Published in final edited form as:

Curr Opin Chem Biol. 2010 October ; 14(5): 568–573. doi:10.1016/j.cbpa.2010.07.023.

Microfluidic Landscapes for Evolution

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Summary

Evolution at its heart is an iterative algorithm composed of three steps: selection, amplification and mutagenesis. This algorithm can be applied to complex inputs such as populations of whole organisms and viruses, or mixtures of bare nucleic acids and proteins. The output is the same: evolutionary adaptation of new and improved function subject to selection. Recent breakthroughs in microfluidic technology have introduced automation and process monitoring to in vitro evolution, and reproducible preparation of emulsions and other multi-phase reaction landscapes. It is at this intersection of compartmentalization and in vitro evolution where miniaturization is again redefining experimental design in contemporary chemistry and biology.

Introduction

While studying the bacteriophages Q β and MS-2, Sol Spiegelman isolated the viral replicase proteins responsible for template-directed reproduction of the viral genomic RNA. In a brilliant experiment, he observed in vitro template-directed synthesis of new genomic RNA when the replicase was mixed with the four nucleoside triphosphates and viral RNA input. A small sampling of the genetic information in the reaction transferred to a new vessel containing only replicase and nucleoside triphosphates produced more RNA in less time. The size of the passaged genomic RNA gradually decreased until only a minimal replicase-binding sequence and a few bits of genetic information remained. The most efficiently replicating RNA sequences, now free of the selection burden to produce infectious particles, had shed all functional genes during in vitro enzymatic replication [1]. “Potentially, other selective stresses can be imposed on the system to generate RNA entities which exaggerate other molecular features,” Spiegelman wrote, and so was born the idea that the principles of Darwinian evolution could be directly applied to molecular populations to discover sequences with new functionality.

Evolution either in vitro or in vivo is the iterative execution of three processes: selection, amplification and mutation (Figure 1). From a diverse population of individuals, members with desirable traits enjoy a reproductive advantage under given selection pressures. The genetic information encoding the desired traits is imperfectly inherited by progeny, which constitute a new population adapted to the selection pressure. The key constraint and absolute requirement for any system to undergo evolutionary adaptation is an association of selected trait (phenotype)

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Conflicts

The author declares no conflicts of interest.

and the information encoding that trait (genotype). The pioneering Q β experiments embody the simplest and most efficient evolving system: the molecules are simultaneously phenotype and genotype, and fitness is measured as a replication advantage. However, the confines of the Q β system are narrow, and generalization to selection for any chemical activity was not obvious at the time.

The advent of sequence-specific DNA and RNA amplification technology catalyzed three seminal investigations that taught generalization of Darwin's principles to any nucleic acid sequence with selectable function. RNA sequences that unexpectedly bound organic dyes [2] and polymerases [3], or that catalyzed phosphodiester transfer [4] were the fruits of these expeditions into RNA sequence space. The field exploded with new proof that selected RNA motifs can recapitulate key biochemical activities found in modern biology [5], and the use of display approaches for evolving non-informational proteins and peptides followed shortly.

Regardless of the macromolecular target's composition or the nature of the desired activity, in vitro evolution campaigns share several characteristics. First, key substrates and mutant libraries are synthesized, and selection or screening methods are developed. Then, evolution proceeds in an iterative series of selection or screening steps followed by amplification and mutagenesis steps. These steps are repeated until the population exhibits a desirable level of new activity. Cloning, sequencing and phylogenetic analysis ensue to identify the mutations conferring the new and improved activity. These recursive chemical operations present a key challenge and opportunity for microfluidic technology because automation of repetitive sample handling procedures and real-time quantitation are hallmarks of microfluidic systems. In addition to freeing the human scientist from the burdens of selection, miniaturization is also a route to new landscapes for in vitro evolution. Microfluidic droplet generators, gradient flow chambers, and microfabricated solid supports pose compelling new environments that were once unattainable or unimaginable in the last age of tips, tubes and pipettors.

Continuous Culture

In vitro evolution can be conducted either continuously or in a step-wise fashion. Continuous in vitro evolution most closely resembles evolution in vivo because selection, amplification and mutagenesis occur contemporaneously, and fitness is directly measured by replication rate. Thirty years after Spiegelman's experiments, selective isothermal amplification produced the first catalytic molecules by requiring members of the population to clear a catalysis hurdle in the race to be the best amplifier. RNA molecules were challenged to catalyze single turnover ligation of a promoter-containing oligonucleotide substrate. Ligating the promoter element to the molecule's terminus tags the sequence for transcription of progeny, and evolutionary adaptation occurs as the population is serially passaged [6].

Serial passaging is a method for population maintenance most commonly encountered in microbiology. As a microbial population expands, nutrients are continuously perfused to maintain the population in constant exponential growth. The first demonstration of this principle in microfluidics at the molecular scale was a simple flow-based reactor in which isothermally amplifying DNA sequences were maintained against a steady flow of nucleotide building blocks, polymerase enzymes and primers [7]. Population maintenance was also one of the first demonstrations of very large scale integration in microfluidics. A microchemostat composed of a mixing loop and multiple reagent manifolds was used to maintain a small culture of *E. coli* in log phase. The microfluidic loop was divided into 16 independently addressable segments to purge the reactor loop of biofilms, a tour de force of valve integration architecture that demonstrated long-term propagation of minute bacterial populations. Further studies using the microchemostat focused on population ecology in a model predator-prey system composed

of two *E. coli* strains [8]. On-line process monitoring for bacterial growth, such as dissolved oxygen content and cellular density are also readily integrated [9].

A simpler microfluidic serial dilution circuit (Figure 2A) maintained a population of catalytic RNA molecules undergoing continuous in vitro evolution. The circuit featured inputs for seeding and delivery of fresh enzyme and nucleotide reagents. The 5-valve reactor is programmed to flush growth medium into the loop, preserving a small plug of material for passaging. Once flushing is complete, the carryover is mixed into fresh growth medium using computer-controlled pumping [10]. The device was interfaced with an inverted confocal fluorescence microscope, which monitored the synthesis of new RNA by fluorescence. The population adapted catalytic efficiency by improving the Michaelis constant a commensurate 90-fold over 500 logs of continuous in vitro evolution given the selection constraint of systematically reduced oligonucleotide substrate concentration [11].

Step-Wise & Compartmentalized Evolution

Coupled selection and amplification is a rare experimental luxury for in vitro evolution. Amplification, selection and mutagenesis are much more often executed separately in step-wise fashion. For example, an experiment evolving aptamers, nucleic acid sequences that specifically bind a target ligand, includes: 1) binding the population to a stationary phase presenting the ligand, 2) washing to eliminate non-specific binding, 3) elution with free ligand, and 4) amplification, mutagenesis and regeneration of the adapted progeny aptamers. The benefits of automation in step-wise evolution were recognized very early on, and an “evolution machine” was designed for high-throughput aptamer selections based on pipetting robots and plate readers [12]. Microfluidics promised the same automation benefits coupled with integration and disposability.

The first three steps have been executed nicely in a device that magnetophoretically isolates aptamers to Botulinum neurotoxin type A. Ligand-coated ferromagnetic beads and aptamer population are introduced into a microfluidic flow bed with co-flowing wash buffer. Integrated metallic guides precisely align the beads into the center of the washing flow under an applied magnetic field, partitioning the aptamer-bound beads from the unbound sequences (Figure 2B) [13]. Washing stringency can be quantitatively controlled in these flow-type reactors, as demonstrated in the phage display stringency-dependent enrichment of the HQP streptavidin-binding motif from a randomized library [14].

Evolving molecules with specific binding properties is generally a well understood process. More complex chemical activities, such as multiple turnover catalysis, membrane transport and signaling remain challenging targets because it is difficult to establish the requisite association between genotype and phenotype. In vitro compartmentalization [15] offers an alternative method for associating genotype and phenotype in which a population of genes is dispersed in a stable water-in-oil emulsion with each emulsion droplet housing a single gene. The aqueous phase also contains the biochemical machinery needed to translate the gene into functional nucleic acid or protein molecules, and probe substrates that transduce the enzymatic activity of interest. High-throughput screening or genetic selection can be used to isolate droplets that contain genes encoding highly active enzymes.

There are several problems associated with in vitro compartmentalization. First, conventional bulk emulsification produces highly heterogeneous droplets, which confounds stochastic dilution. As a bulk process, there is little control over droplet size, and procedures generally describe stirring or homogenization, which are not easily parameterized. Second, emulsification proceeds through vigorous mixing or shearing, with more uniform and stable emulsions generated after prolonged emulsification. However, extended homogenization

diminishes biochemical activity. Microfluidic technology has largely addressed these limitations.

Droplet production is the microfluidic analog of emulsification. Unlike bulk emulsification, microfluidic droplet generators produce monodisperse droplets with no mechanical homogenization. Droplet generation is also easily specified by the chemical composition of the aqueous and oil phases, the flow rates of the two phases, and the circuit geometry. All of these parameters are readily quantified and reproduced in other laboratories.

The last two years have delivered significant technological breakthroughs in droplet production and handling. Droplet production has now been studied and quantified at the single droplet level using microfluidics. Individual droplets can be formed in oil/surfactant suspensions and assessed for stability [16] and leakage [17]. Circuit components have been designed for operations directly relevant to compartmentalized *in vitro* evolution, such as on-line droplet merging [18], droplet array incubation [19], and integrated fluorescence-activated droplet sorting [20]. Other circuits have addressed various singular aspects of screening-based evolution, such as compartmentalized *in vitro* bacterial translation [21–23], enrichment of complex eukaryotic enzymes in compartmentalized retroviral display [24], and on-line kinetic analysis of enzymes that operate far from the reaction conditions of *in vitro* translation [25]. These elements have been seamlessly stitched together in an elaborate microfluidic circuit that combines droplet generation, delay line incubation, and droplet sorting (Figure 3A). Single bacterial cells displaying variants of horseradish peroxidase (HRP) were loaded into droplets with fluorogenic HRP substrate, fluorescent droplets were retained and subject to multiple rounds of screening. The device produced several improved mutants, one being seven-fold improved over wild type activity [26].

New Expeditions in Sequence Space

It is a well-known fact in directed evolution that the probability of discovering truly superior mutants is positively correlated with population size. The future challenges facing *in vitro* evolution emanate from this core observation.

Addressing population size is relatively straightforward: droplet generation throughput must increase. Parallelization is an obvious route for achieving higher throughput in microfabricated circuit architectures, and recent papers describe array-based approaches to parallel droplet generation [27,28]. Flow focusing can be parallelized with careful layout of oil and aqueous channels. Topological constraints for constructing a network of 32 or 96 cross-shaped intersections are non-trivial, but surmountable with appropriate chucks and manifolds for addressing multiple common-reagent inputs (Figure 3B) [27]. Large numbers of aqueous streams undergoing parallel emulsification result in higher droplet generation rates, faster aqueous sample processing, and lower back pressure. An example of this approach is a simple two-layer device containing 110 shallow and narrow aqueous phase-conducting nozzles that intersect a deep and wide annular oil manifold. As aqueous phase is pumped through the central reservoir radiating outward to the manifold, the orthogonal oil flow shears droplets off at rates starting in the kHz range, easily scaled to 50–100 fold higher (Figure 3C). The prototype array was used to compartmentalize single catalytic RNA parent molecules, enzymatic replication reaction mix, and aminoglycoside antibiotic inhibitor of RNA-mediated catalysis. Compartmentalization in this experiment prevented the rise of a dominant master sequence, allowing all antibiotic resistant variants to persist and multiply [28].

Commensurate with increased droplet production, droplet volumes must decrease for purely practical reasons. A modest homogeneous solution population of RNA aptamers is $\sim 10^{13}$ molecules, corresponding to 100 μL of 1 μM RNA. To achieve the same population size in 10-pL microfluidic droplets would require an oceanic 100 L of aqueous phase for

emulsification. Reducing droplet capacity to 10 fL, the volume of an average bacterial cell, requires 100 mL aqueous phase; still a large volume, but approachable in scale.

The other matter requiring attention is the process of identifying desirable variants and touches on the fundamental difference between selection and screening. Screening procedures assess each variant individually, and sort according to some parameter. Bacterial plates, chip-based droplet and cell sorters, and their ancestral microplates and readers are examples of screening procedures. Screening by definition requires time based on the method's throughput and the population size. Selections operate on the population in bulk. Selective bacterial culture, affinity chromatography, and electrophoretic separation are examples of selection. The time required to perform selection is *independent* of population size. Ever larger population sizes will demand innovative reaction designs that facilitate selection. Examples of successful selection mode experiments carried out in compartments include compartmentalized self replication [29] of superior polymerase mutants that efficiently and selectively replicate their co-encapsulated gene and RNA-catalyzed Diels-Alder coupling [30] are two examples of the power of selection targeting catalytic phenotypes. The array device of [28] also favors selection over screening; droplets containing antibiotic-resistant catalysts produce progeny and droplets containing susceptible parents fail to replicate. Selection occurs when the droplets are heat-deactivated, pooled and subject to PCR amplification. The chip is nothing more than a tool for making "fancy mayonnaise" to be collected and processed.

The most exciting innovations in microfluidic evolution will be born out of new microchip technology for generating novel environments, or landscapes, and in studying the new molecular fauna spawned from traditional tube-based evolution experiments. For example, a second continuously evolving catalytic RNA motif was recently discovered [31], spurring studies of co-evolution [32] in continuous molecular cultures, and a later study demonstrated enzyme-free self-sustained cross-catalytic RNA replication [33]. Adding systematically-controlled compartmentalization to these systems eliminates the rise of dominant master sequences, allowing broad surveys of catalytic function and diversity in sequence space, and modeling the exchange of genetic components between cell-like compartments [34].

This latter point highlights an area where microfluidic technology is poised to make the biggest impact. Water-in-oil emulsions and droplets are only the first crude steps toward simulating cellular compartmentalization. True cellular compartments are not two-phase systems. Rather, they are single-phase vesicles with miscible intra- and extravesicular phases separated by a bilayer of amphipathic molecules. Controlling and understanding compartmentalization is central to studies of the chemical origins of life and Darwinian evolution [35,36], but there are no rational routes to cell-sized vesicles with controlled size, lamellarity, and defined encapsulation efficiency. Simpler bulk methods accept uncontrolled lamellarity in favor of size exclusion-based panning for micron-scale structures [37]. Microfluidics is making inroads here, too, with circuits that produce sub-micron monodisperse and unilamellar vesicles by flow focusing [38], templated vesicle formation in water/oil/water double emulsions [39], and interfacial micro-jetting [40].

Conclusions

In vitro evolution is blazing new trails in macromolecular discovery and optimization, and in understanding the adaptation and plasticity of biological information. Technological innovations in microfluidic circuitry for water-in-oil droplet generation in particular are playing a central role given the indispensable nature of compartmentalization. And, the possibility of integrated droplet handling and interrogation shows great promise for automating large-scale screening experiments. Open-ended challenges remaining for the future of microfluidics in directed evolution include higher throughput and more complex compartment generation, and

innovations in selection-based approaches for finding the golden needles in the ever larger haystack of droplets.

Acknowledgments

This work was supported by a National Institutes of Health Pathway to Independence Career Development Award (GM083155).

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•• Outstanding Interest

• Special Interest

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Figure 1.

In vitro evolution of macromolecular function mirrors organismal evolutionary adaptation. Members of a population are subject to selection pressure, favoring certain individuals and disfavoring others. Selected individuals reproduce and their progeny inherit the genetic information that conferred fitness to their parents. Amplification of genetic material during reproduction can be imperfect, resulting in contemporaneous diversification and amplification. Alternatively, amplification and mutagenesis can be executed step-wise. Example populations are composed of finches from the Galápagos (genus *Geospiza*) or RNA sequences selected to catalyze phosphodiester transfer.

Figure 2.

Continuous flow microfluidic circuits and biochemical outputs. Reactor channels are shown in black, reservoirs are indicated by black circles and valves are indicated by gray squares. (A) A serial dilution circuit features reagent input, I, and output for analysis, O. Two bus valves control access to the main reactor loop, and three in-line valves effect mixing. Biochemical output of the device was an evolved enzyme with markedly improved K_M (0.4 μM , closed circles) when compared to the parental enzyme (35 μM , open circles). (B) A microfluidic aptamer selection circuit featured magnetophoretic separation in an applied magnetic field with microfabricated field-guiding conductors (magnified, *inset*) that drove bead-bearing aptamers from the peripheral input flows (I) into the center washing flow (wash). By guiding the beads into the center of flow, the trifurcated outlet separated product beads in the central outlet (O) from the peripheral waste streams (W). Biochemical output of the device was a selected sequence with ~30 nM affinity for the *Botulinum* type A neurotoxin (closed circles) compared to the starting library, which had no measurable affinity (open circles). Adapted with permission from [11] and [13].

Figure 3.

Compartmentalization circuits. (A) Compartmentalized evolution can be achieved by flow focusing two aqueous precursor streams, AQ1 and AQ2, with oil to produce monodisperse droplets. Droplets exit the device and enter a delay line, later re-entering the device with oil injection to focus the droplet stream onto a confocal fluorescence detector. Dielectrophoresis electrodes, DEP, activate on fluorescence to deflect droplets either into a retention well, OUT, or otherwise defaulting to waste, W, in the absence of DEP. (B) An array configuration of the oil-sheathing intersection accomplishes monodisperse generation at 32 nodes simultaneously. Aqueous input is introduced into the central AQ reservoir with on-line valve-driven pumping, intersected by the oil manifold (gray), and continuing out to 16 independent emulsion outputs, OUT. (C) Compartmentalization can be achieved at high rates using array approaches, exceeding 10^7 droplets per hour. An array of 110 aqueous phase channels, AQ, empties into a wide and deep annular manifold channel with continuously flowing oil. Droplets are collected at OUT for further processing. The device is fabricated in PDMS using a dual-height mold to specify the two different channel depths, and is scalable to at least ~1,000 nozzles. Schematics are adapted with permission from [26], [27] and [28].