LASER INDUCED LYSIS OF *ESCHERICHIA COLI* CELLS AND *BACILLUS SUBTILIS* SPORES

A Thesis

Presented to the Faculty of Graduate School

of Cornell University

in Partial Fulfillment of the Requirement for the Degree of

Masters of Engineering

by

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August 2005

ABSTRACT

The principle of laser induced cell/spore lysis was explored in this project. *E. coli* K12 and *B. subtilis* spores were chosen as the target analytes. The lasers available for testing were of wavelength 980nm and 1480nm. As a proof of concept, a 2µl droplet of *E. coli* culture in nutrient broth was place on a hydrophobic surface. The tip of the laser fiber was placed directly above the droplet, and the droplet was exposed to 100mW (1480nm) or 200mW (980nm) for 4 minutes. An alternative setup of the droplet testing was also designed to prevent evaporation. The droplet was placed in a well made in 2mm thick PDMS and covered with a glass slide. The laser fiber was placed above the glass slide. The results showed that there was moderated lysis using both lasers. The percentage lysis was greater using 1480nm laser (21%) than the 980nm laser (14%). This was expected since the main cause of cell lysis is due to a sudden increase in temperature. The aqueous nature of cells thus makes the lysis highly dependent on the water absorption coefficient, which is 50 times higher at 1480 nm than at 980 nm. The alternative setup showed slightly less lysis (9% for 980nm). The difference to the droplet experiment results indicates that evaporation occurred during the droplet test which resulted in a smaller testing volume and thus in higher exposure to the laser.

The next step was to test the laser in microfluidic channels in order to determine whether it would be applicable for a micro-bioanalytical system. A 25 parallel-channel design with channel widths of 50µm and depths of 100µm were fabricated on a silicon wafer and subsequently realized in poly(dimethylsiloxane) using soft-lithography. In addition to *E. coli* cells, the effectiveness of lysis of *B. subtilis* spores was also explored. Variable flow rates of 5µL/min, 2µL/min, 1µL/min and 0.5 µL/min were implemented. The results showed a greater percentage of lysis of *E.*

coli cells compared to the droplet method (31% for 5μL/min, 41% for 2μL/min, and 43% for 1μL/min). However, no lysis was observed for *B. subtilis* spores under any flow rate.

BIOGRAPHICAL SKETCH

The author grew up in Kaohsiung, Taiwan where he completed his elementary education. He moved to Worcester, MA during his teen years and completed his secondary education there. The author enrolled at Cornell University in Fall 2000 to pursue a Bachelor of Science in Electrical and Computer Engineering and received such a degree in May 2004. He continued his studies at Cornell University and enrolled in the Master of Engineering program in the field of Biological Engineering under the supervision of Professor Antje Baeumner.

To my parents

ACKNOWLEDGEMENTS

I would like to thank my advisor, Professor Antje Baeumner of the Department of Biological and Environmental Engineering at Cornell University, Ithaca New York, for her invaluable mentoring. She shows extraordinary patience and understanding towards a student, like myself, who comes from a different disciplinary.

I would also like to thank many of the individuals in our research group – Barbara Leonard and Sam Nugen for their instructions on bacterial culturing; John Connelly, Katherine Lee, and Hesed Kim for their help with the laser systems; Vasily Goral and Natalya Zaytseva for their help with microfluidic devices; and Kevin Nichols for fabricating the channel designs.

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Chapter 1
Introduction

1.1 Pathogenic Organisms

Bacterial pathogenesis is one of the most common forms of infectious disease. Worldwide, infectious diseases account for 26% of the estimated total 57 million annual deaths [1]. Early detection of these organisms is crucial to in containment of the diseases. Therefore, not only are these detection systems needed in a clinical setting, they are also important for measurements directly on-site. In addition, environments such as food processing factories and farmlands would benefit from these early detection methods.

Today, there is an even greater need for detection systems in the field. The Anthrax scare showed that post offices and government buildings have a need for an alarm system that is robust and able to detect trace of the antigen [2]. In battlefield, a portable detection system that could be carried around would also be very beneficial to the soldiers.

1.2 Bioanalytical and Micro-total Analysis System

A biosensor is defined by IUPAC to be a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals [3]. Key to the biosensor is the biological aspects of the recognition element. Advantages of using a biosensor, compared to traditional analytical chemistry techniques, include speed, simplicity, cost, ability to detect analytes that would be other wise difficult to detect by other methods, and portability. However, the analyte that is used for

detection may not be readily available or may be too contaminated for the biosensor to detect properly. Thus, sample preparation steps need to be included in the overall analysis. A bioanalytical system is a device that includes the ability to perform all the necessary preparation steps and the detection itself. These steps may include purification, extraction, and amplification. [4]

Since a key feature of a biosensor is its portability, the preparation steps also need to be incorporated into a portable system. Therefore, a micro-total analysis system (μTAS) is an ideal implementation for such a requirement. A μTAS integrates all the instruments used on the bench top onto a chip [4]. Thus, it is also called "lab-on-a-chip". Figure 1.1 shows a generalized structure of a micro total analysis system.

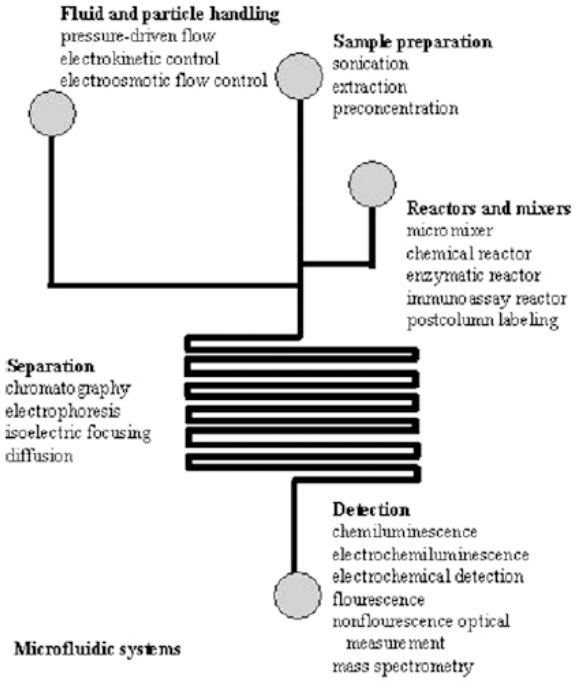

Figure 1.1 A schematic of the common components of a μTAS, adapted from [4]. This microdevice integrates a fluid handling component (ex. micropump), a reactor (ex. micromixer), a separation chamber, and finally, a detector.

In the case that the target analyte of detection is a bacterial organism, two biological elements are often used to form a sandwich with the target analyte, for example, antibodies are frequently used that recognize specific epitopes on the surface of the

cell [5]. This method is simple; however, the specificity could be low if similar strains of bacteria exist bearing similar epitopes and antigens on their outer surface. In other methods, intracellular components such as nucleic acid molecules are used for detection. A complementary probe can be designed that hybridizes to a particular sequence of RNA/DNA. However, in order for the nucleic acid to be avaliabe, the cell itself needs to be lysed in order to release its intracellular molecules.

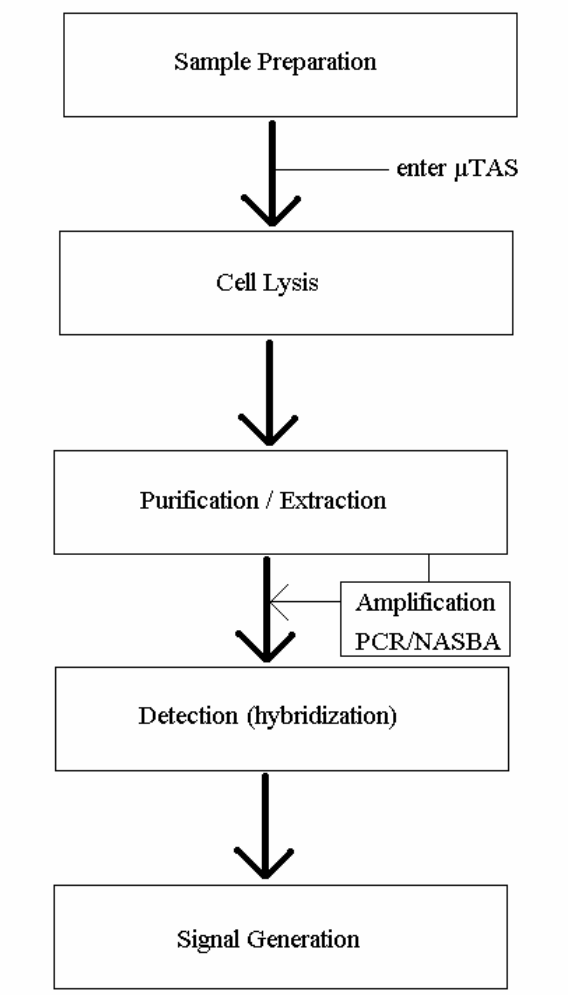

Figure 1.2 Schematic of a bioanalytical system that detects a bacterial organism through via its nucleic acid sequence (DNA or RNA).

A bioanalytical μTAS for the detection of nucleic acid sequences (Figure 1.2) starts off by injecting the sample culture onto the chip. A lysing mechanism is used for breaking up the cells and releasing its contents. Nucleic acid molecules are purified and subsequently amplified using reactions such as the Polymerase Chain Reaction (PCR) for DNA and Nucleid Acid Sequence Based Amplification (NASBA) for RNA. While PCR is still dominating the amplification "market", NASBA will soon become a much more interesting application due to its isothermal nature, which lends itself very well to miniaturization. [6]

1.3 Existing Cell Lysis Methods

Many different means of cell lysis have been explored. Sometimes multiple methods need to be used to achieve sufficient cell lysis.

1.3.1 Thermal Lysis

Thermal lysis is the simplest method for lysing. The cell culture is heated to 95°C to induce lysis. It is also possible to multiplex the lysing step with PCR [7]. In μTAS, a micro heater can be implemented that uses a resistive heater coil. The amount of heat produced is proportional to the voltage applied which in turn can be controlled by a microcontroller coupled with a thermostat. The entire system could easily be implemented on a chip [8]. One other advantage is that no other buffer solution is needed, thus reducing the complexity of the channel design and the possibility of interference for detection. The main disadvantage is that many components (proteins, RNA) would be denatured at such high temperature [9]. In addition, thermal lysis is not effective against all organisms, especially spores [10].

1.3.2 Mechanical Lysis

In general, mechanical is carried out using two different strategies: using a homogenizer and ultrasonic disruption. A homogenizer pumps the sample solution through a restricted space building up very high pressure. The pressure is suddenly released, causing cavitation and creates a liquid shear powerful enough to lyse cells [11]. Similarly, ultrasonication uses rapid sinusoidal movement of a probe in the liquid sample to create high acceleration that creates powerful liquid shear. Both methods can achieve a very high yield of lysis (>99.9%) and are standard methods in microbiology and molecular biology labs. The major disadvantage here is the cost. In addition, it is unlikely to create high pressure in microfluidic devices. A miniaturized sonicator, however, has been developed [12]. Another drawback for mechanical lysis is that the cells are broken up completely so all intracellular materials are released. This makes the purification and extraction process more difficult.

1.3.3 Detergent Lysis

Detergents promote the breakdown of the cell membrane structure by disrupting the hydrophobic interaction that holds the membrane together. Detergent lysis is fairly simple since the procedure generally involves mixing lysis buffer with the sample culture. However, the properties of the detergent or its products may cause problems in purification and extraction of the desired target analyte [13].

1.3.4 Enzymatic Lysis

Enzymatic lysis is a biological method that is highly efficient and also minimizes denaturizing of the desired molecule. Lysozyme is commonly used and has shown to be effective even against *Bacillus anthracis* spores [14]. However, enzymatic lysis has one common disadvantage with detergent lysis when implemented in microfluidic

channels. A second channel for inject the lysis buffer is needed. The bigger problem is to get the two liquid, buffer and the culture, to mix properly. Due to low Reynolds number in microfluidics, the liquid will stay in laminar flow and form a characteristic barrier at the interface [15]. Therefore, a micro-mixer would need to be designed, adding greater complexity to the system.

1.4 Laser Lysis

1.4.1 Theory

The proposed cell lysis method in this study is laser-induced cell lysis. Energy from a laser beam that shines on a cell or spore should be able to be absorbed causing the cell walls to break apart similar to heat-induced cell lysis. The phenomenon is not completely understood, but there are a few proposed mechanisms.

Light falling on an object would exert forces on this object. Calculations showed that 1mW of light energy absorbed would create a force of roughly 5pN. This force exerts on a bacterial organism, which has very small mass (~1pg) and diameter (~1μm), translate to a pressure of 30 times the gravitational force [16]. Nevertheless, ultrasonication, an almost complete lysis method, creates a g-force that is in the tens of thousands range above gravitational force, which makes light pressure insignificant.

Light is also a carrier of an electric field. The electric field generated is very small and is not sufficient to lyse a cell. But it may be an aid in lysing.

The major contributor of laser lysis is the thermal effect. The liquid medium, often water, can absorb light energy and convert it to thermal energy. The concept is similar to thermal lysis. However, with the use of a laser, it would be possible for heating a much smaller region for a short period of time. Thus, the hope would be that the

temperature in a cell/spore heats up dramatically as it crosses into the path of the laser beam. This should cause the membrane to, at least partially, lose its integrity. The exposure time is relatively short. And once it exits the beam path, temperature should drop relatively quickly since the surrounding would cool it down with ease. This could reduce any denaturing effect that would have been caused by traditional thermal lysis.

1.4.2 Water absorption spectrum

In order for laser lysis to be effective, the power of the laser needs to be transferred to the medium. Hence, the medium must be able to absorb light effectively. In this case, water is the medium. Like all material, its absorption strength varies with the wavelength of the incident light. Figure 1.3 shows the absorption spectrum between 400nm and 2400nm. The Lambert absorption coefficient, α, is defined to be:

$$dP = - \alpha P \, dx$$

P is power and dP is the differential power change across the material in direction x. Solving the equation yields:

$$P = P_o \exp(-\alpha x)$$

P_o is the initial power of the light incident on the material. Therefore, a larger coefficient correspond to a lesser penetrating power of light. Hence, more power is being absorbed by the material as light passes through.

When designing laser lysis experiments, the wavelength of the laser should correspond to the medium it intends to penetrate. In addition, the thickness of the medium should be as thin as possible. This is where microfluidic channels and soft lithography becomes beneficial. The channels can be made very thin so that the laser beam can penetrate easily. Using Polydimelthyl Siloxane (PDMS) to encase the channels would

allow the laser to penetrate through transparent walls so little power would be lost due to the material of case structure.

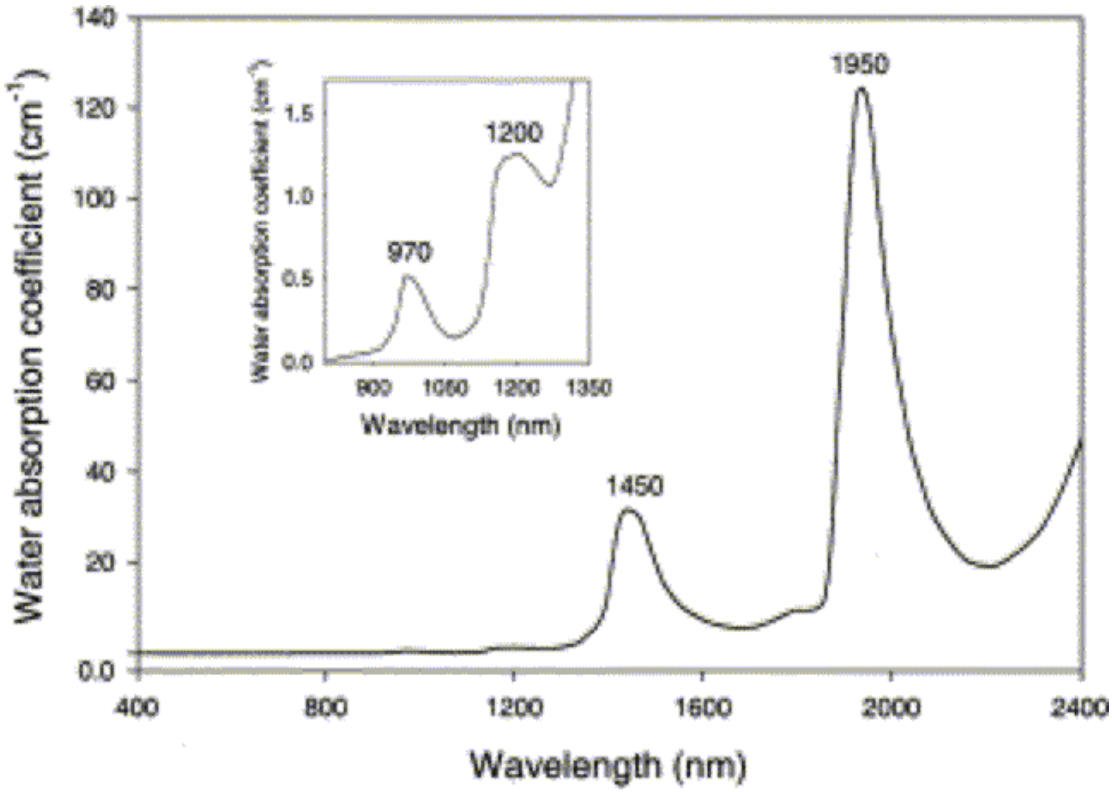

Figure 1.3 Wavelength of incident light versus water absorption coefficient. Note that the y-axis values in the insert are much smaller than the main plot [17].

It is easy to tell from Figure 1.3 that for water, highest absorbance of light occurs at 1950nm (within the limit of 400nm to 2400nm). The lasers available for this project are 980nm and 1480nm. These wavelengths occur close to two local peaks in the absorbance spectrum, although the peak at 1450nm is roughly 50 times greater than the peak at 950nm. Table 1.1 summarizes the noteworthy wavelength and the corresponding absorption wavelength [18].

Wavelength (nm)	Absorption coefficient (cm -1)
980	0.502
1480	~25
1950	109

Table 1.1 Water absorption coefficient and corresponding wavelength

The 1480nm laser is therefore expected to produce much better lysis efficiency then the 980nm laser.

1.4.3 Current scope

An on going project at Cornell University is attempting laser lysis on *Mycobacterium tuberculosis* cells. The instruments used are the same as in this project.

Another group has attempted to laser lyse *Bacillus globigii* spores. The spores were mixed with a laser light absorbing matrix and co-crystallized into a 200μm wide and 20μm deep nanovial formed in a PDMS plate. A 6mW UV nitrogen laser with pulsed output at 337nm was employed [19]. After laser enshrinement, the crystal was dissolved and PCR was carried out on the sample. Its results showed that DNA from the spores was released. SEM images further showed deformed and deflated spores after the laser treatment. However, the spore count before and after laser lysis was not quantified so the efficiency of such a setup was not known. The use of light absorbing matrix and UV laser is also not ideal in the implementation of a portable biosensor system.

1.5 *Escherichia coli* cell membrane

E. coli belongs in the category of gram negative bacteria. These organisms have three layers of barrier between the cytoplasm and the surrounding: cytoplasmic membrane, the cell wall, and the outer membrane [20].

The cell wall of gram negative bacteria is composed of 20% peptidoglycan. The outer membrane is an asymmetric membrane bilayer, i.e. the inner layer contains only phospholipids with nearly the same composition as found in the cytoplasmic membrane, while the outer layer contains exclusively one type of molecule, lipopolysaccharides and various pore forming proteins (porins) [20].

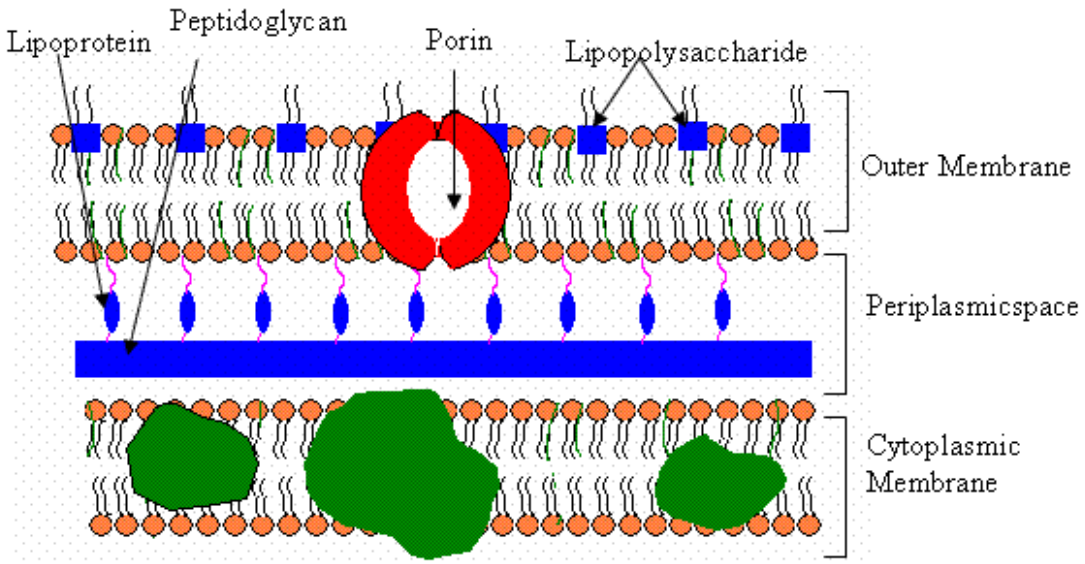

Figure 1.4 Figure above demonstrates the outer membrane, periplasmic space and cytoplasmic membrane composition of gram-negative bacteria. For gram negative bacteria cell wall is composed of 20% peptidoglycan. The outer membrane of Gram-negative bacteria contains exclusively one particular type of molecule, the lipopolysaccharides and the various pore forming proteins (porins) [20].

A gram positive bacterium consists only of the cytoplasm, the cytoplasmic membrane, and the cell wall. However, its cell wall has a distinctly thick layer of peptidoglycan, which is 90 % of the composition of the cell wall. Thus, gram negative bacteria, although more complex structurally, are easier lysed than gram positive bacteria due to the less rigid compositions of the surrounding layers.

1.6 *Bacillus subtilis* spores

Spores are designed by nature to survive in harsh, nutrient-limited conditions. Therefore, compared to *E. coli* cells, *B. subtilis* spores are much more resistant to outside forces. Its first line of defence is a thick proteinaceous coat on the surface. A second protective barrier is an inner membrane that has very low permeability to small hydrophilic molecules [21]. These characteristics make spores harder to be lysed by traditional methods that try to digest the cell walls (although still possible by detergent lysis as mentioned before), but should have little effect on laser lysis. The spore core has an extremely low water content and this is the major concern that spores may be difficult to be lysed by laser since water is needed to absorb light energy and transfer it into thermal energy.

1.7 Design

This project intends to explore the possibility of using laser as a mean of lysing cells for the application in a bioanalytical microsystem. A biosensor needs to be simple, robust, and portable. The implementation of laser into such a device will meet all these requirements. Using laser eliminates any extra channels or complicated design needed for the channel carrying the sample. The laser can also be easily miniaturized. A droplet test is designed to prove the validity of laser lysis. *E. coli* cell culture will be made into 2μL droplets and subjected to laser enshrinement from above. The laser employed are of wavelength 980nm and 1480nm.

Subsequently, lysis experiments in microfluidic channels made of PDMS will be performed with the lasers. The flow rate will be varied to simulate different exposure times. *Bacillus subtilis* spores will also be subjected to the same laser treatment in the microfluidic channels.

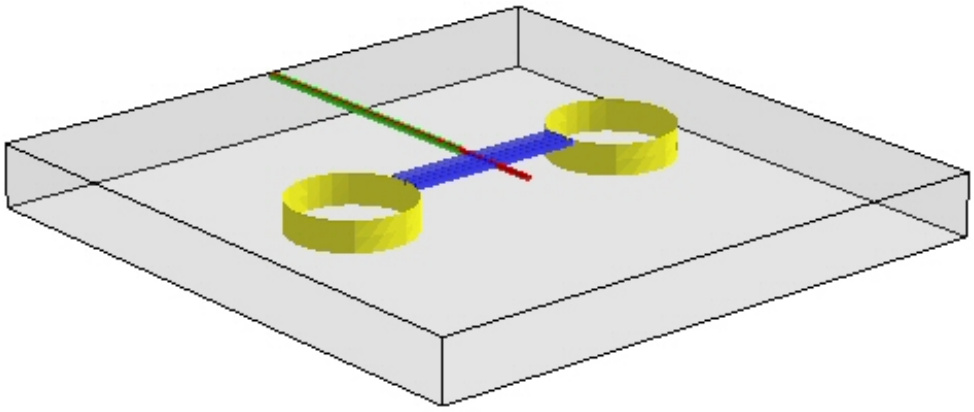

Figure 1.5 Schematic of the PDMS device. Shown in blue are the channels. Shown in yellow are the input and output wells. Shown in green is the laser trench. Shown in red is the simulated laser beam.

Chapter 2
Materials & Methods

2.1 Materials

Difco® Nutrient agar and broth were obtained from Becton, Dickinson and Company, Sparks, MD. The chemicals for synthesizing the sporulation media – Potassium Chloride, Calcium Nitrate, Manganese Chloride, Magnesium Sulfate, Ferrous Sulfate, and Sodium Hydroxide (pellets) – were purchased from Mallinkrodt, St. Louis, MO. Bovine Serium Albumin was obtained from Sigma, St. Louis, MO. The Sylgard® Silicone Elastomer Kit, used for soft lithography, was obtained from Dow Corning, Sparks, MD.

Beckman (Fullerton, CA) DV 520 General Purpose UV/Uis Spectrophotometer was used for obtaining optical density. The syringe pumps were obtained from KD Scientific, Holliston, MA. The 1mL plastic syringes were from Becton, Dickinson and Company, Franklin Lakes, NJ. Silicon wafers were made in Cornell Nanofabrication Facility (CNF), Ithaca, NY. The two lasers, 1480nm and 980nm, were obtained from Dr. Frank Wise and Dr. Joe Ballantyne, Ithaca, NY. Rain·x® was purchased from a local store.

Petri dishes, glass slides, flexible tubings, pipet tips, micro-centrifuge tube, centrifuge, vortexer, and other common laboratory supplies were purchased from VWR Scientific Products New York, NY.

2.2 Microfluidic Channel Design

The two designs of microfluidic channels were obtained from Mohit Dhawan's M.S. project [16]. The schematics are shown in Figure 2.1.

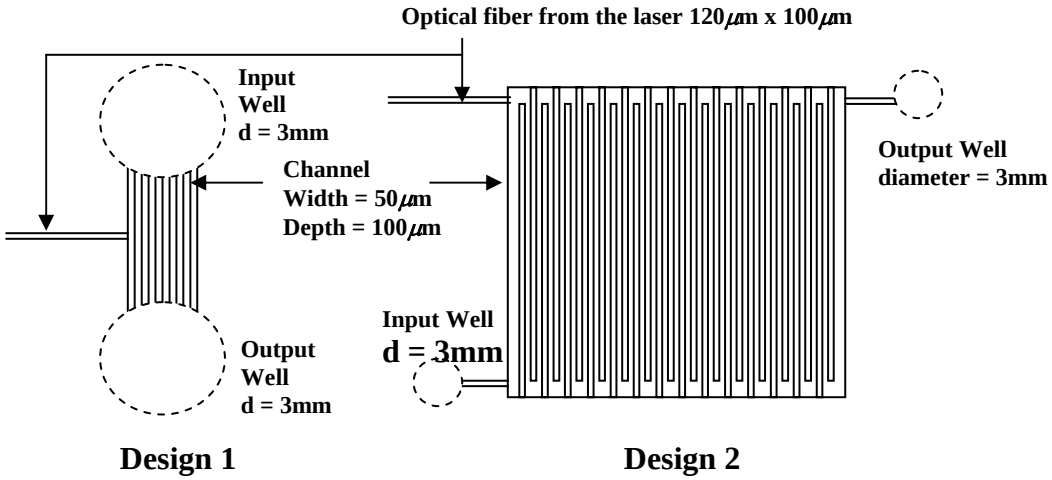

Figure 2.1 The two channel design. Design 1, referred to as the parallel design, has 25 channels of length 2mm each. Design 2, referred to as the serial (serpentine) design, has channels of the same width. The total area of the serial channels is 10mm x 10 mm. The input and output well, noted by the dotted circles, are not part of the pattern etched on to the silicon wafer.

Design 1, the parallel design, uses 25 parallel channels of 20mm length. The laser trench is placed perpendicular to the channels at the center (10mm from each end). Design 2, the serial design, uses one channel zigzagged into a square shape. The area of the square is 10mm x 10mm. The laser trench is placed 10mm from the input well. In both cases, the microfluidic channels are 50μm wide and 100μm deep. The laser trench is 120μm wide and 100μm deep. A small gap (~50μm) exists between the microfluidic channels and the laser trench.

Two silicon wafers of the parallel design were made by John Connelly. Each wafer has 8 replicated designs of the channels. The serial design wafer was made by Kevin Nichols.

A 3D picture of the parallel channels in action is presented in Figure 2.2. The culture solution is loaded to the input well on the left; then travels through the channels and gets collected in the output well on the right. The laser beam, shown in red, would ideally penetrate all of the channels.

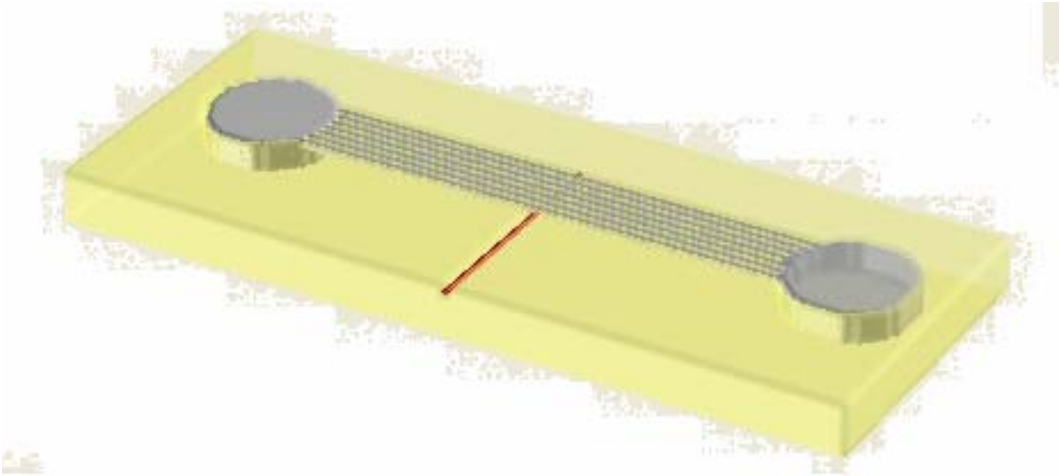

Figure 2.2 3D model of the parallel channel design. The yellow region represents the PDMS device. Grey area is where fluid flow occurs. The two cylinders on the ends represent the input and output wells. The laser beam is represented by the red line bisecting the channels.

An intuitive representation of the lysing process is presented in Figure 2.3. The cell/spore (black sphere) on the left hand picture enters the laser beam as a whole and exits as debris. However, in the real world, the laser beam would scatter. Moreover, the bursting of the cell is not what occurs in the real world. Rather, a loss of membrane integrity, similar to thermal lysis rather than mechanical lysis, is the expected result.

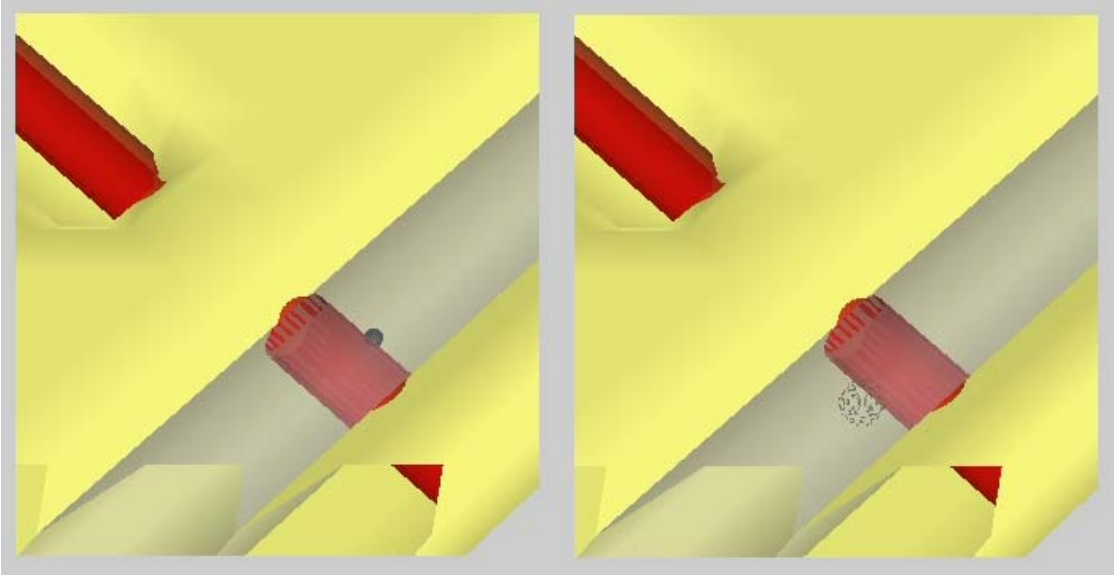

Figure 2.3 Laser lysis in action. Picture on the left shows a cell/spore (black sphere) entering the laser beam. As it exits the beam, it is lysed and thus separated into pieces, as shown on the picture on the right.

2.3 Preparation

2.3.1 Agar Plates & Dilution Tubes

5.75g of nutrient agar is dissolved in 250ml of water. The solution is stirred and heated to near boil. Then it is autoclaved at 121°C for 15 minutes. After cooling, the solution is poured into plates. A total of 10 to 12 plates are made each containing approximately 20mL of nutrient agar.

Dilution tubes made with nutrient broth are needed for *E. coli* only since *B. subtilis* spores can survive in water. 0.8g of nutrient broth is dissolved in 100ml of water. The solution is subsequently transferred into culture tubes with 9ml in each, and then autoclaved at 121°C for 15 minutes. The tubes are stored at 4°C. The plates are stored at room temperature for 24 hours to make sure no contamination occurred during the process of making them, then stored at 4°C until use.

2.3.2 *E. coli* culture

A streaked plate with *E. coli* K12 strain was obtained from Sam Nugen. For the preparation of each experiment, colonies from the plate are transferred into a nutrient broth culture tube. The culture tube is placed in an incubator-shaker set at 37°C and 175rpm for 2 to 3 hours. The optical density of the medium is obtained through a spectrophotometer. An OD_{600nm} value of 0.025 to 0.05 is ideal since it translates to roughly 10,000 to 25,000 cfu per micro liter. After obtaining the desired concentration, the culture medium is diluted down 1/1000 times with fresh medium in order to obtain 100 – 250 cfu/10µL.

2.3.3 *B. subtilis* spores

A streaked plate with *B. subtilis* colonies was obtained from Barbara Leonard. 200ml of Shaeffer's (Difco) sporulation medium was prepared [22]. After inoculation tubes are placed into an incubator shaker (37°C, 125rpm) for 96 hours. The culture solution is then centrifuged and the remaining pellet is washed. The pellet is resuspended and allowed to sit overnight. This process is repeated 3 times (3 times over night). The final solution with a resuspended pellet is diluted and placed in an 80°C heating block for 10 minutes.

The ideal concentration for the spores is only obtainable by doing serial dilution of the culture solution (by 1/10 each time) and plating onto the agar plate. Ideally, the number of colonies per plate should be above 100 and below 250.

2.3.4 Silicon wafer

Dust and PDMS residuals can easily accumulate on the surface of a silicon wafer. To clean the wafer, it is dipped into hot concentrated sulfuric acid and let it sit until it cools down (1 – 2 hours). The wafer was rinsed thoroughly with DI water. Subsequently, Rain·x® was applied to the wafer in order to create a hydrophobic surface. Any residual Rain·x® was wiped off with lint-free wipes. Note that the wafer should be completely dry before pouring PDMS on it, or else it will stick to the surface irreversibly.

2.3.5 Polydimelthyl Siloxane (PDMS)

PDMS is a mixture of linear polymers of methyl silicone and is commercially packaged by Dow Corning as a siloxane base polymer and a curing agent. The manufacturer recommended mixture of base and curing agent with a ratio of 10:1 by volume. However, it was discovered experimentally that this ratio did not cure the

polymer very well. A 3:1 ratio is used instead although this increases the elasticity of the PDMS device [4]. 3ml of the base polymer and 1 ml of the curing agent is mixed in a 15ml centrifuge tube and vortexed for 5 minutes. The mixture is poured on to a wafer and placed into a 60C oven for 1 hour. After it is completely cured, the PDMS layer is peeled off the silicon wafer and ready to be used for microfluidic experiments.

2.4 Laser Lysis Setup

2.4.1 Droplet Method

The droplet method involves placing a drop of the cell culture medium on a surface with the laser shining through it from the top. 2µl were used to form the drop. The surface is created by wrapping Para-film on a glass slide. The hydrophobic surface of the Para-film will force the liquid to condense into a drop. The laser fiber is stationed directly above the droplet using a stand with a clamp. The tip of the fiber should be as close to the droplet as physically possible. An IR card is placed under the glass slide so the horizontal position of the laser fiber can be easily adjusted.

An issue with the setup above is that the heating of the laser caused great amount of evaporation of the droplet. Thus, an alternative method is to create a thick layer of PDMS and use the hole puncher to create a 3mm diameter well to place the droplet in. The well is then covered with a glass slide, and the laser fiber is placed above the glass slide.

2.4.2 Microfluidics

Microfluidic channels are made in PDMS using the parallel design as described in chapter 3.2. Using the hole puncher, the input and output wells (3mm in diameter)

were created on the PDMS. The device was then placed on a glass slide. The side with the channels should face the slide. Carefully, the laser fiber was inserted into the trench. A two-piece platform made of Plexiglas was used to hold the device together. The top piece has two metal tubes that should be lined up with the input and output wells. The two pieces are screwed together tightly to prevent any leaking in the PDMS device. The tube associated with the input well is connected to a syringe on a pump, and the output well is connected to an Eppendorf tube for collecting the solution. The device is shown in a schematic (Figure 2.4) and an actual photograph of the experimental set up (Figure 2.5).

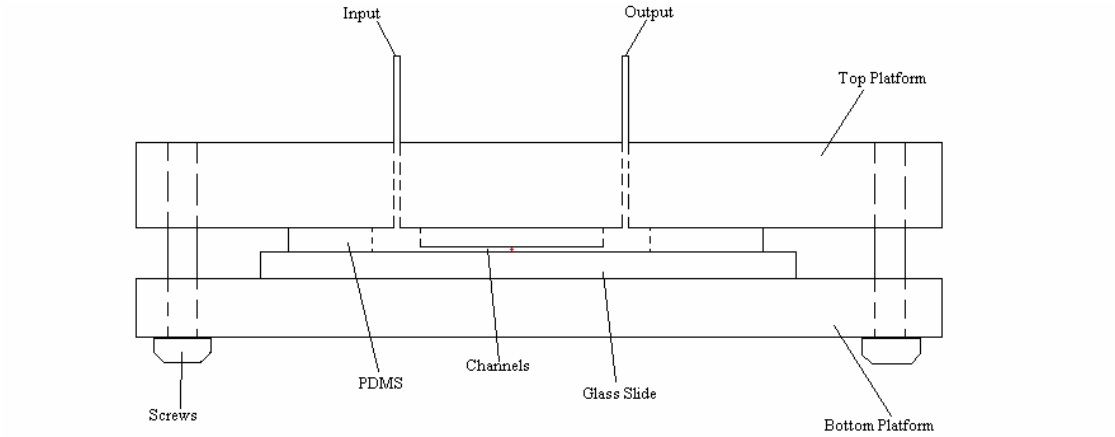

Figure 2.4 Schematic of microfluidic setup. The PDMS and the glass slide are sandwitched between the top and bottom platforms. The top platform includes the 2 tubes connecting input and output wells respectively. The channels face the glass slide. The laser beams comes out of the surface as shown by the red dot in the middle of the channels. The schematic is not drawn to scale.

Figure 2.5 Laser lysis in action. The syringe attached to the pump injects cells/spores into the channels held in the platforms. The output is collected with Eppendorf tubes. The laser fiber comes in from the top.

2.5 Experimental Procedure

2.5.1 Lysis using Droplet Method

A 2μL droplet of *E. coli* culture solution is subjected to a laser of wavelength 1480nm at 100mW and 980nm at 200mW. The exposure time was set at 4 minutes and was monitored manually by a stopwatch. The resulting solution was collected in an Eppendorf tube containing 90μL of nutrient broth. The contents in the tube were plated on to an agar plate and left over night in a 37°C incubator cabinet.

2.5.2 Lysis using Microfluidic Channels

2.5.2.1 *E. Coli*

The experiments are accomplished in the following steps:

1 The channels are pre-flushed with 20μL of nutrient broth at 40μL/min. This step is to ensure that there is no leak in the device and flush out possible contamination. The liquid at the output is not collected.

2 A total of 10μL of cell culture is loaded into the device. The flow rates used are: 5μL/min, 2μL/min, and 1μL/min.

3 Flush the channels with 10μL of nutrient broth at the flow rate specified in step 2. This ensures that all of the culture solution passes through the laser beam at the desired flow rate.

4 Post-flush the channels with 40μL of nutrient broth at 40μL/min.

All the output from step 2, 3, and 4 are collected in Eppendorf tubes that are pre-filled with 40μL of nutrient broth. The final amount of liquid should be 100μL which is ideal for plating.

Microfluidic experiments were only carried out using the 980 nm laser since the 1480 nm laser lost power most likely due to age.

2.5.2.2 *B. subtilis*

The procedure for lysis of *B. subtilis* in PDMS was mostly the same as *E. coli* with the major differences noted below.

- It is expected that spores are much harder to lyse than cells, therefore an even slower flow rate of 0.5μl/min is added.
- Instead of using nutrient broth to wash the channels, DI water is used. However, the initial experiments showed that even without applying laser, the

spore count at the output is much lower than expected. This indicated that they are lost in the channels possibly due to a higher affinity for the PDMS walls. Thus, Bovine Serum Albumin (BSA) is introduced as a blocking agent. A 0.5% BSA in DI water was found to be sufficient in reducing the spores lost in the channels. Therefore, BSA solution is used instead of pure DI water for the washing steps.

- The Eppendorf tubes, with their output solution, are placed in 80C heat block for 10 minutes. This step should eliminate any bacteria in the solution besides spores.

Chapter 3
Results and Discussion

3.1 Effectiveness of lysis of E. coli using the droplet method

E. coli K12 strain was subjected to both 1480nm and 980nm laser using the droplet method. The exposure time was set at 4 minutes. Tables 3.1 and 3.2 summarize the results.

Table 3.1 **Results of the droplet method for lysis of E. coli cells using 1480nm laser at 100mW exposure for 4 minutes. The number of *E. coli* cells counted on nutrient agar plates with and without laser treatment are given.**

Trial	No treatment	Laser treatment
1	109	96
2	123	97
3	147	113
4	115	113
5	133	71
6	152	108
7	133	120
Average	130	102.57
Std dev	15.84	16.44

Table 3.2 **Results of the droplet method for lysis of E. coli cells using 980nm laser at 200mW exposure for 4 minutes. The number of *E. coli* cells counted on nutrient agar plates with and without laser treatment are given.**

Trial	No treatment	Laser treatment
1	113	102
2	121	105
3	123	94
4	112	106
5	108	85
Average	115.4	98.4
Std dev	6.35	8.85

In Table 3.3, the relative percentage lysis for the 1480nm and the 980nm laser are given.

Table 3.3 The percentage lysis vs. laser wavelength and power using the droplet method

	1480nm, 100mW	980nm, 200mW
% Lysis	21.10%	14.70%

As expected, the 1480nm produced a greater percentage of lysis (21% vs. 15%) even at half of the power. However, in both cases, the percent lysis determined is lower than what was found previously by Dhawan. The most likely reason was the difference in experimental setup. The setup used here did not include a lens that focused the beam directly on the center of the droplet. Therefore, the power of the laser was much more scattered and also misaligned. In addition, as mentioned before, the 1480 was deemed unusable later on in the microfluidic experiments. But, it was also likely that an insufficient, but less noticeable, power output was generated by this laser during these experiments.

The alternative droplet method of using a PDMS well and a glass slide cover did not prove to be advantageous (Table 3.4).

Table 3.4 Results of the droplet method (alternative) for lysis of E. coli cells using 980nm laser at 200mW exposure for 4 minutes. The droplet is placed in a PDMS well with a glass slide cover. The number of *E. coli* cells counted on nutrient agar plates with and without laser treatment are given.

Trial	No treatment	Laser treatment
1	106	104
2	124	107
3	126	93
4	101	109
Avg	114.25	103.25
Stdev	12.6062	7.13559
% Lysis	9.63%	

It is possible that the extra glass layer created more scattering and possibly absorbed some laser power. However, it is also possible that in the uncovered experiments, some cells were simply lost due to evaporation of the medium. It was estimated visually that the droplet has shrink by one third in volume. In Dhawan's experiments, since the laser beam was more focused and well aligned, more evaporation would have likely occurred. This also could have contributed to the higher percentage of lysis observed by Dhawan.

3.2 Effectiveness of lysis of E. coli using microfluidic channels

E. coli K12 strain was subjected to the 980nm laser in the microfluidics channels. Silicon wafers of both the parallel and serial design were made. However, the CAD drawing of the serial design did not allow for enough space between the microfluidic channel and the laser trench. The analysis of the wafer showed that this wall was only about 75% in height compared to the rest of channel walls. Experimenting with the wafer resulted in liquid leaking into the laser trench. Unfortunately, due to time constraint, a redesigned wafer was not produced. and lysis experiments were only carried out with the parallel design.

Tables 3.4, 3.5 and 3.6 summarize the results of using the parallel design with the 980nm laser at 200mW power setting with different flow rates (5 μL/min, 2 μL/min, 1 μL/min) resulting in different exposure times, i.e. 0.18 seconds, 0.45 seconds, and 0.9 seconds, respectively.

Table 3.5 **Investigation of the dependence of laser-induced cell lysis on the exposure time, using the 980nm laser at 200 mW power. The flow rate of 5μL/min was used, translating into 0.18s exposure time. The number of *E. coli* cells counted on the nutrient agar plate after flowing through the parallel design micro channels with and without treatment are shown.**

Trial	No treatment	Laser treatment
1	78	53
2	69	46
3	77	55
4	64	43
5	64	46
Average	70	49
Std dev	6.8	5.1

Table 3.6 **Investigation of the dependence of laser-induced cell lysis on the exposure time, using the 980nm laser at 200 mW power. The flow rate of 2μL/min was used, translating into 0.45s exposure time. The number of *E. coli* cells counted on the nutrient agar plate after flowing through the parallel design micro channels with and without treatment are shown.**

Trial	No treatment	Laser treatment
1	90	64
2	94	52
3	95	66
4	99	48
5	97	48
Average	95	56
Std dev	3.4	8.8

Table 3.7 **Investigation of the dependence of laser-induced cell lysis on the exposure time, using the 980nm laser at 200 mW power. The flow rate of 1µL/min was used, translating into 0.9s exposure time. The number of *E. coli* cells counted on the nutrient agar plate after flowing through the parallel design micro channels with and without treatment are shown.**

Trial	No treatment	Laser treatment
1	112	50
2	105	63
3	114	67
4	100	69
5	leaked	60
Average	108	62
Std dev	6.4	7.5

In Figure 3.1, the relative percentage lysis between the flow rates in the parallel design channel is shown.

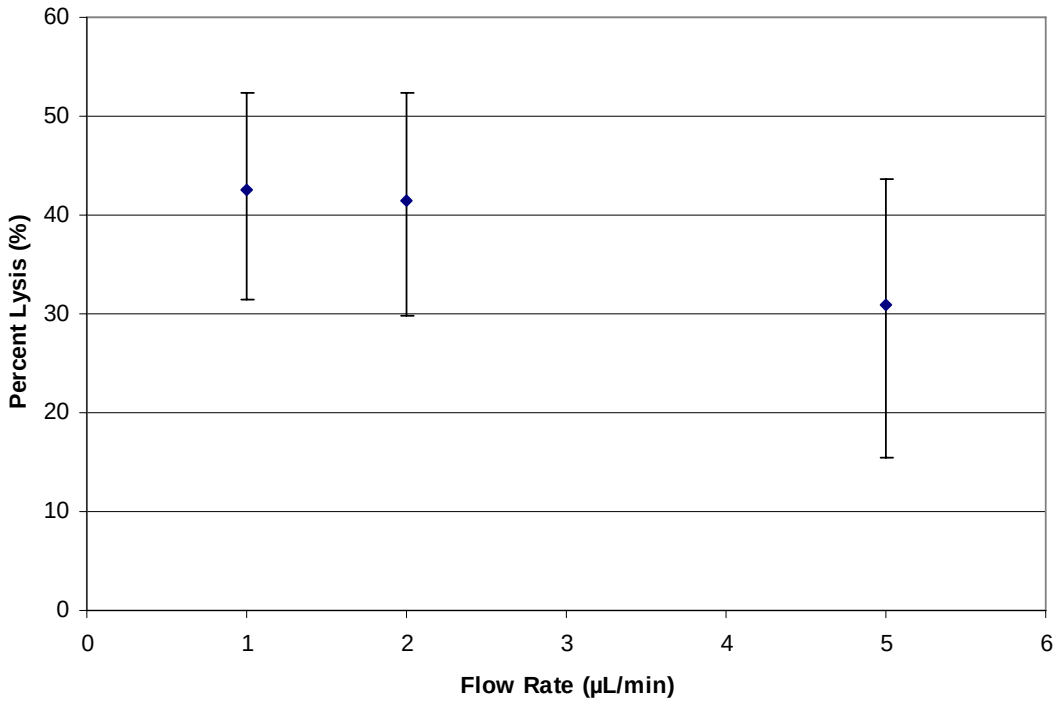

Figure 3.1 **The percentage lysis versus flow rate in the parallel-design micro channels using the 980nm at 200mW. Each analysis was done with five replicates. The flow rates translate into exposure times of 0.18s (5µL/min), 0.45s (2µL/min), and 0.9s (1µL/min)**

The data shows that 980nm laser at 200mW will lyse a certain percentage of *E. coli* cells depending on the exposure time to the laser. As expected, longer exposure time created more absorbance in the laser energy, hence, higher lysis. However, the overall percentage was much lower when compared to Dhawan's results who reported 67.5% lysis at 5μL/min and 98.5% lysis at 1μL/min.

3.3 Effectiveness of lysis of B. subtilis spores using microfluidic channels

Since *B. subtilis spore* had much higher affinity to PDMS channel wall than *E. coli* cells, testing the effectiveness of a blocking agent, 0.5% BSA in water, in order to prevent the loss of spores in the microchannels was first done. Table 3.7 summarizes the results.

Table 3.8 **Blocking of the PDMS microchannels in order to prevent loss of spores by adhesion. 0.5% BSA was used to pre-flush the channel. Data comparing the use of the blocking agent with no blocking agent present were carried out Direct plating used 10μL of spore culture that were directly plated on the Petri dish, in the case of the microchannel experiments, 10μL of spore solution was flown through PDMS channels at 5μL/min.**

Trial	Direct plating	Thru PDMS, w/o BSA	Thru PDMS, w/ BSA
1	103	73	92
2	99	24	90

BSA was deemed to be an effective blocking agent. It was found that 0.5% was a good blocking reagent. While without BSA 52% spores were lost through adhesion to the PDMS channels, a yield of 91% spores was found after blocking the channels with the BSA solution.

Subsequently, *B. subtilis spore* were subjected to the 980nm laser at 200 mW power in the microfluidics channels. Again, only the parallel-design microfluidic channels

were used. Tables 3.8, 3.9, 3.10, and 3.11 summarize the results for flow rates of 5μL/min, 2μL/min, 1μL/min, and 0.5μL/min, respectively, resulting in exposure times of 0.18 seconds, 0.45 seconds, 0.9 seconds, and 1.8 seconds respectively.

Table 3.9 **Investigation of laser-induced spore lysis of B. subtilis spores. 10 μL of spore solution was flown through the parallel design microchannels at 5 μL/min and exposed to the 980nm laser at 200 mW power, resulting in 0.18s of exposure. Viable spores were counted subsequently on nutrient agar plates.**

Trial	No treatment	Laser treatment
1	Leaked	146
2	141	149
3	180	176
4	172	168
5	180	175
Average	168.	163
Std dev	18.6	14.3

Table 3.10 **Investigation of laser-induced spore lysis of B. subtilis spores. 10 μL of spore solution was flown through the parallel design microchannels at 2 μL/min and exposed to the 980nm laser at 200 mW power, resulting in 0.45s of exposure. Viable spores were counted subsequently on nutrient agar plates.**

Trial	No treatment	Laser treatment
1	94	77
2	65	96
3	101	101
4	80	83
5	60	38
Average	80	79
Std dev	17.8	24.9

Table 3.11 **Investigation of laser-induced spore lysis of B. subtilis spores. 10 μL of spore solution was flown through the parallel design microchannels at 1 μL/min and exposed to the 980nm laser at 200 mW power, resulting in 0.9s of exposure. Viable spores were counted subsequently on nutrient agar plates.**

Trial	No treatment	Laser treatment
1	147	150
2	135	116
3	115	77
4	103	118
5	99	95
Average	119.8	111.2
Std dev	20.6	27.4

Table 3.12 **Investigation of laser-induced spore lysis of B. subtilis spores. 10 μL of spore solution was flown through the parallel design microchannels at 0.5 μL/min and exposed to the 980nm laser at 200 mW power, resulting in 1.8s of exposure. Viable spores were counted subsequently on nutrient agar plates.**

Trial	No treatment	Laser treatment
1	158	67
2	62	45
3	120	16
4	14	94
5	124	143
6	175	80
7	61	114
8	131	129
9	121	155
Average	107	94
Std dev	51.5	46.3

It was found that with no flow rate investigated, laser-lysis of B. subtilis spores could be determined. While in all cases a lower number of spores were viable after laser

treatment, the numbers were insignificant taking standard deviations into account. This becomes especially obvious with the data for the slowest flow rate of 0.5 μL/min in which adhesion of spores still caused a high scatter of the data. A total of 9 analyses were carried out. Here, 12% lysis was obtained, however, with standard deviations near 50% the data become insignificant. Therefore, it is likely this channel design (or the setup, as will be discussed in more detail in chapter 3.4) is not suitable for laser lysis that requires slow flow rates.

3.4 Possible improvements toward the current design

The results of laser lysis of *E. coli* showed that the 980nm laser was much less effective when compared to results obtained by Dhawan [4]. One possibility may be the different setup used. Here, we used a positive flow pressure in order to more precisely control the flow rates vs. a negative pressure used by Dhawan.

Also, the position of the laser fiber tip in the trench was of concern. The beam coming out of the fiber was 12μm wide and yet the depth of the channels was 100μm wide. The laser beam would disperse to reach the entire length. Calculation showed that with a wavelength of 980nm, it would take 5mm for the beam width to reach 100μm [4]. The width of all 25 channels was about 2.5mm, therefore, the laser fiber should be placed about 2.5mm from the wall. However, in this experiment, the fiber was placed as close to the channels as possible. Thus, it is likely that many organisms passing through the channels were never exposed to the laser.

The use of input and output wells created reservoirs that were difficult to completely empty and wash. This added more variability to the system. The flow in these wells was not as well defined as in the channel. The input and output tubes on the top Plexiglas platform have a set distance between them, so it was not possible to place those openings at the ends of the wells. Therefore, after the organisms are injected

into the input well, they did not necessarily enter the channels as expected. This would be a more prominent phenomenon at lower flow rate. So it would have been possible that some of the organisms did not enter the channels until the post-flush washing step at much higher flow rate. At this time, the laser would have been turned off. Even if it was on, the exposure time on such high flow rate would be so low that lysis would not have been expected. Therefore, a design should be used that eliminates the need of these wells. One such design could be as simple as a wider starting channel that would branch off to numerous thinner parallel channels.

It would be possible to increase the lysis yield of *E. coli* with the improvements mentioned above. *B. subtilis* spores, however, showed that they were much more resistant to laser-induced lysis. Therefore, either more energy or a more appropriate wavelength is needed in order to induce lysis. Using a laser that emitted light near 1950nm would most likely have resulted in lysis even with using a much lower power output. However, the downfall of using a higher wavelength laser is portability. The lysis method is designed for a micro-total analysis system, and as the wavelength of the laser increases, so does size. Therefore, finding the right balance between, wavelength, power, and size would be the goal of this area of research in the future.

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