ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
ΔΙΑΤΜΗΜΑΤΙΚΟ
ΠΡΟΓΡΑΜΜΑ ΜΕΤΑΠΤΥΧΙΑΚΩΝ
ΣΠΟΥΔΩΝ
ΟΠΤΙΚΗ ΚΑΙ ΟΡΑΣΗ

«3D RECONSTRUCTION OF CORNEA»

Leoni Georgiadi

ΤΡΙΜΕΛΗΣ ΕΠΙΤΡΟΠΗ ΕΠΙΒΛΕΨΗΣ

Παναγοπούλου Σοφία, Φυσικός PhD

Κυμιωνής Γεώργιος, Αν. Καθ. Οφθαλμολογίας

Μαρία Φαρσάρη, ερευνήτρια, ΙΤΕ

ΤΡΙΜΕΛΗΣ ΕΠΙΤΡΟΠΗ ΑΞΙΟΛΟΓΗΣΗΣ

Τσιλιμπάρης Μιλτιάδης, Καθ. Οφθαλμολογίας

Κυμιωνής Γεώργιος, Αν. Καθ. Οφθαλμολογίας

Σιγανός Χαράλαμπος, Επίκ. Καθ. Οφθαλμολογίας

Ηράκλειο 2016

Acknowledgement

I would like to thank Dr. Maria Farsari, Dr. Sofia Panagopoulou, Dr. Ranella Anthi, Dr. Ntina Terzaki, Aleka Manousaki, Nonlinear Lithography Group, my family and my friends for their precious help and their support, all this time.

Contents

Περίληψη

Ο κερατοειδής είναι ένας διαφανής ιστός στο μπροστινό μέρος του ματιού, ο οποίος διαθλά το φως και διευκολίνει την όραση. Μια πολύ μικρή αλλαγή στη γεωμετρία του κερατοειδούς έχει αξιοσημείωτη επίδραση στη δύναμη της όρασης. Αν το σχήμα του κερατοειδούς αλλάξει εξ΄αιτίας τραυματισμού ή ασθένειας, τότε μειώνεται η οπτική οξύτητα και η όραση χάνεται σε μεγάλο βαθμό, ανάλογα και με το σφάλμα.

Ο σκοπός αυτής της εργασίας είναι να δημιουργήσουμε ένα τρισδιάστατο μοντέλο του κερατοειδούς σε υψηλή ανάλυση χρησιμοποιώντας βιοσυμβάτα και φωτοευαίσθητα υλικά. Αυτο επιτεύχθηκε με τη μέθοδο της απευθείας γραφής με Λειζερ (DLW), μιας τεχνολογίας για την κατασκευή σύνθετων τρισδιάστατων κατασκευών υψηλής ανάλυσης. Το ενδιαφέρον για την παραπάνω μέθοδο έχει αυξηθεί σημαντικά τα τελευταία χρόνια εξ΄αιτίας των ποικίλων πιθανών εφαρμογών, όπως στο πεδίο των μηχανικών, ηλεκτρονικών και οπτικών μικροσυσκεύων, των βασισμένων σε πολυμερή οπτικών κυματοδηγών, των οπτικών μέσων αποθήκευσης, των βιοιατρικών εφαρμογών και άλλων συναφών πεδίων.

Ένα υλικό από πολυφωτονικό πολυμερισμό πρέπει να περιέχει δυο βασικά συστατικά:

α) ένα πολυμεριζόμενο υλικό

β) φωτοεκκινητή

Γι΄ αυτήν την εργασία χρησιμοποιήθηκε ένα υδρικό υλικό με οργανικά και ανόργανα κομμάτια.

Στο πρώτο μέρος της εργασίας προσπαθήσαμε να φτιάξουμε μια τρισδιάστατη κατασκευή που αναπαριστά τις ίνες του κολλαγόνου στον κερατοειδή.

Στο δεύτερο μέρος της μελέτης προσπαθήσαμε να φτιάξουμε πιο περίπλοκες κατασκευές, όπως ένα σκακι, με την ίδια μέθοδο (DLW).

Αρχικά η κατασκευή σχεδιάστηκε με τη βοήθεια ενός λογισμικού CAD και στο δεύτερο βήμα κατασκευάστηκε με τη μέθοδο DLW χρησιμοποιώντας την τεχνική του διφωτονικού πολυμερισμού.

ABSTRACT

The cornea is a transparent tissue in front of the eye that refracts light and facilitates vision. A slight change in the geometry of the cornea remarkably affects the optical power. When the shape of the cornea changes due to an injury or a disease, then visual acuity is reduced and eyesight is lost in a large proportion, depending on the fault.

The aim of this project was to construct a 3D model of cornea in high resolution, using biocompatible photosensitive materials. This was achieved with the method of Direct Laser Writing (DLW), a technology for the fabrication of 3D complex structures with high resolution. The interest in DLW has highly increased during the last years because of its various potential applications, such as in the field of mechanical, electronic, and optical microdevices, polymer based optical waveguides, optical data storage, biomedical applications, and other similar fields.

A material for structuring by multiphoton polymerization must contain two basic components:

a) a polymerizable material

b) photoinitiator

For this project, an inorganic-organic material was used.

In the first part of the project we tried to make a 3D srtucture that represents the fibers of collagen of the cornea.

In the second part of the study we tried to make more complex chess-like structures, with the same process (DLW). Firstly, the stucture was designed with the help of a CAD software and in a second step the structure was fabricated by Direct Laser Writing via the two-photon polymerization technique.

1.Introduction

Lasers have many uses in processing biomaterials. Two-photon polymerization is a very promising technique that uses ultra-fast (femtosecond) lasers for the fabrication of micron and sub-micron scale structures. When the laser beam is tightly focusing into the volume of a photosensitive material, the pulses interact with the material through two-photon absorption and the two-photon polymerization process, transferring liquid material into the solid state. The result is the fabrication of a 3D structure. After illumination, the unmodified material is removed by an appropriate developer, and the fabricated structure is revealed.[1]

In this study, we explore the production of well-defined macroscopic scaffolds with two-photon polymerization (2PP). The interest in two-photon polymerization has highly increased during the last years because of its various potential applications such as in the field of mechanical, electronic, and optical micro-devices, polymer based optical waveguides, optical data storage, biomedical applications and the like.[1],[6]

The cornea is a clear, transparent tissue which, when in healthy state, transmits almost 100% of the visible radiation. Under normal physiological conditions, the cornea is able to maintain indefinitely its integrity and transparency. However, the cornea is the most vulnerable portion of the outer tunic of the eye, and in certain situations its defence capability is overwhelmed. [7]

For this project we decide to approach the model of cornea with different ways. Initially, we wanted to see if the cells follow the orientation given by the structure. Afterwards, more complex stuctures are designed:

a) a chess- like stucture
b) stucture with two levels of lines

The 30% DMAEMA hybrid were used to produce scaffolds by 2PP . Here, we describe the synthesis and preparation of these materials and show that they can be structured accurately in three dimensions via DLW . Our next step was the investigation of cell growth on the three dimension scaffolds, made by these processes. The cells which were used, were fibroblats. The reason we used these cells, was because the cornea stroma mostly consists of fibroblasts which create the collagen fibrils.

CHAPTER 2

Theoretical part

2.1 : Cornea

2.1.1 About Cornea

The cornea is a natural, transparent and outer lens, located at the front of our eye that covers the iris, pupil, and the anterior chamber. The cornea has no blood supply it gets oxygen directly through the air. Oxygen first dissolves in the tears and then diffuses throughout the cornea to keep it healthy.

In humans, the cornea has a diameter of about 11.5 mm and a thickness of 0.5–0.6 mm in the center and 0.6–0.8 mm at the periphery.

It is an ocular tissue which acts as a window and has a number of basic properties for the quality of our vision. The cornea permits the light to enter the eye and it plays an important role in focusing images on the retina, therefore any alteration in its structure has adverse effects on our vision.

At the same time, the cornea becomes, by its very nature, a layer of protection for the internal ocular structures against possible external threats, whether physical, chemical or as a result of microbes.

The cornea also performs a photoprotective function by absorbing ultraviolet radiation of wavelengths between 200 and 300 nm (most of UV-C and UV-B), preventing them from reaching and damaging the posterior ocular elements.[1],[3]

In figure 1 we can see the anatomy of human eye, and where the cornea is located.

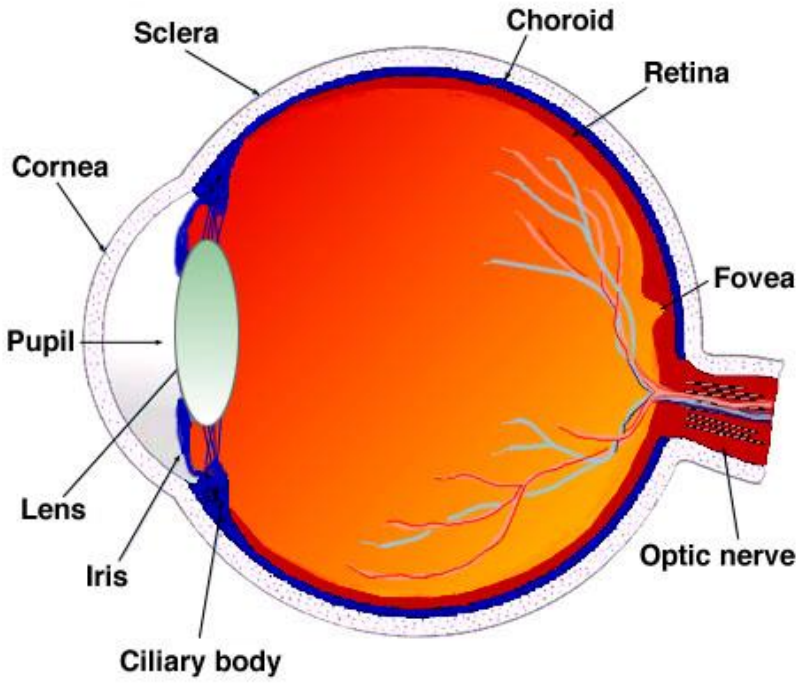


Figure 1: human eye

2.1.2 Structure of the Cornea

The complex structure of the cornea, consisting of five discrete layers (epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium), all within about 600 μm of tissue, is an indication of the variety of functional requirements that the cornea must meet. In figure 2,3 we can see the structure of the Cornea

***Corneal epithelium**:*

An exceedingly thin multicellular epithelial tissue layer (non-keratinized stratified squamous epithelium) of fast-growing and easily regenerated cells, kept moist with tears. Irregularity or edema of the corneal epithelium disrupts the smoothness of the air/tear-film interface, the most significant component of the total refractive power of the eye, thereby reducing visual acuity. It is continuous with the conjunctival epithelium, and is composed of about 6 layers of cells which are shed constantly on the exposed layer and are regenerated by multiplication in the basal layer.[1]

Bowman's Layer

Lying directly below the basement membrane of the epithelium, is a transparent sheet of tissue known as Bowman's layer. It is composed of strong layered protein fibers called collagen. Once injured, Bowman's layer can form a scar as it heals. If these scars are large and centrally located, some vision loss can occur.[8],[7]

Stroma

Beneath Bowman's layer is the stroma, which comprises about 90 percent of the cornea's thickness. It consists primarily of water (78 percent) and collagen (16 percent), and does not contain any blood vessels. Collagen gives the cornea its strength, elasticity, and form. The collagen's unique shape, arrangement, and spacing are essential in producing the cornea's light-conducting transparency.The corneal stroma consists of approximately 200 layers of, mainly, type I collagen fibrils. Each layer is 1.5-2.5 μm. Up to 90% of the corneal thickness is composed of stroma.[7],[10]

There are 2 theories of how transparency in the cornea comes about:

1) The lattice arrangements of the collagen fibrils in the stroma. The light scatter by individual fibrils is cancelled by destructive interference from the scattered light from other individual fibrils.[7],[10]
2) The spacing of the neighboring collagen fibrils in the stroma must be < 200 nm for there to be transparency. [7],[10]

Descemet's Membrane

Under the stroma is Descemet's membrane, a thin but strong sheet of tissue that serves as a protective barrier against infection and injuries. Descemet's membrane is composed of collagen fibers (different from those of the stroma) and is made by the endothelial cells that lie below it. Descemet's membrane is regenerated readily after injury.[8]

Endothelium

The endothelium is the extremely thin, innermost layer of the cornea. Endothelial cells are essential in keeping the cornea clear. Normally, fluid leaks slowly from inside the eye into the middle corneal layer (stroma). The endothelium's primary task is to pump this excess fluid out of the stroma. Without this pumping action, the stroma would swell with water, become hazy, and ultimately opaque. In a healthy eye, a perfect balance is maintained between the fluid moving into the cornea and fluid being pumped out of the cornea. Once endothelium cells are destroyed by disease or trauma, they are lost forever. If too many endothelial cells are destroyed, corneal edema and blindness ensue, with corneal transplantation the only available therapy.[7],[8]

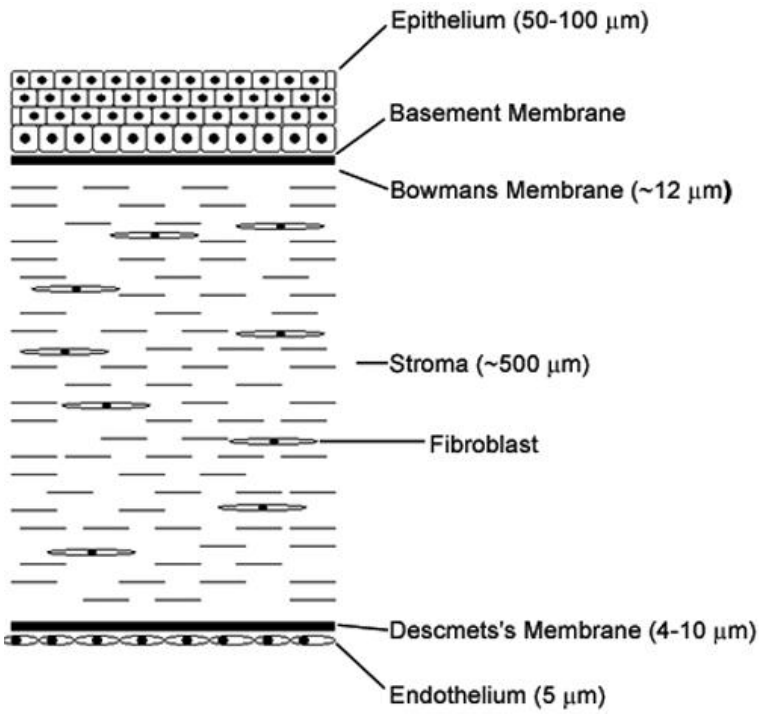


Figure 2:structure of Cornea

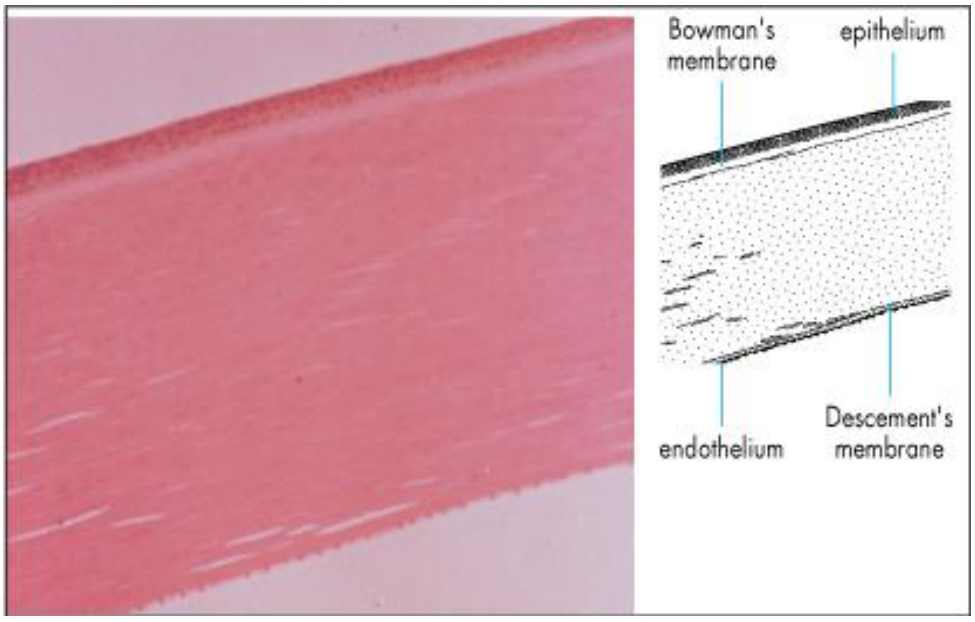


Figure 3:structure of Cornea

CHAPTER 2.2: Tissue engineering

About Tissue Engineering

Tissue Engineering is the study of the growth of new connective tissues, or organs, from cells and a collagenous scaffold, to produce a fully functional organ for implantation back into the donor host. This technique will allow organs to be grown from implantation (rather than transplantation) and hence free from immunological rejection[46]. The method of tissue engineering is still the subject of intensive research, therapeutic applications mainly focus on the cultivation of tissues from one cell type as the cartilage tissue. Other examples are the synthesis of heart valves and vascular prostheses. The skin growth is already used therapeutically. Tissue engineering utilizes living cells as engineering materials. Examples include using living fibroblast in skin replacement or repair, cartilage repaired with living chondrocytes, or other types of cells used in other ways.[47]
In figure 4 we can see the basic principles of Tissue engineering.[49]

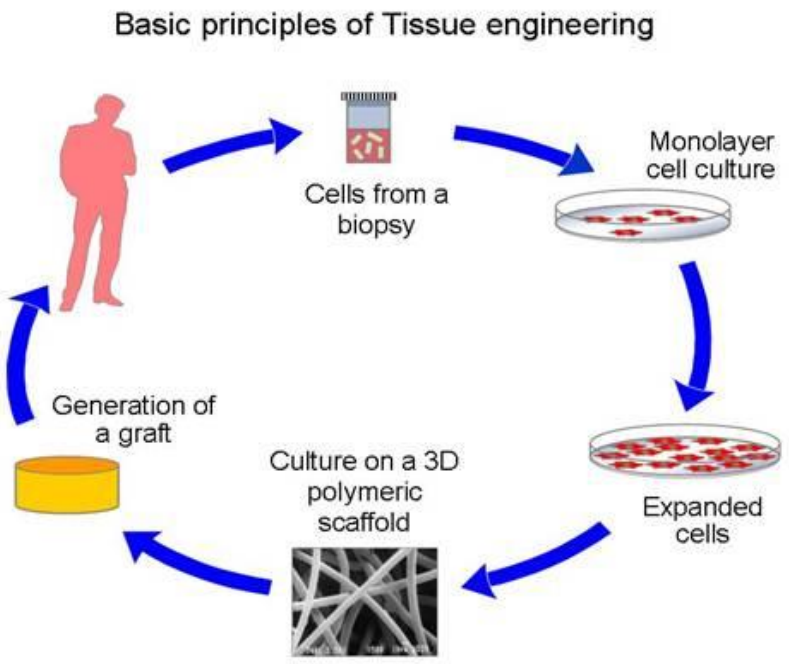


Figure 4: Basic principles of Tissue engineering.

How does tissue engineering work

The challenge of tissue engineering is to mimic what happens in nature. Attempts are being made to engineer *in vitro* practically every tissue and organ in the body. Work is proceeding in creating tissue-engineered liver, nerve, kidney, intestines, pancreas and even heart muscle and valves. There are some steps to be followed to achieve this result. Cells used for tissue engineering may come from cell lines from patients themselves (autogenous), from donors (allogenic), from a genetically identical donor (syngenic) and from animal sources (xenogenic).

Tissue engineering is a promising science field, because in the future it can help many patients who are unable to get a transplant.

Biomaterials

The choice of the appropriate biomaterial is an important factor in tissue engineering. Biomaterials can be derived either from nature, or synthesized in the laboratory, using a variety of chemical approaches, utilizing metallic components, polymers, ceramics or composite materials. Biomaterials are also used every day in dental applications, surgery, and drug delivery. For example, a construct with impregnated pharmaceutical products can be placed into the body, which permits the prolonged release of a drug over an extended period of time. [53]

Biomaterials are used in:

- Bone plates
- Bone cement
- Dental implants for tooth fixation
- Blood vessel prostheses
- Heart valves
- Skin repair devices
- Contact lens
- Drug delivery mechanisms
- Sustainable materials

CHAPTER 3

Materials and Methods

3.1: Materials

In this chapter, we describe the materials used in this study. For our structures we used an organic-inorganic hybrid, containing silicon and zirconium alkoxides and 30 mole% 2-(dimethylamino)ethyl methacrylate (DMAEMA).

3.1.1 DMAEMA (poly(2-(dimethylamino ethyl)methacrylate)

In this study, we used a hybrid material containing 30% DMAEMA, an organic monomer. This is a pH responsive cationic polyelectrolyte with tertiary amino group, and it has also thermosensitive property with a phase transition temperature of around 50^{o}C. These materials have been extensively used on surfaces and coatings and were shown to exhibit antibioadherent properties against bacteria, macrophages, and fibroblasts similarly to other cationic biocides.

Zirconium propoxide (ZPO) is a particularly suitable bio- material, due to its advantageous mechanical properties such as high strength, toughness, and stability. Although our work considers ZPO as a component in a hybrid biomaterial for a tissue engineering application, most prior work using it derives from the area of prosthetic substitution.[9],[11]

A recent study on DMAEMA proposed that polymers with a branched architecture and an intermediate molecular weight are promising candidates for efficient gene delivery, since they combine low cytotoxicity with acceptable cell transfection. *[Figure 5 Chemical structures of poly(DMAEMA)]*[9],[12].

Hybrid organic–inorganic materials combining the above mentioned mechanical, chemical, and biological material properties have emerged recently as a new class of materials in tissue engineering [9]. Biocompatibility studies, mechanical property measurements, made by "Konstantina Terzaki, Maria Kissamitaki et al., “Pre-osteoblastic cell response on three-dimensional, organic-inorganic hybrid material scaffolds for bone tissue engineering” DOI: 10.1002/jbm.a.34516"

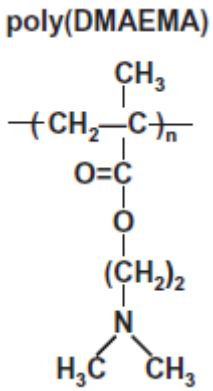


Figure 5: Chemical structures of poly(DMAEMA)

3.1.2 Fibroblasts Cells

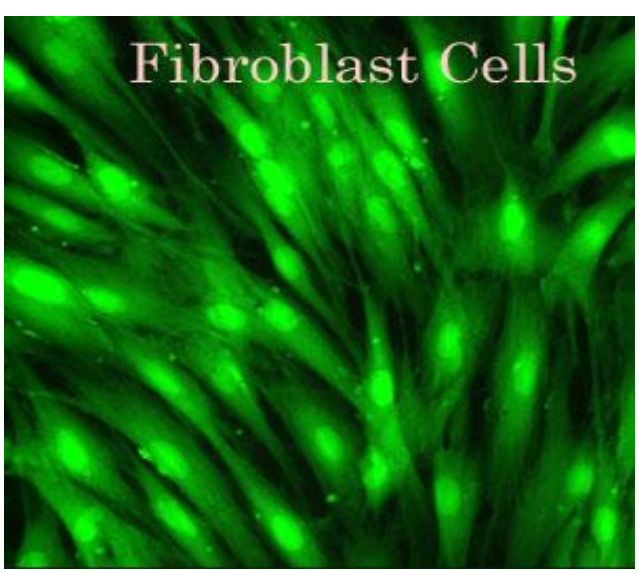
A fibroblast is a type of cell that synthesizes the extracellular matrix and collagen, the structural framework (stroma) for animal tissues, and plays a critical role in wound healing. Fibroblasts are the most common cells of connective tissue in animals.

Fibroblasts make collagens, glycosaminoglycans, reticular and elastic fibers, glycoproteins found in the extracellular matrix , and cytokine TSLP. Growing individuals' fibroblasts are dividing and synthesizing ground substance. Tissue damage stimulates fibrocytes and induces the mitosis of fibroblasts.[15]

Fibroblasts also produce glycoproteins and polysaccharides for the ground substances, a gel-like material that surrounds collagen fibers of dense connective tissue, forming an “extracellular matrix”, which contributes to the structural integrity of ligaments and tendons, determines the physical properties of connective tissue. In addition, fibroblasts have a tissue repair function, and wounds stimulate fibroblast production. Collagen’s ubiquity makes fibroblasts the most common cells of connective tissue in mammals.[18]

In this study specifically **3T3 cell lines** were used.

NIH 3T3 cells are established from a NIH Swiss mouse embryo. These cells are highly contact inhibited and are sensitive to sarcoma virus focus formation and leukaemia virus propagation. Cells have now lost their contact inhibition. This cell line was established from NIH Swiss mouse embryo cultures in the same manner as the original random bred 3T3 and the inbred BALB/c 3T3. The established NIH/3T3 line was subjected to more than 5 serial cycles of subcloning in order to develop a subclone with morphologic characteristics best suited for transformation assays. It is therefore used for DNA transfection studies[16],[17] (Figure 6: Fibroblasts cell)



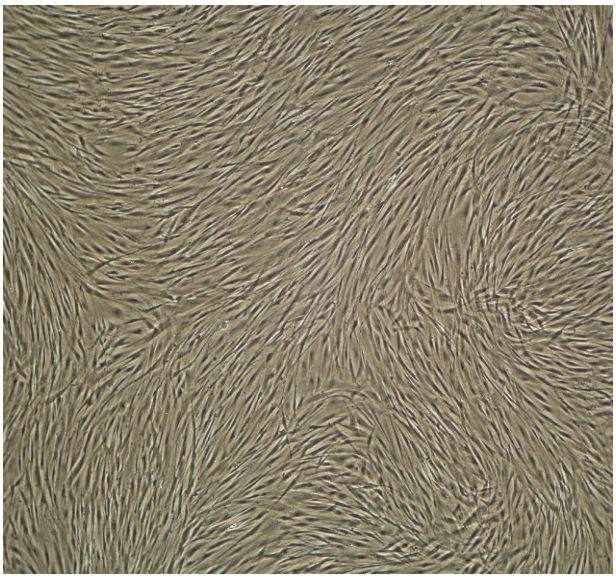


Figure 5: Fibroblasts cell[18]

3.1.3 Cell culture media

The medium that was used for fibroblasts growth, consisted of DMEM 10% v/v FBS (Fetus Bovine Serum) and 1% antibiotics (PenStrep-GIBCO). The cell cultivation was performed in cell culture flasks 25cm^2 and 75cm^2 as well as in6 well plates. The fibroblasts were cultured at 37^oC in an atmosphere of 5% CO_2 (incubator -Forma Scientific USA). Before seeding the cells on different surfaces, cells were grown to confluency, detached with 0.05% trypsin/EDTA (GIBCO). Hemocytometer Neubauer was used for the calculation of the concentration of cells. Finally, Optical Laser Scanning Confocal and Scanning Electron microscopy were used for cell imaging , Scrapers, DMEM serum free (GIBCO)+Trypsin 0,05%,.

3.2 Methods

In this chapter the sol-gel methods, two-photon polymerization, cell culture, sputtering and scanning electron microscopy are described.

3.2.1 The Sol-Gel Methods

The idea behind sol-gel synthesis is to "dissolve" the compound into a liquid state, in order to bring it back as a solid, in a controlled manner. The sol-gel method prevents the problems with co-precipitation, which may be inhomogeneous, be a gelation reaction.

A **sol** is a **stable dispersion of colloidal particles or polymers in a solvent**. The particles may be amorphous or crystalline. An aerosol is particles in a gas phase, while a sol is particles in a liquid,

A **gel** consists of a **three dimensional continuous network**, which encloses a liquid phase, In a colloidal gel, the network is built from agglomeration of colloidal particles. In a polymer gel the particles have a polymeric sub-structure made by aggregates of sub-colloidal particles. Generally, the sol particles may interact by van der Waals forces or hydrogen bonds. A gel may also be formed from linking polymer chains. In most gel systems used for materials synthesis, the interactions are of a covalent nature and the gel process is irreversible. The gelation process may be reversible if other interactions are involved.

The process is based on the phase transformation of a sol obtained from metallic alkoxides or organometalic precursors. This sol, which is a solution containing particles in suspension, is polymerized at low temperature in order to form a wet gel. This one is going to be densified through a thermal annealing to give an inorganic product like glass, polycrystals or a dry gel. By using this method, inorganic-organic hybrid materials offer properties better than those prepared alone. Sol-gel materials have been applied in many fields, such as membranes, chemical sensors and catalysis[51][52].

3.2.2 Two photon polymerization

The polymerization is based on the phenomenon of multiphoton absorption and subsequent polymerization. Two-photon excitation provides a means of activating chemical or physical processes with high spatial resolution in three dimensions and has made possible the development of three-dimensional fluorescence imaging, optical data storage,and lithographic microfabrication. These applications take advantage of the fact that the two-photon absorption probability depends quadratically on intensity, so under tight-focusing conditions, the absorption is confined at the focus to a volume of order [37]. The basis of two-photon polymerization is the phenomenon of two-photon absorption (TPA). There are two types of two photon absorption: sequential and simultaneous. In sequential, the absorbing species is excited to a real intermediate state: then a second photon is absorbed. The presence of the intermediate energy state implies that the material absorbs at this specific wavelength; it will therefore be a surface effect and will follow the Beer–Lambert law. In simultaneous absorption, there is no real intermediate energy state, i.e. the material is transparent at that wavelength. Instead, there is a virtual intermediate energy state and two-photon absorption happens only if another photon arrives within the virtual state lifetime. Photopolymerization is a light-induced reaction which converts a liquid or gel monomer into a solid polymer.[1],[26] Two photon polymerization has many advantages as a technique. A femtosecond laser pulse can be closely focused onto liquid- state monomers and the resulting TPP used to initiate chemical processes and the formation of features close to 100 nm in size. [50] Multiphoton absorption and multiphoton ionization can lead to laser damage of optical materials and be used to write permanent index structures into the interior of optical materials.

Direct Laser Writing (DLW) has been demonstrated as a technology for the fabrication of 3D structures with high resolution. The technique is based on the phenomenon of multi-photon polymerization. When the beam of an ultrafast infrared laser is tightly focused into the volume of a photosensitive material, the polymerization process can be initiated by nonlinear absorption within the focal volume. By moving the laser focus three-dimensionally

through the photosensitive material, 3D structures can be fabricated.

By simply immersing the sample in an appropriate solvent, the unscanned, unpolymerized area can be removed, allowing the 3D structure to reveal. A variety of applications have been proposed including microfluidics , micro-optics , scaffolds for biomolecules and cells , and photonics and metamaterials.[34],[35]

3.2.3 Cell culture

Cell culture is the complex process by which cells are grown under controlled conditions, generally outside of their natural environment[38]. In practice, the term "cell culture" now refers to the culturing of cells derived from multi-cellular eukaryotes, especially animal cells. The historical development and methods of cell culture are closely interrelated to those of tissue culture and organ culture. For our experiments the 3T3 cell line was used. The 3T3 cells have the capacity to adhere to the plastic surface of the flask, the 6 well plate and cover slips. The NIH/3T3 cells were suspended to a concentration of 10^5 cells/mL in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% antibiotic solution and 1-2 ml of cell suspension was added in the 6 well plate and cultured at 37 _C, for 24, 48 or 72 h in an atmosphere of 5% CO2.The cell culture was performed in a concentration $5x10^5$ cell/ml in flasks, $2.5x10^3$ cell/ml in 6 well plates and the cover slips, in the incubator Forma Scientific at 37^0C and in the presence of 5% CO_2. The culture media were changed every 2-3 days.Before seeding the cells on the different surfaces, cells were grown to confluency, detached with 0.05% trypsin/EDTA and diluted in complete medium at an appropriate density. All experiments were done in triplicates to ensure reproducibility and obtain better statistics.

3.2.4 Sputtering

Sputtering is a process whereby atoms are ejected from a solid target material due to bombardment of the target by energetic particles [48].It only happens when the kinetic energy of the incoming particles is much higher than conventional thermal energies (≫ 1 eV). This process can lead, during prolonged ion or plasma bombardment of a material, to significant erosion of materials, and can thus be harmful. On the other hand, it is commonly utilized for thin-film deposition, etching and analytical techniques.

The sputtering process begins when a substrate to be coated is placed in a vacuum chamber containing an inert gas - usually Argon - and a negative charge is applied to a target source material that will be deposited onto the substrate causing the plasma to glow.

3.2.5 Scanning electron microscopy (SEM)

A scanning electron microscope (SEM) is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons. The electrons interact with electrons in the sample, producing various signals that can be detected and that contain information about the sample's surface topography and composition. The electron beam is generally scanned in a raste scan pattern, and the beam's position is combined with the detected signal to produce an image. SEM can achieve resolution better than 1 nanometer. [44]

The SEM is routinely used to generate high-resolution images of shapes of objects (SEI) and to show spatial variations in chemical compositions: 1) acquiring elemental maps or spot chemical analyses using EDS, 2)discrimination of phases based on mean atomic number (commonly related to relative density) using BSE, and 3) compositional maps based on differences in trace element "activitors" (typically transition metal and Rare Earth elements) using CL. The SEM is also widely used to identify phases

based on qualitative chemical analysis and/or crystalline structure. Precise measurement of very small features and objects down to 50 nm in size is also accomplished using the SEM. Backescattered electron images (BSE) can be used for rapid discrimination of phases in multiphase samples. SEMs equipped with diffracted backscattered electron detectors (EBSD) can be used to examine microfabric and crystallographic orientation in many materials. In figure 6 we see the SEM

To create an SEM image, the incident electron beam is scanned in a raster pattern across the sample's surface. The emitted electrons are detected for each position in the scanned area by an electron detector. The intensity of the emitted electron signal is displayed as brightness an a display monitor and/or in a digital image file. By synchronizing the position in the image scan to that of the scan of the incident electron beam the display represents the morphology of the sample surface area.

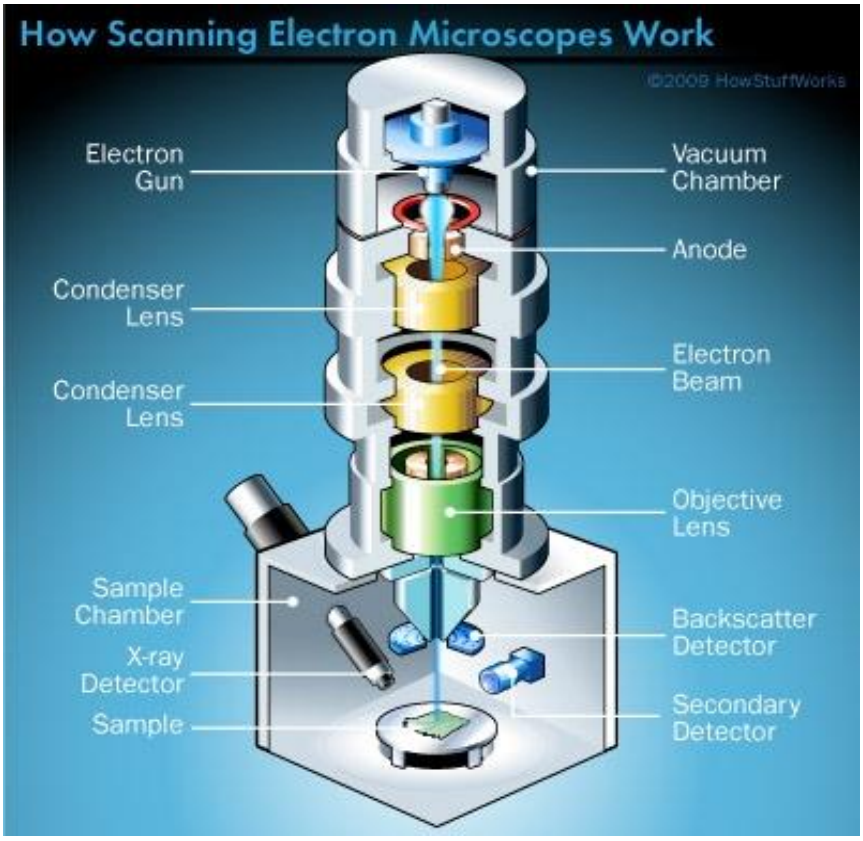


Figure 6: SEM

CHAPTER 4

Experimental

4.1 (3D) structure fabrication

The structure which we constructed was made in order to allow the 3T3 cells which would be placed to the structure, to form networks and be developed. These structures were prepared in DLW. For these 3D structures 30% DMAEMA – 1%BIS was used. For the creation of the structure one small drop was placed on a glass (15mm diameter) for overnight in room temperature. After that the glass was transferred under the laser. The laser beam was focused into the photopolymerizable polymer. The laser beam was expanded 20× using a telescope lens to illuminate the full back aperture of the microscope objective and to achieve optimal focussing. The structures were fabricated in a layer-by-layer fashion with the last layer on the surface of the coverslip. After the completion of the component build process, the sample was rinsed in2-propanol: 1-propanol(7:3). The samples were characterized by scanning electron microscopy (SEM).

4.2 Direct laser writing (DLW)

A Ti:Sapphire femtosecond laser was used operating at 800nm (Femtolasers Fusion).
This source is a compact diode-pumped femtosecond laser oscillator with integrated dispersive mirrors that pre-compensate the beam delivery and focusing optics to achieve sub-20 fs duration pulses into the sample. The beam was focused into the photopolymerisable composite.

The structure was generated using an x-y galvanometric mirror digital scanner (Scanlabs Hurryscan II), controlled by SAMLight (SCAPS) software. The scanner was adapted to accommodate high numerical aperture focusing microscope objective lenses(20X, N.A. = 0.65,40x, N.A. = 0.95, 100x, N.A. = 1.4, Zeiss, Plan Apochromat). To achieve better focusing, the laser beam was expanded 2x using a telescope lens arrangement. Movement on the z-axis and large-scale movement on the x-y plane was achieved with a three-axes linear encoder stage. Beam control was achieved using a mechanical

shutter. A CCD camera was mounted behind a dichroic mirror for online monitoring of the polymerization process.

In figure 7,8,9 DLW set up and figure 10 how the beam work.

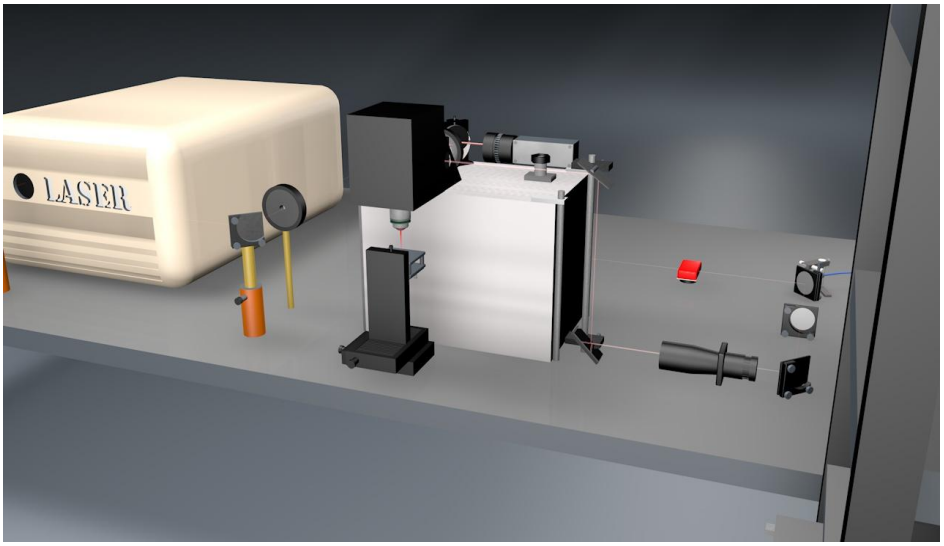


*Figure 7: DLW (*Direct laser writing), Galvo system

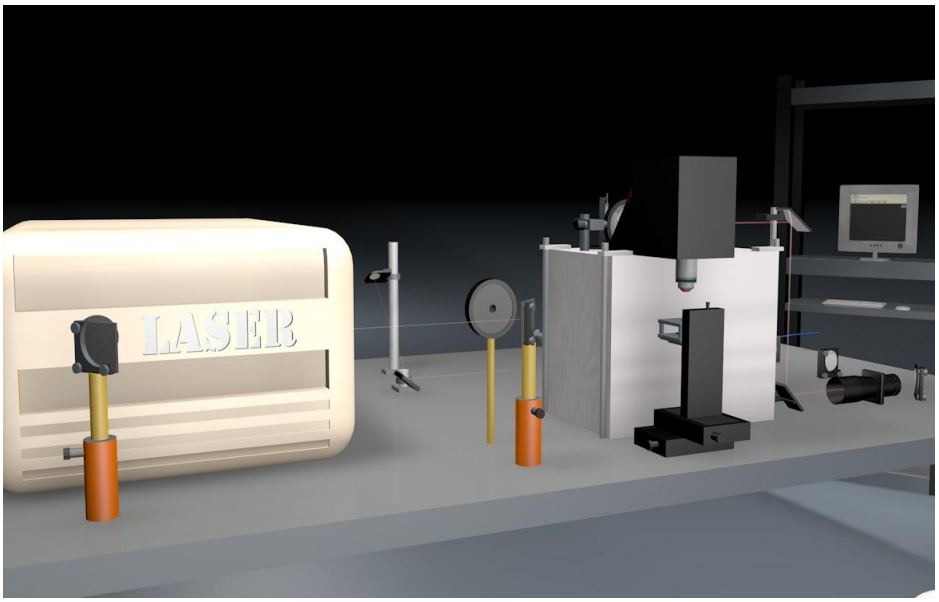


*Figure 8 DLW (*Direct laser writing), Galvo system

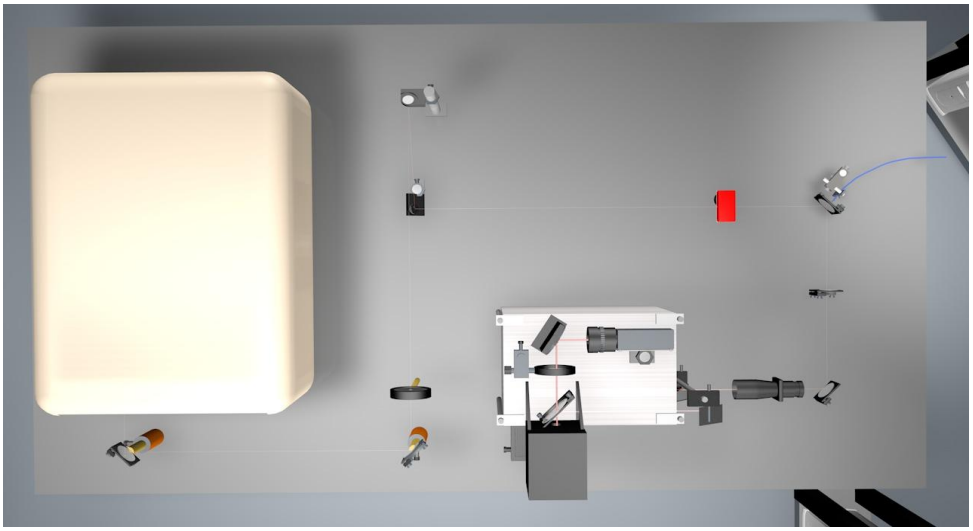


*Figure 9: DLW (*Direct laser writing), Galvo system

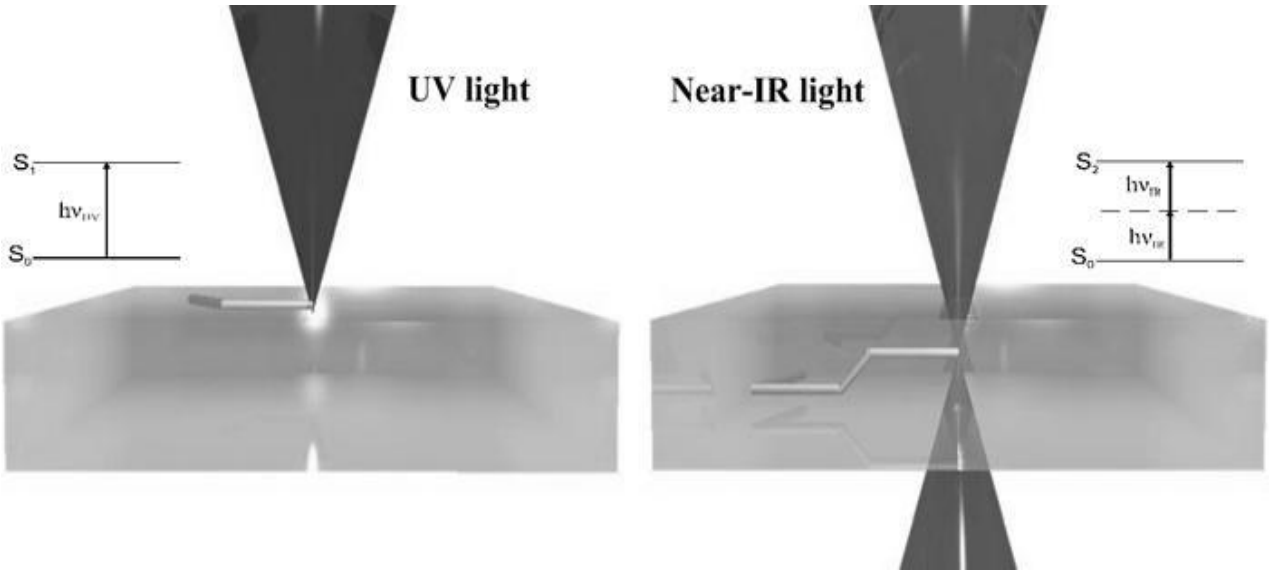


Figure 10: the beam of the laser

4.3 Preparation DMAEMA hybrid

This is an organic-inorganic composite, produced by the addition of methacryloxypropyl trimethoxysilane (MAPTMS, 99%) to zirconium propoxide (ZPO, 70% in propanol). 2-(dimethylamino)ethyl methacrylate (DMAEMA, >99%) was also added which was copolymerized with MAPTMS upon photopolymerization. ZPO and the alkoxysilane groups of MAPTMS served as the inorganic network forming moieties. 4,4-bis(diethylamino) benzophenone (BIS) was used as the photoinitiator.

MAPTMS was first hydrolyzed using HCl solution (0.1 M) at a 1:0.1 ratio. After 5 minutes, the ZPO was slowly added to the hydrolyzed MAPTMS at a 3:7 ZPO:MAPTMS molar ratio. After stirring for 15 minutes, DMAEMA was added at a DMAEMA/MAPTMS molar ratio 5:5. Following another 30 minutes, water was added to the mixture at a 2.5:5 MAPTMS:H_2O molar ratio. Finally, the photoinitiator, at a 1% w/w concentration with respect to photopolymerizable methacrylate moieties was added to the mixture. After stirring for a further 15 minutes, the composite was filtered using a 0.22 μm syringe filter.[22]

4.4 Fixation Protocol

1. Rinsing the samples with SCB 0.1M 2times for 5minutes
2. After that we remove the SCB solution and we add GDA 2% + PFA2% / SCB for 30minutes
3. We repeat the first step
4. Now in order to gradually dehydrate the cells we have to start some successive rinses with ethanol. The concentrations will be : 30% , 50%, 70%*, 90% and 100%. The estimate time of each rinse is 7minutes.
5. In this step we have to be very careful in order to transfer our samples in a vessel with Dry Alcohol. Since our final rinse was with 100% ethanol we have to protect our cells from a rapid ethanol evaporation. To avoid this, we have already prepared a new vessel with Dry Alcohol and quickly we transfer our samples there. Estimated time 10minutes
6. Critical Point Drying
7. Sputtering

CHAPTER 5

Results

5.1.1 In this study we first decided to fabricate a 6-layer structure (6μm height). Dimensions are (300x300) μm, distance between lines is 10μm and thickness of every line is 2μm.

For the 3D structuring of the 30%DMAEMA the direct laser writing parameters were first optimized for this material. The 3D structures were fabricated with a 20× objective lens. SEM images of the 3D structures are included in figures 11,12,13.

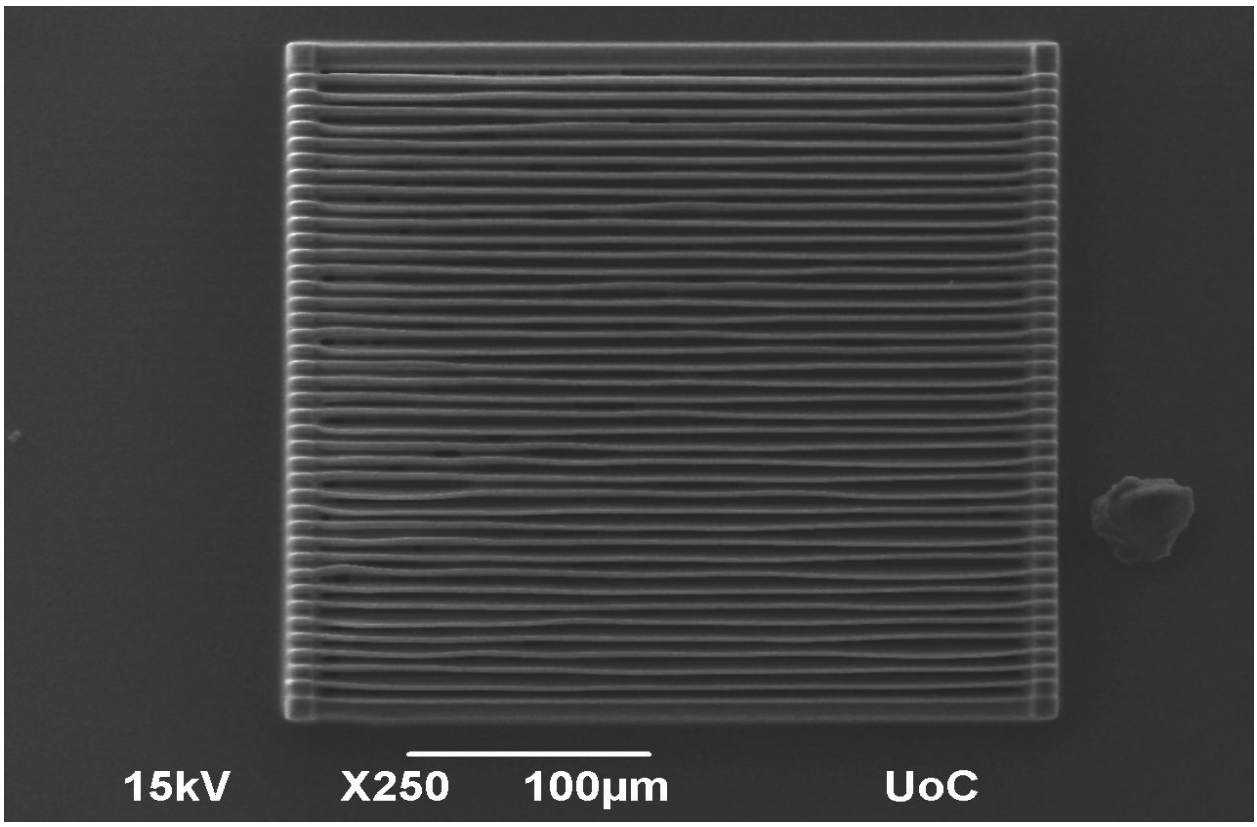


Figure 11

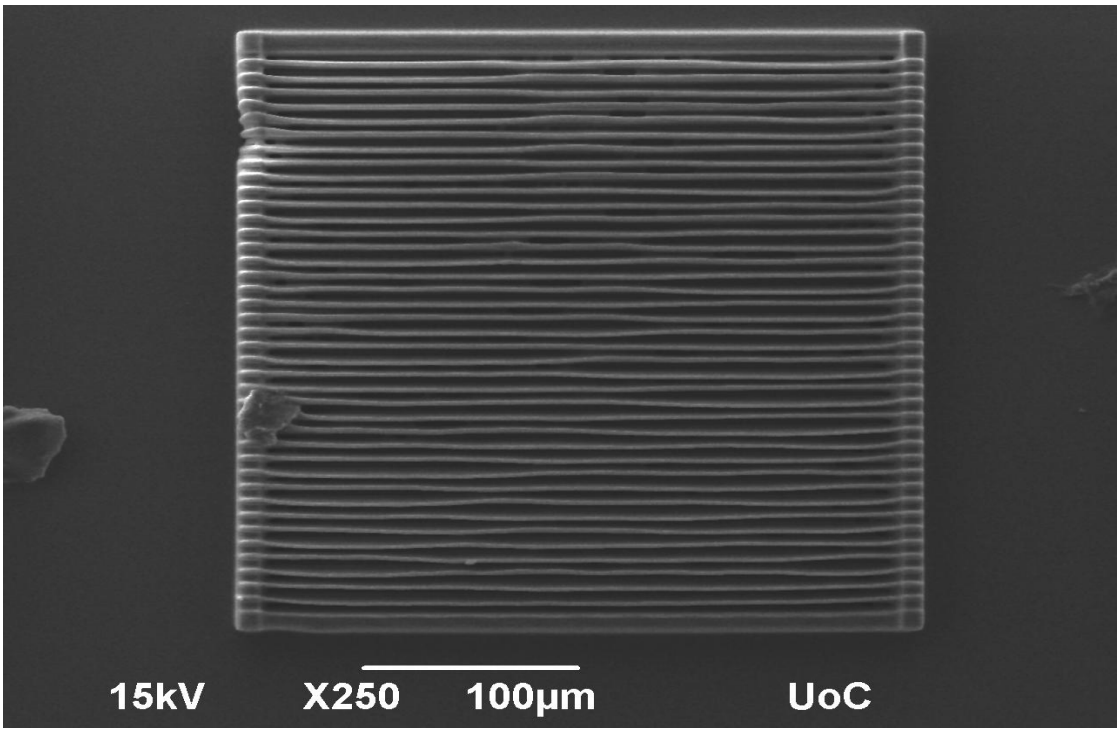


Figure 12

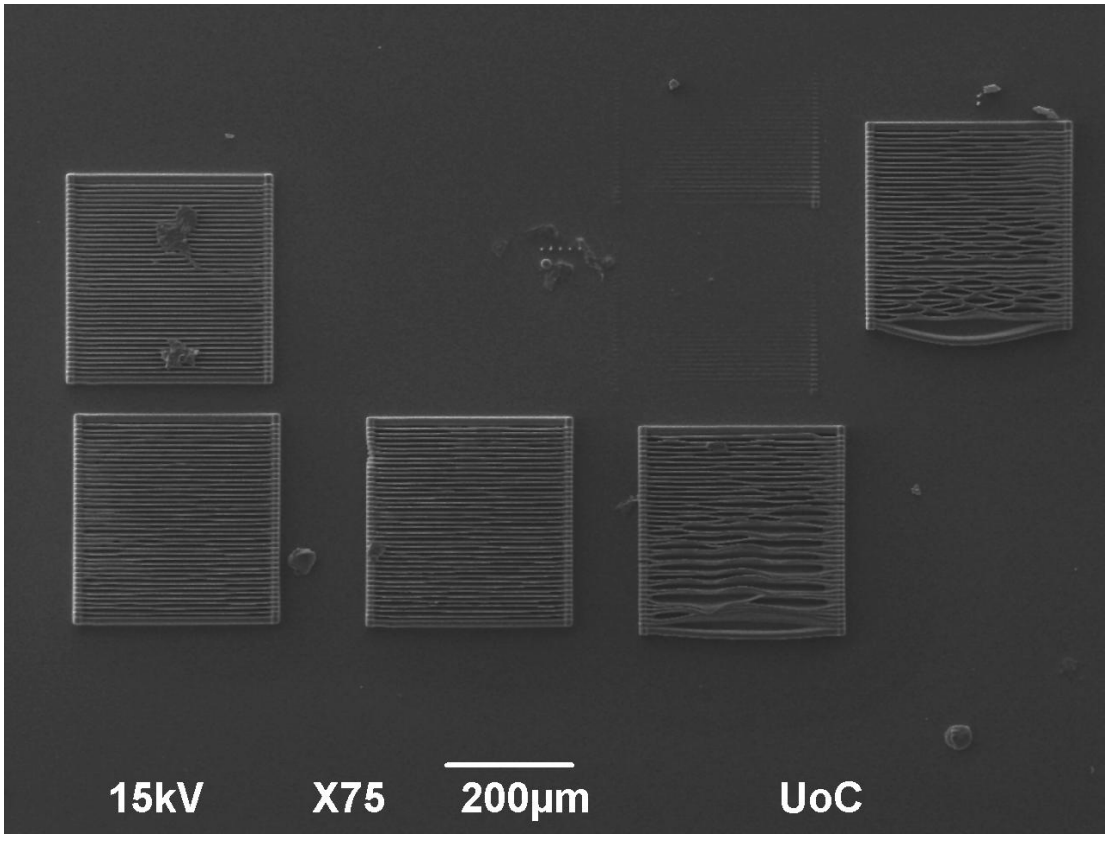


Figure 13

In this structure, fibroblast cells were cultured for 3 days. The number of cells per ml cultured were 10^5.The following photos show the structure with cells. Figures 14,15,16,17,18,19. We can notice that the cells have developed as expected. They have also followed the structural orientation.

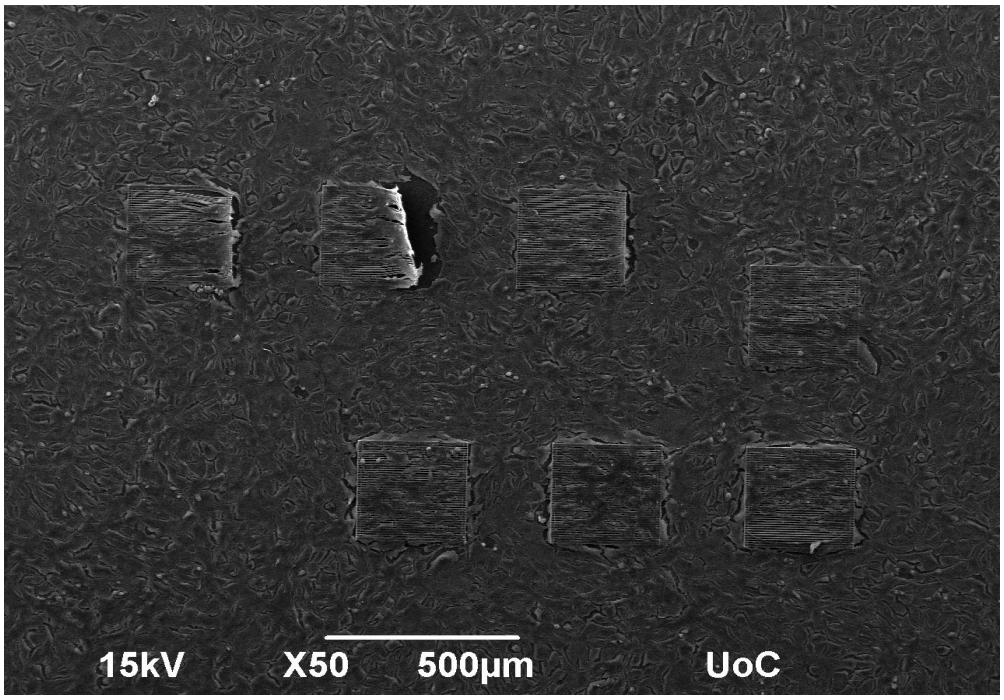


Figure 14

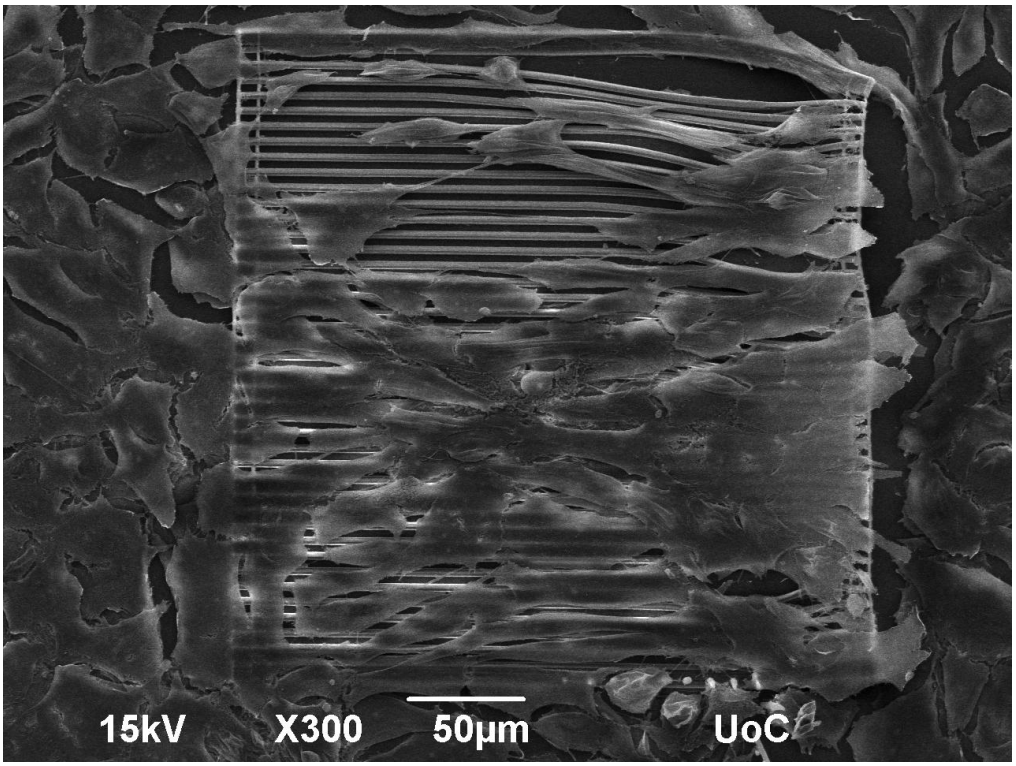


Figure 15

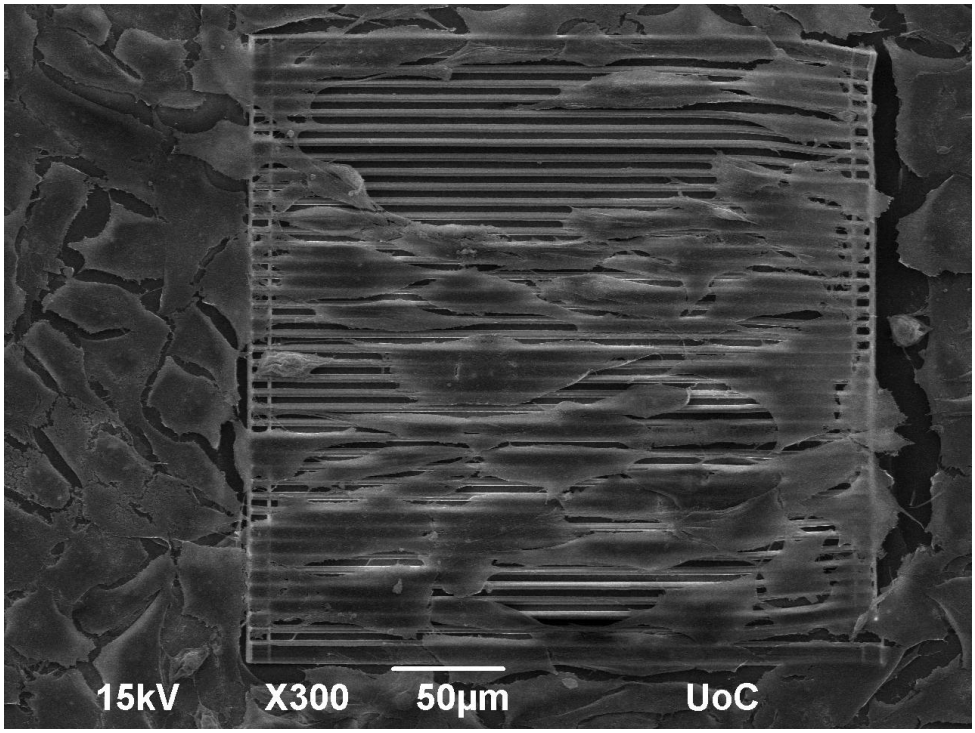


Figure 16

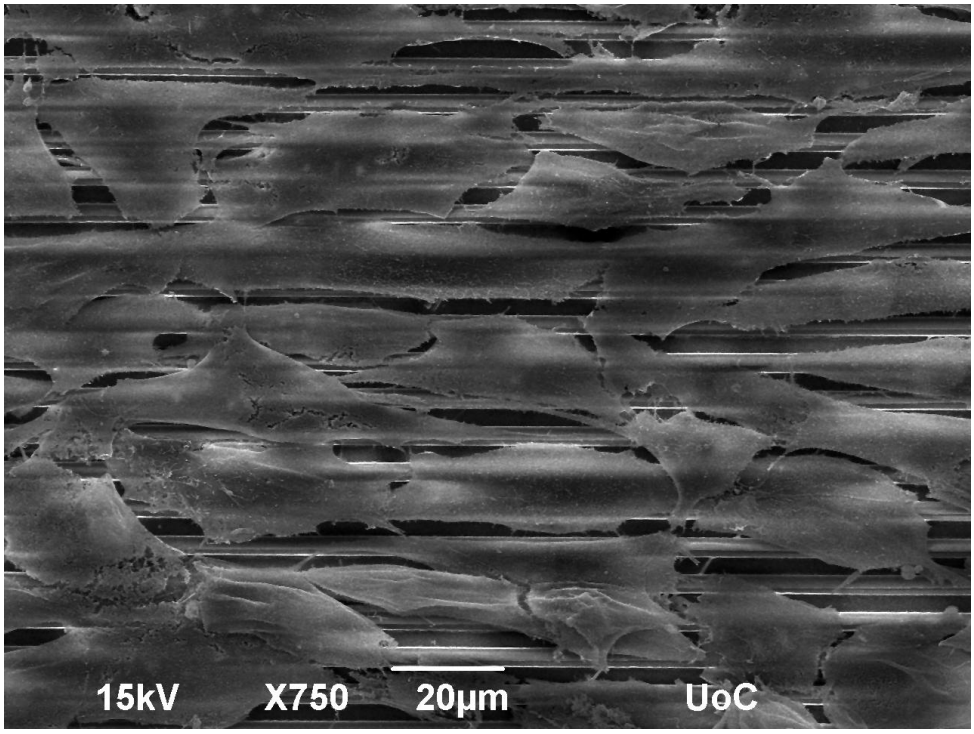


Figure 17

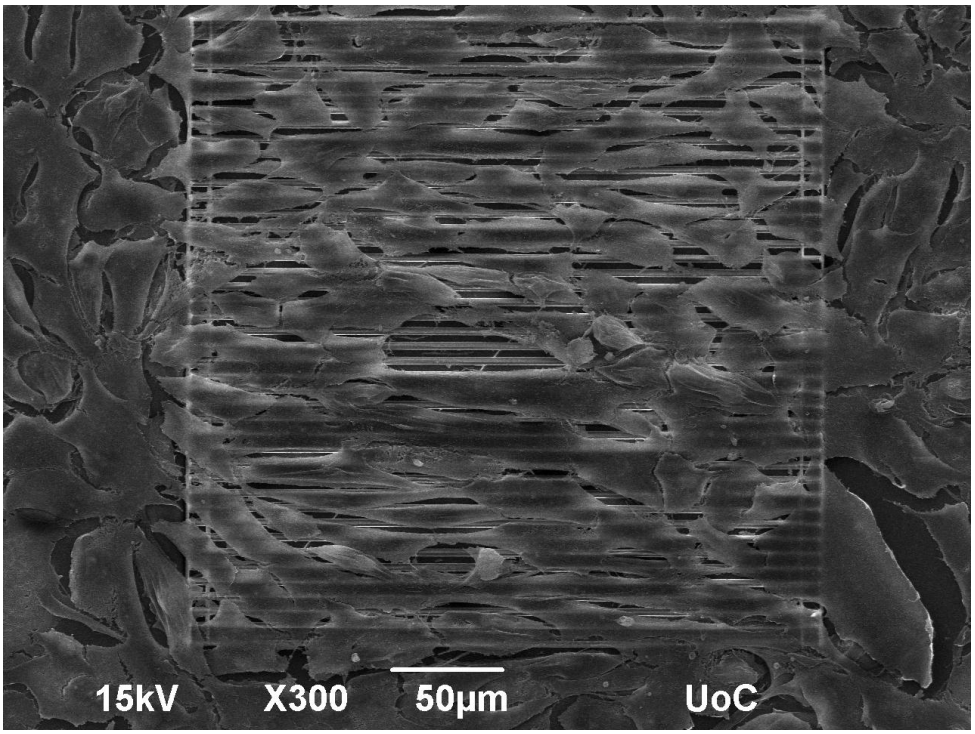


Figure 18

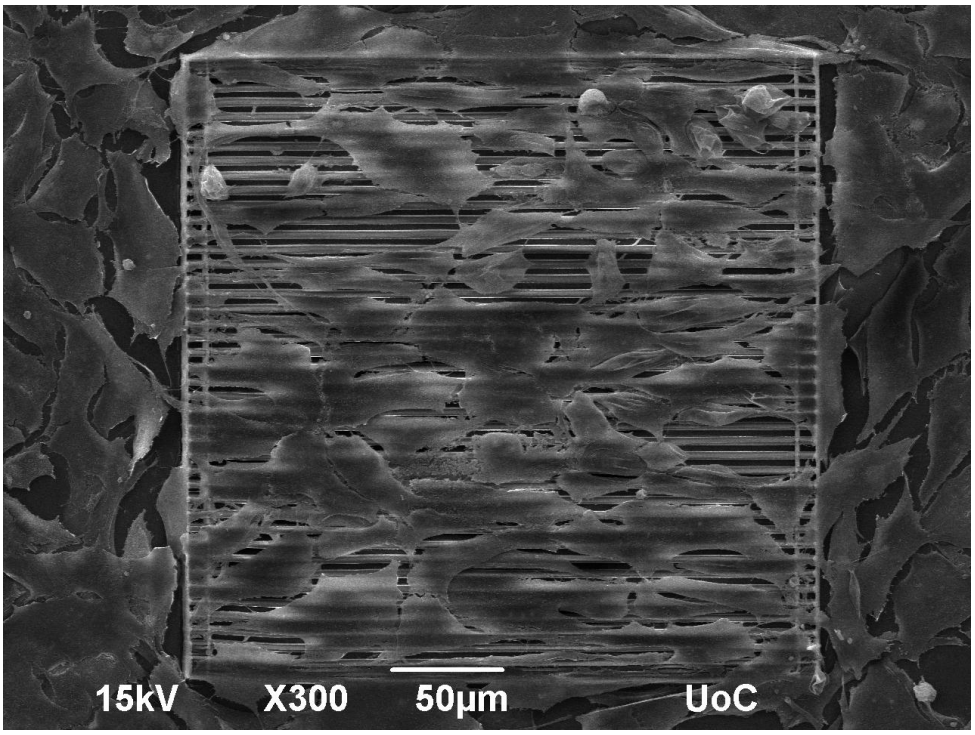


Figure 19

5**.1.2** Afterwards, structures with distance between line 15μm and 30μm were fabricated. In figure 20,21,22 fibroblast cells were cultivated for 3 days. The numbers of cells per ml are 10^5 cells in structure with line distance 15μm.

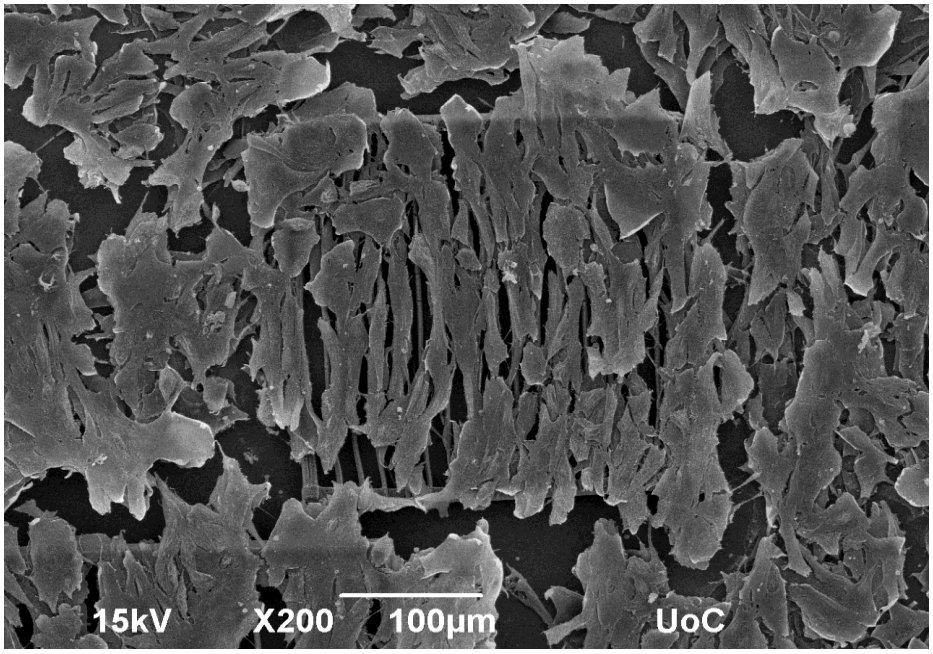


Figure 20

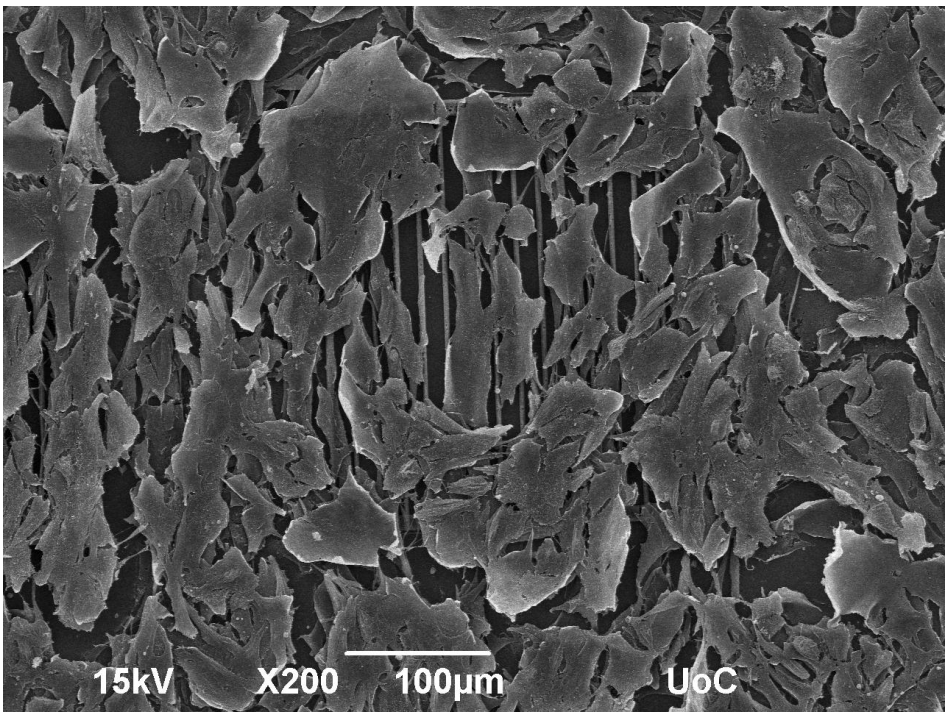


Figure 21

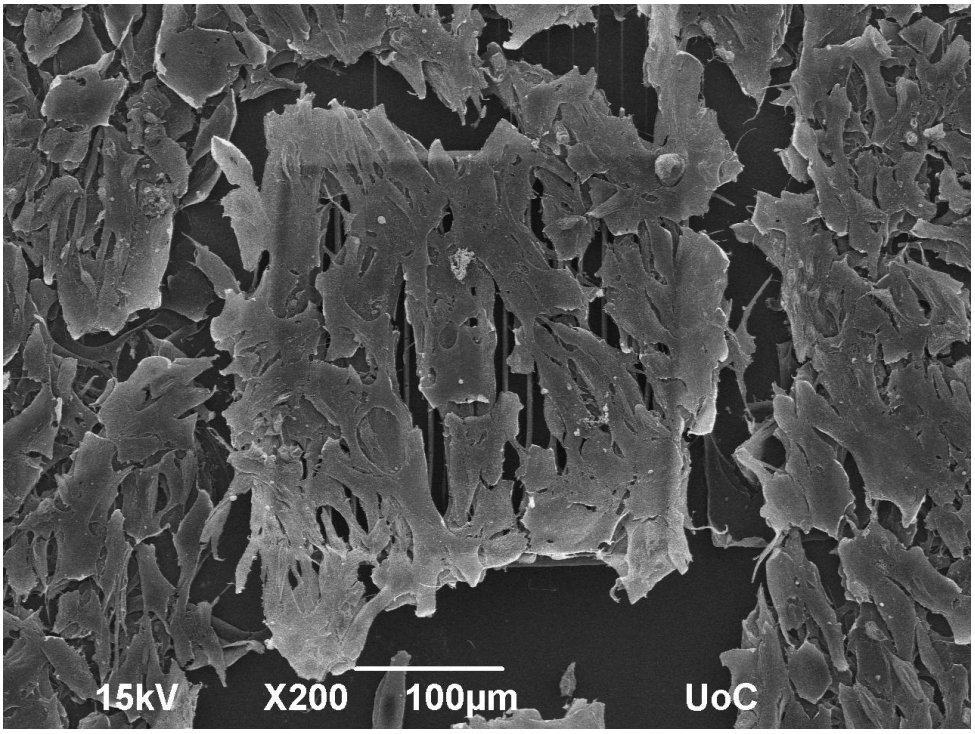


Figure 22

In figure 23,24,25 fibroblast cells were cultivated for 3 days in structure with the distance between lines being 30μm.

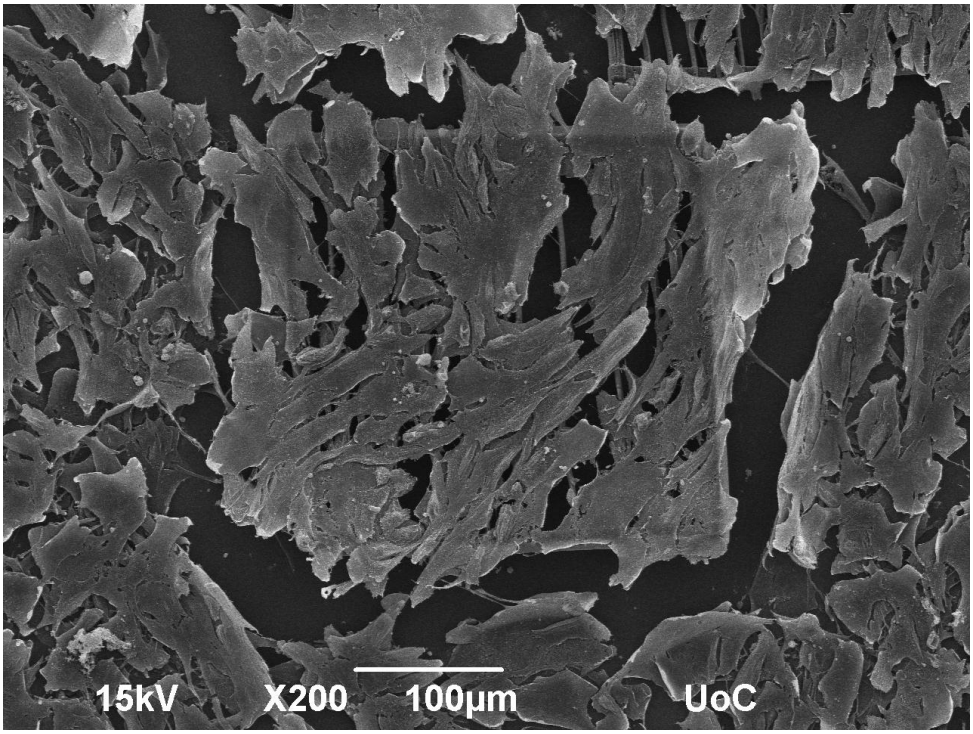


Figure 23

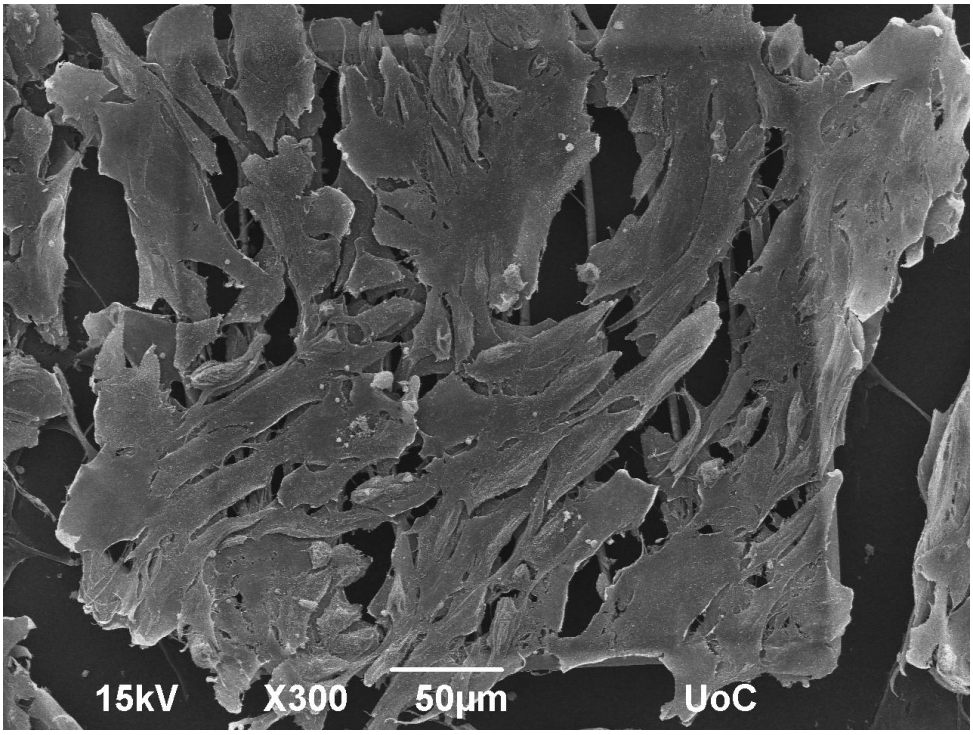


Figure 24

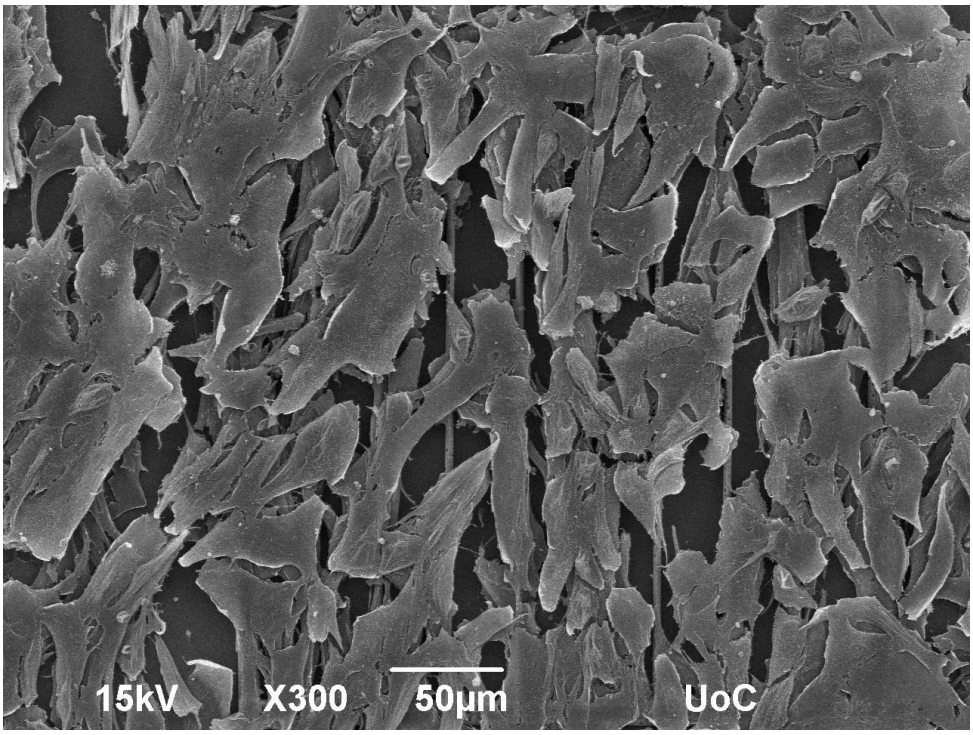


Figure 25

5**.1.3** Next step, structures like a chess were fabricated.Dimensions are (400x400) μm, distance between every line is 10μm and thickness of every line is 2μm. Photos of 3D structures in figures 26,27

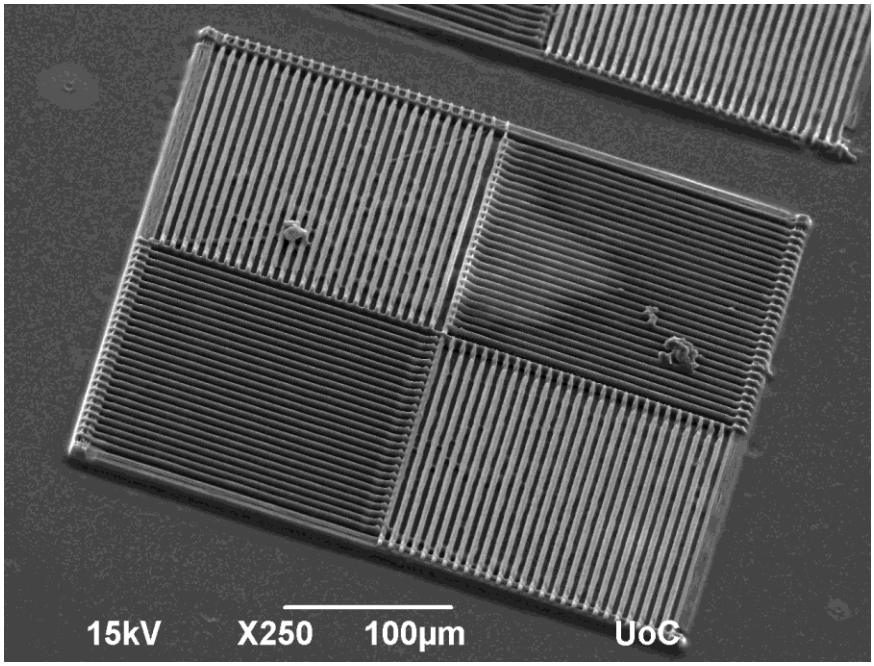


Figure 26

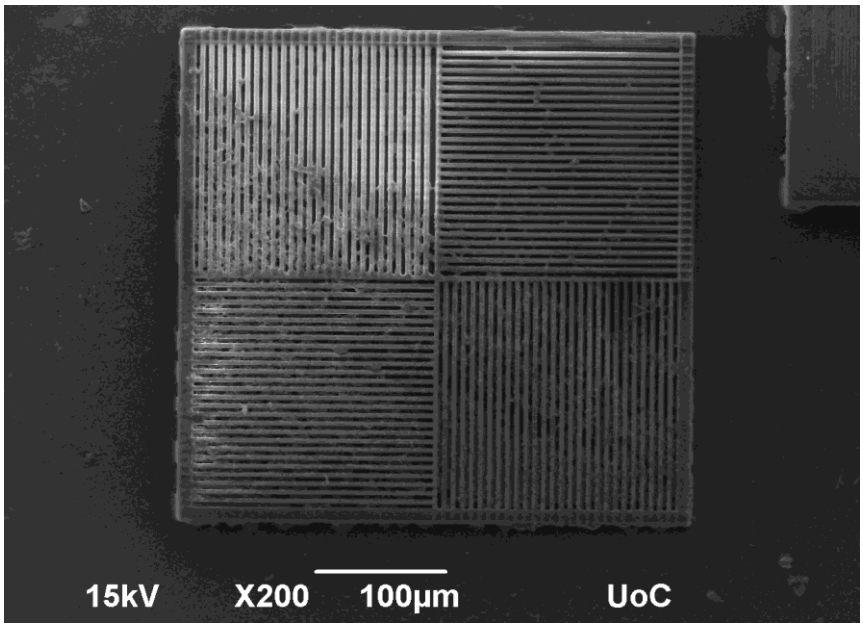


Figure 27

In this structure fibroblast cells were cultured for 3 days. The number of cells were used are 10^5 cells. Next photos show the structure with cells. Figures 28,29,30,31,32

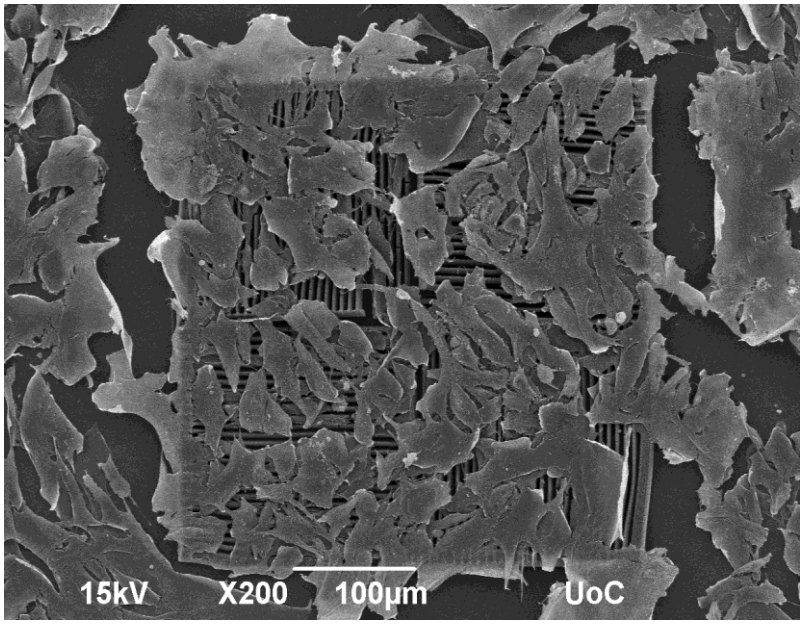


Figure 28

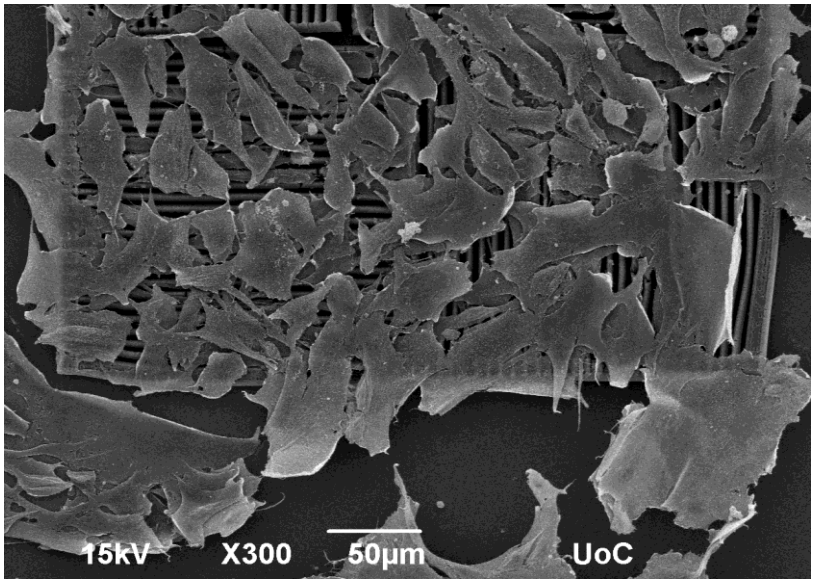


Figure 29

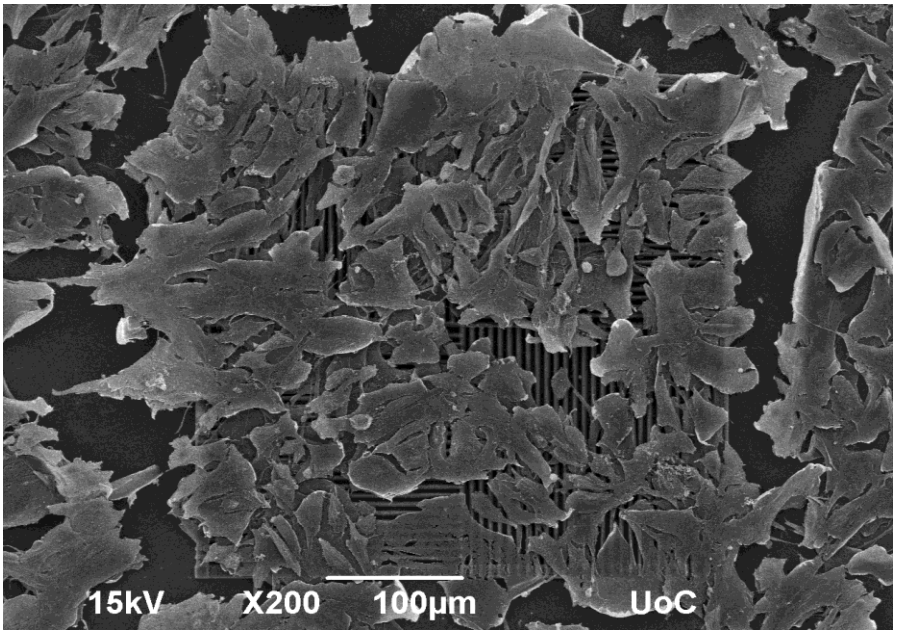


Figure 30

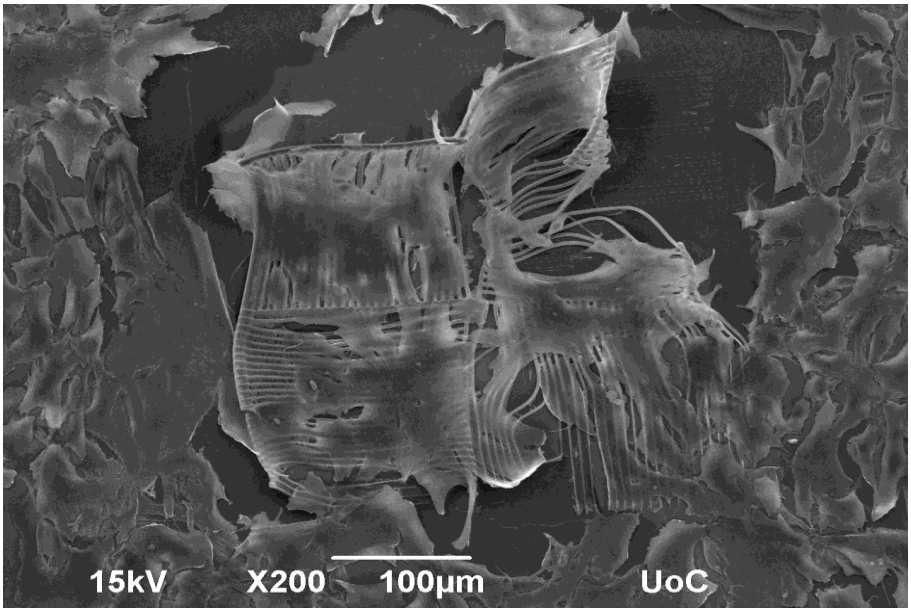


Figure 31

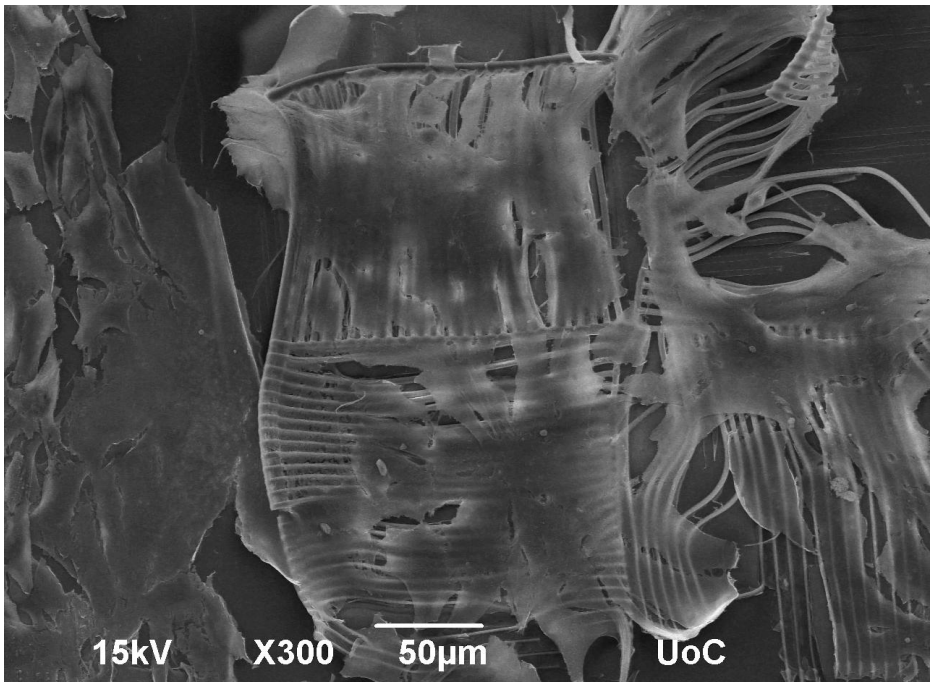


Figure 32

6. Conclusions

The primary aim of the experiments was to find out if the cells were able to follow the destination we had originally made them to follow. Then we had to figure out if a possible change of the distance among the lines could differentiate their direction. The scaffolds were created by the MPP technique and the biomaterial were used for these experiments. In the first phase of our experiment we had one layer of cells over the cornea stroma that has many layers of the cells. Examining the results, we conclude that in the structures we made with 10μm distance between the lines, the cells follow a specific direction. When the distance increases, as in our structures with 15μm and 30μm distance between the lines, cells begin to lose their orientation. Especially in the structures with 30μm distance the cells form no lines at all. In the chess-like structures we observe that the cells tend to follow the direction we specify. Quite possibly, the cells (Fibroblasts) we used have this response to our structures. The size of fibroblasts is 20μm approximately. What we can conclude, is that it is possible when we have a thick layer of cells, we will not be able to witness the movement of the cells within this structure. Therefore, in the next experiments we should use a smaller number of cells.

As a **future work**, we can attempt to create a two-level structure, in order to examine if the cells will then be able to follow a certain direction.

References

1. V. Melissinaki, A. A. Gill, I. Ortega et al., “Direct laser writing of 3D scaffolds for neural tissue engineering applications,” *Biofabrication*, vol. 3, Article ID 045005, 2011.

2 . A Koroleva, A A Gill et al., “Two-photon polymerization-generated and micromolding-replicated 3D scaffolds for peripheral neural tissue engineering applications” Biofabrication 4 (2012) 025005 (11pp)

3. Christopher N. LaFratta, Tommaso Baldacchini et al., “Replication of Two-Photon-Polymerized Structures with Extremely High Aspect Ratios and Large Overhangs” *J. Phys. Chem. B* 2004, *108*, 11256-11258

4. Brad A. Bryan, Yi Cai et al., “The Rho-Family Guanine Nucleotide Exchange Factor GEFT Enhances Retinoic Acid- and cAMP-Induced Neurite Outgrowth” Journal of Neuroscience Research 83:1151–1159 (2006)

5.http://el.wikipedia.org

6. Master thesis. ‘’Two-photon Polymerization Process for Tissue Engineering and Medical Application”. Vasileia Melissinaki.

7..https://en.wikipedia.org/wiki/Cornea

8. https://nei.nih.gov/health/cornealdisease

9. http://www.healthline.com/human-body-maps/cornea

10. en.wikipedia.org/wiki/Stroma_of_cornea

11.Train V. Chirilaa, Celia R Hicksa, Paul D. Daltonet al."Artificial cornea" 1998, Pages 447–47

12.Linda J Muller, Elizabeth Pels, at al. "A new three-dimensional model of the organization of proteoglycans and collagen fibrils in the human corneal stroma" March 2004, Pages 493–50

13.http://www.molvis.org/molvis/v9/a56/clout-fig2.html

14. Jeffrey W. Ruberti, Abhijit Sinha Roy, et al. "Corneal structure and function "

15. https://en.wikipedia.org/wiki/Fibroblast.

16.Todaro GJ, Green H . Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. 17: 299-313, 1963.

17.http://www.abcam.com/nih-3t3-mouse-embryonic-fibroblast-cell-line-whole-cell-lysate-ab7179.html.

18.http://www.fibroblast.org

19.Selva Çavuş, Gülten Gürdağ "Synthesis and Swelling Behavior of poly(2-dimethylaminoethyl methacrylate-co-N-hydroxymethyl acrylamide) Hydrogels" Istanbul University, Faculty of Engineering, Department of Chemical Engineering, 34320, Avcilar

20.Aleksandr Ovsianikov, Sabrina Schlie et al., "Two-photon polymerization technique for microfabrication of CAD-designed 3D scaffolds from commercially available photosensitive materials" *J Tissue Eng Regen Med* 2007; 1: 443–449.

21."N,N-Dimethylaminoethyl methacrylate" TECHNICAL DATA SHEET 213.

22. Konstantina Terzaki, Maria Kissamitaki et al., "Pre-osteoblastic cell response on three-dimensional, organic-inorganic hybrid material scaffolds for bone tissue engineering" DOI: 10.1002/jbm.a.34516

23.Gabija Bickauskaite, Maria Manousidaki et al., "3D Photonic Nanostructures via Diffusion-Assisted Direct fs LaserWriting" Volume 2012, Article ID 927931.

24. Lim HB, Oh KS, Kim YK, Lee DY. "Characteristics of hydrothermal stability and machinability of t-ZrO2/Al2O3 composites as a femo- ral head for total hip replacements." Mat Sci Eng a-Struct 2008; 483–84:297–301.

25. Synatschke CV, Schallon A, Jerome V, Freitag R, Muller AHE. "Influence of polymer architecture and molecular weight of poly(2- (dimethylamino)ethyl methacrylate) polycations on transfection efficiency and cell viability in gene delivery." Biomacromolecules 2011;12:4247–4255.

26. A Koroleva, A A Gill, I Ortega et al., "Two-photon polymerization-generated

and micromolding-replicated 3D scaffolds for peripheral neural tissue engineering applications" Biofabrication 4 (2012) 025005 (11pp)

27. Aleksandr Ovsianikov, Sabrina Schlie et al., "Two-photon polymerization technique for microfabrication of CAD-designed 3D scaffolds from commercially available photosensitive materials" *J Tissue Eng Regen Med* 2007; 1: 443–449.

28. Gabija Bickauskaite, MariaManousidaki et al "3D Photonic Nanostructures via Diffusion-Assisted Direct fs LaserWriting" Volume 2012, Article ID 927931, 6 pages

29. Canet Acikgoz, Mark A. Hempenius, Jurriaan Huskens et al., "Polymers in conventional and alternative lithography for the fabrication of nanostructures" European Polymer Journal 47 (2011) 2033–2052

30. Linjie Li and John T. Fourkas "Multiphoton polymerization" June 2007 Volume 10 Number 6

31. Farsari M, Vamvakaki M et al., "Multiphoton polymerization of hybrid materials" J Optics-Uk 2010;12.

32.Juodkazis S, Mizeikis V et al., "Three-dimensional microfabrication of materials by femtosecond lasers for photonics applications" J Appl Phys 2009;106.

33. LaFratta C N, Fourkas J T et al., "Multiphoton fabrication" *Angew.Chem. Int. Edn*

34. Juodkazis S, Mizeikis V et al., "Three-dimensional microfabrication of materials by femtosecond lasers for photonics applications" *J. Appl.*

35. FarsariM and Chichkov B "Two-photon fabrication" 2009

36. Hong-Bo Sun Satoshi Kawata "Two-Photon Photopolymerization and 3D Lithographic Microfabrication" APS (2004) 170:1

37. Brian H. Cumpston, Sundaravel P. Ananthavel, Stephen Barlow, Daniel L. Dyer et al.,"Two-photon polymerization initiators for three-dimensional optical data storage and microfabrication" *Nature* **398**, 51-54 (4 March 1999).

38.https://en.wikipedia.org/wiki/Cell_culture

39. Mikael Karlsson, Tommy Haraldsson et al., “Fabrication and transfer of fragile 3D PDMS microstructures”. J. Micromech. Microeng. 22 (2012) 085009 (9pp)

40. Gabija Bickauskaite, MariaManousidaki et al., “3D Photonic Nanostructures via Diffusion-Assisted Direct fs LaserWriting” Volume 2012, Article ID 927931.

41. Juodkazis S, Mizeikis V et al., “Three-dimensional microfabrication of materials by femtosecond lasers for photonics applications” J Appl Phys 2009;106.

42. Selva Çavuş, Gülten Gürdağ “Synthesis and Swelling Behavior of poly(2-dimethylaminoethyl methacrylate-co-N-hydroxymethyl acrylamide) Hydrogels” Istanbul University, Faculty of Engineering, Department of Chemical Engineering, 34320, Avcilar

43. Aleksandr Ovsianikov, Sabrina Schlie et al., “Two-photon polymerization technique for microfabrication of CAD-designed 3D scaffolds from commercially available photosensitive materials” *J Tissue Eng Regen Med* 2007; 1: 443–449.

44. https://en.wikipedia.org/wiki/Scanning_electron_microscope

45. Smith KCA, Oatley, CW (1955). "The scanning electron microscope and its fields of application". *British Journal of Applied Physics* **6** (11): 391–399.

46. http://www.rpi.edu/dept/chem-eng/Biotech-Environ/Projects00/tissue/What%20is%20Tissue%20Engineering.htm

47. https://en.wikipedia.org/wiki/Tissue_engineering

48. R. Behrisch(ed)(1981). Sputtering by Particle bombardment:, Springer, Berlis ISBN 978-3-540-10521-3

49. http://www.wikilectures.eu/index.php/Tissue_engineering_principle

50. Kwang-Sup Lee1*, Dong-Yol Yang2, Sang Hu Park2 and Ran Hee Kim1 "Recent developments in the use of two-photon polymerization in precise 2D and 3D microfabrications"

51. J. P. Boiloty, J. Biteauy, F. Chaputy, T. Gacoiny, A. Brunz, B. Darracqz, P. Georgesz, Y. Levyz, " Organic–inorganic solids by sol–gel processing: optical Applications," Pure Appl. Opt. **7,** 169–177, (1998).

52. D. Sangeeta, J. R. LaGraff, "Inorganic Materials Chemistry, Desk Reference," Second Edition, ed, (CRC Press)

53. Phd thesis Konstantina Terzaki University of crete Department of Materials Science and Technology. "Non-linear micro/nanolithography with short-pulse lasers: applications on biomaterials and biosensors".