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Probing pathways of adaptation with continuous evolution

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Introduction

Directed evolution is an effective approach to engineering enzymes and proteins for industrial, medical, and biotech applications [1,2] and was recently recognized with a Nobel Prize in Chemistry for its extraordinary practical impact. What is perhaps less well-known is the role directed evolution has played in elucidating and testing evolutionary mechanisms and theories of gene adaptation [3–6]. Understanding how a gene evolves is traditionally done retrospectively, by examining natural sequences and structures and working backwards to reconstruct descent and key evolutionary intermediates [7–11]. However, inferred histories are incomplete, can never be fully validated, and usually represent an N=1 experiment, as the conditions of natural evolution do not systematically repeat. By accelerating the process of evolution in the laboratory, directed evolution offers a way to study the evolution of genes in the forward direction, enabling researchers to observe adaptation in controlled environments, often in many replicates, and armed with the ability to sample and characterize the entire "fossil record" of each experiment. Such studies have yielded critical insights into the mechanisms by which genes, particularly proteins, evolve [12–15], the importance of stability in protein evolvability [16–21], the catalytic promiscuity of enzymes in the evolution of new activities [22–25], the complex fitness landscapes of proteins [26–35], and the role of neutral drift and fluctuating environments in crossing fitness valleys [36–39], to name only a few – and has solidified directed evolution as a powerful tool for understanding adaptation [5,6,40–42].

Despite these significant successes, there are limitations to using classical directed evolution techniques to study evolutionary mechanisms. Commonly, directed evolution mimics natural evolution by subjecting one or more genes of interest (GOIs) to multiple rounds of *ex vivo* diversification (*e.g.* error-prone PCR), transformation into cells, and selection [2]. Each

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round of this process represents a step in an adaptive trajectory but requires significant manual intervention that restricts the extent and scale of experiments. This keeps three tantalizing categories of experiments largely outside our reach. First are experiments requiring the traversal of long mutational pathways such as ambitious adaptations or studies aimed at probing gene evolution under varying conditions over extended periods of time. Indeed, most directed evolution experiments reach outcomes less than 5–10 non-synonymous mutations away from the parent sequence [16,38,43], with some exceptions that study the effects of extensive mutagenesis in one or few rounds [44–47]. Second are experiments requiring high statistical power through replication, such as studying drug resistance pathways, comparing the effects of different conditions on adaptation, detecting rare outcomes and rare adaptive trajectories, or mapping rugged fitness landscapes. Currently, most directed evolution experiments are limited to only a few replicates [39,43,48]. Third are experiments that wish to capture or test complex population dynamics, since the technical idiosyncrasies of transformation and *ex vivo* diversification can cause population bottlenecks and perturb dynamics in artificial ways that influence evolutionary trajectories.

To address these limitations, synthetic biologists are working to establish a new paradigm in directed evolution through the construction of so-called continuous evolution systems. Continuous evolution achieves diversification of GOIs *in vivo* such that manual rounds of *ex vivo* diversification, transformation, and *in vivo* GOI expression and selection are not needed [49–52]. Instead, rapid diversification of GOIs occurs concurrently with their expression and functional selection, converting labor-intensive stepwise directed evolution processes into ones requiring only the serial passaging of cells under selection conditions. This allows for evolution experiments that require long mutational pathways, large-scale replication, and the ability to capture complex population dynamics, categories particularly useful for testing evolutionary mechanisms and theory (Figure 1). In this opinion, we will briefly discuss the current state of continuous evolution systems and present their early successes and potential in reinvigorating the use of directed evolution to study basic questions in gene and protein adaptation.

State of continuous evolution systems

We will center our discussion in this section around three key properties that continuous evolution systems should have: targeting, durability, and scalability. There are others, discussed in depth elsewhere [52,53], but targeting, durability, and scalability are uniquely important if the goal is to study mechanisms of how a GOI evolves, as these three properties enable the rapid exploration of long mutational trajectories with statistical power.

First, targeting. Certainly, if one wishes to explore how a GOI evolves, one will not want other loci contributing to the evolved function. But beside this practical reason for targeting is a deeper one. Very high rates of diversification are needed to see adaptation at the gene level on laboratory timescales, but such high rates harm or destroy host genomes, since there is a general inverse relationship between the rate at which an information polymer can be mutated under selection for function and its size [54–57]. For example, a GOI of size 1 kb can likely withstand a continuous mutation rate on order ~10^{-3} substitutions per base (s.p.b.)

while a host genome of size 10^7 bp (*e.g.* for *Saccharomyces cerevisiae*) will likely accumulate a lethal mutation every generation at mutation rates around ~10^{-6} s.p.b. and experience clear fitness defects at mutation rates above ~10^{-8} s.p.b. [53,55]. Indeed, mutation rates of microbes and mammalian cells are evolutionarily optimized to be in the 10^{-9}-10^{-10} s.p.b. range to prevent deterioration of fitness through high mutational loads over time [57–59]. Therefore, to evolve a GOI rapidly *in vivo*, mutations must be targeted to the GOI with extreme specificity. Most continuous evolution systems achieve incomplete targeting of GOIs relative to host genomes and other DNA [60–62], but two systems have either managed to achieve complete targeting or avoid the problems of genomic mutation physically [49,53]. OrthoRep, developed in our lab, consists of an orthogonal DNA polymerase (DNAP)-plasmid pair in *S. cerevisiae* that can mutate target GOIs at ~10^{-5} s.p.b. without any increase in host genomic rates (~10^{-10} s.p.b.) [53]; and phage assisted continuous evolution (PACE) elevates phage genome mutation rates along with host mutation rates, but ingeniously disregards host mutation effects by removing host *Escherichia coli* cells fast enough to prevent host propagation but slow enough to ensure phage propagation [49,63].

Second, durability. Ideally, a continuous evolution system will mutate target GOIs indefinitely so that long mutational paths (*e.g.* >10 non-synonymous mutations) can be traversed over extended periods of strong selection or more complex sequences of selection that mimic natural evolution [64,65]. So far, both OrthoRep and PACE have proven to be quite durable – we have used OrthoRep in several evolution experiments to evolve GOIs for >300 generations and still observe rapid adaptation, accumulating 10–20 mutations (manuscripts in preparation); and PACE has been used in experiments that adapt over hundreds of phage generations, accumulating 10–20 mutations [64,65]. Durability in other continuous evolution systems [60–62,66–73] remain untested, but one can predict durability based on the architecture of the system. For example, in OrthoRep, the only way a GOI gets replicated is through an error-prone DNAP, encoded on a host plasmid or genome that doesn't experience elevated mutagenesis. This, combined with the fact that OrthoRep achieves complete mutational targeting to avoid selection against elevated mutation rates through mutational loads on the genome, favors durability. Likewise, in PACE, durability is favored, because the only way a phage genome encoding a GOI is replicated is through error-prone means.

Third, scalability. Especially important for experimental evolution, a continuous evolution system should ideally be scalable in nature. Evolving a GOI with a large number of replicates is crucial for observing low frequency events [53,74,75], inferring beneficial mutations [76,77], and determining the extent to which evolutionary trajectories are reproducible [31,78]. Continuous evolution systems that are fully *in vivo*, such as OrthoRep, offer scalablity, because evolution experiments can be carried out simply through serial passaging, amenable to extensive replication or parallelization [60,61,71,72]. Although PACE usually requires chemostat or turbidostat setups that limit scale, recent experiments demonstrate that PACE may be conducted via bulk passaging without such setups and should be amenable to extensive replication [79].

In short, a number of continuous evolution systems, including OrthoRep and PACE, are at a stage of development where they should be able to routinely drive GOI evolution at the speeds, durations, and scale required to study mechanisms of gene evolution through forward evolution experiments.

Early applications of continuous evolution to studying evolutionary pathways and mechanisms

PACE has been the most successful continuous evolution system for proteins to date. In PACE, a GOI is encoded on a phage's genome and through coupling an improvement in the GOI's function to phage survival and infectivity, GOIs with beneficial mutations rapidly propagate in a pool of *E. coli*. By having a continual influx of *E. coli* at a rate that is between the doubling time of phage and *E. coli*, GOIs can rapidly accumulate mutations while mutated host cells are removed. Although most PACE experiments have focused on protein engineering applications, some have aimed to understand the details of evolutionary mechanisms. In 2013, Leconte and colleagues examined the effect of selection stringency and rate of mutagenesis, key parameters in evolutionary theory, on the evolution of T7 RNA polymerase (RNAP) towards recognition of the T3 promoter [80]. While the effect of mutation rate on adaptive pathways has been studied in other contexts [81–87], PACE enabled Leconte *et al.* to isolate the effects of mutation rate on a single gene in freely evolving replicate cultures [80]. They demonstrated that high mutation rates resulted in more reproducible fixation, possibly by increasing the frequency in which superior mutations are accessed, as consistent with predictions made *in silico* [84]. Further, Leconte *et al.* showed that the strength of selection resulted in substantial differences in adaptive trajectories with stronger selection favoring lower diversity and higher reproducibility as expected. Significantly, only with the benefit of replicate experiments were Leconte *et al.* able to show that while mutational patterns indeed appeared across replicates, both the specific adaptive mutations undergoing fixation, and even successful adaptation itself, can be stochastic in nature.

In a separate study, Dickinson *et al.* used PACE to explore contingency in evolution [88]. Previous research has shown that while the stepwise evolution of a single gene or a small set of genes could be practically deterministic [26,31,89], convergent evolution from dissimilar proteins and histories can lead to vastly different sequences, structures, and activities [32,90,91]. In their 2013 study, Dickinson *et al.* asked just how much dissimilarity in the history of a protein's evolution was needed to result in significant changes in evolutionary outcomes. With PACE, they were able to conduct replicate evolution experiments where T7 RNAP was first diverged to recognize either the T3 or SP6 promoter, and subsequently pressured to recognize the same final promoter, a hybrid T3/SP6 promoter. Dickenson *et al.* impressively showed that the divergent evolutionary steps were sufficient to drastically alter the mutational trajectory as well as the maximum catalytic efficiency of the final evolved enzymes. Specifically, populations of T7 RNAP that were first evolved for T3 promoter recognition evolved lower activity for the final promoter compared to populations first evolved for the SP6 promoter, with differences persisting even after extensive continued selection (~40 generations at high mutational load) for recognition of the final promoter.

This study elegantly shows the importance of contingency in evolution and how historical effects are not easily forgotten through strong selection alone. Further, through characterization of mutations present in different experiments, Dickenson *et al.* identified a key epistatic interaction between two mutations that act to isolate two outcomes from each other. With these results, PACE gives us a sense of the types of questions in basic evolutionary biology that continuous evolution can address, questions that would be difficult to study with traditional directed evolution techniques. Although these examples focus only on T7 RNAP, PACE is generalizable to the evolution of any function that can be linked to the expression or activity of an essential phage coat protein [63]. Indeed, in molecular-engineering-motivated PACE experiments, many other proteins have been evolved [2,49,64,65,79], and additional basic evolution experiments on proteins besides T7 RNAP are surely underway.

Another continuous evolution technology, OrthoRep, has recently enabled a detailed mapping of adaptive trajectories on a fitness landscape, including low probability events, and demonstrated the effects of epistasis and clonal interference on the reproducibility of adaptation [92]. OrthoRep uses an orthogonal error-prone DNA polymerase-plasmid pair in *S. cerevisiae* to achieve targeted mutagenesis of GOIs [53,92]. By encoding *Plasmodium falciparum* dihydrofolate reductase (*Pf*DHFR) on OrthoRep, Ravikumar *et al.* rapidly evolved resistant pyrimethamine-resistant *Pf*DHFR variants simply by passaging 0.5 mL yeast cultures in media containing increasing concentrations of pyrimethamine. Owing to the scalability of OrthoRep, this experiment was easily repeated 90 times to abundantly sample adaptive trajectories. From this, Ravikumar *et al.* uncovered a more complex fitness landscape than previously realized, including new mutants as resistant as those widely studied. One mutant occurred frequently due to a highly adaptive first-step mutation (S108N) that exhibited a conflict with a highly-adaptive later mutation (D54N), making most sequences containing both S108N and D54N non-functional. Yet C59R and/or Y57H were able to resolve this conflict between S108N and D54N. This led to convergence of adaptive trajectories across replicates. However, in a few replicates, rare mutations steered populations towards other equally-fit outcomes, including ones lacking S108N, and suboptimal local fitness peaks. Since these alternative variants are expected to respond differently to secondary drugs, population structures and strategies that favor rare mutational pathways may be important for drug schedule design, which we are currently exploring. In short, by exploiting rapid and scalable continuous evolution, one can explore adaptation on rugged fitness landscapes to tease out both the stochastic and deterministic nature of evolution.

Future potential

Continuous evolution systems hold great promise in studying the mechanisms and pathways of gene adaptation. The early studies described above give a glimpse into how continuous evolution can be used to carry out controlled forward evolution experiments that discover and map interesting regions of fitness landscapes, test the reproducibility of adaptation, and compare how different parameters of evolution and selection schedules result in different mutational trajectories and outcomes. As more researchers use continuous evolution to carry out forward evolution experiments with previously inaccessible speed, depth, and scale,

significant insights should be made. These should not only include exquisite details of how specific genes adapt through the interplay among mutations, but also general insights into the most fundamental questions in molecular evolution – the reproducibility of adaptation [93,94], how fitness valleys are crossed [95,96], the importance of fluctuating environments or population structure in adaptation [97], the prevalence and role of epistasis in protein evolution [98–100], the existence of tradeoffs among different gene functions [19,41], the determinants of evolvability [40,101], the high prevalence of certain folds or structures in enzymes [102–104], the evolutionary basis of protein-protein interactions [105,106], and the role of both intracellular and environmental conditions in dictating how a gene adapts [107,108]. With the number of powerful systems available and ongoing development in each, such as the inclusion of gene-specific sexual recombination into OrthoRep (unpublished data), continuous evolution should become a staple technology for probing the fundamentals of adaptation.

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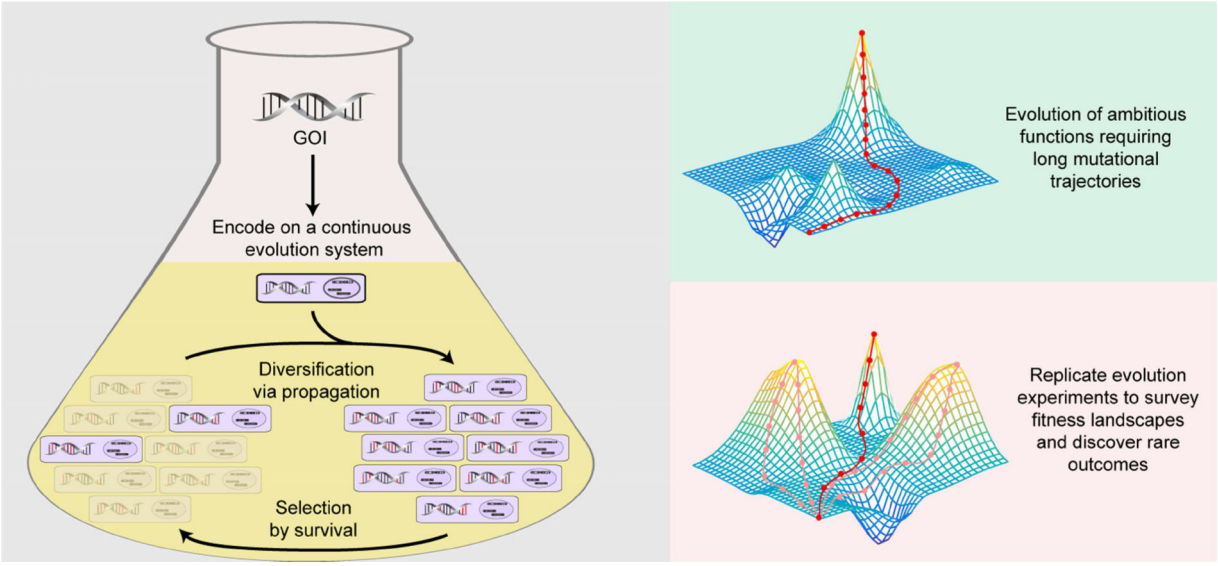

Figure 1.

Continuous *in vivo* evolution systems enable the rapid continuous diversification of genes of interest in multiple replicate cultures. Through coupling continuous diversification with selection, simply passaging cultures can drive protein evolution on laboratory timescales. This allows proteins to achieve ambitious functions that may require high numbers of mutation (>10–20). Further, the ability to run replicate evolution experiments allows for the detailed mapping of fitness landscapes, discovery of rare outcomes, exploration of multiple environmental conditions and population structures, and statistical power in testing evolutionary reproducibility and basic evolutionary theories.