Nano Fabricated 3D Extracellular Matrix (ECM) Scaffolds to alter the Cancer Cell Behavior

BY

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THESIS

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LIST OF ABBREVIATIONS

μCP	micro Contact Printing
μΤΜ	micro Transfer Molding
ACS	American Cancer Society
AFM	Atomic Force Microscopy
CAT	Computerized Axial Tomography
CDC	Center for Disease Control and Prevention
CLL	Chroniclumphocytic Leukemia
CNT	Carbon Nanotubes
СТ	Computed Tomography
DAC	Digital to Analog Converter
DRE	Digital Rectal Exam
EBL	Electron Beam Lithography
ECM	Extracellular Matrix
EMT	Epithelial Mesenchymal Transformation
FAK	Focal Adhesion Kinase
FOBT	Fecal Occult Blood Test
HSQ	Hydrogen silsesquioxane
IPA	Iso Propyl Alcohol
LIGA	Lithographie, Galvanoformung, Abformung
MBE	Molecular Beam Epitaxy
MFM	Magnetic Force Microscopy
MIBK	Methyl Isobutyl Ketone
MIMIC	Micromolding in Capillaries

LIST OF ABBREVIATIONS (Continued)

MPA	Multi Photon Absorption
MRI	Magnetic Resonance Imaging
NCI	National Cancer Institute
NSAID	Non Steroidal Anti Inflammatory Drugs
PDMS	Polydimethylsiloxane
PMMA	Poly(methyl methacrylate)
RIE	Reactive Ion Etch
RMS	Root Mean Square
SAM	Self Assembly Monolayers
SEER	Surveillance Epidemiology and End Result
SEM	Scanning Electron Microscopy
SFK	Src Family Kinase
SPM	Scanning Probe Microscopy
STM	Scanning Tunneling Microscopy
vdW	van der Waal

SUMMARY

There are three major factors that govern cell behavior: chemical, cellular and mechanical. Recent studies showed that the cellular microenvironment is capable of changing the cell behavior. Simple, engineered microenvironment, such as an array of holes and pillars were used for pilot studies, and the results turned out very interesting.

The studies were extended to replicate the exact biological environment using FDA approved bio-compatible material, such as PMMA and PDMS. State of the art tools, equipment and cutting edge technology were required to replicate and reproduce the environment.

Current lithographic techniques are not capable of producing sophisticated 3D structures suitable for this application. Therefore using electron beam lithography as a tool, a modern technique was required to reproduce the microenvironment, referred to as extracellular matrix. Extensive studies were carried out to develop unique technique that can convert a grayscale image to advanced 3D structure. SEM images of the biological tissue samples were obtained and converted to reproduce the required ECM scaffolds.

1. INTRODUCTION

1.1 Nanotechnology

Matter in nanometer scale has an unusual profile unlike properties of the bulk, and this modern scientific knowledge is applied in a number of different fields. Understanding, modeling, constructing and controlling matter in such small scale is encompassed by nanotechnology. Although nanotechnology is still a developing field, and scientists are still discovering and testing new ideas, it has a wide range of potential applications. The first concepts of the nanotechnology were introduced by the Nobel Prize winning physicist, Richard Feynman, in his world famous lecture "There is a plenty of room at the bottom", which was presented at an American Physical Society meeting. His idea was to manipulate the matter within an atomic scale with the aid of a new technology. There are two approaches in which the nanofabrication processes being done: "top down" and "bottom up". "Top down" means digging into the nanoscale regime starting from the bulk matter. Photolithography is the best example for top down approach. In the "bottom up" approach, process start with the atomic or molecular level and manipulate them in a controlled manner, such as in Molecular Beam Epitaxy (MBE) and Molecular Self Assembly. Nanotechnological approaches were first used in electronic device fabrication, but later on scientists realized it's capabilities and started to use the techniques in medical applications [1], like cancer therapy [2, 3], targeted drug delivery [4, 5], chemotherapy for tuberculosis [6] and controlled drug delivery using microchips [7].

1.2 Nanotechnology in Medical and Biological Applications

Nanotechnology has opened a wide variety of alternative paths and approaches in modern medicine and biology. Most of the approaches are capable of providing better results compared with the old methods.



Figure 1: Applications of Nanotechnology in Biology and Medicine.

Drug and gene delivery is one of the approaches studied since the concept of the nanotechnology became popular, and it is a promising technique in modern medicine. Conventional drug delivery techniques like oral pills, IV, intradermal or intramuscular mix with blood stream and circulate all over the body. Only a small portion of the drug delivers to the exact location in the body which needs it. The rest leads to toxicity because, at the same time, healthy organs/tissues also get exposed to drugs, which ultimately decreases the life time. Moreover, higher dosage is required at a time to make the treatment effective and counterbalance the drug loss. Use of nanodevices and nanoparticles as drug and gene delivery vehicles [8] is a promising treatment strategy compared with the old methods because the drugs can be delivered to the exact location ,which it requires, thus reducing dosage, drug loss and toxicity. Because the particles are very small, they do not sediment or block the microvascular system and can easily travel through the blood stream which adds more value to the approach. Use of polymeric nanoparticles for treatments is widely studied due to the bio-compatibility and degradability. Microneedles can also be used to deliver drugs with nanoparticles because they do not activate the sensory nerves due to their low outer skin penetration depth up to 10-20 µm. Although the technique is a good treatment strategy, it has some drawbacks too [9]. For example, these nanoparticles can be captured inside the liver or spleen and sediment when time progresses. This could cause serious side effects, but the use of bio-degradable material is a better solution. Use of functionalized carbon nanotubes (CNTs) is also becoming more promising technique because of their structural properties [10]. Oxidative and synthetic protocols can be used to attach functionalized groups to the end and/or to the side walls of CNTs, which make them highly soluble in organic solvents.

Fluorescent probes fabricated with semiconductor nanocrystals are narrow, tunable and have a symmetric emission spectrum compared with the conventional fluorophores [11]. Moreover, they are photochemically stable. These nanocrystals are available in different sizes ranging from 400 nm to 2 μ m. Also this type of crystals can be coated with different materials and depending

on the application. Semiconductor quantum dots are widely been used as bioconjugates [12]. Bio-molecules are attached to the quantum dots so that analyte, which attaches to the biomolecule, changes. For example, by changing the bio-molecule, it is possible to attach proteins, DNA and viruses.

Protein detection became more sensitive and efficient with nanoparticle based techniques [13] when compared to conventional clinical assays. When bones and joints need to be replaced, Titanium is the material, which was recently used because of its unique properties, but sometimes the body rejects the replacement because replacement interface is not accepted due to insufficient roughness. Introducing a bioactive appetite coating [14] can be used to reduce the rejection of replacement and opens a new path for nanotechnology applications in tissue engineering.

1.3 History of Cancer and Oncology

When the cells in a part of the body start to grow abnormally or out of control, it could be the beginning of a cancer or tumor. Although the cancer can occur any part of the body there are several common cancer types, namely they are, lung, colon, breast, pancreatic and prostate cancer. According to the statistics, cancer is the second leading cause of death in United States. Among them, lung cancers are responsible for most of the deaths. Colon cancer is identified as second leading cause of deaths from cancers.

The history of cancers goes back to about 3000 BC, even though it was not called cancer at that time. According to the records, it was first discovered in Egypt but categorized the disease as "No Treatment". The origin of the word cancer comes from ancient Greek from the terms called *carcinos* and *carcinoma*. Until the 15th century there wasn't any deep studying or understanding

about the cancer. Time to time in the history, people postulated different theories to explain the cause of cancer. Namely, they are, Humoral Theory, Lymph Theory, Blastema Theory, Chronic Irritation Theory, Trauma Theory and Infectious Disease Theory. The real development in scientific oncology started in 19th century after the discovery of modern microscope, so that it led to study the sample tissues of patients who died with cancers. By the middle of 20th century, with huge new discoveries and developments in science and medicine, scientists had a good understanding about biological systems. The discovery of the exact chemical structure of DNA led to uncover lots of hidden areas. They found out that the cause for cancer is genetic mutation due to chemical or radiation effect. Moreover, they also discovered that sometimes the damaged, mutated or defective genes can be inherited [15-17]. Today, there are a lot of causes of cancer, which could be explained by the lifestyle of humans becoming more complex and chaotic.

In general, the term *Oncology* means the study of cancers. First of all, the patient is directed for screening tests and then based on the results doctors can diagnose the stage cancer that the patient has. Depending on the stage, doctors can direct the patient to the most appropriate therapy. Most of the time, the early detection of a cancer is very hard [18]. When a patient starts to show the symptoms, the cancer has already spread into the major parts of the body. Therefore, early detection of a cancer is very important because it is the main factor that decides whether the patient will recover or not.

There are several types of techniques that are used to screen cancers. Incisional or excisional biopsy is the most common technique. In this technique, a tissue sample is extracted from the patient and will be used to identify whether there is an abnormal growth of the cells. Endoscopy is another technique, which is used to screen cancers, especially to examine the interior of a hollow organ, for example, the human colon. Moreover, there are some advanced techniques,

like ultrasound, Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) scan that, depending on the nature of the cancer, doctors can decide which is most appropriate. Some invasive techniques, like the use of radio isotopes have also rarely been used. With the development of nanotechnology, scientists have introduced novel techniques to detect cancer or tumors [19-22], but most of them are still need to be optimized to apply to a patient.

Once the diagnosis is over, the next step is cancer treatment. This step also depends on the nature of the cancer or tumor. Most of the time, surgery is used to remove the cancer or tumor completely. This will only be successful if the cancer or tumor has not spread over large area of the body. After the surgery, chemotherapy and radiotherapy can be used to remove some parts of the cancer or tumor, which is surgically hard to remove, or to reduce the risk of reoccurrence. Sometimes surgery does not guarantee a complete recovery; the cancer or tumor can spread over all the organs of the body. Surgery will only increase the survival period. Recent cutting edge research has introduced genetically modified cells that belong to the patient itself and are capable of destroying malignant cells [23]. This new technique was applied to chroniclymphocytic leukemia (CLL), which is the most common type of blood disease.

Chemoprevention [24, 25] is another way to reduce the risk of having a cancer. Here, the idea is to reduce the mutagenic damage to DNA that ultimately creates malignant cells. There are a number of chemoprevention, such as the use of healthy foods and drinks [26, 27], reduced the use of and exposure to carcinogenic chemicals or constituents and use of botanical dietary supplements [28-30] or drugs [31, 32] which modulates the regulatory pathways and reduce the DNA damage.

After centuries of research, the cancer or tumor is still a type of disease that remains as critical and does not have a specific treatment or therapy capable to cure completely. Chemoprevention is the best solution for cancer, however it is challenging for present lifestyles. Some chemopreventive drugs can have severe side effects when there is a long term exposure. Although there are therapies and surgical treatments for cancer or tumor, all of them are invasive and some modern approaches seem promising. Still there are a lot of areas which need to be modified. Therefore, a new less invasive and effective therapeutic approach for cancer or tumors is a timely requirement.

1.4 Cancer Statistics, Anatomy, Risk Factors and Colon Cancer Prevention



Figure 2: Percentage prevalence of colorectal cancer among adults 50 years or older in USA in 2006-2008. Image courtesy: Center for Disease Control (CDC), public domain.

As briefly outlined earlier, cancer remains the second leading cause of deaths in USA according to the Center for Disease Control and Prevention (CDC). Statistics indicate that colon and rectum cancer deaths are the second leading cause of cancer deaths in USA, and in numbers it was 49,380 in the year 2011. The death rate due to colon cancer from the years 2004 to 2008 is 20.7% in males and 14.5% in females. Moreover, it is estimated that during the year of 2012 there will be 51,690 deaths. Even though statistics shows that there are declines of the incidents in each ethnic category, overall it shows 1.7% increment per year since 1992 for adults younger than age of 50 years. Among the general population, the lifetime risk of colon cancer is 2.5%-5%.



Figure 3: Colon cancer incident rate and mortality rate in different ethnic groups Image courtesy: SEER NCI, public domain.

When it comes to anatomy, colon is located in the abdominal cavity and is the last organ of the digestive system. Although it does not play a major role along the digestive process, it extracts the water from the waste and absorbs some nutrients. The colon in mammals has four different sub-sections: they are ascending colon, transverse colon, descending colon and sigmoid colon. When it comes to the microscopic anatomy of the colon, there are four different layers of tissues can be identified. They are mucosa, submucosa, muscle layer and serous layer. Studying of the microscopic anatomy is very important, especially the mucosal epithelium because that is where the majority of colon cancers begin. Long, thin tubular glands known as crypts, which can be found in mucosal epithelium are the locations that undergoes cell division. Typically, the epithelium is completely replaced every four to six days. Cell division is considered an ideal process in occurrence of genetic mutation or damage. Therefore, this epithelium repair is vulnerable to genetic damage or mutation, which ultimately turns into a cancer by changing the regular cell reproduction or differentiation pattern.



Figure 4: Anatomy of colon; showing parts of the colon and its anatomical position in human body.

With other types of cancers, mainly colon cancers, occur because of inherited genetic abnormalities. Even with the main factor being inheritance, the carcinogenesis is a slow process and takes 10 to 15 years to develop. Mainly, there are few steps in colon carcinogenesis. The first phase is known as *initiation*, which is the stage that normal cells initiate towards a genetic mutation pathway. In *clonal expansion* stage, normal cells have not transformed to cancerous cells, but they have accumulated as clones, which are ready to move ahead. When further genetic changes occur and have turned cells cancerous, it is known as *benign tumor formation*, and this is when the polyps are formed. In *malignant tumor*, polyps convert to malignant tumor, and finally the malignant tumor acquires all the properties. Clinically, this cancer is referred to as *clinical cancer*. When it comes to the metastasis of colon cancer, soon after it penetrates the muscularis mucosa, it becomes invasive. Thereafter it grows deeper into the bowel wall and tissues. Lymphatic is one way in which the colon cancer spreads. Blood is another form, and the direct expansion to other organs is also a common way to spread, especially to the liver because the blood veins are a rich environment.



Figure 5: Microscopic view of a cross section of a colonic wall.

Research indicates that normally it takes five years to develop a polyp and in average, it will take the same time to transform a polyp to cancer. The next risk factor in line after the genetic inheritance is the influence of environmental factors such as the diet. Studies showed that red. processed meat can increase the risk and that the cooking method, such as the meat is being cooked at high temperature for long time, is also a factor in determining the risk. Eating of vegetables, fruits, natural whole grain, calcium and milk can lower risk of colon cancer development. According to the research, fatty acids and bile in diet caused the irritation in bowel mucosa, which proliferate cells with damage DNA. Highly reactive free radicals can also be easily combined with cellular substances that cause DNA damage. Therefore, a healthy diet is very important because dietary fats and dietary fibers play totally opposite roles when it comes to colorectal cancer. Smoking and alcohol consumption also increase the risk of having colon cancer. If the alcohol consumption two to three drinks per day, the risk can be increased by 23%. Obesity is another factor that leads to a colon cancer development. When it comes to age, incidence rate is 15% higher for the ages above 50 years. Also when the incident rate is compared between men and women, men have a 35%-40% higher chance of getting a colon cancer than women.



Figure 6: Polyp formation and different stages of colon cancer. (Image courtesy: NCI)

Physical activity is a major factor that can prevent most of the colon cancer incidences. Engaging in moderate activity for more than 30 minutes for five days is highly recommended. Studies indicated that high levels of physical activity decrease the risk by 50% for both men and women. People who become active later in life can also reduce the risk of cancer. On the other hand, aspirin like drugs or non steroidal anti inflammatory drugs (NSAID) can lower the risk of colorectal cancer but is not recommended due to side effects.

It is very rare to have any symptoms in the early stages of colorectal cancer; therefore, screening is very important. Most of the symptoms occur when there is a polyp. A few warning signs are;

bleeding from the rectum, blood in stool, discomfort feeling and need of bowel movement when there is none and unintentional weight loss. Blood in the stool is the most common warning sign because this mostly occurs due to polyps or cancer in colon. Lower abdominal pain, cramping and bloating is can also be a symptom and in most cases, the discomfort can be localized to right side of the abdomen and even is possible for a physician to feel it by touch. The most important physical exam, which can be done to examine colorectal cancer, is called digital rectal exam (DRE). After performing the DRE, samples can be used to perform fecal occult blood test (FOBT), and if the hydrogen peroxide, which is the chemical used to test whether there is a blood in stool, turns to blue indicates that there is a bleeding somewhere in gastrointestinal track. However, this test can provide a negative result in a situation when the presence of undigested food such as meat. After the development of fiber optics, endoscopy is considered as the main approach to diagnose colorectal cancer. The most common, reliable technique, which can examine the whole large intestine, is known as colonoscopy. Colonoscopy uses flexible fiber optics along with air line to inflate the bowel to help to keep it open. During the procedure, the patient is kept under conscious sedation. For this purpose, a drug which can make a person very drowsy and relaxed is being used. According to the data, colonoscopy has 85% detection rate for colorectal cancers.

Computerized axial tomography (CAT) is a technique that can reconstruct the internal anatomy with the aid of x-ray images. Unlike in regular x-rays, CAT scan can distinguish internal organs, blood vessels, lymph nodes and nerves with higher accuracy. Even though the CAT scan is not used for colorectal cancer screening, it is capable of providing indirect evidence of a spread of colon cancer to other internal organs. Therefore, the technique is used to perform surgeries when

the cancer become highly invasive and spreads to other important organs such as liver or kidneys.

Even though it is invasive, surgical treatment is the most common technique that doctors use to treat colon cancers. Pre tests are conducted to identify what stage of the cancer is in and in order to provide the baseline. During the surgery, the entire cancer has to be removed along with tissues, lymphatic and vascular supply. Adequate amount of surrounding tissues should be removed to lower the risk of reoccurrence. The most common complication after the surgery is blood loss. In some cases, blood transfusion is required. Infections are also possible after the surgery, therefore use of antibiotics is recommended. Radiation and chemotherapy can also be used to treat other types of cancers; however, they are still invasive and have a number of adverse effects. Therefore, an effective, less invasive alternate treatment for colon cancer is timely requirement.

1.5 Role of Surface Topography in Manipulating the Cell Fate

1.5.1 Motivation

In present, nanotechnology has a greater impact on finding alternative treatment techniques, especially for cancers, which is one of a leading cause for deaths in all over the world. But, most of them still are in an experimental level, requiring a lot of modification and optimization in order to develop the technique as a potential therapy. Even with alternative drug carriers, such as nanoparticles, the approaches can be toxic up to a certain level. To overcome most of the above problems, here we propose a whole new approach for cancer treatment that is not harmful to the human body at all. It is experimentally proven that the cell behavior is regulated by three factors: Chemical, Cellular and Mechanical.



Figure 7: Different factors affecting the cellular regulation.

The effects of these factors on cells are interrelated, but they can be studied individually because of their complex interrelationship. Mechanical factors include stress and strain exerted on cells. The best way to exert stress and strain on cells is to fabricate soft polymer scaffolds, which have unique topographies and can be used to seed cells. The topographies closely mimic the Extracellular Matrix (ECM), which is an important cellular structure that acts as a supporting membrane for cells. Preliminary studies have already shown that there is an effect on cells when the surface topography changes [33]. As electrical engineers, who are exposed to this different kind of field, there will be brief explanations of biological terms.

The microtopographies used in the preliminary studies had "trough-like' and "peak-like" The idea to choose that topography originated after carefully studying the structures. Extracellular Matrix (ECM) topography under normal cells, well and poorly differentiated cells using Atomic Force Microscopy (AFM). These studies are mainly focused on colon cancer treatment. The varying of normal cells to cancerous cells were chosen and seeded on the fabricated microtopographies. Moreover studies showed that Focal Adhesion Kinase (FAK) is active in well differentiated cells and inactive in poorly differentiated cells. Therefore, FAK active cells on fabricated "trough-like" topography are motile and on "peak-like" topography are immotile. Also when cells undergo mechanical stress, Rho-GTPases activate and generate contractile forces. Based on the above results, we hypothesize that the "Extracellular Matrix Microtopography critically regulates the cancer cell differentiation and invasion". Our aims were to carefully study how the ECM topography changes when cells become cancerous and experimentally prove ECM topography regulates the cell behavior. Eventually, the idea was to find out whether there exists a common ECM topography that is capable of regulating cells. The first step to test the hypothesis was to scan human colon tissue samples with normal, well differentiated, moderately differentiated and poorly differentiated cells using a Scanning Electron Microscope (SEM). Then the 3D structures, which closely mimic the actual ECM, were replicated on a bio-compatible material, mainly Poly di-methyl siloxane (PDMS) and sometimes it was Poly methyl-methacrylate (PMMA).

As engineers, lack of background in biological concepts and terms is regular fact. Therefore to make everything complete, commonly used biological terms and concepts will be introduced and discussed for better understanding.

1.5.3. <u>Cell</u>



Figure 8: Main constituents of a typical animal cell.



Figure 9: Main constituents of a plant cell.



Figure 10: Main constituents of a bacterial cell.

Cells are the fundamental structural and functional unit of all the living organisms. The body of all living organisms has cellular organization characterized by the presence of one or more cells in the body. The organisms with one cell in their body are called **unicellular**, such as bacteria and some viruses. Organisms having more than one cell in their body are called **multicellular**, and most of the animals and plants belong to this category [34]. In a typical animal cell, cytoplasm, a gel like substance found in the interior of the cell, facilitate the anchorage of different organelles such as mitochondria, Golgi apparatus, endoplasmic reticulum, lysosomes etc. Cellular organelles are responsible for carrying out essential biochemical and

metabolic processes such as respiration, macromolecule biosynthesis and transport of different molecules in the cells. Cell membrane separates cytoplasm form the outside environment and serves as a semi permeable barrier [35].



1.5.3.1. Cancer and cellular changes

During the process of transformation of a normal cell into a cancer phenotype numerous cellular changes take place. Genetics, carcinogenic chemicals, infections, radiation, hormones, diet and environmental factors can trigger the initiation, promotion and/or propagation of cancer [36-38] leading to the transformation of a normal cell into a cancer cell. Most of the cellular changes associate with cancer is related to cell proliferation and uncontrolled cell growth. In a benign or non cancerous cell, cellular functions such as cell division, growth and proliferation occurs in a



Image layout adopted from Surh Y.J. [39]

tightly regulated manner. But when a cell becomes a cancer cell, it will show increased uncontrolled growth and cellular functions such as metabolism. Also, there can be numerous morphological changes that occur in a cancer cell such as the cell size and shape, nuclear size, nuclear shape, chromatin content and nucleoli shape. The number will be changed making it distinguishable in appearance under a microscope. In a normal cell, the mutated cells will be either repaired or led to apoptosis (programmed cell death) in a tightly controlled cell cycle. But in the cancer cell, because the cell division is very rapid, there is not enough time for the repair mechanism to activate, and the mutation will carry in to the daughter cells. Also, because the death signals have been suppressed in cancer cells, the apoptosis mechanism can be overcome or bypassed and the cells can keep on proliferating. In most of the cancers the localized cancer is not the main culprit, but the spreading of the cancer cells in to other vital organs in the body, which is known as **invasion and metastasis**. The cancer cells associated with metastasis typically develop morphological changes as well as the changes associated with the attachment of them to the extra cellular matrix (ECM)[40].

1.5.3.2. Epithelial to Mesenchymal Transition (EMT)

Epithelial tissues line the cavities of the body giving them protection from the surrounding environment. Epithelial cell layers are tightly connected to each other and form one or several cell layers around the organs and cavities. Mesenchymal cells on the other hand are capable of migrating from one place to another and are important in the embryonic development. The concept of epithelial to mesenchymal transition (EMT) in relation to cancer is developed by the hypothesis that transformed epithelia cells can acquire the abilities to invade by behaving as mesenchymal cells. In a pathological point of view, EMT is important in the metastasis and invasion of cancers, since mesenchymal cells show high mobility compared to epithelial cells. EMT is associated with loss of adhesion proteins such as E-cadherin and increased expression of some proteins such as Snail, Twist and Zeb 1/2.



Figure 13: Schematic diagram of epithelial to mesenchymal transition.

1.5.3.3. Cancer Stem Cells

Stem cells are characterized by their ability to divide and differentiate into diverse cell types and possess the ability to self renewal in normal development. There are two different types of stem cells found in the development of human body; embryonic stem cells, which are found in the embryonic development, and adult stem cells, which are found in adults in different organs. Cancer stem cell hypothesis came to play recently into the world of cancer, stating that the cell population in a tumor is not homogenous and there is a small portion of cell population called "**cancer stem cells**" that can initiate and maintain the malignant tumors. The adult stem cells are considered to act as the culprit in this scenario[41]. Every normal tissue has adult stem
cell population which can undergo self renewal and can also differentiate into any cell type in that tissue of origin. Cells that can self renew can undergo infinite division. There is a growing body of evidence that the adult stem cells can act as cancer stem cells, which can initiate and maintain cancer. According to this elegant hypothesis, cancer therapy and caner preventive mechanisms can be mediated via these cancer stem cells.

1.5.4 Cell Membrane

The cell membrane is an important, semi permeable membrane with thickness of 4 – 10 nm which separates and protects the cell from surrounding environment. By facilitating and regulating the movement of molecules in and out of the cell, plasma membrane acts as a semi permeable membrane. Cell membrane consists of two layers of phospolipids with hydrophilic head and hydrophobic tails, so it is called lipid bi-layer. There are other lipids, such as cholesterol and glycolipids, in the cell membrane apart from the most abundant phospholipids. Protein molecules (integral proteins and peripheral proteins) are embedded in this lipid bi-layer forming a fluid mosaic structure as described by Singer and Nicolson [42]. Integral proteins can act as transporters and cellular pumps that facilitate the influx and efflux of molecules. There are glycoproteins and protein molecules on the outer surface of the cell membrane that facilitate the cell membrane to play a key role in cell–cell interaction and signaling.



Figure 14: Fluid mosaic structure of cell membrane.

1.5.5 Proteins

Proteins are complex biopolymers made up with α -amino acids, which can be found in all living organisms. Proteins are required to initiate every biochemical process in our bodies. Proteins can be broadly classified in to two categories, depending on their function, viz; functional proteins and structural proteins. Functional proteins may act as enzymes, signaling molecules, hormones and antibodies, which can facilitate important biochemical processes in the body. Structural proteins can be found in cytoskeleton and in extracellular matrix and plays an important role providing rigidity to an otherwise fluid cellular environment. Also, there are other structural proteins known as motor proteins, such as myosin, and fibrous protein, such as actin, involved in the muscle movements and cytoskeleton formation respectively [43]. One of the important behaviors of proteins is folding and unfolding [44, 45], which regulates biological activities. Also, there are some proteins that do not fold in regulating body functions. According to the classical picture, which is correct so far, the process is a result from entropically driven reduction in nonpolar surface areas.



Figure 15: Crystal structure of actin.

PDB ID: 1J6Z

The crystal structure of uncomplexed actin in the ADP state. (2001) Science 293: 708-711

Although the plasma membrane has less protein molecules compared to lipid molecules, they play an important role in cellular functions, cell adhesion and cell-cell interaction. Also, there are changes occurring to the intracellular proteins and membrane proteins during cancer. Therefore, most of the cancer therapies focused on proteins that are involved cellular functions and structure. For example, the most widely used chemotherapeutic drug, paclitaxal blocks progression of cell division (mitosis) by binding to microtubules, which is composed of the structural protein tubulin. Also, the cell-ECM attachment is mainly mediated via the cellular protein β - cadherine and the ECM protein integrine.

1.5.6 Extracellular Matrix (ECM)



Figure 16: Typical structure of extra cellular matrix.

Extracellular Matrix is a complex structural network around cells, which gives support and anchorage. It has the most complex molecular composition compared to other biological ECM plays highly important role in the body influencing the cell survival, assemblies. development, migration and shape. Moreover, it is essential for intracellular communication and segregation of tissues. Depending on the tissue type, ECM composition and abundance can be differed. For an example, ECM is very hard in bones and teeth, yet transparent and soft in ECM macromolecules are capable of organizing diverse forms depending on the cornea. requirement of the functional tissue [46]. Two major types of macromolecules can be found inside the ECM, namely; **polysaccharides**, which are chains of glycosaminoglycans, and **fibrous** proteins including elastin, collagen, fibronectin and laminin. Fibrous proteins are essential to maintain structural and adhesion functions in ECM. For example, collagen proteins are the main constituent of all ECM's. Glycosaminoglycans are another important constituent which are composed of repeating sugar molecules. Since these sugar molecules are negatively charged, entrapment of water will allow it to form a gel like structure allowing the ECM to withstand compressive forces. There are other proteins in the ECM such as elastin and fibronectin that play a role in structural stability of the ECM. For an example, elastin is a type of protein, which evolved to provide flexibility and to withstand stretch. Elastic fiber, which can be found in ECM, is made out of elastin and provides elasticity (resilience) to the tissues. Likewise, each protein in the ECM has a unique role which is why it is very complex and need to be studied a lot.



Figure 17: Structural view of collagen protein.

PDB ID: 2F6A A 'Collagen Hug' model for Staphylococcus aureus CNA binding to collagen. (2005) Embo J. 24: 4224-4236



Figure 18: Primary function of elastin protein, contracting and stretching.

1.5.6.1. Altered ECM and Malignancy

Although the initiation of transformation of a normal cell into a cancer cell occurs inside a cell, during cancer progression significant structural and mechanical changes to the properties of ECM constituents can occur [47]. Changes in cell shape and alterations in the interactions with the ECM are the main characteristics of cancer. Because ECM is in close proximity with the cells, it will affect the growth, survival, motility and angiogenesis of tumor cells. Adhesion of cells into the ECM will induce cellular signal transduction mainly via integrin and cellular receptors [48]. One of the main characteristic of malignant transformation of a normal cell is the loss of anchorage dependent growth. This will ultimately lead to develop more aggressive stage

of cancer, which involves cancer metastasis. Invasive cancer cell migration from the primary cancer site to secondary sites will be facilitated by proteins in the ECM such as integrin. Integrin mediated cell signaling pathways that involves focal adhesion kinase (FAK) and src family kinase (SFK) are the main pathways associated in invasion and migration of cancer cells. Angiogenesis or new blood vessel formation, which increases the O₂ and nutrition supply to the cancer, is a very important adaptation of the cancer to survive in an environment, where cells are growing fast and the nutritional needs are thriving. Increased expression of different classes of integrin in ECM facilitates is angiogenesis.

1.5.7. Focal Adhesion Kinase

Focal Adhesion Kinase (FAK) is a special type of protein found inside the body that has unique functionality in cell adhesion, mortality and many more [49]. FAK can be found in the structures called focal adhesion, which are multi protein structures linking ECM to cytoplasmic cytoskeleton.



Figure 19: Role of FAK in normal cells and in cancer formation.



Figure 20: Role of FAK in cell migration, survival and growth.

FAK is abundant in the cytoplasm of cells that are attached to the ECM [50]. FAK act as a molecule that can activate the integrin related signaling cascade to initiate and propagate adhesion and migration of cells in the ECM. There is evidence that FAK-deficient cells migrate

very slowly compared to FAK containing cells [51, 52]. FAK can be an important protein related to cancer. Since typical cancer cells tend to alter the growth rate, structure of the cytoskeleton, motility, adhesion and survival, it was expected to find highly expressed FAK in cancer cells leading to increased cancer cell motility, adhesion and proliferation; [53, 54] nevertheless, the FAK gene promoter has never being extensively studied.

1.5.8 Rho GTPase

Rho family of GTPase is a G protein that regulates signaling cascades related to cell adhesion [55, 56]. It is also responsible for the regulation of assembly and organization of actincytoskeleton [57] and also for growth and repair of neural pathways and axons. Although it was predicted to have mutated Rho GTPase in cancer cells, only one member of this family found to be mutated in cancer, yet over expression can be observed in different cancer types [58]. Still the exact mechanism of action is unclear whilst over expression of Rho GTPase contribute to increased invasiveness of cancer [59].

1.6 Fabrication of 3D ECM Scaffolds

Because the ECM is very important part of the body and has wide range of influences in cell regulation, it is really important to understand its mechanism. Scientists have not fully uncovered this yet. In order to do that, according to our experimental approach, it is required to extend the studies by characterizing and fabricating ECM. Therefore, a powerful tool is essential to characterize and fabricate ECM's that mimic the actual structure and, at the same time, holds the same mechanical properties. Even though the basic photolithographic techniques can be used, advanced lithographic techniques are desirable to fabricate high accuracy 3D ECM scaffolds.

1.6.1 Basic Lithography Techniques

Photolithography is the most fundamental approach in any micro and nano fabrication but has considerable drawbacks when narrowing down the length scale because it is not possible to reduce the size of the features below the diffraction limit of the UV light. Therefore, the highest resolution, which can be achieved with photolithography, is $\lambda/2 - \lambda/4$ of the light used, where λ is the wavelength of the light. Moreover, it is not a better candidate for advance 3D micro and nano fabrication. Recent studies have shown that standard photolithographic techniques can be used in an advanced manner in such a way that it allows creating very small patterns [60, 61].

1.6.2 Advanced Lithography Techniques

There are a number of techniques capable of fabricating 3D structures. Depending on the application, each technique is different. Dip Pen Lithography [62, 63] (DPL) is one of the newest technique introduced after the rapid development of scanning probe microscopy (SPM). Unlike the standard operational modes in atomic force microscopy (AFM) such as characterization, invention of DPL opens paths for direct draw technique with wide ranges of molecular substances. One of the main advantages is it's capability of placing molecules selectively within a highly localized region or even in a large area.



Figure 21: Dip pen nanolithography using AFM tip.

Development of this technique has cleared lots of pathways in research such as nanopatterning in electronic industry, magnetic particle deposition for storage and nanocrystal growth for biotechnology applications. The basic operational procedure is that the naturally formed water meniscus can transport the ink to the substrate by capillary effect. The ink and substrate should have the appropriate chemical conditions. The ink is transferred to the substrate by the diffusion process and the feature size is totally controlled by the dwell time. For example, to draw a circle the probe goes to the correct location and allows the ink to diffuse. Increase in dwell time, increase the feature size.

Area =
$$\pi r^2 = C\Delta t$$

Where C is the diffusion coefficient and t is the dwell time.



Figure 22: Feature size control by changing the dwell time



Figure 23: Basic process step of Nano imprint lithography

Nano-imprint Lithography (NIL) [64, 65] was introduced as high throughput, low cost nonconventional lithographic technique. Research indicated that the technique is capable of patterning sub-25 nm structures over a large area. The technique has two basic steps; the first one is the imprint on a resist with a mold which creates a thickness contrast pattern on the resist. Then using anisotropic etching process, such as reactive ion etch (RIE), residual resist in the compressed area can be removed. Resist is heated to a certain temperature before the imprinting to make it viscous and for easy deformation. One of the advantages is the resolution limiting conditions, such as scattering and diffraction which are critical in electron beam lithography and photolithography, but do not affect the resolution. Silicon and silicon dioxide can be used to create the mold but there is no restriction. In order to create the mold electron beam lithography and RIE can be used. However, when selecting the resist, it is favorable to use one with low thermal expansion coefficient. Mold release agents should be added in order to reduce the adhesion of the resist to the mold. Therefore, one mold can be used for multiple attempts, which allows reducing the cost. The drawback associated with this approach is that the minimum feature size always limited to the feature size of the mold, and since the standard electron beam lithography and RIE techniques used to create the mold, the sizes are restricted.

Soft lithography [66-68] is another technique which can be used to create 3D structures, and it is known as "poor man's method" because it is relatively low cost technique. Moreover, mass production is easy with this technique. This technique is also ideal for biological and medical applications. PDMS is the main polymer which is used in this technique. Once the PDMS stamp is ready, it can be used for four main different techniques, such as, micro-contact printing (μ CP), micromolding in capillaries (MIMIC), micro-transfer molding (μ TM) and near field lithography.

The LIGA process [69-71] is another approach to fabricate advanced 3D structures. In this process, synchrotron generated radiation is used to pattern the photoresist. The process is capable of creating high aspect ratio structures, but less access to synchrotron radiation prohibits the usage of the method. Therefore this technique is ideal for large scale production with the characteristic dimension in the sub micron range. In general, soft synchrotron radiation with the characteristic wavelength of 2 nm ($\lambda_c = 2$ nm) is being used. However the mask, which needs to use in this technique, should be able to block the x-ray radiation; therefore, it is required to use a thick x-ray mask, typically made out of beryllium foil.

Multiphoton Absorption (MPA) Polymerization is another advanced [72, 73] technique, which is used to create sophisticated 3D structures. The process is a non-liner optical effect created using an ultra fast titanium sapphire laser. In this process, the resist is exposed to the laser and by changing the focusing point inside the polymer the resist can be polymerized. This polymerization can be done according to the desired pattern. After developing the resist, unpolymerized areas can be washed away. Laser excitation can generate features with transverse dimension as small as 80 nm². Among these, few are highly appropriate for high resolution 3D structure fabrication.

1.6.3 Electron Beam Lithography

Electron Beam Lithography (EBL) is another state of the art fabrication technique, which is capable of producing sophisticated high aspect ratio 3D structures with a resolution of less than 20 nm. There are two main types of lithography systems, namely they are, point beam type and variable shape beam type. However, the point beam type EBL is the most popular one because it is capable of creating extremely small dimension patterns. Moreover, point beam EBL can generate high resolution electron beam with large nano-ampere order current which allows high speed patterning on the substrate. An EBL system consists of four main units. They are electron optics control system, high precision stage control system, lithography control system and vacuum control system.



Figure 24: Main units of an EBL system.



Figure 25: Main units of electron beam lithography column

Unlike UV light, electrons have lower wavelength. Therefore, it allows fabricating structures less than sub-20 nanometer regime.

According to the De-Broglie equation, λ (wave length) = h/p, where h is the plank constant ($h = 6.63 \times 10^{-34}$ Js) and p is the momentum of the particle.

Diffraction limit, $d = \lambda / 2$ (*n. sin* θ), where *d* is the slit width (in this context, it is the minimum feature size), λ is the wavelength and *n. sin* θ is called the numerical aperture.

When the writing takes place, the desired pattern is pixilated and assigned a dose to achieve the required height profile. In commercially available EBL system, there are two scanning methods have been used. The vector scan method, in which the beam is applied only over the selected pixels and the raster scan method, in which the beam scans on the substrate at constant speed while turning on/off the beam according to the pattern. An electron beam is generated in the gun at a vacuum of less than ~ 2×10^{-9} mbar, which is located inside the column. Inside the column, all of the electron optical controllers such as electron accelerator, magnetic lens, astigmatism corrector and aperture are present. Electron emission from the filament can be done in two different ways. When using a thermionic source such as tungsten, heat, of about 1800 K, is applied to the material until the electrons overcome the work-function. Sometimes there is a coating material such as zirconium dioxide to reduce the work-function. A field emission source can be used simply by applying electric field sufficient to tunnel the electrons through the barrier. For highly accurate patterning, the beam current should be constant throughout the process, and the stability of the high voltage system is most important because a small fluctuation directly change the current and the deflection angle of the beam, which leads to skewness and out of focusing of the patterns. Typically, the drift is less than 0.5% for 8 hours

therefore the beam current is almost stable because high voltage is controlled with an accuracy of 1/240000 using an 18-bit DAC.



Figure 26: Vector scan (left) and the raster scan (right) in electron beam lithography.

Focusing of the electron beam is achieved by electromagnetic lenses. Typically, the configuration has four lenses which are capable of writing stable patterns within few nanometer resolutions. Axis of the beam is another important factor which affects the high resolution patterning. Four sets of beam axis alignment coils are placed right above the each electromagnetic lens, to perform the alignment. The electron beam turned on and off by the beam blanking electrodes in both vector and raster scan modes. In this process an electric field is applied perpendicular to the optical axis. Ideally, the beam is supposed to be circular but due to

asymmetry of the electromagnetic field, creates astigmatism of the beam. Therefore, astigmatic correctors are placed around the optical axis to eliminate the astigmatism.

The operational chamber vacuum level is $\sim 10^{-6}$ mbar. Electron acceleration voltages differ from 0-100 kV, but in Raith eLine system, it is up to 30 kV; however, it is desirable to use low acceleration voltages to reduce the proximity effect, which will be discussed later.

PMMA is a commonly used positive tone resist and became a better candidate for EBL because of the great structural flexibility and resolution that can be selected according to the molecular weight of the polymer [74]. Also there are some other types of resists like SU-8, ZEP-520 and Hydrogen silsequioxane (HSQ), available for EBL applications [75-77]. Depending on the application one can choose the most appropriate resist. The first SEM based EBL machine was developed in 1960s' soon after the discovery of PMMA. The Raith eLine Electron Beam Lithography system available at the Nanotechnology Core Facility is also equipped with an SEM, which is capable of high resolution imaging with a beam spot of 7 nm, and is a plus for the project. Also, the laser-interferometer controls the stage with 2 nm position resolutions, which is an advantage in creating high accurate patterns.



Figure 27: Beam spot shape with and without astigmatism correction

Although the EBL technique has great deal of advantages, it has some limitations too. Unlike the parallel exposure in conventional photolithography, point to point exposure in EBL takes very long time to complete a job. EBL is very expensive and sophisticated technique, and the high maintenance cost limits the access to use it as a tool. Geometric aberration reduces the maximum write field size. Proximity effect due to the electron scattering broadens the feature dimension, which is the most significant limiting factor in EBL. Generally, proximity function is given by the approximate sum of two Gaussian functions which describe the behavior of forward and backward scattered electrons [78].

$$f(r) = k \left[\exp\left(-\frac{r^2}{\beta_f^2}\right) + \eta_E \beta_f^2 / \beta_b^2 \exp\left(-\frac{r^2}{\beta_f^2}\right) \right]$$

Where r is the distance from the point of the incident beam, β_f and β_b are characteristic width of forward and backward scattered electrons and η_E is the ratio of inter-generated contributions of backscattered to forward scattered electrons, while k is a constant.



Figure 28: Proximity effect in electron beam lithography

The magnitude of the proximity effect can be decreased by two ways. Use of low extraction voltages for patterning can significantly reduce the proximity effect [79-81]. Also, by changing the substrate or resist composition or both [82] can be useful in reducing the proximity effect. Some correction factors such as *Prognosis, Pattern dimension adjustment and Electron exposure adjustment* [78] can also be used to lower the effect due to proximity. Moreover one

can change the parameters depending on the application to reduce the proximity effect as much as possible, because each experiment should be treated individually.



Figure 29: Montecarlo simulation of electron scattering with high and low extraction

2. ELECTRON BEAM LITHOGRAPHY AS A TOOL TO FABRICATE 2D AND ADVANCED 3D STRUCTURES

2.1 Sample Preparation

Silicon substrates were chosen to fabricate the 3D structures and ECM scaffolds. Atomic plane orientation was not a critical factor, thus there was a freedom to choose silicon substrate with any orientation. Substrates were diced into 1 cm x 1 cm pieces with an uncut sample height of around 25%-20% of the total thickness. 495PMMA with anisole (4%) was purchased from Microchem Corporation. Then the PMMA was spun for 60 seconds at 1000 rpm, 1500 rpm and 2500 rpm which gave resist thicknesses roughly varying from 300 nm – 100 nm. Then the prebake was performed at 180 °C for 120 seconds. Values of the physical factors were not common for any application, thus they need to be modified and optimized depending on the application. A critical experiment, which required going down into sub-nanometer regime, needs optimized baking and development conditions. After that the resist was exposed using Raith-100 EBL system available at the NCF.



Figure 30: 495 PMMA 4% Anisole thickness variation with the spin speed

2.2 2D EBL Approach

The most common approach in electron beam lithography is the 2D EBL approach. A large exposure dose is applied on the resist which is enough to expose the resist completely all the way down to the substrate. This approach was used to reproduce the main features of the ECM scaffolds and to add the surface roughness.



Figure 31: Common 2D EBL approach.

SEM images of human colon tissue samples under normal cells, well differentiated, moderately differentiated and poorly differentiated cancer cells were obtained. However, 2D EBL approach was only capable of replicating the main features of ECM under normal colon cells and well differentiated cancer cells. Other ECM's were highly complex; therefore, it was not possible to replicate them with the common approach. GDSII designing software is used to design the ECM templates, which is a built in option to Raith eLine software. Then the resist was exposed with a suitable dosage. Typically for this kind of pattern the writing time was 10-12 minutes. Methylisobutyl-ketone (MIBK) diluted with iso-propyl alcohol (IPA) with the ratio of 1:3 was used to develop the resist for 1 minute. However, for some critical dimensions the developing time should be changed in order to reduce the over development. After that, the sample was soaked in IPA for 30 seconds to stop the development. Measurement of beam current before every run is required in order to calculate the area, line and dot dosage levels.

$$Area Dose = \frac{Beam Current \times Area Dwell Time}{(Area Step Size)^2}$$
$$Line Dose = \frac{Beam Current \times Line Dwell Time}{(Line Step Size)^2}$$

Required dose calculation for a certain exposure is done with the above three equations. The beam current is the parameter which needs to be measured and the step size is the minimum pixel size of the pattern. Although step size critically affects the feature quality, it can be changed but it is important to choose the correct value.



Figure 32: Estimation of main feature locations and size with a custom grid for ECM under well differentiated cells.

In order to use the 2D EBL approach a template had to be designed. Therefore the locations of the main features were roughly estimated. In order to do that, a grid with equal spacing was placed on top of the image. Since the typical write field size is 100 μ m, template size was restricted to 100 μ m x 100 μ m. Then the features were designed in such a way that it closely represents the actual main features. After that, the resist was completely exposed and developed.



Figure 33: Estimation of main feature locations and size with a custom grid for ECM under well differentiated cells.



Figure 34: GDSII view of the ECM image corresponds to the ECM under normal cells.

Similar steps were performed for the ECM image corresponds to the ECM under well differentiated cancer cells. Since the main features were not circular and symmetric, several special customized techniques were used to design the template to make it more similar to the actual image.



Figure 35: GDSII view of the ECM image corresponds to the ECM under well differentiated cells.

Then the next step was to add and increase the surface roughness to the fabricated ECM scaffolds. Surface roughness is an important factor when dealing with biological materials. For example, with the absence of required surface roughness, cells will not attach to the fabricated surface, or in a case like bone or joint transplant, if the required surface roughness is not there, it can be rejected by the body. Therefore the surface roughness should be close enough to the actual biological surface. In order to add and increase the surface roughness a grid of lines wide 1 μ m with a period of 5 μ m was placed on top of the ECM template. Since the grid should not be exposed all the way into the substrate dose level had be chosen accordingly. Therefore a very low dose level was assigned to the grid pattern. After that, the initial grid pattern was modified and replaced a grid with thin lines with the width of 500 nm and the period reduced by half. Moreover, the roughness was further modified by adding overlapped doughnut patterns with



Figure 36: GDSII view of the ECM template with the grid used to increase the surface roughness corresponds to the ECM under (A) normal and (B) well differentiated cancer cells.



Figure 37: GDSII view of the ECM with overlapping doughnut pattern and zoomed view without and with the dose.

2.3 <u>3D EBL Approach</u>

This is a complex EBL technique, and there are very few facilities: less than ten in the whole world who are capable of using it. Among them, the Nanotechnology Core Facility here at UIC is the only facility that uses the advanced 3D EBL technique for biological and medical application. The technique fabricates real 3D structures with less than sub -5 nm z- resolution. Moreover, the technique is capable of creating real 3D structures for wide range of other applications as well.



In gray-scale EBL, the electron beam dose is kept at a constant value, but the required thickness contrast is achieved by changing the transparency of the mask in such a way that change in transparency leads in reduction of the dose. In 3D EBL, the thickness contrast is achieved without a gray scale mask. Dose of the electron beam energy is controlled carefully in such a way that it creates the specific feature with required height. However, to achieve this, it was required to have adequate information of the relationship between the dose of the electron beam and the corresponding thickness of the PMMA resist. To obtain this information, it was required to fabricate large number of test structures. A pyramidal structure was the ideal 3D pattern which we thought that could give us accurate information about the dose depth relationship.

Similarly, as in the previous approach, GDS II designing software was used to make the required templates.



Figure 39: Fabrication steps of 3D EBL technique.

The step size of the fabricated dose pyramids was chosen with five different values starting from $1 \mu m$ to $5 \mu m$. The reason to choose different sizes was to produce a structure with fewer defects which can accurately provide information because when the feature size goes down, proximity effect plays a major role. However, the proximity correction can be added, but it increases the dwell time significantly because it breaks the original features to large number of small features. The dose pyramid templates had to be designed carefully. Since the idea was to obtain thickness contrast over the resist, a special designing method has to be established in order to overcome couple of problems. Therefore three different approaches were used and two of them provided
accurate information. In the first approach, set of squares placed on top of each other in such a way that the center of all the squares share same axis. Dose was carefully assigned to each square, to obtain the thickness contrast by changing the dose of the electron beam. Pyramid can construct either step up or step down configuration by changing the descending or ascending.



Figure 40: Top view (left) and 3D side view (right) of square layer staked pyramid.

The problem associated with this approach was either when writing step up or step down pyramid, the electron beam shoots on top of a common region. Therefore, one region keeps on exposing over and over again, and at the end it will be over exposed. After developing the sample, it was not possible to obtain the required thickness contrast. No matter how much dose of the electron beam has reduced, once the resist was exposed to electron beam, it was very easy to expose the all the way into the substrate. Therefore, a different approach had to be established to overcome the problem.



Figure 41: 2D (left) and 3D (right) AFM images of the fabricated square layer stacked pyramid.



Figure 42: Cross sectional profile of the square layer stacked pyramid.



Figure 43: 2D (left) and 3D (right) AFM images of square layer stacked pyramid fabricated with extremely reduced dose.



Figure 44: GDSII template of tile type dose pyramid without the dose (left) and with dose (right) colors.

The next approach was to use small squares with the required step size to construct the whole pyramid. In this approach it was necessary to assign dose to each square, therefore in a situation with $10 \ge 10$, there are 100 squares which need to treat individually, and thus it takes long time to design the template. The other problem is there is a small discontinuity in between small squares. However, it was not affected to the accuracy of results.

Feature size was varied from 1 μ m to 5 μ m, but when the feature size goes down, there was a tendency of having unwanted artifacts on the pattern. We believe it was mainly due to the proximity effect, because the artifacts were significant where the electron beam dose was high.



Figure 45: Side view (left) and 3D side view (right) of tile type dose pyramid.

In EBL, there are two main methods to change the dose. The first method is to change the dose during the template design, so that the designer has freedom to assign any amount of dose for individual objects. The other method is the overall dose can be increased or decreased by a desired factor, thus it will multiply the assigned dose with the given factor for all the objects in the template.



Figure 46: Cross sectional profile along of the time type dose pyramid along the adjacent squares with a small discontinuity.



Figure 47: Tile type pyramid of 1 µm step size with unwanted artifacts due to proximity effect.

Write field alignment was not required since it was a single exposure and there were not multiple steps involved in the process. Location was assigned for each template with the specific U, V coordinates. Therefore it was easy to locate the pattern during the scan. Moreover the patterns were placed towards the center of the 1 cm x 1cm to facilitate the atomic force microscopy scanning.



Figure 48: Cross sectional profile of tile type pyramid of step size with 3 $\mu m.$



Figure 49: 2D (left) and 3D (right) AFM image of a tile type pyramid.

The last approach was the best approach capable of delivering most accurate results. The data was mainly used to obtain the dose depth relationship during the next set of experiments. Different size closed loop square frame like shapes stacked next to each other in such a way that it creates a pyramid. The close loop for each step was created using line command available in GDS II software. However, the coordinates had to be pre determined for each step before designing the template. Each step consists of eleven points; therefore, it completes the step pattern. In this design, the electron beam does not shine on same region again and again, so that after the development, it is capable of providing the required thickness contrast which is the main goal we wanted to achieve. Also, the continuity of the design was well maintained in the patterns. Since the patterns are relatively large, step size of the software was not required to

change; however, to maintain the consistency, it was kept at 0.016 μ m throughout the patterning process.



Figure 50: Cross sectional profile of tile type pyramid with grayscale levels.



Figure 51: 2D (left) and 3D (right) AFM images of tile type down pyramid.



Figure 52: Top view (left) and 3D side view (right) of frame layer type pyramid

After performing the atomic force microscopy (AFM) scan, the cross section profiles of the each pattern were carefully studied and the values were recorded. The image analysis utility in AFM software is capable of delivering the required information. The dose was assigned to each layer starting by 0.2 μ C/cm² and the value was increased up to 1.2 μ C/cm² with the increments of μ C/cm². It was not possible to increase the dose more than 1.2 μ C/cm², because it was enough to expose the PMMA all the way down to the substrate for a resist thickness around 300 nm. In our final application, the required change in z-value could be within 100 nm. Therefore, the information, which extracted from the studies with 300 nm thick resist, was adequate. Since it was necessary to obtain accurate data, the total size of the pyramids kept at less than 50 µm.

Otherwise during the scanning process, there was a possibility of generating an error along zprofile, because the AFM piezo's need to expand and contract over a large area which can create bended 3D image. AFM software is capable of overcoming the problem by simply flattening the surface, but there is a possibility of losing some important data during the process.



Figure 53: GDS II views of frame layer type pyramid with step size of 2 μ m with (left) and without (right) dose colors.



Figure 54: 2D (left) and 3D (right) view of a frame layer type pyramid with step size of 3 μ m.

Table below represents the dose and depth of PMMA data of four patterns which was observed using AFM.

Dose	Depth (nm)					
	Pattern 1	Pattern 2	Pattern 3	Pattern 4	Average (nm)	Std. Dev (nm).
0.2	4.64	4.51	4.6	4.96	4.6775	0.196022958
0.3	6.66	5.97	8.016	7.38	7.0065	0.885623509
0.4	13.52	11.25	11.67	13.04	12.37	1.082558697
0.5	15.49	13.79	17.48	18.95	16.4275	2.258766256
0.6	22.22	21.3	27.2	27.73	24.6125	3.322181763
0.7	35.17	35.22	37.43	40.57	37.0975	2.543558859
0.8	54.41	53.21	56.48	57.86	55.49	2.078605302
0.9	83.24	79.64	88.99	91.38	85.8125	5.348241923
1	128.25	124.02	135.62	139.77	131.915	7.099091491
1.1	191.11	182.19	189.46	198.97	190.4325	6.885551902
1.2	239.68	238.45	246.19	246.73	242.7625	4.304581861

Table 1: Measured depth of PMMA for different dose values.

Following graph represents the does and depth behavior of the dose pyramid in which the minimum step size was $3 \,\mu m$.



Figure 55: Depth of the PMMA vs the applied dose of the electron beam.

When carefully looking at the graph, it looks like an exponential behavior. But there are two linear regions can be identified in the graph, and it was almost similar for all the dose pyramids with different step sizes. From $0.2 \ \mu\text{C/cm}^2 - 0.7 \ \mu\text{C/cm}^2$, there is one linear region and from 0.8 $\ \mu\text{C/cm}^2 - 1.2 \ \mu\text{C/cm}^2$ the other linear region exists. To perform maskless grayscale lithography, one can choose either upper or lower region of dose depending on the required thickness. It can be used to achieve good step profile as shown in figure 2.19. However, the obtained data can be fitted to the following formula;

$Depth(dose) = 2.2233 exp (4.016 \times dose)$

The exponential behavior of the depth is can also be seen in the cross section profile of the pyramid. Ideally, the step size should be 3 μ m, but the approximate measured value is 3.531 μ m. The discrepancy of the values occurs due to the proximity effect and developing conditions, which is another physical factor which needed to be controlled carefully.



Figure 56: Cross sectional profile of the frame layer type pyramid with the step size of 3 μ m.

However throughout the whole approach it was required to control the conditions very carefully. During the patterning, the electron beam was carefully controlled to get the required thickness. There are several parameters in EBL, which user can manipulate in order to optimize the results. Physical working distance can be adjusted by changing the height, in terms of the local coordinate system which makes the users life easy, it is the w-coordinate. Desired values can be directly entered through the stage control utility. The maximum resolution is 1 µm, since changes in z-direction are not critical. The minimum physical value for the working distance is 3 mm but it is not recommended because the gun is at very close range to the stage. Generally, the beam current is measured at physical working distance of 5 mm, but the standard working distance is 10 mm. However, the user can change this physical working distance as desired, but during these experiments it was maintained at 10 mm to preserve the conditions. There is another working distance which the user can adjust, but it is done electronically. The process focuses the electron optics inside the column in order to get a clear image. Change in physical working distance directly affects the exposed depth of the resist since it changes the electron However there is no better understanding or mathematical explanation about the energy. relationship of the physical working distance and how it changes the electron beam energy, but they are inversely proportional.

Electron beam energy
$$\alpha \frac{1}{Physical working distance}$$

Adjustments of the working distance inside the column can be explained easily. A comparison can be done between a regular lens and a magnetic lens, since the focal point of an electromagnetic lens is defined same as in optical lens and it is given by;

$$\frac{1}{f} = \frac{1}{s_0} + \frac{1}{s_i}$$

Where, f is the focal length and s_0 , s_i are object to and image to lens distances.

The magnification (or de-magnification, 1/M) can be given by;

$$M = \frac{d_0}{d_i} = \frac{s_0}{s_i}$$

And for electromagnetic lens, focal length is related to the current (*I*) as follows via relativistic electron velocity E_0 , $f \propto \frac{E_0}{I^2}$. The applied force on electrons in electromagnets relates to the magnetic field according to following equation;

$$\vec{F} = -e\left(\vec{v} \times \vec{B}\right)$$

Where F is the force on electrons, e is the electron charge, v is the velocity of electrons and B is the magnetic field.



Figure 57: Comparison of Electron optics and optics when focusing an electron beam and light.

The aperture size is another parameter which the user can adjust. There are six different types of apertures available in system. The sizes are 7.5 μ m, 10 μ m, 20 μ m, 30 μ m, 60 μ m and 120 μ m, but the standard aperture is 30 μ m. When the aperture changes, it directly affects the electron beam current. Small size aperture reduces the current, and large size aperture increases the measured beam current because it controls the number of electrons which passes through. Also there is a limit when reducing the aperture size since the wave nature of electrons can lead to generate diffraction patterns. However, in our experiments the standard aperture was used, again to maintain the consistency, but sometimes optimizations were carried out with different size of apertures.

The third parameter, which the user can change, is the acceleration voltage. As outlined in the previous chapter, electron emission in the gun can be constructed in two different ways, namely they are thermionic emission and field emission. In thermionic emission the emission current density is described by the Richardson-Dushman formula, which is;

$$J = A T^2 \exp(-\frac{\varphi}{kT})$$

Where *A* is a constant, *T* is the temperature, φ is the work function of the material and the *k* is the Boltzmann constant. As soon as the electrons jumped out from the filament, it undergoes acceleration through an electric field. This acceleration voltage has to be maintained in a precise value throughout the writing process because slight change can lead to a huge error. Increase of the acceleration voltage energizes the electrons and increases the velocity which is given by;

$$v = \sqrt{\frac{2eV}{m}}$$

Where e is the charge of the electrons, V is the potential difference and m is the mass of an electron.

Changing the acceleration voltage directly affects the exposed depth of the PMMA resist. Highly energized electrons are capable of penetrating deeper into the resist; therefore, it had to be kept at 10kV for all the experiments to make sure in maintaining same conditions. As stated earlier, optimizations were tried with different voltages. However change in acceleration voltage changes a lot of other parameters as well. Therefore, it is a parameter which needs to be handled very carefully.

As briefly outlined earlier, developing in 3D EBL is a crucial step. In 2D EBL, it does not require any control in developing because the exposure was done all the way down to the substrate. However, in 3D EBL, the resist is not exposed all the way to the substrate, and it could be either close to surface or substrate. If the developing step is not controlled, there is a possibility to wash away the regions which need to be protected. The developer was diluted with iso-propyl alcohol, and the dilution can be done with different ratios. Obviously in this approach a rapid development should be reduced. Therefore the developer was diluted with 1:3 ratios to gain more control by reducing the developing speed. There are several parameters associated when characterizing a resist. Contrast (γ) of a resist is the main measurement associated with the development performance and it measures the response of resist solubility to change of exposure dose. The contrast is defined as;

$$\gamma = \log^{-1} \frac{D_0}{D_i}$$

Where D_0 is the critical dose, which is the minimum dose required rendering the resist completely soluble and D_i is the exposure.

Cold development is another technique introduced to gain more control because the temperature of the developer is a critical factor which affects the quality of the patterning resolution, roughness, cross section shape and line edge roughness. However, the argument is valid for thick PMMA (>500nm) more than thin resist. Development time of the exposed resist is determined by the dissolution rate (D) of the PMMA and is given by;

$$D = Aexp\left(\frac{-\Delta E_{ad}}{kT}\right)$$

Where A is a constant, ΔE_{ad} is the PMMA activation energy (depends on the PMMA molecular weight), k is the Boltzmann constant and T is the temperature. In our experiments the average time was 25 seconds – 30 seconds. The value was determined after carefully studying different developing times. The cold development was not performed since the regular method was capable enough to produce the required pattern and the resist thickness was fairly thin.

2.4 One-to-one Image to 3D Fabrication

This is the most advanced and accurate technique which we developed especially to fabricate 3D scaffolds. Using this technique, the image contrast can be directly converted to dose which ultimately gives the corresponding 3D structure. The conversion can only be performed with grayscale image with 8 bit resolution. Therefore along z-direction the technique can produce 256 grayscale levels which are more than enough to get the sufficient thickness contrast to reconstruct the corresponding 3D image. Time consumption and high computer resource requirement is the drawback associated with the technique.

To incorporate the technique to our fabrication process, scanning electron microscopy (SEM) was required. Collaboration with the UIC medical school and Department of Bioengineering lead us to obtain the required images of the ECM's under different stages of colon cells. Grayscale SEM images were ideal candidate for one-to-one reproduction. In general, the obtained images were smoothened and de-noised to increase the quality using freely available image enhancement software called Reshade. This step was not required but is capable of

increasing the quality of the fabricated pattern. Then the resolution was brought down to 8-bit before performing the conversion. Any image analysis software could perform the task, but mostly we used Adobe Photoshop. The number of pixels of the image is another parameter which needs to be considered since it is helpful in determining the area of the fabricated structure. The other parameter is the conversion step size, and it is the minimum size of the GDS II pixel size. Manipulation of the pixels of the image and the conversion step size can be used to get the required area. In a regular image, increase of the number of pixels can increase the quality of the image. The same argument holds in our fabrication method. Resolution of the fabricated structures can be increased by decreasing the step size. However, decreasing the step size consumes lots of resources. Therefore in our experiments we didn't use step sizes less than 100 nm. In order to calculate the area of the pattern should be multiplied with the step size. For example let's consider the following image in which the dimensions are;



Figure 58: 8-bit gray scale image of Albert Einstein (Courtesy of the Library of Congress LC-USZ62-99506). Width = 256 pixels and Height = 256 pixels

Let's assume the step size which we are going to use is 100 nm (0.1 μ m). The size of the fabricated structure is give by

After doing the math for the above picture length and width of the fabricated structure will be $25.6 \ \mu m \ x \ 25.6 \ \mu m$. After the conversion of this image, the converted GDS II template consists of 65,536 squares each with the dimension of 100 nm x 100 nm.

Gamma level is another factor which can determine the writing depth of the whole pattern. By increasing or decreasing the value a desired area can be selected. During the conversion the value was kept it at 0.3 - 0.4 since we wanted to convert the whole image without any elimination. The pictures below are the view of GDS II template without and with dose colors. In the zoomed portion, the number of unit pixels can be seen.



Figure 59: GDS II view of the converted Einstein image without (left) and with (right) dose colors.

The design dose range is from 0.163 to 1.00 and the way that we achieved the dose contrast to reconstruct the image is can clearly be seen. After writing and developing this dose contrast will appear as thickness contrast on the resist. Upon determining optimizing all the physical and chemical parameters, the technique can be used to replicate any surface. However, writing time this technique is comparatively long. Since the EBL is point by point exposure method, for this image it has to expose more than 65,000 squares. Approximately it was taken two hours to write this image. Larger images take up to 10 -15 hours and sometimes even more.

Images below represent the colon epithelia under normal cells and the same image after enhancing the properties. The image on the right is much smoother than the one on left. Also the dimensions were changed to 400 x 400 pixels.



Figure 60: Original SEM image (left) and the smoothened, enhanced and resized image of normal colon epithelia.

After that the same parameters were used to convert the image to GDS II template. Since the image size was different, the converted template consists of 160,000 squares because the step size was increase to 200 nm. Otherwise the file could be too big and take long time to finish writing.



Figure 61: GDS II view of the converted normal colon epithelial image without (left) and with (right) dose colors.

2.5 Fabrication of 3D ECM (EGG Crate) Scaffolds for Stem Cell Experiments

In this part of the project one-to-one image to 3D fabrication techniques were incorporated with another technique in which our lab introduced, especially in fabricating 3D structures. This technique is called thermal reflow, and basically it is considered as a "fine tuning" step in 3D fabrication. In order to increase smoothness of the any fabricated 3D structure, this technique will be the best candidate. However when performing this technique, the overall height also drops down and it tends to spread out as illustrated in the figure below.



Figure 62: Shape of the structures before thermal reflow (left) and after thermal reflow (right).

Few experiments were conducted to determine out the appropriate temperature. It should not be too hot or low. Because it would melt down everything or would not do make any changes to the features. Since the manufacture suggested pre-bake temperature is 180° C, we believed the appropriate temperature should be somewhat around. However after performing several experiments it turned out that 180° C is the best temperature which capable of producing the best result. The next parameter which needs to concern about is the time. In a similar manner the appropriate time was determined. In general, it was 20 seconds – 25 seconds but if the features are ruined with different artifacts, another 10 - 15 seconds could be added.

The process can be approximated by the given empirical equation:

$$\frac{h}{h_0} = 1 - a \log_{10}\left(\frac{t}{t_0}\right)$$

Where h_0 is the initial height, h is the height after thermal reflow, a is a constant which equals to 0.33, t_0 is the time at h_0 and t is the total time of the process.

Fabricating the egg crate like profile was essential for stem cell experiments. However it was quite challenging, and previous 3D fabrication techniques were not capable to produce the ideal profile. In order to use the one-to-one image to 3D technique, it was necessary to obtain an ideal image which can provide the required dose contrast and the image itself has to be created. We tried three different types of software to get the task done, but MATLAB and Mathematica was the only two candidates which provided a better solution. The two dimensional view of the egg crate profile is similar to sine or cosine function. Therefore, addition of two sine and cosine functions along x, y direction was capable to generate the 3D structure in which at the end transformed into the 2D picture. In order to make the profile sharp hills and valleys, squared value of the sine and cosine functions can be used. Later on without 3D to 2D transformation, a simple 2D contour plot was capable in managing the problem. Given below is the MATLAB coding which was used to generate the image. In similar fashion, the same image can be generated in Mathematica too.

[X,Y] = meshgrid(-5:0.0025:5,-5:0.0025:5);

 $Z = (sin(X)).^{2} + (cos(Y)).^{2};$

figure;

[cs,hc]=contourf(X,Y,Z,15);

set(hc,...'EdgeColor','none');

Given below is the generated 3D egg crate profile and 2D contour egg crate profile. Even though the generated images consist with colors, it can be easily converted to grayscale in such a way that it creates a sufficient dose contrast.



Figure 63: MATLAB generated 3D egg crate profile by adding sine and cosine functions.



Figure 64: 2D view from top of the transformed 3D egg crate profile.

The above egg crate profile can be modified in a manner that it looks like the ideal egg crate profile, by modifying the MATLAB code as given below. By comparing the images it is obvious the enhancement of the structures.

[X,Y] = meshgrid(-5:0.0025:5,-5:0.0025:5);

Z = sin(Y-X.*sqrt(3)).*sin(Y+X.*sqrt(3)).*sin(2.*Y);

figure;

[cs,hc]=contourf(X,Y,Z,15);

set(hc,...'EdgeColor','none');



Figure 65: 2D view from top of the enhanced egg crate profile.

In a similar manner we performed one-to-one image in the 3D approach, the egg crate profile was constructed. The AFM image below shows the fabricated egg crate profile. Careful study of the left image illustrate that there is a small discontinuity along the thickness contrast. The error comes with the discontinuity of the dose contrast. However after the thermal treatment which represents in right image, the structures were smoothened and the discontinuity was eliminated. The overall height has been dropped down by roughly 150 nm.



Figure 66: 3D AFM images of the one-to-one image to 3D converted egg crate profile before (left) and after (right) thermal reflow.

Even though this approach gives us the flexibility to fabricate the required structure, it consumes time and lots of computer resources. EBL writing time for only one template with 40 μ m \times 40

 μ m consumes 3 – 4 hours. Also it requires a suitable image which can provide the required thickness contrast, and it is very hard to get rid of the discontinuity. Therefore it was required to continue the studies to find out an alternate method to fabricate the structure with minimum resources. Our studies indicate that the thermal treatment itself is capable of creating the required structure. In order to do this, the resist was patterned with an array of holes with a period of 2 μ m. The diameter of the holes was varied as 900 nm, 1.0 μ m, 1.1 μ m and 1.2 μ m. As we did in 2D EBL, the resist was exposed all way down to the substrate and developed. Then the thermal treatment was applied to the developed sample with an average time of 30 – 35 seconds, which is little higher than the previous value we used. The AFM images showed that after a simple thermal reflow, the holes were totally transferred to egg crate profile and the cross sectional image indicated the ideal sinusoidal curve. The corresponding images are shown below.



Figure 67: 3D AFM images of the array of holes (left) and after the thermal treatment to array of holes in converting to egg crate profile (right).

As we seen before the overall height has been reduced, however this time it was by more than 200 nm because the treatment time was little higher than the previous case.

3. CHARACTERIZATION TECHNIQUES, RESULTS AND DISCUSSION

3.1 Introduction to AFM

In order to study the accuracy of the fabricated samples, high resolution 3D characterization technique was required. For this requirement, Atomic Force Microscopy (AFM) is the best candidate which capable of delivering accurate 3D and 2D information about the fabricated nanostructures. AFM falls into Scanning Probe Microscopy (SPM) family which is called Scanning Tunneling Microscope (STM) developed in early 80's at IBM Research Zurich [83, 84]. AFM is a versatile tool which can be used for wide range of applications including micro/nano device fabrication [85], [86], [87], [88] and biological [89], medical applications [90], [91], [92].

A laser beam shines on the moving cantilever which reflects back to a photodetector provides the required information which is capable of generating the 3D topographical image. Contact and Tapping modes were used to study the topography of the sample surfaces and liquid imaging with both modes were frequently used to study the cell topography. Moreover, AFM can be used to study magnetic, electric and lateral forces too. Nowadays the AFM has evolved from an imaging tool to measuring tool which allows researchers to conduct wide varieties of experiments.

The hypothesis of the project solely based on the mechanical factors which govern the cell behavior, therefore it is an important step to study about the mechanical properties and understand them. AFM was also used to study about the local surface properties of the fabricated samples, such as the Young's Modulus or Elasticity because of its accuracy down to nanometer scale with fine controlled very small applied force in the range of micro or nano newtons. Moreover, it can be used to measure adhesion of surfaces. Therefore it is ideal
technique to study the surface properties, especially on soft polymeric and biological samples. Indentation method is the first technique which was used to measure the interaction forces; however, it has limitations too. In order to perform the indentation, it is required to find out the desired location and it is a challenging step because of the small length scales. Also when it comes to the sticky and soft samples, probability is high in breaking the linearity of the cantilever. Phase imaging can also be used to study the elasticity, thus it is not an accurate method due to lack of quantitative data.

In simple force distance method, cantilever is lowered towards the desired location of the sample which required measuring the force. At a certain distance the cantilever feels the attractive force, and it attracts towards the sample with deflection. When it retracts until there is a certain force the cantilever won't disengage from the sample and just after the applied force is enough, it retracts [93]. When performing indentation, the sample will be held in such a way that it's hanging. Then the cantilever lowered towards the sample and a force is applied on top of the sample. From the deflection of the cantilever the force can be calculated.



Figure 68: Approach and retraction of AFM tip towards and away from a sample.



Z position

Figure 69: Force calibration curve for one approach and retract cycle of the tip.

A qualitative measurement of the adhesion properties and characteristic of a sample can be interpreted by observing the force calibration curves. In general, there are six main types of curves have been discussed. However it is highly likely to observe different kinds of curves depending on the type of the sample. Moreover, the type of force exists between the sample and the cantilever tip can be different too, and there are several types of forces which significant in



Figure 70: Qualitative characterization of samples using the force calibration plot.

nanometer regime. Namely, they are van der Waals (vdW) forces, Capillary forces, Casimir forces, Chemical potential, and Magnetic and Electrostatic forces. Also when the tip is in contact with the sample, it allows measuring Young's modulus or elastic properties and Viscoelasticity. In biology, cell surface interaction can be measured when the tip retracts from the sample. Protein unfolding and DNA stiffness can be measured when the tip is at sub 10-100 nm regime.

Especially when imaging cells using AFM, the height of a cell is a critical factor. In general, cell height can be up to 15 μ m. Therefore, the maximum *z*-resolution of the scanner in AFM should be comparable with the typical cell height. Otherwise, it would be extremely difficult to image cells, especially in contact mode operation. However in tapping mode, it is possible to perform the scanning without number of difficulties. One possible solution to perform cell scan with low z-resolution scanner is to scan different regions of the cells separately. For example, it is easy to scan the edges of the cells and nucleus of the cell, separately, because around the nucleus, the cell has maximum height. By combining the images, it is possible to construct the full picture. BioScopeTM from Bruker AXS is specially design AFM to scan biological samples, especially cells since it has a high z-resolution. Moreover, there are lots of other factors which the scientists want to study on cells other than the topography. For example, it is very important to study about the adhesive properties, molecular interactions, surface charges and surface hydrophobicity [94] of cells to determine wide range of experimental conclusions because malignant cells differ from healthy, normal cells in various aspects like cell growth, morphology, cell-cell interaction and cell-ECM interactions. Studies about surface hydrophobicity are very useful because it plays a major role in protein folding and aggregation. Also the surface hydrophobicity of self assembling monolayer (SAM) with wide variety of functional groups [95] and their interactions can be studied with chemically modified AFM cantilevers which provide valuable information in Chemistry and Biology, especially when using AFM probes with functional bio-molecules. Cell surface characterization using AFM is capable of providing information in order to determine whether the cells are normal or malignant [96] by comparing the indentation data. Recently, there was an interesting study about cells called Sonocytology [97]. In this study the scientists have found out that the cell membrane has its own frequency to

vibrate in kilohertz region and the AFM setup can be used to capture the signal and amplify it to audible sound. The idea is this technique can be used to identify malignant cells because the oscillation frequency is different from the normal cells due to change in cell membrane rigidity. Forces between DNA oligonucleotides can be measured using AFM by chemically attaching ends of double strands to a substrate and the AFM tip, which can easily provide valuable structural properties of DNA. Moreover, elasticity of proteins and domain unfolding can also be studied using AFM. With the aid of interleave or lift mode it is possible to image magnetic and electric forces simultaneously with the height data of the sample. However it is required to use special probes to obtain data related to magnetic and electrical forces. For example for magnetic force modulation (MFM), the cantilever should capable to hold magnetic properties, so it is needed to coat with special ferromagnetic material. In general, interleave or lift mode can be used to extract and compare any two different data types.

Van der Waals forces which arise due to instantaneous polarization play significant role in close proximities and the interactions can be represented by Lenard-Jones potentials which is given by,

$$V(r) = 4\varepsilon \left[\left(\frac{\sigma}{r}\right)^{12} - \left(\frac{\sigma}{r}\right)^{6} \right]$$

Where σ is the diameter of the atom, *r* is the intra-atomic distance and ε is depth of potential well. Therefore AFM can be used to measure the vdW forces, and the colloidal probes will be the best candidate because of its geometry. In certain situations, it is required to get rid of the vdW forces to obtain the required data with high resolution and accuracy. Therefore change in the operational medium will be a solution. Scientist also used AFM to study about surface vdW forces and strength of single and multiwall carbon nanotubes [98, 99] which can estimate the binding energies. In order to study about the capillary forces, especially the interfacial tension

occurs in liquid bridges between two surfaces, using AFM, it is necessary to prepare the AFM tip with the appropriate coating.

However, the AFM available in Nanotechnology Core Facility (NCF) at UIC capable of scanning z-limits up to 4 μ m and therefore it was extremely difficult to use it for biological applications. Special techniques were used to overcome the problems associated with the equipment. The other challenge is to maintain the biological samples alive and in order to do that it is required to keep the appropriate media during the scan, such as phosphate buffer (PBS). Manufacturer suggested method to perform the scanning in a liquid media is to use the specially design liquid cell which allows users to maintain the appropriate environment in both contact and tapping mode operations. The liquid cell comes with a special o-ring which creates a leak free room filled with media, and if necessary it has inlets which can be used to add more media once everything has been setup. The problem with this set up is that when engaging, the o-ring is dragged away with the sample and the cantilever. Even though the movement is very small most probably somewhat in the sub-5 μ m range, it changes the laser focus and leads to a reduction in the received intensity to the photo-detector.



Figure 71: Method of scanning cells with less z-resolution scanners.

Due to the extreme difficulties, it was necessary to find an alternate method to perform the liquid imaging. During the scan, the separation between the cantilever, cantilever holder and the sample is very small. Therefore, it was easy to hold few droplets of the liquid between the sample and the cantilever holder. The reason for this behavior is the capillary forces and the interaction forces between the solids and liquids. However, if the contact angle of the liquid droplet with the sample surface is very high, in other words if the surface does not wet by the liquid this method is rather easy but for the liquids which wet the sample surface can be used carefully without being spilled. Moreover the scanner can move without any resistances, so that there were no unnecessary loads on X&Y piezo. Also it was required to form the droplet in such a way that it covers the cantilever completely in order to avoid any unwanted changes in the optical path due to refraction or partial reflections.

Tapping mode operation is the best method to use, especially when scanning biological samples because of minimal damage. Even though, because of the high non-linear nature, the governing principles associated with this technique are still under debate, scientists always prefer to use this method. It doesn't matter how fast the electronics respond to the feedback loop associated with the electronics, there still can be a chance in false predictions. The modern technique called Peak Force QNM, which was introduced by Bruker AXS, suggested to use force response curves at each pixel in order to increase the imaging speed and the accuracy of the data. In this technique the force applied to the sample can be controlled accurately and allows measuring the elastic modulus properties from mega Pascal to giga Pascal range and the adhesion forces from pico Newton to micro Newton range. When using regular tapping mode, it is required to tune the cantilever. The simple form of the mathematical equation for cantilever oscillation by comparing it as a point mass, can be given as follows;

$$m\ddot{z} = -kz - m\frac{\omega_0}{Q}\dot{z} + F_{ts} + F_0\cos\omega t$$

Where Q is the quality factor, k is the spring constant and ω_0 is the resonance frequency of the cantilever. Mass represented by m, F_0 is the amplitude and ω is the angular frequency of the driving force. Also it is necessary to add the force contribution due to tip surface interactions which represent by F_{ts} . However, considering the cantilever as a vibrating beam, the oscillation can be given as;

$$EI\frac{\partial^4}{\partial x^4} w(x,t) + \mu \frac{\partial^2}{\partial t^2} w(x,t) = F(x,t)$$

Where w(x,t) is the transverse displacement of the cantilever, E is the Young's modulus, I is the moment of inertia, μ is the mass per unit length and F(x,t) includes all the associated forces per unit length.

The most convenient method to tune the cantilever is to use the AFM software because it has the built-in option to serve the purpose. The user needs to input the frequency range and the target amplitude. Then the software will find out the drive frequency, corresponds to an eigenmode and the drive amplitude of the cantilever. In case of tapping mode operation in liquid, it will be very difficult to use the auto tune procedure because the cantilever damps inside the liquid, which describes as below and the damping substantially reduces the quality factor (Q) of the cantilever.

$$EI\frac{\partial^4}{\partial x^4}w(x,t) + \mu\frac{\partial^2}{\partial t^2}w(x,t) + a_0\frac{\partial}{\partial t}w(x,t) = F_{total}(x,t)$$



Figure 72: Tapping mode AFM cantilever.

Where a_0 is the damping coefficient and $F_{total}(x,t)$ represents all the forces including loading component due to damping. The other terms has the usual meaning as outlined earlier. For the

first mode of vibration, the natural frequency is expressed as follows;

$$\omega_n = \left(\frac{k_n}{l}\right)^2 \sqrt{\frac{EI}{m}}$$

Where k_n is the mode constant and l is the length of the cantilever. And other terms has the usual meaning as explained earlier.

In order to get rid of this problem, the manufacturer's suggested method is to start with lower cantilever drive frequency and keep going up until it provides a better image. By starting from a lower frequency will allow the user to find out the first cantilever vibrating eigenmode. The other method is to do a calculation to roughly estimate the drive frequency by taking account the damping force, although there are lot of other interactive, repulsive forces are present. Also, it is possible to tune the cantilever without the liquid to find out the resonance frequency and then by adding the corrections, it is possible to estimate how much the frequency should shift from the original frequency. However, newer AFM use higher eigenmodes to improve the resolution and the contrast of the images. It has been shown that the uses of spherical tips are capable of reducing the vdW forces when operating in liquid media [100]. The vdW interaction force between the plane and spherical objects can be approximated as given below;

$$F_{vdW} = -\frac{A_{12}}{6} \frac{R}{D^2}$$

Where A12 is the Hamaker constant between two surfaces, R is the radius of the spherical tip and D is the distance between two surfaces.



Figure 73: Scanning in a liquid media with and without the o-ring.

There are several models which describes about calculating the adhesion force using the AFM. The simplest approach is described in the Digital Instrument, Nanoscope III user operational manual. Force is given by $F = k \times \Delta z$ where *k* is the spring constant of the cantilever, which is provided by the manufacturer and Δz is the deflection of the cantilever along z-direction. Δz can be calculated by the force curve and the z-piezo sensitivity, provided by the AFM manufacturer.

The other widely used model to describe the elastic deformation of two bodies in contact under load is called the Hertz model. This model considers two different cases of AFM tips; one is a sphere with radius R and the other one is a cylindrical cone with an opening angle of α .

$$F(sphere) = \frac{4}{3} \frac{E}{(1-\nu)} \sqrt{R} \delta^{\frac{3}{2}}$$

$$F(cone) = \frac{\pi}{2} \frac{E}{(1-\nu)} tan(\alpha) \delta^2$$

Where the δ is the indentation and **v** is the Poisson ratio. The elastic constant (*k*) is given by the ratio of, $E/(1-v^2)$.

$$k \text{ (spring constant)} = \frac{E}{1 - \nu^2} = \frac{3}{4} \frac{F}{\sqrt{R}} \frac{1}{\delta_2^3}$$

Viscoelsatic Hertz model is an extended version of the general model which describes the relationship when measuring the forces in a viscous media. Therefore, the equation is modified with a complex term which gives an additional force term due to oscillation.

Therefore k becomes, $k \rightarrow k^* = k' + ik''$ and the force becomes;

$$F \approx \frac{4}{3}k\sqrt{R}\delta_0^{\frac{3}{2}} + 2k^*\tilde{\delta}\sqrt{(R\delta_0)}$$

Where δ is the oscillating indentation.



Figure 74: Change in contact angle from wetting to non-wetting.

Another theory which was proposed by Johnson, Kendall and Roberts [101] includes the adhesion effect by treating the AFM tip as a sphere. The theory was originally proposed to overcome several contradictions in Hertz theory at lower loads. It considers two elastic spheres in contact without any external load. The energy loss during this contact considers for further calculations and in this case the adhesion force can be approximated by;

$$F_{ad} = \frac{\pi \gamma R_1 R_2}{(R_1 + R_2)}$$

Where γ is the energy per unit contact area and $R_1 \& R_2$ are the radiuses of the two spheres.

With the presence of external load the elastic modulus can be approximated as given below;

$$E = \frac{3(1-\nu^2)P}{8} \left(\frac{3}{\delta^3 R}\right)^{\frac{1}{2}}$$

Where *P* is the external load, *v* is the poison ratio, δ is the indentation and the *R* is the normalized radius of the two spheres. The values extracted from the force calibration plots or force volume curves can be used with either two models to calculate the elastic modulus. However, in newer AFMs are equipped with models, therefore just after the measurement is being done, the elastic modulus will be given.

There are two other numerical models which discuss adhesion forces. In contact stress analysis model [102], one or two elastic layers bonded to an elastic homogenous half-space is considered and it is treated as the indentation medium. Obtained results by solving the boundary value problem were then applied for an impact of two solid bodies which allows determining the variation of contact force and surface displacement as a function of time. In the other model

[103], it considered two bodies with different elasticity and curvatures in contact with an elastic plate.

In addition to AFM, Scanning Electron Microscopy (SEM) was also used to characterize the samples. However SEM could only be used to scan PMMA samples but not the PDMS because of the charging effect. PDMS could be characterized using SEM with a thin metal film on top which helps to reduce the charging effect. Even though it is coated with a conducting thin metal film, the conductivity of the metal layer can be destroyed because of the soft PDMS material. Therefore it was little bit challenging for non conducting samples.

3.2 Live Cell Imaging

As briefly described earlier, the AFM available in NCF is not specially designed for biological applications. Therefore it was required to follow some alternate techniques during the scan. The figures given below were obtained using both tapping and contact mode but the quality of the tapping mode images are better. These experiments were carried out to determine the growth rate of carotid artery endothelial cell membranes towards each other.



Figure 75: 2D tapping mode image of boundary of cells (left) and the corresponding 3D image (right).

In the 3D image, maximum height is shown as 663.1 nm. This is not the correct height because the scan was performed in tapping mode and since the cells are very high compared to the maximum z-resolution of the scanner, it mostly includes the data for the topmost surface. The maximum height measured in this image was 512 nm.



Figure 76: Cross section profile along the nucleus region of the cell of tapping mode imaging.

Following figures were obtained with contact mode imaging. The cell height was critical when imaging and high tip deflections were observed.



Figure 77: 2D contact mode image of the carotid artery endothelial cells (left) and the corresponding 3D image (right).

The cross sectional profile along the nucleus of the above image measured the average height of

 $2.5 \ \mu m$ which is much greater than the value obtained from tapping mode imaging.



Figure 78: Cross section profile along the nucleus region of the cell of contact mode imaging.

The images given below indicate the way that cells grow. When time progresses the membranes of the cells connects each other and that is what happens when healing a wound. This is an attempt of an alternate study to investigate about the wound healing properties in cellular level. AFM is a better tool to investigate these properties but it is challenging to keep cells under the AFM cantilever because they are unable to survive in such environment. However rapid transient processes can be easily scanned using modern high speed AFM's.



Figure 79: Membranes of two cells growing towards each other (left), Cell membranes grown towards each other (middle) and the zoomed view of the cell membrane boundary (right).

3.3 Characterization of Fabricated Structures with 2D EBL Approach

Characterization of the structures which fabricated with 2D EBL approach was necessary evaluate the surface roughness. Even though the grid was helpful in increasing the surface roughness, it was necessary to determine the type of grid which required adding the approximate surface roughness.



Figure 80: Cross sectional profile of the structure with increased surface roughness with square grid (A) and the spectral RMS amplitude (B).



Figure 81: Cross sectional profile of the doughnut shaped grid pattern (A) and the corresponding spectral RMS amplitude (B).

The measured RMS amplitude for square grid pattern was around 48 nm and it was 89 nm for doughnut shaped grid pattern. Therefore the surface roughness was almost doubled by introducing the doughnut shaped grid. However it was not possible to achieve the surface roughness close to the actual ECM with this approach. The image shown below is a surface analysis of a real colon tissue. The cross sectional profile shows intense surface roughness all over the surface.



Figure 82: Difference between "roughness' and "waviness." Panel A: ECM underlying normal colonic epithelia, Panel B: Primary curve, Panel C: Roughness curve and Panel D: Waviness curve. Data generated using MeX SEM-S software. Magnification 500x.*

*Image courtesy from Dr.Sarah Glover, Department of Medicine, University of Florida.

3.4 Advanced 3D EBL Structure Characterization

Our unique technique was proven to be the most powerful and ideal approach in reproducing biological samples. Without any restriction, it is capable of reproducing any structure and the Einstein image clearly proves the capability.



Figure 83: AFM scan of the fabricated image on PMMA (2D view on left and 3D view on right) by achieving the thickness contrast

Similarly the ECM scaffolds corresponds to all four stages were fabricated and characterized using AFM.



Figure 84: On top row, SEM image of the normal colon epithelia (left), 2D AFM image of the fabricated PMMA structure (middle), 3D topography of the fabricated structure (right) and similarly on the bottom row, SEM image of the well differentiated colon epithelia (left), 2D AFM image of the fabricated PMMA structure (middle), corresponding 3D topographical image of the fabricated structure (right)

The fabricated structures are the exact replication of the SEM image. In order to fabricate the PDMS stamp, the PMMA should be the inverse of the above image. Using image analysis, it was achieved without any problem. PDMS was mixed with the curing agent with 10:1 ratio and poured onto the PMMA and kept in a vacuum chamber for one day. Change in temperature was not required since the room temperature produced the required results.



Figure 85: On top row, SEM image of the moderately differentiated colon epithelia (left), 2D AFM image of the fabricated PMMA structure (middle), 3D topography of the fabricated structure (right) and similarly on the bottom row, SEM image of the poorly differentiated colon epithelia (left), 2D AFM image of the fabricated PMMA structure (middle), corresponding 3D topographical image of the fabricated structure (right)

Even though we could achieve very small variations along z-directions on the fabricated structures using advanced 3D fabrication techniques it was required to study about the surface properties such as roughness and the elastic modulus. In general, the elastic modulus of the biological surfaces is very small compared to most of the bio compatible polymers. Therefore it has been the main challenge for the scientists so far when reproducing materials for biological applications. Soft tissues in animals have a modulus of less than 1 kPa and bones are as high as

10 GPa. ECM falls in to soft tissue category, and it is very hard to achieve such small value with PDMS in which the average value is 1.8 MPa.



Figure 86: Cross sectional profile of fabricated structure corresponds to normal colon epithelia (top) and well differentiated colon epithelia (bottom).

The above two figures shows the cross sectional profile of the fabricated structures, and by comparing the waviness with the previous figures it is possible to distinguish the increase. Measured roughness was around 100 nm. Qualitative measurements were performed to observe the properties of the PMMA and PDMS samples since it was very difficult to get a quantitative number with the available resources. Following, force calibration plots were obtained while contact mode imaging and by comparing the curves PDMS is much softer than the PMMA.



Figure 87: Force calibration plot for PMMA (top) and for PDMS (bottom).

3.5 In-Vitro Testing

Fabricated samples were handed over to our collaborators in UIC Bioengineering and Department of Medicine at the University of Florida. The testing was designed with two end points and in such a way that it can be constructed multiple tests in between. In one end, normal cells were seeded on cancerous ECM scaffold and in the other end cancerous cells were seeded on normal ECM scaffold. According to our hypothesis, cancerous ECM scaffold should transfer the normal cells to cancerous and the opposite for normal ECM scaffold. However, experiments are still undergoing to prove that the normal ECM scaffold can transfer the cancerous cells to normal cells since the environment conditions and other parameters have to be carefully controlled.



Figure 88: Hypothesis testing strategy for poorly differentiated and normal colon epithelia.

Epithelial to mesenchymal transformation (EMT) assay was conducted to obtain the data. For better cell attachment the fabricated ECM scaffolds were coated with adhesion promotive protein. For this purpose, a thin layer of gold can be used as well. Normal colonic cells (NCM-460) were seeded on glass, planar PDMS, well differentiated colon ECM scaffold and poorly differentiated colon ECM scaffold. In this experiment, glass and planar PDMS act as the control. After 72 hours of incubation confocal images of the cells were obtained.

Images below show the SNAIL protein staining. The SNAIL protein is a method to detect mesenchymal markers which normally can be found in highly metastatic cells or stem cells. Increase in the green color shows that the cells are highly metastatic. On glass and planar PDMS, it shows less green color which means that they are not transformed to cancerous cells. On the other hand, cells seeded on well differentiated and poorly differentiated colon ECM scaffolds were transformed to cancerous cells. Among them, cells seeded on poorly differentiated ECM scaffolds are highly metastatic.



Figure 89: Confocal images of cells seeded on glass (A), planar PDMS (B), well differentiated colon ECM scaffold (C) and poorly differentiated colon ECM scaffold (D).

4. CONCLUSIONS

In present, cancer remains as one of the leading cause of deaths in US, and still leading scientists and doctors are unable to find a complete cure. DNA damage or genetic mutation in cells is the starting point of a cancer. Once this happens, cells start to grow without control, and regular apoptosis cannot be executed. Inheritance is the main path to carry mutated genes. Therefore regular screenings are important to determine if such cases exist in family history. Environmental factors can be responsible for gene damage too. Especially for colon cancer, red meat consumption, smoking and alcohol use are few major factors which can trigger the genetic mutation. Early detection can increase the survival, but prevention is the best way to fight against cancer. Use of balanced and healthy diet, regular exercise at least 1 hour for five days and more fruit and vegetable consumption, especially rich with fibers, specifically reduce the risk of colon cancers.

Current cancer treatment techniques are highly invasive and have lots of adverse effects. None of the techniques prove any promising results so far. Therefore an alternate method for cancer treatment is a timely requirement. In an era in which the core sciences reach to their ends, multidisciplinary research is essential in most fields. Therefore this joint effort of research disciplines in engineering, biology and medicine was to find out an alternate method to treat colon cancer with less invasive nature. Engineers had to play a major role in developing new technique to facilitate biological experiments. The idea was to change the mechanical factors which affect on cells to control the cell growth which ultimately opens path for alternate cancer treatment. Mechanical factors were proposed to change by engineering the cellular environment. In order to conduct this, material selection was a crucial step and on top of that it was necessary to find advanced technique which capable of engineering nanoscale structures.

Electron beam lithography is a state of the art technique which is regularly used for sub -50 nm patterning. However, the use of the equipment as a tool in fabricating 3D structures was limited and there are very few number of facilities conducts such experiments in all over the world. After extensive research, a technique was developed which can fabricated any type of structure with high precision. The technique was then used to fabricate the required structures, which were extracellular matrix scaffolds of colon epithelial tissue which corresponded to different stages of cancer. What happens in this technique is, a grayscale 8-bit resolution image can transfer to a 3D structure by achieving a thickness contrast within range less than 100 nm. In order to fabricate ECM scaffolds SEM images were used. Step size and the gamma value are the two main important parameters during the conversion and step size was not reduced below 100 nm, and the gamma was kept at 0.3 - 0.4. Physical parameters when fabricating were kept at standard values. A 30 µm aperture was always used when fabricating the structures. Extraction voltage was always kept at 10 kV, and the measured beam current was around 0.2 nA. Physical working distance was fixed to 10 mm to maintain the consistency throughout the experiments. Focusing the electron beam by changing the aperture and astigmatism was required since large beam sizes could add unwanted features to the structure. The best beam spot size was obtained while maintaining the above parameters, and it was measured to be around 29 nm. An empirical formula $Depth(Dose) = 2.2233 exp (4.016 \times Dose)$ was introduces to explain the relationship between the dose of the electron beam and depth of the PMMA under above condition. Average developing time has to be kept at 25-30 seconds for better results. MIBK was diluted with IPA (1:3 ratio) to gain high resolution and more control during the developing process. Fabrication was time consuming since the EBL technique is a point to point exposure procedure and in average, to fabricate one structure on 100 μ m x 100 μ m area takes almost 3.5 –

4 hours. The large area patterning is crucial with EBL, and it could take days to finish one pattern. The cost associated with this technique is also high since the EBL itself cost millions. Quality of the fabricated structures was optimized when necessary with the thermal reflow technique. In general, thermal reflow technique made the surface smooth and free of unwanted artifacts. The technique itself was capable in fabricating some complex 3D structures even without one-to-one image to 3D EBL technique. However when performing the thermal reflow, overall height goes down and at the same time feature sizes expand from the bottom since the process is kind of like melting down the resist. Therefore the changes had to be accounted in advance to make sure when fabricating the required structure. When it comes to the results EBL was an ideal tool in fabricating true 3D structures not only for biological applications but also for almost all of other application which requires high precision 3D geometry.

Characterization was a necessary step to optimize the EBL process in determining correct gamma value of the fabricated one-to-one structures. AFM was the best tool to achieve this since its capability in producing accurate information. Both tapping and contact methods were used to characterize the structures. Force curves were obtained for PMMA and PDMS structures and softness of PDMS qualitatively can be seen. PMMA is a hard material and it was an ideal material to use as a mold during this process. To conclude, AFM capabilities were well adapted to characterize the samples and optimize our technique. SEM was also used but extracted 3D information was not accurate as much as in AFM. PDMS structures could not be images properly due to the charging effect even with the presence of a thin metal layer.

When transferring patterns to PDMS, the inverse mold has to be fabricated and the inverse of the image was obtained by simple image analysis. Curing agent was used with PDMS (1:10 ratio)

and kept inside a vacuum chamber for a day. Room temperature was adequate and delivered perfect results. Tapping mode was used to characterize the PDMS samples.

In-vitro testing was designed and performed by our collaborators here at UIC Bioengineering and at the University of Florida to prove the hypothesis. The experiments were planned to seed normal cells on cancerous ECM scaffold and cancerous cells on normal colon ECM scaffold. Normal cells to cancerous cells transformation by cancerous ECM scaffold was proven by epithelial to mesenchymal transformation assay. Glass and planer PDMS used as a control during the experiment. High SNAIL proteins in cancerous cells were detected with the increase in green color of the confocal images. Cancerous cells to normal cells on normal colon ECM scaffolds still not proven since it required well controlled environment and time.

Upon success on *in-vitro* testing, the next goal is to apply the technique to treat cancer patients. The method which can deliver the treatment involves the colonoscopy. In order to change the microenvironment the idea is to use a patterned dissolvable salt which has flexible properties. The only challenge is to transfer the pattern into a large area of this dissolvable polymer. It can also be achieved using multiple replication of the small structure.

The alternate treatment seems to be promising and it will be non-invasive for patients when compared to the current methods. The technique can be extended to treat other type of cancers as well but the only problem is the method of delivery. As a conclusion, our new method of advanced 3D fabrication is unique an ideal solution for replicating biological and any sophisticated 3D structure and our joint effort seems to be producing promising results in next generation cancer research.

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APPENDIX

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APPENDIX (Continued)



Tuesday, July 19, 2012

Ms. Terese Winslow 714 South Fairfax Street Alexandria, VA 22314

Dear Ms.Terese,

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A copy if this letter is included for your records. Thank you for your kind consideration of this request.

Sincerely,

Kasun Punchihewa

Nanotechnology Core Facility (NCF) University of Illinois at Chicago Room 3064, Engineering Research Facility 842 West Taylor St. Chicago, IL 60607-7022

The above request is approved.

Irere M. Winstow Date: July 30, 2012 Approved by

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