Low Volume Bacterial Chemostat in Microfluidic Devices Through the Use of Hexagonal Traps.

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BRIEF. Low volume bacterial chemostat: Novel method aimed at rapid diagnosis of bacterial infections.

ABSTRACT. Bacteria are a type of microorganism (tiny forms of life). Pathogenic bacteria can cause bacterial infections that ultimately give rise to severe medical conditions (*e.g.* sepsis). Obtaining rapid and preferably early diagnosis of sepsis is important for reducing patient mortality rates. Proper diagnosis of sepsis is difficult because symptoms, such as rapid pulse, respiratory difficulties and fever are common symptoms to other conditions. Presently, hospitals require an average of 24-72 hours to identify bacterial pathogens and the associated drug susceptibilities from blood samples via blood culture or counting white blood cells. Reducing the time necessary to culture, identify and determine antibiotic susceptibility of bacteria leads to quicker and possibly more accurate diagnosis of infection. Thus, in this work, through the application of new technology (microfluidics), a device containing numerous hexagonal traps with microbe-sized wall openings and corridor spacing was created. This design allows for bacterial entrance, trapping, growth monitoring and ultimately analysis. Bacteria *Escherichia coli OP50* (2 μm long; 0.5 μm in diameter) were pumped through the device in a suspension of Luria-Bertani (LB) broth, which was re-circulated for three hours using a microfluidic pump in a closed-loop fluidic circuit with approximately total volume, This setup allows the bacteria to enter the hexagonal traps and proliferate *in situ*. We were able to detect the presence of bacteria in the bulk sample from growth in the hexagonal chamber that began with a very small number (perhaps as low as a single cell) of starting cells. Future research will be aimed at bacterial labeling, with the goal of bacterial species identification and antibiotic susceptibility in under 24 hours.

INTRODUCTION.

It is important to obtain rapid determination of potentially harmful pathogens in hopes of reducing mortality rates in patients with bacterial infections. Bacterial infections can give rise to life threatening medical conditions such as sepsis – the presence of a threshold level of bacteria in the blood stream that can ultimately affect all organs in the body. Sepsis can cause the patient's organs to become dysfunctional and fail.. Roughly 28-50% of sepsis patients die within the first month of diagnosis [1]. Well over 18 million cases of sepsis occur worldwide each year, 750,000 of which occur in the United States [2]. Of these people who contract sepsis, 14,000 die from the illness in the United States [3]. This complex illness is difficult to diagnose and treat; therefore making an efficient early diagnosis and pathogen determination is important for the treatment of the disease and saving patient lives.

Of the numerous individuals that are affected annually, there are some who are more susceptible to contracting sepsis. For example, post-transplant, post-operative, and even neutropenia patients that are found in the intensive care unit seem to be the most vulnerable. Very young children (1-12 months) may contract sepsis because their immune systems are not completely developed. The number of patients dying from sepsis has doubled within the last 20 years, and it is estimated that the number of severe cases is set to grow at a rate of 1.5% per year [2]. This is due primarily to the strong medications used to treat cancer patients, organ-transplant patients, etc. that weaken the immune system, making these patients more susceptible to sepsis.

In hospitals, an estimated 24-72 hours is necessary to identify bacterial or fungal pathogens and any associated antibiotic treatment from blood culture techniques [2]. Part of the time required for identification may be eliminated if bacterial culturing were to be accomplished on a platform that enables automated microscopic analysis – since the microscopic bacterial colonies may be detected before the colony becomes visible to the naked eye. A simple, flow-through microfluidic device with recirculation may concentrate colony growth in a single plane that can be interrogated by microscopy, and may enable optimization of growth conditions in rich liquid broth and achieve more rapid bacterial growth than what is possible with simple agar plates. Early diagnosis and adequate treatment of pathogens are critical for saving the lives of sepsis patients. Over the past several years, a variety of rapid methods have been investigated for detecting pathogens, including derived immunological assays [9], physicochemical tests based on bacterial growth [4], nucleic acid-based tests [5] and gene based PCR [6].

Rapid pathogen detection through the use of microfluidic devices may aid rapid diagnosis of bacterial infections. Microfluidics is a new technology that enables precise control and manipulation of fluids that are geometrically constrained to a small scale [7] and often a single or multiple precisely defined planes. These inexpensive transparent devices operate by controlling the flow of liquids or gases through a series of tiny channels and valves which can then be observed under a microscope. A common use for these devices in our group is the trapping of live cells with cell-sized traps which are placed uniformly throughout the center of the trap device [11]. The traps have been used to analyze a variety of cells including primary human cells, yeast, and various mammalian cell lines. Once these traps have retained the cells, cell dynamics can be monitored that allow for the acquisition of innovative information pertaining specifically to the cells used. Generally, cells studied within these devices are mammalian cells which tend to be larger than bacterial cells. We have made a simple alteration in the design of the cell trap itself in order to efficiently trap the smaller sized bacterial cells.

This work presents a specially designed microfluidic device containing a plethora of hexagonal chambers with three micron-wide wall openings. The microfabricated device allows rapid trapping, culturing and ultimately analysis of *Escherichia coli OP50 (E.coli)* and essentially functions as a form of micro-chemostat. A traditional chemostat is a large bioreactor containing growing microbes that is constantly replenished with fresh broth at a carefully determined rate that is matched to cell growth rate. As fresh medium is added to the culture solution older media and cells are removed at the same rate to keep the culture volume constant [8]. In the micro-chemostat the bioreactor is the microfluidic device, which has a constant volume because of its constrained geometry. The chamber contains many hexagonal microchambers and is thus not a simple open chamber, but may still allow minute amounts of bacteria to leave through the exit as it is replenished with fresh medium from the entry. The micro-chemostat studied here is intended for one-time "batch" growth of bacteria rather than long-term "continuous" growth. A single bacteria that leaves the corridors of the device and enters the hexagonal microchamber will receive rich nutrients that enable growth and division. After a sufficient number of generations of growth, the hexagonal microchamber will become partly full or full of bacteria which will change the optical properties of the device in that region and allow detection of the micro colony. Depending on the concentration of bacteria, the volume of the sample and the recirculation volume and time, there will fewer or more micro-colonies detectable within the field of hexagons – which will correlate with the activity level of the bulk sample. In situ analysis of the micro-colony may enable identification of one or more bacterial species present in the bulk sample and is an area of active research in our group. Potentially, these

devices could decrease the time required for culturing and identification of bacteria in clinical samples, which may result in earlier administration of antibiotics appropriate and necessary to combat the infection ultimately decreasing the number of associated mortalities.

MATERIALS AND METHODS.

AutoCAD Software.

AutoCAD® is a program developed by Autodesk, Inc. that is used to create designs that are able to be scaled down for printing at the microscopic level. Each of the hexagons are separated by a total distance of 6 μm (Figure S1).

Photolithography.

Photolithography is the process of transferring a pattern to a substrate via optical means. In essence a two-dimensional process in which each patterned layer has the same thickness [7]. Here, the design created through AutoCAD is engraved in a silicon wafer, creating a positive image mask to be used for the fabrication of a device.SU-8, one of the most commonly used epoxy based liquid negative photoresist [4] was spun in order to obtain channels 5.5 microns in height. The characteristics of the negative photo resist used allow for there to be solidification when exposed to UV light, the next step is to cure the design so that there is cross-linking between the mixtures and the stencil. Thus, the stencil is placed above the newly prepared wafer and the UV light is focused from above in order to harden the SU-8 on the silicon wafer. The excess SU-8 is washed away with Isopropyl Alcohol (IPA) leaving behind a positive image of the stencil.

Microfabrication.

The photoresist (SU-8) master mold (created through Photolithography) is cleaned and a mixture of polydimethylsiloxane (PDMS) and Silicone Elastomer Curing Agent is poured onto the master mold. This mixture is then gently poured over the master mold, covering the mold entirely and placed in the oven to bake. Afterwards holes are punched for the inputs and outputs using sharpened syringe tips. In the end, a Harrick Plasma Cleaner is used to plasma-bond the flat surface of the device onto a glass cover slip. Once secured on the glass slip, the device can be used for experimental purposes. (Methods S1).

The Rotary Planar Peristaltic Micropump (RPPM).

The Rotary Planar Peristaltic Micropump is capable of re-circulatory flow, allowing for its use in various mixing schemes. Initial characterization steps have shown that the RPPM is capable of producing a steady, reproducible flow rate making it an essential component of the experimental setup. This minute micropump components are assembled within the pump apparatus (Figure S2). Here, tygon tubing (Cole Parmer Tygon Microbore EW-06418-02) with a 0.51 mm inner diameter is used to connect the pump to the inputs and outputs of the microfluidic device. The device (created via microfabrication) is then primed with deionized water and attached to the stepper motor drive system. RPPM runs fifteen revolutions per minute for three hours using the Arduino Software. The Arduino-based stepper motor controller regulates the flow rate of bacterial solution within the microfluidic device.

Experiment Execution.

First, a tube attached to the input to the RPPM was placed in a solution of LB medium only. Although an exact measure of flow rates in this range is not easily measured, we estimate that the flow rate was approximately 200-1000 nL per minute. The output of the RPPM was attached to the input of the device, and the entire circuit was flushed for 10-15 minutes. Once the circuit was primed, the input of the RPPM was placed in a clear bulk solution containing a 1:10 dilution of *Escherichia coli OP50* (see supplementary methods) to LB medium, and the RPPM only (detached from the device) was primed with the bulk solution approximately 10-15 minutes to ensure there was a consistent flow of solution. Next, the RPPM was attached to the input of the device, and the output of the device was attached to the input of the RPPM to create a closed circuit for recirculation (approximate volume of the circuit was 1-10 μL) [12]. The bulk solution was then recirculated through the device for one hour to ensure bacteria were present within the device. Finally, the RPPM was disconnected from the device and fresh LB medium only (no bacteria) was flushed through the RPPM and tubing. Once the pump and tubing lines were filled with LB medium only, the RPPM was attached to the device for recirculation of the fresh medium for two hours. Image data were taken during this time interval.

Standard Microscopy and Image Analysis.

The microscope used in this experiment was the Nikon Eclipse 50I; facilitating the acquisition of images. The images were taken throughout a two hour long experiment at 10x magnification. This allowed for more of the traps to be observed. Images were taken at ten minute intervals producing a total of twenty-five Differential Interference Contrast (DIC) images. Proliferation of the trapped bacteria was observable over time. Time-lapse images were analyzed through the program ImageJ (NIH), in order to calculate the difference in pixel intensity between the DIC images.

RESULTS.

Six of the twenty five DIC images that were taken are provided in Figure 1. In the rightmost image of Figure 1, at time 0 minutes, all of the hexagonal traps are free of bacteria, thus there is no visible shading within them. Between times T=20 and T=100 there is an extreme change in the amount of bacteria present, thus resulting in the change in contrast mentioned previously. The changes in contrast amongst these images are indicative of bacteria entering the traps. Once the bacteria have entered the traps, they proliferate resulting in densely populated traps.

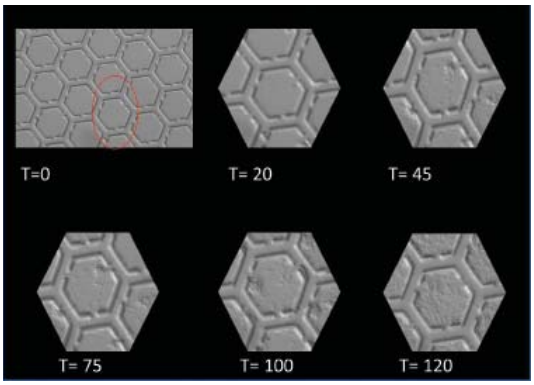

Figure 1. As time progresses, DIC images of trapped *E.coli* are taken for the two hour period while LB medium recirculated through the device. Bacterial cells enter the hexagonal traps through the left and are re-circulated throughout the device, so that the *E.coli* receives the nutrients necessary for division. The area enclosed by the red circle in the first image is indicative of the same trap represented in the other images; shading in trap gets darker with time progression.

To elucidate how much growth occurred within the device over the experiment, Figure 2 depicts an image taken at time zero minutes compared to an image taken at time one-hundred and twenty minutes. It is important to not from the images that the bacterial solution enters from the left and exit from the right.

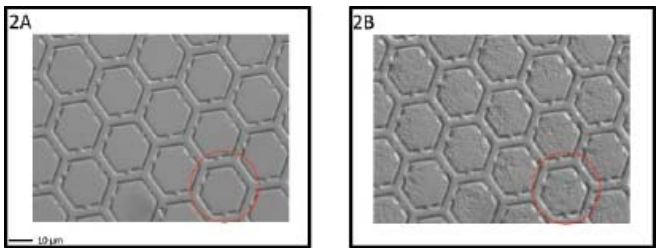

Figure 2. Here, image 2A is representative of the experimental trial at time equals zero minutes and 2B is representative of the experimental trial at time equals one-hundred and twenty minutes Overall, *E.coli* growth within the hexagonal traps is the most evident when comparing images 2A and 2B. At T=0 there is no bacteria present, whereas at the end of the experimental trail, the traps are heavily populated.

From the DIC images taken, a pixel density test allowed for the quantification of bacteria located within each cell. Differences in pixels between each image were then taken and plotted to depict the growth of *E.coli* over the experimental trial (Figure 3).

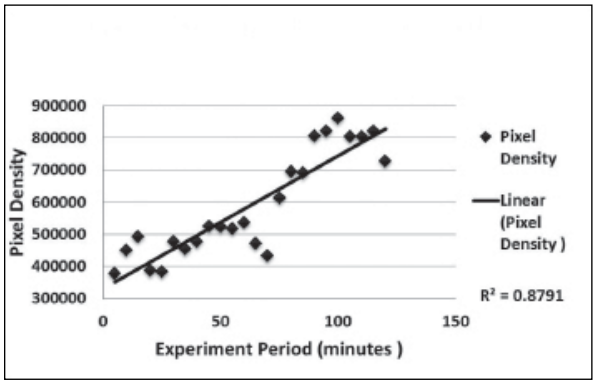

Figure 3. This graph represents the integrated pixel density over a 2 hour period. Each point corresponds numerically to the image that was taken at Image J was used in order to determine the differences in the intensity of pixels amongst each picture taken at the five minute intervals. The fluctuation in intensity levels is due to the un-focusing of the microscope over time. From the data collected, an R^2 value of 0.8791 was formulated.

DISCUSSION.

Although it is difficult to estimate the quantity of bacteria present within each hexagonal trap after the two hour experiment, Figure 1 demonstrates that as time progresses a change in contrast amongst the hexagonal traps with respect to time is notable; between thirty-five and forty percent of the hexagonal traps were occupied by the *E.coli* at the end of the experimental trail. Thus, it can be concluded that the hexagonal traps effectively trapped the bacteria that entered while simultaneously allowing the bacteria to live and proliferate.

Figure 2 is representative of the time lapse DIC images that were taken of the *E.coli* over the two hour experimental trial. Over time, the bacteria that were trapped took in the nutrients that was provided through the LB medium and initiated the proliferation process; the traps became increasingly darker. The progressive change in shading that occurred within the hexagonal traps as time lapsed was indicative of *E.coli* proliferation that occurred as a result of the adequate environmental conditions as well as a constant replenishment of fresh medium solution.

In Figure 3, the fluctuations in integrated pixel density are in essence outliers within the data collected over the two hour period. These fluctuations resulted from microscope issues that caused some images to be out of focus. In order to avoid having images that are unfocused, which makes it difficult to analyze the bacteria at that particular stage of proliferation, additional trials must be carried out to determine the temporal behavior of bacteria. From the data however, an acquired R^2 value of 0.8791 asserting the validity of the changes in shading that occurred amongst images.

CONCLUSION.

The purpose of this study was to determine if bacterial colonies and colony growth could be detected in the hexagonal chambered microfluidic device with an aim toward culturing bacteria quickly for clinical application. The amount of time necessary for bacteria to proliferate within this particular microfluidic device to the point of detection was under two hours. Although the increase in bacteria number is partly due to cell division (proliferation) and partly due to influx following advection from bacteria in the bulk media, it is important that the presence of the colony and its growth were detectable with standard differential interference microscopy techniques. In order to conclusively make a diagnosis of the presence of bacteria in a clinical sample we estimate that an ideal colony size that would be necessary is over fifty percent coverage within each individual hexagonal trap. Over the past several years, a variety of rapid methods have been investigated for detecting pathogens, including derived immunological assays [9], physicochemical test based on bacterial growth [4], nucleic acid-based tests [5] and gene based PCR [6]. It is evident research scientists have a similar goal in mind; developing a novel method that minimizes the amount of time necessary to culturing bacteria. Reducing the amount of time necessary for conventional bacterial culturing processes may lead to much quicker detection of pathogens in human patients which will ultimately allow for both quicker diagnosis of bacteria present and earlier administration of antibiotics necessary to combat harmful pathogens.

In this study, the incorporation of microfluidic devices allowed for the detection of a growing colony of the bacteria *E.coli* in less than twenty-four hours by providing a controlled environment that optimized cell growth conditions. The use of microfluidic devices in order to obtain these novel methods is advantageous in comparison to some of the previous studies because these microfluidic devices require very small volumes of solution. This inexpensive device has allowed for important advances in the development of a novel method useful for both directly observing and analyzing bacterial proliferation. Therefore, this study demonstrated the utility of a microfluidic device with hexagonal traps as a form of low-volume bacterial chemostat for trapping and growing bacteria, specifically *E.coli OP50*, in under 24 hours which may lead to a faster clinical lab test.

Future research includes the incorporation of bacterial labeling by targeting unique proteins with fluorescent markers. It is hypothesized that once the bacterium has proliferated within the device, these trapped bacterial cells can be labeled if their cellular membranes are lysed exposing the cytosol and nuclei containing the necessary proteins and DNA.

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SUPPORTING INFORMATION.

Figure S1. AutoCad Device Design

Figure S2. Rotary Peristaltic Micropump

Supplementary Methods. Cell Culturing

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