AN OPTICALLY ADDRESSED THERMORESPONSIVE MICROFLUIDIC SYSTEM FOR CELL CULTURING AND HARVESTING

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI‘I AT MANOA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ELECTRICAL ENGINEERING AUGUST 2012

By
Swapnil Namekar

Thesis Committee:
Aaron T. Ohta, Chair
Vinod Malhotra
Olga Boric-Lubecke
© Copyright 2012

By
Swapnil Namekar

ii
ACKNOWLEDGEMENTS

I would like to thank all those who have helped and inspired me during my study. I sincerely express my profound sense of gratitude to my thesis advisor Prof. Aaron Ohta for his support, encouragement, and invaluable suggestions, which have made this thesis a great learning experience. I am very thankful to Prof. Olga Boric-Lubecke and Prof. Vinod Malhotra for their participation in the thesis committee and for helpful suggestions.

I am thankful to the National Science Foundation (NSF) for financial support in the form of a Research Assistantship. Special thanks are due to Ms. Gail Hasegawa, the former department secretary for the administrative help, and Clem Spriggs, the former IT system administrator.

I am thankful to Prof. Yi Zuo, Dr. Naresh Pandya, Wenqi Hu, and Madhuri Namekar for their support throughout my work. I would like to acknowledge the support of my fellow students, Michelle Zhang, Kelly Ishii, and all other friends in the Department of Electrical Engineering.

I am indebted to my mom, dad, my elder brother Shailesh Namekar and my wife Renu Namekar who encouraged me to complete this work.
ABSTRACT

The present work demonstrates a “smart” culture platform that can be used for cell culturing and harvesting specific cells, such as stem cells. It is desirable to culture homogeneous specific cell populations for various biomedical applications such as fundamental cellular studies and studies of tissues. Traditional cell culturing techniques lack control over the spatial location of individual cells in a culture, or the types of cells that are seeded. These limitations hinder studies on cell-cell interactions and the creation of completely homogeneous populations of cells. In order to overcome these limitations, a smart culture dish was developed that enables control of specific individual cells in a cell culture by using optical illumination. In addition, a method for seeding specific individual cells in a three-dimensional culture using optical illumination was demonstrated. Both of these techniques can individually be used for achieving control over specific individual cells and their spatial location in a culture. This further enables the culturing and harvesting of specific individual cell types, and the creation of homogeneous specific cell populations.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iv
ABSTRACT ........................................................................................................................... v
LIST OF FIGURES ............................................................................................................. vii
CHAPTER 1 INTRODUCTION ......................................................................................... 1
CHAPTER 2 2D OPTICALLY ADDRESSED THERMORESPONSIVE CELL CULTURING DEVICE ......................................................................................... 10
CHAPTER 3 3D OPTICALLY ADDRESSED THERMORESPONSIVE CELL CULTURING DEVICE ......................................................................................... 15
CHAPTER 4 FUTURE WORK .......................................................................................... 34
CHAPTER 5 CONCLUSION ............................................................................................. 35
REFERENCES ................................................................................................................... 38
APPENDIX ...................................................................................................................... 41
LIST OF FIGURES

Figure 1.1 Effect of trypsinization for cellular detachment from the culture dish surface.

Figure 1.2 PNIPAAm polymer causes altering in its surface properties with respect to the temperature applied to it around its LCST.

Figure 1.3 Schematic of cellular manipulation using thermoresponsive substrate in 2D format.

Figure 1.4 Schematic of cellular manipulation using thermoresponsive substrate in 3D format.

Figure 2.1 Solution exchange method.

Figure 2.2 Contact angle testing setup.

Figure 2.3 Contact angle testing result.

Figure 3.1 Experimental setup for 3D culture device.

Figure 3.2 Schematic of 3D culture device.

Figure 3.3 ITO heater design

Figure 3.4 Effect of PNIPAAm concentration in DI water on Sol-gel transition.

Figure 3.5 Gelling areas with respect to circular pattern diameter

Figure 3.6A Gelling areas with respect to constant area patterns

Figure 3.6B Patterns with different shapes but constant area.

Figure 3.7 Standard cross pattern dimensions.

Figure 3.8 Gelled PNIPAAm area with respect to time.

Figure 3.9 Gelled area with respect to time from 0 to 30 s.

Figure 3.10 Still images captured from the videos of sol-gel experiment.

Figure 3.11 Gelling reduction areas with respect to time.

Figure 3.12 Reproducibility of PNIPAAm gelling with time.

Figure 3.13 Gelling area with respect to optical pattern intensity.

Figure 3.14 Gelling area with respect to PNIPAAm solution flow rate.
Figure 3.15 Time modulation experiment.

Figure 3.16 Microbead trapping in PNIPAAm hydrogel.

Figure 3.17 Sol-gel experiment for cellular manipulation.

Figure 3.18 Cell viability experiment.
CHAPTER 1
INTRODUCTION

Cellular therapeutics have the potential to treat diseases like Alzheimer’s disease, type-1 diabetes, stroke, heart disease, and spinal cord injuries [1]. However, cellular therapies are still in the research and development stages due to difficulties in studying single cell behaviors, controlling cell differentiation, and creating homogeneous populations of specific cells [1]. Some of these difficulties arise due to limitations in traditional cell culturing procedures, which are suitable for large numbers of cells, but offer limited precision and repeatability in the applications where small amounts of cells are used.

Traditionally, cells are cultured in bulk, where billions of cells are grown in one culture. The large quantity of cells in bulk cultures presents a challenge for the isolation and harvesting of specific individual cells.

When adherent cells are cultured, they secrete proteins that anchor them to the surface of culture dishes. These protein attachments need to be dissolved to release the cells from the culture dish surface. Trypsin is commonly used protein-degrading enzyme that can be used to detach cells from the culture dish surface. These detached cells can be used in further experiments, or for harvesting.

However, enzymatic procedures can be hazardous to cell health, and can reduce cell viability due to non-specific protein digestion [2]. To maintain cell viability, the trypsinization time can be controlled. Nevertheless, cellular therapeutic procedures try to avoid any use of enzymatic procedures [2]. Furthermore, control over specific individual cell detachment is not possible using such enzymatic procedures, as trypsin detaches the entire cell sheet from the culture dish surface (Fig. 1.1).
Figure 1.1 Effect of trypsinization for cellular detachment from the culture dish surface. Trypsin attacks the extracellular matrix of the cells and digests cellular protein that helps in detaching them from the culture surface. However, excessive trypsin exposure can affect cell health.

Figure 1.2 PNIPAAm polymer causes altering in its surface properties with respect to the temperature applied to it around its LCST. Above LCST the polymer behaves as hydrophobic surface while below LCST it behaves as hydrophilic surface. Hydrophobic surface assists cell adhesion while hydrophilic surface resists cell adhesion. This property of polymer offers control over cellular detachment without trypsinization, maintaining cell viability.
The limitations associated with trypsin and similar enzymatic procedures can be removed by using thermoresponsive polymer surfaces for cell culturing. One thermosensitive polymer, poly(N-isopropylacrylamide), or PNIPAAm, is biocompatible [3, 4]. For cell culture applications, PNIPAAm polymer films with a thickness of 11 nm to 20 nm are coated on the surface of a culture dish (Fig. 1.2) [5-8].

The lower critical solution temperature (LCST) of PNIPAAm is 32°C [9]. Above the LCST, PNIPAAm is hydrophobic, or water-repellent, but below the LCST, it becomes hydrophilic, or water-attractive. In the hydrophilic state, the PNIPAAm polymer resists cell adhesion, while in the hydrophobic state it assists cell adhesion. This property of the polymer eliminates the need of trypsin to release the cells because the cells can be released or trapped by simply controlling the culture dish temperature. This method has no adverse effects on cell viability [3]. However, the isolation of specific cells cannot be achieved as changes in the temperature affect the entire cell population on a culture dish surface [10-12].

To overcome this issue, Cheng et al. designed a culture device with microheaters beneath a PNIPAAm coating [13]. The microheaters controlled the local temperature of the polymer surface. This altered the polymer surface characteristics from the hydrophilic to the hydrophobic state, and hence control over cellular adhesion was achieved in the microheater-heated regions. The unheated regions remain at room temperature, resisting cellular attachment. By activating specific microheaters, cells were patterned on a surface. This technique provides some flexibility in patterning cells in specific areas, but single cell isolation is difficult to achieve because of an insufficient patterning resolution of approximately 1 mm [13]. In addition, it is challenging to pattern specific cells since this device can only manipulate cells that are present above a microheater. Cellular manipulation is not possible in regions without microheaters.
Localized cellular manipulation can also be done using an opposite approach: locally cooling areas of a PNIPAAm coating [14]. This approach uses an inverted microscope with a hotplate. The PNIPAAm-coated culture dish was placed onto the hot plate. The hot plate maintains the culture dish temperature at about 36°C, which keeps the entire polymer surface in the hydrophobic state. Liquid nitrogen was used as a cooling gas, and was directed onto the bottom of the culture dish to cool only desired portions of the dish. The cooling gas reduces the local temperature of the culture dish below the LCST, causing the surface to become hydrophilic where it is cooled, locally releasing cells. The resolution achieved using this approach was approximately 1 mm, which is not sufficient to isolate specific individual cells in a culture.

In biomedical research, *in vitro* cell culturing plays an important role as a tool to conduct biomedical research without animal testing. Cell culturing techniques can be broadly classified into two different types: 2D cell culturing and 3D cell culturing.

Cell culturing in 2D involves the growth of a single layer of cells on a surface. 2D cell culturing provides a simple way to grow cells *in vitro*, and hence is often used to perform routine assays. Advanced laboratory setups are not required for such cell culturing, reducing experimental costs. However, cells grown in 2D cultures can exhibit abnormal characteristics [15-18], as current 2D cell culturing methods do not adequately mimic in vivo environments. Thus, 2D cell culturing is widely used since it is relatively simple and inexpensive, but 3D cell culturing is preferred if an *in vivo*-like environment is desired.

Cell culturing in 3D mimics the *in vivo* environment by recreating the characteristics of the extracellular microenvironment [15, 19]. 3D cell culturing has the potential to provide conditions that enable greater control of physiological properties of cells in a culture. However, it is difficult to isolate single specific cells using existing 3D culture techniques [20, 21].
To create 3D cultures, hydrogels are used to create scaffolds for cell attachments. Hydrogels are water-permeable gels made of synthetic or natural polymers. There are various hydrogel materials that can be used to perform 3D cell culturing, including PNIPAAm [3, 4, 22], poly(ethylene glycol) (PEG) [20, 23-25], agarose [26, 27], alginate [28, 29], and others. The hydrogel is the basic ingredient involved in 3D cell culturing, and is often seeded with cells after the gel formation process. Otherwise, hydrogels can be formed around cells, using photopolymerization [20] or other techniques.

Lee et al. has fabricated 3D microstructures in a layer-by-layer fashion [21]. The technique features rapid and economical fabrication of hydrogels. The composition of each layer was regulated by microfluidic control of photopolymerized PEG. The same group further used different types of cells during fabrication of 3D microstructures, effectively seeding them in a 3D scaffold [21]. However, the material used is PEG, which does not reversibly change phases, and thus cannot transition from hydrogel to an aqueous solution once polymerized. This presents difficulties in subsequent cell harvesting.

The focus of this thesis is the creation of optically addressed thermoresponsive culture devices for the culturing of cells. We call these devices “smart” culture dishes, because they enable greater control and precision in cell culturing. Using our smart culture dish, homogeneous population of specific cells can be created from mixed populations, such as those obtained when harvesting primary cells. This functionality will facilitate the development of cellular therapeutics, and also provide an important tool for basic cellular research.

We have developed two types of optically addressed devices to manipulate the cells and culture them further in both 2D and 3D formats. The issues associated with existing techniques and technologies, mentioned earlier, are addressed in the smart culture devices. The optically addressed devices absorb light and convert it to thermal energy, which affects the characteristics of thermosensitive polymers that coat the device surface (Fig. 1.3) or that are suspended in solution (Fig. 1.4).
Figure 1.3 (a) A mixed population of cells are introduced onto the culture dish. (b) The culture dish absorbs the light from the optical pattern and converts it into heat. A local hydrophobic surface is formed in the areas heated with the optical pattern, trapping the required cells (green cells). The unwanted cells (violet) can be flushed away using microfluidic flow. (c) Trapped cells can be cultured and subsequently harvested.

In the 2D smart cell culture dish, selected cells can be cultured from a heterogeneous cell sample. A mixed cell population can be introduced with the help of syringe pump at a desired flow rate (Fig 1.3a). The desired cells are indicated by the green color in Fig. 1.3, while undesired cells are indicated by the violet color. The desired cells need to be trapped, while the undesired ones need to be flushed away. Since PNIPAAm changes its surface characteristics if its temperature is changed above or below its lower critical solution temperature, it can be used to control cell adhesion. At ambient temperatures the PNIPAAm surface is in a swollen state, which resists cell attachment the surface due to steric repulsion (Fig 1.2). At physiological temperatures, the PNIPAAm surface is in its deswollen state that allows cells to attach the surface. To enable command over specific individual cells, this temperature control must be very localized. Manipulation at single-cell level can be done by controlling the temperature beneath the surface where desired cells are observed. Therefore the area beneath the desired cells can be heated locally by optical pattern exposure as shown. The light energy from the optical pattern is converted
to heat to change the thermosensitive polymer characteristics. In other words, the transition from hydrophilicity to hydrophobicity is seen where an optical pattern is present, due to an ambient to physiological temperature transition. The optical pattern only heats the surface beneath the desired cell above LCST of PNIPAAm polymer while other areas remain below the LCST of the PNIPAAm polymer. In this manner the desired cells get trapped due to altering of PNIPAAm surface characteristics. The other undesired cells remain in solution, and can easily be flushed from the device using microfluidic flow (Fig. 1.3b). The desired trapped cells can be cultured further (Fig. 1.3c).

A related technique can be used to realize controlled 3D cultures. For 3D culturing, the cells are first suspended in an aqueous PNIPAAm solution. The cell sample in PNIPAAm solution is introduced into a microfluidic chamber (Fig. 1.4a). Similar to 2D culturing device, an optical pattern can be used to trap desired cells. However, the temperature change due to the optical pattern produces a different result. In this case, the PNIPAAm solution changes its state from liquid to gel reversibly in response to the temperature change above or below LCST of PNIPAAm, also known as a sol-gel transition. The sol-gel and gel-sol transition is rapid, which allow us to manipulate cells

Figure 1.4 (a) Cell sample in PNIPAAm solution are introduced into a microfluidic chamber bonded to the absorbing substrate. (b) The substrate absorbs the light from the optical pattern and converts it into thermal energy. In the areas heated by the optical pattern, the PNIPAAm forms a hydrogel that traps the required cells. The unwanted cells can be removed by microfluidic flow. (c) Trapped cells can be cultured and subsequently harvested.
depending upon our requirement of specific cells that need to be trapped or released for further culturing purpose. The gel formed due to temperature change can be used to hold the cells even under flow conditions. The gel area can be controlled using optical pattern size and its exposure time to achieve single cell isolation. Thus, after the introduction of cell sample with PNIPAAm solution the desired cells can be captured in PNIPAAm gel after irradiating area beneath it by optical pattern (Fig. 1.4b). The absorbing surface of the device converts light energy of the optical pattern into thermal energy enabling local heating for the sol-gel transition. The other cells in the solution remain free, so the desired cells can be captured in the gel while free undesired cells can be flushed away (Fig. 1.4c). The captured cells can be cultured further. After cellular trapping, the optical pattern can be turned off. The device temperature can be maintained at 37°C in the absence of the optical pattern using a thin-film indium tin oxide (ITO) heater.

The device is based upon opto-thermal effects. In contrast, the photo-electric effect, or the conversion of light directly into electricity, is a more traditional electrical engineering topic. Devices based on the photo-electric effect include photodiodes and phototransistors [30]. These devices convert light into electrical energy. The light is absorbed at the p-n junction and generates electron-hole pairs in the depletion region, resulting in an increased current flow [30]. However, the opto-thermal effect refers to the conversion of light directly into heat energy. Opto-thermal effects can also be used to generate electricity, but this is an indirect method, as the thermal energy is used to boil water to generate steam that rotates a turbine. One absorbing material used for opto-thermal energy conversion is amorphous silicon due to its approximately 90% light absorption capability [31]. The same opto-thermal effect is used in our technique at the microscale level. The light pattern irradiates the substrate made of amorphous silicon material, which absorbs the light and converts it into heat energy. This heat energy is
further used for sol-gel transition (3D culture device) or altering surface characteristics from hydrophilic to hydrophobic (2D culture device).

The system presented here also uses an electrical thin-film heater designed using basic electrical engineering principles. The heater design is related to the sheet resistance that is obtained with respect to surface area of the heater device. The temperature characteristics depend upon this sheet resistance due to heater surface area. The resistance is directly proportional to the ratio of heater dimensions \((L/W)\) and the sheet resistance is directly proportional to ITO layer thickness. This resistance is more compared to the contact resistance due to electrical contacts hence the contact resistance can be neglected. After application of voltage the temperature of the heater increases due to power loss into the heater. The heater resistance is related to sheet resistance by:

\[
R = R_s \left( \frac{L}{W} \right) \tag{1}
\]

where \(R_s\) is the sheet resistance in units of ohms per square, \(L\) is the distance between the electrical contacts on the thin film, and \(W\) is the width of the thin film. The resistance is directly proportional to the power dissipation in the thin film, and therefore the heat generation. This is described by:

\[
P = I^2 R \tag{2}
\]

where \(P\) is power dissipated in the form of heat and \(I\) is the applied current through the thin film.

Chapters two and three of this thesis discuss the 2D and 3D cell culturing devices, respectively. Chapter two details the design, operation, and setup of the 2D culture device, and the process for coating PNIPAAm polymer onto an optically absorbing substrate. The characterization of fabricated PNIPAAm coatings is included. Chapter three details the design, operation, and setup of the 3D culturing device. Fabrication details, device characterization, and preliminary tests on cells are also included. Chapter four discusses further work that can be done on these projects, and Chapter five concludes this thesis.
CHAPTER 2
2D OPTICALLY ADDRESSED THERMORESPONSIVE CELL CULTURING DEVICE

It is easier to grow cells using 2D cell cultures compared to 3D cultures. Therefore, to perform routine assays, biologists prefer 2D cultures. In 2D cell cultures, cells are grown on flat polystyrene surfaces that are stiff and unnatural. The cells adhere and spread by forming attachments to proteins that are deposited on this plastic surface. To release the cells from the surface, there are several methods as discussed in Chapter 1, including the standard practice of trypsinization, or more exotic methods such as the use of thermosensitive polymer coatings, controlled by changing the temperature across the whole surface [10-12], or locally with microheaters [13] or gas coolants [14]. These current techniques for releasing cells are not precise enough to control the adhesion of specific individual cells. However, single-cell precision is desirable, as it enables the creation of a homogeneous cell population for culturing. The homogeneous cell population can allow the study of cellular growth and of cellular interactions. Moreover, homogeneous cell populations play an important role in gene expression studies [32], as well as in the development of cellular therapies.

Using our optically addressed smart cell culture device, a homogeneous specific cell population can be created on a flat surface. To achieve control over specific individual cells, an optical pattern can be used to heat the culture dish surface locally (Fig. 1.3).

2.1 2D Smart Culture Dish

The cell-culturing device that we developed is a polydimethylsiloxane (PDMS) elastomer chamber bonded to a glass slide coated with the thermoresponsive polymer PNIPAAm. A previously reported solution-exchange method [33] is used to coat the glass slides with PNIPAAm to create a thermoresponsive culture dish. The PDMS chamber is fabricated by using standard PDMS device fabrication procedure, explained in
detail in Chapter 3. An external ITO heater can be fabricated using glass slide with an ITO layer on it, also explained in Chapter 3.

The culture device uses optical patterns to heat the substrate locally to achieve the PNIPAAm surface characteristic transition. The PDMS chamber helps to accommodate cells for manipulation while the substrate will assist or resist cell adhesion, depending upon the location of the optical patterns. The heat from the optical patterns raises the temperature of the PNIPAAm coating above the LCST, causing a localized hydrophilic to hydrophobic transition at the surface of the device.

2.2 2D Culture Dish Fabrication

In order to make the PNIPAAm-coated surface suitable for cell adhesion, the PNIPAAm thickness must be limited to 20 nm [5]. There are a few techniques for creating a PNIPAAm coating with this desired thickness [6, 7], but due to the limitations of the equipment available, the solution-exchange method, developed by Mizukami et al. [33] was determined to be the most practical method.

Based upon the process detailed in reference 33, the following PNIPAAm coating procedure was implemented. Initially, cleaning procedures were performed before the actual coating process. The whole process was done in the College of Engineering’s cleanroom facility to maintain cleanliness. First, the glass slides were cleaned with acetone followed by methanol, isopropanol, and finally deionized (DI) water. The acetone cleaning may cause residue formation, which is minimized if methanol is used immediately after the acetone. The rinsed slides were dried with pressurized air. A subsequent Piranha cleaning was done for 30 minutes immediately after the initial cleaning process. The slides were soaked in Piranha solution (70% H$_2$SO$_4$ and 30% H$_2$O$_2$) for 30 min. The slides were then rinsed with DI water followed by drying with pressurized air. The cleaned slides were transferred to a glass petri dish for performing the solution-exchange method (Fig. 2.1). The first solution of N-isopropylacrylamide (NIPAAm) in chloroform (1:1 weight-to-volume ratio, or 5 g of NIPAAm in 5 ml of chloroform) was added to the glass petri dish containing the glass slides. The solution is
then removed using a pipette before introducing the second solution of NIPAAm in cyclohexane (0.25 g NIPAAm in 5 ml of cyclohexane) to the glass petri dish. The solution is removed again with a pipette. Finally, diluted NIPAAm in cyclohexane (0.005\% mol of NIPAAm in cyclohexane) is introduced into the glass petri dish. After keeping the slides for 1 hour in the solution, the slides were irradiated with a UV lamp with an intensity of 20.1 mW/cm$^2$ for approximately 2 minutes. The slides were then rinsed with DI water, completing the coating procedure.

**Figure 2.1** Solution exchange method. (a) After cleaning process, the substrates were moved to a glass petri dish, and the first solution of NIPAAm in chloroform was introduced. (b) The first solution was removed and exchanged with the second solution of NIPAAm in cyclohexane. (c) The second solution was again removed, and exchanged with the final solution of diluted NIPAAm in cyclohexane for about an hour followed by UV exposure to form the PNIPAAm coating on to the glass substrates.

### 2.3 Experimental Results

Contact angle testing is done after the solution exchange to check the quality of the PNIPAAm coating. Contact angle testing is used to check surface characteristics such as hydrophilicity and hydrophobicity of the PNIPAAm polymer coating. The setup for contact angle testing consists of a CCD camera, a macro lens, a hotplate, a micropipette, and a lamp (Fig. 2.2). A 20-µl water droplet is placed onto a PNIPAAm-coated device on the hotplate. The CCD camera is adjustable in both the vertical and horizontal axes, and the hotplate is adjustable in the vertical axis. This helps in positioning the CCD camera to image the water droplet. The CCD camera is connected to the computer and by adjusting focus using the macro lens, the droplet can be viewed on computer screen. The contact
angle readings were taken at two different temperatures, room temperature (20°C) and near-physiological temperatures (37°C to 40°C). Images of the droplet were captured using the bundled CCD camera software (AVSImage). These images were analyzed using Image J software [34] to measure the droplet contact angle.

![Diagram](image)

**Figure 2.2** Contact angle testing setup. A vertically and horizontally adjustable CCD camera was used to image a water droplet placed on a PNIPAAm-coated glass chip on a vertically adjustable hot plate. The light source helps to capture clear images of droplet.

The PNIPAAm coating should behave like a hydrophilic, or water-loving, surface at room temperature, and a hydrophobic, or water-repellent, surface at 40°C. Based on previous literature [35, 36], the contact angle should be approximately 60° at 20°C, and 90° at 40°C.

As expected, at 20°C, the measured contact angle was 63.3°, which indicates that a hydrophilic surface that can resist cell adhesion is present (Fig. 2.3) [35, 36]. At 40°C, the measured contact angle achieved was 91.3°, indicative of a hydrophobic surface that assists cell adhesion [35, 36].
Figure 2.3 Contact angle testing results from water droplets on a PNIPAAm-coated glass slide at 20°C and 40°C.

2.4 Future Work

The contact angle tests show that the PNIPAAm-coated surface is promising for controlling cellular adhesion. However, experiments with cells remain to be performed. The proposed experimental procedure would be as follows. Microfluidic single-inlet, single-outlet PDMS chambers can be manufactured and can be bonded to the PNIPAAm-coated glass slide. A cell sample can be introduced into the chamber with a syringe pump. The cells can be observed under the microscope, and the desired cells can be manipulated by using optical patterns to heat the culture dish surface locally above the LCST, while the unirradiated portion of culture dish remains at room temperature. The transition from hydrophilicity to hydrophobicity will be seen only at irradiated portion of the culture dish. The hydrophobic surface due to the optical pattern irradiation will attract the desired cells to the substrate while other cells remain suspended into the chamber. In this way the desired cells can be trapped and undesired cells can be flushed out of the chamber.
CHAPTER 3
3D OPTICALLY ADDRESSED THERMORESPONSIVE CELL CULTURING DEVICE

The ability of 3D cell culturing techniques to resemble *in vivo*-like environments makes it an attractive alternative to 2D cell culturing techniques. 3D cell cultures are useful where highly accurate cellular behavior is required. Assays using 3D cultures have results that are closer to human or animal testing results [37]. Thus, use of 3D cultures has the potential to eliminate animal or human testing.

![Diagram of 3D cell culture device](image)

**Figure 3.1** The setup for the 3D cell culture device. Cells in microfluidic device can be observed under microscope and then recorded through camera connected to it. By using a computer, optical patterns can be generated for cellular trapping. A syringe pump (not shown) is used to introduce the cell sample in a thermoresponsive solution at the desired flow rate.

Existing 3D cell culturing techniques involves creation of scaffold-like structures to support cells. The scaffolds are porous to aid in the delivery of nutrients to the cells in a culture. The lack of control over specific individual cells in a culture is the major issue with existing 3D cell culturing technologies.
The smart culture dish presented in this study can tackle these issues effectively. The device consists of a PDMS chamber bonded to a thermoresponsive substrate. Here, an amorphous-silicon-coated glass chip is used as a substrate. This thermoresponsive substrate absorbs light energy and converts it into heat energy. The purpose of the PDMS chamber is to accommodate the cell sample under manipulation. The technique uses cells in a diluted PNIPAAm solution. The culture dish temperature can be controlled using optical patterns that change the temperature to above or below the LCST of the PNIPAAm, triggering the sol-gel transition of the PNIPAAm solution. This transition is an effective tool to trap the desired cells in a gel for further culturing in 3D. The optical pattern size and shape assists the heating of the culture dish in the desired local regions for cellular trapping at the single-cell level.

3.1 3D Culture Platform

The experimental setup of the 3D culture platform consists of a computer, video camera, microscope, objective lens, optical source, optical pattern generator, syringe pump, and a microfluidic device that serves as the culture dish (Fig. 3.1).

The culture dish is placed on the microscope stage and anchored with tape to avoid displacement of the culture dish due to the strain from tubing connections to the culture dish. The inlet of the device is connected to a syringe pump by Tygon tubing (inner diameter = 0.5 mm, outer diameter = 1.5 mm). A syringe pump is used to introduce the cell sample along with a thermoresponsive solution at a predetermined flow rate. The tubing connections to the device are made after the PDMS chamber fabrication but before bonding the chamber to the substrate. The tubing connection between the syringe pump and the inlet of the device offers an effective way to inject a cell sample in PNIPAAm solution into the device at predetermined flow rate. The device outlet drains the undesired cell sample into a centrifuge tube.

The injection of a cell sample in diluted PNIPAAm solution can be done with a syringe pump. After injection, the cells can be observed using the microscope by viewing images of the microfluidic chamber on a computer via a video camera attached to the microscope. The computer is also connected to the optical pattern generator, which is a
standard computer projector (Dell 2400MP). It can be seen from Figure 3.1 that the projection of the optical pattern was done from bottom of the culture dish.

![Figure 3.2 3D cell culturing device. A PDMS chamber with one inlet and one outlet is fabricated and bonded to the amorphous silicon coated substrate. Tubing is connected (not shown) to the inlet and outlet for the introduction of cell sample to be manipulated.](image)

### 3.2 Culture Dish Fabrication

The device fabrication involves creating the PDMS microfluidic chamber, bonding the chamber to an amorphous-silicon coated glass chip, and connecting tubing to the inlet and outlet ports (Fig. 3.2). The layout of the PDMS chamber was designed using AutoCAD. Some of the factors affecting the design include the desired volume of cell sample to be manipulated, and the minimization of dead zones that might cause air pockets in the chamber or prevent sufficient fluid flow when flushing cells. The chamber shape avoids acute angles to prevent the formation of dead zones. Channels with a length of 2 mm, width of 50 µm and height of 50 µm lead from the central chamber with dimensions of approximately 5 mm × 5 mm to the 1.5-mm-diameter inlet and outlet ports. The final design was submitted to the Stanford Microfluidics Foundry [38], and a master mold was created. This master mold was used in the PDMS fabrication process.
The master mold was stored in a 14-cm-diameter sealed petri dish. Without disturbing the master mold, the PDMS solution was poured into the petri dish. The PDMS package includes base agent and curing agent. The PDMS base agent was weighed on a balance, ensuring that there was sufficient PDMS base agent to cover the master mold in the petri dish. A 10:1 proportion of base to curing agent is generally used to fabricate PDMS devices [39-41]. The curing agent was then weighed to obtain the appropriate amount. After ensuring a 10:1 proportion by weight of both base and curing agent, the agents were poured into a new large container for mixing. A glass stirrer rod was used to mix the base and curing agents continuously for at least 10 minutes. The mixture was poured into the petri dish containing the master mold. To get rid of any air bubbles, the petri dish was degassed in a vacuum chamber at a pressure of 550 mm of Hg for about 30 minutes. The petri dish was then kept in an oven at 60°C for about 5 hours. Slow curing is usually preferred to obtain a high-quality PDMS device. The cured device was then covered with the petri dish lid and sealed with the tape.

The remaining processes can be done in a cleanroom or in a regular laboratory. A knife was used to cut the edges of the cured PDMS so that it could be removed from the petri dish. Care was taken to avoid cuts in the fabricated devices. After peeling off the PDMS, the master mold was carefully separated from the PDMS layer. The PDMS layer was stored in a new petri dish. The master mold was cleaned with acetone, methanol, isopropanol, rinsed with DI water, and stored for re-use. Scalpels were used to dice separate devices from the PDMS layer.

A small hole puncher was used to create the holes at inlets and outlets of each device. To ensure that the input and output holes are free from blockages, they were checked by introducing water using a syringe at both the inlet and outlet. Stainless steel tubing connectors were inserted in the input and output holes. Tygon tubing with an inner diameter of 0.5 mm and an outer diameter of 1.5 mm were cut and connected to the steel connectors.

The optical pattern is not required after cell trapping, but the temperature of the device needs to be maintained at 37°C for further culturing. Hence another heat source is required. The device here is designed with a thin-film heater embedded in the substrate, which consists of a 1.1-mm-thick glass slide coated with an indium tin oxide (ITO) layer.
of 200 nm thickness, topped by an amorphous silicon (a-Si) layer with a thickness of 1 µm (Figure 3.3). The amorphous silicon layer was removed from the edges of the substrate, and the electrical connections were made to ITO layer using silver epoxy. By applying a voltage, the heater can be activated to maintain the device at 37°C even in the absence of any optical patterns.

![Figure 3.3 ITO thin-film heater embedded in the substrate of the device.](image)

The PDMS device with the connected tubing can be bonded to the substrate using hand pressure for temporary use while performing experiments. However, a more robust bond can be created by using a corona treater [42]. After treating the substrate with the corona treater for few seconds, the PDMS chamber was immediately bonded to it by applying pressure from both sides. The device is compressed overnight to strengthen the bond.

### 3.3 PNIPAAm Solution

To achieve the sol-gel transition needed to isolate specific cells, we used a diluted PNIPAAm solution consisting of PNIPAAm in DI water or in Phosphate Buffered Saline (PBS). First, gelling as a function of the PNIPAAm concentration in DI water was empirically determined. PNIPAAm was purchased in crystal form (MW = 19,000-30,000), and added to DI water at concentrations of 5%, 10%, 15%, and 20% (weight to volume). When adding the PNIPAAm to the water, it was observed that the PNIPAAm
tends to clump in the solution, making it difficult to dissolve. However, if the DI water with PNIPAAm crystals was kept in a refrigerator at about 4 °C for 15 to 20 minutes, the PNIPAAm completely dissolves with no shaking or stirring needed.

Circular patterns with diameters ranging from 25 μm to 375 μm were created using the optical pattern generator, and projected onto a smart culture dish. PNIPAAm solutions with different concentrations were introduced separately into the device using a syringe. For each circular pattern size, the applied voltage was gradually increased until gelling was observed.

![Figure 3.4 Effect of PNIPAAm concentration in DI water on Sol-gel transition.](image)

As PNIPAAm concentration increases, the voltage required to trigger the sol-gel transition decreases, assuming the light pattern size is held constant (Fig. 3.4). Gelling is observed for a 310-μm-diameter pattern when 10% to 20% PNIPAAm solution is used and no voltage is applied, but not for a 5% PNIPAAm solution. On the other hand, at 2.5 V, gelling occurs in all the PNIPAAm solutions, regardless of the size of the circular pattern.
From these experiments, we selected the 10% PNIPAAm solution for subsequent experiments. Next, circular pattern diameters were varied without an applied voltage, and the area of the resulting gel was quantified (Fig. 3.5). As the pattern diameter increases, the gelled area also increases, after a threshold of a 310-µm-diameter pattern is met. While a diameter of 310 µm represents an improvement in resolution compared to previously reported results [microheater, cooling gas], a smaller gel area is desired to achieve single-cell resolution.

**Figure 3.5** Gelling areas with respect to circular pattern diameter

### 3.4 Characterization of PNIPAAM Gelation

To achieve a smaller gel, different light pattern shapes were evaluated. The light patterns had various shapes, but the same area, in order to keep the optical power delivered to the substrate constant. The light pattern exposure time and location for each pattern was kept constant. The exposure time was 10 s, and device was allowed to cool for 1 minute between tests. In addition, the PNIPAAm solution was set to continuously flow through the microfluidic chamber at a volumetric flow rate of 1.5 µl/min.
Figure 3.6A Gelling areas with respect to constant area patterns

Figure 3.6B Light patterns with different shapes but constant area.
The gelled area observed (Fig. 3.6A) for each light pattern shape (Fig. 3.6B) is different. The largest gel area, about 0.045 mm$^2$, was observed for the cross pattern. This pattern also produced gels in the shortest period of time. The cross pattern was used in further sol-gel experiments, referred to as a standard cross (Fig. 3.7). Two of the patterns caused no gelation, so pattern shape plays important role in gelling. The different pattern shapes that used to perform this experiment are shown in Figure 3.6A. The area for all these patterns was kept constant at 0.1211 mm$^2$.

When using the standard cross pattern, gelling is observed only near the center of the cross. The small gelled area compared to actual size of the light pattern helps in locating and capturing cells. The temperature distribution into the device can be examined using thermochromic paint to check maximum temperature rise that can occur due to irradiation of optical pattern over the period of time while performing experiments. Thermochromic paint with a range of 30-35°C was used. The temperature can measured by observing the color of the thermochromic paint. As temperature increases throughout the range, the color shifts from red to green to blue. If the temperature exceeds 35°C, the paint becomes black. A thermocouple was also used simultaneously with the thermochromic paint to verify the temperature rise. Most of the sol-gel experiments were done using the thermocouple to check temperature rise. It was observed that the temperature did not exceed 37°C throughout these experiments.
Figure 3.7 Standard cross pattern dimensions.

The standard cross pattern is further used to study the gelled area with respect to time, using a solution of 10% PNIPAAm in DI water (Fig. 3.8). The experiment was performed by turning the optical pattern on for some time and then off for 1 minute to cool down the substrate to its initial temperature. Under these conditions, gelling is observed after 3 s of exposure time. From 0 to 30 s, the gelled area increased rapidly (Fig. 3.9), but for longer exposure times the gel area saturates.

Figure 3.8 Gelled PNIPAAm area with respect to time.
Figure 3.9 Gelled area with respect to time from 0 to 30 s.

Gelled area for exposure times from 5 s to 20 s is shown in Fig. 3.10. The gel disappeared within short period of time after turning off the light pattern for exposure times less than 15 s. The tail of the gel in each image can be seen, as the PNIPAAm solution is flowing at a linear flow rate of 100µm/s from the left to the right in the images.

Figure 3.10 Still images captured from the videos of sol-gel experiment.
After turning off the optical pattern, the dynamics of how the gel disappears can be quantified (Fig. 3.11). To find gelling reduction area with time, three different videos were captured after gelling due to exposure of 21.4 s, 27.54 s, and 32.57 s respectively. By using those videos the time required for gelling reduction of the gel formed due to different exposure times is shown in the graph. The gel area reduction with time and its best-fit curve with a corresponding time constant are shown (Fig. 3.11). The gel area reduction vs. time can be used to determine a time-modulation scheme to maintain a constant gel area. This can help to control the increase in gel area as a function of time, and move towards smaller gels to trap specific single cells.
Figure 3.11 Gelling reduction areas with respect to time. Three different exposure times were tested: 21.4 s, 27.54 s, and 32.57 s, respectively. A curve and time constant was fitted to each data set.
Figure 3.12 Reproducibility of gelling with respect to time by using an amorphous-silicon-coated glass substrate.

Reproducibility experiments indicate that the device is reliable, with a standard deviation of 0.0045 mm$^2$.

Gelling area was also measured with respect to the intensity of the light pattern. The light pattern power was measured by using an optical power meter (Newport, model 1830-C). The brightness of the light pattern was changed by adjusting the output color of the projector along the grayscale from completely black to completely white. The pattern with different brightnesses were exposed on a device containing PNIPAAm solution for 10 s to check the PNIPAAm sol-gel transition. The gelling area increases with an increase in intensity (Fig. 3.13).
Another experiment was done to measure gelled area with respect to flow rate (Fig. 3.14). The flow rate is varied using a syringe pump, changing the flow speed of liquid PNIPAAm in the device. It was observed that there is inverse relation between flow rate and the gelled area. Gelled area reduces with increased in flow because of increased cooling of the substrate.
Thus, gelled area increases with time, reduced flow rate, and increased light pattern intensity. This information is used to help maintain constant a gelled area. A gel area of 0.02$\text{mm}^2$ would be sufficient to achieve specific single-cell isolation. To accomplish this, program was written in Processing [43] to expose light pattern for a set period of time (6 s), then turn off the pattern for 0.2 s. These on and off times are varied further to achieve a constant gelling area. This time modulation achieved a gel area of 0.02 mm$^2 \pm 0.001$ mm$^2$ (Fig. 3.15).

![Figure 3.15 Time modulation experiment. The exposure on and off times were varied to maintain a constant gelled area.](image)

3.5 Microbead Trapping

Microbead trapping was done to verify device performance. The trapping of a clump of 20-$\mu$m-diameter polystyrene microbeads in an optically patterned hydrogel was achieved (Fig. 3.16). A PNIPAAm solution containing 10- and 20-$\mu$m-diameter microbeads was introduced into the microfluidic chamber using syringe pump. The solution is flowing from the right to the left at a linear flow rate of 100 $\mu$m/s. An optical pattern is used to heat the substrate, causing a hydrogel to form only in the illuminated area. The optical pattern was then removed to visually verify the trapping of the clump of
microbeads in the PNIPAAm hydrogel. After turning off the optical pattern, the gel reverts back to a solution due to substrate cooling. Eventually, the beads are freed from the gel trap, and flow away in the solution.

Figure 3.16 Trapping of 20-µm-diameter microbeads in a hydrogel. The scale bar is 200 µm. (a) A microbead solution flowing at 100µm/s in a hydrogel solution. The target beads for trapping are indicated by the arrow. (b) An optical pattern is used to heat the substrate, causing a gel to form in the illuminated area. (c) The hydrogel with the optical pattern removed, showing the trapped target particles. (d) Substrate cooling in the absence of optical heating reverses the trap.

3.6 Cell Trapping

A similar trapping experiment was done by using a cell density of approximately about $1 \times 10^5$ cells/mL in a solution of 10% PNIPAAm concentration in PBS. MDCK II cells were introduced in the 10% PNIPAAm solution using a syringe pump at a flow rate of 1.5 µl/min. In this experiment, circular optical patterns were used to trap the cells. The
cell(s) of interest were observed under the microscope, and then an optical pattern was exposed onto the desired cell. The optical pattern triggered the sol-gel transition of the PNIPAAm, trapping the cell(s) of interest into a gel. Single cells can be trapped (Fig. 3.17a), or multiple cells (Fig. 3.17b). Since the flow of the PNIPAAm solution is from left to right, other cells around the trapped cell(s) are flushed out of the PDMS chamber. When the optical pattern was turned off, the cell(s) trapped in the gel can be observed. After trapping desired cells, we can replace the PNIPAAm solution by flushing the chamber with PBS. While this is done, the temperature of the device was maintained at 37°C using the ITO heater. Since optical pattern was removed the required voltage was applied to the device so as to get required temperature.

![Figure 3.17 Sol-gel experiment for cellular manipulation](image)

**Figure 3.17** Sol-gel experiment for cellular manipulation

A cell viability experiment was also done in order to check the effects of device temperature, PNIPAAm solution, and the device itself on cell health. The cell viability experiment was done by using MDCK II cells at a density of approximately about 1 ×
10^5 cells/mL in 10% PNIPAAm solution. The cell sample was introduced into the PDMS chamber along with the PNIPAAm solution. A LIVE/DEAD assay kit (Invitrogen) was used to perform this viability experiment. An optical pattern was used to trap the cells in the device in a PNIPAAm gel (Fig. 3.18). The circle in the diagram indicates the gelled area. The live cells fluoresce green while dead fluoresce red. The fluorescent image on the right indicates all the cells fluoresce green and no red fluorescence was observed, which indicates that the viability is 100%. That means there was no adverse effect of device and the experimental parameters on to the cells.

Figure 3.18 Cell viability experiment [44].

The 3D culturing system developed here can overcome some of the limitations associated with current techniques. The microbead trapping and cellular experiments indicates that our smart culture device has capability to manipulate cells at a single-cell level with no adverse effect on cell health.
CHAPTER 4
FUTURE WORK

There is much room for future work in both the 2D and 3D smart cell culture devices. Fortunately, although there is difference in device fabrication process, the function that both the devices serve is similar. Both devices can be used to trap desired cells and flush out undesired cells; the only difference is that the 2D culturing device cultures the cells on a flat surface while the 3D culturing device does it in a 3D hydrogel. Hence, similar experiments are can be performed as a future work for both types of devices.

For the 2D cell-culturing device, results from the contact angle testing indicate the uniformity of the PNIPAAm coating on to the glass slide. However, cellular testing remains to be done. The cellular testing involves checking device function using actual cells. Cells can be introduced into the device and then the device temperature can be changed from 20°C to 37°C to note the effects on cell adherence. Following this, the trapping of selected cells can be done by heating the device surface locally using optical pattern. This experiment can show the trapping of desired cells and flushing of undesired cells. Viability experiments also need to be done to check for any adverse effects of the device on cell health and its growth.

Temperature distribution on to the device after irradiating the device with an optical pattern over a period of time can be checked by performing experiments using thermochromic paint [45] or rhodamine B [46, 47]. This experiment can give an idea about the maximum temperature rise that can be experienced by cells in the device. The limit of the temperature at which cells behave normally should not be exceeded. Optical pattern exposure times can be adjusted, if necessary, to maintain the temperature within desired limits throughout the cellular experiments. However, based upon temperature measurements done in our 3D culture device, it is anticipated that cells will be exposed to temperatures of less than 37 °C at all times.
The final experiment that needs to be done is 2D cell culturing in the device after trapping the desired cells and flushing out the undesired ones. The cell culturing can be done in controlled culture conditions using a CO\textsubscript{2} incubator. The viability tests and growth rate of the cells in culture should be measured to check the culture performance of the device.

Similar to the 2D culturing device, some experiments need to be done for the 3D culturing device. Cells need to be cultured in the 3D device. The growth rate of the cells and their viability during and after culturing need to be measured.

For device fabrication, the corona treatment procedure needs to be optimized in order to achieve a high-quality, strong bond. This optimization can be done by considering few parameters such as the distance between the substrate and corona treater while bonding, treating time, and the time for which the optimum pressure that need to be applied after corona treatment. The current corona-treated bond breaks if pressure is applied when making tubing connections. After performing several experiments, the tubing connectors had blockages from PNIPAAm residues, necessitating frequent changing of the tubing connections. If the corona treater process is not optimized and if it is used for several times on the same device, there are increased chances of damaging the thermoresponsive coating. This affects experimental results due to uneven heating of the substrate.

Rare cells can be grown using our smart culture devices to make a homogeneous cell population. The rare cells in a mixed sample can be introduced into the device. Using the standard cross pattern with irradiation time controlled by the Processing program, a constant gelled area of 0.02 mm\textsuperscript{2} can be achieved. This gelled area is sufficient to trap cells at single cell level. Trapped specific cells can further be harvested to create a homogeneous population.
CHAPTER 5
CONCLUSION

This thesis described the cell culture applications for our smart culture devices along with
detailed descriptions of the design, fabrication, and testing. Existing techniques and
technologies were discussed, including their limitations that hinder their use in specific
applications such as cellular manipulation and in the development of cellular therapies.

The existing 2D and 3D cell culturing techniques were discussed in detail with
respect to their use in performing biological assays. Some applications are more suitable
to 2D culturing due to its simplicity and low cost. On the other hand, 3D culturing is
preferred where highly accurate cell behavior is desired. The drawbacks associated with
current 2D and 3D culturing techniques that limit their use in development of cellular
therapies were discussed. Further, the properties of our smart culture devices were
discussed that can address these drawbacks and become attractive in development of
cellular therapies such as stem cell therapies.

The 2D culture device was discussed in detail, including its fabrication procedure
and tests to check the performance of the device. Contact angle testing results indicate
that the PNIPAAm coating obtained is of good quality, as indicated by water contact
angles of 63.3° and 91.3° at temperatures of 20°C and 40°C, respectively. The 3D culture
device fabrication procedure, and its testing was also discussed. Several experiments
were done to check performance of the device as well as to check its effects on cell health.
The 100% viability indicates that our technique is cell-friendly and offers effective tool to
manipulate microbeads and cells at single cell level using optical patterns. A time-
modulated setup maintained a constant gel area of 0.020 mm$^2$, which can be used to trap
a specific single cell. The repeatability of the gelled area was also examined and found to
have a deviation of 0.0045 mm$^2$. The testing results from both the devices were as
expected, indicating that they have the potential to be used individually to perform
cellular assays with great precision.

Adherent cells with any size and shape can be manipulated with these smart
culture devices. Generally, human cell sizes range from approximately 5 to 20 µm in
diameter. The smallest gelling area of 0.02 mm$^2$ was achieved using time modulation,
corresponding to linear dimensions of approximately 140 µm × 140 µm. This area is
sufficient to trap a single cell, and the shape and size of the cell will not affect the manipulation technique. The only thing that needs to be controlled to achieve single-cell trapping is the cell density. A cell density of $1 \times 10^5$ cells/mL can be used while performing cellular manipulation experiments to achieve trapping at the single-cell level.

The future work that needs to be done is discussed after performing tests on the fabricated devices. The future work includes some more cellular experiments and actual culturing of cells using both types of smart culture devices.
REFERENCES


Appendix

Processing source code used in the time modulation experiment:

color wht=255;
color blk=0;
int start =0;

void setup() //initialize variables
{
  background(0); //set background to black
  size(1600,1200); //set screen size to 1600 px x 1200 px
  noStroke(); //No pattern border
  rectMode(CENTER); //set pattern location at center
  fill(255); //set fill color to white
}

float msec = millis();

void draw() //begin main program execution
{
  background(0); //set background to black
  if(millis()-start < 6000) //set optical pattern ON time to 6 s
    {
      rect(width/2,height/2,299,75);
      rect(width/2,height/2,84,263); // Optical pattern with desired dimensions
    } 
  if((millis()-start >= 6000)&&(millis()-start < 6200)) //set pattern OFF time to 0.2 s
    {
      background(0); //no optical pattern
    } 
  if((millis()-start >= 6200)&&(millis()-start < 10200))  //set pattern ON time of 4 s
    {
      rect(width/2,height/2,299,75);
      rect(width/2,height/2,84,263); // pattern
    } 
  if((millis()-start >= 10200)&&(millis()-start < 10400)) //set pattern OFF time of 0.2 s
    {
      background(0); //no pattern
    } 
  if((millis()-start >= 10400)&&(millis()-start < 14500)) //set pattern ON time of 4.1 s
    {
      rect(width/2,height/2,299,75);
      rect(width/2,height/2,84,263); // pattern
    } 
  if((millis()-start >= 14500)&&(millis()-start < 14700)) //set pattern OFF time of 0.2 s

if((millis()-start >= 14700)&&(millis()-start < 18800))  //set pattern ON time of 4.1s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}

if((millis()-start >= 18800)&&(millis()-start < 19000)) //set pattern OFF time of 0.2 s
{
    background(0); //no pattern
}

if((millis()-start >= 19000)&&(millis()-start < 22100))  //set pattern ON time of 3.1 s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}

if((millis()-start >= 22100)&&(millis()-start < 22300)) //set pattern OFF time of 0.2 s
{
    background(0); //no pattern
}

if((millis()-start >= 22300)&&(millis()-start < 25400))  //set pattern ON time of 3.1 s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}

if((millis()-start >= 25400)&&(millis()-start < 25600)) //set pattern OFF time of 0.2 s
{
    background(0); //no pattern
}

if((millis()-start >= 25600)&&(millis()-start < 28800))  //set pattern ON time of 3.2 s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}

if((millis()-start >= 28800)&&(millis()-start < 29000)) //set pattern OFF time of 0.2 s
{
    background(0); //no pattern
}

if((millis()-start >= 29000)&&(millis()-start < 31000))  //set pattern ON time of 2 s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}

if((millis()-start >= 31000)&&(millis()-start < 31200)) //set pattern OFF time of 0.2 s
{
background(0); // no pattern
}
if((millis()-start >= 31200)&&(millis()-start < 35300)) // set pattern ON time of 4.1 s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}
if((millis()-start >= 35300)&&(millis()-start < 35500)) // set pattern OFF time of 0.2 s
{
    background(0); // no pattern
}
if((millis()-start >= 35500)&&(millis()-start < 37600)) // set pattern ON time of 2.1 s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}
if((millis()-start >= 37600)&&(millis()-start < 37800)) // set pattern OFF time of 0.2 s
{
    background(0); // no pattern
}
if((millis()-start >= 37800)&&(millis()-start < 40100)) // set pattern ON time of 3.1 s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}
if((millis()-start >= 40100)&&(millis()-start < 43200)) // set pattern OFF time of 0.2 s
{
    background(0); // no pattern
}
if((millis()-start >= 43200)&&(millis()-start < 43400)) // set pattern ON time of 3.1 s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}
if((millis()-start >= 43400)&&(millis()-start < 46500)) // set pattern OFF time of 0.2 s
{
    background(0); // no pattern
}
if((millis()-start >= 46500)&&(millis()-start < 46700)) // set pattern OFF time of 0.2 s
{
    background(0); // no pattern
}
if((millis()-start >= 46700)&&(millis()-start < 48800)) //set pattern ON time of 2.1 s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}

if((millis()-start >= 48800)&&(millis()-start < 49000)) //set pattern OFF time of 0.2 s
{
    background(0); //no pattern
}

if((millis()-start >= 49000)&&(millis()-start < 50800)) //set pattern ON time of 1.8 s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}

if((millis()-start >= 50800)&&(millis()-start < 51100)) //set pattern OFF time of 0.3 s
{
    background(0); //no pattern
}

if((millis()-start >= 51100)&&(millis()-start < 53100)) //set pattern ON time of 2 s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}

if((millis()-start >= 53100)&&(millis()-start < 53400)) //set pattern OFF time of 0.3 s
{
    background(0); //no pattern
}

if((millis()-start >= 53400)&&(millis()-start < 55400)) //set pattern ON time of 2 s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}

if((millis()-start >= 55400)&&(millis()-start < 55700)) //set pattern OFF time of 0.3 s
{
    background(0); //no pattern
}

if((millis()-start >= 55700)&&(millis()-start < 57700)) //set pattern ON time of 2 s
{
    rect(width/2,height/2,299,75);
    rect(width/2,height/2,84,263); // pattern
}

if((millis()-start >= 57700)&&(millis()-start < 58000)) //set pattern OFF time of 0.3 s
{
    background(0); //no pattern
}
if((millis()-start >= 58000) && (millis()-start < 59000))  //set pattern ON time of 2 s
    {
        rect(width/2,height/2,299,75);
        rect(width/2,height/2,84,263);  // pattern
    }
if((millis()-start >= 60000) && (millis()-start < 60300)) //set pattern OFF time of 0.3 s
    {
        background(0); // no pattern
    }
if((millis()-start >= 60300) && (millis()-start < 62300))  //set pattern ON time of 2 s
    {
        rect(width/2,height/2,299,75);
        rect(width/2,height/2,84,263);  // pattern
    }
if((millis()-start >= 62300) && (millis()-start < 62600)) //set pattern OFF time of 0.3 s
    {
        background(0); // no pattern
    }
if((millis()-start >= 62600) && (millis()-start < 64600))  //set pattern ON time of 2 s
    {
        rect(width/2,height/2,299,75);
        rect(width/2,height/2,84,263);  // pattern
    }
}  //end draw() function