Bi 1x
Introduction to microscopy and biological scales

1 Overview

For centuries, curious minds have used microscopes to observe the world which is hidden to the naked eye. In this experiment, you will learn the basics of microscopy and how one can use computing to analyze images. We'll cover a little bit of how the microscopes work, how they should be used, and what to do if things fail. We'll also be introduced to some of the fascinating biology of the damp-wood termite *Zootermopsis nevadensis*.

2 Background

In this section, we present a brief overview of some background information to help you understand the context and techniques of the experiment.

2.1 Biological scales

Much as in physics and astronomy, the size scale of biological inquiry spans well over 10 orders of magnitude. To investigate questions at all size scales requires innovative and diverse techniques and technologies. Microscopes and microscopy techniques are very important components of micro-scale biological investigations.

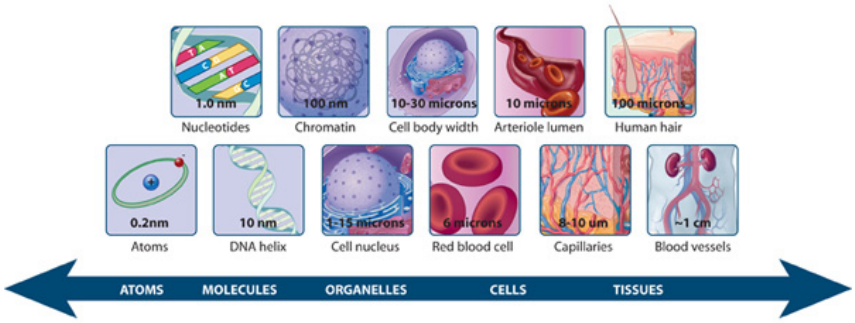

Figure 1: An illustration of the sizes of various biological structures. Image taken from the Book *Essentials of Cell Biology*, published by Nature Education, 2010.

To get a sense of biological scales, check out this nifty animation from the Genetic Science Learning Center at the University of Utah. Another interactive exploration of scales, going both much bigger and smaller than biology is here (though you will need Flash installed or you can watch the video on YouTube). It is also very much worth your while to watch the classic IBM Powers of Ten video from 1977 on YouTube.

2.2 Brightfield, Phase Contrast, and Fluorescence Microscopy

Throughout the course, you will use three main modes of microscopy - brightfield illumination, phase contrast, and fluorescence. While we will leave the details of image formation, magnification, and resolution for more focused classes, it is important that you have a basic understanding of how each modality works.

Brightfield. In brightfield microscopy, an image is produced by shining white light upon an object. The contrast arises from an approximate $-\pi/2$ phase shift of the scattered light relative to the unscattered light. The contrast produced is weak and it is often difficult to see single cells, not to mention their features. However, it is very useful to image larger samples or stained samples, and we will use it in the *Drosophila* embryogenesis module later in the course.

Phase Contrast. Much like brightfield, phase contrast microscopy generates contrast through the phase shift of scattered light relative to unscattered light. However, phase contrast uses a set of optics to induce a $\pi/2$ phase shift only in the unscattered light as it passes through the objective and before it reaches your eyes. The phase shift of the scattered light relative to the unscattered light is then π, causing dark features through destructive interference, and producing a much more noticeable contrast. Phase contrast is very useful for imaging small samples, such as bacterial cells.

Fluorescence Microscopy. This technique is incredibly useful for detecting the subcellular localization of proteins, determining the concentration of a protein in the cell, or even for detecting very specific molecular interactions. In order to use fluorescence microscopy, the cell must contain a fluorophore, a molecule that can be excited by photons of a specific wavelength and relax by emitting photons of a longer wavelength. A (very simplified) schematic of fluorescence microscope can be seen in Fig. 2.

A fluorescence microscope is composed of three main components, a excitation light source, a dichroic mirror, and a detector. The fluorescence light source can be a full spectrum source, such as an Hg arc lamp, or a laser. When using a full spectrum light source, a separate filter must be used to restrict the excitation beam to a specific wavelength. The dichroic mirror can be either reflective or transmitted depending on the the wavelength of the incident light. For fluorescence microscopy, the dichroic mirror is reflective to the excitation beam and permissive to the emission beam. The detectors in our lab are charge-coupled device (CCD) cameras which can produce images of high resolution.

Each microscopy modality has its advantages and disadvantages. In Fig. 3, we see the same field of cells taken with brightfield illumination alone, phase contrast,

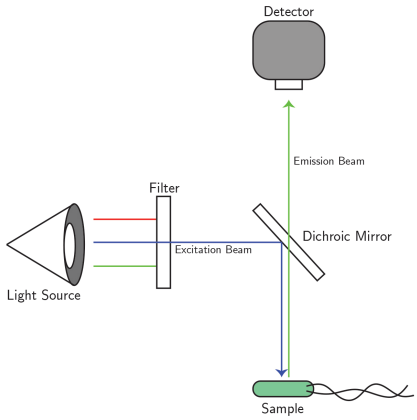

Figure 2: A simplified view of a fluorescence microscope. All lenses have been omitted for clarity.

and fluorescence.

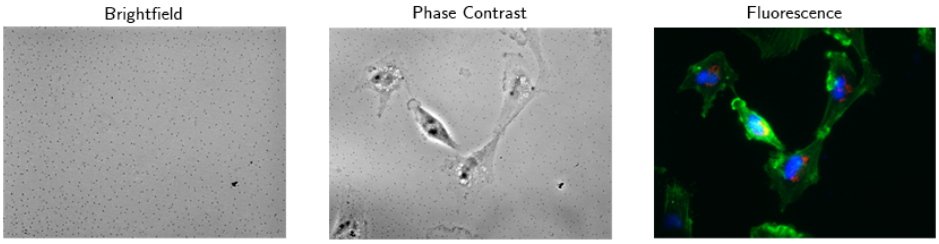

Figure 3: Left to right: the same area of interest imaged with brightfield, phase contrast, and fluorescence microscopy.

2.3 The Graticule

A graticule, or stage micrometer, is a commercially produced slide which is used as a ruler. This is use to calibrate the optics of your microscope setup. The data gathered from the microscope consist of digital images. To correlate distances in the digital images to real distances in your sample, you need to know the *interpixel distance*. Take a picture of a ruler is a great way to do this!

In this experiment, you will take photos of a graticule at $10\times$, $40\times$, and $100\times$ magnification. You will use image processing skills you will learn in the tutorials to process the graticule image and determine the pixel spacing for each objective/camera setup. **Graticules are very expensive**. Please handle them with care!

2.4 The Termite Gut Ecosystem

Many multicellular organisms rely on microbes in order to survive. A prime example of this phenomenon is the remarkable ability for termites to extract energy from wood. In Bi 1x, we will use microscopy to examine the biodiversity of protists in the *Zootermopsis nevadensis* intestinal tract.

While these termites cannot reproduce in the low altitude of Pasadena, they thrive within dead and dying Ponderosa pine trees in the Angeles National Forest, only 30 miles away (and 5000 feet up!). Termites, ants, wasps, and bees are all **eusocial** insects meaning that the colonies are spread into different **castes**. Termites, however, are not closely related to ants, wasps, or bees and have evolved their eusocial behavior independently! Understanding the origin and convergent evolution of eusocial behavior is still an active area of research.

Termites colonies are composed of different castes (Fig. 4):

- **Reproductives:** These members are responsible for laying the eggs and are the "parents" of the colony.
- **Workers:** These are the members who are responsible for eating the wood and generating the large network of tunnels known as **galleys**. By chewing apart the wood, they feed the rest of the members of the colony.
- **Soldiers:** As their name suggests, this caste is responsible for defending the colony from invaders such as ants and pesky Bi 1x TA fingers. These members have large mandibles but do not chew any wood. They feed off of the feces of the workers which contains pre-masticated wood particles.
- **Alates:** These members are winged termites who are reproductively mature. When the colony matures or is removed from the queen, these caste members develop and leave the colony in pursuit of greener pastures. Upon finding a mate, they will become the king and queen (reproductives) and will form a new colony.

Making up one-third of the termite's weight, the gut (Fig. 5) is a fertile ecosystem for microbes and protozoa alike. The gut microbiota is essential for the digestion of cellulose and starch, the main energy and carbon sources for these insects. In many termite species lignocellulose fermentation is predominantly achieved by large single celled protozoa. Protozoa populations may be diverse and interact in complicated ways.

The dominant protozoa in the termite gut belong to the genera *Trichonympha*, *Streblomastix*, and *Trichomitopsis*. The TAs will point out these respective genera while you are looking at the gut contents with the microscopes. There are also myriad bacterial and archaeal species that we will not study intently, but nonetheless carefully look at in this module.

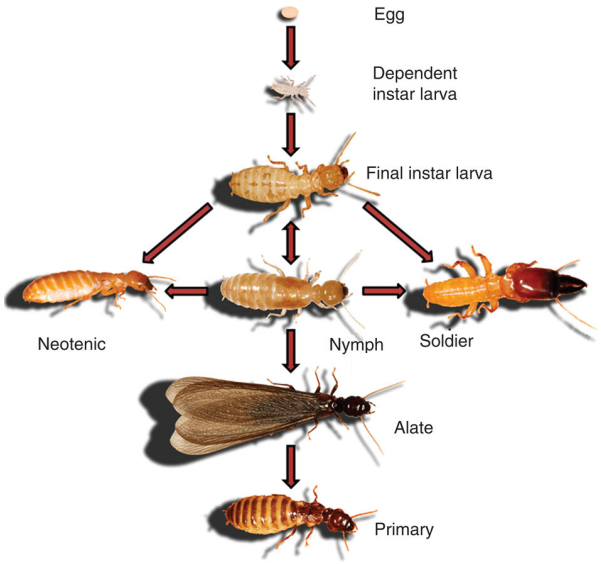

Figure 4: Developmental stages and castes of *Zootermopsis nevadenis*. Arrows represent direction of differentiation. The final instar larvae are the workers and are incapable of sexual reproduction. Notice the nascent wing buds on the nymph. The neotenics, alates, and primaries are reproductives. The primary reproductive caste shown here does not actively reproduce, but differentiates into a king or queen, which can produce massive amounts of eggs. Adapted from Terrapon, et al., *Nature Comm.*, 3636, 2015.

Figure 5: A *Zootermopsis nevadensis* worker (bottom) and its intestinal tract (top). While the protists and microbes are distributed throughout the tract, the large "clump" in the middle of the intestine is responsible for the majority of the cellulose digestion. Image courtesy of Jared Leadbetter.

3 Protocol

The TAs will provide you with a graticule. After you have mastered operating the microscopes and taken pictures of the graticule, you will move out to the lab area to prepare samples of the termite gut for imaging.

3.1 Microscope Operation

We have five microscopes in the lab, two made by Olympus and three made by Nikon. Each microscope has some small differences from the others, e.g., they all have different cameras. Importantly, all use the same software to control the microscope and image acquisition, MicroManager (stylized µManager). It is open source, free software developed in Ron Vale's lab at UCSF and now absorbed into the company Technical Instruments. One microscope also uses Nikon's own software to control some of the lighting. A summary of the microscopes is shown below in the table below.

Name	**Model**	**Software**
Nemesis	Olympus IX71	MicroManager
Wheels	Olympus IX71	MicroManager
Tenjin	Nikon Ti Eclipse	MicroManager/Nikon
Bolt	Nikon Ti Eclipse	MicroManager
Hermes	Nikon Ti Eclipse	MicroManager

In order for the software to control the components of each microscope, the components must all be on before the software is launched. Before launching the software, you will turn on:

- The microscope stage.
- The camera or camera controller.
- The brightfield lamp.
- The microscope itself (Nikon only).

The TAs will point out where each of these switches are. The software can run without the fluorescence lamp turned on. If fluorescence will be used in the experiment, it should be turned on before launching the software. **The fluorescence box produces very high intensity, UV laden light and should be treated with utmost care and respect.** There are several rules one should abide while using the fluorescence boxes.

- Once the fluorescence box is on, it cannot be turned off for a minimum of 15 minutes. **Never turn the box on and off in quick succession. This causes damage to the power source.**

- Once the fluorescence box is turned off, the lamp must be allowed to cool down for at least 30 minutes before being turned back on.
- Finally, **never look into the eyepiece when the fluorescence beam is on.**

Once everything is turned on and ready to go, summon a TA to help you launch the software. Once you get the hang of using the microscopes, you can launch the software by yourself, but for now, you should only operate the microscope with a TA present.

3.2 Brightfield and Köhler Illumination

After you get the microscope turned on, a TA will show you how to get the brightfield illumination on. To produce sharp and well contrasted images on the microscope, you must adjust the illumination such that it is even across the entire field of view. This even illumination is part of Köhler illumination and requires manual adjustment. It is imperative that you adjust Köhler illumination for each focal plane you image. To appropriately set up Köhler illumination, do the following steps.

1. Make sure your slide is in place and your subject in focus. (Your first subject will be a stage micrometer.)
2. Open the condenser lens aperture by moving the slider on the top of the microscope to the left. Find and focus onto an object in your sample. Köhler illumination is dependent on the focal plane that you are observing.
3. Once you have focused on your sample, fully close the aperture. This will restrict the illumination of the sample to a very small octagon of the sample.
4. Adjust the height of the condenser lens until the aperture appears as a sharp polygon. You can adjust the height of the condenser by turning the knob on the back of the condenser lens system.
5. Adjust the location of the polygon by turning the knobs on the left and right of the condenser assembly until it is in the middle. These knobs do not adjust in the x and y directions. Rather, they adjust in a saw tooth pattern. This makes alignment a little tricky, but practice makes perfect.
6. Once the octagon is properly centered and in sharp focus, open the aperture such that the entire field of view is illuminated.

Congratulations! You've correctly aligned Köhler illumination.

3.3 Phase rings

Using only brightfield microscopy generates images of low contrast. To generate a high contrast image, a phase ring must be in place to induce a phase shift in the

unscattered light. Each objective has its own phase ring of a specific diameter. These phase rings are located in the condenser lens system and can be changed by rotating the phase wheel. In general, the objective/phase ring pair are as follows.

- 10× = Phase 1
- 40× = Phase 2
- 100× = Phase 3

The phase rings are not always in the spots indicated above. To check the phase ring turn the wheel on the bottom of the eyepiece to "CT" on the Olympus microscopes or slide the slider on the Nikon microscopes. If the ring from the inside of the objective perfectly coincides with the phase ring, the phase ring is correctly aligned and in place. If the ring is much larger or smaller it is the incorrect phase ring. **If a phase ring appears to be misaligned, ask your TA for help**.

3.4 Focusing and Image Acquisition

As you probably surmised from the discussion of phase rings, each microscope is equipped with objective lenses for 10×, 40×, and 100× magnification. The objectives are very fragile components and must be treated with the utmost respect and care. At no point in time should any objective touch the sample. Both the 10× and 40× objectives are called *air objectives* meaning that no oil or water is needed. **Putting oil on a 10× or 40× objective will ruin it.** For each microscope, the 100× objective is an *oil immersion lens* and requires a dot of oil on the lens to generate a clear image. The oil used is of a refractive index as close the refractive index of the glass as possible, thereby increasing the numerical aperture and allowing for more light to be collected by the lens. Each microscope has a bottle of oil. **It is important to only use the oil for your specific microscope. The Olympus and Nikon microscopes use different oils that are incompatible.** Before focusing an image using the 100× objective, place a small dot of oil on the coverglass on the side facing the objective. For your first oil application, be sure to watch a TA do it first so you know how much oil to use.

To bring a sample into focus, always start with the objective at the lowest possible position. Slowly bring the objective towards the sample using the coarse focus adjustment knob until it comes into focus. With an oil objective, you will see the objective "splash" into the oil when it comes into contact. To prevent damage to the objective, always have a partner keep an eye on the objective to make sure it does not collide with the sample. Once the sample is in focus, you can move along the position using the stage joystick next to the computer.

Setting up image acquisition to get digital images varies depending on which software you are using. A TA will guide you through this process for your first few acquisitions until you get the hang of it.

3.5 Termite gut fluid extraction and mounting for microscopy

The course staff have collected termites from the Angeles National Forrest and have been keeping them in an incubator in the lab. You will need to extract the intestinal gut fluid from a termite. When extracting gut fluid, be sure to record your observations regarding the amount of gut fluid that is released onto the slide. The procedure is shown below, which will also be demonstrated by your TA.

1. Place a glass slide on the CO_2 pad and make sure that CO_2 is flowing. You should hear a slight hiss from the gas flow.
2. Using forceps, place a worker termite onto the glass slide and then quickly cover the glass slide with a petri dish cover. The CO_2 will fill the enclosed space, acting to anesthetize the termite but also causing it to release a large amount of organism-rich gut fluid. Note that the termite's abdomen should be pointed on the glass slide to ensure the gut fluid is released onto the glass slide (and not onto the CO_2 pad!).
3. Once the gut fluid has been released, immediately lift the pertri dish. If there is any solid waste in the gut fluid (it will look like a brown pellet), remove it using forceps. Add 25µL of synthetic **T**ermite **G**ut **F**luid (TGF) onto the released gut fluid. This solution is isotonic to the actual termite gut fluid and will not result in the immediate death of the protozoa. In some cases, the gut fluid will still be at the end of the termite's abdomen. Place the termite into a container of used termites for recovery.
4. Immediately place a glass coverslide on the gut fluid.
5. Take your slide to your assigned microscope and observe the protists and microbes that live in the gut. Be sure to look at the organisms at $10\times$, $40\times$, and $100\times$ magnification and record your observations. Remember that you will need to add oil to the coverslide when viewing with a $100\times$ magnification, so plan to do this last.
6. During your observations, be sure to take snapshots of what you see. If possible, you can also acquire a movie. Be sure to ask the TAs for help with this acquisition.

4 Assignment

Problem 0 (Summary)**.**
Write a brief summary of the Introduction to Microscopy experiment, and what we sought to learn. You summary should be between one paragraph and one page in length.

Problem 1 (Microscope safety (**prelab**))**.**
When is it ok to look into the microscope eye pieces? When is it *not* ok?

Problem 2 (Biological scales in Bi 1x)**.**
In this course, we will work with several model organisms. We will work with *E. coli*, *S. cerevisiae*, *C. elegans*, and the termite *Zootermopsis nevadensis*. (In the past, we have also worked with *Drosophila melanogaster*) Of course, we also have *Homo sapiens* running around lab. Approximately how big are each of these organisms? The excellent BioNumbers website is a great resource for these numbers and many many others.

Problem 3 (Calibrating your measurements)**.**
Use your images of the graticule to determine the interpixel spacing at $10\times$, $40\times$, and $100\times$ magnification. You will need to use some of the image processing skills you learned in the tutorials. Remember to turn in your code for full credit.

Problem 4 (Sizing up *Zootermopsis nevadensis*)**.**
Much can be learned about life simply by watching and making some estimates. Using the observations you made of the termite gut, make the following estimates. For each estimate, be sure to explain your thought process and show any and all approximations you made. For this problem, do not use any resources other than your brain, your experiences in this lab, a paper, and pen (no calculators or internet).

a) What is the volume of the termite gut?

b) What is the concentration of protists and microbes within the gut? Remember that you diluted the protists when you added TGF.

c) What is the ratio of bacteria and archaea to eukaryotic protists in terms of volume and in terms of number?

d) How many different species of *Trichonympha*, *Streblomastix*, and *Trichomitopsis* are in the termite gut? Think about this estimate carefully.

Problem 5 (The art of asking questions)**.**
The termite gut, as you've just seen, is far from boring sludge. While studied for decades, very little is known about how the microbial and protist community works with one another to survive. In fact, the role that many of the protists play in the gut is entirely unknown! Based on your observations in the lab, write three topics of the termite gut you think would be interesting to study and why. There is no right answer to this question, so be creative!

Problem 6 (The termite gut community)**.**
Show an image from each magnification you used to observe the termite gut protists. Write a brief explanation of the type of organisms that are visible in the field of view.

Be sure your axes have appropriate units so that we can tell the scale of the images we are looking at.

Problem 7 (Data)**.**
Attach all data, code, and sample images not specifically asked for in the other problems. Present them neatly and clearly.