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Next generation tools to accelerate the synthetic biology process

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Synthetic biology follows the traditional engineering paradigm of designing, building, testing and learning to create new biological systems. While such approaches have enormous potential, major challenges still exist in this field including increasing the speed at which this workflow can be performed. Here, we present recently developed microfluidic tools that can be used to automate the synthetic biology workflow with the goal of advancing the likelihood of producing desired functionalities. With the potential for programmability, automation, and robustness, the integration of microfluidics and synthetic biology has the potential to accelerate advances in areas such as bioenergy, health, and biomaterials.

Reducing biological systems to their component parts is frequently used to understand these complex systems, but the reductionist approach is fraught with challenges. These difficulties were highlighted in 2002 by Yuri Lazebnik, who imagined the process by which a biologist might attempt to fix a broken radio using the reductionist approach (DOI: 10.1016/S1535-6108(02)00133-2). First, the most important components of a functional radio would be identified by deliberately and randomly removing components from thousands of working radios. Second, those important components would be systematically altered to test their effect on radio function. Finally, with sufficient data it may eventually be possible to determine how the radio works at the component level, and identify a target component for replacement. In this partially tongue-in-cheek example, the reductionist approach is taken to its' ultimate conclusion in which every component of the system is characterized, and a model relating component activity and system function is synthesized from the evidence, enabling the biologist to fix the radio.

The difficulty in applying this approach to even a single biological cell is in the sheer quantity and functional complexity of the system components. While an engineer with functional knowledge of the working principles of the radio would conduct a series of simple tests to isolate the problem and then fix it, how might we determine the working principles of biological systems? For the engineering student, these principles often come from practice projects, in which sub-systems of the radio are first constructed from individual components and then experimented with. Synthetic biology allows us to do just that: design and construct complex biological systems from the ground up, to simultaneously learn more about biology while creating useful technologies. This integrative field has allowed us to create new biomaterials, novel therapeutics, and design environmentally-friendly biorenewable fuels. For example, microbial systems can be engineered to produce artemisinic acid, a precursor for antimalarial drugs, that will provide more patients with affordable treatments (DOI: 10.1038/nbt833). Other microbial systems have been engineered to convert simple sugars into advanced biofuel compounds (DOI: 10.1038/ncomms1494).

As in conventional engineering, the process of realizing new biological systems typically follows an iterative design–build–test–learn (DBTL) cycle (DOI: 10.1039/c4lc00509k). For a given biological process, the DBTL cycle includes selecting biological parts and pathways (design), using genomic modification tools to generate a library of strain variants (build), assessing the performance of the constructed strains (test), and ultimately evaluating the results to determine if the design was successfully realized or requires further improvement (learn). This directed evolutionary approach requires advanced biotechnologies, and one of the major challenges in synthetic biology is workflow acceleration and automation, to find reliable and robust methods to implement new biological designs in living organisms. Microfluidic technologies may address many of the technical challenges in designing and in implementing synthetic biological systems, and in this research highlight, we review several recent microfluidic strategies to develop these advanced workflows.

Engineering a new biological system starts with the "design" phase. It can be thought of as developing the initial specifications for a system to achieve a certain task. This involves two critical design decisions: (1) selecting a biological host (*i.e.* the type of cell) for transplanted DNA, as the host plays a crucial role in functional performance of the engineered system, and (2) deciding which biological pathway to engineer such that a desired output is likely met. Software tools (such as TinkerCell [10.1186/1754-1611-3-19] or

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'gro' [10.1021/sb300034m]) have been developed to integrate knowledge from previous experiments to automatically prioritize those pathways that relate to target bio-molecule production. Once the optimal design(s) are chosen, the system is constructed in a wetlab. This "build" phase assembles individual gene fragments (*i.e.* parts) into plasmids and genomes (*i.e.* bioparts) for specific functions using various assembly mechanisms including one-pot Golden-Gate and Gibson assembly or *in vivo* based assembly conducted in yeast. *In vivo* assembly is favorable over other mechanisms as it does not require the use of expensive enzymes to join DNA parts together and can produce much larger final products (up to 100 kb) compared to other methods.

Assembling these DNA parts into bioparts, pathways, and genomes in the 'Build' phase requires novel technologies to address the sample handling throughput and precision required for these experiments. A recent study in *Nucleic Acids Research* by Yehezkel and coworkers (DOI: 10.1093/nar/gkv1087) demonstrates how technologies might be used to enhance the build phases without the typical one-pot or *in vivo* assembly. They developed a digital microfluidic platform that assembles synthetic genes and clones using existing gene synthesis methodologies in a cell-free system (Fig. 1). Using a conceptually simple approach in which movement of liquid droplets on a surface is controlled by electrical actuation, this automated platform allows reservoirs containing reagents for DNA assembly to be sampled, mixed with other reagents, diluted, stored, and processed in a designated PCR lane to assemble the different parts into bioparts. Using this device, they showed that with an increasing number of parts, performing Gibson assembly increased the number of non-specific construction products compared to their cell-free assembly method. Furthermore, they demonstrated that assembling genes (*via* device) results in high-fidelity clones (an error-rate of 1/450), improving over traditional assembly techniques. Aside from DNA construction, one of the bottlenecks in "building" DNA parts is the cloning process. The authors overcame this limitation by conducting *in vitro* cloning *via* single molecule PCR. To obtain a single molecule, the assembly product is iteratively diluted until one target DNA molecule per droplet is obtained. These single molecules (which have a unique barcode) are further amplified using PCR and then sequenced for verification. In comparison with manual techniques, their platform demonstrated (1) a reduced cost by a factor of 50 (*i.e.* 50-fold reduction in volumes), (2) a reduction in time (*i.e.* cell-free cloning), and (3) an improvement in parallelization of assembly reaction using automation. Each of these advantages enables fast and rapid DNA assembly that could generate libraries of plasmids without manual handling errors and the bottleneck of inserting assembled parts into cells and plating for clones.

Although integrating cell-free DNA assembly and *in vitro* cloning in the "build" process has alleviated several bottlenecks in synthetic biology workflow, engineering biological systems is most commonly performed using assembly and cloning techniques within cells. The process of DNA assembly usually requires combining DNA parts in either a single isothermal reaction using restriction enzymes, overlap-directed homology or using the powerful *in vivo* recombination capabilities of yeast. One of the major challenges when dealing with traditional DNA

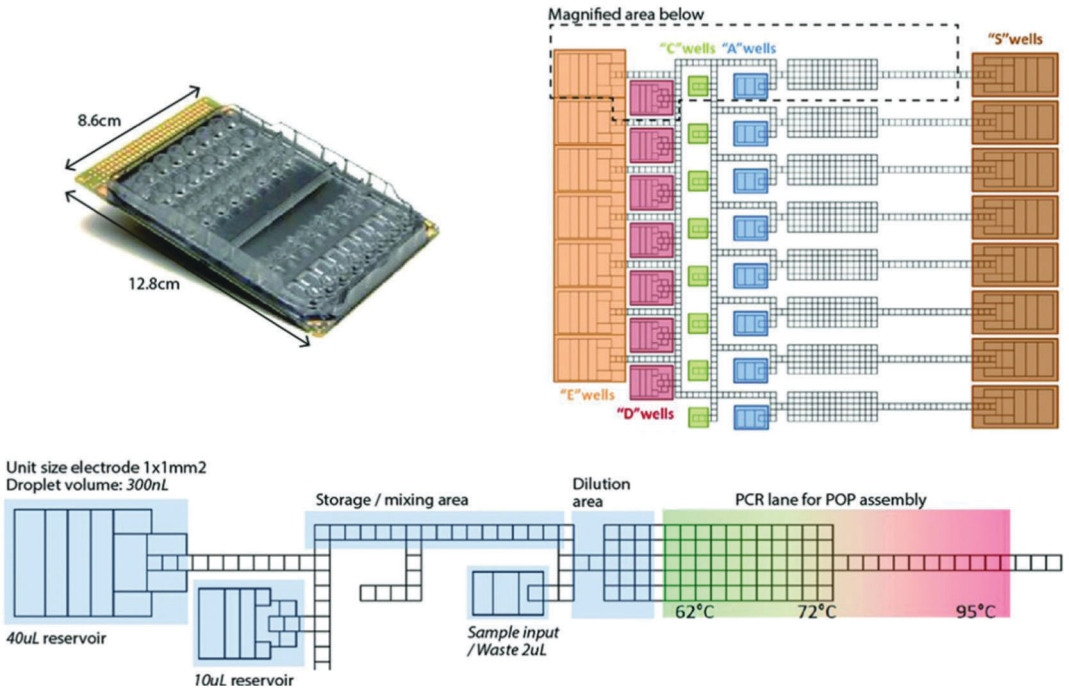

Fig. 1 Overview of the digital microfluidic design. Top-left: Image showing the fully assembled device with cartridge. Top-right: Dimensions and general layout of the cartridge. Bottom (magnified): Annotated schematics of a section of the microfluidics cartridge layout. Reservoirs are used to hold master mixes (*i.e.* enzymes), primers, and DNA oligos. The dilution zone is used to perform the serial dilutions for *in vitro* cloning. The PCR lane consists of three temperature zones: 62 °C, 72 °C, and 95 °C. Figure reused with permission from Yehezkel *et al.* [DOI: 10.1093/nar/gkv1087].

assembly mechanisms is the need for transformation (*i.e.* introducing DNA into the host strain). Traditional approaches for transformation such as electroporation or heat-shock treatment lack automated, standardized, and robust tools. While several microfluidic systems have previously been demonstrated for electroporation and heat-shock techniques, they do not address the required integration between transformation, plating colonies and selecting for single clones. In a recent paper in *ACS Synthetic Biology*, Gach *et al.* [DOI: 10.1021/acssynbio.6b00011] demonstrate a powerful "all-in-one" tool to automate heat-shock transformation that eliminates additional plating procedures. They used a digital microfluidic system to mix assembled plasmids with bacterial cells and integrated small millimeter-scale heaters to apply a heat-shock. To eliminate the "plating" step, the authors transferred the droplets containing the transformed cells into a microfluidic channel to initiate incubation and cell culture. The study tested bacterial transformation for a variety of different conditions (*e.g.*, DNA concentration, temperature changes, mixing frequency, and culture duration) and discovered that reducing the duration of the heat shock temperature at 42 °C gave rise to higher transformation efficiencies. Furthermore, the authors extended their work towards transforming other microbes (*i.e.* fungi and yeast) and determined on-chip culture was a necessity in achieving successful transformation since it allowed time for the cells to produce the antibiotic resistance proteins. This is the first chip to integrate both of these processes and is an automated and versatile method that has the potential to be integrated with other synthetic biology upstream (DNA assembly) or downstream processes (testing for functionality).

Incorporating multiple phases of the DBTL synthetic biology process into microfluidic platforms is extremely challenging as it involves performing several disparate tasks. Assembling DNA and automating transformation on-chip is a less well-established technology due to the complexity of the assay operation. To address this, a paper published recently in *Journal of Biological Engineering* by Linshiz and coworkers (DOI: 10.1186/s13036-016-0024-5) describes a multipurpose microfluidic platform that conducts DNA assembly in bacteria and yeast systems with on-chip transformation (DOI: 10.1021/acssynbio.5b00062). The authors demonstrate automated assembly of a ~750 bp construct combined with a pETBlue expression vector containing GFP to yield a ~6 kb plasmid (Fig. 2). Furthermore, automated transformation and *in vivo* DNA assembly in yeast were also exploited in this device, where a library of multiple variants and promoters with a common vector backbone were created to enable high-throughput DNA assembly and transformation. More importantly, this device enables the integration of both "build" and "test" into one platform. The authors were able to conduct two tests using their microfluidic chip. They showed (1) expression of constructed fluorescent protein GFP with different promoters, and (2) levels of isopentenol production using a colorimetric assay. Isopentenol is an excellent alternative source to fossil fuels and the authors used their platform to screen production of isopentenol using different inducer concentrations. A linear relationship between isopentenol concentration and inducer concentration is shown, which confirms the functionality of the engineered microbe. Although these are preliminary steps for "testing" an engineered cell, their platform shows the potential of implementing a complete synthetic biology design cycle.

As more strains are being "built", large scale analysis of the engineered organisms is needed for the "test" phase of the process. High-throughput assays, such as screens or selections that assay the target molecule are perfectly suited for strain optimization. While increased throughput may enable more

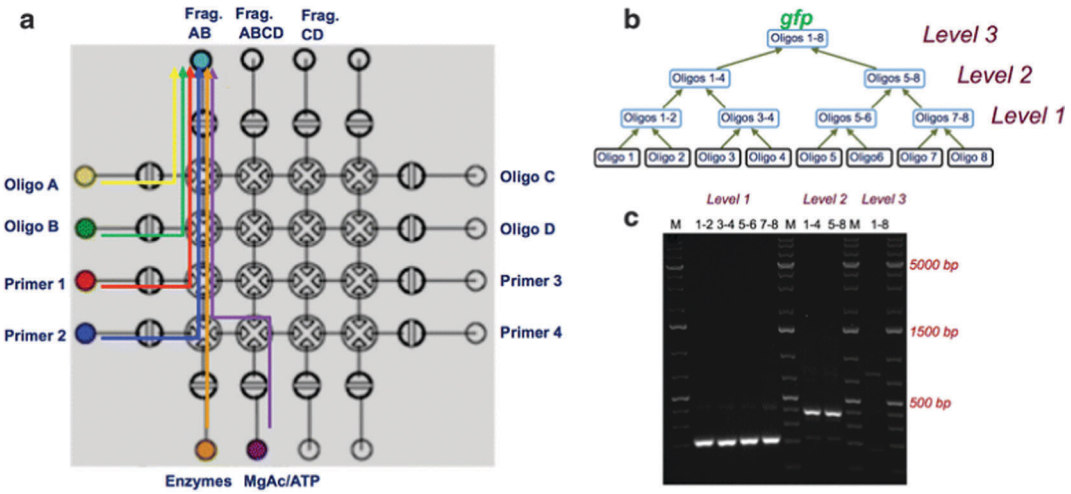

Fig. 2 Overview of the isothermal DNA construction on the valve-based microfluidic platform. (a) Overview of the basic DNA assembly steps on the microfluidics platform. Stage I. Two oligos A and B (as shown) and a mixture of enzymes are transferred to the reactor. Stage II. Primers 1 and 2, and a mixture of enzymes, are transferred to the reactor. Stage III. A mixture of ATP and magnesium acetate, and a mixture of enzymes, are transferred to the reactor. The temperature is increased to 38 °C, and the reaction is incubated for 15 min. As a result, an elongated and amplified DNA fragment AB is produced. (b) Hierarchical construction tree of seven separate synthesis reactions that result in the final product (gfp as shown). (c) Gel electrophoresis image of all the intermediates and the final gfp construct. Lanes as labelled by: M: GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific); level 1 (quarter) fragments: 1–2, 3–4, 5–6, 7–8; level 2 (half) fragments: 1–4 and 5–8; level 3 (full length gfp) fragment: 1–8. Figure reproduced from Linshiz *et al.* [DOI: 10.1186/s13036-016-0024-5] under the terms of the Creative Commons Attribution 4.0 International License.

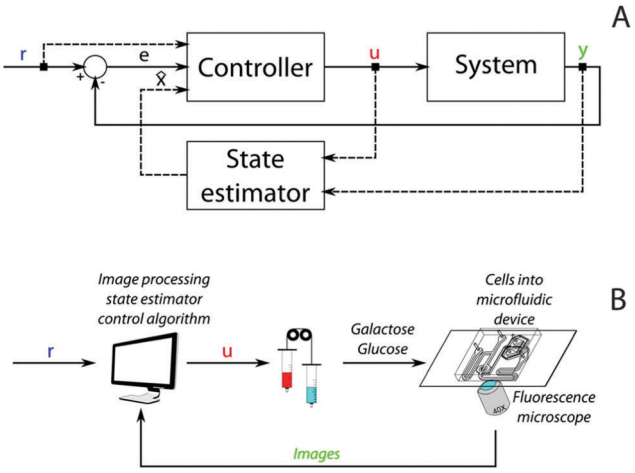

Fig. 3 Control scheme and experimental setup. (A) Generalized control scheme used to implement the three different control algorithms. (B) Experimental setup; a computer controls the entire platform which executes the control algorithm every sampling interval. Yeast cells are trapped on a microfluidic device and their fluorescence is analyzed by an inverted microscope. Images are acquired using a microscope to calculate the output, which calculates the new input u and controls the automated syringes to provide input (*i.e.* galactose or glucose) to the cells. Reprinted with permission from Fiore *et al.* [DOI: 10.1021/acssynbio.5b00135]. Copyright 2016 American Chemical Society.

strains to be analyzed and assayed, there remains an integrational gap between throughput in the "test" and "design and build" phases. Current methods to test functionality are typically low throughput like microscopy or mass spectrometry. These largely manual methods significantly slow down analysis and knowledge generation which will inform further DBTL cycles. Therefore, in addition to high-throughput screening, there needs to be an analysis tool that can perform real-time evaluation of the implemented designs that will rapidly inform future "design/build" decisions. Fiore and coworkers published a paper in *ACS Synthetic Biology* (DOI: 10.1021/acssynbio.5b00135) that successfully created a new microfluidic control loop that automatically reads an output signal and modulates the input signal such that the output reaches a desired level. Specifically, the authors implemented and tested three control algorithms to enable cells to express a desired constant fluorescence level (*i.e.* set-point control) or maintain a time-varying fluorescence level (*i.e.* signal tracking control). They used a channel-based microfluidic device to carry out the experiments, which trapped the yeast cells in a defined region and automate the delivery of reagents (inputs) that will increase or decrease the fluorescence levels of the yeast cells (Fig. 3). *In vivo* control experiments showed that using the difference between the output signal and the desired signal as the input gave the most consistent results in maintaining the fluorescence levels. Although this is only a proof-of-principle study, these approaches may ultimately be used to conduct real-time characterization of the designed biological parts in a single experiment. More importantly, it shows that the "test" and "learn" phases may be integrated on a platform, bringing us one step closer towards a completely integrated synthetic biology workflow.

The main goal of synthetic biology is to rapidly design biological systems following the DBTL workflow. Microfluidic platforms are promising approaches to accelerate the DBTL cycle since it increases throughput and automation, offers precise control of reagents and the spatial environment, and increases the scale of engineered biological systems. The papers described here represent a first-step towards accelerating the synthetic biology cycle and will continue to evolve and to mature towards routine tools used in laboratories and in industrial settings. The integration of microfluidics and synthetic biology is still in its infancy and while much work remains, these approaches seem extraordinarily promising in realizing the underlying dream of synthetic biology: to understand life, by building it.