UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Experiment on Bacterial Growth in Dense Colonies in Microfluidic Device

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Chemical Engineering

by

Yang Wang

Committee in charge:

Professor Alexander Groisman, Chair
Professor Kalyanasundaram Seshadri
Professor Forman Williams

2015

The thesis of Yang Wang is approved, and it is acceptable in quality and form

For publication on micro film and electronically:

_____

_____

_____

Chair

University of California, San Diego

2015

DEDICATION

To my parents, Tianzhong Wang and Yanping Song, thank you for always supporting me to chase my dream and showing me how to become a good person.

我爱你们

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

This thesis would never have been finished without a great deal of help from many people whom I would like to thank.

First of all I would like to thank my advisor, Professor Alex Groisman, for giving me the chance to study in his research group as well as assigning me the very interesting project to work on. No matter busy or not, Alex had patient explanations ready whenever I had difficulty with the experiment. I would also thank Alex for his recommendation letter so that I received the admission to pursue my Ph.D degree in the future.

Dr. Edgar Gutierrez is a veritable panacea in the lab. He always helped me with my work even he is busy doing something else. Meanwhile his shockingly vast knowledge and expertise always helped me solve some annoying problems and saved my day.

Dr. Mya Warren is really nice and always helped me when I met some difficulty in biological area. Due to the lack of equipment, I ran out of experimental material like the bacterial growth medium or bacterial strain plate sometimes. But Mya always gave me supplement as soon as possible so that I have never wasted time due to the finish of these materials.

Also, I would like to thank other people in the group. Kaps is really hilarious and always make the lab full of fun. He is also an accommodating man and always tried to give me help even I did not ask him. Alex Yang and Ward are also very nice

to work with. It was really my pleasure to work with these guys.

Furthermore, I have to thank my roommate Jixia Ren, a really nice guy to live and study with.

He has always been helping me with the problem I encountered in both study and life. Without his help I could have had much more difficulties in my graduate study. I also have to thank Xing Xing and Yuan Chen, and Bowen Zhang, without the support from these friends, my graduate life would not have been so happy.

Many thanks go to Professor Forman Williams and Kalyanasundaram Seshadri for serving as my defense committee and recommending me to the Ph.D programs I applied.

Finally I would like to thank my parents who raised me up and always support my study in every way in the past twenty-four years. For all the years, they not only guide me to work and study hard, but also teach me to be a good person, enjoy my life and help others. Without their firm support and patient education, I would never have been keeping progressing in both my daily life and my academic life.

ABSTRACT OF THE THESIS

The Experiment on Bacterial Growth in Dense Colonies in Microfluidic Device

by

Yang Wang

Master of Science in Chemical Engineering

University of California, San Diego, 2015

Professor Alexander Groisman, Chair

Microfluidics has shown tremendous development in the past years because of its applications in biological and chemical research. Microfluidic devices can be easily and inexpensively fabricated through rapid phototyping and soft lithography with silicone elastomer. Microchannels allow laminar flow and diffusion. With these properties, flow behavior can be predicted accurately and controlled precisely. Thus, microchannels allow new functionalities that are unavailable on macrochannels.

Microfluidic chemostat has been designed and studied,[1,21] and it is able to maintain chemostatic condition and without flushing the cells out.[1] In this thesis, the hydrogel is used as the main function area of the microfluidic device to easily maintain the chemostatic condition for the cell growth and keep the cells from escaping at the same time, which could be utilized as the device to study the response of bacterial cells to mechanical forces.

1. INTRODUCTION

Microfluidics, as the name implies, refers to the fluids flow in the channel with the size of micrometer. In this situation, fluids behave differently from those in traditional macro scale situation. Microchannels allow fluids to flow in a laminar and steady manner with molecular diffusion as the sole mixing process. Thus, microchannels allow accurate prediction and precise control of fluid flow. These features are useful in biological, chemical, and medical applications. Moreover, the extremely small sample volume in microfluidic devices allows the use of small amounts of expensive reagents. Finally, modern microfluidic devices can be fabricated easily, rapidly, and inexpensively, and disposable chips instead of the expensive large fluid system are used.

Despite the above-mentioned advantages, relatively few microfluidic devices are commercially available.[2] The main reason is that, up until now, there is no emergence of so-called “killer app” as known as high value application. For instance, new types of bioassay for monitoring patient response to therapy and for detecting disease biomarkers at an early stage remain to be developed.[2] However, it is highly likely that the researchers can develop some of high value applications for microfluidic technology, because the microfluidic device, as mentioned before, can be fabricated cheaply, disposable, and provide rapid result, for example, the paper based

microfluidic device developed by Whiteside et al.[3] Currently the microfluidics diagnostic for the infectious disease in the developing countries is one field where the microfluidic technology occupies a significant position.[4]

Even though microfluidics has yet to be commercialized, it is an active field of academic research. The application in the research area varies from biological analysis to chemical synthesis and from DNA sequencing to cell Biology. For example, microfluidic devices can be used to screen conditions for protein crystallization.[5,6] As shown in figure 1, multiphase flows can be manipulated to produce monodispersed droplets (Romero's group), which can be utilized as the platform for high-throughput biochemistry. Traditional assay may require a lot more reagent for the same reaction than microfluidic device. Excellent references for this area include conferences (e.g., Transducers, Micro Total Analysis Systems, and High-Performance Capillary Electrophoresis) and journals (e.g., Analytical Chemistry, Sensors & Actuators, Electrophoresis, Microfluidics, Nanofluidics, Biomicrofluidics, and Lab on a Chip).[7]

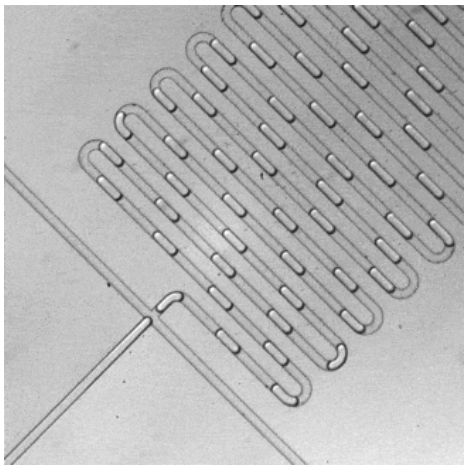

Figure 1. Microfluidic device that can produce monodispersed droplets

Microfluidic technology is still in its infancy; thus, a great deal of work is warranted. The fundamentals of microfluidic technology are strong, and its capability of fluid manipulation, requirement of small reagent volumes, and precise control over concentration make this technology highly desirable worldwide.[2]

This thesis focuses on the design and application of a novel microfluidic device as a chemostat to study the response of bacterial cells to mechanical forces.

1.1. Flow and Diffusion in Microfluidics

Microfluidics is the manipulation of fluids in the channels and chambers with length scale of 1-100 microns. Due to the small scale of these channels and chambers, which ranges from single cell to bacteria colony, the microenvironment of the experiment can be easily controlled.

In the area of fluid mechanics, the most fundamental equations are the Navier Stokes equation, which is the equation of motion, or the expression of Newton's second law in the fluids, together with the continuity equation. With the assumption of the fluid considered as incompressible, which is true in most situations (except some extreme condition, which could never be approached) and Newtonian, which means the shear stress and strain rate are linearly related by viscosity, the Navier–Stokes equation and continuity equation can be respectively expressed as $\frac{\partial \vec{v}}{\partial t} + (\vec{v} \cdot \vec{\nabla})\vec{v} = -\frac{1}{\rho}\vec{\nabla}p + \nu\nabla^2\vec{v}$, and $\nabla \cdot \vec{v} = 0$. Where $\vec{v}$ is the flow velocity, ν is the kinematic viscosity, ρ is the density of the fluid and p is the pressure exerted on the fluid. The Navier-Stokes equation could be nondimensionalized by using the characteristic length scale, L_0 and velocity scale, V_0 to express the physical quantities. The dimensionless Navier-Stokes equation can be expressed as $Re\left[\frac{\partial \vec{v}^*}{\partial t^*} + (\vec{v}^* \cdot \vec{\nabla}^*)\vec{v}^*\right] = -\vec{\nabla}^* p^* + \nabla^{*2}\vec{v}^*$, where the star denotes the variables as dimensionless and $Re \equiv \frac{V_0 L_0}{\nu}$ is the Reynolds number. The Reynolds number is the ratio between inertia and viscous forces in the fluid flow. When the $Re \gg 1$, the left side of the dimensionless Navier-Stokes equation, which is the convective terms, dominates, and

the flow is turbulent, which is hard to simulate and predict. However, when $\mathrm{Re} \ll 1$, the right side of the dimensionless Navier-Stokes equation, which is the inertial terms, dominates. The equation can be simplified into the Stokes equation $0 = -\vec{\nabla}^* p^* + \nabla^{*2}\vec{v}^*$, the flow is a laminar or layered flow in which fluid streams flow in parallel to each other and mix only though molecular diffusion[8]. Typical flows in microfluidic device have velocity, characteristic length, and kinematic viscosity of 0.01-1000 mm/s, 0.01-0.1 mm and 1 mm^2/s respectively, yielding a Reynolds number of $10^{-4} - 10^2$. Even when the flow speeds in microchannel exceed 1 m/s in microchannels, the Reynolds number is likely to remain relatively low (<100), and the flow is likely to remain laminar and stable.

The fluid flow in microfluidic devices is usually laminar and steady. Hence, the mixing of fluids in these devices differs from that in macrofluidic devices; the mixing of fluids with laminar and steady flow also differs from that of fluids with turbulent flow. Fluid streams on a macroscale usually mix uniformly through convection. However, laminar and steady flows in microfluidic devices usually mix through diffusion. For example, two streams of water with red and black dyes that flow side by side in the same channel or chamber of a microfluidic device will continue to flow side by side and mix through diffusion.

The characteristic time for a molecule to move a distance r is given by the relation $t_{diff} = \frac{r^2}{2nD}$, where n is the number of dimensions at where the diffusion occurs, D is the diffusion coefficient. To mix two side-by-side streams well, the residence time in the channel should be several times the characteristic time, with r

equal to half of the channel width. This is an important relation, for instance, in the design of the device, the length of the channel should be long enough, and the motivation should be suitable to provide sufficiently low velocity of the flow, so that the residence time of the fluids in the channel is adequate for well mixing. Table 1 is some relevant diffusion coefficients.

Table 1. Relevant diffusion coefficient of some substances

Materials	Diffusion coefficient (cm^2/s)
Nitrogen, oxygen in air	~ 0.2
Nitrogen, oxygen in water	~ 2×10^{-5}
Nitrogen, oxygen in PDMS[9]	~ 3×10^{-5}
Small molecules in water	~ $(0.5 - 1) \times 10^{-5}$

1.2. General Design and Fabrication Methods

The microfluidic devices are designed and fabricated using the standard soft lithography techniques[10] depending on the target function. Figure 2[11] shows a schematic of the standard fabrication of microfluidic devices. The dimensions of microchannels are dictated by the specific experiment or assay. In cases when microchannels need to have different depths, separate masks are designed for each depth. Other practical issues, such as the desired flow rate, also determine the dimension of the channel.

The design process starts from sketching the device by using vector-based graphic software programs, such as Macromedia Freehand, Solidworks, and Auto CAD. In this project, Professor Groisman designs the device features by using the Freehand 9.0. In general, a single device is designed separately into multiple versions with different capabilities by varying some device parameters, such as channel and chamber sizes. Then, the optimum design is selected from these versions. The different versions are arranged side by side on a large sheet, which is then sent to a high-resolution printing facility to produce high-resolution photomasks. These photomasks are plotted in black ink on transparent mylar films. The printing of the photomasks is not performed in the laboratory. The individual masks are cut out and glued to a 4″×4″ glass slide with the printed side out.

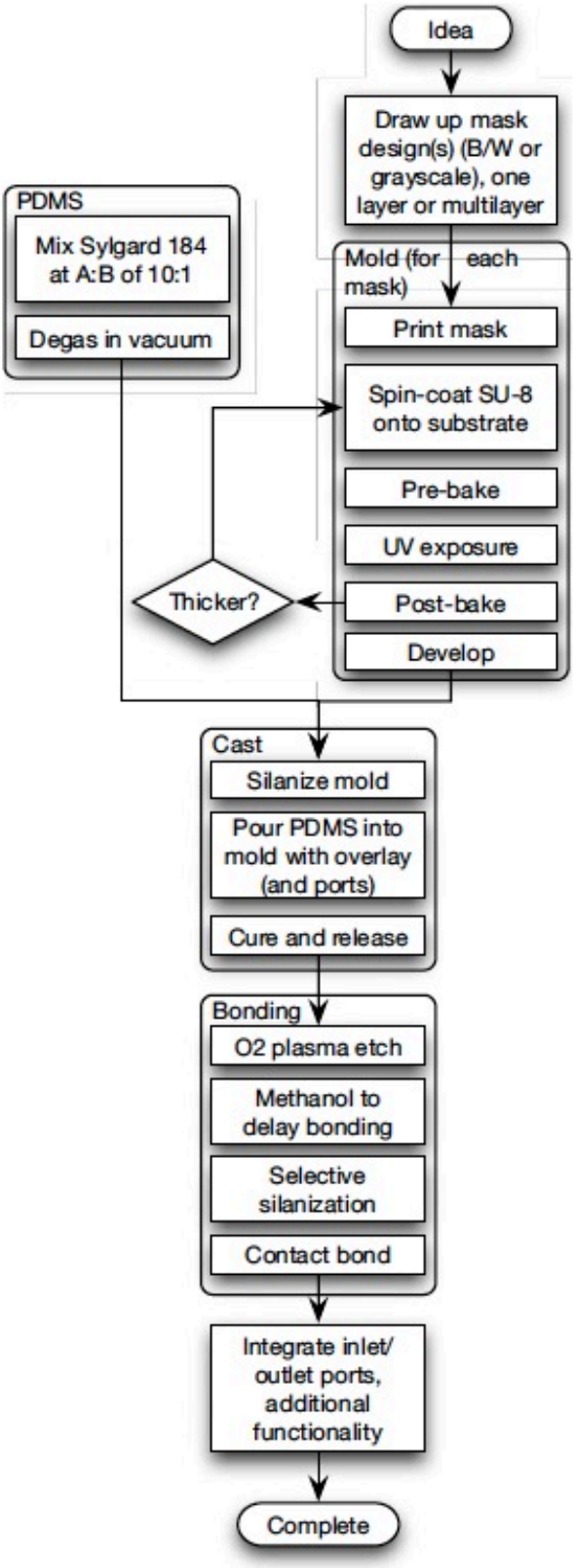

Figure 2. Schematic of a general fabricating microfluidic device with PDMS

Printing of the photomasks has certain limitations, such as the smallest size allowed to print reliably. Chrome masks can be ordered when small or fine features are required, but these materials are difficult to procure and expensive.

The next step in the fabrication process is the preparation of a silicon master mold through contact lithography with the previously printed photomasks. A photoresist layer is spin-coated on a 4” or 5” polished silicon wafer. The desired thickness of the layer is achieved by regulating the spin coating speed. In general, photoresists are of positive and negative types. In positive photoresists, the area exposed to light becomes soluble in the photoresist developer and therefore can be washed. Meanwhile, the portion unexposed to light remains insoluble to the developer and thus remains on the silicon wafer. In negative photoresists (e.g., SU-8 family), the area unexposed to light is soluble to the developer and therefore can be washed. Meanwhile, the exposed area becomes insoluble and thus remains on the silicon wafer. Negative photoresists are usually used because of their wide selection of thickness, and multi-level patterns are easy to implement with these photoresists. After the spin-coated photoresist layer is prepared, the wafer with an uncured-epoxy layer is pre-baked on a hot plate at 65 °C and then at 95 °C. The face side of the photomask is brought into contact with the photoresist, the wafer is exposed to UV light through the photomask, and post-baked on a hot plate at 65 °C and then at 95 °C to cross-link the photoresist in an exposed area. If the microfluidic device has channels with different depths, another photoresist layer is spin-coated on the wafer and placed on a mask aligner to align the mask appropriately with respect to the existing pattern. In this step,

care is taken to perfectly align the feature in two layers; otherwise, the device will not function as expected. The wafer is again exposed, post-baked, cooled, and then immersed in the bath of photoresist developer to remove the uncured photoresist and prepare the master mold. The finished master mold is usually baked in an oven at 150 °C to increase the adhesion between the epoxy and the wafer. To help the mold survive multiple castings, the wafer surface is treated with trimethylchlorosiloxane (TMCS) vapor for 2−5 min. Without this treatment, the surface is adhered to the polydimethylsiloxane (PDMS) pre-polymer, and the photoresist pattern on the wafer might be peeled off during the next step. Finally, the master mold is ready to use.

The final step of fabrication is to cast a PDMS replica from the master mold. The PDMS, as the most common material used to fabricate microfluidic devices, has several important properties. For instance, this material is transparent in the visible spectrum, allowing bright-field illumination of the channels from the top, through the bulk of the chips. It also has a very low auto-fluorescence background, making the chips compatible fluorescence microscopy. Fully cured PDMS is non-toxic and cell-compatible. Finally, PDMS is porous and approximately six times more oxygen permeable than water. In this step, Sylgard 184 by Dow Corning is the commonly used formulation of PDMS. This product has base and cross-linker components. The manufacturer recommends the ratio of 1:10 between the cross-linker and the base, but other ratios ranging from 1:5−1:20 can be employed to achieve different hardness levels of the cured PDMS. Reducing the proportion of the cross-linker in the mixture generally leads to softer formulation of PDMS.[12] Before the cast, an aluminum foil

cup is prepared to place the wafer and hold the PDMS pre-polymer. Then, the two components of the PDMS are mixed with a certain proportion. The mixture is rapidly poured onto the wafer surface. Then, the pre-polymer covered wafer is placed in a vacuum chamber to be degassed to remove the air bubble. Bunsen burner can be used to flame the liquid and rapidly remove the bubble on the top of the liquid. Then, the degassed pre-polymer is baked at 85 °C for 45–90 min to rapidly cure the pre-polymer into a solid. Afterward, the PDMS solidifies into flexible silicone rubber, and the bottom of the PDMS, which has contact to the master mold, becomes a replica of pattern on the wafer surface. The PDMS cast is carefully peeled off from the wafer to avoid damaging the PDMS and the photoresist. Once the PDMS is safely detached, it is cut into pieces to fit a cover glass for microscopic observation. Holes are punched by a sharpened hypodermic needle attached to a luer holder to create inlets and the outlets of the microfluidic device. Then, the chip is bonded to a glass cover slip by baking in an oven at 80 °C for 1–24 h. If the experiment requires a multi-layer device, i.e., when the temperature needs to be controlled by the continuous flow of cool water, the control layer should be prepared using another master mold, and a thin flow layer should be prepared through spin coating. The thin-flow layer of PDMS is cured for 10–20 min to solidify but remain sticky, and the control layer (with the regular thickness of the chip) is placed on the sticky flow layer on the wafer. The alignment is based on the pattern of the flow layer. The two layers are cured at 85 °C to become a monolith. The device is peeled off and cut, and holes are punched for the inlet and the outlet. Finally, the glass slide is sealed onto the chip.

1.3. Standard Experimental Setup

A Nikon TE2000 inverted fluorescence microscope is used to characterize the device and conduct other experimental procedures. A camera is attached to the side port of the microscope to record images through Fire-i software.

Fluids fed into and drawn from the device are stored in separate reservoirs, usually in plastic syringes that are held vertically with the luer connectors down. All syringes are attached to different moving stages sliding along vertical rails next to the microscope. Syringes are connected to the device with plastic tubing and short hypodermic needles (capillary) inserted into the inlet and outlet holes punched in the PDMS device.

The flows in the device are driven by applying differential pressure between the inlet and the outlet. The syringe containing the fluid fed to the device is raised with respect to the outlet syringe. However, both syringes are placed above the device to avoid the formation of air bubbles by negative pressure, given that the atmospheric air is driven into the microchannel through the porous PDMS chip.

1.4. Hydrogel

Hydrogel is polymer with high water content. This polymer is a network of hydrophilic polymer chains and is sometimes in which water is the dispersion medium. This gel can contain natural or synthetic polymeric networks.

In this project, agarose is used as the hydrogel to build the chemostat. Agarose has been used in microfluidic devices for single-cell studies [13] and bacterial chemotaxis analysis[14], Agarose is a polysaccharide polymer generally extracted from seaweed. It has a linear polymer structure with repeating units of the disaccharide agarobiose, which is composed of D-galactose and 3,6-anhydro-L-galactopyranose. Agarose is purified from agar by removing another principal component, agaropectin. Agarose is usually used in molecular biology to separate large molecules, especially DNA, through electrophoresis. In this project, agarose is used for 3 main reasons. First, agarose has strong ability of absorbing water and is more permeable to aqueous solution than PDMS[15], and small numbers of cells can deplete the local nutrient environment over small distance scales[16]. Second, PDMS is relatively rigid and can mechanically damage cells, if pushed against them.[17,18,19]. The gap between the flush channel and the bacterial growth chamber in the old design[1] is not necessary because agarose is permeable to small and medium molecules but not to bacterial cells.

1.5. *Escherichia coli*

E. coli is a Gram-negative, facultative anaerobic, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms).[20]

E. coli is the most widely investigated prokaryotic organism because it can be grown and cultured easily and inexpensively in the laboratory. Furthermore, the reproduction of *E. coli* only takes 40 min under favorable conditions. This organism is used as a carrier for recombinant DNA in biotechnology and microbiology.

1.6. Organization of the Thesis

This thesis focuses on the design and the operation of the microfluidic chemostat for the study of quantitative cell biology.

Chapter 1 introduces the origin and development of microfluidics, the basic physical principles used in microfluidics, the general design and fabrication, the standard experimental setup, and the main innovative material used in the project.

Chapter 2 provides the methodology of the project, especially the design, operation, and the experiment performed to test the microfluidic device.

Chapter 3 shows the data, including the images and the results collected from the experiment.

Chapter 4 discusses the result.

Chapter 5 gives a brief conclusion of the project.

2. METHODOLOGY

2.1. Design of the Device

The microfluidic device designed in this project is used as a special chemostat to confine the growth of bacterial cells in a small chamber with different sizes while keeping the fresh medium flowing in from outside reservoir. Thus, the environmental conditions in each channel are always identical to the nearby channels and favorable to the growth of bacterial cells. The device facilitates the growth of bacterial cells from a single cell to a high density. Hence, the response of cells to the mechanical force can be analyzed through the device.

Professor Groisman's lab had created the microfluidic chemostat allowing the growth of cell to high density and the analysis of cell responding to exogenously added autoinducer.[1] A similar microfluidic chemostat used to study the highly self-organized behavior of bacteria cells response to the cope with the local environmental challenges[21]. In the first design, the entire device is composed of PDMS, which endows flexibility, to facilitate cell loading, the depth of the capillaries connecting the channels and chambers can be controlled by regulating the gauge pressure inside the device to make it permeable (at high gauge pressure) and impermeable (at low gauge pressure) to the cells[1].

The second device has three main differences compared with the first one. First, the depth of the chamber in the second design is much smaller than that of the

chamber in the first one. Thus, the device can create a monolayer of cells and detect cellular responses at a single cell resolution. Moreover, the second device has no capillaries, indicating that no physical barriers for the cells enter and exit the chamber. Although the distances between channels are the same, the chambers considerably vary in shape. Moreover, posts with different shapes are found inside the chamber as the inner boundaries.[21]

Improvements have been made in designing the device in this project on the basis of the two previous devices. Unlike the main functional areas of the devices mentioned before, that of the present device is not made of PDMS. The PDMS chip only holds the device core, which is made of hydrogel (agarose), and provides vacuum pressure to facilitate the attachment of the device on a glass slide. However, the main functional area of the device in this project is still similar to those of the previous two devices in some aspects. That is, the area contains an array of parallel channels for the continuous flow of fresh medium and parallel rows of chambers for the growth of cells (Figure 5). The channels are approximately 5000 μm long and approximately 50 μm wide, whereas the chambers are approximately 1650 μm long and approximately 10 μm wide. The inlet and outlet of the device are circular with a radius of approximately 500 μm. Also, the design in this project contains no capillaries between the adjacent chambers and channels, and the chambers and channels are directly connected at the upstream ends of the chambers. Figure 5 shows that the direction of the flow is downward, with the three circles on the top serving as the inlets (only the one in the middle is punched to use in the experiment) and the one

circle at the bottom serving as the outlet. An array of 12 flowthrough channels and 33 long chambers divided into three levels from top to bottom with 11 chambers are found in each level. The two main inlet channels connected to the middle inlet merge at a T junction. On the inlet and outlet sides, the branching from a single channel into an array of 12 parallel channels is binary and symmetric. This arrangement leads to equal flow rates through the channels and highly balanced pressures at the two inlets of the T junction from where the cells flow into the chambers. In addition, since the chambers is are dead-ended, and the depth of the chambers is much smaller than that of the channels (6 μm versus 30 μm; Figure 3), the fluids flow through the chambers are small, such that the flow of the nutrients depends on the percolation of the medium through the porous agarose hydrogel. In this design, cells are loaded into the device by injecting the cell suspension from the inlet and waiting for the cells enter the channels. When at least one cell reaches the dead end of the chamber, the fresh medium can be flushed in the flowthrough channels.

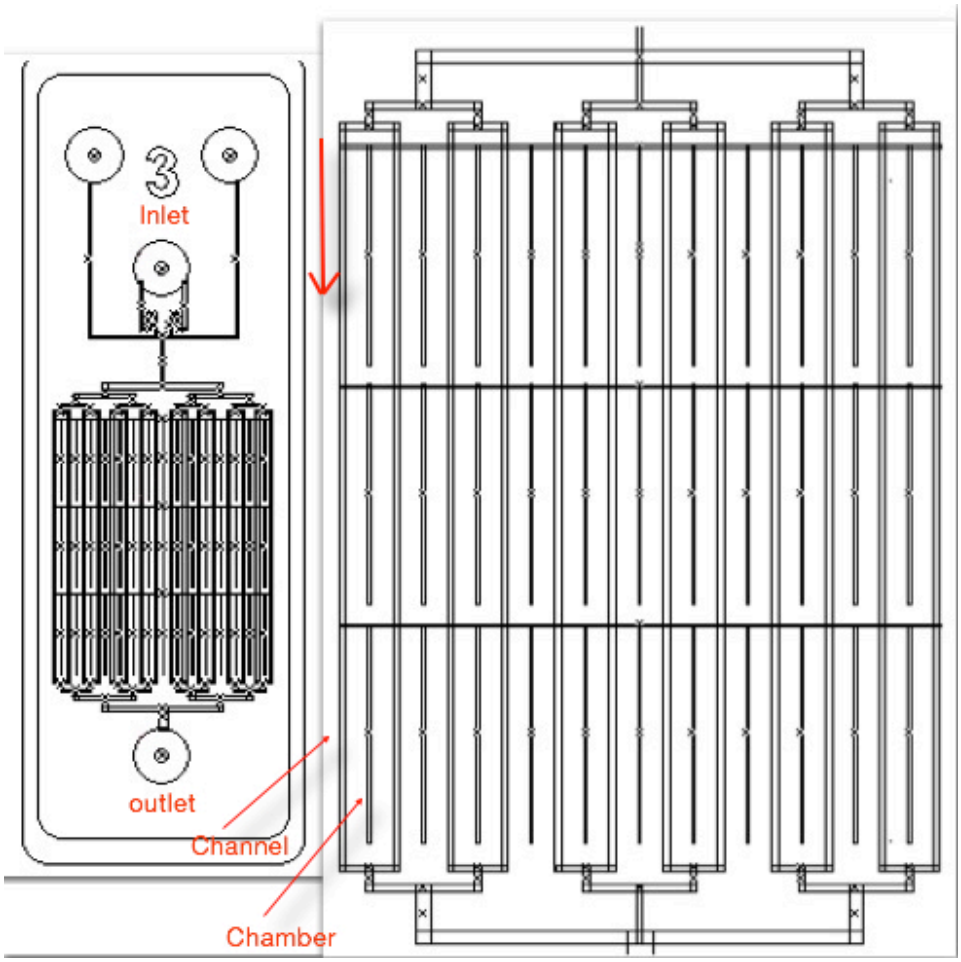

Figure 3. Design of the device

2.2. Fabrication of the Device

The microfluidic chemostat in this project can be divided into two parts: the PDMS frame and the agarose part (main functional area). In addition to the master mold as in the general fabrication process, two PDMS molds (intermediate and final) are needed in this process because the functional area is made of agarose.

2.2.1. Fabrication of the Master Mold

The first part of the fabrication process is to make the master mold. The SU-8 2000 series is used as the photoresist. SU-8 2000 is an improved formulation of SU-8, which is widely used by MEMS (Micro-electromechanical System) and in microfluidic device fabrication. Approximately 0.5–200 μm can be achieved as a single coat process. In addition, SU-8 2000 has high optical transmission, which makes it ideal for imaging near-vertical sidewalls in thick layers with wavelength larger than 165 nm (Figure 4). Before the photoresist is spin coated, the wafer is cleaned with solvent and blow-dried. Then, the wafer is placed in the spin coater and attached to the plate by the vacuum. The process speed and time are selected on the basis of the data shown in Figures 5 and 6.

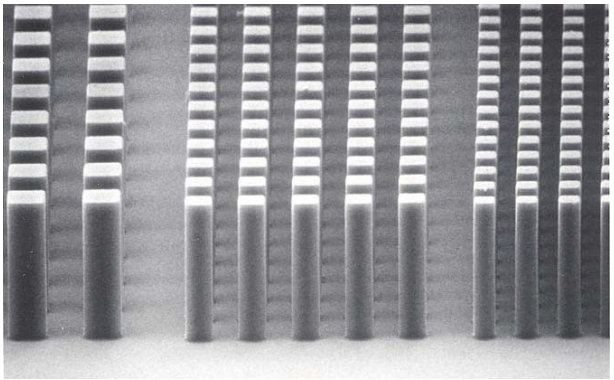

Figure 4. 10 μm features, 50 μm SU-8 2000 coating[22]

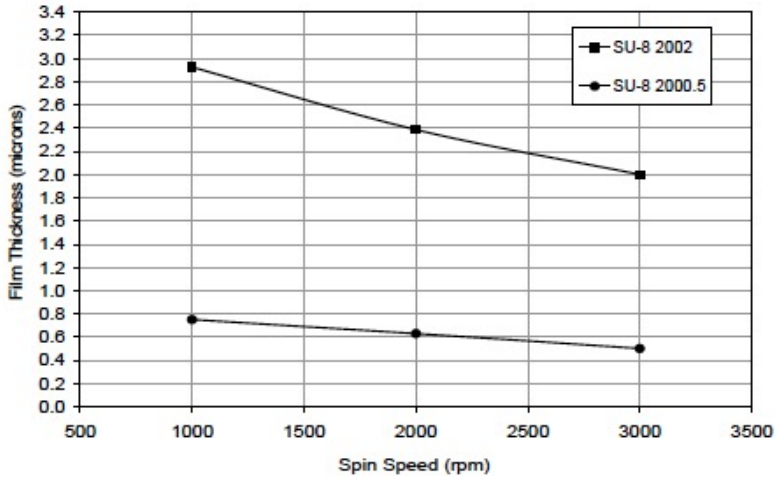

Figure 5. SU-8 2000 Spin Speed versus Thickness a[22]

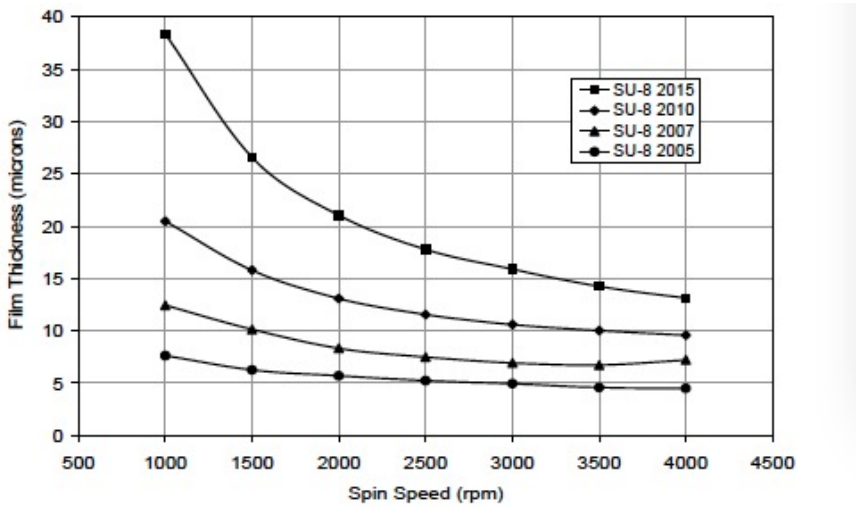

Figure 6. SU-8 2000 Spin Speed versus Thickness b[22]

SSU-8 2005 is used for the first photoresist layer to make a 6 µm-thick layer. The program is set to 500 rpm for the first 5 s and to 2000 rpm for the last 30 s. Then, the wafer is carefully detached from the plate and placed on a hot plate at 65 °C for 1 min and 95 °C for 2 min for the pre-bake. After cooling down, the wafer is placed on the UV mask aligner. The vacuum line is used to attach the wafer to the plate, the air line is used to clean the photomask through blow drying for the first layer, and then the mask is fixed on a metal frame connected with the vacuum line. The photomask is

aligned with respect to the existing relief of cured photoresist on the wafer. The mask height is also adjusted to enable contact with the first photoresist layer. After regulating the position, the wafer is exposed to UV light for 4–5 min through the photomask, and then the mask frame and the wafer are carefully removed from the system. The wafer is placed for 1 min on a 65 °C hot plate and then for 1 min on a 95 °C hot plate for post-bake. After cooling down, the wafer is subjected to the second lithographic process to complete the pattern on the master mold. For the second layer, SU-8 2015 is used as the photoresist, and the program is set to 500 rpm for the first 5 s and to 1100 rpm for the last 30 s. The process on the first layer is repeated to spin coat the second photoresist layer. For this layer, the pre-bake is 1 min at 65 °C and 3 min at 95 °C. The machine for spin coating should be cleaned with acetone to prevent the cap from sticking to the machine. The photomask is aligned with respect to the patterns on the wafer by using the mask aligner and the fiduciary marks on the wafer. In this step, care should be taken to perfectly match the marks; otherwise, the microchannels and microchambers might be misaligned, and the device might malfunction. After the alignment, the wafer is exposed to UV light through the mask for 5 min and then post-baked at 65 °C for 1 min and then at 95 °C for 3 min.

The next step is to develop the wafer with the developer. After cooling from the post-bake, the wafer is placed in a glass container with the developer on a shaker for 3 min and then rinsed with the developer to completely remove the unexposed photoresist (soluble to the developer). Isopropanol is used for the final rinse, and then the wafer is blow dried with air. The final step in making the master mold is to treat

the wafer with TMCS vapor for 3 min to salinize the surface. This treatment prevents the PDMS replica from attaching to the surface in the next step. The remaining photoresist pattern is not peeled off and can be reused. Finally, the master mold is ready to work as shown in Figure 7.

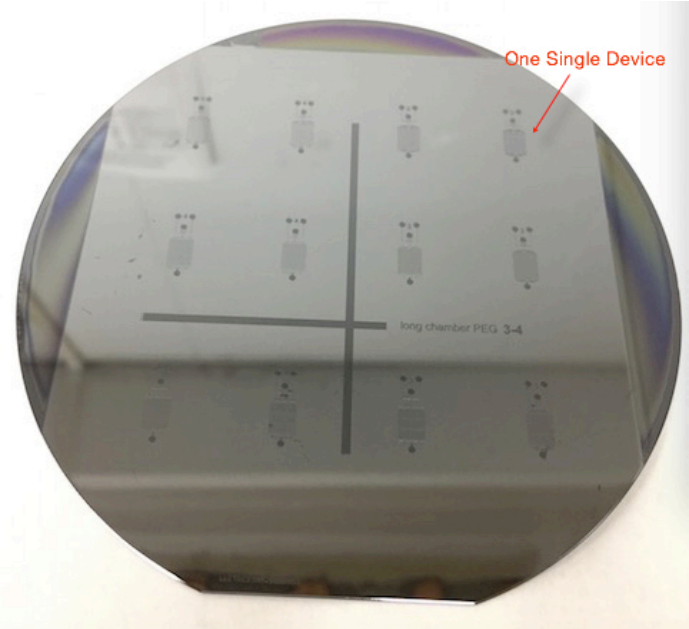

Figure 7. Master Mold

2.2.2. Preparation of the PDMS Mold

The second part of the fabrication process is to make the PDMS mold for the cast of the device. This step is not completely same to the general fabrication process because the main functional area of the device is not PDMS but agarose. Therefore, the device is cast by using the PDMS mold instead of the master mold. To prepare the PDMS replica of the master mold, Sylgard 184 is used as the formulation of the pre-polymer, and the recommended ratio of 10:1 between the base and the cross linker is used. To make the replica with an appropriate thickness (0.5−1 cm), 40−50 g of the base and 4−5 g of the cross linker are mixed in a plastic beaker on the basis of the wafer area. The beaker is placed in a mixer to evenly combine the two components. Before pouring the mixture on the wafer, the wafer is surrounded with aluminum foil to form a “boat” that confines the pre-polymer on the surface and then cleaned with blown air to ensure the absence of dirt on the surface. After pouring the mixture on the wafer, the wafer is placed in a vacuum chamber to degas and remove air bubbles. The disappearance of bubbles on the surface takes several minutes; alternatively, a Bunsen burner can be used to remove these bubbles. Then, the wafer is incubated at 60 °C and 90 °C for 15 and 30 min, respectively, to cure and solidify the PDMS. In this step, the wafer must be positioned horizontally to distribute evenly the thickness of the replica. After the incubation, the PDMS replica is carefully peeled off from the wafer and formed as a square by cutting the edges.

The prepared replica cannot be used as the mold to cast the device yet because

the pattern on the replica is the same as the desired pattern. A mold should have pattern that is the mirror image of the desired pattern. Therefore, the first replica made from the master mold is called the intermediate mold (Figure 8). The intermediate mold is used to prepare the final mold for casting the device.

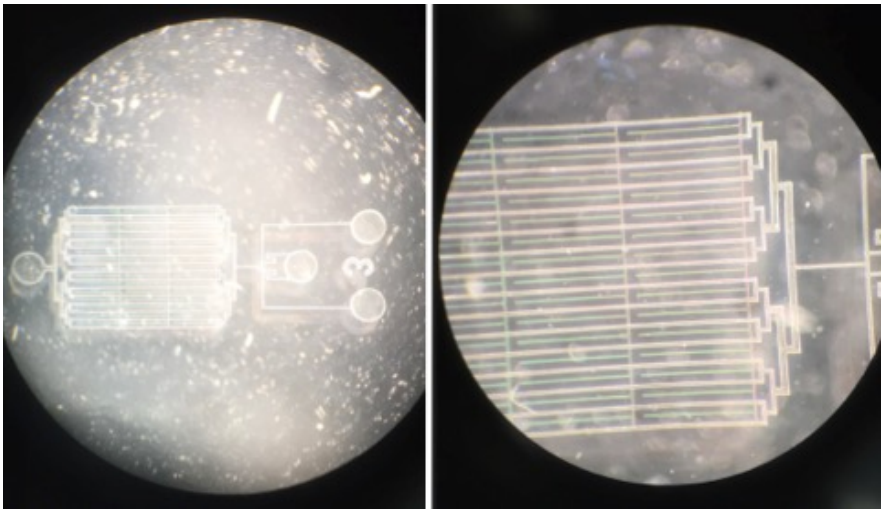

Figure 8. Micrographs of the pattern of a single device on the intermediate mold

Before casting the final mold, the intermediate mold is treated with air plasma for 10 s to functionalize the surface. Plasma is a partially ionized gas; thus, some electrons and ions are free, whereas some are bound to an atom or molecule.[23] Gases such as nitrogen, hydrogen, and oxygen can dissociate and react with the substrate surface during the air plasma treatment. With these gases, the air plasma treatment creates chemical functional groups. This surface treatment greatly differs from the most commonly used method for PDMS surface modification.[24-27] After the plasma treatment, the intermediate mold is again treated with TMCS vapor to prevent the final mold from sticking during peeling off. After the TMCS treatment, the intermediate mold is incubated at 80 °C for 10 min. Then, the PDMS pre-polymer is

prepared for casting the final mold. The thickness of the final mold is still between 0.5 and 1 cm but slightly thicker than the intermediate mold. Therefore, 50 g of the base and 5 g of the cross linker are mixed to prepare the pre-polymer. Before pouring the PDMS pre-polymer, the intermediate mold is cleaned with blown air and adhesive tape. The homogenous mixture is poured onto the surface of the intermediate mold, which is in a foil boat. Then, as casting the intermediate mold, the mixture is degassed in a vacuum chamber to remove air bubbles. The pre-polymer is incubated at 80 °C for about 2 h, and then the final mold is cured and solidified. The final mold is peeled off from the intermediate mold and formed into a square by cutting the edges. Thus, the final mold is ready to use.

2.2.3. Preparation of the PDMS Frame for the Device

The third part is to prepare the PDMS holder or the frame of the device. As shown in Figure 9, the plastic mold with a depth of approximately 4 mm is pasted on a new wafer. This depth allows the vacuum area to provide pressure for holding the device tightly on the glass slide. The plastic mold contains two components. The first component provides the vacuum pressure; the other component is pasted on the wafer in the middle of the first component for the functional area. After being properly pasted and cleaned, the mold is placed in a foil boat as previously mentioned. The pre-polymer mixture (60 g of the base and 6 g of the cross linker to create a 1 cm depth) is poured onto the surface of this wafer. Then, the pre-polymer is degassed and baked as above mentioned. After solidification, the PDMS mold is peeled off and cut into single devices as shown in Figure 10.

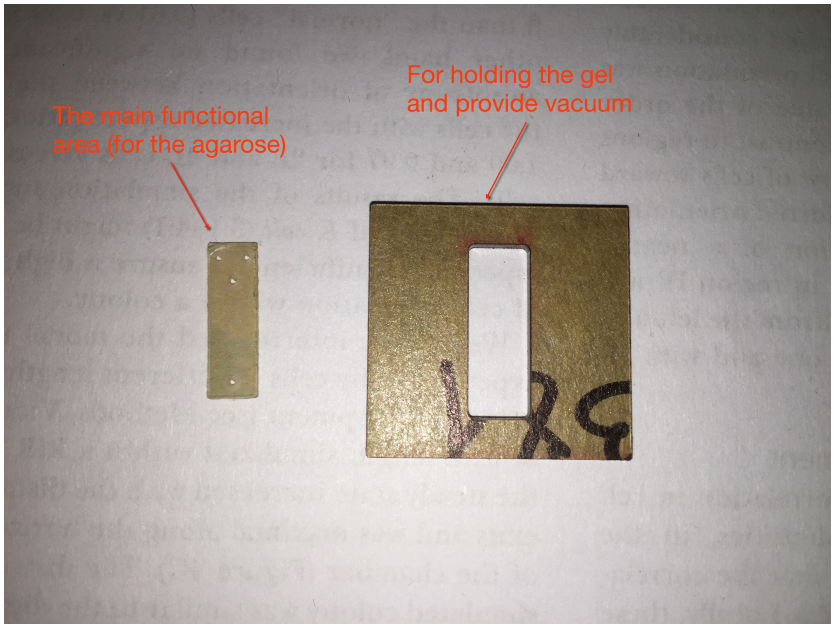

Figure 9. Plastic mold for the PDMS frame of the device

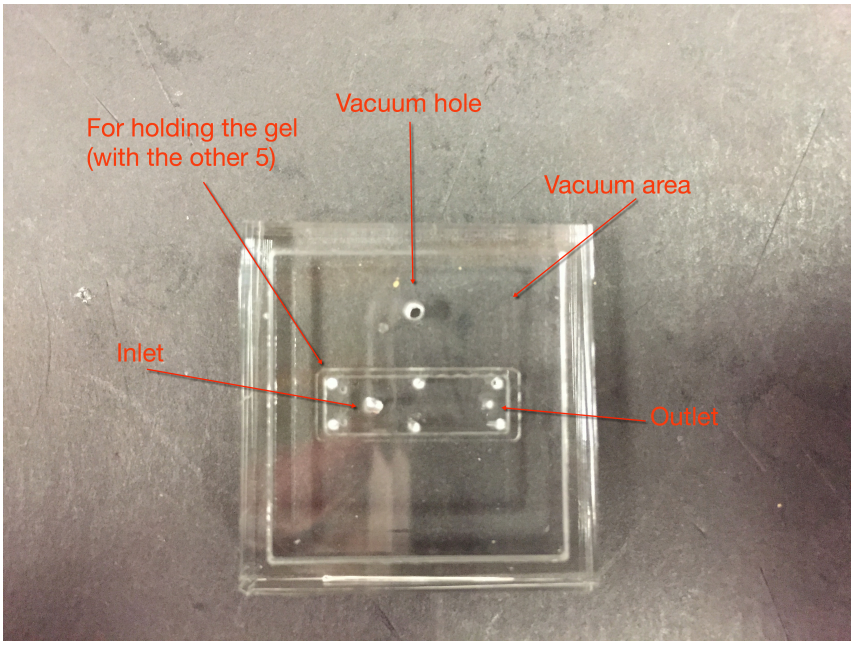

Figure 10. PDMS frame of the device

Holes are punched using a luer stub. The large hole on the top is for the vacuum line, the rectangular area is for agarose casting, the six holes around the rectangular are for holding the agarose with the PDMS frame, the hole at the left side of the two holes in the middle is for the inlet of the fluid, and the hole at the right side is for the outlet.

2.2.4. Casting the Device

The final part of fabricating the device is agarose casting using the PDMS frame and the final mold. Both the PDMS frame and the final mold are treated with air plasma to make the surface hydrophilic. Then, agarose solution is preferred. The agarose powder used is obtained from SIGMA–ALDRICH Co. After several tests, the solution with 10% agarose is selected for the subsequent experiments. Sufficiently low agarose concentration fails to confine the growth of the cells in the chamber; by contrast, excessively high concentration allows the solution to solidify rapidly, making the device hard to cast. In this case, 10 g of distilled water and 1 g of agarose powder are mixed in a beaker. The whole weight of the beaker including the solution is recorded. Then, the beaker is heated on a microwave stove to dissolve the powder in the water. In this step, the beaker is heated for several seconds each time to prevent the solution from boiling and overflowing. Each time after heating, the beaker is placed on the scale and added with water until the total weight reaches the recorded weight without changing the concentration (i.e., 10%). When the powders completely dissolve and the solution becomes transparent, the beaker is placed in a small vacuum chamber to remove bubbles. This step should be quickly conducted because the vacuum lowers the temperature of the solution and makes it solidify. After degassing, the beaker is placed in a hot water bath to keep its content hot and liquid.

When the agarose solution is prepared, the PDMS frame is aligned on the final mold under the microscope in such a way that the rectangular area of the PDMS

frame covers the pattern on the final mold. After the alignment, the frame is connected with the vacuum line, and the vacuum pressure is regulated to be approximately −40–−50 psi. The agarose solution is collected using a 1 mL syringe and rapidly placed on the luer stub. The syringe is inserted into the inlet of the frame, and the solution is slowly pushed into the functional area until air bubbles are removed. After the injection, the syringe is removed from the PDMS frame. The solution is solidified for 3 min before removing the PDMS frame from the final mold, thereby preventing the agarose from sticking to the mold instead of the frame. Finally, the luer stub is used to punch the inlet and outlet holes from the agarose side. The finished novel microfluidic device is shown in Figure 11. For storing, the device is placed in a centrifuge tube with a 0.05% sodium azide solution.

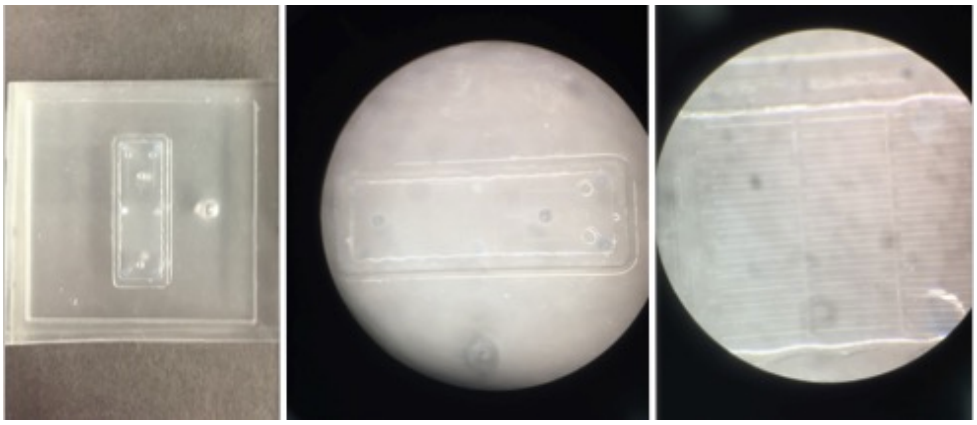

Figure 11. Finished device and micrographs of the microchannel pattern

2.3. Operation and the Experiment of the Device

Doctor Mya Warren has prepared the bacterial culture plate in Terry Hwa's laboratory for this experiment. The strain used is NCM3722 Del MotA with chromosomal ptet-GFP. The plate is stored in the refrigerator for reuse. One plate can usually last for a month. A new plate is needed after a month because the bacteria in the old plate are mostly dead. Before each experiment, a 5 mL centrifuge tube is filled with 3 mL of lysogeny broth (LB) medium by using a 1 mL pipette. Then, the bacterial cells are sampled from the plate and inoculated into the LB medium in the centrifuge tube by using an inoculation needle (Figure 13). The bacteria should be incubated for approximately 2–3 h to achieve the exponential phase of cell growth. The tube is placed on a shaker and incubated at 37 °C for 3 h.

The cell suspension in the tube becomes unclear after the incubation. Another 5 mL centrifuge tube is used to dilute the cell suspension 30 times. Moreover, 0.1 mL of the cell suspension and 3 mL of fresh LB medium are transferred into the new centrifuge tube using the 1 mL pipette.

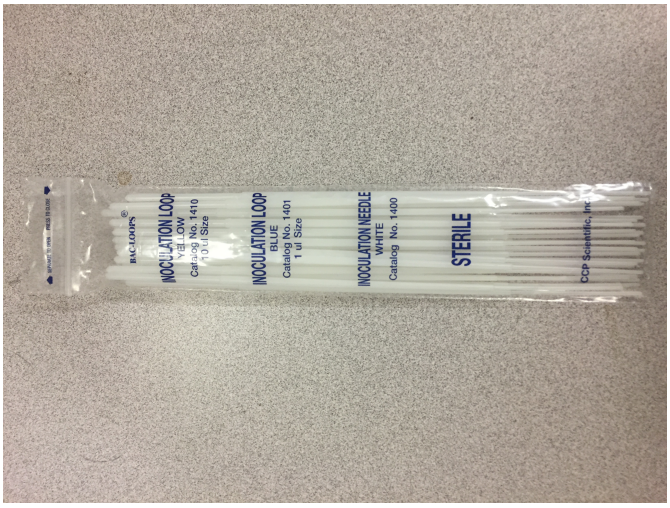

Figure 12. Inoculation Needle

After these processes, the bacterial cells are ready to be used in the experiment. A glass slide is attached on the side of the device with the pattern for microscopic observation. The microscope with camera is connected to the computer. Fire-i software is used for the observation. The channels and chambers can be observed clearly on the computer by regulating the UV exposure and light intensity. The vacuum line is connected to the vacuum hole of the device. Two 50 mL syringes serve as the reservoir and are placed on two moving stages that slide along vertical rails placed adjacent to the microscope. Both syringes have the luer stub at the bottom connected with a plastic tube. One of the syringes is filled with fresh LB medium, and is the other is used for the inlet. The inlet syringe is positioned higher than the outlet syringe to produce differential hydrostatic pressure for driving the flow inside the device. However, both syringes are higher than the device to prevent the formation of air bubbles in the flow.

Before cell loading, the device is flushed with fresh medium to remove air bubbles in the microchannels and microchambers. A syringe with fresh medium is used to inject the medium through the inlet on the device, allowing the fluids to flow out from the outlet. Another syringe with diluted cell suspension is used to load the cells into the device. In this step, the cells are slowly injected from the inlet hole. The loading of the cells into the microchambers is verified by monitoring the computer connected to the camera on the microscope. The injection of the cell suspension into the device is terminated when at least one cell can be observed in the chamber. Afterward, the reservoir with fresh medium is connected to the device by inserting the

plastic tube into the inlet of the device and placing another syringe to the outlet of the device. The growth of the cells in the microchambers is observed and recorded using Fire-i. Even small movements of the cells or colony need to be observed. Thus, the interval for image capturing is set to 3 min.

3. RESULTS AND DISCUSSION

In the microfluidic chemostat of our group in 2005,[1] *E. coli* cells loaded in the chambers started as few as 1–2 cells, formed microcolonies, and then eventually filled the chambers completely.

However, the chambers in the device of 2007[21] have areas that allow the cells to freely exit into the flowthrough channels. As long as the flow of fresh medium is maintained in the flowthrough channels, cell proliferation is continued and not limited. The simultaneous occurrence of cell proliferation and escape enables the cells to collectively transfer from the dense colony to the chamber exit. The flux rate of cell escape from the chambers is proportional to the rate of colony expansion and the average growth rate of the cells. When the cells grow to high density, the orientations of cells are often anisotropic and highly correlated over distances much larger than the cell length.

In the current design, the long chamber between two adjacent ones has one open end that allows the cells to exit freely and a closed end on the other side. These dead ends are also where the cells start growing. The direction from the open end of the chamber to the dead end of the chamber is the same as the direction of the fluid flow in the flowthrough channel. Thus, the cells cannot flow out of the chamber once they are loaded into the chambers. They can only flow out of the chamber when the colonies become very dense and reach the open end of the chamber.

One chamber is selected for observation after loading the cells into the chambers (Figures 13–16). This chamber has only two cells at the end of the chambers. The colony is formed with the gradual growth of the cells. Then, the colony keeps expanding and reaches the open end of the chamber. The cells can exit to the flowthrough channel at the open end of the chamber. Images of the open end (Figure 17) show that the cell movement somehow follows the organization described in 2007[21]; the directions of the cells are basically the same, pointing to the exit of the chamber.

When the colony in the chambers becomes denser, the rate of growth slows down, this can be observed from the images, that the expansion of the colony is not as fast as it was. With the growth keep slowing down, the expansion of the colony almost stopped. Then, however, escaped from the chamber and start growing outwards.

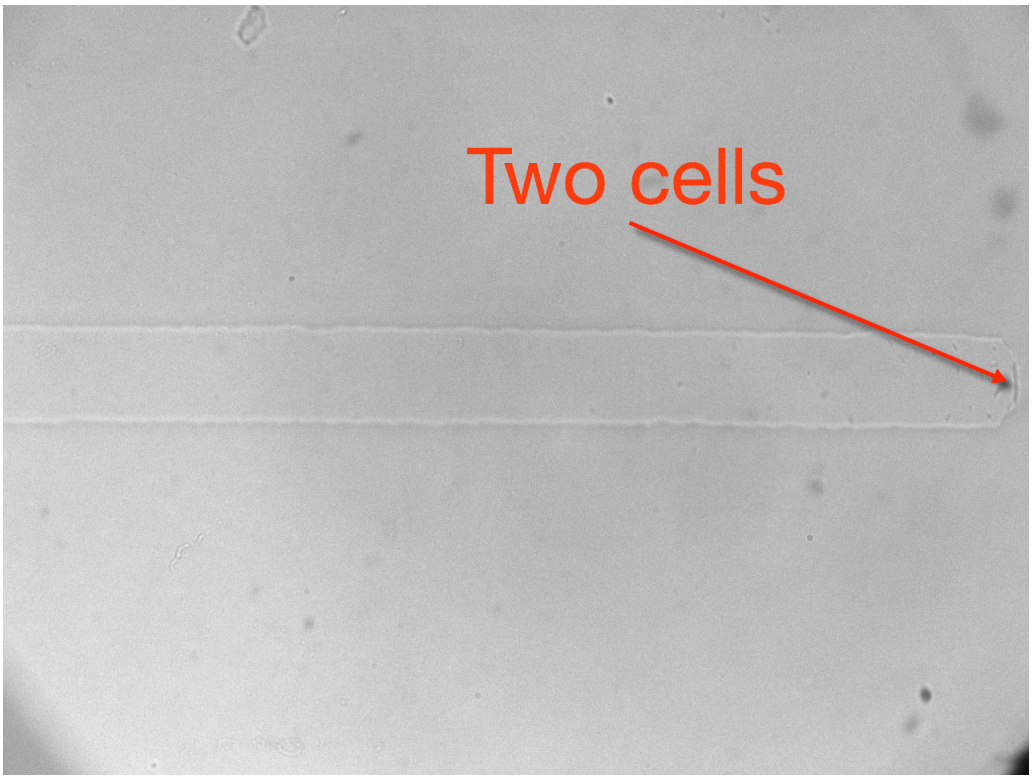

Figure 13. Growth initiation in a chamber loaded with two cells

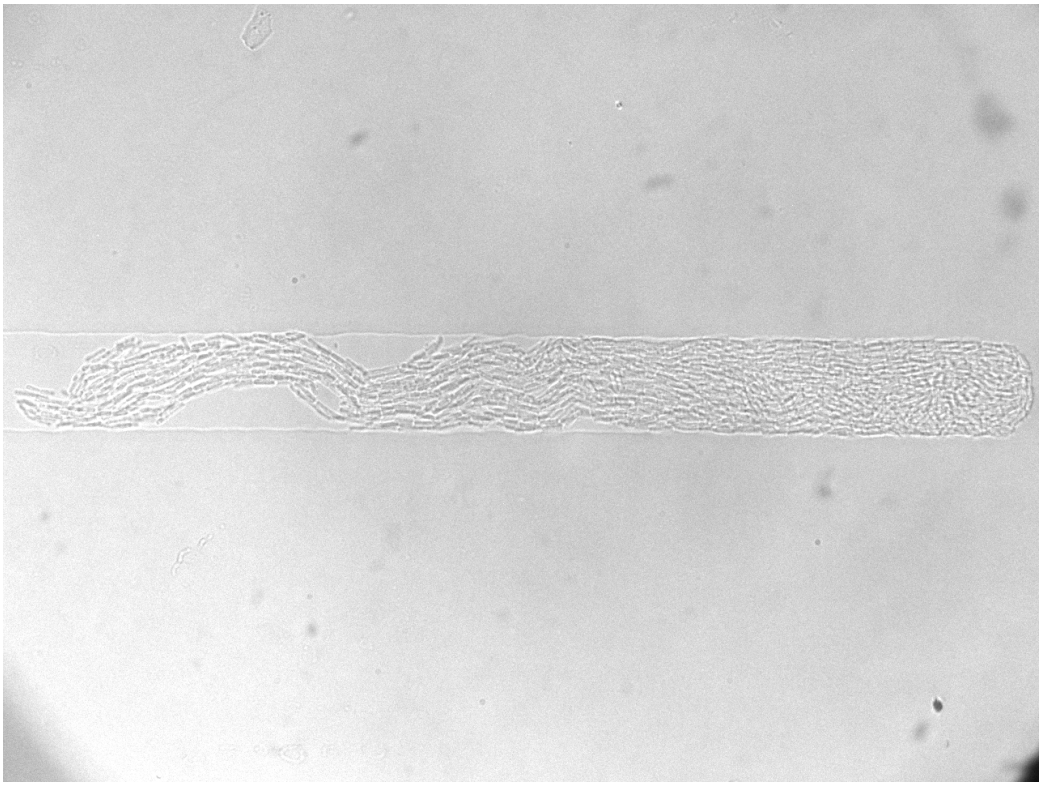

Figure 14. Colony, 8.7 h after growth initiation

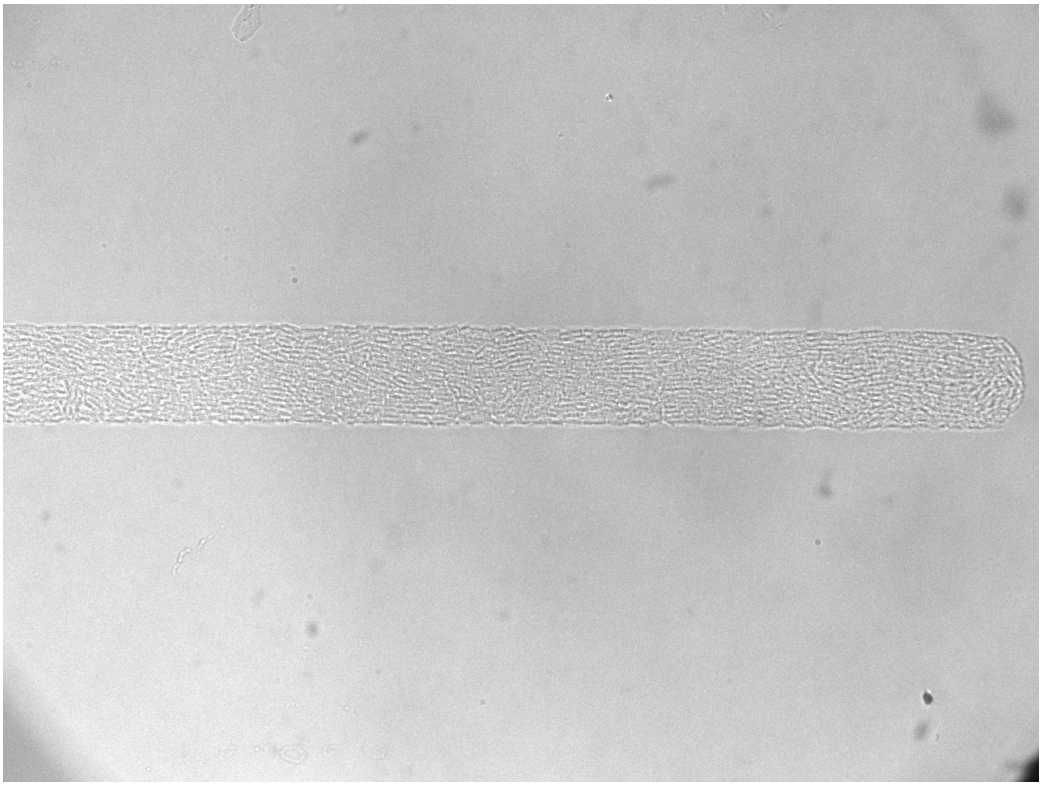

Figure 15. Colony, 9.95 h after growth initiation

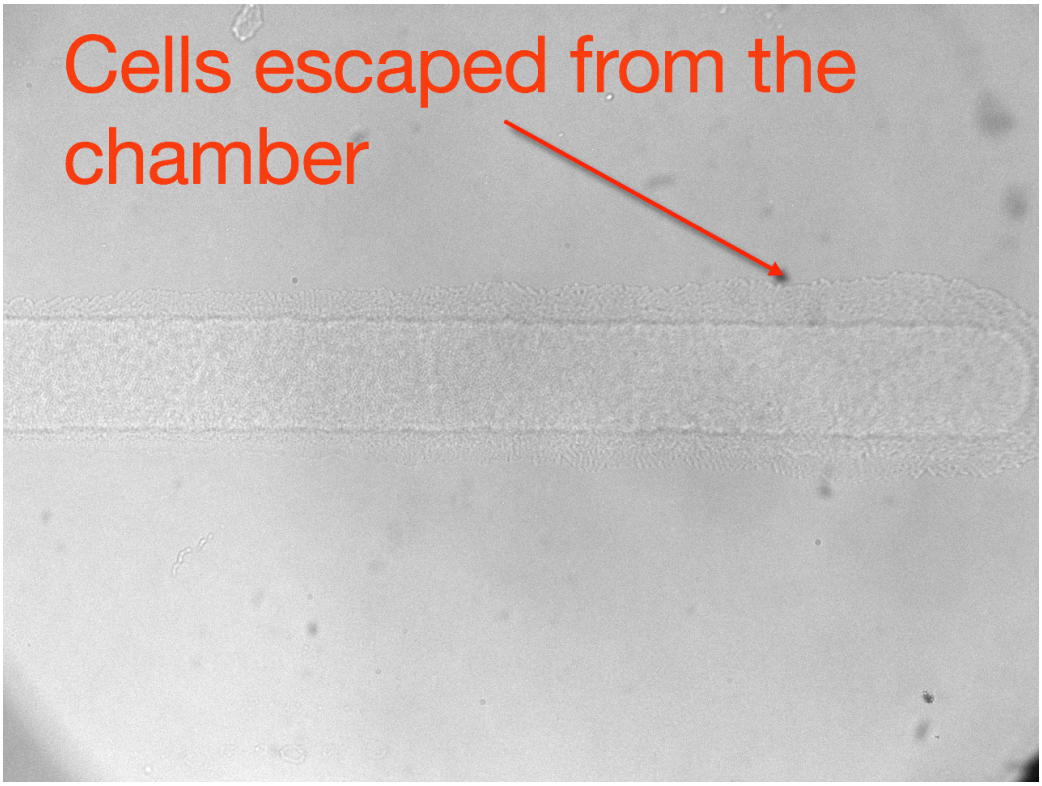

Figure 16. Colony, 13.7 h after growth initiation, and cell escape

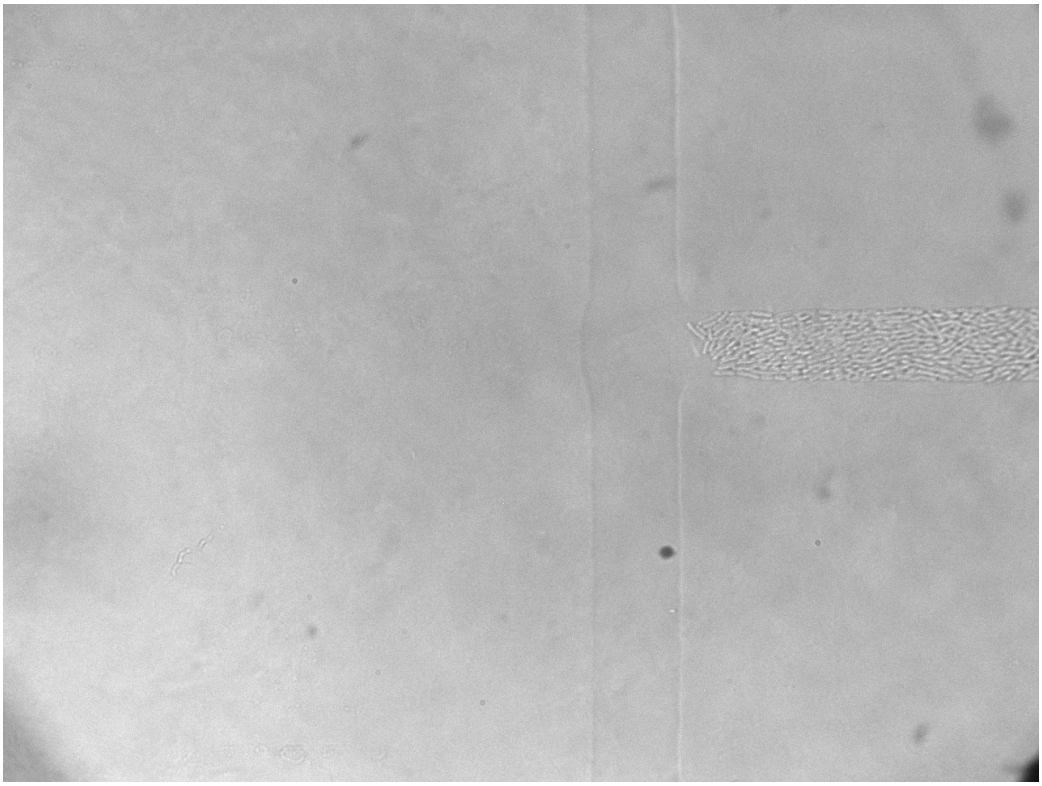

Figure 17. Open end of the chamber during cell growth

The objective of this project, which is not really reached since the lack of time, is to make the colony in the end of the chamber (where the cells are in a really dense condition) stop expanding, or, in another words, to make the cells stop growing. The cell growth at the dead end of the chamber can be measured. Cell growth suppression is supposed to be the response of cells to mechanical forces. Several strategies have been employed to achieve this objective. These strategies include using different concentrations of the agarose solution, regulating the hardness of the PDMS frame by preparing the pre-polymer with different base–cross linker ratios to adjust the vacuum pressure, adding gelatin in the agarose solution, and depositing a thin agarose layer on the glass slide surface to bond the to the glass.

However, since we can clearly observe the stalling of the growth of the cells while the colony expands and becomes denser and denser before the cells escape from the chamber, this observation is close to the expectation. The agarose solution can be used to fabricate the device. The only problem lies in increasing the strength of the device to prevent the cells from breaking through the gel and growing in the chamber.

4. CONCLUSION

Bacteria frequently exist in extremely high cell densities. A chemostat is a biological reactor for microorganism growth; this device continuously provides fresh medium for microorganisms. However, cells in the conventional macroscale chemostat cannot reach ultimately high densities because of the depletion of nutrients and the accumulation of metabolites in the medium. Therefore, a new microfluidic chemostat is designed in this project on the basis of previous works 2005[1]. With agarose used to replace the traditional PDMS as the main functional area, the device enables the cells to grow to extremely high densities. Cells are also easy to be loaded into the new device.

Consequently, the device is feasible for the growth of bacteria cell to high densities. Minimal improvement is required for this technique to be applied in studying the quorum sensing of bacterial cells.

REFERENCES

[1] Groisman, A., Lobo, C., Cho, H., Campbell, J. K., Dufour, Y. S., Stevens, A. M., & Levchenko, A. (2005). A microfluidic chemostat for experiments with bacterial and yeast cells. *Nature methods*, *2*(9), 685-689.

[2] Whitesides, G. M. (2006). The origins and the future of microfluidics. Nature, 442(7101), 368-373.

[3] Martinez, A. W., Phillips, S. T., & Whitesides, G. M. (2008). Three-dimensional microfluidic devices fabricated in layered paper and tape. *Proceedings of the National Academy of Sciences*, *105*(50), 19606-19611.

[4] Lee, W. G., Kim, Y. G., Chung, B. G., Demirci, U., & Khademhosseini, A. (2010). Nano/Microfluidics for diagnosis of infectious diseases in developing countries. *Advanced drug delivery reviews*, *62*(4), 449-457.

[5] Zheng, B., Tice, J. D., Roach, L. S., & Ismagilov, R. F. (2004). A Droplet-Based, Composite PDMS/Glass Capillary Microfluidic System for Evaluating Protein Crystallization Conditions by Microbatch and Vapor-Diffusion Methods with On-Chip X-Ray Diffraction. *Angewandte chemie international edition*,*43*(19), 2508-2511.

[6] Hansen, C. L., Skordalakes, E., Berger, J. M., & Quake, S. R. (2002). A robust and scalable microfluidic metering method that allows protein crystal growth by free interface diffusion. *Proceedings of the National Academy of Sciences*,*99*(26), 16531-16536.

[7] Reyes, D. R., Iossifidis, D., Auroux, P. A., & Manz, A. (2002). Micro total analysis systems. 1. Introduction, theory, and technology. *Analytical chemistry*,*74*(12), 2623-2636.

[8] Schulte, T. H., Bardell, R. L., & Weigl, B. H. (2002). Microfluidic technologies in clinical diagnostics. *Clinica Chimica Acta*, *321*(1), 1-10.

[9] Merkel, T. C., Bondar, V. I., Nagai, K., Freeman, B. D., & Pinnau, I. (2000). Gas sorption, diffusion, and permeation in poly (dimethylsiloxane). *Journal of Polymer Science Part B: Polymer Physics*, *38*(3), 415-434.

[10] Quake, S. R., & Scherer, A. (2000). From micro-to nanofabrication with soft materials. *Science*, *290*(5496), 1536-1540.

[11] Friend, J., & Yeo, L. (2010). Fabrication of microfluidic devices using polydimethylsiloxane. *Biomicrofluidics*, *4*(2), 026502.

[12] Ottosen, R. M. (1999). Replication and compression of surface structures with polydimethylsiloxane elastomer. *Journal of Chemical Education*, *76*(4), 537.

[13] Moffitt, J. R., Lee, J. B., & Cluzel, P. (2012). The single-cell chemostat: an agarose-based, microfluidic device for high-throughput, single-cell studies of bacteria and bacterial communities. *Lab on a chip*, *12*(8), 1487-1494.

[14] Cheng, S. Y., Heilman, S., Wasserman, M., Archer, S., Shuler, M. L., & Wu, M. (2007). A hydrogel-based microfluidic device for the studies of directed cell migration. Lab on a Chip, 7(6), 763-769.

[15] Qin, D., Xia, Y., & Whitesides, G. M. (2010). Soft lithography for micro-and nanoscale patterning. Nature protocols, 5(3), 491-502.

[16] Mather, W., Mondragón-Palomino, O., Danino, T., Hasty, J., & Tsimring, L. S. (2010). Streaming instability in growing cell populations. Physical review letters,104(20), 208101.

[17] Männik, J., Driessen, R., Galajda, P., Keymer, J. E., & Dekker, C. (2009). Bacterial growth and motility in sub-micron constrictions. Proceedings of the National Academy of Sciences, 106(35), 14861-14866.

[18] Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L., & Leibler, S. (2004). Bacterial persistence as a phenotypic switch. Science, 305(5690), 1622-1625.

[19] Wang, P., Robert, L., Pelletier, J., Dang, W. L., Taddei, F., Wright, A., & Jun, S. (2010). Robust growth of Escherichia coli. Current biology, 20(12), 1099-1103.

[20] Singleton, P. (2004). Bacteria in biology, biotechnology and medicine (No. Ed. 6). John Wiley & Sons.

[21] Cho, H., Jönsson, H., Campbell, K., Melke, P., Williams, J. W., Jedynak, B., ... & Levchenko, A. (2007). Self-organization in high-density bacterial colonies: efficient crowd control. *PLoS biology*, *5*(11), e302.

[22] Microchem Inc. http://www.microchem.com/.

[23] Makamba, H., Kim, J. H., Lim, K., Park, N., & Hahn, J. H. (2003). Surface modification of poly (dimethylsiloxane) microchannels. *Electrophoresis*, *24*(21), 3607-3619.

[24] Ren, X., Bachman, M., Sims, C., Li, G. P., & Allbritton, N. (2001). Electroosmotic properties of microfluidic channels composed of poly

(dimethylsiloxane). *Journal of Chromatography B: Biomedical Sciences and Applications*, *762*(2), 117-125.

[25] Peterson, S. L., McDonald, A., Gourley, P. L., & Sasaki, D. Y. (2005). Poly (dimethylsiloxane) thin films as biocompatible coatings for microfluidic devices: cell culture and flow studies with glial cells. *Journal of Biomedical Materials Research Part A*, *72*(1), 10-18.

[26] Vickers, J. A., Caulum, M. M., & Henry, C. S. (2006). Generation of hydrophilic poly (dimethylsiloxane) for high-performance microchip electrophoresis.*Analytical Chemistry*, *78*(21), 7446-7452.

[27] Pinto, S., Alves, P., Santos, A. C., Matos, C. M., Oliveiros, B., Gonçalves, S., ... & Gil, M. H. (2011). Poly (dimethyl siloxane) surface modification with biosurfactants isolated from probiotic strains. *Journal of Biomedical Materials Research Part A*, *98*(4), 535-543.