ENGINEERED BASAL LAMINA EQUIVALENT - FABRICATION AND BIOMEDICAL APPLICATIONS

by

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ABSTRACT

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APPLICATIONS

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A novel technique based on the principle of solvent casting is developed to create uniform, ultrathin and stackable films of Collagen I/IV and ECMgel for use as Engineered Basal Lamina Equivalent. The mixture of these two proteins provides key molecular elements (laminin, collagen type IV, entactin and perlecan) in a basal lamina with strong mechanical properties (collagen type I). Rat tail derived collagen solution is mixed with ECMgel, and the solution is cast onto PDMS molds and air dried in a custom built air tunnel. The dried membranes are briefly UV cross-linked, peeled off from the PDMS mold to be used for its various applications. Thickness of the membrane can be controlled by the amount and concentration of the protein mixture (0.5 - 1 μm) and is very uniform for the entire membrane. The basal lamina membrane has been positively stained for both collagen type I and laminin.

In 2D applications, six different types of cells have been cultured: human aortic endothelial cells, human glioblastoma multiforme, human fibroblasts, human astrocytes, human

smooth muscle cells and E-18 derived rat cortical neurons. All these cell types demonstrated a robust growth on the membrane with a positive staining to specific antibodies corresponding to each cell type.

As 2D applications do not entirely resemble the true native environment, the basal lamina membrane has been used for developing 3D applications. In one application a novel technique has been developed to create a 3D stack of cortical neurons resembling the cortical layers in the mammalian brain. E18 derived rat cortical neurons are cultured on 3 separate layers of the basal lamina membrane and then stacked together allowing the axons from one layer to migrate to the adjacent layers forming a complex network of dense axons in 3 dimensions as seen in the mammalian cortex. This 3D model has been used to study the migration of glioblastoma cells in which an additional layer of human glioblastoma multiforme cells is added onto the cortical neuron stack and the cells are allowed to migrate to the lower layers for specific time points. This application has also been developed to create a blood brain barrier model where endothelial cells are cultured on the basal lamina membrane and stacked above the layers of cortical neurons. This technique provides a unique and easy to fabricate 3D cell culture model which brings us closer to the native environment and can be used to study the interactions between different cell types. Using this technique the cells in each individual layer of the stack can also be easily controlled. The second application developed using the basal lamina equivalent is of a gas exchanger. The permeability of the membrane is controlled by using higher concentrations of collagen type I (6 mg/ml) because of which the membrane is impermeable to water but highly permeable to oxygen. The exchange of oxygen is allowed to take place across the membrane with water running on one side and oxygen on the other. The significant change ($p < 0.01$) in the concentration of dissolved oxygen in the water was measured before and after running the experiment using a dissolved oxygen sensing meter. The membrane being ultrathin brings the distance of exchange of oxygen closer to the native environment.

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CHAPTER 1

INTRODUCTION

1.1 Overall Objective

The primary aim of this project is to fabricate and characterize a basal lamina equivalent membrane to be used for developing various biomedical applications such as cell culture using the membrane as a substrate, for developing a multi-layered 3D culture model and for fabricating an artificial gas exchanger thus bringing the *in vitro* tissue and cell culture environment closer to the *in vivo* conditions.

1.2 Overview of Research

The entire project describes the fabrication, characterization and applications of the basal lamina equivalent using collagen type I and ECM gel. The background and literature review along with the significance of the project are described in chapter 2. The literature study reviews the techniques used to resemble and mimic the interactions between cells and the ECM environment *in vivo.* Further the various 3 dimension cell culture models using ECM proteins are described. The oxygenator models used at present for efficient exchange of oxygen are also reviewed. The various 2 dimension cell culture techniques developed using the basal lamina equivalents are explained in chapter 3. The use of the basal lamina equivalent as a cell culture substrate for various cell types is also described in chapter 3. The 3D applications using the basal lamina equivalent are described in chapter 4. The 3D cell culture model developed for studying glioblastoma multiforme migration is explained in this chapter. The gas exchanger application is also described in chapter 4. The final chapter describes briefly the ongoing and future applications using the basal lamina equivalent where the membrane is being used to study the invasive properties of renal cancer cells. The schematic of the entire overview of the project is as shown below in Figures 1.1 – 1.3.

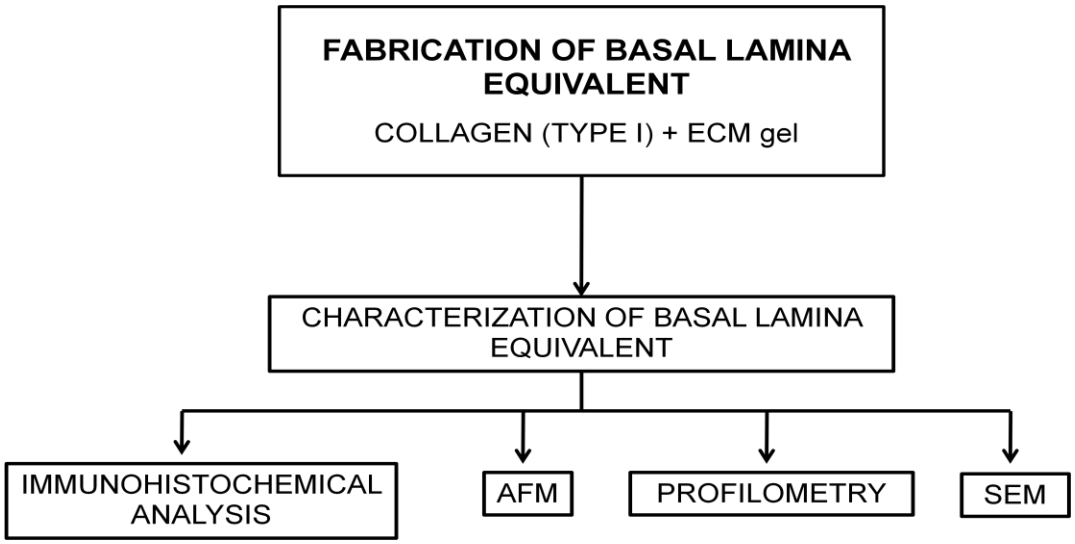

Figure 1.1 Schematic of the fabrication and characterization of the Basal Lamina Equivalent

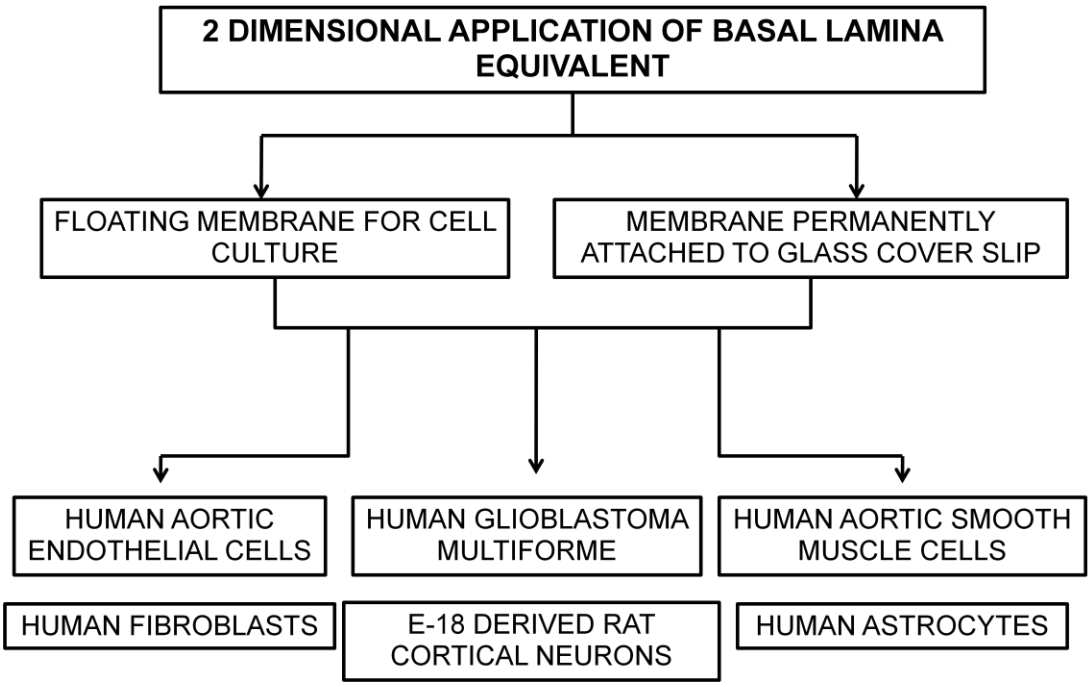

Figure 1.2 Schematic of the 2D applications using the Basal Lamina Equivalent

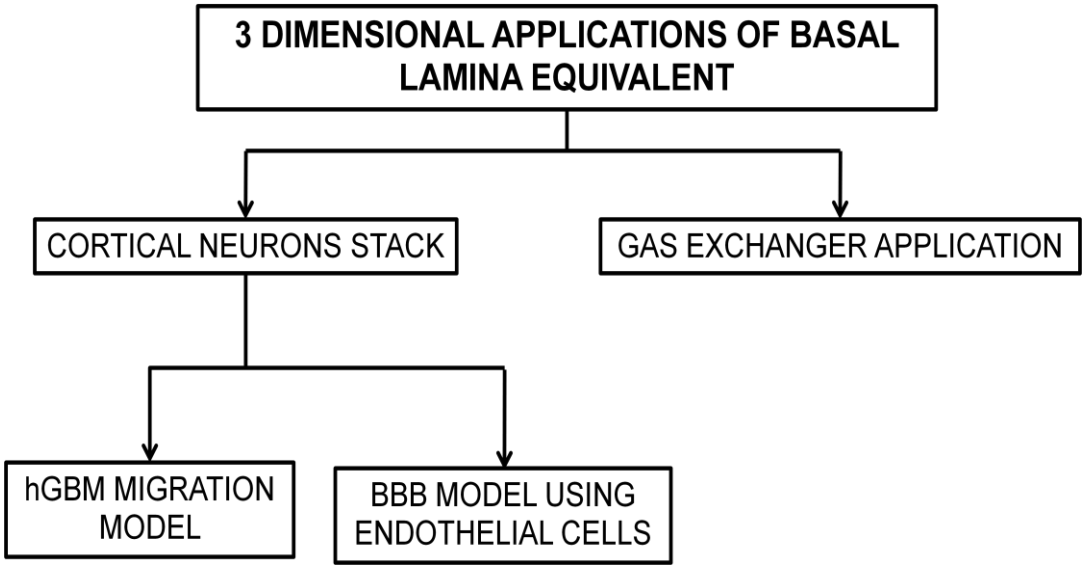

Figure 1.3 Schematic of the 3D applications of the Basal Lamina Equivalent

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

2.1 Introduction

Engineering as a whole aims at creating devices, techniques or systems which assist or improve a way of life. Tissue engineering explicitly aims at creating implantable organs and tissues to regenerate injured or damaged parts. The *in vivo* environment is highly complex and understanding all the processes for the improvement of human health is very challenging. This has lead to the development of *in vitro* techniques and models which simplify the complexity present *in vivo* and help in a better understanding of the in native environment and tissue and organ functioning. The human body is so highly complex that no *in vitro* system can come close to mimicking the true native environment; hence the need to constantly improve the currently available *in vitro* techniques or models always exists.

2.2 Basal Lamina – Structure and Function

The extracellular matrix has several different structural forms; however, the two predominant forms comprise the connective tissue matrix and the basement membrane. The connective tissue matrix is a porous and fibrillar structure surrounding fibroblasts and other cells present (1). The basement membrane has several names like basal lamina, basement lamina (2). It is present throughout the body in association with cells and tissues and the basal lamina and its related structures are collectively called the basement membrane (3). It can be described as a dense, amorphous thin sheet like structure, of a thickness in the range of 50-100 nm observed and identified by transmission electron microscopy (4, 5). The composition of the basal lamina can be divided as round about 90% protein, 8% carbohydrate, and 2% lipids (2). The four primary components are collagen (IV), laminin, entactin and heparin sulphate proteoglycan

(perlecan) (6). The collagen type IV is a heterotrimeric molecule with two α 1 like chains and one α 2 chain where the three α polypeptide chains form a triple helical structure. The molecular weight of collagen is 550-600 kDa (5, 7). Laminin is the most abundant non-collagenous protein found in the basal lamina with a heterotrimeric fork like structure of α, β and Ɣ chains. The α chain has an approximate molecular weight of 400 kDa and a length of 160 nm. The β and Ɣ chains have a molecular weight of around 200 kDa and form the shorter arms of the laminin fork like structure with a length of 60 and 40 nm respectively (8). Nidogen also known as entactin has three globular domains in its core protein composition and makes up 2-3% of the protein composition of the basal lamina. The globular domains are separated by two rod like structures and this protein has binding sites for collagen, laminin and perlecan. Perlecan is a heparin sulphate proteoglycan with a core molecular size of 400-450 kDa. It has five distinct domains and several binding sites for collagen, entactin, integrins and heparin (8, 9). The collagen and laminin are the only basal lamina components known to self assemble into structures and are important for the stability of the basal lamina membrane. The perlecan and entactin bridge the network formed by laminin and collagen improving the stability of the basal lamina (8). Earlier it was thought that collagen forms the scaffold on which the other basal lamina components assemble, however newer studies have shown that laminin assembles first forming the template during the initial assembly of the basal lamina (9, 10). Laminin is now known to spontaneously self assemble into polygonal lattices by calcium dependent interactions with all the three (α, β, Ɣ) short arms *in vivo,* along with the long arm being attached to cell surface receptors. The collagen is also known to self assemble to form networks however interaction with cell receptors has not been reported (10, 11).

2.3 Cell – ECM Interactions

The basal lamina is a part of the extracellular matrix, which provides support to the surrounding tissues and cells along with acting as a medium of communication between cells and also allowing the transit of various growth factors. It has a large variety of biological

functions ranging from providing structural stability to cells, to tissue organization, cell differentiation to name a few (12, 8, 13). It separates the monolayers of cells from the underlying tissues. It is present underlying the epithelial and endothelial cell monolayers and also found surrounding the nerve, fat and muscle cells (14). The formation of the basal lamina is essential for the normal growth, development and function of tissues (15).

The development of the basal lamina is known to proceed in a step wise manner depending on receptor-ligand interaction which provides information essential for its assembly and functioning as shown in Figure 2.1 (1). It is known to contact with the cells by binding with the cell membrane receptors such as integrins which bind to laminin present in it (16). Integrins and dystroglycan are two transmembrane receptors for the extracellular laminin. Studies have shown that both these receptors are essential for the correct assembly of the basal lamina. These receptors bind to the long arm of the laminin molecules of the basal lamina and connect the extracellular matrix to the actin present in the cytoskeleton of the cells (1, 17, 18). This rearranges and regulates the cytoskeleton of the cell and hence is essential in defining the cellular polarity, cell adhesion and migration (19). The integrins receptors are the primary players in the attachment of cells to the basal lamina (20). The main adhesion receptor is the α6β4 integrin receptor which binds to laminin. The controls and maintains the mechanical structure of the epidermis. Along with this the polarity of the cells is highly affected by their attachment to the basal lamina. A good example of cellular polarity would be of the epithelial cells. The attachment of these cells to the basal lamina determines the apical and basal axis of these cells (21).

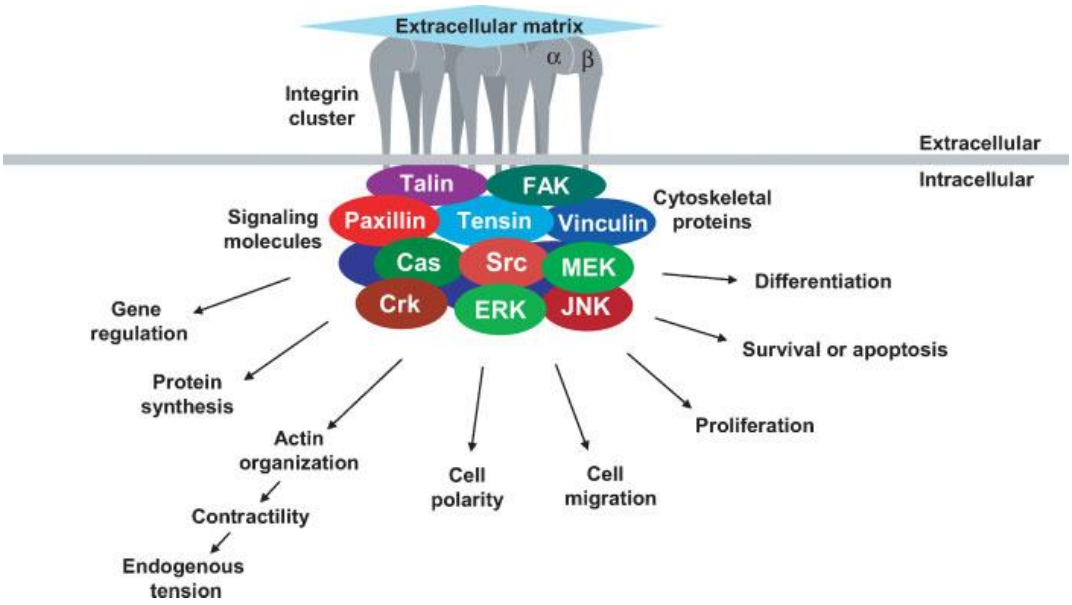

Figure 2.1 Interaction of the extracellular matrix with the integrin receptors (20)

2.4 Current Techniques using Basal Lamina Analogs *in vitro*

The role of the basal lamina in cell attachment, tissue organization, providing structural support to cells, providing a pathway for growth factors has lead to the development of various analogous models of a fabricated basal lamina. Micro fabrication techniques have been previously used to create a basal lamina like structure with controlled topographic features to understand the influence of the topography of the basal lamina on the cells it supports (22). Studies have been done using artificial basement membrane gel made from soluble ECM for studying the differentiation of bone marrow cells (23). A three dimensional reconstruction of the basal lamina has been done in a raft culture using a three dimensional collagen gel matrix. This reconstruction has been used for studying the migration of keratinocytes for a reconstructed skin equivalent (24).

2.5 Cell Culture in 3 Dimensions

The ultimate aim of tissue engineering is to create a 3-dimensional environment using a combination of cells, scaffolds or devices which aid in cell growth and differentiation just to short list a few (25). Although animal models serve as models which are close to the conditions

present in human beings, the complications involved give rise to the need to *in vitro* 3 dimensional models using cells and other extra cellular matrix components. This is the reason behind an increasing need for *in vitro* models which are closer to the *in vivo* environment than that which is provided by the conventional 2 dimensional culture techniques (25). The 2 dimensional cell culture systems like petri dishes, flasks, well plates etc. are indeed very convenient for use, have high cell viability and have helped tremendously in understanding basic cell biology there are several drawbacks in this type of a culture system (26). The 2 dimensional cell culture systems drastically lack the *in vivo* architecture and cause a dramatic adaption by the cells in order to survive on the rigid substrates. The cells develop phenotypes which are very different from the actual cells present *in vivo* (27). These adaptations by the cells alter their original metabolism and also reduce functionality. This leads to a misinterpretation of the results obtained as the cells up to a certain extent have been forced to survive and adapt to an environment different from the native one (26, 28, 29, 30). *In vivo*, the cells are surrounded and supported by a large number of ECM components which help in communication between cells with the help of the large number of cytokines and trophic factors which are secreted by the cells (31). The ECM acts as a medium of communication between the cells present in the body. This however is difficult to mimic in 2 dimensional cell cultures. Even though in the conventional cell culture methods, many of the growth factors, serum and other such components can be provided to the cells via the cell culture medium, the absence of the extra-cellular matrix and the proteins it contains along with water and ions which are continuously supplied to the cells *in vivo* cannot be fulfilled (32). In 2 dimensional monolayers of cell culture, many of the chemical and physical cues which define the identity of the cells *in vivo* are absent. This way of culturing of cells on 2D surfaces does allow the cells to grow or communicate with the neighboring cells in the natural way. Recently, it has been shown that cells behave in an unnatural way when they are grown in 2D monolayers after been taken from their natural 3 dimensional tissue environment (33). Even though the 3D culture systems cannot entirely

mimic or represent the in vivo environment, a great amount of effort is being put into engineering materials, scaffolds and devices for 3D cell culture aiming at providing a spatial environment for cell culture closer to the native *in vivo* environment and to overcome some limitations present in the 2D culture systems (31).

2.6 Current 3 Dimensional Cell Culture Techniques

Since the 1970s researchers were trying to understand the differences between cells cultured on flat surfaces and those on 3 dimensional systems. New systems or cell culture models are being continuously developed and are now available commercially (34). The advantage 3D systems provide over 2D culture is the extracellular adhesions all over the surface of the cells versus the adhesions present only on the flat surface in 2 dimensions (35). The most popular 3D cell culture systems available commercially are the filter well inserts, sponges and gels and the microcarriers. The filter well inserts are micro porous filter membranes which can be inserted into a culture dish or well plate and can be used to culture cells on either sides of the membrane. The filters are made with 4 different polymer membranes and the type of membrane selected for making the filter depends on the application. These filters are used to study cell polarity for epithelial cells and also study cell migration. Another commonly used technique for cell culture is gels and sponges made from purified ECM components (36). The most commonly used proteins are collagen, laminin and gelatin. There are several commercially available gels like Matrigel, Extracel and Algimatrix. These are useful in studying the invasion and migration properties of tumor cells (34, 37). Another option for 3D substrate is small spherical microspheres which are coated on plates and flasks as substrates. Any number of different proteins can be coated as microcarriers on 2D cell culture systems. Beyond these commercially available products for 3D cell culture there are several other models or systems which are being continuously developed to bring *in vitro* culture as close as possible to the native conditions. Among the many developed and used 3D scaffolds or devices for cell culture, hydrogels having cross-linked networks and have a high water content along with being

porous have known to show a high efficacy as matrices for 3D cell culture (33). Also, 3D models using neurons and astrocytes with the help of a bioactive scaffold containing ECM proteins and proteoglycans have been engineered and have been observed to show electrophysiological activity of the neurons. These constructs have been said be a better way for measuring neuron activity than the traditional 2D cell culture (38). Studies have also shown that fibroblasts when cultured in 3D collagen matrices experience a completely different environment than that experienced in the 2D culture. The adhesion domains, cell-matrix integrin dependent interactions present in 3D models is different from that present in 2D affecting the migration, signaling and plasticity of the fibroblasts cultured (39).

2.7 Oxygen Exchange – Functioning and Advances

The lungs are the responsible for the exchange of carbon dioxide and oxygen from the blood to the surrounding atmosphere. The lungs comprise of a large area of branched network of air sacs called alveoli which are densely surrounded with blood capillaries. The blood capillaries are extremely thin allowing only one red blood cell to cross the capillary at one time for exchange of oxygen and carbon dioxide. The total surface area across which the exchange of gases takes place is around 70-80 m^2 and the distance or thickness across which the exchange takes place is 1 micron (40, 41). In just the United States, more than 35 million people suffer from chronic lung disease. The best solution for any lung disease is the lung transplant, however due to the severe shortage of donors patients die on the waiting for the organ donor. Hence the need for developing better artificial gas exchange models is always increasing to provide a substitute to patients till a lung transplant can be done (42, 13). At present artificial lungs are nowhere close to the native lungs as far as functioning is concerned. However they try to maximize mimicking the efficiency of the natural lung with the exchange of gases. The natural lungs have the capacity to exchange oxygen and carbon dioxide at a rate of 2-6 L/min; however the artificial gas exchangers at present have a maximum efficiency of up to 250-400 mL/min (42, 43). The surface area to blood volume ratio and the small distance for gas

exchange make the native lung very efficient (44). Three different types of oxygenators have been developed till date for exchange of gases. These are the film type oxygenators in which gas exchange takes place over a thin film of blood and hence due to the large surface area required, a large priming volume is needed. The second type is the bubble oxygenators in which the oxygen is directly bubbled into the lungs. This technique provides a high surface area as the bubbles are in direct contact of the blood however the trauma is very intense due to the mechanical stress exerted by the bubbles on the blood. The third and most commonly used technique is that of membrane oxygenators in which the exchange of oxygen and carbon dioxide takes place across a semi-permeable membrane (40, 45). Table 2.1 shows the comparison of the gas exchange distance across different membranes in different studies measured in microns.

Table 2.1 Comparison of Gas Exchange Distance with different membrane types

Reference	**Gas Diffusion Distance (microns)**
Human Lung (43)	1-2 microns
Kolobow (46)	100 microns
Burgess (44)	64 microns
Potkay (42)	15 microns
Basal Lamina Equivalent (current study)	1-2 microns

CHAPTER 3

FABRICATION, CHARACTERIZATION AND 2D APPLICATIONS OF THE BASAL LAMINA EQUIVALENT

3.1 Introduction

The fabrication of the basal lamina equivalent from rat tail derived collagen and ECM gel has been explained in this chapter. The characterization studies are performed to measure the thickness, surface topography, and nano structure of the membrane are also explained. After fabricating the membrane, it was used as a substrate for culturing different cell types. Two different techniques developed for using the membrane as a substrate for cell culture have been explained in this chapter along with the different cell culture studies done.

3.2 Fabrication of the Basal Lamina Membrane

The basal lamina membrane was prepared from a mixture of collagen type I and ECM gel. The collagen was obtained from rat tails. The collagen was extracted from rat tails of rats preferably 4-6 months old. A previously optimized protocol was followed to obtain sterile collagen. The ECM gel was purchased from Sigma. The collagen obtained from rat tails was stored at -80^{o}C in the lyophilized form. For making the basal lamina membrane, the lyophilized collagen was dissolved in 0.02N acetic acid (Sigma) to obtain a final concentration of 3 mg/ml. The concentration of ECM gel was 8-12 mg/ml. The concentration of the collagen was optimized to 3 mg/ml. 20 µl of ECM gel was mixed in 1 ml of collagen (I/IV). The vial in which the gel was prepared had to be pre-cooled to prevent denaturation of the proteins. The gel was stored in 4˚C until used to prepare membranes. To prepare basal lamina membranes, the gel (quantity depending on the application which the membrane was being used for) was added on a PDMS mold as shown in Figure 3.1. The PDMS mold had a height of 100 microns to enable

easy peeling of the dried membrane. The gel was spread on the mold with the help of a pipette tip and placed in a custom built air-flow tunnel. The pressure of the air flowing through the tunnel was kept at 1.8 SCFH of air, allowing the membrane to dry in approximately two hours. The membrane after being completely dried was exposed to UV light of wavelength 350 nm for 25 minutes to cross link the proteins. The membrane was not peeled off for approximately 60 minutes after exposing to the UV light allowing it to cool down. The membrane after 60 minutes was gently peeled off from the PDMS mold using a pair of forceps. The peeled off basal lamina membrane was completely fabricated to be used for various applications. The membrane had to be sterilized by placing in UV light in the bio-safety cabinet for 30 minutes if used for cell culture purpose.

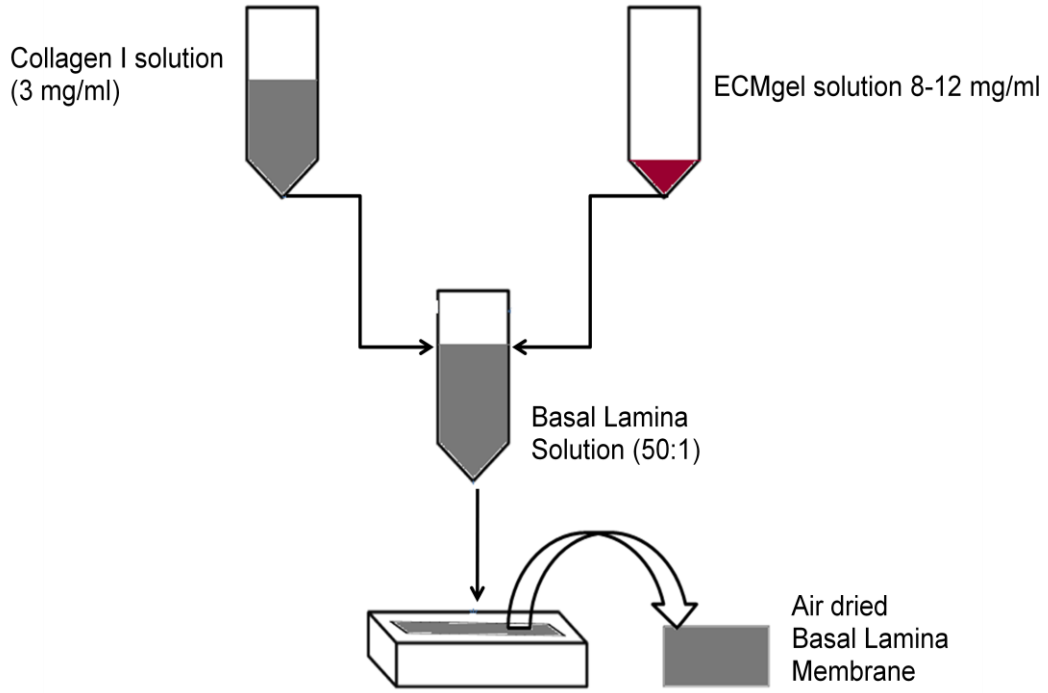

Figure 3.1 Schematic of the Fabrication of basal lamina equivalent

3.3 Characterization of the membrane

After fabricating the basal lamina membrane, it was stained with antibodies specific to collagen and laminin confirm the presence of proteins after drying it. The thickness of the

membrane was measured by profilometry. The surface topography was measured by scanning electron microscopy and the nano texture of the membrane was measured by atomic force microscopy. The samples were prepared in the same manner for all the characterization studies. The membrane was peeled off from the PDMS mold after UV cross-linking. This membrane was placed in a 60 mm petri dish with a glass cover slip (22X22 mm) placed in it. The dish containing the membrane was filled with PBS for 60 seconds to neutralize the acetic acid from the collagen. The PBS was drained out from the dish and deionized water was added to the same petri dish to remove the salts present in PBS. The DI water was slowly drained from the petri dish after one minute, ensuring that the membrane was placed on the cover slip. The membrane was made to stick on the cover slip by drying it with nitrogen gas. The nitrogen gas was gently blown over the entire length of the membrane sticking it permanently to the cover slip.

3.3.1 Immunohistochemical Analysis

The basal lamina membrane after air drying and attaching on the glass cover slip as mentioned earlier, was fixed with 4% paraformaldehyde in PBS and double immunostained for laminin (RbIgG, 1:500, Sigma) and collagen type I (mIgG1, 1:200, Sigma). Secondary antibodies were goat anti-RbIgG Dylight 488 (1:220, Jackson ImmunoResearch) for laminin and goat anti-mIgG1 Dylight 594 for collagen type I.

3.3.2 Profilometry

The thickness of the basal lamina membrane was measured by the Alpha Step IQ profilometer provided by the Nanofab Facility at The University of Texas at Arlington. This membrane attached onto the cover slip was used to measure the thickness of the membrane with the help of the profilometer.

3.3.3 Atomic Force Microscopy

The nano texture of the membrane was measured by the Dimension 500 Atomic Force Microscope in the Nanofab Facility of The University of Texas at Arlington. 6 samples were scanned to obtain an average of the nanostructure of the membrane.

3.3.4 Scanning Electron Microscopy

The surface topography of the membrane was observed with the Zeiss Supra 55VP scanning electron microscope (SEM, Carl Zeiss) in the Nanofab Facility of The University of Texas at Arlington. 3 samples were sputter coated with gold metal of a thickness of 15 nm and scanned at a magnification of 1000X to observe the surface topography.

3.4 2D Applications of the membrane

The membrane has been used in two different ways for culturing cells. In the first technique the membrane was attached on a cover slip as explained previously for the characterization studies. The cover slip was placed in the bio safety cabinet and exposed to UV light (wavelength 350-400 nm) for 30 minutes for sterilizing the membrane. The cover slips were transferred to sterile 35 mm petri dishes and incubated overnight in the cell culture medium at 37^{o}C and 5% CO_2. In the second technique developed, the membrane was peeled off from the PDMS mold, cross-linked with UV light and sterilized in the bio-safety cabinet for 30 minutes under UV light. The cell culture medium was added in a 60 mm petri dish and the membrane was held with a pair of forceps and gently placed in the medium. The membrane was kept floating in the medium without allowing it to curl up or fold. The petri dish was incubated overnight at 37^{o}C and 5% CO_2.

3.5 Cell Culture and Reagents

For cell culture applications, six different primary cells were cultured on the either the floating or attached basal lamina equivalent: human aortic endothelial cells (HAEC), human glioblastoma multiforme (hGBM), human aortic smooth muscle cells (HASMC), human fibroblasts, human astrocytes and E-18 derived rat cortical neurons. hGBM cells were cultured

in serum-free tumor medium (DMEM/F-12 supplemented with B-27 (Invitrogen), Insulin Transferrin Selenium-X (Invitrogen), gentamycin (50 µg/ml, Invitrogen), 20 ng/ml mouse epidermal growth factor and 20 ng/ml basic fibroblast growth factor. HAEC, HASMC, human fibroblast and astrocytes were cultured in DMEM/F-12 medium containing 10% fetal bovine serum. E-18 derived rat cortical neurons were cultured in Neurobasal medium supplemented with B-27, gentamycin, brain derived neurotrophic factor (10 ng/ml, Peprotech) and neutrotrophin-3 (10 ng/ml, Peprotech).

The cells growing on either floated or attached EBL were fixed with 4% paraformaldehyde in PBS and immunostained for following markers: EGFR (mIgG2b, Sigma, 1:500) and laminin (RbIgG, Sigma, 1:500) for hGBM cells; VWF (Von Willebrand factor, RbIgG, Santa cruz biotech, 1:200) for HAEC; Smooth muscle actin (mIgG2a, AbDSerotec, 1:200) for HASMC; Vimentin (mIgG1, Sigma, 1:500) for human fibroblasts; GFAP (RbIgG, Dako, 1:1000) for human astrocytes; and beta III tubulin (mIgG2b, Sigma, 1:500) for cortical neurons.

3.6 Results and Discussion

3.6.1 Fabrication of the Basal Lamina Equivalent

The basal lamina membrane was fabricated by the solvent casting technique. Figure 3.2 shows the entire fabrication process. The concentration of the rat tail derived collagen was optimized to 3 mg/ml. At this concentration, the air dried membranes were easily peeled off from the PDMS mold with a pair of forceps. For concentrations lower than 3 mg/ml, it was unable to peel of the membrane from the mold without tearing it. At this concentration of the collagen solution mixed with the ECM gel solution, ultrathin easily peelable basal lamina membranes were obtained. The PDMS mold used for the solvent casting technique was of the dimensions (1cm* 1.2cm). The size of the mold was varied according to the size of the membrane required for specific applications. The rectangular pattern on the mold was of a height of 100microns which enabled the air-dried membrane to be peeled off easily with a pair of forceps. Figure 3.2 (A) shows the PDMS mold with the rectangular pattern of 100 microns height. Figure 3.2 (B)

shows the basal lamina solution added on the mold with a pipette. The basal lamina solution was spread evenly on the mold with a pipette tip as shown in Figure 3.2 (C). The solution was air dried in a custom built air tunnel at a low pressure of 0.8 SCFH of air to obtain dried basal lamina membranes as seen in Figure 3.2 (D). The air dried membranes were exposed to UV light of the wavelength 350 nm for 25 minutes for cross-linking the proteins for better mechanical strength before peeling off from the mold. After the membrane was cross-linked, it was gently peeled off from the mold as shown in Figure 3.2 (E). The Figure 3.2 (F) shows the basal lamina membranes fabricated in 3 different sizes according to the applications in which they were used.

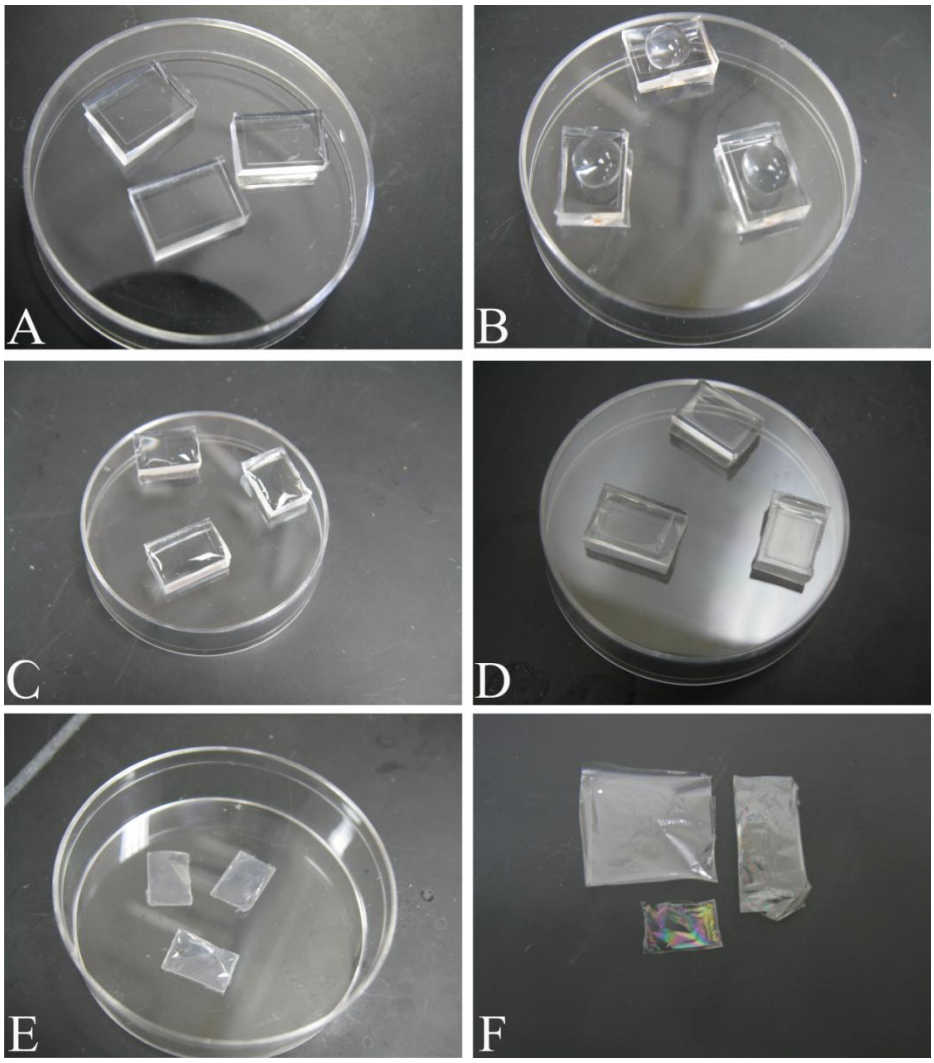

Figure 3.2 Fabrication Procedure of the Basal Lamina Membrane by solvent casting technique in different sizes based on its applications (A) PDMS mold for adding basal lamina solution (B) Basal Lamina solution added with pipette tip (C) Solution spread evenly on mold (D) Air – dried Basal Lamina Solution (E) UV cross-linked membrane (F) Basal Lamina membrane in different sizes based on application

3.6.2 Characterization Studies of the Membrane

The basal lamina membrane was stained with antibodies for laminin and collagen I after it was fixed with 4% para-formaldehyde. The membrane showed positive for the laminin and collagen I antibodies indicating that the proteins were not denatured by the air drying and the UV cross-linking. The Figure 3.3 (A) shows the immunohistochemical result of the basal lamina

membrane. The green color shows the staining for laminin and the red color shows the staining for collagen type I. The thickness of the membranes was measured by profilometry. 100 µl of the basal lamina solution was used to make the membranes which were measured by profilometry. Each sample was measured multiple times from different areas. The thickness of around 0.6 microns with a standard deviation of 0.2 microns was obtained for 6 samples scanned for profilometry as shown in Table 3.1.

Table 3.1 Profilometry of Basal Lamina Membrane

	Thickness (microns)
Average	**0.607**
Standard Deviation	**0.278**

Figure 3.3 (B) shows the profilometer graph for a basal lamina membrane sample. The nano texture of the membrane was measured by atomic force microscopy. The readings for the scanned samples are shown in table 3.2. 6 samples were scanned multiple times.

Table 3.2 Atomic Force Microscopy of the Basal Lamina Membrane

	Nano Texture (nm)
Average	**156.873**
Standard Error Mean	**42.585**

Figure 3.3 (C) shows the AFM reading for measuring the nano texture of the membrane between two random points on the membrane. The surface topography of the membrane was observed by the scanning electron microscope was shown in Figure 3.3 (D).

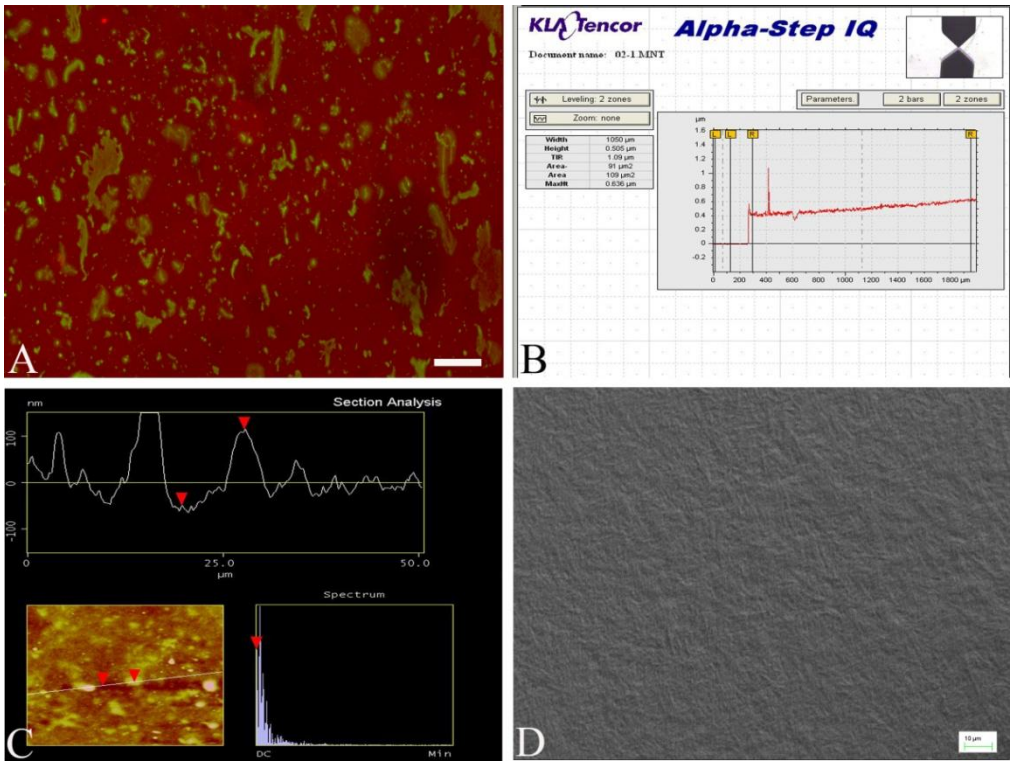

Figure 3.3 Characterization studies of the Basal Lamina Equivalent (A) Immunohistochemical analysis of Basal Lamina Equivalent (B) Profilometry for measuring thickness of membrane (C) Atomic force microscopy for surface nano texture of membrane (D) Surface Topography and uniformity visualized by SEM

3.6.3 2D Applications of the Basal Lamina Equivalent

The basal lamina membrane was used in two different ways for cell culture and 2D applications. Figure 3.4 (A) shows the basal lamina membrane premanently attached on the glass cover slip. This membrane was successfully sterilized and used as a substrate with no other surface treatment or modification for cell culture. Figure 3.4 (B) demonstrates the basal lamina membrane being used as a floating membrane for cell culture. The membrane was observed to float on the cell culture medium without any folding for deformation. The cells showed robust growth on the floating membrane implying that the membrane in spite of floating on the surface of the cell culture medium was semi permeable allowing the cells to receive

nutrition from the cell culture medium. This floating membrane was also further used for various 3 dimensional applications.

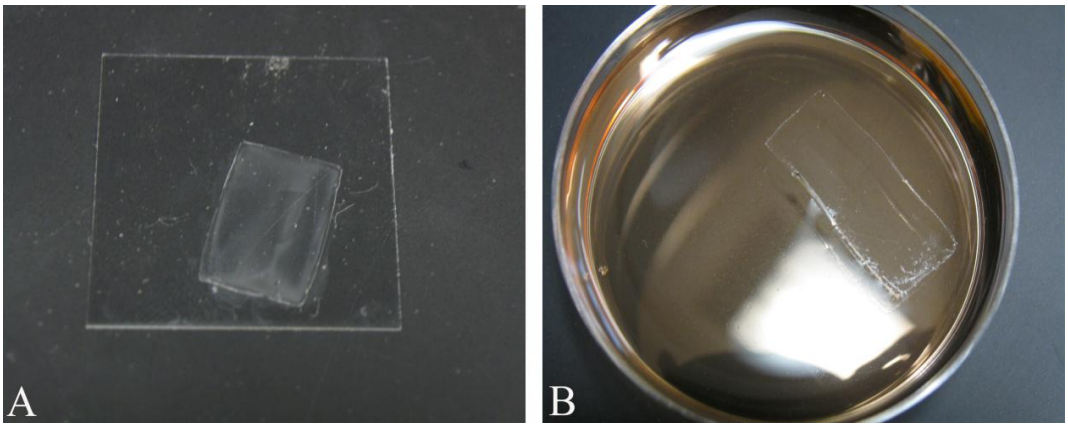

Figure 3.4 2D applications of the Basal Lamina equivalent. (A) Membrane permanently attached on glass cover slip. (B) Membrane floating on cell culture medium

3.6.4 2D Cell culture on the Basal Lamina Equivalent

After characterizing the membrane, it was used for cell culture either attached on the glass surface or in the floating form. 6 different types of cells were cultured on the membrane. The cells were observed to attach and proliferate well on the membrane. All the different types of cells showed robust growth on the membrane as seen from Figure 3.5. Figure 3.5 (A) shows the culture of E-18 derived rat cortical neurons. The cells have been stained for beta-3 tubulin (green). As seen from the figure the neurons attached and grew well on the basal lamina membrane. The figure shows the cell growth in 72 hours after the cells were seeded. The primary human glioblastoma cells (133P) were also cultured on the basal lamina membrane. These cells were found to attach and proliferate well on the membrane. The cells were fixed and stained for laminin (green) and epidermal Growth Factor Receptor (EGFR) (red) which is highly expressed in these cells as shown in Figure 3.5 (B). Human fibroblast cells were cultured on the basal lamina membrane attached to a glass cover slip. The cells were fixed and stained for vimentin (green) and nucleus (DAPI- blue) as observed in Figure 3.5 (C). The culture of human aortic endothelial cells also showed robust growth on the basal lamina membrane. The

cells were stained for Von Willebrand Factor (VWF) (red) and nucleus (DAPI – blue) as shown in Figure 3.5 (D). Human smooth muscle cells were cultured on the membrane attached on glass. The cells were fixed and stained for smooth muscle actin (green) and nucleus (DAPI-blue). The cells were observed to attach and grow well on the membrane as seen from Figure 3.5 (E). The human smooth muscle cells, human astrocytes and human fibroblasts were also cultured on the floating basal lamina membrane. The cells were fixed and the smooth muscle cells were stained for smooth muscle actin and nucleus (DAPI – blue) as seen in Figure 3.5 (F), the human astrocytes were stained for glial fibrillary acidic protein (GFAP) (green) as seen in Figure 3.5 (G) and the human fibroblasts were stained for Vimentin (green) and nucleus (DAPI – blue) as shown in Figure 3.5 (H). The cells when cultured on the floating membrane showed a robust growth similar to the growth seen on the membrane attached on glass. This showed that the membrane was semi-permeable allowing the cells to attach well and proliferate when floating in the medium and also receive nutrients from the cell culture medium in spite of not being completely immersed in the medium. After culturing these different cells on the basal lamina membrane it was observed that this membrane proved to be a good substrate for cell attachment, proliferation and growth. The images in Figure 3.5 (A), (B), (C), (D), (E) and (H) were taken with a magnification of 10X and the scale bar in Figure 3.5 (A) indicates a distance of 100 microns. The images in Figure 3.5 (G) and (H) were taken with a magnification of 20X and the scale bar in Figure 3.5 (G) indicates a distance of 50 microns. The scale bar shown for the 10X images in Figure 3.5 (A) indicates the same distance for all the 10X magnification images as all the images were taken in the exact same manner. The scale bar shown for the 20X images in Figure 3.5 (G) indicates the same distance for all the 20X magnification images as all the images were taken in the exact same manner.

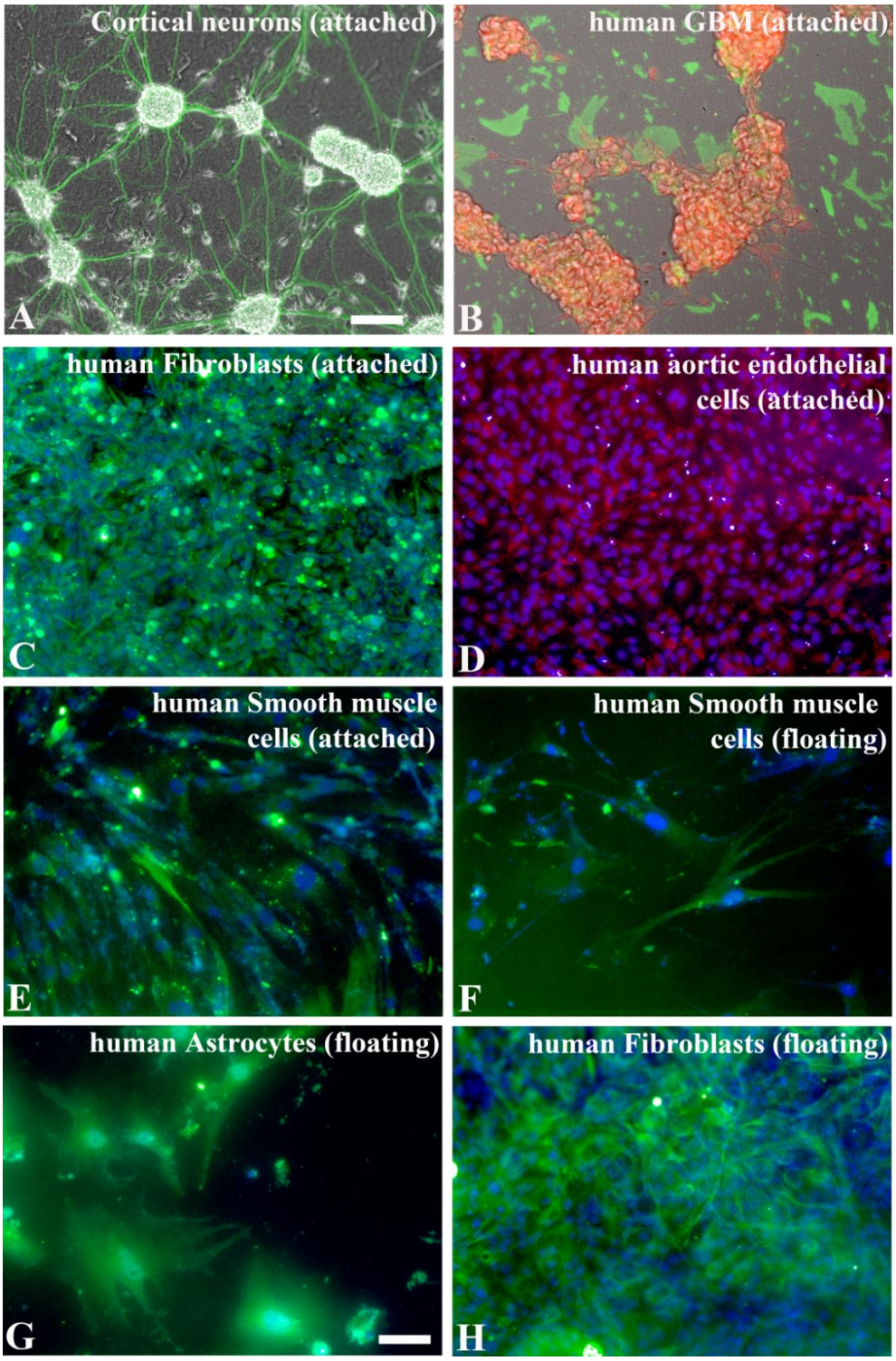

Figure 3.5 Cell culture in 2D using the basal lamina equivalent

As mentioned earlier, the basal lamina is an integral component of the human body. It is composed of nearly 50 different proteins although collagens (primarily type IV) and laminin are the most abundant proteins comprising the basal lamina (4, 6). The basal lamina is responsible for the architecture of the tissues, supporting cells and for the various interactions between cells and between the cells and the ECM (6). The collagen IV is the protein responsible for forming a stable network in the basal lamina. Where collagen adds to the structural stability of the basal lamina, it is laminin which is important for the cell adhesion. It is known to bind to several different molecules, including its self thus acting as a medium for interaction between cells and between cells and the ECM (47). The functions of the basal lamina for providing structural support, for cell differentiation and migration and for acting as a medium of communication make it such an important component in cell culture and study *in vitro*. While culturing cells *in vitro* on the conventional cell culture dishes in 2D, the culture medium and the growth factors added to it do provide the complete *in vivo* environment in which the cells grow (32). This has lead to the development of various ways in which artificially prepared basal lamina can be used for 3D cell culture.

We have proposed to create a Basal Lamina Equivalent by using rat tail derived collagen type I and an ECM gel. The concentration of collagen type I was optimized to 3 mg/ml as at a concentration lower that it was not possible to obtain peelable membranes. The solution was air-dried to form ultra-thin, peelable basal lamina membrane with a thickness which is comparable to the thickness of the basal lamina in our bodies. The ECM gel used provided the proteins collagen (IV) and laminin which are the key components of the true basal lamina. By using the rat tail derived collagen (I) the basal lamina membrane has good mechanical properties which enabled its easy handling for cell culture and various 3D applications. The concentration of the ECM gel was 8-12 mg/ml as provided by the manufacturer. The ratio of mixing the collagen type I and Ecm gel was optimized to 50:1 as at a higher volume of the ECM gel used, the solution could not be air-dried to obtain peelable membranes. The thickness of the

basal lamina *in vivo* is in the range of 50-100nm and the thickness of the basal lamina membrane prepared by us is the range of 0.5-1 microns. The technique proposed by solvent casting and air-drying is very easy and also cost effective and be used in several applications. The proteins were not denatured after air drying as tested by and seen from the immunostaining of the membrane for collagen and laminin which shows that this technique for creating the basal lamina membrane was successful. Another important and unique feature of the membrane is its semi-permeability and easy wetability. This enabled the use for cell culture as a floating membrane on which cells can be cultured and also as a substrate attached onto a cover slip. This membrane when air-dried and UV cross-linked has good mechanical properties which allow easy handling, however once added to the culture medium the membrane is easily wet and semi permeable which allows the cells being cultured on it to grow well. By culturing six different types of cells on the basal lamina membrane we confirmed its effectiveness in supporting cell growth and proliferation. This membrane also being highly transparent could be easily sterilized for cell culture by placing under UV light in the bio safety cabinet for thirty minutes. This technique did not involve the use of any ethanol or other sterilization techniques because of the thinness and transparency of the membrane. It was confirmed to be effective as all the cell culture done using the basal lamina membrane was free of any contamination. This enabled its use for cell culture which was further expanded into 3D applications using the membrane.

3.7 Conclusion

Summing up, the fabrication of the Basal Lamina Equivalent provides an easy and cost-effective technique for making ultra-thin, easy to handle, multi-purpose peelable membranes used for various 2D applications and cell culture. The cells cultured showed a robust growth. The mechanical strength, flexibility and ease of use can be widely exploited for various 3D applications.

CHAPTER 4

3D APPLICATIONS OF THE BASAL LAMINA EQUIVALENT

4.1 Introduction

There are two applications which have been developed using the Basal Lamina Equivalent. A novel 3D model for cell culture has been developed to create easily stackable layers of cells. Rat embryonic cortical neurons have been cultured on three different layers using the basal lamina equivalent as a substrate. The three separate layers of neurons have been stacked into one 3D cell culture model allowing the axons from one layer of neurons to grow into another layer resembling the complex neuronal structure in the cerebral cortex. This model has further been developed to study the migration the human glioblastoma multiforme and also to develop a model resembling the blood-brain barrier with a layer of endothelial cells on the basal lamina.

The second application developed using the basal lamina membrane was a micro fluidic device for exchange of oxygen across the membrane. The basal lamina membrane was sandwiched between two layers of micro-patterned PDMS. One layer of PDMS was used to run water and the second layer was used to run oxygen. The oxygen was allowed to diffuse across the membrane and increase the oxygen concentration in the water flowing on the other side of the membrane.

4.2 Fabrication of the 3D stack

The basal lamina membrane was mounted on an aluminium framework for support for using it to culture cells for preparing a stack. The aluminium foil was cut into rectangles of approximate dimensions (2.5cm X 2cm). The aluminium has a thickness of 0.001 inches. A circular punch of a diameter of 8mm was made in the center of the rectangular aluminium foil. This aluminium framework was sterilized by keeping it in 70% ethanol for 20 minutes. The

aluminium framework was washed with sterilized de-ionized water twice in the bio-safety cabinet and left to dry. The air-dried basal lamina membrane was sterilized by exposing it to UV light in the bio-safety cabinet for 30 minutes. After the sterilization, the membrane was attached to the framework with PBS. The aluminium framework was kept in one petri dish for support. Using a pair of forceps, the membrane was gently lifted and held firmly on the aluminium framework in such a way so as to have the circular punch in the center of the membrane. PBS was added on the 4 corners of the membrane using a micropipette. This firmly secured the membrane onto the aluminium framework. This was repeated for each of the layers of the stack. Each such membrane stuck on the aluminium framework serves as one individual layer of the stack. The membrane with the support was added into a petri dish containing the cell culture medium. The aluminium framework allowed the membrane to float in the medium enabling easy handling of the membrane in the future for stacking purposes. The membrane was incubated in the cell culture medium overnight at 37˚C and 5% CO_2. After the overnight incubation of the membrane, the medium was replaced with fresh cell culture medium. Each stack contained 3 layers of cortical neurons. The cells are seeded onto 3 floating basal lamina membranes and allowed to grow for 3-4 days at 37^{o}C and 5% CO_2. The medium was replaced with fresh medium with growth factors every 2 days. After 3-4 days, the neurons showed adequate growth allowing the 3 layers to be stacked to make the 3D cell culture model. The individual layers were stacked up and held together with the help of a PDMS block. The PDMS block had an opening made by multiple punches using an 8 mm biopsy punch. The PDMS block had an approximate thickness of 5 mm and was sterilized using 70% ethanol. The three floating layers of basal lamina membrane with cortical neurons were stacked up one onto another and held together with the help of the PDMS block. One layer of neurons was lifted and held to the bottom of a petri dish containing medium with the help of a pair of forceps. With the help of another pair of forceps the second floating layer of neurons was lifted and gently added onto the first layer without damaging the cells. The third layer of neurons was also added onto the first

two in the same manner. The three layers were held together with the forceps and the PDMS block was gently placed onto the layers holding them together. The entire cell culture model was then incubated at 37^{o}C and 5% CO_2 for 4-5 days allowing the axons from one layer to grow into the other adjacent layers.

4.3 Cortical Neuron Cell Culture

The membrane attached on the aluminium frame support is incubated overnight at 37˚C and 5% CO_2 one day before seeding the cells. Before seeding the cells, the medium is drained and fresh Neurobasal Medium (Invitrogen) supplemented with B-27 (Invitrogen) and Gentamycin (50µg/ml Gibco) and Neurotrophin-3 and Brain Derived Growth Factor (BDNF) (20ng/ml) is added to the culture dishes containing the floating membrane samples. Well dissociated E-18 rat cortical neurons are then seeded on the floating basal lamina membrane. The cells are placed in the incubator at 37˚C and 5% CO_2 and allowed to grow for 3-4 days. After 3-4 days, the individual layers of the membrane are stacked with the help of the PDMS block. Each stack contains 3 layers of cortical neurons cultured on the basal lamina membrane as shown in Figure 4.1. The stack of layers of cells is incubated and cultured for 4-5 days in order to allow the axons from one layer of the stack to pass to the other two layers. The medium is changed regularly every 2 days and the cells are incubated at 37˚C and 5% CO_2.

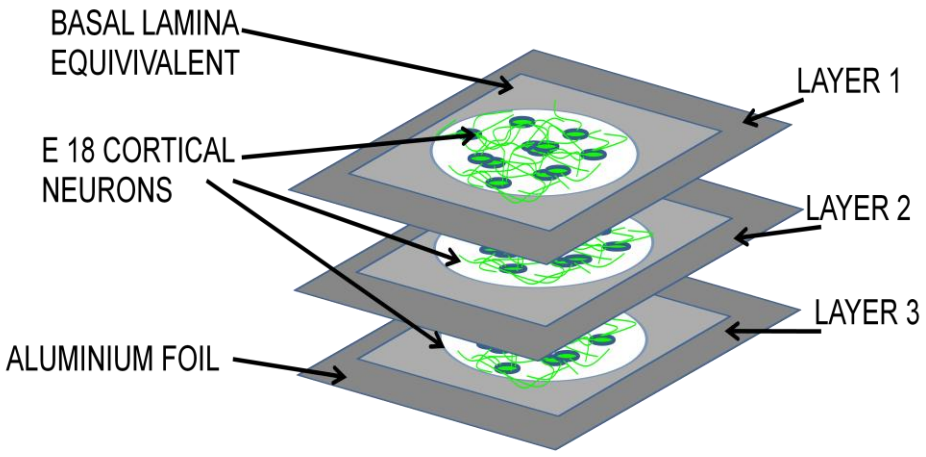

Figure 4.1 Schematic of cortical neurons in 3 layers on the Basal Lamina Equivalent

4.4 Primary Human Glioblastoma Cell Culture

The cells were obtained from The University of Texas at the Southwestern Medical Center in Dallas TX from willing patients. The cells were suspended in serum free Dulbecco's modified Eagle's Medium/F-12 medium (50/50), containing 2% B-27 (Invitrogen) supplement, 0.25 % Insulin-Transferin-Selenium-X, Penicilin-Streptomycin (100 units/ml and 100 µg/ml respectively), 20 ng/ml mouse EGF and basic Human Fibroblast growth factor (20 ng/ml).The cells were transfected with lentivirus having monomeric-cherry (*m-cherry*) fluorescent protein expression. The cells floating in the medium in a cell culture dish were used for the cell seeding. The medium containing the cells was collected and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was chemically dissociated using 1ml trypsin EDTA and 1 ml of 0.2% collagenase (type II) for 5 minutes at 37oC in the water bath. Soya Bean Trypsin Inhibitor (SBTI) was added to the cell suspension and the cells were physically dissociated using a 2 ml pipette. The suspension was centrifuged at 1000 rpm for 5 minutes to obtain a cell pellet. 1 ml of cell culture medium was added to the pellet and the cells were counted and 25,000 cells were seeded on a floating basal lamina membrane sample prepared as mentioned earlier and as shown in Figure 4.2.

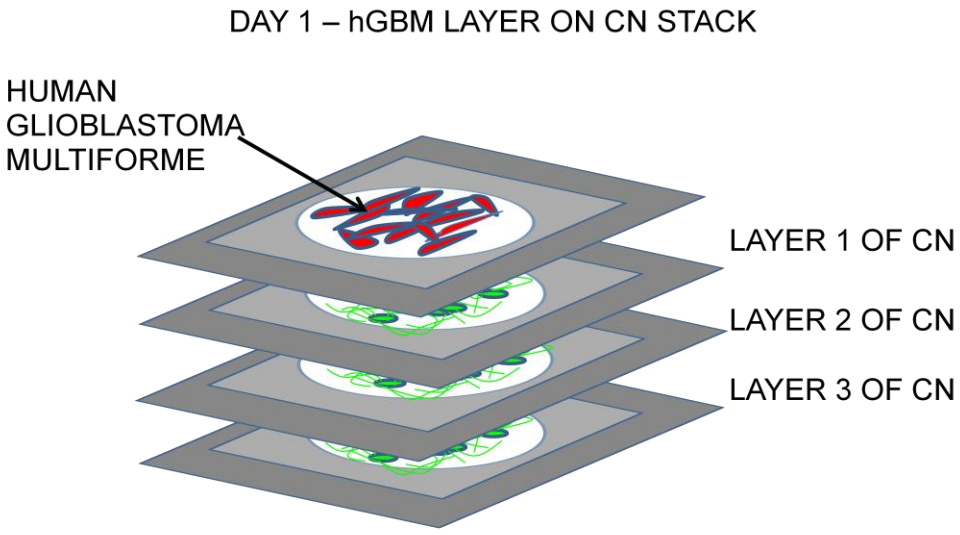

Figure 4.2 Schematic of hGBM layer added on cortical neuron stack for migration study

4.5 Primary Human Glioblastoma Cell Migration Study

The cells were counted and seeded on the basal lamina membrane. The membrane was attached to the aluminium framework and suspended in medium. The cells were seeded and allowed to attach on the membrane for 24 hours. After 24 hours, the floating membrane was lifted with the help of the forceps and placed on the cortical neuron three layer stacks. The PDMS block holding the cortical stack was gently lifted, without disturbing the stack. The cortical stack was held in place with the help of a pair of forceps. The layer of glioblastoma cells on the membrane was placed on the cortical neurons and the PDMS block was replaced on the stack. The glioblastoma cells were allowed to migrate to the lower layers of the stack. The migration study was performed with two time points as depicted in Figure 4.3 and 4.4. One sample of the migration study was fixed after 3 days with 4% para- formaldehyde. The second sample for the migration study was fixed after 6 days with 4% para-formaldehyde.

The imaging of the stack to observe the migration of the glioblastoma cells was done using a Zeiss LSM 510 Meta Confocal microscope. The cortical neurons were stained with beta-3-tubulin antibody and the glioblastoma cells had the m-cherry protein expression hence no staining was required.

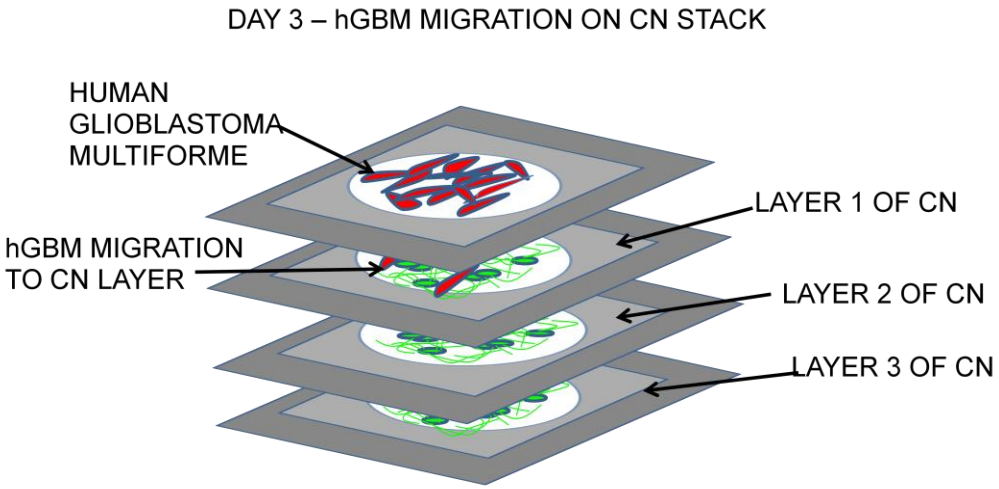

Figure 4.3 Schematic of migration of hGBM in cortical neuron stack in 3 days

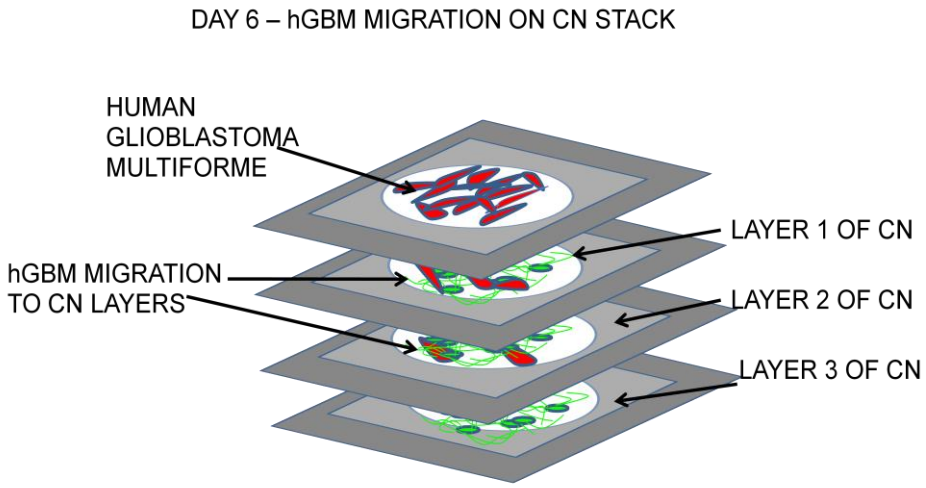

Figure 4.4 Schematic of migration of hGBM in cortical neuron stack in 6 days

4.6 Cortical Neuron Stack with Endothelial Cells

The endothelial cells were obtained from the human aorta from willing patients. The cells were cultured in a low serum medium (2%) in a cell culture flask (T-75). To seed the cells, 4 ml of trypsin EDTA was added to the flask after draining the medium. The cells were incubated in trypsin for 5 minutes at 37^{o}C. The detachment of the cells from the flask was confirmed under a microscope and the cell suspension was added to SBTI. The cells were physically dissociated using a 2 ml pipette and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 1 ml of medium. The cells were counted and 30,000 cells were seeded on the basal lamina membrane. The cells on the floating membrane were allowed to attach and proliferate for 48 hours. After 48 hours, the layer of endothelial cells was placed on the cortical neuron stack in the same manner as mentioned previously for the glioblastoma cells. The entire stack was cultured in Neurobasal medium with 2% B-27 supplement and BDNF and NT-3. The stack was fixed using 4% para-formaldehyde after 2 days. The stack was imaged using the confocal microscope mentioned earlier. The cortical neurons were stained using beta-3-tubulin and the endothelial cells were stained with Von Willebrand Factor (VWF).

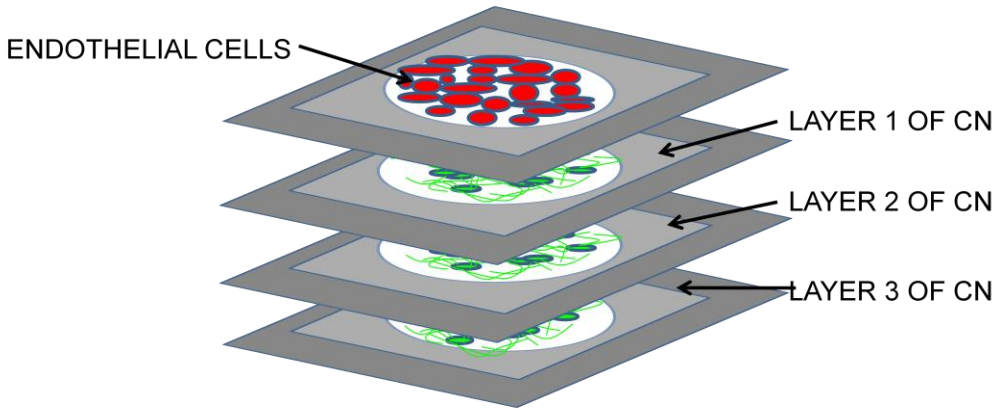

Figure 4.5 Schematic of Endothelial cell layer on cortical neuron stack for Blood Brain Barrier Model

4.7 Designing of mask for fabrication of silicon wafer for gas exchange device

The design for the silicon wafer for the micro fluidic pattern was designed using AutoCAD. The desired dimensions for the device were designed with AutoCAD and the mask was fabricated. The pattern on the mask was used as a mold to transfer the pattern on the silicon wafer with photo-resist coated on it. This mask served as the mold for fabricating the Polydimethylsiloxane (PDMS) devices. The device was designed separately for water and for oxygen. The height for both the devices was fabricated as 100 microns.

4.8 Fabrication of Silicon wafer

The silicon wafer was fabricated in the Nanofab facility in The University of Texas at Arlington. The materials used for fabricating the wafer were – 4 inch silicon wafer, SU8-50 photoresist (Microchem), SU8 developer (Microchem) and acetone. The machines used for fabricating the wafer were – hot plates, spin coater and back-side aligner.

The fabrication of the wafer is explained in detail below:

1. The silicon wafer was washed with acetone (solvent). The wafer was dehydrated by baking at 200^{o}C for ten minutes for removing any moisture. The wafer has to be dehydrated properly for proper adhesion of photo-resist.

2. The wafer was coated with SU8-50 photo-resist by pouring the resist on the wafer and spinning at 500 rpm at 100 rpm/s for 10 seconds followed by spinning at 1000 rpm at 300 rpm/s for 30 seconds.
3. After coating the wafer, it was baked at 65^{o}C for 10 minutes followed by baking at 95^{o}C for 30 minutes.
4. The photoresist used has to be polymerized at a near UV wavelength range of (350 – 400 nm). The exposure energy was in the range of 300 mJ/cm^{2} – 550 mJ/cm^{2}. When the resist is exposed to the UV light, the negative photoresist cross links leaving the pattern on the wafer to be polymerized. The duration for which the resist is exposed the UV light is according to the manufacturer's protocol for obtaining the desired height of the pattern. For obtaining the height of the pattern as 100 microns, the UV exposure was done for 28 seconds.
5. For cross-linking the exposed regions of the resist the wafer was baked at 65^{o}C for 1 minute and at 95^{o}C for 10 minutes.
6. The unexposed parts of the wafer which have not been cross-linked were washed off using the SU8 developer. The wafer was immersed in the developer and washed rigorously for 10 minutes. The wafer was blow dried using nitrogen.
7. The wafer was hard baked at 150^{o}C for 30 minutes after the entire fabrication process for achieving maximum yield and avoiding peeling of the photo-resist after repeated usage.

4.9 Fabrication of micro fluidic devices from the silicon wafer mold

The materials used for the fabrication of the micro fluidic devices were – silicon wafer with the pattern, SYLGARD® 184 elastomer kit (DowCorning), 2 hot plates, scalper blade, aluminium foil and vacuum dessicator. The process for fabrication of the PDMS device is explained step-wise below:

1. The PDMS and the curing agent provided by the manufacturer were mixed in a 1:10 ratio (6 ml of curing agent: 60 ml of PDMS).
2. The mixture was placed in the vacuum dessicator under a pressure of 20 mm of Hg for 1 hour to remove all the air bubbles created in the mixture.
3. The PDMS was removed from the dessicator after all the air bubbles were removed. It is very important to ensure that all the air bubbles were removed because air bubbles present in the PDMS would interfere with the micro fluidic pattern while curing the PDMS.
4. The silicon wafer fabricated by photolithography was wrapped in an aluminium foil and placed on a hot plate at 70^{o}C.
5. The PDMS mixture was poured onto the wafer and any air bubbles introduced into the mixture while pouring it were removed with a sharp forceps
6. The wafer was placed on a hot plate at 150^{o}C for 5 minutes allowing the PDMS to cure and cross-link.
7. After the PDMS cures completely, the wafer was placed on the hot plate at 70^{o}C and the PDMS pattern was cut out using a scalper blade.

The micro fluidic pattern for the device for running the oxygen and the water were fabricated in the above mentioned manner. After the devices were made, a 2 mm punch using a biopsy punch was made.

4.10 Fabrication of the basal lamina membrane

The basal lamina membrane used for this application was prepared by mixing 20 µl ECM gel in the previously mentioned concentration with 1 ml of rat tail derived Type I collagen of a concentration of 6mg/ml. 1 ml of this mixture was spread on a PDMS mold of the dimensions 2 mm X 2 mm and kept in the chemical hood for drying of the mixture. After the mixture was air dried, the membrane was peeled off from the mold and kept under a long wave UV lamp of wavelength (350 nm) for cross-linking the proteins.

4.11 Assembly of the Gas Exchanger Unit

The basal lamina membrane was placed on the PDMS device having the channels for running the oxygen as shown in Figure 4.6. The PDMS device for running the water was placed face up (side of the channels on top) in a UV – ozone plasma chamber, along with the membrane bound air channels device. The water and air (with the basal lamina membrane) were exposed to the UV light for 10 minutes. After exposing the devices to UV, the devices were placed one on top of the other with the membrane being sandwiched between them. The channels for water and air were placed perpendicular to one another with the membrane separating them. The plasma treatment helped in binding the two PDMS layers together. The device was left over night after assembling it, in order to get permanent and strong bonding between the two PDMS layers to prevent and leakages of wate*r* or air.

4.12 Working of the Gas Exchanger

After permanently bonding the device together, the entire unit for running the experiment for the exchange of oxygen across the membrane was assembled as shown in Figure 4.12 (C). The inlet of oxygen was connected to the bottom PDMS layer from the 2 mm punch. The input pressure of oxygen was set to 0.1 SCFH. The outlet for the oxygen was made from the other end of the same layer via a 2 mm punch. A pipe connected to the punch was used to allow the oxygen to flow out. The air was flown from below the membrane and the water was flown from above the membrane. The water was flown above the membrane at a flow rate of 0.6 ml/hour. The outlet for the water was connected from the other end of the device, leading to a beaker where the water after exchange of oxygen was collected. The experiment was run for 20 minutes collecting 2 ml of oxygenated water for measuring the change in the oxygen concentration. The concentration of dissolved oxygen in the filter water was measured before and after running the experiment using a dissolved oxygen meter (Mettler Toledo SG6).

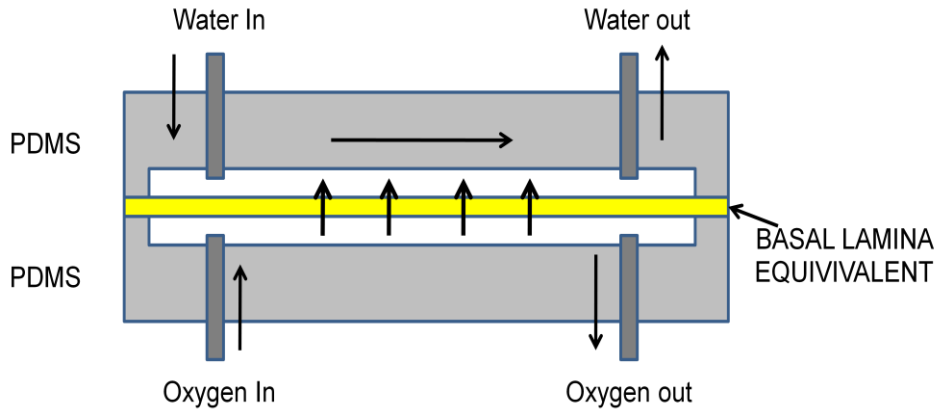

Figure 4.6 Schematic of Exchange of Oxygen across the Basal Lamina Equivalent

4.13 Results and Discussion

4.13.1 Fabrication of the 3D Stack

As mentioned earlier, the basal lamina membrane was prepared by the solvent casting method. The Figure 4.7 (A) shows the air dried Basal Lamina membrane after it was UV cross-linked and peeled off. The Figure 4.7 (B) demonstrates the aluminium frame work on which the membrane was attached with the help of PBS. This aluminium framework with the membrane formed one individual layer of the stack. All the layers of the stack using the membrane were prepared in the exact same manner as shown in Figure 4.7 (C). The small drops of PBS added onto the membrane stuck the membrane well onto the aluminium. The individual layers of the stack were dropped into the cell culture medium as shown in Figure 4.7 (D). The membranes after dropping into the medium did not rupture or peel off and were properly stuck to the framework. The cells seeded on these floating membranes also showed robust growth successfully allowing the membranes to be used for 3D applications. The individual layers of the stack after the cells had grown and attached were stacked into one model and held together with the help of the PDMS block as seen in Figure 4.7 (E). The membranes after stacking together still showed robust cell growth. The stacked layers were seen to support cell growth without any damage to the membrane or the cells thus successfully fabricating the 3D stack model. The PDMS block was successful in holding the different layers of the stack together

allowing the cells from one layer to migrate or interact with the cells in the adjacent layers as shown in Figure 4.7 (E).

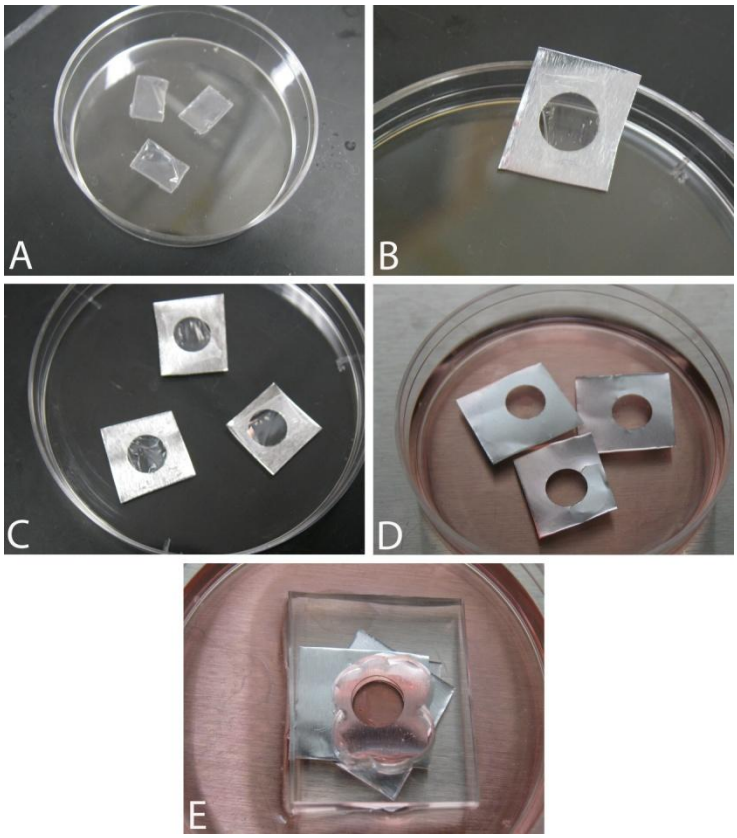

Figure 4.7 Fabrication Procedure of the 3D Stack

4.13.2 Cortical Neuron Stack

The E-18 derived rat cortical neurons were seeded onto the floating membrane samples and showed robust growth. The cells were observed to survive well after the three individual floating membrane layers were stacked up into a single stack held together with the PDMS block. The cells present in the lower most layer of the stack also survived in spite of the other two layers present above it. The cortical neurons were fixed and stained with β- III Tubulin (green) as shown in Figure 4.8. The stack was mounted on a cover slip and imaged using the Zeiss LSM 510 Meta Confocal microscope. The entire stack was scanned with a scan interval of 1 micron. The neurons from one layer were seen in focus for one scan and were observed to

gradually move out of focus for each descending scan. The confocal microscope scanned one plane at a time with a 1 micron interval with only objects in that particular plane being in focus for the scan. The axons which were in focus in one plane were observed to have a continuity with the planes out of focus in that particular image showing the connectivity of the axons within the layers from a dense network of axons as seen in the brain. The figure 4.8 shows the stack of neurons with a magnification of 10X. The first image shows the top most layer of the stack descending to the bottom most layer with the last image. The scale bar in the last image shows a distance of 100 microns. The cortical neurons after being stacked into one layer were cultured for up to 4 weeks. The cells were healthy for 4 weeks with regular change in culture medium. The membrane on which the cells were cultured was also intact for up to 4 weeks of culture.

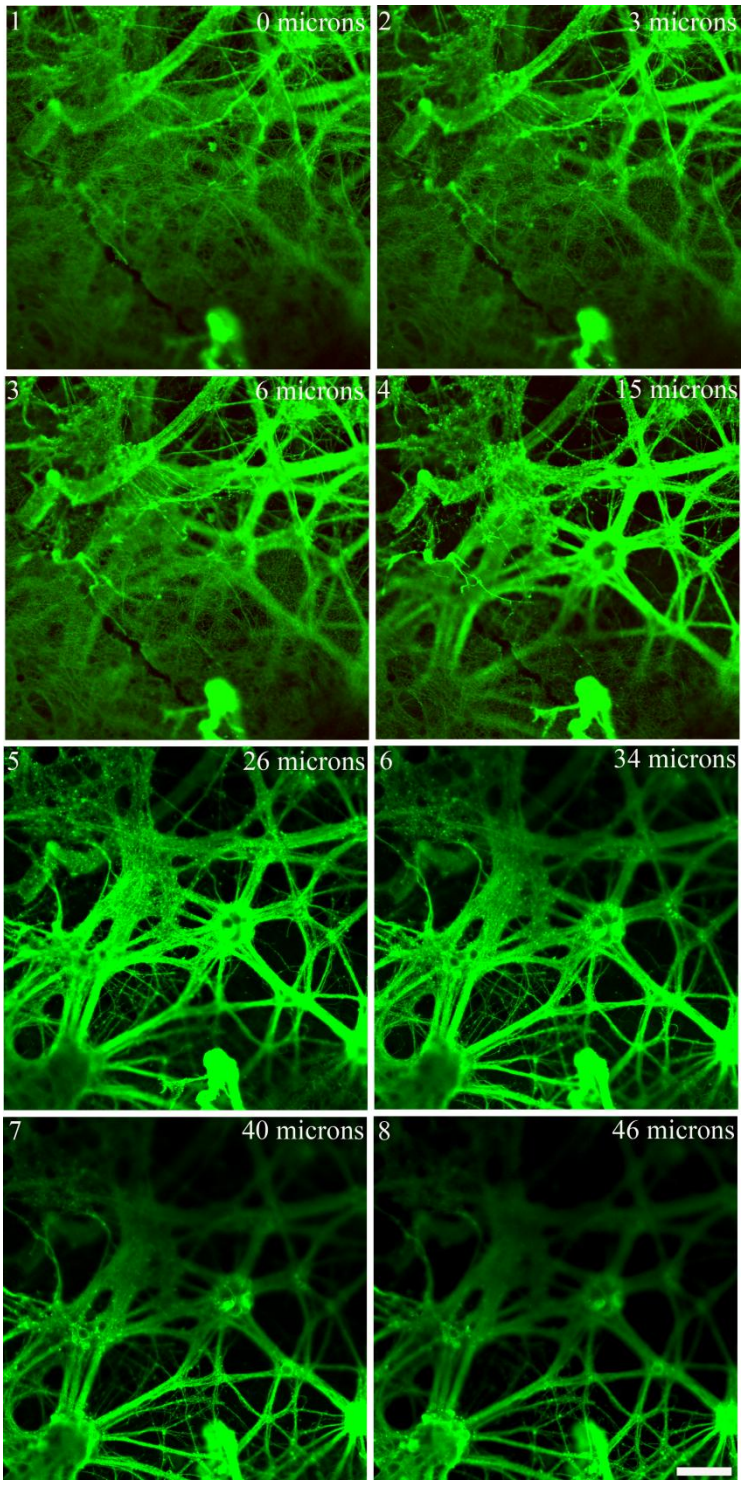

Figure 4.8 Confocal Microscope Imaging of Cortical Neuron Stack in 3 Layers on Basal Lamina Equivalent

4.13.3 Human Glioblastoma Migration Study

The cortical neuron stack was used as a model to study the migration of the human glioblastoma multiforme (hGBM) in 3 dimensions. The glioblastoma cell layer was added onto the stack of neurons and the cells were allowed to migrate. The migration was studied for 2 different time points of 3 and 6 days. The stack was fixed after the 3rd and 6th day and the neurons were stained for β-III tubulin (green) as seen from Figure 4.9 and 4.10. The glioblastoma cells were transfected with lentivirus for the m-Cherry dye and were not stained. The stack was imaged with the Zeiss LSM 510 Meta Confocal microscope. The stack for the 3 day migration of the glioblastoma cells was imaged with a magnification of 20X as seen from Figure 4.9. The stack was scanned with a 3 micron interval for each scan. The first image in figure shows the layer of the glioblastoma cells (red) which was the top most layer in the stack. Each image shows the objects in focus only in that particular plane. With each scan towards the lower layers the neurons were observed to be in focus. The glioblastoma cells were also seen in the same focal plane as the neurons indicating that the cells had migrated through to the lower layers of the stack. The first image shows only the glioblastoma cells and the third and fourth images in Figure 4.9 show the glioblastoma cells in focus with the neurons showing that they are in the same focal plane up to a depth of 12 microns in 3 days. The lower layers show only the neurons in focus indicating that in 3 days the hGBM had migrated up to 12 microns in the stack.

The stack for the 6 day migration of the hGBM was also scanned in a similar manner. The stack was imaged with a magnification of 10X. The scan interval for the stack was 4 microns. The first two images in Figure 4.10 show the layer of the hGBM (red). No cortical neurons were seen in this focal plane. The lower scans in figure 4.10 show the neurons and the hGBM cells. The neurons were stained with β-III tubulin (green). The lower layers of the stack with the GBM and the neurons show that the hGBM cells had migrated to the neuronal layers up to a depth of 24 microns. The layers of the stack below the 24 micron layer showed neurons

only or the hGBM cells out of focus. The migration for the 2 time periods showed that with 6 days of culture time, the GBM cells had migrated to a greater depth of the stack than that for the 3 days of migration. The scale bar in the last image of Figure 4.10 shows a distance of 100 microns for a 10X magnification image. The magnification with which the stack was scanned did not affect the depth of the scan. The scan for the stack of 3 days in Figure 4.8 had a total depth of 27 microns and the scan for the stack of 6 days had a total depth of 40 microns. This variation in depth of the stack was because of difference in cell number, cell attachment, cell spreading and migration. In 3 days, the hGBM cells had migrated up to 12 microns in the stack and in 6 days the hGBM had migrated up to a depth of 24 microns. As seen from Figures 4.9 and 4.10 the glioblastoma cells showed positive staining for β-III tubulin, however the cells were transfected with the *mCherry* dye which enabled the cells to be differentiated from the cortical neurons other than the obvious morphological differences.

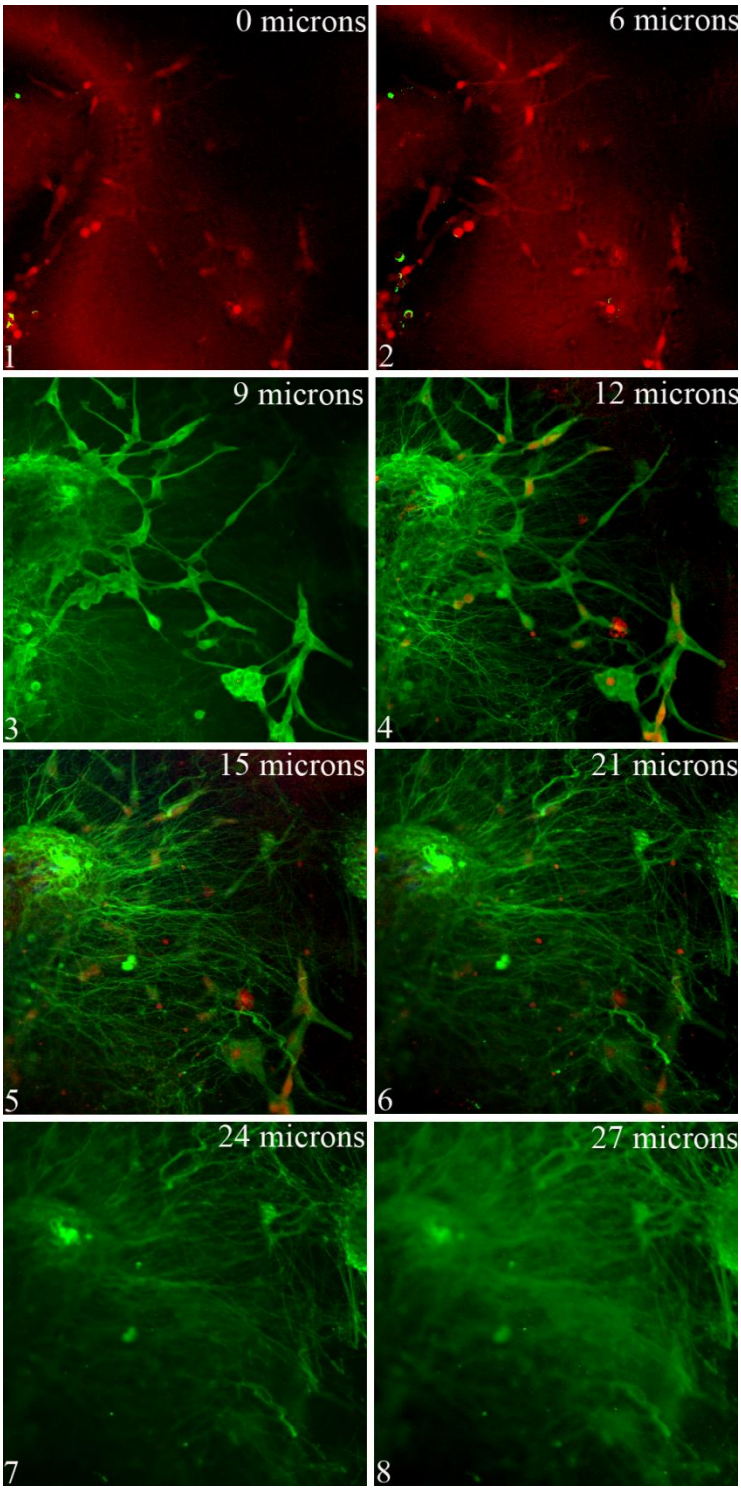

Figure 4.9 Confocal Microscope imaging of Human Glioblastoma Migration through Cortical Neuron layers in 3 days

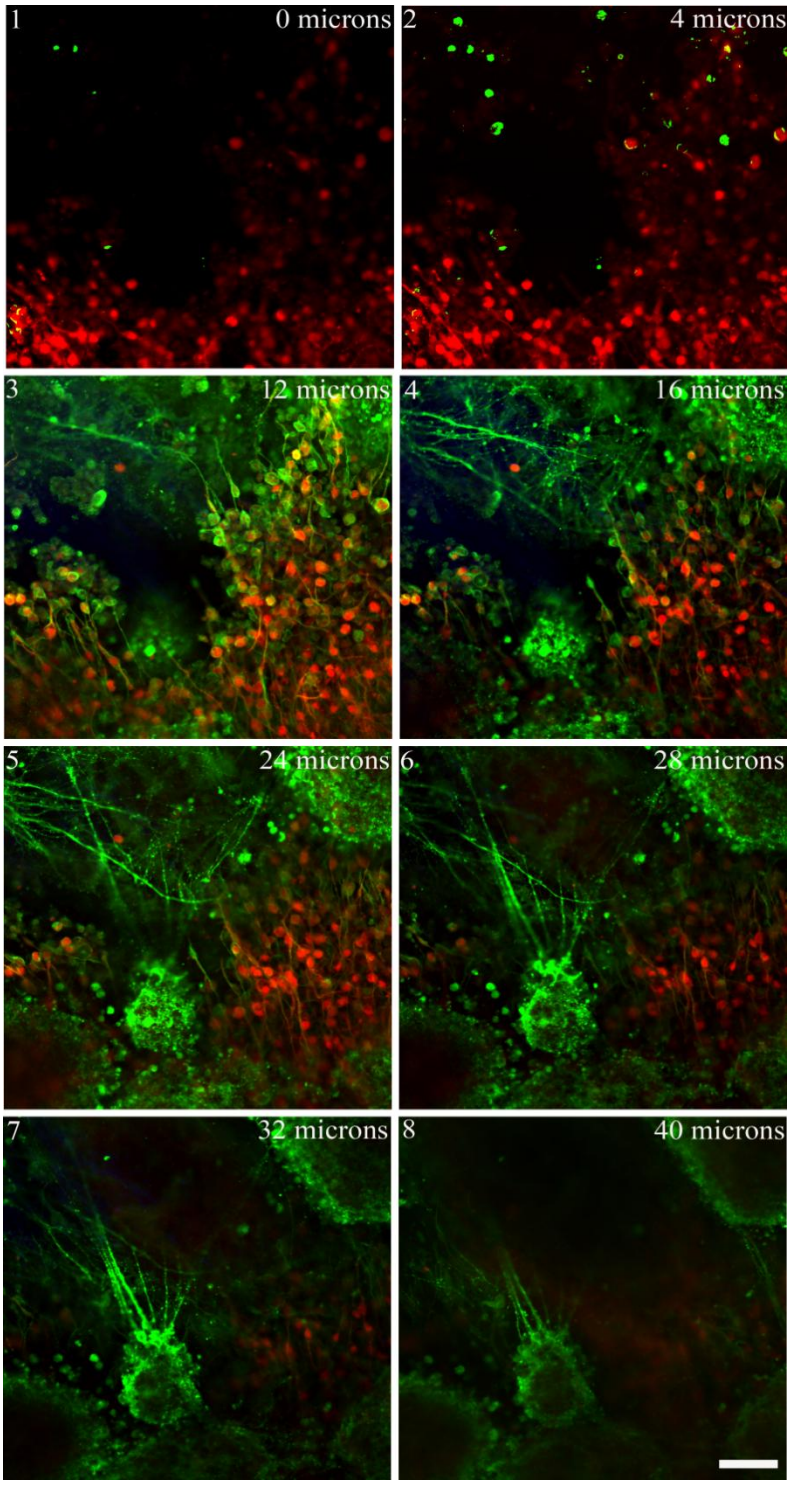

Figure 4.10 Confocal Microscope imaging of Human Glioblastoma Migration through Cortical Neuron layers in 6 days

4.13.4 Cortical Neuron Stack with Endothelial Cells

The cortical neuron stack was used to develop a blood brain barrier (BBB) model by adding a layer of endothelial cells cultured on the basal lamina membrane on the layers of neurons. The stack was imaged in the same manner as mentioned earlier using the confocal microscope. The endothelial cells were stained with von Willebrand Factor (VWF) (red) and the neurons were stained with β-III tubulin (green). The stack was imaged with a magnification of 10X as shown in Figure 4.11. The stack was scanned with an interval of 5 microns. The first image in Figure 4.11 shows the layer of endothelial cells (red). The endothelial cells formed a monolayer on the basal lamina membrane and there were no axons of the neurons seen in that focal plane. Through the lower layers of the stack the neurons were seen in focus and the endothelial cells were seen to move out of focus as seen in Figure 4.11. This model could be used to study tumor metastasis into the brain crossing the BBB where the tumor cells have to cross the layer of endothelial cells and basement membrane to enter the brain. The cortical neuron stack was cultured for 1 week before the endothelial cells were added on the stack. The stack after adding the layer of endothelial cells was cultured for 72 hours before the stack was fixed and stained. The scale bar in the last image of Figure 4.11 shows a distance of 100 microns.

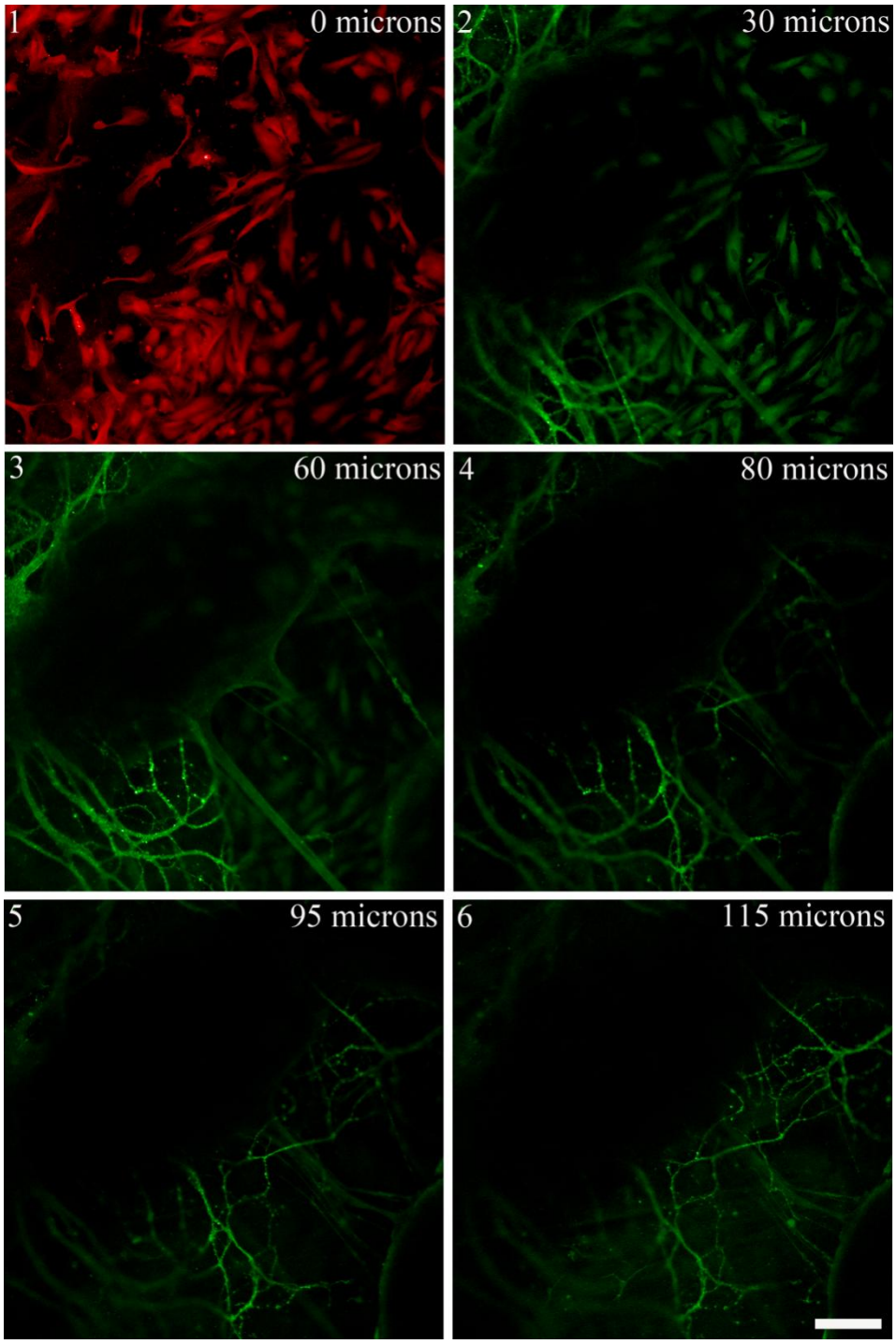

Figure 4.11 Confocal Microscope imaging of Endothelial Cell layer on Cortical Neuron Stack

4.13.5 Gas Exchanger Application using the Basal Lamina Equivalent

Figure 4.12 (A) shows the basal lamina membranes made with 2 different concentrations of the rat tail derived collagen I of 3 mg/ml and 6 mg/ml in order to control the permeability of the membrane. The water run on one side of the membrane was observed to leak through the membrane with a 3 mg/ml concentration of collagen used for other applications described earlier. The concentration of the collagen used to prepare the basal lamina membrane was increased to 6 mg/ml. with this concentration the water did not leak through the membrane as desired for the application of gas exchange across the membrane. Figure 4.12 (B) shows the PDMS device of the 2 layers of water on the left hand side and air on the right hand side respectively before the device is assembled together with the membrane in between. The basal lamina membrane is placed on the PDMS device with the microchannels for air as seen in Figure 4.12 (B). Figure 4.12 (C) shows the entire set up for running the gas exchanger unit.

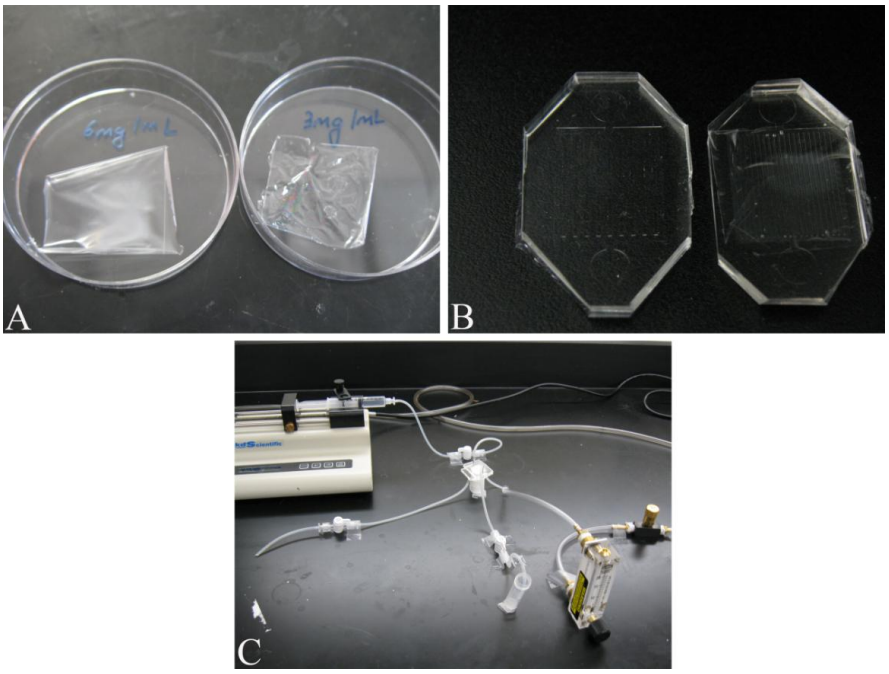

Figure 4.12 Gas Exchanger Application using the Basal Lamina Equivalent

Gas exchange across the membrane was successful with the experiments performed. The change in the concentration of dissolved oxygen in water after it is passed through the membrane showed the exchange of oxygen across the membrane. The change in the concentration of dissolved oxygen was significant with a p – value < 0.01. The table 4.1 shows the values of the dissolved oxygen concentration in the filter water before and after the experiment.

Table 4.1 Concentration of Dissolved Oxygen (PPM) in water

	Before	After
Average	6.93	7.098
Std. Deviation	0.10	0.14
p-value	0.0003	

The figure 4.13 shows the graph of the values of oxygen concentration obtained.

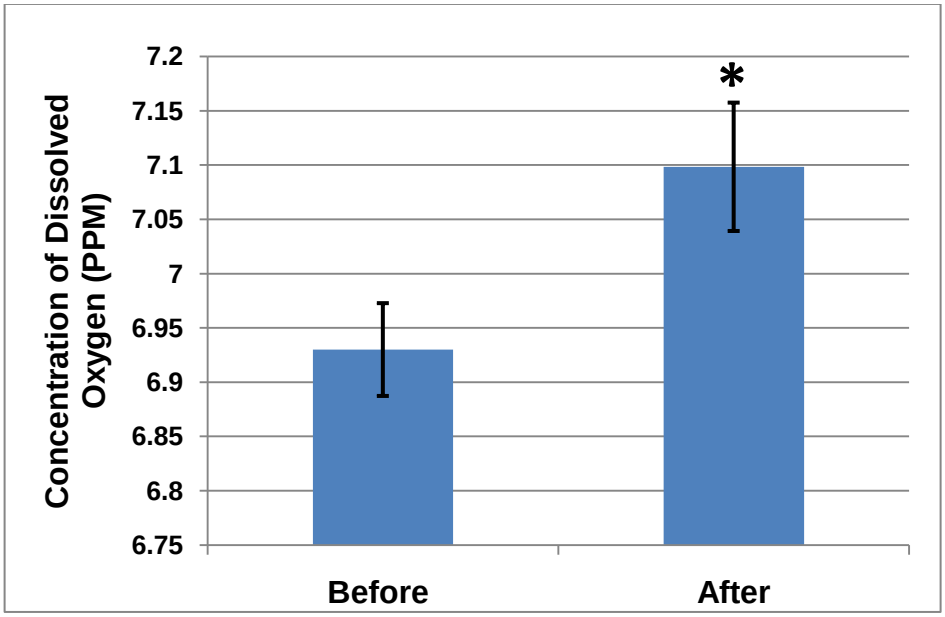

Figure 4.13 Graph representing the concentration of dissolved oxygen (PPM) before and after exchange of oxygen across the basal lamina equivalent (Mean ± SD). *P<0.01 between oxygen concentration before and after exchange across membrane

The 3D stack of cortical neurons developed is very easy to fabricate and cost-effective. The basal lamina equivalent as seen earlier was a good substrate for supporting cell growth and

proliferation. This was the reason for its usage in developing the 3D stack model. The semi-permeability of the membrane allowed cells to be grown on the floating membrane samples. The membrane being made from proteins did not require any further surface modifications or coatings as would be required by other polymer membranes. The thinness of the membrane allowed cells in several different layers to be stacked together to form one model. The other 3D culture systems present like filter well inserts, sponges, gels and microcarriers cannot be used to culture different types of cells in one single model. This technique developed allowed the cells to first be cultured on individual layers and then be stacked into one because of which the different types of cells could be cultured and brought together into one model. This would allow studying the interactions of different cell types with each other and with the ECM scaffold (basal lamina equivalent). The good mechanical strength of the membrane also allowed the easy handling of the different floating layers before being stacked together. The basal lamina membrane provided a good substrate for cell growth because of which after stacking the different layers, the cells on the lowermost layer of stack also survived well. This 3D model because of its porosity allowed the cells from one layer to interact with the cells in the adjacent layers. This model brings cell culture in 3 dimensions closer to the native conditions as the basal lamina membrane is made from proteins and does not require any surface modifications. The cells cultured in different layers and stacked together have ECM and cells on all three dimensions for interaction as present *in vivo.* Finally, this stack model is very easy to fabricate and allows use of multiple cell types in one single cell culture model.

In the second 3D application the membrane has been used for a gas exchange application. The semi-permeability of the membrane made its use in this application effective. The exchange of oxygen into water took place across the membrane where oxygen was run on one side of the membrane and water on the other. The permeability of the membrane was optimized allowing only the air to permeate across the membrane but not the water. This was controlled and optimized by using a higher concentration of the collagen (type I) for making the

solution of the basal lamina before it was air-dried. The increase in concentration of the oxygen (measured in ppm) was observed with a significant difference ($P< 0.01$). This enabled us to use the membrane for the gas exchange application similar to that which takes place at the alveolar level in our lungs across the basement membrane. The thickness of the membrane is in the range of 1-1.5 microns making it close to the gas diffusion distance in the lungs (43). The thickness of the membrane is less than any other commercially available membrane used for gas exchange. Another unique property of this application was the real time monitoring of the change in the oxygen concentration of the water. The water and the oxygen were allowed to flow continuously on the respective sides of the membrane without holding the water and the oxygen in the PDMS device for any time to allow effective exchange. This makes this device very close to the actual exchange of oxygen which takes place in the alveoli where we are continuously breathing and exchanging oxygen with the atmosphere.

4.14 Conclusion

The basal lamina equivalent after being used for cell culture and after observing a robust growth of cells on the membrane, it was developed and used for 3D applications. The semi-permeability of the membrane allowed cells to be grown on floating membrane samples and then stacked up into one model. This technique provided the control over the cell types being cultured into what eventually turned out to be one 3D stack. This technique has provided an easy and cost-effective way to study the interaction between different cell types and the ECM bringing the *in vitro* 3D culture systems closer to the native environment. The use of the basal lamina equivalent for the gas exchange application also provides a platform for the usage of a biological membrane in gas exchange in which the thickness across which gas exchange takes place is as close as it can get to the native environment. Summing up this basal lamina equivalent can be used to develop several 3D applications owing to its mechanical strength, semi-permeability, ease of handling and cost-effectiveness.

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CHAPTER 5

ONGOING AND FUTURE WORK

The basal lamina equivalent is being used to develop a tumor invasion device as shown in Figure 5.1 where the membrane is sandwiched between two blocks of PDMS with an 8 mm punch. The PDMS blocks have a height of approximately 2 mm which allows the device to have separate compartments to contain different cell culture mediums. Endothelial cells are seeded on one side of the membrane. After the cells attach and proliferate the device is flipped to bring the other side of the membrane on top. Invasive cancer cells like renal cancer cells are seeded on this side of the membrane. The renal cancer cells are cultured in low serum medium (2%) whereas the endothelial cells are cultured in medium with a greater percentage of serum (10%). This would cause the renal cancer cells to degrade the layer of basal lamina and migrate through the endothelial resembling the *in vivo* conditions of cancer metastasis.

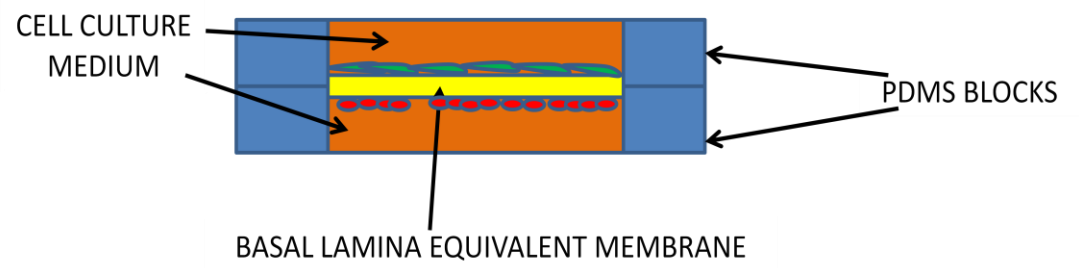

Figure 5.1 Schematic of Tumor Invasion device fabricated using Basal Lamina Equivalent

The multi-layered stack cell culture model will be further developed and used for studying tumor invasion and metastasis. The layer of endothelial cells seeded on top of the cortical neuron stack would be flipped and astrocytes would be seeded on the other side of the basal lamina membrane to form a monolayer thus resembling the blood brain barrier more closely. The layer of an invasive cancer type would be added on top of this model and the

invasiveness of the tumor cells would be studied. This would resemble the metastasis of cancer cells into the brain where these cells in vivo have to cross the layer of endothelial cells, degrade the basal lamina and cross the layer of astrocytes to enter the cortical tissue. This model would thus help in understanding the interactions between the cancer cells and the different cells it interacts with for metastasis. The migration and the interactions between the cells would be studied and different markers would be used for staining the cells to understand any changes in the structure of the cells for migration which might be taking place.

The cortical neuron stack with the layer of human glioblastoma would be used to better understanding the interactions between the neurons and the glioblastoma cells where these cells could be stained with different markers for studying the interactions between these cells. The glioblastoma migration model would be used to study the effects of various drugs on the migration rate of these cells into the cortical tissue.

The preliminary data obtained for the gas exchanger application using the basal lamina equivalent would be used for developing a portable gas exchanger where atmospheric air would be used instead of pure oxygen and the water would be replaced by blood for a high efficiency in exchange of oxygen in the blood because of the oxygen binding to the hemoglobin present in the blood. The gas exchanger developed validated the semi-permeability of the membrane to air which would be further utilized and exploited for developing a portable gas exchanger which would have an increased surface area for the exchange of oxygen thereby increasing the rate of oxygen exchange but still maintaining the gas diffusion distance of 1-1.5 microns.

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BIOGRAPHICAL INFORMATION

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