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BIOTECHNOLOGY PROGRESS

NOTE

Cell Culture and Tissue Engineering

A low-cost, high-throughput microfluidic nano-culture platform for functional metagenomics

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Abstract

Functional metagenomics is an attractive culture-independent approach for functional screening of diverse microbiomes to identify known and novel genes. Since functional screening can involve sifting through tens of thousands of metagenomic library clones, an easy high-throughput screening approach is desirable. Here, we demonstrate a proof-of-concept application of a low-cost, high-throughput droplet based microfluidic assay to the selection of antibiotic resistance genes from a soil metagenomic library. Metagenomic library members encapsulated in nanoliter volume water-in-oil droplets were printed on glass slides robotically, and cell growth in individual drops in the presence of ampicillin was imaged and quantified to identify ampicillin-resistant clones. From the hits, true positives were confirmed by sequencing and functional validation. The ease of liquid handling, ease of set-up, low cost, and robust workflow makes the droplet-based nano-culture platform a promising candidate for screening and selection assays for functional metagenomic libraries.

KEYWORDS
antibiotic resistance, droplet, metagenomic library, screening

Microbial ecosystems are now recognized to harbor massive, diverse, largely unexplored chemical space of interest to biotechnology.[1] Since many of these environmental organisms are unculturable, functional metagenomics serves as a powerful alternative for the discovery of novel, diverse, and previously unknown bioactive entities.[2] Metagenomic libraries can be constructed by cloning DNA fragments obtained from a microbiota sample into an expression vector, and transforming the library into a suitable host such as *Escherichia coli*.[3–5] These libraries are probed for functions of interest such as antibiotic resistance or enzymatic activity, the functional clone is isolated, and the heterologous library gene is annotated. Robust high-throughput approaches are necessary to achieve these goals due to the immense size of the library (10^6–10^7 unique clones). In the past decade, several microfluidics-based screening systems have been developed for the separation and characterization of single clones.[6–9] Although these systems are highly efficient, fast, and accurate, the commercial systems are expensive, costing several thousands of dollars, or demand expertise in sophisticated assembly/microfabrication techniques in order to generate droplets of consistent size distribution. In this work, we report the development of a simple and cost-effective microfluidic high-throughput screening platform. The method is an adaptation of the platform developed by Jiang et al[10] As proof-of-concept, we apply it to the identification of antibiotic resistance genes from a soil metagenomic library; while antibiotic resistant clones can be easily selected in high-throughput on agar plates, in practice this microfluidics method will offer distinct advantages in non-selective screens for functions coupled to molecular reporters or other readouts, specifically in the isolation and recovery of hit library members. Compared to other microfluidics screening platforms, ours offers greater ease of implementation, and a lower cost by orders of

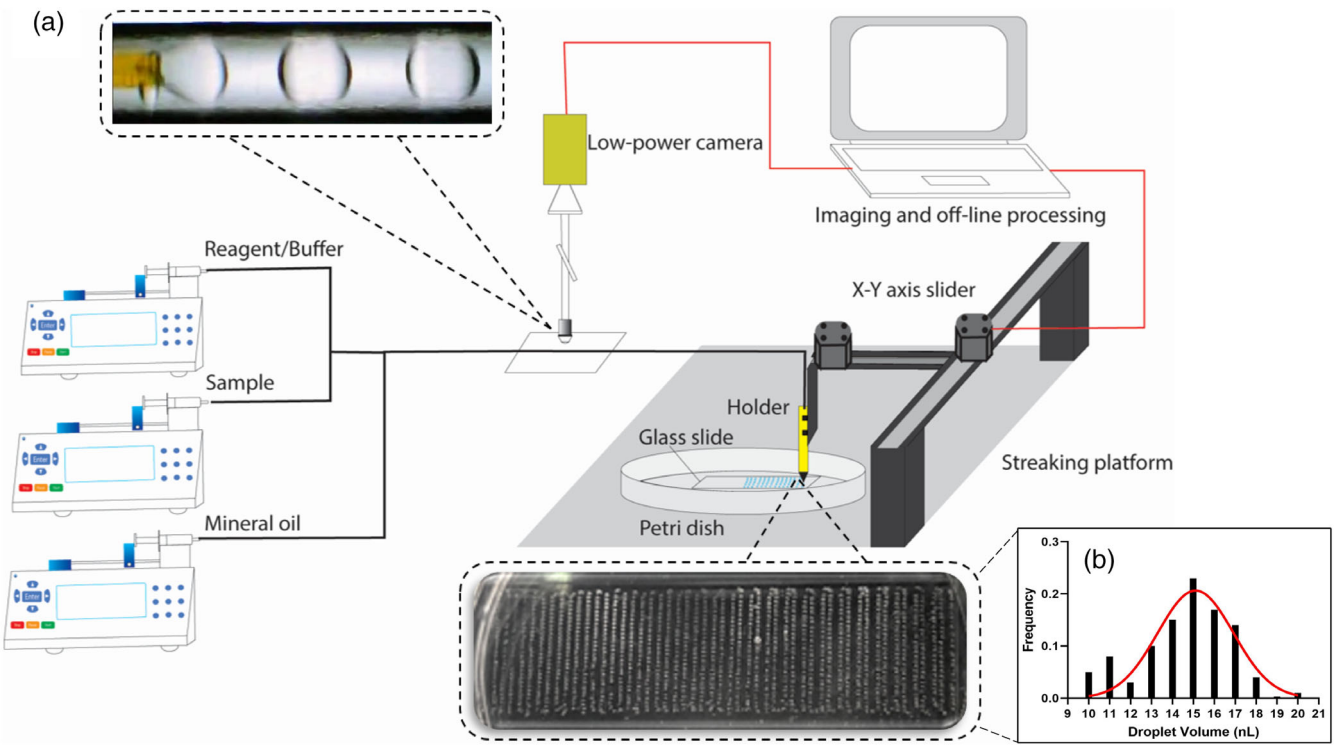

FIGURE 1 Generation of nano-culture droplet arrays. (a) Experimental set-up for nano-culture droplet generation. The system includes three syringe pumps leading into two concentric inlets with the inner and outer diameters of 40 μm and 280 μm, respectively; a low power, light microscope, and a programmable printer head. Nano-culture droplet array in predefined patterns is generated. (b) The droplet volume is normally distributed with a mean of 15 nl

magnitude (~2000 USD set-up costs and ~100 USD running costs, Table S1).

An overview of the method for identification of antibiotic resistance genes (ARGs) against the β-lactam ampicillin is presented in Figure 1a. Nano-cultures of metagenomic library members were arrayed on a chip. The chip was then screened for resistance against ampicillin, and the presence of putative ARGs was confirmed by gel electrophoresis, followed by amplicon sequencing and annotation. Each chip contained 50 rows x 34 columns of droplets, and it took 100 s to array one chip.

The microfluidic devices were fabricated using three types of tubing, vinyl tubing for inlet channels (280 μm I.D., 640 μm O.D., #BB31785-V/1, Scientific Commodities Inc. (SCI)), fused silica tubing for junctions (40 μm I.D., 140 μm O.D., Ibis Scientific) and LDPE tubing for outlet channel (280 μm I.D. and 640 μm O.D., #BB31695-PE/1, SCI). The microfluidic device had three inlet channels joined to the outlet channel using fused silica tubing, and super glue at the junctions. The cell suspension was pumped through channel 1, and media containing ampicillin was pumped through channel 2 using syringe pumps (Harvard Apparatus) at a flow rate of 1 μl/min. Mineral oil of viscosity 10 cSt (#ALK-RTM8, Paragon Scientific) was pumped through channel 3 at a flow rate of 15 μl/min. Once the cells were mixed with media at the first junction, the oil–water interface was formed at the second junction. The droplet generation was visualized using a low power light microscope (Moticom 1080). The outlet channel was connected to a pen through a rubber cork, and the pen was fixed to a programmable printer (Bachin Draw T-A4) on to a substrate.

The droplets generated can be stored on glass Petri dishes or glass microscope slides pretreated with 3-aminopropyltriethoxy silane (APTES), which renders the surface hydrophobic. Glass Petri dishes or slides were washed with acetone for 5 min and filled with 2.5% APTES solution in deionized water and incubated at room temperature for 30 min. The surfaces were washed thrice with deionized water for 15 min each, and dried using compressed air, covered with an aluminum foil, baked in the oven at 120°C for 30 min, and then cooled at room temperature. The dishes were stored in a desiccator until further use. Prior to use, the dishes were filled with a thin layer (typically 2 ml) of mineral oil just enough to cover the droplets, prevent evaporation, and immobilize the droplets on the surface of the slides. Once printing is complete, the droplets were visualized using a bright-field microscope, and the size was quantified using Image J. Under the given flow conditions, the droplet volumes were normally distributed with mean ± SD of 15.0 ± 2.1 nl (Figure 1b).

Mega X strains of *E. coli* at defined concentrations were prepared in LB media. The media, cell suspension, and mineral oil were loaded in 3 ml syringes. The cells were mixed with the media at the first junction of the tubing in at 1:1 ratio, and then this cell suspension was mixed with mineral oil to form water-in-oil droplets with cells being encapsulated inside the droplets. The slides were incubated at 37°C for different durations. The drops containing the cells were imaged at

×20 magnification using an inverted microscope (Leica DMi8). Automated stage control and imaging features were used to collect the images of drops, which were processed offline. Drops without any cells were used as blank controls for background. The cell number inside the drops was counted using ImageJ after thresholding the images, converting to binary images, and automatically analyzing the particles. For each treatment or condition, at least 20 identical spots from each chip were analyzed. All characterization experiments were performed in duplicate.

First, we prepared a calibration curve by altering the cell number in each droplet. We fabricated the nano-culture *E. coli* chip containing 1200 spots, each with 15 nl volume, by varying the cell concentration in the feed cell suspension: 0 (blank), $6.6 \times 10,^{5}$ $1.32 \times 10,^{6}$ $6.6 \times 10,^{6}$ $1.32 \times 10,^{7}$ $6.6 \times 10,^{7}$ 1.32×10^{8} cells/ml. The characterization studies were performed with two different strains of *E. coli*: kanamycin-resistant strain (kanR), kanamycin- and ampicillin-resistant (kanR/ampR), referred as susceptible and resistant strains, respectively. Soon after encapsulation, the cells were visualized, and manually counted. The expected cell distribution matches well with the actual distribution attesting to the reproducibility across the droplet distribution (Figure 2a,b). Next, we characterized cell growth kinetics in the droplets by measuring droplet cell counts over 6 h. The growth follows an exponential trend during this period, and beyond 6 h, the cell number increases to values that could not be counted microscopically (Figure 2c,d). Lastly, we tested both ampicillin-resistant and ampicillin-susceptible strains by exposing the nano-cultures starting with 5 cells per droplet to 0, 1, 5, 10, 50, 100, 500 µg/ml of ampicillin, and counting the increase in cell numbers after 6 h using ImageJ as described above. The nano-cultures showed a classic dose–response profile that fits well with the Hill equation (Figure 2e). The IC_{50} values obtained for the susceptible and resistant cells were 5.952 ± 0.603 µg/ml and 43.27 ± 4.25 µg/ml, respectively. The increase in IC_{50} due to the ampicillin resistance-conferring plasmid prompted us to choose a concentration of ampicillin (30 µg/ml) that is effective for the identification of ampicillin-resistant clones.

Having established the reproducibility of nano-culture assays, we selected a metagenomic library for ampicillin resistance genes. We used a soil metagenomic library (S18-3A) in *E. coli* Mega X strain consisting of 2–5 kb metagenomic fragments, and kanamycin resistance gene as a selective marker in pZE21 plasmid.[3] The diversity estimate of the library is 3×10^{5} unique metagenomic fragments. The library, saved as a 50% (vol/vol in LB) glycerol stock, was kept stored in a freezer at −80°C until use. Just before screening, the frozen stocks were titered to determine the bacterial cell concentration, which was determined to be 10^{9} cells/ml. *E. coli* cell suspension was prepared with fresh LB-amp media such that each 15 nl droplet is expected to contain on average 5 cells, and the final ampicillin concentration is 30 µg/ml.

E. coli metagenomic library members were arrayed at 5 cells per 15 nl droplet, and 1700 droplets per chip. We arrayed a total of 25,500 clones from the library in three chips along with 30 µg/ml ampicillin. The cells were cultured for 6 h, and cell growth was

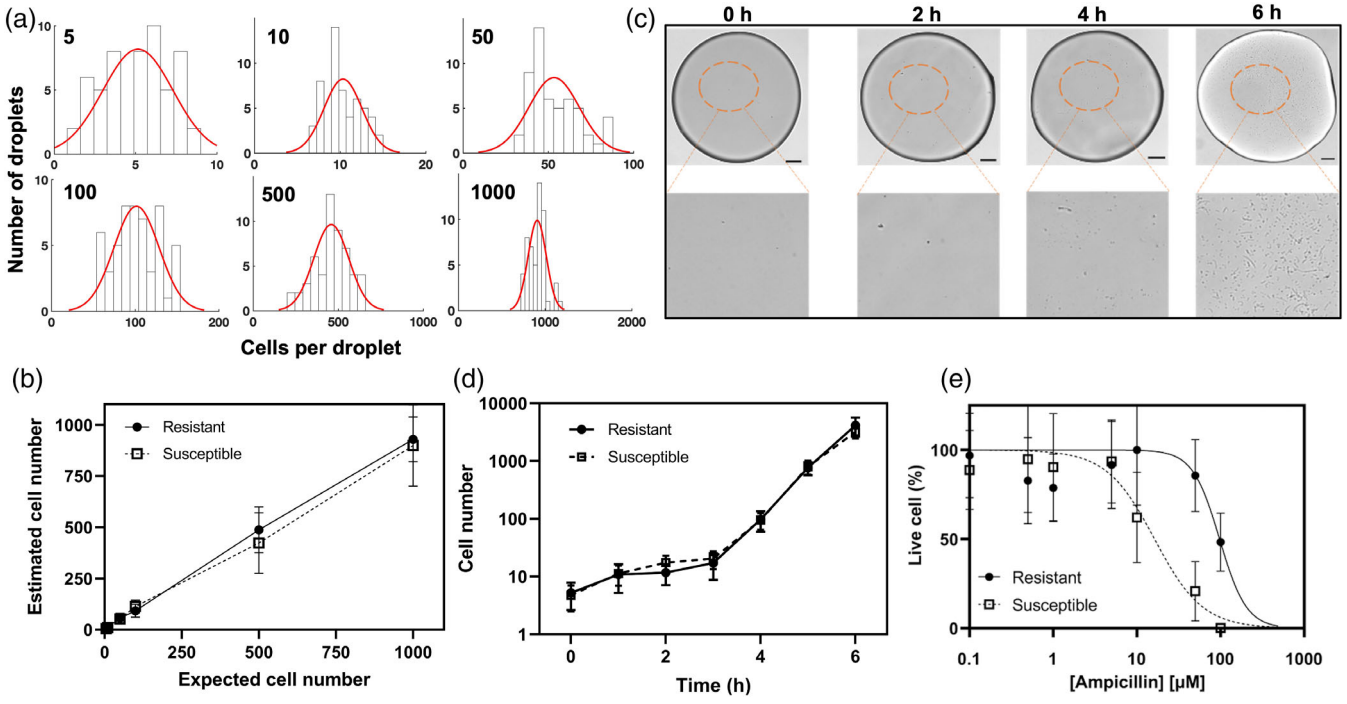

FIGURE 2 Characterization of *Escherichia coli* nano-cultures. (a, b) Expected and actual distribution of cells in each droplet ($R^2 = 0.996$, $n = 54$); (c) Representative images of cells grown in the nano-culture droplets starting with 5 cells per droplet. The scale bar is 10 µm; (d) Quantification of cell growth in the droplets. The results are data averaged from 200 droplets from three independent experiments; (e) The cultures were exposed to various concentrations of ampicillin at the time of seeding, and after 6 h the viability was quantified. The IC_{50} values were estimated to be 5.952 ± 0.603 µg/ml and 43.27 ± 4.25 µg/ml for resistant and susceptible cells ($n = 3$; $p < 0.05$, Student's t test)

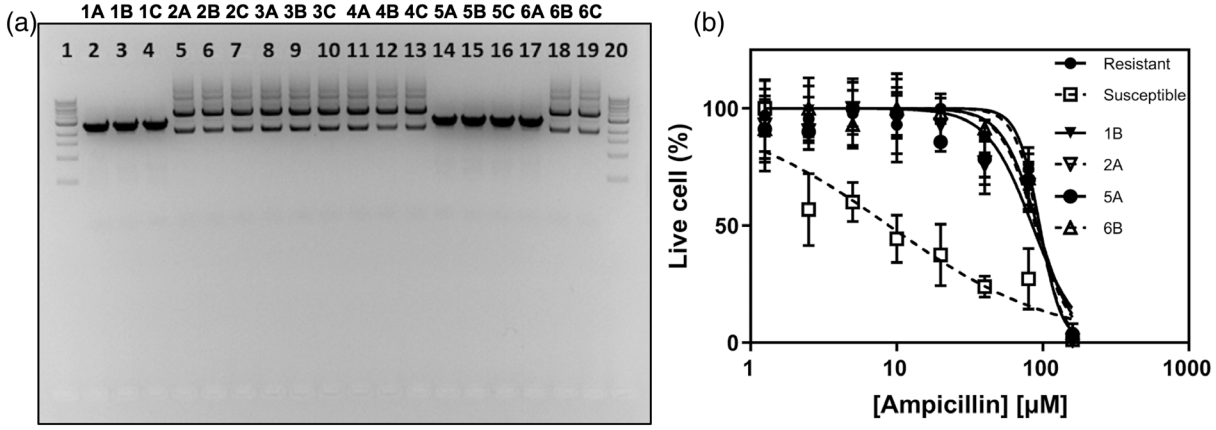

FIGURE 3 Screening of metagenomic libraries with nano-culture droplet arrays. (a) Gel electrophoresis on PCR-amplified metagenomic gene fragments from the hit clones. The lanes are: 1 and 20: DNA ladder; 1–18: Clones 1A, 1B, 1C, 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B, 4C, 5A, 5B, 5C, 6A, 6B, 6C, with numbers 1–6 referring to the 6 quadrants on ampicillin agar plates (renumbered) that exhibited growth after streaking of hit droplets, and A-C referring to individual colonies picked from each quadrant. Observed lane band patterns corresponded with unique fragments identified after Sanger sequencing: Fragment 1: Lanes 2–4, 14–17 (Clones 1A-C, 5A-C, 6A); Fragment 2: Lanes 5–13, 18–19 (Clones 2A-C, 3A-C, 4A-C, 6B-C); (b) Confirmation of representative hit droplets from ampicillin selection (1B, 2A, 5A, 6B) using 96-well plate dose response assay

visualized by microscopy. Based on the results from Figure 2e, we expected that only ampicillin-resistant clones would grow. We identified 15 droplets that showed significant cell growth, and 12 of these droplets were picked successfully. The picked droplets were streaked on agar plates containing 30 μg/ml ampicillin with each quadrant of the agar plate streaked from a distinct droplet. We observed that only six of the 12 quadrants showed cell growth suggesting that other quadrants were likely due to inefficiency in the manual picking of the droplets. Three colonies were picked from each of the quadrants (labeled as clones 1A-C, 2A-C, 3A-C, 4A-C, 5A-C, 6A-C).

To characterize antibiotic resistance genes in these hits, the single colonies were cultured, and the plasmids were purified, amplified using PCR, and analyzed by gel electrophoresis as described in detail.[11] We observed gene fragments corresponding to the hits (lanes 2–19, Figure 3a). The PCR-amplified metagenomic fragments were purified using thExoSAP-IT PCR Product Cleanup Reagent (Applied Biosystems, Cat. No 78205.10.ML) according to the manufacturer's protocol. Approximately 60 ng of each purified PCR product was submitted for Sanger sequencing (Genewiz, New Jersey) with forward primer 5′-cacatcagcaggacgcactgacc and reverse primer 5′-aggagag cgttcaccgacaaacaacag (5 μl each primer at 5 μM). Sanger sequences were aligned in SnapGene® v6.0.2, using the MUSCLE algorithm (v.3.8.1551) with default parameters. From these alignments we observed two unique fragments. Consensus forward and reverse sequences for the two fragments were extracted from the MUSCLE alignments. Low-quality ends (N's) were manually trimmed, and the resulting sequences were submitted to NCBI BLAST® (BLASTN program, using the megablast algorithm for highly similar sequences with default parameters) and queried against the standard Nucleotide collection (nr/nt database). Hits with >95% sequence identity were considered.

It was observed that the 18 samples represent two unique inserts (see SI), and this corresponded with the two band patterns seen on the gel (Fragment 1: Lanes 2–4, 14–17; Fragment 2: Lanes 5–13, 18–19). Fragment 1 Reverse read aligned with 99.20% identity (97% query coverage) to three sequences (GenBank Accessions JX009326.1, JX009232.1, JX009210.1) all of which had been deposited by Forsberg et al. as hit sequences from functional metagenomic selections of the same metagenomic library S18-3A on β-lactam antibiotics piperacillin, carbenicillin, and amoxicillin, respectively.[3,4] Similarly, Fragment 2 Forward and Reverse reads aligned to two sequences (GenBank Accessions JX009231.1, JX009297.1, >96.78% identity and >94% query coverage), again deposited by Forsberg et al. as hits from selections of library S18-3A on β-lactams carbenicillin and penicillin G, respectively. β-lactam resistance of representative clones (1B, 2A, 5A, and 6B). The hit clones from nano-culture screen were tested for ampicillin susceptibility using a 96-well plate dose response assay, and were found to be resistant (Figure 3b).

In summary, in this proof-of-concept demonstration, we have used a simple microfluidics-based system to identify antibiotic resistance genes from a metagenomic library. We have shown that we are able to obtain β-lactam resistance genes that were consistent with what has been previously described by our group using agar plate cultures.[3–5] This method will be particularly useful, compared to well-plates, for the dilution of large libraries into much smaller cell numbers to the limit of one cell per spot. The platform may then be adapted in functional screening applications such as identification of active peptides, such as through use of chromogenic or fluorogenic readouts of cell growth or enzyme activity on a substrate, followed by automated image capture and analysis.[12] Although the throughput of a couple of thousand spots per chip at nanoliter volumes falls short of the pico-liter volumes of droplet-based, automated, and rapid microfluidic sorting; at a cost of only a few hundred dollars, the method can be an optimal test bed before entering sophisticated ultra-high throughput screening.

AUTHOR CONTRIBUTIONS

Manpreet Kaur, Aura Ferreiro, Cheng-Yu Hung designed and performed experiments, analyzed the data, and prepared the figures. Gautam

Dantas and Anand K. Ramasubramanian designed the research, analyzed the data, and procured funding. Anand K. Ramasubramanian wrote the manuscript with input from all authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/btpr.3317.

DATA AVAILABILITY STATEMENT

The gene sequences of hit clones are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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