**Introduction**:

If you had to choose between broccoli and chocolate, would you eat both simultaneously or one at a time? Which one would you eat first? How and what do you think a mouse or a fly would choose? What about a bacterium?

According to the famous Monod's experiment[1], E. coli can choose to eat a certain sugar even when multiple types of sugar are present in the growth media. Today, we are going to focus on one of the most well known examples—the lac operon.

The lac operon controls the production of three different genes associated with the metabolism of lactose. One of these genes is lacZ, which encodes for the enzyme beta-galactosidase which metabolizes lactose. When the growth media has glucose and lactose E. coli will not produce any beta-galactosidase. It will only produce it once it has consumed all the glucose. This means that the cell has a way of determining if there is no glucose and if there is lactose in the media.

In this experiment we will explore the part of the circuit that takes care of detecting the presence or absence of lactose in the media. Instead of lactose, which would get eventually eaten by the cells, we use the inducer IPTG. This small molecule interacts with Lac repressor in much the same way that the real sugar does except that it cannot be cleaved by the enzyme beta-galactosidase, making it a useful experimental substrate.

In the absence of lactose or IPTG, Lac repressor binds to an operator that overlaps the lac promoter. Because of this overlap, RNA polymerase cannot bind to DNA to initiate transcription, resulting in repression. More interestingly, there are three operators in the wild type lac operon with different binding strengths. Tetramerized Lac repressor can even bind to two operators at the same time, resulting in a DNA loop which enhances the repression effect.
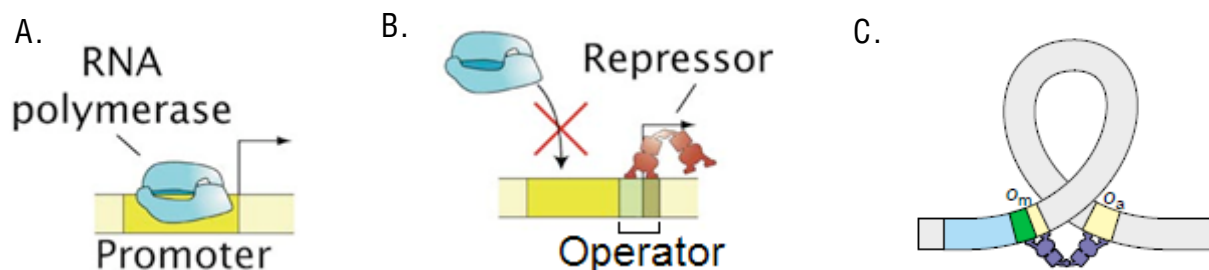

Fig 1. Repression in the *lac* operon. (A) A RNA polymerase molecule binds to its promoter on the DNA to initiate transcription. **(B)** When a repressor molecule binds to its operator, it blocks part of the promoter and leads to repression. **(C)** A tetramerized repressor can bind to two operators at the same time, thus forming a DNA loop and enhancing the repression effect.

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[1] Jacques Monod "The growth of bacterial cultures" Annual Reviews Microbiology. 1949. 3:371-394.

However, no response in biology is really digital. There is no such thing as something being "on" or "off". You can instead ask an analog question: to what extent is something off?

The Construct:
In this experiment, we will use two synthetic sequences for the lac operon: one with a single operator O1, and another one with two operators Oid (an operator with ideally strong binding strength) and O1. In both of the sequences, the target lacZ gene was replaced by a YFP gene controlled by a promoter called lacUV5 (a mutant of the wild type *lac* promoter).
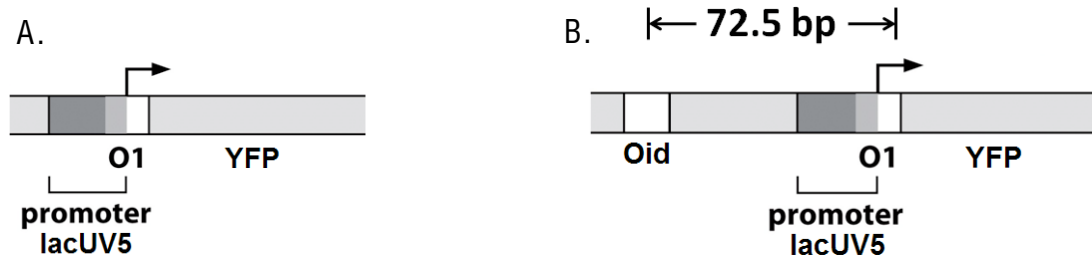
Fig 2. The construct. (A) Sequence with a single operator O1. **(B)** Sequence with two operators: Oid and O1. The length of the spacer DNA between the two operators is 72.5bp. The promoter in both of the sequences is lacUV5 controlling the expression of a YFP gene.

Measuring gene expression:
We will measure the fluorescence expressed by cells containing the construct described above for different concentrations of IPTG. We want to define a fold-change in gene expression, a measure of the relative change in gene expression upon the addition of inducer.

One problem with measuring fluorescence in cells is that the cells are fluorescent themselves! This means that if you measure the fluorescence of a cell expressing YFP you will actually be measuring the fluorescence of the YFP molecules on top of the autofluorescence of the cell. In order to account for this you can measure the autofluorescence of a cell that lacks any YFP.

We will define the fold-change in gene expression as

$$fold\text{-}change([LacI]) = \frac{Fluo([LacI] \neq 0) - AutoFluo}{Fluo([LacI] = 0) - AutoFluo}$$

Here, AutoFluo is the autofluorescence and Fluo([LacI]) is the fluorescence in cells with varied amounts of Lac repressors. However, since we can't directly measure the concentration of Lac repressors, but we can control the amount of IPTG, instead we can express the fold-change as

$$fold\text{-}change([IPTG]) = \frac{Fluo([IPTG] \neq 0) - AutoFluo}{Fluo([LacI] = 0) - AutoFluo}$$

To measure Fluo([LacI≠0]), we use a modified strain of cells with the same YFP gene but no Lac repressor.

Note that Fluo[IPTG] refers to the total integrated fluorescence in a cell. This is because we are interested in the total gene expression in a cell. How will you correct for autofluorescence, given that cells have different areas (i.e., how should you calculate AutoFluo)?

In order to quantify the level of gene expression you will need to write code in MatLab or other language of your choice. You will have to find the cells automatically and use the mask in order to integrate over the fluorescence of each cell.

Experimental protocol:
What happened behind the scenes:
Five hours before you started your experiment we diluted cells from an LB overnight culture into minimal media in the presence of different concentrations of IPTG. This particular minimal media has salts, a carbon source and some amino acids.

We also prepared agar pads now made with PBS (phosphate buffer). Cell cannot grow in this medium. The idea is that we will be taking snapshots of their state when we took them out of the culture.

Due to time limitation, we had mounted the samples on the Wilco dishes for you. For each construct, there are 8 samples: 0uM (no IPTG in the media), 10uM, 50uM, 100uM, 500uM, 1mM, NoLacI, and NoFluo. Thus, you will have two Wilco dishes and 8 agar pads on each plate. Before starting imaging, we had let the pads equilibrate to the temperature inside the scope chamber before sealing the plate.

NOTE: Remember to keep track of what sample is on which pad!! This is extremely important so that you can convert your images into a curve which reflects the level of gene expression as a function of the inducer.

Your mission:
Start with the single operator construct.
1) Set up the plate on the scope. We will take the images at room temperature.
2) Set up Micro-Manager to take a picture of Brightfield (with phase contrast) and FITC (green/yellow fluorescence) at the same time (Ask the TAs about **Multi-D Acquisition** if you are not sure how to use it). Decide on what exposure to use for your snapshots by looking at the brightest sample, the one with NoLacI. Make sure there is no saturation in the fluorescence channel!
3) Take 5 snapshots of the NoFluo control and of the NoLacI sample. You want to shoot for having more than 100 cells per sample. These two are the most important samples as they're fundamental to calculating the fold-change. That is why we want to make sure right from the beginning that everything went OK.
4) Now, take 5 snapshots on each of the remaining strains. Make sure to save all your data!
5) Repeat steps 1-7 for the other construct.

Assignment:

1) Write a code to find the cells in the phase contrast image. Use the function **regionprops** to explore properties such as *MajorAxisLength*, *MinorAxisLength*, *Area*, etc. It is also a good idea to play with the ratio of these two magnitudes, as they set some bounds on the shape of the object. Explain the reasoning behind any of the thresholds that you draw using the **regionprops** properties.

2) Make and show an overlay of one of your favorite phase contrast pictures and the mask (Hint: explore the function **cat**).

3) For each cell, overlay its corresponding mask with the fluorescence image and calculate the total fluorescence per cell. For example, if you want to choose cell number 4 in a labeled image called ImLabel (the one created by the function **bwlabel**) you can do

>> ImCellFluo = immultiply(ImLabel==4,ImFluo);

where ImFluo is your fluorescence image. The total (uncorrected) fluorescence of the cell is

>> sum(sum(ImCellFluo));

and the area can be found using **regionprops**. Note: When calculating the total fluorescence per cell, make sure to subtract the autofluorescence and background (these steps are not shown above). Ask for help if it is not clear to you how to do this.

4) For each construct, repeat the analysis for each snapshot and strain. Make a histogram of the areas per cell for each of the following: the NoFluo sample, the NoLacI sample, and your favorite sample that was grown in the presence of IPTG. Comment on the differences you saw, if there is any.

5) Finally, calculate the fold-change for each one of your samples for both constructs. Use the function **mean** to calculate the mean level of fluorescence corresponding to each strain. Don't forget to include error bars! You can obtain a standard deviation by using the function **stdev** and a standard error by combining your standard deviation with the length of the vector (using the function **length**).

6) Plot fold-change vs. IPTG concentration. Do it on a linear and a log-log plot. Try to fit these plots to the following two formulas (Hint: explore the functions **fittype** and **fit**):

$$(1) \quad fold\text{-}change = \frac{\beta \cdot [IPTG]}{K + [IPTG]} \qquad (2) \quad fold\text{-}change = \frac{\beta \cdot [IPTG]^n}{K^n + [IPTG]^n}$$

where β is the *maximal expression level* of the promoter, K is a *dissociation constant*, and n is called the *Hill coefficient*. Be sure to include error bars in your plots and explain possible sources of the errors.

Which formula gives a better fit? What features of the curve change as you change the value of n ? Compare the fitted values of n between the two constructs. Explain any difference.

7) According to PBoC Chapter 19.2.5, we can express the repression level of Lac repressors as inverse of the fold-change. Using equation 19.28 in PBoC and the binding energy of the operator O1, estimate the number of effective Lac repressors in the cell as a function of concentration of IPTG for the single operator case. Plot the data points. Be sure to include error bars. What is the role of IPTG (positive or negative regulation)?