Design and Verification of a Modified Ichip to Incorporate Coculturing of Soil Microbes

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Abstract

It is widely accepted that the vast majority of microbial species are unculturable, meaning they cannot be grown on standard laboratory media. Of the many attempts to grow such species, one successful strategy has been the ichip, which gives microbes in monoculture access to molecules from their natural environment by means of a porous membrane. However, the environment these microbes were taken from contain a myriad of species, some of which may produce necessary factors others depend on. Indeed, it has been shown that bacteria can produce signaling molecules that can cause uncultivated bacteria to grow. However, the ichip only intentionally includes monoculture. Here we created a modified ichip (michip) by making half of the chambers double the volume of the other half, which leads to one or two cells loaded into each chamber type, on average, given the sample is correctly diluted. Multiple design iterations were undergone to optimize the michip for easier use, and its functionality was verified by loading the michip with *E. Coli* K-12 and detecting growth under a microscope. Given that the ichip has grown an antibiotic producing bacteria, we hypothesize that the michip could be used to grow currently unculturable microbes, some of which might be able to produce novel antibiotics. We also hypothesize that growth on sterilized soil could also increase the diversity of species grown in either the ichip or the michip.

Keywords: Unculturable microbes, ichip, coculture

Introduction

Most microbial species evade cultivation on artificial media, which makes them unculturable. Estimates of total microbial diversity vary, with some as low as 120,000 and others as high as 10^{12}, yet as of 2017 only 12,000 species have been described [1,2,3]. Additionally, increasing this number is relatively slow with only 600 to 800 species being discovered every year [3]. One of the motivations of culturing these currently unculturable bacteria is their potential to produce useful compounds like antibiotics. Given that over half of pharmaceuticals are microbial secondary metabolites [4], we can only wonder what products might be out there waiting to be discovered.

One early strategy to grow these unculturable microbes is called the diffusion chamber [5]. The diffusion chamber consists of two membranes with 0.03 μm pores attached to the top and bottom of a metal ring. The inside of the chamber is loaded with molten agar and multiple microbial cells. Then the diffusion chamber is incubated in a simulated environment that mimics the environment those cells were taken from. Once in this environment, small molecules that may induce growth can diffuse into the diffusion chamber. Colonies forming after this incubation period can be picked and loaded into another diffusion chamber, resulting in the isolation of novel species after many such inoculations. A high-throughput version of the diffusion chamber was made with the invention of the ichip, which consists of a thin piece of plastic with many holes that can each be loaded

with agar and, on average, a single microbial cell [6]. Multiple miniature diffusion chambers are formed after membranes are attached to the top and bottom of this device. This strategy was shown to grow novel microbial species after two weeks of incubation in either soil or marine sediment [6]. Subsequently, 10,000 such chambers were screened for antimicrobial properties, which resulted in the discovery that the previously unculturable species *Eleftheria terrae* produced a novel antibiotic which was named teixobactin.[7]

Ichips can induce up to 50% of inoculated cells to form colonies,[6] yet this begs the question why the other 50% don't grow. Incubation time could be a factor, but another factor could be that of coculture. Since these unculturable microbes must be growing in nature, culturing strategies that do a better job of replicating this environment should grow a larger share of the microbes from environmental samples. It has been estimated that marine sediment samples have an average of 2.4×10^3 unique bacteria species,[8] thus placing cells in monoculture is not replicating this aspect of their environment. Additionally, it has been shown that bacterial strains cultivated in the diffusion chamber can produce signaling molecules that are able to induce uncultivable bacteria found in the same environment to grow[9].

If a microbe in the ichip has a bacteria on the other side producing a factor it needs, that bacteria might not be in a high enough abundance to affect the microbe in the ichip. Thus, one solution to this would be to have two microbes in the same chamber, so signal producing microbes can grow in close proximity to microbes that might depend on such signals. We produced and verified a modified ichip (michip) that can be used to determine whether or not coculturing can lead to the domestication of new microbial species. This is done by changing the volume of half of the chambers (called the large chambers) such that they have double the volume as the other half (called the small chambers). The small chambers are dedicated to monoculture, which can be used as a control and as a way to culture microbes whose growth might be hindered by the presence of other species.

Materials and Methods

Michip Design

Michips were designed in Fusion 360 (educational license to J.A.S) using parameters that allowed for the design to be easily changed. Eventually we incorporated the dimensions of a standard 96 well plate into our prototype design. This was laser cut out of acrylic and tested. After such testing we changed part of the design by changing the parameters (Figure 1). This ended up being the final design, and 25 of these michips were laser cut utilizing the services provided by the company Xometry Inc.

Michip Inoculations

Michips, the membranes (GVS Life Sciences hydrophilic PCTE filter, part number 1239558), and the 96 well cover foils are first sterilized using an Ennva UV light sanitizer. This sterilization method was verified by observing whether it could stop the growth of *E. coli* K-12 cells streaked on an agar plate and immediately exposed to the UV-C light for 3, 6, or 9 minutes (Figure S1). After sterilization, silicone glue (DAP, part number 786) is applied in a thin layer to one side of the michip using a sterilized gloved finger. This side is pressed against a membrane that has slightly more area than the michip, and the glue is left to dry for one hour. After drying, properly diluted microbial samples can be mixed with molten agar and pipetted into the michip, with the membrane side facing down. The volume on the pipette is changed such that large chambers receive double the volume as small ones. After allowing the agar to dry for 30 minutes, the cover foil is applied to the other side of the michip. This protocol was greatly influenced by the one reported by Berdy et. al [10].

Michip Verification Experiment

Specific michip chambers were loaded with sterile LB agar or LB agar contaminated with *E. coli* K-12 (see results section). 60 µL were loaded into the small chambers and 120 µL were loaded into the large chambers. Michips were incubated for over 24 hours in sterile nalgene beakers filled with enough LB media to completely submerge the michip. After this incubation the cover foil was removed and each chamber of the michip was imaged using an EVOS XL Core microscope at 20X magnification. The chambers of the second test case partially dried out by the time they were imaged, yet applying deionized water proved sufficient to see the colonies that formed during the incubation period. The absorbances of 200 µL of LB media surrounding each michip and their respective controls (sterile and *E. coli* K-12 contaminated LB stock) were measured at 630 nm. Three measurements were taken and averaged for each sample.

Imaging and Gram Stains of Soil Bacteria

Soil microbes were dislodged from soil particles as described previously [10]. 5 µL of this sample was pipetted onto a glass microscope slide on top of a ruler (Figure S2A). Once the water in the sample evaporated, it was heat fixed using a lighter and then gram stained following the procedure from Cornell Veterinary College[11] and imaged under 400X total magnification using an Echo Rebel microscope. Using ImageJ to measure the area of a given soil sample with known volume and counting the cells throughout the dried and gram strained sample under a microscope, those counts per area could theoretically be converted to counts per mL.

Results

Design Iterations of the Michip

In order to include coculturing, the radius of the larger chambers were increased such that the resulting volume would be double that of the smaller chambers. Thus, the same microbial sample can be loaded into the michip and chambers will contain, on average, a different number of cells. If the dilution of the microbial sample is done properly, this average can be 1 for the small chambers and 2 for the large chambers. This is important because it eliminates the possibility that nutrient concentration per microbe is a potential explanation for any difference in the species identity found to have grown within the different diffusion chamber types within the michip. Given the unknown nature of unculturable microbes, it is a very real possibility that they have different dividing times that could be changed by nutrient concentration.

The design process was started by incorporating the large and small chambers into an ichip with dimensions similar to that of the original design (Figure 1). This design was later scaled to the dimensions of a 96 well plate and the chamber layout changed in order to allow for multichannel loading of the michip. Multichannel pipetting significantly increases the efficiency of michip inoculation, given the time in between when microbes can be added to molten agar and the time it solidifies. Finally, the chamber sizes were scaled down to allow for easier attachment of the membrane.

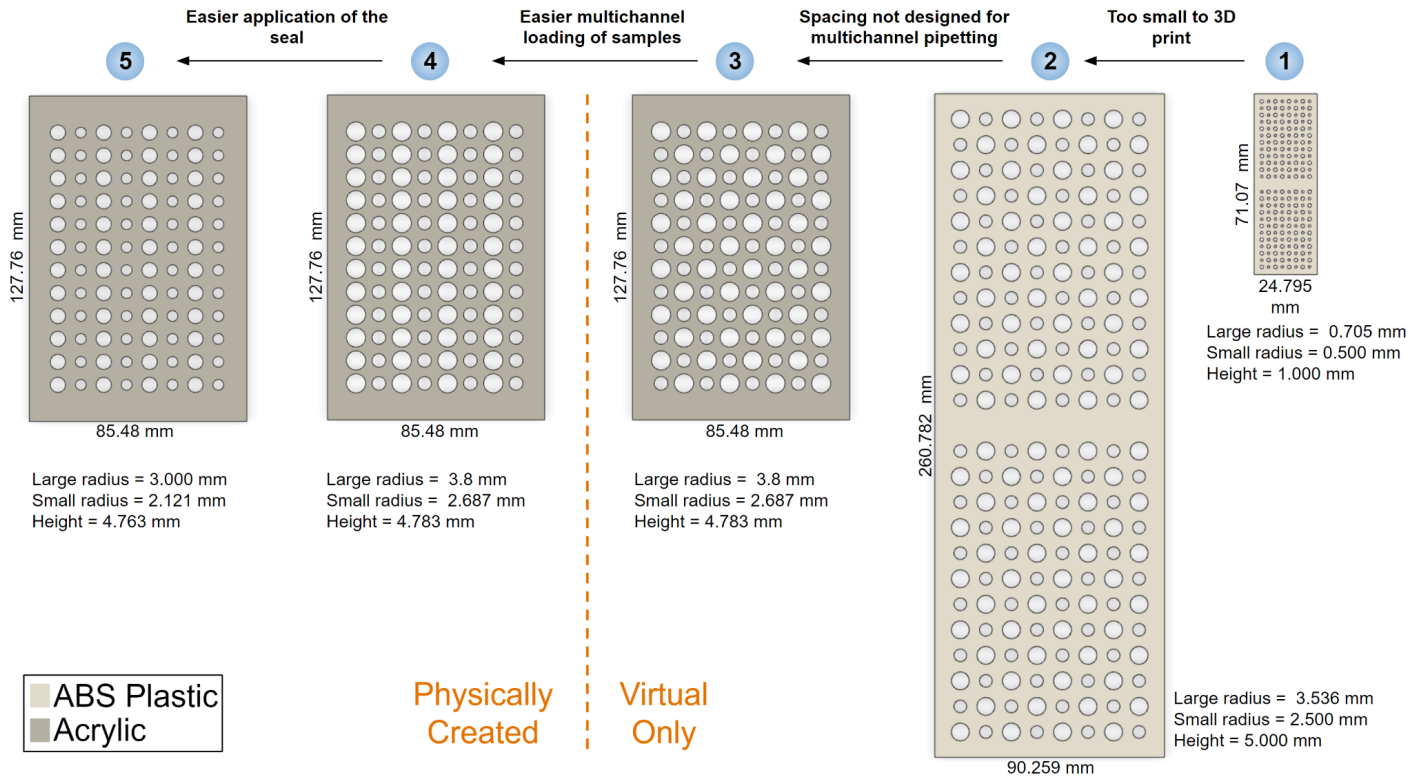

Figure 1: Design iterations of the michip. Five designs were made in Fusion 360, and the main motivation behind changing each design is shown above the arrows between designs.

Verification of the Michip

For interpretation of unculturable microbial growth experiments, it is important to show that nothing can enter, exit, or move within the michip. To show this is the case, two experimental test cases were designed (Figure 2). The first michip was loaded with LB agar contaminated with *E. coli* K-12, except for the last column which was kept as a sterile control, and immersed in sterile LB media. The second michip was loaded with sterile LB agar, except the last column which was loaded with LB agar contaminated with *E. coli* K-12 cells. This second michip was subsequently immersed in LB media contaminated with *E. coli* K-12. These inoculation patterns and the sterility of the surrounding media allowed us to test whether microbes could enter the michip (the first michip) or exit the michip (the second michip). Each chamber was imaged and examined for the presence of colonies, and the absorbance of the surrounding media was measured. Growth was detected only in chambers or surroundings inoculated with *E. coli* K-12, meaning that the seal adequately kept them from entering or exiting the michip (Figure 3). The correct presence of growth in the last column also strongly suggests nothing can move within the michip.

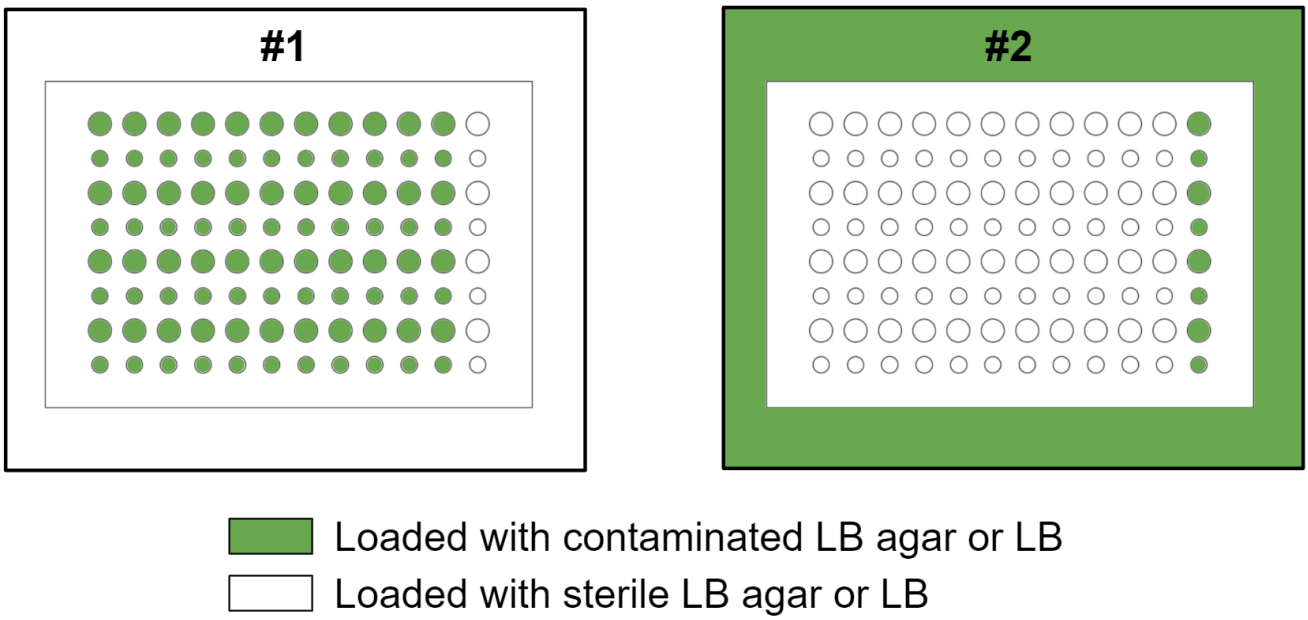

Figure 2: Images show the set up for verifying the seal of the michip. For experiment #1, the michip had 84 chambers loaded with contaminated LB agar or LB media shown in green and 12 chambers contained sterile LB agar or LB media indicated in white. Experiment #2 was the reverse, with 84 chambers loaded with sterile LB agar and 12 inoculated with contaminated LB agar. The contaminated LB agar contains *E. coli* cells to observe growth.

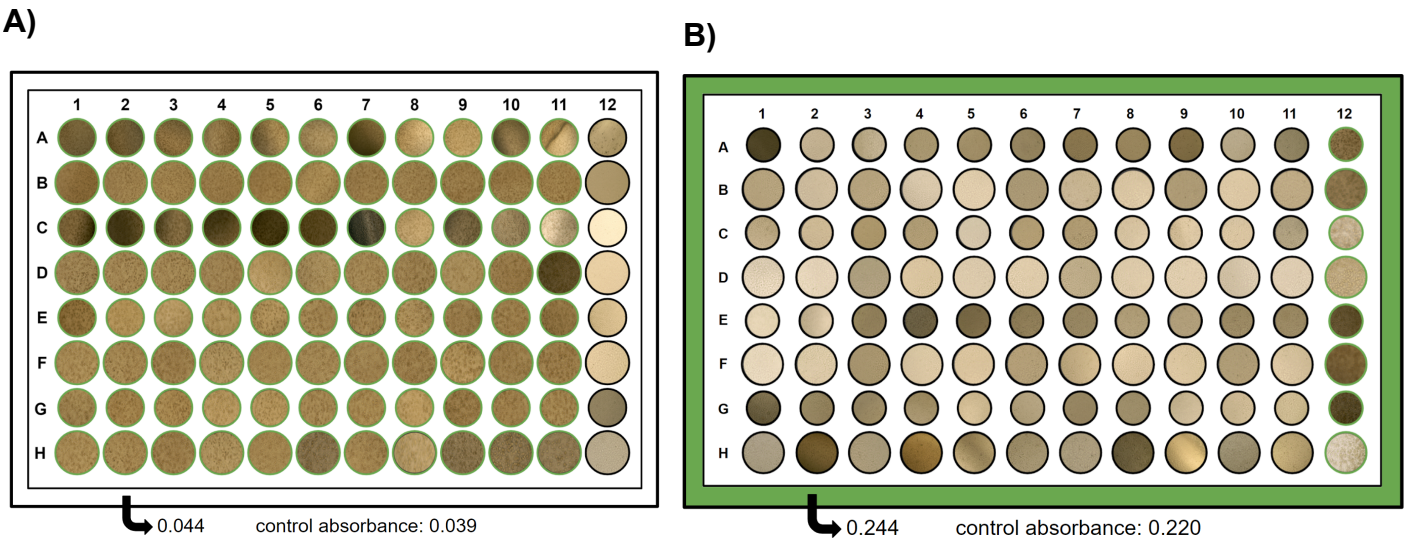

Figure 3: Michip Validation. Two experiments were performed to verify the seal of the michip. The green circles indicate expected growth and black circles represent where growth was not expected. **A)** The first experiment had 84 chambers inoculated with *E. coli* cells and 12 were inoculated with sterilized LB media. **B)** The second experiment was the reverse of experiment 1 with 84 chambers containing only sterilized LB media and 12 had *E. coli* cells. The results indicated growth had occurred in the chambers that were expected and similarly, no growth was observed in the expected chambers for both experiments. Absorbances confirmed whether growth was seen.

Discussion

In this study we successfully designed and verified a modified ichip to include the coculturing of microbes. Growth experiments utilizing *E. coli* K-12 showed that this device is able to keep microbes in their chambers, as well as prevent other microbes from entering. As far as we are aware, no previous methods have been made that can grow currently unculturable microbes in coculture. However, Lodhi et al. have proposed including coculture in the ichip by making the device out of a 0.03 μm porous substance [12]. Our design has two main benefits over this proposed one. First, if attempts to take chamber contents and grow any present colonies in a petri dish are made, they will be able to include potential symbiotic microbes. With this proposed design the signaling molecules could be coming from any of the surrounding chambers. Secondly, by having different species in the same chamber, the concentration of any molecules one species produces will be higher than if they were separated by a membrane.

One limitation of our design is that if it is loaded by dilution, then some small chambers are virtually guaranteed to be loaded with two cells, and some large chambers are virtually guaranteed to be loaded with one cell. This might blur any comparisons of species identity between the large and small chambers. However, the average number of cells in each chamber should allow for a comparison to be drawn. Additionally, Liu et al. used fluorescence activated cell sorting to load a michip [13], which could be of use to better improve comparisons of species identity in the michip.

Due to the difficulty of producing multiple michips, along with the unexpected difficulty in verifying its functionality, microbial cells couldn't be successfully enumerated from soil samples. Previous methods involve using DAPI [6], however a gram staining method was attempted in order to be more cost efficient. Images were taken without soil particles removed in order to test whether a gram stain could distinguish between soil particles and microbes (Figure S2B). Methods exist to remove soil particles [14], so further work could be done to see if a gram stain could be used instead of DAPI.

The future direction for the project would be to enumerate soil microbes and make proper dilutions to inoculate multiple michips. The chamber contents can subsequently be gram stained or be placed on artificial media to determine if they can grow without access to small molecules from their natural environment. Additionally, chambers can be filled with sterilized soil to test the hypothesis of whether soil structure is necessary for some microbes to grow. The most distant future direction would be to screen microbes grown in the michip for antibiotics.

Author Contributions and Notes

J.A.S. Conceptualized the study, J.A.S. and G.D.C. gathered data, and J.A.P provided funding and supervision. The authors declare no conflict of interest.

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Supplemental Figures

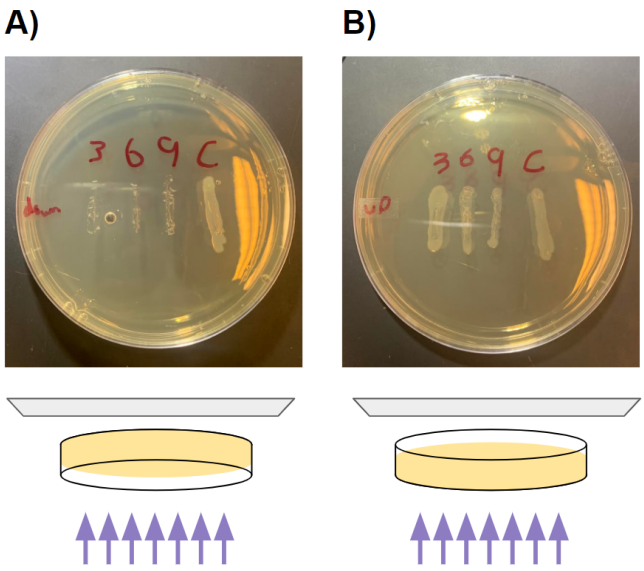

Figure S1. Sterilization Method: wooden applicators were dipped into an *E. coli* K-12 solution, streaked out onto an agar plate, and exposed to UV-C light for 3, 6, or 9 minutes. The control was added with no exposure to the UV-C light. **A)** The agar plate was inserted into the sterilizer faced down and the plate in **B)** was placed face up. Growth was not observed when the petri dish was faced down, therefore objects being sterilized must be flipped over and the sterilization process repeated for the other side. The gray trapezoid represents a mirror that is supposed to reflect UV-C light (the purple arrows).

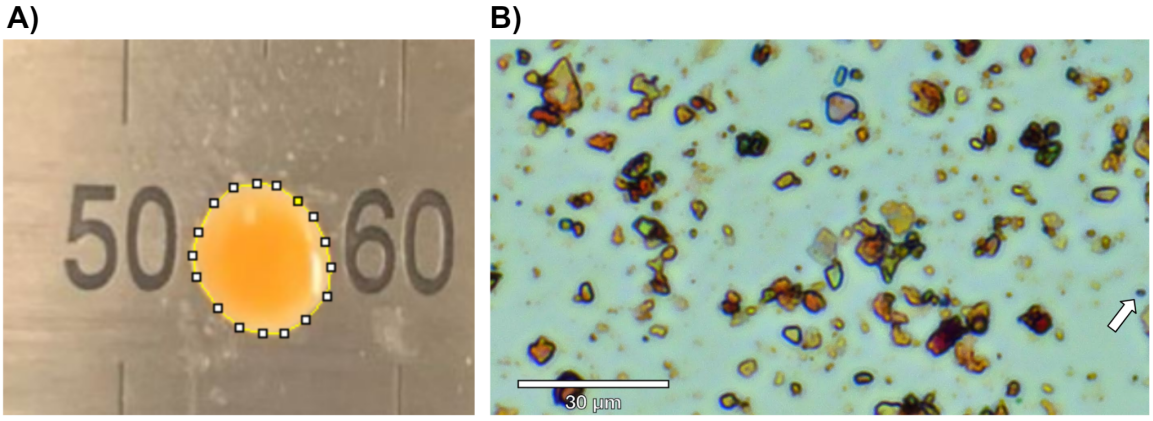

Figure S2. Attempt to Quantify The Concentration Soil Bacteria: A) A drop of a soil sample placed onto a glass slide on top of a ruler (units are in mm) and outlined in ImageJ. **B)** A gram stain of the soil sample in A. The white arrow indicates a potential microbe.