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Perspective: Solidifying the impact of cell-free synthetic biology through lyophilization

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ABSTRACT

Cell-free synthetic biology is an exciting and new branch in the field of synthetic biology. Based on *in vitro* transcription and translation systems, this application-focused domain builds on decades of cell-free biochemistry and protein expression to operate synthetic gene networks outside of cellular environments. This has brought new and perhaps even unexpected advantages. Chief among these is the ability to operate genetically encoded tools in a sterile and abiotic format. Recent work has extended this advantage by freeze-drying these cell-free systems into dried pellets or embedded paper-based reactions. Taken together, these new ideas have solved the longstanding challenge of how to deploy poised synthetic gene networks in a biosafe mode outside of the laboratory. There is significant excitement in the potential of this newfound venue and the community has begun to extend proof-of-concept demonstrations in important and creative ways. Here I explore these new efforts and provide my thoughts on the challenges and opportunities ahead for freeze-dried, cell-free synthetic biology.

1. Introduction

The field of cell-free synthetic biology has undergone tremendous growth in the past few years and is on track to become an important domain of application-based synthetic biology. This builds on years of pioneering efforts by many such as, Swartz, Ueda, Noireaux and Jewett, who established the fundamental "operating systems" for a diverse range of proof-of-concept applications [1–7]. As part of this applied effort, during my postdoctoral studies with Jim Collins, we showed that cell-free systems can be freeze-dried and hosted in porous matrices such as paper [8]. This, along with the work of others [9], has introduced the exciting possibility of deploying poised synthetic gene networks outside of the laboratory in a biosafe mode. These freeze-dried cell-free (FD-CF) reactions also have the important advantage of allowing for distribution and storage at room temperature, and thus avoid the need for a cold chain. In the time since these ideas were first reported, work from us and from across the community has extended this concept to other exciting applications [8,10–16], and there is a growing interest in using FD-CF reactions for the delivery of synthetic biology and biotechnology to new environments.

From my perspective, what makes FD-CF systems so compelling is their potential to extend access to healthcare through the development of freeze-dried platforms for de-centralized diagnostics [10] and the portable manufacturing of protein-based therapeutics [11]. A similar case could be made for many other applications where portable sensing and manufacturing could enhance real world capabilities (e.g. agriculture, national security). Research in general stands to benefit greatly from the potential for FD-CF to enable rapid prototyping of genetic constructs and to make on-demand, small-batch custom molecular reagents broadly accessible [11,15,17]. Below, I will highlight a subset of the most exciting work to date in cell-free synthetic biology and will consider how this new portable format can extend the reach of biotechnology to more diverse and mainstream applications. During these early days, as the field is still emerging, it is the collective effort of the community that will advance the field through the remaining technical challenges to practical implementation. However, given the nature of perspective articles, this is not an exhaustive review and so I apologize in advance to those whose work is not referenced.

Before continuing, I must first define the term "cell-free reaction" for the context of this synthetic biology perspective. Reference to such reactions has traditionally been limited to standard coupled transcription and translation systems; however, as I will discuss, the community has started to augment cell-free systems with other engineered biochemical systems and proteins. This includes the addition of repressors to ensure tight control of genetic switches in cell-free conditions [8,12], as well as the supplementation of cell-free systems with chaperones and other factors for transcription

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and translation [18–21]. The coupled transcription and translation systems themselves can be based on cell extracts, often from *E. coli* [1,2,5–7,21–23], but also increasingly from diverse bacterial sources [24–30] or eukaryotic systems such as mammalian [31–34], insect [35] and plant cells [36,37]. There are also recombinant cell-free systems, comprised of the purified enzymes of transcription and translation. While relatively costly, these recombinant systems offer the advantage of being biochemically defined, containing as few as 35 enzymes, and concomitantly providing low non-specific activity for gene circuits to operate [5,38,39]. Further, although not the focus here, other biochemically defined systems that include isothermal amplification (e.g. LAMP, RPA), as well as CRISPR-related proteins, offer new opportunities for the field to explore [10,13,40].

I will also begin by reviewing the concept of freeze-drying and the protocols that have been used in the preparation of FD-CF. Freeze-drying, also known as lyophilization, is the process of removing water from a frozen solid under vacuum through sublimation. In this process, water molecules are drawn from the solid ice phase, directly to the gas phase without passing through the liquid state. Perhaps most widely recognized as a method of preservation for the food industry (e.g. instant soup or noodles), freeze-drying is also heavily used by pharmaceutical industry to extend the shelf life of drugs. For the preparation of FD-CF, the process begins with freezing the assembled cell-free reactions and then transferring the samples to the high vacuum of a freeze-drier (e.g. Labconco, VirTis) until dry. Once in place, the vacuum will prevent thawing of the samples, but it is important that they remain frozen during this transition. The protocol is simple and is generally similar across the literature; however, there are some variations. In our papers [8,10,11], and some by others [14,16], cell-free reactions were flash frozen in liquid nitrogen (−196 °C) and then placed in a freeze-drier for as little as three hours to overnight. More complex protocols have also been reported that include repeated cycles of shell freezing the cell-free system in an ethanol bath (−40 °C) followed by 20-minute drying periods on the freeze drier [9]. While all of these approaches appear to yield productive FD-CF systems, there has yet to be a careful evaluation that establishes best practices. Such an effort could lead to greater standardization and reproducibility between studies; this theme will be explored more fully in the "Challenges and Opportunities" section below.

2. Current state of the field

2.1. Sensing and diagnostics

Work to date with FD-CF systems has exploited the incredible capacity of biology for sensing and synthesis. Our work began with a focus on the former of these capabilities with the development of FD-CF paper-based genetic switches that could be actuated with small molecules or RNA inducers [8]. The freeze-dried, paper-based reactions were also used to demonstrate the potential for programmable *in vitro* diagnostics with antibiotic resistance gene and strain-specific Ebola virus sensors. Utilizing toehold switches [41], these diagnostic sensors could recognize ~30 bp sequences and could be designed to use a pathogen's genome as a barcode for either diagnosis or identification of other clinically relevant features.

While the potential for deploying such capabilities out of the laboratory was exciting, the sensitivity of these first paper-based systems was not sufficient to detect clinically relevant concentrations of pathogens from samples. In the year that followed, we worked to improve the system's sensitivity and developed field-ready methods for sample preparation. By placing an isothermal amplification step for target sequences ahead of the sensing step by the toehold switches, we were able to improve the detection threshold by several orders of magnitude (~10^6).

These advances were put into use in early 2016, when the outbreak of the Zika virus in Brazil prompted us to apply our improved system for detection of the virus. Here, by merging our paper-based toehold switch reactions with an upstream isothermal amplification step, the resulting biomolecular platform was able to detect clinically relevant concentrations of Zika viral RNA (1–3 femtomolar) directly from infected macaque serum. Furthermore, our platform could distinguish Zika from closely-related Dengue virus sequences [10]. These diagnostic capabilities were also augmented by the development of a CRISPR/Cas9-based module that could discriminate between Zika genotypes with single-base resolution. These sensors were designed with a colorimetric output that allowed users to interpret the results either with their naked eyes or with the use of a purpose-built portable electronic optical reader.

Other groups have also begun to adopt the FD-CF format as a venue for portable diagnostics. An early demonstration of the paper-based scheme included a FD-CF sensor for detecting antibiotic contamination in water supplies. Here authors exploited the inhibition of protein synthesis that many antibiotics share (e.g. macrolides, aminoglycosides, tetracycline, chloramphenicol) to regulate the production of a colorimetric reporter [14]. In work from the Freemont lab, cell-free systems have also been used for the detection of *Pseudomonas aeruginosa* infection from patient samples using gene circuit-based molecular recognition of a pathogen-related quorum molecule, 3-oxo-C12-HSL [42]. This biosensor, which aimed to tackle the challenge of monitoring *P. aeruginosa* infection in cystic fibrosis patients, was capable of quantitative detection at nanomolar concentrations and the results mapped impressively well with parallel measurements using mass spectrometry. This work is notable for its successful screening of patient sputum samples, which required the challenging task of incorporating a preparation step to isolate the analyte from the thick and glycoprotein-rich sample matrix.

Other recent cell-free biosensor efforts have focused on the identification of chemicals that disrupt human endocrine receptors (hERβ or hTHβ) [16,43]. These compounds, such as the contaminant bisphenol A, are associated with a wide range of health concerns that include cancer and developmental problems [44]. Here authors used cell-free reactions to support the on-demand synthesis of an allosteric fusion protein that switches into an active conformation when exposed to water samples containing such contaminants. The system was able to detect target analytes (low nM) from diluted samples of blood, urine and waste water, and could be run from the FD-CF state [16,43]. Based on the mercury resistance operon of transposon Tn21, another group has developed a FD-CF system to also monitor water quality. Here using FD-CF, the transcriptional repressor MerR was used to create mercury(II)-responsive expression of a LacZ reporter gene [12].

In most cases, these sensor demonstrations come from research groups that also have considerable experience in building whole-cell biosensors, and this highlights an important feature of cell-free synthetic biology in general. While not always portable between systems, many synthetic biology applications are compatible with either environment. This potential for tool sharing is good news for both cell-free and cell-based communities and will hopefully lead to more practical implementation of technologies on both sides. Interestingly, similar efforts have aimed to bring greater portability to cell-based tools [45,46]. An example of such work has been demonstrated by the Daunert lab; here, *E.coli*-hosted sensors for the quorum molecule AHL were dried onto paper strips [47]. Upon rehydration, cells responded to the presence of target

molecules with the production of LacZ and a corresponding colorimetric response.

2.2. Synthesis/manufacturing

In parallel with cell-free sensing applications, there has been an effort to use FD-CF for the portable manufacturing of biomolecules [11,15,48]. This work follows on the recent trend of de-centralized manufacturing with fabrication tools such as 3D printing and laser cutting, and promises to similarly distribute the capability of synthesis at the molecular level. Some of the most compelling applications involve the production of protein-based therapeutics (e.g. vaccines and antibodies), which generally require a continuous cold chain for distribution. If brought to scale, this capacity could bring a sea change to global health efforts, public health emergency response (e.g. to infectious diseases) and small-batch production for research or personalized medicine.

The concept of using FD-CF reactions for biomanufacturing owes its origin to cell-based recombinant protein expression and the bioproduction of commodities. The production of recombinant insulin, which has replaced harvesting from animal pancreases, serves as an exemplar of cell-based and centralized manufacturing for many pharmaceuticals and biotechnological products [49–51]. However, as mentioned, challenges to distribution, the unpredictable and urgent need to vaccinate in the face of an outbreak and the customized nature of personalized medicine highlight the need for a mode of de-centralized biomanufacturing. Work by us and others in the community has demonstrated that FD-CF can be used to meet this need through small-pot reactions [11,15,48]. In our proof-of-concept work, we generated over 50 molecules and validated the activity of many of these products in immunoassays, cell-based assays and *in vivo* animal experiments [11]. Perhaps the highlight of this work was the demonstration that a vaccine antigen for diphtheria could successfully induce immunization in mice, along with a corresponding demonstration of scale-up to 33 human doses for a cost of $10 to $18/dose [11].

The Bundy group has demonstrated the production of the therapeutic enzyme onconase in FD-CF, which highlights the potential of FD-CF platforms for the production of normally cytotoxic molecules that pose challenges to conventional cell-based production [15]. This follows on the long recognized potential for cell-free systems in general as an important tool for the production of toxic components [52,53]. More recently, another effort demonstrated the production of pyocin S5 in FD-CF reactions [48]. Pyocins are narrow spectrum bacteriocins from *P. aeruginosa*. Previous *E. coli*-based work demonstrated that these toxic proteins could be used to kill planktonic *P. aeruginosa* and reduce the production of related biofilms [54]. Here, work from the Wolfe group showed that a similar benefit could be provided through the biosafe FD-CF format.

Pioneering laboratories, together with the community as a whole, have demonstrated the value of cell-free expression through the production of hundreds of proteins [18] including, among many others, vaccine antigens [55,56], single- and multi-domain antibodies [18,57–60], antibody conjugates [11], cytokines [57], peptides [61,62] and membrane proteins [63]. These capabilities have also spawned commercial efforts focused on centralized, cell-free manufacturing through start-ups like Greenlight Biosciences and Sutro Biopharma, which have involved partnerships with large pharmaceutical companies such as Merck and Celgene. New players in the commercial space include Synvitrobio and Cell-Free Tech and we can safely expect more to come. With this growing capacity, it seems reasonable to assume that — when needed — this impressive body of work could be translated into de-centralized production through FD-CF formats.

In the context of biomanufacturing, it is also interesting to look at the potential of cell-free systems for self-assembling macromolecular complexes. Products of self-assembly in nature include flagella [64], ribosomes and viruses [65], and many nanobiotechnology efforts have started to explore how self-assembly can be used to create nano-scale tools [66,67]. Given the self-assembling nature of these complexes, the emergent structures simply come together in the presence of sub-units and so researchers have exploited cell-free reactions to essentially create nano-structures directly from DNA inputs alone. Fascinating work has demonstrated the cell-free synthesis of Norwalk virus capsids [68] and bacteriophages T4, T7, ϕ174- and MS2 [69–71] in cell-free systems. The most noteworthy of these are the cell-free synthesis of infectious bacteriophages T7 and T4 from genomic DNA, particularly considering T4's sizeable genome. T4 phage has a genome size of approximately 169 kbp and the particles themselves are comprised of 1500 proteins from 50 genes. Not only does the making of these infective particles require full replication of the genome, it also requires error-free structural assembly and loading of this replicated genome [65,69]. Some other macromolecular assemblies achieved in cell-free systems include those of ATP synthase (25 subunits) [72], *E. coli* RNA polymerase (5 subunits) [73] and the human T-cell receptor (7 subunits) [74].

These macromolecular systems have also been used to make novel assemblies. This includes the cell-free expression of T4 gp15 and gp18 proteins, which have been used to make nano-scale hexameric rings and nanotubes [75]. This concept has been extended further to the cell-free manufacturing of virus-like particles (VLPs) [76–79], which are an emerging class of therapeutics that can be used as vaccines [80], targeted drug delivery vehicles [66], imaging agents and catalysts [80]. These non-infective nanoparticles can be rationally designed to a wide variety of specifications. For example, by selecting different capsid proteins, the diameter of particles can be controlled with precision (between 20 nm and 64 nm) [66,81]. The residues of the VLPs can also be modified using click chemistry, allowing the rational assembly of hybrid assemblies. This includes conjugation of VLPs to growth factors, fluorescent dyes, DNA, cell-specific targeting moieties like antibodies and stealth agents such as PEG [82,83]. Being derived from viruses, VLPs are of course hollow and so can also be loaded internally with cargo (e.g. small molecule drugs, RNAs, proteins and peptides) [66]. With such a modular platform and diverse set of potential applications, FD-CF may provide an interesting mechanism for on-demand, on-site synthesis of VLPs. One can envision FD-CF ultimately helping extend distribution of such future therapeutics to the domains of global health, emergency response, personalized medicine and research.

3. Challenges and opportunities

These early, but compelling, examples suggest the application of FD-CF could bring profound changes to how the benefits of synthetic biology, and biotechnology in general, can be distributed. The potential to extend the accessibility of these new technologies is especially exciting for applications where cost, urgency or the need for personalization limit feasibility. To realize these envisioned benefits, the field needs to tackle technical challenges related to making the biosafe FD-CF format more accessible, scalable, durable and efficient. Below I briefly highlight a few exciting efforts that are "moving the ball forward".

I will begin with one of the challenges where the field is already making excellent progress: improving the accessibility and scalability of cell-free reactions. Most first generation high-yielding *E. coli* extract-based cell-free protocols relied on bead mills or high-pressure homogenizers to crack cells open for lysate extraction [2,84,85]. These labor-intensive and costly methods made cell-free preparation slow and meant that few had the opportunity to explore potential applications. With the goal of making the

cell extract protocols more accessible and scalable, groups started exploring alternative methods of lysis [86]. Sonication emerged as a strong alternative to conventional methods and was fully operationalized in Kwon and Jewett where they demonstrated high productivity of cell-free extracts from as large as 10 L *E. coli* cultures [87]. Sonication also allows for rapid prototyping of small volumes, which facilitates screening of alternate strains or mutants. We use this method ourselves in the lab and have had great success with a relatively small capital investment.

This trend toward more accessible and scalable lysis methods was taken a step further last year with the development of autolysis-based cell-free systems [12]. Here a plasmid encoding phage lambda gene R is introduced into *E. coli* to provide low constitutive expression. Gene R encodes a lysin enzyme that degrades the cell wall; without the companion phage lambda gene S (a pore forming holin) cells remain intact and productive [88]. The authors cleverly then use a freeze-thaw cycle to breach the inner bacterial membrane, allowing the lysin to enter the periplasmic space and degrade the cell wall, which triggers lysis. The resulting extracts have been shown to have higher productivity than a reference commercial S30 extract. Remarkably, transcription/translation activity also appears to be present in non-processed lysin-containing whole cells that are simply freeze-dried onto paper. With autolysate cost at an estimated $1/mL [12], one can start envisioning highly distributed and productive applications where FD-CF input costs are almost incidental.

There are, of course, also decades of biochemical, molecular and application-based improvements that have contributed to the advancement of high-yielding FD-CF. Thus far, *E. coli*-based systems have been created that are capable producing GFP in the range of 2.3 mg/mL or higher in batch mode and over 6 mg/mL in semi-continuous mode [23]. For more challenging proteins, such as multi-domain or eukaryotic proteins, published and ongoing efforts are incorporating, among many other features, chaperones [18–21], *in vitro* glycosylation [89], biohybrids [90–92] and diverse cell sources of cell lysate (e.g. bacterial, plant, mammalian) [1,22–29,31–33,35–37] for the production of next generation FD-CF. Many opportunities remain to be explored in this space and it leaves one to wonder what the limits of cell-free production may be. It also remains to be seen whether given sufficient time, it would be possible for cell-free systems to match or exceed the high reported yields (17.5 mg/mL [93],) from some cell-based recombinant expression systems.

The discussion of cell-based systems also provides an opportunity to briefly address the purification of products generated by FD-CF. While not necessary for nasal, oral or dermal delivery of therapeutics, intravenous injection-based delivery will require a purification scheme and so, as a field, we are faced with the challenge of how to isolate products in a simple, robust and low cost manner. Our proof-of-concept work successfully demonstrated the use of regenerated amorphous cellulose as a matrix to purify target proteins fused to a cellulose binding domain (CBM3) [11]. Such a cellulose-based matrix is orders of magnitude less expensive than convention purification matrices like Ni-NTA or anti-FLAG beads, but much work remains on translating this type of concept to a field-ready state.

Another key challenge to the implementation of FD-CF is durability and field-ready storage. Based on personal experience and conversations with others, recombinant and extract-based FD-CF may have differential stability, with the former being more readily stabilized. This trend seems to be reflected in the literature as well. We have found that recombinant-based FD-CF (PURExpress, NEB) can remain active after a year of storage at room temperature [8], while I have not found published stability data from extract-based FD-CF beyond five months [9,48]. With the affordability of extract-based systems, development of protocols to extend their stability will be critical for advancement of the field. With this in mind, it is interesting to note that a review of related patents suggests that the use of certain cyroprotectants may enable stabilization of extract-based FD-CF, including the complete stabilization of activity during two years of storage at room temperature [94].

A related challenge is that the freeze-drying process will likely serve as a barrier to scale up and wider adoption of cell-free technologies. While large-scale lyophilization for bulk production is technically possible, it requires a significant capital investment. To address this issue, one group has explored substituting freeze-drying with drying under ambient conditions. This method uses the disaccharide trehalose, found in the desiccation-hardy organism water bear, as a cryoprotectant [48]. By doing so, authors were able to dry cell-free reactions under ambient conditions without freeze-drying equipment. Moreover, the resulting reactions remained active after storage at elevated temperatures (37 °C) for months and could also tolerate storage under atmospheric oxygen and humidity. Further exploration of cryoprotectants and their impact on post-preservation stability is a standing challenge for the field and one that promises to greatly advance the goal of low-cost, scalable methods for the distribution of next generation molecular tools.

Finally, the emerging field of cell-free synthetic biology in this early stage has an opportunity to standardize procedures, testing and reporting. As touched on above in the context of lyophilization protocols, such standardization holds great potential to accelerate advancement of the field by reducing duplication of efforts and ensuring reproducibility between labs. While significant work related to parameter searching has been done by many, there is opportunity to curate and consolidate the community experience, perhaps in annual or biennial technical reports. Key features for review, among others, could include preparation of cell-free systems, lyophilization or parallel drying methodologies, reagent sources, materials preparation (e.g. paper substrate), storage conditions (e.g. N_2(g), desiccant), performance metrics for common reporters and standardization of experimental procedures.

4. Conclusion

As population growth continues, issues like food and health security are becoming important challenges for the world. The umbrella of biotechnology, which includes synthetic biology, offers to bring the weight of fundamental biology, chemistry and engineering to bear on these problems. While cell-based solutions will certainly play a key role, de-centralized modes of delivering these benefits will ensure distributed capabilities and greater access. With this aspiration in mind, it will be essential for us to venture beyond the bench and test our FD-CF sensors and manufacturing platforms directly in the field and with patients. Such efforts will not only improve our science, but will also make sure that the benefits of our work are realized. Finally, my hope is that this biosafe FD-CF platform will also bring another key resource to face these challenges: the minds of the next generation of bioengineers. By harnessing FD-CF features for education, this format also holds a great opportunity to bring synthetic biology to the classroom; much in the same way personal computers have introduced students to coding. With the combined creativity of young students, the incredible progress being made in FD-CF applications and advances toward FD-CF access and scalability, I see an exciting future for the field of cell-free synthetic biology.

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