METHOD THAT POSITIONS CELL-LADEN OR CELL-FREE MATRICES AT DEFINED POSITIONS FROM EACH OTHER INSIDE A SINGLE MICROFLUIDIC CHANNEL

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ABSTRACT

METHOD THAT POSITIONS CELL-LADEN OR CELL-FREE MATRICES AT DEFINED POSITIONS FROM EACH OTHER INSIDE A SINGLE MICROFLUIDIC CHANNEL

In recent years, the use of microfluidic has increased in the field of many biological studies. Microfluidic technology has a large area which is a joint product of biology and industry covering all branches of science.

The small size of the microfluidic chip offers many advantages in the use of microfluidic. During the analysis, the microfluidic chip offers many advantages such as, use of less material, less waste generation, temporal control, opportunity of analysis under the microscope and high throughput analysis. In addition to these, while microfluidic chip is providing a safe environment for users, via mimicking the physiological environment, it also provides a suitable environment in order to make cell, tissue and organs based assays.

Microfluidic devices especially use in cancer studies, chemical analysis, tissue engineering, drug screening, immunology and stem cell differentiation.

In this study, we aimed to develop methods depending on the distance to position the MDA-MB 231 breast cancer cells in the microfluidic channels. Firstly, the microfluidic channels were obtained by using the soft lithography and experiments with breast cancer cells were performed using these channels. Breast cancer cells containing matrix was loaded into microfluidic chips and precipitated onto blank matrix by using centrifuge. The aim of repeating this process was to position the breast cancer cells at different distanced locations.

ÖZET

HÜCRELİ YA DA HÜCRESİZ MATRİKSLERİN TEK BİR MİKROAKIŞKAN KANAL İÇİNDE BİRBİRLERİNDEN BELİRLİ UZAKLIKLARDA KONUMLANMASINI SAĞLAYAN YÖNTEM

Son yıllarda, birçok biyolojik çalışma alanında mikroakışkan kullanımı artmıştır. Mikroakışkan teknolojisi biyoloji ve endüstri alanlarının ortak bir ürünü olup tüm bilim dallarını kapsayan, geniş bir alana sahiptir.

Mikroakışkan çiplerin boyutlarının küçük olması, mikroakışkan kullanımda birçok avantaj sağlamaktadır. Yapılan analizler esnasında mikroakışkan çipler, az malzeme kullanımı, az atık oluşumu, zamansal kontrol, mikroskop altında inceleme imkânı ve yüksek çıktılı analizler gibi birçok avantaj sunar. Bunların yanı sıra, mikroakışkan çipler kullanıcılar için güvenli bir ortam sağlamakla beraber, fizyolojik ortamıda taklit ederek hücre doku ve organ bazlı deneylerinin yapılabilmesi için uygun bir ortam sağlamaktadır.

Mikroakışkan cihazlar özellikle kanser çalışmalarında, kimyasal analizlerde, doku mühensliğinde, ilaç taramasında, immünoloji ve kök hücre taramasında kullanılmaktadır.

Bu çalışmada, MDA-MB 231 meme kanser hücrelerini, mikroakışkan kanal içerisinde uzaklığa bağlı olarak konumlandıracak yöntem geliştirmeyi amaçladık. Öncelikle yumuşak litografi yöntemi kullanılarak mikroakışkan kanallar elde edildi ve bu kanallar kullanılarak meme kanseri hücreleri ile deneyler yapıldı. MDA-MB-231 meme kanseri hücrelinin bulunduğu matriks, mikroakışkan çiplerin içerisine yüklendi ve santrifüj kullanılarak hücresiz matriks'in üzerine çöktürüldü. Bu işlem tekrar yapılarak meme kanseri hücrelerini farklı uzaklıklara konumlandırmak amaçlandı.

To my family…

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CHAPTER 1

INTRODUCTION

1.1. Microfluidic Devices

Microfluidic refers to the science and technology of fluid system that process or manipulating small amount of liquids at nanoliter to femtoliter scale (Ren, Chen, & Wu, 2014) .Microfluidic technology is multidisciplinary field of engineering, physics, chemistry, biochemistry, nanotechnology and biotechnology.

Miniaturization and integration of a range of devices supply the many useful capabilities; the ability to use small volume of materials provides increasing safety while using chemical material or biological agents. Moreover, they offer high resolution and sensitivity under the microscopy, high throughput analysis, short time for analysis. Finally, they provide physiologically relevant settings for the cell biology experiments (Manz et al., 1992).

1.2. Microfluidic Devices Fabrication

In order to fabricate microfluidic devices, there are several lithography methods. Photolithography and soft lithography are commonly used methods. Other lithography methods are E-beam lithography, X-ray lithography, and Interference lithography etc. (Xia & Whitesides, 1998). The method is determined according to the aim of the research and the facilities.

1.2.1. Photolithography

In order to fabricate microdevices such as microfluidic devices, microelectronic circuits and micro electro mechanical systems (MEMS), photolithography method is usually used. This method means removing parts of a thin photoresist film (Choi & Park, 2010).

Photolithography method is also called as UV- lithography that is a parallel writing method uses light to transfer geometric pattern on the light sensitive materials, called photoresist. Using this method, 2D and 3D micrometer scale designs are fabricated (Qin, Xia, & Whitesides, 2010).

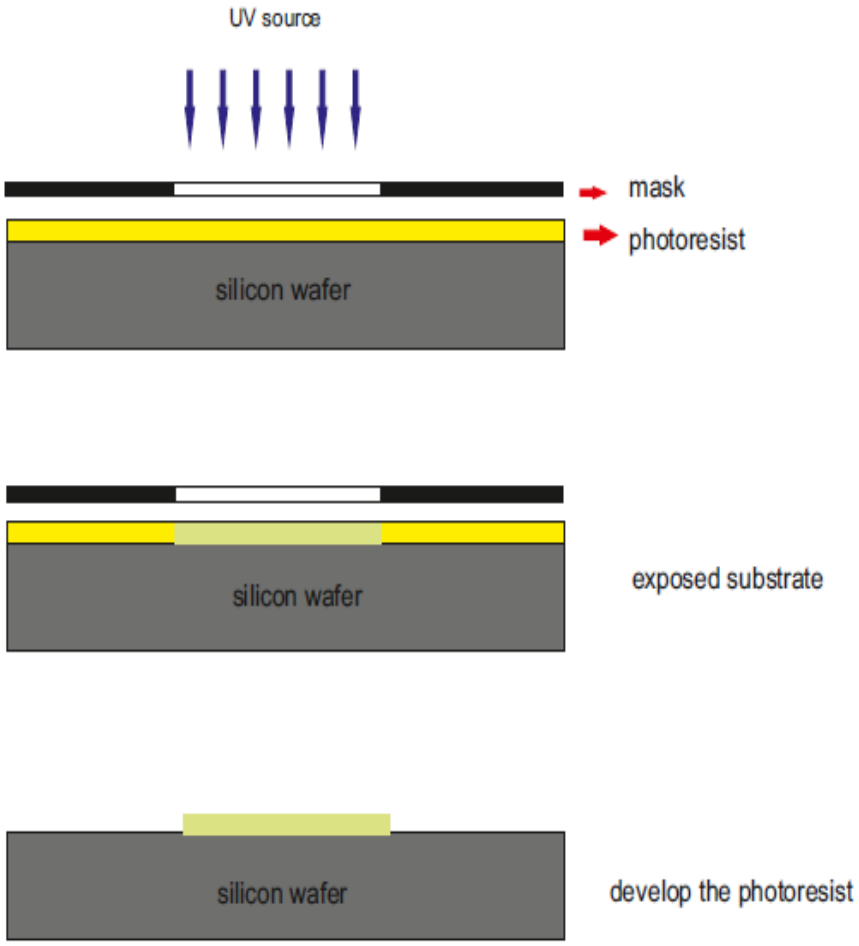

Figure1.1. Schematic of the photolithography steps.

1.2.2. Soft Lithography

Soft lithography symbolizes a non-photolithographic strategy. It is made of self-assembly and replica molding. It is used for both nano and micro-device fabrication. Soft lithography provides transfer of desire pattern to elastomer mold. Transfer can be performed repetitively. The method includes replication of a structure on a master in a soft lithography. That's why; soft lithography provides a convenient, effective and low cost method for the formation and manufacturing of micro and nano-structures

(McDonald & Whitesides, 2002; Qin et al., 2010). Fabricated devices is a single-use devices due to their price and single- use devices eliminate contamination in the analyses (Ng, Gitlin, Stroock, & Whitesides, 2002).

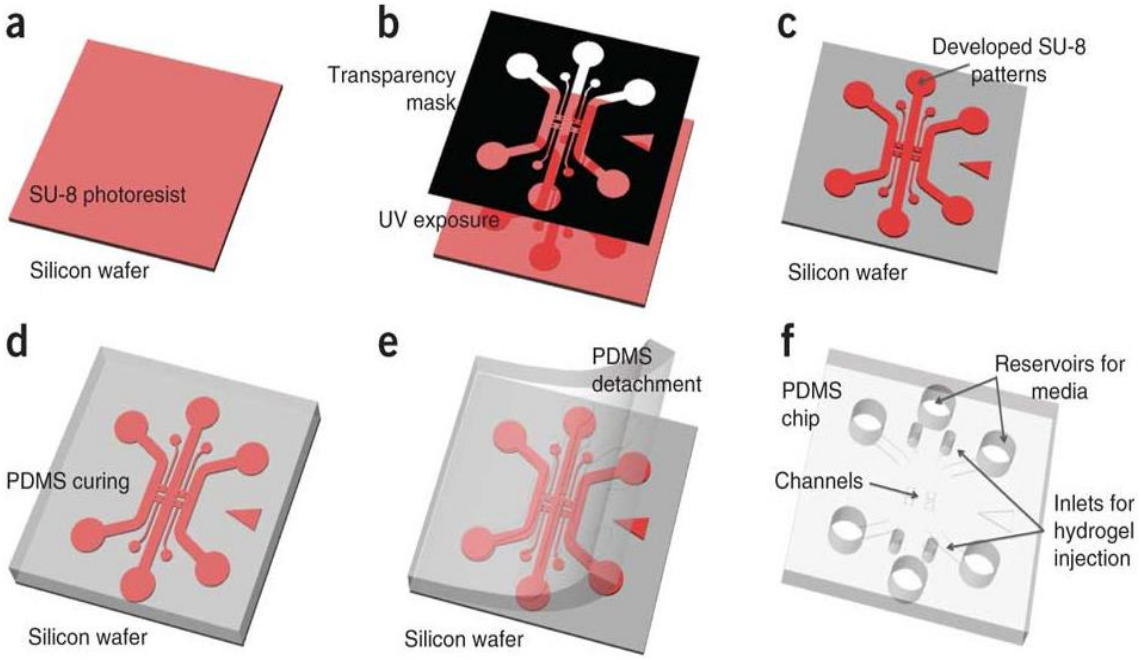

Figure 1.2. (a-f) schematic of the soft lithography (a-c) and soft lithography (d-f) procedure (Source:Shin et al., 2012)

1.2.3. Materials Characteristics in Photolithography

In photolithography method, photoresist, silicon wafer, developer, isopropanol and desired pattern containing mask are used as materials.

Photolithography is an etching process. It supplies to the transfer of a pattern on the substrate. In this method, stringent must be provided to increase aspect ratio and lateral future size shrink by resist materials (Ito, 2005) .

A Photoresist is used usually in photolithography, is a light sensitive material. SU-8 photoresist is formulated for photolithography and it has important mechanical, physical and chemical properties such as high sensitivity, high resolution, low optical absorption, high thermal stability and good chemical resistance. Due to the SU-8 properties, usage area of SU-8 is very large such as MEMS, microfluidic devices, microstructure and basic material for molding and packaging (Lorenz et al., 1997)

. There are two types of SU-8; negative SU-8 photoresist and positive SU-8 photoresist.

Negative SU-8 photoresist means that exposed area to the UV light of SU-8 become polymerized and there are highly cross-links. That's why exposed area does not dissolve in the developer solution.

Positive SU-8 photoresist behaves opposite of negative photoresist. Exposed area of positive SU-8 photoresist dissolves in the developer solution.

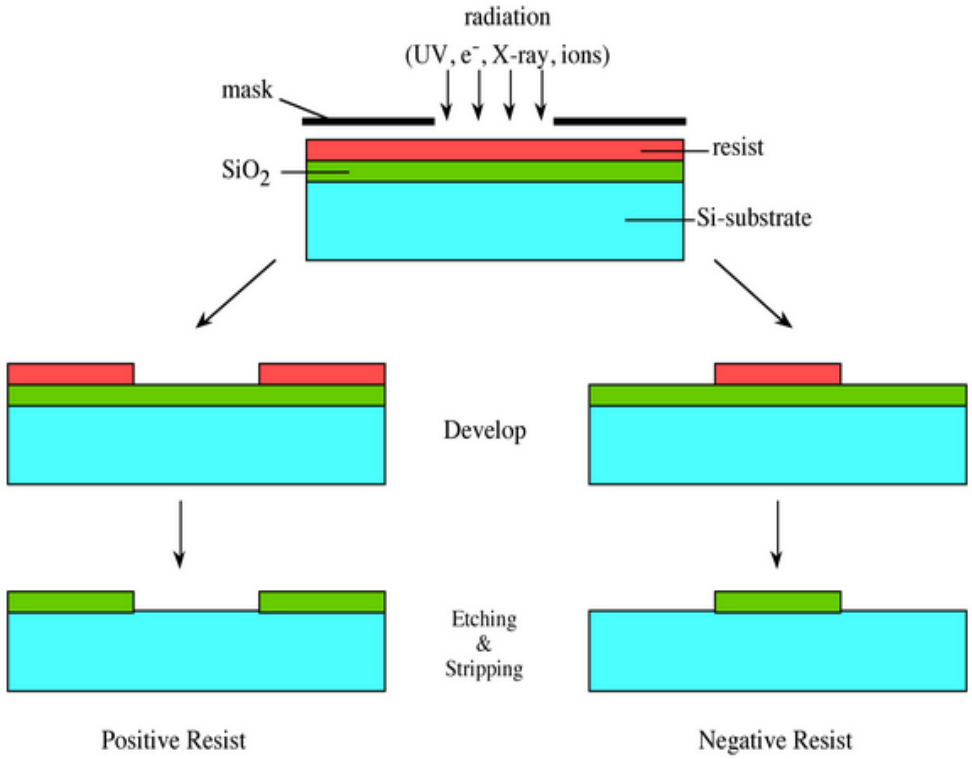

Figure 1.3. Comparison of negative and positive SU-8 photoresist
(Source: Alrifaiy, Lindahl, & Ramser, 2012)

Silicon wafer is generally used in the electronics fabrication industry. Adhesion of SU-8 photoresist and silicon wafer are simple because of expansion coefficient of them. Surface roughness of silicon wafer is small, so the substrate is uniform. And also silicon wafers are fabricated in the clean room so cleaning is not necessary. However, only disadvantage of silicon wafer is price. Although it is more expensive than glass, still it has larger surface area.

In photolithography, 3 types of photo mask are used which are chrome mask, emulsion glass mask and film mask. Chrome mask contains soda lime glass and quartz so it has low thermal expansion and high optical transmission but it is expensive. Emulsion glass mask is soda lime glass covered with a film. In order to require high definition and position accuracy, it is more suitable. Film mask uses pet film base material. The film composition and production processes are almost the same with emulsion glass mask.

1.2.4. Materials Characteristics in Soft Lithography

Soft lithography starts with the production of a Polydimethylsiloxane (PDMS) replica of a master. PDMS and curing agent are components of soft lithography.

For the research and development of the microfluidics, PDMS is the most common material. PDMS is an optically transparent elastomer so it can be easily bended which is provided by controlling of the stiffness. PDMS supplies to fabrication of microfluidic system easily thanks to outstanding properties such as low price, optical transparency, low permeability to water, low electrical conductivity, biocompatibility and flexibility. Additionally, PDMS is used for the demonstration and new concepts facilities also it permits to prototype devices (SindyK.Y. et al., 2009). Moreover PDMS is nontoxic material for proteins and cells so the microstructure which is made from PDMS is convenient for cellular studies (Whitesides, Ostuni, Takayama, Jiang, & Ingber, 2001).

1.2.4.1. Mechanical Properties of PDMS

Mechanical properties of PDMS are crucial for the MEMS applications. PDMS has to give a permanent experimental data on the stress- strain relationship to be useful in MEMS application (Kim, Kim, & Jeong, 2011). The mixing ratio of PDMS and curing agent determines its mechanical properties interestingly (Unger, Chou, Thorsen, Scherer, & Quake, 2000) . For example, the stress- strain experiment was applied on the mixing PDMS base and curing agent at 10:1 ratio (Kim et al., 2011) . The result is that during the first loading and unloading cycle with 100% of strain was observed. And the yield stress was larger at a low applied strain.

1.2.4.2. Chemical Properties of PDMS

PDMS base and curing agents are mixed at desire ratio. After polymerization of PDMS, the surface of PDMS becomes hydrophobic. The reason is that it has repeating units of $–O\text{-}Si(CH_3)_2$. In order to be hydrophilic of the surface, oxygen or air plasma can be exposed. That's why methyl group is destroyed by silanol group and silanol group introduction occurs via exposure to plasma (Ng, Gitlin, Stroock, & Whitesides, 2002) (Kim, Chaudhury, & Owen, 2000).

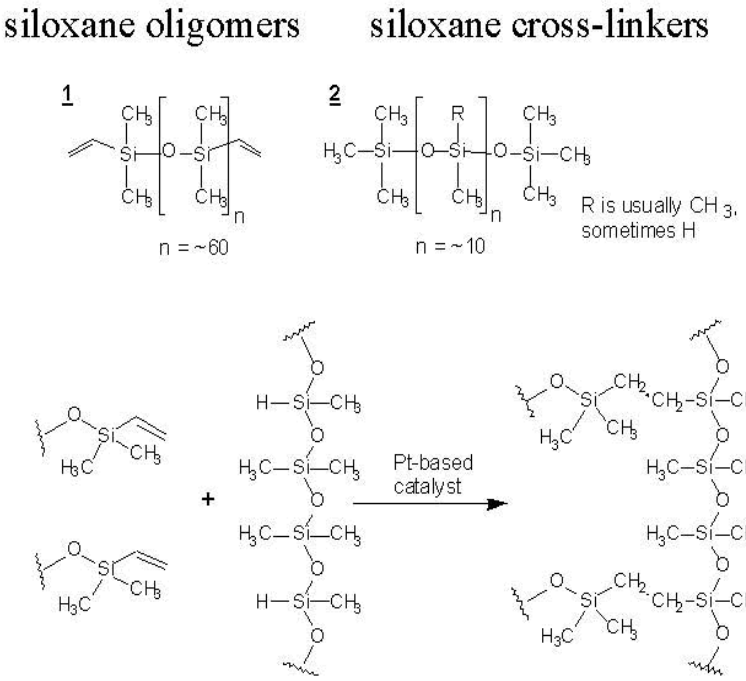

Figure 1.4. Structure of PDMS base and PDMS and curing agent after polymerization (Source: Campbell et al., 1999).

1.2.4.3. Optical Properties of PDMS

Despites PDMS includes nanoparticle which provides the unwanted scattering of light, the optically transparent range of PDMS is 240 to 1100 nm. Refractive index of PDMS is around 1.41. The thickness of PDMS for enclosure of microfluidic components are limited in the devices which have been fabricated, so there is no

significant loss during the coupling of light into and out of the devices even if scattering due to passage of light through PDMS is occurred (Campbell et al., 1999).

1.3. Application of Microfluidic Devices in the Cell Culture Systems

Culturing cells in microfluidic devices have been used to study for many biological process and responses such as comparing with macro scale cell cultures and large scale bioreactors in testing conditions, studying of complex biological process such as stem cell differentiation, growth and proliferation and making experiment in drug testing (Dittrich & Manz, 2006),(Burdick & Vunjak-Novakovic, 2009).

Microfluidic devices can be used in 2D cell culture systems in order to examine monolayer cells, especially in individual cell observation for migration assay, chemotaxis and differentiation of stem cells. Cultivation of cells are favorable to real time analysis of cell behavior, examine large quantities of cells and fluorescence detection method in 2D platforms (Tehranirokh, Kouzani, Francis, & Kanwar, 2013).

In 2D cell culture system, cells are cultured as a monolayer on the surface of the substrate. Monolayer cell culture is made by using petri dish, multiwell plates or culture flask. 2D cell culture systems do not mimic the physiological context so the conditions cannot supplied such as oxygen and shear stress of growth factors, are harmful for cell viability (Meyvantsson & Beebe, 2008). Cells lose their phenotypes in 2D culture environments because *in vivo* conditions are not provided. However, endothelial cells are specific cells so they can mimic the *in vivo* responses. Although endothelial cells can mimic the *in vivo* conditions in 2D, most cells need signals from the 3D environment to mimic the physiological conditions (Griffith & Swartz, 2006).

Development of microfluidic devices or lab-on-a-chips supply the assertive conditions controlled of cell growth or differentiation and these systems allow high repeatability and lower cost while analyzing of cells at a single cell level (Gupta et al., 2010).

In 2 dimensions, human breast epithelial cells develop like tumor cells, but these cells show normal growth behavior when they are cultured in 3D microenvironments (Weaver et al., 1997). And also when stem cells are cultured in 3D as compared to mono layer culture, advanced chondrogenesis of embryonic stem cells has been observed (Tanaka et al., 2004).

CHAPTER 2

EXPERIMENTAL

In these experiments, in order to mimic *in vivo* system, we use 3D controlled *in vitro* microenvironments. 3D controlled *in vitro* microenvironments were constructed with soft lithography which includes fabrication of SU-8 masters and PDMS molds. Creating microenvironments were examined in cell based experiments.

SU-8 master was created with soft lithography method. This method was performed in a Class 1000 clean room at the Applied Quantum Research Center (AQRC) at Izmir Institute of Technology. PDMS molding was performed on bench in laboratory conditions. Cell based experiments was performed in laminar cabinet and standard laboratory conditions.

2.1. Su-8 Master Fabrication and Photomask Creation

Masks were drawn by using Corel Draw. The files were converted to PDFs format and high resolution (3600 dpi) print-outs were ordered with the help of zumtanitim, Ankara, Turkey.

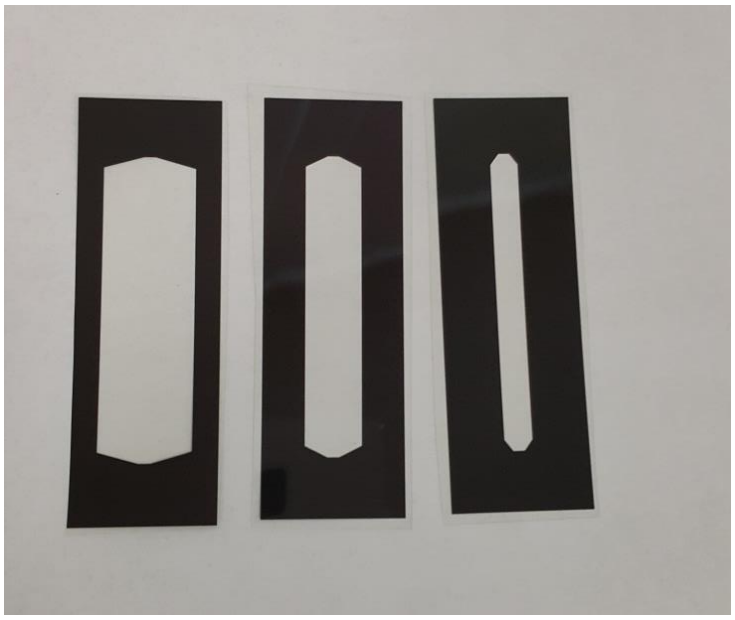

Figure 2.1. Overview of the mask

SU-82075 negative photoresist (provided from Microchem Newton, MA) temperature had to be equal with room temperature so SU-8 was kept waiting on the bench. At the same time, silicon wafer that is (500 ± 25 microns thickness) provided by University WAFER, which was placed on hotplate (Wise Stir® MSH-20D, DAIHAN Scientific, Korea). Hotplate was set up at 65^0C for 5 minutes then SU-8 2075 negative photoresist was poured at the middle part of silicon wafer and photoresist was spread homogeneously by hand.

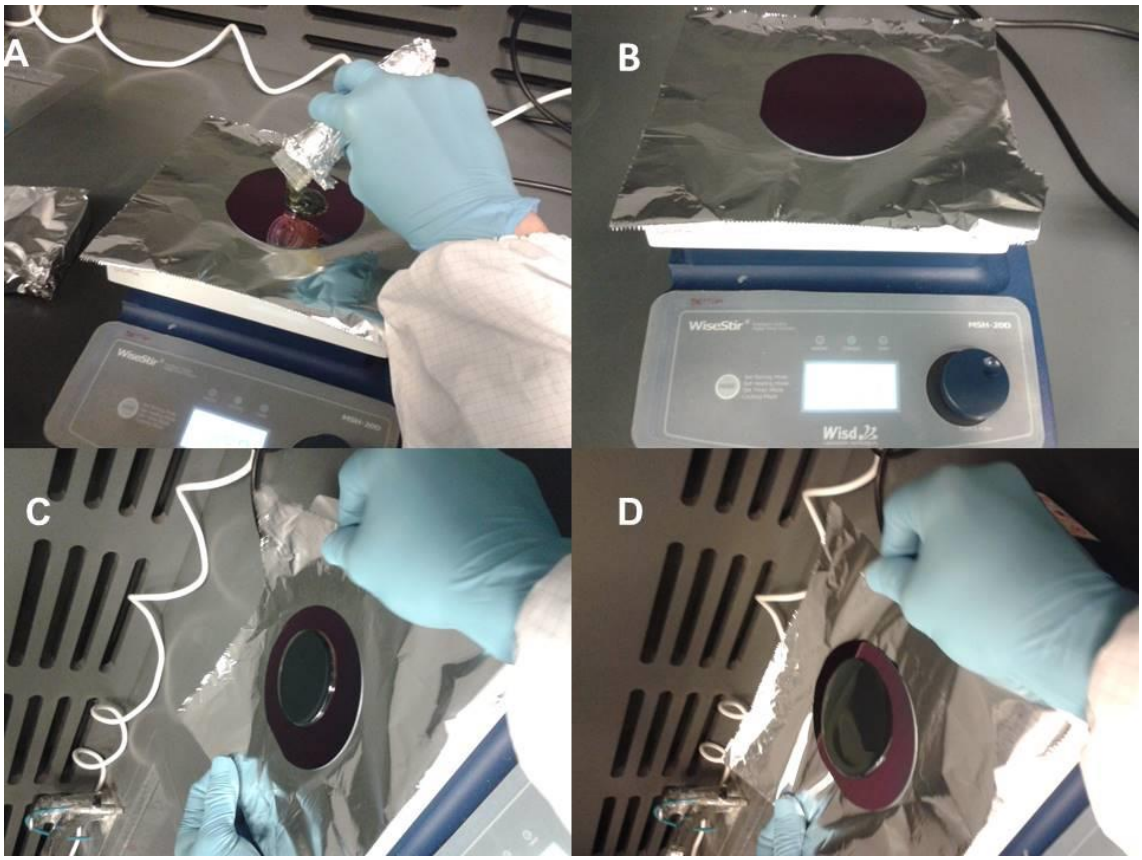

Figure 2.2. a-) wafer was on the hotplate which is set up 65^0C b-) while SU-8 2075 negative photoresist was poured on the silicon wafer c-) after SU-8 negative photoresist was poured. d-) SU-8 negative photoresist was spread by hand.

After spreading, silicon wafer was placed on the bench for 5 minutes. The next step was spin coating, using a spin coater (G3P-8 Desk-Top Precision Spin Coating System, Specialty Coating Systems, Indianapolis, IN) 270µm-620µm layer of SU-8 2075 negative photoresist was created on the silicon wafer (100mm diameter). Before using spin coater, dwell, ramp and rpm were set up according to the thickness of the photoresist layer. After spinning, silicon wafer was kept into the spin coater for 5 minutes.

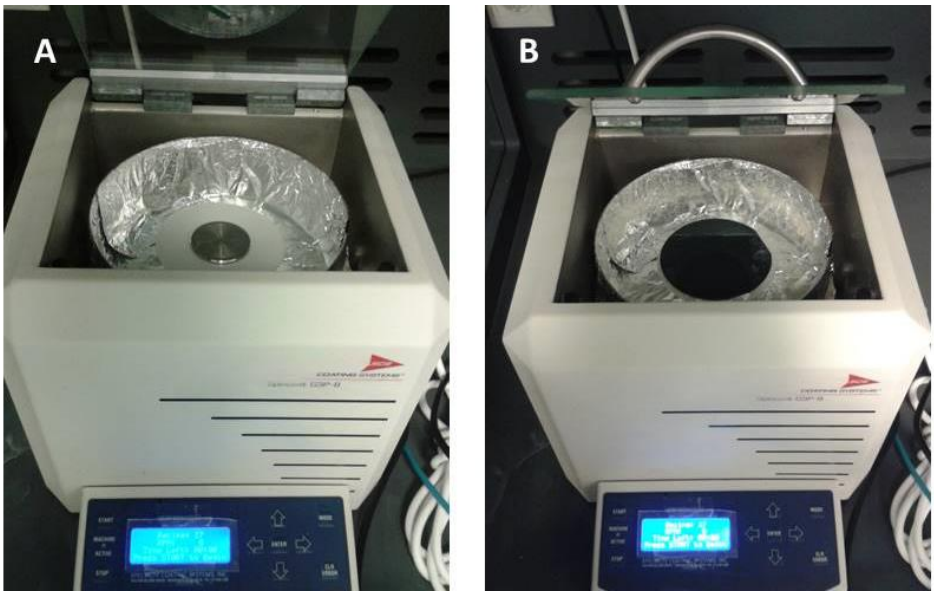

Figure 2.3. a-) Spin coater; b-) after spinning, silicon wafer was in the spin coater.

Following step was soft bake, silicon wafer was covered by SU-8 2075 negative photoresist and it was placed on hot plate at 65º C and 95º C. Soft bake time was dependent the thickness of the SU-8 2075 negative photoresist layer. For example, if the thickness of SU-8 2075 negative photoresist was 340µm, 15 minutes was enough for 65º C and 75 minutes was enough for the 95º C. At the end of soft bake, heater was closed and the silicon wafer was kept on the heater for slow cooling down.

Following day, hot plate was set up at 95º C for wrinkle test. When the silicon wafer was put on hot plate; if any wrinkle was seen, silicon wafer was hold at 95º C about 10 minutes and kept at room temperature for 5 minutes. After that wafer was placed on the heater which was still at 95º C and wrinkle test was applied again until there was no wrinkle. If there was no wrinkle, the exposure step was applied.

In the UV exposure step, mask containing desired pattern was placed surface of the silicon wafer. UV light (400 watt) was given on the silicon wafer by using mask aligner (OAI Hybralign Series 200). Expose time of UV light was depend on the thickness of SU-8 2075 negative photoresist, for example, in order to obtain 340µm thickness of photoresist, UV light was exposed 50 second to be crosslink in the SU-8 2075 negative photoresist.

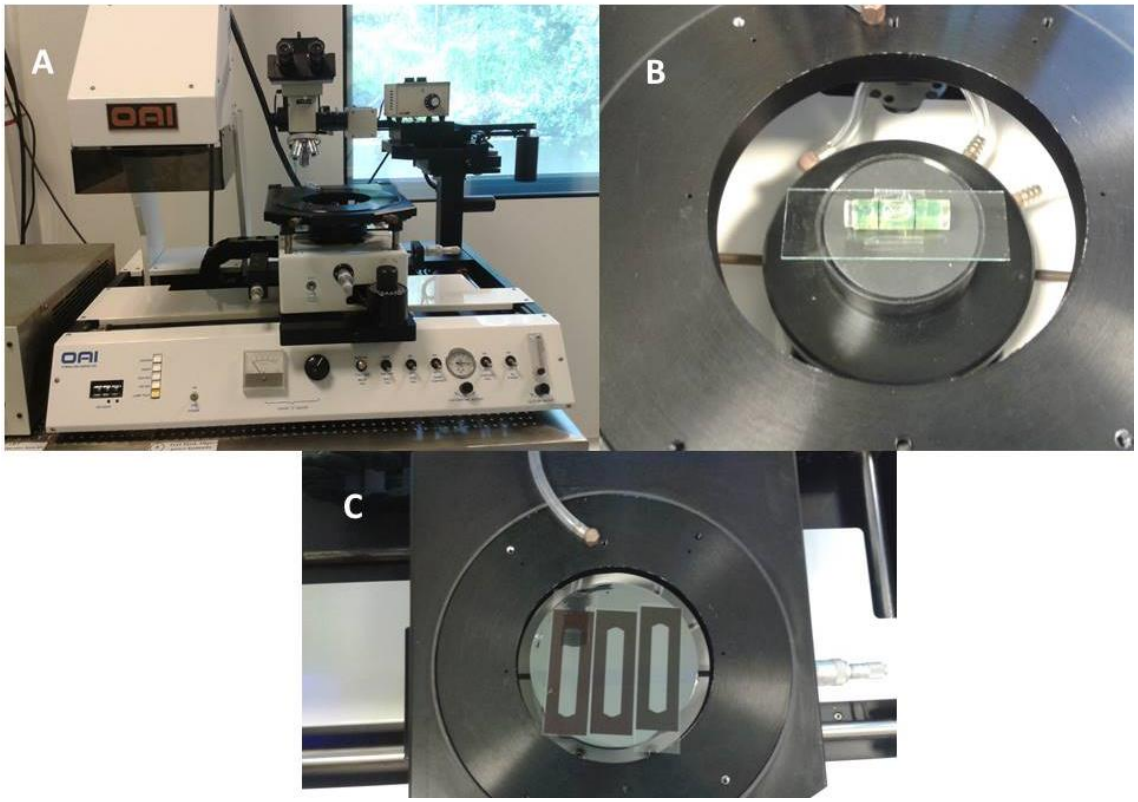

Figure 2.4. OAI Hybralign Series 200 mask aligner and it was used for exposure.

After exposure part, next step was post bake. The mask was split up from the silicon wafer then silicon wafer was left on the heater at 65 ^{0} C. After that temperature was set up 95 ^{0} C and silicon wafer was heated at 95 ^{0} C. Post bake time was depended on the thickness of SU-8 2075 negative photoresist. For example, for 340µm thickness, 5 minutes for 65 degrees and 15 minutes for 95 degrees was enough clearly.

After 24 hours, development step was applied on the silicon wafer, developer solution (Microchem,Newton, MA) temperature had to be about 25^{0} C so it was placed on the bench to be equal its temperature and room temperature. After that developer solution was poured into the petri dish. Then the silicon wafer was put into the petri dish and it was hold on for 5 minutes. After 5 minutes, petri dish was shook for 15 minutes. The non-exposed area of SU-8 2075 negative photoresist dissolved in the developer.

Following action was whether SU-8 2075 negative photoresist was clearly decomposed. SU-8 master silicon wafer was washed with isopropanol in order to control development part was complete or not. While rinsing substrate with isopropanol, there could be seen precipitation (white cloud). If any precipitation was seen, developer

part would be repeated again otherwise silicon wafer was washed with fresh isopropanol.

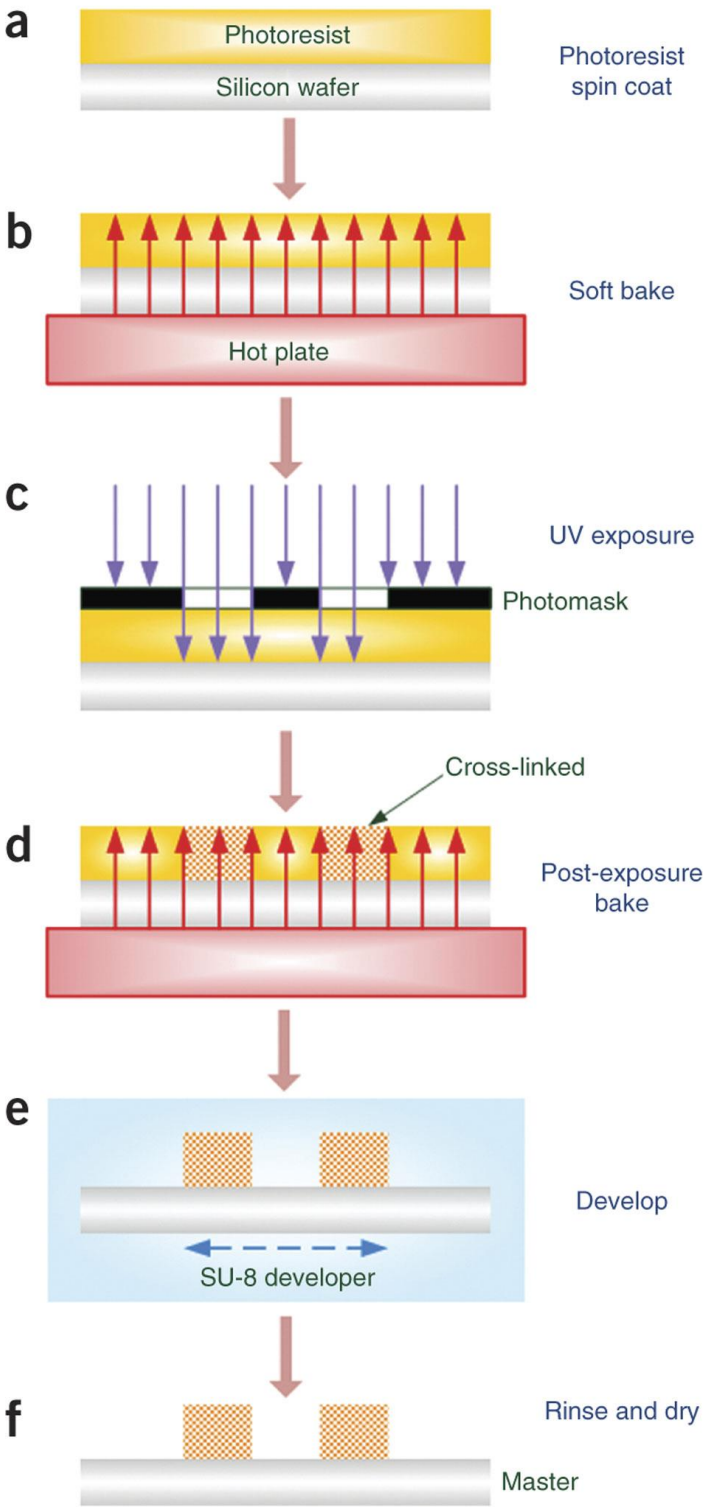

Figure 2.5. Schematic illustration of master fabrication with standard photolithography technique (Source: Geng, Zhan, Wang, & Lu, 2011).

2.2. PDMS Molding

PDMS molds were made of polydimethylsiloxane (PDMS) elastomer. First, 184 SILICONE ELASTOMER KIT PDMS (Dow Corning, Midland, MI, U.S.A) base and curing agent were mixed at a 10:1 weight ratio.

Figure 2.6. Sylgard 184 Silicon Elastomer Kit , base and curing agent

After mixing, the mixture was degassed in a vacuum chamber (PlusMED, 7E-A, Danyang, P.R.C) for 30 (3×10') minutes.

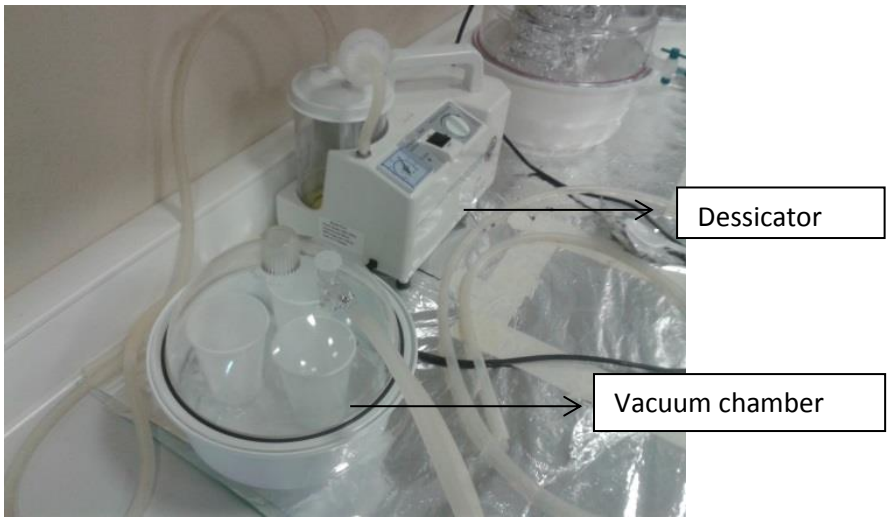

Figure 2.7. Desiccator and vacuum chamber

At the same time, wafer was washed with ethanol (EtOH) and ultrapure (UP) water, then wafer was wet with demolding agent (Triton-X, DH_2O, EtOH; 1:9:40). After that a case was constructed from aluminum foil and double- sticky tape was stuck into the case. Then silicon wafer was placed in the case. PDMS was poured on the foil covered wafer.

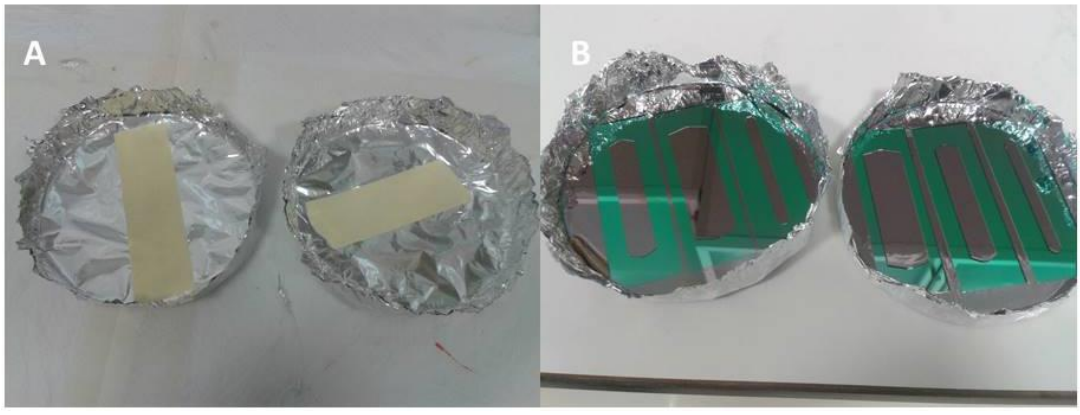

Figure 2.8. Figure A was aluminum foil chamber and double sticky tape into the aluminum foil, figure B was SU-8 master wafer inside in aluminum foil chamber

Next step was waiting to polymerization of PDMS for 2 days. After 2 days, SU-8 master wafer and PDMS were separated from each other. During this action SU-8 master wafer and PDMS were wet with EtOH.

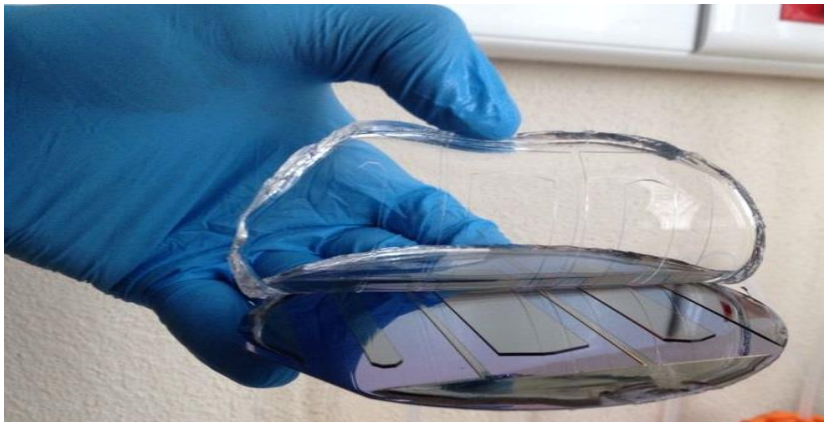

Figure 2.9. While SU-8 master silicon wafer and PDMS stamp were split up from each other

Following step was cutting PDMS. PDMS was cut by knife and, both side and middle part of PDMS was punctured. A hole diameter of both side was 3, 5 mm and middle hole diameter's was 1, 5 mm. And also, both sides of PDMS were punctured as large holes. PDMS were cleaned with scotch tape, EtOH and UPH_2O. First, the dust which were on the PDMS or inside holes, were cleaned with scotch tape and the following cleaning steps were applied by sonicator (WiseClean, WUC-A03H, DAIHAN Scientific, Korea) and hand.

Cleaning of PDMS molds steps were; 5 times rinsing with up and EtOH, 10' in sonicator (UPH_2O), 5 times rinsing with UPH_2O, 5' in sonicator (EtOH), 1 time rinsing with EtOH, 5' waiting in EtOH, 1 time rinsing with UPH_2O, Dry with nitrogen gas.

Figure 2.10. Wiseclean sonicator

Clean PDMS and glass slide (76×26×1mm, Paul Marienfeld, GERMANY) were cleaned and surfaces of them were activated by using UV/OZONE procleaner (BIOFORCE NANOSCIENCES, UV/Ozone ProCleaner™ 220, U.S.A). PDMS and glass slides were placed in the UV/OZONE procleaner and 5 minutes UV light and OZONE gas were given, then PDMS and slide were stuck each other. Then it was placed on the hotplate which was set up at 100º C. Device was waited 10' on the hotplate.

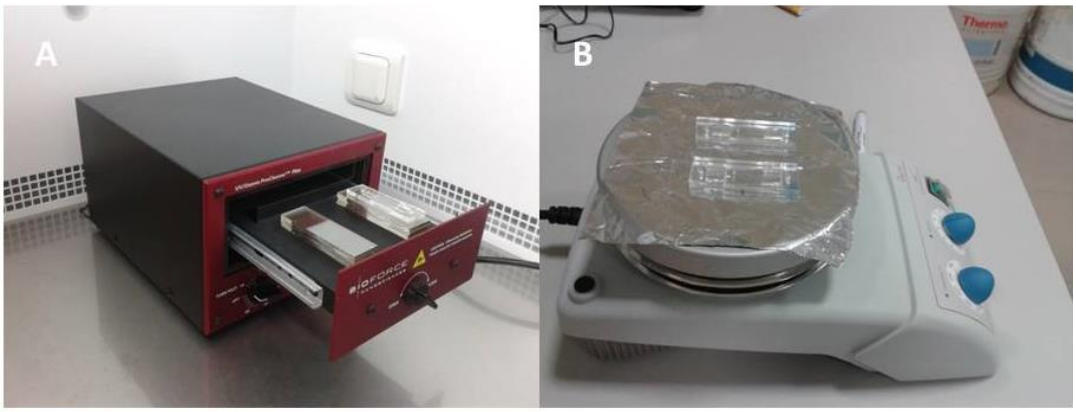

Figure 2.11. A PDMS mold and slide in UV/ OZON and B microfluidic devices on the hotplate

At last, UP and EtOH were injected in microfluidic devices and removed 2 times. Then, these microfluidic devices were placed in the laminar flow cabinet in order to expose UV light for 30 minutes.

2.3. Cell Based Experiments

MDA - MB - 231 breast cancer cells were bought from ATCC. These cancer cells were sheltered in liquid N_2 tank. Cells were cultivated in the MDA-MB-231 complete medium (DMEM with %10 FBS). Breast cancer cells were grown up to 80% confluency, and then they were passaged and prepared for the further experiments in laminar flow cabinet. Before the experiment, matrigel was taken out from -80^{o}C to 4^{o}C in ice previous day.

On the day of experiment first matrigel was diluted with MDA-MB-231 DMEM at a 1:1 or 3:1 volume ratio. Secondly, cancer cells ($3x10^6$ cells in 1 ml MDA-MB-231-DMEM) were mixed with matrigel at a 1:1 or 3:1 volume ratio. All work was performed on the ice.

Gelatin (G2500-100G) and agarose was used as a barrier under the matrigel. Gelatin was mixed with PBS (1x, phosphate, buffer, saline) and 7, 5 % of gelatin-PBS mixture were obtained. Also 1% of agarose was prepared by using PBS. The 7, 5 % of gelatin mixture was put in incubator (Nüve incubator) at 37° C and it was waited 35$'$ to dissolve but to dissolve of agarose, it was placed at 80° C for 25$'$. In the experiment part,

first, sacrificial material (gelatin, agarose) was injected into the microfluidic device and device was waited on the ice for 10 minutes.

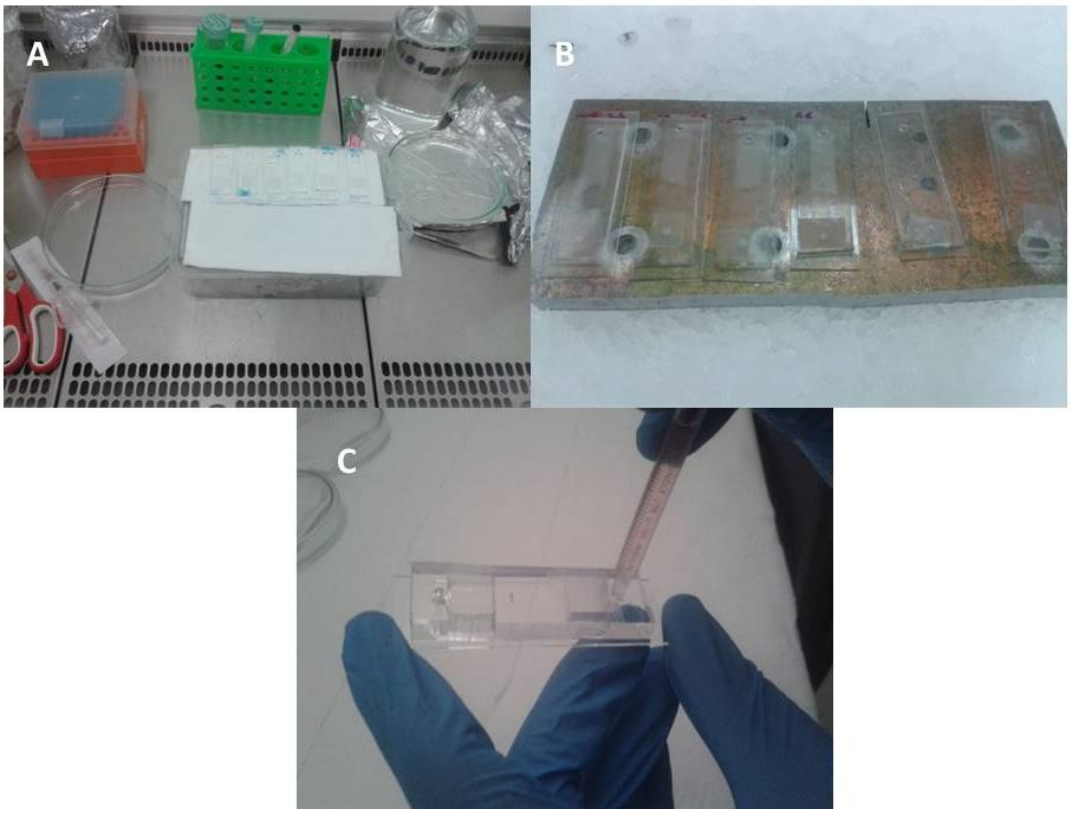

Figure 2.12. A and B gelatin loaded microfluidic devices were placed on the ice to polymerization of sacrificial material C gelatin was loading into the microfluidic device.

After polymerization of sacrificial material, cell free matrigel was injected into the microfluidic devices and devices were placed in the falcon as crosswise. Then falcons were placed in the centrifuged. Centrifuge (Nuve NF400R) was used to fall down cell free matrigel on the sacrificial material barrier. Centrifuged was set up 1000 rpm for 2 minute at 4^{o}C.

Figure 2.13. Devices were centrifuged in the centrifuge.

After centrifuged, falcons were hanged on the centrifuge for 10 minutes. Then, falcons were placed in incubator and hanged on for 35'. After polymerization of cell free matrigel, 231 cell laden matrigel was injected into the devices and centrifuged at 1000 rpm for 1 minute at 4°C. After that falcons were placed in incubator and hanged on for 35' again.

Following step was removing sacrificial material. Gelatin was liquid at 37º C so gelatin was removed easily form inside of devices. Agarose did not dissolve at 37° C so agarose was cut and removed from the large holes. Two side of microfluidic devices were holes so these holes were filled with medium (L15 serum).

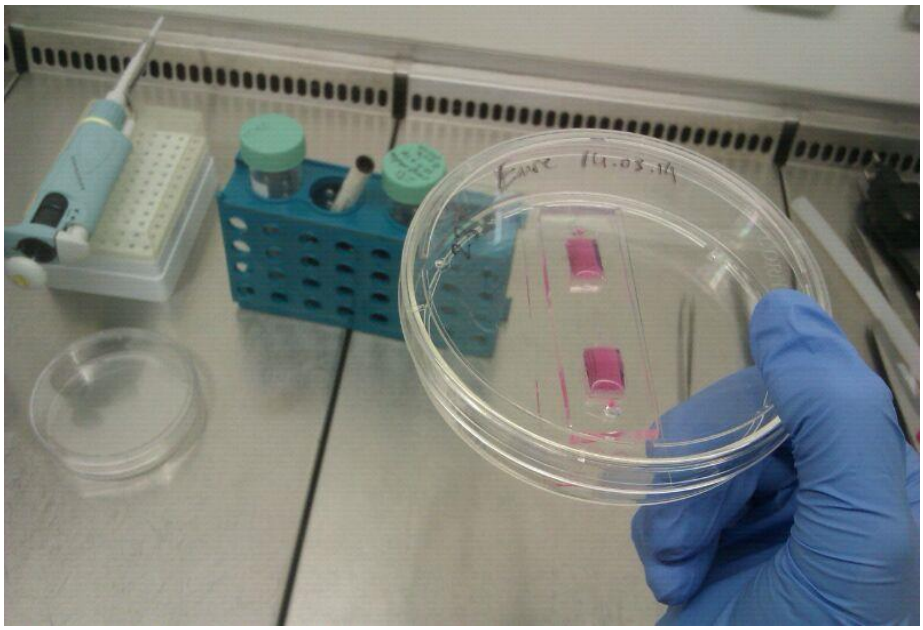

Figure 2.14. Sacrificial material was removed and MBA-MD 231 cell complete medium was filled into the large holes.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Photo Mask and Device Creation

Mask had 3 different channels and channels lengths were 5 cm but widths were variable in the range of 5 mm, 10 mm and 15 mm. Microfluidic channels were punctured from 2 sides and diameters of holes were 3, 5 mm in order to be inlet and outlet, then the middle part of microfluidic channels were punctured and diameter of hole was 1, 5 mm to be inlet for cell laden and cell free matrigel.

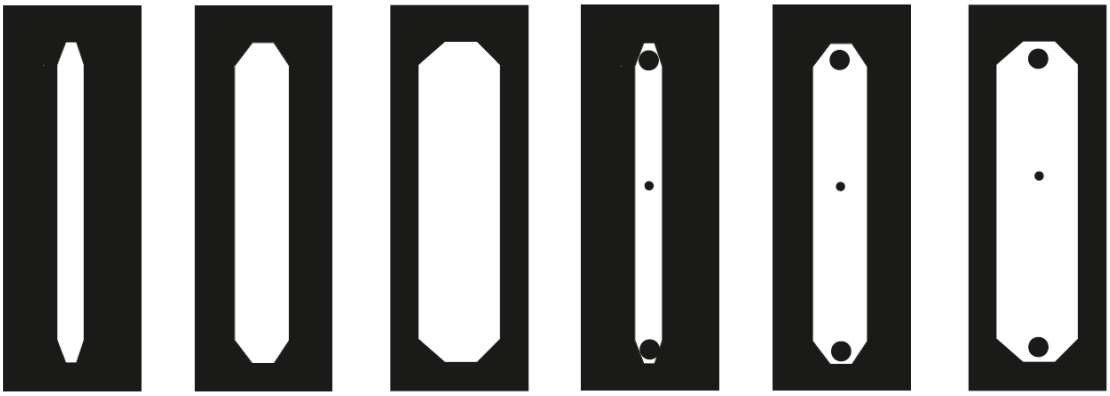

Figure 3.1. The schematic of the mask

First trials were performed to find suitable sacrificial materials with 50%, 25% and 10% of sucrose. Sucrose was tried in order to use as barrier under the cell free matrigel. However, their melting time was too short. Polymerization time of cell free matrigel was normally about 30 minutes in the humidity incubator at 37º C (Soofi, Last, Liliensiek, Nealey, & Murphy, 2009). Melting time of different concentrated sucroses were about 75, 35, 20 seconds for 50%, 25% and 10%; respectively. We tried to use sucrose-gelation combinations and both only gelatin and sucrose at different concentrations, by this approach we calculated their melting time of these mixtures.

At 37℃	Percentage-melting time	Percentage-melting time	Percentage-melting Time
Sucrose	10%- 20"	25%- 35"	50%- 75"
Sucrose-gelatin	2.5%-%7,5%-25'	5%-5% -20'	7.5%-2,5%-16'
Gelatin	10%- 35'	7.5%-30'	5%- 18'

Table 1. Melting time of sucrose, gelatin and their composition was calculated at different percentage.

After all optimization experiments, as seen from the table, sucrose and gelatin combination melting times were not enough to polymerization of cell free matrigel and to be barrier under the cell free matrigel. Most suitable material was seen 10% of gelatin. Its melting time was about 35 minutes and melting time was clearly close with polymerization time of cell free matrigel.

In this study, 10% of gelatin was loaded inside a microfluidic channel and it was left on the ice to polymerize. After that, we tested the gelatin whether it was removed easily or not from the channel. Gelatin melting time and cell free matrigel polymerization time was so close but important thing was removing of gelatin from the microfluidic channel. As a result of this test, we saw that 10% of gelation could not be removed from the microfluidic channel properly. Therefore, we thought, this problem was about the shape of the microfluidic channel. To solve removing problem, 4 holes were punctured from different point on the channel and experiment was repeated by using 10% of gelatin.

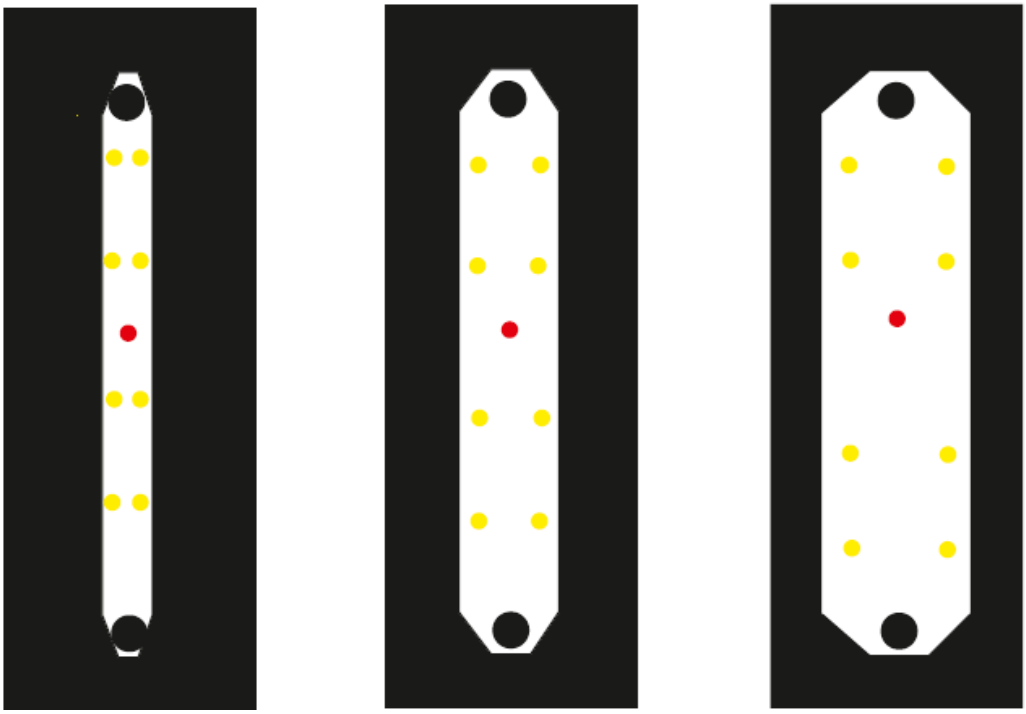

Figure 3.2. The device's width were 5mm, 10mm and 15mm, black holes diameters were about 3,5 mm. Red and yellow holes diameters were about 1,5 mm. Cell free matrigel was loaded form red holes. Loaded gelatin was removed from the yellow holes.

At the end of study, 10% of gelatin could not be removed from the microfluidic devices (figure 4.2). Because of this, shape of device was changed again. Large holes were punctured from the top and bottom of device, and small hole was punctured from at the middle of device, were not changed. Only rectangle holes were punctured instead of 4 holes.

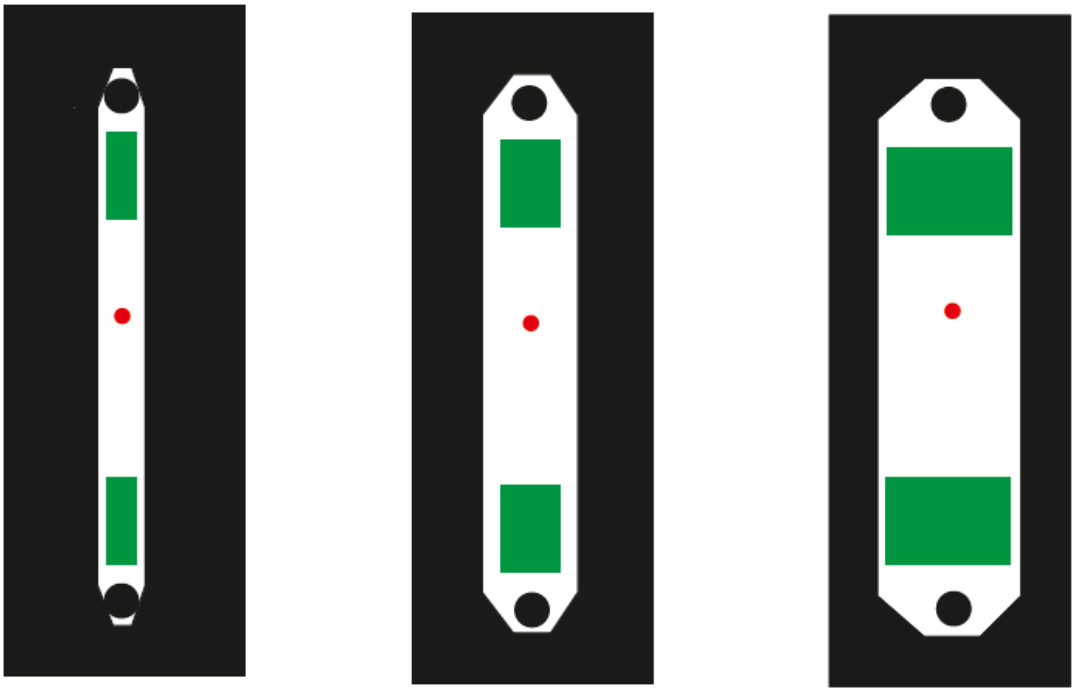

Figure 3.3. The device's width were 5mm, 10mm and 15mm, black hole's diameter were 3,5 mm and red hole's diameter was 1,5 mm. Rectangular holes dimensions was 3mm-9mm, 6mm-9mm and 8mm-9mm.

Experiment was made by using these new devices. At this time, 10% of gelatin could not be removed again because its viscosity was too much. That's why we decided to use 7, 5% of gelatin. Experiment was repeated again by using new devices and 7, 5 % of gelatin.

7, 5 % of gelatin was loaded into the microfluidic channels and devices were left on the ice for 10 minutes to polymerize, and then sample was placed in the incubator to melt gelatin for 30 minutes. After melting of gelatin, it could be removed from inside a microfluidic channel and we optimized the shape and percentage of gelatin to make trouble-free experiment by using microfluidic devices.

After obtained rectangular holes, 1% of low melting agarose was used alternative sacrificial material. Agarose did not melt at 37ºC so other shapes of devices were not suitable to use agarose. Low melting agarose was loaded into the devices like gelatin and devices were left on the ice for the polymerization of agarose. After polymerization of agarose, it was cut and removed. Some part of agarose could not be removed and it was under the cell free matrigel. However; pore size of agarose nearly 650 nm (Narayanan, Xiong, & Liu, 2006) so the medium clearly could penetrate and reach the cells.

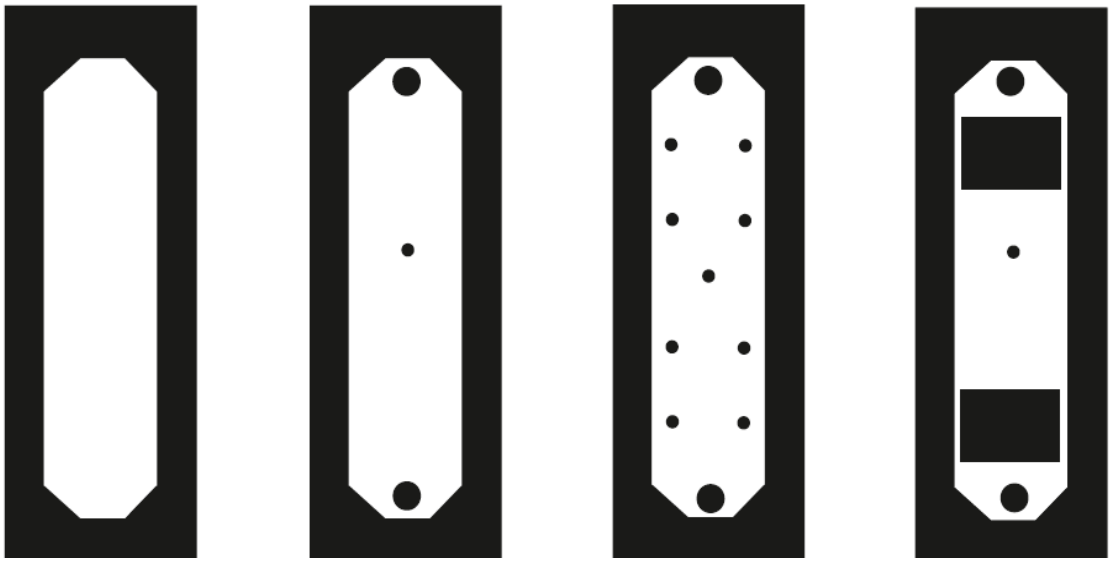

Figure 3.4. The process of the shape of microfluidic devices.

3.2. SU-8 Master Fabrication

In this study, SU-8 height of master was optimized and optimized result was in the height of 340 micrometer. These masters were used in cell based experiments.

Before soft lithography trials, we determined the hot plate temperature set up. Soft lithography depends on the characteristics of photoresist. It was so sensitive to temperature changes, we surveyed hot plate temperature on surface and on screen of the hot plate. We found out appropriate temperatures as 65º C and 95º C on surface of the hot plate and temperatures were used in further experiments. In addition to this, we checked the uniformity of the temperature distribution on the surface of the hot plate.

In the first day, silicon wafer was placed on the hot plate for 5 min which was set up at 65^0 C. Silicon wafer must be clean before use; there must not be any dust on the substrate. Hotplate was set up at 65^0C because silicon wafer had to be hot to spread SU-8 negative photoresist easily and to activate surface of the substrate. Then SU-8 negative photoresist was poured on the substrate and it was spread out by hand homogenously. Good wetting of the substrate by SU-8 negative photoresist was crucial for obtaining homogenous and stable coating. Silicon is the best material for adhesion with SU-8 (Zhang, Du, & Xu, 2013). That's why SU-8 negative photoresist and silicon

wafer were stuck each other strongly. SU-8 master silicon wafer was placed in the spinner and it was waited for 5 minutes to be cooling of SU-8 because SU-8 was too much fluid at 65^0C. Silicon wafer was spun depends on the thickness of SU-8 negative photoresist at different ramp, rpm and dwell.

# of silicon wafer	1	2	3
Spin speed (rpm)	1000	1000	500
Spin time (s)	10	5	20
Soft bake 65^0 C (m)	15	15	15
Soft bake 95^0 C (m)	45	75	90
Expose time (s)	35	50	60
Post bake 65^0 C (m)	5	5	5
Post bake 95^0 C (m)	15	15	15
Develop time (m)	20	20	20
Height (μm)	270	340	620

Table 2. Number of silicon wafer, SU-8 2075 negative photoresist thickness and other properties.

After the coating process, the substrate was soft baked in order to remove the solvent and improve resist–substrate adhesion. After coating, photoresist contains a remaining solvent concentration depending on the photoresist and its thickness. Soft bake plays crucial role in photo-imaging so soft baking temperature and time are also important. The photoresist coating become photosensitive only after soft baking. Over soft baking degrades the photosensitivity of photoresist, under soft baking prevents lights to reach photoresist. Soft bake is also supplied to higher development rate (Zhang, Du, & Xu, 2013).

Soft baking temperature was at 65^0C and 95^0C and its time was depended the thickness of photoresist. After 24 hours, wrinkle test was applied at 95 ºC. The aim of wrinkle test to observe any wrinkle on the substrate or not and to confirm the soft bake was completed. If wrinkle test was not applied on the substrate, the SU-8 master mold could be destroyed or undulating surface could be seen after soft bake part.

After wrinkle test, the mask was placed on the SU-8 coating silicon wafer. The mask and substrate had to be physical contact with each other because we applied

contact printing at the exposure part. In the contact printing, UV light was only touch the open surface of the substrate so this system was properly supplied to very high resolution. After that, UV light was exposed on the substrate to transferred pattern onto the substrate. UV light wavelength was 350- 400 nm and its energy was 400 watts. The most important thing was exposure time at the exposure part. There were 3 different exposure styles which are under expose, over expose and optimal expose.

We needed to over exposure while transferring pattern on the substrate so exposure time was much more than optimal expose time. The reason was that coating substrate and PDMS were split from each other easily and PDMS was not damaged during the separation process. However too much over exposure caused to prevent the solubility of SU-8 in the developer so some patterns could not be opened after development. That's why exposure time was so important and it was depended on thickness of the photoresist.

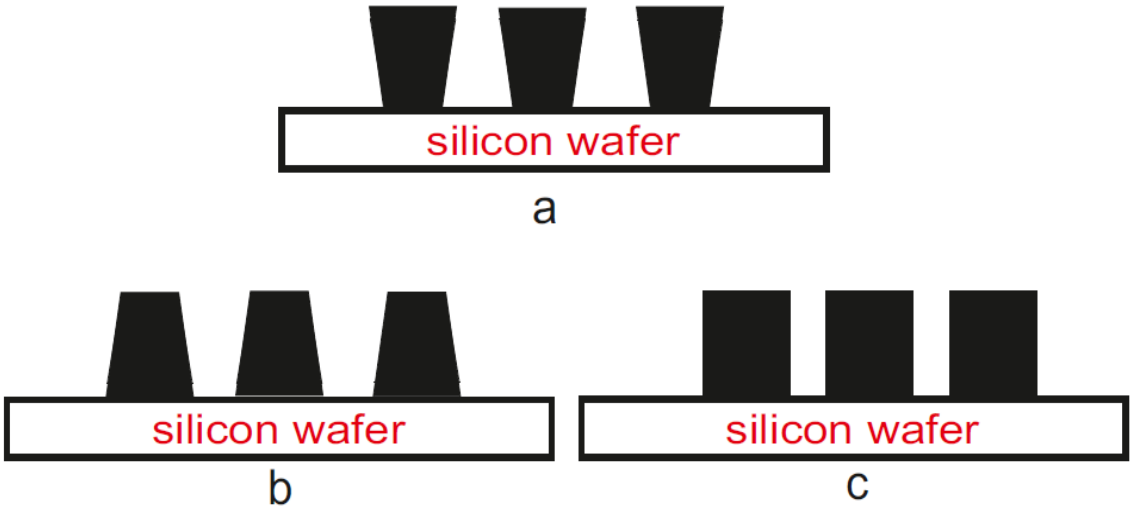

Figure 3.5. Figure a was related to the under expose which means expose time was not enough to create crosslinks in the photoresist. Figure b demonstrated the over expose of substrate and crosslinks were much more in the deep side. Figure c demonstrated optimal expose, deep side and surface of photoresist were getting equal UV light.

After exposure part was completed, substrate was placed on the bench for 5 minutes to relaxation of photoresist then it was placed on the hot plate at 65º C for the post exposure bake for 3 - 5 minutes, then temperature was increased to 95º C for 9 – 15 min. The post exposure bake time was depended on the thickness of the photoresist. The

post exposure bake part was applied to increase the number of crosslinks, remove solvent and adhesion of the photoresist to silicon wafer and to obtain strong photoresist (Microchem, inc. 2013). Post exposure bake time must not be too much, if it was larger than 15 minutes, the photoresist would not dissolve in the developer. At the end of post exposure bake, hot plate was turned off and left the substrate to cool down to room temperature slowly.

In development part, SU-8 coated silicon wafer was placed in the petri dish which was filled with developer. It was kept in developer solution for 5 min in order to penetrate developer solution in to the photoresist. Afterwards petri dish was shaken for 15 minutes. During this time, the non-exposed area was dissolving and the patterns appeared at the end of development step. Temperature of developer solution had to be equal to room temperature therefore before development step, developer solution was kept on the bench for half an hour. If it was not at room temperature, sudden cracks would be seen on the pattern. Duration of development step has also a crucial role. If development time was less than 20 min, non-exposed area did not dissolve clearly so there were seen photoresist particles on the SU-8 master. If development time was higher than 20 min, photoresist would separate from silicon wafer. After all, IP (isopropanol) test was applied to determine end of developer step. White cloud or precipitation indicated that development step was not concluded. If white cloud was seen while doing IP test, the development step was repeated. If there were not observed any white cloud, development step could be finished. After development step, SU-8 masters were rinsed with fresh developer in order to discard any SU-8 residuals, and then substrate was washed with isopropanol in order to inactivate developer solution.

3.3. PDMS Molding

Polydimethylsiloxane base and curing agent were mixed at a 10:1 ratio. If the ratio of PDMS and curing agent was 5:1 weight ratio, it would be stiff. During the cutting and drilling of PDMS, there could be seen crack on the PDMS mold. Therefore PDMS mold was not useful in our experiment. PDMS and curing agent were mixed by using spoon and mixture was placed in the vacuum chamber to degas bubbles. If there were any bubbles in the mixture, small holes would be seen in the PDMS mold after polymerization. The mixture was poured on the substrate and kept on room temperature

for 2 days. To keep on room temperature of mixture and substrate was not only prevented SU-8 master mold but also prevented damage to the PDMS mold.

Fabricated microfluidic devices were well sterilized to prevent any contamination that could be precluded biological application. PDMS molds were cleaned with scotch tape first, then EtOH and UP water to remove dust and prevent contamination by using sonicator. Slides were also cleaned with methanol and UP water.

PDMS molds and slights were placed under the UV-light and ozone gas in the UV-OZONE proclenear to generate surface activation of PDMS molds and slights. Therefore, they were stuck each other easily and their surfaces became hydrophilic which supply the leakage of hydrogels during the loaded. After sticking of PDMS molds and slights, devices were placed on the hot plate at 100º C to be permanent bonding. Then fabricated devices were washed with 70% EtOH and UP water twice then they were placed under the UV-light for half an hour. After exposing UV-light, they were located in the oven in order to them hydrophobic again for 24 hours at 80º C (Shin et al., 2012).

3.4. Cell Based Experiment

The cell based experiments aim was to create distance between the MDA-MB-231 breast cancer cells. Fabricated devices were used in these experiments and the cells were placed as a layer inside microfluidic devices. During the experiment all works performed on the ice and centrifuge was set up at +4º C.

Matrigel was normally stocked at -80º C. Before starting the experiment, matrigel was kept on at +4º C overnight with in ice bucket to become liquid. Liquid matrigel was diluted with MDA-MB-231 complete medium to decrease the stiffness of matrigel before loading. Cells and cell free matrigel were mixed at volume ratio 1:1 or 3:1 because cells were in the complete medium. Therefore cell laden and cell free matrigel's stiffness were equal.

In experimental part, first, rectangular holes were closed down with scotch tape to prevent the escape of sacrificial material outside. If sacrificial material escaped outside, there would not be a barrier for the matrigel. Sacrificial material was loaded in the channel and devices were hanged on ice to polymerization of sacrificial material.

After polymerization of sacrificial material, cell free matrigel was loaded and devices were placed in the falcons transversally-(figure 4.7) because, if devices were not placed into the falcons transversally, there would not be a gap between PDMS mold and matrigel in figure 4.6.

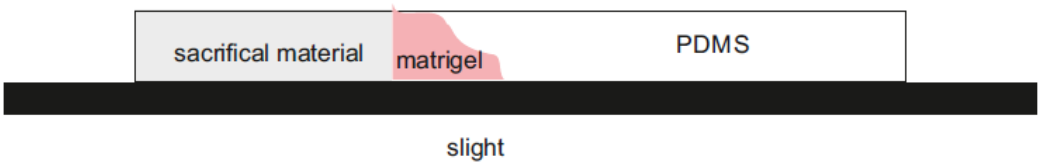

Figure 3.6. The gap between matrigel and PDMS mold.

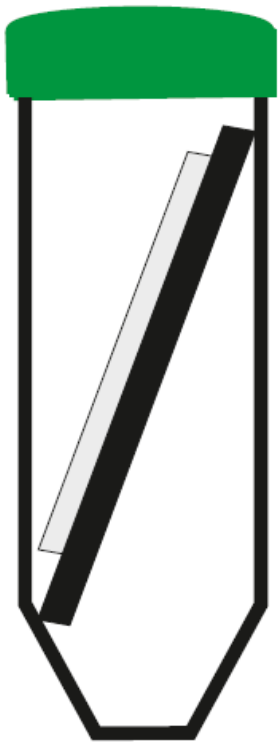

Figure 3.7. Device was placed as crosswire into the falcon.

Falcons were spun by using centrifuge. For the cell free matrigel, the optimal rpm and time were 1000 rpm and 2 times. Otherwise matrigel penetrated sacrificial material barrier or it did not fall down on the sacrificial material. Although devices were placed as crosswire into the falcons, there were possibilities to be gap PDMS mold surface and matrigel. To reduce this problem, devices were kept waiting in the

centrifuge for 10 min. Cell laden matrigel was centrifuged at 1000 rpm but its time was 1 min and 1 min was enough in order to fall down cells on the cell free matrigel. When cell laden matrigel was centrifuged at 1000 rpm for 2 min, penetration would be seen. Cells penetrated into the cell free matrigel and layer was not created.

The matrigel was polymerized for 30 minutes in a humidity incubator at 37º C (Soofi et al., 2009).Polymerization of cell free matrigel and cell laden matrigel were higher than 30 minutes. Because they were diluted with MDA-MB-231 DMEM and our devices were kept on incubator (37º C) for 35'. The reason was that cell laden and cell free matrigels were in the devices and devices were in the falcons. Matrigel had to be well polymerized in order to prevent cell penetration while centrifugation of cell laden matrigel. And also, polymerized matrigel was supplied to physiological environment for the cells.

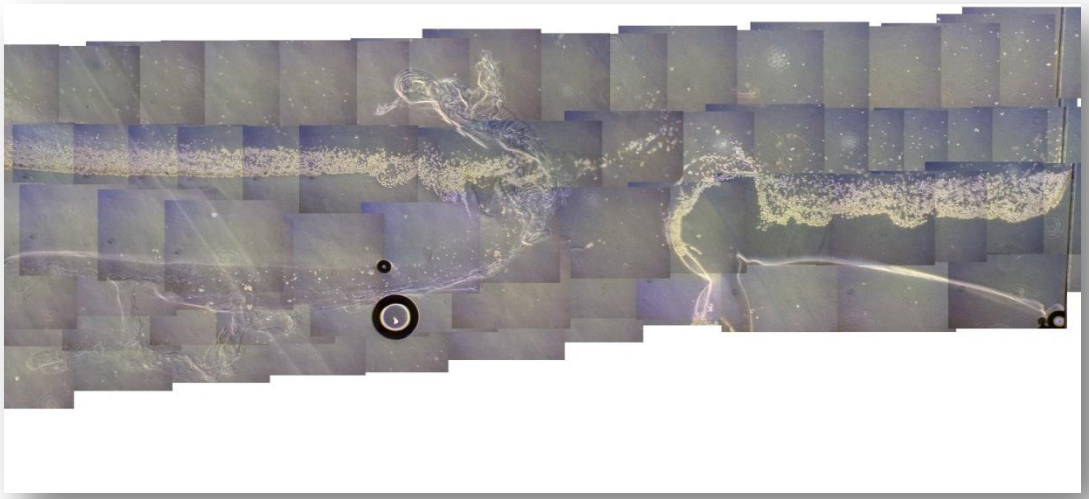

Figure 3.8. A single cell layer into the microfluidic device. 7, 5 % of gelatin was used as a sacrificial material. $3x10^6$ breast cancer cells were used. 1st day photo. This picture was obtained under the microscope.

Figure 3.8 shows the loaded cell laden and cell free matrigel into the microfluidic device. There was a problem with the cell free matrigel because it was not polymerized so the middle part of cell free matrigel was disrupted. That's why, some of cells precipitate to the bottom of the device. Left side of device was useful to analyze the cell migration.

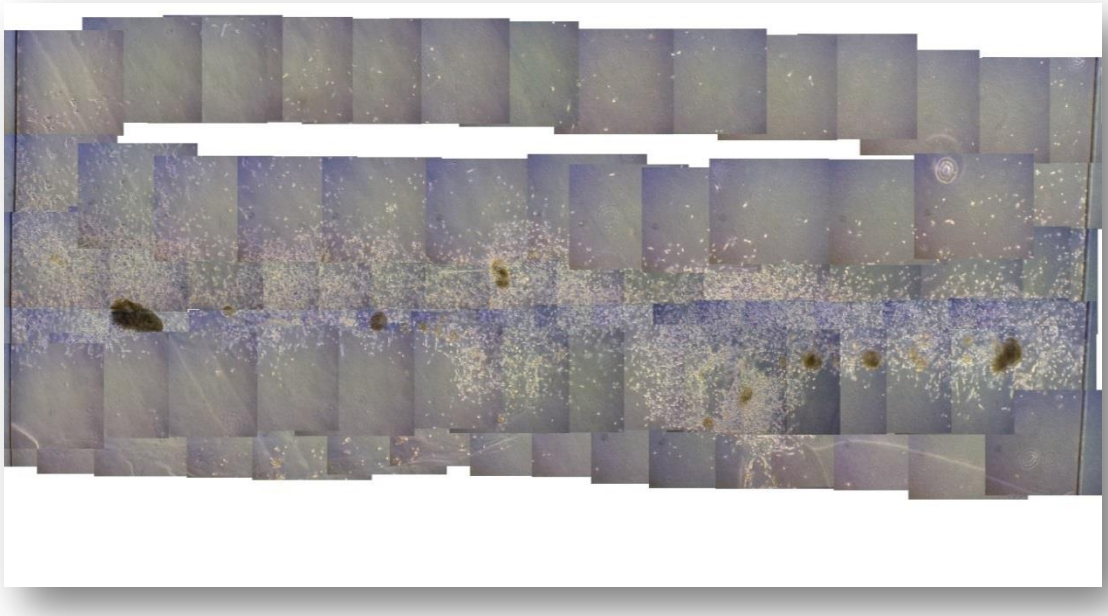

Figure 3.9. A single cell layer was created into the microfluidic device. 7, 5 % of gelatin was used as a sacrificial material. $3x10^6$ breast cancer cells were used. 7^{th} day photo. This picture was obtained under the microscope.

Figure 3.9 is related to 7^{th} day. The cell migration is observed in this figure. Cells stuck in the device and they grew up.

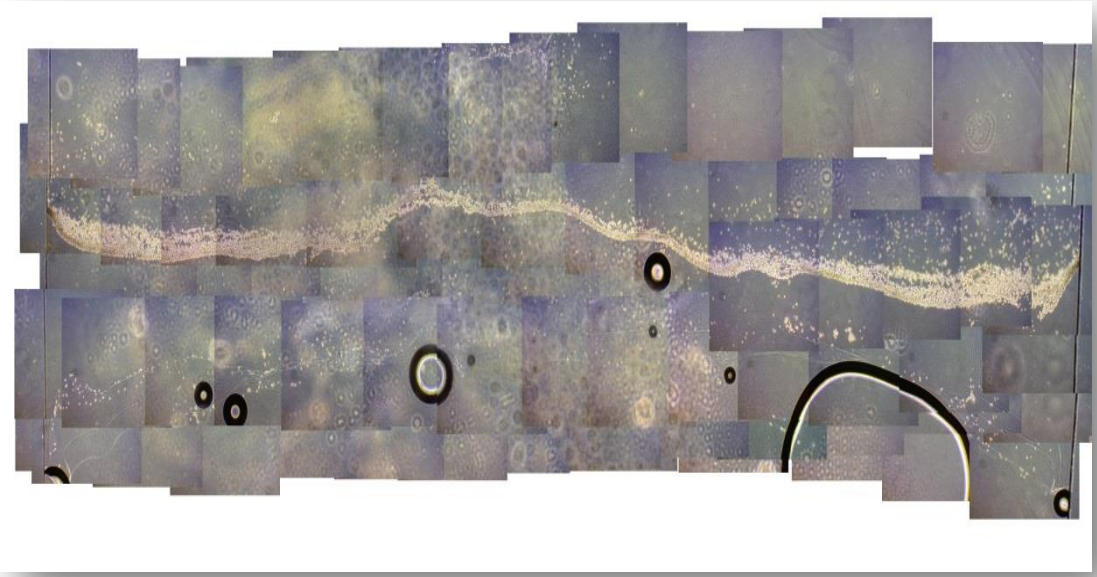

Figure 3.10. A single cell layer was created into the microfluidic device. 7, 5 % of gelatin was used as a sacrificial material. $3x10^6$ breast cancer cells were used.1^{st} day photo. This picture was obtained under the microscope.

Figure 3.10 shows us the single breast cancer cells layer. There was no deformation in the layer. Cell free matrigel surface was a wavy so the cells were not flat as seen. The wavy surface reasons could be polymerization of cell free matrigel while loading it.

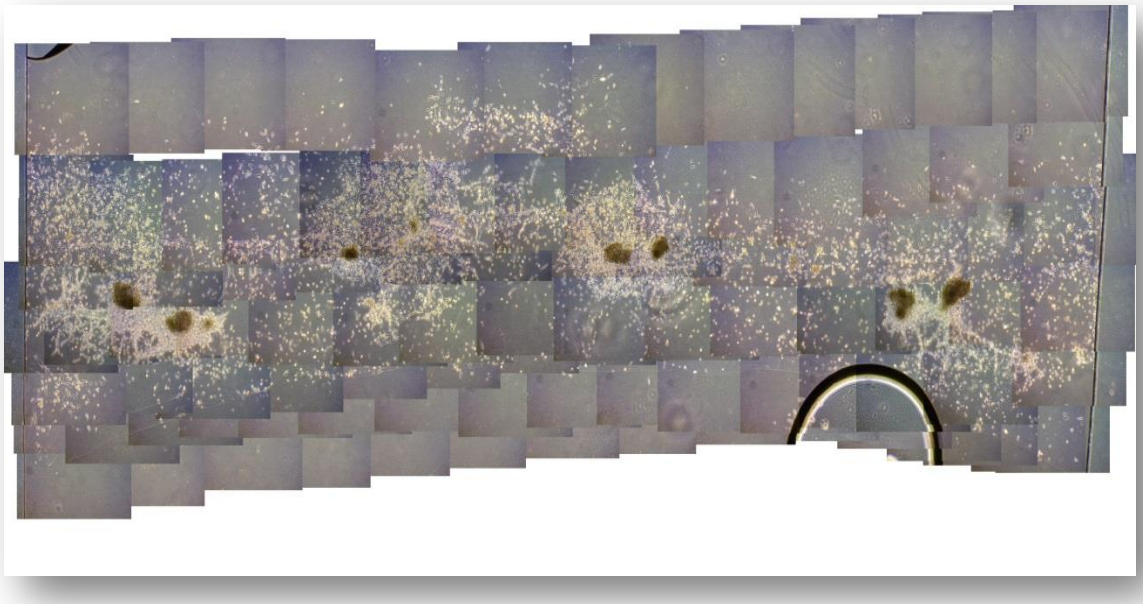

Figure 3.11. A single cell layer into the microfluidic device. 7, 5 % of gelatin was used as a sacrificial material. $3x10^6$ breast cancer cells were used. 7^{th} day photo. This picture was obtained under the microscope.

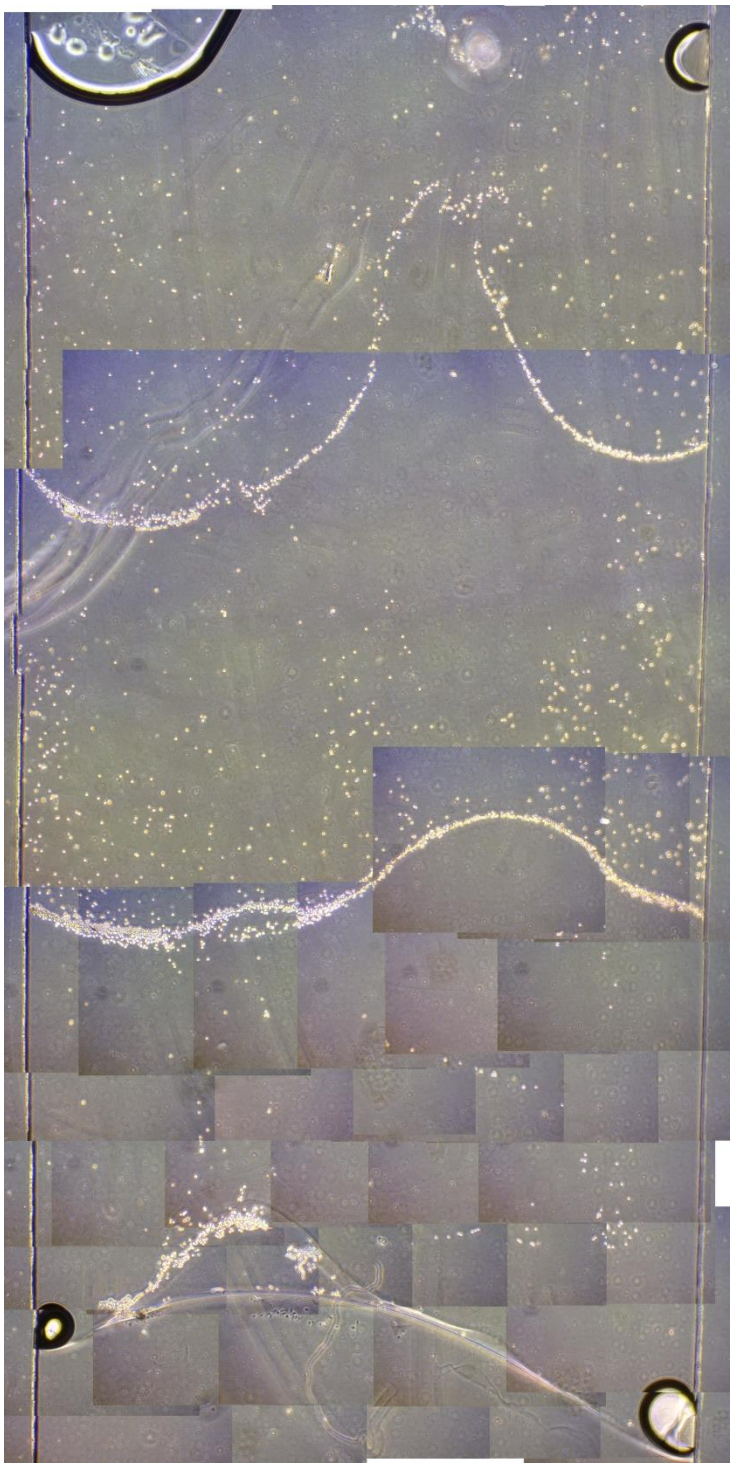

Figure 3.12. Double cell layers were created into the microfluidic channel. $6x10^6$ breast cancer cells were examined. This result was obtained in the 1st day.

Figure 3.12 shows us double cell layers. In this experiment 1% of low melting agarose was used as a sacrificial material. Some of cells were penetrate in cell free matrigel layer and fallen down on the agarose. The reason could be every point of matrigel was not polymerized. As seen the picture layers had a peaks. The reason of

these peaks that matrigel was loaded at the middle part of microfluidic channel and centrifuged. After centrifuged cell free and cell laden matrigel, device was left in the incubator to polymerization. While cell free or cell laden matrigel polymerize, cells returns to their loading position.

The distance between the cell layers are changing between 2, 5 and 2, 7 mm.

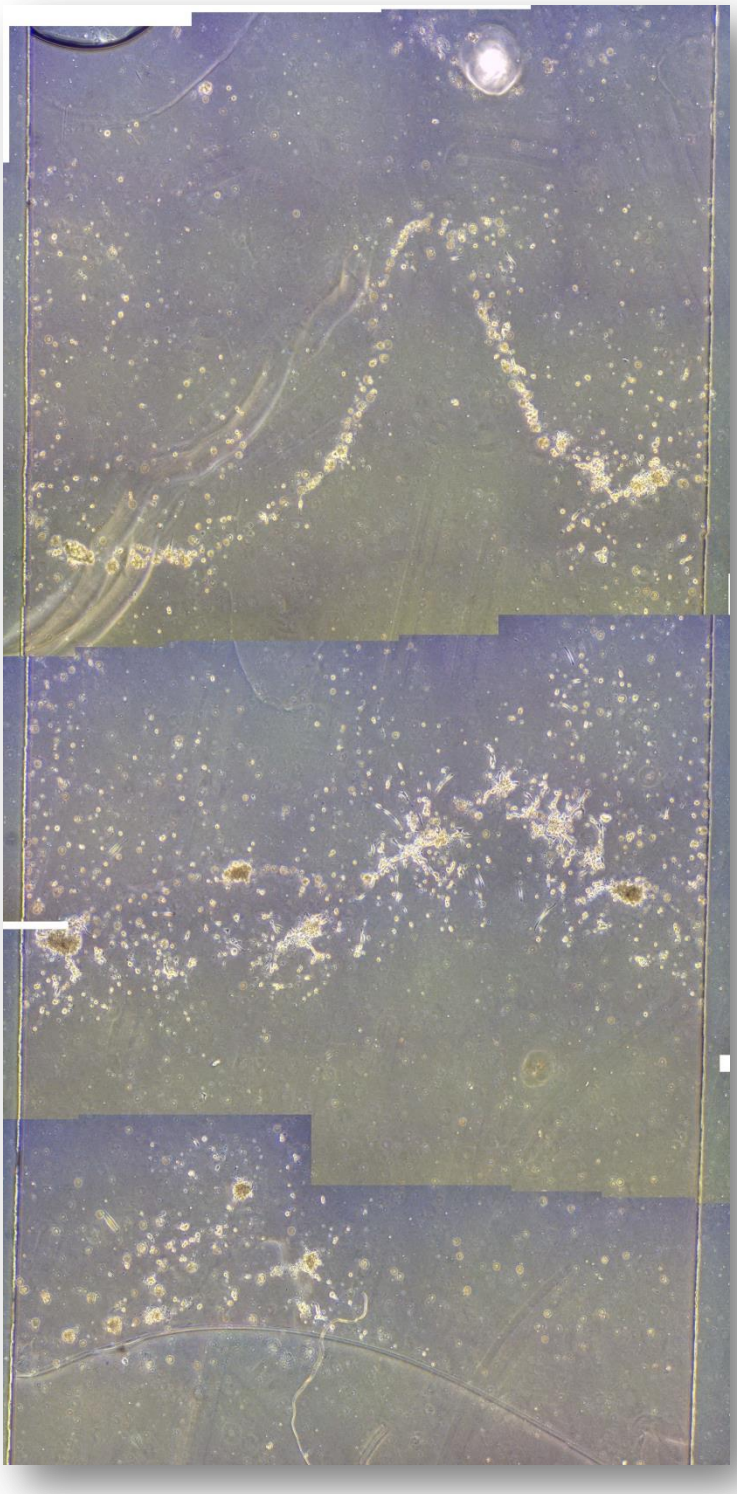

Figure 3.13. 7th day of double cell layer experiment.

As seen from the 7th day of experiment, cells stuck PDMS and glass and also each other. They collected some point of devices (dark area). In the matrigel, breast cancer cells want to collect and hang on each other. If collagen was used in the

experiment instead of matrigel, breast cancer cells were extended into the collagen. They touch every point of device.

CHAPTER 4

CONCULUSION

In this thesis, microfluidic devices were fabricated by using photo- lithography and soft- lithography methods. Fabricated devices were used in the cell- base experiment.

In the photolithography method, first hot plate temperature was optimized and the optimal temperature was found as 65º C and 95 º C. After that, soft bake time, post bake time, spin time, ramp, dwell, rpm, exposure time and developer time were optimized. To obtain 340 μm height of photoresist thickness; we used our optimized parameters which are soft bake time: 15' at 65º C, 45' at 95º C; post bake time: 5' at 65º C, 15' at 95º C; spin time: 5'' at 1000 rpm; exposure time: 50'' and developer time: 20'.

In the soft lithography method, PDMS and curing agent were mixed at 10:1 ratio then mixture was poured on the substrate and it was left on the bench at room temperature for the polymerization of PDMS.

In the cell-based experiment, first, sacrificial materials were found. Many materials were examined in the microfluidic channel. Lastly, 7, 5 % of gelatin and 1 % of low melting agarose was performed as a sacrificial material. The next step was optimization of shape of microfluidic device. Important point was removing sacrificial material from the microfluidic device. That's why; many times device was punctured at different point to easily remove it. At the end of optimization, some part of PDMS was cut from the top and bottom of channel so the rectangular holes were created. These holes were used to remove sacrificial material and load medium.

In the experiment $3x10^6$ breast cancer cells were used. To analyze and observation of cells migration, this cell numbers was clearly enough. Matrigel was diluted with DMEM because cell free and cell laden matrigel stiffness had to be equal. The reason was that breast cancer cells were in complete medium (DMEM) and complete medium and matrigel were mixed to supply physiological content. Therefore, the stiffness of cell laden matrigel was less than pure matrigel.

By using centrifugational positioning process, cells were participated on the sacrificial material and cell free matrigel. For participating cell free matrigel on the

sacrificial material, device was centrifuged at 1000 rpm for 2'. For the cell laden matrigel, rpm was the same but time was decreased for 1' to prevent penetration of cells. After applying this process, cells layer was created into the single microfluidic channel. The distance between the cell layers nearly 2, 6 mm but the distance gets decreased. If channel width or channel height is increased, the distance decreased or if cell laden matrigel volume is decreased, the distance also decreases.

In this work, we developed easy method which supplies to create distance between the cell layers. By using centrufagational positioning process, experimental set up get easier than the other methods. In the other methods, there are many posts and channels in the architecture of microfluidic devices. It is difficult to performed fabrication process and experimental method with channels and posts. Our device contains single channel and any posts.

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