Week 5 Lab: Introduction to Fluorescence Microscopy and Microscope Calibration

Goal:
Identify the optics necessary for fluorescent imaging and use them to observe fluorescent samples.

Introduction:
Fluorescence is a property of certain molecules to absorb a photon of a particular wavelength, then emit a photon at a longer wavelength. Microscopes built for fluorescence imaging use a combination of filters and dichroic mirrors to separate excitation and emission light, which results in images with low background. Fluorescence microscopy detects molecules, proteins, or structures that are inherently fluorescent or are labeled with a fluorophore.

Samples:
Pre-stained Tissue Culture Cells

1. Start as in the previous labs - identify the relevant optics in the fluorescence path of your microscope. What is the excitation light source on your microscope? We have a number of different light sources on the microscopes in the course and they work in different ways. You should learn what kind of a light source is on your scope and how it works.
2. Where is the excitation pathway? Where are the filter cubes? What other optics (irises, etc.) are in the fluorescence light path? What do they do?
3. What fluorescent filter cubes are on your microscope? What dyes are they designed for?
4. Where is the emission pathway? How does the dichroic mirror separate this from the excitation pathway?

Protocol:
1. Mount the slide and focus using brightfield optics.
2. Switch to fluorescence illumination. Use different fluorescence filter cubes to observe different wavelength dyes.
3. Use the imaging software to perform simple tasks including taking timelapses, multiple wavelength composites, multiple positions, etc.

Microscope Calibration:

Dark and Flat Correction:

Cameras don't read zero when there is no light hitting them - they have an offset, known as the camera bias. In addition, there may be other sources of background in your sample. If you're trying to do quantitative imaging, it's necessary to correct for this. [Question: what kind of errors would you get if you fail to correct for camera bias?] Additionally, the image may not be equally bright across the field of view. This can come from either variations in the illumination intensity across the field of view or variations in the detection efficiency across the field of view. There are techniques to distinguish between these two sources of non-uniformity, but fortunately we can lump them together most of the time. [Question: when would lumping these together be a bad idea?] This allows us to easily measure the total variation in intensity across the field of view by imaging a uniformly fluorescent sample, such as a piece of fluorescent plastic or a solution of fluorescent dye.

To generate a flat-field correction image, first acquire a bias image, or just an estimate of the bias value. Then mount a fluorescent slide on the microscope, focus on it (using the edge of the slide makes it easier to get into focus) and acquire multiple images of it, while moving it around (so that any pieces of dust or spatial nonuniformities in the sample are averaged out). Take 5-10 images like this, then average them together and subtract off the camera bias. This is your flat-field correction image. To use it to correct other images you acquire, you should first subtract off the bias, then divide by the flat-field correction image (though you can often skip subtracting the bias if your signal is large and you can tolerate a small error).

$$I_{measured} = \text{Bias} + I_{true} * \text{Flat}$$

Measuring the point spread function (PSF):

The response of your microscope to a point source of light, its Point Spread Function (PSF), characterizes much of the optical performance of your microscope. Since point sources of light are hard to come by, instead we use brightly fluorescent beads smaller than the resolution limit of the microscope. Specifically, we use green fluorescent 100nm beads (FocalCheck Microspheres from Molecular Probes). To measure the PSF, we want to immobilize these beads on a coverslip, as follows:

1. Rinse two coverslips in ethanol. Blot off most of the excess and flame to dry.
2. Add ~5 ul of diluted bead suspension (1:10000 or greater) to one coverslip.
3. Rub coverslips together to disperse beads and allow them to dry.

4. Put a ~10 ul drop of glycerol on a slide, and place the coverslip on it, bead side down.
5. Seal with nail polish.

Focus on your bead slide and center a bead in the field of view. Acquire a Z-stack of the bead using small (.25 um or less) z-steps, setting the exposure time so that the bead is just below saturation at brightest focus. Look at the resulting PSF in all three dimensions. Note any aberrations in the PSF. Measure the full width at half maximum intensity (FWHM) of the PSF, or the distance between zeros. This can be done by measuring the intensity through a line-scan. Does this correspond with the expected size of the PSF for the objective you're using? Is the PSF symmetrical?

For a self-luminous bead much smaller than the diffraction limit:

$$d_{xy} = \frac{1.22\lambda}{2NA}$$

d_{xy} – *resolution limit in X and Y*
λ – *wavelength of emission light*
NA – *numerical aperture of the objective*

$$d_z = \frac{\lambda n}{(NA)^2}$$

d_z – *resolution limit in Z*
λ – *wavelength of light*
n – *refractive index of immersion media*
NA – *numerical aperture of the objective*

Repeat this for one or two other objectives, then record PSFs using an objective with a correction collar and rotate the correction collar to see how this affects the PSF and how you can recognize spherical aberration.

Checking for wavelength-dependent pixel shift:
Prepare a slide as above with TetraSpeck beads (these fluoresce at multiple wavelengths). Take images of these beads at high magnification using different filter sets (for instance: DAPI, FITC, TRITC, Cy5). Determine the amount of shift (in all 3

dimensions: X, Y and Z) between the various images by visual inspection of the color overlay of these images and by determining the centroid of each bead in each channel.