

Bi/BE 227 Winter 2016

## **Assignment #1**

### **Widefield and confocal laser scanning microscopy**

#### **Schedule:**

Jan 4: Lecture and demo

Jan 4-13: Students get trained on how to use scopes, start on assignment

Jan 4-20: Carrying out the assignment

Jan 23: Brief discussion of assignment; group written reports due (note this is a Mon, we'll try to keep it brief to get done on time.)

#### **Goals for this week**

##### **Part A. Widefield microscopy**

- Understand the light path organization in a widefield microscope
- Adjust microscope to achieve Köhler illumination
- Differences between Stereo Microscope and Widefield
- View fluorescent samples in epi-fluorescence mode

##### **Part B. Confocal Laser Scanning Microscopy**

- Get familiar with the LSM scopes: image thin fixed samples.
- Examine the differences between air and oil objective lenses, and the influence of imaging parameters.

#### **Things to watch for...**

1. Be *VERY* careful not to get oil onto an air/water immersion objective lens, especially when switching from oil to air/water objectives as there is usually enough residual oil on the sample slide to corrupt the air/water lens. Before switching from an oil to an air/water objective, be sure to clean the sample slide with an OptiPad cleaning pads (lens paper with isopropanol).
2. *NEVER* clean any objective lens with a Kim wipe or OptiPads! Use only Kodak lens cleaning paper with Zeiss cleaning solution. Clean objective lenses with lens paper by the "drop and drag" method, and never apply direct pressure to the front surface of the objective lens.
3. Be *CAREFULL* to watch out the 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. Objectives with high NA usually have a short working distance. A good objective costs thousands of dollars and hitting the slide may ruin them.

#### **Samples**

Carolina sample slides (pollen, algae, ...). **Clean off oil with OptiPad when done.**

## **Assignments**

### **A. Widefield microscopy**

A1. Brightfield Imaging (Köhler Illumination) (see poster on wall, if you need help remembering)

Adjust objective to focus on sample

- Set field diaphragm to half the size and adjust height of condenser lens for field diaphragm aperture to be in focus and adjust centering with the screws. - Open field diaphragm. - Experiment with opening and closing the condenser diaphragm (also called the aperture

diaphragm), which sits right above the condenser.

This procedure must be repeated for every new objective.

**Q1 for report: What is the influence of opening or closing the field diaphragm?**

**Q2 for report: What is the influence of opening or closing the condenser diaphragm?**

A2. Stereo Microscope adjustment you should always do.

- Zoom to highest magnification.
- Focus on sample.
- Zoom out to lowest magnification
- Close one eye and focus specimen just using eyepiece
- Repeat with other eye and eyepiece

Now you will be able to zoom from highest to lowest magnification without needing to change the focus.

A3. Epi-Fluorescence (using the widefield microscopes attached to confocals)

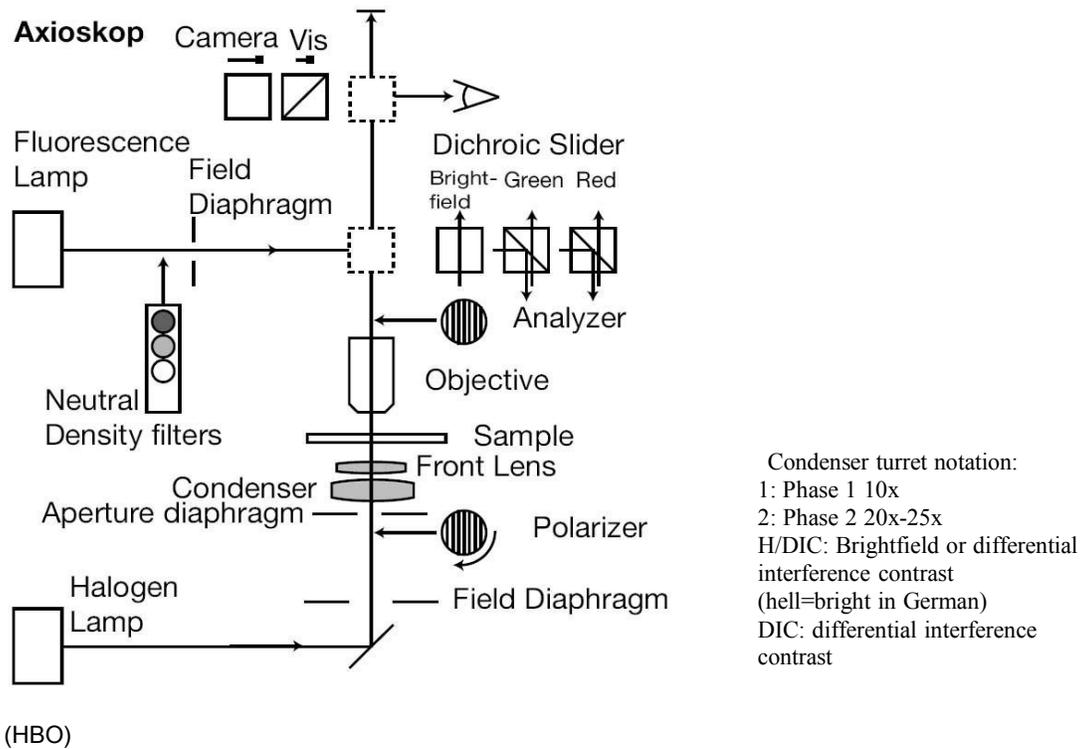
- Choose a dichroic filter (either marked Red or Green).

**Q3 for report: Which color do you see through the eye piece when the HBO is still on? Why?**

- Block the transmission light from the HAL in the software (switch to FL), or physically block the light path with a lens cap (upright scopes only).
- Turn on the fluorescence light (HBO/ Mercury source/ EXFO Excite).
- There is a slider in the path for the FL light with two neutral density filters, one empty slot and one position to block all light to adjust intensity. Set to one of the open positions.

**Q4 for report: this technique is called epi-fluorescence, whereas the brightfield imaging is characterized by a trans-illumination. Based on**

the light path diagram (figure 1), could you explain the difference between “epi” and “trans”?



**Figure 1:** Light path diagram of the Axioskop (widefield microscope)

## B. Confocal Laser Scanning Microscopy

For this part you only need to acquire single channel (one-color) images on the LSM scopes, but be sure to try both the 488nm and 543 lasers, as they will have different optimal parameters (especially laser attenuation). Use Carolina Slides and 40x air (NA=0.75) and 40x oil (NA=1.3) objectives.

B1. Compare differences in brightness and resolution between air and oil objectives. Using one of the Carolina slides, take your best image using the 40x air objective, recording the settings. Now, switch to the 40x oil objective and collect an image using the same settings as for the 40x air lens. Then adjust the settings and take the best possible image with the oil lens.

Q5: What adjustments did you need to make to improve the image taken with the 40x oil? How does the objective's NA fit in to the differences between these imaging parameters?

B2. Try different scan speeds. (Use a 40x oil objective)

Q6: Compare an image collected at a very fast scan speed with an image of the same region collected with a scan speed  $< 8$ . What gives the better image? Why?

B3. Try different averaging schemes. (Use a 40x oil objective)

Q7: Compare images of the same sample collected with or without averaging (try averages of 4, 8, 16). What gives the best image? Why?

B4. Explore different pinhole diameters. (Use a 40x oil objective) Take an image of a sample with the pinhole totally open and then a series of images with a smaller and smaller pinhole.

Q8: Compare an image of the same sample collected with the pinhole of  $\sim 1.2$  airy unit with an image collected with larger and larger pinhole settings. What happens to the image as you decrease the pinhole?

B5. Collect images at varying Zoom settings. (Use a 40x oil objective)

Q9: Compare an image of the same sample collected with the pinhole of  $\sim 1.2$  airy unit at Zoom settings = 1x, 2x, 4x. How does image quality compare at 1x, 2x, 4x?

## Optimal spatial sampling and resolution:

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  $d_{\text{lateral}}$  and  $d_{\text{axial}}$ , then optimally one should have a lateral pixel of size of  $(d_{\text{lateral}}/2)$  and z-sections separated by  $(d_{\text{axial}}/2)$ . (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:

$$d_{\text{lateral}} \approx 0.6 \cdot (\lambda) / \text{NA} \quad d_{\text{axial}} \approx 1.6 \cdot n \cdot (\lambda) / \text{NA}^2$$

where  $\lambda$ =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. Note that 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  $\sim 575\text{nm}$ , pinhole = 1 Airy unit: Lateral resolution = 0.25  $\mu\text{m}$

Axial resolution = 0.7  $\mu\text{m}$  Then, you should have (using the ideal Nyquist's criterion):

pixel size  $\sim 0.12 \mu\text{m}$

interval for Z sectioning = 0.35  $\mu\text{m}$  (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 the radio 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.

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## General Notes

- You can open, view, and prepare your images with Fiji (<http://fiji.sc/Fiji>), which works on most computer platforms and are is freely downloadable.
  - Written report: each group prepares a group written report (2-3 pages) answering the questions (Part A and B) and with a selection of images illustrating the topics covered in Part B. Do not worry about the print quality of your images - we only use them as a guide in the grading, as we should have the high-quality digital versions of them from your submitted digital copy
- (NOTE: no oral presentation for Assignment 1 !!!)
- Reminder: both the written report and digital copy of the presentation have to be submitted at the beginning of class for full credit.