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Cell-free synthetic biology for *in vitro* prototype engineering

Simon J Moore, James T MacDonald and Paul S Freemont

Centre for Synthetic Biology and Innovation, Department of Medicine, South Kensington Campus, London, United Kingdom

All correspondence should be addressed to Paul Freemont (p.freemont@imperial.ac.uk)

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Abstract

Cell-free transcription-translation is an expanding field in synthetic biology as a rapid prototyping platform for blueprinting the design of synthetic biological devices. Exemplar efforts include translation of prototype designs into medical test-kits for on-site identification of viruses (Zika, Ebola), whilst gene circuit cascades can be tested, debugged and re-designed within rapid turnover times. Coupled with mathematical modelling, this discipline lends itself towards the precision engineering of new synthetic life. The next stages of cell-free look set to unlock new microbial hosts that remain slow to engineer and unsuited to rapid iterative design cycles. It is hoped that the development of such systems will provide new tools to aid the transition from cell-free prototype designs to functioning synthetic genetic circuits and engineered natural product pathways in living cells.

Introduction

Cell-free systems represent a historically important component during the founding of the field of biochemistry. Ever since the pioneering efforts of the Nobel laureate Eduard Buchner (Nobel Prize in Chemistry in 1907) and his discovery of fermentation in yeast cell-extracts[1], cell-free systems have been repurposed towards the further understanding of biological processes. Indeed, arguably one of the most notable biological discoveries of the 20th century was the unravelling of the genetic code by Marshall Nirenberg and colleagues[2–4], which was underpinned by the use of *E. coli* cell-extracts to study coupled transcription-translation (TX-TL). Together with Har Khorana and Robert Holley this resulted in a shared Novel Prize in Physiology or Medicine in 1968. On this theme, the efforts of Alfred Goldberg led to the unveiling of an ATP-dependent mechanism for protein degradation by ubiquitin in a mammalian cell-free system[5].

Cell-Free Synthetic Biology

Today, with the rise of synthetic biology and the design and construction of synthetic life[6], cell-free systems have yet again found a niche towards the understanding of biological networks and biosynthetic pathways[7,8]. Indeed, by isolating the cellular components of core metabolism and the TX-TL network within a test-tube, this allows the synthetic biologist to study systems without the regulatory constraints and limitations of a dividing, evolving or adapting living cell. This mini-review summarises the efforts of recent cell-free synthetic biology research and the opportunities it provides for the future.

Cell-free coupled TX-TL uses the core machinery of RNA polymerase holoenzyme, the translation apparatus (ribosomes, tRNA-synthetases and translation factors) and energy regeneration enzymes to amplify a set of DNA instructions into the target protein(s) of choice (Figure 1A). Therefore, the study of cell-free presents an enticing opportunity to the synthetic biologist to design and engineer living systems from the bottom-up as prototype designs. Exemplar demonstrations of cell-free synthetic biology include their use as biomolecular 'breadboards'[9,10], healthcare biosensors[11,12] and enzyme cascades[13–16]. Coupled with the aid of computational

design approaches[10,17–19], these early developments in cell-free synthetic biology will aid the engineering of more complex systems. We shall now summarise the cell-free platforms available, with a specific focus to its use in prototyping genetic circuits.

E. coli cell-free - PURE or crude cell-extract?

The choice of a well characterised cell-free system almost entirely resides with *E. coli* platforms, which are based on either a crude cell-extract[20–23] or a system of purified recombinant elements (PURE)[24,25]. A vital area of importance to cell-free systems is the process of energy regeneration, which represents the major cost factor and limitation for both the PURE and cell-extract based routes. Firstly, transcription requires nucleotide triphosphates (NTPs - ATP, UTP, GTP, CTP), with each mRNA transcript utilised multiple times for protein synthesis[26]. Protein translation is the major energy cost factor and requires two high energy phosphate bond equivalents for tRNA aminoacylation and two high energy phosphate bond equivalents per peptide bond formed[27]. In addition, a single high energy phosphate bond equivalent is required for each of the initiation and termination steps. Therefore, a small sized 25 kDa protein costs approximately 35-44 mM of ATP to synthesise 1 mg/mL under batch synthesis[27].

Firstly, in respect to the PURE system[25], this includes the purified components (108 in total) of the entire *E. coli* translation machinery including ribosomes, 22 tRNA synthetases, initiation factors, elongation, release and termination factors, which when combined with T7 RNA polymerase, tRNA, energy regeneration enzymes, substrates (amino acids, creatine phosphate) and synthetic DNA instructions, this reconstitutes the entire TX-TL network within a test-tube. This rather remarkable engineering feat is commercially available as the PURExpress® kit (New England Biolabs). Whilst the high cost of the system prohibits scaled-up applications, a variety of cell-free researchers use the PURExpress® system to study the dynamics and kinetics of TX-TL[24,28–32]. The major advantage of the PURE system is it's high efficiency due to an absence of competing side-reactions such as nonspecific phosphatases[24], which rapidly degrade the energy source.

In contrast, a crude cell-extract provides an inexpensive route to protein synthesis. In addition, unlike the PURE system, reactions are scalable into high-volume

fermentation conditions [33,34]. However, with the presence of other primary and secondary pathway enzymes (phosphatases, amino acid biosynthesis), this leads to undesirable side reactions during catabolism of the starting energy source. Importantly, based on improvements in energy regeneration schemes by the groups of Swartz[27,35–37], Jewett[38,39] and Noireaux[40–42], powerful cell-extract based batch systems can now reach recombinant protein yields of up to 2.34 mg/mL[33,40], whilst extended steady-state synthesis can be achieved through the use of a semi-permeable dialysis membrane device, thus elevating protein yields up to 6 mg/mL[40]. In addition, inexpensive energy sources such as glucose[27], glutamate[33], maltose/maltodextrin[41] and succinate[43] can be used to reduce the cost of energy regeneration in cell-free systems (Figure 1B). To this end, various cell-extract protocols have been developed and are based on the harvesting of cells at exponential phase, when typically intracellular translation is at its peak. Standardised protocols involve washing the cells, mechanical lysis[38] and activation of the extract through a run-off reaction, a process believed to degrade endogenous mRNA transcripts and genomic DNA that can reduce cell-free translation efficiency[23]. Additional dialysis can also remove inhibitory small molecules, but the requirement of this varies between *E. coli* strains and user preference[38].

Cell-Free Prototyping

Cell-free TX-TL provides the ability to study gene expression in isolation with the timescale from DNA to experimental results taking a few hours[10,44,45], whereas depending on the host chassis, an *in vivo* based approach can take several days to weeks. Thus, cell-free provides a prototyping approach (Figure 2) for rapid cycling between circuit experimental design and debugging[9]. For gene expression, to enable cell-free prototyping, fluorescence tags that monitor both mRNA and protein synthesis can be studied in real-time[29,40,46,47], thus providing dual microscale quantitative data of the TX-TL cascade that can be difficult to achieve within in a living cell. In addition, the starting concentration of the substrates and relative enzyme stoichiometry can be determined[40], thus aiding system identification and mathematical modelling of the chemical reaction dynamics[9,17,18]. These models can be used to inform future circuit designs as part of an iterative design process. *In vivo*, the cellular components are constantly being diluted by cell growth and division as

well as being synthesised. In contrast, batch cell-free reactions are closed systems starting with a limited set of initial resources. These differences make direct comparisons between *in vivo* and cell-free reaction dynamics of complex multi-promoter circuits difficult. One method to combine the rapid prototyping benefits of cell-free while emulating the conditions found in living cells is to use microfluidic devices to allow the continuous dilution and replenishment of the reaction substrates that extend the steady-state of protein synthesis up to 30 hours[29]. This method was used to design three and five node ring oscillators in cell-free, based on the use of PCR products to test initial prototypes, before a model-inspired design-build-test cycle led to circuit designs that were also found to function in cells[48]. In this way, cell-free therefore provides a simplified dynamic biochemical model system that can be accurately described mathematically[49].

In another context, cell-free prototyping can also be useful towards the design of synthetic cells. At the systems level, central to this effort is the further understanding of cellular compartmentalisation. Due to its difficulty, especially at the structural level, a perhaps understudied area of biology is the dynamics of protein folding in the lipid membrane bilayer. Cell-free uniquely provides an opportunity to study the folding of membrane proteins[50], whilst in synthetic microfluidic based liposomes, enzymes and substrates can be transported from one cell to another, demonstrating a simple recreation of membrane trafficking[51]. Towards complexity, cell-free systems have also begun to be implemented for the assembly of large protein complexes. A classically studied system is the T7 bacteriophage that invades *E. coli* cells and hijacks the native host's TX-TL apparatus for replication[52]. Through cell-free, it has now been shown possible that the 40 kbp dsDNA genome of the T7 bacteriophage, which constitutes 57 genes, can be reconstituted *in vitro* to demonstrate the assembly of a natural protein compartment[53]. This is also expandable to other bacteriophage systems[40]. Moving beyond biological compartmentalisation, cell-free has applications at the interface of nanotechnology for studying gene expression and the synthesis of protein nanotube on biochips[54]. Together these examples of compartmentalisation demonstrate an extra level of complexity in cell-free systems for prototype designs, which may aid in the design of new synthetic cells in the future.

Non-model Cell-Free Platforms

Viewed from a different perspective, synthetic biology has begun to examine the prospects of engineering non-model microbial hosts[55] that can provide unique advantages for biotechnological application, such as rapid growth with inexpensive substrates, growth in extreme conditions or unique enzyme machinery, which in some cases can only accessed within non-standard microbial hosts. However, the greatest disadvantage of such cultivatable microbes is a combination of one or more of the following traits, such as a general lack of characterised gene expression tools, poor genetic tractability or insufficient knowledge towards the microbe's metabolism. Whilst cell-free cannot directly address genetic competence, it could provide a starting point to understanding the host's inherent TX-TL kinetics, genetic tools and enzymology, without the time-limitations associated with direct engineering of the host. Noticeably, the methodology for cell-free extract preparation[42,56] has shown universal application to a variety of microbial cell-free platforms such as *Saccharomyces cerevisiae*[57,58], *Streptomyces* spp[59–63] and *Bacillus* spp[43,64]. Such interest in the use and application of alternative cell-free systems as a prototyping device is likely to grow, however, the cooperative development of synthetic biology tools with translational application into live cells may provide the greatest opportunity to access the design space of traditionally difficult to engineer microbes. In particular reference to the *Streptomyces* family, the high G+C (%) soil bacteria, it has long been appreciated that these hosts provide a unique and well characterised platform for the assembly of a rich repertoire of natural products[65]. Focusing on *Streptomyces* cell-free, the recently developed high-yielding *Streptomyces lividans* and *Streptomyces venezuelae* host platforms[59,62] can potentially provide an opportunity to access high G+C (%) enzymes from secondary metabolism directly within a test-tube for combinatorial biosynthesis. With further advances in efficiency and yield, *Streptomyces* cell-free could be used for incorporating non-natural or potentially toxic substrates into natural products, thus expanding the chemical space of biosynthesis. A proof of concept of how cell-free can be used to incorporate non-natural amino acids into protein backbones was demonstrated for creating modified forms of the model protein GFP in *E. coli* cell-free[66]. Whilst this technology is in its infancy, it is also possible to engineer this in living cells in high-yield[67], which has been made available through the multiplex automated genome engineering (MAGE) technology[68]. However, this methodology is currently only accessible in engineered

strains of *E. coli*. Thus with further developments, cell-free potentially provides a novel route to prototype and engineer the application of novel chemistry in natural product biosynthesis[69].

Conclusions

The emergence of cell-free systems from its historical links in foundational biochemistry has provided a platform to this expanding field in synthetic biology. Perhaps the greatest challenge of cell-free studies is to establish and define the boundaries and limitations of mimicking cellular biology within cell-free systems. One understudied area is the impact of molecular crowding on enzyme velocities[70,71] and spatial organisation[72], which can only be artificially controlled in cell-free reactions. Cell-free systems are reminiscent of primordial biology[73], whereby enzymes (or ribozymes) and chemicals once freely tumbled without the restrictions of biological compartmentalisation and the regulatory control of the genome. With the growing interest in the design of a minimal synthetic cell[74–76], cell-free systems can provide a base towards the design of synthetic life from individual components. We anticipate that the prototyping and modelling of gene expression and enzyme machinery from understudied non-model microbes will place important new tools at the cell-free synthetic biologist's disposal.

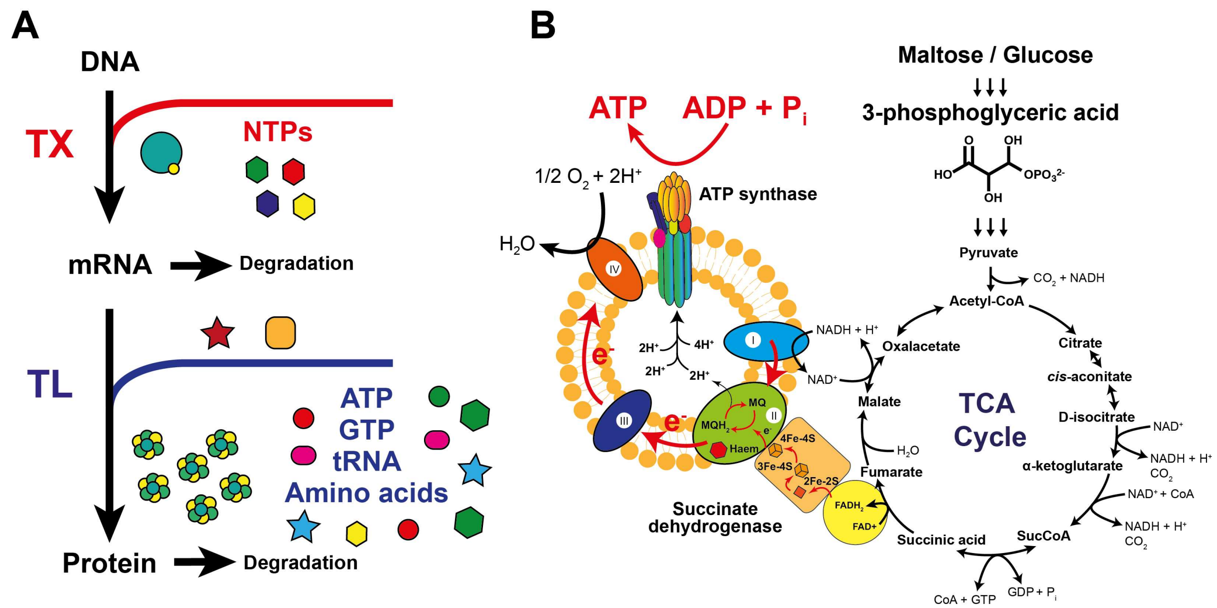

Figure 1. Summary of cell-free transcription-translation. A, Transcription (TX) and translation (TL) process and requirements of nucleotide triphosphates (NTPs) and substrates (ATP, GTP, tRNA and amino acids). **B,** Energy regeneration cycle for central metabolism. ATP is synthesised through the formation of inverted vesicles, which spontaneously form during cell-disruption[37]. Abbreviations: MQ, menaquinone; MQH_2, reduced menaquinone.

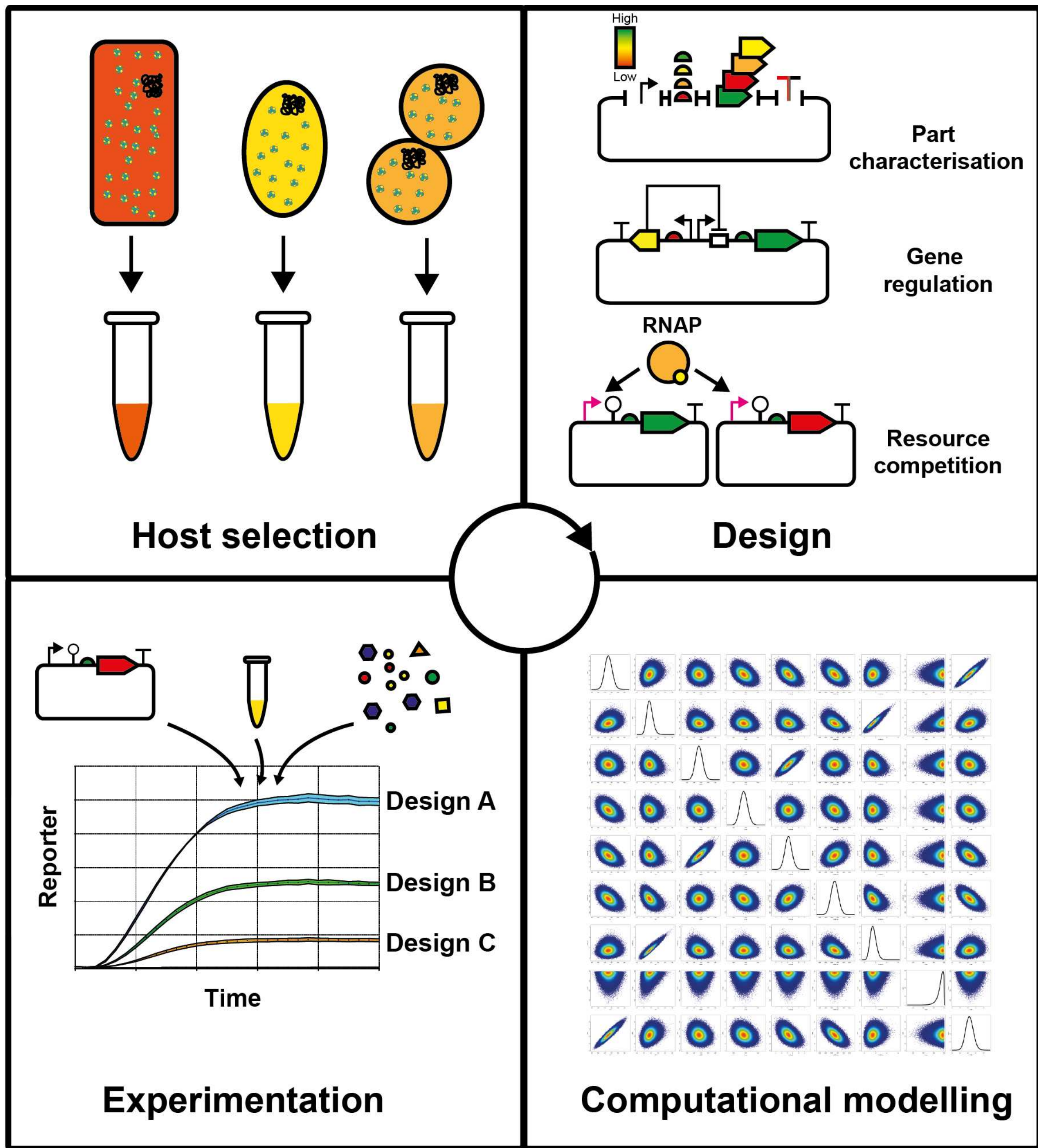

Figure 2. Prototyping cell-free TX-TL systems. A workflow for the prototyping of new microbial platforms, coupled with genetic design, testing and computational modelling.

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