MICROSCOPY AND CELL MOTILITY

I. ABSTRACT

Surrounded by a fluid in thermal equilibrium, all cells move in response to random interactions with their environment according to Brownian motion. Some cells are also "active particles," capable of self-propulsion, perhaps by swimming as driven by their flagella or cilia, or by pushing their way past other cells, changing shape as needed. Bacteria may swim in search of food sources, while the macrophages of our bodies may hunt down and swallow invading cells that could be a threat to our health. Such motion can be studied quantitatively using an optical microscope and CCD camera.

II. OBJECTIVES

- Operate a microscope in bright-field mode
- Calibrate a CCD camera and use it to capture images of cell motion
- Measure the diffusion constant of small spheres undergoing Brownian motion
- Observe and quantitatively characterize the motion of a swimming bacterium

III. BACKGROUND

The theoretical background for Brownian motion and the self-propulsion of cells is covered in an accompanying document. Please read this material and answer the questions it contains before you come to the biophysics lab.

In this lab module, cell motion is studied using a CCD camera attached to an optical microscope. Compound microscopes are part of the equipment for the biophysics lab; the one described here is a Carl Zeiss Microscope. The optical configuration of a conventional bright-field microscope is based on two lenses—an objective lens near the specimen and a second lens at the eyepiece. The distance between the lenses is arranged such that an observer sees an inverted virtual image, as in Fig. 1. You may want to refresh your knowledge of optics from first-year physics if terms such as *inverted* and *virtual* are unfamiliar.

The objective lenses are mounted on a turret or nosepiece that can be rotated as needed. Modern microscopes are parfocal, meaning that the specimen remains in focus if a new lens is rotated into position without having to adjust the height of the microscope stage. Lower magnification lenses (10x, 20x and 40x) are designed for use in air, while higher magnification lenses (50x and 100x) require a drop of oil (with a specific index of refraction) to be placed between the objective and the specimen. In the latter cases, the specimen must be inert to the oil, or be protected from it by a glass coverslip. Note that not all of these lenses will be on the lens turret of the microscope you will be using.

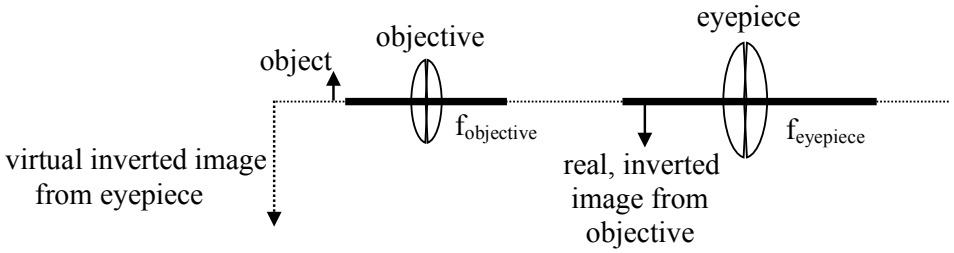


FIG. 1. Images in a compound microscope.

The illumination of the specimen must be adjusted to obtain images with maximum resolution. To understand how and why a microscope must be adjusted to produce high-resolution images, we first recall a basic result from the theory by *Ernst Abbe*, a 19th-century German scientist working at the Zeiss microscope company. According to Abbe's imaging theory, the resolution of a microscope is set by

$$d = \frac{\lambda}{n_c \sin\theta_c + n_o \sin\theta_o}\,, \tag{1}$$

where d is the smallest separation between two general objects in a sample that can be resolved, n_c is the index of refraction of the medium separating the condenser from the sample, n_o is the index of the medium separating the objective from the sample, θ_c is the maximum angle of rays hitting the sample, θ_o is the maximum angle of rays collected from the sample. See Fig. 2. The combination $n \sin\theta$ is often defined to be the *numerical aperature* (NA), and thus we can write Eq. (2) as

$$d = \frac{\lambda}{NA_c + NA_o}\,, \tag{2}$$

where we define the numerical apertures of the condenser and objective. Notice that the resolution is proportional to the wavelength of light used (0.4–0.7 μm for blue–red light). Notice, too, that the key to getting high resolution is to use high-NA condensers and objectives. If we work in air ($n = 1$), the NA ≤ 1, and the maximum resolution is just $d \geq \lambda/2$.[1]

With this idea of resolution in mind, we can understand what we desire from a microscope illumination system:

1. Illuminate the sample plane uniformly, even if the source (e.g., a light-bulb filament) is irregular.

2. Control the area of illumination. (Many biological samples and chemical probes are damaged by light and we should not illuminate parts of the sample unnecessarily.)

[1] It is worth noting that this limit follows from basic ideas in quantum mechanics. In one dimension, Heisenberg's Uncertainty Principle states that $\Delta x\, \Delta p \geq h$. For a single photon diffracted at an angle θ in vacuum (air), the de Broglie relation implies that the change in momentum is $p = \frac{h}{\lambda} \sin\theta$. Putting these together gives $\Delta x\, \frac{h}{\lambda} \Delta(\sin\theta) \geq h$. The maximum change in $\sin\theta$ is 2 (from $+1$ to -1), since a beam can be diffracted from the forward to the backward directions. Simplifying, this gives $\Delta x \equiv d \geq \lambda/2$.

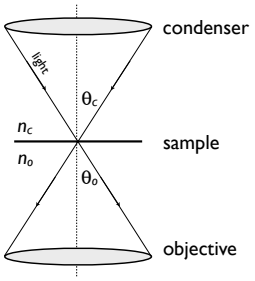


FIG. 2. The cone of rays (NA) produced by the condenser and collected by the objective determines the resolution of an object within the sample.

3. Control the angle of the cone of rays of the condenser and objective. These should be optimized for resolution, as described above.

A system of illumination that responds to all three of these criteria is known as *Köhler illumination* and dates, again, from the 19th c. The basic idea is to place an image of the filament at the back focal plane of the condenser, so that each point on the filament produces a parallel beam of light that uniformly illuminates the sample. See Fig. 3 for a transmitted-light version.

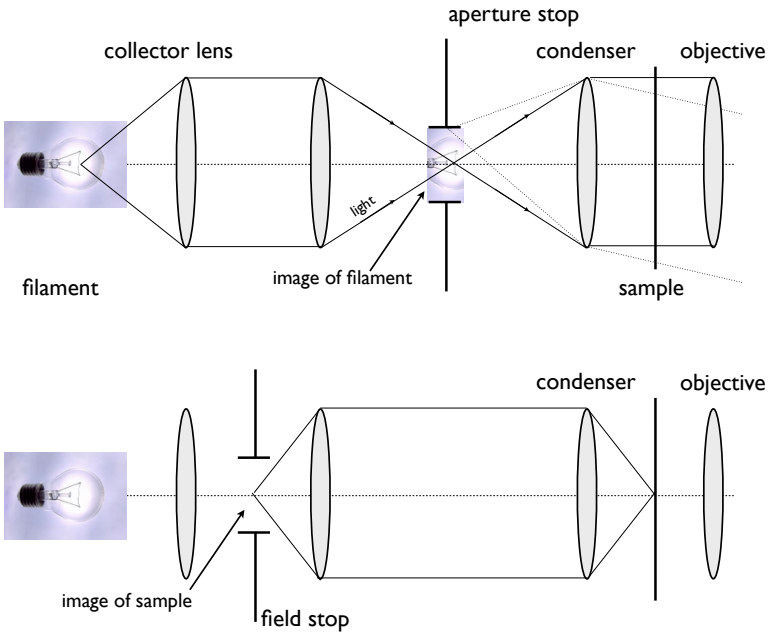


FIG. 3. Köhler illumination. Top. The filament is imaged to an intermediate plane. At that plane, a diaphragm functions as the aperture stop, determining the angle of rays that illuminate the sample. Bottom. The same elements, redrawn to show the field stop, which is imaged onto the sample plane, thus controlling the illumination area.

To translate these ideas into practice, we describe here, rather than in the procedures

section, how to set up Köhler illumination. All parts are labeled in Fig. 4. There are two diaphragms to adjust: a field diaphragm and a condenser diaphragm (field and aperture stops).

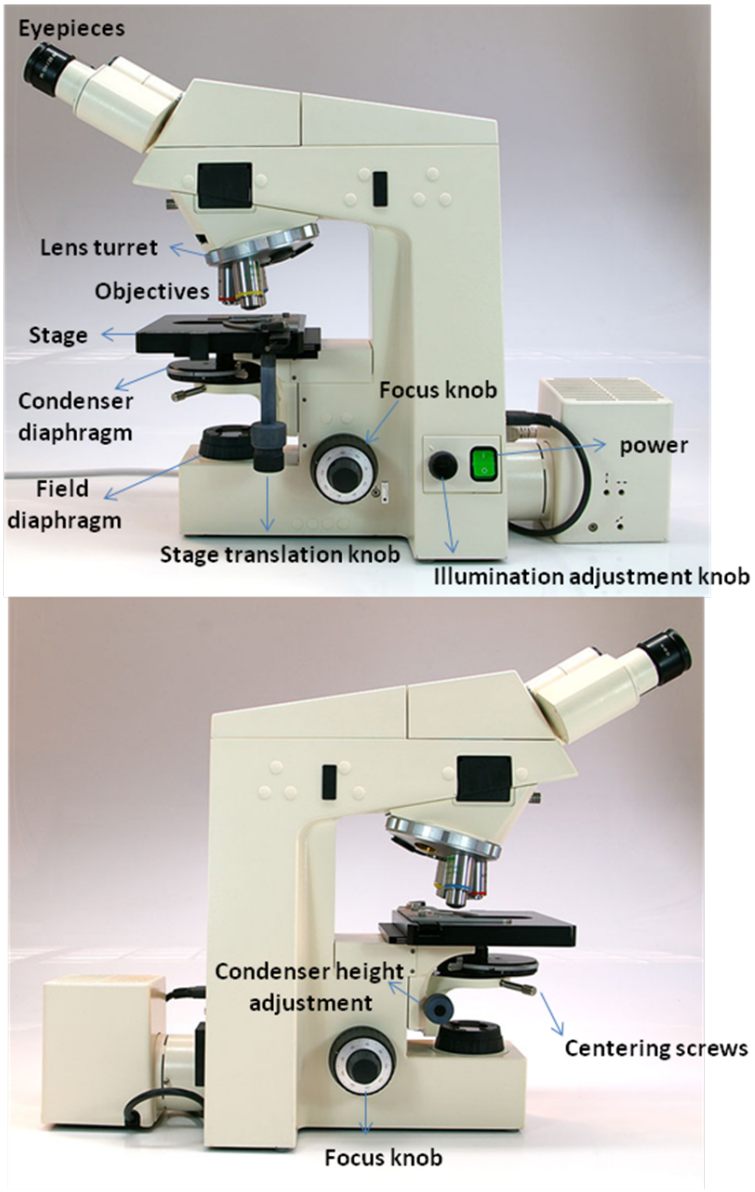


FIG. 4. Typical configuration of a Carl Zeiss upright microscope.

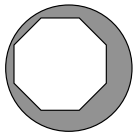
- *The field diaphragm*, which creates the field stop, is located on the base of the microscope, and its adjustment ring shows its open and closed position. It controls the area of the sample that is illuminated.
- *The condenser diaphragm*, which creates the aperture stop, is mounted under the stage and is controlled by a small lever under the condenser between the two centering

screws. Slide the lever to control the aperture; start with the condenser aperture largely closed (lever to the right) and the field diaphragm open.

Also, make sure the neutral density filter, located just below the centering screws, is rotated out of the light path. Last but not least, make sure the illumination lamp is turned on before your start! The on-off switch is located at the right rear of the microscope housing and the brightness of the lamp can be changed by rotating the illumination adjustment knob.

1. Place the specimen on the stage. Use the height adjustment knob at the back of, and under, the stage to raise the condenser lens to its highest position. Rotate the 10x or 20x objective lens into place. Then, use the focus knobs to raise/lower the stage and bring the specimen into focus. DO NOT change the height of the stage any further during this procedure.

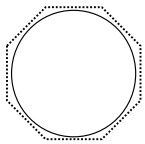
2. Contract the field diaphragm to reduce the amount of light entering the sample until the illuminated spot seen through the eyepiece is smaller than the field of view and you can see the edges of the diaphragm (may be blurry). What you should see through the eyepiece is something like:



3. Adjust the height of the condenser (NOT THE STAGE!) to optimize the focus of the field diaphragm. The diaphragm edges should be sharp, not blurry. Use the small knobs attached to the condenser for this adjustment, not the big focussing knob for the objective. Because the condenser lens has a fair bit of chromatic aberrations, the image goes from having a blue halo to a red halo as you move through the plane of sharpest focus. (The different colours are in focus at slightly different heights.)

4. Use the two centering screws to centre the illuminated spot (which tracks the condenser lens) relative to the circular microscope field of view. (Look directly through the eyepiece rather than using the camera.) The focal region can be moved in two directions using two centering screws, each at a 45° angle with respect to the principal axes of the rectangular stage. Start with the field diaphragm opening fairly small; then gradually open it as you perform the adjustment, making the offset from the centre more obvious. After this adjustment, the image of the diaphragm should look like a polygon inscribed and centred within the circular viewing region of the eyepiece. If the microscope is badly out of alignment, you may need to refocus and adjust the height of the condenser iteratively.

5. Open the field diaphragm more, but just enough to fully illuminate the sample. Now, the circular viewing region inscribes the polygon:



If the image isn't bright enough, increase the brightness of the lamp rather than open the diaphragm any further. Adjust the condenser aperture so that its NA (the angle of the rays focussed on the sample) approximately matches the NA of the objective lens you use. (See Fig. 2 and the microscopy website given at the end of this document.) Thus, for a 100x objective, the condenser diaphragm should be fully open (lever to the left) to collect the largest amount of light; for a 10x objective, the diaphragm should be closed down more.

If you want to work with a high magnification objective lens (100x), it is better to first center the polygon using a lower magnification lens (e.g. 10x) as described above, and then carefully rotate the objective turret to the 100x lens.

IV. MATERIALS AND EQUIPMENT

- Upright, bright-field microscope with attached CCD camera
- Computer running LabVIEW program to control the camera
- Glass microscope slides and coverslips (No. 1), parafilm and heat block
- Stage micrometer for calibration
- Concentrated solution of 1 μm diameter polystyrene spheres (or beads)
- Concentrated solution of *E. coli* strain HCB1274
- *S. cerevisiae* (baker's yeast), water and sugar
- Other cell samples, as provided
- micropipette, tips and microcentrifuge tubes

V. INVESTIGATIONS

Before doing any measurements, familiarize yourself with the microscope by performing the adjustments outlined in Sec. III to align the optics and optimize the illumination of a prepared slide or sample such as the stage micrometer. Next, calibrate the scope and camera using a *stage micrometer*, a special glass microscope slide with precisely cut calibration marks.

A. Image acquisition and calibration

Select the 100x objective by rotating the lens turret. Note the number of lines per cm on the stage micrometer, then place it into the sample holder on the microscope stage and adjust the xy location of the stage such that the micrometer is centered in the field of view. The stage can be translated using the coaxial knobs on its right-hand side (see Fig. 4). Turn on the illumination lamp and adjust the brightness using the illumination adjustment. Fine-tune the location of the micrometer by viewing it through the eyepiece.

Now, run the camera program to see and capture images using the CCD camera. This is described in *Protocol: Image acquisition*. Light must be directed away from the eyepieces and toward the camera using the sliding rod on the microscope housing above the stage (at the base of the eyepiece housing); otherwise, the camera image will appear to be black. Adjust the illumination as needed if the image is under or over-exposed. This is best done using the illumination adjustment knob (Fig. 4), but can also be changed by modifying the default shutter value in the camera program.

Capture an image of the stage micrometer. To calibrate, open the image using an image-viewing program and draw a screen box between the centres of two calibration marks on the micrometers; you can use the edges of the marks if you prefer, just be consistent in your choice. Choosing widely spaced marks reduces the uncertainty of the calibration. Note the dimensions of the screen box in pixels. Divide the known length between calibration marks by the number of pixels to obtain the physical image length per pixel. Note that the eyepiece magnification is irrelevant to the calibration, as light does not pass through it on the way to the camera. Your calibration is valid only for the 100x objective that you will be using.

B. Brownian motion

The preparation of the wet-mount microscope slide needed here involves two initial steps: fabricating a small chamber on the slide and mixing the bead+water solution to be studied.

1. Microscope chamber and bead dilution

For instructions on chamber-making, see *Protocol: Making Sample Chambers*. Make both thick chambers (with parafilm spacers) and thin chambers (with no spacer, as detailed in the protocol). For this experiment, you will be provided with a concentrated solution of 1.27 μm diameter polystyrene beads (0.5% weight/volume), which you will have to dilute before taking images. Calculate the dilution factor first, and check it with the laboratory instructor. The chamber depth is dictated by the thickness of the parafilm ($\gtrsim$ 100 μm thick), and the camera's field of view is 640 $\times$ 480 pixels^2. The depth of field (thickness where objects are in focus) depends on the settings. We will use conditions where it is $\lesssim$ 5 μm. Ideally, you would like to image $\approx$ 20 beads in the field of view. The following prelab question will take you through the calculations needed for this.

Pre-lab question 1: If the microscope's calibration factor is 100 nm / pixel, what dilution of beads do you need to make to achieve the desired density in the viewing area? The density of polystyrene is $\approx$ 1.05 g/ml. Assume that the depth of field $\approx$ 5 μm.

Once you have calibrated the microscope viewing area, you must redo these calculations using your own values. After making the dilute solution of beads, load it into the chamber and seal, as described in *Protocol: Making Sample Chambers*.

Place the bead-bearing chamber on the microscope stage and move into focus, viewing with the 100x objective. Note that the 100x objective is an oil-immersion objective, which means that the medium between lens and sample (the n in NA=$n \sin\theta$) is ≈ 1.5. Using $n > 1$ increases the microscope's resolution. In practice, you need to put a drop of microscope oil between the sample chamber and the lens in order to focus properly into an aqueous sample. VERY CAREFULLY put a small drop of oil there, which will require raising the lens away from the sample chamber. When properly focused into the sample chamber, the objective will be almost in contact with the sample. The depth of focus of this objective is much less than the depth of the chamber, so beads will move in and out of focus with time. Try to focus on the mid-range of the chamber before collecting data. Since the focus of the camera may differ slightly from what you see through the eyepiece, double-check the focus on the screen. Vary the brightness of the illumination lamp until you are content with the image quality.

2. Data collection and analysis

You are now ready to track the motion of a bead and to study its dynamics. Consult *Protocol - Acquiring Movies with Vision Assistant* for details. Take at least five seconds of images as a data set. You need to capture the motion of at least one bead for several seconds before it drifts out of focus. That is, a bead wanders out of focus long before it diffuses out of the xy capture region of the camera. The result of this procedure should be a movie (in .avi format) of the motion of one or several particles.

The next step is to convert the images of diffusing beads into trajectories (x_n, y_n) for each bead, where $x_n \equiv x(n\,\Delta t)$ is the position as measured at time step n, with interval Δt between each image. See *Protocol - Tracking Beads* for information on how to do this.

From the background material on diffusion, we expect that the displacements $\Delta x_n \equiv x_{n+1} - x_n$ are Gaussian random variables with mean 0 and variance $2D\Delta t$. Test this claim by making a histogram of displacements. Some things to try:

- Calculate the mean and variance directly from the time series. (Igor has `Wavestats`, as well as `Mean` and `Variance` functions. Matlab has `Mean` and `Var`.) From the Stokes-Einstein relation (see background material), you should be able to predict the variance. Compare with observations.
- Fit the histogram shape to a Gaussian. The mean and variance should match, approximately, your results above. Is the shape consistent with a Gaussian?

- The expression for the variance neglects a couple of effects. First, each measurement of the position x_n can have its own noise η_n that is independent of the thermal noise. Let us assume its variance is η^2. Also, each measurement is not instantaneous but averages the position over the camera exposure time t_c. The result is that the increment Δx_n is not independent of the increment Δx_{n-1} because both share motion over the camera exposure for x_n. A more detailed calculation then gives

$$\langle \Delta x^2 \rangle = 2\eta^2 + 2D(\Delta t - \tfrac{1}{3}t_c)\,. \tag{3}$$

  In Eq. (3), the measurement error is two η^2 because Δx is the difference of two measurements, each with independent measurement errors of variance η^2. Are these corrections significant? (You can estimate η^2 if you can measure a stuck bead. Otherwise, try to get an approximate value by looking at the bead image and the value reported by the analysis program and roughly guess at the likely error of a measurement.

The second way to estimate the diffusion constant is to calculate $\Delta x^2(\tau) \equiv \langle [x(t+\tau) - x(t)]^2 \rangle = 2D\tau$, as a function of τ by averaging different intervals. This function is known as the *mean-squared displacement* (MSD). We'll provide an Igor function to help calculate it. Then plot $\Delta x^2(\tau)$ vs. τ and fit to find the slope. Although this method seems similar to the histogram and direct variance calculations above, it has some advantages. First, when the interval τ is large, the corrections mentioned above will be truly negligible. Second, motion is often more complicated than diffusion, and looking at dynamics over a range of time scales can tease out such behaviour. For example, $\Delta x^2(\tau)$ vs. τ may be linear over a range of time intevals τ but deviate at either earlier or later time intervals (or both). In practice, just fit the "straight" portion of the MSD.

C. Bacterial motion

Beads are passive objects: they diffuse in a solution and drift in response to flows and other forces. *Active materials* are a more interesting class of objects: they can swim and push and generally show a richer set of dynamics. Of course, they need an energy source to create this motion and thus are intrinsically non-equilibrium systems. Perhaps the nicest examples are living organisms. In this lab, we next study the motion of several types of bactera, all varieties of *E. coli*.

You will be provided with a concentrated solution of mutant *E. coli* (strain HCB1274) which has one component of its motion altered. Normally, *E. coli* self-propel at constant velocity (*run*) for a second or so, before stopping to *tumble* and randomly select a new direction for the next run. In the strain used for this module, the tumble mode has been disabled. The experiment proceeds exactly as in Sec. B for polystyrene beads. Once again, you will have to calculate the dilution factor for the sample to be observed; reduce the number of *E. coli* in the camera's field of view by at least a factor of two compared to the number of beads in Sec. B. When you focus on the specimen, the bacteria will appear as rods of differing length; they may appear to rotate about an axis as well as translate. Different preparations of the cells may show different levels of activity. The density of bacteria in your sample solution should be lower than the bead sample, making it potentially easier to

follow the cells unambiguously; however they move faster than inert beads under Brownian motion, making tracking more difficult. Follow the same analysis as Sec. B through to the end.

Pre-lab question 2 (to be completed for lab period 2): What dilution of *E. coli* do you need to make to achieve the desired density in the viewing area? Assume the *E. coli* solution you are given has an optical density at 600 nm of $OD_{600} = 1.0$, and that this optical density corresponds to 2×10^8 cells/ml. Then calculate the dilution needed to obtain ≈ 10 cells in your field of view.

D. Cell size

Here, you will image eukaryotic cells, to get a sense of the sizes and morphologies (shapes) of a different cell types. You should grow *Saccharomyces cerevisiae* cells by sprinkling some baker's yeast (one teaspoon = 5 ml) in 250 ml of warm water that has had 20 grams (2 tablespoons) of sugar added to it. Stir gently and let sit for five minutes, until the mixture is bubbling. Then prepare a wet mount of this sample. You may need to dilute it to clearly see the cells. Determine their size and shape and compare with *E. coli*.

Image cells from the human cell line HT1080. They are provided as a prepared, fixed mount, either unstained (for comparison with previously imaged samples) or stained blue for enhanced contrast. HT1080 cells are epithelial cells (cells that form the lining of tissues) derived from fibrosarcoma cells, which are malignant tumor cells from connective tissue. Because they are tumor cells, they proliferate easily and are used in laboratories around the world to produce mammalian proteins and study cellular processes.

VI. SUGGESTED TIMELINE

Day 1:

1. learn the operation of the microscope and its attached CCD camera
2. image a stage micrometer and calibrate the camera
3. prepare a wet mount of 1 μm diameter polystyrene spheres
4. capture and analyze the Brownian motion of the spheres
5. come to lab having answered the six pre-lab questions in the background document and the first pre-lab question in this document

Day 2:

1. come to lab having answered the second question in this document
2. prepare a wet mount with the *E. coli*

3. capture the movement of *E. coli* under their self-propulsion
4. quantitatively analyze their motion
5. measure the sizes of eukaryotic cells and note any differences between their appearance and that of *E. coli*

VII. ITEMS TO INCLUDE IN LAB WRITE-UP

Please include in your lab report a description/discussion/tabulation of the following items. Spreadsheets can be submitted electronically, while the rest of the information, including example calculations of each type, must be included in hard copy.

- Answer all eight prelab questions. (6 are in motility paper; 2 here.)
- Present micrometer calibration.
- Phys 833: Describe the tracking algorithm used.
- Prepare a table of raw measurements of xy-coordinates in pixels and microns. Include, for each individual cell or bead studied, t (sec), x (px), y (px), $x(\mu\text{m})$, y (μm).
- Include example plots of
  - y vs. x
  - x vs. t and y vs. t
  - $r^2 = x^2 + y^2$ vs. t

  for one bead and for one bacterium
- Histogram Δx and Δy for one bead, where "Δ" is calculated over one time interval Δt. Include Gaussian fit, estimate of D. (Correct for camera exposure.)
- Define $\Delta\, x(m) \equiv x_{n+m} - x_n$. That is, look at the displacement over a time $\tau = m\,\Delta t$. Then plot $\langle[\Delta\, x(m)]^2\rangle$ vs. $m\,\Delta t$. The ensemble averages are first over the trajectory of a single bead. Then you can decide whether the trajectories of different beads are uniform enough to average over them, as well. Repeat for the y coordinate.
- Analyze whether motion is diffusive or self-propelled by finding the time-dependence of $\langle[\Delta\, x(m)]^2\rangle$ (and y, too): does it scale like t or t^2? Discuss....
- For beads, you now have two estimates of D. Compare to each other and to the Stokes-Einstein prediction.
- Calculate the dilution factors for beads and cells.
- Discuss difficulties encountered in the experimental procedures.

- Discuss how motion in the third dimension (z) affects your results.
- Include images of each cell type (bacterial and eukaryotic, including scale bars) and sizes of cells that you determined from these images
- Discuss differences in morphology between the different cell lines.
- Discuss at least two (Phys 433) or of all (Phys 833) questions from Sec. VII below.

VIII. QUESTIONS TO PONDER

- How would your results change if you used larger beads? What about the same size of beads but made of a metal?
- Why do the speeds of the bacteria vary, and what influences their variation?
- How would your results change if you followed the bacteria for a shorter period of time (but at much higher frame rate)? For a much longer period of time? Please explain your reasoning.
- What dynamics would you expect to observe for a spherical bacterium that had no propulsion mechanism?
- How does the theoretical diffusion constant depend upon cell length? [See Fig. 4.5 of Berg (1993).]

IX. ADDITIONAL READING

- Nikon, manufacturer of microscopes and lenses has an excellent resource for microscopy.
- Howard Berg, *Random Walks in Biology*, Princeton (1993). Ch. 4-6.
- Dennis Bray, *Cell Movements: From Molecules to Motility*, Garland (2001). Ch. 1–3, 16.