Chapter 16

Microfluidic Single-Cell Phenotyping of the Activity of Peptide-Based Antimicrobials

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Abstract

Antibiotic resistance is a major challenge for modern medicine, and there is a dire need to refresh the antibiotic development pipeline to treat infections that are resistant to currently available drugs. Peptide-based antimicrobials represent a promising source of novel anti-infectives, but their development is severely impeded due to the lack of suitable techniques to accurately quantify their antimicrobial efficacy. A major problem involves the heterogeneity of cellular phenotypes in response to these peptides, even within a clonal population of bacteria. There is thus a need to develop single-cell resolution assays to quantify drug efficacy for these novel therapeutics. We present here a detailed microfluidics-microscopy protocol for testing the efficacy of peptide-based antimicrobials on hundreds to thousands of individual bacteria in well-defined microenvironments. This enables the study of cell-to-cell differences in drug response within a clonal population. It is a highly versatile tool, which can be used to quantify drug efficacy, including the number of individual survivors at defined drug doses; it even enables the potential exploration of the molecular mechanisms of action of the drug, which are often unknown in the early stages of drug development. We present here protocols for working with *Escherichia coli*, but organisms of different geometric shapes and sizes may also be tested with suitable modifications of the microfluidic device.

Key words Peptide-based antimicrobials, Microfluidics, Single-cell analysis, Phenotypic heterogeneity, Persisters, Viable-but-nonculturable cells, Antibiotic susceptibility

1 Introduction

Traditional methods for studying antibiotic efficacy rely on bulk measurements performed on populations of cells. The most common technique remains the seemingly ubiquitous Minimum Inhibitory Concentration (MIC) assay [1], which assesses the minimum drug concentration required to inhibit the growth of a culture or lawn of bacteria, commonly via direct visual inspection of the treated culture. A related standard involves determining the Minimum Bactericidal Concentration (MBC), which is the concentration that kills at least 99.9% of the initial bacterial inoculum [2].

Although MICs and MBCs are considered the "gold-standards" for determining antibiotic susceptibility [1], there are

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widespread reports of variability in their determination even for a single antibiotic-strain combination [2, 3]; sources of variability include intra-laboratory and inter-laboratory variability, in addition to inter-strain variability [3, 4]. Furthermore, there are various different types of MIC and MBC assays, and assay conditions can be a very significant source of variability as well, depending on the type of antibiotic used [2, 5]. MIC assays with antimicrobial peptides, in particular, suffer greatly due to the inoculum effect, requiring a reevaluation of the role of the "MIC" in quantifying the antimicrobial efficacy of these drugs [6]. It has been proposed that this inoculum effect with peptide antibiotics such as LL37 is due to the (typically) cationic peptides being retained in target cells; after death, the peptides remain absorbed by the dead cells, and the effective "free" peptide concentration in the surrounding medium is lowered, thus enabling the survival of other cells in the population leading to a heterogeneous response to the antibiotic [7]. With a decline in traditional small molecule antibiotic pipelines and a corresponding increase in antimicrobial resistance across the globe [8], antimicrobial peptides and other peptide-based antimicrobials have seen a resurgence in interest as potential therapeutics [9–13]. Hence, it is imperative to have bio-metrological standards that are fit for the purpose of quantifying the efficacy of these drugs.

As mentioned above, MIC and MBC assays are bulk measurements that provide information at the population level and cannot investigate heterogeneities in drug responses that are inherent in any bacterial population; there is a layer of phenotypic complexity that is additional to the heterogeneity in drug efficacy caused by the cellular retention of drugs after death, discussed above for the case of antimicrobial peptides [14–16]. Heterogeneities in response to antibiotic treatment deserve particular attention, since rare subpopulations of cells such as persister and viable-but-nonculturable (VBNC) cells are typically missed by bulk measurements, and it is now widely accepted that these rare survival phenotypes are a major cause of the relapse of bacterial infections in clinical settings [17, 18]. *Any* antibiotic development pipeline will require tools to investigate the levels of these rare subpopulations of cells that are genetically identical to their neighbors, but which are capable of surviving much higher doses of the drug.

Microfluidic techniques have revolutionized biological assays over the past decade and are being leveraged for single-cell analyses in a range of biomedical studies [19–21]. Since its initial development in 2010, the bacterial "Mother Machine" microfluidic device has become the tool of choice among the biophysics and bacteriology communities for its ability to capture thousands of bacteria on chip, which can then be set into a desired metabolic or experimental state prior to single-cell phenotyping using high magnification objectives [22–29]. The device is a two-level microfluidic chip containing a "main" channel for bacteria, media, drug, or dye

delivery, and thousands of smaller "side channels" to trap individual bacteria. The main channel typically has a width of 100 μm and a height of 25 μm, whereas the side channels have widths and heights optimized for trapping the bacterial species being investigated; with *Escherichia coli*, side channels with dimensions of 1.4 μm × 1.4 μm × 25 μm are typically used. We have repurposed this device into a "Killing Machine" (Fig. 1) for testing the antibacterial efficacy of antibiotics (including peptide-based materials) at the single-cell level [30–32]. This provides a wealth of information currently unavailable using standard techniques, including the quantification of nongrowing "sleeper" cells that survive the drug, the enumeration of subpopulations which grow even in the presence of the drug, or the identification of persister-like subpopulations which survive the drug treatment and regrow once favorable environmental conditions are restored [30]. Furthermore, when testing novel drugs, the single-cell microscopy involved may provide information on possible mechanisms of action of the drugs, which can then be investigated using appropriate knockout or reporter strains on the same platform.

To aid its use, we provide here a comprehensive protocol, detailing the experimental procedures required to perform this microfluidics-microscopy assay to test the efficacy of antimicrobial peptides, using *Escherichia coli* as the model organism. Other organisms may be used in either the same or a related device, depending on the size and motility of the cells, after optimization of their confinement efficiency within the chip.

2 Materials

2.1 Reagents and Consumables

1. Silicon wafer based or epoxy mold of the mother machine microfluidic device (*see* **Note 1**).
2. Sylgard 184 Polydimethylsiloxane (PDMS) kit (Elastomer and Curing Agent, Dowsil).
3. Spoon/mixer and plastic cup for mixing PDMS.
4. Absolute ethanol.
5. Bovine serum albumin (BSA, 50 mg/ml in MilliQ water).
6. Lysogeny broth (LB): 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl, Melford.
7. Flasks, beakers, Petri dishes.
8. LB agar streak plates: 15 g/l agar.
9. Minimal media: 1 × M9 salts, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mg/l thiamine hydrochloride in MilliQ water.
10. Peptide antibiotic: prepare in a solution containing 90% minimal media and 10% fresh LB.

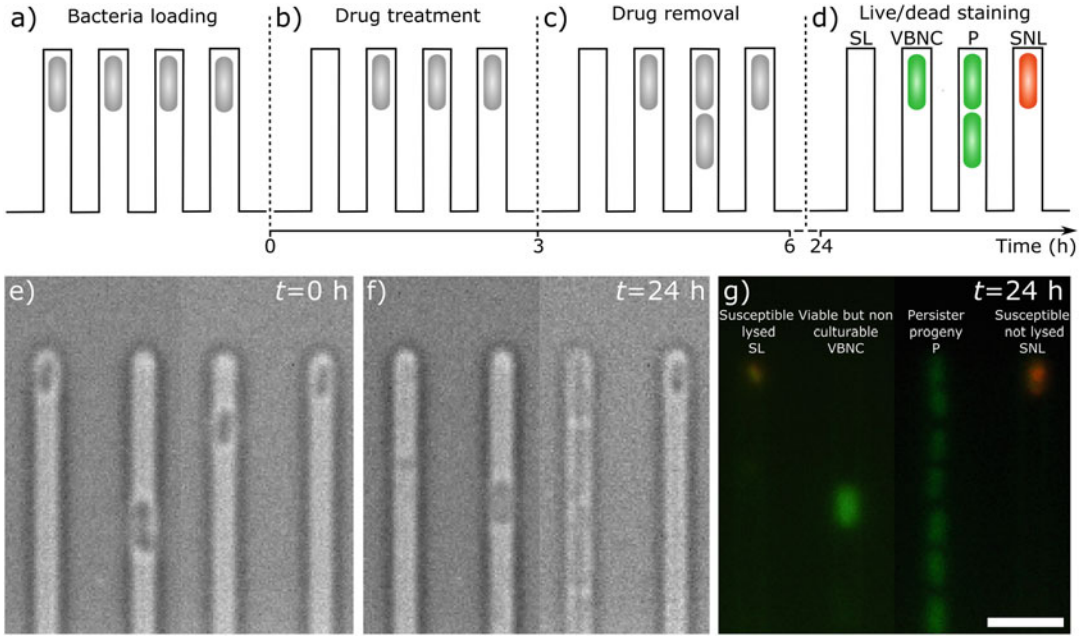

Fig. 1 Schematic describing the working of the microfluidic "killing machine" for testing antibiotic efficacy at the single-cell level. After the bacteria are loaded in the side channels (**a**, **e**), they are treated with the drug for 3 h (**b**) followed by incubation with fresh LB (Lysogeny broth) medium (**c**). Survival phenotypes are determined using appropriate dyes to determine cell survival or death after overnight incubation in the fresh LB medium (**d**, **f**, **g**). Figures (**e**, **f**, **g**) are bright-field (**e**, **f**) and fluorescence (**g**) images of the same set of cells through the course of an experiment, representing examples of the different single-cell phenotypes observed in response to drug treatment. Although the image here shows staining using dyes for both live (green) and dead (red) cells, a single dye (propidium iodide, PI) to determine cell death is sufficient to determine the phenotypes of interest, which simplifies the experimental protocols. Cells that show fluorescence following PI treatment are considered dead, whereas those that remain unstained are classified as having survived the treatment. Scale bar $=$ 5 μm. (Figure reproduced without changes from Bamford et al., 2017, *BMC Biology* **15**, 121 (Publisher: Springer Nature), originally published under a Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/)). Note that the images and paper cited refer to work with ampicillin at 25 $\times$ MIC, and the survivors that do not regrow when incubated with fresh nutrients are termed viable-but-nonculturable (VBNC) cells, whereas those that regrow after removal of the drug are termed persister cells. When using novel peptide-based therapeutics where the MIC is not a suitable standard, it is suggested that the user doses the cells with a defined drug concentration of choice (reporting the exact concentration value rather than a multiple of the "MIC"). We suggest using the terms "nongrowing" or "VBNC" survivors for the nongrowing survivors and "growing survivors" to indicate the survivors that grew either during drug treatment or after removal of the drug; the term "persister" is conventionally used when the drug dose is much greater (typically 25$\times$ to 100$\times$) than the "MIC", which may or may not be the case in these measurements, since the "MIC" is difficult to define for these drugs. Further, as mentioned in the text, with peptide-based therapeutics, one may also observe some cell growth and division during the drug treatment, which is a different phenotype to the conventional "persister" phenotype

11. Protein LoBind 0.5-ml tubes (Fisher Scientific).
12. *Escherichia coli* BW25113 strain (cryo-stocks stored at −80 °C in a 1:1 mixture of stationary phase culture media and glycerol).
13. 1-ml and 10-ml syringes
14. Syringe needles (BD Microlance™ 3, 27G × ½″, 0.4 × 13 mm).
15. 0.22-μm Medical-Millex GS filters (Millipore).
16. 50-ml falcon tubes.
17. Spectrometer cuvette (1 ml).
18. Propidium Iodide (stock diluted 1:1000 in fresh LB).
19. Type I glass coverslip (0.13–0.17 mm thickness, 22 × 50 mm).
20. Tubing A: microfluidic tubing FEP Nat. dimensions 1/32″ × 0.008″ (Fluigent/Thames Restek UK).
21. Tubing B: microfluidic tubing FEP dimensions 1/16″ × 0.030″ (Fluigent/Gilson).
22. Tubing C: microfluidic fine bore polythene tubing 1.09 mm × 0.38 mm (Portex).
23. Aluminum foil.

2.2 Equipment

1. Spectrometer for optical density measurements (Biowave CO8000 Cell Density Meter).
2. Centrifuge (Eppendorf 5810R, A-4-62 rotor).
3. Oven.
4. Pipettes.
5. Shaker.
6. Incubator/warm room (37 °C).
7. NanoDrop 2000.
8. 30 W plasma oven (Zepto, Diener Electric, Germany) with associated vacuum pump and compressed air cylinder.
9. Tweezers (Idealtek 120 mm Anti-Magnetic Stainless Steel Flat; Rounded Tweezers Anti-Acid, Part No. 2A.SA).
10. 4-channel Fluigent pressure pump (MFCS™-EZ) with associated N_2 gas cylinder connection and accessories.
11. Fluiwell-4C holder and associated 1.5-ml vials (Micrewtube) and fluid connectors (Upchurch Scientific).
12. Fluigent flow unit with associated fluid connectors and Flow-Rate platform "Flowboard."
13. 0.75-mm biopsy punch with plunger (WellTech Rapid-Core—0.75).

14. Epifluorescence microscope with an XYZ stage. A suitable example involves an Olympus IX73 body with two piezo stages (M-545.USC and P-545.3C7, Physik Instrumente) controlled with LabView software.
15. High magnification objective (e.g., Olympus UPLSAPO 60× W (N.A. 1.2)).
16. Green LED light source for dead stain measurement with a TRITC filter cube (e.g., pE-300 White LED system).
17. Camera (e.g., sCMOS Zyla 4.2, Andor, Belfast).
18. Vortex mixer.

3 Methods

3.1 Microfluidic Chip Preparation

1. Prepare a 10:1 mixture (by weight) of the elastomer:curing agent components of the PDMS kit. When preparing the mixture, use a spoon or a mixer to ensure thorough mixing of the two components.
2. Post mixing, the PDMS will have a number of air bubbles. Leave the mixture to stand (covered) at room temperature for 1 h so that the air escapes. Alternately, this process may be sped up by placing the mixture under a vacuum for 30 min.
3. Once large air bubbles have dissipated, pour the mixture on to the Silicon wafer based or epoxy mold, aiming for a height of approximately 1.0–1.5 cm above the channel features on the mold (*see* **Notes 2** and **3**). Cure in an oven at 70 °C for 2 h.
4. After curing, use a sharp scalpel to cut out the chip from the mold, ensuring that the scalpel is never near the channel features themselves. Particular care should be taken when using a Silicon wafer based mold, as these are prone to fracture if too much pressure is applied to a point on the surface. Cut away excess PDMS along the perimeter using a blade to ensure a cuboidal PDMS chip with clean side surfaces.
5. Use the biopsy punch to punch inlet and outlet fluid access ports into the chip. In the standard mother machine, there is a single "main" channel that forms a large U shape in the device. The inlet and outlet should be punched at the two ends of the U shape (*see* **Note 4**).
6. Post punching, use a pipette (10 μl) to flush the inlet and outlet columns with absolute ethanol to clear away any PDMS debris that may be left behind. Following this, dry the columns and chip using a jet of N_2. The chips are now ready for bonding to a glass coverslip to prepare the microfluidic device. PDMS chips may be created in a group and stored indefinitely before use.

7. Chips should only be plasma bonded to glass coverslips (creating the microfluidic device) on the day of the experiment.
8. To prepare the chip for bonding, use scotch tape to remove any particulate matter that might have settled on the PDMS chip, particularly on the surface hosting the channels.
9. Separately, use scotch tape to clean a glass coverslip (Type I coverslip, 0.13–0.17 mm thickness).
10. Turn on the plasma oven after first opening the valve to the connected compressed air cylinder (for relevant air pressures, please refer to the plasma oven manufacturer's instructions).
11. Vent the chamber, and on opening, place the coverslip and the PDMS chip (channel hosting surface facing up) in the middle of the metallic plate used within the oven.
12. Switch off the ventilation and pump the chamber for 2 min.
13. Turn on the airflow to approximately 1 Nl/h and keep pumping the chamber for another 2 min.
14. Generate the plasma (30 W) for 10 s.
15. Vent the chamber. After 5 s, stop pumping the chamber.
16. Once the chamber is open, immediately press the two treated surfaces together, and use a flat-bottomed tweezer to gently press the underside of the glass coverslip along the perimeter of the entire device. Apply gentle pressures during this step to avoid collapsing the channels.
17. Hold the device under the light so that the channels are visible, and ensure that the bonding was successful (i.e., only the outline of the channels should be visible). If not, attempt to gently depress the perimeter again with the tweezers, and optionally, place the device in an oven (70 °C) for 5 min to enhance the adhesion.
18. Using a 10-μl pipette, introduce the BSA solution (50 mg/ml in MilliQ water) into the chip at the inlet. Ensure that the BSA has coated the main channel (the filling of the channel is visible under reflected room lights). Once coated, gently detach the pipette tip (containing solution) from the pipette and leave the tip embedded in the inlet of the device.
19. Place the chip at 37 °C for at least 30 min (30–90 min works well).

3.2 Culture Preparation

1. Use good microbiological practices appropriate to the biosafety level certification of your laboratory.
2. Prepare your bacterial culture as per the recommended protocols for the organism, setting up the culture the night before the experiment.

3. For *E. coli* BW25113, prepare streak plates on LB agar and seed an individual colony from the plate into a flask containing 150 ml LB. Use antibiotics as appropriate if required for relevant knockout or reporter strains.
4. The culture is left overnight on a shaker (200 rpm) at 37 °C to grow the cells into stationary phase.
5. On the day of the experiment, once the chip has been prepared, treated with BSA, and left to incubate at 37 °C, remove the culture flask from the incubator and check the optical density (OD at 595 nm) using a spectrometer (use fresh LB as the blank).
6. Take 50 ml of the culture in a falcon tube and centrifuge at (RCF) 3220 × *g* for 5 min.
7. Decant the supernatant into another 50-ml tube. Filter 10 ml of this supernatant solution twice using two separate 0.22-μm filters.
8. The final solution is labeled "spent LB" for the purposes of this protocol.
9. Concentrate the bacterial sediment to an OD of approximately 75 in spent LB and vortex to ensure good mixing.

3.3 Peptide Antibiotic Sample Preparation

1. This will vary depending on the exact properties of the peptide being prepared. We currently work with water-soluble peptides that are stored at −80 °C in powder form. These peptides are also suitable for vortexing.
2. To prepare the peptide solutions, carefully tip a flake of peptide (using, for example, a handheld pipette tip) from the stock vial into a 0.5-ml protein LoBind tube (*see* **Note 5**).
3. Immediately add 50 μl of MilliQ water to the peptide sample and vortex thoroughly.
4. Measure the absorbance of the sample using the UV-Vis settings of a NanoDrop.
5. In the NanoDrop settings, ensure that you have selected the appropriate wavelength (i.e., the wavelength for which the extinction coefficient of your peptide is known) and that the Auto Path Length option is selected. On completing the reading, the true path length (1 mm) will be reported on the Υ-axis of the output absorbance curve.
6. Use MilliQ water as the blank and measure the absorbance of your peptide sample.
7. Since the extinction coefficients may not be measured at the peak of the absorbance spectrum, there may be some variability in the absorbance readings, which would affect your true experimental concentrations. Therefore, perform the blank

and measurement cycle in triplicate at the very least and use the average value to calculate your stock concentration using Beer–Lambert's law (*see* **Note 6**).

8. Separately, prepare 400 μl of a solution containing 90% (360 μl) minimal media and 10% (40 μl) fresh LB.
9. Prepare 400 μl of a 10 μM solution of your peptide (or your concentration of choice) by diluting the peptide stock solution into the minimal media-LB solution prepared in the step above. Vortex thoroughly.

3.4 Setting Up the Microfluidic Pumps and Connections

1. Connect the 4 ports of the Fluiwell to the 4 pressure ports of the Fluigent pump using the appropriate manufacturer supplied tubing.
2. Switch on the Fluigent system and open the valve connecting the pump to the N_2 cylinder. Ensure that the pressure applied never exceeds the pressure rating of the pump. A 1 bar-rated Fluigent pump is suitable for these experiments. Switch on the corresponding Maesflo software and allow it to initialize.
3. Switch on all the microscopy components (light sources, stages, camera) and open the imaging software. An example of a suitable imaging system includes an Olympus IX73 body fitted with Physik Instrumente piezo stages, automated filters, and an Andor sCMOS camera, which are controlled using a custom LabView script. Alternatively, Micromanager [33] is a commonly used ImageJ/Java-based free software that may be used for this purpose.
4. Ensure that the filters are set to bright-field mode and that the LED light sources are off. Only transmitted light for bright-field imaging is required during drug treatment. The LED light source will only be required when performing the staining of dead bacteria with propidium iodide (PI) at the end of the experiment. However, if a fluorescent reporter needs to be tracked during the course of the drug treatment, adjust the imaging and LED settings accordingly.
5. Use a Fluigent Flow unit in conjunction with the pressure pump. This is an additional device that measures the flow rate generated by the applied pressures, and via a feedback system enables the setting of a defined flow rate for the experiments. The Fluigent system can be replaced by syringe pumps as well, but the Fluigent system is more convenient for fluid exchanges when running the system, as will be clear in the protocol below.
6. Connect the appropriate tubing (using tubing A) via fluidic connectors (i.e., nuts/ferrules that connect the fluidic tubing to ports) to the inlet and outlet of the flow unit (both the tubing and connectors are available from Fluigent). Tubing lengths will depend on the distance between the microscope

stage (where the microfluidic device will be mounted) and the positions of the flow unit and the Fluiwell-4C.

7. Using a conical fluidic connector, connect the tubing that is input to the flow unit into one of the ports of the Fluiwell-4C; details for connecting the tubing are available on the Fluigent website, and tubing B may be required as a sleeve for tubing A depending on the connector used. At the same port, attach a 1.5-ml vial (Micrewtube) containing at least 1 ml of spent LB media. Adjust the length of the tubing that is immersed in the vial to ensure that it reaches the bottom of the spent LB vial (*see* **Note 7**).
8. Separately, using another fluidic connector, connect another piece of tubing to an adjacent port in the Fluiwell and attach an empty 1.5-ml vial below the tubing—this will be used to collect the waste fluid while running the experiment.
9. Using the Maesflo software, increase the pressure on the port with the spent LB vial to flow the media through the tubing. Use pressures of 300–400 mbar till approximately 0.5 ml of liquid has flushed through (typically 3–4 min) and then stop the flow. The system is now ready to be plugged into the microfluidic device.

3.5 Seeding the Microfluidic Device with Bacterial Cells

1. Once the microfluidics-microscopy setup is ready for use, remove the BSA-coated microfluidic device from the incubator and gently remove the BSA-containing pipette tip from the inlet.
2. Vortex the concentrated (OD 75) bacterial culture and draw approximately 0.5 ml of this concentrated culture into a 1-ml syringe. Tap the syringe to remove air bubbles.
3. Attach a needle (*see* Subheading 2.1) to the syringe and carefully connect the needle to the appropriate tubing (an approximately 10 cm piece of tubing C). Push out 2–3 droplets of the concentrated culture into a waste container to flush out any dirt particles within the tubing (*see* **Note 8**).
4. Applying pressure, create a small droplet of culture at the end of the tubing (again ensuring there are no air bubbles in the syringe or tubing).
5. Insert the tubing carefully into the inlet punch. This may require some trial and error depending on the flexibility of the tubing and the quality of the punched inlet. A good practice involves using a sharp, clean blade to cut 1–2 mm off the tubing at the end that is being inserted into the chip, which facilitates its smoother insertion into the inlet (*see* **Note 9**).
6. Once the tubing is inserted into the inlet, apply gentle pressure to the syringe until you start observing the BSA solution being

pushed out of the outlet. Once the BSA is removed, the cloudy bacterial solution will start emerging from the outlet.

7. Blot away the bacterial solution at the outlet with tissue, and repeat the application of pressure till the bacterial solution forms another droplet at the outlet. Blot this away. Repeat the process a total of 3–5 times.
8. Gently remove the tubing (and associated syringe) from the inlet, and use a piece of scotch tape to cover both inlet and outlet holes to prevent any evaporation. Leave the filled device at 37 °C for 5–10 min.
9. Check the device under the microscope in bright-field illumination. The main channel should contain a high density of bacteria, and the side channels should start filling with cells. Ideally aim to capture 1 cell in each side channel, and no more than 3 per channel. This filling efficiency may vary slightly per experiment, so it is important to keep checking the device every 5–10 min till a suitable number of channels are filled with between 1 and 3 cells each. If the microscope has an incubation chamber set to 37 °C, this filling process may be monitored continuously, but if the experiment is performed at room temperature, use the incubator to facilitate filling (at 37 °C).

3.6 Microfluidics-Microscopy Assay

1. Once the side channels of the device are suitably populated with bacteria, remove the tape covering the inlet/outlet ports and connect the tubing from the flow unit (containing spent LB) into the inlet of the microfluidic device. When performing this step, make sure there is a liquid droplet at the tip of the tubing before inserting it in the chip to avoid air bubbles in the device.
2. Next, connect the tubing from the waste vial to the outlet of the device.
3. Carefully mount the entire device on the microscope. The stage should ideally have a clamping mechanism to keep the chip in place during the course of the experiment. Locate the channels hosting the bacteria.
4. Use the Fluigent Maesflo software to calibrate the flow control unit. Once calibrated, switch control of the pressure port connected to the flow unit to the flow control software. This will allow the user to set a desired flow rate through the experiment. Ensure that the pressure port connected to the waste is always set to 0 (no applied pressure) throughout the experiment.
5. Once calibrated, flow spent LB through the device at 300 μl/h for 8 min to clear away the bacteria from the main channel.

6. Stop the flow and switch the spent LB vial in the Fluiwell-4C with the vial containing 400 μl of the antibiotic solution. Note that the advantage of using this system over syringe pumps is that these fluid exchanges can be done without disturbing the microfluidic chip itself.
7. Start flowing the drug solution at 300 μl/h for 8 min and then lower the flow rate to 100 μl/h. Start imaging as soon as you start flowing the drug solution through the device—this records the condition of the cells at the initial ($t = 0$ h) time point.
8. The number of imaging positions (fields of view at which images are recorded) depends on the number of cells that the user requires analyzed from an experiment. With the 60 × W objective and camera mounted on our microscope, the field of view captures about 22 side channels. Assuming an average occupation of 1 bacteria per channel, for imaging 100 cells one requires about 5 imaging areas, and correspondingly for 1000 cells one would require around 45 imaging areas. This choice is left to the user's discretion. Acquire images in bright-field with 30 ms exposure times.
9. Image the same regions at hourly intervals for 3 h of drug treatment (so 4 images are recorded per region as the cells are dosed with the drug). Images may be acquired more frequently if required (*see* **Note 10**).
10. After 3 h of antibiotic treatment (this can be adjusted at the user's discretion, though note the volume of antibiotic solution prepared must be modified accordingly), stop the drug flow. Replace the drug solution containing vial in the Fluiwell-4C with a vial full (1.5 ml) of fresh LB media. Restart the drug flows, again at 300 μl/h for 8 min, followed by a lowering of the flow rate to 100 μl/h (*see* **Note 11**).
11. The purpose of incubating the cells in fresh LB after 3 h of antibiotic exposure is to determine whether any cells survived the treatment and to distinguish between survivors that remain dormant and those that regrow when exposed to favorable nutrient conditions. Image the regions of the device as before at hourly intervals for the next 3 h.
12. After 3 h of LB media supply, stop the flow. Empty the waste vial and then reattach the vial. Restart the LB flow at 50 μl/h; this will now be left overnight (*see* **Note 12**).
13. The next morning, typically 14–15 h after the previous imaging time point, check that all the flows were maintained and that there was no leakage and/or blockage of the device. This is noted by inspecting the channels and noting that the flow rate did not deteriorate overnight.

14. Prepare a solution of the dead stain propidium iodide (PI), by diluting 1 μl of PI stock solution (supplied in DMSO) in 1 ml of LB. Vortex this solution thoroughly and protect the vial from light by covering it in foil.
15. Stop the flow of LB in the microfluidic device and replace the LB vial with the vial containing the PI solution. Flush the chip as before for 8 min at 300 μl/h, and then slow the flow to 100 μl/h and run this for 15 min.
16. After 15 min of PI flow at 100 μl/h, start imaging the same regions of the chip as before. However, now additionally use the green LED (at 20% intensity on a pE-300 White LED system, 30 ms exposure times) with a TRITC filter set to alternately record bright-field and fluorescence images for each of the imaging positions. Dead cells and cell debris will stain with PI and show strong fluorescence. Cells that are clearly visible in bright-field and remain dark against the background of fluorescence in the channel are counted as being alive. These will be divided into two populations, those that remained nongrowing and those that regrew and populated the entire side channel that they were initially hosted in.
17. After acquiring this final set of images, stop the flow of PI and remove all the tubing from the microfluidic device. The tubing is reusable and should be flushed with approximately 1 ml of absolute ethanol using the Fluigent pressure pump. Note that the waste tubing and the tubing used to seed the microfluidic chip with cells must also be flushed with ethanol (the latter may be flushed using a syringe and needle). All the Micrewtube vials are also reusable and should be cleaned with ethanol and left to dry before further use.
18. Images may be analyzed either manually or using automated codes [30, 34] that have been developed for the analysis of mother machine data. It is recommended that, especially for novel drugs, the initial analysis be performed manually on ~100 cells before proceeding to using an automated script.
19. The analysis of this data enables the quantification of the division and growth rates of cells during drug treatment, the percentage of survival, the fraction of cells that survived in a dormant (nongrowing) state, and the fraction of cells that regrew despite the drug treatment. Each individual cell is, in principle, a "biological repeat," and the side channels can be considered the equivalent of different compartments ("wells") of a well plate. The single-cell resolution facilitated by this methodology enables the direct investigation of the heterogeneity in response to an antibiotic of a clonal population of cells.

20. The images can also help identify points in the growth cycle when the cells appear to be most susceptible to the drug. This may help with the identification of the drug target(s), if unknown.
21. Using knockout and fluorescent reporter strains may also help in target identification using this methodology.
22. Ultimately, the platform is extremely versatile and can be applied to a range of single-cell level studies in drug development. The protocol presented here provides a basic toolkit for running these experiments in the context of testing peptide-based antimicrobials. Further iterations and modifications are left to the imagination and creativity of potential users of this technology.

4 Notes

1. Details for the construction of the original mother machine mold are available courtesy of the Jun laboratory [22]. Variants of the device (including a Dual Input Mother Machine [34]) may also be purchased commercially from suppliers such as Micro Resist Technology GmbH. The dimensions of the side channels being used are critical to ensure the efficient confinement of the cells on the chip for long-term experiments—this will depend on the size of the bacterial cells being investigated. It is recommended that any Silicon wafer based mold be cast in PDMS and epoxy copies made [25] for long-term use to minimize the risk of damage to the original mold from repeated use.
2. Before pouring the PDMS, ensure that there is no particulate matter on the surface of the mold. This can be ensured by cleaning the mold's surface using scotch tape (epoxy mold) or by cleaning the mold (Silicon) with isopropanol followed by drying it with compressed gas.
3. The optimal PDMS chip height is determined by the size of the biopsy punch being used to punch inlet/outlet holes—the cured chip should be of a smaller height than the biopsy punch tip is in length, such that a clean punch may be effected when creating the fluidic inlet/outlet.
4. Since the channel is small (100 μm width), it is helpful to use two pieces of scotch tape applied to the surface of the chip (channel side facing up) on either side of the locations you wish to punch—this helps align the biopsy punch over the channel. Alternatively, use a benchtop microscope to align the punch with the channels before punching the inlet and outlet. It is also helpful to position the chip on top of a glass slide for this

purpose, and a dark background below the slide helps provide contrast to facilitate accurate punching. Punch through the PDMS chip with a vertical angle of approach, and punch through to the other side of the chip, using the plunger to clear any debris. The PDMS column punched through should be removed before withdrawing the punch from the hole. Avoid punching the columns multiple times, and before each punch, use scotch tape to clean any PDMS debris from the tip of the punch to ensure a smooth cut through the PDMS. Poor punching protocols may lead to chip failure due to poor attachment of the tubing when running the device.

5. Most peptide antibiotics are charged and have strong electrostatic interactions with latex gloves, so use caution when opening the vials containing peptide powders.
6. Ensure your sample is vortexed thoroughly prior to the NanoDrop measurements.
7. It is critical to ensure that the tubing used for flowing solutions through the chip is always fully immersed in liquid when a positive flow rate is applied; otherwise, air will be passed into the microfluidic device, which will compromise the experiment.
8. Be careful when inserting the needle into the tubing; a good safety practice is to not push any bacterial culture through the needle before connecting it to the tubing to minimize the risk of infection in the case of needle injury.
9. Occasionally, the tubing does not fit properly in the inlet, but since this is a different tubing to the one used with the Fluigent pump, it does not necessarily mean that the chip is unusable. In such a case, it is also possible to try filling the chip from the outlet; the difficulty in inserting the tubing at the inlet may have been due to a lower quality punch at the inlet.
10. For water immersion objectives, one should replace the water on the objective prior to each imaging run. By lowering the objective turret and partially rotating it, it is possible (with standard microscopy stages) to access the objective with a pipette to replace the immersion water droplet without disturbing the microfluidic device. If this is not possible, carefully shift the device when refreshing the immersion liquid, ensuring that the tubing connections are not disturbed.
11. During the initial 8-min flush, the tubing between the vial and the chip will still contain drug solution. Therefore, we always image at the start of this flushing step, and hence the final ($t = 3$ h) drug treatment imaging time point should coincide with the start of this flushing step *after* the vials have been switched.

12. If the LB vial was not initially filled to the brim, the user may need to top up the LB vial as well at this step.

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