# Droplet-Microarray: Miniaturized Platform for High-Throughput Screening of Antimicrobial Compounds

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Currently, there are no time-saving and cost-effective high-throughput screening methods for the evaluation of bacterial drug-resistance. In this study, a droplet microarray (DMA) system is established as a miniaturized platform for high-throughput screening of antibacterial compounds using the emerging, opportunistic human pathogen *Pseudomonas aeruginosa* (*P. aeruginosa*) as a target. Based on the differences in wettability of DMA slides, a rapid method for generating microarrays of nanoliter-sized droplets containing bacteria is developed. The bacterial growth in droplets is evaluated using fluorescence. The new method enables immediate screening with libraries of antibiotics. A novel simple colorimetric readout method compatible with the nanoliter size of the droplets is established. Furthermore, the drug-resistance of *P. aeruginosa* 49, a multi-resistant strain from an environmental isolate, is investigated. This study demonstrates the potential of the DMA platform for the rapid formation of microarrays of bacteria for high-throughput drug screening.

## 1. Introduction

The increasing incidence of antimicrobial resistance in bacteria and the lack of new antibiotics that can be used to treat drug-resistant bacterial infections have become a major threat to human health worldwide.[1–3] The development of antibiotic resistance among various bacteria belonging to the "ESKAPE" group of human facultative pathogenic bacteria is a particular cause for concern. The ESKAPE group of bacteria comprises *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* (*P. aeruginosa*), and *Enterobacter spp.*, which are known causes of serious hospital-acquired infections.[2] Several

of these clinically relevant bacteria have developed resistance to most currently available antibiotics.[1] It is estimated that during the last decade, direct cost caused by antimicrobial-resistant bacteria is €1.5 billion per year in the EU, Iceland, and Norway.[4] Consequently, novel agents that control the growth of these human pathogens are urgently required.[5] Evaluation of the synergistic effects of existing drugs and investigation of the inhibitory activity of numerous naturally occurring compounds against pathogenic bacteria are also regarded as important approaches in the search for novel treatment options.

The currently available high-throughput screening methods based on multi-well microplates are time-consuming and costly, requiring expensive robotics for plate handling and pipetting.[6] Furthermore, this type of screening requires relatively large amounts of expensive reagents, and microtiter plates. Most antibiotic resistance analyses are based on defined protocols for routine testing, and the cost of the modifications required to screen newly identified natural compounds and synergistic effects with other compounds are prohibitive for many research and development (R&D) laboratories.

Alternative methods have been developed for specific applications. Choi et al. developed a paper-based array to screen the electricity-producing bacteria.[7] In another study, a growth chip with a porous aluminum oxide layer containing small cavities was used to culture and screen microorganisms. With a cavity size of 7 × 7 µm and up to one million cavities per chip, this method offers the capacity for very high-throughput screening

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although single cavities cannot be used to assess the effectiveness of antimicrobial substance.[8] Despite the advantages of these alternative techniques, the difficulties associated with production and high cost remain.

Recently, we introduced the droplet-microarray platform (DMA) with precisely separated superhydrophobic and hydrophilic areas.[9] By wetting the DMA with aqueous solutions, we can create an array of small (90 nL), spatially separated droplets. These micro-reservoirs contain sufficient liquid to provide an appropriate environment for the growth of eukaryotic cells and prevent cross-contamination, with the additional advantages of ease of handling and few pipetting steps. The DMA platform also facilitates the simultaneous analysis of a library of substances in parallel by sandwiching compounds printed glass slides with DMA slides.[10] Thus, the DMA platform represents a simple, rapid, and highly cost-effective method of screening the antibacterial effects of a variety of substances.

Here, we present the DMA platform as a novel and cost-effective technology for performing miniaturized high-throughput screening of bacteria to accelerate the detection of antibiotic-resistant microbes in samples from patients and environments. In this study, we used *P. aeruginosa* as a target strain since this opportunistic Gram-negative human facultative pathogenic bacterium is known to cause a plethora of hospital infections, including respiratory, urinary tract, and wound infections.[11,12] Moreover, this pathogen is well-known for its high intrinsic resistance against a variety of different antibiotics and disinfectants.[13] Therefore, due to the extensive use of antibiotics in hospitals, acquired multidrug-resistance among *P. aeruginosa* is a major concern.[14] Thus, in this study, we validated the DMA screening platform using clinically applied antibiotics to investigate the antibiotic-resistance of the multi-drug resistant *P. aeruginosa* 49 isolate.

## 2. Results and Discussion

### 2.1. Growth of *P. aeruginosa* PAO1 GFP on DMA

A schematic representation of bacterial seeding and proliferation on DMA slides is shown in **Figure 1**a. Aqueous solutions applied onto this slide spontaneously form an array of separated microdroplets due to the difference in wettability of the hydrophilic square and the superhydrophobic borders (Figure 1b; Table S1, Supporting Information). 1.5 mL droplet of bacterial suspension was placed onto the superhydrophobic-hydrophilic array for 30 s before the slide was tilted to form microdroplets containing bacteria. Each DMA slide (7.5 × 2.5 cm) contains three microarray pattern compartments containing 196 hydrophilic squares (Figure 1c). With one DMA slide, 588 droplets in few seconds were formed, where each droplet representing an individual compartment for subsequent antimicrobial testing.

The distributed volume of droplets on DMA slides was evaluated with a pattern size of 1 mm. Figure 1d shows a Gaussian distribution of the droplet volume, with the volumes of more than 80% of the droplets ranging from 70 nL to 130 nL. Based on this information, single droplets of 90 nL were used in the subsequent experiments. The distribution of the radius and

height of the droplets are shown in Figure S1, Supporting Information.

First, *P. aeruginosa* PAO1 expressing GFP (*P. aeruginosa* PAO1 GFP) was used to evaluate the growth of bacteria after seeding on DMA slides since the expression of this protein facilitates direct microscopic monitoring of bacterial persistence or growth. The distribution of initial bacteria number in each droplet after seeding is shown in Figure S2a, Supporting Information. There were 109 ± 54 bacteria in each droplet on average. DMA slides were placed in a humidity box, which was a sealed plastic box with a piece of wet tissue in it. Figure S2b, Supporting Information, shows that the high humidity in the box could prevent the evaporation of droplets on DMA slides. The mass of droplets on DMA slides placed in air was decreased from 0.066 ± 0.001 g to 0.001±0.001 g in 25 min at room temperature. While the mass of droplets on DMA slides placed in the humidity box was decreased from 0.069 ± 0.003 g to 0.060±0.003 g in 15 min and didn't change much in the next 2 h. The mass change of droplets incubated in the humidity box over 24 h at 37 °C was measured as well. It shows that more than 77% of the volume of droplets remained on the DMA after incubation. To investigate the effect of pattern size on bacterial growth, three hydrophilic square pattern sizes were applied to DMA slides. Bacteria on DMA slides with hydrophilic spots of 1 mm and 3 mm shows both bright green fluorescence after incubation for 24 h, which was visually comparable with the fluorescence of bacteria grown in 96-well plates (Figure 1e). Digital images of the bacterial spots were quantified for the fluorescence intensity using the software ImageJ. Here, the fluorescence intensity of all spots was normalized to the fluorescence intensity of bacteria grown in 96-well plates after 24 h incubation to investigate whether growth of bacteria would be affected in small volume. The fluorescence intensity of bacteria on DMA slides with hydrophilic spots of 0.5 mm was 0.35 ± 0.06 fluorescence units, which was much lower than the fluorescence intensity of the bacteria in 96-well plates. This result suggested that the small volumes of the 0.5 mm hydrophilic spots contained not enough cells of *P. aeruginosa* PAO1 for fluorescence signal evaluation. Therefore, the 1 mm spot pattern was used to form droplets on one DMA slide for further applications, rather than 3 mm pattern for DMA production. The density of bacteria on the DMA slide was $1.8 \times 10^9 \pm 0.9 \times 10^9$ CFU mL$^{-1}$, which was close to the density of bacteria ($2.0 \times 10^9 \pm 0.6 \times 10^9$ CFU mL$^{-1}$) incubated in 96-well plates (Figure 1g; Table S2, Supporting Information). Both fluorescence imaging and bacterial density results confirmed that the DMA slides with hydrophilic spots of 1 mm support the persistence and growth of bacteria in individual microdroplets.

### 2.2. DMA as a Screening Platform

Aiming at a single step screening approach, the sandwiching process was evaluated using nano-liter amounts of antibiotics being transferred into individual bacterial droplets. Antibiotics were preprinted onto a fluorinated glass slide with the I-DOT instrument and then accurately placed into contact with the bacterial droplets on DMA slides using the CellScreenChip (CSC) (**Figure 2**a,b; Figure S3, Supporting Information). Figure 2c–h
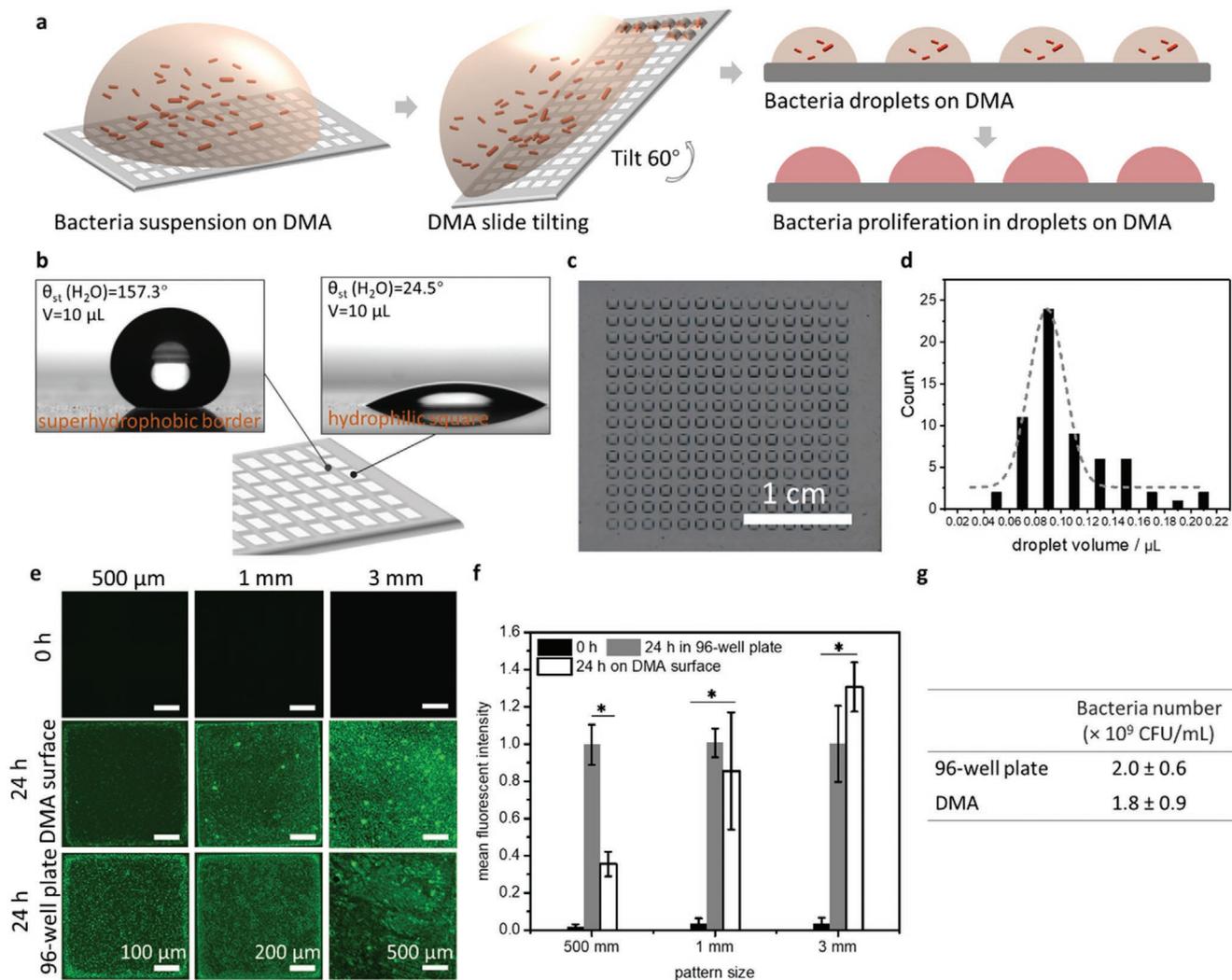
**Figure 1.** Growth of *P. aeruginosa* PAO1 GFP on DMA slides. a) Scheme of bacteria seeding on DMA. b) Photographs of droplets of water on the hydrophobic border (left) and hydrophilic square (right) of the DMA surface with the corresponding static water contact angle. c) Digital image of DMA after droplets of Basal Medium 2 (BM2) medium formed. d) Distribution of droplet volume on DMA slides. e) Fluorescence images of *P. aeruginosa* PAO1 GFP incubated for 24 h on the DMA slide and in a 96-well plate. 500 μm, 1 mm, and 3 mm are the edge length of the hydrophilic square. f) Growth of *P. aeruginosa* PAO1 GFP strain in 96-well plates and on DMA surfaces detected by measuring mean fluorescent intensity per pixel of cultured bacteria. All fluorescence intensity values were normalized against *P. aeruginosa* PAO1 GFP cultured for 24 h in 96-well plates. Data were presented as mean ± SD of three experiments with three repeats each time. *P*-values <0.05 were considered statistically significant. g) Bacterial density in 96-well plate and on DMA surfaces after incubation for 24 h. Data were presented as mean ± SD of three experiments with three repeats each time.

shows the results of the test using vancomycin at 13.5 μM (ineffective for inhibition of *P. aeruginosa* PAO1 growth) and ciprofloxacin at 40 μM (effective for inhibition of *P. aeruginosa* PAO1 growth) printed in on the DMA in a predesigned pattern.[15] Figure 2c confirms the absence of cross-contamination during the sandwiching process between the droplets containing ciprofloxacin (no strong green fluorescence) and the neighboring droplets containing vancomycin (bright green fluorescence). A scan of the fluorescence intensity of each droplet is shown in Figure 2d. Furthermore, we used this sandwiching method to stain the droplets with 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC), which is converted to the red fluorescent molecule CTC-formazan by metabolically active cells. A shown in Figure 2e,f, the bacteria showed bright red fluorescence in droplets

containing vancomycin, which was not observed in droplets containing ciprofloxacin being directed against the sensitive strain of *P. aeruginosa*. The growth of bacteria can also be visually evaluated, with droplets containing actively dividing bacteria appearing opaque after drying, while the droplets without high-density bacteria appear transparent (Figure 2g). We speculate that the difference in transparency is caused by the deposition of living bacteria and as well as the formation of a biofilm on the DMA surface since the printed vancomycin was not able to prevent the multiply of bacteria. Then the layer of bacteria reflects light leading to a brighter, opaquer surface (Figure 2g). This hypothesis was supported by the SEM images shown in Figure 2i,j. A layer of bacteria was observed on the white spots, while there was no such bacterial film on the transparent spots.
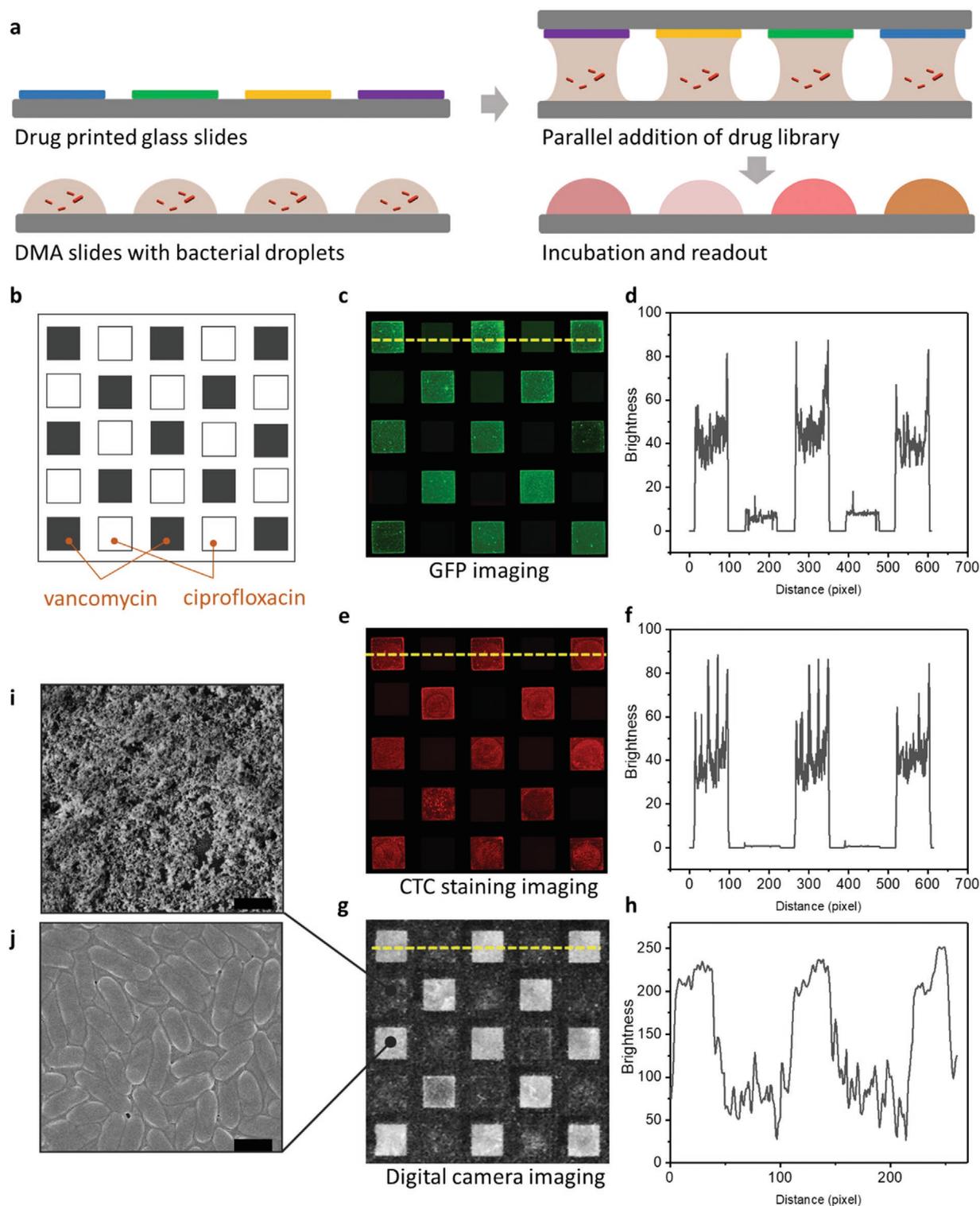
**Figure 2.** Droplet microarray as a screening platform. a) Scheme of the sandwiching process for screening antibiotics. b) Predesigned pattern of printed antibiotics on fluorinated glass slides. c) Image of green fluorescence of the bacteria on DMA with 25 (5 × 5) spots treated sequentially with vancomycin (13.5 µM) or ciprofloxacin (40 µM). d) Scan of fluorescence intensity across the yellow line shown in (c). e) Image of red fluorescence of active bacteria on DMA with 25 (5 × 5) spots treated sequentially with vancomycin (13.5 µM) or ciprofloxacin (40 µM), and stained with CTC using the sandwiching method. f) Scan of fluorescence intensity across the yellow line shown in (e). g) Digital image of DMA surface of the bacteria on DMA with 25 (5 × 5) spots treated sequentially with vancomycin (13.5 µM) or ciprofloxacin (40 µM)). The DMA slide was placed on black color paper. h) Grayscale scan of the yellow line shown in (g). i) SEM image of the transparent hydrophilic spot on DMA surface in (g). j) SEM image of opaque hydrophilic spots of DMA surface in (g).

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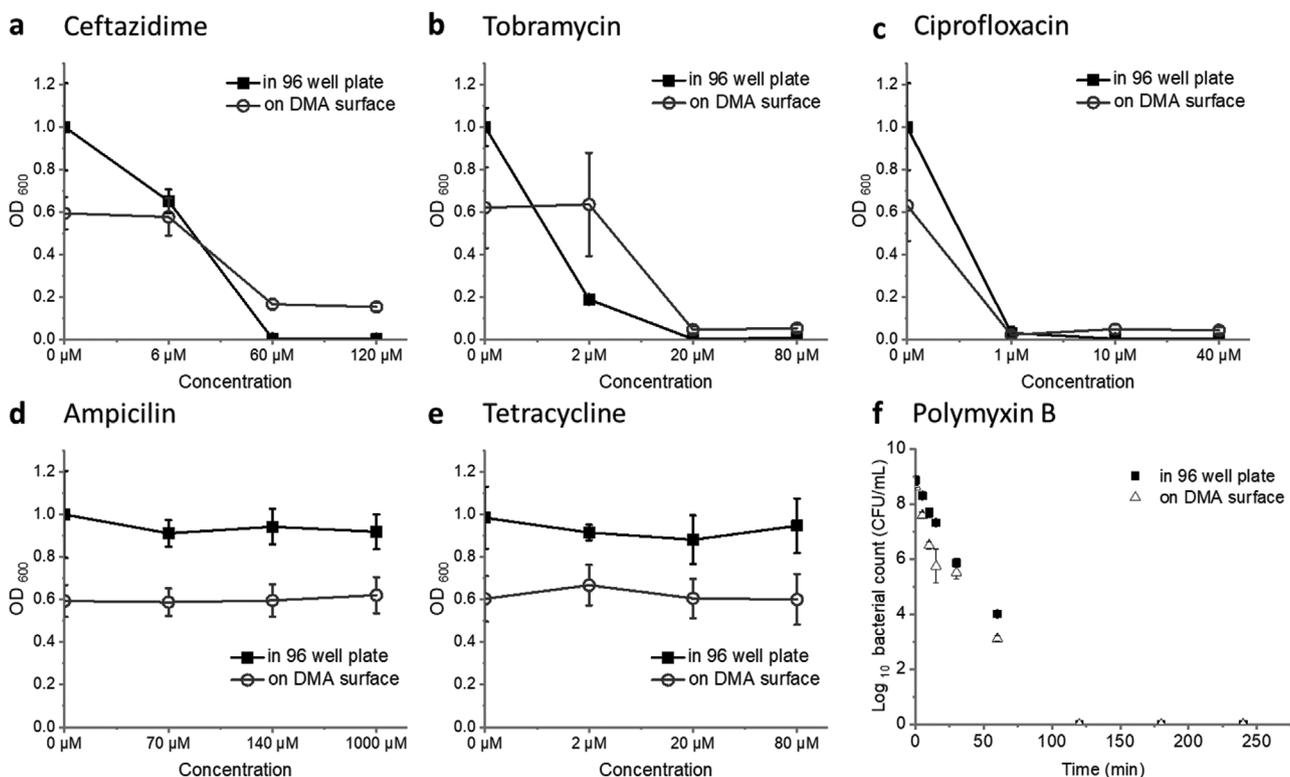
**Figure 3.** a–e) MIC of ciprofloxacin, ceftazidime, tobramycin, ampicillin and tetracycline for *P. aeruginosa* PAO1 GFP assayed in 96-well plates and on DMA surfaces (DMA slides: readout by fluorescence intensity and the intensities were converted into OD values (Figure S4, Supporting Information); 96-well plates: readout by OD measurement). All results were normalized to a blank control (0 μM in 96-well plates). Data were presented as mean ± SD of three experiments with 10 repeats each time. f) Time-course assay of the antibacterial activity of polymyxin B on *P. aeruginosa* PAO1 on DMA slides. Data were presented as mean ± SD of three experiments with 5 repeats each time.

This visually detectable readout of bacterial growth on DMA surfaces has the advantage over the other approaches that no expensive device is required.

As an antibiotic screening platform, the DMA should give comparable results to those obtained using a microtiter plate-based method. Hence, the MIC (Minimal Inhibition Concentration) of five antibiotics were investigated with *P. aeruginosa* PAO1 GFP assayed on DMA surfaces and in 96-well plates. The MIC is the lowest concentration of antimicrobial compounds that is able to inhibit the growth of bacteria in an overnight assay based on the determination of the $OD_{600}$ value. As shown in **Figure 3**, the MIC values of ceftazidime on DMA slides and in 96-well plates were both in the range of 6–60 μM. The MIC values of ciprofloxacin on DMA slides and in 96-well plates were both in the 0–1 μM range. The MIC value of tobramycin on DMA slides was in the 2–20 μM range, while the MIC was in the 0–2 μM range in 96-well plate, although values were consistent with the range of MIC values listed in EUCAST database (0–68 μM). Ampicillin and tetracycline were shown to be ineffective antibiotics for *P. aeruginosa* PAO1 in both the DMA slide and 96-well plate assays. The time dependence of the antimicrobial effect of polymyxin B was also investigated on DMA slides and in the 96-well plate. As shown in Figure 3f, the number of living bacteria was reduced by exposure to polymyxin B in a time-dependent manner inactivating all bacteria

on the DMA slides and in the 96-well plates in the first 2 h incubation. These observations confirm that the small volume of the droplet on a DMA slide does not influence the kinetics of the antibacterial effect of polymyxin B on *P. aeruginosa* PAO1.

### 2.3. Antibiotic Resistance Study of *P. aeruginosa* 49 on DMA Slides

As a new methodology, the DMA platform shows promising potential in facilitating and advancing antibiotic resistance studies of bacteria derived from patients or the environment. We investigated the ability of 18 antibiotics at two concentrations to inhibit growth of *P. aeruginosa* 49 on DMA slides and in a 96-well plate as a proof of principle to identify antibiotic resistance. *P. aeruginosa* 49 were isolated from clinical wastewater from the sewer close to the surgery department and from the clinical wastewater collection pipes Germany.[16] Berditsch et al. reported that *P. aeruginosa* 49 are resistant to gentamicin, ciprofloxacin, imipenem, ceftazidime, amikacin, azlocillin, and piperacillin-tazobactam with using disk diffusion assay.[17] Here, a number of 18 antibiotics have been chosen of various categories of antibiotic; include *β*-lactam antibiotic (cephalosporins, ceftazidime, imipenem, meropenem, amoxicillin, carbenicillin, ampicillin, methicillin), quinolone antibiotic
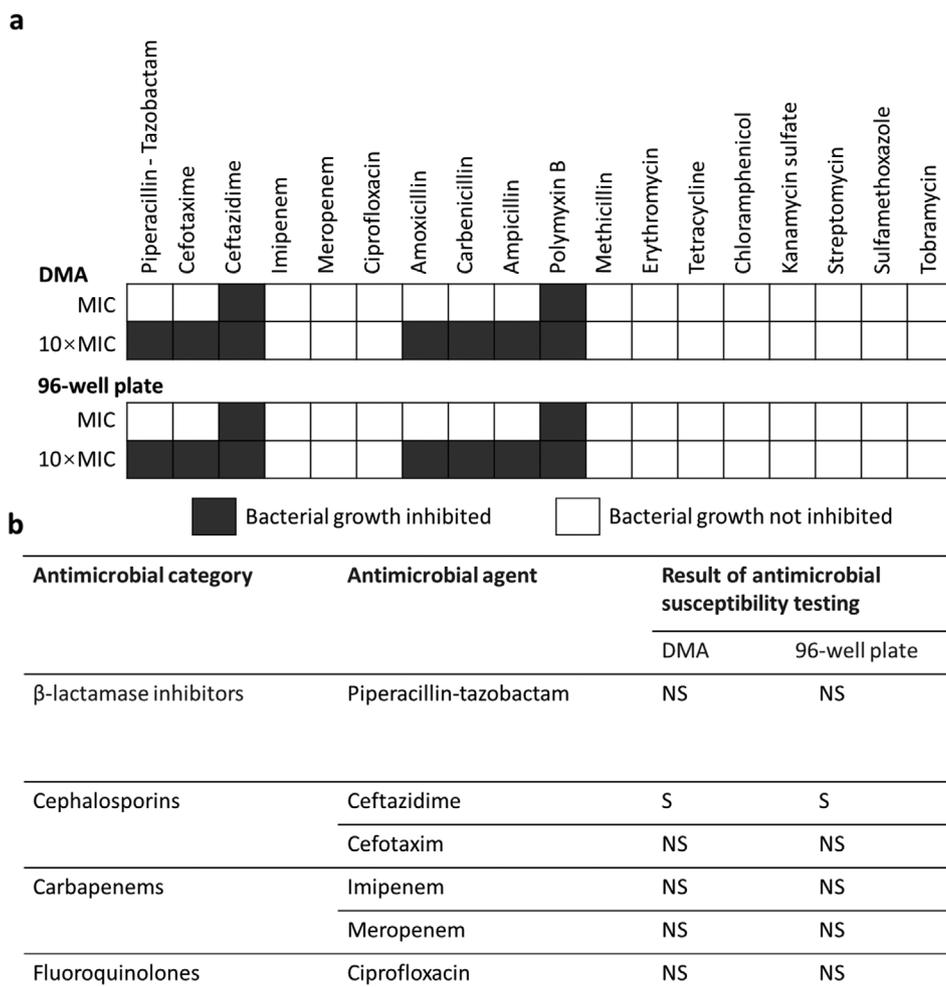
**Figure 4.** Screening result of antibiotic effectiveness against *P. aeruginosa* 49 on DMA surfaces and in 96-well plates. Two concentrations of antibiotics were tested. The MIC value of antibiotics was obtained from the EUCAST database. In 96-well plates, antibiotics were transferred into the bacterial suspension (100 µL per well) with pipetting. On DMA surfaces, antibiotics were transferred into droplets of bacterial solution using the sandwiching method. Initial bacterial density: $OD_{600} = 0.001$. The bacteria were incubated with antibiotics for 24 h at 37 °C. The antibiotic activity was evaluated by visual inspection of the transparency of the wells or droplets (opacity indicates live bacteria). Three experiments with 10 repeats (10 wells and 10 spots) of each concentration of antibiotics were performed. The antibiotic was defined as effective when there were ≥ 8 wells or spots were transparent. S is sensitive; NS is not sensitive.

(ciprofloxacin), antimicrobial peptides (polymyxin B), macrolide antibiotic (erythromycin), tetracycline antibiotics (tetracycline), aminoglycoside antibiotic (kanamycin sulfate, streptomycin, tobramycin), sulfonamides (sulfamethoxazole), chloramphenicol antibiotic (chloramphenicol), and combinations (piperacillin–tazobactam). We used the MIC concentration obtained from EUCAST database of *P. aeruginosa* as reference (Table S3, Supporting Information). We also tested 10-fold MIC concentrations to reveal the sensitivity of *P. aeruginosa* 49 to these antibiotics. The antibiotics were transferred into *P. aeruginosa* 49 droplets using the sandwiching method. After incubation for 24 h, the DMA slides were dried in air. Opaque spots (bacterial growth has not been inhibited) indicated the lack of antibiotic effectiveness, while transparent (bacterial growth has been inhibited) spots revealed that the antibiotic was effective. In 96-well plates, wells with high turbidity suggested the lack of antibiotic effectiveness, while low turbidity/transparent suggested that the antibiotic was effective. All the result was

readout visually. **Figure 4**a shows that, with the exception of ceftazidime and polymyxin B, *P. aeruginosa* 49 was not sensitive to the chosen MIC concentrations of antibiotics. However, piperacillin-tazobactam, cefotaxime, amoxicillin, carbenicillin, and ampicillin inhibited the growth of *P. aeruginosa* 49 at the high concentration (10 × MIC). According to the universal definition of drug-resistance, *Pseudomonas* bacteria are defined as multidrug-resistant bacteria if the strain is resistant to some of antimicrobial agents from the following four categories: penicillins + *β*-lactamase inhibitors, cephalosporins, carbapenems, and fluoroquinolones.[18] As shown in Figure 4b, *P. aeruginosa* 49 isolated from waste-water was identified as a multidrug-resistant bacterial strain. The sensitivity of *P. aeruginosa* 49 to polymyxin B and ceftazidime tested on DMA slides and in 96-well plates is shown in Figure S5, Supporting Information. The MIC value of the two antibiotics read from DMA slides and 96-well plates were the same, that is, 2 mg mL⁻¹ for ceftazidime and 4 mg mL⁻¹ for polymyxin B.

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## 3. Conclusion

A novel platform for culturing bacteria in spatially separated micro-reservoirs filled with a medium was established. This DMA platform can be used for screening the efficiency of clinically used antibiotics against bacterial pathogens. The advantages of the DMA platform are ease of handling, almost no pipetting steps in creating hundreds of micro-reservoirs, and parallel testing of chemical compounds in minute amounts for screening full drug libraries. This platform offers the ability to investigate drug-resistance of bacteria isolated from patients and the environment with minimal cost and effort. As a proof of principle, *P. aeruginosa* PAO1 as well as the multi-drug resistant *P. aeruginosa* PA49 isolate could be grown successfully on the DMA surfaces within 24 h. Here, the different categories of antibiotics were applied by sandwiching a fluorinated glass slide preprinted with the drugs to the DMA containing bacteria. The growth of the bacterial culture on DMA slides can be visualized by microscopy using a GFP expressing strain PAO1::GFP or applying a staining method. Furthermore, bacterial growth can be detected and evaluated by visual examination of the turbidity/transparency of the hydrophilic spots. In parallel and as a control, the obtained DMA screening results were comparable to those using a conventional 96-well plate assay against a multi-drug resistant *P. aeruginosa* strain.

In further studies, the DMA platform will be used to identify potential natural or synthetic drug candidates for the treatment of bacterial infections. In extension, this DMA platform opens the opportunity to study the synergetic effects of combinatorial drug treatment.

## 4. Experimental Section

*Materials and Instruments*: Patterned superhydrophobic-hydrophilic glass slides (7.5 × 2.5 cm) were obtained from Aquarray GmbH (Eggenstein–Leopoldshafen, Germany). Each slide had three compartments, each containing 196 (14 × 14) square-shaped hydrophilic spots (1 × 1 mm). The distance between hydrophilic spots was 500 µm. Ethanol, potassium phosphate, $(NH_4)_2SO_4$, $MgSO_4$, $FeSO_4$, NaOH, HCl, and glucose were from Merck (Darmstadt, Germany). Müller–Hinton (MH) medium was purchased from Merck (Darmstadt, Germany). Glass slides (Nexterion Glass B) were purchased from Schott (Jena, Germany). (1H, 1H, 2H, 2H-perfluorooctyl) silane was purchased from Sigma-Aldrich (Munich, Germany). CTC was purchased from Polysciences Europe GmbH (Hirschberg an der Bergstrasse, Germany). Ciprofloxacin was purchased from Fluka (Seelze, Germany). Ceftazidime and tazobactam were purchased from ACROS ORGANICS (Geel, Belgium). Tobramycin, cefotaxime, amoxicillin, ampicillin, polymyxin B, methicillin, erythromycin, kanamycin sulfate, sulfamethoxazole, and tetracycline were purchased from Sigma-Aldrich (Munich, Germany). Piperacillin was purchased from Alfa Aesar (Kandel, Germany). Imipenem and meropenem were purchased from Cayman Chemical Company (Michigan, USA). Chlorampenicol was purchased from AppliChem (Darmstadt, Germany). Streptomycin was purchased from Carl Roth (Karlsruhe, Germany).

The AxioImage M2 system equipped with an Apotome (Carl Zeiss, Oberkochen, Germany) was used for fluorescence microscopy. A DSA 25 contact angle goniometer (Krüss, Germany) was used for water contact angle measurement. The I-DOT non-contact liquid dispenser was purchased from Dispendix (Stuttgart, Germany).

*Bacterial Strain, Medium Preparation, and Culture Conditions*: *Pseudomonas aeruginosa strain* PAO1 was used as a screening target in this study.[19] This strain was tagged by introducing plasmid *pUCP20::GFP* by electroporation, resulting in the production of the green fluorescent protein (GFP) to facilitate monitoring of *P. aeruginosa* PAO1 (designated *P. aeruginosa* PAO1 GFP) by fluorescence microscopy.[20] *P. aeruginosa* PAO1 GFP was routinely grown in Müller–Hinton (MH) broth medium overnight at 37°C. The bacterial suspension was adjusted to $OD_{600}$ = 0.1 with minimal medium Basal Medium 2 (BM2) and then diluted 1:100 with BM2 medium to obtain a bacterial suspension of $10^6$ colony forming units CFU mL$^{-1}$. *Pseudomonas aeruginosa strain* PA49 (designated *P. aeruginosa* PA49) was cultured in BM2 medium overnight at 37°C.[16] The bacterial suspension was adjusted to $OD_{600}$ = 0.1 with BM2 and then diluted 1:100 with BM2 medium to obtain a bacterial suspension of $10^6$ CFU mL$^{-1}$.

*Seeding and Culture of Bacteria on the DMA Slide*: 1.5 mL of solution was added to one of the three compartments of squares on the DMA slide ensuring that all 196 spots were covered. The droplet was left to stand for 30 s to allow the bacteria to settle. The slide was then quickly tilted and the droplets formed spontaneously as the liquid flowed away. A non-contact liquid dispenser can be used to significantly reduce the amount of bacterial sample, provided the dead-volume of a corresponding dispenser is low.

For incubating bacterial cells, the DMA slide was placed inside a Petri dish within a box with wetted tissues that was closed to prevent evaporation. The box was placed in an incubator at 37°C and the bacteria were cultured for the required period of time.

To calculate the volume of droplets on the DMA, droplets on DMA slides were prepared first. The height (H), and radius (r) of droplets were measured with a DSA 25 contact angle goniometer (Krüss, Hamburg, Germany). The volume of the droplets was then calculated based on the assumption that the droplets formed part of a spherical cap.

To enumerate the bacteria on DMA slides, one of the three compartments in the DMA slide, which contained 196 bacterial droplets on its surface, was immersed into 20 mL BM2 medium and vortexed for 60 s. The suspension was then serially diluted with cell wash buffer and 10 µL of the dilutions were seeded on Luria broth (LB) agar plates. After incubation for 24 h at 37°C, the colony number on LB agar plates was recorded used to estimate the number of bacteria on the DMA slide. Details of the estimation are shown in the supporting information.

*Printing of Antibiotics onto Fluorinated Glass Slides*: Glass slides were cleaned by immersion in 1 M NaOH solution for 1 h, washed with water for 30 s, and then immersed in 1 M HCl for 30 min. After washing with water for 30 s, the cleaned glass slides were fluorinated by incubation overnight with 30 µL trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane in a pressurized (50 mbar) desiccator. Antibiotics were printed onto the slides using the I-Dot non-contact liquid dispenser. The antibiotics ceftazidime, tobramycin, ampicillin, vancomycin, tetracycline, piperacillin, erythromycin, amoxicillin, and carbenicillin were dissolved in DMSO (2 mg mL$^{-1}$) whereas ciprofloxacin, polymyxin B, imipenem, and meropenem were dissolved in sterile water (2 mg mL$^{-1}$). Further dilutions were performed with sterile water to obtain the appropriate amount of antibiotics per square with a printable volume ranging between 5 nL and 100 nL. After printing with antibiotics, the DMA slides were dried in air to remove traces of DMSO.

*Sandwiching DMA with Preprinted Antibiotics*: To expose the bacteria to antibiotics, an antibiotic preprinted slide was sandwiched with the DMA slide using the CellScreenChip (CSC, as described in Figure S3, Supporting Information). This novel instrument allows the precise alignment of two glass slides while controlling the distance between them. The DMA slide and the antibiotic printed slide were clamped into the lower and upper frames of the CSC, respectively. The distance between the two frames was controlled by four micro-screws, fixed at a specific height. The CSC was closed and aligned by four pillars located at the corners of the lower frame that are positioned to align with four reference holes in the upper frame. In this way, the bacteria-containing droplets on the DMA slide were placed in contact with the antibiotic imprinted slide without excess

pressure. Since the antibiotics were printed in a specific pattern correlating to the DMA slide, the mirror image of the printed pattern was observed on the DMA slide after sandwiching. Sandwiching was carried immediately after the bacteria were seeded and to prevent evaporation, the sandwiched slides were placed in a humidified box during the stamping process. All experiments were conducted at 37°C with a stamping time of 20 min.

*Bacteria Staining*: Using the I-Dot non-contact liquid dispenser, CTC solution (4 mм freshly prepared in medium) was printed onto a fluorinated glass slide (90 nL per spot). The CTC-stained slides were dried overnight and then exposed to bacteria using the same method used to transfer antibiotics; the stamping time was 10 min. After the addition of CTC, DMA slides loaded with bacteria-containing droplets were incubated for 3 h at 37°C.[21]

*Imaging and Analyzing Growth of Bacteria*: Before imaging, the DMA slide was dried for 10 min in the dark at room temperature to allow the bacteria to accumulate in a layer on the surface. Images of *P. aeruginosa* PAO1 GFP and CTC-stained *P. aeruginosa* PAO1 GFP were obtained manually with the Zeiss Axio Imager 2 microscope. To compare the fluorescence from bacteria in droplets on DMA slides and in 96-well plates, the bacteria suspension was transferred from the 96-well plate onto DMA slides to form droplets. After drying, squares on DMA slides were imaged.

ImageJ was used for image analysis. The mean fluorescent intensity of hydrophilic squares (the whole hydrophilic square) was measured. The mean intensity per pixel of the background was subtracted from this value to calculate the mean intensity produced by the GFP synthesized inside the bacteria. The background was detected on the superhydrophobic border within a square of $100 \times 100$ pixels.

*Time-Kill Assay of Antibiotic on PAO1 on DMA Surface*: To investigate the kinetics of antibiotic activity on DMA slides, suspensions of *P. aeruginosa* PAO1 (initial bacterial density $OD_{600} = 1$, $10^9$ CFU mL$^{-1}$, BM2 medium) with polymyxin B (40 µg mL$^{-1}$) were incubated for a predetermined time (5, 10, 15, 30, 60, 120, 180, and 240 min) in a 96-well plate (100 mL) and on a DMA slide (90 nL per droplet). To enumerate the bacteria on DMA slides, one of the three compartments in the DMA slide, which contained 196 bacterial droplets on its surface, was immersed into 20 mL BM2 medium and vortexed for 60 s. The suspension was then serially diluted with cell wash buffer and 10 µL of the dilutions were seeded on Luria–Bertani broth (LB) agar plates. After incubation for 24 h at 37°C, the colony number on LB agar plates was recorded used to estimate the number of bacteria on the DMA slide. The number of bacteria per well in the 96-well plate was estimated in the same way following the culture of 17.6 µL of bacteria suspension.

*Screening of Antibiotics on DMA Surfaces with Multi-Drug Resistant Strain P. aeruginosa PA49*: Antibiotics (Table S3, Supporting Information) were printed onto fluorinated glass slides using the I-Dot. The amount printed was calculated according to the MIC and the droplet volume (90 nL for 1 mm squares). *P. aeruginosa* PA49 suspension ($10^6$ CFU mL$^{-1}$) was seeded onto DMA slides, which were then sandwiched with the antibiotic printed glass slides using the CSC instrument. The two surfaces were sandwiched at 37 °C for 20 min before the antibiotic printed glass slide was removed and the DMA surface was incubated at 37 °C for 24 h. The same screen was performed in a 96-well plate, with antibiotics added directly into bacterial solution (100 µL) to obtain the same concentration as that of the bacterial droplets on the DMA surface. The solutions were then incubated at 37°C for 24 h. After incubation, the DMA surface was dried in air for 10 min.

*Statistical Analysis*: All data were represented as mean ± SD of n ≥ 3 individual repetitions for each experiment. Experiments were at least repeated three times. The statistical significance of the experimental data was determined with a two-tailed Student t-test (*p* value < 0.05). The fitting curve of distribution of the volume of droplets in Figure 1d was plotted in OriginPro using "nonlinear curve fit" function: Gaussian model. Scan of fluorescence intensity in Figure 2d,f,h was measured with the "Plot Profile" function of ImageJ.

## Supporting Information

## Acknowledgements

## Conflict of Interest

The authors declare no conflict of interest.

## Keywords

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