

Lab 4: Fluorescence Microscopy and the Boltzmann Distribution

I. Before you come to lab...

A. Read the following material:

- 1. Chapters 33-6; 34-1,2,3,9; 38-2; 40-8 of Giancoli
- 2. The supplementary handout on energy, temperature and the Boltzmann distribution
- B. Read through the entire lab, paying particular attention to the introduction and equipment list.
- C. Answer the questions at the end of this writeup and be prepared to turn them in at the beginning of lab.

II. Introduction

A. Light and the visible spectrum

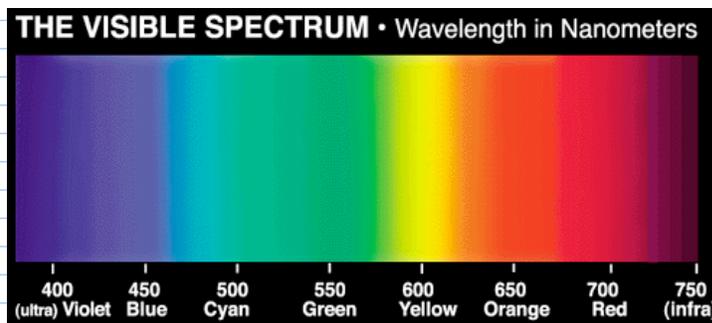
1. Photons

- a. Even though light is a wave phenomenon, careful experiments have shown that light comes in discrete packets called photons.
- b. The energy of a photon is related to its frequency by the equation

$$E = hf$$
 where h is Planck's constant (6.63×10^{-34} J s).
- c. An atom or molecule can absorb a photon by raising an electron to a higher energy level. The change in energy of the electron must be equal to the energy of the photon absorbed.
- d. Similarly, when an electron jumps down from a high energy level to a low one, a photon is emitted which carries away the excess energy.

2. Color and wavelength

- a. 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.
- b. 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.
- c. 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:

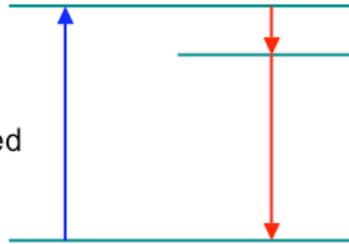


- d. A photon with a wavelength slightly longer than about 700 nm is lower in energy than a photon of red light and is thus called infrared (or IR). A photon with a wavelength slightly shorter than 400 nm is higher in energy than violet light and is thus called ultraviolet (or UV).
- e. White light is 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. 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:

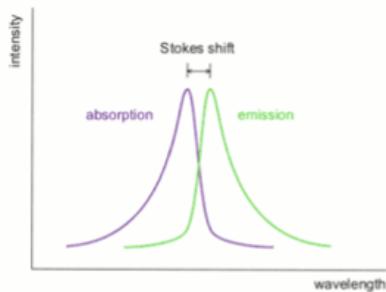
One photon absorbed



Two photons emitted

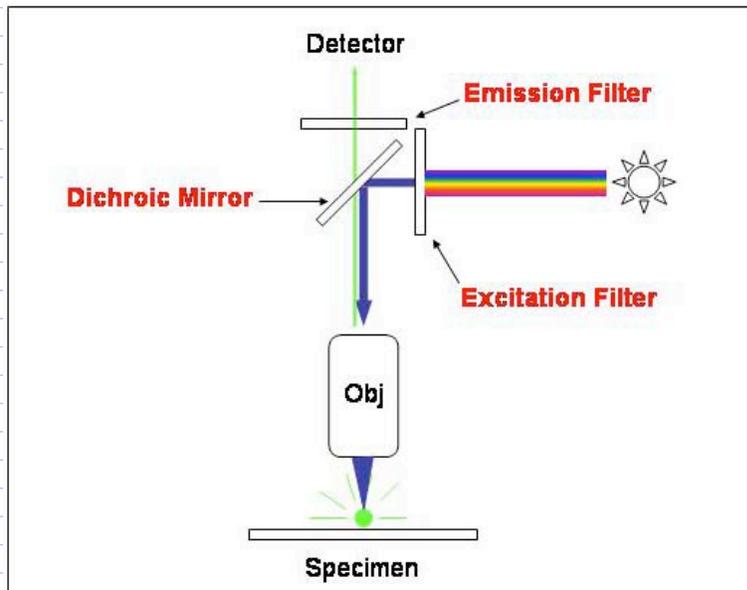
- 2. The absorbed photon must have an energy equal to the sum of the energies of the two emitted photons. Since the energy of a photon is proportional to its frequency, which is inversely proportional to wavelength, the emitted photons are longer in wavelength than the absorbed photon. This phenomenon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

- 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 reaches the detector is from the fluorescent portions of the specimen. 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.

▼ C. Boltzmann distribution

- ▼ 1. Consider a system with many particles, each of which can occupy many different states. We label the states in order of increasing energy: particles in state 0 have energy ϵ_0 (the lowest energy), particles in state 1 have energy ϵ_1 , and so on, so that the particles in state i have energy ϵ_i for any $i \geq 0$. The particles are subject to the following constraints:
- a. There is a fixed total number of particles, N .
 - b. There is a fixed amount of total energy E which is shared by all the particles.
- ▼ 2. We are interested in knowing how many particles are in each state. For every state i , let n_i be the number of particles in that state. Then it turns out that if the system of particles is in thermal equilibrium at a temperature T , the distribution of particles is given by the *Boltzmann distribution*:

$$\frac{n_i}{n_j} = e^{-(\epsilon_i - \epsilon_j)/kT} = e^{-\Delta\epsilon/kT}$$

- a. In fact, the particle energies being distributed according to the Boltzmann distribution is what it means for the system to have a certain temperature. Temperature is expressed in absolute units of Kelvins (K), where a difference of one Kelvin corresponds to a difference of one degree Celsius, but a temperature of 0°C corresponds to 273K.
 - b. In the above equation, k is Boltzmann's constant, and has a value equal to 1.38×10^{-23} J/K.
 - c. Being in thermal equilibrium means the system is not exchanging energy with its surroundings. Later, we will see that when two systems which are not in thermal equilibrium with each other are placed in contact, energy will flow from the hotter one to the colder one until thermal equilibrium is reached at some intermediate temperature.
 - d. The Boltzmann distribution can also be interpreted for a single particle at thermal equilibrium at temperature T : the probability that the particle has a particular energy ϵ is proportional to $e^{-\epsilon/kT}$.
 - e. We can think about kT as being the thermal energy scale of the system: significant variations happen for energies on the order of kT , but if two states are much closer together in energy than kT , they are equally probable. And if two states are much further apart in energy than kT , the higher one will be (almost) unoccupied by any particles.
3. The Boltzmann distribution is pretty much universal, but on a macroscopic level we often don't notice its effects because kT is so small. At room temperature ($T \approx 300\text{K}$), kT is only about 4×10^{-21} joules. So if you have a 1 gram block sitting on the floor, there is a non-zero probability that it is actually moving slightly, or raised somewhat from the floor, but only by some amount so tiny that we don't notice or care. (Its speed is on the order of 10^{-9} m/s and its height off the ground is on the order of 10^{-19} m.)
4. However, for microscopic particles such as molecules and atoms, the Boltzmann distribution is hugely important. It explains why not all of the air molecules in a room are lying motionless on the floor like your 1 gram block. They are so small that for them to have energies on the order of 4×10^{-21} J, they must be whizzing around the room at fantastic speeds... which is exactly what air molecules do.
- ▼ 5. In this lab, we will use objects which fall squarely in between the macroscopic and microscopic: 1 micron spheres suspended in water. The concentration of spheres changes with height because of gravitational potential energy.
- a. Recall from mechanics that an object of volume V immersed in water experiences a buoyant force $F_b = \rho_{\text{water}} Vg$, where ρ_{water} is the density of water and g is the acceleration due to gravity.

- b. This buoyant force reduces the effective weight of the object from $W = \rho Vg$ to $W_{\text{eff}} = (\rho - \rho_{\text{water}})Vg$, where ρ is the density of the object. If the effective weight is still positive (object more dense than water, which is the case for our spheres), it will still sink.
- c. So the gravitational potential energy of an object immersed in water is given by $U_{\text{grav}} = (\rho - \rho_{\text{water}})Vgh$. This is the energy term that goes in the numerator of the exponential.
- d. Therefore, we expect the concentration of spheres to decrease with height: $c(h) = c_0 \exp(-U_{\text{grav}}/kT)$, where c_0 is the concentration at $h = 0$ (which we can define at any height we wish).

▼ 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 yellow LED light source which illuminates the sample from below, and a blue light source which illuminates from above. When the yellow 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. If both light sources are on at once, you will be able to see both the whole field in yellow and the fluorescent parts in green.
- 3. The upper focus knob is for coarse focus adjustments, and the lower knob is for fine focus. The lower knob is calibrated: turning the knob by a single division changes the height of the objective relative to the stage by 2.5 microns.

• B. iSight camera

You'll use this to capture images from the microscope and analyze them in Logger Pro.

▼ C. Vernier light sensor

- 1. This is a probe that measures the intensity of light. The sensitive area is the aperture at the end of the wand.
- 2. You don't really need to worry very much about the units of light intensity (which is the lux); the important thing is that larger numbers correspond to more intense light.
- 3. However, the sensor is not equally sensitive to different wavelengths of light, much like your eye. You will need to account for this when using the sensor to characterize the filters in the microscope setup.
- 4. There are three different ranges of intensity available, which can be controlled with the three-way switch on the black plastic box connected to the detector. For this lab, you should use the 0-6000 lux setting.

▼ D. LED switchbox

- 1. This is a box consisting of 9 LEDs of different colors (wavelengths). The rotary switch on the side of the box controls which LED is turned on.
- ▼ 2. The peak wavelengths (in nanometers) of the 9 LEDs are:
 - a. red 656
 - b. red-orange 631
 - c. orange 605
 - d. yellow 593
 - e. green 525
 - f. aqua 505
 - g. blue 467
 - h. violet 420
 - i. ultraviolet 400

▼ 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. A solution of microspheres in water has been prepared and put onto a well slide for your use.
- 2. Diameter of microspheres: $d = 1.00 \pm 0.05 \mu\text{m}$
- 3. Density of microspheres: $\rho = 1.057 \pm 0.003 \text{ g/cm}^3$

▼ F. Water

- 1. Density of water: $\rho = 0.9973 \pm 0.0005 \text{ g/cm}^3$
- 2. Viscosity of water: $\mu = 8.9 \times 10^{-4} \text{ Pa s}$
- 3. Index of refraction of water: $n = 1.33$
- 4. You may assume that the room temperature is $24 \pm 1^\circ\text{C}$, or $297 \pm 1\text{K}$.

▼ IV. Procedure

▼ A. Before you begin...

- ▼ 1. Camera setup
 - a. Open the file called Lab4.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.
- ▼ B. Filter properties
 - 1. Place the LED switchbox such that the LEDs are at the same height as the light sensor and about 5 cm away.
 - 2. With all of the LEDs off, zero the sensor (Apple-0 in Logger Pro, or choose Zero from the Experiment menu) to account for the background level of light.
 - 3. One at a time, turn the LEDs on and align the light sensor with each one in succession. Record the intensity values in the column labeled I_0 on page 1 of the Logger Pro file. These numbers represent the unfiltered intensities of each LED.
 - ▼ 4. 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.
 - 5. Now shield the light sensor with the emission filter and re-zero it (since the background light level is different with the filter in place). Again, measure the intensity of each LED in succession and record the values in the column labeled I_{em} .
 - 6. Do the same thing for the excitation filter, again re-zeroing before you begin. Record the intensity values in the column labeled I_{ex} .
 - 7. Qualitatively, what do you observe about the amount of light transmitted by each filter at different wavelengths?
 - 8. Create a new calculated column which shows the percentage of light transmitted by the emission filter. Make a graph of this column vs wavelength and paste it below:

 - 9. Do the same for the excitation filter and paste that graph here:

 - 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. Save your Logger Pro file.
- ▼ C. Boltzmann distribution
 - ▼ 1. Re-attach the emission and excitation filters to the microscope apparatus.
 - a. First, screw the emission filter back into the top of the apparatus and lower the camera back down to its original position.
 - b. Next, put the narrow end of the light guide on top of the light bulb and then screw the excitation filter onto it. Then take the combined unit and screw it back into the side of the microscope apparatus.
 - c. 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 from one of your TFs and load it into the microscope stage. Turn on the yellow LED light source below the microscope.
 - ▼ 3. Turn to page 2 of the Logger Pro file and from the Insert menu, select Video Capture.

- 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 (yellow) 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. When you have found the microspheres, turn on the blue light source. You should see the microspheres glow green. Turn off the yellow light source, and you will see only the spheres, with no background light. (Ooh and aah appropriately.)
 - c. You can also adjust the intensity of the blue light (using the dimmer switch).
 - d. 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.
 - e. Take a snapshot of the field of view at height 1 by clicking on Take Photo.
 - f. Look at the fine focus knob and adjust the height upward by 5 microns. Turn to page 3 and take another photo.
 - g. Repeat step f five more times until you have a photo at each of heights 1 through 7, spanning a range of 30 microns. Each photo should have slightly fewer microspheres than the previous one.
 - h. Save your Logger Pro file.
- ▼ 5. 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 9 (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 seven rows of data for height values ranging from 0 to 30 microns. (You don't have to paste 7 screenshots.)
- ▼ 6. Analysis
 - a. Make a graph of the natural log of the number of spheres as a function of height. Fit a line to the graph and determine the slope. Paste a copy of the graph below:
 - b. What is the scale height of the distribution? In other words, how far do you have to move in order to reduce the concentration of microspheres by a factor of e ? (Warning: you will have to convert your height from apparent depth into true depth. See pre-lab question B.)
 - c. Based on this 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.
 - ▼ d. Calculate the uncertainty of your value of k .
 - ▼ (1) The full calculation is extremely messy, but you can greatly simplify it by identifying which sources of uncertainty are significant and which are negligible. Here is a comprehensive list of quantities with uncertainty which might propagate into the uncertainty of k :
 - (a) the scale height calculated from your graph
 - (b) the conversion factor from apparent depth to true depth
 - (c) the density of the microspheres

- (d) the density of water
- (e) the radius of the microspheres
- (f) the absolute temperature
- (g) the acceleration due to gravity
- (2) Talk to a TF if you are unsure how to do this calculation.
- (3) uncertainty of $k =$
- (4) Does the accepted value of k (1.38×10^{-23} J/K) fall within your range of uncertainty? If not, how far outside is it?

▼ V. Conclusion

- A. You've reached the end of the lab. Congratulations!
- B. Save your work in this file and in Logger Pro.
- C. Submit the electronic copy of your lab report as you did for Lab 3. The instructions for doing so are on a laminated sheet by each computer. They are slightly different this time because you will want to save your images in addition to the Logger Pro file.

▼ VI. Pre-lab assignment.

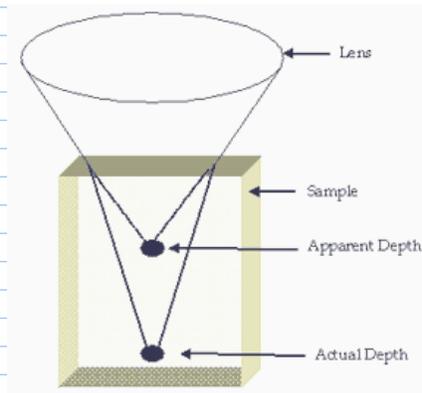
Answer the following questions on a separate sheet of paper *before* coming to lab. Remember to write your name and lab time on the sheet.

▼ A. Terminal velocity, diffusion, and the Boltzmann distribution

- 1. The weight and the buoyant force are not the only forces on a falling microsphere. There is also a drag force which opposes the motion of the sphere (i.e. it points upwards) given by $F_{\text{drag}} = 6\pi\mu r v$, where μ is the viscosity of water, v is the speed of the sphere, and r is its radius. Using this equation, calculate the terminal velocity of a sphere falling in water, and estimate its numerical value for the microspheres we will use in the lab.
- 2. If you took PS2, you learned that the flux of particles, J , is related to the concentration gradient by Fick's equation: $J = -D \, dc/dh$ where D is a constant called the diffusion coefficient. In the presence of external forces (like gravity), the flux becomes $J = -D \, dc/dh + (DF/kT) c$ where F is the net external force in the $+h$ direction. Show that the Boltzmann distribution, $c(h) = c_0 \exp(-U/kT)$ where U is the potential energy as a function of h , is an equilibrium solution to Fick's equation (i.e. show that the flux is zero).
- 3. For a well slide of depth $350 \mu\text{m}$ which initially contains a uniform concentration of microspheres, estimate how much time it would take to reach the equilibrium concentration. (Hint: use the terminal velocity.) Can you see why you will be using slides prepared a day in advance?

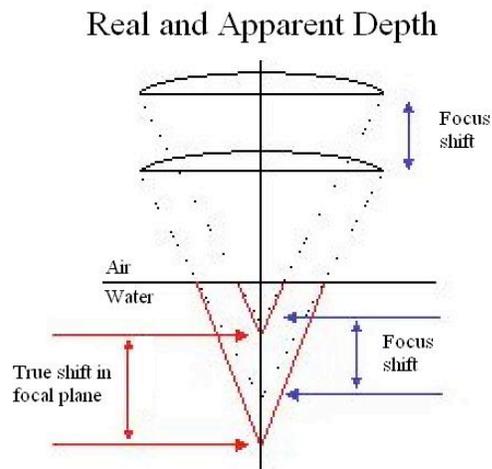
▼ B. Apparent vs true depth of the microscope focal plane

- 1. When you look through a microscope, you see whatever happens to be located at the focal point of the microscope's objective lens. The purpose of adjusting the focus knobs on the microscope is to move the focal point up and down until the objective is focused on whatever you are trying to observe. However, because of refraction, the focal point isn't where you might expect it to be:



The diagram is exaggerated for effect, but you can see that because the sample is immersed in water, the actual depth of the focal plane differs from the apparent depth. If the index of refraction of the water is n , and all of the lines in the diagram are nearly vertical, calculate the actual depth below the surface in terms of the apparent depth.

2. Now consider the problem of adjusting the focus:



When we raise or lower the objective by $1\ \mu\text{m}$, by how much does the actual focal plane shift? You will need the answer to this calculation to do the analysis of your data.

3. Does your answer to part 2 change if you add a glass cover slip between the lens and the water surface?