Mini-Review **Open Access**

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Are the biomedical sciences ready for synthetic biology?

https://doi.org/10.1515/bmc-2020-0003
received November 19, 2019; accepted January 2, 2020.

Abstract: The ability to construct a functional system from its individual components is foundational to understanding how it works. Synthetic biology is a broad field that draws from principles of engineering and computer science to create new biological systems or parts with novel function. While this has drawn well-deserved acclaim within the biotechnology community, application of synthetic biology methodologies to study biological systems has potential to fundamentally change how biomedical research is conducted by providing researchers with improved experimental control. While the concepts behind synthetic biology are not new, we present evidence supporting why the current research environment is conducive for integration of synthetic biology approaches within biomedical research. In this perspective we explore the idea of synthetic biology as a discovery science research tool and provide examples of both top-down and bottom-up approaches that have already been used to answer important physiology questions at both the organismal and molecular level.

Keywords: engineering approaches; design principles; mechanisms; bottom-up discovery; top-down discovery

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Introduction

Discovery and engineering are two research approaches commonly used to understand the natural world [1]. To date, much of our knowledge of physiology is derived from research using a discovery mindset, which focuses on unraveling a system to understand how it works. Individual components of a system are removed or modified, and observations of how the system changes are recorded and synthesized to create a model of the system. Due to the complexity of biological systems, oftentimes it is not possible to measure all effects simultaneously, thus this strategy can be riddled with potential side effects and limitations (technical, metrological, and biological). Yet, this approach is essential and the initial step on the path to mechanistic understanding as it is difficult to infer how a system works without first identifying the individual components. An engineer takes the opposite approach; using well-characterized individual components to build the system from scratch [2]. This approach is structured on well-known design principles and iterative improvements that are implemented over time. In theory, as the design evolves, we develop a greater understanding of how the overall system works. One common theme that defines the synthetic biology field is the application of this engineering mindset to biology. Biological systems have been redesigned by repackaging individual parts into novel combinations with unique or desirable properties, often with applied science implications. Scientists are only now beginning to take action and use synthetic biology as a tool for research discovery [1–6].

What is synthetic biology?

Synthetic biology is a broad field that is difficult to define. Functionally, it encompasses (1) the design and construction of new biological entities such as enzymes, genetic circuits (i.e. genetic logic gates that mimic electrical circuits) and cells, (2) the reconstruction of existing biological systems with the goal of understanding

mechanism, and/or (3) the redesign of existing biological systems. In the first point, synthetic biology is similar to synthetic organic chemistry [7]; its main goal is to harness biological processes and knowledge to create new materials or capabilities not found in nature. Those adopting the second and third point view synthetic biology as the next generation of biological reconstitution as its new approaches and capabilities allow researchers to ask deeper questions pertaining to molecular mechanisms, the origins of life, and the design of a minimal cell capable of harnessing the basic principles of life [8]. Another view is that synthetic biology represents the promise of systems biology, providing the tools necessary to interpret and interrogate the molecular insights derived from increasingly complex systems-level data. Regardless of definition, the tools and concepts afforded by synthetic biology have great potential to contribute to the biomedical sciences.

Top-down synthetic biology

The field of synthetic biology can be divided into two broad domains based on research strategy - top-down and bottom-up [9, 10]. Top-down approaches aim to harness biological phenomenon to engineer a predictable response to an input. Compared to traditional biomedical research approaches, this allows for improved experimental control over how biological processes are measured and/or modulated. To accomplish this, researchers often redesign and modify well-characterized biological components for their advantage. This could be expressing optogenetically sensitive ion channel to modulate neuron function, or designing a molecular sensor to monitor cell signaling events in real-time. Early examples of this methodology include the development of a genetic toggle switch [11] and a genetic oscillator in bacteria [12, 13]. More recently, researchers have focused on modifying organisms to have new capabilities by designing genetic circuits that integrate multiple signals via logic gates and couple sensing with a biological readout such as fluorescence or biological activity [14–17]. From a biotechnology viewpoint, considerable advancements in chimeric antigen receptor T cells (CAR-T) and other smart immunotherapies that contain self-regulating control elements have been made possible by similar top-down synthetic biology approaches [18, 19].

Examples of top-down synthetic biology

Many investigators within the physiology and biomedical sciences community have already begun to take advantage of top-down synthetic biology tools to advance their research. This is most evident in optogenetics. Pioneered by Deisseroth, Boyden, and colleagues [20–22], optogenetics is one example where top-down approaches have been implemented at both the organismal and molecular levels to control and modify signaling activities in individual cells [23, 24] as well as to gain insight into specific neuronal circuitry and brain activity function in rodents [23, 25] and zebrafish [26]. Recently, Swift *et al.* utilized channelrhodopsin to modulate *locus coeruleus* activity in rats and showed they can predictably control sleep spindle formation and consequently interfere with REM (rapid eye movement) and NREM (non rapid eye movement) sleep. They found that these changes directly impacted rat memory formation and that by preventing sleep spindle formation, rats were no longer able to reliably recall learned tasks [25]. These experiments would not have been possible without the use of their synthetic biology approach as the degree of accuracy and precision required to modulate a specific part of the brain is not feasible using other methods such as drug perturbation or genetic knockdown.

While the aforementioned optogenetic approaches have played a major role in linking neuron activity to specific actions, prior knowledge of the underlying neuronal circuitry is necessary. To overcome this limitation, multiple groups have devised strategies that utilize calcium flux (commonly observed upon neuron activation) and light activation to investigate activity-dependent neuronal processes. An initial iteration of this was CaMPARI, an engineered photo-switchable fluorescent protein that irreversibly switches from green to red light emission upon binding calcium (i.e. neuron activation) in the presence of light (Figure 1A) [27]. As a proof-of-concept, researchers expressed CaMPARI ubiquitously in the *Drosophila* brain. By applying light to specific brain regions while exposing flies to a panel of odors, researchers were able to identify specific neurons involved with the odor sensory response by looking for neurons that had switched from green to red fluorescence (Figure 1B, C) [27].

One limitation of this approach is that it cannot be coupled to a downstream effector. Recently, researchers in the Ting group developed a similar tool that couples neuron activity to a transcriptional response [28]. Similarly

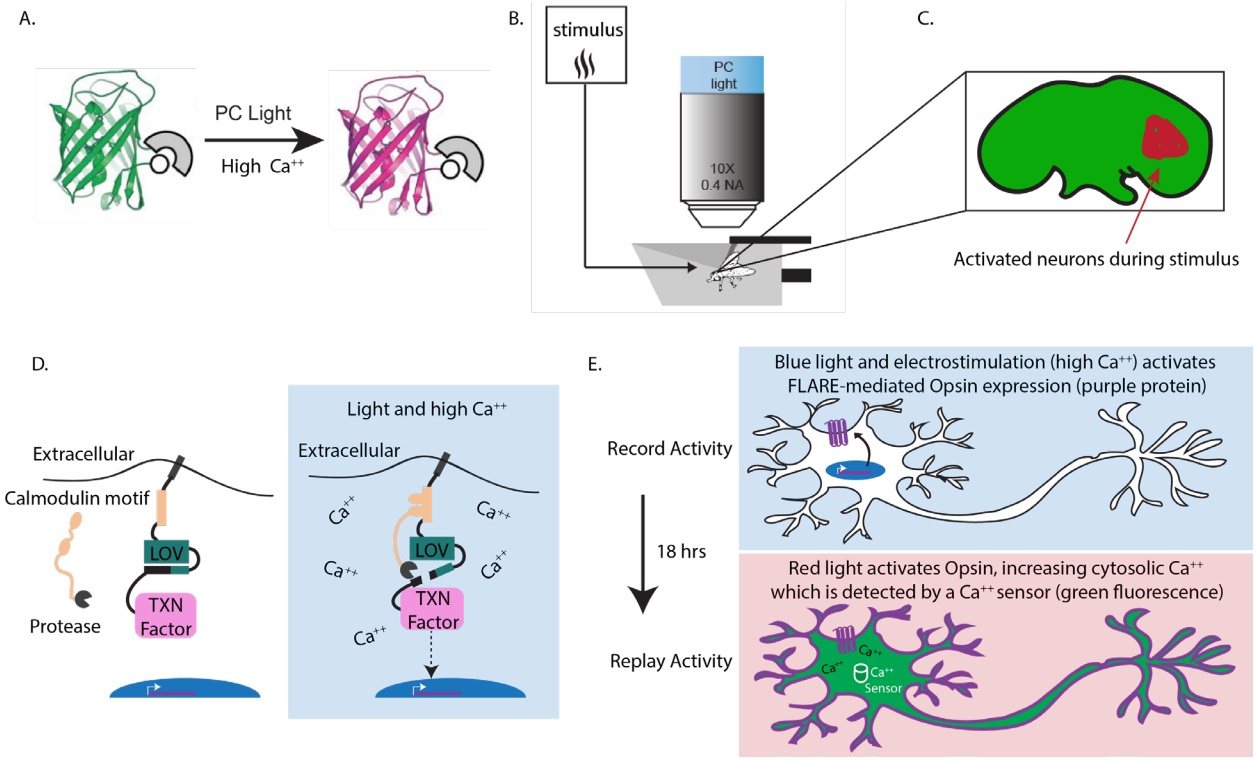

Figure 1: Recent top-down synthetic biology approaches to study biology. **A.** Schematic representation of CaMPARI technique. **B.** Proposed experimental design to use CaMPARI to study activity dependent neuronal circuitry in *Drosophila*. **C.** Cartoon of Drosophila brain illustrating potential result from CaMPARI study depicted in B. As is illustrated, the entire brain is fluorescently green however, upon activation of CaMPARI specific regions of the brain responsible for a response to a stimulus are switched to red fluorescence emission. **D.** Schematic of the FLARE technology. **E.** Proposed experimental strategy illustrating FLARE's record and replay ability in neurons. Panels for this figure were adapted from Fosque et al 2015 (panels A-C) [27] and Wang et al 2017 (panels D&E) [28].

to CaMPARI, FLARE (Fast Light- and Activity-Regulated Expression) requires high calcium and light for activity. However, unlike CaMPAIR where activation is solely linked to a change in fluorescence, FLARE activation leads to a change in downstream transcription. Briefly, neuron activation results in a calcium influx, which leads to the coupling of a TEV protease to the membrane tethered LOV domain-protected transcription factor (Figure 1D). At this time, steric hindrance prevents protease cleavage and functional transcription factor release (Figure 1D). However, upon light activation, this is relieved and protease cleavage results in the release of a functional transcription factor that is able to activate transcription of a desired target (Figure 1D) [28]. Compared to CaMPARI, FLARE allows researchers access to additional tools beyond fluorescent proteins to study downstream neuron activities. An interesting application of this is illustrated in Figure 1E. In this example, researchers coupled FLARE activity to the transcription of an Opsin (light sensitive protein) and after 18hrs Opsin was activated using red light to trigger an increase in calcium indicator fluorescence – indicative of an activated neuron (Figure 1E) [28]. This is a powerful proof-of-concept as FLARE allowed the researchers to not only identify activated neurons but also the ability to manipulate them afterwards [28].

In addition to optogenetic approaches, researchers recently developed a cellular memory system (MEMOIR) that records cellular history using a fluorescent readout [29]. One of the major limitations of lineage tracing experiments is that spatial information is lost. To overcome this issue, the researchers introduced a series of identical DNA sequences (i.e. coined scratchpads) that each had an associated unique barcode identification sequence. Next they used the stochastic gene editing capabilities of CRISPR-Cas9 to randomly and irreversibly mutate the originally identical scratch pads to generate of record of cellular history. As a readout, single molecule RNA fluorescence in situ hybridization probes (smFISH) were used to simultaneously detect scratchpads and unique barcodes in single cells allowing them to determine the lineage of individual cells within a mouse embryo stem cell population simultaneously via direct imaging

[29]. Future applications of this approach could include modifying this system to record other cellular events and could be accomplished by coupling CRISPR-Cas9 activity to biological sensor to measure a specific biological process.

At the single cell physiology level, several groups have engineered sensors and other synthetic constructs in mammalian cells to detect changes in cell signaling [30–33], as well as interrogate the role of protein localization in regulating these processes [34, 35]. One clever use of synthetic biology to study cell signaling dynamics, pattern formation, and differentiation was the development of the synthetic Notch (SynNotch) circuit [36]. Using the Notch receptor as a base (due to the simplicity and direct transcription response to ligand activation), the Lim lab replaced the extracellular ligand-binding motif and intracellular transcription-regulating motif with user-defined protein domains that recognize "synthetic ligands". The result was a chimeric receptor capable of sensing an exogenous extracellular signal and translating it into a desirable transcriptional output [36]. Due to the direct transcription response of these chimeric receptors, co-expressing different circuits is readily possible. Applications of this approach have facilitated research into cell signaling circuitry, pattern formation and cell differentiation in both cell culture and organoid models [36–41].

Bottom-up synthetic biology

In contrast to top-down approaches, bottom-up synthetic biology aims to assemble biological systems from scratch. Unlike natural systems where it can be difficult to predict experimental crosstalk and outcomes, bottom-up approaches provide superior experimental control by clearly defining experimental inputs and assumptions. Organoids are one area of bottom-up synthetic biology in which groups of cells rather than proteins are utilized as building blocks to reconstitute a synthetic tissue or organ [42]. Introducing control elements via synthetic gene circuits within organoids allows researchers the ability to more closely mimic developmental processes such as differentiation and pattern formation [3, 42, 43]. By comparing synthetic to natural processes, researchers are able to better understand the evolutionary origins and conserved mechanisms underlying these processes with greater control than methods that rely on systematically breaking the system to understand how components interact. Recent advancements using synthetic biology approaches within the organoid space are nicely review by Morsut [42] and Davies [3].

At the molecular level, recent research has focused on developing liposomes (asymmetric or symmetric phospholipid double emulsion vesicles) with defined input-output relationships [44, 45]. This is the initial phase in the development of synthetic organelles or cells and represents a major first step in overcoming the limitation of traditional bulk *in vitro* reconstitution experiments. In contrast to traditional approaches, by conducting experiments within liposomes, researchers are able to better recapitulate the spatiotemporal regulation of a cell or organelle, which is necessary for understanding more complicated biological mechanisms that have spatiotemporal elements. In addition to democratizing liposome generation, microfluidic devices have also been used to generate nested liposomes that more closely resemble cellular structures [46]. For more information, a nice review of the properties of liposomes and polymersomes as well as generation methods was recently published by Ridear et al. [47].

Improved reliability and utility of cell-free expression systems (i.e. coupled transcription-translation reactions (TXTL)) [48], are a second major advancement within bottom-up synthetic biology that has great potential to impact biomedical research. While cell-free lysates have been used for more than 50 years [49], recent advancements have made these approaches increasingly feasible [48]. Cell-free expression systems are composed of a crude cell lysate without the nucleus or plasma membrane fractions. Lysates are then supplemented with genetic circuits, RNA polymerase as well as metabolic building blocks (NTPs/Amino Acids) that allow for the simultaneous transcription and translation of a target gene (Figure 2A). The inclusion of lipid membranes and organelles, such as ER microsomes, in mammalian cell-free lysates have made the synthesis and functional reconstitution of difficult-to-purify cell membrane proteins increasingly possible (Figure 2A, B) [44]. Additionally, while *in vitro* reconstitution has been used for years [49], the ability to encapsulate cell-free lysate with specific genetic circuits, within liposomes or other artificial structures, have made this technology increasingly useful as a model to study minimal cells or membrane bound organelle-like structures with spatiotemporal resolution.

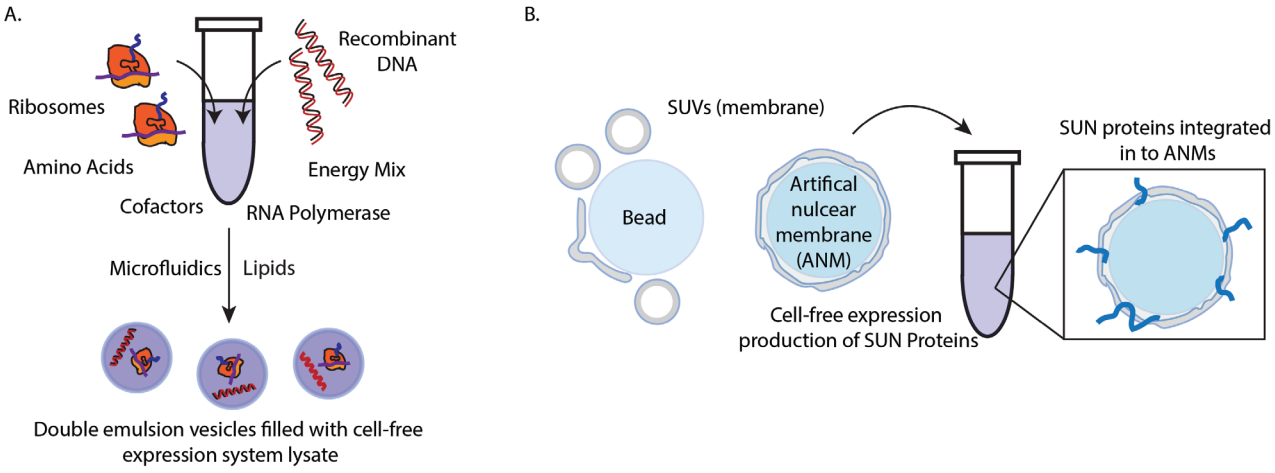

Figure 2: Bottom-up synthetic biology approaches to study biology. **A.** Cell-free expression system schematic. Cell lysate is collected from a desired cell type – bacterial or mammalian – and supplemented amino acids, energy mix (NTPs), cofactors, salts, RNA polymerase, and your desired cell-free expression system compatible plasmid. Incubation a specified temperature results in the production of the desired protein (encoded by the plasmid). Experiments can be conducted in bulk or encapsulated within double emulsion vesicles (i.e. liposomes) **B.** Experimental strategy for the investigation of the SUN1 and SUN2 topology on artificial nuclear membranes. Initially silica beads are incubated with SUVs to generate model artificial nuclear membranes (ANMs). Afterwards, ANMs are incubated with cell-free lysate containing plasmids encoding the SUN1 or SUN2 genes, which upon protein synthesis, leads to the functional reconstitution of the SUN proteins on ANMs. Panel B was adapted from Majumder et al 2018 [50].

Examples of bottom-up synthetic biology

Bottom-up synthetic biology approaches have been used to create artificial biological functions in liposomes that mirror natural biological processes. By encapsulating cell-free lysate within liposomes, researchers have been able to generate artificial input and output relationships within a synthetic vesicle. Early advances in this space have used mechanical sensing as a proof-of-principle application. In one example, cell-free lysate with a nested genetic circuit was encapsulated within phospholipid liposomes. The genetic circuit encoded a mechanosensitive channel membrane protein (MscL) and G-GECO, a genetically encoded calcium sensor. When liposomes were exposed to hypo-osmotic shock, an influx of calcium through activated MscL channels was reversibly detected by G-GECO fluorescence [44]. Due to the membrane lipid charge, diffusion of calcium into liposomes was solely dependent on MscL expression and osmotic shock [44]. This example highlights two important proof-of-principle concepts that exemplify of the utility of bottom-up synthetic biology. Firstly, it showcases that difficult-to-purify membrane proteins can be functionally produced using the cell-free expression system. Secondly, while this is a simple mechanism, it is an example of where an external stimulus is translated to intra-vesicular information. For many applications, this is critical as the ability to transfer information between different compartments is foundational to many biological systems.

Bottom-up synthetic biology has also been used to recapitulate natural processes to better understand the molecular mechanism of a particular process. Researchers recently used a mammalian cell-free expression system to study the nuclear membrane architecture of the LINC complex (Linker of Nucleoskeleton and Cytoskeleton) on artificial nuclear membranes (ANMs) not readily feasible using traditional cell biology techniques [50]. WT and mutant SUN1 and SUN2 proteins (components of LINC complex) were individually and co-expressed using mammalian cell-free lysate and allowed to integrate into ANMs (Figure 2B). Utilizing a protease protection assay and comparing WT and mutant receptors, the authors were able to reveal topology differences between SUN1 and SUN2 [50]. To test the functionality of this artificial model of the LINC complex, the authors reconstituted KASH-binding SUN proteins for the first time. This study showcases the potential of using synthetic platforms to better understand molecular mechanisms and highlights how recent advancements in synthetic biology are responsible for growth in this area.

Cell-free expression systems have also been used for rapid prototyping. Recent work from the Noireaux lab showcased the utility this technology to rapidly test CRISPR-Cas efficacy *in vitro* before verifying results in a more complex experimental system [51]. To do so, CRISPR-Cas machinery was expressed using the cell-free expression system with linear DNA targets and guide RNAs. Using a fluorescent readout for CRISPR efficacy they were able to quickly screen guide RNAs for efficacy. This approach was further used to characterize the effects of CRISPR inhibitory proteins as well as to quickly identify CRISPR PAM sequences without the need for transfection optimization - a common nuisance when characterizing new Cas proteins [51]. Furthermore, by removing an additional layer of variability when conducting experiments in cells or an organism, this approach is able to distinguish between CRISPR-Cas or application-specific issues such as transfection efficacy or unintended side effects.

Future applications of bottom-up synthetic biology may permit investigation of proteins that are lethal or to limit crosstalk between signaling cascades that are difficult to untangle. Additionally, immunodepletion of specific proteins or isolation of specific organelles from cell-free lysates (pioneered with *Xenopus* egg extracts [52, 53]) may provide researchers with the ability to more easily study how a protein or process interacts within the larger system without long-term side effects such as transcriptional reprogramming possible with knockdown or genetic knockout experiments.

Are we ready to adopt synthetic biology in basic science? Lessons from the rise of high-throughput sequencing technologies

As noted by others [1–5, 54], the biotechnology applications of synthetic biology only capture a subset of its scientific utility and that the transition from being a niche field to widely used scientific approach is necessary for the realization of its full potential. A similar evolution occurred with rise of high-throughput sequencing (genomics, RNA sequencing etc.). Although the human genome project was completed in the early 2000s, for many, high-throughput sequencing technologies remained out of reach because of limited access to technology, required expertise, and cost. As these methods have become increasingly accessible over the intervening years, many research hypotheses either include or have been influenced by RNA sequencing data sets and the power of this technology is beginning to be realized within the biomedical sciences.

There are many parallels between the research environment that enabled the rise of high-throughput sequencing technologies and the current environment in the synthetic biology. Facilitated by the development of software tools that aid in genetic circuits and sensors design [55, 56], new molecular cloning strategies [57] to generate difficult-to-construct plasmids, and increased utility of high-throughput screening technologies, have all enable researchers ability to test new ideas with unprecedented speed [56, 58]. Decreasing costs of these services via commercial suppliers have also played a role in this trend. Additionally, for bottom-up synthetic biology, reproducible and accessible cell-free expression system lysates are now widely accessible through both commercial suppliers (NEB PURExpress and Thermo Fisher Expressway) as well as via standardized protocols that make producing a variety of cell lysates in-house feasible for every lab [48, 59–65]. These developments have rapidly increased the user-friendliness of using synthetic biology methodologies for academic researchers. Given these recent advancements, it is surprising that synthetic biology approaches are not more commonly used research tools within the biomedical research community.

Challenges facing synthetic biology approaches within physiology

There are several challenges that we still face when applying synthetic biology methodologies to new systems. As is true with any engineering process or assay, standardization and component modularity remain areas of improvement. Central to this is the ability to measure things accurately and reliably. Recognizing the importance of measurement accuracy and reproducibility to synthetic biology, a new joint initiative between NIST, Stanford, and industry (JIMB - https://jimb.stanford.edu/) has recently been established with the goal of improving metrology within the biological sciences and initially focusing in synthetic biology and genomics [66].

For top-down approaches, off-target effects as well as specificity of reagents or genetic circuits are particularly important to consider. The leakiness of expression and switch-like behavior of sensors can have consequential implications on the accuracy of measurements. Researchers developing these technologies have spent significant time investigating these areas to limit the effects

of these challenges by including additional "insulators" to genetic circuits (commonly accounted for in sensor design software tools) as well as modeling the compatibility of multi-layer circuit designs [55, 58]. Recognizing these limitations, researchers have utilized irreversible methods events such as cleavage or DNA mutagenesis as readouts to decrease measurement stochasticity.

Areas of improvement in bottom-up synthetic biology can be further sub-divided. To improve liposome stability, the use of buffers with potential compatibility issues with other components remains an area of improvement. One report found that the use of polyvinyl alcohol precipitated some of the cell-free expression components in liposomes [67]. Oftentimes this limitation can be overcome by making predictable changes to buffer composition. Robustness of liposome generation protocols is also an area from improvement as the strengths and weaknesses of each approach need to be considered for each application. For cell-free expression systems, while the list of different cell-free lysates generated from different cell types continues to grow, it is still limited and can become an issue if cell type specific processes are studied. Additionally, the efficacy of the cell-free systems to synthesize all proteins (very large or difficult to fold) remains largely unknown and may require special modifications to ensure proper folding or modification for protein activity.

Conclusion

As stated by Elowitz and Lim, to understand life we need to create it [2]. An essential part of transitioning from observation to understanding is the ability to predict and harness knowledge to create or resynthesize important concepts. The potential to use synthetic biology methodologies to answer outstanding questions in physiology is great. Continually improving methodologies, decreasing investment, and access to resources have democratized synthetic biology and transformed what was initially a niche field, into a powerful tool to study biomedical science. The goal of this manuscript was to present evidence for why the rise of synthetic biology applications within physiology is imminent. Whether you study electrophysiology, pattern formation, stem cell differentiation, or cell and molecular physiology, it is time to consider synthetic biology as a useful technique to advance your science.

Acknowledgements: MSD thanks support from the National Science Foundation Graduate Research Fellowship Program and University of Michigan Rackham Pre-Doctoral Fellowship. APL acknowledges support from National Science Foundation (1612917, 1844132, and 1817909). Additionally, we would like to thank Mariana Schnell for help with making the figure illustrations. We would also like to thank Sagardip Majumder, Matthias Truttmann, Carol Elias, and Suzanne Moenter for constructive comments during the writing of the manuscript.

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