Introduction

Directed Evolution is a branch in synthetic biology which aims to hijack the evolution selection process in our favour to direct a DNA sequence to a desired phenotype. This protocol describes the pipeline we intended to follow to make our approach to direct evolve our IMT3_sfGFP sensor. From the variability induction suing the Mutagenesis Plasmid 6 (MP6) [1] to the different bacteria selection using cell sorting. The final objective is to be able to reconnect this process to make different evolutionary cycles (fig 1).

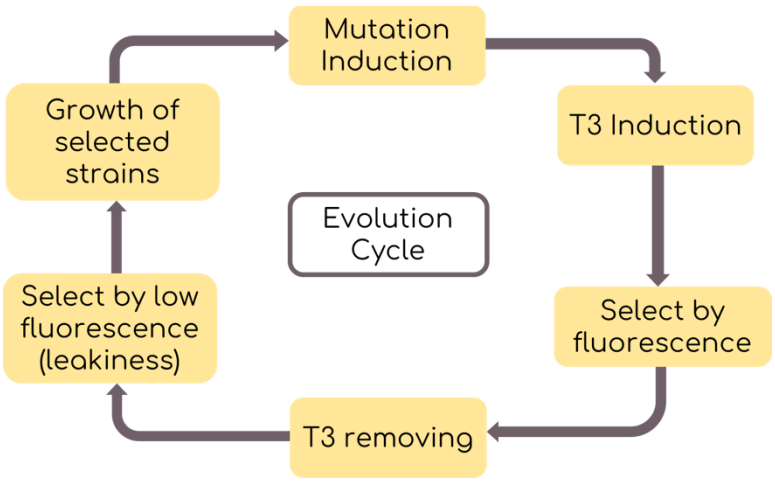

Figure 1: Evolution Cycle

Materials

- MP6 plasmid
- Target plasmid to evolve
- Arabinose
- Glucose
- LB + glucose Plates
- Electro competent cells
- Cell cytometry and cell sorting facilities

Pipeline:

Mutation Induction: creating variability

The first thing we need in order to evolve anything is to generate variability to see which one is better than the others. There are different approaches to creating variability in a DNA sequence: mutagenic compounds, ionizing radiation, duplication errors, etc. Here we found a study where they induced mutagenesis in bacteria using the MP6 plasmid [1]. Broadly speaking, this plasmid encodes a DNA polymerase subunit that has two properties: being more prone to errors and having more affinity to create the DNA polymerase construct, and other proteins that stabilize and/or increase the mutagenesis effect of the plasmid. This leads to an increased random mutagenesis, in the case of MP6 up to 10^{-5} mutations per bp per generation.

A good thing about the MP6 is its inducibility, the mutagenesis effect is governed by the promoter Pbad. This promoter is activated by arabinose and inhibited by glucose. Meaning that we have a tuneable control over the intensity of the mutations and a temporal control over the time these mutations are happening.

In practise, we already had the miniprep MP6 with Chloramphenicol resistance and the CloDF13. This is important because we want it to be compatible with our IMT3_sfGFP_pUC-Amp, witch it is. Firstly, we have different selection markers (two different resistances) which makes easier to select the cells that have both plasmids. And secondly, the pMB1 (from pUC) ori and the CloDF13 ori are compatible, meaning that they don't compete for the same machinery to duplicate, ensuring the continuity of both plasmids in the cell.

1. Co-Transform BL21 electrocompetent cells with MP6-Clor and IMT3_sfGFP_pUC-Amp via cell electroporation.
2. Since we are looking for diversity, inoculate the cells in liquid LB media with Chloramphenicol, Ampicilin and 100mM Arabinose (to induce the mutation plasmid) and cultivate overnight at 37ºC shaking.

This makes that in every cell there is the MP6 that will randomly mutate the bacteria genome and the plasmid, meaning it mutates the IMT3_sfGFP sequence. The next day, we will have a populations of cells that each one will have different versions of the IMT3_sfGFP_pUC-Amp plasmid. To unify a plasmid version per cell, we will perform a miniprep to extract all the variants of plasmids, and repeat the electroporation transformation, this way each bacterium will have a single plasmid variant.

3. Using the miniprep product, transform BL21 cell via electroporation.
4. Part of the transformed cells will be plated in Ampicilin + Glucose LB plates and the rest of transformed cells in a LB liquid culture with ampicillin and glucose. Let grow overnight at 37ºC.

The glucose is used to stop the mutagenesis in the case that a bacteria had been co-transformed with MP6. The colonies that will grow in the plates, will be used to sequence the IMT3_sfGFP. Each colony represents a variant of the sequence, and the results will be used to make a study on how the mutations appear, how are distributed or any relevant information about the mutagenesis process. The liquid LB cultures will be used to perform cytometry studies.

5. Prepare liquid cultures of each colony to miniprep the next day and sequence.
6. For the liquid culture, prepare it for cell cytometry and induce it with an arbitrary 80mM of T3.

In cell cytometry we will be looking for two things. First with traditional cell cytometry, we will evaluate the fluorescence of our sensor for each cell at 80mM of T3. This will map a distribution of fluorescence. Additionally, a cell cytometry of our non-mutated control IMT3_sfGFP_pUC-Amp strain can give us the mapping of fluorescence before mutations. Comparing these two fluorescent profiles, we would be able to see if any subpopulation is brighter than the control, meaning that exists mutations that enhance the fluorescence of the sensor.

The second thing we want to obtain from cell cytometry, specifically from cell sorting, is the ability to sort the cells by its fluorescence. We would have to set a threshold that separates the bacteria that bright from the fluorescent-related mediocre. The Flow Cytometry Core Facility (PRBB) of our institution gave us the option to sort the bacteria and place it in an agar plate forming a grid of selected bacteria, that we will be able to grow and know the sequence, to see if there is any predominant mutation that gives the sensor the ability to bright more.

This way, we would select the bright specimens when T3 is in the media. However, a problem arouses from this. Showing more fluorescence when T3 is added does not mean a better performance on the sensor, is should not emit fluorescence (or the minimum as possible) when not induced with T3. That's why a new liquid culture will be created including all the bacteria that reached the threshold (apart from the ones plated).

These new culture will be prepared for cell sorting but will not be induced with T3. Then, when sorting, the condition of selection will be that emits less fluorescence as possible (meaning it has less leakiness). Again, these selected bacteria (double selected to be precise) will be individually plated in agar plates forming a grid. This way we will have single colonies from the "best" sensing bacteria, that can be sent to sequencing to make a study on what changes have had been produced.

What have we seen is a full cycle of directed evolution. Ideally, these selected bacteria will be grown and co-transformed again with the MP6 plasmid, inducing once again mutagenesis. Then will be treated as the first batch of mutant cells, repeating the cycle as many times as we would want.

The theory says that more fit sensing bacteria will arouse from diversity, making us to believe that by doing this process of directly evolving our sensor, we will actually get a better Intein Mediated T3 (IMT3) biosensor. A very strong and versatile tool indeed.

References:

[1]: Badran, A., Liu, D. (2015). **Development of potent in vivo mutagenesis plasmids with broad mutational spectra.** *Nat Commun*, 6, 8425. https://doi.org/10.1038/ncomms9425