THE REPUBLIC OF TURKEY

BAHCESEHIR UNIVERSITY

DEVELOPMENT AND TESTING OF A PROTOCOL FOR CREATING LIVE CELL ARRAYS TOWARDS MEASURING FUNCTIONAL CELL RESPONSE AND CELL-CELL INTERACTIONS IN HIGH-THROUGHPUT

Master's Thesis

RANDA SABOUNI

İSTANBUL, 2018

THE REPUBLIC OF TURKEY
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GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
BIOENGINEERING

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ABSTRACT

DEVELOPMENT AND TESTING OF A PROTOCOL FOR CREATING LIVE CELL ARRAYS TOWARDS MEASURING FUNCTIONAL CELL RESPONSE AND CELL-CELL INTERACTIONS IN HIGH- THROUGHPUT

Randa Sabouni

Bioengineering

Thesis Supervisor: Assist. Prof. Ayca YALCIN OZKUMUR

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Testing the effect of the environment and the effect of different antibodies and titers on the cells is a required technique to discover new treatments for many diseases. Many of the therapeutic strategies need the integration of more than one element, such as the integration of antibody with specific type of cells. Identification of such antibodies has been accomplished with the cell-based screening assays which has many limitations. To overcome those limitations, living cell arrays technique has been created. In our work we aimed to develop a technique for producing arrays of live cells on a glass slide that allow for high throughput measurements of cell response and cell-cell interactions. That requires the adhesion of the target cells on the glass slide and forming a confluent monolayer in order to be patterned. In this thesis, we have tested various cell adhesion matrices (ECM gel, Lam, VTN and FN) on silane-functionalized glass slides with two cell lines, HT-29 and HT-1080, towards developing an assay for bi-specific antibody enhanced T-cell attachment to cancer cells. We have created a robust, controlled, and repeatable coating protocol for producing cell-coated slides that can be used for creating live cell arrays for testing functional responses of cells such as migration, proliferation or secretion, and coordinated interactions with other cells.

Keywords: Cell Patterning, Cell-Coated Slides, HT-29 Cell Line, HT-1080 Cell Line, ECM.

ÖZET

FONKSİYONEL HÜCRE TEPKİLERI VE HÜCRELER ARASI ETKİLEŞİMLERİN YÜKSEK KAPASİTELİ ÖLÇÜMÜ İÇİN CANLI HÜCRE DİZİLERİ OLUŞTURMA PROTOKOLLERİNİN GELİŞTİRİLMESİ

Randa Sabouni

Biyomühendislik

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Hücrelerde çevrenin, farklı antikor ve titrelerin etkisini test etme birçok hastalık için yeni tedavilerin keşfinde gerekli tekniktir. Tedavi edici stratejilerin birçoğu birden fazla unsurun birleşimine ihtiyaç duyar; mesela belirli bir hücre tipinin ilgili antikor ile birleşimi gibi. Bu antikorların tanımlanması birçok sınırlaması olan hücre bazlı tarama testleri ile gerçekleştirildi. Bu sınırlamaların üstesinden gelmek için, canlı hücre testleri tekniği geliştirildi. Çalışmamızda biz, hücre-hücre etkileşimi ve hücre yanıtına yüksek veri ölçümü sağlayan, lam üzerindeki canlı hücreleri tahlil etmek için bir teknik geliştirmeyi hedefledik. Bu da, örneklemek için tek katman konflüent kültür oluşturulması ve istenen hücrelerin cam yüzeye tutunmasına ihtiyaç duyar. Bu tezde biz, bispecifik T-cell hücresinin kanser hücresi ile etkileşimini gösteren tahlil geliştirmek için HT-29 ve HT- 1080 iki hücre tipini silan işlevli lam üzerinde çeşitli hücre tutunma şekillerini test ettik. Böylece biz diğer hücrelerin fonsiyonel hücre yanıtları olan hücre göçü, yayılması, sekresyonu ve koordineli etkileşimini test etmek için, canlı hücre dizileri oluşturulmasında kullanılan hücre kaplı lamlar üretiminde tutarlı, kontrol edilebilir, tekrarlanabilir kaplama protokolleri oluşturduk.

Anahtar Kelimeler: Hücre Dizileme, Hücre Kaplı Yüzeyler, HT-29 Hücre Hattı, HT-1080 Hücre Hattı, Hücre Dışı Matris

CONTENTS

FIGURES

ABBREVIATION

APTES	:	(3-Aminopropyl)triethoxysilane
ASC	:	Antibody Secreting Cells
BiTE	:	Bi-specific T-cell Engager
CEA	:	Carcinoembryonic Antigen
Coll	:	Collagen
ECM	:	Extracellular Matrix
FA	:	Focal Adhesion
FAK	:	Focal Adhesion kinase
FN	:	Fibronectin
GAG	:	Glycosaminoglycan
GF	:	Growth Factor
HIV	:	Human Immunodeficiency Virus
HTS	:	High Throughput Screening
Lam	:	Laminin
NWAP	:	Nanowell-assisted Cell Patterning
PDMS	:	Poly(dimethylsiloxane)
PEG	:	Polyethylene Glycol
PEL	:	Polyelectrolyte
PG	:	Proteoglycan
VTN	:	Vitronectin

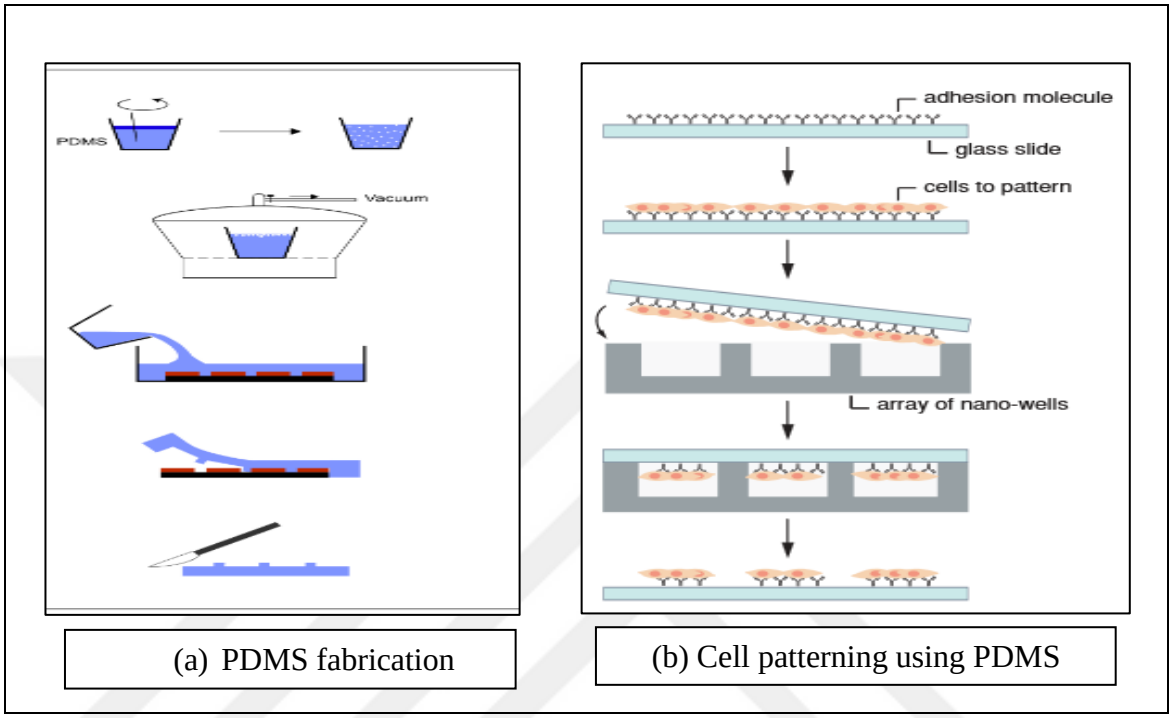
1. INTRODUCTION

In today's world, discovering new drugs for treating many types of diseases became a demand. Powerful techniques and tools are being developed in order to gain insight into the effect of a specific drug on its target and its mechanism in treating the disease in *in vitro* studies. As therapeutics, antibodies play a huge role in treating many diseases such as specific types of cancers or viral illness. Identifying antibodies in drug discovery is a complex procedure; because in many cases antibodies require the integration of other elements for implantation of their functional activity, as example of such antibodies is bi-specific antibody. In order to identify the therapeutic effect of those antibodies, cell-based screening assays and droplet-based screening techniques have been used. They facilitated the identification of many therapeutic drugs despite their limitations such as the complexity and high cost (Ozkumur et al., 2015).

Recently, living cell arrays have been implemented to overcome the limitations of the conventional methods. In this technique, cells are patterned on a substrate, subsequently; measuring functional cell response and cell-cell interactions can be measured in high-throughput, thus the effect of a specific drug such as antibody on the cells can be detected and controlled (Ozkumur et al., 2015).

In this work, we use soft-lithography-based stenciling to pattern the target cells on glass slides. This technique can be summarized by creating a confluent monolayer of the target cells on a pre-treated glass slide, then bringing the cell-coated slide in contact with an array of nanoliter size-well created out of poly(dimethyl siloxane) (PDMS). This physical contact disrupts the target cells in the contacting area on the glass slide, and removal of disrupted cells through washing reveals a living cell microarray (Figure 1.1). This technique will allow us to observe the functional activity such as migration or proliferation of patterned cells under different conditions such as before and after introduction of a stimulus (e.g. therapeutics) to investigate its effect on the cells.

Figure 1.1: Breif illustration of steps of PDMS fabrication and patterning using PDMS.



Source: (Ozkumur et al., 2015, Théry & Piel, 2009).

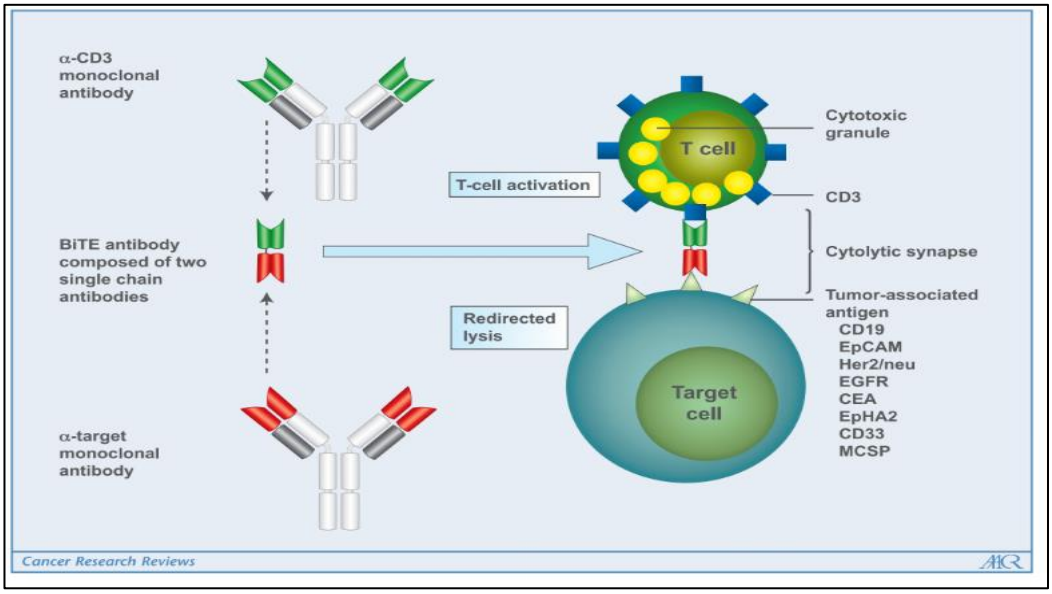
In my thesis, I focused on producing confluent monolayer of different target cells on glass slides to create living cell arrays. Within the scope of my thesis I have tested various cell adhesion matrices and I optimized a robust, controlled, and repeatable protocol for creating a uniformly distributed layer of live cells on a glass slide. This optimized protocol will be used in future studies for measuring variety of functional responses and cell-cell interactions in high-throughput. To optimize the protocol I have tested four types of extracellular matrices (ECMs) (ECM gel, vitronectin, fibronectin and laminin) with two cell lines (HT-29 and HT-1080) and determined the optimal ECM type and concentration for enhanced cell attachment while maintaining cell viability for each cell line.

The two cell lines that were chosen, colorectal adenocarcinoma (HT-29) and fibrosarcoma (HT-1080), correspond to the target and the control cells respectively, for a cell-cell interaction we are interested in monitoring in array format. The target cell

line HT-29 expresses a specific receptor, carcinoembryonic antigen (CEA), on its surface, whereas the control cell line HT-1080 does not. This protein has many functions in cells such as enhancing the adherence of the cells on the ECM. In our application of interest, bi-specific antibody directed T-cell binding and cell killing in cancer therapy, it plays a role as a receptor for the bi-specific antibody (Figure 1.2) (Rezik et al., 2018, Oberst et al., 2014).

Bi-specific antibodies are manufactured fusion proteins composed of two single chain antibodies produced for treatment of many types of cancers. The treatment mechanism relies on binding the T-cells with the tumor cells to initiate killing action by allowing T-cells to exert their cytotoxic compounds into the tumors. The antibodies called bi-specific T-cell engager (BiTE) antibodies bind CD3 receptors of any type of cytotoxic T-cells with a specific antigen found on cancer cells (Figure 1.2). Recently, two types of bi-specific antibodies have been tested clinically, MT110 and MT103. More than 10 types of antigens can be recognized and bound to those antibodies including CEA which is expressed in our target HT-29 cells (Baeuerle & Reinhardt, 2009).

Figure 1.2: Illusturation of BiTE function to direct killing of the tumor cells.



Source: (Baeuerle & Reinhardt, 2009).

Our proposed patterning protocol can be used in an assay to detect bi-specific T-cell engager (BITE) directed killing of cancer cells by T-cells. Once tumor cells are patterned with the developed protocol in an array format, functional responses such as migration, proliferation, and secretion can be tested under varied conditions. After the introduction of BiTE antibodies, the engagement of T-cells and the BiTE antibody directed killing of cancer cells can be detected. Our patterning protocol developed within this project ultimately allows for isolating each element of the array and measuring coordinated events of cell-cell interactions at each isolated element. This aspect suggests that the outcomes of this study may be useful in future development of a platform that allows for testing the effect of the environment and the effect of different antibodies and titers at each isolated element of the array, and may even enable testing of different antibodies produced from individual cells in engineered cell lines.

2. LITERATURE REVIEW

2.1 LIVING CELL ARRAY

High-throughput cell-based assays have been used in many applications, such as drug discovery, cytotoxicity assays, testing the effect of many stimuli on the cells' behavior. The conventional method for cell-based assays is in well-plate format (usually 96- or 348- well plates) has limitations such as the high cost due to use of high quantities of reagents and lack of quantitative information on cell functions. Living cell arrays have been invented to overcome those limitations. The main advantages of living cell arrays are that they can provide quantitative information on cell functions and have the potential to integrate many elements in one assay. Additionally, they are low cost since they consume reagents in nano-scale and can provide high-throughput assays in short time scales (Fernandes et al., 2009).

Living cell arrays can be defined by positioning cells in specific small locations on a substrate. Creating those arrays typically require functionalizing of the substrate by specific materials such as polymer, antibody or protein in the required locations in order to allow the cells to attach and maintain their viability. Such cell arrays allow us to monitor cells' behaviors in high-throughput screening (HTS). In addition, this technique can be used to create 3D microenvironment for the cells (Fernandes et al., 2009).

Living- cell array techniques can be divided into two main categories (Fernandes et al., 2009):

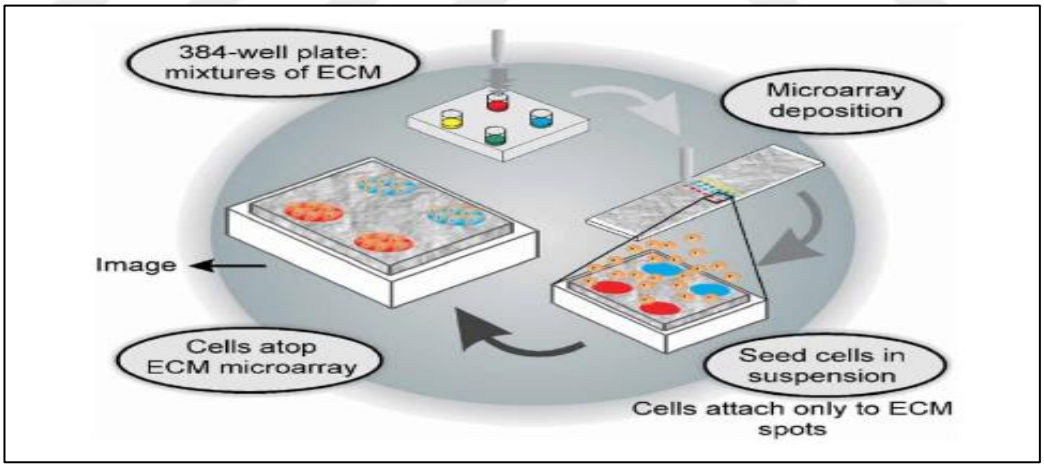
a. Automated spotting for screening applications.
b. Microfabrication for controlling various aspects of the local cell environment.

Automated spotting technique to create cellular microarray is defined as dispensing of the required liquid such as cell suspension or biomaterials in specific location on the solid base using robotic fluid-dispensing devices. Creating this microarray allows the researchers to monitor the cells' behavior toward specific stimuli and their interaction

with specific biomaterials which can be used to screen a library of biomaterials such as antibodies. This technique can be used to create 3D microenvironments for the cells (Fernandes et al., 2009).

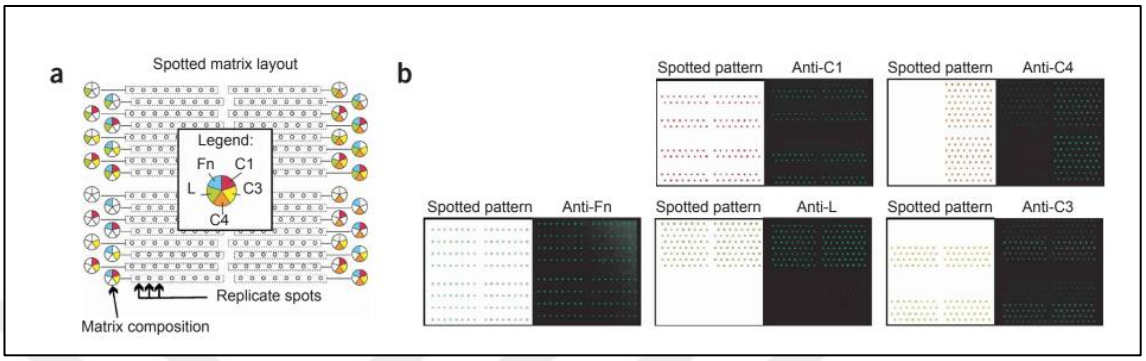
An example of using cellular microarrays in biomaterials research is using a standard DNA microarray hydrogel slide spotted with different types of ECM to study primary rat hepatocytes and mouse embryonic stem cells reactions with 32 combinations of 5 different types of ECMs. Different ECM combinations were spotted using a standard DNA microarray spotter that leads to create islands of the seeded cells on the ECM area since the cells do not attach to acrylimade gel in non-spotted areas. In this study the cell arrays could be maintained for 7 days. After day 7, the destroying of the cell arrays was due to ECM detachment from the substrate not because of the cells' migration, since the non-adhesive acrylimade gel areas prevent the cells from migrating to their sides for more than 28 days (Figure 2.1) (Figure2.2) (Figure 2.3) (Flaim et al., 2005).

Figure 2.1: Schematic illustrates creating cellular microarray to study the effect of ECM on the cells.



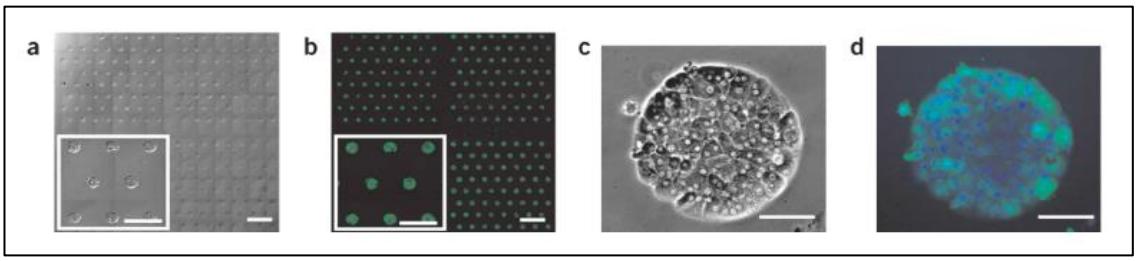
Source: (Flaim et al., 2005).

Figure 2.2: Illustration of ECM spotting and immunofluorescence. (a) Mixtures of 32 different ECMs have been spotted separately and each mixture has been repeated 8 times. (b) Immunofluorescence of the corresponding pattern is shown for each replica.



Source: (Flaim et al., 2005).

Figure 2.3: Cellular microarray of primary rat hepatocyte on different compositions of ECMs. Images were taken by: (a) Hoffman contrast microscope, (b) fluorescence microscope, and high magnification images are shown for one island in (c) and (d).



Source: (Flaim et al., 2005).

Cellular microarrays have many applications in many fields of the research. They can be used in drug discovery since they have superiority over conventional well-plate assays used in the pharmaceutical factories. Also, they can improve drug discovery by facilitating cytotoxicity assays to the drugs under study and they can be used for screening biomolecules. Cellular microarrays have also been used in gene transfection studies where the virus encoded with the gene of interest is spotted on a substrate and resulting infection is monitored upon seeding of mammalian cells on the same substrate.

In stem cell research, cellular microarrays can be used to test the effect of the library of molecules on the stem cells' fate (Fernandes et al., 2009).

Microfabrication is a technique that used to control cellular microenvironment in living cell assays. In this technique, microfabricated cell-based devices are constructed by soft-lithography. Briefly, a photomask is designed by computer-aided design tool and printed, then a photo-lithography is performed by exposing a silicon wafer coated with photo-resist material through the photomask, resulting in creating silicon master mold that holds the photomask design. After that, an elastomeric material such as Polydimethyl-siloxane (PDMS) or Poly(ethelynglycol) (PEG) is casted on the silicon master mold, thermally treated and peeled from the mold to create a flexible and transparent film. Microfabricated cell-based devices can be created with desirable characteristics such as size and shape. They can be used directly as a stamp, as an open microwell or as three-walled microchannel. (Yoshimoto et al. 2013, Fernandes et al. 2009, Yarmush & King 2009).

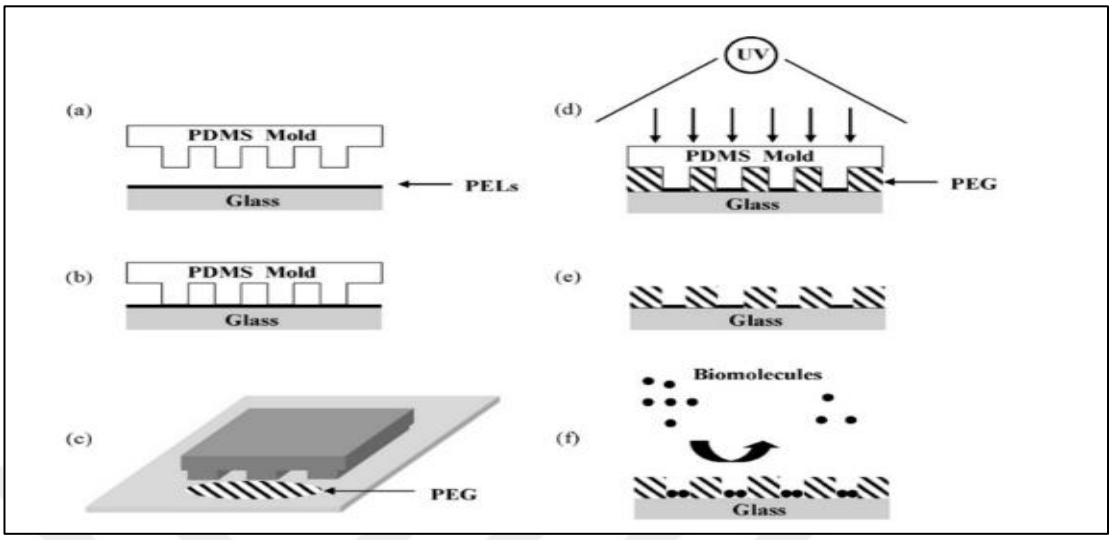
Microfabricated arrays can be created by mechanical patterning, chemical patterning or electromagnetic patterning. Those patterning techniques can be used for studying single cells or multicell colonies for understating the intercellular communications (Fernandes et al. 2009, Yarmush & King 2009, Albrecht et al. 2004, Whitesides et al. 2001).

Mechanical patterning is the technique that relies on creating mechanical barriers to capture the cells. The mechanical barriers are created by using PDMS microstructures as a stencil, fluidic channel or microwell traps. In the mechanical patterning, the cells are located in pre-defined wells, subsequently; they can be quantified by microscopy or by automated image analysis software (Yarmush & King, 2009). An example for mechanical patterning is a method called nanowell-assisted cell patterning (NWAP), in this method a nano-well device that created out of PDMS is used to disrupt the cell monolayer and create a pattern of cells to detect different cellular activities. This method is explained in detail in chapter 3.

Chemical patterning is a technique that relies on creating different adhesive areas, more adhesive area such as FN and less adhesive area such as PEG, in which the cells localize on the adhesive areas and avoid the non-adhesive areas. Coating the substrate with different adhesive molecules can be conducted either by physisorption or by covalent binding strategies that based on self-assembled monolayer chemistry. This technique is achieved by combining the microfabrication and surface modification strategies (Yarmush & King, 2009). Below is an example for creating microwell array based on chemical patterning.

An example of creating microwell arrays for living cells is the fabrication of polyethylene glycol (PEG) with photolithography to create a substrate with two different regions, the first region which is PEG inhibits the attachment of biomolecules such as cells, proteins or bacteria, and the other region is polyelectrolyte (PEL) which enhances the attachment of biomolecules by electrostatic interaction. Creating the microwell arrays is conducted by firstly coating a glass slide with PEL, then a pre-fabricated PDMS mold is brought in contact with the PEL-coated glass and PEG is poured on the PEL-coated glass and allowed to introduce to the microwell by capillary action. After that, the whole assembly is exposed to the UV light to fabricate the PEG to work as a barrier between biomolecules array. Finally, the PDMS mold is removed and the biomolecules are added. In this way a microarray for biomolecules is created by a technique called micromolding in capillaries (MIMIC) (Figure 2.4) (Shim et al., 2007).

Figure 2.4: Microfabrication of PEG by photolithography to create a microarray of biomolecules.



Source: (Shim et al., 2007).

Electromagnatic patterning is a technique that uses electromagnatic forces to manipulate the cells based on their polarizability (dielectrophoresis) or their charge (electrophoresis) resulting in localizing the cells in space and constructing living cell arrays (Yarmush & King, 2009).

2.2 CELL ADHERENCE TO SUBSTRATE THROUGH ECM PROTEINS

The previous chapter has shown the importance of cell adhesion in certain locations to create living cell arrays. In this chapter, the importance of cell adhesion in general for the cells' state in both *in vivo* and *in vitro* are explained. The adhesion material discussed in detail in this chapter is extracellular matrix (ECM). At the end of this chapter, a chemical procedure for enhancing ECM functions in *in vitro* studies is explained.

2.2.1 The Importance of Cell Adhesion in *in vivo* Tissues and *in vitro* Studies

Cell adhesion is defined by the ability of the cells to stick to another cell or to another material. Its role is not limited to mechanical support for the cells, but it also influences and controls most of the cellular activities: it has an effect on cell survival, cell

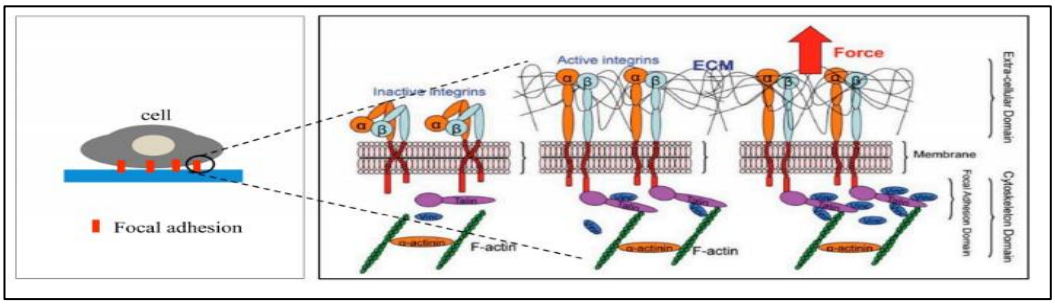
migration, cell cycle and signal transduction that control cell differentiation. All these activities participate in development and maintenance of tissues, which indicates the significance of cell adhesion on the overall multicellular organism (Khalili & Ahmad, 2015).

Since cell adhesion has a huge impact in cells' activities, many of studies need the introducing of ECM to understand the cellular activities more clearly. For example, determination of the effect of a biomechanical factor or any other environmental factor on the cells' response needs attaching the cells on ECM protiens. Most of the mammalian cells in culture need to adhere to their substrate and to each other in order to perform their cellular function properly. Increasing cell attachment to the substrate will increase the bonding between cells' surface receptors and their ligands (Khalili & Ahmad, 2015).

2.2.2 Phases of Cell Adhesion in *in vitro*

The cells undergo a three-stage process in order to attach completely to the substrate. In the first stage, initial attachment will start between the cell and ECM via binding of integrin heterodimer receptor with one pair of mediated adhesion molecule, and this binding initiates other similar bindings resulting in increasing of the number of integrin-mediated adhesion bindings with the time. Increasing in integrin-ligand bindings results in strengthening the adhesion of the cell to ECM, which flattens the cell because the contacting area between the cell and its ECM is increased. The optimal time to allow the cell to attach completely via its integrins to the substrate needs to be examined practically. In the second stage, reorganization of the cytoskeleton proteins will occur since the actin is converted into microfilament bundles. Finally, in the last stage, focal adhesion (FA) molecule will be formed between the cell cytoskeleton and its substrate resulting in a maximized interaction area between the cell and its substrate to its extremity (Figure 2.5). The relationship between the strength of cell adhesion and the length of the time that cells adhere to the substrate is a positive one (Khalili & Ahmad, 2015).

Figure 2.5: : Illustration of the formation of FA between ECM and cell cytoskeleton.



Source: (Khalili & Ahmad, 2015).

2.2.3 Extracellular Matrix

Extra cellular matrices (ECMs) play essential roles in human body, they participate in biochemical and biomechanical properties of the cells which affect morphogenesis, cell differentiation and homeostasis. Also, they play important roles as scaffold proteins. Any mutation alters their genes will lead to severe diseases in human body which reveal their importance for human body (Frantz et al., 2010).

The orientation of ECMs that produced by the cells in the matrix is affected by the orientation of cytoskeleton proteins inside the cells. The production of ECMs in most connective tissues is the responsibility of fibroblasts or fibroblast family cells such as osteoplasts in bone tissues and chondroplasts in cartilage tissues (Alberts et al., 2002).

2.2.4 ECM Molecular Components and Structure

ECM consists mainly of two macromolecules, polysaccharide glycosaminoglycan (GAG) which bind in most cases with core protein forming protoeglycan (PG) and fibrous proteins which are divided into two main categories based on their functions, structural proteins (collagen and elastin) and adhesion proteins (fibronectin, vitronectin, etc.) (Teti, 1992).

2.2.5 Protoeglycans (PG)

Protoeglycan is the main component of ECM and has many important roles that make ECMs work properly. PG is composed mainly of two molecules, glycosamino glycan (GAG) and specific protein core (except hayluronic acid) (Frantz et al., 2010).

2.2.6 Collagen (Coll)

Collagen is the most important ECM; it is secreted by fibroblasts that is found either in stroma or recruited from neighboring tissues. It constitutes the majority of fibrous proteins in ECM and the most abundant protein that reach up to 30 percentage of the total amount of proteins in all multi-cellular animals. Collagen plays many important roles such as providing the tensile and strength to the tissue, regulate cell adhesion, support chemotaxis and migration and direct tissue development. There are 28 types of Coll found in vertebrates. The variation in Coll types occur while synthesis procedure because of enzymatic post-translation modification (Frantz et al., 2010).

2.2.7 Laminin (Lam)

Laminin is one of the adhesive proteins, and contains three polypeptide chains (α, β and γ) that have many domains to interact either with the integrins on cell surfaces or with other ECM components. The three polypeptide chains bind to each other through disulfide bonds forming asymmetric cross shape. It is the main constituent of the basal lamina. Each type of Lam polypeptide chains has many isoforms, and different combinations of those isoforms result in many different types of Lam. Cells attach to the Lam through two different receptors; integrin and dystroglycan (Alberts et al. 2002, Teti 1992).

2.2.8 Vitronectin (VTN)

Vitronectin plays essential roles in the tissues for all multicellular organisms. Its main roles appear in cell adhesion and cell migration. The main primary place of VTN production is in liver, but it can be synthesized also locally by tissues especially by cells involved in migration. VTN binds to the cells through RGD sequence to cell receptors called integrin. VTN is expressed from a single gene with no alternative splicing hence there is only one type of VTN protein (Longo, 1996).

2.2.9 Fibronectin (FN)

Fibronectin is one of the most important ECM components due to the crucial roles it plays in the tissue. It has roles in cell attachment, functions and migration. FN is composed of two homogenous peptides binding to each other via two disulfide bonds. FN interacts with syndecan4 which is a transmembrane receptor to modulate the function of integrin in cell-matrix interaction. FN is coded from a single gene that produces twenty different FNs by alternative splicing of RNA transcription (Frantz et al. 2010, Teti 1992).

2.2.10 Integrin Receptors

The adhesion molecules in ECM bind to the cell surfaces through specific transmembrane receptors called integrins. Integrins are located on cell surface, and link the cytoskeleton in the cytosol of the cell to the ECM macromolecules. This interaction results in inducing an immediate signal transduction and influencing cellular activities. Integrins are a family of heterodimer cell surface receptors that share a general structure in all cells. This structure is composed of two subunits called α and β, and each subunit has three domains: extracellular domain which is the long N-terminal, transmembrane domain and cellular domain which is the short C-terminal. Each integrin binds to specific ECM components. One cell can have more than one integrin to bind to different ECM molecules (Teti, 1992).

In some cases, the cells have more than one integrin on their surfaces which are specialized to bind with only one specific ECM ligand. In other cases, there are some integrins which can bind with more than one ECM ligand. Different ligand regions can be recognized with different integrins which result in stimulating different cell functions. In addition to the diversity in the integrin subunits and in their combinations, the intracellular domain of alpha subunit has varying sequences that can also contribute to activating different intracellular functions (Jouliano & Haskill, 1993).

2.2.11 Roles of ECM in Tissues

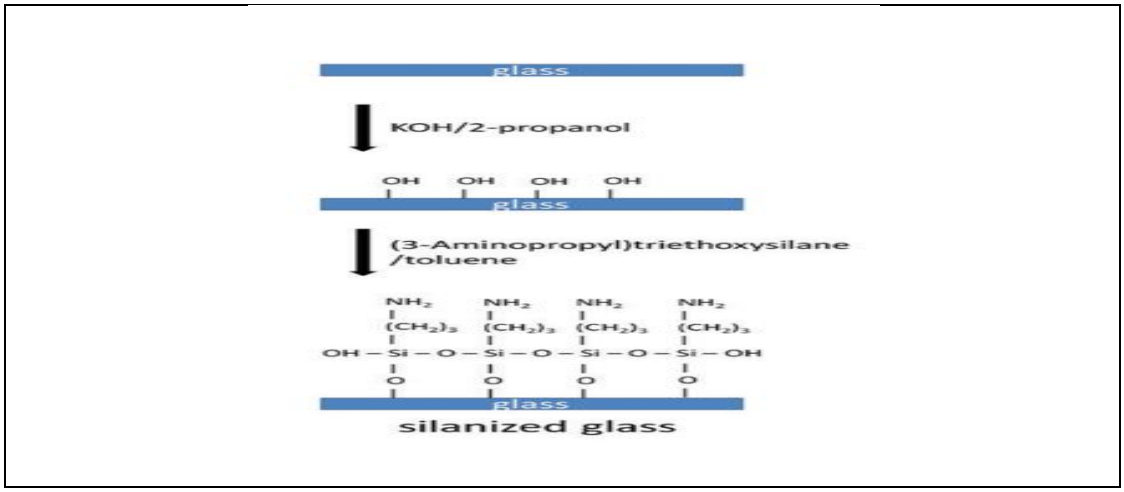
ECMs are more than just inert structures surrounding the cells. They have crucial roles in tissues. They provide distinct mechanical support for all tissues depending on their type and functions (Teti, 1992). They control signal transduction through governing the growth factors (GF) (Frantz et al. 2010, Hynes 2009). They participate in cell migration through releasing the migrated cells from their ECM by ECM degradation (Alberts et al., 2002). They enhance cell proliferation *in vitro* (Gospodarowicz et al., 1980), and they promote cell adhesion through forming FA molecules with intracellular proteins (Teti, 1992).

2.2.12 Role of (3-Aminopropyl)triethoxysilane in Enhancing ECM Coating and Cell Attachment

(3-aminopropyl)triethoxysilane is a chemical reagent that interacts with the hydroxyl group found on the glass surface and introduces amino groups to the glass surface which interact laterally with ECM proteins (Figure 2.6) (Masuda1 et al., 2014).

According to a study (Masuda1 et al., 2014), (3-aminopropyl) triethoxysilane has improved cell culture when the cells were maintained on ECM-silanized glass slide. Silanized glass slides show higher water contact angles compared with non-silanized glass slides. That means the silanized glass has a more hydrophobic surface than non-silanized glass. In the same study, MDCK cells were seeded on both silanized glass coated with laminin and non-silanized glass coated with laminin and observed for three days. Laminin under the cell area on the silanized slide was more stable indicated by a higher fluorescence intensity compared with laminin on non-silanized glass. Also, the results showed that cells on the silanized slide had spread morphologies and high migrating rates while cells on the non-silanized slide had cobblestone-like shapes and low migration rates. Overall, treating the glass slide with a silane reagent before coating the slide with ECM has been shown to improve the stability of ECM and enhance cell culture properties (Masuda1 et al., 2014).

Figure 2.6: Illustration of (3-aminopropyl)triethoxysilane chemistry on glass slide.



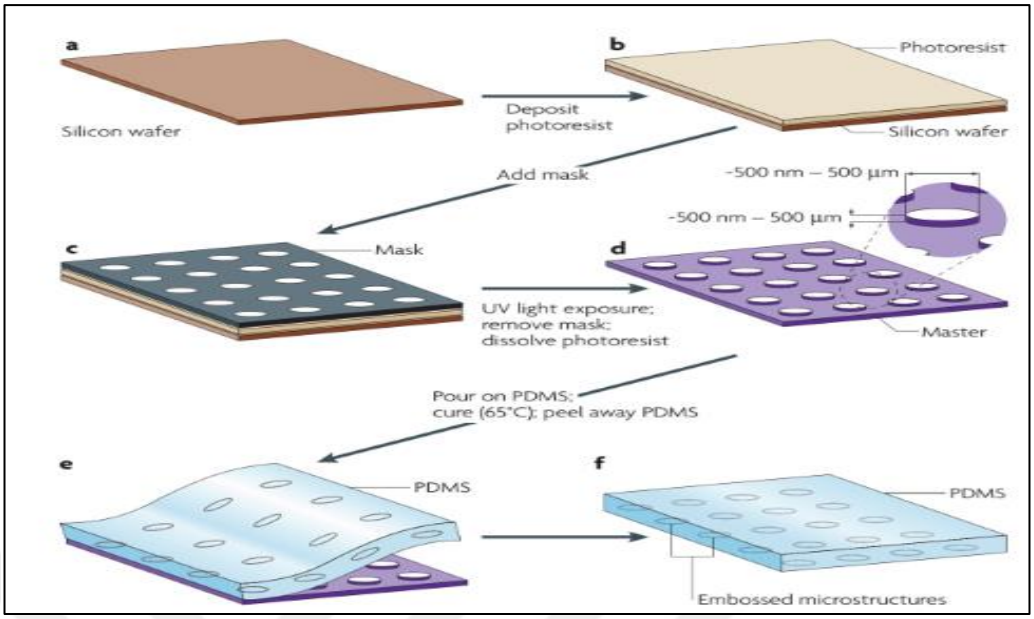
Source: (Masuda1 et al., 2014)

2.3 CELL PATTERNING USING PDMS STAMPING FABRICATED BY SOFT-LITHOGRAPHY

Cell patterning using PDMS stamps requires fabrication of a stamp using polymeric material. The stamps are fabricated by the soft-lithography technique that requires using an elastomeric material which is mostly PDMS. PDMS is poured on a master mold (generally fabricated via photolithography) with the desirable design and size and cured. After that, the PDMS stamp is peeled off from the rigid master (Whitesides et al., 2001).

PDMS has many advantages over other polymeric materials that make it the most used material in microfabrication, it is gas permeable, it is a transparent material and it has a hydrophobic surface which can be converted into hydrophilic based on the application either by oxygen plasma or by chemicals (Madadi & Terre 2013, Whitesides et al. 2001).

Figure 2.7: PDMS fabrication by soft-lithography.



Source: (Weibel et al., 2007)

3. PRELIMINARY WORK ON CREATING LIVING CELL ARRAYS

In this chapter, earlier work on creating living cell arrays via a new method called nanowell-assisted cell patterning (NWAP) is explained briefly. This technique employed specific attachment of cells on the surface via antibodies directed to surface markers. In this thesis, we are investigating whether a universal, non-specific method may be used for attachment of cells to the surface and for subsequent patterning.

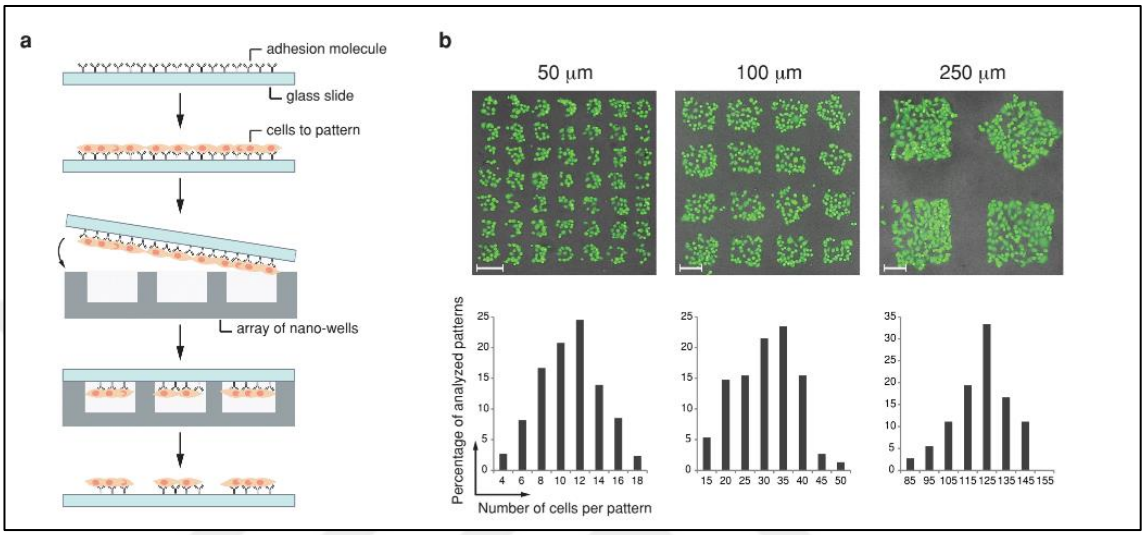
3.1 NANOWELL-ASSISTED CELL PATTERNING

In earlier work Yalcin Ozkumur et al. (Yalcin Ozkumur et al., 2015) have developed nanowell-assisted cell patterning (NWAP) technique for use in HIV neutralization assays with GHOST cells. To apply this technique, firstly a confluent monolayer of the cell of interest has been formed by attaching the cells on a glass slide that has been coated with a specific adhesion molecule (mouse anti-human CD4 antibody) that is specific to the CD4 expressed on the cell surface to promote cell attachment. A pattern of cells is then created by sealing the cell-coated slide against an array of nano-wells that has been created out of PDMS by soft lithography. Then, the array of nano-wells is removed which results in disrupting the cells in the contacting area. This mechanical disruption results in creating a pattern of cells with specific size depending on the design of the array of the nano-wells. Nano-well devices can be created with different sizes by altering their designs. Each well has sub-nanoliter to nanoliter volume depending on the size. In this work three different sizes of nano-well devices have been used (50 μm, 100 μm, and 250 μm) for cell patterning (Figure 3.1).

The cell arrays can be used to measure different cell responses. Figure 3.2 illustrates using cell arrays to measure cell migration and cell proliferation with and without adding of focal adhesion kinase (FAK) inhibitor which is a molecule that inhibits cell motility. The array of cells has been captured at time 0 hr and 24 hrs to monitor cell migration and cell proliferation in the both cases (+FAK inhibitor and –FAK inhibitor). The cells have been stained with calcein green and the background is shown in purple.

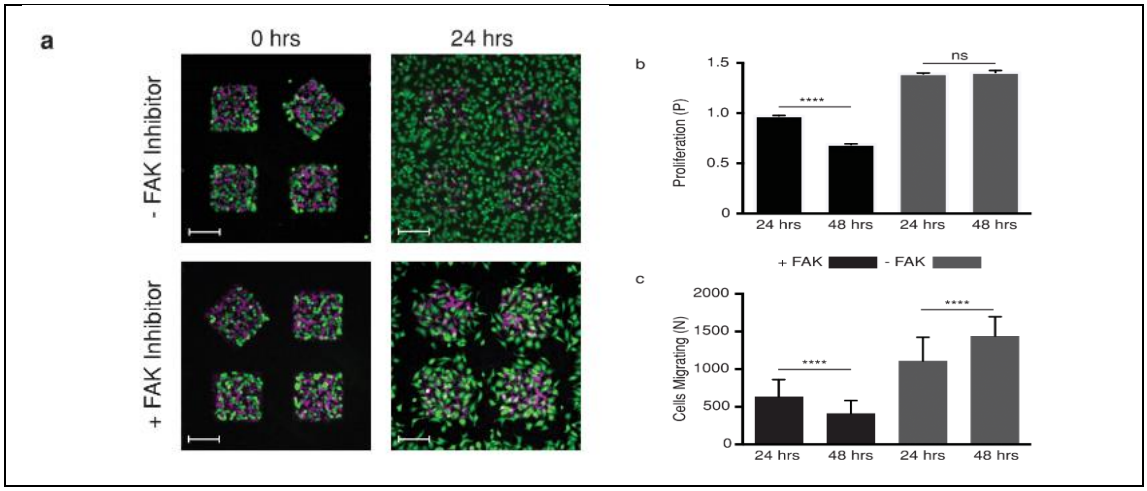
The results showed that the addition of FAK inhibitor inhibit both cell migration and cell proliferation in the patterned cells which contributes to the maintenance of the patterned cells for 48 hrs.

Figure 3.1: Cell patterning by NWAP method.



Source: (Ozkumur et al., 2015)

Figure 3.2: Images for patterned cells at 0 hrs and 24 hrs with and without FA inhibitor.

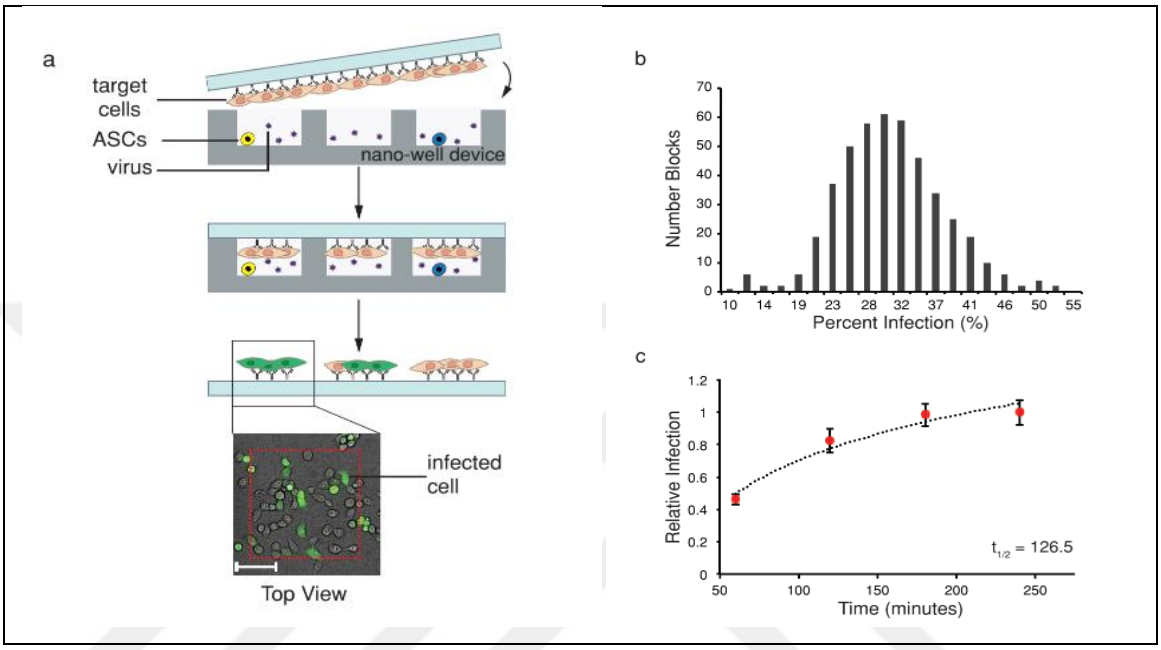


Source: (Ozkumur et al., 2015)

This technique also has been used in a virus neutralization assay. In this application several types of antibody secreting cells (ASCs) have been loaded with the HIV virus in the nano-well device, then the device has been sealed against of monolayer of cells of

interest to detect the infection activity of the virus in the presence of the ASC to determine whether the secreted antibodies are neutralizing antibodies or non-neutralizing antibodies (Figure 3.3).

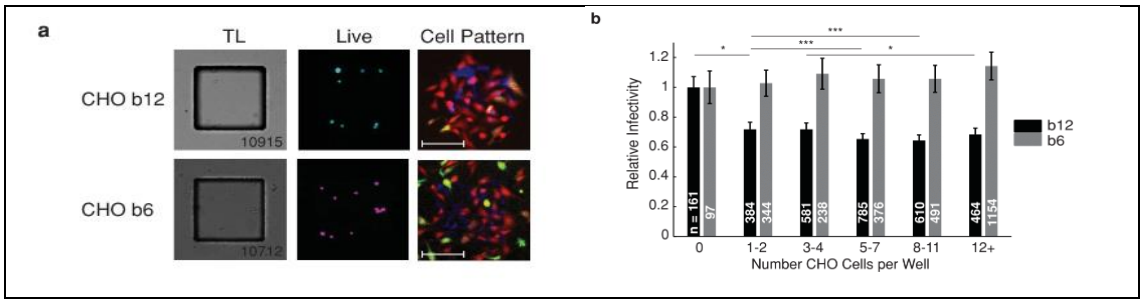
Figure 3.3: Nano-well device for virus neutralization assay.



Source: (Ozkumur et al., 2015)

Figure 3.4 illustrates the use of two different kinds of ASCs (CHO b12 and b6 cells) in the neutralization assay. The results show that CHO b12 cells are secreting neutralizing antibody since the percentage of infected GHOST cells are decreased in the array that contains CHO b12 cells compared with the array that contains CHO b6 cells. The green color indicates the infected cells in each pattern.

Figure 3.4: Neutralization assay after integration of b12 and b6 .



Source: (Ozkumur et al., 2015)

In this work, nano-well devices have been successfully used for creating live cell arrays, and different cell responses such as migration, proliferation and virus neutralization via antibody secretion have been measured.

3.2 LIVING CELL ARRAYS FOR BI-SPECIFIC ANTIBODY ASSISTED CELL KILLING

This chapter explains how living cell arrays can be used in identifying bi-specific antibodies for application in drug discovery.

Bi-specific antibodies are artificial proteins that can bind with two different proteins simultaneously to activate specific cellular activity. One type of bi-specific antibody is the bi-specific T-cell engager (BiTE) antibody which links CD3 receptor on T-cells with CEA which is one of tumor associated antigens that are expressed on tumor cells such as colon colorectal adenocarcinoma. This binding leads to activation of the cytotoxic function of T-cells by secreting their cytotoxic compounds in the tumor cells resulting in killing of cancer cells (Baeuerle & Reinhardt, 2009).

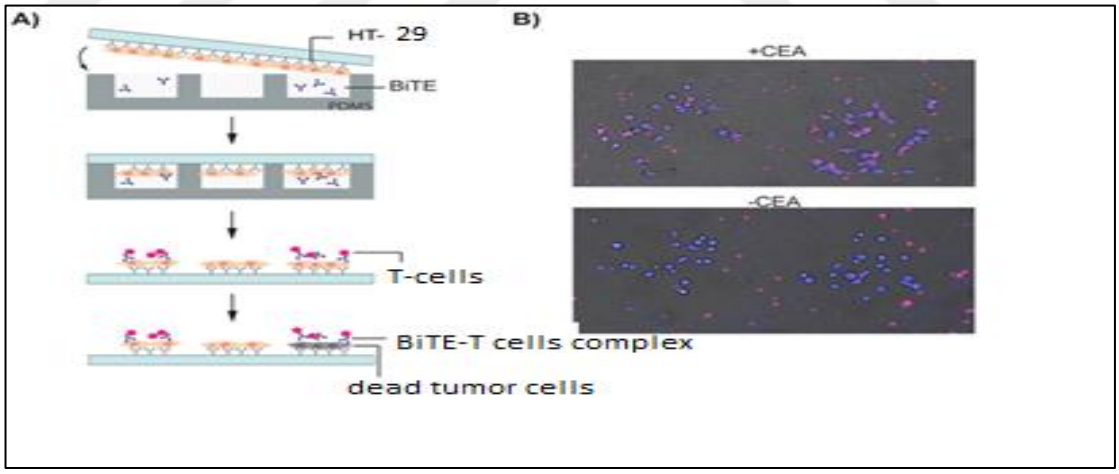
The NWAP technique may be used for performing the BITE assay and assessing efficiency of the antibodies in exerting their effect. The planned method to demonstrate the use of NWAP for this assay is shown in Fig 3.5a. After producing a confluent monolayer of the cells on glass slide, the BiTE antibody will be loaded on a pre-fabricated PDMS stamp. After that, the BiTE loaded PDMS stamp will be brought in contact with the monolayer of the cells on the glass slide which leads to the disruption of the cells in contacting area. This mechanical disruption of the cells will create an array of living cells, at the same time, BiTE antibody will bind to the cells of interest in the loaded wells. Then, T-cells will be added to the patterned cells in order to bind with BiTE and initiate death of the cancer cells by apoptosis.

It is not trivial to determine highly expressed markers on the cells that will be used for this assay (HT-1080 and HT-29), and these cell lines (particularly the HT-29 cells) are difficult to maintain in culture or on treated glass surfaces. On poly-l-lysine coated

slides, the generation and maintenance of a monolayer of cells has been difficult. After creating the cell patterns via NWAP, the binding of T-cells to the CEA expressing cells has been observed, however, killing activity could not be determined because the few patterned cells could not be maintained on the surface. Fig 3.5b shows and example for the comparison of binding of T-cells (red cells) to CEA expressing (blue cells) and BITE treated cell patterns (top panel) vs. BITE treated negative control (blue cells) cell patterns (bottom panel). The images show that T-cells bind only to CEA presenting cells.

In order to increase cell attachment and patterning, the glass slide should be treated with specific biomolecules. For this application, we hypothesized that ECM proteins could work since all tumor cells have receptors on their surfaces that can interact with ECM proteins.

Figure 3.5: Using living cell array in measurement of bi-specific antibody assisted cancer cell killing activity of T-cells.



4. DATA AND METHOD

In this chapter, the protocols used for cell culture, glass surface functionalization, surface coating with extracellular matrix, cell seeding, and scratch assays are given in detail. The procedure for inspecting the cell-coated glass slides and data analysis are also included in the end.

4.1 MAMMALIAN CELL CULTURE

Two cell lines have been used in this study, the target cell line and the control cell line which are HT-29 and HT-1080, respectively. HT-29 cell line (ATCC® HTB-38™) is a colorectal adenocarcinoma cell line maintained in McCoy's 5a Medium Modified (Catalog No. 30-2007). HT-1080 cell line (ATCC® CCL-121™) is a fibrosarcoma cell line maintained in Eagle's Minimum Essential Medium (Catalog No. 30-2003). All cell culture methods have been taken from ATCC protocols and performed under aseptic conditions. All the cells that have been used in the experiments had passage numbers that were less than 15.

4.1.1 Cell Thawing

The cryovial is removed from liquid nitrogen, then thawed at 37^{o} C in water bath for two minutes. Under the cell culture hood, the cryovial content is transferred to a 15 ml falcon tube that contains 9 ml of complete media (fresh media supplemented with 10% fetal bovine serum (FBS) (PAN Biotech)). The cells in the tube are centrifuged for 7 min, then the supernatant is discarded, and 4 ml of pre-warmed complete media is added to the cells pellet, suspended very well and transferred to a T-25 flask. Finally, the flask is checked under the microscope and transferred to the CO_2 incubator (Panasonic MCO-170AICUV-PE CO2 INCUBATOR) that provides 5% CO_2 and 37^{o} C.

4.1.2 Cell Passaging and Maintenance

Cell passaging was performed when cells reach 70-80 percentage confluency. The flask is removed from the incubator and checked under the microscope. The old media is discarded and the cells are washed with Dulbecco's Phosphate Buffered Saline (D8537 SIGMA) three times to remove any traces of FBS. Trypsine (p10-029100 Pan Biotech) is added to the cells for cell dissociation. The flask is incubated for 5 min in the incubator to enhance the detachment of cells, then complete media (fresh media supplemented with 10% of FBS and 1% of Penicillin-Streptomycin Solution (30-2300 ATCC)) is added to neutralize the trypsine effect, and the cell suspension is transferred to a 15 ml falcon tube and centrifuged for 5 min. After centrifuging, the supernatant that contains trypsine is discarded and complete media is added to the cells pellet and suspended very well. The cell suspension is transferred to a desired number of new labeled flasks. Finally, the new flasks are checked under the microscope and transferred to the incubator.

4.1.3 Cryopreservation of Cells

Cells are trypsinized (standard protocol) and neutralized with complete media, and transferred to a 15 ml falcon tube. The cell suspension is centrifuged for a 5 min, and the supernatant is discarded and replaced with cold freezing media (90% FBS+ 10% DMSO (CP-10 OriGen)). The cell suspension is re-suspended quickly and transferred to the desired number of pre-labeled cryovials with 1×10^6 cells per 1 ml in each vial. The cryovials are transferred quickly to -20 °C for two hours, then to -70 °C overnight and finally to liquid nitrogen the next day.

4.1.4 Cell Counting Using Hemocytometer

Cell suspension is prepared (standard protocol) and 10µl from this suspension is transferred to each chamber of the hemocytometer (Cat. No. 06 400 10 MARIENFELD), each chamber was observed under the microscope at 10X magnification and the cells in the middle square were counted twice, then the average of the cell number in both chambers is calculated and recorded. Cell density and cell number were calculated using the following formulas.

Cell Density (Cells/ml) = Number of Cells × Dilution Factor × 10^4

Total Cell Number = Cell Density × Total Volume

4.2 GLASS SILANIZATION

The silanization with (3-Aminopropyl)triethoxysilane (APTES) (A3648 Sigma-Aldrich) procedure was performed according to the manufacturer protocol. First, the glass slide was soaked in 10% NaOH for 10 min, then washed three times with distilled water, and allowed to dry. The slide was then soaked in acetone for two min and dried. Next, the clean and dried slide was soaked in 2% APTES in acetone for two min then washed with distilled water twice. Finally the slide was dried completely at 100 °C for 30 min. The slides were used immediately after preparation or within two months of storing them in the desiccator at room temperature (Masuda1 et al., 2014).

4.3 ECM COATING

All the ECM reagents have been purchased from Sigma-Aldrich and used according to Sigma-Aldrich protocols and concentration recommendations. All the procedures have been conducted under aseptic conditions. All the lifter-slips (Cat. NO. 72184-60) used either in ECM coating or in cell seeding were blocked in 3% (v/w) milk in PBS for 30 min, then washed with DI-water twice and air dried before using. The ECM working solutions are prepared according to the protocols detailed below.

4.3.1 ECM Gel

ECM Gel from Engelbreth-Holm-Swarm murine sarcoma (E1270 SIGMA) stock solution was removed from -20 °C and thawed overnight at +4 °C. ECM gel manipulation was conducted on ice to prevent forming the gel before the coating. ECM gel has been diluted to 1:2 ratios by adding one part of ECM gel to one part of the cold serum-free media to have a working solution.

4.3.2 Vitronectin

Vitronectin from human plasma (V8379 SIGMA) stock solution was removed from -20 °C and thawed at room temperature. VTN has been diluted to 1:3 ratios by adding one

part of VTN to two parts of DI-water (Cat. NO. 809-115-CL WISENT) to have a working solution with a final concentration 16.13µg/ml.

4.3.3 Fibronectin

Fibronectin human plasma (F2006 SIGMA) stock solution was removed from -20 °C and thawed at room temperature. FN has been diluted to 1:2 ratios by adding one part of FN to one part of PBS to have a working solution with a final concentration 484 µg/ml.

4.3.4 Laminin

Laminin from mouse Engelbreth-Holm-Swarm (EHS) sarcoma (11243217001 ROCHE) stock solution was removed from +4 °C. Lam stock solution has been used directly as a working solution without dilution at 500 µg/ml concentration.

4.3.5 Coating Procedure

After preparing the working solutions according to the protocol for each type of ECM, 85 µl of ECM working solution was added to the silanized glass slide, covered with a blocked lifter-slip, and incubated in a closed and hydrated chamber for one hour at room temperature. After the incubation, the glass slide was washed three times with PBS to remove excess ECM, then blocked with 3% (v/w) milk in PBS for 30 min. Finally, the glass slide was washed twice on a rocker for 5 min, firstly with PBS and then with DI-water (Cat. NO. 809-115-CL WISENT). The glass slide was air dried before seeding the cells (Masuda1 et al., 2014).

4.4 CELL SEEDING

The cell suspension was prepared, and cells were counted according to the standard protocols described previously. The cell suspension was centrifuged and the supernatant was discarded. The necessary volume of serum-free media to reach the desired cell density was added to the cell pellet, and cells were suspended very well. A volume of 85 µl of each cell density was seeded on the ECM coated glass slide under a blocked lifter-slip and incubated for 30 min at room temperature. The cell-coated slide was washed twice with serum-free media for 5 min on a rocker to remove unbound cells, and complete growth media was added to the cell-coated slide. Cells were monitored under a light inverted microscope (Leica DM IL LED) with 10X magnification to detect initial attachment to the surface. Images of cell-coated slides were taken 30 min after incubation in serum-free media, 2 hours after incubation in complete media, and next day.

4.5 SCRATCH ASSAY

The scratch assay has been applied when the cells reach 90%-100% confluency in the next day of seeding on ECM coated slides. A sterilized 10 µl micropipette tip was used to create a scratch in a straight line on the confluent monolayer. The surface was then washed with PBS to remove debris, and then the slides were soaked in complete growth media. Images were taken immediately after creating the scratch, after 4 hours, after 8 hours and the next day to monitor the migration of the cells and closure of the scratch.

4.6 IMAGING AND DATA ANALYSIS

Images were taken with an HD Digital microscope camera (Leica MC170 HD) mounted on the light inverted microscope (Leica DM IL LED) and connected to the computer in order to document the images via the compatible Leica application suite (LAS) software.

The number of attached cells in each image was counted manually for 24 hrs images.

5. FINDINGS

In this chapter, the findings from the ECM coating optimization experiments are presented for the two cell lines of interest, HT-29 and HT-1080.

5.1 DETERMINING THE OPTIMAL ECM FOR CELL ATTACHMENT

The cells have been seeded on ECM-coated slides for all ECM types at 1000 000/ 4.84 cm^2 cell seeding density to determine the optimal ECM type. At the next day of the seeding, the number of attached cells has been counted in three different areas on the same slide for each ECM type and the average was calculated. The results are shown in Figure 5.1 and Figure 5.2 for HT-29 and HT-1080 cell lines, respectively. As shown, HT-29 cell line has a higher number of attached cells with well-maintained morphology when seeded on ECM gel compared to other types of ECMs or un-coated slides (Figure 5.1). For the HT-1080 cell line, VTN gave the best cell attachment with well-maintained morphology in comparison to other types of ECM or uncoated slides (Figure 5.2).

Figure 5.3 and figure 5.4 exhibits images for HT-29 cells and HT-1080 cell lines, respectively at 1 000 000 cells/ 4.84 cm^2 on different substrates. The images have been taken next day of the seeding at 10X magnification.

Figure 5.3 illustrates the changing in morphology for the cells on both Lam and FN (Figure 5.3 (a) and (b)) respectively. It also illustrates the poor attachment for the cells on both VTN and silanized glass (Figure 5.3 (c) and (e)) respectively. Figure 5.3 (d) shows higher number of attached cells that form a confluent monolayer with well-maintained morphology on ECM gel which reveals that ECM gel is the optimal ECM for HT-29 cell line. Figure 5.3 (f) is an image for HT-29 cell in T-75 flask work as a reference for the morphology of the cells.

Figure 5.4 illustrates the maintenance of HT-1080 cells of their morphology on all types of ECMs which are FN, VTN, ECM gel and Lam (Figure 5.4 (a), (b), (c) and (d) respectively) plus the silanized glass and the non-treated glass (Figure (e) and (f) respectively). The figure also illustrates that the largest number of attached cells is on VTN-coated slide in which the cells form confluent monolayer which reveals that the VTN is the optimal ECM for HT-1080 cells. Figure 5.4 (g) is an image for HT-1080 cell in T-75 flask work as a reference for the morphology of the cells.

Figure 5.1: Graph illustrates the number of attached HT-29 cells on different substrates at 1 000 000 cells/ 4.84 cm^2 cell seeding density.

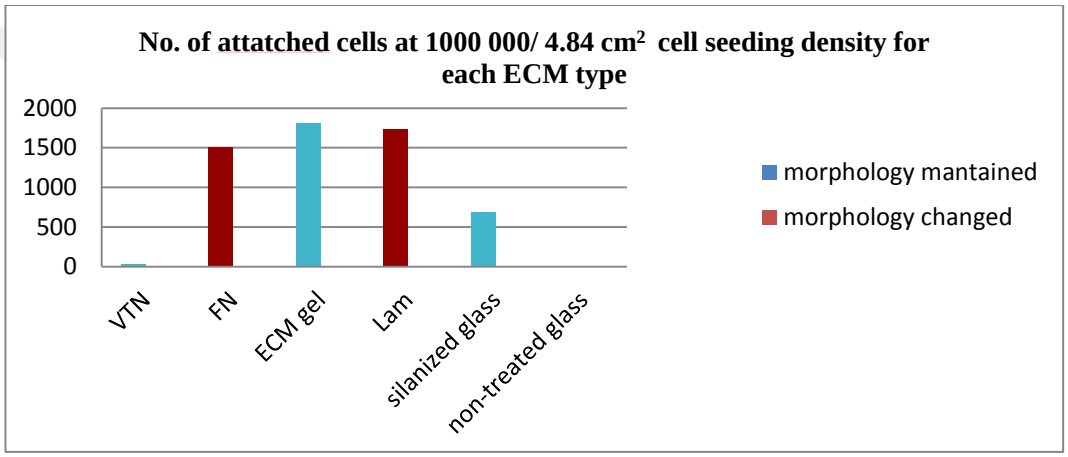


Figure 5.2: Graph illustrates the number of attached HT-1080 cells on different substrates at 1 000 000 cells/ 4.84 cm^2 cell seeding density.

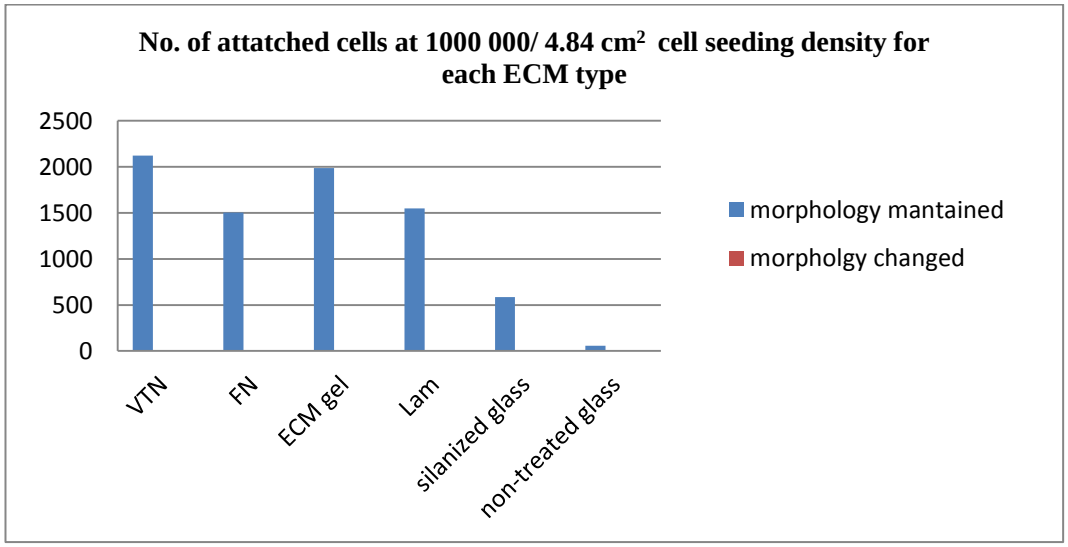
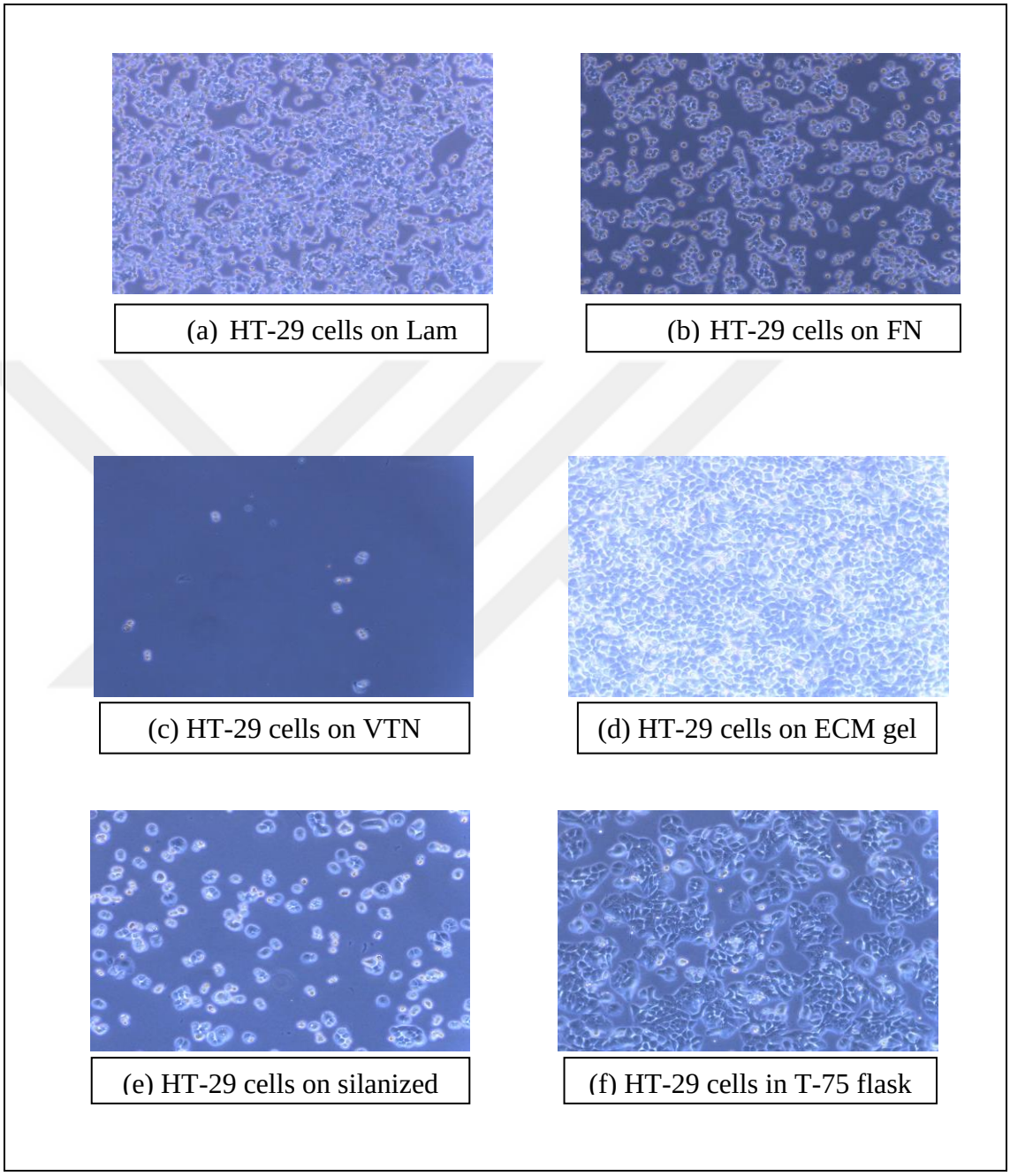


Figure 5.3: Images for HT-29 cells (10X) seeded at 1000 000 cells/ 4.84 cm2 on different substrate (image size (285.76 µm, 214,32 µm)).



(a) HT-29 cells on Lam

(b) HT-29 cells on FN

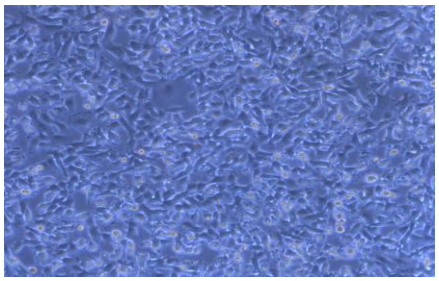
(c) HT-29 cells on VTN

(d) HT-29 cells on ECM gel

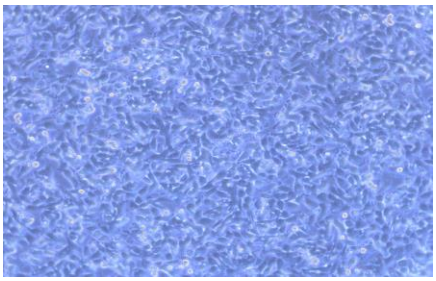
(e) HT-29 cells on silanized

(f) HT-29 cells in T-75 flask

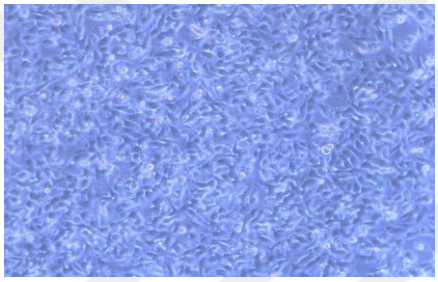
Figure 5.4: Images for HT-1080 cells (10X) seeded at 1000 000 cells/ 4.84 cm2 on different substrates (image size (285.76 µm, 214,32 µm)).



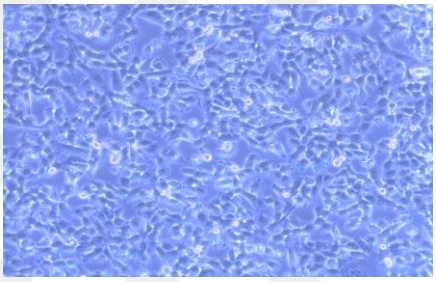
(a) HT-1080 cells on FN



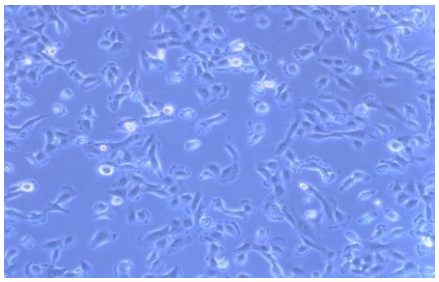
(b) HT-1080 cells on VTN



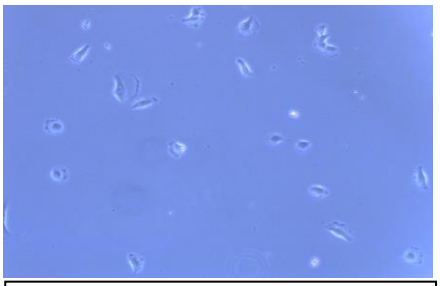
(c)HT-1080 cells on ECM gel



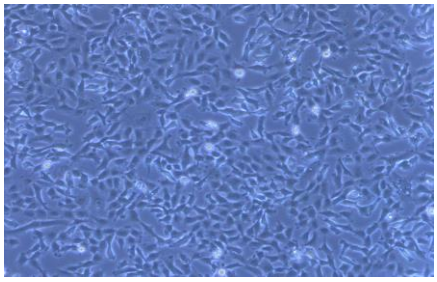
(d) HT-1080 cells on Lam



(e) HT-1080 on Silanized glass



(f) HT-1080 on Non-treated glass



(g) HT-1080 cells in T-75 flask

5.2 CELL DENSITY OPTIMIZATION

In order to determine the optimal cell density that allows cells to reach 90%-100% confluency next day with the optimal ECM (ECM gel, VTN, FN or Lam) for both cell lines, HT-29 and HT-1080. A range of cell densities have been seeded on all ECM types for both cell lines. The cell densities were as following:

a. 37 500 cells/ 4.84 cm^2
b. 300 000 cells/ 4.84 cm^2
c. 600 000 cells/ 4.84 cm^2
d. 1 000 000 cells/ 4.84 cm^2

The result showed that 1000 000 cells/ 4.84 cm^2 is the optimal cell density for HT-29 on ECM gel and HT-1080 on VTN, since at this cell density cells reach 90%-100% confluency next day that form confluent monolayer.

Figure 5.5 shows images for HT-29 cell line at different cell densities next day of the seeding on silanized glass coated with ECM gel.

Figure 5.6 shows images for HT-1080 cell line at different cell densities next day of the seeding on silanized glass coated with VTN.

Figure 5.5: Images for HT-29 cell line seeded at different cell densitits on ECM gel coated slides (image size (285.76 µm, 214,32 µm)).

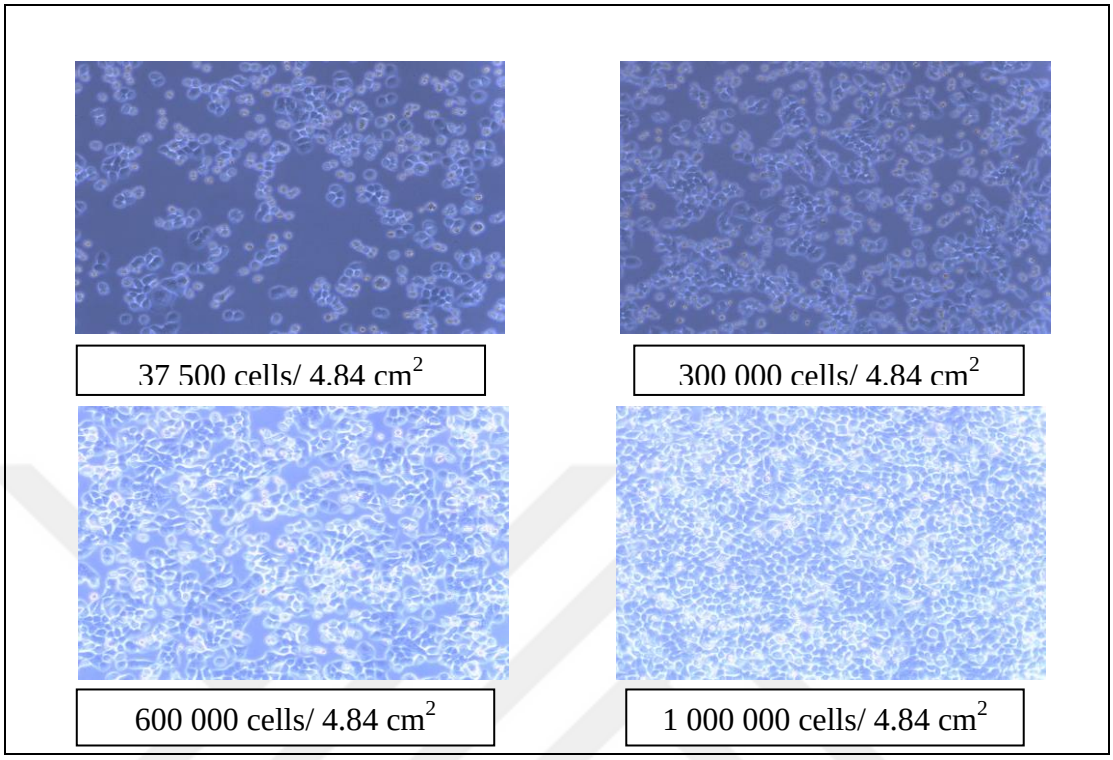
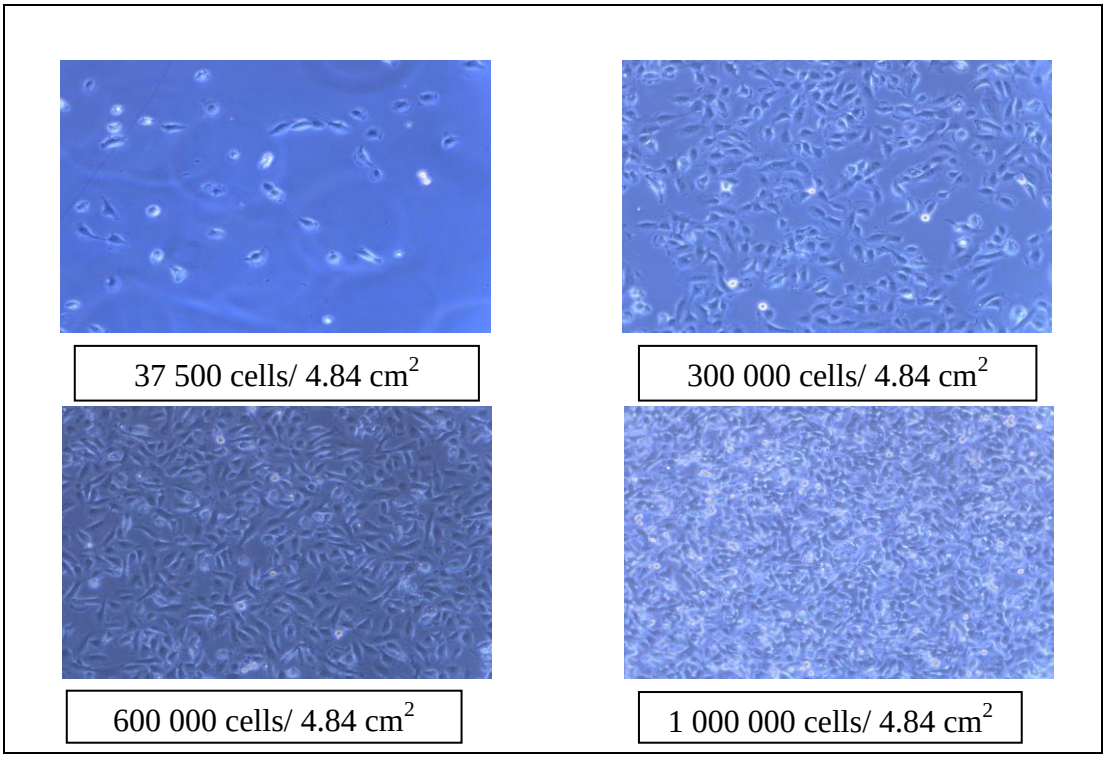


Figure 5.6: Images for HT-1080 cell line seeded at different cell densities on VTN-coated slides (image size (285.76 µm, 214,32 µm)).



5.3 CONSISTENCY OF THE CELL COVERAGE ON SILANIZED GLASS SLIDE COATED WITH ECM

In order to show the consistently of the produced slides for each cell line with its optimal ECM, three different cell densities have been applied, three different slides for each cell density have been produced, and for each slide three areas have been captured and analyzed. The three following cell densities used are:

a. 500 000 cells/ 13.2 cm^2
b. 1 500 000 cells/ 13.2 cm^2
c. 3 000 000 cells/ 13.2 cm^2

The cell coverage variation between different areas on the same slide and variation between different slides when cells seeded at the same cell densities has been illustrated in (Figure 5.7, 5.8 and 5.9) for HT-29 cell line, and in (Figure 5.10, 5.11 and 5.12) for HT-1080 cell line, when the cells are seeded at 3000 000 cells/ 13.2 cm^2 cell density, 1 500 000 cells/ 13.2 cm^2 cell density and 500 000 cell/ cm^2 cell density respectively.

Figure 5.7: Variation in the number of attached cells when HT-29 cells seeded on ECM gel at 3,000,000 cells/ 13.2 cm2 cell density.

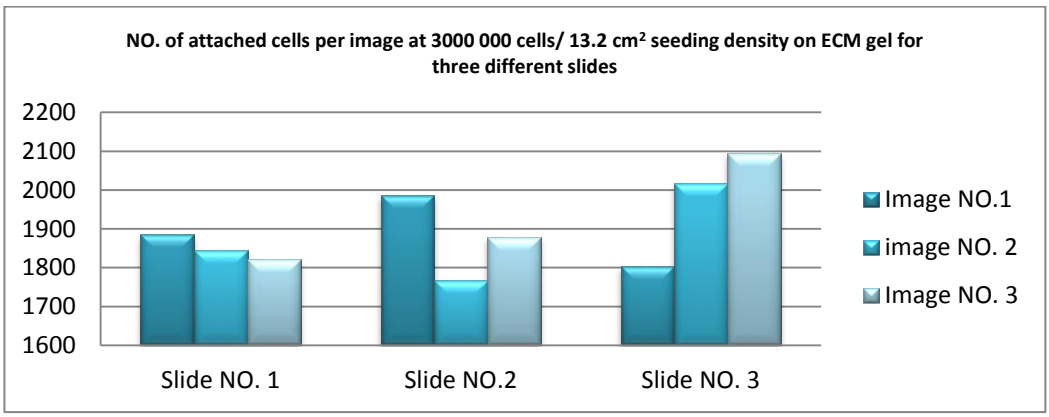


Figure 5.8: Variation in the number of attached cells when HT-29 cells seeded on ECM gel at 1,500,000 cells/ 13.2 cm2 cell density.

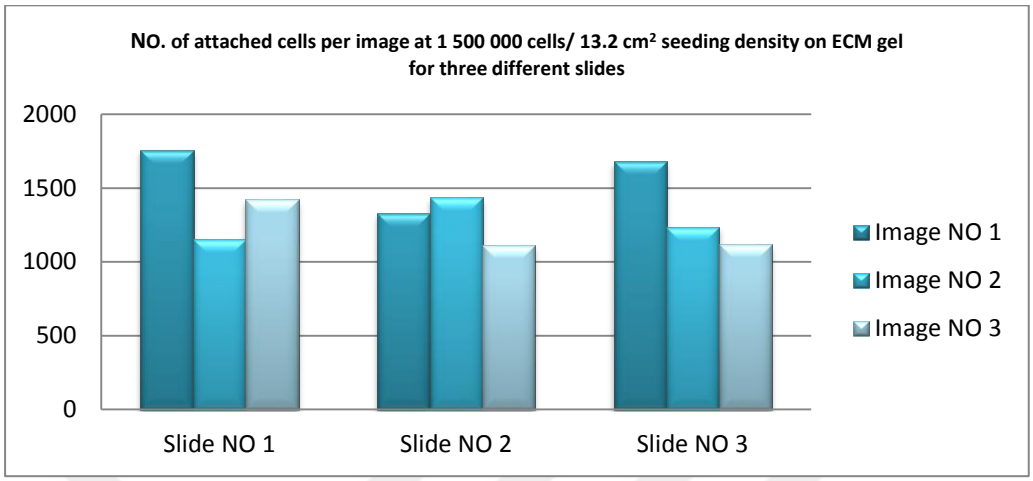


Figure 5.9: Variation in the number of attached cells when HT-29 cells seeded on ECM gel at 500,000 cells/ 13.2 cm2 cell density.

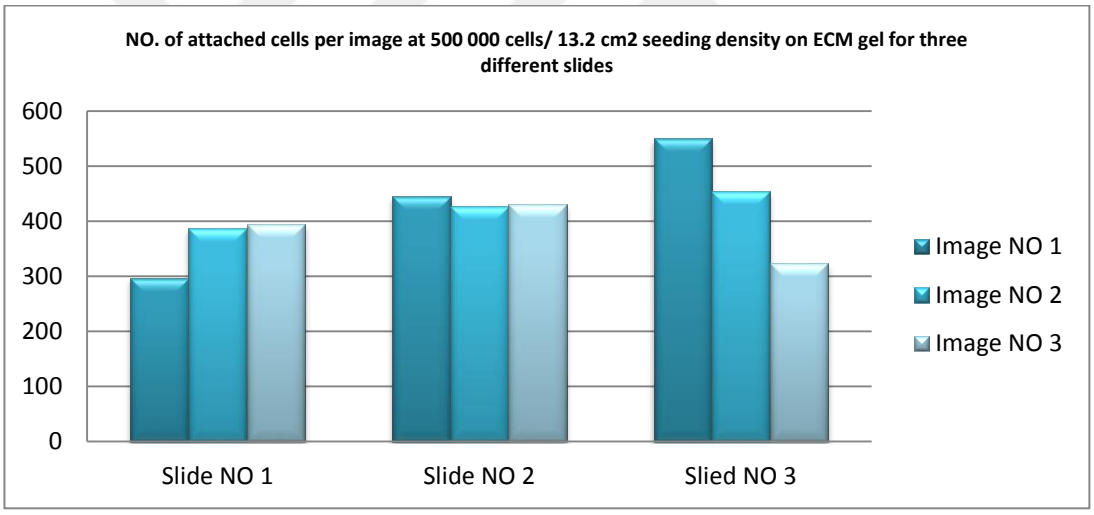


Figure 5.10: Variation in the number of attached cells when HT-1080 cells seeded on VTN at 3,000,000 cells/ 13.2 cm2 cell density.

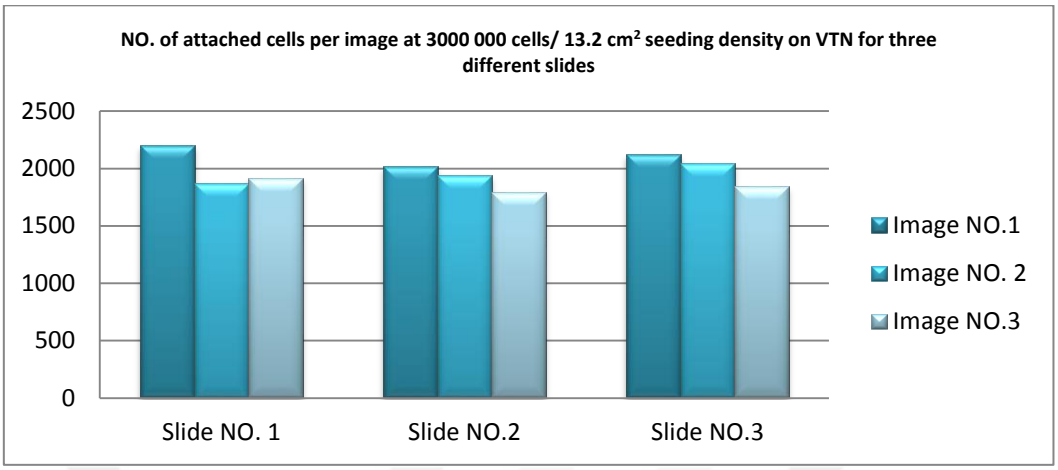


Figure 5.11: Variation in the number of attached cells when HT-1080 cells seeded on VTN at 1,500,000 cells/ 13.2 cm2 cell density.

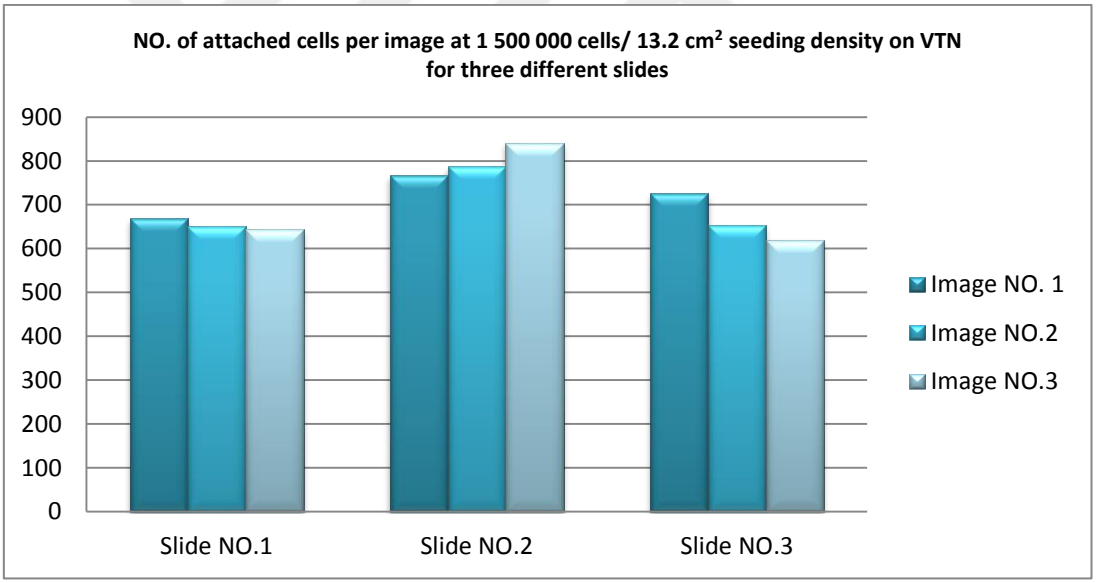
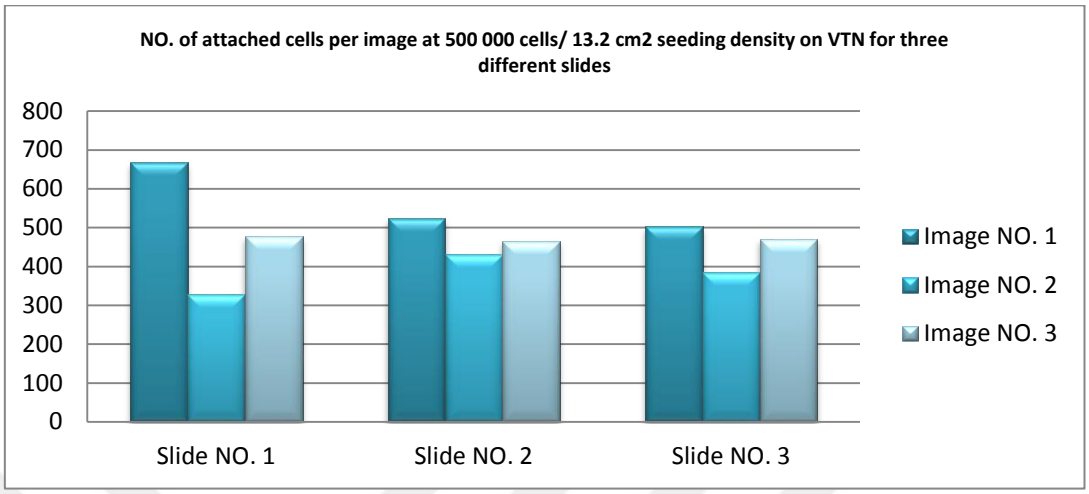


Figure 5.12: Variation in the number of attached cells when HT-1080 cells seeded on VTN at 500, 000cells/13.2 cm2 cell density.



5.4 SCRATCH ASSAY

Scratch assay has been performed for HT-29 and HT-1080 cell lines seeded on silanized glass slides coated with ECM gel and VTN, respectively, at the optimal cell density. The experiment was performed next day of the seeding, at which time the cells form confluent monolayers. Images were taken right after the scratch has been made at time t=0, 4, 8, and 24 hours. The scratch assay on the HT-29 cells showed that the scratch did not close after 24 hours, meaning that the cells have not been migrated (Figure 5.13). The scratch assay on the HT-1080 cells showed that the cells have migrated to close the scratch after 4 hours (Figure 5.14).

Figure 5.13: Images show scratch assay for HT-29 cells seeded on silanized glass slide coated with ECM gel.Images have been captured (10X) at different interval times.

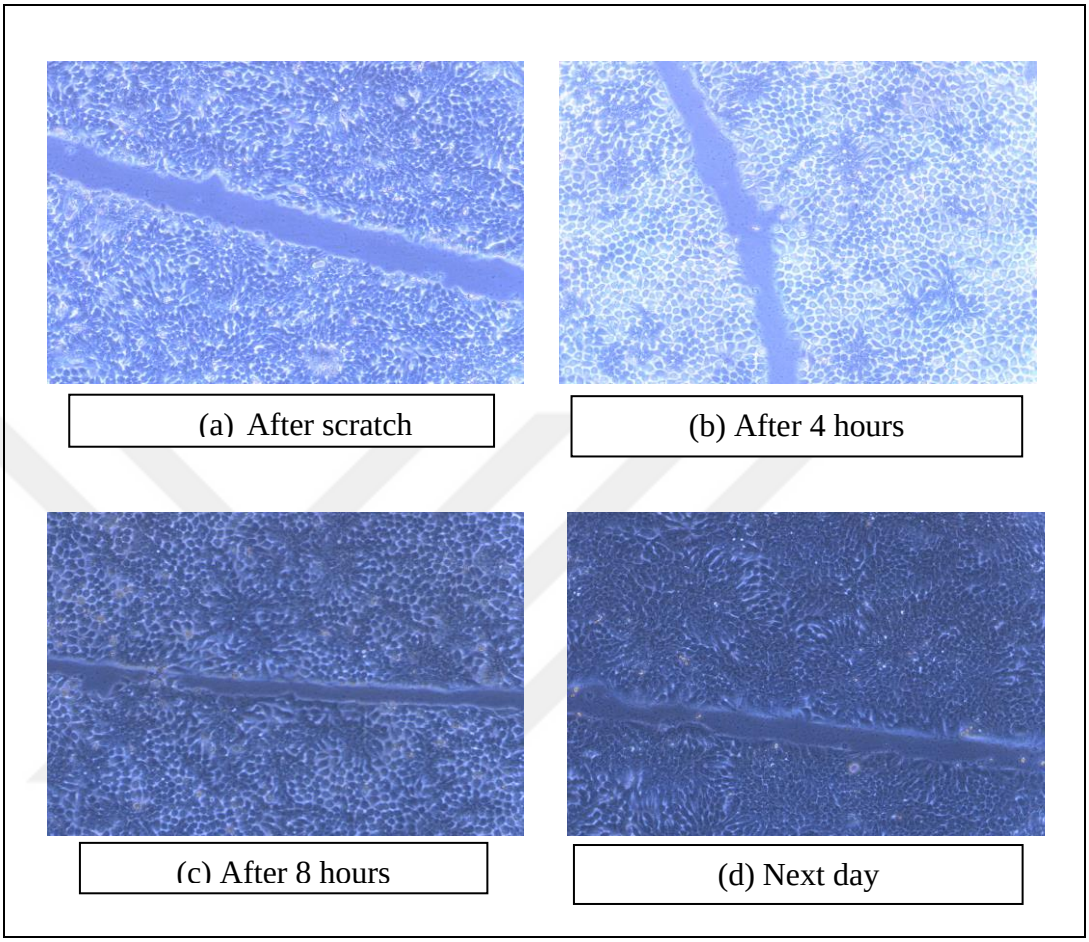
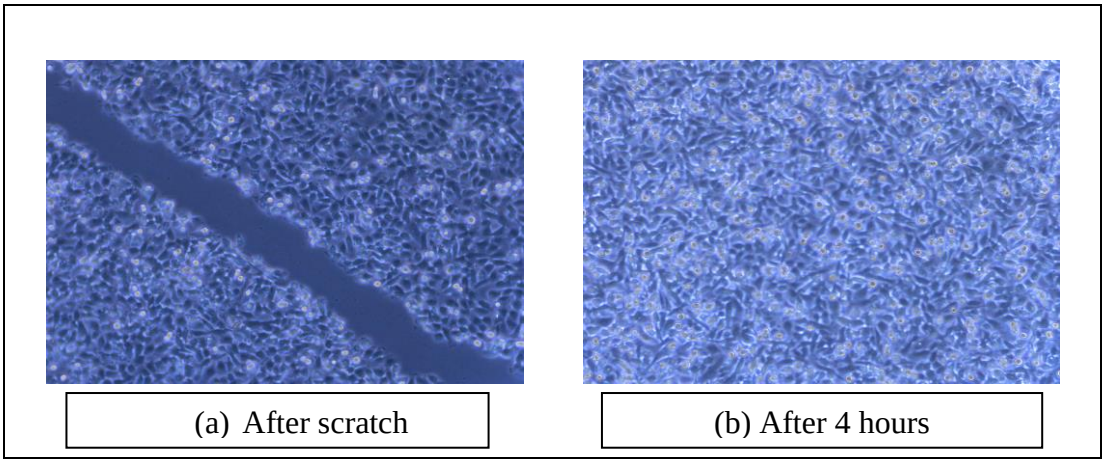


Figure 5.14: Scratch assay for HT-1080 cells on VTN. (a) Image was taken after the creating the scratch. (b) Image was taken after 4hours which illustrate the closure of the scratch.



6. DISCUSSION AND CONCLUSION

In this thesis a protocol for preparing live cell arrays by using proteins to adhere the cells to the substrate has been optimized. Four different types of ECM proteins (VTN, Lam, FN and ECM gel) have been tested with two different cells, HT-29 and HT-1080.

To optimize this protocol, each cell line has been tested for attachment with four different ECM types coated on silanized glass slides together with control slides (non-treated slides and silanized glass slidse without ECM coating) to determine the optimal ECM for cell adherence.

For HT-1080 cells, all ECM types have been shown to work for attachment and maintaining morphology. The cells have a higher number of cell attachments after 24 hrs of seeding on VTN compared with the other ECM types when seeded at the same cell density. The cells also have been attached on control slides but in a very low number. The cells have attached by 75.5 % lower on silanized glass slides than on silanized-VTN slides and 97.3% lower on non-treated slides than on silanized-VTN slides, which illustrate the importance of VTN coating in enhancing cell attachment.

For HT-29 cells, most ECM types work for attachment, but only ECM gel worked for maintaining morphology. The cells had a round morphology on both FN and Lam which differ from their original fibroblast-like morphology. They attached in a very low number on silanized-VTN slides and on silanized slides. They did not attach on the non-treated slides. The cells have been attached by 62.3 % lower on silanized glass slides than on silanized-ECM gel slide and 99.5% lower on non-treated slides than on silanized-ECM gel slides, which also illustrate the importance of ECM gel coating in enhancing cell attachment.

Controlled cell coverage on the surface for the optimized protocol has been demonstrated by seeding the cells with variant cell densities (37 500 cells/ 4.84 cm^2, 300 000 cells/ 4.84 cm^2, 600 000 cells/ 4.84 cm^2 and 1 000 000 cells/ 4.84 cm^2) for each

ECM type. The result showed that at the 1 000 000 cells/4.84 cm^2 cell density, the cells form a confluent monolayer next day of the seeding.

Repeatability of the method and protocol has been demonstrated by determining variability between slides and between different areas on the same slide. Each cell line has been seeded on the optimal ECM protein with 3 different slides at the same cell density and three different areas from each slide have been captured and analyzed for number of cells attached after 24 hrs. Three different cell densities have been tested for each cell line, 500 000 cells/ 13.2 cm^2, 1 500 000 cells/13.2 cm^2 and 3 000 000 cells/13.2 cm^2. The variation in the number of attached cells between different slides or different areas on the same slide was low, which illustrates the strength of the protocol and the high consistency of the cell coverage.

Scratch assay has been performed for both cell lines on the optimal ECM protein to demonstrate proliferation and migration functionality of cells attached on ECM substrates. The result showed that HT-1080 has migrated well on VTN and closed the scratch after 4 hrs. For HT-29, the cells did not migrate on the ECM gel and failed in closing the scratch after 24 hrs confirming low migration rate as shown in an earlier work (Mclnroy & Maatta, 2007). Although HT-29 cells do not migrate on the ECM gel, the produced slide of a confluent monolayer of cells can be used in identifying bi-specific antibody assisted T-cell killing application since the aim of this application is to detect cell apoptosis not cell migration. Also, the inhibition of their migration on the produced slides facilitates the maintenance of the array for longer periods of time. This advantage is important when the assay is not performed on the same day of creating the array.

In conclusion, this new non-specific cell attachment protocol can now be used for the bi-specific antibody assisted T-cell killing application by allowing the cells to attach to the substrate using ECM proteins and creating living cell arrays for detecting many cellular activities. In future work, this non-specific cell attachment protocol for cell patterning can be used to test a single BiTE antibody across the array at high-throughput. This patterning protocol also allows for isolating each element of the array

and measuring coordinated events of cell-cell interactions at each isolated element. This aspect suggests that the outcomes of this project may be useful in future development of a platform that allows for testing the effect of the environment and the effect of different antibodies and titers at each isolated element of the array, and may even enable testing of different antibodies produced from individual cells in engineered cell lines.

Different applications that run assays on cell substrates or living cell arrays, can adopt this protocol for other cell types. The protocol together with the patterning method can be used for identifying new therapeutics which can potentially facilitate drug discovery research.

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