

Bi 1x Spring 2020

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, within a certain dynamic range. 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

Bacteria can grow and divide very rapidly. 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

$$\frac{dN}{dt} = rN, \quad (1)$$

$$\Rightarrow N(t) = N_0 e^{rt}, \quad (2)$$

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, temperature, and 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 led 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 $I = I_0e^{-\epsilon lc}$, where ϵ is the wavelength-dependent molar absorption coefficient of the species in question, l is the path length through the cuvette (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 $A = \ln(1/T) = \epsilon lc$. 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 OD₆₀₀ 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 ϵl), 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 OD₆₀₀ and cell concentration. A good rule of thumb is that an OD₆₀₀ 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 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 *E. coli*.
- Determine the correspondence between OD₆₀₀ 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 1 mL of LB. Remember that absorbance is always measured relative to a blank.
3. Remove a 1 mL sample of your culture using a 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 OD₆₀₀ and measure the absorbance. Record the reading and the EXACT clock time.
5. Bleach your cuvette for 15 minutes before dumping the sample down the drain and throwing the cuvette in the biotrash.
6. Prepare a series of eight ten-fold dilutions of your culture (i.e., 10⁰ to 10⁻⁷) in 1 mL volumes using the provided Eppendorf tubes.
7. Each person in your group then takes four sequent dilutions (i.e., 10⁰ through 10⁻³). Label your plate with your initials, time, OD₆₀₀ measurement, and label the indicated spots with your dilutions—one per spot. Spot 10 µL of each of your dilutions at the indicated marks. To do this, go to the first stop of your pipette before touching the agar and allow the droplet itself to touch the agar, fully expelling the volume. Do not stab the agar with your tip; if you do, ask for a new plate. Close the plate between each sample.
8. Gently tilt the plate until vertical, allowing the droplets to streak to the opposite edge of the plate. Avoid mixing the droplets.
9. Place the plate back flat onto the benchtop until the liquid dries.
10. 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 OD₆₀₀ and cell density.
11. 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.
12. After your final OD measurement, make serial dilutions and a new set of drip plates.
13. 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 compute the growth rate based on the movie you

will obtain. This serves 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 pads, just in case there is a problem with one.

1. Prepare LB with 2% agarose, keep in water bath to prevent it from solidifying (temperature depends on agarose used; for ours, this is 42 C).
2. Align three microscope slides in parallel on the benchtop.
3. On each of the outer two slides, place two coverslips on top of each other at the center of the slide. These will determine the height of your agarose pad.
4. Pipette 250 μL of LB with 2% agarose onto the middle slide.
5. Lower another slide across the agarose and coverslip spacers, creating an even pad.
6. When the agarose solidifies, split the slides, revealing the pad on one of them. Use a coverslip to cut the pads into approx 3×3 mm squares.

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 10-100 \times more concentrated). While not strictly necessary, the latter is used to make finding the correct focal plane easier (see below).
2. 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.
3. 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.
4. Gently transfer the pads to a glass-bottomed 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!).
5. 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.
6. You will need to use a petri dish adapter for your microscope's stage to mount the dish.
7. Begin by finding your focal plane using 10 \times and 40 \times objectives. When you are ready to move to 100 \times , remove your plate from the stage, make sure that there is a TA supervising, and rotate in the 100 \times objective.
8. Put a very small drop of oil on the 100 \times objective and 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. Use the high-concentration “helper” pad to find a bacteria and get a rough estimate of the focal plane for your more dilute pad that you will use for imaging. You will need to apply oil to the glass bottomed dish, since you will be using a 100× oil objective. You should use brightfield illumination for phase contrast microscopy while doing this. The TAs can help you with this, and also setting up Köhler illumination.
2. While still on the concentrated pad, determine a good exposure time for the fluorescence channel. This is done by trial and error. Different scopes will vary, but most will have reasonable times are between 100 ms and 2000 ms. Begin with shorter exposures.
3. Move over to a more dilute pad, again imaging using phase contrast (not fluorescence). You should now only need to make fine adjustments to the focus to find the bacteria. You want to find one to four bacteria that are far from others for your growth movie, since you do not want other colonies encroaching.
4. When you have found the cells you want to watch grow, you will acquire images over time. Set up an acquisition where you take a brightfield (phase) image and a fluorescent image for each time point. We will be manually focusing and acquiring the images, so set a timer to remind you when to take the images. Think carefully about how often you want to take pictures (see problem 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).

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).

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).

When acquiring your growth movies, how long should you wait between frame acquisitions? What is your reasoning?

Problem 4 (Plots of bulk growth curves).

You have access to the data sets of the bulk assays from the entire class, so you have growth data for a range of kanamycin concentrations. Plot all of the curves (OD_{600} vs. time) on the same plot.

Problem 5 (Regression and doubling times).

Take the logarithm of the data you plotted in problem 4 and perform a regression to get the growth rate. 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. *Hint:* While you could use `scipy.optimize.curve_fit()` to do the regressions, to compute the slope from a linear regression, you can use the `np.polyfit()` function. This fits x - y data to a polynomial. To fit to a polynomial of degree one (a line), use `np.polyfit(x, y, 1)`. The first entry in the returned Numpy array is the slope and the second entry is the intercept.

Problem 6 (Effective kanamycin concentrations).

Look at your curves from the above problem. What minimum concentration of kanamycin is necessary to prevent bacterial growth?

Problem 7 (Doubling time from movies).

Estimate the cell doubling time from your movies acquired on the microscope by using the segmentation techniques we discussed in class and again performing a regression. Do you get the same division rate that you got for the bulk measurements in the absence of antibiotics?

Problem 8 (Data and code).

Attach all code used for data analysis. Also include a few representative images displaying how well your segmentation method performed. Don't forget that your axes in your image displays should be in units of microns.