3D PRINTED THREE-FLOW MICROFLUIDIC CONCENTRATION GRADIENT GENERATOR FOR CLINICAL E. COLI-ANTIBIOTIC DRUG SCREENING

Eric C. Sweet, Joshua C.-L. Chen, Ilbey Karakurt, Alison T. Long, and Liwei Lin*
Berkeley Sensors and Actuators Center, University of California, Berkeley, USA

ABSTRACT

This paper presents a *symmetric three-flow* microfluidic concentration gradient generator, made possible by a 3D microchannel network fabricated *via* ultra-high resolution Multijet 3D printing. The prototype fluidic device is utilized as an effective tool for the expedited screening of multi-drug combinations. It rapidly generates 15 discrete combinations of three input drugs which are used to determine the minimum inhibitory concentration (MIC) value of the three individual antibiotics, as well as to perform simultaneous clinical three-way antibiotic interaction studies on ampicillin-resistant *Escherichia coli* (E. coli) bacteria. The results from individual antibiotic experiments are used to determine the discrete MIC values, and the multi-drug study reveals clinically relevant antagonistic, synergistic and suppressive interactions between the three different families of antibiotics commonly used to combat antibiotic resistant infections. Furthermore, a simple and effective method of visual interpretation of the experimental results is employed, demonstrating this device's use as an effective multi-drug screening platform for applications in point-of-care biomedical research and clinical medicine.

INTRODUCTION

Conventional Microfluidic Gradient Generators

Microfluidic concentration gradients are widely used in various biomedical testing applications [1], for example for the high-throughput screening of antimicrobial drugs against various pathogens [2,3]. Certain pairwise antibiotic combinations are known to inhibit the growth of single-antibiotic resistant bacteria, and the combinations of three or more antibiotics are becoming increasingly clinically important for drug combination therapies (Figure 1) [3].

However, conventional 2D microfluidic devices fabricated *via* soft lithography techniques are limited by their tedious fabrication process and planar structure that and can only produce a symmetric concentration gradient between two inputs. This has ramifications in the field of microfluidic drug screening, as combinations of only two drugs can be tested at once, and critical combinations of three or more drugs cannot be practically elucidated using conventional devices. To overcome this limitation, here we employ an alternative design methodology and additive manufacturing approach [4] to fabricate a 3D microfluidic concentration gradient generator that symmetrically combines three fluids and produces 15 discrete outputs.

Determination of Antibiotic Minimum Inhibitory Concentration (MIC) Value

Minimum inhibitory concentration (MIC), defined as the lowest concentration of an antibiotic that is necessary to inhibit the growth of a specific bacteria, is a highly clinically relevant value that guides how physicians prescribe treatment and is considered the gold standard for testing the susceptibility of microorganisms to antimicrobials. MIC is not the minimum bactericidal concentration, however, the lowest concentration of antimicrobial that will kill the pathogen. Current MIC techniques such as broth dilution and agar plate methods involve manual pipetting and fluid handling, which is both manual labor and time intensive. Microfluidic devices are currently used to streamline this process, by minimizing required reagent volumes; however, due to fundamental limitations of 2D lithographic processes used to fabricate these devices, it is difficult, if not impossible, to study the effects of more than two antibiotics simultaneously [2, 7].

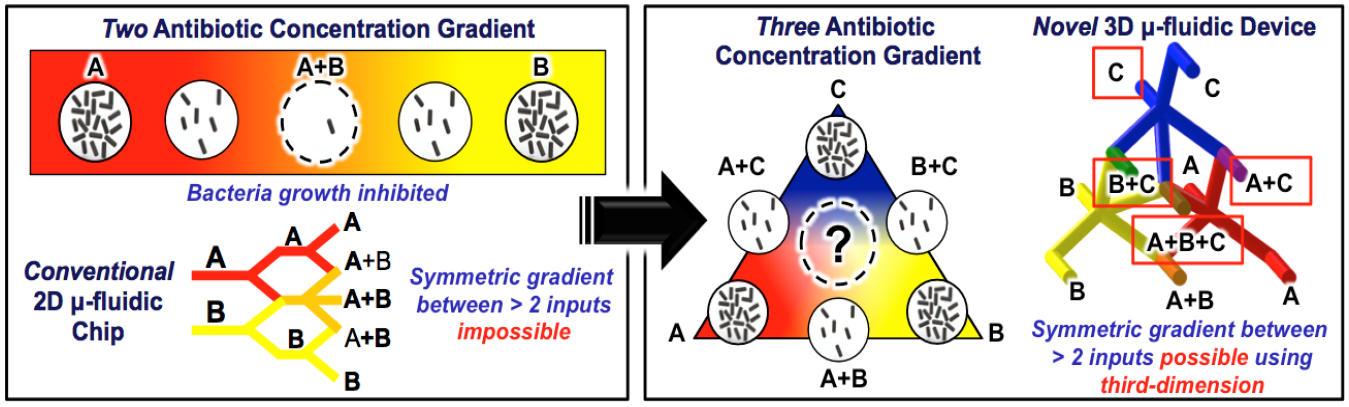

Figure 1: Concept of a three antibiotic concentration gradient and the 3D printed three-fluid gradient generator. (Left) Certain combinations of two antibiotics known to better inhibit bacterial growth than individual drugs. Conventional 2D microfluidic chips can only produce symmetric gradient between two inputs. (Right) Combinations of three antibiotics are clinically useful, novel 3D device produces symmetric gradient of three antibiotics.

Importance of Multiple Drug Interactions

Combination drug therapy, the simultaneous use of multiple growth-inhibiting drugs to treat bacterial and viral infections, is a promising method in the fight against antimicrobial resistance of specific pathogens such as HIV and tuberculosis. The issue with antimicrobial resistance is further exacerbated by the fact that only a few antibiotics have been discovered in the past few decades. In many clinical cases, multidrug resistant bacteria, such as tuberculosis, can be successfully treated with the correct combination of drugs [5]. However, rapid clinical screening of the effects of antimicrobial combinations on specific bacteria cultures must be performed in order to elucidate any drug resistance or toxic adverse effects due to patient variability and in order for the treatment to be successful.

When multiple drugs are combined into a "drug cocktail" and administered to kill a particular microbe, their combined effectiveness is compared to that of either of the two drugs on their own: the combination can either be more effective (synergistic), requiring the use of a lesser volume of drugs; less effective (suppressive), requiring the use of a greater volume of drugs; or no more or less effective (antagonistic). Remaining unanswered fundamental questions regarding specific biochemical mechanisms of multi-drug interactions necessitates the comprehensive clinical screening of drug combinations on a case-by-case basis given a particular pathogenic culture [6].

FABRICATION AND DEVICE

The actual 3D printed microfluidic device (Figure 2) is designed in SolidWorks solids modeling software and fabricated using ultra-high resolution Multijet 3D printing (3D Systems Projet 3000HD). The 3D printer simultaneously deposits micro-droplets of a UV-photocurable sacrificial wax support material and the structural polymer (Visijet EX200 resin), which enables the fabrication of 3D enclosed microchannels and internal geometries of high complexity. The sacrificial wax is removed from the device interior using a simple procedure involving flow with heated mineral oil [4]. This process is used to fabricate a 3D network of horizontal and vertical thin-walled microchannels.

Figure 2: Actual 3D printed device, US dime for scale.

The microchannels are 750 µm in diameter and are interconnected by hollow bulbs that mix (aided by chaotic adjective mixing) two or three flows at each junction. These junctions are arranged to form two main layers of horizontal microchannels interconnected by vertical microchannels, a 3D arrangement that can only be achieved *via* additive manufacturing. The network generates various concentration gradients between the three input species and produces 15 distinct outputs. Droplets from each of the outlets are collected via external tubing and routed into a 3D printed 15-well triangular droplet collector (Figure 3a) for visual analysis.

RESULTS AND DISCUSSION

Verification of Flow Characteristics

A COMSOL Multiphysics simulation was run on the CAD model to predict the theoretical concentration of each species from one of the inlets at every outlet; subsequently, the actual generated gradient was verified with an experiment using one fluorescein and two lysogeny broth (LB) solutions as the inputs and UV fluorescence imaging of the triangular droplet collector (Figure 3a). The predicted and experimental results follow a similar trend as illustrated in Figure 3b. The normalized fluorescence intensity per-well was calculated using ImageJ, which is indicative of the concentration of any of the species present per-outlet. The percentage values in Figure 3b were multiplied by the loading concentration in order to determine the actual concentrations of antibiotic in each well in all experiments.

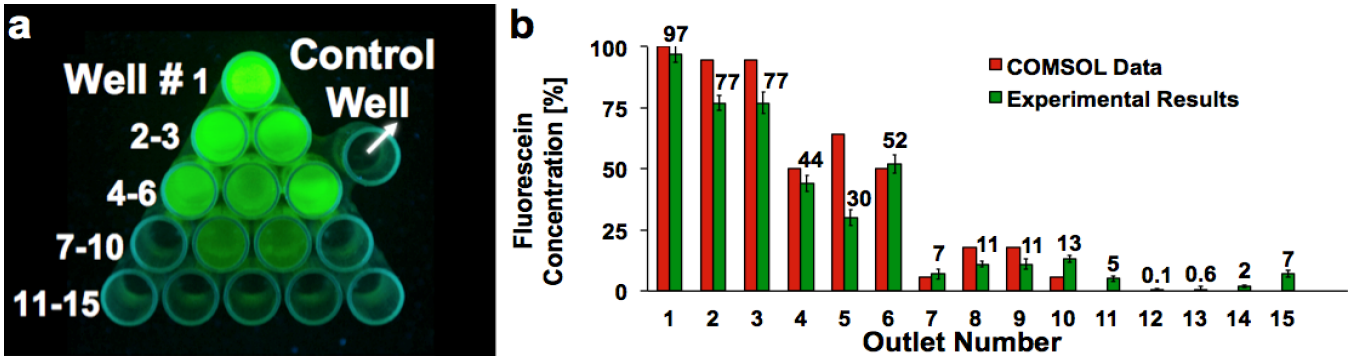

Figure 3: Experimental validation of flow characteristics via *fluorescein intensity analysis, fluorescein in well #1, LB media in wells #11&15. (a) Image of triangular droplet collection wells showing generated fluorescein gradient. (b) Fluorescein concentration per-well, values from COMSOL simulation and experimental fluorescence imaging.*

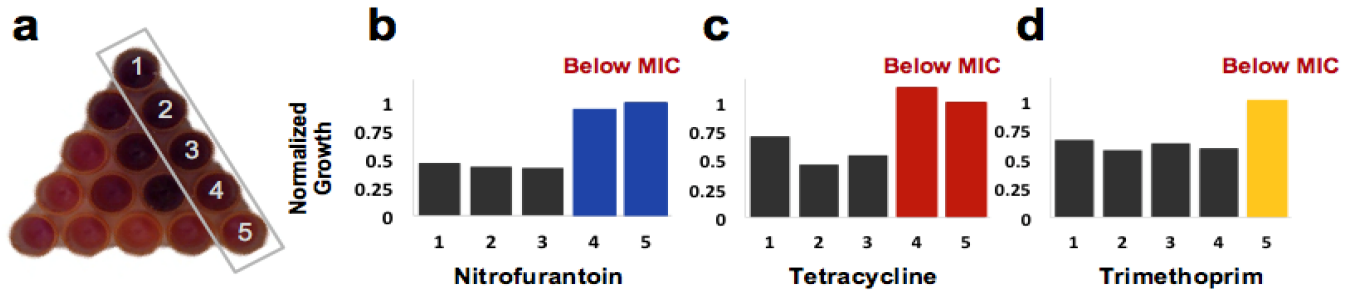

Figure 4: Individual antibiotic MIC trials. (a) Colorimetric image of trial with trimethoprim and LB solutions. Normalized bacterial growth to determine the MIC of (b) trimethoprim, (c) tetracycline and (d) nitrofurantoin where colored bar demonstrates bacterial proliferation when the concentration is below the MIC.

Determination of Individual Antibiotic Minimum Inhibitory Concentration (MIC) Values

We chose to analyze three commonly clinically used antibiotics, each with a unique mechanism of action: trimethoprim, nitrofurantoin, and tetracycline. Each of these antibiotics has well documented MIC values and clinical efficacy against antibiotic resistant E. coli.

Individual MIC determination trials were performed by loading one antibiotic and two lysogeny broth (LB) solutions into each of the three inputs to the device. Then resazurin sodium salt powder (44 µM concentration, 60 µL volume) and 60 µL of ampicillin-resistant K12 E. coli (~$2*10^8$ cells/mL) were added into the wells. The cell density of the bacteria culture was determined using a UV spectrophotometer to read the OD600 absorbance measurement, and an online converter [7] used to determine cell density. The doubling time for E. coli is approximately 20 to 30 minutes [8]. Thus, after the antibiotics, resazurin and bacteria were loaded into each well, we incubated the cultures for 1 hour at 37°C to encompass roughly two growth cycles.

As the E. coli bacteria proliferate and metabolize nutrients in the LB media, they metabolize the resazurin salt, which is dark blue in color, into resorufin, a mitochondrial metabolic indicator that is red/pink in color. After incubation, the color change in each well is directly dependent on the cellular growth of the bacteria at different drug cocktail concentrations. As a result, the color change was quantified to determine the degree of inhibition of cellular growth in each well.

Figure 4a shows the visual results of the MIC trial using trimethoprim. A colorimetric image of each triangular droplet collector was analyzed using ImageJ image analysis software, whereby the surface average of the red intensity value of each well was normalized to that of the control well, which had the maximum value, and then compared to one another in order to quantify relative cellular growth. To determine the MIC value, we looked for a binary output of growth versus no growth. When bacterial proliferation occurs, the red value is roughly on the same order of magnitude as the control, whereas the red values in wells that show bacterial growth inhibition are significantly lower than the control. The quantity of normalized growth (from normalized red intensity values) for each well is graphed for individual MIC trial: nitrofurantoin in Figure 4b; tetracycline in Figure 4c; and trimethoprim in Figure 4d.

Six-times each literature-derived antibiotic MIC value was loaded into one fluid input for each experimental condition. An equal volume of LB and E. coli solution were subsequently added to each well to give an effective loading concentration of two times the MIC value. Thus, the actual loading concentrations of antibiotic were 0.485 mg/L trimethoprim, 3.88 mg/L tetracycline, and 15.52 mg/L nitrofurantoin. These concentrations were optimized to deliver antibiotic concentrations above and below the literature MIC values. The experimental MIC values for each antibiotic trial are tabulated in Table 1. MIC values for nitrofurantoin and tetracycline showed a high correlation to the literature values, within a 5% difference. The MIC value for trimethoprim, however, varied significantly from the literature value. This may suggest a lower MIC value for trimethoprim than that reported in literature, or may be due to bacterial mutations or aberrations with the trimethoprim stock solution itself. Nevertheless, concrete MIC values were calculated for each of the three antibiotics individually, validating the gradient generator's use for clinically relevant antibiotic screening.

Table 1: MIC values, literature vs. experimental

MIC Value	Nitro.	Tetra.	Trimeth.
Literature [9]	8 mg/L	2 mg/L	0.25 mg/L
Experimental	8.32 mg/L	2.08 mg/L	0.065 mg/L
% Difference	4%	4%	74%

Study of Multi-Drug Interactions

We next explored combinatorial antibiotic interactions using trimethoprim, tetracycline and nitrofurantoin as the three fluid inputs for the microfluidic concentration generator. Figure 5a shows the experimental image of this three-way trial. A similar protocol was employed for the three-way interaction studies and individual MIC experiments, with equal volumes of antibiotic, E. coli, and resazurin added to each well. Studies have shown that nitrofurantoin and tetracycline are antagonistic, tetracycline and trimethoprim are suppressive, and trimethoprim and nitrofurantoin are synergistic [6].

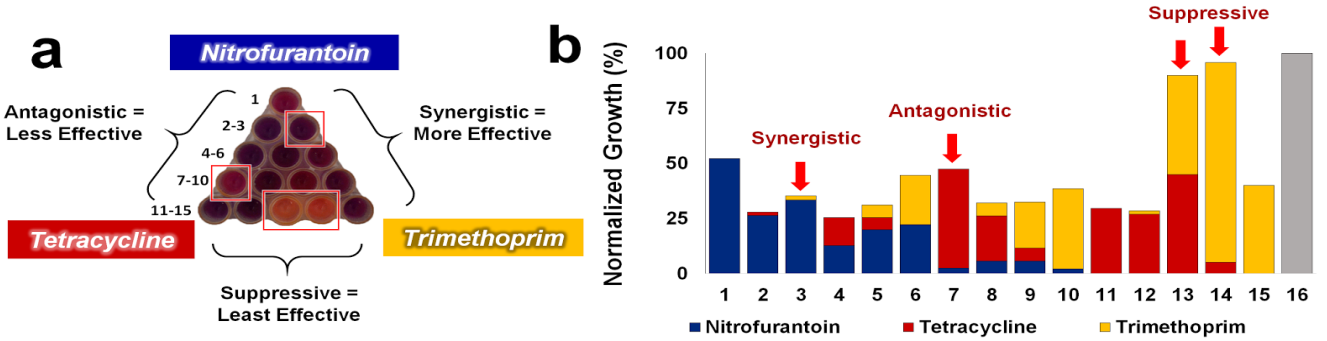

Figure 5: Three-way antibiotic interaction trial. (a) Colorimetric image of all three antibiotics. Red boxes emphasize wells that have the strongest antibiotic interaction effects. (b) Normalized bacterial growth in all visualization wells. Each bar demonstrates a percent composition of each antibiotic in the respective well. Bar 16 is the control well not depicted in the image.

Table 2: Multiple antibiotic interactions, literature vs. experimental

Combined Antibiotic Effects	Nitrofurantoin + Tetracycline	Nitrofurantoin + Trimethoprim	Trimethoprim + Tetracycline	All Three Antibiotics
Literature [9]	Less Effective	More Effective	Less Effective	???
Experimental	Less Effective	More Effective	Less Effective	More Effective

Our results, shown in Figure 5b and Table 2, indicate validated pairwise interactions, where wells experiencing synergy are brighter than neighboring wells and wells experiencing antagonism or suppressive effects are darker than neighboring wells. The inner wells of the collection chamber (wells 5, 8 and 9) in which all three antibiotics were combined in moderate concentrations show similar or more effective inhibition of bacterial growth than each antibiotic alone. These results suggest that three-way combinations of trimethoprim, tetracycline, and nitrofurantoin may have a greater antimicrobial synergistic effect than various pairwise antibiotic treatments.

CONCLUSIONS

In summary, we have demonstrated the effective fabrication, characterization and application of our 3D printed microfluidic concentration gradient generator for use as a biomedical drug screening tool. The utility of the three-antibiotic gradient was demonstrated by determining the MIC value for three clinically-relevant antibiotics: trimethoprim, tetracycline and nitrofurantoin, which showed correlation with established literature values. The medical relevance of 3D gradient generation was then demonstrated with two- and three-way antibiotic interaction studies. The observed synergistic, antagonistic and suppressive effects of pairwise-combined antibiotics agree with descriptions in literature, and the three-way results suggest the benefit of three-way antibiotic treatment for ampicillin-resistant E. coli. The data presents a novel 3D microfluidic design that provides a relevant multi-drug concentration gradient for applications in research, biology and medicine.

ACKNOWLEDGEMENTS

We greatly appreciate the contributions from various members of the *M*^{3}*B* Program and the Liwei Lin Lab, UCB.

REFERENCES

[1] A. G. Toh *et al.*, "Engineering microfluidic concentration gradient generators for biological applications", *Microfluid Nanofluid*, vol. 16(1), pp. 1-18, 2014.

[2] K. Churski *et al.*, "Rapid screening of antibiotic toxicity in an automated microdroplet system", *Lab Chip*, vol. 12(9), pp. 1629-1637, 2012.

[3] E. Tekin *et al.*, "Enhanced identification of synergistic and antagonistic emergent interactions among three or more drugs", *J. R. Soc. Interface*, vol. 13(119), pp. 1-11, 2016.

[4] R. D. Sochol *et al.*, "3D printed microfluidic circuitry via multijet-based additive manufacturing", *Lab on a Chip,* vol. 16, pp. 668-678, 2016.

[5] S J. A. Caminero *et al.*, "Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis", *Lancet Infectious Diseases*, vol. 10(9), pp. 621-629, 2010.

[6] T. Bollenbach, "Antimicrobial interactions: mechanisms and implications for drug discovery and resistance evolution", *Current Opinion in Microbiology*, vol. 27, pp. 1–9, 2015.

[7] C. H. Collins, P. M. Lyne, J. Grange, and J. Falkinham, *Collins and Lyne's microbiological methods*, 8th ed. London: Hodder Arnold, 2004.

[8] G. Sezonov et al., "Escherichia coli Physiology in Luria-Bertani Broth," *Journal of Bacteriology*, vol. 189, no. 23, pp. 8746–8749, 2007.

[9] J. M. Andrews, "Determination of minimum inhibitory concentrations", *Journal of Antimicrobial Chemotherapy*, vol. 48, pp. 5-16, 2001.

CONTACT

*Eric C. Sweet, tel: +1-978-996-6799; ericsweet@berkeley.edu