Bi 1x Spring 2014: *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 a light microscope. 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, which is around the entire human population, overnight. As with human or animal populations growing under optimal conditions, the rate of population increase will be 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 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 a number of 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 antibiotic resistant strains. To better understand the concepts involved in combating bacterial infection, we will measure the bulk growth rate of *E. coli* in growth medium after challenge with various concentrations of the antibiotic kanamycin. We measure cell growth using a light scattering, as outlined in the following section. Knowing growth rates provides useful information about the fitness of the bacteria in the medium of interest.

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 (scattering with no change in momentum). 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 $I = I_0e^{\epsilon lc}$, 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 translation efficiency of the sample is the ratio of the final and initial light intensity. $T = I_1/I_0$, and the reported quantity is called the absorbency $A = \ln(1/T) = \epsilon lc$. The general rule of thumb is that the accurate assonance 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, absorbance is often called optical density, abbreviated OD$_{600}$ 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 later count the number of bacterial colonies present. Because the initial solution was dilute, 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$_{600}$ and cell concentration. A good rule of thumb is that an OD$_{600}$ of 0.1 corresponds to about $10^8$ cells/mL.

2.5 Bacterial growth

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. We use phase contrast microscopy, which we learned about in last week’s lab, to visualize the bacteria.

2.6 Your Mission

- Measure baseline bulk growth rate in LB medium.
- Measure how an anti-bacterial substance (Kanamycin) affects actively growing *E. coli*.
- Determine the correspondence between OD$_{600}$ and cell density 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. Groups of two will be assigned a particular antibiotic concentration with which to assess growth rate.
2. Overnight cultures of wild type *E. coli* were inoculated in 20 mL of LB medium, and incubated on a shaker at 37°C for 30 min 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. Add the appropriate amount of stock kanamycin (which is 10 mg/mL) to your culture tube to get the desired total kanamycin concentration. (See prelab problem 1.)

2. 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.

3. 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.

4. NOTE: You will have a blank sample, a sealed cuvette with sterile medium. Absorbance is always measured relative to a blank. Be sure to measure the blank before every culture measurement!

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 dilute your cells $10^3$ times, 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 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 2 through 5 every 10 to 15 minutes. Be sure to record the exact time and OD of each measurement.

10. When the experiment is over, either you or your lab partner should email Bill (wireland@caltech.edu) a comma separated value (CSV) file with your growth curve data. The first column is the time in minutes of each measurement, and the second column is the measured OD$_{600}$ value. Include the concentration of kanamycin you used in your email.

### 3.2 Growth Movie

As a complement 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 to introduce the single cell measurements that can be done using an automated microscope.

#### 3.2.1 Preparing the agar pads

1. Stretch a piece of parafilm on the bench. Place a 24×50 mm or 24×60 mm coverslip on the parafilm.

2. Pipette 2-3 mL of LB with 1.5% low melting temperature 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. Cut two 3 to 4 mm squares of agarose and put them on the slide. Cover the pads while they are drying (without touching them!). The idea is to make sure the pad is not too wet. Your cells would float in the drop of water, and not too dry at the same time, cells need moisture to divide. (We’re making two pads in case something goes wrong with one.)

2. Spot 2 µL of cells on each pad. Remember that you should NOT touch the pad with the pipette tip!

3. Cover the pads with 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.

4. Gently transfer the pads to a Wilco petri dish as demonstrated by your TA. Remember that the side where you spotted the cells should face the bottom of the Wilco petri dish.

5. Place the dish on the microscope stage leaving the lid open. Make sure that the environmental chambers are properly closed. The idea behind this step is to let the pad equilibrate with the temperature of the environmental chamber. If you close the lid and see condensation forming then open it again and wait some more time.

6. After around 15 minutes, seal the dish using parafilm. Make sure the parafilm does not interfere with the placement of the dish on the stage. This step is meant to reduce evaporation from the pad which would result in drying.

7. Put a drop of oil on the 100× objective and on the bottom of the dish where the pads are located. Load the dish onto the stage with a petri dish adapter.

8. Make sure the phase ring corresponds to Ph3.

### 3.2.3 Setting up the movie

1. Launch Micro-Manager.

2. Find the focal plane of the cells on the pad and set up Köhler Illumination in bright-field live.

3. Go to the Multi-D Acquisition window and open the XY list (next to the use XY list option).

4. 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 some areas with only one or two cells in the middle and some other areas with a lot more cells.

5. Set up the channels to be used. You should use the brightfield setting without binning. Make sure that you have a reasonable exposure! (75-100 ms)

6. Set up the autofocus by selecting “Autofocus” and open its option dialogue. Choose the following options:
7. Choose “Save files to acquisition directory” and create a sub folder in “Bi1X2014” folder on the desktop to save your images. Also, choose “Single Window” in the “Display” option. This is important for long movies; otherwise you will run out of RAM and Micro-Manager can crash. NOTE: Always save your files in the local drive first!

8. 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 one of the frames fails to focus go back to the position and make sure it has not drifted out of the range of the autofocus search. If you are having issues, ask your TA!

9. 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)).
How much stock kanamycin should you add to your culture tube?

Problem 2 (Serial dilution (prelab for growth curves)).
Say you measure an OD\textsubscript{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 Micro-Manager 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\textsubscript{600} and absolute number of bacteria. How does this calibration factor compare to our rule of thumb that OD\textsubscript{600} = 0.1 corresponds to 10\textsuperscript{8} cells/mL?

Problem 5 (Molar extinction coefficient for \textit{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? Make sure you report the units of $\epsilon$.

**Problem 6** (Plots of bulk growth curves).
Download all of the bulk assay data from the class. This provides growth data for a range of kanamycin concentrations. Plot all of the curves ($\text{OD}_{600}$ vs. time) on the same plot.

**Problem 7** (Linear regression and doubling times).
Take the logarithm of the data you plotted in problem 6 and fit linear 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** (Logistic equation as a growth model).
In the previous problems we assumed the bacteria grew exponentially as described by equations (1) and (2). Instead the bacteria could be following a logistic growth model, or

$$\frac{dc}{dt} = rc \left(1 - \frac{c}{K}\right), \tag{3}$$

where $K$ is the carrying capacity, or the maximum number of bacteria that can be present and still have growth. Typically, the carrying capacity for *E. coli* in LB media occurs at a concentration corresponding to $\text{OD}_{600} \approx 7$. The logistic equation has a $c^2$ term describing cell death (negative cell growth). What might be the physical basis for this term? If the logistic growth model is correct, your calculated growth rate will have some error. Can this source of error be ignored? Explain.

**Problem 10** (Doubling time from movies).
Estimate the cell doubling time from your movies acquired on the microscope by using the segmentation technique we discussed in class. Do you get the same division rate in all positions on the pad?

**Problem 11** (Division times).
Look at the movies corresponding to one or two positions and manually track the different cell division times. How do you decide that a cell has divided? Make sure to comment on your criteria for division. Plot a histogram of the division times. Make sure to label your axes. Find the mean division time and its standard deviation. Make sure to include units. How does the division time compare to the bulk results? If there is a difference, why do you think this could be?

**Problem 12** (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 $\text{OD}_{900}$. 

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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 12