3D Microfluidic Concentration Gradient Generator for Combination Antimicrobial Susceptibility Testing

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ABSTRACT

Microfluidic concentration gradient generators (μ-CGGs) have been utilized to identify optimal drug compositions through antimicrobial susceptibility testing (AST) for the treatment of antimicrobial-resistant (AMR) infections. Conventional μ-CGGs fabricated via photolithography-based micromachining processes, however, are fundamentally limited to two-dimensional fluidic routing, such that only two distinct antimicrobial drugs can be tested at once. This work addresses this limitation by employing Multijet-3D printed microchannel networks capable of fluidic routing in three dimensions to generate symmetric multi-drug concentration gradients. The three-fluid gradient generation characteristics of the fabricated 3D μ-CGG prototype were quantified through both theoretical simulations and experimental validations. Furthermore, the antimicrobial effects of three highly clinically-relevant classes of antibiotic drugs, tetracycline, ciprofloxacin, and amikacin, were evaluated via experimental single-antibiotic minimum inhibitory concentration (MIC), pair-wise and three-way antibiotic combination drug screening (CDS) studies against model antibiotic-resistant Escherichia coli bacteria. As such, this 3D μ-CGG platform has great potential to enable expedited combination AST screening for various biomedical and diagnostic applications.

Keywords: 3D printed, additive manufacturing, microfluidics, concentration gradient generator, minimum inhibitory concentration, combination drug screening, antibiotic, antimicrobial susceptibility testing
1 Introduction

2 Treatment of antimicrobial resistant (AMR) infections places a significant economic burden on the worldwide economy, upwards of $35 billion per year in the United States alone, and is projected to be the cause of over 10 million people per year by the year 2050\textsuperscript{1–3}. In the context of antibiotic resistance for instance, more than 18 distinct bacteria, including pathogenic Escherichia coli (E. coli), Listeria monocytogenes (L. monocytogenes) and Staphylococcus aureus (S. aureus), have developed biological resistance to one or more of the world’s essential first-line-of-defense antibiotic agents\textsuperscript{4,5}. The susceptibility of AMR-organisms to antimicrobial compounds is assessed in clinical and biomedical research settings through antimicrobial susceptibility testing (AST) methods, predominantly minimum inhibitory testing (MIC) and combination drug screening (CDS), which are particularly useful in the fight against antibiotic-resistant infections, such as urinary tract infections (UTIs)\textsuperscript{6}.

Conventional MIC testing\textsuperscript{7} (Supplementary Materials Fig. S1a) involves overnight incubation of a patient-collected bacteria sample in the presence of growth media and dilute antibiotic solutions\textsuperscript{8} in order to determine the lowest dose of a single antibiotic, known as the MIC value, required to inhibit the proliferation of bacteria and increase in bacteria colony density\textsuperscript{9,10}. The MIC value therefore represents the lowest recommended antibiotic dose effective in treating a particular infection without encouraging further antibiotic resistance\textsuperscript{11–13}. Furthermore, the only effective treatment against certain AMR infections is the use of combinations of multiple antimicrobial agents\textsuperscript{14–17}. Conventional antibiotic CDS (Supplementary Materials Fig. S1b) is performed in a similar manner to MIC testing, yet involves bacterial incubation in the presence of solutions containing specific ratios of different antibiotic compounds\textsuperscript{8,18} to ascertain the combined effects of the antibiotics; whereby, certain synergistic antibiotic combinations are more effective at inhibiting bacterial proliferation than either of the two antibiotics on their own, whilst different combinations are either antagonistic, less effective, or additive, exhibiting neither combined effect\textsuperscript{19,20}.

Conventional AST techniques, while well-established, generally require multiple independent manual labor-intensive fluidic handling procedures, involving a minimum of \textasciitilde16-24 hours for sample enrichment and dilution, followed by \textasciitilde24-72 hours for complete AST analysis\textsuperscript{8,21}. As a result, the duration from sample collection to delivery of definitive AST results in clinical settings can take anywhere from two days to one week\textsuperscript{10}. A standard clinical procedure whilst a clinician awaits AST evaluation, therefore, is to prescribe a large dose of a broad-spectrum antibiotic anyway to stop the infection from worsening, which often contributes to the very emergence and propagation of AMR in the first place\textsuperscript{22–24}. Moreover, whilst MIC values\textsuperscript{9,11} and CDS results\textsuperscript{16,20,25} for specific antibiotics and bacterial strains can be found in the literature, antibiotic sensitivity can evolve over the lifetime of a bacterial colony\textsuperscript{26}; therefore, frequent MIC and CDS testing is recommended in clinical settings\textsuperscript{10,27,28}, posing a considerable limitation on AST throughput and overall cost, for screening more than two antibiotics at one time, especially\textsuperscript{29,30}. 

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Various microfluidic-based AST platforms\textsuperscript{23,31} have demonstrated miniaturized and multiplexed fluid handling\textsuperscript{32,33} to increase the throughput of AST analysis\textsuperscript{29,34,35} and decrease the mortality rate and healthcare costs\textsuperscript{36,37} associated with treating clinical AMR-related infections\textsuperscript{38,39}, for novel drug development\textsuperscript{40,41}, for point-of-care clinical dosage recommendations\textsuperscript{42–45}. Microfluidic concentration gradient generators (\(\mu\)-CGG), the most widely-adopted class of microfluidic AST technologies\textsuperscript{46} for MIC and CDS studies, employ branching microchannel networks comprised of nodal units to produce diluted concentrations representing a gradient between input species. Discrete concentration gradients, for example one antibiotic and one buffer solution\textsuperscript{47,48}, are represented by the independent outputs from the \(\mu\)-CGG network itself\textsuperscript{49}, often referred to as discrete \(\mu\)-drug cocktails\textsuperscript{15}. Several discrete \(\mu\)-CGG devices have been demonstrated towards both single-antibiotic MIC testing and CDS studies\textsuperscript{29,50} of clinically-relevant antibiotics against laboratory-standard bacteria\textsuperscript{51}, bioengineered strains\textsuperscript{52} and cells isolated from biological fluid samples\textsuperscript{40,50,53}. The \(\mu\)-drug cocktail solutions are either used to perform on-chip bacteria culture\textsuperscript{54} or are collected as discrete \(\sim\mu\text{L}\) volume antibiotic solutions for use in off-chip bacteriological experiments\textsuperscript{39}. As a result, a typical duration for rapid AST analysis is on the order of \(~6\text{–}8\text{ hours}\)\textsuperscript{55}.

The application of \(\mu\)-CGG enabled devices towards AST involving more than two antimicrobial compounds, however, is fundamentally limited. Traditional MEMS-based microfluidic fabrication approaches are monolithic in nature; as a result, conventional microchannels have inherently two-dimensional geometric complexity and are therefore capable of fluidic routing in essentially only two dimensions\textsuperscript{56}. Symmetric fluidic gradients, those capturing all possible combinations of the inputs, are limited to only two distinct fluidic species at a given time (Fig. 1a). Previous \(\mu\)-CGG’s have demonstrated handling of more than two input fluids, however such gradients do not produce any combinations of non-adjacent fluidic species and are non-symmetric (Fig. 1b). Alternative manufacturing approaches, including the tedious and error-prone manual alignment and bonding of PDMS layers, have been demonstrated towards generating quazi-3D microfluidic structures, however numerous limitations of such processes limit the 3D geometric complexity and practical functionality of such CGG designs\textsuperscript{57,58}.

Given the ever-advancing capabilities, cost reduction and widespread commercial availability of high resolution (\(\leq 100\mu\text{m}\)) 3D printing technology, additive manufacturing has garnered significant interest recently towards various microfluidic applications\textsuperscript{59}; yet, previously demonstrated 3D printed microfluidic devices have fairly limited applicability towards AST applications, and in particular none have demonstrated the generation of discrete gradients of more than two antibiotics for AST\textsuperscript{50–62}. Since conventional \(\mu\)-CGG devices are therefore limited to producing \(\mu\)-drug cocktails that capture the greatest range of possible combinations of only two antibiotics simultaneously, \(\mu\)-CGG enabled CDS of three or more antibiotics demonstrates significantly lower throughput and is fundamentally limited\textsuperscript{29,63}. 

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This paper reports the design and development of a truly 3D \( \mu \)-CGG prototype, employing a unique 3D microchannel network which is only possible to fabricate using an additive manufacturing-based approach. The only way to accomplish a symmetric gradient of three or more fluids is to perform fluidic routing in truly three dimensions (Fig. 1c). A tetrahedrally-arranged network of nodal microchannel units, geometrically symmetric in 3D space and capable of generating inherently symmetric three fluid gradients, is modeled and fabricated. The concentration gradient generation characteristics of the 3D \( \mu \)-CGG are first theoretically simulated and used to optimize the design through the use of different integrated 3D microfluidic mixing (\( \mu \)-mixer) structures to best elucidate the analytically predicted behavior, then the performance of the fabricated prototype are experimentally validated through fluorescence imaging. Finally, antibiotic gradients collected from the device are used to demonstrate its proof-of-concept utility as an AST tool towards MIC testing, pair-wise and three-antibiotic CDS bacteriological experiments for three clinically relevant antibiotics against antibiotic-resistant \( \textit{E. coli} \) bacteria.

Materials and Methods

Design of the 3D microchannel network

All microfluidic designs were modeled using Solidworks computer aided design software (\textit{Dassault Systemes}, Velizy-Villacoublay, France). All inlet and outlet geometries enabled device-to-world interfacing via standard 20 gauge stainless steel catheter couples (SC20/15, \textit{Instech}, PA, USA). All microchannels, bulbs and solid bodies were saved as individual part files, then arranged in a single assembly file to produce the positive-feature network (Fig. 2a) for use in all theoretical simulations. Subtraction of the model with a solid body was used to produce the final manufacturable design (Fig. 2b), saved as a single part file, exported as an .STL file and imported to 3D printing software for manufacturing.

Prototype fabrication and post-processing

Additive manufacturing of the prototype was accomplished using a Projet 3000UHD Multi-jet modeling 3D printer (\textit{3D Systems}, SC, USA) (Fig. 2c). The materials employed in this work were Visijet M3 crystal polymer \(^{64} \textit{(3D Systems)}\) and Visijet S100 hydroxylated wax \(^{65} \textit{(3D Systems)}\). Both materials were deposited simultaneously in an inkjet-like process in roughly 35\( \mu \)m thick layers with a lateral feature resolution as low as 50\( \mu \)m\(^{66} \). The support material, which was necessary to reinforce and successfully resolve overhanging geometries such as the 3D \( \mu \)-mixer rifling and hollow spherical cavities, was removed from the device after 3D printing using a previously demonstrated post-processing protocol\(^{67,68} \). Briefly, the prototype was placed inside a pre-heated oven at 75°C for 15 minutes with the outlets facing downwards on top of paper towels, in order to facilitated drainage of support material from the microchannels through capillary action. The device was then submerged in beaker...
containing food-grade Bayes mineral oil pre-heated to roughly 60°C for roughly 10 minutes. The heated mineral oil was flushed through each device outlet thrice using a 10mL syringe (Cole-Palmer, IL, USA) attached to a 20-gauge Luer stub (model LS20, Instech) until all of the interior support material was removed. Finally, the process was repeated using a soapy water solution and potable water to remove any residual mineral oil (Fig. 2d). For further details see Supplementary Material Sec. S2.3.

Theoretical simulations

Computation fluid dynamics (CFD) simulations were performed using COMSOL Multiphysics (Version 4.5a, COMSOL, Inc., CA, USA) finite element analysis software to determine the theoretical performance of all 3D models. The theoretically predicted normalized concentration \( N \) of the species from one inlet \( (C_1) \) at each outlet for a range of input fluid flow rates was calculated as the average \( N \) value over the entire microchannel outlet face (Fig. 3). Due to the 3D symmetry of all simulated microchannel networks, the \( N \) values for \( C_1 \) assigned to device Inlet #1 was identical to the results if \( C_1 \) was assigned to Inlets #2 & #3. Therefore in this work, only \( C_1 \) assigned as the Inlet #1 input was simulated. For further details see Supplementary Material Sec. S4.

Preparation of cell solutions

Ampicillin-resistant BL21(DE3) gram-negative Escherichia coli (E. coli) bacteria was procured from Agilent Technologies, CA, USA in 2 mL cryovials as glycerol stock and stored at -80°C. A scraping from the glycerol stock was added to 10 mL of Lysogeny Broth (LB media), a bacteria-specific nutrient-rich solution, in a 25 mL T25 flask with a breathable filter cap (#169900, ThermoFisher Scientific, MA, USA), and enriched in a bacteria incubator at 37 °C and 4% CO\(_2\) overnight. The following day, \(~10\mu\text{L}\) was transferred to agar media to create a solid bacteria colony plate\(^{10}\), which was stored at -4 °C for use up to one month. Before each day of bacteriological experiments, a fresh bacterial inoculation was created by adding a single bacterial colony harvested from the agar plate to 10 mL of LB media and incubating overnight at 37°C. The cell density of the solution was measured the next day using a UV-VIS Spectrophotometer (Vernier, OR, USA) following the OD600 method\(^{8,70}\), then a serial dilution was performed in order to create an inoculation with an initial cell density of \(~5*10^5\) cfu/mL, following cell viability\(^{71-73}\) and AST\(^{38,50,52,74,75}\) conventions. LB media was prepared by dissolving LB powder (LB Miller Broth #L3522, Sigma-Aldrich, MO, USA) in de-ionized (DI) water to make a 25g/L solution in a 1000mL Pyrex autoclave bottle (ThermoFiser-Scientific), followed by autoclave sterilization at 120 °C for 25 minutes. For further details see Supplementary Material Sec. S5.2.
**Preparation reagent and antibiotic solutions**

Whenever solids were added to liquids or multiple solutions were combined to create new solutions, sterile 50 mL conical polypropylene centrifuge tubes (#339652, ThermoFisher Scientific) were used, aggravated using a vortex mixer (Vortex-Genixe 2, Scientific Industries, NY, USA) for roughly 30 seconds to ensure a homogeneous solution, then wrapped in aluminum foil and stored at -4 °C for up to one week of use. Rhodamine fluorescent dye solution was created by adding 50 drops of 0.04% Fluorescent Red Dye Rhodamine B Solution (OnlineScienceMall) to 50 mL of DI water. Resazurin metabolic indicator solution was prepared to a concentration of ~4.4 mM by adding ~5 mg of resazurin salt powder (Sigma-Aldrich) to 50 mL of sterile LB media. Antibiotic solutions were prepared by combining the required mass of antibiotic powder (each acquired from Sigma-Aldrich) to 500 mL of sterile LB media to create the desired antibiotic stock solution concentration, roughly twice the approximate average of published MIC values for a specific antibiotic against *E. coli*. For all concentrations of antibiotic stock solutions used throughout this work, see Supplementary Material Fig. S12.

**Device operation and collection of output gradients**

Discrete fluidic outputs from the fabricated 3D µ-CGG prototype were collected using the experimental setup conceptually illustrated in Fig. 2e. Briefly, for each experiment, fluids from three independent fluidic reservoirs attached to a MAESFLO microfluidic control platform (Fluitgent, Paris, France) were pressure-driven through the three device inlets at a steady state volumetric flow rate of 1000 µL/min for one minute. The device output solutions, each contained in discrete segments of Tygon microbore tubing (#06420-03, Cole-Palmer, IL, USA) for which the length was cut to isolate the desired solution volume, were then routed to discrete wells on a standard 96 well plate (#07-200-720A, ThermoFisher Scientific). See Supplementary Material Sec. S6.1 for further details.

**Experimental fluid flow characterization**

The 3D µ-CGG device was used to generate gradients between one rhodamine fluorescent dye solution and two DI water inputs, the 90 µL output solutions collected on a 96 well plate. Briefly, using the fluorescence imaging setup (Fig. 2f), the normalized concentration of rhodamine in each solution, averaged over three experiments, was determined by imaging each solution under UV light excitation inside a custom-build light isolation box with a red bandpass optical filter (610-700nm, #W6308, Omega Optical, VT, USA) and DSLR camera (Canon EOS 1000D, Canon, Tokyo, Japan), as the excitation and emission energy peaks of rhodamine are 540 nm and 625 nm, respectively. Rhodamine fluorescence emission in each solution was quantified using the image analysis protocol detailed in Supplementary Material Sec. S6.3.
Bacteriological AST experiments

For each experiment, the 3D \(\mu\)-CGG device was used to generate gradients between antibiotic and buffer solutions. The output 30 \(\mu\)L \(\mu\)-drug cocktail solutions were collected on a 96 well plate, then 30 \(\mu\)L each of resazurin metabolic indicator solution and \(E.\ coli\) inoculation were pipetted into each well. A 0% antibiotic (fully uninhibited proliferation) control, consisting of 30 \(\mu\)L each of bacterial inoculation, resazurin solution and LB media, was pipetted into one well, and no-proliferation control, consisting of 30 \(\mu\)L of resazurin solution and 60 \(\mu\)L of LB media, was pipetted into another well. For further details see Supplementary Material S6.4. Each plate was first incubated at 37 °C (Figure 2f) until a visible gradient between blue and pink from the 0% antibiotic control across the device outputs was observed, for an average of \(~5\) hours. The normalized bacterial proliferation in each solution, averaged over three experiments, was determined by imaging each solution under excitation from green LED light\(^{74}\) using a 585 nm optical filter (#W6308, Omega Optical)\(^{77}\). The fluorescence emission of resorufin in each solution was quantified following the image analysis protocol detailed in Supplementary Material Sec. S6.4.

Results

3D \(\mu\)-CGG design

The 3D \(\mu\)-CGG microchannel network (Fig. 2a) features three fluidic input channels and thirteen discrete output channels. The manufacturable design, consisting of microchannels as hollow structures in a solid body, is shown in Fig. 2b. Briefly, the network is comprised of a truly symmetric 3D arrangement of tetrahedrally arranged nodal combination-mixing-splitting units (Fig. 2b,c, insets). Fluids enter each nodal unit through a vertical channel, here shown with a channel-penetrating 3D rifled microstructure (3D rifled \(\mu\)-mixer) integrated into the side-wall, into a hollow spherical bulb, then flow symmetrically through independent outlet microchannels to the next nodal units. The network features three distinct layers of vertical microchannels accomplishing truly symmetric fluidic routing in all three dimensions, generating equivalent proportions of the fluidic inputs to device Inlets #1&#2, #2&#3, #1&#3 and most critically #1,#2&#3 at discrete outlets, which is impossible to achieve with planar fluidic routing. Comprehensive analyses of pressure-driven CGG networks using electric circuit analogies were published in seminal reviews in Refs.\(^{49,78}\). Mathematical approaches traditionally used in the design of conventional 2D CGG’s\(^{48,79}\) were extended in this work to develop a nodal analysis methodology (Supplementary Material Sec. S1.5) which was used to analytically calculate the expected output flow rates \((Q_i)\) and concentration of each input fluidic species \((C_i)\) at each nodal unit (Fig. 2b, inset) and device outlet. The fabricated and post-processed (Fig. 2c) 3D \(\mu\)-CGG prototype is shown in Fig. 2d, along with the experimental setups used to collect (Fig. 2e), incubate and analyze (Fig. 2f) the device output solutions.
Simulated concentration gradient generation performance

One important assumption made during the analytical calculations of $Q_i$ and $C_i$ at each device outlet is that all fluids are completely mixed when they split off from a given nodal microchannel unit. The accuracy of the $Q_i$ and $C_i$ parameters of each fluidic output of the fabricated 3D $\mu$-CGG prototype to the analytically calculated values is therefore highly dependent on the microfluidic mixing efficiency achieved by each nodal unit. To enhance mixing quality inside each nodal unit, the effect of integrating intra-channel 3D $\mu$-mixer structures into the side-wall of each vertical microchannel between the upper bulbs (where fluids combine) and the lower bulbs (where the fluids split off from) was investigated by studying three different 3D $\mu$-CGG designs incorporating: (i) smooth-walled vertical channels, serving as a reference, and imbedded (ii) 3D bulbous and (iii) 3D rifled microstructures, both based on a previously demonstrated 3D printed $\mu$-mixers to induce chaotic advective fluid motions for enhanced mixing efficiency. The analytically calculated normalized concentrations ($N_c$) of a single input fluidic species ($C_1$) at each outlet of a conceptual device (Fig. 3a) were independent of input flow rate into the device. The theoretically predicted $N_c$ values from the COMSOL simulation results for all 3D $\mu$-CGG models over a range of symmetric input fluid flow rates from 0 to 4000 $\mu$L/min (Fig. 3b-d) become independent of input flow rate for all devices around $\sim$1000 $\mu$L/min; therefore only the $N_c$ values at $\sim$1000 $\mu$L/min were selected for comparison between devices. Additionally, all analytical and theoretical results, and the percent errors between the two, are tabulated in Table 1.

The reference $\mu$-CGG design (Fig. 3b) exhibited the highest percent errors in theoretical $N_c$ at device Outlets #2 & #3 and Outlets #6 & #8, averages of $\sim$24% and $\sim$57% error, respectively, due to the inefficient microfluidic mixing inside the smooth-walled vertical microchannels. Furthermore, the 3D bulbous $\mu$-mixer integrated $\mu$-CGG design (Fig. 3c) exhibited a comparable degree of inaccuracy at Outlets #2 & #3 and Outlets #6 & #8, averages of $\sim$26% & $\sim$60%, respectively; likely the result of still incomplete fluidic mixing inside these particular 3D microstructure designs. The 3D rifled $\mu$-mixer integrated $\mu$-CGG design (Fig. 3d) on the other hand demonstrated the most accurate results, as the $N_c$ values at all outlets are within 10% of the analytically calculated values. A conventionally-accepted metric for the acceptable error of CGG output concentrations for AST applications is a maximum of 10%\textsuperscript{39,49}. From this study, the 3D rifled $\mu$-mixer integrated $\mu$-CGG model was deemed capable of generating accurate ($\leq$10% error) output concentrations representing practically useful proportions of each input fluidic species, i.e. $\sim$1, $\sim\frac{7}{10}$, $\sim\frac{5}{10}$, $\sim\frac{3}{10}$ & $\sim$0, in addition to an output capturing a nearly equivalent proportion of all three input fluidic species, i.e. $\sim\frac{1}{3}$, and was therefore chosen as the most appropriate design for for prototype fabrication, experimental characterization and bacteriological demonstrations.
**Experimental fluid flow characterization**

The experimental characteristics of the fabricated 3D µ-CGG prototype were assessed by using the device to generate a gradient between one rhodamine fluorescent dye solution and two DI water fluidic inputs. Three experiments were performed with the rhodamine solution input into one of the device inlets, i.e., Inlet #1, then the process was repeated with the rhodamine solution input into each of the other two inlets, i.e., Inlets #2 & #3, respectively (corresponding to the inlet labeling convention shown in Fig. 2a). With the device outputs collected on a 96 well plate, fluorescence imaging was used to measure the fluorescence emission of rhodamine in each solution in order to ascertain the distribution of rhodamine solution from the device Inlet of interest at every output of the device.

With rhodamine solution used as the input to Inlet #1 (Fig. 4a), the mean experimental rhodamine \( N_c \) values of all device output solutions were within 10% of the theoretically predicted values from the COMSOL simulation results, exhibiting an average standard deviation of \(~4.8\%\). Likewise, the distributions of rhodamine solution from Inlets #2 (Fig. 4b) & #3 (Fig. 4c) amongst the device outlets produced \( N_c \) values within 10% of the theoretically predicted values as well, exhibiting average standard deviations of \(~3.9\%\) & \(~3.1\%\), respectively. For further discussion see Supplementary Material Sec. S.6.3. Finally, the experimental \( N_c \) values from each experiment were used to quantify the concentration of each of the three fluidic input species contained in every device output solution, in terms of a percentage of the concentration of each input stock solution, as presented in Supplementary Material Fig. S11. Regarding practical utility for three-fluid studies, the prototype demonstrated the ability to simultaneously generate three distinct gradients between only two of the three input fluidic species, produced at the outlets along each side of the bottom of the device (e.g., Outlets #1, #3, #5, #8, #13 capture a gradient between fluidic inputs to Inlets #1 and #3, without contamination from that from Inlet #2). Furthermore, 100% of each fluidic input was produced at Outlets #1, #9 & #13; \(~50\%\) each of only two fluidic inputs were produced at Outlets #4, #5 & #11; and an approximately equivalent proportion (average of \(~34\%)\) of each of the three fluidic inputs was produced at from Outlet #7.

**Bacteriological AST demonstrations**

The ability of the developed 3D µ-CGG prototype to serve as a microfluidic platform for AST applications was demonstrated through proof-of-concept bacteriological experiments evaluating the antimicrobial efficacy of tetracycline, ciprofloxacin and amikacin, three different clinically relevant antibiotic compounds commonly used to combat AMR-related infections⁹ (Supplementary Material Sec. S6.4.1), against an ampicillin-resistant strain of \( E. \) coli bacteria used as a demonstrative model. Briefly, all bacteriological experiments involved generation of a gradient between antibiotic stock solutions and growth media.
using the fabricated µ-CGG prototype; the addition of metabolic indicator solution and *E. coli* inoculation to each discrete
device output µ-drug cocktail solution collected on a 96 well plate; followed by incubation and detection. Resazurin salt
(further discussed in Supplementary Material Sec. S5.1) was employed in these studies as a redox indicator of biological
metabolism. Resazurin (dark blue in appearance) is readily metabolized to produce a bright pink molecule, resorufin, whereby
the rate of resorufin production in solution is proportional to the rate of respiration of viable cells. Fluorescence microscopy is
a well-suited method to detect resorufin production\(^{81}\), as resofurin is highly fluorescent (peak \(\lambda_{ex} = 579\) nm, \(\lambda_{em} = 584\) nm),
whereas resazurin is weakly fluorescent. Resazurin-based cell viability protocols have proven simple, accurate and reproducible
methods to quantify assess the metabolic activity of organisms, particularly bacteria\(^{38,74,82}\). In this work, normalized emission
of resorufin was measured and used to produce a Normalized Growth (\(N_g\)) value elucidating the degree of bacterial proliferation,
and thereby antibiotic-induced bacterial growth inhibition, in each solution\(^{83,84}\).

**Single-antibiotic MIC testing**

Experimental single-antibiotic MIC testing results are presented in Fig. 5. For each antibiotic, triplicate experiments were
performed with the antibiotic solution into device Inlet #1 and growth media solutions into Inlets #2 & #3. Mean \(N_g\) values
for solutions containing µ-drug cocktails from 100% antibiotic to 100% buffer are plotted from outlets along one edge of the
device, i.e., Outlets #1, #3, #5, #7, #8 & #13. The MIC value of the antibiotic was determined with the first apparent step-wise
reduction in mean \(N_g\) value, i.e., enhancement in growth inhibition. The diagram in Supplementary Material Fig. 11a was then
used to calculate the MIC value in mg/L. All experimentally determined MIC values, as well as MIC values from the literature,
are presented in Supplementary Material Fig. S12.

Tetracycline has been one of the safest and most effective antibiotics used to treat serious conditions such as syphilis,
cholera, malaria and the plague, and is also useful in multi-drug treatments for AMR-related infections, such as bacterial
peptic ulcers\(^{20,85}\). The tetracycline-buffer-buffer gradient (Fig. 5a) results indicated that the MIC value was represented by
the µ-drug cocktail from Outlet #5. It exhibited normalized growth of \(\sim 20\%\) corresponding to a tetracycline concentration
of \(\sim 0.26\) mg/L, which is near the range of for similar strains of *E. coli* in prior works\(^{9,86}\). The µ-drug cocktails containing
higher concentrations of tetracycline than that from Outlet #5 showed equivalent or lower amounts of growth, whereas those
containing lower concentrations of tetracycline showed higher amounts of growth, between \(\sim 60\%-100\%\).
Ciprofloxacin is commonly used antibiotic to combat UTI’s, respiratory infections and gastroenteritis, and is often effective in combination with other antibiotics to treat AMR-related infections in CDS applications\textsuperscript{16,25,30,87}. The experimental ciprofloxacin-buffer-buffer gradient (Fig. 5b) results indicated that the MIC value corresponded to the \( \mu \)-drug cocktail from Outlet #5. It exhibited normalized growth of \( \sim 5\% \), corresponding to a ciprofloxacin concentration of \( \sim 50 \mu g/L \), which is in agreement with the documented range for similar known multi-drug AMR strains of \( E. coli \)\textsuperscript{86,88}. Solutions containing higher concentrations of tetracycline showed roughly the same amount of growth, whereas those containing lower concentrations of ciprofloxacin show increased amounts of growth, \( \geq \sim 50\% \).

Amikacin is a particularly effective antibiotic in combination treatments used to combat infections such as serious UTI’s, tuberculosis and bacterial meningitis, and AST evaluation of amikacin is frequently performed in clinical and drug development settings\textsuperscript{16,88–91}. Results from the amikacin-buffer-buffer gradient (Fig. 5c) study indicated that the MIC value was represented by the \( \mu \)-drug cocktail from Outlet #8. It exhibited normalized growth of \( \sim 30\% \), corresponding to a ciprofloxacin concentration of \( \sim 11 \text{mg/L} \), which agrees well in the documented range of amikacin MIC values for similar strains of \( E. coli \)\textsuperscript{86,91,92}. Solutions containing higher concentrations of amikacin showed equivalent or lesser growth, whereas the solution from Outlet #13 containing no antibiotic showed the highest amount of growth.

**Pair-wise antibiotic CDS studies**

The fabricated 3D \( \mu \)-CGG prototype was also proposed for use as an AST tool to perform pair-wise CDS studies. Triplicate experiments were performed with two different antibiotic solutions into device Inlets #1 & #3 and growth media solution into Inlet #2. Mean \( N_g \) values for solutions representing the gradient between 100\% of both antibiotics collected from Outlets #1, #3, #5, #7, #8 & #13 are plotted. The combined effects of each antibiotic pair follow similar trends from prior AST studies with related bacterial strains\textsuperscript{16,25,45,93,94}. The specific ampicillin-resistant \( E. coli \) used in this work were experimentally assessed by comparing the \( N_g \) values of each solution as shown in pair-wise antibiotic CDS results in Fig. 6.

The gradient between tetracycline and ciprofloxacin (Fig. 6a) exhibited the expected antagonism\textsuperscript{25} in the solution from Outlet #3 (\( \sim 0.34 \text{mg/L} \) tetracycline, \( \sim 28.8 \mu g/L \) ciprofloxacin). This specific chemistry would therefore be avoided in treating an infection caused by this specific strain of bacteria. Furthermore, the gradient between amikacin and ciprofloxacin (Fig. 6b) demonstrated the expected synergism\textsuperscript{16,45} in the solutions from Outlets #5 (\( \sim 7.68 \text{mg/L} \) amikacin, \( \sim 48.8 \mu g/L \) ciprofloxacin) & #8 (\( \sim 6.08 \text{mg/L} \) amikacin, \( \sim 65.3 \mu g/L \) ciprofloxacin); these specific chemistries would therefore be highly recommended in treating such an infection. Moreover, the gradient between tetracycline and amikacin (Fig. 6c) revealed the expected additivism\textsuperscript{93,94} in all solutions, from Outlets #3, #5, #7 & #8, therefore none of these solutions would serve as inherently...
beneficial treatments. All other solutions not explicitly mentioned demonstrated additive effects and therefore none of these chemistries would be recommended as effective treatments.

**Three-antibiotic CDS assessment**

Finally, the ultimate capability for AST evaluation of more than two antimicrobial agents was demonstrated using all three antibiotic solutions. Here, each single operation of the device generated three distinct pair-wise gradients, between amikacin and tetracycline (Outlets #1, #3, #5, #8 & #13); amikacin and ciprofloxacin (Outlets #1, #2, #4, #6 & #9); and ciprofloxacin and tetracycline (Outlets #9, #10, #11, #12 & #13), in addition to one three-antibiotic µ-drug cocktail chemistry, simultaneously.

The mean N₂ data from the three-antibiotic CDS experiments (Fig. 6d) revealed the expected additivism between amikacin and tetracycline (Outlets #3 & #5); synergism between amikacin and ciprofloxacin (Outlets #2, #4 & #6); and antagonism between ciprofloxacin and tetracycline (Outlets #10, #11 & #12). Furthermore, an unexpected synergistic pair-wise interaction was exhibited between amikacin and tetracycline from Outlet #8. As a result, this specific chemistry would be recommended in treating an infection caused by the ampicillin-resistant *E. coli* strain as demonstrated in this work. Moreover, the µ-drug cocktail containing a nearly equivalent proportion of all three input antibiotic compound concentrations (Outlet #7) demonstrated synergism as compared to the 100% solutions of amikacin and ciprofloxacin, and additivism as compared to the 100% solutions of amikacin and tetracycline, as well as ciprofloxacin and tetracycline. In this demonstration, the µ-drug cocktails from Outlets #2, #4, #5, #8 & #13 exhibited more effective inhibition of bacterial proliferation than those of the three-antibiotic µ-drug cocktail, and would therefore represent more effective dosages for treatment. Regardless, the three-antibiotic µ-drug cocktail is the direct result of the unique 3D fluidic routing capability demonstrated by the 3D µ-CGG microchannel network which is otherwise impossible using planar fluidic routing processes.
Conclusions

The throughput of multi-drug AST applications using conventional $\mu$-CGG devices is fundamentally limited by the inability of such systems to generate symmetric concentration gradients of more than two antimicrobial solutions at a time. In this work, we developed a 3D $\mu$-CGG prototype employing a truly 3D microchannel network through the use of an additive manufacturing approach to accomplish fluidic routing in three-dimensions in order to generate symmetric three-fluid concentration gradients. Analytical modeling and theoretical simulations were used to design and optimize the microchannel network via inclusion of embedded 3D $\mu$-mixing structures in order to produce thirteen distinct output $\mu$-drug cocktail solutions for bacteriological studies. Experimental characterizations validated the generated concentrations to within 10% of the predicted values, justifying the use of the proposed 3D $\mu$-CGG system for multi-drug AST evaluations.

As a proof-of-concept, the fabricated prototype was used evaluate the efficacy of three clinically relevant antibiotic compounds against model antibiotic-resistant *E. coil* bacteria. The MIC values of the individual antibiotics were characterized to be in agreement with the documented ranges for each compound. Furthermore, the known synergistic, additive and antagonistic effects of each combination of antibiotics were experimentally observed through individual pair-wise CDS studies. As such, the unique 3D fluidic routing capabilities enabled a three-antibiotic CDS study to simultaneously generate three distinct pair-wise antibiotic concentration gradients including one $\mu$-drug cocktail containing all three antibiotic species in 5 hours from a single operation for enhanced throughput over conventional multi-antimicrobial CDS approaches.

Moving forward, additive manufacturing permits straightforward and on-demand modification of 3D $\mu$-CGG designs to produce tailored concentration gradient characteristics. Such devices can be rapidly prototyped and fabricated in clinical point-of-care settings using commercially available 3D printers to reduce the time-to-deployment and manufacturing costs. Ongoing technological advances in additive manufacturing resolution, material variety and scalability will enable engineering of evermore advanced 3D $\mu$-CGG designs that can be incorporated into more complex micro-total analytical systems to significantly increase the throughput of AST to combat emerging antibiotic-resistant bacterial infections in clinical and drug development settings.
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Author contributions statement

E.S. and J.C. conceived and modeled the designs; E.S., B.Y. and J.C. designed the experiments; E.S., B.Y., J.C., R.V., Y.L., A.L., E.J., T.W., C.M., S.L. and A.C. performed the experiments; E.S., R.V., Y.L., E.J., T.W., R.J. and Y.A. analyzed the data; E.S. wrote the manuscript; L.L. supervised the project and edited the manuscript.

Additional information

The authors declare no competing interests.
Figure 1. Conceptual microfluidic concentration gradient generators (µ-CGG’s). (a) Conventional two input planar devices (e.g. PDMS-based systems fabricated via soft-lithography) generate symmetric gradients between both fluids, capturing all possible combinations of both species. (b) Limited to 2D fluidic processing due to the two-dimensionality of monolithic microchannel networks, such devices are unable to generate symmetric fluidic gradients between three or more inputs (i.e. no combinations of fluid inputs 1&3 are produced). (c) Only a truly 3D microchannel network capable of 3D fluidic routing, impossible to achieve using planar microfluidic fabrication methods, can generate of symmetric 3D gradients of three or more input fluids (i.e. 1&2; 2&3; 1&3; and 1,2&3).
Figure 2. Developed 3D $\mu$-CGG prototype and experimental setup. (a) 3D microchannel network design, dimensions and indication of labeling convention of device Inlets #1, #2 & #3; positive solids model of all hollow structures comprising the truly symmetric 3D arrangement of nodal tetrahedral units (blue inset). (b) Reverse solids model, representing the manufacturable 3D microfluidic design comprised of a single solid body with imbedded hollow microchannel structures; (red inset) flow rates ($Q_{in}, Q_{out}$) and input species concentrations ($C_{in}, C_{out}$) into and out of each nodal unit, variables used in all analytical device output calculations; fluid inputs indicated by colored arrows. (c) Concept of (top) 3D printing fabrication and (bottom) post-processing method to remove internal support material. (d) Fabrication results, actual 3D $\mu$-CGG prototype after post-processing; hollow interior structures are visible through the semi-translucent structural material, with US quarter for scale. Conceptual illustrations of the experimental setups to (e) collect the antibiotic gradient outputs from the fabricated 3D $\mu$-CGG device and to (f) perform biological incubation and fluorescence imaging to quantify bacterial proliferation.
Figure 3. Theoretical performance of various 3D µ-CGG designs from COMSOL CFD simulations. (a) Analytical calculations for all 3D microchannel network designs are independent of fluidic flow rate, outlet numbering convention shown in right, bottom. Theoretical results for (b) a design incorporating smooth-walled vertical channels, (c) a design incorporating vertical channels with repeating hollow spherical sidewall indentations, and (d) a design incorporating vertical channels with imbedded 3D rifling in the sidewalls. Plots (left) of normalized concentration of a single input fluidic species from one of three device inlets at all 13 device outlets versus input fluid flow rate from 0 to 4000 µL/min, highest percent differences between analytical and simulated results are indicated in red, results at ~1000 µL/min are also tabulated in Table 1; visualizations (right) of the concentration distribution on all surfaces of the positive solids model of the 3D microchannel network. The 3D rifled µ-mixer integrated design in (d) exhibits the lowest error (within 10%) at all outlets and is the design chosen for prototyping, experimental characterization and demonstration.
Table 1. Theoretical results from COMSOL CDF simulations, normalized concentration of a single input fluidic species from one of three device inlets at all 13 device outlets (row 1) at input flow rate of 1000 µL/min. (Row 2) Analytical calculations and theoretical results (top), with percent error from analytical values (bottom), for the (row 3) 3D rifled µ-mixer, (row 4) 3D bulbous µ-mixer and (row 5) smooth-walled vertical channel integrated 3D µ-CGG designs. Outlets with percent error higher and lower than 10% are indicated in red and green, respectively.

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Figure 4. Experimental flow verification results validating concentration of each fluidic input species at each device outlet. Normalized concentration of rhodamine fluorescent tracer dye input into (a) Inlet #1, (b) Inlet #2 and (c) Inlet #3 (DI water input into the other two device inlets). (Left) Isometric view of microchannel network and device input species, and bottom-up view of the microchannel network outlets displaying device outlet numbering scheme, outlets containing to 100% of each input species are indicated. (Right) Plot of mean normalized rhodamine concentration (green) with theoretical COMSOL simulation results (yellow) for each outlet solution, error bars signify standard deviation between triplicate experiments. Experimental results at all outlets are within 10% of the theoretically simulated values.
Figure 5. Experimental single-antibiotic MIC testing bacteriological results, antibiotic solution input into device Inlet #1 and growth media input into Inlets #2 & #3 for all experiments. (a) Tetracycline, (b) Ciprofloxacin and (c) Amikacin antibiotic MIC experiments, performed in triplicate. (Top) Mean normalized growth values and standard deviation error bars of fluid outputs capturing a gradient from 100% antibiotic to 100% buffer along one edge of the device, data representing the MIC value indicated by colored bars and calculated concentration. (Bottom) Illustration of the inputs into the microchannel network, device outlet numbering convention and indication of the outlets containing 100% antibiotic and 100% buffer solutions.
Figure 6. Experimental CDS bacteriological results revealing inhibitory interactions between all combinations of antibiotics, experiments performed in triplicate. Pair-wise interaction studies with antibiotics input into Inlets #1 & #3 and growth media input into Inlet #2: (a) Tetracycline+Ciprofloxacin, antagonism (red arrow); (b) Amikacin+Ciprofloxacin, synergism (green arrow); and (c) Tetracycline+Amikacin, additivism (grey arrow). (Top) Mean normalized growth values and standard deviation error bars of fluid outputs capturing a gradient between 100% of each antibiotic, data representing known antibiotic interaction indicated by colored arrows. (Bottom) Illustrations antibiotic inputs, device outlet numbering convention and indication of outlets containing 100% of each antibiotic and buffer. (d) Three-antibiotic interaction study with antibiotics input into Inlets #1, #2 & #3; (left) mean normalized growth values and standard deviation error bars of all thirteen fluid outputs, data representing known antibiotic interactions indicated by colored arrows; (right) illustrations of the antibiotic inputs, device outlet numbering convention and indication of outlets containing 100% of each antibiotic.