BACTERIO'CLOCK

Horloge Biologique

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Summary

Our project aims at biologically devising a *"oscillating FIFO behaviour, synchronized at population level"*. Such a setup will trigger periodic events and, therefore, can be considered as a *"biological clock"*. To completely deserve this appellation, the system has to fulfill the following specifications :

- **Oscillatory system** : It will consist in providing a periodic output for the duration of the experiment. To do so, we will use a genetic cascade, initiated by a specific inducer which last step will inhibit the previously mentioned inducer.
- ***FIFO* System** : The period of the oscillation is even more interesting if it allows the sequential switching on and off of several genes. Our setup involves three genes which will get activated and desactivated successively as a *"FIFO : First In, First Out"*. This sequence is monitored by a logic structure called *Feed-Forward Loop* (*FFL*).
- **Synchronization** : Yet, being able to control this sequential activation within a single cell can be seen as a "first step" in biological clock devising. In order to amplify this phenomenon (to observe it in an easier way or even to find future applications), it has to be extened to a whole population of bacteria. Here comes the synchronyzation issue: we will use methods based on the *"quorum sensing"* phenomenon.

We will base our project on an already existing structure, partly fulfilling the evoked specifications: the system that leads to the production of E. Coli *flagella*.

1 Oscillations

The system must successively activate and inhibite at least one genic expression, continuously along time. In this objective we will use a very simple logical system, based on a structure with two genes : X and Y. X activates Y and Y represses X, in such a way that we get the following *cascade* :

X starts to express
↓
the expression of X activates Y
↓
the expression of Y represses X
↓
X is no more expressed
↓
Y is no more activated
↓
X is no more repressed
↓
X can restart to express...

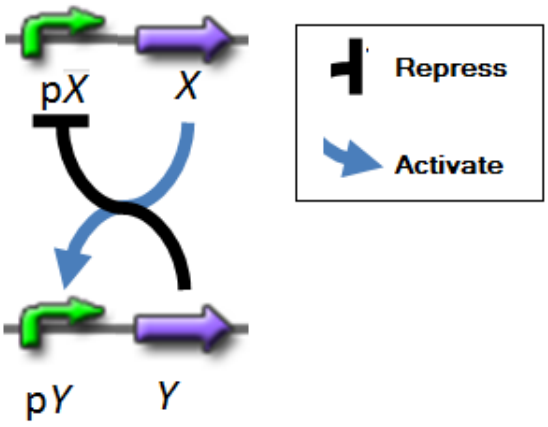


Figure 1: Basical principal to create oscillations

In practice, this type of cascade is very difficult to implement biologicaly. The competition between the expressions of X and Y often leads to a steady-state : the proteins from X and from Y stabilise themselves to constant concentrations, in such proportion that their formation and degradation rates cancel each other.

To solve this problem, we will intend to :

- realise a *"Feed-Forward Loop"* (*FFL* — see section **2 Séquence FIFO**), which introduces a delay between the repression of X and the disappearance of Y. It allows, at least, the existance of several periods before the stabilisation in a steady-state.

- repress indirectly X by Y, by the mediation of a small diffusive molecule A (built by an enzyme that results from the expression of Y — see section **4.1 Synchronisation**). A will diffuse in the whole medium, as an intercellular messenger. Moreover, its action is easily tunable (by introducing a "destructor" of this molecule, or by inhibiting the specific receptor).

- tune the parameters of production, action and degradation of A, until it causes the existance of two incompatible states ("presence/action of A" *vs.*

"absence/inaction of A") in the cycle. That will avoid establishment of a steady-state (which would involve co-existance of the previous states).

2 FIFO order

To describe orders of appearance/disappearance of signals in a sequence, we can use the terms of *"queue"* and *"stack"*. In the former, also called FIFO, the order respects the principal *"First In = First Out"*: the first person arrived in the queue will leave first. In the latter, also called LIFO, the order respects *"Last In = First Out"*: the last object put on the stack will be removed first.

If the expressions of several genes are under the control of only one inductor, whose influence raise then decrease, the "activation thresholds" of this genes (that we assume different) are successively overpassed, then underpassed, in the *LIFO* order: the one whose threshold is the highest is activated last, and is shut down first.

Now, as it is the case for a clock, which counts hours from 1 to 12 and then restarts from 1, our device must control the expression of several gene in the *FIFO* order (see Fig 2).

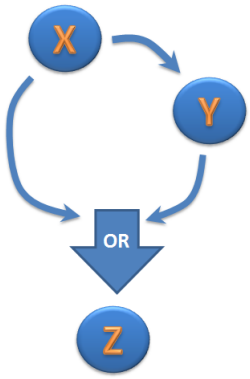
LIFO clock (3 signals)			hour	FIFO clock (3 signals)		
signal1	signal2	signal3		signal1	signal2	signal3
•	•	•	0	•	•	•
☼	•	•	1	☼	•	•
☼	☼	•	2	☼	☼	•
☼	☼	☼	3	☼	☼	☼
☼	☼	•	4	•	☼	☼
☼	•	•	5	•	•	☼
•	•	•	↪ 0	•	•	•
Confusion h1/h5 and h2/h4			(6 hours)	No confusion		

Figure 2: Order for a clock

Conceptually, in the "control theory," there are two kind of systems that can adapt themselves to environmental variations (for example, to ensure a steady–state) according to whether they integrate the output signal in the computations (Feed–Back) or not (Feed–Forward).

There exists combinations of 3 gene regulatory interactions that are considered as *Feed–Forward Loops* (*FFL*). Given the nature of regulatory interaction between these 3 genes, different FFL can be defined. In our system, we use an *OR gate C1–FFL* (see Fig 3, page 5). With *multiple output*, it should allow us to implement

the FIFO behavior, if the kinetic constants are correctly fixed. We will control the successive expression of three genes (see Fig 4, page 6).



X is the master regulator : it directly acts on Z, and indirectly by controlling Y. These two actions are *coherent* (both "induction relationships"). They are combined by an *OR* logical gate : if X or Y are active, then Z will be activated.

Figure 3: *OR* gate Coherent type 1 *Feed-Forward Loop* (C1–FFL).

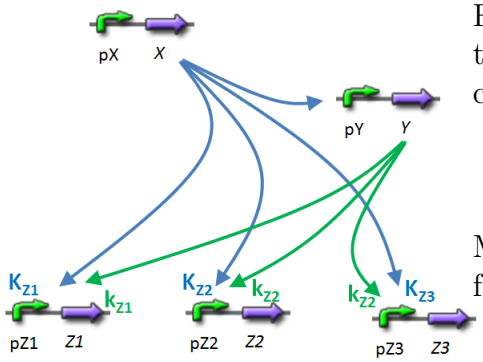
3 The fagella construction system of *E. coli*

Biologically implementing a *FIFO* system appears to be complicated, given the fact that we have to control the different activation/inhibition constants. Hopefully, the bacterium *E. coli* has a fagella construction system in which the proteins involved are assembled successively in a determined order to ensure the correct structure formation and functioning of the flagella. As a consequence, we can use the genes coding for these proteins to implement our *FIFO* system. Considering previous sections notation, "X" will be *flhDC*, "Y" will be *fliA*, and "Z_i" will be, among others, *fliL*, *fliE*, *fliF*, *flgA*, *flgB*, *flhB*, *fliA*, *fliD*, *flgK*, *fliC*, *meche*, *mocha* and *flgM*. It seems that their expression follows a FIFO dynamics [1] (see section **4 Difficulties of our implementation**).

We selected for *Z1*, *Z2* et *Z3* (partially because of their well adapted activation thresholds [1, 2] — see section **2 FIFO Order**) the promoters of *fliL*, *flgA* (eventually replaced by *flgB*) and *flhB*.

Moreover, in the natural system, the presence of FlgM inhibits the expression of *fliA* – thus preventing further flagella assembling; FlgM is then expulsed from the cell by the basal part of the flagella, and yet allowing the continuation of the remaining process[1]. We do not take into account this phenomena in our project and we ensure the control of the actvation order by using a *FFL*. Furthermore, the flagella formation is not necessary for our *FIFO* implementation but we just consider the right set of genes and promoters that is useful for our project.

(a)



From the graph (b) we observe that the activation constants must respect the following conditions:

$$K_{Z1} < K_{Z2} < K_{Z3}$$
$$k_{Z1} > k_{Z2} > k_{Z3}$$
$$(\text{here, } K_Y < K_{Z1}$$

Moreover, the following conditions must be fulfilled :

$$tk_{Z1} < \tau K_{Z1} \qquad tk_{Z2} < \tau K_{Z2}$$
$$tk_{Z3} < \tau K_{Z3} \qquad \tau K_{Z1} < \tau k_{Z1}$$

(b)

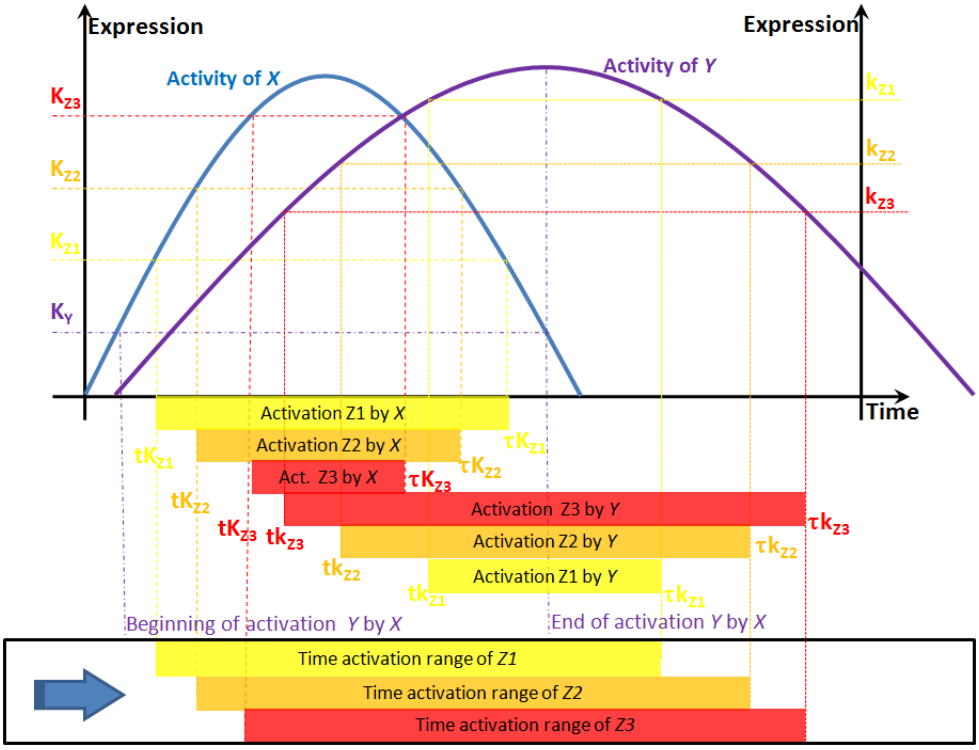


Figure 4: Multiple Output OR gate C1–FFL *FIFO*. (a) Genetic design (b) Kinetics of gene expression

For a gene Z, we call K_Z the activation threshold of Z by X and k_Z that of Z by Y. We denote tK_Z (resp. tk_Z) the time at which the expression of X (resp. Y) overpasses K_Z (resp. k_Z) and τK_Z (resp. τk_Z) the time at which the expression underpasses that threshold.

We must notice that the induction of *fliA* by *flhDC* comes after the inductions of *Z1*, *Z2* et *Z3* ($K_Y > K_{Z3}$ — see Fig 4, page 6); and *fliA* activates itself in combination with *flhDC*[3], in order to avoid a premature drop of *fliA* expression

(see Fig 5, page 8).

4 Inherent problems for our system

The natural system present in *E. coli* does not show such a loop device described in the section **1 Oscillations**. Moreover, previous descriptions of the systems are only valid at the individual level. And yet, oscillations should be performed by the *whole population* in the culture. Finally, other important problems related to the implementation of the system are described in the following.

4.1 Synchronisation

The best way to synchronize a population of cells regarding to a periodic phenomenon is to introduce a *check point* at the end of every cycle. For that, we will use a feedback loop (see section **1 Oscillations** — action of Y on X) that is under the control of *quorum sensing* system. This system consists in a little molecule (that we name A) that diffuse in the medium and can only be active at high concentration — said in another way, A is considered active when only the major part of the population is at the end of the cycle.

The introduction of a destructing enzyme of A would enhance and eventually precise its action. We don't yet represent this alternative in the Fig 6, page 9.

4.2 Growth in a constant exponential phase

The formation of a flagella is an event regulated by many different environment factors. For our project, we will need to minimize as much as possible those outside effects. One of those factors is the state of the growth phase. The flagella system is only active in the *exponential phase of growth*[1]. This is why we must maintain the population in this latter. For this, we will re-use the genetic system engineered by the Ron Weiss team in Princeton called *"program population control"*[4].

This program also goes through *quorum sensing* communication, therefore we must check for crosstalking between both quorum sensing systems. We propose then to use two compatible systems that have already been described recently working together in the same population [5].

4.3 Pratical details and feasability

The diverse interactions described in our models (see figure 6, page 9) must be tuned as precisely as possible in order to get the best efficiency and robustness for our system. In particular, we will have to check that promoter we use behave

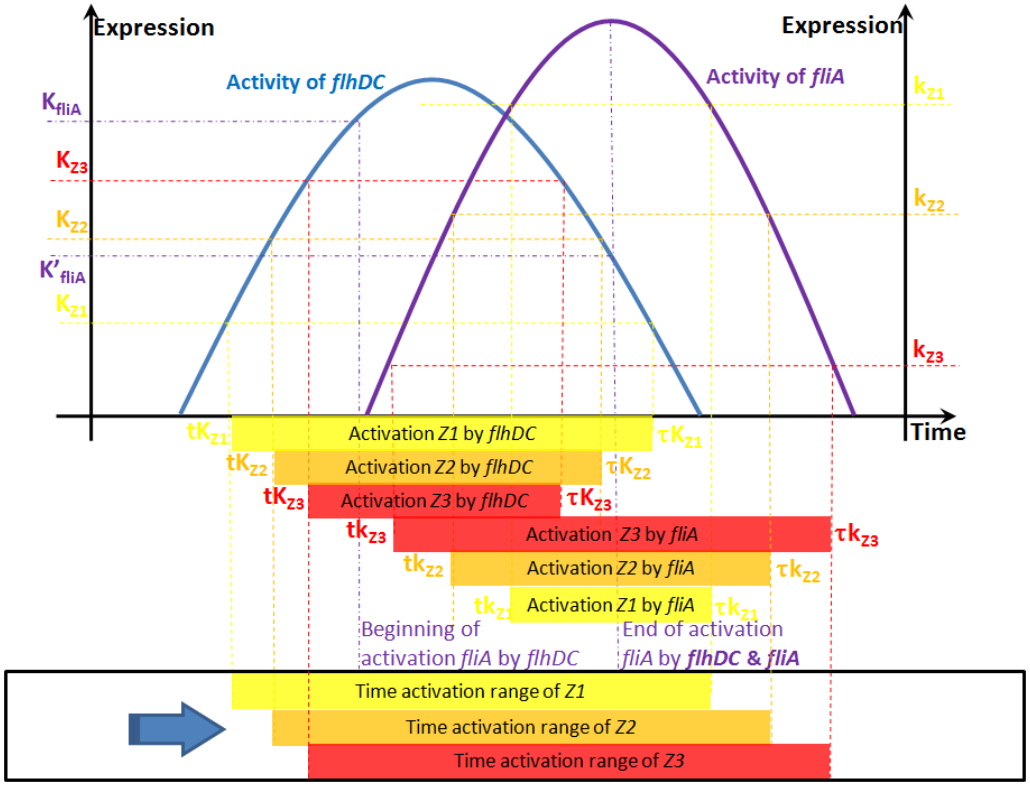


Figure 5: FIFO sur le modèle *E. coli*
For a gene Z, we call K_Z the activation threshold of Z by X and k_Z that of Z by Y. $K_{fliA} > K'_{fliA}$ correspond to the auto-activation of *fliA* (see section **3 The fagella construction system of *E. coli***)

as described in the corresponding references (*SUM* logical gates instead of *OR* and *FIFO* sequence for gene expression order – see section **2 Séquence FIFO**). Then, previous considerations – for instance, the models with the constants K_Z et k_Z – are simplifications.

The diagram on Fig 6, page 9, introduces the most functionnal of our models. We keep in mind other alternatives, like the use of the natural promoter of *flhDC* (inhibited by OmpR) instead of P_{Tet} (inhibited by TetR).

On the diagram, near the interactions with P_{FliL}, P_{FlgA} et P_{FlhB}, are written the "affinities" from the target with its activators (the greater is the number, the lower is the corresponding "activation threshold"), quantified in [2].

Lastly, we keep the opportunity to introduce in the medium molecules of aTc (anhydrotetracycline), which reduce the action of TetR on P_{Tet}, in order to tune the "negative Feed–back".

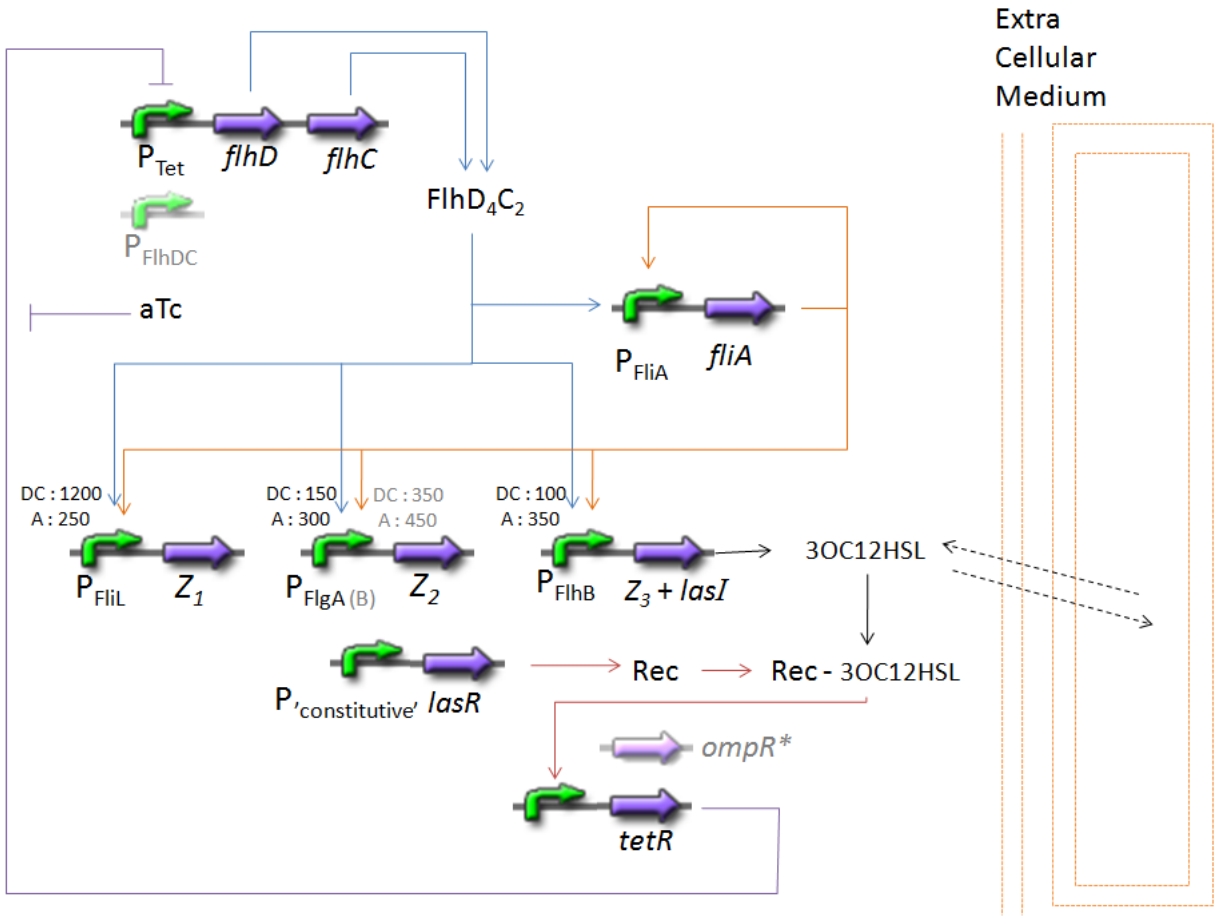


Figure 6: General structure of our project
Only the parts that concern strict implementation of the fonctions that we defined are represented : for example, the *program population control* system is not described.

Conclusion

Each aspect of this project represents, on its own, important challenge of synthetic biology. Performing oscillations along time, ordering gene expressions and synchronise a population of bacteria, once achieved, would surely become basical elements (because needed) for many further creations of genetical engineering. Beyond the surname *"clock"*, such a system, gathering these functionnalities, would most probably get applications in a lot of fields of biology. For example in medicine, it is the way onto the development of "smart drugs". By activating different products successively each at the right point, such a drug could coordinate the effects in order to enhance or secure them.

References

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