MICROFLUIDICS BASED DROPLET GRADIENT GENERATOR FOR SCREENING MICROALGAE UNDER VARIOUS CULTURE MEDIA CONDITIONS

A Thesis

by

SHENYANG YANG

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Chair of Committee,	Arum Han
Committee Members,	Timothy Devarenne
	Xiaoning Qian
	Pao-Tai Lin
Head of Department,	Miroslav M. Begovic

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ABSTRACT

Microalgae has attained great interest as a future renewable energy source. However, the production cost of microalgae based biofuel is still not economically competitive compared to fossil fuel and food crops and oil seeds. As a result, microalgae based second generation biofuel requires a significant improvement. One strategy to achieve this is the optimization of their cultivation conditions that can provide both high cell growth and oil production. Here, this thesis will present a versatile droplet microfluidics based gradient generator, it is capable of adjusting culture media composition, which allows for studying the effect of nutrient conditions on microalgal growth. The developed platform entails droplet microfluidics where a large number of single-cell encapsulated droplets can be generated and utilized as independent bioreactors. The platform consists of a droplet gradient generator and a downstream culture chamber. In the droplet gradient generator, a mixture of two different culture media solutions, each containing cells, was used as input solution. While maintaining a total flow of the mixture into the droplet generator, only the mixing ratio of those two solutions was changed by adjusting flow rates between them, droplets of time dependent concentration could be generated. This flow rate change is automatically controlled by a LabVIEW program, enabling to manage a step size and time interval between each change. In the downstream culture chamber, a serpentine channel structure is utilized to store droplets in sequence and track their growth and oil production over time. The

gradient generation in the platform was first characterized with FITC solution where a linear profile of fluorescent intensity distribution was observed. Also by manipulating the step size and time interval, a gradient of concentration with different concentration range was easily adjusted. This platform is currently being utilized to analyze the growth of *Chlamydomonas reinhardtii* under different nitrogen concentrations.

DEDICATION

Thanks to my mom, dad and my grandparents for their enormous love, support and trust.

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I am very grateful for having Dr. Arum Han as my advisor and committee chair for my master study and my thesis. I would like to express my deepest gratitude to Dr. Arum Han for his guidance, support, mentorship and his patience all the way through my project and my master study.

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NOMENCLATURE

MEMS	Micro-electromechanical Systems
FITC	Fluorescein isothiocyanate
PR	Photoresist
ERB	Edge Bead Removal
PDMS	Polydimethylsiloxane
IPA	Isopropyl alcohol
TAP	Tris–Acetate–Phosphorus

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CHAPTER I

INTRODUCTION

1.1 Objective and Motivation

Microalgae based biofuel has been a heated topic over the past decades. Supporters think it's a clean, sustainable and high efficiency energy. Protesters, on the other hand, say algal based biofuel is too expensive and has a negative energy balance. But with the strong need to reduce green house gas emissions, and the decreasing reserves of crude oil[1],people have been investigating in biofuel for a sustainable and clean energy alternative.

The first generation biofuel is the crops and vegetable oil including rapeseed oil, sugarcane, sugar beet, and maize.[2] They do release less air pollutant than fossil fuels but most of them are also consumed by human beings and livestocks. The second generation biofuels are derived from lignocellulosic agriculture and forest residues together with non-food crop feedstocks.[3] They do not compete with human beings but there is also a competition of resource. To grow them , it takes a lot of agriculture land and it also causes some environmental problems such as extinction of bio-species due to deforestation.

Then recently people began to see microalgae as the third generation of biofuel. Microalgae is a single cell organism live and grow by photosynthesis, it could convert

the green house gas and sun light into chemical energy, part of which is what we used as biodiesel. Plus microalgae grows relatively quickly and it could live in very harsh condition, therefore there's no direct competition of agricultural land. But for now production cost for the third generation biofuel from microalgae is high and it is not commercially competitive compared to other fuel energy. '*Algal biofuels aren't sustainable, are too expensive, and need too much water, nitrogen, phosphorous, and carbon dioxide.'* stated by Alice Friedemann on March 29, 2015 on Energyskeptic.[4]

In anyways, microalgae still has great potential for exploitation, further investigation of increasing their ability to produce lipid in order to reduce cost is urgently needed. The motivation of this work is by understand how cells preform under different culture environment and compare their ability to reproduce, we could be able to optimize the culture condition and maximize cell growth as well as oil production, ultimately lower the production cost by improving microalgae based biofuel productivity in massive cultivation. Still, a lot of work needs to be done to make microalgae biofuel commercially competitive. Here in this work, we focused on the optimization of microalgae culture media in terms of chemical concentration. In the following chapters details will be explained.

1.2 Microalgae Growth and Oil Production

Algae is one of the oldest life forms on the earth.[5]. Without any development beyond cells, algae structures still serve their primarily goals of energy conversion. This

enables them to adapt to harsh environment conditions and reproduce in the long term. [6]

Eukaryotic cells, which comprise of many different types of common algae are categorised into a variety of genres mainly defined by their pigmentation, life cycle and basic cellular structure.[7] The most important classes are: green algae (Chlorophyta), red algae (Rhodophyta) and diatoms (Bacillariophyta).Table 1 show different type of microalgae cells and their ability to produce oil on a dry matter basis (%).[8]

Species	Proteins	Carbohydrate	Lipids
Scenedesmus obliquus	50-56	10-17	12-14
Scenedesmus quadricauda	47	-	1.9
Scenedesmus dimorphus	8-18	21-52	16-40
Chlamydomonas reinhardtii	48	17	21
Chlorella vulgaris	51-58	12-17	14-22
Chlorella pyrenoidosa	57	26	2
Spirogyra sp.	6-20	33-64	11-21
Dunaliella bioculata	49	4	8
Dunaliella salina	57	32	6
Euglena gracilis	39-61	14-18	14-20
Prymnesium parvum	28-45	25-33	22-38
Tetraselmis maculata	52	25	3
Porphyridium cruentum	28-39	40-57	9-14
Spirulina platensis	46-63	8-14	4-9

Table 1 Composition of microalgae on a dry matter basis (%)

Species	Proteins	Carbohydrate	Lipids
Spirulina maxima	60-71	13-16	6-7
Synechoccus sp.	63	15	11
Anabaena cylindrica	43-56	25-30	4-7

Table 1 Continued

In this work, the wild type microalgae *Chlamydomonas reinhardtii* (Figure 1)was used, because this is a well studied algae type, many of the mutant of *Chlamydomonas reinhardtii* have been documented by the researchers all over the world.

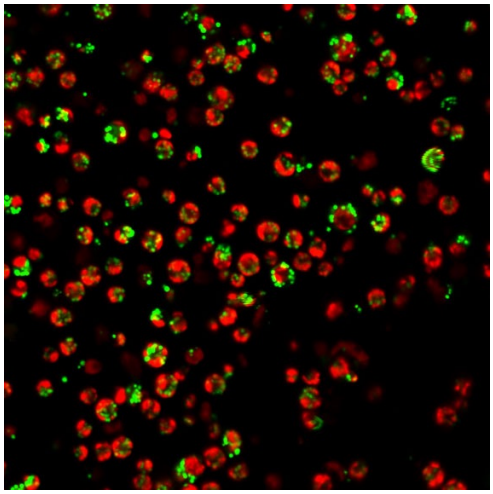

Figure 1. *Chlamydomonas reinhardtii* cells under microscope,red indicates cell body; green indicates lipids.

For massive biodiesel cultivation, the major factors that would determine their economically production acceptance are: productivity which include strain engineer, strain screen and strain selection; photosynthetic and growth efficiency; and productivity of oil. Besides that mechanism and method of production and harvesting would also affect the over all costs of biodiesel.[3]

Besides from the development of new strain, while many microalgae strains naturally have high lipid content (refer to Table 1 20%-40%), [9] more works could be designed to increase the cell reproduction rate by optimizing the factors that would affect growth such as the control of nitrogen and phosphors level in the culture media, light intensity and light cycle ,temperature, salinity , CO_2 concentration and so on .[10, 11]

1.3 Conventional Method

Many researches have been trying to investigate in microalgae growth behavior. Conventionally, studies of how microalgae cells grow and reproduce under different nutrition conditions are operated in macro scale. Most of the experiments use flasks or tubes as cell cultivation container . Growth rate characterization is done by using centrifug or drying equipment to measure dry weight of the cells or by taking samples from the cell suspension and measuring cell density overtime with hemocytometer.

There are many flaws with these common practice to analyze microaglae:

First taking samples for the container may introduce contamination to the remaining culture. Because of human handing and repeatedly open the culture container, cell growth may be affected by air borne pathogen and bacterias.

Second the sample cannot accurately represent the cell density of the cell suspension due to human operation mistakes, such as not mixing the suspension throroughtly and the observation are all based on behaviors of cells as a colony not single cell.

Third, the number of concentration suspensions is limited. It will require too many flasks and place for the flasks if multiple data are needed for a more accurate growth curve and it's also every time consuming. A micro fluidic culture and observation platform is introduced in this thesis, that could work perfectly to resolve the problem mention previously.

1.4 Microfluidics

Microfluidic devices could provide a number of advantageous features for micro-scale bio/chemical analysis systems. [12] In this structure, design in micro level is patterned on the PDMS chip, will then be used as liquid/air channels as well as reaction chambers. This structure could also be fabricated easily and cost effectively. [12]

Microfluidic systems is popular among biochemical application due to their capability to precisely control small flow,[13]monitor and manipulate samples at a nano-

liter to pico-liter scale.[14, 15] Microfluidic culture systems used for examine microalgal lipid production, density changes, or growth kinetics have also been developed.[13]

In recent years, in some novel works, the use of microfluidic systems have been extended to multiphase flows, in which a special design of the formation of micro droplets have been published. [16] These systems enable the formation of dispersions with highly attractive features, particularly the control over the size and volume of the dispersed phase(water) and narrow distribution of the sizes(spacing) of individual droplets or bubbles. [17] The use of immiscible fluids to generate fluidic segment , especially the controlled formation in a micro-scale, new method to perform chemical reactions and biochemical analyses is made possible. Reactions could be carried out directly on chip, and using extremely small volumes of reagents.[18]

Mono-cell level studies is crucial for better understanding of the performance of algal growth under various conditions.[13]The high-throughput photo bioreactor as well as incubator introduced in this thesis overcomes the shortcomings of conventional systems and methods by providing mono cell resolution for photosynthetic micro-organism under an extremely well controlled environment.[19]

Compared to the conventional practice,the main functions of this microfluidic device is it could generate hundreds of droplets with a range of concentrations in terms of a certain component in the culture media. Microalgae cells could be trapped in the droplets. The cultivation and observation could all be done on this same device.By

analyzing the fluorescent intensity, cell growth rate could be calculated from the change of cell numbers in the droplets.

1.5 Thesis Organization

This thesis will introduce the development of high-throughput droplet generation platforms, including the designs and working principles, material snd fabrication. And also the experimental and tests results.

Chapter 2 will present the gradient generator device design used for high-throughput screening for microalgae under various culture media conditions. A novel design of two-inlets gradient generator integrated with long serpentine cell culture channel will be demonstrated. Fabrication steps and materials used in the fabrication will also be introduced in chapter 2. Test results of the device will be shown and explained in the end of chapter 2.

Chapter 3 will conclude the project and the thesis and give a rough idea about the future research plan to exploit the application of the platform. Possible collaborate of this platform with other devices will be proposed.

CHAPTER II

DROPLET MICROFLUIDICS PLATFORM

2.1 Design and Working Principle

In this project, a versatile droplet microfluidics based gradient generator capable of adjusting culture media composition is designed and fabricated. The platform allows for studying the effect of nutrient conditions on microalgal growth. In the design a novel two-inlet droplet generation structure is used to form a range of concentration. Each of these droplets contains a unique mixture of solutions and the droplets are generated linearly in terms of time.

A mixing channel is followed by the droplet generation. This mixing channel allows the solution in the droplets to thoroughly blend with each other. Then the droplets are directed into a downstream culture chamber. The chamber is capable for storing 500 droplets that could be utilized as independent bioreactors, it is also capable for observation and analysis under microscope. More details will be explained in the following paragraphs.

2.1.1 Droplet Generator

To enable a single cell level observation and analysis of cell performance, special technique is needed to provide this micro level bioreactor . A T-junction is used in this work to generate water droplets with diameter of hundreds of microns. By trapping the

microalgae cells in this micro droplet, we could be able to eliminate many of the impact of the surrounding environment and cross contamination.

A simplest scientific fact that we all know is water and oil do not mix with each other. Based on that, by using oil as continuous phase carrier and inject water in to the oil, bubbles of water could be formed in the oil carrier as the dispersed phase.[18] Oil and water are injected into two micro channel, this is called a T-junction because the two channels are perpendicular to each other. The micro channels are designed with a inlet at the end of each channel. Connected with 1 ml BD syringe with micro tubing.

To realize the function of changing the concentration component inside the micro droplets, two water inlets are used instead of one. They then merged into one channel and form a T-juncton with the continuous phase oil.(Figure 2)

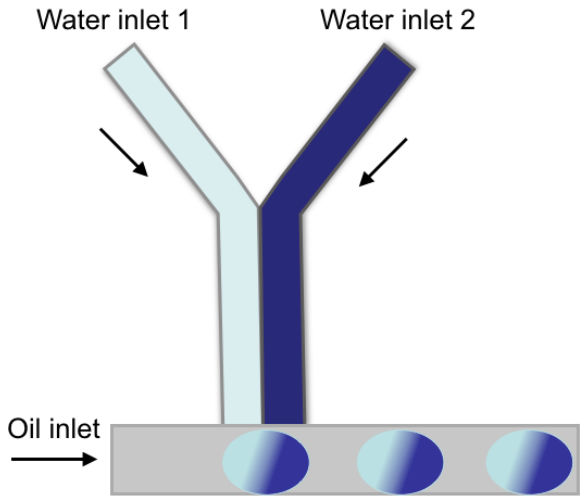

Figure 2 Design of droplet generation

In this two inlet design, two different water solution is injected to form the dispersed phase.(Figure 3) By changing the flow rates of the two water inlets the concentration of the mixture of solution in the droplet is therefore changed.

In order to generate droplets of consistent size, the flow rate combination of the continuous phase and dispersed phase should be fixed. Therefore while keeping the flow rate of the oil as a constant, the flow rate of water should also be as a constant. In order to change the water solution concentration under this condition, we kwwp the flow rate of one inlet increasing while keep another water inlet decreasing. Figure 3 show the laminar flow between the two water inlets, DI water and FITC (fluorescein isothiocyanate 0.5 mol/ml) solution is used for better discrimination between the flows.In this chase the flow rate of FITC is 20 µl/h DI water is 120 µl/h. The different between the width of two flows could be clearly seen from the image.

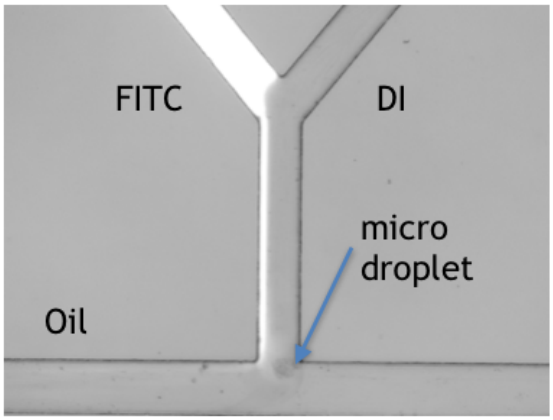

Figure 3 Laminar flow between DI water and FITC.

For a certain flow rate of the water inlet, the droplet size will decrease as the oil flow rate increase. But while the oil flow rate keep increasing the droplet size will reach up to a limit. This is due to the design of the channel width. In this device the width of the water inlets are 190 μm as well as the merged water channel, the width of the oil channel is 218 μm. The hight of the channel is 165 μm.

Cell suspension is use in this design as water solution, details of cell preparation and encapsulation is explain in 2.2.3 Cell encapsulation.

2.1.2 Mixing Channel and Incubation Chamber

Followed by the droplet generator is a mixing channel. (Figure 4) Semi arc shaped channels are oppositely connected with each other. When droplet flows by these semi arcs, the two solutions could completely mixing with each other. The solution inside of the droplets is then ready for the subsequent cell culture.

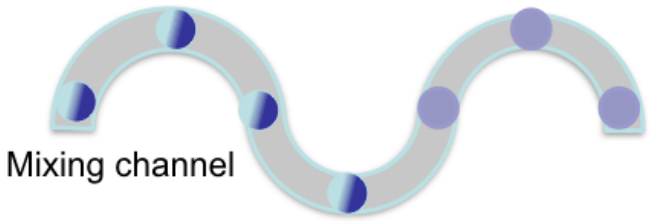

Figure 4 Mixing channel design

The downstream is a culture channel contains 42 serpentine lines of channels, which are capable of storing 500 droplets that could be utilized as independent bioreactors. It is also capable of observation under microscope. The height of the culture chamber is 165 μm. Figure 5 is the overall design of the versatile droplet generation chip. The height of the channel is determined by the thickness of the SU-8 master on the silicon wafer. Which is then determined by the spin speed of the SU-8 photoresist coating. Informations about the device fabrication and material that has been used will be introduced in the subsequent chapter. Details of the fabrication procedure is also presented in Appendix B.

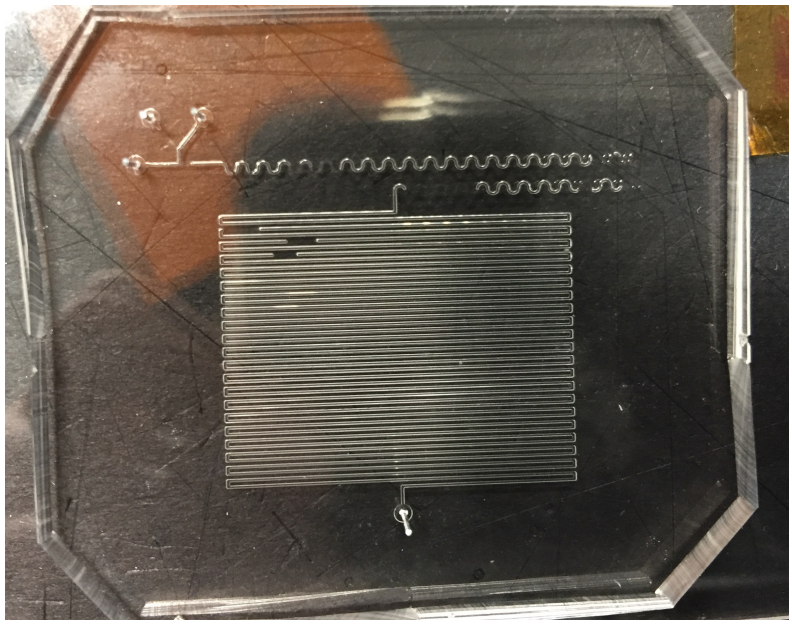

Figure 5 Versatile droplet generation chip

2.2 Material and Method

PDMS-based microfluidic devices have been widely used in biochemical and biomedical applications due to it's small feature size. Applications such as micro-reactors, microchips for capillary gel electrophoresis, and micro valves are successfully designed, fabricated and used in research.[12]

2.2.1 Device Fabrication

Figure 6 and Figure 7 shows the overall fabrication steps for this microfluidic device. Silicon wafer was used as substrate of the mold. Negative photoresist and UV (Ultra Violet) was used to cast the pattern on SU-8 negative photoresist master on the silicon wafer. PDMS (Polydimethylsiloxane) was later poured on the silicon wafer and cured. After peel off the PDMS layer from the substrate, a piece of glass is used to bond with PDMS layer to from the micro pattern between them.

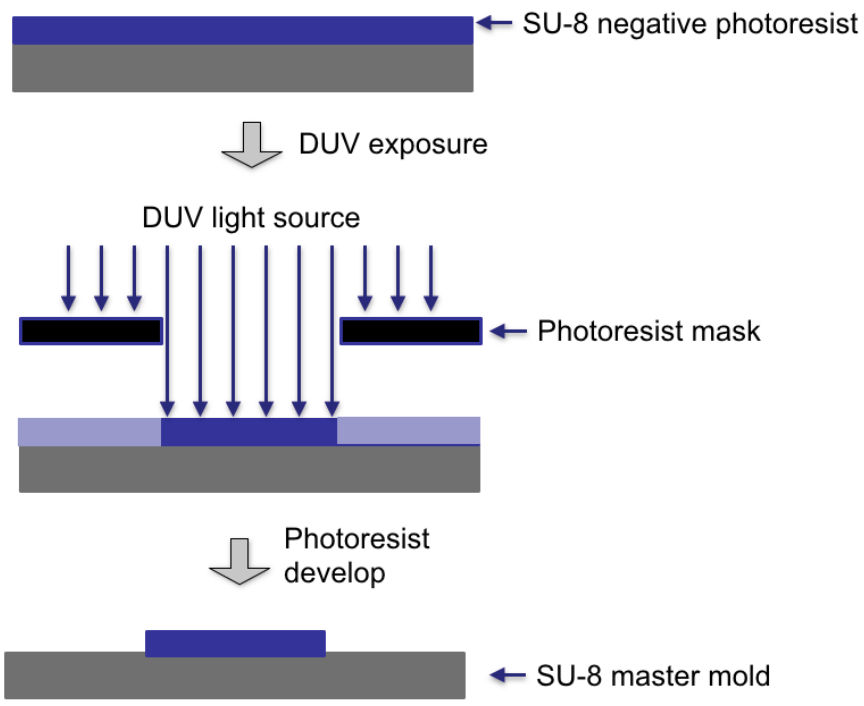

Figure 6 Steps for SU-8negative photoresist fabrication

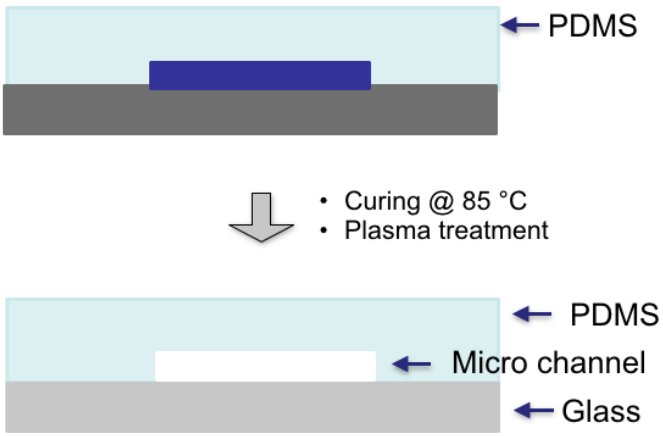

Figure 7 Steps for microfluidic device fabrication and bonding

Photoresist: SU-8 has attracted great interest for MEMS devices applications. [20] Due to it's good chemical and mechanical properties, many novel microfluidic devices have been designed and fabricated.[21] SU-8 photo resist is said to be a better alternative of LIGA (lithographie, galvanoformung und abformung) in microsystem applications especially for thick designs.[22] Advantages of using SU-8 photoresist include high aspect ratio,which could give a vertical side wall on the developed pattern and faster drying for higher throughput.

For the device used in this work, a master with height of 165 μm is fabricated. SU-8 2075 was used to pattern the channels on the silicon wafer. Edge bead removal (EBR) was used to develop the SU-8 pattern after UV exposure.(Figure 6)

PDMS: Polydimethylsiloxane as an organic polymer is commonly referred to as silicones. It is widely used in medical cosmetic as well as microfluidic devices. PDMS is inert, non-toxic and thermally stable, it severs the purpose of this work perfectly.[23].

PDMS 184 (Dow Corning Corporation) was used in fabricating the main part of the droplet generation chip. It is a heat curable PDMS with cross-linker (curing agent). [23] The mixing ratio recommend by the company is 10:1. Before cursing it is a vicious liquid, after baking it becomes elastic solid, therefore the material could envelope around the SU-8 mold and form micro level channel on the cured surface. (Figure 7)

Surface treatment:

Silane coating: surface silanization is used in the mold casting step on the silicon wafer. The Self-assembled monolayers (SAM) surface contain —Si—O—CnH(2n

+1)groups, which after hydrolysis into reactive silanol groups —Si—OH, form a covalent bonded layer on the silicon surface. When PDMS is pure on the substrate and cured it will be easily peeled off without adhesions.[24]

Plasma: the PDMS and glass are treated with a oxygen plasma surface cleaning before bonding together. By oxidizing both the of surfaces of the PDMS and the glass, the atoms, chemical groups and bonds are changed. After bringing them in to conformal contact, a strong chemical bond is formed between the PDMS and glass. This would conceal the micro pattern on PDMS and provide a negatively charged sidewall inside the channel that support electroosmotic flow (EOF).[25] Here another step,aquapel coating, is needed to change the micro channel into hydroponic property.

Aquapel coating: aquapel is used after PDMS bonding, modifying inside of the micro channel, changing it into hydrophobic.Details of these surface treatment procedures is demonstrated in appendix B: Device fabrication procedure.

2.2.2 Flow Rate Control Program

A Labview program is used in this work to control the syringe pumps in order to control the flow rate. The interface of the programs is showed in Figure 8. To generate droplets of consistent size the flow rate of the oil water should be fixed. This requires the total flow rate of the water mixture to be a constant, at the same time the mixing ratio of two solutions need to be changed to change the solution concentration inside the droplets. This is obtained by increasing the flow rate of one solution while decreasing

another one at the same rate, droplets of time dependent concentration could them be generated in sequence.

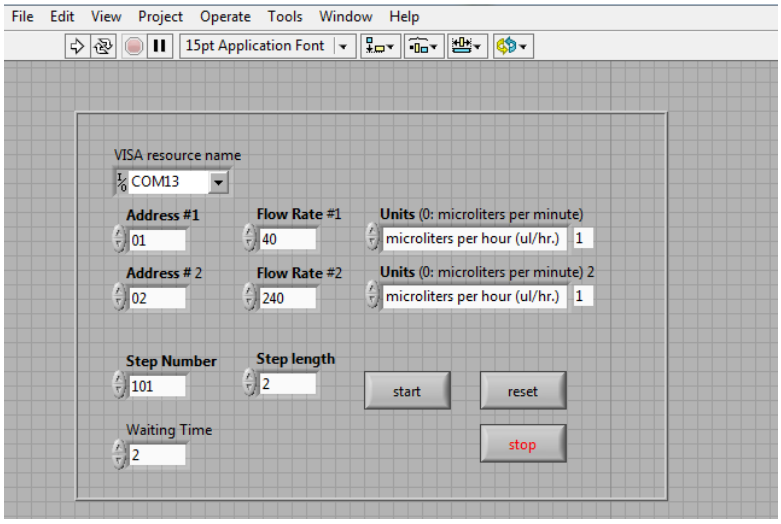

Figure 8 Labview control program interface

The two syringe pumps used in this work are *70-2213 Pico Plus* (by Harvard Apparatus, Inc., MA). This is a micro-stepping pump with 1.8° step angle geared 36:1 motor. The minimum step rate and maximum step rate are 1 pulse in 27.6 sec and 200 steps per second, correspondingly.[26] Even thought the maximum step rate seem too be fast enough, human operating cannot achieve that step rate without the help of computer program. Labview here serves the purpose. It is a software designed to control the hardware using a visualized programming interface.

In the program show in Figure 8, we could set the initially flow rate of the two water inlets and the step length of each change, the time interval between each step and also the step number in total. Ideally if the step interval of the program is ultimately small and the the step number to be big enough we could get a linear profile with out any step or pulse. But in reality, due to the limitation of the communication speed between the syringe pumps and the computer and the mechanism inside the pump related with step moter, the shortest step interval we could get is 2 seconds between each steps. None the less, with the help of this Labview program, it is good enough to provide a smooth range of concentration compared with human operating. (Figure 9)

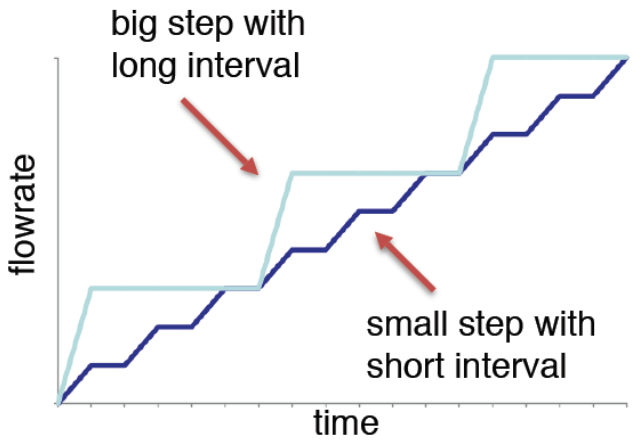

Figure 9 Illustration of the Labview program design principle

2.2.3 Cell Preparation

The cell used in this work is CC406 (*Chlamydomonas reinhardtii),* which is a wild type with less mutation. The cell sample is first taken from a nearby pond and then

washed and cultured in Tris–Acetate–Phosphorus (TAP) media. Detailed components of TAP media is in the following table(Table 2):

Trace Elements Solution	Concentration	Solid Ingredients	Concentration	Liquid chemicals	Concentration
Na_2EDTA	50 g/L	NH_4Cl	400 mg/L	glacial acetic acid	1 mL/L
$ZnSO_4 \cdot 7H_2O$	22 g/L	$MgSO_4 \cdot 7H_2O$	100 mg/L		
$CaCl_2 \cdot 2H_2O$	0.05 g/L	$CaCl_2 \cdot 2H_2O$	50 mg/L		
H_3BO_3	11.4 g/L	K_2HPO_4	108 mg/L		
$MnCl_2 \cdot 4H_2O$	5.06 g/L	KH_2PO_4	56 mg/L		
$FeSO_4 \cdot 7H_2O$	4.99 g/L	Tris(hydroxymethyl)aminomethane	2420 mg/L		
$CoCl_2 \cdot 6H_2O$	1.61 g/L				
$CuSO_4 \cdot 5H_2O$	1.57 g/L				
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.10 g/L				
KOH	16 g/L				

Table 2. Recipe of TAP media. [27]

After reculture of the cell for 3 days, the cell is now ready for the experiment. Before the experiment cells are suspended in TAP media of $2x10^4$ cell/mL. The cells are transferred in to fresh TAP media, the concentration are counted using hemacytometer. Two 1 ml BD syringe are used. One prepared with TAP media with cell suspended in it,

noted as 100% nitrogen solution. Another is prepared with N-limited media, noted as 0% nitrogen solution. Figure 10 shows the Chlorophyll autofluorescence image of the cell encapsulation. In which the cells are showed in white dot (and white lines because of fast flow rate and relatively long exposure time).

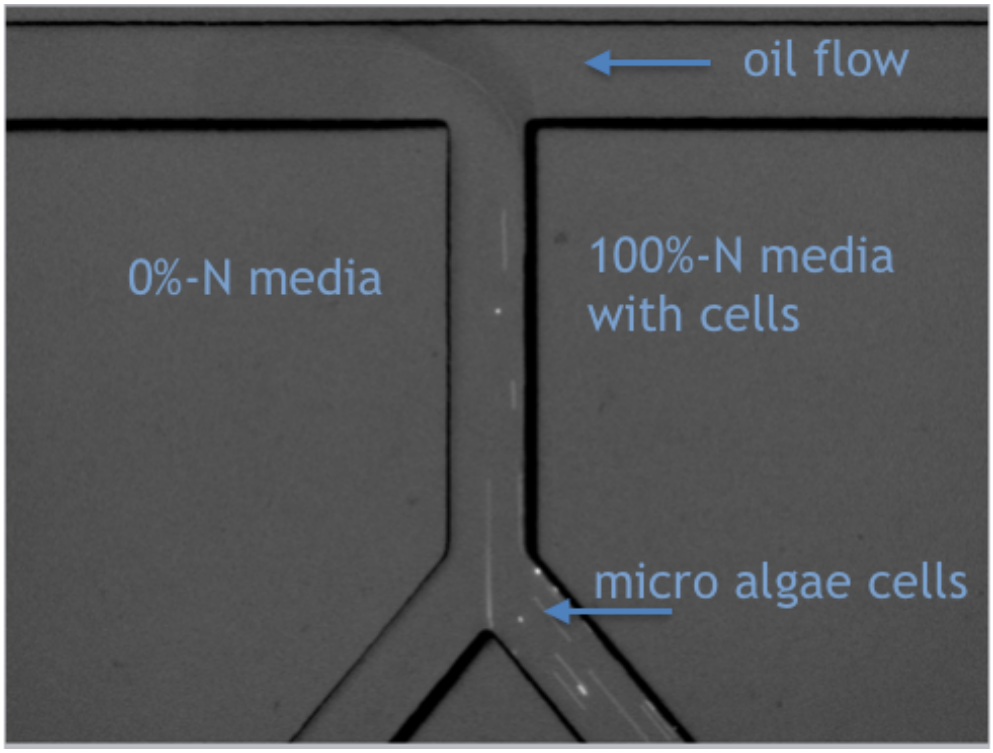

Figure 10 Chlorophyll autofluorescence image of the cell encapsulation

2.3 Result and Analysis

Before the device could be used for cell culture and cell growth analysis, multiple tests about the basic functions and the experiment accuracy and it's

repeatability are conducted. The following block diagram(Figure 11) shows the steps of device testing and characterization :

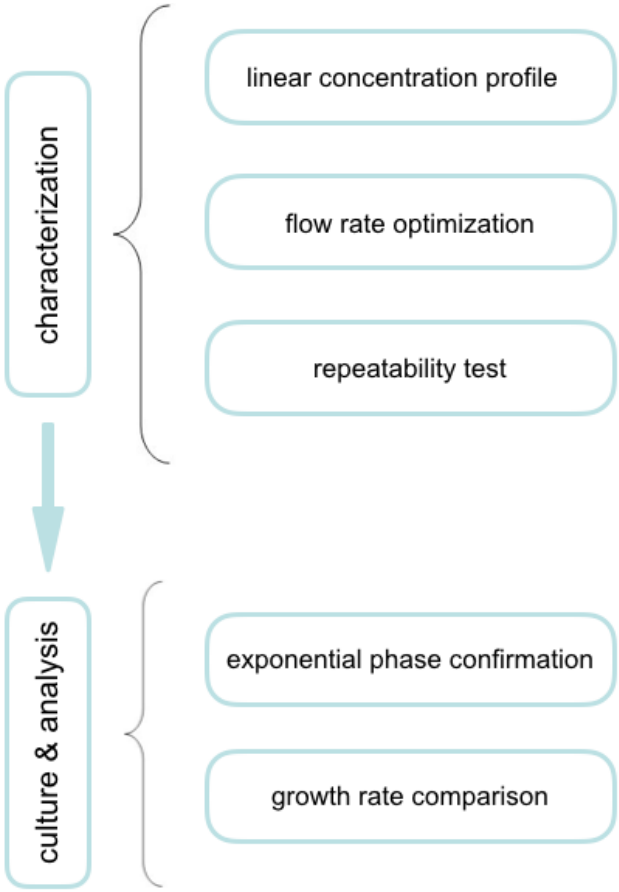

Figure 11 Block diagram of device characterization and test steps

2.3.1 Device Characterization with Fluorescent Solution

In this work, Fluorescein isothiocyanate (FITC) is used as a flow characterization tool. FITC is a derivative of fluorescein, widely used in applications related with flow

cytometry.[28] FITC is the original fluorescein molecule functionalized with an isothiocyanate reactive group (-N=C=S), replacing a hydrogen atom on the bottom ring of the structure. FITC has excitation and emission spectrum peak wavelengths of 495 nm.[29] The FITC solution used in characterize the concentration is 0.5 mol/L. Starting with a concentration of 128.4 mol/L condensed solution, the FITC was diluted with ratio 1:225.8 with DI water. This FITC concentration is proofed to be able to linearly represent the actual within the mixed flow concentration without saturation.

2.3.1.1 Linear Concentration Profile Characterization

The concentration profile was tested with 0.5 mol/L FITC water solution and DI water. the starting flow rate was FITC: 20 μl/h versus DI water: 120 μl/h and oil flow rate of 350 μl/h. The droplet diameter of flow rate combination is 245 μm. Because the hight of the channel is smaller than the the droplet diameter, the measurement is done by directing the droplet out of the device to a piece of bare glass. By letting the droplets floating on the glass freely without squashing, the size measurement is much more accurate.

The step rate control program was set to be 2 μl/h/step and the step interval is 6 seconds between each two steps in Labview. With a step number of 50, the total flow rate change will then reach just as the opposite of the beginning flow rate: FITC: 120 μl/h versus DI water: 20 μl/h. During the experiment, images were taken at the same spot by Zeiss camera at the droplet generation speed . Also a picture of the overall chamber is

taken after the experiment. Image J was used to analyze the fluorescent intensity inside the droplets which could be used to represent the concentration of FITC solution. Figure 12 is result after fluorescent intensity analyze.

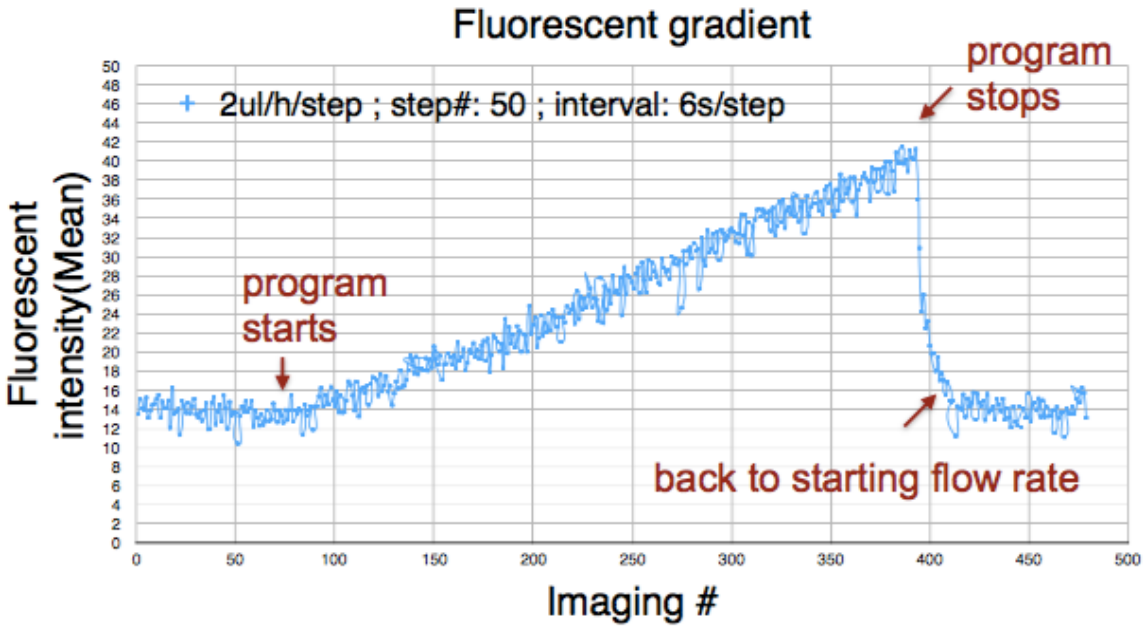

Figure 12 FITC florescent gradient generated with the Labview program

After starting the program (around 70th droplets), the concentration begins to increase gradually till the program stops. When the program stops the flow rates change back to the beginning flow rate. From figure 12, we could see that the concentration drops right after the program stops, and goes back to the starting concentration. By manipulating the step length and step interval, gradient with different slope could be generated.

2.3.1.2 Flow Rate Optimization

Have been proved that a linear, controllable concentration profile could be generated by the flow rate control program. The next step is to optimize the gradient profile to make it more flat and smooth. As you may see from figure 12, small fluctuations (around 10%) exist all the way along the curve. This is due to the nature of the pump. The pico plus syringe pump is driven by a stepper motor, which is connected with a sophisticated treaded rod. When changing the flow rate the angular velocity of the stepper motor is changed, therefore changing the pitch of the stepper motor per unit time. Even though the motor and threaded rod is precise, the flow rate changing system is still a discrete procedure.

To minimize the fluctuation in the concentration profile, I doubled the flow rate and tested different flow rate combinations in the Labview program. It turns out that after doubling the flow rate, the system is less vulnerable to disturbance and discrete control. Also by having smaller step length and shorter time interval it will result in a more smooth concentration profile. But due to the communication delay between the computer and syringe pump as well as within the pump, the minimum time interval that we could use is 2 seconds between each steps. The final flow rate begins with s FITC: 40 μl/h versus DI water: 240 μl/h and oil flow rate of 3500 μl/h. The droplet diameter of flow rate combination is 315 μm.

2.3.1.3 Repeatable Test

Before running the experiment for microalgae cell culture, we have to make sure that the concentration profile of different runs and different devices are the same. The test experiment is done by using two different devices fabricated form tow different master. (Figure 13).

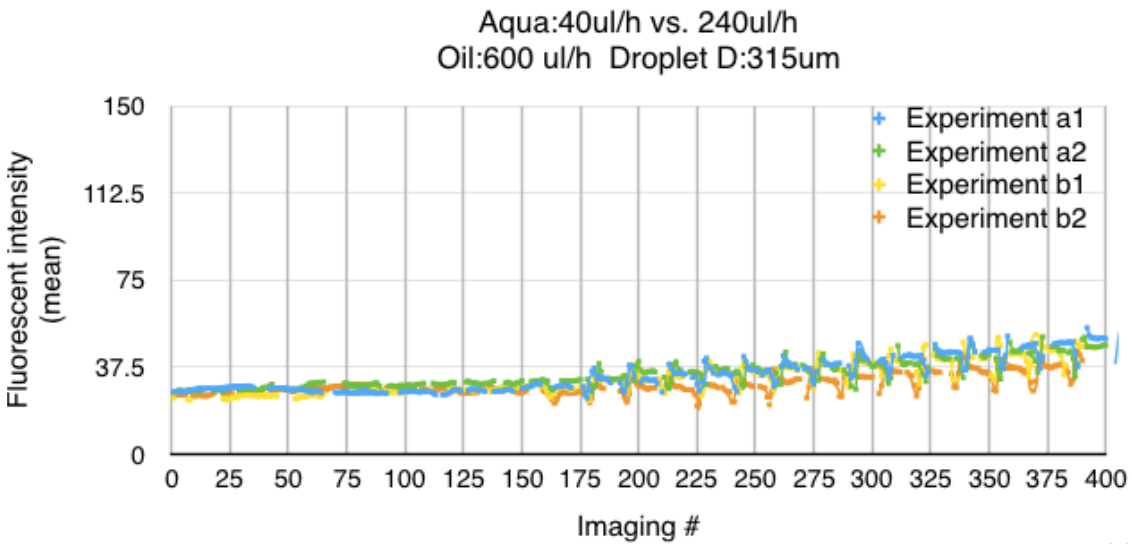

Figure 13 Concentration comparison between different runs

In this figure experiment a1 and a2 were operated on the same device but different runs and experiment b1 and b2 are from another device fabricated with a different master. From the figure we could conclude that the concentration generated in each runs are uniform and the experiment is repeatable. Besides that, we also need to

make sure droplets with the same concentration are always at the same positions inside the channel. Another experiment is then conducted to test this.(Figure 14).

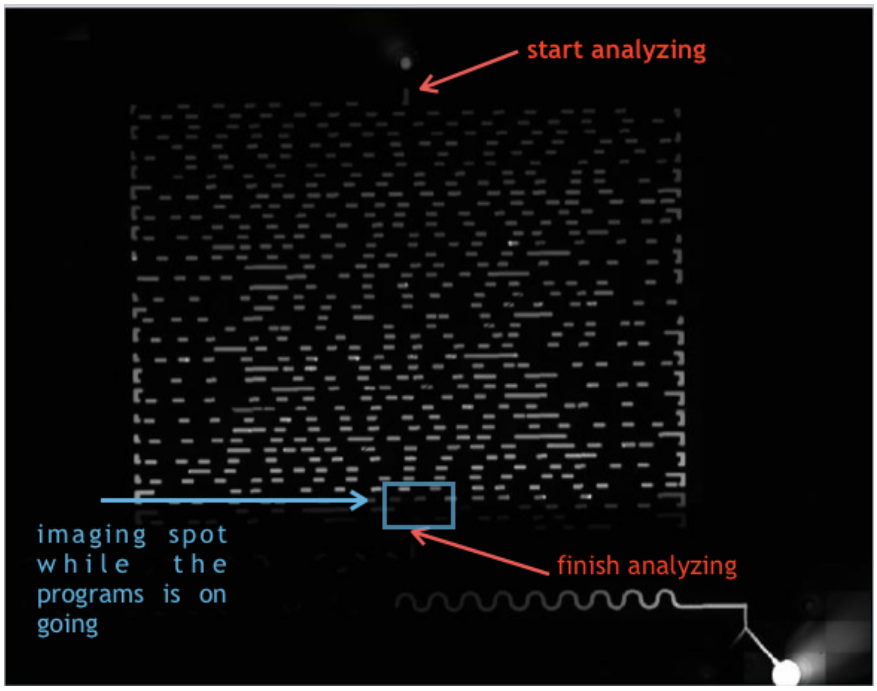

Figure 14 Microscope image of the incubation chamber. Droplets are filled with fluorescent dye.

Images were first taken at a fixed spot of the channel and than images of the whole chamber was taken after the experiment. Figure 15 shows the analysis of these two sets of images. The blue curve is the result of the fluorescent analysis of the all the droplets generated. The red curve on the other hand is the fluorescent intensity from the beginning of the channel all the way down to the end of the channel.

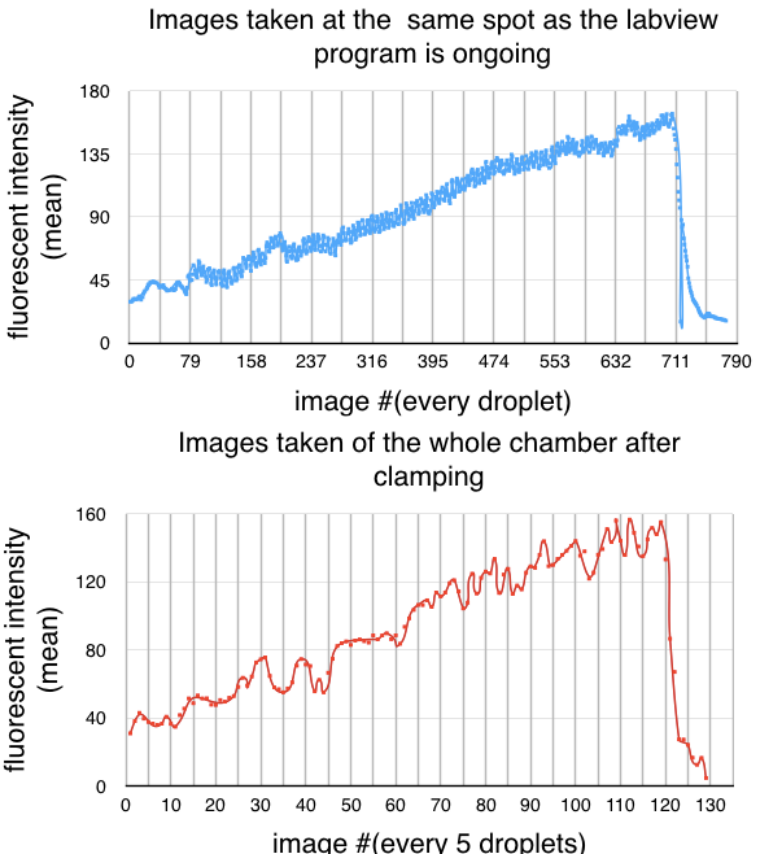

Figure 15 Droplets position characterization:(above):images taken at the same spot as the Labview program is ongoing. (bottom): mages taken of the whole chamber after clamping

After comparing the two figures above we could conclude that we are able to generate a concentration gradient and trap the profile in the channel. Also the gradient profile is occupying most of the channel without wasting too much of the spaces. And the lowest concentration will always be located at the beginning of the chamber.

2.3.2 Cell Growth Rate vs. Nitrogen and Phosphors Concentration

2.3.2.1 Culture Condition

To eliminate the interfere of other factors that may affect the cell growth, multiple control methods was used to make sure the cells are in the same culture condition except culture media concentration. This is to make sure the cell growth rate variation is solely a function of nitrogen or phosphors concentration in culture media.

Light Source: in this experiment the light intensity applied on the cells is 80 μmol /cm2. The light intensity is very important in photosynthesis cell culture . If the light intensity is too low it might inhibit logarithmic cell growth. In the wild nature, the light intensity is just above saturation level and if the light intensity is too high it will slow down the growth as well. The threshold intensities level in case of saturation or inhibition also depend on other factors such as temperature, CO2 concentration, or nutrient supply.[30] The light intensity we used is proofed to be below saturation and well enough for logarithmic growth.(by Shih-Hsin Ho et al.) [31]

The lamp used in the project is model T1468 from Ael Lighting Co., Ltd., 120V 60Hz with light bulb from Philips Lighting. The power the light bulb is 60 watt and the brightness is 860 lumens. The uniform light intensity area under the lamp is tested using a terrestrial quantum sensor i.e. light meter from LI-COR Inc., model number LI-250. Based on the light intensity meter, 80 μmol /cm2 is observed on the plane 20.3cm below the light bulb. An illustration of the uniform light intensity area is shown in Figure 16.

In which the light purple circle indicates that the area in which light intensity is within 80 µmol /cm2 ± 1 µmol /cm2. Whereas the dark purple circle indicates that the area in which light intensity is within 80 µmol /cm2 ± 0.5 µmol /cm2. The gray sphere in dictates the lampshade.

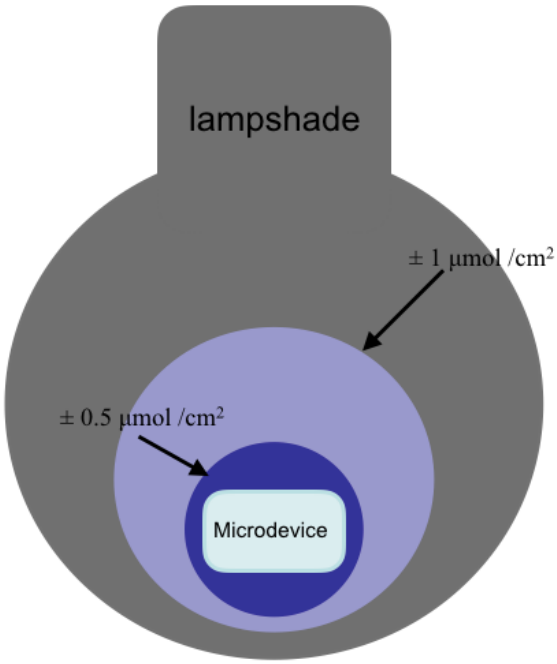

Figure 16 illustration of the uniform light intensity area

Temperature: temperature might be the most widely measured factor regarding microalgae growth. Temperature is almost invariably measured and controlled in researches to study microalgae growth and lipid production. [32] The reason that temperature is important to algal performance is that it affects many chemical reactions

and transportation process inside the microalgae cell. Most of the reactions are enzyme-catalyzed processed that temperature here strongly affect the activity of the enzymes. The microalgae cells could live in a wide range of temperatures between 10°C to 30°C. And the optimal temperature for biomass cultivation and lipid production is proofed to be 20°C.[33] The temperer for our culture is room temperature in our lab: 21°C ± 1°C, which is very close to the optimal temperature.

The PDMS device is completely kept in DI water during the culture to prevent droplet evaporation through the PDMS layer.

2.3.2.2 Microalgae Growth Rate

If algal cells are grown under unregulated conditions, characterization of it's growth could be represent by the equation:

$$N_t = N_0 e^{\mu t}$$

Where in this equation, N_0 is the initial cell number, μ is the growth rate, N_t is the cell number at time t and t is the amount of time between N_t and N_0.

If the living condition is limited, cell reproduction will decreases as growth rate in no longer an exponential factor. Instead the growth rate deviates from being a linear relationship .And finally the growth curve will gradually go into a stationary phase . The maximum growth rate for a culture is calculated based on the linear part of the growth curve or i.e. the logarithm part of the growth rate curve, which occurs before any type of limitation on growth occurs. The logarithmic growth curve is first used in this

experiment to find out the exponential growth phase of the microalgae culture. The growth rate analysis is based on the exponential growth phase regardless of the stationary part.

2.3.2.3 Results and Image Analysis

In four days of culture, images were taken every 12 hours to analyze cell growth.Figure 17 is the microscope images of the same droplet observed over 4 days. The column on the left is a droplet sample taken from the high nitrogen concentration(~75%); while the column on the right is the droplet from low nitrogen concentration(~25%).

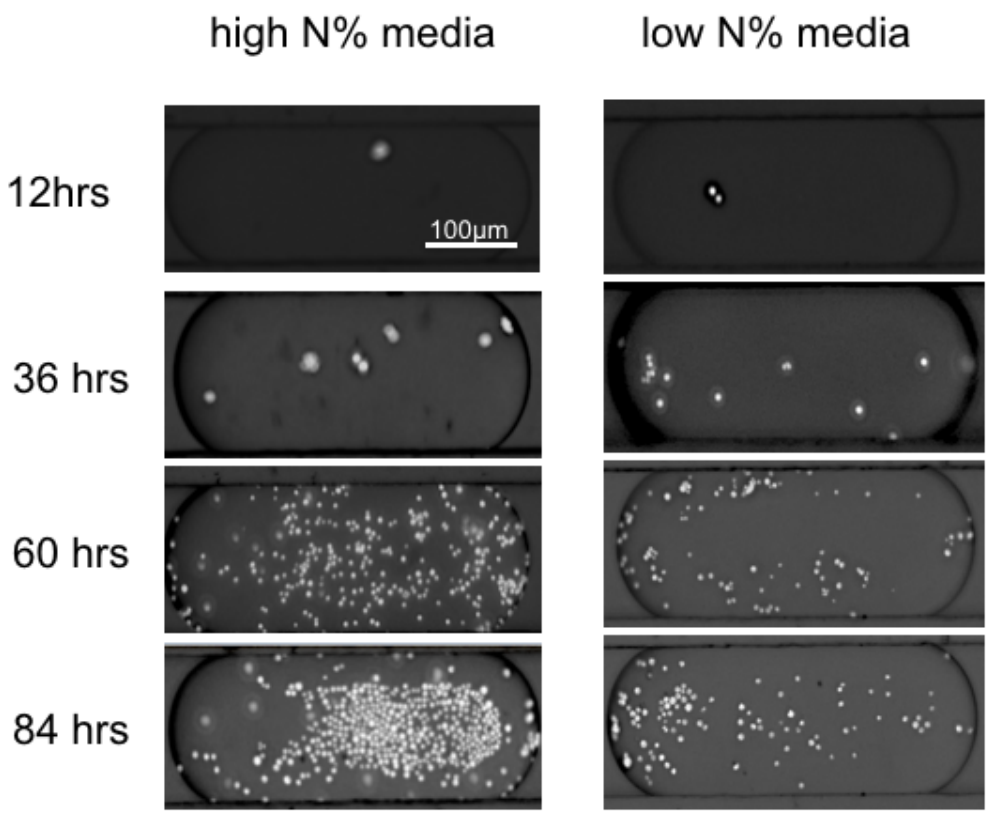

Figure 17 Compare of microscope images of cell growth under different nitrogen concentration

All the data samples are first gathered to form the cell growth curve. The cell number is represented by the fluorescent intensity, which was analyzed using Image J. *Chlamydomonas reinhardtii* cell is chlorophyll auto-fluorescent, it don't not need extra florescent dye for detection. the microscope used here is Zeiss Axio Observer Z1

microscope (Carl Zeiss MicroImaging, LLC), installed with a digital camera (Orca Flash2.8 CMOS Camera) and a filter set (excitation: 460–500 nm, emission > 600 nm).

Figure 18 is the growth curve of cells with different nitrogen concentration.We could see from the figure that, cells cultured in low nitrogen media reach there stationary phase faster. Cells cultured in high nitrogen media grow for the longest time and saturate the slowest. Also a threshold concentration was observed, under threshold concentration of nitrogen, cell growth increase gently, above threshold concentration of nitrogen, cell growth begins to hike.

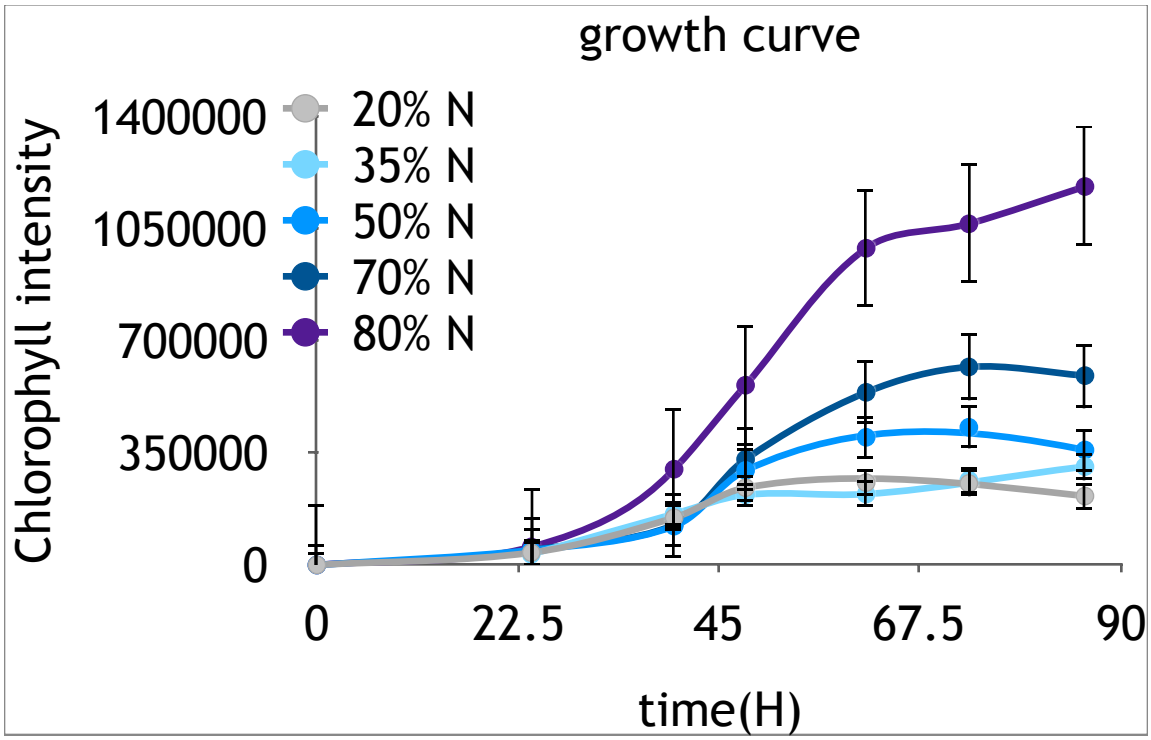

Figure 18 Growth curve comparison of different nitrogen % media

Figure 19 is the log scaled growth curve, where the exponential growth phase as represent by a linear slope. We could see form the curve that between 48 hours and 24 hours the log growth curve are showed a leaner increase, namely all in the exponential growth rate phase. This time window was used to analyze the cell growth rate, which is showed in figure 20.

We could see from figure 20 that cells grown in low nitrogen concentration media slow down their growth earlier than those grown in high nitrogen concentration media. Growth rate comparison of different concentration media is then showed in figure 20. Where the Y axis is the ratio between the cell number after 60 hours and cell number of 48 hours.

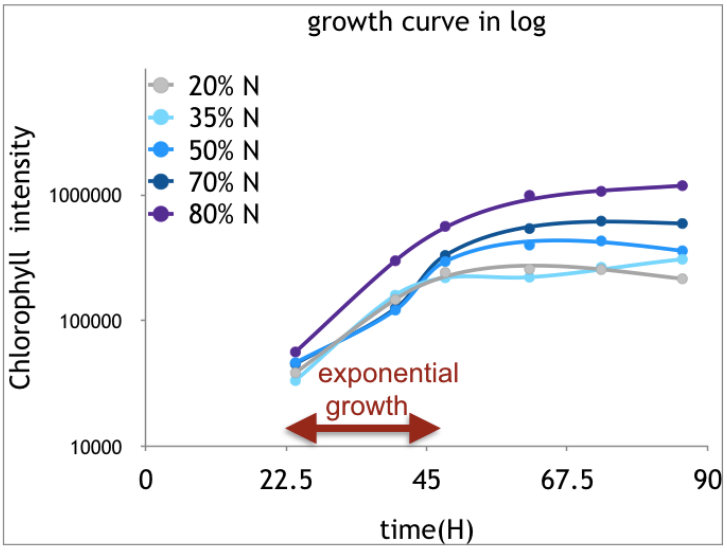

Figure 19 Log scale growth curve

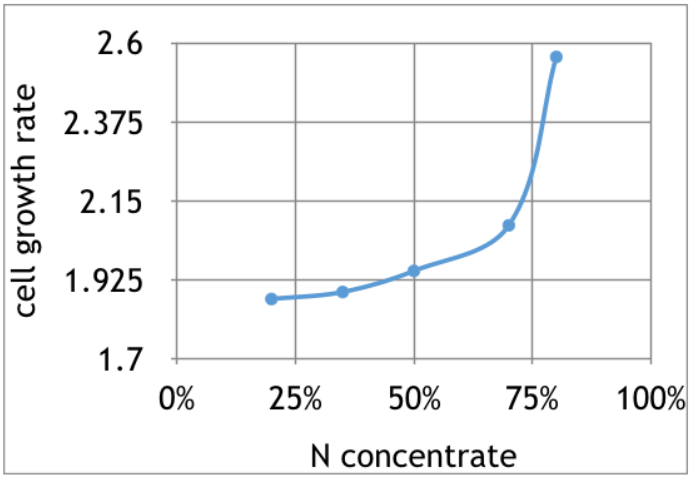

Figure 20 Cell growth in different nitrogen concentration

After calculating the growth rate with the equation showed before, an increase in growth rate was observed. The growth rate is defined as a feature when the cells are in their exponential growth phase, which they are cultured in unlimited nutrition. But because the sample point is not enough to show where exactly is the exponential growth phase, after analyzing the result with student T test and P value the detailed trend is unclear but at lease we could conclude that the growth rate is increasing when the nitrogen concentration is increasing.(Table3, 4)

concentration(%)	growth rate	stdev	stder
20%	1.87	0.23	0.10
35%	1.89	0.24	0.11
50%	1.95	0.52	0.23
70%	2.08	0.27	0.12
80%	2.56	0.71	0.32

Table 3 Nitrogen concentration vs. growth rate

H	20%<35%	35%<50%	50%<70%	70%<80%
P	0.527	0.882	0.155	1

Table 4 Student T test and result

CHAPTER III

SUMMARY AND FUTURE WORK

3.1 Project Summary

In this work, a developed microfluidics device is designed, fabricated and tested that this micro droplet generation device could be used for gradient generation and cell culture. In the device testing a linear concentration range of culture media is generated in the culture chamber and cells are successfully trapped in the droplets. A Labview program is used to control the step number, step length and step interval of the flow rate changes. The number of droplets and slope of the gradient,i.e., the gradient profile could be manipulated by changing the Labview program settings.

The device has been tested multiple times and the gradient results is statistically uniform. 100% nitrogen TAP media and 0% N-limited media are used in the project the generate a nitrogen gradient profile of 13.5% ~ 86.5%. And multiple methods are used to improve the stability of the concentration sequence. *Chlamydomonas reinhardtii* cells are trapped in the droplets and cultured for four days and images are took every 12 hours from cell encapsulation. The effect of nitrogen concentration in cell culture nutrition compositions on microalgal growth is investigated. *Chlamydomonas reinhardtii* cells in lower nitrogen condition saturate earlier and increased growth rate was observed in higher nitrogen concentration condition. The result in this work could be used as reference in future massive microalgal cultivation.

3.2 Future Work

There are many factors in the culture media that would affect microalgae cell growth . In addition to nitrogen concentration. If time allows the next step after this work might be the characterization of other element component in cell culture media, such as phosphorus. Hence, the ratio between phosphorus and nitrogen is also a subject that worth being studied.

In addition to the cell growth rate characterization, this platform would also be use for lipids production observation. Modification of the chip design is needed. Because the detection of lipid that the microalgae cells produced requires staining step with fluorescent dye. Extra inlet might be added to the present design to realize this function.

For the ultimate gaol of this project: by improving the productivity of third generation biofuel and lower the cost of production to make microalgae based biofuel more commercially competitive. Combination and integration of this versatile gradient generator with other functions is preferably needed. Such system like a high-throughput photo-bioreactor array, which is capable of applying 64 different light conditions to the microalgae cells to investigate how light conditions influence algal growth. (by Hyun Soo,Kim *et al.*)[13] And a droplet microfluidics-based screening platform for investigating and screening oil production of large numbers of algal strain. (by Hyun Soo, Kim *et al.*) [34]. These platform all working as different functions.If they are combined together and collaboratively working as a powerful tool, it would be a great improvement of the efficiency of single cell level microalgal performance analysis. It

will help the industry to have a better understanding of how the cells growth and their ability to produce lipid under different culture conditions, as well as understanding of how cell performances are different with different DNA strain, ultimately help commercialize this third generation microalgae based biofuel.

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APPENDIX A

MASK DESIGN: DROPLET GENRATION PLATFORM

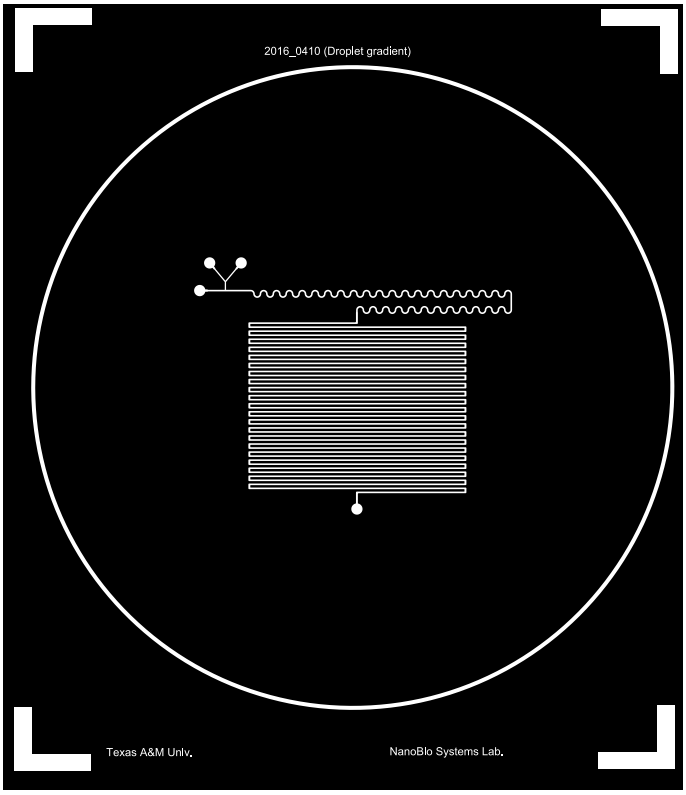


Figure A.1. Mask layout of the micro-droplet platform device.

APPENDIX B

DEVICE FABRICATION PROCEDURE

SU8 Photoresist Preparation Procedure

1. Rinse bare silicon wafer with acetone, and DI water, and blow dry with N2 gun.

2. Pour a appropriate mount of negative photoresist. (SU-8 2075, Microchem, Inc., Newton, MA)

3. Dehydrate the silicon wafer in the oven at 200°C for 10 minutes.

4. Load the silicon wafer on the vacuum plat inside the spin coater.

5.Spin coating at at 1000 rpm for 60 seconds, with a accurate time for 20 seconds and slow down time for 10 seconds to get a layer of photoresist at 165 μm.

6. Soft baking the wafer on a hot plate at 65°C for one hour, followed by 95°C baking over night. After baking, cool down to room temperature before exposure.

UV Exposure and Pattern Develop

1. Expose UV using a mask aligner (MA6, SUSS MicroTec Inc., Waterbury Center, VT) at 2.8 mW/cm^2 for 110 second with the photomask showed in appendix A.

2. Post exposure, put the wafer on the hot plate at 65°C for 20 minutes, followed by 95°C baking for 2 hours, cool down to room temperature before develop.

3. Develop the patterns using Edge Bead Removal (EBR) (Microchem, Inc., Newton,

MA) for 10 minute. Gently agitate the silicon wafer when developing.When observe no residual photoresist on the wafer, rinse with fresh ERB and IPA

4. Rinse with Isopropyl alcohol (IPA), and blow dry with N2 gas.

PDMS Fabrication Procedure

1.Place the wafer with the SU-8 master mold on the surfer inside the vacuum pump together with a weight boat containing 6 ~ 7 drops of tridecafluoro-1,1,2,2-tetrahydrooctyl. (trichlorosilane, United Chemical Technologies, Inc., Bristol, PA)

2. Vacuum the desiccator for 1.5 hours to allow trichlorosilane vaporization and evenly sprayed over the surface of wafers.Until a blue foggy coating is visible on the silicon wafer.

3.Clean the silicon wafer with IPA and blow dry with N2 gas.

4. Mix PDMS (Sylgard ® 184, Dow Corning, Inc., Midland, MI) prepolymer with matching curing agent at 10 : 1 ratio in a weight boat. And first degassing in vacuum pump for 15 minutes.

4. Pour the degassed PDMS prepolymer mixture onto the trichlorosilane coated master wafers for 25 g per 3 inch wafer.Followed by the second degassing by putting the wafer with PDMS back to the vacuum pump for 20 min.

5.Remove any visible bubble if there'a any after the degassing with plastic dropper.

6. Cure in an 65°C oven for 2 hours.

PDMS Bonding Procedure

1. After cured for 2 hours, slowly peel off the PDMS from the wafer.

2. Based on the design, cut the PDMS sheet to a proper size, punch the inlets and outlet holes on the PDMS with a gauge 19 needle.

3. Open via holes on the PDMS with a sharp tweezer.

4. Clean a piece of clear glass with ethanol and blow dry with N2 gas.

5.Put the glass and the PDMS sheet(pattern side up) into the plasma chamber. Vacuum the chamber for 2 minutes.

6. Apply oxygen plasma treatment (100 mTorr, 100W) for 2 minutes, bond the PDMS sheet on to the clear glass.

7. Bake in the oven of 65°C for 2 hours.

Aquapel Coating Procedure

1. After baking let the device cool down to room temperature.

2. Block the two water inlets with plastic tubing, inject Aquapel (Pittsburg Glass Works LLC. PA), using 1ml BD syringe into the oil inlet until Aquapel is filled in all the channels.

3. Push out the Aquapel with air using gauge 19 syringe.

4.Refill the chamber with FC-40 (3M Electronics Material Solutions Division, MN), push out the FC-40 with air using gauge 19 syringe.

Device Degassing Procedure

1. Fill in all the channels with FC-40. clamp all the inlets and the outlet.

2. Put the device in to a container, add DI water till the device is completely underwater.

3. Put the container in to the vacuum pump for at lease 12 hours.

4. Till this time the device is done with fabrication and is ready for cell culture.

APPENDIX C

LABVIEW PROGRAM

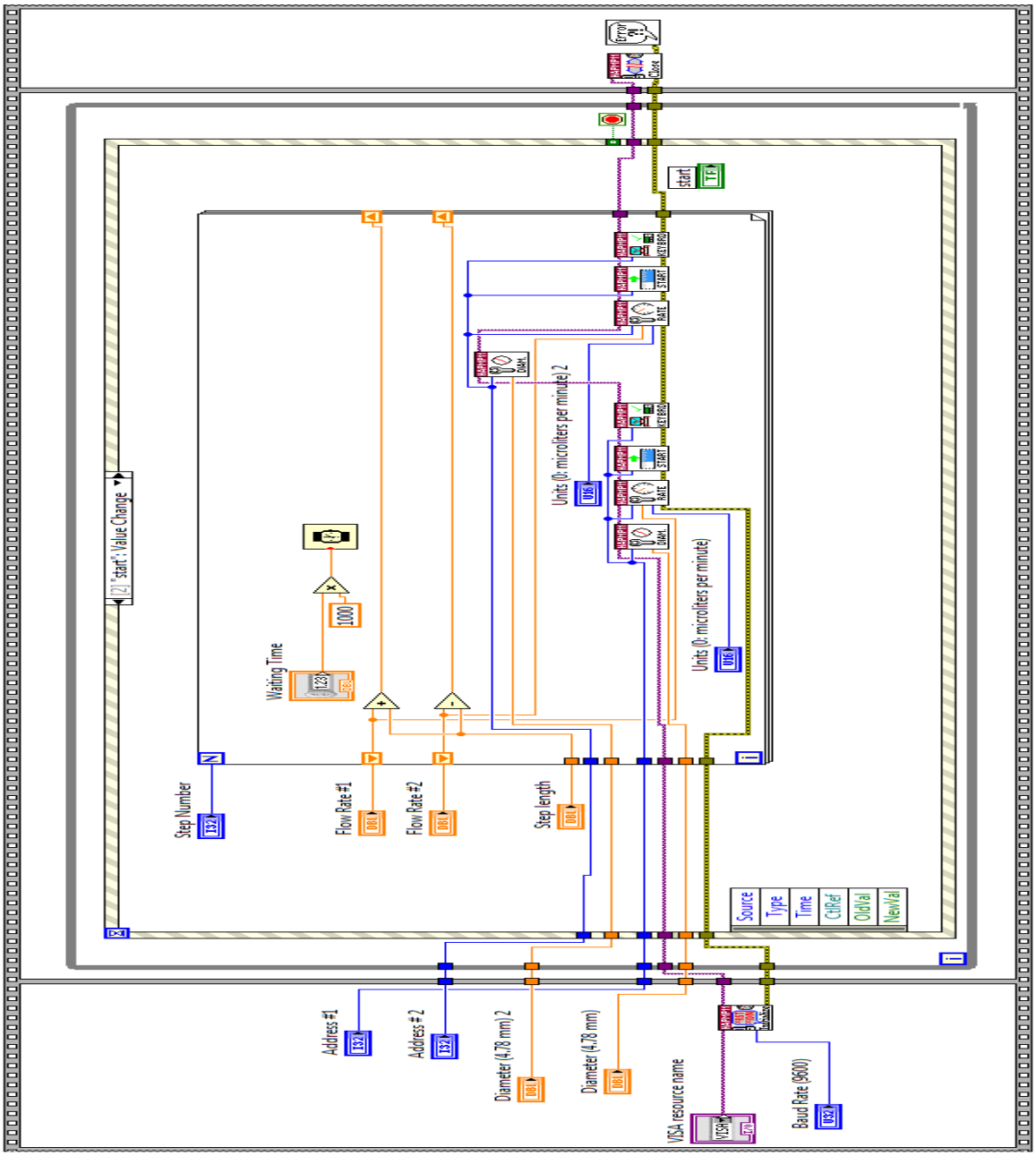


Figure C.1 Block diagram of the labview program

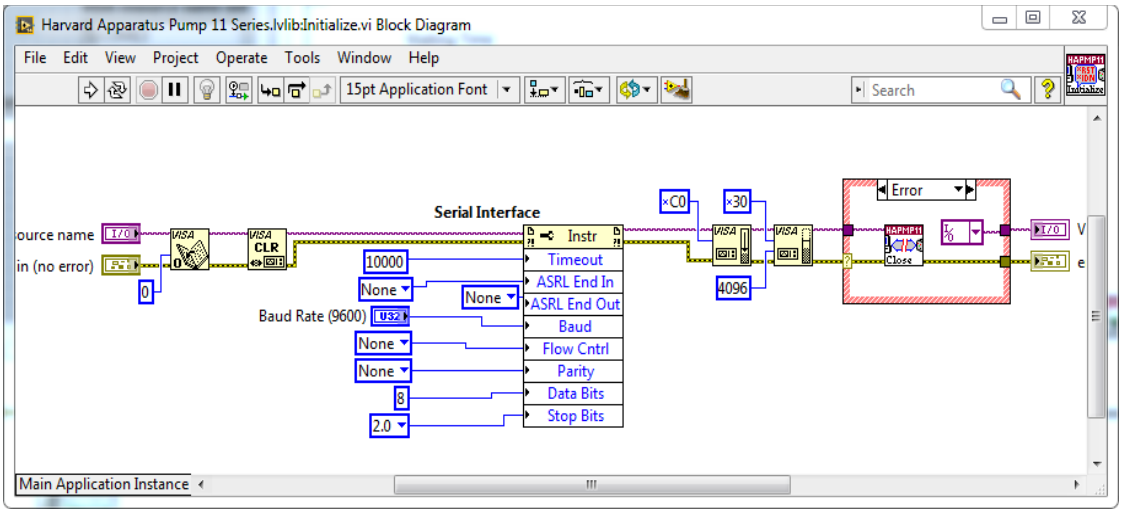


Figure C.2 Initialize block diagram of the labview program

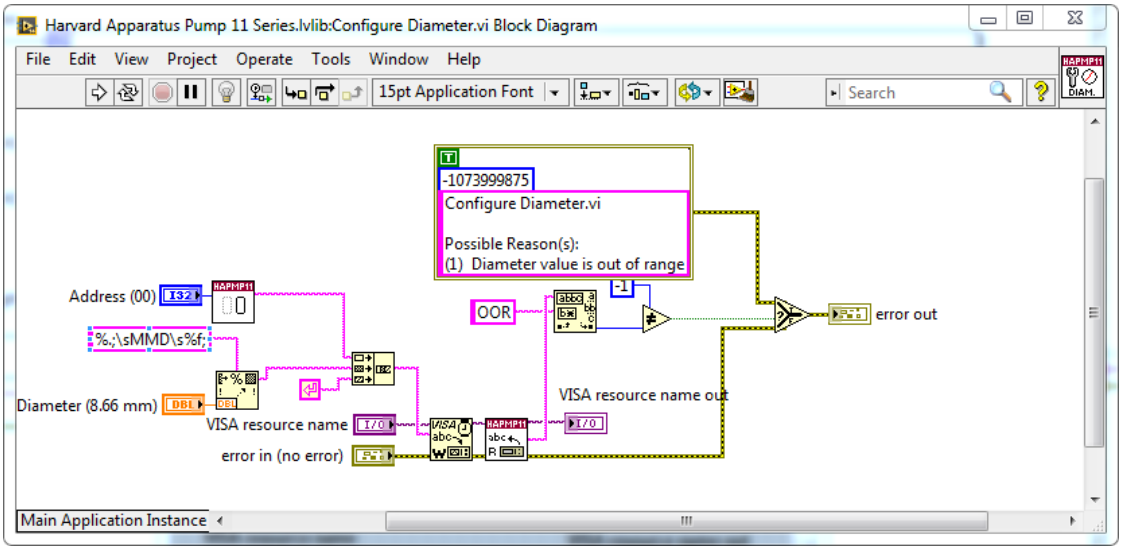


Figure C.3 Configure diameter diagram block diagram of the labview program

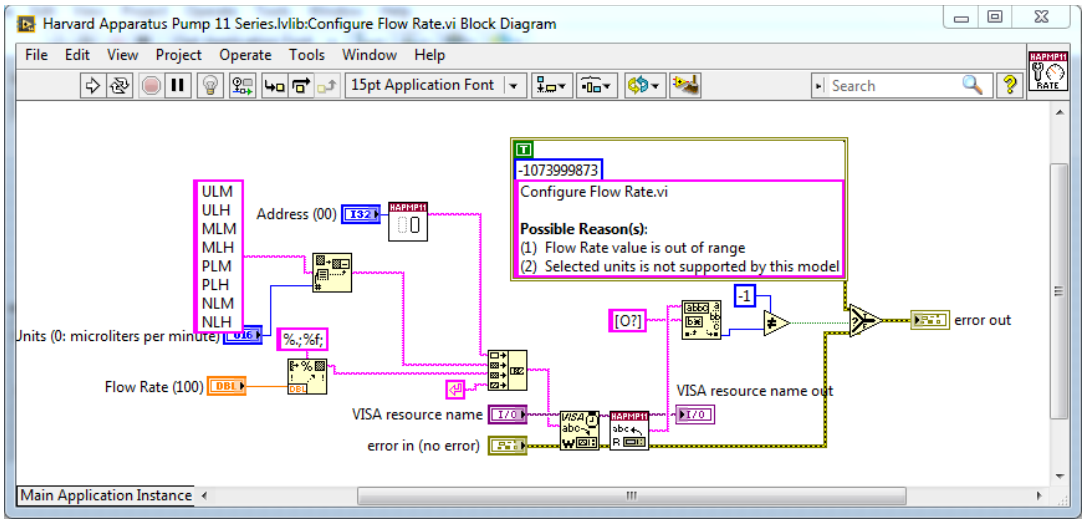


Figure C.4 Flow rate block diagram of the labview program

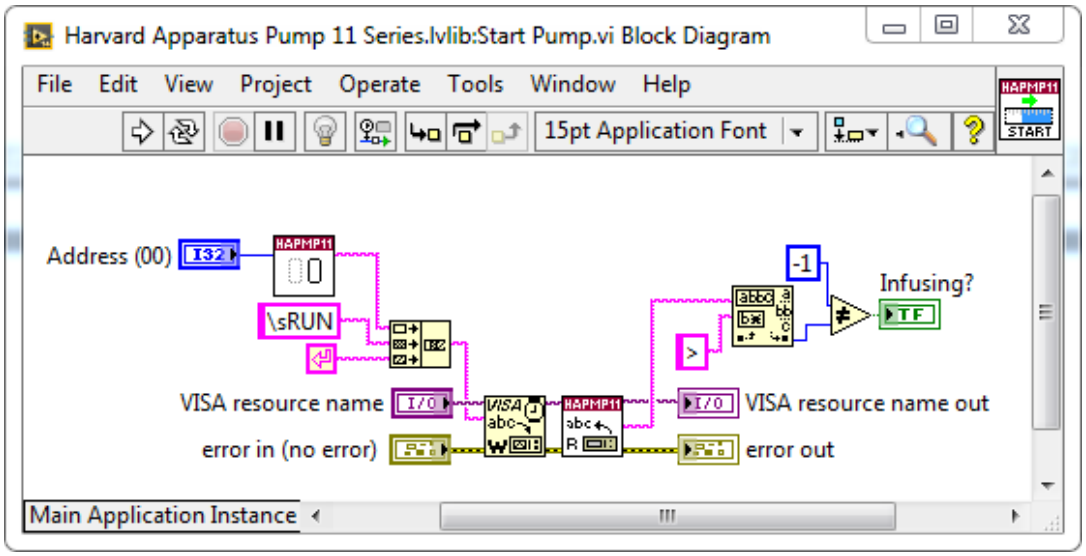
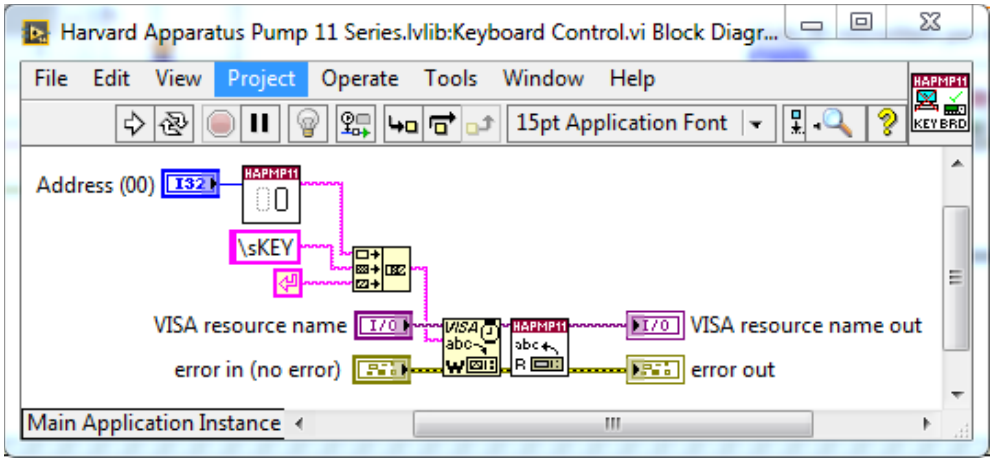


Figure C.5 Start pump block diagram of the labview programFigure



C.6 Keyboard control block diagram of the labview program

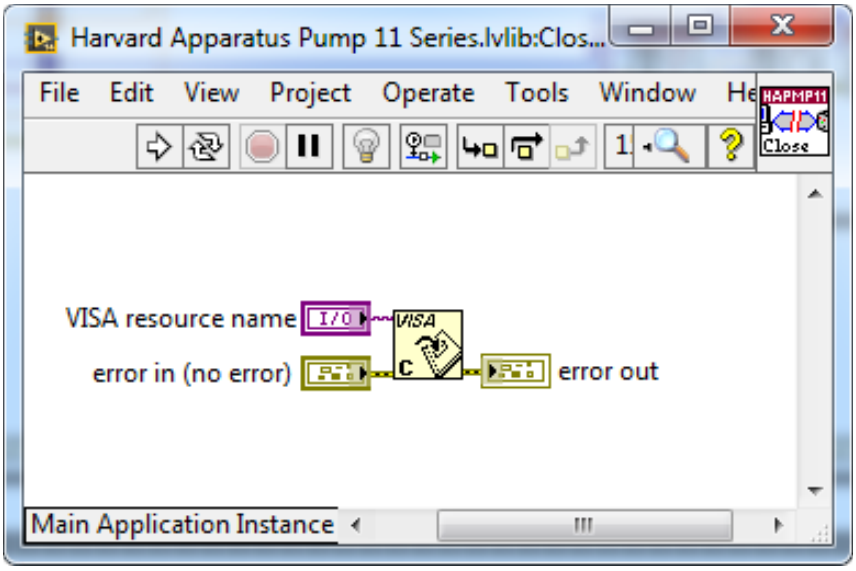


Figure C.7 Close control block diagram of the labview program

APPENDIX D

CELL PREPARATION PROTOCOL

Cell Reculture

1. The cell sample, CC406, used in this project is collected directly from local pond. The sample is washed and cultured in normal TAP media.

2. Take 1ml of cell sample from the old sample into the flask and add 50ml of fresh TAP media.

3. After reculture for 3 days the sample is ready for encapsulation.

Cell Washing

1.Take 5ml cell suspension solution form the reculture sample into a 15ml tube.

2. Centrifuge the sample at 3500 rpm for 5 minutes.

3. Change the culture media to fresh TAP media, gently shake the tube till the cells are resuspensioned.

4. Second centrifuge at the same condition.

5.Repeat step 3.

6.The cell is then ready for droplet encapsulation experiment.

APPENDIX E

EXPIRMENT AND CLAPING PROTOCOL

1. Start 2 inlets with with flow rate: 1400 µl/h vs. 1400 µl/h for 30sec to push the cells out from the syringe.

2. Change the flow rate back to 140 µl/h vs. 140 µl/h for 1~2min to stabilize the pressure inside the chamber.

3. Change the flow rate to: cell suspension 40 µl/h vs. N-limited media 240 µl/h and wait the cell speed to stabilize for 2 minutes.

4. Start Labview program.

5. After the program is finished wait for 20 seconds.

6. Clamp the inlet and outlet all together.