

Lab on a Chip

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Integration of microfluidics into the synthetic biology design flow[†]

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One goal of synthetic biology is to design and build genetic circuits in living cells for a range of applications. Major challenges in these efforts include increasing the scalability and robustness of engineered biological systems and streamlining and automating the synthetic biology workflow of specification-design-assembly-verification. We present here a summary of the advances in microfluidic technology, particularly microfluidic large scale integration, that can be used to address the challenges facing each step of the synthetic biology workflow. Microfluidic technologies allow precise control over the flow of biological content within microscale devices, and thus may provide more reliable and scalable construction of synthetic biological systems. The integration of microfluidics and synthetic biology has the capability to produce rapid prototyping platforms for characterization of genetic devices, testing of biotherapeutics, and development of biosensors.

1 Introduction

Over the last decade, synthetic biology has emerged as a field with potential applications in diverse fields including pharmaceuticals, biofuels, and materials. The engineering of a microbial production pathway for artemisinic acid (a precursor for antimalarial drugs)¹ has lowered the production costs of those drugs from \$2.40 to \$0.40 per dose, expanding the number of patients who can afford the treatment for a disease that kills millions². Several companies from around the world (Gevo in Englewood, Colorado, and Butamax in Wilmington, Delaware, Butalco in Fuerigen, Switzerland) have all developed methods to increase the yield of isobutanol or butanol for commercial biofuels using various strains of yeast,³ while Amyris has adapted the mevalonate and deoxyxulose phosphate metabolic pathways in yeast to ferment farnesene⁴. Engineered bacteriophages (viruses targeting bacteria) have been used to destroy biofilms⁵ and resensitize otherwise antibiotic-resistant strains of bacteria⁶, which could extend the effectiveness of current drug therapies in the face of rising antibiotic resistance. However, despite these success stories, the field of synthetic biology faces challenges in workflow acceleration and automation as it seeks to scale from single prototypes to commercial enterprises. The field of microfluidics has proved successful in a wide range of applications in biology and has the potential to address these challenges.

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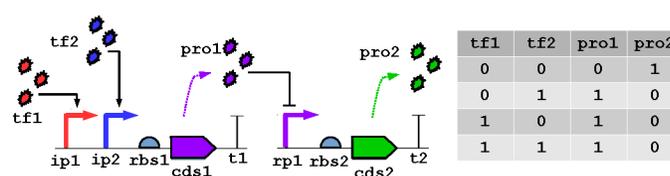


Fig. 1 A genetic NOR gate, and accompanying truth table. The presence of transcription factors *tf1* or *tf2* activate the inducible promoters *ip1* and *ip2* respectively and allow for production of *pro1*, coded for by *cds1*. The protein *pro1* acts as a transcription factor on repressible promoter *rp1* and blocks production of *pro2*, coded for by *cds2*. The final output, *pro2* is only produced when both transcriptional factors *tf1* or *tf2* are absent.

1.1 Engineering biology

Synthetic biology seeks to manipulate the structure and functions of DNA to create new biological systems according to engineering principles. The primary paradigm in the field is to identify biological primitives involved in the transformation of DNA to a protein and encapsulate these individual DNA sequences as “parts”. Synthetic biology has taken advantage of the modularity in the structure of bacterial open reading frames (ORFs) to abstract portions of the sequence as “parts”. These “parts” include sequences for promoters, ribosome binding sites, coding regions, and terminators, and can be combined to create functional “devices” which can be introduced into living organisms such as bacteria, yeast, or mammalian cells^{8–10}. As logical frameworks such as repressible systems exhibiting the behavior of inverters already exist in gene expression systems, synthetic biology seeks to harness and re-engineering these natural logic systems for other applications.

A genetic device that implements the Boolean NOR function and the corresponding truth table are shown in Figure 1. This device is designed to function in prokaryotic systems. In this circuit, the inputs are the transcription factors *tf1* and *tf2*, which control the inducible promoters *ip1* and *ip2* such that transcription only takes place when one or both of the transcription factors are present. Transcription continues until terminator *t1*, and the resulting mRNA is translated into the protein *pro1* (coded for by the coding region *cds1*). The protein *pro1* acts as a transcription factor for the repressible promoter *rp1*, preventing transcription starting at *rp1* if it is present. The output of this system is the protein *pro2* (coded for by the coding region *cds2*), which is only expressed if neither *tf1* or *tf2* are present. In the absence of *tf1* and *tf2*, *pro1* is not expressed. Transcription starts at *rp1* and continues through *t2*, allowing *pro2* to be expressed. This behavior of this device emulates that of the Boolean NOR function, in which the output is true if and only if both inputs are false. NOR and NAND functions are functionally complete and can be used to build all other Boolean logic functions, thus allowing these devices to be the basis for biological computation. As such, many different implementations of these functions exist in synthetic biology^{11–13}.

While many different classes of biological devices such as oscillators¹⁴, filters¹⁵, noise generators¹⁶, and the beginnings of analog computation¹⁷ exist, we focus here instead on the biological devices implementing Boolean logic¹⁸ and other extensions of the digital abstraction found in electronics. We use the digital abstraction not to replicate silicon based computing, but as an alternative method for designing robust biological circuits that are insensitive to noise and can be tuned to specific input conditions. Additionally, the digital abstraction is well understood, and numerous techniques have been developed for its description, synthesis, and verification. The introduction of Boolean logic and memory devices into biological systems leads to new applications and potential methods of computation for solving otherwise computationally intensive and complex problems^{19,20}.

1.1.1 Boolean logic in biological devices

Biological logic devices can be used to detect specific combinations of chemical or environmental triggers for targeted pharmaceutical and biotechnology applications^{21,22}. One application for biological logic circuits is in the field of cancer research^{23,24}, where the use of digital logic provides the necessary specificity for targeting strains of cancer cells while leaving other cells unharmed^{25,26}. Nissim *et al.*²⁷ introduce a tunable dual promoter system that implements the Boolean function AND that targets cancer cells while ignoring pre-malignant cells.

While devices implementing two input boolean logic functions are useful in synthetic biology, more complex computa-

tion would allow for applications such as the biological sensing of multiple chemical species in the same device, the identification of specific genetic markers, and environmentally tailored drug dosage responses²⁸. One way of constructing more complex functions is to increase the layers of logic in the genetic device. This method was used by Moon *et al.* to create a four input transcriptional AND gate²⁹ with 11 orthogonal (non-interfering) regulatory proteins made from two layers of two input AND gates. Another tactic is to separate the larger function into smaller functions and place devices implementing the smaller functions into different cells. These cells then communicate with each other through intercellular signaling pathways. All sixteen two input functions have been built from *E. coli* cells containing NOR gates that communicate through the quorum sensing pathway²¹. Distributed computing³⁰ has also been implemented in yeast with the development of both a 2-to-1 multiplexer and a basic addition circuit³¹.

1.1.2 Memory and state in biological devices

The next step in increasing complexity of computation is to generate the concept of memory or “state”, such that the cell remembers what has previously happened and takes that into account in new calculations. One approach is to use recombinases, enzymes used by bacteria-infecting viruses to manipulate their host’s genome, to turn specific DNA sequences on and off by switching the orientation of the DNA³². Memory devices and counters have been integrated into cells through the use of recombinase based circuits^{33,34}. The use of recombinase has also provided synthetic biologists with a form of rewrite-able and addressable data storage capable of information storage through over 100 cell divisions and through repeated switching without losing performance³⁵. More recently, Siuti *et al.* have used recombinase based circuits to implement all 16 two-input Boolean logic functions with stable DNA-encoded memory of events in *E. coli* without requiring cascades of multiple gates³⁶.

1.1.3 Specify-Design-Assemble-Verify workflow

The practice of synthetic biology typically follows an iterative process of specification, design, assembly, and verification. The process begins with the specification of the function of the novel genetic device either by hand or with one of the new description languages such as Eugene^{37,38}, GEC³⁹, or Proto⁴⁰. In the design phase, biological parts are selected from repositories to implement the specified function. Tools such as GenoCAD⁴¹, j5⁴², or Clotho⁴³ may aid the design process. Assembly of a novel genetic device starts with obtaining the parts of interest by isolating segments of DNA from natural sources or by *de novo* synthesis through companies such as DNA2.0, GeneArt², or Gen9⁴⁴. Parts are assembled into devices by joining the segments of DNA together through the use of restriction enzymes (proteins that cut DNA at certain se-

quences) and ligases (proteins that create new bonds between DNA bases). Common assembly techniques, including BioBricks⁴⁵, BglBricks⁴⁶, Gibson⁴⁷, GoldenGate⁴⁸, the Modular Overlap-Directed Assembly with Linkers (MODAL)⁴⁹, and modular cloning (MoClo)⁵⁰, are reviewed in detail elsewhere⁵¹. Software tools are being developed to automate and optimize the assembly process^{52,53}.

The completed device is then inserted into a host organism, commonly *E. coli* for prokaryotic systems and yeast for eukaryotic systems, and the organism is grown under a variety of conditions to test the function of the device as compared to the original specifications. For verification purposes, the expression of fluorescent proteins is often used as a substitute for the gene of interest as they are more easily measured through flow cytometry to determine the efficiency of the device under test. The fluorescent protein is replaced by the protein of interest in the final application. At this stage, the genetic device may be further refined based on the gathered data, or the host cells may be grown for harvesting more copies of the device.

As new techniques in synthetic biology generate large libraries of thousands of part variations and combinatoric devices, increased throughput and automation are needed to test and characterize these constructs to allow the acceleration of the specify-design-assemble-verify development cycle for synthetic biology. To lower the barrier of entry into the field, synthetic biology needs to take advantage of the improvement in automation and computer aided design tools. Software tools for synthetic biology have grown in the past years to encompass a variety from rules based constraint languages to gene designers to basic simulators. Standards such as the Synthetic Biology Open Language (SBOL)⁵⁴ are being developed to facilitate information exchange between various tools in the toolchain and will be vital in building end-to-end workflows⁵⁵ in synthetic biology from high level languages to compilation from the language to biological parts to DNA assembly. While the available tools are still lagging behind the current state of the art of biological research, the development of these tools helps with refining the rules of genetic design for future applications⁵⁶.

1.2 Microfluidics

Microfluidics is comprised of the analytical systems and tools for the study and manipulation of small volumes of liquids, typically at micro and nano liter scales. The advantages of microfluidics come from the decrease in scale, which allows for more predictable fluid flow, decreasing the amount of reagents needed for reactions, and smaller devices and experiment setups. Microfluidics have been used for chemical analysis⁵⁷ and PCR⁵⁸ as well as a variety of applications in molecular biology⁵⁹, systems biology⁶⁰, stem cell studies^{61,62}, tissue engineering⁶³, point of care diagnostics⁶⁴, pathogen detection⁶⁵,

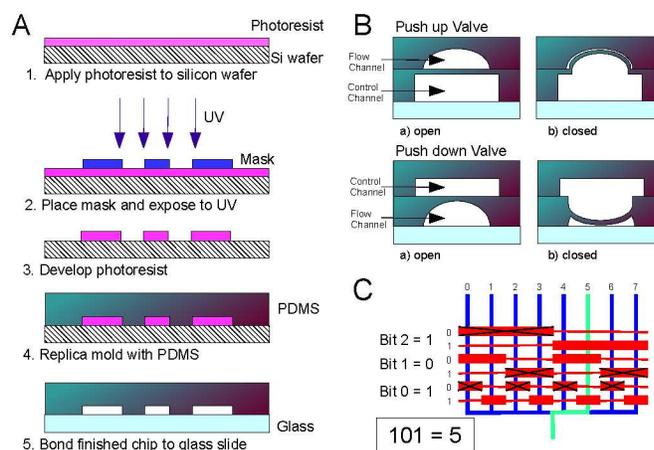


Fig. 2 (A) Fabrication process for soft lithography (adapted from Ref.⁶²). (B) 2 possible structures (push-up or push-down) for valves in multilayer soft lithography (adapted from Ref.⁷⁸). (C) Multiplexer for selection of 8 possible fluid lines, using 6 control lines to represent the 3 bit binary number of the selected line (adapted from Ref.⁷⁹).

and systematic toxicity studies⁶⁶.

Recently, synthetic biologists have been developing and using microfluidics to study synthetic gene networks and network dynamics⁶⁷. Due to the potential in microfluidic systems for the precise control over input stimuli^{68,69} and the ability to track single cells⁷⁰, there has been an increased interest in microfluidic platforms to further synthetic biology⁷¹. While there are a number of reviews postulating the benefits of microfluidics for synthetic biology, those reviews have focused on specific applications^{71–73} or high level overviews of many competing microfluidics technologies^{74,75}. We instead present the list of challenges facing synthetic biology as the field matures and describe how microfluidics could be used to find solutions to those challenges as well as potential future applications for systems and workflows integrating both synthetic biology and microfluidics.

The field of microfluidics covers a wide range of technologies such as lateral flow tests, linear actuated devices, pressure driven laminar flow, microfluidic large scale integration, segmented flow microfluidics, centrifugal microfluidics, electrokinetics, electrowetting, surface acoustic waves, and dedicated systems for massively parallel analysis^{76,77}. For the purposes of this paper, we will focus on the subset of microfluidics that offer the most relevance to current problems in the synthetic biology workflow, particularly in spatial and temporal gradient generation, microfluidic large scale integration (mLSI) high throughput screening, DNA synthesis, and cell culture.

1.2.1 Device physics

In pressure driven systems, the reduced scale of microfluidic devices results in deterministic fluid flow. The low Reynolds number (a comparison of the forces acting on the flows) of the flow means that the nonlinear and chaotic effects due to turbulence caused by inertial forces are removed, and the flow is restricted to the laminar region. In this flow regime, the behavior of the fluids can be predicted by the size of the fluid channel and the viscosity of the fluid in a manner analogous to Ohm's Law in electrical engineering⁸⁰, making it easier to simulate and verify the function of the device. The mixing of parallel flows is dominated by diffusion instead of convection, so that the flows only interact at their boundary. This effect can be exploited to generate spacial gradients of chemicals of interest⁶⁹. Mixers^{81,82} can be used to speed up integration of flows. A detailed synopsis of the physics and fluid mechanics specific to microfluidic devices may be found elsewhere^{83,84}.

1.2.2 Design and fabrication

A widely used fabrication material for microfluidic devices is polydimethylsiloxane (PDMS). The properties of PDMS make it well suited for use in biological applications as it offers flexibility for fabrication, scalability, and potential for longterm growth and monitoring of cells⁸⁵. PDMS is optically transparent, chemically inert, impermeable to water while being permeable to gases, and non-toxic to cells⁸⁶. The cost of the raw material is around \$0.05 per cm^3 ^{87,88}, making it suitable for rapid prototyping and quick design iteration. PDMS based microfluidics have been used for a variety of purposes in recent years, including as an alternative platform for computation⁸⁹. Development of the microchemostat⁹⁰ has allowed for cells to be grown in microfluidic chips for long periods of time, thus allowing for more complex, long term experiments. However, the low elastic modulus of PDMS makes it unsuitable for high pressure applications as high pressure causes channel deformation in PDMS based devices⁸⁸.

Microfluidic chips are fabricated from PDMS through soft lithography. As many detailed reviews of the process exist^{86,91,92}, we will only provide a brief description. Photoresist (typically SU-8) is spun out over a substrate of silicon, and a transparency with the chip design is placed over it as a mask. The sandwich of mask, photoresist, and substrate is then exposed to UV light. The mask is removed, and the photoresist washed in developing agent to obtain the master mold. PDMS layers are cast from the master through replica molding. The channels are then sealed against a substrate suitable for imaging and connected to input and control structures. A summary of the process is shown in Figure 2A. The entire fabrication process, from the creation of the photomask to the molding of the chip, takes no more than a few days including the turnaround time for printing the photomask. This process does require the experimenter to have access to a high qual-

ity cleanroom, purchase specialized fabrication equipment for soft lithography⁹³, or else contract out the fabrication process to a dedicated microfluidic foundry.

1.2.3 Multilayer Soft Lithography and Microfluidic Large Scale Integration

An extension of soft lithography, multilayer soft lithography, allows devices to be built of multiple layers of PDMS, typically with one layer as a fluid flow layer and another layer as a control layer with channels pressurized by external actuators⁸⁷. Fluid flow is controlled by strategic placement of valves in the control layer, which restrict fluid flow when pressurized by causing the PDMS to deform and create a seal across the channel to impede fluid flow⁹⁴. Two types of valves described by Melin and Quake⁷⁸, push up and push down, are shown in Figure 2B. For work with cells, push down valves are preferred as they allow for easier cleaning of the flow channels and chip reuse⁹⁵. Multiple valves may be controlled by the same pressurized control line, and the optimization problem lies in minimizing the number of control lines needed to operate a chip.

The interaction of the control and flow layer through valves form the basic building block of microfluidic large scale integration (mLSI). As devices made from multilayer soft lithography grow more complicated, an increased number of external pressure lines are needed to control fluid flow. Microfluidic multiplexers, developed by Quake and colleagues⁷⁹, contain combinatorial arrays of binary valve patterns and allow increased fluid manipulation with a minimal number of control inputs such that only $2 \log_2 n$ control lines are needed to access the valves to select from one of n fluid channels. This makes them very suitable for high throughput applications that require manipulation of hundreds or thousands of fluid elements. The multiplexer shown in Figure 2 uses 6 control lines to represent the 3 bit binary number for selecting the fluid channel. Recent advancements in mLSI architecture focus on increasing the number of control elements on the chip through component miniaturization or additional layers, decreasing the reliance on external pneumatic lines through on-chip logic, and increasing reusability through programmable chips⁹⁶. Work has also been done on reducing contamination and back flow through the use of a microfluidic serial digital to analog pressure converter⁹⁷.

While CAD and automation have been primarily focused on droplet based digital microfluidics^{98–101} rather than mLSI, a new subset of tools are being developed for layout and optimization of mLSI devices. Earlier tools for mLSI include Biostream, a tool for designing GUIs and control valves for multilayer devices^{89,102} (freely available at¹⁰³) and Micado⁹⁴, which automated control valve placement and routing for a given flow layer. Extensions of that work have led to developments in a microfluidic description language similar to hard-

ware description languages used in electronics¹⁰⁴, algorithms for laying out the flow layer based on a high level description of chip function¹⁰⁵, and better algorithms for valve placement and control routing^{106,107}.

1.3 Using microfluidics to solve challenges in synthetic biology

Introducing complex engineered systems into cells presents numerous challenges at different levels of the synthetic biology workflow. We present in Figure 3 a sampling of the current challenges in synthetic biology and the microfluidic technologies most applicable to solving those challenges. We begin with the problems facing accurate specification of the function of novel genetic devices as the specification is only as useful as the understanding of the underlying biological behavior. A major challenge when designing new biological devices is that synthetic biology still suffers from a lack of well characterized parts that do not interfere with each other when used together to construct larger systems. Important factors in the assembly of a device include potential unintended changes in function introduced when joining two segments of DNA and the time and cost efficiency of the currently available assembly methods. Verification of device function and stability requires accurate monitoring and measurement of protein expression at both the population and single cell level for extended periods of time. Microfluidics technologies in environmental control, high throughput assays, DNA synthesis, and cell culture can be used to augment and supplement existing work in synthetic biology to address these challenges.

2 Specification

Accurate specification of biological device function requires prediction of future behavior of combinations of biological parts and a standardized input/output model to share behavioral data across different designs. However, with the exception of some well studied systems such as the quorum sensing system¹¹⁰, not all biological behavior in synthetic biological parts is well understood or predictable. In many cases, a device that performs well in one host system fails to perform when transplanted into a different host. In addition, different parts are characterized with different experimental methods such that there is no one standard of input/output measurement to specify interfaces between devices. As a result, many new devices fail to function without extensive trial-and-error, which is costly in both time and materials. To build more predictable systems, we require better characterization of part behavior over a wide range of environmental conditions to achieve the necessary understanding to build the correct models.

2.1 Challenges

In electrical engineering, simulation is used to predict device behavior and debug potential design flaws without spending time and resources in the lab. Whereas we have a solid understanding of semiconductor device physics and can create accurate models for electronic parts for simulations, we are still developing similar knowledge to create models of biological parts. Chen *et al.* characterized terminator efficiency for 582 natural and synthetic transcriptional terminators and from that data generated a predictive model of terminator behavior given the sequence¹¹¹. Similar models have been generated for ribosome binding sites¹¹², but these sequence based models alone cannot predict behavior of combinations of parts in a biological system. Likewise, new software tools developed for simulating synthetic biological systems^{113,114} are based on models of biological process of cell growth, diffusion, and protein interactions and degradation but may not take into account the details of part function based on DNA sequence.

Datasheets for electronic parts contain the information needed to create accurate behavioral models of those parts for use in simulations, including the valid input and output ranges, the switching characteristics, and frequency response for noise analysis. Canton *et al.*⁸ postulates the creation of similar datasheets for biological parts and produces a datasheet for BioBrick BBa_F2620, a device that produces the transcription factor LuxR and is controlled by a regulated operator. Datasheets for biological parts must include different information than their electrical engineering counterparts due to issues such as host context and degradation rate of inputs and outputs that have no electronic parallels. Biological parts need to be characterized for orthogonality and multi-component behavior as well as the more usual single component behavior. The long-term behavior of a part is dependent on such factors as the strain and growth stage of the host cell, the mutation rate, as well as environmental conditions such as temperature, pH, and culture media. For example, degradation rate of acylhomoserine lactone (AHL), the key signaling molecule in the popular and commonly used bacterial quorum sensing system, varies with both pH and temperature, making devices using this system sensitive to environmental changes¹¹⁵. The addition of biological parts and device into the host cell introduces large amounts of foreign DNA that may impact host cell metabolism¹¹⁶. To produce useful datasheets and models for simulation would require massive amounts of characterization data. The spatial and temporal environmental control that microfluidics provides combined with high throughput cell culture assays (described in 3.2) could be used to develop platforms characterization and test platforms for synthetic biology devices.

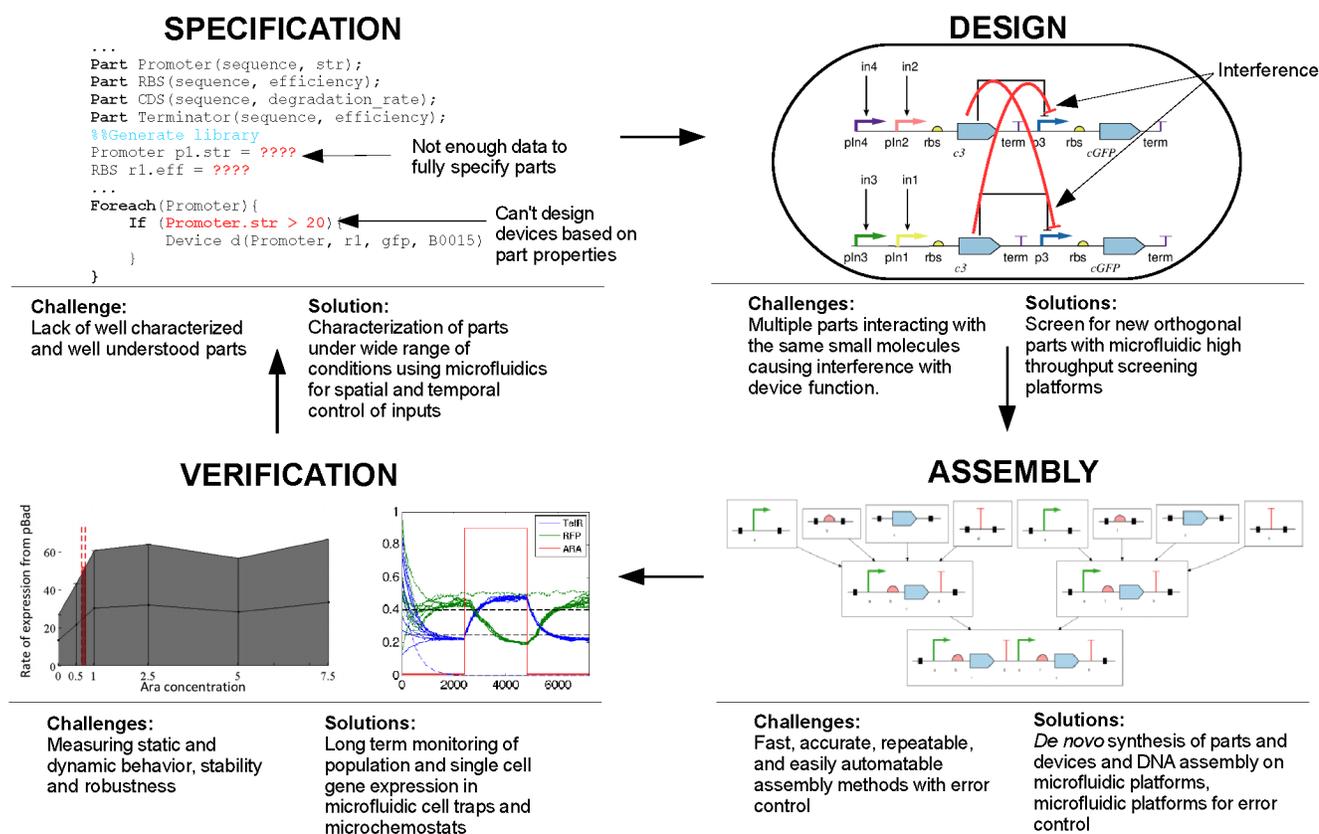


Fig. 3 The synthetic biology workflow of specification-design-assembly-verification, and the challenges at each step in the workflow. The use of microfluidics in environmental control, high throughput screening, DNA synthesis, and cell culture may be used to augment current work in synthetic biology and address these challenges.

2.2 Current Solutions

Static analysis of biological devices involve determining the valid input and output ranges and generating the input output curve that shows the range of outputs for any given input. Inputs to a biological device are transcription factors at various concentrations, and outputs are fluorescent reporter proteins⁸. A wide range of inputs is applied to the device under test to generate the full range of outputs. Additional characterization includes testing for interactions with other similar transcription factors and testing the effects of simultaneously applied multiple inputs.

Microfluidic resistance networks for generating complex spatial and temporal gradients (Figure 4A)^{69,117} are well suited to generating the input ranges needed to characterize basic device behavior. Combining these networks with the ability to select multiple inputs through multiplexing allows for testing interoperability and orthogonality¹¹⁸. This tech-

nique has been used for combinatorial drug screening¹¹⁹. The microfluidics chip by Cooksey *et al.* (Figure 4B) provides the ability to generate complex spatial gradients in a central chamber from combinations of up to 16 unique inputs by using segments of high fluid resistance and outlets to control flow¹⁰⁸.

Dynamic characterization requires the ability to trigger inputs on and off at a given frequency and measure the delay between the change in input and change in output as both valid and invalid inputs require time to propagate through the system. Faults may occur in a system when inputs are changed before the output is stable, when unstable outputs are used in a downstream function, or when the outputs of unstable inputs are used. Determining these timing characteristics of a biological part requires precise temporal control over the inputs to that system.

Wang *et al.* used valves to control inputs through a T junction to create square waves and ramp functions (Figure 4C) to characterize the dynamic signaling behavior of social amoeb-

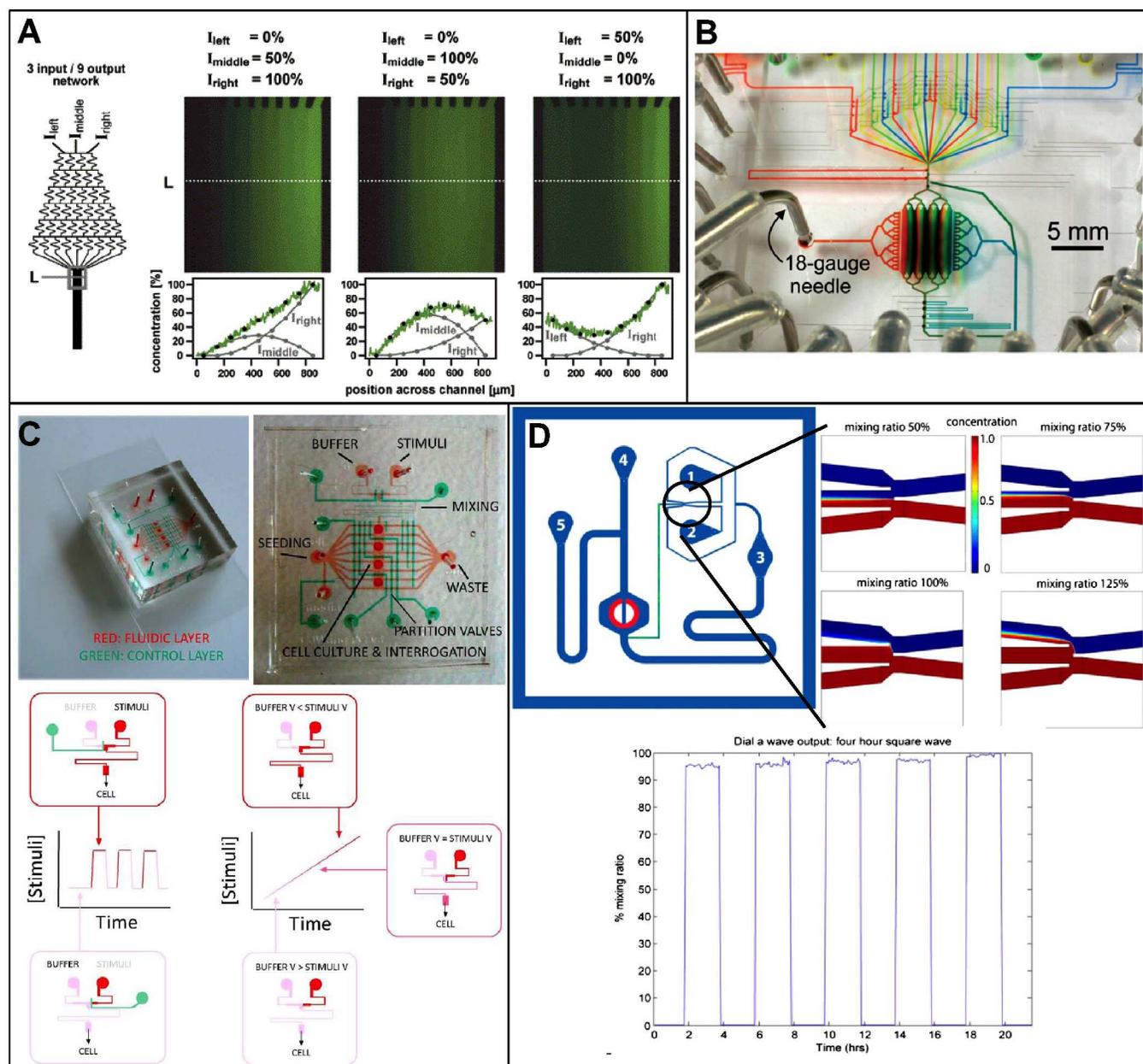


Fig. 4 (A) Resistance network used to generate complex spatial gradients in fluid channels. (Reprinted with permission from S.K.Dertinger, D.T.Chiu, N.L.Jeon and G.M.Whitesides, *Analytical Chemistry*, 2001, 73, 1240–1246. Copyright (2001) American Chemical Society.”) (B) Microfluidic chip for multipurpose testing using a combination of 16 inputs and outputs to generate spatial gradients in the central test chamber. (Reproduced from Ref. ¹⁰⁸ with permission from The Royal Society of Chemistry.) (C) Dial-a-Wave device for generation of temporal stimuli via adjustment of flow interface for study of synthetic oscillators. (Used with permission from ¹⁰⁹. Copyright 2011 Buamgartner *et al.*) (D) Microfluidic function generator using valves to control flow through a T junction. (Used with permission from ⁶⁸.)

bae *Dictyostelium discoideum* as it transitions from a single cell to a multicellular form during its life cycle⁶⁸. A different method of function generation uses laminar interface guidance to direct the laminar interface between input flows

by adjusting the ratio of input flows¹²⁰. Further refinement of this method led to the development of the ‘Dial-a-Wave’ device (Figure 4D) used to study dynamics of environmental effects on the galactose metabolism network in yeast¹⁰⁹.

These microfluidic function generators can be also used for frequency domain analysis, which allows for applications of control theory techniques¹¹⁸ such as block modeling and provides additional insights on noise and stability in biological devices^{121,122}. Advances in microfluidic fabrication means that on-chip microfluidic oscillators^{123,124} could also be used to generate complex input stimuli waveforms for device characterization while relying less on external hardware.

2.3 Future Work

Currently, part characterization experiments for static and dynamic behavior are carried out individually, and as such, are costly in both time and reagents. The expense of these experiments no doubt contributes to the lack of available characterization data. As a next step in the progression of designs useful for part characterization, we suggest a microfluidics platform for multi-dimensional characterization of biological parts. Such a device would allow for simultaneous experiments and data collection of the input/output dose response behavior, timing characteristics, and noise analysis through measuring single cell gene expression¹¹⁸.

A starting point for such a platform could begin with a design similar to the gradient generator by Cooksey *et al.* A number of cell traps or microchemostats could be placed in the central chamber for monitoring cell growth and gene expression at the single cell level. A full discussion of single cell analysis in microfluidics is provided elsewhere¹²⁵ while we describe some of the key microfluidic devices in single cell trapping and cell culture in Section 5.2. The input to the central chamber could then be switched between a gradient generator and a waveform generator to allow for multiple types of experiments on the same device. Ideally, multiple experiments could be run on the same biological part simultaneously with the same microfluidics setup on this device.

For example, the biological part in this microfluidics device could be subject to a gradient of inputs to measure the dose response. The inputs could be turned on and off at will to generate the temporal waveforms needed to measure the timing characteristics. Finally, as the cell traps could support single cell analysis, noise analysis could be performed on the part. Being able to perform many different experiments using one setup could allow for rapid characterization of new biological parts and devices.

3 Design

The nature of biology and evolution results in many homologous biological parts and pathways. Using homologous parts in a device leads to unwanted molecular interactions in the cell and interfere with the intended function of the device. We refer to these unwanted interactions as biological crosstalk. The

likelihood of crosstalk increases as biological devices grow more complex and involve more regulatory networks⁷. The use of orthogonal parts (parts that do not interfere with each other) reduces crosstalk, but there is lack of these parts in the current repertoire of synthetic biology. To increase the scalability of biological devices, we must find new orthogonal parts and regulatory systems¹³⁰ or reuse the currently available parts and systems²¹.

3.1 Challenges

New orthogonal parts can be discovered by surveying known genomes for novel regulatory networks. By mining the genomic database at European Bioinformatics Institute, Stanton *et al.* curated a collection of 73 homologs to TetR (a commonly used gene comprised of a repressible promoter and the repressor protein), and from those homologs, screened and isolated 16 orthogonal promoter-repressor pairs for use in new genetic devices¹³⁰. The limiting step in this process is the final screening of pairwise interactions as over 5000 individual experiments were required to screen the homolog library. Microfluidic high throughput screening platforms would allow for hundreds if not thousands of parallel experiments and reduce the time needed to discover novel orthogonal parts.

New parts may also be obtained through directed evolution, a method of applying selective pressure to a library of variants to engineer for specific functions without prior knowledge of the system¹³¹. Conventional methods for cycles of mutation, cell growth, and selection require frequent human intervention and several days per cycle, but new automation techniques such as Multiplexed Automated Genome Engineering (MAGE)¹³² reduce both time and human attention required to generate large libraries. Using MAGE to optimize the pathway in *E. coli* that produced isoprenoid lycopene required screening 10^5 colonies after 5-35 evolution cycles. The scale of microfluidic devices is too small to screen for level of diversity produced by MAGE, but perhaps may be used as a secondary screening platform on a subset of the optimized colonies.

One method of part reuse, as demonstrated by Tamsir *et al.*, is to separate large circuits into smaller circuits, each in a different cell colony, which communicate with each other through intercellular signaling chemicals. Using this technique, they built all possible two input boolean functions from biological NOR gates. The colonies are spatially separated on a plate by hand, with the intercellular signaling chemicals spreading through diffusion. However, the diffusion of these signals is not directed towards specific colonies and may reach unintended targets and cause crosstalk between circuits. Microfluidics could be used to physically isolate each colony and restrict intercellular signaling to specific colonies via controlling the media flow to reduce this crosstalk.

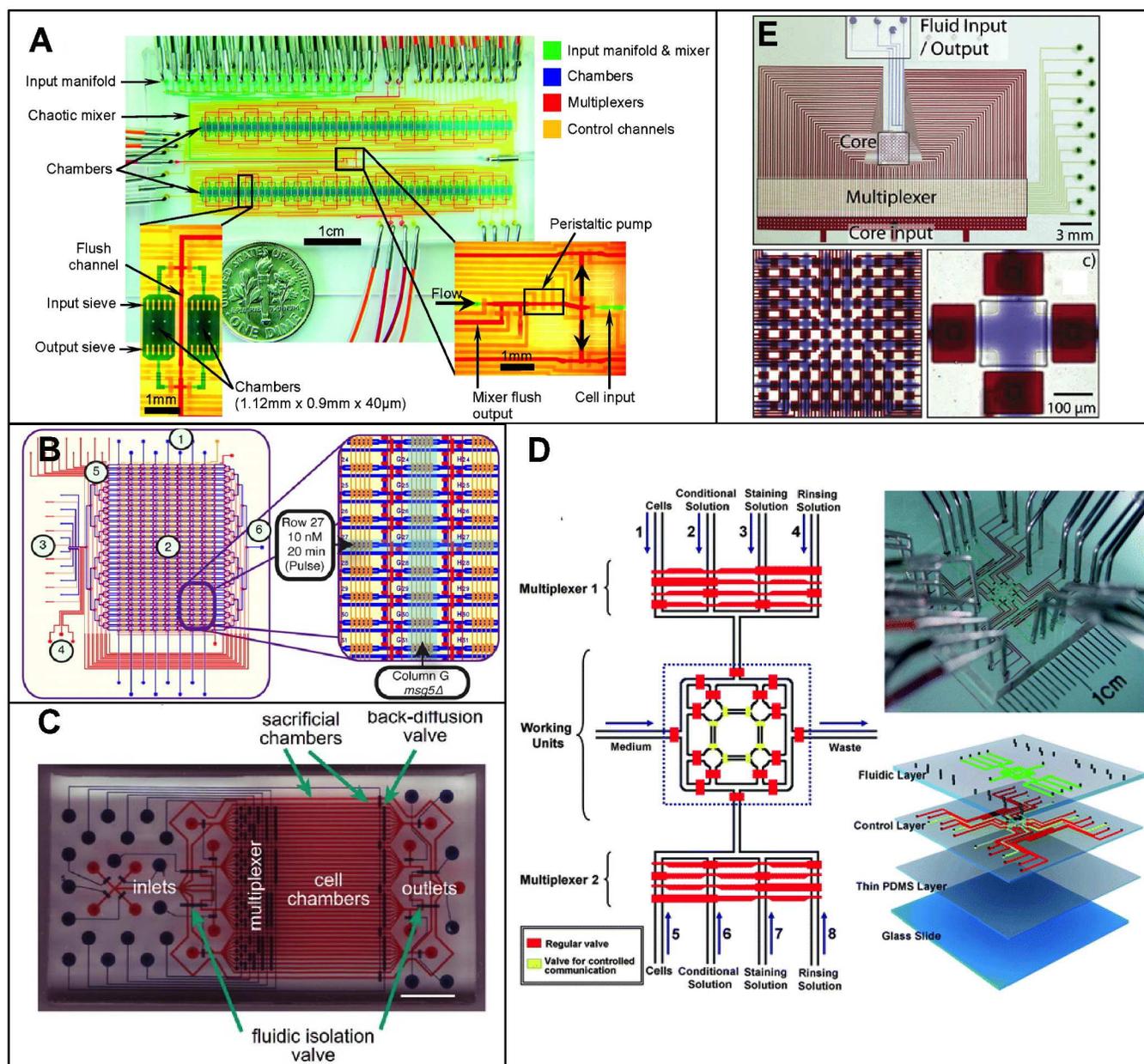


Fig. 5 (A) 96 chamber cell culture device that allows for individual culture conditions in each chamber. (“Reprinted with permission from R. Gómez-Sjöberg, A. A. Leyrat, D. M. Pirone, C. S. Chen and S. R. Quake, *Analytical chemistry*, 2007, 79, 8557–8563. Copyright 2007 American Chemical Society.”) (B) High throughput screening device with 2048 cell traps capable of 256 simultaneous screening experiments. (Used with permission from¹²⁶. Copyright 2009 Taylor *et al.*) (C) Device for high content cell screening used to study cell signaling dynamics. (Used with permission from¹²⁷. Copyright 2009 Cheong *et al.*) (D) Device for control and monitoring of intercellular communications. (Reproduced from Ref.¹²⁸ with permission from The Royal Society of Chemistry.) (E) Programmable general purpose microfluidic architecture with 64 300pl nodes for reactions and liquid storage. Insets show the array of nodes and the valves surrounding each node. (Reproduced from Ref.¹²⁹ with permission from The Royal Society of Chemistry.)

3.2 Current Solutions

An early device by Gómez-Sjöberg *et al.* contained 96 cell culture chambers, each with the capability for unique culture

conditions, for automated screening and assays (Figure 5A) and was used to study the effects of transient stimulation on

human stem cells¹³³. Increased miniaturization of components and a focus on single cell imaging have led to higher density screening platforms. A device by Cheong *et al.* (Figure 5C) allows high throughput analysis of single cell signaling dynamics in 32 cultures^{95,127}. Taylor *et al.* developed a device with 2048 cell culture chambers capable of conducting 256 simultaneous screening experiments (Figure 5B) for studying mating hormone responses in yeast (*Saccharomyces cerevisiae*, a model organism for eukaryotic studies)¹²⁶ while Denervaud *et al.* developed a parallel microchemostat array for growing and observing 1152 strains of yeast-GFP strains¹³⁴. These devices could be adapted for the screening and observation of part libraries developed through genomic mining and directed evolution.

An example of using microfluidics to control intercellular signaling is described by Liu *et al.*¹²⁸ with a four chamber network connected by thin channels for communications which could be opened or closed with valves (Figure 5D) used to study the response of NIH 3T3 fibroblasts to soluble signals from hepatocellular carcinoma cells. A network similar to this could be used to isolate cells and control signaling in biological distributed computing. A general purpose software-programmable architecture of an array of nodes surrounded by individually addressable valves similar to the one developed by Fidalgo *et al.* (Figure 5E) could be used to increase the size of circuits used in distributed biological computation. Incorporating basic Boolean logic directly into the chip through the use of pressure gain valves^{135–137} could allow further scaling of the microfluidic architecture by reducing the number of external control lines needed for larger experimental setups. Preliminary work has also been done on integrating microfluidic devices with liquid handling robots to increase automation and decrease the reliance on external control lines¹³⁸.

3.3 Future Work

We can expand on the idea of distributed biological computing^{21,30,31} with the high throughput arrays and control of intercellular signaling through valves to produce a hypothetical platform for biological computing. This platform might leverage technologies already present in electrical engineering and digital design to further biological circuit design in synthetic biology. The architecture for this platform may contain both the microfluidic architecture and the software for integrating biological circuit design with generating microfluidic valve controls.

The microfluidics architecture for this hypothetical platform could contain banks of ports for inputs and outputs and an array of microchemostats to house the cells used for computing. Each port and microchemostat could be individually accessible through the use of multiplexing, and each microchemostat could house a cell colony containing a biological

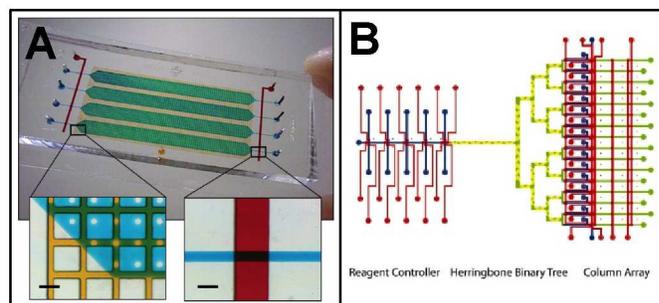


Fig. 6 (A) Microfluidic chip capable of 4 parallel 500nl synthesis reactions, with the gene synthesis chamber shown on the left in yellow and green and the fluid channel and valve overlay on the right in blue and red. (Kong, David S, Carr, Peter A, Chen, Lu, Zhang, Shuguang and Jacobson, Joseph M, “Parallel gene synthesis in a microfluidic device”, *Nucleic acids research*, 2007, 35, 8, e61, by permission of Oxford University Press) (B) 16 column microfluidic DNA synthesizer comprised of the reagent controller, a herringbone mixer, and a reaction column array. (Lee, Cheng-Chung, Snyder, Thomas M, and Quake, Stephen R, “A microfluidic oligonucleotide synthesizer”, *Nucleic acids research*, 2010, 38, 8, 2514–21, by permission of Oxford University Press).

device for the basic unit of computation in the larger biological circuit. Chemical signals could be passed from one stage of computation to the next through the opening and closing of specific valves. An extra isolated microchemostat housing a biological oscillator circuit could be used to synchronize the other biological devices on the chip if the quorum sensing system is used for intercellular communications¹³⁹. Synchronization could prevent logic faults caused by timing such as incorrect inputs being used in calculations or incorrect outputs being read.

4 Assembly

A vital part of synthetic biology is the technology used to assemble individual DNA parts into complex devices. The ideal DNA assembly method would allow for arrangement of parts in a specific sequence without scarring that would interfere with device function, easy generation of combinatorial libraries of constructs, and automation of the process¹⁴⁰. However modern assembly methods still fall short of the ideal.

4.1 Challenges

BioBricks and its variants BglBricks and 2ab assembly¹⁴¹ depend on standardized flanking restriction enzyme cut sites for pairwise assembly and create constructs with 8 base pair (bp) scar sites between parts. This class of methods is time intensive for large constructs as each reaction requires several days,

and automation has been limited by the sequential nature of the assembly process⁵¹. More promising are the one pot reactions such as GoldenGate⁴⁸, GoldenBraid¹⁴², and MoClo⁵⁰, which allow for combination of multiple parts in a single reaction with smaller scar sites. However, both types of assembly methods use restriction enzymes to cut and join parts, so all parts used in these methods must first have all internal restriction enzyme sites removed through targeted mutations¹⁴³. This adds another step and another point of potential failure in the construction of large devices. *De novo* synthesis of complete devices could eliminate the problems with DNA assembly, but the price point of current DNA synthesis at \$0.40 to \$1.00 per base pair¹⁴⁴, with an increase in price to dollars per base pair for large constructs of thousands of base pairs⁴⁴, and inconsistent turn-around time¹⁴⁵ makes the technology unsuitable for large devices. In addition, the price of DNA synthesis has plateaued at \$0.40 per base pair, and is unlikely to decrease further without a drastic improvement in technology⁴⁴.

4.2 Current Solutions

As the major component of the price of conventional DNA synthesis is based on reagent use and sample handling¹⁴⁶, the reduction in scale provided by microfluidic chips could lower the price of DNA synthesis to be viable for *de novo* synthesis of devices. An early example by Kong et al (Figure 6A), consists of four 500nl parallel reactors, each capable of synthesizing genes of up to 1 kilobase (kb) in length from starting concentrations two orders of magnitude lower than conventional reactions. A similar device by Lee et al¹⁴⁷ (Figure 6B), provides up to a 100 fold reduction in reagent use while producing 16 oligonucleotides in parallel at concentrations that did not require amplification before assembly. Combining this with the technology for the mass production of oligonucleotides in microarrays¹⁴⁴ and the development of microfluidic chips for two-step gene synthesis¹⁴⁸ could lead to an decrease in price of DNA synthesis to under \$0.05 per base pair, and reach the point where synthesis of large constructs becomes viable. Microfluidic devices have also been developed for the purification of oligonucleotides, which increases the fidelity of synthesized DNA¹⁴⁹.

4.3 Future Work

While much work has been done in DNA sequencing and amplification⁵⁹, little literature exists on importing existing DNA assembly techniques used in synthetic biology to microfluidic devices. Implementing processes such as BioBrick or MoClo assembly on microfluidic platforms would increase throughput and automation and decrease reagent use. Hillson *et al*¹⁵⁰ have proposed and patented a flexible microfluidic system for

both pairwise and one-pot DNA assembly based on existing procedures for DNA ligation and amplification and cell transformation¹⁵¹ in microfluidic systems. The company Genabler has also developed a proprietary one-pot assembly method for use with a modular microfluidic system¹⁵². However, little to no literature exists on the efficiency of commonly used DNA assembly techniques when performed in microfluidic devices.

5 Verification

The goal of verification is to compare the performance of the assembled device to the previously written specification, and from that comparison, determine the modifications needed refine the design rules and models used in the initial specification. This requires subjecting the new device to a set of testing conditions and monitoring the expression of proteins of interest under those conditions. Where as we focused on the behavior of individual parts in previous sections, we focus here on the analysis of systemwide behavior. The microfluidic devices described in Section 2.2 can be easily used to test the dynamics of novel biological devices and systems in addition to testing biological parts.

5.1 Challenges

One factor affecting the long term performance and reliability of biological devices not present in electronic devices is the natural mutation rate present in the host cell¹¹⁵. Long term monitoring of biological devices via microchemostats and other similar cell culture setups is essential for characterization of the effects of mutation rate on stability and robustness of novel biological circuits. Cell traps in these devices must allow for easy loading of cells and media, distribution of nutrients under high cell density, growing of cells in defined patterns to assist with tracking for single cell observations, and removal of cells without clogging the device⁷⁰. Data at the single cell level as well as the population level is required as important variations in single cells may be masked by the population data¹⁵⁴. Monitoring device behavior at the single cell level also provides data on noise in the biological devices caused by random timing of biochemical reactions and discrete molecules, which is important for accurate modeling of circuit dynamics^{67,125}.

Microfluidic platforms are well suited for and have been used in a variety of single cell studies¹⁵⁵, including investigating the mystery of antibiotic persistence in bacteria⁸⁵. Behavior of the cells in these devices are monitored through time-lapse fluorescent microscopy (TLMF), and images of the cells are processed with segmentation algorithms to track cell lineage and gene expression at the single cell level¹⁵⁶.

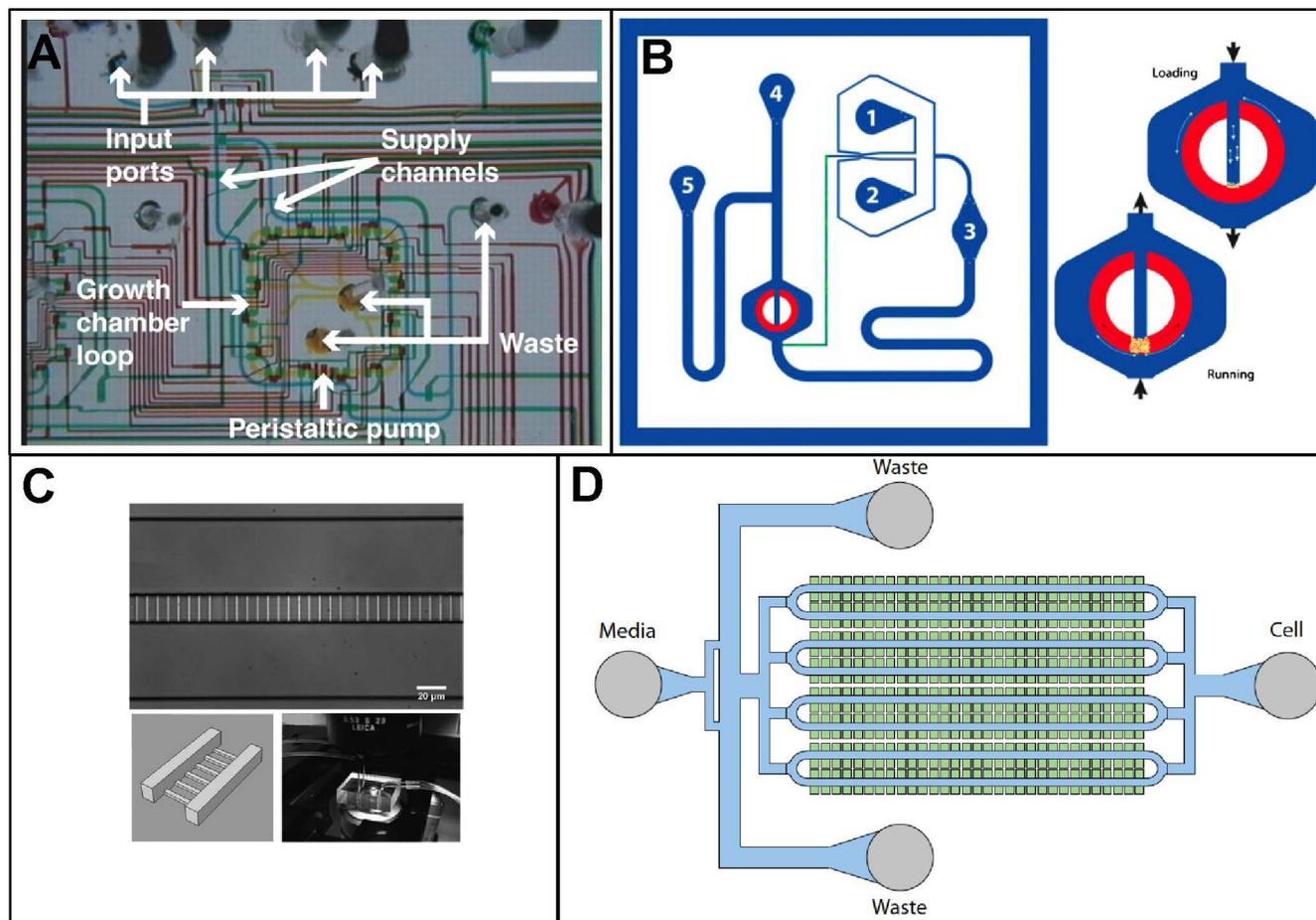


Fig. 7 (A) Microchemostat for long-term study of *E. coli*. (Used with permission from Ref.⁹⁰) (B) Donut cell trap for study of yeast regulatory networks. (Used with permission from¹⁰⁹. Copyright 2011 Baumgartner *et al.*) (C) Microchemostat that forces cell growth in lines for observation of single cell dynamics. (Reproduced from Ref.¹⁵³ with permission from The Royal Society of Chemistry.) (D) Cell trap array for the study of synchronizing synthetic oscillators. (Reprinted by permission from Macmillan Publishers Ltd: Nature. Ref.¹³⁹, copyright 2013)

5.2 Current Solutions

Hasty *et al* pioneered the use of microfluidics in the study of dynamics of synthetic biological circuits and the synchronization and entrainment of genetic oscillators in *E. coli*^{139,157,158}. The main device used in these studies (Figure 7D) consists of a main feeding channel flanked by rectangular trapping chambers that are sized for the ideal distribution of cell density, nutrients, and signaling enzymes for intercellular oscillators¹⁵⁷ and allows for exponential growth of a colony for up to 4 days. Growing cells are pushed outside the chambers and swept away to the waste ports. For yeast studies, the Hasty lab has developed a microchemostat with a doughnut trap to control cell growth and the dial-a-wave junction (as described in Section 2.2) to control input flow (Figure 7B) that

allowed cell growth for up to several days under various input conditions¹⁰⁹. The construction of both this device and the version designed to optimize resource use with 8 parallel microchemostats capable of running individual experiments are described in detail as a case study of using microfluidics to study synthetic systems⁷⁰.

The microfluidic bioreactor chip (Figure 7A) developed by Balagadde *et al* was designed to inhibit biofilm formation and allow for long-term continuous cell growth in 6 16nl microchemostats. This device was used study the long term growth of *E. coli* programmed with a genetic kill switch regulated by cell density through quorum sensing and the dynamics of a synthetic *E. coli* predator-prey system.⁹⁰ A microchemostat for constraining cell growth in lines for easy tracking (Figure 7C) developed by Long *et al.* consists of 600

sub-micron growth chambers connected to two feeding channels that traps *E. coli* and was used to observe growth rate and GFP expression at the single cell level¹⁵³. These microfluidic chips, combined with complex input generation schemes and multiplexing, can be used to develop sophisticated setups for characterization of long-term static and dynamic behavior of biological parts and devices

5.3 Future Work

One unexplored avenue in using microfluidics to study dynamics of gene expression is using feedback to influence and control the device of interest. Feedback may be used to stabilize device behavior, remove non-linearities, or account for system fluctuations, all of which would enhance the understanding of novel biological systems. Feedback systems already exist in microfluidic devices in the form of on-chip oscillators, and *in silico* feedback combined with optogenetics¹⁵⁹ have been used to implement feedback systems regulating intracellular signaling in fibroblasts¹⁶⁰ and gene expression in yeast¹⁶¹. A new system combining *in silico* feedback with microfluidic control of gene expression could be useful in characterizing and studying system-level behavior of biological devices.

6 Conclusion

We present in Table 1 a summary of the challenges facing different steps in the synthetic biology workflow and the key microfluidic technologies of interest in solving those challenges. The ability for fine tuned spatial and temporal control over inputs in microfluidic devices could be of great use in characterization of biological parts, leading to better specification of biological device behavior. Microfluidic large scale integration can be used for high throughput screening of potential new orthogonal parts or to control intercellular signaling for distributed biological computing in order to reuse existing parts. Automated construction of large biological devices could be achieved with a combination of microfluidic platforms for *de novo* synthesis of biological parts and microfluidic platforms for common DNA assembly techniques such as MoClo or Bio-Bricks. Finally, long term cell culture in microfluidic cell trap and microchemostats could assist with accurate measurements of gene expression at both the population and single cell level for verification of the performance of biological devices.

Both synthetic biology and microfluidics are building on the foundations laid by electrical engineering, in particular, the digital abstraction. The development of mLSI technology, particularly in the areas of on-chip logic and control, mimics that of digital electronics with the valve taking the place of the transistor as the basic unit of computation. The digital abstraction is used in synthetic biology to increase robustness,

decrease noise sensitivity, and allow specific tuning of inputs biological devices. Both fields have also developed their own versions of the functionally complete NOR gate^{21,137}, thus allowing for basic computation on platforms other than silicon. As both fields are already using similar abstractions for devices and computation, the digital abstraction provides an easy interface for integration of biological and microfluidic computation to develop novel systems that benefit from the advantages of both fields. Of note in this integration process is the new microfluidics track at the International Genetically Engineered Machines (iGEM) competition¹⁶² designed to introduce high school and undergraduate students to using microfluidics in designing and studying synthetic biological circuits.

The goal of synthetic biology is to use naturally existing logical constructs in biology (such as repressible and inducible genes) for novel applications in biosensing, therapeutics, and biomaterials. Microfluidics provides reduction in reagent use, increase in high throughput and automation, and precise control over the spatial and temporal environment necessary increase the scale and robustness of synthetic biology. The integration of microfluidics and synthetic biology has the capability to increase the scale of engineered biological systems for applications in cell-based therapeutics and biosensors, expand on the idea of distributed biological computation, and produce new rapid prototyping platforms for the characterization of genetic devices.

7 Acknowledgements

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Workflow Domain	Challenge	Potential Solution	Applicable Microfluidic Technology	References
Specification	Accurate and standardized models of parts needed for composition of composite devices and prediction of device behavior in simulations	Characterization of parts and devices over wide range of environments and operating conditions	Large scale spatial and temporal fine-grained control over chemical inputs in microfluidic devices	68,69,108,117,120
Design	Small number of available orthogonal parts result in unwanted molecular interactions in large devices	Engineering large libraries of new parts or reuse of existing parts	Microfluidic large scale integration for high throughput screening assays for new parts and controlling intercellular signaling for distributed biological computing	79,95,126,128,129,133,134
Assembly	Fast, accurate, repeatable construction of large genetic devices with minimal interference of part/device function	De novo synthesis of genetic parts and devices	Microfluidics DNA synthesis and assembly	10,144,146–149
Verification	Accurate measurements of gene expression at population and single cell level for multiple generations	Long term monitoring of single cells for part/device stability	Microchemostats and cell traps supporting long term cell growth and single cell monitoring	70,90,153,156,157

Table 1 Key microfluidic technologies for investigation of challenges present in the synthetic biology workflow

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