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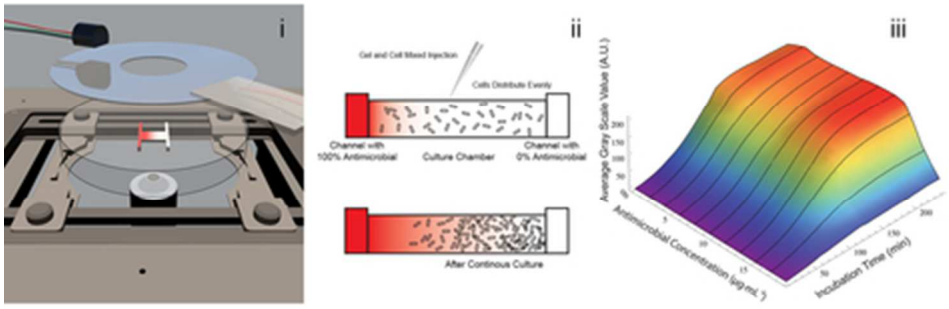

Time Lapse Antibiotic Susceptibility Investigation
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Time Lapse Investigation of Antibiotic Susceptibility using a Microfluidic Linear Gradient 3D Culture Device

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Abstract

This study reports a novel approach to quantitatively investigate the antibacterial effect of antibiotics on bacteria in a three-dimensional microfluidic culture device. In particular, our approach is suitable for studying pharmacodynamics effects of antibiotics on bacterial cells temporally and with a continuous range of concentrations in a single experiment. The responses of bacterial cells to a linear concentration gradient of antibiotics were observed with time-lapse photography, by encapsulating bacterial cells in an agarose-based gel located in a commercially available microfluidics chamber. This approach generate dynamic information with high resolution, in a single operation *e.g.*, growth curves and antibiotic pharmacodynamics, in a well-controlled environment. No pre-labelling of the cells is needed and therefore any bacterial sample can be tested in this setup. It also provides static information comparable to standard techniques for measuring Minimum Inhibitory Concentration (MIC).

Five antibiotics with different mechanisms were analysed against wild type *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* Typhimurium. The entire process, including data analysis, took 2.5-4 h and from the same analysis, high-resolution growth curves were obtained. As a proof of principle, a pharmacodynamic model of streptomycin against *Salmonella* Typhimurium was built based on the maximal effect model, which agreed well with the experimental results. Our approach has potentials to be a simple and flexible solution to study responding behaviours of microbial cells under different selection pressures both temporally and at a range of concentrations.

1 Introduction

In recent years microfluidics has gained significant advances in mammalian cell studies, due to a similar size range as the cells[1]. However, compared to mammalian cell studies, the use of microfluidics to analyse bacteria is less common[2-3]. Bacterial cells strongly impact our daily life and are in human bodies estimated to be around ten times more frequent than "our" (mammalian) cells[4]. An interesting and important growing global problem is antibiotic resistant bacteria[5], causing intractable infections with increasing personal suffering and medical costs[6].

The traditional antibiotic susceptibility testing, as recommended by the Clinical and Laboratory Standard Institute (CLSI), has mainly been limited to Mueller Hinton agar plates containing predefined concentrations of antibiotics or dilution series in liquid Mueller Hinton broth[7]. An alternative technique is the Etest[8], consisting of a rectangular strip with a set of pre-defined antibiotic concentrations within a certain concentration range. The Etest eases practical operation significantly but it still needs a long incubation time and only records the final time point of the bacterial response, the minimum inhibitory concentration as measured by no visible growth after 18-20 h incubation. For more in-depth pharmacodynamics studies of the response of bacterial cells to antibiotics, time-kill experiments, where the fraction of surviving cells are measured at intervals during treatment with fixed concentrations of drug, are conventionally used. In general, these methods are time-consuming and laborious, especially when a larger number of antibiotic concentrations are required for testing. Microtiter plate based assays, using multiple wells with different antibiotic concentrations, provide temporal data and

increase the number of tested concentrations but has basically the same shortcomings as previous methods[7, 9].

Besides smaller amounts of sample and reagent volumes, microfluidics offers faster procedures and significantly decreases the time for analysis to only a few hours, due to shorter diffusion distance in the channel[3, 10-19]. Several microfluidics approaches have been tested for bacterial studies. A droplet based digital device was first used to investigate bacterial susceptibility by mixing nutrient, a viability indicator, bacterial cells and antibiotics[3]. Later, magnetic beads were introduced to enhance its performance[10], and recent work aimed to make this digital based approach automated[11]. Another approach has been to use micro-fabricated micro-wells to capture droplets with bacteria inside[12]. However, these droplet-based approaches can only test a single antibiotic concentration. Testing numerous concentrations will make the device too complex to implement. A polydimethylsiloxane (PDMS) device with a simple straight channel was shown to have similar performance as traditional liquid and solid methods, owing to the excellent gas permeability of PDMS[13]. Furthermore, by fixating the bacteria on the channel surface, mechanical stress as well as chemical (antibiotic) stress was studied in a similar design[14]. Recently, the principle of the Etest was transferred to a microfluidic device with a limited set of drug reservoirs connected to a straight channel[15]. Studies have also been focused on single cells assays via various trapping strategies such as DEP[16] (dielectrophoretic) or agarose-based encapsulation[17]. In addition, the straight channel approach was further extended to analyse antibiotic susceptibility of bacterial biofilms[18]. However, their microfluidic system required 24 h of incubation and that the tested bacteria contained a plasmid expressing green fluorescent protein (GFP), limiting the approach to molecularly

engineered lab strains. Recently, a microfluidic device was developed to determine MIC with a 3D fluorescent visualization technique[19].

There are several limitations to previous approaches. First, they can only analyse a single antibiotic concentration or a set of a few selected antibiotic concentrations. Second, they only offer static results and provides no information on how bacterial cells respond to the antibiotics during the incubation. Third, most approaches require the bacteria to be studied to be internally labelled by for example green fluorescent protein and can therefore only be used with genetically modifiable lab strains and not with clinical isolates. Instead of static values, pharmacodynamics, that investigates concentration dependent effects of a drug against the targeted microorganism over time, can provide a link between antimicrobial activity and the susceptibility of a bacterial strain[20]. A recent study demonstrated a microfluidic device for pharmacokinetics-pharmacodynamics modelling, which requires a few days for analysis[21]. Further, mathematic models based on chemical kinetics have been built according to the mechanism of the antimicrobial and the population evolution of the strain[22]. These models are the quantitative frameworks for interpreting the results of antimicrobial susceptibility testing and for the generation of novel hypotheses on the mechanism of antimicrobials targeting bacterial strains[21]. This kind of information is very important to evaluate the antibacterial effects of novel drugs and guide the usage of antibiotics[20]. There is a need for fast methods and systems that could overcome the disadvantages of previous works.

One promising solution is to use a microfluidic 2D gradient system[23]. In much, it is similar to the technology used in this work but a 2D cell culture in a small area reduces

the cell numbers and, hence, give less statistics. Still, this system complements well ours and is especially useful when studying single cells and their cell growth.

In this work, our aim was to develop a rapid and simple way to investigate antibiotic susceptibility temporally and at different concentrations and to offer high-resolution readouts with numerous concentrations and dynamic information on bacterial response under these concentrations. To achieve this, a continuous linear antibiotic gradient with gel-encapsulated bacteria was used to generate high-resolution readouts, which in principle can produce unlimited numbers of concentrations in a given range; and time-lapse microscopy was used to obtain dynamic information. Finally, the grey scale intensity in the micrographs is assumed as linear to the local cell concentration and continuous data analyses are made in regard to time and antibiotic concentration. A schematic representation of the procedure is shown in Fig. 1. In our tests, five antibiotics with different mechanisms against three common wild type strains of Gram-positive and Gram-negative bacteria were investigated. Static measurements (Minimum Inhibitory Concentration[24]) with the traditional methods were used as reference, and we systematically investigated the bacterial growth curves at various concentrations of drug. Finally, a pharmacodynamics model of streptomycin against *Salmonella* Typhimurium LT2 was generated and validated.

2 Materials and Methods

2.1 Preparation and experiments with microfluidics

Cell preparation: Bacterial strains used were *Escherichia coli* K12 MG1655, *Salmonella enterica* serovar Typhimurium LT2 and *Staphylococcus aureus* SH1000 from the strain

collection of the Department of Medical Biochemistry and Microbiology, Uppsala University. Single colonies were picked from Lysogeny broth agar (LA) plates and cultured in 5 mL Lysogeny broth (LB) medium for 5-6 h until OD_{595} reached 0.3-0.4. The final amount of seeding bacteria was obtained with centrifugation for 1 min at 4,000 rpm followed by re-suspending of the bacterial pellet in phosphate buffered saline to an OD_{595} of 0.1, approximately 1×10^8 cells·mL^{-1}.

Antibiotic preparation: A syringe containing 5 mL of Mueller-Hinton medium (DIFCO) with one of the following antibiotics from Sigma-Aldrich, final concentration indicated within parenthesis, ampicillin (20 μg·mL^{-1}), spectinomycin (50 μg·mL^{-1}), streptomycin (20 μg·mL^{-1}), tetracycline (5 μg·mL^{-1}) and vancomycin (2 μg·mL^{-1}), was prepared. In parallel, a syringe containing 5 mL Mueller-Hinton medium without antibiotics was also prepared.

Cell Loading and Device Set-up: 50 μL of Mueller-Hinton medium including approximately 1×10^8 cells·mL^{-1} was mixed with 50 μL of 1% low melting temperature agarose (Sangon Biotech) at 37ºC. Eight μL of the mixture was injected into the chamber of the microfluidic gradient generator chip (Ruby, Gradientech), Fig. 1A. The chamber is 4 mm in length, 3 mm in width and 500 μm in depth, which makes a volume of 6 μL. Extra mixture was pushed out through the outlets and washed away. The chip containing the agarose-bacterial mixture was left at room temperature for 30 min for the agarose to solidify. After the gel solidified, the two syringes were connected to two different inlets of the chips as shown in Fig. S1 in the Electronic Supplementary Information (ESI). To establish a linear gradient, one of the syringes contained Mueller-Hinton medium and the other syringe Mueller-Hinton medium with an antibiotic added. As a control for uniform

growth in the chamber, tests were also made with only growth medium without antibiotics in both syringes. The time for the formation of an approximately linear antibiotic gradient was estimated to be 45 min, and at that time, according to reference data and classical diffusion theory a maximal error in the middle of the chamber of -3% for ampicillin and -10% for vancomycin are present (more details in the ESI)[25-27]. Both syringes were placed in a syringe pump (PHD2000, Harvard apparatus) with the flow rate of 5 $\mu L \cdot min^{-1}$ for the first 30 min to make the medium with and without antibiotics diffuse into the agarose-bacterial gel to form a linear concentration gradient as fast as possible. After that, the pump rate was set to 2 $\mu L \cdot min^{-1}$.

Temperature regulation: The temperature of the chip was maintained at 37°C with a thermostat to enable the time-lapse photography with the inverted microscope. To build a proportional-integral-derivative (PID) feedback control system a digital thermometer (DS18b20, Dallas company), a chromium alloy resistance wire heater and a single-chip microcomputer (Arduino UNO R3, Arduino) were used. The detailed design is shown in Fig. S2 in the ESI. A photo of the microfluidic device including the temperature regulation positioned on the microscope is shown in Fig. S3 (ESI).

Data acquisition, Quantification and Analysis: The culture device (chip) was placed on an inverted microscope (Eclipse TE-2000, Nikon Instruments Inc.) and the 10X objective lens with a phase contrast condenser was used for observation. A digital CCD camera (Spot RL Mono, Diagnostic Instruments) connected to a computer was used to record the images every 30 s after the placement of the chip. The exposure time was set to 12 ms. A heater and a thermometer were attached to the chip's container to ensure a stable temperature during the incubation under the microscope (Fig. 1A). The grey scale value

of the phase contrast images was used to indicate the growth of the bacterial cells. The recorded images were saved as 8-bits grey scale in tif-format and the grey scale values were transferred to between 0 to 255 A.U. Images were cropped and rotated to focus on the chamber part with 1,500 pixels in length and 400 pixels in width (Figure 1B). The grey scale value of each pixel was read in to a matrix. All rows of the matrix were added together into an array, which contained 1,500 data points. The background was eliminated before the data analysis, using the first micrograph as the background of the test. Each grey scale sum of a row in the array is presented as a grey scale value, which indicates the cell concentration of the corresponding location of the length. Hence, in theory the resolution of concentration will be the range of concentrations over the length of the linear gradient divided by the number of pixels. That is, if the lowest concentration is zero, the dynamic range will be comparable to a study of dilutions down to 1,500 X of the original (highest) concentration.

With a linear distribution of the antibiotic concentration, a specific concentration could be mapped to a certain location of the chips length. With this information the relation between cell growth and the antibiotic concentration could be determined. Series of arrays were constructed into a matrix, where the time is determined along the columns while the width location-concentration was along the rows, Fig. 1C. Each column indicates the growth of the cells under a certain concentration of the antibiotics, and each row indicates the cell concentration of the whole chip at a certain time. Cell growth data under four concentrations were picked evenly and were plotted as continuous growth curves. These curves show a temporal distribution of cell growth (Fig. 1D, left panel). Also, the column value could be used and plotted as a growth distribution curve of cells

at different concentrations, e.g., for MIC determination (Fig. 1D, middle panel).

2.2 Reference studies with traditional methods

CLSI Protocol and Etest: In order to compare this on-chip test with traditional static protocols, the MIC was determination by the CLSI protocol, which is a dilution susceptibility testing method standardized by The Clinical and Laboratory Standard Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards, or NCCLS) and by the Etest according to the instructions from the supplier (AB bioMérieux). Each antibiotic stock solution was diluted to 1 $mg \cdot mL^{-1}$, 0.1 $mg \cdot mL^{-1}$ and 0.01 $mg \cdot mL^{-1}$ with Mueller-Hinton medium. For the liquid MIC test, a two-fold serial dilution of the antibiotics was used, and the total culture volume was 1 mL. Single overnight cultures of 5 mL *E. coli*, *S.* Typhimurium LT2 and *S. aureus* were grown separately at 37ºC in Mueller-Hinton medium. After diluting the cells according to the 0.5 McFarland standard (~10^8 $CFU \cdot mL^{-1}$) with Mueller-Hinton medium, the solution was further diluted a 100-fold in Cation-adjusted Mueller-Hinton broth (CAMHB). A bacterial suspension of 1 mL was added into 1 mL of a diluted antibiotic solution, as mentioned above. The bacterial cells with antibiotics were cultured at 37°C for 20 h.

2.3 Pharmacodynamics modelling

The concentration (growth) of the bacterial cells in the chamber without antibiotics can over time be described by,

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{N_{max}}\right) \qquad \textbf{(Equation 1)}$$

where N is the cell concentration, indicated by the grey scale value in our system; t is time; r is the growth rate constant; N_{max} is the maximum concentration of the cells that can grow in the chamber. This logistic equation describes a typical sigmoid growth of bacterial cells in a continuous and space limited culture condition. We use a sigmoid E_{max} model (Effect model equation) to describe the effect of antibiotics, which assumed a non-linearly relation to the concentration[28],

$$E = \frac{E_{\max} C^p}{EC_{50}^p + C^p} \qquad \textbf{(Equation 2)}$$

where E_{max} is the maximal effect of a certain antibiotic against a specific bacterial strain; C is the concentration of the antibiotic; p is the sigmoidal factor; and EC_{50} is the concentration of the antibiotic that decrease cell growth to 50%. The effect model is an ordinary hill equation that describes the binding of antibiotic molecules to their targeting molecules. The factor p defines the shape of the concentration against effect, which represent whether the binding of one antibiotic molecule with one target molecule facilitates or inhibits other binding reactions. For different mechanisms of antibiotics, the effect of the antibiotics was incorporated into the equation (growth) in different ways. In this demonstration, we focus on streptomycin. Streptomycin binds to the S30 sub-unit of the ribosome and interrupts the initiation of translation. This effect significantly reduces the growth rate of the target cells. The model can be further modified as,

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{N_{\max}}\right)(1 - E) \qquad \textbf{(Equation 3)}$$

where the growth rate parameter r has been reduced by the effect model function related to the concentration of antibiotics. Parameters r, N_{max}, E_{max}, EC_{50} and p characterized the growth of the cells.

The data was analysed with a non-linear model fit algorithm in Mathematica (Version 9.0.0.0). All data from the streptomycin experiment with *S*. Typhimurium LT2 and the control test (without antibiotics) were included in the data analysis. The cross-validation was used to perform model validation. One hundred concentration values were randomly chosen and the trials with these concentration values were excluded from the estimation of parameters. Then these excluded concentrations values were predicted by the model. Data from the test and the prediction from the model with the same concentration were plotted and presented graphically, Pharmacodynamics, Fig. 1D.

3 Results and Discussion

3.1 Cell growth under an antibiotic gradient and MIC determination

Control experiments were performed to validate the growth of *E. coli* and *S.* Typhimurium in the microfluidics device without antibiotic addition. Both species showed logarithmic growth during a majority of the incubation time. The cell growth was uniform, showing that the growth of the bacteria was not influenced by their position in the chamber (Fig. S4 in the ESI). We tested the microfluidics gradient setup with four different classes of mechanistically different antibiotics on three different bacterial species. Figure 2 shows growth curves of *S.* Typhimurium and *E. coli*, cells in the presence of three translation-inhibiting antibiotics. Similar shapes of growth curves were obtained for *S.* Typhimurium LT2 with addition of streptomycin (A) or tetracycline (B) and for *E. coli* with addition of spectinomycin (C). All three antibiotics inhibit bacterial growth by binding to the ribosome, thereby inhibiting bacterial protein synthesis.

From the growth curves, there are indications on the uncertainty of the optical readout. There are no indications to why the grey scale should change at very high concentrations of antibiotic. Hence, the uncertainty is approximately ± 10 a.u. grey scale.

The output, cell growth against the antibiotic concentration gradient over time (Fig. 2A-C), indicates the relation of the concentration-effect between the antibiotic and the growth of the bacterial cells, where the steeper the drop in cell growth is the higher the concentration-effect relation is, *i.e.* the so-called all-or-nothing effect[29]. From these curves we can extract several parameters that are important for studies of the antimicrobial action of antibiotics. First, the bacterial growth rate at every concentration of drug within the gradient can be extracted from the plot of growth versus time (Fig. 2). Second, the minimum inhibitory concentration can be determined from the breakpoint where 90% of the growth is inhibited. Third, any effect of antibiotic on the bacterial growth at concentrations below the MIC can easily be assessed and monitored over time. All translation inhibiting antibiotics gave nice curves with expected shape. The reduction of growth was directly dependent on the concentration of drug with marked drops in growth around the MIC. The effect of antibiotic concentration on growth was evident already after 120 min.

We also used antibiotics with a different mode of action, inhibition of the bacterial cell wall: ampicillin against *E. coli*, and vancomycin against *S. aureus* (Fig. 3). These antibiotics have different targets, vancomycin binds the terminal D-alanyl-D-alanine residue of peptidoglycan, blocking its elongation[30], while beta-lactams like ampicillin bind as irreversible inhibitors of the penicillin binding proteins (transpeptidases) and inhibits cross-linking of the peptidoglycan forming a weaker cell wall[31-32]. For *S. aureus*

with the addition of vancomycin there was a clear threshold effect of growth inhibition as seen previously[33]. For *E. coli* with the addition of ampicillin we observed a marked difference in the growth curves, reflecting the different mode of action of this antibiotic. Before 100 min, there was no significant difference in growth between cells at different ampicillin concentrations. Cells subjected to higher ampicillin concentrations even showed slightly increased growth, Fig. 3 (A). However, after 120 min, cells growing at concentrations higher than 5 $\mu g \cdot mL^{-1}$ began to decrease in number (due to cell disruption), yielding a decrease of the average grey scale value. At 240 min there was a clear breakpoint at which the bacteria most probably lysed. This breakpoint coincides very well with the MIC measured on plates by standard methods. This variation in growth curves is an example of the mechanistic difference between ampicillin that still allow growth until the cells lyse, and the protein synthesis inhibiting antibiotics where the growth rate is directly affected by the inhibition of translation. In our study, the optical intensity is used to present the growth of the bacteria, which will reflect increased cell size or cell number indiscriminately. In previous studies with flow cytometry, it was found that the bacteria increased in cell size when treated with high concentrations of ampicillin[33-35]. We believe that an enlarged cell size was the dominant reason for the increased optical intensity early in the experiment.

The growth to concentration distribution may also have potential in determining the MIC value. For instance, from Fig. 3 (B'), it is easy to determine the *S. aureus* MIC for vancomycin to 1.5 by finding the breakpoint at the antibiotic concentration where inhibition was observed in the growth curve. When using the same relative grey scale value of 75 of 255 at 240 min as an indicator for MIC at the different chips, each MIC

value determined by the chip is between the values for MIC determined by broth dilution and Etest, Table 1. Not surprisingly, most of the MIC values determined by our method are more in agreement with Etest than with broth dilution, given the slight differences in bacterial growth in liquid (broth dilution) and on solid media (Etest and our ship). Compared to other microfluidic approaches to determine MIC such as that in ref. 19, our approach requires significantly less time, without introducing any fluorescent labelling and dedicated fluorescent microscopy.

By comparing the time-cost of our microfluidic approach and traditional analysis, it is possible for significant savings in time for our microfluidic approach. Noticeable differences in cell growth were observed already after 2 h incubation. To determine the MIC break points, our approach may give a result with incubation as short as 2 h 30 min when streptomycin and tetracycline were used for *S.* Typhimurium LT2 and vancomycin was added to *S. aureus*. For measuring the MIC of ampicillin for *E. coli* a longer incubation was needed but still only 4 h. The data analysis is fast and only a few seconds are needed to provide the full data from the images. However, both the Broth Dilution method and Etests need overnight incubations for at least 18 h.

As mentioned above, a continuous concentration gradient offers higher resolution readouts than traditional approaches. Furthermore, with a continuous concentration and temporal observation, a significantly larger amount of information was obtained as compared to the standard methods (Fig. 2). For instance, in traditional approaches, the concentrations are predefined and to decrease the large amount of laborious operations a limited set of concentrations are used, which somewhat decrease the details observed. This is especially true when steep concentration-effect relation happens. Replacing the

traditional 2-fold-dilution based concentration series to a linear concentration gradient will generate a more detailed picture of growth inhibition. With the high resolution of the data we can extract from a single group of experiment, we created 3D diagrams of bacterial growth curves as shown in Fig. 4. This helps in finding more detailed information without redoing the experiments. For instance, comparing Fig. 4 (A) and Fig. 4 (B), we'll find an intuitive and detailed description of the growth difference between antibiotics with different mechanisms. With our camera, the resolution of this method can reach the highest antibiotic concentration present on one of the sides of the linear gradient divided by 1,500. In Fig 4, each pixel-step on the Y-axis represents a step of 13.3 $ng \cdot mL^{-1}$.

In summary, besides giving researchers a more detailed and faster method to look at the interaction between antibiotic and bacteria, our proposed approach has potential in offering fast high-resolution static results.

3.2 Pharmacodynamics study

As a demonstration, a pharmacodynamics model for the impact of streptomycin to *S.* Typhimurium LT2 growth was built and also cross validated to experimental data, Fig. 5. The parameters used are listed in Tables 2 and 3. The concentration-effect relation of streptomycin against *S.* Typhimurium LT2 has a steep sigmoid shape according to the parameters and with a *p* value of 6.97, i.e. significantly greater than 1. This steep relation can also be revealed from the concentration of cell growth distribution, Fig. 5 (A). The EC_{50} value estimated from the model gives us the efficiency of the antibiotic and for streptomycin against *S.* Typhimurium LT2 it is estimated to 18 $\mu g \cdot mL^{-1}$, Table 3. The value for EC_{50} is close to the MIC value of 18 $\mu g \cdot mL^{-1}$ as determined from three

different methods, indicating that with the addition of a small amount of streptomycin the level of cell death can move from 50% to close to 100% efficiency. This also confirms that the concentration-efficiency curve has a sigmoid-like shape, indicating that streptomycin and the target ribosome molecules have a cooperative binding effect. With this simple device, we demonstrate the possibility of utilizing the large amount of dynamic data provided to estimate and validate the mathematical model and obtain growth and pharmacodynamics parameters that are important for evaluation of the antibacterial effect on an antibiotic.

4 Conclusions

Time-lapse microscopy using a microfluidic linear gradient 3D culture device with bacterial cells was proposed for investigating antibiotic susceptibility. A large amount of dynamic data on the bacterial behaviour was obtained in this process, *e.g.* growth curves and cell behaviours at the range of concentrations of the antibiotics gradient at any given time point. The presented results show that this approach can rapidly obtain MIC values and total yield of bacterial growth. We also show that the produced data could be used for pharmacodynamics studies that are important to evaluate an antibiotic candidate or further study the effect of antibiotics on bacteria. In addition, this approach is flexible, and can easily be extended to any kind of bacterial strain and for studies such as antimicrobial combination therapy or multi-resistant bacteria with minor revision of the device.

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List of Figures

Fig. 1 A schematic presentation of our approach: (A) A low melting temperature agarose with homogeneously dispersed bacteria was injected into a 3D microfluidic culture device, placed above an inverted optical microscope with a phase contrast condenser. After the gel has solidified, medium with or without antibiotics are introduced into the two channels located on opposite sides of the gel, forming a stable and continues gradient. (B) Time lapse digital optical images were recorded with a high resolution CCD camera and saved in an 8-bit grey-scale .tiff format. Figure 1B shows three such micrographs of the incubation chamber, from left to right. The first represent the background when the experiment starts, the second a lighter micrograph after some time of incubation and the third a micrograph where the nearly white is representing high cell concentrations, which ends abruptly (grey) at a concentration close to the MIC. (C) After verification and pre-processing, the images were quantified as a 2D optical intensity graph against time and antibiotic concentration, presenting the growth information of the embedded bacteria. (D) Analyses based on the extracted data show the temporal and concentration information of the antibiotic effect on growth of bacterial cells. These quantitative data could be used to determine static information such as MIC. The large amount of dynamic data, allows for building a pharmacodynamics model.

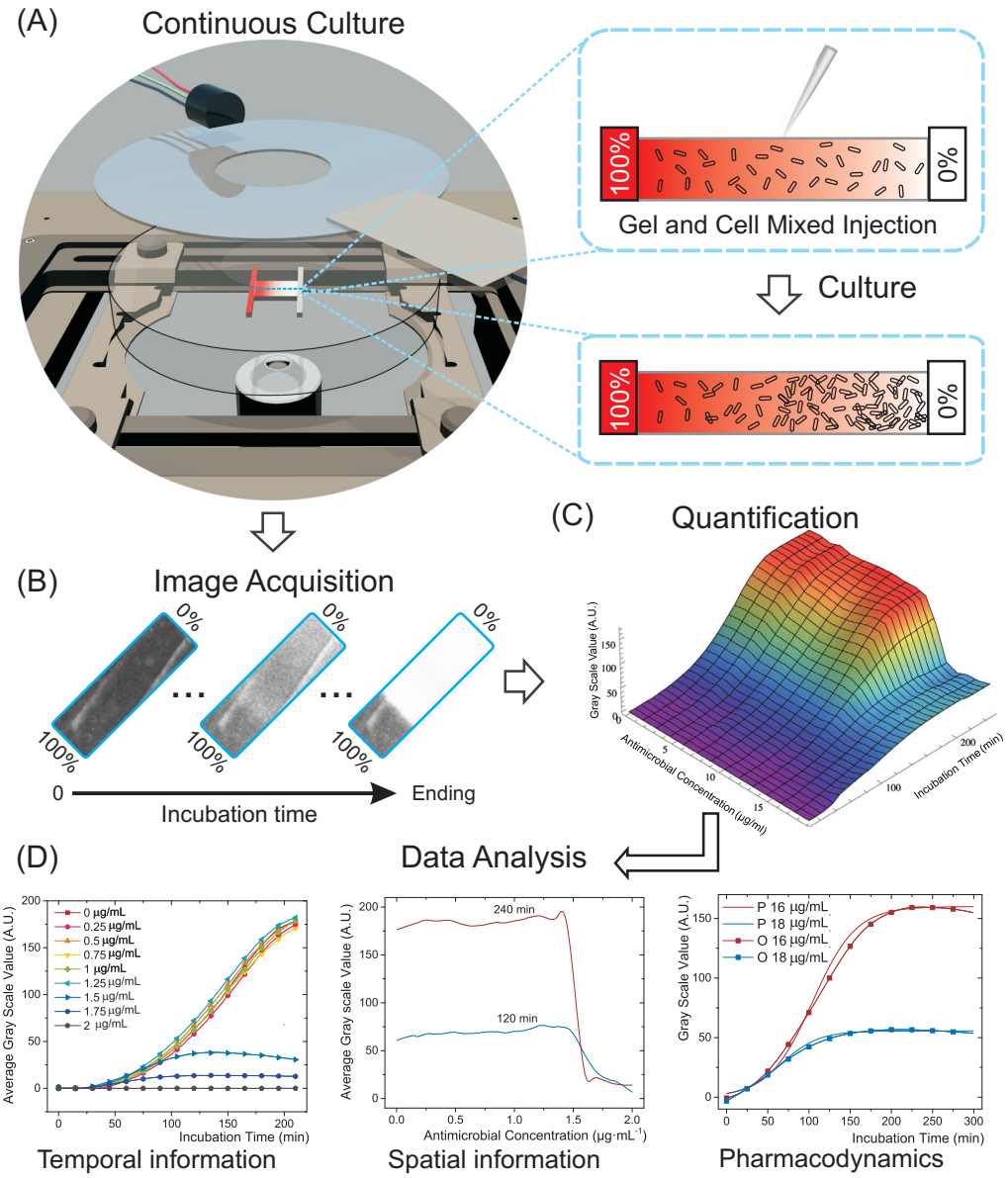
Fig. 2 Temporal and antibiotic concentration results of cell growth from three different bacterial strains incubated with three different antibiotics. The left column represents the temporal growth curves at different antibiotic concentrations, while the right column is the antibiotic concentration curves at different times. In all figures the Y-axis is the average grey scale value, which indicates growth accumulation of cells. (A) and (A') are

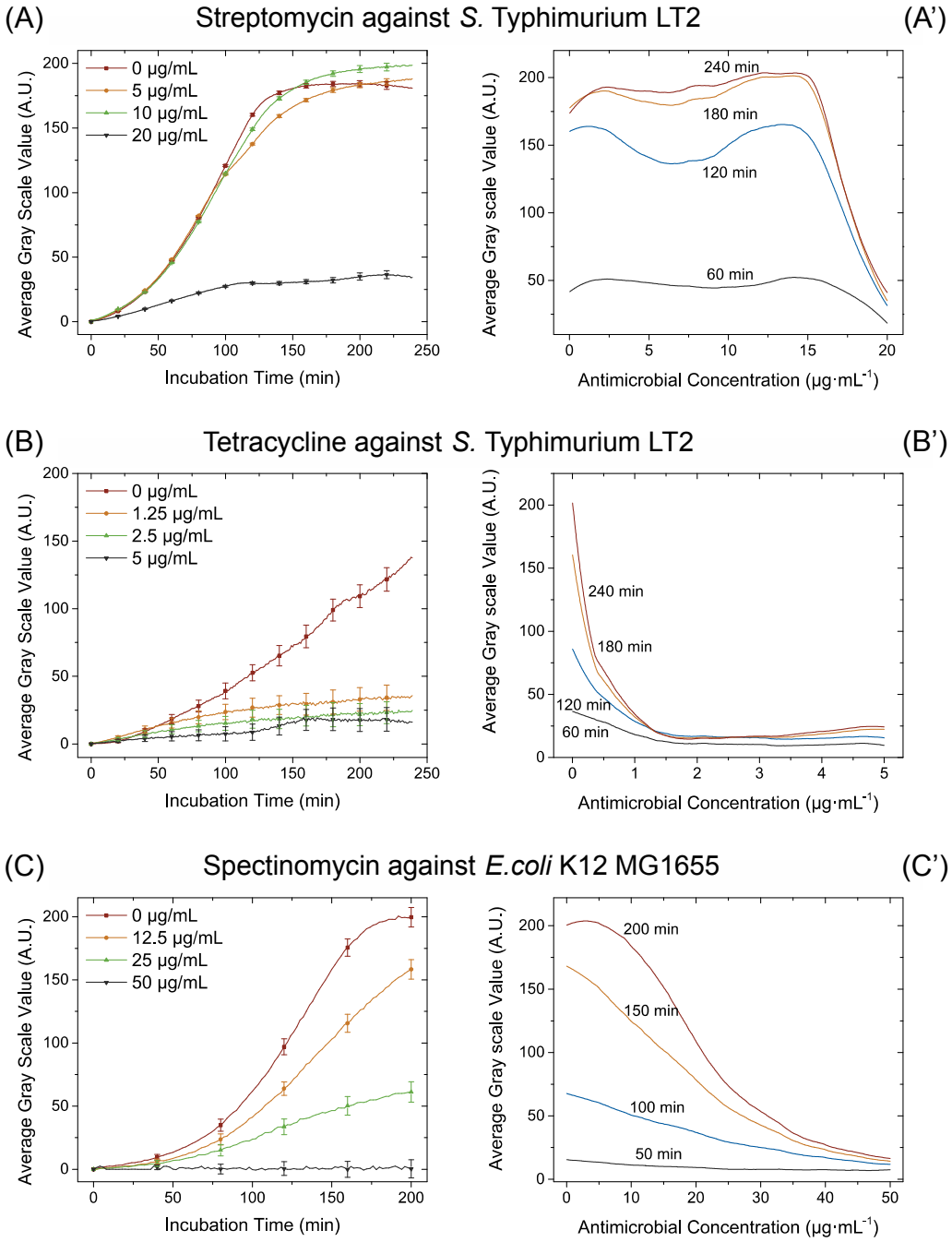
diagrams of *S.* Typhimurium LT2 with streptomycin. (B) and (B') are diagrams of *S.*. Typhimurium LT2 with tetracycline. (C) and (C') are diagrams of *E. coli* with spectinomycin.

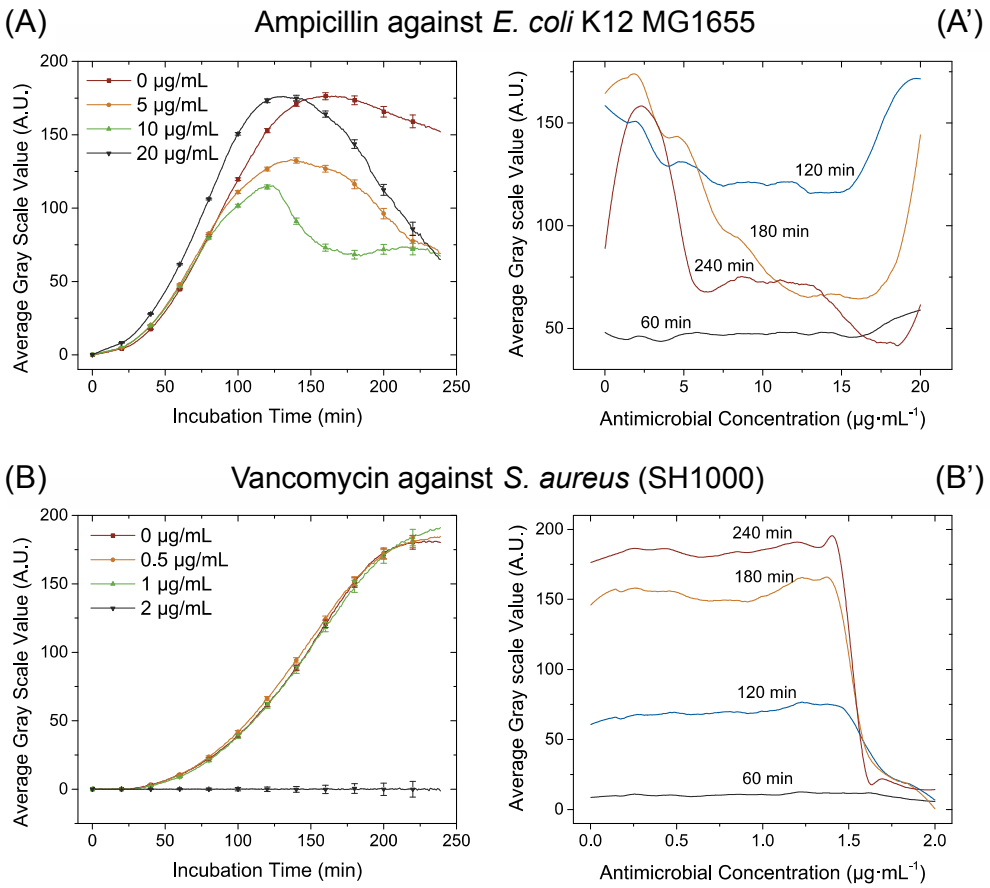
Fig. 3 Comparison between two different antibiotics for two different bacterial strains. The upper panels represent growth curves (A) and concentration distribution (A') of *E. coli* with ampicillin. The lower panels represent growth curves (B) and concentration distribution (B') of *S. aureus* with vancomycin.

Fig. 4 3D diagrams of bacterial growth curves with an antibiotic gradient. (A) and (B) are 3D diagrams of growth curves from the experiment with ampicillin (0 to 20 $\mu g \cdot mL^{-1}$) against *E. coli* and streptomycin (0 to 20 $\mu g \cdot mL^{-1}$) against *S.* Typhimurium LT2. The X-axis is the incubation time, the Y-axis is the antibiotic concentration gradient and the Z-axis is the average grey scale value.

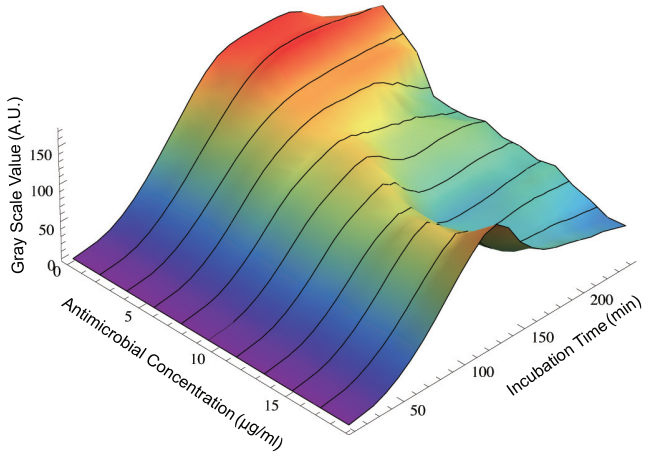
Fig. 5 Simulation and validation of a pharmacodynamics model for streptomycin against *S.* Typhimurium LT2. (A) Predicted (P) and observed (O) growth curves. (B) A linear fit cross validation of the model. Correlation coefficient R= 0.978.



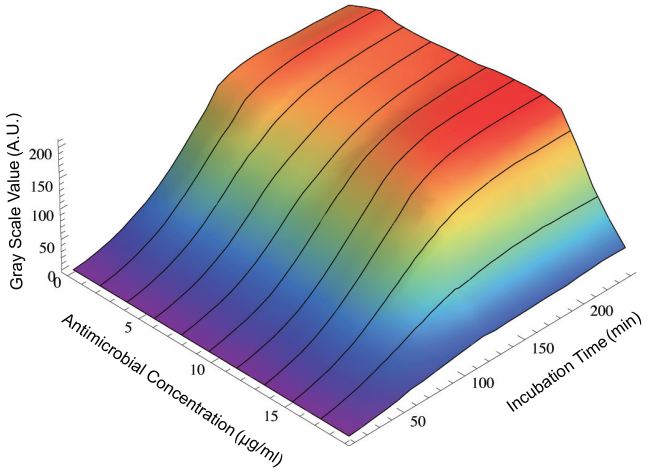




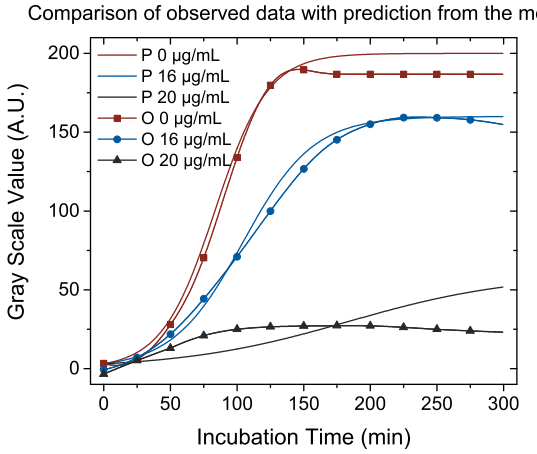
(A) Ampicillin against *E. coli* K12 MG 1655



(B) Streptomycin against *S.* Typhimurium LT2



(A)



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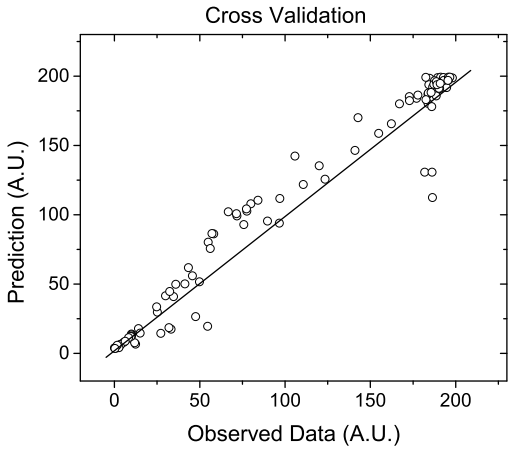

Table 1. MIC determination of antibiotics against a set of bacteria[‡]

E . coli				*S.* Typhimurium LT2			
Antibiotic	CLSI	Etest	Chip	Antibiotic	CLSI	Etest	Chip
Ampicillin	4-8	4	5	Streptomycin	16-32	16	18
Spectinomycin	16-32	16	18	Tetracycline	1-2	0.5	0.5

S. aureus			
Antibiotic	CLSI	Etest	Chip
Vancomycin	1-2	1.5	1.5

[‡] Unit: $\mu g \cdot mL^{-1}$

Table 2 Estimates of bacterial cells growth parameters and relative standard errors

Parameters	Estimated value	Standard error (%)
r	0.025	0.059
N_{max}	202.003	3.84

Table 3 Parameters for cross validation

Parameters	Estimated value	Standard error (%)
p	6.97	4.26
EC_{50}	16.00	2.89
E_{max}	1.33	0.72