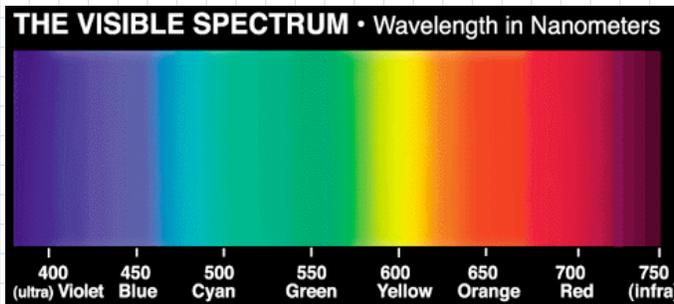


Lab 6: Microscopy

I. Background

A. Light and the visible spectrum

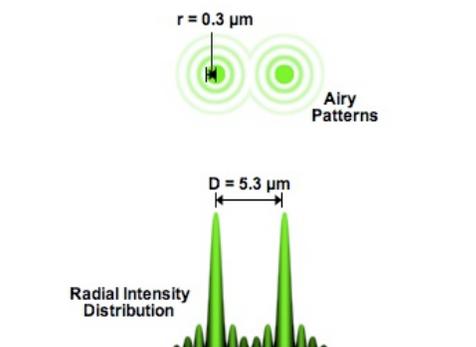
1. What we perceive as color is related to the different wavelengths of light. For example, light of 650 nm wavelength is seen as red, whereas 450 nm is seen as blue.
2. Of course, each wavelength also corresponds to a given frequency, given by $f = c/\lambda$, but it is more common to refer to colors by their wavelength rather than their frequency.
3. The only wavelengths that our eyes can detect are between roughly 400 and 700 nm. This is referred to as the visible portion of the electromagnetic spectrum and is depicted below:



4. A photon with a wavelength slightly longer than about 700 nm is lower in energy than a photon of red light and is thus called infrared (or IR). A photon with a wavelength slightly shorter than 400 nm is higher in energy than violet light and is thus called ultraviolet (or UV).
5. White light is a combination of all of the wavelengths in the visible spectrum. When an object appears, say, red under white light, that means it is reflecting, or re-emitting, the red light, and absorbing all of the other (shorter) wavelengths.

B. Resolution in microscopy

1. Recall that diffraction limits the resolution of any image formed using waves, including light. In the field of microscopy, one natural question to ask is, "What is the smallest feature that can be imaged on a microscope?" A more precise way of asking this question is, "What is the minimum distance between distinguishable objects in an image?"
2. **Rayleigh's criterion** for resolvability states that two diffraction patterns are indistinguishable *if the peak of the central maximum of one lies within the central maximum of the other*. In other words, the angular separation of the two sources must be greater than or equal to the angular half-width of either diffraction pattern for the two sources to be resolvable as separate.
 - a. For diffraction from a single long slit of width D , the diffraction pattern consists of narrow fringes, with the bright central maximum in the middle. The angular half-width θ of the central maximum is given by $\sin \theta = \lambda/D$, which for small values of θ gives the simple result $\theta \approx \lambda/D$.
 - b. For diffraction through a circular aperture of diameter D , the pattern consists of concentric rings, with a bright central disc called an **Airy disc** corresponding to the central maximum.

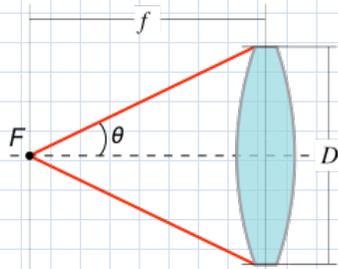


c. The angular half-width θ of the Airy disc is given by
 $\sin \theta = 1.22 \lambda/D$,
 where D is the diameter of the aperture. This can again be simplified using the small-angle approximation, to give
 $\theta \approx 1.22 \lambda/D$.

3. In the particular case of a microscope, we can solve for the smallest distance between two resolvable features by assuming that the sample lies approximately at the focal point of the objective lens. Then the linear distance between the two is equal to the distance to the lens times their angular separation, which gives a distance of about
 $s = 1.22 f\lambda/D$

for the minimum spatial resolution. Here D is the diameter of the objective and f is its focal length. So to resolve very fine features, you want to use short-wavelength light (blue or violet), and you want the lens to have a short focal length but a large diameter. (You can also gain a little bit by immersing the objective and sample in oil or some other high-index-of-refraction material, since that has the effect of reducing λ by a factor of n .)

4. Lensmakers use the term **numerical aperture** (or NA) to characterize lenses. The NA, which is dimensionless, is equal to the sine of the angle θ in the following diagram (note that this is not the same θ we were talking about above, which was related to the size of the central maximum of a diffraction pattern):



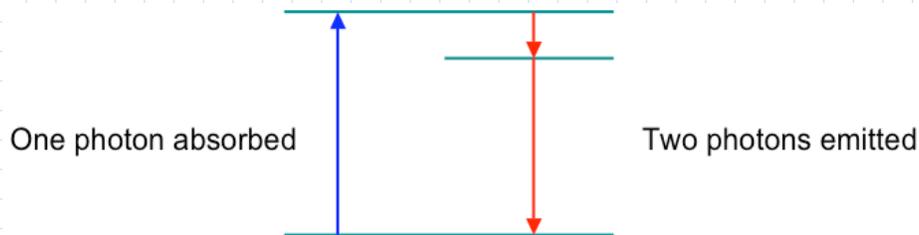
The NA is determined by the curvature of the lens; for small θ , NA is roughly $D/2f$ (since $D/2f$ is $\tan \theta$, and $\sin \theta \approx \tan \theta$ for small θ). But as you can see from the definition, the NA cannot exceed 1 (without oil immersion) and in practice is limited by the fact that lenses cannot have arbitrarily short focal lengths without being extremely small in diameter. (The shorter the focal length, the more curved the lens must be; but the more curved it is, the smaller it has to be.)

5. Lenses with large NA not only have better resolution, but they also accept more light (i.e. a greater percentage of the light emitted from the focal point is gathered by the lens) and thus provide brighter and cleaner images. In terms of the NA, the minimum spatial resolution is
 $s = 0.61 \lambda/NA$.

This is an important equation to know for microscope resolution.

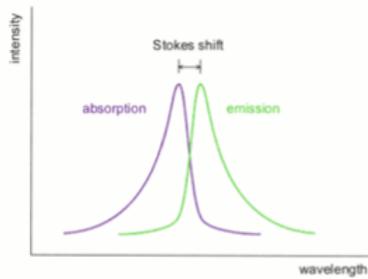
C. Fluorescence microscopy

1. Most of the time, when an atom or molecule absorbs a photon to raise an electron to an excited (higher-energy) state, it immediately re-emits a photon of the same wavelength as the electron drops back its original state. However, certain molecules (which are called **fluorescent**) have the property that when an electron is excited, it prefers to drop back down to its original state via an intermediate level rather than making the whole jump at once. Because of this, two photons are emitted for every one that is absorbed, as seen in the following energy level diagram:



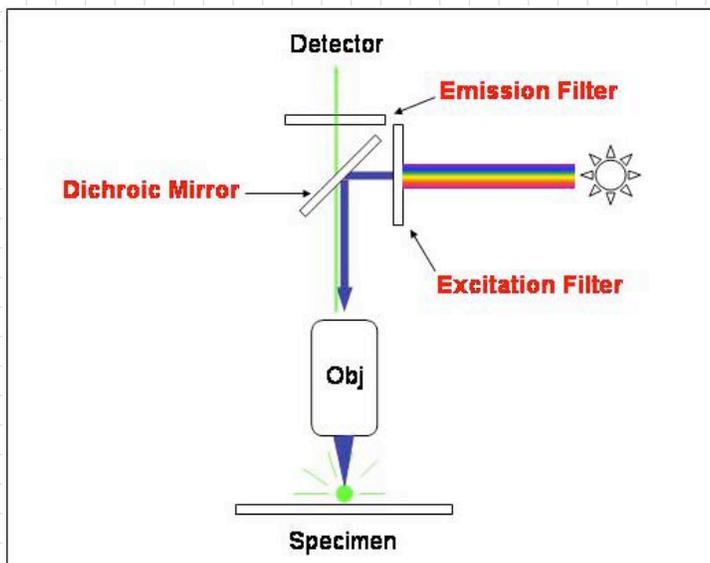
2. The absorbed photon must have an energy equal to the sum of the energies of the two emitted photons. Since the energy of a photon is proportional to its frequency, which is inversely proportional to wavelength, the emitted photons are longer in wavelength than the absorbed photon. This phenomenon is called the **Stokes shift**:

Stokes shift



Typically, one emitted photon is *slightly* longer in wavelength than the absorbed photon (with most of the energy) and the other is *much* longer (with very little of the energy) and hence not visible. The Stokes shift refers to the slight lengthening of the wavelength between the absorbed photon and the more energetic of the two emitted photons.

3. One common application of fluorescence is to use UV light to stimulate the emission of visible light. This is the principle behind the operation of fluorescent lamps, which are much more energy-efficient than incandescent lamps.
4. The application we are most interested in is **fluorescence microscopy**:

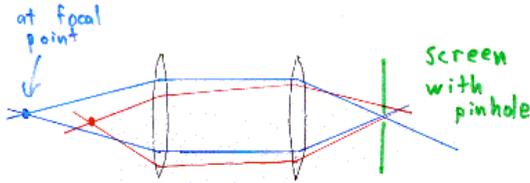


5. In the schematic diagram above, the specimen contains a fluorescent material which emits green light when illuminated with blue light (green is longer wavelength—and hence lower energy—than blue). The operation of the microscope is as follows:
 - a. The *excitation filter* takes white light from the light source and transmits only blue light.
 - b. The *dichroic mirror* reflects the blue light down into the objective.
 - c. The *objective* is a converging lens that focuses the light down onto the sample under observation.
 - d. The fluorescent portions of the specimen then emit green light. The non-fluorescent portions of the specimen merely reflect the blue light.
 - e. Both the green light and then blue light then pass back through the objective. The dichroic mirror allows the green light to pass straight through, but not the blue light.
 - f. Just in case, there is an *emission filter* next which blocks blue light but allows all longer wavelengths to pass through.
6. Thus, the only light which illuminates the sample is blue, but the only light which reaches the detector is green. So the only things that are visible are the parts of the sample which emit green light when excited by blue light; i.e., the fluorescent portions of the specimen.
7. This makes fluorescence microscopy a powerful technique for marking specific details of a specimen. For example, a biologist might tag certain parts of a cell with fluorescent protein and then be able to view the shape and size of those structures without having to physically remove them from the cell.
8. Even for samples which can be viewed using ordinary (bright-field) microscopy, fluorescence can help enhance the contrast

between the sample and its surroundings by drastically reducing the amount of background light in the microscope image.

▼ D. Confocal microscopy

1. Another technique that is used to improve the quality of microscope images (often in conjunction with fluorescence microscopy) is **confocal microscopy**. In a confocal microscope setup, pinholes are placed strategically so that only light from a certain focal plane will actually be used to form the image, and other light is blocked out:



Imagine that the sample is located at the blue focal point. Light emitted from that point is focused by the lenses to an image point on the right, as represented by the blue rays in the diagram. The red rays represent light from another point in the sample (the red rays are *not* intended to indicate a different wavelength of light; for now, wavelength is irrelevant); they are focused to some other image point.

2. If we are particularly interested in the image of the blue focal point, then we can put a screen with a pinhole at the image point of the blue light. At that location, all of the light from the blue focal point is focused down to a very narrow region, and so will pass through the pinhole. But the light from the red point is out of focus there, so most of it gets blocked by the screen. If you put your detector (or eye) to the right of the screen, the only light that will get there is the light from the original (blue) focal point.
(Diagram and explanation courtesy of Eric Weeks, <http://www.physics.emory.edu/~weeks/confocal/>.)
3. In the lab today, you won't be using a confocal microscope setup. But you will be able to play around with a collection of lenses and light sources which illustrates how a confocal microscope works.

▼ II. Introduction

- A. 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. Finally, you'll use a microscope and some knowledge of optics to observe the Boltzmann distribution in a sample of microspheres and derive a value for Boltzmann's constant.
- B. Objectives for this lab:
 1. Understand the limit that diffraction imposes on the resolution of microscopes
 2. Visually explore samples of different sizes under the microscope and see the difference that resolution makes
 3. Understand the operation of a fluorescent microscope, including the spectral properties of the filters in it
 4. Understand the principles behind confocal microscopy
 5. Use the microscope to demonstrate the Boltzmann distribution and measure Boltzmann's constant

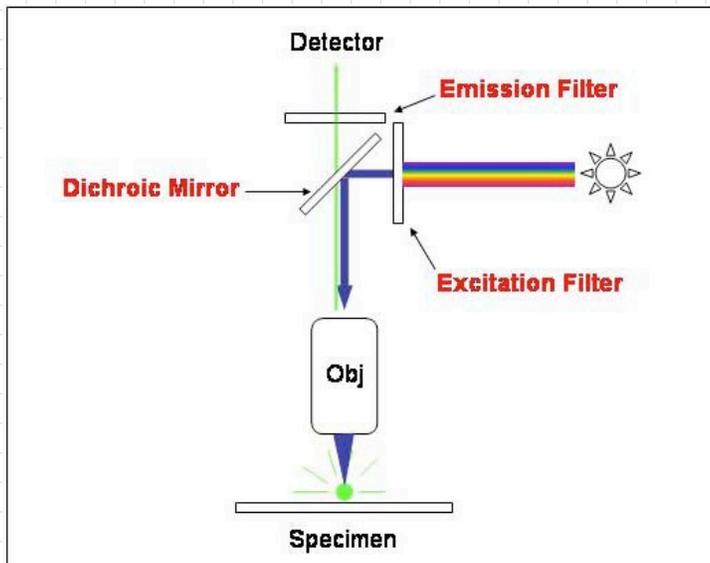
▼ III. Materials

▼ A. Spencer microscope

1. You might remember this microscope from the Brownian motion lab in PS2. It is an ordinary microscope with a 40x objective (labeled in blue) and a 10x objective (labeled in yellow). However, this time it has been outfitted to work as a fluorescence microscope instead of just a regular bright-field microscope, by means of the apparatus attached at the top where the eyepiece normally goes.
2. There are two light sources: a green LED light source which illuminates the sample from below, and a blue light source which illuminates from above. When the green light source is on, the microscope can be used as a bright-field microscope. When the blue light source is on, the microscope acts as a fluorescence microscope, which means that fluorescent parts of the specimen show up as glowing green. To enhance the contrast in fluorescence mode, turn off the green light source.
3. The upper focus knob is for coarse focus adjustments, and the lower knob is for fine focus. The lower knob is calibrated to tell you how much the height of the stage moves when you turn the knob. Some of the microscopes are calibrated in microns; others are in arbitrary units, but a Post-It note with the calibration factor is attached to each scope.

▼ B. Fluorescence microscope apparatus

1. This is a unit that fits on top of the microscope, where the eyepiece would go. It consists of a light source, a dichroic mirror, and two filters, as diagrammed in the fluorescence microscope part of the background information. For clarity, we'll repeat the diagram here:



- 2. The light source is a very bright blue LED attached on the side of the apparatus. It is controlled by an on/off switch and dimmer knob. There is also a clear plastic cone which acts as a focusing lens for the wide-beam LED.
 - 3. The **excitation filter** goes between the blue light source and the dichroic mirror. It makes sure that all wavelengths longer than blue are filtered out, so that the sample is illuminated only with blue light.
 - 4. The **emission filter** goes at the top, just below the iSight camera. It blocks all blue light, so that only the fluorescing parts of the sample can be seen.
 - 5. 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.
- C. iSight camera
You'll use this to capture images from the microscope and analyze them in Logger Pro.
- ▼ D. Vernier spectrometer
- 1. This is a device you may have seen in PS1. Basically, it 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.
 - 2. The spectrometer is very useful for characterizing the filters that comprise the fluorescence microscopy setup.
 - 3. We only have enough spectrometers for every other group to have one. Arrange with your neighbors to share the spectrometer.
- ▼ E. Fluorescent microspheres
- 1. These are polystyrene microspheres which have been coated with fluorescent dye. When the dye is excited with blue light, it emits green light. Several solution 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.
 - ▼ 2. There are three different sizes of microspheres: 0.3, 0.5, and 1 micron (diameter). For the 1-micron spheres, you'll need a little bit more information to do a calculation in the final part of the lab:
 - a. Diameter of microspheres: $d = 1.00 \pm 0.05 \mu\text{m}$
 - b. Density of microspheres: $\rho = 1.057 \pm 0.003 \text{ g/cm}^3$
 - ▼ 3. You might also need the following information about the water in which the microspheres are placed in solution:
 - a. Density of water: $\rho = 0.9973 \pm 0.0005 \text{ g/cm}^3$
 - b. Index of refraction of water: $n = 1.33$
 - c. You may assume that the room temperature is $24 \pm 1^\circ\text{C}$, or $297 \pm 1\text{K}$.
- ▼ F. Confocal "microscope" setup
- 1. This consists of two light sources, a lens, and a viewing screen. The setup is intended to demonstrate the principle behind confocal microscopy, although there is no actual microscope involved.
 - 2. Again, we only have enough setups for every other group. So while your neighbors are using the spectrometer, you could look at the confocal setup, and vice versa.

IV. Procedure

A. Before you begin...

1. Camera setup

- a. Open the file called Lab6.cml in Logger Pro.
 - b. From the Insert menu, select Video Capture. You should be prompted to select a camera; choose IIDC FireWire Video. If it then asks you which resolution you want, select 800x600.
 - c. A video capture window will open. Leave it open for now.
2. Open the Photo Booth software and take a picture of yourselves. (Because Logger Pro is using the external iSight camera, Photo Booth will automatically use the built-in camera, which is what you want.) Drag the photo into the space below:

3. Tell us your names!

4. Go back into Logger Pro and close the video capture window.

5. Put on a pair of protective gloves and carefully remove the emission and excitation filters from the microscope setup. Always use gloves when handling the filters, and please *PLEASE* be careful with them.

- a. The **excitation filter** is the one between the light source and the dichroic mirror. In this apparatus, as in the [diagram](#) from the introduction, the light source connects to the side of the apparatus. Unscrew the black cylinder from the main body of the apparatus, and then carefully unscrew the light from the black cylinder. A small cone of clear plastic (the light guide) will be left inside the cylinder; remove this and you will be left with the excitation filter.
- b. The **emission filter** is the one between the top of the apparatus and the iSight camera. Slide the camera about halfway up the housing of four vertical bars in which it sits, and then carefully unscrew the short black cylinder which contains the emission filter.

B. Characterizing the filters in the fluorescence microscope

1. For this part, you'll need the spectrometer connected to the computer. Since you'll be sharing spectrometers, arrange with your neighbors that one group will do part B while the other does part C (confocal microscopy), and then you can switch. Each part should take about 15-30 minutes.

2. Plug the USB cable of the spectrometer into one of the empty slots in the back of the iMac, and turn to page 1 in Logger Pro.

3.

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.)

4. 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

5.

When the spectrum is the right height, click  Stop and the graph should freeze in place with the spectrum of the light source. Press **Apple-L** to store that spectrum.

6. Now shield the detector with the **excitation** filter and take another spectrum in the same way (but you don't have to store this one using Apple-L). Compare it to the unfiltered spectrum. What's the difference between the two?

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

8. Now remove the excitation filter and put in the **emission** filter. Click on Collect, 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?

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

10. 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.

11. Now put both filters together (you can screw one on to the end of the other) and take another spectrum using the double filter. What happens? Is this what you expected?

- 12. Save your Logger Pro file and move on to the next part.

C. Confocal "microscope" setup

- 1. This is a demonstration in which two light sources at different places produce images through a converging lens. In a real confocal microscope, there would be at least two lenses, but this is a simplified scenario.
- 2. Turn on the LEDs by connecting the clip leads to the battery. You'll see the red and blue lights turn on. **Note:** the lights are different colors only for the sake of viewing clarity; nothing in this section of the lab is critically related to the wavelength of light.
- 3. Position the lens about 6-10 inches away from the circuit board holding the LEDs. On the other side of the lens, put an index card to act as a viewing screen. You should see both red and blue spots on the card. As you move the screen back and forth along the axis of the lens, you will see the red and blue spots grow and shrink.
- 4. Position the screen so that the blue spot is minimized. This is the location of the blue image. Using a ruler, measure:
 - a. object distance (distance from blue LED to lens) =
 - b. image distance (distance from lens to screen) =
- 5. Using the two distances you've measured, and the lens equation, calculate the focal length of the lens. (Don't worry about uncertainty.)
Focal length =
- 6. Now let's consider the red LED.
 - a. Measure the red object distance:
 - b. Use the lens equation again to calculate where the red image should be formed:
 - c. Move the screen until you locate the red image (the place where the red spot is focused to its smallest point). Measure the image distance. Is this close to your calculated value?
- 7. Now move the screen back further. You should see both red and blue spots on it. Take the card with the pinhole in it, and position it at the blue image distance. (Try to center the hole on the blue spot there.) What happens to the spots on the screen?
- 8. Move the pinhole to the red image distance and repeat the observation. What happens now?
- 9. In your own words, explain how this phenomenon could be used to reduce the background light in a microscope setup.
- 10. Turn off the LEDs by unclipping one of the leads from the battery terminals.

D. Fluorescence

- 1. After you have completed parts B and C (in either order), take the very bright blue LED light source from the fluorescence apparatus, and point it at the index card with a dab of fluorescent paint on it. What happens?
- 2. While still illuminating the fluorescent spot, look at the spot through the excitation filter.
 - a. What do you see?
 - b. What can you conclude about the wavelength of the light emitted from the fluorescent spot? (Refer back to part B if you don't remember what the excitation filter does.)
- 3. Now put away the excitation filter, and place the emission filter between the LED and the fluorescent spot.
 - a. What do you see?
 - b. What can you conclude about the wavelength of light needed to excite the fluorescent molecules?
- 4. Now screw the LED back into the cylinder of the excitation filter, along with the clear plastic cone. (Put the narrow end of the cone on top of the LED bulb, and then screw the excitation filter on top of both so that the cone is inside of the cylinder.) Shine it on the fluorescent spot, and look at the spot through the emission filter.
 - a. What do you see?
 - b. Why is this a useful setup for microscopy purposes?

5. Turn off the LED light source and move on to the next part.
- E. Looking at microspheres**
1. Re-attach the light source, emission and excitation filters to the microscope apparatus. If you don't remember what connects to what or need assistance reassembling your microscope, ask a TF for help.
 2. Use the 40x microscope objective. Obtain a prepared slide of 1-micron spheres from one of your TFs and load it into the microscope stage. Turn on the green LED light source below the microscope.
 3. 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.
 - a. If you are prompted for which camera to use, choose IIDC FireWire Video.
 - b. If you are prompted for resolution, choose 800x600.
 4. Locate the microspheres
 - a. You should see a green image of the microscope focal plane. Using the coarse focus adjust, 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.
 - b. If you can't find the microspheres, raise the objective (using the coarse adjust) until it is far from the sample and then try switching to the 10x objective. Then locate the spheres using the 10x and switch back to the 40x without touching the coarse adjust.
 - c. If you still can't find them, ask a TF for help.
 5. Once you have found the microspheres, can you see the Airy discs? What about the Airy rings surrounding the discs?
 6. Now turn on the blue light source and turn off the green one. What happens? (Ooh and aah appropriately.)
 7. 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:
 8. Look at markings on the 40x objective and locate the numerical aperture. It should be after the "40" and a slash.
 - a. What is the numerical aperture?
 - b. Using it, calculate the minimum spatial resolution of the objective. What is it?
s =
 - c. Are your observations of the three different sizes of microspheres consistent with this calculation?
- F. Boltzmann distribution**
1. Go back to the 1-micron sphere sample and locate the microspheres as before. Switch to the fluorescent light mode.
 - a. You can also adjust the intensity of the blue light (using the dimmer switch).
 - b. Adjust the fine focus until you think you have the largest number of microspheres in your field of view. We'll call this height 1.
 - c. Take a snapshot of the field of view at height 1 by clicking on Take Photo.
 - d. Look at the fine focus knob and adjust the height upward by 5 microns. Turn to page 3 and take another photo.
 - e. Repeat step c four more times until you have a photo at each of heights 1 through 5, spanning a range of 20 microns. Each photo should have slightly fewer microspheres than the previous one.
 - f. Save your Logger Pro file.
 2. Count the microspheres
 - a. Now return to page 2. Double-click on the photo and select Standard Analysis. You can use the image analysis features of Logger Pro to help you count the spheres.
 - b.  Click on the  Add Point icon and start marking each sphere in the photo with a colored dot.
 - c. You may have to make some judgment calls about what constitutes a sphere in the focal plane and what does not. It does not particularly matter which criteria you apply as long as you are consistent about it.
 - d.  When you have marked every sphere, click on the  Statistics button in the toolbar. A box will appear on your graph which tells you the statistical features of the dots. The only number we care about is at the very end: samples. That indicates how many total dots there are.
 - e. Record the number of spheres in the appropriate column on page 7 (Analysis) of the Logger Pro file, along with a height of 0 microns.
 - f. Paste a screenshot of your photograph (with your added dots for each sphere) here:

- g. Repeat steps a-e for each of your other photos, filling in the table on page 9 with five rows of data for height values ranging from 0 to 20 microns. (You don't have to paste all of the screenshots.)

3. Analysis

- a. Make a graph of the number of spheres as a function of height. Fit an exponential function to the graph and determine the value of the exponential coefficient. Paste a copy of the graph below:
- b. How far do you go up to move in order to reduce the concentration of microspheres by a factor of e ? This is called the **scale height** of the distribution.
- c. Note that the apparent height changes by less than the actual height of the focal plane by a factor of n (you'll see why on next week's homework assignment). So multiply your scale height by $n = 1.33$ to get a corrected scale height:
- d. According to the Boltzmann distribution, the number of spheres at a given height should be proportional to $\exp(-U(h)/kT)$ where $U(h)$ gives the potential energy of the spheres at height h . In this case, the potential energy is gravitational, but we need to take into account the buoyancy, so
$$U(h) = (\rho_{\text{spheres}} - \rho_{\text{water}}) \left(\frac{4\pi r^3}{3} \right) gh$$
 where the ρ 's are the densities of the spheres and the water, r is the radius of the spheres, and g is the gravitational acceleration.

Based on your value of the scale height and what you know about the microspheres (size, mass, etc.), calculate the value of Boltzmann's constant:

$k =$

What are the units of Boltzmann's constant?

If your value is not at least on the same order of magnitude as the accepted value, re-check your calculation, and talk to a TF if necessary.

- e. Without calculating an uncertainty, how does the accepted value of k (1.38×10^{-23} J/K) compare to your calculated value?

V. Conclusion

- A. Save your work in this file and in Logger Pro.
- B. Submit the electronic copy of your lab report as usual.
- C. You've reached the end of the lab. Congratulations! It's been a pleasure doing labs with you this semester. Good luck in the rest of the course!