UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Application of Protein and Small-Molecule Microarrays to Study Stem Cell Differentiation, Cancer Growth, and Personalized Therapy

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Bioengineering

by

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The Dissertation of Dayu Teng is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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## DEDICATION

This dissertation is dedicated to my parents, whose boundless love nourished my growth and whose sacrifice granted me the material and mental enjoyments and opportunities.

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## ABSTRACT OF THE DISSERTATION

Application of Protein and Small-Molecule Microarrays to Study Stem Cell Differentiation, Cancer Growth, and Personalized Therapy

by

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The DNA microarray technology has contributed widely in biological studies. Recently, a method to detect the effects of extracellular matrix (ECM) proteins on cells using microarray was developed. In this dissertation, the microarray technology was applied to study the effects of ECM proteins on human mesenchymal stem cell (hMSC) differentiation, and to identify the best growth-supporting ECM conditions for glioblastoma, and to create a method for efficient small molecule drug screening.

The importance of ECM on hMSCs is well known. Numerous studies have shown that the ECM deposed by specific types of cells could induce differentiation in

hMSCs. However, clinical application of cell-deposited ECM could carry risks such as contamination, immune rejection and batch-to-batch variation. An artificial ECM could avoid these problems. In this dissertation, the effects of ECM proteins were screened on the hMSCs differentiation using the microarray technology. The myogenic and neurogenic differentiation profiles are reported. This information can contribute to the selection of optimal differentiation-inducing coating on the muscle or nerve tissue engineering scaffolds.

Cell culture has always been a fundamental tool in cancer research. However, most primary cancer cells are very difficult to maintain. In this dissertation, ECM protein combinations were tested for their efficacy in supporting the growth of glioblastoma cells. Twelve best combinations were identified. The result may lead to successful maintenance of the primary cells of glioblastoma and other types of cancerous tumors. The results may yield larger quantities of the primary cancer cells, which are heretofore limited in supply, and thus facilitate the advancement of cancer research.

Genetic marker-based personalized medicine has attracted much attention. However, our understanding of the genes is still limited. A direct drug efficacy screening on the diseased cells of an individual patient may complement the gene-based personalized medicine. In this study, a small-molecule drug microarray method is reported. This method is able to screen a large number of drugs and their combinations by using a very small mount of patients' diseased cells, making it possible to design personalized drug cocktails.

# Chapter I Introduction

## I.A Brief history of Stem Cell Research

Stem cells, with their ability to differentiate into various types of adult cells and their potential to replace diseased organs, have captured the public's attention in recent years. The discovery of stem cells came shortly after the invention of microscopes in the 1800's when the cells were studied as the basic building blocks of life. In the mid 1800's, the concept of "stem cell" was developed when researchers observed that some cells have the ability to become other types of cells [1, 2]. More thorough studies in the early 1900's demonstrated that various types of blood cells could branch from certain bone marrow cells [2]. These cells in the bone marrow were later named the hematopoietic stem cells and were the first well-established stem cells. Based on this knowledge, bone marrow transplant has been introduced into the clinics to replenish the blood-making stem cells after chemotherapy or radiation since the 1950's, which were decades before the development of a thorough scientific understanding of these cells [3]. The self-renewal activities of mouse bone marrow cells were first quantitatively described by Canadian researcher James Till and Earnest McCulloch in 1963 [4]. In the past, most work in this area had been focusing on the adult stem cells that come from the bone marrow and other tissues in post-fetal phase and can only become a limited number of cell types. It was only after the quantum leap in biotechnology of the 1980's and 1990's, an American scientist James Thomson was able to create the first embryonic stem cell line by removing and growing cells from a spare embryo at the blastocyst stage in 1998 [5]. Since then, the availability of

embryonic stem cells and their potential to become and replace any cell types in a human body appeared in the center stage of the scientific research.

However, the application and the scientific study of the embryonic stem cells face many controversies and difficulties. One major controversy lies over the fact that isolating the embryonic stem cells involves destroying the embryo. The source embryos are typically the leftover and abandoned ones after an in vitro fertilization process. Many believe that because these embryos still have the potential to become a fetus if implanted in a mother's womb, destroying an embryo is against their religious or moral belief. The other point of view suggests that because the source embryos were abandoned and would never be implanted to become a fetus, it is acceptable to use these embryos for research and treatment. In addition to the controversy, there are many practical difficulties to bring embryonic stem cells to clinical application. One of such difficulties is the ability of the embryonic stem cells to proliferate indefinitely, a characteristic very similarly to that of cancer cells. Therefore, applications in human must proceed with great caution. Also, because the embryonic stem cells are genetically different from the cells of the patients, the potential therapeutic products derived from them will likely be rejected by the patients' immune system. Because of the controversies and difficulties mentioned above, the embryonic stem cells will probably have direct clinical impacts much later after thorough and long investigates. The adult stem cells are, however, not associated with such controversies and difficulties.

One type of these adult stem cells is the mesenchymal stem cells. Like the successfully clinically applied hematopoietic stem cells which generate blood cells, mesenchymal stem cells are also mostly found in the bone marrow. They were first identified as fibroblast-like osteogenic cells by Friedenstein and colleagues from guinea pig bone marrow and spleen in 1970 [6]. During the following decades, many researchers contributed to the realization that MSCs were capable of differentiating into multiple connective tissue lineages. Caplan first introduced the concept of mesenchymal stem cells in 1994 [7]. The tri-lineage, with adipogenic, osteogenic and myogenic potentials, was first described in detail by Pittenger in 1999 [8]. Thereafter, mesenchymal stem cells were reported to differentiate into numerous tissue types, including some non-mesenchymal lineages such as adipose, skin, retinal pigment, cardiac, neuronal, skeletal muscle, and other tissues [9-20], Figure I.1.

## I.B Clinical Applications of Mesenchymal Stem Cells

Many studies have shown promising therapeutic potentials of MSCs in lung injury [19], kidney repair [21], diabetes [22], graft-host diseases [23, 24], and cardiovascular repair [18, 25] in human patients, as well as diseases in animal models. A large number of clinical trials have shown interesting results in recent years. Chen et al. reported significant improvement in functional markers resulting from coronary artery injection with autologous MSCs post PCI in acute myocardial infarction patients [26]. In 2004, it was shown that injection of autologous bone marrow cells may enhance left ventricular functional recovery in patients with acute heart infarction [27]. Howitz et al. showed promising results on allogenic bone marrow cell therapy in

osteogenesis imperfecta patients who were characterized by production of defective collagen I in 1999 and 2001 [28, 29]. In 2000, it was shown that MSC infusion may enhance the effects of peripheral blood progenitor cell transplantation to prompt hematopoietic recovery of patients receiving high-dose chemotherapy [30]. Over 40 ongoing clinical trials involving MSCs are registered with NIH ClinicalTrials.gov. One of the studies conducted at UCSD is examining whether "adult stem cells [Provacel™(PUMP1)] are safe and possibly effective in the treatment of acute myocardial infarction", which is sponsored by Osiris Therapeutics. UCSD Medical and Cancer Centers have also been involved in many trials using adult stem cells as in the study of "Combination Chemotherapy Followed by Bone Marrow and/or Peripheral Stem Cell Transplantation in Treating Patients With Recurrent Medulloblastoma or CNS Germ Cell Tumors" sponsored by NCI.

### I.C Extracellular Matrix Environment Effects on MSC Behavior

The ability of adult stem cells to differentiate into multiple cell types carries tremendous potential in cell therapy and regenerative medicine. Controlled differentiation of these stem cells is crucial for clinical applications. It is well recognized that the self-renewal or differentiation of stem cells depend heavily on their microenvironment [31-34]. Many studies have shown that the microenvironment, including the soluble factors and extracellular matrix (ECM) proteins of a particular tissue, can direct stem cells to differentiate towards that tissue type [33, 35-37]. In addition to the chemical factors, recent studies suggested that mechanical aspects of the microenvironment also play important roles in the adult stem cell fate [32, 38-40].

In certain situations, optimal substrate elasticity may be needed to produce the desired result [39]. Artificial matrices that mimic the natural microenvironments may offer unique advantages regulating adult stem cells self-renewal and differentiation for tissue engineering. We believe a combinatorial microarray study may contribute to defining artificial chemo-mechanical microenvironments for the self-renewal and differentiation of human mesenchymal stem cells (hMSCs).

Despite many promising advances in the field, much more remains to be learned. MSCs have been isolated from skeletal muscle [41], adipose tissue [42], umbilical cord blood [43], the circulatory system [44], as well as fetal blood, liver, bone marrow, lung [45, 46] and other sources. Studies mentioned above have demonstrated that MSCs extracted from the source tissue can differentiate when transplanted in target tissues. One important question is what signals from the source tissue maintain MSCs in undifferentiated state, while the factors from the target tissues direct MSCs to a specific differentiation. These signals include neighboring cells, chemical signals such as soluble factors and ECM proteins, passive mechanical properties of the environment such as elasticity and active mechanical signals such as stretch and flow. These signals are encompassed by a widely supported concept: the microenvironment or niche, introduced by Schofield in 1978 [47].

Although all aspects of the niche remain to be elucidated, some studies suggested that ECM from native tissue or in vitro deposition can maintain self-renewal or regulate differentiation [37, 48-50]. A study by Chen et al. recently suggested that the ECM made by bone marrow cells promote MSC self-renewal [49]. Datta et al. in

2005 showed that decellularized ECM synthesized by osteoblasts can enhance osteoblastic differentiation of MSCs [37]. Recent evidence even suggests that ECM deposited by endothelial cells can direct MSC towards endothelial and smooth muscle lineages. Some efforts have been made to design artificial scaffolds such as nano-materials and synthetic polymers that mimic native ECM for MSC support [51, 52]. A comprehensive profile of the effects of various ECM protein compositions on MSC self-renewal and differentiation may offer new avenues for designing the artificial niche.

An important aspect overlooked by many in the past is the mechanical environment. A recently proposed concept by Engler et al. that the elasticity of the substrate alone can regulate MSC differentiation generated much excitement [39]. Incorporating the elasticity of the substrate may provide further optimization of the artificial niche.

Both chemical and mechanical information of the ECM are transmitted through integrins. There have been many studies in this area in other systems. Recent evidence also indicated that integrin expression and activity are involved in MSC differentiation [53]. In 2001, β1 integrin was shown to play an important role for differentiation of hMSCs into osteoblasts [54]. In 2006, Goessler et al. demonstrated that α5β1 and αIIbβ3 were down-regulated while V3 integrin was not changed when hMSCs were induced towards the chondrocyte lineage [55]. Single or small numbers of integrins have been studied in relation to hMSC self-renewal and differentiation;

however, a comprehensive profile of ECM-integrin interaction for hMSCs has yet to be mapped out.

## I.D Primary Cancer Cell Culture

Cancer has remained as one of the top killer diseases in the world. Its prevalence is only rising as the life expectancy increases in recent years. Even though there has been great progress made in cancer treatment, much more work remains to be done.

In spite of the develop of advanced biotechnology such as bioinformatics, culturing and expanding primary cancer cells is still one of the most direct and effective methods in cancer research. One of the key characteristics of cancer cells is their immortality, the ability to grow indefinitely. One would expect that cancer cells can be easily grown in vitro. However, contrary to intuition, most primary cancer cells have been surprisingly difficult to maintain in vitro. The lack of effective culture and expansion methods in many types of cancers such as glioblastoma (neuro-cancer) significantly hinders the study of these diseases, [56-58].

As most other cells, malignant tumor cells also attach and grow on ECM in vivo. The effects of their surrounding microenvironments can be critical for their growth. Previous studies have demonstrated certain degrees of success using pre-coat ECM proteins on the culturing substrates to improve primary cancer cell culture, [57, 59]. However, the ECM conditions used were very limited. A well defined and cancer-specific ECM profile will great facilitate the expansion of the primary cancer cells.

In recent years, studies suggest among the numerous types of cells in a tumor, there could be a small subpopulation of cells exhibiting stem cell-like features such as unlimited proliferation. It is believed that this subpopulation of cells in a tumor is responsible for the growth of the tumor and thus plays an important role in the pathogenesis of cancer [60]. These cells are referred as cancer stem cells or cancer-initiating cells [33, 60, 61]. In addition to the difficulty of primary cancer cell culture, the small size of cancer stem cell population makes it even more challenging to isolation and maintain. A method to isolate and characterize small population of cells from a multi-type cell mixture can greatly facilitate the development of this young field.

## I.E Personalized Medicine in Cancer Therapy

The importance of individual variations has been long recognized in medicine. It is not surprising to see a drug treating one patient with great efficacy while being ineffective for another. This phenomenon may be due to many factors such differences in the pathogenesis, drug metabolism and tolerance to the drug-toxicity. Therefore, it is a common practice for a physician to switch between similar drugs when treating a patient. Most of the treatment adjustments in drugs today are based on experience and trial-and-error rather than hard evidence because of our lack of understanding in these individual variations.

The scientific advancement such as the human genome project has shed light onto solving the puzzle of individual variations. If we are able to use the information of an individual’s genetic makeup as a roadmap, it is possible to design medicinal

treatment specifically for that patient, to generate the maximal efficacy and minimum adverse effects. Thus, the concept of personalized medicine recaptures the attention of the medical community. However, understanding the significance of the individual genetic differences in relation to the responses to certain drugs is a daunting task, let along the enormous cost to sequence a complete human genome. Furthermore, genetic information is only one part of the individual differences. The gene-based personalized medicine can only be effective in a small portion of the diseases.

One of the more direct ways to find the best drug for an individual patient is to testing the patient's disease-causing cells against all potential drugs for that disease. In fact, testing disease-causing cells against chemical agents is the primary method for current drug discovery. Typically, the cells of interest are cultured in multi-well plates with 384 – 3456 wells per plate. A liquid handling robot then transfers the drugs into each well. Finally, the efficacy of each drug is determined by the response of the cells exposed to each drug. Such system requires a large number of cells, ~10,000 cells per well for 384-well plate, and 200 cells per well for 3456-well plate. The process also requires a sophisticated liquid handling robot and adequate cell processing facility. Apart from the capital cost of the instruments and facility, the difficulty lies in the limited amount of cells that can be extracted from a patient. A cost-effective method that can screen a number of drugs by using a small number of cells from a patient can be very valuable in the personalized medicine screening.

## I.F Microarray Technology

The microarray technology was first developed in 1995 to monitor the change in expression of many genes in a high-throughput and miniaturized format [62]. The technology transfers a library of cDNAs onto a glass slide using a robotic pin-based printer forming an array of spots with a diameter of 100 – 150 μm. The microarray of cDNA library allows the corresponding fluorescently labeled DNAs sample to bind. The fluorescent intensity of each spot represents the quantity of DNAs and therefore shows the expression level of each gene of interest. The commercially available microarray instruments and analysis software make it convenient for other applications.

The protein and small molecule microarray methods have also been developed [63-69]. However, these methods have been applied mainly to study the protein-protein interaction and protein-small molecule interactions. Only one study showed the effects of small molecules on live cells in a microarray format [70]. In this study, the cells were cultured on the entire area of the microarray including the empty spaces between the spots. The effects of the drugs in the microarray were observed from responses of cells near the microarray spots. This method required a large quantity of cells which must cover the microarray area. The cells on one spot could influence the cells on another because the cells were all interconnected and free to migrate on the microarray. There was not a clear border distinguishing the cells on one spot from the neighboring cells. A method that can keep the cells only on the microarray spots and isolated from neighboring spots may address some of the concerns.

## I.G Figures

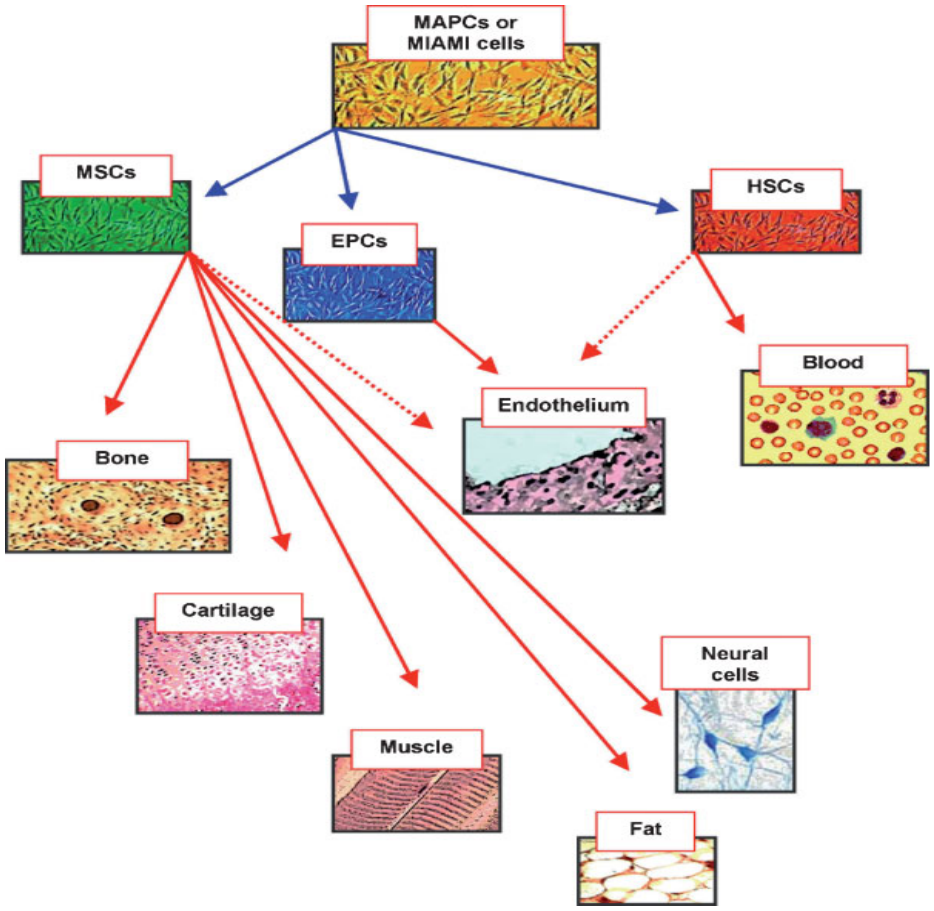



**Figure I.1: Differentiation hierarchy of bone marrow stem cells.**
The multi-potent adult progenitor cells (MAPCs) or marrow isolated adult multi-lineage inducible (MIAMI) cells may differentiate into mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs) and hematopoietic stem cells (HSCs). The MSCs can then differentiate into multiple lineages of tissues including bone, cartilage, muscle, fat, neural cells and likely endothelium. The EPCs can differentiate into endothelium. The HSCs can differentiate into various blood cells and likely endothelium.

GIORDANO et al., J. Cell. Physiol. 211: 27–35, (2007)

# Chapter II Extracellular Matrix Protein Profile for Mesenchymal Stem Cell Differentiation

## II.A Abstract

As an important part of the microenvironment, the ECM protein plays an important role in supporting and regulating the behavior of cells. Previous studies have shown that the ECM proteins deposited by various types of cells may induce mesenchymal stem cell differentiation. However, the composition of the differentiation-inducing ECM proteins remains unclear. A well-defined ECM protein condition may be advantageous for standardized tissue engineering. We utilize the microarray technology to screen the best from sixty-three ECM protein combinations for myogenic and neurogenic differentiation in mesenchymal stem cells. Six commonly found ECM proteins collage I, collage III, collage IV, collage V, fibronectin and laminin are mixed in all possible sixty three combinations with each component at equal concentration, i.e., in the presence of more than one protein components in a combination mixture, the concentration and quantity of each of those protein components are the same, with the total concentration equal to that for a single protein. These ECM protein combinations are then deposited onto a glass slide coated with a thin layer of dehydrated poly-acrylamide in a microarray format. A commercially available DNA microarray robotic system is used for this procedure. Then the suspended cells are seeded on the ECM protein microarray. Because the poly-acrylamide surface prohibits cell attachment, cells only grow on the microarray

spots creating isolated cell islands. The differentiation markers of the cells are fluorescently labeled. The resultant signals are then analyzed using standard microarray software. The best ECM protein combinations for myogenic or neurogenic differentiation are identified. These well-defined conditions will help produce more standardized mesenchymal stem cell-based tissue engineering.

## II.B Introduction

The ability of stem cells to become other functional cells has brought excitement to the scientific community in recent years. The potential of the stem cell-based cell therapy and tissue engineering in treating and even curing devastating diseases has also captured the public attention. Between the two major categories of stem cells, adult stem cells may have more near future clinical applications than embryonic stem cells because of factors such as autologous source and less complexity. In fact, the hematopoietic stem cells isolated from the bone marrow have been successfully applied to restore patients' blood generating capacity for decades. Another group of cells from the bone marrow that may have important clinical applications is the mesenchymal stem cells. The mesenchymal stem cells have been shown to have tri-lineage: myogenic, osteogenic and neurogenic potentials [8]. Because of their abundant availability, stability and multi-tissue generation capability, the possible clinical applications of mesenchymal stem cell are being heavily explored. Numerous clinical trials have been initiated and completed to assess the safety and efficacy of mesenchymal stem cell-based cell therapies, e.g., for improving cardiac function after myocardial infarction and reducing the adverse effects of chemotherapy.

However, these therapies are mostly administrated by injecting the mesenchymal stem cells intravenously or locally with little pre-manipulation to optimize the therapeutic efficacy. Though valuable, such therapies are still far from the cell-based tissue engineering the public hopes. One of the possible methods for such tissue engineering is through surface-induced differentiation of the mesenchymal stem cells. Several surface differentiation factors have been demonstrated, including substrate rigidity [39], substrate surface pattern [71] and cell-deposited ECM proteins [37, 49]. Because of the biological friendliness and ease of handling, the ECM proteins have certain advantages as a differentiation inducing surface factor in tissue engineering. Most current studies focus on the cell-deposited extracellular matrices. There are variations in the matrices secreted by different types of cells. The matrices deposed by allogenic cells may lead to immune rejection. An artificially synthesized ECM would be valuable for such surface-induced differentiation. To avoid the difficulty of identifying the exact natural compositions, we employed the high-throughput screening technology to identify an optimal combination of ECM proteins as a starting point for the artificially synthesized differentiation-inducing surface.

## II.C Materials and Methods

In order to create isolated cell islands in ECM microarrays, poly-acrylamide, which prohibits cell-attachment, is chosen. In addition to preventing cell attachment, poly-acrylamide has no cell toxicity and appropriate porosity for protein absorption. In order to effectively use the existing microarray fabrication and imaging instruments,

the poly-acrylamide is coated on standard 25 x 75 mm glass slides. A chemical cross-linking process is used to covalently bond the poly-acrylamide to the glass.

### II.C.1 Glass Slide Activation

The glass slides were modified based on a protocol previously established by Flaim et al. [72]. The glass microscope slides (75 × 25mm, Corning) were cleaned with detergent followed by five complete rinses in deionized water ($dH_2O$). After washing in fresh Millipure water (18 M-ohm/$cm^2$, $MQH_2O$), the slides were washed with 100% acetone, 100% methanol, and finally with five rinses of $MQH_2O$. The slides were next etched in 2 M NaOH solution for 1 hr, followed by thorough $MQH_2O$ rinsing. The slides were dried with filtered compressed air and then further dried in a vacuum oven for 1 hr. The dried slides were then functionalized in a 2% solution of 3-(trimethoxysilyl)propyl methacrylate (Sigma) in 95% ethanol solution over 1 hr, rinsed in toluene or 100% ethanol, dried with compressed air, and baked for 15 min at 80°C in a vacuum oven.

### II.C.2 Poly-Acrylamide Gel Preparation

Method 1: This method was modified from a protocols previously established by Pelham and Wang [73]. The reaction is illustrated in Figure II.1. Poly-acrylamide solutions composed of 5 – 10% acrylamide ($-CH_2CHCONH_2-$) (Bio-Rad) in $MQH_2O$, 0.05 – 0.2% bis-acrylamide (N,N'-methylenebisacrylamide) (Bio-Rad) in $MQH_2O$. 0.6 mg/ml of ammonium persulfate in water was added to initiate the polymerization process. The free radical generated from the persulfate molecule was responsible to

initiate the polymerization process. The process was rapidly accelerated by adding 4 μl/ml of Tetramethylethylenediamine [$(CH_3)_2NCH_2CH_2N(CH_3)_2$, TEMED (Invitrogen)], a catalyst to the reaction. Once the TEMED was added, the casting process must be carried out within 2 min before the solution gelled. The acrylamide-bis monomer solution was quickly delivered onto the silanated glass slides. 25 x 75 mm No.1 cover glasses (Bellco Glass) were placed over the acrylamide-bis solution. The capillary effect spread the solution into a thin layer. After polymerizing for 20 min, the surfaces of the slides were flooded with MQ $H_2O$. The cover glasses were removed with a razor blade or fine-tip tweezers. The gel covered slides were soaked in MQ $H_2O$ for 24 – 48 hr to remove the excess acrylamide.

Method 2: The reaction is illustrated in Figure II.2. Five ml of acrylamide and bis-acrylamide were mixed in a 15-ml conical tube according to the dilution scheme. The solution was subjected to vacuum / degassing for 15 minutes to remove oxygen, which inhibits acrylamide polymerization. The vacuum pressured was controlled at -10 inches of Hg. Too high of a vacuum could induce severe evaporation. The final volume of the solution was checked for loss of volume. One hundred μl of Irgacure 2959 solution was added to the 5 ml acrylamide-bis solution (200 mg/mL I2959 in 100% methanol, a light sensitive free radical initiator). The acrylamide-bis monomer solution was delivered onto the silanated glass slides. 25 x 75 mm No.1 cover glasses (Bellco Glass) were placed over the acrylamide-bis solution. The capillary effect spread the solution into a thin layer. The solution-covered slides were exposed at a 2-inch distance under the UV (312 nm) lamp for 7 min to induce polymerization. The

surfaces were flooded with $H_2O$. After removing the cover glasses with razor blade or fine tipped tweezers, the gel covered glass slides were soaked in $H_2O$ for 24 hr to remove the excess acrylamide.

### II.C.3 Extracellular Matrix Proteins Spotting

*Protein printing buffer.* 2X protein printing buffer consisted of 200 mM acetate (Sigma S2889), 10 mM EDTA, 40% glycerol, and 0.5% triton X-100 in MQH2O, with pH adjusted to 4.9 using glacial acetic acid.

*ECM protein preparation and mixing.* Purified ECM stock solutions were prepared at 1 mg/ml (rat collagen I, human collagen III, human collage IV, human collagen V, human fibronectin and mouse laminin, and stored appropriately. ECM stock proteins were diluted 50:50 with 2X printing buffer, mixed, and loaded into a 96-well plate. Biomek 2000 liquid handling system was used to deliver the six types of proteins into a 384-well polypropylene source plate (Greiner). The six proteins were mixed into 63 equal-concentration combinations, with the same total concentration. For example, in the combination composed of collage I, fibronectin and Laminin, the concentrations of collage I, fibronectin and laminin are each 1/3 of the concentration when only a single protein is used. There are 63 possible equal-concentration combinations from six proteins.

$$\sum_{r=1}^{6} \frac{6!}{r!(6-r)!} = 63 \quad \text{Equation 1}$$

where r = number of proteins in a combination set. Thus, for example, for three proteins, r = 3, and the number of all possible 3-protein combinations from the choice of 6 proteins is $\frac{6!}{3!(6-3)!} = 20$.

Each pure protein was delivered into the preplanned well positions in the 384-well plate. For example, the wells contain collage I is marked black in Figure II.3.

*ECM arraying.* SMP 3.0 spotting pins (Telechem) were prepared by oxalic acid treatment according to manufacturer's directions. Slides were prepared by dehydration on a 40°C hotplate for 15 min. All printing was done with a SpotArray 24 (Perkin Elmer) using the recommended motion-control parameters (Telechem) at room temperature with humidity controlled to ~65% RH. Each of the 20 ECM was deposited with five replicates at a 450-μm separation between each spot. After printing, the ECM arrays could be stored at 4°C for ~48 h in a sealed box containing slurry of NaCl to maintain the humidity.

### II.C.4 Cell Culture

Human mesenchymal stem cells (hMSC, Lonza) isolated from human bone marrows were cultured in Mesenchymal Stem Cell Growth Medium (MSCGM, Lonza). The cells were initially seeded at ~5,000 cells / $cm^2$. The cells were maintained in 37°C and 5% $CO_2$ in humidified culture incubators. The media were changed every 3 days. The cells were subcultured to 70-80% confluency, dissociated by 0.05% Trypsin EDTA, and centrifuged at 600g for 5 min. The subcultures were

conducted at ~5000 cells / $cm^2$. The differentiation potentials of the hMSC were confirmed using the hMSC adipogenic differentiation medium and hMSC osteogenic differentiation medium (Cambrex). The adipogenic cells induced from hMSCs were confirmed by staining the lipid vacuoles with Oil Red O. The osteogenic cells induced were confirmed by Koss staining.

hMSCs in passages 4 and 5 were used in the experiments. Prior to cell seeding, the multi-well chamber gaskets and slide culture apparatus were sterilized. The microarray slides were exposed to UV for 5 min before being assembled into the culture apparatus with the gaskets. The cells were dissociated from the culture dish by 0.05% Trypsin EDTA. Then 150 μl of cells were seeded into each well at ~1000 cells / $cm^2$. The cells were allowed to attach to the ECM protein microarray for 30 min. The loose cells were washed away, and the initial images were acquired.

### II.C.5 Immunofluorescent Staining

*Confirmation of Differentiation Potential*

The human mesenchymal stem cells were first cultured to confluency. The media were changed into osteogenic differentiation media or adipogenic differentiation media. After 7 days of differentiation induction, the cells were fixed with 4% paraformaldehyde. The adipogenically induced hMSCs were stained with Oil Red O. The osteogenically induced cells were processed with Koss staining. The images were then acquired.

*Staining of Differentiation Markers*

After 5 – 7 days of culturing, the cells on the microarray were stained with the differentiation marker antibodies of interest. Thus, 20 μg / ml of MyoD (Chemicon) for myogenic differentiation and 20 μl/ml of neuro-specific Tubulin β III (Sigma) for neurogenic differentiation were added to individual microarrays. The fluorescent signals were generated by adding 20 μg/ml TRITC secondary antibody, which was washed away before imaging.

*Nuclear Staining*

After fixing, the cells were stained with POPO-3 iodide nuclear acid stain (Invitrogen) or Hoechst 33342 nuclear stain (Sigma) for 10 min. POPO-3 was prepared at 0.2 μM in 5X SSC (3 M sodium chloride, 0.3 M sodium citrate) at pH 7. Hoechst 33342 was prepared at 10 μg / ml in PBS. The sample was incubated with either solution for 30 min in dark. The staining agents were thoroughly washed away before imaging. The POPO-3 stain requires excitation wavelength of 543 nm, whereas Hoechst is excited at 350 nm.

### II.C.6 Extracellular Matrix Protein Microarray Characterization

The fabricated ECM microarrays were labeled with antigen-specific antibodies anti-collagen I, III, IV and V (ABR-Affinity Bioreagents), anti-fibronectin (Abcam), anti-laminin (Sigma) at 20 μg/ml. The anti-mouse TRITC secondary antibody at 20 μg/ml was added to give the fluorescent signal. The slides were allowed to dry in dark before imaging.

## Image Acquisition

### *Microarray Imaging*

The microarray slides. were first fixed with 4% paraformaldehyde, and the cells were then stain for the markers of interest. The slides were allowed to dry in dark for 1 hr before imaging using ScanArray 4000 (GSI Lumonics), a multi-color laser confocal scanner for glass slides Three excitation wavelengths are available at 543 nm, 594 nm, and 633 nm. Various levels of laser power and detector sensitivity were chosen to optimize the image quality.

### *Semi-Automated Microscopy Imaging*

Though convenient, the ScanArray 4000 (GSI Lumonics) can only take fixed and dried samples. In order to perform live-cell imaging, the traditional microscopy imaging was employed. Because of the large quantity of the images, an automated stage was programmed with IPLab to scan the desired areas of the microarray.

## Data Analysis

The quantitative microarray signals from the ScanArray 4000 scanner were analyzed using Genepix Pro 3.0 (Molecular Devices). A standardized spot size was chosen in Genepix Pro to analyze the intensity of fluorescent signal on each spot. The final signal obtained was the overall intensity minus the background intensity. In every set of experiments, all the imaging acquisition parameters such as exposure time, laser power and detector sensitivity were kept the same. The differentiation marker signal is normalized by the amount of cells represented by the nuclear stain signal. Three

biological repeats of experiments were conducted to obtain the mean value and the standard error of the mean.

## II.D Results

The multi-potency of hMSCs (Lonza) was characterized by differentiation induction. The confluent, early-passage, undifferentiated hMSCs appeared spindle shaped (Figure II.4 **A**). The induced adipogenic cells produced lipid vacuoles clearly stained by Oil Red O (Figure II.4 **B**). The $Ca^{2+}$ deposited by the induced osteogenic cells was stained dark by Kossa staining (Figure II.4 **C**).

The ECM proteins microarray was labeled with anti-collagen I, III, IV and V, or anti-fibronectin (Figure II.5). The image was imaged using ScanArray 4000 (ex, 546 nm; em, 617 nm). Each ECM protein combination is printed with five repeats. Therefore, the complete 63 conditions plus one extra repeat of condition No. 63 require 320 spots. The 320 spots are separated into four subarrays due to the culturing chamber dimension.

One subarray stained by anti-fibronectin is shown in Figure II.6. The exact composition of each condition is labeled next to the spots. The spots without fibronectin show no detectable fluorescent signal. The quality of the spots is shown in Figure II.7. Each individual protein of the six ECM proteins was separately printed and stained.

The ECM protein microarray slides were assembled into a culturing apparatus with multi-well gaskets (Figure II.8). The wells are sealed from one another. The lower two rows were not used in this case.

The cellular microarray is shown in Figure II.9. The bright field image shows the isolated cell islands on each microarray spot (Figure II.9 A). The fluorescent image of these cells is shown in Figure II.9 B.

The effect-profile, the profile of the differentiation-inducing effects of ECM combinations, of the ECM protein combinations on the hMSCs for 7 days is presented in an increasing order in Figures II.10 and II.11. For myogenic differentiation, the combination of collagen I, III, V and fibronectin induced the highest expression of MyoD, an early muscle lineage marker. For neurogenic differentiation, the combination of collagen III + IV, and collagen IV + V induced the highest expression of neuro-specific Tubulin βIII, a neural lineage marker.

## II.E Discussion

The potential of stem cells to become other functional cell types captured the scientific and public imagination. The hope to repair and replace injured or diseased tissues using stem cells inspired numerous studies to apply them in cell therapy and tissue engineering. Because of their abundance, ease of extraction and stability, adult stem cells such as MSCs have certain advantages in becoming clinically applicable soon. One approach for MSC differentiation is to grow them on a scaffold with a differentiation-inducing surface. Many studies have showed that the ECM deposited by a particular type of cells may effectively induce differentiation. However, applying cell-deposited ECM is associated with some practical problems such as contamination from the depositing cells, immune reaction and batch-to-batch variation. A well-defined ECM prepared artificially using purified components may be a solution to

these problems. In this study, we applied the microarray technology to screen 63 ECM combinations to create an effect-profile of mesenchymal stem cells towards myogenic and neurogenic differentiations.

The DNA microarray technology was made available by the robotic printing system, reading instrument, and data analysis software. The live cell microarray was developed based on these existing instruments and software. Six commonly found ECM proteins: collagen I, collagen III, collagen IV, collagen V, fibronectin and laminin were mixed into 63 combinations with equal total concentrations. Five repeats of 63 combinations plus an extra combination No. 64 were printed into one set of microarray composed of four subarrays on one glass slide (Figure II.5). This format was chosen because of the dimension of the multi-well culturing gaskets (Figure II.8). The multi-well gasket could isolate the subarrays from each other, allowing independent soluble factors to be added or stained separately. The protein microarray format was printed on a poly-acrylamide coated glass slide. Each protein spot in the microarray was specific and uniform (Figures II.5, II.6 and II.7). One subarray was stained fluorescently with anti-fibronectin antibody (Figure II.6). The spots containing fibronectin expressed fluorescent signal, whereas the ones without fibronectin were very dim. The protein spots were very specific to its presence in the combination. In Figure II.7, collagen I, collagen III, collagen IV, collagen V, fibronectin and laminin were printed individually into 10 spots each, and then stained with their own corresponding antibody. The protein spots were very uniform in shape and intensity.

The characterization ensured the reliability of the variations in intensity resulting from differences of protein combinations.

In the present study, I have demonstrated the differentiation potentials of hMSCs on different ECM protein combinations on microarray slides. The cells were cultured separately in adipogenic and osteogenic differentiation media. The ability of these cells to become adipogenic and osteogenic are shown in Figure II.4. The microarray glass slides were assembled into the culturing apparatus with the multi-well gaskets (Figure II.8). After seeded onto the ECM protein microarray in the culturing apparatus, the cells only attach to the ECM microarray spots, forming isolated cellular islands on these spots (Figure II.9). The Hoechst fluorescently labeled cells in Figure II.9 allowed us to quantity the amount of the cells.

After 7 days of culturing, the cells were fixed and stained for the differentiation markers. MyoD and neuro-specific Tubulin βIII were chosen as markers for myogenic and osteogenic differentiations, respectively, based on previous studies [39]. The nuclear stain was also applied to quantify the cell number.

The stained slides were then scanned using the ScanArray. The profile showed that the combination collagen I, III, V and fibronectin and the combination collagen III and IV had the most myogenic and neurogenic differentiation-inducing effects, respectively. It should be noted that differentiation is a complicated process and that one differentiation marker alone may not be sufficient to characterize the degree of differentiation. Other markers, genetic profile and functional assays will be needed to verify and quantify the degree of differentiation. The advantage of the microarray

technology is its high throughput. The goal of this study is to demonstrate the use of the microarray technology for the high-throughput screening to select matrix combinations that are most favorable for the desired differentiation, thus reducing the large number of combinations to a few that become manageable for further detailed analysis to identify the most optimal differentiation conditions with these artificial matrices. Thus, the microarray approach provides a valuable starting point for identifying the optimal differentiation matrix and significantly reduces the time and efforts in search of such conditions.

## II.F Figures

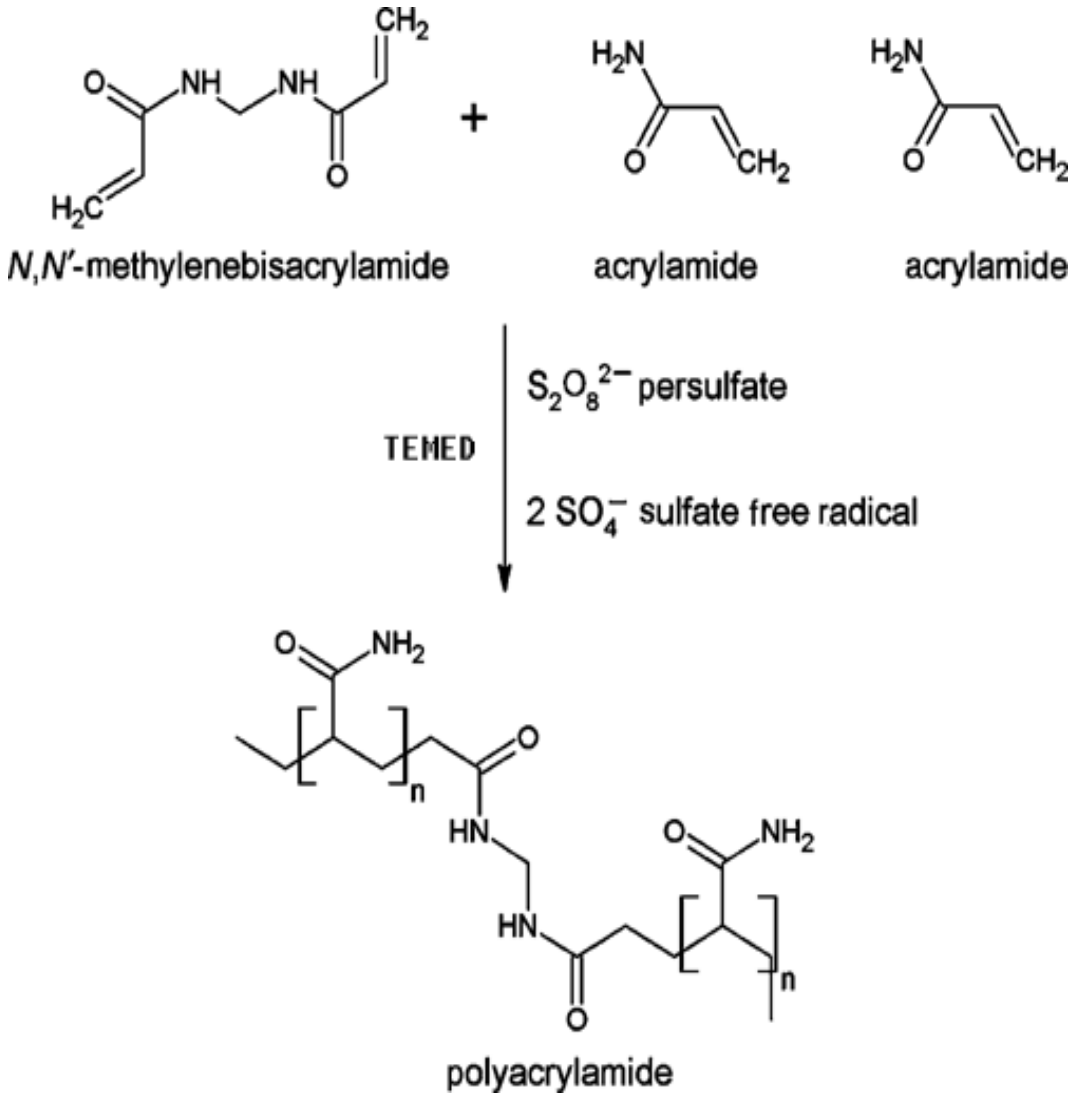



**Figure II.1: Acrylamide and bis-acrylamide polymerization reaction 1.**
The polymerization process is initiated by the free radical generated from persulfate and catalyzed by TEMED.

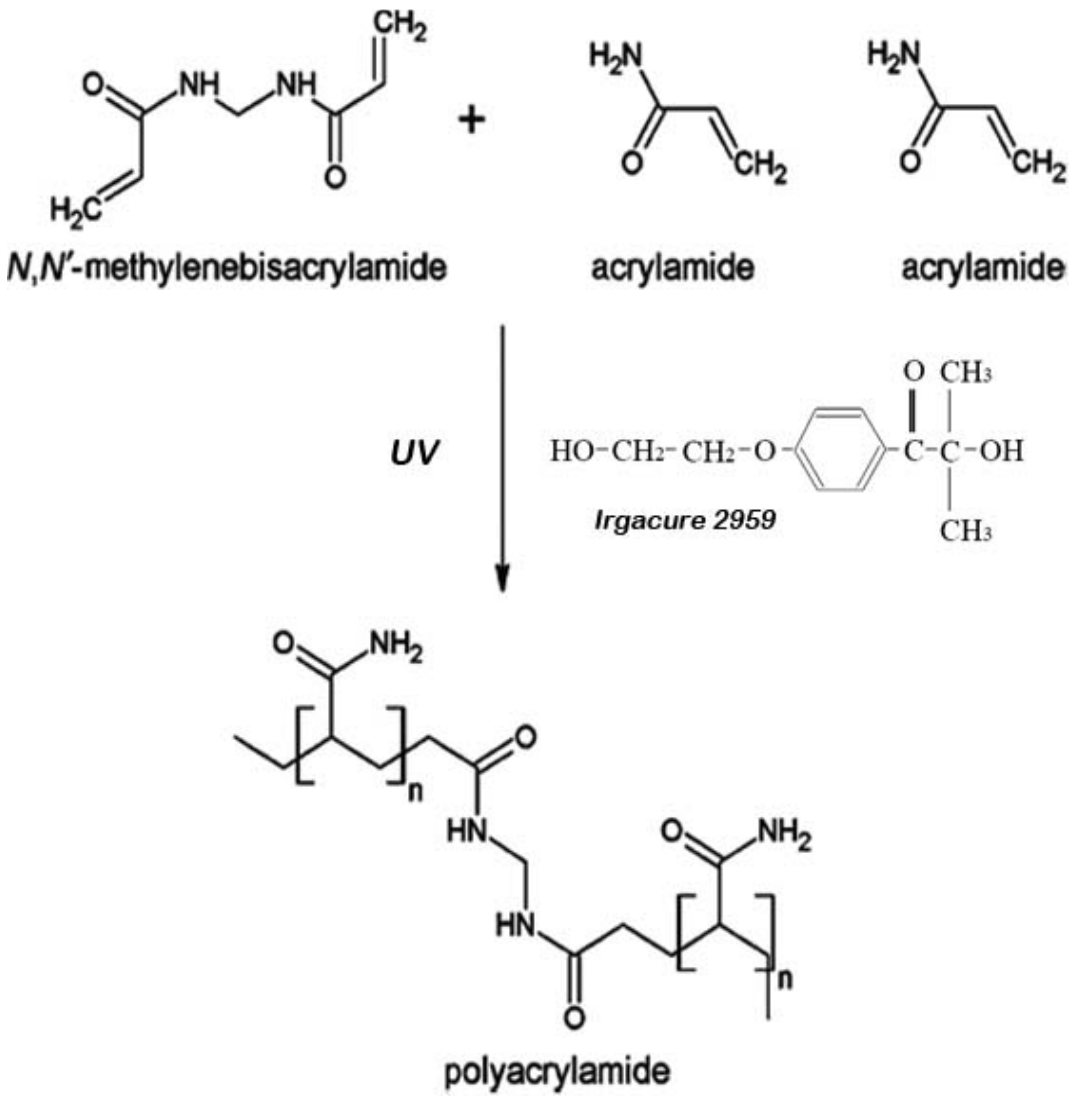
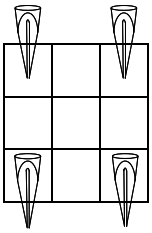



**Figure II.2: Acrylamide and bis-acrylamide polymerization reaction 1.**
The polymerization process is initiated by the free radical generated from Irgacure 2959, a photo-initiator activated by UV.

**A**



**B**

| | a | b | c | d | e | f | g | h | i | j | k | l | m | n | o | p | q | r | s | t | u | v | w | x |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1 | | 3 | 4 | | 5 | | | | | | | F | | L | 13 | | 14 | | | | | | |
| B | | | | | | | | | | | | | | | | | | | | | | | | |
| C | 15 | | 1F | 1L | | 34 | | | | | | | 35 | | 3F | 3L | | 45 | | | | | | |
| D | 4F | | 4L | 5F | | 5L | | | | | | | FL | | 134 | 135 | | 13F | | | | | | |
| E | | | | | | | | | | | | | | | | | | | | | | | | |
| F | 13L | | 145 | 14F | | 14L | | | | | | | 15F | | 15L | 1FL | | 345 | | | | | | |
| G | 34F | | 34L | 35F | | 35L | | | | | | | 3FL | | 45F | 45L | | 4FL | | | | | | |
| H | | | | | | | | | | | | | | | | | | | | | | | | |
| I | 5FL | | 1345 | 134F | | 134L | | | | | | | 135F | | 135L | 13FL | | 145F | | | | | | |
| J | 145L | | 14FL | 15FL | | 345F | | | | | | | 345L | | 34FL | 35FL | | 45FL | | | | | | |
| K | | | | | | | | | | | | | | | | | | | | | | | | |
| L | 1345F | | 1345L | 134FL | | 135FL | | | | | | | 145FL | | 345FL | 1345FL | | 1345FL | | | | | | |
| M | | | | | | | | | | | | | | | | | | | | | | | | |
| N | | | | | | | | | | | | | | | | | | | | | | | | |
| O | | | | | | | | | | | | | | | | | | | | | | | | |
| P | | | | | | | | | | | | | | | | | | , | | | | | | |

**Figure II.3: Protein Loading Map in 384-Well Plate**
The pattern of protein loading consists of four wells in the corners of a 9-spot gridone, with these wells all separated by one space because of the geometry of the four pins used to print the microarray (**A**). The protein combination map in the 384-well plate is shown in (**B**). 1: collage I, 3: collage III, 4: collage IV, 5: collage V, F: fibronectin, L: laminin. As an example, the combination of collage I, III, V and fibronectin is represented as 135F. Black highlighted wells are those that contain collage I.

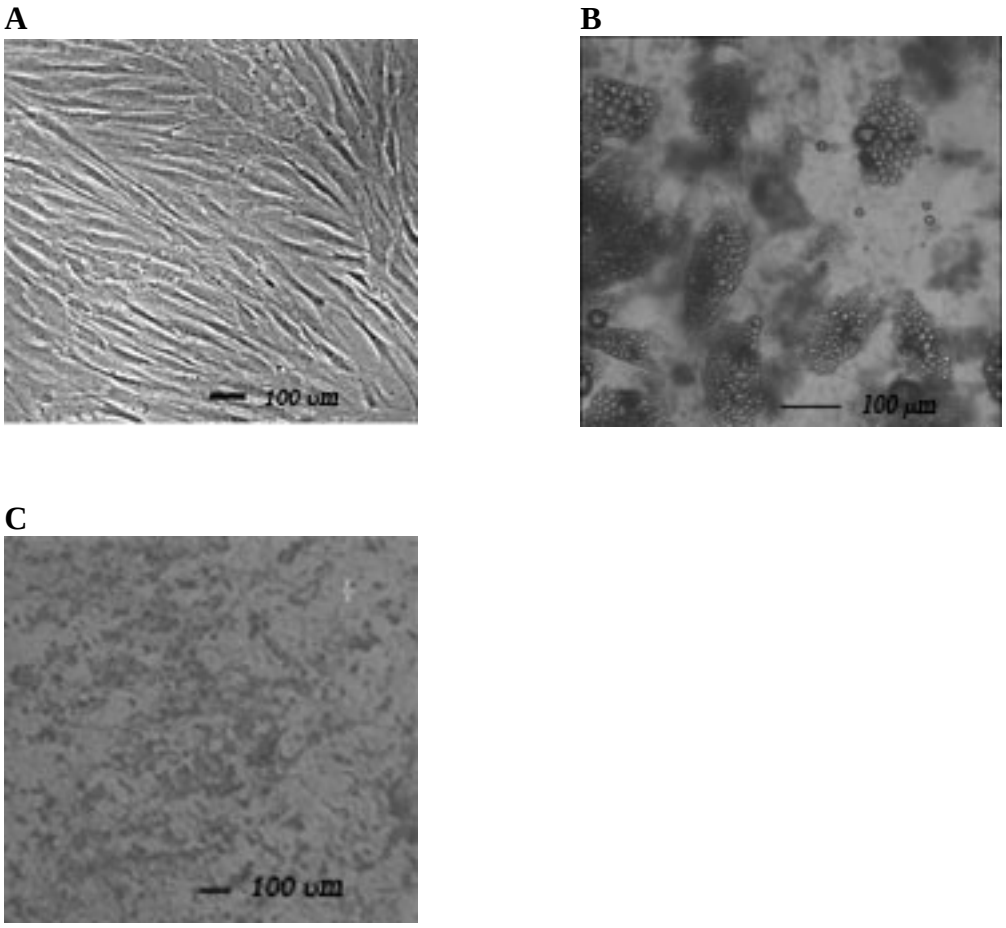



**Figure II.4: MSCs Characterization.**
**A.** Undifferentiated MSCs. **B.** Adipogenic induced MSCs. Oil droplets are clearly visible. **C.** $Ca^{2+}$ deposits stained dark with $AgNO_3$ indicates the osteogenic induced MSCs.

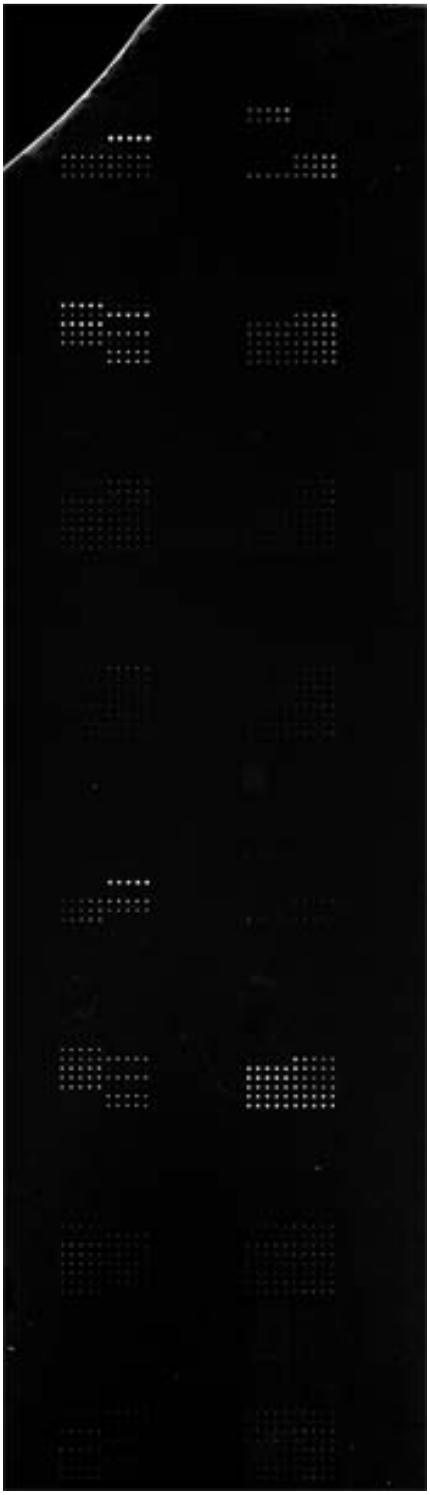


**Figure II.5: Whole slide (25 x 75 mm) microarray stained with anti-fibronectin and anti-collagens.**

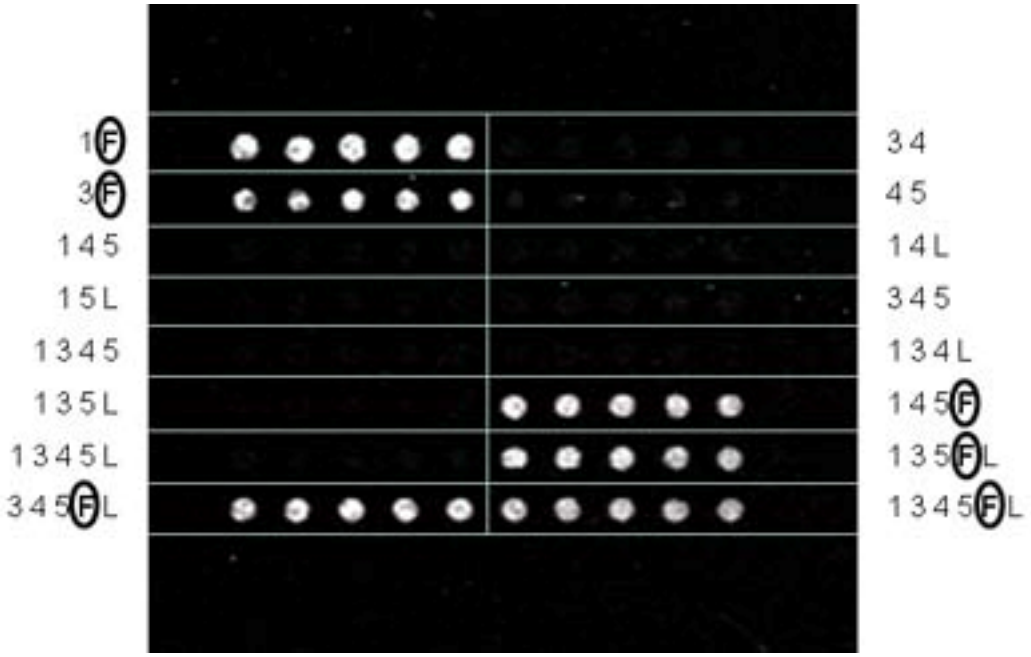



**Figure II.6: Anti-fibronectin stained subarray.**
One array containing 16 extracellular matrix protein combinations with 5 repeats stained with anti-fibronectin. 1, 3, 4, 5, F, L represent collagen I, III, IV, V, fibronectin and laminin respectively. Anti-fibronectin antibody stained only the spots of combinations that contain fibronectin.

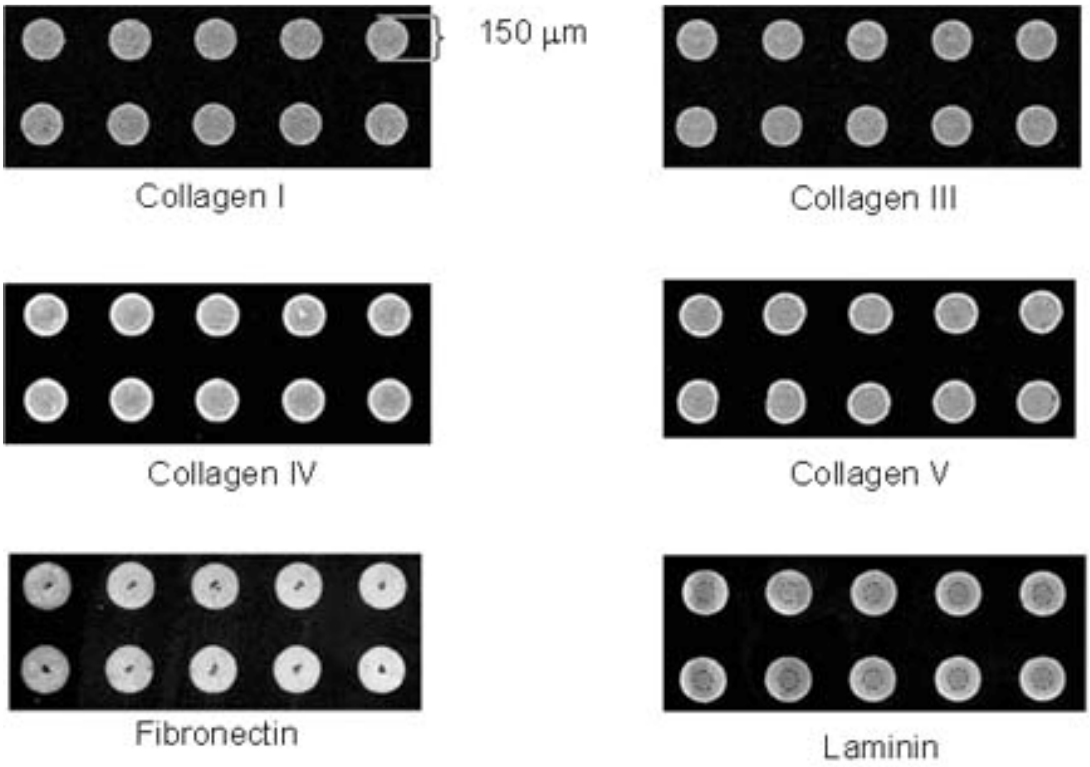



**Figure II.7: Individually stained ECM protein spots.**
Each set of 10 spots contain only one type of extracellular matrix protein. Each set was stained with the respective antibodies.

**Figure II.8: Multi-Well Microarray Culture Apparatus.**
The apparatus contains 3 microarray slides. Three sets of 16-well gaskets were pressed on top of the slides by the apparatus. Each well surrounded one subarray of 80 spots. The bottom four wells were not in use. The clean and dry unused wells indicate the complete seal and separation provided by the 16-well gaskets.

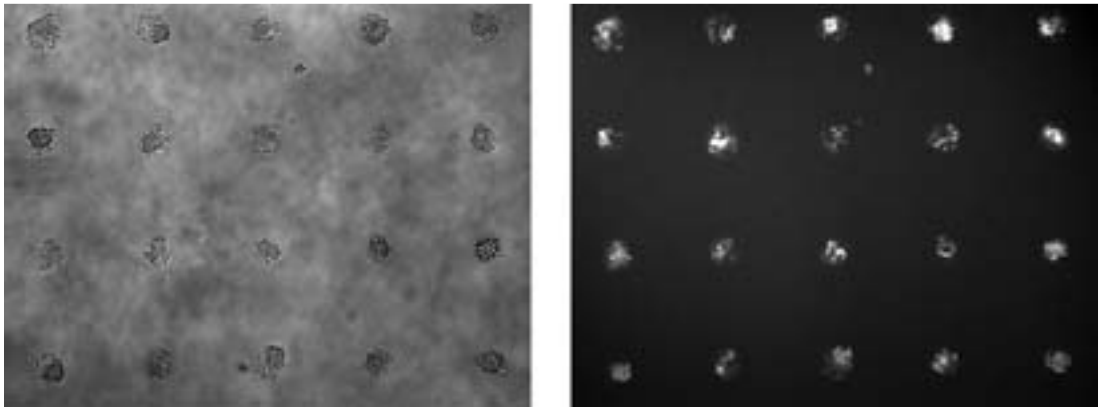


**Figure II.9: Cells seeded on a 5x4 microarray with 20 spots.**
**A**: Bright field image with 4x objective. **B**: Cells with Hoechst nuclear stain on microarray with 4x objective.

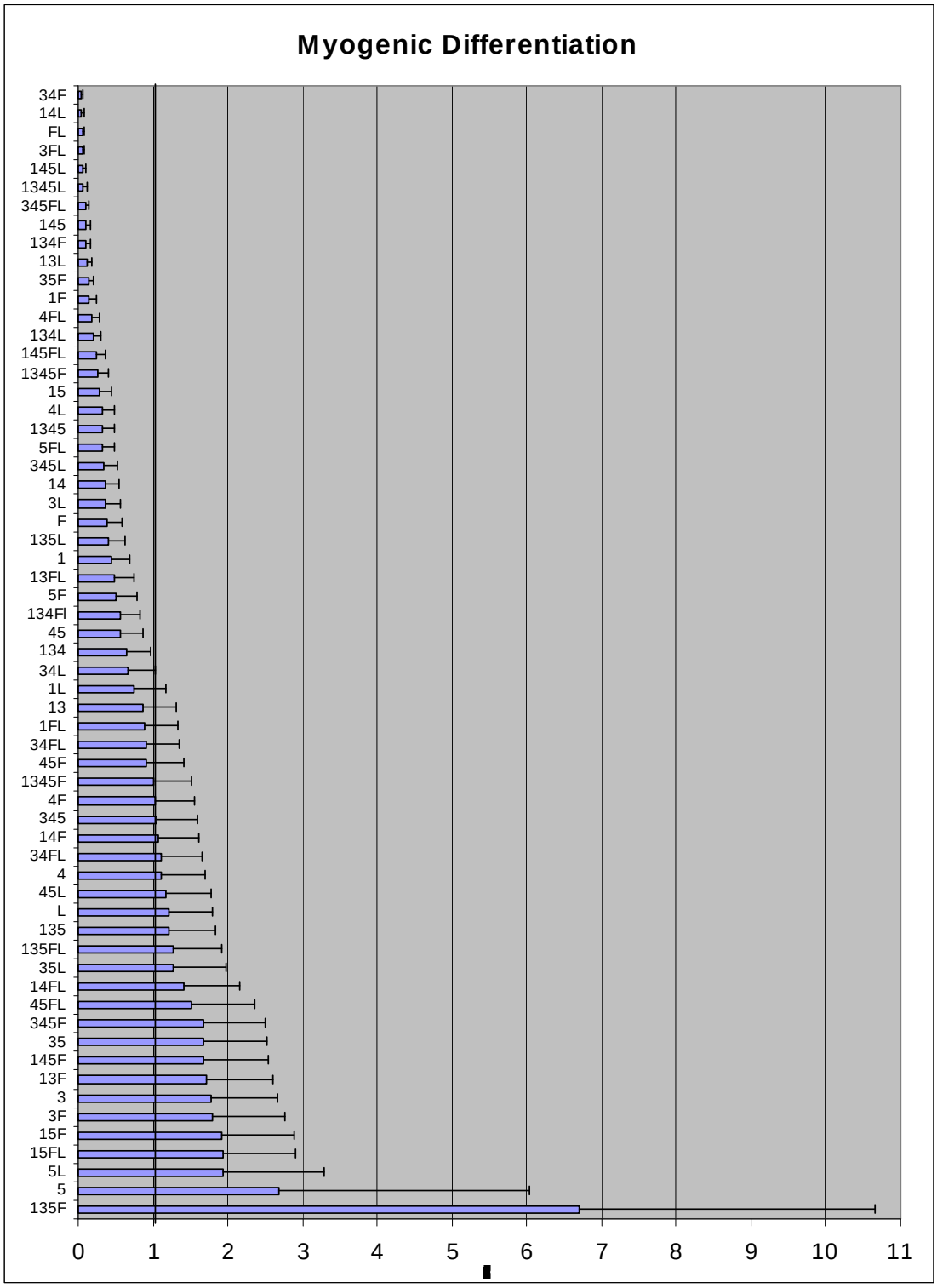



**Figure II.10: The effects of the 63 ECM combinations on hMSC towards myogenic differentiation.**

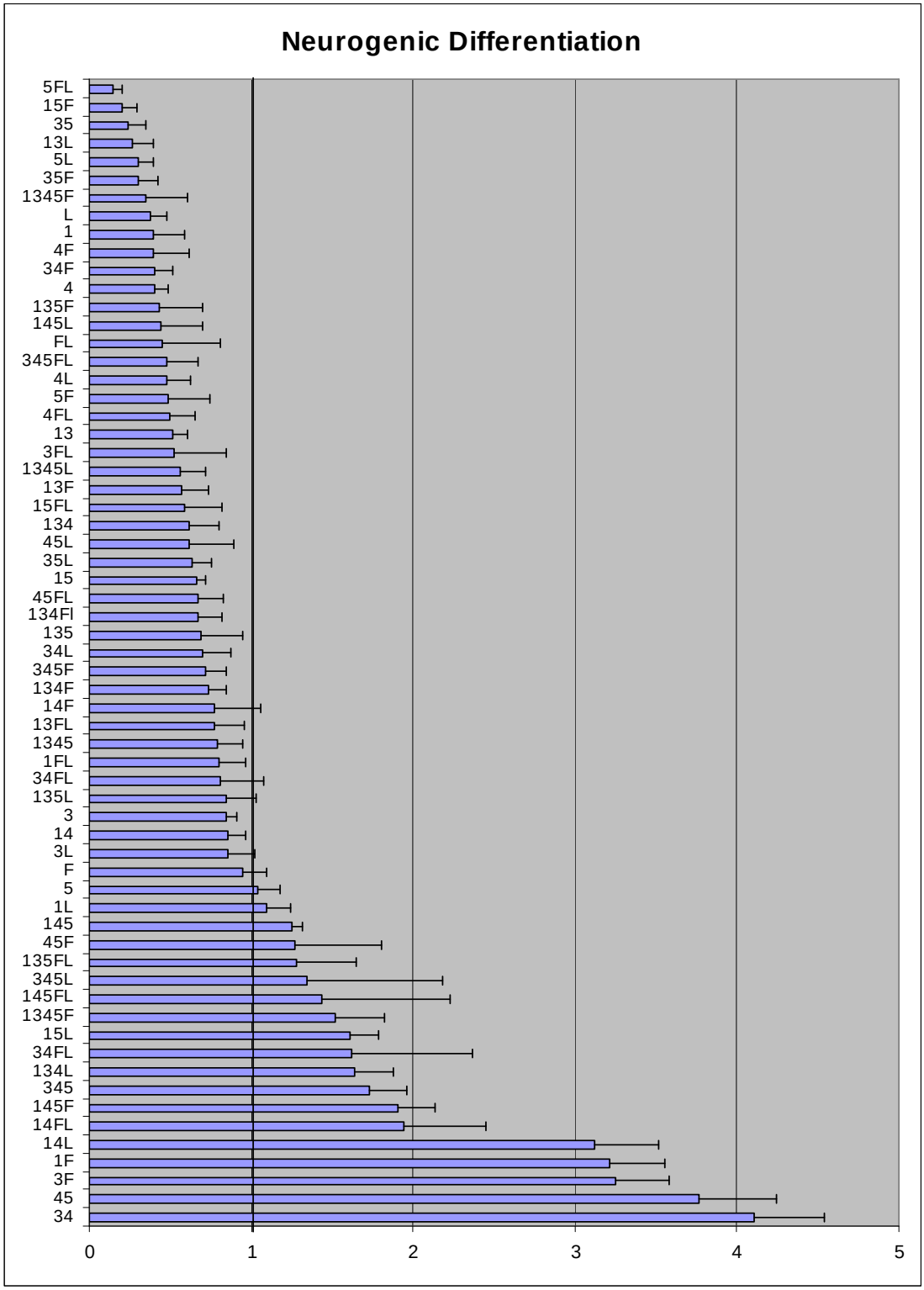



**Figure II.11: The effects of the 63 ECM combinations on hMSC towards neurogenic differentiation.**

# Chapter III Application of Extracellular Matrix Protein Microarray and Small-Molecule in Cancer Cell Culture and Personalized Therapy

## III.A Abstract

The difficulty to maintain and expand primary cancer cells has been a hindering factor in cancer research. The importance of the ECM in regulating cancer cell behaviors such as proliferation has been well-recognized. A growth-supporting ECM may be a necessary ingredient to maintain and expand the primary cancer cells.

In this study, the ECM microarray technology was applied to identify positive growth-supporting conditions for cancer cells. In these experiments, sixty three combinations from six commonly found ECM proteins were spotted onto a microarray using. As an example, the four glioblastoma (brain cancer) cell lines were seeded onto the ECM microarray. The effects of each of the 63 ECM combination on glioblastoma proliferation were determined. The 12 combinations that best support the growth of the glioblastma cells were identified. The ECM microarray method was also applied to the primary cancer cells isolated from patients. The best growth-supporting combination: collagen I, III and IV for the patient cancer cells was one of the 12 conditions identified using the cancer cell lines.

In addition to determining culture conditions, the microarray technology can also be used to isolate subpopulations for cells. In this study, unusually large cell aggregates were observed occasionally. It is likely that a small subpopulation of the highly proliferating cells were in those large aggregates. In recent years, it is believed

that only a small subpopulation of highly proliferating and invasive cells is responsible for the malignancy of the cancer. These cells are referred as the cancer-initiating cells or cancer stem cells. The ability to isolate the highly proliferating cells may be an effective tool in studying the cancer-initiating cells.

Heterogeneity exists not only in tumor cells but also in the patient population. Much similar to the different ECM preferences of the glioblastoma cell lines, patient-to-patient variations in drug-response have been well-known. The importance of an individually-designed treatment or personalized medicine has been long recognized.

In this study, a small-molecule microarray method was used to screen many drugs and their combinations against patient's diseased cells for the best treatment for that patient. Unlike the ECM proteins, the small-molecule drugs have very high diffusivity. The small molecules must be immobilized on a microarray format to maintain the effective time. An immobilization method using polymers such as was poly-acrylamide developed. The experiments showed the fluorescent small-molecule drugs were effectively retained on the spots after the microarray was immersed in media for hours. The growth-supporting ECM for the diseased cells was then spotted over the small molecule microarray, allowing the diseased cells to attach on the drug microarray. The effects of the drugs and their combinations could then be observed.

This ECM microarray method not only can be applied to maintain the primary cancer cells but also be used to isolate the possible cancer-initiating cells. The expanded cancer cells can then be applied in the small-molecule drug microarray

screening. The patient-specific drug-response data may help the physicians to design a personalized therapy for each individual patient.

## III.B Introduction

Cancer, as one of the top disease killers of all times, has devastating effects on human health and quality of life through the history. It not only tortures the bodies of the patients, but also brings mental suffering to their family and friends. After decades of efforts in basic research, developments in drug and surgical technology, and progresses in clinical care, significant advancements have been made in cancer treatment. Now many types of cancer have become manageable. The prognosis of many successfully managed types has become years and decades, instead of months. Having a cancer is no long a death warrant in many cases. In spite of the successes against cancer, however, much remains to be done. There are still aggressive types of cancer such as the glioblastoma, a brain cancer that has the prognosis of 6-18 months.

In cancer research, a primary method is the study of cancer cells in vitro. Much of the successful advancements against cancer were based on the modern molecular studies, drug screening and biotechnologies, which all heavily rely on the use of cancer cells in vitro, often needing a large number in the millions. Therefore, the availability of cancer cells with adequate amount and good quality is a fundamental requirement for cancer research. One of the characteristics of cancer is uncontrolled growth and division beyond normal limits. One would expect the culturing and expansion of cancer cells in vitro should be easier than most other primary cells. However, counter-intuitively, most primary cancer cells cannot be grown in vitro. A

few that do may stop growing after a very short time, making the cell source very scarce. Some cell lines have been developed for this purpose. The cell lines are immortal cells that can be easily cultured and expanded. However, the inserted genes that make the cell lines immortal may have significantly altered their characteristics, which are critical in cancer treatment research. In addition, the process of creating a cell line carries many uncertainties and the production yield is very low. A method to effectively culture and expand the primary cancer cells extracted from a tumor sample will be very valuable for development of cancer research.

Like most other types of cells, cancer cells with the exception of blood cancers are also attached to the ECM. The interactions between cancer cells and the ECM are very influential to the behaviors of the cancer [74, 75]. The cancer cells may need the proper ECM to attach and grow on. Since various types of cancerous tumors have very different extracellular matrices, the matrix environments vary from cancer to cancer. A cancer-specific ECM may support the growth of the primary cancer cells. A high-throughput screening method may be effective for identifying such supportive conditions. The development of such a method is presented in this study.

The primary cancer cells expanded using the identified growth-supporting ECM may have meaningful research and clinical applications. One of such clinical applications is to use the expanded cancer cells extracted from a patient towards designing a personalized treatment for that particular patient.

Personalized medicine has been receiving much attention in recent years. The concept that treatment should be tailored for every individual patient is not new. It is

well known that patients with the same disease may respond to the same drug differently. This may be caused by the different drug transport, drug metabolism and drug sensitivity. However, because of the complexity of the problem, there is still not an effective solution, and many of the therapeutical approaches are mainly trial-and-error. The development in biotechnology in recent years such as the human genome project has made it possible to map the genetic differences among individuals. Such information may provide insights into how an individual may react to a particular drug, thus providing a possibility to render personalized treatment. However, extracting treatment-relevant data from the complicated and vast amounts of genome information has been challenging.

While much effort has been focused on the genetic-based personalized medicine, another avenue is to apply direct drug efficacy screening on patients' own cells. This has not been done because of the source of the patients' cells, especially diseased cells, are very limited. The traditional microwell drug-screening methods require large number of cells. Typically, the standard drug screening is conducted in 384 – 3456 well plates. Even in the 3456-well plate, a minimum of 200 cells are needed in a well / drug. Then there is the high capital cost of the robotic liquid handling system to process 3456-well plates. A more resource-efficient method is needed for drug screening on patient's diseased cells.

For its miniaturization and high throughput, the microarray technology, which is a technology that has risen to meet the challenge of genomic studies, could be adopted to conduct drug screening with a small number of cells. One previous study

reported the development of a small molecule microarray [70] by culturing the cells over the entire slide covering both the small molecule microarray spots and empty spaces. The cell density would decrease on the spots containing effective drugs. However, there were a few shortcomings of this method. The cells grown on one microarray spot were not isolated from other spots. There could be potential cell migration that complicates the results. The affected cells on separate spots were all connected by the cells between the spots. There lacks a distinct border. Also, the quantity of the cells needed to cover the microarray and empty spaces was not necessarily small.

The cellular microarray technology described in the previous chapters of this dissertation may effectively address these problems. There are several advantages to using the ECM microarray. The optimized ECM may enhance the survival and growth of the patients' diseased cells which can often be difficult to maintain. The isolated cell islands can produce distinct and independent results. Only a few cells are needed to attach onto each spot instead of the entire area, lowering the cell quantity requirement significantly. However, incorporation of the small molecule drugs into the microarray may be challenging. The general cut-off molecular weight of the small molecule drug is ~800 Da. Unlike the ECM proteins with molecular weights of kilo-Daltons, these small molecules can rapidly diffuse into the solution. The small-molecule drugs must be effectively immobilized in the microarray and slowly released. In this study, a method was developed to immobilize small-molecule drugs in a microarray format.

## III.C Materials and Methods

### III.C.1 Glass Slide Surface Treatment

The glass slides were chemically treated to provide the necessary substrates for the microarray. The glass slides were placed in a 25-slide stainless steel rack (Electron Microscopy) throughout the chemical treatment process until gel casting. All washing process was carried out in a magnetic stirring system. The slide rack was prompted up by a plastic ring spacer, allowing a stirring bar to spin freely in a glass beaker. The glass beaker was then placed on a stirrer to provide the mixing in the washing process. The Glass microscope slides (75 mm x 25 mm x 1 mm, Premium Precleaned, Fisher) were first washed with a detergent solution (Sparkleen) at 20 g/L in warm tap water for 30 min in the stirring system, and this was followed by five complete rinses in deionized water (dH2O) and another 30 min of Millipore water (18M-ohm/$cm^2$). The slides were then placed in a separate glass jar with 100% acetone on a shaker for 30 min. The same process was performed with 100% methanol. The slides were then rinsed 5 times with MQ water wash and placed in the 2 M NaOH solution for 1 hr heated to 70°C followed by 3 times of MQ water wash. The slides were placed without drying in a 2% 3 – (Trimethoxysilyl)Propyl Methacrylate (Sigma) solution in 95% ethanol prepared immediately before use. After remaining in the solution on a shaker for 1 hr, the slides were washed by dipping the slide-rack 5 times into the 100% ethanol. The liquid layer on the glass slides should be uniform, signaling only one solution is present on the surface. The slides were then transferred into a vacuum

oven to allow the silane to be cured under 80°C heat for 20 min. The slides could be stored for two weeks in a vacuum desiccator.

### III.C.2 Poly-Acrylamide Gel Casting

A 10% acrylamide and 0.5% bis-acrylamide solution was prepared from the 40% acrylamide and 2% bis-acrylamide stock solutions (Bio-Rad Laboratories). Typically, 1 ml of each of the stock solutions were mixed thoroughly with 2 ml of MQ water. 100 μl of the photoinitiator (Irgacure I2959) in 100% methanol at 200 mg/ml was added into the acrylamide-bis solution. The final working solution was placed in vacuum for 15 min to remove the oxygen which could interrupt the polymerization process. Air bubbles would appear on the wall of the solution container at the end of the degassing.

During the degassing process, the silanized glass slides were placed on the reverse side of a 96-PCR tube pack. The bottom of the tubes provides a firm support without touching the sides of the glass slides. Four slides were fitted onto each PCT tube rack. Keeping the sides of the glass slides isolated was important to avoid leaking of the solution due to capillary effect. 150 μl of the degassed working solution was placed on each glass slide. A 75 x 25 mm No.1 cover glass (Bellco Glass) was used to cover the working solution over the glass slides. A gentle dabbing with a Kimwipe would clean off the dusts. Over-cleaning might lead to gel attachment and thus causing difficulty when removing the cover glass. The placement was performed slowly to avoid trapping air bubbles. The capillary effect would fill the space between the cover glass and glass slide with a uniform layer of acrylamide-bis working solution.

The cover glass covered slides were then placed 2 inches under a UV lamp with 365-nm bulbs. The slides were exposed to the UV for 7 min to induce the polymerization and crosslinking of the acrylamide-bis solution. The slides were soaked in MQ water for 5 min. The cover glasses were then removed by a razor blade. A layer of 100-μm think poly-acrylamide gel would be covalently bonded to the glass slides. The gel-coated glass slides were then placed in fresh MQ water for 24 – 48 hr in order to remove any excess acrylamide monomers. The remaining acrylamide solutions were disposed according the safety regulation. Any equipment in touch with the acrylamide solution was handled with care. Acrylamide monomers are toxic whereas the polymers are inert.

### III.C.3 Protein Printing Buffer (2X)

105.5 mg of sodium acetate (Sigma) and 37.2 mg of EDTA were added to 6 ml of MQ water. The solution was vortexed and sonicated for 5 min to ensure complete dissolution. A 100-ml bottle of 100% triton X-100 was heated in a microwave oven for 40 sec to lower the viscosity for easier transfer. The sodium acetate and EDTA solution (6 ml) was also heated in a microwave for 20 sec for easier mixing with triton X-100. 50 μl of heated triton X-100 was added and mixed into the 6-ml solution. Although the solution might appear cloudy at first, it would become clear once cooled to room temperature. Glycerol was added to make the final solution to 10 ml. 40 – 80 μl of acetic acid was added to adjust the pH of the solution to between 4.5 and 5.0.

The solution was sterile-filtered with a 0.45-μm filter. The final solution was placed in a 4°C refrigerator for storage.

### III.C.4 Extracellular Matrix Protein Preparation

The ECM proteins: rat collagen I isolated from rat tails, human collagen III, IV, V and fibronectin isolated from human placenta were purchased from BD; mouse laminin was purchased from Sigma. The proteins were diluted to 1 mg / ml with sterile MQ water. To prepare 200 μl of the final protein solution at 0.5 mg/ml, 100 μl of the 1 mg/ml protein solution and 100 μl of the 2x protein printing buffer were mixed together in individual wells of a 96-well plate. The mixing was done thoroughly and gently. Over-agitation may induce certain proteins to polymerize and hinder the microarray printing quality. The six protein-printing-buffer solutions were then transferred to the designated wells in a 384 well plate. This transferring was performed by a Biomek 2000 liquid handling system. The six protein-printing-buffer solutions were mixed into 63 combinations and an additional repeat of combination No. 63 as the $64^{th}$ to make an even number.

### III.C.5 Microarray Printing

The SMP 3.0 spotting pins (Telechem) were treated with oxalic acid and sonicated in 5% microclean (Telechem) solution for 20 min, rinsed in MQ water, and then dried using Kimwipe paper by gently touch the surface of the pin with the edge of the paper without any supporting force. The pins were allowed to dry completely before loading into the instrument because capillary effect from any residual water

might impede the sliding of the pins when printing, resulting in immobilized pins that do not make appropriate contact with the substrate surface.

The gel-coated glass slides soaked in water were placed on a hotplate at 40°C for 15 min, resulting in completely dehydrated polymer-coated slides. The dehydrated slides were then loaded into the substrate positions in the SpotArray 24 (Perkin Elmer). The printing process was performed at a loading time of 3 sec, printing approaching speed at 25, and print contact time of 50 ms. The humidity in the printing chamber was controlled at 65%. The 64 ECM protein mixtures was printed at repeats of 5, with the total of 320 spots divided into four subarrays, each with 10 x 8 spots. The spot center-to-center distance was 450 μm. The subarrays were separated by 3 mm. The dimensions of the array/subarray were designed on the basis of the multi-well culturing gasket. The printed ECM protein microarray slides could be stored in a box containing NaCl surry to maintain the humidity for 48 hr.

### III.C.6 Multi-Well Microarray Culture

The multi-well gaskets (Whatman) and the culture apparatus were cleaned and soaked in 70% ethanol, washed in MQ water, and then autoclaved prior to the culture. The microarray slides and the culture apparatus were sterilized under UV in the tissue culture hood for 30 sec and 5 min, respectively. The multi-well gaskets were placed on the top of the microarray slides. Each subarray was left exposed in the center of the well. The slide-contacting side of the gasket was lined with a silicone material for sealing purpose. The gaskets and the microarray slides were pressed firmly together with a culturing apparatus, sealing and isolating each well from each other. The

assembled culture apparatus was then sterilized under UV in the tissue culture hood for 30 sec. 100 μl of media was transferred into each well using a multi-channel pipette. The apparatus was then covered with a standard 96-well plate lid and moved to the culture incubator.

### III.C.7 Cell Culture

Four different types of glioblastoma (brain cancer) cell lines DK, U87, WT and V3 were cultured in DMEM supplemented with 10% FBS. The culture conditions were kept the same to observe the effects of the ECM. The cells were kept in a 37°C and 5% $CO_2$ humidified incubator. The four cell lines were transfected with GFP in order to quantify the number of live cells fluorescently as a function of time. The cells were subcultured at 80-90% confluency.

In order to extract cancer cells from a patient sample, the surgically removed tumor tissue was quickly cut into small pieces under sterile condition. The pieces were then placed in a dissociation solution for 5 min to remove the cells from the attached ECM. The cell solution was then centrifuged at 500x g for 10 min. After the removal of the supernatant, the cells were resuspended in 10% FBS-supplemented DMEM and prepared into a density of $10^4$ to $10^5$ cells / ml.

### III.C.8 Small-Molecule Microarray

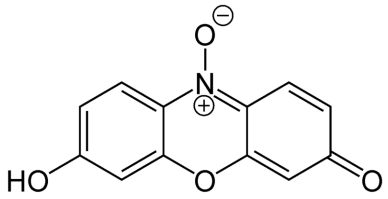
There are two methods immobilizing the small molecules in the microarray.

*Small-Molecule Immobilization Methods*

Method 1: In principle, the water-soluble small-molecule solutions were mixed with the desirable monomer solutions. Because the monomer solutions are typically water based, the DMSO-based small molecule drugs may not be applicable in this method, but will be addressed with Method 2. The small-molecule / monomer mixture was then transferred into a 384-well plate. The microarray robotic system would then print the mixture into a microarray on the substrate. The polymerization process was then initiated on the microarray spots. Once the monomer polymerized, the 3D mesh formed by the polymer chains would trap the small molecules and prevent them from rapidly diffuse away (Figure III.11). This process can be repeated to construct multi-layers of the drug immobilizing polymer. The properties of the polymer such as the polymer chain and crosslink density, chemical affinity and the degradation of the polymer may all be engineering to have the desired drug retaining and releasing characteristics.

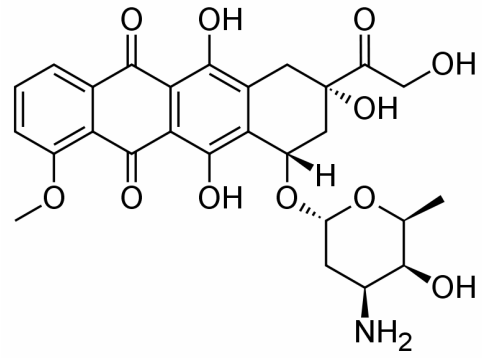
In this case, poly-acrylamide was chosen as the immobilizing polymer. The acrylamide, bis-acrylamide and the photo-initiator were mixed at an appropriate concentration. It was found that 10% acrylamide, 0.25% bis-acrylamide and photo-initiator at 5mg/ml as a good starting point. Specifically, 50 μl of the fluorescent small molecule Resazurin (Sigma), 20 μl of the 40% acrylamide solution (Biorad), 10 μl of the 2% bis-acrylamide solution (Biorad) and 0.4 mg of photo-initiator were mixed together. Resazurin is not a drug but a small molecule fluorescent stain. It was chosen because its fluorescent property makes the detection and quantification convenient. It

is also not toxic like most other drugs [76]. Resazurin has a molecular weight of 251 Da, much lower than the 800 Da cut-off line for small-molecule drugs. Therefore, it was used as a demonstration molecule.




**Resazurin (m.w. 251 Da)**

After the conditions were tested using resazurin, an anti-cancer drug, doxorubicin was used to confirm the method.




**Doxorubicin (m.w. 580 Da)**

The resazurin or doxorubicin - acrylamide mixture was loaded into a 384-well plate. The mixture solution was then printed on the dehydrated poly-acrylamide coated glass slides using SpotArray 24 (Perkin Elmer). Two different pin SMP3 and WCMP were employed to print spots with different quantities and sizes. The SMP3 pin

delivers 0.7 nl of the solution to form spots with the diameter of 100 μm. The WCMP pin delivers 1.5 nl of the solution to form spots with the diameter of 200 μm.

Method 2: In the second method, the small molecule solution and the monomer solution were printed separately (Figure III.12). The small molecule solution was first printed at the desired concentration into the microarray on the substrates. The small molecule microarray slides were allowed to dry for 10 min. The monomer solution e.g., acrylamide-bis solution was prepared with 10% acrylamide, 2.5% bis-acrylamide, and 5 mg/ml of photo-initiator. The acrylamide solution was then printed precisely on top of each small molecule spots. The slides were then transferred 2 inches under UV to initiate polymerization. The polymerized acrylamide gel covered over the small molecule spot and formed a capsule enclosing the small molecule. This process can be repeated for several times to obtain various levels of drug contents and polymer coating. The retention and release mechanism could be engineered based on the requirements of the cellular assay. For example, if the cells require a longer period of attachment, the covering poly-acrylamide cap could be made of thicker layer and higher density of the polymer chains.

*Extracellular Matrix Cover*

After the small molecule and polymer microarray was completed, 30 min of drying was allowed before the final layer of ECM proteins was printed on the top. The protein printing process was much similar to that of the previous two chapters except

only one type of ECM combination was printed on top of all spots. The type of diseased cells to be screened determined the covering ECM condition, e.g., one of 12 growth-supporting ECM conditions for glioblastoma cells identified in Chapter II. Or any other growth-supporting ECM such as the native ECM from the diseased tissue can be dissociated into a solution and printed. This layer of ECM provides the substrate for the cells to attach to. Because the unspotted poly-acrylamide surface does not allow cells to attach, the cells would only be present on the ECM covered small molecule spots.

### III.C.9 Cell Seeding

Prior to cell seeding, the small molecule microarray slides were assembled into the culture apparatus with the multi-well gaskets as described in the Chapter II. Differently from before, 150 μl of media was transferred into each well containing the small molecule microarray. The media was removed after 10 min of incubation in 37°C before cell seeding. This step removed the excess small molecule drugs what were not immobilized. After the cells were seeded, a multi-layer microarray was formed (Figure III.13). The immobilized small molecule drugs were on the bottom along with the immobilizing polymer. The ECM proteins were over the small molecules. The cells were on the top of the ECM proteins. The efficacy of the drugs could be determined based particular cellular assays. In the case of cancer, the most effective drugs would be the ones that can lead to cell death. Therefore, the small molecule spots with the largest amount decrease in number of cells would the most effective.

### III.C.10 Data Analysis

To quantitatively analyze the degree of growth supported by the ECM microarray, the GFP-transfected glioblastoma cells were cultured on the microarrays for two days. The fluorescent images were taken at 16 and 48 hr. The images were analyzed using GenePix Pro 3.0. The differences between the signal and background were recorded. The data from three biological repeats were statistically compared. The ECM combinations that support cell growth the best were identified.

## III.D Results

The U87 glioblastoma cells were cultured on an ECM protein microarray for 3 days. The bright-field pictures were taken at 3 hr, 24 hr and 3 days after seeding. Figure III.1 shows a 5 x 4 array chosen as an example to illustrate the differences in the effects of four ECM protein combinations out of 63 tested. The cell densities were very similar on every spot in Figure III.1 at 3 and 24 hr. At 3 days, the sizes of the cell aggregates in the same row were still similar. However, there were differences in the sizes of the cell aggregations for different ECM proteins (across the rows). While the first two rows were similar, the third row was slightly larger, and the sizes of the cells aggregations in the fourth row (C1,C4,C5,F,L) were significantly larger than the first three, indicating a larger number of cells with this particular ECM combination. For U87 glioblastoma cells seeded over the ECM spots containing eight other ECM protein combinations for 24 hr and 3 days (Figure III.2), there were almost no cells present on the conditions of collagen I, fibronectin and laminin (individually) after 3 days.

The GFP-transfected cell lines of U87, DK, V3 and WT were cultured for 2 days. The live-cell fluorescent images were taken at 16, 36, and 48 hr post seeding (Figure III.3). The fluorescent signals of each spot were processed using GenePix Pro 3.0, and the intensity ratio between day 2 (48 hr) and 16 hr were calculated for all four cell lines. The intensity ratios, which reflect the degree of cell growth from 16 to 48 hr, are plotted in Figures III.4 – III.7 for U87, DK, V3 and WT, respectively. For each cell type, the ratio (in an increasing order) is plotted against the ECM protein combinations. The best growth-supporting ECM combinations are not the same for these different cell lines. The top 10 best growth-supporting ECM combinations were chosen from every cell line. Among these 40 conditions, 12 combinations appeared in the top 10 of more than one cell lines. These 12 are listed in Table III.1, with 3 combinations appearing in the top 10 of three ("Frequency" column of Table III.1) out of the four cell lines studied. Calculation of the total times each ECM protein appeared among the 40 top-10 conditions shows that collagen IV has the highest rate of appearance, followed by collagen III and collagen V.

The primary cancer cells were also tested on the ECM microarray. The cells isolated from a patient's glioblastoma tumor were cultured on the ECM microarray. One of the best conditions that supported the growth of these cells was the combination of collagen I, III and IV (Figure III.8). This combination was also one of the top 12 combinations selected from the four glioblastoma cell lines.

In addition to screening for the best conditions for cellular functions, the ECM microarray was also used to isolate cells with different growth rate in a population.

Figure III.9 on U87 glioblastoma cell line shows that the growth rate of the individual cell colonies has considerable variations, with the presence of a few very large cell aggregations, although the fluorescence intensities (cell number) on these spots at the initial seeding were quite similar to the neighboring spots which were much smaller at 5 days. The ECM combinations were identical in the same row. The cells from the large aggregates were extracted and cultured in a petri dish. These cells were stained with nestin. The cells from the large aggregates had higher expression of nestin compared with the control cells (Figure III.10).

The effects of the immobilization of the small molecules are shown in Figure III.14. The top two rows of fluorescent small molecule microarray spots at two different concentrations were immobilized by another layer of poly-acrylamide gel; the bottom two rows of fluorescent small-molecule spots were only printed and not immobilized. The microarray slides were then immersed into the media solution. After 10 min, the bottom two rows of the non-immobilized small-molecule spots lost most of their fluorescent intensity. The top tow rows of the immobilized spots effectively retained their fluorescent intensity.

Doxorubicin, an anti-cancer drug, was printed onto a microarray and immobilized using 10% acrylamide and 0.1% bis-acrylamide at different concentrations (Figure III.15). The doxorubicin microarray retained the fluorescent intensity after being immersed in the media for 30 min.

## III.E Discussion

Primary cancer cell culture is a fundamental tool in cancer research. However, primary cancer cells are very difficult to grow and expand. Most research studies on basic cancer biology and drug screening rely heavily on a limited number of cell lines. It is well known that cancer cells have interactions with their ECM. It is reasonable to expect that cancer cells require the proper ECM to grow. While the exact components in the native microenvironment of a particular cancer are difficult to determine, the modern high-throughput screening technology may help us to identify an artificial condition that would support the growth and expansion of a particular type of cancer cell.

In this study, the glioblastoma cell lines were cultured on the ECM protein microarray for 3 days. The bright field images revealed the obvious differences in the cells grown on different ECM combinations. In Figure III.1, the cell aggregates in the fourth row were significantly larger than the cell aggregates else where. This phenomenon was not caused by the variation in microarray spots since the microarray characterization showed consistent spot size and protein content. This was not due to the uneven cell seeding, either, because the cell quantities over the spots were very similar at 3 hr and 24 hr (Figure III.1). The present results indicate that certain ECM combination, e.g., collagen I, IV, V, fibronectin and laminin, could support the growth the glioblastoma cells much better than the other combinations. In contrast to the growth-supporting combinations, there were also conditions that can hinder the growth of the glioblastoma cells (Figure III.2). There was almost no cell left on the spots

containing a single type of protein: collagen I, fibronectin or laminin. This suggests that individual collagen I, fibronectin or laminin either prevented the cells from being attached at the beginning or could not provide the survival requirement of the cells. These data further confirmed the importance of the ECM in supporting the survival and growth of cancer cells. The cell lines used in the experiment were immortalized and were very robust in various culturing conditions. The cells extracted from a patient sample could be more sensitive to the microenvironments.

Time-dependent studies were conducted on GFP-transfected glioblastoma cell lines U87, WT, DK and V3 cultured on the ECM microarrays for 2 days. The GFP-fluorescent signals emitted from the cells were used to determine the quantity of cells on each spot (Figure III.3). This experiment gave us a quantitative method to find the ECM combinations best support the growth of the cells. Fluorescent images were taken at 16, 36 and 48 hr. Examination of growth differences between 48 and 16 hr (Figures III.4 – III.7) yielded the best 10 growth-supporting combinations for each of the four cell line. Among these 40 combinations, there were 9 combinations that appeared in the best 10 for 2 cell lines and 3 of them appeared in the best 10 for 3 cell lines (Table III.1). These 12 combinations could serve as the starting points to be chosen for maintenance and expansion of the primary cancer cells.

When the primary cells extracted from patient samples were cultured on the ECM microarray, there were few cells on many combinations. However, there were a few conditions that supported the growth of the cells. One of the best was the combination collage I, III and IV (Figure III.8). This was also one of the twelve

combinations for the glioblastoma cell lines (Table III.1), although this condition was not the highest ranked for the four cells lines. There significant differences between the primary cells and the cell lines indicate their different responses to the ECM conditions. The primary cells extracted from different patients may also respond to the ECM differently due the individual variations. The data from more patients may help further identify the optimal condition or a panel of conditions.

In addition to identifying the growth-supporting conditions, the ECM microarray can also be used to isolate particular cell subpopulation. It is known that a small percentage (1-2%) of cells in glioblastoma cell line may undergo mutations and exhibit different behaviors, and therefore become a subpopulation different from the rest of the population. During seeding cells on the ECM microarray, we could choose to seed a small number (~5) of cells on each spot by controlling the seeding density. If one of the cells on a spot belonged to a subpopulation, the percentage of that cell is 20% compared to the 1-2% in the overall population. The behavior of the subpopulation isolated on a spot would then be much easier to identify by comparing to the average spot. In Figure III.9, there were a few distinctively large cell aggregates. As mentioned previously, the microarray spots were uniform in size and content to begin with when the cells were first seeded. In addition, the ECM combinations were identical in the same row. It is very likely that a subpopulation of cells that led to the formation of large cell aggregates had a much higher growth rate compared to the average. The cells extracted from the large cell aggregates expressed higher level of nestin, a neuro-cancer initiating cell marker, than the control cells (Figure III.10). This

is a possible indication that the cells from the large aggregates express more cancer-initiating behavior. This method could be particularly valuable for isolating specific types of cells from a tumor sample because a tumor may contain numerous types of cells. The ability to isolate and concentrate the cells of interest can greatly improve the efficiency of primary cancer cell culture. More importantly, it is believed that there is a small population of cells that is truly responsible for the malignancy of a tumor. These cells are commonly referred as the cancer-initiating cells or cancer stem cells. The ability to isolate these cancer-initiating cells may carry significant values in studying their behavior and developing more effective treatment against cancer.

The difficulty in primary cancer cell culture has limited the progress of cancer research. Finding a way to effectively extract, maintain and expand primary cancer cells in vitro may contribute tremendously to the research and treatment of cancers that still devastate human lives and their families. One of the ways is to modify the culture microenvironment of the primary cancer cell. This study has demonstrated a method using the ECM microarray technology to screen for the growth-supporting matrix conditions for the glioblastoma cells. Based on the initial success, such a method could be applied to many other types of cancers. Different types of cancers would likely require different ECM support similar to the findings that the four cell lines of the same cancer have different preferences. The same type of cancer in different patients might also exhibit different responses. The best combination for one might not fit others. It might be necessary to find the best in every case.

If the cancer cells from different patients may respond to the growth-supporting ECM differently, it is likely that they may also respond to drugs differently. A small-molecule drug microarray method was developed in this study to screen the effects of hundreds of drugs and their combinations for a particular patient using a small amount of cells extracted from that patient, making it possible to conduct personalized therapy.

Small-molecule drugs typically have molecular weights less than 800 Da. Such molecules have much higher diffusivity compared to the ECM proteins. Therefore, simply printing the small molecules onto a substrate would not work. The small molecules must be effectively immobilized. This could be achieved by two methods: mixing the small molecules with a polymer (Figure III.11) or covering the small molecule spots with a polymer (Figure III.12). The second method requires an extra step but is more universal as the small-molecule drugs may be in various solvents that should not be mixed with water. The polymers could prevent the rapid diffusion of the small molecule but allow it to slowly dissipate towards the cells cultured on top. The ability to retain the small molecules may be calibrated by modifying the properties such as degradability, pore size which is governed by the polymer chain and cross-linking density, and the chemical affinity for the small molecules. Various types of polymers such as alginate, PLGA, PEG and others can be applied to immobilize the small molecules. Poly-acrylamide was chosen in this case because its polymer chain and cross-linking density can be easily modified, and there is also a strong bond between the immobilizing poly-acrylamide and the poly-acrylamide substrate.

To demonstrate the utility of this method, a fluorescent small molecule, resazurin was used to represent the small-molecule drugs. Resazurin has a molecular weight of 251 Da, much lower than the general cut-off of 800 Da for small-molecule drugs. It is commonly used for cell growth / viability assay with little harm to the cells, contrary to most of the drugs with significant toxicity [76, 77]. The polymer-immobilized small-molecule microarray spots were compared to the non-immobilized ones (Figure III.14). The immobilize spots showed significantly stronger ability to retain the small molecules after 10 min of immersion in media. The slight variation in the spot morphology between Figure III.14 **A** and **B** was due to the rehydration and swelling of the poly-acrylamide gel covering the small-molecule spots.

The validity of this method was verified using a real drug, doxorubicin, an anti-cancer drug with a molecular weight of 580 Da. Conveniently, this drug is also intrinsically fluorescent. Various concentrations of the doxorubicin was printed into a microarray and immobilized with 10% acrylamide and 0.25% bis-acrylamide. The microarray slide was then immersed in the media for 30 min. The fluorescent intensity strongly suggests that doxorubicin was effectively immobilized and retained in the microarray.

This technology may be used to design personal drug cocktails by screening the effects of a panel of drugs and their combinations on the patient's own diseased cells. For example, when a tumor is surgically removed from a cancer patient who would undergo chemotherapy, the cells could be isolated from the removed tumor and cultured in larger scale in the growth-supporting ECM condition identified using the

ECM microarray. These cells could then be seeded on the microarray created from a panel of applicable anti-cancer drugs and their combinations. The effects of these drugs on the cancer cells could then be determined using the small-molecule drug microarray. An optimal cocktail of these drugs tailored for that specific patient could be formulated, providing a personalized drug therapy. Cells used in the screening may also be extracted using biopsy from the diseased tissues of patients who are not suitable for surgery. Even cells from healthy tissues such as the liver or muscle can be extracted using biopsy to screen the adverse side effects of the drugs.

It is our hope that the ECM microarray can become an effective tool to maintain and expand primary cells, and to isolate the disease-causing cell subpopulation from a heterogeneous source such as a tumor, while the small-molecule drug microarray can offer the physicians with the information on how a patient would respond to a panel of drugs without applying all the drugs on the patient, and therefore help the physician to provide personalized therapy.

## III.F Tables and Figures

**Table III.1: The top 10 best growth-supporting ECM combinations. for glioblastoma cells.** Among these 40 conditions, 12 combinations listed here appeared in the top 10 of more than one cell lines. Three combinations appeared in the top 10 of three ("Frequency" column of Table III.1) out of the four cell lines studied. Calculation of the total times each extracellular matrix protein appeared among the 40 top-10 conditions shows that collagen IV has the highest rate of appearance, followed by collagen III and collagen V.

| Collagen I | Collagen III | Collagen IV | Collagen V | Fibronectin | Laminin | **Combination** | **Frequency** |
|---|---|---|---|---|---|---|---|
| X | X | X | X | | | **1345** | **3** |
| | X | X | | | | **34** | **3** |
| | X | X | X | | | **345** | **3** |
| X | X | X | X | X | X | **1345FL** | **2** |
| X | X | | X | X | X | **135FL** | **2** |
| | X | X | | X | X | **34FL** | **2** |
| | | X | X | X | X | **45FL** | **2** |
| X | X | X | | | | **134** | **2** |
| | | X | X | | | **45** | **2** |
| X | | | | X | | **1F** | **2** |
| X | | | | | X | **1L** | **2** |
| | | | X | X | X | **5FL** | **2** |
| | | | | | | | |
| 13 | 17 | 19 | 16 | 12 | 12 | **Total** | |

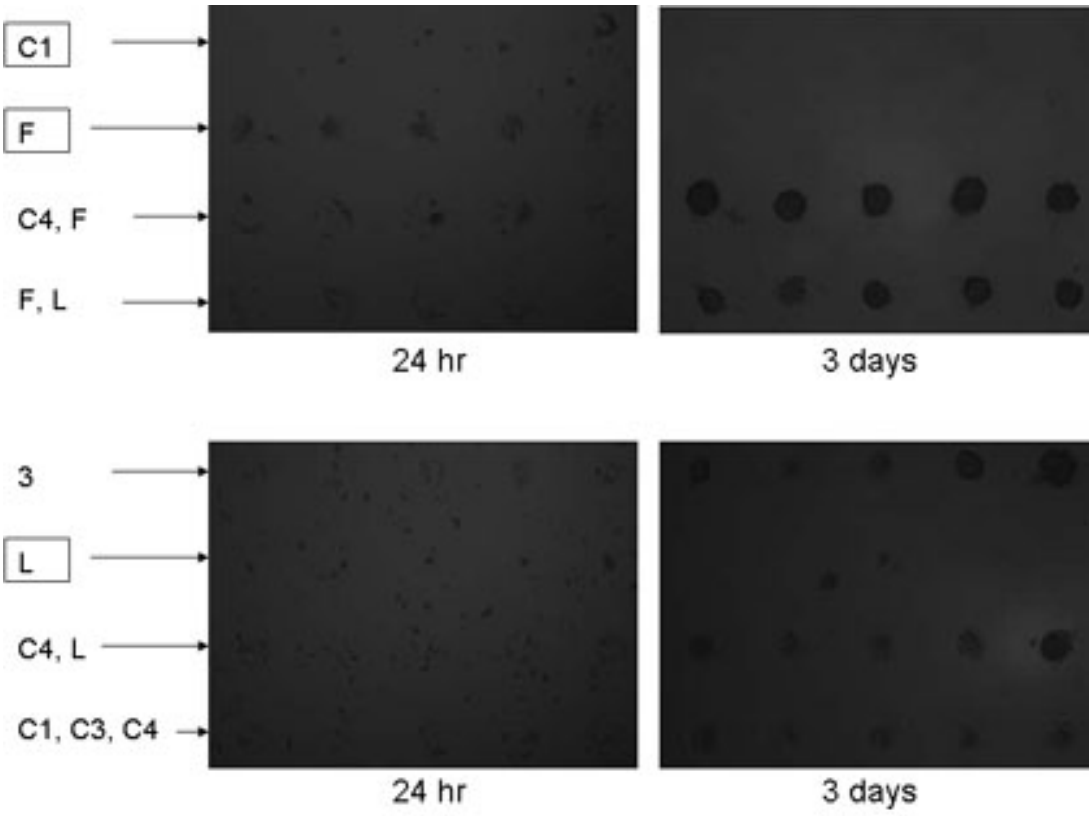



**Figure III.1: U87 cells cultured on EMC microarray.**
The bright field pictures were taken at 24 hr and 3 days post seeding. The ECM combinations are labeled on the left. C1 = Collagen I, C3 = Collagen III, C4 = Collagen IV, C5 = Collagen V, F = Fibronectin, L = Laminin. There were few cells left on the spots of single proteins collagen 1, fibronectin and laminin.

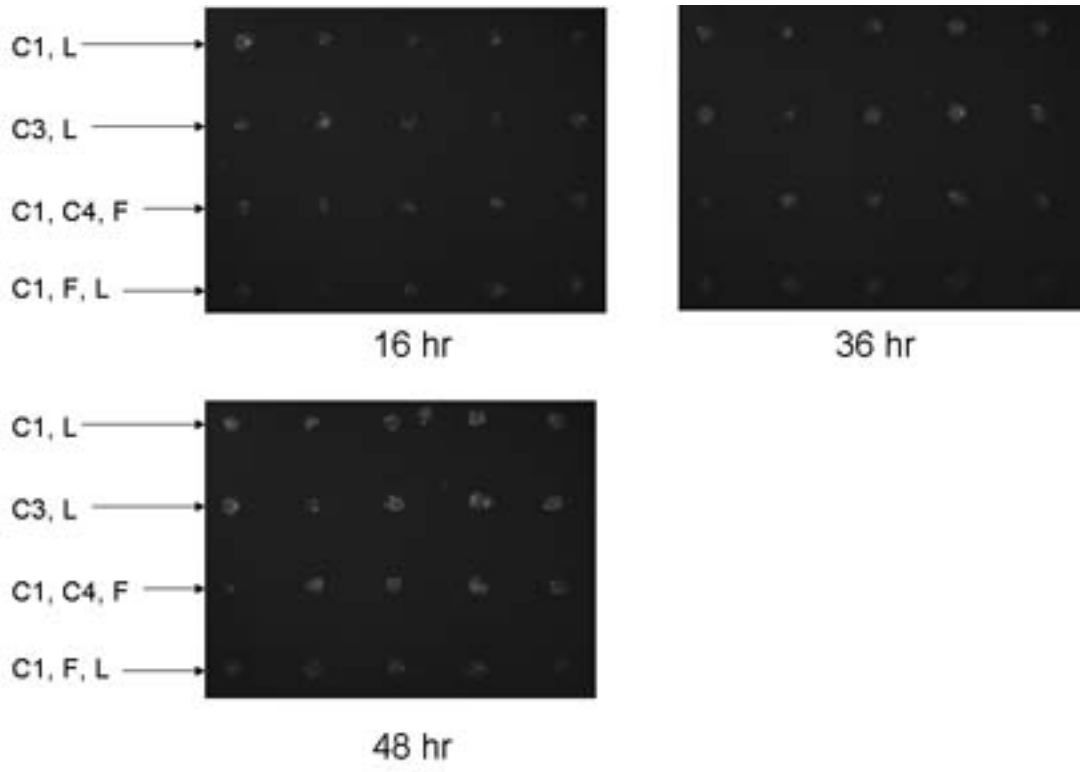



**Figure III.2: GFP V3 cells ultured on ECM microarray.**
The fluorescent images were taken at 16 hr, 36 hr and 2 days post seeding. The ECM combinations were labeled on the left. C1 = Collagen I, C3 = Collagen III, C4 = Collagen IV, C5 = Collagen V, F = Fibronectin, L = Laminin.

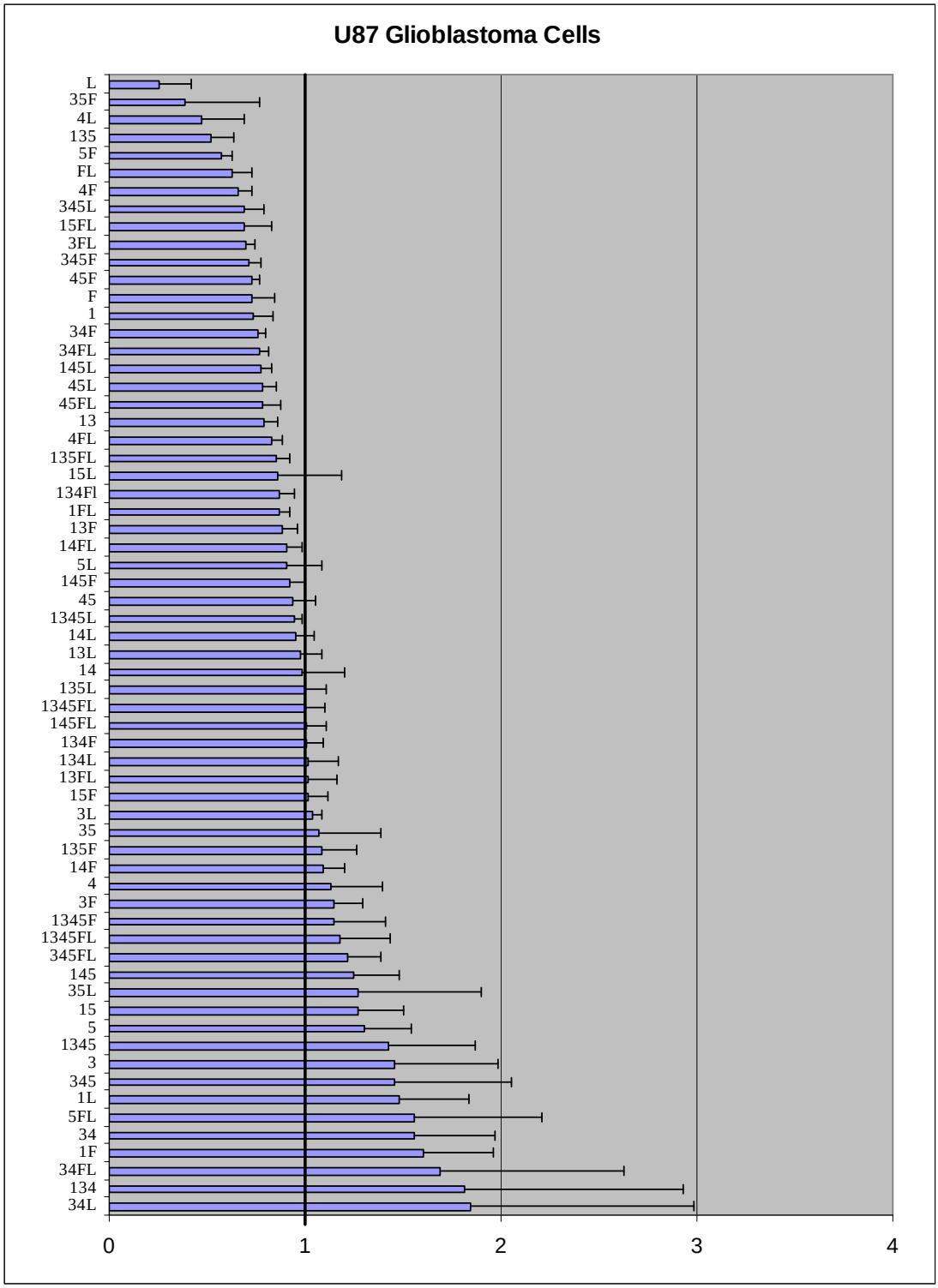



**Figure III.3: The effects of the 63 ECM combinations on the growth of U87 cells.**

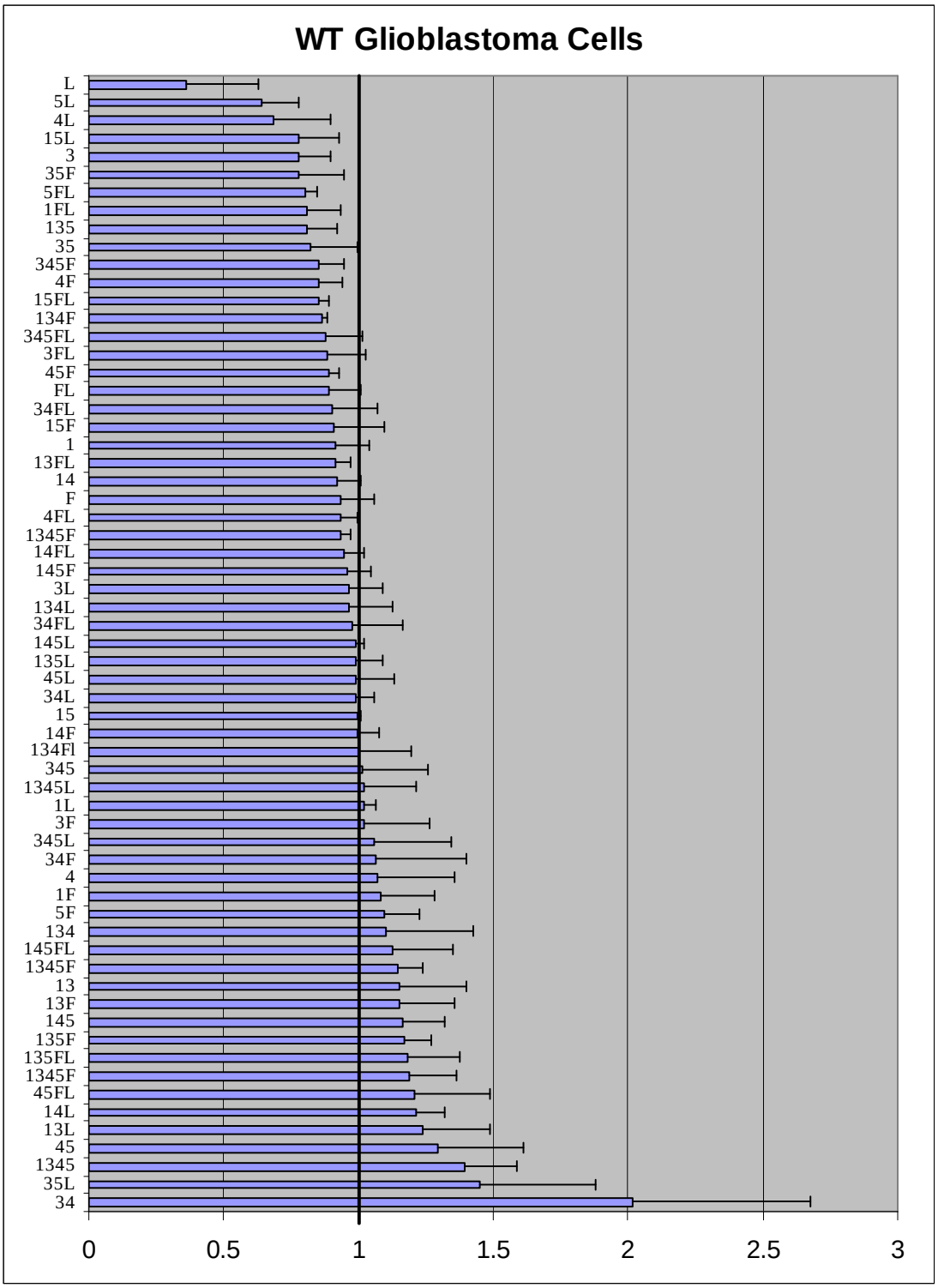



**Figure III.4: The effects of the 63 ECM combinations on the growth of WT cells.**

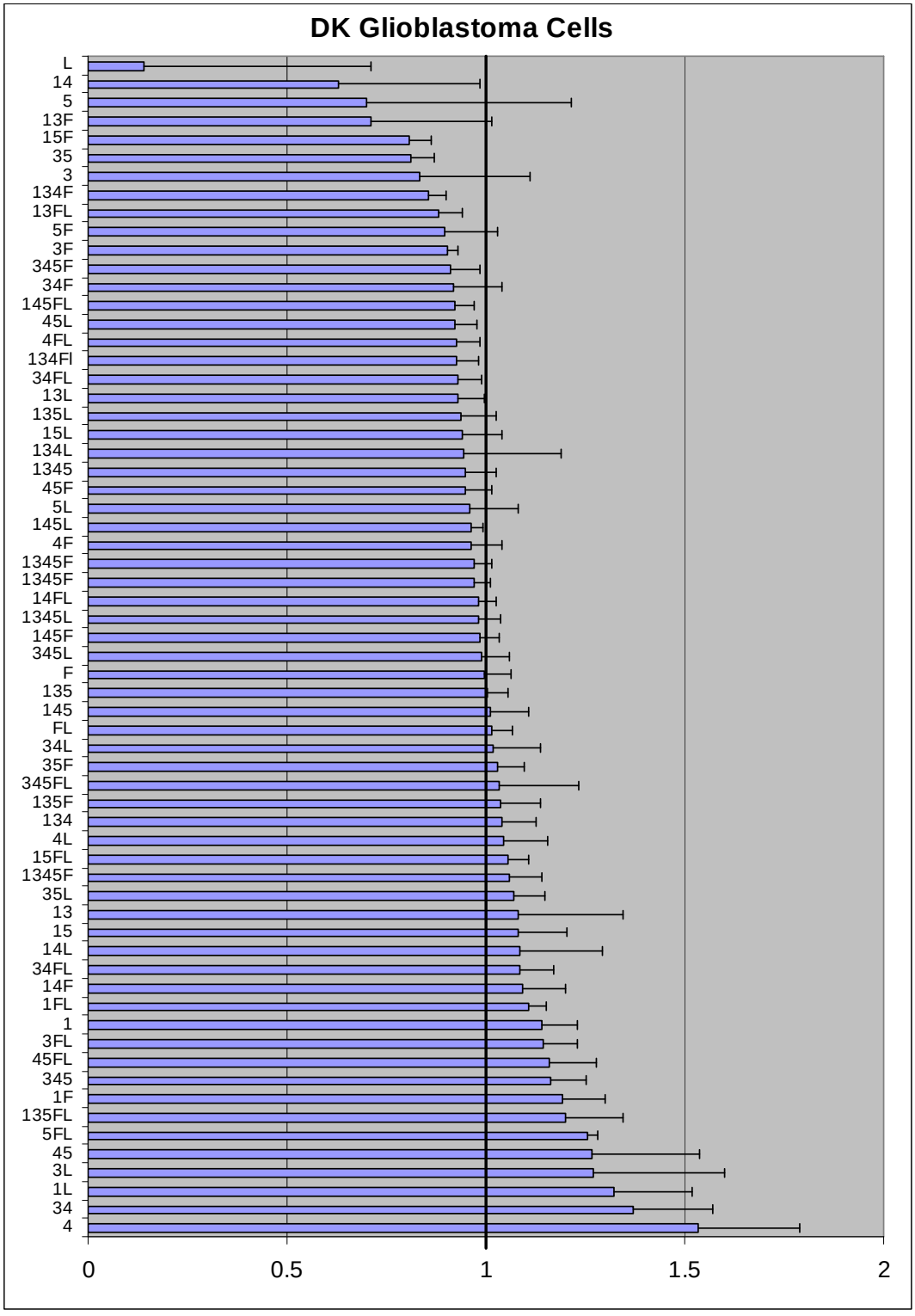



**Figure III.5: The effects of the 63 ECM combinations on the growth of DK cells.**

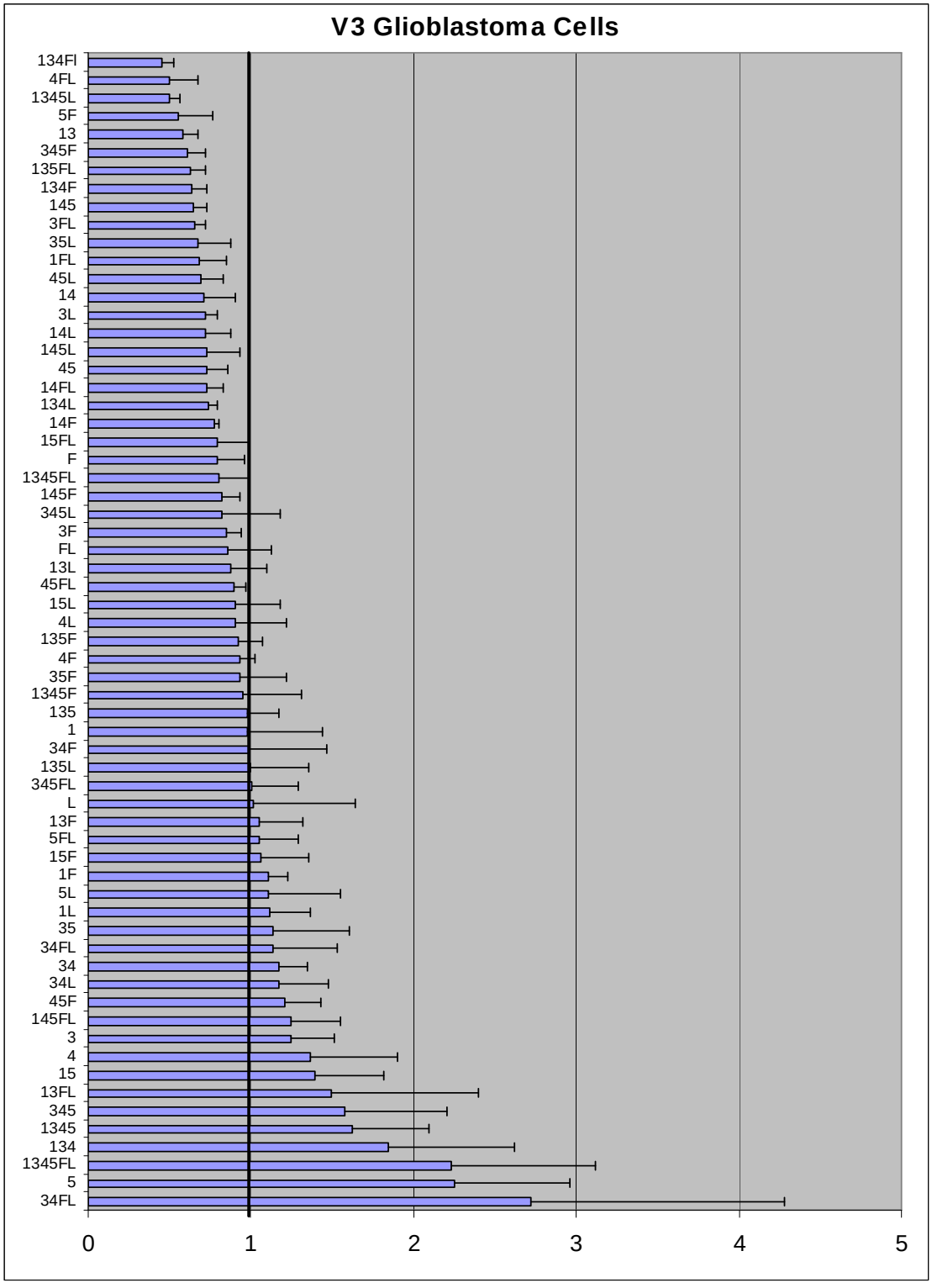



**Figure III.6: The effects of the 63 ECM combinations on the growth of V3 cells.**

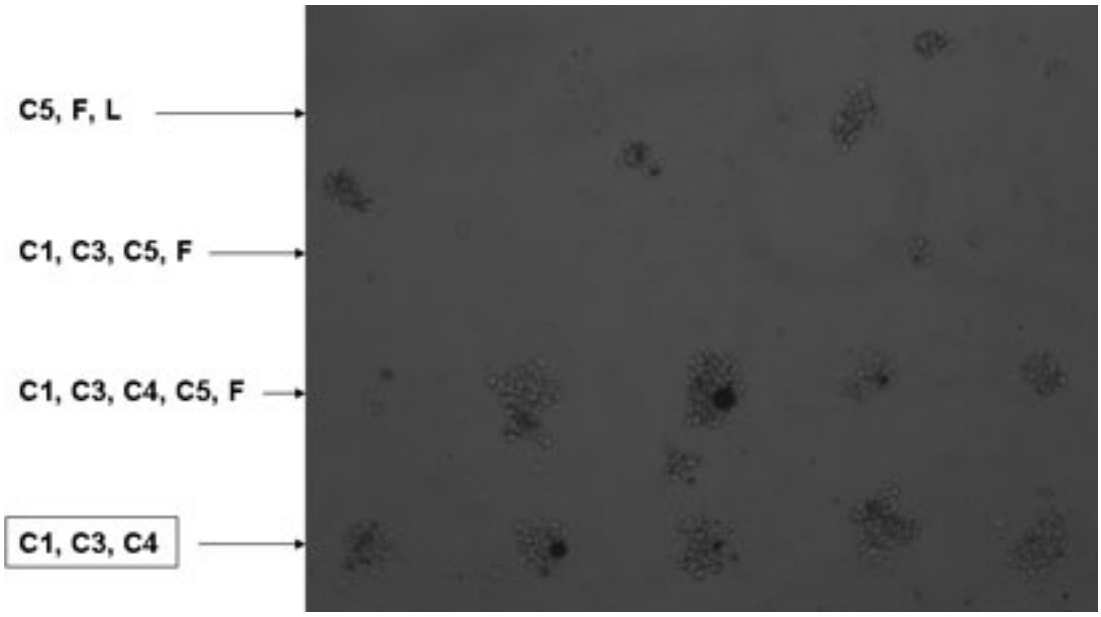



**Figure III.7: Patient's glioblastoma cells cultured on the ECM microarray.**
The ECM combinations were labeled on the left. C1 = Collagen I, C3 = Collagen III, C4 = Collagen IV, C5 = Collagen V, F = Fibronectin, L = Laminin. One of the best growth-supporting conditions was the combination collagen I, III and IV.

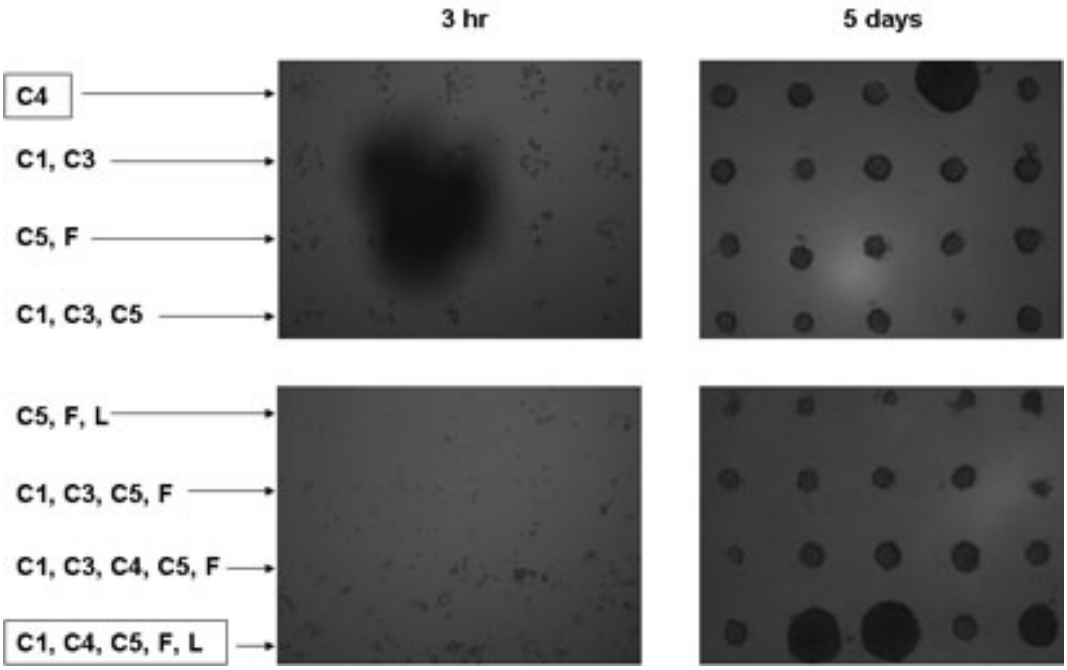



**Figure III.8: Large cell aggregates appeared after 5 days of culture.**
The ECM combinations are labeled on the left. C1 = Collagen I, C3 = Collagen III, C4 = Collagen IV, C5 = Collagen V, F = Fibronectin, L = Laminin. The combination was identical among the 5 spots in the same row. The initial seeding densities at 3 hr were fairly similar among all spots. At 5 days, however a few significantly large cell aggregates appeared (indicated with white arrows).

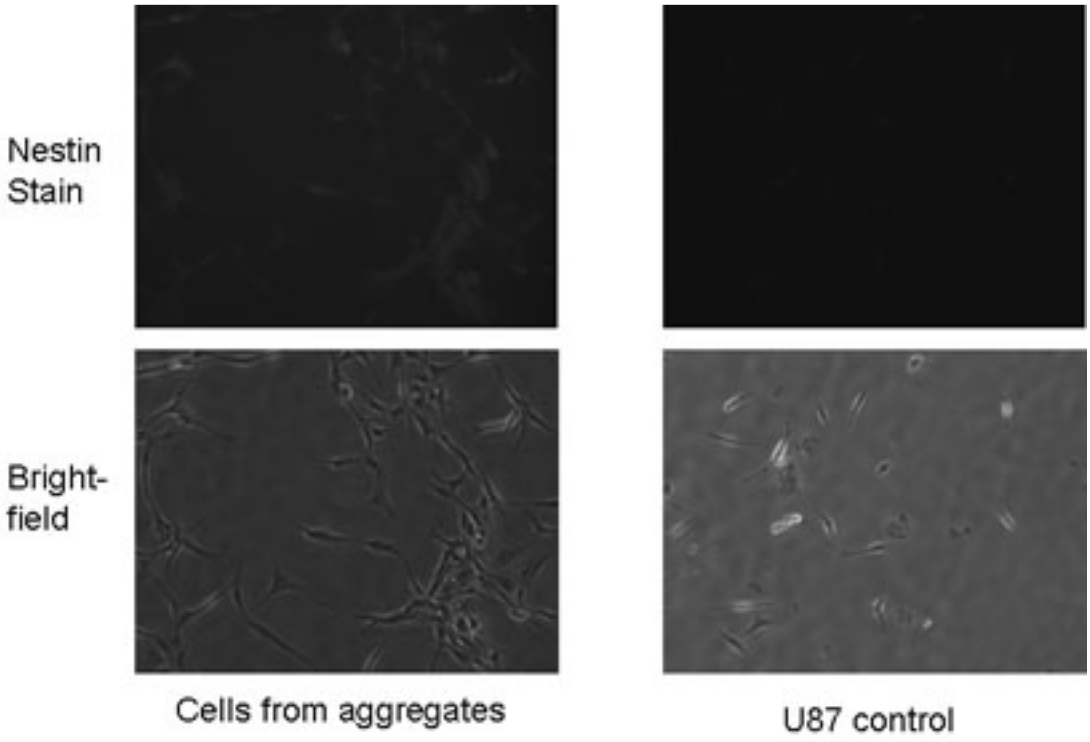



**Figure III.9: Nestin stain on cells from large aggregates.**
The cells extracted from the large aggregate in Figure 21 and the U87 controls were stained with Nestin. The upper two images were fluorescently stained with nestin. The lower two are the bright field images of the one above. The cells extracted from the large cell aggregates had higher expression of nestin.

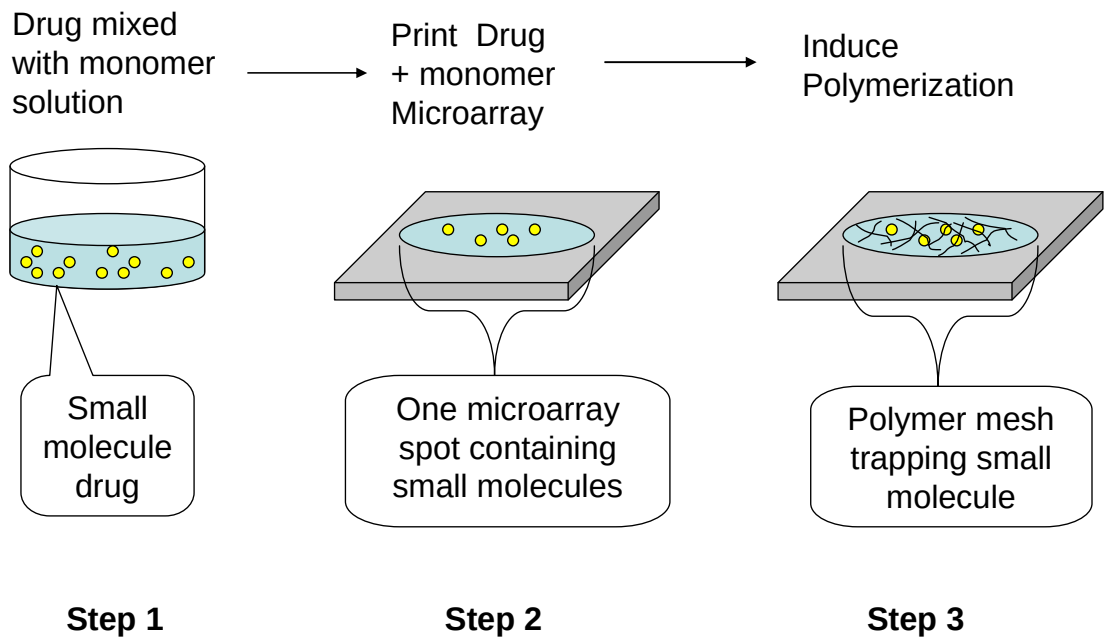



**Figure III.10: Small-molecule immobilization method 1.**
**Step1:** The small-molecule drugs are mixed with the monomer solution of choice. **Step 2:** The drug + monomer solution is printed into the microarray. **Step 3:** The polymerization process is induced. The crosslinked polymer chains form a mesh immobilizing the small molecules.

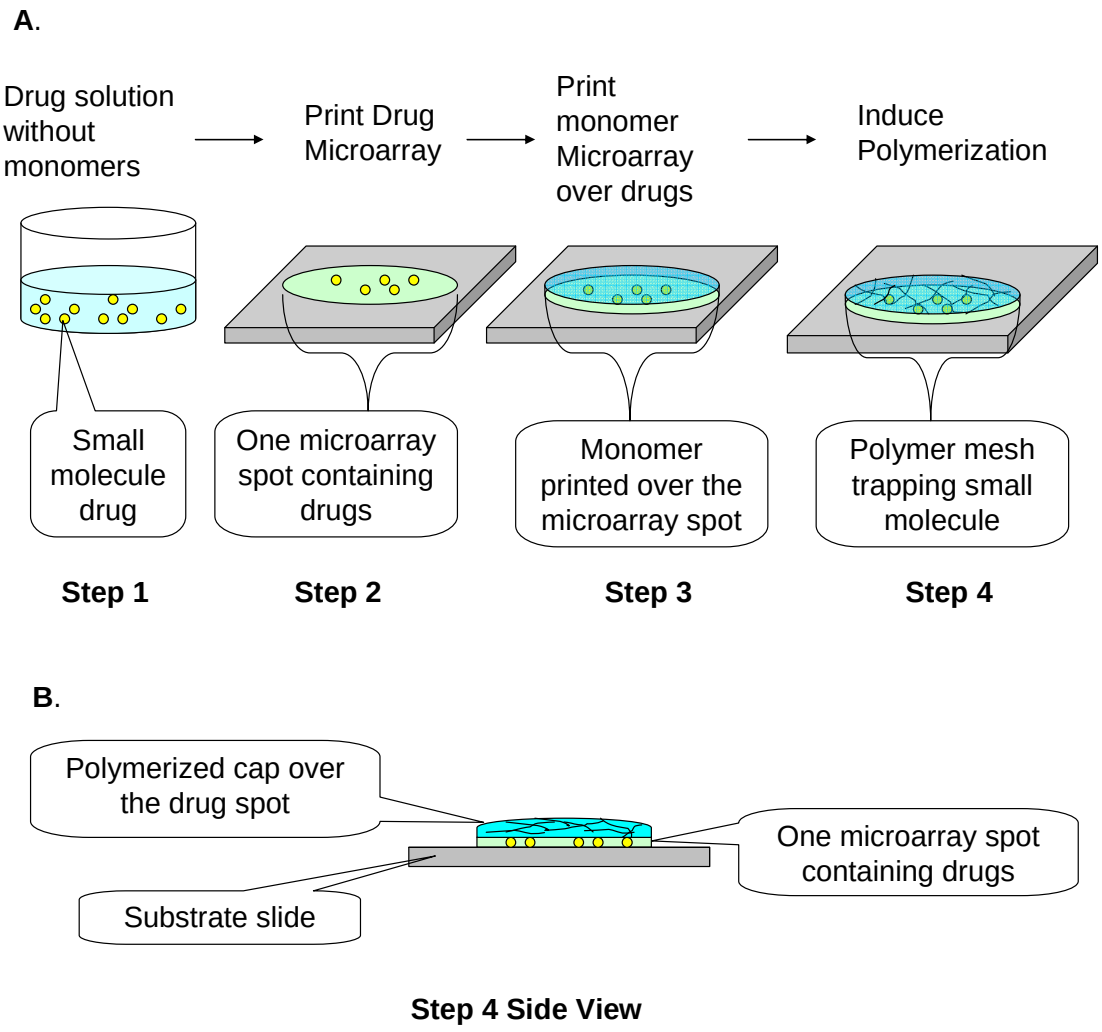



**Figure III.11: Small-molecule immobilization method 2.**
**A. Step 1:** The small-molecule drug solution is obtained without monomers. **Step 2:** The drug solution is printed into the microarray. **Step 3:** A layer of monomer solution is printed exactly over the drug microarray spots. **Step 4:** Polymerization was induced. The polymerized gel forms a cap covering and immobilizing the drug spots.
**B** provides a side view of the Step 4. One layer of drug microarray spot is printed on the substrate slides. The polymerized cap is covering and immobilizing the drug spot.

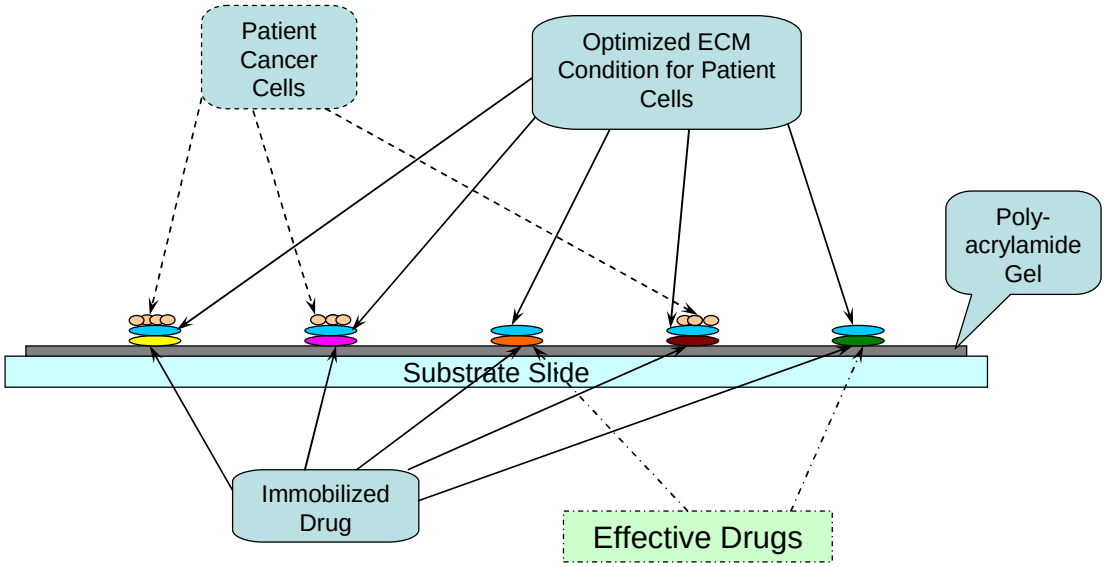



**Figure III.12: Small-molecule microarray screening method.**
The small-molecule drugs spots immediately above the poly-acrylamide gel indicated by the arrows from “Immobolized Drug”. The growth-supporting ECM spots indicated by the arrows from “Optimized ECM Conditions for Patient Cells”, for the diseased cells, e.g., cancer cells are printed over the immobilized drug. The patient diseased cells, e.g., cancer cells indicated by the dashed arrows from “Patient Cancer Cells” are seeded on the spots. No or less cells would be left on the spots indicated by the dashed arrows from “Effective Drugs”. The drugs on these spots may be effective inducing apoptosis in the diseased cells.

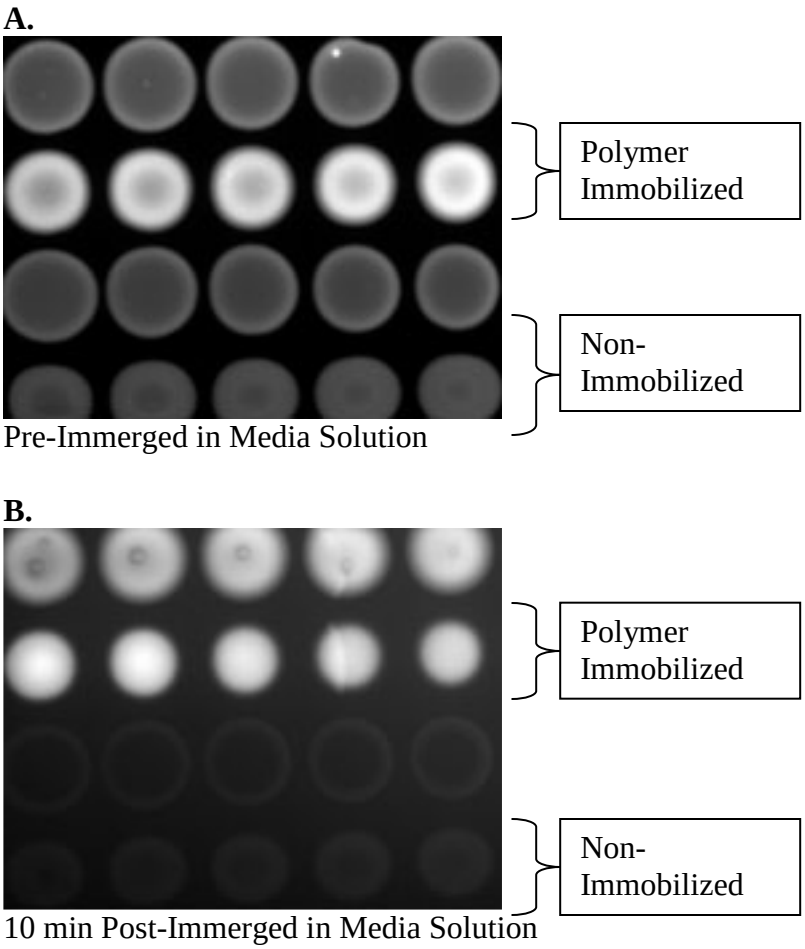



**Figure III.13: Immobilized vs. Non-Immobilized Fluorescent Small Molecule.**
The immobilized small molecule spots (top two rows) maintained fluorescent signal after immersed in the media; the non-immobilized small molecules quickly dissipated in the media.

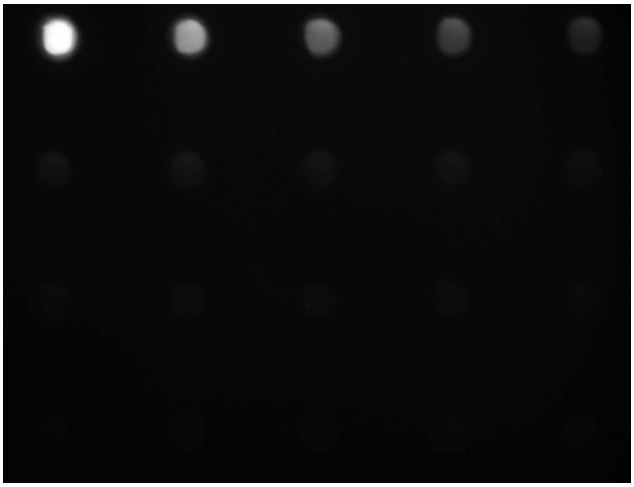


**Figure III.14: Doxorubicin immobilized microarray spots.**
Doxorubicin, an anti-cancer drug printed into microarray at different concentrations showing the retained fluorescent signal after immersed in media for 30 min.

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