

DESIGN AND FABRICATION OF A MICROBIOREACTOR FOR YEAST
CULTURING

by

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to my precious family...

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ABSTRACT

DESIGN AND FABRICATION OF A MICROBIOREACTOR FOR YEAST CULTURING

From the beginning of the 1980, a new platform called “microfluidics” has been appeared. Microfluidics generally means the miniaturization of devices and systems, and this field covers engineering, biochemistry and biotechnology with several applications. As an advantage of microfluidics, small reagent or sample volumes are employed through the systems, therefore excess consumption of materials can be prevented. Since microfluidic devices can handle many processes, they can decrease the dependency on conventional equipment, which are always expensive and time consuming. Therefore, biosystems engineering heads toward the microfluidic systems with yeast applications. Within the framework of this thesis, the design and fabrication of a microbioreactor for yeast culturing was performed. A design for yeast culturing was drawn via L-Edit layout program and a master was obtained. Microbioreactor was made of polymethyl methacrylate (PMMA) polymer, which is biocompatible material, and fabricated via hot embossing by employing the master and solvent-assisted bonding techniques. In order to optimize the parameters such as fluid flow, concentration distribution within the designed chip, COMSOL Multiphysics program was utilized. Before yeast experiment, polystyrene beads of 2 and 10 μ m diameter were tested through the fabricated chip to check its functionality for yeast culturing. The ultimate goal of this thesis was to get knowledge about the uncharacterized YOR060C protein in yeasts. Yeast preculture at optical density (OD) of 0.5 was fed through the microbioreactor and brightfield and fluorescence images of yeast cells within the chip were taken with CCD camera on the microscope. The experiment was lasted for approximately 28 hours and the images were processed via Fiji image processing package program. Cell count, cell area and cell perimeter were estimated from brightfield microscopy images and cell integrated density, the indicator of fluorescence luminescence, was obtained from fluorescence microscopy images. According to the obtained results, cell proliferation within the microbioreactor was successfully achieved, which approves the usage of the designed microbioreactor for further yeast culturing.

ÖZET

MAYA KÜLTÜRLENMESİ İÇİN MİKROBİYOREAKTÖR TASARIMI VE ÜRETİMİ

1980 yılı başından itibaren, “Mikroakışkan teknolojiler” olarak adlandırılan yeni bir platform karşımıza çıkmaktadır. Mikroakışkanlar genel olarak cihazların ve sistemlerin minyatür boyutlarını içerir ve çeşitli uygulamaları ile mühendislik, fizik, kimya, biyokimya, nanoteknoloji ve biyoteknolojiyi kapsar. Mikroakışkan sistemlerde reaktif veya numune miktarının az olması bir avantajdır ve böylece malzemenin aşırı tüketimi önlenabilir. Ek olarak, mikroakışkan cihazlarla birçok işlem yapılabilir ve bu da her zaman pahalı ve zaman alıcı olan geleneksel ekipmanlara olan bağımlılığı azaltabilir. Bu nedenle, mikroakışkanların avantajlarından yararlanabilmek için, biyosistem mühendisliği çeşitli hücre sistemleri ve özellikle maya uygulamalarında mikroakışkan sistemlere yönelmiştir. Bu tez çerçevesinde, maya kültürlenmesi için bir mikrobiyoreaktör imalatı üzerine çalışılmıştır. Maya kültürlenmesi için uygun bir tasarım L-Edit programı ile çizilmiştir ve bir kalıp elde edilmiştir. Mikrobiyoreaktör, elde edilen kalıbı kullanarak sıcak presleme ve çözücü yardımcı yapıştırma teknikleri kullanılarak canlılık ile uyumlu olan polimetil metakrilat (PMMA) polimerinden üretilmiştir. Tasarlanan çip (mikrobiyoreaktör) içindeki optimum akış profilini ortaya çıkarmak için, COMSOL Multiphysics programı kullanılmıştır. Maya deneyinden önce, 2 ve 10µm çaplı polistiren boncuklar üretilen çipe beslenmiş ve çipin maya kültürlenmesine uygun olup olmadığı incelenmiştir. Bu tezin ana hedefi, mayalarda flüoresan etiketli YOR060C proteininin işlevi hakkında bilgi edinmektir. Optik yoğunluğu 0.5 olan maya ön kültürü mikrobiyoreaktöre beslenmiş ve mayaların ışık ve flüoresan görüntüleri mikroskobun CCD kamerası ile çekilmiştir. Deney yaklaşık olarak 28 saat sürmüş ve elde edilen görüntüler Fiji görüntü işleme paket programı ile analiz edilmiştir. Işık mikroskobundan elde edilen görüntüler ile hücre sayısı, hücre alanı ve hücre çevresi hesaplanmış ve flüoresan mikroskobundan elde edilen görüntü ile flüoresan ışımasının göstergesi olan entegre hücre yoğunluğu elde edilmiştir. Bu bulgular ışığında nanolitre hacimli mikrobiyoreaktör içerisinde hücre büyümesinin gerçekleştiği, dolayısı ile bu mikrobiyoreaktörün hücre kültürü çalışmalarına uygun olduğu onaylanmıştır.

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LIST OF SYMBOLS

c	Concentration
D	Diffusion constant
F	Body forces
h	Height
L	Length
p	Pressure
u	Velocity
w	Width
x_i	Weight fraction
ρ	Density
μ	Dynamic viscosity
ρ_{mix}	Mixture density

LIST OF ACRONYMS/ABBREVIATIONS

3D	Three dimensional
CAD	Computer aided design
CAN	Chronological aging network
CDK	Cyclin-dependent kinase
CFD	Computational fluid dynamics
CNC	Computer numerical control
COP	Cyclo olefin polymer
CP	Cytoplasm
DB	Dense body
EDM	Electrical discharge machining
EGFP	Enhanced green fluorescent protein
G	Glucan
GFP	Green fluorescent protein
IB	Inner halve of the plasma membrane bilayer
IMB	Integral membrane protein
μ CP	Microcontact printing
μ TM	Microtransfer molding
M	Mannoprotein
MAPK	Mitogen-activated protein kinase
MEMS	Microelectromechanical systems
MIMIC	Micromolding in capillaries
nDEP	Negative dielectrophoretic
OB	Outer halve of the plasma membrane bilayer
OD	Optical density
ORC	Origin-recognition complex
PAC	Photoactive compound
PC	Poly (carbonate)
PCB	Polycomplex body
PDMS	Poly (dimethylsiloxane)

PE	Poly (ethylene)
PET	Poly (ethylene terephthalate)
PLA	Poly (lactic acid)
PMMA	Poly (methyl methacrylate)
PMP	Peripheral membrane protein
PP	Poly (propylene)
PPE	Periplasmic enzyme
Pre-LC	Preloading complex
Pre-RC	Prereplicative complex
PS	Poly (styrene)
PVC	Poly (vinyl chloride)
REM	Replica molding
SAMIM	Solvent-assisted micromolding
SC	Synaptonemal complexes
SPB	Spindle pole bodies
Tg	Glass transition temperature
WOS	Wall outer surface
YPD	Yeast extract-peptone-dextrose
YPDA	Yeast extract-peptone-dextrose-agar

1. INTRODUCTION

Over the past two decades, miniaturization of devices and systems has become very popular issue in scientific advances, thus a new platform “microfluidics” has emerged. The definition of the microfluidics is the discipline of regulation and manipulation of fluids and particles within a system that has micron and submicron dimensions, and the microfluidics technology collaborates in developing the methods and devices. Particularly, the electronics, chemical, biological and medical fields have benefited from the developing microscale technologies. With the help of microfluidics, conventional laboratory handling, processing and analytical techniques are revolutionized. Since the quantity of reagent or energy consumed in the operations are independent from the results, information assembling and data processing applications can be handled in miniaturized systems [1]. Microfluidic systems need low-cost fabrication methods for high-throughput productions. Reaction, separation or detection can be conducted in microfluidic devices by using small sample volumes. In addition, high-throughput drug screening, single cell or molecule analysis and manipulation, drug delivery and therapeutics, biosensing, and point-of-care diagnostics are some of biological applications that can be processed via these devices. Due to high surface area to volume ratio of microfluidic devices, heat and mass transfer operations can be done efficiently. With these devices, many processes can be conducted in an easy and faster way than the conventional equipment, which are always expensive and time-consuming. Since the microfluidic devices are cheap, disposable and portable handheld tools, they can make life easier in many areas. Moreover, by employing microfluidic devices, diffusion time can be decreased at least a million-fold due to proportionality of diffusion time and system length. Consequently, microfluidic devices have many advantages over conventional ones [2-5].

The microfluidic and nanofluidic systems’ characteristic length scales associated with various biological entities are given in Figure 1.1, which depicts that, it is logical to design the systems and devices in accordance with the characteristic dimensions of cells, molecules or other biological entities. In addition to functionality and advantages of microfluidic devices, fabrication of a device is an important phenomenon. Modelling of a microfluidic device is implemented by computer aided design (CAD) tools such as L-Edit, AutoCAD or

SolidWorks. After designing the chip, scientists employ many fabrication methods to obtain a desired device functioning properly. Wet and dry etching, 3D printing, photolithography and hot embossing are the most widely used techniques for device fabrication. Moreover, in order to prevent contamination within the device, produced chip is covered with another piece via bonding, and adhesive bonding, thermal fusion bonding and solvent bonding are commonly used bonding techniques in industry [6].

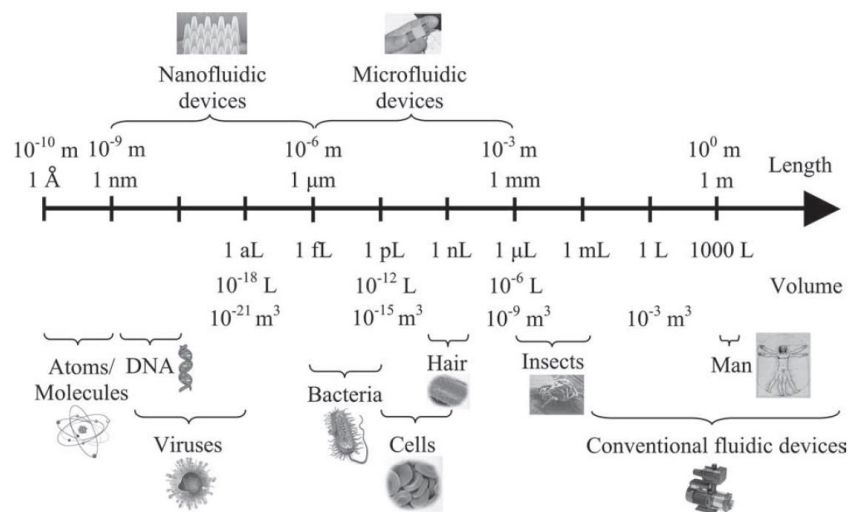


Figure 1.1. Characteristic length scales for microfluidic and nanofluidic systems [2].

As mentioned earlier, microfluidic devices are used in many research areas and biosystems engineering is one of them. Yeast cells, which belong to fungi family, are commonly employed as a model system for eukaryotes. They are frequently used to reveal the cellular organization in higher organisms, including humans. Yeast dimensions can change from species to species but they generally are 3 or 4 μm in diameter being suitable for microfluidic study [7] [8]. The usage of microfluidic devices for yeast cell culturing can provide high-throughput analysis with small sample volumes.

Within the scope of this thesis, a microfluidic device (a microbio reactor) for yeast culturing was modelled in L-Edit program and a mask was produced on the basis of model. By using the mask, a positive master was created via electrochemical wet etching. A polymethyl methacrylate (PMMA) polymer work piece, which had 3×5cm dimensions, was put onto master and the designed model was transferred to the PMMA work piece with the

help of hot embossing technique. Another PMMA piece was employed to cover the hot embossed piece by means of solvent-assisted bonding. The in-house fabricated microfluidic device (microbioreactor) was placed on the microscopy stage and yeast cells were loaded into the microbioreactor and let to grow there with the aim of the investigation of their behavior in approximately 1.5 nanoliter chambers present in this Lab-on-a-Chip platform. The ultimate aim was to obtain a broader understanding of the function of YOR060C protein, which is an uncharacterized protein.

The second chapter of this thesis, entitled as “Theoretical Background” explains the yeast cell characteristics, applications of microfluidic chips to yeast cells and fabrication methods of microfluidic devices. The next chapter gives device fabrication, yeast culturing and image processing steps used in the present study. The fourth chapter focuses on both computational and experimental results and further discussions on yeast culturing in a Lab-on-a-Chip platform. The last chapter includes the main conclusions drawn from this study along with the contributions to the research and the recommendations for future work.

2. THEORETICAL BACKGROUND

2.1. Yeast

Yeast is originated from the words of the gyst in Old English and yes roots, which means boil, foam or bubble. Yeasts are eukaryotic microorganisms classified under the taxon Fungi and constitute about 1500 out of total more than 50000 fungus species. Yeast and yeast-like forms normally have one nucleus per cell and they have spherical, oval or cylindrical shapes as shown in Figure 2.1.

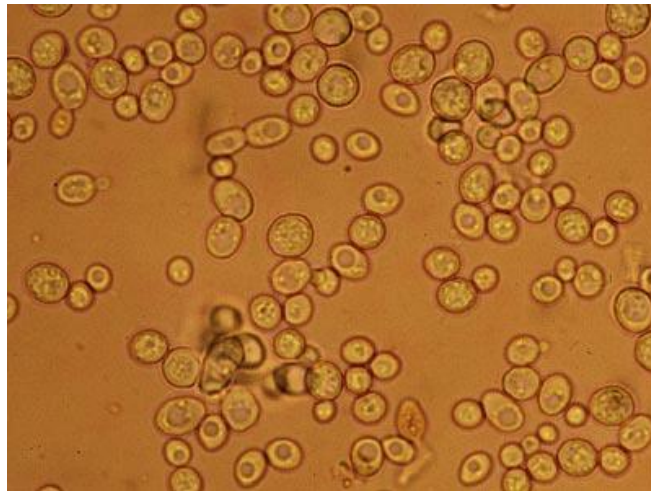


Figure 2.1. Yeast cells [9].

Yeast cells produce alcohol and carbon dioxide from sugar, therefore these living organisms are mostly attracted by beer brewers, wine makers and bread bakers since the earliest time of history. Yeast cells have porous double-layered wall for taking in the nutrients and giving out the metabolites. The optimum conditions for yeast growth are about 20 to 30°C, pH 7 and sugar for enriched environment.

Figure 2.2 summarizes the fermentation process and ethanol and carbon dioxide are produced at the end of the reactions, which take place in the cell. Maltase, invertase and zymase enzymes can be produced by yeast cells during fermentation. Maltase is used to convert maltose to glucose, invertase is utilized for sucrose conversion to glucose and

fructose, and zymase catalyzes the conversion of sugar into ethanol and carbon dioxide. Due to their complex genetic structure, rapid growth ability in optimum conditions, easy replication, having both haploid and diploid states and well defined genetic system properties, yeast cells are widely used as a model organism in most of the biological studies [12 – 14].

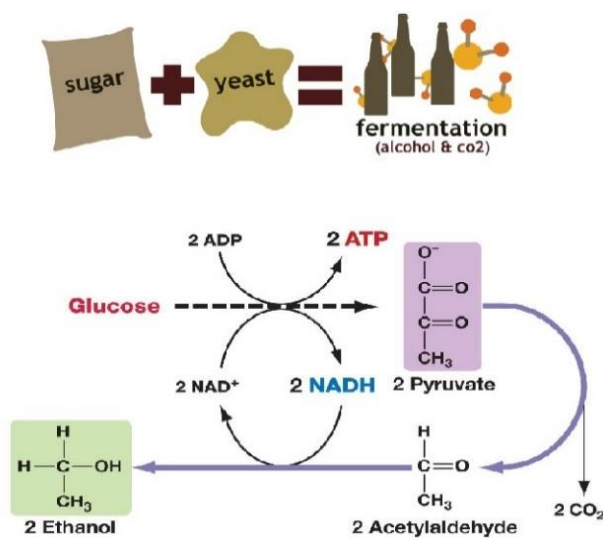


Figure 2.2. Fermentation in yeast cells [10, 11].

2.1.1. Cell Structure of Yeast

Being a eukaryotic organism, yeast includes a set of specific subcellular components. Nucleus, mitochondria, endoplasmic reticulum, Golgi complex, secretory vesicles and vacuoles are the organelles, which exist in yeast cells. All of the organelles are dependent on each other with their individual duty to sustain yeast lifetime and they are surrounded by a cell envelope which has an inelastic cell wall isolated from the plasma membrane by the periplasmic space. Figure 2.3 shows the organized structure of the yeast cell.

The diffusion barrier is created between the cytoplasm and the culture medium for the intake of low molecular weight materials by the plasma membrane. Enzymes produced by yeast cells exist between plasma membrane and cell wall in the periplasmic space and they do not pass through the cell wall. Mannoprotein and glucan are the main components of the cell wall. Cell wall undergoes several morphological changes due to budding, mating and

sporulation events despite its rigid structure. In addition, immunochemical properties of the yeast cells are regulated by the carbohydrate moieties of the mannoproteins [15].

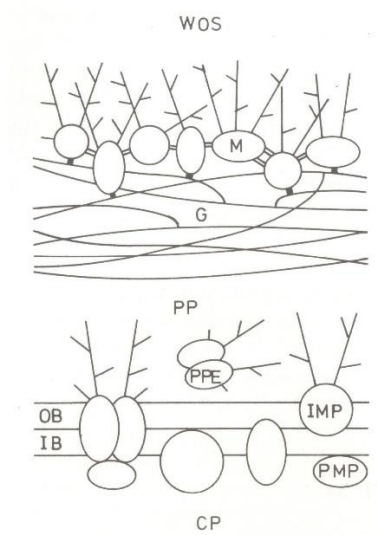


Figure 2.3. Yeast cell surface illustration. CP is cytoplasm, IB and OB are inner and outer halves of the plasma membrane bilayer, PMP is peripheral membrane protein, IMB is integral membrane protein, PPE is periplasmic enzyme, PP is periplasmic space, G is glucan, M is mannoprotein and WOS is wall outer surface. (=) is S-S thioester bond and (-) is covalent bond [15].

The plasma membrane has intrinsic membrane proteins, phospholipids and sterols, present in a lipid bilayer and extrinsic proteins cover the bilayer surface. Lipid composition of yeast cells and their membranes change from cell to cell and it is easy to manipulate. Therefore, this situation makes yeast cells ideal model organism for studying the properties and functions of lipids in eukaryotic cell membranes.

Vacuoles are present in yeast cells in different sizes during their life cycle. Large vacuoles shrink and break into small vesicles when the budding starts and they can combine to form large vacuoles as the budding process proceeds. Protease and hydrolase enzymes are gathered in the vacuoles and it can be said that, vacuoles play a similar role in yeast cells to that of lysosomes in animal cells. In addition, vacuoles are also used as storage of metabolites because 25% and more of amino acid pool and most of the arginine exist in these organelles.

In yeast cells, secretion is accomplished by the following pathway: Endoplasmic Reticulum → Golgi → Vesicles → Cell surface and this is similar to mammalian secretion. However, unlike the mammalian cells, a receptor for signal recognition for protein existence is not clear.

Yeast cells can do both respiration and fermentation. Respiring yeast cells contain more than 50 mitochondria but, glucose repressed cells have only a few mitochondria in their body. Although glucose repressed cells have mitochondria, their cristae is poor and respiratory enzymes show low activity. Mitochondria has two membranes and inner one constitutes the cristae. There are phospholipids on both of the membranes and inner membrane contains more cardiolipin. Sterol to phospholipid ratio is 1 to 30 in both membranes. By using nuclear genes, most mitochondrial proteins are encoded, synthesized on cytoplasmic polysomes and imported into the mitochondria [15].

2.1.2. Life Cycle of Yeast Cells

Yeast cells can exist in both haploid and diploid forms having one and two chromosomes, respectively. A diploid cell is formed from the merge of two opposite mating types (alpha and α) haploid cells in the natural sexual cycle. The diploid cell, which represents the heterozygous generation with respect to mating type, is unable to mate and it cannot secrete or response the mating elements. At this situation, the cell experiences meiosis under malnutrition conditions, such as lack of nitrogen, and ascus is produced with four haploid spores. Next, spores are transferred to the nutrient medium for germination and rapid fusion within the ascus to form two diploid alpha/ α -cells [15]. Figure 2.4 summarizes the yeast cell cycle.

In order to carry out the mating, haploid cells have to be conditioned by mating factors. Opposite mating types are mixed and in response to mating factors, agglutination increases in G1. Mating arises between unbudded cells in G1 because cells in G1 have different cell surface than others. In addition, agglutination material is found only on cell surface of haploids and zygotes, not on diploids or spores. Pheromone induction results in agglutination increase, needs carbon and nitrogen supply and also dependent on RNA and protein synthesis. Mating factors stimulated cells have increased the glucan/mannan ratio on the cell

wall and cells take the elongated shape. In order to undergo the meiosis, and generate ascospores, diploid alpha/a-cells are taken into the nitrogen-free acetate medium (sporulation medium). When the cells are in the sporulation medium, they will finish their cell cycle to G1 and get into the meiotic pathway.

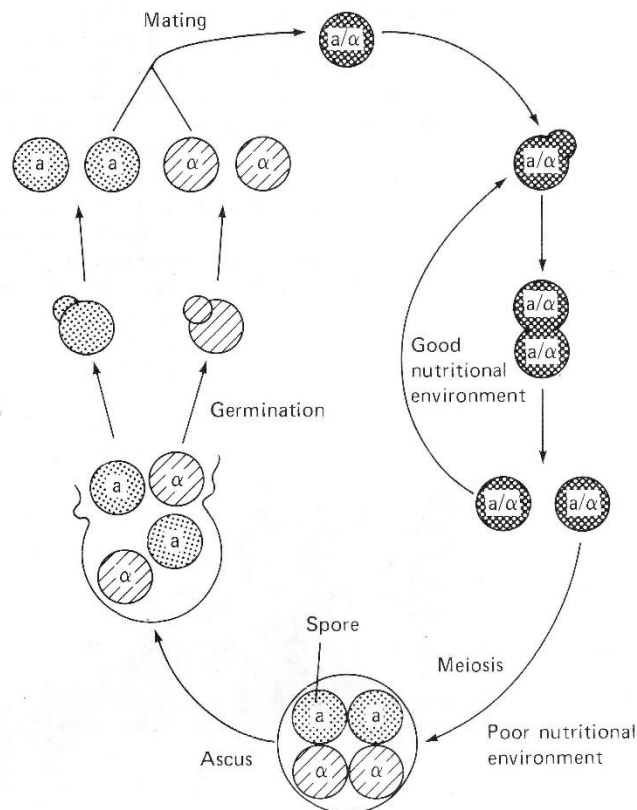


Figure 2.4. Life cycle of yeast cells [16].

Yeast meiosis event, described in Figure 2.5, is similar to that of other eukaryotes, the DNA is replicated about 4 to 10 hours before the meiotic prophase (Figure 2.5a and 2.5b). Spindle pole bodies (SPB), found in the nuclear envelope, are duplicated and before the premeiotic S-phase, dense body (DB) emerges in the nucleus (Figure 2.5b). Synaptonemal complexes (SC) become visible in the DB and at leptotene unpaired lateral elements and zygotene short SCs become detectable. At pachytene, DBs disappear or decrease in size and SCs take their maximum length (Figure 2.5c). In diplotene, a single polycomplex body (PCB) is created and SC cannot be seen (Figure 2.5d). Next, SPBs are separated by the spindles in the opposite direction and when the spindle reaches its maximum length, SPBs have enlarged cytoplasmic sides and PCBs disappear (Figure 2.5e). This shows that the

meiosis I is finished. Afterwards, every SPB duplicates and these pairs separate from each other with lobed shape. Two spindles are created between two SPBs and located in the opposite direction (Figure 2.5f and 2.5g). The prospore wall dissociates and the space between them is filled with cytoplasmic material such as mitochondria (Figure 2.5g). Once the second nuclear division is finished, the spindles break down, the prospore wall closes and ascospores emerge. With the help of deposition of the substances between the two membranes of spore wall, the wall growth progresses and the plasmalemma of the ascospore constitutes the inner layer of the ascospore wall (Figure 2.5h).

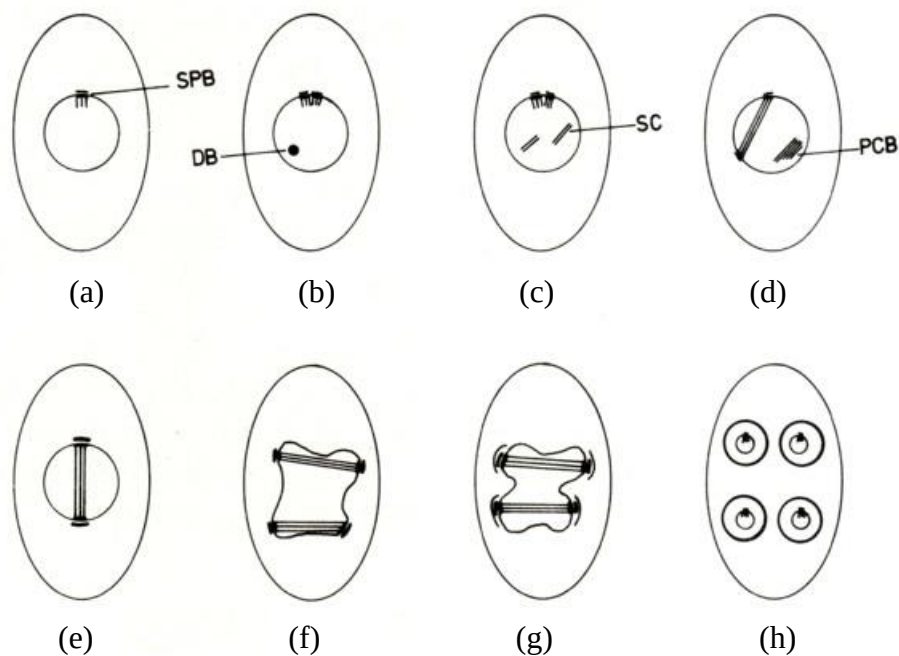


Figure 2.5. Stages of yeast meiosis [15].

Yeast cells also grow by budding in the optimum nutrient medium conditions. If the cell has a bud, this shows the new division cycle of the cell. Genetic material duplicates and other cellular constituents increase. Total cell mass and total volume also increase due to bud formation. Chromosome separation occurs mitotically within an intact nuclear membrane. Subsequent to the cytokinesis, wall septa is formed and daughter cell leaves with its all cellular components and functions needed for its survival. Figure 2.6 summarizes the budding event of the yeast cell [15][16].

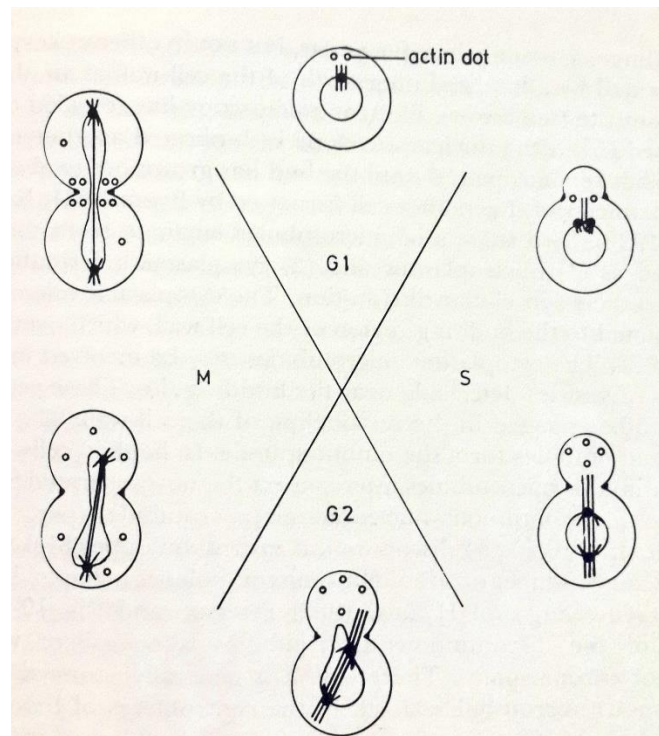


Figure 2.6. Budding of the yeast cell [15].

2.1.3. Cytological Methods for Yeasts

Acquiring structural knowledge about unstained yeast cells with light microscopy is very difficult. By using 1000-fold magnification, yeast vacuole and several cytoplasmic material forms may be obtained. Therefore, phase-contrast microscopy with several labeling techniques makes the visualizing of cellular structure of yeast cells possible. The information about the cell surface and the cell itself can be derived from fluorescence microscopy by employing fluorochromic dyes. Green fluorescence protein (GFP) extracted from jelly fish (*Aequorea victoria*) is utilized as a reporter for intracellular localization and gene expression studies in yeast cells [17 – 20]. The gene to be investigated is tagged with GFP and subcellular density of the expressed proteins can be monitored by fluorescence microscopy. In addition, functional analysis of yeast genome can also be performed by the GFP-tag technique. If fluorescein isothiocyanate and Rhodamine B are combined with monospecific antibodies raised against yeast structural proteins, the range of cellular properties of the cells monitored can increase and immunochemical reactions can be distinguished by brilliant fluorescence using a dark-ground microscope. Wu et al. (1996) have used confocal scanning

laser immunofluorescence microscopy to reveal the intracellular distribution of proteins. They have detected the spatial organization of mitosis-regulating protein kinases and phosphates in fission yeast [21]. In order to monitor the organellar morphology of yeast cells, confocal microscopy can be employed. Visser et al. (1995) discovered high-resolution three-dimensional images of fluorescently labeled yeast mitochondria by using this technology [22]. Protein localization in the cytoplasm nucleus and vacuole can be identified by immunofluorescence microscopy and higher resolution ultrastructural knowledge about yeast cells can be acquired with immunoelectron microscopy.

Electronic particle counters, size analyzers and digital image processing are employed to get information about yeast cell morphology. The geometry of cells and filaments, morphological heterogeneity and vacuolar formation during growth can be monitored by using image processing techniques [23][24][25].

2.2. Microfluidic Chips for Yeast Applications

Yeasts are the well-known and most preferred model organisms for cell biology research. They represent the eukaryotic cell field and researchers ultimately can get information about human biology by working with yeast cells. In the literature, it is seen that microfluidic devices are widely used with several cell types and microorganisms and yeast is one of them. With small sample volumes, high throughput analysis can be done via microfluidic devices. The following table gives the summary of microfluidic devices utilized in yeast cell culturing:

Table 2.1. Summary of microfluidic devices used in yeast cell culturing.

Ref	Chip Type	Material	Fabrication method	Immobilization technique	Application
[26]	Micro-chemostat	PDMS	Photolithography, oxygen plasma	Tesla diode	Gene expression and cellular growth (aging)

Table 2.1. Summary of microfluidic devices used in yeast cell culturing (cont.).

[27]	Multi-chamber microfluidic device	PDMS, glass	One level soft-lithography, oxygen plasma	Microwells	Cell loading, culturing and phenotypic study
[28]	Microfluidic chip	PDMS	Multilayer soft-lithography	Sieves	Mating pheromone response, cell number, cell size, cell morphology and GFP concentration measurements
[29]	Microfluidic chip	PDMS	Multilayer soft-lithography	PDMS columns	Pheromone induced MAPK signaling network study, cell tracking and segmentation, total fluorescence amount, cell statistics calculations
[30]	Micro-electrode system	PDMS, glass, SU-8	Simple hybrid glass-SU-8-PDMS process, oxygen plasma	A negative dielectrophoretic force	Trapping, cultivation and release of selected cells

Table 2.1. Summary of microfluidic devices used in yeast cell culturing (cont.).

[31]	Wheatstone-bridge-analogous arrangement	PDMS, glass	Standard photolithography, oxygen plasma	None- adjusted roof height to obtain monolayer	Live-cell imaging of several yeast strains
[32]	Microfluidic dissection platform	PDMS, glass	Replica molding, photolithography, UV irradiation	PDMS micropads	Long-term live-cell imaging of the complete replicative lifespan of budding yeast cells.
[33]	Microfluidic chip	PDMS, glass	Replica molding, oxygen plasma	Sulfo-NHS-LC-biotin chemical modification and adjusted roof height	Revealing the cell cycle dynamics, cell/organelle morphologies and several fluorescently labeled proteins through the lifespan of a single yeast cell
[34]	Microfluidic chip	PDMS, glass	Standard multilayer photolithography, plasma treatment	PDMS pensile columns	Single cell gene expression analysis

Table 2.1. Summary of microfluidic devices used in yeast cell culturing (cont.).

[35]	Micro-chemostat	PDMS	Photolithography, oxygen plasma	Shallow-depth region for monolayer	Monitoring and quantifying budding yeast at different dilution rates
[36]	Micro-chemostat	PDMS	Standard photolithography	Monolayer design	Investigating single yeast cell dynamics, protein abundance, localization, cell size and growth of yeast cells
[37]	Microfluidic chip	PDMS	Soft-lithography	V-shaped pockets	Trapping and keeping track of yeast cells exposed to chemical stress with the help of hydrodynamic forces
[38]	Microfluidic chip	PDMS, glass	Soft-lithography, oxygen plasma	Hydrodynamic pressure application and PDMS pillars	Replicative life span, cell cycle dynamics and history-dependent behavior

Cookson *et al.* [26] worked with yeast cells and they fabricated a Tesla microchemostat to reveal the single-cell gene expression over cellular generations. Their device was used to capture the cells and feed them by nutrient in the trapping region. Yeast cells were fluorescently tagged and fluorescent images were used to extract the time-series data. The microchemostat had three port chip design as shown in Figure 2.7a. One of the ports was used for cell loading, the other one was used for media supply and final one was used for waste. After cell loading, cells which were not trapped were removed with the waste channel to prevent clogging. Nutrient supply through the trapping side was done via diffusion and advection (Figure 2.7b).

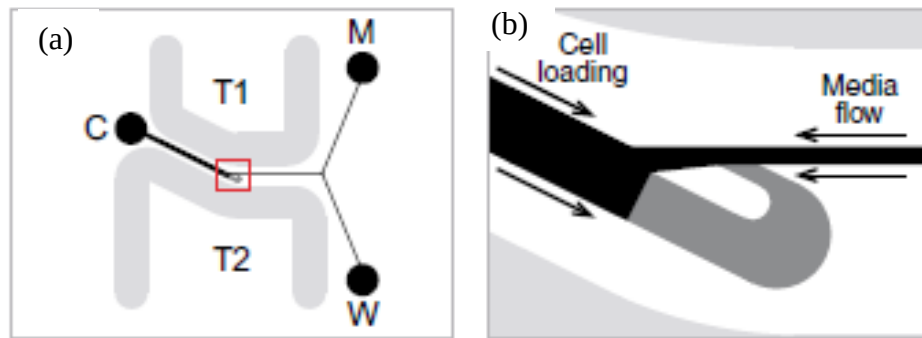


Figure 2.7. Chip design (a) three-port chip design (b) cell loading and media flow [26].

The microfluidic device was fabricated by using poly(dimethylsiloxane) (PDMS) polymer from a patterned silicon wafer template with the 4 μ m-high cell trapping chamber in a monolayer. Chip did not contain any actuators or valves. Nutrient and cell loading flow controls were carried out by gravitational forces. By using this microchemostat, single-cell gene interpretation and cycle time as a function of generation were discovered [26].

Luo *et al.* [27] designed a multi-chamber microfluidic device for fast yeast cell loading, culturing and phenotypic research. The device was fabricated from the PDMS to get benefit from its high gas solubility feature. Cell loading procedure can be done in 1 min via degassing induced aspiration utilization of PDMS. Figure 2.8 shows that, the device had an array of triangular cavities to trap the yeast cells and these cavities were connected to the main channel. Before cell loading, device was degassed and with the help of gas absorption property of PDMS yeast cells in buffer solution were fed to the device. Since flow velocity

inside the triangular cavities was almost zero, medium exchange can be performed without any cell loss. Yeast cell growth was carried out in one layer, so monitoring of cell number can be done easily, phenotypic changes with cell doubling time can be observed. The level of enhanced Green Fluorescent Protein (EGFP) expression of the cells were monitored at seven different Cu^{2+} concentrations [27].

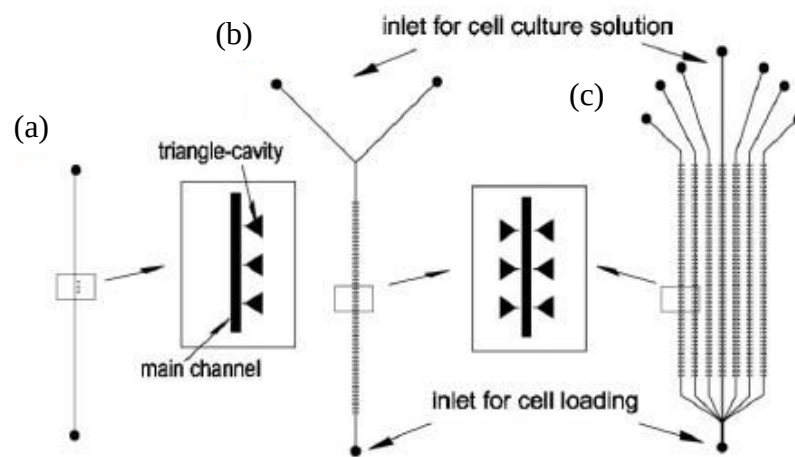


Figure 2.8. Chip design (a) main channel and triangle cavities at one side (b) cell culture solution and cell loading inlets at the top and at the bottom respectively (c) seven parallel channels and triangle cavities at two side of the main channel [27].

A microfluidic imaging platform, developed by Taylor *et al.* [28], was used to conduct single yeast cell experiments. Device was made of polydimethylsiloxane (PDMS) and fabricated by using multilayer soft lithography method with three layer design. Cells and the chemical channels form the “flow layer” which was on top of the device, channels for pneumatic valves form “control layer” which was in the middle of the device and the bottom or “blank layer” was for the sealing the device. Their platform allowed 256 simultaneous time-lapse live-cell imaging analysis in a 32 stimulant-concentration sequences with programmable on-chip mixing and perfusion in one device. Mating pheromone response in yeast cells were examined with eight dissimilar genotypes in dynamic environmental situations along the matrix columns and rows (Figure 2.9). With the help of the row-valves, every different yeast strain was loaded through the unique column. Cells were trapped at the vertex of the matrix in a perfusion chamber and during the media exchange in order to prevent cell loss, cells were immobilized with the help of partially closed valves. The

perfusion chamber of the device had 3.5 μm height to keep the cells in monolayer, so cells can be examined during long-term imaging. Cell number, cell size, cell morphology and green fluorescent protein (GFP) concentration of the cells were monitored by image analysis pipeline and complete analysis of wild type and 11 deletion mutants are done [28].

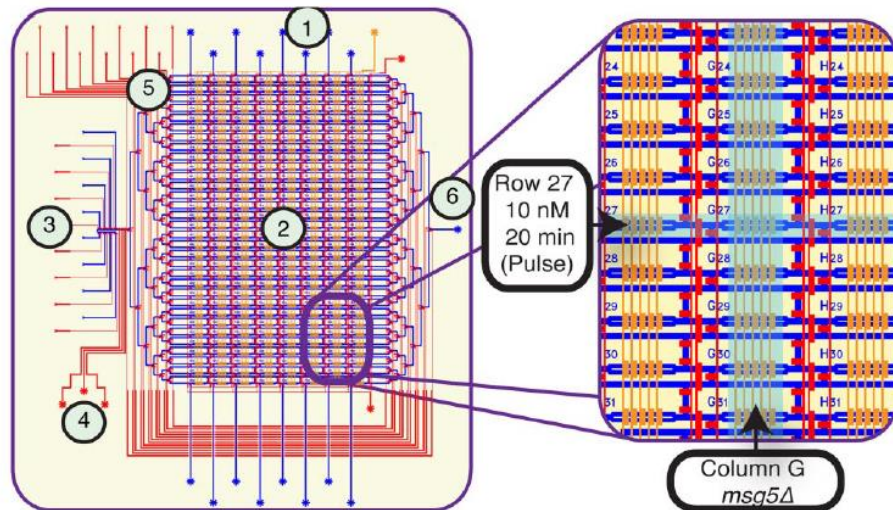


Figure 2.9. Microfluidic imaging platform schematic, blue lines show flow structure and red lines show control structure (1) inlet for cell loading (2) columns and rows of the device (3) inlet for chemicals and control (4) peristaltic pump (5) multiplexer (6) outlet [28].

In 2010 Taylor's group improved their system by high-throughput tracking of single yeast cells in a microfluidic imaging matrix. They have overcome some limitations encountered in their previous study. They have again used PDMS to manufacture the microfluidic device by multilayer soft lithography technique. Device comprises a top layer, which includes channels for pneumatic valving and a bottom layer, which includes flow channels and chambers, thus these layers allow 128 independent experiments. By mechanical clamping and polymerization in agarose gel, single cell tracking and immobilization of yeast cells can be achieved in one device. Pheromone induced mitogen-activated protein kinase (MAPK) signaling network in *Saccharomyces cerevisiae* was studied through the device with 8 genotypes under 16 conditions. Figure 2.10a and 2.10b describe the chip design:

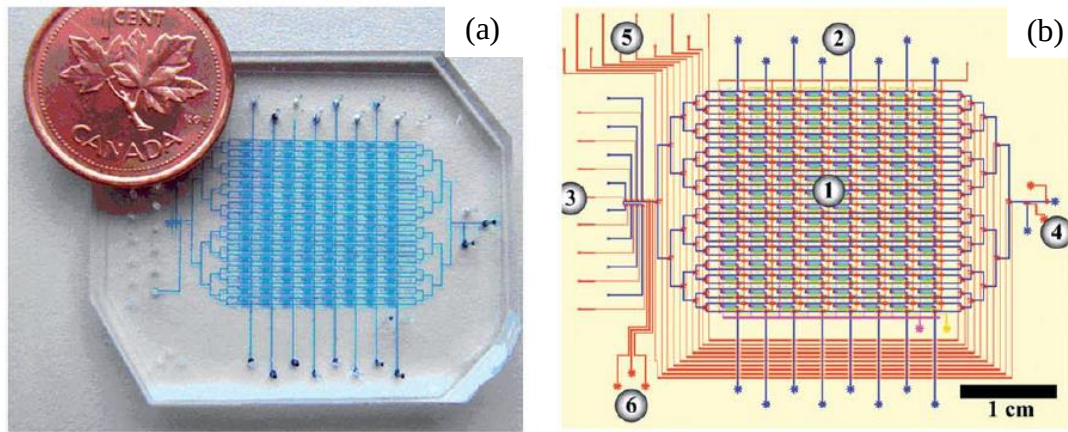


Figure 2.10. Chip design (a) microfluidic chip (b) numbered parts of the device: (1) 8 columns and 16 rows for chambers (2) cell loading inlets (3) inlets for chemicals (4) outlets (5) multiplexer for reagent delivering (6) peristaltic pump [29].

Through the device, the application of mechanical clamping with in situ polymerization provides long-term tracking and immobilization of single cells. With the help of diffusion and column and row valves, the cell loading, medium flushing and perfusion of the cells were accomplished successfully. Cells were loaded as a monolayer in $684 \times 260 \times 4.4 \mu\text{m}^3$ chambers which contains 6000 cells. One wild type and seven deletion mutants in 16 various α -factor concentrations were monitored for 24h experiment. All recorded images were processed with a custom image analysis pipeline in MATLAB. Cell tracking and segmentation, total fluorescence amount, cell statistics calculations were done via this program, and the yeast cell pheromone response was analyzed [29].

In “Microfluidic single-cell cultivation chip with controllable immobilization and selective release of yeast cells” study of Zhu *et al.* [30], selective cell trapping and releasing and cultivation experiments were performed. With the application of hybrid glass-SU-8-PDMS technique, microfluidic device at high optical transparency was fabricated. Mild suction and negative dielectrophoretic (nDEP) force were employed through the device to trap or release the cells, respectively. Once the cell was trapped in immobilization orifices, cultivation of cell is handled there. By using COMSOL Multiphysics 3.5a, computational fluid dynamics (CFD) simulations were done to reveal the necessary parameters for trapping and releasing. In addition to simulations, polystyrene beads and yeast cells were practiced to examine the characteristics of the device.

Figure 2.11 describes the device which has two suction channels of 300 μm width, a culturing channel of 150 μm width, orifices of 5 μm width and all channels have 30 μm height. Cells were loaded via the syringe pump and negative pressure was implemented through the orifices to immobilize the cells. In order to do the cultivation of single cells, side channels of various sizes were arranged on the main perfusion channel and microelectrodes were placed in each section to create the nDEP force.

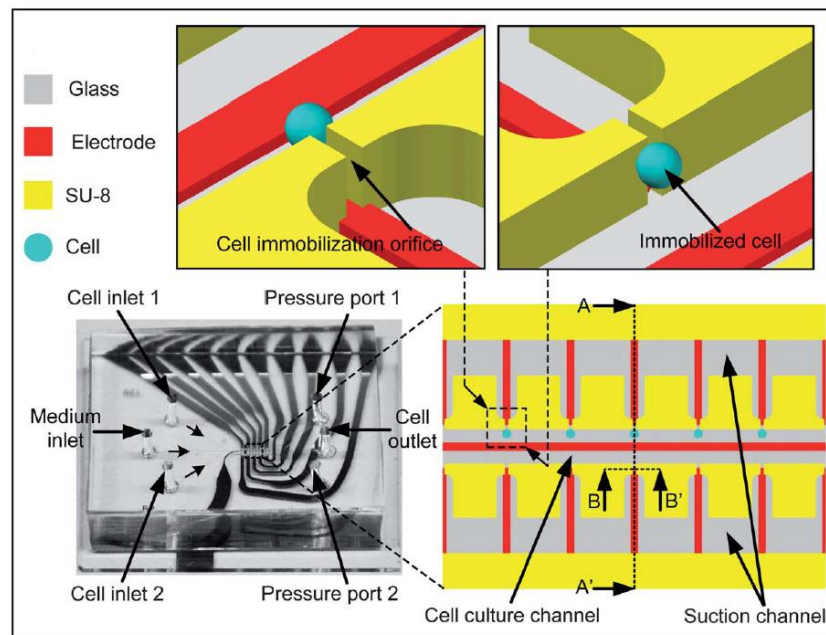


Figure 2.11. Microfluidic device of Zhu *et al.* [30].

With the help of the hydrodynamic and nDEP forces, budding yeast cells of interest (*Saccharomyces Cerevisiae*) were monitored during 70 min long experiments and compatibility of the device was proved [30].

Hierlemann's group [31] developed another microfluidic device, which can perform both aerobic and anaerobic yeast cell cultivation for more than 3 days. Cell-cycle timing, stress-pathway activation and the protein aggregation events can be observed under several metabolic conditions by using image analysis. The microfluidic device ensures easy cell loading and subsequent operations. High-resolution time-lapse images are taken via programmed microscope when the yeast cells were placed in a horizontal plane.

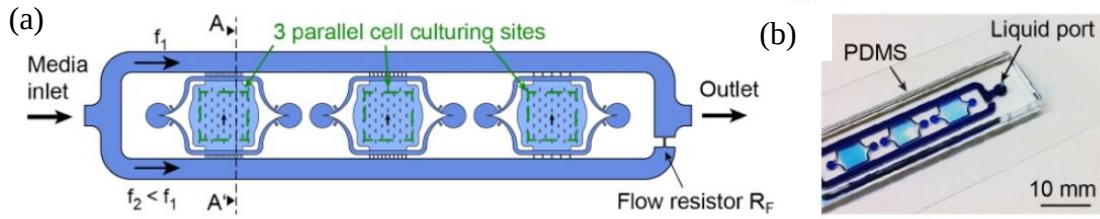


Figure 2.12. Chip design (a) top view of the microfluidic device schema (b) general view of the device [31].

Figure 2.12. A shows the top view of the device. Three distinct yeast strains can be grown in three different chambers without any intervention between them. A channel, required for perfusion and nutrient supply, was located around the chambers. PDMS was used as a source material to produce the device and standard photolithography was applied. O_2 plasma was employed to activate the PDMS surface and glass was used to cover the device. According to the flow rate and cell clamping force, the height of the channels were customized. Fluorescence and bright-field images of the yeast cells in the device were taken in the 5 to 10 min intervals. Through the experiment, several metabolic conditions were applied and replicative aging of the yeast cells was studied [31].

In the study of Lee *et al.* [32], continuous and high-resolution imaging of single yeast cell was accomplished in a microfluidic dissection platform. The microfluidic device consists of a single focal plane and an array of soft PDMS micropads. Since device was designed as a dissection platform, only mother cells were collected in micropads to observe the aging alterations in cells during the whole lifespan, and daughter cells were removed throughout the experiments. Figure 2.13a depicts the general working mechanism of the microfluidic device and 2.13b shows array of micropads which have $30\ \mu\text{m} \times 15\ \mu\text{m}$ dimensions. There was 4 to 5 μm height between the micropads and glass cover slide, hence trapping of mother cells was ensured. PDMS was used to produce the device and microstructures were created by utilizing photolithography. The platform permits the observation of aging mechanism of a single cell from young to death. Since PDMS is soft elastomer, fabrication of the device was made of this material and yeast mother cells were gathered easily under the PDMS micropads. Daughter yeast cells were removed from the device by applying continuous medium flow. Time-lapse images were taken in 10 min

intervals and replicative and chronological aging of a single cell were studied using this microfluidic dissection platform [32].

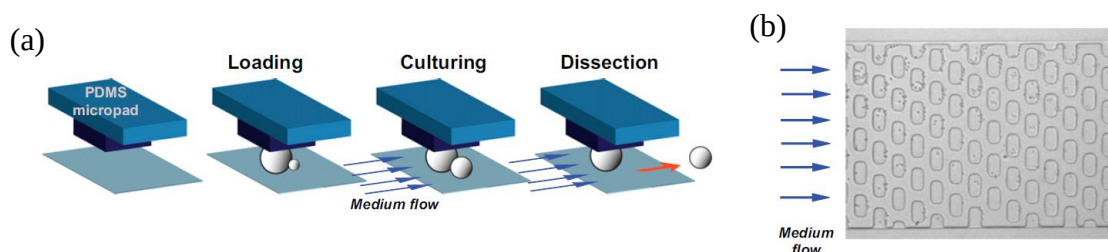


Figure 2.13. Chip design (a) the illustration of loading, culturing and dissection processes in the microfluidic device (b) micropad arrays [32].

There are several studies in the literature about yeast cells, but molecular causes of aging and death was ambiguous until the study of Xie and his colleagues [33]. They developed mother cell tracking system in a microfluidic device to reveal the cell cycle dynamics, cell/organelle morphologies and several fluorescently labeled proteins through the lifespan of a single yeast cell by using time-lapsed microscopy. Microfluidic device utilized in this study was competent to keep mother cells in the chambers while removing daughter cells by applying two general rules: (i) most mother cells are bigger than the daughter cells in size and (ii) daughter cells have newer cell wall than mother cells. Figure 2.14 shows the microfluidic device's channels and chambers where mother cells were confined in the direction perpendicular to the flow plane. Device height was adapted to the size of mother cells but flow speed may flush away mother cells with daughter cells that cause clogging inside the chip during experiments. Therefore, in order to prevent flushing away and clogging, surface treatment of the both mother cells and glass slide of the device was employed. Sulfo-NHS-LC-biotin and biotinylated-BSA followed by neutravidin treatment were used for surface modification of mother cells and glass slide respectively. Figure 2.14b describes the adherence mechanism of mother cells to the glass surface due to creation of biotin-avidin complex. Since daughter cells have newly synthesized cell walls, they cannot hold on to the glass surface. In the main channel and chambers, flow velocity was $300\mu\text{m/s}$ and 0 to $30\mu\text{m/s}$, respectively, that were sufficient to keep mother cells inside the chambers. The microfluidic device was produced by using PDMS which was poured on the mold, and then it was cured. Subsequently, access holes were opened and the chip was

bonded to the cleaned glass slide after plasma surface activation process. There were 20 chambers with $4 \times 100 \times 200 \mu\text{m}$ (depth $\times$ width $\times$ length) dimensions and fluid pumping was done through the main channel where pressure difference was created between the two sides of the chamber. The chip was operated up to 3 days under time-lapsed microscopy in order to observe the life span of mother cells and data were taken at 10 min intervals. Cellular and molecular phenotypes of the cells, lifespan of cells and the stresses that cause aging were investigated with this system [33].

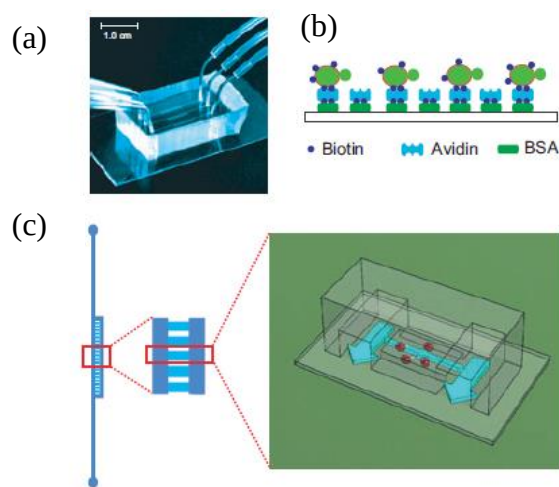


Figure 2.14. Chip design (a) general view of the chip (b) mother cell and glass (at the bottom of the chip) surface modification (c) channels and chambers inside the chip, arrows show the flow directions and yeast cells (red) are confined in the chamber [33].

Another study on yeast application in microfluidic device is: “Single Cell Analysis of Yeast Replicative Aging Using a New Generation Microfluidic Device”. In this study of Zhang *et al.* [34], removal of daughter cells in the device was handled again. The chip includes microfluidic channels with pensile columns that confine mother cells and flush away daughter cells with the introduction of the fresh media. Figure 2.15 shows the design of the device. It consists of 4 parallel modules, which can expand to 10 modules, operating independently that permits concurrent experiments with different strains. One main channel and two side channels with 100 pensile columns exist in each module and there was a bridge between main channel and side channels to prevent the blocking of air bubbles. In one experiment, 10 distinctive strains can be analyzed and 100 mother cells can be monitored. It would take a lot of time with the traditional methods to perform these experiments but with

this microfluidic platform this limitation has been overcome. In addition, the device provides consistent temperature and nutrient supply preventing variations from experiment to experiment.

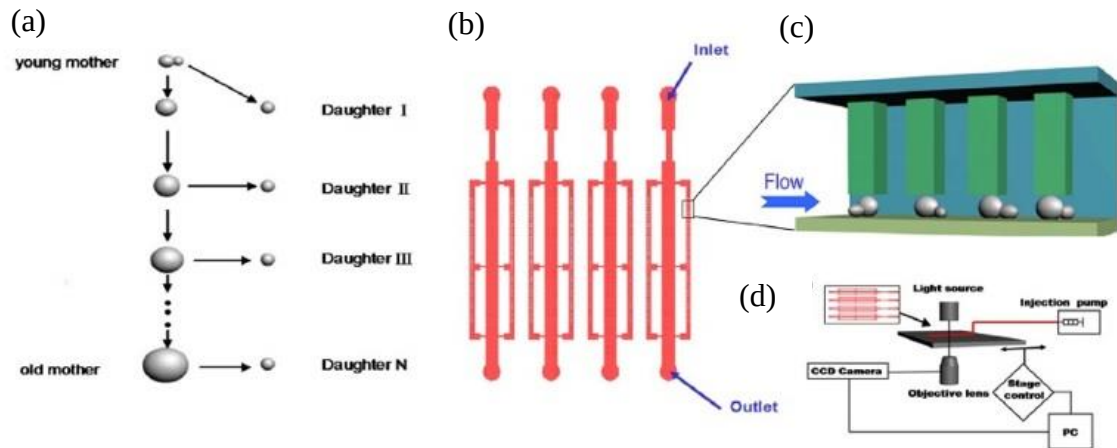


Figure 2.15. The chip (a) schematics of the yeast cell replicative aging (b) microfluidic device design (c) mother cell trapping between the pensile column and glass slide (d) experimental setup [34].

The required mold, for the production of the chip, was fabricated by using standard multilayer photolithography process. When the mold was ready, PDMS is poured on the mold and it was left for curing at 70°C. Inlet and outlet ports were opened and after surface treatment with plasma, chip was bonded to the glass slide. The reason for using PDMS was to get benefit from the PDMS's elastomeric property. During cell loading, yeast cells were injected with high velocity to swell the gap between the pensile columns and glass surface, therefore cells were loaded into this gap. After a while, flow velocity was lowered, pensile columns take their original shape and 1 or 2 cells were confined under these columns as shown in Figure 2.15c. Fluorescence and brightfield images were taken and single cell gene expression analysis was done. This system enabled to reevaluate the known long and short-lived mutants' lifespan and the change in translation elongation factor TEF2 expression was discovered with the help of fluorescently tagged proteins in the mother cells. Moreover, mother-daughter inheritance and age dependent asymmetric partitioning of a general stress response reporter between mother and daughter cells were investigated via trapped mother cells' daughters [34].

Park *et al.* [35] designed a two-depth culture chamber in their microchemostat array. The system was capable of the replenishment of culture medium 1% per replenishment cycle in a 250nl volume. There was an array of 8 microchemostats on a $40 \times 60 \text{ mm}^2$ footprint that were operated automatically by a controller unit. During the experiment, different yeast cell densities were monitored at different dilution rates and that approves the chemostatic functionality of the system. In addition, budding index quantification, correlated with the dilution rate, resulted in growth-dependent cell division and this also confirms the chemostatic functionality of the device.

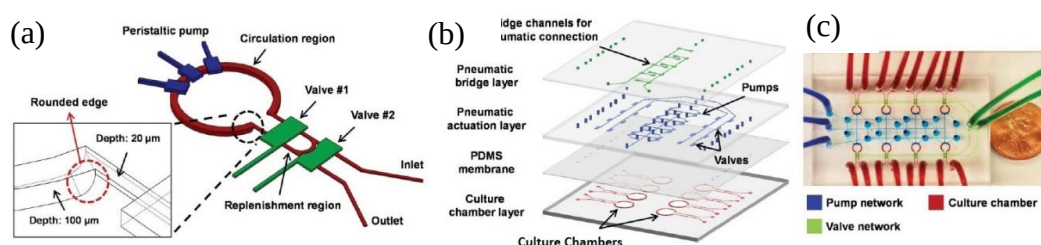


Figure 2.16. Chip design (a) the single small-volume fraction replenishment microchemostat unit composed of a two-depth culture chambers, pneumatically actuated peristaltic pumps and two valves (b) 4 PDMS layers consists of 8-unit microchemostat array (c) colored general view of the 8 microchemostats on a $40 \times 60 \text{ mm}^2$ footprint [35].

This microbioreactor was divided into small sections, fresh culture medium replenishment was done in these sections and the sections were combined again to turn into microbioreactor during the operation of the device. The device includes loop-shaped culture chamber having two regions, deep and shallow regions with $100 \mu\text{m}$ and $20 \mu\text{m}$, respectively, and the replenishment cycle was done via these two regions as shown in Figure 2.16a. A loop-shaped cell culture chamber, pneumatically actuated peristaltic pumps and two pneumatic valves were the parts of this microfluidic device. Cell circulation inside the chamber was done via peristaltic pumps, and the isolation of replenishment region during the culture medium replenishment cycle was done via two valves. Cell growth occurs inside the main loop region which has $100 \mu\text{m}$ depth and culture medium was supplied through these region via peristaltic pumps. Valve 1 and 2 were used to disconnect the replenishment region from the main loop and inlet/outlet ports, respectively and these valves regulated the replenishment volume of the device. As seen in Figure 2.16b and 2.16c, the microbioreactors

can be operated simultaneously by a single controller and the device can work under 8 different biomolecular conditions and run experiments in 8 microchemostats in parallel. Moreover, the system was the combination of four layers of PDMS which were a culture chamber layer, a membrane layer, a pneumatic actuation layer and a pneumatic bridge layer from bottom to top. With the application of conventional photolithography processes, PDMS replication master molds were fabricated. Next, fabricated layers were surface treated with oxygen plasma and they were arranged under microscope. After fabrication of the device, cells were fed to the system via the inlet port and during loading, valve 1 and 2 were kept open and peristaltic pump was turned off. When the cell loading was completed, valve 1 is closed and inlet/outlet channels and the replenishment region were flushed with culture medium to remove the remaining cells. Subsequent to the discharging of the cells, valve 1 was opened and valve 2 was closed to start the growth cycle and cell circulation inside the chambers with the help of peristaltic pump. Valve 1 was closed to separate the shallow replenishment region from circulation region during the replenishment cycle and the medium of separated replenishment region was changed with fresh medium. Finally, valve 1 was opened and valve 2 was closed to rejoin the replenishment and circulation regions. In this study, fully automated microchemostat system was employed to investigate the yeast cell density under different dilution rates with low cost and high throughput [35].

The study of Denervaud *et al.* [36] reported a parallel microchemostat array system that was used to investigate single yeast cell dynamics. GFP-tagged yeast strains were examined on the single cell level with 20min time resolution. In 4085 GFP-tagged strains, protein abundance and localization in response to methyl methanesulfonate were measured, and 576 GFP-tagged strains were further analyzed under five additional conditions. Eventually, more than 10000 unique experiments were handled to get information about the yeast proteome. Protein abundance, localization, cell size and growth of single yeast cells were determined via this microfluidic system. Figure 2.17 describes the microfluidic system which had 1152 microchemostats in one single device. The device was modeled by using Clewin software. Since the chip consists of a flow and control layer, two different mold were produced and standard photolithography processes were applied to fabricate these molds. In the flow layer, flow channels, $260 \times 300\mu\text{m}$ small microchemostats and shallow sieve channels were established with the heights of $15\mu\text{m}$, $5\mu\text{m}$ and $1.7\mu\text{m}$ respectively. In the control layer which had $12\mu\text{m}$ height, channels, chamber button and a chamber outlet valve

existed. Channels were used for creating microvalves, the chamber button was employed for generating pressure difference across the chamber to force cells to grow in a monolayer and chamber outlet valve was utilized for avoiding the contamination during the experiment [36]. With the help of DNA array spotter, yeast strains were arrayed on a microscope coverslip as shown in Figure 2.17c. Next, microfluidic device was aligned on the cell array and device priming was started with the medium supply. There was no fluid flow inside the growth chambers and nutrient supply was done via diffusion, therefore cross contamination of microchemostats was prevented. After a while, yeast cells began to proliferate and filled the accessible area of the microchemostat. When the microchemostat was full of yeast cells, cells were sent out at the top of the microchemostat and flushed away by the medium stream. Cells cannot go out from the bottom of the chemostat because there were shallow sieve channels which were used for diffusion of nutrients and chemical compounds from the flow channels to the microchemostats. Cells cannot go out from the bottom of the chemostat because there were shallow sieve channels which were used for diffusion of nutrients and chemical compounds from the flow channels to the microchemostats.

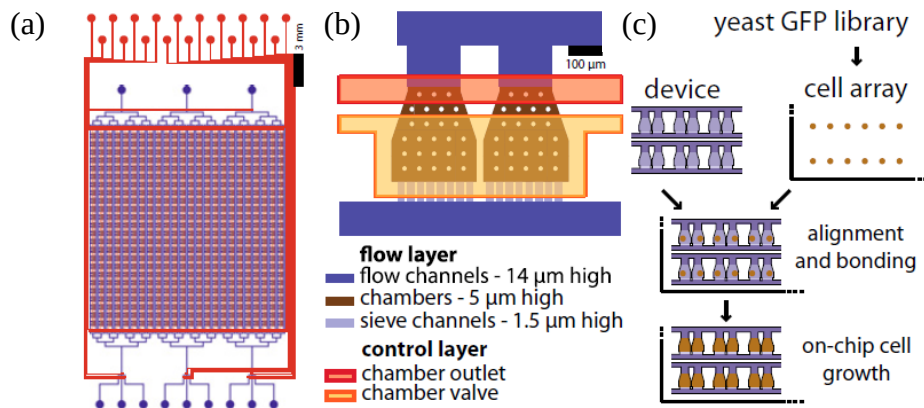


Figure 2.17. 1152 microchemostat in a single device (a) microchemostat array design (flow and control layer are shown in blue and red respectively) (b) a closer look to a pair of microchemostats (c) chip programming steps [36].

In order to obtain high-quality images, yeast cells were enforced to growth in 5 μm height microchemostats to arrange the cells as a monolayer. The device was located on an automated fluorescence microscope and phase contrast and fluorescence images are taken in 20 min time intervals. After the experiment, chamber and cell segmentation, background correction, signal deconvolution and protein localization analysis were done via custom written image analysis software [36].

The microfluidic device shown in Figure 2.18 was designed by Goksor's group [37] for managing high-throughput single-cell experiments [37]. Soft lithography methods were employed to fabricate the chip in PDMS and pure hydrodynamic forces dominated the device for cell trapping. V-shaped pockets were placed in the cell-trapping region and pockets can entertain at most 6 yeast cells with a diameter of $4\mu\text{m}$. Arsenite (As(III)) uptake of yeast cells was followed at different flow rates using GFP-tagged heat shock protein Hsp104.

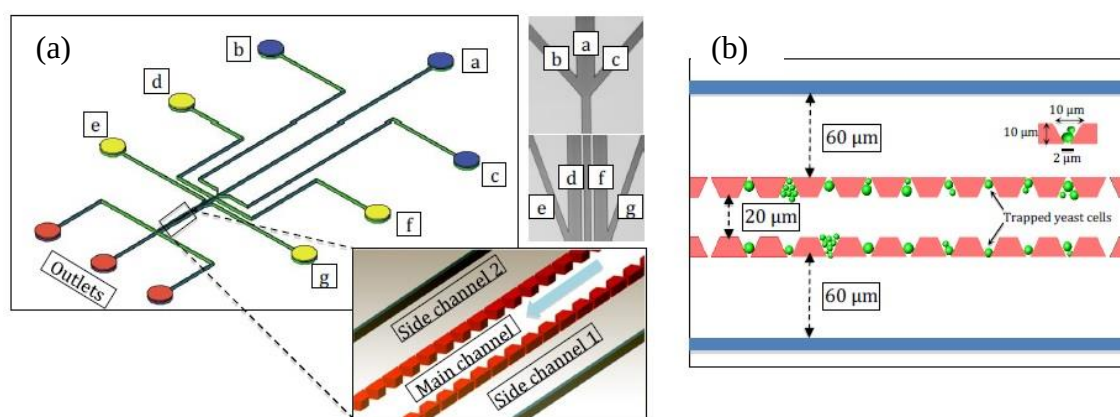


Figure 2.18. Chip design (a) schematic illustration of the microfluidic device with inlet and outlet ports. Yeast cells and stress solutions were introduced in to the chip via inlets “a”, “b” and “c” and they were connected to the main channel. Hydrodynamic cell loading and reagent injection through the side channels were done via inlets “d” and “f”. Sheath flow inlets were “e” and “g” (b) top view [37].

The device was designed to trap and keep track of yeast cells exposed to chemical stress with the help of hydrodynamic forces. Fluorescence microscopy was employed to monitor the response of the cells under imposed perturbations. Figure 2.18a describes the general view of the chip with cell and reagent inlets and outlets. There were two side channels and one main channel of $20\mu\text{m}$ width inside the device and cell trapping region was located in the main channel. The objective of the inlets “b” and “c” were cell loading and inlet “a” was utilized to feed the stress solution through the device. Inlets “d” and “f” were the main side inlets and they were employed to load the cells or introduce the stimuli. Sheath flow inlets were “e” and “g” and additional reagents can be fed through these inlets. V-shaped pockets of $10 \times 10\mu\text{m}$ dimensions were present between main channel and side channels of $60\mu\text{m}$ width, as shown in Figure 2.18b. The chip height was $4.8\mu\text{m}$ for placing

the cells in monolayer and V-shaped pockets can accommodate at most 6 cells of 4 μ m diameter approximately. The device was modeled by using AutoCAD program and with the application of photolithography processes, the chip was produced from PDMS. Produced chips were separated from their masters, access holes through the device were opened by punching and PDMS replicas were bonded to coverslips by oxygen plasma treatment. In order to analyze the fluid flow behavior in each cell trap, COMSOL Multiphysics program was used by applying incompressible Navier-Stokes equations and No-slip boundary conditions at channel walls. The chip was located on an inverted epifluorescence microscope stage and cell responses under chemical exposure were recorded. During the experiments, brightfield and fluorescence images were taken with 100 $\times$ oil immersion objective. The chip was segmented into 13 pieces and images were taken in 30s intervals between two neighboring segments. After the experiment, images were processed in an open source image analysis custom software [37].

In their study, Crane *et al.* [38] introduced ALCATRAS (A Long-term Culturing And TRApping System), a microfluidic device which hosts budding yeast cells. Up to 1000 cells can be observed inside the device in a stable and precisely determined environment. During the 60 hour long experiment, daughter cells were flushed away by fluid flow and extracellular media can be changed regularly in seconds. Replicative life span, cell cycle dynamics and history-dependent behavior in the response to stress were studied. The benefits of this system were the simplicity of device fabrication, environmental switching, high scalability and daughter cell discharging. Hydrodynamic pressure was utilized in the trapping operation and more than 90% of cells were trapped by continuous flow during the cell loading. Culture media can be switched in a short time and cell response to the media change can be monitored. In addition, cells underwent their cycle times normally without sign of stress. General view of the device is shown in the Figure 2.19. There were three inlet ports on the device; the middle one was used for cell loading and the other ones were used for medium supply from syringes to the device. Relative flow rates were changed during the experiment to refresh the medium in the flow chambers. More than 1500 cell traps are located in the chamber and the cell traps were formed by two PDMS pillars. Media supply was done from the top of the pillars and pillars have larger width at the trap entrance as shown in Figure 2.19e.

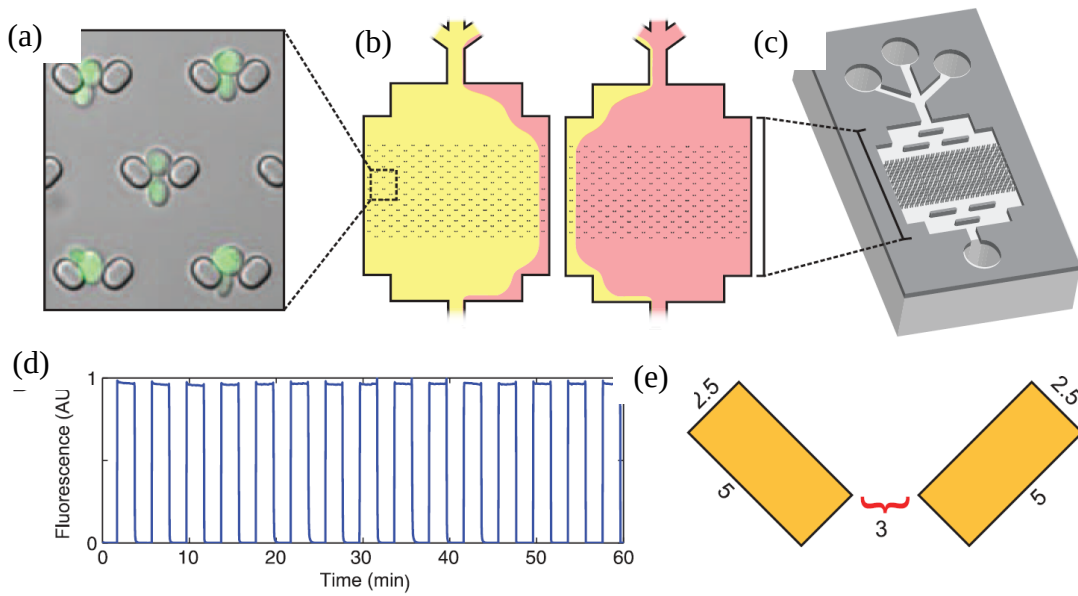


Figure 2.19 (a) Pillars for cell trapping (b) media switching flow in the chamber (c) general view of the device. Inlets at the top, cell trapping area in the middle and outlet at the bottom of the device (d) media switching rate vs time graph according to the fluorescein in the media (e) the pillar shape and dimensions [38].

This device was designed to keep the mother cells in the pillars while removing daughter cells from the trapping area. When the cell was trapped, hydrodynamic forces start operation and the pressure difference was formed by directing the fluid flow from the top to the bottom of the pillar. Therefore, mother cells were prevented to escape from the trapping zone. In order to flush away the daughter cells from the device, two options were used relying on the polarity of the trapped mother cells. First option was to push the bud to the side and remove it with the help of the directed flow when the bud was at the top of the trap. Second option to remove the daughter cell was to separate the mother cell quickly from the bud during the step of the cytokinesis, when the bud was formed at the downstream of the mother cell. During the experiments, lifespan was determined in terms of the reduction in cell viability as a function of replicative aging. In addition, heat-shock protein Hsp 104p-GFP level was also reported. The device fabrication was accomplished by employing conventional soft lithography techniques and AutoCAD software program was used to design the chip. When the design was finalized, it was printed on a chrome-on glass mask and a mold was produced. The chip was made of PDMS and bonded to coverslips after oxygen plasma treatment. The device was placed on the inverted microscope and there was

an incubation chamber for providing constant temperature of 30°C. Images for approximately 1000 cells were taken in 10 min intervals for a 50 hour long experiment. After image acquisition, cell segmentation, cell tracking and data extraction were performed by using MATLAB [38].

2.3. Microfluidic Device Fabrication Methods

Microfluidic systems have various potential applications. Inkjet printers, blood-cell separation equipment, biochemical assays, chemical synthesis, genetic analysis, and drug screening are some examples of systems and processes that are operated via microfluidic platforms. However, fabrication of these microfluidic devices is as important as processed systems. Device construction and dimension, smoothness and bonding are significant issues that should be handled during fabrication. In this section, wet and dry etching, computer numerical control (CNC) machining, photolithography, electrical discharge machining (EDM), injection molding, laser ablation, soft lithography, wire imprinting, hot embossing and 3D printing will be introduced in chronological order.

2.3.1. Wet and Dry Etching

2.3.1.1. Wet Etching. Wet etching, which is also known as chemical etching or liquid etching, is a process to remove undesirable substance from the surface. Anisotropic wet etching and isotropic wet etching are the types of wet etching processes and their difference is shown in Figure 2.20. Liquid chemicals or etchants are used to perform wet etching on a wafer. Masks are necessary to characterize the determined patterns on the wafer. Before the fabrication, masks are transferred onto the wafer by using lithography. During the etching process, by using liquid chemicals, materials covered by masks are protected and other parts are etched away.

Several chemical reactions occur in wet etching processes to produce the desired device. Generally three fundamental steps are followed in each etching operation:

- (i) Liquid chemical diffuses through the structure that must be removed.
- (ii) Liquid chemical and material, which will be etched away, react via reduction oxidation (redox) reactions.

- (iii) Byproduct diffuses from the surface [39].

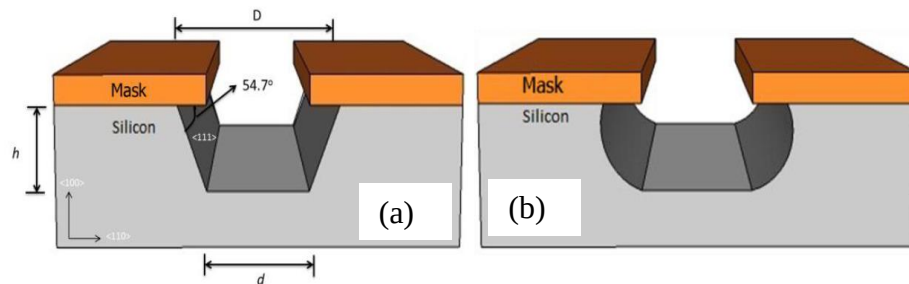


Figure 2.20. The difference between the (a) anisotropic and (b) isotropic wet etching [39].

2.3.1.2. Dry Etching. Dry etching is also known as gas etching. In this process, substrate material is etched away by using plasmas or etchant gases. Applying high kinetic energy of particle beams or chemical reaction or both of them, the reaction occurs. In physical dry etching (Figure 2.21); ions, electrons or photons are employed to remove the substrate atoms via high kinetic energy. During physical etching, particles collide the substrate surface and disappear after the collision. Only physical reaction occurs in this type of dry etching.

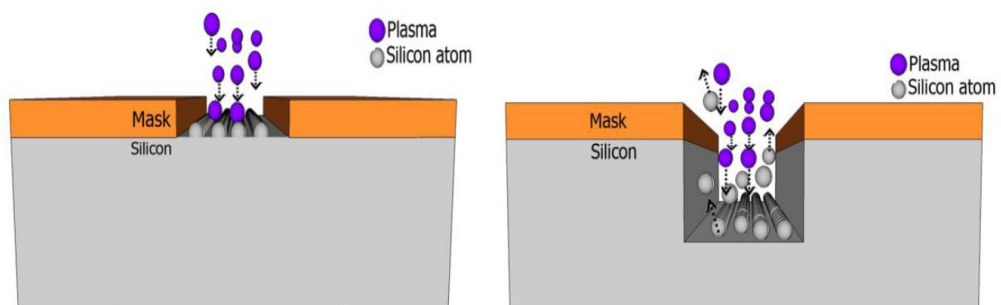


Figure 2.21. Physical dry etching process [40].

Another type of dry etching is chemical dry etching also called vapor phase etching, as shown in Figure 2.22. Liquid chemicals or etchants are not used in chemical dry etching. Surface of the device is knocked out via chemical reaction, which takes place between the etchant gases and the surface. CH_4 , SF_6 , NF_3 , Cl_2 or F_2 are used in chemical dry etching processes.

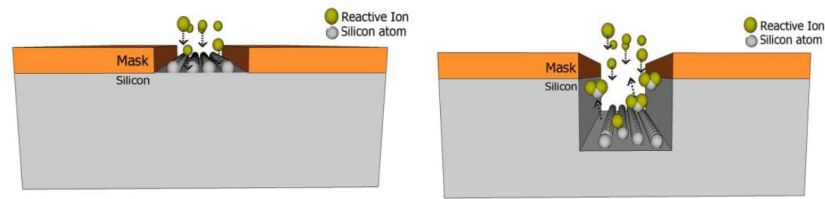


Figure 2.22. Chemical dry etching process [40].

Reactive ion etching is another type of dry etching method which is shown in Figure 2.23. In this process, physical and chemical treatments are combined with each other to attain high level of resolution. In industry and research, reactive ion etching is the commonly used process. Reactive ion etching process responses faster than other types since both physical and chemical treatments are utilized. CF_4 , SF_6 and $\text{BCl}_2 + \text{Cl}_2$ are widely employed reactive ion etching gases [40].

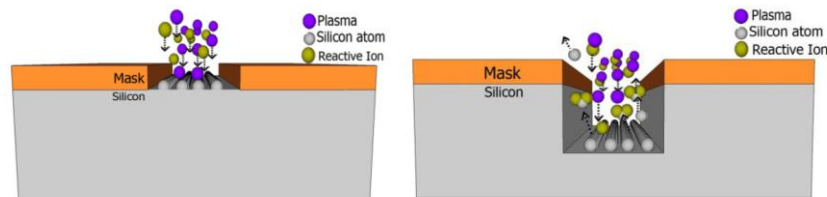


Figure 2.23. Reactive ion etching process [40].

2.3.2. Computer Numerical Control (CNC) Machining

Computer numerical control (CNC) is a program, which is installed in a machine that produce the device by using numerical data. In order to produce the desired device, required numerical data is converted into proper electrical signals. Then, these electrical signals are used as input of the machine that manufacture the device. The program, program input device, machine control unit, drive system, machine itself and the feedback system are the six components that CNC systems include [41]. CNC is a direct-write micromachining method that does not need masks to produce the device, and fabricated microchannels have less surface roughness than those manufactured by other methods such as hot embossing, imprinting or injection molding. CNC machining systems can provide quick responses to

produce the devices due to their high accessibility and rapid prototyping capability. CNC machining technique can be employed for production of microdevices that have dimensions larger than 50 microns [42].

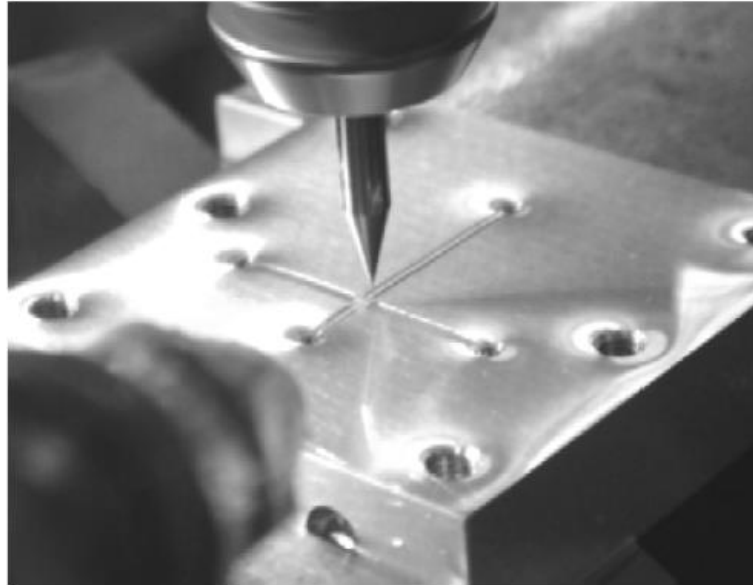


Figure 2.24. Computer numerical control machining [42].

2.3.3. Photolithography

Photolithography is also known as optical printing process. Here, again the aim is to constitute and transfer desired patterns onto the substrate properly. Photolithography fabrication steps start with photoresist coating. Photoresist is a light-sensitive material used in several industrial processes. It is composed of three elements, which are a base resin (polymer), a photoactive compound (PAC) and a solvent. A base resin is needed to provide structural characteristics of the resist and photoactive compound is necessary for light curing applications.

Two types of photoresist are available in the industry. One of them is negative photoresist that turns into less soluble material when the light is applied. The other one is positive photoresist that turns into more soluble material when the light is implemented.

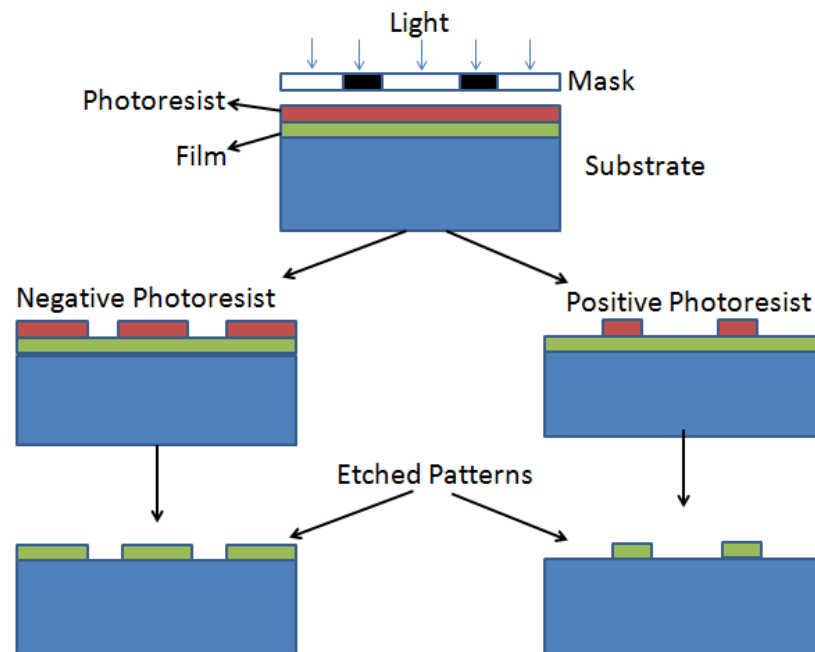


Figure 2.25. Photolithography procedure [43].

By using mask, which is a transparent material with opaque regions on it, is used to conduct the photolithography procedure. Light cured photoresist layer is subjected to developer which is a chemical material. The purpose of the developer is making treatments on light cured and uncured regions at different rates. When the negative photoresist is utilized, light uncured regions are treated by developer and when the positive photoresist is utilized light cured regions are treated. In addition to production steps, clean environment is very important to implement the fabrication [44].

2.3.4. Electrical Discharge Machining

Electrical discharge machining (EDM) method was first utilized by Lazarenko and Lazarenko in 1946. In this method melting and evaporation are important steps to fabricate the device. Two distinctively charged conductive surfaces are employed to generate sudden discharge via spark. With this spark, substances are melted and evaporated by the created localized heat regions. In addition to fabrication via conductive materials, one electrode as a conductive material and the device to be produced as a nonconductive material can also be machined via EDM technique. In order to have stable dielectric environment during fabrication, production is done within the dielectric fluid. Sinker and wire EDM are the two

types of the EDM process. Sinker EDM is a two-step fabrication method since complementary shape of the tool is produced. In wire EDM, structure of the device is machined by removing the bulk material. Eventually, EDM technique is applicable to fabricate difficult materials [45].

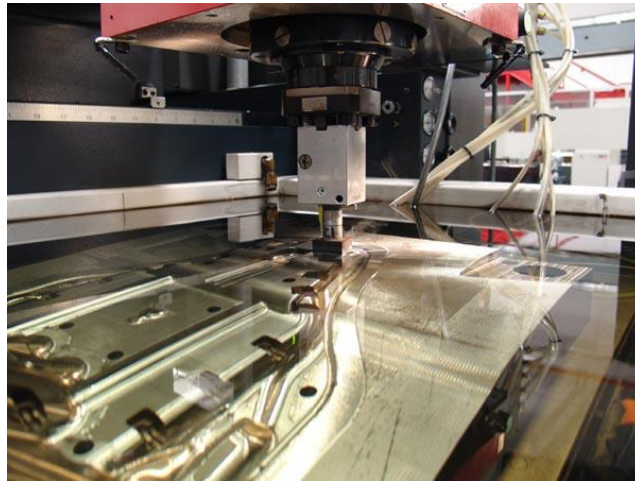


Figure 2.26. Electrical discharge machining [46].

2.3.5. Injection molding

Injection molding is a widely used fabrication method in industry to produce different kinds of objects. In this process, firstly; prepolymerized pellets such as polycarbonate (PC) or PMMA are melted. After melting, substances are fed to the heated mold cavities via injection by applying high pressure. Substances are cooled down under the glass transition temperature (T_g) and they take the shape of the mold. After cooling, desired objects are separated from the mold. Melting ability of the plastics provide increasing contact area with the mold to have definite replications. Operating temperature, pressure and residence time must be optimized to obtain precisely fabricated devices. Figure 2.27 depicts the injection molding process.

Because of complexity of the molding instruments and master productions, the application of injection molding to microfluidic devices are generally done in industry rather than research laboratories. Lastly, production via injection molding provides higher

throughput because most devices can be produced in a few seconds and this method allows large-volume production of objects [47].

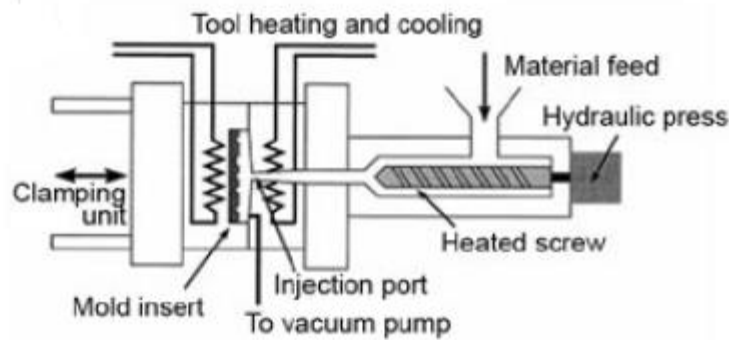


Figure 2.27. Injection molding process [47].

2.3.6. Laser Ablation

In laser ablation method, device to be produced absorbs the pulsed laser light, and chemical bonds in the polymer chain break via electronic transitions. C_2H_2 , CO_2 or CO are dissociation products that are produced during electronic transitions, and must be removed. Channel dimensions are specified by the pulse energy applied through the substance. Polystyrene, nitrocellulose, poly (methyl methacrylate) (PMMA), poly (ethyleneeterephyhalate) and poly (tetra-fluoroethylene) are the polymers that can be processed by laser ablation method. Figure 2.28 describes the laser ablation method:

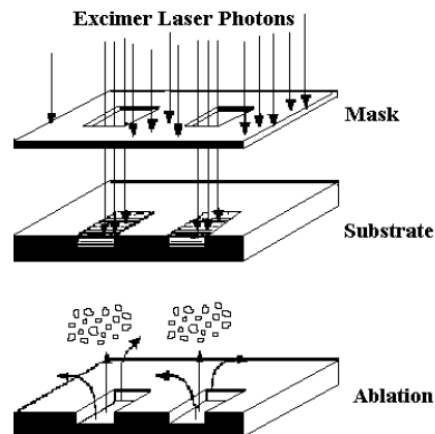


Figure 2.28. Laser ablation process [48].

Serial (direct) write and scanning mode are the two fabrication ways of laser ablation. In serial (direct) write mode, device to be produced or mask is fabricated under the write beam. The beam width and the laser repetition rate determine the ablation region, and production time is characterized by the needed channel dimensions. In the scanning mode, mask movement and substrate operate simultaneously. Patterning time is specified by the number of laser shots and laser shots determine the channel dimensions [48].

2.3.7. Soft-lithography

Non-photolithographic techniques are gathered under a topic called soft-lithography which employs organic flexible materials for transferring patterns to the substrate material by using an elastomeric stamp or mold. Soft-lithography covers microcontact printing (μ CP), replica molding (REM), microtransfer molding (μ TM), micromolding in capillaries (MIMIC) and solvent-assisted micromolding (SAMIM). Figure 2.29 describes the general procedure of the soft-lithography process which is also called rapid prototyping. Manufacturing with soft-lithography does not need expensive investments due to simple procedure applied. Optical diffraction and optical transparency are not of concern in this method and dimensions smaller than 100nm can be fabricated without advanced lithographic techniques. Devices, that are difficult to produce by photolithography, can easily be produced by soft-lithography.

In pattern transfer, contact printing is a skilled way to produce devices. In order to have successful fabrication, conformal contact between the stamp and substrate surface is an important issue to have desired device. Simplicity and convenience are the advantageous aspects of printing because if the stamp is readily available, one can produce more than one pattern using experimental methods. Waste is minimized since printing is an additive technique and this technique is applicable in many areas. Three-dimensional fabrication cannot be implemented via printing which is the worst drawback. In replica molding, three dimensional structures can be duplicated by a single step. Diffraction gratings, holograms, compact disks and micro tools are fabricated via replica molding since this method allows mass production. Many complicated designs in the master can be fabricated in reliable, simple and cheap way and smaller than 100 nm resolutions can be obtained [49].

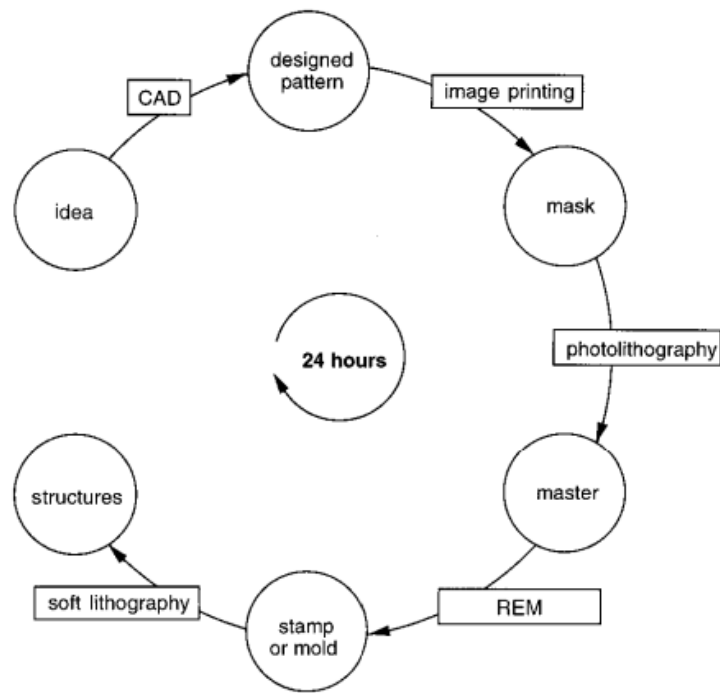


Figure 2.29. Soft-lithography manufacturing steps [49].

2.3.8. Wire Imprinting

In wire imprinting fabrication method, amorphous polymers are used due to their low glass transition temperatures (T_g), which range from 120 to 180°C. The definition of the glass transition temperature is given as; amorphous areas of a polymer take tough and glassy form at a determined temperature. Figure 2.30 describes the wire imprinting method [48].

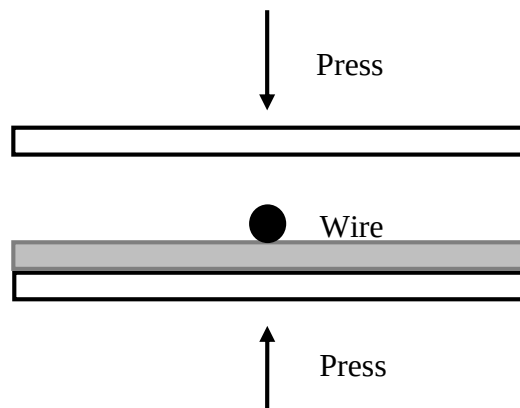


Figure 2.30. Wire imprinting [50].

In this method, in order to create microchannels, small-diameter chrome wires are utilized. Firstly, the polymer substance to be treated is heated above the glass transition temperature. Then, chrome wires are pressed into the substance and force is implemented to produce the microchannels. The channel dimensions are specified by the features of the chrome wires, and the device takes shape accordingly. However, reproduction of the same device via wire imprinting technology is very difficult due to deformations occurred during the fabrication process.

2.3.9. Hot Embossing

Hot embossing is another microfluidic device production technique, which is very convenient method for microfluidic device fabrication. Polymer, thin material is exposed to heat, which is higher than the polymer's glass transition temperature to implement the desired design. Mold is employed in this process and it has the reverse shape of the wanted device. Mold and polymer workpiece are superimposed and pressure is applied through them. In order to have the designed pattern on the workpiece, sufficient time, optimum pressure and temperature have to be provided. Polymer deformation and then mold feature replication occur during the embossing process. After sufficient time, polymer workpiece is cooled down to demolding temperature while maintaining pressure on it. At the demolding temperature, fabricated piece is separated from the mold. Figure 2.31 describes the hot embossing process.

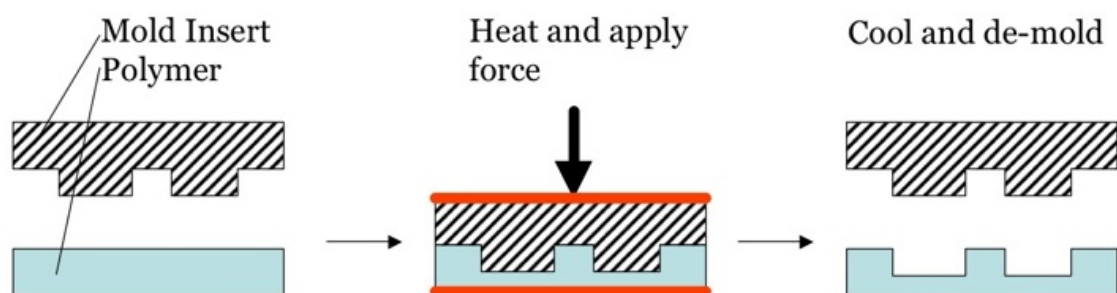


Figure 2.31. Hot embossing process [51].

In order to conduct the embossing processes, high initial costs are required to buy the needed instruments and producing molds. However, since only two production steps (blank

production and embossing) are necessary for this fabrication method, cycle time is shorter than other methods. In addition to this, embossing instruments can be used in input/output port punching or thermal bonding processes. Since embossing machines assure multi-purpose usage, higher initial costs can be considered less important [52].

2.3.10. 3D Printing

Lab-on-a-chip device production systems generally require expensive conventional equipments to fabricate the desired tools and production with these equipments are time consuming. In addition, three-dimensional (3D) device production, alignment and bonding are difficult to perform via traditional methods. Since concept-to-chip time becomes an important issue in productions, one-step fabrication mechanisms come into prominence. Scientists have begun to search new ways to fasten the production steps. Device production by purchasable 3D printers are started to be used in manufacturing. The usage of 3D printers provides minimized resources and fabrication skills. There is no need to use masks via this production method since 3D printers are able to convert digital designs into physical models in an easy and faster way. Models to be fabricated are created by the computer aided design (CAD) programs such as AutoDesk, AutoCAD, SolidWorks or Creo Parametric. Then, these CAD files are transformed into .STL (Standard Tessellation Language) files. Moreover, since model designs are computer-based, they can be shared among other research laboratories [53]. Although 3D printers have prominent and notable features to construct the microfluidic devices, they have also limitations that prevent their widespread usage. Firstly, most 3D printers' resolution is unable to produce the desired channel dimensions. Due to resolution problems, processing time and final print size may change. Secondly, resin is another important problematic issue. Resin should be cheap and capable of mechanical, optical, electrical and chemical characteristics. Opaque materials are generally employed in many printers but these materials do not allow the imaging processes. Finally, price is another limitation of 3D printer's usage. As resolution capability of the printer increases, its price also increases. As a result, these three problems prevent the 3D printer utilization in many areas [54][55].

3. MATERIALS AND METHODS

3.1. Chip Fabrication

3.1.1. Creating a Mask

Designing a microfluidic device for a specific purpose or an experiment is a very important step of microfluidic device fabrication. The design should meet all kinds of requirements to achieve the intended goal. In this work, a microchemostat is intended to be created in order to study the chronological aging of yeast cells. 8 chambers along with inlet and outlet channels for yeast and nutrient flow are designed. Every chamber has its own C-shape for trapping the yeast cells as shown in Figure 3.1.

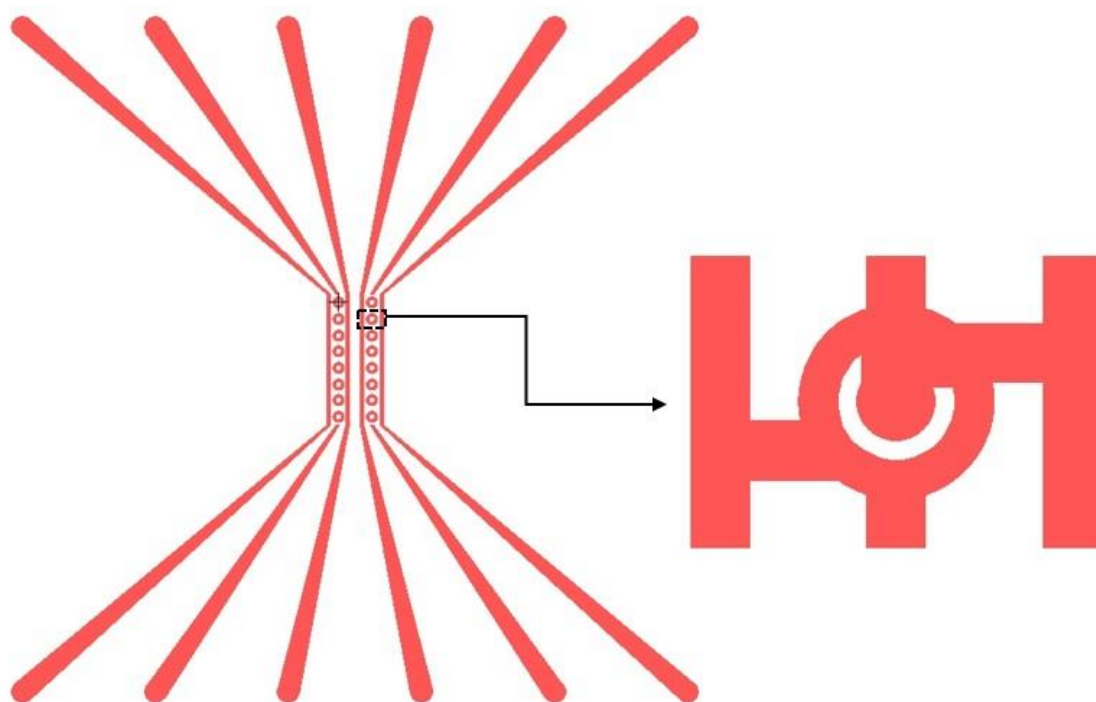


Figure 3.1. General view of mask and chambers.

After deciding on the shape of the microbioreactor the chambers and channels are modeled by a computer aided program (CAD), L-edit, which is a layout editor. The mask is drawn in two dimensional form by using L-edit program. Every chamber's C-shaped

trapping region has a diameter of 400 μm . The channels for yeast and nutrient inlet and outlets have 300 μm width and 12 μm height (Figure 3.1). When drawing is finalized, file format is converted into .pdf and it is sent to a high resolution printer (Çözüm Tanıtım, Ankara) to be printed on an acetate paper. Printed mask is used in the photolithography process.

3.1.2. Photolithography

Photolithography process is performed on a stainless steel which has 10x10cm dimensions. First, steel is cleaned with deionized water and dried with nitrogen gun to wipe out dusts on the surface. Steel is positioned in the spin coater (SPS SPIN 150, Netherlands) and fixed with the help of vacuum. Adhesion promoter is dropped to promote adhesion of the photoresist onto the steel. Spin coater is set to 2000 rpm and adhesion promoter is spread over the steel for 30 seconds. PR 1828 photoresist is used to cover the steel surface after the treatment with adhesion promoter. Photoresist is dropped on the steel surface and steel is coated with photoresist at 2000 rpm for 30 seconds in spin coater. After photoresist coating, steel is soft baked on a hot plate at 95°C for 2 minutes. The mask is superimposed on the photoresist coated steel to apply positive photoresist and then it is exposed to UV light. UV light exposure process takes 2 minutes and it causes chemical change in the photoresist. The mask is separated and steel is submerged in a special solution, which is called developer, for 50 seconds. UV light exposed part of the photoresist disappears since positive photoresist is applied. After all, the photoresist mask pattern stays on top of the steel and steel is cleaned with deionized water. In order to strengthen the photoresist hold on the steel, it is hard baked at 95°C for 3 hours.

3.1.3. Molding via Electrochemical Etching

Electrochemical etching method is employed to create the mold. Photolithography applied steel is attached to the insulator plate. 2 kg NaCl and 8 liters distilled water is mixed to prepare a saline (salt-water) solution. Steel attached insulator plate and another steel piece are placed in a container and saline solution is poured into it. Steel plate, which will be etched, is anode and the other steel piece, on which ions from the electrolyte gel are accumulated, is cathode electrode. DC power supply is set to 57 A and it is used to generate

current to electrochemical cell. Anisotropic etching is performed for 1.5 minutes to create the mold. At the end of this process, positive mold is obtained. Figure 3.2 and 3.3 show the etching steps and created mold respectively.

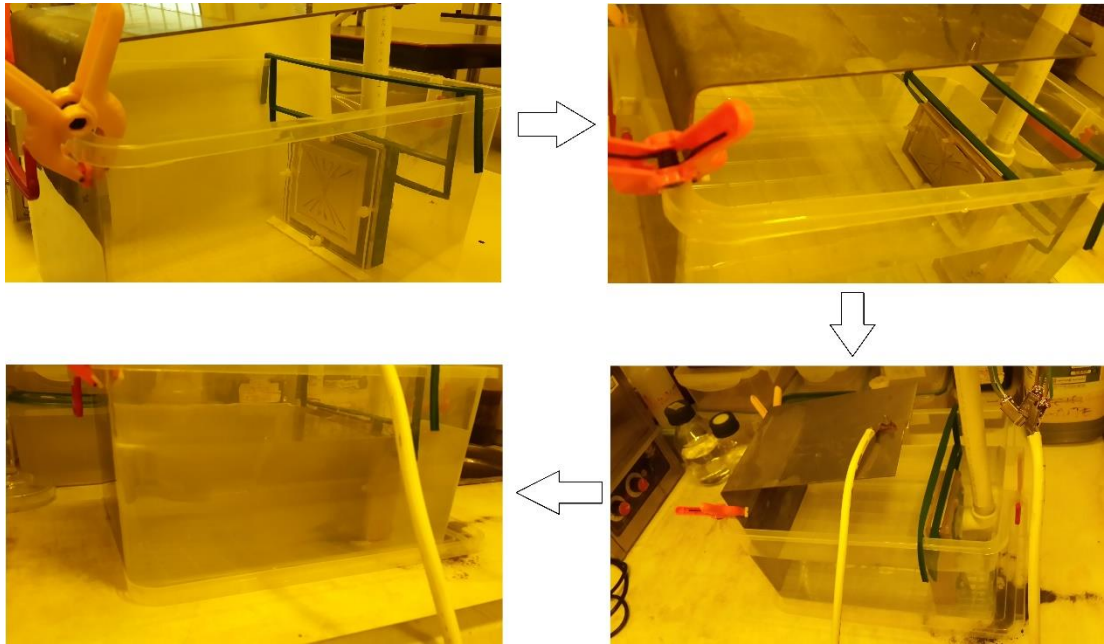


Figure 3.2. Placing steel pieces into the container, pouring NaCl solution, connection of power supply and etching.

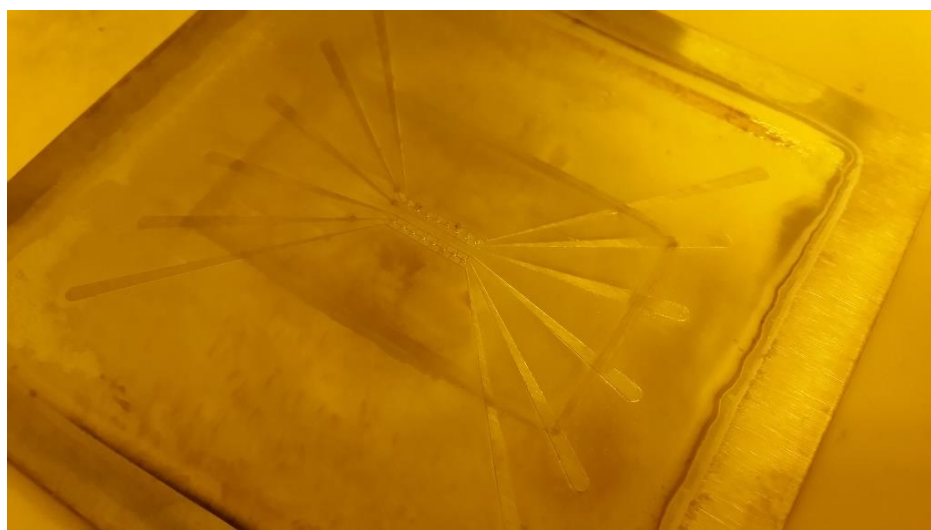


Figure 3.3. Mold.

3.1.4. Hot Embossing

Microfluidic device fabrication is done via hot embossing technique. In this technique, fabricated master or mold is used to generate the microstructures on a polymer work piece. Poly (methyl methacrylate) (PMMA) polymer sheets are employed to produce the device. The height of the sheets are 3mm, the width and height are 3cm and 5cm, respectively. First, cleaning of the PMMA is carried out by using detergent, isopropyl alcohol and distilled water. Then, substance is dried with the help of nitrogen gun. Dried PMMA is superimposed on the mold. The bottom of the mold and the top of the polymer piece are covered with aluminum foil. Hot plates of the hydraulic press machine (Carver 3851CE-0, USA) are set to 120°C (Figure 3.4). Aluminum foil covered mold and polymer piece are inserted in between the hot platens and 1250 lbs pressure is applied for 10 minutes. After 10 minutes, temperature of the hot platens are set to 70°C and PMMA is left for cooling under pressure. At the end of 1 hour, hot embossed PMMA is demolded from the mold.



Figure 3.4. Hydraulic press machine (Carver).

3.1.5. Bonding

Hot embossed PMMA piece is covered with another PMMA piece which has 1 mm height, 3 cm width and 5 cm length. The inlet holes for the fluidic connections are drilled on the cover piece with 1.52mm diameter. Before bonding, hot embossed PMMA piece and

cover PMMA piece are cleaned again by the detergent, isopropyl alcohol and deionized water. Cover and hot embossed bottom PMMA pieces are immersed in isopropyl alcohol for 2 minutes to activate the PMMA surface. Then, both pieces are dried by nitrogen gun. Cover piece and hot embossed piece are aligned and put in between the glass sheets. Glass pieces are used to prevent defects on the PMMA sheets. Consequently, taking images under microscope is expected to be easier with smooth PMMA surfaces. Hydraulic press unit is heated and aligned pieces are settled in to the press machine. Applied temperature is in between 50-90°C, pressure is 750 lbs and duration is 10 min. After 10 min, the fabricated device is left to cool under pressure inside the press machine and taken out at room temperature. Figure 3.5 and 3.6 show the general view of the fabricated device, microbio reactor and microscope photo of the chambers inside the chip respectively.

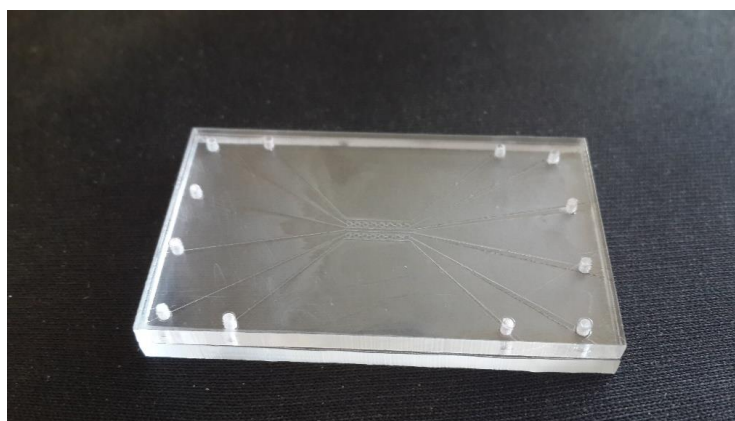


Figure 3.5. Fabricated microfluidic device.

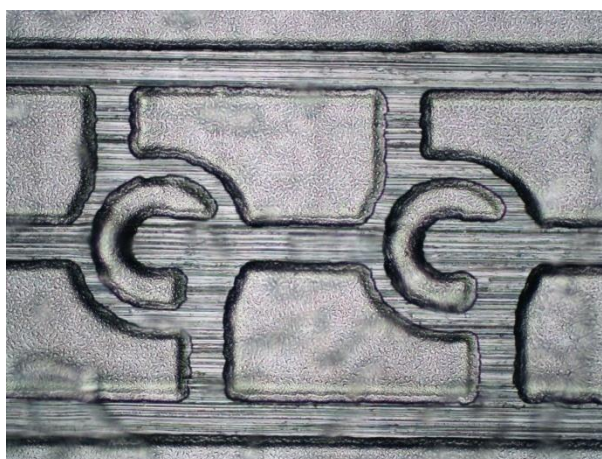


Figure 3.6. The chambers inside the chip.

6 silicon tubings of the same length of are cut from the tubings roll to use as yeast and nutrient inlets and outlets. Figure 3.7 shows that, these tubings are connected to fabricated microfluidic device with the help of epoxy and left for drying at room temperature.

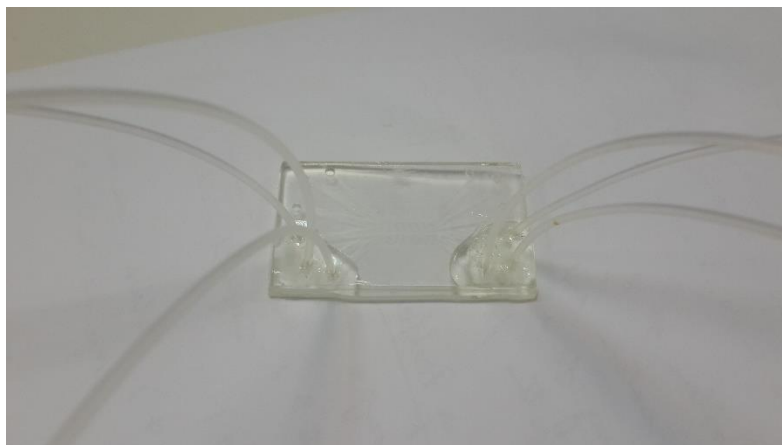


Figure 3.7. Tubings connected microfluidic device.

3.1.6. Preliminary Experiments on Lab-on-a-Chip Platform by Beads

In the first stage, commercial polystyrene beads with standard sizes of $2\mu\text{m}$ (white) and $10\mu\text{m}$ (blue) diameter were employed as a test model for cellular experiments. They were used to evaluate the proper functioning (e.g. cell trapping, protein secretion etc.) of the in house fabricated microfluidic Lab-on-a-Chip platform (microbioreactor system). Before loading the bead suspension into the syringe, the polystyrene beads were mixed into the medium. Then, these bead samples are loaded into syringes and then injected into the cell culture channel with a controllable continuous-flow provided by syringe pumps. During the experiment, continuous imaging of the bead behavior are recorded by a CCD camera.

3.2. Culture Preparation

3.2.1. Yeast Strain

The strain used in the experiments is wild type yeast strain (BY4741) where its YOR060C gene was tagged with GFP (an *Aequorea victoria* GFP (S65T) tag at the C-terminus end) to visualize the protein expression. The GFP fusion proteins are integrated

into the yeast chromosome through homologous recombination and are expressed using endogenous promoters. The GFP tagged strain was kindly given by Prof. Steve Oliver and Dr. Ceyda Kasavi.

3.2.2. Sterilization

First, laminar flow hood and the necessary laboratory equipments are sterilized by using technical grade ethanol. Cotton pieces of different sizes are wrapped by gauze and they are used to plug the erlenmeyer flasks of different sizes (50ml, 100ml, 1L and 2L). Next, aluminum foils are capped on the plugged erlenmeyer flasks. Materials such as micropipette tips and capped erlenmeyer flasks are placed in autoclave and they are sterilized at 121°C for 15min.

3.2.3. Preparation of Inoculum

In order to prepare YPD medium, 20gr yeast extract, 10gr peptone and 700ml purified water are put into the 2L erlenmeyer flask, capped and sterilized in autoclave at 121°C for 15min, then left cooling at room temperature in the sterile hood. 20gr glucose and 300ml purified water are put into the 1L erlenmeyer flask, again capped and sterilized in autoclave at 121°C for 3min to prevent caramelization and then left for cooling in the sterile hood at room temperature.



Figure 3.8. Growth of yeast strains.

Yeast extract-peptone solution and glucose solution, which are kept at room temperature, are mixed to create the YPD medium. 10ml of YPD medium is poured into each flask of 50 ml volume (1:5 ratio), then filter papers (1x1cm) containing yeast strains (10microliter) are put into each flask containing YPD the medium. Next, gauze wrapped cotton and aluminum foil are capped on these flasks. Finally, they are placed into the shaking incubator at 28°C temperature and 180rpm rotation, until turbidity in the flasks is observed. Turbidity (Figure 3.8) indicates growth of yeast strains inside the flasks. These flasks are used as inoculum for further yeast culturing.

3.2.4. Preparation of Frozen Stock Cultures

In order to prepare 30 wt% glycerol solution, 13 ml glycerol and 31ml purified water are mixed in a schott bottle and sterilized in autoclave at 121°C for 15min. In order to prepare frozen stock, conical screw cap tubes are filled with 0.5ml yeast culture (taken from inoculum culture) and 0.5ml 30 wt% glycerol solution. The solution inside the tubes are mixed expeditiously with the help of vortex and thereafter the tubes are stored at -80°C.

3.2.5. Preparation of Solid YPDA Medium and Stock Culture

10gr agar, 10gr yeast extract and 5gr peptone are mixed with 500ml purified water in a 2L sterilized schott bottle and placed in autoclave for sterilization at 121°C for 15min. Later, 10gr glucose is added to this solution, stirred and sterilized in autoclave at 121°C for 3min.

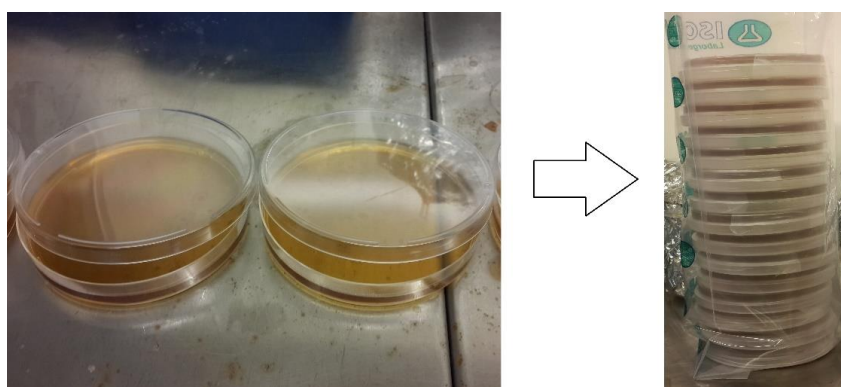


Figure 3.9. Petri dishes containing solid YPDA medium and stored in bags at 4°C.

Once the YPDA medium is cooled to about 70°C it is distributed into the petri dishes in 30ml portions in the laminar flow hood under sterile conditions. Petri dishes are kept under the sterile hood half closed for 2h to prevent humidification. The petri dishes with solidified medium are sealed with parafilm around the lid and placed into their plastic bags. The bag is taped closed and stored upside down at 4°C in a refrigerator until use (1-2 months).

3.2.5.1. Spreading of Yeast onto Agar Plates. Yeast cultures (inoculum), which were grown in erlenmeyer flasks, are pipetted in 200µm portions onto the solidified YPDA medium. These cultures are thinly spread over the solid medium with the help of L-shaped glass (cell spreader) under sterile conditions in laminar flow hood. Parafilm sealed petri dishes are placed upside down in an oven at 30°C for 2 days for culture growth. Once the yeast cultures are grown, petri dishes are taken out of the oven and put in the refrigerator at 4°C until use (1 month).

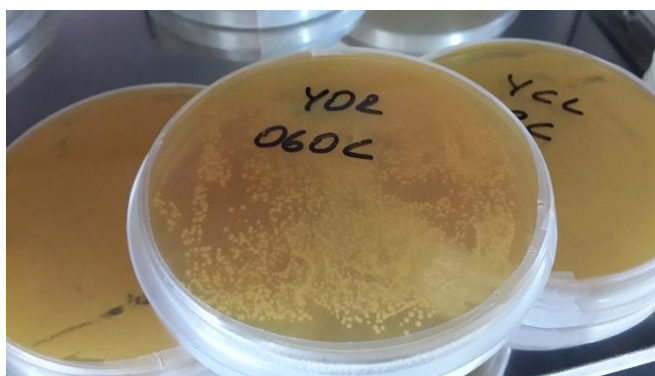


Figure 3.10. Yeast cells growing on the solidified YPDA medium.

3.2.5.2. Making a Streak Plate. Laminar flow hood is sterilized with technical grade ethanol. The inoculation loop is sterilized at the tip of the flame of the bunsen burner. Then, in order not to kill the cells, this small wire loop is cooled down at the edge of the solid YPDA medium. Next, the inoculation loop is dipped into the yeast culture in the erlenmeyer flask. Yeast culture collected by small wire loop is dropped onto the solidified YPDA medium. Inoculum is smeared backwards and forwards across a quarter of the area of the solid medium. Petri dish is then turned 90° in clockwise direction and inoculum is smeared again. This process is repeated until all quarters are done, with the last part being the most dilute

one. Afterward, the lid of each petri dish is sealed with parafilm and the petri dishes are placed upside down in the oven at 30°C for culture growth for 1-2 days.



Figure 3.11. Streak plates.

3.2.6. Yeast Culturing in Liquid YPD Medium

Laminar flow hood and required laboratory equipment are sterilized with technical grade ethanol. Erlenmeyer flasks of different sizes (100ml, 300ml, 500ml, 1L and 2L) are plugged by gauze wrapped cotton pieces. Aluminum foils are used to cover the cotton pieces of the erlenmeyer flasks. Materials such as micropipette tips, graduated cylinders and capped erlenmeyer flasks are sterilized at 121°C for 15min. 20gr yeast extract and 10gr peptone are dissolved in 700ml purified water. The solution is sterilized in autoclave at 121°C for 15min and left to cool at room temperature in sterile hood. 20gr glucose is dissolved in 300ml purified water. Glucose solution is sterilized in autoclave at 121°C for 3 min to prevent caramelization of glucose and this solution is also left for cooling at room temperature in sterile hood. In order to prepare 100ml YPD medium, 30ml cooled glucose solution and 70ml cooled yeast extract-peptone solution are mixed with the help of vortex in 500 ml erlenmeyer flask. 20ml YPD medium is put into each 100 ml erlenmeyer flask. Yeast precultures grown on solid YPDA medium are used to inoculate these liquid medium containing flasks. By using Bunsen burner, the inoculation loop is sterilized at the tip of the flame and the small wire loop is cooled down at the edge of the solid YPDA medium. Yeast cells grown on the YPDA medium are scrapped with the help of inoculation loop and the loop is dipped into the 20ml YPD medium in the erlenmeyer flask. This procedure is used to obtain liquid cultures of YOR060C yeast strain. The precultures are placed into the shaker

and kept at 28°C and 180rpm rotation, until turbidity in the flasks is observed. When the turbidity is observed, flasks are taken from the shaker and put into the refrigerator at 4°C until usage.

3.2.7. Yeast Experiment in the Microfluidic Device

Liquid yeast cell culture (20 ml) in the 100 ml erlenmeyer flask, inoculated by solid culture on YPDA medium, is used for loading the microbioreactor. The optical density (OD) of the liquid culture is measured by using spectrophotometer at 600nm wavelength and the culture is diluted by YPD medium at 1:10 ratio to obtain an OD of 0.5 in order to prevent possible cell clogging inside the microfluidic device. 60ml YPD medium are loaded into each of the 3 sterile syringes and diluted yeast culture is loaded into another sterile syringe. Syringes with YPD media are fitted to the syringe pumps (New Era, The USA) and pump flow rates are set to 1 μ L/min. Microfluidic device, microbioreactor, is placed on the microscope, silicon tubings are connected to the syringes and microbioreactor is perfused for 2 hours with YPD medium. At the end of the 2 hours, yeast culture is fed through the middle inlet into the microbioreactor. The flow rates for yeast cell loading and nutrient (YPD medium) loading are 1ml/h and 0.1 μ L/min, respectively. When the yeast cells are trapped in each of the 8 chambers (chambers of 1.5 nanoliter volume), cell loading is stopped and nutrient supply is resumed through all of the 3 channels. Throughout the experiments, fluorescence and bright field images are taken at certain time intervals and the experiment lasted about 28 hours. Figure 3.12 shows the general and close views of the experimental setup.

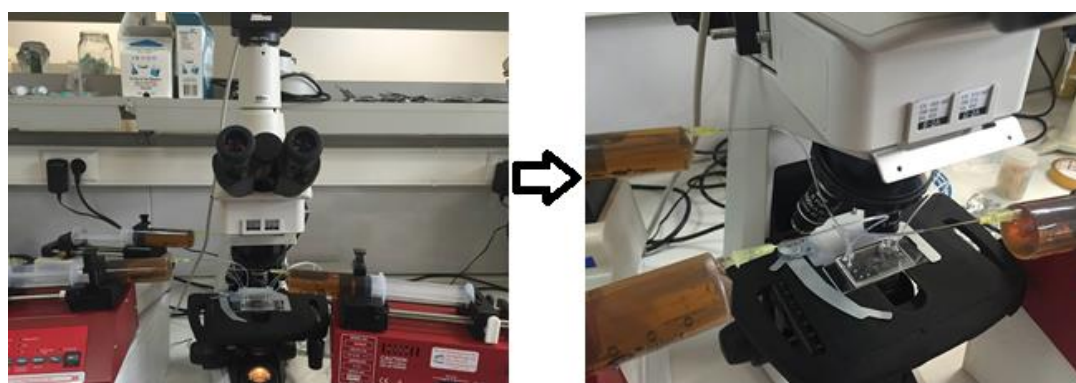


Figure 3.12. General and close views of the experimental setup.

3.3. Image Processing

3.3.1. Brightfield Image Processing

Brightfield images taken during the experiments with yeast cells are processed by using image processing and analysis software Fiji – ImageJ. First program is started and brightfield image of yeast cells for a specific chamber is opened. The area containing yeast cells is selected with *Freehand selection* tool on the Main toolbar and outside of the area is cleared. Selected area is processed with *Measure* under the *Analyze* section and the result is recorded in the excel sheet. *Smooth* and *Sharpen* tools are used sequentially in the *Process* section to have better image quality. Next, *Process* → *Filters* → *Variance* path is followed to filter the image and in the *Variance* box radius is set to 2 pixels. In order to clear the unnecessary shapes around the yeast cells, *Process* → *Math* → *Subtract* path is followed and in the *Subtract* box, the value is set to 50. Figure 3.13 shows the filtered image.

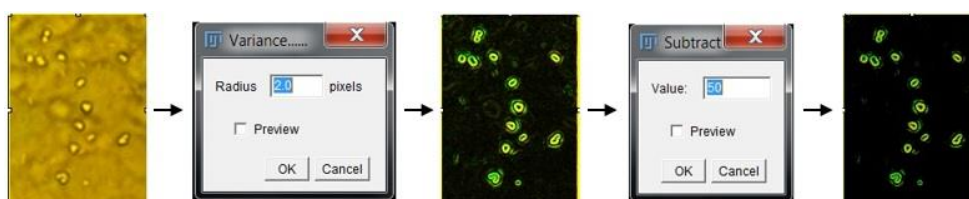


Figure 3.13. Steps of image filtering.

After filtering, image is converted into 8-bit image from red green blue (RGB) image by *Image* → *Type* → *8-bit*. Thresholding is applied through *Image* → *Adjust* → *Threshold* path. In the threshold box *Dark background* is checked, *Otsu* Thresholding is selected and *Apply* button is clicked. Figure 3.14 describes the threshold applied image.

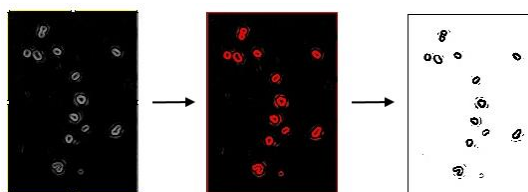


Figure 3.14. Threshold applied image.

Analyze Particles is selected under *Analyze* on the Main toolbar. In the *Analyze Particles* box, *Size (pixel²)* is set to 100-Infinity, *Overlay Outlines* is selected from *Show* menu and *Display results*, *Clear results*, *Summarize* and *Add to Manager* are checked. Finally, the results in terms of *Cell number*, *Area*, *Mean*, *Perimeter*, *Integrated Density*, *%Area* and *Raw Integrated Density* are recorded in the excel sheet.

3.3.2. Fluorescence Image Processing

First, fluorescence image is opened via ImageJ computer program. Since image is in RGB format, this image is split into its red, green and blue channels via *Image* → *Color* → *Split Channels*. Since yeast cells are green fluorescent protein (GFP) tagged, green channel is chosen. The area containing yeast cell is analyzed by using *Analyze* → *Measure* command. Next, image is sharpened by *Sharpen* command under *Process* tool. Filtering is applied two times; first *Process* → *Filters* → *Maximum* with radius at 2 pixels, then *Process* → *Filters* → *Variance* at radius 2 pixels. In order to eliminate the noise in the image, *Smooth* command is used under *Process* tool. *Process* → *Find Edges* path is followed to reveal the yeast cells on the image. Subtraction is applied via *Process* → *Math* → *Subtract* way and in the *Subtract* box, the value is set to 50. Figure 3.15 summarizes the image processing steps until thresholding.

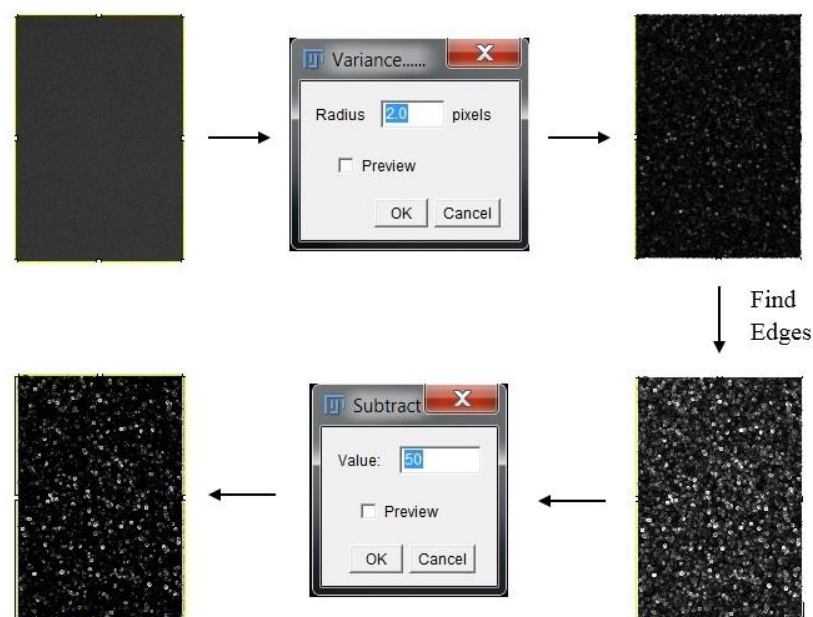


Figure 3.15. Image filtering steps until thresholding.

Otsu thresholding is implemented on the image by using *Image* → *Adjust* → *Threshold* path and *Dark background* is checked on the *Threshold* box. Figure 3.16 shows thresholding steps of the image.

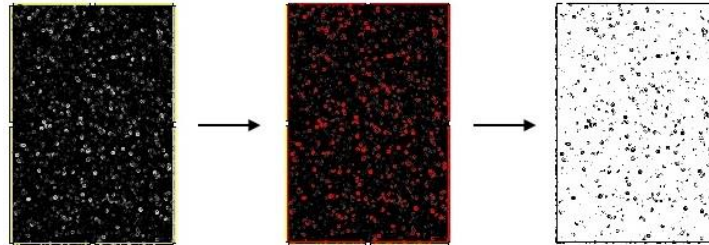


Figure 3.16. Image thresholding steps.

Eventually, yeast cells are analyzed by using *Analyze* → *Analyze Particles* command. On the *Analyze Particles* box; *Size (pixel²)* is entered as 0-Infinity, *Overlay Outlines* is selected under *Show* list and *Display results*, *Clear results*, *Summarize* and *Add to Manager* are checked. Results are recorded in the excel sheet.

3.4. Simulation Studies by COMSOL Multiphysics

In order to identify the fluid flow inside the individual chambers, a series of 2D and 3D computational fluid dynamics (CFD) simulations were performed by COMSOL Multiphysics.

Microfluidic device, microbioreactor, is simulated in COMSOL Multiphysics 3.5a using Mems module with the ‘incompressible Navier-Stokes’ and ‘convection and diffusion’ application modes to analyze the environment within the device. The procedure is as follows: First, COMSOL Multiphysics is started. *Space dimension* is set to 3D on the *New* page. Following the *MEMS Module* → *Microfluidics* → *Flow with Species Transport* → *Incompressible Navier-Stokes* path, application mode is selected. *Multiphysics* button is clicked and application mode is added to the model. By using *Options* menu, *Constants* of the model is given in Table3.1.

Table 3.1. Constants of the model.

Name	Expression	Description
rho	1013.2	Density (kg/m ³)
eta	0.000894	Viscosity (Pa*s)
V_y	0.0069444	Yeast velocity (m/s)
V_n	0.00069444	Nutrient velocity (m/s)
D	6.334e-10	Diffusion constant (m ² /s)
c0	111	Inlet concentration (mol/m ³)

In *Subdomain Settings* under the *Physics* menu, Subdomain 1 is selected and settings are entered in the related areas as the following (Table 3.2):

Table 3.2. Subdomain settings of incompressible Navier-Stokes mode.

Property	Value
ρ	rho
η	eta

Boundary settings are entered as given in Tables 3.3 and 3.4.

Table 3.3. Boundary settings of incompressible Navier-Stokes mode.

Settings	Boundaries 6, 36, 66	Boundaries 2, 32, 62	Boundary 4	All others
Boundary type	Inlet	Outlet	Symmetry boundary	Wall
Boundary condition	Velocity	Pressure, no viscous stress		No slip
U_0	V_y or V_n	0	-	-

Table 3.4. Boundary settings of convection and diffusion mode.

Settings	Boundary 36	Boundaries 6, 66	Boundaries 2, 32, 62	All others
Type	Concentration	Concentration	Convective flux	Insulation/Symmetry
c_0	c_0	0	-	-

The investigated parameters are flow velocity, concentration profile and amount of cell loading within the microbio reactor.

3.5. Simulation Studies by LTspice

LTspice IV simulator was used to reveal hydraulic resistances in the microbio reactor. Fluid flow profile within the chip was also validated via hydraulic resistances. Probable resistance locations were determined in the chamber and nodes were placed on the inlets, outlets and connection points between the resistances. The following figure (Figure 3.17) shows resistances and nodes (red points) of a chamber:

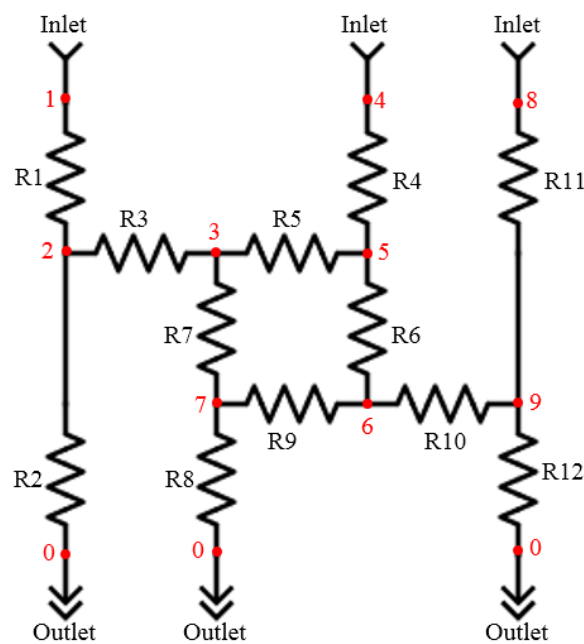


Figure 3.17. Resistances and nodes of a chamber.

Hydraulic resistances (according to rectangular cross section) in the chamber were calculated by using the following equation:

$$R_h = \frac{12\mu L}{wh^3(1 - 0.630h/w)} \quad (3.1)$$

Where, μ (N.s/m²) is dynamic viscosity, L (m) is length, w (m) is width and h (m) is height. The calculated resistances were entered into the LTspice program. The following figure (Figure 3.18) shows the workspace of simulator:

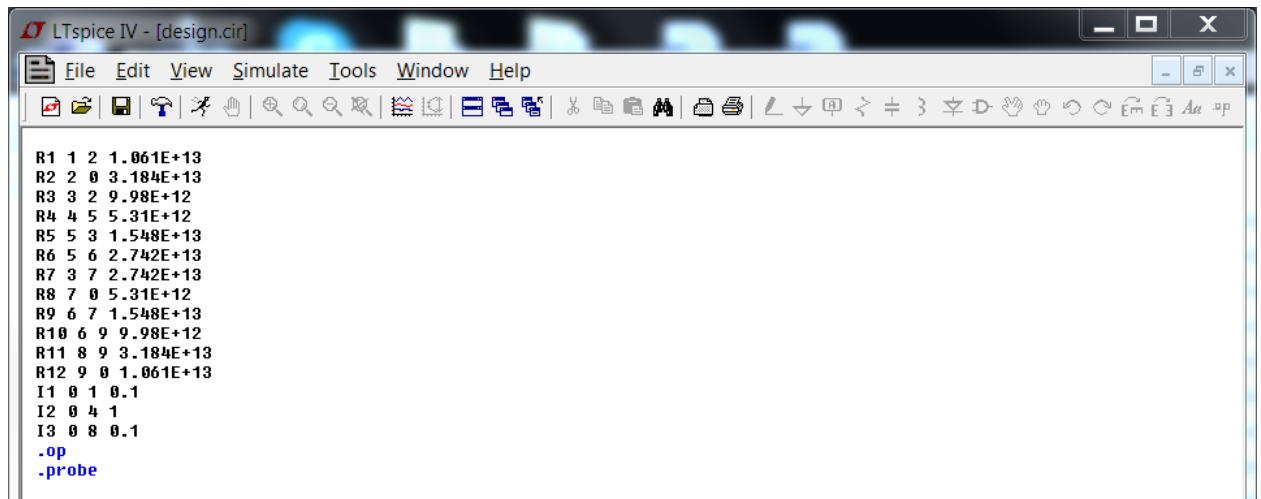


Figure 3.18. Workspace of LTspice.

In Figure 3.18, resistances were entered by using nodes and I1, I2 and I3 showed fluid flows through the inlets. As an example, resistance 1 was entered as R1 1 2 1.061E+13, 1 and 2 were the nodes that R1 was placed in between and 1.061e+13 was the hydraulic resistance value. The fluid flow profile was consequently obtained.

4. RESULTS AND DISCUSSION

The aim of the present thesis study was to design and fabricate a microfluidic device (a microbioreactor), which can be used for yeast culturing. This in-house developed Lab-on-a-Chip platform will ultimately be used to obtain a broader understanding of the function of YOR060C protein in yeast, which is an uncharacterized protein.

4.1. Design of Microfluidic Device (Microbioreactor)

Figure 4.1 shows the here developed microfluidic system with its chambers and channels. The ports a and c are used for nutrient inlets, port b is used for cell loading or nutrient inlet case by case and ports d, e and f are outlets of the device. All of the channels have $300\mu\text{m}$ width and each chamber has a radius of $500\mu\text{m}$, the height of the device is $12\mu\text{m}$. 8 chambers are sequentially placed in the microfluidic device. Each chamber has its own c-shaped region which has a radius of $200\mu\text{m}$ and the purpose of this C-shape is to trap and hold the yeast cells during cell loading and nutrient flow. The microfluidic device used in the experiments is produced from polymethyl methacrylate (PMMA) polymer by following hot embossing and solvent (isopropyl alcohol) assisted bonding techniques via hydraulic press machine.

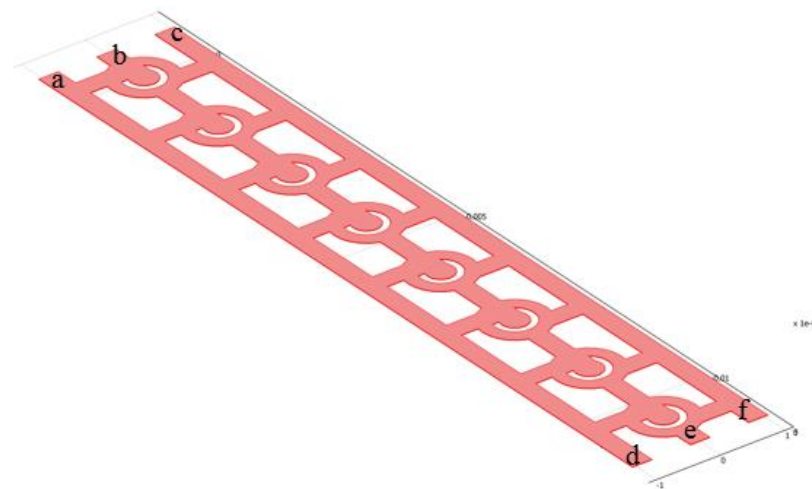


Figure 4.1. General view of the channels and chambers. a and c stand for nutrient inlets, b is used for cell loading or nutrient inlet and d, e and f are outlets of the device.

4.2. Choice of Materials for the Fabrication of Microfluidic Device: Polymers and Solvents

4.2.1. Polymers

This era is named as polymer age since people are largely get involved in polymer science or technology. The word polymer, which is introduced by Greeks, comes from poly and meros meaning many and parts, respectively. Macromolecule or large molecule is also used by some scientists rather than polymer. Polymers have very high molecular weights since they include a number of constitutional units that are connected by covalent bonds. The chemical reactions between monomers create polymers.

Polymers can be classified as biopolymers such as cellulose, collagen and synthetic polymers such as polyethylene and polyamide (nylon). Intermolecular and intramolecular forces between the molecules and within the molecules create the differences between the polymers, for example; plastics, fibers, elastomers or rubbers share general polymer properties but they show distinct characteristics [56]. In addition to natural and synthetic classification of polymers, they are also categorized as thermoplastics or thermosets based on their thermal response. Thermoplastic materials can be softened and let to flow by applying heat and pressure. Nylon and polystyrene are thermoplastic polymers. However, when the heat is applied to these thermoset polymers, chemical reaction starts and cross-linked, solid polymers are created. Urea-formaldehyde and phenol-formaldehyde are the examples of thermoset polymers [57]. Polymers represent high strength and rubber elasticity. They have their own glass transition temperatures and high viscosities.

In order to apply hot embossing, polymer work piece should be exposed to a high temperature, which is higher than the polymer's glass transition temperature. Working above the glass transition temperature allows polymers to soften. The following table (Table 4.1) summarizes general properties, which are important for hot embossing process of polymers. Polysiloxane, polymethyl methacrylate, polyacrylamide or polydimethylsiloxane are some polymeric substances that are utilized in biomaterials industry [58].

Table 4.1. Parameters of polymers used in hot embossing process.

Polymers	Glass Transition Temperature (°C)	Melting Temperature (°C)	Thermal Expansion Coefficient (°C⁻¹)
Poly(lactic acid) (PLA)	55-65	130-230	7.4×10^{-4}
Poly(methyl methacrylate) (PMMA)	85-165	160	$50-101 \times 10^{-6}$
Poly(dimethyl siloxane) (PDMS)	(-125)	(-40)-(-48)	3.1×10^{-6}
Poly(carbonate) (PC)	145	150-267	$22-75 \times 10^{-6}$
Poly(ethylene terephthalate) (PET)	67-81	250-270	$20-80 \times 10^{-6}$
Poly(styrene) (PS)	100	100-241	$67-151 \times 10^{-6}$
Poly(vinyl chloride) (PVC)	80-85	100-260	50×10^{-6}
Poly(ethylene) (PE)	-	120-180	200×10^{-6}
Cyclo olefin polymer (COP)	70-163	190-230	$60-70 \times 10^{-6}$

In the present thesis, among these polymeric materials, Polymethyl methacrylate (PMMA) was used due to its chemical inertness to many solutions and solvents and its low fluorescence background. In addition, PMMA has many advantages such as; high mechanical strength, hardness and rigidity. PMMA materials exhibit excellent transparency and polishing of PMMA is easy. They have good thermal stability and insulation properties. Moreover, they display low water absorption and excellent water resistance. 92% of light can pass through a typical PMMA grade, which is more than those of glass and other plastics. PMMA materials can be found easily and they are cheaper alternative to polycarbonate. Most importantly, PMMA is a biocompatible polymer, thus it is selected in this work [59][60][61].

4.2.2. Choice of Solvent

In order to bond the polymers with each other, generally solvents are used to activate the surface of the polymer pieces. Cyclohexane, acetone, isopropanol, acetonitrile, ethanol dimethyl sulfoxide, methanol and water are the most common solvents used for solvent-assisted polymer bonding. If the solvent is implemented on the polymer surface, the polymer chains become mobile and they can diffuse across the solvated layer. Therefore, strong bonding can be created between the surfaces via this method. Vapor or liquid solvents are

used and generally liquid solvents are employed. Polymer/solvent systems should have different solubility parameters to prevent the excessive solvent uptake by the polymer. If, the solubility parameter of solvent and polymer are close each other, channel deformation during bonding on the polymer surface may occur.

Table 4.2. Solubility parameters of polymers.

Polymer	$\Delta[(\text{J}/\text{cm}^3)]$
Polytetrafluoroethylene (PTFE)	12.6
Polyethylene (PE)	16.3
Polypropylene (PP)	16.3
Cyclic olefin copolymer (COC)	17.7
Poly(methyl methacrylate) (PMMA)	20.1
Polystyrene (PS)	18.7
Polyvinyl chloride (PVC)	19.4
Polycarbonate (PC)	19.4
Polyethylene terephthalate (PET)	21.8

Table 4.3. Solubility parameters of solvents.

Solvent	$\Delta[(\text{J}/\text{cm}^3)]$
Cyclohexane	16.7
Methylened ichloride	19.8
Ethylened ichloride	20.0
Acetone	20.4
n-hexanol	21.8
Isopropanol	23.4
Acetonitrile	25.1
Ethanol	26.0
Dimethyl sulfoxide	26.7
Methanol	29.6
Water	47.7

Table 4.2 and 4.3 show the solubility parameters of polymers and solvents, respectively [62]. In the present study, isopropanol is chosen as the proper solvent for bonding because it is most appropriate and abundant solvent in many laboratories among others. In addition, PMMA-isopropanol solubility parameters are different in each other and this makes isopropanol as a good choice for bonding.

4.3. CFD Simulations by COMSOL

The designed microfluidic device (microbioreactor) is modeled by COMSOL Multiphysics 3.5a software and series of fluid dynamic simulations were performed to analyze the system within the microfluidic chip, i.e., the fluid flow inside individual traps and concentration distribution in chambers and channels. Microelectromechanical Systems (MEMS) module is utilized with ‘incompressible Navier-Stokes’ and ‘convection and diffusion’ modes. Incompressible Navier-Stokes mode is employed for identifying the fluid flow and pressure drop inside chambers, and convection and diffusion mode is employed for revealing the glucose distribution within the microfluidic device. Steady-state incompressible Navier-Stokes equations and the continuity equation with the constant fluid density and the mass conservation assumptions are applied to run the simulations (Equation 4.1 and 4.2):

$$-\nabla \cdot (\mu(\nabla u + (\nabla u)^T)) + \rho(u \cdot \nabla)u + \nabla p = F \quad (4.1)$$

$$\nabla \cdot u = 0 \quad (4.2)$$

Equation 4.2 represents the Navier-Stokes continuity equation, where u is the velocity (m/s), p is the pressure (Pa), μ is the dynamic viscosity (Pa.s), ρ is the density (kg/m³) and F is the body forces such as gravity. During the simulations, body forces are taken as zero. Mass transport equation used in the simulations is (Equation 4.3):

$$\nabla \cdot (-D\nabla c + cu) = 0 \quad (4.3)$$

Where D represents the diffusion coefficient (m²/s) and c is the concentration (mol/m³). Several flowrates (0.1, 1, 10 μ L/min) are applied through the inlets by using

velocity component, and pressures were set to zero at the outlets. No-slip boundary conditions ($u=0$) are used for the walls and convective flux ($n \cdot (-D\nabla c) = 0$) is set to the outlets.

A glucose concentration of 2 percent by weight (20g/L, 111 gmol/m³) is fed through the middle channel and glucose concentration is set to zero in the two other channels. Parameters such as the density of glucose solution, dynamic viscosity and diffusion constant are calculated according to the supplied glucose concentration, and the density is found as 1013.2 kg/m³, dynamic viscosity is 0.000894 Pa.s and diffusion constant between glucose and water is 6.334×10^{-10} m²/s. Calculation of parameters is shown in Appendix B.

Two different cases are handled in the model; one of them is cell trapping, the other one is nutrient feeding.

4.3.1. Velocity Distribution in Microbioreactor

In order to feed and trap yeast cells in the c-shaped region, several flow rates are practiced through the inlets and the flow conditions inside the device were analyzed (Table 4.4).

Table 4.4. Summary of the flow rates and velocities of models.

Model	Flowrate in the middle channel (μL/min)	Flowrate in the side channel (μL/min)	Flow in C-shaped region	Chamber 8: velocity at center (x=0) (m/s)
1	10	10	Rapid	2.8×10^{-3}
2	1	1	Rapid	2.8×10^{-4}
3	1	10	Inefficient	1.96×10^{-3}
4	0.1	1	Inefficient	1.96×10^{-4}
5	10	1	Rapid	1.14×10^{-3}
6	1	0.1	Optimum	1.14×10^{-4}

In the first simulation study, equal flow rates are used in three channels for cell trapping. Figure 4.2 shows the general velocity field at $10\mu\text{L}/\text{min}$ flow rate, Figure 4.3 shows the first and last (eighth) chambers' speed distributions and Figure 4.4 shows speeds inside the c-shaped region of chambers.

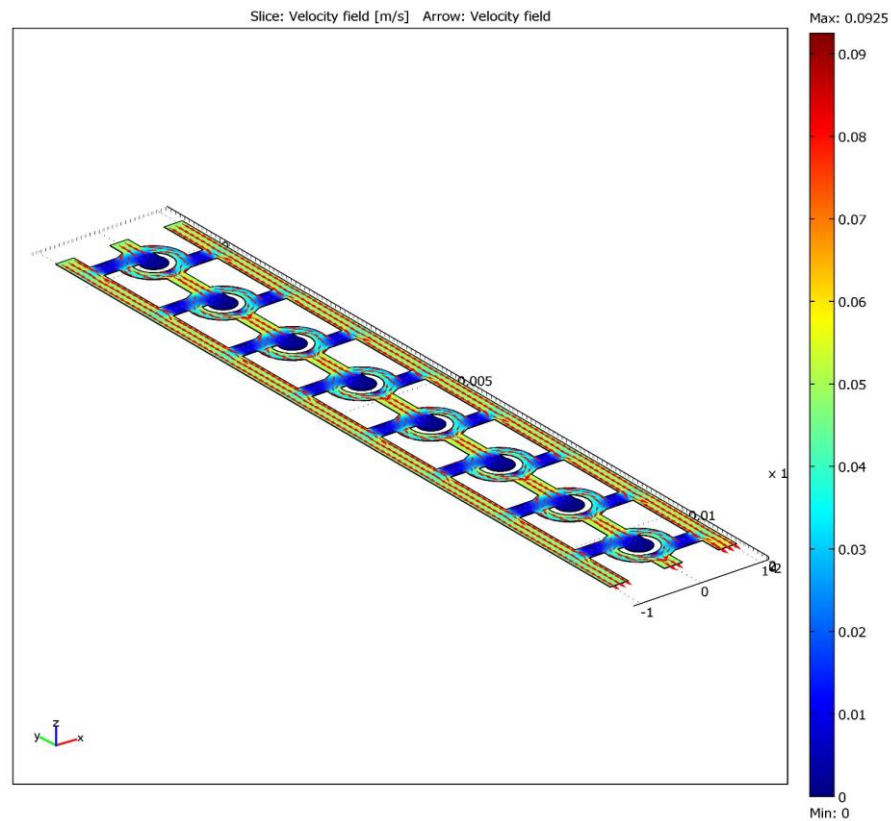


Figure 4.2. Velocity field at $10\mu\text{L}/\text{min}$ flow rate.

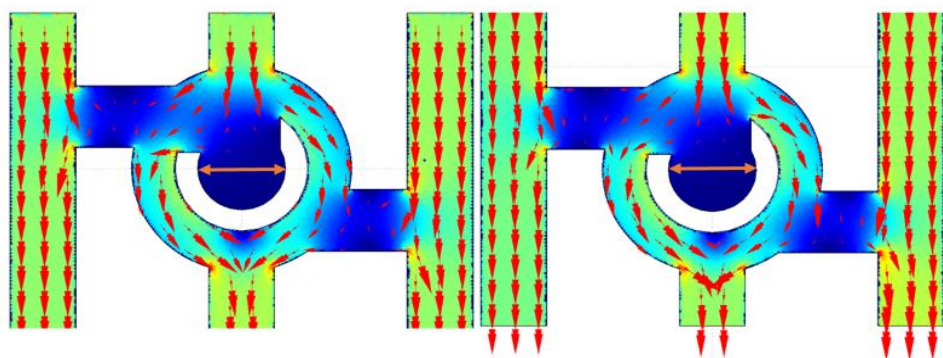


Figure 4.3. Flow distributions inside the 1st and 8th chambers ($10\mu\text{L}/\text{min}$ flowrate through all inlets).

Arrows in Figure 4.3 and speeds in Figure 4.4 indicate that the fluid flow through the c-shaped region (orange horizontal two-sided arrows indicate the region) is too rapid. Fluid flows towards the c-shaped region, circulates and leaves or flushes away around the chambers without entering the c-shaped region. Therefore, flow rate at $10\mu\text{L}/\text{min}$ is not efficient for cell loading into the chambers, at this flowrate, cells will probably enter and then immediately leave the chambers. Figure 4.4 supports this claim because the speed inside the c-shaped region ($x=0$) is too fast (around $3 \times 10^{-3} \text{ m/s}$). At this flow rate yeast cells cannot be sustained in the chambers and they may be flushed away. Thus, cell trapping at $10\mu\text{L}/\text{min}$ is not possible.

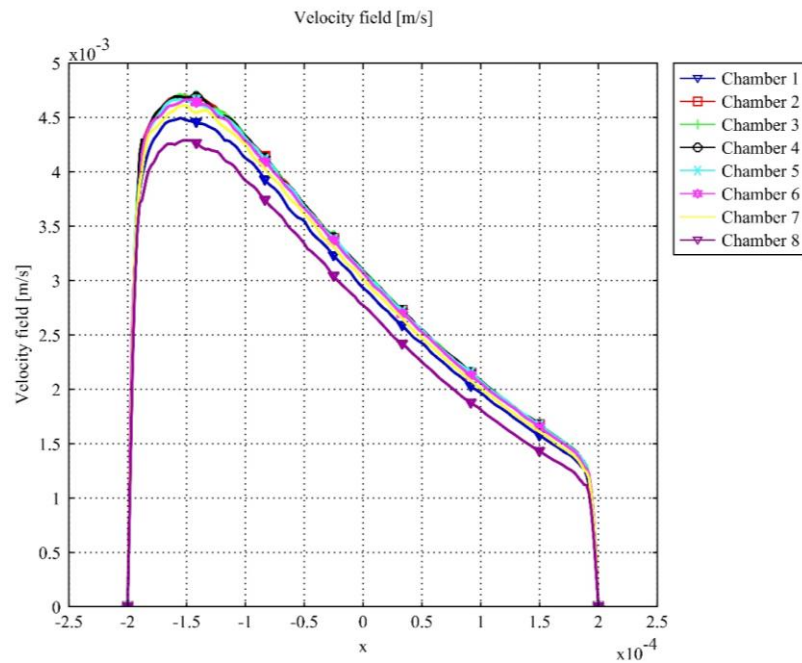


Figure 4.4. Velocities inside the c-shaped region of chambers ($10\mu\text{L}/\text{min}$ flowrate through all inlets).

Next, a flow rate of $1\mu\text{L}/\text{min}$ is set to the three inlets, and Figure 4.5 shows the general fluid flow distribution in the device. The flow distribution inside the device at $1\mu\text{L}/\text{min}$ is same as at $10\mu\text{L}/\text{min}$. It is observed that, again flow rate inside the chambers is too rapid for cell trapping. Fluid enters the chambers, circulates and leaves or flushes away without entering the c-shaped region. Figure 4.7 also describes the speed inside the chambers and according to this graph the speed inside the chambers is still high (around $3 \times 10^{-4} \text{ m/s}$ at $x=0$) for cell trapping.

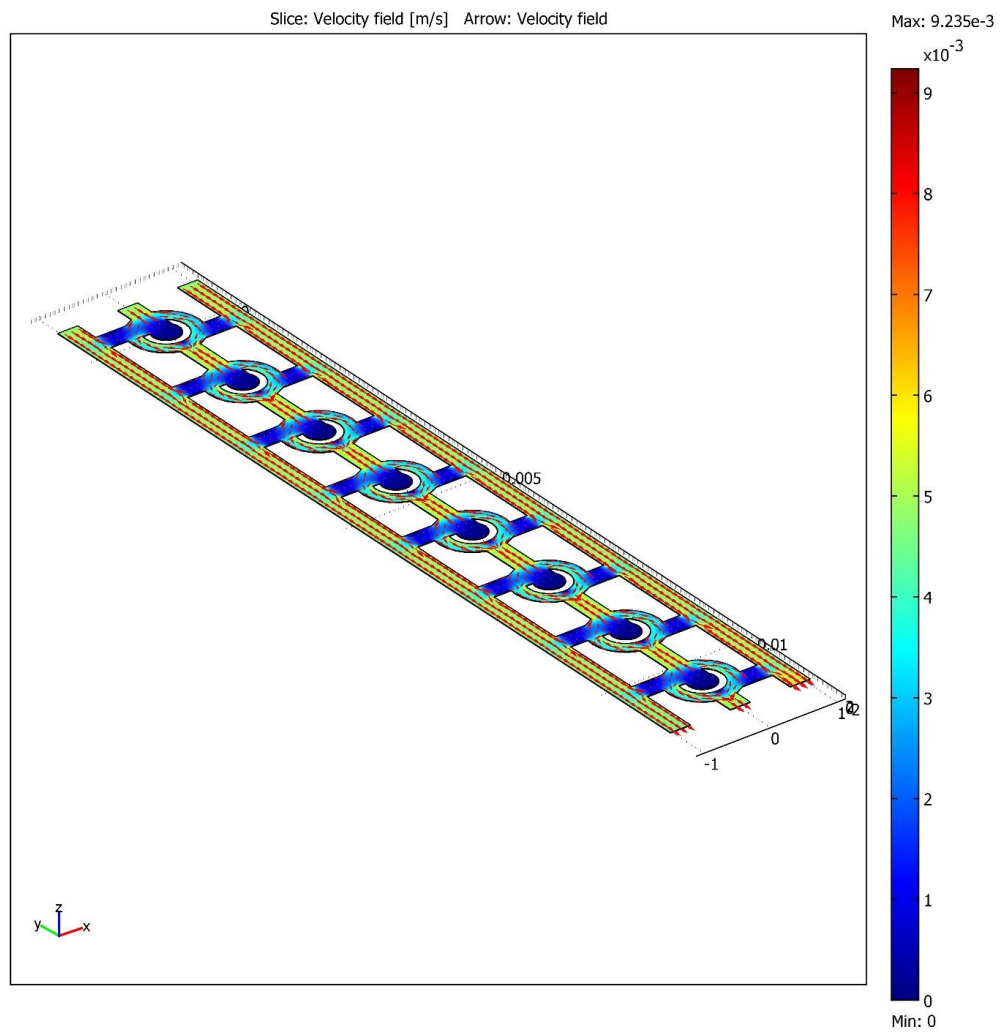


Figure 4.5. Velocity field at $1 \mu\text{L}/\text{min}$ flow rate.

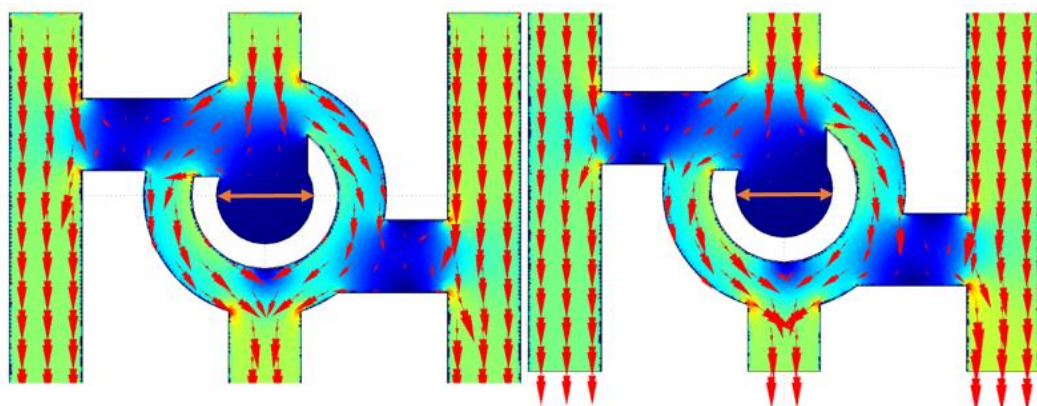


Figure 4.6. Flow distributions inside the 1st and 8th chambers ($1 \mu\text{L}/\text{min}$ flowrate through all inlets).

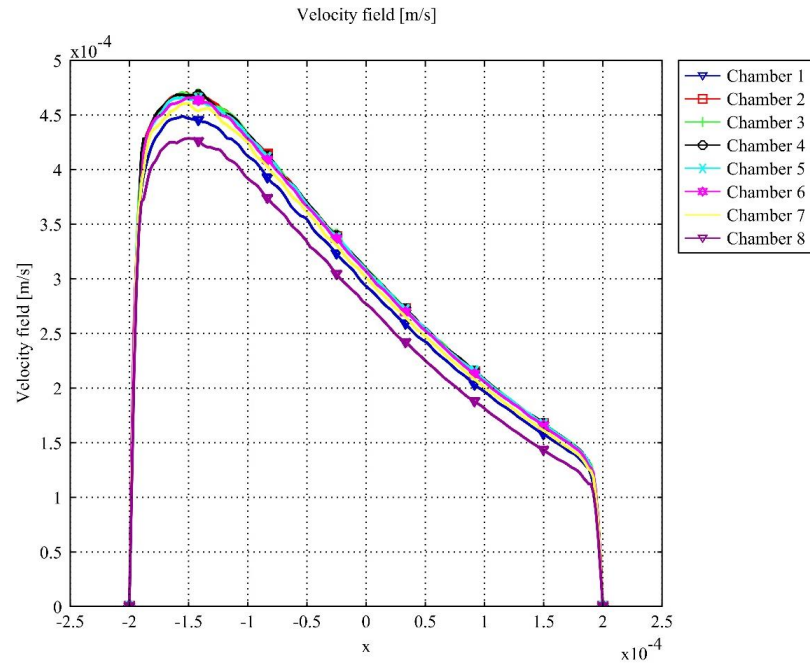


Figure 4.7. Velocities inside the c-shaped region of chambers ($1\mu\text{L}/\text{min}$ flowrate through all inlets).

In order to enforce the flow of fluid into the c-shaped region, the flowrates of the side channels are increased by 10 fold, i.e., $10\mu\text{L}/\text{min}$ and $1\mu\text{L}/\text{min}$ flow rates are chosen for side and middle channels, respectively. Figures 4.8-10 show the flow distributions and velocity profiles along horizontal axis within the chambers. As it can be seen from these figures, the application of the higher flow rates in the side channels improved the flow distribution in the chambers. Figure 4.9 reveals that the fluid flow is directed towards the chambers but entry to the chambers is still difficult in the c-shaped region (indicated by orange horizontal two-sided arrows) due to the hydrodynamic forces, the fluid stream flows around the c-shaped region and moves away. According to Figure 4.10, the speed in the first chamber is smaller than those in other chambers. Therefore, it can be said that cell trapping may be efficient in the first chamber. Then, since the speed increases in the following chambers, cells may enter these chambers but can leave immediately due to the circulation inside the c-shaped region. As a consequence of these findings, i.e., cell trapping is not efficient due to high speeds (around $2 \times 10^{-3} \text{ m/s}$ at $x=0$), the flow rate is further decreased in the following models, and 1 and $0.1\mu\text{L}/\text{min}$ flow rates are used in the side and middle channels, respectively (Figures 4.11-13).

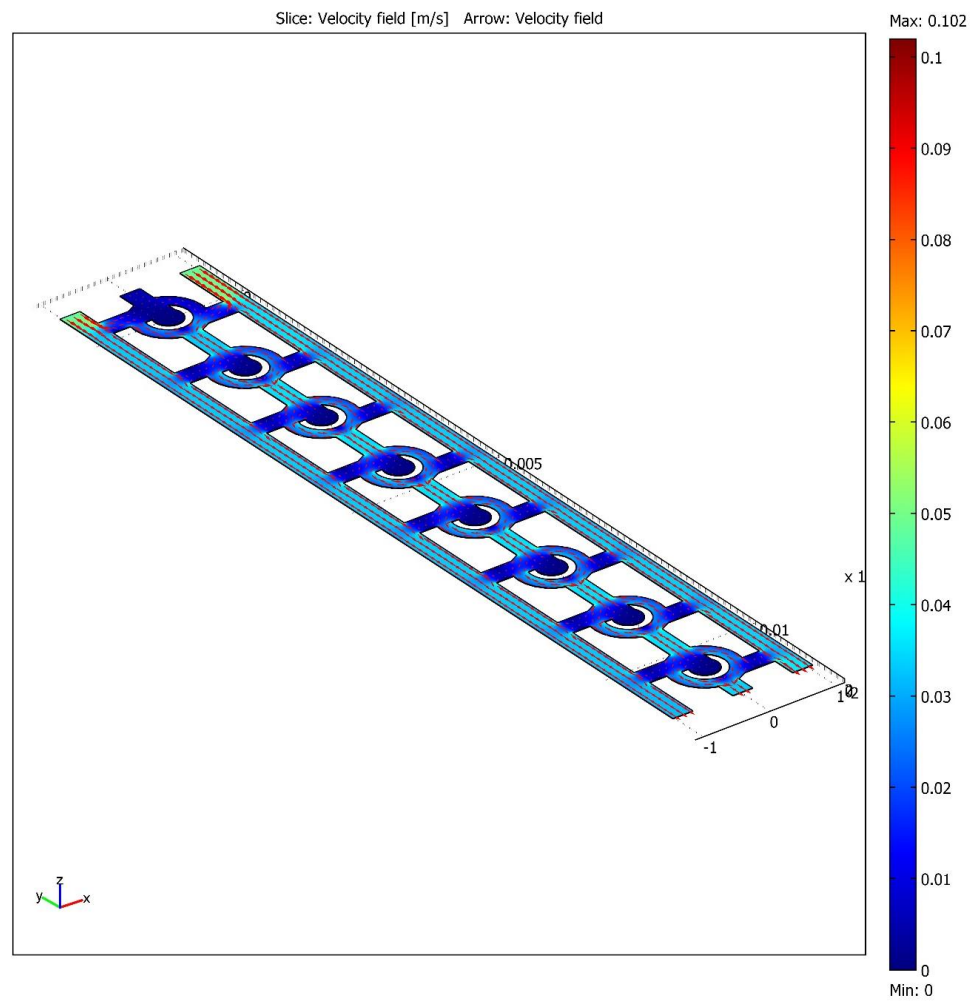


Figure 4.8. Velocity field at flow rates of 1 and $10\mu\text{L}/\text{min}$ for the middle and side channels, respectively.

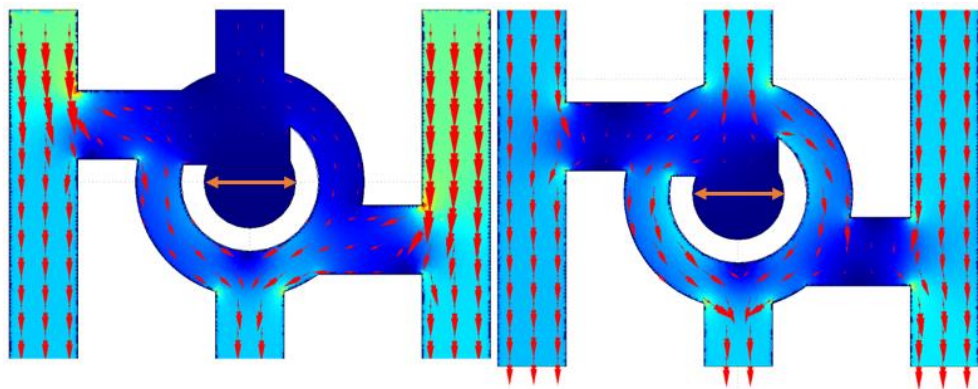


Figure 4.9. Flow distributions inside the 1st and 8th chambers (1 and $10\mu\text{L}/\text{min}$ flowrates through the middle and side inlets, respectively).

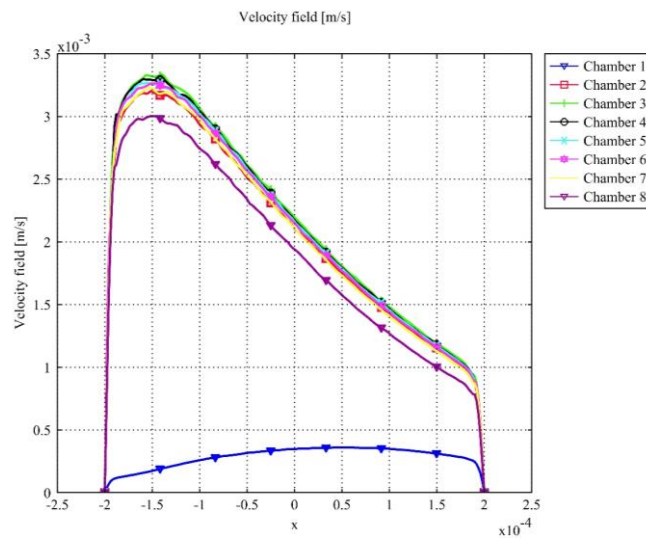


Figure 4.10. Velocities inside the c-shaped region of chambers (1 and 10 $\mu\text{L}/\text{min}$ flowrates through the middle and side inlets, respectively).

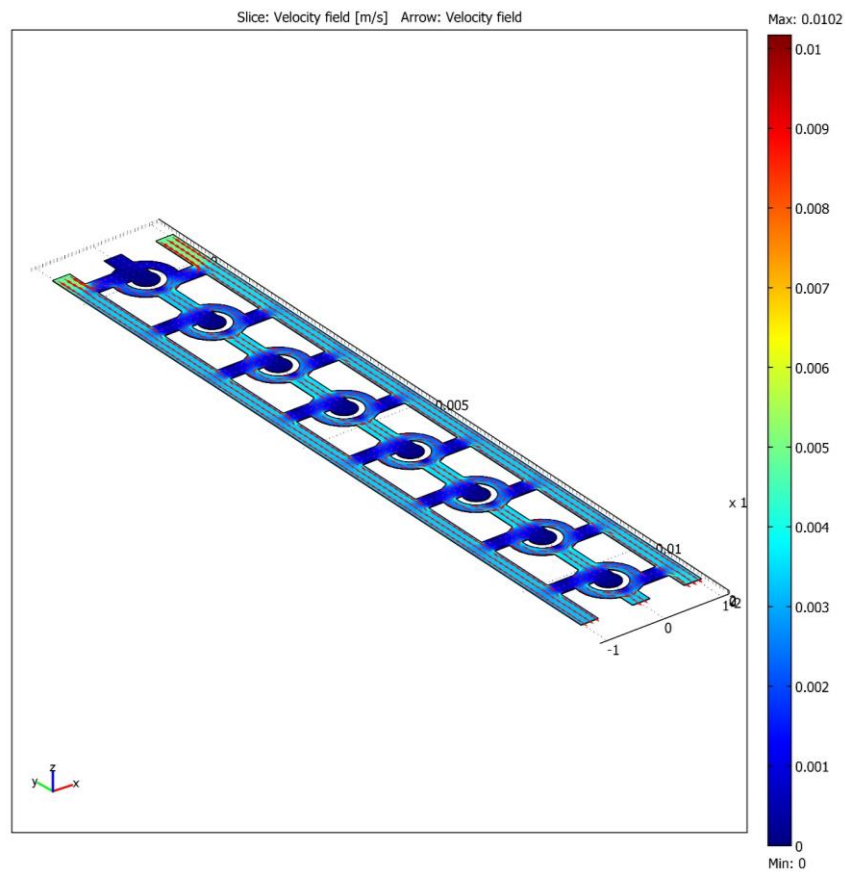


Figure 4.11. Velocity field at flow rates of 0.1 and 1 $\mu\text{L}/\text{min}$ for the middle and side channels, respectively.

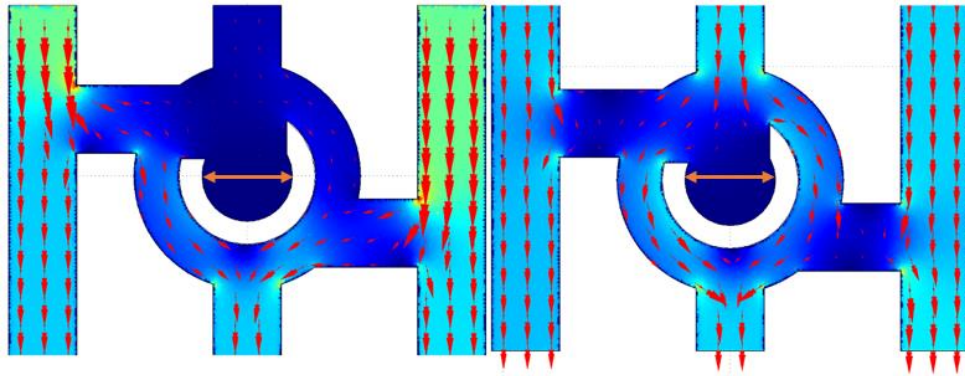


Figure 4.12. Flow distributions inside the 1st and 8th chambers (0.1 and 1 μ L/min flowrates through the middle and side inlets, respectively).

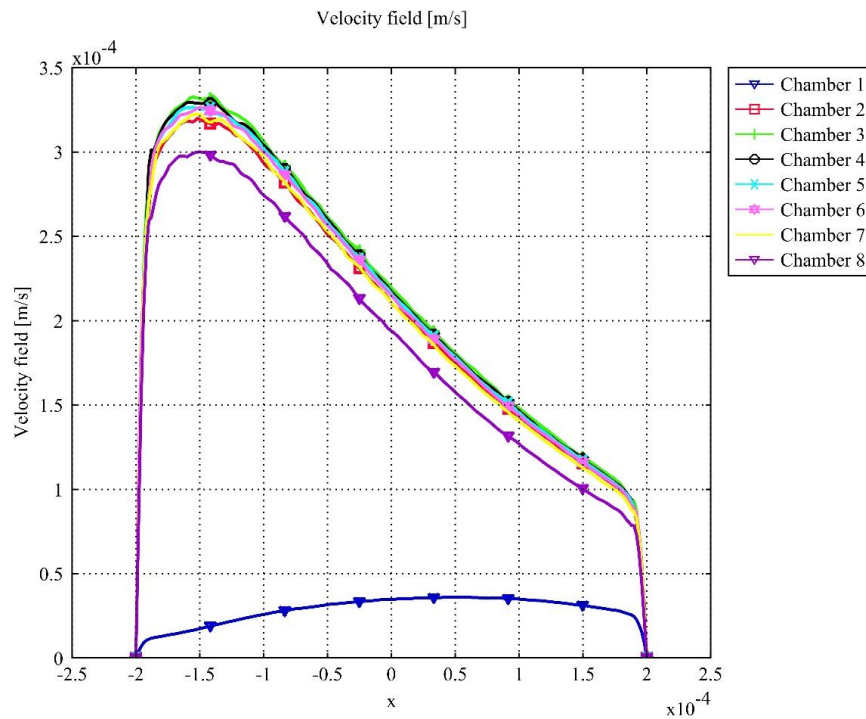


Figure 4.13. Velocities inside the c-shaped region of chambers (0.1 and 1 μ L/min flowrates through the middle and side inlets, respectively).

The application of the lower flow rates in the channels has not changed the flow distribution within the device significantly. Figure 4.11 and 4.8 resemble each other. In addition, Figure 4.12 and 4.9 are also similar to each other. The fluid stream again flows around the chambers. Although, the speeds inside the c-shaped region (according to Figure

4.13) are decreased (around 2.3×10^{-4} m/s at $x=0$), the model is still not reliable and convenient for cell trapping. Therefore, a flow rate increase in the middle channel (keeping the flowrates of side channels constant) and then a decrease both in middle and side channels are tested in order to have effective and operative cell trapping. 10 and $1 \mu\text{L}/\text{min}$ flow rates are employed in the middle and side channels, respectively and the flow distribution is analyzed (Figures 4.14-16).

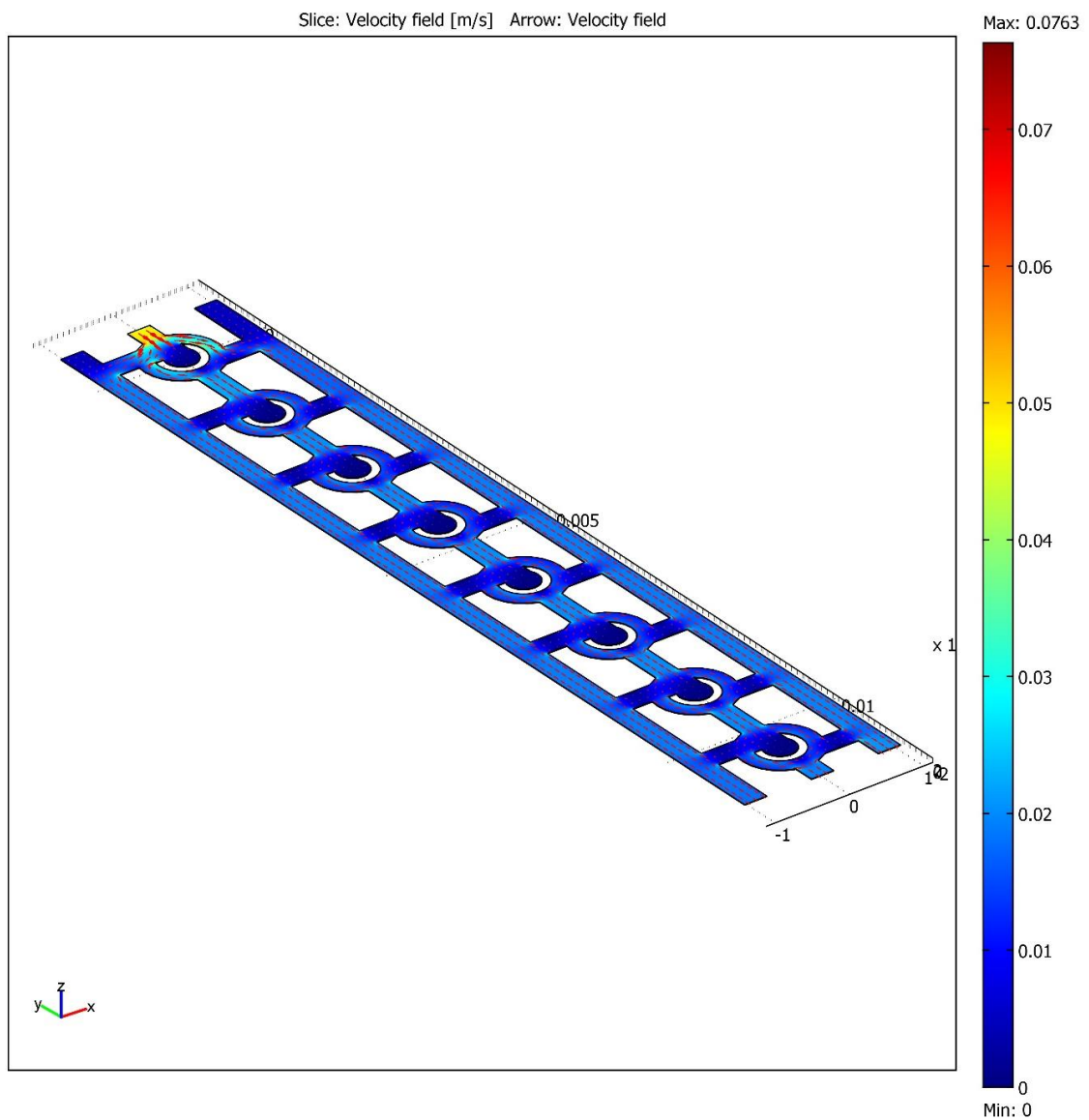


Figure 4.14. Velocity field at flow rates of 10 and $1 \mu\text{L}/\text{min}$ for the middle and side channels, respectively.

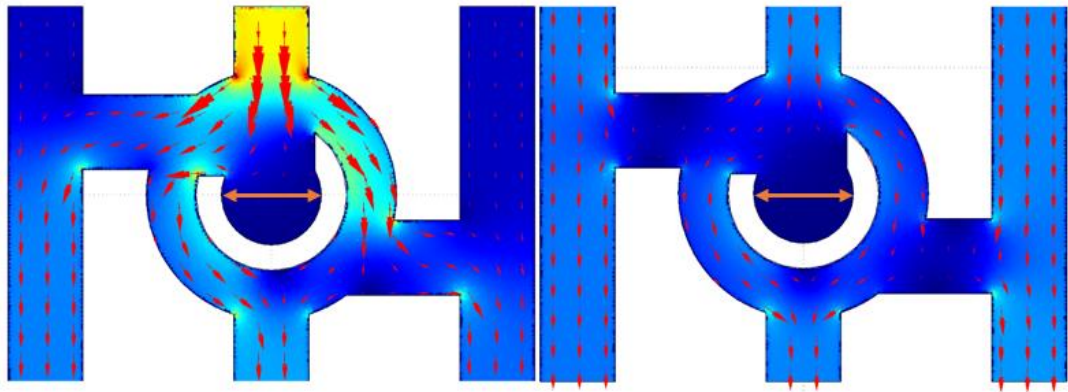


Figure 4.15. Flow distributions inside the 1st and 8th chambers (10 and 1 μ L/min flowrates through the middle and side inlets, respectively).

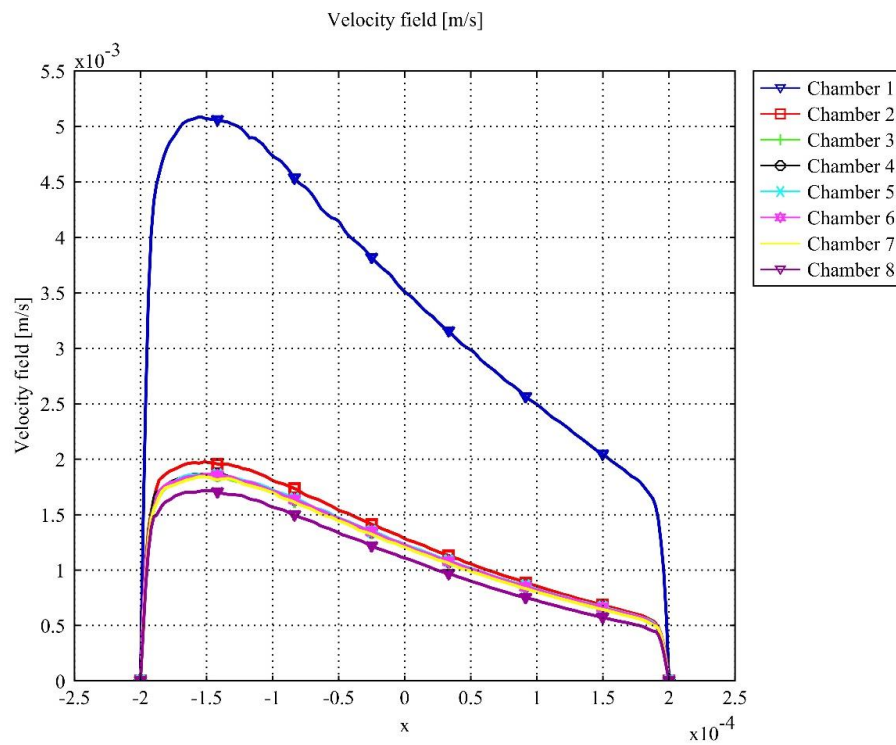


Figure 4.16. Velocities inside the c-shaped region of chambers (10 and 1 μ L/min flowrates through the middle and side inlets, respectively).

Figure 4.14 shows that, the flow distribution is focused in the middle channel and that is a desired situation. The flow in middle channel runs through the c-shaped region (Figure 4.15) and the side streams sweep the excess fluid in the chambers. Hence, yeast cells can be trapped in the c-shaped region and untrapped/remaining cells will be discarded via side

channels. It is decided that this model is an effective one for cell loading, but, according to Figure 4.16, the speeds inside the c-shaped region is still high (around 1.3×10^{-3} m/s at $x=0$), especially in chamber 1 (around 3.5×10^{-3} m/s at $x=0$). This may cause circulation in the area as it was the case in the previous models and may remove the loaded cells from the chambers. Therefore, in order to prevent the cell loss, the flow rates are further decreased in the following model, i.e., 1 and $0.1 \mu\text{L}/\text{min}$ flow rates are used through the middle and side channels, respectively.

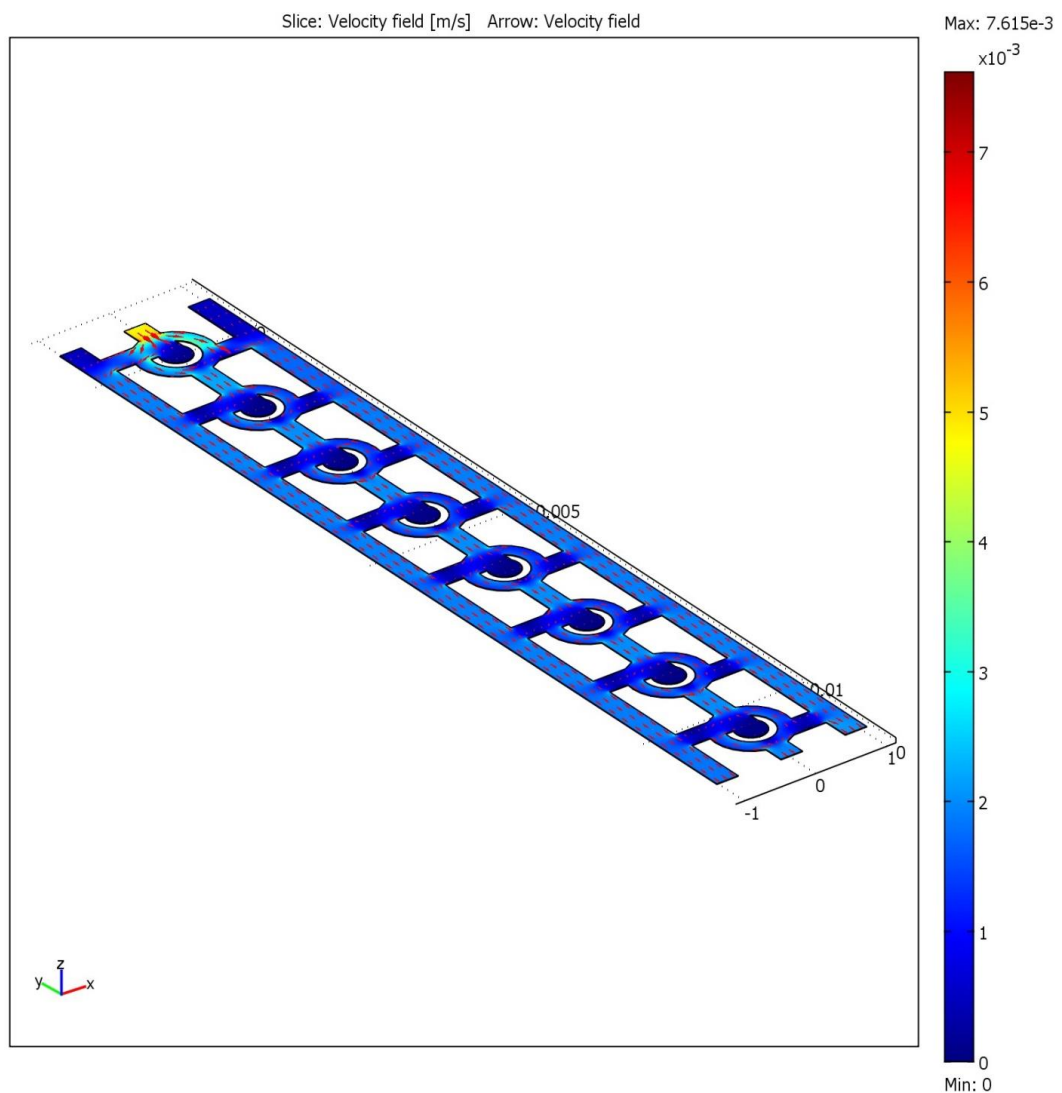


Figure 4.17. Velocity field at flow rates of 1 and $0.1 \mu\text{L}/\text{min}$ for the middle and side channels, respectively.

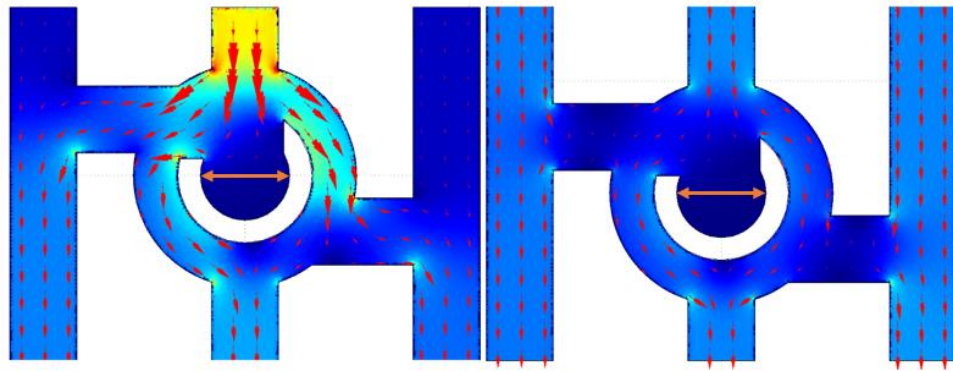


Figure 4.18. Flow distributions inside the 1st and 8th chambers (1 and 0.1 $\mu\text{L}/\text{min}$ flowrates through the middle and side inlets, respectively).

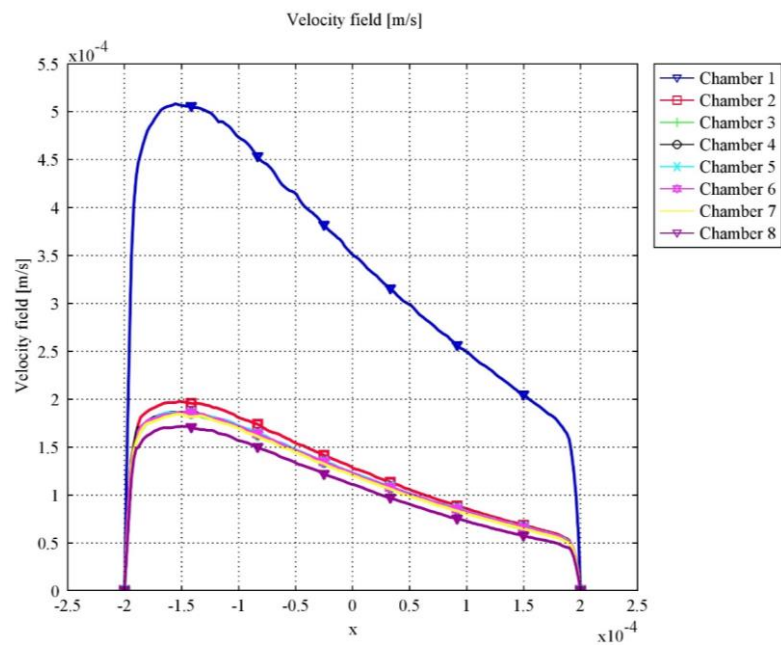


Figure 4.19. Velocities inside the c-shaped region of chambers (1 and 0.1 $\mu\text{L}/\text{min}$ flowrates through the middle and side inlets, respectively).

According to Figure 4.17, the flow in the middle channel, bearing cells, goes towards the center of the c-shaped region. In Figure 4.18, the first chamber is expected to be loaded with the cells and excess cells are flushed away by the side channels. In the following chambers, again the flow in the middle chamber progresses through the center of the chambers, and arrows show that the flows in the side channels enforce the flow towards the middle channel to go into the c-shaped region. In this model, the speeds are not too much

(around 1.2×10^{-4} m/s at $x=0$), as can be seen in Figure 4.19. Since the middle inlet is just above the first chamber, the speed in this chamber is higher than the others, and other chambers maintain their stable environment. Beside the middle channel, side channels are used functionally in this model both for keeping stable flow in the chambers and flushing away the untrapped cells from the device. Eventually, this model is decided to be used for cell loading experiment.

In order to be sure of the reliability of the model, additional analyses are performed. The speeds at the chamber entrances (both left and right) are collected from the model and results are shown in Figure 4.20.

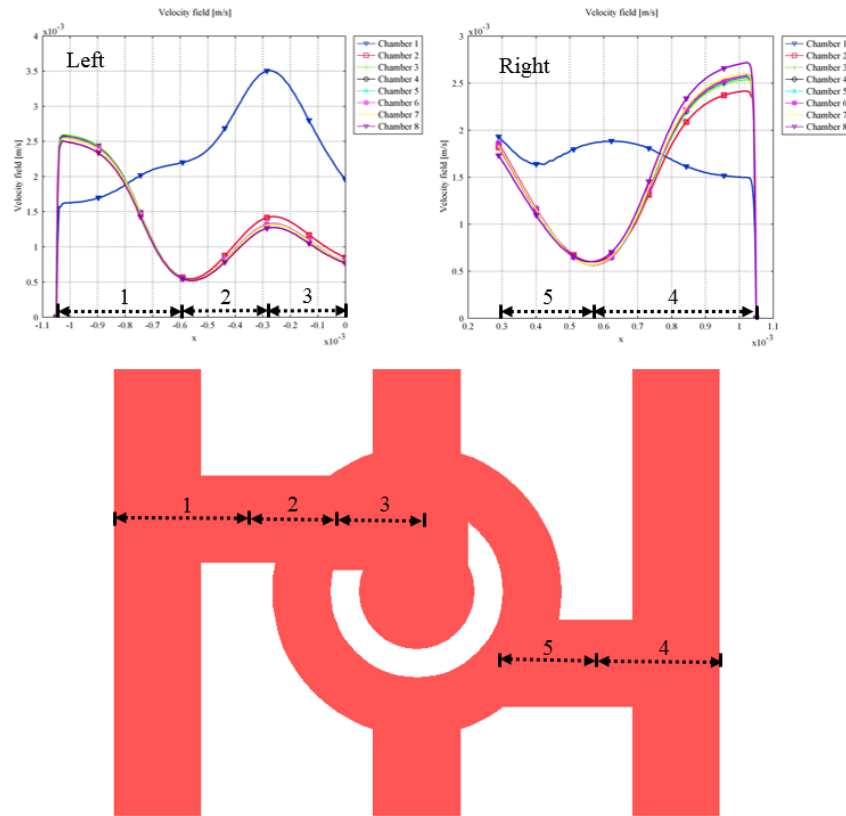


Figure 4.20. Velocity profiles at the left and right entrances of the chambers.

According to Figure 4.20, the velocity field in the first chamber is different from the others and it has higher values due to the location of the middle inlet of the device. The following chambers (2-8) have the same trend within each other. At the left entrance of chambers 2-8, the fluid speeds are first high, but then decrease along the channel that

connects the side channel and chambers (dotted line 1), and finally increase through the chamber (dotted line 2). The reason of the second increasing trend is that the flows in both the connecting channel and middle channel intermix with each other due to the chamber design shown in Figure 4.20. The speeds decrease again through the c-shaped region (dotted line 3). At the right entrance, which is located below the c-shaped region, by looking from right to left (entrance starts from the right) speeds are again high, and then decrease due to connection channel between the side channel and chambers and increase towards the middle of the chamber. Therefore, it can be said that there are always flows in all the chambers, connective channels and side channels in the device to perform the cell loading efficiently.

4.3.2. Pressure Distribution in the Microbioreactor

The pressure profile along the channels is shown in Figures 4.21 and 4.22. Since the outlet of the device is open to the atmosphere, the pressure at the outlets is zero and the simulation results confirm that. The pressure difference between the inlets and outlets reveals the progress of fluid flow within the chip. Since higher flow rate $1\mu\text{L}/\text{min}$ is set to the middle inlet ($0.1\mu\text{L}/\text{min}$ =side inlets), maximum pressure is seen there and it is around 2430Pa .

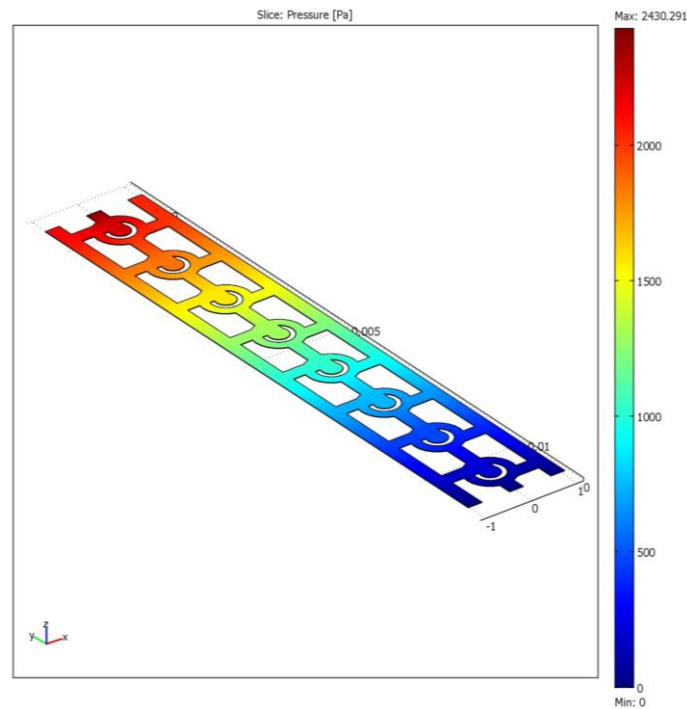


Figure 4.21. The distribution of pressure drop along the microfluidic device.

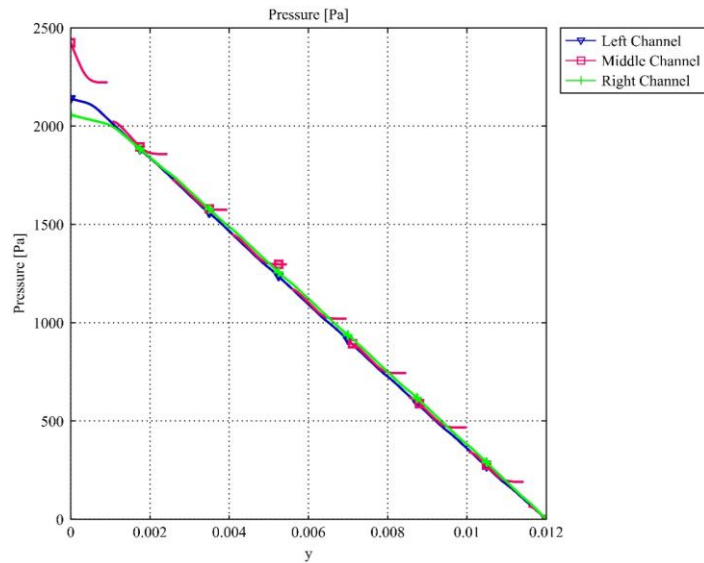


Figure 4.22. Pressure drop throughout the microfluidic device (y-vertical distance).

4.3.3. Nutrient Distribution in the Microbioreactor

In addition to the flow and pressure distribution in the microfluidic device, concentration distribution of a nutrient (main carbon source- glucose) is also studied. Although, in the experiments with yeast cells, nutrient medium of the same glucose concentration will be fed through the three inlets of the chip, in the model only middle inlet is used for loading glucose solution in order to see the effect of concentration distribution of the carbon source along the device. Therefore glucose distribution along the device is given in the following figure with 1 and 0.1 $\mu\text{L}/\text{min}$ flow rates through the middle and side inlets respectively.

Figure 4.23 shows the concentration distribution of glucose in the microbioreactor. During cell loading, yeast cells are fed through the middle inlet, and it is expected that they are trapped in the c-shaped region of the chip and side channels are used to sweep the untrapped excess cells. The concentration profile in Figure 4.23 approves this hypothesis. The color distribution on the surface of the chip shows that the glucose concentration in the c-shaped region is almost same in each chamber (Figure 4.24). Therefore, it can be said that stable and convenient environment for cell trapping can be maintained during cell loading.

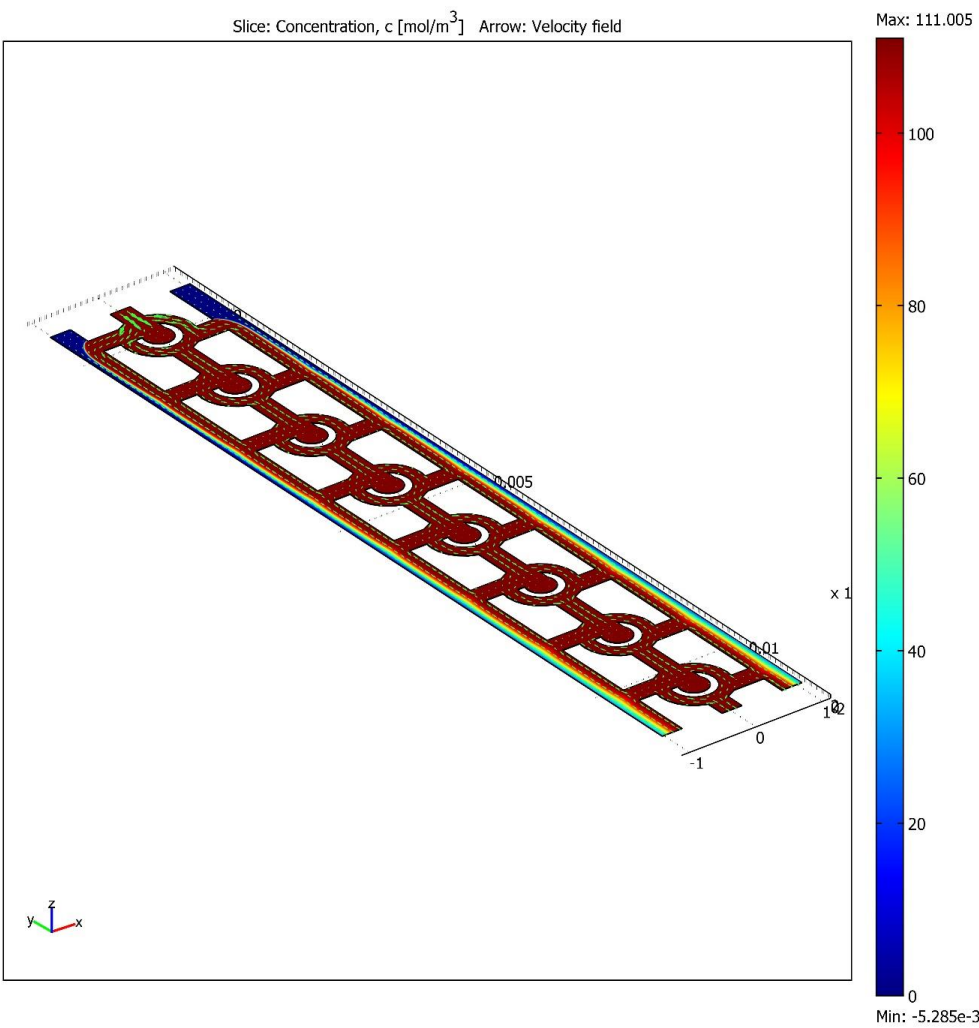


Figure 4.23. Concentration distribution of glucose along the device.

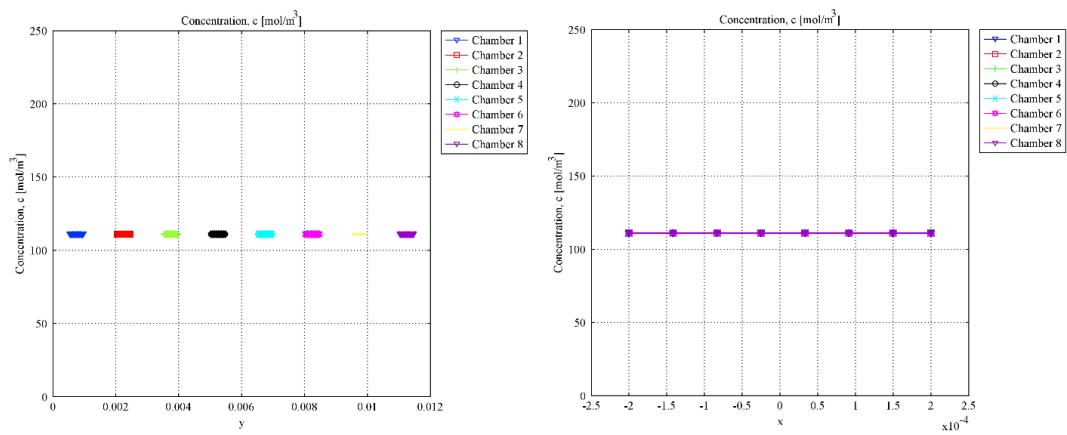


Figure 4.24. Concentration profile in the c-shaped region of the chip both in y (vertical) and x (horizontal) directions.

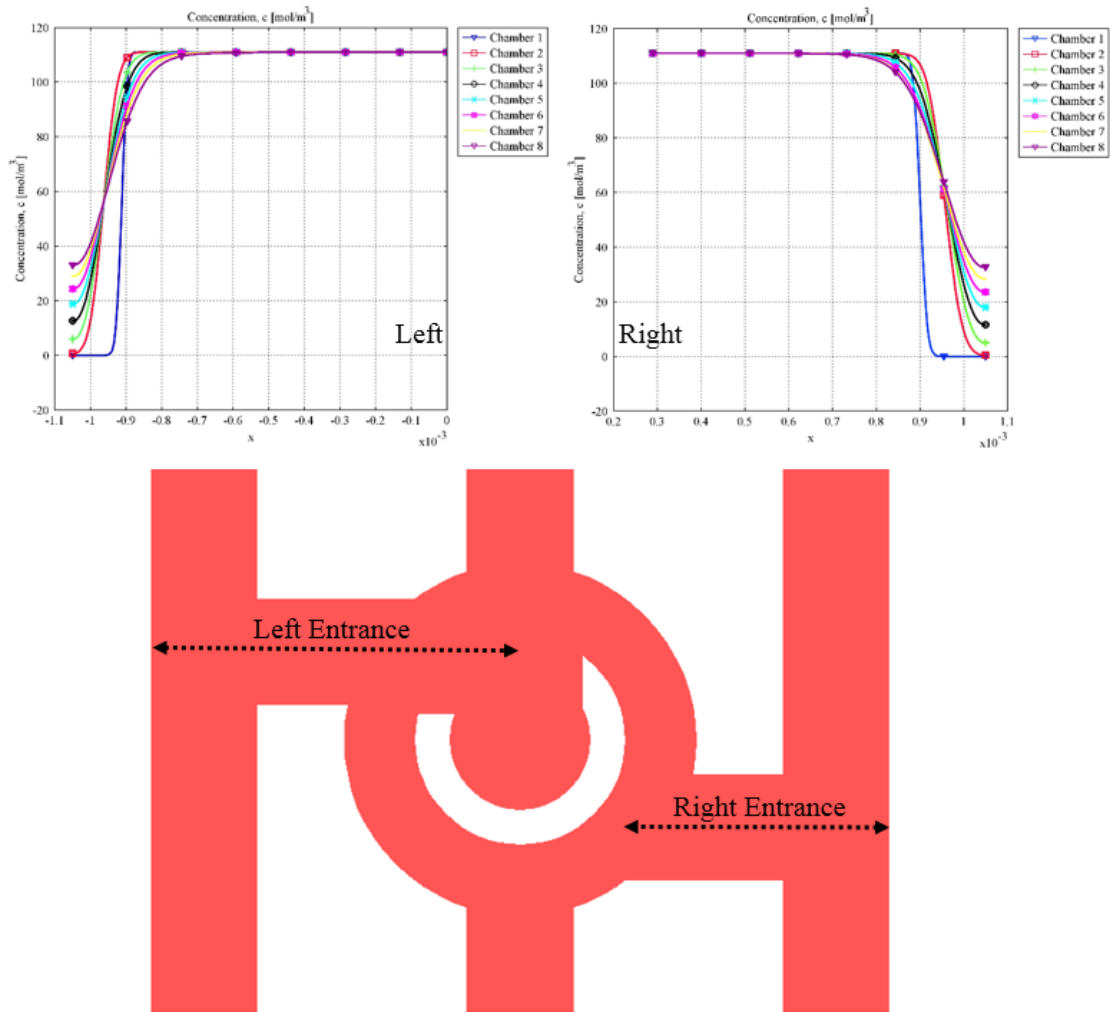


Figure 4.25. Concentration profiles at the left and right entrances of the chambers.

The concentration profiles at the chamber entrances (Figure 4.25) show that the glucose concentration of 111 mol/m^3 is successfully sustained through the chambers providing stable environment at the feed concentration. Therefore, it can be said that proper diffusion of glucose occurs in the chambers. Moreover, the concentration profile of glucose at various heights at the entrance of the first chamber is obtained in order to see the effect of height on nutrient diffusion inside the chip (Figure 4.26). There is no concentration change at different heights of the chip suggesting stable environment within the microfluidic system. For other chambers the trend in Figure 4.26 is almost same and therefore only chamber 1 is selected for representation.

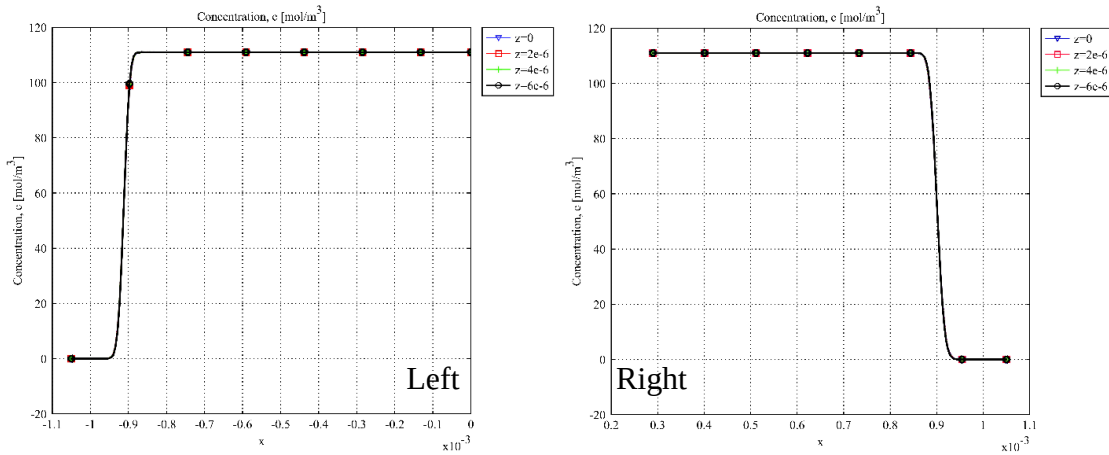


Figure 4.26. Concentration profiles in z-direction at the left and right entrances of chamber 1.

4.3.4. Optimized System: Operating Conditions for Nutrient Feeding

In the light of the above mentioned simulation studies, $1\mu\text{L}/\text{min}$ flow rate is selected for cell inlet and $0.1\mu\text{L}/\text{min}$ is chosen for nutrient inlet in order to load and trap yeast cells within the microfluidic device. When the cell loading is finished, nutrient feeding through the three inlets is started in the experiments. In order to create a stable environment within the chip and prevent cell loss from the c-shaped region as a result of flow circulation, nutrient streams are fed at the same flow rates. Previously (Figures 4.2 and 4.5), it was seen that higher flow rates such as $10\mu\text{L}/\text{min}$ and $1\mu\text{L}/\text{min}$ caused circulation and cell loss in the system. Therefore, flow rates are reduced by tenfold. The following figures illustrate the environment in the chip at $0.1\mu\text{L}/\text{min}$ flow rates.

In Figure 4.27, blue colored area shows the minimum speed and that is the desired result for nutrient feeding to keep the yeast cells stable in the c-shaped region of the device. Figure 4.28 illustrates the flow between the side channels and the chambers to maintain the stable environment in the chambers. Figure 4.29 shows that the speeds in all the chambers in the c-shaped region is similar and this is also desired for the experiments. Therefore, $0.1\mu\text{L}/\text{min}$ flow rate is selected for nutrient feeding for all the three inlets.

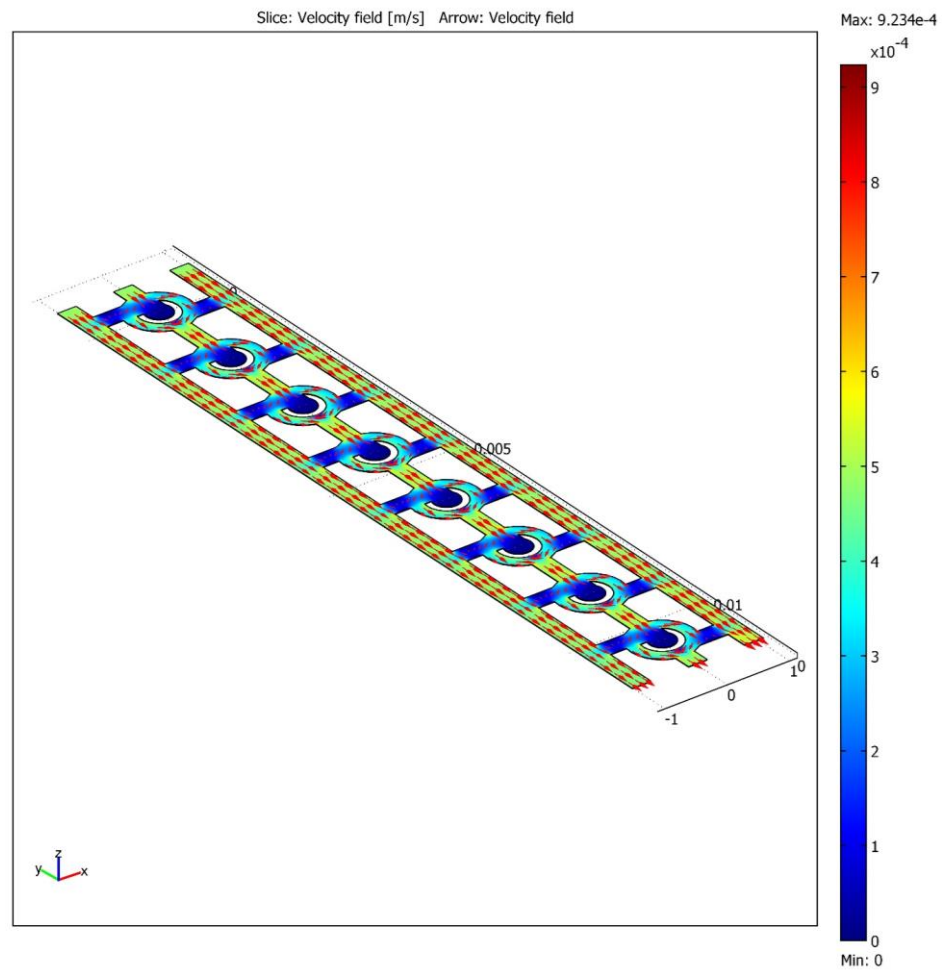


Figure 4.27. Velocity field at a flow rate of $0.1 \mu\text{L}/\text{min}$.

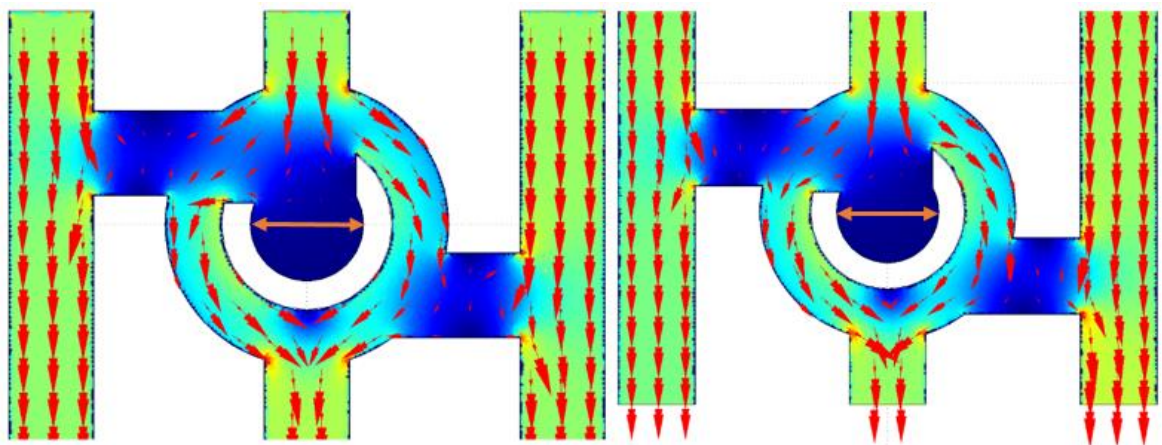


Figure 4.28. Flow distributions inside the 1st and 8th chambers ($0.1 \mu\text{L}/\text{min}$ flowrate through all inlets).

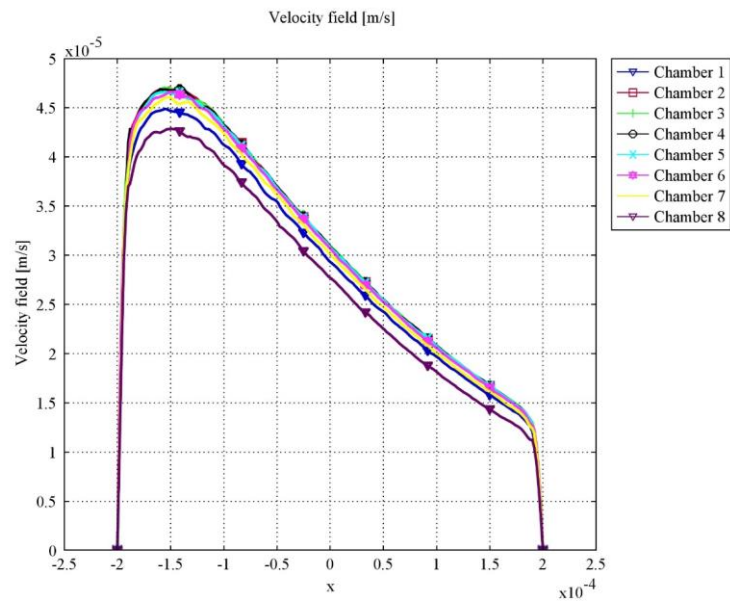


Figure 4.29. Velocities inside the c-shaped region of chambers ($0.1 \mu\text{L}/\text{min}$ flowrate through all inlets).

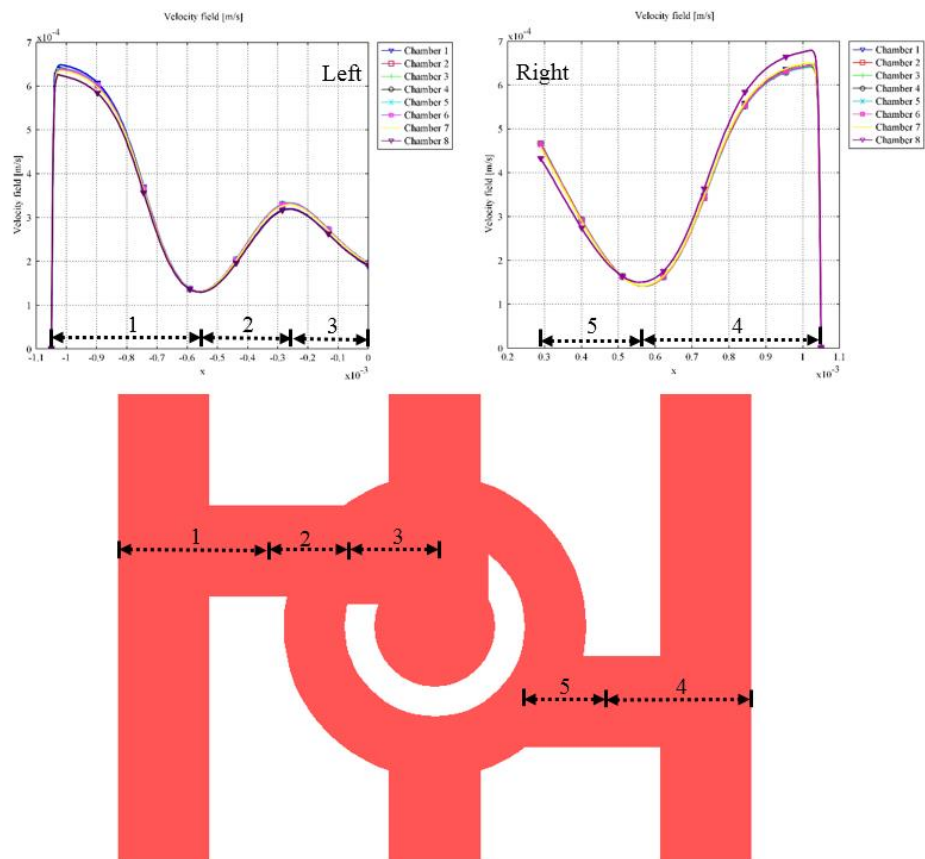


Figure 4.30. Velocity profiles of the left and right entrances of the chambers.

In order to be sure of the reliability of the model, additional analyses are performed. First, the speeds at the chamber entrances are obtained and given in Figure 4.30. The speed profiles for both left and right chamber entrances have the same trend due to equal flowrate application through the inlets. In the left entrance, the speed starts from zero as a result of no-slip condition at the walls, then increases sharply towards the center of the left side channel. When the fluid enters the connection channel between the side channel and chamber, its speed decreases (dotted line 1). Next, the speed again increases (dotted line 2) due to encountering with the flow coming from middle channel and then decreases through the c-shaped region (dotted line 3). In the right entrance, similarly, the speed increases sharply towards the center of the right side channel, and decreases at the connection channel (dotted line 4). Since right entrance is located below the c-shaped region (Figure 4.30), the fluid from channel meets with the fluid coming from the main channel in the chamber and the velocity increases in all the chambers (dotted line 5) (Figure 4.30).

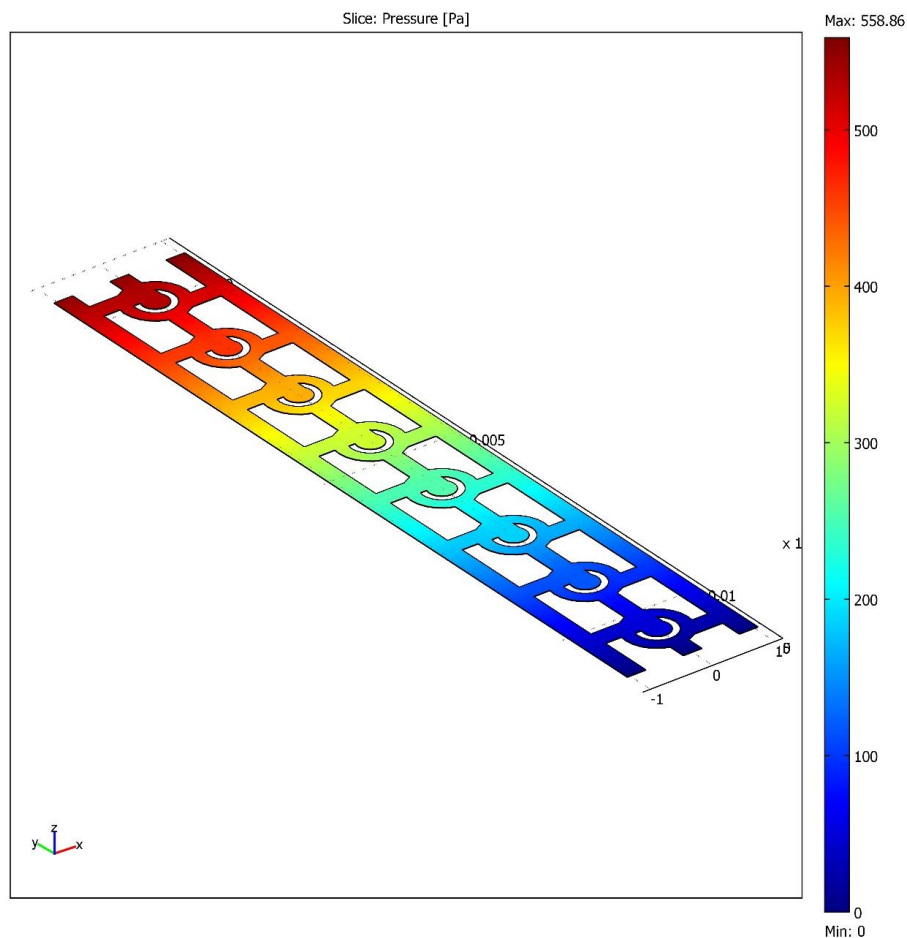


Figure 4.31. Pressure drop distribution along the device.

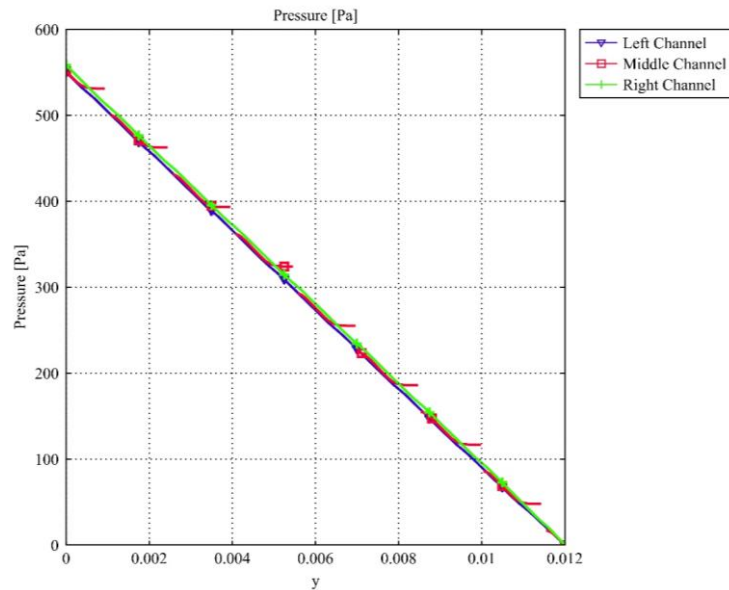


Figure 4.32. Pressure drop along the microfluidic device.

The pressure drop distribution along the chip is demonstrated under the optimized flowrates of $0.1 \mu\text{l}/\text{min}$ in Figures 4.31 and 4.32. Pressure is zero at the outlets, which are open to the atmosphere, and it is approximately 0.5% of atmospheric pressure at the inlets. Pressure difference is important in the microfluidic system to provide the flow within the chambers and channels. As smaller speeds are set to the model in the nutrient feeding part, the pressure difference is smaller at a fluid flowrate of $0.1 \mu\text{l}/\text{min}$ (Figure 4.31) than that at $1 \mu\text{l}/\text{min}$ (Figure 4.21).

Concentration profile within the microfluidic device is also obtained for nutrient feeding part. In fact, nutrient medium of equal glucose concentration ($111 \text{mol}/\text{m}^3$) should be fed through the three inlets of the microfluidic device, but in order to see the diffusion effect among the channels and chambers, nutrient medium is supplied only to the middle channel and others are set to zero glucose concentration. Figure 4.33 shows both the concentration profile and velocity profiles in the microfluidic device. It is observed that there is a gradual color change from the inlets to the outlets. In the chambers located near the inlets, concentration is higher than those in chambers located near the outlets. Therefore, it can be said that diffusion successfully occurs among the side channels and chambers within the microfluidic device.

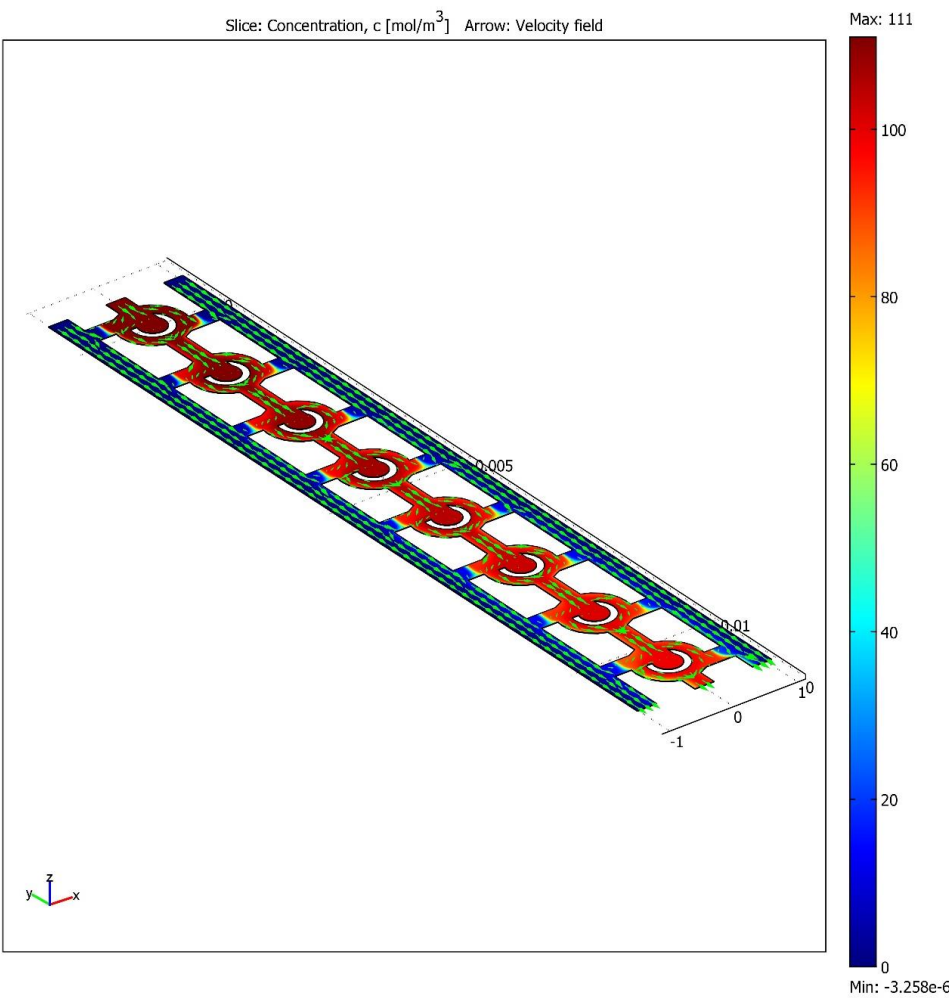


Figure 4.33. Concentration distribution along the microfluidic device.

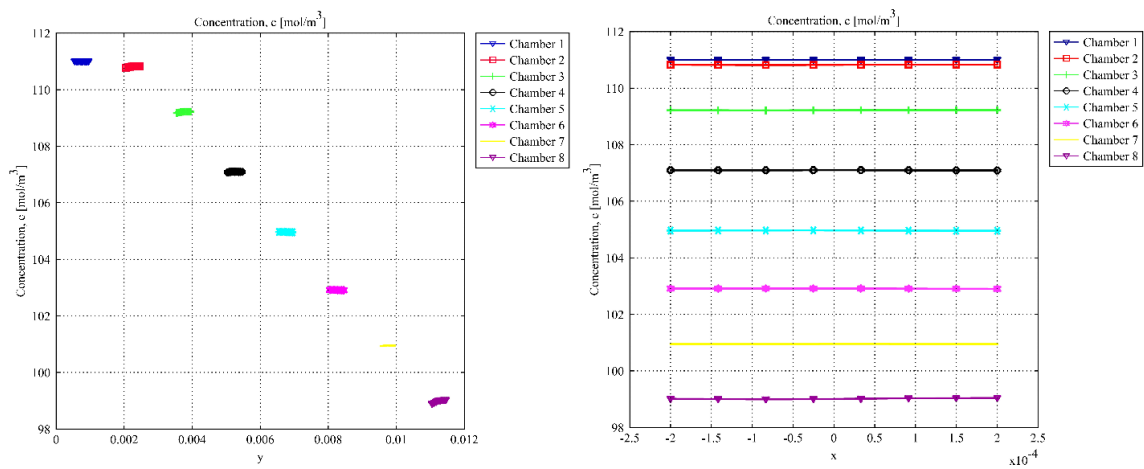


Figure 4.34. Concentration profile in the c-shaped region of the device both in y and x directions.

Figure 4.34 also confirms that, as the flow progresses from the inlets to the outlets, glucose concentration decreases and this can be considered as the evidence of the diffusion within the chip. Therefore, during nutrient feeding, when the medium of the same glucose concentration is fed to the microbio reactor, stable environment can be created providing equal conditions to yeast cells.

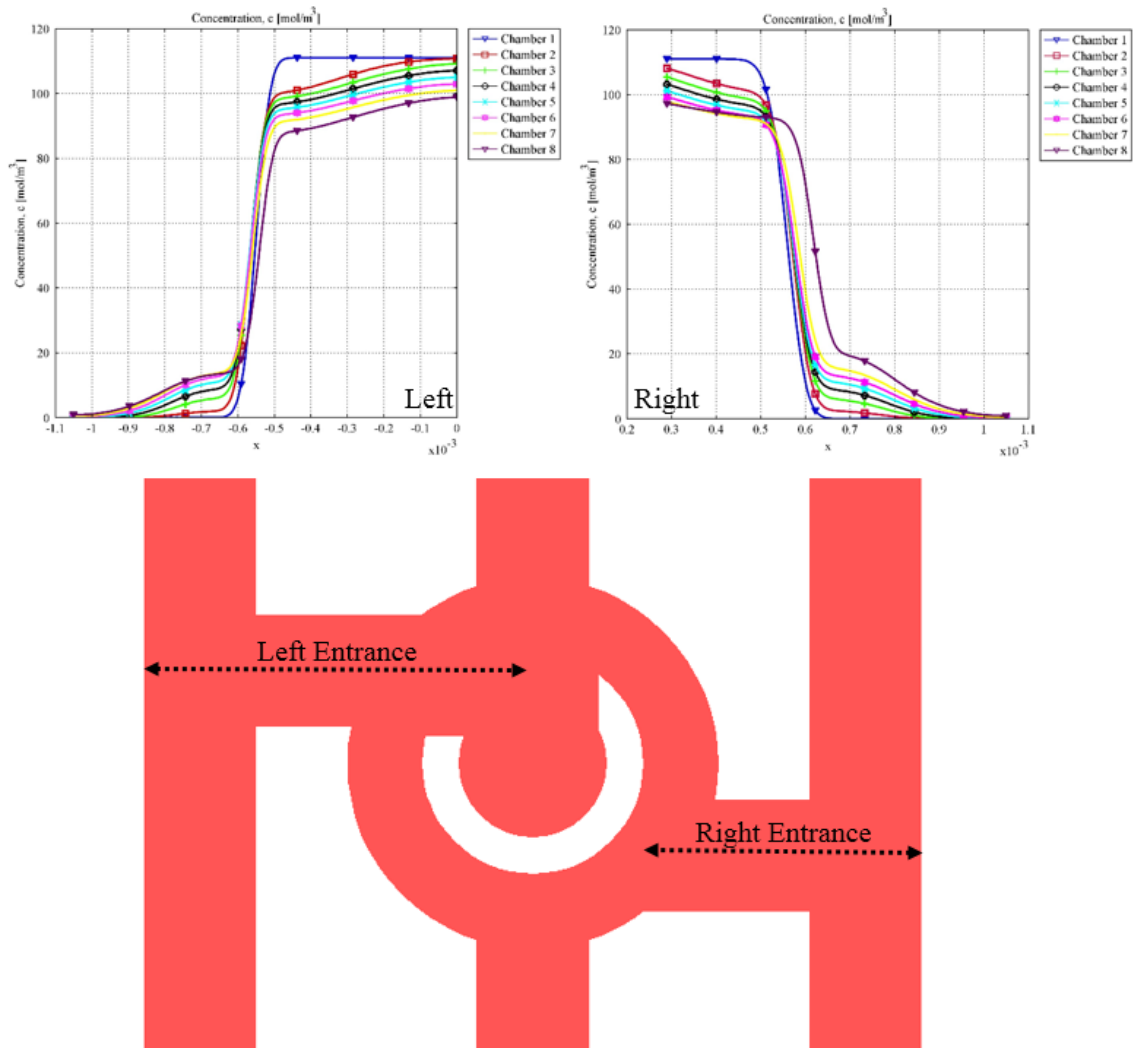


Figure 4.35. Concentration profiles at the left and right entrances of the chambers.

Figure 4.35 is obtained to analyze the concentration profiles at the chamber inlets. The left and right entrances of the chambers have the same trend lines. When progressing from chamber 1 to chamber 8, a concentration difference is observed.

As mentioned before, the channels have $12\mu\text{m}$ height. The concentration profile at the chamber entrance is also obtained at different heights for chamber 1. Figure 4.36 shows that there is no difference in concentrations at different heights. Therefore, stable environment is also attained in the z direction of the chip to feed and sustain the yeast cells.

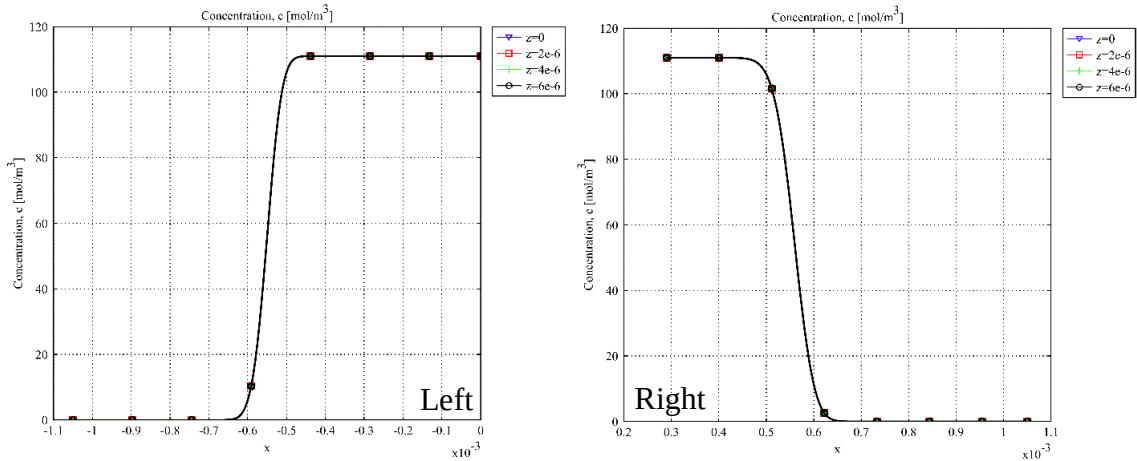


Figure 4.36. Concentration profiles of the left and right entrances of chamber 1 at different heights of the microbioreactor.

As a conclusion, 1 and $0.1\mu\text{L}/\text{min}$ for middle and side channels, respectively, during cell loading model and $0.1\mu\text{L}/\text{min}$ through the three channels during nutrient feeding model are chosen to be used as optimum flowrates in the experiments.

In the above mentioned simulations, the presence of beads and yeast cells in the chambers are neglected. Consequently, their effect on flow is also neglected. Therefore, these simulations should be considered as the first approximations of the system.

4.4. Validation of the Flow Profile in the Microbioreactor

In order to validate the fluid flow profile within the microbioreactor, the hydraulic resistances within the chamber was estimated by LTspice IV simulator. By considering the inlets of the device, the resistances were located according to possible flow profile in the chamber. Electrical circuit network representing the fluid flow in the device (the following

scheme) was adapted to simulator. Although the actual microfluidic device contains 8 chambers, the model studied is scaled to one chamber only. By using rectangular cross-section, the hydraulic resistances shown in the Figure 4.37 were calculated using the following Equation 4.1.

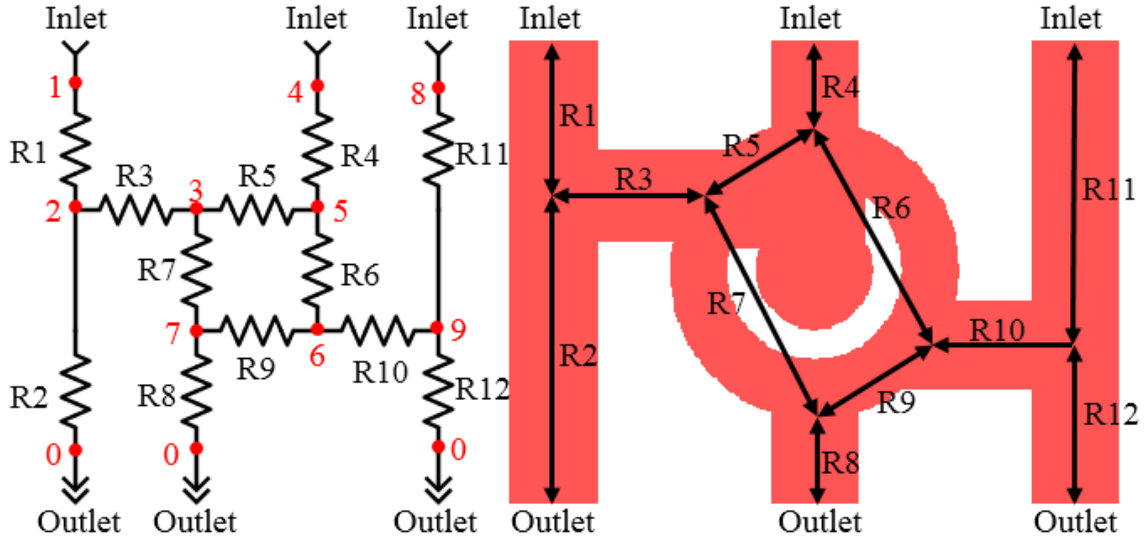


Figure 4.37. Resistance illustration in the chamber.

$$R_h = \frac{12\mu L}{wh^3(1 - 0.630h/w)} \quad (4.1)$$

Where, μ (N.s/m²) is dynamic viscosity and equals 0.000894 for 2% glucose solution, L (m) is total length, w (m) is width and h (m) is the height. As it can be seen from the resistances on the device, R1 equals to R12, R2 equals to R11, R3 equals to R10, R4 equals to R8, R5 equals to R9 and R6 equals to R7 because their lengths, widths and heights were same. Necessary calculations were done and the results were presented in Table 4.5.

After obtaining the resistances, the nodes were determined (shown in Figure 4.37 as red points) and these resistance values were entered to the simulator by using nodes. For example, since R1 was between node 1 and 2, it was written as R1 1 2 1.061E+13. 1.61E+13 was the resistance value of R1 and the following figure (Figure 4.38) shows the simulator's

workspace and results. The simulation of cell loading was considered, therefore I2 was taken as 1 and I1 and I3 were taken as 0.1.

Table 4.5. Resistance calculations.

	R1=R12	R2=R11	R3=R10	R4=R8	R5=R9	R6=R7
Length (m)	0.0005	0.0015	0.00047	0.00085	0.00048	0.00025
Width (m)	0.0003	0.0003	0.0003	0.0002	0.0002	0.0003
Height (m)	1.20E-05	1.20E-05	1.20E-05	1.20E-05	1.20E-05	1.20E-05
Viscosity (N.s/m2)	0.000894	0.000894	0.000894	0.000894	0.000894	0.000894
Rh (N.s/m5) or (Pa.s/m3)	1.06E+13	3.18E+13	9.98E+12	2.74E+13	1.55E+13	5.31E+12

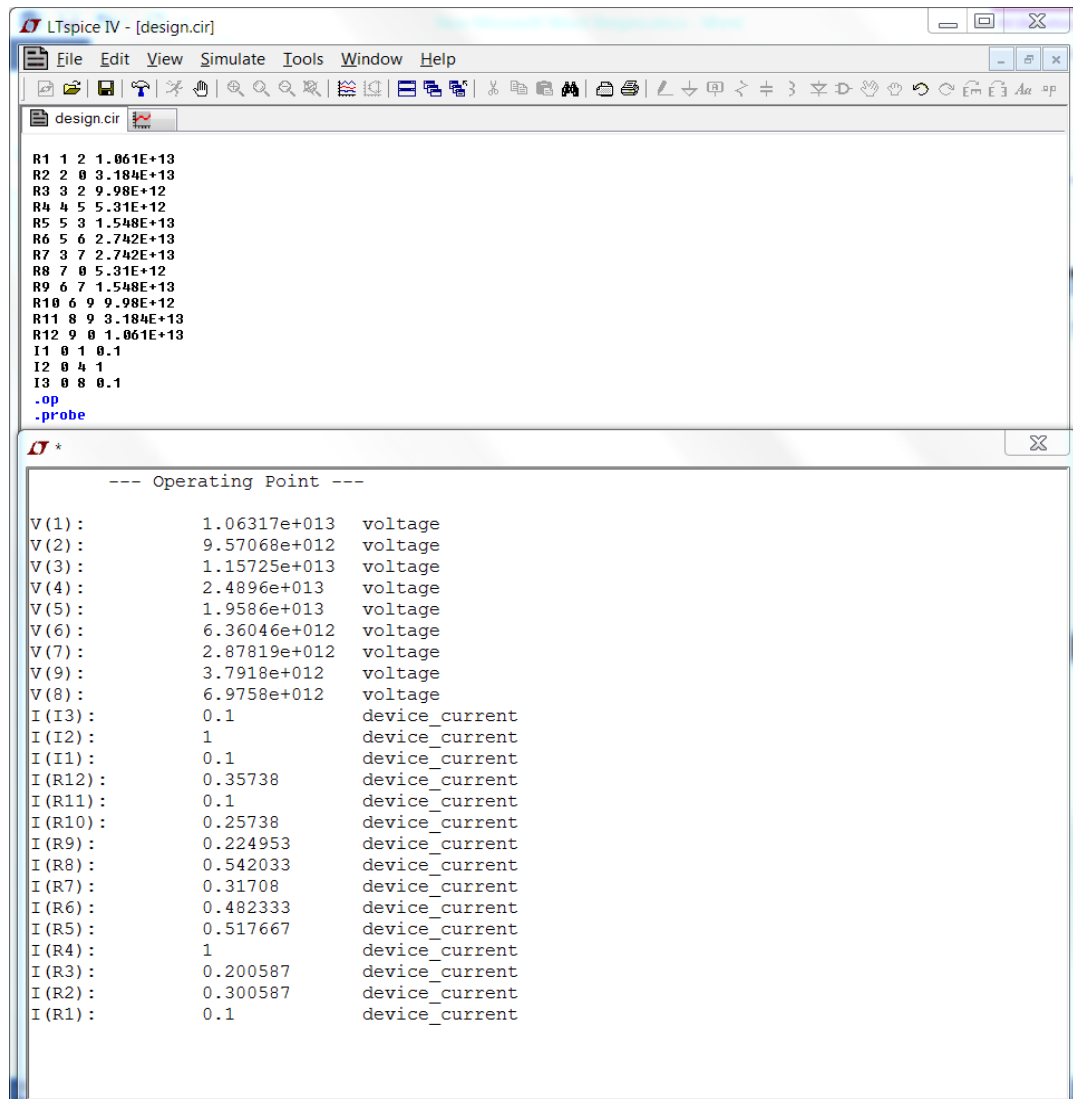


Figure 4.38. LTspice workspace and results.

The flow profile obtained is shown on the following figure with red arrows (Figure 4.39):

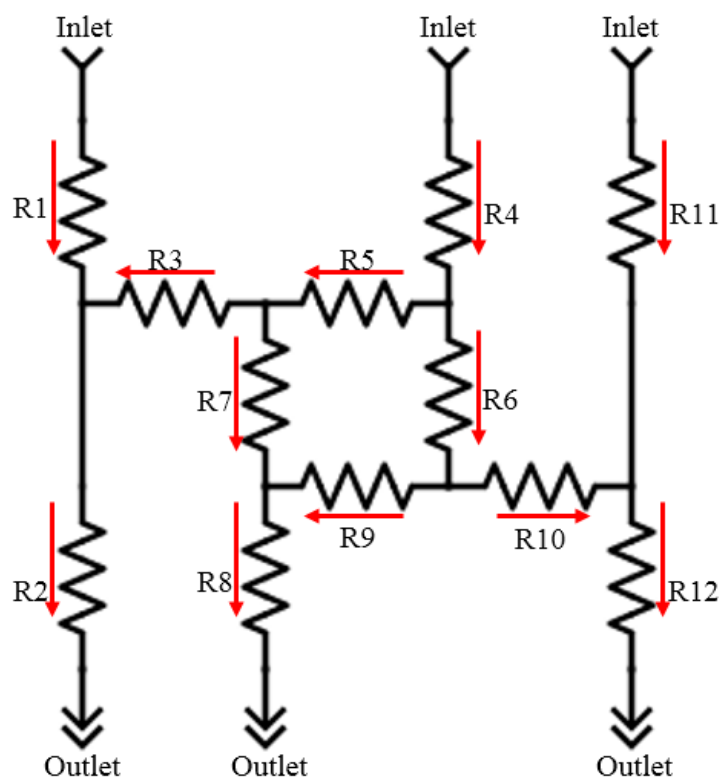


Figure 4.39. Flow profile in the microfluidic device.

According to Figure 4.39, yeast cells were loaded from the middle inlet at $1\mu\text{L}/\text{min}$ flow rate and passed through the resistance, R4. Nutrients were fed from the other inlets at $0.1\mu\text{L}/\text{min}$ flow rates and passed through R1 and R11. Yeast cells entering the c-shaped region passed through R5 and R6. R5 is split into R3 and R7 and R6 is split into R9 and R10. Since R3 and R10 represent the connection channels between the chamber and side channels, as in the COMSOL simulation, the fluid on R3 and R10 flows towards the side channels. Therefore, the accuracy and reliability of results on fluid flow obtained by COMSOL were here validated. In order to test these simulation results as well as the operation of the fabricated microbioreactor, preliminary experiments are done by using polystyrene beads of white $2\mu\text{m}$ diameter and blue colored beads of $10\mu\text{m}$ diameter instead of yeast cells. In the following section, the results of these experiments with beads will be given.

4.5. Preliminary Experiments with Polystyrene Beads

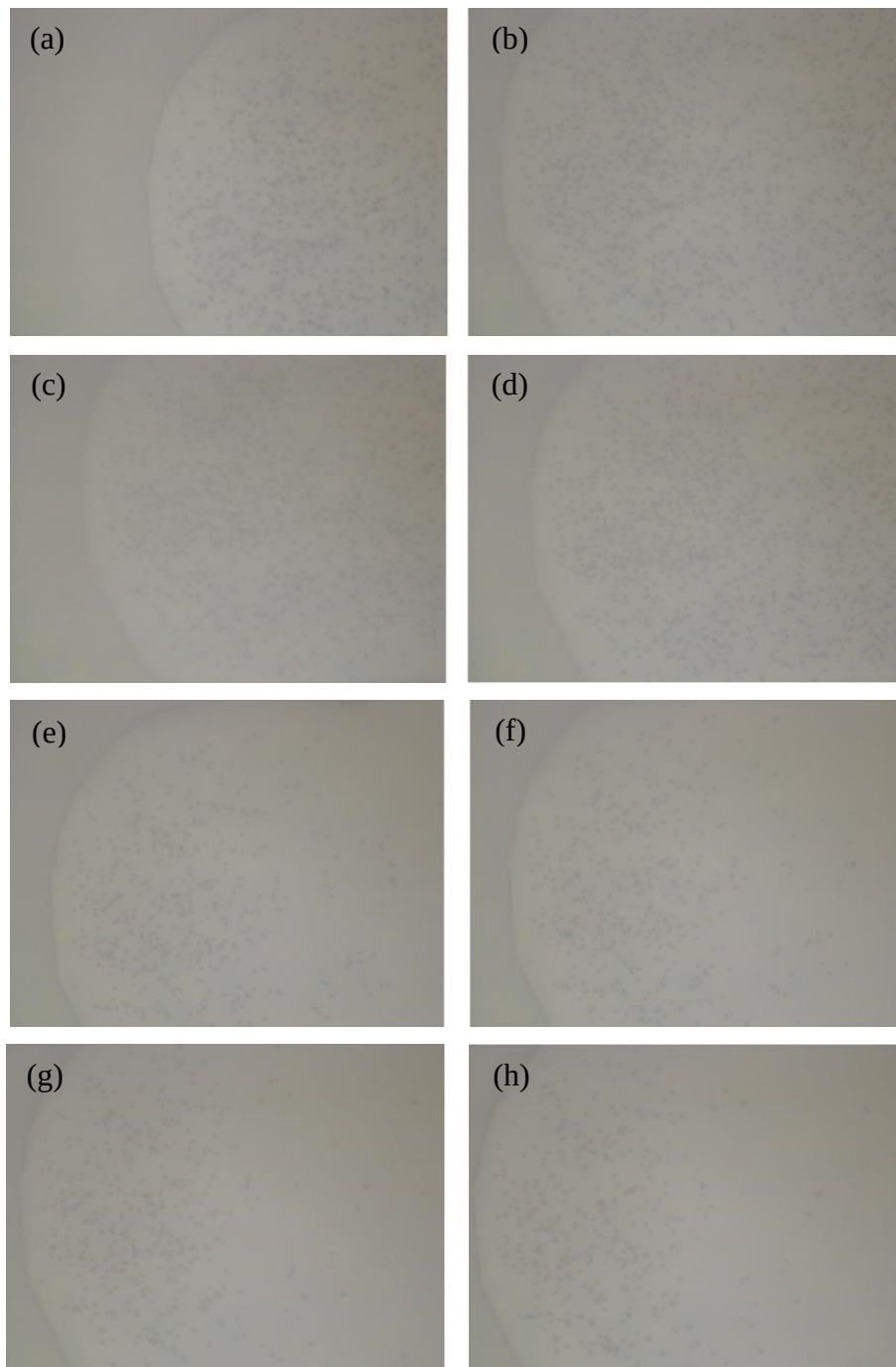
The preliminary experiments are carried out by using two different types of beads, one of them is white-transparent beads of $2\mu\text{m}$ diameter and the other one is blue colored beads of $10\mu\text{m}$ diameter. During the experiments, bead movements are monitored and several photos are taken to evaluate the conditions within the microfluidic system. The first experiment is accomplished by white beads to analyze the cell trapping and nutrient feeding operations within the device. The reason of selecting smaller beads initially is to prevent the clogging of the device as the chip height is $12\mu\text{m}$. However, yeast cells may create multi-cell clusters. Therefore, blue beads (bigger ones) are used to predict and observe the behavior of yeast cell clustering during the experiments.

4.5.1. Experiments with Beads of $2\mu\text{m}$ Diameter

6 tubings made of silicon were connected to the fabricated microfluidic chip with the help of epoxy and left drying at room temperature. During drying process, bead solution was prepared. 1mL white beads were taken from the 5mL bead solution (10% solids concentration) with the help of the syringe, and diluted in sterile distilled water. Since there are three inlets in the device, the bead solution was charged into a syringe connected to the middle channel and another two syringes were filled with pure water. Syringes were fitted to the syringe pumps and pumps were set to the values optimized by COMSOL Multiphysics program. According to the simulation results, the flow rate of the bead carrying syringe pump was set to $1\mu\text{L}/\text{min}$ and other pumps were set to $0.1\mu\text{L}/\text{min}$ in order to mimic cell loading step. Beads were fed through the middle inlet for trapping them in the c-shaped region and other inlets were charged with pure water. Bead loading was continued until all chambers (1.5 nanoliter volume), were loaded. Next, when the bead loading was finished, pure water was fed through the middle inlet at $0.1\mu\text{L}/\text{min}$ flow rate together with other side inlets and the experiment was conducted for at least 6 hours. During the bead loading and water feeding, photos were taken at 30min time intervals utilizing CCD camera, and chamber 3 was selected for demonstrating the situation inside the chip.

As it can be seen from Figure 4.40, in the beginning of the experiment, too much beads were trapped in the c-shaped region due to bead loading. Then, the number of beads

decreased during water supply and finally stable environment was created (Figure 4.40, photos j, k and l). Therefore, it can be said that due to continuous bead supply during loading, many beads were trapped in the c-shaped region and excess beads were flushed away during water supply. Afterwards, as time goes by, trapped beads conserved their positions in the chip and no bead loss was observed from the outlets. However, these beads could not be removed from the chip, bead precipitation may have occurred in the microfluidic device.



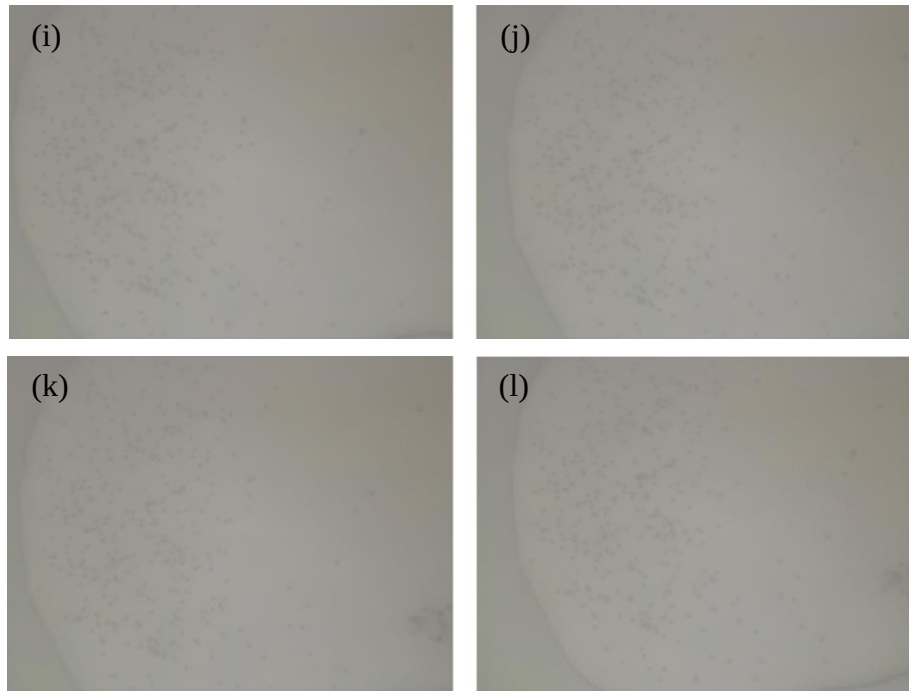


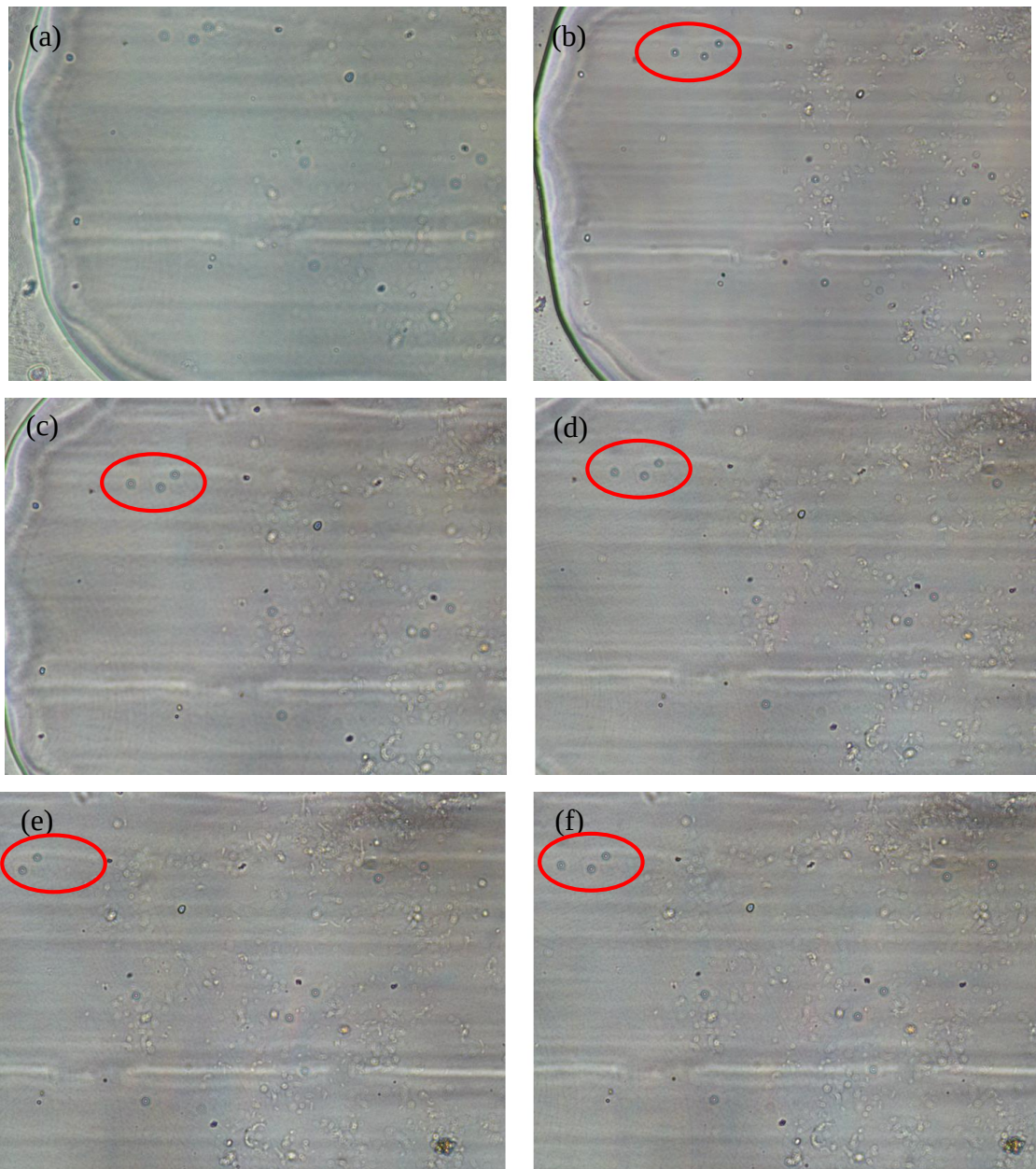
Figure 4.40. White beads trapped in the c-shaped region (time passes through (a) to (l)).

4.5.2. Experiment with Blue Colored Beads of 10 μ m Diameter

The experiment with bigger beads was conducted with another in-house fabricated microfluidic device. Again, 6 silicon tubings were connected to the chip with epoxy and left drying at room temperature. 1mL blue beads (10 μ m diameter) were taken from the 5mL bead solution (5% solids concentration) and diluted with pure water. Diluted beads were transferred into a syringe. 3 syringes (one with blue beads and the other two with pure water) were fitted to the syringe pumps. A flow rate of 1 μ L/min was set for the bead syringe pump and flow rates of 0.1 μ L/min were set for pure water syringe pumps and bead loading through the middle inlet was started. During bead loading, pure water was also fed to the device via side inlets and image capturing was initiated. When all the chambers contained blue beads, bead supply was stopped and water supply was started at the middle inlet at a flow rate of 0.1 μ L/min in conjunction with side inlets. The photos of chamber 3 are shown in the following figure (Figure 4.41).

In this experiment, blue beads were more diluted than previous white beads to see the beads separately in the c-shaped region. Therefore, more controlled environment was created

from start to end of the experiment. The beads surrounded by a red circle on Figure 4.41 is the indicator of the stable environment in the chip, since they conserved their positions during the experiment. In addition, the aim of using blue beads (10 μ m diameter) is to monitor whether clogging appears or not. It can be seen from Figure 4.41 that clogging was not occurred and the experiment was finished successfully. Therefore, during the experiment with yeast cells in the fabricated microfluidic chip, clogging is not to be observed even though multi-cell clusters occur within the device. As a conclusion, it can be said that, designed microfluidic chip (microbioreactor) is suitable for yeast cell culturing.



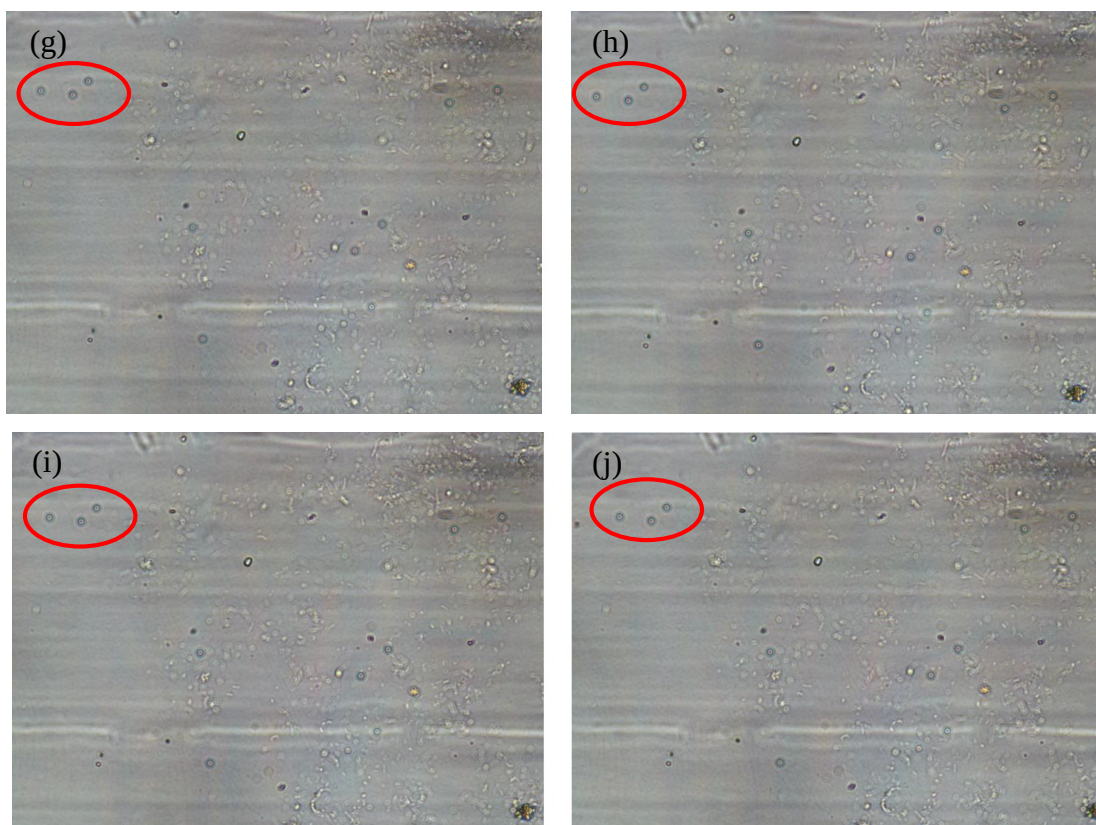


Figure 4.41. Blue beads trapped in the c-shaped region (time passes through (a) to (j)).

4.6. Yeast Culturing in Microfluidic Device (Microbioreactor)

In order to demonstrate the functionality of the in-house developed microfluidic device for living cells, yeast (*Saccharomyces cerevisiae*) was used as the model organism. The yeast strain used in this study was obtained from Prof. Steve Oliver, Cambridge University, Department of Biological Sciences, UK. The wild type (WT) BY4741 strain was derived from the parental S288C strain and has the following base genotype: MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*. The WT strain was transformed with the gene coding for enhanced green fluorescent protein (GFP) and GFP tagged YOR060C strain was obtained.

The Lab-on-a-Chip platform was prepared for yeast culturing experiments. The microfluidic device (microbioreactor with chambers of 1.5 nanoliter volume) was mounted onto a Nikon E200 fluorescent microscope. 6 connection tubings were stuck to the device by epoxy and left drying at room temperature. Diluted yeast preculture (OD=0.5) was filled into the syringe (60 ml) and three other syringes (each 60 ml) were filled with YPD medium.

Tubings connected to the chip were located under the microscope, nutrient syringes (containing YPD medium) were fitted into the syringe pumps and perfusion of the chip with medium was started.



Figure 4.42. Experimental setup.

When all the chambers were filled with YPD medium, yeast cell loading was initiated through the middle inlet. After cell loading, continuous nutrient feeding through all channels was accomplished and the experiment lasted for 28 hrs. In order to follow the growth of yeast cells in microbio reactor, yeast cells were imaged with a 40x air objective ($NA=0.65$), and both brightfield and fluorescence microscopy images were taken at certain time intervals during the experiment. All the photos taken were processed via Fiji/ImageJ software and the results are given in the following sections. The parameters such as cell count, cell area and cell perimeter of GFP tagged yeast strain (YOR060-GFP) were determined by processing the brightfield and fluorescence images. Integrated density of cells was additionally obtained for fluorescence images to measure the amount of luminescence.

4.6.1. Chamber 1

As mentioned above, the yeast cells were monitored in 8 chambers of the microbioreactor. As the sign of cell growth, the change in cell count, cell area and cell perimeter with respect to time were determined in each chamber. The following figures (Figures 4.43-46) show the results of the brightfield microscopy images for chamber 1:

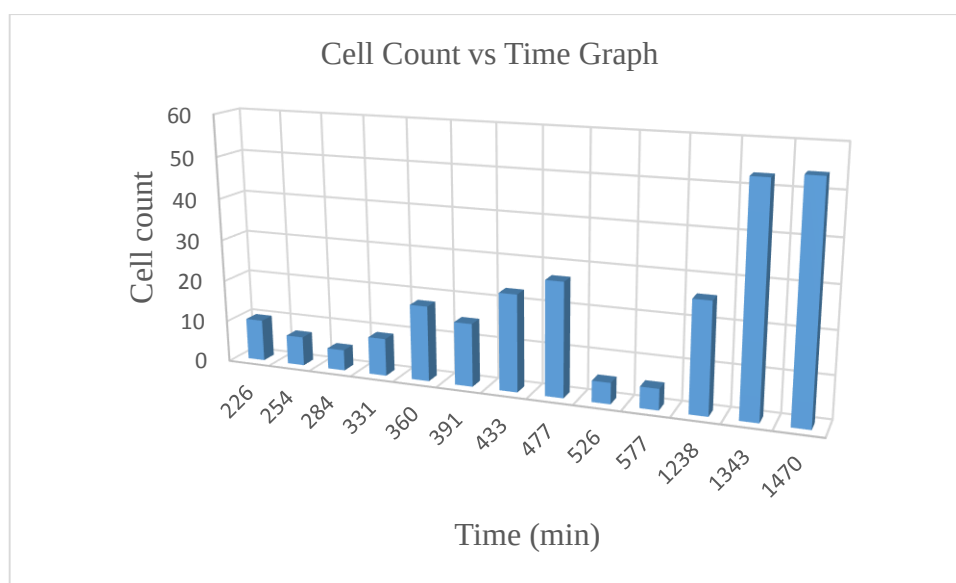


Figure 4.43. Cell count vs time graph for chamber 1 (brightfield images).

According to Figure 4.43, the cell number in the first chamber shows an increasing trend. Although, image taking was started at zeroth time, no cell was counted in the first 226 minutes due to cell loading. In between 226th and 477th minutes there was an increase in cell number with some fluctuations. Sharp decrease was observed in 526th and 577th minutes but as time goes by, the number of cells was increased in the chamber. Therefore, it can be said that, since number of cells increase throughout the experiment, yeast cells proliferated and culturing was performed successfully.

In Figure 4.44, the cell area vs time was presented. The trend on cell area was similar to that in cell number in Figure 4.43, and there was sharp decrease again in 526th and 577th minutes. Although an increasing trend was observed after the sharp decrease, the final area value was smaller than the value at 477th minutes. This situation might be the result of cell

sedimentation in the chip. As the number of cells increase in the chambers, cell sedimentation was the inevitable result.

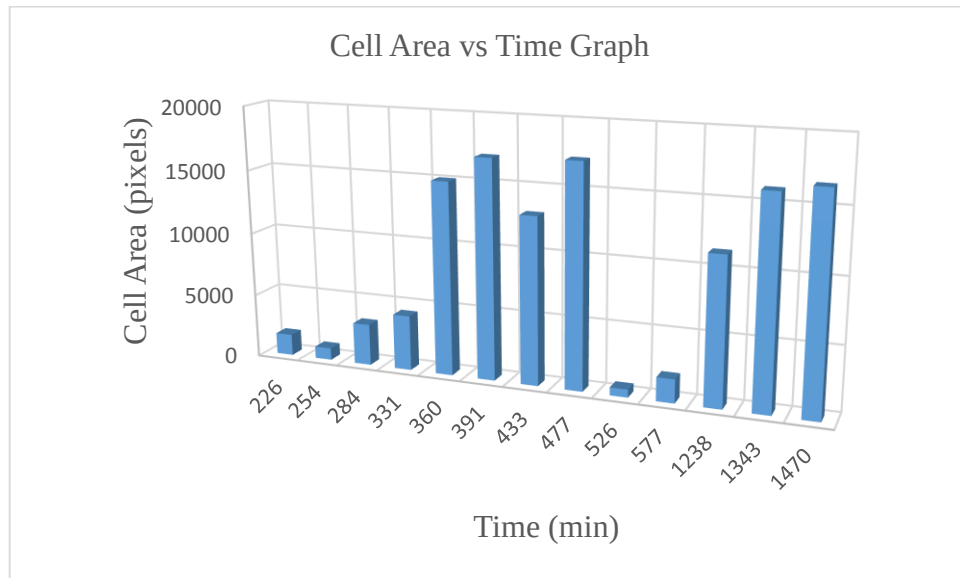


Figure 4.44. Cell area vs time graph for chamber 1 (brightfield images).

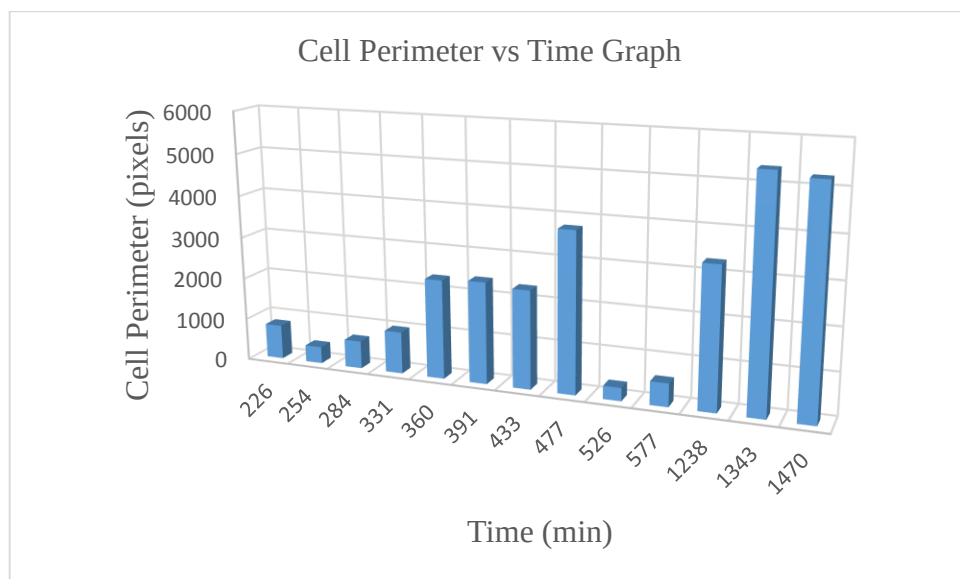


Figure 4.45. Cell perimeter vs time graph for chamber 1 (brightfield images).

Figure 4.45 shows the cell perimeter vs time graph in chamber 1. There was an increasing trend towards the 477th minute and then a sharp decrease was observed at 526th

and 577th minutes as in the previous figures. Cell perimeter was increasing after this sharp decrease, but its final value was smaller than the previous one and this can again be explained by cell sedimentation in the device. Due to the sedimentation, the monitoring of the cells gets difficult.

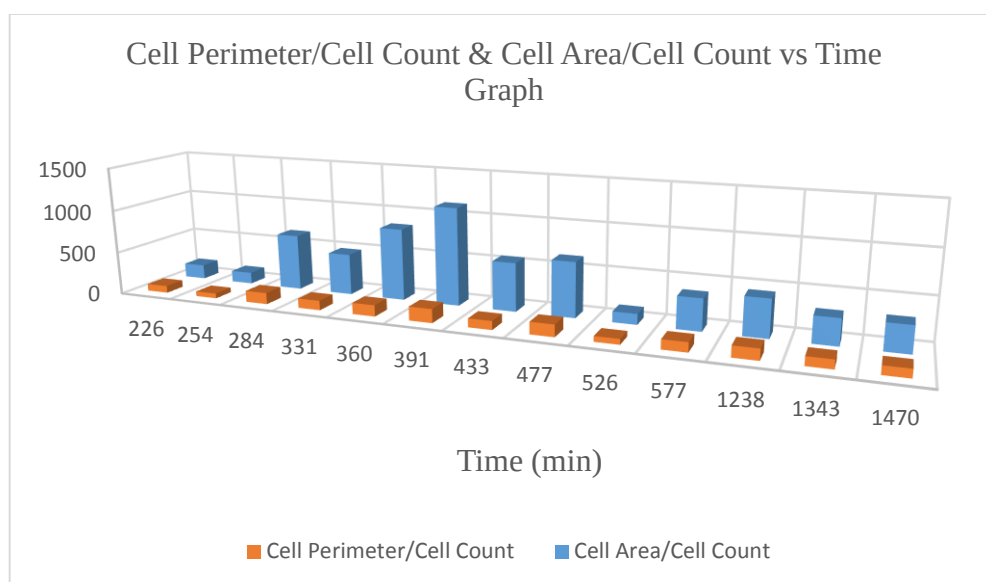


Figure 4.46. Cell perimeter/cell count and cell area/cell count vs time graph (brighfield images).

In addition to the former figures, the graphs of cell perimeter/cell count and cell area/cell count vs time were also given. In the graph of cell perimeter/cell count, the trend is almost same but in the graph of cell area/cell count there were excessive fluctuations. As the cell number increases, cell area and cell perimeter should also increase and there must be constant relation between them. This situation was achieved successfully in cell perimeter/cell count. Towards the end of the experiment, the values of both cell area/cell count and cell perimeter/cell count reach a relatively stable level.

Finally, since the yeast strain is GFP tagged, the integrated density of the cells vs time graph was obtained to see the fluorescence luminescence during culturing and Figures 4.47 and 4.48 show the results. At the beginning of the experiment, a high luminescence was observed due to excess yeast cells. After 332nd minute, an increase in the cell density (fluorescence intensity) could still be monitored.

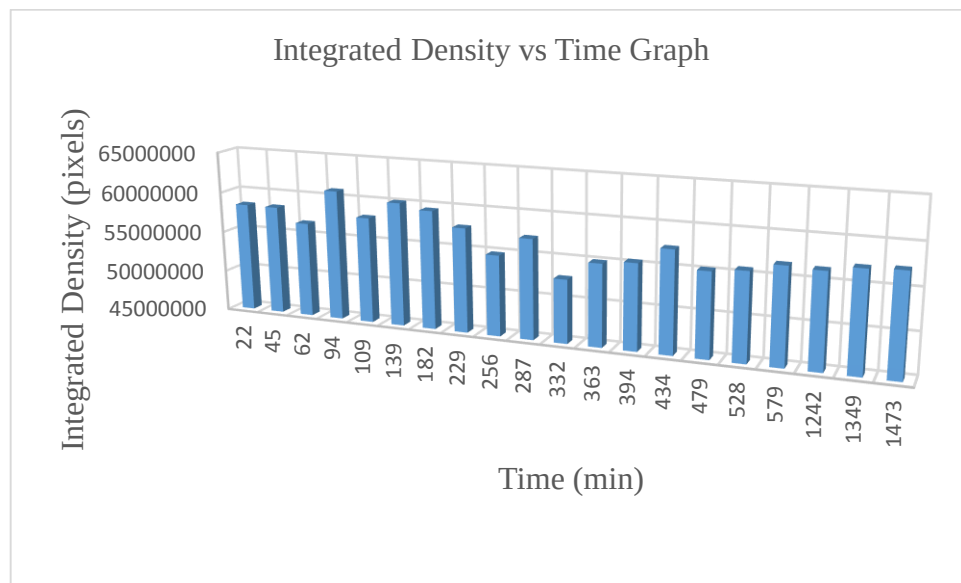


Figure 4.47. Cell integrated density vs time graph for chamber 1 (fluorescence images).

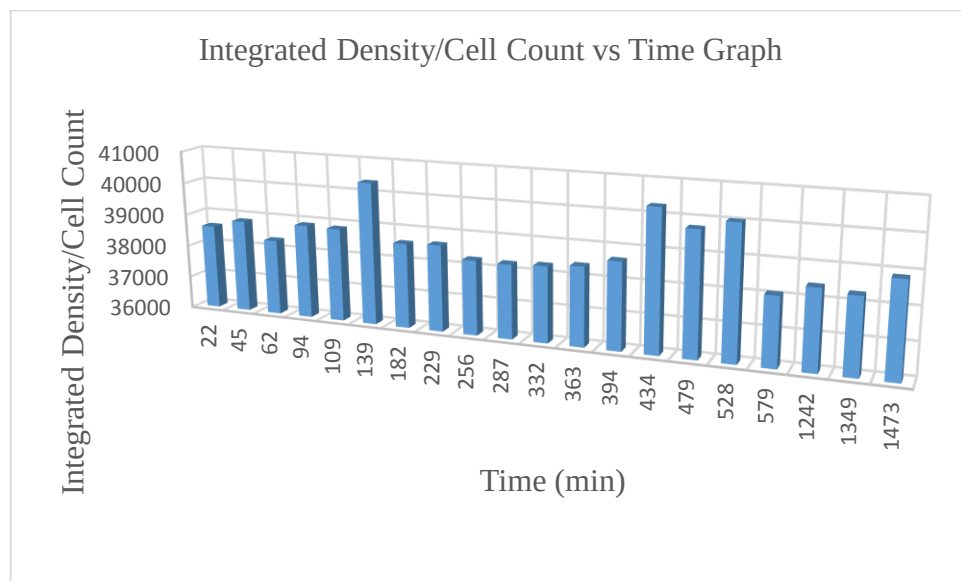


Figure 4.48. Cell integrated density/cell count vs time graph (fluorescence images).

The graph of integrated density/cell count vs time has also a stable trend around 38000 except a few outliers. It can be summarized for chamber 1 that, due to its closeness to the middle inlet (cell loading inlet), at the beginning of the experiment too much cells were trapped but after a while, by removing excess cells via nutrient flow, the conditions within the chamber had reached a stable level.

4.6.2. Chamber 2

The results of brightfield microscopy images processing are given in the following graphs (Figures 4.49-52) and cell count, cell area and cell perimeter are evaluated via the taken images. First, cell count vs time graph is shown (Figure 4.49):

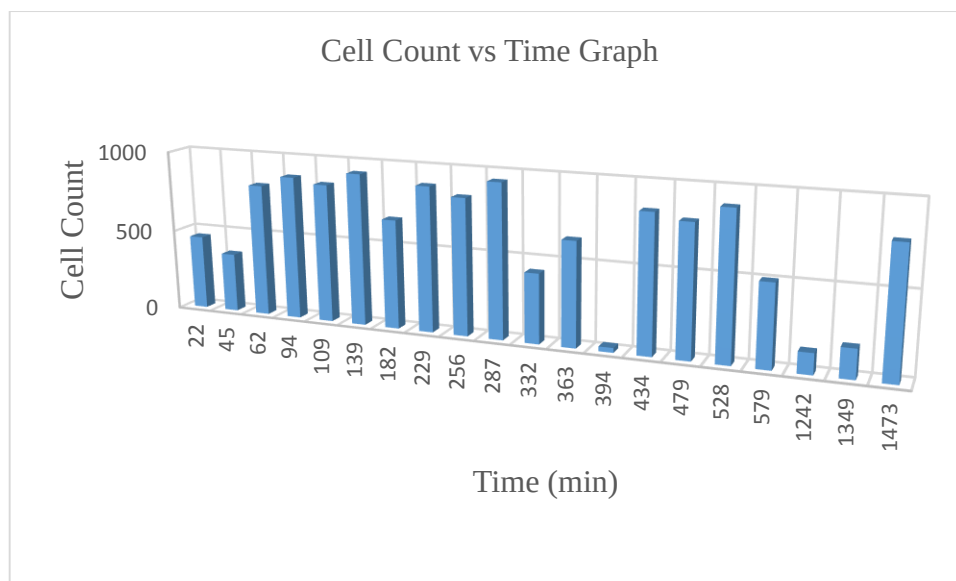


Figure 4.49. Cell count vs time graph for chamber 2 (brightfield images).

According to Figure 4.49, there was a fluctuating (increasing and decreasing) trend in cell number. The maximum value was obtained at 287th minutes. Cell number should be increasing towards the end of the experiment and after 332nd minutes, there was an increase except a few outliers and then after 528th minute sharp decrease was observed. Since the final cell number is smaller than the maximum value, cell sedimentation within the chip may have occurred.

Figure 4.50 represents the cell area vs time graph of chamber 2. Maximum cell area is observed at 22nd minutes due to cell loading through the chamber. Again, after 332th minutes increasing trend in cell area was detected except some outliers such as the data at 1242nd and 1349th minutes. Although, ups and downs were seen in the graph, towards the end of the experiment increasing trend was observed.

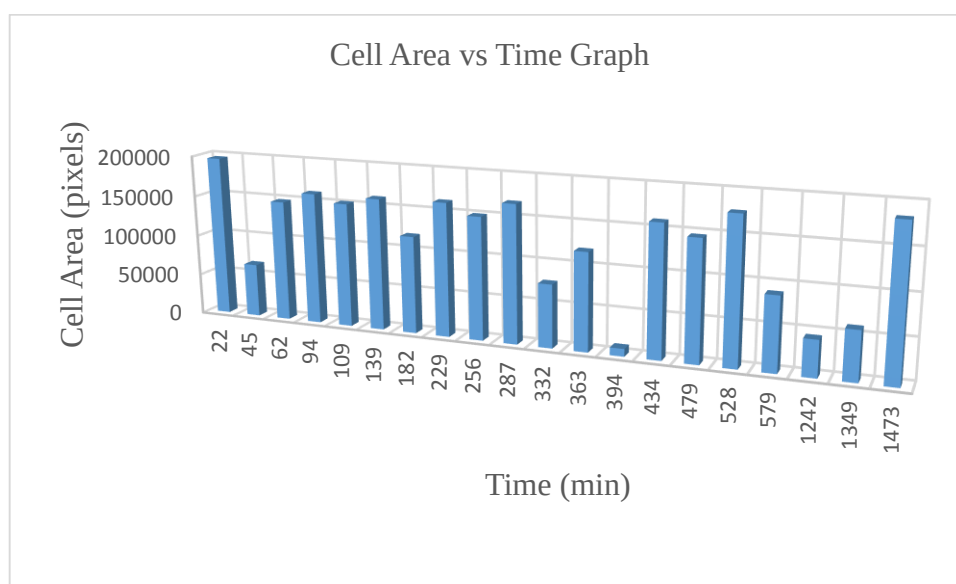


Figure 4.50. Cell area vs time graph for chamber 2 (brightfield images).

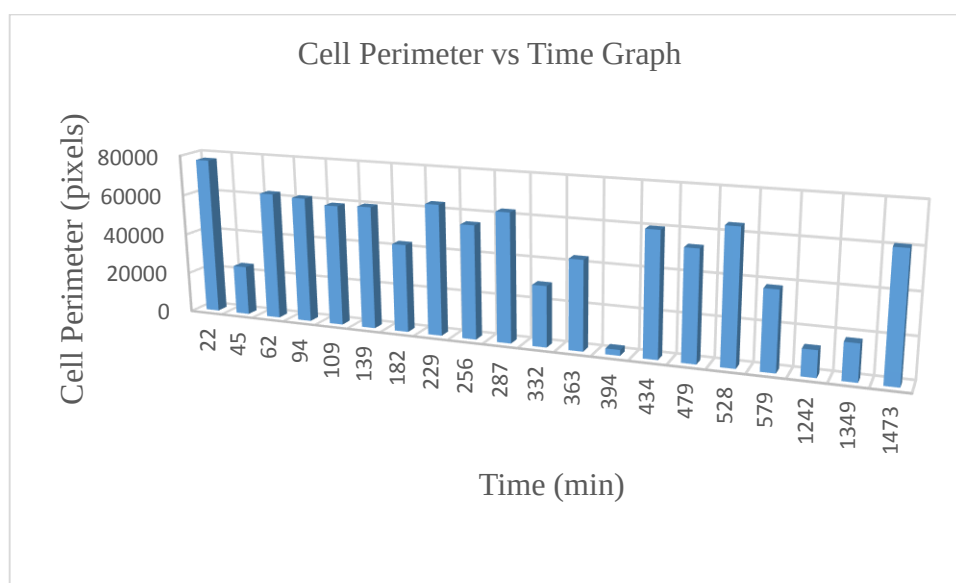


Figure 4.51. Cell perimeter vs time graph for chamber 2 (brightfield images).

Cell perimeter vs time graph also shows up and down trend like the previous two graphs. It can be said that 332nd minute was the turning point of chamber 2 as it was the case for chamber 1. When some of the outliers such as data at 394th or data at 1242nd minutes were eliminated, increasing trend could be observed. In addition, cell sedimentation effect was also detected in this graph. Ups and downs can be explained by the mobility of the yeast

cells in the chamber. There must be times that cells move towards the top (closer to the microscope lens) or bottom of the chip and that might affect the results.

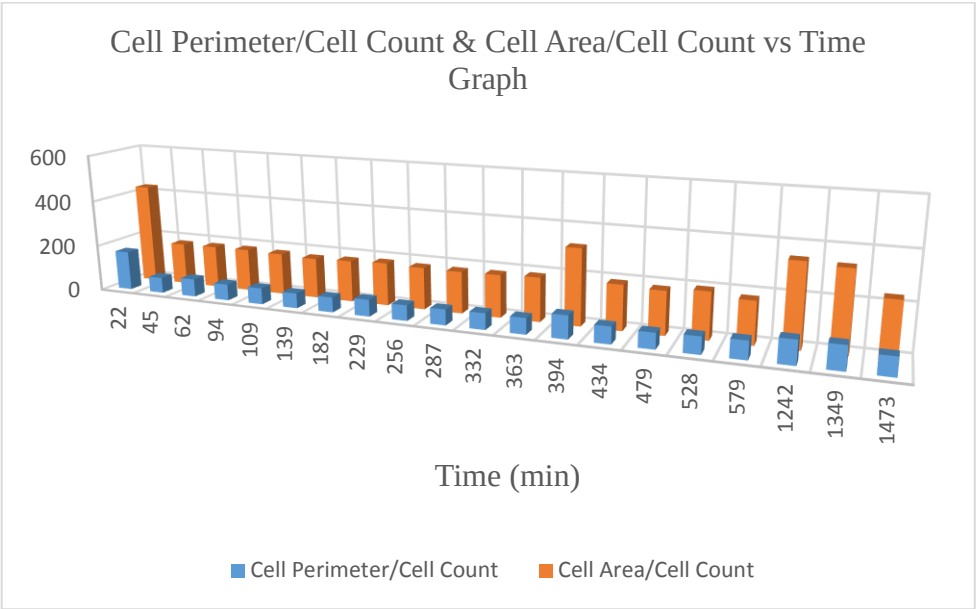


Figure 4.52. Cell perimeter/cell count and cell area/cell count vs time graph (brightfield images).

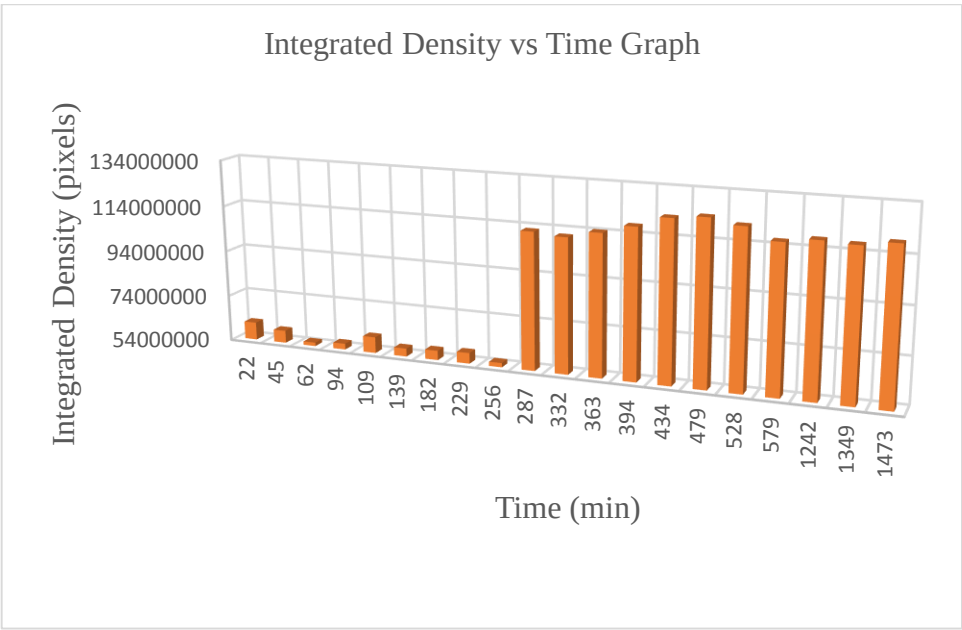


Figure 4.53. Cell integrated density vs time graph for chamber 2 (fluorescence images).

Although previous graphs did not display the expected trends due to highly fluctuating results, Figure 4.52 is a satisfying graph. Cell perimeter/cell count was almost fixed at 67 and cell area/cell count was fixed around 180 with some outliers. According to this graph, it can be said that, stable environment was created in chamber 2.

After analyzing brightfield microscopy images of chamber 2, fluorescence microscopy image processing results are given in the following figures (Figure 4.53-54). Figure 4.53 displayed that integrated density of the cells also increased throughout the experiment. Cell integrated density/cell count vs time graph fluctuated between 37000 and 41000. Decreasing trend was observed at the end of the experiment, luminescence of the cells seemed to decline while the cell number was increasing.

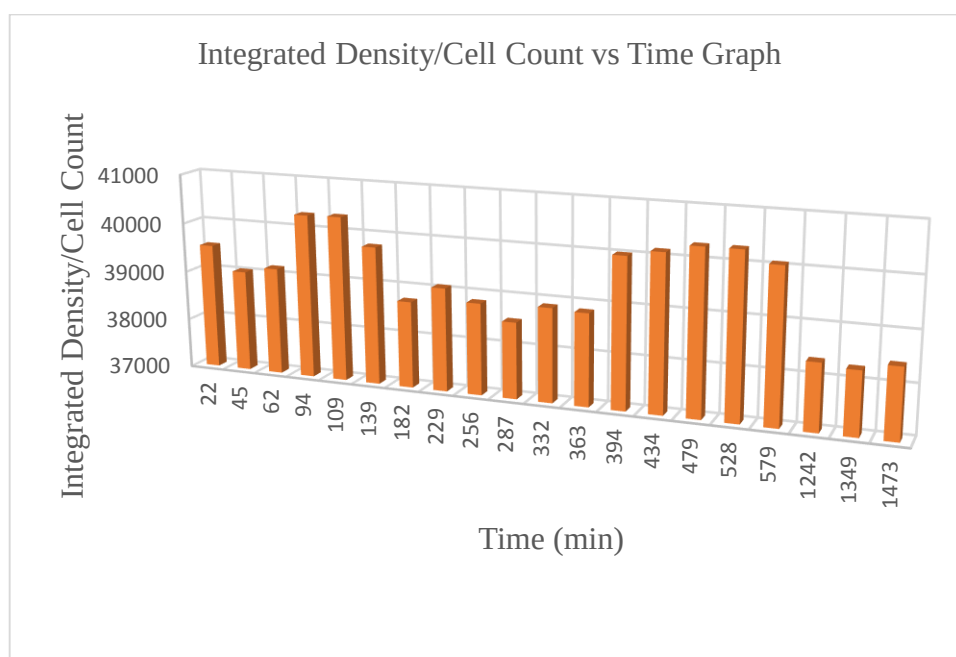


Figure 4.54. Cell integrated density/cell count vs time graph (fluorescence images).

4.6.3. Chamber 3

In this section, the results of brightfield and fluorescence microscopy images for chamber 3 were analyzed. First brightfield images were handled and cell count, cell area and cell perimeter graphs were given (Figures 4.55-57):

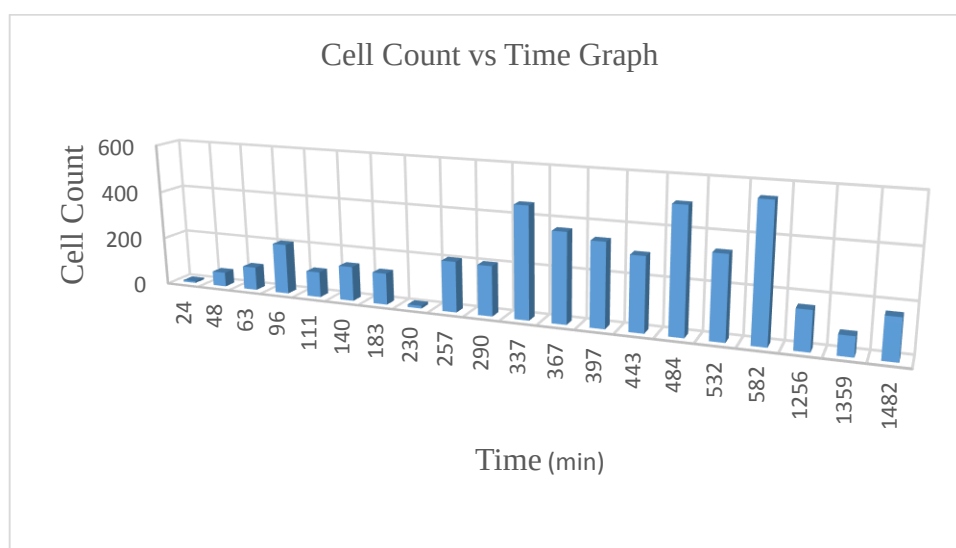


Figure 4.55. Cell count vs time graph for chamber 3 (brightfield images).

According to Figure 4.55, cell number was roughly increasing until 582nd minutes with some outliers. Sharp decrease was observed in data taken at 1256th minutes. Generally, a decrease in cell number was seen in the last data taken in first and second chambers, but in this chamber this decrease took place early. This might be the result of the cell movements in the chambers, because sometimes, during the experiment, the adjustment of the focus of the microscope was difficult, therefore cells may not be monitored properly.

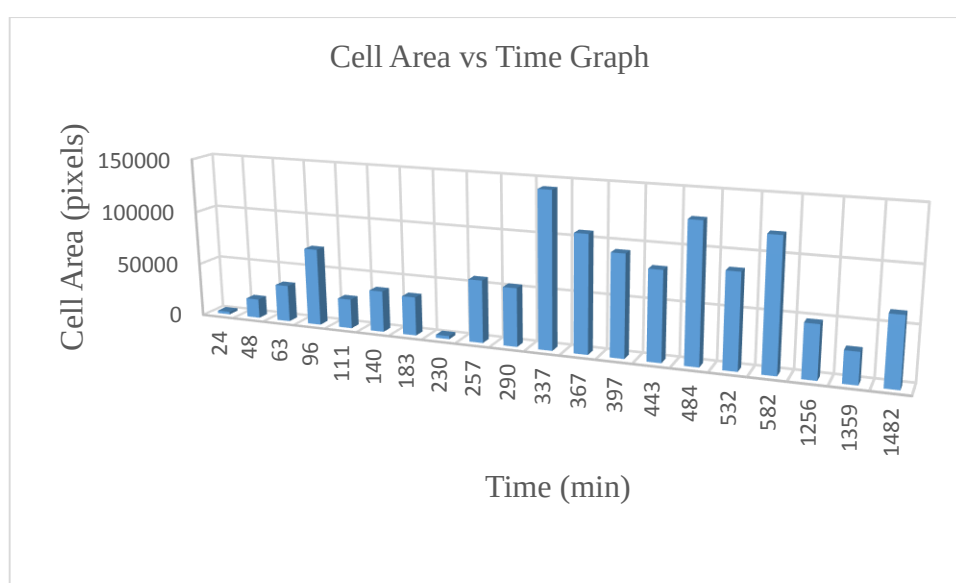


Figure 4.56. Cell area vs time graph for chamber 3 (brightfield images).

In Figure 4.56, cell area also exhibits an increasing trend up to 532nd minute with its ups and downs. Again a decrease was seen at the last three data points. The reason of unstable data trend might be cell movements inside the chip or improper focusing of the microscope.

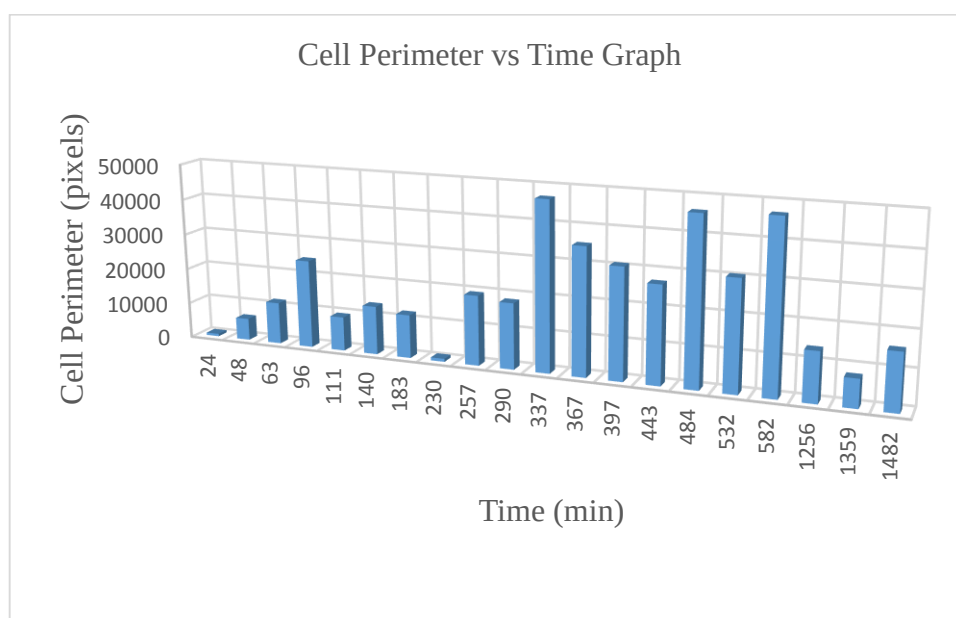


Figure 4.57. Cell perimeter vs time graph for chamber 3 (brightfield images).

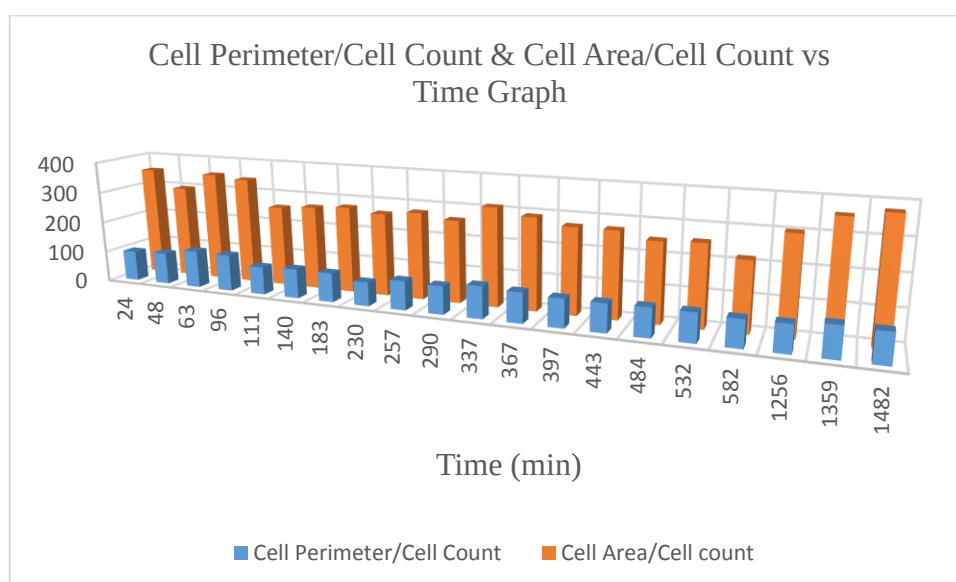


Figure 4.58. Cell perimeter/cell count and cell area/cell count vs time graph (brightfield images).

Nonlinear trend was also observed in cell perimeter (Figure 4.57). If the data at 96th, 337th, 484th and 582nd minutes were considered, yeast perimeter showed increasing trend as expected. In addition, a sharp decrease after 582th minutes was seen due to cell movement or poor focusing on the cells. According to Figure 4.58, an irregular trend was followed in cell area/cell count, however, cell perimeter/cell count data showed almost linear tendency.

During the image processing, integrated density of the yeast cells were analyzed using fluorescence images revealing the amount of emitted light. Figure 4.59 showed the results for chamber 3. There was an increase until the 579th minute and then a decrease towards the end of the experiment. It can be said that, although there was a decrease in the final values, all of the parameters (cell count, cell area etc.) showed almost same relationship with time. Therefore, coordination in parameters was achieved in chamber 3.

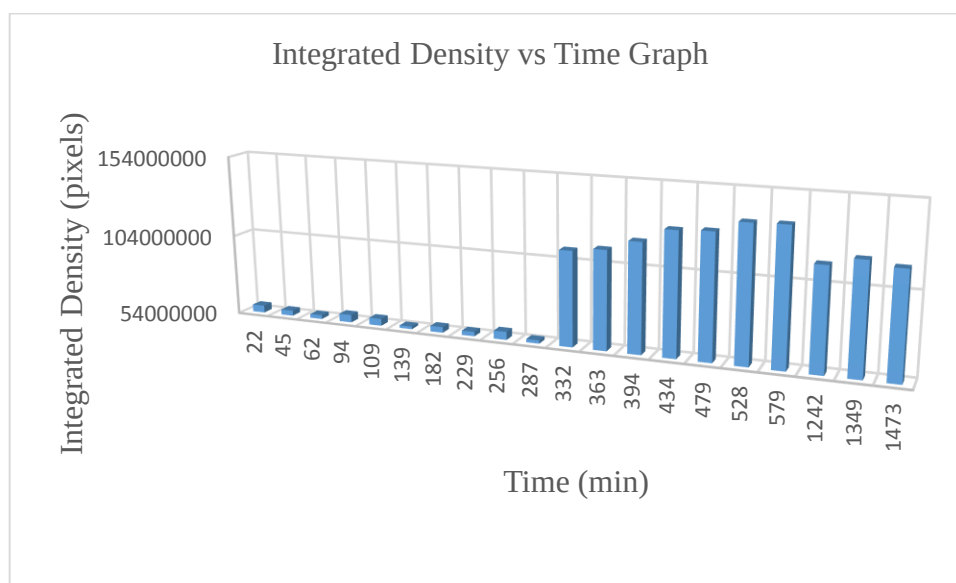


Figure 4.59. Cell integrated density vs time graph for chamber 3 (fluorescence images).

According to Figure 4.60, although rise and fall was observed in integrated density/cell count vs time graph the values are in between 37000 and 41000. Therefore, it can be said that, linearity was obtained approximately. As a conclusion, compatible results were collected for chamber 3. Although a decrease was monitored in all parameters through the end of the experiment, the results of brightfield and fluorescence microscopy images matched up with each other.

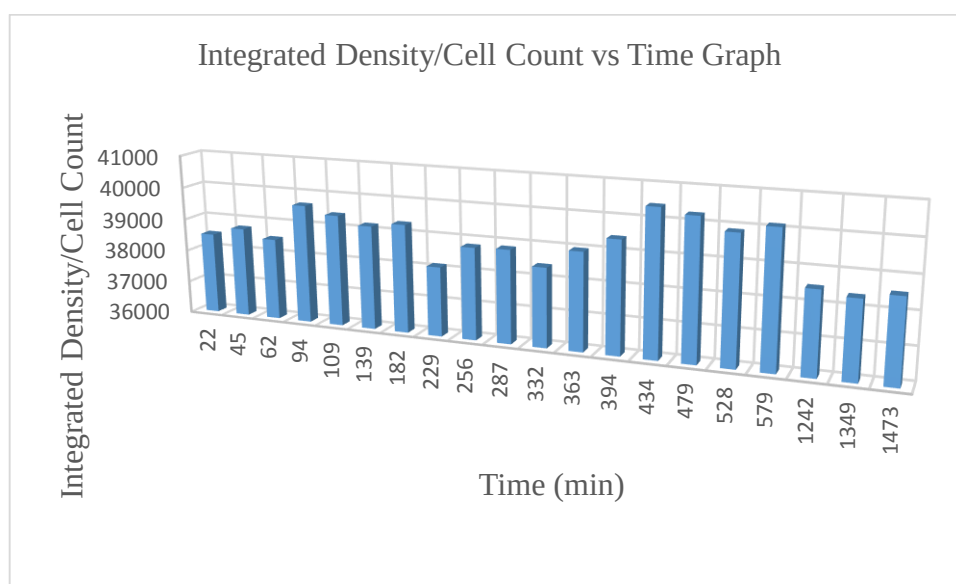


Figure 4.60. Cell integrated density/cell count vs time graph (fluorescence images).

4.6.4. Chamber 4

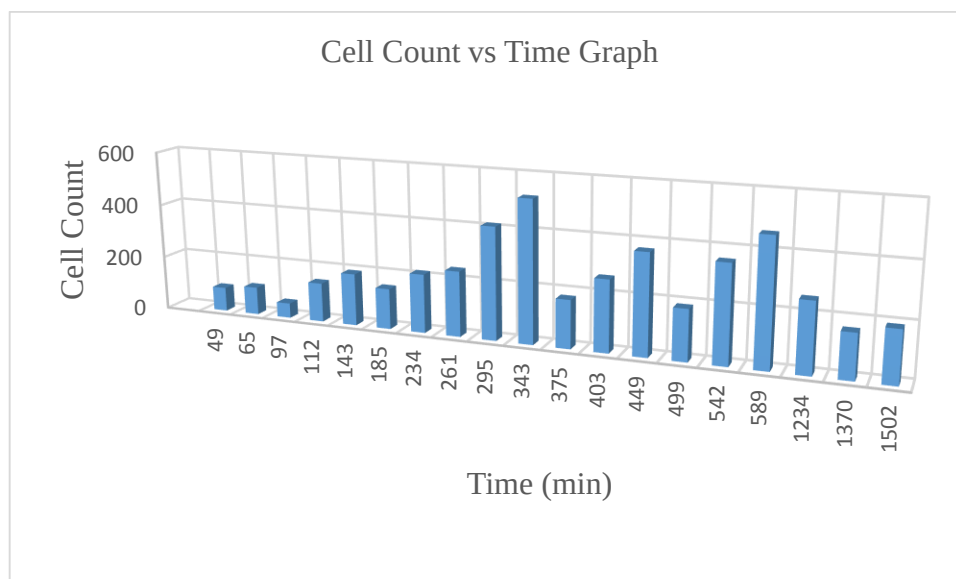


Figure 4.61. Cell count vs time graph for chamber 4 (brightfield images).

An increasing trend in cell count was observed in Figure 4.61, although there were some outliers. Interestingly, the data taken at 343rd minutes was a little higher than the data taken at 589th minutes. Like chamber 3, final three data underwent a decrease in chamber 4.

The reason of this decrease may be explained by the cell sedimentation or erroneous focusing of the microscope lens.

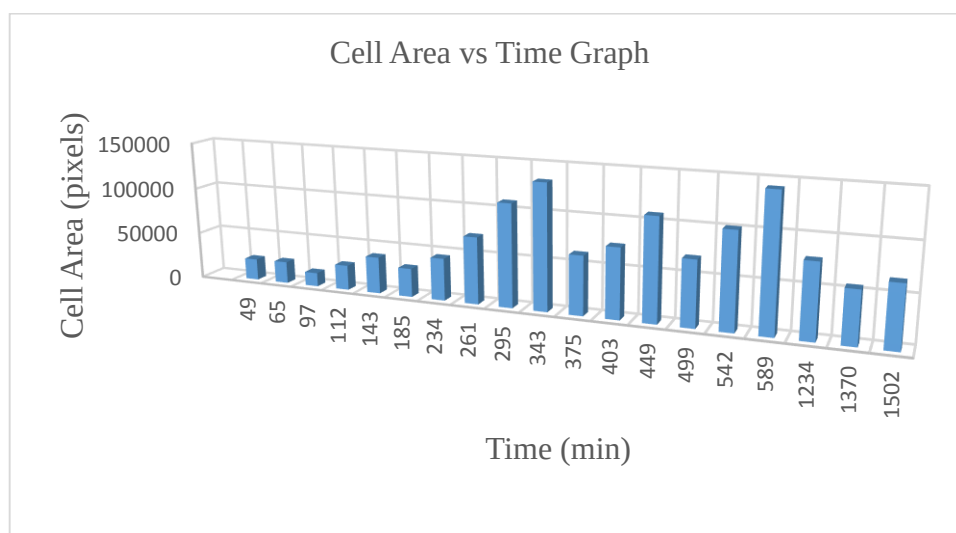


Figure 4.62. Cell area vs time graph for chamber 4 (brightfield images).

Yeast cell area showed an increasing trend up to 589th minute with some outliers (Figure 4.62). In contrast to cell count graph, where the data taken at 343rd minutes had high value, the area obtained at 589th minute was greatest. Again, decrease in final three data was observed.

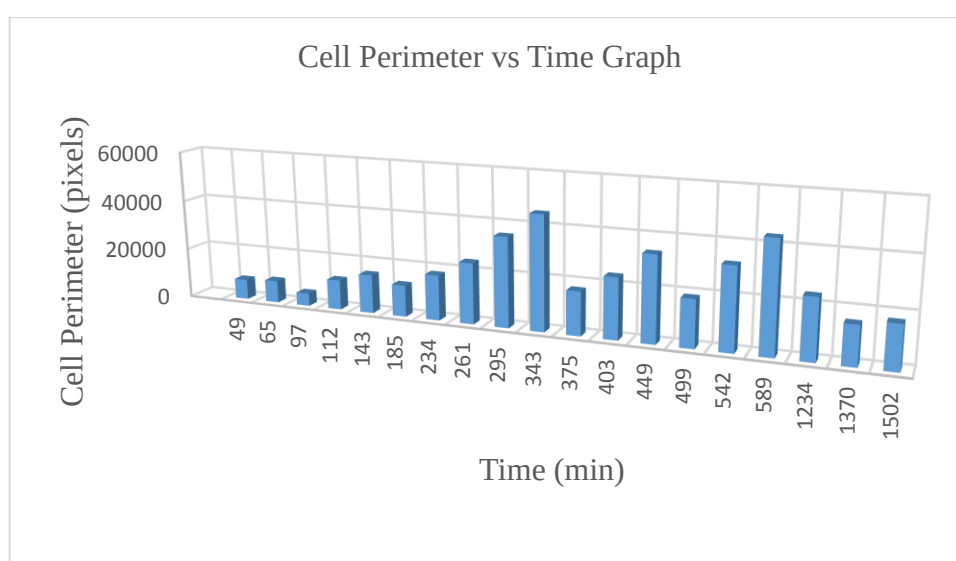


Figure 4.63. Cell perimeter vs time graph for chamber 4 (brightfield images).

The cell perimeter vs time graph (Figure 4.63) showed the same trend like cell count graph in Figure 4.61. It is observed that, again, the data at 343rd minutes was higher than the data at 589th minutes. Although, outliers stood out in the graph, general trend was increasing from the beginning to 589th minutes. At the end of the experiment, a sharp decrease was observed in cell perimeter.

In Figure 4.64, the graphs of cell perimeter/cell count and cell area/cell count vs time are given. According to the plot, cell perimeter/cell count showed almost constant trend which was an expected result but cell area/cell count have nonlinear trend with respect to time. The reason of these rise and falls in cell area/cell count graph may be the result of disparity between the cell area and cell count values.

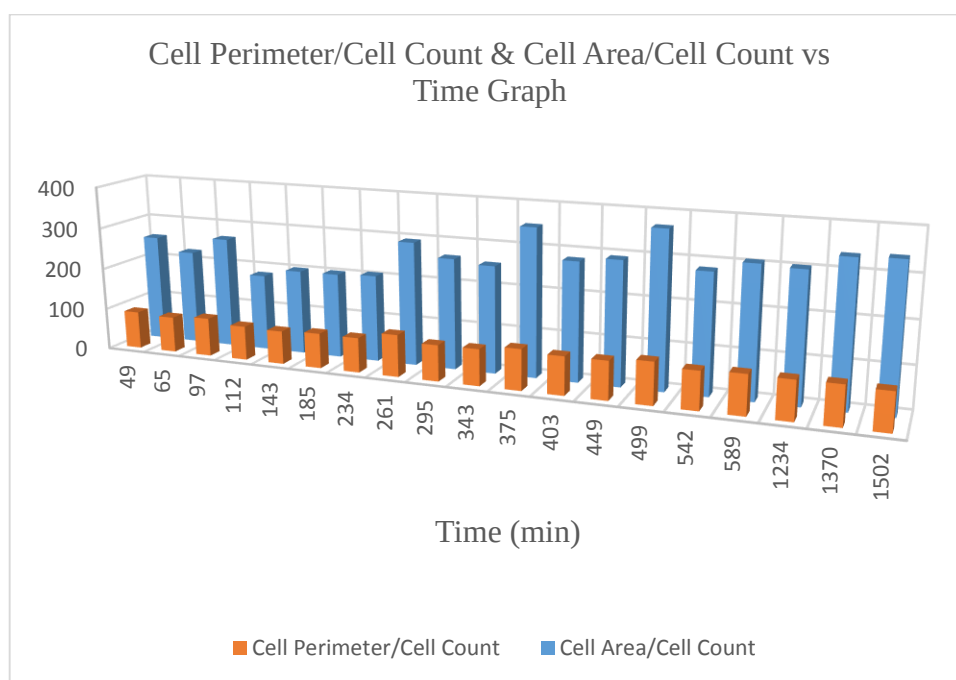


Figure 4.64. Cell perimeter/cell count and cell area/cell count vs time graph (brightfield images).

Figure 4.65 had rising trend in cell integrated density. Although there were some outliers, sharp increase was seen in 256th minutes. The maximum values were obtained at 528th and 1242th minutes.

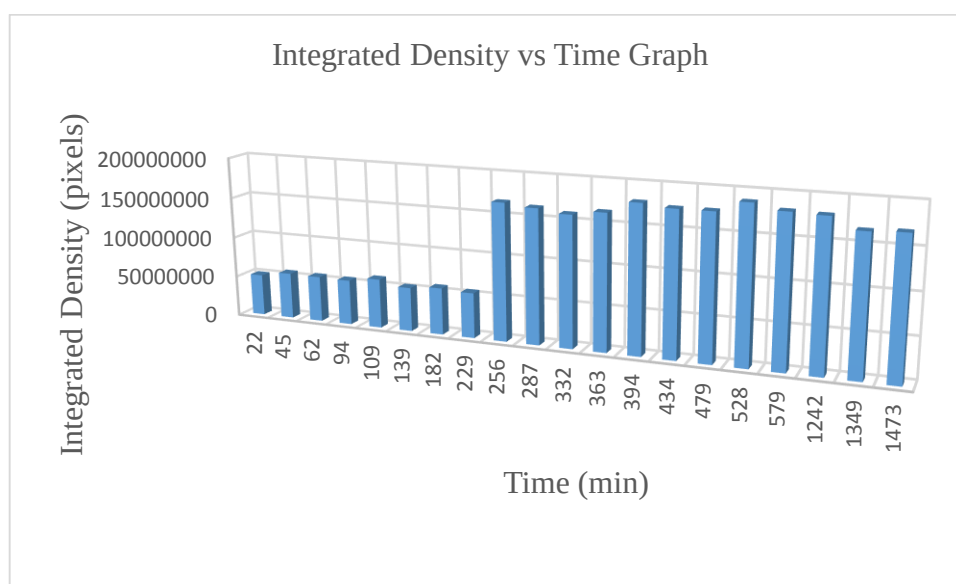


Figure 4.65. Cell integrated density vs time graph for chamber 4 (fluorescence images).

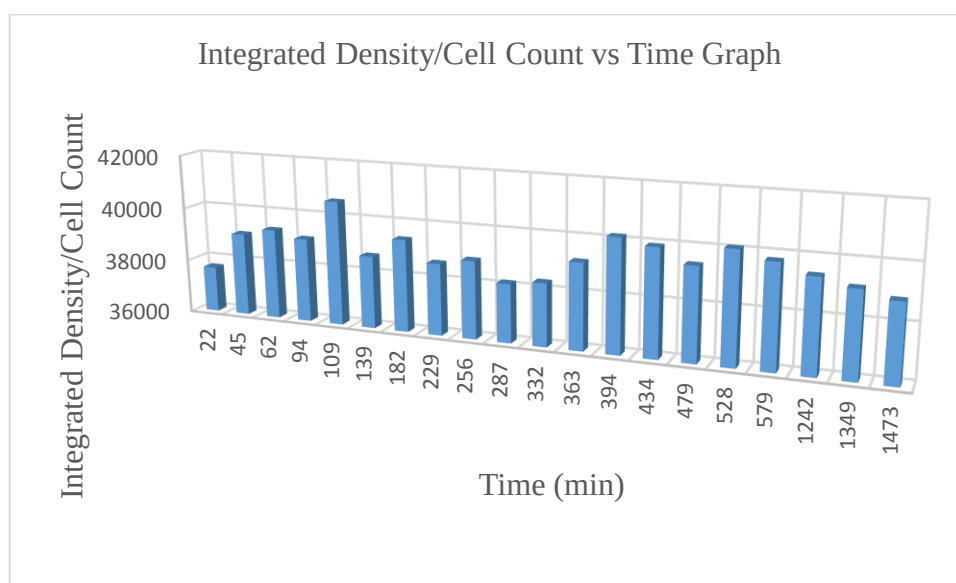


Figure 4.66. Cell integrated density/cell count vs time graph (fluorescence images).

In integrated density/cell count vs time graph, almost nonlinear trend was observed. The range was between 37000 and 41000 which was not a big difference.

In summary, the parameters (cell count, area, perimeter and integrated density) analyzed for chamber 4 were compatible with each other. Sharp increase at 256th minutes

and decrease after 1242th minutes were seen in all four variables. In addition, brightfield and fluorescence microscopy images showed harmony with each other with increasing time.

4.6.5. Chamber 5

Unlike the results of the previous brightfield microscopy images for cell count, chamber 5 displayed two separate rising trend lines. Except some outliers, cell count was increasing with respect to time. At the final value, a decrease in the amount of cells was observed and this was clearly the result of cell sedimentation within the chamber.

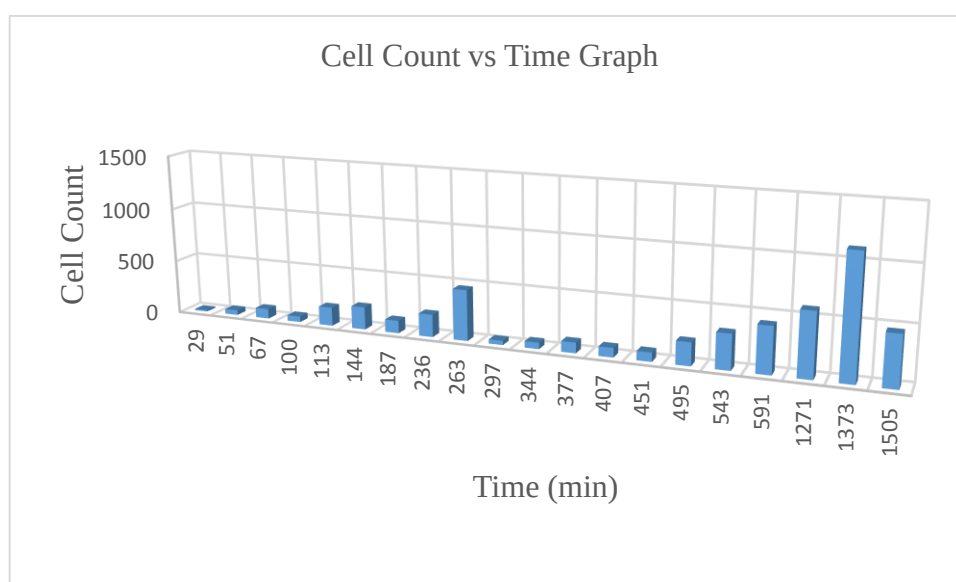


Figure 4.67. Cell count vs time graph for chamber 5 (brightfield images).

Like the cell count plot, cell area vs time graph (Figure 4.68) also had rising tendency (2 increasing trend lines) with respect to time. Higher values were attained through the end of the experiment as expected. Again, there was a sharp decrease in the final data due to cell sedimentation. Moreover, cell monitoring within the chip got difficult towards the end.

Cell perimeter vs time graph in Figure 4.69 had the same tendency with those of cell count and cell perimeter. Except some outliers, generally increasing trend was observed. Again, due to cell sedimentation within the chip, final data taken at 1505th minute (25th hour) was decreasing.

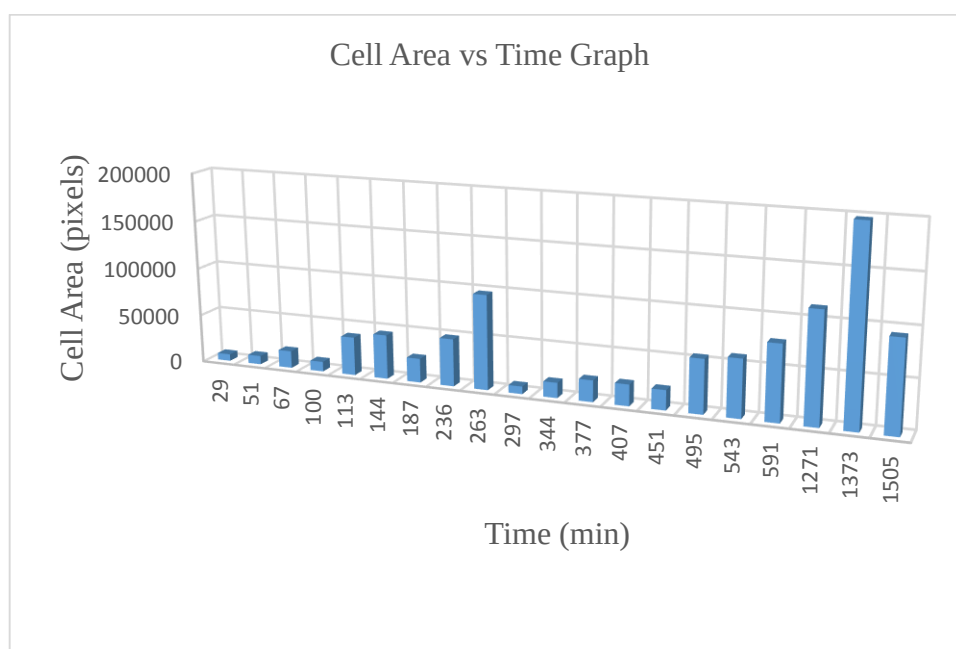


Figure 4.68. Cell area vs time graph for chamber 5 (brightfield images).

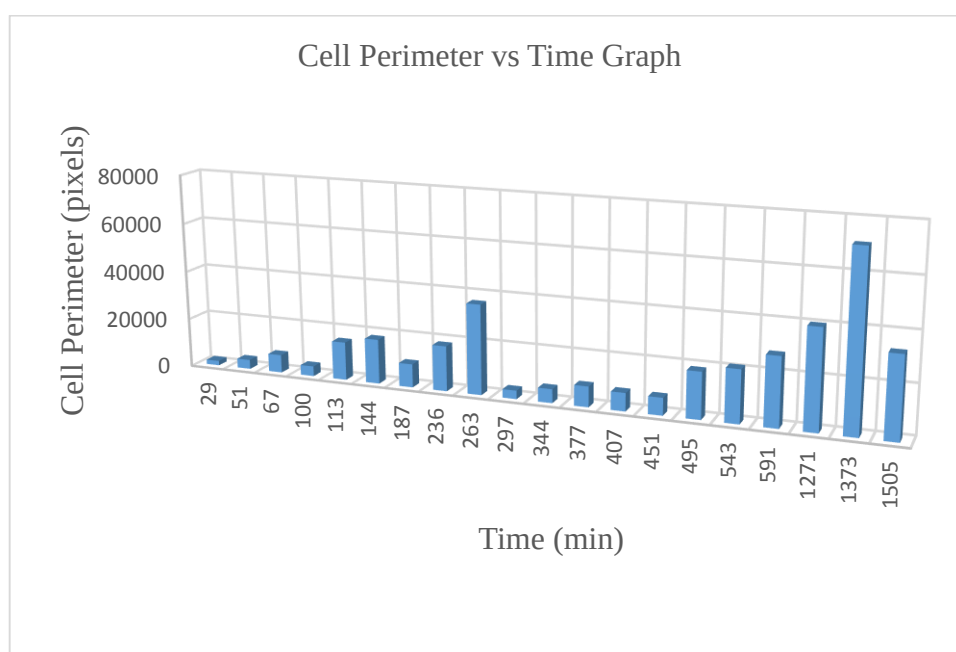


Figure 4.69. Cell perimeter vs time graph for chamber 5 (brightfield images).

Figure 4.70 shows an expected outcome in terms of cell perimeter/cell count and cell area/cell count vs time. Almost constant trend was seen except the first data for cell area.

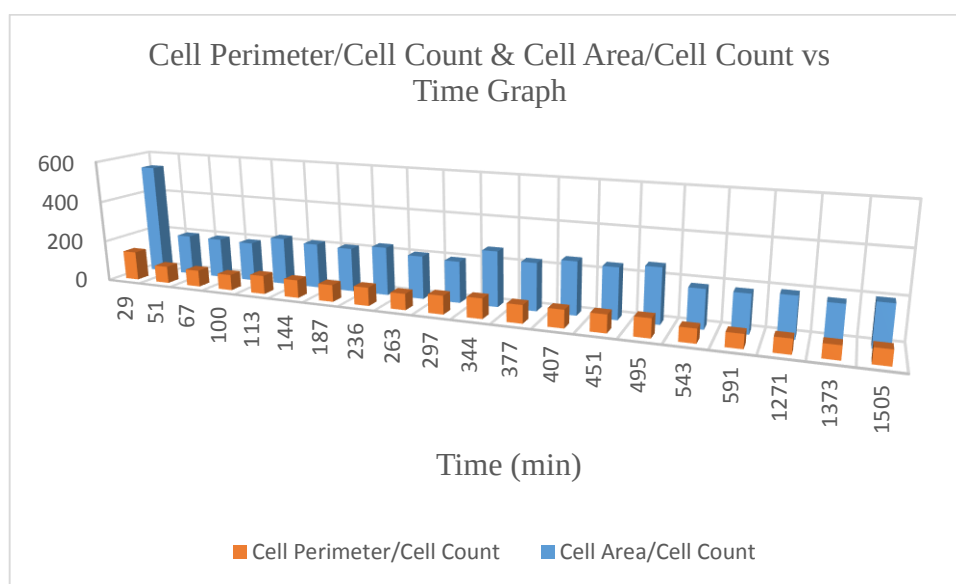


Figure 4.70. Cell perimeter/cell count and cell area/cell count vs time graph (brightfield images).

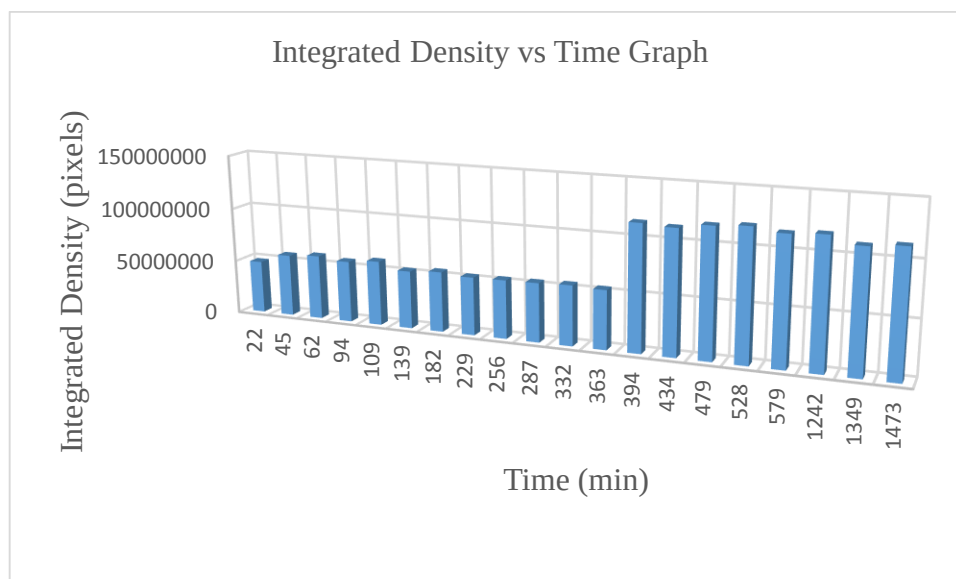


Figure 4.71. Cell integrated density vs time graph for chamber 5 (fluorescence images).

In Figure 4.71, cell integrated density vs time graph was depicted. Although, a slight decrease was seen in the last two data, the rising trend was observed from the beginning. Sharp increase was seen at 394th minutes.

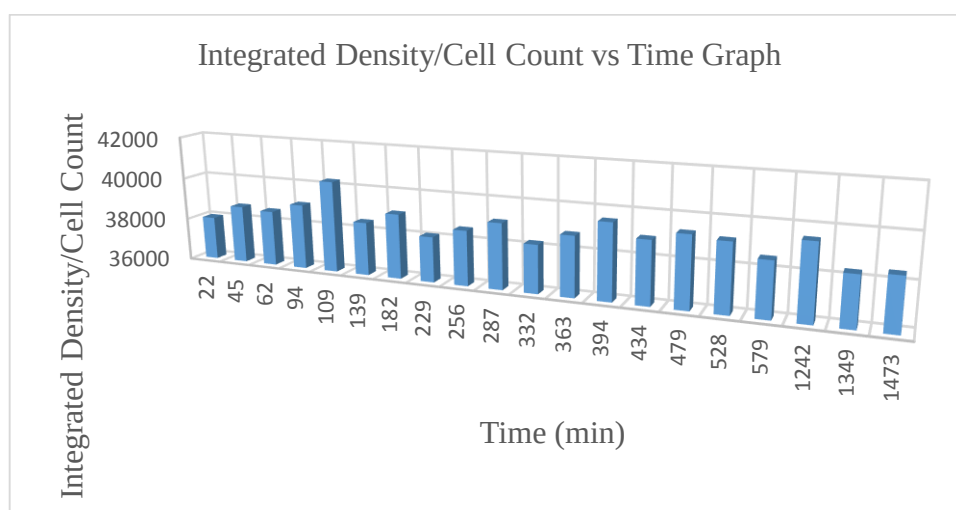


Figure 4.72. Cell integrated density/cell count vs time graph (fluorescence images).

Cell integrated density showed almost linear trend with some outliers. The range was between 38000 and 41000. Yeast cell proliferation and culturing were handled successfully in chamber 5.

4.6.6. Chamber 6

In this section, image processing results for chamber 6 will be given. As in the former cases, cell count, cell area, cell perimeter and cell integrated density will be considered.

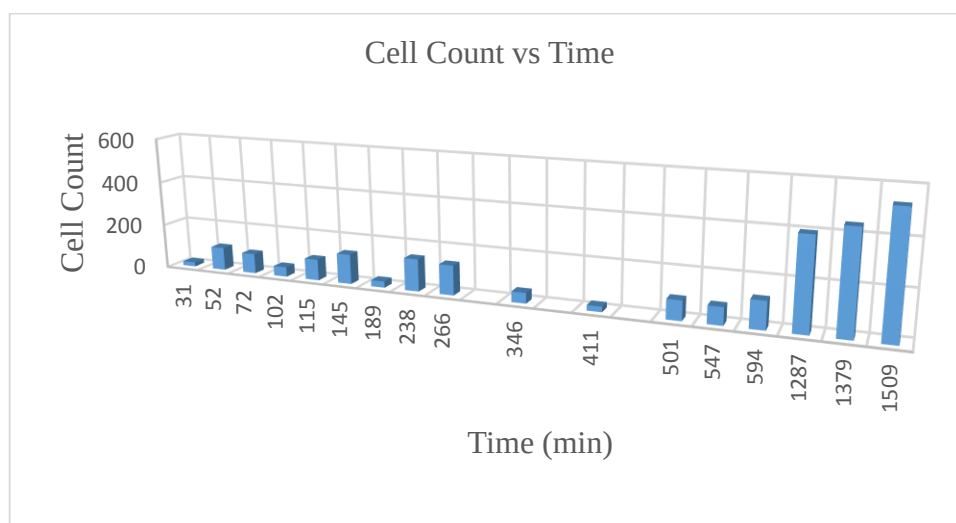


Figure 4.73. Cell count vs time graph for chamber 6 (brightfield images).

During the experiment, chamber monitoring was done frequently, but as seen in Figure 4.73, there seems to be some missing data points. Due to cell movements inside the chamber or improper focusing of microscope lens, yeast cells could not be observed in those times. Nevertheless, cell count had an increasing trend through the experiment.

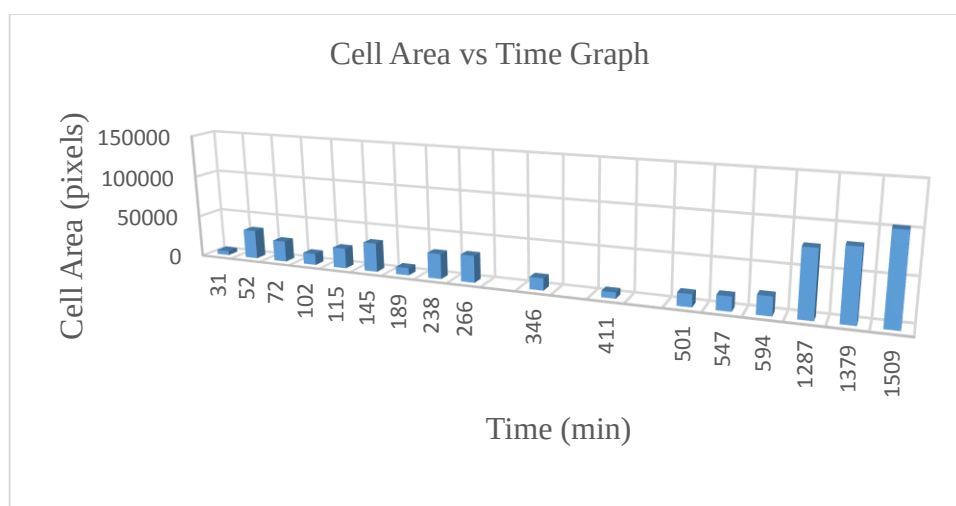


Figure 4.74. Cell area vs time graph for chamber 6 (brightfield images).

Since no cells were found in Chamber 5 in certain times, there were also missing data points in Figures 4.74 and 4.75. Except some rise and falls, both cell area and perimeter were increasing through the experiment and sharp increase was seen in 1287th minutes.

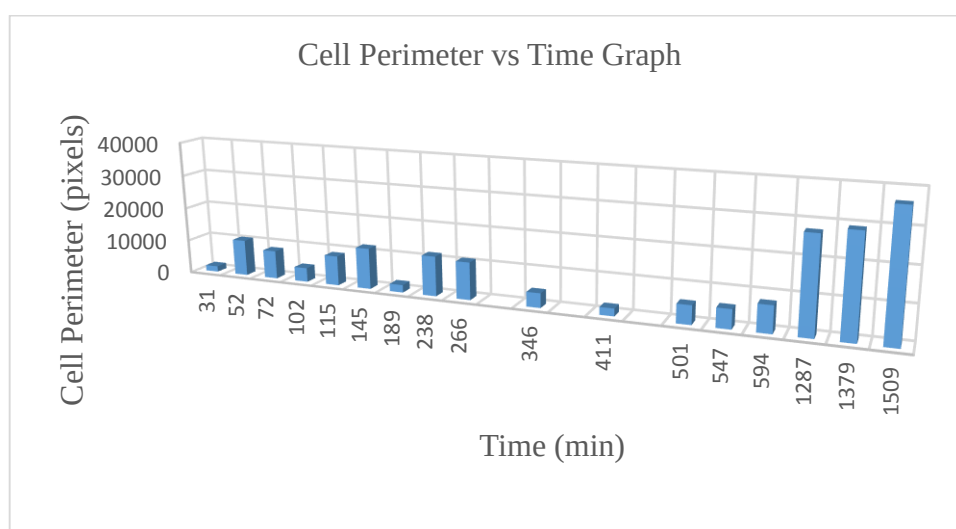


Figure 4.75. Cell perimeter vs time graph for chamber 6 (brightfield images).

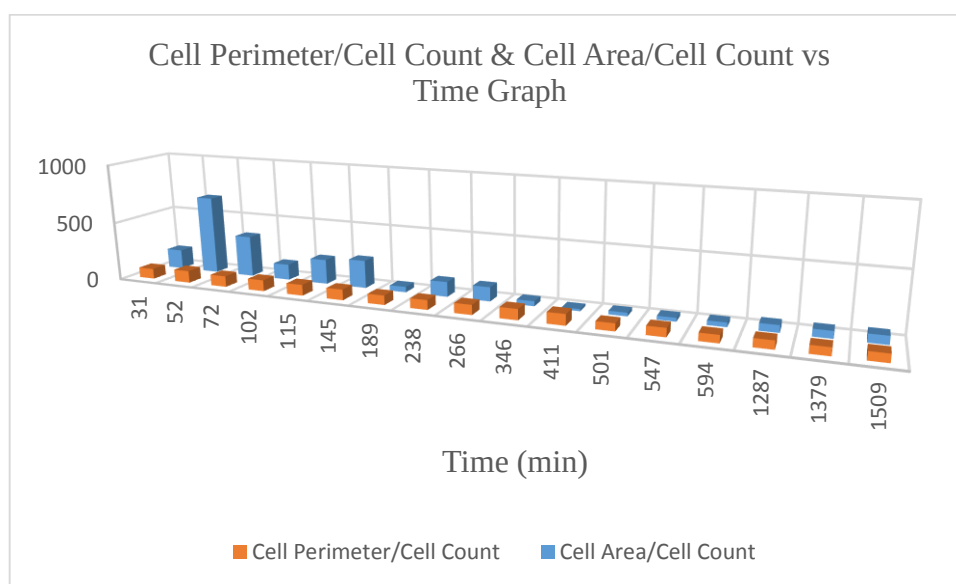


Figure 4.76. Cell perimeter/cell count and cell area/cell count vs time graph (brightfield images).

Although, compatible results were obtained in previous graphs, perfect harmony was not seen in cell area/cell count graph. In contrast to cell area/cell count graph, cell perimeter/cell count graph had almost a linear trend with respect to time.

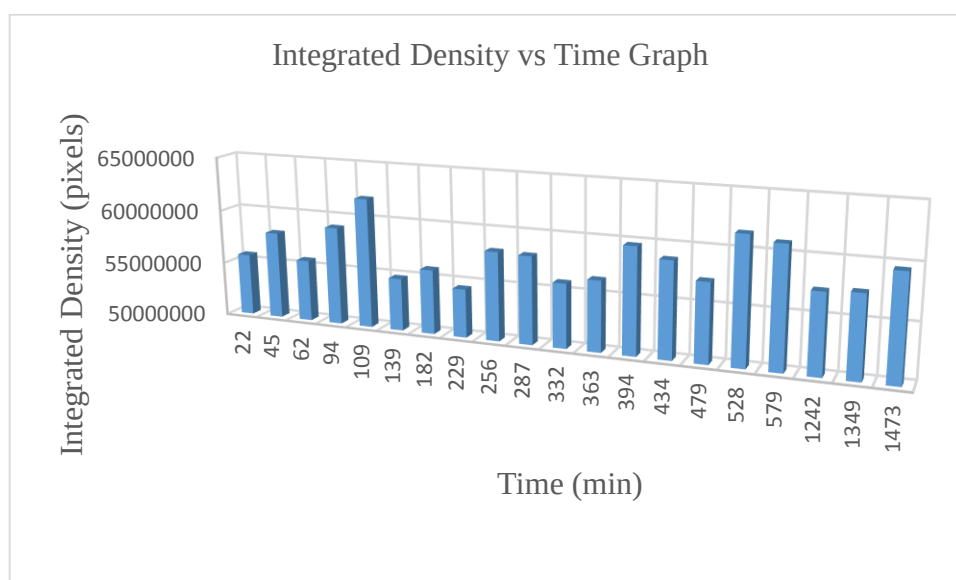


Figure 4.77. Cell integrated density vs time graph for chamber 6 (fluorescence images).

Cell integrated density seems to have a nonlinear trend with respect to time as given in Figure 4.77. After 139th minutes, a rising trend was observed until 529th minute in chamber 6.

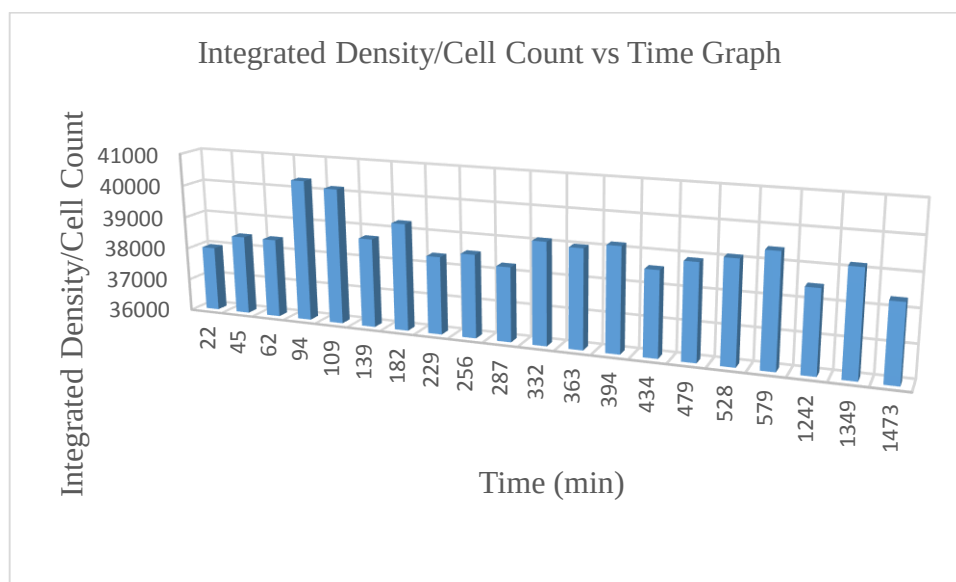


Figure 4.78. Cell integrated density/cell count vs time graph (fluorescence images).

Similar to the former graphs of cell integrated density/cell count, Figure 4.78 had almost linear relation with respect to time. No big difference among the data points were seen, except two outliers which appeared at 94th and 109th minutes. Otherwise, expected result was obtained in this plot too. As a conclusion for chamber 6, brightfield microscopy images showed generally increasing trends in considered parameters. When the fluorescence microscopy images were analyzed, cell perimeter/cell count, cell area/cell count and cell integrated density/cell count values reached constant levels and this was the evidence of created stable environment within the chamber.

4.6.7. Chamber 7

In Figure 4.79, similar to Figure 4.73 (chamber 6), some data points were missing on the graph, although images were taken frequently. Nevertheless, cell count had an increasing trend with respect to time with no cell sedimentation.

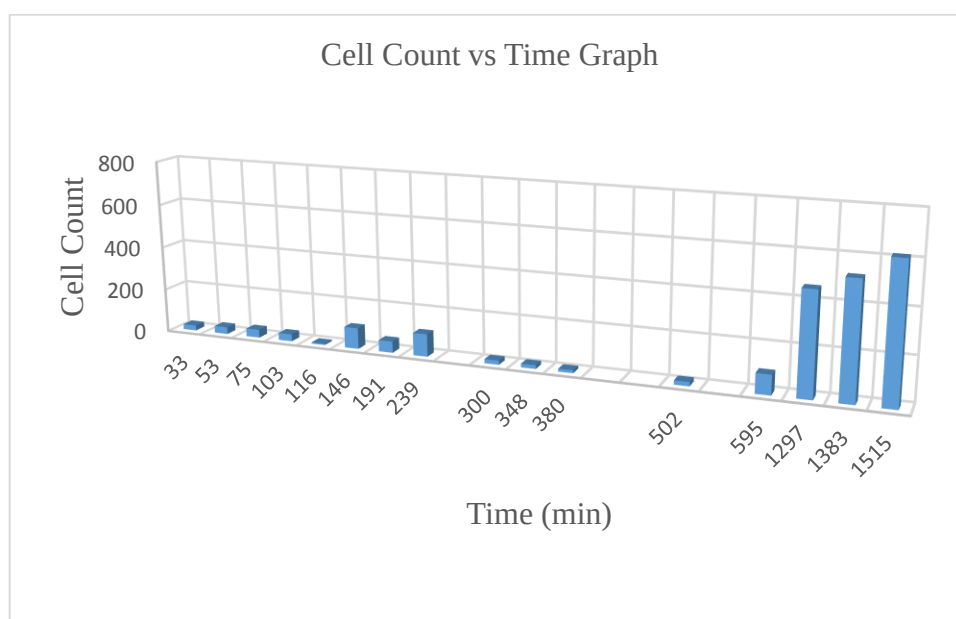


Figure 4.79. Cell count vs time graph for chamber 7 (brightfield images).

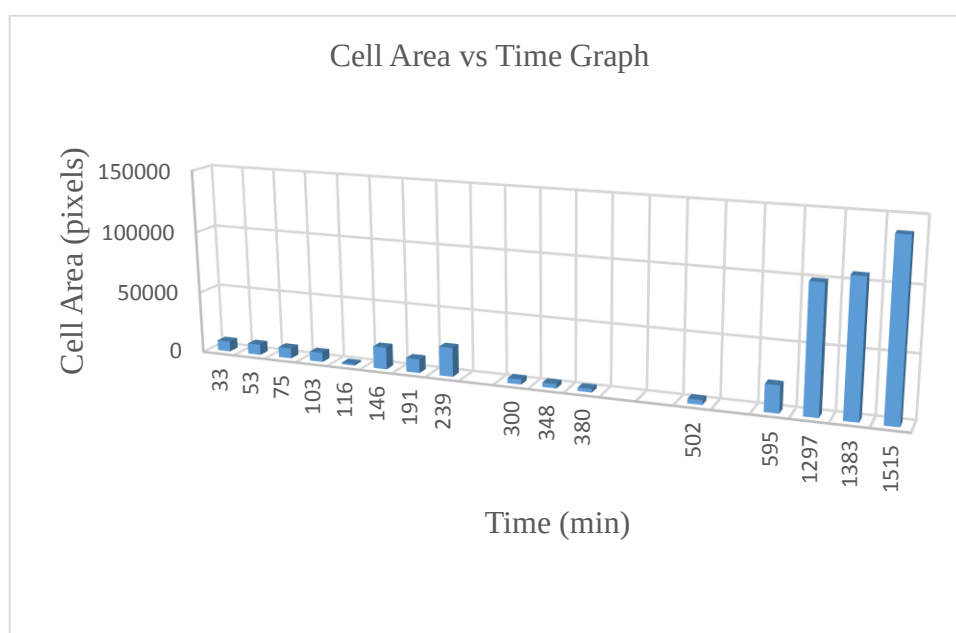


Figure 4.80. Cell count vs time graph for chamber 7 (brightfield images).

Except some outliers and missing data, cell area and perimeter also had a rising trend with respect to time and this was an expected result (Figures 4.80 and 4.81). Maximum area and perimeter values were attained at the final data points. From 595th minutes to the end, rising tendencies in values can be seen (Figures 4.80 and 4.81).

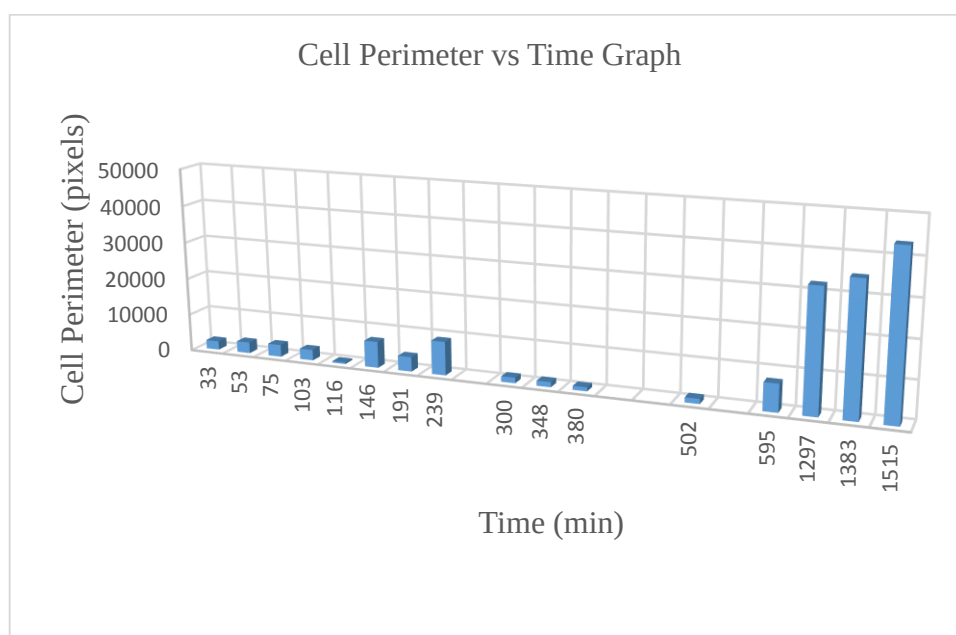


Figure 4.81. Cell perimeter vs time graph for chamber 7 (brightfield images).

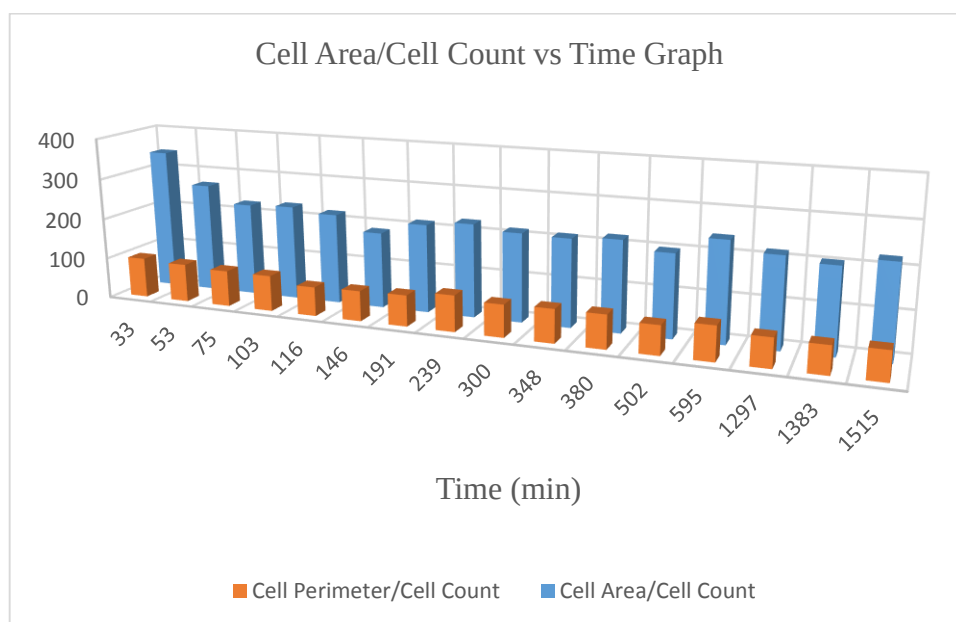


Figure 4.82. Cell perimeter/cell count and cell area/cell count vs time graph (brightfield images).

Except the data at 33rd minutes, cell perimeter/cell count and cell area/cell count vs time values exhibited almost constant trend. This graph was the evidence of stable outcomes obtained during the experiment.

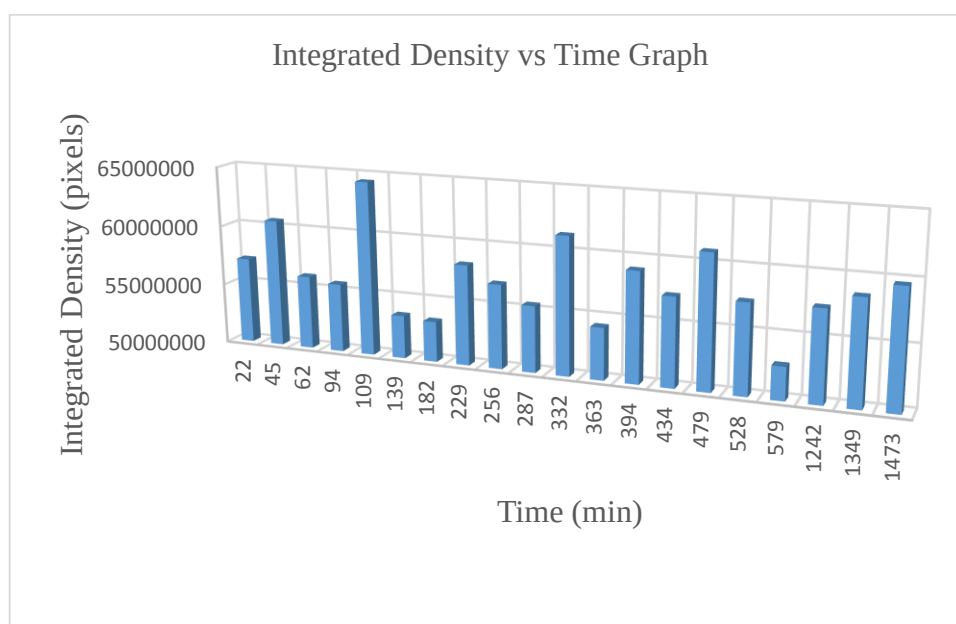


Figure 4.83. Cell integrated density vs time graph for chamber 7 (fluorescence images).

Cell integrated density vs time graph showed improper trend. Increasing trend could be seen at the final four data points and before them, many ups and downs were observed. This was not an expected result, the outcome should have an increasing trend towards the end of the experiment.

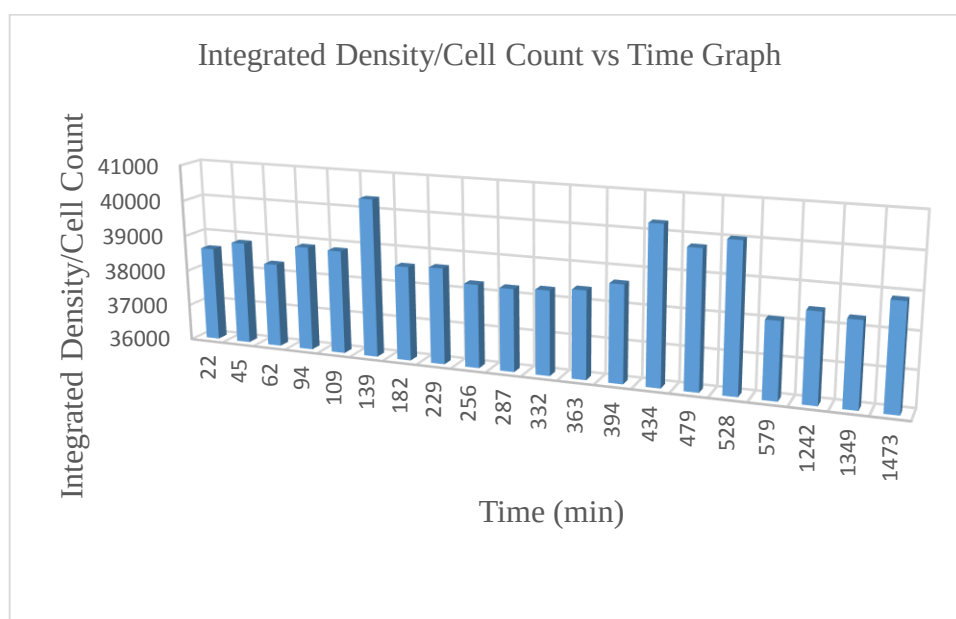


Figure 4.84. Cell integrated density/cell count vs time graph (fluorescence images).

Although some outliers stood out in integrated density/cell count vs time graph (Figure 4.84), the trend was almost constant as expected. Therefore, it can be summarized for chamber 7 that, despite the missing points in brightfield images, all parameters showed increasing trend through the experiment. In case of fluorescence images, many outliers were observed in parameters with respect to time.

4.6.8. Chamber 8

In this section, final chamber of the fabricated microfluidic device will be analyzed. As in the previous chambers, the results of brightfield and fluorescence microscopy images will be given in terms of cell count, cell area, cell perimeter and cell integrated density (only for fluorescence images).

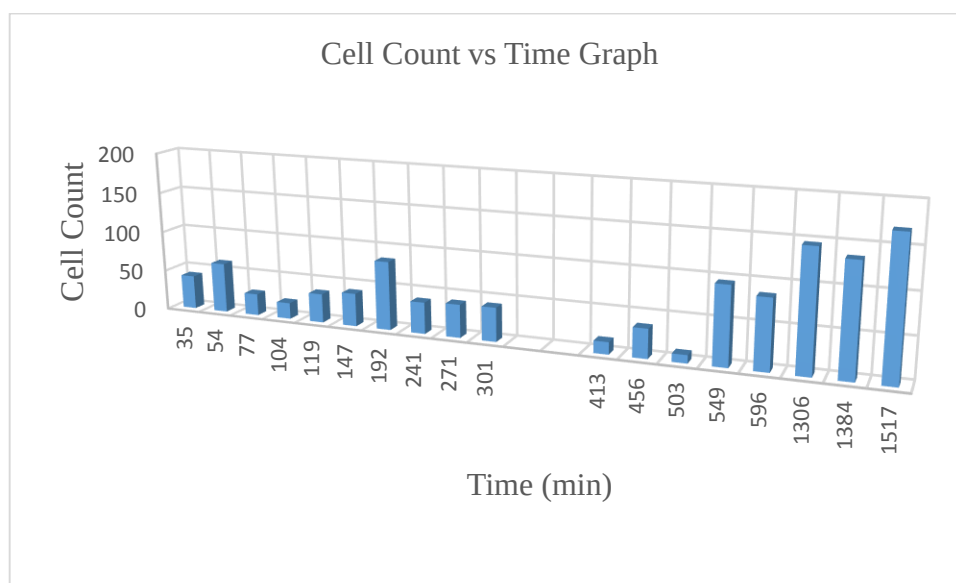


Figure 4.85. Cell count vs time graph for chamber 8 (brightfield images).

The graph of cell count vs time was given in Figure 4.85 and cell count exhibited a generally increasing trend. Some data points were missing and outliers were seen in the graph but, towards the end the increment in data was clearly monitored.

Despite the missing data points and outliers, cell area showed a rising trend with respect to time (Figure 4.86). An increase was seen in cell area while a decrease was

observed in cell count at the same time (1384th minutes) interestingly. The final value of data was slightly lower and this should not be explained by cell sedimentation because cell sedimentation was not monitored in cell number.

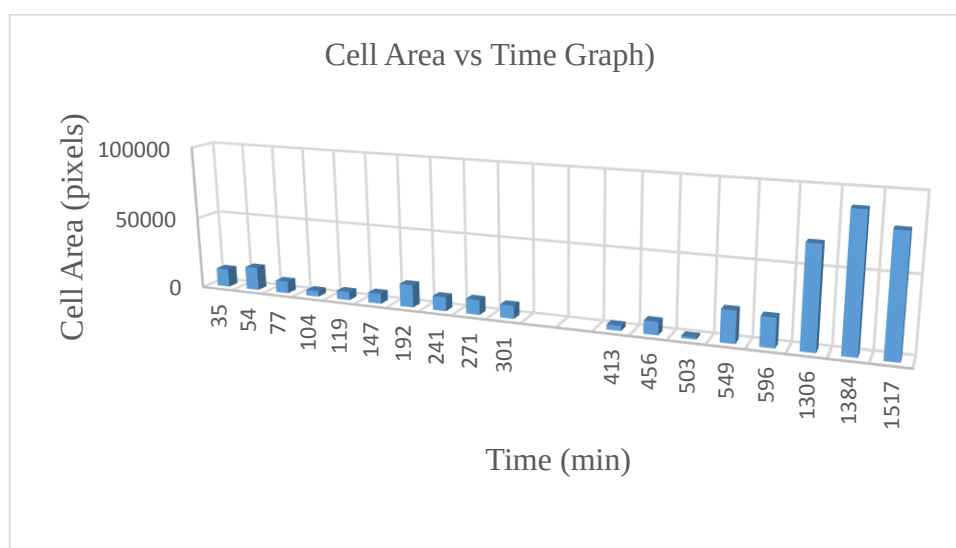


Figure 4.86. Cell area vs time graph for chamber 8 (brightfield images).

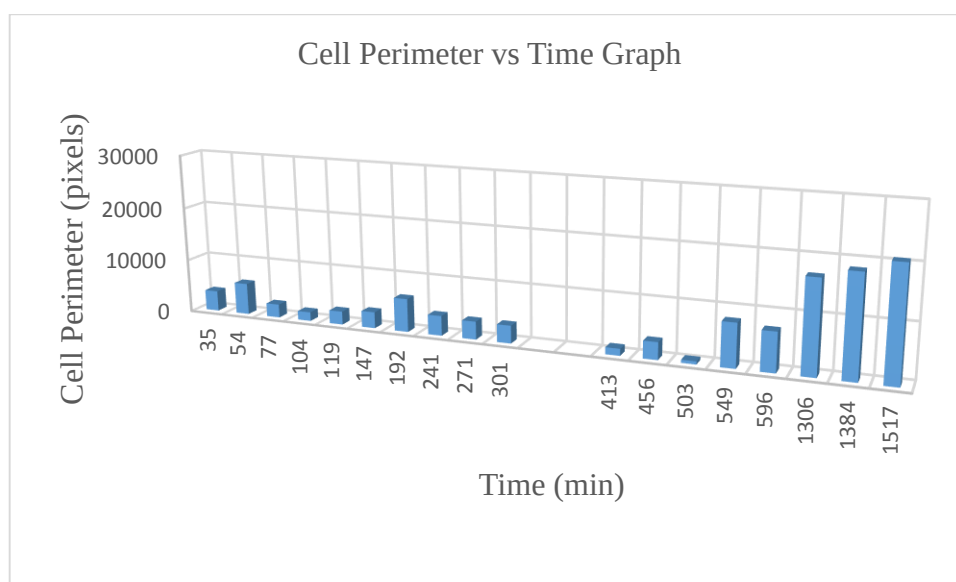


Figure 4.87. Cell perimeter vs time graph for chamber 8 (brightfield images).

The graph of cell perimeter vs time also exhibited an increasing trend. Unlike the cell area, an increment in cell perimeter was observed at the end of the experiment.

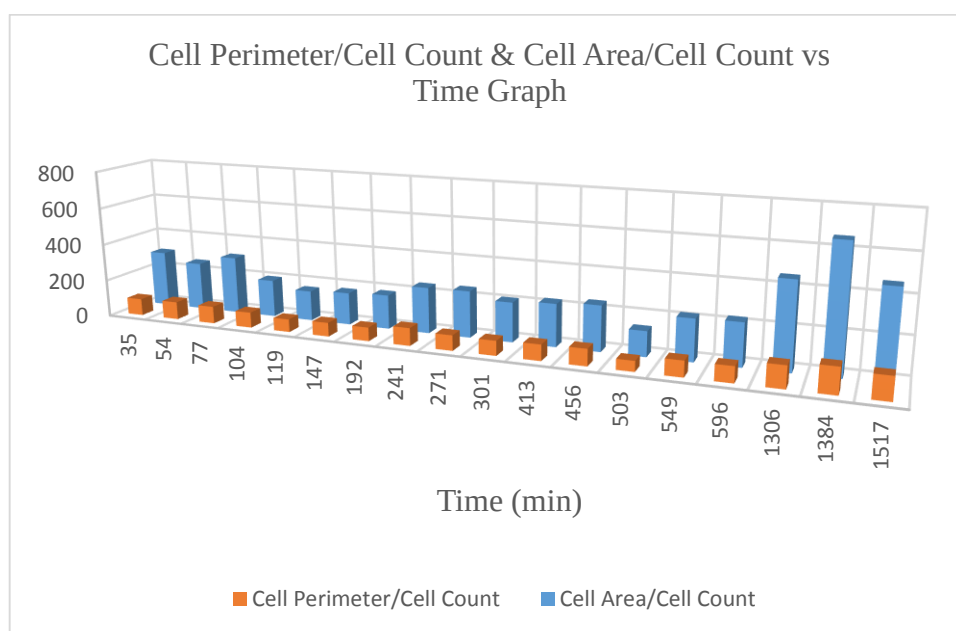


Figure 4.88. Cell perimeter/cell count and cell area/cell count vs time graph (brightfield images).

According to Figure 4.88, cell perimeter/cell count graph had mostly a constant trend with respect to time, whereas cell area/cell count graph exhibited a nonlinear trend. It can be said that, cell area and cell count did not have consistent relationship with each other.

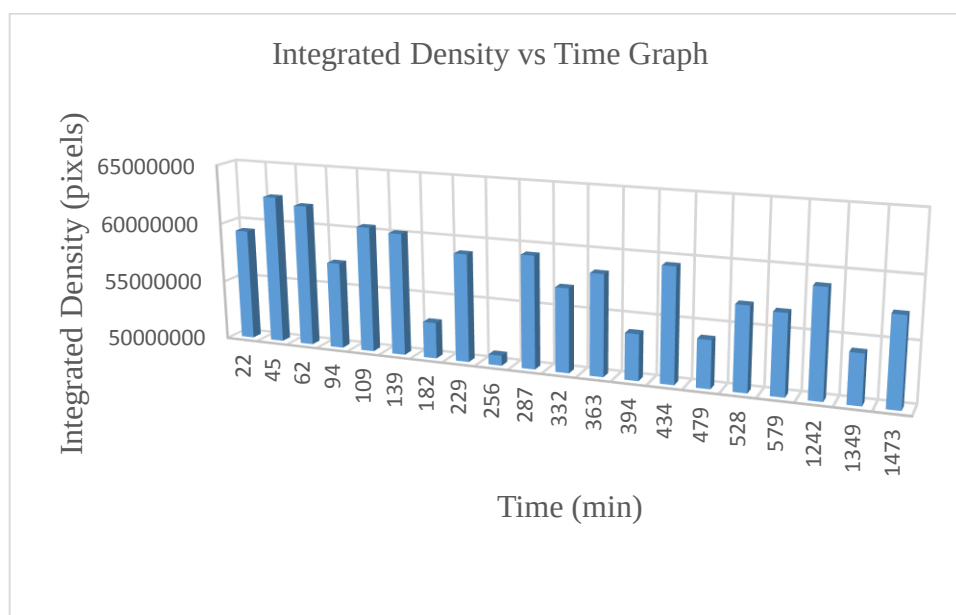


Figure 4.89. Cell integrated density vs time graph for chamber 8 (fluorescence images).

An inconsistent trend was obtained in cell integrated density vs time graph. Again, maximum value appeared at 45th minutes and many rises and falls were seen in the plot. Towards the end of the experiment, there should be an increasing trend but it was not acquired for this chamber.

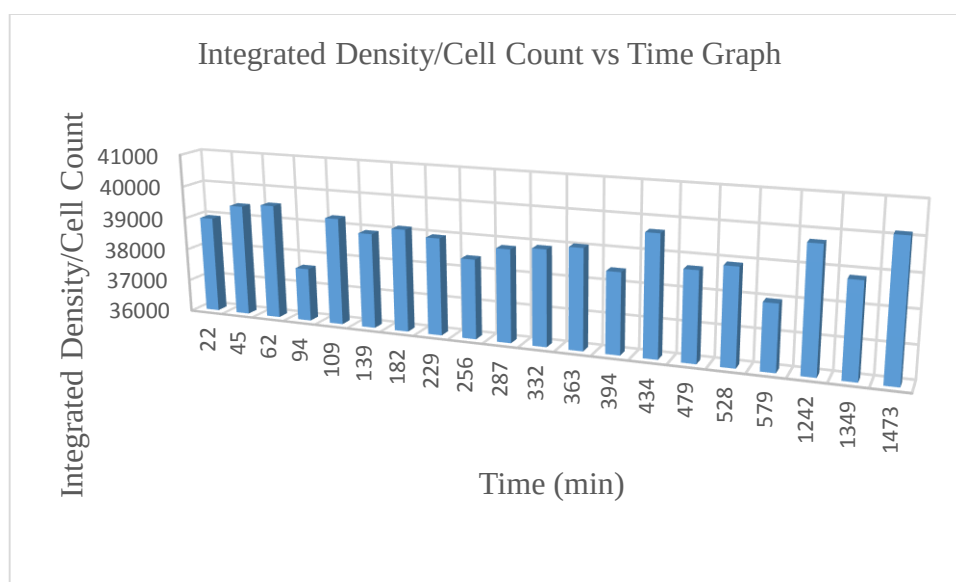


Figure 4.90. Cell integrated density/cell count vs time graph (fluorescence images).

The graph of cell integrated density/cell count displayed almost a constant trend with respect to time. Although some outliers were seen in the graph, the values were between 37000 and 41000.

4.7. Discussion

4.7.1. YOR060C/SLD7 Protein Function in Yeast

The ultimate aim of this study is to obtain a broader understanding of the function of YOR060C protein (which is an uncharacterized protein) and its relation to chronological aging pathway. The chronological aging network (CAN) in *Saccharomyces cerevisiae* was reconstructed by integrating protein–protein interactions with Gene Ontology terms [63]. Many terms related to cell cycle (S phase, interphase etc.) and especially to its mitotic (M) phase (DNA replication initiation, cytoskeleton organization, chromosome segregation and

organization, cytokinesis, etc.) stand out among the enriched categories of proteins in aging network. These observations suggest that DNA replication factors have a role in chronological aging. These DNA replication factors usually participate in several biological processes, such as mitosis and genomic stability maintenance, known to be related to chronological aging.

In cells with a nucleus (eukaryotes) the cell cycle is generally divided into two periods: interphase and mitotic phase:

- Interphase – the cell grows, accumulates nutrients required for mitosis, duplicates its DNA and prepares for cell division. It is divided in G1, S and G2 phases.
- Mitotic phase (M) – Mitosis and cytokinesis together define the mitotic phase of a eukaryotic cell. During mitosis the cell divides its nucleus. Chromosomes (duplicated in interphase) in the cell nucleus are separated into two identical sets of chromosomes, each in its own nucleus. Generally the division of the nucleus is followed by cytokinesis, the division of cytoplasm, organelles and cell membrane. After cytokinesis the division is concluded and the two cells are completely divided.

Chromosomal DNA replication in eukaryotes initiates from multiple specific sites termed replication origins. DNA replication that takes place at the replication origins is also termed origin firing. Replication origins characterized in *S. cerevisiae* are about 200 base pairs (bp) long.

To initiate DNA replication, multiple replicative proteins assemble on and dissociate from these replicative origins. Initially the origin-recognition complex (ORC) associates with replication origins throughout the cell cycle. The Mcm complex (a DNA helicase comprising Mcm2–7) is loaded in all of the potential replication origins to form the prereplicative complex (pre-RC) from late M phase to G1 phase. Two different protein kinases are required for the activation of pre-RC-formed origins: cyclin-dependent kinase (CDK) and the Dbf4-dependent Cdc7 kinase. Once cells reach S phase of the cell cycle, Sld2 and Sld3 are phosphorylated by cyclin-dependent kinase (CDK). Phosphorylated Sld2 interacts with the C-terminal BRCT pair of Dpb11 and promotes the formation of a complex, called a pre-loading complex (pre-LC), which contains phosphorylated Sld2, Dpb11, DNA

polymerase epsilon, and a GINS complex. Phosphorylated Sld3 interacts with the N-terminal BRCT pair of Dpb11 and associates with replication origins together with another essential replication protein, Cdc45, in a mutually dependent manner. Once an origin has been activated, the pre-RC disassembles, and its reassembly is prevented until the next cell cycle [64][65].

In fact, SLD7 (YOR060C) protein does not exhibit an essential role in DNA replication cycle, but if SLD7 protein does not exist in the environment, delayed chromosomal DNA replication occurs. It was demonstrated that, SLD7 and SLD3 are interacting physically and it is claimed that when the SLD3 and SLD7 interact, SLD3 experiences a conformational change and this situation affects its stability. SLD7 affects SLD3's resistance to proteases by altering its conformation. As a result, efficiency of the initiation of DNA replication may be disturbed by this conformational change. In addition, the interaction between the SLD3 and CDC45 is weakened by SLD7 and this may lead to more efficient DNA replication due to displacement of SLD3 from origins. On the other hand, the absence of SLD7 decreases the cellular SLD3 level in the environment, and that results in the delay of the dissociation from origins. Thus, SLD7 is necessary for SLD3 to initiate the DNA replication [64].

Although SLD7 is not essential for cell growth, its absence reduces the level of cellular SLD3, delays the dissociation from origins of GINS, and slows S-phase progression. Cells lacking SLD7 exhibit delayed chromosomal DNA replication. These results suggest that SLD7 is required for the proper function of Sld3 at the initiation of DNA replication [64].

If the amount of SLD7 that is associated with Sld3 decreases, free SLD7 may disperse inside or outside the nucleus, where it can interact with other factors to perform other functions. Mitosis event and SLD7 relation was discovered by Tanaka et al. (2011). Another experimental evidence that SLD7 has other roles apart from DNA replication is provided by the study performed by Altmann and Westermann (2005). Four mutants lacking proteins of unknown function (including SLD7 mutant) showed severe mitochondrial defects and aberrant mitochondrial morphology. The study suggests that SLD7, as well as the other 3 proteins with unknown function, might encode novel essential proteins required for mitochondrial morphogenesis but the molecular mechanism is still unknown [66].

With the help of both high-throughput and low-throughput experiments, 12 genetic and 3 physical interacting partners have been identified for SLD7 so far (Table 4.6). Almost all the interactors are directly related in DNA replication according to their GO terms.

Figure 4.91 shows the functional and physical interactions among the genes involved in DNA replication:

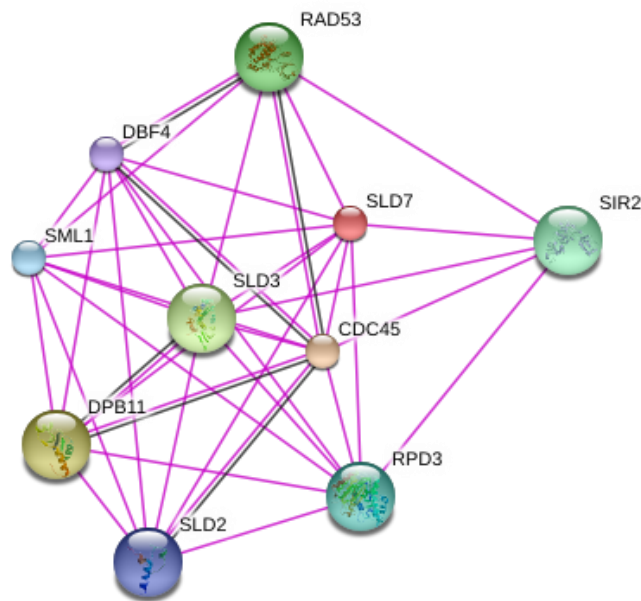


Figure 4.91. DNA replication initiation factors related to SLD7 (obtained from STRING database).

Table 4.6. List of genetic and physical interactor for SLD7 (retrieved from Biogrid).

Genetic Interactor	CDC45, CDC73, DBF4, DBP11, MAD1, RAD53, RPD3, SGS1, SIR2, SLD2, SLD3, SML1
Physical Interactor	HEK2, NAB2, SLD3

The main biological processes described for the above mentioned genes (Table 4.6), that can be related to chronological aging, fall into the following categories: genome stability

(DNA damage repair, telomere maintenance), mitosis, mitochondrial organization. Among these interaction partners of SLD7, SIR2 is directly involved in chronological aging. It also plays roles in silencing telomeres and negatively regulates initiation of DNA replication. Its genetic interaction with SLD7 was detected by phenotypic suppression assay. In this assay, SLD3, SLD7 and CDC45 were overexpressed in wild-type and sir2 deleted cells. In wild-type cells these factors induced a premature activation of late origins while in sir2 deleted cells the overexpression restored the activation of all Sir2-dependent origins.

A genome-wide study showed that SLD7–GFP protein is localized to the nucleus and spindle pole [67]. The genes coexpressed with SLD7 were retrieved from the SPELL database. Most of these coexpressed genes localize in the nucleus. Among the top 100 coexpressed genes, NSL1 is the only one that localizes in spindle pole and therefore colocalizes with SLD7, but no physical interaction between these two proteins was yet identified. Among the top 100 coexpressed genes only CKA2 is directly involved in chronological aging. It is the alpha catalytic subunit of casein kinase 2 (CK2), CK2 is a Ser/Thr protein kinase with roles in cell growth and proliferation. CK2A has an established role in chronological aging. CKA2 was found to be one of the genes whose deletion significantly extended life span. Among the top 100 coexpressed genes, RTG1, which is a transcription factor, and therefore localized in nucleus like SLD7, contributes to communication between mitochondria, peroxisomes, and nucleus. RTG1 involvement in mitochondrial functions and osmoadaptive response (both processes with a major role in chronological aging) make it a gene of interests for further studies in chronological aging. Beyond all these information, protein abundance is an important criterion to visualize the GFP-tagged gene encoded protein expression. Unfortunately, SLD7 protein has a very low protein abundance which makes its detection very difficult. Under the experimental conditions used in this study, the visualization of localization of YOR060C/SLD7 in nucleus was not possible. Although, during the experiment, the subcellular localization of SLD7 protein could not be monitored properly, the following figures still approve the cell proliferation within the chambers.

In Figure 4.92, the first picture is taken at the beginning of the experiment, and the last picture 4 (bottom right hand corner) is taken at the end of the experiment displaying cell

proliferation within the device. It can be said that the optimum proliferation conditions for yeast cells were created in the chip.

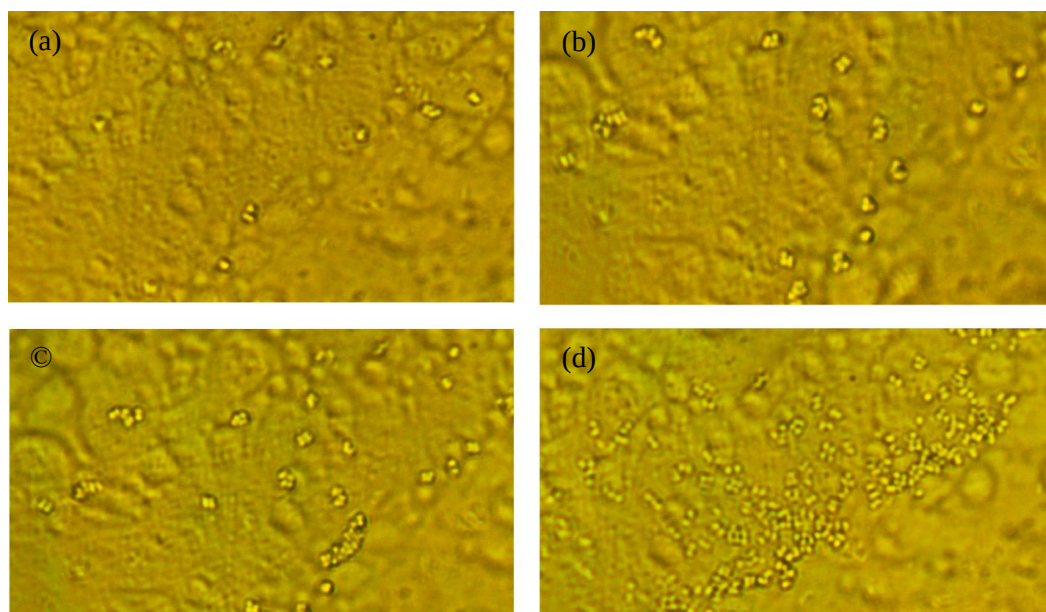


Figure 4.92. Cell proliferation within the device (time passes through (a) to (d)).

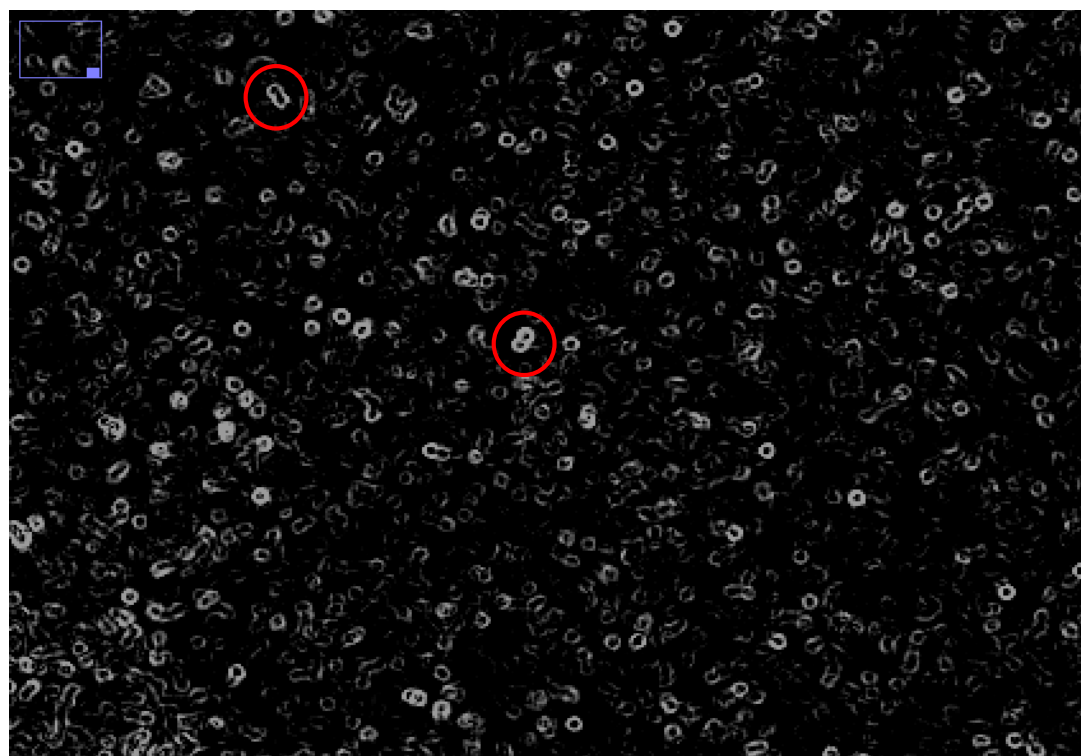


Figure 4.93. Budding (shmooing) cells.

Budding, conjugation and sporulation constitute the yeast life cycle, and shmooing yeast cells are shown in red circles in Figure 4.93. Therefore, again, cell proliferation within the chamber was achieved successfully.

4.7.2. Cell Growth in Microbioreactor

In the previous sections (4.6.1-8), images were analyzed chamber by chamber. In order to make general evaluations about the microbioreactor containing 8 chambers, the data were summarized in the following figures and discussed further.

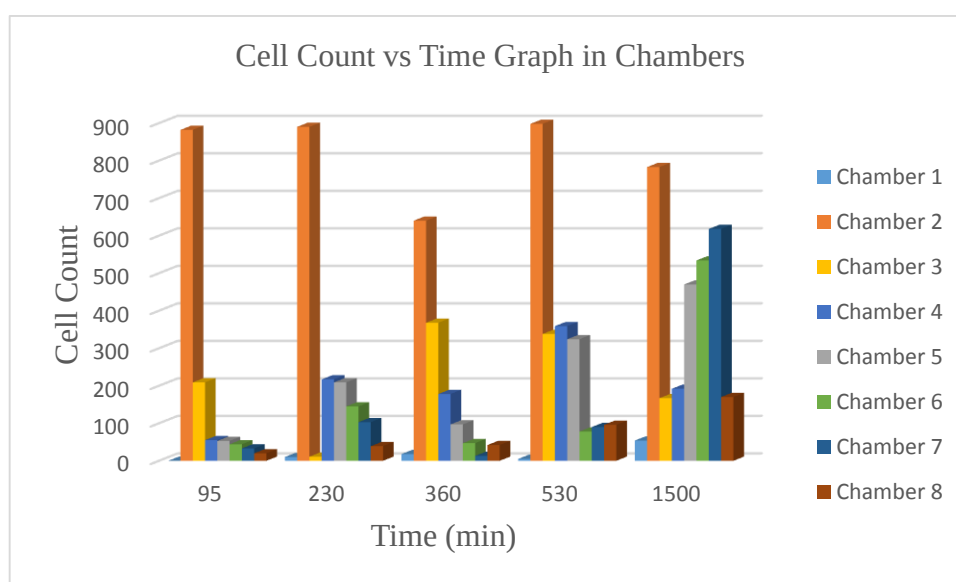


Figure 4.94. Cell count vs time graph for all chambers (brightfield images).

In Figure 4.94, cell count in all chambers was summarized at 5 different time intervals. In chamber 1, a considerable increase in cell count was seen at the end of the experiment, but in chamber 2, cell count neither increased nor decreased, i.e. rises and falls were seen in the trend line of chamber 2. In chamber 3, represented by yellow colored bars, cell count was increasing up to 360th minutes and then it was decreasing towards the end. One can say that the cell count has an increasing trend till 360-530 minutes for Chambers 2-4 whereas it takes continuously increasing values in Chambers 5-8 except some outliers. In addition to the cell count trend of chambers with respect to time, maximum cell number was obtained in chamber 2 and minimum cell number was seen in chamber 1. It can be claimed that; due

to closeness of chamber 1 to the middle inlet, fast circulation may have occurred within the c-shaped region of chamber 1, therefore most of the cells may be swept out of the zone to the next chamber, Chamber 2. In addition, chambers 3 and 4, and chambers 5 and 6 almost had same number of cells but chambers 5 and 6 had higher values of cells than those obtained in chambers 3 and 4.

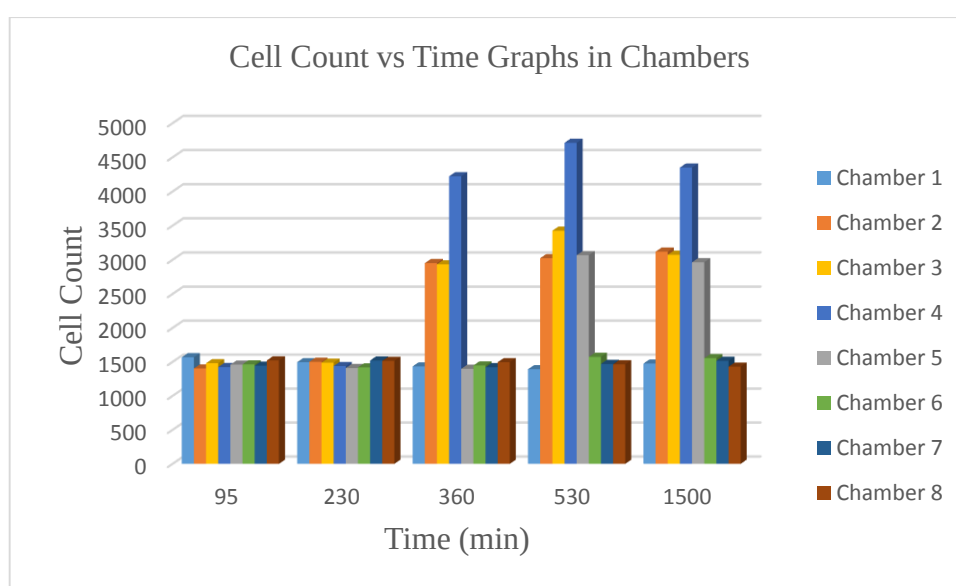


Figure 4.95. Cell count vs time graph for all chambers (fluorescence images).

In Figure 4.95, cell counts obtained by processing the fluorescence microscopy images were given for all the chambers at 5 different time points. According to the figure, almost equal number of cells was seen in chamber 1. This situation can be explained by cell loading because, chamber 1 was the closest chamber to the middle inlet during cell loading, in the experiment cells first visited this chamber. After a while, when the nutrient was fed to the microfluidic device, cells conserved their positions in the chamber, any alteration in their location was not seen. In chambers 2-5, increasing trend was observed throughout the experiment. There were some decreases in the final data for these chambers and this can be explained by cell sedimentation. Maximum numbers of cells were attained in chamber 4. In chambers 6, 7 and 8 the increasing trend of cell count was not obtained, they all exhibited almost same trend with respect to time.

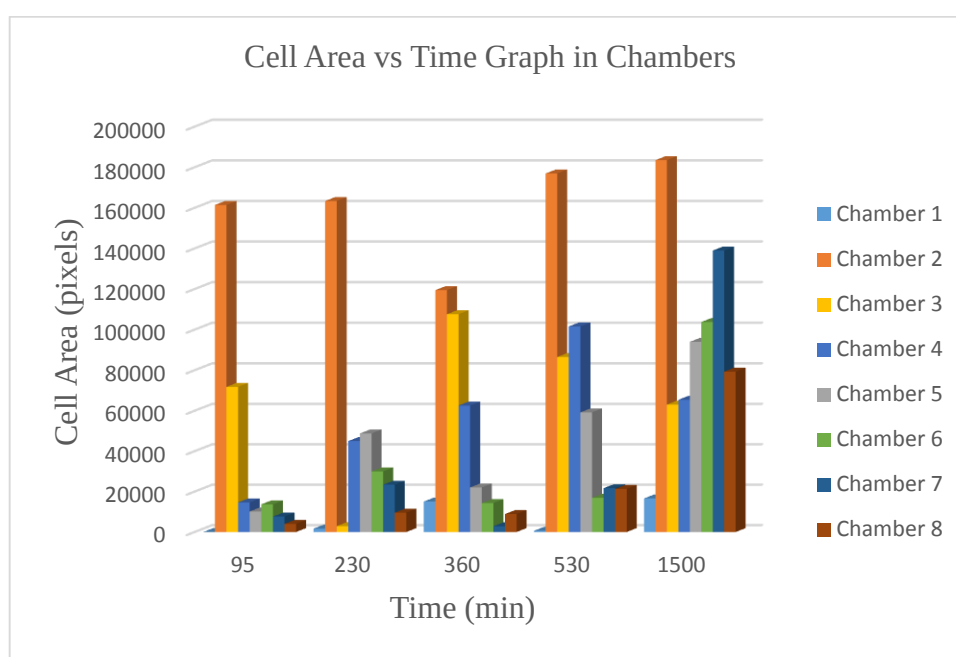


Figure 4.96. Cell area vs time graph for all chambers (brightfield images).

The area and the perimeters of cells in all chambers (Figures 4.96 and 4.97) follow similar trends. According to Figures 4.96 and 4.97, cell area and perimeter exhibited increasing trends in chamber 1 except the outlier seen at 530th minutes. Although, chamber 2 had non-uniform cell count distribution in Figure 4.94, area and perimeter of cells in chamber 2 displayed rising trends throughout the experiment except the data taken at 360th minutes. In chamber 3, ups and downs in cell area and perimeter were monitored resulting in uneven distribution. In addition, cell sedimentation effect can be mentioned in chamber 3.

Cell area and perimeter in chamber 4, represented by blue colored bar on the charts, showed increasing trends towards the final data, but the final data taken at 1500th minutes had smaller value than the maximum value attained. Therefore, cell sedimentation effect may again be thought for chamber 4. Cell area and perimeter in chambers 5-8 had increasing trends throughout the experiment with some outliers like the result on cell count, chamber 2 stood out with its maximum values in cell area and perimeter. After chamber 2, chambers 7 and 4 also attained maximum cell area values, respectively. To sum up, cell area and perimeter in chambers 1, 2, 5, 6, 7 and 8 showed rising trend lines throughout the experiment.

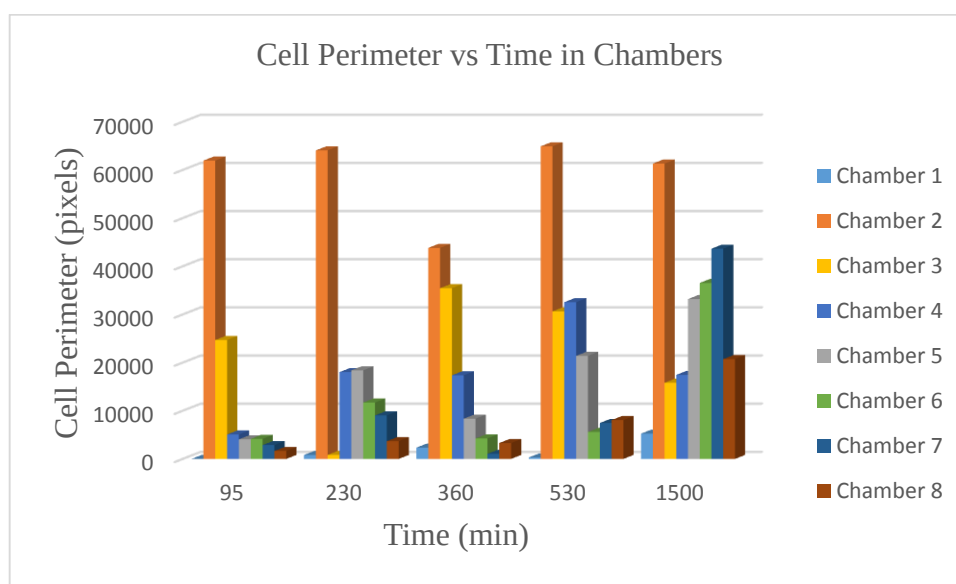


Figure 4.97. Cell perimeter vs time graph for all chambers (brightfield images).

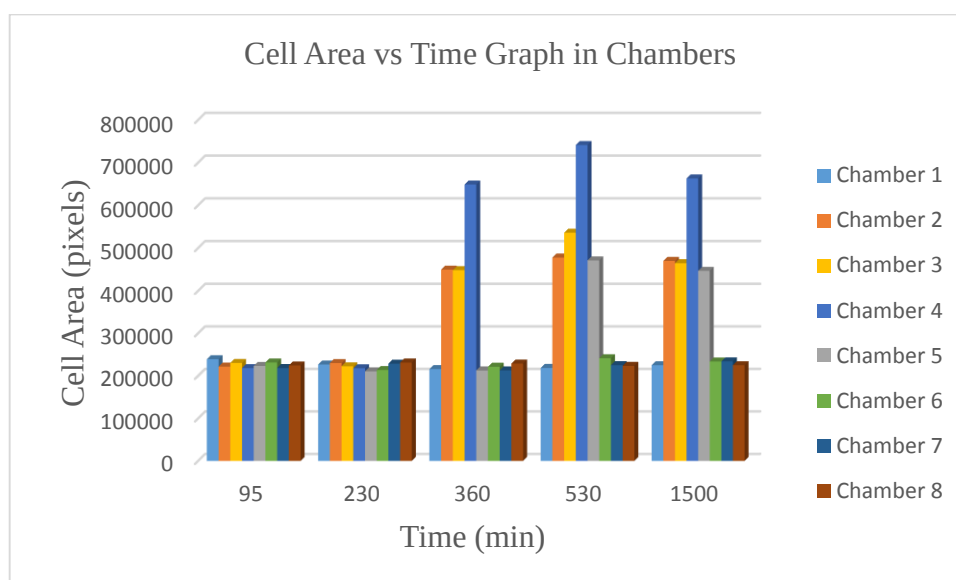


Figure 4.98. Cell area vs time graph for all chambers (fluorescence images).

According to the results obtained by processing the fluorescence microscopy images (Figures 4.98 and 4.99), cell area and perimeter did not change significantly in chamber 1. Unlike chamber 1, chambers 2-4 exhibited increasing trends in both cell area and perimeter. As time goes by, cell area expanded without any outliers. As it was the case for the cell count (Figure 4.95), a slight decrease was observed in the final data of chambers 3 and 4 due to

cell sedimentation. When chamber 5 was considered, an incrementing trend was seen in the graphs after 360th minutes but again a decrease occurred at the end. Finally, the last three chambers, Chambers 6, 7 and 8 have almost linear trends in the parameters of cell area and perimeter (Figures 4.98 and 4.99).

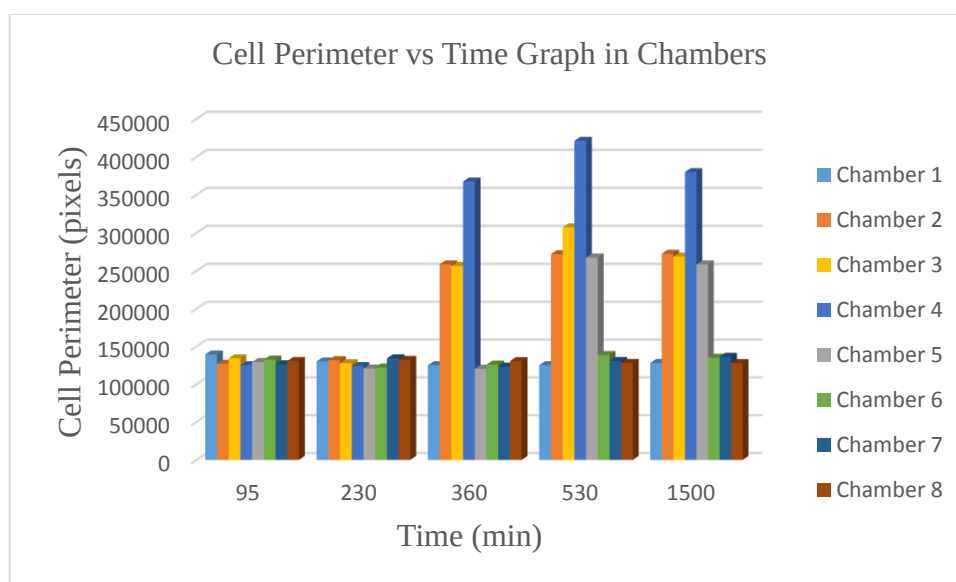


Figure 4.99. Cell perimeter vs time graph for all chambers (fluorescence images).

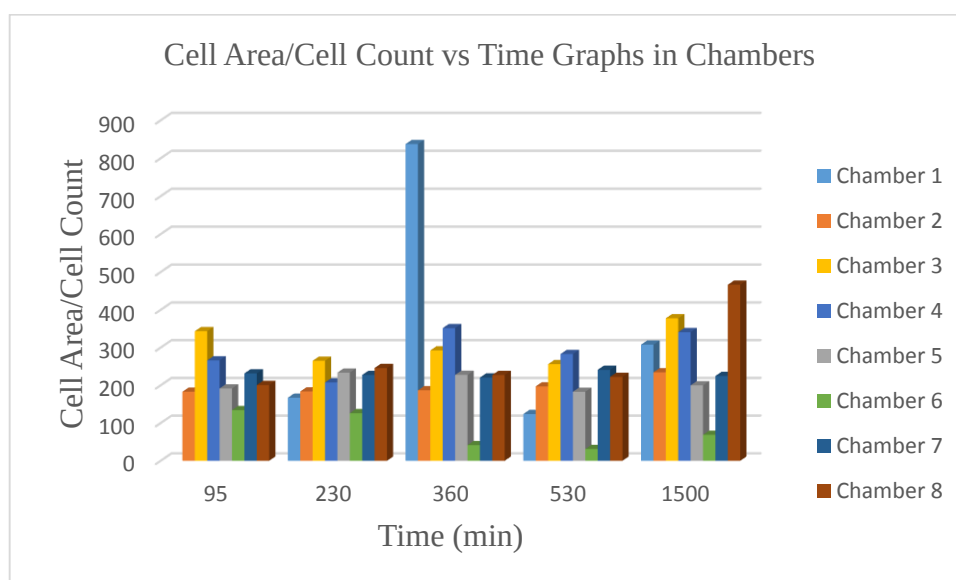


Figure 4.100. Cell area/cell count vs time graph for all chambers (brightfield images).

The cell area/cell count for all chambers was plotted with respect to time in Figure 4.100. At first glance, the outlier was seen at 360th minutes for chamber 1. Except that outlier, all chambers had approximately similar values with each other. Although rises and falls were seen in Figure 4.100, almost linear trend can be seen. Maximum values were found in chamber 3, chamber 4 and chamber 8 and the minimum value was found in chamber 6. Since the cell area/cell count values were close to each other, it can be said that, stable environment under equal conditions were created for all the chambers during the experiment.

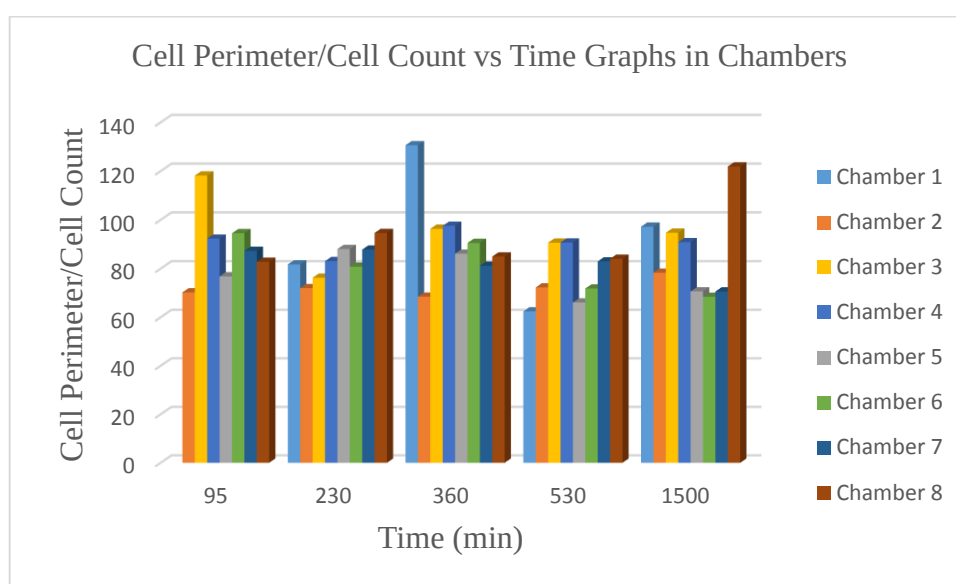


Figure 4.101. Cell perimeter/cell count vs time graph for all chambers (brightfield images).

More regular trend was seen in the Figure 4.101 for cell perimeter/cell count. Every chamber had close values of cell perimeter with each other and as time goes by, the values did not show extreme changes. It can be said that, by looking at both Figure 4.100 and Figure 4.101, permanent habitat was constituted for yeast cells from beginning to the end of the experiment under optimum conditions.

The graphs of cell area/cell count and cell perimeter/cell count obtained by processing fluorescence microscopy images were given in Figures 4.102 and 4.103. According to the graph, except some outliers, the parameters (cell area/cell count and cell perimeter/cell count) evaluated had same values. The range for cell area/cell count was between 148 and

158. The cell perimeter/cell count range for all chambers was between 85 and 91. In addition, maximum values were seen in chambers 2, 6 and 8.

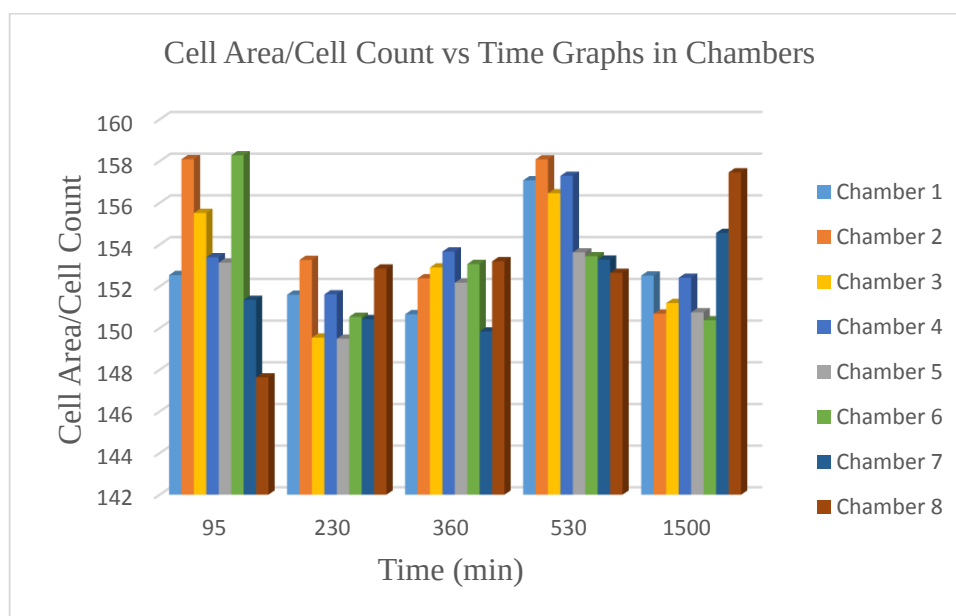


Figure 4.102. Cell area/cell count vs time graph for all chambers (fluorescence images).

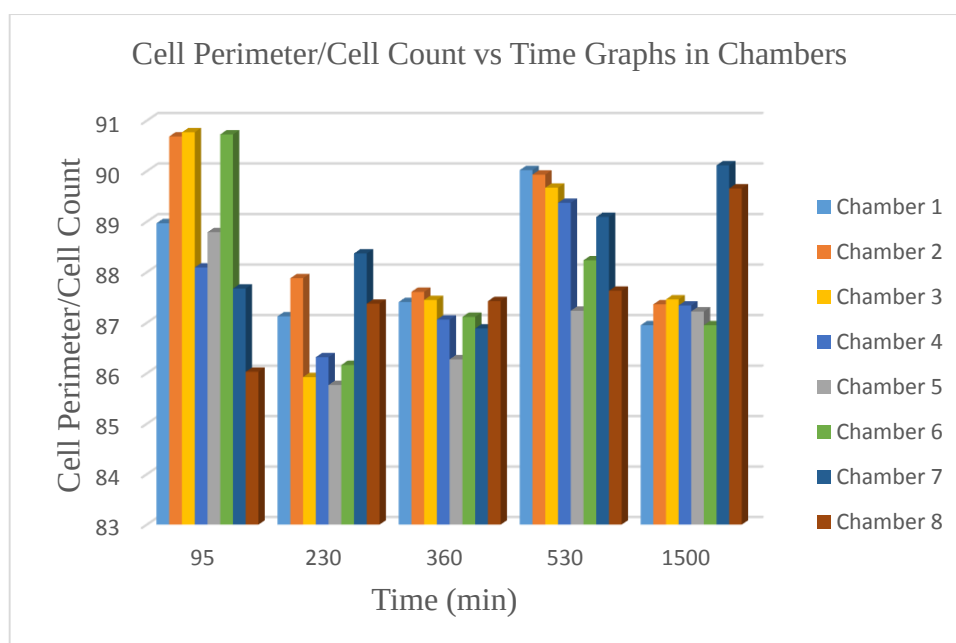


Figure 4.103. Cell perimeter/cell count vs time graph for all chambers (fluorescence images).

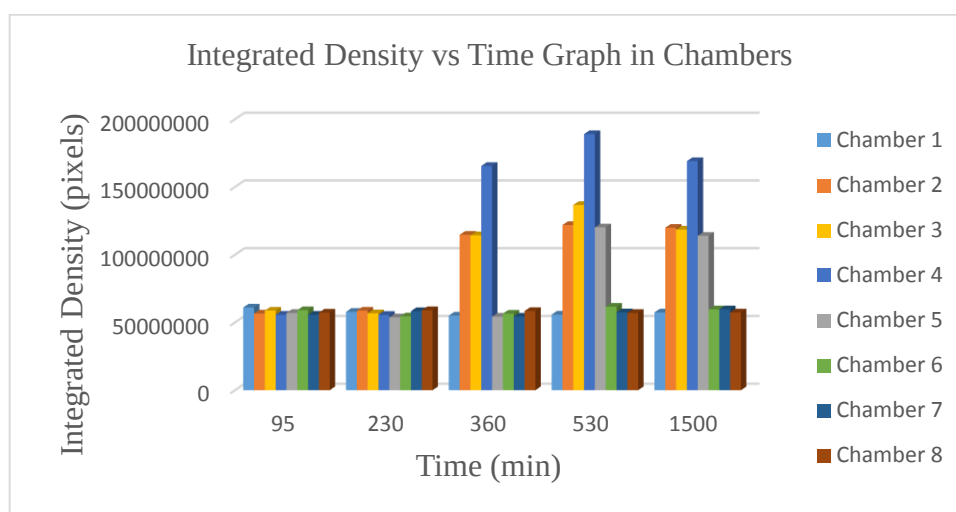


Figure 4.104. Cell integrated density vs time graph for all chambers (fluorescence images).

Cell integrated density with respect to time was displayed for all chambers in Figure 4.104. Chamber 1, chamber 6, chamber 7 and chamber 8 showed no change. They all had almost linear trend in cell integrated density parameter throughout the experiment. Chamber 2 had a rising trend in cell integrated density for all times. Chambers 3-5, represented by yellow, blue and gray colored bars, showed increasing trends up to 530th minutes but at the final value, a decrease was seen due to cell sedimentation or improper focusing. Maximum values in this parameter were attained in chamber 4.

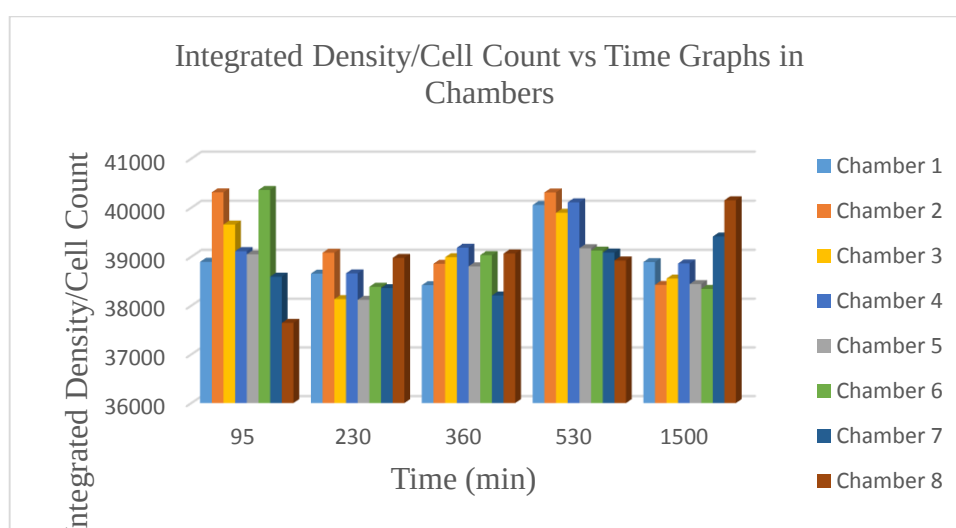


Figure 4.105. Cell integrated density/cell count vs time graph for all chambers (fluorescence images).

The graph of cell integrated density/cell count was displayed in Figure 4.105, where almost similar values are obtained in the evaluated parameter. Maximum values were attained in chambers 2, 3, 4, 6 and 8. Despite the rises and falls seen in the graph, the range was between 37500 and 40500, which were close to each other. Finally, stable environment was created for yeast cells under optimum conditions during the experiment.

In conclusion, the growth of yeast cells was successfully accomplished in approximately 1.5 nanoliter chambers of the fabricated microbio reactor, and the behavior of cells was investigated in the present Lab-on-a-Chip platform.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

In conclusion, the following achievements were obtained on the basis of the thesis work:

- The designed microfluidic chip was successfully produced by employing hot embossing and solvent-assisted bonding techniques.
- The velocity and concentration profiles within the chip were revealed via COMSOL Multiphysics program and appropriate flow rates were determined for the experiment with yeast cells.
- The performance of the fabricated chip was tested by feeding polystyrene beads (2 and 10 μ m in diameter) into the device.
- Yeast cell trapping and culturing operations were successfully performed within the chip (chambers of 1.5 nanoliter volume), confirming that the chip served the intended purpose.
- The experiment with yeast cells was carried on for approximately 1600 minutes (16 generations) and the microbioreactor was found to operate properly.
- The brightfield and fluorescence microscopy images of all the chambers were taken at regular time intervals during the experiment and they were processed via Fiji image processing package program. The parameters such as cell count, cell area, cell perimeter and cell integrated density within the chambers were calculated and it was found that they show an increasing trend throughout the experiment confirming the growth of yeast cells within the microbioreactor.

- In brief, the here designed and in-house fabricated Lab-on-a-Chip platform is operating successfully and can be used for yeast culturing experiments.

5.2. Recommendations

The ultimate aim of this thesis study was to fabricate and operate a microfluidic device for yeast culturing. Within the framework of this thesis, a GFP tagged strain of *Saccharomyces cerevisiae* was used through the produced chip and cell count, cell area, cell perimeter and cell integrated density results were estimated. Nonetheless, a lot more studies should be done in order to deeply investigate the function of YOR060C protein.

The following recommendations can be implemented for the future work:

- During the experiment, yeast cells at some locations could not be properly detected within the device. Their appearance was almost blurred in the chambers. In addition, many yeast cells disappeared toward the bottom of the chip due to cell sedimentation. In order to provide clearer cell images and prevent the sedimentation, inverted fluorescence microscope with phase contrast attachment has to be used.

- Since PMMA was a biologically compatible and optically clear polymer, it was safely employed in this work. Other polymers such as PDMS can be used to benefit from its elastic property. PDMS can undergo reversible deformations, therefore, single-cell experiments can be performed in a chip made of PDMS.

- In the experiment, 2% glucose solution was used as the main carbon source. Different concentrations of glucose can be used to create perturbations (environmental stress) within the system to monitor the yeast cells' behavior against the changed environment.

- In order to have thorough insights about YOR060C protein, the experiment should be repeated with improved conditions for visualization of its localization in nucleus as wells as in spindle pole.

APPENDIX A: RESULTS OF FLUORESCENCE MICROSCOPY IMAGES FOR CHAMBERS

A.1. Results of Fluorescence Microscopy Images for Chamber 1

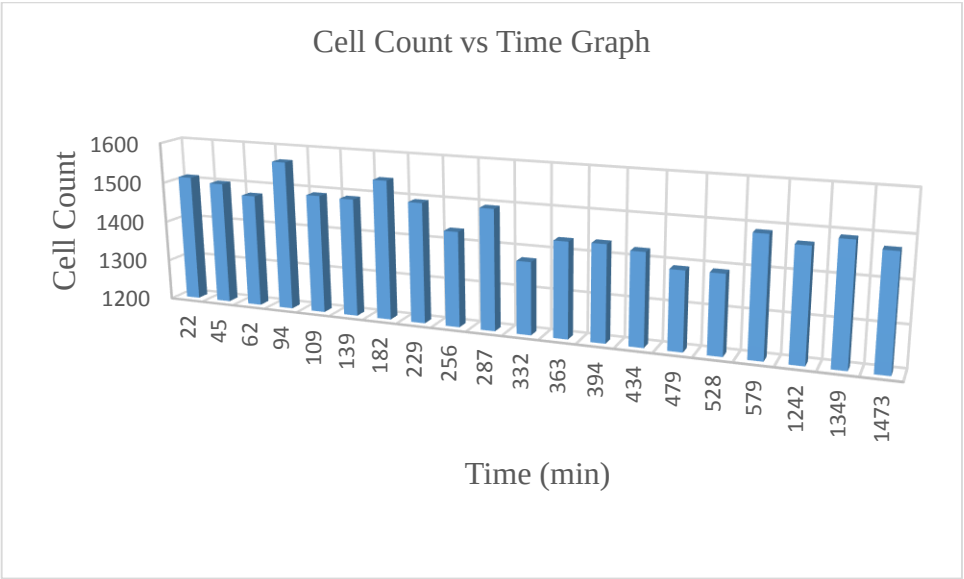


Figure A.1. Cell count vs time graph for chamber 1.

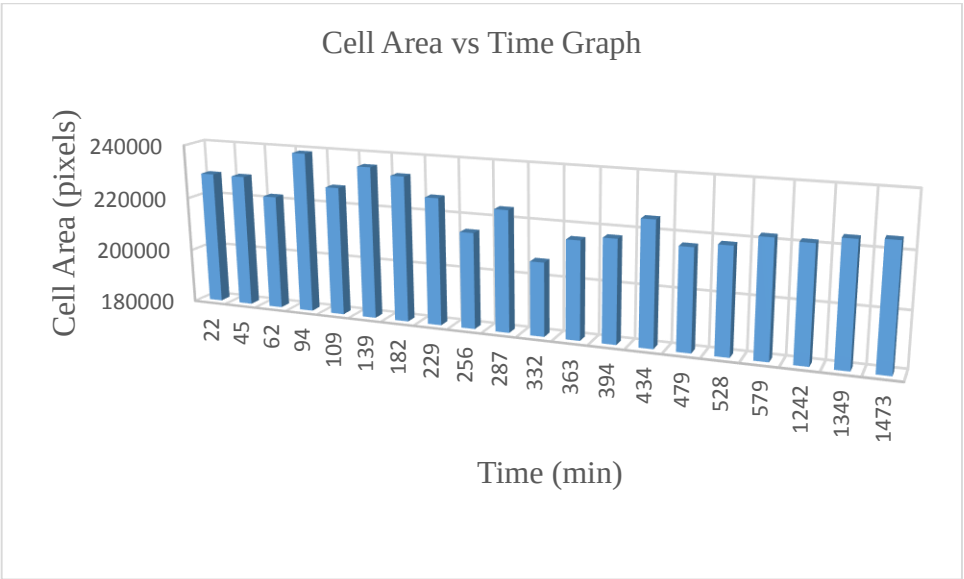


Figure A.2. Cell area vs time graph for chamber 1.

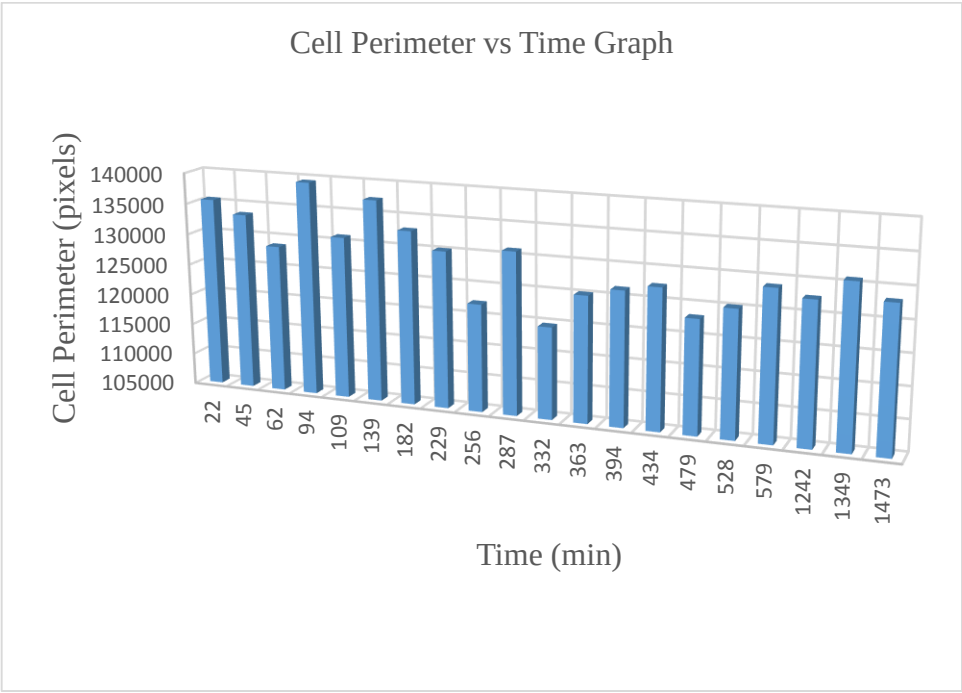


Figure A.3. Cell perimeter vs time graph for chamber 1.

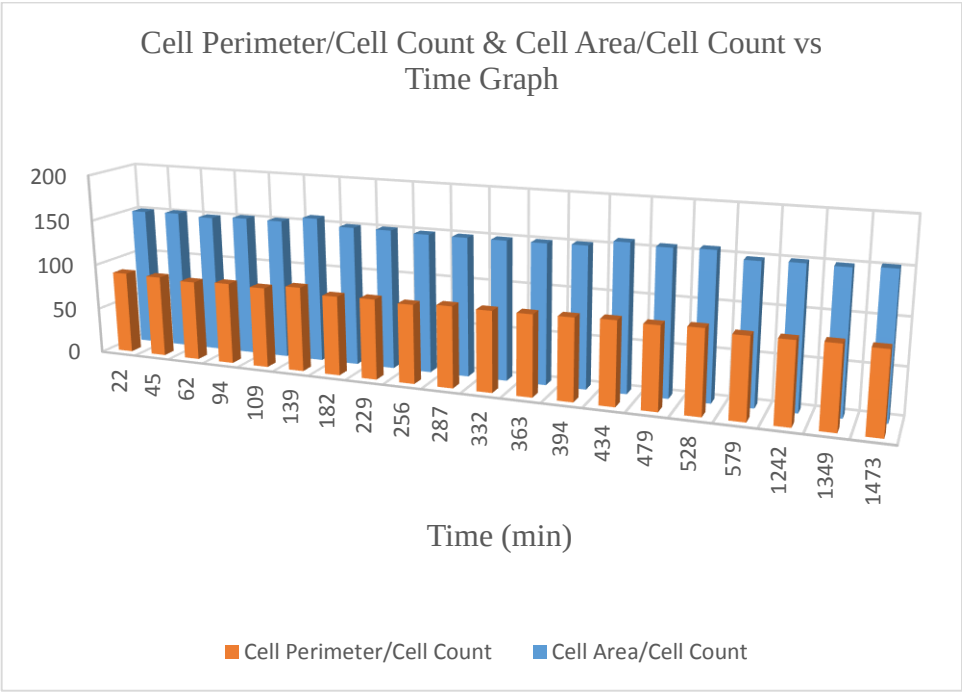


Figure A.4. Cell perimeter/cell count and cell area/cell count vs time graph for chamber 1.

A.2. Results of Fluorescence Microscopy Images for Chamber 2

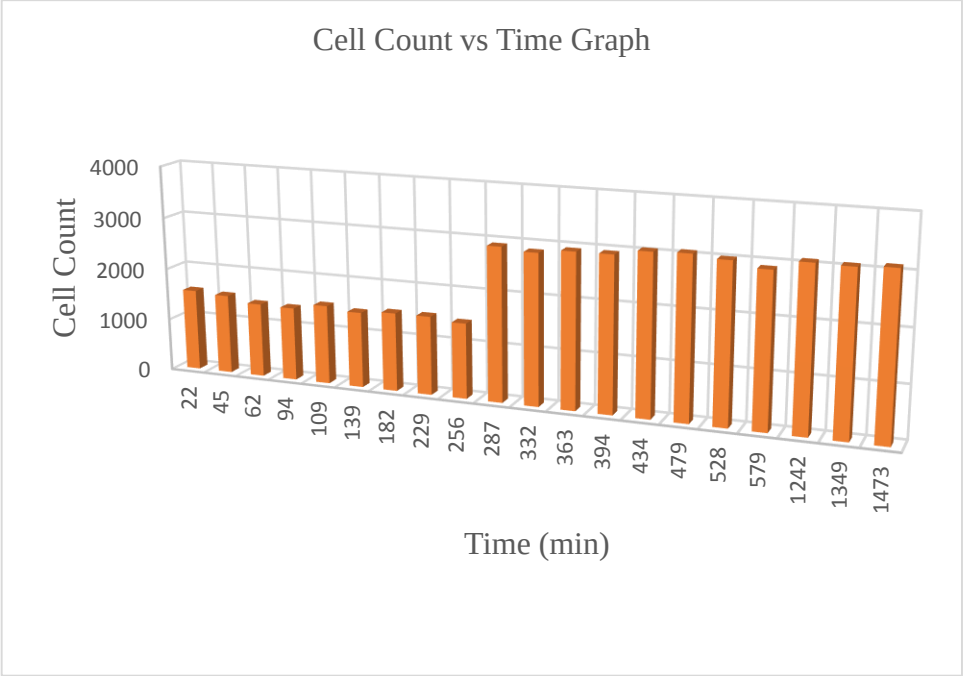


Figure A.5. Cell count vs time graph for chamber 2.

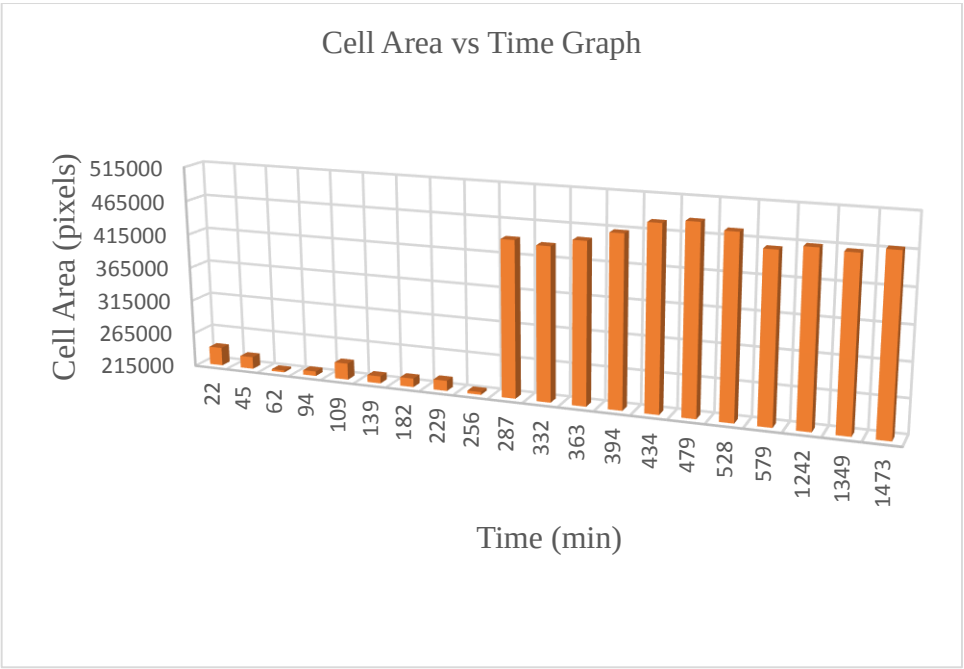


Figure A.6. Cell area vs time graph for chamber 2.

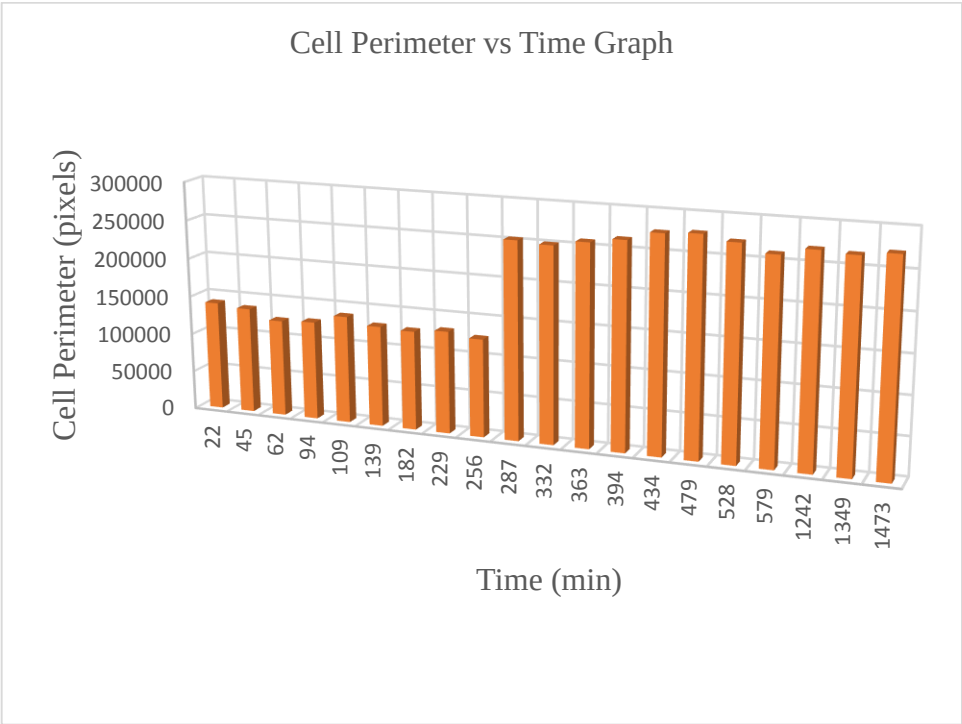


Figure A.7. Cell perimeter vs time graph for chamber 2.

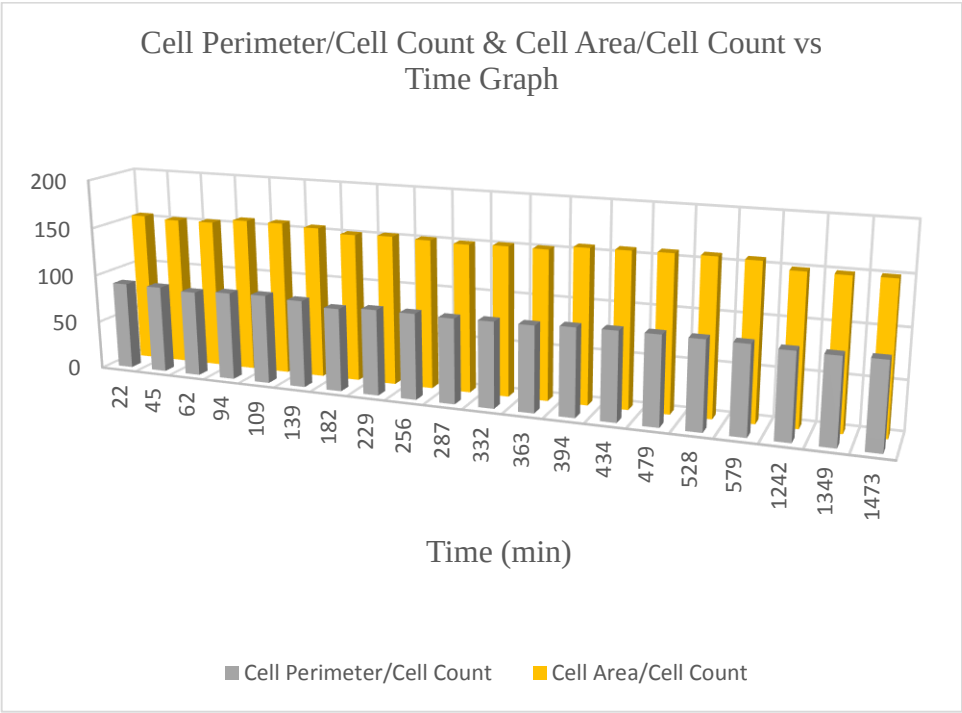


Figure A.8. Cell perimeter/cell count and cell area/cell count vs time graph for chamber 2.

A.3. Results of Fluorescence Microscopy Images for Chamber 3

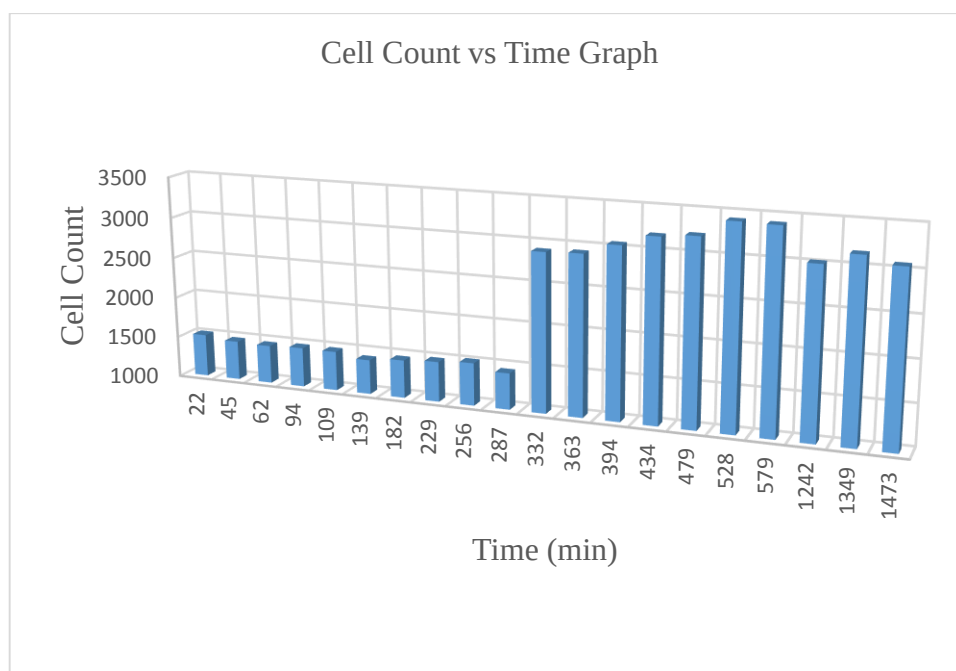


Figure A.9. Cell count vs time graph for chamber 3.

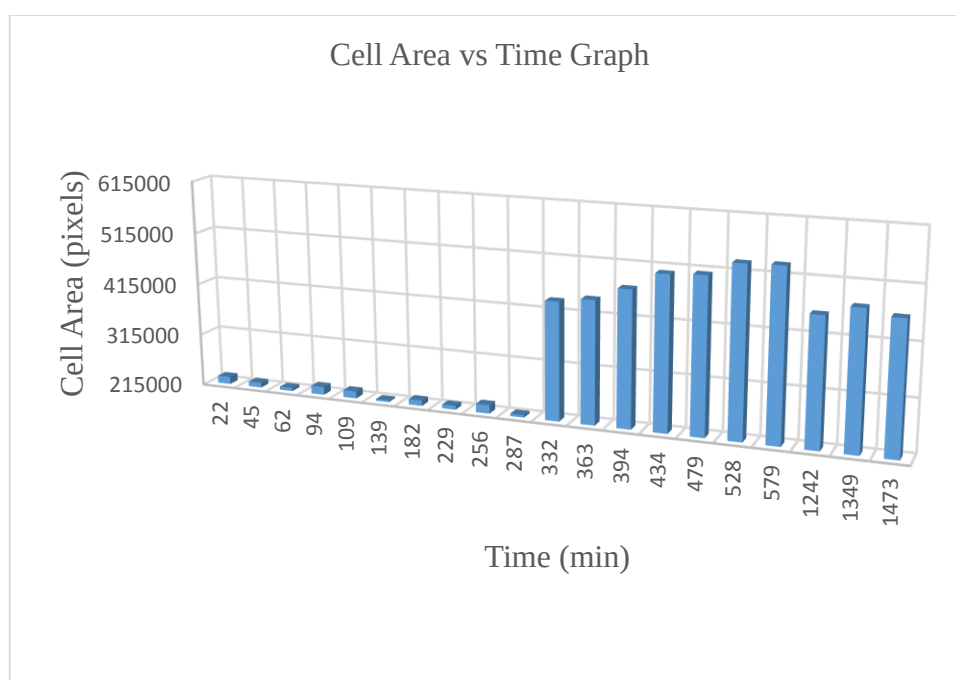


Figure A.10. Cell area vs time graph for chamber 3.

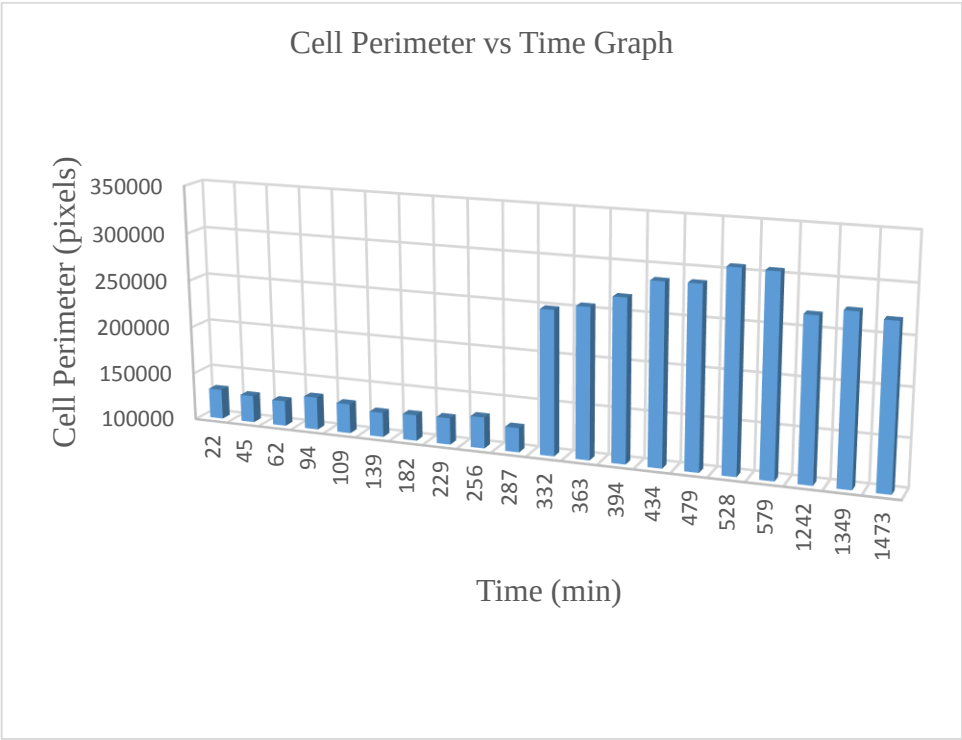


Figure A.11. Cell perimeter vs time graph for chamber 3.

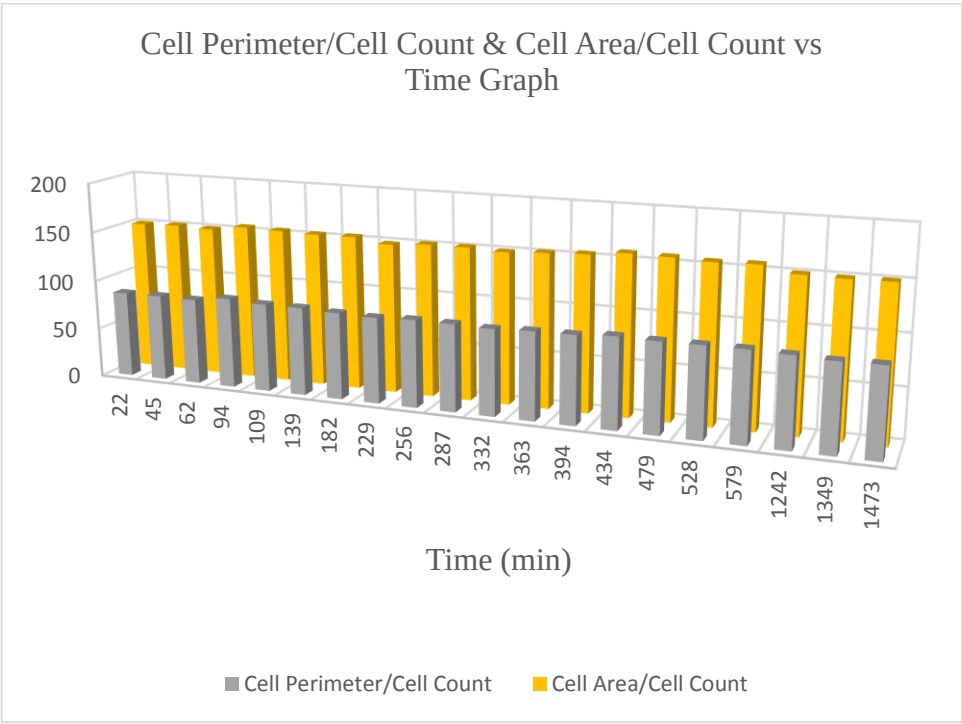


Figure A.12. Cell perimeter/cell count and cell area/cell count vs time graph for chamber 3.

A.4. Results of Fluorescence Microscopy Images for Chamber 4

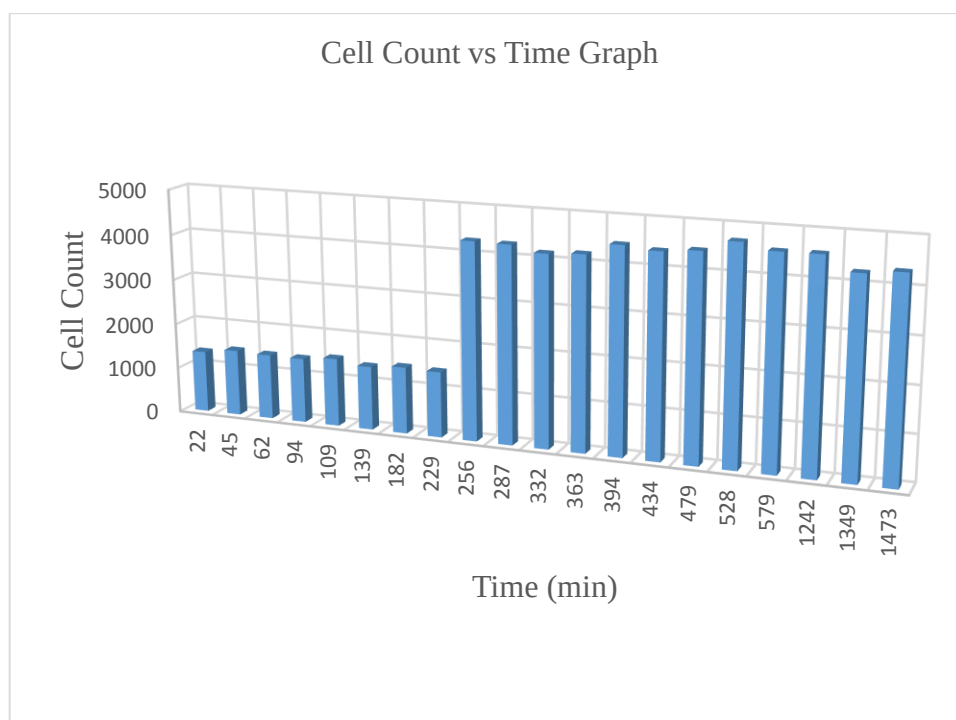


Figure A.13. Cell count vs time graph for chamber 4.

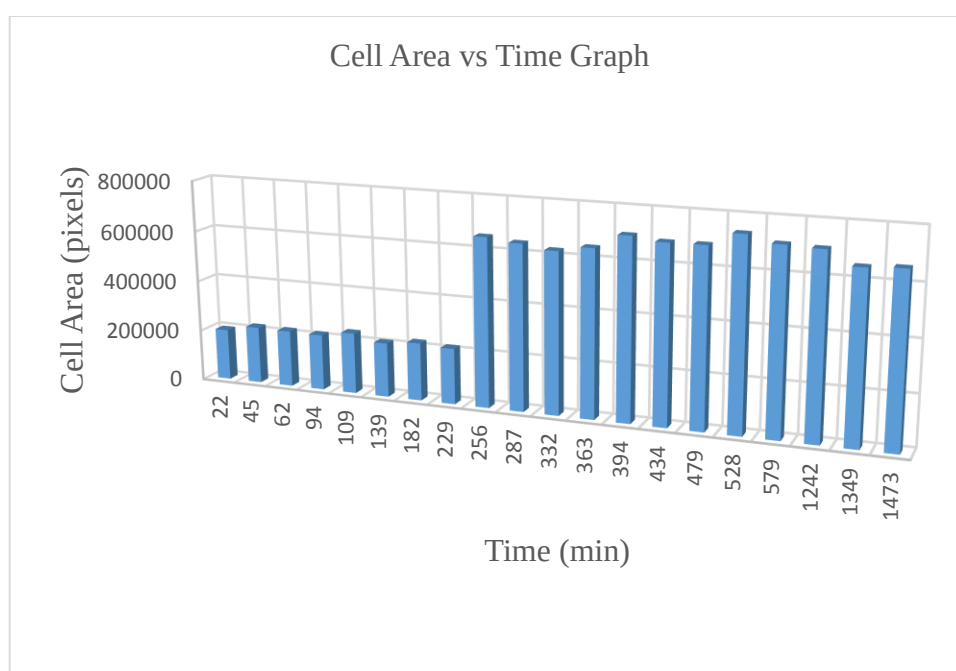


Figure A.14. Cell area vs time graph for chamber 4.

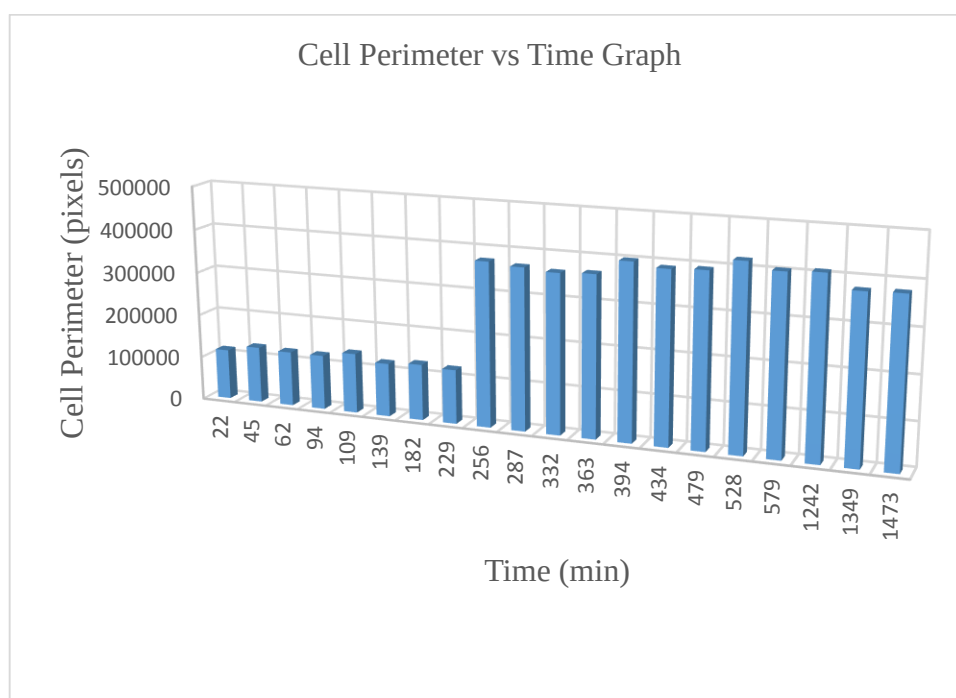


Figure A.15. Cell perimeter vs time graph for chamber 4.

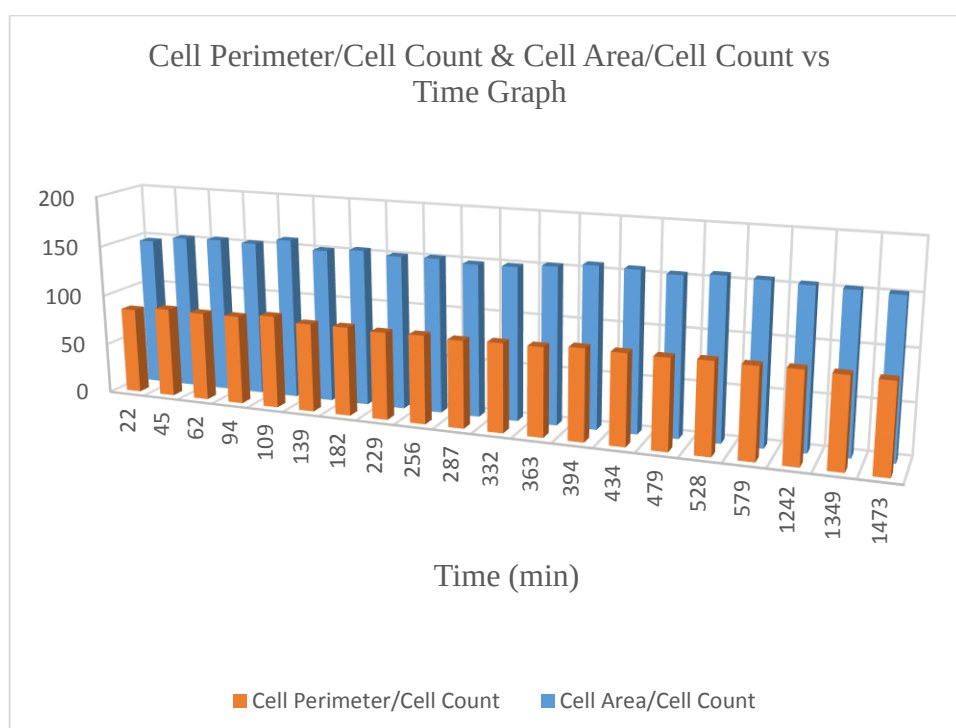


Figure A.16. Cell perimeter/cell count and cell area/cell count vs time graph for chamber 4.

A.5. Results of Fluorescence Microscopy Images for Chamber 5

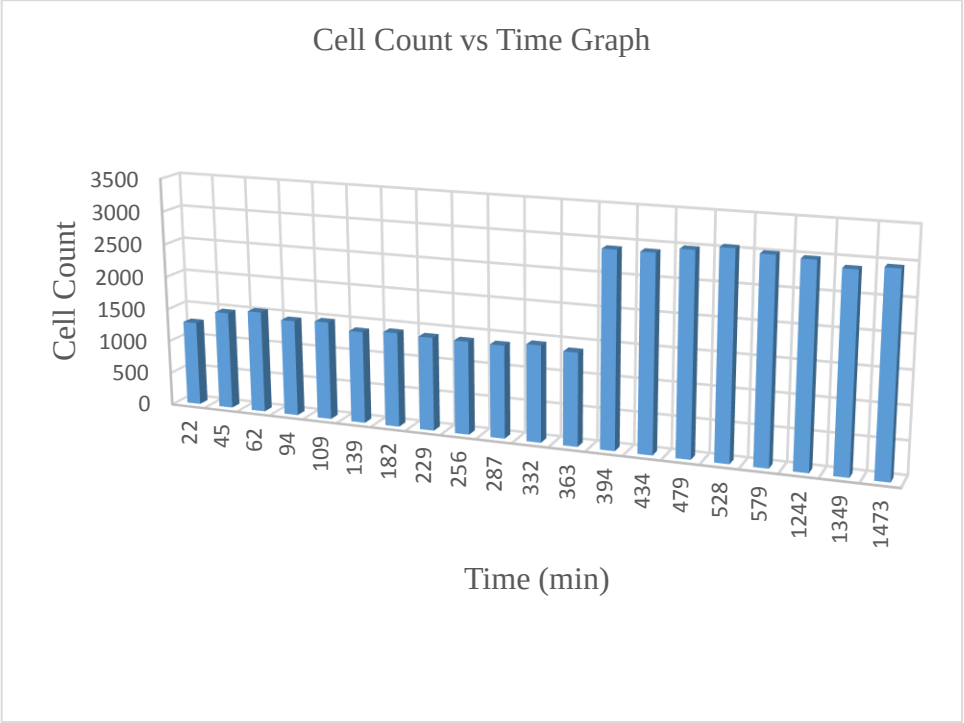


Figure A.17. Cell count vs time graph for chamber 5.

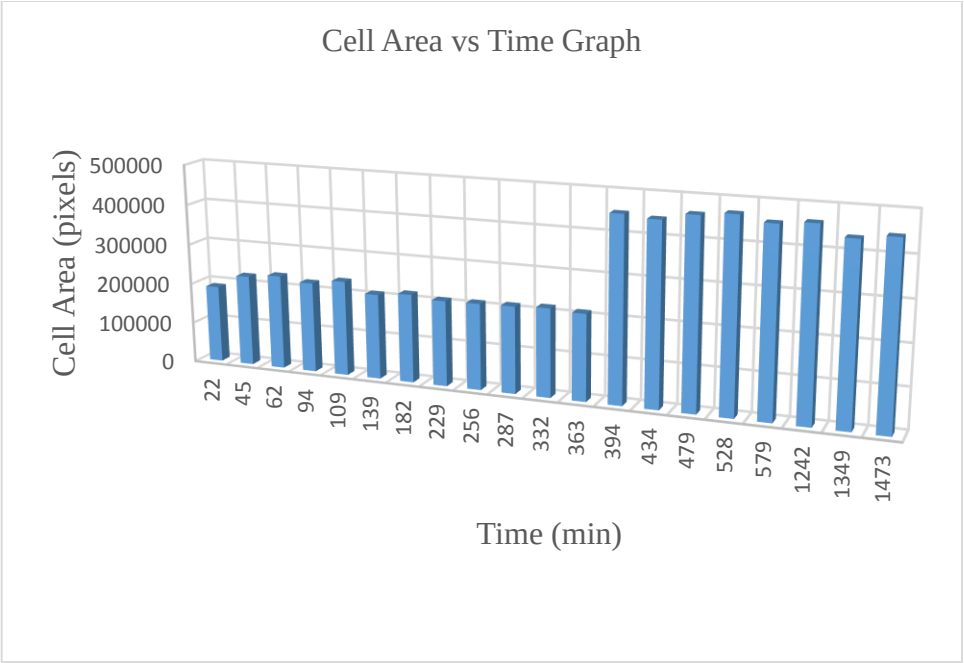


Figure A.18. Cell area vs time graph for chamber 5.

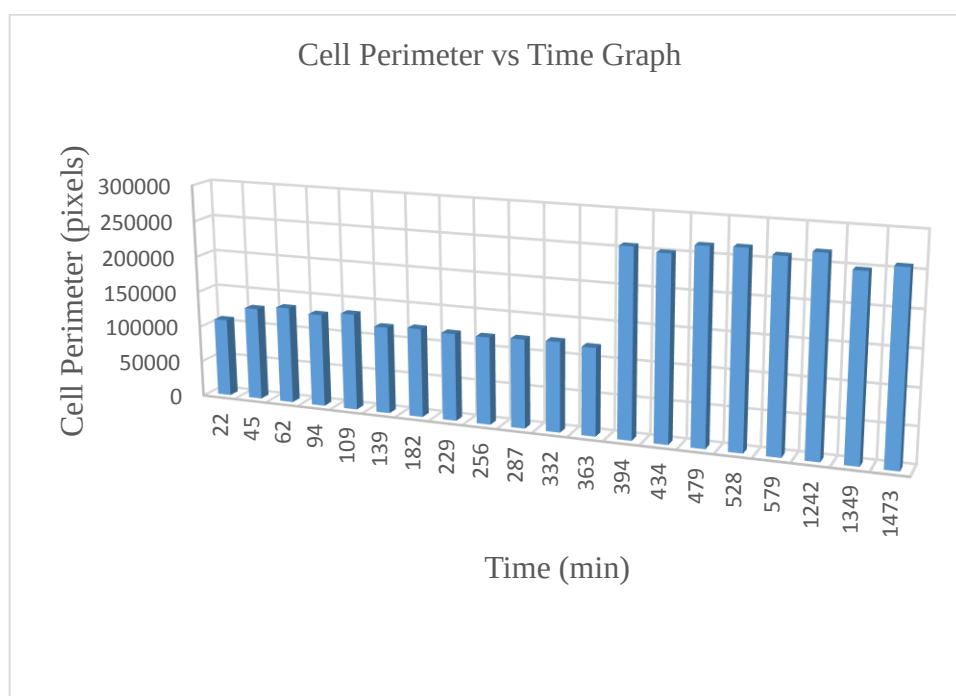


Figure A.19. Cell perimeter vs time graph for chamber 5.

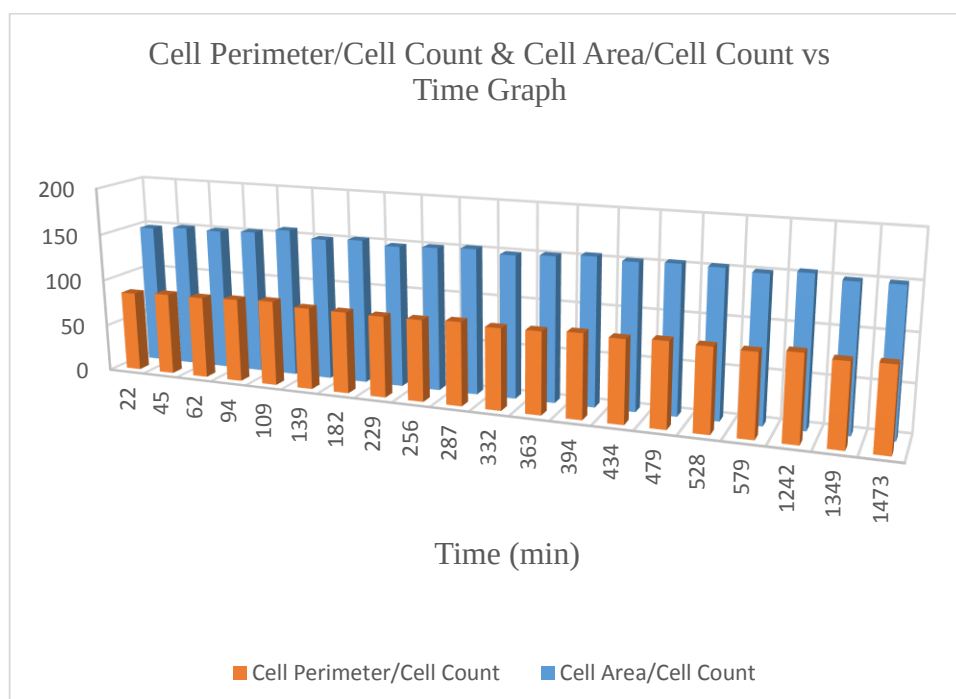


Figure A.20. Cell perimeter/cell count and cell area/cell count vs time graph for chamber 5.

A.6. Results of Fluorescence Microscopy Images for Chamber 6

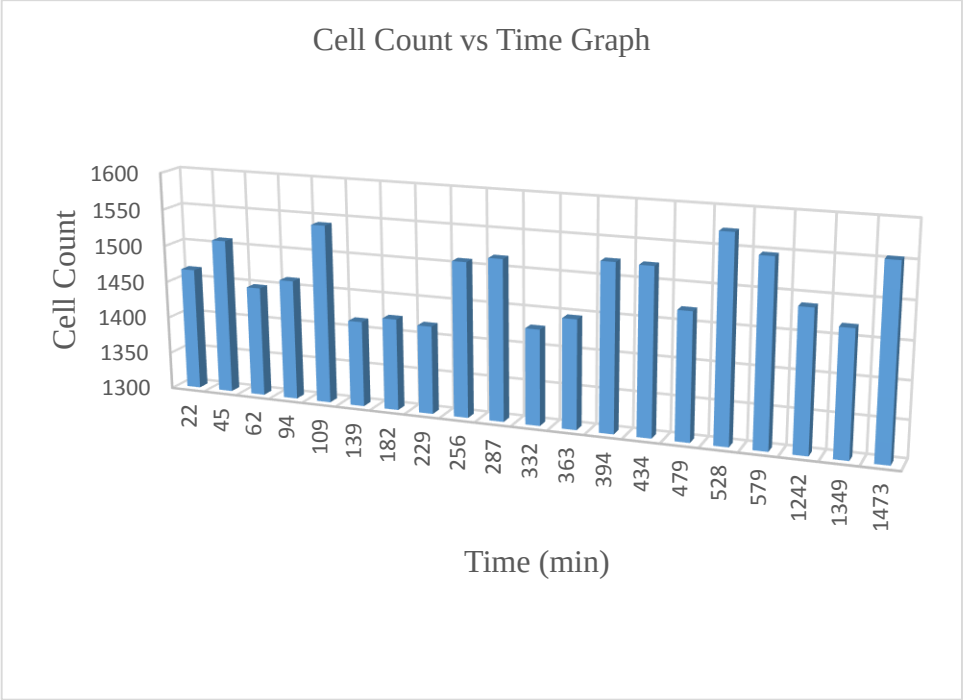


Figure A.21. Cell count vs time graph for chamber 6.

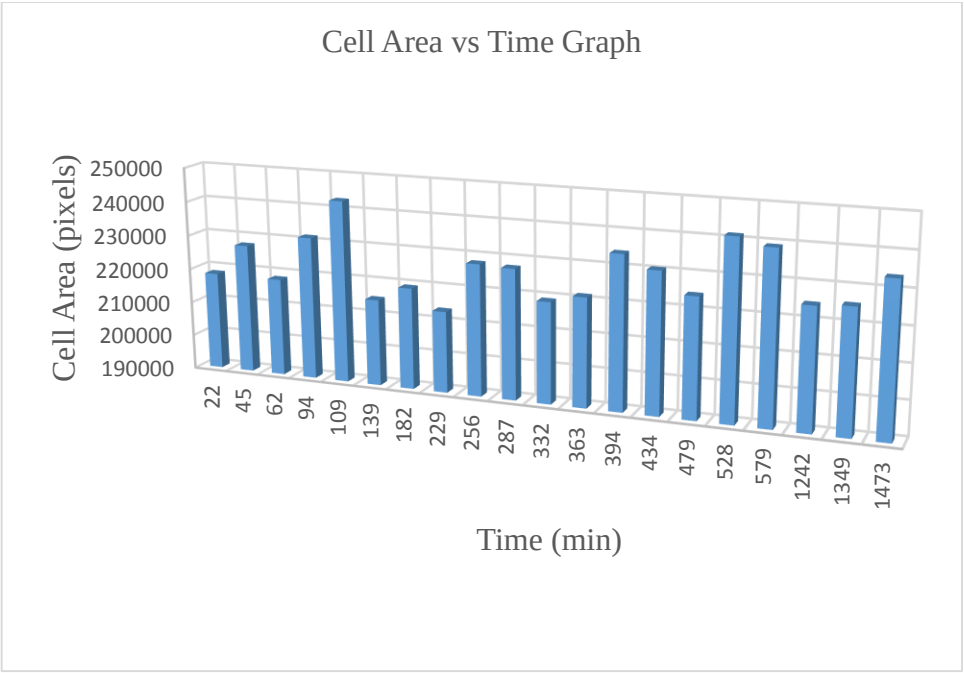


Figure A.22. Cell area vs time graph for chamber 6.

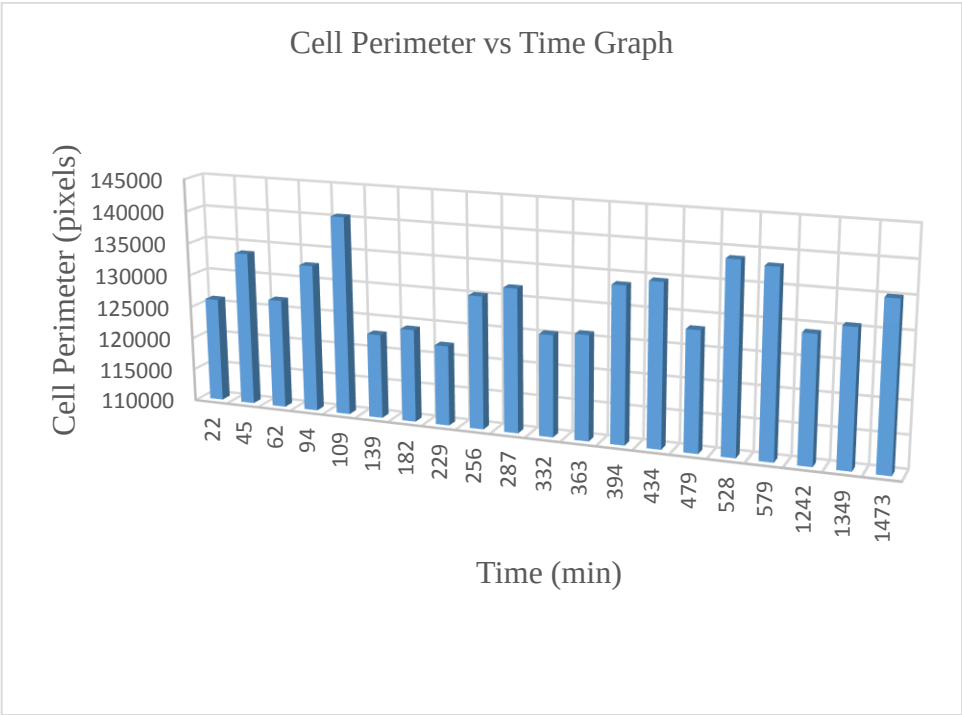


Figure A.23. Cell perimeter vs time graph for chamber 6.

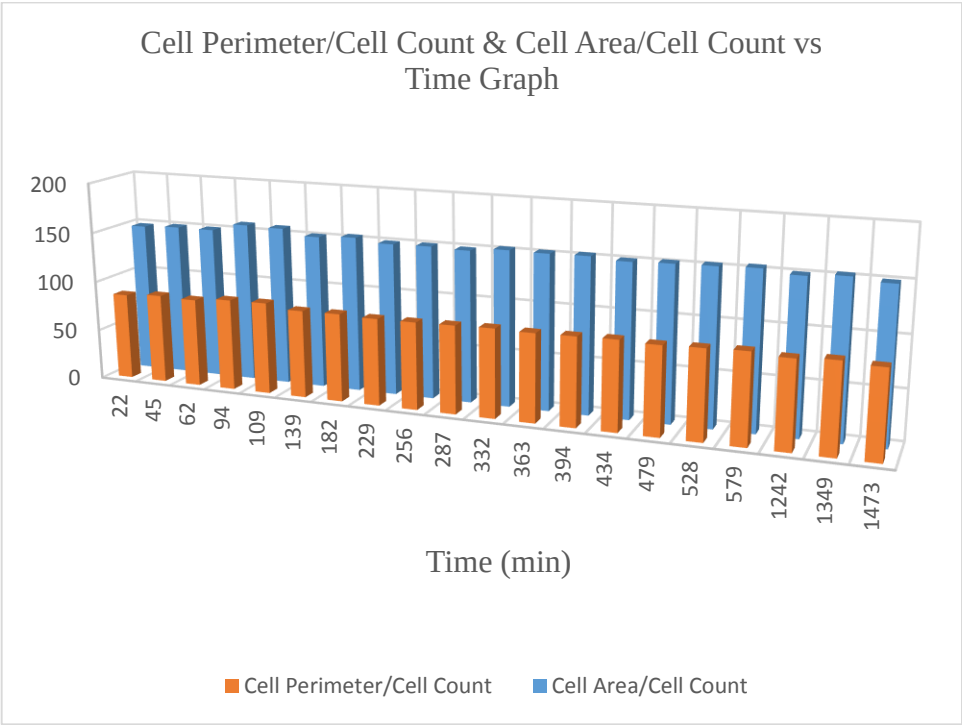


Figure A.24. Cell perimeter/cell count and cell area/cell count vs time graph for chamber 6.

A.7. Results of Fluorescence Microscopy Images for Chamber 7

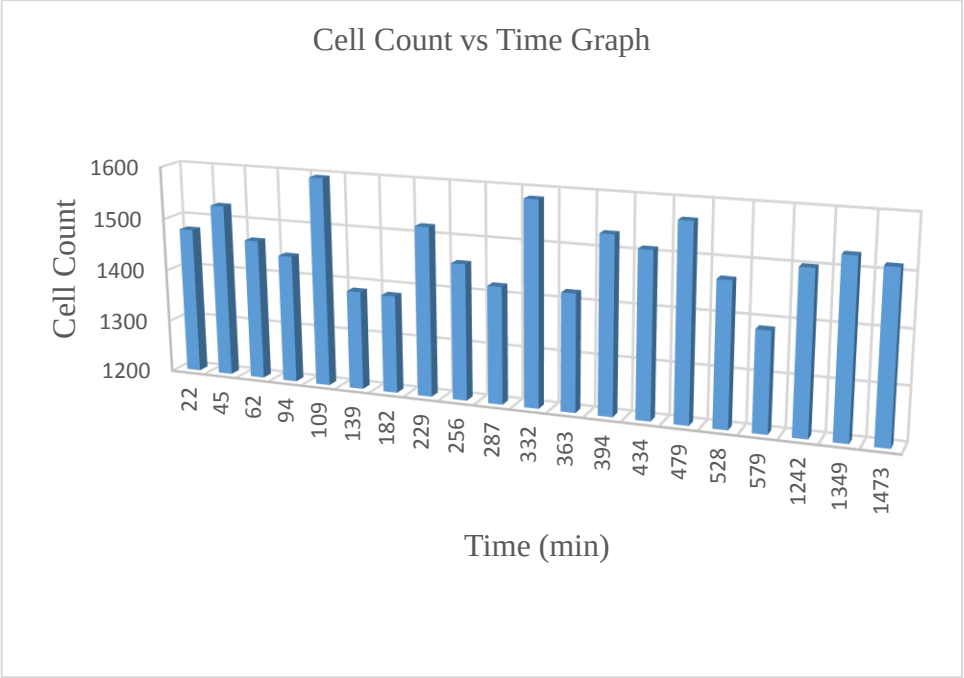


Figure A.25. Cell count vs time graph for chamber 7.

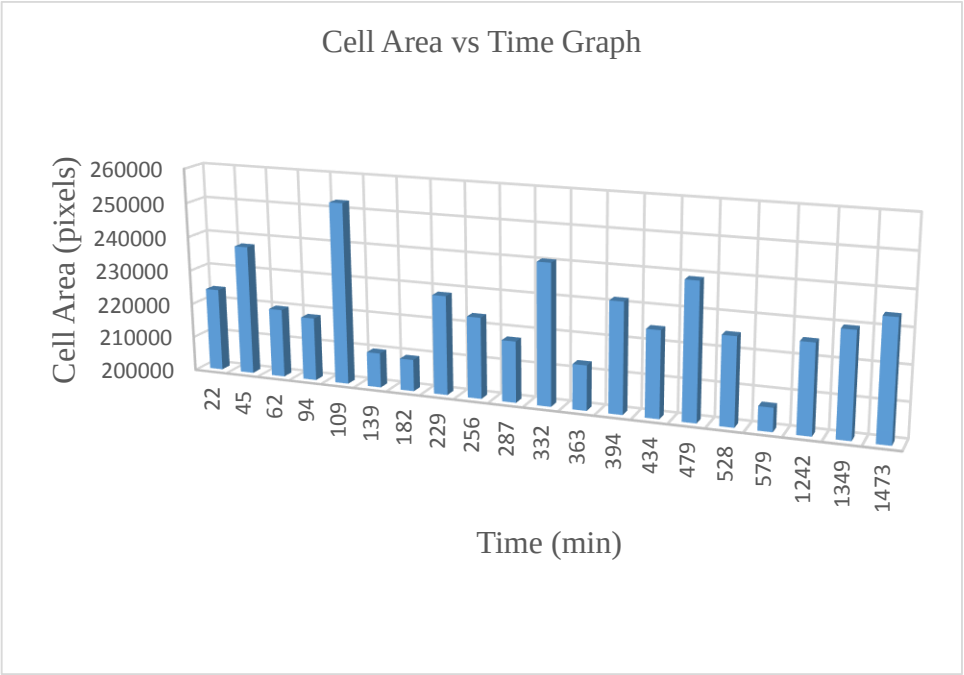


Figure A.26. Cell area vs time graph for chamber 7.

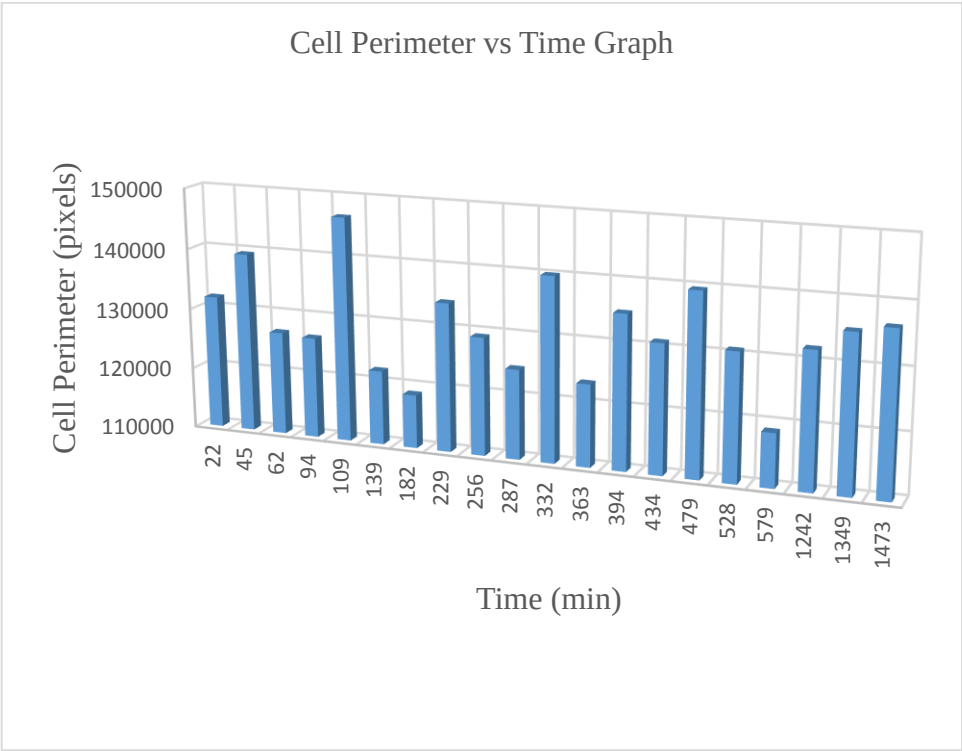


Figure A.27. Cell perimeter vs time graph for chamber 7.

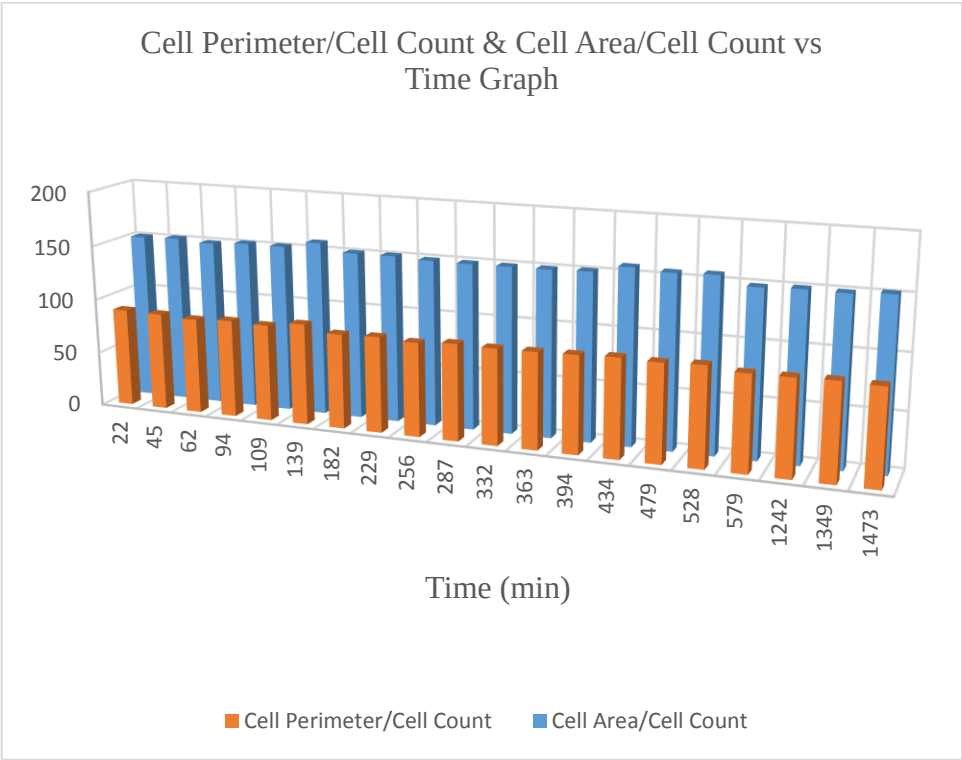


Figure A.28. Cell perimeter/cell count and cell area/cell count vs time graph for chamber 7.

A.8. Results of Fluorescence Microscopy Images for Chamber 8

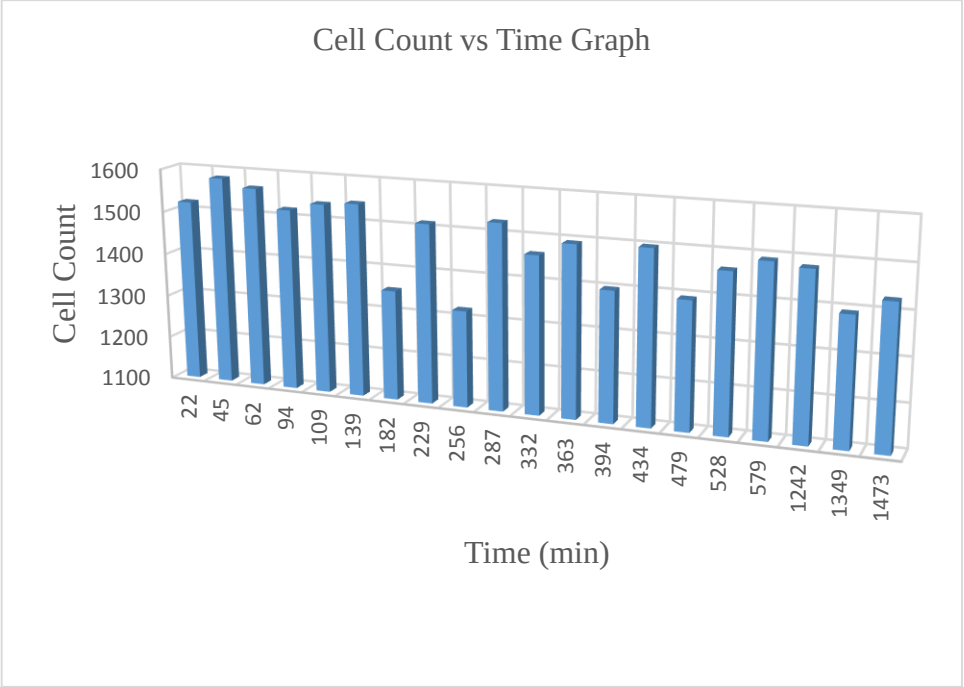


Figure A.29. Cell count vs time graph for chamber 8.

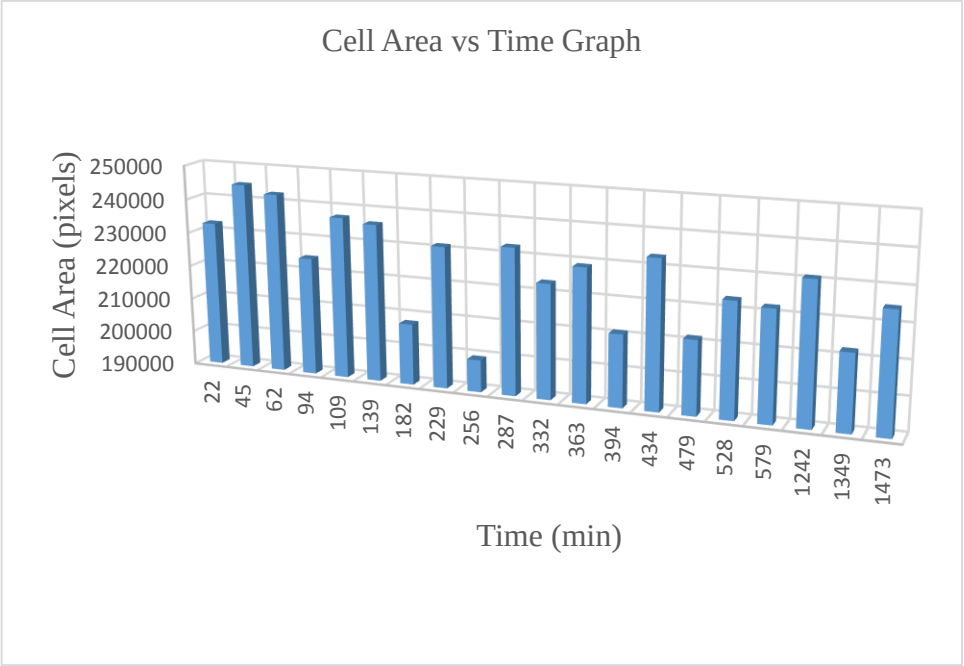


Figure A.30. Cell area vs time graph for chamber 8.

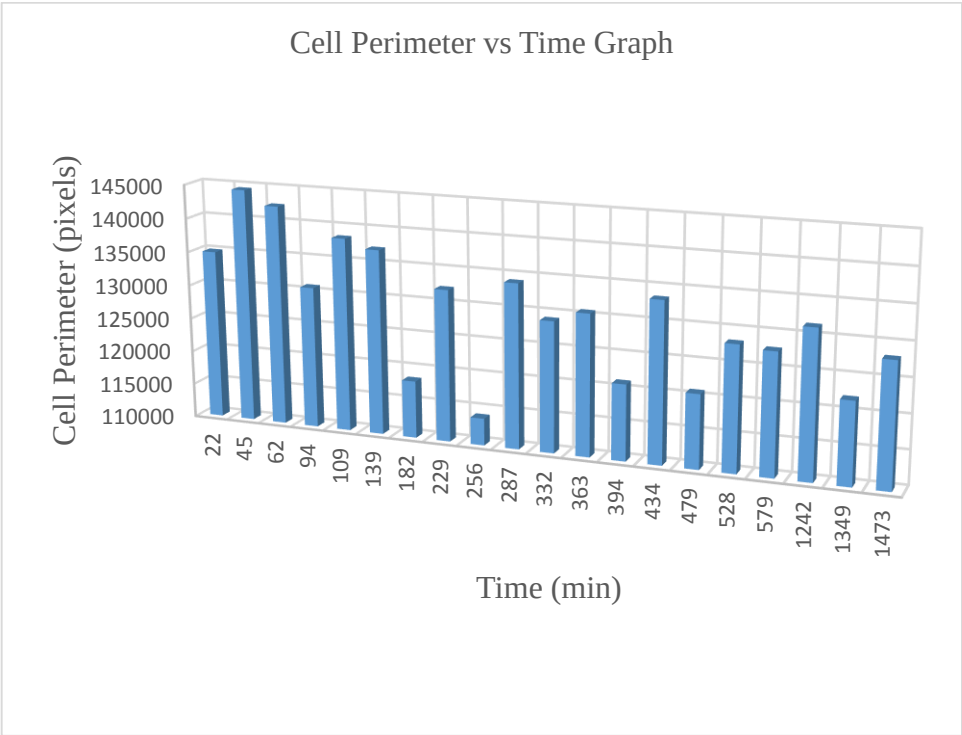


Figure A.31. Cell perimeter vs time graph for chamber 8.

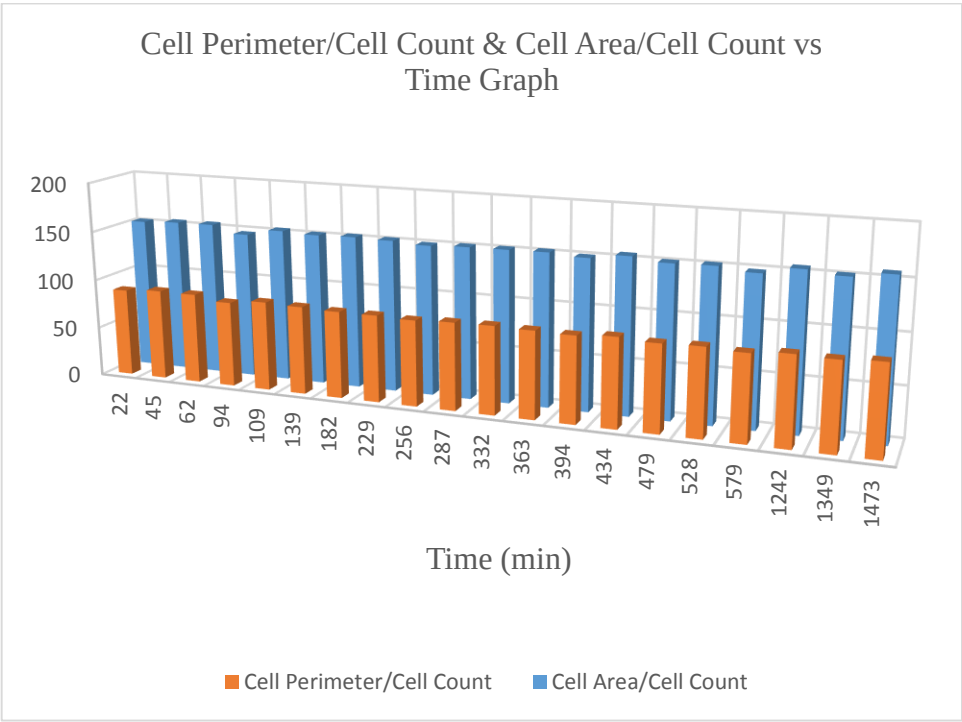


Figure A.32. Cell perimeter/cell count and cell area/cell count vs time graph for chamber 8.

APPENDIX B: DENSITY AND DIFFUSION CONSTANT CALCULATIONS

B.1. Density Calculation

2% glucose solution was used as nutrient in the experiments and needed density for COMSOL simulations were calculated by using the following equation:

$$\rho_{mix} = \frac{1}{x_1/\rho_1 + x_2/\rho_2} \quad (B.1)$$

Where, ρ_{mix} is mixture density, x_1 and x_2 are weight fractions of the components and ρ_1 and ρ_2 are densities of components.

B.2. Diffusion Constant Calculation

Diffusion constant of the 2% glucose solution was calculated by using the following equation and the equation was derived from constants taken from “Binary Mutual Diffusion Coefficients of Aqueous Solutions of Sucrose, Lactose, Glucose, and Fructose in the Temperature Range from (298.15 to 328.15) K” paper [68].

$$D = -3 * 10^{-10}c + 7 * 10^{-10} \quad (B.2)$$

Where, D is diffusion constant and c is concentration of the solution.

REFERENCES

1. Haraldsson, K. T., *Fabrication of Polymeric Devices via Photocurable Liquid Monomers*, Ph.D. Thesis, KTH-Royal Institute of Technology, 2005.
2. Yeo, L. Y., H. Chang, P. P. Y. Chan, and J. R. Friend, "Microfluidic Devices for Bioapplications", *Small*, Vol. 7, pp. 12-48, 2011.
3. Becker, H. and U. Heim, "Hot Embossing as a Method for the Fabrication of Polymer High Aspect Ratio Structures", *Sensors and Actuators A: Physical*, Vol. 83, pp. 130-135, 2000.
4. Mark, D., S. Haeberle, G. Roth, F. V. Stetten, and R. Zengerle, "Microfluidic Lab-on-a-Chip Platforms: Requirements, Characteristics and Applications", *Chemical Society Reviews*, Vol. 39, pp. 1153-1182, 2010.
5. Mauk, M. G., R. Chiou, V. Genis, and M. E. Carr, "Image Analysis of Microfluidics of Flow at the Microscale", *120th ASEE Annual Conference & Exposition*, Atlanta, GA, USA, 2013.
6. Tsao, C., and D. L. Devoe, "Bonding of Thermoplastic Polymer Microfluidics", *Microfluidics and Nanofluidics*, Vol. 6, pp. 1-16, 2009.
7. Walker, K., H. Skelton, and K. Smith, "Cutaneous Lesions Showing Giant Yeast Forms of *Blastomyces Dermatitidis*", *Journal of Cutaneous Pathology*, Vol. 29, pp. 616-618, 2002.
8. Legras, J. L., D. Merdinoglu, J. M. Cornuet, and F. Karst, "Bread, Beer and Wine: *Saccharomyces cerevisiae* Diversity Reflects Human History", *Molecular Ecology*, Vol. 16, pp. 2091-2102, 2007.

9. Yeast-lab, “What are the Optimum Conditions for Yeast Reproduction?”, 2015, <http://yeast-lab.wikispaces.com/>, [Accessed July 2015].
10. Tedx Vienna, “Fermentation 101: The Pursuit of Happiness”, <http://www.tedxvienna.at/blog/fermentation-101-the-pursuit-of-hoppiness/>, [Accessed July 2015].
11. Biology Pictures, “Alcohol Fermentation in Yeast”, <http://biology-pictures.blogspot.com.tr/2012/12/alcohol-fermentation-in-yeast.html>, [Accessed July 2015].
12. Madigan, M.T., J. M. Martinko, and J. Parker, *Brock Biology of Microorganisms*, Prentice Hall, New Jersey, 9th edition, 1949.
13. Sherman, F., “An Introduction to the Genetics and Molecular Biology of the Yeast *Saccharomyces cerevisiae*”, *The Encyclopedia of Molecular Biology and Molecular Medicine*, Vol. 6, pp. 302-325, 1997.
14. Amjid, M. R., A. Shehzad, S. Hussain, M. A. Shabbir, M. R. Khan, and M. Shoaib, “A Comprehensive Review on Wheat Flour Dough Rheology”, *Pakistan Journal of Food Sciences*, Vol. 23, pp. 105-123, 2013.
15. Tuite, M. F., and S. G. Oliver, *Saccharomyces*, Plenum Press, New York, USA, 1998.
16. Wiseman, A., *Genetically-engineered Proteins and Enzymes from Yeast: Production Control*, Ellis Horwood, New York, USA, 1991.
17. Niedenthal, R. K., L. Riles, M. Johnston, and J. H. Hegemann, “Green Fluorescent Protein as a Marker for Gene Expression and Subcellular Localization in Budding Yeast”, *Yeast*, Vol. 12, pp. 773-786, 1996.

18. Waddle, J. A., T. S. Karpova, R. H. Waterson, and J. A. Cooper, "Movement of Cortical Actin Patches in Yeast", *The Journal of Cell Biology*, Vol. 132, pp. 861-870, 1996.
19. Cormack, B. P., G. Bertram, M. Egerton, N. A. R. Gow, S. Falkow, and A. J. P. Brown, "Yeast-enhanced Green Fluorescent Protein (yEGFP): a Reporter of Gene Expression in *Candida albicans*", *Microbiology*, Vol. 143, pp. 303-313, 1997.
20. Sawin, K. E. and P. Nurse, "Identification of Fission Yeast Nuclear Markers Using Random Poly-peptide Fusions with Green Fluorescent Protein", *Proceedings of the National Academy of Sciences*, Vol. 93, pp. 5146-5151, 1996.
21. Wu, C., S. F. Lee, E. Furmaniak-Kazmierczak, G. P. Côté, D. Y. Thomas, and E. Leberer, "Activation of Myosin-I by Members of the Ste20p Protein Kinase Family", *The Journal of Biological Chemistry*, Vol. 271, pp. 31787-31790, 1996.
22. Visser, W., E. A. van Spronsen, N. Nanninga, J. T. Pronk, J. Gijs Kuenen, and J. P. van Dijken, "Effects of Growth Conditions on Mitochondrial Morphology in *Saccharomyces cerevisiae*", *Antonie Van Leeuwenhoek*, Vol. 67, pp. 243-253, 1995.
23. O'Shea, D. G. and P. K. Walsh, "Morphological Characterization of the Dimorphic Yeast *Kluyveromyces marxianus* var. *marxianus* NRRLy 2415 by Semi-automated Image Analysis", *Biotechnology and Bioengineering*, Vol. 51, pp. 679-690, 1996.
24. Zalewski, K. and R. Buchholz, "Morphological Analysis of Yeast Cells Using an Automated Image Processing System", *Journal of Biotechnology*, Vol. 48, pp. 43-49, 1996.
25. Walker, G. M., *Yeast Physiology and Biotechnology*, John Wiley and Sons, New York, USA, 1998.

26. Cookson, S., N. Ostroff, W. L. Pang, D. Volfson, and J. Hasty, "Monitoring Dynamics of Single-cell Gene Expression over Multiple Cell Cycles", *Molecular Systems Biology*, Vol. 1, pp. 1-6, 2005.
27. Luo, C., X. Zhu, T. Yu, X. Luo, Q. Ouyang, H. Ji, and Y. Chen, "A Fast Cell Loading and High-throughput Microfluidic System for Long-term Cell Culture in Zero-Flow Environments", *Biotechnology and Bioengineering*, Vol. 101, pp. 190-195, 2008.
28. Taylor, R. J., D. Falconnet, A. Niemistö, S. A. Ramsey, S. Prinz, I. Shmulevich, T. Galitski, and C. L. Hansen, "Dynamic Analysis of MAPK Signaling Using a High-throughput Microfluidic Single-cell Imaging Platform", *Proceedings of the National Academy of the United States of America*, Vol. 106, pp. 3758-3763, 2009.
29. Falconnet, D., A. Niemistö, R. J. Taylor, M. Ricicova, T. Galitski, I. Shmulevich, and C. L. Hansen, "High-throughput Tracking of Single Yeast Cells in a Microfluidic Imaging Matrix", *Lab-on-a-Chip*, Vol. 11, pp. 466-473, 2011.
30. Zhu, Z., O. Frey, D. S. Ottoz, F. Rudolf, and A. Hierlemann, "Microfluidic Single-Cell Cultivation Chip with Controllable Immobilization and Selective Release of Yeast Cells", *Lab-on-a-Chip*, Vol. 12, pp. 906-915, 2012.
31. Frey, O., F. Rudolf, and A. Hierlemann, "High-Resolution Live Cell Imaging Of The Yeast Life Cycle", *16th International Conference on Miniaturized Systems for Chemistry and Life Sciences*, Okinawa, Japan, 2012, pp. 1582-1584, 2012.
32. Lee, S. S., I. A. Vizcarra, D. H. E. W. Huberts, L. P. Lee, and M. Heinemann, "Whole Lifespan Microscopic Observation of Budding Yeast Aging Through a Microfluidic Dissection Platform", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 109, pp. 4916-4920, 2012.
33. Xie, Z., Y. Zhang, K. Zou, O. Brandman, C. Luo, Q. Ouyang, and H. Li, "Molecular Phenotyping of Aging in Single Yeast Cells Using a Novel Microfluidic Device", *Aging cell*, Vol. 11, pp. 599-606, 2012.

34. Zhang, Y., C. Luo, K. Zou, Z. Xie, O. Brandman, Q. Ouyang, and H. Li, "Single Cell Analysis of Yeast Replicative Aging Using a New Generation of Microfluidic Device", *PLoS ONE*, Vol. 7, pp. 1-10, 2012.
35. Park, J., J. Wu, M. Polymenis, and A. Han, "Microchemostat Array with Small-volume Fraction Replenishment for Steady-state Microbial Culture", *Lab-on-a-Chip*, Vol. 13, pp. 4217-4224, 2013.
36. Dénervaud, N., J. Becker, R. Delgado-Gonzalo, P. Damay, A. S. Rajkumar, M. Unser, D. Shore, F. Naef, and S. J. Maerkl, "A Chemostat Array Enables the Spatio-temporal Analysis of the Yeast Proteome", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 110, pp. 15842-15847, 2013.
37. Banaeiyan, A. A., D. Ahmadpour, C. B. Adiels, and M. Goksör, "Hydrodynamic Cell Trapping for High Throughput Single-cell Applications", *Micromachines*, Vol. 4, pp. 414-430, 2013.
38. Crane, M. M., I. B. N. Clark, E. Bakker, S. Smith, and P. S. Swain, "A Microfluidic System for Studying Ageing and Dynamic Single-cell Responses in Budding Yeast", *PloS ONE*, Vol. 9, pp. 1-10, 2014.
39. Jaeger, R. C., G. W. Neudeck, and R. F. Pierret, *Introduction to Microelectronic Fabrication*, Prentice Hall, New Jersey, USA, 2001.
40. Madou M.J., *Fundamentals of Microfabrication: The Science of Miniaturization*, Chemical Rubber Company Press, Boca Raton, Florida, USA, 2002.
41. Akar B., M. Erdi, D. Gokce, N. Kaynak, "Computer Numerical Control (CNC)", Hacettepe University, Ankara, Turkey, 2010.
42. Lin Y. S., C. H. Yang, C. Y. Wang, F. R. Chang, K. S. Huang, and W. C. Hsieh, "An Aluminum Microfluidic Chip Fabrication Using a Convenient Micromilling Process

- for Fluorescent Poly(DL-lactide-co-glycolide) Microparticle Generation”, *Sensors*, Vol. 12, pp. 1455-1467, 2012.
43. Khaleel, H. R., H. M. Al-Rizzo, and A. I. Abbosh, *Advancement in Microstrip Antennas with Recent Applications*, InTech, Rijeka, Croatia, 2013.
 44. Adams T. M. and R. A. Layton, *Introductory MEMS: Fabrication and Applications*, Springer, New York, USA, 2010.
 45. Waldbaur, A., H. Rapp, K. Lange, and B. E. Rapp, “Let There Be Chip-towards Rapid Prototyping of Microfluidic Devices: One-step Manufacturing Processes”, *Analytical Methods*, Vol. 3, pp. 2681-2716, 2011.
 46. Wikipedia, “Electrical Discharge Machining”,
https://en.wikipedia.org/wiki/Electrical_discharge_machining, [Accessed June 2015].
 47. Fiorini G. S. and D. T. Chiu, “Disposable Microfluidic Devices: Fabrication, Function and Application”, *Biotechniques*, Vol. 38, pp. 429-446, 2005.
 48. Ford, S. M., *Fabricating Microfluidic Devices in Polymers for Bioanalytical Applications*, Ph.D. Thesis, Louisiana State University, 2002.
 49. Xia Y. and G.M. Whitesides, “Soft Lithography”, *Angewandte Chemie International Edition*, Vol. 37, pp. 550-575, 1998.
 50. Li, P. C. H., *Microfluidic Lab-on-a-Chip for Chemical and Biotechnology Analysis and Discovery*, Chemical Rubber Company Press, Boca Raton, Florida, USA, 2005.
 51. MEMS & Nanotechnology Exchange, “Hot Embossing”,
<https://www.mems-exchange.org/capabilities/embossing>, [Accessed June 2015].
 52. Dirckx, M. E., *Demolding of Hot Embossed Polymer Microstructures*, Ph.D. Thesis, Massachusetts Institute of Technology, 2010.

53. Gross B. C., J. L. Erkal, S. Y. Lockwood, C. Chen, and D. M. Spence, “Evolution of 3D Printing and Its Potential Impact on Biotechnology and the Chemical Sciences”, *Analytical Chemistry*, Vol. 86, pp. 3240-3253, 2014.
54. Comina, G., A. Suska, and D. Filippini, “PDMS Lab-on-a-Chip Fabrication Using 3D printed Templates”, *Lab-on-a-Chip*, Vol. 14, pp. 424-430, 2014.
55. Shallan A. I., P. Smejkal, M. Corban, R. M. Guijt, and M. C. Breadmore, “Cost-Effective Three-Dimensional Printing of Visibly Transparent Microchips within Minutes”, *Analytical Chemistry*, Vol. 86, pp. 3124-3130, 2014.
56. Carraher, C. E., *Seymour/Carraher's Polymer Chemistry*, Chemical Rubber Company Press, Boca Raton, Florida, USA, 2007.
57. Ebewele R.O., *Polymer Science and Technology*, Chemical Rubber Company Press, Boca Raton, Florida, USA, 2000.
58. Kumar A. and R. K. Gupta, *Fundamentals of Polymer Engineering*, Marcel Dekker, New York, USA, 2003.
59. PMMA Acrylic Sustainable Solutions, “Acrylic Sheets – PMMA: Benefits”, <http://www.pmma-online.eu/benefits>, [Accessed July 2015].
60. AZO Materials, “Polymethylmethacrylate – Acrylic – PMMA General Purpose”, <http://www.azom.com/article.aspx?ArticleID=788>, [Accessed July 2015].
61. Suu, Y. G., “Studies on Mechanical Properties of Poly(Methyl Methacrylate) and Poly(Methyl Methacrylate)-Modified Natural Rubber Blend”, [http://eprints.usm.my/10333/1/STUDIES_ON_MECHANICAL_PROPERTIES_OF_POLY\(METHYL.pdf](http://eprints.usm.my/10333/1/STUDIES_ON_MECHANICAL_PROPERTIES_OF_POLY(METHYL.pdf), [Accessed July 2015].
62. Herold, K. E. and A. Rasooly, *Lab-on-a-Chip Technology: Fabrication and Microfluidics*, Caister Academic Press, Norfolk, England, 2009.

63. Borklu Yucel, E. and K. O. Ulgen, “A Network-based Approach on Elucidating the Multi-faceted Nature of Chronological Aging in *S. cerevisiae*”, *PLoS ONE*, Vol. 6, pp. e29284, 2011.
64. Tanaka, S., R. Nakato, Y. Katou, K. Shirahige, and H. Araki, “Origin Association of Sld3, Sld7, and Cdc45 Proteins is a Key Step for Determination of Origin-firing Timing”, *Current Biology*, Vol. 21, pp. 2055-2063, 2011.
65. Enserink, J. M., *DNA Replication-Current Advances*, InTech, Rijeka, Croatia, 2011.
66. Altmann, K. and B. Westermann, “Role of Essential Genes in Mitochondrial Morphogenesis in *Saccharomyces cerevisiae*”, *Molecular Biology of the Cell*, Vol. 16, pp. 5410-5417, 2005.
67. Huh, W., J. V. Falvo, L. C. Gerke, A. D. Carroll, R. W. Howson, J. S. Weismann, and E. K. O’Shea, “Global Analysis of Protein Localization in Budding Yeast”, *Nature*, Vol. 425, pp. 686-691, 2003.
68. Ribeiro, A. C. F., O. Ortona, S. M. N. Simoes, C. I. A. V. Santos, P. M. R. A. Prazeres, A. J. M. Valente, V. M. M. Lobo, and H. D. Burrows, “Binary Mutual Diffusion Coefficients of Aqueous Solutions of Sucrose, Lactose, Glucose, and Fructose in the Temperature Range from (298.15 to 328.15) K”, *Journal of Chemical & Engineering Data*, Vol. 51, pp. 1836-1840, 2006.