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Microbial experimental evolution in a massively multiplexed and high-throughput era

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Experimental evolution with microbial model systems has transformed our understanding of the basic rules underlying ecology and evolution. Experiments leveraging evolution as a central feature put evolutionary theories to the test, and modern sequencing and engineering tools then characterized the molecular basis of adaptation. As theory and experimentations refined our understanding of evolution, a need to increase throughput and experimental complexity has emerged. Here, we summarize recent technologies that have made high-throughput experiments practical and highlight studies that have capitalized on these tools, defining an exciting new era in microbial experimental evolution. Multiple research directions previously limited by experimental scale are now accessible for study and we believe applying evolutionary lessons from *in vitro* studies onto these applied settings has the potential for major innovations and discoveries across ecology and medicine.

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Introduction

If decades of studying evolution have taught us anything, it is that life finds a way. But how? Understanding the pace and variety of evolutionary solutions, the predictability and repeatability of adaptation, and the interplay between evolution and complex cellular networks has been central to evolutionary biology. Yet, given the complexity of the natural world and the large uncertainties about distant evolutionary pasts, these questions are impossible to directly probe outside the context of carefully controlled experiments. The field of experimental evolution, born roughly 50 years ago, was aimed at addressing precisely this concern.

Experimental evolution, wherein laboratory populations are evolved *in vitro*, is an exercise in constraints: isolating variables to observe how simple experiments can lead to surprising insights about the mechanistic basis of evolution. The first serious incursion into the field was led by pioneers in microbial evolution. The Long-Term Evolution Experiment (LTEE), started in 1988 by Rich Lenski, aimed at testing the fundamental concepts of repeatability and parallelism in evolution [1]. It became the apotheosis of the 'first era' of experimental evolution. Leading up to the 2000s, experiments explored the basic frameworks of evolution and shaped our understanding of core evolutionary concepts that had so far only been theorized: fitness landscapes and trajectories [1–3], the dynamics of clonal interference [4,5], niche partitioning and specialization [6,7], and eco-evolutionary and host-pathogen dynamics [8–10]. Coupled with classical genetics, this work unraveled the genes and pathways relevant for adaptation in a wide variety of contexts and even led to an early appreciation of epistasis and pleiotropy [8,11,12].

By the early 2000s, experimental evolution saw a complete makeover thanks to next-generation sequencing and advances in molecular and synthetic biology. This 'second era' was dominated by evolve and re-sequence experiments that allowed scientists to observe the dynamics of genomic evolution in real-time [13–16]. Easier cloning across model organisms led to direct tests of evolved mutations in ancestral genetic backgrounds [17,18] and increasing computational power enabled sophisticated evolutionary simulations with 'digital organisms' [19–22]. This era sharpened questions about how biological systems are continuously tuned during adaptation — evolution did not appear to be limited by lack of beneficial mutations. Improvements in genome engineering techniques paved the way to more complex experimental designs, which could now be coupled with problems that were previously only amenable to theoretical treatments [23,24]. For instance, McDonald et al.

evolved populations with and without recombination [25,26], confirming theoretical predictions that sex promotes adaptation by both decoupling beneficial mutations from hitchhiking mutations and by alleviating the strength of clonal interference.

Over the last few years, the portrait of organismal physiology and evolutionary trade-offs painted by experimental evolution has been remarkably comprehensive. We now have a framework to analyze a host of complex evolutionary phenomena. Epistasis, pleiotropy, quantitative trait loci, co-evolution, symbiosis, drift, and mutational biases have all been explored and codified, even if not fully understood. We stand the cusp of a new era in experimental evolution. With the recent explosion of modern high-throughput technologies, new biological and evolutionary mysteries can be specifically targeted and addressed. In this perspective, we begin by describing novel technological developments that allow thousands of populations to be evolved in a wide array of complex evolutionary scenarios and their adaptation to be tracked at high resolution. We then highlight exciting open areas and challenges that are ripe for exploration and suggest ways in which new discoveries and syntheses from the past decades can extend beyond the explorative philosophy of the field.

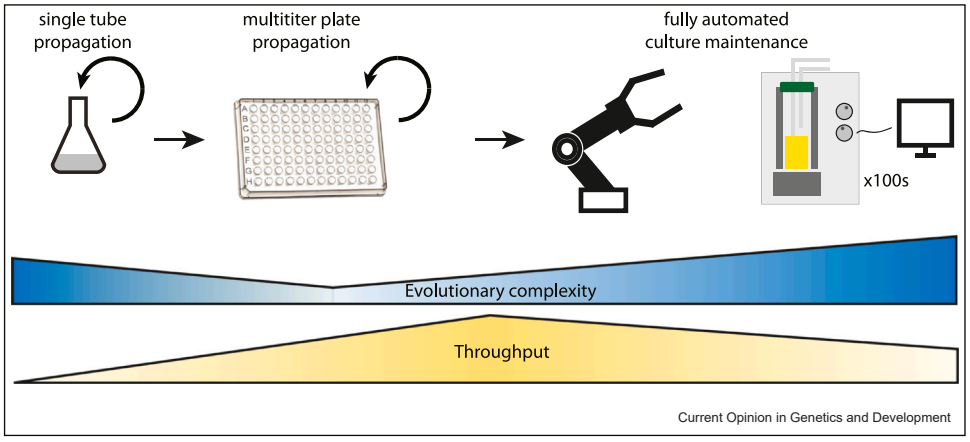
High-throughput genetics and evolution

The use of liquid-handling automation

Two major technical advances in experimental evolution relate to improvements in scale and complexity. As statistical questions about evolution become more amenable to experimental approaches, numbers of replicate cultures and the frequency of culture passaging have increased extensively. Key to this success has been the increased adoption of sophisticated liquid handling techniques. Once a suitable biological system has been constructed, the main task of all evolution experiments is controlled liquid handling. Passaging 12 lines in a simple environment for an extended time requires patience and rigor (which is by no means an easy task) but passaging hundreds of lines in complex environments has made automation essential. The first generation of automation made use of either small-volume chemostats [27,28] or large liquid handling robots [16,29,30]. Experimental throughput has improved dramatically thanks to the advent of these robots, allowing researchers to maintain thousands of cultures in multi-well plates with relative ease (Figure 1).

One drawback of these first-generation approaches is the difficulty in performing complex evolutionary scenarios, such as cross-mixing of cultures across wells or non-traditional transfer regimes. Thus, experimental evolution has traditionally not investigated anything that remotely approaches the environmental complexities of our world, sometimes bringing in question the relevance of the biological findings of the field [31] (though we note that evolution in agriculture and breeding has striking parallels to studies in microbial experimental evolution [32]). Although it is possible to set up robotic arms to move plates from incubators to the deck and write programs that account for short and varying transfer regimes, it is challenging and impractical to setup unless a lab is willing to dedicate a single machine for just one experiment, though there are success stories [33,34]. Recently, exciting developments in open-source millifluidics systems now allow parallel cultures to be maintained in extremely complex scenarios [35]. These second-generation systems can manage dozens of

Figure 1



Technological progress in culture maintenance. Microbial experimental evolution has improved from a single tube in a single environment to hundreds of tubes in complex environments. Modern millifluidics systems allow increased evolutionary complexity while also increasing throughput.

populations at modest population size with complex in-line changes in evolutionary environments, mixtures of independent cultures at precise volumes, and real-time tracking of growth rate, making them a key instrument in experimental evolution labs.

Another recent development that has powered the second generation of automation in this area is the advent of microfluidics and liquid droplet chemistry [36]. These systems can monitor hundreds of populations at minute population sizes (e.g. the mother machines [37]), which can investigate non-adaptive processes such as drift and mutational accumulation in exquisite detail and throughput. Liquid droplets, in a similar spirit, consist of few nanolitres that can encapsulate individual cells or enzymatic reactions and have revolutionized parallelization of high-throughput experiments and phenotyping. For instance, van Raay et al. performed an evolution experiment of *E. coli* within droplets where they selected for growth yield rather than growth rate, showing reversal of adaptation to an environment is possible [38].

Next-generation phenotyping

With massively parallel population maintenance, the challenge moves to data collection and analysis. Two key recent innovations have made phenotyping dramatically easier: 1) miniaturization and liquid-handling automation of enzymatic reactions involved in next-generation sequencing, and 2) the use of DNA barcodes for parallelized growth assays. With these strategies, analysis of evolving population has mostly kept pace with the increased throughput and complexity of culture maintenance.

Currently, a single Novaseq lane can sequence ~10^5 microbial genomes. This allows systematic evolve-and-resequence experiments for thousands of populations. However, the cost of sample preparation relative to the cost of sequencing remains high for most applications. The second era of experimental evolution saw the miniaturization and homebrewing of the genomic sequencing process to reduce sample preparation cost to approximately 10$ per sample [39]. Coupling these strategies with liquid handling robotics reduced the cost even further: at less than 0.1$ per sample, Nguyen Ba et al sequenced the genomes of 100 000 yeast strains in a few weeks [40].

A challenge that is often underappreciated is measuring the phenotypic changes of evolving populations. Fitness measurements can be obtained through in-line growth rate sensors, or by competitive-fitness assays [41]. However, measuring the fitness of a few thousand strains using these methods is laborious, taking hours of analysis on a flow-cytometer or manual counting on agar plates. The defining phenotyping technology for the new era of experimental evolution has been the adoption of DNA barcodes. This technology allows simultaneous competitive fitness assays by tracking changes in barcode frequencies using next-generation sequencing (Figure 2) [42–44]. Recently, this technique was modified to allow repetitive barcoding allowing the tracking of evolution for extended periods of time at extremely high resolution [45], while others leveraged targeted sequencing to observe the evolutionary potential in key loci [46,47].

High-throughput genetics

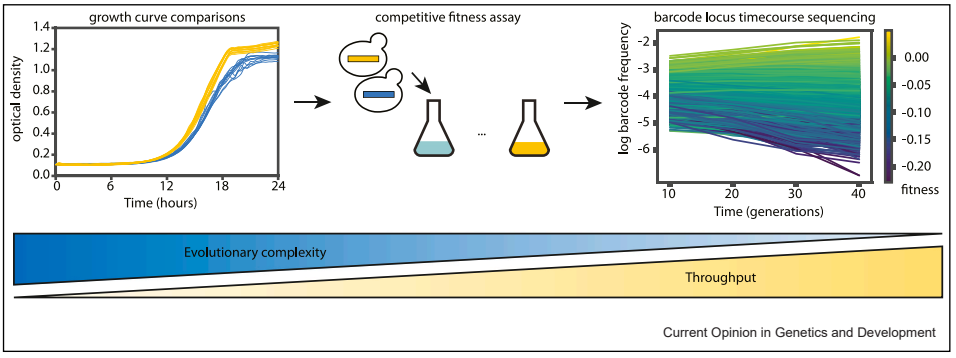
Genotype-phenotype mapping is crucial to experimental evolution. Unfortunately, even in the genetically tractable yeast, the ability to reintroduce genetic modifications from an evolution experiment back into the ancestor can be limited, especially given the complex number of mutations accumulated during evolution. Implementation of new synthetic biology techniques that enable rapid introduction of mutations in 'wild-type' genomes solves this problem. These techniques include transposon mutagenesis [48,49] (usually coupled with the addition of barcodes for later phenotyping), automated Multiplex Automated Genomic Engineering [50], which use cycles of oligonucleotide mutagenesis, and CRISPR-Cas9 which allows genetic editing in many species and even within communities [51–53]. One breakthrough in understanding fitness landscapes came from improvements in *in vitro* cloning techniques, and particularly CRISPR-Cas9 gene drives and hierarchical mating that now allow systematic assembly of combinatorically complete landscapes *in vivo* [54,55].

Open problems in the new era of experimental evolution

The essence of experimental evolution has been replication in controlled laboratory studies. However, many of the big open questions in evolutionary biology lie in the realm of evolution in natural environments. While the first two eras of experimental evolution focussed on basic questions about evolutionary dynamics in controlled settings, several recent evolution experiments have blurred the distinction between observing 'real-world' evolution and observing 'laboratory' evolution. Ground-breaking in this area has been the incorporation of technologies and insight from experimental evolution. The similarities between *in vitro* and *in vivo* experiments are now stronger than ever.

We note that it is still the dawn of *in vivo* evolution experiments, as we are capitalizing on tools and insights from *in vitro* experimental evolution. As the field develops, we expect a synergy will form where *in vivo* evolutionary studies will contribute to our understanding of core evolutionary principles (or to new technologies enabling more complex laboratory evolution), and this will feed back into further exciting areas. In this section,

Figure 2



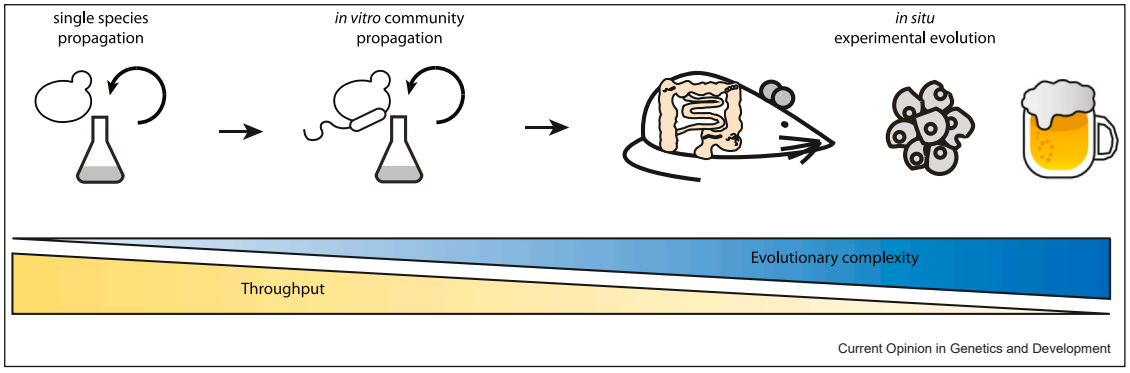
Technological progress in population phenotyping. With the increased throughput in culture maintenance, phenotyping throughput has kept pace by increasing phenotyping accuracy and by performing bulk measurements. However, bulk measurements are more challenging to establish for some complex phenotypes.

we discuss how combining novel current era technologies such as massively parallel barcoding, high-throughput robotics, and DNA/RNA sequencing with experimental evolution in more natural contexts has led to important insights in evolutionary biology, ecology, and human health, all of which offer a stunning array of ripe open questions amenable for investigation (Figure 3).

Synthetic communities

Most of the last 50 years of experimental evolution focused on constraining the system to one species. Indeed, an unattributed but popular saying is that experimental evolution with two species is simply called 'contamination'. However, recent work in microbial ecology, coupled with our enhanced control over biological systems, has renewed interests in studying the evolutionary outcome and potential of biological communities. These studies are reviewed in depth elsewhere [56], as they range immensely from mutualism and co-evolution of yeast and bacteria to cross-kingdom interactions between virus, algae, plants and fungi [57–61]. We highlight a few creative and original systems that are currently being explored. For instance, Goldford et al. assembled hundreds of naturally occurring soil bacteria *in vitro* and found their community assembly and within culture taxonomic variation to be highly predictable [62]. Bajic et al. showed that a community Flux Balance Analysis model was highly predictive of bacterial interaction and community assembly [63], and more recently, Gowda et al. were able to use a consumer-resource model inferred solely from genome sequencing data to predict interaction modes between bacteria in a soil community [64]. Yet other work has focused on

Figure 3



Increased complexity of experimental evolution. As our handle on biological processes improves, experimental evolution is now exploring evolution of microbial communities *in vitro* but is also exploring evolving processes *in vivo*. Experimental evolution in natural settings has lower throughput but benefits strongly from the technological progress that was refined by *in vitro* experiments.

engineering communities based on single species (for example, mutualistic cross-feeding communities), which removes complications due to evolutionary divergence of species [65,66].

Evolutionary innovations and transitions

Questions about evolution are deeply rooted in our fascination for the natural world. Evolutionary novelties, such as the emergence of multicellularity, endosymbiotic relationships, and novel genes have all recently been adopted as the focus of many experimental evolution studies, with many benefitting from high-throughput workflows. Some have looked at ecosystems in the real world that mimic the dynamics of experimental evolution systems [67–70], while others attempt to recreate it in the lab. For example, Ratcliffe et al. used a simple approach to select heavier yeast cells [71], which evolved yeast clusters that resembled snowflakes. Snowflake yeasts represent independent emerging multicellular 'organisms' whose evolution and properties can be studied in parallel. This and other carefully engineered systems have led to remarkable insight into how multicellular organisms might have evolved [72–76]. Similarly, Mehta et al. pioneered a system where they engineered auxotrophic *E. coli* cells to grow inside yeast cells lacking mitochondria [77,78], with the goal of studying the evolutionary dynamics of obligate endosymbionts in eukaryotes. Given the absence of ancestral fossils for microbial life, experimentally recreating important evolutionary events in a variety of contexts is our only strategy to make sense of our past. Innovative selection pressures from a 'naïve' context allow scientists to probe the fundamental rules of life and evolution while controlling for confounding factors due to historical contingency, and thus, when accompanied by new high-throughput tools, these experiments can provide an intimate and high-resolution view of previously unanswerable processes.

Human health

One very important extension of experimental evolution is the study of evolutionary dynamics of cells in the context of human health. Investigations into antibiotic resistance [79,80], the spread and evolution of infectious diseases [81,82], and the evolutionary dynamics of human microbiomes [83] are paramount to understanding human health. Indeed, a renewed understanding of human physiology and its tight coupling with the microbiome has emerged in recent years [84].

Gut and skin microbiomes offer an exciting avenue for exploration using tools developed from experimental evolution. These microbiomes offer constancy in terms of strain diversity, which allows for reproducibility and simplicity that has often been espoused by the field of *in vitro* experimental evolution. Moreover, spatial structure inside and on the body provides niches for hundreds of 'independent' populations simultaneously. By leveraging our abilities to observe evolutionary dynamics in real time, longitudinal sequencing in the human gut has shown evidence for rapid adaptation and diversification in line with observations from evolution experiments [85]. Indeed, Lieberman et al. sampled the microbiomes of healthy humans over time and found evidence for negative frequency dependence and coexistence among gut inhabitants [86], dynamics that have been experimentally characterized in the LTEE. These evolutionary stories also extend to the skin microbiome, where pores act as islands, following the ecological rules of island biogeography [87,88]. Finally, our understanding of founder effects and mutational biases from experimental evolution suggests that human microbiomes are seeded by a handful of starting strains, and that the community frequently turns over during the course of a lifetime [89]. Evidently, theory initially developed from observing the evolution of microbial populations have carried over.

Another topic that has received tremendous attention in recent years is the eco-evolutionary dynamics in host–pathogen interactions. Some studies have even looked at evolution on a global scale, with recent focus on HIV [90,91] and Covid19 [92]. The area of study is too broad for us to cover here, but one remarkable use of insights from experimental evolution has been in combinatorial phage therapy [93]. With the right phage and antibiotic combinations, evolutionary 'dead-ends' can be created by exploiting fundamental trade-offs discovered through simple laboratory evolve and re-sequence experiments [94,95]. Indeed, this has already been deployed in end-of-the-line situations [96].

The rapid spread of creative and complex model systems is pushing the boundaries of evolutionary dynamics. Evolution of cancer cells, and the drivers that mediate evolutionary pressures amongst them, has been gaining focus as cancerous cells eventually become resistant to therapies [97]. The predictability of this resistance is of immense importance for the care of patients, and similar attempts to measuring the evolutionary propensities of antibiotic resistance in a variety of bacterial systems has paved the way for similar experiments in cancer cells [98,99]. Similarly, modern DNA barcoding technologies developed in microbes are now being used in complex novel systems to track evolutionary dynamics and to study local interactions amongst cells. In a recent example, barcoded tumors in mice were used to study tumor progression across different chemotherapeutics [54].

Outlook and conclusion

The advent of modern technologies makes it tempting to follow the intellectual direction of the first two eras of experimental evolution: explore evolutionary space to see

the many surprises that drift and selection can together orchestrate from mere mutations. Yet, there is a new opportunity to shift gears towards focussed evolutionary questions and to take the road less traveled by. Thanks to explorers before us, we now have a solid foundation of molecular evolution and population genetics, and synergistically combining past framework with massively parallel and high-throughput technologies will lead to new discoveries in underexplored areas. Judicious use of technologies and their subsequent improvements, combined with a solid understanding of evolutionary dynamics and theory, will be crucial to gain insight amidst the fundamental limitations of throughput and complexity. We have provided a short but non-exhaustive summary of a few open areas in this perspective. Fundamentally, the intersection of experimental evolution with these fields has the greatest potential for pushing the boundaries of evolutionary biology.

Conflict of interest statement

The authors declare no conflict of interest.

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