

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

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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 the 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 2–3 weeks by biologists with some electronics experience or engineers familiar with basic microbiology.

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 laboratory by exposing a bacterial culture to an environment in which growth is inhibited by antibiotics and by characterizing the resistant mutants that emerge^{5,6}. However, as bacteria are typically subjected to a fixed drug concentration, those studies are generally limited to only one or at most a few mutational steps 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 experiments using environments in which drug concentrations are determined beforehand is almost impossible, as 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 the evolution of drug resistance⁷. 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}.

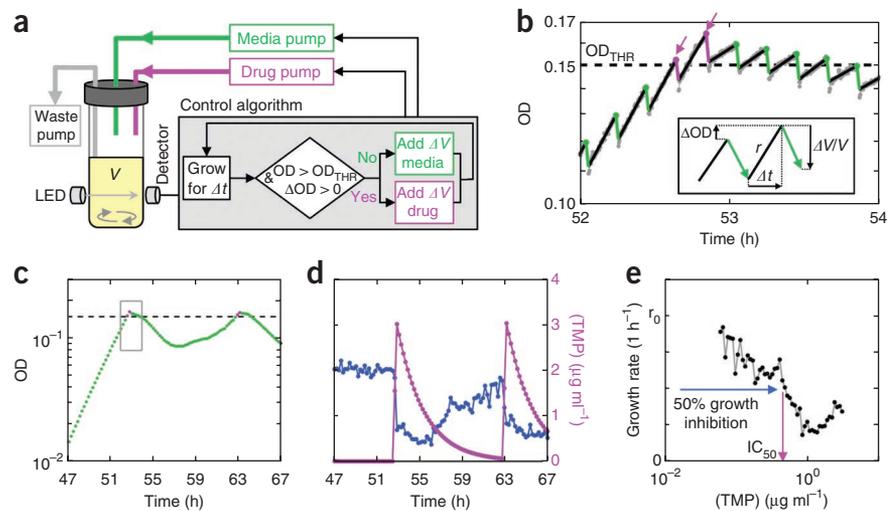
We recently introduced an automated continuous culture device, the morbidostat, for studying evolution of drug resistance in a controlled environment containing an antibiotic at a concentration that is dynamically adjusted such that the bacterial population is constantly challenged (Fig. 1)⁵. The bacterial population is growing in a 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 medium or fresh medium containing dissolved drugs. Similar to a chemostat, in which cell cultures are periodically diluted

at a constant rate lower than the maximal growth rate of the population, the dilution rate of the morbidostat $r_{\text{dilution}} \equiv \Delta V / (V \cdot \Delta t)$ is fixed. In steady state, the bacterial growth rate must reach a value that matches this dilution rate. However, in contrast to a chemostat, in which 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; 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 on the basis of optical density (OD) measurements (Fig. 1b, gray dots). Next, depending on the calculated growth rate and the current OD of the culture, the morbidostat decides whether to add fresh medium or fresh medium plus drug (in either case, the same fixed volume ΔV is added). Fresh medium with the drug is injected only if the OD exceeds a threshold (OD_{THR}) and if the growth rate is higher than the dilution rate (Fig. 1b, magenta filled circles); in all other cases, fresh medium is injected. Drug concentrations inside the culture vials increase with drug injections and are gradually reduced by dilution with successive fresh medium injections (Fig. 1c, magenta line). The value of OD_{THR} is chosen to be small enough such that the population is never nutrient limited, typically $\text{OD}_{\text{THR}} = 0.15$. Choosing the values of Δt , V and ΔV such that the dilution rate r_{dilution} is substantially 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 a maximal rate of $r_0 = 0.8 \text{ h}^{-1}$, the morbidostat is set to $V = 12 \text{ ml}$, $\Delta V = 1 \text{ ml}$ and $\Delta t = 12 \text{ min}$, such that the dilution rate of the system is half of the growth rate of bacteria in the absence of drugs ($r_{\text{dilution}} \equiv \Delta V / (V \cdot \Delta t) = 0.4 \text{ h}^{-1}$; Fig. 1b, green filled circles). Throughout the entire experiment, the volumes of the cultures are kept constant using a suction pump (Fig. 1a).

As the bacterial 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

PROTOCOL

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 gray dots. Growth rates (r) of bacterial populations are periodically calculated by fitting exponential growth functions (black lines). Markers with magenta and green colors represent dilutions with drug solution and fresh medium, respectively. **(c)** Final OD values at the end of each growth cycle. **(d)** Growth rates (blue) and drug concentrations (magenta) between two consecutive drug solution injections. **(e)** The drug concentration that inhibits growth by 50% (IC_{50}) is calculated by analyzing growth rates and corresponding drug concentrations. TMP, trimethoprim.



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 3 weeks⁵.

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 mid-sized commercial incubator (inner dimensions: 24 × 24 × 20 inches); 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, in which cells are grown at a constant growth rate, or as a turbidostat, in which cells are grown at a constant density, by making only simple modifications to the control algorithm. Therefore, constructing a morbidostat enables a diverse range of experiments to be carried out in a biology laboratory.

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 1-s intervals, the morbidostat can perform precise growth rate measurements for each growth period (Δt). For $\Delta t = 12$ 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 medium that will inhibit the growth of evolving bacteria at a desired rate⁶. However, the drug concentrations must be manually adjusted on a daily basis by quantifying the resistance of the evolving population⁶. 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 multistep increases in drug resistance in real time. In contrast, microbial selection experiments that are carried out using antibiotic-containing agar plates or growth medium have the main advantage of relative simplicity compared with 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}.

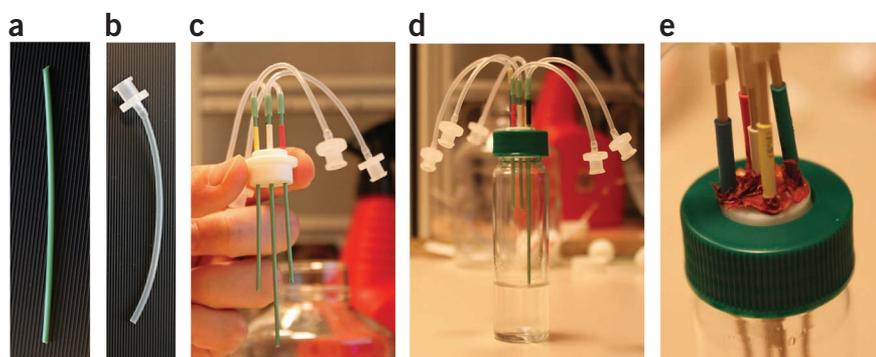
Other potential applications

The experiments that can be done with the morbidostat are not limited to studying the evolution of bacterial drug resistance. We speculate that without any time-consuming modifications to the system the morbidostat could potentially be used to carry out long-term adaptation experiments that address evolution of stress response in bacteria and yeast, host-parasite interactions in microbial communities and the 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 (Chemglass) with open-top screw caps (Chemglass) are used for growing bacterial cells in the morbidostat. A magnetic stir bar (Stirbars.com) 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 (Chemglass) 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 the following: injection of fresh medium; two separate drug solutions; filtered air intake; and removal of the excess liquid for keeping the culture volume constant. Autoclavable PEEK (polyether ether ketone; Fisher Scientific) tubing is inserted into these openings for adding or removing liquids. High

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 it just touches the top of the culture in the vial, the volume of which is 12 ml. (e) High temperature-resistant silicone is applied to the entire upper face of the Teflon insert.



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 (VWR) and male/female Luer thread style connectors (Value Plastics). The entire tube assembly can be sterilized by autoclaving 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 ODs. A Plexiglas adaptor (Supplementary Fig. 1) is machined and mounted on the magnetic stirrer using L brackets. In all, 15 tube holders are machined from black Delrin material (Supplementary Fig. 1). Every tube holder has two openings drilled for LED light sources and photodetectors. These openings are positioned at a 135° angle to maximize the detection of scattered light. An LED light source and a photodetector are mounted to each tube holder and connected to the circuits feeding them (Radio Shack; Fig. 4a). A circuit is built for measuring the voltage across the photodetector (Fig. 4b), and the voltages are recorded using a data acquisition (DAQ) card (Measurement Computing). After doing a calibration for converting voltage readings to OD, the entire tube holder array (Supplementary Fig. 2) is placed in a temperature-controlled incubator.

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. 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, we found that the sizes of the evolved strains with or without drugs were very similar to the size of

wild-type bacteria in the absence of drugs⁵. 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 medium and drug solutions. In all, 45 peristaltic pumps (Clark Solutions) 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 (Measurement Computing). Modified autoclavable screw cap glass bottles are used as medium reservoir bottles and are connected to these pumps. A 16-channel peristaltic pump is used as a waste pump for maintaining fixed culture volumes in the culture vials (Harvard Apparatus). The activation of the waste pump is also done via the relay device. Pumps are connected to culture vials with autoclavable silicone tubing (VWR). After ensuring 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 12 ml of growth medium, and then 10 μl of bacterial cells from glycerol stock are added. These cultures are grown overnight

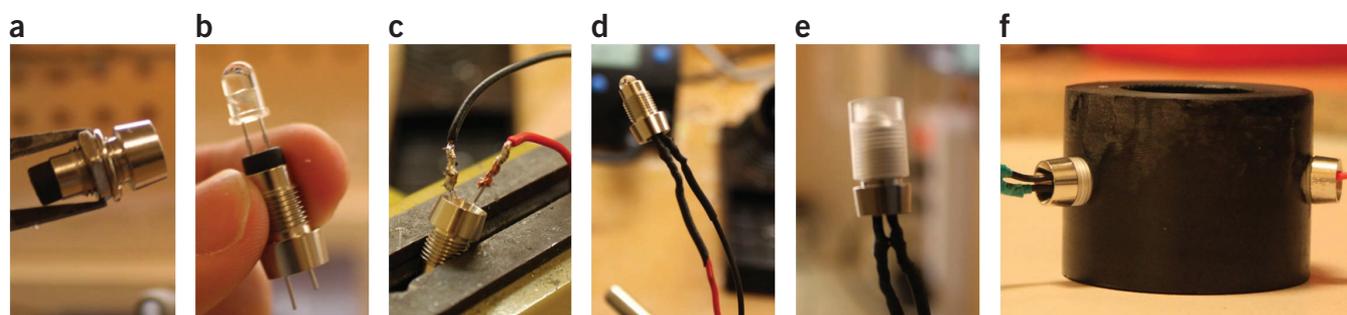


Figure 3 | Construction steps for tube holder assembly. (a) Commercial LED holder. (b) The LED and the photodetectors are inserted into the LED holder. (c) Legs of the LED are soldered to wires. (d) Wire connections are insulated with heat-shrink insulators. (e) Custom-machined LED housing is attached to the LED holder. (f) LED and photodetector assemblies are inserted into the custom-machined Delrin tube holders.

PROTOCOL

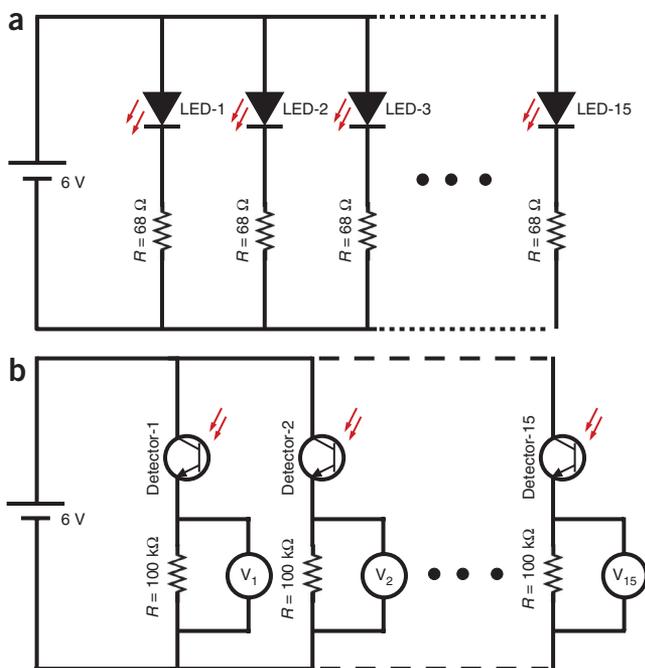


Figure 4 | Circuit diagrams for the OD detection system. (a) Each LED is connected to a 68- Ω resistor in series. All LEDs are connected in parallel. (b) Each photodetector is connected to a resistor in series. All photodetectors are connected in parallel.

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 at which cells grow exponentially. This range can vary depending on the strains and growth medium used in the experiments.

Measurement of dilution rate. After sterilizing all culture- and medium-interfacing components of the system and initializing all of the peristaltic tubing, the culture vials are filled with 12 ml (V) of growth medium and 10 μ l of bacterial cells from glycerol stock. The morbidostat setup is operated using the chemostat

algorithm such that fresh medium 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 medium containing glucose and ampicillin at 30 °C, cells double every ~50 min ($r_0 = 0.8 \text{ h}^{-1}$). Under these settings, we dilute cultures by ~8% every 12 min (Δt) by adding ~1 ml (ΔV) of fresh medium into the culture. The theoretical dilution rate can be calculated using the following formula: $r_{\text{dilution}} \cong \Delta V / (V \cdot \Delta t) = 0.4 \text{ h}^{-1}$. By using the chemostat data, we extract the experimental dilution rate and compare it with the theoretical dilution rate. Such comparison is necessary, as actual dilution rates can slightly vary between morbidostat vessels owing to inherent imperfections of the commercial parts such as tubing and peristaltic pumps.

Measuring the minimum inhibitory concentration (MIC). The MIC of each drug is measured before the experiments. These measurements can be done using 96-well plates or sterile cell growth tubes, depending on the availability. We generally prefer using 96-well plates, as they require less volume and measurement with multiple replicates is convenient. If the drug is used for the first time and the MIC is not known, we make a drug gradient on the 96-well plates in which 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 1,000 cells to each well and incubate the plate on a plate shaker for 24 h at the desired temperature. At the end of 24 h, the minimum drug concentration at which bacteria cannot survive (the well shows no increase in OD) is assigned as the MIC. A measurement using a linear drug gradient, which covers the MIC value found in the previous measurement, is used if a more precise MIC value is necessary. For MIC measurements, fresh drug solutions from powder stocks are prepared according to the instructions provided by the suppliers.

Estimation of drug concentrations for growth inhibition. Both morbidostats and chemostats feed growing cell cultures with fresh medium at a fixed dilution rate. The main operational difference between the morbidostat and a chemostat is that the former adds fresh medium containing drug solution to the bacterial cultures

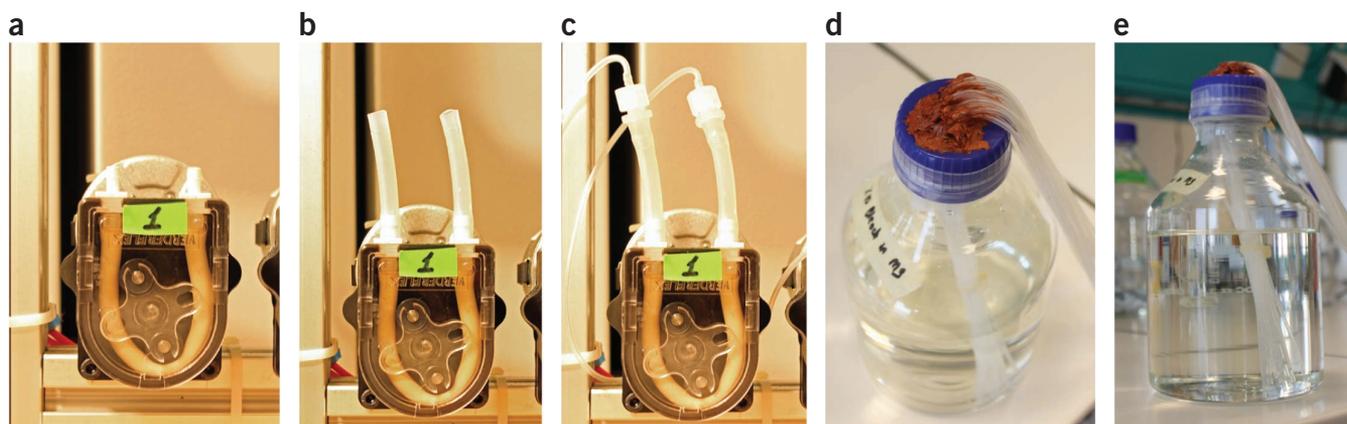


Figure 5 | Construction steps for pump array and medium reservoir assemblies. (a) A peristaltic pump is attached to the pump stand (Supplementary Fig. 4). (b) Two pieces of silicone tubing are attached to the pumps. (c) An assembled peristaltic pump with Luer connectors and silicone tubing. (d) Silicone 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. (e) The assembled medium reservoir.

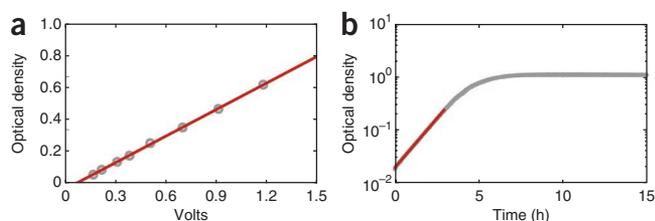


Figure 6 | Calibration of the detectors. (a) The voltages (gray circles) created by cultures with known OD values are recorded. A line (red line) is fitted for finding the calibration factor. (b) An *E. coli* culture is grown overnight and OD values are recorded (gray line) every second. Red line shows the range in which the growth is exponential.

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. 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 ten times higher than the 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 medium are prepared (stock A and stock B). The drug concentration of stock A is typically $10\times$ MIC and the drug concentration of stock B is $50\times$ MIC (or $5\times$ stock A). 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 medium and drug solution bottles are connected to the peristaltic pumps and morbidostat algorithm is tested (**Supplementary Fig. 4**). We generally aim to slow down the 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, $5\times$ (Sigma-Aldrich, cat. no. M6030)
- Glucose (Sigma-Aldrich, cat. no. G7021)
- Amicase (Sigma-Aldrich, cat. no. 82514)
- *E. 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 (Reagent Setup)
- Bleach
- Ethanol
- Sterile water

EQUIPMENT

Glass vial assembly

- PEEK tubing (Fisher Scientific, cat. no. 05-701-6)
- PEEK cutter (Fisher Scientific, cat. no. 05-701-84)
- Silicone tubing (1 mm inner diameter (i.d.), VWR, cat. no. 60985-708)
- Female Luer connector (Value Plastics, cat. no. FTLL004-6005)
- Male Luer connector (Value Plastics, cat. no. MTL004-6005)
- Flat-bottom glass vial, 40 ml (Chemglass, cat. no. CG-4902-08)
- Teflon insert for 24–400 open-top GPI cap (Chemglass, cat. no. HMS-0909-151GC)
- 24–400 open-top GPI cap (Chemglass, cat. no. CV-3750-0024)
- Permatex 81878 no. 101 sensor safe Ultra Copper hi-temp RTV silicone
- Male Luer thread style cap (Value Plastics, cat. no. MTL004-6005)
- Female Luer thread style cap (Value Plastics, cat. no. FTLLP-6005)
- Magnetic stir bar (Stirbars.com, cat. no. SBM2003MIC)
- Syringe filter (0.2 μm ; VWR, cat. no. 514-0066)
- Syringe filter (0.2 μm ; VWR, cat. no. 514-0068)

Tube holder array assembly

- Machined Plexiglas sheet (**Supplementary Fig. 1**)
- 15 position magnetic stirrer (Neutec, cat. no. VL - F203A0178)
- Machined L brackets
- Machined Plexiglas cover (**Supplementary Fig. 1**)
- Custom-machined Delrin tube holder
- O-rings for supporting tubes inside the holder

- LED holder (Radio Shack, cat. no. 276-080)
- IR LED emitter and detector (Radio Shack, cat. no. 276-142)
- Machined housing for LED holder
- Colored 22-gauge stranded wire (Amazon.com)
- Resistor, 68 Ω (Radio Shack, cat. no. 271-1106)
- Various resistors (Radio Shack, 10–100 k Ω)
- High-power AC adaptor (Radio Shack, cat. no. 273-318)
- Temperature-controlled incubator (VWR, cat. no. 1535)
- DAQ card (Measurement Computing, cat. no. USB-1616FS)
- Uninterruptable power supply (UPS; Amazon.com)
- Windows computer with MATLAB software 32-bit installed (for control software)
- Wire stripper
- Soldering tools
- Electrical tape and/or heat shrink insulation
- Wire zip ties (for organizing things)
- Eppendorf tubes
- Serological pipettes
- Spectrophotometer and cuvettes

Pump array construction

- Peristaltic pumps (Clark Solutions, cat. no. m451605)
- USB controller relay interface device (Measurement Computing, cat. no. USB-ERB24)
- 16-channel peristaltic pump (Harvard Apparatus, cat. no. 73-3154)
- Silicone tubing (0.125-inch i.d., VWR, cat. no. 89068-474)
- Male Luer connector (Value Plastics, MTL025-6005)
- Erlenmeyer flask, 6 liters (VWR, cat. no. 1500-6000)

Constructing medium reservoir bottles

- Medium storage bottle with GL45 screw cap, 5 liters (VWR, cat. no. 1395-5L)
- Medium storage bottle with GL45 screw cap, 1 liters (VWR, cat. no. 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 ensure that the efficacy of the drugs is not decreased. 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.

PROTOCOL

PROCEDURE

Assembling morbidostat vials ● TIMING ~9 h plus drying overnight

- 1| Cut three pieces of PEEK tubing, each 3 inches (~8 cm) in 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 one piece of PEEK tubing 1.5 inches (~4 cm) in length. This will be used for filtered air intake.
- 3| Cut one piece of PEEK tubing 5 inches (~12 cm) in length. This will be used for extraction of excess culture in morbidostat vials.
- 4| Cut five pieces of silicone tubing (1 mm i.d.), each 4 inches (~10 cm) in length.
- 5| Insert each piece of PEEK tubing into one piece of silicone tubing to a depth of ~0.5 inches (~1 cm).
- 6| Insert female Luer connectors into the other end of the piece of silicone tubing (**Fig. 2b**).
- 7| Fill a 40-ml flat-bottom glass vial with 12 ml of 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-inch (~12-cm) PEEK tubing through one of the holes until 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-inch (~8-cm) PEEK tubing should be at least 1 inch (~2.5 cm) above the liquid level inside the glass vial in order 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 min.
- 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 h

- 19| Mount the machined Plexiglas sheet on the 15-position magnetic stirrer using L brackets.
- 20| Take two LED holders and discard the washers and the nuts that came with them (**Fig. 3a**).
- 21| Push the legs of the IR LED all the way into the LED holder from the small opening (**Fig. 3b**).
- 22| Push the legs of the photodetector all the way into the LED holder from the small opening.
- 23| Solder four pieces of 22-gauge stranded wires to the legs of the LED holder and photodetector (**Fig. 3c**).
▲ **CRITICAL STEP** The use of wires with different colors (i.e., red, green, blue, black) is helpful to keep track of devices and polarities. The length of the wires depends on their organization.

- 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| Repeat Steps 20–25 to prepare 15 pairs of LED and photodetector assemblies.
- 27| Place all of the LED and photodetector 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 avoid confusion and aid in debugging.
- 28| Connect a 68-Ω 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 Plexiglas sheet attached to the magnetic stirrer.
- 30| By following the circuit diagram given in **Figure 4a**, connect all of the LEDs in parallel and feed them with a power supply. Set the voltage to 6 V. Ensure that all the LEDs are emitting by using a digital camera that can detect IR (e.g., webcam or mobile phone camera).
- 31| By following the circuit diagram given in **Figure 4b**, splice the wires coming from the short leg of the photodetectors.
- 32| By following the circuit diagram given in **Figure 4b**, connect a resistor to the other leg of the photodetectors.
- ▲ **CRITICAL STEP** The resistance of all resistors should be chosen such that 1 OD change should correspond to 2 V. These resistances are generally ~100 kΩ 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 Plexiglas 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-inch (~5-cm)-diameter port on the right side of the incubator.
- 35| Connect all the photodetectors to a power supply and set the voltage to 6 V.
- ▲ **CRITICAL STEP** All of the photodetectors should be connected in parallel.
- 36| Connect wires to both sides of the photodetector resistors for measuring the voltage across them using a multichannel DAQ card. Repeat this for all 15 tube holders.
- 37| Connect these pairs of wires to the screw terminals of the DAQ card.
- ▲ **CRITICAL STEP** The DAQ card has 16 screw terminals, numbered 0–15. Connect tube holder no. 1 to terminal 0, tube holder no. 2 to terminal 1 and so on.
- 38| By using the software provided by the DAQ card supplier, ensure that all of the photodetectors are responding to variations in incident light. The DAQ card we use is set to acquire data at an acquisition rate of 500 Hz, and the voltage readings are median-filtered every second to eliminate noise. Typically, signal-to-noise ratio (mean/s.d.) 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 ~2 h**
- 39| Dilute an overnight bacterial culture to ~OD = 0.75 in minimal growth medium.
- 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 r.p.m.

PROTOCOL

- 42| Put the culture vial in the first tube holder and wait for 10 s. Record the voltage for 10 s 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 this for all tube holders.
- 44| After completing the voltage recording for all 15 tube holders, take out 5 ml of the culture using a serological pipette and transfer it to a spectrophotometer cuvette.
- 45| Measure the OD of the removed culture using a standard spectrophotometer. Record the OD 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 and 47) until the OD 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 ($OD = \text{calibration factor} \times \text{voltage} + \text{offset}$) for finding the calibration factor. We typically use a MATLAB command called 'robustfit' for fitting a line (**Fig. 6**). Calibration factors may change over time. In our experience, the difference in calibration factors is ~5% after 1 month of continuous use.

? TROUBLESHOOTING

Assembling the pump array ● **TIMING** ~24 h

- 50| Assemble the pump stand (**Supplementary Fig. 3**).
- 51| Attach all of the peristaltic pumps to the pump stand (**Fig. 5a**).
▲ **CRITICAL STEP** Forty-five pumps are necessary, as 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-V AC; 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's gloves is advised.
- 53| Connect the C contact of relay switch no. 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.
! **CAUTION** Unplug the power source before making the connections.
▲ **CRITICAL STEP** Two relay boxes will have a total of 48 relay switches. We use all of the 24 relay switches in the first relay box and 21 relay switches from the second relay box.
- 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 neighboring relay switches. For example, relay switches nos. 1–3 on the relay box control the fresh medium pump, the drug A pump and the drug B pump of culture 1. The MATLAB code (GUI.m) provided with this protocol (**Supplementary Data**) must be modified if the connections are made differently.
- 56| Use one of the remaining three 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 24th 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 ensure that the code controls the pumps properly (**Supplementary Fig. 6**).
- 58| Cut 90 pieces of silicone tubing ~4 cm in length (0.125-inch i.d.).
- 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 (medium pump, drug A pump, drug B pump and suction pump) and cut the silicone tubing (1 mm i.d.) to the appropriate length.
- 64| Insert one male and one female Luer connector to the ends of each silicone tubing piece 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, medium 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 tubing such that the 16-channel pump will draw liquid away from the culture vials.
- 69| Fill an Erlenmeyer flask with 1 liter of pure bleach. This will be used for the 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 to the measured length (1 mm i.d.).
- 72| Insert a female Luer connector to one end of all of the pieces of silicone tubing from Step 71.
- 73| Connect all of the silicone tubes to the 16-channel pump using Luer connectors.
- 74| Bundle all of the 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 medium reservoir bottles ● TIMING ~6 h plus drying overnight**
- 76| Drill 16 holes of 2.5 mm in diameter on GL45 bottle caps using an electrical drill.
- 77| Cut 15 pieces of silicone tubing (1 mm i.d.) 2 feet (~60 cm) in length.
- 78| Cut a piece of silicone tubing (1 mm i.d.) 4 feet (~1.2 m) in length.

PROTOCOL

- 79| Close a bottle tightly using the drilled GL45 cap.
- 80| Push all 15 pieces of silicone tubing (2 ft. in length) through the holes on the cap such that the silicone tubing touches the bottom surface of the bottle.
- 81| Push the 4-foot (~1.2-m) length of silicone tubing about 1 inch (~2.5 cm) through the remaining hole 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 min.
- 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 two reservoirs of 5-liter volume and four reservoirs of 1-liter volume. Smaller reservoirs are used for drug stocks; you may need more drug solution reservoirs depending on the experimental design.

Sterilizing the tubing ● **TIMING** ~4 h

- 88| Fill four reservoirs with 10% (vol/vol) bleach, 70% (vol/vol) ethanol, sterile water and growth medium, respectively.
- 89| Connect the 10% (vol/vol) bleach reservoir to medium pumps using Luer connectors.
- 90| Spray 70% (vol/vol) ethanol on all the Luer connections.
- 91| Run all of the medium pumps and the suction pump for 5–10 min.
▲ **CRITICAL STEP** Closely watch the process and ensure that tubes and connections are not leaking.
- 92| Let the bleach stand in the system for 5 min with the pumps turned off.
▲ **CRITICAL STEP** Bleach may clog the tubes if it stands for too long.
- 93| Swap the 10% (vol/vol) bleach reservoir with the sterile water reservoir.
- 94| Run all of the medium pumps and the suction pump for 5 min.
- 95| Swap the sterile water reservoir with the 70% (vol/vol) ethanol reservoir.
- 96| Run all of the medium pumps and the suction pump for 5 min.
- 97| Let the ethanol stand in the system for 15 min with the pumps turned off.
- 98| Swap the 70% (vol/vol) ethanol reservoir with the sterile water reservoir.
- 99| Run all of the medium pumps and the suction pump for 5 min.
- 100| Swap the sterile water reservoir with the growth medium reservoir.
▲ **CRITICAL STEP** To avoid contaminating the medium reservoir, this step has to be done very carefully using sterile gloves and an open flame, if available.

101| Insert sterile syringe filters between the male Luer connectors attached to the peristaltic medium pumps and female Luer connectors attached to the medium reservoir.

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

102| Run all of the medium pumps and the suction pump for 5 min 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 medium reservoir at Step 100.

Running the morbidostat assay ● **TIMING 2–4 h per day**

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

▲ **CRITICAL STEP** We did all experiments with filtered minimal M9 medium with 0.2% (wt/vol) ampicillin and 0.4% (wt/vol) glucose. However, LB or other medium types can be used. Extra calibration of the detectors might be needed if different media are used. Stock A has a drug concentration that is ten times higher than MIC. Stock B is 5 times more concentrated than stock A. If alternative media are used, the dilution rates in the control code may need to be adjusted to accommodate changes in growth rate.

▲ **CRITICAL STEP** Growth medium should be prepared in advance. Prepare the medium and ensure that there is no contamination in the medium for at least 24 h.

105| Fill all of the morbidostat vials with 12 ml of cell culture. Label the vials (culture no. 1, culture no. 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. Next, run the morbidostat software (**Supplementary Data**). You can run GUI.m for basic pump controls, automated calibration of the detectors and 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 medium every 12 min. 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 five 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 h and transfer 500 µl of the cells to a new sterile glass vial that contains ~12 ml of growth medium.

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

108| Transfer 500 µl of the cells to a sterile 1.5-ml Eppendorf tube, add 250 µl of sterile 50% (wt/vol) glycerol and freeze/store it at –80 °C. Eppendorf tubes must be properly labeled.

109| Resume the experiment.

▲ **CRITICAL STEP** Several problems, such as contamination, may occur in morbidostat experiments. Keeping the glycerol stocks from each day of the experiment is crucial. 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 can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

| 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 medium injections | Biofilms on the inner wall of the cell vial | Replace the glass vial and resume the experiment from the previous day |

● TIMING

Steps 1–18, assembling morbidostat vials: ~9 h plus drying overnight

Steps 19–38, assembling tube holder array: ~24 h (inexperienced users may need more time)

Steps 39–49, calibration of the detectors: ~2 h

Steps 50–75, assembling the pump array: ~24 h (inexperienced users may need more time)

Steps 76–87, constructing medium reservoir bottles: ~6 h plus drying overnight

Steps 88–103, sterilizing the tubing: ~4 h

Steps 104–111, running the morbidostat assay: 2–4 h 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 3 weeks. One of the biggest advantages of the morbidostat is the ability to follow the evolution of drug resistance almost in real time. As illustrated in **Figure 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 are 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.

Note: Supplementary information is available in the [online version of the paper](#).

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