Welcome to Caltech’s BE Bootcamp 2011. Today you will be exploring the size of bacteria with microscopy and the rate of bacterial growth with spectrophotometers. Please read relevant protocols in their entirety before starting!

**Part 1 – The Size of Things**

**Background:**
Through these exercises we hope you will learn about the typical sizes of microbes, multi-cellular organisms, organelles, their shapes, “colors”, and modes of growth and feeding. Necessarily, this requires that we learn about microscopy. In particular, you will acquaint yourself with modes of light microscopy: bright-field (BF) and phase contrast (PC). Additionally, certain samples are either naturally fluorescent or they have been modified chemically or genetically to have specific fluorescent markers. Fluorescence microscopy is a mainstay of modern biological microscopy. For additional tutorial on basics of microscopy, check out the course website: [http://www.rpgroup.caltech.edu/courses/PBL/bootcamp2011/protocols_and_references/](http://www.rpgroup.caltech.edu/courses/PBL/bootcamp2011/protocols_and_references/)

**Goals:**
1. Go over the Micro-Manger Manual attached at the end of this handout with your TA.
2. Understand the basic mechanisms and light paths of light and fluorescent microscopy, know the location of: eyepieces, objectives, stage, focus adjustments, condenser aperture diaphragm, field stop, phase ring, lumen, fluorescent filter block turret, and intensity filters (excitation and emission).
3. Be able to setup Köhler Illumination in bright-field.
4. Familiarize yourself with the basics of objectives: magnification, depth-of-field, working distance, aberration correction, numerical aperture (objective and condenser), and the effect of imaging medium (air vs. oil) on resolution.
5. Have a working understanding of how to capture images with a CCD camera, including how contrast, gamma, bit depth, saturation, binning, exposure time, frame rate and image format are all related to the quality of video or still images.
6. Spatially calibrate your microscope at multiple magnifications:
   a. Using a “graticule” to directly correlate pixel size with spatial dimension, estimate the error in your calibration.
   b. Using the known pixel size on the CCD and objective magnification, estimate the calibration.
   c. Verify that magnification and spatial calibration have a linear relationship.
7. Take images of the various organisms that we have on hand, using the different microscopy methods, and save images for possible future analysis or show-and-tell. Time constraints will likely limit each group to two or three organisms, but please try to take at least one fluorescent sample. Your TA will show you how to mount these specimens.
8. Having a notion of the time scale of various biological processes can be quite valuable – now we will focus on the time scale of cell division and photo-bleaching. You will use your knowledge of microscopy to take real-time and time-lapse videos of these processes.
   a. Making agarose pads for single cell microscopy as demoed by your TA:
      i. Place a piece of parafilm on a glass plate and put a 24mm×60mm coverslip on top of it.
      ii. In a conical tube make up 1.5% (w/v) low-melting agarose in desired media:
         • LB – Luria-Bertani; rich media for growth of bacteria
         • PBS – Phosphate-Buffered Saline; lowest autofluorescence but no carbon source
      iii. Microwave until agarose has dissolved, forming a viscous fluid.
      iv. Pipette 2-3mL of the melted agarose onto the coverslip to cover the whole surface, gently lay another coverslip on top, and let it sit for 5-10 minutes until the agarose harden.
      v. Cut hardened agarose into 1cm×1cm square pads.
   b. Add 2µL of your sample on top of an agar pad. Be aware not to poke the pad with your pipette tip! Let the sample dry on the pad for 15 minutes at 37°C.
   c. Transform the pad onto a Wilco dish that was made to be specialized for microscopy. If you are using an inverted microscope, make sure your sample is facing downwards on the dish! Ask a TA for help if necessary.
   d. Experiment 1 – Photobleaching of fluorescent proteins in E. coli:
      i. Set up the microscope:
         • Objective: 100x magnification
         • Phase: 3
         • Fluorescent Turret: YFP or mCherry
      ii. Mount your sample onto the stage. Add a drop of oil on the 100x objective lens and a drop of oil on the back of the Wilco dish where your sample is located.
      iii. Adjust focus and set up Köhler illumination.
      iv. Take continuous snapshots in the corresponding fluorescent channel until the fluorescent protein is completely bleached. For example, 200 frames at 1 sec/frame interval.
   e. Experiment 2 – Bacterial cell division:
      i. Equilibrate the Wilco dish that is loaded with your sample in the 37°C culture chamber for 15 minutes. Be sure to leave the lid open.
      ii. Set up the microscope:
         • Objective: 100x magnification
         • Phase: 3
      iii. Mount your sample onto the stage. Seal the Wilco dish with a strip of parafilm. Add a drop of oil on the 100x objective lens and a drop of oil on the back of the Wilco dish where your sample is located.
      iv. Adjust focus and set up Köhler illumination.
      v. Look for single cells at 5 different positions use the “XY-List” option. Take enough snapshots to observe 2-3 division events in BrightField only. For example, 20 frames at 3 min/frame interval.
Part 2 – The Rate of Things

Cell Growth and Spectroscopy
While there are a multitude of important time scales in biology, arguably one of the most important is concerned with the rate of cell division. In the bacteria E. coli, rates of cell division are closely linked with evolutionary fitness and hence our basic understanding of natural selection. To better understand the concepts involved in cell division, we will measure the bulk growth rate of E. coli in media with different amounts of antibiotic. We measure cell growth using spectroscopy and optical density as outlined in the following section. The included formulas will prove useful in analyzing your spectroscopic data.

Beer's Law and Optical Density
It is often extremely useful to know the concentration of cells in a liquid culture, and can be used in various kinetic measurements of fitness and protein production. Light scattering is the primary method by which we measure cell density. In the following section, we will discuss the basic physical concepts that allow determination of concentration as well as the formulas that relate optical properties to concentration.

The basic physics are as follows:
1. Light, with a wavelength comparable to the size of the object being measured (600 nm), enters the sample at an initial intensity ($I_0$).
2. 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 amount 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 serially diluted to get an accurate reading.)
3. Upon exiting the sample, the light intensity is reduced by $I_i = I_0 e^{-abc}$, where $a$ is the wavelength-dependent molar absorption coefficient of the species in question, $l$ is path length – usually 1 cm in modern spectrophotometers, and $c$ is the concentration, this is known as Beer's Law. See the figure below:

4. The transmission efficiency of the sample is the ratio of the final and initial light intensity, $T = I_i/I_0$, where the measured quantity, called “absorbance” is $A = \ln(1/T) = acl$. The general rule of thumb is that accurate absorbance readings lie in the range $0.01 < A < 1$. (Serial dilutions of the sample will be necessary to bring $A$ within this range.)

Measuring E. coli Growth
For more see:
This experiment will take ~3 hours and everyone will participate, working in groups of two or three.

Goals:
- Measure baseline growth rate in rich media.
- Measure growth with added antibiotic.
- Determine the correspondence between OD\textsubscript{600} and cell density.

Prior to your involvement:
1) Groups will be assigned a particular mixture in which to assess growth rates.
   i) Growth 1 – LB + 0 µg /mL Kanamycin
   ii) Growth 2 – LB + 2 µg /mL Kanamycin
   iii) Growth 3 – LB + 4 µg /mL Kanamycin
   iv) Growth 4 – LB + 6 µg /mL Kanamycin
   v) Growth 5 – LB + 8 µg /mL Kanamycin
   vi) Growth 6 – LB + 10 µg /mL Rifampicin
   vii) Growth 7 – LB + 20 µg /mL Rifampicin
   viii) Growth 8 – LB + 30 µg /mL Kanamycin
2) Cultures of wild type (MG1655) \textit{E. coli} were inoculated into 50mL of each of these media, and incubated on a shaker at 37°C for one half-hour prior to the start of the experiment. This ensures that the so-called “lag phase” has passed, and that (slow) exponential growth has begun.

Procedure:
1. Every 10-15 minutes (as time and coordination allows), remove a 1mL sample of your culture using a sterile pipette. Deposit this into a 1mL cuvette and immediately bring to the spectrophotometer (the “spec”).
2. Carefully insert the cuvette into the spec in the correct orientation. Make sure the spec is set to read OD\textsubscript{600}, and measure the absorbance. Record the reading and the EXACT clock time.
   a. You should have been given a “blank”, i.e. a sealed cuvette with sterile media. Absorbance is always measured relative to the blank. Be sure to measure the absorbance of the blank with every culture measurement!
   b. You may want to take readings in more than one of the available specs, to check their consistency.
3. Properly dispose of the sample cuvette, and repeat these steps for the next ~3 hours.
4. When you reach OD\textsubscript{600} ~0.1 and at your maximum OD\textsubscript{600}, remove 100µL from the culture and put it in a labeled eppendorf tube.
   a. For the moment, let us assume OD\textsubscript{600} 0.1 = 10^8 cells / mL. With that in mind, properly dilute your sample with LB so that 500µL contains on the order 50 cells (~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\textsuperscript{3} times, do another 10\textsuperscript{2} and 10\textsuperscript{4} dilutions.
   b. For each of the diluted samples, evenly spread 50µL with beads on a LB plate.
   c. Incubate the plates overnight at 37°C. Later, we can count colonies and determine the exact correspondence between OD\textsubscript{600} and cell density.

Questions:
   i. What is the cell density in units of CFUs/mL at OD\textsubscript{600} 0.1? (Hint: CFU = Colony Forming Unit)
   ii. What is the margin of error on this figure and how do you determine it?
   iii. Calculate the molar extinction coefficient of \textit{E. coli} at 600 nm?
5. Afterwards you should have a list of times and absorbances. With the blank’s absorbance subtracted from each sample value, make a plot of absorbance vs. time.
Questions:

a. What do you expect to see in such a plot?

b. Is there anything strange about your plots? Can you calculate a doubling time(s)?

c. Can you estimate the MIC (minimum inhibitory concentration)? It may help to share your data with other students.

d. What is the clinical significance of the MIC and what can sub-MIC doses of antibiotic lead to in the long run?