UNIVERSITY OF CRETE

MATERIALS SCIENCE AND TECHNOLOGY DEPARTMENT

Undergraduate Thesis

Scaffold fabrication using Laser Irradiation for Cell Cultivation

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ABSTRACT

One of the most promising techniques of 3D scaffold fabrication for cell culture is the two-photon polymerization technique. This method allows the fabrication of micrometer size structures with submicron resolution. In this study, a photosensitive sol-gel hybrid material, zirconium doped silicate has been studied for its suitability in three dimensional scaffold fabrication. The fabrication of 3D scaffold for cell alignment, directional growth and glial-like carpet formation, is presented. This hybrid material provides a matrix for cell growth (organic part) and mechanical stability to the structure (inorganic part).

The use of femtosecond laser structuring permits the fabrication of a wide range of mechanically stable scaffolds of different sizes and shapes to be tested in terms of cell viability, proliferation and orientation.

ACKNOWLEDGMENTS

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"This Thesis is dedicated to my two beloved persons who passed away and I missed them. I will always remember you! Farewell"

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THE MAIN GOAL AND OUR WORK

It is widely known that the neural tissue cannot regenerate itself and this might have fatal impacts in the life of a human. In the context of the study of neurogenesis, in the field of the tissue engineering, our goal is to establish a 3D experimental cell culture system. In the framework of this diploma thesis, we co-cultivated two types of cells on 3D scaffolds fabricated by the direct laser writing technique. The first one is the cell line SW10 (Schwann Glial Cells) and the second one is the cell line N2A (Neural Cells).
In this work we studied the viability and the proliferation of the SW10 and N2A cell lines on the 3D scaffolds. Furthermore, we showed that axonal growth of the N2A and the formation of a glial carpet from the SW10 can be achieved on top of our 3D scaffold.

BRIEF CONTEXT

In the first part we are going to give the definition of Tissue Engineering and we are going to talk about its applicability and its main principles and why it is necessary.
Moving forward to the second part, we are going to introduce the direct laser writing technique, the method we used in order to fabricate the 3D scaffold. In the third part, we will present the fabrication of the 3D structure, starting from the materials we used and ending with the physical phenomenon that governs this technique. The main subject of the fourth part will be the cell lines we used and the biological procedures we followed in order to observe the cells on our structure. In the last part, our results will be presented.

TISSUE ENGINEERING & NEURAL SYSTEM

CHAPTER 1

Tissue Engineering is an interdisciplinary scientific field within the broader area of regenerative medicine. More specifically, tissue engineering seeks to develop functional tissue or even organ substitutes to replace biological functions. For this purpose, different scientific fields have to meet together: biology, engineering, physics, chemistry and materials science.

The future of tissue engineering seems to be quite promising since it has been estimated that millions of people worldwide could benefit from its therapies. There is huge scientific interest in a specific area of the regenerative medicine that deals with neurogenesis. In neurogenesis the scientists try to find a way to treat neurodegenerative and autoimmune neural diseases, such as Parkinson's disease, Alzheimer's disease and many others. The ultimate goal of tissue engineering is the *in vitro* fabrication of a fully functional organ on a three dimensional scaffold.

The cells in the brain can be divided into two groups: Neurons and Neuroglia cells. In this thesis work the neuron cells used were the Neuron2A cell line and the Neuroglia cells used were Schwann Cells.

1.1 Neuron Cells

A typical neuron is an electrically excitable cell that processes and transmits information through electrical and chemical signals. A neuron differs from other body cells, in its unique structural elements. It consists of the following regions: the cell body, the dendrites, the axon, and the synaptic terminals. The region of the neuron that is responsible for intercellular information transfer is called axon.

The dendrites are designed to receive incoming information and the synapse transmits information across physiologically long distances. When an electrical signal

reaches the end of a neuron, it triggers the release of a chemical signal, which travels rapidly across the short gap between cells (the synapse) (Fig.1). [1],[2]

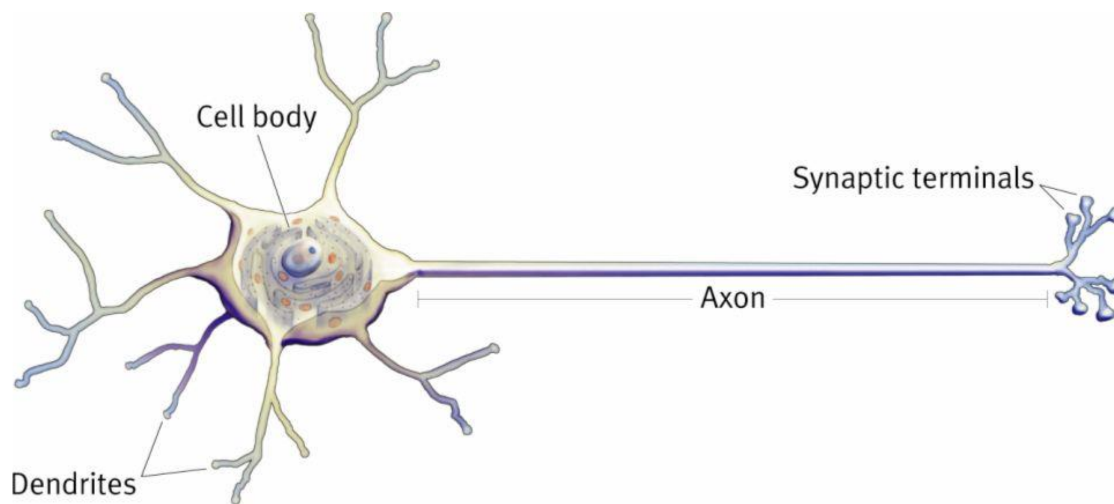

Figure1. *Schematic view of a nerve cell. Image from: bio3520.nicerweb.com/Locked/chap/ch03/neuron.htm*

1.2 Glial Cells

Based on their appearance, glial cells can be divided in two groups: Astrocytes and Oligodendrocytes, in the central nervous system, and, Schwann cells, enteric glial cells and satellite cells, in the peripheral nervous system. Astrocytes have a star-like appearance and control the chemical composition of the fluid surrounding the neurons. They maintain appropriate concentrations of ions and neurotransmitters in the neuronal environment. Also, they control the growth of neurons, regulating synaptic communication between neurons and altering blood flow in regions of the brain that are highly active. Oligodendrocytes have a central cell body with arms all around their body and they form the myelin sheath around axons in the central nervous system, allowing the fast conduction of signaling, which is essential for nervous system function(Fig. 2). [1],[3],[4]

In the peripheral nervous system (PNS), the major glial cells are Schwann cells and may occupy up to about 1mm of the length of an neural axon. They have a supporting role for the neuron, wrapped around the axons of the peripheral nervous system. They lack the ability to transmit synaptic messages and can divide indefinitely throughout life. They are found in two types, myelinating and non-myelinating. The myelinating Schwann cells form insulating sheaths around axons that are comparable in structure and function to those made by oligodendrocytes. The non-myelinating cells show similarities with astrocytes and are likely to have metabolic and mechanical

support functions. Schwann cells produce molecules that influence the expression of neural proteins important for neural survival and differentiation. They have a central role in numerous aspects of neuronal homeostasis and are associated with a number of pathologies [5],[6]

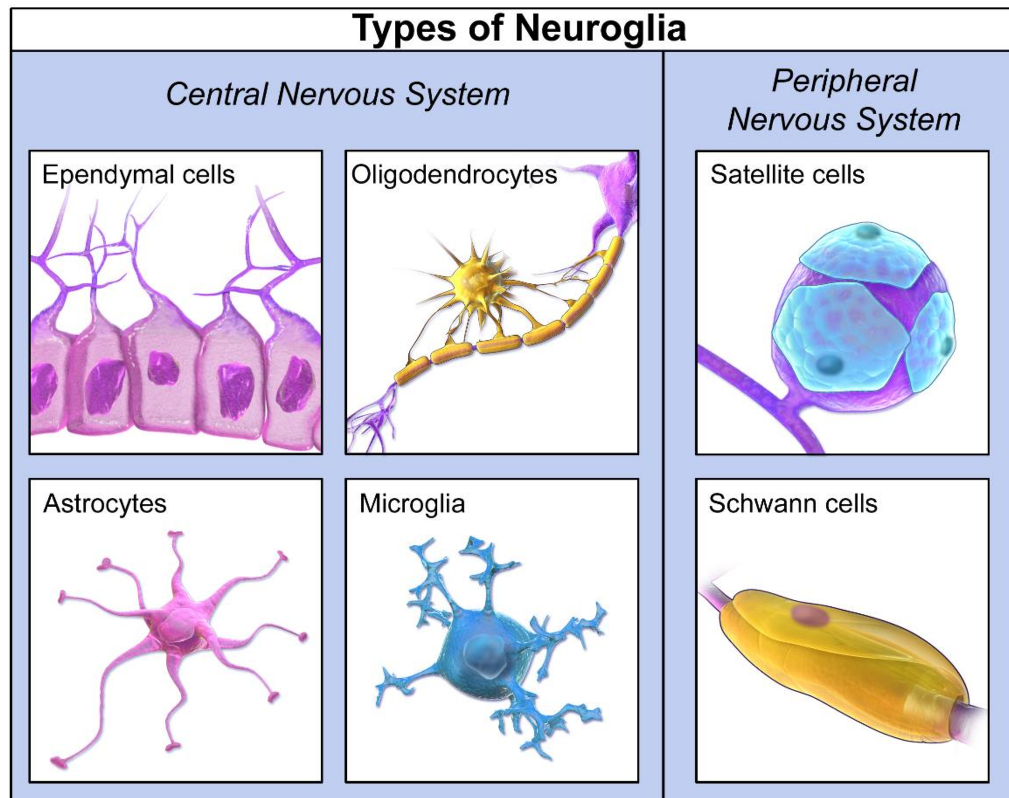

Figure2. *Image showing diversity of glial cell types found in the central and peripheral nervous system. Image from: https://canvas.brown.edu/courses/851434/pages/neurons-and-glia*

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[1] Irwin B. Levitan, Leonard K. Kaczmarek "**The Neuron cell and molecular biology**", Oxford University Press 1997, Second Edition.

[2] http://www.biologyreference.com/Mo-Nu/Neuron.html#ixzz3ySUT0SzR

[3] Glial cells Kristjan R. Jessen∗ Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK Received 17 September 2003; received in revised form 12 February 2004; accepted 16 February 2004

[4] http://www.ucl.ac.uk/cdb/research/jessenmirsky/publications/IntJBiochem.pdf

[5] http://www.sciencedirect.com/science/article/pii/S1357272506001634

[6] Schwann cells: Origins and role in axonal maintenance and regeneration
Kanav Bhatheja, Jeffrey Field, doi:10.1016/j.biocel.2006.05.007

DIRECT LASER WRITING SCAFFOLD FABRICATION

CHAPTER 2

Direct Laser Writing, is a unique, non-linear stereolithographic technique based on two-photon polymerization allowing the fabrication of 3D micro structures. This technique was used for a 3D scaffold fabrication to be used in 3D cell culture. In general, the technique is applied in optoelectronics, biomaterials, telecommunications, sensors and microfluidics.

The DLW offers some unique advantages compared to other stereolithographic methods. For example, the DLW permits the fabrication of a 3D computer designed structure with resolution beyond the diffraction limit and causes no thermal damages to the material. In the (Fig. 3) bellow, 3D microstructure models fabricated in IESL/FORTH are presented [1].

***Figure 3**. 3D model of (a) an ancient Greek temple (made by Giannis Melissinakis), (b) a guitar and (made by Vasileia Melissinaki)(c) an airplane (made by Angeliki Zafeiropoulou)*

2.1 The 3D Scaffold

The final scaffold (Fig. 4) composed of two symmetric arm-like cubic structures connected by eight equidistant and of different length guidelines. In order to study the migration, adhesion and orientation of the cells pendulous guidelines were fabricated.

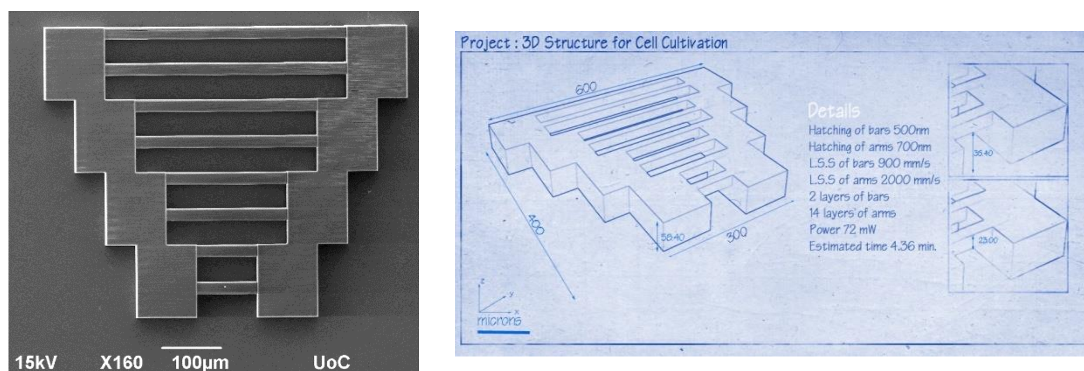


Figure. 4. *In the left side, SEM image of a top view image of our scaffold. In the right side, a schematic of the scaffold with its properties.*

2.2 Scaffold fabrication (a schematic approach)

The fabrication process (Fig. 5) of the 3D scaffold could be described in ten steps. First (Fig. 5-A), we synthesize the chemical solution and then (Fig. 5-B) we transfer, with a pipette, a small amount of the solution on a silanized cover slip. Both the cover slip and the droplet remain in a vacuum chamber in order for the excess solution to evaporate and the solution to become a gel (gelation process) (Fig. 5-C). In the next step, our sample is placed upside-down in a metallic base below the lens which focuses the laser beam (Fig. 5-D). Once the laser beam is focused inside the volume of our sample, the photopolymerization is triggered and a green fluorescent light is emitted (Fig. 5-E). Below the sample, we placed a red led light and using a CCD camera behind the galvo mirrors, we can have a live view of the polymerization process in an external monitor due to the refrective index change of the polymerized material (Fig. 5-F). The laser set up is described in detail in the next chapter. Once the photopolymerization ends, the sample is removed from the metallic base (Fig. 5-G,H) and is placed inside a Petri dish with the proper solvent and the non-polymerized areas are removed. (Fig. 5-I). Finally, the structure is strongly attached upon the cover slip and it is chemically and mechanically stable (Fig. 5-J).

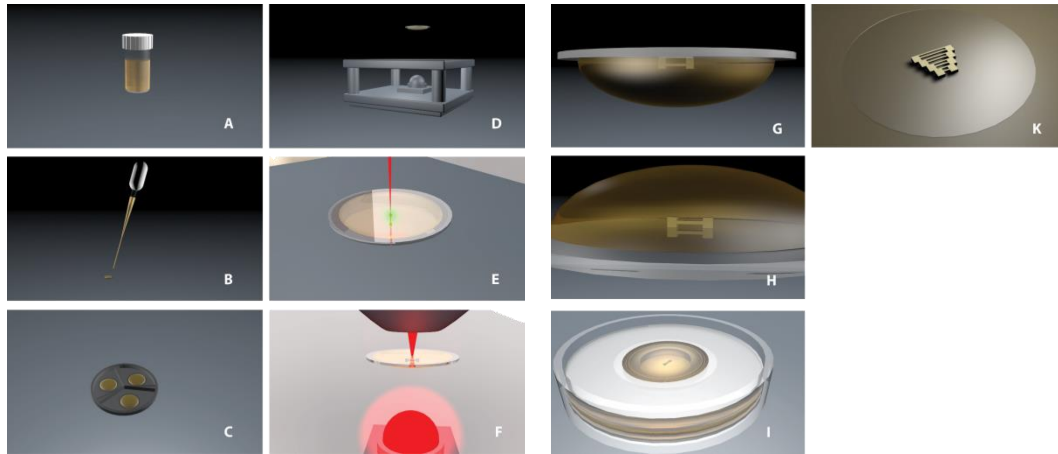


Figure. 5 *Step by step fabrication of our 3D scaffold (schematic). Images generated by Cinema4D*

References Chapter 2

[1] Elmina Kabouraki, Konstantina Terzaki, Vasileia Melissinaki, Maria Manousidaki, Maria Vamvakaki and Maria Farsari, "**Chapter 8, Direct fs Laser Writing of 3D Nanostructures**", Springer International Publishing Switzerland 2015, S. Sakabe et al. (eds.), *Progress in Nonlinear Nano-Optics, Nano-Optics and Nano-photonics*, DOI 10.1007/978-3-319-12217-5_8

CHEMICALS AND CHEMICAL SYNTHESIS OF THE SAMPLE

CHAPTER 3

The basic materials used (Fig.6) in the fabrication of the 3D scaffold were a photo initiator, two organic monomers and zirconium Isopropoxide as the inorganic component. The final 3D structure is a hybrid material composed of organic – inorganic elements. Different ratios between the organic and inorganic components may change the mechanical properties of the scaffold.

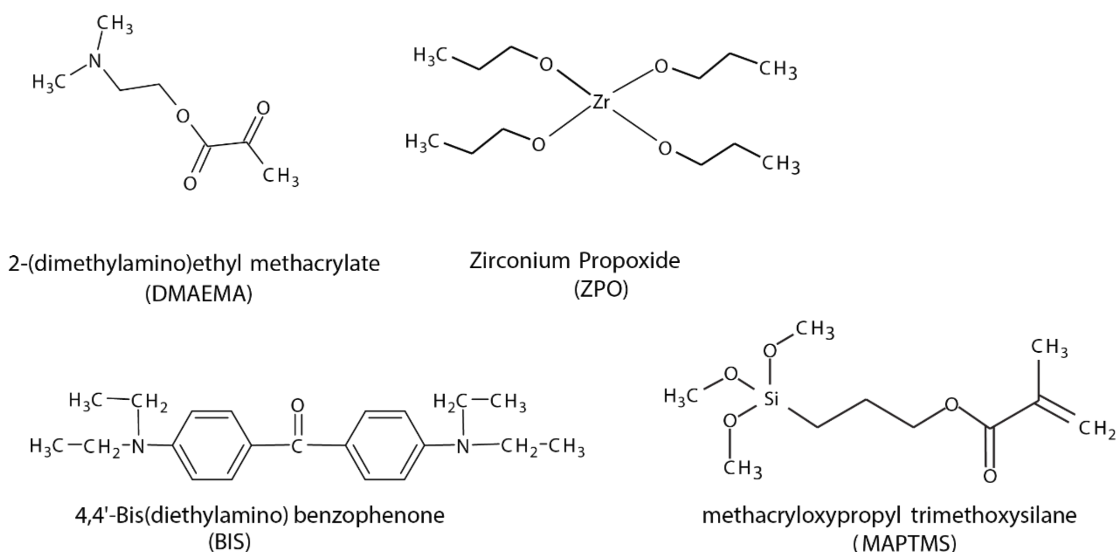


***Figure 6**. The chemicals used*

The inorganic part if formed by the hydrolysis of MAPTMS and by the condensation of MAPTMS with ZPO via the sol-gel process. In addition, the organic network is formed by the polymerization of DMAEMA and MAPTMS using the free radical polymerization technique; in which a photoinitiator, BIS, generates two active radicals

by absorbing the photon energy (hv).All the above chemicals were purchased from Sigma Aldrich. The synthesis of the current material has first introduced K. Terzaki, N. Vasilantonakis, A. Gaidukeviciute, C. Reinhardt, C. Fotakis, M. Vamvakaki, and M. Farsari "3D conducting nanostructures fabricated using direct laser writing" *Opt. Mater. Expr.* **2011,** *1,* 586-597.

3.1 The Sol-Gel Process

The sol-gel process is a method for producing a solid material from small molecules by the transition from a liquid – a sol- to a material with non-zero shear modulus – a gel [1].

3.2 The Photopolymerization Process

The photopolymerization process follows a free radical polymerization method according to which a polymer is formed by the successive addition of a free macroradical to the double bond of the monomer. In general, free radicals can be generated via a number of different mechanisms; in our case, free radicals are generated by photon energy absorption. The mechanism of the free radical polymerization has three main steps, the initiation, the propagation and the termination of the polymerization.

Initiation

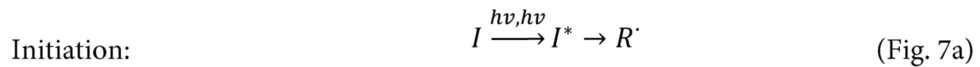
Initiation is the first step of the free radical polymerization process. During this step, a labile bond of a photoinitiator is cleaved and two free radicals are produced (Fig. 7a). After the formation of the two free radicals, each one of them attacks a monomer to form a new molecule composed by the segment of the initiator and a monomer repeat unit.

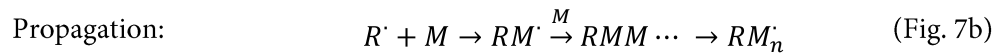
Propagation

The above radical attacks the double bond of another monomer and a new radical is formed by a free radical with two monomer repeat units. This process continues until a very long macroradical is formed. (Fig. 7b).

Termination

The termination of the free radical polymerization may occur by two main ways, combination (Fig. 7c), and disproportionation.


Initiation: $I \xrightarrow{h\nu,h\nu} I^{*} \rightarrow R^{\cdot}$ (Fig. 7a)


Propagation: $R^{\cdot} + M \rightarrow RM^{\cdot} \xrightarrow{M} RMM \cdots \rightarrow RM_n^{\cdot}$ (Fig. 7b)

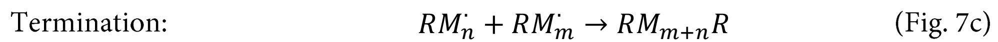

Termination: $RM_n^{\cdot} + RM_m^{\cdot} \rightarrow RM_{m+n}R$ (Fig. 7c)

Fig 7. *The free radical polymerization process (Image generated with Illustrator CS5, [2])*

In photopolymerization process, the use of light instead of heat, in order to cleave the initiator bond, has certain advantages: (a) The elimination of the solvent (not necessarily), (b) high reaction rates at room temperatures and (c) spatial control of the polymerization [2].

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[1] Richard A. L. Jones " Soft Condensed Matter", First Edition 2002, Oxford Master Series in Condensed Matter Physics, 95

[2] Maria Farsari, Maria Vamvakaki and Boris N Chichkov "Multiphoton polymerization of hybrid materials" J. Opt. **12** (2010) 124001 (16pp)

TWO PHOTON ABSORPTION PHENOMENON AND LASER SET-UP

CHAPTER 4

Following the chemical synthesis of our material, for the fabrication of our scaffold inside the volume of the material we rely on the use of the two-photon absorption phenomenon. This allows to fabricate geometrically complex 3D models.

The two-photon absorption phenomenon is a non-linear optical phenomenon that occurs when two photons of same energy (hv/2) excite an electron from the ground state to an excitation state. On the contrary, upon a linear absorption, the energy of a single photon is enough to transcend the energy gap.

Analytically, a photon of energy (hv/2) excites the electron from the ground state to an intermediate virtual state below the first excitation state. This virtual state has a very short life time (of the order of a few femtoseconds). If a second photon of the same energy arrives during this fs time period, it will be absorbed and the electron is excited to an elevated energy state. (Fig. 8).

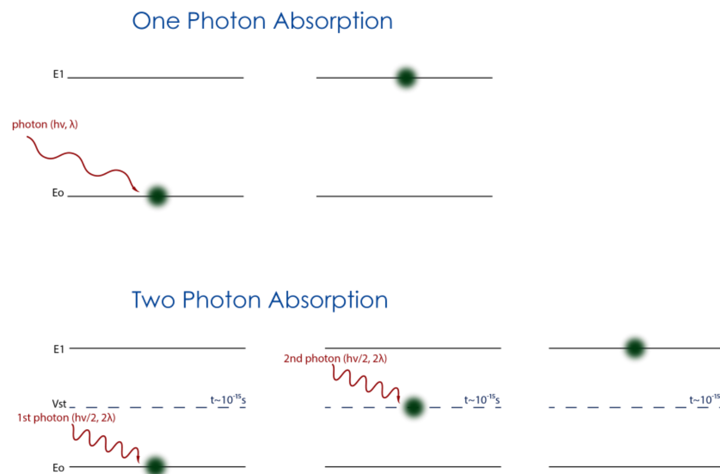

Figure 8. *The two ways of electron absorption (Image generated with Illustrator CS5, [1])*

4.1 Laser Types

A laser type that is widely used for the two-photon polymerization process is the Ti: Shapphire laser. These lasers have two advantages: (a) They have a short pulse duration (picosecond or less) (c) Their wavelength is 800nm, which is twice the wavelength of polymerization of a plethora of photopolymers and at 800nm most photopolymers are transparent allowing the in-volume focusing of the laser beam with minimal scattering [1].

4.2 Two-photon polymerization process

Once the laser beam is tightly focused in-volume of the sample, the polymerization starts with two active radicals of the photoinitiator being generated. The two photon absorption rate is proportional to the square of the laser intensity, thus a high spatial resolution can be obtained beneath the diffraction limit [2].

4.3 Laser Set-up

The laser set-up used in the lab is represented in figure 10 below. The laser source was a Ti:Shapphire femtosecond oscillator operating at 800nm, with pulse length at 200fs and a repetition rate of 50-80 MHz. The photopolymerization process follows a layer–by-layer formation and each layer is formed using a x-y galvometric mirror scanner and a x-y-z piezoelectric stage. Furthermore, we use a microscope objective lens in order to achieve a tightly focused laser beam in the volume of the material and the intensity of

the beam can be controlled by neutral density filters, an attenuator or a combination of polarizer and a waveplate. Moreover, using a monitor and a CCD camera mounted after a dichroic mirror, we can watch in real time the photopolymerization process. This is possible since the refractive index of most photopolymers changes upon polymerization, so that the irradiated structures become visible during the fabrication [3].

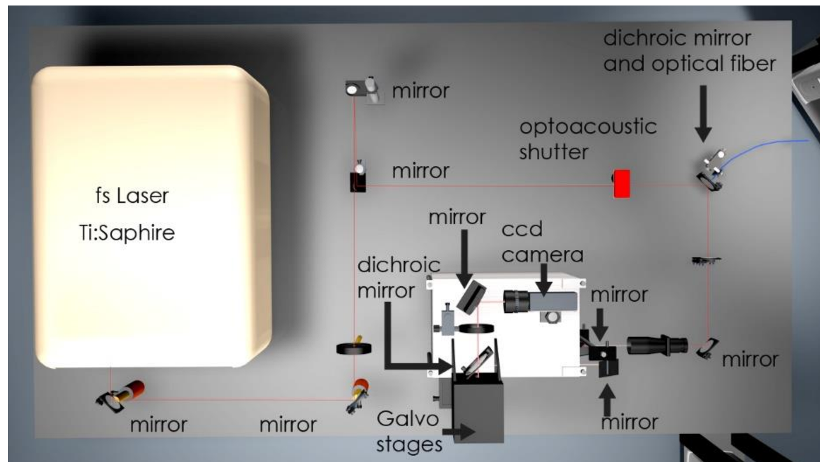

Figure 9. *The two ways of electron absorption (Image generated with Illustrator CS5)*

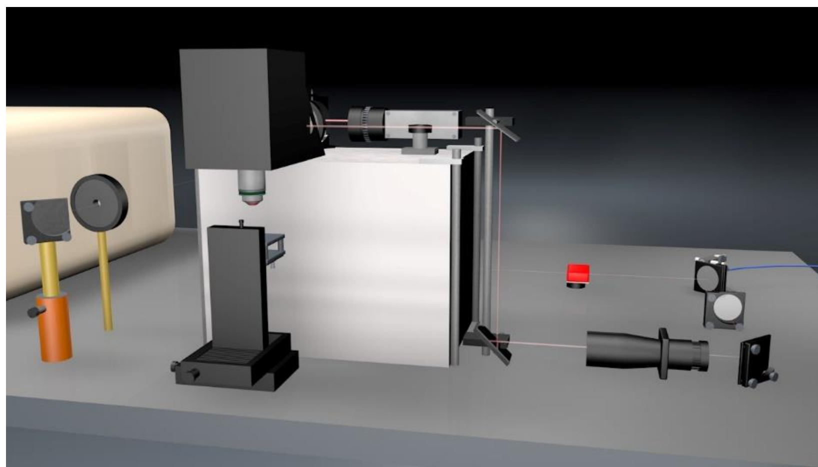

Figure 10. *The two ways of electron absorption (Image generated with Illustrator CS5)*

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[1] Maria Farsari, Maria Vamvakaki and Boris N Chichkov "Multiphoton polymerization of hybrid materials" J. Opt. **12** (2010) 124001 (16pp)

[2] Satoshi Kawata, Hong-Bo Sun, Tomokazu Tanaka & Kenji Takaba "Finer features for functional microdevices" Nature **421**, 697-698 (16 August 2001)|doi: 10.1038/35089130

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EXPERIMENTAL PROCESSES AND RESULTS

CHAPTER 5

This Chapter is divided in two parts. The first part is dedicated to the fabrication of the 3D structure while the second part refers to the cell culture and results. The material we used for the fabrication of our 3D structure has previously been tested for other types of cell culture [1]. The aim of this work is the establishment of a 3D neural cell cultivation.

5.1 Structure

The software used for the design of the 3D structure fabrication was the SolidWorks while the software used to make the structure editable for 3D printing was Viscam. Furthermore, we set the parameters for the layer-by-layer 3D fabrication via SAMLight and we control the piezoelectric stage via NANO V10. The 3D scaffold fabrication is shown at the following images.

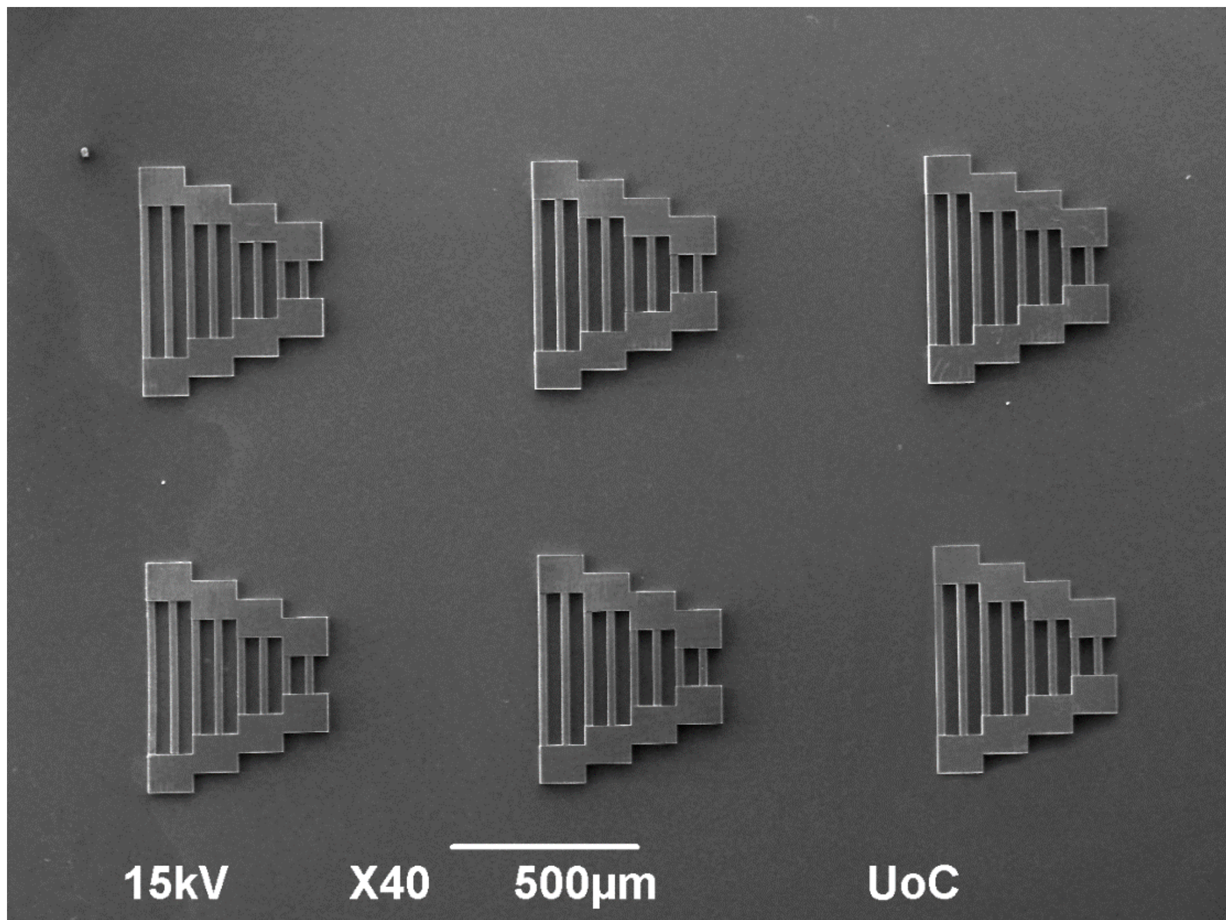


Figure 11. *SEM top view images of our3D structures*

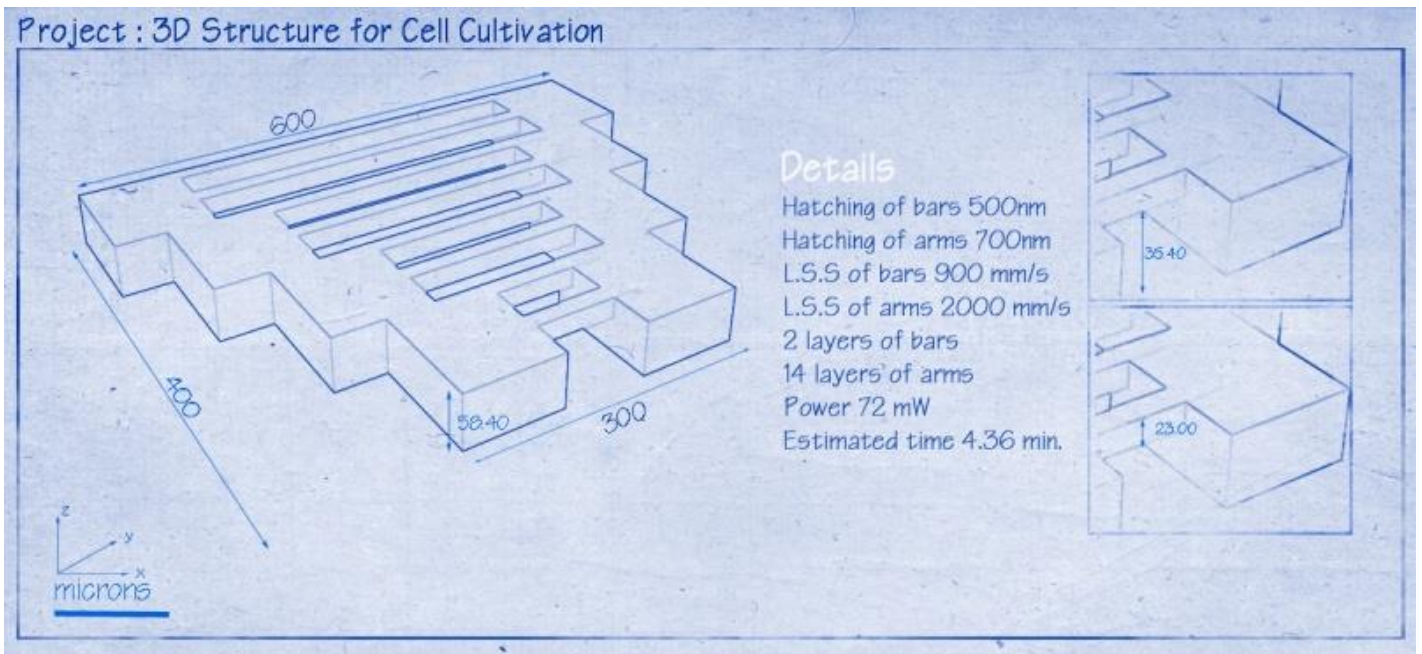


Figure 12. *Sketch of the structures.*

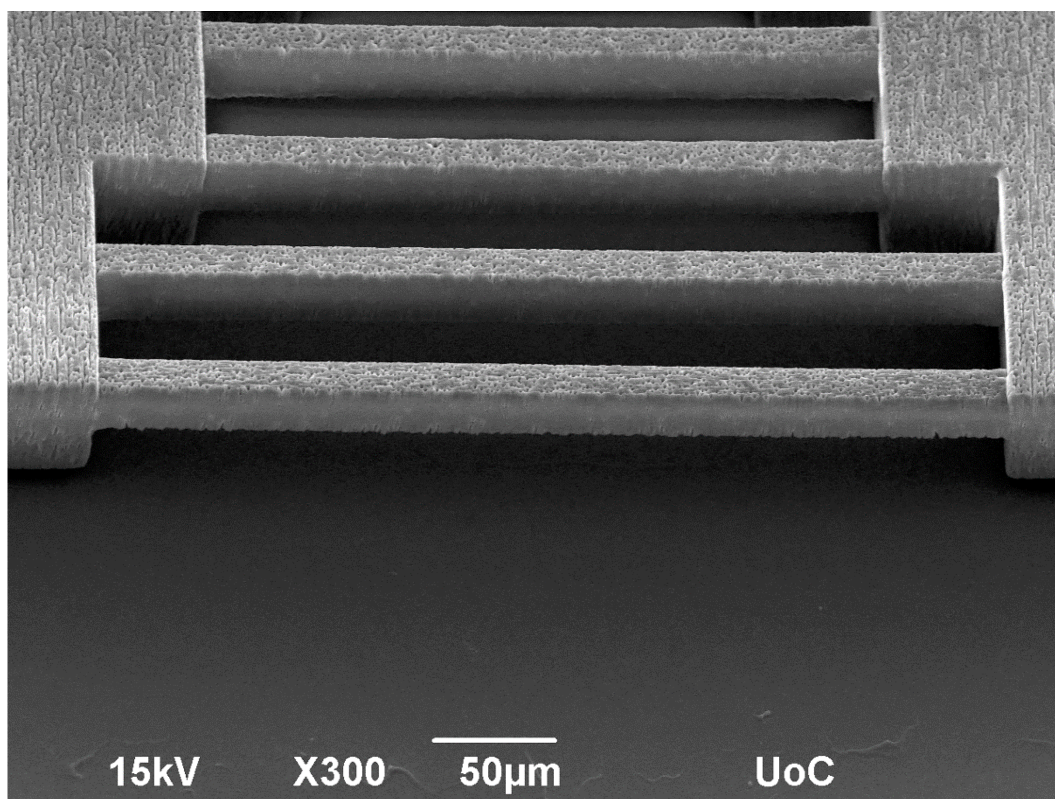


Figure 13. *Tilted view of a magnified 3D structure*

The experimental procedure of the material synthesis is described below.
A 30% DMAEMA–ZPO material was prepared as described below.

30% DMAEMA – ZPO			
ORDER	**CHEMICALS**	**VOLUME (mL)**	**MASS (g)**
1	MAPTMS	1.3037	1.36178
	HCl	0.13037	0.13037
2	ZPO	0.99571	0.999702
3	Water	0.189	0.189
4	BIS	-	0.0165
5	DMAEMA	0.37292	0.3527
Total Volume of The Solution			
2.9917 mL			

The procedure step by step:

First, we start with the hydrolysis of MAPTMS using an aqueous solution of HCl 0.1 M. We have to stir the solution because HCl and MAPTMS are immiscible before the hydrolysis. As the procedure continues we carefully add ZPO (70% w/v in propanol) avoiding the interaction with atmosphere humidity. We let the solution to homogenize with constant stirring. In addition, water is added and the solution is left

to stir for approximately 20 minutes. Furthermore, the photoinitiator and the proper amount of DMAEMA are added.

Then, the solution was filtered and stored until use. This solution is now the final material with which we are going to fabricate the 3D scaffold within the volume of a gel on a cover-slip. That means, that our final solution has to partially cover the surface of a cover-slip. Before that, we have to treat its surface in order for the structures to attach on the glass.

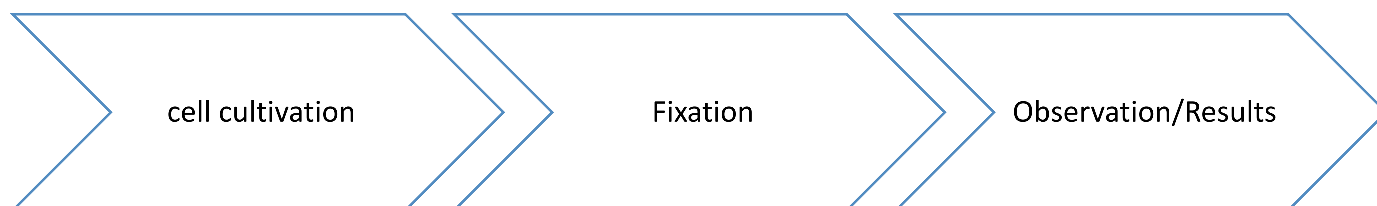
A small amount of the material is placed on the surface of a cover slip and is dried under vacuum in order to remove any solvent.

Glass Preparation

Cover slips with a 13 mm diameter are used. First they are placed in a beaker filled with ethanol and immersed in an ultrasonic bath for at least 1 hour. After that the ethanol is removed and 20mL of CH_2Cl_2 with 250μl of MAPTMS are added to the beaker. The cover slips are once more immersed into the ultrasonic bath for 3-4 hours. Then, we remove the MAPTMS and the CH_2Cl_2 and rinse with ethanol. Finally the glasses are stored in ethanol. The purpose of this process is to attach MAPTMS molecules on the surface of the glass so that the polymer is linked with them during the scaffold fabrication.

5.2 Cell Cultivation

In order to study the morphology, the proliferation and the migration of the cells around and upon our 3D scaffold, we have to follow some universal process first.



A cell cultivation is a process that is used to prolong the life and/or expand the number of cells or microorganisms in the culture. In order to cultivate cells or microorganisms, all the solutions and the materials used have to be sterile.

Step by step the cell cultivation:

1. Remove and discard the spent cell culture media from the culture flask.
2. Cover the cells in the flask with 2ml of Trypsin and leave the solution to act for about 2-5 minutes.
3. To stop the action of the Trypsin we add 2ml of fetal bovine serum.
4. We centrifuge it for 3 minutes at 2500 rpm, re-suspend the cell pellet in a new test tube adding about 5-6 ml of growth medium and transfer to a new culture flask.
5. After that we keep the new passage of cells in the right temperature inside the incubator.

If we want to set an experiment and we need to know exactly the number of the cells we will use, then we have to count them using the hemocytometer (Figure 14.).

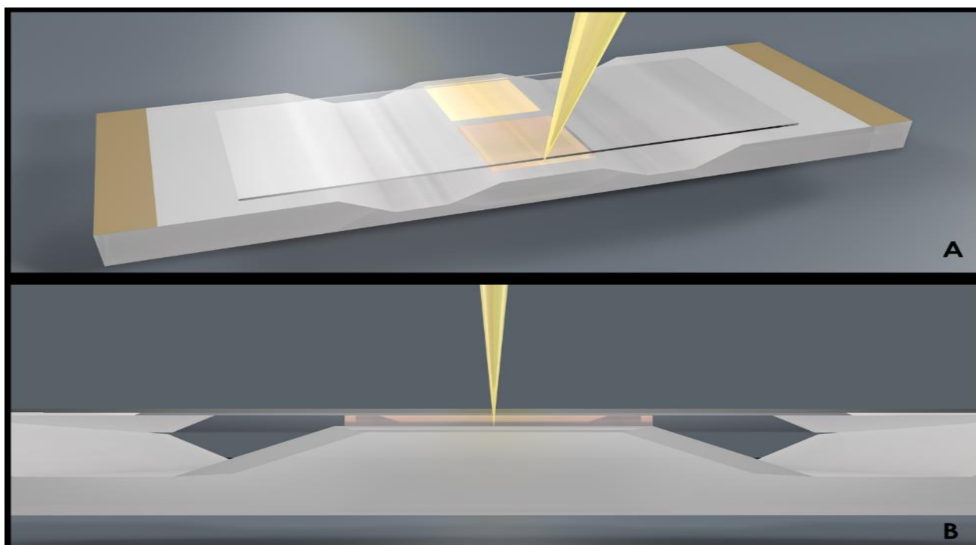


Figure 14*. A hemocytometer consists of a thick glass microscope slide. Created by me using Cinema 4D.*

Fixation:

In order to study a sample in SEM, the specimen needs to be completely dry, since the sample has to face extremely vacuum. For a biological sample, a strict fixation protocol needs to be followed. Usually, a fixation protocol, performed by incubation in a solution of a buffered chemical fixative, such as glutaraldehyde, sometimes in combination with formaldehyde [2][3][4] and other fixatives, [5] and optionally followed by post-fixation with osmium tetroxide[2]. The fixed tissue is then dehydrated. Because air-drying causes collapse and shrinkage, this is commonly achieved by replacement of water in the cells with organic solvents such as ethanol or acetone, and replacement of these solvents in turn with a transitional fluid such as liquid carbon dioxide by *critical point drying*. Finally, the carbon dioxide is removed while the sample remains in a supercritical state, so that no gas–liquid interface is present during

drying. The dry specimen is usually mounted on a specimen stub using an adhesive such as epoxy resin or electrically conductive double-sided adhesive tape, and *sputter-coated* with gold or gold/palladium alloy before examination in the microscope.

Fixation for Scanning Electron Microscope:

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. 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 estimated 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 the 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.

For Ultraviolet microscopes, in order to observe biological samples (i.e. cells) we have to somehow dye the samples. The procedure is called Immunocytochemistry (ICC).

Fixation Protocol

1. Rinsing with PBS pH 7.4 (phosphate buffer saline)
2. Adding PFA (paraformaldehyde) 4%
3. PBS
4. PBS – Triton (0.1%) for 5-10 min. The triton added is a detergent which creates porous in the cytoskeleton in order for the antibodies pass into the cytoplasm and attach to the proteins.
5. PBS
6. PBS – BSA 2% for a half hour.
7. Now we add the first antibody in PBS-BSA 1% for 1h. *Phalloidin is a toxin that belongs to a class of toxins called phalotoxins, which are found in the death cap mushroom. Phalloidin is binding to the F-actin in the cytoskeleton and it can provide information on the morphology and the borders of the cells. Using phalloidin allows to observe actin filaments of the fixed cells with a red fluorescent colour.*
8. Last rinsing with PBS

9. If we want to count nucleus rather than observe the cytoskeleton only, we can transfer our sample to a microscope slide glass and we carefully add a coverslip sealing solution of the last antibody, DAPI and we cover it with a coverslip slide. We can also wait for DAPI to act for 1-5 minutes at room temperature in a dark place. We make sure than no light coming to the sample. *DAPI is a fluorescent stain that binds strongly to A-T rich regions in DNA. As DAPI passes through the cell membrane, it can be used to determine both the viability and the position of the cell.*
10. Fluorescence images can be visualized with a fluorescence microscope. We make sure to use the correct filter for visualizing fluorescent – labelled cells.

5.3 Observation/Results:

SW10 cell line cultivation

We cultivated the SW10 cells for 4 days at 33°C and with a concentration of $0.5x10^5$ cells/ml. Then we studied the morphology of cells using scanning electron and fluorescence microscopy. In figure 16 the 3D cell culture is presented in which we can see that the material we used is compatible with the SW10 cell line.

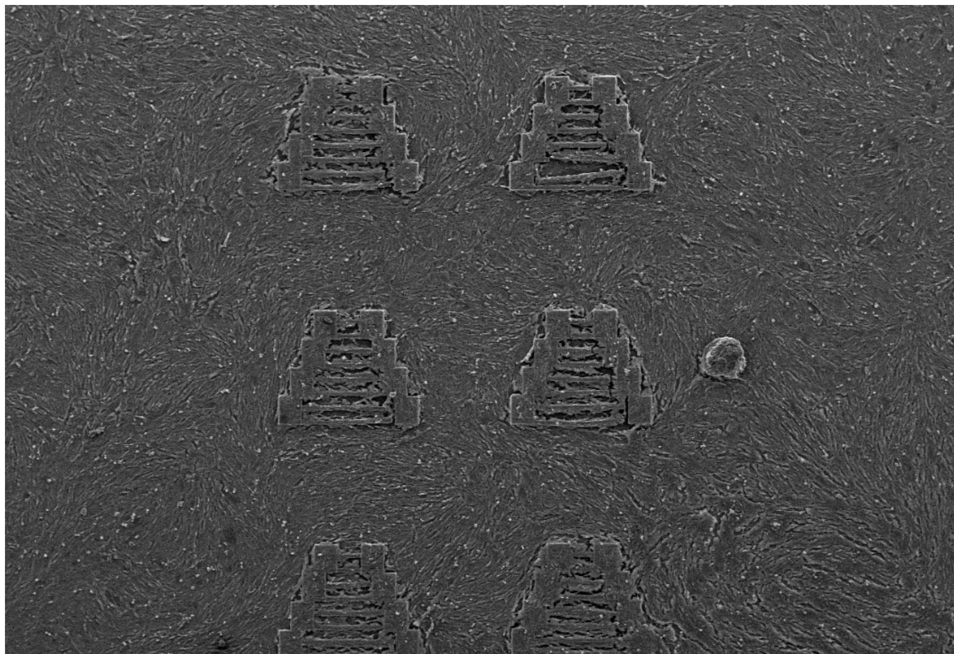


Figure 15. *SEM image of the 3D SW10 cell culture.*

High magnification SEM images showed that glial like cells (SW10) can migrate, proliferate and form the characteristic glial carpet on the guidelines of our 3D scaffold.

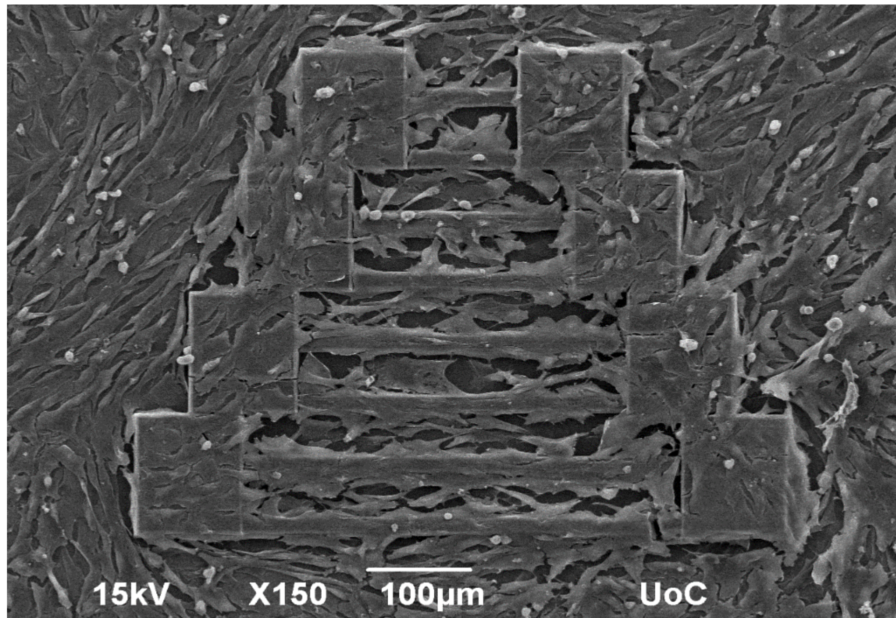


***Figure 16**. SEM magnified image of the 3D SW10 cell culture.*

In figure 17 we present a magnified image in which we can notice that the SW10 cells are wrapped around the guideline in an oriented pattern.

Figure (a)

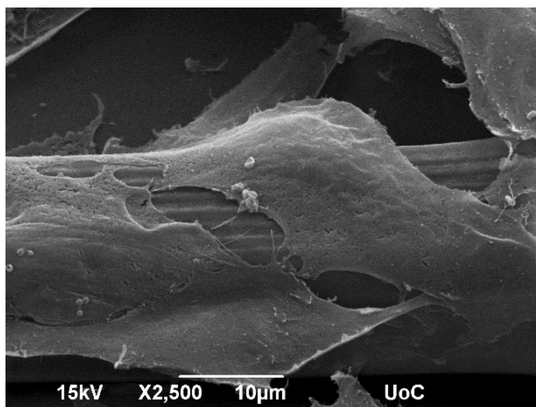


Figure (b)

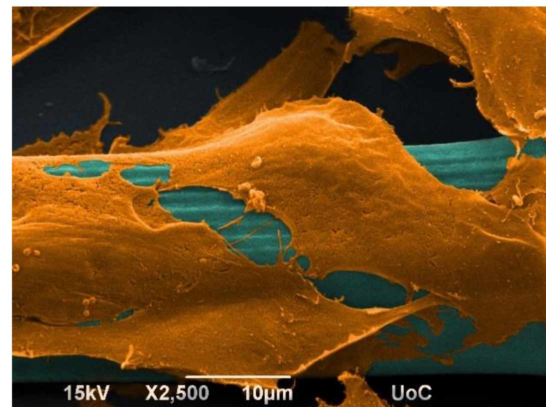


***Figure 17.** High magnification SEM image (a) from a 3D scaffold with SW10 cells and the same image (b) with pseudo-colors using Photoshop.*

Using fluorescent dyes, under a UV fluorescence microscope the cytoskeleton (stained red) and the nucleus (stained blue) can be observed.

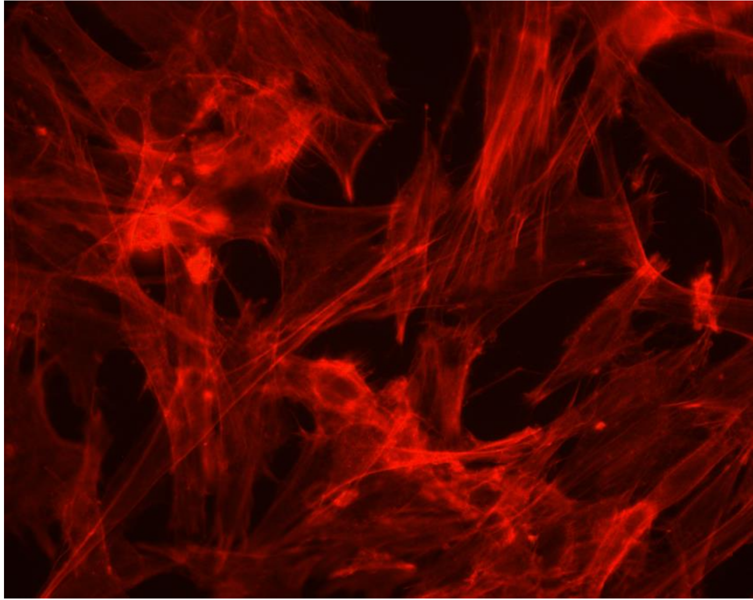


Figure 18. *SW10 cells cultured on cover slip and stained with phalloidin*

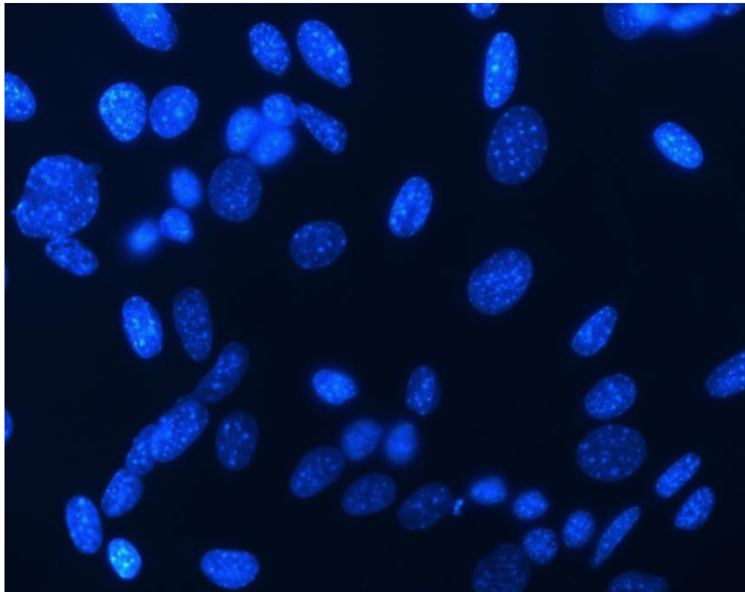


Figure 19. *SW10 cells cultured on cover slip and stained with DAPI*

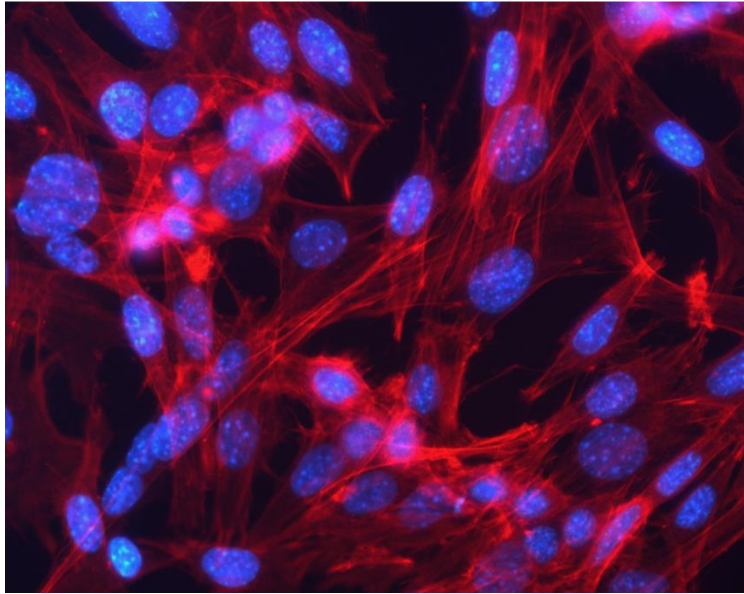


Figure 20. *An overlay image from the two previous images.*

Moreover, we observed our 3D cell culture under the fluorescence microscope. The 3D structure strongly fluoresces at any UV filter due to the photoinitiator we used. As it is shown at the SEM images, the SW10 cells were wrapped around the guidelines forming the characteristic glial like carpet.

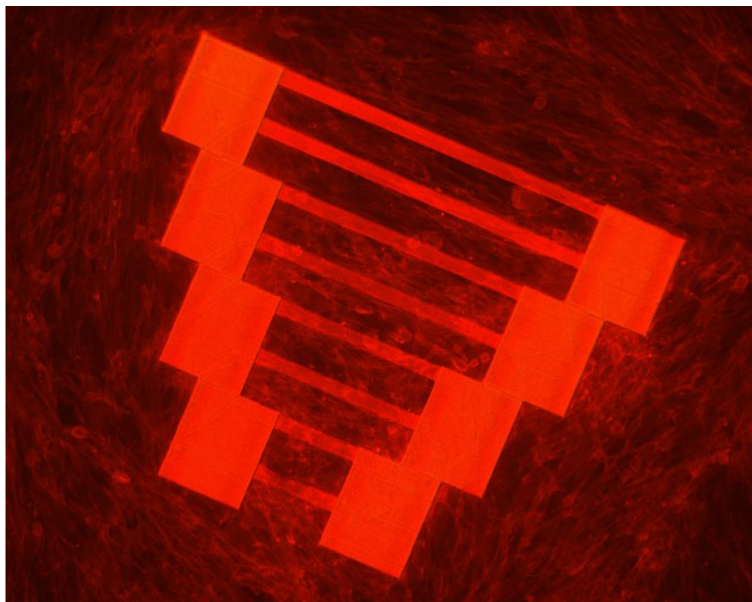


Figure 21. *An image from a 3D SW10 cell culture with emphasis on the actin filaments of the cells.*

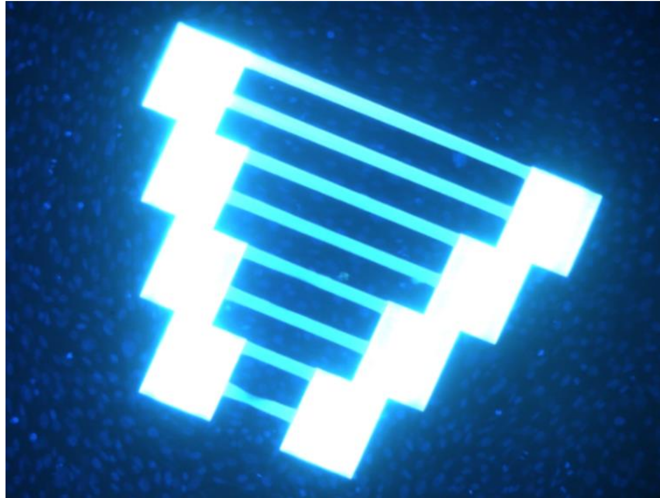


Figure 22. *An image from a 3D SW10 cell culture with emphasis on the nuclei of the cells.*

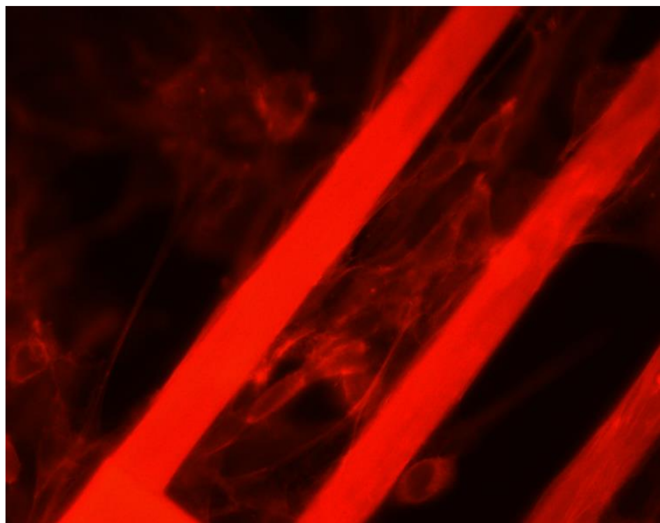


Figure 23. *A magnified image from a 3D SW10 cell culture with emphasis on the actin filaments of the cells.*

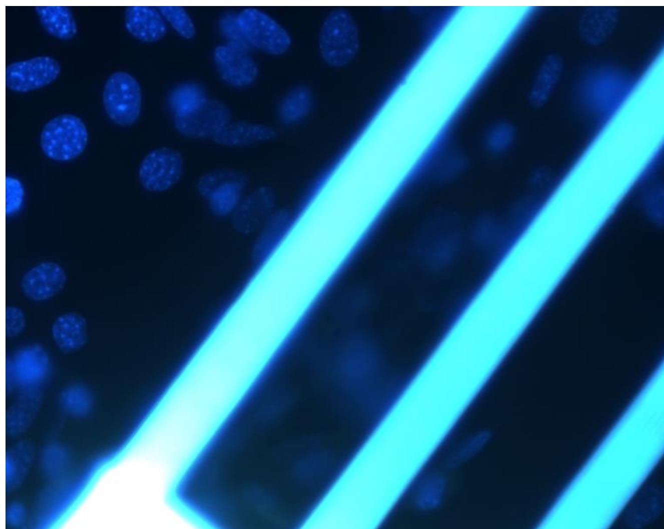


Figure 24. *A magnified image from a 3D SW10 cell culture with emphasis on the nuclei of the cells.*

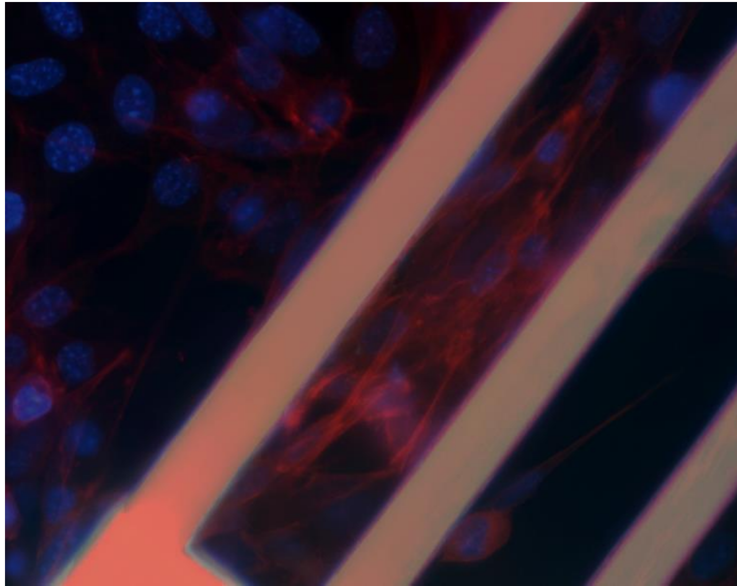


***Figure 25**. A merged image from the two previous images shows the existence of the cells around the guidelines.*

N2A cell line cultivation

We studied the morphology of N2A cells after 3 days of cultivation using scanning electron microscopy. In figure 26, a N2A cell cultivation on a cover slip is presented. High magnification images (figures 26, 27 and 28) revealed that N2A can grow and proliferate upon and along the 3D structures. Furthermore there is an axonal grow along the guidelines.

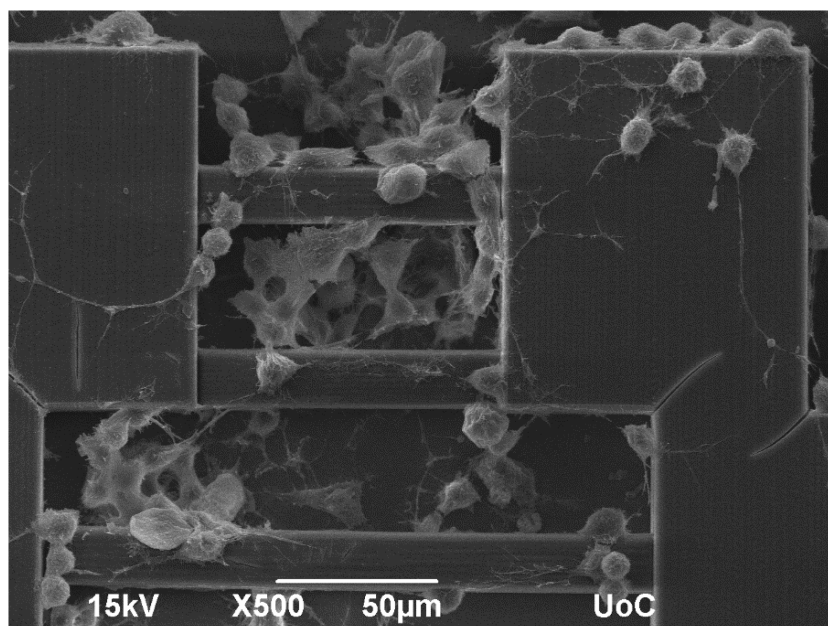


***Figure 26.** An image from SEM showing the migration and proliferation of the N2A cells upon and around the scaffold.*

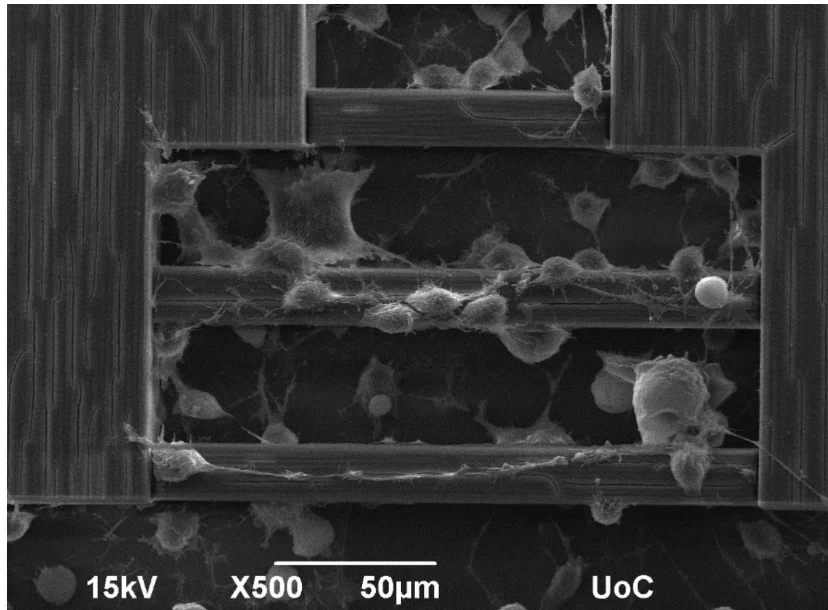


Figure 27. *An image from SEM showing axonal growth of a N2A cell along a structure's guideline.*

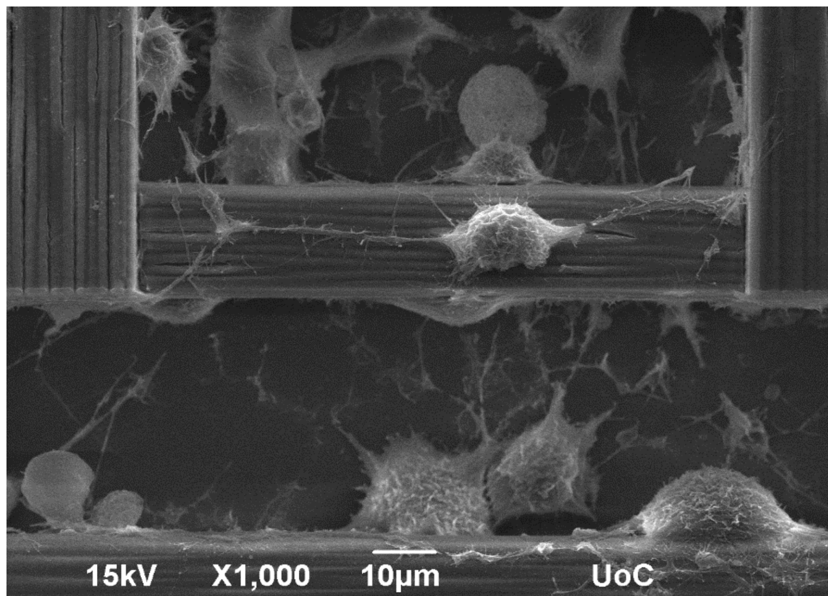


Figure 28. *An image from SEM showing another type of axonal growth of a N2A cell along a structure's guideline.*

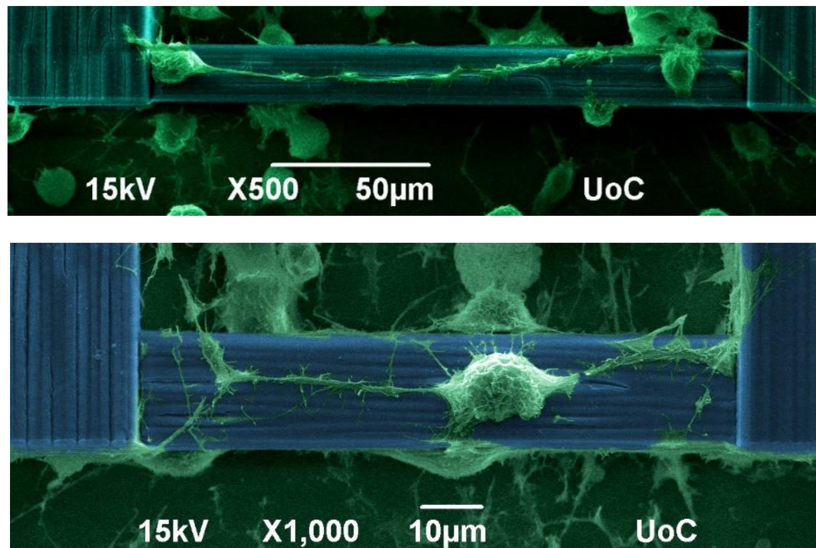


Figure 29. *Details of the previous images 26 and 28 with pseudo-color using Photoshop. We can easily see the axonal growth of the cells.*

5.4 Results and Discussion

The main objective of the present study focuses on critical challenge met in the interdisciplinary field of tissue engineering, namely the development of an experimental model for neurogenesis studies. Toward this aim, the fabrication of 3D scaffolds with different geometrical characteristics using a given photostructurable material, was carried out and the response of SW10 and N2A cells on these scaffolds was assessed.

As a structure design, suspended guidewires of different length were used which were constructed between two parallel rectangular blocks in order to study the potential directed growth of neuronal cells. The fabrication technique used was 2PP and the 3D scaffolds were made using a hybrid organic-inorganic photostructurable material. The scaffold fabrication was successful and the 3D constructs exhibited a high degree of accuracy and resolution. In addition, the high-reproducibility of the fabrication technique is very promising for its broader use in tissue engineering applications.

The cells culture studies and the cell viability and proliferation revealed that the structuring material is biocompatible for neural and glial cell cultivation. These results are in accordance with previous studies concerning fibroblast cell cultivation on the same hybrid organic-inorganic material [8]. That is to say, the cells were shown to migrate, adhere and proliferate on the laser fabricated bridge-bearing structures. In particular, the SEM and fluorescence microscopy images collected from the SW10 cell line cultivation showed a well oriented glial-like carpet formed along every guideline of

the scaffolds (Fig. 17, 21 and 22). In addition, it was shown that the SW10 cells can be wrapped around the guideline exhibiting a well oriented pattern (Fig. 18, 23-25). Immunofluorescence experiments confirmed the adhesion of SW10 cells and the glial carpet formation. However, it should be mentioned that the 2PP fabricated structures fluoresce strongly under the UV microscope due to the photoinitiator used for the structuring of the hybrid material.

As far as the N2A cells are concerned, it was shown that they can be grown and proliferate successfully upon and along the 3D structures (Fig. 26-28). Furthermore, their neurite extensions were also observed by SEM and immunofluorescence analysis. This conclusion is supported by the experimental observation that the length of the neurite depends on the guideline length (Fig. 28). In particular, the longer the guideline, the longer the neurite body.

The above experimental results are encouraging for the fabrication of an experimental model by 2PP for neurogenesis studies via the 3D co-cultivation of SW10 and N2A cell lines. The ultimate goal would be the examination of the cell lines interaction under co-cultivation conditions and the creation of a neural network along the guidelines of the 3D structure. Such an experimental model would also allow studying the neural network functionality via the myelination and demyelination process.

References Chapter 5

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