Hexagonal-Chambered Microfluidic Device: A New Method and Device for Capturing and Culturing Environmental Microbes

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ABSTRACT. Although the current knowledge of microbial life is vast, there still exist a practically uncountable number of bacteria, fungi and other microbes that have yet to be identified and categorized. Part of this deficiency is due to current methods for culturing bacterial colonies, most commonly, the petri dish. In this setting, where all different species of bacteria are placed together, competition arises and the slower growing, less adaptive bacteria that may even be prey to other, larger, bacteria die out and are never identified. This research presents a new method for the capturing and culturing of different bacteria from an environmental sample that may enable colony growth and observation of low-abundance species by separating bacterium into different 'niches'. A microfluidic device with small hexagonal chambers was formed and used to trap free-living bacteria. The bacteria were nourished with a media derived from the original sample and observed growing in the hexagons, demonstrating that naturally-occurring bacteria can be cultured this way.

INTRODUCTION.

Bacteria and microbes are an important part of our natural world, living within the food chain, degrading pollutants, and suppressing plant disease. A diverse bacterial flora is essential for human health as well, and it has been estimated that a healthy adult human maintains up to three pounds of bacterial cells in their microbiome [1]. Furthermore, it has been postulated that a decrease in human microbial flora due to antibiotic overuse and other medical interventions is associated with long-term disease [1]. Bacteria are used in many applications, including pharmaceutical and food production [2], pest control [3], and water treatment [4]. As more bacteria are discovered, applications for those bacteria become apparent and our knowledge of the tiny microorganisms and their ecosystems expands. In short, microbes and bacteria are ubiquitous in the external and internal human environment and it is important to learn more about their diversity and relative abundances in their native environment

The traditional petri dish smear, while inexpensive and simple, has significant limitations which we think may be overcome with new technology for microfabricated habitats. The smear groups bacteria together which allows competition among bacteria for resources within the dish, limiting the ability of all bacteria to survive. The larger, more adaptive, or predatory bacteria, who have therefore likely already been identified, will outgrow and may consume the smaller, less adaptive bacteria. This eliminates their chance to be identified. By using a microfluidic device instead of a petri dish, it may be possible to avoid this competition. Microfluidic devices, colorless PDMS (polydimethylsiloxane) chips with channels for fluid flow-through, are becoming more prominent in the scientific community with their ability to control fluid motion and the total environment of an organic body within the device. They present real-time sampling which allows for quick disease diagnosis [5] and observation of cell manipulation, including current chemotherapy drugs [6].

This research aims to create a new microfluidic device (Figure 1) that filters and separates all of the individual bacterium into different hexagonal chambers, thereby capturing and culturing bacteria that may typically be out-competed. In these private 'niches', the bacteria can grow without having to compete for resources with other microorganisms. Furthermore, the ability of this device to capture and culture bacteria is tested with visualization techniques. Rather than using lab strands of bacteria, "wild" microbial samples from the environment are tested for the first time. From here, measures will be taken to isolate and identify those bacteria that may eventually have potential use in a research or other beneficial settings.
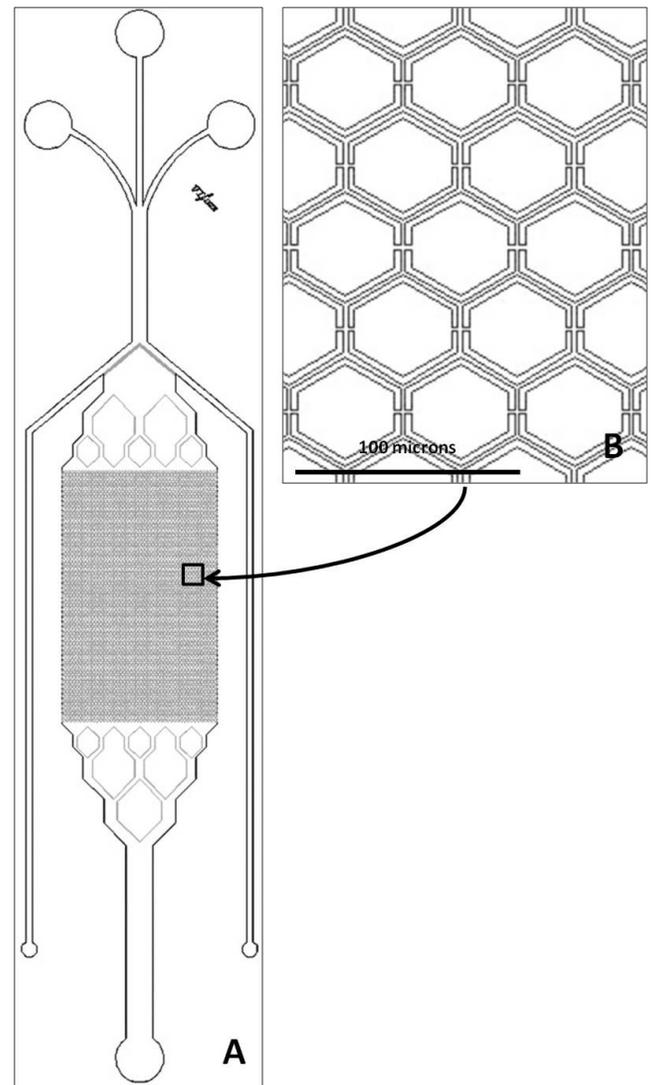
Figure 1. (A) Microfluidic device design [7] with inputs positioned at top and outputs at bottom, (B) larger image of portion of hexagonal traps (grey midsection of Figure 1.A).

MATERIALS AND METHODS.

The mask for the microfluidic device was made using standard photolithography methods, previously described [8] and imaged (Figure S1), with the negative resist technique. A disk is cleaned and covered with a resist coat and mask. The mask is the inverted version of the trap design and covers all areas which are to be left open. UV light illuminates the master and exposes the uncovered area which remains on the disk after baking and development (Figure S1). A final hard bake is completed and the master, which has a raised pattern will be used to make multiple devices, is ready.

The conventional method for the formation of PDMS microfluidic devices [9] uses a 1:10 ratio of curing agent and base, respectively. PDMS newly formed in this way is poured into the master and placed into a vacuum to remove any bubbles within the PDMS mix. The PDMS-covered master was incubated at 65°C for a minimum of 4 hours before being cut out and hole-punched with a 23 thin wall (TW) gauge needle on the inlet and outlet side the of the trap design, which fits polyethether ketone (PEEK) tubing. The hexagonal trap pattern was originally designed to trap free-floating bacteria in diluted blood samples [7]. Traps are densely packed together to encourage trapping by limiting hydraulic flow [7].

Samples were collected from the southwestern shore of a standing-water 'Hidden Pond' in Shelby Bottoms Park in Nashville, TN (as indicated in Figure S2). Hidden pond once served as a holding pond for agricultural activities in the area, but now receives water primarily from residential sources. The pond is approximately 2,500 square meters and surrounded by Newark silt loam soil type and populated with many oblite anaerobic plants. Nashville has a humid subtropical climate with cold winters and hot summers. Temperatures of the day and time that the samples were collected (June 29, 2012 starting at 11:30 AM) ranged from 39°C to 42°C. A map of the site is provided in Figure S2.

Samples from the Hidden Pond site were stored in parafilm-wrapped FALCON tubes at a temperature of 1°C. One part of this sample was vacuum filtered at 2.7 microns (Whatman 50) in order to retain live bacteria while excluding larger microbes and soil particles that may inhibit visualization or growth of bacteria or clog the microfluidic device (measurements imaged below in Figure 2.) This sample was used as the 'Live Pond Sample' (HP_1) and was vortexed before each use to homogenize and ensure a bacterial population that was an accurate reflection of the original pond-water sample. Another part of the original Hidden Pond sample was isolated and manipulated to serve as a nutritional media for the bacteria in HP_1. The advantage of the nutritional media having been derived from the original sample being that the bacteria stay in the original environment of the pond water rather than a commercial carbon-and-nitrogen rich solution. This part, HP_2, was autoclaved in order to sterilize the sample and burst cell walls to release nutritious organelles into the sample before being vacuum filtered at 2.7 microns (Whatman 50) to produce a nutritious, familiar media for the bacteria in HP_1. Equal Part of HP_1 and HP_2 were combined for a final solution HP_{MIX}. Separate petri dishes were swabbed with HP_1, HP_2, and HP_{MIX} before re-storage. A complete flow diagram of this process is shown in Figure S3.

The device was prepped with isopropyl alcohol before having HP_{MIX} pumped (Harvard Apparatus) at 250 nl per minute for one hour before switching to 100% HP_2 at 50 nl per min for 16 more hours. Throughout this process, the device area was incubated at a constant temperature of 37.5°C and water droplets were present at the inlet and outlet of the device to prevent evaporation through the device.

The device was imaged using an inverted microscope and image software (Zeiss, Metamorph) every 2.5 minutes to visualize the capture and culture of bacteria in each separate hexagonal well. These images show if bacteria were properly trapped in the hexagons and if growth was due to bacterial culturing.
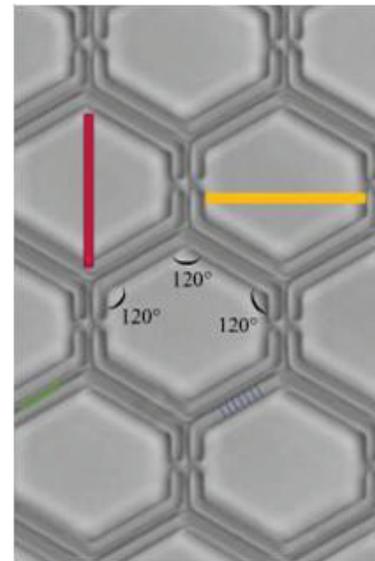

Figure 2. Hexagonal well measurements: height (red) is 43.072 μm, width (yellow) is 44 μm, channel width (green) is 2 μm, wall width (blue) is 3 μm, and depth of device is 4.5 μm. Approximate volume within each well is 6,681.4 μm3.

RESULTS.

After incubating at 37.5°C for 66 hours, the streaked sample of HP_1 showed signs of growth by producing 4 different colonies within the petri dish, verifying the presence of live bacteria that are able to be cultured. No growth was visible where HP_2 was streaked after 64 hours of incubation at 37.5°C (Figure S4, A and B respectively) affirming that this nutritional media is sterile.

From 0 to 60 minutes of HP_{MIX} influx, bacteria were trapped by the device (Figure 3, A - D). At 60 minutes, approximately 29% of the hexagonal traps were populated with at least one bacterium (Figure 3, D). There was positive growth in the device from 60 to 850 minutes, while the sterile growth media, HP_2, was steadily pumped through the device. Hexagon population increased from 29% to 77% (Figure 3, D and H). Because HP_2 does not contribute bacteria or any other particles that could accumulate within the device, the trapped bacteria from HP_{MIX} continue to grow with the aid of the nutritional value of HP_2. Multiple hexagons overflowed with bacterial growth and moved into adjacent hexagons, raising this percentage.

A stitched image of all parts of the hexagonal portion of the device shows that most of the bacterial population is concentrated towards the input side (Figure S5).
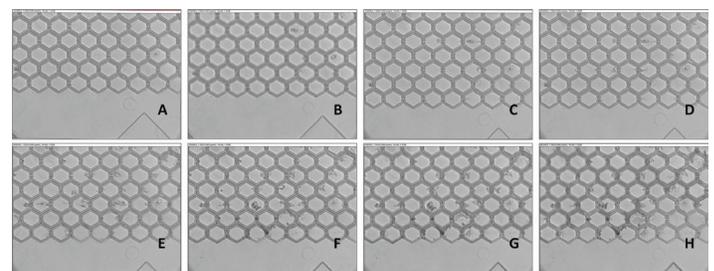
Figure 3. Time-lapse images of HP_{MIX} at 20x magnification, at minutes (A) 0, (B) 20, (C) 40, (D) 60. Time-lapse images of bacteria with nutritional media (HP_2) at 20x magnification, at minutes (E) 95, (F) 320, (G) 585, (H) 850. All photos show same location on the input side of the device.

DISCUSSION.

The presence of bacterial colonies in the HP_{MIX} streaked petri dish (Figure S4,

A) shows that there are live bacteria within the HP_{MIX} sample that will flow through, and potentially grow within the microfluidic device. The absence of bacterial colonies in the HP_2 streaked petri dish (Figure S4, B) means that there will be no additional growth or bacterial accumulation inside of the device due to bacteria originating from the nutritional sample. HP_2 can also act as a diluting solution to HP_1 because it adds no more living bacteria or full-sized bacterial mass (due to all cells being ruptured during the autoclave process) to the final HP_{MIX}.

Bacteria from HP_{MIX} was trapped within the hexagonal cells of the microfluidic device from 0 to 60 minutes (Figure 3, A-D) which is consistent with the original purpose and results of the device design [7]. In comparing visualization of the device at 60 minutes (Figure 3, D) and 850 minutes (Figure 3, H), it is clear that the bacteria has cultured as, between these times, only nutritional media has entered through the device, not new bacteria, dismissing the idea that the growth is caused by accumulation for this amount of time.

Future research includes further confirmation of these conclusions. Potential clogging and the presence of air bubbles within the device were consistent threats, so to visualize any clogging of the device, a fluorescent solution will be pumped through the device, following HP_{MIX} and will be monitored uniformly. The fluorescent solution should flow through the device evenly if unclogged, but a skewed and bending fluorescent solution pattern would be an indication of clogging. In order to avoid these issues in the future, the same experiment will be conducted with varied dilutions of HP_{MIX} and the redesign of the hexagonal portion of the device (with wider 'hallways', fewer traps, and re-scaling of the traps). Varied dilutions should also be tested to observe culture patterns and ensure that only single bacterium occupy a hexagonal niche.

The possibility of bacterial communication through chemical pulses, and intimidation or bacterium death due to that communication, is not addressed in this paper's presented method. Despite a constant, one-directional flow in this paper's device, it is still possible for bacteria to communicate this way within the microfluidic device. If this is the case, not all bacterial species within the original sample will have the same chance of surviving once trapped in the device.

With this specific microfluidic device, where each bacterium is allowed its own niche, it is likely that unidentified bacteria have survived within this culture method. The later stage of sequencing one of these bacterial colonies is will be possible after extracting a sample from the device, which would truly reveal if this method for bacterial culturing can better promote for non-adaptive bacteria than previous methods. Future work also aims to develop a technique for securely sampling specific bacterial colonies from within the microfluidic device, possibly with a small pulled-glass or femto-tip needle or by altering PDMS adhesion methods for a removable device. Because this culture method for bacteria is new, there are many directions for further research.

SUPPORTING INFORMATION.
Figure S1. Photolithography and Microfabrication Graphic
Figure S2. Map of Sample Area
Figure S3. Sample Procedure Flow Diagram
Figure S4. Swabbed Petri Dish Results
Figure S5. Full Microfluidic Device Image

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