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ABSTRACT
Microfluidics continue to gain traction as an inexpensive alternative to standard multi-well plate-based, and flow cytometry-based, assay platforms. These devices are especially useful for the types of ultra-high throughput screens needed for enzyme discovery applications where large numbers (>10^6) of unique samples must be screened rapidly[1]. Coupled with cell-free protein synthesis[2], microfluidics are being used to identify novel enzymes useful for a variety of applications with unprecedented speed. However, these devices are typically produced using PDMS, and require considerable infrastructure and artisanal skill to fabricate, limiting their accessibility. Likewise, enzyme hits obtained from a screen are often validated manually and would benefit from automation of downstream validation processes. To address these limitations, we propose a workflow which leverages software tools to automate the rapid design and fabrication of low-cost polycarbonate microfluidic devices for use as high-throughput screening platforms for enzyme discovery, as well as an automated DNA assembly tool to streamline validation of screening candidates. Using this workflow, we aim to identify novel oxidoreductase enzymes from environmental metagenomic DNA libraries, for use in electrochemical biosensors.

Keywords
Synthetic biology; cell-free; screening; microfluidics; CAD

1. INTRODUCTION
Environmental microbes possess an incredibly diverse set of enzymes and small molecules that they produce to thrive and interact with their environment. This resource can be tapped, using high-throughput functional screens, to discover novel biomolecules with numerous applications, from biosensing to biomanufacturing. These types of screens have relied heavily on lower throughput microtiter plate-based assays, as well as higher throughput flow cytometry, but microfluidics are emerging as a cheaper, faster, ultra-high throughput alternative[3]. These devices are often designed ad-hoc using graphic design software such as Adobe Illustrator, which does not allow easy parameterization of device components or iteration of designs. These devices are also typically fabricated using polydimethylsiloxane (PDMS), which requires specialized equipment and personnel training, limiting its accessibility in many academic labs. However, emerging software tools which automate the design of microfluidic devices from a high-level functional specification, as well as fabrication of devices using CNC-milled geometries in polycarbonate, are beginning to address this issue.

A second bottleneck in these enzyme screens lies in the downstream validation of positive hits. Putative enzymes identified in a screen are usually cloned into expression vectors, transformed into expression hosts such as *E. coli* or yeast, and used to produce and purify the protein for downstream analysis. Depending on the number of positive hits identified in a screen, this can lead to a non-trivial number of protein expression vectors that need to be cloned, especially when trying to assess the optimal position of the affinity tag itself (N- or C-term). Modular DNA assembly strategies like MoClo[4,5], together with software tools like mocloassembly.com[6] offer a scalable and automatable DNA assembly workflow that can address the need to generate combinatorial protein expression vectors via liquid handling robots. Coupled with cell-free protein synthesis, a larger number of expression vector variants can be assembled and tested with unprecedented speed.

2. MICROFLUIDIC DESIGN & FABRICATION
Microfluidic device design has largely been artisanal, with researchers often resorting to graphical design software tools to manually draw out microfluidic device geometries. This process makes it difficult to iterate on new designs since individual components of the device design must be changed manually and non-parametrically. Our software tools, however, allow us to specify high level microfluidic functionality parametrically, and fabricate devices via CNC milling in thermoplastics on the order of hours[7]. This is in stark contrast to more traditional PDMS-based device fabrication which can take days. This workflow has enabled us to design, fabricate, and test many iterations of devices to rapidly identify key geometry parameters important for functional screening of enzyme libraries. These parameters dictate processes like droplet generation rate & size, droplet merging & splitting, on-chip PCR, cell-free protein expression, and fluorescence/colorimetric-based droplet sorting.

3. CELL-FREE PROTEIN SYNTHESIS
Cell-free (CF) protein synthesis is experiencing a new renaissance as a versatile, fast, and inexpensive biological prototyping platform[2]. These CF mixes typically use cellular extracts from various organisms which provide the machinery necessary for *in-vitro* transcription and translation from a DNA template. This allows researchers to simply add DNA circuits which encode for proteins of interest, to achieve high amounts of expressed protein. This circumvents several steps in more traditional protein expression workflows where DNA for expression vectors must first be assembled, then transformed into the expression organism of choice. With CF mixes, even linear DNA fragments generated via PCR can be used as protein expression templates, further shortening prototyping time[8].

For enzyme screening we are using *E. coli* cell extracts to express protein within water-in-oil droplets generated in our microfluidic

device. At the initial point of droplet generation these droplets encapsulate single members of a microbial metagenomic DNA library which enables screening of individual members in high throughput. Screening reagents and enzyme substrates are later added to each droplet via droplet merging, and droplets which exhibit a fluorescent/colorimetric signal above a predefined threshold value are isolated for downstream sequencing. We are particularly interested in identifying novel oxidoreductase enzymes since they couple well with electrochemical sensors, similar to the ubiquitous blood glucose meter. This class of enzymes catalyzes the transfer of electrons from one molecule to another, oftentimes generating hydrogen peroxide as a byproduct of catalysis of an analyte of interest. To monitor enzyme activity in droplets we will use the fluorometric chemical probe Amplex UltraRed which is oxidized to a fluorescent product in the presence of hydrogen peroxide. Droplets exhibiting a fluorescence signal intensity above a predetermined threshold, presumably as a consequence of analyte degradation, will be sorted for downstream sequencing and validation.

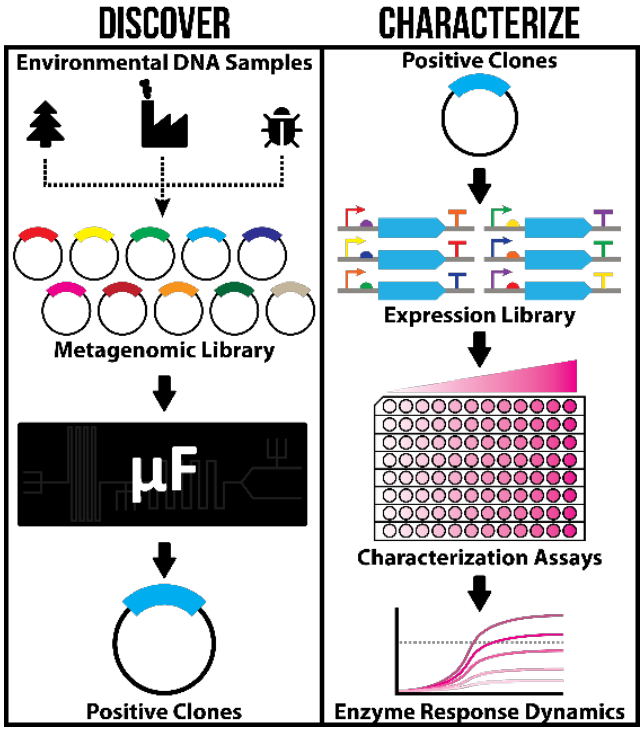

Figure 1. Automated microfluidic design & DNA assembly for rapid enzyme screening and downstream validation. [Discover] Using our software tools we can rapidly iterate across the microfluidic design space to generate variants of devices via CNC-milling. These devices take complex DNA libraries as an input, and selects droplets which respond to a molecule of interest. [Characterize] Positive hits from the screen are then sequenced, cloned into a MoClo destination vector, and expression libraries are automatically generated by liquid-handling robots using the software tool mocloassembly.com.

4. AUTOMATED DNA ASSEMBLY FOR ENZYME CHARACTERIZATION

In high-throughput screens, enzyme hits must be validated to test substrate specificity, and identify optimal parameters such as temperature, pH, and catalytic rate. This typically requires the generation of new DNA circuits for expression and purification of candidate enzymes, often via affinity chromatography. This can quickly become laborious as the number of hits from a screen increase, and the optimal position (N- or C-term) for a genetically encoded affinity tag is unknown. To address this scaling need, we are using Modular Cloning (MoClo) and the software tool mocloassembly.com to automatically build various instances of protein expression circuits from these screens via liquid handling robots. These circuits are then used in new CF reactions to rapidly generate protein for validation tests in an effort to quickly characterize new enzymes obtained from the functional screen.

5. ACKNOWLEDGMENTS

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