

MICROFLUIDIC DEVICE FOR CYTOTOXICITY SCREENING

BY

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THESIS

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ABSTRACT

Cytotoxicity screening is important for the identification of different toxins and the quantification of toxin concentrations that can be detrimental to the proliferation of mammalian cells. Current cytotoxicity screening method requires initial cell seeding in 96-well cell culture plates and a large volume of culturing media, overall a time-consuming and resource-intensive process. We have developed a microfluidic device with internal structures that can evenly distribute cells across five chambers and requires only microliter volume of cell culturing media. In this study, we performed fluid dynamic simulations of different microfluidic designs with internal structures that can enhance the uniformity of distribution of cells in the initial seeding. We fabricated microfluidic devices with the optimal internal structures and performed several cell seeding experiments that showed high uniformity distribution of cells across all chambers. We have also performed cytotoxicity-screening experiments, using dimethyl sulfoxide and ethanol as toxins, to compare the performance between the microfluidic device and 96-well plate and the results showed close agreement between the two systems. Due to the generalizable design, this microfluidic device can be utilized in *in vitro* studies such as the response of cells to water-soluble factors such as anti-cancer drugs as well as the dynamic binding of anti-bodies to adherent cells.

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LIST OF SYMBOLS AND ABBREVIATIONS

f	Body Force
BAA	Bromoacetic Acid
CO ₂	Carbon Dioxide
HeLa	Cervical Cancer Cell Line
CHO	Chinese Hamster Ovary Cells
ρ	Density
DIC	Differential Interference Contrast
DMSO	Dimethyl Sulfoxide
DBP	Disinfection By-products
EPA	United States Environmental Protection Agency
SU-8	Epoxy-based Negative Photoresist
HAA5	Haloacetic Acids
μm	Micro-meter, $1/10^6$ of a meter
<i>P</i>	Partition Coefficient
Pa	Pascal or Newton/meters ²
PBS	Phosphate Buffered Solution
PDMS	Polydimethylsiloxane
P	Pressure
r_p	Radius of particle
Re	Reynolds Number
R6G	Rhodamine 6G
TIFF	Tagged Image File Format

T	Temperature
TRITC	Tetramethyl Rhodamine Isothiocyanate
THM	Trihalomethanes
$\mathbf{v}, u$	Velocity of Fluid
η	Viscosity

CHAPTER 1: INTRODUCTION

1.1 Disinfection By-products

Disinfection by-products (DBP) are formed from the reaction between chemical disinfectants (e.g. chlorine and chloramines) and organic constituents (e.g. decaying vegetation) in the treatment of wastewater. The major classes of DBP that have identified and regulated by Environmental Protection Agency (EPA) are trihalomethanes (THM), haloacetic acids (HAA5), bromate, and chlorite [1, 2]. The class of THM includes of chloroform, bromoform, bromdichloromethane and the class of HAA includes chloroacetic acid, dichloroacetic acid, and bromoacetic acid [3]. The regulation of human exposure to DBP by EPA does not differentiate the level of exposure to the individual species (i.e. chloroform vs. bromoform) of the major DBP classes but instead considers the total exposure to a major class of DBP. The current maximum containment levels for THM, HAA5, bromate, and chlorite are 0.080 mg/L, 0.060 mg/L, 0.010 mg/L, and 1.0 mg/L respectively [4].

Humans are exposed to DBP in treated water through ingestion, inhalation and skin absorption on a daily basis. Life time exposure to chlorinated water and the related DBP is associated with an increased risk for urinary bladder cancer in large-scale epidemiological studies. For exposure higher than 50 µg/L trihalomethanes (THM), there has been an average increase of odds ratio of 1.44 in the incidence of urinary bladder cancer in the population, see Figure 1.1 [5]. Systemic *in vitro* studies of prolonged exposure of DBP to both prokaryotes and eukaryotes have been conducted to identify the relative cytotoxicity of each of the DBP. The exposure of selected DBP, the most common types in drinking water, to *Salmonella Typhimurium* indicated brominated acetic acids to be more much more cytotoxic than the chlorinated analogs and provided a quantitative comparison of cytotoxicity of the DBP [6]. While cytotoxicity

screening studies using prokaryotes offered relevant quantitative information, the exposure of DBP to humans is better represented by *in vitro* studies using mammalian cells. Chinese hamster ovary (CHO) cells were exposed to the same set of selected DBP's utilized in the *Salmonella Typhimurium* study and there was no correlation between the cytotoxicity of DBP in the bacterial study using *Salmonella Typhimurium* and the mammalian cell study using CHO cells [7]. The difference in the level of cytotoxicity of DBP between the bacterial and mammalian systems is indicative of the differences in the underlying biological mechanisms such as membrane transport of DBP and reactivity of DBP with intercellular organelles. Mammalian cell assays can provide more relevant toxicological information than microbial assays in understanding the risks to human from exposure to DBP. When combined with high throughput screening methods, mammalian cell-based assays can provide rapid quantitative assessment of different concentrations and types of DBP relevant to human exposure.

1.2 Cytotoxicity Screening using 96-well Plate

The current method of cytotoxicity screening utilizes a 96-well plate for a systematic study of cell growth in the presence of different concentrations of toxin. The 96-well plate screening method allows for a relatively quick screening of the cytotoxicity of the toxins with sufficient number of wells for statistical significance. The general protocol for the 96-well screening method is summarized based on the reports from Plewa *et al.* [8, 9].

Approximately 3,000 CHO cells are seeded in each of the 96 wells first. Next, different concentrations of toxin diluted in cell culturing media are dispensed into each of the 96 wells such that there is an increase in the concentration of toxin along the length of the 96-well plate, see Figure 1.2. The plate allows for a screening of twelve different concentrations of toxin and eight individual wells contain the same concentration of toxin. The 96-well plate with cells and

toxin-added culturing media is placed in an incubator and the cells are continuously cultured for 72 hours, exposed to the toxin in the entire duration. After 72 hours of culturing, the cells in the 96 plates are fixed with methanol, rinsed with the culturing media, and then stained with crystal violet stain. Due to the fixation and rinsing steps, only the viable cells remain stained in the wells. The relative density of stained cells in each well is quantified using an absorbance measurement. The cytotoxicity of the toxin can be then determined by comparing the relatively densities of viable cells after the 72-hour exposure with respect to different concentrations of toxin.

While the 96-well plate screening method offers a quantitative assessment of cytotoxicity of toxin, the entire process is time-consuming and resource-intensive. The individual well of the entire plate must be loaded manually, once for seeding the cells and once for dispensing toxin-added media. Likewise, for the absorbance measurement, all of the wells must be measured individually. In terms of resources, more than 100 mL of culturing media is needed per trial of the screening. Due to the low-concentration level of toxin present in contaminated samples, a large volume of the sample is required to distill a high concentration of the toxin such that there is a sufficient volume to dilute in order to perform one trial of the 96-well screening experiment.

An alternative method of cell toxicity screening must address the deficiencies of the current 96-well screening method. Specifically, it must alleviate the time-consuming processes of cell loading and the viability assessment of cells by automating the processes or running the individual processes in parallel. In addition, it must reduce the required volume of toxin-contaminated source and provide the same quantitative information to assess the viability. Lastly, the alternative method must be compatible with the protocol used in 96-well plate for the purposes of comparison and validation.

1.3 Microfluidic-based Cell Culturing

1.3.1 Background

Microfluidics has been utilized to transport, contain, and manipulate micro-liter to nano-liter volume of fluid [10]. The usage of microfluidics in cell culturing application inherently lowers the required volume of fluid and satisfies the low volume requirement of cytotoxicity screening. Moreover, the size of a microfluidic device, with the same number of individual wells, is much smaller than the macro-scale devices, the largest dimension being on the order of tens of millimeters. Microfluidic devices are often coupled with a programmable syringe pump to transport the fluid in a controllable manner and such system can be used to automate the cell and culturing media loading processes. Due to the ability to manipulate fluid more dynamically in microfluidic devices than in macro-scale devices (e.g. 96-well plate), the usage of microfluidics can enable more physiological-relevant conditions such as a constant flow environment and a lack of waste build-up in a cell-culturing application. A literature review of microfluidic devices for cell culturing presents several different methods and designs to create a compatible environment for biological samples. The major categories of microfluidic device design include: (1) patterned surfaces, (2) single-layer micro-channels and micro-wells, (3) multi-layer micro-channels, and (4) micro-chambers with internal structures.

1.3.2 Cell-Patterned surfaces

One of the simplest methods of cell culturing using microfluidics is patterning micro-scale regions on a surface to define where cells can adhere and grow, see Figure 1.3. The patterning process is accomplished by exposing a cell-culture surface to an oxygen-plasma treatment through a blocking mask. The regions of the surface that are blocked by the mask are not exposed to oxygen plasma and the regions that are exposed subsequently react with oxygen

plasma to become hydrophilic regions. Since cells preferably adhere and grow on hydrophilic surfaces, they can adhere only to the regions that are exposed to oxygen plasma. The usage of a blocking mask in oxygen plasma-modification of a surface allows spatial definition of micro-scale regions that can accommodate cell adhesion and growth [11].

1.3.3 Micro-channels and micro-wells

Soft lithography technique has been used extensively in the field of microfluidics to create micro-scale features such as channels and chambers. The lithography technique consists of fabricating a negative master of microfluidic features using photolithography and replicating the positive features on an elastomer, the most common being polydimethylsiloxane (PDMS). The usage of soft lithography allows rapid fabrication and accurate replication of micro-scale channels and chambers on an elastomer, which can be used to maintain the cell culture.

Using the soft lithography technique, micro-channels and micro-wells have been fabricated to localize the regions of cell adhesion and growth for biological studies. The largest dimension (e.g. diameter) of the micro-channels and micro-wells is on the order of 100 μm , which is sufficient to maintain tens of mammalian cells [12]. In contrast to the patterned surface where the cells are localized on a planar surface, the three-dimensional geometry of micro-channels and micro-chambers confines the adhesion and the subsequent growth of cells to a micro-scale region without the need for a patterning step. The differences in the feature height of micro-channels and chambers inherently prohibit the cells from growing outside of the features to a larger height. Moreover, the three-dimensional structures can be fully sealed by bonding to a fluid-impermeable material such as glass and form enclosed spaces with micro-liter to nano-liter volumes. The ability to fabricate fully sealed micro-scale chambers and channels using soft

lithography enables the possibility of long-term cell culturing, which requires constant suspension and immersion of cells in a culturing media for maintenance and growth.

1.3.4 Multi-layer microfluidics and micro-chambers with internal structures

The capability of the microfluidic device can be improved with the usage of multi-layer designs to allow more complex control over fluid transport. For example, multi-layer microfluidic devices can have built-in pneumatic valves by pressurize one channel to deform and occlude the adjacent channel [13]. One layer of channels can be used to load cells into the microfluidic device and a different layer of channels can be used to introduce culturing media during the period of cell culturing [14]. Internal structures such as micro-sieves can be used to capture a desired number of cells in a moving fluid, see Figure 1.4 [15]. It is evident that with increasing complexity in fabrication, microfluidic devices can be designed to incorporate a variety of control mechanisms to manipulate the fluid transport and the particles in the fluid. In the following chapter, the requirements and constraints of cell culturing are discussed in order to formulate the design of the microfluidic device that can address those issues.

1.4 Figures

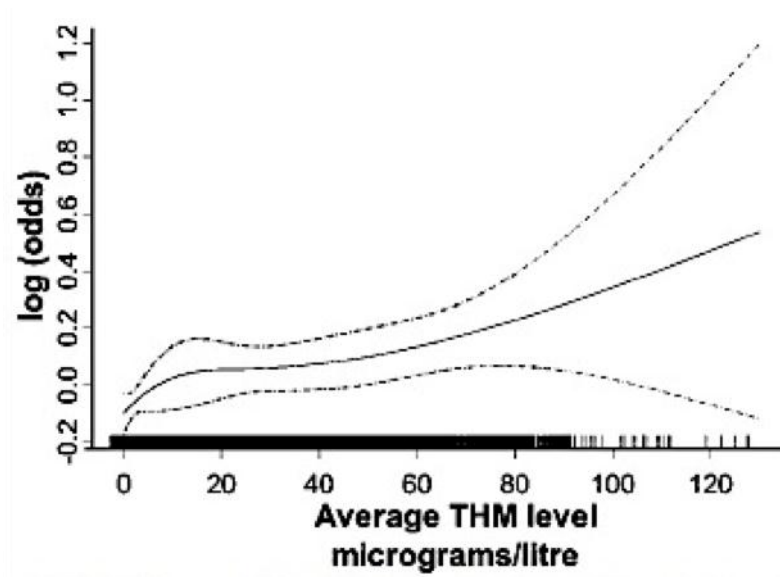


Figure 1.1 Association between the lifetime level of exposure to chlorinated water (Trihalomethanes) and the risk for urinary bladder cancer. Modified from [5].

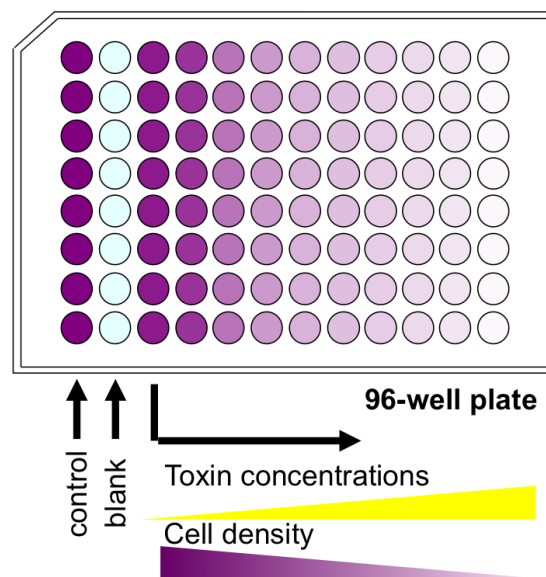


Figure 1.2 Schematic configuration of a cytotoxicity screening method based on a 96-well plate. Modified from [8].

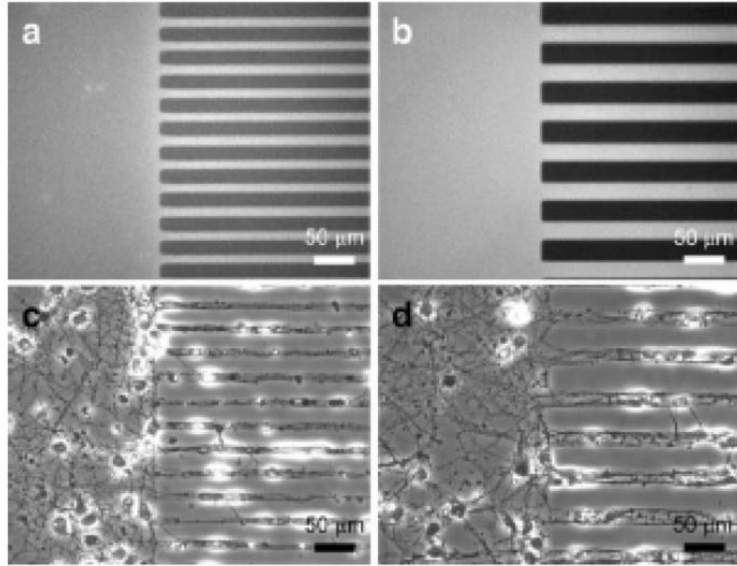


Figure 1.3 Patterned surfaces for defining cell attachment and growth area. Line patterns with different periodicity fabricated using exposure to oxygen plasma (a, b). Preferential attachment and growth of neuronal cells in micro-scale regions exposed to oxygen plasma (c, d). Modified from [11].

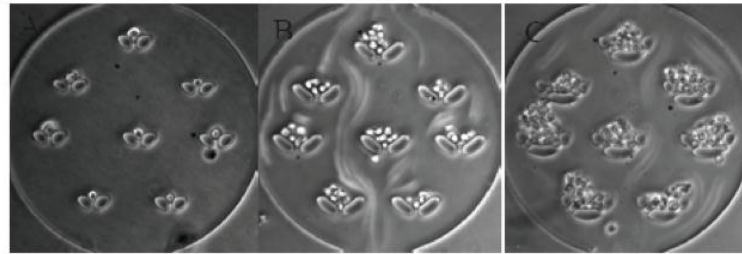


Figure 1.4 Micro-sieve structures constructed to load the same number of cells into each chamber. Different sizes of micro-sieves capture different number of cells in each chamber. Modified from [15].

CHAPTER 2: MICROFLUIDIC DESIGN REQUIREMENTS AND SIMULATION

2.1 Requirements for Microfluidic Cytotoxicity Screening

2.1.1 Biocompatibility and cell model

All materials involved in the fabrication of the microfluidic device for cell culturing must be biocompatible. Biocompatibility is defined as “the ability to of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient ... of that therapy ...” [16]. The biocompatibility of material in cell culturing applications ensures that the functions and growth of cell are unaffected by the surrounding environment. That assurance is important for studies such as cytotoxicity screening where the effect of the toxin concentration on the functions and growth of cells is under examination.

Mammalian cell line such as Chinese hamster ovary (CHO) cells is a good model to study the detrimental effects of toxins since mammalian cells are more biologically similar to human cell lines than bacterial organisms. CHO cell lines are immortalized (i.e. able to divide indefinitely) and non-cancerous (i.e. express cell contact-inhibition and cell apoptosis), both of which are biologically relevant for assessing the risk of exposure to toxins in human population [17]. While cancerous cell lines (e.g. HeLa cells) are common in cell studies, they are not appropriate for cell toxicity screening application due to the genetically altered pathways of cell apoptosis and cell contact inhibition, both of which affect the function and growth of cells. Such phenotypes associated with cancer cell lines alter the interaction and effect of toxin on the cells and are difficult to account for in the resulting data. Thus, for cell toxicity screening, it is important to utilize an immortalized cell line, which is non-cancerous, to accurately assess the effect of toxin on the function and growth of cells.

2.1.2 Uniform cell number across wells

The uniformity in the number of cells loaded across the individual chambers is important for a cytotoxicity assay due to the comparison assessment of relative toxicity in the mammalian cells. The uniformity must be maintained to ensure statistical significance and similar milieu for cell growth in terms of nutrient consumption and waste production. Moreover, by the nature of microfluidics, the initial number of cells inside the chambers is much lower than that of the macro-scale assay; therefore, a difference of tens of cells can represent a larger standard deviation in uniformity than in a macro-scale assay where the initial number of cells exceeds 3,000. The intent of this research project is to create a cell-culturing microfluidic device that can replace the traditional method of cell toxicity screening. For validation, the microfluidic device must be compatible with the protocol utilized in the traditional macro-scale cell toxicity screening.

2.2 Simplicity in Fabrication and Operation of Microfluidic Devices

2.2.1 One-layer fabrication

The end users of this microfluidic device will be life-science researchers, who are unfamiliar with the design and fabrication techniques. Therefore, it is crucial for the practicality of this device to be simple in fabrication, operation, and maintenance. The final design of the microfluidic device must be appropriate for the skill and knowledge of the end users in order for the device to be practical in research environment. With those considerations in mind, the design of the microfluidic device was considered to be a one-layer device, which is simpler in fabrication. Multi-layer microfluidic devices require multiple photolithographic masks and alignment in fabrication.

2.2.2 Fluid stoppage and gravity-driven flow for long-term culturing

Traditionally, fluid transport into a microfluidic device is coupled with a syringe pump or a control pump system to deliver the required volume and flow rate of the fluid. A syringe pump is ideal for this application due to its portability, simplistic operation, and accuracy in volume control. However, it cannot be used to reliably stop the fluid in transport due residual pressure difference between inlet and outlet and/or height difference between inlet and outlet, both of which can continue to drive the fluid even after the syringe pump has been stopped. Therefore, another system must be in place to ensure a complete stoppage of fluid in order to allow the cells in the fluid to settle and attach to the substrate. If the cells remain in suspension in the moving fluid, there is no opportunity for the cells to be loaded inside the microfluidic device.

Once the cells are loaded, the microfluidic device must take kept inside an incubator, which maintains the required conditions for cell growth. Due to the long-term nature ($t=72$ hours) of cell culturing required, there is a constraint in the available space and compatible environment for a syringe pump inside an incubator. Moreover, the incubator is a completely sealed space and there is no electrical connection inside the incubator for a syringe pump. Active fluid transport through the microfluidic device using a syringe pump inside an incubator is not a viable option. Therefore, passive fluid transport, a gravity-driven process, was considered. The obvious advantage is the lack of requirement for a source of power; however, the disadvantages are several: (1) minimal control of the rate of fluid transport, (2) additional tubing/coupling to the microfluidic device, and (3) manual refilling of the fluid. Despite its disadvantages, gravity-driven fluid transport was sufficient for the requirements of this research project.

2.3 Finite Element Analysis of Microfluidic Device Designs

2.3.1 Motivation for performing finite element analysis

The two major requirements of the microfluidic device design for cell culturing application are cell-loading uniformity and de-aggregation of cells at loading. The desire for cell-loading uniformity is for statistical advantage, where uniformity will allow direct one-to-one comparison between the growth of cells in different microfluidic devices. Moreover, the uniformity of cell loading ensures the cells are growing in a similar environment, which includes the available amount of nutrient or the level of waste in the culturing media.

Aggregation of cells at loading is undesirable for microfluidic cell culture since CHO cells, and most adherent mammalian cells, express cell contact inhibition, a cellular mechanism that disallows the continual growth of cells when the cell culture is confluent. If the contact inhibition effect is in place, the growth of cells will be retarded and the effect is difficult to account for in the resulting cell growth measurements. For the purpose of this project, the effect of toxin on the growth of the cells in the microfluidic device is important; therefore, the design of the microfluidic device must minimize or eliminate the effect of contact inhibition.

Finite element analysis offers a cost-effective to study the performance of microfluidic designs, allowing quick assessment of tens of designs without any fabrication. Fluid dynamic simulations have been used frequently to study the fluid transport in microfluidics. Moreover, the microfluidic device designs drawn up to be simulated using finite element analysis can be further used in the fabrication of the devices. With the two major requirements in mind, the potential designs of the microfluidic device were generated and simulated in a commercial software package (COMSOL, Burlington, MA). The following designs were simulated in COMSOL: (1) simple channel, (2) simple cell chamber, and (3) cell chamber with micro-posts, see Figure 2.1.

2.3.2. Microfluidic simulation set up

The microfluidic simulation was completed using a two-dimensional incompressible, isothermal

Navier-Stokes model and a simpler Stokes model. The general equation of fluid dynamics is given by the Navier-Stokes equation (Equation 2.1) where the velocity field is described by the gradient of pressure, the divergence of the stress tensor, and the body forces acting on the fluid. The laminar or turbulent nature of a fluid flow is defined by the unit-less Reynolds number, Re , see Equation 2.3. The fluid flow in microfluidics is characteristically laminar, that is the Reynolds number is less than 2300 since the characteristic length, L , is generally micrometers. For this microfluidic device, L can be considered to be approximately 50 μm . Due to the laminar flow, a simpler model, Stokes model, can be used to simulate the fluid flow in microfluidics. In the Stokes model, the inertial effects are neglected due to their diminished influence in the microfluidic regime. The simplified Stokes model is only concerned with the divergence of the pressure field in the fluid and other body forces acting on the fluid, see Equation 2.2.

$$\rho \frac{D\mathbf{v}}{Dt} = -\nabla p + \nabla \cdot \mathbb{T} + \mathbf{f} \quad (2.1)$$

$$\nabla \cdot \mathbb{P} + \mathbf{f} = \mathbf{0} \quad (2.2)$$

Theoretically, the Stokes model fully captures the fluid dynamics in the microfluidic regime, which is viscosity-dominant ($Re \ll 1$), fully laminar flow. Computationally, the Stokes model offers a reduction in the number of calculations to be made and allows more simulations due to the decrease computational time. The Stokes model simulation has been verified against the more complex Navier-Stokes model simulation and for the microfluidic design presented in this research project, the difference of velocity fields between the two models was negligible. Additional assumptions made to the simulation were that the fluid is incompressible and isothermal. The fluid under study in simulation and eventually utilized in experiment was Phosphate buffered solution (PBS) and under experimental conditions, the assumptions made were computationally advantageous due to a reduction in variables to be solved.

$$Re = \frac{\rho VL}{\mu} \quad (2.3)$$

$$dh = 4 \frac{A}{p} \quad (2.4)$$

The models consist of a 50 μm -wide channel and 1 mm-diameter cell chambers with and without micro-posts. The choice of circular chambers, as opposed to other geometries, is to obtain the highest value of hydraulic diameter, dh . As shown in Equation 2.4, the hydraulic diameter is a ratio of the wetted area, A , and the wetted perimeter, p . The circular geometry gives the maximal value of dh with the minimum value of the wetted perimeter, which is proportional to the resistance of fluid transport and with the maximal value of surface area, which is desirable for maintaining a large number of cells. The boundary settings include an inlet uniform velocity of 1 mm/s and the outlet set as the atmospheric pressure. All other walls of the model are set as no-slip surface ($\mathbf{v} = 0$ m/s at the wall). The physical properties of the simulated fluid were described as having a density of $1\text{e}3 \text{ kg/m}^3$ and a dynamic viscosity of $1\text{e}-3 \text{ Pa}\cdot\text{s}$.

Once the velocity distribution of the fluid flow through the microfluidic device has been simulated, a particle tracing simulation was performed to observe the travel of the particles through the microfluidic channels and chambers. The individual particles have a mass of $1\text{e}-10$ kg and a radius of $7.5 \mu\text{m}$. The particles are introduced to the microfluidic device at a rate of 6 per second. The motion of the particles, or the force exerted on the particles, in the microfluidic device is described as a function of the velocity of the particle, u_p , relative to the velocity of the fluid, u , and the physical properties of the particle (i.e. the radius of the particle, r_p) and the fluid (i.e. density, ρ ; viscosity, η) see Equations 2.5 and 2.6 [18].

$$F = \pi r_p^2 \rho (u - u_p)^2 (1.84 Re_p^{-0.31} + 0.293 Re_p^{0.06})^{3.45} \quad (2.5)$$

$$Re_p = (|u - u_p| 2r_p \rho) / \eta \quad (2.6)$$

All of the microfluidic simulations were conducted in COMSOL version 3.5a under 64-bit

Windows Server Standard (Service Pack 1) with 2.83 Giga-hertz Intel Xeon CPU and 8.00 gigabytes of memory.

2.3.3 Microfluidic simulation results

The average Reynolds number across the entirety of the microfluidic device was less than 1, indicating the pure laminar nature of the fluid inside the device. We observed three phenomena from the microfluidic simulation that addressed the requirements of a microfluidic cell-culturing device. First, there was an initial cell separation due to a parabolic velocity profile in the channel, which is advantageous since it reduces the probability of cell aggregation in the microfluidic channel, see Figure 2.2. Second, each of the micro-posts inside the microfluidic chamber bifurcates the incoming fluid and physically separates the particles embedded in the fluid, which further discourages the aggregation of cells in the microfluidic device, see Figure 2.3. Third, the steady-state distribution of particles in a series of three microfluidic chambers shows uniform cell number and the steady-state distribution can be reached in less than one minute, see Figures 2.4 and 2.5. However, for the microfluidic chambers with internal micro-posts, the distribution of particles inside the chambers was more spread out, as opposed to being concentration in the center of the chamber, see Figure 2.5. This separation of particles in the microfluidic chamber was observed in all of the chambers in the simulation.

While the particle simulation showed the transient spatial distribution of particles, it must be noted that the simulation did not consider all of the forces that act on the particles in the real world. It is obvious from Equation 2.5, gravitational force is not considered although its influence would be small, relative to other forces (e.g. viscosity). Likewise, the normal forces from the structures and surfaces inside the microfluidic device are not considered in the particle

simulation. Therefore, the particle simulation is useful only for understanding the spatial distribution of the particles in a steady state fluid condition inside the microfluidic device.

Due to the success of the microfluidic device in addressing the requirements of a microfluidic cytotoxicity screening, the designs of the microfluidic devices from simulation were utilized in the fabrication of the designs.

2.4 Figures

Structure	Geometry
Simple Channel	
Simple Cell Chamber	
Cell Chamber with Micro-posts	

Figure 2.1 Schematic of microfluidic structures studied in COMSOL fluid dynamic simulation. Arrows indicate the direction of the fluid flow.

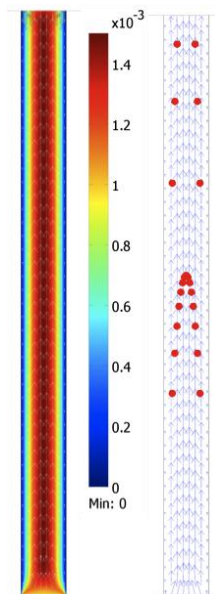


Figure 2.2 Fluid dynamic simulation results of a microfluidic channel. Velocity map of the fluid flow inside a microfluidic channel (left) and the position and the distribution of micro-particles (red dots) traveling through the microfluidic channel (right). The unit of the color map is given in m/s. The direction of the flow is from the bottom to the top. The width of the microfluidic channel is 100 μm .

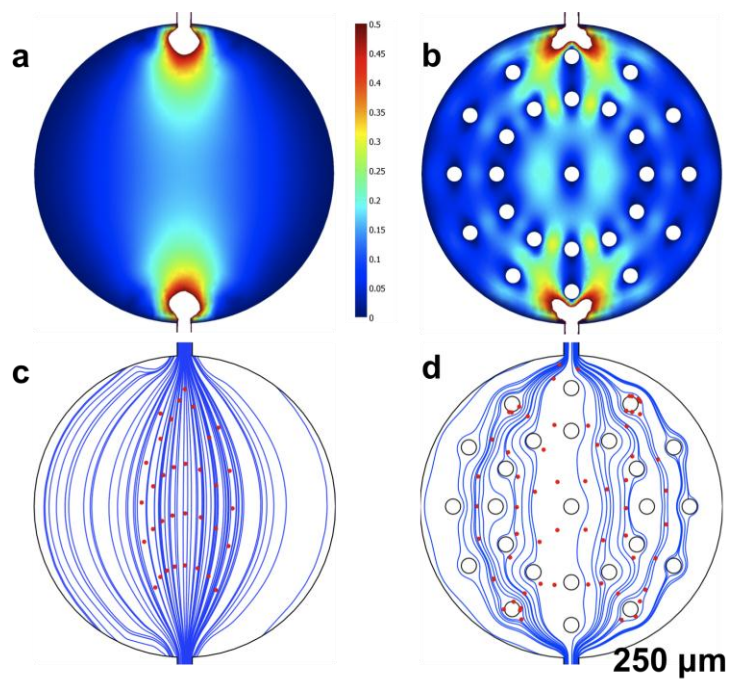


Figure 2.3 COMSOL fluid dynamic simulation result comparison of microfluidic chambers with and without internal structures. Velocity profile of microfluidic chamber with no internal structures (a) and microfluidic chamber with internal structures (c). Streamline and particle tracking inside microfluidic chamber with no internal structures (b) and microfluidic chamber with internal structures (d).

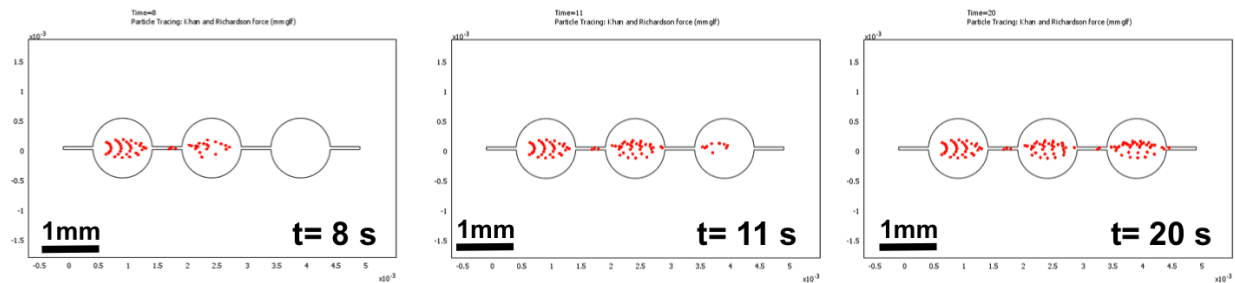


Figure 2.4 Three microfluidic chambers with no internal structures connected in series. Distribution of particles (red dots) flowing through the chambers over time = 8 seconds (left), 11 seconds (middle), and 20 seconds (right).

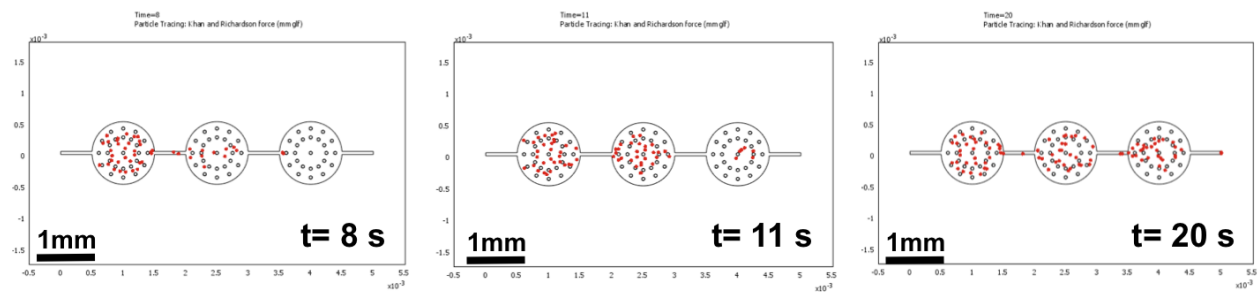


Figure 2.5 Three microfluidic chambers with internal structures (micro-posts) connected in series. Distribution of particles (red dots) flowing through the chambers over time = 8 seconds (left), 11 seconds (middle), and 20 seconds (right).

CHAPTER 3: EXPERIMENTAL METHODS

3.1 Microfluidic Device Fabrication using Soft Lithography

3.1.1. Fabrication of SU-8 master and PDMS device

Master wafers were prepared by spin coating a 30- μm layer of SU-8 2025 photoresist (MicroChem, Newton, MA) on silicon wafers and patterning the chambers and micro-post structures using a Quintel Aligner exposure system. PDMS (Dow Corning, Midland, MI) was prepared using a 10:1 v/v ratio of base to curing agent was then cast against the master and left to degas overnight followed by a 1 hour cure at 65 °C to complete the cure. PDMS devices were cut out by a razor blade and the fluidic connection ports were punched using an 18-gauge blunt-ended needle, see Figure 3.1. The PDMS was treated with oxygen plasma along with degreased glass slides for 0.9 minutes, after which both were pressed together to bond permanently.

3.1.2. Device visualization and characterization

The optical inspection of the microfluidic devices was accomplished using a 10X objective inverted microscope with bright field illumination and phase contrast mode, see Figure 3.2. Due to the fidelity of PDMS, the replication of microfluidic chambers, channels, and micro-posts was consistent over tens of devices. The only degradation observed over several replications was the micro-posts distortion or destruction due to the normal wear and tear of the replication process. New SU-8 masters were fabricated consequently to replace the old masters.

Passive pumping (gravity-driven) of a solution through the microfluidic chambers was qualitatively assessed using a time-elapsd, fluorescent imaging technique, see Figure 3.3 (right). A 200- μL pipette tip was filled with 200 μL of Rhodamine 6G solution and the pipette tip was inserted into the inlet of the microfluidic device. Fluorescent images of chamber 1 (the chamber closest to the pipette tip) and chamber 5 were taken for 0 minute, 5 minutes, 15 minutes, and 30

minutes using the TRITC setting with a 10x objective inverted microscope. The samples were kept in a dark container when not being imaged and the exposure of fluorescent imaging was <5 ms. The intensity levels across the chambers 1 and 5 were analyzed and plotted using Matlab (Natick, MA).

3.2 Microfluidic Cell Culturing Protocol

3.2.1 Cell culturing protocol

Chinese hamster ovary (CHO) cells are maintained in Ham's F12 medium containing 5% fetal bovine serum (FBS), 1% antibioticantimycotic solution (10 units/mL penicillin G sodium, 10 µg/mL streptomycin sulfate, 25 µg/mL amphotericin B, 0.85% saline; Invitrogen, Carlsbad, CA), and 1% glutamine and grown in 100 mm glass culture plates at 37 °C in a humidified atmosphere of 5% CO₂. The cells exhibit normal morphology, express cell contact inhibition and grow as a monolayer without expression of neoplastic foci. CHO cells are transferred when the culture becomes confluent.

3.2.2. Microfluidic device set up and cell loading experiment

Prior to cell loading, the microfluidic devices were first flushed using 75% ethanol and 1X PBS to sterilize, degas, and remove any leftover debris from fabrication. All the solutions used were filtered with 0.22-µm syringe filter prior to usage. Following the flushing step, a 100-µg/mL solution of fibronectin was flowed into the microfluidic device and the device was incubated for 1 hour to allow fibronectin to attach to the microfluidic surfaces. Post-incubation, the microfluidic devices were stored in 4°C environment for 24 hours prior to the cell culturing experiment.

Using 3×10^6 cells/mL cell solution, CHO cells were flowed through the microfluidic device using a syringe pump or manual pumping at approximately 100 µL/s, see Figure 3.3 (left).

During the cell loading process, the transit of the cells through the microfluidic chambers was monitored real-time to ensure there is no blockage or aggregation. Once there is a steady flow of cells through the microfluidic chambers, the flow was stopped using a clamping device to occlude the inlet and outlet tubing. Once the flow has stopped, the cells were allowed to settle and attach, and after five minutes of flow stoppage, the inlet and outlet tubing were disconnected.

A 200- μ L pipette tip was filled with the appropriate media and the pipette tip was inserted into the inlet port of the microfluidic device. The microfluidic device then was placed in a petri dish, covered with a large beaker to maintain high humidity, and placed inside an incubator with 70%-80% relative humidity and 5% CO₂. The cells inside the microfluidic devices were incubated continuously for 72 hours and imaged every 12 hours for cell enumeration.

3.2.3 Assessment of cell loading uniformity

The cell loading uniformity has been assessed by comparing the number of cells in each chamber immediately after loading. Cell-loading uniformity is determined by finding the ratio of the mean of the number of cells loaded in all the chambers and the standard deviation of the number of cells loaded in all the chambers. If the uniformity is high, the ratio will have a low value and vice versa for low uniformity.

3.3 Cell Growth Assessment and Automated Cell Enumeration

3.3.1 Cell visualization

During the 72-hour culturing period, the individual chambers of the microfluidic device were imaged every 12 or 24 hours using a 10X objective with bright field illumination in an inverted microscope (Olympus, Center Valley, PA). Closer inspection images of the cells were taken using the 40X DIC objective with the same microscope. Each imaging session lasts approximately five

minutes per microfluidic device and the devices were immediately returned to the cell-culturing incubator after the imaging sessions. The resulting images were saved as 8-bit gray scale files in TIFF format.

3.3.2. Cell enumeration

Manual counting of the cells was accomplished using an open-source image processing software, ImageJ (Bethesda, MD). Due to the distinct elongated morphology of the viable CHO cells, manual inspection was one possible method of identifying cells in the microfluidic chambers. All viable cells in view were marked with a black dot (5 pixels in diameter) using the Pencil tool in ImageJ, see Figure 3.4 and using intensity thresholding, the black dots were isolated from the rest of the features in the image. After intensity thresholding, the black dots, which represent the number of cells in the image, were enumerated using the Analyze Particles function in ImageJ using the default settings. The above procedure was repeated for each of the microfluidic chambers to determine the number of cells in each device.

Due to the repetitive nature of manual cell enumeration, an image-processing program was developed in-house using the image processing tools included in Matlab (Natick, MA). The purpose of the program was to eliminate the need for manual counting while providing the same level of accuracy in identifying and enumerating the viable cells. The algorithm of the program follows a similar protocol as the manual counting, which includes intensity thresholding, blob detection and enumeration. The inputs required from the user are the location of a particular image file or a folder that contains multiple image files to be processed, a straight line to identify the center of the microfluidic image, and a location to save the processed image files, see Appendix A for Matlab scripts. All of the automated image processing was conducted in Mac OS X 10.6.7 with 2.1 GHz Intel Core 2 Duo with 4 GB 667 MHz DDR2 SDRAM.

3.4 Figures

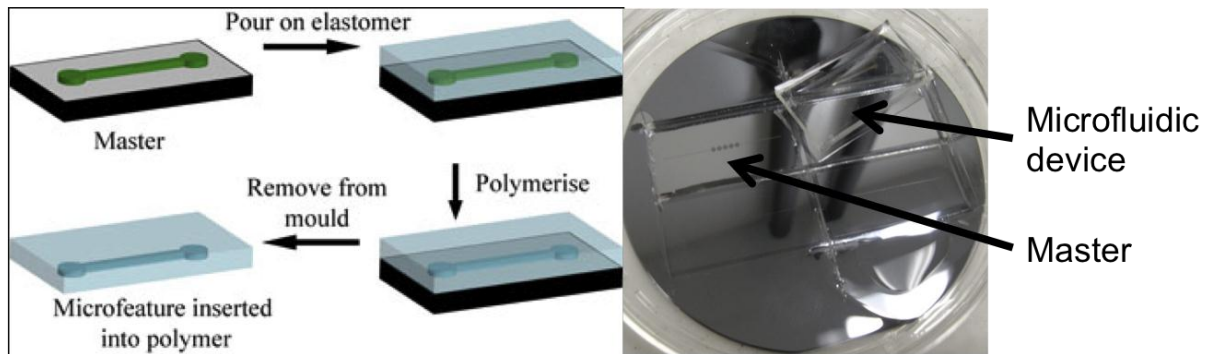


Figure 3.1 Fabrication of microfluidic device using soft lithography. Step-by-step process schematic of soft lithography fabrication (left) and a photograph of a microfluidic device fabricated from a master (right).

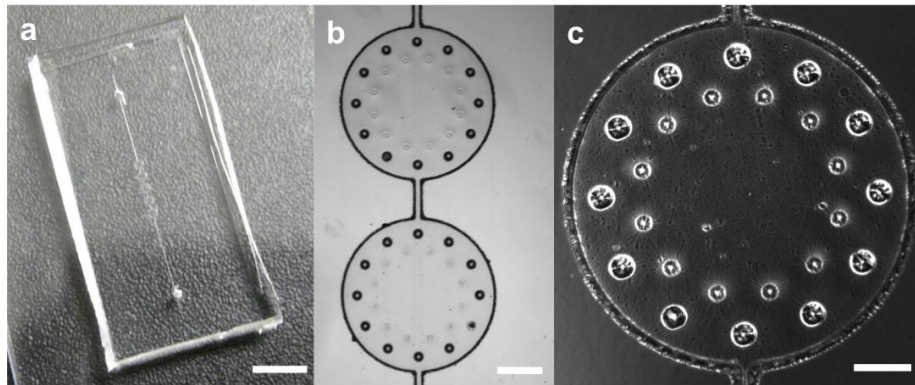


Figure 3.2 Visualization of microfluidic device with internal structures. Macro image of a finished microfluidic device (a), bright field illumination microscope image of the microfluidic chambers with internal structures (b), and a closer view of the micro-post array inside the microfluidic chamber (c). Scale bars are 1 cm, 250 μm , and 100 μm respectively.

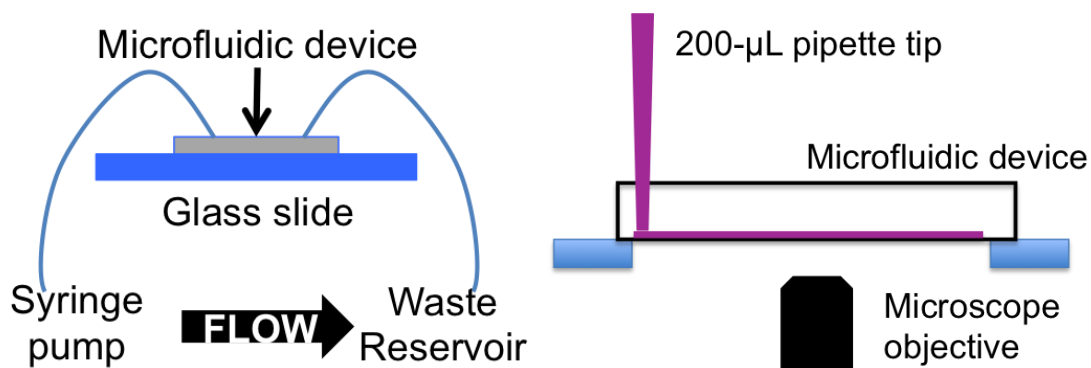


Figure 3.3 Experimental set up of the microfluidic cell loading and imaging. The cells enter the microfluidic device from the tubing attachment (thin blue line) to the syringe pump and exit to the waste reservoir (left). The imaging of the microfluidic device is done by placing the microfluidic device on a sample holder over the inverted microscope objective (right). Note the drawings are not to scale.

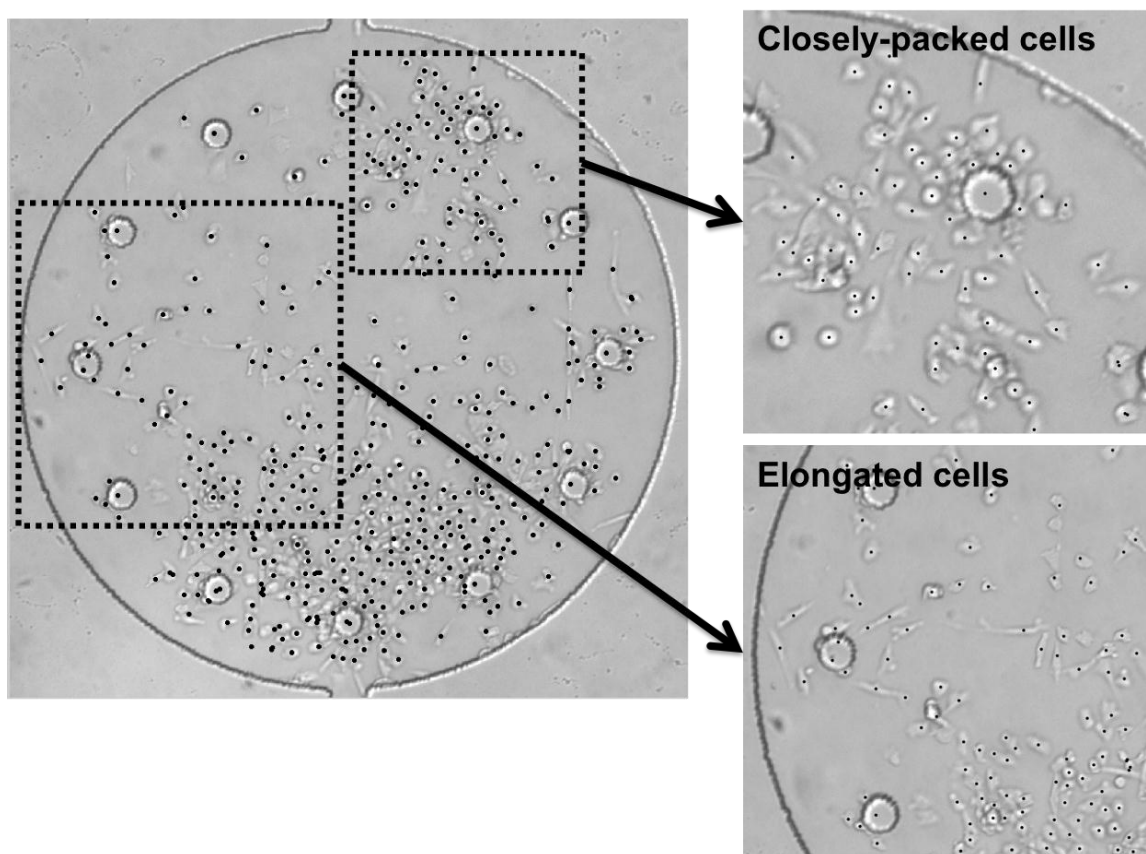


Figure 3.4 Image processing to detect the seeded cells in the microfluidic chamber. Detected cells (black dots) are marked and enumerated in a post-processing step.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Uniformity and Localization of Cell Loading

4.1.1. Spatial distribution of cells inside the microfluidic device

In independent trials of cell loading ($n=6$), the mean and one standard deviation of the percent uniformity are 0.19 and 0.07 respectively, see Table 4.1. Another word, on average, one standard deviation of the cell loading over all the chambers is less than 20% of the mean value. It must be noted that due to the low number of cell loading, the difference in the number of cells seeded in each chamber actually vary by less than ten cells on average. The cell loading uniformity has been observed to be higher with a higher concentration of cells than that of a lower cell concentration because the difference in number of cells loaded per chamber is independent of the cell concentration.

In the loading trials, cell aggregation was minimal due to the physical separation of cells inside the microfluidic chamber via the micro-posts. Less than 5% of the total cells inside a microfluidic chamber have been observed to attach to another cell. Individual cells and the lack of aggregation of cells inside each of the microfluidic chambers can be observed in Figure 4.1. Cell de-aggregation was also supplemented by gentle pipette agitation prior to loading the cells inside the microfluidic device. The average distance between the cells inside a microfluidic chamber after loading was measured to be approximately 50-100 μm , depending on the concentration of cells that was used. Cell aggregation was only observed when using very high concentrations of cells ($> 10^7$ cells/mL) in the loading solution. Therefore, the concentration of cells utilized in all of cell culturing experiments was 3×10^6 cells/mL to minimize cell aggregation.

4.1.2. Localization of cell loading and growth inside the chambers

The differences in the cross-sectional area and surface area of the microfluidic chambers and channels give rise to the localization of cells inside the chambers. According to the principle of

conservation of mass, the flux of fluid through the channel must be same as the chamber and for the flux to be constant the fluid must travel with a higher velocity through the channel with a smaller cross-sectional area than the chamber with a greater cross-sectional area. Therefore, the cells in the loading solution travel faster through the channel, unable to settle and attach to the substrate. In addition, more of the cells localize in the chamber due to a greater surface area and travel slower through the chamber area compared to that of the channel due to a greater cross-sectional area. The combined effect of greater surface area and cross-sectional area contributes to more cells localizing in the chambers and the low velocity of the fluid to allow the cells to adhere and grow.

Due to the design of the microfluidic device, the majority of the loaded cells localizes consistently inside the microfluidic chambers and not in the microfluidic channels between the chambers, see Figure 4.2. Consequently, the growth of cells is localized inside the microfluidic chambers and does not block the gravity-driven flow of media throughout the entire device.

4.1.3 Gravity-driven fluid transport for long-term cell culturing

Gravity-driven fluid transport is a simple method for delivering a steady flow of nutrients and culturing media to the microfluidic chambers. The gravity-driven flow delivered an average of 100 μL every 12 hours after which the media was replenished to maintain the same average volume flow rate. The fluid transport through the microfluidic chambers using gravity-driven flow was visualized using a fluorescent solution, see Figure 4.3, and the average intensity levels of the first and last microfluidic chambers equalized within five minutes, see Figure 4.4. The amount of time required to achieve equalization ($t=0.08$ hours) across all the chambers is much smaller than the total time of cell culturing ($t=72$ hours) or media replenishment ($t=12$ hours).

Therefore, there should be no differences in the level of exposure of nutrients and toxin diluted in the cell culturing media that the cells are suspended in.

4.2 Cell Growth and Viability in Comparison Experiments

4.2.1 Cell growth in microfluidic devices

The growth of cells in the negative control microfluidic devices (n=5) has a mean of 12 folds increase over the initial number of cells loaded in the 72-hour culturing duration, see Figure 4.5. The cells inside the microfluidic chamber express normal CHO cell morphologies as compared to the morphologies observed in the 96-well plate system. The majority of the cells in the microfluidic chambers are adherent, displaying an elongated morphology and a minority of the cells display a spherical morphology, which has been observed in cells that are undergoing cell division, see Figure 4.6. Cells at the stage of cytokinesis have been observed in the microfluidic device as well. The cell growth across the five microfluidic chambers shows the same level of increase over the 72-hour period of cell culturing, see Figure 4.7. The independence of cell growth rate to the proximity of the source of cell culturing media indicates the gravity-driven fluid flow rate is high enough to replenish the nutrients and the toxin in each microfluidic chamber.

4.2.2 Exposure of cells to DMSO

Cell morphology observed in the cytotoxicity screening of DMSO was similar to the morphology of cells in negative control in the initial loading. When the cells were initially loaded in the microfluidic chambers, the majority of the cells displayed round morphology. Within five minutes of flow stoppage, the cells settled and attached to the bottom surface of the microfluidic chamber and initial membrane protrusion can be observed.

The viability of cells exposed to DMSO in the microfluidic device closely matched that of the viability of cells in the 96-well plate over a wide range of concentration of DMSO (1.0%, 1.5%, and 2.5%). A visualization inspection of the growth of cells in the presence of DMSO shows no major differences in the cell morphology, see Figure 4.8. However, the difference in growth rate can be observed over the duration of the 72-hrs cell culturing between the cells in the negative control microfluidic device and the DMSO-exposed microfluidic device, see Figure 4.9. The standard deviation of the number of cells in the microfluidic device was higher than that of the 96-well plate due to the low number of cells loaded initially in the microfluidic chambers. Moreover, there may be an edge effect that may skew the distribution. Overall, the cytotoxicity of DMSO concentrations varying from 1% to 2.5% has been shown to be in close agreement between the 96-well screening method and the microfluidic screening method, see Figure 4.10.

4.2.3 Exposure to cells to ethanol

The morphology of cells exposed ethanol in the microfluidic device did not show any deviation from the morphology of cells in the negative control microfluidic device. In the presence of ethanol, the cells still express adherent, elongated morphology as well as the spherical morphology when undergoing cell division. When the concentration of ethanol was too high (> 4%, data not shown), the cells did not adhere well to the glass substrate, often getting removed from the microfluidic chambers due to the moving fluid and did not grow, undergoing apoptosis as noted by cell membrane blebbing.

For the concentrations of ethanol that were studied (2.5%, 3%, 4%), the level of cytotoxicity expected from the 96-well screening method was not observed. The cells in the microfluidic device exposed to the same concentration of ethanol consistently showed larger growth than those in the 96-well plate over the 72-hour culturing period, see Figure 4.11. The

uptake of ethanol by cultured cells may have altered due to the dynamic fluid nature of gravity-driven fluid flow in the microfluidic devices as opposed to a static fluid in the 96-well screening method. Moreover, ethanol may be adsorbed or absorbed by the exposed materials in the microfluidic device, lowering the local concentration, which may give rise to the lowered cytotoxicity.

4.3 Discussion

4.3.1. Device design discussion

The method of fabrication of the microfluidic device is a two-step process once the SU-8 master has been made. The fabrication process consists of a casting/curing step and bonding step. The accuracy of replication was consistently high with the master showing little wear after tens of replication cycles. Since the microfluidic device is single layer, there is no need for additional alignment or bonding in the fabrication process. Due to the simplicity of fabrication, tens of devices can be fabricated simultaneously within an hour by a single researcher.

The uniform cell loading across all chambers was observed consistently throughout the cell loading experiments. Several preparation steps such as filtration and gentle cell agitation ensured the uniformity of the cell loading by removing any unwanted particles inside the microfluidic chambers. Occasionally, the uniformity of cell loading was disrupted by the presence of small particles such as insoluble fibers and broken pieces of PDMS lodged inside the microfluidic channels. Careful sample preparation and handling can be implemented to avoid introducing small particles into the microfluidic channels. The uniformity of cell loading also improved when the volume flow rate of the cell solution was low ($< 250 \mu\text{m/s}$).

One major obstacle to microfluidic cell culturing was the accidental introduction of bubbles into the microfluidic channels and chambers. Bubbles can be inadvertently introduced

when switching the tubing and they are very difficult to remove from the device without damaging the cells. We recommend the immersion of any opening or potential opening with PBS solution to avoid accidental introduction of gas and the immediate replacement of microfluidic devices with any bubbles inside for cell culturing proposes.

Likewise, the stock solution of cell culture may contain small particles or even cell lysate that can potentially clog the microfluidic device. The removal of those particles or cell lysate can be tricky since they are mixed well with the cells. Light centrifugation may be a possible separation method if the particles are denser than cells. Shear stress in high velocity fluid flow can disrupt the cell membrane and lyse the cells. Avoiding high velocity of fluid flow with cells suspended through the tubing and the syringe can prevent cell lysing. Cell lysate can be observed as viscous streaks in the microfluidic devices and the entire volume of fluid must be replaced since cell lysate contains degradation enzymes such as proteases that are detrimental to cell culturing.

4.3.2. Cell growth and viability in microfluidic cell culturing

In 96-well plate cell culturing, the cell media is not changed during the 72-hour period, allowing for the buildup of waste in addition to the depletion of nutrients over the duration. In the microfluidic device, fresh cell media is continually introduced to the cells via the gravity-driven fluid transport during the 72-hour period. Therefore, in the microfluidic device, not only are the cells exposed to fresh nutrients (and toxin for non-negative control devices) but also, the wastes produced by the cells are removed from the microfluidic chambers. In addition, for the cells that undergo apoptosis, the soluble and insoluble factors released into the surrounding media are also removed in the microfluidic device. The combinatorial effect of constant-replenishment of fresh nutrients and removal of waste in microfluidic device may lead to a lowered susceptibility to the

toxins that the cells are exposed to. Moreover, the dynamic nature of the fluid inside the microfluidic device may represent a more realistic interaction between the toxin and epithelia cells inside a human body.

The continuous fluid flow inside the microfluidic chambers induces shear stress on the apical surface of the adherent cells. The effect of shear stress on the growth of cells can be observed as a re-alignment or orientation of the cell growth parallel to the direction of the fluid flow inside the microfluidic chambers. Similar observation has been made in a microfluidic environment using vascular endothelial cells [19]. We have observed that the effect on several occasions as a number of cells aligning vertically or along the direction of fluid flow over the course of 72-hour cell culturing. Based on observations, the shear stress does not affect the growth rate of cells in the microfluidic chambers and there was no preferential apoptosis in the cells that aligned to the direction of fluid flow. However, the fluid flow does affect only the alignment of the cells, not their spatial distribution, and the alignment of cells occur in a small number of microfluidic chambers.

4.3.3 Cell growth and viability in the presence of DMSO and ethanol

Dimethyl sulfoxide (DMSO) is a small polar, aprotic molecule, commonly utilized in the cryo-freezing method of preservation of biological samples and as a delivery agent in transporting non-ionized, low-molecular weight molecules across the skin barrier. The partition coefficient, P , which is a measure of the relative solubility of a compound in a mixture of two immiscible solvents, is given as the ratio of concentration of solute in octanol versus the concentration of solute in un-ionized water. The logarithm of partition coefficient of DMSO is reported to be -1.35 [20]. At low concentrations (< 5 % v/v), DMSO has been observed to facilitate membrane thinning and with increasing concentrations, DMSO is able to induce transient water pores to

allow the diffusion of hydrophilic solutes into the cytoplasm [21]. With sufficient concentration of DMSO, there is a total breakdown of the phospholipid bi-layer of the cell membrane and release of intracellular organelles to the extra-cellular environment, which leads to cell death.

The agreement of cell growth between the 96-well screening method and the microfluidic screening method for dimethyl sulfoxide demonstrates that the uptake or the interaction of DMSO between the cell membrane is unaltered by the differences in the dynamics of the culturing fluid that surrounds the cells. The cellular membrane permeability of DMSO has been simulated using molecular dynamics and the DMSO molecules have been shown to be more penetrating than protic solvents such as ethanol [22]. Therefore, regardless of the dynamic nature of the fluid in the microfluidic device, DMSO can induce the same level of cytotoxicity as observed in the static culturing of the 96-well screening method.

Ethanol is a small, uncharged polar molecule, which can passively diffuse across the phospholipid cell membrane without the aid of transport proteins [23]. The logarithm of partition coefficient of ethanol is reported to be -0.30 [24]. The cellular membrane permeability of ethanol has been determined from micropipette aspiration measurements [25]. However, ethanol, a polar protic solvent, does not induce the same level of the formation of pores in membranes as DMSO because of their strong hydrogen bonding to polar lipid head groups. In a dynamic fluid environment, the diffusion of ethanol across the cellular membrane may be decreased due to the high miscibility of ethanol in water due to the readily formation of hydrogen bonds with water molecules [21]. Although the level of cell growth differs between the two screening methods, the trend of decreasing cell growth with increasing concentration of ethanol is still consistent. Ethanol absorption in PDMS is also one possible mechanism that can lower the local concentration of ethanol that is exposed to the cultured cells inside the microfluidic device.

However, the absorption of ethanol in PDMS blocks, extracted using solvent, has been quantified using gas chromatography and the quantity of absorbed ethanol is too low to affect the growth rate of the cells [26].

PDMS requires multiple solvent extraction steps to remove any leftover reagents from the cross-linking process of PDMS and the left-over PDMS oligomer residue can be cytotoxicity to the cells that are exposed [27]. However, both DMSO and ethanol were found to have low solubility (i.e. low extraction of PDMS oligomer) in a solvent compatibility assessment with PDMS [28]. Therefore, the material compatibility for cell culturing cannot account for the differences in the cell growth between the 96-well screening method and microfluidic screening method.

4.4 Figures

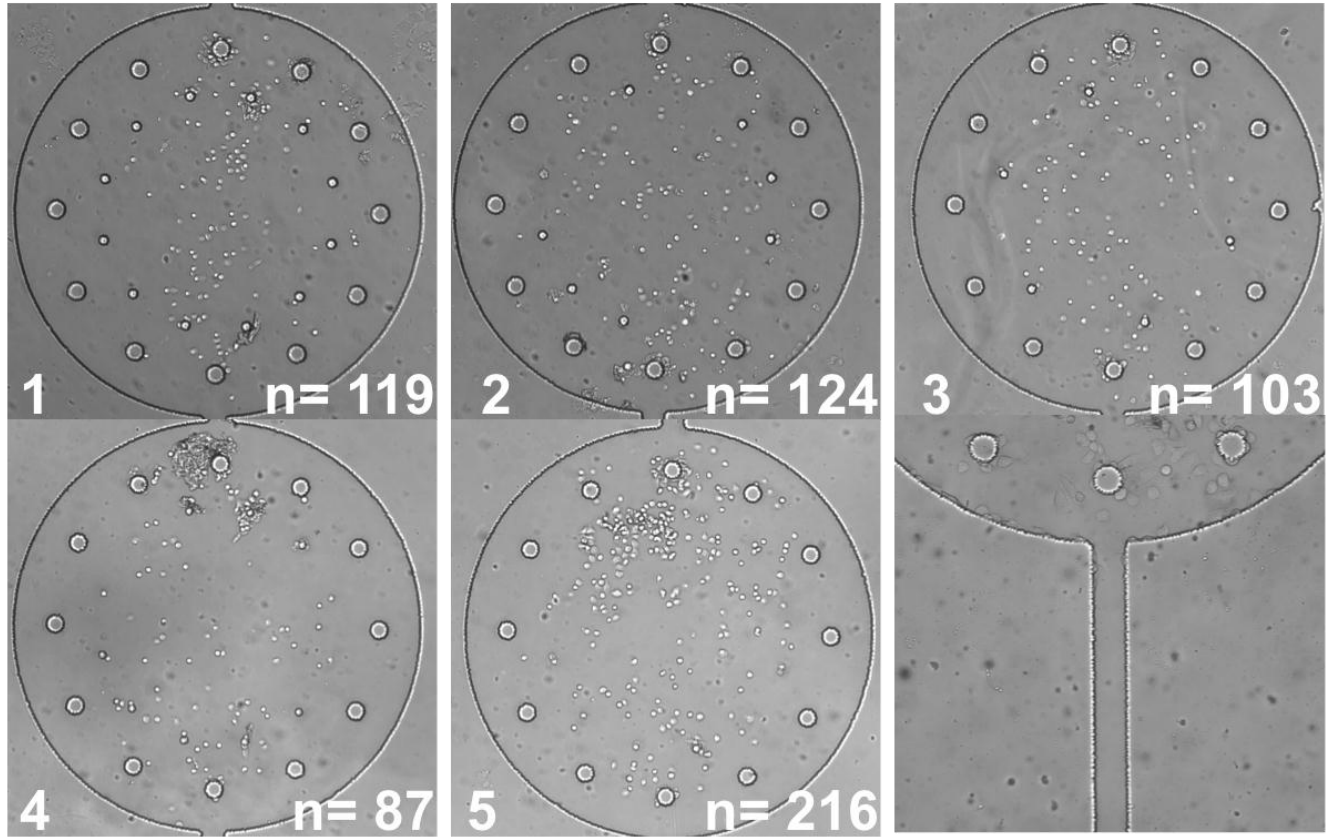


Figure 4.1 Microscope images of the microfluidic chambers (1-5) after the cells (bright dots) have been seeded. Manually counted number of cells indicates uniformity of the cell loading across the chambers. Localized cell seeding inside the microfluidic chambers can be observed in the lack of cells in the microfluidic channel (bottom right).

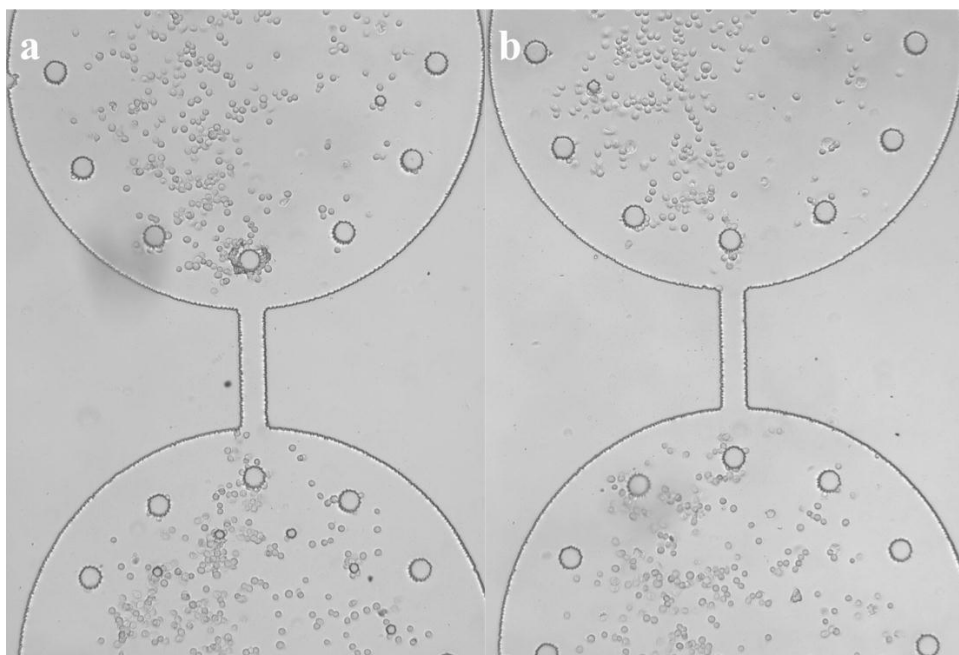


Figure 4.2 Visualization of the microfluidic channels connected two microfluidic chambers. Localization of cells inside the microfluidic chambers can be observed with minimal or no cells in the microfluidic channels (a, b).

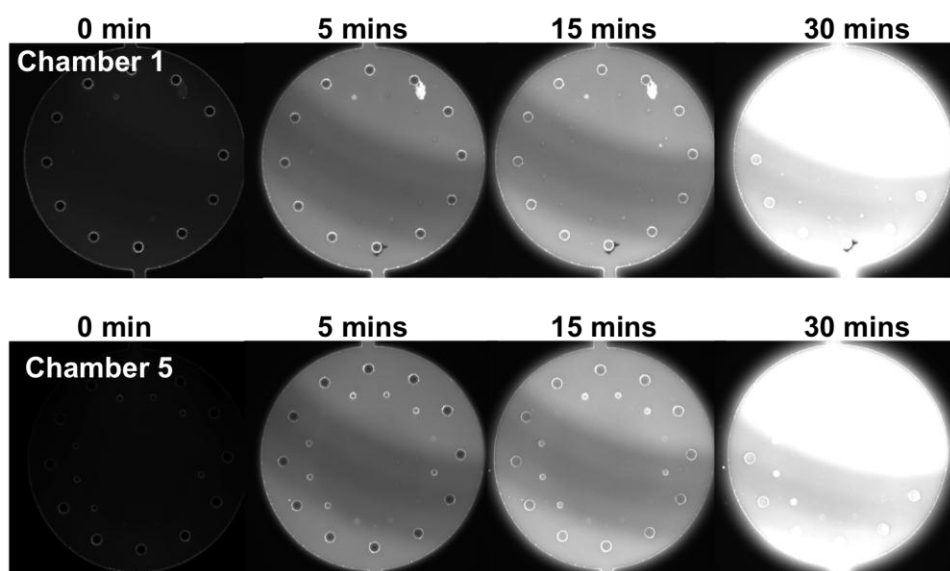


Figure 4.3 Time-elapsd images of passive flow of fluorescent solution into the microfluidic chambers. Chamber 1 is the chamber closest to the source of the fluorescent solution (top) and chamber 5 is the chamber farthest from the source of the fluorescent solution (bottom).

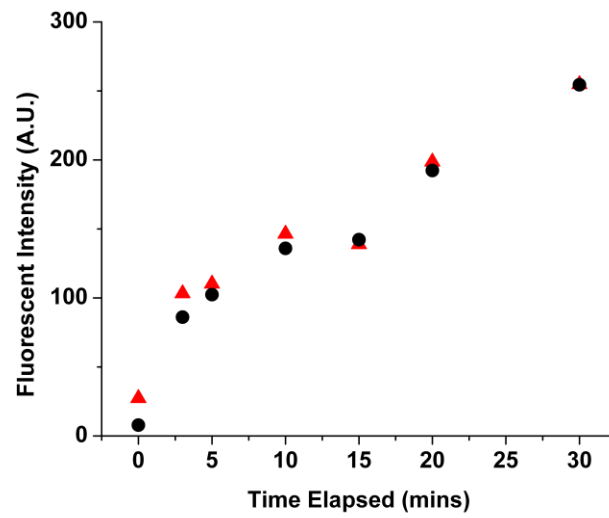


Figure 4.4 A comparison between the average intensity levels between chamber 1 (red triangles), the chamber closest to the source of fluorescent solution, and chamber 5 (black circles)

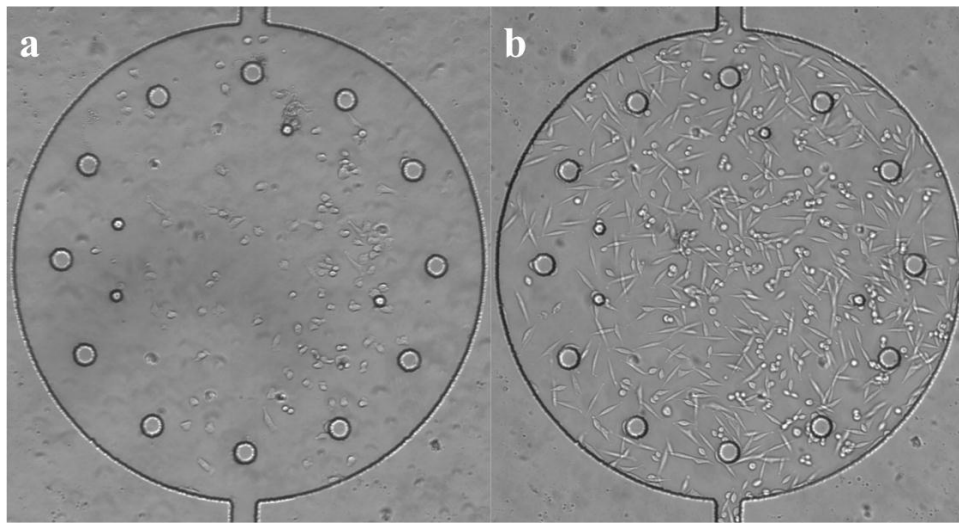


Figure 4.5 10X bright-field microscope images of a microfluidic chamber at t=0 hour (initial loading) (a) and at t=72 hours of incubation (b). This microfluidic device was a negative control that is the cells were not exposed to any toxin.

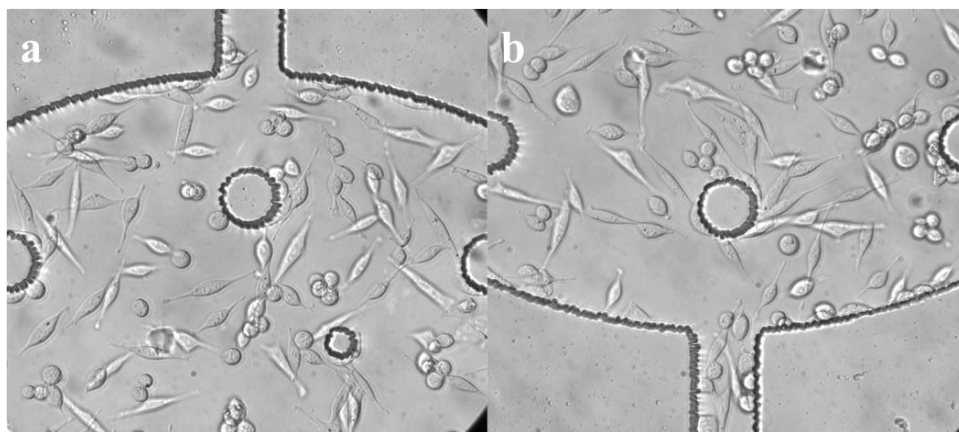


Figure 4.6 40X DIC microscope images of CHO cell residing in a microfluidic chamber (same chamber as Figure 4.) at $t=72$ hours of incubation. Top portion of the microfluidic chamber (a) and bottom portion of the microfluidic chamber (b). Note the elongated morphology of adherent cells and spherical morphology of cells undergoing cell division. This microfluidic device was a negative control that is the cells were not exposed to any toxin.

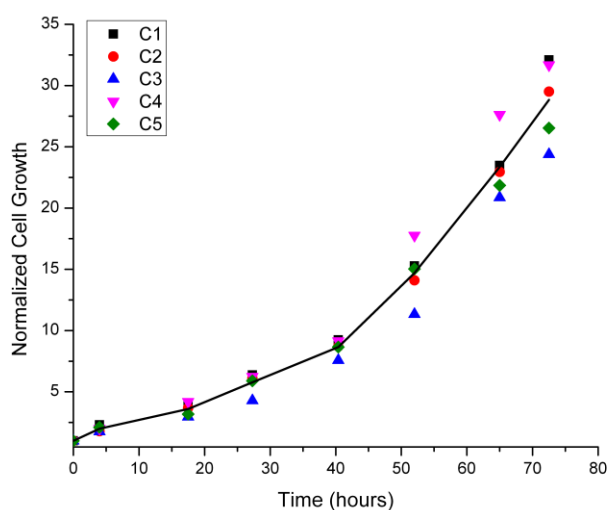


Figure 4.7 Normalized cell growth versus cell culturing time of a negative control microfluidic device across five chambers. C1, C2, C3, C4, and C5 denote chambers 1, 2, 3, 4, and 5 respectively. The line is the mean of the normalized cell growth of the five chambers.

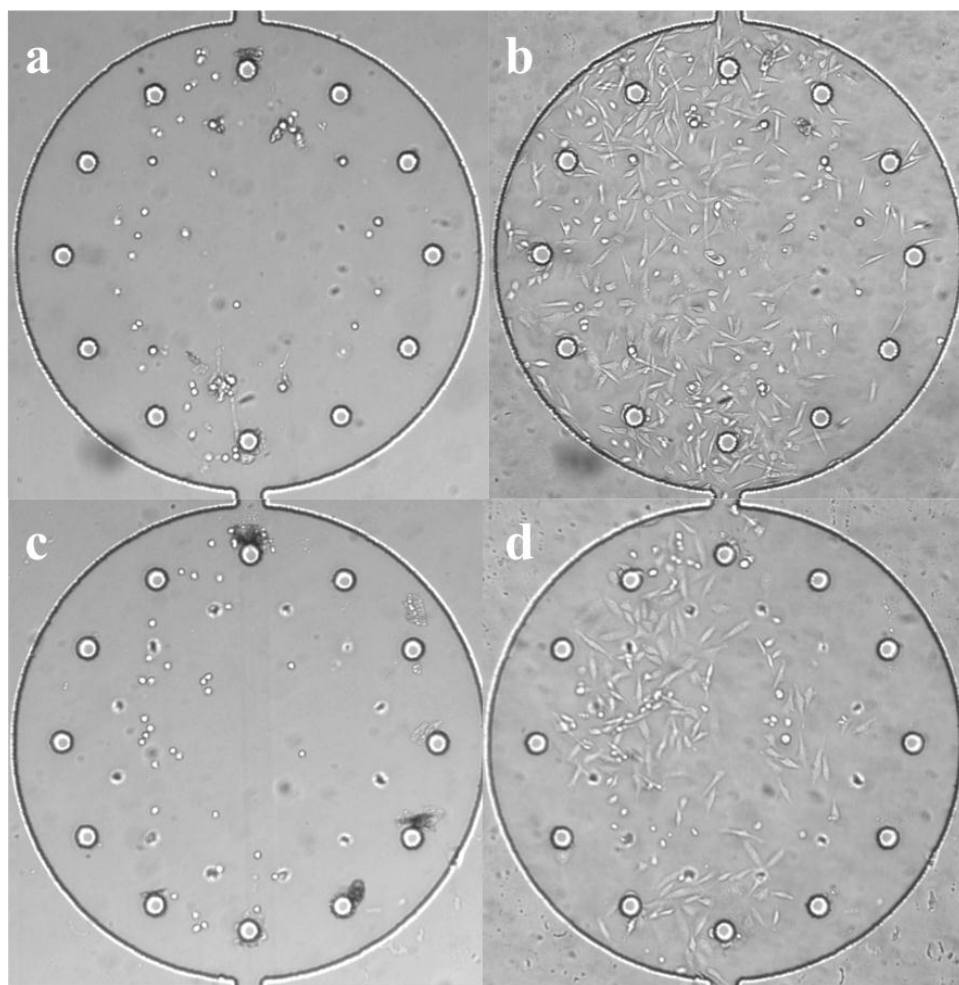


Figure 4.8 Visualization comparisons between cell growths exposed to no toxin and cell growth exposed to 1.5% DMSO. Microfluidic chamber with cell growth exposed to no toxin visualized at $t=0$ hour (a) and $t=72$ hours (b). Microfluidic chamber with cell growth exposed to DMSO visualized at $t=0$ hour (c) and $t=72$ hours (d). Cell growth in the presence of 1.5% DMSO was an average of 43% of the negative control cell growth.

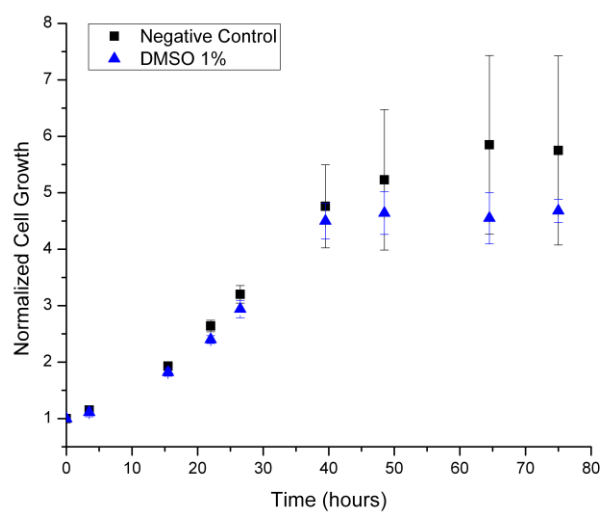


Figure 4.9 Normalized cell growth versus cell culturing time of a negative control (blue squares) and DMSO 1% (black triangles) microfluidic devices. The mean of the normalized cell growth of five chambers is plotted with one standard deviation.

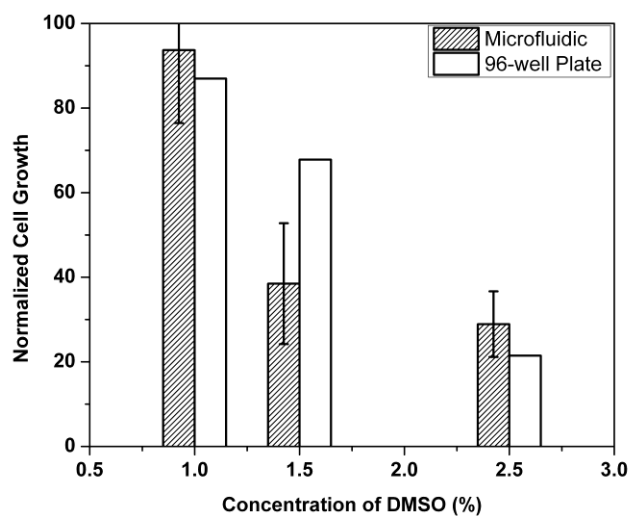


Figure 4.10 A comparison of normalized cell viability as a result of exposure to DMSO in microfluidic screening (blue triangles) and 96-well plate screening (black squares).

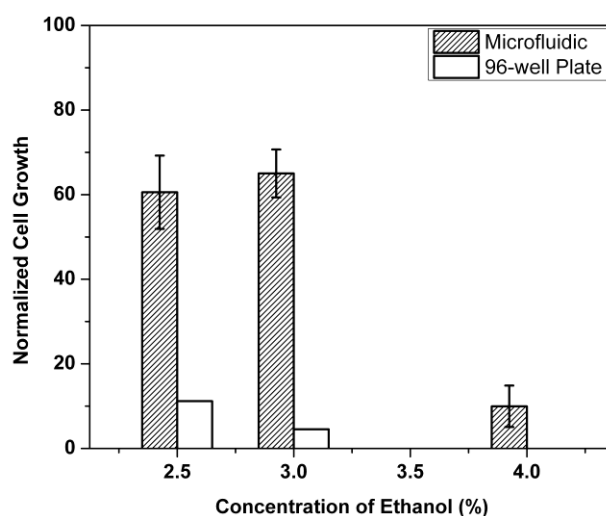


Figure 4.11 A comparison of normalized cell viability as a result of exposure to ethanol in microfluidic screening (blue triangles) and 96-well plate screening (black squares).

4.5 Tables

Table 4.1 Assessment of cell loading uniformity over six independent microfluidic devices. The mean number of cells and one standard deviation are calculated based on five separate microfluidic chambers per microfluidic device. The percentage of uniformity is calculated as a ratio of standard deviation and the mean multiplied by 100.

Microfluidic device	Mean number of cells	Std. Dev.	% Uniformity
1	27.2	5.4	19.7
2	41.0	10.1	24.7
3	55.4	11.3	24.7
4	47.2	6.5	13.8
5	26.5	2.4	9.0
6	35.0	9.2	26.3

CHAPTER 5: CONCLUSION

5.1 Microfluidic Device for Cytotoxicity Screening

Localization and entrapment of mammalian cells in microfluidic chambers have been demonstrated for long-term cell culturing in cytotoxicity screening application. The localization in microfluidic chambers has shown to be highly uniform and dispersive for very low number of cell loading using micro-posts structure in the microfluidic chambers. The microfluidic device is ideal for immortalized, non-cancerous cell lines that express cell contact inhibition and apoptosis pathways. The compatibility with current cytotoxicity screening method has been demonstrated by comparing the toxicity of dimethyl sulfoxide and ethanol over a range of concentrations. Close agreement in the level of cytotoxicity between the microfluidic device and the 96-well plate methods was shown for dimethyl sulfoxide but ethanol was less cytotoxic in the microfluidic cytotoxicity screening experiments. Dynamic fluid environment in the microfluidic device may contribute to the decreased susceptibility of the cells to the toxicity of ethanol. The simplicity in fabrication and operation of the microfluidic device enables high usability for other similar biological studies using microfluidics.

5.2 Recommended Future Studies

5.2.1 Investigate the growth rate of CHO cells in collected environmental samples

The toxins that have been utilized in this research project are representative of the actual toxin in interested, disinfection by-products. As observed in the experimental results, the interaction between the toxins and the cells are not the same and depends highly on the chemical properties of the toxic molecules (i.e. solubility, reactivity, net charge, membrane permeability). Due to the dynamic nature of the microfluidic device, the interaction between the toxins and the cells is altered compared to the static 96-well plate design and the difference in mechanism of toxin

uptake leads to the difference in the cytotoxicity of the toxin in different environment. Future studies will incorporate samples collected from the environment (treated sources of water such as swimming pools and drinking water) to assess the toxicity of those sources to CHO cells.

5.2.2 Investigate the genetic effects of exposure to DBP

While the cellular basis of the effect of toxin has been investigated in this study, potential genetic defect caused by toxin is the next item worthy of studying. The logic behind this progression is that the genetic defects are not immediately detrimental to the livelihood of the organisms; they manifest only in the long-term over many cycles of cell growth. This chronic effect, compared to the acute toxic effect, may be the most relevant for understanding the effect of DBP on human population since DBP is exposed through water consumption and inhalation over the entire life time of a human. The chronic exposure to DBP may be most detrimental on the genetic level and that should be investigated through even longer culturing periods, over hundreds of cycles of cell divisions.

5.3 Microfluidic Device Design Improvements

5.3.1 Fabricate a large array of chambers

The capability of the current microfluidic design can be enhanced with the inclusion of a larger array of chambers (e.g. 10 chambers by 10 chambers array) compared to the current five-chamber design. The array design will enable an increase in number of chambers to culture wells as well as an increase in the number of samples that can be studied. The same fabrication method can be utilized while the photolithography mask must be changed to the desired array design.

5.3.2 Incorporate gradient generator

An integrated, on-chip gradient generator is an ideal device to couple with the current microfluidic chamber-channel device [29]. In a typical toxicity screening experiment, increasing

concentrations of toxins are exposed to the cells and the resulting density of viable cells is assessed after 72 hours of culturing. The different concentrations of toxin are generated by manual dilution and mixing and depending on the required number of the concentrations, the dilution process can be labor-intensive. The advantage of on-chip gradient generator is that the different concentrations of toxin can be generated in a single step, simply by passing the buffer solution and the stock toxin solution (via two inlets) through a series of baffles to mix and dilute the stock toxin solution, see Figure 5.1. This proposed system is compatible for the cell cytotoxicity screening application because of the simplicity and practicality of the design.

5.3.3 Improve the implementation of gravity-driven fluid transport

As noted in the design requirement chapter, gravity-driven fluid transport method has several advantages over the active pump-driven fluid transport and it is suitable for the needs of this research project. However, the level of control over the fluid transport (e.g. volume flow rate, inlet pressure) will need to increase as the complexity of the microfluidic device increases as well. Gravity-driven fluid transport can be improved by integrating an adjustable valve for control over the start and stop of the fluid transport as well as the volume flow rate of the transport. The volume of the reservoir for the gravity-driven fluid transport can be increased as well to accommodate the increase in the number of microfluidic chambers.

The method of integrating the gravity-driven fluid transport can also be improved by using interlock-able plugs in the inlet and outlet to ensure a complete seal and avoid any accidental introduction of bubbles into the fluid. The presence of bubbles inside a microfluidic is highly detrimental and compromises microfluidic experiments due to the high resistance of bubble movement through the microfluidic channel, thereby blocking all fluid movement.

5.4 Figures

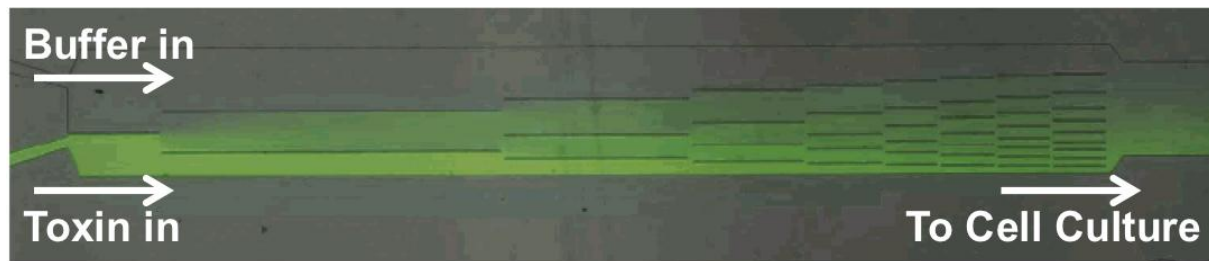


Figure 5.1 A microfluidic gradient generator. A fluorescent solution is flowed through the bottom inlet and a buffer solution is flowed through the top inlet. The two solutions are mixed and diluted through a series of horizontal baffles, which breaks up the streamlines. Modified figure from [29].

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APPENDIX A: MATLAB SCRIPT FOR AUTOMATED CELL ENUMERATION

A.1 Instruction for running Matlab image processing script

call_cc2func function

For clarity, the comments that accompany the Matlab have been italicized to distinguish between the two different syntaxes. The purpose of the inclusion of the Matlab script is

```
clear all; close all;
```

```
% Parameters to identify the name of the files and the range of level of  
% threshold to scan  
name =2:5;  
threshold = 150:5:250;
```

```
% Locate the center of the chamber and identify the diameter of the chamber  
% by drawing a straight line through the center of the chamber  
for j=1:length(name);  
file_name = ['Image',num2str(name(j)),'.jpg'];  
I2 = imread(file_name);  
I = I2;  
figure; imshow(I); hold on;  
h=imline;  
position = wait(h);  
hold off;
```

```
% Calculate the coordinates of the center and the radius of the chamber  
% based on the two end points of the drawn line  
center = [(position(2,1)+position(1,1))/2,(position(2,2)+position(1,2))/2];  
radius = sqrt((position(2,1)-position(1,1))^2+(position(2,2)-position(1,2))^2)/2;
```

```
% Call cellcount2_func to return the number of detected cells for each  
% level of threshold  
for i=1:length(threshold)  
count(i) = cellcount2_func(I,0,threshold(i),center,radius);  
end  
close all;
```

```
% Identify the level of threshold that gives the highest number of detected  
% cells and calculate the second highest number of detected cells  
x=find(count==max(count));  
cc2(I2,threshold(x+1),center,radius,file_name);  
close all; clc;  
end
```

A2. Cellcount2_func function

```
function [count] = cellcount2(file,option,pixCol,center,radius)

thres_area_max = 400;
thres_area_min = 10;
threshold = 0.1; %Roundness threshold
I=file;
imshow=size(I);

%% Start of function

c(1) = center(1,1);
c(2) = center(1,2);
r=radius;

for i = 1:imshow(1)
    for j = 1 : imshow(2)
        if(((i-c(2))^2) + ((j-c(1))^2) > (r)^2)
            I(i,j) = 0;
        end
    end
end

%% Identify brighter cell like structures

% use a for loop to run through the matrix to find pixels with color
% greater than 199. The cells are significantly brighter white than the
% other entities in the chamber. One issue with this is the that outer walls
% of the chamber are also bright white. We will deal with that later.

for i = 1:imshow(1)
    for j = 1:imshow(2)
        if(I(i,j) > pixCol)
            I(i,j) = 255;
        else
            I(i,j) = 0;
        end
    end
end

%% Noise reduction and hole filling

I = bwareaopen(I,20); % remove noise (anything less than 20 pixel)
I = imfill(I,'holes'); % fill up all the remaining cells
```

```
%% BOUNDARY
```

```
[B L] = bwboundaries(I);
```

```
stats = regionprops(L,'Area','centroid'); %obtain the centroid of each block
```

```
count = length(B);
```

```
for k = 1:length(B)
```

```
    boundary = B{k};
```

```
    % compute a simple estimate of the object's perimeter
```

```
    delta_sq = diff(boundary).^2;
```

```
    perimeter = sum(sqrt(sum(delta_sq,2)));
```

```
    % obtain the area calculation corresponding to label 'k'
```

```
    area = stats(k).Area;
```

```
    % compute the roundness metric
```

```
    metric = 4*pi*area/perimeter^2;
```

```
    if (area > thres_area_max) || (area < thres_area_min) || (metric < threshold)
```

```
        count = count - 1;
```

```
        stats(k).Centroid = [0,0];
```

```
        for i = 1:imsz(1)
```

```
            for j = 1:imsz(2)
```

```
                if(L(i,j) == k)
```

```
                    L(i,j) = 0;
```

```
                end
```

```
            end
```

```
        end
```

```
    end
```

```
end
```

A3. cc2 function

cc2 function is the same as the Cellcount2_func function and the sole difference was in name to avoid confusion between the loop over the different levels of threshold.