

Microscopy and Cell Motility¹

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 digital camera.

Objectives

- Operate a microscope in bright-field mode
- Calibrate 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 swimming bacteria; contrast with motion of similarly sized beads
- Design and carry out an experiment of your choice

1 BACKGROUND

The theoretical background for Brownian motion and the self-propulsion of cells is covered in an accompanying document *Background on Cell Motion*. Please read this material and answer the questions it contains as you to prepare to work on the microscopy lab.

In this lab module, cell motion is studied using a digital camera attached to an optical microscope. Compound microscopes are part of the equipment for the biophysics lab; the one shown in Fig. 1 is a Carl Zeiss Microscope, but other microscopes are similar. 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 objective forms a magnified, real image as shown in Fig. 2. The eyepiece forms a magnified image of the image from the objective. The distance between the lenses is arranged so that an observer sees an inverted virtual image. You may want to refresh your knowledge of optics from first-year physics if terms such as *inverted* and *virtual* are unfamiliar.

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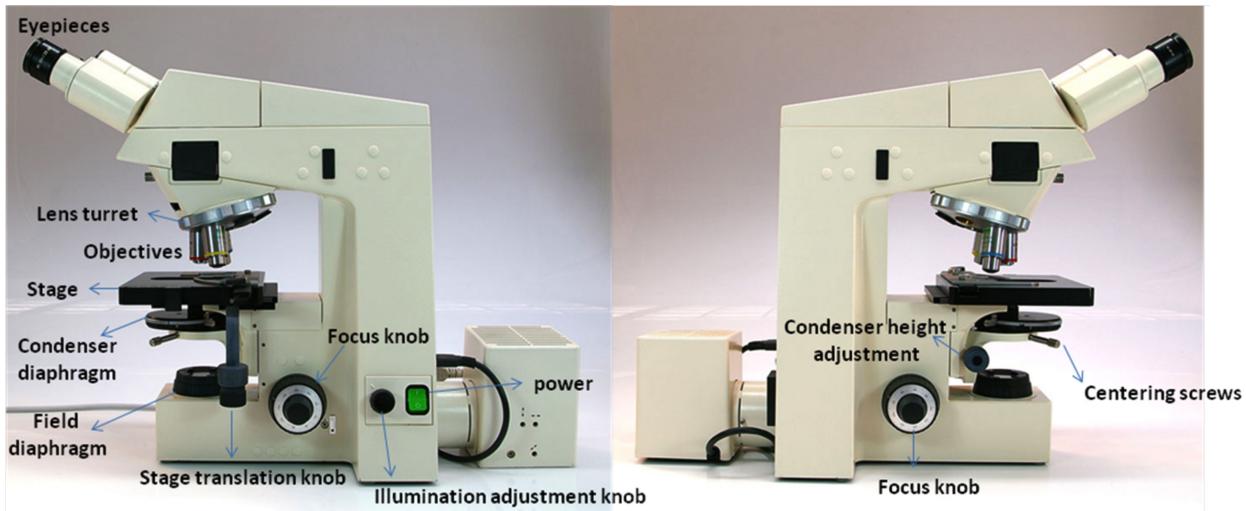


Figure 1: Typical configuration of an upright microscope, as seen from both sides of the microscope. Identify these components on your microscope.

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. Not all of these lenses may be on the lens turret of your microscope.

1.1 Resolution

The specimen illumination must be adjusted to maximize the resolution of your images. 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

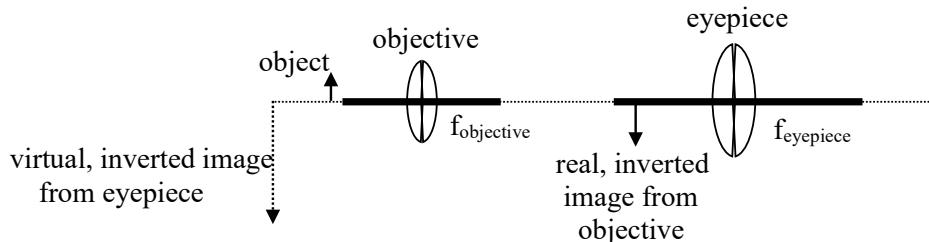


Figure 2: Images in a compound microscope.

is set by

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

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, as shown in Fig. 3. The combination $n \sin \theta$ is known as the *numerical aperture* (NA); we thus rewrite Eq. (1) as

$$d = \frac{\lambda}{\text{NA}_c + \text{NA}_o}, \quad (2)$$

in terms of 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 high resolution requires high-NA condensers and objectives. If we work in air ($n = 1$), then $\text{NA} \leq 1$, and the maximum resolution is just $d \geq \lambda/2$.²

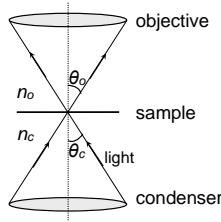


Figure 3: The cone of rays (NA) produced by the condenser and collected by the objective determines the resolution of an object within the sample.

1.2 Illumination

With this idea of resolution in mind, a microscope illumination system must

1. Illuminate the sample plane uniformly, even if the source (e.g., a light-bulb filament) is irregular.
2. Control the area of illumination. (Since many biological samples and chemical probes are damaged by light, we should not illuminate more of the sample than we need to.)
3. Control the angle of the cone of rays of the condenser and objective. These should be optimized for resolution, as described above.

²It is worth noting that this limit follows from basic ideas of quantum mechanics. In one dimension, Heisenberg's Uncertainty Principle states that $\Delta x \Delta p \geq \hbar/2$. For a single photon diffracted at an angle θ in vacuum (air), the de Broglie relation implies that the change in momentum is $\Delta p = \frac{\hbar}{\lambda} \Delta(\sin \theta)$. Putting these together gives $\Delta x \frac{\hbar}{\lambda} \Delta(\sin \theta) \geq \hbar$. 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 $\Delta x \equiv d \geq \lambda/2$.

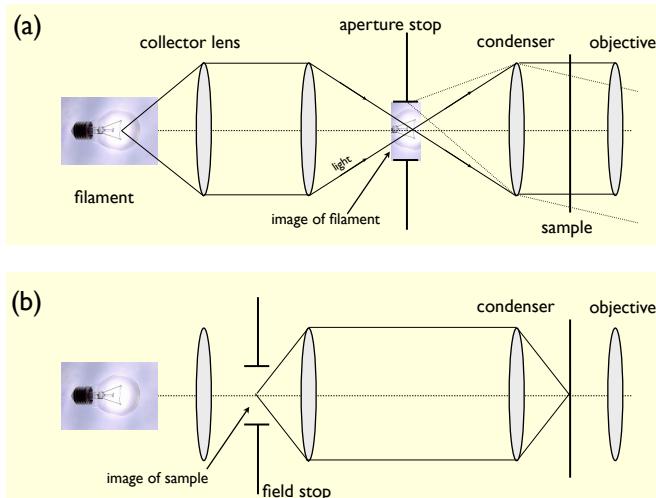


Figure 4: Köhler illumination. (a) The collector lens images the filament of the light source onto the back focal plane of the condenser. The condenser projects the light onto the sample. In the sample plane, light from the filament is defocused. A variable diaphragm placed in the focal plane of the condenser functions as the *aperture stop*, determining the angle of rays that illuminate the sample, thus impacting the sample contrast. (b) The same elements are redrawn to show the *field stop*, which is imaged onto the sample plane, thus controlling the illumination area.

Köhler illumination, first developed in the 19th century, responds to all three of these criteria. The key 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. 4 for a transmitted-light version. Try this [tutorial](#) on Microscopy U.

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 $\approx 1.05 \text{ g/ml}$. Assume that the depth of field $\approx 5 \mu\text{m}$ and that you are doing your first measurements with beads with diameter 1 μm . Assume, also, that the concentration is as specified in Sec. 2.2.

For 833 students: The problem here implicitly assumes that the concentration of beads is uniform. Is it? What does the Boltzmann distribution for bead concentration imply? Calculate the condition implied by the Boltzmann distribution and see whether it is relevant. Assume a chamber thickness of 100 μm .

Pre-lab Questions 2–7: See the Cell Motility script.

Pre-lab Question 8 (to be completed for Lab Period 3): Assume the *E. coli* solution you are given has an optical density at 600 nm of $\text{OD}_{600} = 1.0$, and that this optical density corresponds to 2×10^8 cells/ml. If you want ≈ 10 cells in your field of view, should you dilute? If yes, by what factor? If no, why not?

2 MATERIALS AND METHODS

2.1 Equipment

- Upright, bright-field microscope with attached digital camera
 - A typical microscope is shown in Fig. 1
 - We are currently using FLIR BlackFly cameras on all microscopes; these contain a CMOS chip that is 1/3" on the diagonal and has a field of view of 1288×964 pixels²
- Computer running LabVIEW program to control acquire images using the program Vision Assistant
- Software
 - Vision Assistant. See *Protocol: Acquiring Movies with Vision Assistant* for information on use of the program Vision Assistant.
 - ImageJ. See *Protocol: Tracking Particles* for information on how to use the MTrack2 plugin to track particles.
 - Python. See the Python Demos folder on Canvas for examples to make a histogram, calculate mean and variance, and to do MSD analysis
- Stage micrometer for calibration

2.2 Samples and Sample Chambers

You will need to make at least one sample chamber for each solution studied. See *Protocol: Making Sample Chambers* for information on how to prepare these.

Materials for sample making:

- Glass slides
- Number 1 cover glasses
- Parafilm and heat block or double-sided tape and nail polish
- Micropipette, tips and microcentrifuge tubes
- Concentrated solution of $\approx 1 \mu\text{m}$ diameter polystyrene spheres (0.5 wt%)
- Concentrated solutions of other sizes of polystyrene spheres (0.5 wt%)

- Concentrated solution of mutant *E. coli* strain HCB1274
- Concentrated solution of wild type *E. coli* strain DH5 α
- *S. cerevisiae* (baker's yeast), water and sugar
- Other cell samples, as provided
- Various materials for short projects

Be sure to dispose of all *E. coli* samples appropriately. They are considered to be biohazardous materials and should be placed in the small bucket labeled "Biohazard Waste" on the table opposite the sink.

2.3 Setting up the microscope

Follow the *Protocol: Microscope Setup ...* appropriate for your microscope in order to achieve maximum contrast and definition. You should run through the set up procedure each lab period to ensure that the alignment is optimized.

3 INVESTIGATIONS

Before doing any measurements, familiarize yourself with the microscope by performing the adjustments outlined in the setup protocol. Next, calibrate the microscope and camera using a *stage micrometer*, a special glass microscope slide with precisely printed calibration marks. After you are familiar with the microscope and you have calibrated it, please complete the required modules on Brownian and bacterial motion. In your remaining lab periods, investigate a short project of your choice.

3.1 Calibration and image acquisition

You need to calibrate the objective that you plan to use for your experiment, typically the 100x objective. Note that you will need to use **microscope oil** for this objective (see note on use of oil-immersion lenses above), but first set up the micrometer with a lower magnification objective.

Note: Please minimize the amount of oil you use. If oil gets into the objective, it can compromise the optics. Please wipe the surface of the objective to remove excess oil at the end of the day.

Place the stage micrometer 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. 1). Once you have the object in the field of view, switch to the objective you wish to calibrate. Fine-tune the location of the

micrometer by viewing it through the eyepiece. Adjust the brightness of the illumination lamp using the illumination adjustment (Fig. 1).

Now, run the camera program to view and capture images using the digital camera. This is described in *Protocol: Acquiring Movies with Vision Assistant*. If the camera image is black, make sure that light is reaching the camera: in some cases 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). Adjust the illumination as needed if the image is under or over-exposed. This is best done using the illumination adjustment knob (Fig. 1), 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 such as *ImageJ* and draw a straight-line selection between the edges of two calibration lines on the micrometer. Be consistent in your choice of reference and make sure that your calibration lines are perpendicular to the straight-line selection. For *ImageJ*, if you double-click on the selection tool, you can set the width of the line. The plot profile is averaged over this width, which can be helpful. 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. Then **Control-k** brings up the **Plot Profile**, which can be exported so that you can also graph it (and analyze it for more careful calibration, if you wish). Be sure to estimate an uncertainty for your calibration. Note that the eyepiece magnification is irrelevant to the calibration, as light does not pass through it on the way to the camera. Also, your calibration is valid only for the selected objective.

3.2 Brownian motion

The goal of this investigation is to study Brownian motion of spherical particles. The particles we will use are composed of polystyrene; they are commonly used because they can be made with a very uniform size and sphericity. They also have a density close to water so that effects of gravity on the motion are minimized. Aim to carefully characterize particles of at least one size, starting with the $1 \mu m$ spheres.

3.2.1 Sample preparation

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.

- Make a sample chamber. For instructions on chamber-making, see *Protocol: Making Sample Chambers*. This protocol includes instructions for making both thick chambers (with parafilm spacers) and thin chambers (with no spacer). You can use the protocol for thin chambers for quick tests - to see if you can focus on the particles for example - but for any quantitative investigation, you will need to prepare a thick chamber.

- Dilute your sample. The concentrated solution of polystyrene spheres that you are provided with will need to be diluted so that you can image ≈ 20 beads in the field of view. Calculate the dilution factor and check it with the laboratory instructor before preparing your sample. The chamber depth is dictated by the thickness of the parafilm ($\sim 100 \mu\text{m}$ thick), and the camera's field of view is 1288×964 pixels². The depth of field (thickness where objects are in focus) depends on the settings. We will use conditions where it is $\lesssim 5 \mu\text{m}$. Redo your prelab calculations using the calibration data you obtained in Part 3.1. Load a dilute solution of beads into the chamber and seal, as described in *Protocol: Making Sample Chambers*.

3.2.2 Sample imaging

Place the bead-bearing chamber on the microscope stage and move into focus, first viewing with a lower magnification objective before switching to higher magnification. When properly focused on the sample chamber, the objective will be almost in contact with the sample. The depth of focus of the higher magnification objectives 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.

3.2.3 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 on how to make a movie. The result of this procedure should be a movie (in .avi format) of the motion of one or several particles. You need to collect sufficient data to be able to make a decent histogram of the displacement of one or more beads. Adjust your data collection procedure to optimize your analysis.

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 Particles* 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 a 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. Calculate the diffusion coefficient. From the Stokes-Einstein relation (see background material), predict the diffusion coefficient. Compare with your observations.
- Fit a Gaussian function to your histogram. The mean and variance should match, approximately, your results above. Is the shape consistent with a Gaussian? Calculate the diffusion coefficient and compare with the expected value.
- To compare the motion of several beads, subtract the mean values.

The third way to estimate the diffusion constant is from the slope of a plot of the square of the end-to-end distances as a function of time. The square of the end-to-end distance $\Delta x_n^2 \equiv \Delta x^2(\tau) \equiv \langle [x(t + \tau) - x(t)]^2 \rangle$ is also known as the *mean-squared displacement* (MSD). The angle-brackets represent an ensemble average - in this case you can calculate the MSD as a function of $\tau = n\Delta t$ by averaging different intervals of your trajectories. We'll provide an Python demo 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. For example, 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 intervals τ but deviate at either earlier or later time intervals (or both). In practice, just fit the “straight” portion of the MSD.

For example code for your analysis, see the *Python Demos* folder on Canvas.

Refinement to consider if you have time:

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

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

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.

3.3 Cell size

Here, you will image eukaryotic cells, to get a sense of the sizes and morphologies (shapes) of a different cell types. First you should grow *Saccharomyces cerevisiae* (baker’s yeast) cells. Add 10 mL of warm water (body temperature) to a 15 mL Falcon tube, add 2 g sugar and 2 g baker’s yeast.³ Stir gently and let sit for five minutes, until the mixture is bubbling. Then prepare a wet mount (thin sample without a spacer) of this sample. You may need to dilute it to clearly see the cells. Determine their size and shape and compare with *E. coli*.

You should also 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

³The amounts do not need to be exact; use these numbers for guidance.

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.

3.4 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 will study the motion of several types of bacteria, which are 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 and a concentrated solution of wild-type *E. coli* (strain DH5 α). 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 mutant 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. We recommend that you 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 lower density of bacteria in your sample as compared to the bead sample, should make it 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. If you have time, measure the motion of the wild-type bacteria and compare. Compare as well to the motion of comparably sized beads. In particular, if you see a diffusive regime in wild-type *E. coli*, how does the diffusion coefficient compare?

3.5 Short projects

Use the microscope to investigate a short project of your choice; this project should be the focus of your report. Please choose from the following:

- Intracellular movement in onion cells. An onion is made up of layers that are separated by a thin membrane that is one cell-layer thick. The membrane can easily be obtained by peeling it from any layer of the onion using tweezers, and then mounted on a microscope slide. See the UCB Advanced Lab BMC for background, on Canvas.
- Anomalous diffusion in polymer solution (PEO) or gel (agarose) (see article by Banks and Fradin for background, on Canvas).
- Chlamydomonas (algae) swimming; compare the algae swimming to diffusion of mutant *E. Coli*.

Or consider a project that addresses one of the following:

Points to ponder:

- How would your results change if you were to follow the bacteria for a shorter period of time (but at much higher frame rate)? For a much longer period of time?
- How does the expected diffusion constant depend upon cell length? [See Fig. 4.5 of Berg (1993).]

4 SUGGESTED TIMELINE

Lab Period 1:

- come to lab having read the labscrip, the document “Background: General Aspects of Cell Motion,” and answered Pre-lab Questions 1–5
- learn the operation of the microscope and its attached camera
- image a stage micrometer and calibrate the camera
- prepare a sample of 1 μm diameter polystyrene spheres
- capture a video of the polystyrene spheres undergoing Brownian motion and analyze their motion
- if you have time, consider the impact of the two effects discussed at the end of the data collection section of Investigation B (Eq. 3).

Lab Period 2:

- come to the lab with a completed analysis of the motion of the beads you measured in Lab Period 1
- capture the Brownian motion of larger beads, quantitatively analyze their motion, and compare the diffusion coefficient of beads of different sizes
- qualitatively examine the fixed cells (HT1080) and yeast cells

Lab Period 3:

- come to lab having answered Pre-lab Questions 6–8
- prepare a sample with mutant *E. coli*, strain HCB1274
- capture the movement of mutant *E. coli* under self-propulsion
- quantitatively analyze their motion
- if you have time: look at the wild-type *E. coli* strain (DH5 α) and compare to the mutant HCB1274 strain used above.

Lab Period 4–6:

Complete a short project of your choice. Bring a project plan to Lab Period 4.

5 Items to include in your documentation

In addition to a record of day-to-day happenings, calculations, diagrams, essential equations, lists of equipment, filenames, etc. please make sure you include at least the following in either your lab notebook or your analysis notebook.

- The micrometer calibration.
- References to data files. Record the name of every file saved. Record the location of the saved files.
- Example plots. Set your coordinate system so that $x = y = 0$ at $t = 0$ for each trajectory that you analyze. You do not need to include the raw data in your lab note-book, but you should include some example plots:

- 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

- A histogram Δx and Δy for one bead, where “ Δ ” is calculated over *one time interval of the camera*, Δt . Include a Gaussian fit and estimate D . (Correct for camera exposure.) Note that you can estimate D directly from the variance of the displacements and from a fit of the data to a Gaussian distribution. Do these methods agree?
- Define $\Delta x(m) \equiv x_{n+m} - x_n$. That is, look at the displacement over a time $\tau = m \Delta t$, averaging over all displacements that you can measure in the single trajectory (over all available n). 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 $\langle [\Delta y(m)]^2 \rangle$: does it scale like t or t^2 ? Compare motion of living systems (bacteria, cells) with non-living ones (beads, dead bacteria or cells, if you have them).
- For beads, you now have up to three estimates of D . Compare to each other and to the Stokes-Einstein prediction.

For 833: look up discussions about the effect of a nearby wall or substrate on the diffusion constant and discuss in connection to your experimental results.

- Calculations of the dilution factors for beads and cells.
- Discussion of difficulties encountered in the experimental procedures.
- Discussion of how motion in the third dimension (z) affects your results.
- Images of each cell type (bacterial and eukaryotic, including scale bars) and sizes of cells that you determined from these images
- Discussion of differences in morphology between the different cell lines.
- Project plan, day-to-day recording of experimental work and analysis for your project.

6 ADDITIONAL READING

- Nikon, manufacturer of microscopes and lenses has an [excellent resource](#) for microscopy.
- B.A. Scalettar and J.R. Abney, “Biomedical imaging in the undergraduate physics curriculum: module on optical microscopy,” *Am. J. Phys.* **83**, 711–718 (2015). Good overview of the basics.
- J. Mertz, *Introduction to Optical Microscopy*, 2nd ed. Cambridge Univ. Press (2019). An advanced but surprisingly readable treatment of all aspects of microscopy, from the basics to almost every modern technique.
- 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.