MICROFLUIDIC DARWINIAN EVOLUTION
Brian M. Paegel and Gerald F. Joyce
The Scripps Research Institute, USA

ABSTRACT
Directed evolution is the application of Darwinian principles to populations of molecules. Repeated rounds of selection, mutagenesis, and amplification yield macromolecules with desired chemical functions. Conventional approaches are necessarily ad hoc, relegating the control of experimental parameters such as selection pressure and mutagenesis to guesswork. We have constructed the first chip-based system that interfaces computer control with Darwinian evolution to eliminate manual sample manipulation and to provide real-time feedback describing evolutionary progress.

KEYWORDS: evolution, RNA, catalysis, dilution

INTRODUCTION
Darwin’s theory of evolution is the unifying principle that codifies biological organization from organismal morphology to genome structure. In the laboratory, directed evolution is the application of Darwinian principles to populations of molecules. Repeated rounds of selection, mutagenesis, and amplification yield macromolecules with desired chemical functions. These principles were applied to populations of RNA molecules to select for sequences with RNA ligase activity by way of continuous in vitro evolution [1]. In this reaction, amplification is selective for RNA sequences that can catalyze the ligation of a promoter-bearing oligonucleotide substrate to their own 5'-triphosphate termini. This transformation marks the molecules for replication by reverse transcriptase and RNA polymerase. Progeny RNA are produced with new 5'-triphosphate termini, and can participate in a new round of catalysis and replication.

As the population expands, those individuals exhibiting the most advantageous combination of catalysis and replication rate propagate their genetic material most rapidly and dominate the reaction. When the population has exhausted the substrate materials in the reaction mixture, a small aliquot of the material is diluted into fresh reaction mixture, allowing the population to propagate. Computer control and online monitoring of this process was achieved using a glass-PDMS-glass microfluidic serial dilution circuit [2], eliminating manual pipet-based sample handling and allowing real-time visualization of Darwinian evolution at the molecular scale.

EXPERIMENTAL
The microfluidic serial dilution circuit was modified to accept two input sample streams for continuous in vitro evolution (Fig. 1). The substrate input stream (promoter-containing oligonucleotide substrate, cDNA primer, dNTPs, NTPs) and enzyme input stream (T7 RNA polymerase, reverse transcriptase, inorganic pyrophatase) mix 1:1 at the in bus valve. Each input stream contains common reagents, including mono- and divalent cations, DTT, nonionic detergent, and thiazole orange intercalating dye. Thiazole orange is used for homogeneous fluorescence monitoring of polynucleotide synthesis. Reagent inputs and product output are connected to the device via PEEK capillary tubing and Nanoport fittings (Upchurch Scientific).
The circuit was initially seeded with a mutagenized population of one billion individuals derived from a previously isolated catalytic RNA [3] and challenged to amplify under conditions of reduced substrate concentration (1 µM). When the population reached the desired 10-fold increased concentration as monitored by homogeneous fluorescence, the in, out, a and b valves were opened while keeping valve c closed, flushing fresh enzyme and substrate reagents through the loop portion containing a and b, and isolating one tenth of the reaction as carryover in the loop portion containing c. The spent reaction materials were collected in EDTA quenching buffer. Once flushing was complete, in and out were closed and a, b, and c were serially actuated to mix the carryover into the fresh reagents, initiating a new burst of selective 10-fold amplification.

RESULTS AND DISCUSSION

The RNA population was challenged to catalyze the ligation of the oligonucleotide substrate under conditions of progressively lower substrate concentrations. The serial dilution circuit automated an exponential growth phase followed by a 10-fold dilution, which was repeated for 500 log-growth iterations (Fig. 2, top). The population was periodically removed from the device and mutagenized to introduce diversity. The time required for the population to achieve 10-fold growth was plotted as a function of run time, a measure of the instantaneous fitness of the population (Fig. 2, bottom). Evolution was observed in real time as the population adapted and achieved progressively faster growth rates over time.

The final evolved enzyme contained a set of 11 mutations that conferred a 90-fold improvement in substrate utilization, $K_M$, which allowed the molecule to catalyze substrate ligation efficiently under conditions of low substrate concentration (Fig. 3). Analysis of the final evolved molecule revealed a new mode of substrate binding, and confirmed the function of a substrate-proximal region of the RNA [4].

CONCLUSIONS

The system presented here was used to evolve a RNA molecules with superior substrate utilization by pressuring the population to amplify under progressively diminished substrate concentration. This miniaturized approach reduces evolution to a microfluidic algorithm, allowing the experimenter to observe and manipulate evolutionary adaptation.
REFERENCES


Figure 2. Chip-based evolution of an RNA enzyme over 500 logs of selective amplification. The population was monitored in real time based on fluorescence intensity (middle). After each log of growth (i), a 10-fold dilution was executed by microfluidic manipulation. The substrate concentration was reduced periodically to maintain selective pressure on the population ([S] in µM). With each reduction in substrate concentration, sample was removed from the chip, amplified using error-prone PCR, and re-introduced into the circuit. The time between dilutions (Δ) was measured as a function of time and plotted to display the instantaneous fitness of the population at each log growth cycle (bottom). Adapted from [4].

Figure 3. Michaelis-Menten analysis of the final evolved enzyme. The evolved enzyme (●) exhibited a $k_{cat}$ of 21 min$^{-1}$ and $K_M$ of 0.4 µM. The starting enzyme (○) exhibited a $k_{cat}$ of 20 min$^{-1}$ and $K_M$ of 35 µM. Adapted from [4].