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Synthetic *in vitro* circuits

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Abstract

Inspired by advances in the ability to construct programmable circuits in living organisms, *in vitro* circuits are emerging as a viable platform for designing, understanding, and exploiting dynamic biochemical circuitry. *In vitro* systems allow researchers to directly access and manipulate biomolecular parts without the unwieldy complexity and intertwined dependencies that often exist *in vivo*. Experimental and computational foundations in DNA, DNA/RNA, and DNA/RNA/protein based circuitry have given rise to systems with more than 100 programmed molecular constituents. Functionally, they have diverse capabilities including: complex mathematical calculations, associative memory tasks, and sensing of small molecules. Progress in this field is showing that cell-free synthetic biology is a versatile training ground for understanding native biological circuits and engineering novel functionality.

Introduction

Breakthroughs in our ability to read and write DNA have enabled researchers to construct sophisticated, circuit-like behavior in living systems [1]. More than ten years ago, pioneering efforts by Elowitz and Leibler and Gardner *et al.* [2,3] provided a conceptual framework for viewing cells as a system of interacting circuits – structures composed of several elements that are capable of information processing by accepting an input, executing a series of logical computations, and producing an output. This framework, championed by many synthetic biologists [4], has inspired new applications and shed light on our understanding of fundamental biological phenomena. Nevertheless, *in vivo* circuitry is often unpredictable. Even if the functions of individual `parts' are known, they may not work as expected in different cellular contexts due to intertwined circuit dependencies and crosstalk between biomolecules. Thus, the complexity of successfully implemented *in vivo* circuits to date is limited in size and scope to tens of molecules [4]. This pales vastly in comparison to

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the simplest living organisms, which consist of several hundred to thousands of genes, non-coding RNA species, and small molecules.

As a complement to *in vivo*-based approaches, *in vitro* systems provide an unprecedented freedom to modify and control biochemical systems for technological applications and to understand the design principles of biological circuits. Removing genetic regulation, for instance, drastically reduces complexity by negating a cells' ability to adapt and evolve [5]. For decades, *in vitro* systems have made integral contributions to biological research in both discovery and technology - from discovery of the genetic code [6] to the development of core techniques such as the Polymerase Chain Reaction [7] as well as disruptive technologies for manufacturing-scale cell-free protein synthesis [8]. Now, the study of complex biological circuits is emerging as a novel application of *in vitro* systems that can complement and guide *in vivo* studies.

This review will focus on recent developments in the field of synthetic *in vitro* circuits over the last 3 years. We first examine minimal nucleic-acid based systems that offer unparalleled experimental flexibility and predictability. Next, we discuss circuits created in hybrid systems – containing purified enzymes and nucleic acids. We end our discussion of *in vitro* circuits on complex systems that are capable of transcription and translation.

Nucleic acid systems

While the classical sense of the word “circuit” conjures up images electricity flowing through transistors, DNA is capable of both carrying information and performing computations on that information [9,10]. Circuits constructed in this manner have relatively few possible interactions and points of control (Figure 1 **(**top**))** making their quantitative description more manageable and comprehensive. They provide theoretical insight into the generalized function of chemical circuits based on diffusion and stochastic binding – principles that must be understood to make genetic circuit design a predictive science.

Dramatic increases in circuit complexity in DNA systems over the last three years partly stem from the development of `seesaw' gates that make use of toehold exchange reactions (Figure 2A,B) [11–13••]. In classical toehold displacement reactions, a single-stranded DNA (ssDNA) molecule displaces one strand of a double-stranded DNA (dsDNA) complex by binding to a complementary short overhang (toehold) region on the dsDNA. The ssDNA (input) displaces the previously bound strand of DNA (output), which can then serve as an incoming signal for another reaction. From this starting point, researchers have built a diversity of nano-scale devices and circuits capable of complex computation (see [14•] and [15] for more comprehensive reviews).

In the case of toehold exchange reactions, the reverse reaction is made possible by including a toehold design on both sides of the dsDNA molecule (Figure 2A). Only two cascaded seesaw gates are required to produce the OR and AND logical functions (Figure 2B) and more complex cascades can compute NOT, NAND, NOR, and XOR. To show the scalability of these gate units, Qian *et al.* constructed a neural network consisting of 112 DNA strands that demonstrates associative memory capable of answering 81 possible questions [16], as well as a 130-stranded DNA circuit capable of computing the square root of a 4 digit binary number [17•].

Models derived from experimental investigations [11,12] are increasingly accurate at predicting the kinetics of strand displacement reactions and have allowed construction of increasingly complex implementations. Further, experiments guided by thermodynamic models have verified designs that perform within a 20°C range of temperatures, 1–5 μM concentrations of nucleic acid, and 1–47 mM Mg^{2+} [18•]. This robustness illustrates that the

understanding gained from these systems may be generalized to a range of conditions and not limited to particular experimental set-ups. A suite of computational tools, theoretical frameworks and even dedicated programming languages can be used to design and analyze DNA circuits [19–23]. Concurrently, novel theoretical possibilities [24] and practical implementations are continually being developed such as those utilizing hairpins [25,26], cooperative binding [27], deoxyribozymes [28,29], photochemical activation [30] and associative toehold activation [31].

Importantly, studying strand displacement circuits *in vitro* has led to practical uses that would have been difficult to anticipate prior to the expansion of this largely theoretically motivated work. Researchers have shown that *in vitro* DNA circuits can amplify signals and detect RNA and small molecule analytes using different reporting methods [32]. Further, an area of research that is likely to expand involves using DNA circuits as visualization tools for imaging DNA based reaction cascades [33], mRNA's [34] and reversibly imaging proteins [35].

Hybrid systems

Understanding and exploiting transcriptional logic is a complex problem that unites many fields of biology, but the vast number of interacting species and circuit cross talk within cells makes it difficult to characterize individual transcriptional gates. *In vitro* hybrid systems composed of DNA templates and purified enzymes, however, allow researchers to isolate the performance of small circuits before exploring how they interact within larger biochemical systems [36–39]. DNA still acts as the logic gate for signal integration (see Figure 3A for sample AND gate implementation), but the inputs and outputs can be DNA, RNA, or metabolites.

Hybrid systems are able to amplify signals, produce new signals, and degrade waste products – functionality that is difficult to implement in nucleic acid systems. Pioneering studies in 2011 highlight the diversity of these circuits, and the fact that similar high-level designs can be implemented in different ways. Montagne *et al.* created a system (the “Oligator”) capable of sustained oscillations using DNA polymerase, a nicking enzyme, and an exonuclease that were responsible for amplification, propagation, and destruction of information carrying DNA oligonucleotides [40••]. This circuitry used only three template strands and three enzymes but was able to couple a positive feedback and a delayed negative feedback loop in a computationally predictable manner that used readily attainable parameters.

Using a different molecular implementation, Kim and Winfree produced a system capable of sustained oscillations by using RNA polymerase and RNaseH to produce and degrade cascading DNA/RNA signals [41••]. They showed oscillations using three distinct designs: a seven-stranded two-switch negative feedback oscillator, an amplified negative feedback oscillator which included the use of a positive feedback system, and finally a novel molecular implementation of a three-switch ring oscillator that was the focus of pioneering *in vivo* studies [2]. Franco *et al.* later expanded on this oscillating system and displayed potential applications by using the negative feedback oscillator as a timing device to separately activate DNA nanotweezers and produce an RNA aptamer [42••]. As with Montagne *et al.*, modeling was integral to understanding system behavior and parameters – including concentrations and sequences – that would produce desired results. Merely by introducing the novel interaction that was required to time their device, the oscillations were severely restricted compared to the original system. This problem of coupling distinct systems was elegantly overcome through the use of an insulator circuit that isolated the output signal and prevented it from disrupting the timing device.

While the previous studies showed successful implementations of designs, they varied in their predictability and quantitative understanding of the systems. Deviations from kinetic models, especially with regards to the repressilator implementation, highlight the shortcoming of our knowledge and make a strong case for studying these systems before attempting to explain more complex cellular oscillators. For instance, incomplete degradation products may partially inhibit reactions in ways that are difficult to predict. In addition, small differences in transcription and degradation rates can compound over time and result in unstable oscillations with growing amplitudes or mono-stable behavior. Nevertheless, studies aimed at predicting and modeling more complex systems show a clear path forward for creating autoregulatory switches [43], neural networks [44], incoherent feed forward loops [45], pattern formation reactions [46•], as well as transcriptional rate regulation based on self-repression [47] and positive feedback [48].

As with DNA circuits, hybrid circuits have a variety of potential applications that are just beginning to emerge. In work that stems partly from initial theoretical and *in vitro* research[49], hybrid circuits were demonstrated inside *Drosophila* embryo lysates [50] providing insight on the functioning of circuits in a much more complex chemical environment. Several studies showed successful *in vivo* implementations of logical computing for potential applications in diagnosis and therapeutics [51,52].

Complete systems

Although previously mentioned studies may include the use of proteins, complete (either purified, or extract based) systems produce proteins *in vitro* and integrate them into circuits. Logical integration occurs at the DNA level but inputs and outputs may be DNA, RNA, protein, or metabolites – all of which may be produced, degraded, or modified in response to signals. Importantly, the diversity of interactions means that there are many more steps that must be understood for predictive implementation of complex circuits (Figure 1). However, these extra steps are also novel points of control that, once thoroughly characterized, may be exploited by researchers to construct highly complex logic systems.

Complete cell-free circuits began with the seminal work in 2003 of Noireaux *et al*. who showed that activating and inhibitory proteins could be produced *in vitro* and that one-, two-, and three-stage cascades could be created using these proteins as circuit elements [53]. Rather than relying on orthogonal promoters from these previous studies, Shin and Noireaux have recently recreated [54] and comprehensively characterized the endogenous sigma factor-based regulation system for use in *Escherichia coli* extracts [55••]. This allowed for the construction of the logical AND gate (Figure 3B), negative feedback loops, and multiple stage cascades. They also demonstrated the modularity of their toolkit for future applications of *in vitro* metabolic engineering and as a possible testing ground for *in vivo* recombinant circuit design. In complementary work, Asahara and Chong expressed promoter subunits and sigma factors in a single tube and showed promoter specific transcription initiation from solely *in vitro* translated and assembled products [56]. Taking an alternative route towards a similar goal, Karig *et al.* expanded on the original work of Noireaux *et al.* by showing that tool-kits based on orthogonal T7 promoters are capable of complex computations by characterizing several inducible and repressible variants and creating a negative feedback loop *in vitro* [57].

An overarching theme of these studies is the precise characterization of individual circuit elements, which are then implemented into complex cascades where their interactions may be controlled. While the search for biological `parts' has proven fruitful for *in vivo* synthetic biologists, many of these parts are still highly context dependent. In cell-free systems, these parts exist in a context outside of cellular adaptation and evolution and the results are

therefore expected to be more tunable and reproducible. However, before controlling protein levels based on logic integration, we must understand the range of possible points of control and parameters that are important to steady-state protein levels. Karig *et al.* looked at the effects of ribosome binding sites and plasmid usage on target protein levels that were essential components of their circuit [57]. Though they did not explicitly recreate circuits, in separate studies Ahn *et al.* used cell-free systems to characterize the effects of 3' transcript stability, fusion partner leader sequences, and initial codons on protein production rate and steady state levels [58–60]. Many of these studies look at determinants of single protein production, but Du *et al.* have taken this work one step farther and studied the complications that arise when trying to express multiple proteins *in vitro* [61]. Karzbrun *et al.* took a step towards integrating the effects of these different components by creating a computational framework for predicting protein levels [62•]. With this knowledge, researchers may be able to implement logical control at specific processes to enable diverse circuit designs and some even dream of minimal cells [63].

Finally, in what we believe will be an increasing trend during the coming years, several studies have designed and analyzed circuits *in vitro* and successfully implemented those designs *in vivo*. Saito *et al.* developed a translational regulatory system based on L7Ae, an archael ribosomal protein, binding to mRNA molecules [64••]. They describe different mRNAs that are capable of activation and repression in response to the same protein input. This elegant study highlights the diversity of possible inputs that can be accepted by circuits composed in complete systems and exploits a unique control point that is only available in systems capable of translation. Importantly, their design was first tested in a purified cell-free translational system before being successfully implemented inside of a human cell-line. Similarly, Karig *et al.* tested their T7-driven negative feedback constructs in a purified cell-free translation system, cell extracts, and *E. coli* cells and showed the expected functionality across all systems [57]. These studies indicate that in addition to an increasing number of *in vitro* applications [65,66], *in vitro* environments can be a training ground for *in vivo* circuit design.

Conclusion

There has been dramatic growth in the construction, complexity, and predictability of cell-free biological circuits over the past three years. This growth underscores the importance of cell-free systems as a novel environment for engineering and analyzing complex biological circuits at the molecular level. In addition, designs that rely on DNA strand displacement reactions are yielding tremendous insight into the emergent behavior of complex chemical systems that lies at the heart of cellular life. Recent growth in this field also reflects an increasing interest in exploiting the unprecedented freedom of design afforded by cell-free biology for compelling applications [5,65–67]. The *in vitro* circuits described above, being structurally and functionally diverse, are poised to impact chemical, physical, biological, and computational research in diverse and unpredictable ways. This is precisely what makes their continued development necessary and exciting.

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Highlights

Simple *in vitro* biochemical circuits can provide insight into biological design.

Greater complexity of interactions in *in vitro* circuits decreases predictability.

Unanticipated applications derive from theoretically motivated work.

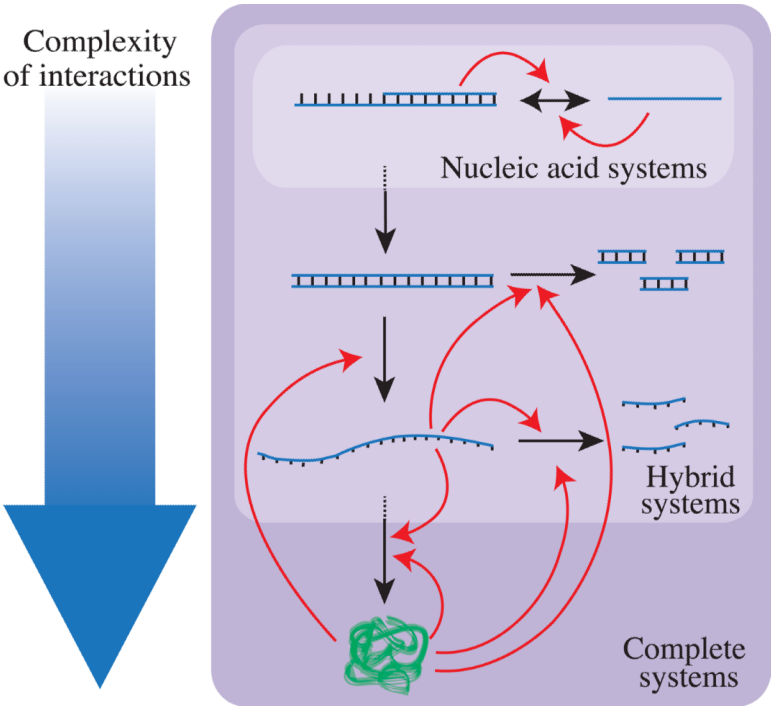

Figure 1.
The reactions and molecular composition of different synthetic *in vitro* circuits. Black arrows represent routes of information flow while red arrows represent interactions capable of controlling or modulating this flow. Nucleic acid based systems contain relatively few types of interactions, resulting in more predictable behavior. Hybrid systems, which are capable of producing and degrading nucleic acids, are intermediate on this scale with several new points of control and information flow. Complete systems can replicate the entire `central dogma', but their increased complexity of interactions makes them less predictable. Note: small-molecule inputs and outputs via enzymes, ribozymes, and deoxyribozymes can, in principle, occur in all systems.

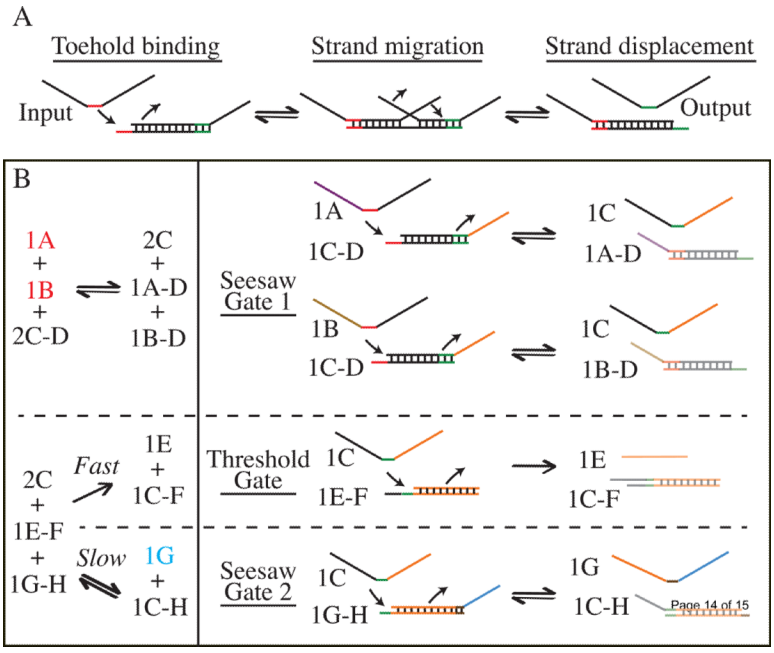

Figure 2.

Toehold exchange and seesaw gate function and implementation [13]. **(A)** A ssDNA input binds to a complementary toehold (red) on a seesaw gate and – through strand migration – replaces an output strand of the dsDNA that is then capable of the reverse reaction via a separate toehold (green). **(B)** A functional AND gate that takes input molecules A and B and outputs G. The left side presents a simplified overview while the right includes molecular detail. At seesaw gate 1, inputs (A and B) displace a strand of DNA (C) that then encounters threshold gate 1(E–F). This gate quickly and irreversibly turns C into waste by having a larger complementary overhang than that of molecule G–H. Only after threshold molecules have been depleted will C bind to G–H and produce the output strand G.

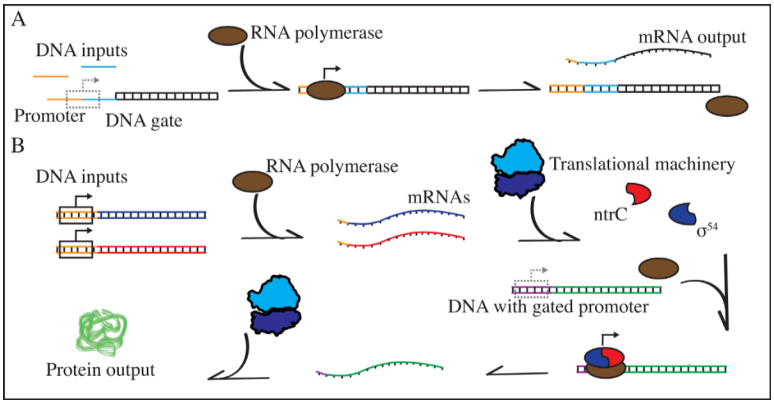

Figure 3.

Sample AND gate implementation in hybrid and complete systems. **(A)** Hybrid systems may use DNA, RNA, or small molecule inputs and outputs. Here, an incomplete promoter (dashed box) is bound by two ssDNA inputs that complete the promoter region. The polymerase enzyme (brown) then binds and transcribes an mRNA output. **(B)** Complete systems have a diverse array of possible inputs and outputs. Here, two DNA input signals with intact promoters (solid box) are transcribed into mRNA and translated into functional protein products ntrC (red) and σ^{54} (blue). These protein products then bind to a polymerase and allow it to transcribe a gated promoter (dashed box), leading to production of a protein output [55].