Bi 1x Spring 2016: *E. coli* Growth Curves

1 Overview
In this lab, you will investigate growth of the bacterium *E. coli*, watching the growth in two ways. First, you will determine growth rate in a solution of media by measuring optical density over time. The optical density is proportional to the mass of bacteria in the solution. Second, you will watch individual bacteria grow and divide using fluorescence microscopy. In this case, the mass of bacteria is proportional to the fraction of your images occupied by bacteria. Through these two different approaches, you will learn about the rates that bacterial populations convert media into new life.

2 Background
2.1 Bacterial growth
As you have already seen in our introductory experiments, bacteria grow and divide at an amazing rate. A solution containing approximately 100 bacteria can grow to well over $10^9$ bacteria—roughly equal to the entire human population—overnight. As with human or animal populations growing under optimal conditions, the rate of population increase is proportional to the current population. This means that

$$\frac{dN}{dt} = rN,$$

$$N(t) = N_0 e^{rt},$$

where $N(t)$ is the number of bacteria in your sample at time $t$ and $N_0 = N(0)$. The growth rate $r$ depends on many features of the environment, such as the growth medium or presence of antibiotics.

2.2 Cell challenge with anti-bacterial substances and spectroscopy
While most bacteria pose no threat to humans, there are many species that can cause very serious infections. One major challenge facing biomedical scientists is how to kill off these bacteria after an infection has taken hold. This challenge has become more and more difficult as the abuse of antibiotics has lead to the widespread occurrence of strains with antibiotic resistance. To better understand the concepts involved in combating bacterial infection, we will measure the bulk growth rate of *E. coli* in growth medium at various concentrations of the antibiotic kanamycin.

2.3 The Beer-Lambert Law and optical density
To determine the density (concentration) of bacteria in liquid media, we load a cuvette with the suspension of cells. In the spectrophotometer, light with a wavelength comparable to the size of the object being measured (600 nm) enters the sample at an initial intensity ($I_0$). Cells, having a different index of refraction as the surrounding medium, randomly reflect and scatter light out of the incident light path. The amount of scattering is proportional to the mass of cells at any given point in the sample. (Caveat: to get an accurate reading, cell density must be low enough that light is only scattered once in its journey through the sample. Often, this means that cells must be diluted to get...
an accurate reading.) Upon exiting the sample, the light intensity is reduced by the cells from \( I_0 \) to \( I = I_0 e^{-\epsilon lc} \), where \( \epsilon \) is the wavelength-dependent molar absorption coefficient of the species in question, \( l \) is the path length (usually 1 cm in modern spectrophotometers), and \( c \) is the concentration. This is known as the Beer-Lambert Law. The transmission efficiency of the sample is the ratio of the final and initial light intensity, \( T = I/I_0 \), and the reported quantity is called the absorbance \( A = \ln(1/T) = \epsilon lc \). This is known as the Beer-Lambert Law. The transmission efficiency of the sample is the ratio of the final and initial light intensity, \( T = I/I_0 \), and the reported quantity is called the absorbance \( A = \ln(1/T) = \epsilon lc \). The general rule of thumb is that the accurate absorbance readings lie in the range of \( 0.01 < A < 1 \). (Serial dilutions of your sample may be necessary to bring the absorbance into this range.) In the context of bacterial growth, the absorbance \( A \) is often called optical density, abbreviated OD\textsubscript{600} for absorbance of light at a wavelength of 600 nm.

### 2.4 Calibration of optical density

In the previous section, we established that the optical density is proportional to cell concentration. To determine the constant of proportionality (the product \( \epsilon l \)), we dilute a sample of known OD to a very low concentration of bacteria. We then spread a small volume of this diluted sample onto a plate with a good growth medium. We incubate the plate and count the number of bacterial colonies present. Provided the initial solution was dilute enough, each colony should correspond to a single bacterium. Since we know how much we diluted the original sample, we can then back-calculate its concentration. This gives us the constant or proportionality between OD\textsubscript{600} and cell concentration. A good rule of thumb is that an OD\textsubscript{600} of 0.1 corresponds to about \( 10^8 \) cells/mL.

### 2.5 Measuring growth at single-cell level

While the scattering experiments provide bulk information of growth rates, we can also look at small colonies under a microscope and investigate growth rates of individual cells. Here, we simply mount a freshly inoculated agar pad containing LB medium on a microscope slide and capture images over time. If our initial concentration of bacteria is sufficiently low, we can watch isolated, individual bacteria proliferate into massive colonies of cells over the course of just a few hours. Though we could do this with phase contrast microscopy, this module will introduce you to modern fluorescence microscopy to acquire time lapse movies.

### 2.6 Your Mission

- Measure bulk growth rate under optimal conditions in LB medium.
- Measure how an anti-bacterial substance (kanamycin) affects actively growing \textit{E. coli}.
- Determine the correspondence between OD\textsubscript{600} and cell concentration in liquid medium.
- Determine the growth rate of cells on an agar pad with LB medium by acquiring images of individual cell colonies.

### 3 Protocol

#### 3.1 Bulk Assay

Prior to your involvement:

1. You and your partner will be assigned a particular kanamycin (antibiotic) concentration with which to assess growth rate.
2. Overnight cultures of wild type E. coli will be inoculated in 40 mL of LB medium, and incubated on a shaker at 37°C for 2 hours prior to the start of the experiment. This ensures that the lag phase has passed, and that (slow) exponential growth has begun.

3.1.1 Bulk Assay Experimental Procedure

1. Based on your assigned kanamycin concentration, find the corresponding flask in the water bath shaker. The appropriate amount of kanamycin has already been added to each flask. (See prelab problem 1.)

2. NOTE: The TAs will have blanked the spectrophotometers using 1mL of LB. Remember that absorbance is always measured relative to a blank.

3. Remove a 1 mL sample of your culture using a sterile pipette. Deposit this into a 1.5 mL cuvette and immediately bring it to the spectrophotometer.

4. Carefully insert the cuvette into the spectrophotometer in the correct orientation (so that the beam goes through the thin side). Make sure the spectrophotometer is set to read OD$_{600}$ and measure the absorbance. Record the reading and the EXACT clock time.

5. Properly dispose of sample cuvette.

6. Considering your first OD$_{600}$ measurement and the rule of thumb relating OD$_{600}$ to cell concentration, properly dilute your sample with LB to about 100 cells/mL. This will require serial dilution. Try to be accurate in your pipetting! Bracket your dilution by a factor of 10 on either side. For example, if you decide to make a $10^3 \times$ dilution, also make a $10^2 \times$ and a $10^4 \times$ dilution. Make sure to write down your dilution factors on the respective vials.

7. For each of the three diluted samples, evenly spread 200 µL with beads on a LB plate. Make sure to clearly label your name, date, OD reading, and dilution factor on the plate.

8. Place the plate upside down in the 37°C incubator. We will let it incubate overnight and then count colonies and determine the exact correspondence between OD$_{600}$ and cell density.

9. Continue to take OD measurements for the rest of the class period by repeating steps 3 through 5 every 15 to 20 minutes. Be sure to record the exact time and OD of each measurement.

10. When the experiment is over, enter your time points and OD measurements on the spreadsheet on the laptop in lab. Be sure that your kanamycin concentration is entered correctly.

3.2 Growth Movie

As a complement to the bulk measurements you did, you will measure cell division at the single cell level under the microscope. The idea is to not only compute the cell doubling time based on the movie you will obtain, but also to introduce the single cell measurements that can be done using an automated microscope.

3.2.1 Preparing the agar pads

The agar pads will be prepared by your TAs, but the protocol for making them is displayed below. Each group should have two or three, just in case there is a problem with one.

1. Stretch a piece of parafilm on the bench. Place an 18×18 mm coverslip on the parafilm.
2. Pipette about 500-750 µL of LB with 2-3% agarose.

3. Drop another coverslip on top in order to “sandwich” the agarose. This step might be easier to do with your gloves off.

4. Let it dry for 30 minutes.

3.2.2 Mounting the sample

1. Your TAs will provide you with two samples of cells: one at an appropriate concentration for our growth movies (about $10^6$ cells/mL), and another at a higher concentration (about 10-100x more concentrated). While not strictly necessary, the latter is used to make finding the correct focal plane easier (see below).

2. Cut three 3 to 5 mm squares of agarose. Cover the pads with a lid of a plate or a tip box lid while they are drying (without touching them!). The idea is to make sure the pad is not too wet and not too dry. If it is too wet, your cells would float in a drop of water. At the same time, you want to make sure the pad is not too dry, since cells need moisture to divide. (We’re making multiple pads in case something goes wrong with one.)

3. Spot 1 µL of cells on each pad. Remember that you should NOT touch the pad with the pipette tip! Two pads should get cells from the low concentration and one pad from the high concentration cultures.

4. Cover the pads with a lid of a plate or a tip box lid and let them dry for a few minutes until the water spot is no longer visible. Depending on how dry the pad is, we might put it in the 37°C incubator to speed up drying.

5. Gently transfer the pads to a petri dish as demonstrated by your TA. Remember that the side where you spotted the cells should face the bottom of the petri dish. Be sure to mark the edge of the dish with a Sharpie and note in your notebook the orientation of the pads in the dish so you know which is which when you go under the microscope (remember to account for the mirror image flip when inverting the dish!).

6. Seal the dish using parafilm. Make sure the parafilm does not interfere with the placement of the dish on the stage. The parafilm is meant to reduce evaporation from the pad which would result in drying.

7. Put a drop of oil on the bottom of the dish where the pads are located. Load the dish onto the stage with a petri dish adapter.

3.2.3 Setting up the movie

1. Launch MicroManager.

2. Make sure you have the correct phase ring for the 100x objective.

3. Find the focal plane of the cells on the pad and set up Köhler Illumination in bright-field live. (Finding the focal plane can be difficult on the low-concentration pads. We had you prepare the high concentration “helper” pad simply to help you find a rough focal plane on a pad with many cells; then, only fine adjustments will be needed after moving to the others.)

4. Go to the Multi-D Acquisition window and open the XY list (next to the use XY list option).
5. Move around the pad to mark 5 to 10 different positions on the XY list where you see interesting things. For example, you might want to include at least a couple areas with only one or two cells in the middle and some other areas with a lot more cells. The number of positions you can image is limited by the frame rate (from Problem 3) and the autofocusing time for each frame at each position (see item 8 below), so don’t choose too many.

6. Next determine a good exposure time for the fluorescence channel. This is done by trial and error. To avoid bleaching the cells you want to image for the movies, find a position with some cells far away (at least several fields of view) from the positions you marked in the XY list. Stop live and switch to the mCherry channel (if your microscope does not have an mCherry channel, use TRITC instead). Set an exposure time and take a snap. Iterate until satisfied (photobleaching means you don’t have unlimited iterations!). Different scopes will vary, but most will have reasonable times are between 100 ms and 2000 ms. **Important:** Switch the channel back to brightfield when done.

7. Set up the channels to be used. You should add the brightfield channel without binning. Make sure that you have a reasonable exposure (75-100 ms). Also include the mCherry (or TRITC) channel, with the exposure time you determined above. **Important:** the fluorescent channel must be listed above the brightfield channel (or else µManager may photobleach all the cells and ruin the movie).

8. Set up the autofocus by selecting “Autofocus” and open its option dialogue. Choose the following options:

   - 1st number of steps: 12
   - 1st step size: 1.5
   - 2nd number of steps: 8
   - 2nd step size: 0.3
   - Threshold: 1
   - Crop ratio: 0.75
   - Channel: Brightfield

   Close and reopen the dialogue box for changes to take effect. With these settings autofocusing takes about 20 sec on the Olympus scopes and about 30-40 sec on the Nikons.

9. Choose “Save files to acquisition directory” and choose the appropriate subdirectory in the Bi1x2016 folder on the desktop. Also, choose “Single Window” in the “Display” option. This is important for long movies; otherwise you will run out of RAM and MicroManager can crash.

10. Before you start taking your movie make sure that everything works. Do a Multi-D acquisition for a single time point. This results in a single picture for each of your stored positions. If you are having issues, ask your TA!

11. Now you are ready to take your movie. Think carefully about how often you want to take frames (See prelab question 3.)

4 **Assignment**

**Problem 0 (Summary).**

Write a summary of this experiment, its objectives, and conclusions between one paragraph and one page in length.
**Problem 1** (Kanamycin dilution (prelab for growth curves)).
Suppose you are given a 40 mL flask and you want to add stock kanamycin (at a concentration of 10 mg/mL) so that the final solution has kanamycin concentration of 2 µg/mL. How much stock kanamycin should you add?

**Problem 2** (Serial dilution (prelab for growth curves)).
Say you measure an OD$_{600}$ of 0.05 for your first measurement in a growth curve. Write down a sensible protocol for the serial dilution to get 1 mL of a solution of about 100 cells/mL in LB medium.

**Problem 3** (Microscope frame rate (prelab for growth movie)).
When acquiring your growth movies, how long should you tell MicroManager to wait between frame acquisitions? What is your reasoning?

**Problem 4** (OD calibration).
Count the colonies on your plates and use this value to determine a calibration factor between OD$_{600}$ and absolute number of bacteria. How does this calibration factor compare to our rule of thumb that OD$_{600} = 0.1$ corresponds to $10^8$ cells/mL?

**Problem 5** (Molar extinction coefficient for *E. coli* in LB medium).
What is the molar extinction coefficient $\epsilon$ of *E. coli*, in LB medium, given that the path length through the cuvette is 1 cm? Express your answer in units of cm$^2$/pmol (square centimeters per picomole).

**Problem 6** (Plots of bulk growth curves).
The Bi 1x staff will email you the data sets of the bulk assays from the entire class. This provides growth data for a range of kanamycin concentrations. Plot all of the curves (OD$_{600}$ vs. time) on the same plot.

**Problem 7** (Regression and doubling times).
Take the logarithm of the data you plotted in problem 6 and fit curves. What feature of these curves corresponds to the bacterial growth rate? Write the growth rates in terms of bacterial doubling time. Comment on these doubling times.

**Problem 8** (Effective kanamycin concentrations).
Look at your curves from the above problem. What minimum concentration of kanamycin is necessary to prevent bacterial growth?

**Problem 9** (Doubling time from movies).
Estimate the cell doubling time from your movies acquired on the microscope by using the segmentation and regression techniques we discussed in class. Do you get the same division rate in all positions on the pad?
Problem 10 (Data and code).
Attach all code used for data analysis. Also, include the following:

a) Write down the exact serial dilution protocols you did calibrate your OD$_{\text{g00}}$.

b) Show the initial and final frames of two of the positions you took data from. Make sure to include scale bars, time stamps and captions. You should burn in your scale bar as we discussed in class. You may label the time points in the figure caption, but you can also easily add time stamps using Fiji’s time stamper tool. Follow the menus: Image → Stacks → Time Stamper. Comment on anything particularly notable about these images.

c) Show segmented (binary black-and-white) images for the images you showed in (b).