacks are collected using optimal intervals. Sections spaced too far apart will leave gaps. Sections placed too close means oversampling and bleaching due the excessive illumination. These best intervals are ~50% of the thickness of the optical slice that you are collecting. Remember that the thickness of the optical slice is related to the NA of the objective and the size of the pinhole used. Below are listed the approximate thickness of optical sections collected with our objectives.

**Objective: 40x Air (NA=0.75)**
Optical section thickness = 1.7µm
Optimal interval for Z sectioning = 0.84µm

**Objective: 40x Oil (NA=1.3)**
Optical section thickness = 0.8 µm
Optimal interval for Z sectioning = 0.41µm

**Objective: 63x Oil (NA=1.4)**
Optical section thickness = 0.7 µm
Optimal interval for Z sectioning = 0.35µm

All @ pinhole = ~1Airy unit

**Choosing Top and bottom boundaries of a Z-stack**
*There are few options to set this parameter:*
**1) Do it by eye**. Using epifluorescence or a fast LSM scan - focus down and up. Note the Z-position at bottom on the parameter windows. Then focus up until you get slightly above the specimen region of interest and note that Z position. Leave the scope focus at that top position. Make sure that you designate your current section position to 1. Then figure the total size of the stack you want to collect and divide by the interval to get the number of sections, add 1 and put that information into the dialog box for number of sections. Then start the Z-stack collection and it should go from the top ->down to the bottom that you selected (one section at a time).

**2) Software based boundary setting.** Click on the Z-scan radio button. The scope will do a fast vertical scan. Then it will display this vertical scan with the current focus position in a dotted line and the top and bottom (as shown in the Z section dialog box) as solid lines. The user can move the top and bottom boundaries accordingly.

**NOTE** beware that the software isn't intelligent and it will change the interval size as you move the top and bottom boundaries. So you will have to go back and change the number of sections in order to get you desired interval. There isn't a way to keep the interval parameters constant while changing the boundaries.

**Final Note:** The output from a Z-stack is going to be a series of images. Make sure you save the output to a folder or you will have a mess on your data disk.

## Z Sectioning in Zeiss software

***Z Interval***: Step size in microns

***Number of Sections:*** User defined based on sample size.

***Current Section Pos:*** Take care to always make sure that you are at the beginning of your stack

***Refractive Correction:*** N2/N1
(Example: Oil/Water =1.518/1.0= 1.518)

***Destination:***

Video Memory: internal video memory buffer, **do not use.**
**File: Use this option**
Screen: Display only one image on the image screen
Host Memory: Save into computer's RAM, must be saved through the File menu.

***Z-Scan:*** Line Scan through your sample at the center of the image window. Users can define boundaries for z-sectioning through the use of this function. **NOTE** that adjusting the top and bottom boundaries will change the interval size; you'll have to readjust it.

***Move to X***: Single scan at first, middle, or last positions.

***Ok:*** starts the image acquisition.