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In vitro regulatory models for systems biology

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ABSTRACT

The reductionist approach has revolutionized biology in the past 50 years. Yet its limits are being felt as the complexity of cellular interactions is gradually revealed by high-throughput technology. In order to make sense of the deluge of "omic data", a hypothesis-driven view is needed to understand how biomolecular interactions shape cellular networks. We review recent efforts aimed at building *in vitro* biochemical networks that reproduce the flow of genetic regulation. We highlight how those efforts have culminated in the rational construction of biochemical oscillators and bistable memories in test tubes. We also recapitulate the lessons learned about *in vivo* biochemical circuits such as the importance of delays and competition, the links between topology and kinetics, as well as the intriguing resemblance between cellular reaction networks and ecosystems.

Contents

1. The molecular revolution of biology

The publication of the double-helix structure of DNA ushered the molecular revolution of biology. In the last 50 years, biologists have broken cells into finer and finer components in an attempt to unravel their inner mechanisms. This reductionist approach has relied on three complementary paradigms: *in vivo*, *in vitro* and *in silico*. *In vivo*, for example, genetic studies have uncovered genes and mutations underlying numerous physiological and pathological pathways—such as cystic fibrosis (Riordan et al., 1989) or oncogenesis (Hanahan and Weinberg, 2011). Genetic studies often discover the function of an unknown gene by mutating or knocking out its protein. On the one hand, *in vivo* studies offer the advantage of studying proteins in their natural environment. On the other hand, the complexity of cells often obfuscates the role of a given protein.

The second approach, based on *in vitro* protocols, aims to isolate a protein from its environment to observe its action in detail. For example, enzymology uses *in vitro* assays, sometimes very elaborate (Rondelez et al., 2005), to discover the mechanism and measure the kinetic and thermodynamic parameters of various key biochemical transformations. Crystallography is another technique that epitomizes reductionism, seeking to explain the role of proteins based on their atomic arrangements. Hypotheses about cellular mechanisms are often not completely accepted until their molecular basis has been validated by crystallographic

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studies. However, *in vitro* studies offer a controlled albeit artificial environment, which may lead to artifacts or omissions of crucial mechanisms.

Lastly, *in silico* techniques or the building of theoretical frameworks have seconded experimental approaches. For example, protein folding algorithms link the 3D structure of a protein—and potentially its function—to the sequence of its amino acids (Dill et al., 2008). In drug discovery, docking simulations are a valuable tool to assess the binding of drugs to their target (Kitchen et al., 2004).

Yet, numerous lines of evidence point to the limits of what can be understood by breaking cells into finer and finer components and looking individually at each of these elements (Hartwell et al., 1999; Kitano, 2002). Challenges to reductionism were for example raised by bioengineers who sought to alter metabolic pathways to boost the production of useful molecules such as ethanol. They realized that in order to tailor metabolism locally, they first needed to understand it globally (Nakatsui et al., 2010; Westerhoff and Palsson, 2004). Similarly, drug discovery increasingly requires a systems approach to predict far-reaching and off-target effects (Hood and Perlmutter, 2004).

The limitation of reductionism is not surprising given that cells are defined not only by their components (proteins, genes, factors…) but also—and mostly—by the interactions between these components (repression, activation, allostery…). In other words, most cellular processes are not performed by a dedicated molecular compound, but orchestrated by networks of interdependent chemical events. Gene regulatory networks provide a prominent example. They can be seen as directed networks of transcriptions and translations. Their nodes are proteins and genes, and their edges are chemical transformations or interactions between them. Transcription networks participate in the regulation of virtually all biological processes, ranging from cellular differentiation (Herskowitz, 1989) to apoptosis (Haupt et al., 2003) or immune response (Calvano et al., 2005; Eulgem and Somssich, 2007).

Reductionism typically apprehends genetic regulation with knockout assays, in which a studied protein is temporarily or permanently repressed. By observing the effect of the knockout on the phenotype, assumptions are drawn on the role of the missing element. But knockout assays are crude because they focus more on proteins than on their interactions. In fact, knockout assays not only remove a node from a collection of proteins, but also prune all the edges of the regulatory network that lead to or originate from this node (the regulations). The limitations of the reductionist approach are creatively illustrated by Yuri Lazebnik (Lazebnik, 2002). The author wonders whether reductionism would help a biologist to fix a broken radio and concludes that an integrated and functional language—similar to that used by engineers—is required to capture the complexity of cellular behaviors.

2. Systems biology

Given this necessity to understand biological functions as emerging from fully integrated systems, a purely descriptive approach is no longer efficient. At some point one must make informed guesses about the kind of general architectures that could provide a given function, and then submit this hypothesis to the filter of experimental facts.

This "hypothesis-driven" systems biology emerged concomitantly to the realization of the human genome project (Furusawa and Kaneko, 2012; Huang, 2009) and is now a powerful driving force to our understanding of biological systems (for recent examples see (Dodd et al., 2007; Salmena et al., 2011). It asks whether there exist design principles for cellular networks—which is not obvious in the first place since biological networks are evolved rather than engineered (Alon, 2003; Jacob, 1977). Typical examples of questions it addresses are as follows. What kind of topology ensures concentration–robustness (the property that a species has an identical concentration for all legitimate steady states) (Shinar and Feinberg, 2010)? What is the simplest way of making a biochemical oscillator (Novak and Tyson, 2008)? What is the interplay between the dynamics of a network, its topology and the degree of nonlinearity of its chemical reactions (Novak and Tyson, 2008)? What are the fail-safe mechanisms that cells use to compensate for the failure of some of their components (Kitano, 2004)?

This "hypothesis-driven" systems biology draws many of its foundations from the theory of dynamical systems. Cellular networks are described as biochemical instantiations of these mathematical concepts, forming out-of-equilibrium systems that display dissipative spatiotemporal behaviors (multi-stability, oscillation, spatial patterns…). This approach proposes experimentally testable hypotheses in order to validate putative mechanisms, or verify commonly accepted assumptions. Like its reductionist counterpart, it relies on *in vivo*, *in silico* and *in vitro* methods to put to a test the proposed design principles about biochemical circuits.

2.1. In vivo systems biology

In vivo, "hypothesis-driven" systems biology is supported by the rise of synthetic biology, whose birth dates back to two papers in 2000. In the first one, Elowitz and Leibler synthetically engineered an oscillator by expressing three mutually repressing proteins into *E. Coli* (Elowitz and Leibler, 2000). In the other paper, Gardner et al. engineered a bistable switch with two mutually repressing proteins (Gardner et al., 2000). Their work departed from reductionism because it sought to alter edges rather than nodes in a network of cellular components. The success of the approach strongly anchored key concepts of dynamical systems theory (including bifurcations, attractors and so on) to the study of cellular behaviors. Since then, the *in vivo* synthetic approach to systems biology has shed a new light on genetic regulation and provided a wealth of re-wired cellular devices (Qi et al., 2013). For example, synthetic circuits helped to understand the role of noise in gene expression (Eldar and Elowitz, 2010; Elowitz et al., 2002; Suel et al., 2007), or highlight the minimal units required to drive cell cycles (Coudreuse and Nurse, 2010)

2.2. In silico systems biology

Mathematical toy models are often used in physics to capture essential features of a complex system. Similarly, toy models have proved indispensable in biology to sharpen intuition and verify assumptions, because they condensate in a few molecular components and reaction steps the essence of a biological process. Classical toy models include: kinetic proofreading (which drastically reduces error rates in biosynthesis or antigen recognition (Hopfield, 1974; Ninio, 1975)), ultrasensitivity (which bestows a digital response to some circuits (Goldbeter and Koshland, 1981; Buchler and Louis, 2008) or morphogenesis robustness (which ensures stability of morphogen gradients against perturbations (Eldar et al., 2002).

Conversely, fully descriptive simulations are equally needed to rigorously verify and predict the integrated dynamics of cellular networks—provided a corpus of their mechanisms already exists (Tomita, 2001). *Mycoplasma genitalium* proved small enough (~500 genes) to be tackled by a "whole-cell" approach. Karr et al. gathered 1900 parameters from 900 publications in order to simulate in greatest detail the interactions between the metabolome, transcriptome, genome and proteome of *Mycoplasma genitalium* (Karr et al., 2012). In some sense, "whole-cell" simulations are the systems biology's pendants to atomistic simulations.

In silico simulations often make predictions that are experimentally verifiable. Mather et al. (2010) analytically studied competition of substrates for an enzyme using queuing theory. They predicted a striking effect (correlation resonance) in which the levels of competing substrates suddenly correlate around a balancing point. Correlation resonance was subsequently verified *in vivo* with a synthetic circuit that saturated the degradation machinery of *E. Coli* (Cookson et al., 2011).

Data mining—which eschews strong biological hypotheses about the structure of its data—has also unearthed salient principles from the deluge of information made available by high-throughput experiments. Jeong et al. (Jeong et al., 2000) have discovered that the statistical distribution of nodes in metabolic networks is not random, but often follows approximately a scale-free law: a few species, called hubs, are connected to many others, while most species are connected to few others. Milo et al. (Milo et al., 2002) have exhibited motifs that appear more often than random in database descriptions of cellular networks (feed forward, overlapping regulons or multi input nodes), and are candidate bricks from which regulatory networks could be formed.

2.3. In vitro regulatory networks

With regard to the complexity of cells, the study of *in vitro* networks stands as the "enzymology" of systems biology. It extracts networks from their cellular context, or replicates their topology, in order to study their dynamics, kinetics, and thermodynamics in detail. For the same reasons that *in vitro* studies provide a controlled environment to reductionism, *in vitro* regulatory networks are toy models that offer a flexible test bed for the design principles of biochemical networks. But while *in vitro* reductionism investigates natural proteins transposed to artificial settings (for example in a crystal structure), *in vitro* regulatory studies decipher the working principles of biological regulatory systems by reproducing, with various levels of abstraction, prominent dynamic features of these reaction networks (Fig. 1).

The *in vitro* approach to understanding genetic circuits fundamentally seeks to reproduce inside tubes the central dogma of transcription/translation regulation: "DNA makes RNA makes proteins, which control DNA" (Fig. 1a and b). The most obvious way is to recreate *in vitro* this circular flow of information using the very enzymatic machinery that actuates this process within cells. While transcription (DNA to RNA) is relatively straightforward to perform *in vitro*, translation (RNA to protein) is more challenging because it relies on hundreds of components. Yet, cell-free protein synthesis is now commonly available, initially driven by the need of biotechnologists to produce proteins on a large scale (Katzen et al., 2005). Cell-free expression systems typically use a crude cell extract for translation, coupled to a bacteriophage RNA polymerase for transcription. To overcome the limitation of crude cell extracts (presence of nucleases and proteases, poor characterization of components…) Shimizu and colleagues introduced a purified set of components obtained by recombination (Shimizu et al., 2001). But while biotechnologists only need to produce proteins, synthetic biologists also need to degrade them so as to achieve a true dynamic behavior. Indeed a system that only produces components without removing any cannot display complex self-organizing behaviors (Halley and Winkler, 2008). Degradation of proteins is often obtained by tagging them for degradation by a dedicated enzymatic machinery (Elowitz and Leibler, 2000; Fung et al., 2005). *In vitro* regulation such as activation, repression or cascading was demonstrated by (Noireaux et al., 2003) with commercial cell-free systems. Noting the paucity of regulation mechanisms of conventional cell-free synthesis, (Shin and Noireaux, 2012) recently reported *in vitro* regulatory networks that combined sigma factors of the *E.Coli* transcriptional regulation machinery with bacteriophage polymerases. They used this enriched repertoire of regulation to construct feedforward, recurrent and logic circuits. They also demonstrated the working of synthetic circuits inside a liposome, paving the way to the construction of a protocell (Noireaux et al., 2011).

A more abstract approach to *in vitro* regulation dispenses with the machinery for protein synthesis and protein-based regulation, while preserving the key concepts of biochemical networks. This is because systems biology is more concerned by the interactions of components than their precise nature. The possibility to overlook structural chemical details while keeping kinetic interaction patterns is an important, if not the major, difference between systems biology and reductionism.

DNA offers an ideal material to replace protein-based signaling because in addition to its information-storage role, it is a well understood, programmable and easily available polymer. Progress in DNA nanotechnology in the last two decades has emphasized the versatility of this molecule: it can be folded into convoluted 2D and 3D structures (Douglas et al., 2009; Han et al., 2010; Rothemund, 2006), reproduce hallmarks of enzymatic processes such as allostery, catalysis and cooperativity (Lohmann et al., 2012; Seelig et al., 2006a; Stojanovic et al., 2002; Zhang and Winfree, 2008; Zhang et al., 2011), compute logic functions (Seelig et al., 2006b; Elbaz et al., 2010; Qian and Winfree, 2011; Genot et al., 2011a; Orbach et al., 2012; Genot et al., 2013), perform autonomous locomotion (Bath et al., 2005; Green et al., 2008; Lund et al., 2010; Omabegho et al., 2009; Wickham et al., 2011; Yin et al., 2008), and even serve as a template to direct chemical reactions (Kanan et al., 2004; McKee et al., 2010).

Kim, White and Winfree pioneered an alternative approach to *in vitro* translation that bypasses proteins for signaling while greatly improving modularity (Kim et al., 2006). In their "genelet" networks, nucleic acids not only encoded the topology of a network, but also carried its signals (Fig. 1c) in a DNA to RNA to DNA loop. The role of enzymes is limited to the production (RNAP) and degradation of those signal strands

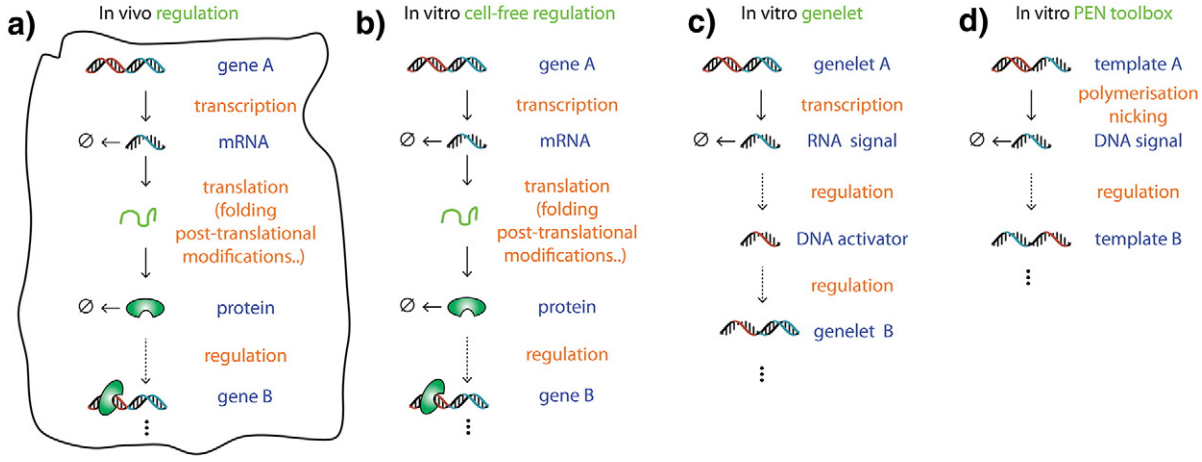

Fig. 1. *In vivo* and *in vitro* regulatory networks. All the approaches use DNA to encode the topology of a network and reproduce the central dogma with different levels of abstraction. a) The flow of information in *in vivo* regulation: DNA makes RNA makes proteins, which control DNA. b) Cell-free regulation faithfully reproduces the central dogma *in vitro*, relying on the same enzymes that actuate it *in vivo* (Shin and Noireaux, 2012). c) The genelets replace protein signaling by RNA signaling, removing the complex chemical step of translation (Kim et al., 2006). d) The PEN toolbox uses three enzymes (Polymerase, Nicking enzyme, Nuclease) and relies on DNA only, eschewing transcription into RNA (Montagne et al., 2011).

(RNAse). More specifically, the nodes of their networks are dsDNA templates. Their extremity contains a nicked T7 RNA polymerase promoter whose labile strand is called the "activator". The T7 promoter maintains its transcriptional activity despite the nick, provided that it is in full duplex form. However, "inhibitor" strands compete for the activator, with the effect of reducing the transcriptional activity. The core concept is that now the RNA transcripts of one genelet can bind the DNA machinery and thus arbitrarily affect the balance of activation/inhibition of another genelet. This provides a means to build activation/inhibition networks of arbitrary topology. Moreover, these circuits exhibit strong nonlinearity due to an ultrasensitive mechanism: since threshold strands sequester RNA signals, the level of signal must exceed that of the threshold to inhibit or activate a promoter sequence. The resulting transfer function between the level of input signal and RNA is almost digital, with fitted Hill coefficients ($n = 5–6$) in the order of those observed for synthetic or biological circuits with multimeric transcription factors.

Those *in vitro* transcription networks are in theory capable of elaborate computations, similar to those of neural networks (Kim et al., 2004). The same authors also experimentally demonstrated the modularity of their genelets by rationally engineering a typical dynamic nonlinear network: a bistable circuit. In this design, two nodes mutually inhibit each other by (transiently) sequestering their respective activators (Kim et al., 2006). The resulting system has two stable states and forms the simplest component of a dynamic memory. Experimental results agree with modeling based on a dynamical systems approach (systems of coupled nonlinear ordinary differential equations). (*Subsoontorn* et al., 2012) subsequently refined the circuit and presented an autoregulatory bistable switch.

Recently, (Kim and Winfree, 2011) reported the construction of oscillators based on their genelets. Several topologies yielded oscillations: negative feedback, positive/negative feedback and circular repression of three switches (Fig. 2e). Uncertainty on the degradation of signal strands proved a major impediment to the quantitative modeling of the oscillators. However, several mechanisms such as the introduction of delays partly stabilized oscillations (Novak and Tyson, 2008).

In addition to providing lessons on biochemical circuits, synthetic clocks offer new ways to dynamically control molecular systems. Clocks are essential to orchestrate complex sequences of actions: computers use an electronic clock to arrange their computations and the human body uses a molecular clock—the circadian cycle—to regulate its metabolism. However molecular engineers lacked an embedded clock to orchestrate chemical reactions. (Franco et al., 2011) took an important step when they reported the use of synthetic transcriptional oscillators to drive DNA–nanomechanical systems (the load). A major obstacle was the retroaction exerted by the load on the oscillator; in order to drive large amounts of load, equally large amounts of oscillators are needed. This is because the load sequesters driving strands from the oscillator, thereby altering or even suppressing the oscillations. The detrimental retroaction of the load was alleviated by an insulating circuit, which amplified the signal, thus reducing the level of driving compounds drained from the clock. This clock opens the road to a new family of autonomous systems that require synchronous operations: for example multistep chemical synthesis (Gartner et al., 2004; He and Liu, 2010, 2011; McKee et al., 2010) or sequential computations (Pei et al., 2010; Stojanovic and Stefanovic, 2003; Stojanovic et al., 2002). This work also suggests that similar mechanisms might exist to insulate biological clocks (such as the circadian clock) from the downstream operations that they control (Del Vecchio et al., 2008; Loriaux and Hoffmann, 2013).

An alternative approach to *in vitro* transcription networks was proposed by Suyama and colleagues with the RTRACS system (Ayukawa et al., 2011; Nitta and Suyama, 2004; Takinoue et al., 2008). Their basic mechanism, which turns an RNA input into an RNA output, relies on three polymerization steps (by RNA polymerase, DNA polymerase, and reverse transcriptase) and one degradation step (by Ribonuclease H). They mathematically demonstrated the feasibility of an oscillator based on a network of such reactions. Experimentally they demonstrated the modularity of their system, exhibiting for example an AND gate. The modular design of RTRACS offers the potential to integrate various exogenous functions, such as the production of aptamers or the downstream regulation of cell-free synthesis.

Montagne and colleagues took the *in vitro* abstraction a step further with the Polymerase Exonuclase Nickase (PEN) toolbox (Fig. 1d). They replaced transcription of RNA by a DNA polymerization step and thus worked with networks entirely based on DNA oligonucleotides (Montagne et al., 2011). In their design, a DNA signal strand binds to a

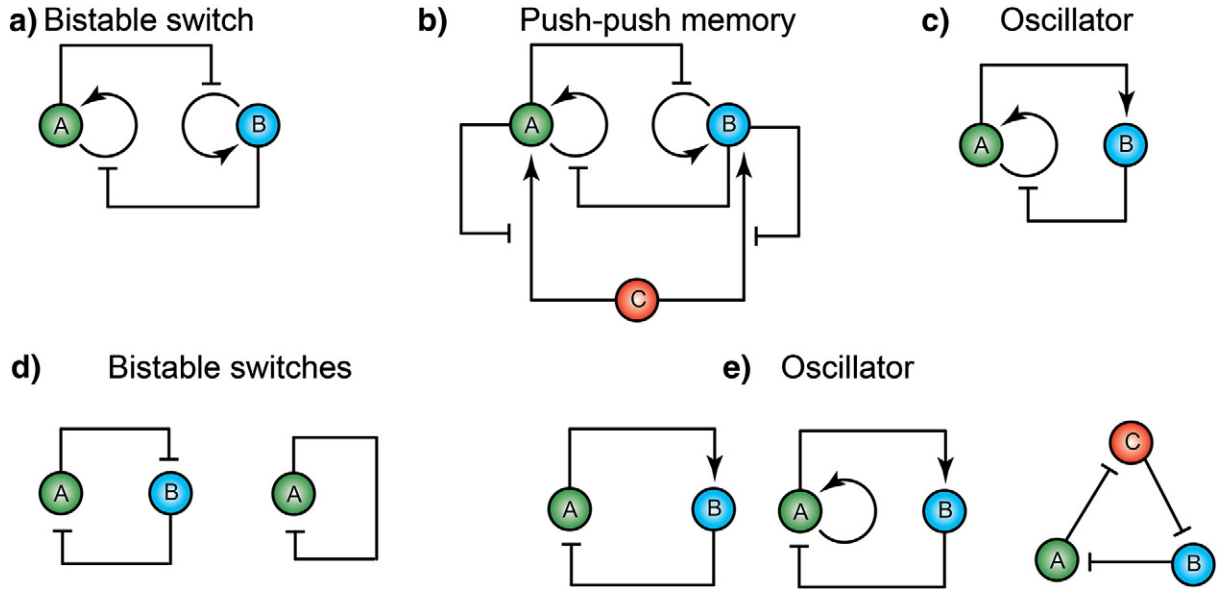

Fig. 2. Overview of the major topologies implemented in vitro. a) Bistable switches from the PEN toolbox (Padirac et al., 2012a). Two autocatalytic loops mutually inhibit each other. b) Toggle memory. Based on the previous design, an input *c* alternatively switches the memory into the A or B state. c) Oscillator based on an autocatalytic loop and delayed inhibition (Montagne et al., 2011). d) Bistable switches based on genelets (Kim et al., 2006). The first switch comprises mutually inhibiting nodes (which are not autocatalytic). The second is an autoregulatory switch that inhibits itself. e) Oscillators with delayed inhibition, delayed inhibition plus autocatalysis, and circular inhibition (repressilator) (Kim and Winfree, 2011).

template, which directs its elongation by a DNA polymerase. A nicking enzyme recognizes this product and nicks the elongated duplex, liberating the primer and a new signal strand. A thermophilic exonuclease continuously degrades signal strands. Signal strands can also act as inhibitors by binding in the middle of a template, preventing hybridization of activating strands. Besides the difference in the molecular nature of the dynamic species (DNA, degraded by a DNA exonuclease, for the PEN toolbox and RNA, degraded by a RNAse for the genelets) the PEN toolbox and the genelets approaches differ markedly in their inhibition mechanism: The genelets sequester activators in order to inhibit the production of signal, whereas the PEN toolbox sequestrates templates (this difference has important dynamic consequences). Montagne et al. used this simple system to assemble an oscillator based on auto-activation and delayed auto-repression (Fig. 2c). The oscillator (which was named *Oligator*, following the traditional nomenclature of chemical oscillators) was initially monitored with an unspecific intercalating dye. Later Padirac et al. (2012b) reported the development of fluorescence methods to directly monitor reactions at particular nodes of interest, without overly perturbing the function. Padirac et al. (2012a) also reported the use of bistable switches to implement toggle memories, which can be switched ON and OFF by repeated addition of the same input.

These hybrid systems—which use DNA for encoding topology and carrying signals and enzymes for actuation—are truly dynamic, powered by a continuous flow of energy from a source (dNTP or NTP) to a sink (dNMP or NMP). However, even with the reduced set of reactions used in such studies, it appears that precisely predicting the behavior of enzymes remains challenging. Qian et al. (2011) followed an even more abstract approach to understand biochemical circuits. They implemented neural networks using a mechanism that relied only on the hybridization and displacement of DNA: toehold-mediated strand displacement (Chen, 2012; Genot et al., 2011b; Xing et al., 2011; Yurke and Mills, 2003; Zhang et al., 2007). Departing from the traditional Boolean approach to computing circuits, Qian and colleagues reported a Hopfield memory (which recognizes and completes memorized patterns) that stands as the most advanced example of emergent behavior based only on DNA hybridization. While they do not resort to enzymes, circuits based solely on DNA hybridization may be relevant to cells. Indeed the discovery of RNAi has shown that simple hybridization events involving only short nucleic polymers (i.e., the flow of information does not pass through the central dogma) are important mechanisms in the global regulation of genetic information (Salmena et al., 2011).

3. Lessons from *in vitro* regulation

If the study of *in vitro* regulation networks seeks to elucidate design principles, then what lessons have we learned? In view of the body of results accumulated in the last 10 years, a handful of recurrent themes keep popping up.

3.1. Degradation is as important as production regarding the functioning of complex reaction networks

The balance between them is a recurrent concern in the literature (Beutel and Peacock-Lopez, 2006). For example, Karzbrun et al. (2011) studied a coarse-grained model of *in vitro* transcription/translation/degradation which was expected to lead to steady states. However the quick saturation of degradation led to a constant accumulation of proteins. Kim and Winfree (2011) investigated in fine detail the effect of abortive production and incomplete degradation on oscillations (for example by testing different nucleases). With (biological) hindsight, the importance of degradation is not surprising as organisms like *E. Coli* devote as much as 3% of their enzymatic activity to proteolysis (Maurizi, 1992) and protein half lives can span a range going from years to minutes (Eden et al., 2011; Loriaux and Hoffmann, 2013)

3.2. Delays are essential drivers of dynamics

Montagne et al. (2011) noted that an intermediate species was necessary to delay auto-repression and enable robust oscillations. Kim and Winfree (2011) observed that designs with delays gave rise to more robust oscillations. Those experimental facts confirm theoretical models pointing to the importance of delays for oscillations. For example, Novak and Tyson (2008) suggested that such delays occur due to the nuclear trafficking of RNA and proteins, and may enable cellular oscillators.

3.3. Competition for enzyme alters dynamics

An overarching theme is the subtle influence of competition (Genot et al., 2012; Nandagopal and Elowitz, 2011; Rondelez, 2012; Yeung et al., 2012). Enzymes, which drive most of the chemical steps in cellular networks, are limited resources. This fact is classically embodied by the use of the Michaelis–Menten equations to describe enzymatic kinetics. Since enzymes take a finite time to perform their catalytic function, there exists a maximal processing speed (Vm), reached when the concentration of substrate far exceeds the Michaelis constant of the enzyme (Km). This saturation leads to bottlenecks in cellular pathways which are well known to metabolic engineers (Tyo et al., 2007).

Competition appears when at least two different substrates compete for the same enzyme. Because saturation depends on the *total* concentration of *all* substrates, the processing of one type of substrate invariably slows down the processing of all other types of substrates, creating hidden layers of interactions between substrates (Rondelez, 2012).

In vitro studies magnify the effect of competition, which potentially goes unnoticed *in vivo* (and hence is involuntarily neglected in *in silico* models). Indeed, competition may be masked *in vivo* by the large number of substrates competing for the same enzyme, rendering the processing kinetics pseudo-first order with respect to a given substrate (increasing the apparent Km) (Wong et al., 2007). *In vitro* studies, by contrast, have fewer substrates, unmasking this pseudo-first order. For example Fujii and Rondelez (2013) studied the dynamics of *in vitro* oscillators when they are run individually or collectively. They observed that two oscillators, with distinct frequencies when run individually, might synchronize when run in the same tube. This coupling may seem surprising since the oscillators are unrelated in sequence. However they compete for the same set of enzymes to actuate their oscillations, which potentially couples their frequencies. In this specific case, the coupling was reported to result mostly from competition for the degradation enzyme.

While competition creates hidden and non-linear interactions, it is not necessarily a nuisance. Shin and Noireaux (2012) observed that competition of sigma factors for the polymerase could be engineered to regulate gene expression. Kim et al. (2004) proposed to use competition for a polymerase to efficiently compute Winner-Take-All functions. This approach was extended to show how competition could be used *in vitro* (Genot et al., in press) and *in vivo* (Genot et al., 2012) to process information, for example by amplifying small differences in concentrations, or digitizing the result of a molecular computation.

3.4. Competition may deeply influence gene transcription

It is often assumed that coexpressed proteins share a common regulation mechanism (Allocco et al., 2004). The correlation resonance of Cookson et al. (2011) and Mather et al. (2010) shows that the expression of proteins degraded by a common pathway may be correlated, even if they do not share any upstream regulation mechanism. This correlation was generalized *in silico* by Genot et al. (2012) who noted that species

do not even need to compete to be correlated, and that it suffices that they share a common competitor. These claims concerning the importance of competition in synthetic systems echo the increasing appreciation of its repercussions by molecular biologists. For example, mapping of the transcription landscape in *S. Cerevisiae* has shown that on average 40 binding sites compete for each transcription factor in this organism (Lee et al., 2002). Competition for miRNA has been proposed as an extra layer of interactions that profoundly impact regulation (Salmena et al., 2011)

3.5. Topology matters, so do kinetics

Regulatory networks are inherently dynamical systems with rich long-term dynamics: bifurcations, unstable points, attractors, limit cycles (Furusawa and Kaneko, 2012). While a wealth of information is retrieved by inspecting the topology of cellular network, *in vitro* studies point to the equal importance of their kinetic details (Sorger, 2005). Indeed, some network topologies cannot give rise to oscillations or bistability if their underlying kinetics is too linear (Novak and Tyson, 2008). For example in (Kim et al., 2006), mutual inhibition of transcription is sufficient to achieve bistability. By contrast, this topology would not work in the *in vitro* networks of (Montagne et al., 2011) because their kinetics are inherently linear. To implement bistable states, they compensated the linear kinetics with two additional autocatalytic loops. They essentially traded kinetic nonlinearity (large Hill coefficient) for topological complexity (the addition of edges to a node). In turn, the same autocatalytic topology was reused by Huang (2009) with different kinetics to propose a theoretical model of cellular differentiation that displays tri-stability. The intimate but equivocal relationship between topology and function was noted *in vivo* by Guet et al. (2002) who identified synthetic networks with identical topologies but different responses. Conversely, they exhibited networks with similar responses but harboring different topologies. The topology of the bistable switch presented by Padirac et al. (2012a) can also lead to a tristable switch, as shown by (Huang, 2009).

3.6. Resemblance with ecosystems

Cellular networks are complex systems; complex behaviors emerge from the collective function of simple components. The engineering of cellular networks is often compared to the design of electronic circuits, but one may wonder if cellular networks do not look more like ecosystems. Firstly, the competition for enzymes that we mentioned above is reminiscent of the competition for resources in ecosystems (Huisman and Weissing, 1999). Secondly, emerging behaviors—such as complex spatiotemporal population dynamics—are more the hallmark of ecological networks than electronic circuits. For example Marshall and Ellington (1999) observed the emergence of molecular parasites—a recurrent theme in ecology—during the isothermal *in vitro* amplification of nucleic acids. *In vitro* parasites were reported on several other occasions (Ehricht et al., 1997; Urabe et al., 2010). Recently, two of us (Y.R and T.F) constructed a synthetic molecular ecosystem that displays predator–prey dynamic (Fujii and Rondelez, 2013). The system is based on a variant of the chemistry of (Montagne et al., 2011). The molecular ecosystem exhibits sustained oscillations over more than 30 periods and reproduces predatory, mutualistic and competitive interactions. This oscillatory behavior emerges from a simple recipe: three enzymes and a single strand of DNA, in a standard buffer. The remarkable robustness and simplicity of this predator–prey oscillator shows that much inspiration ought to be taken from the subtle dynamics of ecosystems.

4. Conclusion and perspectives

We have highlighted how the design and study of *in vitro* regulatory networks shed light on the workings of cells. In view of the emergence of systems biology, *in vitro* networks offer an opportunity to explore new hypotheses, but also to verify previous assumptions or theoretical propositions (Eigen and Schuster, 1978; Kauffman, 1969; Schuster and Sigmund, 1983). Notably, we have stressed that, from a dynamic point of view, the precise chemical nature of a network is not very important: what really matters is the topology and the kinetics of the interactions. This has allowed investigators to abstract natural regulatory networks with *in vitro* mimics, keeping the essence of biological regulation while allowing a simpler implementation and observation. Another point to note is the importance of dynamic systems theory to understand *in vitro* networks, which is also increasingly used to explain *in vivo* processes such as the differentiation of stem cells (Furusawa and Kaneko, 2012; Huang, 2009; Yamanaka, 2009). Progress in DNA nanotechnology will offer increasingly elaborate methods to regulate *in vitro* reactions, reproducing faithfully the subtle molecular choreography orchestrated by cellular networks. For example, well-mixed dilute solutions are only an ideal approximation of the careful hierarchical spatial organization found in cells. For *in vitro* systems, DNA origami may provide a scaffold to localize various molecular components of a given network in order to tune their cooperative behavior (Pinheiro et al., 2011). Man-made DNA walkers and motors (Bath et al., 2005; Green et al., 2008; Lund et al., 2010; Omabegho et al., 2009; Wickham et al., 2011; Yin et al., 2008) may be used to translocate specific compounds or obtain biased diffusion patterns. And the recently reported origami membrane pores by Langecker et al. (2012) may open the way to the mimicry of cellular compartmentalization and its important dynamic effects (Novak and Tyson, 2008). We therefore expect that *in vitro* systems will progressively incorporate more and more molecular devices to provide instrumental dynamic models of cellular processes, while at the same time staying tractable enough to allow quantitative modeling and analysis. This will position them as an invaluable tool to address basic questions related to systems biology.

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