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Building a Morbidostat: An automated continuous-culture device for studying bacterial drug resistance under dynamically sustained drug inhibition

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Abstract

We present a protocol for building and operating an automated fluidic system for continuous culture that we call the "morbidostat". The morbidostat is used to follow evolution of microbial drug resistance in real time. Instead of exposing bacteria to predetermined drug environments, the morbidostat constantly measures the growth rates of evolving microbial populations and dynamically adjusts drug concentrations inside culture vials in order to maintain a constant drug induced inhibition. The growth rate measurements are done using an optical detection system that is based on measuring the intensity of back-scattered light from bacterial cells suspended in the liquid culture. The morbidostat can additionally be used as a chemostat or a turbidostat. The whole system can be built from readily available components within two to three weeks, by biologists with some electronics experience or engineers familiar with basic microbiology.

Keywords

Laboratory adaptation experiments; antibiotic resistance; microbial stress response; morbidostat; chemostat; turbidostat

INTRODUCTION

Antibiotic resistance is an important public health problem rendering currently available drugs useless and threatening millions of lives[1–4]. Evolution of resistance by spontaneous mutations can be studied in the lab by exposing bacterial culture to an environment where growth is inhibited by antibiotics, and characterizing the resistant mutants that emerge[5,6]. However, since the bacteria are typically subject to a fixed level of the drug concentration,

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those studies are generally limited to only one or at most a few mutational step conferring resistance. Once such single mutations emerge and sweep through the population, the inhibition by the drug is relieved and there is no additional pressure to evolve higher levels of resistance. To be able to follow the evolution of resistance through sequential accumulation of multiple mutations, we must thus be able to keep increasing the drug concentration such that the evolving bacterial population is constantly challenged. However, carrying out such experiments using environments where drug concentrations are determined beforehand is almost impossible since the phenotypic effects of emerging mutations on complex evolving populations cannot be predicted. Experimental systems that can automatically adjust drug concentrations to maintain a fixed level of growth inhibition on evolving bacterial populations are therefore useful for studying evolution of drug resistance[7]. Such experimental systems become particularly important for studies that aim to quantify the evolutionary dynamics of bacterial populations in different drugs or drug combinations[8,9].

We recently introduced an automated continuous culture device, the “morbidostat”, for studying evolution of drug resistance in a controlled environment containing an antibiotic at a concentration that is dynamically adjusted such that the bacterial population is constantly challenged (Fig. 1a)[5]. The bacterial population is growing in fixed volume (V) with continuous stirring, and at fixed time intervals (Δt) the culture is diluted by injection of a fixed amount (ΔV) of fresh media, or fresh media containing dissolved drugs. Similar to a chemostat, where cell cultures are periodically diluted at a constant rate smaller than the maximal growth rate of the population, the dilution rate of the morbidostat $r_{\text{dilution}} \cong \Delta V/(V \cdot \Delta t)$ is fixed. In steady state, the bacterial growth rate must reach a value that matches this dilution rate. However, in contrast to a chemostat, where the bacterial growth is inherently limited by nutrient availability, in the morbidostat the cell density is kept low such that the population is not nutrient limited and instead its growth rate is controlled by externally adjusting drug concentration. At the end of each period Δt, the growth rate (r, Fig. 1b, black lines) is calculated based on the optical density measurements (OD, Fig. 1b, grey dots). Then, depending on the calculated growth rate and the current OD of the culture, the morbidostat decides whether to add fresh media, or fresh media plus drug (in either case the same fixed volume ΔV is added). Fresh media with drug is injected only if the OD exceeds a threshold (OD_{THR}) and the growth rate is higher than the dilution rate (Fig. 1b, magenta filled circles) in all other cases fresh media is injected. Drug concentrations inside the culture vials increase with drug injections and are gradually reduced by dilution with successive fresh media injections (Fig. 1c, magentaline). The value of OD_{THR} is chosen to be small enough such that the population is never nutrient limited, typically OD_{THR}=0.15. Choosing the values of Δt, V and ΔV such that the dilution rate r_{dilution} is significantly lower than the maximal growth rate of the bacteria, forces the system to adjust the drug concentration to reduce the growth rate accordingly. Typically, for bacteria growing at maximal rate of r_0=0.8 hr^{-1}, the morbidostat is set to V=12ml, ΔV=1ml and Δt=12 minutes, such that the dilution rate of the system is half of the growth rate of bacteria in the absence of drugs ($r_{\text{dilution}} \cong \Delta V/(V \cdot \Delta t)$=0.4 hr^{-1}; Fig. 1b, green filled circles). Throughout the entire experiment, the volumes of the cultures are kept constant using a suction pump (Figure 1a).

Since the population growth is limited by drug inhibition and not by nutrient depletion, the evolutionary changes occurring in the populations are likely to be associated with drug resistance. Once the population evolves and becomes more resistant, it starts to grow faster in the presence of the drug, and in response the drug concentration is adjusted such that the population is again inhibited using a higher drug dose and the growth rate converges to the fixed dilution rate of the system. The continuous drug inhibition challenges the bacterial population to keep evolving by accumulating multiple resistance mutations. We typically continue morbidostat experiments until a diminishing rate of increase in resistance is

observed. For all three drugs used in our recent studies, all of the cultures attained high, steady resistance levels within three weeks[5].

Construction of the morbidostat involves assembly of the morbidostat culture vials, assembly of the optical detection system, and assembly of a computer controlled array of peristaltic pumps used for liquid transfer. Our design currently allows growing 15 independently controlled cultures in parallel in a midsized commercial incubator (inner dimensions: 24″x24″x20″), however this capacity is easily increased if the setup is built in a larger incubator or in an environmental room with temperature control. If necessary, many other improvements to the system can be made by using more sensitive cell density detection optics or replacing the peristaltic pumps with formulators. The morbidostat apparatus can be used as a chemostat, where cells are grown at a constant growth rate, or as a turbidostat, where cells are grown at a constant density, by only simple modifications of the control algorithm. Therefore constructing a morbidostat enables a diverse range of experiments to be carried out in a biology lab.

Comparison with other methods

The morbidostat has the advantage of enabling laboratory evolution experiments in a dynamic environment that can be systematically perturbed with high time resolution. As OD measurements are taken continuously at one second intervals, the morbidostat can perform precise growth rate measurements for each growth period (Δt). For Δt = 12 min, this results in five growth rate measurements per hour, allowing nearly continuous maintenance of growth rate inhibition by dynamically adjusting the drug concentrations in evolving populations. Alternatively, bacteria can be evolved in a bioreactor using drug containing growth media that will inhibit growth of evolving bacteria at a desired rate[6]. However, the drug concentrations must be manually adjusted on a daily basis by quantifying the resistance of the evolving population[6]. Unlike conventional selection techniques such as the disc diffusion assay, or selection on agar plates or liquid with fixed drug concentrations, which selects just once for bacteria capable of surviving particular drug levels, the morbidostat does not relax selection as the bacteria become increasingly resistant [7,9,10]. Instead, it follows the changes in drug sensitivity and adjusts the drug concentration accordingly to maintain bacterial growth inhibition (fig. 1d). This feature makes it possible to follow the evolution of multi-step increases of drug resistance in real time. On the other hand, microbial selection experiments that are carried out using antibiotic containing agar plates or growth media have the main advantage of their relative simplicity compared to the morbidostat. However, bacterial mutants that are selected in constant drug environments tend to develop only one mutation that will release the selection pressure unlike clinical isolates that carry multiple resistance-conferring mutations[5,11].

Other potential applications

The experiments that can be done with the morbidostat are not limited to evolution of bacterial drug resistance. In the morbidostat, without any time consuming modifications to the system, it is potentially possible to carry out long term adaptation experiments that address evolution of stress response in bacteria and yeast, host-parasite interactions in microbial communities and evolution of drug resistance in cancer cells.

Overview of the Procedure

Construction of the morbidostat vials—The procedure for building the morbidostat starts with constructing 15 morbidostat culture vials (Fig. 2). Flat bottom glass vials (www.chemglass.com, CG-4902-08) with open top screw caps (www.chemglass.com, CV-3750-0024) are used for growing bacterial cells in the morbidostat. A magnetic stir bar (http://www.stirbars.com, SBM2003MIC) is dropped into the bottle for mixing the culture

with a magnetic stirrer. A custom-designed, but now commercially available, Teflon insert with five through holes (www.chemglass.com, HMS-0909-151GC) and the open top screw cap are used for secure sealing of the bottle opening. The five openings on the Teflon insert are reserved for injection of fresh media, two separate drug solutions, filtered air intake, and removing the excess liquid for keeping the culture volume constant. Autoclavable PEEK (polyether ether ketone; www.fishersci.com, 05-701-6) tubing is inserted into these openings for adding or removing liquids. High temperature-resistant silicone is applied to the upper face of the Teflon insert to fix the PEEK tubing to the Teflon insert. All of the connections between pumps and culture vials are made using autoclavable silicone tubing (www.vwrlabshop.com, 60985-708) and male/female Luer thread style connectors (Value Plastics, MTLL004-6005 / FTLL004-6005). The entire tube assembly can be sterilized by autoclave when necessary.

Construction of the tube holder array—We construct an array of tube holders, which sits on a 15 position magnetic stirrer (Fig. 3). The tube holder array is used to continuously mix bacterial cultures and to read their optical densities (OD). A Plexiglass adapter (Supplementary Fig. 1) is machined and mounted on the magnetic stirrer using L brackets. 15 tube holders are machined from black Delrin material (Supplementary Fig. 1). Every tube holder has two openings drilled for LED light sources and photo-detectors. These openings are positioned at a 135° angle to maximize the detection of scattered light. An LED light source and a photo-detector is mounted to each tube holders and connected to the circuits feeding them (www.radioshack.com, 276-0142, Fig. 4a). A circuit is built for measuring the voltage across the photo-detector (Fig. 4b) and the voltages are recorded using a data acquisition card (www.mccdaq.com, USB-1616FS). After doing a calibration for converting voltage readings to optical density, the entire tube holder array (Supplementary Fig. 2) is placed in a temperature controlled incubator (VWR, cat # 1535).

Measuring the growth rate by directly using OD values may inherently have some complications because of long filamentous cells or cell lysates in the culture vials. In order to minimize these effects in our measurements, the morbidostat assay is designed such that cells always grow in a nutrient-rich environment. Furthermore, in such cases, we perform control measurements to verify the extent of such effects. For instance, the sizes of the evolved strains with or without drugs were very similar to the size of wild type bacteria in the absence of drugs[5]. Such control experiments are especially important when using bactericidal drugs or drugs causing filamentation.

Construction of the pump array—The final step is to assemble the pump array that will be used to inject fresh media and drug solutions. 45 peristaltic pumps (http://www.clarksol.com, m045) are mounted on a custom-made aluminum pump rack (Fig. 5, Supplementary Fig. 3). These pumps are activated via two computer-controlled 24-channel electromechanical relay interface devices (www.mccdaq.com, USB-ERB24). Modified autoclavable screw cap glass bottles are used as media reservoir bottles and are connected to these pumps. A16-channel peristaltic pump is used as a waste pump for maintaining fixed culture volumes in the culture vials (www.harvardapparatus.com, 73-3154). The activation of the waste pump is also done via the relay device. Pumps are connected to culture vials with autoclavable silicone tubing (www.vwrlabshop.com, 60985-708). After making sure that all tubing connections are secure and electrical components work properly and safely, the system is sterilized and is ready for use. The entire system is controlled with a custom Matlab code with a Graphical User Interface (GUI.m).

Initial tests to optimize the morbidostat assay

After the entire apparatus is assembled, the following initial tests are performed before each new long term experiment.

Exponential growth rate measurements—All of the culture vials are filled with 12ml of growth media and 10 μl of bacterial cells from glycerol stock is added. These cultures are grown overnight and the OD values are recorded throughout the experiment while all of the pumps are turned off. The resulting growth curves (Fig. 6) are used to characterize experimental parameters such as growth rate in the absence of drugs and the OD range where cells grow exponentially. This range can vary depending on the strains and growth media used in the experiments.

Measurement of dilution rate—After sterilizing all culture and media interfacing components of the system and initializing all of the peristaltic tubing, the culture vials are filled with 12 ml (V) of growth media and 10 μl of bacterial cells from glycerol stock. The morbidostat setup is operated using the chemostat algorithm such that fresh media is periodically added to the vials. The dilution rate (r_{dilution}) of the system should be less than the exponential growth rate (r_0) of the cells. When we grow wild type MG1655 *Escherichia coli* cultures in M9 minimal media containing glucose and amicase at 30°C, cells double every ~50 minutes (r_0=0.8 hour^{-1}). Under these settings, we dilute cultures by ~8% every 12 minutes (Δt) by adding ~1 ml (ΔV) of fresh media into the culture. The theoretical dilution rate can be calculated using the following formula $r_{\text{dilution}} \cong \Delta V/(V \cdot \Delta t)$ =0.4 hour^{-1}. Using the chemostat data, we extract the experimental dilution rate and compare it with the theoretical dilution rate. Such comparison is necessary since actual dilution rates can slightly vary between morbidostat vessels due to inherent imperfections of the commercial parts such as tubings and peristaltic pumps.

Measuring the Minimum Inhibitory Concentration (MIC)—Minimum Inhibitory Concentration (MIC) of each drug is measured prior to the experiments. These measurements can be done using 96 well plates or sterile cell growth tubes depending on the availability. We generally prefer to use 96 well plates since they require less volume and measurements with multiple replicates is convenient. If the drug is used for the first time and MIC is not known, we make a drug gradient on the 96 well plates where each consecutive well on a row is three-fold diluted, with the first well having the strongest drug concentration and the last well having no drugs. Later, we add roughly 1000 cells to each well and incubate the plate on a plate shaker for 24 hours at the desired temperature. At the end of 24 hours, the minimum drug concentration where bacteria cannot survive (the well shows no increase in optical density) is assigned as the MIC. A measurement using a linear drug gradient which covers the MIC value found in the previous measurement is employed if a more precise MIC value is necessary. For MIC measurements, fresh drug solutions from powder stocks are prepared following the instructions provided by the suppliers.

Estimation of drug concentrations for growth inhibition—Both morbidostats and chemostats feed growing cell cultures with fresh media at a fixed dilution rate. The main operational difference between the morbidostat and a chemostat is that the former adds fresh media containing drug solution to the bacterial cultures when the OD of the culture exceeds a threshold and the growth rate is higher than the dilution rate of the system. Therefore, being able to add the proper amount of drugs is important. In order to estimate how much drug has to be added into the cultures, we grow several drug-sensitive *E. coli* cultures until they reach the threshold (OD_{THR}) and then manually add 1 ml of drug solution with various concentrations. The growth rates of these cultures tend to decrease depending on the drug

type and concentration. We generally found that drug solutions at concentrations 10 times higher than the Minimum Inhibitory Concentration (MIC) inhibited growth sufficiently.

Trial run of the experiment—After quantifying the concentration of the drug solution that should be used, two drug solutions in fresh media are prepared (stock-A and stock-B). The drug concentration of stock-A is 10xMIC, and the drug concentration of stock-B is 50xMIC. All of the drug injections into morbidostat tubes are initially made from stock-A. However, if the cells acquire resistance and inhibiting growth with injections from stock-A takes too long, the morbidostat starts making injections from stock-B. All fresh media and drug solution bottles are connected to the peristaltic pumps and morbidostat algorithm is tested (Supplementary Fig. 4). We generally aim to slow down growth of *E. coli* cells with one or two consecutive drug injections (Fig. 1b). If these conditions are met, we start the long-term experiment.

MATERIALS

REAGENTS

- M9 minimal salts, 5X (Sigma Aldrich, M6030)
- Glucose (Sigma Aldrich, G7021)
- Amicase (Sigma Aldrich, 82514)
- *Escherichia coli*, MG1655 wild type laboratory strain. Other bacterial strains such as *Bacillus Subtilis* can also be grown in the morbidostat. Bacterial strains are kept as glycerol stocks at −80°C.

  ! CAUTION Both drug sensitive and drug resistant bacteria should be handled by strictly following the biosafety rules for microbiological practices. Note that special permissions may be required for experiments with pathogenic bacteria.
- Antibiotic(s) to be tested (see Reagent Setup)

EQUIPMENT

Glass vial assembly

- PEEK Tubing (www.fishersci.com, 05-701-6)
- PEEK cutter (www.fishersci.com, 05-701-84)
- Silicone tubing (1mm ID, www.vwrlabshop.com, 60985-708)
- Female LUER connector (Value Plastics, FTLL004-6005)
- Male LUER connector (Value Plastics, MTLL004-6005)
- 40 ml flat bottom glass vial (Chemglass, CG-4902-08)
- Teflon Insert for 24–400 Open Top GPI Cap (www.chemglass.com, HMS-0909-151GC)
- 24–400 Open Top GPI Cap (Chemglass, CV-3750-0024)
- Permatex 81878 #101 Sensor Safe Ultra Copper Hi Temp RTV silicone
- Male LUER thread style cap (Value Plastics, MTLLP-6005)
- Female LUER thread style cap (Value Plastics, FTLLP-6005)
- Magnetic stir bar (www.stirbars.com, SBM2003MIC)

- Syringe filter (0.2 μm, www.vwrlabshop.com, 514-0066)
- Syringe filter (0.2 μm, www.vwrlabshop.com, 514-0068)

Tube holder array assembly

- Machined plexiglass sheet (Supplementary Fig. 1)
- 15 position magnetic stirrer (Neutec, VL-F203A0178)
- Machined L Brackets
- Machined plexiglass cover (Supplementary Fig. 1)
- Custom machined Delrintube holder
- O-rings for supporting tubes inside the holder
- LED holder (Radioshack, Catalog #: 276-080)
- Infrared LED Emitter and Detector (Radioshack, Catalog #: 276-142)
- Machined housing for LED holder
- Colored 22 gauge stranded wire (amazon.com)
- 68Ω resistor (Radioshack, Catalog**#:** 271-1106)
- Various resistors (Radioshack, 10kΩ–100kΩ)
- High-Power AC Adapter (Radioshack, Catalog # 273-318)
- Temperature controlled incubator (VWR, cat # 1535).
- Data acquisition card (www.mccdaq.com, USB-1616FS).
- Uninterruptable power supply (UPS, amazon.com)
- Windows computer with Matlab 32-bit installed (for control software)
- Wire stripper
- Soldering tools
- Electrical tape and/or heat shrink insulation
- Wire zip ties (for organizing things)

Pump array construction

- Peristaltic pumps(www.clarksol.com, Catalog # m451605)
- USB controller Relay Interfavce device (www.mccdaq.com, USB-ERB24)
- 16 channel peristaltic pump (www.harvardapparatus.com, 73-3154)
- Silicone tubing (0,125″ ID, www.vwrlabshop.com, 89068-474)
- Male LUER connector (Value Plastics, MTLL025-6005)
- 6L Erlenmeyer flask (www.vwrlabshop.com, 1500–6000)

Constructing media reservoir bottles

- 5L Media Storage Bottle with GL45 Screw Cap (www.vwrlabshop.com, 1395-5L)
- 1L Media Storage Bottle with GL45 Screw Cap (www.vwrlabshop.com, 1395-1L)

REAGENT SETUP

Handling antibiotics—Throughout the morbidostat experiments, we measure the MIC of our drug solutions on a daily basis using wild type drug sensitive strains to make sure that the efficacy of the drugs is not decreased. In order to extend the lifetime of these solutions, we strictly follow the instructions provided by the suppliers. For example, we wrap drug solution bottles with aluminum foil when we use light sensitive drugs.

PROCEDURE

Assembling morbidostat vials ●TIMING~ 9 hours, plus drying overnight

1 Cut 3 pieces of PEEK tubing with 3″ length. After cutting these pieces, bevel one end of each piece using a PEEK cutter. These pieces will be used for liquid injections (Fig. 2a).

2 Cut 1 piece of PEEK tubing with 1.5″ length. This will be used for filtered air intake.

3 Cut 1 piece of PEEK tubing with 5″ length. This will be used for extraction of excess culture in morbidostat vials.

4 Cut 5 pieces of silicone tubing (1mm ID)with 4″ length.

5 Insert each piece of PEEK tubing into one piece of silicone tubing to a depth of ~0.5″.

6 Insert female LUER connectors into the other end of piece of silicone tubing (Fig. 2b).

7 Fill a 40 ml flat bottom glass vial with 12 ml water.

8 Close the opening of the vial with the Teflon Insert for 24-400 open top GPI cap.

9 Close the opening of the bottle using a 24-400 open top GPI cap.

10 Push the 5″ PEEK tubing through one of the holes till the tubing just makes contact with the water inside the glass vial (Fig. 2c).

11 Push all of the other PEEK tubing pieces through the holes on the Teflon insert (Fig. 2d).

▲ **CRITICAL STEP** The bevel shaped end of the 3″ PEEK tubing should be at least 1″ above the liquid level inside the glass vial to avoid contamination by droplets.

12 Apply high temperature-resistant silicone to the entire upper face of the Teflon insert to fix the PEEK tubing to the Teflon insert (Fig. 2e).

13 Repeat steps 1–12 to make a total of 15 culture vials.

14 Wait overnight until the silicone is completely dried.

15 Close all of the female LUER connectors using male LUER thread style caps.

16 Transfer the assembled vial caps to clean glass vials, each containing a magnetic stir bar.

17 Autoclave all of the assembled vials at 121°C for 20 minutes.

18 Attach syringe filters to the female LUER connectors reserved for air inlet, taking care to keep the vial side of the filters sterile.

Assembling tube holder array ●TIMING~ 24 hours

19 Mount the machined plexiglass sheet on the 15 position magnetic stirrer using L brackets.

20 Take two LED holders and discard the washers and the nuts that come with them (Fig. 3a).

21 Push the legs of the infrared LED all the way into the LED holder from the small opening (Fig. 3b).

22 Push the legs of the photo-detector all the way into the LED holder from the small opening.

23 Solder 4 pieces of 22 gauge stranded wires to the legs of LED and photo-detector (Fig. 3c).

▲ **CRITICAL STEP** Using wires with different colors (i.e. red, green, blue, black) is helpful to keep track of devices and polarities. The length of the wires must be selected depending on the organization of the wires.

24 Insulate all of the wire connections using electrical tape or heat-shrink insulator (Fig. 3d).

25 Mount the LED holder to the machined housing for LED holder (Fig. 3e).

26 Prepare 15 pairs of LED and photo-detector assemblies following steps 19–25.

27 Place all of the LED and photo-detector assemblies into the openings on the sides of the machined Delrin tube holders (Fig. 3f).

▲ **CRITICAL STEP** At this point, there will be 15 tube holders and 60 wires. Labeling the tube holders and wires will help to avoid confusion and aid indebugging.

28 Connect a 68Ω resistor to the wire from the LED anode and insulate the connection using electrical tape or heat shrink insulator.

29 Mount all of the tube holders to the circular openings on the plexiglass sheet attached to the magnetic stirrer.

30 Following the circuit diagram given in Fig. 4a, connect all of the LEDs in parallel and feed them with a power supply. Set the voltage to 6V. Make sure that all the LEDs are emitting using a digital camera that can detect infrared (e.g. webcam or mobile phone camera).

31 Following the circuit diagram given in Fig. 4b, splice the wires coming from the short leg of the photo-detectors.

32 Following the circuit diagram given in Fig. 4b, connect a resistor to the other leg of the photo-detectors.

▲ **CRITICAL STEP** The resistance of all resistors should be chosen such that 1 OD change should correspond to 2 Volts. These resistances are generally around 100kΩ and may differ for each detector. We prefer to keep these resistors outside the incubator to measure the voltages across them. Therefore, keep the connections as long as necessary.

33 After all the connections are made, affix the machined plexiglass cover to protect the wires from possible liquid spills (Supplementary Fig. 2).

34 Guide all the wires coming from the tube holder array to the outside of the incubator using the 2″ diameter port on the right side of the incubator.

35 Connect all the photo-detectors to a power supply and set the voltage to 6V.

▲ **CRITICAL STEP** All of the photo-detectors should be connected in parallel.

36 Connect wires to both sides of the photo-detector resistors for measuring the voltage across them using a multichannel data acquisition card. Repeat for all 15 tube holders.

37 Connect these pairs of wires to the screw terminals of the data acquisition card.

▲ **CRITICAL STEP** The data acquisition card has 16 screw terminals, numbered 0 to 15. Connect tube holder #1 to terminal 0, tube holder #2 to terminal 1, and so on.

38 Using the software provided by the DAQ card supplier, make sure that all of the photo-detectors are responding to variations in incident light. The DAQ card we use is set to acquire data at 500Hz acquisition rate and the voltage readings are median filtered every second to eliminate noise. Typically signal-to-noise ratio (mean/standard deviation) in our voltage readings is better than 100.

▲ **CRITICAL STEP** The most common reasons for having a nonresponsive detector are broken connections or reversed polarity.

Calibration of the detectors ●TIMING~ 2h

39 Dilute an overnight bacterial culture to ~OD=0. 75 in minimal growth media.

40 Add ~15 ml of the diluted culture in a glass vial and drop a magnetic stir bar in the vial.

41 Turn on the magnetic stirrer that sits under the tube holder array, and set the stirring speed to ~200 rpm.

42 Put the culture vial in the first tube holder, wait for 10 seconds. Record the voltage for 10 seconds and calculate the median voltage value (Supplementary Fig. 5).

43 Move the culture vial to the next tube holder and record the median voltage as described in step 42. Repeat for all tube holders.

44 After completing the voltage recording for all 15 tube holders, take out 5ml of the culture using a serological pipette, and transfer to a spectrophotometer cuvette.

45 Measure the optical density of the removed culture using a standard spectrophotometer. Record the optical density value.

46 Dilute the cell culture in the glass vial by adding 5 ml of fresh medium and mix well.

47 Measure the voltage values for the diluted cell culture in all tube holders and record the corresponding OD value using the spectrophotometer.

48 Repeat the dilution and voltage recording process (steps 46–47) until the optical density drops below 0.03.

49 After finishing voltage and OD recordings for all 15 cultures, plot voltage values against corresponding OD values for all 15 tube holders and fit a line (OD=calibration factor x Voltage + offset) for finding the calibration factor. We

typically use a Matlab command called “robust fit” for fitting a line (Fig. 6). Calibration factors may change over time. In our experience, the difference in calibration factors is ~5% after a month of continuous use.

? TROUBLESHOOTING

Assembling the pump array ● TIMING~24 hour s

50 Assemble the pump stand (Supplementary Fig. 3).

51 Attach all of the peristaltic pumps to the pump stand (Fig. 5a).

▲ **CRITICAL STEP** 45 pumps are necessary since three pumps are reserved for each morbidostat culture vial. Label all of the pumps properly.

52 Connect one of the two wires of each pump to one of the poles of the AC power source.

! CAUTION The pumps we use work with 110 VAC, therefore one can directly use a power outlet on the wall. Precautions should be taken for safe handling of high voltage. All of the electrical connections should be safely insulated with electrical tape or similar material. Wearing electrician gloves is advised.

53 Connect the C contact of the relay switch #1 to the other pole of the AC power source.

54 Connect all of the C contacts of the two relay boxes together using insulated wires.

▲ **CRITICAL STEP** Two relay boxes will have total of 48 relay switches. We use all 24 of the relay switches in the first relay box and 21 relay switches from the second relay box.

! CAUTION Unplug the power source before making the connections.

55 Connect the second wire of the pumps to the NO contacts on the two relay boxes.

▲ **CRITICAL STEP** In our current configuration, the three pumps feeding a culture vial are controlled by three neighbor relay switches. For example, Relay switches #1, #2, and # 3 on relay box control fresh media pump, drug A pump, and drug B pump of culture 1. The Matlab code (GUI.m) provided with this protocol (Supplementary Data) has to be modified if the connections are made differently.

56 Use one of the remaining 3 unoccupied relay switches to control the 16-channel peristaltic suction pump.

▲ **CRITICAL STEP** Turning this type of peristaltic pump on and off with an electronic control board should be done by strictly following the instructions provided by the pump supplier in order to protect the electronic pump circuits. In our current configuration, we use the 24^{th} relay switch of the second relay box for controlling the suction pump. The Matlab code provided with this protocol (Supplementary Data) has to be modified if the connection is made differently.

57 Test all of the connections and make sure that the code controls the pumps properly (Supplementary Fig. 6).

58 Cut 90 pieces of silicone tubing with ~4 cm length (0,125″ ID).

59 Attach silicone tubing pieces to the tubing connectors of each pump (Fig. 5b).

60 Insert a male LUER connector to each piece of silicone tubing attached to the pumps (Fig. 5c).

61 Insert a male LUER connector to the ends of each piece of silicone tubing installed in the 16-channel suction pump.

62 Place all of the morbidostat culture vials in the tube holders.

63 Measure the distance between the culture vials and the pumps (media pump, drug A pump, drug B pump, and suction pump) and cut silicone tubing (1mm ID) with appropriate length.

64 Insert one male and one female LUER connector to the ends of each silicone tubing pieces from step 63.

65 Group the four sections of tubing prepared for each culture vial and bundle the tubes together using electrical tape. All male LUER connectors should be on the same end of the bundle. Label every tube carefully by including the culture number and the pump names (i.e. Culture 1, media pump: 1M).

66 Connect the male LUER connectors of the bundle to the female LUER connectors on the morbidostat tubes.

67 Connect the female LUER connectors of the bundle (the ones reserved for liquid injections) to the male LUER connectors on the exit port of the peristaltic pumps.

68 Connect the last female LUER connector of the bundle that will be used to draw excess volume from the culture vial to the male LUER connectors on the 16-channel suction pump.

▲ **CRITICAL STEP** Double check the flow direction of the suction pump and properly connect the tubings such that the 16-channel pump will draw liquid away from the culture vials.

69 Fill an Erlenmeyer flask with 1 liter pure bleach. This will be used for collection of bacterial culture removed from the culture vials.

70 Place the Erlenmeyer flask in a safe place that is sufficiently close to the 16-channel pump.

71 Measure the distance between the 16 channel pump and the mouth of the Erlenmeyer flask. Cut 15 pieces of silicone tubing with the measured length (1mm ID).

72 Insert a female LUER connector to one end of all of the pieces of silicone tubing from the step 71.

73 Connect all of these silicone tubes to the 16-channel pump using LUER connectors.

74 Bundle all of these silicone tubes together using plastic cable ties or electrical tape.

75 Put the free end of the bundle into the Erlenmeyer flask. Seal the mouth of the Erlenmeyer flask using parafilm.

▲ **CRITICAL STEP** Empty the flask periodically and always keep enough bleach to kill the bacterial cells.

Constructing media reservoir bottles • TIMING~ 6 hours, plus drying overnight

76 Drill 16 holes with 2.5mm diameter on GL45 bottle caps using an electrical drill.

77 Cut 15 pieces of silicone tubing (1mm ID)with 2 feet length.

78 Cut a piece of silicone tubing (1mm ID)with 4″ length.

79 Close a bottle tightly using the drilled GL45 cap.

80 Push all 15 pieces of silicone tubing (2′ length) through the holes on the cap such that the silicone tubing touches the bottom surface of the bottle.

81 Push the 4″ length of silicone tubing about one inch through the remaining h ole on the cap.

82 Insert a female LUER connector to the top ends of the pieces of silicone tubing pieces (these ends stay outside the bottle).

83 Apply high temperature resistant silicone to the entire upper face of the GL45 cap to fix the silicone tubing to the cap (Fig. 5d).

84 Wait overnight until the silicone is completely dried (Fig. 5e).

85 Close all of the female LUER connectors using male LUER thread style caps to keep the bottle sterile.

86 Autoclave the assembled reservoir at 121°C for 20 minutes.

87 Replace the male LUER cap attached to the silicone tubing reserved for air intake with a syringe filter taking care to keep the bottle side of the filter sterile.

▲ **CRITICAL STEP** We recommend assembling at least 2 reservoirs with 5lt volume and 4 reservoirs with 1 lt volume. Smaller reservoirs are used for drug stocks and one may need more drug solution reservoirs depending on the experimental design.

Sterilizing the tubings • TIMING~ 4 hours

88 Fill four reservoirs with 10% bleach (vol/vol), 70% ethanol (vol/vol), sterile water, and growth media respectively.

89 Connect the 10% bleach reservoir to media pumps using LUER connectors.

90 Spray 70% ethanol on all the LUER connections.

91 Run all of the media pumps and the suction pump for 5–10 minutes.

▲ **CRITICAL STEP** Closely watch the process and make sure that tubes and connections are not leaking.

92 Let the bleach stand in the system for 5 minutes with the pumps turned off.

▲ **CRITICAL STEP** Bleach may clog the tubes if it stands for too long.

93 Swap the 10% bleach reservoir with the sterile water reservoir.

94 Run all of the media pumps and the suction pump for 5 minutes.

95 Swap the sterile water reservoir with 70% ethanol reservoir.

96 Run all of the media pumps and the suction pump for 5 minutes.

97 Let the ethanol stand in the system for 15 minutes with the pumps turned off.

98 Swap the 70% ethanol reservoir with sterile water reservoir.

99 Run all of the media pumps and the suction pump for 5 minutes.

100 Swap the sterile water reservoir with growth media reservoir.

▲ **CRITICAL STEP** This step has to be done very carefully using sterile gloves and open flame if available, to avoid contaminating the media reservoir.

101 Insert sterile syringe filters between the male LUER connectors attached to the peristaltic media pumps and female LUER connectors attached to the media reservoir.

▲ **CRITICAL STEP** This step has to be done very carefully using sterile gloves and open flame if available, to avoid contaminating the media lines.

102 Run all of the media pumps and the suction pump for 5 minutes to initialize the tubing.

103 Repeat steps 88-102 for sterilizing the drug pumps. Drug pumps are finally connected to stock-A and stock-B reservoirs instead of the media reservoir at step 100.

Running the Morbidostat Assay • TIMING 2–4 hour/day maintenance

104 On the first day of the experiment, add 100 μl of wild type, drug sensitive cells into 200 ml of minimal M9 media with 0.2% amicase (wt/vol) and 0.4% glucose (wt/vol). Incubate the cells for 30 minutes in a flask at 30°C using a shaker. At this stage, the OD of the culture is not detectable.

▲ **CRITICAL STEP** All of our experiments were done with filtered minimal M9 media with 0.2% amicase and 0.4% glucose. However, LB or other media types can be used. Extra calibration of the detectors might be needed if different media is used. Stock-A has a drug concentration that is 10 times higher than MIC. Stock-B is 5 times more concentrated than stock-A. If alternate media are used, the dilution rates in the control code may need to be adjusted to accommodate changes in growth rate.

▲ **CRITICAL STEP** Growth media should be prepared in advance. Prepare the media and make sure that there is no contamination in the media for at least 24 hours.

105 Fill all of the morbidostat vials with 12 ml of cell culture. Label the vials (Culture #1, Culture #2 etc.).

▲ **CRITICAL STEP** Use autoclaved sterile glass vials every time. Glass vials are autoclaved with one magnetic stir bar inside them.

106 Seal the vials. Place all of the vials in the tube holders. Then run the morbidostat software (Supplementary Data). You can run GUI.m for basic pump controls, automated calibration of the detectors, quick simulations, as well as to start the experiment with custom parameters that you specify. The computer will continuously record the OD values of the cultures and feed the culture with fresh media every 12 minutes. No injections will be made into a vial until the OD inside the culture exceeds 0.03. If the OD of a culture exceeds OD_{THR} (currently 0.15) and the net growth of the culture is positive, drug solution from stock-A will be added. If the morbidostat cannot inhibit growth by adding drug solution from stock-A, it will start injecting drug solution from stock-B.

▲ **CRITICAL STEP** If drug solution is being added from stock-B, prepare a drug solution that is 5 times more concentrated than stock-B. On the next day, replace stock-A with stock-B and replace stock-B with the new more concentrated drug solution.

? TROUBLESHOOTING

107 Pause the experiment after 24 hours, transfer 500 μl of the cells to a new sterile glass vial that has ~12ml growth media.

▲ **CRITICAL STEP** Switching to fresh vials every day is necessary to avoid biofilm formation. Otherwise, bacterial biofilms become visible in 2–3 days.

108 Transfer 500 μl of the cells to a sterile 1.5 ml Eppendorf tube, add 250 μl sterile 50% glycerol and freeze/store at −80°C. Eppendorf tubes must be properly labeled.

109 Resume the experiment.

▲ **CRITICAL STEP** Several problems such as contamination may take place in morbidostat experiments. Keeping the glycerol stocks from every day of the experiment is very critical. Restart the experiment using the glycerol stock from the previous day if anything goes wrong with a culture.

110 Analyze the data on a daily basis to calculate IC_{50} values (Fig. 1d).

111 Continue the experiment until a diminishing return in the rate of increase of resistance of the evolving populations is observed.

TROUBLESHOOTING

Troubleshooting advice is provided in Table 1

TIMING

Steps 1–18: Assembling morbidostat vials: ~9 hours, plus drying overnight

Steps 19–38: Assembling tube holder array: ~24 hours, inexperienced users may need more time.

Steps 39–49: Calibration of the detectors: ~2h

Steps 50–75: Assembling the pump array: ~24 hours, inexperienced users may need more time.

Steps 76–87: Constructing media reservoir bottles: ~6 hours, plus drying overnight

Steps 88–103: Sterilizing the tubings: ~4 hours

Steps 104–111: Running the Morbidostat Assay: 2–4 hour daily maintenance

ANTICIPATED RESULTS

In the morbidostat, for all of the antibiotic compounds we have used so far, drug sensitive *E. coli* populations evolved strong drug resistance in less than three weeks. One of the biggest advantages of the morbidostat is following evolution of drug resistance almost in real time. As illustrated in Fig. 1b-e, the amount of drug that is necessary to inhibit bacterial growth by fifty percent (IC_{50}) can be calculated by directly analyzing the data acquired during the morbidostat experiment. In general, we adjust the parameters of the assay such that one or two injections of drug solution is adequate to slow down the bacterial growth. However, as the bacteria evolve resistance, the number of drug injections noticeably increases. When the

assay is successfully carried out for several weeks, drug resistance of bacterial populations increases either gradually or in a stepwise manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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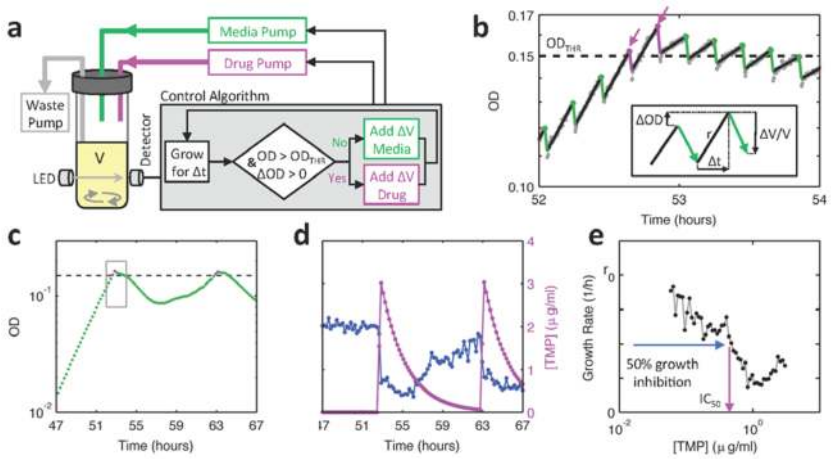

Figure 1. The morbidostat is an automated continuous-culture device that maintains a constant level of growth inhibition on evolving bacterial populations

a, The working algorithm of the morbidostat. **b**, Representative bacterial growth in the morbidostat. OD values are shown with grey dots. Growth rates (*r*) of bacterial populations are periodically calculated by fitting exponential growth functions (black lines). Markers with red and green colors represent dilutions with drug solution and fresh media, respectively. **c**, Growth rates (blue) and drug concentrations (magenta) between two consecutive drug solution injections. **d**, The drug concentration that inhibits growth by 50% (IC_{50}) is calculated by analyzing growth rates and corresponding drug concentrations.

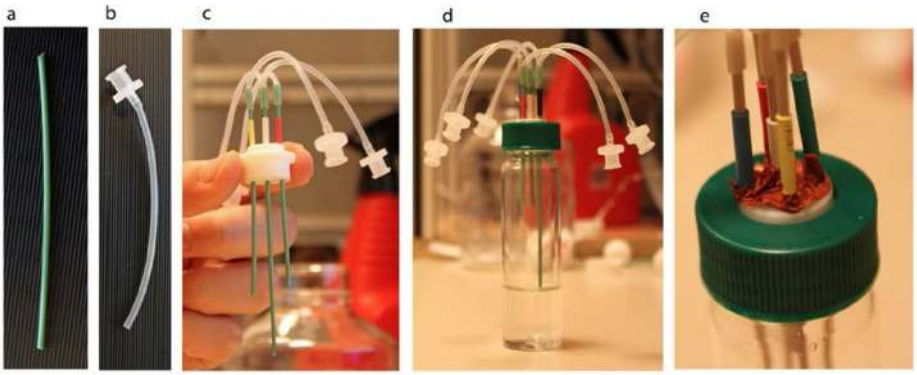

Figure 2. Construction steps for morbidostat vial assembly

a, PEEK tubing pieces. **b**, Assembled silicone tubing and LUER connector. **c**, Teflon insert assembled with PEEK tubing pieces. **d**, Assembled morbidostat vial. The height of the longer PEEK tubing is adjusted such that the volume of the culture in the vial is 12 ml. **e**, High temperature-resistant silicone is applied to the entire upper face of the Teflon insert.

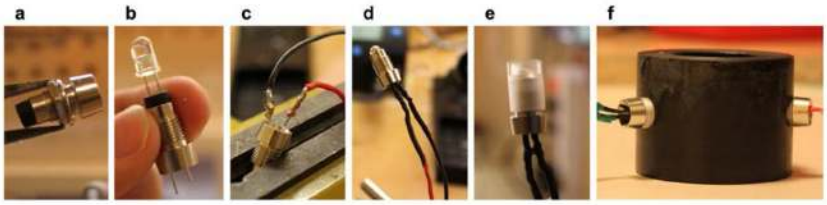

Figure 3. Construction steps for tube holder assembly

a, Commercial LED holder. **b**, LED and the photo-detectors are inserted into the LED holder. **c**, Legs of the LED are soldered to wires. **d**, Wire connections are insulated with heat shrink insulators. **e**, Custom machined LED housing is attached to the LED holder. **f**, LED and photo-detector assemblies are inserted into the custom machined Delrin tube holders.

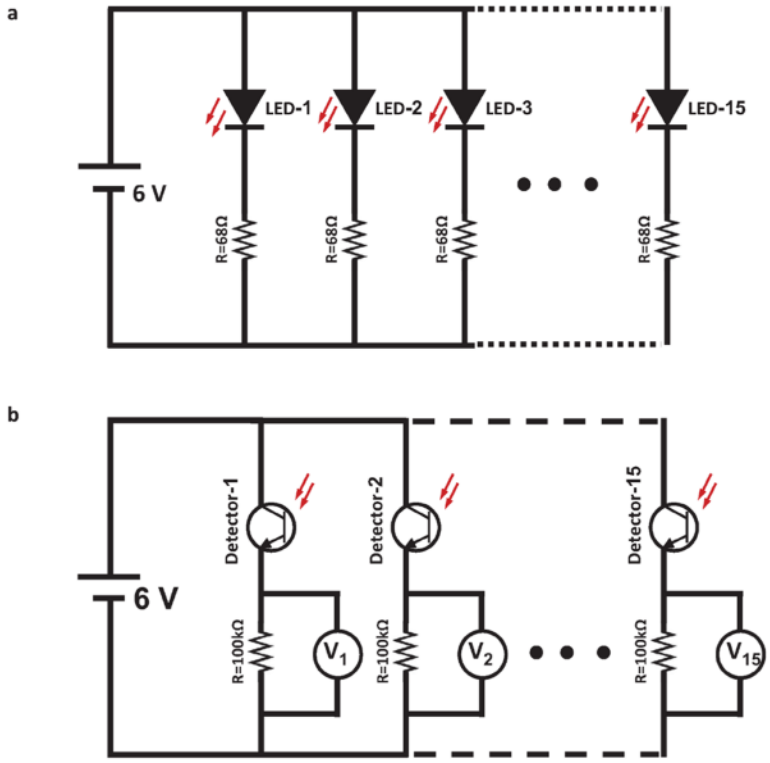

Figure 4. Circuit diagrams for the OD detection system

a, Each LED is connected to a 68Ω resistor in series. All LEDs are connected in parallel. **b**, Each photo-detector is connected to a resistor in series. All photo-detectors are connected in parallel.

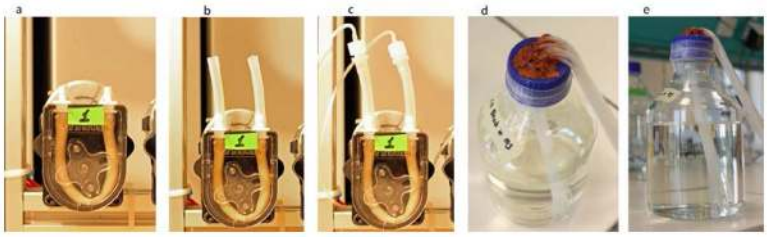

Figure 5. Construction steps for pump array and media reservoir assemblies

a, A peristaltic pump is attached to the pump stand (Supplementary Fig. 4). **b**, Two pieces of silicone tubing is attached to the pumps. **c**, An assembled peristaltic pump with LUER connectors and silicone tubing. **d**, Silicone tubing pieces are inserted through holes on a GL45 screw cap. High temperature-resistant silicone is applied to the entire upper face of the screw cap. **f**, The assembled media reservoir.

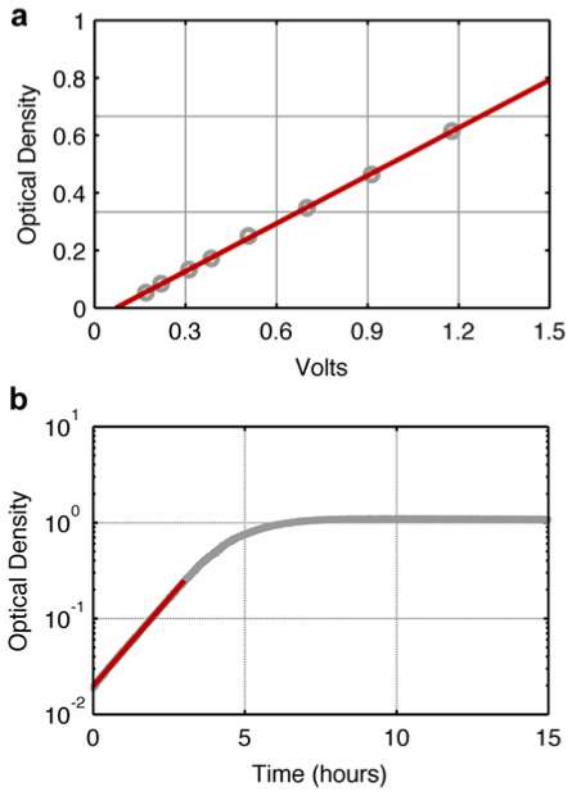

Figure 6. Calibration of the detectors

a, The voltages (black asterisk) created by cultures with known OD values are recorded. A line (red line) is fitted for finding the calibration factor. **b**, An E. coli culture is grown overnight and OD values are recorded (grey dot) every second. Red line shows the range where the growth is exponential.

Table 1

TROUBLESHOOTING

Step	Problem	Possible reason	Possible solution
49	Dynamic voltage range of a photo-detector is small.	The resistor connected in series for amplification is not appropriate.	Try a range of new resistors with different resistances.
106	Tubing is contaminated.	Working in conditions that are not sufficiently sterile.	Wash the tubing using 10% bleach solution and sterile water. Autoclave all the tubing.
	Unexpected spikes in voltage readings.	Bacterial clumps in the liquid culture.	Increase stirring speed or resume the experiment from the previous day.
	Bacterial growth is not inhibited by antibiotics.	Drug stock is not concentrated enough or old.	Make stronger fresh drug solutions from powder.
	Bacteria do not grow after injections of antibiotics.	Drug stock is too concentrated.	Dilute the drug stock.
	OD does not change after media injections.	Biofilms on the inner wall of the cell vial.	Replace the glass vial and resume the experiment from the previous day.