A Low-Cost, Open Source, Self-Contained Bacterial EVolutionary biorEactor (EVE)

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Abstract Evolutionary forces shape bacterial population characteristics in a matter of days. Here, we present a framework that allows students and scientists to study bacterial evolution of antibiotic resistance over a few days. This approach is in line with recent efforts to study evolution of drug resistance in organisms at increasingly smaller time scales and in a high-throughput manner. One effective approach is through the use of customized bioreactors – devices that can continuously culture bacteria and monitor population growth in real time. These devices are often technically challenging and expensive to implement for scientists, let alone students or teachers who seek an innovative and intuitive way of studying evolution. Here, we present a continuous bacterial culture device that can be easily and inexpensively constructed, flexibly configured for various evolution experiments, and used in both academic and educational environments. As this EVolutionary biorEactor (EVE) is capable of replicating the functionality of many prominent and expensive bioreactors on the market today, we hope the greater accessibility of EVE will allow interested high school and college students to study biological concepts such as population dynamics and evolution using innovative technology. Within the educational environment, we also hope to foster interaction and interest between the engineering and biological fields by allowing teachers and students to build their own systems and share experimental designs and results based on our proposed open framework.

Introduction

Continuous microbial culture is a powerful, high-throughput method of observing and directing evolution in both research and industrial settings. Bioreactors are continuous culture devices that simultaneously passage media into and remove volume from active cultures to maintain volume homeostasis ***Novick and Szilard*** (***1950***). The rate of media inflow and outflow is called the dilution rate; various types of bioreactors dynamically adjust the dilution rate to keep a desired parameter constant. Chemostats, the simplest bioreactors, maintain a constant dilution rate ***Voulgarelis et al.*** (***2019***). Turbidostats adjust the dilution rate to maintain a constant turbidity. Finally, auxostats refer to bioreactors that use any other parameter to control the dilution rate. Examples of parameters used in auxostats include pH and oxygen tension.

Here, we present a protocol for the assembly and operation of a continuous culture device called the the EVolutionary biorEactor (EVE). It is a framework for a system which can support multiple individual bioreactors, which we call culture units (CUs). Each of these CUs are capable of simultaneously running their own experiment with different antibiotics, rates of passaging media and removing waste, population density sampling rates, and many other customizable parameters. The EVE system began by openly and cost-conservatively replicating and expanding upon the morbidostat functionality using the methodology outlined by Toprak *et al.*. The morbidostat keeps the cell density low by exposing the population to selective pressures such as cytotoxic drugs. To prevent extinction of the culture, the morbidostat control algorithm temporally modifies the concentration of drug delivered to the in accordance to the adaptive dynamics of the population under study. ***Toprak et al.*** (***2013***).

The physical functionality of the EVE is centered around a culture vial and is inspired by prominent bioreactors featured in the literature ***Toprak et al.*** (***2013***); ***Yoshida et al.*** (***2017***); ***Wong et al.*** (***2018***); ***Milias-Argeitis et al.*** (***2016***); ***Dößelmann et al.*** (***2017***). Traditionally, costs of commercially-available bioreactors range from approximately $10,000 for academic units to $25,000 for the general public. These bioreactors are often built specifically for one field of research and are difficult to modify for other applications. However, a recent shift towards open-source science has brought about a new wave of do-it-yourself, customized continuous culture devices to accommodate a wide variety of experimental designs across several research areas ***Nash*** (***2005***); ***Matteau et al.*** (***2015***); ***Gill*** (***2018***); ***Cressey*** (***2017***); ***Perkel*** (***2017***); ***Pilizota and Yang*** (***2018***). At the same time, the proliferation of maker-spaces throughout high schools and colleges have increased student access to engineering and design resources. As a result, bioreactors are an ideal educational tool to foster interest in experimental evolution for college, high school, and even elementary school students.

A few such designs with detailed protocols have already been published in the literature. However, barriers in electrical, software, and biological expertise, as well as cost, still leave the assembly and operation of these machines out of reach for students or scientists in resource-poor settings ***Yoshida et al.*** (***2017***); ***Toprak et al.*** (***2013***); ***Wong et al.*** (***2018***). In addition, increasing the functionality of these machines while maintaining cost-effectiveness is an enduring challenge ***Takahashi et al.*** (***2014***); ***Liu et al.*** (***2016***); ***Miller et al.*** (***2013***); ***Hoffmann et al.*** (***2017***).

Description of the EVE

The primary purpose of the EVE is to record data from sensors that take measurements of growing cultures. Recorded data fed into programmed logic with the intention of controlling the growth of the culture over time. Control over the culture is accomplished through the use of pumps which control the flow of fresh media and drugs into the culture. To do this, a culture is started in a vial and placed in our custom holder. This holder is positioned on a stand which contains a small fan. The fan has magnets glued to it so that when the magnets rotate, a magnetic stir-bar at the bottom of the culture vial is induced to stir. Stirring is useful in two ways: first, to facilitate diffusion of oxygen into the culture media and second, to keep the culture's turbidity uniform across the culture vial.

The vial holder also houses two diodes (one LED and one photodiode). Throughout the experiment, the LED shines a light through the culture. Depending on the growth and corresponding

turbidity of the culture, light is variably scattered through the culture, changing the amount of light that reaches the photodiode (PD). The LED and PD are connected to a circuit board (either a breadboard or PCB) that powers the devices and converts the analog information from the PD into digital information. Digital information is recorded by the Raspberry Pi, a single board computer that controls EVE operation by running the EVE software package. The circuit board is connected to the Raspberry Pi with a ribbon cable, enabling the two boards to interact digitally. The culture vial is also connected to three liquid pumps with three plastic tubes. Each pump in turn connects to either a fresh media reservoir, a media reservoir containing cytotoxic drugs (antibiotics in this paper), or a waste reservoir. Similar to other bioreactor designs, the culture vials have an air intake tube that is fitted with an air filter. When powered by the circuit board, a specific pump activates and pumps either media or media mixed with drug into the vial. The waste pump also activates whenever the media pumps activate to prevent culture overflow. The waste pump can also provide the opportunity for easy temporal sampling. The software on the Raspberry Pi is responsible for triggering these pumps at specific points during the culture to achieve the (user defined) goal of the experiment. A schematic of the setup is shown in Fig 1.

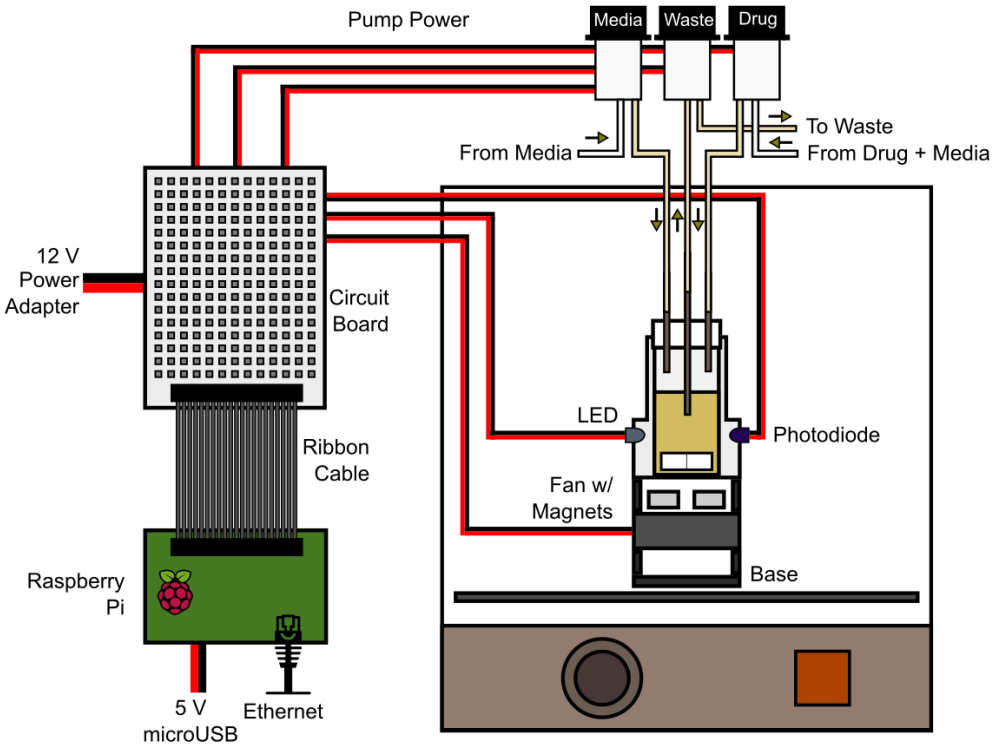

Figure 1. Hardware schematic of the EVE system. The culture vial is located inside the incubator. As the culture grows, an LED shines a light through the culture. Light that is reflected by the culture is received by the photodiode. A circuit board interprets and conveys photodiode readings to the Pi which runs control software. Based on user-configured directions, and optical density readings, the Pi uses the circuit board to power pumps connected to the vial. This strategy enables precise and dynamic control of the culture.

Before the experiment, the culture vials and reservoirs are sterilized in an autoclave, and the silicone tubing is sterilized with bleach, ethanol, and sterile water solution, as described in the supplemental information. The voltage information recorded from the PD reflects the degree to which light is scattered within the culture vial - a surrogate of the absorbance of the culture. In many scenarios, the voltage value provides a clear picture of the state of the culture. However, if a true absorbance is needed, a simple calibration curve correlating the voltage value with an optical density can be performed. Steps to generate calibration curves, as well as sample calibration curves are included in the supplemental information.

Overview of Functionality

Prominent bioreactors featured in the literature are quite versatile and can be used to perform a variety of experiments. The EVE aims to follow suit by being capable of performing a variety of experiments and is pre-programmed to replicate morbidostat, turbidostat, or chemostat functionality. Additional functionality can be programmed for customized needs. To demonstrate the full functionality of EVE we performed an experiment of bacterial growth under a selective pressure of ampicillin mixed into media.

Per the morbidostat selection algorithm, drug is introduced to the culture vial as the bacteria cross a manually set or automatically detected growth threshold. The drug concentration is then lowered to allow for the recovery of the population to initial population size. This is done by repetitively adding fresh media to the culture while removing waste. In the experimental data below, the bacterial population is able to recover in 12 hours. The drug solution is then re-introduced to the culture. After this second introduction, the optical density decreases 15%, indicating development of tolerance/resistance to the drug. The population is again allowed to recover for a shorter period – around 6 hours. After this recovery period, the addition of more ampicillin does not kill the population. At the end of the experiment, the cells are effectively resistant to ampicillin. Resistance of the resultant population can be confirmed with a minimum inhibitory concentration (MIC) assay ***Andrews*** (***2001***). The experiment can be performed with a variety of different drugs, however drug stability in cell culture should be considered. For instance, ampicillin is stable in culture for 3 days ***Perlman*** (***1979***). If the experiment lasts longer than the stability of the drug in culture, the drug solution can be refreshed by replacing the drug reservoir with one that is freshly made.

As the experiment progresses, users can monitor the bacteria's growth via the live web interface or through the included Slack integration. At the end of the experiment, users can parse the recorded data and visualize the data using their own methods. Graphs of a trial we performed are included in Fig 2.

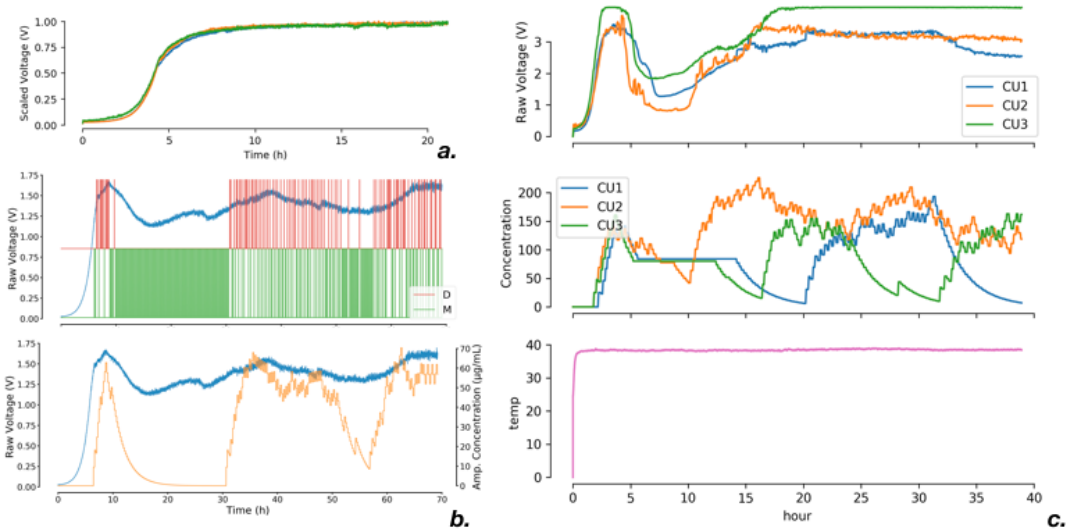

Figure 2. Experimental data generated from the EVE. (a) Biological replicates (individual CUs) show bacterial growth as voltage measurements. As bacteria grows, more light is scattered on the photodiode and allows precise measurement of growth over time. (b) A single CU grows bacteria (E. coli) into log phase and then introduces the culture to drugs (Ampicilin). The culture is allowed to recover after initial exposure and then is exposed again without allowing ampicilin washout. Slowly, over 72 hours, resistance is evolved in the culture. (c) Even though the same experiment is performed in each of the culture units, each culture behaves differently, allowing users to see the stochasticity of evolution.

Conclusions

We present a low-cost, open-source, extensible, and functionally-flexible bioreactor design. An EVE build used for simple growth experiments with triplicate CUs can be built for approximately $72, while an EVE build for directed growth experiments with selective agents will cost approximately $154.This cost is an estimate for all the parts excluding the incubator, 3D printer costs, and any glassware needed to prepare the solutions for the experiment. These estimates are included in the Github repository. []

This is particularly relevant for those who wish to implement the system in the educational setting. This low cost enables labs to explore concepts of evolution without investing much capital in tools. A further, novel feature of our system is the ability to manage multiple experimental protocols at once. The capability of the EVE system to manage multiple protocols in this manner makes it the only system of its kind that is fully open source and of low-cost.

While current restrictions include limited processing power from the Raspberry Pi, support for a maximum of 4 simultaneous CUs with the current circuit design, and autonomous passage of only one drug solution at a time, future modifications can easily be made to the system to address these limitations. The proposed framework has many applications to the study of evolution for educational and research purposes as well as directed evolution for industrial applications, and beyond. Indeed, this device may be used to study a wide variety of biological concepts, even outside the field of evolutionary biology. The open source nature of the project welcomes modification to the hardware and the software for more specialized applications of the technology. We hope the versatile and accessible nature of this device makes it useful for scientists and educators from all areas.

Materials and Methods

Assembly of the EVE can be separated into hardware and software sections. Hardware setup influences software configuration, so it is beneficial to start with hardware setup.

Hardware

A list of required hardware components can be found in the GitHub repository. To summarize, the list of required broad categories of equipment are as follows:

- Incubator
- 3D printed hardware to contain the vial and electronics
- Pumps
- Vials, silicone tubes, and reservoirs
- Circuit board and related electronics
- Raspberry Pi

The GitHub repository contains the following pieces of information in the “Start Building Folder”:

- Circuit schematics and PCB files
- 3D print-able hardware to hold the vials, diodes, caps, magnet-holders, and fans
- Build instructions for experiment equipment
- Experimental procedures

This cost is an estimate for all the parts excluding the incubator, 3D printer costs, and any glassware needed to prepare the solutions for the experiment.

Circuit board files are packaged in the Github repository and ready to be sent to a PCB manufacturer. We used a Chinese manufacture who charged approximately $35 for each PCB. This cost includes assembly of the PCB with 90% of the surface mounted components as well as shipping costs.

Software

Software is based on the Python language and can be run from a single Raspberry Pi. Users interact with a simple web interface that is capable of controlling the hardware, running experiments, and editing configuration files. The web interface used in this project is forked from another open source script server (https://github.com/bugy/script-server) and modified to integrate with EVE control applications. This web interface can be utilized by multiple users connected to the local network, allowing for remote monitoring and control of experiments. This network capability of the device also allows it to save data to network locations, preventing lack of local space on the Pi from limiting experiment duration. The software allows data to be saved to a USB attached to the Pi or to network locations mounted to the Pi's file system. A schematic representation of the software is in Fig .

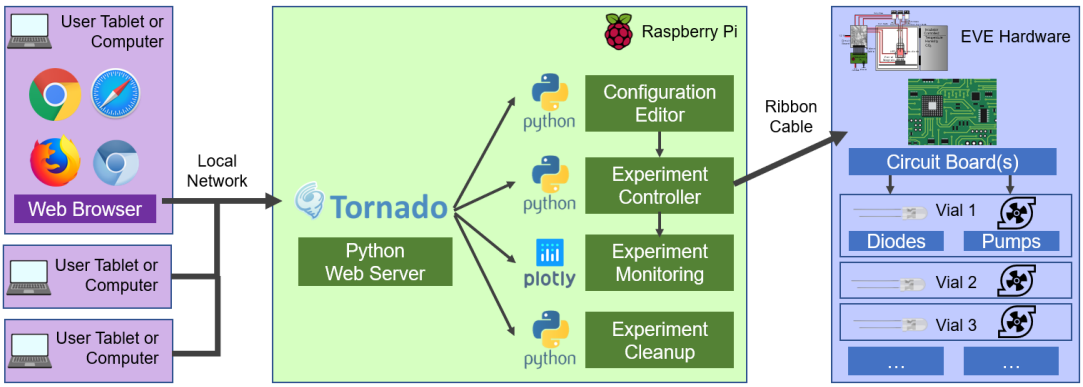

Figure 3. Software schematic of the EVE system. The device's software resides on the Raspberry Pi and centers around a Python-based web-server, which serves as the GUI for experimental design and control. The experiment controller application directly interfaces with the circuit board. All of these applications are open source and are built with extensibility in mind.

Further instructions on the setup and use of the software are provided in the Github repository wiki section. Within the repository, users will be able to download the full software packages and read about how to use the software. Instructions will also be included along with the software. There already exists a growing community of users who ask questions within Github and through email. Some users have modified EVE's code to work with their projects and are working to contribute code to the base repository. As the user base grows, we expect more code to be made available and extend the device to specific applications.

Calibration

Calibration can be performed to map voltage of the photodiode (a surrogate of the optical density of the culture) to the true absorbance of the culture at 600nm. Steps to calibrate the EVE and sample graphs will be included in the Github repository's wiki section. The spectrophotometer that we used to measure the true optical density of the samples was the Biotek Synergy H1 Hybrid Reader. For the types of LED and photodiodes that we are using, we see a linear relationship between the voltage and the OD600. Since we are using inexpensive pumps, flow rates should be measured regularly and updated within the configuration file. By controlling the duration of pump activation, information bout the flow rates can be used to ensure volume homeostasis.

Supplemental Information

- **Github Repository.** Contains all the information needed to construct an EVE. All resources are open-source and under the MIT licence.: https://github.com/vishhvaan/eve-pi
- **Start Building Folder.** Contains parts list, circuit designs, configuration file definitions, and printable hardware: https://github.com/vishhvaan/eve-pi/tree/master/Start%20Building

- **EVE Wiki.** Protocols for biological and experimental setup: https://github.com/vishhvaan/eve-pi/wiki

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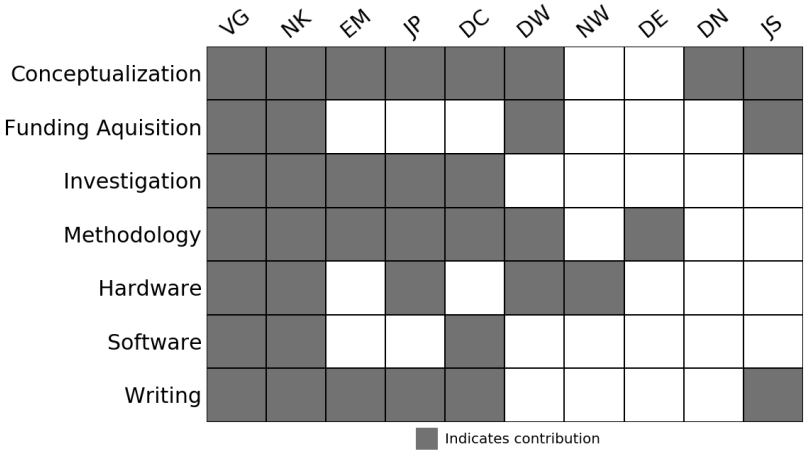
Contributions



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