Precise, automated control of conditions for high-throughput growth of yeast and bacteria with eVOLVER

Brandon G Wong[1,2,6], Christopher P Mancuso[1,2,6], Szilvia Kiriakov[1,3], Caleb J Bashor[4] & Ahmad S Khalil[1,2,5]

Precise control over microbial cell growth conditions could enable detection of minute phenotypic changes, which would improve our understanding of how genotypes are shaped by adaptive selection. Although automated cell-culture systems such as bioreactors offer strict control over liquid culture conditions, they often do not scale to high-throughput or require cumbersome redesign to alter growth conditions. We report the design and validation of eVOLVER, a scalable do-it-yourself (DIY) framework, which can be configured to carry out high-throughput growth experiments in molecular evolution, systems biology, and microbiology. High-throughput evolution of yeast populations grown at different densities reveals that eVOLVER can be applied to characterize adaptive niches. Growth selection on a genome-wide yeast knockout library, using temperatures varied over different timescales, finds strains sensitive to temperature changes or frequency of temperature change. Inspired by large-scale integration of electronics and microfluidics, we also demonstrate millifluidic multiplexing modules that enable multiplexed media routing, cleaning, vial-to-vial transfers and automated yeast mating.

Living organisms exist in complex environments that select for evolved phenotypes[1–3] (**Fig. 1a**). In the laboratory, experiments using growth selection in liquid culture have been integral to studying the relationship between genotype and phenotype. These include functional genomic library screening and selection[4,5], characterization of natural and synthetic cellular systems[6–8], and directed evolution to either study evolutionary processes[9–11] or evolve new functions[12–15]. Development of technologies for precise control of growth conditions has not kept pace with improvements in genotype characterization using sequencing technologies. For example, bioreactors are highly controllable but are low-throughput, whereas batch cultures are difficult to control but can be high-throughput. This has hampered understanding how genotypes evolve under selective pressure[16] and constrained experimental designs.

The main challenge to designing growth selection experiments is balancing the tradeoff between control and throughput (**Fig. 1b**). Batch cultures maintained by serial passage permit parallel testing of many strains and conditions, but are inherently discontinuous and offer limited temporal control[17]. Although automated cell growth systems can maintain constant growth rates under precisely defined conditions, they are difficult to parallelize due to cost, space, and design complexity[16–18]. The convergence of several open-source technologies—inexpensive additive manufacturing, DIY software/hardware interfaces, and cloud computing—has enabled facile fabrication of custom laboratory platforms[19–22]. Various examples of automated cell culture include devices for automated dilution routines to investigate acquisition of antibiotic resistance[23], real-time monitoring of bulk fluorescence[7], light-based feedback control of synthetic gene circuits[24],

and chemostat parallelization[25]. Unfortunately, the *ad hoc* design of these systems limits their scalability and restricts their reconfiguration for other experimental purposes.

We present eVOLVER, a DIY framework that offers users the freedom to define the parameters of automated culture growth experiments, for example, temperature, culture density, and media composition, and scale them to any size. eVOLVER is constructed using highly modular, open-source wetware, hardware, electronics, and web-based software that can be rapidly reconfigured for virtually any type of automated growth experiment. eVOLVER can continuously control and monitor hundreds of individual cultures, collecting, measuring, and recording experimental data in real time, for any timescale. Facile programming of algorithmic culture 'routines' is possible, such that feedback between the growing culture and the system couple the status of a culture (e.g., high optical density (OD)) to its automated manipulation (e.g., dilution with fresh media). By combining this programmability with arbitrary throughput scaling, eVOLVER can be used for fine resolution of fitness landscapes, or determination of phenotypes that arise during selection.

We apply eVOLVER to carry out diverse growth and selection experiments. First, we evolve yeast populations in multiple selection conditions at high throughput and measure evolved fitness in several conditions to assess adaptive outcomes. Next, by performing growth selection for a yeast knockout library under temporally variable temperature stress conditions, we show that eVOLVER can be used to explore the relationship between environmental fluctuations and adaptive phenotypes. Finally, by integrating millifluidic multiplexing modules, we show that eVOLVER can carry out complex fluidic

---

[1]Biological Design Center, Boston University, Boston, Massachusetts, USA. [2]Department of Biomedical Engineering, Boston University, Boston, Massachusetts, USA. [3]Program in Molecular Biology, Cell Biology and Biochemistry, Boston University, Boston, Massachusetts, USA. [4]Department of Bioengineering, Rice University, Houston, Texas, USA. [5]Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, Massachusetts, USA. [6]These authors contributed equally to this work. Correspondence should be addressed to C.J.B. (caleb.bashor@rice.edu) or A.S.K. (khalil@bu.edu).
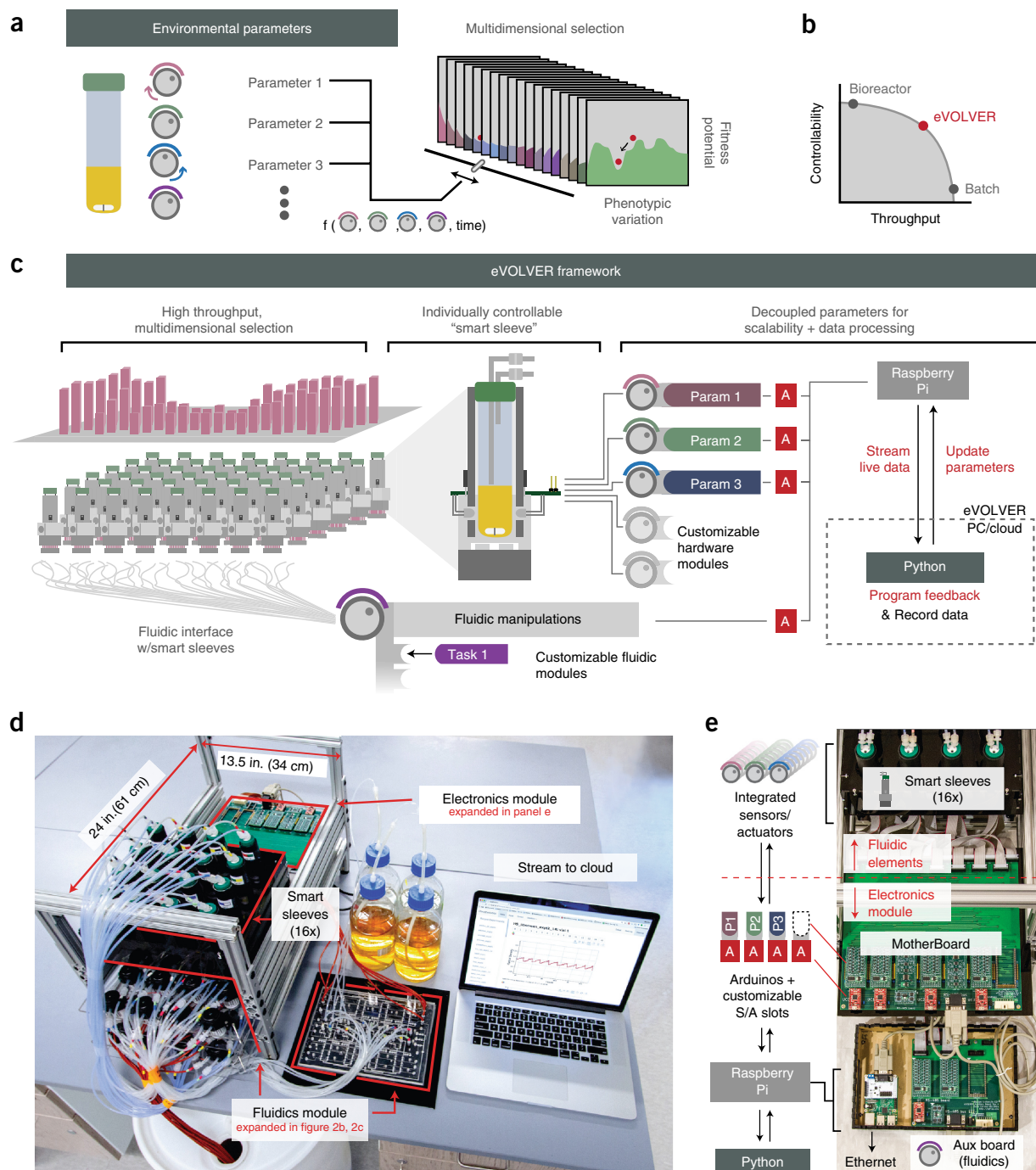
Figure 1 eVOLVER: an integrated framework for high-throughput, automated cell culture. (**a**) Understanding how cellular phenotypes arise from multidimensional selection gradients requires multiparameter control of culture conditions. (**b**) Growth fitness experiments face a tradeoff between precision control of culture conditions and throughput. eVOLVER enables reliable scaling along both axes. (**c**) eVOLVER hardware, fluidic, and software modules. System design is modular and synergistic. Left: eVOLVER is designed to scale to high-throughput. Center top: smart sleeve unit. Smart sleeves integrate sensors and actuators needed to measure and control parameters of individual cultures. Center bottom: eVOLVER fluidic manipulation system (peristaltic pumps or millifluidic devices) controls movement of media and culture within the system. Right: a modular, scalable hardware architecture interfaces with smart sleeve and fluidic modules to achieve individually addressable, real-time culture control. The hardware functions as a bidirectional relay, streaming live data (via Raspberry Pi) collected from each smart sleeve to the external computing infrastructure running control software (written in Python). This software records and processes data and returns commands to the hardware in order to update culture parameters. System customization can be achieved by swapping fluidic handling devices, adding new parameter control modules, or programming new feedback control routines between culture and software. A, Arduino microcontrollers. (**d**) 16-culture eVOLVER base unit. Fluidics (media input, waste output) are physically separated from the electronics. The base unit can be cloned and parallelized to increase experimental throughput. (**e**) eVOLVER hardware architecture. Smart sleeves communicate with electronics module via a motherboard. Control modules, which control single parameters across all smart sleeves within a 16-culture unit, are composed of Arduino-connected control boards occupying motherboard sensor/actuator (S/A) slots. Arduinos are programmed to interpret and respond to serial commands from the Raspberry Pi, which communicates with software run on a user's computer or server.
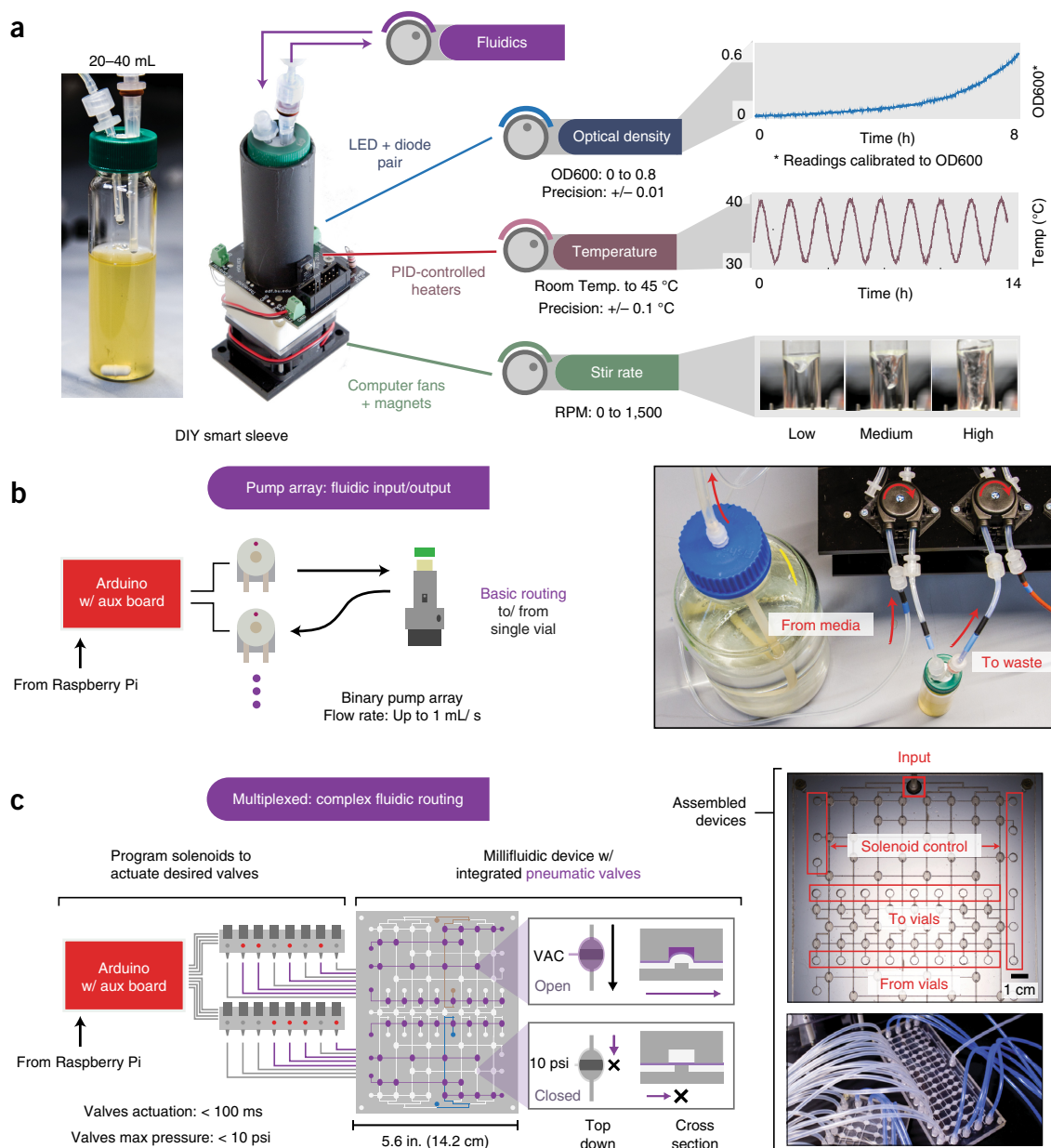
Figure 2 Design and performance of eVOLVER modules. (**a**) Generalizable configuration of smart sleeves for continuous culture; control of fluidic input/output, optical density, temperature, and stir rate. Left: smart sleeves are designed to accommodate 40 mL autoclavable borosilicate glass vials. Efflux straw length determines culture volume. Center: smart-sleeve-integrated electronic components. LED–photodiode sensor pairs perform OD_{900} readings. Thermistors and heaters attached to a machined aluminum tube maintain PID temperature control. Magnet-attached computer fans rotate stir bars inside the vials. Components are wired to a printed circuit board and mounted on an inexpensive 3D printed chassis. Individual sleeves cost ~$25 and can be assembled in ~10 min. Right: Specifications of smart sleeve parameters: optical density, temperature, and stirring. Device measurement precision varies with experimental conditions (e.g., cell type, room temperature) but can be adjusted to achieve necessary precision and range (e.g., tuning temperature PID constants, or filtering OD measurements) (**Supplementary Note 4**). Reported values are typical for experiments described in **Figures 3** and **4**. Calibration may be performed as often as desired, though settings are largely invariant over thousands of hours of use. (**b**) Basic fluidic handling in eVOLVER utilizes pumps with fixed flow rates of ~1 mL/s and can be actuated with a precision of ~100 ms. (**c**) Millifluidic multiplexing devices enable novel, customized liquid routing. Devices are fabricated by bonding a silicone membrane between two plastic layers with laser-etched flow channels. Integrated pneumatic valves actuate on the membrane to direct fluidic routing from media input to output ports (to or from vials).

manipulations, thereby extending the scope and range of possible growth and selection experiments.

RESULTS
Design of an automated cell culture framework
Most automated culture systems are designed to address specialized needs and control just one or more predetermined culture parameters.

We designed eVOLVER so that it could be configured to measure and control an arbitrary set of user-defined parameters. In order to accomplish this, the hardware design enables rapid, cost-effective scaling and customization, including with future technology (**Fig. 1c** and **Supplementary Figs. 1–3**). eVOLVER hardware comprises three modules (**Fig. 1d**). First, a customizable 'smart sleeve' houses and interfaces with individual culture vessels. Second, a fluidic module

controls movement of liquid in and out of each culture vessel. Third, the modular hardware infrastructure simplifies high-volume bidirectional data flow by decoupling each parameter into individual microcontrollers (**Fig. 1e**). A detailed description of eVOLVER design and construction can be found in **Supplementary Notes 1–11**, **Supplementary Code**, and https://www.fynchbio.com.

The smart sleeve mediates monitoring and control of growing cultures (**Fig. 2a**). Each sleeve is composed of a machined aluminum tube (for temperature control), printed circuit board mounted with sensors, actuators, and other electronic components, all attached to a custom three-dimensional (3D) printed mount. The smart sleeve can be readily mass-produced for high-throughput experiments, or reconfigured to meet custom experimental needs, such as larger culture volumes, pH or oxygen sensors for bioprocess applications, or light-emitting diodes for optogenetic studies. In this article we use a smart sleeve configuration that accommodates 40-mL glass vials, and is configured to control three experimental parameters: stirring, temperature, and culture density (**Fig. 2a** and **Supplementary Figs. 4–8**).

eVOLVER's fluidic module controls movement of media, culture, and liquid reagents in the system (**Fig. 2b,c** and **Supplementary Fig. 9**). It can be configured to two fluidic handling modes: either a basic mode, which uses peristaltic pumping to control media influx and efflux for each culture[26] (**Fig. 2b**), or a complex mode in which multiplexed routing enables sophisticated fluidic manipulations (**Fig. 2c**). User-actuated peristaltic pumps are robust, simple to use, and can be scaled to carry out media dilution routines for multiple parallel continuous cultures[25,26] (**Supplementary Fig. 10**). However, they scale poorly to more sophisticated routing where the number of required connections and control elements expands nonlinearly with the number of cultures. Our complex fluidic mode applies principles of large-scale integration (LSI) to overcome this limitation. Originally developed for electronic devices, and then adopted in microfluidics[27,28], LSI uses combinatorial multiplexing to expand the number of input-output paths per control channel[29,30]. Inspired by LSI, we created physically compact, millifluidic multiplexing devices by adhering a silicone rubber membrane between two clear sheets of laser-etched plastic, each patterned with desired channel geometries and aligned to form an intact device (**Fig. 2c** and **Supplementary Fig. 11**). Devices can be designed as needed to carry out custom fluidic protocols, including complex media-dispensing routines, transfer of liquid between cultures, or periodic cleaning protocols to maintain sterility. Millifluidic device design and fabrication protocols are presented in **Supplementary Note 6**.

We developed a hardware and software infrastructure for eVOLVER that complements the smart sleeve and fluidic modules (**Fig. 1c**). Our design uses a central organizing printed circuit board (motherboard) that controls core functionalities (e.g., serial communication, signal routing) and contains slots for plug-in boards that manage specialized features (e.g., temperature control; **Fig. 1e** and **Supplementary Figs. 1–3**). The control modules—custom-printed circuit boards wired to Arduino microcontrollers—read and power smart-sleeve-mounted components. During an experiment, individual control modules manage each culture parameter (e.g., temperature, stirring). A motherboard distributes control of up to four independent parameters across a set of 16 smart sleeves, comprising a single eVOLVER base unit (**Fig. 1d**). Microcontrollers associated with each base unit are coordinated by a single Raspberry Pi, which is a small, low-cost, single-board computer that serves as a bidirectional relay to a user's computer or a cloud server (**Fig. 1c,e**). The modularity of this design facilitates repurposing and scaling; users can easily modify or augment eVOLVER's experimental capability by connecting new control modules, while additional base-units can easily be cloned to achieve higher throughput (**Fig. 1d** and **Supplementary Fig. 12**).

An important feature of eVOLVER's design is the use of network connectivity to coordinate and run experiments over the internet. eVOLVER's distributed hardware architecture enables efficient transmission of large packets of high-dimensional, real-time data (**Fig. 1c,e**) such that a single computer, located anywhere with an internet connection, can monitor hundreds of cultures in real time (**Supplementary Fig. 3**). Python scripts manage the acquired data and execute the control algorithms that define a selection scheme.

For example, during a typical data acquisition/control protocol, a scripted routine can query the Raspberry Pi every 30 s for smart-sleeve-acquired culture status data, such as temperature or optical density (**Fig. 1c,e**). Recorded data are algorithmically converted into higher-order data (e.g. growth rate), which can be used to update individual smart sleeves or fluidic channels with new settings, such as adjusted temperature or media dilution. By modifying scripts, a user can change selection criteria (**Supplementary Fig. 13**), or specify a selection pressure design by subtle iterations of the same algorithm across multiple smart sleeves.

The eVOLVER system presented in this article can run long-term (250+ h) experiments without electronic or software failure (**Fig. 3** and **Supplementary Fig. 14**). Most components have a lifetime of >4,000 h, so hardware replacement is unlikely during most experiments. Hardware calibration can be done as much as needed, but we found that settings were unaltered during dozens of experiments that comprised more than 1,700 h of operation (**Supplementary Figs. 4**, **6**, **8**, and **10**). eVOLVER is designed to withstand liquid spills, with smart sleeves and fluidic components physically separated from control hardware (**Fig. 1e**). Additionally, the system can be set up to avoid microbial contamination. In a control experiment, we found that the system remained sterile for a ~10-d experiment while incubating uninoculated, antibiotic-free media alongside passaged *Escherichia coli* cultures (**Supplementary Fig. 14**).

Experimental yeast evolution

We first configured eVOLVER to function as a turbidostat in order to study the relationship between culture density and fitness in yeast populations. Culture density was maintained in a constant, defined window bounded by lower and upper OD thresholds ($OD_{lower\ threshold} - OD_{upper\ threshold}$). Using continuously recorded OD data (**Supplementary Figs. 7** and **8**), an algorithm activates dilution when the upper threshold is exceeded. Dilution continues until the lower threshold is reached (**Fig. 3a**). Population growth rates are calculated in real time by segmenting and fitting the OD trace between dilution events. We varied the upper and lower OD thresholds across 78 yeast populations grown in parallel, thereby defining a two-dimensional selection space based on minimum and maximum culture density (**Fig. 3b** and **Supplementary Note 12**). Cultures were grown in glucose-limited media for 500 h (40–280 generations, depending on the culture), resulting in diauxic shifts with OD windows that exceed ~0.35 (**Supplementary Fig. 15**). The frequency with which the culture experiences the shift is a function of density-dependent selection prescribed to individual cultures (**Fig. 3b**). OD measurements allowed us to generate maps of population and evolution parameters across the selection space, including population growth rates (**Supplementary Fig. 16**) and average genome replication events as a function of OD and growth rate (**Fig. 3b** and **Supplementary Fig. 17**).

We used eVOLVER to evaluate fitness parameters of isolates from each of the evolved populations by having isolated strains compete against a fluorescently labeled ancestor strain at low- or high-density
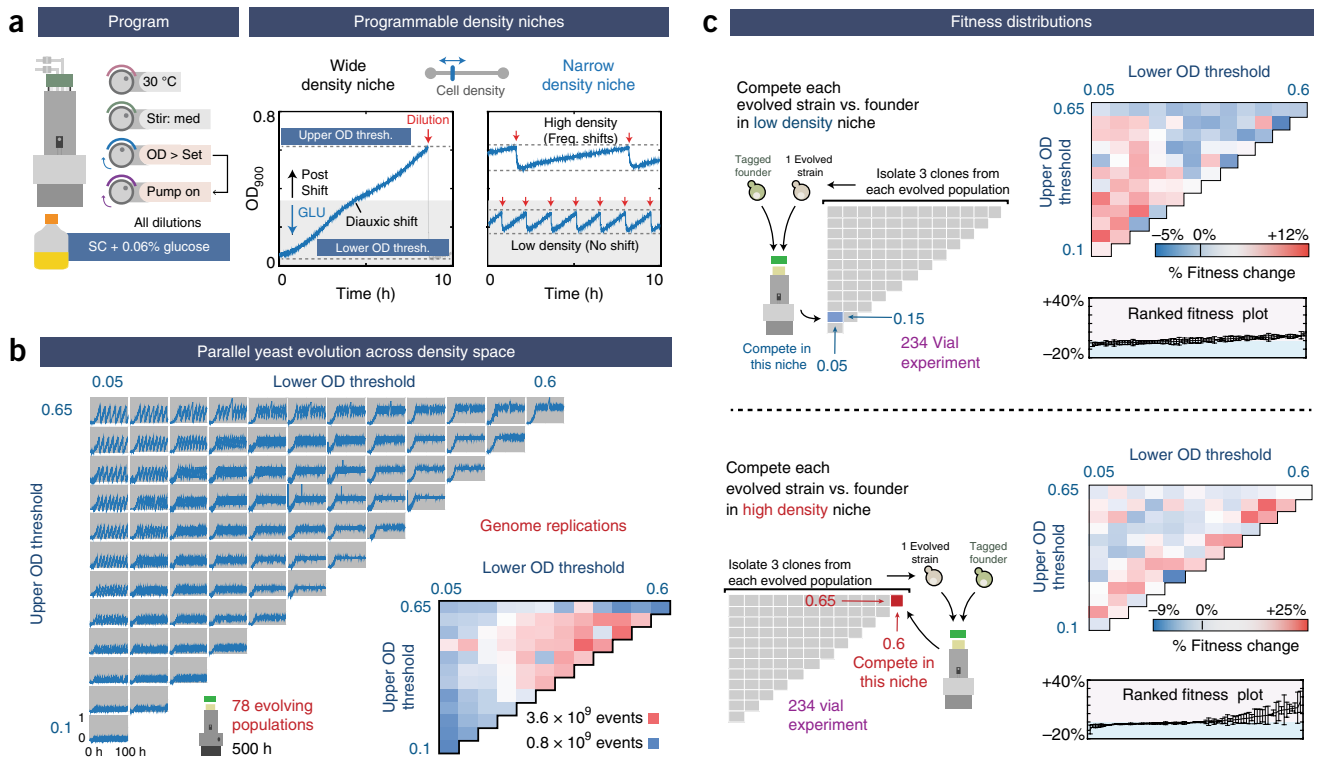
Figure 3 High-throughput experimental evolution across a multidimensional selection space. (**a**) Programming eVOLVER to maintain culture density selection routines during yeast evolution. Left: eVOLVER was configured to maintain cultures within defined density niches using a feedback between OD measurements and dilution events (turbidostat function). Right: representative growth traces for yeast (*Saccharomyces cerevisiae* FL100) cultures growing under wide and narrow density niches. For each culture, the programmed OD window determines population size, and the consequent dilution rate and diauxic shift frequency. (**b**) Parallel evolution of 78 yeast populations in distinct density niches. Culture OD traces are shown for populations evolved for 500 h in density windows with varied lower (0.05–0.6) and upper (0.1–0.65) OD thresholds. Lower right: heat map of the estimated genome replication events for the 78 populations. Values were calculated by multiplying average number of cells by the number of doublings, both estimated through segmentation of the OD trace. (**c**) Fitness distributions of evolved strains. Three clones from each evolved population in competition with the ancestral strain under low-density (OD 0.05–0.15, top) and high-density (OD 0.60–0.65, bottom) growth regimes. Right: heat maps for mean fitness change relative to the ancestor (top) and ranked fitness with standard error bars (s.e.m.) representing competitive fitness for each clone (bottom).

continuous growth conditions (468 total cultures), and assaying for population ratio over time using flow cytometry[31]. The fitness distributions in low- and high-density growth conditions were distinct (**Fig. 3c**). K-means analysis of the fitness phenotypes yielded three distinct groups: low-density specialists, high-density specialists, and the remainder, which have low fitness in both measured niches (**Supplementary Fig. 18**). Low-density specialists evolved from most of the cultures maintained at lower OD thresholds (**Fig. 3c** top, and **Supplementary Fig. 18**). In contrast, high-density specialists were isolated only from cultures maintained within narrow, high-OD windows (**Fig. 3c** bottom, and **Supplementary Fig. 18**). These results demonstrate that by systematically varying the features of a selective environment, eVOLVER can be used to precisely resolve adaptive niches.

Selection under temporally varying pressure regimes

There is growing interest in understanding how temporal changes in parameters of an environment shape adaptation[32]. Fluctuating conditions may yield adaptations distinct from those selected under monotonic pressure[33,34]. Prior studies often use either highly controlled microfluidic systems[35] to study a few strains at a time or tedious bulk techniques[36] for limited perturbation on a pooled population of thousands of strains. eVOLVER can provide both control and throughput, enabling temporal changes of one selective pressure on

a large population (>10^7 cells) while holding other culture conditions constant. To demonstrate this, we carried out growth selection experiments on a pooled yeast knockout library[4,5,37] (5,149 unique members) under conditions in which a single environmental variable—temperature—was temporally varied. A two-dimensional selection space was programmed by varying the magnitude and period of square-wave temperature oscillations (**Fig. 4a,b**). Cultures were maintained for 6 d in turbidostat mode, and samples collected every 48 h (**Supplementary Note 13**). At the conclusion of the experiment, next-generation sequencing was performed on each selected population to determine library member frequency[38,39], which was used to calculate the fitness of each member[31] (**Supplementary Figs. 19–21** and **Supplementary Note 13**).

We computed a two-dimensional (2D) weighted centroid for each library member by transforming its measured fitness into coordinates of temperature magnitude and frequency, which allowed us to compare the library phenotypes across the 2D temporal selection space (**Fig. 4c**, **Supplementary Fig. 22** and **Supplementary Note 13**). To justify the centroid as a representative phenotypic metric, we picked four library members from different regions of the distribution (Δ*HSP104*, Δ*KAP120*, Δ*AHA1*, and Δ*SWA2*) for experimental validation in four temperature selection conditions. We also chose a control strain (Δ*HO*) whose centroid is near the population mean, suggesting the knockout had no adaptive value. Fitness
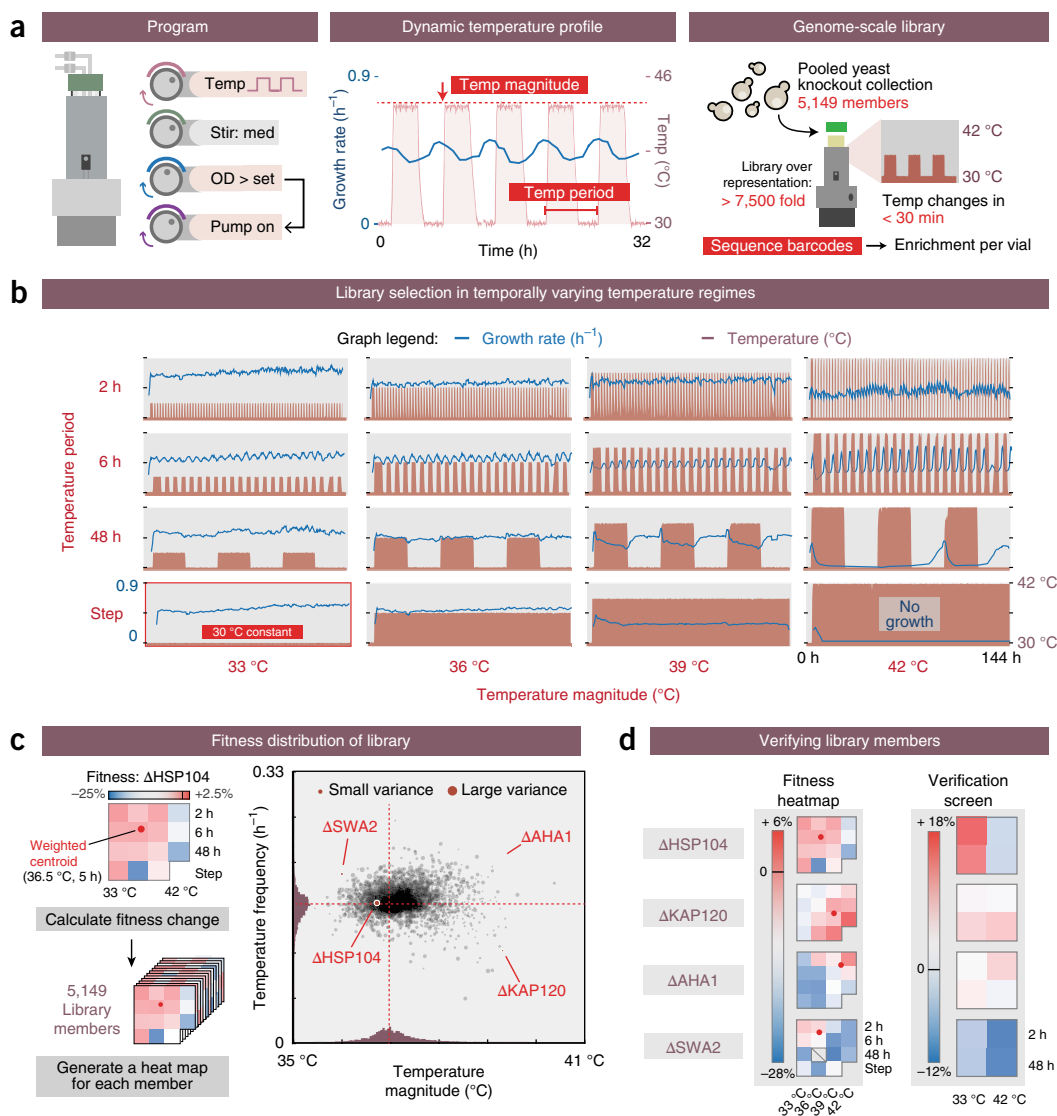
Figure 4 Genome scale library fitness under temporally varying selection pressure. (**a**) Programming temporally varying temperature regimes. Left: eVOLVER configuration for conducting turbidostat experiments (OD window: 0.15–0.2) under fluctuating temperature stress. Middle: snapshot of temperature waveform (red) alternating between 30 °C and 39 °C on a 6-h period, and corresponding culture growth rate (blue). Right: parallel cultures of pooled yeast knockout collection were grown. Selection-based enrichment of library members was quantified at various time points using next-generation sequencing. (**b**) Full set of dynamic temperature regimes. Temperature magnitudes (33 °C, 36 °C, 39 °C, or 42 °C) were varied against periods (2 h, 6 h, or 48 h, or a constant step), and run against a 30 °C control culture. Recorded temperature (red) is plotted with culture growth rates calculated between dilutions (blue). (**c**) Mapping fitness of library members to dynamic selection space. Left: for each library member, fitness heat maps were generated in each selection regime, and used to calculate weighted fitness centroids within temperature magnitude/frequency coordinate space. Right: scatter plot of fitness centroids for the full library. (**d**) Validation of library selection. Four strains with distinct profiles were chosen for verification and set in competition against a neutral control strain (ΔHO) under four different temporal selection regimes. Population ratios were measured using quantitative PCR.

measurements for these strains in pairwise competition against ΔHO were consistent with their centroid positions from the pooled library experiment (**Fig. 4d**), supporting our use of the fitness centroid as an appropriate phenotypic metric. To compare these results with prior literature, we statistically compared fitness centroids of previously annotated gene sets[40] to the fitness centroid distribution of all knockout members. We expected and observed gene sets associated with relevant annotations, like heat sensitivity, to be significantly skewed from the population mean (**Supplementary Fig. 23**). Similar analysis on individual knockout strains identified several chaperone and chaperone cofactor genes,

which are known to have a role in responding to thermal stress[41] (**Supplementary Fig. 24**).

To characterize similarities between environments, we used cross-correlation and principle component analysis on the library composition over time to quantify the degree to which different conditions bring about the same fitness changes in the knockout library. We observed three distinct groupings. First, a high-temperature, high-frequency group was composed of 42 °C fluctuations with a period of ≤6 h. Second, a high-temperature, low-frequency group was composed of 42 °C fluctuations with 24-h period and constant 39 °C conditions. Finally, a mild temperature group was composed of all
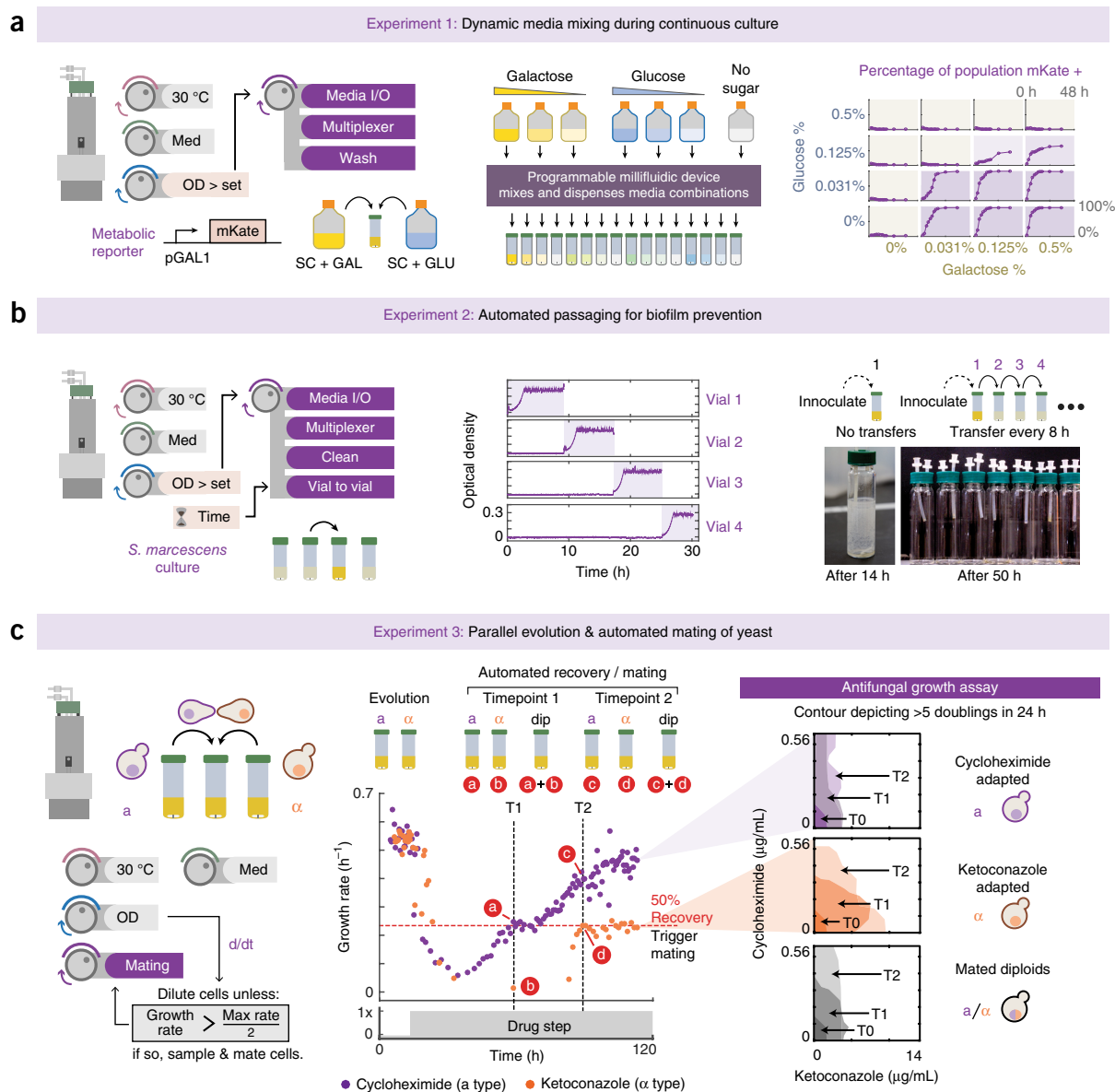
Figure 5 Integrated millifluidic devices enable scaling of complex fluidic manipulation. (**a**) Demonstrating dynamic media mixing in continuous culture. Left: eVOLVER program for maintaining cells in turbidostat mode using millifluidic device to mix and dispense appropriate dilution volumes. A yeast galactose-inducible reporter (*pGAL1*-mKate2) was used to validate the device by maintaining cultures in turbidostat mode at different ratios of glucose and galactose. Center: any combination of seven media inputs can be mixed and dispensed into any of the 16 culture vessels. Right: reporter induction (by population percentage) for 16 cultures containing different glucose/galactose ratios, as measured by flow cytometry. (**b**) Preventing biofilm formation with automated vial-to-vial transfers. Left: a millifluidic device can enable interculture transfers between any of the 16 cultures. Center: *Serratia marcescens* cultures were maintained in turbidostat mode, with culture transfer events triggered every 8 h. Right: a culture maintained in a single vessel forms a thick biofilm after 14 h, while automated transfer prevents visible biofilm formation. (**c**) Using millifluidic devices to automate yeast mating. Left: haploid strains containing opposite mating types are maintained as turbidostat cultures under antifungal selection. Vial-to-vial transfers are triggered by growth rate feedback control, used to sample haploids and form diploids within the device using an automated mating protocol. Center: growth rate of haploid cells evolved under cycloheximide (0.2 μg/mL, purple) or ketoconazole (6 μg/mL, orange) selection was monitored continuously following drug exposure. Once growth rates of either drug-evolved culture equals 50% of the wild-type growth rate under no selection, automated mating and transfer is carried out. This was performed at two time points: $t_1 = 68.7$ h and $t_2 = 98.1$ h. Right: antifungal resistance was assayed for recovered haploid and diploid populations. Contours correspond to an antifungal concentration range in which at least five generations of growth were observed in 24 h (on average).

remaining conditions, with temperatures ≤39 °C (**Supplementary Figs. 25** and **26**). We identified gene ontology (GO) term annotations that are linked to fitness defects in one or more of these groups (**Supplementary Fig. 26**). We observed that genes controlling functions known to affect growth rate (e.g., mitochondrial function, ribosome biogenesis) significantly altered fitness in the mild temperature

group. Interestingly, ribosome components and processing factors also showed high-frequency sensitivity at 42 °C, supporting a potential role for ribosome biogenesis in transitions in and out of stress[42]. We further analyzed potential sources of frequency dependence. We found that the high- and low-frequency groups were characterized by annotations associated with cell cycle checkpoints (e.g., DNA damage

response, organelle fission), which temporally regulate cellular processes and therefore might be expected to affect cellular response to fluctuating stresses at different frequencies.

Automated culturing with fluidic multiplexing devices

In order to show the potential of the fluidic multiplexing framework to automate movement of reagents and cells in eVOLVER, we designed devices for three experiments (**Supplementary Note 14**). These included dynamic media mixing during continuous culture to track yeast response to varied ratios of sugars, preventing bacterial biofilm formation by automated passaging, and programming sexual reproduction between adapting yeast populations.

We show that fluidic multiplexing can be used to manage media composition for multiple cultures maintained by eVOLVER by constructing an eight-channel media selector device that draws media from multiple input sources and dispenses a defined mixture to a culture of choice (**Fig. 2c** and **Supplementary Figs. 27** and **28**). Galactose utilization in yeast is regulated by the ratiometric sensing of available galactose and glucose[43]. We maintained and tracked a yeast strain harboring a galactose-inducible fluorescent reporter (*pGAL1*-mKate2) in continuous cultures featuring 16 different sugar compositions (**Supplementary Figs. 29** and **30** and **Supplementary Note 15**). Dyed media, tracking relative sugar levels in each culture, confirmed that the device can dynamically mix and dispense components in correct ratios for extended periods of time (**Supplementary Fig. 29**). We used flow cytometry measurements to track galactose-induced population fractions, confirming maintenance of sugar-dependent gene induction (**Fig. 5a**).

The utility of the millifluidic system for mediating liquid transfer between cultures was demonstrated using a device that overcomes biofilm formation during long-term continuous growth experiments (**Supplementary Figs. 28, 31**, and **Supplementary Note 16**). *Serratia marcescens*[44], a bacterial species that readily forms biofilm on glass surfaces, was grown in turbidostat mode. Using a vial-to-vial transfer device, inoculation of a fresh vial was performed at 8 h intervals, followed by automated sterilization of culture-exposed fluidic paths (**Fig. 5b**). While thick biofilm deposition was observed after only 14 h in a culture grown continuously in a single vial (no transfers), no deposition was observed in vials maintained under the transfer routine (**Fig. 5b**). With daily replacement of used vials, four smart sleeves could be used to passage a culture, biofilm-free, for an indefinite period of time (**Supplementary Fig. 32**).

Finally, we demonstrate that multiple functionalities can be combined to automate complex fluidic handling routines. We designed a fluidic routine for automated sexual reproduction of adapting yeast populations by integrating multiplexed media selection, vial-to-vial transfer, and post-transfer device cleaning (**Supplementary Figs. 27, 28, 31** and **33**). This routine was used to conduct mating between separate, opposite-mating-type haploid yeast evolving under different antifungal selections: cycloheximide and ketoconazole (**Fig. 5c** and **Supplementary Note 17**). Rather than manually sampling at arbitrary time points, once haploid cultures reached user-defined growth-rate milestones, automatic sampling and mating were carried out on the device (**Supplementary Figs. 33** and **34**). A minimum inhibitory concentration assay performed on both diploid and parental populations indicated that the haploid-evolved cycloheximide resistance was transferred to diploids in a dominant manner (**Fig. 5c** and **Supplementary Figs. 35** and **36**). Conversely, the general antifungal resistance that emerged under ketoconazole selection appeared to be recessive. Further sequencing of ketoconazole-evolved haploid strains revealed nonsense mutations in *ERG3* (**Supplementary Fig. 37**), which has been shown to confer recessive resistance to azole antifungals[45].

DISCUSSION

We report the development of eVOLVER, which is a DIY framework for automated cell growth experiments. Our design is customizable and provides researchers with the ability to design, build, and share both experimental configurations and data (**Figs. 1** and **2**). With straightforward modifications, eVOLVER can be reconfigured to conduct any of the recently reported continuous growth studies (**Supplementary Table 1**), and can replace batch culture techniques used in several recent experimental evolution studies[9,11,32,46]. It is straightforward to add hardware components to the platform as they become available. For example, integration with open-source pipetting robots would automate culture sampling, thereby enabling fluorescence-activated cell sorting (FACS) for assaying gene expression, or droplet microfluidics for single-cell studies. The configuration we report in this article is designed for well-mixed liquid cultures, but eVOLVER can be adapted for the coordination of multiple arrayed sensors to capture spatial distributions in static liquid cultures or phototrophic cultures. We have reported use of eVOLVER for growing lab-adapted suspension cultures of bacteria and yeast. We note that while it is feasible to use our system for growing mammalian cell lines, additional attention to sterility and removal of residual cleaning agents would be needed, and bead/matrix systems might be required for adherent cells.

The multiple possible configurations enable precise specification of individual culture environments. By systematically co-varying parameters, eVOLVER can be used to investigate cellular fitness along multidimensional environmental gradients, potentially allowing for experimental decoupling of overlapping selection pressures. The ability to arbitrarily program feedback control between culture conditions and fluidic functions allows the user to algorithmically define highly specialized environmental niches.

We showcased the versatility of our system using an experimental evolution study (**Fig. 3**) of yeast in 78 different culture-density windows. We then generated fitness distributions by testing fitness of evolved clones in low- and high-density niches, identifying low- and high-density specialists. Interestingly, high-density specialists were most often derived from evolution in narrow OD windows. Since the prescribed culture density windows are related to the frequency of diauxic shift at limiting glucose concentrations[47], it is interesting to speculate that the strains selected for were those with metabolic programs that facilitate rapid metabolic shifts. Further work is needed to confirm that the differences observed in the fitness distributions are relevant. For example, a baseline fitness distribution generated from a large number of replicate evolutions could be used to rule out the possibility that stochastic events dominate the observed fitness differences. Additionally, comparing the fitness of whole evolved populations in each condition could help isolate true adaptation from variation due to clonal differences. Finally, assaying fitness in additional niches is required to determine how well fitness distributions correlate with the assayed niche, as well as to ascertain the existence of generalists.

In a second experiment, we assessed growth fitness of a yeast genome-wide knockout library under systematically varied temperature fluctuations, demonstrating that eVOLVER could be used to extract unique fitness information across a temporally varied selection surface (**Fig. 4**). In a similar approach—using sublethal stress administered under temporally diverse selection conditions—selection experiments could be conducted for other types of libraries at a resolution beyond what is available for standard growth selection schemes[4,38,39]. Using this approach, algorithms with additional parameters could be used to perform fitness and selection experiments investigating biological phenomenon associated with temporal adaptation, such as bet-hedging, noise, and transcriptional feedback.

Accurate fluidic manipulation is a requirement of continuous culture automation, but past approaches to fluidic routing have been tedious to customize and difficult to control, imposing limitations on experimental design. We presented a method to build and control millifluidic devices that plugs into the eVOLVER framework for programmatic routing of fluids during continuous culture. To highlight the utility and robustness of the devices, we performed three experimental demonstrations: sophisticated fluidic mixing and dispensation, vial-to-vial transfers, and integration of multiple devices for more complex culture routines (**Fig. 5**). These experiments illustrate the potential of custom millifluidics with this platform.

In the future we hope that our framework could be applied to studying contributions of individual species to community fitness in microbial consortia, designing synthetic circuits that minimize fitness costs to the host cell[3], identifying circuit designs for producer strains to maximize stability over time in industrial bioreactors[48], or in optimization of synthetic microbial genomes[49,50]. We hope that eVOLVER will serve as a democratic platform for research by a broad community of users to build, execute, and share experiments.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS
B.G.W., C.J.B., and A.S.K. conceived the study. B.G.W. developed the system with guidance and input from all authors. B.G.W. and C.P.M. performed and analyzed experiments. S.K. generated reagents. C.J.B. and A.S.K. oversaw the study. All authors wrote the paper.

COMPETING INTERESTS
The authors declare no competing interests.

1. Elena, S.F. & Lenski, R.E. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nat. Rev. Genet.* **4**, 457–469 (2003).
2. Nichols, R.J. *et al.* Phenotypic landscape of a bacterial cell. *Cell* **144**, 143–156 (2011).
3. Nevozhay, D., Adams, R.M., Van Itallie, E., Bennett, M.R. & Balázsi, G. Mapping the environmental fitness landscape of a synthetic gene circuit. *PLoS Comput. Biol.* **8**, e1002480 (2012).
4. Giaever, G. *et al.* Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418**, 387–391 (2002).
5. Li, Z. *et al.* Systematic exploration of essential yeast gene function with temperature-sensitive mutants. *Nat. Biotechnol.* **29**, 361–367 (2011).
6. Zuleta, I.A., Aranda-Díaz, A., Li, H. & El-Samad, H. Dynamic characterization of growth and gene expression using high-throughput automated flow cytometry. *Nat. Methods* **11**, 443–448 (2014).
7. Takahashi, C.N., Miller, A.W., Ekness, F., Dunham, M.J. & Klavins, E. A low cost, customizable turbidostat for use in synthetic circuit characterization. *ACS Synth. Biol.* **4**, 32–38 (2015).
8. Feist, A.M., Herrgård, M.J., Thiele, I., Reed, J.L. & Palsson, B.Ø. Reconstruction of biochemical networks in microorganisms. *Nat. Rev. Microbiol.* **7**, 129–143 (2009).
9. Lang, G.I. *et al.* Pervasive genetic hitchhiking and clonal interference in forty evolving yeast populations. *Nature* **500**, 571–574 (2013).
10. Maddamsetti, R., Lenski, R.E. & Barrick, J.E. Adaptation, clonal interference, and frequency-dependent interactions in a long-term evolution experiment with *Escherichia coli*. *Genetics* **200**, 619–631 (2015).
11. Yona, A.H. *et al.* Chromosomal duplication is a transient evolutionary solution to stress. *Proc. Natl. Acad. Sci. USA* **109**, 21010–21015 (2012).
12. Wang, H.H. *et al.* Programming cells by multiplex genome engineering and accelerated evolution. *Nature* **460**, 894–898 (2009).
13. Esvelt, K.M., Carlson, J.C. & Liu, D.R. A system for the continuous directed evolution of biomolecules. *Nature* **472**, 499–503 (2011).
14. Ravikumar, A., Arrieta, A. & Liu, C.C. An orthogonal DNA replication system in yeast. *Nat. Chem. Biol.* **10**, 175–177 (2014).
15. Crook, N. *et al.* In vivo continuous evolution of genes and pathways in yeast. *Nat. Commun.* **7**, 13051 (2016).
16. Bull, A.T. The renaissance of continuous culture in the post-genomics age. *J. Ind. Microbiol. Biotechnol.* **37**, 993–1021 (2010).
17. Gresham, D. & Dunham, M.J. The enduring utility of continuous culturing in experimental evolution. *Genomics* **104**, 399–405 (2014).
18. Piper, M.D.W. *et al.* Reproducibility of oligonucleotide microarray transcriptome analyses. An interlaboratory comparison using chemostat cultures of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **277**, 37001–37008 (2002).
19. Cressey, D. The DIY electronics transforming research. *Nature* **544**, 125–126 (2017).
20. Kong, D.S. *et al.* Open-source, community-driven microfluidics with Metafluidics. *Nat. Biotechnol.* **35**, 523–529 (2017).
21. Adamo, A. *et al.* On-demand continuous-flow production of pharmaceuticals in a compact, reconfigurable system. *Science* **352**, 61–67 (2016).
22. Perkel, J.M. The Internet of Things comes to the lab. *Nature* **542**, 125–126 (2017).
23. Toprak, E. *et al.* Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nat. Genet.* **44**, 101–105 (2012).
24. Milias-Argeitis, A., Rullan, M., Aoki, S.K., Buchmann, P. & Khammash, M. Automated optogenetic feedback control for precise and robust regulation of gene expression and cell growth. *Nat. Commun.* **7**, 12546 (2016).
25. Hope, E.A. *et al.* Experimental Evolution Reveals Favored Adaptive Routes to Cell Aggregation in Yeast. *Genetics* **206**, 1153–1167 (2017).
26. Toprak, E. *et al.* Building a morbidostat: an automated continuous-culture device for studying bacterial drug resistance under dynamically sustained drug inhibition. *Nat. Protoc.* **8**, 555–567 (2013).
27. Thorsen, T., Maerkl, S.J. & Quake, S.R. Microfluidic large-scale integration. *Science* **298**, 580–584 (2002).
28. Melin, J. & Quake, S.R. Microfluidic large-scale integration: the evolution of design rules for biological automation. *Annu. Rev. Biophys. Biomol. Struct.* **36**, 213–231 (2007).
29. Unger, M.A., Chou, H.P., Thorsen, T., Scherer, A. & Quake, S.R. Monolithic microfabricated valves and pumps by multilayer soft lithography. *Science* **288**, 113–116 (2000).
30. Grover, W.H., Skelley, A.M., Liu, C.N., Lagally, E.T. & Mathies, R.A. Monolithic membrane valves and diaphragm pumps for practical large-scale integration into glass microfluidic devices. *Sens. Actuators B Chem.* **89**, 315–323 (2003).
31. Kryazhimskiy, S., Rice, D.P., Jerison, E.R. & Desai, M.M. Microbial evolution. Global epistasis makes adaptation predictable despite sequence-level stochasticity. *Science* **344**, 1519–1522 (2014).
32. Mitchell, A. *et al.* Adaptive prediction of environmental changes by microorganisms. *Nature* **460**, 220–224 (2009).
33. Ketola, T. *et al.* Fluctuating temperature leads to evolution of thermal generalism and preadaptation to novel environments. *Evolution* **67**, 2936–2944 (2013).
34. Sæther, B.-E. & Engen, S. The concept of fitness in fluctuating environments. *Trends Ecol. Evol.* **30**, 273–281 (2015).
35. Bennett, M.R. *et al.* Metabolic gene regulation in a dynamically changing environment. *Nature* **454**, 1119–1122 (2008).
36. Salignon, J., Richard, M., Fulcrand, E. & Yvert, G. Genomics of cellular proliferation in periodic environmental fluctuations. *Mol. Syst. Biol.* **14**, e7823 (2018).
37. Giaever, G. & Nislow, C. The yeast deletion collection: a decade of functional genomics. *Genetics* **197**, 451–465 (2014).
38. Smith, A.M. *et al.* Quantitative phenotyping via deep barcode sequencing. *Genome Res.* **19**, 1836–1842 (2009).
39. Gibney, P.A., Lu, C., Caudy, A.A., Hess, D.C. & Botstein, D. Yeast metabolic and signaling genes are required for heat-shock survival and have little overlap with the heat-induced genes. *Proc. Natl. Acad. Sci. USA* **110**, E4393–E4402 (2013).
40. Cherry, J.M. *et al.* Saccharomyces Genome Database: the genomics resource of budding yeast. *Nucleic Acids Res.* **40**, D700–D705 (2012).
41. Morano, K.A., Grant, C.M. & Moye-Rowley, W.S. The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. *Genetics* **190**, 1157–1195 (2012).
42. Loar, J.W. *et al.* Genetic and biochemical interactions among Yar1, Ltv1 and Rps3 define novel links between environmental stress and ribosome biogenesis in *Saccharomyces cerevisiae*. *Genetics* **168**, 1877–1889 (2004).

43. Escalante-Chong, R. *et al*. Galactose metabolic genes in yeast respond to a ratio of galactose and glucose. *Proc. Natl. Acad. Sci. USA* **112**, 1636–1641 (2015).
44. Rice, S.A. *et al*. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. *J. Bacteriol.* **187**, 3477–3485 (2005).
45. Anderson, J.B., Sirjusingh, C. & Ricker, N. Haploidy, diploidy and evolution of antifungal drug resistance in *Saccharomyces cerevisiae. Genetics* **168**, 1915–1923 (2004).
46. González, C. *et al*. Stress-response balance drives the evolution of a network module and its host genome. *Mol. Syst. Biol.* **11**, 827 (2015).
47. Brauer, M.J., Saldanha, A.J., Dolinski, K. & Botstein, D. Homeostatic adjustment and metabolic remodeling in glucose-limited yeast cultures. *Mol. Biol. Cell* **16**, 2503–2517 (2005).
48. Alper, H., Fischer, C., Nevoigt, E. & Stephanopoulos, G. Tuning genetic control through promoter engineering. *Proc. Natl. Acad. Sci. USA* **102**, 12678–12683 (2005).
49. Hutchison, C.A. III *et al*. Design and synthesis of a minimal bacterial genome. *Science* **351**, aad6253–aad6253 (2016).
50. Richardson, S.M. *et al*. Design of a synthetic yeast genome. *Science* **355**, 1040–1044 (2017).

ONLINE METHODS

General methods and analysis used throughout the study are described here. A detailed description of eVOLVER device design is presented in **Supplementary Notes 1–11**. For a more complete description of experiments featured in **Figures 3–5**, see **Supplementary Notes 12–17**, which include methods and results.

Strain construction. Genotypes for yeast and bacterial strains used in this study are listed in **Supplementary Table 2**. Plasmids were constructed using standard molecular biology techniques. Strains were generated using standard lithium acetate transformation. When appropriate, non-isogenic pooled population samples were given unique designations for clarity (**Supplementary Table 2**).

Routine cell culture techniques. Culture conditions varied according to the needs of particular experiments (see methods in **Supplementary Notes 10–17**). Cultures used to seed eVOLVER experiments were prepared as follows: *Saccharomyces cerevisiae* (obtained from frozen stock or single colonies) was grown in 2 mL of YPD media (2% glucose) at 30 °C in a shaking incubator (300 r.p.m.) for at least 36 h. For routine overnight culture of *Escherichia coli* or *Serratia marcescens*, cells obtained from frozen stock were used to inoculate 2 mL of LB Miller broth grown at 37 °C for 12 h in a shaking incubator (300 r.p.m.).

Flow cytometry. Flow cytometry was used to measure single-cell fluorescence throughout the study. Prior to measurement, 200 µL of yeast culture (see methods for experiment-specific growth conditions) was diluted with 100 µL of filter-sterilized PBS supplemented with cycloheximide to a final concentration of 20 µg/mL, then incubated at 4 °C in the dark for no less than 3 h to allow for fluorophore maturation. An Attune NxT Flow Cytometer (Invitrogen) equipped with an autosampler was used to acquire data. For a typical experiment, at least 10,000 events were acquired. Cells were analyzed using FlowJo (Treestar Software). Intact cells were gated using forward and side scatter, followed by gating on fluorescence channels (green and/or red, as appropriate) to determine the fractional distribution of each population.

Fitness calculations. Competitive fitness, in which a strain of interest is co-cultured in competition with a reference strain, was assayed in the same fashion throughout the study. The ratio of the two strains was determined at multiple time points—generally at the beginning and end of an experiment—either by flow cytometry or qPCR. Fitness values, F, were calculated using the following equation[31]:

$$F = \frac{1}{t_2 - t_1} \log\left(\frac{n(t_2)}{n_r(t_2)} \middle/ \frac{n(t_1)}{n_r(t_1)} \right)$$

where t is number of generations, and n and n_r are cell counts for the strains of interest and reference strain, respectively.

Setup procedure for eVOLVER experiments. Prior to each eVOLVER experiment, 40-mL borosilicate glass vials outfitted with a stir bar and capped with an influx port and an efflux port were sterilized by autoclave. Media and waste lines were sterilized by pumping 10% bleach (20 mL), followed by 70% ethanol (20 mL). Lines were cleared by pumping air for 20 s, followed by media (20 mL). Media and waste lines were then attached to the influx and efflux ports of each vial, and each vial filled by pumping 25 mL of the appropriate media through the influx port. At this point, Python control code was initiated, triggering a blank media measurement, and activating smart sleeve heating elements. Prior to seeding with cells, fresh media was incubated in the device for a length of time sufficient for the first 15 optical density recordings to be taken (~2.5 min) and for media to reach the programmed temperature.

Extracting yeast genomic DNA. To extract genomic DNA, ~2 × 10⁶ yeast cells (roughly 30 µL of overnight culture) were pelleted by centrifugation (5 min, 1,000 r.c.f.). Supernatant was removed, and pellets were resuspended in 30 µL 0.2% sodium dodecyl sulfate (SDS), followed by vortexing for 15 s. Suspensions were transferred to PCR tubes and heated in a thermal cycler (37 °C) for 5 min, followed by 98 °C for 5 min before cooling to 4 °C. Extracts were diluted with H_2O to a final volume of 75 µL before being used as PCR template. Primers used in this study are listed in **Supplementary Table 3**.

Library preparation and barcode sequencing. Libraries for next-generation sequencing were prepared by PCR-amplification of genomic DNA (LightCycler 480 Instrument II, Roche) and purification (Zymo Research). Indexing (culture, time point) and sequencing adapters were added via PCR. NextSeq sequencing (Harvard Biopolymers Facility) was used to sequence the culture index, the time point index, and a 55-bp single-end read of the barcode construct. PhiX was added at 50% to increase sequence diversity. Alignment was performed using custom code harnessing MATLAB's Bioinformatics Toolbox and Boston University's parallel computing cluster. Alignment scores were calculated using the Smith–Waterman algorithm (swalign function) and assigned based on best score above a minimum threshold. In total, we assigned more than 244 million reads to 5,149 unique library members to track frequency across the four time points for each of the 16 conditions. A more detailed description of the library preparation and sequencing can be found in **Supplementary Note 13**.

Life Sciences Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. BioProject: accession code PRJNA450093. Data are available from the corresponding author upon reasonable request. Detailed eVOLVER protocols, tutorials, and instructions will be provided at https://www.fynchbio.com.

natureresearch

Corresponding author(s): Ahmad S. Khalil and Caleb J. Bashor

☐ Initial submission ☐ Revised version ☒ Final submission

Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

▶ Experimental design

1. Sample size

Describe how sample size was determined.

When appropriate, triplicate samples were chosen, as is customary in biological experiments. Sample sizes across conditions were guided by the footprint of the device (16-vial units) and scope of the conditions being tested.

2. Data exclusions

Describe any data exclusions.

Data was excluded from fitness measurements due to low sequencing depth for two out of the 16 samples (in Figure 3, Heat Shock Library Screen). Additionally, libraries members present at less than 10^{-10} were not measured due to low frequency counts.

3. Replication

Describe whether the experimental findings were reliably reproduced.

Growth in the Smart Sleeve was replicated across 96 cultures and plotted. Evolution experiments were not replicated, customary in the field. Fitness measurements from the evolved strains were measured in biological triplicates. Measurements from the library screens were verified using a similar, but not identical, assay to the original screen. MIC measurements were also run in technical triplicates.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Single clones from streaked plates were randomly picked when necessary. Randomization was not deemed critical, thus specific culture conditions was assigned in sequential order.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Samples were anonymized during data collection but not during analysis, across all experiments.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed	
☐	☒	The <u>exact sample size</u> (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒	A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	A statement indicating how many times each experiment was replicated
☐	☒	The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☐	☒	A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒	The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒	A clear description of statistics including <u>central tendency</u> (e.g. median, mean) and <u>variation</u> (e.g. standard deviation, interquartile range)
☐	☒	Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

▶ Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.	FlowJo (Treestar Software) was used to extract and process flow cytometry data. MATLAB (Mathworks) and Microsoft Excel were used for data analysis, sequencing analysis, and data visualization. The Python Bokeh library was used for fitness centroid visualization. Code generated in this study for eVOLVER was deposited in GitHub and is made available at https://www.fynchbio.com/documentation/

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

▶ Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.	Materials and reagents are available upon request.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	N/A

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	FL100 (ATCC 28383), Pooled YKO Collection (Transomic TKY3502P), W303 strain from YJW509
b. Describe the method of cell line authentication used.	FL100 - From ATCC YKO Collection - From Transomic W303 strain - authentication was not deemed relevant
c. Report whether the cell lines were tested for mycoplasma contamination.	N/A
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	N/A

▶ Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

N/A

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

N/A

natureresearch

Corresponding author(s):   Ahmad S. Khalil and Caleb J. Bashor

☐ Initial submission   ☐ Revised version   ☒ Final submission

Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

▶ Data presentation

For all flow cytometry data, confirm that:

☒ 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ 3. All plots are contour plots with outliers or pseudocolor plots.

☒ 4. A numerical value for number of cells or percentage (with statistics) is provided.

▶ Methodological details

5. Describe the sample preparation.	Flow cytometry was used to measure single-cell fluorescence throughout the study. Prior to measurement, 200 uL of yeast culture (see specific methods for growth conditions) was diluted with 100 uL of filter-sterilized Phosphate Buffered Saline supplemented with cyclohexamide to a final concentration of 20 ug/mL, then incubated at 4°C in the dark for no less than 3 h to allow for complete fixation.
6. Identify the instrument used for data collection.	An Attune NxT Flow Cytometer (Invitrogen) equipped with an autosampler (Invitrogen) was used to acquire data.
7. Describe the software used to collect and analyze the flow cytometry data.	Data was collected using NxT Flow Cytometer V2.5 (Invitrogen) and analyzed using FlowJo (Treestar Software).
8. Describe the abundance of the relevant cell populations within post-sort fractions.	All reported populations were greater than 0.5% of the population across all experiments.
9. Describe the gating strategy used.	Gates were placed based on wild type cells (dark cells) to determine population fraction that expresses the fluorescent reporter.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☒