

T.C.
DOKUZ EYLUL UNIVERSITY
İZMİR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

**DEVELOPMENT OF 3D BIOMIMETIC
PLATFORM FOR CORNEA ORGANOID
CULTURE THROUGH TISSUE ENGINEERING
APPROACHES**

EMİNE KAHRAMAN

MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCES THESIS

İZMİR-2020

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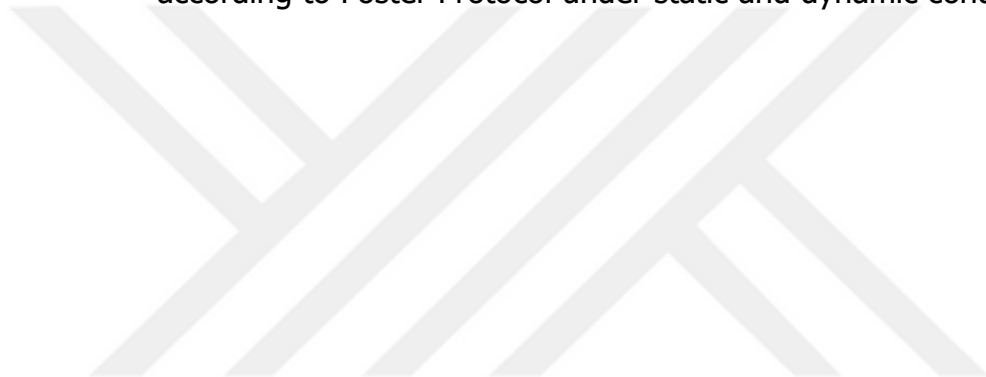
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LIST OF ABBREVIATIONS

2D	2 Dimensional
3D	3 Dimensional
BMSC	Bone Marrow Stem Cells
CV	Collagen Vitrigel
CSH	Concave Shaped Hydrogel
CDM	Corneal Differentiation Medium
CECs	Corneal Epithelial Cells
CSSC	Corneal Stromal Stem Cells
DC	Decellularized Cornea
DIW	De-Ionized Water
DAPI	Diamidino-2-Phenylindole
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
ESCs	Embryonic Stem Cells
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
FT	Freeze-Thaw
GelMA	Gelatin Methacryloyl
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
H&E	Hematoxylin – Eosin
IPSCs	Induced Pluripotent Stem Cells

LSCD	Limbal Stem Cell Deficiency
LSCs	Limbal Stem Cells
MA	Methacrylic Anhydride
NDM	Neural Differentiation Medium
NDC	Non-Decellularized Corneas
Pen/Strep	Penicillin Streptomycin Antibiotic
PAS	Periodic Acid–Schiff
PBS	Phosphate-Buffered Saline
PMMA	Poly (Methyl Methacrylate)
PEG	Poly(Ethylene Glycol)
PDMS	Polydimethylsiloxane
PI	2-Hydroxy-1- (4-(Hydroxy Ethoxy) Phenyl)-2-Methyl-1-Propanone
PVA	Polyvinyl Acetate
qPCR	Quantitative Polymerase Chain Reaction
RDM	Retinal Differentiation Medium
RPCs	Retinal Progenitor Cells
SEM	Scanning Electron Microscopy
SDS	Sodium Dodecyl Sulfate
TE	Tissue Engineering
TAC	Transient Amplifying Cell
TEM	Transmission Electron Microscopy
UP	Ultrapure

UV

Ultraviolet

WHO

World Health Organization



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DEVELOPMENT OF 3D BIOMIMETIC PLATFORM FOR CORNEA ORGANOID CULTURE THROUGH TISSUE ENGINEERING APPROACHES

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ABSTRACT

Human cornea is outermost layer of the eye and acts as a physical and biological barrier for the eye. It has a great role on vision by reflecting light to retina owing to its refractive index. According to World Health Organization (WHO), corneal diseases are one of the major reasons for blindness. Corneal transplantation and removing infected part from the cornea are two main methods for treatment of corneal diseases. However, limited number of donors have been resulted with limited amount of treatment. Hence, researchers have been working on development of alternatives to donor corneas, however current developed structures need to additional improvements to obtain more reliable corneal tissue products. For this purpose, utilizing tissue engineering approaches together with induced pluripotent stem cells (IPSCs) biomaterials and microfluidic systems may lead promising strategies to *in vitro* generate corneal like tissues in a native like micro environment.

This thesis study aims to develop 3 dimensional (3D) biomimetic microfluidic platform to contribute forming of more reliable 3D cornea structures in *in vitro* under dynamic conditions.

To address these objectives different biomaterials have been investigated to find most suitable scaffold for cellular behaviors comparing the SEM images and biocompatibility of materials. Fabrication and validation of microfluidic platform that could mimic the microenvironment of corneal tissue was performed using computer software. IPSCs and cornea organoids were cultured under dynamic conditions for long term.

Keywords: Microfluidic platform, biomaterial, 3D dynamic and static culture, cornea, organoid, IPSCs,

DOKU MÜHENDİSLİĞİ YAKLAŞIMI İLE CORNEA ORGANOİD MODELLERİNİN GELİŞTİRİLMESİNE YÖNELİK 3B BİYOMİMETİK PLATFORM GELİŞTİRİLMESİ

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ÖZET

Kornea, göz için fiziksel ve biyolojik bir bariyer görevi gören gözün en dış tabakasıdır. Kırılma indisi sayesinde ışığı retinaya yansıtarak görme duyusu üzerinde büyük bir rolü bulunmaktadır. Dünya Sağlık Örgütü'ne (DSÖ) göre, kornea kaynaklı hastalıklar körlüğün başlıca nedenlerinden biridir. Tedaviler, o bölgedeki iltihabın kaldırılması ya da kornea nakli ile yapılabilmektedir. Ancak, sınırlı sayıda donör bulunması nedeniyle kısıtlı sayıda hasta tedavi edilebilmektedir. Araştırmacılar donör, kornealara alternatif ürünlerin geliştirilmesi üzerinde çalışmaktadırlar. Bu amaçla, uyarılmış pluripotent kök hücreler (UPKH), *in vitro* koşullarda gerçeğine yakın mikro çevre oluşturmaya yardımcı olan biyomalzemeler ve mikroakışkan sistemler aracılığı ile kornea benzeri doku üretmek için umut vadeden bir seçenektir. Ancak, mevcut olarak geliştirilmiş kornea yapılarının, yapısal ve fonksiyonel olarak gerçeğine daha benzer şekilde üretilebilmesi için ek çalışmalara ihtiyaç vardır.

Bu tezin amacı, geliştirilen dinamik platform ile 3 boyutlu (3B) kornea yapılarındaki kollajen liflerinin gerçek düzenine daha benzer bir şekilde oluşmasını sağlayarak bu alandaki çalışmalara katkıda bulunmaktır.

Bu doğrultuda, hücreSEL davranışlar için en uygun iskeleyi bulmak adına, farklı biyomalzemeler araştırılmış ve bu malzemelerin SEM görüntüleri ve biyoyumlulukları karşılaştırılmıştır. Kornea dokusunun mikro çevresini taklit edebilen mikroakışkan platformun üretimi ve validasyonu yapılmıştır. UPKH'ler ve kornea organoidleri, dinamik koşullar altında uzun süreli olarak kültür edilmiştir.

Anahtar Sözcükler: Mikroakışkan sistemler, biyomalzeme, 3B dinamik ve statik hücre kültürü, kornea, organoid, UPKH

1. INTRODUCTION and GOALS

1.1. Statement and Importance of the Problem

Human cornea, the outermost layer of the eye, mainly comprised of three different layers: epithelium, stroma and endothelium and acts as a physical and biological barrier of the eye. It plays a great role on vision by refracting light to retina owing to its refractive index ¹. According to WHO, corneal diseases are one of the major reasons for blindness ². For corneal diseases, there are two common treatment methods: removing of infected part of the tissue and transplanting cornea allograft to patient depending on the infection side and level. In this regard, there are some commercially available products in the clinic ³. However, further researches are still required to understand working mechanism of cornea and fabricate alternative products to corneal allografts.

Researchers developed first 2 dimensional (2D) cell sheet products as treatment only one layer of cornea or diseased part of the cornea using limbal stem cells ⁴. Then these studies were followed by construction of more complex cornea grafts ⁵. However, new alternatives were needed for patients who suffer from limbal stem cell deficiency (LSCD) to form patient specific tissue constructs. For those people, iPSCs are promising alternatives to generate cornea like structures (cornea organoids) to mimic cornea in *in vitro* for finding better solutions ⁶. Even though early 3D cornea organoid studies, reported by two different research groups using iPSCs, in both developed cornea organoids, orientation of collagen fibers are not similar to native human cornea and air liquid interface of cornea was neglected. ⁷ So, they need additional developments to obtain more reliable native like structure. It has been reported that structural and functional differences of static and dynamic cultures have been observed when the construct were transplanted to the animals ⁸. Hence, studies have been moved to 3D dynamic culture using polymer based hydrogel scaffold to mimic nature of extracellular matrix (ECM).

In this thesis, iPSC derived cornea organoid culture was studied within microfluidic platforms to mimic native like cornea microenvironment in the presence of decellularized cornea and polymer based hydrogels ^{9,10}.

1.2. Purpose of the Research

The purpose of this thesis is to develop 3D biomimetic microfluidic platform for generation of human induced pluripotent stem cells derived cornea organoids. Through this platform, we aim to provide uniform alignment of collagen fibers of generated cornea organoids.

1.3. Hypothesis of the Research

In our study, our hypothesis states that microfluidic platforms can be a promising alternative to generate more reliable in vitro cornea organoids models.

2. GENERAL INFORMATION

2.1. Tissue Engineering

Tissue engineering (TE) term was firstly suggested by Professor Yuan-Cheng Fung, from the University of California, San Diego in 1987 ¹¹. Humans have been suffering from acute diseases and traumas which alter cells, tissues and organs ¹². Hence, there has been increasing demand for construction of functional tissues that can mimic architecture and functions of human body including crucial microenvironment ¹³. To meet these demands, TE combines life sciences and engineering technologies through imitation of nature ¹⁴. Main idea behind the TE is to develop reliable and sustainable therapies for patients. Basically, TE approach can be compartmentalized into four different subtopics: isolation of cells, expansion of cells in bioreactor systems, seeding and culturing these cells onto/into different types of biomaterials to construct new tissues and integration the final construct into the living system ¹⁵. These main principles of TE are shown in Figure 1. However, integration of developed tissue construct into clinics is extremely challenging and there are many criteria to be considered.

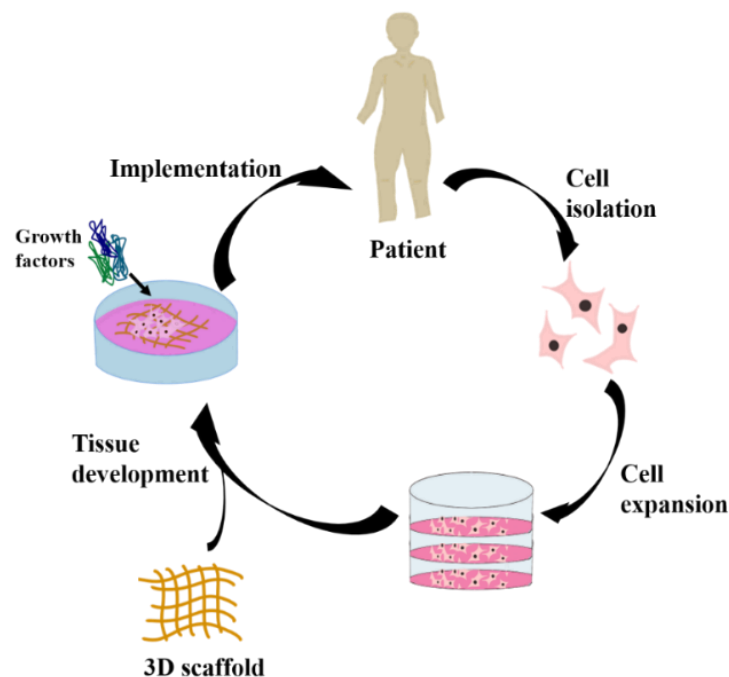


Figure 1. Schematic representation of tissue engineering approach.

In tissue engineering applications cell source must be selected considering the final tissue construct. Cells could be autologous, allogeneic and xenogeneic depending on the cell source. Regarding to that, the immune acceptance and animal sourced contaminant must be concerned. Additionally, different cell types such as stem cells and muscle cells, may be involved to construct a tissue which also need immune acceptance for both cell types ¹⁶.

Cells may be integrated into biomaterials to be used as complementary products for dysfunctional tissue and organs. Researchers have been continually developing new biomaterials that could contribute to tissue regeneration. Regarding these studies, regeneration mechanism of biomaterials in TE application rely on two approaches, cell-based and cell-free regeneration ¹⁷. Cell-based regeneration is integration of engineered device or any kind of product with living cells. Cell-free regeneration is implementation of engineered products into target tissue, which triggers the migration of host cells to inflammation site for healing. For either cell-based or cell-free type, stimulation of tissue growth is depending on the physiological, biological, chemical, and mechanical properties of biomaterials which serve as 3D scaffold for cells ¹⁸. Additionally, soluble molecules or any adequate triggers to induce controlled cellular behaviors may be used to construct 3D tissue that could mimic the native tissue functionally and morphologically.

Final and most critical point of TE is integration of this system into living organism. This step mostly starts with long-term animal experiments, then followed by clinical trials. However, diversity of experimental animals for different disease models is not enough to obtain accurate results for human diseases. Even though some diseases are successfully treated using animal models, many methods may not work with humans. Additionally, most challenging point is immune acceptance. Implementation of foreign construct into human body may results with inflammation, destruction of host tissue or rejection of implanted construct ¹⁹. If researchers successfully overcome this issue, the barrier for treatments of many diseases would be eliminated.

2.2. Tissue Engineering in Ophthalmology

Ophthalmology is a discipline in medicine that carries out researches and treatments for ocular system. Ocular system is comprised of eyes, optic nerve and ocular components such as eyelids, lacrimal apparatus and ocular muscles. Anatomy of eye is composed of sclera, cornea, iris, lens, retina, aqueous and vitreous humor, arteries and veins which supply oxygen ²⁰ (Figure 2). Histologically, eye consists of epithelial, mesenchymal, connective, and neural tissue and alignment of these tissues directly regulates the vision.

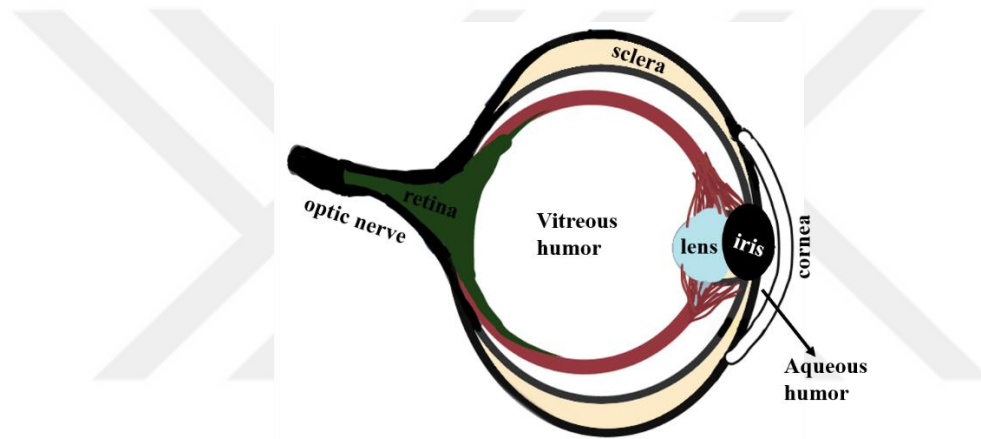


Figure 2. Schematic representation of the eye.

Researchers have been investigating tangible solutions to repair defects in ocular system. Common purpose of these studies is to form tissue constructs, which are similar to native tissue functionally and morphologically. To obtain engineered constructs in ophthalmology, cells were manipulated with growth factors, signaling molecules and other bioactive agents. This is followed by combination of biomaterials to mimic native tissue ²¹. For this purpose, stem cells have been studied for a long time to repair damage ocular tissues using their differentiation capacity. Corneal epithelial stem cells, retinal progenitor cells (RPCs) and bone marrow stem cells (BMSCs) are most common studied cell types for ocular repair ²².

Limbal stem cells (LSCs) are responsible for tissue regeneration of cornea due to their high stemness property ²³. LSCs are found in limbus, which is located

between cornea and conjunctiva. Corneal and LCSs are able to change their phenotype based on the cellular signals due to their high cellular plasticity ²⁴. So, one unipotent LSC is able to produce a transient amplifying cell (TAC) which can proliferate easily by standing in the G0 phase to provide cornea regeneration during wounding ²⁵. In clinic, corneal transplantation is not a solution to recover the vision loss in the absence of LCSs. In such cases, extra LCSs were transferred to patient to trigger formation corneal epithelium ^{26,27}.

Other cell type, retinal progenitor cells, located ciliary marginal zone, are multipotent and capable to self-renew ²⁸. Discovery of RPCs is a hope to form and transplantation of engineered retina for people who suffer from retinal degenerations. However, isolation of RPCs was may be challenging due to limited number of them. Studies reported that, grafting of RPCs into degenerating retina of a mice resulted with differentiation of these cells into neurons and specific cell types that express some photoreceptor markers ²⁹. BMSCs are can be alternatives to limitation of RPCs. BMSCs are founded in mainly stromal layer of cornea. Even though transplantation of these cells may arise to stromal repair, they are not able to differentiate into corneal epithelial cells ³⁰. Additionally, BMSCs are commonly used for treatment of retinal damages. Researches revealed that stem cells are promising source for treatment of ocular diseases in tissue engineering applications due to their plasticity.

Cellular studies have been combined with biomaterials to produce tissue constructs that can mimic native tissue in eye. This tissue engineered constructs have been produced mainly for cornea, lens and conjunctiva. As reported by Wolter and co-workers, cornea and retina respond to foreign materials ³¹. According to Wolter's studies, poly (methyl methacrylate) (PMMA) based prosthesis (for optical zone) with Teflon edge was successfully inserted into corneal stroma and did not cause any inflammation in eye. He also reported that eye was able to form newly membrane to allocate the prosthesis from the anterior chamber. Another study of ophthalmic tissue engineering showed that constructed of 3D human native like

cornea was biocompatible and consist of three layers of human cornea ⁵. Tissue engineering studies for cornea will be discussed in the next chapter.

2.2.1. Cornea Tissue Engineering

Human cornea, located in front of eye, is outermost layer of eye and acts as a physical and biological barrier for eye. It is an avascular tissue and connected with the sclera and nerve endings, which provide to sense mechanical stimuli and temperature changes. Diameter of human cornea is approximately 11.5 mm horizontally, 10.5 mm vertically and 7.8 mm anterior radius of curvature. It has a great role on vision by refracting light to retina owing to its refractive index (1.3375) ¹. It is comprised of three layers and supportive membranes between them: epithelium (outmost layer), basement membrane, Bowman's layer, stroma (middle layer), Descemet membrane and endothelium (innermost layer) (Figure 3).

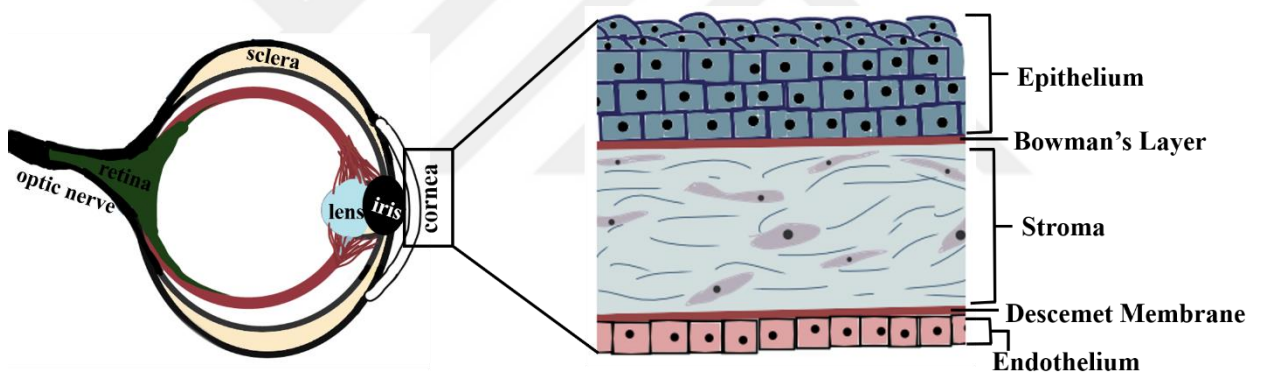


Figure 3. Schematic representation of cornea tissue.

Epithelium, 50 μm in thickness, consists of around four and six layers of non-keratinized epithelial cells which are unstable cells and able to grow rapidly to stabilize protective barrier ^{1,32}. Inner two or three layers are flat with microvilli and micro plicae at top. Epithelium is responsible for refraction property of cornea for vision. Corneal epithelial cells (CECs) have 7-10 days lifetime and they systematically go through apoptosis. Some factors, such as and transforming growth factor (TGF- β 1) and platelet-derived growth factor (PDGF) regulate differentiation of LSCs into CECs to replace the apoptosis cells. Tight junctions are located around the cells in

epithelium and act as waterproof barrier along with the blocking pathogen passages into stroma.

Basement membrane, located between epithelium and stroma, consists of basal epithelial cell and acts as a barrier. Transmission electron microscopy (TEM) images revealed that basement membrane comprised of collagens, laminin and heparan sulfate proteoglycans. Following component is Bowman's layer, which is an acellular, consisting of collagen fibers and about 8–12 μm in depth.

Stroma, largest part of cornea, is about 500 μm thickness in humans and plays a role on structural support of cornea. Stroma also gives cornea its transparency owing to crystalline proteins, which obstructs light scattering. Additionally, stroma composed of Type I and Type IV collagen in a lattice-like alignment, proteoglycans (especially lumican and keratocan), glycoproteins and keratocyte cells that produce collagens in the stroma.^{32,33} Proteoglycans are responsible to support structure, neutrophil movement while infection and arranging of distance between collagen fibers to diminish scattering of the light³⁴. Alignments of collagen fibers and concentration of proteoglycans in stroma differs with depth. Additionally, stroma includes dendritic cells and macrophages to support corneal immunity^{35,36}.

Descemet membrane, about 10 μm in thickness, is located between stroma and endothelium and helps to preserve corneal dehydration. This membrane is mainly comprised of collagen lamellae (collagen type IV and VIII fibrils) and proteoglycans. Collagen Type VIII is Descemet membrane specific collagen in the human body. Collagen fibrils in the cornea exhibit lattice-like alignment with periodically 110 nm intervals between each other¹. Additionally, low amount of laminin, fibronectin, keratan sulfate and heparan sulfate are found in Descemet membrane to preserve transparency.

Endothelium consists of cornea endothelial cells and responsible for dehydration of stroma over the ionic pumps to expand constancy of light through cornea³⁷. Tight junctions and gap junctions are present around cell membrane of

endothelial cells to balance hydration gradient inside the cells through ion changing³⁸. Additionally, cornea endothelial cells do not undergo mitosis in vivo and they are replaced through migration mediated by actin filaments that protect cell shape.

Cornea lies in an air-liquid interface which comprised of tear films and aqueous humor that protects cornea and carries sufficient amount of nutrients for cells³⁹. In adult human, flow rate of the aqueous humor is approximately 1.5–3.0 $\mu\text{L}/\text{min}$ by ciliary⁴⁰ and flow rate of tear films into cornea is almost 1.2 $\mu\text{L}/\text{min}$ (ranges from 0.5-2.2 $\mu\text{L}/\text{min}$)⁴¹. Hence, corneal cells are subjected to fluidic flow to balance homeostasis and to promote recovery of injuries in cornea³⁸.

Approximately 285 million of people suffer from blindness and corneal diseases are one of the major reasons behind it². There are two common approaches as cure for corneal diseases: removal the source of disease such as infection and corneal transplantation²⁰. However, corneal transplantation may not be frequent due to limited number of donors. In this regard artificial corneas could be potential alternatives to donor corneas in clinic. They can be organized as whole cornea or only to treat diseased part of the cornea using corneal tissue engineering approaches. Corneal tissue engineering mainly involves that scaling up corneal cells using biomaterials as a scaffold to, cellular engineering to form different layers of the cornea.

Researchers have been working on cell-based cornea regeneration for a long time. Initially, reconstruction of only one layer of cornea was performed using stem cells or patient derived cells to form cell sheets as monolayer 2D structures. In traditional 2D cell culture, removing of the adherent cells from a culture plate is performed with enzymes and it cause detachment of cells from each other. Okano and co-workers have solved this issue by covalently bonding *N*-isopropyl-amide to culture plate to peel cell layer from the plate without any disruption⁴. They cultured corneal epithelial cells to form corneal cell sheets and transplanted to the patients successfully in clinic. Another reconstruction of corneal epithelium cell sheet was performed by Nishida and co-workers for patients who suffer from bilateral LSCD⁴³.

In this study, patient's own oral mucosal cells were cultured on 3T3 fibroblast cells (as a feeder layer) with presence of mitomycin C and after 14 days cultivation, temperature was reduced to 20 °C from 37 °C to detach oral epithelial cells from the feeder layer. The final cell sheet was successfully transplanted to patients after removal of scar tissue from corneal epithelium without need of suture. Resulting corneal epithelium cells sheets might be an excellent alternative treatment method for patients who have deficiency on their corneal epithelium. Recently, similar method was performed to produce corneal epithelial cell sheet using oral mucosal epithelial cells and transferred to patients who have also bilateral LSCD by Kim and co-workers. This study showed that entire epithelization on ocular surface was possible and visual ability can be developed with this cell sheet ⁴⁴. The final success rate of this study was 75 %. Another study was performed by Kaluzhny and co-workers to mimic drug permeability of cornea in *in vitro*. 3D corneal epithelium tissue was formed using culturing corneal epithelial cells on the porous membrane at air-liquid interface and it has been reported the final cornea tissue model showed gene expression and permeability similar to native human cornea epithelium ⁴⁵.

Reconstruction of stroma was achieved by Syed-Picard and co-workers using corneal stromal stem cells (CSSCs) without using any scaffold or coating material ⁴⁶. CSSCs were cultured on micro patterned Polydimethylsiloxane (PDMS) substrates, which assist cells to produce ECM in an alignment like native tissue. Final cellular and collagenous stromal sheet was separated manually from the PDMS substrate and transferred into mice. After 5 weeks of *in vivo* culture, Syed-Picard and co-workers accomplished similar collagen fiber structure and transparency of native cornea.

Corneal endothelium has been reconstructed using different originated stem cells. Hatou and co-workers showed that corneal stroma successfully promotes formation of tissue engineered cornea endothelium ⁴⁷. Cornea-derived precursor cells were isolated from corneal stroma and cultured in presence of retinoic acid and glycogen synthase kinase to form cornea endothelium sheet. Then, it was

successfully transplanted into rabbit and support corneal transparency and shape without causing edema. Another cutting-edge approach was accomplished by Bourget and Proulx using self-assembly method to create endothelialized stromal substitute ⁴⁸. This final substitute expressed endothelial cell markers, Na⁺/K⁺-ATPase and Na⁺/HCO₃⁻ transporter pumps, which makes it potential treatment for endothelial diseases.

Fabrication of engineered complete cornea construct was successfully achieved by Proulx and co-workers. Corneal epithelial and endothelial cells were cultured two sides of stromal cells to mimic whole corneal structure. The final construct revealed that engineered human cornea was shown same characteristics with native human cornea, comparing expression of tissue specific markers and TEM images. However, the final results showed that there was not any sign for presence of Descemet's or Bowman's membrane ⁵.

2.3. Biomaterials in Cornea Regeneration

Biomaterials have significant role on interactions between targeted cells/tissues and their environment. Suitable biomaterials must have desired mechanical properties and allow cell-cell and cell-material interactions, release of therapeutics such as soluble growth factors, low molecular weight drugs, nucleic acids, peptides, and proteins through material depending on purpose of use ⁴⁹. For corneal regeneration, there are some commercially available products in clinic such as PMMA, Polyhydroxyethylmethacrylate (PHEMA), PDMS-PVP and Poly(phenyl ethyl acrylate-co phenyl methacrylate) ³. However, further researches are still required to overcome immune rejection of materials and to fabricate alternative products to corneal allografts. Hence, cornea tissue engineering studies aims to produce new biomaterials that can mimic native cornea. There are some ongoing researches on development of natural and synthetic biomaterials such as collagen, gelatin, Polyvinyl acetate (PVA), Poly(ethylene glycol) (PEG), keratin, silk and amniotic membrane and decellularized corneas using biomimetic approaches ^{50,51}. These polymer based soft materials have been commonly used as hydrogel forms to mimic 3D structure of ECM. Their hydrophilicity enables to absorb water or biological fluids under traditional

culture conditions. Polymer based hydrogels are sensitive to different stimulus such as heating, pH, light, and chemicals depending on their concentration and chemical structure. Therefore, they have been used as drug carriers and self-healing materials⁵². Soft materials for corneal tissue engineering have been dominating future corneal tissue engineering materials due to their biocompatibility, tunable biodegradability and porous structure. However, their use may be limited due to their low mechanical stability and strength. Current studies have been focusing on improving mechanical strength of hydrogels and responses to different stimuli's by modifying polymer chains or changing the crosslinking methods^{53,54}. So, these developed new biomaterial based cornea products can be an alternative to donor corneas¹⁸.

2.3.1. *Gelatin Based Hydrogels*

Collagen is the most abundant protein in human body with a complex hierarchical conformation within ECM. It mainly founds in skin, bone, cartilage and tendons⁵⁵. Its hydrolysis product is called gelatin which is water soluble protein and contains RGD and MMP sequences which promote cell-matrix interactions⁵⁶. Therefore, it has been commonly used in tissue engineering applications as scaffold. These gelatin based hydrogel scaffolds are biodegradable materials and promote cell survival, adhesion, differentiation and proliferation due to microporous structure of the scaffold⁵⁶.

The addition of methacrylate groups to gelatin provides a photo-crosslinkable hydrophilic polymer which referred as gelatin meth-acryloyl (GelMA)⁵⁷. Photo-crosslink ability of GelMA is desirable for cell culture studies to fabricate tailored scaffolds instantly. GelMA based 3D hydrogel scaffolds are permeable structures that allow flow of biochemical factors and oxygen through it⁵⁶. Hence, gelatin-based hydrogels have been extensively used within the microfluidic systems to generate complex native like tissue constructs under dynamic conditions.

In cornea regeneration, gelatin based hydrogel materials are used as carriers for controlled release of drugs and to form cornea tissue layers in *in vitro*⁵⁸. A study proved that gelatin hydrogels can be used to form native like aligned cornea

endothelial cell sheet owing to transparency, permeability and elastic modulus ⁵⁹. However, these hydrogels are not mechanically stable due to lack of fibrous structure. Tonsomboon and co-workers reported that combination of alginate and gelatin fibers may increase elastic modulus of developed structure for cornea regeneration ⁶⁰. Another study also reported that gelatin scaffolds promotes formation of cornea endothelial cell sheet ⁶¹. All of these studies proves that gelatin based hydrogels can be an alternative to construct different layer of cornea for further transplantations.

2.3.2. *PEG Based Hydrogels*

PEG is one of the most well-known hydrogel material. Molecular structure of PEG is $\text{H}-(\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{OH}$. PEG hydrogels can be formed by crosslinking either via physically or chemically interactions. PEG based hydrogel structures have desirable permeability, elasticity, modulus and degradation rate ⁶². PEG hydrogels are commonly synthesized by photo-polymerization method. These hydrogels are biocompatible and support cellular behaviors owing to their porous structure, which allows diffusion of nutrients and biomolecules. Therefore, they have commonly preferred in tissue engineering applications especially in the drug delivery systems ⁶².

In cornea regeneration, PEG based hydrogels films have thriving impact due to their transparency, biocompatibility and biodegradability ⁶³. It has been reported that PEG can be combined with PHEMA and this developed hydrogel may be an alternative to corneal allografts ¹⁸. PEG have been commonly modified with different peptides and molecules that improve the cell-matrix interactions to promote cellular behaviors. As reported by two study, conjugation of PEG and collagen-like peptides resulted with increasing corneal tissue regeneration and innervation ^{64,65}.

2.3.3. *PVA Based Hydrogels*

PVA is a hydrophilic polymer, containing hydrogen bonds between polar hydroxyl groups which gives PVA water solubility and ability to adhere ⁶⁶. PVA-based hydrogels are commonly formed through physical and chemical crosslinking ⁶⁷.

Physical crosslinking method is repeating freezing and thawing cycles and resulted with higher stability of hydrogen bonds between polymer chains to form crystallites. Properties of PVA based hydrogel are depends on the polymer content, temperature, number of freeze-thaw (FT) cycles and duration of FT. Chemical crosslinking forms covalent bonds between hydroxyl (-OH) groups of polymer chain. For that purpose, there are different crosslinking agents such as glutaraldehyde, borates, DMSO, DMF^{66,68}. However, these chemical agents may cause toxicity of hydrogel for cell culture studies. Recent studies showed that PVA based hydrogels can be produced by combination of different crosslinking methods to improve properties of hydrogel⁶⁹.

PVA has been used as hydrogel material for cornea tissue engineering applications as artificial cornea matrix owing to its transparency, biocompatibility, mechanical stability, porous structure and degradation capacity^{66,70}. Despite these well out-standings, PVA has some limitations, such as, lack of cell-adhesive property⁶⁸. However, it is reported that addition of other type of polymers such as alginate, cellulose, gelatin and heparin to PVA during gelation can overcome such limitations and enhance mechanical stability, porosity, non-toxicity and degradation capacity of PVA based hydrogels⁶⁶. In corneal regeneration, PVA can be also conjugated with different polymers, commonly with collagen to produce suitable hydrogel scaffolds for corneal regeneration. Combination of PVA with collagen resulted with higher mechanical stability of developed scaffolds, improved cell adhesion and proliferation^{71,72}.

2.3.4. *Decellularized Corneas*

Other native like biomaterial is decellularized cornea, showing similar structure of native corneas. Decellularized corneas recapitulate ECM structure and orientation of collagen fibers which is mainly responsible ocular functions in native cornea⁵¹. Decellularization of corneas can be performed by different methods: lyophilization, hydrostatic pressure and chemicals, mostly detergents⁷. Detergents disturb the hydrophobic associations and lipid bilayer to solubilize membrane proteins while maintaining the stromal structure of the cornea. However, decellularization of

corneas has not been achieved completely (100 %), small amount of cells have been observed on the decellularized corneas. This was proved in the study of Gary Yam and co-workers. Use of sodium dodecyl sulfate (SDS) as chemical agent for decellularization successfully removed cells from cornea while maintaining collagens and glycosaminoglycan ⁷³. Additionally, decellularized cornea was recellularized with keratocytes and successfully transplanted to rabbit with no immune response. Another decellularization study performed by Zhang et al., used Triton X-100. Then, these decellularized corneas were successfully transplanted to the patients who suffer from keratitis ⁷⁴. During decellularization, corneas lose some of their transparency, then they have been incubated in glycerol for a while to obtain transparency back and to reduce swelling amount of corneas ⁷⁴. Glycerol also enables long term storage of cornea due to its antimicrobial ability ⁷⁵.

2.4. Microfluidic Systems

Main purpose of tissue engineering applications is development of tissue constructs and whole organs to evolve newly alternatives for people who suffer from organ shortage ⁷⁶. However, traditional 2D cell culture setups cannot fulfill the requirements to mimic native like tissue structures and organs due to inability of supplying oxygen and nutrients to cells ⁷⁷. This revealed need of newly designed platforms, called microfluidic systems, which enable to incorporate conventional cell culture setups with up-to-date techniques to develop 3D biomimetic organ models in *in vitro* dynamic conditions ^{78,79}. Microfluidic systems, also called organ-on-chip systems, comprised of different sized and shaped micro channels to deliver oxygen and nutrients to cells. This systems overcome hypoxia and nutritional deficiency of 3D cellular structures to trigger cell adhesion and proliferation ⁸⁰. Recently, organ-on-chip models have been developed to mimic liver, lung, kidney, heart, blood vessels, intestine, bone marrow, cartilage, bladder, skin to understand of biological processes like disease modeling, drug testing ^{77,81}. Additionally, these devices can be also an alternative to animal models in *in vitro*. In that way, less amount of animal might be sacrificed in the near future.

There are some criteria to design an ideal microfluidic system: cell types, biomimetic scaffolds, forming micro channels, readout of data from the platform ⁸¹. Primary cells, stem cells, cell lines (mostly isolated from same source with the organ to be mimicked inside the microfluidic platform) can be used depending on the purpose of the research. Organ specific supporting cells, such as fibroblasts as feeder layer, can be also cultured with main cell types to increase maturation of developed tissue ⁸².

In dynamic platforms, cells need to be cultured on/in biomimetic scaffold to support them to create tissue structures ⁷⁷. Structure and chemical composition of scaffold need to ensure the necessary cellular microenvironment to promote cellular behaviors such as differentiation and maturation of cells, cell adhesion to construct a tissue. Moreover, scaffold should be mechanically stable under fluidic pressure and allow carrying the electrical or optical signals and biochemical agents depending on the application ⁷⁸. In an ideal perfusion system, porosity of scaffold is highly important to allow continuous flow of culture medium and biochemical triggers through chambers ⁸³. For example, functional biomimetic 3D dynamic platform has been successfully developed under electromechanical stimulation to mimic heart muscle construct ^{84,85}. Developed functional lung-on-chip device also offer stable microenvironment during mimicking mechanical stretch of lung alveoli ⁸⁶.

Micro channels in organ-on chip system must be formed regarding formulations of fluid mechanics. If there are more two or more streams into same channel, fluids inside these channels must stream without turbulent mixing ⁸⁷. Diffusion through interface of these two streams enables to culturing two different cell types in one platform to evaluate interactions of these cells with each other under different drug tests.

Final criteria to design suitable microfluidic platform is obtaining readouts of how constructed tissues give feedback to physical, chemical or molecular triggers in long term. So, microfluidic systems must be fabricated using transparent materials to allow to optical imaging with suitable biocompatibility. It also must be convenient for

handling and forming micro channels to deliver culture medium continuously ⁸¹. Therefore, PDMS and PMMA have been commonly used to fabricate microfluidic systems ⁸⁸.

In ophthalmology, microfluidic systems have been used for a long time mainly to mimic cornea, retina, contact lens for *in vitro* therapeutic drug tests (drug absorption and permeability) to obtain more reliable results comparing to animal studies due to characteristic differences between human and animal models ^{8,89}. Another purpose of use of microfluidic platforms is to evolve new strategies to form organoids under dynamic conditions and increase their reproducibility in *in vitro* ⁹⁰. However, these *in vitro* models fail to recapitulate the ocular microenvironment for pre-clinical studies and detailed investigations should be performed.

In cornea microfluidic systems, cornea structure and its microenvironment have been developed to mimic layer by layer form of cornea with specific function or whole complex corneal structure in one platform ⁸⁹. First, whole cornea structure was fabricated by Minami *et al.* in collagen matrix ⁹¹. These studies followed by another nifty research that showed development of epithelium and stromal layer of cornea in one dynamic microfluidic platform using transparent collagen vitrigel (CV) membrane as scaffold to support cell growth to be used in permeability tests of cornea ⁹². Fluidic pressure is main router to form bilayer tissue construct on both side of CV membrane. Bennet and co-workers also developed a system to evaluate absorption and permeability of formed corneal structure which comprised of corneal epithelium, basement membrane and Bowman's layer under dynamic conditions ⁸. In this platform, human corneal epithelial cells were cultured on functionalized porous polycarbonate membrane, which acts as basement membrane and Bowman's layer under continuous and pulsatile flow. And, it has been reported that pulsatile flow can be an alternative model to mimic drug permeability of cornea on chip systems. Recently, another 3D cornea-on-chip model was fabricated using a cutting-edge method by Bai and co-workers to contribute corneal drug trial studies ¹⁰. Primary epithelial and endothelial cells were cultured separately in two different channels to act as stromal layer. Between collagen stromal layer and epithelial layer,

there was another thin collagen layer in epithelial channel to serve as Bowman's membrane.

2.5. Induced Pluripotent Stem Cell (IPSC) derived cornea organoids

Pluripotent stem cells are primordial cell that can differentiate into wide range of cells, renew themselves and stabilize their potency level ⁹³. Stem cells are divided into categories depending on their potency capacity. Embryonic stem cells (ESCs) are the ones, which can differentiate into any cell types. However, use of ESCs in the research and clinic brings ethical issues due to being embryonic sourced cells. This revealed need of new cell type, which has same self-renewable and potency capacity with ESCs. These cells are called IPSCs and reprogrammed fibroblast cells into ESC-like cells by Yamanaka and Takahashi ⁹⁴. It has been reported that IPSCs can differentiate any cell types in human body such as islet cells, cardio myocytes, endothelial, epithelial, neural cells etc. ^{95,96}. Discovery of IPSCs overcome ethical issues of ESCs, immune rejection risk in clinic and become a groundbreaking opportunity for cellular therapies to generate tissues and organs ⁹⁶. Even though, differentiation of IPSCs into different cell lineages is promising source, this 2D cells may not be able to mimic tissue and organ morphogenesis in standard petri dishes. In this respect, researchers have formed 3D cell structures called organoids. Organoids are self-organized 3D structures which enable to recapitulate physiology and function of represented organ *in vitro* ⁹⁵. Organoids can be formed by differentiation of ESCs, PSCs but mainly IPSCs under ideal culture conditions (extrinsic signals, growth factors, extracellular matrix and culture media) ⁹⁷. Initially, IPSCs differentiate into one of the main three germ layers (endoderm, mesoderm and ectoderm) based on the developmental process of represented organ. Then appropriate culture conditions direct further differentiation into 3D organoid structures ⁹⁸. It has been reported that retinal organoids are derived from ectoderm layer; stomach, liver and lung organoids are derived from endoderm layer; cardiac muscles are derived from mesoderm layer ⁹⁵.

In eye development, IPSCs have been mostly used to form 3D retina and cornea organoids ⁹⁶ while a few lens organoids have been developed ^{99,100}. For

corneal structure development *in vitro*, limbal stem cells are responsible for different corneal cell formation. However, iPSCs are one of the main source for corneal epithelial cell differentiation for people who suffer from LSCD both in clinics and in research ⁶. Besides that, it has been reported that iPSCs can be used to generate more cell types in cornea such as endothelial cells and keratocytes for corneal disease modeling and development new therapeutic like cell sheets ^{7,101}. Corneal organoids must be comprised of corneal epithelium, stroma including collagen fibers, Bowman's membrane/Descemet's membrane, and endothelium layer ¹⁰. Recently, a couple of innovative study have been published on generating of corneal organoids derived from human iPSCs ^{102,103}. In these studies, corneal organoids, approximately 7 mm in size, have been developed under appropriate growth factors and small molecules that inhibits Notch and Wnt signaling pathways to recapitulate cornea in petri dishes. Gene expression level of these corneal organoids proved the presence of corneal epithelium, stroma including collagen fibers, Descemet's membrane and endothelium like mature native cornea. However, in both developed structure, orientation of collagen fibers are not similar to native human cornea and their size and shape are not homogenous like collagen fibers on native stromal layer ⁷ and they need further developments to obtain complete native like structure.

Combination of organoids and microfluidic technologies have been reported by Seo *et al.* use of microfluidic systems resulted with more accurate microphysical and chemical microenvironment for organoids. In this study, cells were successfully cultured on 3D printed cornea shaped porous scaffold to mimic cornea and conjunctiva under dynamic conditions and final construct pave the way to mimic blinking of the eye with organization of gelatin based hydrogel which acts as eyelid ¹⁰⁴. Combination of organoid and microfluidic technologies may be groundbreaking progress to create whole native like eye structure and ocular microenvironment *in vitro* to understand biological process in the eye and to form alternative allografts ⁸.

3. MATERIALS & METHODS

3.1. Type of Research

This study encompasses *in vitro* experiments and based on cell culture techniques.

3.2. Time and Location of Research

All of the research was conducted between September 2017 - March 2020 at Izmir Biomedicine and Genome Center.

3.3. The Universe and Sample of Research

In this thesis primary human cornea samples were used.

3.4. Materials of Research

In this thesis, 3T3 mouse fibroblast cells were obtained from Assoc. Prof. M. Kasım Diril. Transfected GFP (+) 3T3 cells, LX2 Azure cells and hIPSCs were obtained of Prof. Dr. Esra Erdal Bağrıyanık.

3.5. Variables of the Research

Independent Variables: DMEM high glucose medium, FBS and Pen-strep concentration, IPSC differentiation medium components and duration of culture.

Dependent Variables: structural properties of polymer based hydrogel scaffolds, flow rate of microfluidic system, gene expression in protein and RNA level.

3.6. Data Collection Methods

3.6.1. PDMS Mold Fabrication

Silicone elastomer kit (SYLGARD,184) was used to fabricate PDMS molds with a concave shape. Molds were used for shaping of hydrogels. 10 % of curing agent was combined with polymer base were mixed homogenously. Later, air bubbles inside the mixture were eliminated using a desiccator. Master mold was designed using 10 mm and 5 mm diameter marbles and PMMA to yield a depth of 3 mm and 5 mm concave shaped molds (Figure 1A). Final mix was incubated at 80 °C for 3 hours and fabricated PDMS mold is shown in the Figure 4B and 4C.

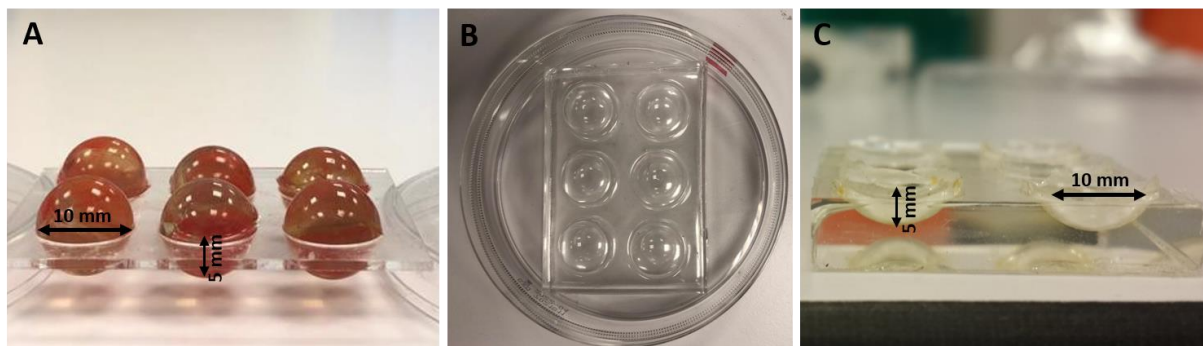


Figure 4. Concave shaped PDMS mold. A) Design of master mold, B) Fabricated PDMS mold, C) Side view of fabricated PDMS mold.

3.6.2. *Hydrogel Synthesis*

GelMA, PEG and PVA based hydrogels were synthesized using different protocols.

3.6.2.1. *GelMA Based Hydrogel*

According to Loessner's protocol, GelMA was synthesized in three steps: reaction, dialysis and lyophilization⁵⁷ (Figure 5).

Reaction step aimed to yield a 10 % (w/v) GelMA concentration. Firstly, PBS was preheated to 50 °C and dissolved completely on a magnetic stirrer for ~1 hour; until the solution became homogenous. Next, gelatin from bovine skin Type B (Sigma-Aldrich, G9382-100G) was slowly added into 100 ml 1X phosphate buffered saline (PBS, pH 7.4, 10 mM). Next, 10ml methacrylic anhydride (MA, Sigma-Aldrich, 276685) was added into the gelatin solution to obtain a final MA concentration of 10 % under a fume hood (in a containment BSL 2 laboratory).

Importantly, glass pipettes delivered 5x2 ml portions of MA carefully and successively. Plastic pipettes were avoided due to the liquid's toxicity. Solution was then stirred for 4 hours at 40 °C. Post methacrylation, solution was transferred into 50 ml falcon tubes and centrifuged at 3500 g for 3 minutes. Supernatant was collected into a clean glass beaker in order to remove pellet; containing unreacted

methacrylic anhydride. Preheated (40 °C) Ultrapure (UP) water as much as two volumes of collected supernatant was added into supernatant solution.

In dialysis step, final solution was transferred into dialysis membrane (Membra-Cel, 14-kDa MWCO). Then, solution inside dialysis membrane was placed into a plastic bucket which was filled with 40 °C UP water. Dialysis process was performed at 85 rpm at 40 °C on a linear shaker for 7-10 days. Aluminum foil was secured to lid of the bucket to observe unreacted methacrylate during dialysis. UP water in plastic bucket was refreshed daily and this process was continued until no unreacted methacrylate was present in the solution and pH value of the UP water was 7-8.

In lyophilization step, resulting GelMA solution was aliquoted into 50 ml falcon tubes and frozen at -80 °C for 2 days. Then, solution was lyophilized for 3-5 days until GelMA was completely dehydrated. Final GelMA was stored at +4 °C until further use. GelMA synthesis was shown in figure 5.

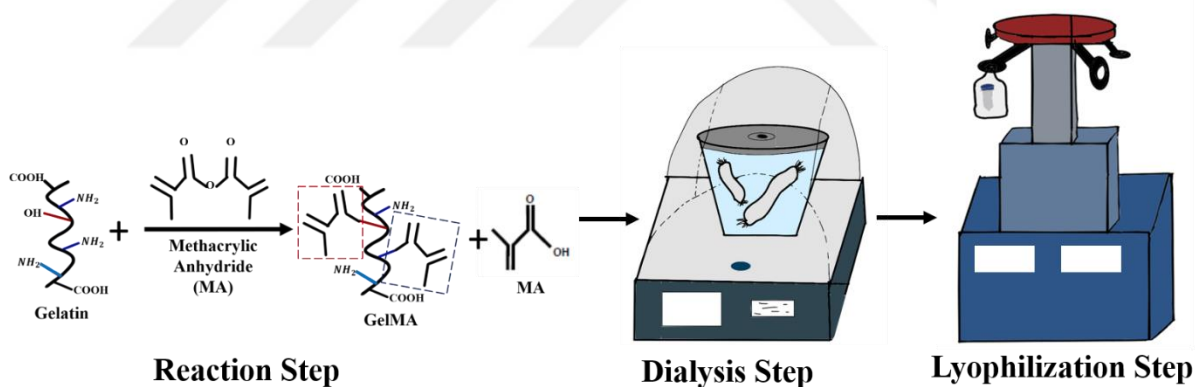


Figure 5. Schematic representation of GelMA synthesis steps.

In order to obtain a GelMA based hydrogel, a photo initiator (PI) (2-hydroxy-1-(4-(hydroxy ethoxy) phenyl)-2-methyl-1-propanone, Sigma Aldrich, 480896 - 10G) was used to provide cross-linking of GelMA under ultraviolet (UV) light. Firstly, precursor solution, 0.5 % photo initiator was prepared in pre-warmed 1X PBS (pH 7.4, 10 mM). A vortex was used to dissolve PI in the solution. Later, synthesized GelMA was dissolved in precursor solution to yield 5 %, 10 % and 20 % (w/v) GelMA

concentration. Final GelMA solution was filtered using a 0.22 μm syringe for future *in vitro* work. Then, pre-prepared concave shaped molds (Figure 4) were filled with final solution of GelMA and exposed to 500 mW/cm^2 UV light for 1 min to form a GelMA based hydrogel.

3.6.2.2. PEG Based Hydrogel

In order to provide cross-linking of PEG under UV light, PI (2-hydroxy-1- (4-(hydroxy ethoxy) phenyl)-2-methyl-1-propanone, Sigma Aldrich, 480896) was used. Firstly, precursor solution, 1 % and 2 % (w/v) photo initiator was prepared in pre warmed 1X PBS (pH 7.4, 10 mM). A vortex was used to dissolve solution. Later, PEG (Sigma Aldrich) was dissolved in pre-prepared 1 % and 2 % precursor solution to yield 20 % (w/v) PEG concentration. Final PEG-precursor solution was filter sterilized again with 0.22 μm syringe to obtain sterile for future *in vitro* works. Then, pre-prepared concave shaped molds were filled with final solution of PEG and then exposed to 500 mW/cm^2 UV light for 1 min to provide complete gelation of PEG.

3.6.2.3. PVA Based Hydrogel

PVA hydrogels were synthesized according to a combination of Ma and Kobayashi's protocols^{66,69}.

Dimethyl sulfoxide (DMSO) (Merck Millipore, 116743) was used as a crosslinking agent of PVA. Firstly, crosslinking solutions, 25 %, 50 % and 75 % (v/v) DMSO were prepared in de-ionized water (DIW) inside capped opaque glass bottles and heated at 110 $^{\circ}\text{C}$ under fume hood. Later, PVA powder was added slowly into the crosslinking solution to obtain final PVA concentration of 12 % under fume hood. And then, solution was stirred for one hour at 110 $^{\circ}\text{C}$ until PVA was completely dissolved. PVA-DMSO solution was transferred into concave shaped PDMS molds and cooled at -20 $^{\circ}\text{C}$ for overnight to promote crystallization and cross-linking of PVA molecules. Lastly, PVA based hydrogels were extracted from molds and immersed into phosphate-buffered saline (PBS) for 48 hours to remove DMSO inside hydrogels with PBS renewed every 24 hours. The final PVA based hydrogels were sterilized under UV for one hour without any PBS. Hydrogels were stored inside 1 %

Penicillin Streptomycin Antibiotic (Pen/Strep) in PBS buffer at +4 °C for further cell culture studies.

3.6.2.4. PVA and Gelatin Based Hydrogel

The crosslinking solution of PVA was prepared according to protocol, which was previously described in 3.6.2.3. Firstly, gelatin from bovine skin Type B (Sigma-Aldrich, G9382) was added into one-third of 110°C heated crosslinking solution and stirred for 30 min at 110 °C until gelatin was completely dissolved. Later, PVA was added into two-third of crosslinking solution and mixed with solved gelatin solution to yield a 12 % of PVA and 5 % of gelatin (w/v) concentration. Final solution was stirred for 1 hour at 110 °C until PVA was completely dissolved and transferred into concave shaped PDMS mold and cooled at -20 °C for overnight. Lastly, PVA-Gelatin based hydrogels were extracted from mold and immersed into PBS for 48 hours to remove DMSO inside hydrogels. Again, PBS was renewed every 24 hours. Final PVA-Gelatin based hydrogels were sterilized under UV for 1 hour without any PBS. Hydrogels were stored inside 1 % Pen/Strep in PBS buffer at +4 °C for further cell culture studies.

3.6.2.5. Agarose Gel Preparation

3 grams of agarose was weighted and dissolved into 100 ml DIW to yield 3 % of agarose solution. Solution was autoclaved in order to be used for coating of cell culture plates and stored at room temperature.

For coating cell culture well plates, agarose solution was heated in microwave and adequate amount of agarose solution depending on type of well plate (1 ml of agarose for 6 well plate) was added into wells and aspirated immediately to ensure that only the surface of well was coated with thin layer of agarose.

3.6.3. Isolation of Mice Corneas

C57BL/6 type mice were used for this study. Mouse cornea was used to optimize decellularization protocols due to limited number of human corneas. C57BL/6 type caged mice were transferred into an operating room for euthanasia.

Mice were carefully picked up by their tails to remove them from their cage and transferred into CO₂ gas chamber. Up to 3 mice were euthanized at one time. After euthanasia, an operation was performed under a stereomicroscope. Mice were individually placed under the adjusted microscope to focus on the eye. All procedures were performed under the microscope and each step is shown in Figure 6. Firstly, eye lobe was dislodged from eyelid by holding sclera on optic nerve with 0.5 μ m type surgical forceps. A small scratch was made at the edge of cornea with lancet number 11 to take it out cornea precisely. Then, cornea was removed gently from the iris by peeling with same lancet. Critical point was not to disrupt lens, which makes removal of whole cornea almost impossible. Contaminants of iris were inevitable but removed by peeling extracted cornea with lancet number 11 under stereomicroscope. Extracted cornea was stored in corneal chamber solution (Alchimia, CTC 001-01).

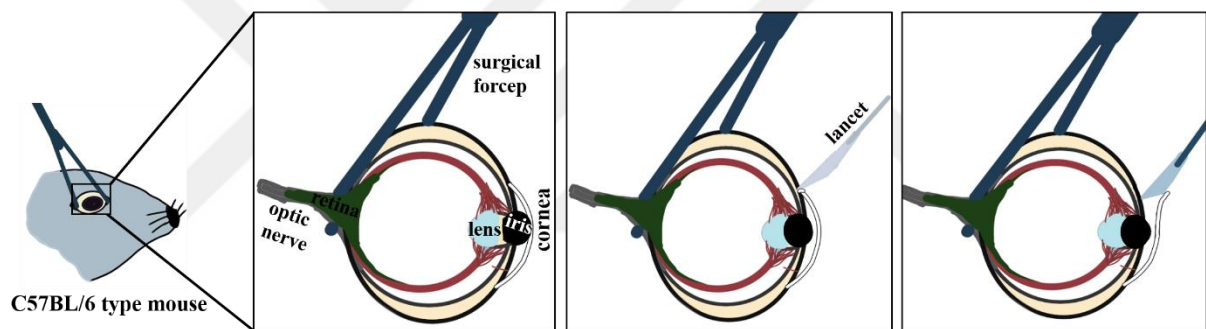


Figure 6. Schematic drawing of cornea extraction from mouse.

3.6.4. *Extraction of Sheep and Cow Corneas from the Whole Eye*

Whole eyes were supplied from a local slaughterhouse. Firstly, eyes were washed with PBS and a scratch was cut vigorously at the edge of cornea with a sterile lancet to take cornea out precisely. Then, cornea was removed from iris by peeling with the same lancet. Extracted cornea was stored in the aforementioned corneal chamber solution. Undesired iris, located at outer side of cornea were removed with lancet.

3.6.5. *Decellularization of Corneas*

Decellularization of corneas were performed with 0.5 %, 1 % and 2 % of SDS solution and 1 % and 2 % Triton-X solution based on the following published

protocol ¹⁰⁵. Mice corneas were decellularized with each chemical agent mentioned above. Sheep, calf and human corneas with different disease backgrounds which is shown in Table 1 were decellularized using SDS solution.

Before decellularization, corneas were kept in corneal chamber solution (Alchimia, CTC 001-01). Corneas were washed with 1X PBS (Gibco, 10010023) at RT and transferred into 5ml Eppendorf tube containing of approximately 2 ml decellularization solutions prepared in DIW. Eppendorf tubes were incubated and shaken at 200 rpm inside 37 °C incubator shaker for 2 days and SDS solution was renewed daily. At the end of the decellularization, corneas were washed with PBS for 1 hour. Later, for sterilization, corneas were transferred into new 5 ml Eppendorf tube which was filled with 70 % Ethanol and incubated and shaken at 200 rpm inside 37 °C incubator shaker for 2 days. 70 % ethanol was also renewed once a day. Then, corneas were washed with sterile 1X PBS for 1 hour and they were transferred into 1 % Pen Strep in PBS buffer at +4 °C. Before cell culture studies, the corneas were exposed to UV light for 1 hour.

Table 1. Information about decellularized human cornea samples.

Sample Code	Condition	Gender	Age
S1	Keratitis Scar	M	10
S2	Healthy	M	57
S2	Healthy	M	57
S3	Congenital Glaucoma	M	
S4	Decompense Graft	F	41
S5	Decompense Graft	M	47

3.6.6. Cell Culture

3.6.6.1. 3T3 Cell Culture

Dulbecco's Modified Eagle Medium (DMEM) high glucose (Gibco, 41965039) was used in cell culture for 3T3 cells. Complete DMEM high glucose medium

containing 10 % heat-inactivated Fetal Bovine Serum (v/v) (FBS, Gibco, 10500064) and 1 % Pen-Strep (Gibco, 15140-122).

In order to start a culture, frozen cells were thawed in 37 °C water bath quickly to avoid damaging cell membrane with presence of DMSO (Sigma-Aldrich, D2650). Once cells inside cryotube were thawed, 1 ml of complete DMEM high glucose medium was added into the cryotube and transferred into 15 ml falcon tube which contained 3-4 ml of pre-warmed complete DMEM high glucose medium. Later, cells were centrifuged at 1200 rpm for 3 minutes. DMSO containing supernatant was removed. Fresh complete medium was added into falcon tube for resuspension of cells. 3T3 cells were seeded on standard polystyrene T75 cell culture flasks in complete DMEM high glucose and incubated at 37 °C incubator with 5 % CO₂. Medium was refreshed within 2 days. When confluency was around 80-90 %, cells were passaged.

For sub-culturing, firstly, medium was removed from T75 cell culture flask and cells were gently washed with 1X PBS (Gibco, 10010023) to remove left over medium inside flask. Then, 2 ml trypsin-EDTA (0.25 %) (Gibco, 25200056) which was enough to cover the surface of the flask was added into flask and incubated (37 °C with 5 % CO₂) for 3-4 minutes to remove adherent cells from flask surface. Cells were examined under a microscope to see whether cells were removed or not. When all cells were removed from surface of flask, two volume of medium was added into flask to inactivate trypsin-EDTA. Suspended cells were transferred into a 15 ml falcon tube, centrifuged at 1200 rpm for 3 minutes and the supernatant was removed. Fresh medium replenished tube. After mixing cells and medium gently, 100 µl of cell suspension were resuspended in 200 µl of Trypan Blue (Biological Industries, 03-102-1B). A hemocytometer was used to count cells.

Cells were frozen in freezing medium which contained 10 % DMSO (Sigma-Aldrich, D2650) and 90 % FBS. To freeze, cells were trypsinized and centrifuged according to protocol above. After, supernatant was removed and an adequate volume of freezing medium was added into a falcon tube for resuspension of cells to

yield approximately $1-2 \times 10^6$ cells per ml. 1 ml of cell suspension was transferred into cryotubes and placed into Mr. Frosty Freezing Container (Nalgene, 5100-0001) and stored at $-80\text{ }^{\circ}\text{C}$. For long-term storage, vials were transferred to a liquid nitrogen tank.

3.6.6.2. LX2 Azure Cell Culture

Adherent LX2 Azure cell line, a human hepatic stellate cell line, was supplied from Erdal Stem Cell and Organoid Technologies Laboratory. DMEM high glucose (Gibco, 41965039) was used in cell culture for LX2 cells. Complete DMEM high glucose medium contains 10 % heat-inactivated FBS (v/v) (Gibco, 10500064) and 1 % Pen-Strep (v/v) (Gibco, 15140-122). LX2 cells were cultured according to the same protocol with 3T3 cell culture.

3.6.6.3. Spheroid Culture

To optimize hydrogels and microfluidic system, all experiments were repeated with spheroid culture as 3D structure like organoid culture. 3T3 cell line and LX2 cell line were used to form spheroids using the same hanging drop protocol (AU - Foty 2011).

In order to obtain 3D spheroids, cells were trypsinized, centrifuged and counted according to standard cell culture protocol above. Then, surface of 100 mm petri dishes were covered with PBS to avoid evaporation of drop-shaped medium. Cells were resuspended in adequate amount of medium to yield a 2×10^4 cell number per 10 μl droplet. 10 μl of medium-cell mix was taken with 10 μl pipette and dropped gently onto cover of petri dishes (Figure 7A). Approximately 50 droplets were placed onto cover of 100 mm petri dishes. Cover was gently enveloped without shaking cells (Figure 7B) and incubated at $37\text{ }^{\circ}\text{C}$ incubator with 5 % CO_2 for 5 days. Formation of spheroid culture was controlled using light microscopy imaging method (Figure 8A) and viability of cells was checked under fluorescence microscopy considering GFP expression of cells (Figure 8B) at day 3.

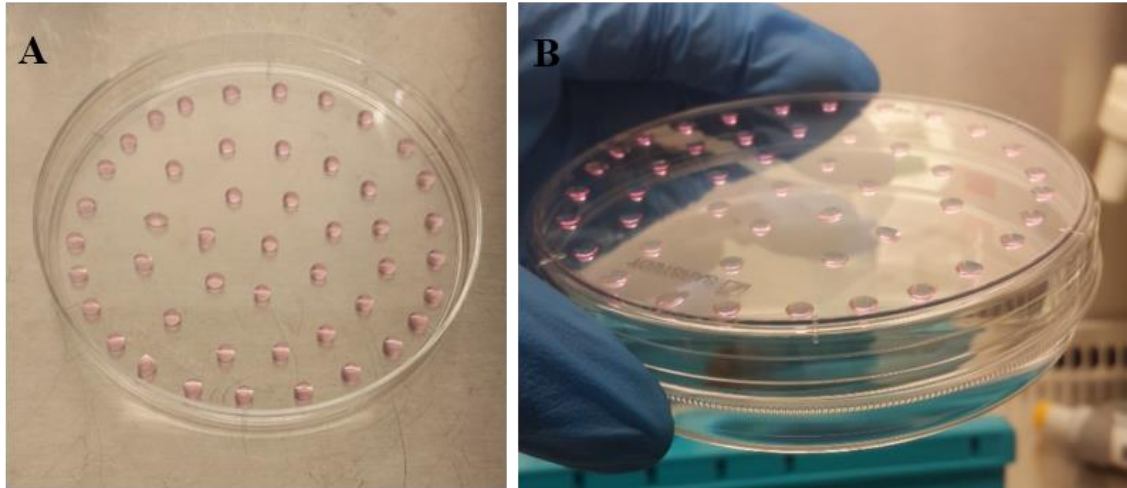


Figure 7. Cell spheroids with Hanging drop method A) Cell droplets on cover of petri dishes, B) Cell droplets before incubation.

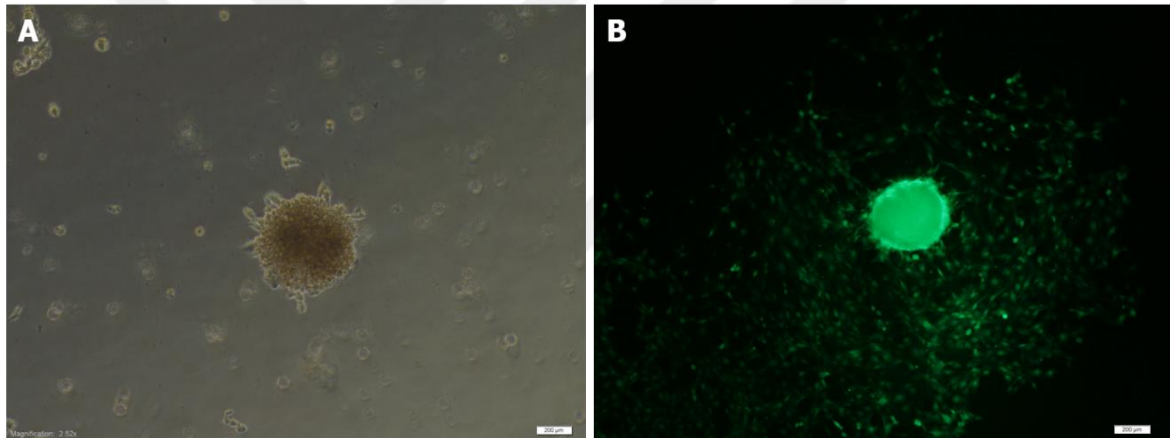


Figure 8. A) Light microscopy images of GFP (+) 3T3 cell spheroids at day 3, B) Fluorescence microscopy images of GFP (+) 3T3 cell spheroids at day 3.

3.6.6.4. *IPSC Culture*

IPSCs were cultured on decellularized human cornea, which was placed onto permeable concave shaped hydrogel inside microfluidic platform (Figure 9). IPSC differentiation was performed according to two different protocols using same microfluidic platform since each has its own advantages regarding culturing time and collagen fibril orientation ^{102,103}.

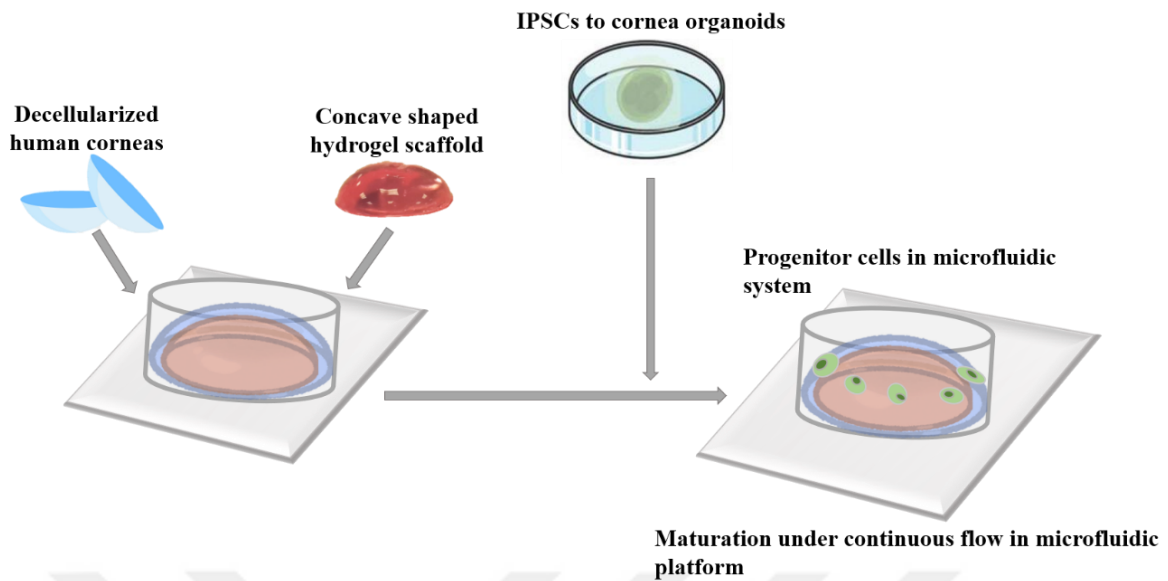


Figure 9. General flow chart of seeding and differentiation of iPSCs into corneal organoids inside microfluidic platform.

As reported by Susaimanickam, newly seeded iPSCs were cultured in complete DMEM/F12, named neural differentiation medium (NDM) and contained 4 % of KOS, 4 % FBS, 1 % NEAA, 1 % Glutamax and 1 % Pen-Strep. Culture medium induces neural ectoderm differentiation after two days. On the 2nd day of neural differentiation, cells were removed from plate and dissociated in order to transfer into microfluidic system (Figure 10). Approximately 1×10^6 cells inside 10 μ l medium were seeded on middle of the decellularized cornea which was placed on the PVA-Gelatin based hydrogel inside microfluidic platform. Cells were incubated at 37 °C with 5 % CO₂ for 3 hours to ensure attachment of cells on decellularized cornea under static conditions. After 3 hours, 80 μ l of NDM was added into platform and cells were cultured inside microfluidic platform under static conditions for 1 day. Then, once neural differentiation was completed, microfluidic platform connected to syringe pump. Cells were cultured under continuous dynamic conditions with presence of retinal differentiation medium (RDM). RDM contained NDM with addition of 2% B27 and was maintained for 30 days. At the end of 30 days, retinal differentiation was completed and induction of corneal differentiation induced with the corneal differentiation medium (CDM) which is obtained by addition of 1 % N2, 5

µg/ml insulin, 5 ng/ml FGF and 10 ng/ml EGF into NDM. However, dynamic culture was stopped on the 24th day of retinal differentiation at the latest, due to global COVID-19 pandemic.

As stated in Foster's research, once iPSCs culture was reached to confluency level (70-80 %), neural induction was started using NDM which comprised of 1 % B27 Vitamin A (-), 28 µg/l sodium selenium, 128 mg/L L-ascorbic acid and ITS which included 38.8 mg/L insulin, 21.4 mg/L transferrin and 38.8 mg/L NaHCO₃ into DMEM/F12. On the 5th days of neural differentiation, cells were removed from plate and dissociated in order to transfer into microfluidic system (Figure 11). Approximately 1x10⁶ cells inside 10 µl NDM were seeded on the middle of decellularized cornea which was placed on PVA-Gelatin based hydrogel inside microfluidic platform and incubated at 37 °C incubator with 5 % CO₂ for 3 hours. Incubation ensured attachment of cells on decellularized cornea under static conditions. After 3 hours, 80 µl of NDM was added into platform and cells were cultured for 1 day inside microfluidic platform under static conditions. After 1day, microfluidic platform connected to syringe pump, which gave cells NDM continuously and differentiation was continued under dynamic conditions. At the end of the 2nd day of dynamic culture, once neural differentiation was completed, RDM was giving through tubing to induce retinal differentiation. RDM, which consisted of same supplement with NDM and smooth agonist. This was maintained for 12 days. Afterwards, RDM was shifted to CDM comprised of 1 % B27, 10 % FBS 1 % Glutamax, 1 % NAEF and 500 nM retinoic acid inside DMEM/F12 medium for 10 days to induce corneal differentiation. However, dynamic fluid was stopped on the 6th day of corneal differentiation, at the latest, because of global COVID-19 pandemic.

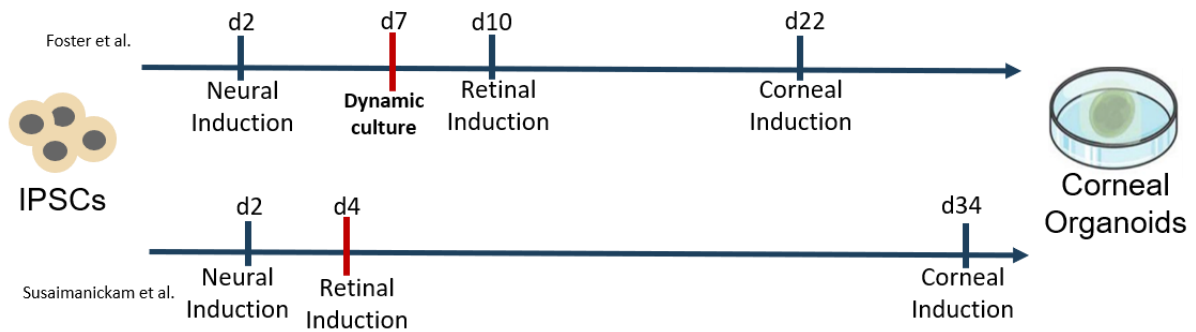


Figure 10. Timeline of iPSCs differentiation into cornea organoids and moving the experimental set-up from static culture to dynamic culture. Red line represent beginning of dynamic culture.

3.6.7. *Fabrication of Microfluidic System*

A microfluidic platform to mimics fluidic pressure inside eye was designed through engineering software, CorelDraw. Microfluidic platform was comprised of 2 different types of PMMA sheets, double-sided adhesive tapes (0.13 mm thickness, 3M™ High-Performance Acrylic Adhesive Transfer Tape 468MP) and tubing, which was used to transfer cell culture medium to system. Bottom part and top part of platform and ring applied pressure on decellularized matrix and was created with a 1.5 mm PMMA sheet. Middle part where hydrogel and decellularized matrix were located was fabricated with 3 mm PMMA sheet. There was a hole on bottom and top part, which was used to connect tubing in order to be served as an inlet and outlet. Then, there are reference holes on every part of platform to assist assembling of pieces precisely. Two different platforms were fabricated for different cell lines. At the beginning of the study, a larger microfluidic system was used. However, the size of the system was decreased due to limited number of cornea samples. Design of the microfluidic platform stage was shown in Figure 11.

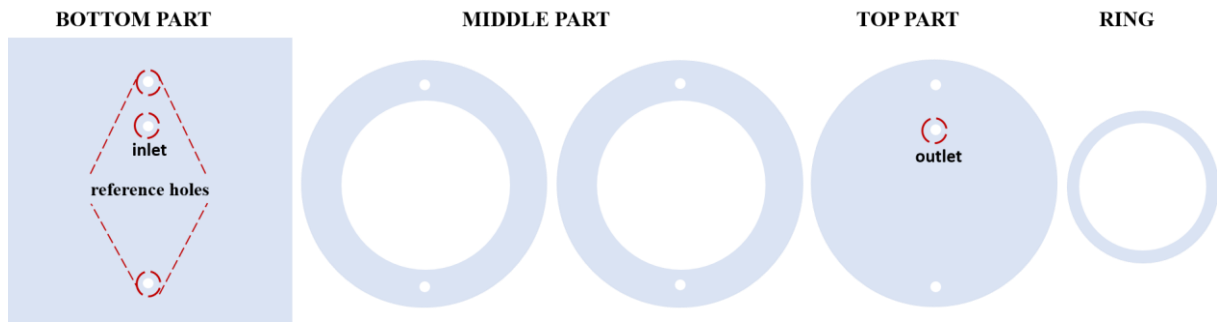


Figure 11. Design of microfluidic platform.

Before assembling microfluidic platform, protective gelatin on PMMA sheets was peeled under a sterile laminar hood. PMMA pieces were washed with 70 % ethanol, left to dry in an upright position and sterilized with UV light for 20 min inside laminar hood. Afterwards, each part was manually built using double-sided adhesive tapes and sterile syringe needles passing through reference to assemble precisely (Figure 12 A and B). To ensure that all pieces were stuck to each other, assembled microfluidic platform was prepared one day before cell culture experiment and stored inside sterile petri dishes under laminar flow. Before starting cell culture experiment, tubing was connected to bottom part of platform to serve as an inlet. Then, hydrogel and decellularized matrix was placed into middle part of the platform. Once cells were seeded on decellularized matrix, top part of platform was constructed using double-sided adhesive tape and another outlet tube was connected to top part of the platform.

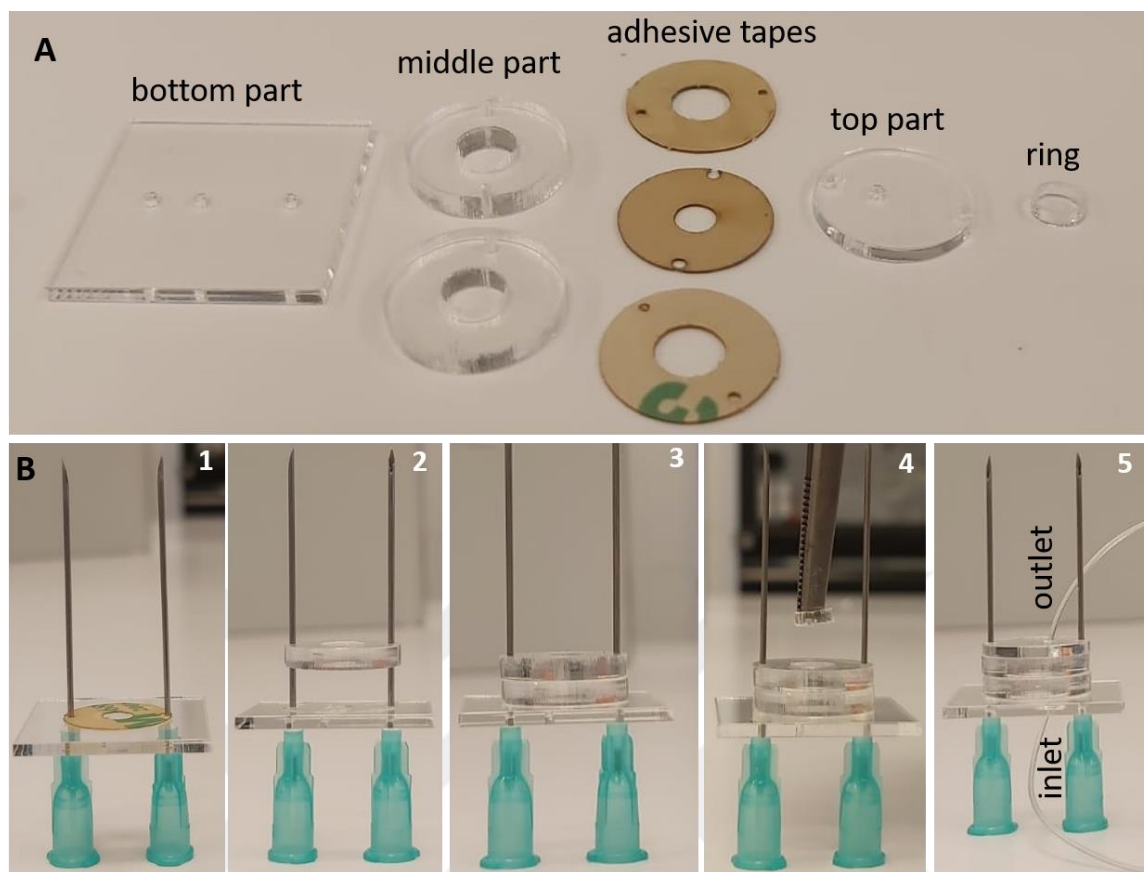


Figure 12. Assembling steps of microfluidic platform.

Assembled microfluidic platform including cells seeded according to protocols above was connected to a syringe pump to perfuse cell culture medium continuously (Figure 13). This created a fluidic pressure inside microfluidic system. Flow rate of syringe pump was optimized as 0,5 $\mu\text{l}/\text{min}$ for iPSC differentiation to create native like microenvironment.

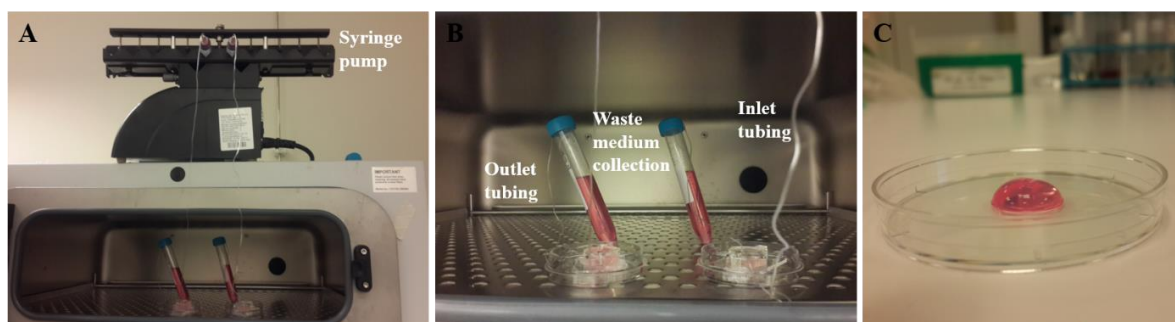


Figure 13. Final fabricated platform. A) Final fabricated platform which was connected to syringe pump, B) Closer image of the platform which shows waste

medium collection inside falcon tubes, C) Permeable hydrogel inside microfluidic platform.

3.6.8. *Characterization of Hydrogel and Cells*

3.6.8.1. *Scanning Electron Microscopy*

Porous structure of hydrogels was analyzed by SEM (Zeiss, GeminiSEM 500), which used Smart SEM software.

Firstly, synthesized concave shaped hydrogels were removed from PDMS mold and placed into 5 ml eppendorf tubes. Then, hydrogel scaffolds were frozen at -80 °C freezer for 1 day to solidify. Frozen hydrogel scaffolds were lyophilized for 24 hours. Afterwards, dried concave shaped hydrogels were divided into 1 mm diameter pieces using surgical lancet. Samples were placed facing upwards onto platform to ensure proper imaging of surface and porous structure of hydrogels. Small part of samples was covered with carbon tapes to avoid excessive loading and samples were coated with thin layer (5 nm) of gold using Quorum, Q150R. Finally, samples were ready for SEM imaging.

3.6.8.2. *Biocompatibility of Hydrogels*

In order to analyze biocompatibility of hydrogels, genetically transfected LX2 azure (blue), GFP+ 3T3 cells (green) and cell spheroids were seeded on different concave shaped hydrogel scaffolds and cultured for different periods. Two different cell lines were used to show that biocompatibility of hydrogel did not change regarding cell type. Cell viability on hydrogels were evaluated with color intensity of seeded cells using ImageJ software. To avoid any confounding factors, same seeding protocol and analyzing method were used for both cell type.

Sterilized concave shaped hydrogels (CSH) were placed into 3 % agarose coated sterile 24 well plate and cells were cultured on CSHs (Figure 14). For single cell experiments, 2×10^5 cells were seeded on CSHs and for spheroid experiment, approximately 50 spheroids were seed on CSHs. GFP+ 3T3 cells were cultured for 7 days and LX2 azure cells were cultured for 15 days. Imaging of cells at certain

specific time points (day1, day3, day7 and day15) was performed using Fluorescence Microscopy (Olympus, IX71).

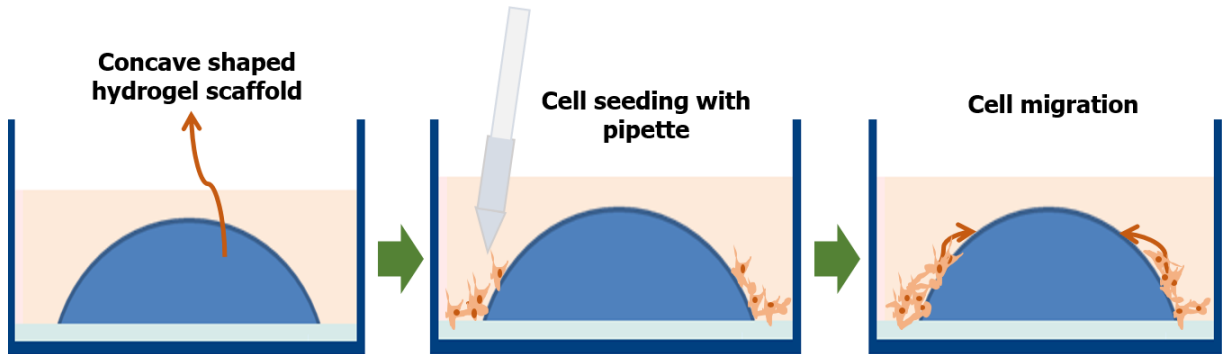


Figure 14. Seeding procedure of GFP+ 3T3, LX2 azure cells and cell spheroids onto CSHs.

3.6.9. Characterization of Decellularized Corneas

Decellularized corneas (DC) were compared with non-decellularized corneas (NDC) which were used as the control group. To characterize decellularized corneas, three criteria were evaluated.

3.6.9.1. DNA Quantification

PureLink Genomic DNA Mini Kit (Invitrogen, K182002) was used to quantify DNA amount of decellularized cornea samples and DNA quantification was performed based on the kit protocol. Aim of DNA quantification was to prove successful decellularization of corneas by comparing DNA amounts.

In digestion step, all steps were performed at room temperature. To provide a balance between weight of DC, NDC pieces and further solutions inside the kit, corneas were weighted firstly. Weight of each piece was measured as approximately 8 mg for mice and 24 mg for sheep and cow. Then, DCs and NDCs were incubated into 180 μ l Digestion Buffer and 20 μ l Proteinase K mixture (called lysis buffer) at 55 $^{\circ}$ C water bath for 2-4 hours until corneas were digested completely. Then, lysate was centrifuged at maximum speed for 3 minutes and supernatant was transferred into a new, sterile eppendorf tube. 20 μ l RNase A was added into lysate, vortexed and

incubated for 2 minutes. Afterwards, 200 µl PureLink Genomic Lysis/Binding Buffer and 200 µl 96–100 % ethanol was respectively added into lysate and vortexed.

For DNA binding step, approximately 640 µl lysate was transferred into spin column in a collection tube and centrifuged at $10.000 \times g$ for 1 min. Later, spin column was transferred into clean collection tube.

For washing DNA, firstly, Washing Buffer 1 and Washing Buffer 2 were prepared by adding 96–100% ethanol according to instructions. 500 µl of Washing Buffer 1 was added into column and centrifuged at $10.000 \times g$ for 1 minute. Then, spin column was transferred into clean collection tube and 500 µl of Washing Buffer 2 was added into spin column, centrifuged at maximum speed for 3 min. To wash more DNA, this step was performed two times using the same spin column and collection tube with same washing solution inside collection tube.

For eluting DNA, spin column was transferred into sterile 1.5 ml Eppendorf tube and 50 µl of Genomic Elution Buffer was added into the spin column, incubated for 1 min. The column was centrifuged at maximum speed for 1 min. This elution step was also performed two times using the same column, tube and elution buffer. Final solution inside Eppendorf tube, which was filtered out of spin column, contains purified genomic DNA.

Once DNA extraction was completed, DNA was quantified in each sample using Nanodrop apparatus. Ratio of absorbance values was calculated at 260 and 280 nm. Genomic DNA was stored at -20 °C.

3.6.9.2. Gel Electrophoresis

1 % agarose solution was prepared in 1X 100 ml Tris-acetate-EDTA buffer (TAE) (Thermo Fisher, B49) and dissolved homogenously inside microwave oven for 30-45 sec. Once gel was cooled for a while, 5 µl of the SafeView Classic product (abm, G108), which provides binding of DNA and visualization DNA under UV light was added into agarose gel. Agarose solution was poured into gel tray without any bubbles. Then, well comb was placed on agarose gel and cooled at room

temperature for 30 min. Well comb was removed from gel and agarose gel was placed into gel box, which was filled with prepared TAE buffer next. In order to prepare loading mix, loading dye (Thermo Fisher, R1161) was mixed with pipetting on parafilm. Loading dye helps to visualize loading gel and increases the concentration of DNA samples and DNA samples of DCs and NDCs. Gene Ruler 1 kb DNA Ladder (Thermo Fisher, SM0311) which consist of 14 DNA fragments was loaded into first lane of the gel. Subsequently, the loading mix for each DNA samples was loaded into other lanes carefully. This was run at 110 V for 2 hours with principle of negatively charged DNA running to positive electrode. At the end, fragments were visualized under a UV light.

3.6.9.3. Hematoxylin – Eosin Staining (H&E)

H&E is used to localize nuclei and extracellular proteins. Hematoxylin (blue) stains nucleic acids inside nuclei and eosin (pink) stains cytoplasm and extracellular matrix.

Sample preparation:

Cryosectioning was performed by histopathology unit of IBG. Before cryosectioning, NDCs were fixed with 4 % PFA in order to be used as the control group. Then, NDCs and DCs washed with 1X PBS for 3 times and they were immersed into 30 % sucrose solution for overnight until corneas were reached to bottom of 1,5 ml Eppendorf tube. Then, sucrose was removed from tube. Corneas were embedded into optimal cutting temperature compound inside cover of 5 ml Eppendorf tube and frozen at -80 °C. These can be stored until sectioning.

For staining, samples were incubated at room temperature for 30 min. and washed with 1X PBS for 3 times. Small amount of Mayer's Hematoxylin Solution (Sigma Aldrich, MHS32) was added onto the samples to cover tissue for 3 min. Samples were washed with distilled water. Afterwards, eosin was added onto the samples for 1 min. Then, sections were washed with 70 %, 80 %, 96 % and absolute ethanol respectively. Stained sections were stabilized with mounting

medium (ProLongTM Gold Antifade Mountant, Thermo Fisher, P36930). Imaging was performed under light microscope (Olympus CKX41).

3.6.9.4. Periodic acid–Schiff (PAS) Staining

PAS Staining (purple) is used to detect localization of glycogens, glycoproteins and glycolipids in fixed and sectioned tissues. PAS stain kit (Abcam, ab150680) was used with Hematoxylin stains which localized nuclei. Decellularized and non-decellularized corneas were stained with PAS.

Samples were prepared using same method of Hematoxylin – Eosin staining. For staining, samples were incubated at room temperature for 30 minutes and washed with 1X PBS 3 times. Slides were immersed into Periodic Acid Solution for 5 minutes and rinsed with distilled water for 4 times. Afterwards, slides were immersed into Schiff's Solution for 15 minutes and washed with hot running tap water first and distilled water next. Small amount of Hematoxylin was poured onto slides for 3 minutes before being washed with distilled water. Finally, slides were dehydrated with 70 %, 80 %, 96 % and absolute ethanol, respectively, and covered with mounting medium for light microscopy imaging.

3.6.9.5. Biocompatibility of Decellularized Corneas: Recellularization

Before cell culture studies, corneas were exposed to UV light for 1 hour. DCs were placed into sterile 24 well plate. GFP (+) 3T3 cells with number of 2×10^5 were seeded on sterile DCs and cultured for 3 days. Imaging of cells at certain specific time points (day1, day3) was performed using Fluorescence Microscopy (Olympus, IX71).

3.6.10. Characterization of Cell Culture Studies

3.6.10.1. Gene Expression with Immunofluorescence of Cornea Organoids Cultured in Microfluidic System

6 weeks old cornea organoids were cultured on the PVA-Gelatin based hydrogels inside microfluidic platform for 5 weeks. At the end of the experiment, organoids were characterized with Ki-67 antibody (Bioss Antibodies, BS2130R) which

is proliferation marker and Cytokeratin 3 (CK3) (Abcam, ab68260) which is cornea epithelial marker. Finally, DAPI was used to localize the nuclei.

At the end of the experiment, top part of microfluidic platform (cover) was disassembled with a sterile lancet to take the organoids out of the platform. However, once the PVA-Gelatin based scaffold moved inside the platform, the organoids were removed from the hydrogel scaffold, which proved that organoids were not adhered to the scaffold like nature of static organoid culture. This confirms previous findings of Foster and Susaimanickam^{102,103}. Hence, staining was performed inside microfluidic platform, which required 150 μ L of reagents to cover the organoids.

For staining, organoids were washed with 1X PBS 3 times and fixed with 4 % PFA for 30 minutes inside microfluidic platform at room temperature. PFA was removed from platform and organoids were washed with PBS 3 times. Subsequently, cells were incubated with staining solution for 2-3 hours at room temperature. Primary antibodies, Ki-67 was diluted in a 1:100 ratio while CK-3 was diluted in a 1:50 ratio together in same staining solution and incubated for overnight at +4 °C. This ensured staining of the inner part of the organoids. Alexa fluor 488 (Abcam, ab150077) and Alexa fluor 594 (Jackson Immuno, 715-585-150) were diluted 1:500 in same staining solution as secondary antibodies. Afterwards, organoids were washed with PBS and secondary antibodies were added into platform and incubated overnight at +4 °C. Later, organoids were washed with PBS 3 times and 1 μ g/mL diamidino-2-phenylindole (DAPI) was added into platform and incubated for 15 minutes. Finally, cells were washed with PBS 3 times and the organoids were placed onto coverslip and mounted with mounting medium for confocal microscopy imaging.

3.6.10.2. Gene Expression with Quantitative Polymerase Chain Reaction (qPCR)

qPCR was performed to analyze gene expression of PIEZO2 which is mechanosensitive ion channel component and expressed under mechanical pressure (Coste et al. 2010; Zeng et al. 2018). The expression of PIEZO 2 gene was showed on 3T3 cell line, which was cultured under dynamic conditions for 3 days. Expression

level of PIEZO 2 was checked at day 1 and day 3 for both static and dynamic culture. As a static control, 3T3 cells were cultured in fabricated microfluidic platform without top part of the platform. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression was used as reference gene to analyze qPCR results since it is known as common housekeeping gene in glycolysis. Forward and reverse sequences of GAPDH and PIEZO 2 are shown in table 2. qPCR experiment was performed in four steps which were RNA isolation, cDNA synthesis, primer design and qPCR analysis.

Samples:

S1= day1, static culture

D1= day1, dynamic culture

S3= day3, static culture

D3= day3, dynamic culture

SC1= day1, static control GAPDH

SC3= day3, static control GAPDH

DC1= day1, dynamic control GAPDH

DC3= day3, dynamic control GAPDH

RNA isolation was performed based on RNA isolation kit (Macherey-Nagel, NucleoSpin RNA XS, 740955.50) protocol. Medium was removed from the culture and 3T3 cells were washed with 1X PBS. 200 μ L, total volume of channel, Trizol (Invitrogen, 15596026) was added into channels to remove cells from surface of channel. Later, cells were collected into 2ml Eppendorf and vortexed for complete homogenization. After 2-3 minutes incubation at RT, Eppendorf was placed onto ice. 40 μ L of chloroform (0.2 volumes relative to Trizol) was added into the 200 μ L of Trizol, mixed thoroughly by shaking robustly and incubated at RT for 2 minutes. Samples were centrifuged at 12.000g for 15 minutes at +4 $^{\circ}$ C to separate 3 phases. Top, transparent phase was called aqueous phase and contained the RNA was transferred into new, sterile 2ml DNase, RNase-free Eppendorf. Same volume of transferred aqueous phase, 100 μ L 70 % ethanol was diluted in Nuclease free water was added into Eppendorf and incubated at RT for 10 minutes. Afterwards, lysate was transferred into NucleoSpin RNA XS Column (blue ring) was placed into

collection tube and centrifuged at 11.000 g for 30 seconds. 100 µl Membrane Desalting Buffer was added and centrifuged at 11000g for 1 minute. Then, 25 µl rDNase reaction mixture was added into column and incubated for 15 minutes at RT to digest DNA. To wash silica membrane, Wash Buffer 1, 2 and 3 was added into column and centrifuged 11.000 g for 30 seconds respectively. Lastly, to collect the pure RNA, 60 µl RNase-free water was added into column and centrifuged at 11.000 g for 30 seconds. Quantity of RNA was recorded with Nanodrop apparatus. Ratio of absorbance values was calculated at 260 and 280 nm. Genomic DNA was stored at -20 °C.

cDNA synthesis was performed according to OneScript Plus cDNA Synthesis Kit (abm, G236) protocol. Calculations for each sample were determined based on the concentration of isolated RNA. According to calculations, 1 µl Random Primers (10 µM), 1 µl dNTP Mix (10 mM), calculated amount of RNA for each sample and Nuclease-free water were added to a final total volume of 14.5 µl into PCR tubes. Then, tubes were mixed through spinning and incubated at 65 °C for 5 minutes, followed by immediate incubation on ice for 1 minute. Afterwards, 4 µl of 5X RT Buffer, 0.5 µl of RNaseOFF Ribonuclease Inhibitor and 1 µl of OneScript Plus RTase were mixed and added into PCR tubes to yield 20 µl of final concentration. Thermal cycler was set up to incubate mixture at 25 °C for 10 minutes, 50 °C for 50 minutes and finally to stop reaction 85 °C for 5 minutes.

Table 2. Forward and reverse sequences of used primers.

Gene	Organism	Forward Sequence (5' 3')	Reverse Sequence (3' 5')
PIEZO2	mouse	CCATGCCAAAGTCAATGGTCG	GCTTATCAAGGCTGGCCGTC
GAPDH	mouse human	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTG TA

Once cDNA synthesis was completed, Syber-Green protocol was used in order to perform qPCR. 10 μ l final mixture per well was prepared inside white 96 well plate which was supplied in the kit. Final mixture was comprised of 5 μ l Mastermix (Promega, GoTaq A6001), 2 μ l nuclease-free H₂O, 1 μ l forward primer, 1 μ l reverse primer, 1 μ l cDNA sample. For each case, 3 final mixtures were prepared and transferred into 3 different wells for repetitive experiment. qPCR run protocol was shown in Table 3 using Roche Light cycler 480.

Final CP values for each sample were calculated based on the $\Delta\Delta$ CP method using this formula. The Δ CP value = CP_{mRNA} – CP_{GAPDH} (average GAPDH) on Excel (Microsoft, USA). Average value of Δ CT_{GAPDH} was used as control to analyze change in expression $\Delta\Delta$ CT values of each sample.

Table 3. qPCR run protocol

Program	Cycle	Temperature °C	Time (s)
Pre-incubation	1	95	120
PCR	40	95	10
		60	10
		72	30
Melting	1	95	10
		65	30
Cooling	1	40	30

3.7. Research Plan

	September 2017- July 2018	August 2018- April 2019	April 2019- March 2020	April 2020- May 2020
Literature Review	X	X	X	X
Planning the Experiment		X	X	
Data Collection and Experimenting		X	X	
Evaluation of Data			X	X
Thesis Writing				X

3.8. Evaluation of Data

Within the scope of this thesis study, GraphPad Prism 7 program was used to analyze qPCR findings. Fluorescence and confocal microscopy images were analyzed through ImageJ program.

3.9. Limitation of Research

Obtaining limited amount of human donor corneas is one of the most restrictive factors of this study. Decellularization of different disease sourced corneas may cause variable changes in cornea structure, which can't be controlled in the laboratory.

Developed microfluidic system has provided successfully culturing of cornea organoids. However, differentiation of iPSCs into cornea organoids inside microfluidic platform couldn't be completed due to global COVID-19 pandemic, which caused to shut down research facilities.

3.10. Ethics Committee Approval

This study was approved by the Ethic Committee of IBG-GOEK at May 16, 2020. Ethic committee approval number is 2020-020. Human corneas were used after taking Patient Consent Form from patients.

Ethic Committee Approval and example of Patient Consent Form were attached on the Appendix section.



4. RESULTS

4.1. Fabricated Hydrogel Images

In order to synthesize permeable concave shaped hydrogels, different polymers (GelMA, PEG, PVA) were used. Mechanical stability, transparency and gelation capacity of polymers with different concentrations were compared.

4.1.1. GelMA based Hydrogels

Different concentrations of GelMA (5 %, 10 % and 20 %) were compared regarding criteria mentioned above (Figure 15 A, B and C). Increase in GelMA concentration resulted in mechanically more stable hydrogel scaffolds and in higher amount of gelation of polymer inside PDMS mold under same power of UV light and exposure time.

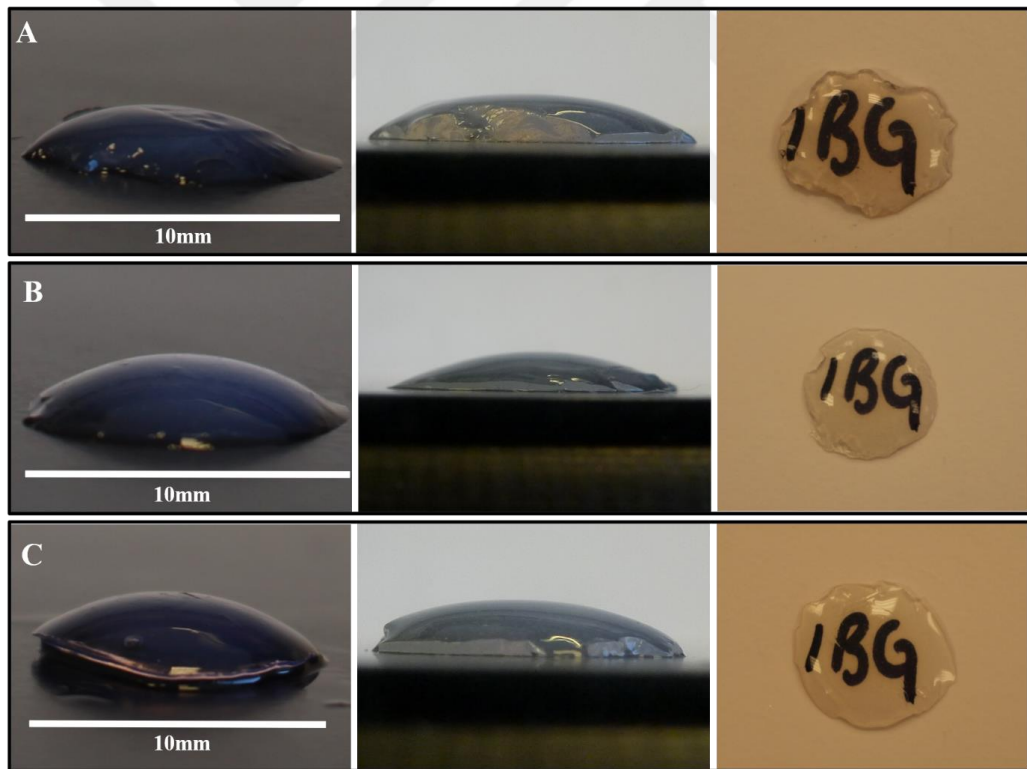


Figure 15. Synthesized GelMA-based hydrogel scaffolds from front view to show diameter and from top view to show transparency of hydrogel scaffolds. A) 5 % GelMA based hydrogel scaffold. B) 10% GelMA based hydrogel scaffold. C) 20 % GelMA based hydrogel scaffold.

4.1.2. PEG based Hydrogels

PEG based permeable CSHs were pre-prepared in 1 % and 2 % precursor solutions to yield 20 % (w/v) PEG concentration. Their mechanical stability was checked manually and transparency levels of hydrogels scaffolds were compared (Figure 16A and B). Increasing concentration of PI (2 %) resulted with mechanically more stable hydrogel and higher amount of gelation of polymer inside mold. However, this caused to less transparent hydrogel formation, especially in middle of CSH due to depth of scaffold.

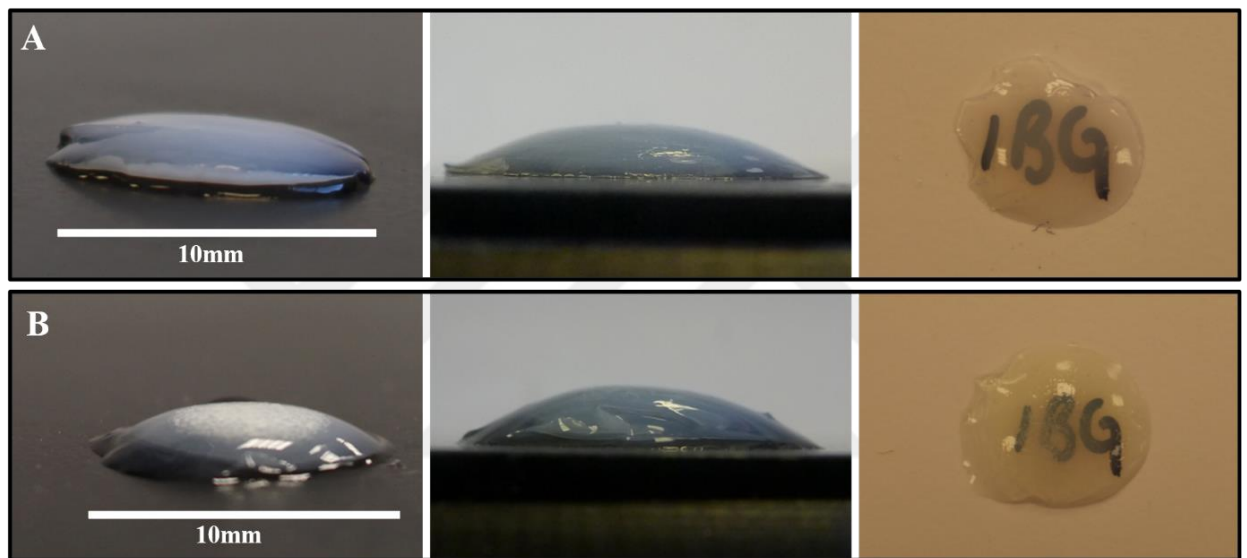


Figure 16. Synthesized PEG-based hydrogel scaffolds from front view to show diameter and from top view to show transparency of hydrogel scaffolds. A) 20 % PEG solution was synthesized using 1 % PI solution. B) 20 % PEG solution was synthesized using 2 % PI solution.

4.1.3. PVA based Hydrogels

Another polymer based CSH was fabricated with PVA using different concentrations of DMSO (25 %, 50 % and 75 %) (Figure 17 A, B and C). Thinnest CSHs synthesized using PVA and cross-linked in 25% DMSO solution, teared due to low mechanical strength. Although increasing DMSO concentration provided more effective crosslinking of polymer, and higher mechanical stability, it also led to loss of transparency of hydrogel scaffolds. Transparency is only important to be used inside microfluidic platform for imaging.

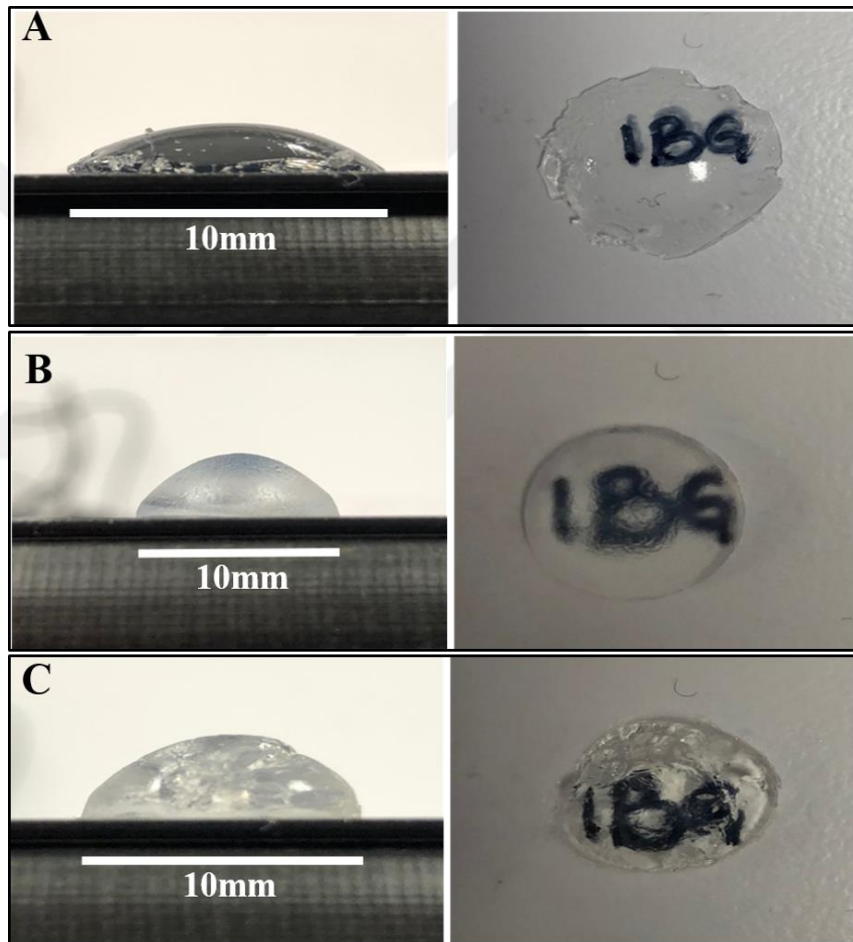


Figure 17. Synthesized 12 % PVA-based hydrogel scaffolds from front view to show diameter and from top view to show transparency of hydrogel scaffolds. A) 12 % PVA solution was synthesized using 25 % DMSO solution, B) 12 % PVA solution was synthesized using 50% DMSO solution, C) 12% PVA solution was synthesized using 75 % DMSO solution.

4.1.4. *PVA-Gelatin based Hydrogels*

Gelatin has been known as favorable material supporting for cellular physiology. In this regard, another hydrogel which had a final 12 % of PVA and 5 % of gelatin (w/v) concentration inside 25 % DMSO was synthesized (Figure 18). This CSH was mechanically more stable than 12 % PVA based CSHs using 25 % DMSO. There were no noticeable differences between transparencies of these two hydrogel scaffolds and gelation capacity of polymers inside mold.

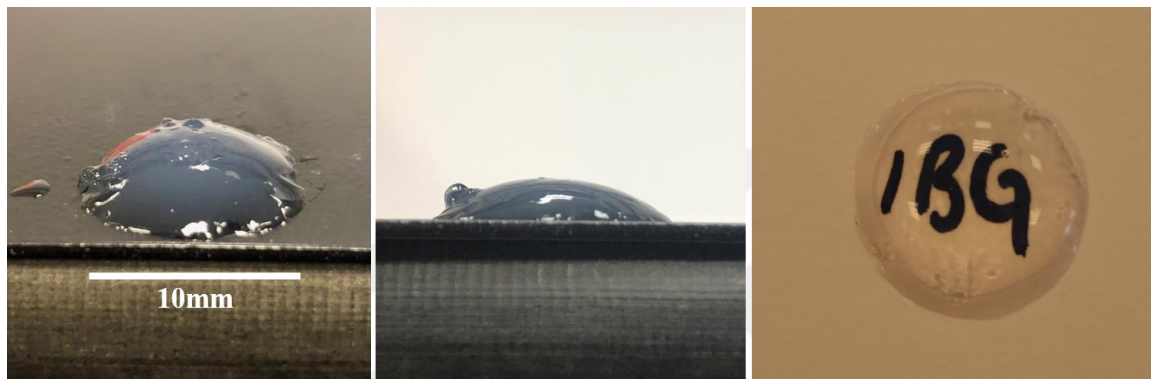


Figure 18. Synthesized 12 % PVA- 5 % Gelatin based hydrogel scaffolds from front view to show diameter and from top view to show transparency of hydrogel scaffolds.

4.2. Characterization of Hydrogels

To evaluate permeability of synthesized hydrogel scaffolds, their porous structures were investigated using electron microscopy technique. Surface structure and cross section of CSHs were compared to find suitable polymer for cell culture studies.

4.2.1. SEM Imaging of GelMA Hydrogels

As expected, increasing concentration of GelMA resulted with higher porosity along with smaller pore size in cross section of hydrogels (Figure 19 A, B and C). This led to formation of more compact structures. However, smoother surface of CSHs was observed with increasing concentration of GelMA.

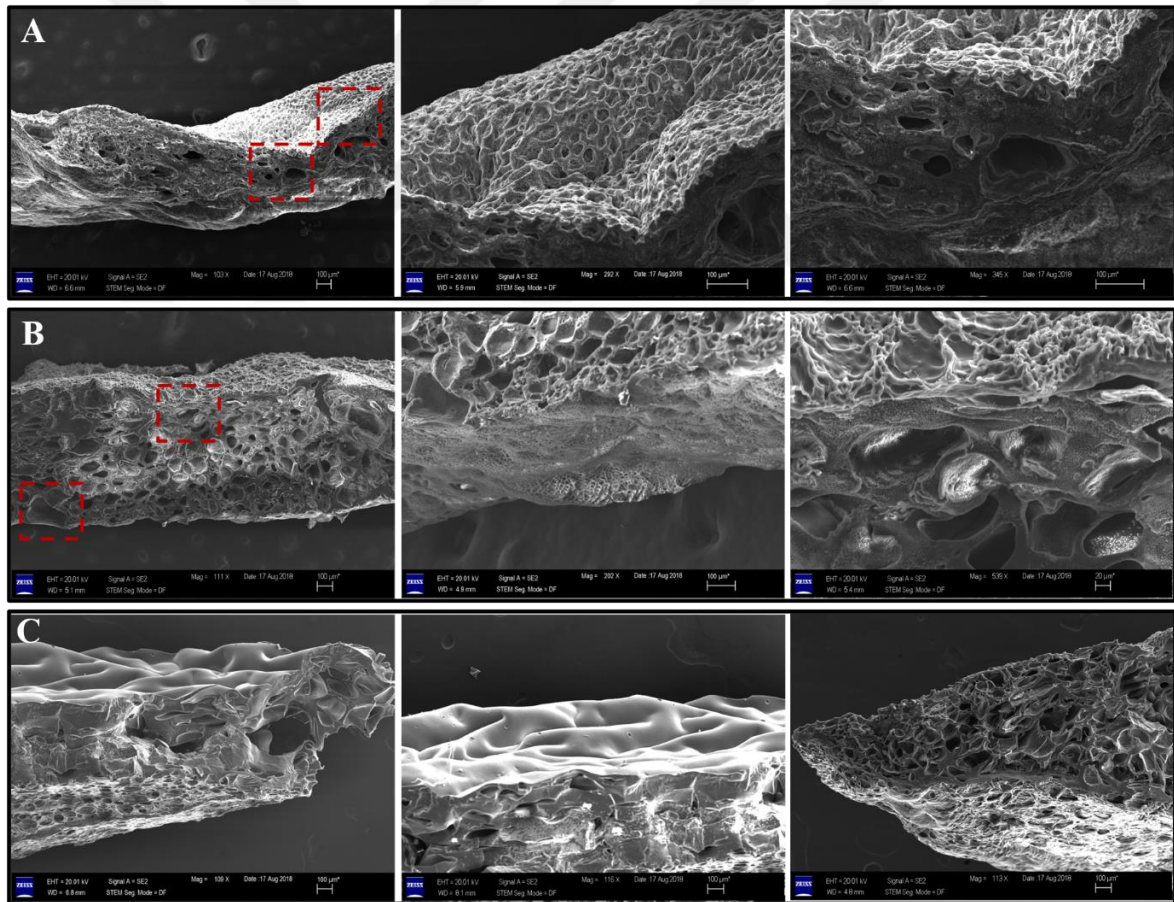


Figure 19. SEM images of GelMA based hydrogel scaffolds in order to investigate surface and porous structure of hydrogels. A) 5 % GelMA based hydrogel scaffold, B) 10 % GelMA based hydrogel scaffold, C) 20 % GelMA based hydrogel scaffold.

4.2.2. SEM Imaging of PEG Hydrogels

Surface structure and porosity of 20 % PEG based CSHs which cross-linked in 1 % and 2 % PI solution were analyzed (Figure 20 A and B). Increase in PI concentration resulted in higher porosity but smaller pore size. Additionally, surface of PEG hydrogel cross-linked with 5 % PI was smoother.

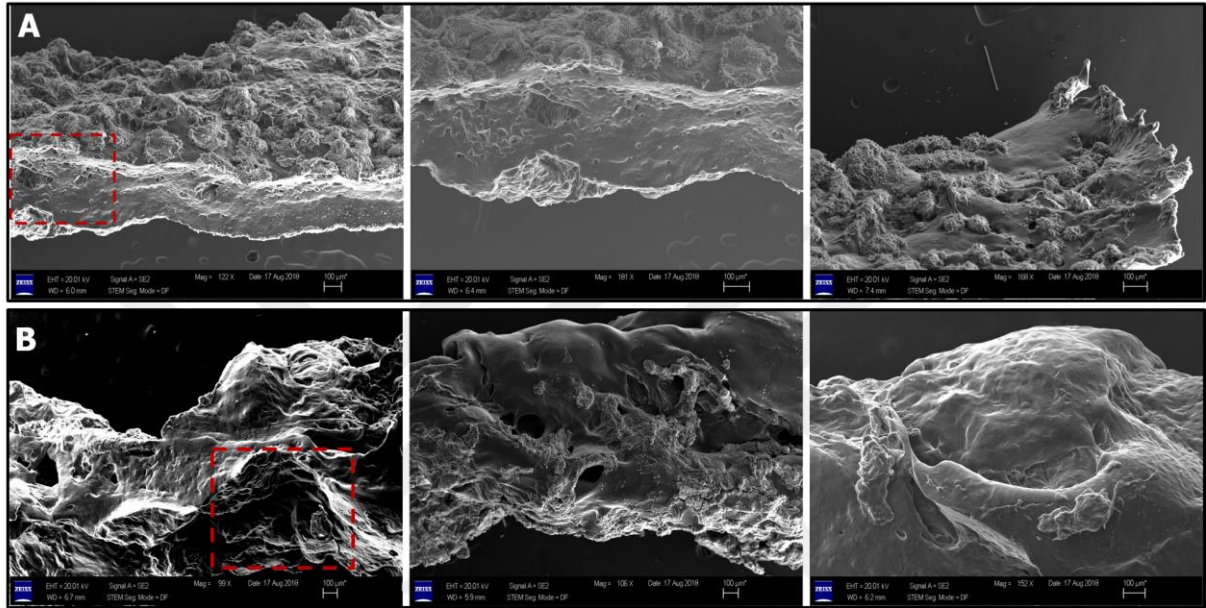


Figure 20. SEM images of PEG based hydrogel scaffolds in order to investigate surface and porous structure of hydrogels. A) 20 % PEG solution was synthesized using 1 % PI solution. B) 20 % PEG solution was synthesized using 2 % PI solution.

4.2.3. SEM Imaging of PVA Hydrogels

Surface and porous structures of PVA based CSHs cross-linked in 25 %, 50 % and 75 % DMSO were compared (Figure 21 A, B and C). As shown in figure 21, increasing concentration of DMSO provided smoother surface and less porous structure in the cross section of the CSHs. It is clear that 25 % DMSO cross-linked PVA hydrogels have more visible pores than the other two concentrations. On the other hand, it is shown that PVA based hydrogels have less porous structure compared to GelMA and PEG based hydrogels. However, these polymers were preferred due to higher mechanical stability than other two polymers.

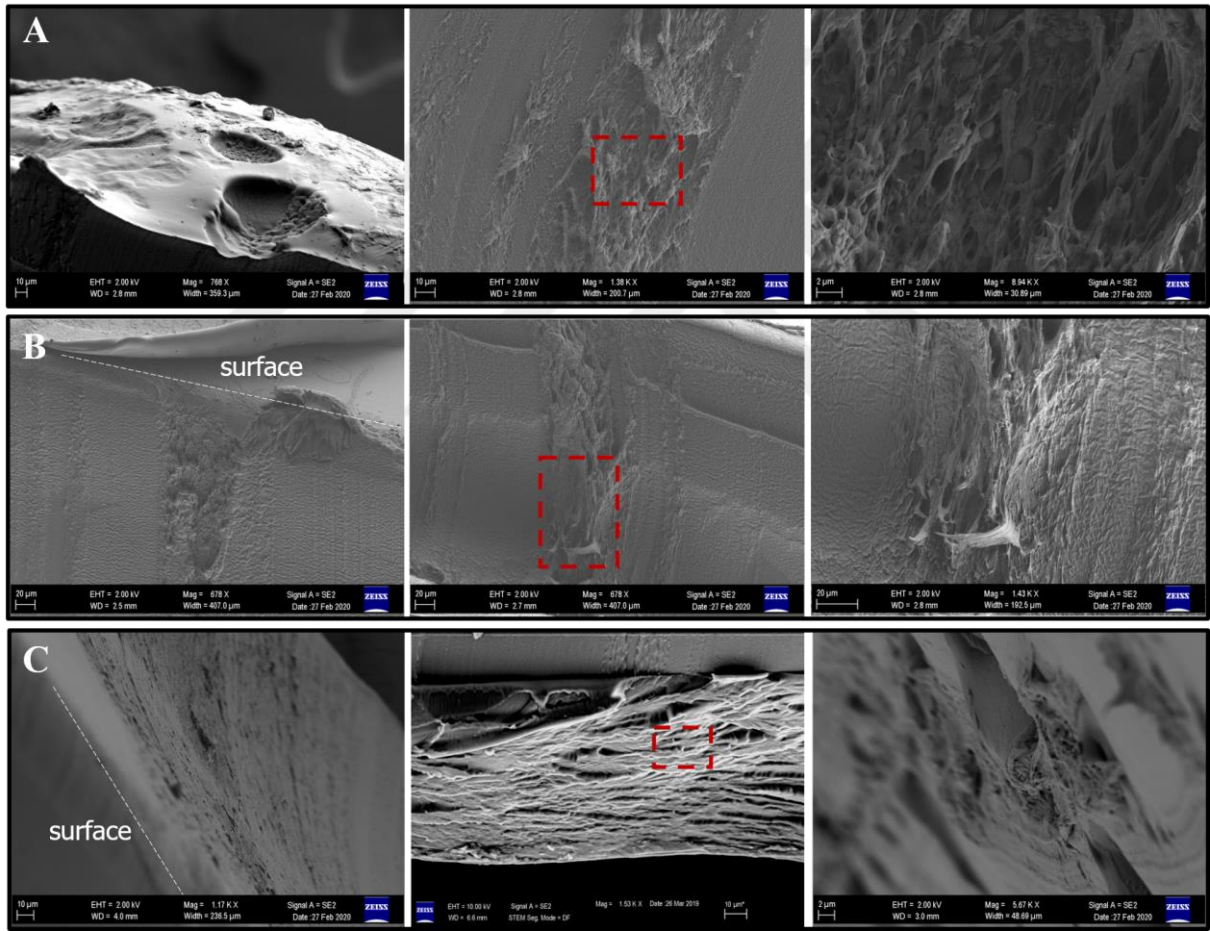


Figure 21. SEM images of PVA based hydrogel scaffolds in order to investigate surface and porous structure of hydrogels. A) 12% PVA solution was synthesized using 25 % DMSO solution, B) 12 % PVA solution was synthesized using 50 % DMSO solution, C) 12 % PVA solution was synthesized using 75 % DMSO solution.

4.2.4. SEM Imaging of PVA-Gelatin Hydrogels

For promoting cellular behaviors, porous structure of PVA based hydrogel cross-linked with 25 % DMSO (the most porous structure) was advanced with addition of gelatin to yield 12 % PVA and 5 % gelatin concentration (Figure 22). As we expected, increased porosity and pore size were observed both in surfaces and cross sections with presence of gelatin. Similar pore structure to GelMA based hydrogel scaffolds was observed.

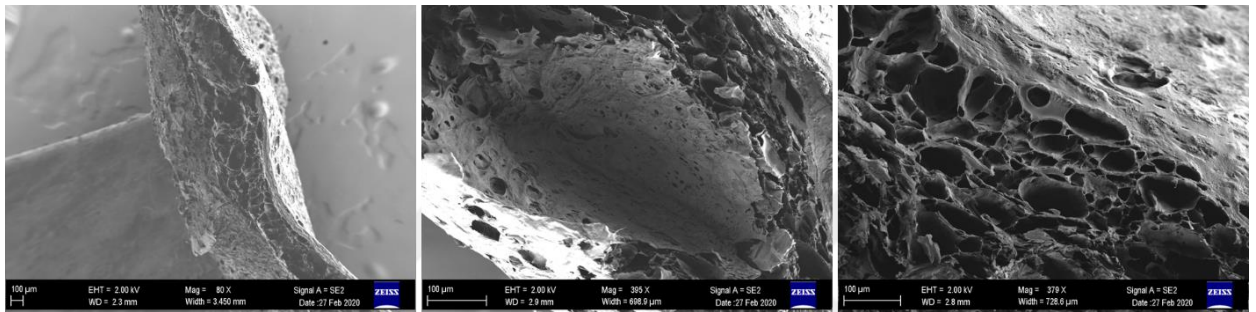


Figure 22. SEM images of 12 % PVA – 5 %Gelatin based hydrogel scaffolds which was solved in 25 % DMSO-DI water in order to investigate surface and porous structure of hydrogels.

4.3. Static Cell Culture on Hydrogel Scaffold

In order to investigate the biocompatibility of hydrogel scaffolds, GFP (+) 3T3 and Azur LX2 cell lines and spheroids were cultured on synthesized hydrogel scaffolds under static conditions.

4.3.1. Cell Viability on GelMA

2×10^5 3T3 GFP (+) cells were seeded on sterilized GelMA based CSHs and cultured at 37 °C incubator with 5% CO₂ for 7 days. Cell proliferation was observed during 7 days of culture and cell viability was controlled at three time points (day1, day3 and day7) regarding GFP expression of cells (Figure 23). It is shown that cells were still alive at the end of the 7 days of culture.

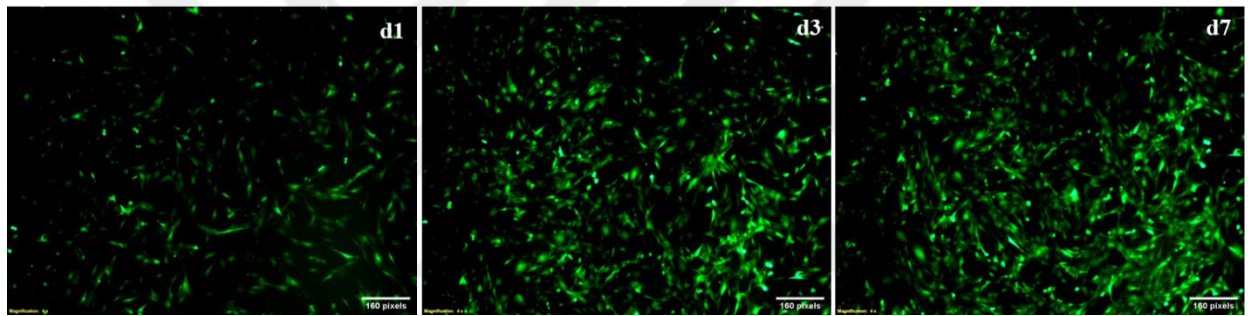


Figure 23. Cell viability of 3T3 GFP (+) cells on 10 % GelMA based CSHs for 7 days.

4.3.2. *Cell Viability on PVA*

2D Static Cell Culture on PVA

To evaluate biocompatibility of PVA based hydrogels and compare effect of DMSO concentration (25 %, 50 % and 75 %) on cell viability, 2×10^5 LX2 Azure cells were seeded on sterilized CSHs and cultured at 37°C incubator with 5 % CO₂ for 7 days (Figure 24). At the end of 7 days of culture, cell proliferation and cell viability were remarkably higher than other two groups in 12 % PVA based CSHs which was cross-linked with 25 % DMSO. This finding also promotes results of SEM that 25 % DMSO concentration had highest porosity and size to allow permeation of cell culture medium. Therefore, following cell culture studies were performed with this concentration.

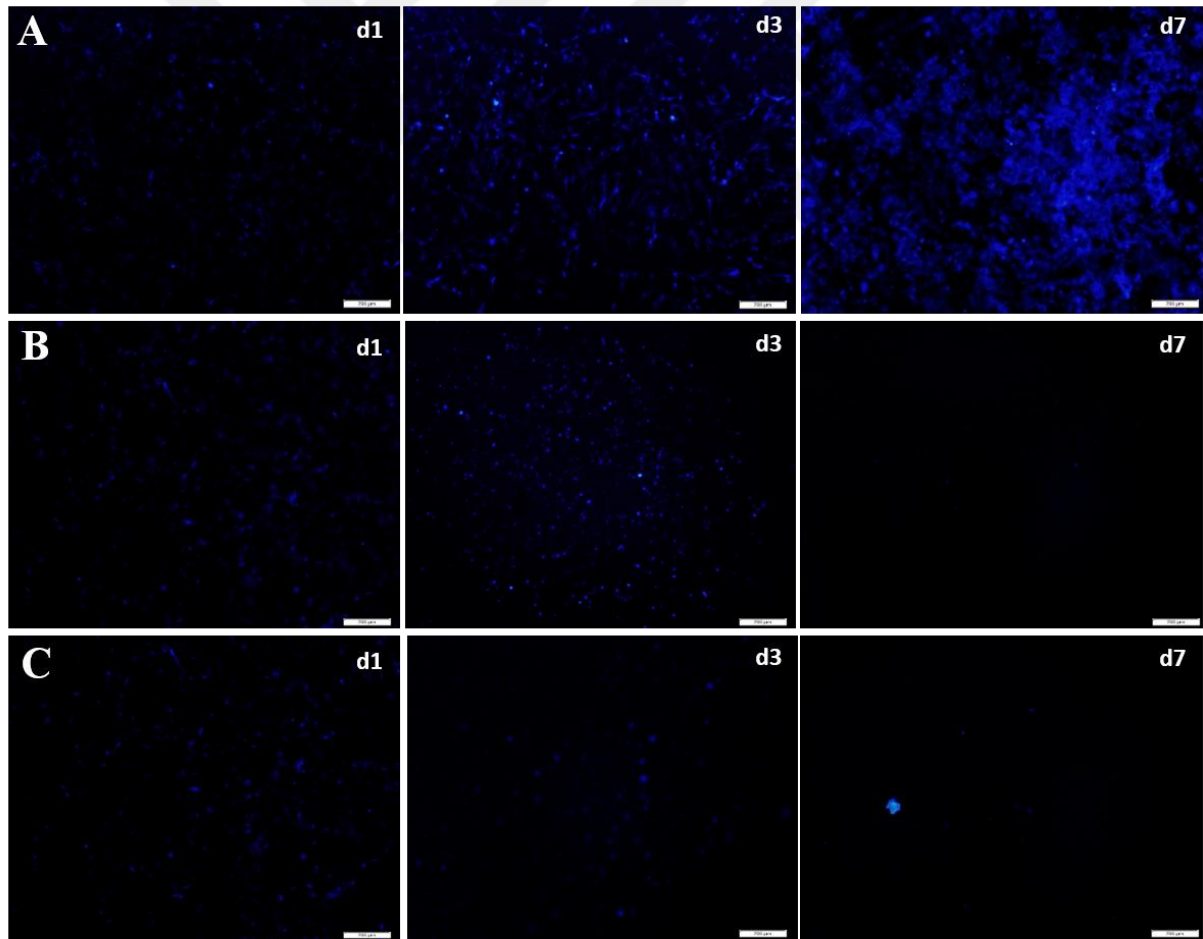


Figure 24. Cell viability of LX2 Azure cells on CSHs for 7 days. A) Cell viability on 12 % PVA based CSH, solved in 25 % DMSO solution, B) Cell viability on 12 % PVA

based CSH, solved in 50 % DMSO solution, C) Cell viability on 12 % PVA based CSH, solved in 75 % DMSO solution. Scale bar: 200 μm .

3D Static Cell Culture on PVA-based Hydrogels

Next step was to investigate survival of 3D cellular structures on 12 % PVA based CSHs, dissolved in 25 % DMSO solution. Approximately 50 spheroids, obtained with 2×10^4 cell number per 10 μl droplet were cultured on 12 % PVA based CSHs at 37 °C incubator with 5 % CO_2 for 15 days. Cell viability of single cells and cell spheroids on PVA based CSHs was observed for 15 days at three different time points (day1, day7 and day15) and compared with cells cultured on standard cell culture plate (Figure 25 A, B and C). For single cells and spheroids on PVA based CSHs, cell viability was lower than control group at the end of the 15 days of culture. However, it was observed that cell spheroids formed a ring shaped structure on hydrogel scaffold (Figure 26). This indicates that alignment of 3D cell groups on PVA based CSHs is possible.

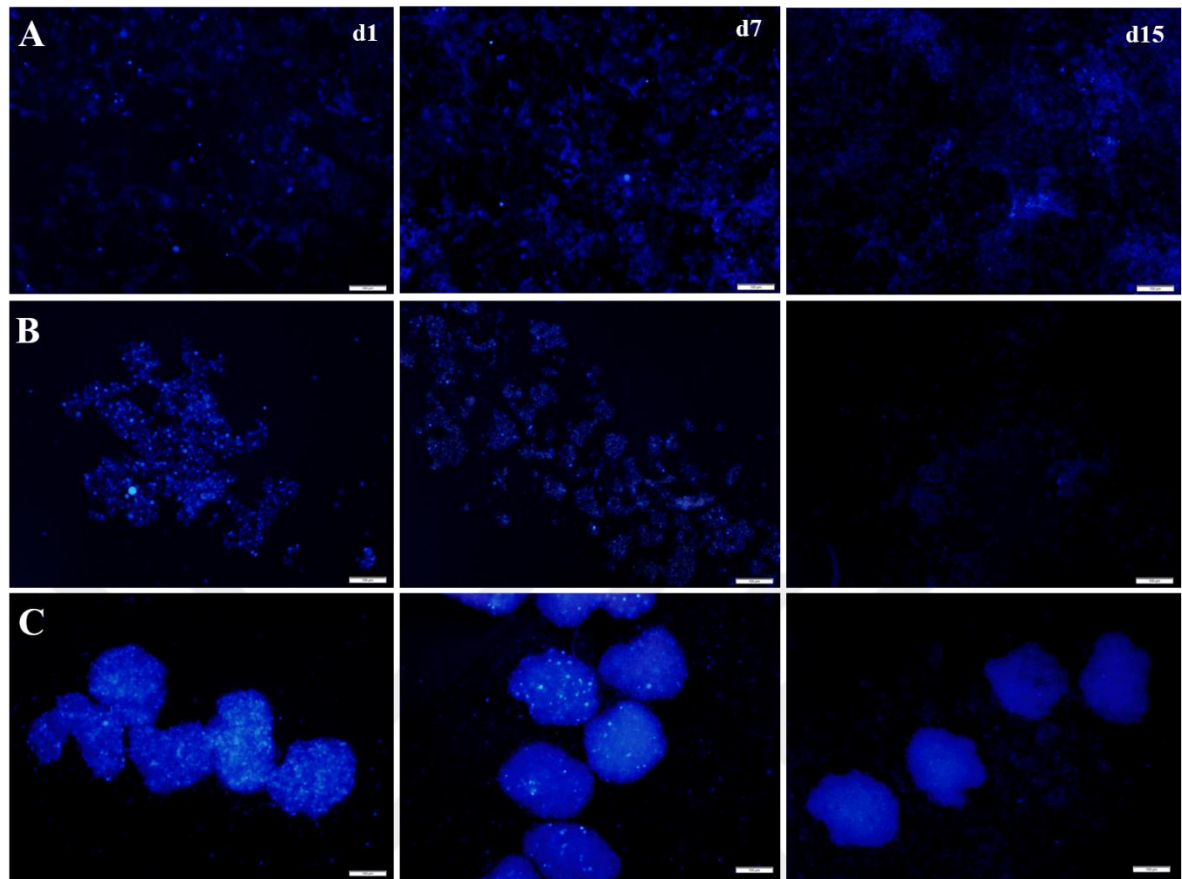


Figure 25. Cell Viability of LX2 Azure single cells and spheroids on 12% PVA based CSHs and standard 2D LX2 cell culture was selected as control group. A) Control group: 2D LX2 Azure cells on 24 well plate. B) LX2 Azure cells on 12% PVA based CSHs. C) LX2 Azure cell spheroids on 12% PVA based CSHs. Scale bar: 200 μ m.

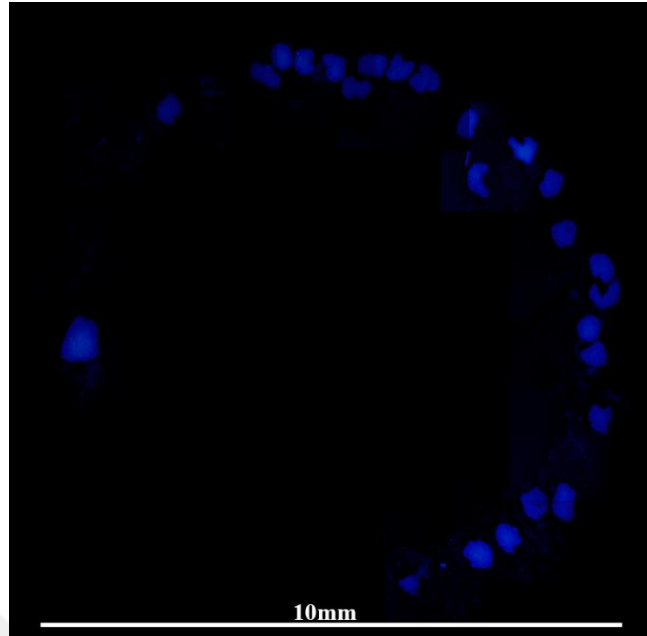


Figure 26. Ring shaped structure which was created by LX2 Azure spheroids on 12% PVA based CSH at day 15.

4.3.3. *Cell Viability on PVA-Gelatin*

According to SEM imaging results of hydrogel scaffolds, considering the porous structure of materials, newly synthesized CSHs were synthesized with addition of gelatin to yield final 12% PVA concentration and 5% Gelatin concentration (Figure 22). To evaluate biocompatibility of CSHs, 2×10^5 3T3 GFP (+) cells and approximately 50 spheroids, obtained with 2×10^4 cell number per 10 μ l droplet, were seeded on PVA-Gelatin based CSHs and cultured for 7 days. At the end of the 7 days of culture, GFP (+) 3T3 cells were still alive on hydrogel but with low morphological similarity compared to control group (Figure 27 A and B). Spheroids also showed lower morphological similarity compared to control group and they moved towards each other at the end of 7 days of culture (Figure 28 A and B).

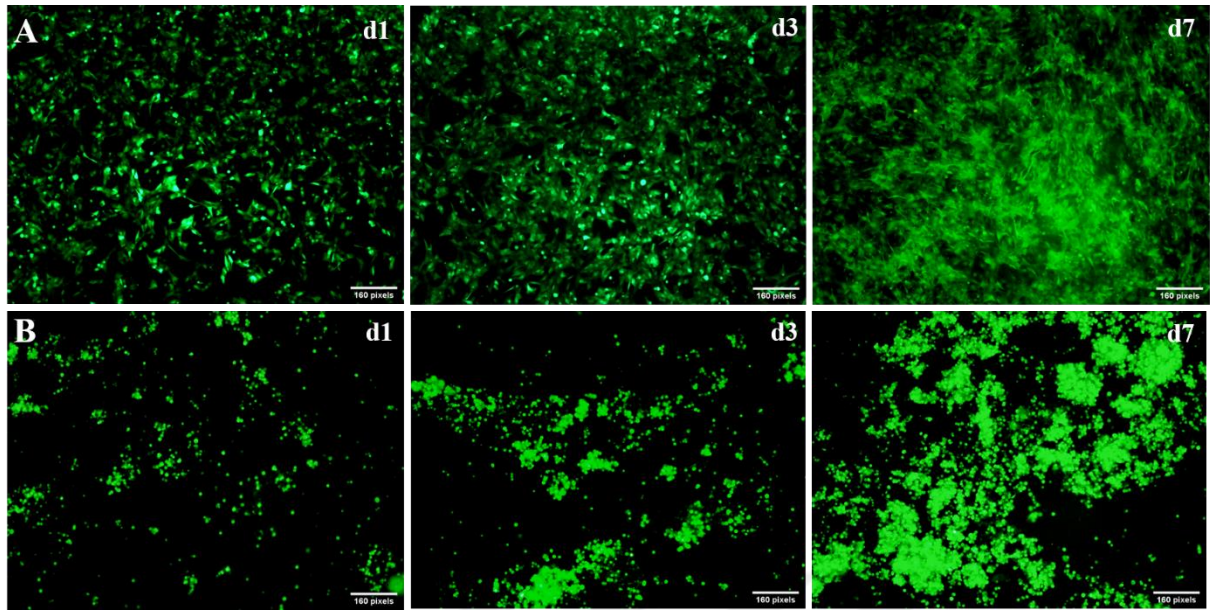


Figure 27. Cell viability of GFP (+) 3T3 cells on 12%PVA-5%Gelatin based CSHs for 7 days. A) Control group: 2D GFP (+) 3T3 cells on 24 well plate. B) GFP (+) 3T3 cells on 12%PVA-5%Gelatin based CSHs. Scale bar: 160 μ m.

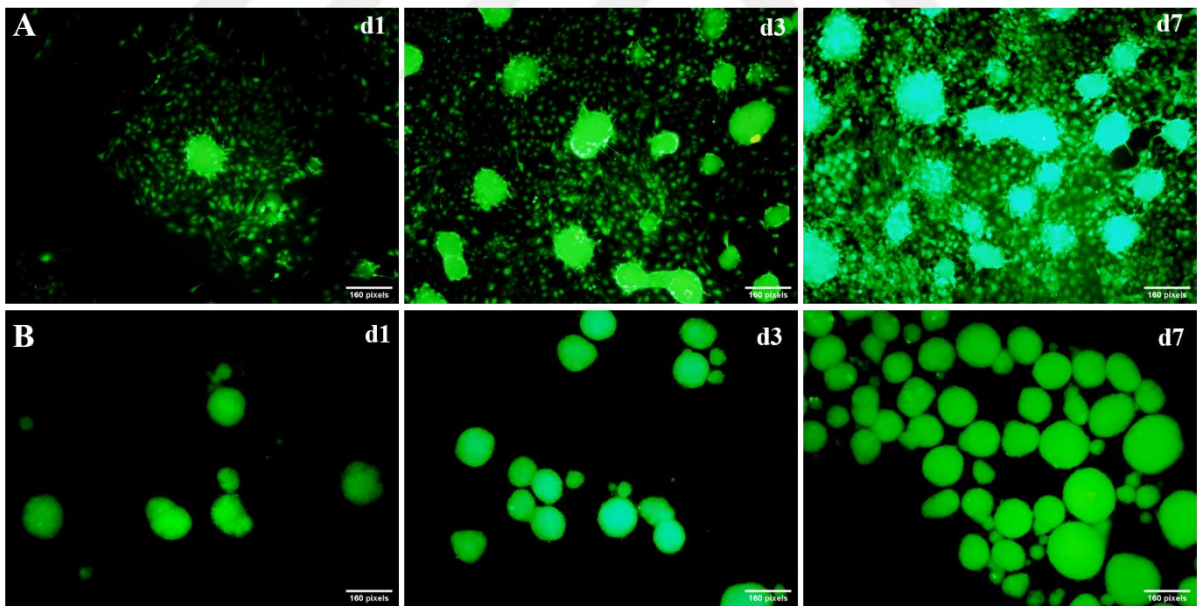


Figure 28. Cell viability of GFP (+) 3T3 cell spheroids on 12% PVA-5% Gelatin based CSHs for 7 days. A) Control group: 2D GFP (+) 3T3 cells on 24 well plate. B) GFP (+) 3T3 cells on 12% PVA-5% Gelatin based CSHs. Scale bar: 160 μ m.

4.4. Characterization of Decellularized Corneas

Decellularized corneas (DC) were compared with non-decellularized corneas (NDC) which were used as control group.

4.4.1. DNA Quantification Results

Decellularization 1:

According to Sasaki et al., mouse corneas were decellularized using 1% and 2% of sodium dodecyl sulfate (SDS) solution, 1% and 2% Triton-X solution and 30% Acetic acid to optimize most successful concentration. Leftover DNA amount on mice DCs decellularized with different chemical reagents were compared to native mouse cornea (Figure 29 A). It was found that decellularization with 1% SDS removed almost 85% of cells from cornea. To support this finding genomic DNA of these samples were run on agarose gel electrophoresis (Figure 29 B) and it was shown that most of the DNA was denaturated with 1% SDS.

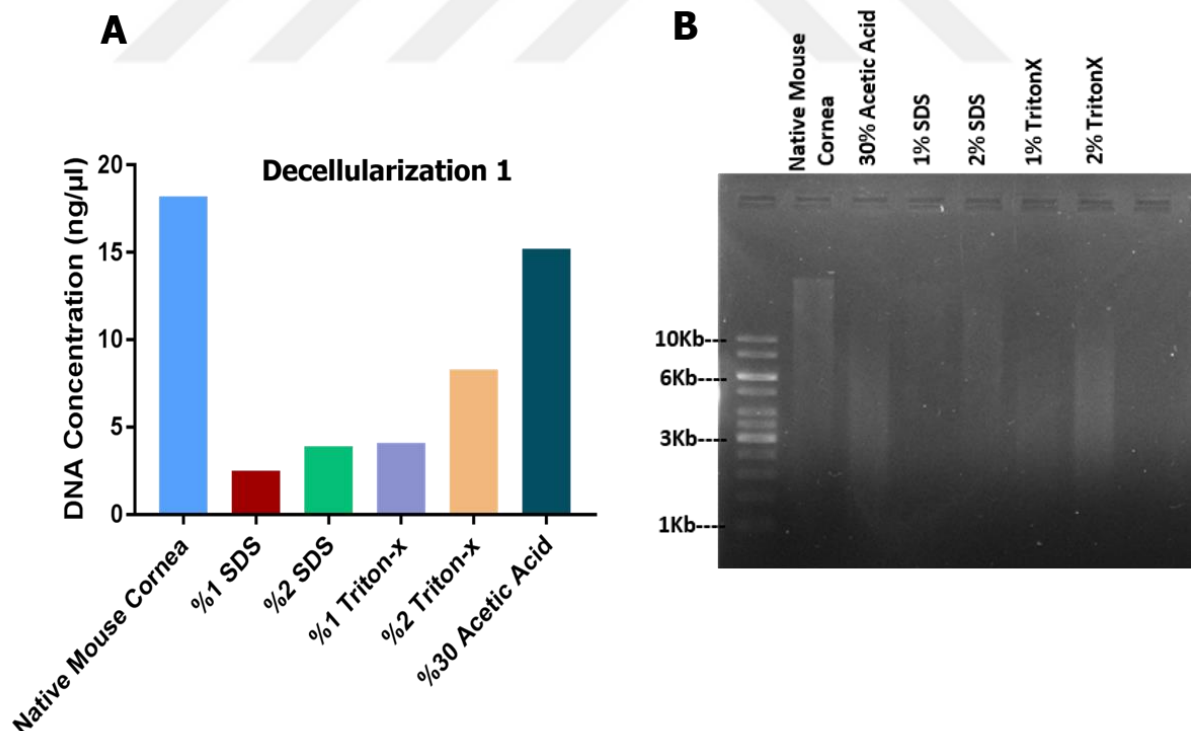


Figure 29. A) Leftover DNA amount of decellularized mouse corneas using different chemical agents. B) Genomic DNA on agarose gel electrophoresis.

Decellularization 2:

According to results of first decellularization experiment, 1% SDS solution was most effective solution on decellularization of cornea. So, next decellularization experiments were performed to investigate effect of lower SDS concentration on sheep and calf corneas (Figure 30). 1% SDS was still most effective decellularization concentration leftover DNA amounts of both mouse and sheep cornea were compared.

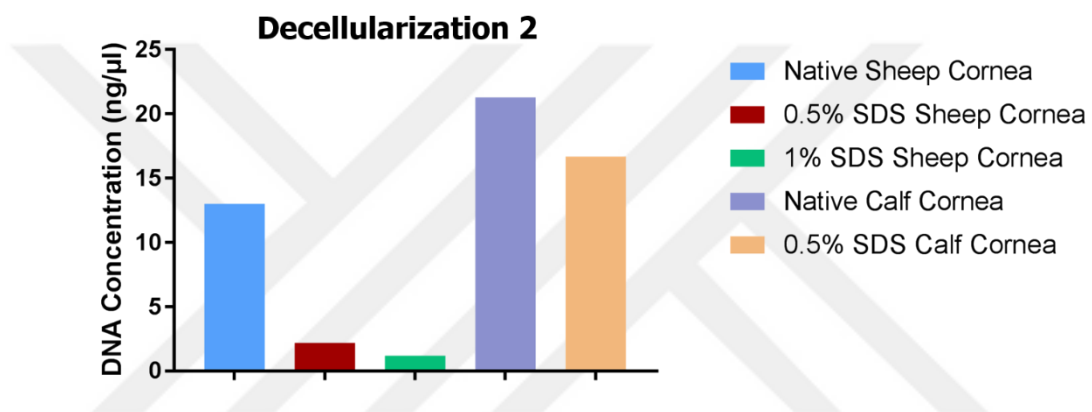


Figure 30. Leftover DNA amount of decellularized sheep and calf corneas using different concentration of SDS reagent.

Decellularization 3:

As reported above, left over DNA amount of decellularized corneas was lowest at 1% SDS concentration. Thus, final decellularization of mouse and human corneas were performed using 1% SDS concentration to evaluate decellularization capacity on human corneas (Figure 31). There was an outstanding difference between DNA amount of DCs and NDCs in both human and mouse corneas. Almost 95% of cells were removed from human cornea and almost 90% of cells were removed from mouse cornea.

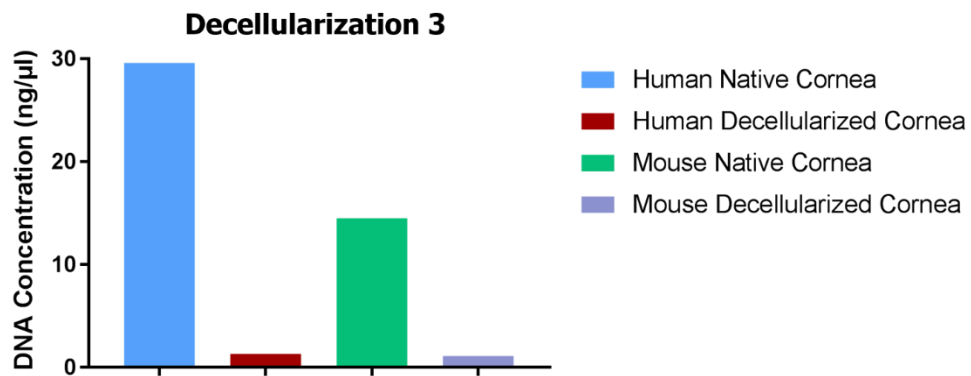


Figure 31. Leftover DNA amount of decellularized mouse and human corneas using 1% SDS.

4.4.2. *Hematoxylin – Eosin Staining*

Hematoxylin-Eosin staining was used to localize nuclei and cytoplasm of cornea cells. H&E staining of decellularized corneas was performed on sheep and calf corneas due to lack of human donor corneas. Decellularization was performed using of 0,5% SDS and 1% SDS concentrations on sheep corneas, 1% SDS on calf corneas. As control group, non-decellularized corneas were analyzed.

Sheep Corneas

Analyzing localization of nuclei and cytoplasm of cells on non-decellularized sheep corneas and decellularized sheep corneas with 0.5 % and 1% SDS were performed to support findings of DNA quantification results (Figure 32 A, B and C). It was indicated that there were still a few cells on DC. Hence, H&E staining also showed that a few nuclei was located on the DCs comparing NDCs. Since 1% SDS concentration was more successful than 0.5% SDS concentration, more amount of cell nuclei was observed on DCs, decellularized with 0.5% SDS.

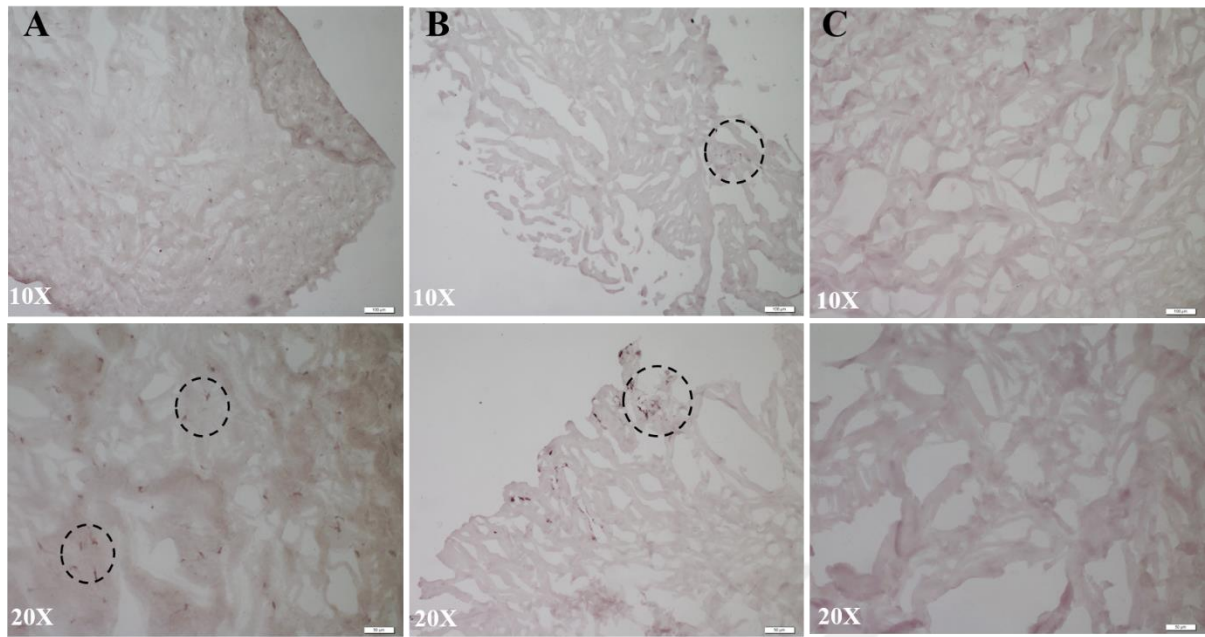


Figure 32. Hematoxylin-Eosin staining images of sheep cornea sections. Hematoxylin stains nuclei which are marked circularly, Eosin (pink) stains cytoplasm of cells. A) H&E staining of non-decellularized sheep cornea as control group. B) Decellularized sheep corneas using 0,5% SDS concentration. C) Decellularized sheep corneas using 1% SDS concentration. Scale bar (10X): 100µm, Scale bar (20X): 50µm.

Calf Corneas

Calf corneas were decellularized using only 0.5 % SDS due to lack of sample amount. H&E staining was performed on these corneas and as control group, non-decellularized corneas were analyzed. Nuclei and epithelium structure of DCs and NDCs were compared in Figure 33 A and B. It is observed that a few nuclei (marked circularly) was located on the DCs comparing the NDCs. Additionally, epithelium of DC was crucially disrupted with the SDS compared to NDC.

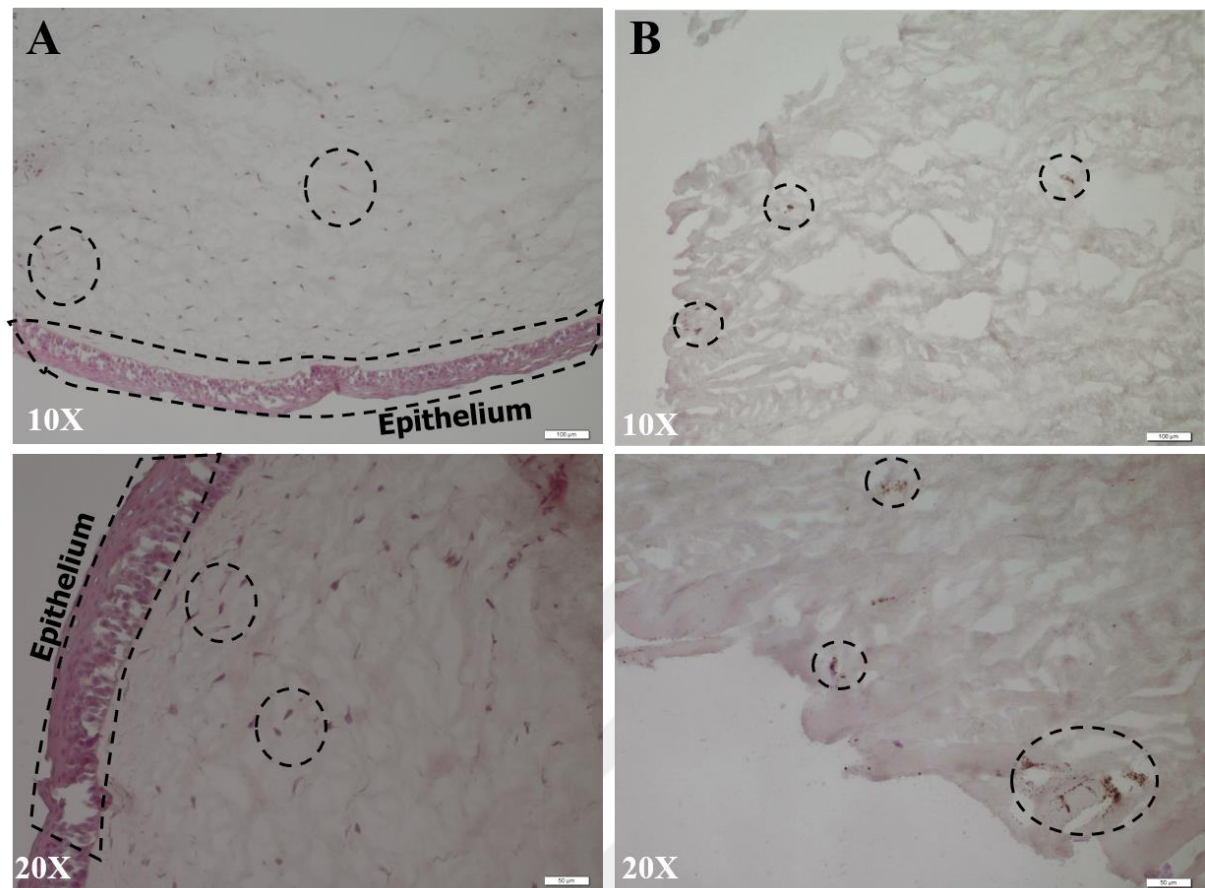


Figure 33. Hematoxylin-Eosin staining images of calf cornea sections. Hematoxylin stains nuclei which are marked circularly, Eosin (pink) stains cytoplasm of the cells. A) H&E staining of non-decellularized calf corneas as control group. B) Decellularized calf corneas using 0,5% SDS concentration. Scale bar (10X): 100µm, Scale bar (20X): 50µm.

4.4.3. *Periodic acid–Schiff Staining*

PAS staining of non decellularized sheep cornea and decellularized sheep corneas with 1% SDS was analyzed to localize glycogens, glycoproteins and glycolipids (Figure 34 A and B). Even though cells were removed from cornea through decellularization, the protein structure of cornea was maintained.

Sheep Corneas

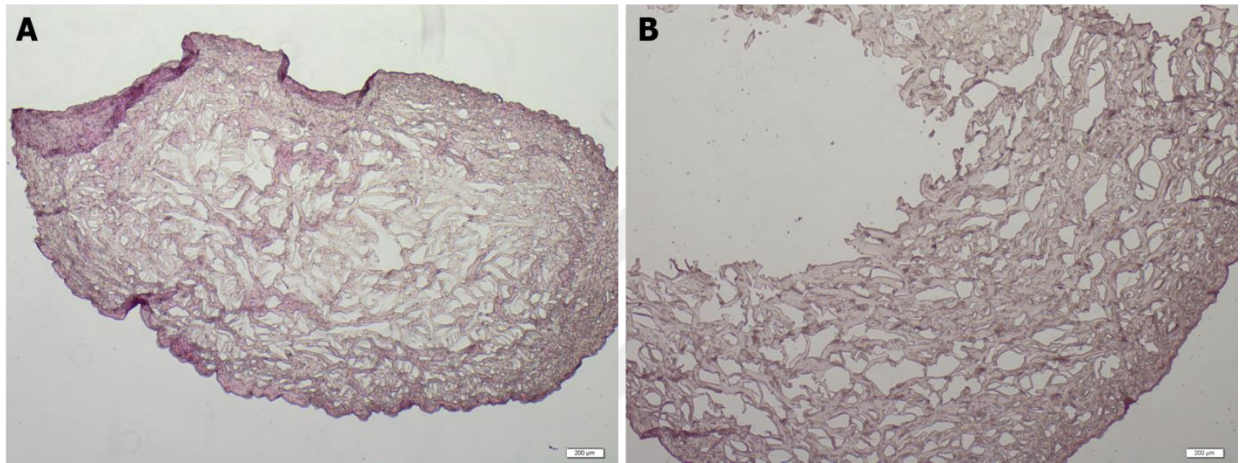


Figure 34. PAS staining images of sheep cornea sections. A) Non-decellularized sheep cornea as control group. B) Decellularized calf corneas using 1% SDS concentration. Scale bar: 200µm.

Calf Corneas

PAS staining of non decellularized calf cornea and decellularized calf corneas with 0.5% SDS was analyzed to localize glycogens, glycoproteins and glycolipids (Figure 35 A and B). Same epithelium disruption was also observed during PAS staining through decellularization. Additionally, protein structure of cornea was maintained even though most of cells were removed from cornea.

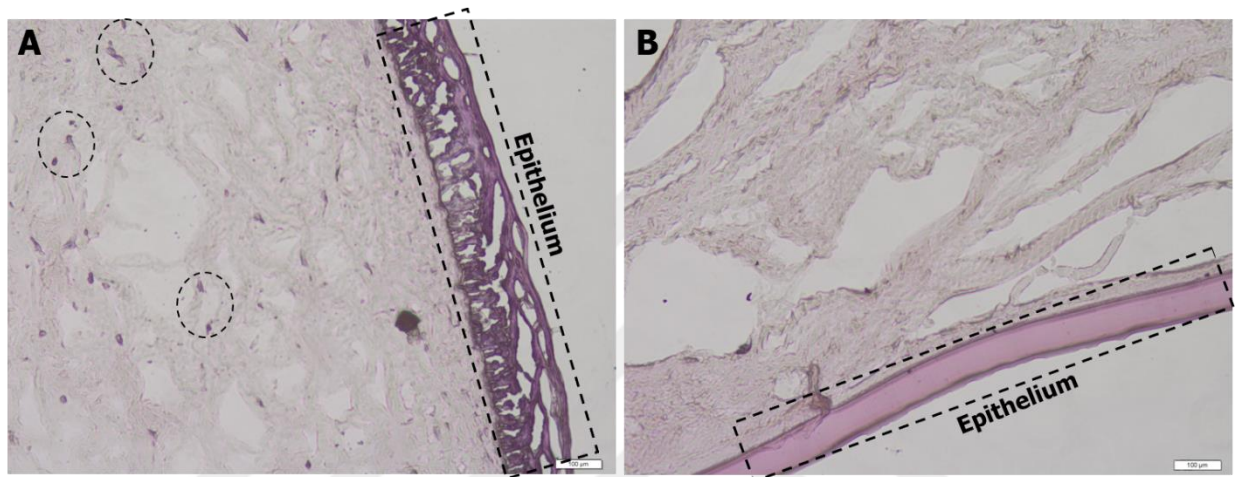


Figure 35. PAS staining images of calf cornea sections. A) Non-decellularized calf cornea as control group, showing structure of epithelium and nucleus localization. B) Decellularized calf corneas using 0.5% SDS concentration. Scale bar: 100µm.

4.4.4. *Biocompatibility of DCs : Recellularization*

To evaluate biocompatibility of decellularized corneas, 2×10^5 3T3 GFP (+) cells were seeded on DCs and cultured successfully for 3 days and compared with 2D standard cell culture plate cultures (Figure 36 A and B). Cells were located at lower side of DC and there were almost no cells in middle part of decellularized cornea.

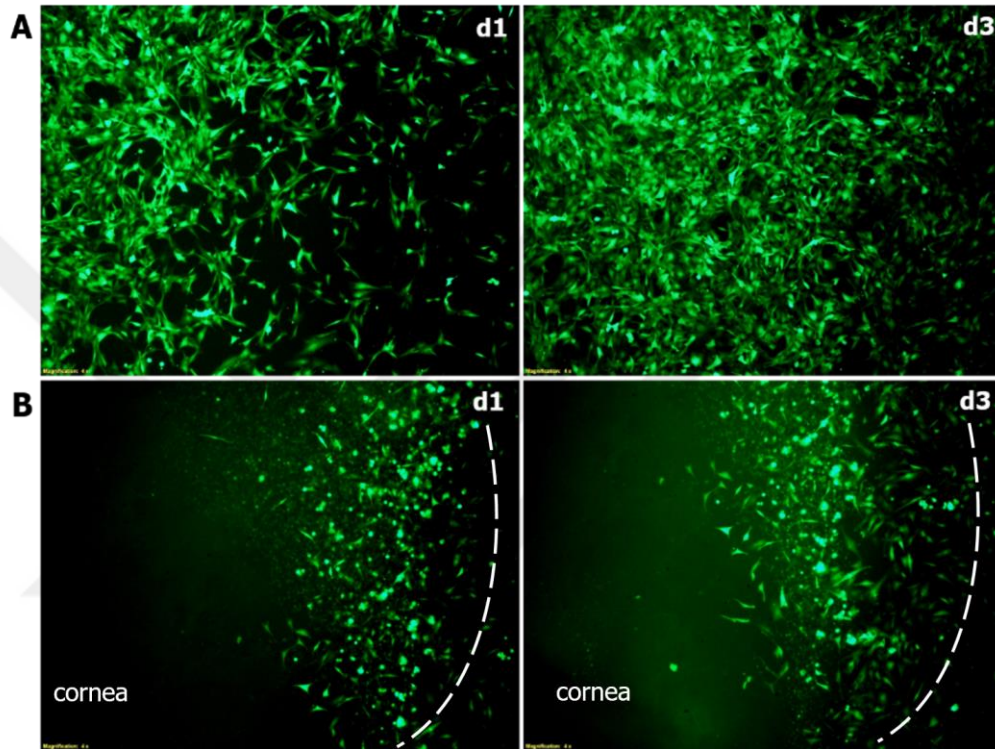


Figure 36. Cell viability of GFP (+) 3T3 cells on DCs for 3 days. A) Control group: 2D GFP (+) 3T3 cells on 24 well plate. B) GFP (+) 3T3 cells on DCs.

4.5. Fabrication and Validation of Microfluidic System

Fabricated microfluidic platform was validated with GFP (+) 3T3 culture in order to be used in iPSC culture. 2×10^5 3T3 GFP (+) cells were seeded on 12% PVA – 5% Gelatin based CSHs and cultured in microfluidic platform with 1 μ l/min flow for 7 days. It was mentioned before that less morphological similarity was observed on PVA-Gelatin based CSHs. However, dynamic culture conditions have solved this problem. As its shown in Figure 37 A and B, cells which were cultured on CSHs under dynamic conditions have same morphological structure with static control group. Additionally, there were no remarkable differences on cell proliferation of the two groups.

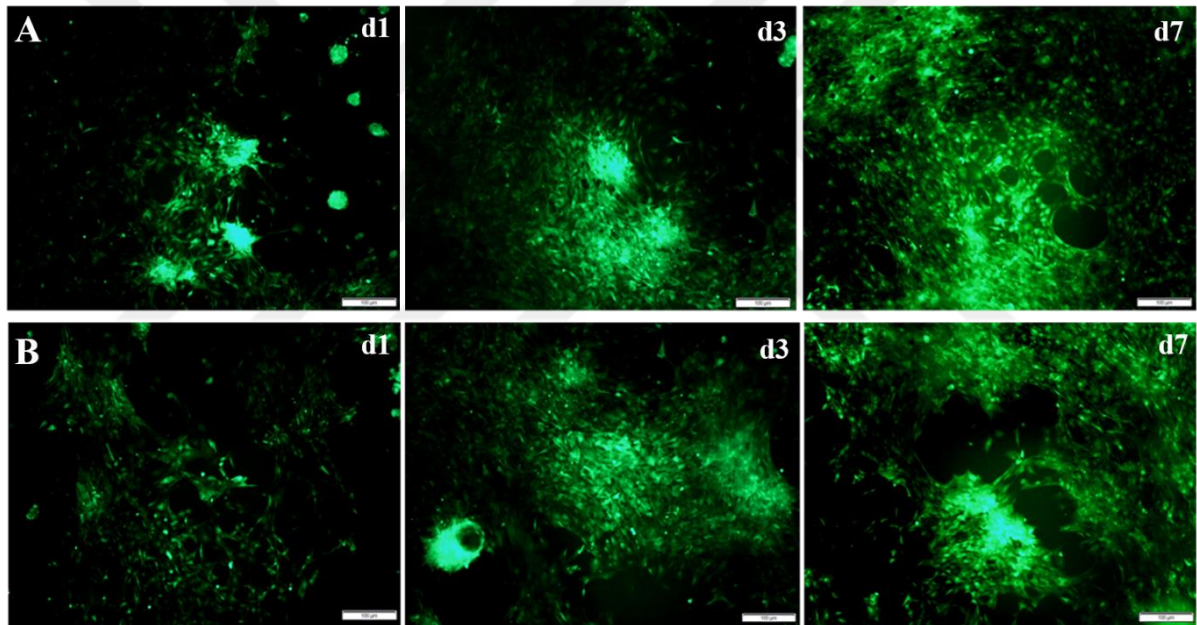


Figure 37. Cell viability under dynamic conditions for 7 days. A) Static control group: GFP (+) 3T3 cells on 24 well plate. B) GFP (+) 3T3 cells on 12%PVA-5%Gelatin based CSHs under dynamic conditions. Scale bar: 200 μ m.

4.5.1. *PIEZO2* Expression

Previously indicated development on cell morphology was obtained by effect of fluidic pressure created by dynamic conditions. Hence, to investigate effect of this pressure, 3T3 cells were cultured under static and dynamic conditions for 3 days and expression of PIEZO 2 gene was analyzed at the end of 3 days of culture (Figure 38). PIEZO 2 expression was low on dynamic culture at first day. There was tremendous change in PIEZO 2 expression of cells which were cultured under dynamic conditions at 3rd day. This data supports the data shown in Figure 37.

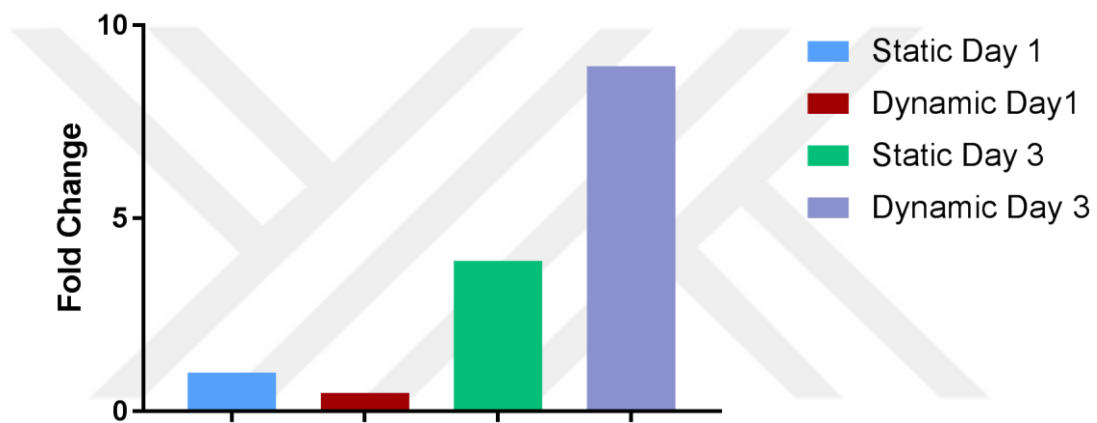


Figure 38. The expression of PIEZO2 gene in GFP (+) 3T3 cells under static and dynamic conditions for 3 days.

4.6. Culturing of Corneal Organoid inside Microfluidic System

Developed cornea organoids (at their 6 weeks of cultivation) were matured. Then they were disaggregated into single cells and were cultured inside fabricated microfluidic system under 0,5 $\mu\text{l}/\text{min}$ flow rate for 35 days (Figure 39). It was shown that single cells and bigger organoid pieces started to adhere to the surface of hydrogel scaffold.

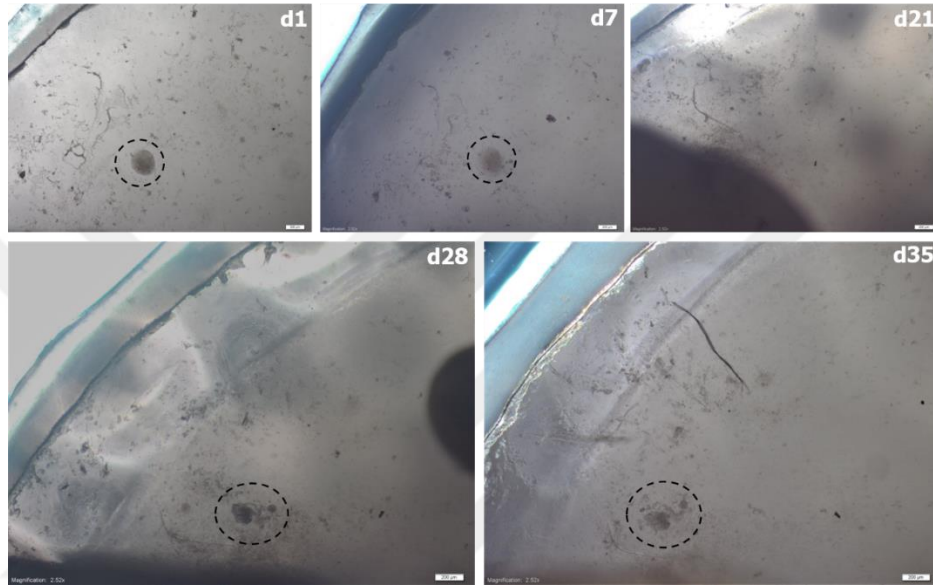


Figure 39. Light microscopy images of cornea organoids disaggregated into single cells inside microfluidic system under 0.5 $\mu\text{l}/\text{min}$ flow rate for 35days. Scale bar: 200 μm .

Compact structure of cornea organoid (at their 6 weeks of cultivation) was cultured inside microfluidic platform under 0.5 $\mu\text{l}/\text{min}$ flow rate for 35 days and its light microscopy images were taken at different time points (day1, day7, day21, day 28 and day35) (Figure 40). It was observed that cornea organoid penetrated on hydrogel and matured.

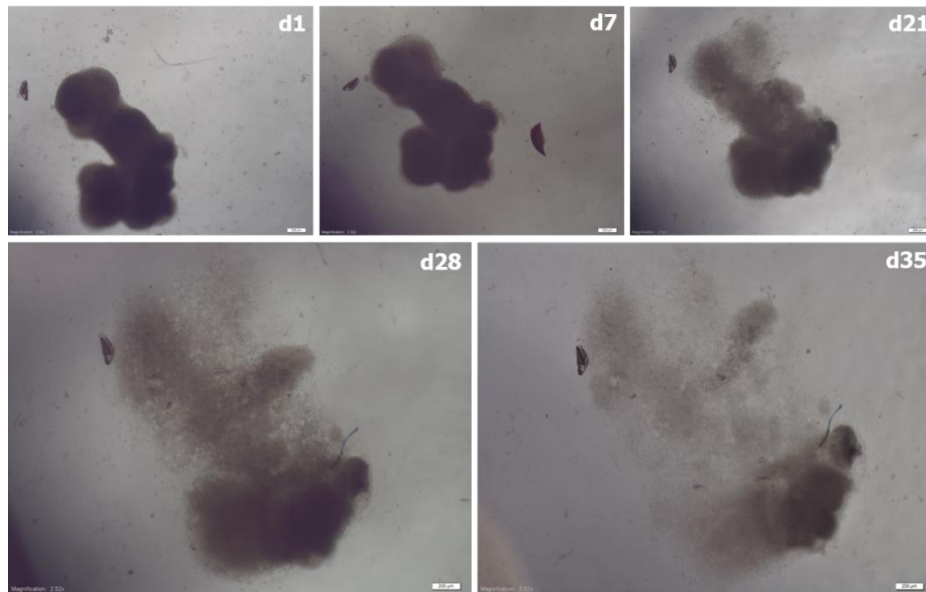


Figure 40. Light microscopy images of cornea organoids at their 6 weeks cultivation inside microfluidic system under 0.5 $\mu\text{l}/\text{min}$ flow rate for 35days. Scale bar: 200 μm .

4.6.1. *Gene Expression of Cornea Organoids Inside the Microfluidic Platform*

At the end of the maturation for 5 weeks of 6 cornea organoids inside microfluidic platform, organoids were characterized with Ki-67 Antibody (proliferation marker) and Cytokeratin 3 (CK-3, corneal epithelium marker) (Figure 41). DAPI was used to localize nuclei. As it is shown in Figure 41, Ki-67 indicates that cells were able to proliferate successfully and CK3 expression shows that cells of organoids kept their corneal organoid formation.

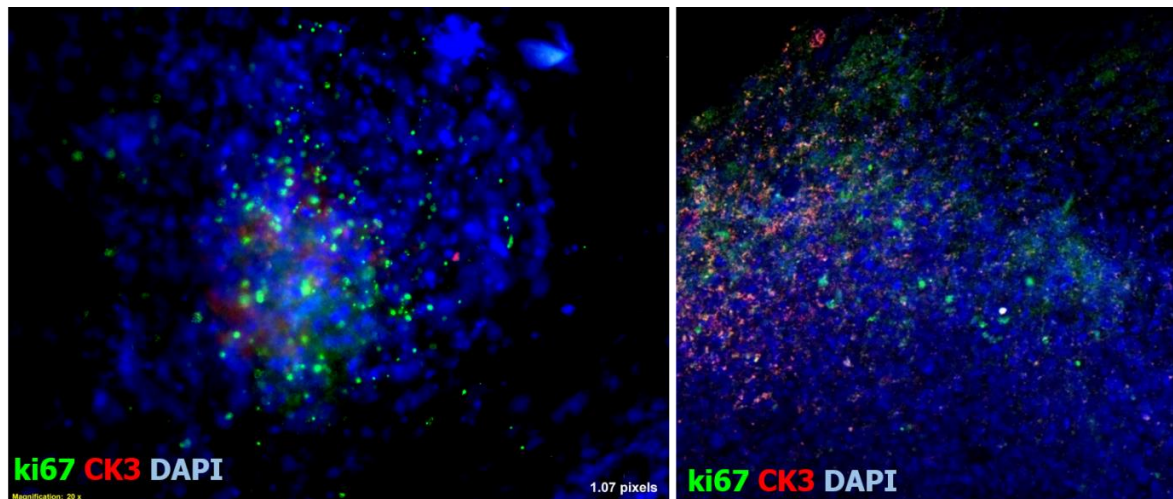


Figure 41. Confocal microscopy images of cornea organoids which were matured in microfluidic platform for 5 weeks. Ki67 (green) is proliferation marker, CK3 (red) is corneal epithelium marker, DAPI (blue) stains nuclei.

4.7. Differentiation of IPSC Cells into Corneal Organoid Inside Microfluidic System

IPSCs directed to corneal differentiation were cultured inside microfluidic system as described in section 3.6.6.4. Different experiments were set up at different dates in order to investigate results with different characterization methods. Light microscopy imaging was performed by starting third day of cultivation to prevent disturbing cells. Localization of hydrogel, DCs and cells, and how images were taken is shown in Figure 42 A and B. In the middle part of the system where cornea and gel overlapped, the images are less clear due to distance from objective. Further light microscopy images were taken at 10X.

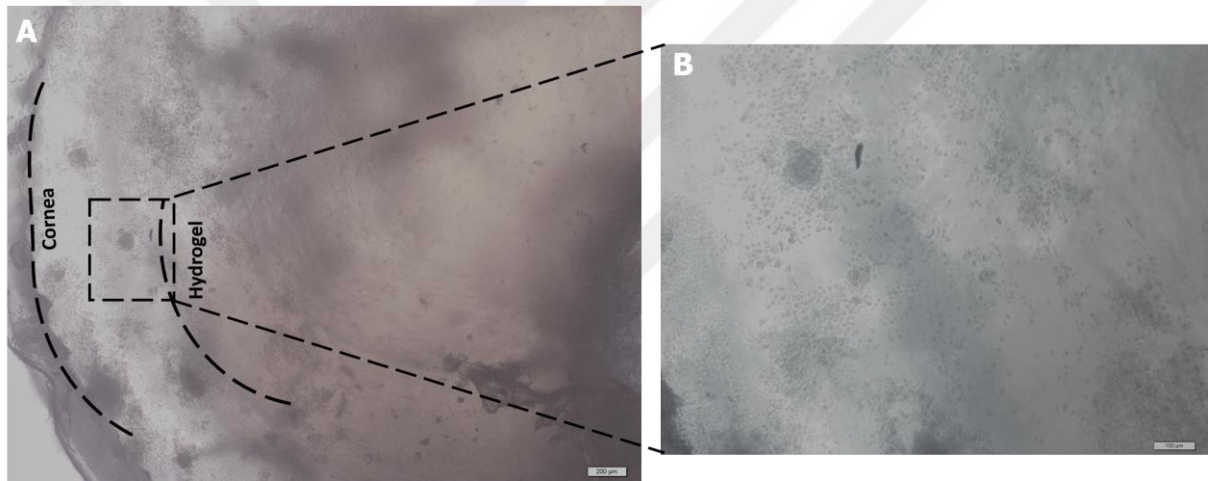


Figure 42. General view that shows how light microscopy images were taken. A) Cellular location of hydrogels showing hydrogel and cornea borders using lines at 4X image. B) Closer look to cells at 10X image.

4.7.1. *3D Progenitor Cells According to Susaimanickam Protocol*

According to Susaimanickam protocol, dynamic culture was started at retinal differentiation phase and compared with static culture of same conditions and light microscopy images were taken at different time points of dynamic culture (day 3, day 7, day 14 and day 21) (Figure 43). At the end of 21 days of culture (25th day of retinal differentiation) cells started to form aggregates (shown in the squares) and express ECM or possibly collagen fibers (shown with arrows) under dynamic conditions. However, these findings were not proved with experimenting methods due to COVID-19 pandemic.



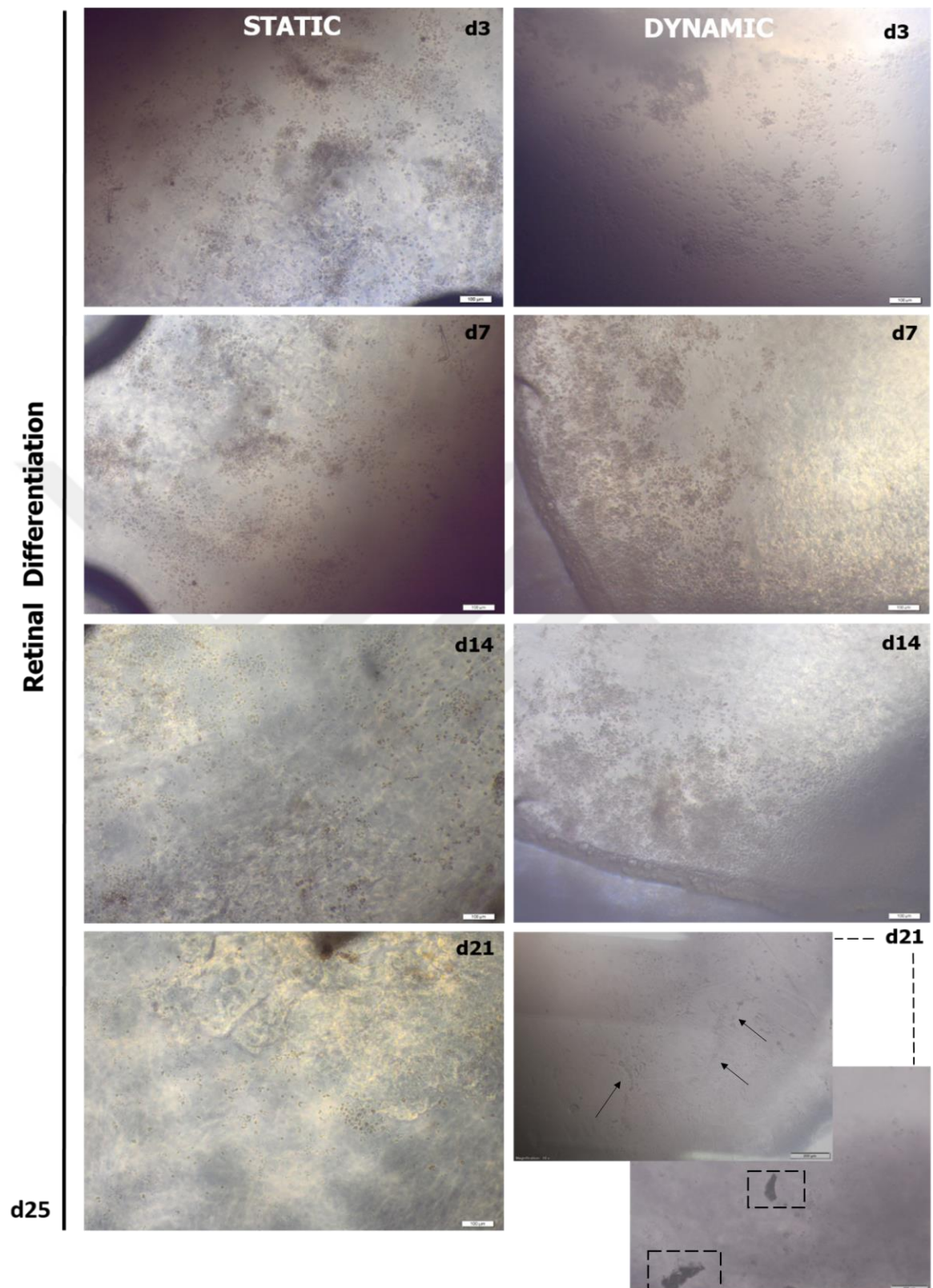


Figure 43. Light microscopy images of progenitor cells which were cultured according to Susaimanickam Protocol under static and dynamic conditions.

4.7.2. *3D Progenitor Cells According to Foster Protocol*

According to Foster protocol, dynamic culture was started at the 5th day of neural differentiation phase and compared with static culture of the same conditions and light microscopy images were taken at different time points of dynamic culture (day 3, day 7, day 14 and day 21) (Figure 44). At the end of 21 days of culture (6th day of corneal differentiation), cell aggregates under static conditions were disrupted. Even though there were small cell aggregate formations, there were no remarkable changes under dynamic culture conditions.



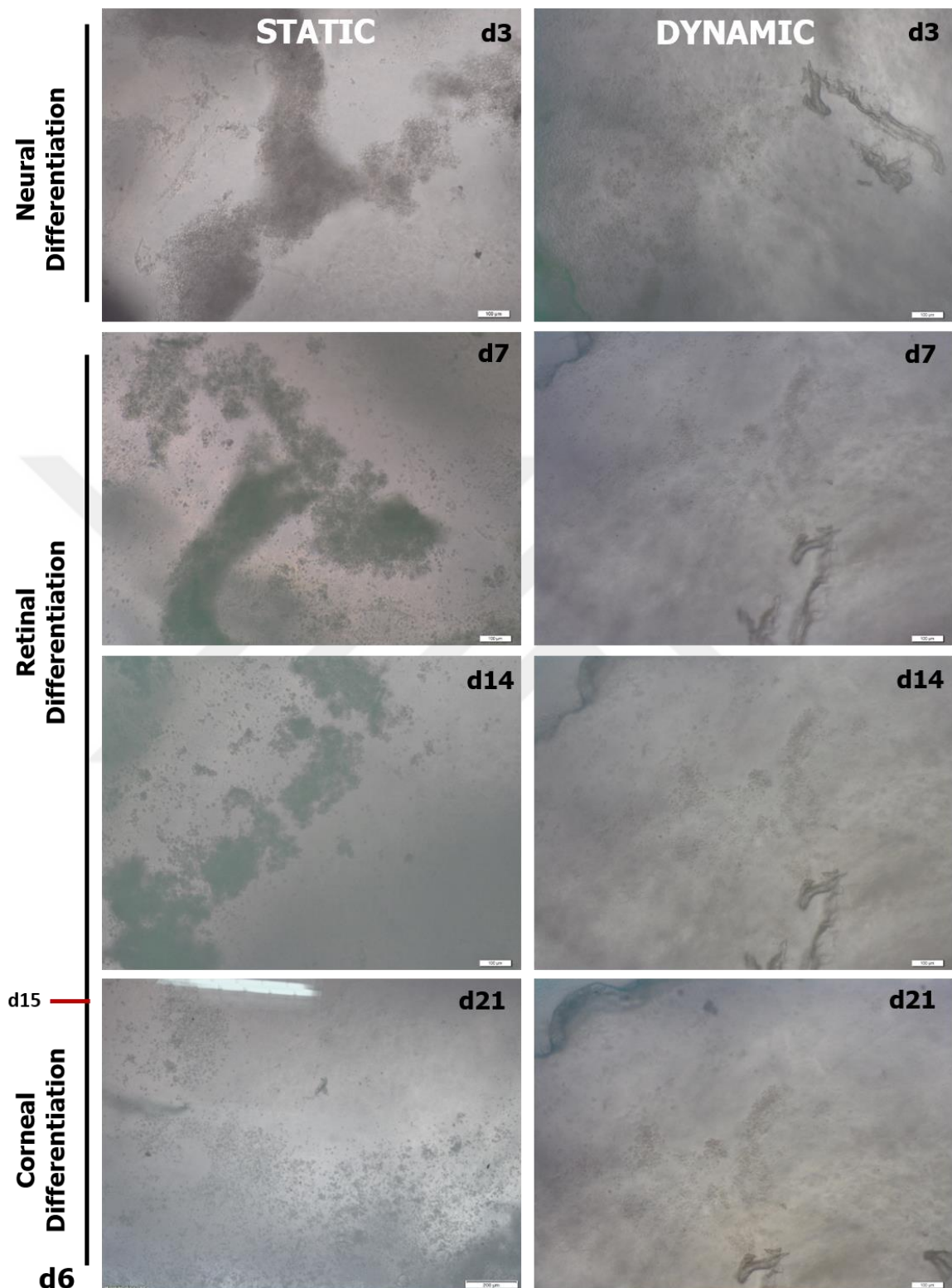


Figure 44. Light microscopy images of progenitor cells which were cultured according to Foster Protocol under static and dynamic conditions.

5. DISCUSSION

In this thesis, different polymer based hydrogel scaffolds were synthesized, donor corneas were successfully decellularized and 3D microfluidic platform was designed and fabricated to create native like microenvironment for cornea organoid culture.

Among different polymers, most suitable CSH scaffold for cellular behaviors was PVA-Gelatin mixture polymer. Comparing different chemicals for decellularization of corneas, SDS was most successful chemical agent.

Hydrogels have been commonly used to support cell proliferation and differentiation due to their resembling structure to ECM and they been used as drug carriers on account of permeable structures⁵². Hence, different polymers (GelMA, PEG PVA, PVA-Gelatin) were cross-linked inside the concave shaped PDMS mold to develop more suitable permeable hydrogel for cell culture studies.

Crosslinking of three different concentration of GelMA was compared: 5%, 10% and 20%. Gelatin was performed using 0.5% PI. Increasing GelMA concentration resulted with mechanically more stable CSHs and more viscous liquid form of GelMA which makes it harder to sterilize through the 0.22 μ m filter. According to SEM images of GelMA based hydrogels, increasing ratio of GelMA resulted with smoother surface of CSH, larger pore size in diameter but lower number of pores. So, most suitable hydrogels was 10% of GelMA due to its mechanical stability and porous structure. Then GFP (+) 3T3 cells were successfully cultured on 10% GelMA based CSH (Figure 22).

PEG based hydrogels were synthesized using different concentrations of PEG (5%, 10% and 20%) and PI (1%, 2%). However, due to depth of concave shaped mold, only 20% concentration of PEG using 1% and 5% PI were homogeneously gelled. Crosslinking of other concentration of PEG resulted with heterogeneous gelatin and rupturing easily while removing hydrogels from mold.

Chemical crosslinking of 12% PVA was performed using different concentrations of DMSO inside deionized water (25%, 50% and 75%). Three concentration of PVA was successfully gelled. Mechanically most stable hydrogel was achieved using 75% of DMSO. However, among these three DMSO concentration, according to SEM images and cell culture studies, 25% DMSO concentration was choose suitable hydrogels. There was higher amount of pore inside 25% DMSO used PVA based hydrogel (Figure 20) and LX2 Azure cells were alive only 25% DMSO used PVA based hydrogel for 7 days (Figure 23). Then, single cells and spheroids were successfully cultured on 12% PVA cross-linked with 25% DMSO based CSH for 15 days and same experiments was performed with GFP (+) 3T3 cells. However, cells didn't show their morphological structure similar to control group. Then 12% PVA- 5% gelatin was cross-linked on heater to form PVA-Gelatin based CSH due to gelatin's ability to promote cellular behaviors. SEM images also revealed that PVA-based hydrogels had numerous number of pore and pore size. But, there were still morphological differences between control group and experimental group. This was solved within microfluidic platform.

In first trial of decellularization of mouse corneas, different chemical agents (SDS, Triton X and Acetic Acid) and their different concentrations (SDS: 1%, 2%; Triton X: 1%, 2%; 30% Acetic acid) were compared. According to Nanodrop results, least amount of leftover DNA was achieved by using 1% SDS solution, which means almost 87% of cells were removed from cornea. Then, 1% SDS was compared with 0,5% SDS solution using sheep and calf corneas and best result was still obtained using 1% of SDS. Finally, decellularization of mouse and human corneas were successfully performed using 1% SDS concentration. Later, all decellularization experiments were done with 1% SDS.

Removal of cells from sheep and calf corneas was also proved through Hematoxylin-Eosin staining. HE staining results also revealed that great majority of cells were successfully removed from both sheep and calf corneas with less amount of left over cells nuclei (Figure 31, Figure 32). Structural changes in decellularized corneas were checked with H&E and PAS staining. In calf corneas, structure of

epithelial layer was disturbed because of that SDS solubilize membrane proteins while maintaining stromal structure of the cornea ⁷³. (Figure 32 & Figure 34).

Initially, GFP (+) 3T3 cells were seeded on 12% PVA-5% Gelatin based CSHs and cultured for 7 days under static and dynamic conditions using developed microfluidic platform. Problem about morphological changes that was mentioned above was solved under dynamic culture conditions (Figure 35). This means that dynamic culture conditions (applied flow rate) had powerful effect on cellular behaviors. This hypothesis was proved comparing PIEZO2 gene expression level of 3T3 cells under static and dynamic conditions. Tremendous change was observed at the end of the 3 days of culture, gene expression level of cells which were cultured under dynamic conditions expressed was higher than static culture (Figure 36).

Later, cornea organoids which were developed under static culture conditions were successfully matured inside the microfluidic platform for 35 days (Figure 38). Matured cornea organoids expressed proliferation marker (ki67) and corneal epithelial differentiation marker (CK3) at the end of the 35 days of culture (Figure 39).

Finally, iPSCs derived progenitor cells which were aimed to differentiate according to two different protocol were cultured inside developed 3D biomimetic microfluidic platform for 21 days. Less amount of human corneas leads to minimize microfluidic platform size and cell seeding area. Hence, small size of microfluidic platform sometimes caused imaging problems. The use of epoxy to integrate inlet and outlet tubing of the platform was covered small part of bottom of platform which resulted with blockage of some part of the areas in platform. To overcome this imaging problem, only a small amount of epoxy was used to hold tubing.

In Susaimanickam protocol, progenitor cells were cultured in dynamic platform starting their retinal differentiation phase. At the end of 21 days of dynamic culture, cells were reached 25th days of retinal differentiation and cells started to form aggregates (Figure 41). There were also ECM or collagen fibers similar structures were observed on the 21st day of dynamic culture. However, these structures could

not be analyzed and progenitor cells were not differentiated until corneal maturation because of COVID-19 pandemic. In Foster's protocol, progenitor cells were cultured in their 5th days of neural differentiation till the 6th days of corneal differentiation (21days in total). Cells formed aggregates at 3rd day of static culture and these aggregated cells were disintegrated over 21 days of culture (Figure 42). There were also small cell aggregations were observed in dynamic culture, but not as much as other protocol. But, cells could not be cultured until fully corneal maturation due to pandemic.



6. CONCLUSION and FUTURE PERSPECTIVES

As a result, in this thesis, development of 3D biomimetic microfluidic platform for cornea organoid culture was successfully completed. PVA-Gelatin based permeable concave shaped hydrogel scaffold was synthesized comparing different polymers to provide diffusion of culture medium through hydrogel and decellularized cornea. Polymer based hydrogel scaffold may be characterized in more detail. Decellularization of corneas was successfully performed optimizing different protocol and chemicals. In developed microfluidic platform, decellularized cornea was used to promote cellular behaviors since decellularized corneas still includes native cornea's proteins and other molecules even though cells were removed. 3D microfluidic platform was designed and fabricated to create native like microenvironment for cornea organoid culture. Cornea organoids which were cultured in static conditions were successfully matured inside this platform. iPSCs were differentiated under dynamic conditions using developed microfluidic system. iPSCs differentiation into cornea organoids further should be performed one more time to prove that this system works and collagen fibers orientation must be checked.

This developed microfluidic environment may be further used in drug permeability tests of 3D cornea structures and such platform can be confirmed using computational simulations, in addition to wet-lab experiments.

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8. APPENDIX

8.1. Ethic Committee Approval



**İZMİR BİYOTIP VE GENOM MERKEZİ
GİRİŞİMSSEL OLMAYAN ARAŞTIRMALAR ETİK KURULU (İBG-GOEK)
KARARI**

Toplantı Tarihi	: 16/05/2020	Toplantı Günü	: Cumartesi
Toplantı Sayısı	: 6	Toplantı Saati	: 13:00

Sayın Sinan GÜVEN,

2020-020 Protokol No'lu; sorumlusu olduğunuz "Doku Mühendisliği Yaklaşımı ile Kornea Organoid Modellerinin Geliştirilmesine Yönelik 3B Biyomimetik Platform Geliştirilmesi" başlıklı araştırmanın uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

Prof. Dr. H. Alper BAĞRIYANIK
Başkan

Pandemi süresince online ortamda gerçekleştirilen toplantımızda alınan kararlar tek imzalı olarak düzenlenmektedir. Pandemi sona erdikten sonra ıslak imzalı karar belgesi teslim edilecektir.

8.2. Patient Consent Form

BİLGİLENDİRİLMİŞ GÖNÜLLÜ OLUR FORMU

Araştırmanın Adı: İndüklenmiş Pluripotent Kök Hücre (İPKH) Kaynaklı Kornea Organoid Modellerinin Oluşturulması

Araştırmayı yapan kişi/kurum:

Araştırma Dokuz Eylül Üniversitesi Tıp Fakültesi Göz Hastalıkları Anabilim Dalı'na "Kornea Nakli" için başvuran hastalardan oluşacaktır. Çalışma Göz Hastalıkları Anabilim Dalı'dan Doç.Dr Aslı YILDIRIM ve İzmir Biyotıp ve Genom Merkezi'nden Prof.Dr. H.Alper BAĞRIYANIK tarafından yürütülecektir.

Araştırmanın Amacı Nedir?

Korneanın keratokonus gibi şekilsel anomalileri, kalıcı kornea ödemi, keratitler veya kazalar, kimyasal yanıklar ve yaralanmalar sonucu gelişen lekeler (opasiteler), kalıtsal kornea lekeleri, uzun süreli kontakt lens kullanımına bağlı kornea hasarları gibi çok sayıda patolojik durumda korneada geri dönüşümsüz hasar oluşabilmektedir. Bu hastalarda sıklıkla tek tedavi seçeneği cerrahi, yani kornea nakli (keratoplasti) ile olmaktadır. Kornea nakli, hastalıklı korneanın kadavradan alınan kornea dokusu ile değiştirilmesidir.

Dünya Sağlık Örgütü 2009 yılı verilerine göre dünya üzerinde yaklaşık 8 milyon insan kornea kaynaklı kördür. Bunların yaklaşık 1.5 milyonu çocuk hastalardır ve acilen kornealarının değiştirilmesine ihtiyaç vardır. Ülkemizde de kornea nakli bekleyen çok sayıda hasta bulunmaktadır.

Çalışmamızda kornea nakli için alternatif bir seçenek olacağını düşündüğümüz yöntemi geliştirebilmemiz için sizlerden halihazırda alınacak olan ve bir daha kullanılmayacak olan korneaları çalışmamızda kullanmak üzere talep etmekteyiz.

Katılma Koşulları Nedir?

Bu çalışmaya Dokuz Eylül Üniversitesi Tıp Fakültesi Göz Hastalıkları Anabilim Dalı'na başvuran ve kornea nakli yapılacak olan hastalar dahil edilecektir.

Nasıl Bir Uygulama Yapılacaktır?

Enfekte olmayan kornea patolojileri nedeniyle görme tashihi amacıyla gerçekleştirilen kornea nakilleri sonrası açığa çıkan morfolojik yapısı bozulmamış kornea dokuları kullanılacaktır. Hali hazırda nakil işlemi için operasyona alınan hastalardan cerrahi işlem esnasında zaten çıkarılacak ve bir daha kullanılmayacak kornealar bu çalışma için ayrılacaktır.

Sorumluluklarım Nelerdir?

Araştırma ile ilgili olarak sizin sorumluluklarınız yoktur.

Katılımcı Sayısı Nedir?

Dokuz Eylül Üniversitesi Göz Hastalıkları Anabilim Dalı'na kornea nakli için başvuran hasta sayısı kadar katılımcıdan oluşacaktır.

Katılımım Ne Kadar Sürecektir?

Bu çalışmada yer almanız için ön görülen süre yoktur.

Çalışmaya Katılma ile Beklenen Olası Yararlar Nelerdir?

Bu çalışmada sizin için beklenen yararlar yada zararlar yoktur. Bilime katkı sağlayabilecektir.

Çalışmaya Katılma ile Beklenen Olası Riskler Nelerdir?

Ameliyat esnasından yapılacak girişim dışında ekstra bir müdahalede bulunulmayacaktır. Bu yüzden olası bir risk yoktur.

Hangi Koşullarda Araştırma Dışı Bırakılabilirim?

Çalışma materyali yetersiz olduğunda, çalışmacı sizin izniniz olmadan sizi çalışmadan çıkarabilir.

Çalışma Kapsamında Giderler Karşılanacak Mı?

Çalışma sırasında gerekli olan işlemlerin masrafı size veya güvencesi altında bulunduğunuz resmi ya da özel hiçbir kurum veya kuruluşa ödetilmeyecektir.

Çalışmayı Destekleyen Kurum Var Mıdır?

Çalışmamız TÜBİTAK 1001 projesi kapsamında TÜBİTAK tarafından desteklenmektedir.

Çalışmaya Katılmam Nedeniyle Tarafıma Herhangi Bir Ödeme Yapılacak Mıdır?

Bu çalışmada yer almanız nedeniyle size hiçbir ödeme yapılmayacaktır.

Katılmama İlişkin Bilgiler Konusunda Gizlilik Sağlanabilecek Mi?

Bu çalışmayla bağlantılı olarak elde edilen ve sizinle özdeşleşmiş her bilgi gizli kalacak, 3. kişilerle paylaşılmayacak ve yalnızca sizin izniniz veya kanunun gerektirdiği ölçüde ifşa edilecektir. Gizlilik tanımlanmış bir kodlama prosedürüyle sağlanacak ve kod çözümüne erişim yalnızca çalışmanın sorumlusu olan araştırmacıyla sınırlı kalacaktır. Tüm veriler, sınırlı erişime sahip güvenli ve şifreli bir bilgisayarda tutulacaktır.

Araştırmaya Katılmayı Kabul Etmemem Veya Araştırmadan Ayrılmam Durumunda Ne Yapmam Gerekir?

Bu çalışmanın içinde olmak isteyip istemediğinize etki altında kalmadan, bağımsızca karar verebilirsiniz. Bu çalışmaya gönüllü olarak katılmaya karar vermeniz halinde dahi, sahip olduğunuz herhangi bir hakkı kaybetmeden veya herhangi bir cezaya maruz kalmadan istediğiniz zaman çekilebilirsiniz. Çalışmadan çekilmek istemenizin ceza karşılığı yoktur ve sahip olduğunuz faydaları kaybetmiş olmazsınız. Bu çalışmaya katılmayla ilgili kararınız, tedavinizi veya doktorunuzla olan ilişkinizi hiç bir şekilde etkilemeyecektir.

Hastanın Beyanı

Yukarıda yer alan ve araştırmaya başlanmadan önce gönüllüye verilmesi gereken bilgileri gösteren 2 sayfalık metni okudum ve sözlü olarak dinledim. Aklıma gelen tüm soruları araştırmacıya sordum, yazılı ve sözlü olarak bana yapılan tüm açıklamaları ayrıntılarıyla anlamış bulunmaktayım. Çalışmaya katılmayı isteyip istemediğime karar vermem için bana yeterli zaman tanındı. Bu koşullar altında, bana ait tıbbi bilgilerin gözden geçirilmesi, transfer edilmesi ve işlenmesi konusunda araştırma yürütücüsüne yetki veriyor ve söz konusu araştırmaya ilişkin bana yapılan katılım davetini hiçbir zorlama ve baskı olmaksızın büyük bir gönüllülük içerisinde kabul ediyorum. Bu formu imzalamakla yerel yasaların bana sağladığı hakları kaybetmeyeceğimi biliyorum. Bu formun imzalı ve tarihli bir kopyası bana verildi.

Gönüllünün (veli/vasi):

Adı- soyadı:

Telefon:

Adres:

İmzası:

Açıklamaları yapan araştırmacının:

Adı- soyadı: Doç. Dr. Aslı YILDIRIM

Telefon:

İmzası:

Rıza alma işlemine başından sonuna kadar tanıklık eden kuruluş görevlisinin:

Adı-soyadı:

Görevi:

İmzası:

Tarih:/...../.....

8.3. Curriculum Vitae

EMİNE KAHRAMAN

ID No / Passport No	26992236302
Date of Birth	01.03.1995
Contact Address	Bahariye Mh. 1987 sokak No:12 D:7 Safa Apartmanı Karşıyaka / İZMİR
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EDUCATIONAL BACKGROUND

Country	University	Faculty/Institute	Department	Degree	Year of Graduation
Turkey	Dokuz Eylül University	Izmir International Biomedicine And Genome Institute	Molecular Biology and Genetics	Master of Science	2020
Turkey	Izmir Katip Celebi University	Engineering and Architecture	Biomedical Engineering	Bachelor	2017

ACADEMIC / PROFESSIONAL EXPERIENCE

Company	Country	City	Unit	Position	Year
Belgian Nuclear Research Center, SCK • CEN	Belgium	Antwerp	Microbiology	Intern	August 2016- October 2016
Etkin Medical Devices	Turkey	Izmir	Research & Development	Intern	January 2016- June 2016

Specialized Field
Tissue engineering Biomaterials Microfluidic Systems Molecular Biology and Genetics

CONFERENCES and AWARDS

Poster Presentation: Awarded to Best Poster Kahraman E., Ersoy N., Akbari S., Erdal E., Bağrıyanık A., Yıldırım C.A., Guven S. (2019). Development of Microfluidic Platform for Cornea Organoid Culture, BIOMED 2019, İzmir, 17-20 October
Poster Presentation: Kahraman E., Ersoy N., Akbari S., Erdal E., Bağrıyanık A., Yıldırım C.A., Guven S., (2018). Development of Microfluidic Platform for Cornea Organoid Culture, BIOMED2018. Biomedical Science and Technology Symposium, İstanbul, 15-16 December
Poster Presentation: Kahraman E., Ersoy N., Akbari S., Erdal E., Bağrıyanık A., Yıldırım C.A., Guven S. (2018). Development of Cornea Organoid Models using Tissue Engineering Approaches, 5. BiomaterialsDays, İstanbul, 22-23 October
Poster Presentation: Kahraman E., Demirci E.A., Yaralı Z.B., Karaman O., (2016). Effects of RGD Peptide Sequence on the Wound Healing of L929 Fibroblast Cells, National Meeting of Biomedical Engineering (BIYOMUT),Izmir, 3-5 November

PUBLICATIONS

Karaman O., Onak G., Demirci E.A., Kahraman E. (2017) Integrin binding peptide promotes in vitro wound closure in the L929 mouse fibroblasts. The European Research Journal, DOI:10.18621/eurj.318606.
