

Combining microfluidics and synthetic biology[†]

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One goal of synthetic biology is to design and build genetic circuits in living cells for a range of applications. A major challenge in these efforts is to increase the scalability of engineered biological systems. In particular, it is quite challenging to construct multiple layered logic circuits within a single cell because of metabolic and “cross-talk” effects. Microfluidic technologies allow precision control over the flow of biological content within microscale devices, and thus may provide more reliable and scalable construction of synthetic biological systems. We present here a summary of the advances in microfluidic technology, particularly microfluidic large scale integration, used to address the challenges facing synthetic biology as well as two potential workflows for combining microfluidic and 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 from 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, Butamax in Wilmington, Delaware, Butalco in Fuerigen, Switzerland) have all developed methods to increase the yield of isobutanol or butanol using various strains of yeast for commercial biofuels³ 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 increase the effect of antibiotics on otherwise antibiotic-resistant strains of bacteria⁶, which could allow for extending the effectiveness of current drug therapies in the face of rising antibiotic resistance. However, despite these success stories, the field of synthetic biology faces significant challenges as it moves from single prototypes to commercial

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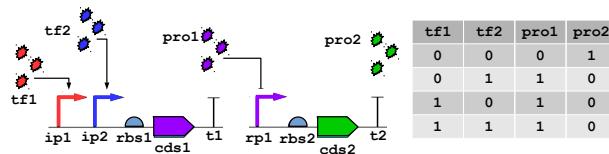


Fig. 1 A genetic NOR gate, and accompanying truth table. The presence of transcription factors *tf1* or *tf2* activate the inducible promoters *ip1* and *ip2* respectively and allow for production of *pro1*, coded for by *cds1*. The protein *pro1* acts as a transcription factor on repressible promoter *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.

scale enterprises.

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 “parts” as individual DNA sequences⁷. DNA consists of four nucleotides adenine (A), cytosine (C), thymine (T), and guanine (G), and triples of nucleotides (codons) encode for amino acids that are the building blocks of proteins. Each strand of DNA is directional, and the strands of a DNA in a double helix run antiparallel to each other. DNA is converted to mRNA through transcription by RNA polymerase, and the mRNA is translated by ribosomes into a sequence of amino acids that fold into a protein.

The basic structure of a prokaryotic (bacterial) DNA se-

quence that expresses a protein consists of the following segments: promoter, ribosome binding site, coding region, and terminator. RNA polymerase binds to the promoter to start transcription. Several classes of promoters exists, and their behavior depends on the presence or absence of small molecules known as transcription factors. Inducible promoters allow transcription only in presence of certain transcription factors while repressible promoters allow transcription only in their absence. Constitutive promoters allow transcription without requiring transcription factors and are useful for providing controls for determining baseline expression of a protein and for establishing constant expression of proteins. The protein to be expressed is coded in the coding region. Transcription of the mRNA stops at the terminator, and the ribosome binds at the ribosome binding site on the mRNA to begin translation of the mRNA into a protein. We abstract these structures (promoter, ribosome binding site, coding region, and terminator) as modular “parts” that can be combined to create functional “devices” which can be introduced into living organisms such as bacteria, yeast, or mammalian cells^{8,9}.

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 as eukaryotic systems have a different mechanism for binding ribosomes to DNA and require a more complicated regulatory structure⁹. In this circuit, the 2 input transcription factors *tf1* and *tf2* control the inducible promoters *ip1* and *ip2* such that transcription only takes place when at least one of the transcription factors is 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 behavoir of this device matches that of the Boolean NOR function, in which the output is true if and only if both inputs are false. NOR and NAND functions can be used to build all other Boolean logic functions, thus allowing these devices to be the basis of biological computation. As such, many different implementations of these functions exist in synthetic biology^{10–12}.

While many different classes of biological devices such as oscillators¹³, filters¹⁴, noise generators¹⁵, and the beginnings of analog computation¹⁶ exist, we focus here instead on the biological devices implementing Boolean logic 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^{17,18}.

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^{19,20}. One application for biological logic circuits is in the field of cancer research^{21,22}, where the use of digital logic provides the necessary specificity for targeting strains of cancer cells while leaving other cells unharmed^{23,24}. Nissim et al²⁵ 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 computation would allow for applications such as the differentiation of molecular species, the identification of specific genetic markers, and environmentally tailored drug dosage responses²⁶. 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 gate²⁷ with 11 orthogonal (non-interfering) regulatory proteins made from two layers of two input AND gates. Another way is to separate the larger function into smaller functions, place devices implementing the smaller functions into different cells, and have the cells communicate 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 pathway¹⁹. Distributed computing²⁸ has also been implemented in yeast with the development of both a 2-to-1 multiplexer and a basic addition circuit²⁹.

1.1.2 Memory and state in biological devices

The next step in increasing complexity of computation is to generate the idea of memory or “state”, such that the cell remembers what has previously happened and takes that into account in new calculations. This can be accomplished with the use of 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 DNA³⁰. Memory devices and counters have been integrated into cell through the use of recombinase based circuits^{31,32}. 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 performance³³.

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 gates³⁴.

1.1.3 Specify-Design-Assemble-Verify workflow

Synthetic biology 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^{35,36}, GEC³⁷, or Proto³⁸. In the design phase, biological parts are selected from repositories to implement the specified function. Tools such as GenoCAD³⁹, j5⁴⁰, or Clotho⁴¹ 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 Gen9⁴². Parts are assembled into devices by joining the segments of DNA together through use of restriction enzymes (proteins that cut DNA at certain sequences) and ligases (proteins that create new bonds between DNA bases). Common assembly techniques, including BioBricks⁴³, BglBricks⁴⁴, Gibson⁴⁵, GoldenGate⁴⁶, and modular cloning (MoClo)⁴⁷, are reviewed in detail at⁴⁸. 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 verify the function of the device. For verification purposes, the output of the device is a fluorescent reporter protein which is 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 also be grown for harvesting more copies of the device.

1.2 Microfluidics

Microfluidics is uniquely poised to solve the problems arising in synthetic biology. 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 a variety of applications in molecular biology⁴⁹, systems biology⁵⁰, stem cell studies^{51,52}, tissue engineering⁵³, point of care diagnostics⁵⁴ and pathogen detection⁵⁵, and systematic toxicity studies⁵⁶. Recently, synthetic biologists have been looking to microfluidics to study synthetic gene networks and network dynamics⁵⁷. Due to the potential in microfluidic systems for the precise control over input stimuli^{58,59}, and the

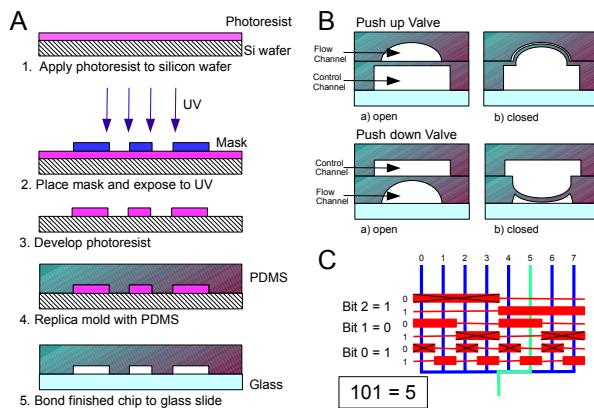


Fig. 2 (A) Fabrication process for soft lithography (adapted from Ref.⁵²). (B) 2 possible structures (push-up or push-down) for valves in multilayer soft lithography (adapted from Ref.⁷⁰). (C) Multiplexer for selection of 8 possible fluid lines, using 6 control lines to represent the 3 bit binary number of selected line (adapted from Ref.⁷¹).

ability to track single cells⁶⁰, there has been an increased interest in microfluidic platforms to further synthetic biology⁶¹. While there are a number of reviews postulating the benefits of microfluidics to synthetic biology, those reviews have focused on the specific applications^{61–63} or high level overviews of many competing microfluidics technologies^{64,65}. 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.

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^{66,67}. For the purposes of this paper, we will focus on the use of polydimethylsiloxane (PDMS) based pressure driven laminar flow and microfluidic large scale integration (mLSI) for the study and advancement of synthetic biology as the physical properties of PDMS, described further in 1.2.2, offer the most flexibility for fabrication, scalability, and potential for longterm growth and monitoring of cells^{68,69}.

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 engineering⁷², 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 interest⁵⁹. Mixers^{73,74} 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 at⁷⁵.

1.2.2 PDMS and soft lithography

The properties of PDMS make it well suited for use in biological applications. PDMS is optically transparent, chemically inert, impermeable to water while being permeable to gases, and non-toxic to cells⁷⁶. PDMS based microfluidics have been used for a variety of purposes in recent years, including as an alternative platform for computation⁷⁷. Development of the microchemostat⁶⁸ has allowed for cells to be grown in microfluidic chips for long periods of time, thus allowing for more complex, long term experiments.

Microfluidic chips are fabricated from PDMS through soft lithography. As many detailed reviews of the process exist^{76,78,79}, 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 and costs around \$0.05 per cm³⁸⁰, making this suitable for rapid prototyping and quick design iteration.

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 actuators⁸⁰. A chip made with this method consists of a flow layer and a control layer. Channels on the flow layer carry signals of interest while channels in the control layer are pressurized with external actuators. 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 flow⁸¹. Two types of valves described in⁷⁰, 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 chan-

nels and chip reuse⁸². 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 multiplexors, developed by Thorsen et al⁷¹ 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 hundreds or thousands fluid elements. The multiplexor 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 chips⁸³.

2 Using microfluidics to solve challenges in synthetic biology

Introducing complex engineered systems into cells presents numerous challenges at different levels of the design process. We present in Table 1 a summary 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 good as the understanding of the underlying biological behavior. In the design phase, synthetic biology still suffers from a lack of parts that do not interfere with each other when used together to construct larger systems. As these devices are assembled, problems arise concerning unintended changes introduced to the sequence 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. As new techniques in synthetic biology result in the generation of large libraries of parts with variations and combinatoric devices, increased automation is needed to test and characterize these constructs to allow the acceleration of the specify-design-assemble-verify development cycle for synthetic biology.

Workflow Domain	Challenge	Potential Solution	Applicable Microfluidic Technology	References
Specification	Accurate models of parts needed for prediction of device behavior in simulations	Characterization of parts and devices over wide range of environments and operating conditions	Spatial and temporal control over chemical inputs in microfluidic devices	58,59,93–95
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 and controlling intercellular signaling	82,88–92
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	84–87
Verification	Accurate measurements of gene expression at population and single cell level for multiple generations	Long term monitoring of cells for part/device stability	Microchemostats and cell traps supporting long term cell growth	60,68,96,97

Table 1 Microfluidics for solving challenges in synthetic biology

2.1 Specification

Accurate specification of biological device function depends on being able to predict future behavior of combinations of biological parts. Currently, however, not all of the behavior is well understood or predictable, and 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 models for part behavior, and to generate such models, we require more and better characterization of part behavior over a range of environmental conditions.

2.1.1 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 the semiconductor device physics and can create accurate models for electronic parts for simulations, we are still developing the 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 sequence⁹⁹. Similar models have been generated for ribosome binding sites¹⁰⁰, but these sequence based models alone cannot predict part behavior in a biological system. Likewise, new software tools developed for simulating synthetic biological systems^{101,102} are based on models of bi-

ological process of cell growth, diffusion, and protein interactions and degradation but do not take into account the DNA sequence of the device and part behavior.

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 more information than their electrical engineering counterparts due to the inherent dynamics of biology. 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, 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 changes¹⁰³. The addition of biological parts and device into the host cell introduces large amounts of foreign DNA that may impact host cell metabolism¹⁰⁴. To produce useful datasheets and models for simulation would

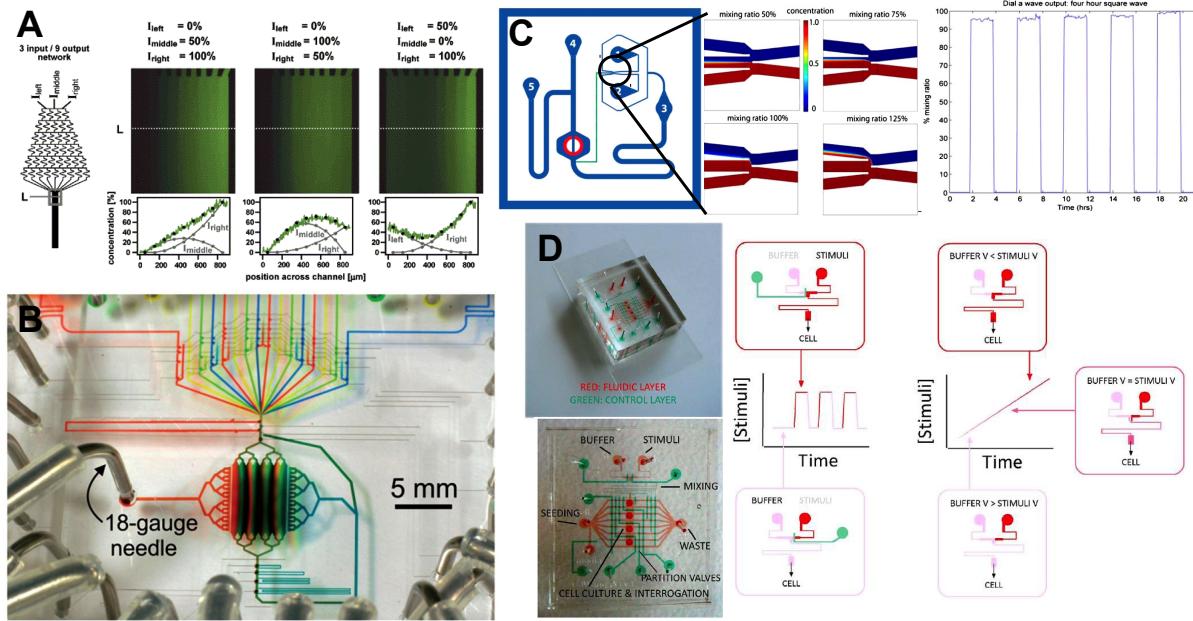


Fig. 3 (A) Resistance network used to generate complex spacial gradients in fluid channels. Used with permission from Ref.⁵⁹. (B) Microfluidic chip for multipurpose testing using a combination of 16 inputs and outputs to generate spacial gradients in the central test chamber. Used with permission from Ref.⁹⁵. (C) Dial-a-Wave device for generation of temporal stimuli via adjustment of flow interface for study of synthetic oscillators. Used with permission from⁹⁸. (D) Microfluidic function generator using valves to control flow through a T junction. Used with permission from⁵⁸.

require massive amounts of characterization data. The spatial and temporal environmental control that microfluidics provides combined with high throughput cell culture assays could be used to develop platforms characterization and test platforms for synthetic biology devices.

2.1.2 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 include testing for interactions with other similar transcription factors and testing the effects of simultaneously applied multiple inputs.

Microfluidic resistance networks for generating complex spacial and temporal gradients (Figure 3A)^{59,93} 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 orthogonality¹⁰⁵ and have been used for combinatorial drug screening¹⁰⁶. The microfluidics chip by Cooksey *et al.* (Figure 3B) provides the ability to generate

complex spacial gradients in a central chamber from combinations of up to 16 unique inputs by using segments of high fluid resistance and outlets to control flow⁹⁵. Replacing the central chamber in the initial design with a screening array such as one of those shown in Section 2.2.2 would adapt it for high throughput biological device characterization.

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. These timing characteristics are vital if we wish to avoid faults in the function caused by inputs changing before outputs are stable and by using unstable outputs. Wang *et al.* used valves to control inputs through a T junction to create square waves and ramp functions (Figure 3D) to characterize the dynamic signaling behavior of social amoeba⁵⁸. A different method of function generation uses laminar interface guidance to direct the laminar interface between input flows by adjusting the ratio of input flows⁹⁴. Further refinement of this method led to the development of the 'Dial-a-Wave' device (Figure 3C) used to study dynamics of environmental effects on the galactose metabolism network in yeast⁹⁸. These microfluidic function generators can be also used for frequency domain analysis, which allows for applications of control theory techniques¹⁰⁵ such as block modeling and provides additional insights on noise and stability in biological devices^{107,108}. On-chip mi-

crofluidic oscillators^{109,110}, which could be used to generate more complex input stimuli waveforms for device characterization.

2.2 Design

The nature of biology and evolution conserve important functions in DNA, resulting in many biological parts that code for similar genes 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 systems¹¹¹ or reuse the currently available parts and systems¹⁹.

2.2.1 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 devices¹¹¹. 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 system¹¹³. 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)¹¹⁴ reduce both time and human attention required to generate large libraries.

A limiting factor for both genomic mining and direction evolution is the final screening of the library of part variants. To screen the homolog library for pairwise interactions involved over 5000 individual experiments, and using MAGE to optimize the pathway in *E. coli* that produced isoprenoid lycopene required screening 10^5 colonies after 5-35 evolution cycles. Microfluidic high throughput screening platforms for these libraries would allow for faster discovery of novel orthogonal parts.

One method of part reuse, as demonstrated in¹⁹, is to separate large circuits into smaller circuits, each in a different cell colony, which communicate with each other through intercellular signaling chemicals. Tamsir *et al.* built all possible two input boolean functions from biological NOR gates in this manner. 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. We envision using microfluidics to physically isolate each colony and restrict intercellular signaling to specific colonies via controlling the media flow to reduce this crosstalk.

2.2.2 Solutions

An early device by Gomez *et al.* contained 96 cell culture chambers, each with the capability for unique culture conditions, for automated screening and assays (Figure 4A) and was used to study the effects of transient stimulation on human stem cells⁹⁰. 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 4C) allows high throughput analysis of single cell signalling dynamics in 32 cultures^{82,112}. Taylor *et al.* developed a device with 2048 cell culture chambers capable of conducting 256 simultaneous screening experiments (Figure 4B) for studying mating hormone responses in *Saccharomyces cerevisiae*⁸⁸ while Denervaud *et al.* developed a parallel microchemostat array for growing and observing 1152 strains of yeast-GFP strains⁸⁹. 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 in Liu *et al.*⁹¹ with a four chamber network connected by thin channels for communications which could be opened or closed with valves (Figure 4D) 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. An 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 4E) 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 valves¹¹⁵⁻¹¹⁷ would allow further scaling of the microfluidic architecture.

2.3 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 process¹¹⁸, but modern assembly methods have yet to reach that pinnacle of achievement.

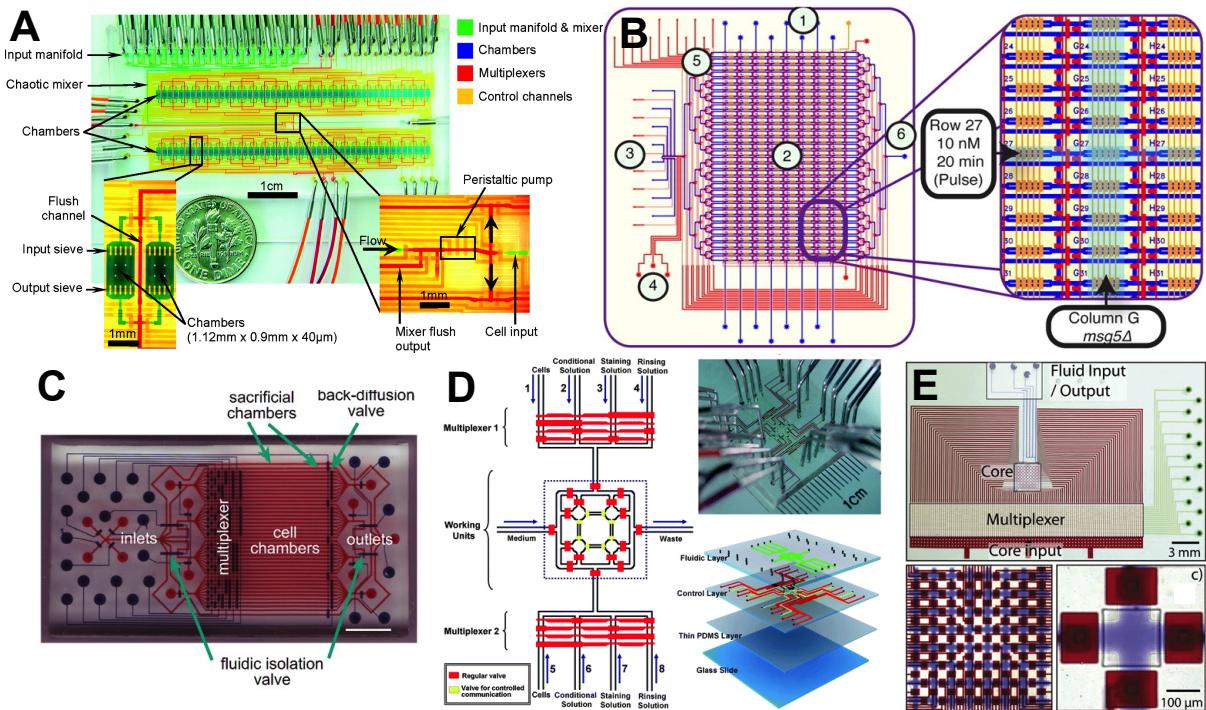


Fig. 4 (A) 96 chamber cell culture device that allows for individual culture conditions in each chamber. Used with permission from⁹⁰. (B) High throughput screening device with 2048 cell traps capable of 256 simultaneous screening experiments. Used with permission from⁸⁸. (C) Device for high content cell screening used to study cell signalling dynamics. Used with permission from¹¹². (D) Device for control and monitoring of intercellular communications. Used with permission from⁹¹. (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. Used with permission from⁹².

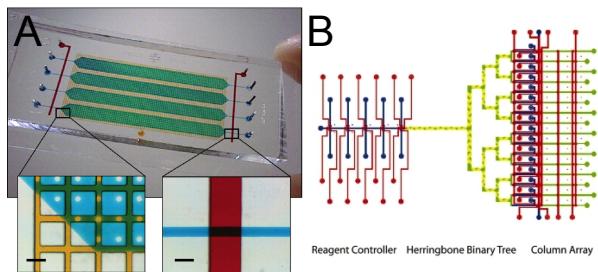


Fig. 5 (A) Microfluidic chip capable of 4 parallel 500nl synthesis reactions, with the gene synthesis chamber shown on the left in yellow and green and the fluid channel and valve overlay on the right in blue and red. Used with permission from Ref.⁸⁴. (B) 16 column microfluidic DNA synthesizer comprised of the reagent controller, a herringbone mixer, and a reaction column array. Used with permission from Ref.⁸⁵.

2.3.1 Challenges

BioBricks and its variants BglBricks and 2ab assembly¹¹⁹ 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 are time intensive for large constructs as each reaction requires several days, and automation has met with limited success. More promising are the one pot reactions such as GoldenGate, GoldenBraid, and MoClo, 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 mutations¹²⁰. 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 pair⁸⁷, with an increase in price to dollars per base pair for large constructs of thousands of base pairs⁴², and inconsistent turn-around time¹²¹ 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 technology⁴².

2.3.2 Solutions

As the major component of the price of conventional DNA synthesis is based on reagent use and sample handling⁸⁴, 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 5A), 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 al*⁸⁵ (Figure 5B), 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 microarrays⁸⁷ and the development of microfluidic chips for two-step gene synthesis⁸⁶ could lead to a decrease in price of DNA synthesis to under \$0.05 per base pair, and reach the point where synthesis of large constructs becomes viable.

2.4 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.

2.4.1 Challenges

Unlike electronic devices, biological devices are inherently unstable due to the natural mutation rate present in the host cell¹⁰³. 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 device stability and function. 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 data¹²³. 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^{57,124}. Microfluidic platforms are well suited for and have been used in a variety of single cell studies¹²⁵, including solving the mystery of antibiotic persistence in bacteria⁶⁹.

2.4.2 Solutions

Cell traps in microchemostats 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 device⁶⁰. Balagadde *et al.* developed a microfluidic bioreactor chip (Figure 6A) designed to inhibit biofilm forma-

tion that allows for long-term continuous cell growth in 6 16nl microchemostats which could be monitored by microscopy for real-time data and used it to study the long term growth of *E. coli* programmed with a genetic device for population control that regulates cell density through quorum sensing and the dynamics of synthetic *E. coli* predator-prey system.⁶⁸. A microchemostat with a doughnut trap to control cell growth and the dial-a-wave junction (as described in Section 2.1.2) to control input flow (Figure 6D) that allowed cell growth for up to several days under various input conditions was used by Baumgartner *et al.* in yeast studies⁹⁸. 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 systems⁶⁰.

A microchemostat for constraining cell growth in lines for easy tracking (Figure 6C) 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 level⁹⁶. Arrays of cell traps have been used to study the synchronization and entrainment of genetic oscillators in *E. coli*^{97,122,126}. The main device (Figure 6D) consists of a main feeding channel flanked by rectangular trapping chambers that are sized for are sized for the ideal distribution of cell density, nutrients, and signaling enzymes for intercellular oscillators⁹⁷ 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. 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.

3 Workflows for combining synthetic biology and microfluidics

To lower the barrier of entry into the field, both synthetic biology and microfluidics need to take advantage of the improvement in automation and computer aided design tools. Software tools for synthetic biology has 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) are being developed to facilitate information exchange between various tools in the toolchain and will be vital in building end-to-end workflows 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 bleeding edge of biological research, the development of these tools helps with refining the rules of genetic design for future applications¹²⁷.

In microfluidics, CAD and automation have been primar-

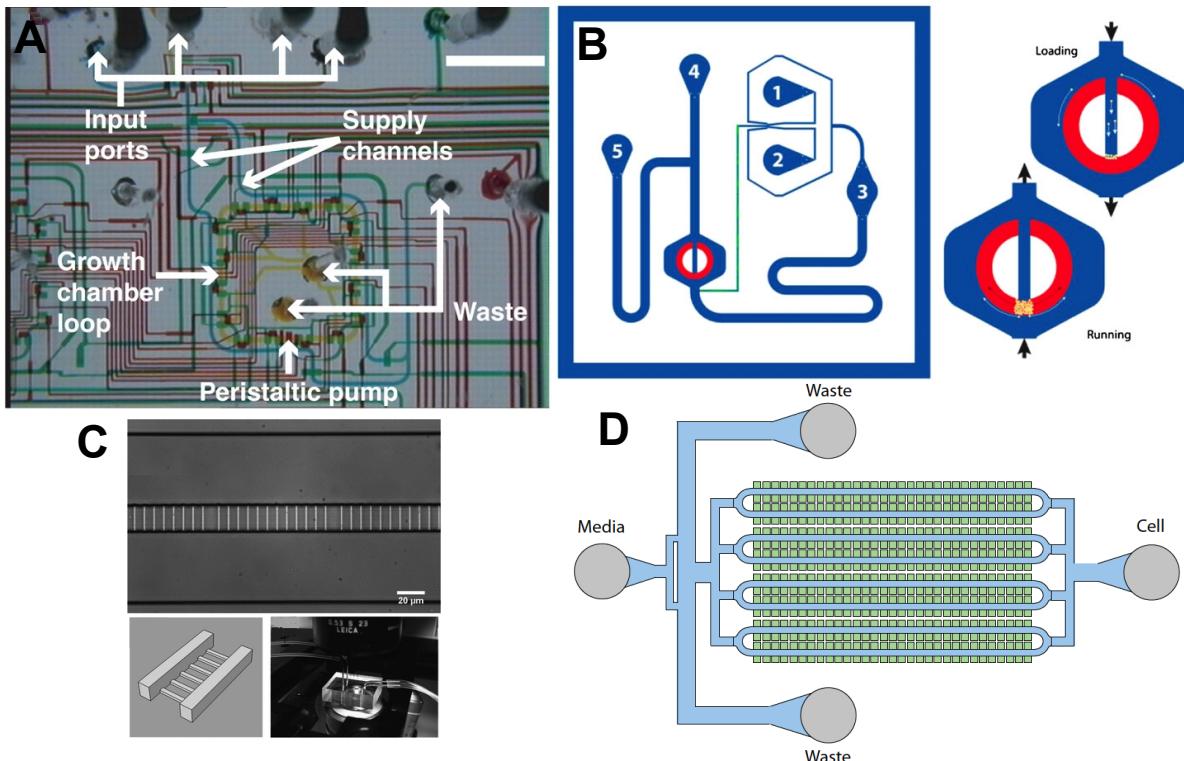


Fig. 6 (A) Microchemostat for long-term study of *E. coli*. Used with permission from Ref.⁶⁸. (B) Donut cell trap for study of yeast regulatory networks. Used with permission from⁹⁸. (C) Microchemostat that forces cell growth in lines for observation of single cell dynamics. Used with permission from Ref.⁹⁶. (D) Cell trap array for the study of synchronizing synthetic oscillators. Used with permission from¹²².

ily focused on droplet based digital microfluidics^{128–131} rather than microfluidic large scale integration. Earlier tools for mLSI include Biostream, a tool for designing GUIs and control valves for multilayer devices^{77,81,132} (freely available at¹³³) and Micado, 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 hardware description languages used in electronics¹³⁴ and algorithms for laying out the flow layer based on a high level description of chip function¹³⁵, and better algorithms for valve placement and control routing^{136,137}. However, there are currently no CAD tools that combine, formalize, and optimize the workflows for synthetic biology and microfluidics. We now propose two potential workflows for using microfluidics to augment and assist developments in synthetic biology.

3.1 Microfluidics augmented distributed computing

Based on the high throughput arrays shown in Figure 4 and the biological devices for distributed computing described in^{19,28,29}, we present a hypothetical platform for biological computing similar to an field programmable gate array

(FPGA) with a microfluidic chip as the underlying structure. This platform will leverage technologies already present in electrical engineering and digital design to further biological circuit design in synthetic biology and consists of both the microfluidic architecture and the software for integrating biological circuit design with generating microfluidic valve controls.

The microfluidics chip will consist of a bank of signal input ports, a bank of signal output ports, a bank of ports for loading cells and media, and an array of microchemostats. Each port and microchemostat should be individually accessible with through the use of multiplexing. Each microchemostat would house a cell colony containing a biological device for the basic unit of computation in the larger biological circuit. Chemical signals would be passed from one stage of computation to the next through of the opening and closing of specific valves. A slight modification of 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 communications¹²². Synchronization would prevent logic faults caused by timing such as incorrect inputs being used in calculations or incorrect outputs being read.

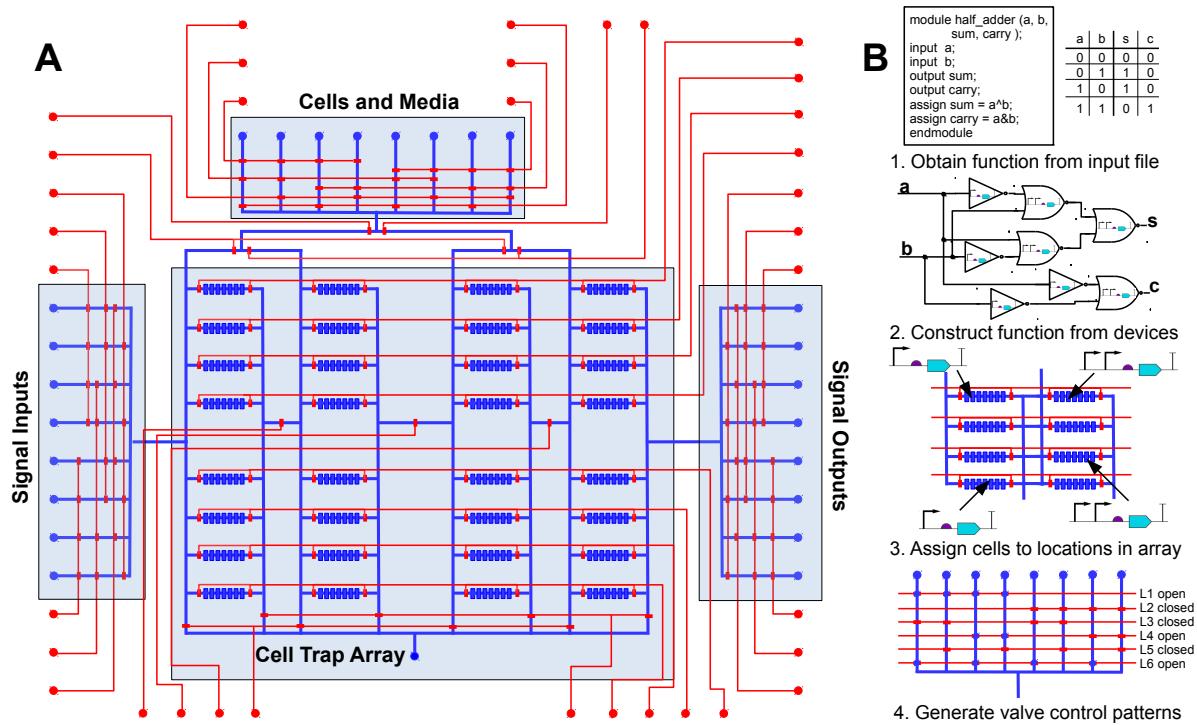


Fig. 7 (A) Hypothetical microfluidics chip for distributed biological computing consisting of an array of 32 microchemostats, 8 loading channels for different cell strains and media, 8 input channels, and 8 output channels. Each input or output channel and each microchemostat can be accessed individually, and signaling molecules can be flushed from one chemostat to another. (B) Workflow for design and execution of biological programs, starting with parsing the function from an input file, and converting the function to a canonical form. The function is built from a selection of available biological devices, and those devices are assigned to microchemostats in the array. Finally, the valve control patterns needed to execute the function are generated.

The system will perform as follows. We begin with a function defined in a hardware description language such VHDL or Verilog and a library of existing biological devices. This library will contain, at a minimum, a biological NAND or a biological NOR gate, and will ideally contain a variety of 2 and 3 input boolean logic functions as well as flip-flops and latches for sequential logic. The software will parse the input file and convert the logic function described within into a canonical form. The function will then be implemented from a selection of available devices in the library. Each device will be mapped to a microchemostat, and the sequence of valves needed to execute the function by routing signals from one microchemostat to the next will be determined. This workflow is similar to the FPGA logic synthesis workflow, with the microchemostat taking the place of the look-up table block in digital design. The placement of biological devices in the microchemostats is analogous to the placement and routing of blocks in the FPGA, and existing algorithms can be leveraged for this system.

3.2 Integrated microfluidics control feedback from microscopy

We present a system for real-time control of a microfluidics chip based on time-lapse fluorescent microscopy (TLMF) of the chip's content by taking advantage of the fact that a typical microfluidics setup uses the same computer for both microfluidics and microscopy control. By combining the software for those two tasks, we can create a system that ties the result of TLMF back into controlling the microfluidics chip and give us unparalleled ability to micromanage experimental setups based on current conditions on-chip.

We assume that the microfluidic chips used with this system have a computer controlled pneumatic system for opening and closing valves¹³³ and that the chips used are for high throughput screening or characterization for biological parts and devices. Expression levels will be measured by intensity of the fluorescent reporters in the biological devices. Behavior of the cells in the chip will be monitored through microscopy and images of the cells will be processed as they are taken to track cell lineage and gene expression at the single cell level

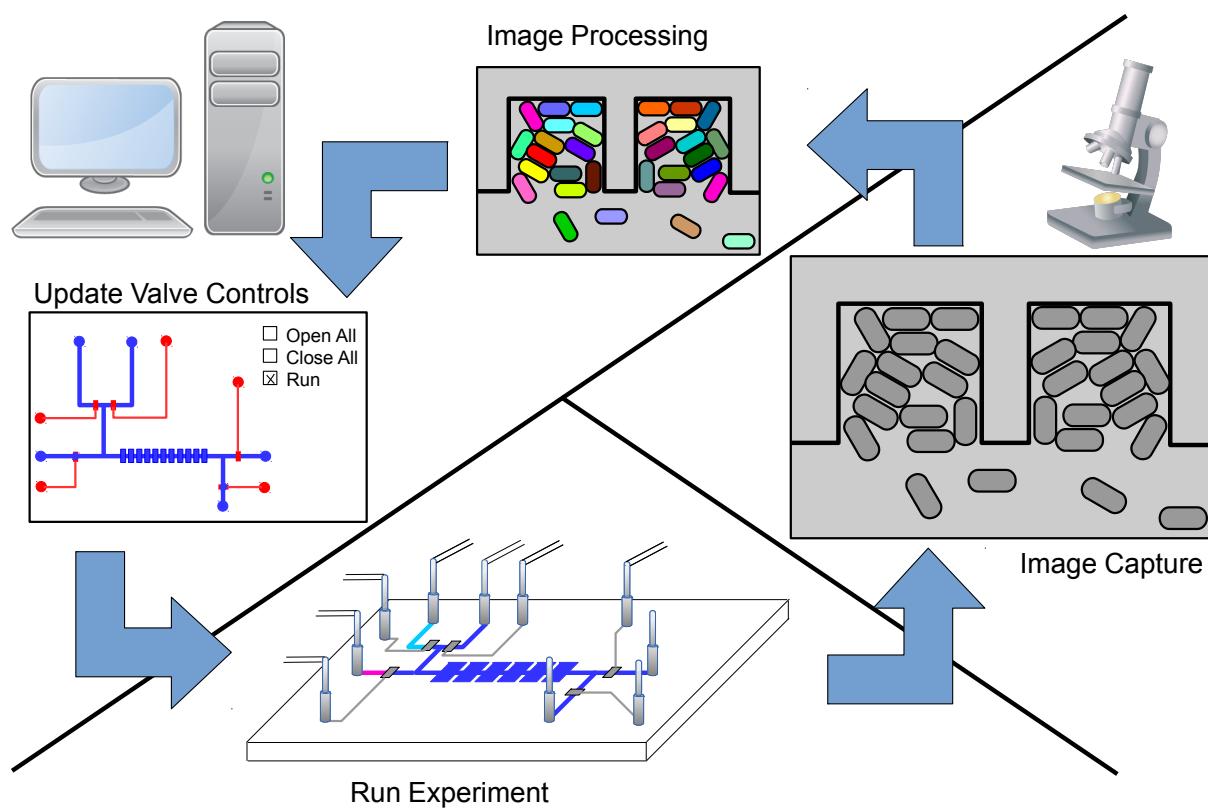


Fig. 8 Real-time updating of experimental conditions on a microfluidic chip based on microscopy data. Images are processed as they are captured via time lapse fluorescent microscopy during the experiment. The resulting data is used to determine the next state of the control lines on the chip.

with segmentation algorithms¹³⁸. The results of the image processing then feeds back to the microfluidic chip controller to determine the next phase of the experiment. As cell growth is much slower than the time required for image processing and triggering a change in the state of valves, this provides us with effectively real-time control of the microfluidics device based on cell behavior.

We propose an extension of the automation work done by Theis *et al.*¹³³ for software programmable microfluidic control to encompass programmable control of the imaging equipment and image processing as well as programmable control of the microfluidic valves. A scripting language that implements the interfaces between the microfluidic setup, the microscope and camera¹³⁹, and the image processing suite¹⁴⁰ will be used to allow the user to define the parameters for the experiments. The language will contain commands for opening and closing valves, for microscope movement and image capture, and for segmentation algorithms and will allow use of conditionals and loops to further define complex experiments. A typical experiment will involve setting up initial growth conditions and monitoring locations of interest on the chip at

defined intervals with future conditions defined by the results of the monitoring. This system could easily be expanded to include remote monitoring of the experimental setup to further reduce the burden on the experimenter.

4 Conclusion

We have presented here a summary of the basic technologies present in synthetic biology and microfluidics. The challenges that face synthetic biology as it seeks to emerge as an engineering discipline in the areas of DNA synthesis, part discovery, and system characterization may be mitigated through the use of microfluidics, particularly the increase in scalability provided by microfluidic large scale integration. As the basic tools for workflow automation exist in both fields, we describe two potential platforms for combining the synthetic biology and microfluidics workflows. 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, and produce new rapid prototyping platforms for the characterization of genetic de-

vices.

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