

Biology 190H
Quantitative Biology
of the Cell
LABORATORY
MANUAL

Fall 2013

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UMASS
AMHERST

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Introduction to Bio 190H Laboratory

Fall 2013

When and where

ISB 364
Tuesday (section 1) and
Thursday (section 2) 12:30-3:30pm

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TA:

Course Materials:

- (1) Purchase a Student Lab Notebook, with 100 bound, carbonless duplicating pages. It is available at the textbook annex in the section for chemistry courses (111, 121H, 269).
- (2) Purchase a lab manual from Collective Copies at 71 S. Pleasant St. on the Amherst common.

Laboratory Schedule 2013

Day	Date	Lab	page
Tu	9/03	Lab 1.1: Welcome to the Lab	15
Th	9/05		
Tu	9/10	Lab 1.2: Absorbance of Fluorescein	19
Th	9/12		
Tu	9/17	Lab 1.3: Fluorescence, Absorbance, Data Mining	25
Th	9/19		
Tu	9/24	Lab 2.1: Viewing and Counting Bacteria	33
Th	9/26		
Tu	10/01	Lab 2.2: Bacterial Growth	37
Th	10/03		
Tu	10/08	Lab 3.1: Qualitative analysis of regulation	43
Th	10/10		
Tu	10/15	<i>Monday Schedule – no lab</i>	
Th	10/17	Lab 3.2: Quantitative Analysis of the Kinetics and Extent of Induction	45
Tu	10/22		
Th	10/24	Lab 3.3: Gene Expression Data Analysis	46
Tu	10/29		
Th	10/31	Unit 4: Protein Folding & Stability	49
Tu	11/05		
Th	11/07	Lab 5.1: Observing Swimming Bacteria	65
Tu	11/12		
Th	11/14	Lab 5.2: Mutations and Aberrant Signaling	69
Tu	11/19		
Th	11/21	Lab 6.1 Gene expression in <i>Drosophila</i>	TBA
Tu	11/26		
<i>UMASS THANKSGIVING BREAK – no Thursday lab</i>			
Tu	12/03	Lab 6.2 Gene expression in <i>Drosophila</i> , continued	TBA
Th	12/05		

Lab Grade

The lab grade is based on 100 pts, 25% of the total course grade (400 pts).

The lab grade is divided into:

- (1) Attendance (10%)
- (2) Lab Notebook (40%)
- (3) Lab Reports (50%)

Attendance (10%):

We expect you to attend every lab meeting prepared to do the work and to interact cooperatively with your colleagues. Notify us of any problems getting to lab in advance of class time.

Lab notebook (40%):

One of the useful skills you will acquire in the laboratory is the proper use of a laboratory notebook. A notebook that accurately details the goals, methods, results, and conclusions of an experiment allows others to continue your work in your absence or replicate and expand upon your results. Such a notebook also makes it possible for you to review your experiments in preparation for writing a report, modify future ones as necessary, and, in the unlikely event of an accusation of misconduct, defend yourself against charges of fraud. You should purchase and use a Student Lab Notebook, write in it with pen, and hand the copy to lab TA at the end of the lab period.

Your notebook records will be evaluated for:

- (1) A statement of purpose. What were you trying to accomplish, and by what means?
- (2) The relevant equations and calculations
- (3) A description of your methods. Could another student understand your work, repeat it, or pick up where you left off?
- (4) Results, including data, observations and commentary. What did you see? What was especially hard or time-consuming? What did you have to modify? What did you have to repeat? What results did you have to get from someone else?
- (5) Reference to errors. Did anything go wrong? What did you do about it? Can the writer's voice be heard (is there a casual, chronological account of what happens, not a repeat of the lab manual with some numbers added in)?
- (6) A summary. What did you find out? What's the overall take-home message?
- (7) Legibility
- (8) Prompt submission
- (9) Conciseness (2 pages or less unless otherwise specified)

Lab Reports (50%)

The enterprise of science depends on good communication. The reports assigned in this course are meant to teach you to illustrate your results and explain them to others. These reports will not be graded solely on the quality of your results, but the quality of your explanation and illustrations. You will collaborate with other students in collecting and analyzing your data. Some reports will be collaboratively written, others, individually.

Explicit instructions will be given for each report. These reports will each include some combination of the following: abstract, introduction, results, discussion, references, and acknowledgments. Not every paper will require every section. See the section on preparing a lab report beginning on page 99, and the sample report on page 110.

Laboratory Basics

Your Station, Equipment and Materials for Daily Use:

You will be provided with a variety of materials and equipment for use during the semester, some for exclusive use by you and your lab partner, and some to be shared with another pair at the same bench. You will be responsible for keeping track of these things.

Computer: At your bench there is a web-connected iMac computer that you and your partner share. It will allow you to (i) open datafiles collected on the automated plate readers, (ii) manipulate data through programs like Excel, (iii) print graphs and short documents on the lab printer. Your station drawer should have the following items: hand counter, set of three micropipettors, three sizes of disposable micropipette tips, microcentrifuge tube rack, container of microcentrifuge tubes, test tube rack, marking pen, label tape.

You will be sharing these things with the group that has the same number as your group on the other lab day; return these items to your station drawer at the end of the lab period.

Shared Equipment, includes:

Scientific balances; incubator/shakers for growing liquid cultures of bacteria; 2 incubators for growing bacteria on agar plates; 2 plate readers under computer control that measure the absorbance, optical density, fluorescence, phosphorescence or luminescence of liquid samples; a compound microscope with phase contrast optics for observing micro-organisms (1 microscope per station); digital cameras for collecting images and movies from the compound microscope; a dissecting microscope for viewing samples (e.g. *Drosophila* embryos) under lower magnification (1 microscope per station).

This shared equipment will be used in some of the lab units, but not others.

Organization and Care of Reagents

Label every plastic tube with a permanent marker so you know that it's yours and what's in it. Label glass containers with tape.

Don't make a liar out of the label. Don't contaminate the stock reagents. This means not to reuse pipette tips, and not to pour any leftover reagent back into the stock bottle. Always use a fresh sterile pipette tip—not one that was used previously, and never one that you have accidentally touched. Even if you can't see liquid in a used tip, microdroplets are in there, and would cross contaminate your solutions if the tip were used more than once. If you have any doubt about the contents of a tube, assume it to be contaminated.

Mark the label of anything you have opened – its sterility or integrity has been compromised, and it should not be put back on the shelf as if it were unused.

Laboratory Safety

We do very little in this course that could result in a fire or an explosion. We will, however, be using a few reagents (in very small amounts and low concentrations – be reassured) that have the potential to cause harm. We will try to alert you to whatever dangers we see in the lab.

Your best bet, however, for protecting yourself is not to get anything in your mouth, eyes, or breaks in your skin. Do not eat or drink or apply lip balm or fix your contacts in the lab.

Wash your hands: after you handle reagents, before you touch your face, before you leave the room, before you have a snack. **Wear gloves:** while you handle anything that might hurt

you, or that you might contaminate. ***Take your gloves off:*** whenever you move from wet bench activities to computer keyboard, or whenever you have touched something you don't want to spread around the room. Remember that if you touch your face with a gloved hand that has just touched a toxic reagent, you might as well not have worn the gloves! (The same applies if you touch barehanded any surface previously touched with a contaminated glove.)

Discarding Waste

There are many different types of waste in this lab:

- recyclable paper and recyclable containers
- broken glass (unrecyclable, dangerous to handle)
- sharp trash (small sharp objects like razor blades)
- biohazard waste (biological specimens that might be infective or invasive)
- hazardous liquid waste (toxic, corrosive, or flammable waste)
- regular trash (solid waste that is not any of the above)
- benign liquid waste (liquid waste that is not hazardous or infective)

Please ask whenever you have the slightest doubt as to how to dispose of something!

Using Wahoo server space

The ISB server Wahoo has space set aside for storage of Quantitative Biology of the Cell files. This is a very useful place for you to keep your files. You can access these files from anywhere that has an internet connection.

There should be a link on the desktop that takes you to the Wahoo Quantbiol space. Find the folder already set up there for your group. Make a new folder with an informative and unique identifying name e.g., "Unit 1 – Tom & Jerry" (if your names are Tom and Jerry, of course). Save your files there, creating identifying subfolders as needed, and keeping a record of what goes where.

From room 364

Obviously, you can readily locate these files from any computer in room 364.

On a networked Macintosh on campus

Open a finder window, and pull down the Go menu. Choose "connect to server" and type "smb://wahoo.nsm.umass.edu/quantbiol". Sign in with your BCRC username and password.

From anywhere else

Download and install FileZilla following the instructions on the OIT web-hosting support page (<http://www.oit.umass.edu/support/web-hosting>).

- Open FileZilla.
- Open Site Manager from the File menu, and enter the following:
 - Select Entry: New Site
 - Hostname: wahoo.nsm.umass.edu
 - Port: 22
 - Protocol: SFTP – SSH File Transfer Protocol
 - Logon Type: Ask for password
 - User: your BCRC username
- Click Connect.
- Enter your BCRC password, and click OK
- Note: The first time you log in, a one-time screen warning you about an unknown host key may appear. Check the box next to Always trust this host, add this key to the cache and click OK.
- The remote site is something like this: /u1/home/bio/username (personalized with your username, of course).
- Clear the Remote site box, and type this: /export/quantbiol
- Enlarge the absurdly small window under the remote site bar and scroll to find your folder. Move files between local and remote folders by drag-and-drop, or by right click and upload (local to remote) or download (remote to local).
- Voilà!

Finding Files You Forgot to Put on Wahoo

The ISB computers clean themselves up every night if they have been untouched long enough. They sweep the files you scattered all over the desktop into the one week backup folder.

You can access this space this way: Macintosh HD/Users/yournetid/OneWeekBackup. It helps to know which member of your group was signed into the computer at the time you put the file on the desktop.

If you put your files into the private space accessible via the shortcut on the desktop, you should be able to find them there later.

Your files will be easier to find if you make a folder on the desktop with a distinctive name, and store them there.

Printing in Biology ISB space

First, spend some time making the file look the way you want. It's hard enough to print without wasting time and effort printing something that looks ridiculous.

Warning: there are many steps to this process. Much frustration, heartbreak, and wasted paper can be avoided by mastering these steps now.

- Select “print” from the file menu. ***This does not send your job to the printer!***
- Notice the name of the printer, as this will tell you where to look for your printout. (The printers in ISB biology space are named after Pacific islands.)

Printer name	room
Nifiloli	264
Nupani	360
Nukapu	364
Ngawa	368

- You may be able to get a menu that lets you change image quality by looking at your options under “copies and pages”.
- Use the preview function to make sure you will be printing the right thing. Check which pages will be included.
- Click print. ***This does not send your job to the printer!*** This will produce an authentication dialog box. Sign in with your BCRC username and password. ***This does not send your job to the printer!***
- You should be directed to the print queue page. (If not, you can find the ISB print release page here: <https://wahoo.nsm.umass.edu/printrelease/>.) Refresh repeatedly until your job shows up. Check the box for your job and click “release”. (If you have made a mistake, this is a good time to cancel a job.) ***This does not send your job to the printer!***
- After you release your job, you get a confirm release page. Click “really print”, and ***this will finally send your job to the printer!***

Unit 1: Light, color, and concentration

How do you know if you've made a solution correctly, and why does it matter?

How mad are your instructors' lab skills?

Absorbance of light

The interaction of light with matter is a fundamental phenomenon of central importance to chemistry, biology and physics.

Light consists of electromagnetic radiation of various wavelengths. The visible spectrum of light includes wavelengths from about 400 nm (blue) to 700 nm (red). Substances that take on a color in broad spectrum (i.e., white) light do so because they absorb part of the spectrum. That part of the spectrum that is not absorbed is what we see. (For example, most naturally occurring yellow objects absorb the blue part of the spectrum, transmitting or scattering the red and green. We experience stimulation of our eyes by equal intensity of red and green light as yellow.) A device that measures the absorbance of light, usually at a narrow band of wavelengths, is called a spectrophotometer, or spectrometer.

Concentration

The quantitative biological sciences require, as a matter of practice, knowing (and therefore the need to determine) the concentrations of molecular species – in the cell, in biological fluids (blood, lymph, etc.), and in laboratory samples.

A pigment is a substance that absorbs visible light. The more pigment dissolved in solution, the darker the solution, and the more light it absorbs. If we know the relationship between concentration and absorbance for a particular pigment, we should be able to figure out the concentration of a solution by measuring its absorbance with a spectrophotometer.

If the substance of interest is not a pigment, we may still be able to use this property of light absorbance by binding it to a pigment, or letting it react with and change the light absorbing properties of some other substance.

Beer's Law

Beer's law quantifies the absorbance of light by a solution, and gives us the means to find the concentration of a dissolved pigment. This relationship gives biologists the means to quantify the invisible. For example, a pigment bound to the substrate of a particular enzyme permits measurement of enzyme activity; a gene specifying a pigment engineered to be transcribed whenever another gene of interest is transcribed permits measurement of gene expression.

In this lab we take a very pragmatic, experimental approach toward exploring the limits of Beer's law for a substance (fluorescein) in solution:

$$A_{\lambda} = \epsilon_{\lambda}cl$$

This equation is read as “The absorbance (of a molecule in solution) at wavelength λ is equal to the product of the molar extinction coefficient, the concentration, and the pathlength.”

(*Reading equations is a practical skill that reinforces the mathematical relationship, the definitions of the variables involved, and helps pinpoint what you may need to know.*)

- ϵ : (epsilon) The molar extinction coefficient is a property of the absorbing species (molecule) being measured. ϵ has units of $M^{-1}cm^{-1}$. For fluorescein, $\epsilon = 92,300$ at 500.75 nm (the peak absorbance wavelength), and 50,358 at 485 nm (the wavelength our plate readers use) in a basic solution (pH ~ 9).
- c : The concentration of the absorbing species. The units are usually molarity (M), moles/L.
- l : Solution pathlength (how far the light has to travel through the solution), usually in cm.
- A : Absorbance. Unitless! (Note that the units of the left hand side must equal the units of the right hand side – the units of ϵ , c and l cancel.)

Spectrometer vs. Plate Reader

In a typical spectrometer, the pathlength is determined by the geometry of the cuvette (see Figure 1). In a plate reader the pathlength depends on the sample volume (proportional to its depth).

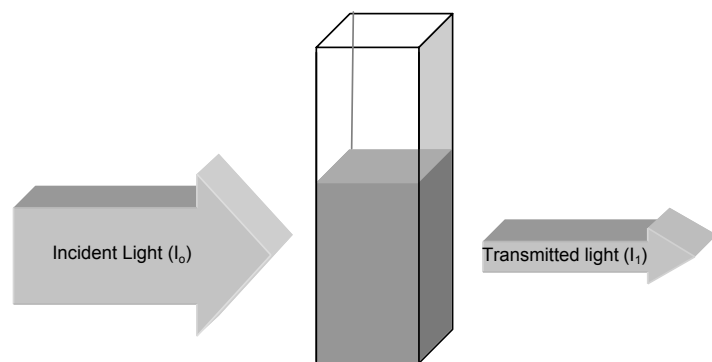


Figure 1. Illustration of absorption, showing incident light intensity (I_0) from left, the cuvette of pathlength, l , with solution of concentration, c , and the transmitted light intensity (I_1).

Limitations of the Beer-Lambert Law

The Wikipedia page lists the limitations of Beer's Law as a failure to satisfy any one of five conditions of linearity*:

1. The absorbers must act independently of each other.
2. The absorbing medium must be homogeneous and must not scatter light.
3. The incident radiation must consist of parallel rays, and experience the same pathlength.

* Wikipedia Page on the Beer-Lambert Law (http://en.wikipedia.org/wiki/Beer-Lambert_law).

4. The incident radiation should be monochromatic (a single wavelength, or color) or have a wavelength range that is narrower than the absorption band.
5. The incident flux must not influence the atoms or molecules; it should only act as a non-invasive probe of the species under study. In particular, this implies that the light should not cause optical saturation or optical pumping, since such effects will deplete the lower level and possibly give rise to stimulated emission.

Fluorescence

Biologists have been using pigments that bind to particular cellular structures to see the inner organization of tissues and cells for over a century. As shown in Figure 2, staining a thin section of trachea from a monkey with hematoxylin (which, because it is basic, binds preferentially to nucleic acids) and eosin (which, because it is acidic, binds to many proteins) renders cells and their nuclei quite visible, even in a black and white micrograph.

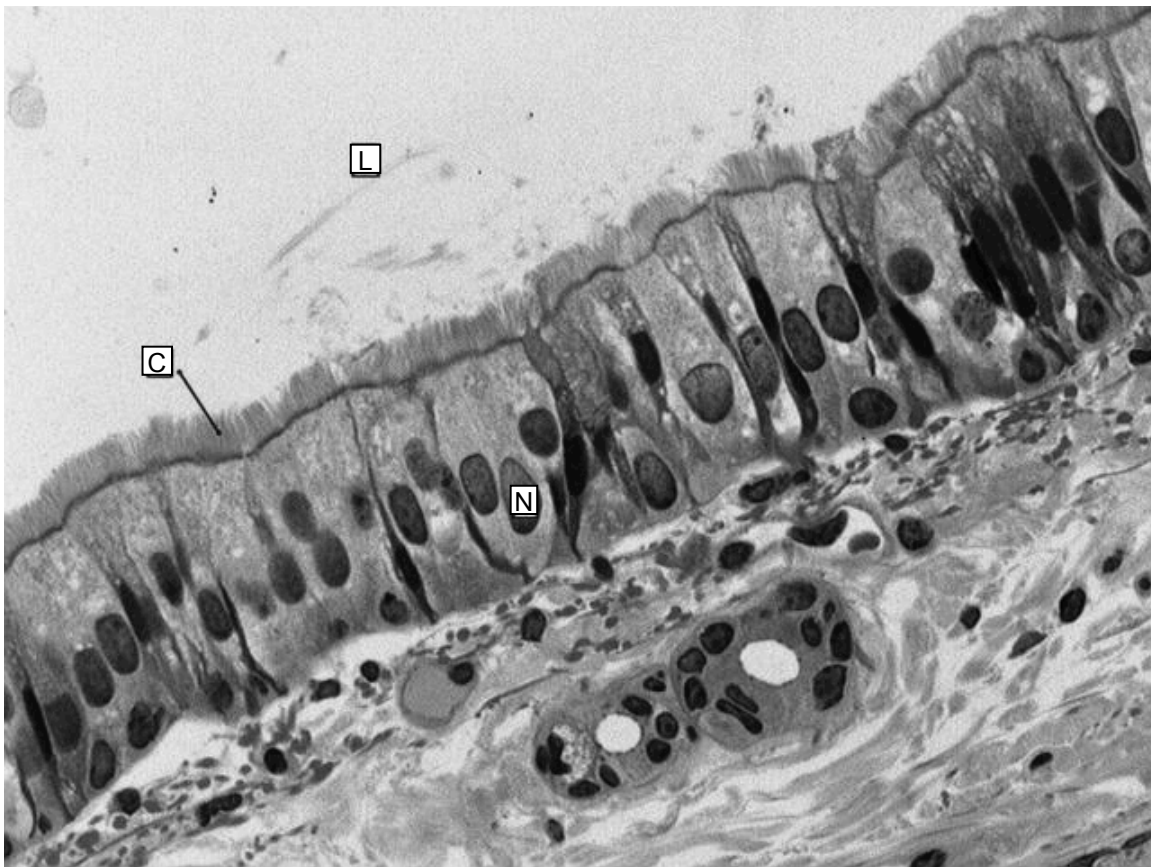


Figure 2. Pseudo-stratified columnar epithelium. C: cilia; L: lumen; N: nucleus. Photo by Biology 523 students, Spring 2009.

Many modern imaging techniques rely on fluorescence, which is the process by which certain substances emit characteristic wavelengths of light when excited by other, shorter, wavelengths. See p. 79 for a fuller explanation of fluorescence.

This special property of fluorescent molecules leads to a unique labeling strategy; attached to the specific targeting agent (an antibody), the fluorescence becomes colocalized with specific subcellular structures, as shown in Figure 3. Alternatively, it is possible to engineer a gene so that it produces not only its usual protein, but also a fluorescent one, as shown in Figure 4.

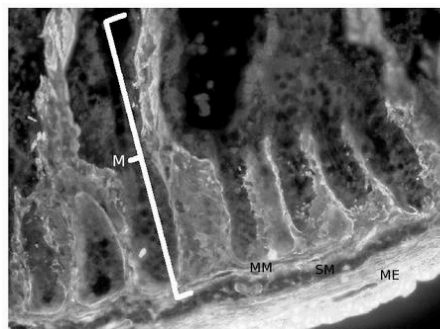


Figure 3. Composite image (left) of a section of rat small intestine, immunostained for connexin (top), laminin (middle), and nucleic acid (bottom). Photo by students in Bio 523, Fall 09.

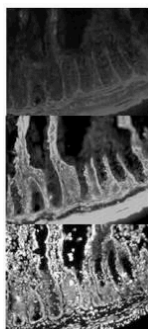


Figure 4. Image of two cells in anaphase. Spindles are made of GFP-tubulin, and are visible by fluorescence. Photo by students in Bio 497H, Spring 08.

Fluorescein.

Apart from its uses in medicine, *e.g.* corneal exams, fluorescein, illustrated in Figure 5, is one of the most widely-used chromophores in biological research. Many of the fluorescent images on the “*Immunofluorescence*” poster (from Cell Signaling Technologies, www.cellsignal.com) in your lab are obtained with fluorescein-labeled antibodies.

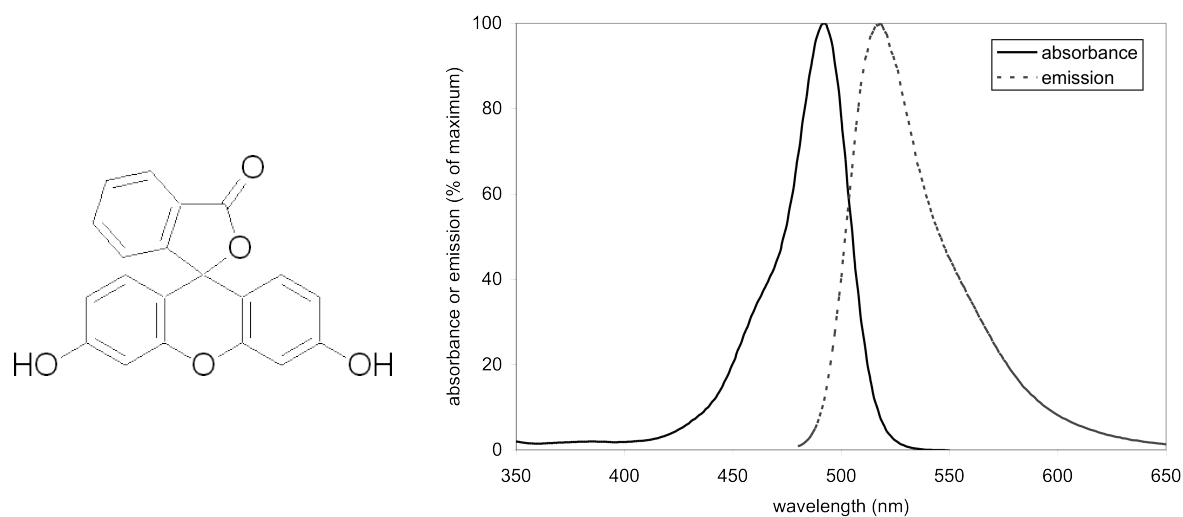


Figure 5. (Left) The structure of fluorescein. (Right) The absorbance (solid line) and the fluorescence emission (dashed line) of fluorescein as a function of wavelength. The maxima in absorption and emission are in the blue and green region of the spectrum, respectively. Structure diagram from [Sigma-Aldrich](http://www.sigmaaldrich.com/thumb/structureimages/50/mfcd00005050.gif) (<http://www.sigmaaldrich.com/thumb/structureimages/50/mfcd00005050.gif>) Spectra plotted from data points from the Fluorescence SpectraViewer at Invitrogen.com (<http://probes.invitrogen.com/media/spectra/data/1359ph9.txt>)

Relevant to this lab exercise, the intensity of fluorescent light can be proportional to the concentration of the (fluorescent) molecule.

$$\text{Fluorescence Intensity} \propto \text{Molecule Concentration}$$

For this reason we have instructed you to measure the absorbance and the fluorescence of fluorescein, and to determine the range of concentrations over which each property (absorbance and fluorescence) is linear.

Lab 1.1: Welcome to the Lab

Micropipettes and Serial Dilution

Welcome

- Introductions
- Expectations
- Pictures
- BCRC accounts
- CURE pre-course survey

Goals for the lab

Familiarization with:

- Rainin LTS micropipettors
- Repeatability
- Mean and standard deviation in Excel
- Scientific balances
- Calculating concentration
- Serial dilutions
- Proper glove use

Qualitative assessment of absorbance and fluorescence of fluorescein.

Micropipettors

These are precision scientific instruments, and must be treated with respect. The pipettor is used to draw liquid up into a cheap disposable tip. The three pipettors you will use take up and deliver liquids in the volume range from $\sim 0.5 \mu\text{L}$ to 1.0 mL. Your instructor will show you how to use this device. More guidance can be found on p. 77. Read and follow these guidelines to maintain the accuracy and precision of your pipettors.

Measurement Repeatability

An essential property of good science is that an experiment gives the same results even in different hands. Repeated measurements of the same thing should give the same value, no matter who makes the measurement.

Our first job, therefore, is to make sure our measurements are reliable. We will be checking both inter- and intra-observer reliability, as well as the reliability of our instruments.

Figure out what each of these volumes of water should weigh, and which pipet should be used to dispense that volume:

μL	mL	weight (g)	weight (mg)	pipet
1000 =		=		
100 =		=		
10 =		=		

Choose one of these volumes for pipet calibration.

Put a weigh boat on the balance pan, and zero the balance.

Have one member of your group dispense the appropriate volume of water onto the pan. Record your result.

Re-zero the balance and repeat 10 times.

Repeat the exercise with the other member(s) of the group.

Data Analysis

Examine your data by eye. Does anything stand out? Any strange values? Next week we will learn a statistical test to see whether you can confidently omit one outlying value. See p. 85 for a sneak peak.

Using the built-in functions in Excel, calculate the mean and standard deviation of each of your sets of repeated measurements.

Describe any differences between measurements made by different people, or with different instruments. Make a few repeat measurements as necessary to distinguish between technique and instrument. (Hint: the biggest source of discrepancy is likely to be either using the second stop on the pipet plunger or letting go of it suddenly during filling. Think about which technical error would give you a measure higher than expected, and which, lower.)

Concentration and dilution

We will use varying concentrations of things (fluorescein, green fluorescent protein, bacteria, sugar) in this lab, so it is important to be comfortable with making dilutions and measuring concentration.

Safe handling of fluorescein

Fluorescein is relatively benign. It is used in eye drops to help doctors find scratches on the cornea. It is added to beer for Saint Patrick's Day festivities.

As you will see, the 1 mM solution* we start with is rather highly concentrated, and it can stain various items. In addition, to get maximum fluorescence from it, we have made it up in 10 mM NaOH, which has a pH 12, which is caustic. (For reference, aqueous solutions of

* (M stands for molar, or moles per liter; m stands for milli, or one thousandth. See p. 85 for more on concentrations, and Table 2 on page 106 for metric prefixes.)

benign substances can only be safely poured down the drain if their pH is between 3 and 11.) For today's qualitative assessment of the light absorbing and emitting properties of fluorescein, we are diluting it with water; next week we will use NaOH.

For these reasons, you should **wear gloves** when you are handling fluorescein solutions. You must **remove your gloves** before you touch your computer.

Preliminary observations

Before you begin your dilutions, observe your 1 mM fluorescein solution with the naked eye in room light and through the special filter in blue light. Record your observations.

Unit Conversions

Whenever you perform calculations on measurements, it is useful to write the units on every number. If you measured two things with different units, you will have to convert from one to the other, and labeling everything is the best way to guard against errors. In the following example, we convert from mM to M by taking advantage of the fact that 1 M = 1000 mM. Dividing a number by itself equals 1, so we can multiply a number by this fraction without changing its value, although we will change its units. Writing the units on the M-to-mM conversion factor guarantees we have not multiplied when we were supposed to divide, or vice versa.

$$1 \text{ mM NaOH} \times \frac{1\text{M}}{1000\text{mM}} = 0.001 \text{ M NaOH}$$

Concentrations

Given that the molecular weight of fluorescein is 332, calculate the grams of fluorescein needed to make a liter of 1 mM fluorescein. Do the indicated conversions.

$$\begin{aligned} 1 \text{ mM fluorescein} &= \frac{g \text{ fluorescein}}{L} \\ &= \frac{mg \text{ fluorescein}}{L} \\ &= \frac{mg \text{ fluorescein}}{mL} \end{aligned}$$

Dilutions

Make a 0.1mM solution

Begin by figuring out how to make 5 mL of 0.1 mM solution from your 1 mM solution. Remember from chemistry that initial concentration times initial volume equals final concentration times final volume:

$$c_i v_i = c_f v_f$$

You need to calculate how much (v_i) of your 1 mM (c_i) solution is needed to make 5 mL (v_f) of a 0.1 mM (c_f) solution.

$$v_i = \frac{c_f v_f}{c_i}$$

Read more about dilutions on page 79.

Make a 2-fold serial dilution

One of the easiest ways to examine a wide range of concentrations is by *serial dilution*. We will use this technique repeatedly throughout the semester.

Make a 2-fold serial dilution of your 0.1mM fluorescein solution:

- Line up 12 3-mL glass tubes in a rack, and put 1 mL of 1 mM water into all but the first tube.
- Pipet 2 mL of 0.1 mM fluorescein into the first tube.
- Transfer 1 mL from the first tube into the second, mix by gently pipetting up and down, then transfer 1 mL of that mix into the second tube.
- Repeat this procedure with tubes 3-11. Discard the last mL into your liquid waste bucket. Your twelfth tube should contain only water. (If you absent-mindedly made the twelfth tube into a fluorescein dilution, just get a 13th tube and put water in it.)
- Make a table in your notebook showing the concentration of fluorescein in each of your tubes.

Absorbance and Fluorescence

Observe your dilution series in room light. Note which is the first tube that you cannot distinguish from water. It may help to put your tubes against a solid white background.

Observe your dilution series in blue light. It may help to put your tubes against a black background. Note which is the first tube you cannot distinguish from water.

Your notebook

In your notebook you should have:

- Your repeated measures data, including means and standard deviations for measurements by each member of your group.
- Your observations about the repeatability of your measurements, or the differences between partners or instruments, and your interpretations as to technique, instrument calibration, etc. (Note: “experimental error” is too vague a term to be useful. Explain what you did to figure out where the differences in accuracy or precision lay.)
- Your observations on the appearance of the fluorescein solution in room light and in blue light.
- Your observations of your dilution series, and a qualitative assessment of absorbance vs. fluorescence.

Clean up

Dump your fluorescein solutions into the hazardous waste container in the fume hood.

Empty your desktop trash bucket into the room trash, along with all your used gloves.

Throw used glass tubes into the glass trash.

Throw used plastic tubes into the room trash.

Return all your equipment to its proper place.

Lab 1.2: Absorbance of Fluorescein

Goals for the lab

Familiarization with:

- The hardware and software of a modern research plate reader spectrophotometer.
- Data management in a spreadsheet: transposing and sorting data, writing equations
- Dealing with outlying data points
- Calculating mean and standard deviation
- Comparing means
- Illustrating the difference between two data sets

Materials

Gloves

Waste buckets

Two different fluorescein solutions nominally at 1mM.

NaOH 10 mM

Today's Major Equipment:

The plate readers

These are multifunctional spectrometers that measure absorbance and fluorescence on many samples almost simultaneously. Throughout the semester you will be using plates with a 96 well format, as illustrated in Figure 6. The rows are labeled A – H, and the columns, 1- 12, so there is a unique label for each well. Plates the same size with 384 and 1536 wells are also available and are used in high-throughput analyses.

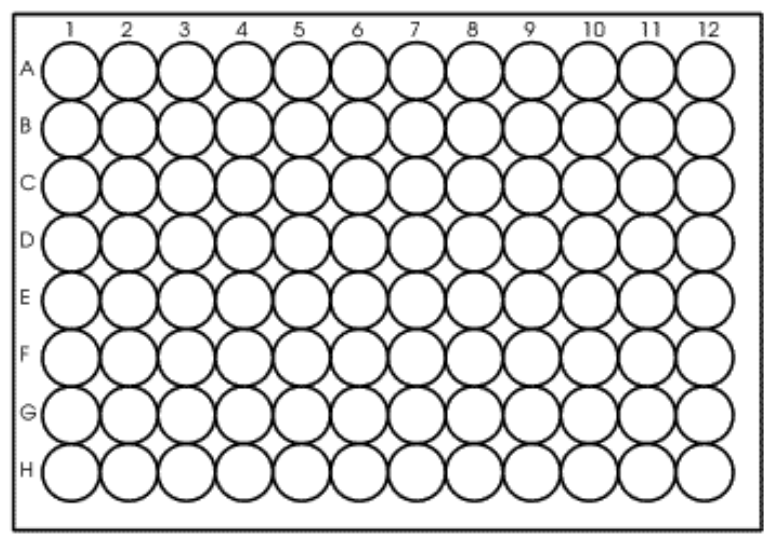


Figure 6. A 96-well plate, with 8 rows labeled A to H; each row has 12 wells; each well holds about 350 μ L.

Procedures

Measurement Repeatability

Last time we checked the repeatability of our measurements by weighing the amount of liquid dispensed.

This time, we will do another quick check using the plate reader.

When the plate reader functions as an absorbance spectrometer, the light passes through the liquid in the well (top to bottom). Therefore, dispensed volumes should be accurate repeatedly to get accurate measurements of absorbance. Remember, in Beer's law, $A = \epsilon cl$, l is the pathlength (see page 12); therefore, variations in volume will lead to variations in pathlength.

1. Make 1 mL of 10 μ M solution of fluorescein from your 1 mM stock, using 10 mM sodium hydroxide (NaOH) as your diluent.
2. Dispense 150 μ L of the diluted fluorescein stock solution into each of the first ten wells of rows A and B. (A1-A10, B1-B10; you fill row A, your partner row B, or vice versa).
3. In the last two wells of each row (A11 and A12, B11 and B12), dispense 150 μ L of the blank solution (10 mM NaOH).
4. Take your plate to the plate reader, where the instructor will assist you in taking the measurements. The measurement method generated for this experiment is called '**Repeatability**'.
 - Open OPTIMA, click your group from the users menu, then click "RUN".
 - Open plate reader by pushing the button on the machine and place your plate on the tray. Push the button on the machine again to have the tray retract back into the machine.
 - When ready to take a reading, click the traffic light on the top left side of the program window.
 - It will now ask you to select a test program: select the test program you wish to use for your plate reading; select ok.
 - On the next menu screen, click start measurement.
 - Where the traffic light was, there will be an absorbance graph. Click this once to see your data being collected in real time.
 - Make sure the values that are showing up are: Absorbance values. This is on the bottom right hand side of the screen.
 - When the reader has concluded collecting data, simply click close. (Do not push save – it doesn't do what you hope it does. It turns your data into a jpeg file!).
 - Your data should be in your Wahoo folder waiting for you to analyze it! Make a note in your notebook of the file name and what's in it.

Questions arising from your data

As you examine your data by eye, many questions undoubtedly will come up. During this period, you will work in pairs of small groups to dig deeper into such topics as those listed below. Guidelines for using Excel to help you answer your questions are found on page 88.

1. **Blanks.** What do I do with all the blank readings? Why aren't they zero?
2. **Accuracy.** How do your (and your partner's) absorbance measurements for fluorescein compare to the theoretical value? How close are the two different fluorescein solutions to that value? Is the difference significant?
3. **Inter-observer reliability.** Is the difference between my partner and me significant? How do I illustrate the differences?
4. **Precision and repeatability.** Note the difference between precision and accuracy, as illustrated in Figure 7. How much variation is there from one measurement to the next? Is it the same for both absorbance and fluorescence? How do we report variation? Are there any inexplicably odd readings? Can I throw them out?

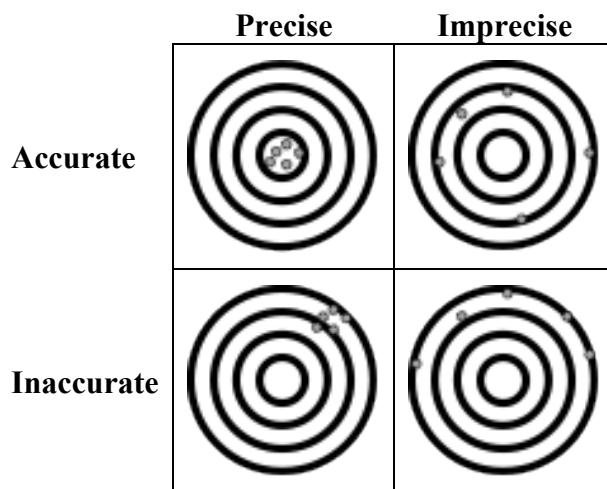


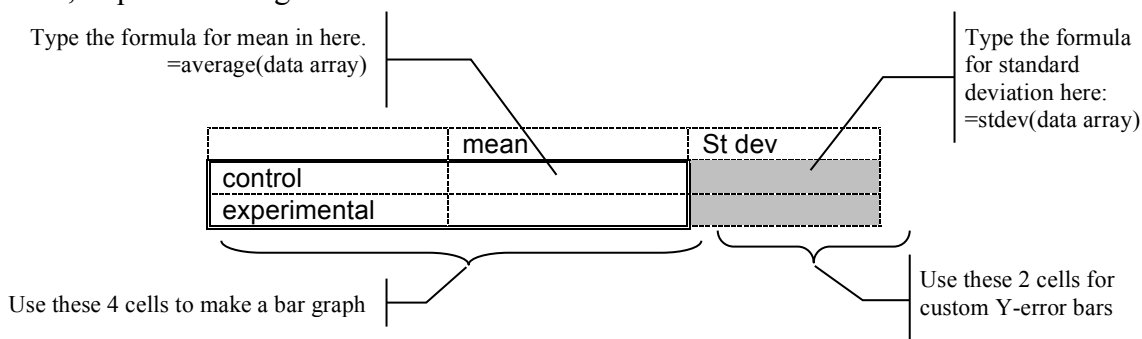
Figure 7. Comparison of precision and accuracy.

Data Analysis

Examine your data by eye. Does anything stand out? Any strange values? Use the Q-test to see whether you can confidently omit one outlying value. See p. 85 for how to do this.

Using the built-in functions in Excel, calculate the mean and standard deviation of each of your sets of repeated measurements.

The easiest way to create a histogram comparing the means, with standard deviation as the error bars, requires making a little table in Excel that looks like this:



Highlight the cells containing the labels and the averages, and use the chart wizard to make a bar graph. Then highlight the bars and format the data series. Make *custom* Y-error bars, using the two cells in which you have calculated standard deviation. Note: the “standard deviation” choice inside the Y-error bar dialog box is not what you think it is! Don’t use it! You must calculate the standard deviation in a cell. (The dialog box has no idea which data went into making the means you are graphing, and cannot possibly calculate their standard deviation. Instead, it calculates the standard deviation of the values you are plotting – in this case your mean and your partner’s mean, and plots an error bar that length starting from the average of those values. I don’t know when this would be useful.) See “Illustrating the relationship” on page 89.

Create a second histogram comparing the absorbance values for the two different fluorescein solutions.

Sensitivity of the plate reader.

Create a 2-fold serial dilution

1. Add 150 μL of blank solution to C1 and C2 of your plate.
2. Dispense 300 μL of 1 mM stock into well D1.
3. Fill D2 to D12 with 150 μL of the blank solution.
4. You are ready to dilute. Using a *transfer volume* of 150 μL , withdraw the transfer volume from D1, dispense into D2 and mix.
5. Repeat this process (D2 to D3, D3 to D4, *etc.*) until all 11 wells (D2 to D12) have had the transfer volume added, mixed, and removed. Discard the 150 μL removed from D12 to liquid waste.
6. Read the absorbance of your solutions in the plate reader.
7. Transfer the data from the plate reader to your computer desk top.

Analyze your data

Organize your measurements.

Put the concentrations of the solutions adjacent to the cells containing the measurements (i.e., if your data are listed in a row, put the concentrations in the row above the data). Your instructors will show you how to get Excel to calculate these concentrations. Do not calculate concentrations with a calculator and type them in! That is like painting a wall with a Q-tip!

You will ultimately be much happier with your file if you put your data into columns, rather than rows. Use the paste special command. Get all your absorbance readings in a single column, with their concentrations to the left of them.

Correct for the blank

Is the absorbance of the NaOH blank zero? If not, subtract that value from each of the other values. Write the equation once, then use the fill function to enter it into the rest of the cells.

Plot the relationship between concentration and absorbance.

Plot concentration vs. absorbance using a scatterplot (not a line graph, which isn’t what it sounds like). Inspect your plot.

Add a trendline by right-clicking on a data point. Make it a line, and ask for the equation and the R^2 to be displayed. (If time permits, you can try eliminating points from the top and/or bottom of the range to create a better fit between line and points. You can also see what happens if you force the line through the origin. We will work on this more next week.)

Assess, at least qualitatively:

- whether the relationship is linear along the entire range of concentrations
- whether there is a minimum concentration required by the reader to distinguish signal from noise
- whether there is a maximum concentration above which there is little rise in absorbance.

Clean up

- Save your plate – the unused wells can be used next week. Label it with tape and put it in your drawer.
- Pour NaOH and fluorescein solutions into the waste container.
- Throw all dirty tips and used tubes in the regular trash.
- Return your pipettors, tips, racks, etc. to your station drawer.
- Never return a partially used stock tube to the rack.

Lab 1.2 notebook:

Either copy your values into your notebook, or attach a printed copy (adequately notated!) to the removable copy of your notebook entry after you have finished writing it.

Calculate the theoretical absorbance of a 10 μ M solution of fluorescence. (See p. 12.)

The molar extinction coefficient of fluorescein is given this way: $E_{485nm}^{1M} = \frac{50358}{M \cdot cm}$.

This means that when light of wavelength 485 nm passes through 1 cm of a 1M solution of fluorescein, the absorbance should be 50,358 (theoretically – we don't actually have a spectrophotometer that can give us this high a reading). The values as reported by our plate reader are already corrected for pathlength, so you can use 1 cm.

For the comparisons you made between your repeated values and your partner's, and between the two fluorescein solutions,

- Write a one sentence comparison, paying attention to similarity, variability, and closeness to the theoretical value.
- Indicate what you did about any potential outliers.
- Write a 1 sentence summary of the statistical evaluation you performed.

Tabulate the means \pm standard deviation. See page 113 for an example of a table that presents means and standard deviations.

For the absorbance measurements of the dilution series, either draw a qualitative graph showing the relationship between concentration and absorbance, or paste in a print-out of your Excel "chart". Write a sentence or two about the relationship.

Lab 1.3: Fluorescence, Absorbance, Data Mining and the art of the standard curve

I have mountains of data! Now what?

Pre-lab homework:

Come to lab with the calculations for doing a 1:5 dilution already complete. See page 26.

Goals for the lab

In this lab, we will use Excel to answer questions arising from our qualitative assessment of our data during the previous lab. You will learn various techniques, such as:

- Serial dilution
- Comparing fluorescence and absorbance
- Sorting data
- Plotting curves
- Using a standard curve to find the concentration of a solution.

Dilution series

Here, you and your partner will prepare a 2-fold, then a 5-fold serial dilution series, and measure the absorbance and fluorescence as a function of concentration. Using the absorbance results, you can determine if Beer's law is *universal* (true under all conditions), or if there are limits to its validity. You can also find out the relative sensitivity of fluorescence and absorbance to concentration. More on dilutions can be found on page 79.

Serial Dilution by 2.

1. Add 150 mL of blank solution to E1 and E2 of last week's plate.
2. Dispense 300 μ L of 1 mM stock into well F1.
3. Fill F2 to F12 with 150 μ L of the blank solution.
4. Proceed as last time (page 22) until all 11 wells (F2 to F12) have had the transfer volume added, mixed, and removed.
5. Read your plate and download the values to your desktop computer.

Serial Dilution by 5.

Here, you and your partner will prepare a 5-fold serial dilution series, according to the protocol described for serial dilution by 2, *with the appropriate modification*. The relevant question is: What is the *transfer* volume for a 5-fold dilution series when the final volume of all the samples in the series is 150 μL .

1. Calculate, and double-check the transfer volume before you start these dilutions. (**Hint:** Use $c_1v_1 = c_2v_2$ with $c_2 = 0.2 \times c_1$, $v_1 = v_t$, where v_t is the transfer volume, and $v_2 = 150 \mu\text{L} + v_t$; solve for v_t . What does v_2 equal? See p. 79 for more help with dilutions.)
2. Create the duplicate dilution series (starting with 1 mM on down) in row G.
3. If you need to start over, use row H.
4. If you have used other wells, ask your instructor to help change the plate layout on the plate reader.

Measure Absorbance and Fluorescence

1. Read the absorbance with the method “**Serial2&5_Abs**”. (This will read both the 2-fold and 5-fold dilution series.) Save the data as an Excel file, transfer the data to your Wahoo folder for analysis at your iMac.
2. Read the fluorescence with the method ‘**Serial2&5_FI**’.
3. If any reading is at the maximum (65000), adjust the gain according to that well, and read the plate again.
4. Retrieve your data, and correct for the blanks.
5. Put the concentrations of the solutions adjacent to the cells containing the measurements. Your instructors will show you how to get Excel to calculate these concentrations. Do not calculate concentrations with a calculator and type them in! That is like mowing the lawn with a scissors!

Compile and sort the data

You will probably like columns better than rows for this activity.

To turn your row data into column data, you will need the paste transposed command (Edit/paste special/transpose).

Get all your absorbance readings in a single column, with their concentrations to the left of them. Do the same thing for your fluorescence readings.

If your concentrations are created by a formula (as they should be), turn them into values with the paste values only command (Edit/paste special/values)

Sort the data from lowest concentration to highest.

The relationship between concentration and absorbance or fluorescence

Plot concentration vs. absorbance using a scatterplot (not a line graph, which isn't what it sounds like). Repeat for fluorescence.

Inspect your two plots.

The art of the standard curve

Determine the range of concentrations over which absorbance is linearly related to concentration, and use "source data" in the chart menu to include only those points in the graph. Add a linear trendline. Think about whether you should force this line through the origin by setting the intercept to 0 (you can do this in the options menu of the trendline dialog box). (Hint: what should the absorbance of no fluorescein be?) One of your trendline options is to include the equation of the line on the graph. (Another option, that may be useful in deciding which points to discard, is to display R^2 . This value is a measure of the goodness of fit of the points to the line. R^2 is 1 for a perfect fit, and 0 for no discernable trend at all.)

This is now a standard curve, that is, a graph you can use to estimate the concentration of an unknown solution, as shown in Figure 8. You can also use the equation of the line to calculate X from Y.

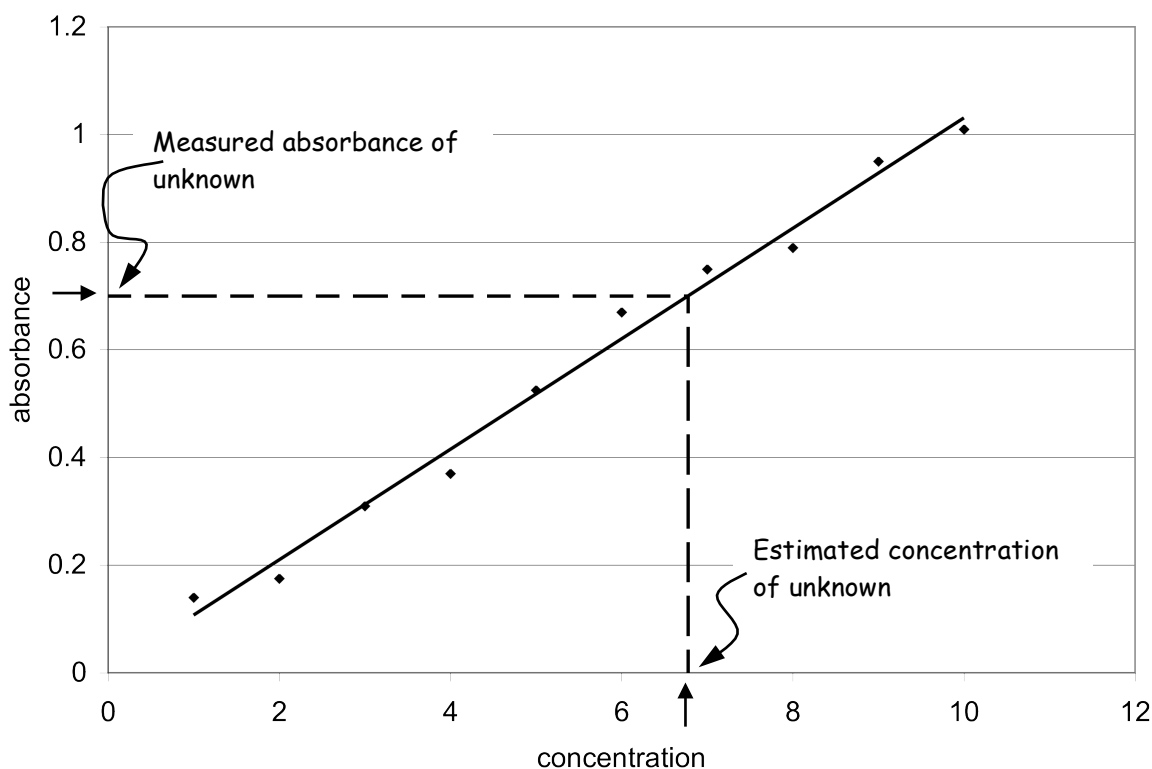


Figure 8. Hypothetical standard curve. A straight line is fitted to points plotted of the absorbance readings from solutions of known concentration. The dashed line shows how to estimate the concentration of a solution giving a reading of 7. Note that the line is used, not the individual data points.

Repeat the process for fluorescence.

Flourescence vs. absorbance. Compare the effective range of concentrations that can be accurately measured with fluorescence to the range that can be measured by absorbance.

Unknowns. There are three fluorescein solutions of unknown concentration in the classroom. Over the course of the afternoon, you should measure both the absorbance and fluorescence intensity of each.

Use your standard curves to estimate the concentration of all three samples.

Lab 1.3 Notebook

Your notebook should include

- Your calculations.
- A map of your 96-well plate
- Qualitative graphs illustrating the relationship between absorbance and concentration, and between fluorescence and concentration, with linear regions highlighted. (Revisit the concept of qualitative graphs, if necessary.) How does the relationship between concentration and absorbance differ from that between concentration and fluorescence?
- The theoretical slope of the line made by plotting fluorescein concentration vs. absorbance. What is the slope of your line (linear region only, of course.)
- Your estimates of the concentration of the three unknowns, along with a description of how you found each one, and why you did it as you did. Under what circumstances should you use absorbance to determine concentration, and when should you use fluorescence?

Unit 1 Lab Report

Write this report as an individual. Choose **one** question that can be addressed with your data from these two weeks' experiments, for example:

- The difference between fluorescence and absorbance.
- The reliability of your pipetting.
- The difference between your pipetting and that of your partner.
- The relative accuracy of your instructors' solution making.
- Something else that has occurred to you in this unit.

Refer to the section on writing reports starting on page 88 for specific advice on writing the different sections of the report.

Write an abbreviated report of your chosen experimental question, as follows:

Title

Write a title that accurately reflects the project you are reporting. See examples on page 102.

Abstract

Write a 5-sentence abstract that summarizes your experiment or your test. Abstracts are described on page 103, and there is an example on page 110. Make every sentence count.

Results

Describe what you found in another 2 or 3 sentences, referring to your illustration or table by its number.

Present a figure or table (or two if necessary) illustrating the point you wish to make about your experiment. Make sure your figure or table is properly labeled, and follows the conventions given beginning on page 105, and illustrated on pages 111 and 113.

Don't bother with the other report sections for this assignment.

Unit 2: Counting Bacteria & Measuring the Rate of Bacterial Cell Division

“Not everyone is mindful of it, but all cell biologists have two cells of interest: the one they are studying and *Escherichia coli*.”*

“What is true for the bacterium, is true for the elephant.”†

Introduction & Background

As you’re learning in lecture, modern cell biology has made tremendous strides toward understanding the inner workings of cells. Historically, bacteria have been organisms of choice for understanding the genetics and molecular & cellular biology of cell function, including metabolism and replication, although the focus has shifted toward eukaryotes in recent years. Nonetheless, studies of prokaryotes remain an important component of the overall picture, including biodiversity (millions of bacterial species, estimated), disease (microbial pathogens, e.g. *E. coli*/*Salmonella* outbreaks), and biotechnology (*E. coli* is a workhorse of genetic and protein engineering). *E. coli* is small, ~ 1 μm across and ~ 2 to 4 μm in length (Figure 9, right). It uses flagella for swimming, which facilitates chemotaxis (Figure 9, left).

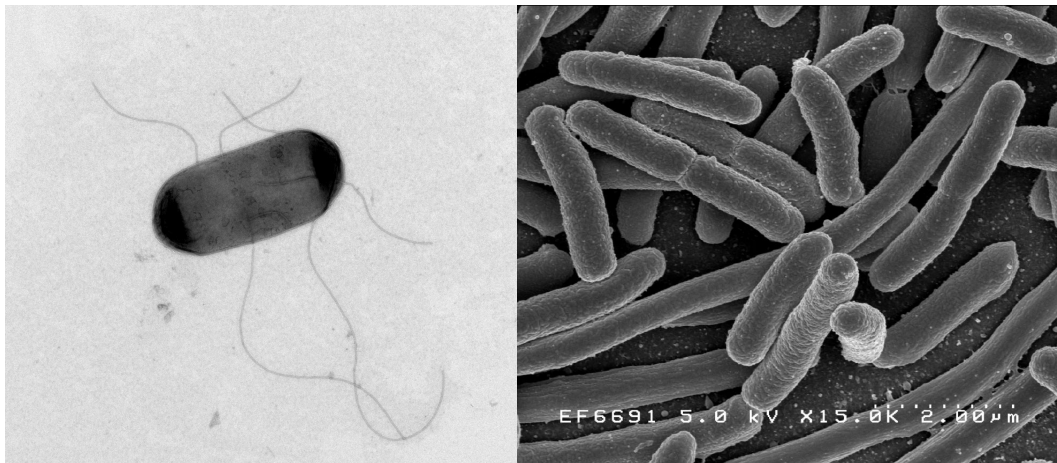


Figure 9. Electron microscope images of *Escherichia coli*. *At left:* transmission electron micrograph of (a deflated) *E. coli* stained with uranyl acetate, to generate contrast. The filaments are flagella. *At right:* A scanning electron micrograph of *Escherichia coli*, grown in culture and adhered to a cover slip (Rocky Mountain Laboratories, NIAID, NIH, http://en.wikipedia.org/wiki/File:EscherichiaColi_NIAID.jpg, accessed 9/3/12).

* Schaechter M, Niedhardt FC in *Escherichia coli* and *Salmonella typhimurium: Cellular and Molecular Biology*. (Niedhardt FC, editor) Washington, DC: American Society of Microbiology, 1987, p 2.

† This quote, or something like it, is attributed to Jacques Monod, who by it implied that the cellular machinery of bacteria is used by eukaryotes. However, the reverse may not be true as frequently; see Wolin, SL (1994) From the Elephant to *E. coli*: SRP-Dependent Protein Targeting 77 (6), 787-790.

Goals for the lab

Fundamental questions are addressed in this unit, as well as technical objectives. These include:

- How fast do bacteria divide when given (most) all the nutrients that are necessary for cell division?
- What is the mode of growth and how does this mode influence the rate of cell division?
- What are the advantages and disadvantages of the three different methods for counting cells?

These objectives are addressed over the next two lab periods:

First Week (Lab 2.1):

Week one has the technical objective to introduce three methods for counting cells: (1) with phase-contrast microscopy, (2) in the plate reader, (3) by spreading diluted cultures on nutrient agar and counting the colonies that appear after incubation.

Second Week (Lab 2.2):

These methods will be used to measure the rate of cell growth under nutrient-rich conditions. How can these methods be used to assess the fundamental requirements for cell division?

Pre-Unit Assignment

- *Learn about phase-contrast microscopy.* Visit ‘Microscopy U’, a website hosted by Nikon Corporation (www.microscopyu.com). Under ‘microscope tutorials’ you will find thorough descriptions (with animations) of basic microscopy and phase-contrast optics. Despite its complexity, try to gain a rudimentary (instinctive) understanding about how phase contrast optics work. Also read the section on phase-contrast microscopy and the use of the Nikon microscope on page 114.
- Review the instructions for using the Motic microscope Camera (page 114) to be ready to take pictures of bacteria.
- Read the application note on the disposable hemocytometer (page 34).
- Ponder lowly *E. coli* and all that it must do to divide. (Do a web search?)
- Do the calculations for next week’s lab, found on page 34.

Lab 2.1: Viewing and Counting Bacteria

How dense is the starting culture?

The phase-contrast microscope: Today's major new equipment.

In Unit 1 we used three pieces of equipment (micropipettors, plate reader, computers) to quantify the fluorescein concentration. In this unit, you will quantify bacteria with the plate reader, but also count bacteria directly using microscopy. Microscopy is a central tool in the study of cells. You can read about phase microscopy on page 116.

Materials

***E. coli* cultures.** Tubes containing stationary phase *E. coli* are in the refrigerator. Your team will withdraw samples from these flasks to make your observations. Keep them cold to prevent cell division.

Pipette Tips.

Luria-Bertani (LB) growth medium. LB is a complete growth medium made from tryptone (trypsin-digested milk protein for amino acids), yeast extract (carbohydrates and vitamins), and NaCl. It will also be used as a diluent and a spectrometer blank. Keep it cold to prevent cells from dividing until you want them to.

Disposable hemocytometers are cell-counting chambers that have calibrated volumes (the name derives from the fact that they are used to count blood cells). Hemocytometers allow you to determine the concentration of cells in solution under the microscope.

Motic camera. Attaches to the microscope eyepiece to take snapshots of cells for a permanent record of your raw data.

96 well plate. To measure the optical density (OD) of cell cultures.

LB-agar petri dishes and glass bead spreaders. Petri dishes, ~10 cm in diameter, are filled with 30 mL of LB-agar. LB-agar hardens as it cools after heat sterilization, into a stiff Jell-O (97% LB, 3% agar by weight). The spongy substrate is used to grow visible colonies of bacteria from single cells in an incubator overnight.

Cell counting techniques

The *technical objective* this week is to learn and compare different means for measuring the cell concentration (cells/mL). Some are more convenient than others; each method makes different assumptions. We will measure cell density by three techniques: (1) OD measurements in the plate reader, counting (2) cells in a hemocytometer and (3) colonies on an LB-agar plate.

Procedures

Get cell cultures.

Take a tube of cells from the refrigerator and carry it *on ice* to your bench.

Make 2 dilution series.

(From practical experience, it has been found, sadly, that the culture has to be diluted much more for plating on agar than for reading in a plate reader. That's why we need two dilution series.)

Use cold LB and keep all tubes on ice at all times. Label 2 sets of tubes: 1A-10A and 1B-10B.

A. 2-fold dilution series:

- Put 0.5 mL of cold LB into each of 9 tubes (labeled 2A-9A), and put them on ice.
- Put 1 mL of cold cell culture to a tube (tube 1A) and place on ice.
- Transfer 0.5 mL from tube 1A to tube 2A. Mix gently.
- Repeat for the rest of the tubes in the series. Keep tubes on ice.

B. 10-fold dilution series

- Put 0.9 mL of cold LB into each of 9 (labeled 2B – 9B) tubes, and put them on ice.
- Put 1 mL of cold cell culture to a tube (tube 1B) and place on ice.
- Transfer 0.1 mL from tube 1B to tube 2B. Mix gently. Keep cold.
- Repeat for the rest of the tubes in the series. Keep tubes on ice.

2. Measure cell optical density (OD) using the plate reader

OD is a more general property of a solution that could refer to either the *absorption* of light (as measured with fluorescein in Unit 1), or light *scattering*, which also reduces the intensity of transmitted light and makes solutions cloudy or *turbid*. Bacterial cells scatter light. The amount of scattering is proportional to the cell concentration. Typically, the OD of a bacterial culture is measured at 600 nm, but our plate reader measures OD at **595 nm**. When the response is linear, the cell concentration will be proportional to OD through a constant (akin to ϵ in Beer's law). The protocol **BUG_OD** is set up to measure OD₅₉₅. Then, using the same sample to count cells directly in the hemocytometer, you will be able to calculate the proportionality constant ((cells/mL)/OD₅₉₅).

- Dispense 250 μ L of each culture in the 2-fold dilution series to wells A1 – A10 of your plate.
- Put 250 μ L of LB into wells A11 and A12.
- Dispense 250 μ L of each culture in the 2-fold dilution series to wells B1 – B10 of your plate.
- Put 250 μ L of LB into wells B11 and B12.
- If you have to wait in line for a plate reader, put your plate in the refrigerator until it is your turn.
- Read the OD at 595nm (OD₅₉₅) with the protocol Bug_OD.
- Open the file at your desktop computer.

3. Count cells in the hemocytometer

The hemocytometer has precisely calibrated volumes that are very small, as illustrated in Figure 10. We can count how many cells there are in one of these areas and calculate cell density from that number.

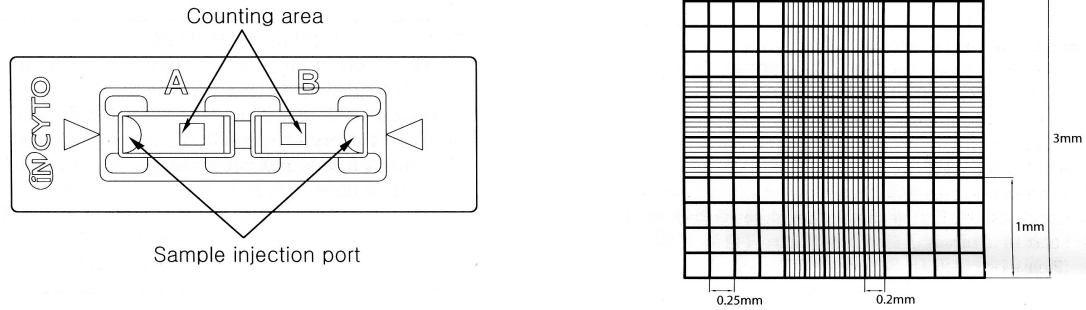


Figure 10. The iN Cyto disposable hemocytometer is a 1 x 3-inch microscope slide (left), but has an attached coverslip with gridlines that define the counting volume. Each slide has two **counting areas** (for two samples). When 10 μL of liquid cell suspension is introduced at the **sample injection port**, capillary action draws the culture liquid into the counting area (expanded at right). The counting area is an array of squares and rectangles. Volumes are calibrated in mm^3 (length \times width \times depth). The depth of the counting area is 0.1 mm. (Image: <http://www.incyto.com>).

Calculate the volume of the entire counting area. **Calculate** the volume of the tiniest area.

See the section beginning on page 77 for instructions in using the phase microscope. After you have successfully examined an empty hemocytometer with your microscope, proceed to the counting procedure:

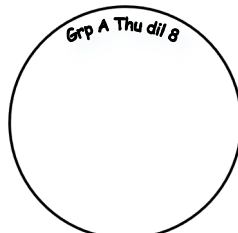
Keep all tubes with cells on ice!

- Pick a culture with the highest OD that seems to be down in the noise.
- Withdraw 10 μL of that culture and dispense it into the sample injection port of the hemocytometer (see Figure 10).
- Locate the counting area in the microscope and bring it into focus under low magnification (10 \times objective). Make sure the appropriate phase ring is in place.
- Increase the magnification to 40 \times (remember also to switch to the matching phase ring). **Important tip: Do not touch the focus knob before switching lenses, as these microscopes are parfocal. If you are afraid of hitting the slide with the lens, push the stage down before you switch lenses. See diagram on page 126.**
- Count the cells in the square(s) you have chosen, keeping careful record of width (w) and length (l) of the grid section. (Make a table in your lab notebook with headings of w, l and cell number for each grid section.)
- Repeat this procedure until you have counted at least 100 cells. (The object is to gain a precise estimate of the number of cells, and the more you count, the greater your confidence in the concentration you calculate.)
- Compute the volume ($l \times w \times d$) for each grid section in which cells were counted, using recorded values of l and w and $d = 0.1$ mm.
- Calculate the cell concentration in units of cells/mL. (Useful conversion factors: 1 $\mu\text{L} = 1 \text{ mm}^3$, 1 mL = 1000 μL .)
- Repeat this procedure with a second 10 μL aliquot. Choose a higher or lower cell density according to how crowded the first sample was. (If it was hard to find cells, choose a more concentrated sample; if they were too numerous to count, go down.)

- (Optional) Take one or more pictures of the (counted) fields using the Motic camera as a permanent record of the raw data. Detailed instructions on using this camera can be found on page 114. Record in your lab notebook the grid section photographed, noting its dimensions.

4. Plate cells on an agar plate to count colonies.

Spreading cells onto an LB-agar petri dish is a counting method in which each colony that becomes visible after an overnight incubation corresponds to one *viable* cell. For cell plating, the desired number of colonies per plate is ~ 50 to 200. Since we don't yet know just how concentrated our cells are, we will have to plate from more than one dilution.

- Label 8 agar plates around the margins of the *bottom* dish, leaving plenty of room for you to view the colonies, and no doubt as to whose plate it is, which dilution.
- 
- Dispense 100 μL of each of the 8 most dilute cell cultures from the 10-fold dilution series onto its own agar plate, and add a few sterile glass beads. Stack the plates right side up, and shake back and forth until the liquid has dried.
 - Remove the beads: Turn each dish upside down in turn, tapping it to knock the beads into the lid, remove the lid while keeping the bottom upside down, and tap the beads into the waste receptacle. Replace the lid.
 - Put the plates in the 37C incubator, *bottom side up* to incubate overnight.
 - Come back the next morning to count colonies (or put the LB-plates in the refrigerator to stop growth). Don't bother trying to count the colonies on overcrowded plates.

Notebook

Your notebook should contain:

1. A table with the dilution factor in each well of your multi-well plate, and the OD_{595} values of each well.
2. Cell counts, volume and cells/mL calculations for each hemocytometer sample, as well as an indication of which tube was used (and its dilution factor)
3. Colony counts for the countable agar plates (entered the next morning, obviously), and the dilution factor for each.

Clean up

Throw empty or LB microtubes in the trash.

Give your liquid waste to your instructor for disposal.

Put all plastic items contaminated with bacteria into the red biohazard waste bin.

Add bleach to bacterial cultures in glass, rinse once, and put in the glass trash.

Throw out your bench pad and all your gloves.

Clean your bench top with 70% ethanol.

Wash your hands.

Lab 2.2: Bacterial Growth

The Cell Concentration – OD₅₉₅ Conversion Factor.

In Week 1, the OD₅₉₅ of a culture of *E. coli* was measured. By analogy to Beer's law, we expect that blank-corrected OD measurements are proportional to cell concentration (with similar assumptions and limitations). Thus, to determine a cell 'extinction coefficient', that is the conversion factor between cells/mL and OD₅₉₅, the cell concentrations were determined directly in parallel to the OD measurement with a hemocytometer and on petri plates. For both methods, the conversion factor has the form:

$$\text{conversion factor} = \frac{\text{cells/mL}}{OD_{595}}$$

$$\text{which is akin to} = \frac{1}{\epsilon_{595}}$$

We can explore the equivalence between this relationship and Beer's Law:

$$\text{Beer's Law: } A_{595} = \epsilon_{595} \times c \times l$$

$$\text{By analogy: } OD_{595} = \epsilon_{595} \times \text{cells/mL} \times l$$

$$\text{The plate reader scales OD to the equivalent } l = 1 \text{ cm} \quad = \epsilon_{595} \times \text{cells/mL}$$

$$\text{Rearrange to show "extinction coefficient": } \epsilon_{595} = \frac{OD_{595}}{\text{cells/mL}}$$

$$\text{Rearrange to show how to obtain concentration from OD: } \text{cells/mL} = \frac{OD_{595}}{\epsilon_{595}}$$

Calculate the density of the initial cell culture from your hemocytometer counts. You calculated the cells/mL for two tubes in your dilution series. Calculate backward from these to estimate the cell density in the initial culture. (For example, if the tube you took your hemocytometer sample from was 1/16 the initial culture, multiply the count by 16 to estimate the concentration in the first tube.)

If you successfully counted cells from two different dilutions, do this operation twice.

Calculate the density of the initial cell culture from your colony counts. As above, figure out the concentration of the initial cell culture.

Discuss the range of values obtained within and between groups. Pick one to use, and give a justification for your choice. (Your justification may depend on your trust of your own measurements, your desire to "split the difference", your respect for your colleagues, your concern about including or excluding dead cells, etc.)

Set up your Excel file. Convert your plate reader data to columns instead of rows with the paste special command. Put all well ID's in one column and OD's in the next.

Make a column to the left of your OD values and enter the appropriate dilution (e.g., 1, 0.5, 0.25, etc.) next to each value. Change equations to values with paste transposed.

Sort the data so the values from the 2-fold dilution series and the 10-fold dilution series are interposed in order.

Insert a blank column between the dilution and the OD values, and convert the dilution values to cells/mL by writing an appropriate equation in the top cell, and filling down.

Plot cell density vs. OD. Highlight your two columns and insert a scatterplot. Right click on a point and add a trendline. Display the equation and the R^2 value (find them under options).

Correct for the absorbance of LB. Does the trendline go through the origin? Why or why not? Correct for that by subtracting the blank or the calculated Y intercept from the OD in a new column inserted between the initial OD and the cell concentration.

Find the linear region of this relationship. R^2 is a measure of how well the regression line fits the data points, and it varies from 1 (perfect fit) to 0 (no relationship between the 2 variables).

Restrict the range of the data points by excluding the high concentrations and try to find a region with an R^2 near 1.

This tells you which OD values will likely be directly related to cell density values. It gives you a way to judge whether an OD value is too high or too low to be a useful indicator of cell density.

Calculate a conversion factor from OD to cell density. Our goal in this exercise is not to do this again! Putting an aliquot of cell culture in the plate reader and getting an OD is much more convenient than doing all this counting over and over. Calculate your conversion factor:

$$\text{conversion factor} = \frac{\text{cells/mL}}{OD_{595}}$$

From the linear region of your OD vs cell density plot, figure out the conversion factor. From now on, you will be able to get cell density by multiplying this factor times OD.

We will discuss the estimates for the cell concentration of the initial undiluted culture, as well as the conversion factors obtained from your data.

Bacterial Growth Curves – 181 time points

Between week 1 and 2, OD's have been measured in a 96-well plate at 6 minute intervals over an 18 hour time span of several different 150 μL *E. coli* cultures. Excel files of these data, streamlined for your convenience, are available on the Wahoo server.

Take this file and do the following manipulations in Excel:

- Correct ODs with spectroscopic blanks (or the Y intercept).
- Convert the ODs to cells/mL.
- Convert cells/mL to millions of cells/mL (this makes the Y axis easier to read).
- Calculate the population size in each 150 μL volume.
- Convert minutes to hours (this makes the X axis easier to read).
- Plot cell concentration as a function of time.
- Add an exponential trend line and restrict
- Start restricting the source data until you identify the region of exponential increase.

- Choose two points on the curve near the beginning and the end of the region of exponential increase, and read the time and population size of each.

Calculating Doubling Time and Reproductive Rate

Bacteria multiply by dividing. Each cell gives rise to two daughters. Under ideal conditions, therefore, the size of the population should grow exponentially, doubling each time the cells divide:

# of generations	0	1	2	3	4	5	6
# of cells	$2^0 = 1$	$2^1 = 2$	$2^2 = 4$	$2^3 = 8$	$2^4 = 16$	$2^5 = 32$	$2^6 = 64$

For single celled organisms that simply divide in two, the generation time is how long it takes for the population to double (i.e., the time per single generation), and the reproductive rate is the number of generations per unit time.

G = generation time = doubling time = $\Delta t/g$ = elapsed time per generation

r = reproductive rate = $1/G$

t_1 = beginning time

t_2 = end time

Δt = elapsed time = $t_2 - t_1$

g = # of generations

N_1 = # of bacteria at t_1

N_2 = # of bacteria at t_2

$N_2 = N_1 \times 2^g$ (growth by binary fission)

solve for g :

$$N_2 = N_1 \times 2^g \text{ (growth by binary fission)}$$

$$\text{Rearrange: } \frac{N_2}{N_1} = 2^g$$

$$\text{Take the } \log^* \text{ of both sides: } \log\left(\frac{N_2}{N_1}\right) = \log(2^g)$$

$$\text{Rearrange: } \log N_2 - \log N_1 = g \log 2$$

$$\text{Divide both sides by } \log 2: \quad g = \frac{\log N_2 - \log N_1}{\log 2} = \frac{\log N_2 - \log N_1}{0.301}$$

$$\text{by definition, } G = \frac{\Delta t}{g}$$

$$\text{Solve for } G \text{ by substitution } G = \frac{\Delta t \times 0.301}{\log N_2 - \log N_1}$$

$$\text{calculate } r \quad r = \frac{1}{G}$$

* Forget how to do logs? See page 104.

Notebook assignment

Clearly indicate the following in your notebook, showing how you obtained each one:

- Hemocytometer cell counts, the volumes of culture counted, and cell concentration (cells/mL) of that sample and of the initial culture.
- Colony counts on the usable plates, with dilution factors, and estimate of cell concentration of the initial undiluted culture .
- Your choice of initial culture concentration
- A plot of OD vs concentration
- The linear region of that plot
- The conversion factors obtained in this way. (Be prepared to give this estimate to the instructor.)
- The doubling time and reproductive rate of *E. coli* at room temperature. Show how you obtained your values.

We will discuss the estimates for the cell concentration of the initial undiluted culture, as well as the conversion factors obtained from your data.

Unit 2 Lab Report

Collaborating with your lab partner(s), write a report describing your conversion factor and your growth curve (refer back to the section on writing that begins on page 88).

The sections should be included in this unit's report:

A **title** that captures the essence of the report. (The title of this chapter may not be suitable.)

An **abstract** that summarizes your work and your findings.

A **methods section** that explains how you obtained your conversion factor, and how your growth experiment was set up.

A **results** section that describes your graph(s) and refers to it (them) in the customary manner. The results section should include graphs of the OD vs. concentration calibration and bacterial growth curves, as well as your calculated reproductive rate and doubling time.

Unit 3: Regulation of gene expression by the lac repressor

Introduction – prokaryotic operons

Some genes are expressed *constitutively* – all the time. The products of constitutively-expressed genes are needed all the time, *e.g.* enzymes for glycolysis, or the RNAs that are the structural components of the ribosome. Other gene products are not needed (or wanted) at all times – the expression of these genes is *regulated*. You have heard about some regulated genes in lecture, *e.g.* those involved in cell differentiation.

The *Escherichia coli lac* operon was one of the first gene regulation system to be studied. It led Jacob and Monod to enunciate the principles of *operon* organization and regulation*, for which they were awarded the Nobel Prize in 1965†. Examples of these original ideas, essentially unchanged, are found in all cells. It gives authenticity to the statement ‘what is true for bacteria is true for the elephant.’

In prokaryotes, genes are often arranged into a functional group called an **operon** (Figure 11). Operons consist of:

- a **promoter** – the binding site on the DNA for RNA polymerase
- an **operator** – the binding site for the repressor protein, in this case LacI. When LacI binds to the operator (*lacO*), RNA polymerase cannot carry out transcription.
- **structural genes** that are co-transcribed as a single mRNA molecule, coding for proteins that function together. In the case of the *lac* operon, these genes code for
 - β -galactosidase (LacZ), which breaks the disaccharide lactose into galactose and glucose
 - Lac permease (LacY), a pore protein that facilitates the transport of lactose across the inner membrane
 - β -galactoside transacetylase (LacA).

The **regulatory gene**, *lacI*, encodes the LacI **repressor protein**. The binding of certain sugars to LacI that causes it to dissociate from the operator site, which then allows transcription of the structural genes (Figure 11).

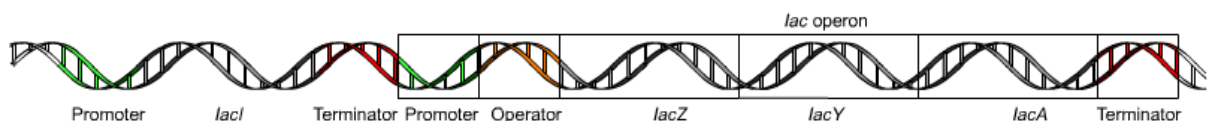


Figure 11. A schematic representation of the *lac* operon

After lactose is taken up by the cell, β -galactosidase catalyzes its isomerization to allolactose (structure in Figure 12). Allolactose binds to Lac I, which lowers its affinity for DNA. The dissociation of LacI allows RNA polymerase to begin transcription.

* Jacob, F, J Monod. 1961. Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* **3**, 318-356.

† Nobel Web, The Nobel Prize in Physiology or Medicine, 1965, available from http://nobelprize.org/nobel_prizes/medicine/laureates/1965/.

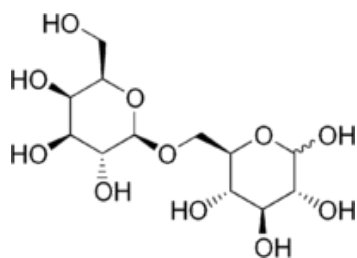


Figure 12. The disaccharide allolactose.

E. coli makes more efficient use of glucose than any other carbon (and energy) source. So, the availability of glucose can inhibit lactose utilization. This is mediated through cAMP (cyclic adenosine monophosphate), an inverse indicator of glucose availability, *i.e.* the smaller the glucose concentration, the greater the concentration of cAMP. cAMP binds to cAMP-dependent catabolite activator protein (CAP). The cAMP-CAP complex binds to the promoter region increasing the affinity of binding of RNA polymerase. Therefore, glucose starvation should increase *lac* structural gene transcription.

Detecting *lac* operon activity.

A significant experimental tool in studies of gene regulation is the *reporter* gene, which produces a signal in proportion to transcriptional activity, when it is placed downstream of the promoter under investigation. In fact, the product of the *lacZ* gene, β -galactosidase, is a widely-used *reporter* when combined with synthetic substrates of LacZ that are chromogenic or fluorogenic (these, respectively, generate pigmented or fluorescent reaction products).

In this lab, we will use an *E. coli* strain that harbors an *expression plasmid*, a genetically-engineered DNA element in which the operator/promoter region has been optimized for protein expression. (This is illustrative of an important biotechnological application of the *lac* operator/repressor system.) The plasmid drives the expression of green fluorescent protein (GFP) under control of LacI and *lacO*. GFP is fluorescent, which, like fluorescein, can be excited by blue light and emits green light. For practical purposes, this means the same settings can be used to detect GFP fluorescence in the plate reader.

Lactose analogues

Lactose is a disaccharide, that is, a two-part sugar consisting of galactose linked to glucose with glycosidic bond. Analogs of lactose, *i.e.* chemicals that are similar, but not identical to lactose, can also bind to and deactivate the repressor. Most analogs are galactosides with another chemical group in place of the glucosyl group of lactose. (See Figure 13.) We will use isopropyl β -D-1-thiogalactopyranoside (IPTG) in this lab. IPTG can bind to the repressor protein, but it is not digested by β -galactosidase. Thus, IPTG is not consumed and it is not a food source.

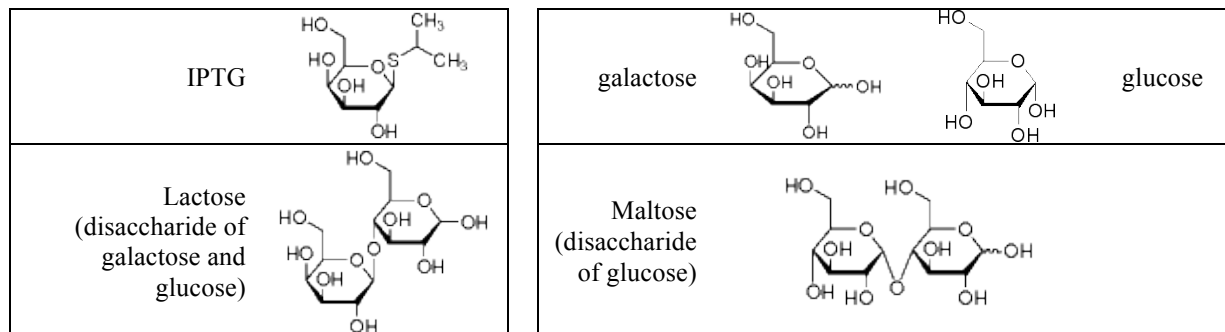


Figure 13. Chemical structures of sugars and sugar analogs available in this lab. All images from Sigma Aldrich Chemical Co. (sigmaaldrich.com).

Lab 3.1: Qualitative analysis of regulation

During the first week we will measure the fluorescence from GFP produced in *E. coli* under control of the *lac* operator. You seek ‘yes/no’ answers to the following questions: Which molecules induce expression, which do not? How closely related are the structures of these molecules? Do any of the sugars actively inhibit the action of the inducer?

To answer these questions you and your partner will select from cultures of E. coli grown with a variety of sugar or sugar-analogues the ones that you hope will address one of these questions. The chemical structures of these sugars are illustrated in Figure 13.

Materials

- Actively growing cultures of bacteria harboring the *gfp* gene under the control of the *lac* operator and the *lac* repressor protein, grown in LB with:
 - a. No additives
 - b. Lactose (0.25 M)
 - c. IPTG (0.1 mM)
 - d. Glucose (0.25 M)
 - e. Galactose (0.25 M)
 - f. Maltose (0.25 M)
 - g. IPTG (0.1 mM) + glucose (0.25 M)
 - h. IPTG (0.1 mM) + maltose (0.125 M)
 - i. Lactose (0.25 M) + glucose (0.25 M)
 - j. Lactose (0.25 M) + maltose (0.125 M)
 - k. Galactose (0.25 M) + glucose (0.25 M)
 - l. Galactose (0.25 M) + maltose (0.125 M)
- Access to a handheld blue light and a suitable filter to observe GFP fluorescence.

Methods

- Formulate a question that can be answered by comparing the fluorescence of particular culture samples from the list above.
- Choose up to six samples of cultures that might answer that question about *lac* operon induction and inhibition. Make sure one of your cultures is LB only and one is either LB + lactose or LB + IPTG. The others should help answer the question.
- Check for the induction of GFP expression using the special blue flashlight (holding it as far away as necessary to see the green fluorescence) and the filter (or goggles).
- Record your observations.
- Demonstrate your results to the rest of the class, in 2 minutes or less. Say what your question was and what you thought would happen, and then show what did happen.

Plan next week's quantitative experiments

Read the instructions beginning on page 45, and plan carefully.

Before you leave, your group should know exactly the volumes of each solution to pipet into each well of your plate.

Clean up

All sugar solutions should be disposed of down the drain, and the tubes thrown in the trash.

E. coli in plastic microfuge tubes should go straight into the red biohazard bin.

All *E. coli* cultures in glass tubes or flasks should be killed with bleach, and then poured down the drain. The flask goes with dirty dishes, and the glass tube goes in the glass trash.

Lab 3.2: Quantitative Analysis of the Kinetics and Extent of Induction

Pre-Lab Planning

The qualitative observations made in Lab 3.1 suggest experiments that you and your labmates will design to quantitatively test the specificity and potency of inducing and anti-inducing agents. You may ask questions such as the following, or whatever can be tested with our system.

- *What concentration of inducer (or inhibitor) is required to induce (or inhibit) expression?*
- *How does the potency of different inducers compare?*
- *What is the time course of induction (slower or faster than growth rate)?*
- *How many molecules of an inducer are required **per cell** to induce expression?*
- *How well does glucose work as an inhibitor?*

To do this you and your partner will be given one row in a 96 well plate (12 wells), to conduct a quantitative experiment. If you and your partner wish to work with a second group, then 2 rows will be at your disposal. Three groups working together will have 3 rows. Collective work requires more planning and group participation, but it offers the opportunity to conduct a more comprehensive set of experiments.

Both machines will be used to collect data for the experiments that you have designed. Samples will be loaded into 96-well plates at the beginning of the period. The data will be collected during and after the lab period. The results will be distributed digitally for analysis.

The experiments that you and your colleagues plan will measure:

- GFP fluorescence *versus* time.
- Cell concentration (OD₅₉₅) *versus* time.

Planning ahead is imperative. The plan should use the plate format effectively, including dilution series and control samples.

Before you leave Lab 3.1, you and your partner(s) should decide what question(s) to address, and what other colleagues to work with.

You should have written out exactly what you plan to dispense into each well.

Before you begin Lab 3.2, you and your coworkers must receive approval for your planned experiments. You need to submit this plan for approval. The plan should include a plate layout and describe (1) the questions addressed, (2) the composition of each well, and (3) the methods of well preparation

In-Lab Experiments:

At the beginning of Lab 3.2, the plates will be constructed in the following manner:

1. You and your coworkers will load 100 μ L of solution into each of your wells.
2. You will pass the plate to the next group and they will do the same.

3. After all the groups have loaded the wells, the instructors will add 100 μL of active *E. coli* culture into each of the wells. Each well will then have a total volume of 200 μL (1 part solution, 1 part cells).
4. The instructors will load control samples into reserve wells. These controls will be serially diluted cells (without any added sugar) to produce a calibration curve and an on-plate blank.

Solutions supplied to you:

- 0.5 M sugar solutions (lactose, maltose, galactose, glucose)
- 0.1 mM IPTG solution
- medium to make dilutions

Data collection

The plates containing the experimental solutions will be loaded into the plate readers, and a program will run which takes OD and fluorescence in rapid succession every 15 minutes for several hours.

Clean up

All sugar solutions should be disposed of down the drain, and the tubes thrown in the trash.

E. coli in plastic microfuge tubes should go straight into the red biohazard bin.

All *E. coli* cultures in glass tubes or flasks should be killed with bleach, and then poured down the drain. The flask goes with dirty dishes, and the glass tube goes in the glass trash.

Lab 3.3: Gene Expression Data Analysis

The data will be made available to you as a compiled Excel spreadsheet. You will use this period to mine the data and make decisions about controls, time-points, comparisons, etc., so that you can tease the answer to your question out of your mountain of data.

Suggestions for Mining the Data

1. Manage your data file.
 - a. Copy the file to your desktop, and work on it there. Delete unneeded columns.
 - b. Make quick-and-dirty scatterplots of OD vs. time and fluorescence vs. time, for all the plate rows you hope to use in your comparison, just so you get an overview of population growth and induction. (You may discover wells that have to be omitted for one reason or another.)
 - c. Convert minutes to hours
 - d. Correct for the OD of LB
 - e. Convert OD to cell concentration in millions of cells/mL
 - f. Calculate the number of millions of cells in each 0.2 mL well.
 - g. Calculate the fluorescence per million cells in each well.
 - h. Calculate the concentration of each sugar in each well.
 - i. Calculate the number of sugar molecules in each well at the start of the experiment.
2. Choose the index or indices that will best answer your question(s). For example, you might choose to compare fluorescence at a given population size, or fluorescence per million cells at a given time, or time needed to reach a given fluorescence per million cells, or time to reach maximum fluorescence per million cells, or maximum fluorescence per molecule of inducer per cell, depending on your particular investigation.
3. Make a variety of graphs to try to illustrate the behavior of the cells under the different conditions in your experiment. Try tabulating data, too.

Suggestions for Writing Unit 3 Reports

Get Organized.

Have a meeting of the group (*early!*) to discuss the report content and develop the plan for writing it . . .

1. What are the major question(s) and finding(s) addressed in the report? *What is the story?*
2. Data analysis. Transform the raw data into a publication-ready form. This final form *should best convey* support for your conclusions.
3. Decide what figures and tables you want in the report. Once you have decided on ‘the story’, then it’s worthwhile to decide the figures and tables to include in your report. It’s easier to write a report with the tables and figures in near final form, since they are like a ‘scaffold’ on which the report is constructed.
4. Share the writing responsibilities. Everyone should read through the report to catch typos, and to improve accuracy, completeness and clarity.

Report Format*

This will be a complete report, written collaboratively by the entire team.

Abstract

Introduction (background and significance)

Materials and Methods

Results

Discussion

Acknowledgements – indicate the contributions of the group members here

Literature References

Pertinent Lac I references†

These papers investigate the regulation of *lac* operon expression at the level of the single cell and molecule:

1. Johan Elf, Gene-Wei Li and X. Sunney Xie. 2007. **Probing transcription factor dynamics at the single-molecule level in a living cell.** *Science*. **316**:1191–1194.
2. Paul J. Choi, Long Cai, Kirsten Frieda, X. Sunney Xie. 2008. A stochastic single-molecule event triggers phenotype switching of a bacterial cell. *Science*. **322**:442-446.

* A couple of comments: (i) While you are writing the report, refer to pages 88 - 112 often, in fact have this document open while you are writing the report. Journal articles also provide a good guide (it is easier to emulate than innovate). (ii) Don't write the report in the order the sections appear. The abstract is usually written last. Materials and methods and results are among the first to be written, etc.

† These papers are written in the style of a 'letter' to *Science* magazine, so unfortunately they are not good examples of the format you have been asked to use in your reports.

Unit 4: Protein Folding & Stability

Summary:

This week you will study the stability of GFP and the reversibility of its folding/unfolding reaction. First, you will unfold (denature) GFP using heat and a chemical denaturant (urea). Then, you will measure the extent of refolding as a function of the urea concentration (high to low) by measuring the reappearance of fluorescence (which is lost upon unfolding and regained upon refolding). From these data and with a simple theory, you will calculate the energetic *stability* of GFP. The stability of the 'native' state of a protein is a property of all folded proteins. It depends on many factors, including the stabilizing molecular interactions that are present in the native state – interactions common to all molecules.

Significance.

Proteins are folded into a functional form called the native state as they exit or after they have exited the ribosome. This is a complex process, given the intricate nature of protein structures. Moreover, in the cell, protein folding can be guided by 'chaperones' – a family of proteins first identified as 'heat shock' proteins, because their expression is induced when a cell is exposed transiently to an elevated temperature (heat shock). Chaperones help the cell to recover from chemical and physical trauma (such as heat shocks).

Some proteins cannot fold into the native state without the assistance of chaperones. On the other hand, some proteins, usually small proteins, fold spontaneously – without the assistance of chaperones, and reversibly – which is to say that they can refold after being induced to unfold. This property of reversible folding has allowed chemists and biologists to study the protein folding process as a chemical reaction equilibrium and from the study of this process, determine the factors that influence the difference in energy between the folded and unfolded forms of a protein.

As it turns out, GFP is one such protein that folds reversibly. Its additional special property – that it is fluorescent, provides a convenient reporter of the extent of 'foldedness', and thus allows us to measure the extent of folding/unfolding in the plate reader.

A BRIEF HISTORY OF PROTEIN FOLDING.

Christian B. Anfinsen was awarded the 1972 Nobel Prize in Chemistry for his work on protein folding – that the amino acid sequence of a protein uniquely determines the 3-d structure adopted by the folded state, also stated as the 'one sequence – one structure' hypothesis. But all generalizations have exceptions, the dogma that came into being from the success of the 'one sequence – one structure' hypothesis was later found to have important exceptions, which Stanley Pruisner discovered. 35 years later Pruisner was awarded the 1997 Nobel Prize in Medicine and Physiology for his prion hypothesis, which states that proteins can sometimes adopt more than one folded state. Besides the native state, prions proteins have a 'prion' state that is the causative agent of various infectious diseases, such as bovine spongiform encephalopathy (mad cow disease). Collectively, these diseases are known as protein misfolding diseases.

It should be clear to you by now that GFP (Figure 14) is an important tool in biological research. Some background information about GFP that you should read before lab is posted at the course website (Lab Handouts Unit 4), including the proteopedia webpage. This site uses molecular graphics to illustrate the structure of GFP and the chromophore buried inside it (<http://www.proteopedia.org/wiki/index.php/GFP>); information from the Nobel Foundation on the 2008 Prize in Chemistry: ‘The green fluorescent protein: discovery, expression and development’, and a tutorial from the Protein Data bank: ‘Bioinformatics of green fluorescent protein.’

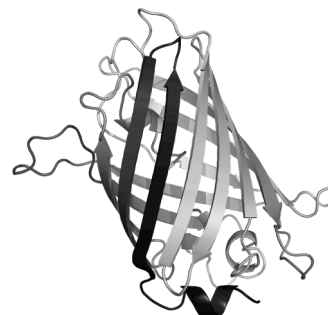


Figure 14. Ribbon structure of GFP

Goals

It is the objective of this lab to measure the equilibrium constant for the folding—unfolding reaction of GFP as a function of the urea concentration. (Urea is a small molecule chemical denaturant that destabilizes folded protein. See Figure 15.) From the measurement of the equilibrium constant, K , the difference in energy between the folded and folded states (ΔG°) can be calculated.

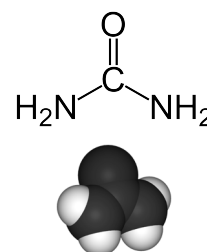


Figure 15. Urea

Because the folding reaction is reversible, we can measure the equilibrium constant for folding (or unfolding). For reversible folding, GFP continually folds and unfolds. We *imagine* that just two states exist, the folded state (N) and the unfolded state (D), which are interconnected by the folding/unfolding pathway (which is unknown!), as depicted in Figure 16.

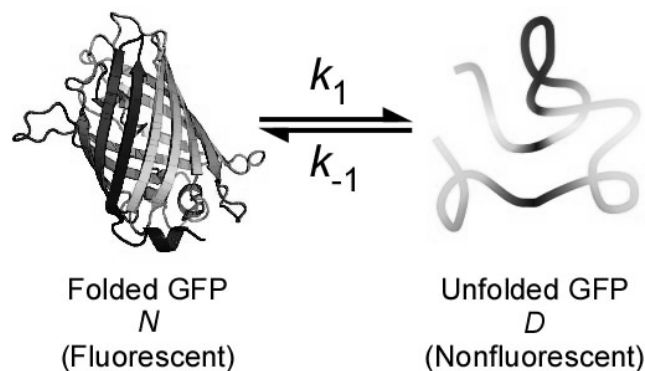


Figure 16. The GFP folding-unfolding reaction equilibrium. The forward reaction rate is characterized by the rate constant (k_1) and the reverse reaction rate by k_{-1} .

A chemical reaction like Figure 16 is written in shorthand as



When the forward and reverse reactions occur at equal rates, the system is at equilibrium.

$$K = \frac{[D]}{[N]} \quad (2)$$

The ratio of the concentrations D and N ($[D]$ and $[N]$) at equilibrium determines K , the equilibrium constant. As strange as it sounds, K is constant only when physical parameters

such as temperature, pressure, and solution composition are kept constant. Changes in any of these can affect the relative stability of the N and D forms, and K will change.

The native (folded) state predominates under cellular conditions ($K \ll 1$). This means that N is substantially lower in energy than D . When this is the case, the folded protein is the *stable* state. The relative energies of N and D can be altered, such that D becomes the more stable form ($K \gg 1$). In this lab, urea (Figure 15), a chemical denaturant, will be used to gradually alter the relative energies of N and D – that is, gradually adjust K . Figure 17 depicts what happens to K as the relative energies of N and D are changed:

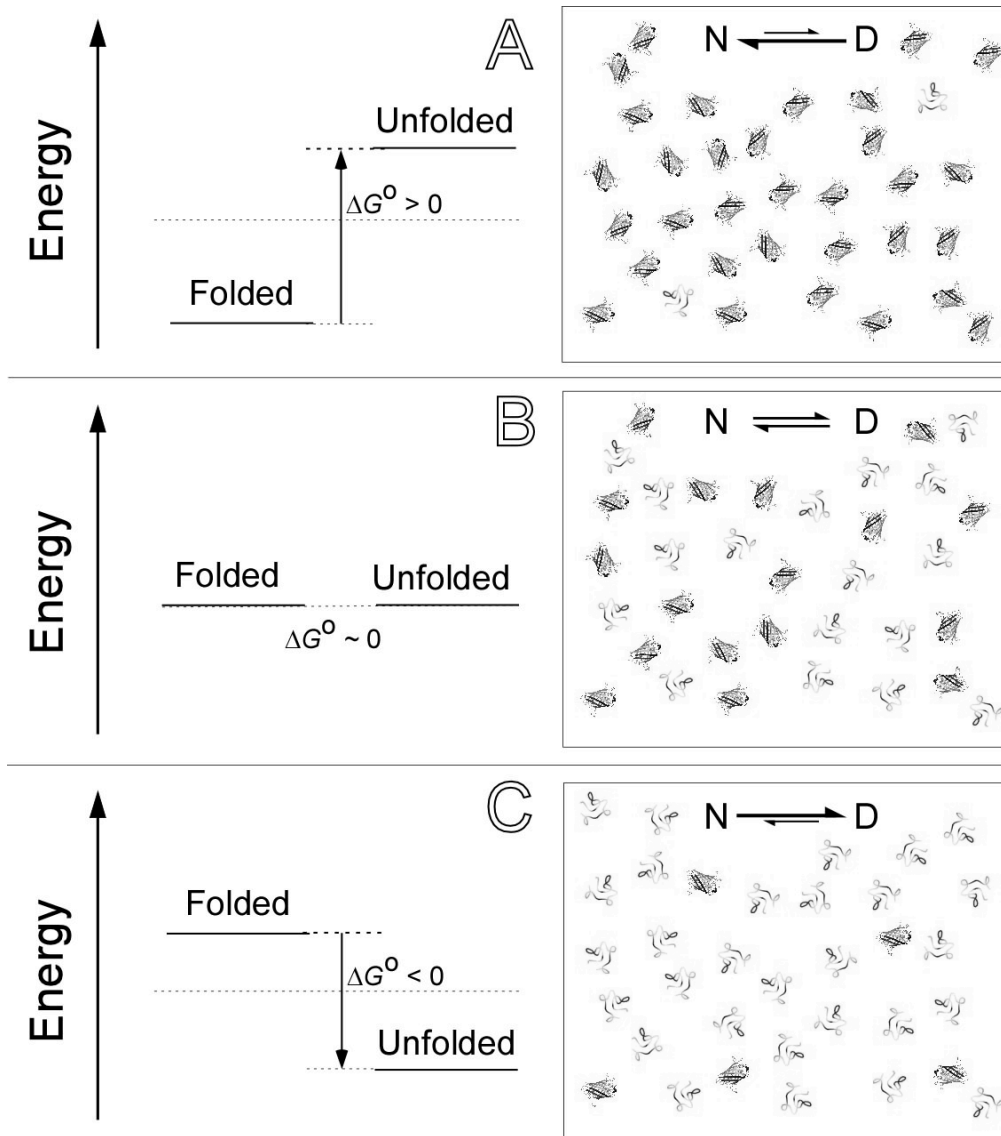


Figure 17. Relative energies of folded (N) and unfolded (D) protein as a function of the urea concentration. A: folded protein is more stable, and thus more prevalent, than unfolded protein at low/zero [urea]. $K \ll 1$. B: unfolded and folded protein equally stable and present in equal abundance at an intermediate [urea]. $K \sim 1$. C: D is more stable and more prevalent than N at large [urea]. $K \gg 1$.

Urea changes the structure of water and binds to unfolded protein and in this way it decreases to the energy of the unfolded protein molecules relative to the folded protein molecules. In other words, the chemical *denaturant* (urea) stabilizes the unfolded state.

How to measure K .

Green Fluorescent Protein (GFP) becomes *nonfluorescent* when it unfolds. Thus, by measuring fluorescence, you measure the fraction of GFP in the *folded* and *unfolded* states. From such a measurement you can determine K . K is related to the energy difference between the folded and unfolded states. The energy difference in the absence of denaturant is estimated (through extrapolation) with measurements and calculations of the difference in energy as a function of the urea concentration. This ‘zero denaturant’ energy difference is a fundamental property of the protein related to the molecular interactions that are present in the native structure.

Model of the Two-State Protein Folding Equilibrium

The folding – unfolding process is a chemical reaction, in which the folded state is the reactant and the unfolded state is the product. Using ‘N’ to stand for native (folded) protein, and ‘D’ for denatured (unfolded) protein, the chemical reaction equilibrium is represented as



When the reaction is at equilibrium, the concentrations of the native and denatured forms remain, *by definition*, constant over time. The ratio of their concentrations, product over reactant, has special significance, and is known as the equilibrium constant, K .

$$K = \frac{[D]}{[N]} \quad (2)$$

In this lab, you shall see that the equilibrium constant, K , depends on the concentration of urea. To make the connection with the dynamic nature of the equilibrium, write the net rate of formation of denatured protein. (Also, refer to the references mentioned on page 55 as part of your pre-lab assignment.) The rate of formation of denatured protein is proportional to the concentration of N :

$$\text{Rate of formation of denatured protein} = k_1[N] \quad (3)$$

The net rate of formation of denatured protein takes into account the formation of native (folded) protein from denatured protein (the reaction in the opposite direction):

$$\text{Net rate of formation of denatured protein} = k_1[N] - k_{-1}[D] \quad (4)$$

When the reaction is at equilibrium, the net rate of formation equals zero.

$$\text{Net rate of formation of denatured protein} = k_1[N] - k_{-1}[D] = 0 \quad (5)$$

In other the rates of the forward and reverse reactions are equal. This equation can be rearranged to obtain the equilibrium constant as the ratio of the two rate constants.

$$\frac{k_1}{k_{-1}} = \frac{[D]}{[N]} \quad \text{where it should be clear that} \quad K = \frac{k_1}{k_{-1}} \quad (6)$$

This formula underscores the dynamic reversibility of the reaction equilibrium. The value of the equilibrium constant reflects the relative stability of the unfolded (denatured) to folded (native) forms, and is related to the difference in energy of the two forms, which given by:

$$\Delta G^\circ = -RT \ln K \quad (7)$$

In this equation, ΔG° is the difference in energy (energy of product minus energy of reactant), R is the ‘Universal Gas Constant’ and T is the temperature in Kelvin units. When

the equation for ΔG° is rearranged to give the equilibrium constant as a function of ΔG° , R and T , the resulting equation is

$$K = e^{-[\Delta G^\circ/(RT)]} \quad (8)$$

It is worth pondering the meaning of this equation. Equation (8) tells us that $[D]/[N]$ is *exponentially* related to the difference in energy between the folded and unfolded forms: $\Delta G^\circ = (\text{energy of the folded form}) - (\text{energy of the unfolded form})$. It is also important to reflect upon the significance of RT . Because ΔG° has units of energy, so will RT (the exponent of e is a pure number, no units). RT is sometimes referred to as the *thermal energy*, the energy that surrounds and permeates everything everywhere (as long as $T > 0$); it is both the reservoir and sink for energy-dependent processes.

Through your experiments during this lab, you can determine values of K , and therefore ΔG° . In the experiment, you delivered the same amount of GFP to each well containing sample in the 96-well plate; it is the amount (concentration) of urea that will be different. To illustrate the fact that the total amount of GFP is the same in each well, consider the following:

$$n_{\text{GFP}(\text{total})} = (\text{Volume of solution per well}) \times [\text{GFP}] \quad (9)$$

$n_{\text{GFP}(\text{total})}$ is total number of moles of GFP per well. As you see below, the volume of solution in each well will be the same (200 μL) and the concentration of GFP, $[\text{GFP}]$, will be the same in each well ($\sim 1 \mu\text{M}$). Therefore, $n_{\text{GFP}(\text{total})}$ will be ~ 200 picomoles. (*Question: How many GFP molecules is this?*)

Now that we have established the number of GFP molecules in each well and, *by design*, it is the same in each well, consider what happens when the urea concentration is changed. First, without urea we can assume safely that all the GFP molecules are in the folded state: $n_{\text{GFP}(\text{folded})} \approx n_{\text{GFP}(\text{total})}$ and $n_{\text{GFP}(\text{unfolded})} \approx 0$. However, as urea is introduced, some GFP molecules denature (unfold), and so $n_{\text{GFP}(\text{folded})} < n_{\text{GFP}(\text{total})}$ and $n_{\text{GFP}(\text{unfolded})} > 0$, **but**

$$n_{\text{GFP}(\text{total})} = n_{\text{GFP}(\text{folded})} + n_{\text{GFP}(\text{unfolded})} \quad (10)$$

Equation 10 is a statement of the fact that the total amount of GFP ($n_{\text{GFP}(\text{total})}$), which is constant, is the sum of the moles GFP present in the folded and unfolded forms ($n_{\text{GFP}(\text{folded})}$ and $n_{\text{GFP}(\text{unfolded})}$, respectively). The *proportion* of GFP in the folded and unfolded forms will change as the urea concentration is changed from one well to the next, but the total amount of GFP will remain the same!

We are almost ready to make the connection to K . A curious thing happens when you divide Equation 10 by $n_{\text{GFP}(\text{total})}$:

$$1 = n_{\text{GFP}(\text{folded})}/n_{\text{GFP}(\text{total})} + n_{\text{GFP}(\text{unfolded})}/n_{\text{GFP}(\text{total})} \quad (11)$$

$$1 = \alpha_{(\text{folded})} + \alpha_{(\text{unfolded})} \quad (12)$$

$$\alpha_{(\text{folded})} = 1 - \alpha_{(\text{unfolded})} \quad (13)$$

Here, $\alpha_{(\text{folded})}$ and $\alpha_{(\text{unfolded})}$ are defined to be the *fractions* of GFP in the folded and unfolded forms. Finally, K is given by:

$$K = \frac{\alpha_{(\text{unfolded})}}{1 - \alpha_{(\text{unfolded})}} \quad (14)$$

It is left as an exercise for you to prove the equivalence of the definition for the equilibrium constant, K , in Equation (2) and the definition given in Equation (14). Equation (14) is significant, because K can be now estimated experimentally from the fluorescence intensity of the GFP.

BOTTOM LINE: By measuring the GFP fluorescence as a function of the urea concentration, the extent to which folded GFP is more (or less) stable than unfolded GFP can be determined. The ‘stability’ of the protein in the absence of urea, obtained by extrapolation of the data to zero urea concentration, provides insight into the fundamental properties of proteins.

Lab 4. Stability of Green Fluorescent Protein

In this lab, you will measure the equilibrium constant of the folding-unfolding reaction of GFP as a function of the concentration of urea, a potent denaturant.

Pre-Lab Assignment.

Learn about GFP using the links at the course website (on the Lab Handouts page for this unit):

- Proteopedia website on GFP (<http://www.proteopedia.org/wiki/index.php/GFP>)
- Information from the Nobel Foundation on the 2008 Prize in Chemistry: ‘the green fluorescent protein: discovery, expression and development.’
- The tutorial from the Protein Data bank: ‘Bioinformatics of green fluorescent protein.’

In your notebook record the results of calculations for volumes of reagents for Urea Dilutions series and control wells. Be Prepared!!! We may check this when you arrive in lab.

- The volume of GFP stock solution necessary for unfolding and refolding (wells A1-A12 and B1-B6)
- The volumes of 9 M urea and TNG buffer needed for the urea dilution series (see below)
- Any dilution calculations necessary for the control wells: B7-B12

Lab Reagents.

You will be provided with:

- 100 mL of a GFP Stock solution in TNG. [GFP] \approx 4 mg/mL
- 5 mL of a concentrated urea. (9 M Urea, 1 mM dithiothreitol (DTT))
- 5 mL of Tris, NaCl, Glycerol buffer (TNG buffer). (100 mM Tris, pH 7.5, 150 mM NaCl, 10% Glycerol, 5 mM DTT)
- One 96-well plate for fluorescence readings

Well Layout for Urea Dilution Series and Controls.

- Total volume in each well = 200 μL
- Volume of denatured GFP added to each well = 10 μL
- The total volume of concentrated urea and TNG buffer added to each well = 190 μL

Row	[urea]	1	2	3	4	5	6	7	8	9	10	11	12
A	[urea], molar	6.00 M	5.00 M	4.75 M	4.50 M	4.25 M	4.00 M	3.75 M	3.50 M	3.25 M	3.00 M	2.75 M	2.50 M
B	[urea], molar	2.25 M	2.00 M	1.75 M	1.50 M	1.25 M	1.00 M	0.00 M	0.00 M	blank	blank	blank	Blank

Before Lab

Calculate the amounts of TNG Buffer and 9 M urea that are necessary to give the final urea concentrations shown above. (*Excel can be used to calculate these volumes.*)

In every well:

$$V_{\text{total}} = 200 \mu\text{L}$$

$$V_{\text{den. GFP}} = 10 \mu\text{L} \quad (\text{made as described below, } [\text{GFP}] = 0.4 \text{ mg/mL, } [\text{urea}] = 8.1 \text{ M})$$

The calculation for urea dilution series:

$$n_{\text{urea}} = [\text{urea}]_{\text{Well}} \times 200 \mu\text{L} = 9 \text{ M} \times V_{9\text{M urea}} + 8.1 \text{ M} \times 10 \mu\text{L}$$

$$V_{9\text{M urea}} = ([\text{urea}]_{\text{Well}} \times 200 \mu\text{L} - 8.1 \text{ M} \times 10 \mu\text{L}) / 9 \text{ M}$$

$$V_{\text{TNG Buffer}} = 200 \mu\text{L} - 10 \mu\text{L} - V_{9\text{M urea}}$$

		Column											
Row		1	2	3	4	5	6	7	8	9	10	11	12
A	[urea]	6.00 M	5.00 M	4.75 M	4.50 M	4.25 M	4.00 M	3.75 M	3.50 M	3.25 M	3.00 M	2.75 M	2.50 M
→	$V_{9\text{ M urea}}$	μL	μL	μL	μL	μL	μL	μL	μL	μL	μL	μL	μL
→	$V_{\text{TNG buffer}}$	μL	μL	μL	μL	μL	μL	μL	μL	μL	μL	μL	μL
	$V_{\text{den GFP}}$	10 μL	10 μL	10 μL	10 μL	10 μL	10 μL	10 μL	10 μL	10 μL	10 μL	10 μL	10 μL
	V_{Total}	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL

		Column											
Row		1	2	3	4	5	6	7*	8*	9**	10**	11**	12**
B	[urea]	2.25 M	2.00 M	1.75 M	1.50 M	1.25 M	1.00 M	0.00 M	0.00 M	6.00 M	6.00 M	0.00 M	0.00 M
→	$V_{9\text{ M urea}}$	μL	μL	μL	μL	μL	μL			μL	μL	0 μL	0 μL
→	$V_{\text{TNG buffer}}$	μL	μL	μL	μL	μL	μL	190 μL	190 μL	μL	μL	200 μL	200 μL
	$V_{\text{den GFP}}$	10 μL	10 μL	10 μL	10 μL	10 μL	10 μL						
	$V_{\text{native GFP}}$							10 μL	10 μL				
	V_{Total}	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL

*Note: Wells B7 & B8 are 'full-fluorescence' control samples, *which are prepared with diluted GFP (0.4 mg/mL) that has not been denatured.*

**Note: Wells B9-B12 contain 6 M urea and TNG buffer blanks (two each).

Lab Period Procedures.

During lab you and your lab partner will (i) prepare a GFP sample that has been denatured with urea and elevated temperature, (ii) introduce aliquots of denatured GFP into wells that contain different concentrations of urea, (iii) prepare full-fluorescence control samples and spectrometer blanks, (iv) measure the GFP fluorescence to determine the fraction *refolded*, and (v) calculate K (and ΔG°) as a function of the urea concentration.

1. Make Denatured GFP in a 1.5 mL Tube *

- Dilute an aliquot of your GFP stock solution 10-fold with 9 M urea, so that you have enough diluted/denatured GFP solution to place 10 μL in each well of the urea concentration series (wells A1-A12 and B1-B6).
- Close the lid of the 1.5 mL tube.
- Label it.
- Put it in the 90°C hot block for 10 minutes.
- After 10 minutes, remove the tube from the hot block and cool to room temp.

2. The Urea Concentration Series. (Mixing at Each Step is Critical!!!)

- Use the 20-200 μL pipettor to dispense the pre-calculated volumes of 9 M urea into wells A1-A12 and B1-B6. Use a different pipet tip for each well.
- Use the 2-20 μL pipettor to dispense 10 μL of denatured GFP solution into each wells A1-A12 and B1-B6. Gently pipet in and out several times to completely dispense the GFP. Use a different pipet tip for each well.
- Use the 20-200 μL pipettor to dispense the pre-calculated TNG buffer into wells A1-A12 and B1-B6. Use a different pipet tip for each well, and mix the TNG with the urea by gently pipetting in and out several times.
- At this point, the total volume in each well (A1-A12 and B1-B6) should be 200 μL .
- With the 20-200 μL set to 150 μL and a fresh (unused) tip for each well, use a gentle in-out pipetting action to mix the contents of the well (GFP, urea, TNG).
- Prepare the spectroscopic blanks (no GFP): 6 M urea (B9 & B10) and TNG buffer (B11 & B12). The blanks, like all samples, are 200 μL .

3. Undenatured GFP Standards.

- Wells B7 & B8 are reserved for undenatured (fully-fluorescent) standards.
- Prepare a 10-fold dilution of GFP, as you did in making denatured GFP, but use TNG to dilute the GFP instead of 9 M urea. (For example, 5 μL GFP stock plus 45 μL TNG.)
- DO NOT HEAT DENATURE.
- Place 10 μL of this 10-fold diluted GFP solution into B7 and B8.
- Add 190 μL of TNG; mix well with gentle pipetting action.

* Hint: Some liquid is always lost with pipetting. Make 10 – 20% more than the minimum needed to dispense into your wells.

4. Incubation Time and Plate Reading.

- By diluting the urea-denatured GFP into smaller concentrations of urea, the GFP will partly refold. But when has it reached equilibrium? As soon as you are finished with the dilution series, read your plate in the plate reader.
- Measure GFP absorbance in the plate reader using the method GFP_Abs.
- Measure GFP fluorescence in the plate reader using the method GFP_Fluor.
- After one hour measure your samples again. Why? Changes (or no changes) in the fluorescence intensities will help you decide whether or not the system has reached equilibrium.
- If the fluorescence changes between the 1st & 2nd readings, what do you conclude? What should you do?

5. 'Extra-Mile Experimental Activity' (Highly Encouraged but not required.)

- Instead of making one series of GFP fluorescence as a function of the urea concentration, make two: one by you, and one by your partner – this will help establish the repeatability of the observations made within your group.
- Carry out steps 1 through 3 in duplicate, dispensing the second urea series in rows C1-C12 and D1-D8.
- Incubate samples as above, but read the absorbance and fluorescence with GFP_Abs_plus and GFP_Fluor_plus. (These protocols read samples in all four rows.)

Data Analysis.

Use Excel to organize your data before you do any calculations, transposing your data into columns so you can see all the paired values of urea concentration and fluorescence in two columns.

Calculate the following:

- Background-corrected fluorescence versus urea concentration.
- The fraction of folded GFP versus [urea].

Normalize by dividing all the values by the largest value of fluorescence.

This largest value of fluorescence intensity might be either the average of the non-denatured standards or the 1 M urea sample. You will have to make the call and justify the choice in your lab report.

Note that this normalized reading, which ranges between 0 and 1.0, is assumed to be the fraction of protein in the folded state.

- The fraction of unfolded GFP versus urea concentration. Calculate the fraction of unfolded GFP as $1 - \text{the fraction of folded GFP}$.
- K versus urea concentration. (See the Appendix, page **Error! Bookmark not defined.**)
- ΔG° versus urea concentration. Use the value of $R = 0.0083145 \text{ kJ}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$ and $T = 298$ Kelvin. (See the Appendix, page **Error! Bookmark not defined.**)
- From the linear fit (trend line) of ΔG° versus [urea], determine ΔG° at [urea] = 0.

Lab Report

Write this report by yourself.

Abstract.

Summarize the experiment: a tiny bit of background, the goal of the experiment, the approach to achieving the goal, the results and conclusion.

Introduction.

Introduce GFP *briefly*, explain the relationship between its structure and the use of fluorescence as a reporter of protein folding, and give a single-sentence rationale for experimental design.

Methods: Summarize *the method*.

Results:

- a. **Percent GFP refolded.** Calculate the maximum percent of GFP that refolded after denaturation.
- b. **Analysis of Protein Stability.** Summarize the analysis of protein stability that led to the estimate of the Gibbs energy change at $[\text{urea}] = 0$. This should include plots of
 - Blank-corrected fluorescence *vs.* $[\text{urea}]$
 - The fraction of native protein *vs.* $[\text{urea}]$
 - The natural logarithm of the equilibrium constant *vs.* $[\text{urea}]$
 - The Gibbs energy change *vs.* $[\text{urea}]$
- c. **Gibbs Energy Change in the Absence of Denaturant ($[\text{urea}] = 0$).** Also, what is the equilibrium constant in the absence of denaturant? How many molecules of folded protein will be present for every molecule of unfolded protein?

Discussion:

You may wish to address some of the following in your discussion.

- What assumptions were made in the data analysis?
- The significance of the exponential relationship between K and ΔG° ?
- How does the absorbance of GFP change as a function of $[\text{urea}]$ compared to fluorescence? Is this a significant observation?
- What do you think about the estimate of ΔG° in the absence of urea? Do you consider it a 'large' energy?
 - How does it compare to the energy of a covalent bond?
 - How does it compare to the energy of an H-bond? (Cite literature sources)
 - How does it compare to the energy present in our surroundings – the thermal energy (Thermal energy is equal to RT , the product of the Ideal Gas Constant, R , in units of kJ/mol/degree and T in Kelvin, 298 at room temperature.)

Unit 5: Chemotactic Signal Transduction in *E. coli* A Rapid Response & Adaptation System

Signaling pathways and networks are ubiquitous in cell biology. One of the earliest sensory pathways studied, the bacterial chemotaxis pathway, has been the object of observations and investigations in the scientific literature for over a century, when Pfeffer (1884, 1888) observed the accumulation of bacteria and other microorganisms near bits of food in liquid media. Julius Adler initiated modern investigations of bacterial chemotaxis, when he established the detection of, and response toward, nutrients and repellents occurred with a genuine signal transduction pathway independent of the cellular uptake and metabolism of nutrients (reviewed in Adler, 1975). Through studies of chemotaxis in *Escherichia coli*, spanning five decades, Adler and other investigators have produced a detailed understanding of the signaling pathway, its genetics, biochemistry and structural biology, and how the molecule-level properties are related to the cellular physiology of the chemotactic response.

For the purpose of this lab, you need to become familiar with *E. coli* swimming behavior prior to the laboratory period. The pre-laboratory assignment for Unit 5 posted at the course web site will help to prepare you for week one, a quantitative analysis of *E. coli* swimming and its responses to chemo-attractants. *E. coli* swims in a ‘random walk’, which is characterized as alternating ‘runs’ and ‘tumbles’ (Berg & Brown, 1972). *E. coli* is propelled by several helical flagella that are attached at their base to a small rotary motor in the cell membrane – the world’s *second smallest* rotary motor. (*What is the smallest?*) When flagella rotate counterclockwise (CCW), they form a bundle that propels the cell forward in a ‘smooth-swimming run’. When the motors reverse direction and the flagella begin to rotate clockwise (CW), the bundle falls apart and the cell tumbles. Upon reforming the bundle, the cell swims in a different, random, direction. See Figure 18.

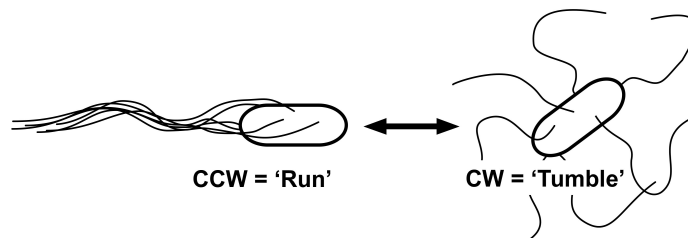


Figure 18. Swimming modes of *E. coli*. Each flagellum is attached to a motor that can rotate either clockwise (CW) or counterclockwise (CCW). When the motors rotate CCW the flagella form a bundle and the cells are propelled forward in a run. When the direction of rotation is CW, the bundle falls apart and the cell tumbles. Alternation between runs and tumbles generates the random walk swimming.

In the absence of chemical gradients, runs are ~ 1 s in duration and tumbles are ~ 0.1 s. In other words, the *frequency of tumbling* is ~ 1 per second (1 s^{-1}). The signaling pathway influences the tumble frequency – the frequency of the transition from CCW to CW motor rotation, and in that way extends the motion of cells up attractant gradients (Figure 19).

Automated tracking of individual cells allows direct observation of the biased walk (Berg & Brown, 1972), but simpler experiments can be used to assess the chemotactic responses toward attractants (Figure 19). When cells and attractant solutions are mixed together quickly, the effect on the frequency of tumbling can be measured. An abrupt increase in the attractant concentration (depicted at top) results in a rapid decrease in the tumble frequency (bottom). The cells become smooth swimming, yet over time cells *adapt*, that is the tumble

frequency returns to the prestimulus value, despite the persistence of the larger attractant concentration. Conversely, a rapid decrease in the attractant concentration increases the tumble frequency, but soon after the cells adapt and the tumble frequency returns to the prestimulus level.

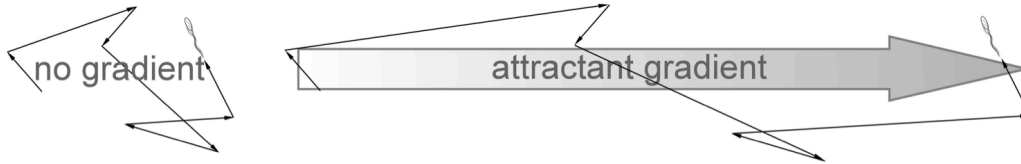


Figure 19. Random walk swimming in the absence of a gradient (*left*), and a biased random walk in an attractant gradient (*right*). Run lengths average ~ 1 s without a gradient. In gradients the runs lengthen (to ~ 3 s) when the movement is in the direction of increasing concentration, but not otherwise. Tumbles are ~ 0.1 s in both situations.

For small changes in concentration (a few μM), the time scale of adaptation is short (~ 5 s), but it becomes significantly longer (~ 1 to 2 min.) for large concentration changes, e.g. $0 \rightarrow 100 \mu\text{M}$. A second feature of large stimuli is response saturation: the tumble frequency essentially reaches zero and stays at a low level until adaptation nears completion.

The biochemical process that mediates adaptation is receptor methylation (Figure 20, *middle trace*). Receptors become more highly methylated in the presence of attractant. Conversely, the methylation level decreases when the attractant concentration is lowered. In this way, receptor methylation represents a short-term memory generated by the signaling system in which *E. coli* compares the current attractant concentration with that of the past 3 to 4 seconds.

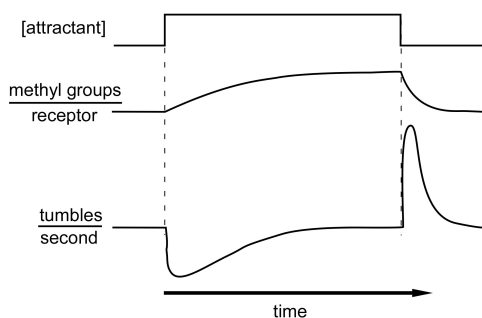


Figure 20. Swimming behavior with changes in the attractant concentration *versus* time. You'll be able to complete the first part of this figure in lab. When the attractant concentration (depicted at top) is increased quickly (by mixing cells with attractant), the tumble frequency decreases (bottom, cells become smooth swimming). Over time, cells *adapt*, that is the frequency of tumbling returns to the prestimulus value, even in the presence of the larger attractant concentration. Conversely, a rapid lowering of attractant increases tumbling. The process of adaptation is mediated by post-translational modification of receptors: Receptors become more highly methylated in the presence of attractant, and methylation level decreases as attractant concentrations are lowered.

The Components and Circuitry of the Signaling Pathway.

The identities of all the components in the pathway and their biochemical roles in the signaling pathway are known. The receptors continuously monitor the concentrations of attractants and repellents in the extracellular environment. The receptors form a complex with and thus regulate CheA, a histidine kinase. Generally, the receptors stimulate CheA activity, but the binding of attractants inhibits CheA activity. In addition as noted above receptor methylation increases their kinase stimulating properties, while demethylation lowers the stimulation of kinase activity by the receptors. The stimulation of CheA activity induces cell tumbling, through the action of one of the CheA substrates, CheY. Phosphorylated CheY (CheY-P) promotes CW motor rotation and tumbling when it binds to the flagellar motor. Tumbles are terminated by CheZ, a phosphatase that acts on CheY-P.

CheA also phosphorylates CheB. CheB-P actively demethylates receptors and is part of a negative feedback loop: CheA stimulation promotes the formation of CheB-P that, through receptor demethylation, *inactivates* CheA. Also, the negative feedback is essential for adaptation. The arrangement of the signaling pathway is illustrated in Figure 21. Clockwise motor rotation results in tumbling.

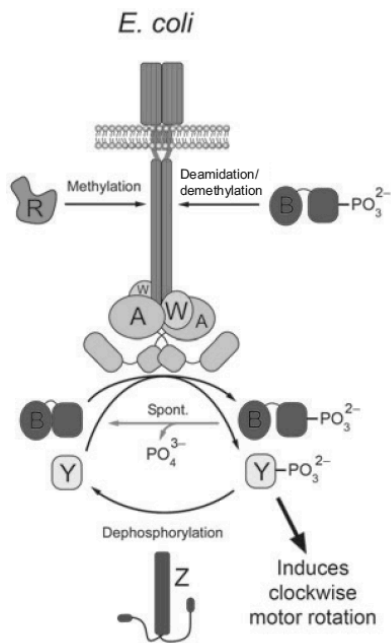


Figure 21. Components of the *E. coli* signaling pathway. Attractants and repellants bind to the extracellular part of the receptor (pink), at top. This binding reaction inhibits the kinase activity of CheA (A), which in turn results in lower levels of phosphorylation on the response regulator protein, CheY (Y) and the methyl-erasing enzyme, CheB (B). The phosphorylated form of CheB actively demethylates the receptor. In addition to inhibiting the kinase activity of receptor-associated CheA, attractant binding increases the rate of post-translational methylation by the methyltransferase, CheR (R). Receptor methylation increases the kinase-stimulating properties of the receptor.

CheA – Kinase, uses ATP to phosphorylate CheY and CheB.

CheB – Methyl-erasing enzyme, phosphorylated CheB is the active form that removes methyl groups from the receptor.

CheY – the Response Regulator protein; the phosphorylated form binds to the motor and elicits tumbling.

CheR – Methyltransferase, uses the methyl group on s-adenosyl-l-methionine to modify the receptor. The rate of receptor methylation increases when attractant binds.

CheW – an Adaptor Protein, required to couple CheA to the receptor through shared binding interactions.

CheZ – a Phosphatase, which catalyzes the dephosphorylation of CheY

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Lab 5.1: Observing Swimming Bacteria

In week 1 of this unit you will use the phase-contrast microscope to observe the swimming behavior of bacteria and their response to small molecule attractants.

Your objective is to determine which attractants are potent stimulators of a chemotactic response. It is of special interest to investigate the relationships between structure and function, that is, why is it that certain molecules elicit a response while other structurally similar molecules do not. Examine the three amino acids illustrated in Figure 22.

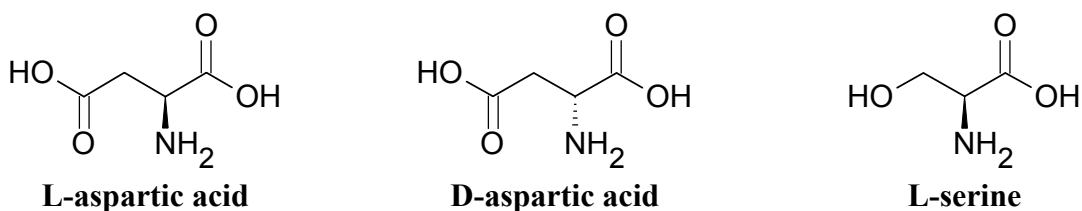


Figure 22. Three structurally similar amino acids

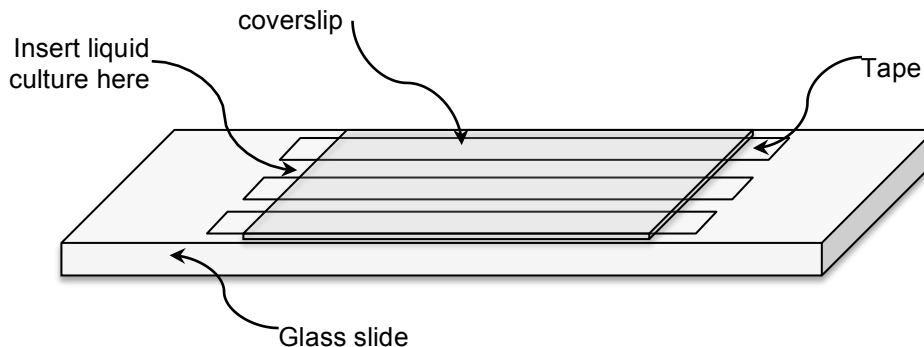
Materials

- Actively growing cultures of wild type *E. coli** that are able to respond to attractants and repellents
- Solutions of potential attractants and repellents.
- Slides, coverslips, and double-stick tape.

Methods

Basal Tumble Frequency

Use double-sided tape to make two or three linear chambers for bacteria on a glass microscope slide, as shown below. The tape creates separate chambers and raises the coverslip high enough off the glass slide to give the cells room to move, but is thin enough to allow the microscope lens to get within its focal distance of the cells.



Make sure the tape adheres completely to both slide and coverslip. You can see this best against the dark bench surface. Label your slide by writing on the glass itself.

Introduce ~10 μL cell culture (of the appropriate dilution) into one of the spaces. Find the cells first with low power, then with higher magnification.

* Wildtype refers to a cell or more specifically a property of the cell that exhibits full function. In this case, wildtype for chemotaxis refers to a cell with a fully functioning chemotaxis system.

Make sure you can follow a single cell and distinguish smooth swimming from tumbling, then start counting the tumble frequency, as described below.

Student One

Use a clock or watch that can count off the seconds. When your lab partner says 'Go' or 'Start', count off ten seconds. At ten seconds, you say 'Stop'.

Student Two

Observe the bacteria in the microscope. When you have found a cell to track, tell your lab partner 'Go' or 'Start' and then count the tumbles until your partner says stop. *The number of tumbles divided by ten is the tumble frequency.* (If you have trouble tracking cells for 10s, try 5s, or count the time necessary to get five tumbles, *etc.*)

Together

Experiment with the method until you and your partner find an approach that you can use well together, and then apply it consistently. Just remember, the tumble frequency is always computed as the number of tumbles observed divided by the time interval over which the observation took place.

Repeat ~ ten times to get an average and standard deviation, exchanging roles from time to time so you both get the experience tracking cells and counting tumble events. How many times will you have to do this? *Until you feel that you have become good at it.*

Keep track of your counts in your notebook while you are taking the data, then transfer it to Excel so you can calculate means and standard deviations.

Response to chemical attractants and repellants

Count the tumble frequency, before as above, under the following conditions.

(How do the cells respond to amino acids acid? How long is the response?)

1. Response to L-serine

- Add ~ 10 μL of cells to a clean subchamber. Determine the pre-stimulus tumble frequency. (Not necessary to repeat if you just completed this.)
- Introduce ~5 μL of 0.01M **L-serine** into the same opening where you injected cells. Note the time
- Move the microscope stage so that you are looking at the region where the two liquids have mixed.
- Find some cells, but wait until (and only until) the cells are no longer carried along by convection (or flow).
- Record the tumble frequency in the presence of serine for several 10 second intervals.
- Make repeated measurements until the tumble frequency returns to the prestimulus value.

2. Response to L-aspartate

- Introduce ~5 μL of 0.01M **L-aspartate** into the same port as above and repeat the procedure.

4. Additional experiments (given time and your interest)

- There are several additional solutions other than L-ser, L-asp provided for you to test. You may want to investigate the response of the bacteria to these. Even ‘qualitative’ observations (response, yes or no?) may prove informative
- Did you conduct the appropriate negative control experiments? For example, did you inject 5 μL of medium (without chemical stimulant of any kind), and measure the effect on tumble frequency?
- What is the potency of the compounds that you found to generate a response? Can you detect responses starting with 10- or 100-fold diluted stock solutions?
- How many times can you observe responses to the same cells with the same or different attractants. (Notice in **1.**, **2.** and **3.**, that L-asp and L-ser were added to the same sub-chamber, but D-asp was added to a different subchamber.)
- Other experiments that you think of (?)

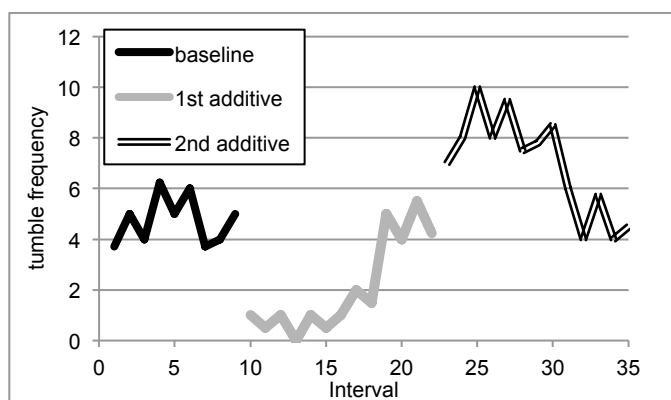
5. Examine responses over time

You may find it helpful to keep a running tally of tumbles per 10 second interval, so that you can make a graph of tumbling changes over time.

If you enter your data like this:

Interval #	Baseline	1 st add	2 nd add
1	-		
2	-		
3	-		
4	-		
5		-	
6		-	
7		-	
8		-	
9			-
10			-
11			-
12			-

It is easy to make a graph like this:



Notebook assignment

By the end of lab period, you should have recorded the following in your notebook:

- a description of your experiments
- sketches of the cell movement
- a record of your observations
- baseline tumble frequency
- your method of dealing with a time-sensitive phenomenon like change in tumble frequency
- graph of tumble frequency over time, showing responses and recovery (or not)
- a description of any additional experiments and findings (from part 4)
- a brief summary or conclusion saying what you think it means.

Before next week

Do the pre-lab assignment for Lab 5.2, starting on page 72.

Clean up

Glass slides should be rinsed, then thrown in the *glass trash*.

Cell cultures should be killed and poured down the drain.

Unused medium can go down the drain.

Glass tubes go in the *glass trash*.

Plastic tubes go in the regular trash.

Lab 5.2: Mutations and Aberrant Signaling

This week you will identify the mutations that lead to defects in chemotactic signaling by the effects of these mutations on the cell swimming phenotype. You will be able to identify the mutation with (fair) confidence for some mutants, for others you may only be able to narrow down the possibilities. To accomplish this, you will use skills from week 1: (i) recognizing runs and tumbles, (ii) quantifying the basal tumble frequency, and (iii) assessing responses and adaptation to chemoattractants, to study these mutants. In addition, you will use *swarm* plates, a simple measure of chemotactic ability, to assess the severity of the mutation on the signaling pathway.

Altogether there are six mutants. After completing the PreLab assignment for week 2, you should have a good understanding how to predict the impact of a mutation on signaling. Today you will put this ability to correlate phenotype with the underlying genetic defect (genotype) in the organism.

You and your partner are responsible for investigating three mutations, either mutants A, D & E (set 1) *or* mutants B, C & F (set 2). You know what set you have based on the swarm plate diameters that you measured this morning. You and your partner may elect to collaborate with the group across the table – they have the mutant set that you do not; each table has all six mutants. By your collective efforts you may develop a more efficient strategy for determining what these mutations are.

General information about the Mutants

All the mutants are deletion mutants, that is to say the mutant genomes have lost most, or all, of one or more genes in the chemotaxis pathway. For example a $\Delta cheA$ mutant lacks the gene for the kinase CheA. $\Delta cheR\Delta cheB$ lacks the genes for both the methyltransferase CheR and the methylesterase CheB. The deletion mutants you are studying can lead to one or more changes in behavior

- A change in the basal run-tumble frequency by, for example, overstimulating or understimulating the kinase CheA, or by disrupting the pathway altogether.
- Become blind to certain attractants, but not others.
- Have little effect on chemotactic ability.
- Lose some chemotactic ability.
- Lose all chemotactic ability.
- Lose the ability to adapt to stimuli.

Bottom Line: A logical analysis of the signaling pathway, based on an ability to predict the effect that the loss of a pathway component (deletion) will have on signaling, combined with measurements of (1) the basal tumble frequency, (2) the ability to respond to attractants, (3) the ability to adapt to attractants, and (4) chemotactic ability, will allow you to identify some or all of the mutations.

Pathway Components

Receptors – There are five chemoreceptors in *E. coli* pathway:

- Aspartate receptor (Tar) – a major receptor
- Serine receptor (Tsr) – a major receptor
- Ribose/galactose receptor (Trg) – a minor receptor
- The peptide receptor (Tap) – a minor receptor
- The aerotaxis receptor (Aer) – a minor receptor

Attractant binding (aspartate, serine, sugars and peptides) to the receptors inhibit the activity of CheA, which forms a complex with receptors and CheW.

CheA – The receptor-regulated kinase that phosphorylates CheY and CheB. Formation of a three-way complex between the receptors, CheW and CheA is required to regulate CheA activity. (*Stimulates activity by forming the receptor-W-A complex, inhibits activity when attractants bind to the receptor-W-A complex.*)

CheW – An adaptor protein that is required to form the complex between CheA and receptors together through shared binding interactions.

CheB – The receptor methylesterase. Phosphorylated CheB is the active form that removes methyl groups from the receptor.

CheR – A methyltransferase that uses the *s*-adenosyl-Lmethionine to methylate receptors. Receptor methylation stimulates kinase activity. The rate of receptor methylation increases when attractants bind.

CheY – The ‘response regulator’ protein. Phosphorylated CheY (CheY-P) binds to the motor and promotes CW rotation, which increases the tumble frequency.

CheZ – (Z) a phosphatase that inactivates CheY-P by catalyzing dephosphorylation.

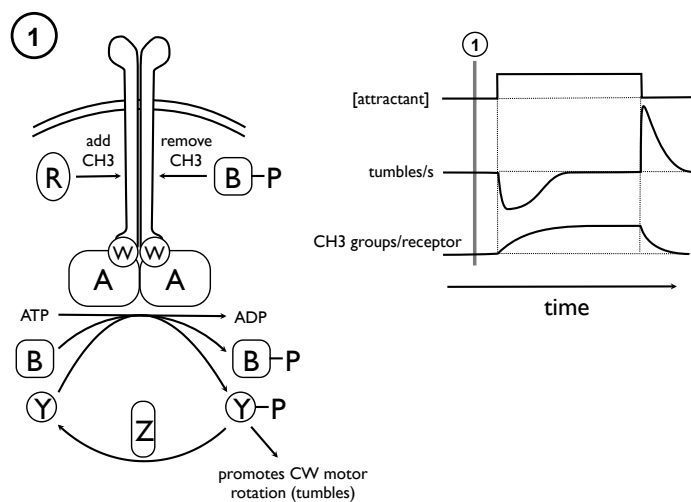
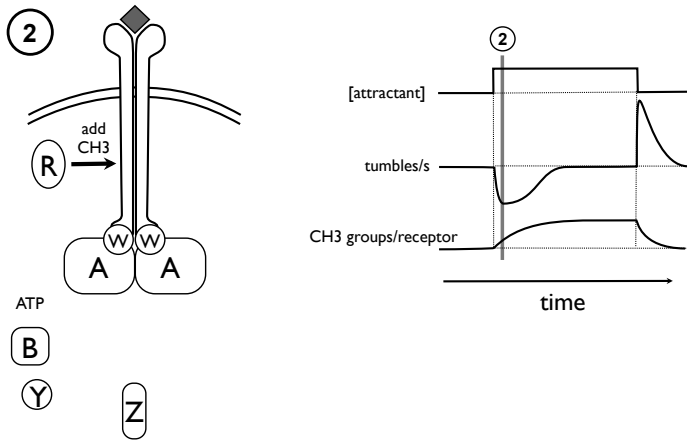


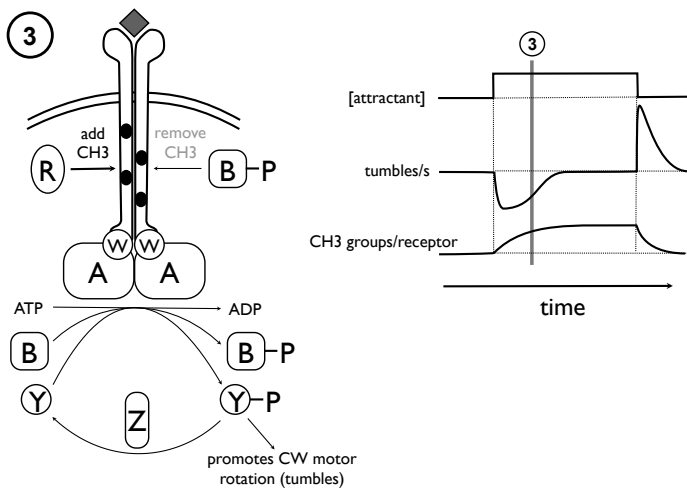
Figure 23

1. The adapted signaling state in the absence of attractant. In the *prestimulus* adapted state, the rate of methyl group (CH₃) addition by CheR (R), and methyl group removal by phosphorylated CheB (B-P), are equal. The basal kinase activity of CheA (A) produces a level of phosphorylated Y (Y-P) that results in an average basal tumble frequency of ~ 1 tumble/s.

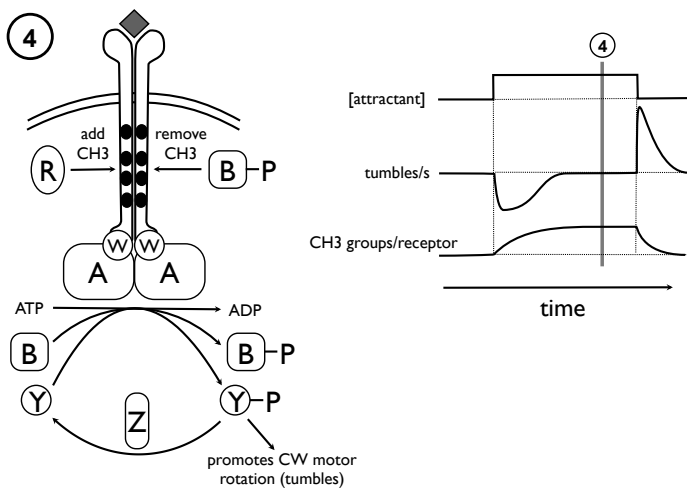
The CheZ (Z) catalyzes Y-P dephosphorylation. B-P decomposes spontaneously with a half-life less than 1 s.



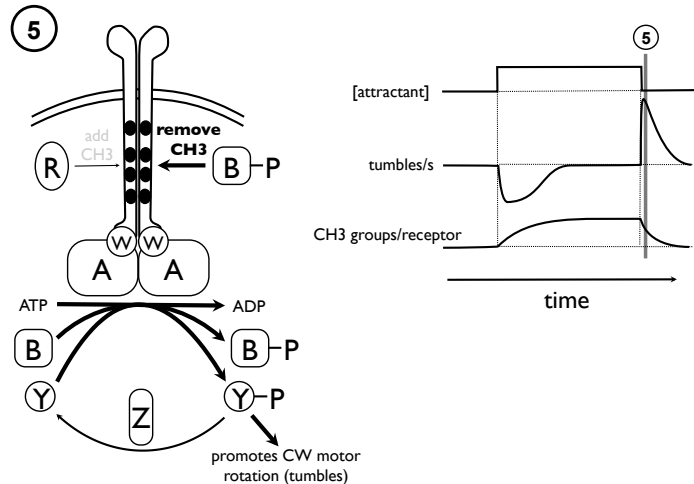
2. The attractant-stimulated (non-adapted) signaling state. The bacterium has just experienced an increase in the attractant concentration. This leads to an increase in the amount of receptor molecules with attractant bound. Attractant-binding inhibits the kinase activity of A (when the receptor has a low methylation level, indicated as *CH3 groups/receptor*). Attractant binding generates two effects simultaneously: it inhibits the activity of receptor-associated CheA and it increases the rate of methyl group addition by CheR. Thus, attractant binding lowers the B-P and Y-P levels, and as a result tumbling (via Y-P) and receptor demethylation (via B-P) are suppressed.



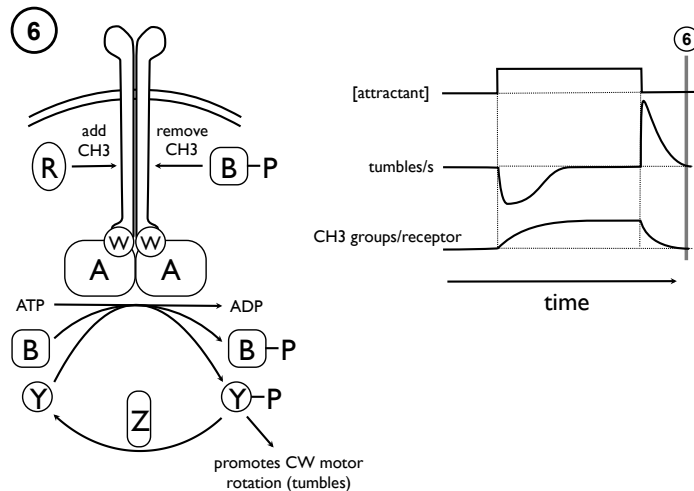
3. The partly adapted state. Over time, as cells adapt, the tumble frequency falls rises above the level just after stimulus (2), yet still below the prestimulus level (1). In state (3), the receptor has been partly methylated (methyl groups added are shown as black ovals). Partial methylation leads to a partial recovery of A activity and a rise in Y-P and B-P. Also, the receptor methylation rate slows and the receptor demethylation rate increases, but there is still net methyl group addition.



4. The adapted state in the presence of attractant. The rates of methyl group addition (by R) and removal (by B-P) are equal in this adapted state despite the higher concentration of receptor-bound attractant and the increased level of receptor methylation. These two factors influence kinase activity differently, such that the effect of the larger attractant concentration (to decrease kinase activity) and the higher methylation level (to increase kinase activity) counterbalance, and produces an activity that matches the prestimulus activity. Consequently the tumble frequencies (directly proportional to [Y-P]) are the same.



5. The stimulated state following attractant removal. Attractant removal leads to a reduction in the attractant bound to receptor. Kinase activity is stimulated (bold arrows) leading to elevated levels of B-P and Y-P. The elevated B-P level increases the rate of receptor demethylation (decreasing the receptor methylation level). Y-P leads to tumbling.



6. Return to baseline

Properties of swarm agar

Agar is a polysaccharide derived from red algae. When it sets up, it creates a matrix of long linear molecules. The plates on which we grew colonies of bacteria contained a concentration of agar that resulted in a relatively solid medium, and individual cells grew on top of it.

Swarm agar is made with a much lower concentration of agar, so it is looser, and more liquid. *E. coli* can actually swim inside it. They don't have as much freedom of movement as they do in liquid culture, because of the agar matrix of tunnels and cul-de-sacs. In order to make outward progress from the point of inoculation, they must be able not only to move in a direction, but also to change direction if they get stuck in a little blind alley.

Swarming is largely driven by nutrient gradients. The only nutrient source in swarm agar is tryptone, which is a trypsin digest of casein (milk protein). The agar therefore contains amino acids and small peptides, which the bacteria use not only to make their own proteins, but also as fuel for the Krebs cycle. The cells stay where you put them until they have consumed the amino acids in the agar, at which point they start to swarm outwards (if they can).

Pre-Lab 5.2: Assignment – Impact of Mutations on Signal Activity

This assignment will help you prepare for the week 2 experiments of unit 5, where you will examine signaling mutants. They build on the methods and concepts developed in week 1: measuring the tumble frequency and measuring the response and adaptation to the addition of attractant.

In the first week, by measuring the pre-stimulus and post-stimulus tumble frequencies, you characterized (i) the basal (and adapted) signaling state, before attractant addition, (ii) the attractant-stimulated state (just following the addition of attractant) and (iii) the return to the adapted state – this adapted state is achieved in the presence of attractant.

The background material below introduces the signaling pathway and the underlying biochemical events that (i) generate the responses that take place upon the addition and removal of attractant and (ii) maintain the adapted state at different attractant concentrations.

Study Assignment:

Study Figure 23, parts 1 to 6, to gain an understanding of the signaling system. If necessary, seek out information from the Internet.

Assignment to Turn In at the Beginning of Lab:

After studying the figures, answer the questions below. Print a clean copy of the table from our website to turn in.

1. In state (2), the kinase, CheA, is *inhibited* or *stimulated* (circle one).
2. The effect of attractant binding in state (2) is to *increase the rate of methylation*, *increase kinase activity*, *inhibit kinase activity* (circle all that apply).
3. If CheR, the methyltransferase, is overexpressed, the cell will adapt to the addition of attractant *more quickly*, *more slowly*, *in the same time period* as no overexpression (circle one).
4. The biochemical states of the signaling pathway in state (1) and state (4), (circle all that apply):
 - a. *are exactly the same*
 - b. *are different – which is observed as different tumble frequencies in states (1) & (4)*
 - c. *are different – which is observed as different levels of receptor methylation in states (1) & (4)*

5. Complete the table. Predict the effect of mutations on the basal tumble frequency and the ability to respond to stimuli. All of these mutations are gene deletions leading to a loss in the expression of the protein.

Check all the boxes that apply:

Gene(s) Deleted	Basal Tumble Frequency (no attractant present)			Responses to Attractants			
	like wildtype (run/tumble)	run	tumble	Like wildtype	Completely Unresponsive	Unresp. to asp only	Unresp. to serine only
<i>cheW</i>							
<i>cheA</i>							
<i>cheR</i>							
<i>cheB</i>							
<i>cheR</i> and <i>cheB</i>							
<i>cheY</i>							
<i>tar</i> (asp. rec.)							
<i>tsr</i> (serine rec.)							
all receptor genes							

Obtain a clean copy of this page from the course website, fill it out, and turn it in.

Reagents and Equipment Available

1. Actively growing cultures of bacteria in motility medium for observations of tumble frequency and attractant responses in the phase contrast microscope
 - wildtype strain (RP437)
 - four mutant strains
2. 4 soft agar swarm plates (tryptone broth: 1% tryptone, 0.5 % NaCl; agar: 0.3%) inoculated with RP437 or one of a variety of motility mutants. These were inoculated at 7 PM last night.
3. Motoc camera with attachment for taking pictures of swarm plates.
4. Phase contrast microscope.
5. Supplies for viewing cells in the phase contrast microscope. Microscope slides, coverslips, and double-stick tape.
6. Minimal medium for diluting cells, as necessary. Solutions of L-Asp and L-Ser.
7. Tubes, racks, pipettors, etc.

Approach

Develop a strategy to investigate each mutant. Start with simple observations of swimming behavior and progress to the more quantitative (and time-consuming) measurements. Outline this strategy in your notebook. Measurements methods in your strategy will include:

1. Basal tumble frequency – as you did last week.
2. Response (and adaptation) to attractants – as you did last week (note you may want to use smaller increases in the attractant concentration to reduce the adaptation time).
3. Swarm rate (speed). Measure the diameter of the swarm rate at three or more points in time to determine the swarm rate (mm/h). Swarm rate is determined from the slope of the swarm diameter plotted as a function of time.
4. Qualitative Observations of Swarm Colony Morphology. Swarm colony morphology gives you clues into nature and severity of the mutation. You can record images of the swarm colonies in the soft agar plate with a camera. Figure 24 illustrates some variations in swarm morphology.

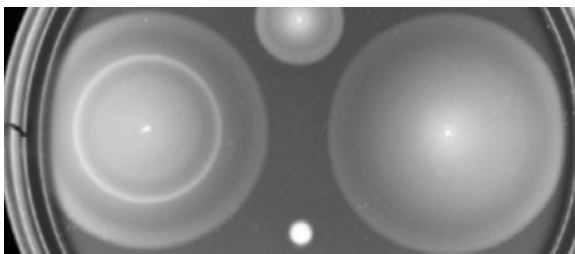


Figure 24. Swarm morphology of wildtype cells (at left) and mutants (top, right, bottom). This single plate was inoculated with the four strains at the same time. Note that some mutations are more severe than others.

Unit 5 Report Format (Individual report)

I. Title – be specific

II. Abstract – high points, not too many details

III. Introduction – Background, context, rationale

IV. Methods – General methods, be accurate, but be no more specific than necessary. Do not report *Results* in the *Methods* section. *Suggested Method Subsections*: Tumble frequency measurements. Responses to attractants. Swarm plates.

V. Results

Week 1 – Swimming behavior of wildtype cells.

- Basal tumble frequencies of wildtype cells (tumbles/sec). Report averages and std devs.
- Results of tumble frequency following L-aspartate, L-serine and D-aspartate stimuli. Did you detect responses? (Compare prestimulus tumble frequency to tumble frequency immediately following stimulation.) Did you detect adaptation? (Tumble frequency returning toward prestimulus value.)

Week 2 – Analysis of mutants

- Observations of swimming behavior (run, tumble, wildtype-like), including basal tumble frequency. (Report average and standard deviations of the basal frequencies. Are there statistically significant differences by t-test?)
- Quantitative (and qualitative) analysis of responses to attractants, e.g., amplitude of the response, or the time to reach half maximum response.
- Observations of swarm colony morphology, and swarm rates. Rate severity of mutation on chemotactic ability: e.g. no/small effect, moderate severity, severe.

VI. Discussion

- Week 1 - Are there differences in the responses to l-asp, l-ser and d-asp that allows you to conclude that one is more potent? Is there any support in the literature for your conclusion?
- Week 2 – Identify the mutants based on arguments constructed with your results. If you cannot unequivocally identify a mutant, then what choices have you narrowed it (them) down to? What additional experiment(s) would you do to identify the mutant.

Hint: Recall that there are 6 deletion mutants possible altogether:

$\Delta cheA$	<i>kinase defective</i>
$\Delta[tartaptsrtrg]$	<i>defective in all chemoreceptors</i>
$\Delta cheB$	<i>methylesterase defective</i>
$\Delta[cheRcheB]$	<i>defective in methylesterase (CheB) and methyltransferase (CheR)</i>
Δtsr	<i>defective in the serine receptor</i>
Δtar	<i>defective in the aspartate receptor</i>

- How is the analysis of mutants consistent with your knowledge of the signaling pathway?
- What experimental uncertainties, e.g. poor cell motility or limited optical resolution, influenced the analysis, and how might these be addressed/improved upon in the future?

VI. Acknowledgements

VII. References

General Techniques and Useful Information

How to use a micropipettor

Rotate the volume adjustor to the desired setting. Note the change in plunger length as the volume changes. Be sure to properly locate the decimal point when reading the volume setting. (Your instructors will demonstrate.)

You have three sizes of pipets in this lab: LTS20s, which can measure between 1 μL and 20 μL ; LTS200s, which can measure between 21 μL and 200 μL ; and LTS1000s, which can measure between 200 μL and 1000 μL (1 mL).

There are three numbers on the display of each pipettor. Look at the top of the pipet to see which one you are holding, then look at the display. The numbers represent volumes as shown below. The color change represents crossing the decimal place or changing units.

L 20		L 200		L 1000	
1	10 μL	1	100 μL	1	1000 μL = 1.0 mL
0	1 μL	0	10 μL	0	100 μL
0	0.1 μL = 100 nL	0	1 μL	0	1 μL

Firmly seat a proper-sized tip on the end of the micropipettor. The tips boxes are color-coded to match the label on the plunger.

When withdrawing or expelling fluid, always hold the tube firmly between your thumb and forefinger, keeping it nearly at eye level to observe the change in the fluid level in the pipet tip. Do not pipet with the tube in the test tube rack or have another person hold the tube while you are pipetting.

Hold the tube in your hand during each manipulation. Open the top of the tube by flipping up the tab with your thumb. During manipulations, grasp the tube body (rather than the lid), to provide greater control and to avoid contamination of the mouth of the tube.

For best control, grasp the micropipettor in your palm and wrap your fingers around the barrel; work the plunger (piston) with the thumb. Hold the micropipettor almost vertical when filling it.

Notice the friction “stops” on the two-position plunger with your thumb. Depressing to the first stop measures the desired volume. Depressing to the second stop introduces an additional volume of air to blow out any solution remaining in the tip.

To withdraw the sample from a reagent tube:

1. Depress the plunger to **first** stop and hold it in this position. Dip the tip into the solution to be pipetted, and draw fluid into the tip by gradually releasing the plunger. Be sure that the tip remains in the solution while you are releasing the plunger.
2. Slide the pipet tip out along the inside of the reagent tube to dislodge any excess droplets adhering to the outside of the tip. To avoid future pipetting errors, learn to recognize the approximate levels to which particular volumes fill the pipet tip.
3. If you notice air space at the end of the tip or air bubbles within the sample in the tip, carefully expel the sample back into its supply tube. Collect the sample at the bottom of the tube by pulsing it in a microcentrifuge.

To expel the sample into a reaction tube:

1. Touch the tip of the pipet to the inside wall near the bottom of the reaction tube into which the sample will be emptied. This creates a capillary effect that helps draw fluid out of the tip.
2. Slowly depress the plunger to the first stop to expel the sample. Depress to second stop to blow out the last bit of fluid. Hold the plunger in the depressed position.
3. Slide the pipet out of the reagent tube with the measurement plunger depressed, to avoid sucking any liquid back into the tip.

Use the ejector button (located at the back and different from the plunger) to remove the tip into a waste container.

Important pipettor don'ts:

- Never rotate the volume adjustor beyond the upper or lower range of the pipet.
- Never use the micropipettor without the tip in place; this could ruin the piston. Pipettors use disposable plastic tips. Every molecular biology lab circulates its own version of the story of the not-too-bright grad student who did not use a tip. Do not be this student!
- Never invert or lay the micropipettor down with a filled tip; fluid will run back into the piston.
- Never let the plunger snap back after withdrawing or expelling fluid; smooth motions are the key to success.
- Never immerse the barrel of the micropipettor in fluid. Only the disposable tip touches the liquid.
- Never reuse a tip. Tips are pretty cheap (about \$0.59 per rack). The risk of cross contaminating your solutions is too great to get tricky with tips. Just use a new one every time unless there is no possibility of cross contamination—like if you are pipetting the same solution into multiple empty tubes.

Fluorescence*

Fluorescence is a quantum mechanical phenomenon in which a molecule absorbs a photon of light, stores the energy briefly, and then releases the energy in the form of another photon. Not every molecule can do this efficiently, and thus there are a limited set of fluorescent molecules – fluorophores – that are available for microscopists. Fluorescent molecules can be attached covalently to molecules of interest or to antibodies that recognize molecules of interest, or noncovalently to molecules of interest. Fluorescent molecules can also be designed to interact with specific structures in the cell or to report on conditions within the cell.

For a molecule to be fluorescent, it must be able to capture a photon, to store energy and then to emit a photon. Such interaction with electromagnetic waves is best accomplished by molecules that have delocalized electrons, thus most fluorophores contain ring structures with pi electrons. A photon arriving at the fluorophore can cause electron rearrangement leaving the fluorophore's electrons in a higher energy (excited) state as the photon's energy is absorbed by the fluorophore. Quite often, the fluorophore has its molecular vibrations and bond rotation energized by this absorption event. These vibrations and rotations rapidly dissipate as heat into the surrounding medium, thus lowering the total energy of the fluorophore. After a while (typical times are 0.5 to 20 nsec; i.e. $0.5^{20} \times 10^{-9}$ sec), the fluorophore's electrons will rearrange to their original low energy (ground) state. The fluorophore often has vibrations and rotations energized during this process, and these quickly dissipate. The excess energy between the excited and ground electronic states is released in the form of a photon, the fluorescence emission photon. See Figure 25 for an energy level diagram of a fluorescence absorption and emission cycle.

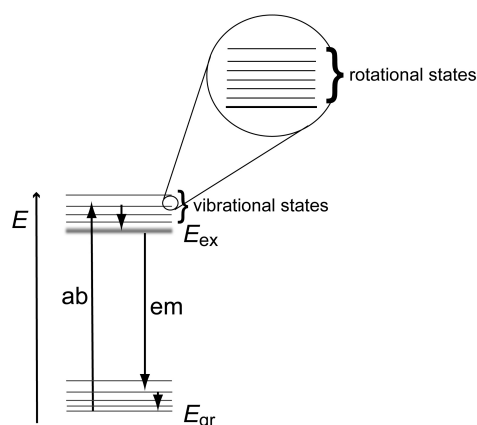


Figure 25. Absorption and emission of photons by a fluorophore. The absorption event, from the ground electronic energy state (E_{gr}) to the excited electronic energy state (E_{ex}), involves interaction of a photon with more energy than the energy gap between E_{gr} and E_{ex} . The excess energy appears as molecular vibrations and rotations in the fluorophore. These quickly dissipate into the surrounding medium. Later, the fluorophore transitions from its excited electronic energy state to a vibrational and rotational state above the electronic ground state, leading to an emitted photon of energy less than the energy gap between E_{gr} and E_{ex} .

Individual fluorescence absorption and emission events can occur many times over for a single fluorophore. When many fluorophores are present, a very large number of photons are absorbed and emitted. Each of these events can lead to a slightly different amount of

* Written by David Gross, Biochemistry Department, for Bioimaging, Spring 2008

(photon) energy absorbed or emitted by the fluorophore. Macroscopically, this appears as a continuous spectrum of photon energy absorbed and emitted (see Figure 26). The absorption spectrum is of higher energy (or shorter wavelength) than the emission spectrum, though there can be a small overlap in the two spectra. Therefore, fluorescence excitation is of shorter wavelength than fluorescence emission.

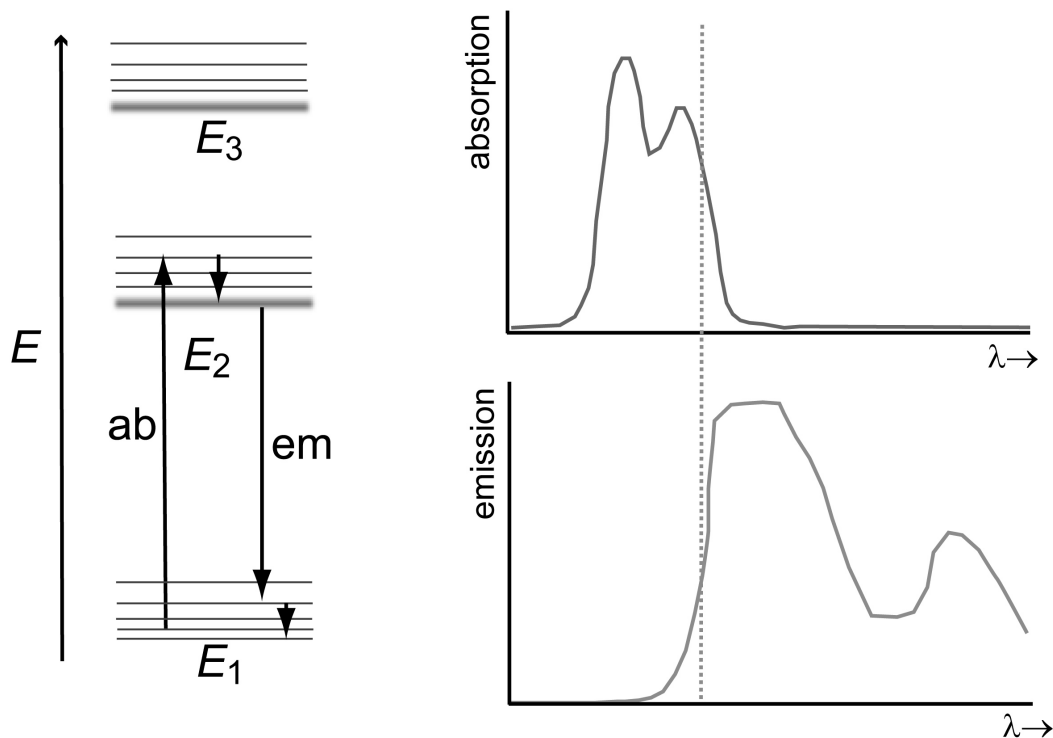


Figure 26. When large numbers of individual photon-fluorophore pairs interact, the specific vibrational and rotational excitations upon absorption or emission can differ for each fluorescence event. This leads to a variety of possible energies for photon absorption and emission. There are so many absorption and emission events that the individual events blend together into apparently continuous absorption and emission wavelength (or energy) spectra. The small overlap in the spectra happens because some particular absorptions are of rather low energy photons (leading to even lower energy emitted photons) and some particular emissions are of rather high energy photons.

Dilutions

It is often very important to know the precise concentration of some chemical you are using in your experiment. Various units of concentration are used in biology and chemistry:

Molarity (M) moles/Liter

mg/mL g/L

% w/vol g/100 mL (because 1 mL of water weighs 1 g)

% vol/vol mL/100 mL

Sometimes you can start from scratch, that is, weigh out the substance and dissolve it in the appropriate amount of solvent (usually water for biological applications).

What if I need 10 mL of 5M NaCl (MW = 58)? You can calculate how much NaCl to dissolve in 10 mL water this way (notice how all the units but g cancel out):

$$\frac{5 \text{ mol}}{\text{L}} \times \frac{58 \text{ g}}{\text{mol}} \times 10 \text{ mL} \times \frac{1 \text{ L}}{1000 \text{ mL}} = 2.9 \text{ g}$$

What if I need 1 mL of 5mM NaCl?

$$\frac{5 \text{ mmol}}{\text{L}} \times \frac{1 \text{ mol}}{1000 \text{ mmol}} \times \frac{58 \text{ g}}{\text{mol}} \times 1 \text{ mL} \times \frac{1 \text{ L}}{1000 \text{ mL}} = 0.00029 \text{ g}$$

Unfortunately, 0.00029 g is an almost imaginary amount of sodium chloride. Even if you made 10 times what you needed, you'd have to weigh out 0.0029 g, and most of our prep room balances only report three decimal places. You could make a liter of 10mM NaCl with 0.29 g, because sodium chloride is cheap to buy and legal to pour down the drain, but many reagents are much harder to come by and dispose of, so we need other ways of making solutions.

If you already had your 10 mL of 5 M NaCl, you could make your 1 mL of 5 mM NaCl by dilution, that is, by taking a small volume of your 5 M NaCl and diluting it in water. To calculate how, we use the lab preparer's best friend:

$$c_1 v_1 = c_2 v_2$$

c_1 = the concentration of the initial solution used to make the more dilute solution

v_1 = the initial small volume of the first solution used to make the dilute solution

c_2 = the concentration of the second, more dilute solution

v_2 = the final volume of the second solution after the dilution is carried out

So we need to figure out what volume (v_1) of the 5 M (c_1) solution to use to make 1 mL (v_2) of a 10 mM NaCl (c_2) solution.

$$v_1 = \frac{c_2 v_2}{c_1} = \frac{5 \text{ mM} \times 1 \text{ mL}}{5 \text{ M}} \times \frac{1 \text{ M}}{1000 \text{ mM}} = 0.001 \text{ mL}$$

Another way to look at this is to calculate the dilution factor, that is, the ratio between the initial and final concentrations. Diluting a 5M solution to a 5mM solution is a 1000-fold dilution:

$$\frac{c_2}{c_1} = \frac{5 \text{ mM}}{5 \text{ M}} \times \frac{1 \text{ M}}{1000 \text{ mM}} = \frac{1}{1000}$$

Therefore, you need 1 part stock solution to make 1000 parts of your final solution.

$$v_1 = \frac{c_2}{c_1} \times v_2 = \frac{1}{1000} \times 1\text{mL} = 0.001\text{mL}$$

Fortunately, I have a micropipettor that can deliver 0.001mL (=1 μ L). So I can make the dilute solution by mixing 0.001mL of my concentrated solution with 0.999 mL of water.

What if I don't really trust* my pipettor down in the single μ L range?

Worse, what if my concentrated sodium chloride stock solution is 1M, rather than 5M?

The initial volume would be 0.0002 mL (=0.2 μ L), and I *really* don't have a pipettor I trust to deliver that small a volume.

I can solve the problem of a v_i too small to deliver by using a *series* of dilutions, each transferring a volume I *can* accurately deliver, to achieve my intended volume and concentration. For this procedure, I use a modification of $c_1v_1=c_2v_2$:

$$c_1v_i = \left(\frac{c_1}{d}\right)(v_f + v_i)$$

c_i = the concentration of the initial solution used to make the next dilution in the series

v_i = the transfer volume

d = the dilution factor (*e.g.*, 2 for a 2-fold dilution series, where the concentration of each solution in the series is half that of the previous one)

$c_2 = \frac{c_1}{d}$ = the concentration of the second solution in the series

v_f = the desired final volume of each solution

$v_f + v_i$ = the initial volume of a diluted solution, until the transfer volume is removed to make the next dilution in the series

This procedure is illustrated in Figure 27.

* When I say I don't trust the pipettor, I mean that I worry about tiny errors in delivery representing a large percentage of the volume I'm transferring.

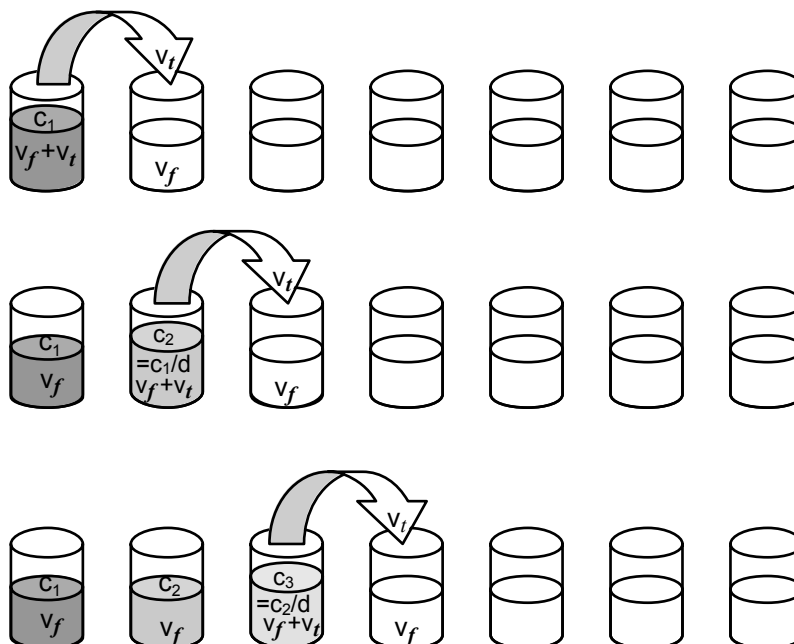


Figure 27. A serial dilution. A small volume (v_t) of solution is transferred to a vessel containing a volume (v_f) of solvent. After these are mixed, v_t of the second solution is transferred to the third vessel, also containing v_f of solvent. The concentration of each solution is $1/d$ the concentration of the previous one in the series.

So if I didn't think I could trust a $1\ \mu\text{L}$ transfer volume, I might try a 10-fold serial dilution ($d = 10$) (both because calculations with 10 are easy, and because I want the last dilution in my series to be one one-thousandth of the initial concentration). Suppose that the $1\ \text{mL}$ volume in the example above was more than enough for my experiment. An easy 10-fold dilution series could be done with a $0.1\ \text{mL}$ transfer volume, as follows:

Starting with the general equation:

$$c_1 v_t = \left(\frac{c_1}{d}\right)(v_f + v_t)$$

Substituting the initial concentration, the dilution factor, and the transfer volume:

$$1\text{M} \times 0.1\text{mL} = \left(\frac{1\text{M}}{10}\right)(v_f + 0.1\text{mL})$$

Solving for v_f :

$$\left(1\text{M} \times 0.1\text{mL} \times \frac{10}{1\text{M}}\right) - 0.1\text{mL} = v_f = 0.9\text{mL}$$

So by starting with $1\ \text{mL}$ ($v_f + v_t$) of my 1M (c_1) solution, and transferring $0.1\ \text{mL}$ (v_t) of it to $0.9\ \text{mL}$ (v_f) of solvent gives me $1\ \text{mL}$ ($v_f + v_t$) of 0.1M $\left(c_2 = \frac{c_1}{d} = \frac{c_1}{10}\right)$ solution.

Repeating this process will give me a series of solutions, each one-tenth the concentration of the one before it.

What if I don't know what concentration range of a particular substance is suitable for use in my experiment or in my measuring device? You need to test a wide range of concentrations. Serial dilution is the best way to achieve such a range. With a 2-fold dilution series of 10, you can take a solution down to less than a 1000^{th} of its original value:

$$\frac{1}{2^{10}} = \frac{1}{1024} = 0.000977$$

With a 10-step 10-fold dilution series, you can get down to 10^{-10} (less than a billionth!) of the original concentration.

What if the manufacturer gives me exactly 1 mg of the chemical? You can take advantage of the manufacturer's precision by dissolving the chemical, still in its original package to prevent loss of material, in a small, known volume of suitable solvent (which the package insert should identify for you), thereby making a stock solution of known concentration (*e.g.*, 1 mg/mL). From that, you can make whatever dilute concentrations you need.

The Q-test for the Outlying Data Point

Sometimes you collect data by repeated measurements that seem to cluster around a certain value, except for one odd data point. You want to be honest; you really did get that measurement, and you don't want to cheat. However, it does seem to be from another universe. For example, suppose these are the readings from your experiment:

25.1 21.2 27.5 22.7 23.8 26.3 40.6 22.9

That 40.6 not only looks out of place, but it has an effect on the mean and standard deviation of your data set:

	mean	standard deviation
including maximum value	26.1	6.3
excluding maximum value	24.0	2.1

It may also have an effect on statistical significance when you compare this distribution to another one by a t-test.

Is it a mistake? Is it noise? Did your recording device hiccup? What is the likelihood of getting an outlier this far from the next value when you are picking from a population with these characteristics?

Chemists (and other scientists) often test for outliers with the Q-test. This test calculates the ratio between the putative outlier's distance from its nearest neighbor and the range of values:

Notice that as the distance between the potential outlier and its nearest neighbor increases, so does Q.

$$Q = \frac{|\text{potential outlier} - \text{nearest neighbor}|}{\text{maximum value} - \text{minimum value}}$$

For our hypothetical data listed above,

$$Q = \frac{|\text{potential outlier} - \text{nearest neighbor}|}{\text{maximum value} - \text{minimum value}} = \frac{40.6 - 27.5}{40.6 - 21.2} = 0.675$$

The significance test consists of comparing your calculated Q to the theoretical Q that is expected to occur 5% of the time if you were sampling from a population with this mean and standard deviation. The null hypothesis is that all your data points come from the same pool. If your Q is as big as or bigger than the critical Q, then you can reject that null hypothesis with 95% confidence, and safely exclude the one odd data point.

Table 1, on page 86, lists critical values of Q at the 5% significance level. Notice that the smaller the number of data points, the larger Q must be for you to reject a data point. For a set of 8 data points, according to the table, Q would be greater than 0.526 just by luck of the draw 5% of the time or less. Since our experimental Q (0.675) is larger than 0.526, we may safely omit the 40.6 value from our data set, because such a large value is unlikely to occur by chance alone. We can conclude that that data point measures something other than what all the others are measuring. To maintain honesty, we can report something like this: "One very large (or small) value was rejected from the data set by the Q-test with 95% confidence."

You may only reject one data point from a data set by this method.

Excel Tricks to help you calculate Q

Sort

Select your data, then choose Sort from the Data menu. This makes visual inspection for outliers easier. *Note: if there are identifiers adjacent to the data values, be sure to include them in the sort, or you will lose the connection between the labels and the values!*

MAX

From the f_x menu, under statistical. This returns the maximum value from a list of values. The result goes in the cell where you typed the equation. You can specify the data array either by typing the range of cells where your data are found, or by clicking and dragging over them.

=MAX(data array)

MIN

This function works just like MAX, but tells you the minimum value in a series.

=MIN(data array)

COUNT

This tells you how many numerical values there are in an array. (If you use COUNTA, it will return the number of non-empty cells, rather than the number of data points.)

=COUNT(data array).

LARGE

This function tells you the kth largest value in a series. Set $k = 2$ to find the second largest value. Set $k = n-1$ to find the next-to-last value in a set of n data points.

=LARGE(data array, k)

SMALL

This function tells you the kth smallest value in a series. Set $k = 2$ to find the second smallest value.

=SMALL(data array, k)

Table 1. Critical values of Q at the 95% confidence ($\alpha = 0.05$) level, for data sets up to $n = 30$

number of data points[*]	Q_{0.05}
3	0.970
4	0.829
5	0.710
6	0.625
7	0.568
8	0.526
9	0.493
10	0.466
11	0.444
12	0.425
13	0.410
14	0.396
15	0.384
16	0.374
17	0.365
18	0.356
19	0.349
20	0.342
21	0.337
22	0.331
23	0.326
24	0.321
25	0.317
26	0.312
27	0.308
28	0.305
29	0.301
30	0.298

* Table from David B. Rorabacher, 1991. Statistical treatment for rejection of deviant values: critical values of Dixon's "Q" parameter and related subrange ratios at the 95% confidence level. *Anal. Chem.*, 63 (2): 139–146

<http://pubs.acs.org/doi/abs/10.1021/ac00002a010>, accessed 9/10/09.

Comparing Means: The t-Test

The Mean (Average).

This is probably the most common measure of central tendency. It is calculated by dividing the sum of all the data values by the number of such values:

$$\text{mean} = \bar{x} = \frac{x_1 + x_2 + x_3 \cdots x_n}{n}$$

Excel will calculate the mean for you with the following formula:

$$\text{=average}(data\ array).$$

data array: You type in (or click and drag over) the address of the data you want averaged.

Function wizard: You can also use the function wizard (f_x) to calculate the average: pick a cell to contain the average, click on f_x , choose AVERAGE (you may have to hunt for it in the statistical menu), highlight the values to average, and click OK. The cell will contain the formula given above.

The Standard Deviation

The standard deviation is one of the most commonly used and easiest to understand measures of spread. It also has some nice properties that will be described below.

The standard deviation is something like the average of all the individual deviations from the mean. This is a tedious calculation to do, so we usually ask a computer to do it for us (although generations of students before you managed with nothing more than calculators, and slide rules before that).

The calculation is done as follows: each datum is subtracted from the mean of all data (these are the individual deviations). About half of these deviations will be negative, and half positive, and if you add them together, they cancel each other out. To correct this, the deviations are squared so they will all be positive. These squares are added together, and their sum is divided by the number of data (actually, one less than the number of data – sorry) to get the “average”. Finally, the square root of this average is taken to correct for the squaring done earlier.

$$sd = \sqrt{\frac{\sum (\bar{x} - x_i)^2}{n - 1}}$$

Where

sd is the standard deviation,

n is the number of data,

x_i is each individual measurement,

\bar{x} is the mean of all measurements, and

Σ means the sum of

Excel will calculate the standard deviation for you with the following formula:

$$\text{=stdev}(data\ array).$$

One nice feature of the standard deviation alluded to above is that it is measured in the same units as the mean, so it is meaningful to add it to or subtract it from the mean.

A second nice feature is that when the data are distributed symmetrically around the mean (that is, when they fall into the famous bell curve of song and legend), between one standard deviation above the mean and one below are found 68% of the data, and between two deviations above and two deviations below the mean are found 95% of the data. This property is illustrated in Figure 28.

The bigger the standard deviation, the wider the spread of data around the mean, as illustrated in Figure 29.

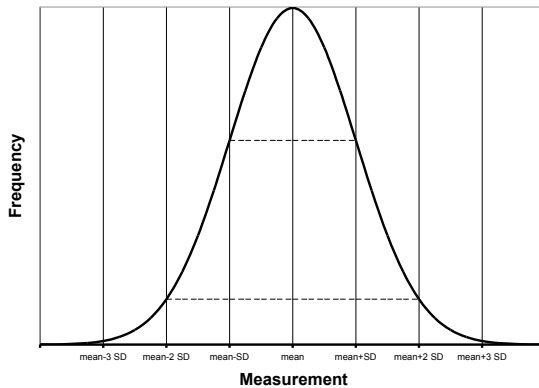


Figure 28. A “bell curve”, showing the symmetrical distribution around the mean. Horizontal lines indicate 1 and 2 standard deviations from the mean.

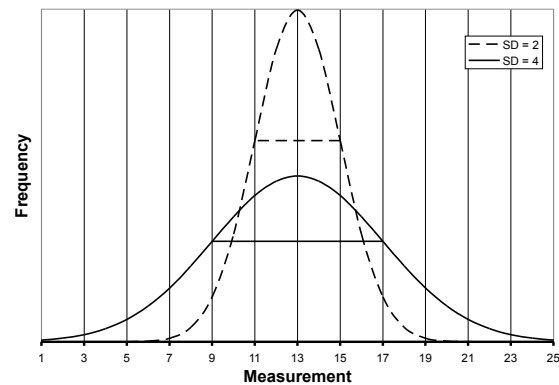


Figure 29. Two “bell curves”, with the same mean (13), but different standard deviations. Horizontal lines indicate 1 standard deviation from the mean.

Comparing The Means.

Usually, even when the means of two groups differ, there is some overlap between the two distributions. How different the two groups “really” are depends, therefore, not only on the difference between their means, but also on the extent of the overlap between their distributions.

Illustrating the relationship

The standard deviation can help to make a more complete comparison between two sets of data. (Here’s where another nice feature of the standard deviation comes into play: it is in the same units as the mean, so they can be added together.) Figure 30 shows why it is important to report not only the difference in the means between your two groups, but also some measure of the variation in each one. The means of groups 1 and 2 differ from each other by the same amount as do 3 and 4, yet the error bars, illustrating the size of the standard deviation, indicate a much greater degree of overlap between groups 3 and 4.

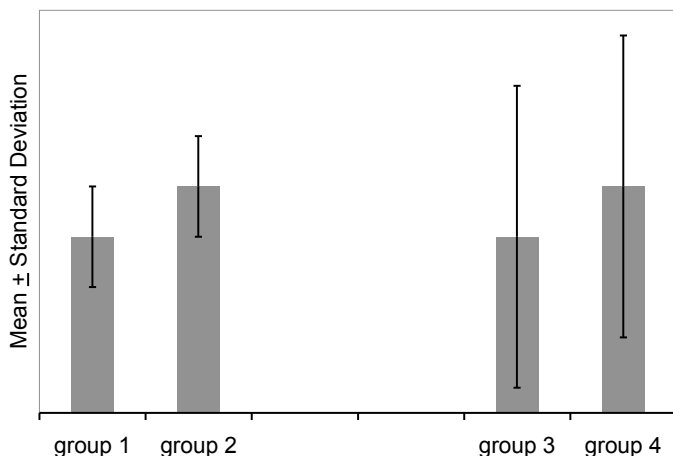


Figure 30. The effect of standard deviation on a comparison between means. Approximately two thirds of the values in each group lie within the one standard deviation error bars. There is much greater overlap between the measurements in groups 3 and 4 than between those in 1 and 2.

The easiest way to create a histogram comparing the means, with standard deviation as the error bars, requires making a little table in Excel that looks like this:

	mean	St dev
control		
experimental		

Choose appropriate descriptive labels for your two groups, as these will appear in your graph. Type the formula for the mean and standard deviation in the appropriate cells, i.e., =average(data array) and =stdev(data array), respectively.

Highlight the cells containing the labels and the averages, shown above with a double outline, and use the chart wizard to make a bar graph. Then highlight the bars and format the data series. Make *custom* Y-error bars, using the two cells in which you have calculated standard deviation. Note: the “standard deviation” choice inside the Y-error bar dialog box is not what you think it is! Don’t use it! You must calculate the standard deviation in a cell. (The dialog box has no idea which data went into making the means you are graphing, and cannot possibly calculate their standard deviation. Instead, it calculates the standard deviation of the values you are plotting – in this case the control mean and the experimental mean, and plots an error bar that length starting from the average of those values. I don’t know when this would be useful.)

Quantifying the relationship: calculating “t”.

The statistic “t” is a measure of the difference between two means, divided by the geometric mean of the standard errors of the population means (a sort of average of the standard deviations of the two populations). (The manner of calculating t depends on various characteristics of the experiment and the data, so this is why you must specify a “test type” before you ask Excel to calculate t for you.)

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{SD_1^2}{n_1} + \frac{SD_2^2}{n_2}}}$$

The value of t gets larger as the difference between the means gets larger; but this is counterbalanced by this measure of spread in the denominator. The greater the standard deviations, or the smaller the sample sizes (n), the bigger a difference in means is required to make t large. You can think of this as a ratio of signal (the difference between the means) to noise (the variation within the population).

Figure 31 illustrates the effect of standard deviation on the t statistic. The same difference between means can be significant or not, depending on the amount of variation in the populations being compared.

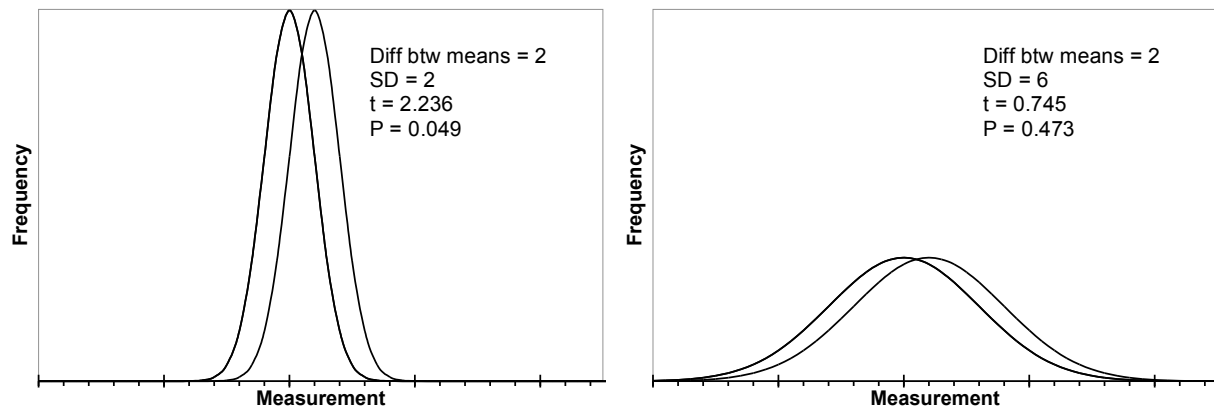


Figure 31. The effect of standard deviation on the t statistic. The means of both pairs of distributions differ by the same amount, yet the t statistic is 2.236 for the pair with the smaller standard deviations, and only 0.745 for those with the larger standard deviations. Notice the greater overlap between the curves on the right. (T values calculated for population size of 10 in each group.)

Interpreting the relationship.

Tests of significance always set up a straw man, the *null hypothesis*, which is that there is no difference between the groups, or no relationship between the variables. Then we find out how likely we are to get a result like our actual result if the null hypothesis were true. From this, we decide to accept or reject the null hypothesis.

If the category in question (*e.g.*, control or treatment) had no effect on the variable being measured (*e.g.*, number of cells in a suspension), you might as well be assigning categories at random. Imagine putting stickers on tubes of cells at random: some get labeled “group 1”, others get labeled “group 2”. Occasionally, such a process would result in all the tubes with dense populations being labeled “group 1”, and all those with very few cells, “group 2”. Such a result would give a rather large t.

How often would a t as large as the one you got in your actual experiment be expected to occur if the stickers were put on at random? The probability of getting a t as large as or larger than yours is called ‘p’, and fortunately, we can look up the p for a given t and number of measurements in any statistics book, on the internet, and inside of Excel.

If a t like yours is rather likely to occur by the chance procedure, you cannot rule chance out, and you must accept that there is no difference between the means of the two groups.

However, if such a t as yours would be exceedingly *uncommon* in a chance process, you may reject the idea that there is no difference between the means, and conclude that there is a *statistically significant difference* between the two groups. The statistic tells you how often a result like yours might occur by chance alone; *it cannot tell you the probability that chance actually caused the difference you observed.*

Conventionally, if a t as big as or bigger than yours can be expected to occur less often than 5% of the time ($p < 0.05$) when there is nothing other than chance acting, we reject the null hypothesis, and conclude that there is a difference between the groups.

By this criterion, the two distributions illustrated on the left in Figure 31 are statistically significantly different from each other, while those on the right are not.

Calculating T With Excel (the quick and dirty method)

If you have both sets of data to compare, use the built-in t-test in Excel (which you can find the statistics category of the function menu:

=ttest(*data array 1, data array 2, number of tails, test type*)

data array 1: the first set of data (enter the addresses, or click and drag over them)

data array 2: the second set

number of tails: 2 if you can't predict how one group will differ from the other, and think the means might vary in either direction, 1 otherwise. (Use 2 most of the time.)

test type: 1, 2, or 3


- 1 for paired data *i.e.*, two measurements on the same thing. In this test type, Excel takes the differences between the paired measurements, rather than between each one and the mean of the population. This works if you are comparing, for example, the absorbance at two different times *of the very same culture* (as opposed to the absorbance at the same time in two different cultures).
- 2 for unpaired data, but where both sets have the same standard deviation (don't use this one – it is unlikely to have two sets of data with identical standard deviations).
- 3 for unpaired data, with unequal standard deviations (two separate sets of measurements, such as number of cells in treated cultures vs. number of cells in control cultures). This is the most likely situation.

Excel will return the value of p that goes with this, but not tell you the actual t. In other words, Excel gives you just the punch line: what is the probability of grabbing two handfuls of data at random from a single population and winding up with two subsets as different from each other as your two data sets are from each other.

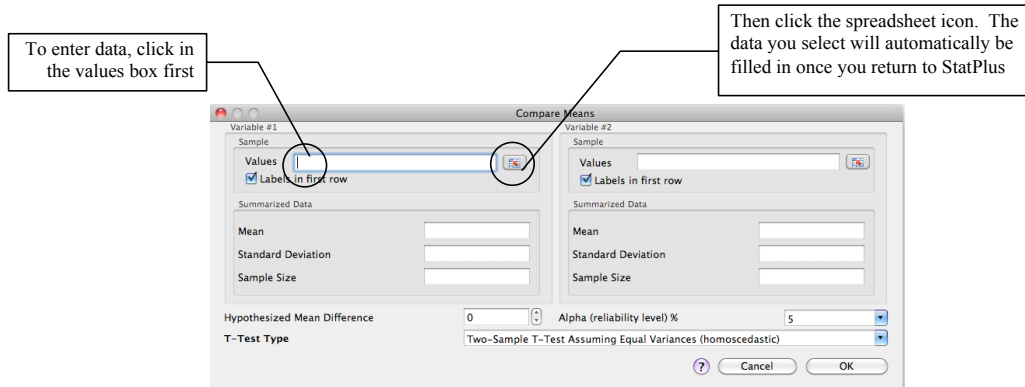
If you don't have both sets of data (if you are comparing your measurements to a standard reported in the literature only as mean plus or minus standard deviation), you have to do more work. Ask your instructor for guidance.


Calculating T With StatPlus.

If StatPlus is in the dock, open it by clicking on it.

If it isn't in the dock, open it from the applications folder. Doing so will put an icon for it  in the dock, and put its main menu bar (StatPlus Spreadsheet Statistics Data Charts Help) at the top of the screen.

Choose Basic Statistics and Tables from the Statistics menu, and Comparing Means (t-test) from that menu. That should pull up a dialog box that looks like the one below.



To enter your data, click in the Values box, then click on the little spreadsheet icon , which should take you to your spreadsheet if it is open, or to a new Excel file. From that new blank file, you can open your data file. Select the data for the first variable, return to StatPlus, and repeat for the second variable.

Check the settings:

If you included labels in the selection make sure StatPlus knows there are labels in the first row.

Leave the summarized data blank.

Set the hypothesized mean difference to zero, (because the null hypothesis is that the two collections of data were chosen at random from the same population) and

Set the alpha (the maximum probability at which you will reject the null hypothesis) to 5% (= 0.05) by convention.

For T-Test Type, choose the two-sample test assuming unequal variances (heteroscedastic). You are not comparing paired data, and your two sets of values almost certainly do not have the same variance (standard deviation squared).

When you click OK, it chugs for awhile, then opens a new file called StatPlusMacResults.xlt, with something like this on a tab called Comparing Means:

Comparing Means [t-test assuming unequal variances (heteroscedastic)]

<i>Descriptive Statistics</i>			
<i>VAR</i>	<i>Sample size</i>	<i>Mean</i>	<i>Variance</i>
	9	6.77778	1.94444
	8	9.375	1.125
<i>Summary</i>			
<i>Degrees Of Freedom</i>	15	<i>Hypothesized Mean Difference</i>	0.E+0
<i>Test Statistics</i>	4.34884	<i>Pooled Variance</i>	1.56204
<i>Two-tailed distribution</i>			
<i>p-level</i>	0.00057	<i>t Critical Value (5%)</i>	2.13145
<i>One-tailed distribution</i>			
<i>p-level</i>	0.00029	<i>t Critical Value (5%)</i>	1.75305
<i>G-criterion</i>			
<i>Test Statistics</i>	#N/A	<i>p-level</i>	#N/A
<i>Critical Value (5%)</i>	#N/A		
<i>Pagurova criterion</i>			
<i>Test Statistics</i>	4.34884	<i>p-level</i>	0.99938
<i>Ratio of variances parameter</i>	0.60573	<i>Critical Value (5%)</i>	0.0255

In this output table,

VAR: Probably short for *variable*. This should have the labels from your two columns of data. If not, type them in before you forget which comparison this is.

Sample Size: Sample size tells you the number of values in each group. Use it to do a reality check: do the sample sizes reported by StatPlus match the number of values in each of your categories? If not, you should repeat your data selection.

Mean: Is this the same as what you had Excel calculate for you on your spreadsheet? (Another reality check.)

Variance is standard deviation squared. It is another measure of the spread of data around the mean.

Degrees of freedom is the total number of data points minus 2 (it's the number of data points that are free to vary before the remaining ones are set).

The **Hypothesized Mean Difference** should be 0 (given as 0.E+0 in Excel-style scientific notation).

Test Statistics is your t value.

The Pooled Variance is an estimate of variance that assumes the true variance of the two samples is the same. You can ignore it.

The p-level (the probability of getting results this different from each other if the two sets of values were really drawn from the same pool) is given twice, once for a one-tailed distribution, and once for a two-tailed distribution.

In general, you should use the p value for the **two-tail** test, since this tests for both positive and negative differences between the means. The one-tail test gives you greater power to

detect a difference in only one direction, but increases the possibility of getting a false indication of significant difference.

The **t Critical value (5%)** is the t associated with that probability. If your t is greater than the critical value, your p-value is less than 0.05. Remember, the bigger the difference between the two groups, the higher the t and the lower the probability of getting a t that big by random factors alone.

It is OK for our purposes to ignore the **G** and **Pagurova criteria**.

This output table is static. If you change anything about your data, you must re-do the analysis.

Excel reports the values with up to nine decimal places. ***This in no way obligates you to do the same!*** Round the values to the number of significant digits of your least precise measurement. You can do this by formatting the cells in Excel, or correcting the number when you paste a result into Word. See page 101 for more discussion of significant digits.

Multiple comparisons – a caution

What if you have more than two sets of data to compare (e.g., two experimental treatments and one control)? It is tempting to do multiple t-tests to find out which means are different from each other. However, this should only be undertaken with great caution, because the more comparisons you make, the more likely you are to find high t-values by chance alone. Remember, the p-value is a calculation of the likelihood of getting a t as high as yours even if the values in both groups were actually drawn from the same population (*i.e.*, by chance), so if you did 100 comparisons, you can predict that chance alone would produce about 5 “significant” results. See Adjusting the critical α , on p. 97.

Analysis of Variance

One improvement on multiple comparisons is to begin with an analysis of variance (ANOVA) on all the data first, to find out whether the variation between groups differs from the variation within each group. ANOVA tests the null hypothesis that there are no differences between the groups, that in fact, all the data are pulled from what is effectively a single population.

ANOVA uses the F-statistic, which is the ratio between the mean square (a measure of variation) between groups and the mean square within groups. The null hypothesis is that the variation between groups is no bigger than the variation within groups, or that $F \leq 1$. As with the t-test, a large F statistic occurs rarely when the groups are the same, so the associated p-value is a measure of how likely it is to get an F as large as yours by chance alone.

Use the one-way ANOVA in StatPlus, assuming you have only one variable (e.g., “treatment”), which consists of different conditions (e.g., “control”, “treatment 1”, “treatment 2”, etc.). Set your data up in columns, with labels in the first row.

Select one-way ANOVA from the StatPlus Statistics menu, click in the “variables” box, click on the spreadsheet icon, find your datafile, and select your data including the labels. Let it run. It will produce a spreadsheet with a table like this:

Analysis of Variance (One-Way)

Summary

Groups	Sample size	Sum	Mean	Variance
control	7	20.	2.85714	1.80952
treatment 1	7	4.	0.57143	0.28571
treatment 2	7	9.	1.28571	0.57143

ANOVA

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	19.14286	2	9.57143	10.76786	0.00084	4.90007
Within Groups	16.	18	0.88889			
Total	35.14286	20				

In this output table:

Groups should accurately label your sets of data. If not, repeat your data selection.

Sample size should accurately reflect the number of data points in each group. If not, repeat, with correct selection of data from your spreadsheet.

Sum should be what you get if you add up all the values in one group. The **Mean** should be the average of the data in the group, and **Variance** is standard deviation squared.

SS stands for the sum of (deviation) squares. It should remind you of the standard deviation. Squaring the difference between the individual values and the mean or between the group means and the overall mean gets rid of the negative values and magnifies the impact of a large deviation. SS_{between} is the sum of the squares of the differences between each group mean and the total mean, times the number of scores in the group, and SS_{within} is the sum of the squares of the differences between every raw score and its sample mean.

$$SS_{\text{between}} = \sum (\bar{x}_i - \bar{x}_{\text{total}})^2 N$$

where

$$\begin{aligned} \bar{x}_i &= \text{the mean of group } i \\ \bar{x}_{\text{total}} &= \text{the mean of all raw scores combined} \\ N &= \text{the number of scores in group } i \end{aligned}$$

$$SS_{\text{within}} = \sum (x_i - \bar{x}_i)^2$$

where

$$\begin{aligned} x_i &= \text{raw score } i \\ \bar{x}_i &= \text{the mean of } i\text{'s group} \end{aligned}$$

Df, or degrees of freedom, is the number of values that are free to vary and still give you the same statistic. Within groups, this should be 1 less than the number of groups. Between groups, this should be the total number of samples minus the number of groups.

MS stands for mean square, or variance, and it is the ratio between SS and df. This yields something like an average, and corrects for the number of scores.

F is the ratio between MS_{between} and MS_{within} ($\frac{MS_{\text{between}}}{MS_{\text{within}}}$). The null hypothesis is that the variation between groups is no bigger than the variation within groups, or that $F \leq 1$. A larger F indicates greater variance between groups than within each group.

The **p-level** is the probability of getting an F as big as yours by chance alone, even if all the data come from the same population.

The **F crit** is the F associated with the p-value (probably 0.05) you specified when you set up the test. It is the smallest F that would let you conclude your groups differ statistically from each other.

If you get a statistically significant result (i.e., a p less than 0.05) with ANOVA, you can test the treatment groups in pairs by the t-test. Doing so, however, increases the chance of finding spurious “significance.” You can guard against that in various ways. The two simplest are to adjust the α (that is, the maximum p-value you will use to reject the null hypothesis of no difference between the groups), or caution your reader that you have performed multiple comparisons.

Adjusting the critical α

The probability of getting a large t rises with the number of t-tests you perform. Therefore you should tighten your decision criterion a little. The easiest way is to correct the critical α by dividing your usual α (0.05 for most purposes) by the number of pairwise comparisons you made. This protects you very well against claiming statistical significance where none exists, but not so well against claiming no difference when there is one.

For small numbers of comparisons, this is nearly identical to the next simplest correction, calculated as $1-(1-\alpha)^{1/n}$. As the number of comparisons grows, this correction stops the α from slipping into infinitesimal territory. This helps prevent you from being unable to detect any differences at all.

You can be very confident of any difference that passes either of these corrected α tests.

There are other ways to guard against spurious significance that are beyond the scope of this chapter, but which a statistician would be delighted to teach you.

Reporting Your Statistical Results.

Use the criteria above, that is, a significance level of $p \leq 0.05$, to decide whether the values in two sets of data can be called statistically different from one another.

Remember that the p value only tells you how likely it is to get a t as big as yours if the two samples really were picked from the same population, so resist the urge to say you’ve proved chance did or did not cause the difference you observe. Here are a few conventional expressions of such a result:

The mean [whatever was measured] of Group 1 (5 ± 2 , $n = 10$) is statistically lower than that of Group 2 (7 ± 2 , $n = 10$) by the t-test ($t = 2.236$, $p = 0.049$).

Group 2 had significantly higher [whatever was measured] by the t-test ($t = 2.236$, $p = 0.049$).

There was no statistically significant difference between Groups 3 and 4 by the t-test ($t = 0.745$, $p = 0.475$).

Logarithms

For $y > 0$, there is a number x such that $a^x = y$, and $x = \log_a y$.

The log of y is the power to which a must be raised to give y .

Common logarithms (what you get with the *log* key on a scientific calculator) use base 10.

When no base is indicated, assume the log is common.

If $10^x = y$, then $\log y = x$

$$10^2 = 100, \quad \text{so} \quad \log 100 = 2$$

$$10^1 = 10, \quad \text{so} \quad \log 10 = 1$$

$$10^0 = 1, \quad \text{so} \quad \log 1 = 0$$

$$10^{-1} = 0.1, \quad \text{so} \quad \log 0.1 = -1$$

$$10^{-2} = 0.01, \quad \text{so} \quad \log 0.01 = -2$$

Rules for manipulating exponents

Rules for manipulating logs

(logs are exponents, too):

$$a^m + a^n = a^{m+n}$$

$$\log(A \cdot B) = \log A + \log B$$

$$\frac{a^m}{a^n} = a^{m-n}$$

$$\log \frac{A}{B} = \log A - \log B$$

$$(a^m)^n = a^{m \cdot n}$$

$$\log A^n = n \cdot \log A$$

$$\sqrt[n]{a} = a^{1/n}$$

$$\log \sqrt[n]{A} = \frac{\log A}{n}$$

$$a^0 = 1$$

$$\log 1 = 0$$

What good are logs? Try to solve $4^x = 5$ without using logs!

Laboratory Report Format

Strive to write clear and concise, but accurate and thoughtful reports. A practical guide for organizing/writing lab reports can be found at the Union College Biology Department website (http://www.union.edu/academic_depts/biology/ResearchReports.php). You can find writing help nearby (<http://www.umass.edu/writingcenter/index.html>).

Do not plagiarize. Plagiarism is wrong, even if it is unintended. Simply put, do not copy other people's words, ideas or accomplishments (data) without permission and/or appropriate credit. The best way to avoid plagiarism is to write your reports entirely in your own words. Understanding just what plagiarism is also helps, and there are many web resources. Here is one: <http://www.indiana.edu/~wts/pamphlets/plagiarism.shtml> (A short and easy to understand pamphlet can be viewed or downloaded from this site.)

Write for a general reader. Write for someone who knows what you knew when you started to read the lab handout, but who isn't in the class and doesn't know the lab number, the instructor's name, or what the ID numbers on the samples mean. You can safely omit things this reader will assume (e.g. that you did the calculations, or measured the volumes), or find unimportant (e.g., when you did the calculations, or what brand of pipette you used).

Formatting

Take advantage of the power of Word. If you haven't learned how to do these things, take this as an opportunity to do so. You'll thank me later.

- Make your headings stick to the section they head (and your figures to their captions below). Use Format/Paragraph/Line and page breaks/keep with next. Even better, choose and use a style (Format/Styles) for all your subheadings and include the keep-with-next formatting as a part of the style.
- Make tables with Table/Insert, not with tabs. (Tables have word wrap.)
- Find and use the superscript command. You can put it on your formatting toolbar (x^2) or get it from Format/Font.
- Find and use the μ (Greek mu, in the symbol font, or available with alt-m on the Mac) to stand for the prefix micro-.
- Use the automatic captioning function: Insert/Caption or Insert/Reference/Caption (depending on the version of Word you have), then refer to all the things you captioned with Insert/Reference/Cross-reference. Everything will renumber itself correctly even if you insert or remove a table or figure.
- If you are going to use a numbered system for your references, use Insert/Reference/Footnote/Endnote.
- If you don't have a stapler, put a running header or footer with your name and the page number on every page, with View/Header and Footer. You can choose to omit it from the first page with Page (or Document) set-up/Layout. You may also use the staplers found in the lab.
- Put two spaces between sentences. It's amazing how much this practice can improve the readability of your paper!

Scientific terminology

Plurals

It is worth learning the correct singular and plural forms of scientific terms. A selection of the most common follows:

one bacterium;	two bacteria
one chiasma;	two chiasmata
one cilium;	two cilia
one criterion;	two criteria
one datum;	two data
one flagellum;	two flagella
one genus;	two genera
one helix;	two helices
one index;	two indices
one larva;	two larvae
one medium;	two media
one mitochondrion;	two mitochondria
one nucleus;	two nuclei
one phenomenon;	two phenomena
one species;	two species (not a typo! – singular and plural are the same)
one stigma;	two stigmata

Acronyms.

Many scientific acronyms are capitalized, but the phrases they stand for are not. For example, **DNA**, **OD**, and **GFP** stand for *deoxyribonucleic acid*, *optical density*, and *green fluorescent protein*, respectively. The names of the elements are capitalized when abbreviated, but lower case when written out: NaCl stands for sodium chloride.

Binomial nomenclature

Scientific names of organisms take this form:

Genus species.

The genus name is capitalized, and the species name is lower case. Both are italicized. For very common species, or after you have first written out the entire name, you may abbreviate the genus name with just its first initial. E.g., *Escherichia coli*, *Drosophila melanogaster*, *Homo sapiens*, *Mus musculus*, can be abbreviated *E. coli*, *D. melanogaster*, *H. sapiens*, *M. musculus*. (You do have to be careful, however, that the reader wouldn't think you were referring to the intestinal ameba *Entamoeba coli* instead of the bacterium *Escherichia coli*.) Getting this right raises your esteem in the eyes of a scientifically literate reader.

Specificity

Use the most specific term you can, even if it is repetitious and your English teacher told you not to repeat the same word too often. For example, "hydrochloric acid" is more specific than "acid", which is more specific than "chemical". Write "lactose" when you mean lactose in particular, and "sugar" when the reader couldn't possibly think you mean any other sugar in that sentence, or when you do mean any sugar.

Miscellaneous good writing

Not scientific exactly, but important nonetheless, clear writing represents clear thinking (and makes a favorable impression on the reader). Watch out for common errors; if you're not sure, look it up. The OWL at Purdue has a good selection of brief explanations of English grammar and mechanics: <http://owl.english.purdue.edu/owl/> Some of my favorites:

- *it's* is short for *it is* or *it has*. There is no apostrophe in *its* (meaning belonging to it) any more than there is in *his* or *hers*.
- The whole *comprises* its parts; the parts *compose* the whole.
- To decide whether to use *me* or *I*, think about what you would write if only one person were involved. For example, since you would never write "Me did an experiment," (or at least I *hope* you wouldn't), you shouldn't write "Me and my partner did an experiment." In addition, for the sake of politeness, put yourself second: "My partner and I did an experiment." Do the same mental analysis for she/her, he/him, they/them, we/us.
- If you count them, you have a *number* of them. If you weigh it, or measure its volume, you have an *amount*.

Reporting numbers

Decimals. Write the zero in front of a decimal, e.g., 0.05.

Significant figures. Don't report more significant figures than are found in the least precise of your measurements. Put your least precise measurement into scientific notation to find the appropriate precision. When you report cell concentration as 50139652 cells/mL (calculated from an OD of 1.013, say), you are suggesting that you can distinguish between 50139653 cells/mL and 50139652 cells/mL; it is more honest to give the concentration as 5.014×10^8 cells/mL.

Just because your calculator or Excel calculated a number to 9 decimal places doesn't justify reporting all those significant digits. In Excel, use the Format/Cells/Number menu to adjust the number of decimal places. Do it yourself if you are copying a number from your calculator display.

It is very hard to read a list of numbers embedded in a sentence. If you want to report more than two numerical values, put them in a table so that the reader can carefully compare them. In the written part of your paper that refers to the table, you need only point out the most important values, or the range, or how many times larger the maximum is than the second largest, or whatever else you think is important.

Choose your units in a way that will make it easiest for your reader to understand your measurements. In your introductory chemistry and biology labs, you probably used only a few of the most common metric prefixes, such as milli-, centi-, and kilo-. Various textbooks and lab manuals contain longer lists of prefixes, but not many contain a complete list. There is no point in memorizing all of them, but it is nice to be able to look them up when you encounter them as you read the scientific literature. The smaller prefixes, such as nano-, pico- and femto-, are becoming increasingly common as analytical chemistry and biotechnology develop more sensitive methods. The larger prefixes, such as mega-, giga-, and tera-, are becoming more common in computing as engineers find ways to pack more and more memory into tiny devices. Table 2 presents prefixes ranging from 10^{24} to 10^{-24} . To

help you visualize the effect of these prefixes, the column labeled “a sense of scale” gives some examples of the magnitudes represented.

Table 2. Metric Prefixes *

Prefix	Abbreviation (note upper and lower case)	Meaning	Synonym	A sense of scale (approximate)
yotta-	(Y-)	10^{24}	1 septillion	Mass of water in Pacific Ocean ~1 Yg Energy emitted by sun per second ~400 YJ Volume of earth ~1 YL Mass of earth ~6000 Yg
zetta-	(Z-)	10^{21}	1 sextillion	Radius of Milky Way galaxy ~1 Zm Volume of Pacific Ocean ~1 ZL Annual world energy production ~0.4 ZJ
exa-	(E-)	10^{18}	1 quintillion	Age of universe (12 billion yr) ~0.4 Es
peta-	(P-)	10^{15}	1 quadrillion	1 light-year ~9.5 Pm
tera-	(T-)	10^{12}	1 trillion	Sun-to-Jupiter distance ~0.8 Tm
giga-	(G-)	10^9	1 billion	Human life expectancy ~3 Gs 1 light-second ~0.3 Gs
mega-	(M-)	10^6	1 million	Two weeks ~1.2 Ms
kilo-	(k-)	10^3	1 thousand	
hecto-	(h-)	10^2	1 hundred	
deka-	(da-)	10	1 ten	
deci-	(d-)	10^{-1}	1 tenth	
centi-	(c-)	10^{-2}	1 hundredth	
milli-	(m-)	10^{-3}	1 thousandth	
micro-	(μ -)	10^{-6}	1 millionth	Diameter of human ovum ~1 μ m Volume of mosquito blood meal ~2 μ L Volume of wood frog egg ~3 μ L
nano-	(n-)	10^{-9}	1 billionth	Radius of chlorine atom ~0.1 nm
pico-	(p-)	10^{-12}	1 trillionth	Mass of bacterial cell ~1 pg
femto-	(f-)	10^{-15}	1 quadrillionth	Radius of proton ~1 fm Volume of <i>E. coli</i> cell ~1 fL Volume of red blood cell ~100 fL
atto-	(a-)	10^{-18}	1 quintillionth	Time for light to cross an atom ~1 as Bond energy of C=C ~1 aJ
zepto-	(z-)	10^{-21}	1 sextillionth	600 atoms or molecules ~1 zmol
yocto-	(y-)	10^{-24}	1 septillionth	Mass of proton or neutron ~1.7 yg

The parts of a lab report

(A sample lab report containing all of these sections begins on page 110.)

Title

Scientific research reports usually have very specific titles. “Lab 1” is uninformative (and dull), especially to the general reader mentioned on page 99. “Fluorescence” is better, but it sounds like the title of a chapter in a textbook on geology, biochemistry, or microscopy. (It’s hard to tell which, isn’t it?) A better title might actually refer to the particular investigation you are writing about. Below are some titles collected at random from various scientific journals.

* adapted from Greg Pearce, chemformula bbcmb@sigmaxi.org,
<http://www.unc.edu/~rowlett/units/prefixes.html>

When do domestic dogs, *Canis familiaris*, start to understand human pointing? The role of ontogeny in the development of interspecies communication. Dorey, Nicole R., Udell, Monique A.R., Wynne, Clive D.L., 2009. *Animal Behaviour*, 79 (1), 37-41.

Ear and eye in subterranean mole-rats, *Fukomys anselli* (Bathyergidae) and *Spalax ehrenbergi* (Spalacidae): progressive specialisation or regressive degeneration? Burda, Hynek. 2006. *Animal Biology*, 56 (4), 475-486

Structure of a Bacterial Dynamin-like Protein Lipid Tube Provides a Mechanism For Assembly and Membrane Curving. Low, Harry H., Sachse, Carsten, Amos, Linda A., Löwe, Jan, 2009. *Cell*, 139,(7), 1342-1352.

Inbreeding depression on beef cattle traits: Estimates, linearity of effects and heterogeneity among sire-families. Carolino, Nuno, Gama, Luis T., 2008. *Genetics of Selection and Evolution*. 40(5): 511–527.

Discovering genetic ancestry using spectral graph theory. Ann B. Lee, Diana Luca, Lambertus Klei, Bernie Devlin, Kathryn Roeder, 2010. *Genetic Epidemiology* 34 (1): 51-59.

Genomic profiling of breast tumours in relation to BRCA abnormalities and phenotypes. Olafur Andri Stefansson, Jon Gunnlaugur Jonasson, Oskar Thor Johannsson, Kristrun Olafsdottir, Margret Steinarsdottir, Sigridur Valgeirsdottir, and Jorunn Erla Eyfjord. 2009. *Breast Cancer Research* 11(4): R47.

Abstract

Most scientific journals require the author to provide a short (~250 word) summary of the experiment, which gives the reader just enough information to decide whether to read the entire paper. A good abstract gives the **motivation** behind the experiment (or some background for context), poses the **question** or problem the experiment was designed to address, summarizes the **method** or approach to answering that question or solving that problem, presents the **results**, and says what **conclusions** can be drawn from those results or what implications they have for further work.

Writing an abstract is an excellent way to force yourself to get to the heart of your project. If you have written more than about 5 sentences (one for each of the concepts listed above), you have probably over done it. See example on page 110.

Introduction

The introductions to scientific papers *almost never* open with this phrase: “the purpose of this experiment ...”. Rather, they start with some background information. Which particular part of the background material you open with depends on the emphasis you wish to give your paper. If you have done any library research, you should summarize it here, in order to provide context.

The introduction also *introduces the experiment*, that is, gives the rationale behind the work and poses the question the experiment is designed to answer. For example, this might be the particular question you sought to address by dredging through the data. Make it prominent – it is the *raison d’être* for your experiment. Here is where you describe your expectations, if you want to include them. See example on page 110.

Methods

Explain how you did your test. Here you can be more specific than you were in your introduction, answering such questions as:

- What concentration(s) of what substance(s) did you test? How was that range of concentrations achieved?
- What measurements did you make? At what wavelengths? Over what intervals? For how long? At what incubation temperature?
- How did you manipulate the data? Did you normalize it in some way (*e.g.*, report some value as % of maximum), or correct for population size, etc.? Did you compare values at a single time point or did you compare rates over time? Did you compare maximum values or average values?

Note: only cookbooks and lab manuals list the ingredients and equipment or give instructions. The methods section of a scientific paper is an account of what you did, written in the past tense, (because it was over before you sat down to write). Although you don't give directions, a reader should be able to repeat what you did from the information you give.

Many details that are important to you during the experiment are irrelevant or even confusing to the reader. Which sample was in which well of your plate, for example, is unimportant unless you think the position affects the reading (as a source of bias, for example, that you intend to explore in your discussion). The reader doesn't know what's special about "Tube 1A", but might be interested in the fact that you rejected one sample as an outlier.

It is important to be concise, and to convey only the most important information. Compare the following two pairs of paragraphs from methods sections:

To begin our experiment, we poisoned the cells with sodium azide. Sodium azide kills cells and prevents them from increasing in number before we can count them. After we killed them with sodium azide, we put some in a hemocytometer. Then we looked at the hemocytometer in the microscope. First we used the 10X lens in order to find the grid. Then we switched to the 40X lens for greater magnification. Then we counted the number of cells in one section of the hemocytometer. That section was 1 mm by 1 mm, and the height was 0.1 mm, so the volume was 0.1 mm³. We divided the number of cells we had counted by the volume of that section, and came up with a concentration. Meanwhile, we put some of the cells in well A1 of our 96 well plate and got the plate reader to tell us the absorbance, using the program called "bug_OD". We subtracted that absorbance from the absorbance of some medium we put in well A2. We divided the hemocytometer concentration by the OD of that sample, and we calculated a conversion factor of 1.6 x 10⁸ cells/mL/OD.

vs.

To calculate our conversion factor (1.6 x 10⁸ cells/mL/OD), we divided the cell concentration achieved with a hemocytometer by the OD of the same sample of cells killed with sodium azide.

To measure the swarm speed of the various strains, we came in the morning of lab to inoculate swarm plates. This was done by using a sterile stick, dipping it into a liquid culture of cells, and stabbing the center of a petri dish containing 0.3% agar. They were then labeled with our names, the strain number, and the time. We carefully placed the plates into an incubator at 30 degrees Celsius, keeping our plates separate from those of other groups so we could find them easily later in the day. During lab, four and a half hours later, we recorded the diameters of the swarms in millimeters with a blue metric ruler approximately every forty-five minutes to an hour.

vs. We inoculated a swarm plate (0.3% agar) by stabbing the center with a sterile stick dipped in liquid overnight culture, then incubated it at 30 °C. We measured the swarm diameter at approximately 5, 6, and 7 hours after inoculation.

See example methods section on page 111.

Results

The results section of a scientific paper consists of text describing the data you collected, and graphs, diagrams, photographs, or tables illustrating those data.

It is your job as the author to guide the reader through your results, showing what you saw or what measurements you made. Toward that end, write this section so that it presents the findings of the experiment *in narrative form*, supplemented when appropriate with tables, diagrams, graphs, illustrations, etc. The narrative must make reference to the figures and tables (referring to them as *Figure 1* or *Table 1*, etc.), pointing out salient features relevant to the hypothesis or rationale of the experiment. ***Never let the reader encounter an illustration without reading about it first.***

Do not include raw data.

As with your methods section, be concise. Compare the following two descriptions of the same figure. The shorter one actually gives more information than the longer one.

The values achieved in the experiment are given in Figure 1. These values are the OD measurements. They were collected over a 720-minute period. We plotted these values against time. The plate reader gave the time in minutes, but we converted that to hours because it is easier to understand “12 hours” than “720 minutes”.

vs. The rapid rise in cell concentration between 2 and 4 hours of a 12-hour incubation is shown in Figure 1.

Figures and Tables

Figures and tables are a crucial and integral part of the Results section. A **figure** is a graph, photograph, or diagram (i.e., a *picture*). A figure will ***always*** have a detailed, descriptive caption ***at the bottom***. The caption is a title, and does not begin: *Figure 1 shows that...*

A **table** is a list of numbers or words. By convention, tables have a detailed title ***at the top***. Tables and figures are numbered separately. Follow the convention for presentation of figures and tables, illustrated in Figure 32.

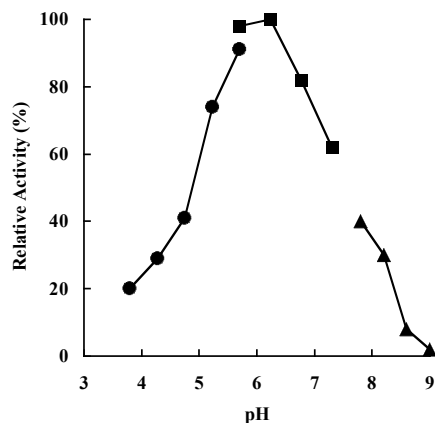


Fig. 2. Effect of pH on the amylase activity. The following buffers were used at 50 mM: sodium acetate buffer between pH 3.8 and 5.7 (circles), sodium-potassium phosphate buffer between pH 5.6 and 7.3 (squares), and glycine-NaOH buffer between pH 7.7 and 9.0 (triangles). All the pH values were adjusted at 70°C.

TABLE 1. Summary of purification of amylase S

Treatment	Total activity (U)	Protein (mg)	Sp act (U/mg)	Yield (%)	Purification (fold)
(NH ₄) ₂ SO ₄	608	434	1.4	100	1
DEAE	582	3.4	171	96	122
Superdex	160	0.14	1,143	26	816

Figure 32. The difference between a figure and a table in a scientific publication (from Chung, Y.C., *et al.*, 1995. *Applied and Environmental Microbiology* 61(4), p. 1502-1506). The figure's caption is below (like the plaque beneath a painting in a museum); the table's heading is above (like the page header in a book of mathematical tables).

This is what the authors had to say about their graph (in the text *before* the graph itself):

The enzyme exhibited a pH optimum at 5.5 to 6.0 with a rapid decline of the activity at the higher and lower pH ranges (Fig. 2).

After reading this description, we can look at the graph and see just how much higher the enzyme activity was at pH 6 than anywhere else along the pH scale. Then we can return to the text to read some more details about the enzyme and the buffers. (Note that they did not write out a boring list of all the activity values at every pH level, but directed our attention to the peak of enzyme activity. Apparently, they didn't think the fact that there was no detectable activity at pH 9 was important, so they didn't mention it.) All this description came before the figure.

Your figures and tables must contain all the information necessary to comprehend them, including descriptive captions and proper labeling.

- Make sure the columns and/or rows of your table are labeled, including units.
- Make sure that the independent variable (the one you set or chose) is on the horizontal axis of your graph, and the dependent variable (the one you found out by doing the experiment) is on the vertical axis. Before you plot anything with Excel, draw a qualitative graph of what you hope it will look like, and use this as a reality check.
- Label your axes. (In Excel, use Chart/Chart options/Titles.) Make sure the chosen units are sensible (fix the number of significant digits in the spreadsheet itself with Format/Cells/Number/decimal places; fix the divisions on the axis with Format/Axis/Scale); fix the font with Format/Chart area/Font. If you can't fix the values on your X axis, make sure you have specified the correct column or row as your X values, and that you have made a scatter plot, not a line graph.
- Show the legend (Chart/Chart options/Legend) on your graph only if there are two or more series of data on the graph, as illustrated in Figure 33. If necessary, you can fix the labels on your legend inside the Source Data dialog box or on the spreadsheet

itself. Alternatively, you can follow what Chung *et al.* do and explain the symbols in the caption (Figure 32).

- Give your illustration an explicit, descriptive caption. Think of this caption as a book title: just as book titles do not begin “This book is about ...”, a figure title should not begin “This figure shows ...”. Omit the giant titles that Excel encourages you to put above the “chart”. These are sometimes suitable in Power Point presentations, or on posters, but they almost never appear in scientific journals, except in a multi-part figure, when they label the individual illustrations grouped together into one figure. This last situation is illustrated in Figure 33, where 4 sets of data requiring different axes are illustrated in a single figure.

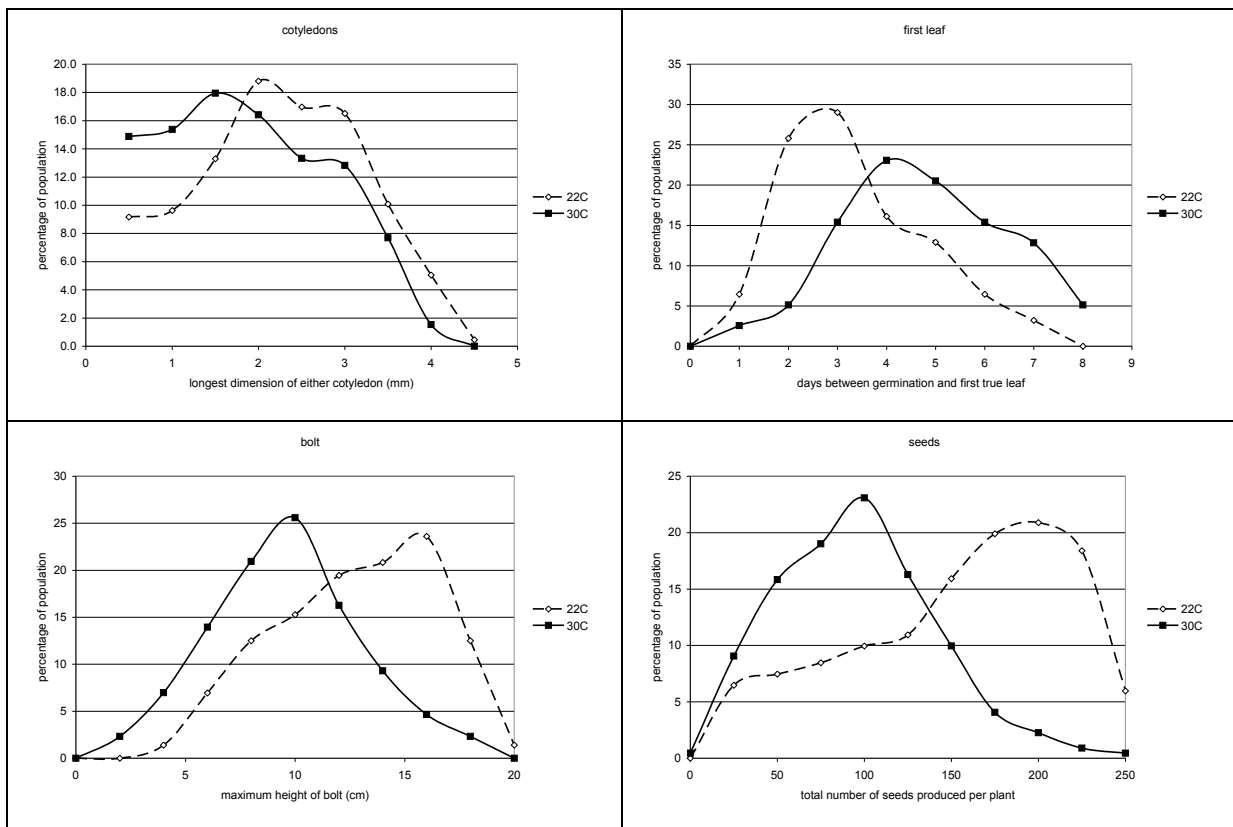


Figure 33. Comparison between wild type *Arabidopsis* plants grown at 22°C and at 30°C. Plants grown at 22°C produced bigger cotyledons, put out their first true leaves sooner, grew taller, and set more seed than those grown at 30°C.

One of the best ways to prepare a results section is to graph your data in different ways to find the arrangement that best shows the answer to your experimental question. (You may not even know the answer until you have looked at different graphical representations of your results.) Once you have decided on the best representation, then you must write out what you want the reader to see in those figures, *and put the written part before the graphs!* See example results sections on page 111 (with figures) and page 113 (with tables).

Discussion

Here's where you get to say *why* you think you got the results you did. If in your results section, you said under which condition the cells grew fastest, here you say why. Save all interpretation and explanation for this section.

Resist the temptation to say that you *proved* anything. Save that for your math or logic class. Your results may be *consistent with* a particular conclusion, or *indicate that* an inference is correct, etc., but they don't *prove* it.

If you think you should have gotten different results, explain why you are surprised by what you found. However, don't say anything about errors unless you can be specific. It is always true that you could have been more careful, so that's uninformative. If you think you made a *particular* mistake (e.g., omitted a particular reagent, left your culture at room temperature instead of on ice, doubled the volume of solution at a particular step, etc.) specify how that mistake would have skewed your results *in the particular direction* you observed.

If you think something should have been done differently (e.g., more replicates, different temperature, higher or lower concentration, etc.) say how this change would have improved your results or given you greater confidence in your conclusions. See page 112.

Note: there is no need to stretch the significance of your results to apply them to the quest for world peace or a cure for breast cancer.

References

You must acknowledge any articles, books, or websites that you used. There are many styles for doing so. In this course, we will use the style required by the *Journal of Cell Biology*, which can be found at their website: <http://jcb.rupress.org/misc/ifora.shtml>.

Give credit where it is due. Scientific papers give credit very precisely, that is, at the very place in the text where you use someone else's idea or picture, by giving the author's name and the date of publication in parentheses. Don't just list all sources at the end and expect the reader to figure out which was used as the source for what. See examples on page 110.

Did you draw the sugar molecules yourself? If not, you must give credit to whoever did for providing the illustrations. The library web site offers this for help on citing electronic sources: <http://www.bedfordstmartins.com/online/citex.html>. The URL alone is insufficient.

Cite the ideas, not the words. If you learn something from an article, summarize the information and restate it in your own words. Don't cut and paste, and don't try substituting a single synonym instead of actually putting the idea into your own words. Don't use terms you don't understand.

List your references in alphabetical order by the first author's last name at the end of your paper. List all the authors, include the title of the book or article, the journal or publisher (and place), and the date. If you used just part of a book, indicate the chapter or pages used. For web sites, give the not only the URL, but any other information you have about it. It is best if you have an author (if one is listed) and the name of the organization that sponsors the site, as well as the date you accessed the site.) Again, you want to give any information necessary for another researcher to find the facts you did. Remember—do not include references that you did not cite.

Examples:

Book: Author(s). Year. *Title*. Location: Publisher. Number of pages, or pages cited.

Hille, Bertil. 1992. *Ionic Channels of Excitable Membranes*. Second Edition. Sunderland, MA: Sinauer Associates, Inc. 607pp.

Article: Author(s). Year. Title of Article. Journal, volume number, pages.

Huxley, A.F. and R. Stämpfli. 1949. Evidence for salutatory conduction in peripheral myelinated nerve fibres. *J Physiol. (Lond.)* 108: 315-339.

Web page: Name of web page. Creator or publisher. Subject. Web address, date accessed.

The Animated Brain. Brainviews, Ltd. Saltatory conduction.

<http://www.brainviews.com/abFiles/AniSalt.htm>, accessed 8/14/07.

Acknowledgements

Did anyone help you with anything not published? Did someone help you learn a new technique, or share data with you, etc.? Give credit to those people, too.

Sample Lab Report

A comparison of maximum *E. coli* population density and tryptophan operon induction in minimal medium, and minimal medium supplemented with either tryptophan or 5-bromotryptophan.

Ann A. Student

Abstract

E. coli are most efficient when given a rich medium, in part because they repress production of unnecessary enzymes, such as those needed for tryptophan synthesis.¹ If these cells can be tricked into repressing their trp operon when tryptophan is not actually present, will the population grow more slowly?² We compared population size and trp operon expression (using a GFP gene inserted into and under the control of the trp operon) of *E. coli* grown in minimal medium, and minimal medium supplemented either with tryptophan or a structural analog of tryptophan (5-bromotryptophan).³ The population of cells was densest in tryptophan supplemented medium ($\sim 3.8 \times 10^6$ cells/mL, almost double that in minimal medium and more than double that of 5BT-supplemented medium), and gene expression was 10 times higher in minimal medium than in tryptophan supplemented medium.⁴ *E. coli* grows more efficiently in the richer medium, and its efficiency is lowest when it is tricked into repressing its trp operon in the absence of tryptophan.⁵

Introduction

Wild type *E. coli* can synthesize all the necessary amino acids in minimal medium containing only sodium phosphate, potassium phosphate, ammonium chloride, sodium chloride, magnesium sulfate, calcium chloride, plus a carbon source such as glucose or glycerol. (Bauman, 2003, p. 182). If a particular amino acid is introduced into the medium, the operon whose structural genes specify the enzymes necessary for synthesis of that amino acid should be repressed, increasing efficiency of growth (Bauman, 2003, p. 220). The trp (tryptophan) operon, for example, includes the gene for tryptophan synthetase, which catalyses two steps in the production of tryptophan (Griffiths, et al., 1999, Fig 11-17). The trp repressor protein, when bound to tryptophan, attaches to the tryptophan operator and prevents RNA polymerase from binding to the promoter site and initiating transcription. Several structural analogues of tryptophan are known to bind to the repressor protein, as well (Pauley, et al., 1978).

Adding tryptophan to minimal medium should prevent expression of the *E. coli* trp operon and increase population growth. Adding a structural analog of tryptophan should also prevent expression of the trp genes, but also slow the growth of the population by reducing the availability of tryptophan.

To test this, we grew an *E. coli* culture in minimal medium to which was added either tryptophan or 5-bromotryptophan (a structural analog of tryptophan), or neither, and measured population density as well as trp operon induction (via an engineered reporter gene – GFP – inserted into the trp operon).

The **title** is specific – it mentions the species, the phenomenon, and the experiment. Genus and species names are in the correct format.

The **abstract** is 5 sentences long, one for each of the following:

1. Motivation (background)
2. Question or problem
3. Approach or method
4. Results
5. Conclusion or implication

The **introduction** provides background, to put the experiment in context. The sources used for information are cited in situ.

It states the experimental question or makes a prediction.

It explains the approach to solving the problem or answering the question

Methods

We incubated 30 1 mL aliquots of a mid-log culture of an *E. coli* strain expressing GFP in their *trp* operon (grown in minimal medium), 10 in minimal medium plus tryptophan (0.1 M), 10 in minimal medium plus 5-bromotryptophan (0.1 M), and 10 in minimal medium alone, for 8 hours in a shaking incubator at 37°C, killed them by addition of 0.1 mL of 10% sodium azide, and measured OD (at 595 nm) and fluorescence (excitation at 395 nm, emission at 509 nm), blanked with minimal medium.

We calculated population size and fluorescence per 10⁶ cells, based on the conversion factor (previously determined by colony counts):

$$1OD = 5 \times 10^8 \text{ cells/mL}$$

We used the Q test to reject one outlying low value in the 5-BT group at the 95% confidence level, then calculated the means and standard deviations of the remaining values.

We determined whether the population sizes or fluorescence per million cells were different from each other by ANOVA.

Results

The cell population was approximately twice as dense in the tryptophan-supplemented medium than in either the minimal or the 5-BT-supplemented medium. This difference was significant at the 0.05 level; the two lower cell densities were not statistically different from each other. See Figure 1.

As can be seen in Figure 2, fluorescence per million cells was highest in minimal medium. Fluorescence was very low in the medium containing tryptophan, and intermediate in the medium containing 5-bromotryptophan. All three values are statistically different from one another at the 0.5 level.

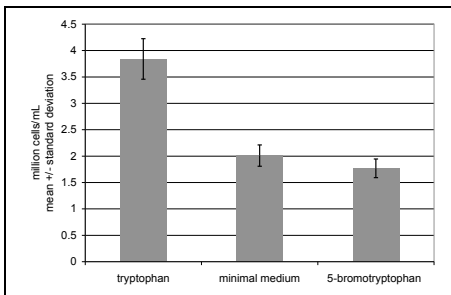


Figure 1. Cell density after 8 hours in different media: minimal medium plus tryptophan, 5-bromotryptophan, or nothing

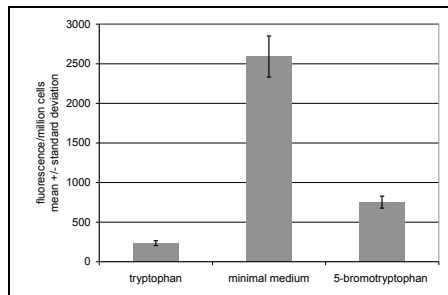


Figure 2. Fluorescence per million cells after 8 hours in different media: minimal medium plus tryptophan, 5-bromotryptophan, or nothing

The **methods** explains how the experiment was done. It is written in past tense, since the experiment was over before the author sat down to write it. It is a narrative, not a set of instructions.

It gives necessary details, but omits extraneous ones.

It tells what data were collected, and how they were analyzed.

The **results** describes the findings in words, then refers to the figures by their numbers.

All pictures (diagrams, photos, and graphs) are called figures. See alternate results section with tables instead of figures, following this report.

The figures are numbered, and they have a descriptive title below.

The axes are labeled (and they match). The means have been plotted to show the overall trends, and error bars (in this case standard deviation) have been plotted to show the variability in the data.

Since there is only one data series in each graph, there is no legend on either figure.

There is no interpretation in this section.

Discussion

As expected, the cell population was highest in medium containing tryptophan, indicating either that this medium supports higher cell densities, or that the populations grew faster in it.

Fluorescence, when corrected for population size, was lowest in the tryptophan-supplemented medium. This indicates that the trp operon was indeed repressed by the presence of tryptophan, because our reporter gene for GFP is under the control of the trp operon. Cells given 5-bromotryptophan, however, also fluoresced significantly less than those in minimal medium, suggesting that 5-bromotryptophan inhibits expression of the trp operon, but it does not do so completely. This is consistent with the reduced cell density, as these cells were prevented from synthesizing tryptophan, and not given any in the medium.

This experiment gives us little information about population growth rate. We don't know the initial population sizes, although we did try to inoculate the same amount of initial culture into each experimental medium. Although the final population density was greatest in the tryptophan-supplemented medium, we don't know whether this represents the maximum population the medium can support or a faster rate of cell division. Taking absorbance and fluorescent readings at intervals over the incubation period would help to answer these questions.

This might also let us figure out if expression of the trp operon changed over time as concentrations of nutrients went down.

References

Bauman, Robert W., 2004. *Microbiology*. Pearson Benjamin Cummings, San Francisco.

Griffiths, Anthony J.F.; Miller, Jeffrey H.; Suzuki, David T.; Lewontin, Richard C.; Gelbart, William M., 1999. *Introduction to Genetic Analysis*. New York: W.H. Freeman & Co.

Retrieved January 17, 2010. Available from:

<http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=iga&part=A2010&rendertype=figure&id=A2011>

Pauley, Robert J., Walter W. Fredricks, and Oliver H. Smith, 1978. Effect of Tryptophan Analogs on Derepression of the *Escherichia coli* Tryptophan Operon by Indole-3-Propionic Acid. *J Bacteriol.* 1978 October; 136(1): 219–226. Retrieved 17:24, December 9, 2009, from <http://www.ncbi.nlm.nih.gov/pmc/articles/instance/218652/>

Acknowledgements

This work was made possible in part by the assistance of Dr. Statistics Prof, who taught me how to do analysis of variance. I also wish to thank Dr. Lab Coordinator for letting me set up the experiment in the biochemistry lab space, and my lab partner, Stu Dent, for helping me put error bars on my bar graph.

The **discussion** interprets the results.

It says whether the results were as expected.

It gives an interpretation and further explanation of the results.

It also criticizes the experiment: what questions were left unanswered, and how might they be addressed? In other words, what would the author do differently, knowing what she now knows?

Complete bibliographic information is given for each of the **references** used earlier in the paper. The reader should be able to find these references from the information given.

The assistance provided by others is credited in an **acknowledgements** section

Alternate Results section, using tables instead of figures

Results

The cell population was approximately twice as dense in the tryptophan-supplemented medium than in either the minimal or the 5-BT-supplemented medium. This difference was significant at the 0.05 level; the two lower cell densities were not statistically different from each other. See Table 1.

As can also be seen in Table 1, fluorescence per million cells was highest in minimal medium. Fluorescence was very low in the medium containing tryptophan, and intermediate in the medium containing 5-bromotryptophan. All three values are statistically different from one another at the 0.5 level.

Table 1. Population density and trp operon expression (GFP fluorescence) in *E. coli* grown in different media. Means \pm standard deviation.

	Medium		
	Minimal	+ Tryptophan	+ 5-bromotryptophan
Millions of cells/mL	2.01 \pm 0.19	3.84 \pm 0.39	1.77 \pm 0.18
Fluorescence/million cells	2592 \pm 261	235 \pm 31	752 \pm 78

The **results** section describes the findings in general, and refers the reader to the table by its number. Tabulating the actual values (rather than listing them all in the text) makes them easier to read.

A list of words or numbers is a **table**, not a figure. **Its number and title go at the top.**

The rows and columns are labeled. As the title indicates, the means have been presented to show the overall trends, and variation is indicated with standard deviation.

If there are both tables and figures in the same paper, they are numbered separately.

Using the Motic Camera

Important Points:

- Do not wipe/touch any of the lenses with anything except lens paper. Kimwipes are NOT lens paper!
- Remove the right eyepiece from the microscope and set it down on the table on the end that goes into the microscope, NOT the end you look into. This could scratch the lens.
- When removing the eyepiece, grip the polished black ring that is beneath the plastic cap.
- Put the camera over the eyepiece socket. Fasten the camera with the set screw.
- Put all camera/eyepiece equipment BETWEEN the computer and the microscope, not near the edge of the table! You run the risk of knocking the eyepiece off the table if it is set near the edge!
- Turn the focusing knob (front side) up to move the microscope stage closer to the objective.
- Turn the focusing knob (front side) down to move microscope stage farther from the objective.

Focusing between your eyes and the camera display:

4x Objective (RED):

When this is focused for your eyes, you need to turn the knob (front part) DOWN to focus the camera display

10x Objective (YELLOW):

Same as for 4x.

40x Objective (BLUE):

When focused for your eyes, it should be close to the correct focus for the camera display, make small adjustments.

Step-by-Step Guide to Taking a Photo:

1. Attach the camera to the RIGHT eyepiece and plug it into a USB port on the back of the Mac.
2. Click the Motic Images Plus icon on the desktop to open the program.
3. Go to File > New
4. Choose “Live Video” and click “Ok.”
5. The window will open to show what the camera sees in the microscope. Go to File > Video Settings to open up the camera adjustment controls.
6. To adjust the exposure (lighting) for the camera, click “Auto Exposure” and wait about 5 seconds. If you want the computer to do this automatically from now on, check off “Full Auto.” If you want to adjust the settings manually, turn off “Full Auto” and move the sliders to tweak the various lighting/color functions.

7. The image on the computer screen will probably not be in focus, because the camera and your eyes are at different “distances” from the slide.
8. To adjust the focus for the computer image, **SLOWLY** turn the focus knob on the microscope in the correct direction for the objective you are using (4×, 10×, or 40×). It is **IMPORTANT** to turn the knob **VERY SLOWLY**, because it takes time for the camera image on the computer to refresh. By focusing too quickly, you may go right past the point where the image is focused.
9. To make a photo, look at the top of the window at the three icons. Click the middle icon (it looks like a small digital camera with a clock next to it).
10. Change the ‘save’ location to the desktop.
11. To the right of where “File Name” appears in the dialog box, click the drop down box to save the image in the jpg format and NOT in the “.mix” format.
12. Leave the capture interval settings alone for now, but change the number of Total Images to “1” instead of “5.” You don’t need five copies of the same picture, right?
13. Click “OK” and the new file will appear on the desktop.

Phase contrast

Introduction to Phase-Contrast Microscopy*

Bright-dark light contrast is produced by exploiting the interaction of the illuminating light with the specimen, and using the changes produced by the specimen in clever ways. One such way is called *phase contrast*. This method was invented by Fritz Zernike, for which he received a Nobel prize.

Phase contrast takes advantage of the fact that all microscopic samples diffract light and images are built up from the recombination of the un-diffracted (so-called *zero order*) and diffracted beams. Think of the zero-order beam as the background. Even absorbing, e.g. black, samples diffract light. You could see this by blocking the zero order beam and noting a dark object becomes bright. Absorbing samples look dark in part because the diffracted light is 180° out of phase with the zero order light and hence destructive interference ensues. Transparent samples diffract light too, but introduce a phase shift that does not cause appreciable interference. Phase contrast is a method to introduce a phase shift between the diffracted and zero-order beams, which let them interfere destructively and gives rise to an intensity difference we can see.

This is accomplished in the phase-contrast microscope by two circular, optical elements (Figure 34). In the front focal plane of the condenser, there is an annulus that restricts the illumination to a ring. In the rear focal plane of the objective, there is a phase ring, onto which the ring of illuminating light is focused. The illuminating light is the zero-order beam, un-deviated by diffraction; when it traverses the phase ring, its phase is advanced or delayed (depending on the instrument design) relative to light passing through the rest of the rear focal plane. It is also customary also to reduce the intensity of the zero order beam by reducing the transmission of the ring. The annulus is built as an opaque sheet with an annular opening through which light passes; the phase ring is a flat plate, which is etched away or built up in a ring shape, at the appropriate diameter. Phase contrast requires the illuminating annulus and the objective phase ring to be superimposed precisely.

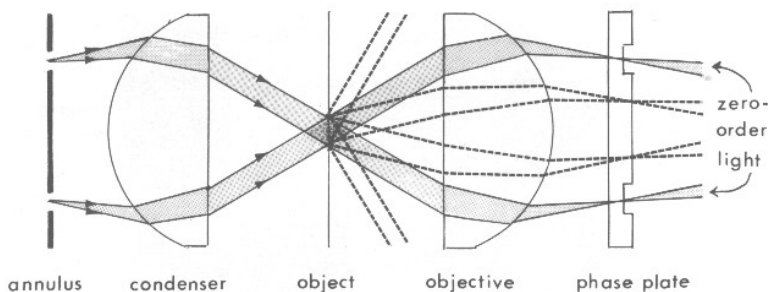


Figure 34. Main features of the phase contrast microscope. The illumination is restricted to a ring by the annulus. The object (sample) diffracts light, giving rise to diffracted (dashed lines) and unaffected ("zero order") light. These beams recombine in the image (not shown). The phase of the zero order beam relative to the diffracted beam is altered by a

quarter wavelength at the phase plate, which allows destructive interference to occur, making the object appear dark. (Figure 24 from Spencer M., 1982. *Fundamentals of Light Microscopy*, Cambridge University Press, London.)

* Written by Tobias Baskin, Biology Department, for Biology 497BH, Fall 2009.

The Nikon E200*

Notes on Handling the System

(1) Installation

This microscope is a precision instrument. Using the microscope in an unfavorable environment could result in malfunctions or degraded performance. Consider the following conditions when choosing the installation location.

- Observation conditions are better if light from windows and bright room light can be avoided.
- Install the microscope in a location with a room temperature of 0° to 40°C and with a maximum relative humidity of 85%. High temperature and humidity are to be avoided because they promote mold growth and condensation, which may damage the microscope.
- Dirt and dust degrade optical performance and are to be avoided.
- Vibrations in the environment will degrade the image. Install the microscope in a location free of vibrations.
- Install the microscope on a solid table and keep the microscope level.
- Select a layout that allows easy detachment of the power cord from the AC inlet of this microscope in the event of emergency.
- This microscope emits a feeble electromagnetic wave. Do not place a precision electronic device near the microscope as precision could be degraded. Also, avoid placing a radio or TV near the microscope as reception of sound and images may be hampered.

(2) Carrying the Microscope

This microscope is a precision instrument. Handle it gently. Strong shocks and forcible operation will damage the instrument. Shocks to the objectives, especially, could degrade image precision.

- When carrying the microscope, hold it at its upper rear and lower front ends.
- Do not hold the focus knobs, the eyepiece tube, or the stage. These parts could easily come off and could result in malfunctions.



(3) Handling the Lamps

Do not touch the glass part of the lamp with bare hands. Wear gloves or use a cloth when handling the lamp so as not to leave fingerprints on the surface. Wipe off any fingerprints or stains using a clean cloth moistened with alcohol. Fingerprints will etch into the hot surface of the lamp and reduce the brightness, damage the lamp or reduce its service life.

Handle the lamp gently. Shocks and vibrations will damage the lamp or reduce its service life.

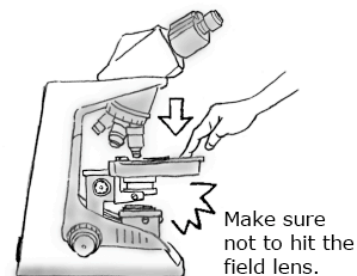
When changing the lamp, be sure that the contact is not damaged. If the contact is damaged, the lamp may not light up or may overheat. Insert the lamp's contact pins fully into the socket holes. If the pins are loose, the lamp could come off or result in a contact failure, which will cause overheating or smoke. Also, make sure that the field lens unit is securely attached.

Do not break the used lamps ; instead dispose of them as special industrial waste or according to the laws applicable to your municipal waste system.

* From *Nikon Eclipse E200 Instructions*, Nikon Instruments, Inc., 1300 Walt Whitman Road, Melville, NY 11474-3064, reproduced with permission.

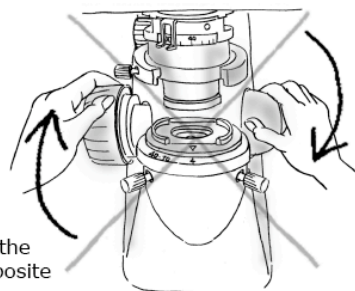
(4) Refocusing

When changing specimens using the refocusing mechanism, gently lower the stage by hand taking care not to hit the field lens with the condenser holder (p. 60).



(5) Focus Knobs

Do not turn the right and left focus knobs simultaneously in opposite directions. Do not turn the coarse focus knob any further after the stage has been moved up or down to its limit. These operations will damage the focusing mechanism. (The coarse focus knob has a protection device. The knob turns freely for a while after it has reached its upper limit.)



(6) Oil-Immersion Observation

Use only a minimum quantity of oil. If too much oil is applied, surplus oil could flow out to the stage and the condenser which could lead to degraded performance.

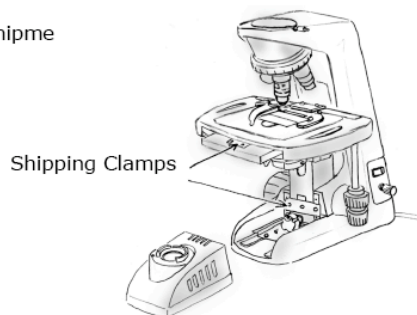


WARNING

When using petroleum benzine or absolute alcohol to wipe off immersion oil or to clean the lenses, follow the instructions provided by their manufacturers. Absolute alcohol and petroleum benzine are inflammable. Take great care when handling them.

(7) Shipping Clamps

The microscope is held tightly by the clamps during shipment before use. For details, see p. 69.



1

Nomenclature of Each Part

The microscope is made up of the following components.

(1) Basic unit

(2) Eyepieces

Screwed on to the eyepiece tube.

(3) Eyepiece Tube

This is a binocular eyepiece tube. A trinocular eyepiece tube is available for photomicrography and TV microscopy.

(4) Objectives

Objectives with various magnifying powers are available.

(5) Condenser

Used for condensing light.

The condenser should be positioned slightly lower than its upper limit.

Adjust the aperture diaphragm lever according to the objective.

(6) Field Lens Unit

Draw out the field lens unit when changing lamp.

The microscope may have a field diaphragm. A field diaphragm is used to control the illumination range and should be adjusted according to the objective. (Note that there are two types of microscopes; the one with the field diaphragm, and the one without.)

(7) Lamp

Halogen lamp 6 V-20 W or 6 V-30 W is used.

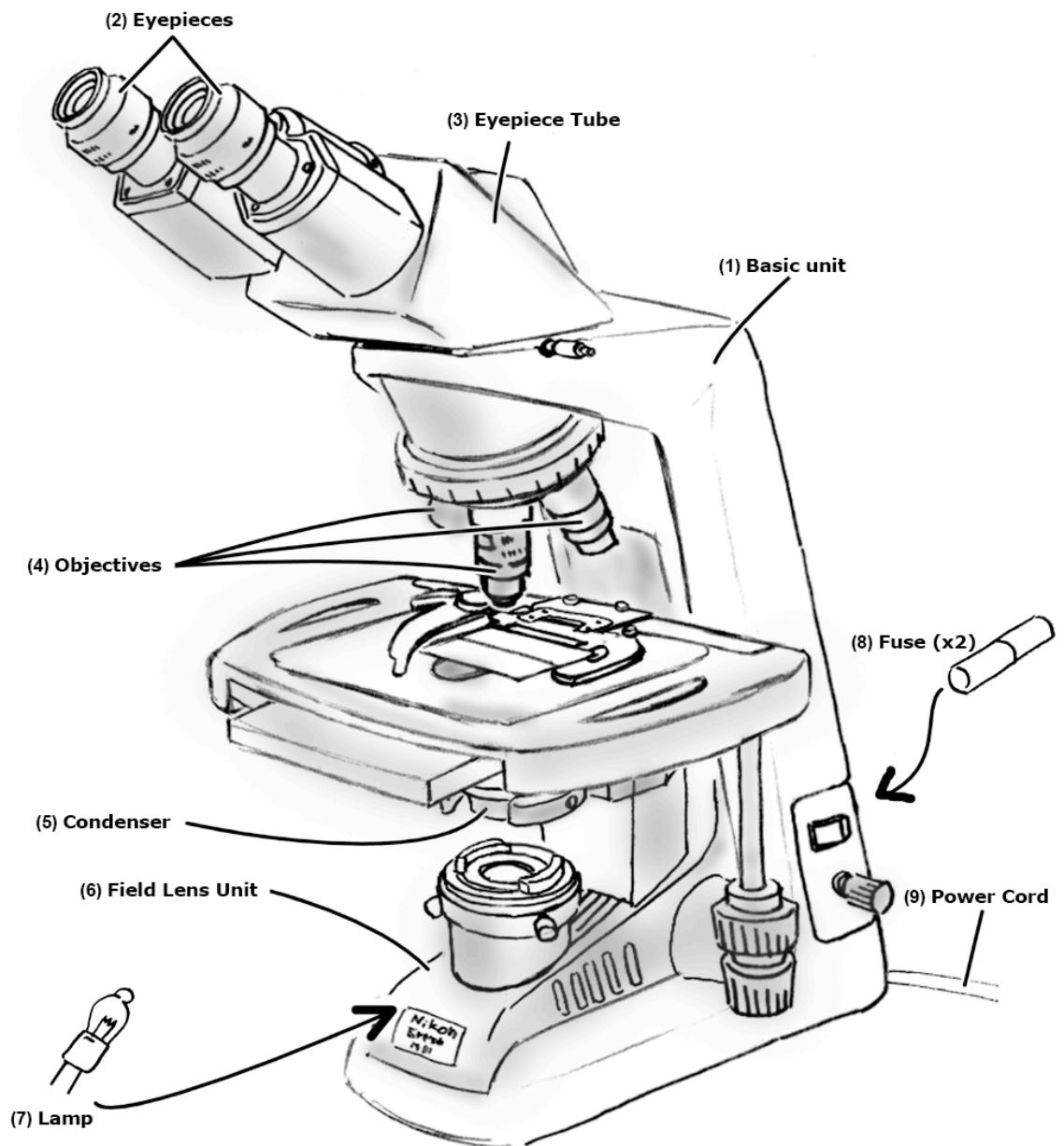
(8) Fuse

Two 250 V 1A time-lag low-breaking type fuses are used.

(9) Power Cord

Use the power cord provided.

Chapter 1 Nomenclature of Each Part

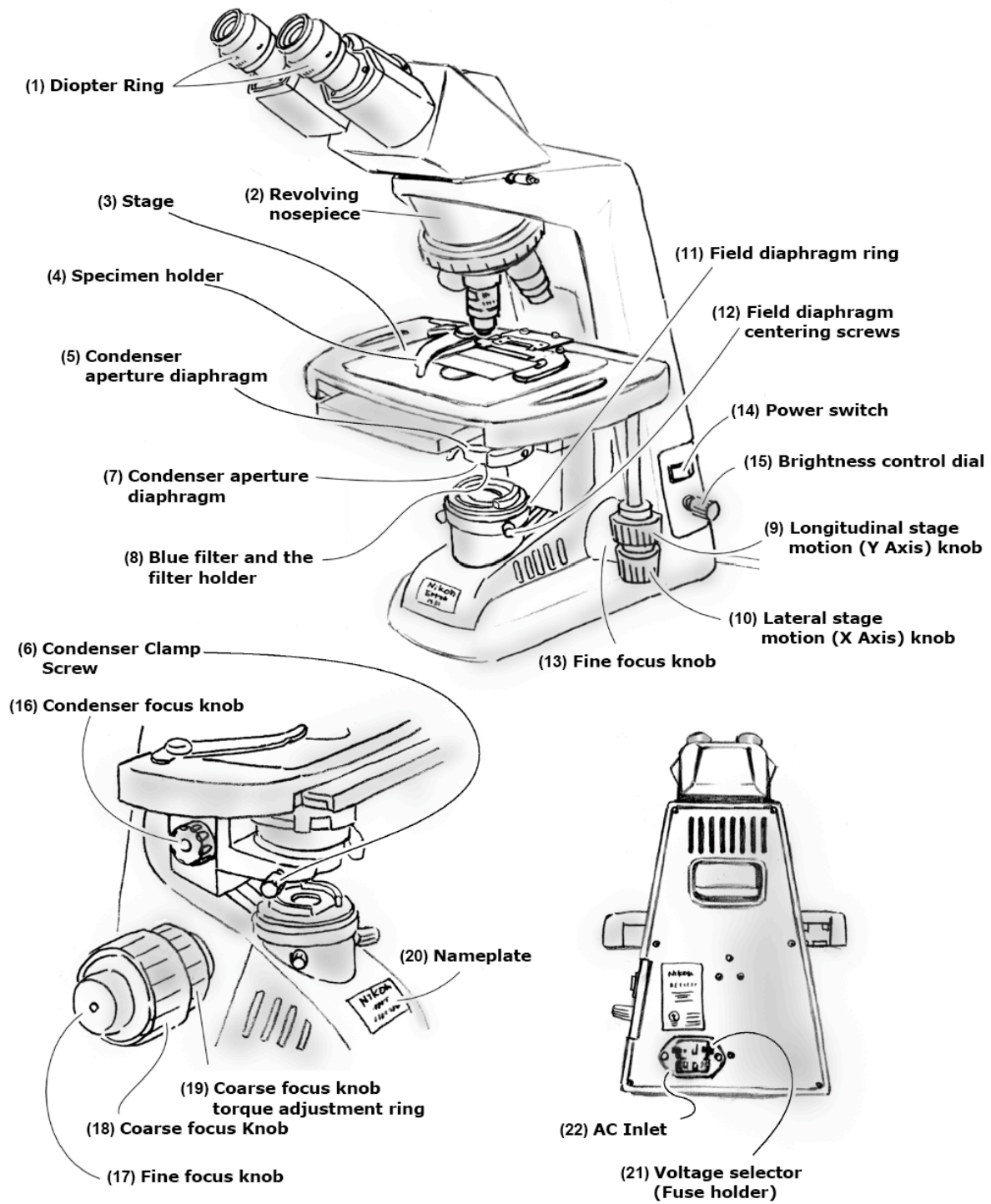


2

Switches and Controls

- (1) Diopter Ring**
Adjust the diopter ring to compensate for the difference between your right and left eyesight. (p. 57)
- (2) Revolving nosepiece**
Can hold up to four objectives.
- (3) Stage**
- (4) Specimen holder**
Put your finger at the root or the tip tilt of the claw to open the claw. (p. 55, 71)
- (5) Condenser aperture diaphragm lever**
Set the lever to match the magnifying power of the objective. (p. 59)
- (6) Condenser Clamp Screw**
- (7) Auxiliary lens for the condenser**
Screw on to the bottom of the condenser.
- (8) Blue filter and the filter holder**
- (9) Longitudinal stage motion (Y Axis) knob**
- (10) Lateral stage motion (X Axis) knob**
These knobs are located either to the right or the left of the stage.
- (11) Field diaphragm ring**
Set the ring to match the magnifying power of the objective. (p. 60)
This ring is equipped only on the microscope with a field diaphragm.
- (12) Field diaphragm centering screws**
Used to center the field diaphragm image. (p. 57)
These screws are equipped only on the microscope with a field diaphragm.
- (13) Fine focus knob**
Used for focusing. There is no coarse focus knob on the side with the stage motion knobs.
- (14) Power switch**
When turned to |, power is turned on and the lamp lights.
When turned to "O", power is turned off and the lamp goes off.
- (15) Brightness control dial**
When turned clockwise, the lamp voltage increases and the viewfield becomes brighter.
When turned counterclockwise, the lamp voltage decreases and the viewfield becomes darker.
- (16) Condenser focus knob**
Use this knob when focusing the field diaphragm image on the specimen. (p. 57)
The condenser focus knob is located on the opposite side of the stage motion knobs.
- (17) Fine focus knob**
- (18) Coarse focus Knob**
There are both coarse and fine focus knobs on the opposite side of the stage motion knobs.
- (19) Coarse focus knob torque adjustment ring**
Used to adjust the tension (torque) of the coarse focus knob. (p. 64)
- (20) Nameplate**
Indicates the input voltage.
- (21) Voltage selector (Fuse holder)**
Use this selector to match the input voltage of the microscope to the voltage provided in your region. (p. 68)
- (22) AC Inlet**
Plug the power cord into this inlet. Make sure that the power switch is off (turned to "O") before plugging the cord in.

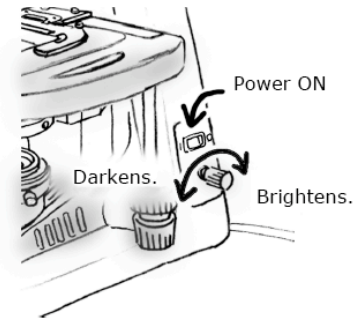
Chapter 2 Switches and Controls



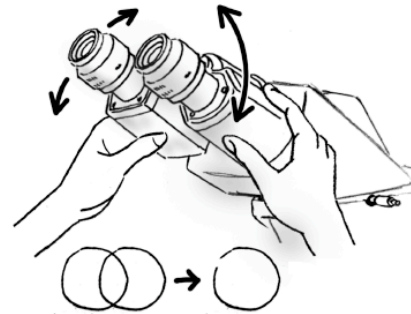
4

Microscopy (Detailed Procedure)**1 Lamp Illumination**

Turn on the power switch (turn to |) and the lamp will come on. Turn the brightness control dial to adjust the brightness of the viewfield. (Turning the dial clockwise increases the brightness; turning the dial counterclockwise decreases it.)

**2 Interpupillary Distance Adjustment**

Adjust the distance between the eyepieces to merge the right and left viewfields into one. (This is an adjustment to match the distance between eyepieces with the distance between your eyes).



Merge the right and left viewfields into one.

Try!!**Changing the Eye Level**

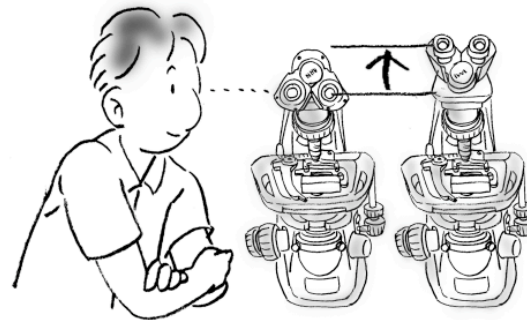
Turn the binocular part of your microscope 180 degrees, and you will get the microscope with higher eye level. *1

If you feel uncomfortable in observing the image due to its size, you may find this convenient.

There is one condition, however, that should be met. Before returning the microscope in the cabinet, put back the binocular part to its original low position. Since left high, the tip of the eyepiece will become the highest point of the microscope and may be damaged easily when bumped against the shelf. In addition, the microscope may be higher than the shelf.

The eye level can be raised even higher (till 50 mm) if an "Eye level raiser (optional)" is installed between the basic unit and the eyepiece tube. See p. 65 for details on the eye level raiser.

*1: When the interpupillary distance is 64 mm, the eye level is raised about 30 mm.



3 Align the Diopter Ring with the Engraved Base Line

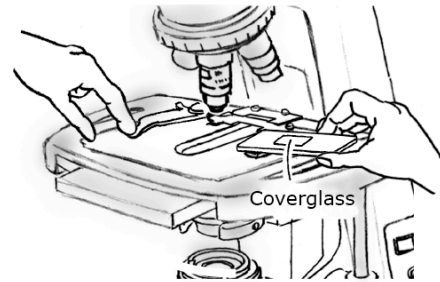
Turn the diopter ring on the right eyepiece to align its bottom edge with the engraved base line. Turn and align the diopter ring on the left eyepiece in the same way.



Match the bottom edge of the diopter ring with the engraved base line.

4 Specimen Mounting

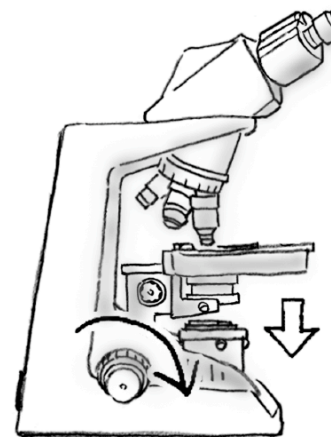
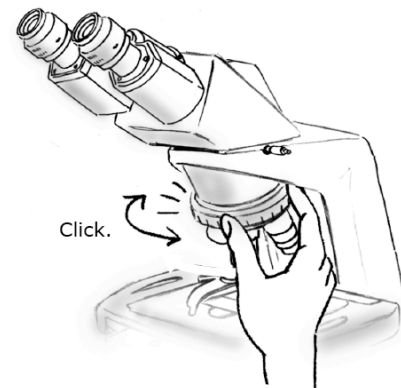
Place specimen slide on the stage with the coverglass facing upward. Open the claw of the specimen holder with your finger at the root or the tip tilt and fix the specimen slide with the claw.



5 Focus with the 10x Objective

Rotate the revolving nosepiece to bring the 10x objective into the optical path. (The objective will click into place when rotated into position.) Bring the specimen image into focus by turning the coarse focus and then fine focus knob.

- Direction of stage movement relative to focus knob rotation is shown in the figure.
- There is no coarse focus knob on the side with the stage motion knobs. There are both coarse and fine focus knobs on the opposite side of the stage motion knobs.
- Do not turn the right and left focus knobs simultaneously in the opposite directions. Do not turn the coarse focus knob further after the stage has reached its lower or upper limit. These operations could result in a malfunction.
- Turn the ring below the stage to set the appropriate condenser: C for bright field, A for dark field, and the appropriate Ph for phase. Match the Ph (1, 2, or 3) to the objective.

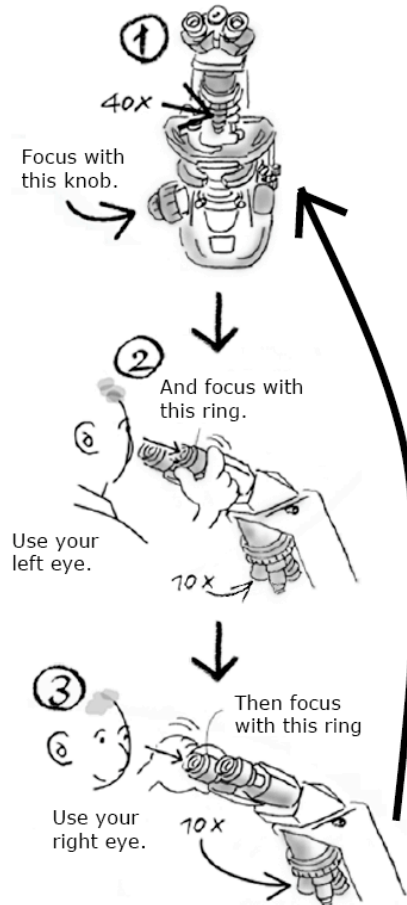


6 Eyepiece Diopter Adjustments

Adjust the diopter ring on the eyepieces according to the difference between your left and right eyesight. This adjustment enables the user to take full advantage of the high-quality objectives, including their parfocality.

- (1) **Swing the 40x objective in the optical path. Rotate the coarse and then fine focus knobs to bring the specimen in focus.**
- (2) **Switch back to the 10x (or 4x) objective. While looking into the right eyepiece with your right eye, focus on the specimen by rotating the right diopter ring and not using the focus knob.**
- (3) **While looking into the left eyepiece with your left eye, focus on the specimen by rotating the left diopter ring and not using the focus knob.**
- (4) **Repeat the steps ② to ③.**

* The next procedure is for microscopes equipped with field diaphragm. If the microscope does not have a field diaphragm, confirm the position of the condenser and skip to procedure 8. (The condenser should be a little lower than its highest position.)



8 Objective Selection

Rotate the revolving nosepiece to the desired objective magnification. (The objective will click into place when rotated into position.)

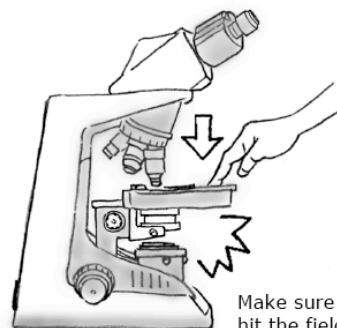
Adjust the aperture diaphragm lever according to the selected objective. If the microscope is equipped with a field diaphragm, also adjust the size field diaphragm (see the procedures 9 and 10).



Replacing a Specimen Using the Refocusing Mechanism

Try focusing on the specimen with 40x or higher magnification objective. You will find the specimen is brought very near to the objective.*¹ It will be very difficult to change the specimen without moving the focus knob. In a case like this, use the refocusing mechanism for easy specimen replacement.

- (1) Use one hand to gently press down the stage.*²
- (2) While holding the stage at that position, change the specimen.
- (3) Gradually release the stage so that it rises slowly. The stage will return to the focal position.



Make sure not to hit the field lens.

*1 : The distance between the front of the objective and the specimen when the specimen is in focus is called the "working distance" of the objective. For details, see p. 74.

*2 : When lowering the stage, take great care not to hit the field lens with the condenser and the parts under the condenser

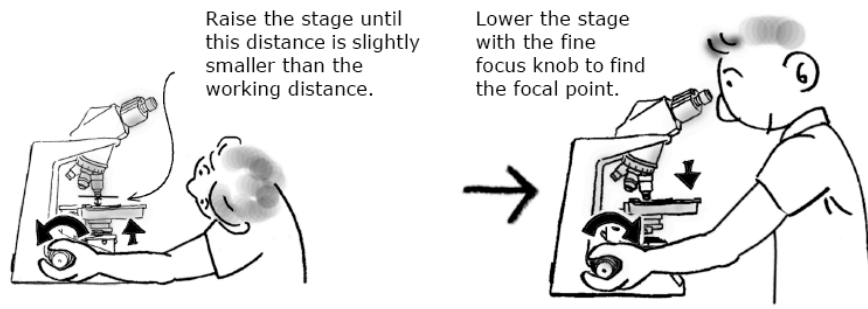


Using the Working Distance for Focusing

Each objective has its working distance indicated on its side. The working distance is the distance between the front of the objective and the specimen when the specimen image is in focus. If you have difficulties in focusing with the standard procedure described on p. 56, try one of the following methods using the working distance for focusing.

Method 1:

While looking at the microscope from the side, rotate the coarse focus knob to bring the specimen close to the objective. When the distance between the specimen and the front of the objective becomes slightly smaller than the working distance, take your hands off the coarse focus knob. The specimen is now almost in focus. Look into the eyepieces and rotate the fine focus knob in the direction that lowers the stage.



Raise the stage until this distance is slightly smaller than the working distance.

Lower the stage with the fine focus knob to find the focal point.

Method 2:

Swing the 40x objective into the optical path. While looking at the microscope from the side, rotate the coarse focus knob until the specimen almost touches the objective (about 0.5 mm apart from the front of the objective). The specimen is now almost in focus. Switch to the 10x objective, look into the eyepieces, and rotate the fine focus knob slightly to find the focal point. Be careful not to hit the objective with the specimen.

11 Turning Off the Lamp

Turning off the power switch (turn to "O") switches off the lamp.
When storing the microscope:

- Unplug the power cord.
- Wait until the field lens unit is cool enough to touch.
- Return the binocular part to its lowest position.
- Cover the microscope with the vinyl dust cover. (Before covering the microscope, make sure that the field lens unit is cool enough to touch.)
- When carrying the microscope, hold it at its upper rear and lower front ends.

1

Oil-Immersion Observation

The “Oil” mark on the side of an objective indicates that it is an oil-immersion type objective. (The oil-immersion objective also has a black band around the barrel end.) An oil-immersion objective is used with the immersion oil applied between the front of the objective and the coverglass. For an oil-immersion objective with a numerical aperture of 1.0 or more, use of an oil-immersion type condenser is required to take full advantage of its performance. An oil-immersion type condenser, like an oil-immersion type objective, needs immersion oil to be applied between the front of the condenser and the coverglass.

The abbe condenser included in the bright viewfield set can be used for oil immersion observation. The condenser has an oil receptacle around its front lens.

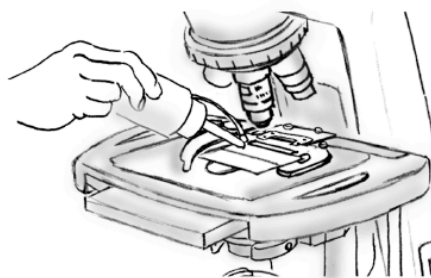
● Example of Oil-Immersion

Condenser:

Move the specimen toward the back and lower the condenser slightly. Add a drop of oil on the front of the condenser from the long hole on the stage. Bring the specimen back over the condenser and slowly raise the condenser.

Objective:

Rotate the revolving nosepiece to move the objective out of position. Add a drop of oil to the specimen. Slowly rotate the revolving nosepiece to bring the objective back into position.



● Eliminate Air Bubbles

Make sure that air bubbles are not trapped during oil application. Air bubbles degrade the image. To see if any air bubbles are trapped in the oil, remove one eyepiece and fully open the aperture diaphragm (and field diaphragm, if the microscope has the field diaphragm). Look into the eyepiece tube and check the objective pupil (a bright round part). If you cannot see it well, replace one of the eyepieces with the adapter and the centering telescope (both optional) and look through the eyepieces of the centering telescope while rotating the eyepiece part of the centering telescope.

Do any of the following to eliminate air bubbles:

- Rotate the revolving nosepiece to move the objective back and forth.
- Gently rotate the condenser focus knob to move the condenser up and down.
- Add another drop of oil.
- Wipe off the oil and apply again.

● Handling of the Immersion Oil

Use a minimum quantity of oil. If too much oil is applied, surplus oil could flow out onto the stage and the condenser and degrade performance.

After completing oil-immersion observation, be sure to clean the objective, condenser, and any other parts that may be stained by oil. Any oil residue left on the lenses of oil-immersion type objectives or adhesion of oils on the front lens of dry type objectives will degrade image quality. Use petroleum benzine to wipe off oil and finish with absolute alcohol (ethyl or methyl alcohol). If petroleum benzine is not available, use methyl alcohol instead. In that case, wipe off the oil several times (generally 3 or 4 times) as the detergency of methyl alcohol is weaker than petroleum benzine.



WARNING

When handling petroleum benzine and absolute alcohol, be sure to follow the instructions provided by the manufacturers. Since they are highly flammable take great care when handling them.

● Cautions on Handling the Immersion Oil

- Close the container cap tightly after use. Make sure that the cap is closed tight after refilling the container. Check the cap periodically to make sure it has not come loose, allowing oil to leak out.
- Do not press the container hard. Oil could splash out.
- If you find an oil drips around the container, wipe them off.
- Avoid contact of immersion oil with eyes or skin. In the event of contact with eyes or skin, take one of the following measures although Nikon immersion oil does not contain any toxic ingredients.
 - ◇ **Contact with skin: Rinse your skin thoroughly with soap and water.**
 - ◇ **Contact with eye: Rinse your eye thoroughly with water (more than 15 minutes) and see a doctor.**
- Do not leave immersion oil in the sun (ultraviolet rays can damage it).