

## I. Before coming to lab

Read this handout and the background.

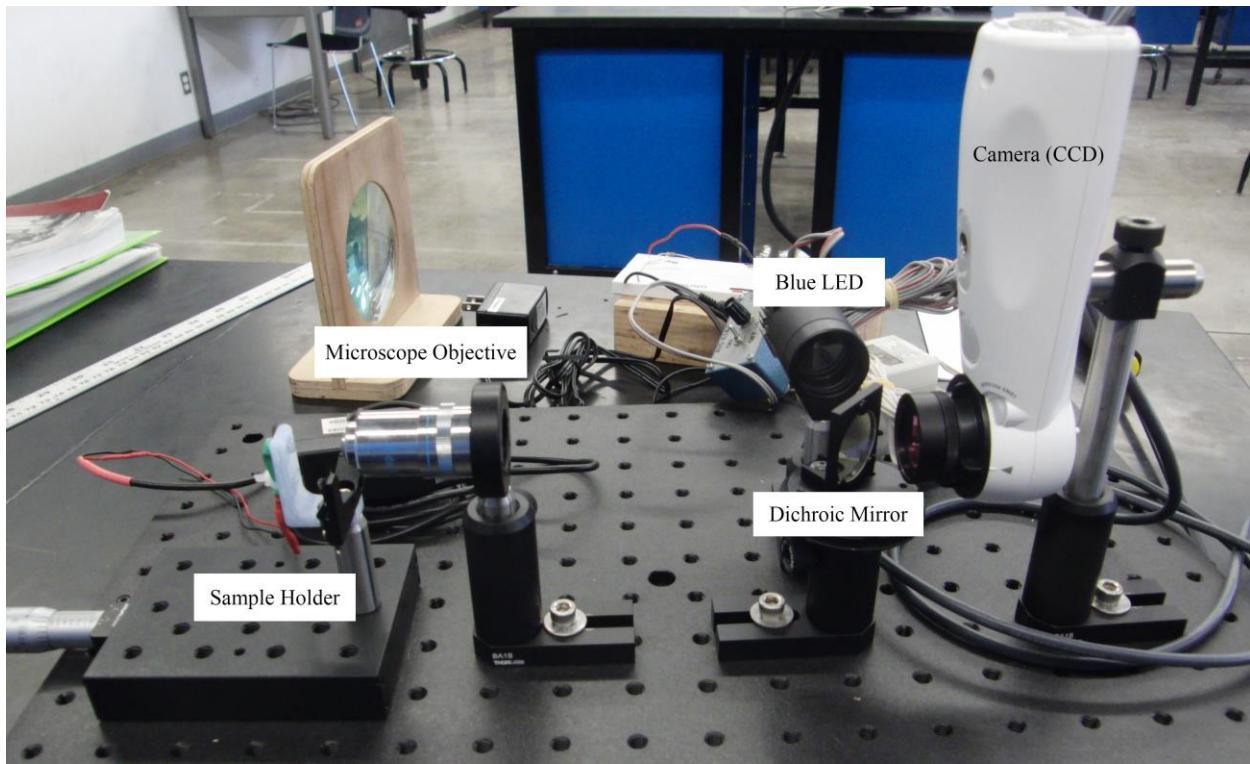
## II. Learning Objectives

In this lab, you'll investigate the physics of microscopes. The main idea is to understand the limitations of a microscope and some of the techniques that are used to overcome these limitations. You'll directly explore bright-field and fluorescent microscopy and work with a demonstration of a confocal setup outside of a microscope.

## III. Materials

### Microscope

You will be using a home-built microscope that shows all of the important components of a fluorescence microscope, built on an **optical breadboard**: a rigid board with threaded holes to which optical components can be attached. Here is what the microscope looks like:



From left to right on the photograph above:

The **sample holder** is where the specimen (on a microscope slide) will be mounted. There is a green LED behind the slide that can provide “bright field” illumination of the slide. The sample holder has a threaded knob (in silver at the far left) that can move the slide back and forth until the image is in focus.

The **objective** is a 40x objective. You will need to get the specimen quite close to the end of the objective—when it is in focus the objective will almost be touching the coverslip of the slide.

The **dichroic mirror** is an important component of the fluorescent scope: it reflects blue light while transmitting green light.

The **blue LED** shines on the dichroic mirror. Blue light reflects off of the mirror and is focused by the objective onto the sample. Fluorescent objects in the sample emit green light, which comes back through the objective, passes through the dichroic mirror, and forms an image on the **camera** (CCD) at the right of the setup.

There are a few other details that are important:

The very bright blue LED is controlled by an on/off switch and dimmer knob. **PLEASE TURN THE BLUE LED OFF WHEN NOT IN USE!** These have a tendency to overheat and fry themselves. There is also a clear lens that acts as a focusing lens for the wide-beam LED.

The **excitation filter** is attached directly in front of the blue LED (it is screwed on to the end). It makes sure that all wavelengths *longer* than blue are filtered out, so that the sample is illuminated only with blue light.

The **emission filter** is attached directly to the camera. It blocks all blue light, so that only the fluorescing parts of the sample can be seen.

Both filters, as well as the light source, can be removed from the apparatus by unscrewing. Please be careful when handling the filters, as they are precision optical instruments. We'll provide you with gloves so that you don't smudge them with fingerprints.

### Vernier spectrometer

This device analyzes the wavelengths that comprise the light that is shone into it and produces a plot of the spectrum. The detector is the small hole in the notched side of the spectrometer. The spectrometer is very useful for characterizing the filters that comprise the fluorescence microscopy setup.

### Fluorescent microspheres

These are polystyrene microspheres that have been coated with fluorescent dye. When the dye is excited with blue light, it emits green light. Several solutions of microspheres in water have been prepared and put onto a well slide for your use. In addition, there is an index card with a drop of fluorescent microsphere solution on it.

There are three different sizes of microspheres: 0.3, 0.5, and 1 micron (diameter). Here is some more information on the 1-micron spheres:

$$\text{Diameter of microspheres: } d = 1.00 \pm 0.05 \mu\text{m}$$
$$\text{Density of microspheres: } \rho = 1.057 \pm 0.003 \text{ g/cm}^3$$

### Camera setup

- Open the “**Lab5.cml**” file in Logger Pro

## V. Procedure

Tell us who you are. (Picture screenshot from Photobooth, full names, and emails please)

**A:**

### Characterizing the filters in the fluorescence microscope

Have one person put on a pair of gloves and carefully remove the emission and excitation filters from the microscope setup. The filters are attached to the blue LED and to the front of the camera. Only the person wearing gloves should handle the filters in order to minimize the number of fingerprints left on them, and please *PLEASE* be careful not to drop/break them.

The **excitation filter** is the one attached to the blue LED. Unscrew the black cylinder from the main body of the LED, and then carefully unscrew the filter from the end of the cylinder.

The **emission filter** is the one attached to the camera. Carefully unscrew the short black cylinder that contains the emission filter.

In order to make sure you haven't mixed the two filters up, the **excitation filter** looks like a mirror under ambient light, whereas you can see through the **emission filter**.

Plug the USB cable of the spectrometer into one of the empty slots in the back of the iMac (unplug LabQuest Mini if it's plugged into a USB slot) and turn to page 1 in Logger Pro. Click on "Collect" in Logger Pro and the spectrometer should automatically begin taking data. See what happens to the spectrum when you point the detector at the LED light source. (The LED is very, very bright; don't look directly into it.) You can also try pointing the detector at the computer screen to see what colors it detects.

Set the LED light source on the bench and set the spectrometer next to it so that the light source shines into the sensor. Leave some space between the LED and the sensor. The sensor is very sensitive to direction; try to line it up so that the spectrum doesn't saturate (at the top of the graph), but the intensity is high enough that you can actually see it. When you have it lined up correctly, leave it in place so that you can add or remove filters without changing the alignment. You might want to use a piece of paper or fabric to shield the sensor from ambient light. When the spectrum is the right height, click "Stop" and the graph should freeze in place with the spectrum of the light source. Press Command-L to store that spectrum.

Now shield the detector with the **excitation filter**. Click "Collect" again; if it asks you if you want to save the data select "Save latest run." Once the spectrum looks clean, click "Stop". Compare it to the unfiltered spectrum. **What's the difference between the two?**

**A:**

Paste a screenshot of the graph with both spectrum samples below:

**A:**

Now remove the excitation filter and put in the **emission filter**. Click on Collect, but this time **don't** save the latest run. Once the spectrum of the emission filter has settled down, click "Stop" and you should see the spectrum of the filtered light (and the original, unfiltered spectrum should still be there in a different color). **How does the new filtered spectrum differ from the unfiltered?**

**A:**

Take a screenshot showing both the unfiltered and emission-filtered spectra and paste it below:

**A:**

**What do these graphs tell you about the behavior of each filter? Is this consistent with what you know about the function of these filters in a fluorescence microscope setup? Explain.**

**A:**

Now put both filters together and take another spectrum using the double filter. **What happens? Why does this make sense?**

**A:**

Save your Logger Pro file and move on to the next part. Leave the microscope apparatus dismantled; you will need to use the individual components in later parts of the lab.

### **Fluorescence**

After you have characterized the filters, take the very bright blue LED light source from the fluorescence apparatus and point it at an index card with fluorescent stickers on it. **What happens?**

**A:**

While still illuminating the fluorescent spot, look at the spot through the excitation filter. **What do you see?**

**A:**

**What can you conclude about the wavelength of the light emitted from the fluorescent spot?**

**A:**

Now put away the excitation filter, and place the emission filter between the LED and the fluorescent spot. **What do you see?**

**A:**

**What can you conclude about the wavelength of light needed to excite the fluorescent molecules?**

**A:**

Now screw the LED back into the cylinder of the excitation filter and screw that back onto the lens, so it's pointing at the dichroic mirror. Put the fluorescent stickers between the lens and mirror so there is a blue dot illuminating one of the stickers (so that half the dot is on the sticker, and half is on the white card). Now look at that dot through the emission filter. **What do you notice about the blue light? And about the fluorescent light?**

**A:**

**Why is this a useful setup for microscopy purposes?**

**A:**

Turn off the LED light source and move on to the next part.

### **Looking at microspheres**

Ask a TF to help you reattach the blue LED light source, emission, and excitation filters to the microscope apparatus. The blue LED should be turned off.

## Microscopy

Obtain a slide of 1-micron spheres and load it into the microscope stage. If there isn't a slide available, you can make one: take one of the slides with the "well" facing up. Shake the suspension of microspheres. Add 3-4 drops of the suspension to the well; try not to get any bubbles. Gently place a cover slip over the well and blot away the excess fluid with a paper towel.

Load the slide into the sample holder. The cover slip should face towards the microscope objective. Be careful not to agitate the slide too much. Plug in the **green LED** light source located on the sample holder.

Turn to page 2 of the Logger Pro file and from the Insert menu, select Video Capture. You won't actually be capturing any video, but the screen will show you the microscope image.

- If you are prompted for which camera to use, choose the USB camera.
- If you are prompted for resolution, choose 800x600.

**Locate the microspheres.** You should see a green image of the microscope focal plane. Using the focus adjustment, try to locate the microspheres. However, be very careful when focusing, as it is very possible to break the microscope slides by trying to bring the objective too close. Don't do that. You will need to bring the objective very close to the cover slip, but **not touching it**.

Cover the entire microscope setup with a dark cloth or a box to block out the ambient light.

**Once you have found the microspheres, can you see the Airy discs? What about the Airy rings surrounding the discs? Paste a screenshot here:**

**A:**

Now turn on the blue light source and turn off the green one. **What happens?** You may need to adjust the angle of the dichroic mirror to ensure that the blue light is focused on the sample; ask a TF to help if needed.

**A:**

Repeat the observations for the samples containing 0.5- and 0.3-micron spheres (use bright-field to locate the spheres, and then switch to fluorescent). **Summarize your results here and include screenshots:**

**A:**

Look at markings on the 40x objective and locate the numerical aperture. It should be after the "40" and a slash. **What is the numerical aperture?**

**A:**

Using the definition of numerical aperture from the Background section, calculate the minimum spatial resolution of the objective. **What is it?**

**s =**

**Are your observations of the three different sizes of microspheres consistent with this calculation? Explain.**

**A:**

Turn off the LED by removing the leads.

Turn off and unplug all power supplies, save your data, and upload it!

## VI. Conclusion

Microscopy

**What is the most important thing you learned in lab today?**

**A:**