Bi/BE 227 Winter 2020

Assignment #2

Adding the third dimension: 3D Confocal Imaging

Schedule:
Jan 13: Assignment
Jan 13-Jan 29: Work on assignment
Jan 29: Student PowerPoint presentations.

Goals for this week
   A. Collect 3D Confocal data set: Z-stack.
   B. Document effects of refractive Index mismatch
   C. Determine linear range for Detectors at different gains.

Samples
   A. Carolina sample slides with pollen grains. **Clean off oil with Lens paper after use**.
   B. Carolina sample slides with pollen grains. **Clean off oil with Lens paper after use**.
   C. Autofluorescent plastic slides from Chroma Technologies.

INSTRUCTIONS:

A. Single channel 3D imaging of Carolina pollen slides. (40x oil, or 63x oil objective)
Before starting any 3D acquisition, read the section "*Considerations for Z-sectioning*" at the end of section B.

A1. Find Settings for 3D Imaging
Collect *Z*-stacks of Pollen grains by exciting with either the 488nm, 543 nm or the 633 laser. Compare stacks collected with different intervals (~50% overlap versus larger intervals).

Q1: 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?

A2. Compare Effect of Pinhole Aperture
Acquire two Z-stacks of the same sample (Carolina pollen grain) with different pinhole size settings (wide open, and ~1 Airy unit), adjusting the intensity, exposure time, brightness, and contrast such as to fill the whole dynamic range. Make sure to record your *X*-scale, *Y*-scale, and *Z*-interval values; visualize the sample in 3D using the Zeiss software or FIJI (Image J) software.

Q2: What is the effect of changing the pinhole size on the 3D renderings?

B. Single channel 3D imaging of Carolina pollen slides. (40x dry and then 40x oil objective)

Before starting any 3D acquisition, read the section "*Considerations for Z-sectioning*" at the end of this section.

B1. Acquire Z-stacks using optimal settings as determined in A2.
Collect *Z*-stacks of Pollen grains by exciting with either the 488nm, 543 nm or the 633 laser and the pinhole set at 1 Airy unit. Make sure that several round spiky pollen are in your field of view. Collect enough optical sections at a small Z-interval (smaller than your Z-section thickness in microns, see below regarding typical objectives). Use the Dry object first and then collect a comparable stack using the oil objective.

NOTE: *Z interval will be smaller and there may be more sections with the oil objective*.

B2. Compare Z-stacks taken with dry and oil objectives.
Compare the Z-stack collected with a dry objective to the one collected using an oil objective in 3 dimensions using the Zeiss confocal software or FIJI. Look at the round pollen in 3D, observing its shape in different planes. Compare the shape in the X-Y plane to cross sections in the X-Z or Y-Z planes.

Q3: How does the shape of the pollen differ between dry and oil objectives? What do you think the refractive index of the material the pollen grains are embedded in is most similar to?

B3. Compare Z-stacks taken with different RI oils.
Using the 40x oil objective, collect two z-stacks as close to the same as possible of a sample but using two different RI oils. Use the standard 518F (RI: 1.518) and the water immersion oil W 2010 (RI: 1.3339). Be very careful to thoroughly clean the objectives and slide between the oils.

Q4: Produce Z projections of both datasets. Comment on the differences between the oils and why it is important to ensure proper RI oil is used.

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. Be careful that your z-stack is not too large to cause the objective to slam into the coverslip toward the end of the stack.*

Choosing Top and bottom boundaries of a Z-stack. There are a couple ways to set this parameter:

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.

All @ pinhole = ~1 Airy unit

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

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

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

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: Be aware 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 =1.518/1.33= 1.14)

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.

C. Determine the linearity of different Confocal detectors.
For this assignment you will compare a detector (PMT) of one of the confocal microscopes in the B168 Church to the GaAsP detector in the LSM 800 in the Biological Imaging Facility (BIF).

C1. Plot gain versus intensity for a PMT (LSM 410 or 310).
Using the autofluorescent slide from Chroma that matches your laser wavelength use the 488 nm or 543 nm laser line and focus on the very bright slide. Use the 20x objective with 0.8 NA that will be provided. Place the pinhole at 1 airy unit to achieve an optical section. Start at the highest

gain (or brightness in older software) and measure the intensity of a typical point in the image. Now decrease the detector gain and measure the pixel intensity again. Repeat several times until you have enough values for a good X-Y plot. Plot the gain versus the pixel intensity on a graph. **NOTE**: *you have to add the 20x 0.8 NA objective to the objective parameter in the Zeiss confocal software*.

Q4. How linear are the PMTs? How broad is the linear range?

C2. Plot gain versus intensity for a GaAsP detector (LSM 800).
NOTE: *GaAsP detectors are very light sensitive so be very careful to start with very low laser power!* Use the same laser line you used for the PMT or as close as you can get (488 nm may be best). Use the 20x lens with 0.8 NA already on the microscope. Again place the pinhole at 1 airy unit. Raise the gain to maximum and measure the intensity of a typical point in the image. Lower the gain and measure the pixel intensity again. Repeat several times until you have enough values for a good X-Y plot. Plot the gain versus the pixel intensity on a graph. **NOTE**: *the gain on the LSM 800 will not go below 500*.

Q5. How linear are the GaAsP detectors? How do they compare to a PMT?

Assignment presentation: You can work in groups. Only oral presentation is required. Just make sure to specifically indicate the answers to the questions somewhere on a slide.

- Answer Qs and present select images illustrating the topics and tasks covered in this assignment.
- Use the Zeiss confocal software or ImageJ/Fiji to visualize your data and select images. Explore the various ways to visualize and analyze your data, for example: single-plane view; z-projection (average, maximum intensity); orthogonal-plane views in xy, yz, or xz.
- Remember to annotate all of the presented images, with relevant imaging parameters, descriptions of the sample, scale bar, visual aids (eg arrows pointing to feature that you want to highlight), etc.
- Discuss any problems and successes you had during imaging.

Optimal spatial sampling and resolution (From Assignment 1):

The optimal spatial sampling density (i.e. size of pixel in the lateral xy plane, and thickness of axial z-section) depends on the optical resolution of the imaging setup. Under-sampling leads to loss of information; over-sampling leads to excessive illumination and unnecessary photobleaching and phototoxicity. The so-called Nyquist's criterion states that the minimal sampling frequency, for no loss of information, is twice of the highest frequency present in the signal.

Applied to imaging, this means that if the microscope has resolutions of d_lateral and d_axial, then optimally one should have a lateral pixel of size of (d_lateral/2) and z- sections separated by (d_axial/2). (The ideal factor of 2 in Nyquist's criterion becomes 2.3 for a real microscope, due to certain practical considerations.)

The resolution of the microscope could be estimated by the following formulas:

d_lateral ≈ **0.6*(λ)/NA**
d_axial ≈ **1.6*n*(λ)/NA2**

where λ=wavelength (of the fluorescence), NA=numerical aperture, n=refractive index of immersion medium.

Using these expressions, you could calculate what optimal voxel size (lateral and axial) you should use. To reach the axial resolution stated above, the pinhole has to be set at 1 Airy unit.

Example:

Objective: 63x Oil (NA=1.4)
For fluorescence ~ 575um, pinhole = 1 Airy unit:

Lateral resolution = 0.25 um Axial resolution = 0.7 um

Using the ideal Nyquist's criterion to determine sampling frequency: pixel size (X,Y) ~ 0.12 um
Interval for Z sectioning = 0.35 um (In practice, you over-sample by a further 30-50% if you want a really nice image, and you under-sample by up to 100% if you're concerned about photodamage/imaging speed.)

The Zeiss LSM software automatically calculates the resolutions described in the above expressions, for a given objective lens. Click on the radio button next to pinhole slider in the main control window, which would bring up a control window for the pinhole, then click on theradio button labeled "Resolution info", toward the lower right corner of the window. You should get a window listing the expected lateral and axial resolutions, for the pinhole size and the lens in use (make sure the software lists the correct lens being used), at the listed excitation and emission wavelengths.