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Directed Evolution: An Evolving and Enabling Synthetic Biology Tool

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Abstract

Synthetic biology, with its goal of designing biological entities for wide-ranging purposes, remains a field of intensive research interest. However, the vast complexity of biological systems has heretofore rendered rational design prohibitively difficult. As a result, directed evolution remains a valuable tool for synthetic biology, enabling the identification of desired functionalities from large libraries of variants. This review highlights the most recent advances in the use of directed evolution in synthetic biology, focusing on new techniques and applications at the pathway and genome scale.

Introduction

In its broadest terms, synthetic biology can be described as the engineering of biological entities not found in nature. The applications for such entities are numerous, including bioremediation, biosensing, and synthesis of value-added chemicals. In the past decade, research interest in the field of synthetic biology has grown significantly, as exemplified by a number of recent reviews [1-10, 11•]. As the field has matured, focus has shifted from simple parts and circuits to more complex pathways and systems. While the goal of designer organisms rationally assembled from a toolbox of standard biological parts remains at the core of synthetic biology, our current understanding of the interactions between these parts is limited. As a result, significant tuning and troubleshooting are often required to achieve the desired functionality of a designed construct, which can be difficult through strictly rational means. To this end, directed evolution has proven vital to the success of many synthetic biology efforts, allowing for the rapid screening or selection of a library of construct variants. In this review, we first discuss some of the most recent advances in directed evolution strategies, focusing on techniques that aid the researchers by limiting intervention required in the experiment and by guiding library design. We then survey the most recent applications of directed evolution to the field of synthetic biology, including advances in the evolution of biosynthetic and signal transduction pathways as well as genome-scale strategies targeting multiple loci.

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Advances in directed evolution strategies

Directed evolution refers to the application of selective pressure to a library of variants of a target biological entity with the intent of identifying those with desired properties. While a powerful tool, directed evolution is inherently limited by the size of the libraries that can be prepared, the rate at which screening or selection can be carried out, and the requirement for significant and time-intensive intervention by the researcher at every cycle of library generation and screening/selection. Nevertheless, a number of recent advances have helped to mitigate these limitations through intelligent library design and *in vivo* evolution.

Intelligent library design

Traditional approaches to library construction involve the random introduction of variation to the target entity. While this method accesses the largest possible solution space for a protein target, it can often result in a large proportion of inactive variants. Thus, new techniques have been developed to generate smaller libraries enriched in active variants. One method, called “reconstructed evolutionary adaptive path” analysis (REAP), has been used to evolve DNA polymerases capable of accepting a new class of modified nucleotide substrates [12]. In this strategy, sites of conservation and variation are identified in a family of related enzymes to reveal targets for mutagenesis. From a library of only 93 polymerase variants, two were identified that performed well with the new substrates. Similarly, Yamashiro and coworkers applied the ancestral mutation method to improve the thermostability and activity of the β -amylase enzyme from *Bacillus circulans* by screening a library of only 18 mutants heterologously expressed in *Escherichia coli* [13]. Both of these examples require knowledge of the sequences of multiple related proteins to identify target mutation sites. If such information is not available, a new technique known as truncated metagenomic gene-specific PCR (TMGS-PCR) can be used [14]. In this method, functional screening of environmental DNA is used to identify a target for evolution. Next, sets of primers are used to amplify homologs of the target gene from environmental metagenomic isolates. By applying a traditional DNA shuffling approach, libraries of significant genetic and functional diversity can be obtained.

In vivo directed evolution

To streamline the evolution process and minimize intervention by the researcher, *in vivo* directed evolution has become a promising strategy. An excellent example is provided by Esvelt and coworkers with their phage-assisted continuous evolution, or PACE, method (Figure 1) [15•]. To demonstrate the power of this method, a T7 RNA polymerase was evolved that recognized a unique promoter sequence. In a period of only 8 days, 200 rounds of evolution were carried out. While the PACE method functions in *E. coli*, other *in vivo* evolution methods have been developed for other hosts. In *Saccharomyces cerevisiae*, a homologous recombination-based technique has been demonstrated to allow library generation and screening without the need for PCR [16•]. In this approach, yeast is co-transformed with linearized vector containing the gene of interest and mutagenic oligonucleotides targeting particular regions of the gene, which can be combined via the native homologous recombination machinery. As proof of concept, this method was used to target an individual codon in an inactive *trp1* gene, allowing repaired mutants to be selected on tryptophan-deficient media. In mammalian cells, *in vivo* evolution can be achieved via the somatic hypermutation method [17]. Somatic hypermutation harnesses the natural process by which the great diversity of immunoglobulin (Ig) genes is produced by B cells to enable *in vivo* library construction. As an example of this technique, Arakawa and coworkers inserted the enhanced green fluorescent protein (eGFP) gene into the Ig light chain locus of B cell line DT40. After three rounds of fluorescence activated cell sorting (FACS), eGFP variants were identified with superior fluorescent intensity. For each of the *in*

vivo evolution methods above, it is important to note that coupling of the target functionality to a readout easily detectable in whole cells (e.g., survival or fluorescence) is critical. For example, in the PACE method, the desirable properties of successful mutants must be able to be coupled to pIII expression; otherwise, they will not be perpetuated to future generations.

Advances in the evolution of pathways

The vast majority of processes of interest to the synthetic biologist require the coordinated action of multiple gene products under the control of complex regulatory networks. Traditionally, biologists have taken a reductionist approach to isolate, study, and engineer individual components of such pathways. In recent years, however, a holistic, systems-level approach has become more prominent. As a result, a number of recent evolutionary strategies have been developed and advances have been made in the engineering of pathways both for compound biosynthesis and signal transduction.

Biosynthetic pathways

As biosynthetic genes are studied in the context of entire pathways, the scope of targets for evolution has expanded (Figure 2). For example, Alper and coworkers have demonstrated pathway optimization through the engineering of promoters [18]. By generating libraries of promoters with different strengths, screening can be carried out in the context of a desired pathway to maximize flux to a desired target, such as lycopene. Similarly, Salis and coworkers have focused on ribosome binding sites as engineering targets through *in silico* design [19]. They have recently developed a computational tool for designing libraries of ribosome binding sites for any gene of interest, which can subsequently be screened in the context of a target pathway to determine the optimum protein expression levels for that pathway. To balance the expression levels of a three-gene mevalonate synthesis operon in *E. coli*, Pfleger and coworkers targeted the intergenic regions, leading to the identification of many operon constructs with improved mevalonate production [20]. Finally, Bikard and coworkers focused on gene order to improve tryptophan biosynthesis in *E. coli* by 11-fold [21]. They first designed a non-functional construct with recombination sites flanking each of the five genes in the *trp* operon, as well as regulatory elements, flanked by recombination sites. Following integrase expression, the *trp* genes were recombined *in vivo*, generating a library of different constructs.

In order to screen libraries of biosynthetic pathways, new techniques are required to efficiently assemble large DNA molecules. For *in vitro* assembly, the method of Gibson and coworkers can be applied [22•]. This method has been used to assemble constructs of >100 kb in length, and thus would be readily applicable to the assembly of pathway libraries. To generate pathway libraries *in vivo*, homologous recombination-based strategies in *Saccharomyces cerevisiae* can be employed. In the DNA assembler method of Shao and coworkers, for example, yeast cells are transformed with several DNA fragments with overlapping ends, covering both the pathway of interest and a vector backbone [23•]. Through the inclusion of multiple fragment variants with compatible homologous ends, libraries can be generated for subsequent screening or selection (H. Zhao, unpublished data). Seeking to increase recombination efficiency, Wingler and Cornish developed a reiterative recombination technique that utilizes homing endonucleases to introduce double-strand DNA breaks at the recombination site [24]. As proof of concept, a mock three-gene construct library of greater than 10⁴ members was prepared.

Beyond the small molecule and secondary metabolite pathways described above, directed evolution has also been applied to more fundamental biosynthetic processes, such as gene expression. The entities involved in such fundamental processes, however, are necessarily

vital to cell survival, and thus are generally recalcitrant to engineering strategies. To remedy this situation, orthogonal mRNAs (o-mRNAs), which contain a modified Shine-Dalgarno sequence unrecognized by native ribosomes, and orthogonal ribosomes (o-ribosomes), which recognize these o-mRNAs, have previously been evolved that operate independently of the cell's native metabolism [25]. An impressive recent example in this field is the evolution of an o-ribosome that recognizes several quadruplet codons as well as the amber codon, thus providing a means for expansion of the genetic code to include unnatural amino acids [26]. In a closely related example, directed evolution proved particularly vital in the *de novo* creation of an orthogonal aminoacyl-tRNA synthetase (aaRS)/tRNA pair to recognize a quadruplet codon [27•]. In this multi-step process, a tRNA was first evolved to be unrecognized by all endogenous aaRSs. Next, a cognate aaRS was evolved that aminoacylates both the evolved tRNA and its progenitor. Finally, multiple rounds of positive and negative selection were used to improve the specificity of the evolved aaRS for the evolved tRNA, and to switch the anticodon of the evolved tRNA from CUA to UCCU, yielding the orthogonal aaRS/tRNA pair.

Signal transduction pathways

In addition to pathways that synthesize target molecules, pathways that transmit information, both in the form of gene circuits and cell communication systems, are of significant interest in synthetic biology. For example, Lou and coworkers were able to tune a complex push-on push-off switch, which responds to a UV input by synthesizing one of two fluorescent reporter genes, through the evolution of ribosome binding sites for two key circuit components [28]. Sayut and coworkers, on the other hand, evolved a transcriptional activator with the guidance of mathematical modeling to enhance the functionality of an AND logic gate [29], while An and Chin utilized the o-mRNA and o-ribosome components to create feed-forward loops for transcription and translation [30]. Beyond the tuning of gene circuitry components, directed evolution can also be used to alter the input and output behaviors of gene switches. For example, McLachlan and coworkers evolved estrogen receptor-based gene switches to respond to new input compounds while simultaneously losing specificity for their native ligand, 17 β -estradiol [31]. Edwards and coworkers, on the other hand, used a random domain insertion approach to fuse cytochrome *b*₅₆₂ to a β -lactamase, thus coupling binding of the native ligand (in this case, heme) to the novel functional output of antibiotic resistance [32]. To facilitate the evolution of gene switches and circuits, powerful new dual-selection strategies utilizing such genes as herpes simplex virus thymidine kinase [33] or the tetracycline transporter TetA [34] have been developed that provide different selectable responses in the “on” or “off” state.

In natural cell signaling pathways, phosphorylation events and protein-protein interactions play critical roles. As a result, evolutionary strategies have recently been employed to engineer these occurrences. For example, a robust signaling pathway between the sensor kinase CpxA and the response regulator OmpR in *E. coli*, which show no cross-talk *in vivo* and only weak cross-talk *in vitro*, was constructed via saturation mutagenesis of CpxA at five amino acid sites [35]. Additionally, Mody and coworkers rewired the mitogen-activated protein kinase (MAPK) signaling network in *S. cerevisiae* by constructing and screening a library of 64 Fus3 and Hog1 chimeras [36]. To evolve protein-protein interactions, Ernst and coworkers selected Erbin, a single peptide recognition module of the PDZ family, from the human genome [37]. Through random mutagenesis, they were able to generate libraries of variants that recognized peptide ligands as diverse as those recognized by the entire PDZ family, with comparable specificity. A particularly good example of the benefits of directed evolution in tandem with rational design was recently provided by Karanicolas and coworkers [38•]. They first designed a pair of proteins *in silico* with a heterodimerization interface. When tested *in vitro*, the K_d was determined to be 130 nM. After applying a

combination of phage and yeast display methods to separately evolve the two proteins, the final mutant pair was determined to have a K_d of 180 pM, representing almost 1000-fold improvement in affinity.

Intercellular communication systems represent another area of significant interest in synthetic biology. Particularly well studied is the *lux* quorum sensing operon of *Vibrio fischeri*, which is predicated on the synthesis of 3-oxo-hexanoyl homoserine lactone (3OC6HSL) by LuxI and its subsequent recognition by the receptor LuxR. Directed evolution has been applied to LuxI to increase its 3OC6HSL productivity [39], as well as to LuxR to broaden its ligand specificity [40,41] and to improve its potency as a transcriptional activator [42]. This last example utilized a two-species *E. coli*/phage M13 selection scheme akin to the PACE method, but in batch mode, improving LuxR activity by 17-fold. To exhibit better control over quorum-sensing systems, a lactonase can be used to degrade the acyl homoserine lactone signaling molecule. Directed evolution has been applied to improve the catalytic efficiency of such enzymes from *Bacillus* sp. and *Geobacillus kaustophilus* HTA426 by 7-fold and 72-fold, respectively [43,44].

Advances in the evolution of genomes

The advances in DNA synthesis and assembly techniques have enabled researchers to construct a whole bacterial genome [45••] or a partial eukaryotic chromosome [46]. But the complexity and unpredictability of living systems are still beyond our capability for *de novo* genome design. Thus directed evolution proves to be a more effective approach for genome engineering than rational design. For example, adaptive evolution has been widely used to improve complex cellular phenotypes for decades. Nevertheless, such methods are time-consuming, and it is difficult to identify beneficial mutations. Newer approaches such as genome shuffling [47] and global transcription machinery engineering (gTME) [48] were developed to significantly accelerate the evolutionary process. Since all of these methods have been extensively reviewed elsewhere [11•,49,50], here we will only focus on the new approaches for directed evolution of genomes that were developed in the past two years.

By coupling parallel DNA synthesis and recombineering, Multiplex Automated Genome Engineering (MAGE) was developed to target many loci in an *E. coli* genome [51••]. The huge combinatorial diversity created by the optimization of 24 genes simultaneously enabled the isolation of *E. coli* variants with a 5-fold increase in lycopene production. MAGE also enabled the replacement of all 314 TAG stop codons with synonymous TAA codons across 32 sets of *E. coli* strains, which were then merged into genomes with up to 80 precise changes by hierarchical conjugation [52]. However, one limitation of this approach is that the target loci were predefined by the design of the recursively introduced homologous oligonucleotides, requiring prior knowledge about the genotype-phenotype correlations.

While MAGE greatly expands the depth of genetic perturbation, TRackable Multiplex Recombineering (TRMR) provides a highly efficient approach to create and track variation over the entire genome [53••]. TRMR permits the modification of upstream sequences before nearly all genes in the *E. coli* genome by insertion of synthetic cassettes with molecular barcodes. A collection of strains that either increase or decrease the expression of a particular gene were constructed. In principle, additional mutations can be readily introduced by performing TRMR recursively to create libraries containing two or more genetic modifications. Such libraries are very important to elucidate genetic interactions when aiming at different phenotypes.

Conclusions and future perspectives

In the face of our limited knowledge of living systems, directed evolution continually contributes to the field of synthetic biology through the creation of genetic parts and circuits, the optimization of biosynthetic pathways, and the multiplex genome engineering for complex traits. Not only useful in achieving desired functions or phenotypes beyond rational design, directed evolution can also greatly advance our understanding of life. On the other hand, the powerfulness of this approach is inherently limited by the library size and screening/selection throughput, which depends upon our ability to couple the desired output to an easily detectable signal. When moving to systems with higher complexity, our experiences gained from protein engineering might not be readily applied. Thus novel tools and new principles are constantly in need to fulfill the potential of directed evolution for the engineering and understanding of living systems from protein, circuit, pathway to genome levels.

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Abbreviations

3OC6HSL	3-oxo-hexanoyl homoserine lactone
aaRS	aminoacyl-tRNA synthetase
eGFP	enhanced green fluorescent protein
FACS	fluorescence activated cell sorting
gTME	global transcription machinery engineering
Ig	immunoglobulin
MAGE	multiplex automated genome engineering
MAPK	mitogen-activated protein kinase
o-mRNA	orthogonal messenger RNA
o-ribosome	orthogonal ribosome
PACE	phage-assisted continuous evolution
REAP	reconstructed evolutionary adaptive path
TMGS-PCR	truncated metagenomic gene-specific polymerase chain reaction
TRMR	trackable multiplex recombineering

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- Recent advances in directed evolution applied to synthetic biology are reviewed.
- New strategies facilitate library creation and *in vivo* evolution.
- Recent applications target biosynthetic and signal transduction pathways.
- New tools enable multiplex genome evolution.

Figure 1.

A PACE experiment. *E. coli* cells continuously flow through a 'lagoon' containing replicating phage, which carry the target gene of interest (1). Following phage infection of an *E. coli* cell (2), functionality of the target protein is linked to production of pIII, a protein that the phage requires to produce infectious progeny. Thus, when a phage encoding a protein with the desired function infects *E. coli*, pIII can be synthesized, and so further infectious phages are produced harboring the functional target gene (3). When a phage encoding a nonfunctional target protein infects *E. coli*, pIII is not synthesized, and so that cell does not produce any infectious phage with the nonfunctional target gene (4). The result is a phage population in the lagoon encoding functional target genes that can re-infect host cells (5), while nonfunctional genes are washed out with the noninfectious progeny (6).

Figure 2. Recent targets for the evolution of biosynthetic pathways include (a) promoters, (b) ribosome binding sites, (c) intergenic regions, and (d) gene order.

Figure 3. MAGE can achieve targeted multiplex modification of a genome by introducing synthetic oligonucleotides recursively to an evolving cell population.