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Review

Digital and analog gene circuits for biotechnology

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Biotechnology offers the promise of valuable chemical production via microbial processing of renewable and inexpensive substrates. Thus far, static metabolic engineering strategies have enabled this field to advance industrial applications. However, the industrial scaling of statically engineered microbes inevitably creates inefficiencies due to variable conditions present in large-scale microbial cultures. Synthetic gene circuits that dynamically sense and regulate different molecules can resolve this issue by enabling cells to continuously adapt to variable conditions. These circuits also have the potential to enable next-generation production programs capable of autonomous transitioning between steps in a bioprocess. Here, we review the design and application of two main classes of dynamic gene circuits, digital and analog, for biotechnology. Within the context of these classes, we also discuss the potential benefits of digital–analog interconversion, memory, and multi-signal integration. Though synthetic gene circuits have largely been applied for cellular computation to date, we envision that utilizing them in biotechnology will enhance the efficiency and scope of biochemical production with living cells.

Keywords: Analog gene circuit · Digital gene circuit · Memory · Metabolic engineering · Synthetic biology

1 Introduction

Biotechnology has benefited from tools that allow researchers to introduce heterologous pathways into microbes [1–3]. However, ensuring that a microbe has all of the machinery for the production of a chemical is often not enough for practical applications. The objectives of the cell rarely match the objectives of the engineer, and so it is the goal of biotechnology to re-wire the cell in such a way that the objectives are aligned and chemical production is optimized. There are many strategies for engineering enzyme levels to avoid production pathway bottlenecks and resource misallocations, including codon optimization [4], tuning promoters [5] and ribosome binding sites (RBSs) [6], and manipulating intergenic regions [7], knockouts [8], and knockdowns [9]. Typically, these methods are used to set the expression levels of different enzymes in a chemical production pathway to their optimal values, which can be determined for a given condition through predictive modeling and/or combinatorial screening [10, 11].

Such optimization can be successful in the laboratory, but difficult when scaling microbial cultures for industry because conditions inevitably become more variable and difficult to control in scaled-up real-world settings [12–14]. Industrial bioprocesses take place in large bioreactors where diffusion is limited and culture mixing is slow and uneven. As a result, cultured cells in bioreactors have different metabolic configurations depending on their loca-

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Abbreviations: A-to-D, analog-to-digital; **ADP,** adenosine diphosphate; **ATc,** anhydrotetracycline; **ATP,** adenosine triphosphate; **CoA,** coenzyme A; **D-to-A,** digital-to-analog; **DNA,** deoxyribonucleic acid; ***E. coli*,** *Escherichia coli*; **FAEE,** fatty acid ethyl ester; **HCP,** high-copy plasmid; **IPTG,** isopropyl β-D-1-thiogalactopyranoside; **LCP,** low-copy plasmid; **mRNA,** messenger ribonucleic acid; **NAD+,** nicotinamide adenine dinucleotide; **NADH,** nicotinamide adenine dinucleotide (reduced); **NADP+,** nicotinamide adenine dinucleotide phosphate; **NADPH,** nicotinamide adenine dinucleotide phosphate (reduced); **PCR,** polymerase chain reaction; **RBS,** ribosome binding site; **RDF,** recombinase directionality factor; **RNA,** ribonucleic acid; **TF,** transcription factor

tion in space and time [14]. Sometimes, undesirable conditions can also arise that create cellular stress. For instance, a common problem results from the zonal formation of low oxygen and/or excess glucose conditions that induce cells to produce organic acids, such as acetate, which inhibit cell growth [15]. Zonal heating, which can occur from physical agitation or from certain metabolic processes, is also a challenge [16].

To overcome the unpredictable and variable nature of industrial bioprocessing, the ideal regulation of a production pathway would not be statically set at the optimum for a single condition, but rather dynamically regulated for continuous adaptation to different conditions. An early example of dynamic regulation in biotechnology comes from Farmer and Liao [17], where intracellular acetyl phosphate was used to signal excess glycolytic flux and subsequently divert that flux to lycopene production in *Escherichia coli*. More recently, Zhang et al. [18] improved titers of fatty acid ethyl ester (FAEE) from *E. coli* by dynamically regulating the FAEE production pathway in response to a key pathway intermediate.

Another beneficial application of synthetic dynamic regulation to microbial production is in bioprocesses that involve multiple steps. For example Dedhia et al. [19] used a synthetic gene circuit (from Chen et al. [20]) to demonstrate that batch cultivation of *E. coli* can yield more biomass if the cells are engineered to switch metabolic programs from glycogen accumulation to glycogen degradation during growth. Other studies indicate that the productivity of a bioprocess can be optimized if microbes are first grown to a critical density under growth-maximizing conditions, and then induced to produce the target chemical in a separate phase [21, 22]. The type of dynamic regulation needed for abrupt state transitions (e.g. switching from a growth objective to a production objective) is different from the aforementioned type of dynamic regulation required for continuous adaptation to changing conditions. The ideal implementation of each regulatory strategy can leverage digital and analog circuits, respectively.

In this review, we focus on different strategies for creating synthetic digital and analog circuits in living cells, each with their respective advantages and utility in various situations. These strategies differ in whether they have memory or not, whether they have one input or integrate multiple inputs, and whether the computational paradigm used is digital or analog. The application of these circuits to metabolic engineering has the potential to not only improve traditional methods of industrial-scale microbial production, but also to enable complex and fully automated production programs that were not previously possible.

2 Digital circuits

In biological circuits, signals can be represented as chemical concentrations and digital signals are those that take on discrete values. Typical digital circuits in metabolic engineering and synthetic biology have single outputs with two possible states, denoted "on" or "off". These circuits could potentially be used to improve efficiencies in multi-step bioprocesses and allow for autonomous state transitions that could facilitate multi-stage chemical production without requiring external oversight. The following subsections discuss: (i) digital memory; (ii) digital computation; and (iii) multi-input digital logic.

2.1 Digital memory

A circuit with memory is one in which the output depends not only on the current input state, but also past states. Natural biological circuits leverage both epigenetic and direct deoxyribonucleic acid (DNA)-encoded memory in association with various applications. Specific examples include lactose sugar metabolism in *E. coli* [23], cell-cycle regulation [24, 25], cell-fate determination in *Xenopus* oocytes [26], and host immune system evasion in *Salmonella* [27, 28]. In the interest of biotechnology, memory enables the ability to turn a transient input into a sustained, digital output, which can reduce the potentially expensive costs associated with supplying industrial-scale microbial processes with exogenous inducers [29]. Memory is also capable of setting up production programs that are conditional on transient events that have occurred in the past. For example Friedland and Lu et al. [30] engineered *E. coli* with memory circuits to count pulses of input inducers. Extensions of this concept could be used to create organisms that autonomously track cell density during a bioprocess by counting cell cycles.

An early example of synthetic memory was implemented epigenetically through a toggle switch that relies on two mutually repressing genes, hereafter referred to as gene A and gene B, to achieve bistability (Fig. 1A and 1C) [31]. In one stable equilibrium, the "on" state of gene A suppresses the expression of gene B, thus antagonizing its own repression by the product of gene B. But if enough inducer of gene B expression is added (relieving repression of gene B by the product of gene A), a critical point is reached and the genes reverse their roles. After this transition, inducers can be removed without reversing the switch. However, if reversal is desired, then it can be achieved with a transient pulse of an inducer for gene A expression. It should be noted that this particular circuit from Gardner et al. [31] represents a form of memory where an inducer, after causing a switch in cell state, can be entirely removed without causing a reversal of state. Variations of the mutual-repression-based epigenetic toggle switch have been made since this publication

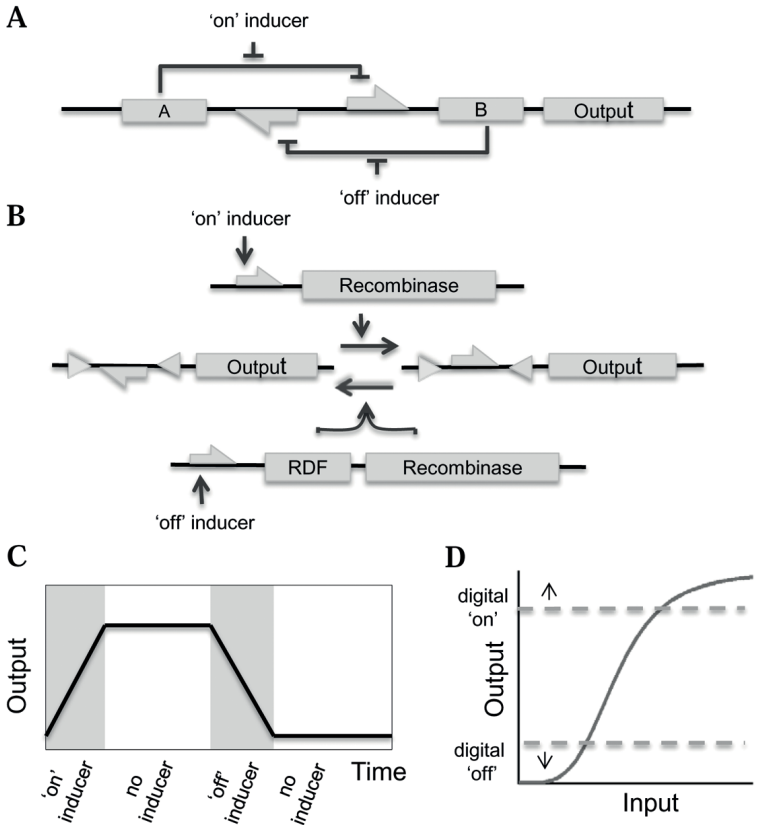


Figure 1. Implementing memory of inducer inputs. (**A**) Schematic of an epigenetic toggle switch, adapted from Gardner et al. [31]. (**B**) Schematic of a unidirectional recombinase-based switch. The reverse transition requires both RDF and recombinase. Half arrows represent promoters, which point in the direction of transcription. Rectangles are genes. Horizontal triangles are unidirectional recombinase recognition sites. (**C**) Simplified example of the output from (A) and (B) over time with "on" inducer and "off" inducer exposure periods followed by respective periods of no inducer to demonstrate memory. (**D**) Input–output transfer functions are analog in nature, but the output can be abstracted by digital "on" or "off" states if it is either above or below defined output thresholds, respectively. This subfigure is adapted from Daniel et al. [97].

[32, 33]. Synthetic positive-autoregulation-based epigenetic memory has also been achieved [34–38].

An alternative strategy for creating memory is to encode digital events directly into a cell's DNA via recombinase systems [30, 39–42]. There is a wide diversity of recombinase systems, both bidirectional and unidirectional, that differ in their underlying biochemical mechanisms [43, 44]. Unidirectional recombinase systems are composed of recombinase enzymes (and sometimes enzyme cofactors) that catalyze one-time recombination between two recognition sites. Depending on the orientation of recognition sites to each other on the same DNA strand, the region between them is either inverted or excised. One strategy for controlling gene expression with recombinase-based memory circuits is to put a promoter inside of an invertible DNA region upstream of an output gene. In one state, the promoter points away from the gene, and in the other state, the promoter points toward the gene and output is produced (Fig. 1B and 1C). This circuit switches states when expression of the cognate unidirectional recombinase is induced, and it remains there even after the recombinase is removed. To make memory switches reversible, bidirectional recombinases can be used, although care must be taken to halt recombinase activity after the desired switching event has occurred [30]. In addition, recombinase directionality factors (RDFs) can be used to reverse the activity of unidirectional recombinases [42].

Compared to epigenetic-based memory, DNA-based memory storage presents potential advantages in terms of stability and scalability [40]. For instance, epigenetic memory requires constant energetic input (e.g. for the transcription and translation of feedback proteins). The resulting metabolic burden may make it difficult to scale the amount of memory that can be engineered into a single cell and can lead to selection against synthetic circuits by host cells. Moreover, because epigenetic memory relies on the maintenance of component protein levels above (or below) a critical threshold, memory could be lost due to noise or events that cause extreme changes in protein levels (e.g. cell division, changing environments, or switching growth phase). On the other hand, DNA-encoded memory imparts minimal metabolic burden because it requires no active transcription or translation to maintain its state. This facilitates the scalability of DNA-based memory modules in living cells and it contributes to the stability of the memory state, which persists even after cell death (and can be determined by polymerase chain reaction (PCR) or sequencing [45]). Further, the memory state is naturally inherited from one generation to the next. In terms of production efficiency during a bioprocess, the decreased metabolic burden associated

with DNA-encoded memory (vs. epigenetic memory) may help to increase production efficiency.

However, the potential scalability of recombinase circuits is currently compromised by a lack of comprehensive characterization of different recombinases, including information on their efficiencies and orthogonality to one another and their host cells. For example only one unidirectional recombinase-RDF pair (BxbI gp35 with gp47) has so far been validated for re-writable memory [42]. To advance the field, further efforts must be made to broaden recombinase characterization and validation for synthetic systems.

2.2 Digital computation

External (user-defined) inputs can be used to change the dynamics of microbes during production bioprocesses. In order to leverage these signals to create and control digital behavior and synthetic state transitions in living cells, a system of transduction is required. In other words, there must be a system capable of converting the chemical make-up and concentration of an input signal (selected for its ability to reliably report on a particular condition) to that of an output signal (selected for its ability to actuate a desired response). For engineering external control of bacteria in synthetic biology, tightly regulated and orthogonal promoters based on small-molecule inducers arabinose, tetracycline, and isopropyl β-D-1-thiogalactopyranoside (IPTG) are commonly used [46]. And for yeast, promoters regulated by copper, methionine, and galactose are also common [47, 48]. As an alternative to chemical inducers, light-sensitive gene expression systems have been developed [49–59].

However, the utility of using external inputs to control cellular transitions is limited by the capacity of the exogenous source (often a human user or machine) to know when and what state transitions are supposed to occur. Alternatively, circuits that detect inputs that are endogenous to a cell or its environment can be used for autonomous sensing and regulation. By enabling transitions that do not require exogenous intervention, these circuits can extend bioprocessing to applications where cellular control and monitoring is not easily achieved (e.g. in the human microbiome or in complex multicellular conditions). Zhang and Keasling [60] reviewed strategies for sensing input molecules that are naturally produced inside of the cell or in the environment. Many metabolites already have natural transcription factor (TF) sensors that change their DNA-binding properties upon being bound. Thus, one approach to biosensing is to identify an existing TF sensor for a desired input, and to re-purpose this TF to target a desired output, which can be achieved by expressing the output from a promoter engineered to interact with the TF [17, 18]. An alternative strategy utilizes regulatory ribonucleic acid (RNA) molecules as biosensors [60, 61]. RNA molecules have been engineered to regulate gene expression at the transcriptional level by activating [62], blocking [9], or terminating [63] transcription, as well as at the post-transcriptional level by affecting messenger ribonucleic acid (mRNA) translation or stability [64–67].

Still, even with the appropriate tools for sensing an input, a significant challenge in generating synthetic digital responses is that chemical signals in nature are analog – their concentrations can vary over a continuous set of values. Although the input–output transfer of any signal transduction system is inherently analog, it can be viewed as digital if appropriate thresholds can be set to distinguish between "off" and "on" (Fig. 1D). Analog-to-digital (A-to-D) converter circuits translate analog input signals to digital output signals, with advanced A-to-D converters capable of mapping multiple analog levels to multiple digital levels. For example a 4-bit A-to-D circuit would convert an analog signal into 16 different output levels, each defined by a different 4-bit binary representation. A bit is defined as a single "off" or "on" value.

Ultrasensitivity, characterized by a nonlinear input–output response curve where a small relative change in input creates a large relative change in output, can provide a basis for A-to-D conversion. The Hill coefficient of a circuit, measured by empirically fitting its response curve to a Hill function, can be used as a quantifiable measure of ultrasensitivity [68]. A response with a Hill coefficient above unity is generally considered to be ultrasensitive, and the higher the Hill coefficient, the steeper the sigmoidal response curve, and therefore the more digital the output is across a certain input range (Fig. 2A–C). It should be noted, however, that there are exceptions when the Hill coefficient is not a valid indicator of ultrasensitivity, and other more rigorous methods of analyzing sensitivity should be used [69].

On a biochemical level, ultrasensitivity can be created through several strategies, including multimerization or cooperative binding of proteins at a promoter [70]. One promising strategy for engineering ultrasensitivity is through molecular titration, wherein an input molecule is sequestered away from its target by another molecule. If the sequestration is strong enough, this creates a threshold (at around the same input level as the sequestering molecule) below which there is no output actuation, and above which there is output actuation. Both natural [71, 72] and synthetic [38, 73, 74] systems have leveraged molecular titration for ultrasensitivity. When combined with autoregulated positive feedback (or mutual repression), ultrasensitivity can lead to bistability (Fig. 2D–F). For example the epigenetic toggle switch discussed in Section 2.1 is a type of bistable 1-bit A-to-D converter that relies on cooperativity for ultrasensitivity. As another notable example, Chen et al. [38] coupled positive autoregulation with a molecular-titration-based ultrasensitive response to create a bistable switch. The input threshold for the switch was tunable via the expression level of the sequestering (titrating) agent.

A

Parameters, Variables	Description
O	output concentration
I	input concentration
Vmax	maximum output flux
K	Michaelis constant
D	dilution and degradation rate
n	Hill coefficient

B

Hill function model

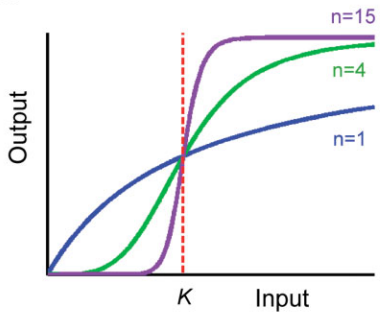
dynamic →

$$\frac{dO}{dT} = Vmax \cdot \frac{I^n}{K^n + I^n} - D \cdot O$$

steady state →

$$O_{ss} = \frac{Vmax}{D} \cdot \frac{I_{ss}^n}{K^n + I_{ss}^n}$$

C



D

Hill function with feedback model

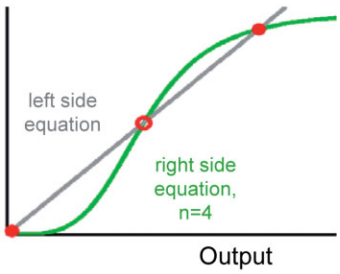
dynamic →

$$\frac{dO}{dT} = Vmax \cdot \frac{O^n}{K^n + O^n} - D \cdot O$$

steady state →

$$D \cdot O_{ss} = Vmax \cdot \frac{O_{ss}^n}{K^n + O_{ss}^n}$$

E



F

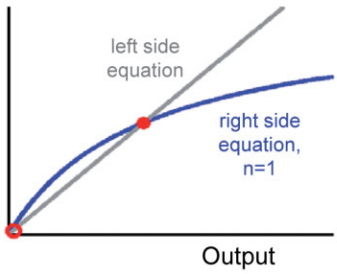


Figure 2. Biochemical Hill models and the role of ultrasensitivity in analog-to-digital conversion. (**A**) A list of parameters and variables used in the models. (**B**) A model where the output production rate is a Hill function of the input, and the output degradation (or dilution) rate is constant per unit output. The steady-state output as a function of the input is determined by setting the time derivative in the dynamic model to zero. (**C**) Results of the steady state model from (B) with three different Hill coefficients (*n*), where all other parameters are constant. Notice that as the Hill coefficient increases, the output becomes more digitized. The constant, *K*, determines the input threshold (red line) across which the output switches states. (**D**) The same model as in (B) except the output is fed back to become its own input (positive feedback). The steady-state output can have more than one solution, so a non-explicit steady state solution is given. (**E**) A plot of the left-hand side and right-hand side of the steady-state equation from (D) for $n = 4$. Steady-state solutions can be found at the intersection points. For this particular choice of parameters, there are three solutions, where the filled red circles represent stable equilibria and the open circle represents an unstable equilibrium. More generally, bistability can occur whenever $n > 1$. (**F**) A plot of the left-hand side and right-hand side of the steady state equation from (D) for $n = 1$. For this particular choice of parameters, there are two steady-state solutions and no bistability. More generally, there will never be more than two solutions when $n = 1$ because the blue curve is not sigmoidal. All plots were made using MATLAB R2012b.

Memory is a property of bistability that can be useful for reducing the cost associated with supplying exogenous inducers as well as creating history-dependent production programs. However, if cells are expected to move back and forth between different conditions and respond accordingly, then using memory may be disadvantageous because memory, without a reversal signal, can create an irreversible state. To this end, future synthetic efforts could be applied toward creating highly cooperative circuit intermediates and reliable sequestering agents for memory-less A-to-D conversion (Fig. 2C, purple line).

Additional circuit modifications can be used to fine-tune the discrete levels of digital output signals from input signals. Specifically, it is important that a system is responsive over the intended input range and has a suitable output range to satisfy the desired application. Modulation of RBS strength is one strategy for tuning input–output functions [6]. Another strategy, albeit one less amenable to predictable design compared with RBS engineering, is to create promoter mutations that affect gene expression [5, 75]. Insulation-oriented design principles that minimize the unpredictable interactions between the promoter, 5′ untranslated region (including the RBS), and gene can further enable the design of constructs with reliable transcriptional expression [76–79].

With the preceding strategies, digital circuits can be made for biotechnology to induce different microbial pathways at each step of a bioprocess to improve its overall efficiency. For example bioprocesses that separate growth from production have been associated with increased productivity [21, 22]. This two-stage bioprocess could be achieved by externally inducing the "on" state of a production pathway at a critical cell density. Alternatively Kobayashi et al. [32] demonstrated that cell-density-dependent gene activation can be achieved autonomously by linking a transcriptional toggle switch to a

synthetic quorum-sensing system. As another example, industrial bioprocesses typically contain a final stage where the product is separated from the culture. Inducing synthetic pathways that create either microbial aggregation or immobilization could facilitate biphasic separation of the product in this stage [80–82].

2.3 Multi-input digital logic

In some cases, a single endogenous or environmental input does not provide sufficient information to determine whether or not there should be a transition in circuit state. A motivating example of this can be found in the catabolic sugar pathways of *E. coli*. Even though *E. coli* can grow on many sugars, they prefer glucose. Thus, activation of particular alternative sugar metabolizing enzymes are not only dependent on whether the alternative sugar is present, but are also dependent on glucose not being present [83]. The digital "AND" operation that can exist between the inputs "alternative sugar present" and "glucose not present" is an example of a dual-input logic gate. A similar synthetic gate could be used for biomedical applications. Consider an engineered cell designed to sense a disease state and subsequently produce a pharmaceutical product. Proper sensing of the disease state might require corroborating multiple inputs that represent hallmarks of the disease to achieve the desired level of specificity [84–86]. Furthermore, proper operation of the chemical production pathway may depend on substrate availability, in which case, the desired logic function for turning on a specific production pathway would be "disease AND substrate".

Synthetic gene circuits have been created that can integrate multiple digital input signals with dual-input logic gates. Tamsir et al. [87] built all 16 two-input one-output Boolean (digital "on" or "off") logic gates. They achieved this by layering "NOR" and "OR" gates as well as single input "NOT" and "Buffer" gates in different *E. coli* cells and then connecting their inputs and outputs through cell-membrane-permeable signaling molecules (Fig. 3). "OR" gates are executed by putting two different inducible promoters upstream of an output gene such that the presence of either inducer will generate expression. The "NOR" operation is then simply executed by attaching a "NOT" gate to the output of the "OR" gate. A limitation of this multi-input strategy is that it requires careful spatial and temporal arrangements of the different cellular gate layers in order to work properly. Regot et al. [88] created a similar multi-cellular logic system in yeast by using components from the yeast pheromone and competence systems. More recently, Moon et al. [89] introduced a novel dual-input "AND" gate in *E. coli* that involved the expression of an activator TF and its chaperone (derived from type III secretion pathways) from sepa-

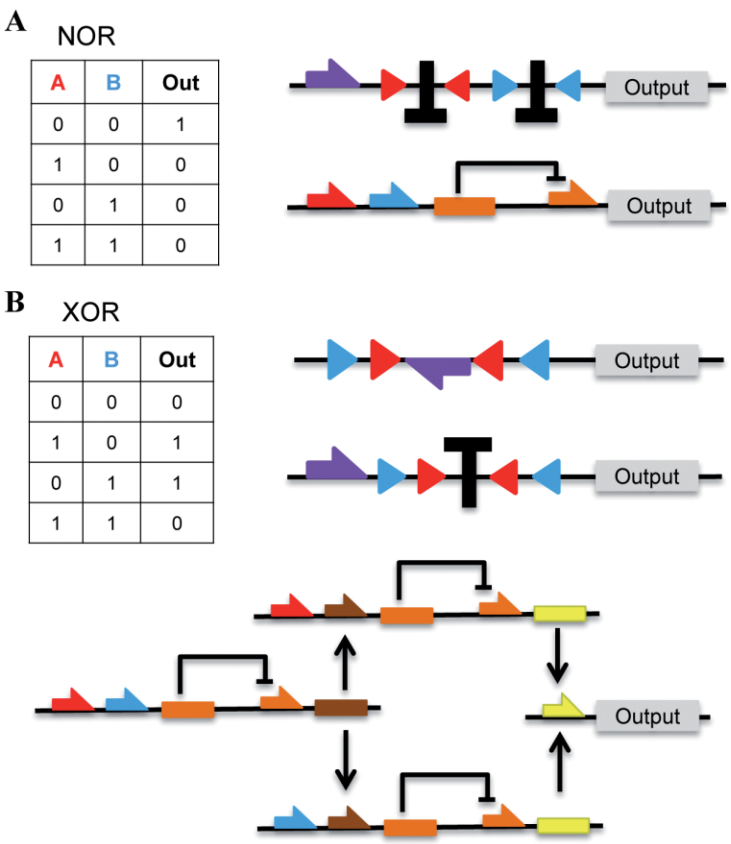


Figure 3. Examples of NOR and XOR digital logic gates. (**A**) The NOR truth table (left), and schematics of published NOR gate circuits (right) from both Siuti et al. [45] and Bonnet et al. [92] (top) and Tamsir et al. [87] (bottom). (**B**) The XOR truth table (left), and schematics of published XOR gate circuits (right) from Siuti et al. [45] (top), Bonnet et al. [92] (middle), and Tamsir et al. [87] (bottom). The large "T" represents a unidirectional terminator. Input A inverts the region flanked by red recombinase-recognition sites on the recombinase-based constructs, and otherwise induces the red promoter. Input B inverts the region flanked by blue recombinase-recognition sites on the recombinase-based constructs, and otherwise induces the blue promoter. Orange gene–promoter pairs interact within the cell, and brown and yellow gene–promoter pairs interact between cells via quorum sensing. Purple promoters are constitutive.

rate inducible promoters. They further demonstrated that by using orthogonal TF/chaperone sets, it is possible to layer these gates in single cells to create up to a 4-input "AND" gate. Other multi-input gates have been successfully generated via RNA regulators [64, 90], one particular example being a riboregulator-based, 3-input AND gate used to program a microbial kill switch [91]. All of the preceding gating strategies integrate all input signals combinatorially, where combinatorial logic refers to digital computation without memory such that the output only depends on the present inputs.

Recombinase-based logic gates that operate with memory and integrate all signals sequentially have been developed [45, 92], where sequential logic refers to digital computation in which the output depends on the past and present inputs. These gates work by expressing unidirectional recombinases from different inducible promoters and arranging their respective recombinase-recognition sites to invert the output gene or different regulatory elements (promoters and unidirectional terminators) upstream of the output gene. With this paradigm, all 16 two-input one-output Boolean logic gates can be implemented in a single layer (Fig. 3) [45, 92].

A key distinguishing feature between recombinase-based circuits and the aforementioned TF and RNA based circuits is that, with exceptions, the former integrate memory with logic and thus enable sequential computations while the latter execute combinatorial state-less logic. The exceptions are that the latter TF- and RNA-based circuits could utilize bistability (as discussed previously) to remember transient inputs and thus compute sequential logic, and the former recombinase-based circuits could potentially employ RDFs to erase memory and thus compute combinatorial logic. Sequential logic is advantageous over combinatorial logic when the computation requires a dependence on history. For example sequential logic gates would be useful for probing the heterogeneous environment of bioreactors where particular conditions do not necessarily exist together, but might be spread over time and space. However, combinatorial logic is advantageous over sequential logic when the computation requires integrating only the current state of multiple inputs, for example when computing which forms of carbon (e.g. glucose, lactose, maltose, etc.) are available to a cell at a given time.

3 Analog circuits

As opposed to digital circuits, analog circuits create graded output curves in response to input signals [93, 94]. During bioprocesses, externally applied analog inputs can be used to tune pathway enzyme levels over an entire microbial population. However, a more powerful use of analog computation for biotechnology is in dynamic sensor-regulator systems that autonomously and continuously sense endogenous input levels and respond accordingly to balance metabolism and maintain high product titers [18]. The following three subsections discuss: (i) basic analog circuits, (ii) multi-input analog circuits, and (iii) digital-to-analog (D-to-A) converters.

3.1 Analog circuits

It is straightforward to map analog inputs to analog outputs. Indeed, biology is inherently analog and any inducible promoter connected to an output can be viewed as an analog circuit. However, one major challenge is in creating analog circuits that are operable over a large dynamic range of inputs. Avoiding signal saturation is the main principle in this endeavor. Madar et al. [95] show that *E. coli* naturally use negative auto-regulation of AraC to broaden the dynamic range of its response to its inducer, arabinose. Free AraC represses its own production, but when it binds the input arabinose, the repression is relieved and more AraC is produced. Because regulator (AraC) levels increase with inducer (arabinose) levels, the input range over which the system responds is extended.

Nevozhay et al. [96] linearized the dose-response curve from a tetracycline repressor (TetR) repressed promoter ($P_{GAL1\text{-}D12}$) in *Saccharomyces cerevisiae* by expressing the TetR gene from $P_{GAL1\text{-}D12}$, rather than from a constitutive promoter, thus creating negative auto-regulation that allowed TetR to avoid saturation from the input molecule ATc. More recently, Daniel et al. [97] built synthetic circuits for analog computation in living cells that exhibit log-linear behavior over an input dynamic range spanning up to four orders of magnitude (Fig. 4A and 4B). These circuits were composed together to create more complex analog computations, as discussed in Section 3.2. The two key components to these synthetic circuits were: (i) a positive-feedback loop on a low-copy plasmid (LCP) to avoid saturation of the sensor TF by the input by generating more TF as more input is added (similar to the strategy in the aforementioned arabinose and synthetic ATc induced systems) and (ii) a high-copy plasmid (HCP) "shunt" to delay saturation of the output promoter by the activated TF by providing additional DNA-binding sites for the TF and reducing the strength of the LCP positive-feedback loop. The transfer function slopes of these circuits were demonstrated to be tunable and could be either positive or negative, thus enabling the computation of positive or negative logarithms, respectively.

For biotechnology, analog circuits could be engineered to respond to endogenous inputs in order to create microbes that autonomously and continually adapt their production programs to changing intracellular conditions. For this type of dynamic sensor-regulator-based FAEE production in *E. coli*, Zhang et al. [18] used a key intermediate (fatty acyl-CoA) in the FAEE production pathway as an input ligand to a re-purposed TF, FadR. In its unbound form, FadR repressed downstream processing at engineered promoters (promoters with FadR binding

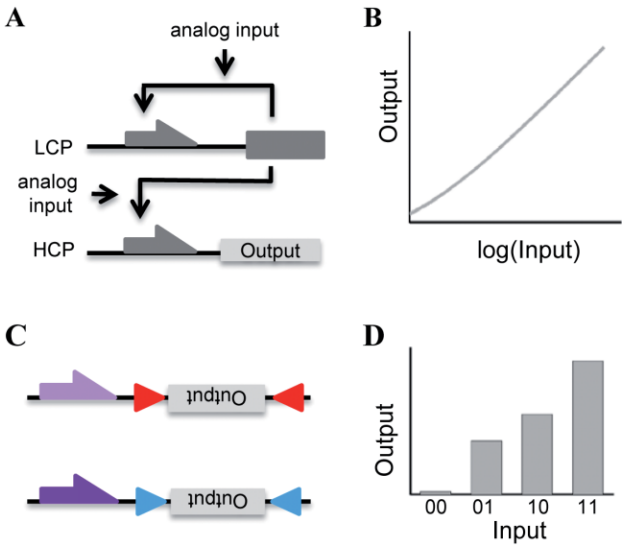


Figure 4. Implementing synthetic analog circuits in living cells. (**A**) Schematic for a synthetic analog circuit with wide-dynamic-range log-linear operation, adapted from Daniel et al. [97]. The same analog input acts to induce expression from promoters on two different plasmid types, a low-copy plasmid (LCP) and a high-copy plasmid (HCP). This circuit motif includes a positive-feedback loop on the LCP and a "shunt" promoter on the HCP, where transcription-factor binding to the shunt promoter produces the output but does not increase production of the transcription factor. (**B**) The input–output transfer curve of the circuit from (A) produces a logarithmically linear response over a wide dynamic range. (**C**) Schematic for a D-to-A converter, adapted from Siuti et al. [45]. Each output gene is flipped to an actively expressed orientation in the presence of its respective recombinase input (represented by red or blue). The two promoters are constitutive, but have different strengths. The net output gene expression level is the sum of the output gene expression level from both circuits. (**D**) The D-to-A converter from (C) generates various output levels corresponding to different Boolean input combinations.

sites that interfere with RNA polymerase binding), thus preventing wasteful resource allocation toward enzyme production during periods of insufficient substrate.

Generally, the choice of input, biosensor, and analog circuitry plays a large role in whether a synthetic dynamic sensor-response system will function properly. First, the input needs to be chosen such that it relays key metabolic information regarding production. Second, as discussed in Section 2.2, there needs to be an appropriate biosensor for the input [60]. Lastly, the analog response should be engineered such that the dynamic input range matches the range over which the input is expected to vary. This is one area where the aforementioned wide-input-dynamic-range design principles could be beneficial for the development of dynamic sensor-regulator systems in biotechnology.

3.2 Multi-input analog circuits

Much like with digital logic gates, multiple sources of analog information from the environment will often need to be integrated for proper adaptive responses. To this end, arithmetic operations and complex functions integrating multiple inputs can be efficiently implemented via the strategies of Daniel et al. [97]. The sum of the logarithm of inputs (or the logarithm of the product of inputs) can be achieved by combining two positive-slope wide-dynamic-range analog circuits with different inputs but a common output. Among many possible applications, this function could be useful when measuring the level of a cofactor that cycles between different forms (e.g. nicotinamide adenine dinucleotide (NAD+), coenzyme A, and ATP) [98]. In this case it would be beneficial to sense several metabolites and respond accordingly to their total pool. In addition, the subtraction of the logarithm of inputs (or the logarithm of the ratio of inputs) can be implemented by combining a positive-slope analog circuit with a negative-slope analog circuit, each with different inputs but the same output. This function could be useful for measuring informative metabolic ratios. For example measuring NADH with respect to NAD+ (as well as NADPH with respect to NADP+) gives important information regarding redox state [99, 100], and measuring ATP with respect to the multiplicative product of adenosine diphosphate (ADP) and free phosphate gives phosphorylation potential, an important indicator of cellular energy status [98]. Sensing and responding to these ratios could be especially important for biotechnology because microbes for bulk chemical production are typically grown under low or no oxygen conditions [101, 102]. Without oxygen, cells cannot oxidize NADH to produce ATP [98], and so significant changes to both the redox and energy state of the cells are expected. Analog circuits might be implemented to sense these changes and respond by pushing flux through pathways to restore appropriate redox and energy values for production.

3.3 Digital-to-analog converters

There may be applications in bioprocessing where it is advantageous for the user to control gene expression in an analog fashion. One strategy would be to simply use an inducible promoter with a graded output. However, maintaining the exogenous inducer input to this promoter could be difficult and expensive. On the other hand, one can use a constitutive promoter to achieve gene expression at a stable level without any inducer, but then the gene expression would not be tunable, which could be troublesome for expressing toxic proteins. D-to-A converters offer a compromise between constitutive and inducible promoters by enabling the programming of different output levels with transient inputs [45]. One general strategy for building D-to-A converters is to use a bank of memory-storing digital circuits, each controlled by a different input but generating the same output molecule with different magnitudes [103]. More specifically, Siuti et al. [45] built a D-to-A converter by using unidirectional

recombinases to independently turn "on" the expression of an output gene from multiple constitutive promoters with different strengths (Fig. 4C and 4D). The result was a circuit that could achieve four different output levels based on the combinations of just two Boolean inputs. Theoretically, this circuit can be scaled to program 2^n stable output levels from n input inducers.

4 Conclusion

The application of synthetic gene circuits to metabolic engineering will enable cells that can dynamically respond to a user and/or environment for enhanced control of the chemical production process. Both digital and analog circuits can be implemented separately for dynamic regulation. We anticipate that sophisticated bioprocesses will utilize both strategies: digital circuits to define and switch between specific cellular states and analog circuits to continually balance these states for efficient production. As a simple example of this digital–analog integration, Daniel et al. [97] built an analog circuit with front-end digital control such that when the digital signal is "off," the analog circuit is "off," and when the digital signal is "on," the analog circuit becomes responsive to its inducer in a log-linear fashion. Optional features of memory, analog–digital interconversion, and multiple-signal integration can add to the versatility of analog and digital computation for biotechnology.

As mentioned in the main text, additional work is needed to advance the application of synthetic gene circuits to metabolic engineering. For example, efforts to expand the characterization of recombinases and recombinase RDFs would be useful. The knowledge of recombination efficiencies and of which recombinase systems are orthogonal to each other and host cells will enable researchers to access the potential of scaling up recombinases into larger circuits that could be used as state machines or binary counters for complicated production programs. Another area for additional work is in the construction of biosensors for intracellular metabolites, and in the construction of analog circuits that can receive these biosensors as inputs. The junction of these two efforts will potentially enable fine-tuned dynamic sensing and regulation for any or multiple metabolites that could be crucial in relaying information regarding cell state.

Although new tools and approaches are being developed, there still exists a gap between efforts to create and characterize new synthetic gene circuits for cellular computation versus the application of these circuits to biotechnology and metabolic engineering. To realize synthetic gene circuits that can scale to real-world applications, synthetic biologists need to have a deeper understanding of current problems in metabolic engineering, gain a greater appreciation of the need for robustness of their genetic constructs at industrial scale, adopt model organisms and systems that are more relevant for biotechnology [13], and incorporate multi-level models into the gene circuit design process [104–106]. Closer interactions between synthetic circuit designers and chemical engineers as well as between academic and industry scientists will be important to addressing these issues and expanding the application of synthetic circuits to biochemical production. We envision that such interdisciplinary interactions will be highly beneficial for metabolic engineering, biotechnology, and synthetic biology.

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