Dynamic DNA-toolbox reaction circuits: A walkthrough

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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.

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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.
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 5’ end of the template but have some mismatches at the 3’ end, hence do not provide 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 5’ end of the template.

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'-mismatched 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.

In their free, single-stranded form, inputs and outputs are recognized and degraded by the exonuclease. The exonuclease 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, and 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 (4^5) 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.

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In a similar way, it is always possible to define an inhibiting module targeting any activation template. This modularity opens the road to the rational molecular encoding of a large variety of dynamic behaviors.

3. Methods

3.1. From a desired behavior to a DNA toolbox-based network

The first step in the process of molecular programming with the DNA toolbox (Fig. 2) is to define the target behavior. What function should the wet system perform? Since a variety of dynamic approaches or circuit architectures can lead to the same behavior, we currently focus only on the macroscopic role of the system, meaning what kind of information processing it can perform. The set of reactions included in the DNA toolbox provides a general framework to construct arbitrary networks where nodes are able to activate or inhibit the production of other nodes; and where the system is continuously updating its state (in chemical terms, it is kept out of equilibrium). It is thus a priori possible to construct an infinite variety of dynamical systems [3,6,13,14]. Molecular approaches (not limited to the DNA toolbox) have already yielded experimentally validated oscillators [4,6,7,15], switches [3,7,16], memories [7], game players [17], robots [18,19], calculators [20] and image processors [21].

The second stage is then to find a relevant network organization. The original concepts of the DNA toolbox were taken from gene regulation, and thus biological networks provide a preeminent source of inspiration to link function and network structure [1] such as oscillators, bistable switches, decision switches, gradient detection, etc. However, because most dynamical systems are based on the same fundamental combination of activating, inhibitory and non-linear processes, many other sources of inspiration can be tapped into to select an architecture (for example, electronic circuits, either Boolean [20] or analog [22], or neural networks [23,24]). Recently, interaction networks from ecosystems have also been taken as models and reproduced as abiotic molecular systems, providing, for example, periodic and chaotic oscillators [15].

For simple cases, mathematical tools such as linear stability analysis or chemical reaction network theory [25] can be used to directly link the structure of the network to its asymptotic dynamic behaviors. It is important to note however, that in most realistic and interesting cases, it is difficult to reduce the actual chemical system to a mathematical description that is compact enough to be handled by such techniques.

For larger systems, like in electronics, combination of modules gives access to new functions [2]. An example based on a bistable memory is given in Fig. 3. Note, however, that insulation of “modules” is generally not guaranteed in a chemical context, and their combination might lead to unexpected cross-talks or load issues, possibly jeopardizing the function [26].

To predict the behavior of these larger networks, which involve a higher number of DNA strands or variables, the alternative consists of brute-force numerical integration of the equations (provided one is able to write a correct set of equations accurately describing the system at hand). In such a case the design of the circuit, taking into account all relevant interactions (i.e. introduced by the designer or inherent to the chemistry itself) can be assisted by computational tools [27].

Once an apparently consistent circuit has been settled with the target function, the last step is to convert this network into effective reactions using the DNA toolbox. If some functions can be straightforwardly obtained with the components (activation cascades or inhibition), others will require the cascading of smaller motifs into an integrated network. These motifs can be reused in a variety of contexts, and may be considered as multi-component operators. For instance, if a second-order amplification is required (i.e. the production rate of output scales quadratically with the input level for low concentrations), generating it with a direct amplification is impossible (single input templates only provide a first-order amplification), but it might be implemented with a bistable module, since second order and bistability are functionally related. This process is facilitated by a graphical representation of the DNA toolbox, where each dynamic DNA species is a node and each template is an edge (see Fig. 3).

When no a priori knowledge on the circuit/function relationship is available (for example, while trying to design a network displaying a specific, arbitrary time trace), stochastic optimization strategies – such as evolutionary algorithms – are reliable to discover a relevant network structure and parameters. Even if numerical prediction are efficient enough in that case, the relevance of this approach rests on the accuracy of the mathematical model describing the system in the general case. Softwares that automate this search process are currently becoming available [28].

3.2. Sequence design

The design starts with the selection of the sequences of the dynamic activator species, from which the sequence of the inhibitors can be deduced following simple rules. Once all the nodes (activators and inhibitors) have been attributed a sequence, the construction of the templates linking those nodes is straightforward. A general requirement concerning the sequence design is to avoid cross-hybridization as much as possible.

The largest systems constructed with the DNA toolbox to date contain less that ten DNA sequences and thus manual design is generally used. Note however that automatic sequence design tools for oligonucleotides are available and could be adapted [20,29,30].

3.2.1. Input sequences

The input sequences correspond to the triggers that will prime any DNA polymerization reaction of the DNA toolbox. Their length ranges from 10 to 15 bases, with the upper limit set by the working temperature (they have to dynamically hybridize and de-hybridize) and the lower limit by the sequence requirement of the nicking enzyme (a duplex that does not extend at least one bp beyond the recognition site is poorly processed by the nicking enzyme Nt.BstNBI, which cuts 4 bases downstream of its 5 base cognate sequence; in other words inputs should have at least 10 bases). We typically use templates that are 11 bases long, in which 5 bases – required for the nicking enzyme recognition site – are fixed. Considering two DNA species to be distinct if at least two of their bases are different, we can virtually design more than 1000 different sequences from which a number can be discarded, using the following filters:

1. The sequence (or the concatenation of two sequences linked by an activation edge in the target network) should not contain a parasitic nicking enzyme recognition site.
2. The sequence (or the concatenation of two sequences linked by an activation edge in the target network) should not allow stable secondary structures to form (this could lead, among other uncontrolled behaviors, to self-triggering in the case of a self-fold with a matched 3’ end) or unwanted interactions between two input species (primer dimerization). Several softwares can be used to predict the formation of secondary structures, such as the NUPACK software suite [30].
3. The melting temperature of the sequence should be neither too high nor too low, and typically close to the experimental temperature (i.e. should not be composed of too many C/G nor A/T).
In fact, adjusting the melting temperature of the various DNA species involved in the network is one way to tune the circuit parameters at a local level (together with changing the templates’ concentrations). Therefore, those adjustments can be very important for the proper function of the network. However, it has been reported that the performance of a given primer in terms of amplification is not simply related to its thermodynamic stability (see Fig. 5). The sequence dependence of exponential DNA amplification [12] is still not well understood, but has been the subject of a recent study [31], in which the authors characterized the performance of about 400 autocatalytic templates. They notably observed that GA or AG dimer-rich sequences were poorly performing. The rules proposed by Qian et al. have to be considered for future design of autocatalytic templates [31].

For each design of a new activator sequence, we experimentally evaluate its relative performance by conducting the following procedure.

1. We first perform a simple autocatalytic amplification (Fig. 5, left panel), using a template that bears the new sequence both as input and output. Since we only want to check the amplification potential, the mixture contains the polymerase and nicking enzyme, but no exonuclease.

2. For all sequences that do yield a reasonable exponential amplification, we conduct an evaluation of their dNTP consumption rate in steady state (therefore in a mixture including the exonuclease) in the presence of a limited amount of dNTPs, e.g. 40 μM (Fig. 5, right panel). To compare the autocatalytic modules, it is possible to calculate their normalized plateau time, \( Pt \), relative to the template \( X \):

\[
Pt = \frac{N \cdot T}{(N_x \cdot T_X)}
\]

with \( N \) the number of limiting dNTPs per polymerized oligonucleotide and \( T \) the plateau time. This number provides a global estimation of the ability of one particular sequence to provide fast turnover (which in turn is dependent on many factors, such as the sequence’s stability, but also its affinity for the various enzymes). For instance, this gives, for templates \( d, e, n, r, w \) and \( x \) in Fig. 5, the following values: \( Pt_w = 0.73, Pt_r = 0.86, Pt_n = 0.87, Pt_x = 1, Pt_e = 1.21 \) and \( Pt_d = 2.66 \).

In the present case, it might be wise to avoid working with template \( d \) (which is slow) or \( r \) (which shows a tilted plateau, for unknown reasons).
3.2.2. Inhibitors

We design inhibitors so that they hybridize to the targeted template with a dissociation constant about two orders of magnitude higher than that of the input strand, enough to outcompete them, but not too high in order to maintain a reasonable rate of spontaneous dehybridizing.

Inhibitors should not be sensitive to nicking and hence their design will depend on the geometric peculiarities of the nicking enzyme that is used, for example, whether it nicks within, before or after the recognition site. In the following we discuss the case of the nicking enzyme Nt.bstNBI, which recognizes a 5 base duplex or after the recognition site. The two unpaired bases at the 3’ end prevent the extension of the template-inhibitor partial duplex by the polymerase. Other designs (8-6, 8-5, etc…) are possible. However, too high coverage of the input sequence will lead to an unwanted recognition site for the nicking enzyme, while too low coverage will not result in a high enough affinity for the inhibitor compared to the input.

3.2.3. Template design

The sequence of the template is the concatenation of the input (trigger) and the output’s (product) complementary sequences (in the order 5’-output complementary sequence-input complementary sequence-3’). After the sequence has been determined, certain chemical modifications of the templates are necessary, as described in the following section.

3.2.4. Modifications

3.2.4.1. Phosphorothioate bonds. All templates are protected from the exonuclease by the addition of 2–4 phosphorothioates to their 5’ end. The number of phosphorothioates needed to prevent enzymatic activity depends on the exonuclease that is used, with 2 being enough in the case of mesophilic RecJ but 3 being necessary for the thermophilic enzyme. Note that in the case of the nicking enzyme Nt.bstNBI, the use of more than two phosphorothioates requires the masking of the 5’ end (complementary to the output) nicking recognition site by a dU modification [7] (see Fig. 6).

3.2.4.2. DeoxyUridine. DeoxyUridine can be substituted for dT in the templates since these two bases are not distinguished by the Bst polymerase (and hence a dA is incorporated in the nascent strand). On the contrary, we have found that the nicking enzyme Nt.bstNBI recognizes the dU-containing site with a much lower affinity. This modification thus serves to minimize the competitive inhibition effect (see Fig. 6).

3.2.4.3. 3’ Phosphate modification. Templates are phosphorylated at their 3’ end in order to prevent any unexpected polymerization which may be triggered upon hybridization to another template or the formation of a secondary structure.

3.2.4.4. Labeling. Labeling a template with one fluorophore allows us to monitor the activity of this template during time-lapse experiments (see the monitoring section for further details). This labeling is generally made on the 3’ end of the templates (though 5’-end labeling is also possible) because the fluorescence shift due to nucleobase quenching will appear as soon as an input hybridizes to the template. By specifically labeling each template present in the reaction, the separate monitoring of each effector in the system becomes feasible [8]. Several fluorophores have been tested and only some of them show a detectable intensity shift upon hybridization. The results are summarized in Table 2. It is also important to note that, since these fluorophores are attached to one end of the template, the melting temperature as well as the affinity of the enzymes (polymerase and nicking enzyme) for the template will possibly be affected, leading to some changes in the Pt value as defined above. Some tuning of the experimental conditions (including temperature and template concentration adjustments) may be necessary when trying out a new dye and.
ideally, all tuning steps (see part 3.6.) should be performed with a consistent labeling of all templates.

### 3.3. Buffer

The main purpose of the reaction buffer is to provide suitable working conditions for the three enzymes. The buffer contains a consensual amount of mono- and bivalent ions, and dNTPs, the basic monomers for the polymerization process. Table 1 details the concentration of each component.

We usually prepare 1 mL of a 4x stock solution of this buffer. During the setup of an experiment, the 1x buffer is supplemented with 4 mM of freshly diluted Dithiothreitol (DTT) (Sigma–Aldrich) and 100 μg mL⁻¹ BSA (NEB). When using the commercial,

![Buffer Diagram](image)

**Table 1.** The concentration of each component.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono- and bivalent ions</td>
<td>XXXX</td>
</tr>
<tr>
<td>dNTPs</td>
<td>XXXX</td>
</tr>
</tbody>
</table>

**Fig. 5.** Comparing the performance of various autocatalytic templates by measuring the turnover of dNTPs. The left panel shows the signal amplification phase using autocatalytic templates d, e, n, r, w and x. The right panel shows typical plateaus in the fluorescence signal indicating that the production and destruction of DNA species by the module has reached a dynamic equilibrium, which ends when the dNTPs are exhausted and the signal drops to its initial value. The duration of the plateau gives an indirect estimation of the productivity of the sequence. The table lists the sequences of the templates (modifications are not shown) and Tm of their products.

**Fig. 6.** Template modifications and effects on enzyme activities [8,48,49].
components of the basic DNA toolbox buffer. The concentrations are starting concentrations and may be adjusted if needed. DTT and BSA are added together with the enzymes and DNA species.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration in 4 times concentrated pre-assembled buffer</th>
<th>Final concentration in solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris–HCl (pH ~8.4)</td>
<td>180 mM</td>
<td>45 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>200 mM</td>
<td>50 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>40 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>Mg (MgCl₂ + MgSO₄)</td>
<td>28 mM</td>
<td>7 mM</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>40 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>dNTPs</td>
<td>200–800 μM</td>
<td>50–200 μM</td>
</tr>
<tr>
<td>Synermonic F108 (Sigma–Aldrich)</td>
<td>0.4% (v/w)</td>
<td>0.1% (v/w)</td>
</tr>
<tr>
<td>Netropsin (Sigma–Aldrich)</td>
<td>8 μM</td>
<td>2 μM</td>
</tr>
<tr>
<td>DTT (Sigma–Aldrich)</td>
<td>4 μM</td>
<td></td>
</tr>
<tr>
<td>BSA (NEB)</td>
<td></td>
<td>100 μg mL⁻¹</td>
</tr>
</tbody>
</table>

Table 2: Nucleobase-quenching performance of common fluorophores. The “++” indicates a shift superior to 25%, “+” stands for below 25%, and “-” means that this fluorophore showed no shift for the sequences tested. Note that these shifts can strongly depend on the sequence.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Nucleobase excitation</th>
<th>Emission/excitation (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM</td>
<td>++</td>
<td>494/520</td>
</tr>
<tr>
<td>JOE</td>
<td>++</td>
<td>520/546</td>
</tr>
<tr>
<td>DY-523XL</td>
<td>++</td>
<td>523/668</td>
</tr>
<tr>
<td>DY-530</td>
<td>++</td>
<td>539/561</td>
</tr>
<tr>
<td>TAMRA</td>
<td>++</td>
<td>546/576</td>
</tr>
<tr>
<td>Cy3.5</td>
<td>++</td>
<td>581/594</td>
</tr>
<tr>
<td>Alexa Fluor 594</td>
<td>++</td>
<td>590/617</td>
</tr>
<tr>
<td>TEX 615</td>
<td>–</td>
<td>596/613</td>
</tr>
<tr>
<td>Atto 633</td>
<td>–</td>
<td>629/657</td>
</tr>
<tr>
<td>Dy-631</td>
<td>+</td>
<td>637/658</td>
</tr>
<tr>
<td>TYE 665</td>
<td>+</td>
<td>645/685</td>
</tr>
<tr>
<td>DY-636</td>
<td>++</td>
<td>647/671</td>
</tr>
<tr>
<td>Cy5</td>
<td>–</td>
<td>647/670</td>
</tr>
<tr>
<td>DY-681</td>
<td>++</td>
<td>691/708</td>
</tr>
</tbody>
</table>

non-thermophilic RecJ exonuclease (RecJf from NEB), 410 mM of trehalose (final concentration) is also added to the buffer. A few remarks:

1. BSA, DTT and Synermonic F108 (a surfactant) act as stabilizing agents for the enzymes. Their inclusion in the buffer allows us to run experiments for up to several days in closed tubes without excessive loss of enzymatic activity. The 100 μg mL⁻¹ of BSA also coats the reactor surface and reduces the possible parasitic surface chemistry. The BSA and DTT are not included in the 4× buffer stock solution because of their shorter lifetime and potential for contamination.

2. Netropsin is an oligopeptide antibiotic able to bind the minor groove of AT-rich double stranded DNA sequences. We use it as a prevention against the occurrence of parasitic DNA species, which tend to appear stochastically when mixtures of polymerase and restriction enzymes are incubated for extended periods of time [32]. Such species, which emerge – and are exponentially amplified – in isothermal amplification schemes [33], have the potential to disrupt experimental setups [34] or even to contaminate entire labs [35], and maximum precautions should be taken to minimize their emergence and spreading. These parasites typically contain long AT repeats, interspaced by sequences that serve as a target for the restriction enzyme present in the buffer [32]. We observed that netropsin at a concentration of a few μM tends to delay the development of such species in a contaminated experiment, while having, at most, a marginal effect on the DNA toolbox reaction. It is possible that netropsin locally stabilizes such AT-rich parasites [36] and inhibits their polymerase-dependent growth [32]. However, we have not yet investigated this phenomenon in depth.

3. Exception for Synermonic, which can be omitted without important consequences, this buffer has been carefully optimized and any change in the composition should be followed by control experiments.

3.4. Enzymes

Our systems require a constant, quantifiable activity of three different enzymes to maintain dynamic and stable behaviors. However, commercial suppliers do not provide a standardized activity for each enzyme batch. Consequently, it is not unusual to see variations in enzyme activity between different batches. These fluctuations are fatal to the behavior of the network because they affect the kinetic balance of the reactions. In order to maintain the same range of enzymatic activity, we consistently check and adjust the activity of every new batch compared to the old one; this can be carried out by testing the old and new enzyme’s activity with the same substrate and adjusting the concentration of enzyme after comparing the measured kinetic parameters. Standard enzymatic assays have been reported in [4].

3.4.1. Polymerase

Polymerization is performed by Bst DNA polymerase, large fragment (New England Biolabs). This enzyme is thermostable and has a good strand displacement activity which is essential for the functioning of the toolbox: it allows the production of inhibiting strands even if the working temperature is well below their Tm. We generally use it from a diluted stock solution of enzyme at 1600 units mL⁻¹ in 10 mM Tris–HCl, 1 mM DTT, 0.1 mM EDTA, 50% Glycerol, 200 μg mL⁻¹ BSA, 50 mM KCl and 0.1% Triton X100 (Sigma–Aldrich), at a concentration ranging from 0.3% to 2% (i.e. 4.8–32 units mL⁻¹).

3.4.2. Nicking enzyme

Regarding the nicking endonuclease, we use Nt.BstNI (New England Biolabs) at a concentration of 1–2% of the commercial stock solution (i.e. 100–200 units mL⁻¹). Counterintuitively, higher concentrations tend to inhibit the polymerase and slow down the reaction. This enzyme is not pre-diluted and the stock is used as provided.

3.4.3. Exonuclease

The exonuclease is used to degrade single stranded DNA species (inputs and outputs) while sparing the templates. RecJf (New England Biolabs) was originally chosen and typically used at 30 units mL⁻¹. This enzyme is not thermostable and requires the addition of 410 mM of trehalose to the reaction mix. A thermo philic ortholog isolated from Thermus Thermophilus HB8, called tTRecJ [37], was also used successfully without trehalose at temperatures up to 50 °C. The plasmid is available upon request.

3.5. Basic protocol

In a PCR tube at room temperature, all the templates are diluted into a solution of ultrapure water (Millipore), 4× buffer (25% of the final volume), DTT (from a freshly made stock dilution), BSA and, optionally, EvaGreen. The resulting solution is thoroughly mixed by vortexing for 10 s before and after adding the enzymes. This final solution is then distributed into optical white PCR tubes, avoiding...
and carefully sealed with a cap or tape. It is possible to put oil on the top of the reaction medium to prevent evaporation. Note that after the vortex steps and the final assembly, a quick centrifugation is performed (10,000 rpm, 5 s) to preserve the integrity of the reaction solution by gathering it at the bottom of the tube. The usual reaction volume is about 10–20 μL, depending on the optical performances of the apparatus. The thermocycler is set at the constant working temperature (typically between 38 and 42 °C for RecJf and 38–50 °C for tRecJ) and programmed to perform repetitive fluorescence measurements to monitor the evolution of the system over a long period. We are currently using CFX96, MiniOpticon or iQ5 real-time thermocyclers from BioRad and Rotor-Gene Q from Qiagen.

3.5.1. Comments on the experimental protocol

1. Nucleotides were custom-made and purified (HPLC grade) by Biomers (Ulm, Germany), re-suspended in Tris–EDTA buffer (100 μmol L\(^{-1}\)) and stored at –25 °C. From these stocks, dilutions of up to 1 μmol L\(^{-1}\) were used as working samples.

2. The 4× buffer composition is designed to contain as many chemicals as possible. For instance, DTT should not be added to the 4× buffer because of its shorter lifetime, as it is eventually oxidized by the air. Fresh DTT solution should be prepared regularly and kept at 4 °C.

3. The enzymes, buffer, and oligonucleotides are stored at –20 °C. The other chemicals are kept at 4 °C.

4. Since dynamical systems rely on enzyme kinetics and activity, the concentrations of enzymes and their substrates need to be as precise as possible. Vortexing steps are therefore essential to the success of the experiments, because they allow the homogenization of the reaction medium, and especially the mixing of viscous and heavy enzyme stock solutions.

5. Concentrations are adjusted for each network, but typical working concentrations for templates are from a few nM to several tens of nM.

6. Enzyme concentrations can be also adjusted for each network. For a given polymerase concentration, the quantity of nicking enzyme is chosen to provide the fastest reaction, as measured for example by the dNTP consumption rate of a simple amplification reaction (Section 3.2.1). The exonuclease concentration controls the lifetime of single stranded species in solution and therefore, the responsiveness of the network. Finally, because it is necessary to avoid saturating the nicking enzyme (which would lead to the accumulation of double stranded templates and a loss of function), the polymerase concentration is chosen as small as possible, while still sustaining non-zero steady states of autocatalytic species. Note that the activity of the exonuclease on its substrate is strongly substrate-dependent, with longer oligonucleotides being degraded much faster, so measuring the activity of the exonuclease on each of its substrates is highly recommended.

7. Once the proportion of each enzyme has been set for the network, in order to decrease the amount of pipetting steps, it is recommended to mix all enzymes together in a stock tube, at the exception of the polymerase, which cannot stand a different buffer from the one provided.

3.5.2. Standard conditions

While the tuning of experimental conditions is a central part of the current molecular programming workflow, some basic, starting conditions can be defined.

- Bst DNA polymerase, Large fragment (NEB) is used at 16 units mL\(^{-1}\) (i.e. 1/500 of the commercial solution).
- Nt.BstNBI nickase (NEB) is used at 100 or 200 units mL\(^{-1}\) (depending on the specific activity of the current batch).
- The exonuclease RecJ (NEB) is used at 30 units mL\(^{-1}\); the thermostable tRecJ is used at a concentration of 30 nM.

3.6. Experimental tuning of a reaction network

The dynamic behavior of a reaction network is the product of both its topological architecture and numerical parameters. The various behaviors associated with a given topology can be sketched in a bifurcation diagram, which shows the qualitatively different dynamics that may arise upon changing the parameters of a given network. Moreover, given a particular behavior, e.g. oscillations, one may still want to change some quantitative aspect, for example the period of the cycle. In the context of the DNA toolbox, one should manage several experimental control parameters that can be used to reach the targeted behavior and tune the time trace, each with its assets and drawbacks.

3.6.1. Global parameters

3.6.1.1. Temperature. In contrast with many other in vivo or in vitro approaches, the DNA toolbox networks can perform over a relatively large range of temperatures, especially when the thermophilic RecJ is used. This range is one of the most readily available control parameters, since it is easy to use the temperature gradient function included in most thermocyclers to explore, in parallel, a full set of different working temperatures. On the flip side, the effects of changing the temperature are not readily forecasted quantitatively. An increase in temperature will weaken the binding of oligonucleotides to their complementary templates, but at the same time, it will possibly increase their enzymatic processing. The Michaelis–Menten constant K\(_m\) may also decrease, leading to less pronounced competition effects. On the quantitative side, while DNA binding constants and rates can be theoretically estimated for any temperature, the enzymatic parameters will need to be re-measured for every new condition (this also applies to changes in the buffer salt content), or plausible mathematical functions linking Michaelis–Menten parameters and temperature should be found for every enzyme [38]. This task is left for future improvements of the model.

Higher temperatures also lead to additional issues such as faster loss of enzyme activity, increased evaporation and increased sensitivity to parasite emergence.

3.6.1.2. Enzyme concentrations. Enzyme concentrations provide more precise tuning of the reaction conditions because it is possible to independently modify the production (by the polymerase and nickase) and degradation (by the exonuclease) processes.

However, regarding production, it has been noted that the kinetics is not as straightforward as one may expect [12]. In fact, for a given polymerase concentration, there generally exists an optimal concentration of nickase above which the amplification reactions slow down again, probably because of competition between the two enzymes.

At constant polymerase/nicking enzyme concentrations, increasing the exonuclease concentration will lower the amount of free oligonucleotides present in the solution, leading potentially to less enzymatic saturation and less side reactions, but it might also slow down the reactions to an unacceptable extent.

3.6.1.3. Buffer composition. Adjusting the buffer composition is less practical as it will require a recalibration of all network parameters, and should only be done for particularly awkward cases. Moreover, counterintuitive effects may also occur: for example,
dNTPs at high concentrations slow down the behavior, and salts can have opposite effects on different enzymes.

3.6.2. Local parameters

3.6.2.1. Melting temperatures. Melting temperatures can be easily adjusted by modifying the sequence of the DNA species. Higher melting temperatures (Tm) result in stronger nodes but will also probably yield a slower relaxation time (as illustrated by the faster oscillations when DNA species with lower Tm are used with the Oligator, Fig. 7).

Many softwares are available online, including programs on the DINAMelt Server [39] and Tm calculators found on the website of many oligonucleotide suppliers. Some modifications, as well as some buffer components (such as intercalating agents or the trehalose used to stabilise the mesophilic RecJf), may modify the Tm of the oligonucleotides; it may be necessary, when in doubt, to measure the Tm directly by performing melting curve experiments.

3.6.2.2. Template concentrations. Adjusting template concentrations is a simple way to modify a node’s strength. The Fig. 8 shows a modelization of such variations on a given network. Note, however, that it is not a good idea to increase the template concentrations above a few tens of nanomolars, since free linear templates inhibit the tRecJ enzyme with a Ki around 40 nM.

3.7. Monitoring

Various strategies have been used to monitor the behavior of the system while minimally disturbing the reaction mix.

3.7.1. Gel electrophoresis

DNA species can be visualized by denaturing polyacrylamide gel electrophoresis (PAGE) after labeling the DNA species with radioactive nucleotides (we previously used 32P dCTP). In order to limit the disturbance of the reaction, the reaction volume can be increased to 200 μL and small aliquots (5 μL) are taken periodically from the reaction mix; the enzyme activity must be immediately quenched by adding EDTA at a final concentration of 50 mM and the aliquots stored at −20 °C. The DNA samples are then loaded in 7 M urea polyacrylamide gel, separated by PAGE and the gels are analyzed with a phosphorimager.

This tried-and-tested technique [4] is easy to implement and enables semi-quantitative analyses of the samples. It presents, however, several drawbacks: it is relatively time-consuming; DNA species of the same length cannot be distinguished; it requires protection from harmful radiation.

DNA species separated by PAGE may also be imaged after staining by fluorescent dyes like SYBR green; however, fluorescent staining is much less sensitive than radioactive labeling (especially for single stranded short oligonucleotides) and we have not been able so far to obtain quantifiable images with such a direct protocol.

3.7.2. Isothermal DNA amplification

Small single-stranded DNA, such as the dynamic signals exchanged by the toolbox templates, can be quantified individually by isothermal amplification [12]. The reaction is a simple autocatalytic amplification monitored in real-time, and simply requires an autocatalytic template specific to the species under study, a DNA polymerase and a nicking enzyme. The template 5' and 3' ends
correspond to the quantified species' complementary sequence and its sequence should present a recognition site to a nicking enzyme; this site, if not present in the analyzed species' sequence, can be inserted between the two repeats. We have previously quantified individual DNA species present in the reaction mix using Nt.BstNBI and Bst DNA polymerase, large fragment in a buffer containing 45 mM Tris–HCl, 50 mM NaCl, 10 mM KCl, 10 mM (NH₄)₂SO₄, 7 mM MgCl₂, 1 mM DTT, 0.1% Triton X-100 and 1 × EvaGreen. The concentrations of enzymes should be optimized for each template in order to resolve small concentrations of analyte, but are typically between 100 and 200 units mL⁻¹ for Nt.BstNBI and between 2.5 and 10 units mL⁻¹ for the Bst DNA polymerase. As for gel electrophoresis, small samples are taken periodically from the reaction mix and immediately quenched by EDTA at a final concentration of 50 mM before storing at −20 °C. The amplification curves of the diluted unknown samples are compared to standard curves obtained from calibrated concentrations of the target oligonucleotides (5' phosphate-modified synthetic oligonucleotides are used to minimize bias) in order to extract the initial concentration. Since the amplification is expected to be exponential, analysis can be done by simply plotting Ct versus the logarithm of the initial concentration (in a process very similar to the analysis used in real-time PCR). The samples are used diluted (typically 100 times) resulting in a low final EDTA concentration; even though, it is strongly recommended that EDTA, at the same final concentration as in the quenched samples, be added to the standard samples to compensate for the loss of enzyme activity that may be caused by the diluted EDTA.

This technique has the huge advantage of being quantitative: it yields the total concentration of each dynamic compound in the mixture. It also has a larger dynamic range than direct measurement strategies. However, it requires some optimization for each species and may perturb the reaction when samples are taken.

3.7.3. Fluorescence measurements

One major drawback of the previous approaches is that a sample of the reaction medium is needed for the analysis. Such repeated and discrete perturbations of the system are not negligible for long-term experiments: temperature and species concentrations may vary and affect the global dynamics.

Real-time thermocyclers used in molecular biology are convenient devices to monitor DNA-toolbox based networks, because they provide a way to maintain small (10–50 μL) samples at a given temperature, and can perform repetitive fluorescent measurements in parallel (e.g., 96 simultaneous measurements). Real-time monitoring of DNA-based reactions is possible thanks to the development of fluorescent dyes that allow the detection and quantification of nucleic acids. In isothermal conditions, DNA-binding fluorophores, such as EvaGreen, become highly fluorescent when bound to double-stranded DNA [40], although some fluorescence is also observed with ssDNA. EvaGreen is typically used at 1 × or 0.5 × (noting that, as most intercalating agents, its presence increases the melting temperature of the DNA duplexes, and that in most cases 0.5 × provides enough signal). However, such DNA-intercalating dyes only enable the global, non-specific monitoring of the total amount of double-stranded DNA in solution.

As mentioned in Section 3.2.4.4, it is also possible to label individual templates at their 5' or 3' end with a specific fluorophore in order to allow real-time monitoring of the hybridization status of the template [8]. Provided the excitation and emission spectra don't overlap, it is possible to perform multiplexed monitoring of all the templates in the reaction. The detection of whether a template is hybridized or not to a complementary oligonucleotide is based on Nucleobase Quenching (NQ). This phenomenon is well documented [41] and relies on the fact that nucleobases can quench a fluorescence signal. For NQ, templates carry a fluorophore on their 3' or 5' end; when the corresponding input hybridizes or is elongated upon polymerization, the fluorescence signal shifts, either up or down depending on the conjugated fluorophore; upon de-hybridization, it returns to its basal level. Above all, NQ has the huge advantage of being sequence-specific: DNA species that hybridize a few bases away from the dye or that are mismatched near the template's fluorophore-bearing 3' end can be readily distinguished from complementary DNA species.
Furthermore, for short oligonucleotides, NQ can be slightly more effective than DNA-intercalating dyes. NQ is currently the most useful monitoring technique compatible with dynamic DNA reaction networks. Fluorescent probes attached to DNA generally modify the thermodynamic characteristics and (probably) their affinity for enzymes as well. The single modification used here is less invasive than the double modification used in other reporting strategies, but such effects need to be taken into account in the design process (i.e. the replacement of a native template by its labeled version will always impact the dynamics of the circuit, in some cases very strongly). It is also possible, for a given target sequence, to label all the corresponding templates (all the templates that use this sequence as an input) with the same fluorescent dye, or alternatively label only one template. In the latter case, templates that are not targeted by any inhibitor may be preferred, because the binding of an inhibitor can also shift the fluorescence, complicating the analysis.

Note that the use of template-bound reporters and an intercalating dye are not mutually exclusive, provided that the wavelengths can be correctly resolved. The combination of dyes provides more information for analysis and debugging.

3.8. Troubleshooting

The behavior of the network qualitatively depends on the combination of 3 enzymatic activities. Moreover, for the system to perform reliably, it is essential that these 3 activities are kept precisely stable over extended periods of time (oftentimes more than a day). This is quite different from the “classic” usage of enzymatic tools in molecular biology, where – typically – reactions are carried to completion with an excess of – possibly unstable – catalyst, and performed sequentially.

To obtain an accurate and reproducible combination of enzymatic activities, it is therefore essential to follow some basic rules, such as avoiding small volume pipetting as much as possible (i.e. less than 0.8 μL for enzymes or chemicals in 50% glycerol, 0.5 μL otherwise). Be aware that the very last microliters of enzyme stocks may not be as active as the original stock, as condensation may have accumulated since the tube was first opened and during repeated round-trips from the freezer to the workbench, leading to a slow drift in the fluorescence signal, possibly due to evaporation or inhomogeneous temperature in the reaction medium (as adding mineral oil on top of the medium improved the signal). In any case, it may be useful to measure the activity of all new enzyme tubes before starting experiments. Because of the delicate balance of activities required by the DNA toolbox, it is not possible to just put an excess of each catalyst.

In the following, we give a list of frequent problems encountered together with their possible causes, and the actions that may be taken in the building of in vitro circuits with fluorescent monitoring. These recommendations are based on accumulated experience in using the DNA toolbox and training new users. It should be carefully considered before starting actual experiments.

3.8.1. Flat curves

3.8.1.1. Faulty buffer. The absence of any shift in the fluorescence should first prompt questions about the composition of the medium. Have any essential chemicals, such as dNTPs or enzymes, been omitted? Are the dilutions correct? Are the enzymes still active?

- Adjust reagent concentrations.

3.8.1.2. Overdose of exonuclease. Too much exonuclease may prevent amplifications from occurring or being detected.

- Reduce exonuclease concentration.

3.8.1.3. Wrong thermocycler settings. Setting the temperature too high will prevent DNA hybridization and might inactivate the enzymes; a too low temperature will reduce enzyme activity and prevent DNA de-hybridization; also, measuring fluorescence using the wrong channel will prevent any signal from being detected.

- Check the run settings.

3.8.1.4. Labeling problem. The dye may not be correctly positioned to observe the reaction: for instance, we generally avoid the labeling of templates that are the target of inhibition, since the replacement of an activator by an inhibitor can lead to some confusion in the interpretation of fluorescent shifts (even if the inhibitor, which binds approximately 4 bases away from the template’s 3’ end, is not expected to produce the same intensity and/or polarity in the fluorescent shift [8]).

- Remove the fluorophore or change its position to another template.

Some sequence/dye pairs do not yield strong fluorescent shifts (see Table 2) and may not be detectable, depending on the thermocycler’s sensitivity.

- Replace the chosen fluorophore; add an intercalating dye to check whether some reactions are occurring.

3.8.2. Drifts in fluorescence signal

3.8.2.1. Bubbles. The presence of bubbles in the tubes caused by careless pipetting of the reaction mix or poor centrifugation of PCR tubes can lead to sudden jumps in the fluorescence signal as bubbles pop or move during the experiment.

- Re-centrifuge the tubes or add a reference dye (e.g. ROX) to the reaction mix to normalize the fluorescence signals.

3.8.2.2. High lid temperature. We have found that heating the thermocycler lid to 100–105 °C (which is very often the default temperature) leads to a slow drift in the fluorescence signal, possibly due to evaporation or inhomogeneous temperature in the reaction medium (as adding mineral oil on top of the medium improved the signal); in fact, setting the lid temperature a few degrees above the reaction temperature is enough.

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3.8.2.3. Secondary DNA structures. The presence of secondary structures in the DNA may lead to a slow fluorescence shift as the secondary structures gradually disappear upon heating and hybridization to other species during the reaction.

- Redesign the oligonucleotides to minimize secondary structures.
- Re-anneal the reaction mix containing the DNA (but not the enzymes!) by heating at 80 °C and slowly cooling back to room temperature on the workbench.

3.8.2.4. Exhausted or unstable reagents. As reagents (particularly dNTPs) are used up and near their exhaustion, polymerization reactions may start to slow down and destruction by the exonuclease become dominant, leading to a slow decrease in the total unprotected single-stranded DNA species in the medium and a subsequent drift in the fluorescent signal.

- Add more dNTPs to increase the autonomy of the reaction (but note that too much might affect the enzymatic rates).

3.8.2.5. Disturbed reaction during sampling or addition of reagents. Adding inputs or removing samples from the solution during the reaction will almost inevitably result in a sudden shift in the fluorescence, which may hinder the analysis.

- Add a reference dye (e.g. ROX) to the reaction mix to normalize the fluorescent signals.

3.8.3. Day-to-day non-reproducible results
The reproducibility is also a frequent problem, which may result from a variety of reasons:

3.8.3.1. Insufficient mixing. Insufficient mixing is often the cause. This trivial procedure is of the utmost importance and should be performed properly. Tried-and-tested methods include vortexing for about 10 s (over-prolonged vortexing, on the other hand, may be detrimental) or, especially for viscous solutions, multiple (20 times) pipetting or inversion of the tubes.

- Vortex for 10 s or pipet the full content of the tube up and down repetitively. Use always the same (validated) mixing protocol.

3.8.3.2. Mistakes during reaction assembly.
- Check and adjust the reagent concentrations.

3.8.3.3. Batch-to-batch variations in enzyme activity.
- Check whether the enzymes have the same activity as before and adjust their concentration if necessary.

3.8.3.4. Degraded DTT. DTT, in particular, is unstable and aged DTT may lead to unusual behaviors (mostly premature divergence of the system).

- Prepare a fresh stock solution of DTT.

3.8.4. Tube-to-tube non-reproducible results
In some case the same master mix, incubated in two different PCR tubes, can lead to two different traces.

3.8.4.1. Variations inherent to the thermocycler’s optical system. First recall that, depending on their optical setup, thermocyclers based on multiwell plates typically do not provide traces that are quantitatively identical from tube to tube. Even properly calibrated, differences in both the baseline and the fluorescent coefficients have to be expected between tubes.

- Some machines (e.g. Rotor-Gene Q), that do not require optical scanning, can minimize this problem.

3.8.4.2. Different tube capping. Differences in the fluorescent signal may be caused by the tubes themselves (e.g. due to varying cap thickness); in this case, some variations may be observed even when performing the same experiment at the same plate position.

- Make sure that the PCR tube caps are all from the same supplier and are properly inserted.

3.8.4.3. Thermocycler temperature homogeneity. If the differences in time trace when running the same reaction in different positions cannot be explained by a linear transformation (adjusting the baseline and the optical coefficient), the tubes in the multiwell plate may not be heated at exactly the same temperature.

- Rotary thermocyclers (e.g. Rotor-Gene Q), which maintain all the samples in the same heated chamber, can minimize this problem; for multiwell plate-based systems, it may be necessary to have the heating block checked.

3.8.4.4. Insufficient mixing. As mentioned above, poor mixing will lead to an inhomogeneous solution, which, if distributed in several tubes, may result in different fluorescent signals. This is the best way to check that the mixing is correct.

3.8.5. Premature death of the reaction
3.8.5.1. Exhaustion of dNTPs. As discussed earlier, the fuel, i.e. the limiting reactant in our system are the dNTPs. Running out of dNTPs will prevent any polymerization process, leading to a gradual degradation of all the dynamic DNA species by the exonuclease and ultimately to a flat fluorescence curve.

- Add more dNTPs.

3.8.6. Unexpected time traces (qualitative differences)
Differences between expected time traces and experimental results can occur. Here again, the sources of error are legion, and the resulting time traces are frequently a combination of these rather than only one.

The predictable exhaustion of the reaction (mentioned above) is yet not the most frequent.

3.8.6.1. Incorrect concentrations. As often, mistakes during the preparation of the reaction mix may result in unexpected time traces.

- Check the protocol and adjust the reagent concentrations.

3.8.6.2. Sub-optimal working temperature. Selecting the correct working temperature is not a trivial task as the enzymes have different temperature requirements. For example, if working with the non-thermophilic enzyme RecJ, the limitation is up to 42 °C even with stabilizing agents such as trehalose in the buffer. On the contrary, the reported optimal temperatures for Bst and Nt.BstNBI are...
The emergence of parasitic species in polymerizing-nick-Bst polymerase, Nt.BstNBI and RecJf values for their As can be expected for unreactive substrate are generalist en-

to minimize their influence:

combined activity of a polymerase and a nickase and are difficult to

cultivated from the signals of template-bound dyes). In the DNA

They generally yield an easily recognized time trace in the signal

fluorescence intensity on the EvaGreen channel after 15 h, revealing the emergence

of parasitic species and their hijacking of the system. Note the strong difference

between the parasitic intensity shift and the amplitude of the original signal. Note also that the oscillations may persist during the initial phase of parasitic emergence.

respectively 65 and 55 °C (although in our hands Nt.BstNBI loses activity very quickly at 55°C); ttRecJ is also more active at 50 than 37 °C. Another point to take into consideration is that a lower tem-

tperature may lead to less side reactions (such as the emergence of parasitic species).

3.8.7. Sudden increase in DNA concentration

Some errors during enzymatic processes (polymerization, nicking) can lead to unexpected species that were not designed in the network. Some of them may be able to create a self-amplifying loop and will then grow out of control. Such parasites eventually occur in many isothermal amplification schemes [35]; the resulting fluorescence signal is a strong shift in the intercalating dye channel (due to the formation of a huge amount of double-

stranded DNA) and erratic variations in the reporter channels (probably due to couplings of the target species dynamics with that of the parasite, via enzymatic saturation).

3.8.7.1. Combined activity of the DNA polymerase and the nicking enzyme.

The emergence of parasitic species in polymerizing-nick-

ing isothermal amplification reactions is well documented [32]. They generally yield an easily recognized time trace in the signal provided by the intercalating fluorescent dye (they are more difficult to spot from the signals of template-bound dyes). In the DNA toolbox, such species seem to appear stochastically from the comb-

ined activity of a polymerase and a nickase and are difficult to completely remove. However, the following actions can be taken to minimize their influence:

- Decrease the concentration of polymerase (Fig. 9a).
- Since the accumulation of double-stranded templates seems to be related to the emergence of parasitic species, make sure to use enough nicking enzyme.
- Use anti-parasite components: DTT, in our case, effectively prevents such divergence, as does netropsin; BSA, by coating the reactor surface and reducing possible parasitic reactions, is also required.
- In the same way as for PCR, avoid contamination from para-

site-laden tubes and avoid the contamination of stocks.

3.8.8. Quantitative differences with model predictions

The toolbox has been set up to provide a conceptually simple, while universal, chemical networking framework. The mathemati-

cal models described in [27] and implemented automatically by the software DACCAD (downloadable at http://www.yannick-ron-delez.com) have been constructed to provide a mathematical description of the system that is as accurate as possible and include most of the side reactions listed below; however, one can only expect to include the most significant fraction of the chemical reactions occurring in the tube and additional interactions may actually take place in vitro. Moreover, a more complete model requires more parameters, which may not be easily available and in any case would involve additional measurements and complexity. Some of the possibly counterintuitive phenomena that may occur in vitro are listed below:

3.8.8.1. Load effect. This effect is arguably the primary source of discrepancies between naive designs based on the assumption of per-

fect modularity and experimental results. It comes from the fact that downstream templates that use a signal produced by another part of the network will in essence sequester a fraction of that signal and hence modify the dynamics of the upstream “module”. This is not specific to the DNA toolbox, but, on the contrary, is a general feature of in vitro [26] or in vivo [42] molecular networks.

- Take these interactions into account when experimentally tuning subparts; use insulation strategies [26,43] (but they will increase the enzymatic load mentioned below); use in silico optimization of the full network with a realistic model.

3.8.8.2. Competitive inhibition between the various substrates of each enzyme. Bst polymerase, Nt.BstNBI and RecJf are generalist en-

zymes with relatively small Michaelis–Menten Km values for their substrates. They are thus prone to competitive effects in the tenth of nanomolar range. Such competition introduces additional regulation layers into the system, with potential qualitative changes in the dynamics [44,45].

- Increase the enzyme concentrations or take the competition into consideration in the mathematical model.

3.8.8.3. Competition between the nicking enzyme and the polymerase for double-stranded DNA.

- Adjust enzyme concentrations (in our systems, Nt.BstNBI seems particularly stable when bound to its recognition site) or take competition into consideration in the mathematical model (this is not the case in the current implementation); increase the temperature to avoid product inhibition of the nicking enzyme.

3.8.8.4. Competitive inhibition of the exonuclease by free single-

stranded templates. As can be expected for unreactive substrate analogs, phosphorothioate-modified templates in their free form
tend to inhibit the single strand-specific exonucleases like RecJ. This can affect the behavior of the network.

- Decrease template concentration; increase exonuclease concentration or take the competition into consideration in the mathematical model.

3.8.8.5. Non-specific polymerization from non-primed templates or non-complementary primers. Non-specific or unprimed amplification in isothermal amplification reactions has also been documented [32,46].

- Reduce polymerase or increase exonuclease concentrations; increase the temperature to reduce spurious binding; this "leak" can also be taken into account in the mathematical model if appropriate parameters are available.

3.8.8.6. Incomplete protection of the templates (leading to their slow degradation).
- Increase the number of phosphorothioate bonds (3 or 4 are usually sufficient in the case of thermophilic RecJ).

3.8.8.7. Sequence-dependent enzyme affinity to different substrates. Enzymes working on DNA may have subtle sequence preferences. For example, GA or AG-rich primer sequences have been reported to be unfavorable to Bst polymerase [31] (Section 3.2.1).

- It may be necessary to measure enzyme activities on each separate template before inputting the parameters to the mathematical model. Alternatively changing the template concentrations may be efficient to compensate for these discrepancies.

3.8.8.8. Sequence cross-talks.
- check the design of the oligonucleotides for unwanted binding or folding

3.8.8.9. Dye influence on the reactivity of the template. As mentioned in Section 3.2.4.4, the fluorescent label used to report on the presence of a given template’s input signal will also affect the affinity of this template for the enzymes and hence the activity of this particular link of the network. Such effects cannot be easily predicted and have to be managed empirically.

- It may be necessary to re-characterize a template and readjust its concentration if a conjugated dye has been added or replaced. Use labels only when needed.

3.8.8.10. Template poisoning. The possibility of forming mismatched, hence non-nickable, but stable template duplexes would lead to a form of poisoning and an apparent decrease in their effective concentration. The occurrence and prevalence of this type of reaction has not been explored yet.

4. Implementation

To illustrate the whole process, we here demonstrate the implementation of an inversion module: with this simple architecture, an activated autocatalyst producing the species α is temporarily switched off upon the addition of an input β. In Boolean terms, this would be called a NOT gate.

4.1. Network design

Fig. 10 shows how the connection of an inhibiting reaction to a self-activating template can generate the desired function.

4.2. Oligonucleotide design

To implement the inversion module, two templates, the autocatalyst α to α and the inhibitor-forming template β to iα, the input β (as well as some trigger α to start the autocatalytic loop) are necessary. The sequences are given underneath. Both templates have three phosphorothioate backbone modifications (indicated by an asterisk) at their 5’ end to protect them from degradation by the exonuclease ttRecJ. The autocatalyst α to α has a TAMRA modification at its 3’ end and a uracil instead of a thymine in the 5’ end nickase recognition site to prevent unproductive binding of the nicking enzyme to its output side and increase the nicking rate. The inhibitor-forming template β to iα is phosphorylated (Phos) at its 3’ end to prevent any polymerization. The recognition sequence of the Nt.BstNBI nicking enzyme is in bold.

α to α: 5’-A*A*C*AGACUCGAAAAACAGACTGA-TAMRA

β to iα: 5’-T*GACTGGT TT C-3’

β: 5’-CTGAGTCAAGG-3’

4.3. Experimental conditions

The reaction was assembled in the buffer containing 45 mM Tris, 50 mM NaCl, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 5 mM MgCl₂, 4 mM DTT, 100 μg mL⁻¹ BSA, 0.1% Syneronic F108, ROX reference dye (1 × from Invitrogen), EvaGreen at 1 μM each). Bst DNA polymerase, Large fragment was used at 24 units mL⁻¹, Nt.BstNBI at 200 units mL⁻¹ and the exonuclease ttRecJ at 50 nM. 30 nM of template α to α and 60 nM of template β to iα were added to the reaction buffer and the reaction was triggered with 150 pM of α. Regarding the tuning of the concentrations, 30 nM of α to α were deemed sufficient to produce a clear shift in TAMRA fluorescence. For β to iα, 60 nM was eventually chosen to allow a wide range of inhibition, depending on the amount of input β; lower concentrations failed to lead to sustained inhibition of α to α, whatever the concentration of input β. Bst and ttRecJ concentrations were slightly higher than the standard starting concentrations (see paragraph 3.5.2) to allow a more dynamic switching between the activated and inhibited states. Nt.BstNBI was also used at a relatively high concentration after finding that more nicking enzyme stabilized the inhibited state.

The fluorescence signals of ROX and TAMRA were recorded with an iQ5 real-time thermocycler set at 39 °C. The reporter dye TAMRA, which is attached to the autocatalyst, monitors the hybridization status of the α to α and, therefore, provides a readout on the quantity of output α. When injecting the input β to the reaction, it was diluted in a maximum volume of 2 μL of the same reaction buffer and injected while the run was paused for a minimal period. Signal artifacts caused during the injection (e.g. due to condensation or slight displacement of the tubes) were corrected by
normalizing the TAMRA signal to the ROX signal. No modifications to the input strands. Upon adding the input \( \beta \), the autocatalytic module is temporarily inhibited, which is observed here through a fluorescence decrease. The dynamic decay of the input and of the inhibitor (not seen here) leads to a recovery of the autocatalytic module and the fluorescence returns to its high level. The inset shows the strength of the inhibition, measured as the time spent at the bottom of the well, as a function of the input concentration. (B) The system can be actuated multiple times.

4.4. Results

Fig. 11A shows the fluorescent signals observed with the implemented inversion module; the gray arrows indicate the injection of input strands. Upon adding the input \( \beta \), the autocatalyst is temporarily inhibited, which is observed here through a drop in TAMRA fluorescence. The dynamic decay of the input \( \beta \) and of the inhibitor (not seen here) leads to a recovery of the invariant module and the fluorescence returns to its original level. The inset shows the strength of the inhibition, measured as the time spent at the bottom of the well, as a function of the input concentration. As shown in Fig. 11B, the system can be actuated multiple times.

Several other behaviors have been studied and implemented in vitro. This includes the oscillatory system known as the Oligator [4] and various chemical memory circuits based on a bistable core [7].

5. Conclusions

We have presented here a general framework, the “DNA toolbox”, which should allow the experimenter to implement, in vitro, networks reproducing the basic dynamic features of genetic regulation. This set of tools enables the design of basic chemical networks that are modular and readily lend themselves to mathematical simulation. Using this simple scheme, it may be possible to build powerful information processing circuits in molecular systems [7,15]. Moreover, the chemical versatility of nucleic acids, as well as the huge number of chemical or biochemical tools that have been developed for their manipulation, will permit the interfacing of these devices with real world applications in the field of nanotechnology [26,47], molecular programming [23] and biological modeling [45].

Disclosure

The authors state that they have no conflicts of interest.

References


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