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Dynamic DNA-toolbox reaction circuits: A walkthrough

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ABSTRACT

In living organisms, the integration of signals from the environment and the molecular computing leading to a cellular response are orchestrated by Gene Regulatory Networks (GRN). However, the molecular complexity of *in vivo* genetic regulation makes it next to impossible to describe in a quantitative manner. Reproducing, *in vitro*, reaction networks that could mimic the architecture and behavior of *in vivo* networks, yet lend themselves to mathematical modeling, represents a useful strategy to understand, and even predict, the function of GRN. In this paper, we define a set of *in vitro*, DNA-based molecular transformations that can be linked to each other in such a way that the product of one transformation can activate or inhibit the production of one or several other DNA compounds. Therefore, these reactions can be wired in arbitrary networks. This approach provides an experimental way to reproduce the dynamic features of genetic regulation in a test tube. We introduce the rules to design the necessary DNA species, a guide to implement the chemical reactions and ways to optimize the experimental conditions. We finally show how this framework, or "DNA toolbox", can be used to generate an inversion module, though many other behaviors, including oscillators and bistable switches, can be implemented.

1. Introduction

In cells, Gene Regulatory Networks provide a general framework for the implementation of computing tasks at the molecular level [1–5]. Indeed genes can be wired in networks of cross interactions through, for example, the expression of regulatory proteins or transcription factors. Only very recently have similar general chemical reaction networking frameworks been described *ex vivo* [3,5]. Similar to what has been done for neural networks, whose fundamental features have been abstracted into the computational framework of Artificial Neural Network circuits (ANN), these approaches focus on the most important dynamic properties of biological GRN to define functional *in vitro* models. Therefore it has become possible to construct *in vitro* reaction networks with well-controlled topologies [4], targeting a precise dynamic function and reproducing biological architectures.

Extracting the essential dynamic features of GRN from the viewpoint of dynamical systems, one is left with a set of collective molecular transformations that can be linked in such a way that the product of one either activates or inhibits the production of another [3,6]. A second important feature is that the network linking these reactions is hardcoded in the sequence of stable DNA strands (genes and promoters). The long-term stability of these DNA species stands in sharp contrast with the dynamic behavior of their products (RNA and proteins) that are constantly produced and degraded/diluted. Therefore, the maintenance of a constant flux of energy through the system, together with a precisely controlled reactivity landscape are also essential features of GRN. We reproduce these three essential characteristics in a set of *in vitro* biochemical transformations: modularity, dynamism and template control of the reactivity.

Here we briefly introduce the general theoretical and experimental framework of a specific approach, the Polymerase/Exonuclease/Nickase Dynamic Network Assembly toolbox (or PEN DNA toolbox), and discuss its properties. We show how activating reactions can be connected together in arbitrary cascades and linked to some inhibiting reactions. Since the basic aspects have been described elsewhere [4,7,8], this paper seeks to address bulk experiments and will not treat the spatially resolved systems also developed in our lab [9,10], as the programming of reaction-diffusion systems is still in its very early stages. The main issues encountered during the construction of a targeted network will be discussed.

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Section 2 begins by laying out the DNA toolbox, and looks at its elementary operating processes. Section 3 describes in detail the workflow, molecular components, design and construction rules, the monitoring and evaluation methods, and wetlab concerns. Section 4 summarizes and exemplifies the previous sections by implementing an inversion module.

2. The DNA toolbox

The system uses a reduced set of only three DNA polymerizing–depolymerizing enzymes: a polymerase, a nicking enzyme (a restriction enzyme that cleaves only one side of a DNA duplex, upon binding to a specific recognition site) and an exonuclease. Globally, the concerted activity of these three enzymes starts from deoxynucleotide triphosphate (dNTP) as substrates, that are converted to deoxynucleotide monophosphate (dNMP) through a polymerizing–depolymerizing process (direct conversion from dNTP to dNMP is kinetically blocked). It thus corresponds to a dissipative process going down a chemical potential; because our experiments are set in a closed tube (no mass transfer), the system will stop working once all the dNTPs are exhausted (Fig. 1).

The second element of the system consists of non-degradable DNA templates (they are protected against the exonuclease by backbone modifications [11]). The 5′ half of these templates directs the sequence of the DNA strands that will be produced by the polymerase (see Fig. 1A). Moreover, because the polymerase requires a primer to initiate its catalytic activity, the 3′ part of the template provides a region that is used to regulate the reaction: a template becomes active (i.e. acts as a support for the polymerase) only if it can find and bind the complementary sequence of its 3′ end, with the additional requirement of a matched terminus for the primer. On the contrary, a primer whose 3′ end does not match perfectly the template will tend to block polymerizing activity. These templates are designed with the recognition sequence of the nicking enzyme positioned in such a way as to introduce a nick in the middle of the nascent strand, thereby releasing the primer (*input*) and a short DNA, the output [1,2,12]. Moreover the temperature of the experiment is set above the melting temperature of the input or output strands but below that of full duplexes. Therefore, while inputs and outputs dynamically hybridize and melt, only the nicking step can release the template and its product at the end of the catalytic cycle.

The system – nicknamed ‘DNA toolbox’ in reference to the fact that it allows the rational building of networks of arbitrary topology – functions as follows:

- First, an input strand transiently binds a template on its input site.
- Second, the primer/template structure is recognized by a polymerase, and elongated to a stable full duplex.
- The nicking enzyme then releases the input and output from the template: they are now able to diffuse and bind other templates.

In their free, single-stranded form, inputs and outputs are recognized and degraded by the exonuclease. The exonuclease we use is a processive enzyme and does not lead to the accumulation of partially degraded intermediates, which would be detrimental to the reaction. The concentration of any species that is not actively produced by a template will therefore eventually decay; the concentration of a species involved in a positive feedback loop of sufficient strength will reach a non-zero steady-state (assuming the exonuclease is not saturated).

Templates can also be temporarily sequestered in an inactive form by inhibitor strands that are partially complementary to the template but have some mismatches at the 3′ end, hence do not trigger polymerization (see Fig. 1B). These inhibitor molecules are also dynamically produced (by the action of the polymerase and nicking enzyme on other templates) and subject to degradation (by the exonuclease, when they are in single-strand, free-floating form).

The modular construction of the system allows the designer to “wire” the templates together so that they control each other’s activity by exchanging small activators or inhibitors. This modularity is central to the building of complex systems; it allows the cascading of elementary modules into precise network topologies. The cascadability is greatly facilitated by the fact that the DNA strands being exchanged are small (typically 10–20 bases long) and do not form complicated secondary structures. Arguably, this range of length theoretically limits the number of species that can be mixed together before unwanted interactions start to occur: of the 11 bases that constitute the shortest oligonucleotides (see Section 3.2 for further details on sequence design), 5 are dedicated to the nicking enzyme restriction site, leading theoretically to more than 4000 (4^6) sequences. After excluding the sequences that may produce cross-talks, secondary structures, G-repeats and other concerns (such as parasitic nicking sites), the realistic number of available sequences is approximately 150. In other words, for medium scale, biologically relevant networks, choosing DNA sequences while avoiding unwanted interactions is entirely feasible. With a correct selection of the sequences, the product of any reaction can be used as the activator of any other module. For that, it is enough to design (and order to a DNA-synthesizing company) the corresponding templates with the *ad hoc* sequences and modifications.

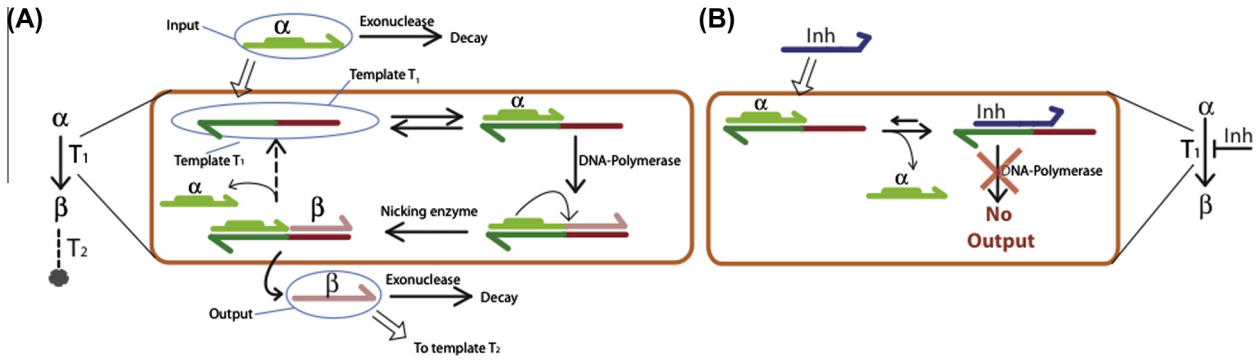

Fig. 1. Building of *in vitro* networks. (A) The biochemical machinery used to implement one edge of the reaction network, that is, an activation link between two DNA species: the template is activated by an oligonucleotide that is 3′-matched to the template and triggers the production of an output oligonucleotide of similar length. The reaction is performed above the melting temperature of these sequences, so de-hybridization happens spontaneously. (B) Inhibition of these activation links is done with oligonucleotides that bind in the middle and are 3′-mismatched to their target template. Consequently, they sequester the template without producing outputs.