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DOKUZ EYLUL UNIVERSITY
IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

GENERATION OF FUCNTIONAL 3D LACRIMAL GLAND IN MICROFLUIDICS

ALİ KEMAL BAŞ

MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCES THESIS

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Supervisor: Assoc. Prof. Dr. Sinan Güven

TÜBİTAK 117S264

Dokuz Eylül Üniversitesi İzmir Uluslararası Biyotıp ve Genom Enstitüsü Genom Bilimleri ve Moleküler Biyoteknoloji Anabilim Dalı,
Moleküler Biyoloji ve Genetik Yüksek Lisans programı öğrencisi Ali Kemal BAŞ
'GENERATION OF FUCNTIONAL 3D LACRIMAL GLAND IN MICROFLUDICS' konulu Yüksek Lisans tezini 28-05-2019 tarihinde başarılı olarak tamamlamıştır.

Doç. Dr. Sinan Güven
BAŞKAN

ÜYE
Doç. Dr. Canan Aslı Yıldırım
Dokuz Eylül Üniversitesi

ÜYE
Prof. Dr. Özlem Yeşil Çeliktaş
Ege Üniversitesi

YEDEK ÜYE
Prof. Dr. Alper Bağrıyanık
Dokuz Eylül Üniversitesi

YEDEK ÜYE
Dr. Öğr. Üyesi Ozan Karaman
İzmir Kâtip Çelebi Üniversitesi

Dokuz Eylul University Izmir International Biomedicine and Genome Institute
Department of Genomics and Molecular Biotechnology,
Molecular Biology and Genetics graduate program Master of Science student Ali Kemal BAŞ has successfully completed her Master of Science thesis titled

'GENERATION OF FUCNTIONAL 3D LACRIMAL GLAND IN MICROFLUDICS'

on the date of 28.05.2019

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CHAIR

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Assoc. Prof. Canan Aslı Yıldırım
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Prof. Dr. Özlem Yeşil Çeliktaş
Ege University

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Dokuz Eylül Üniversitesi

SUBSTITUTE MEMBER
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İzmir Kâtip Çelebi University

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2D	2-Dimension
3D	3-Dimension
APC	Allophycocyanin
AQP5	Aquaporin 5
DAPI	(4',6-Diamidino-2-Phenylindole, Dihydrochloride)
DES	Dry Eye Syndrome/D
DMEM/F12	Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 Nutrient Mixture
DMSO	Dimethyl sulfoxide
DSA	Double Sided Adhesive
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
FTIR	Fourier transform infrared spectroscopy
GelMA	Gelatin Methacryloyl
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
ICC	Immunocytochemistry
LC	Lacrimal Chip
LG	Lacrimal Gland
MAA	Methacrylic Anhydride
OCT	Optimal Cutting Point
PE	Phycoerythrin
PBS	Phosphate Buffered Saline
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PEGDA	Poly(ethylene glycol) diacrylate

PFA	Paraformaldehyde
PI	Photo-initiator
PMMA	Poly(methyl methacrylate)
SEM	Scanning Electron Microscopy
UV	Ultraviolet
ZO-1	Tight junction Protein 1

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GENERATION OF FUNCTIONAL LACRIMAL GLAND IN MICROFLUDICS

Ali Kemal Baş, Izmir International Biomedicine and Genome Institute, Dokuz Eylul University Health Campus, Balcova, 35340, Izmir / Turkey

ABSTRACT

Dry eye syndrome (DES) is a disease caused by a chronic lack of sufficient lubrication and moisture on the surface of the eye because of various reasons. Current methods for treatment of DES are not efficient enough to overcome disease completely. Especially inflammatory cases of this disease lack a proper treatment method. In this project, we aim to develop an artificial functional lacrimal gland from lacrimal gland cells/tissues in the precisely controlled microfluidic system environment. The artificial lacrimal gland can be a potential treatment for dry eye syndrome especially in inflammatory cases.

Tissue engineering is the practice of a combination of cells, engineering, materials, and suitable biochemical and physicochemical factors to repair, improve or replace biological tissues or organs. The aim of tissue engineering is designing controlled artificial organ or tissue models to help medical treatment tools.

Microfluidics are interdisciplinary field which that are based on the control of very low volumes of fluids and have many applications in the fields of basic sciences (such as physics, chemistry and biology) as well as in engineering.

The objective of the project is to generate an artificial functional lacrimal gland inside of a 3D custom designed microfluidic system with lacrimal gland cells and tissue parts. In this microfluidic system, different compartments of lacrimal gland cultured within the natural biocompatible and biodegradable 3D hydrogel. This photo-polymerizable hydrogel provides an extra cellular matrix properties for the construct and keep functioning as in its natural environment.

Keywords: Dry Eye Syndrome, Lacrimal Gland, Tissue Engineering, Microfluidic System, Hydrogel.

MİKROAKIŞKANLARDA FONKSİYONEL LAKRİMAL BEZİN GELİŞTİRİLMESİ

Ali Kemal BAŞ, İzmir Uluslararası Biyotıp ve Genom Enstitüsü, Dokuz Eylül Üniversitesi, Sağlık Kampüsü, Balçova, 35340, İzmir / Türkiye

ÖZET

Kuru göz sendromu (KGS), çeşitli sebeplerden ötürü gözün yeterince ıslak ve kaygan kalamamasından kaynaklanan bir rahatsızlıktır. Hali hazırda uygulanan tedavi yöntemleri hastalığın tamamen tedavi edilmesinde yeterli olmamaktadır. Özellikle yangı ve iltihap kaynaklı vakalarda hastalığı tamamen tedavi etmek mümkün olamamaktadır. Bu projede kontrollü bir mikroakışkan sistem içerisinde yetiştirilen lakrimal bez hücre/dokularından yapay fonksiyonel lakrimal bez geliştirmektir. Bu geliştirilmesi hedeflenen yapay lakrimal bezin kuru göz sendromunun tedavi edilmeyen (özellikle yangı kaynaklı) vakalarına potansiyel bir tedavi yöntemi oluşturulması hedeflenmektedir.

Doku mühendisliği hücre, mühendislik, malzeme ve uygun biyokimyasal ve fizyokimysal faktörler yardımıyla biyolojik doku ve organların tedavi edilmesi geliştirilmesi ya da değiştirilmesi bilimidir. Doku mühendisliğinin hedefi tedavi yöntemlerine destek olmak amacıyla kontrollü bir şekilde yapay doku ya da organ modelleri üretmektir.

Mikroakışkan sistemler düşük hacimlerde sıvı mekaniklerine dayanan ve temel bilimler (fizik, kimya, biyoloji) ve mühendislik alanında bir çok uygulaması olan disiplinler arası bir bilimdir.

Bu projede spesifik 3 boyutlu mikroakışkan sistem içerisinde lakrima bez hücre ve doku parçaları kullanılarak yapay fonksiyonel lakrima bezin geliştirilmesi hedeflenmektedir. Bu sistemde lakrimal bezi oluşturan farklı kompartmanlar biyouyumlu ve biyobozunur hidrojel içerisinde kültür edilecektir. Bu ışıkla polimerize

olabilen hidrojel yapının oluşması için gerekli olan hücreler arası matrix özelliklerine sahip olup yapının kendini doğal ortamında gibi dhissetmesini sağlamaktadır.

Anahtar Sözcükler: Kuru Göz Sendromu, Lakrimal Bez, Doku Mühendisliği, Mikroakışkan Sistem, Hidrojel

1. INTRODUCTION and GOALS

1.1. Statement and Importance of the Problem

The current approach in the treatment of DES includes artificial tear drop, punctual plugs and cyclosporine A, which increases tear secretion via its anti-inflammatory properties in lacrimal gland.(Utine, Stern, & Akpek, 2010) However, in DES that occurs due to inflammatory reasons because of aqueous defect secreted from the lacrimal gland such as Sjögren's Syndrome, mucous membrane pemphigoid, Stevens Johnson syndrome, it is worse. As the disease progresses progressive inflammatory mediators and lacrimal gland atrophy come to life with the accumulation of cells and permanent loss of function in the lacrimal gland and loss of glandular units that leave the site of fibrosis occurs.(Utine, Biçakçigil, Yavuz, & Çiftçi, 2011) In these patients, systemic anti-inflammatory and biological treatments intended to control the systemic course of the disease are often not sufficient to prevent lacrimal gland atrophy.(Utine, Tzu, & Akpek, 2011)

The aim of tissue engineering is designing controlled artificial organ or tissue models to help medical treatment tools.(Güven et al., 2011) Designed tissues are implanted in damaged organs and regions, thereby contributing to the healing of the body by accelerating regeneration. For the best regenerative effect, designed tissue has to mimic material, cell type, mechanical strength and other biological properties at maximum level.

Recent studies show that microfluidic approaches on tissue engineering applications show promising results in terms of artificial tissue reconstruction. With the help of controlled kinetic conditions, mimicking of the natural environment is achieved better. Continuous flow and controlled factor release enables culturing bioengineered tissues in various conditions. These systems are relatively cheap to manufacture and easy to maintain.

1.2. Purpose of the Research

Here in this thesis, we proposed to design and fabricate a 3D tissue engineering platform that will allow us to culture a bioengineered lacrimal gland to open a way to alternate treatment method for severe cases of dry eye syndrome. Aim of this study is to create precursor data for further bioengineering approaches for dry eye syndrome.

1.3. Hypothesis of the Research

In this study, our hypothesis predicts that bioengineered lacrimal gland can be generated in a microfluidic system.

2. GENERAL INFORMATION

2.1. Lacrimal Gland

The lacrimal glands are paired, exocrine glands, one for each eye, that secrete the aqueous layer of the tear film (Figure 1). These secretory acinar glands empty their secretions into excretory ducts which drain onto the ocular surface.(Tiwari, Ali, & Vemuganti, 2014) The size of an average lacrimal gland is approximately 20mm long and 12mm wide with the orbital and having a thickness of 5mm and 3 mm.(Conrady, Joos, & Patel, 2016) These tubuloacinar tissues are responsible for secretion of the proteins, electrolytes and water. Secreted tear film helps to protect and nourish the epithelial cells in the ocular surface(You, Tariq, Kublin, & Zoukhri, 2011).

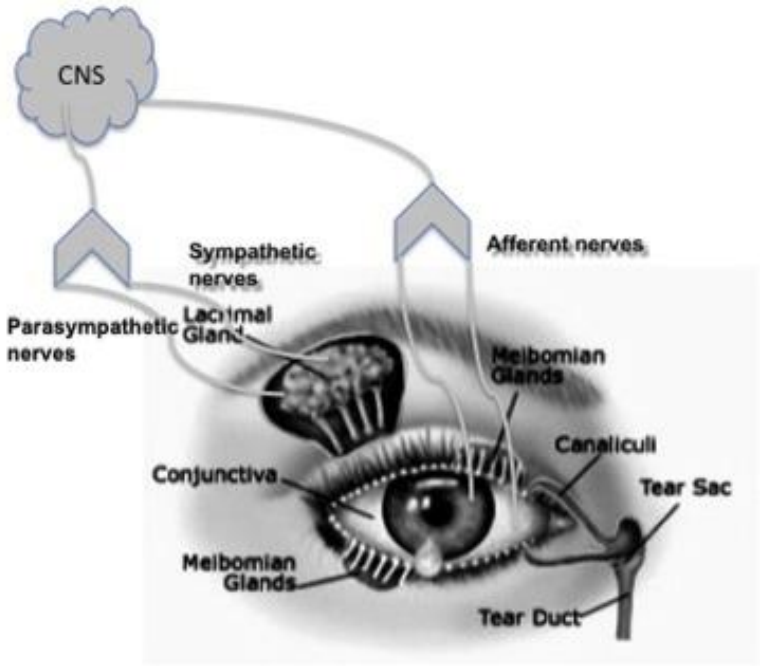


Figure 1: Lacrimal gland on human body.(Tiwari et al., 2012)

Lacrimal glands include acini, ducts and myoepithelial cells in its organization. Glands are developed during the epithelial mesenchymal interactions at embryogenesis.(Hirayama et al., 2013) Mesenchymal sheet surrounds epithelial acinar part and acts as repair mechanism due to stem cell properties. Lacrimal gland cells have shown stem cell like properties in study that aimed to characterize the isolated murine lacrimal gland cells under 2D and 3D conditions. The stemness of these cells was close to embryonic stem cells and progenitor cells in RNA/protein patternwise. They observed that these cells can differentiate into cells types from all 3 different germ lines.(Ackermann et al., 2015)

There are two types of lacrimal glands present in human body, intraorbital and extraorbital. LG development begins with the primary bud coming from the conjunctival epithelium. This bud extends as a tubular sinus into the periorbital mesenchyme, where it branches into 2 locations to form the intraorbital and exorbital parts of the LG that eventually develop into the mature intraorbital LG and exorbital LG.(Liu & Lin, 2014)

2.2. Dry Eye Syndrome

Dry eye syndrome (DES), is a multifactorial disease of the tears and the ocular surface that results in discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface.(Ackermann et al., 2015)

DES is studied in two subclasses as ocular failure and evaporative type in ophthalmology practice. DES due to aqueous insufficiency is due to lacrimal gland injury/absence and has clinically more serious consequences. In Sjögren's syndrome, Stevens Johnson syndrome, mucous membrane pemphigoid, lacrimal gland tumors, as well as after radiotherapy, surgery and trauma aqueous defect related DES is seen. Evaporative type dry eye is more common and it is caused by reasons like unclosed eyelids caused by decrease in the blinking reflex or other reasons which cause eye lids to remain open, or like lack of outermost oily layer of tear due to dysfunction of meibomian glands in eyelash diploma which eventually lead to over evaporation of tears.

Dry eye syndrome (DES), is the condition of having dry eyes due to insufficient tear film production or preservation. Associated symptoms include irritation, redness, discharge, easily fatigued eyes. More severe cases can results in blurred vision or even blindness.(Wilkinson, 2003) DES is a common eye disease with affecting 5-34% of the people depending on the population where the older people affected more.(Messmer, 2015)

There are couple of predominant reasons for dry eye to occur such as Sjörgen’s syndrome, allergies, contact lens usage, deficiencies (e.g. vitamin A deficiency) and side effects of various medications. Treatment methods depends of the cause and severity of the syndrome. Most common methods are teardrops and punctual plugs however on some cases like Sjörgen’s syndrome caused DES available treatment methods perform insufficient. For severe cases and situations like lacrimal gland being unrepariable due to damage, new treatment methods are required.

Transplantation approach is one of the most focused emerging treatment method for dry eye disease. Using tissue engineering principles, creating a functional artificial lacrimal gland is very promising solution against the challenges that were mentioned. A group aimed to engineer a functional 3D lacrimal gland construct by recellularizing the decellularized lacrimal gland tissue with cells isolated from secretory compartments of lacrimal gland. Seeded cells have formed correct lacrimal gland morphology and shown secretory functions as well.(Spaniol et al., 2015) Another group created a functional bioengineered lacrimal gland and transplanted it into lacrimal gland defect mouse model. Transplanted lacrimal glands were developed in vivo and showed functional characteristics suchs as tear production and ocular surface protection.(Hirayama et al., 2013).

In order to successfully to create functional artificial lacrimal gland, the microenvironent of the construct is one of the important factors that affect the lacrimal gland generation. Mechanical and chemical stimulation affecting the cells or tissues have considerable amount of impact on the tissue/organ generation. In a work the morphological and physiological characteristics of isolated rabbit lacrimal gland cells that were cultured on different polymeric coatings with and without the presence of Matrigel. They shown that poly-L-lactic acid (PLLA) coated culture conditions were supporting the morphological and physiological properties of isolated lacrimal gland epithelial cells successfully.(SH, YS, FH, JM, & KS, 2007)

Another source rather than lacrimal gland cells/tissues themselves, is stem cells to create functional artificial lacrimal gland. Through differentiation stem cells can become lacrimal gland cells to form a tissue structure.

Apart from tissue engineering, gene therapy studied were done recently showing that specific genes or pathways affect lacrimal gland development and repair. A group investigated the lacrimal gland epithelial cells for expression pattern and role of Runx1, Runx2 and Runx3 during lacrimal gland development and regeneration. They found that both 3 transcription factors were important for lacrimal gland development and regeneration. Especially Runx1 was abundantly found in ductal and acinar cells. Runx1 and Runx3 expressions were slightly increased during the regeneration and

Runx2 was modestly increased during lacrimal gland differentiation.(Voronov et al., 2013) Another research was done on the role and importance of Smad4, a TGF-b pathway mediator during the lacrimal gland development in mouse. They found that inactivation of Smad4 resulted in the reduction in the size and number of acini as well as pigment accumulation in the embryonic lacrimal gland. Without the presence of Smad4, lacrimal glands was developed considerably smaller than normal lacrimal glands and eventually replaced by adipose tissue.(Liu & Lin, 2014)

2.3. Microfluidics

Microfludics is the science of control and manipulation of fluid flows in small volumes (micro and less). It is a multidisciplinary field combines that combines basic sciences (physics, chemistry and biology) with engineering and technology. Microfludics take the advantage of 3 main concepts; laminar flow, surface tension and capillary forces.(Sackmann, Fulton, & Beebe, 2014) These 3 concepts cause the microenvironment act different than macroenvironment conditions. Gaining advantage of these features enable creating systems that mimics physical, chemical and biological processes that not possible to create under macroenvironment conditions.

There are couple advantages that microfluidic systems offer. First being cheap in both manufacturing and sustaining compared to conventional systems. Microfluidics can be manufactured from simple plastic (e.g. Poly(methyl methacrylate)) with an industrial grade laser cutter or from silicon (e.g. Polydimethylsiloxane) with soft litography techniques. Either material have their advangtages and disadvantages against each other. PMMA systems are easier and cheaper to manufacture than PDMS systems, however PDMS systems have much more precise design options and gives more control overall on the systems. Designs can be completely custom according to the usage purpose and this eliminates limitations that conventional methods have. Also microfluidics offer more controlled environment compared to conventional methods. More variables can be controlled like flow rate, pressure and input intake. Sustaining

these systems are much more cheaper than conventional macro systems in terms of material usage as well as energy consumption of the system.

Application areas of microfluidics have very wide range due to being multidisciplinary science. However, here we focus on biological applications of microfluidics. Microfluidic systems are widely being used as single cell measurements, live cell imaging, rapid molecular events, high throughput screening and tailored contexts (microenvironments).(Duncombe, Tentori, & Herr, 2015) For example a group proposed a rapid and accurate lab on a chip microfluidic system for single cell measurement. The force that required to move a single cell (drag force) through the channels of microfluidics was measured. Then this calculated drag force was related with Newton's law of motion to determine the mass of a single cell.(Rahman et al., 2015)

Also these microfluidic systems can be combined with various biomaterials for tissue engineering approaches to achieve more realistic culturing conditions compared to 2D conventional culture methods.(Torisawa et al., 2014)

Microfluidics are also used in other fields than research. For example a group reported a novel method for differential extraction of sperm specifically for forensic cases. Developed method differentially isolates the sperm and extracts the sperm DNA on microfluidic chip for further genetic analysis. This method reduces the total time for analysis, reduces the manual labor force, increases the capture efficiency immuno-based separation of sperm assays and works at high efficiency rates even samples older than 15 years.(Inci et al., 2018)

Microfluidic systems are being widely used as cancer research platform recently. Using microfluidics for characterization of cancer cells, specialized microfluidic platforms for studying cancer migration and metastasis, isolation of cancer cells from blood on microfluidics and state of art platforms for single cell analysis purpose enables the new diagnostic systems.(Chaudhuri, Ebrahimi Warkiani, Jing, Kenry, & Lim, 2016) A group generated a protocol for ultra fast label free isolation of circulating tumor cells from blood by using spiral microfluidics. Spiral micrifoluidics allow size based isolation

of viable circulating tumor cells. The system takes advantage of hydrodynamic forces that are present in curvilinear microchannels. The system is cheap, easy to use and has a high output compared to the similar systems.(Warkiani et al., 2016)

Microfluidic approaches are also used in developmental studies such as zebra fish oriented research. Fish manipulations, zebra fish imaging platforms for and zebra fish phenotypic readout platforms are couple of examples.(Yang, Gao, Wang, Zhang, & Chen, 2016)

2.4. Biomaterials

Biomaterials are engineered substances that support, treat, repair or replace biological systems of functions.(Dziki et al., 2018) Biomaterials are generally used for therapeutic (augment, repair) and diagnostic (detection platforms) purposes. Also with the recent advancements, biomaterials are commonly used in wide range of research areas such as, drug delivery tools and system modification substances and 3D culture systems.

Biomaterials can be either natural or synthetic depending of the chemical composition. Also natural biomaterials can be modified to synthesize hybrid biomaterials. Many substances are used in the production of biomaterials: metals (titanium), ceramics (alumina), synthetic polymers (polyurethanes, silicones, polyglycolic acid (PGA), polylactic acid (PLA), copolymers of lactic and glycolic acids (PLGA), polyanhydrides, polyorthoesters) and natural polymers (chitosan, glycosaminoglycans, collagen).(Chevallay & Herbage, 2000) Depending on the chemical composition of biomaterial, its usage may differ. For example while titanum is biocompatible but not biodegradable, so it is used as implants for severe skeletal injuries.

Biomaterials, natural or synthetic must be biocompatible at least for the application are that they are used for. They must support, and sustain the biological environment that they reside. Rest of the characteristics may vary and depend on the

usage of biomaterial. Medical implants (stents, dental implants) hydrogels (PEG, GelMA), drug delivery systems (biomaterials that carry drugs for precise delivery) can be given as examples for biomaterial usage.

2.4.1. Hydrogels

Hydrogels are hydrophilic polymer networks that can absorb large amounts of water in their structure.(Feksa et al., 2018) Hydrogels are highly used in tissue engineering platforms due to being biocompatible and biodegredable. Also hydrogels can successfully mimic native ECM with its mechanical and chemical functions. Hydrogels like all biomaterials either can be natural or synthetic derived. Natural polymers like collagen, hyraluronic acid, fibrin, agarose and chitosan are the most commonly used ones for fabrication of hydrgels for tissue engineering and regenerative medicine approaches. Synthetic polymers like poly(ethylene glycol) (PEG), poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) are used for bioenginered scaffolds. (Geckil, Zhang, Moon, & Demirci, 2013)

2.4.2. GelMA

Gelatin-methacrylyol (GelMA) is one of the most commonly used photopolymerizable hydrogel in research field. It is mainly composed of gelatin which is the most abundant protein in the human body. Gelatin enables cell attachment due to its RGD (Arg-Gly-Asp) branches and it supports cell proliferation, function and differentiation. However, gelatin alone has very strict conditions to form a physical hydrogel and has relatively low strength.(Shirahama, Lee, Tan, & Cho, 2016) GelMA modifies gelatin by a chemical modification of Methacrylic Acid (MAA) to support photo corsslinking to stabilize its structure and increased stiffness.(Loessner et al., 2016)

GelMA is crosslinked under UV light with a help of a photoinitiator (PI). This photo initiator has to be water soluble in order to show effect on GelMA. Also low toxicity values for chosen PI is desired.

2.5. Tissue Engineering

Tissue engineering is the science that combines material, biochemical factors and engineering to manipulate cells/tissues (Figure 2).(Langer & Vacanti, 1993) The main purpose of tissue engineering is to maintain, treat or improve the functions of injured or diseased tissue or organ parts with appropriate alternatives.(Khademhosseini & Langer, 2016) Aim is to achieve true morphology and functionality of the target tissue/organ.

Tissue engineering can be briefly summarized as follows: a biopsy is taken from the relevant healthy tissue of the patient and the cells are isolated. From the obtained cells, the stem cell precursor cells are isolated and multiplied in the culture medium. In addition, the stem / precursor cells can also be transformed into another type of cell line. The resulting cells are then loaded onto the carrier scaffold, which is specifically tailored to the target tissue/organ, and loaded into the hydrogel, and subsequent maturation steps are performed. Finally, the developed artificial tissue is implanted in the patient/damaged region. The tissue engineering method also allows for the development of a model tissue / organ for clinical research and the pharmaceutical industry. The most important benefits of this method are 1) providing the most healthy and correct dose of medication without harm to the patient by using the patient's own cells, 2) minimizing ethical concerns by reducing animal experiments, 3) creating appropriate platforms for the development of new drugs and treatment modalities, 4) In addition to existing surgical methods, alternative and innovative techniques can be developed to improve damaged tissues and organs.(Guven et al., 2015)

Besides clinical purposes, tissue engineering approaches are widely used for research areas such as culture platforms, screening platforms and hybrid culture setups. For example a group reported fabrication of a scaffold that supports parenchymal cells assembly on a mechanically tunable matrix which surrounds a perfusable, branched, three-dimensional microchannel network coated with endothelial cells. Group showed the vascularized hepatic and cardiac tissues engineered by their platform, AngioChip.(Zhang et al., 2016)

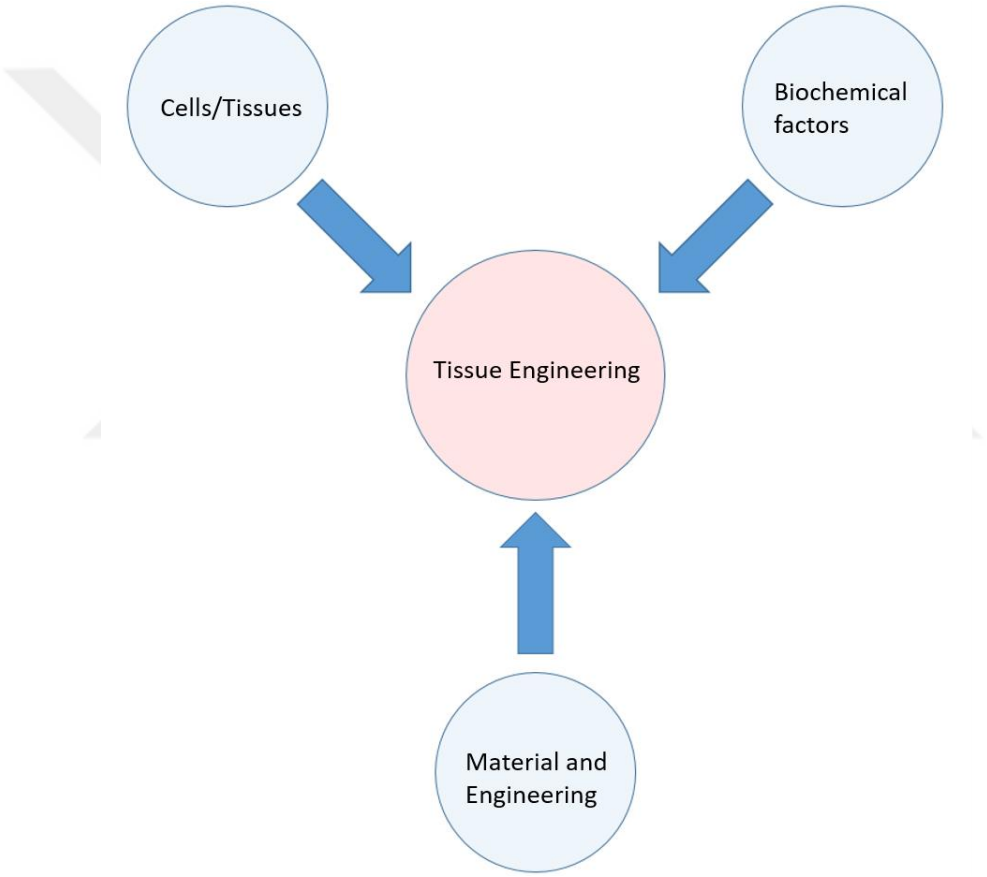


Figure 2: Tissue Engineering

By its default nature, tissue engineering is a form of a 3D system. This brings couple of necessities that must be met like, providing continuous supply of nutrient and oxygen while removing the waste, maintaining necessary pyhsical and chemical conditions for growth and functioning and enabling outer intervention when nessecary.

In this thesis project, we focused on research part of the tissue engineering. We aimed to build a tissue engineering system to mimic the native conditions of our target organ (lacrimal gland) and successfully culture it while maintaining its functionality. Our biomaterial GelMA acts as microenvironment (extracellular matrix) for our bioengineered lacrimal gland while our microfluidic system forms our culture platform.

As mentioned above, to achieve true morphology and functionality, the strucutre of the 3D construct must be controlled to be succesful in tissue engineering. Here in this project this is provided by; photocrosslinkable hydrogel GelMA, custom made microfluidic system and controlled culture environment settings.

3. MATERIALS AND METHODS

3.1. Type of Research

This study is an *in vitro* experimental study

3.2. Date and Location of Research

All research was conducted between 2016 September – 2019 May in Izmir International Biomedicine and Genome Institute (iBG-izmir)

3.3. Universe and Sample of Research

In this thesis scope, primary human samples were not used.

3.4. Materials of Research

In this study, NIH/3T3 mouse fibroblast cells and primer adult/embryonic mouse lacrimal gland cells were used. NIH/3T3 mouse fibroblast cells were obtained from Prof. Dr. Neşe Atabey on 2016. Primary cells were isolated from lacrimal gland of adult and embryonic mice by research team on various dates which can be found in detail under discussion section.

3.5. Variables of Research

Independent variables: DMEM/F-12 cell culture media, FBS and Pen-Strep ratio.

Dependent variables: Flow rate of microfluidic system, volume of microfluidic system, cell seeding density, mechanical properties of the GelMA, UV parameters, mice species.

3.6. Data Collection Methods

3.6.1. Hydrogel Synthesis, Characterization and Properties

3.6.1.1. Synthesis of Photocrosslinkable Hydrogel Gelatin Methacryloyl (GelMA)

In this study a gelatin based hydrogel, Gelatin Methacryloyl (GelMA) was used as cell/tissue carrier for 3D microfludic tissue culture system. GelMA was synthesized as previously described method(Khademhosseini & Langer, 2016) with minor modifications.

Reaction Step

Briefly, we dissolved gelatin in 1X PBS until 10% w/v final concentration is reached and heated it on a magnetic stirrer until it reaches to 50 °C. Then we kept solution at 50 °C for 1 hour while stirring it. After 1 hour, when the solution becames clear and transparent (which means the gelatin is fully dissolved), we slowly added 1.0 ml of methacrylic anhydride (approximately 1 drop of methacrylic anhydride per 10 seconds) per 1 g of dissolved gelatin while stirring it vigorously. After the addition of methacrylic anhydride, reaction was left at 50 °C for 3 hours while stirring it on a magnetic stirrer. After 3 hours of reaction, we transferred the solution into the 50 ml centrifuge tubes and centrifuged it at 3.500 g for 10 min at RT to remove unreacted methacrylic anhydride. After centrifugation, we took the supernatant and diluted it with two volumes of 40 °C UltraPure water to make it ready for dialysis.

Dialysis Step

Then we placed the diluted solution into the 12-kDa MWCO dialysis bags. We put those bags into the large volumes of UltraPure water (e.g. water bucket) and dialyzed it at 40 °C while stirring it for 2-3 weeks(Figure 3). Water was changed daily and after 1st week we started to check the pH of the water on daily basis. When the pH is ~ 7.4 the solution is frozen and lyophilized.

Figure 3: GelMA dialysis step taking place in orbital shaker.

Freeze Drying and Stocking

We put all the dialyzed solution into the 50 ml centrifuge tubes and froze them to -80 °C for 1 day. After the solutions in tubes were completely frozen, we transferred them to the freeze dryer without allowing them to thaw. After that the solution was left to be lyophilized until it is fully dehydrated (~ 1 week). Then lyophilized GelMA solution were stored at -20 °C until further use (Figure 4).

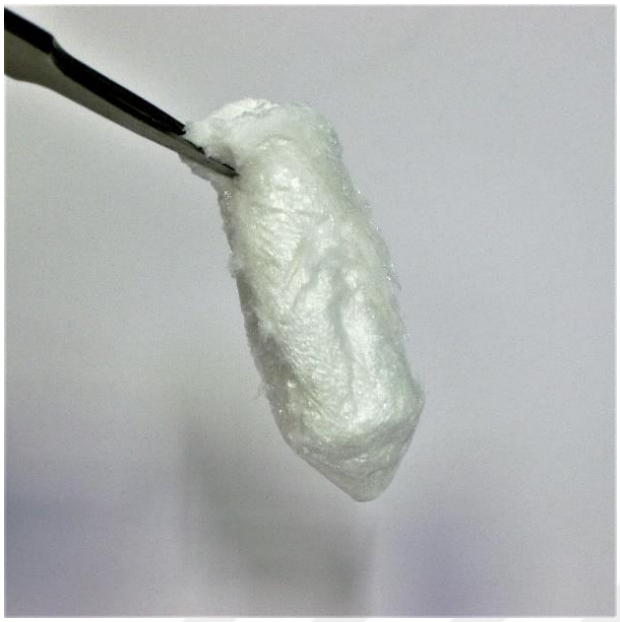


Figure 4: Lyophilized GelMA.

3.6.1.2. Preperation of GelMA solution

First a fresh photo-initiator (IC2959) stock solution was prepared at desired concentration. Photo-initiator was dissolved in preheated (70 °C) 1X PBS by vortexing it right after the addition of photo-initiator. After it fully dissolved, we soaked the desired amount of lyophilized GelMA into the photo-initiator solution and dissolved it completely by mixing it. During our experiments we used GelMA concentrations ranging from 5% to 10% and PI concentrations ranging from 0.1% to 0.5%. After that GelMA solution was ready for further use.

3.6.1.3. Characterization of GelMA solution

To verify the characteristics of our GelMA solution we analyzed our crosslinked and non corsslinked GelMA samples on fourier-transform infrared spectroscopy (FTIR). Also we took scanning electron microscopy (SEM) images of our crosslinked GelMA to confirm the porous structure of crosslinked GelMA.

3.6.1.4. Crosslinking and Cell Encapsulation

Due to nature of our hydrogel GelMA, gelation via crosslinking occurs in the presence of ultra violet (UV) light (photo-initiation) . We used an adjustable UV light source (OmniCure 2000) for this purpose. We built a custom made UV box for safety and adjustment issues. Box was covered with an opaque black material to make it lightproof and it can be opened completely from the front side. Our UV source was connected to the box from the top and the box contained an adjustable stage for different distance settings(Figure 5). Power and duration settings on UV source that we used throguh our experiments were 4 to 5 power and 60 to 90 seconds respectively.

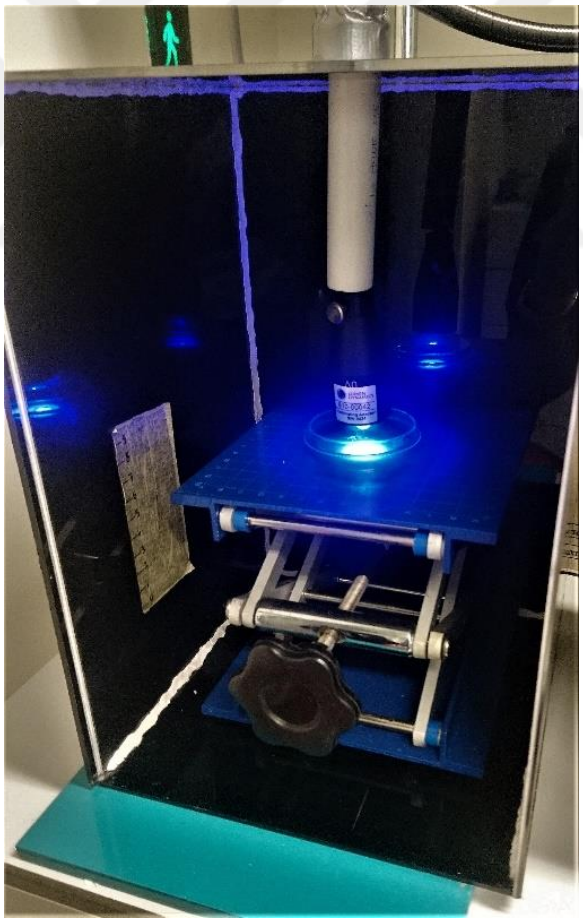


Figure 5: Custom made UV curing box during the photo-initiation process while the lid is open.

To print specific patterns, designed photomasks that are compatible with our static and kinetic culture setups. These photomasks were made from acetate paper and designed at the CorelDRAW x7 software like other components (Figure 6).

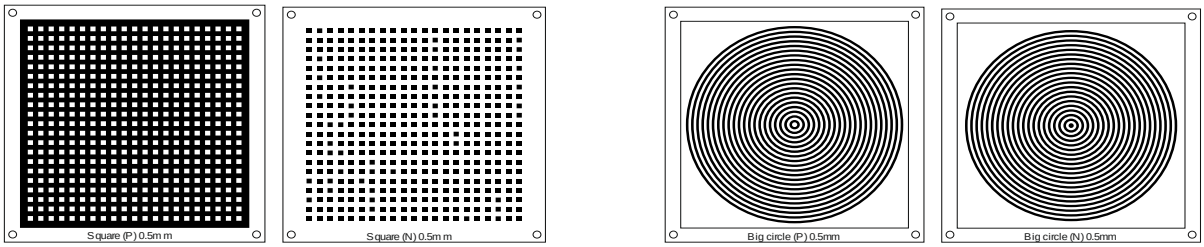


Figure 6: Sample square and ring patterned photomasks and their negatives.

These photomasks were placed onto prepared GelMA solution before the UV exposure. Through the UV exposure the hydrogel under uncovered areas were crosslinked while the uncovered areas were washed in 1x PBS after each corsslinking process.

3.6.2. Microfludic System Design and Fabrication

3.6.2.1. Microfludic System Design

Microfludic system was designed in order to provide two different types of culture media to the two different cell types of the lacrimal gland, epithelial and mesenchymal. The system and its components were designed on CorelDRAW X7 software. In this study the microfluidic system is named as Lacrimal Chip (LC).

3.6.2.2. Microfludic System Fabrication

Our microfludic system is made of medical grade polystyrene and 1.5 mm thick poly(methyl methacrylate) (PMMA) main parts as well as double sided adhesive (DSA), epoxy glue and borosilicate microtubings. Medical grade polystyrene, PMMA and DSA components were cut at laser cutter (Epilog Mini) in our laboratory according to our design and further assembled together after photolitographically bioprinting of 3D artificial tissue inside. Then microtubes were attached to the system with industrial grade fast curing epoxy adhesive. All materials used for microfluidic system fabrication were sterilized with 70% ethanol and UV exposure inside a cell culture grade laminar flow hood prior final use.

Lacrimal Chip (LC) Final Design

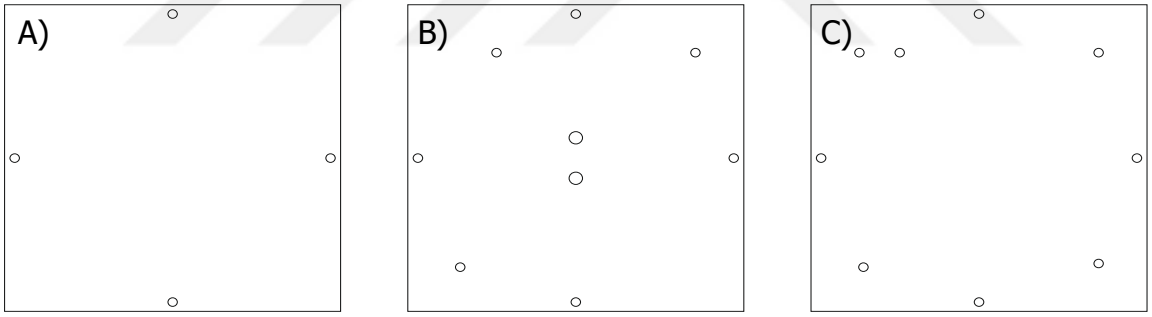


Figure 7: A) Bottom (1st level)), B) middle (3rd level) and C) top layers (5th level) respectively. Top and middle parts were made from PMMA bottom part was made from tissue culture grade polystyrene.

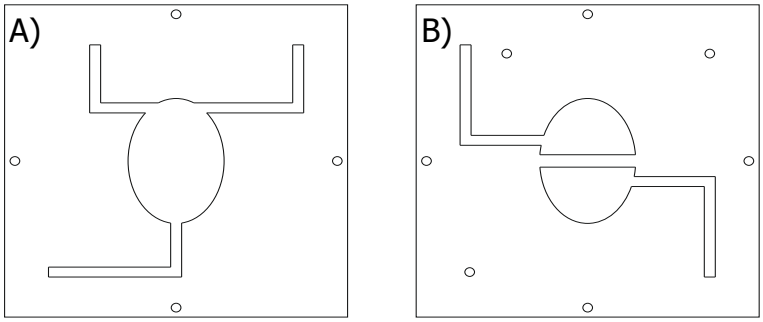


Figure 8: Double sided adhesive for assembly of the chip. A) First part (2nd level) gives the height to the 3D construct and forms the mesenchymal routes whereas B) second part (4th level) forms the epithelial routes.

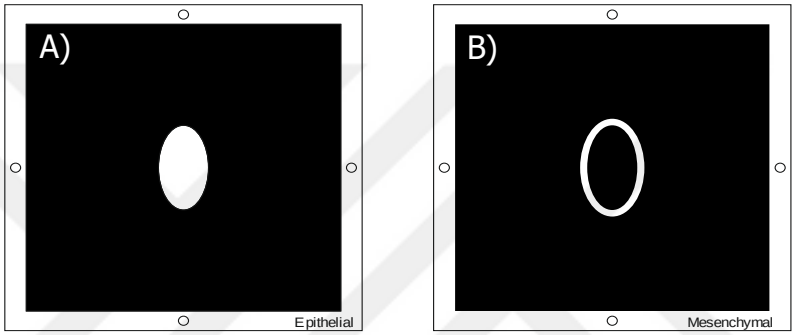


Figure 9: Corresponding photomasks for printing A) epithelial and B) mesenchymal parts respectively. Photomasks were made from acetate paper. Photomasks were fabrication components of the microfludic system but were used to precisely print the 3D artificial tissue compartments to the corresponding spots on the LC.

3.6.2.3. Microfludic System Working Principle

Our system consists of 5 main levels. From bottom to top; 1st level is made from medical grade polystyrene and acts as a base of our system. It is a plain surface and 3D printing is done on this level. 2nd level is made from DSA which contains main culture space and mesenchymal channel routes. This level contains 5 layers of DSA. At 1st layer, epithelial part is printed with its corresponding photomask and at 5th layer, outer mesenchymal part was printed with its corresponding photomask. Because of this difference in height between epithelial and mesenchymal compartments, mesenchymal compartment acts as a wall that surrounds the epithelial compartment providing an isolated culture conditions (Figure 10).

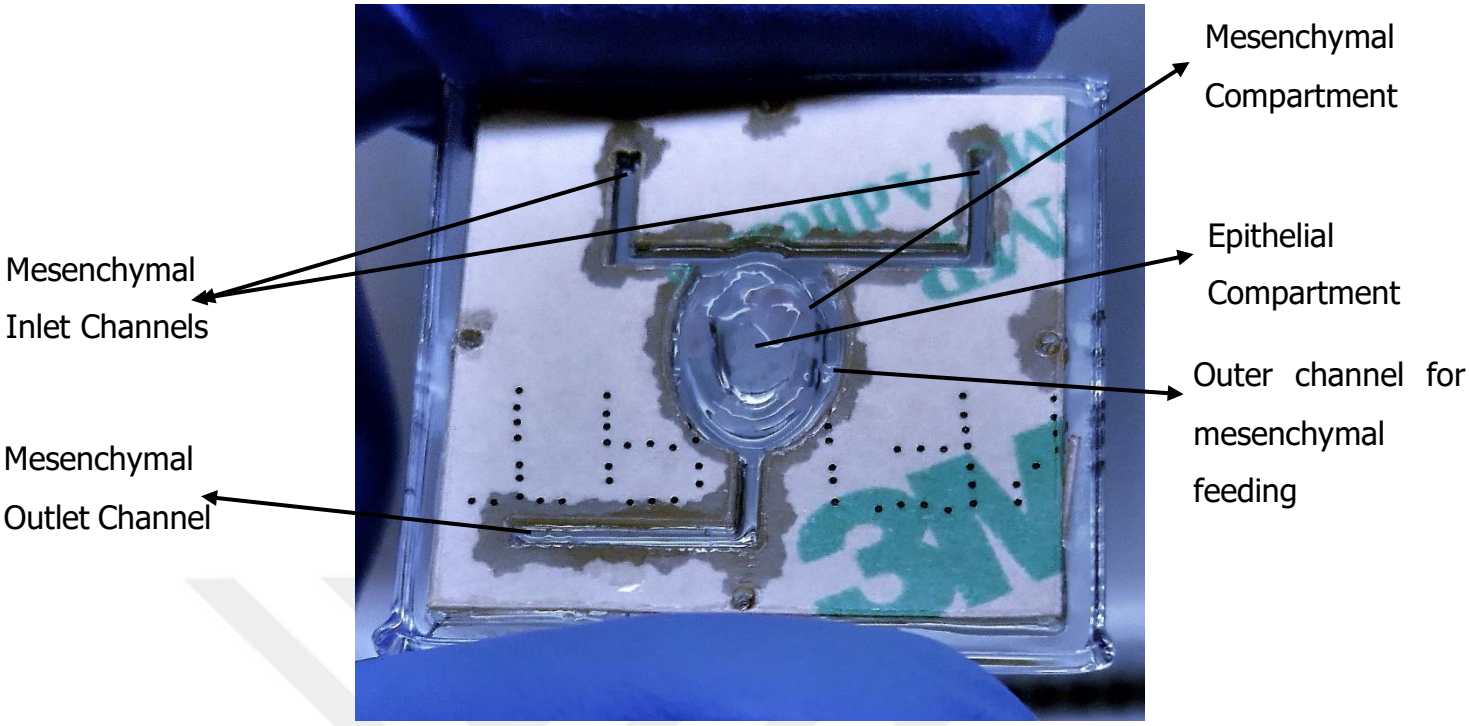


Figure 10: Final design of LC after building 2nd level and GelMA encapsulation of both parts. Inner epithelial compartment is surrounded by outer mesenchymal compartment which acts as an oval wall for isolation of epithelial culture media. After closing the chip mesenchymal part was supplied from outer channels and epithelal part was supplied from the 4th level routes which flowed down through the 3rd level and showered the inner epithelial part from center top hole and drained from the center bottom hole of the 3rd level.

3rd level which is made from PMMA contains culture holes for epithelial compartment. 4th level has epithelial channel routes and is made from 2 levels of DSA and above that there is 5th level which acts as cap of the system is made also from PMMA. All levels except 1st one contains tubing entry holes. There are 5 tubing connections in our system. 3 for mesenchymal compartment (2 inlet, 1 outlet) and 2 for epithelial compartment (1 inlet, 1 outlet). 3 of them act as inlet channels whereas 2 outlets are responsible for disposal.

The system must be aligned precisely for assembly and crosslinking of hydrogel. To achieve the presicion alignment, we built a custom made holder from a petri dish

cover, 4 pieces of 0.5G needles and industrial grade epoxy glue. Each level of the LC as well as photomasks had 4 aligning holes at the middle of each sides. Starting from 1st level, levels were put on the system one by one and assembled there. Also printing via crosslinking of the epithelial and mesenchymal compartments were done on the holder as well.

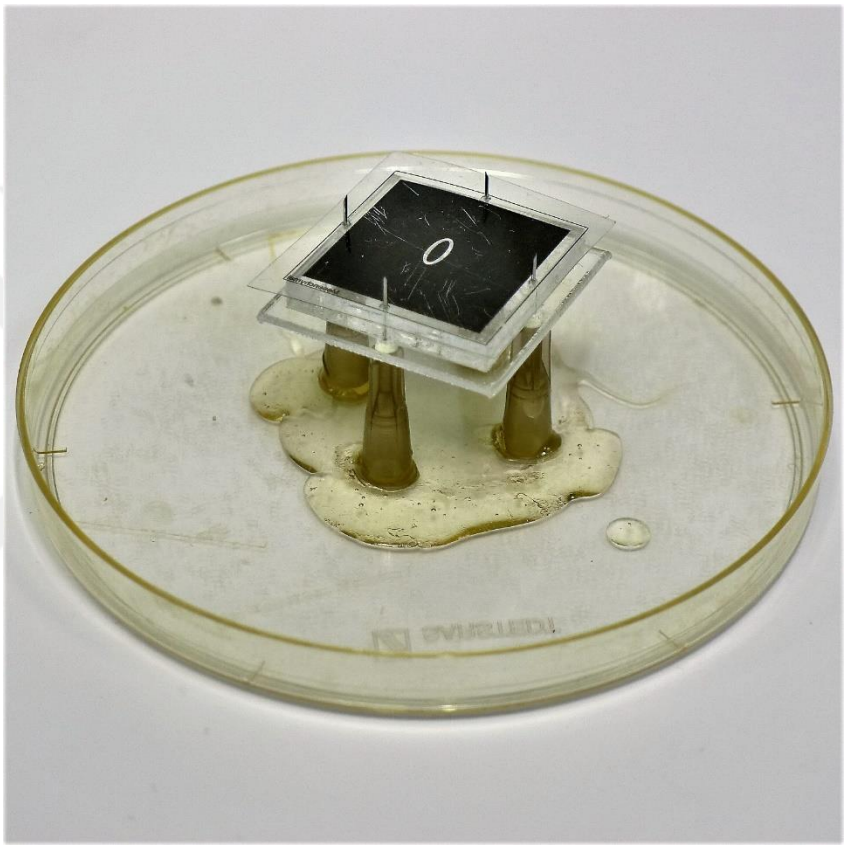


Figure 11: Custom made holder with LC and mesenchymal photomask on it.

Visual Representation of Final LC Design

After finishing the design and fabrication of the LC, we tested it with red and blue food dyes before using in tissue culture experiments.

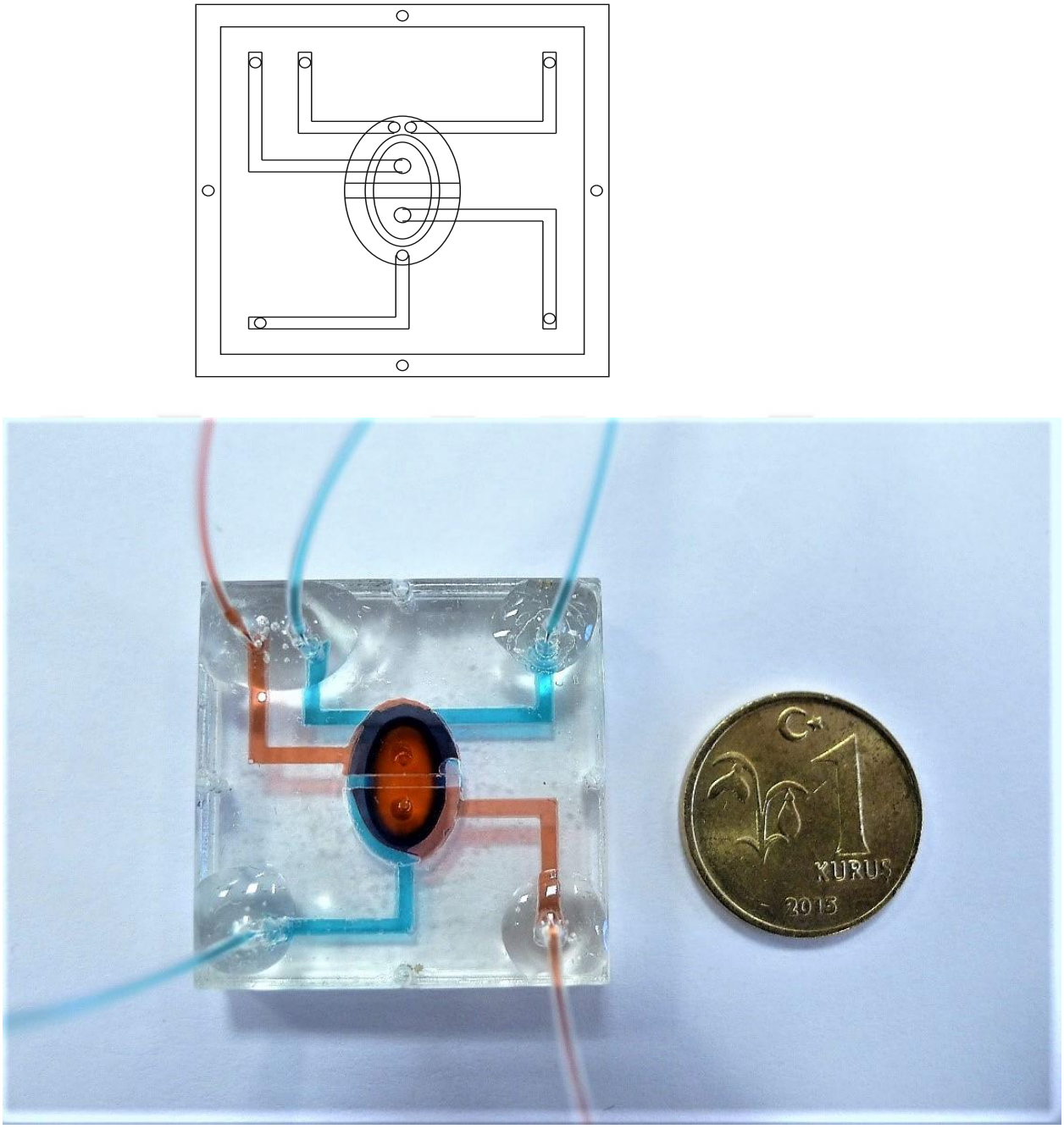


Figure 12: Final Design of LC tested for non-mixing simultaneous flow performance with blue (mesenchymal compartment and routes) and red (epithelial compartment and routes) food dyes. Top channels act as inlets (feeding channels) and bottom channels act as outlets (drain channels). 1 Kuruş for scale (17mm in diameter). Design version is present above for comparison.

3.6.3. Animal Experiments

Adult and embryonic BALB/c mice were used as lacrimal gland tissue source according to ethical committee approval of DEÜ-İBG HADYEK (16/2016). All animals were obtained (purchased) from IBG Vivarium core facility. Adult and pregnant mice were kept under standard conditions in IBG Vivarium core facility and sacrificed prior to the dissection of lacrimal glands. Both adult and pregnant mice were sterilized with ethanol, then sacrificed in CO2 chamber followed by cervical dislocation confirmation. After sacrification both adult and pregnant mice were sterilized with ethanol to avoid any potential tissue contamination. All sacrification and dissection procedures of adult and pregnant mice were done inside a laminar flow hood. For the excision of lacrimal gland tissue;

Figure 13: Sacrification and dissection setup of adult and pregnant mice.

3.6.3.1. Lacrimal Gland Dissection From Adult Mice

The lacrimal gland of adult mouse is a very distinct organ and easy to locate. So after removing the skin between the eye and the ear with scalpel and surgical scissors, it can be easily detected at the lower part of the removed area. After removal of the skin, exposed lacrimal gland tissue is easily removed with a forceps. Collected lacrimal glands are pooled in a centrifuge tube on ice, which contains DMEM/F-12 media and HEPES buffer.

3.6.3.2. Lacrimal Gland Dissection From Embryonic Mice

For embryonic mouse gland, we used embryonic day 16.5 mice as tissue source. Detection of pregnancy plug on female mouse counts as day 0.5. After 16 days embryos are ready for lacrimal gland extraction. However in embryonic mice lacrimal gland is rather hard to detect and localize. For detection and excision, we used previously described protocol.(Finley, Farmer, Emmerson, Cruz Pacheco, & Knox, 2014).

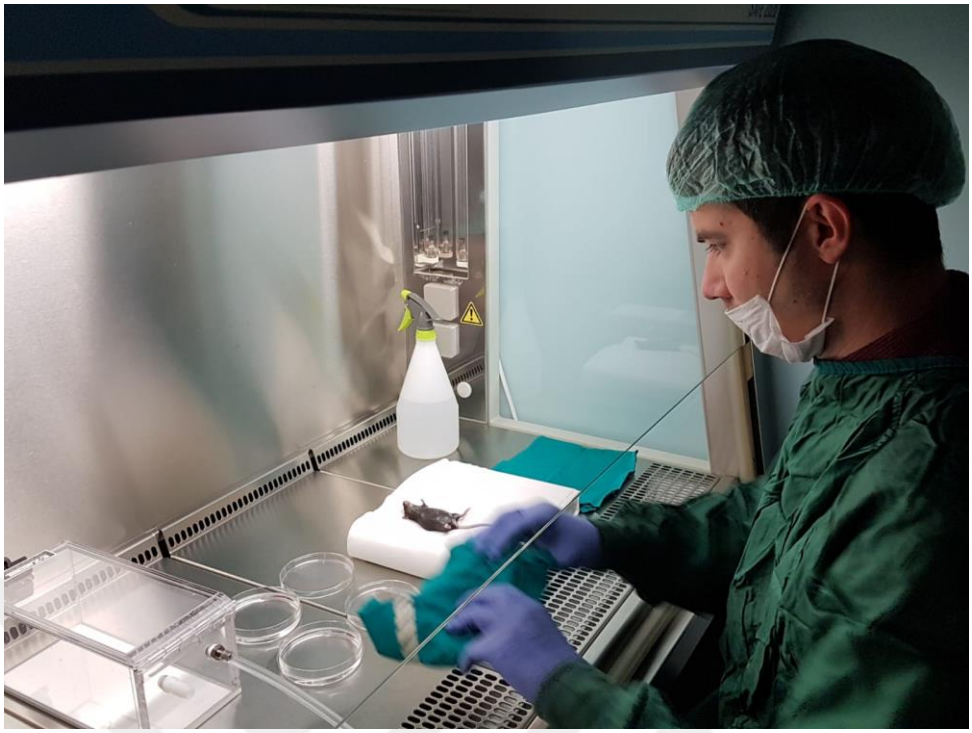


Figure 14: Sacrificed pregnant mice being prepared for dissection.

After sacrification of pregnant mouse, abdomen is cut through the middle line with small surgical scissors. Then uterus is resected and washed in 1X PBS on ice. Embryos are dissected from uterus, which was still in PBS on ice.

Figure 15: Dissected embryos being washed in 1X PBS.

Embryos, were decapitated under the stereo microscope. After that embryonic lacrimal glands are dissected from the ocular part of the fetal head under stereo microscope with the help of small forceps and 25 G needles. All isolated lacrimal glands are put into the 1.5ml centrifuge tube on ice which contains complete DMEM/F-12 culture media and HEPES buffer.

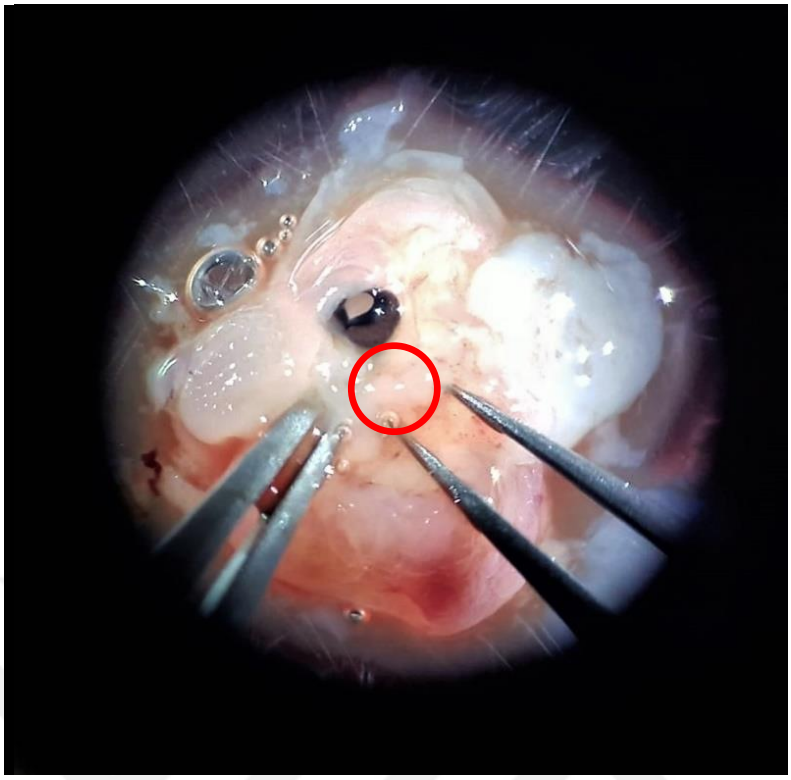


Figure 16: Location of the lacrimal gland in mouse embryo head.

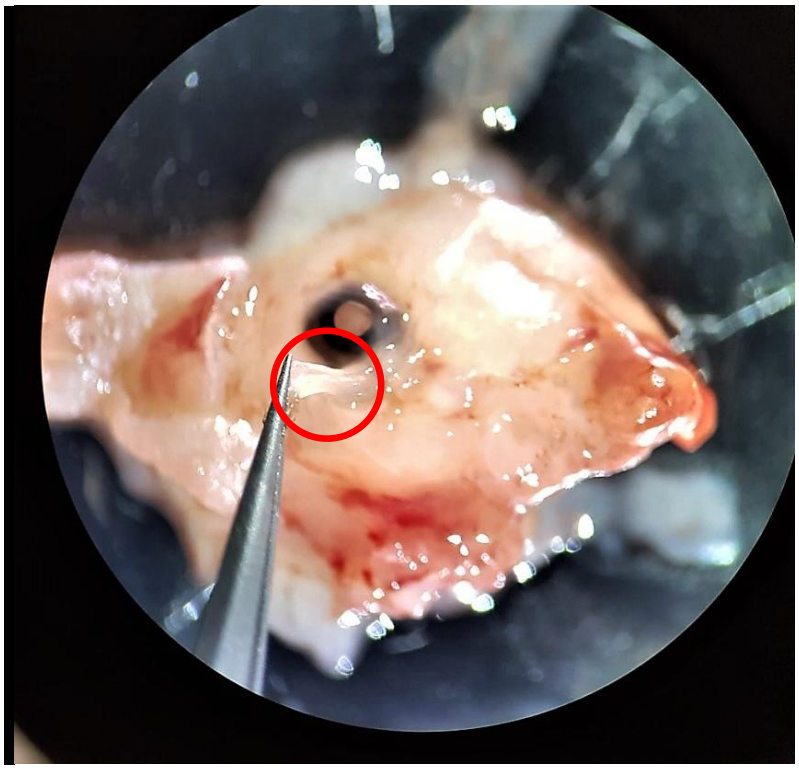


Figure 17: Lacrimal gland of mouse embryo.

3.6.3.3. Epithelial and Mesenchymal Compartment Seperation

To perform tissue encapsulation experiments, we tried to separate the epithelial and mesenchymal parts of the lacrimal gland mechanically under stereo microscope (Zeiss). These separated parts are collected in 1.5 ml centrifuge tubes on ice for further use.

3.6.3.4. Cell Isolation from Adult and Embryonic Lacrimal Glands

To isolate cells from lacrimal gland tissue we used our own variation of previously described method.(Finley et al., 2014) Only difference between adult and embryonic lacrimal gland isolation protocol is that tissues from adult mice require a mechanical dissection step before enzymatic digestion step.

Mechanical Dissection

Due to their size, lacrimal gland tissues that are obtained from adult mice needs a mechanical dissection step before the enzymatic reaction. Obtained pieces are put into a petri dish on ice with the medium inside and chopped quickly into smaller pieces with the help of scalpel and tweezers.

Enzymatic Digestion

Tissue pieces are collected and put into 1.5 ml centrifuges tubes for enzymatic reaction steps. Pieces are treated twice with 100 U/ml collegenase II for 15 min and then with trypsin-EDTA for 10 min. All reactions were done with tube rotator inside a culture incubator at 37 °C. After every enzymatic reaction, the pieces were centrifuged

at 0.7 g for 5 minutes then supernatant is discarded and fresh prepared enzyme is added. After final digestion, the mixture is filtered through a 70 µm cell strainer. Mixture was washed with complete culture medium through the strainer and collected into 50 ml centrifuge tube. The mixture is centrifuged at 0.7 g for 5 minutes. Then supernatant is discarded and fresh culture medium is added for cell counting. After counting cells with the hemocytometer cells are ready for further use.

3.6.4. Histological Experiments

All histological sample preperations (paraffin enbedding, sectioning and histological staining) were done by IBG histopathology core facility technician. Sample preparation for cryosection was done by us. Briefly, tissue parts were embedded in OCT compound inside of a cap of 5ml centrifuge tubes. After aligning the tissue parts at the middle of poured OCT compound, samples were frozen at -80 °C for 1 day. Then prepared samples were cryosectionined and stained.

3.6.5. Cell and Tissue Culture

In this project, our focus was to bioengineer a tissue culture platform assisting the recapitulation of native environment. Cells that were used through this study were, NIH/3T3 mouse fibroblast cell line (as model cell), primary adult mouse lacrimal gland cells and primary embryonic mouse lacrimal gland cells. All cell and tissue cultures were done inside cell culture incubator (Memmert) and all methods were carried on in laminar flow hood (Thermo) under sterile conditions.

Culturing Materials

Cell culture studies were done with sterile pipettes (10 µl – 1 ml), centrifuge tubes (1 ml – 50 ml), serelogical pipette, petri dish plates (60 mm and 100 mm) and culture flasks (25 cm^2 and 75 cm^2). For culturing NIH/3T3 cells complete DMEM culture medium (GIBCO), for culturing both adult and embryonic primary lacrimal gland cells complete DMEM/F-12 culture medium (GIBCO) was used. For culture plate cleavage trypsin/EDTA (GIBCO) was used. As buffer we 1X PBS (GIBCO) in all cell culture applications.

3.6.5.1. Cell Culture

During our cell culture studies we used 3 main methods, passaging (subculturing) of cells, freezing cells for stock (cryopreservation) and thawing frozen cells. We used same protocols for 3 methods on both cell lines and primary cells.

For passaging of cells, first we preheated the complete medium, trypsin/EDTA and 1X PBS to 37 °C. When they are heated, we took the cell culture vessel from the cell culture incubator and put into the laminar flow hood. Then the spent medium was discarded and culture vessel was washed with sufficient amount of PBS 1X to cover the floor of vessel. After washing we discarded the wash solution and added adequate amount of preheated trypsin/EDTA (approximately 0,5 ml per 10 cm^2 of culture vessel). Then the vessel was incubated in cell culture incubator for ~ 2 minutes. When incubation was over, culture vessel was observed under a microscope to confirm high amounts (more than 90%) of deattachment. If not the vessel was incubated in same conditions for 30-60 seconds until the deattachment ratio is met. When deattachment is complete, we added complete culture medium (twice of the trypsin/EDTA volume) into the culture vessel to inactivate trypsin/EDTA. After that we collect the cells with serelogical pipette (by doing up and down for maximum cell collection) and transfer them to 15 ml centrifuge tube. Then we centrifuge them at 600 g for 5 minutes. After

centrifugation, the supernatant is discarded and cells were resuspended with desired amount of fresh complete culture medium for cell counting. After counting the cells with hemocytometer and tryphan blue (Sigma), cells are ready for future use.

To freeze cells, first cells that are going to be frozen need to ready and counted prior to freezing procedure. So for cultured cells it is done after the passaging protocol and for isolated cells it is right after the counting the cells. After our cells were ready, we prepared a fresh freezing medium which contains 90% fetal bovine serum (FBS) (GICBO) and 10% Dimethyl sulfoxide (DMSO). Counted cells were centrifuged again with same settings and supernatant was discarded. For each 1 million of cells 1 ml of freezing medium was added into the 15 ml centrifuge tube and cells were mixed via up and down. Then we transferred the mixture into cryo vials (Isolab) as 1 ml of the mixture per vial. For slow freezing cryo vials were put into the Mr Frosty (Isolab) then put into -80 °C for 1 day. After vials were completely frozen we transferred them into the liquid nitrogen tank (140 °C) for stocking.

Thawing cells is relatively short and fast procedure compared to the first two ones. After taking cryopreserved cells from liquid nitrogen tank, we quickly thawed them in the water bath at 37 °C. Then we transferred the cells that are in freezing medium (1 ml) into a 15 ml centrifuge tube and added 2 ml of preheated (37 °C) complete culture medium. To get rid of DMSO we centrifuged the cells at 600 g for 3 minutes to avoid damage to freshly thawed cells. After centrifugation supernatant was discarded and fresh complete culture medium was added. After mixing the cells with up and down, cells are ready for future use.

3.6.5.2. Tissue Culture

In this study two main tissue culture methods were used, explant culture and bio engineered tissue culture. While explant culture is known method, we generated our method for bio engineered tissue culture.

Explant culture was done only with dissected mouse adult mouse lacrimal glands. After mechanical dissection step, lacrimal gland parts were seeded into petri dish (60 mm and 100 mm) and single drop of FBS were added onto them. After waiting for 3 hours for tissues to hold onto the petri dish surface, we added complete DMEM/F12 culture medium and observed the progress.

On the other hand bio engineered tissue culture, which is one of the main components of this study was done by growing cells encapsulated by an engineered bio material, GelMA hydrogel in both static and dynamic conditions. For culture preparation, the methods are similar in both systems, however culturing methods differ due to different nature (static vs dynamic) of setups. To successfully do a bio engineered tissue culturing three things needed to be prepared; GelMA solution, culturing place (conventional culture vessel or glass slide for static culture and microfluidic system for dynamic culture) and ready to use cells.

Bio Engineered Tissue Culture

We conducted two types of culturing methods on our bioengineered tissues. Static and dynamic culture. Static culture is simply conventional culture of a bioengineered 3D structure. However dynamic culture involves continuous flow of culture medium for feeding the cells/tissue instead of static feeding.

Whether static or dynamic, the first step of bio engineered tissue culture is encapsulation of cells in GelMA hydrogel. Counted cells were centrifuged at 600 g for 5 minutes and supernatant was discarded. Then cells were resuspended with pre-prepared GelMA hydrogel at desired concentrations. Concentrations that we used were 1 million cells per ml, 5 million cells per ml and 10 million cells per ml. After resuspension and mild mixing, the solution is ready for UV activated crosslinking.

Static Culture

If culture will be done on static conditions, the mixture was either put into a conventional culture vessel or infused into a special setup for thinner structures, like a *glass stage.* This glass stage is made by gluing 2 glass microscope slides (Isolab) parallel to each other and have a less than 1 glass slide distance between them, onto a petri dish cap. Another 3rd glass slide will take its place onto preglued 2 glass slides. After placing the 3rd slide, a small space will be left between 3rd slide and petri dish cap surface. This is the place where we infuse our GelMA mixture with cells.

Either in conventional culture vessel or in a *glass stage* setup, our mixture is ready for crosslinking and total encapsulation. As mentioned before corsslinking step is done by UV light under right settings. On conventional culture vessels, crosslinked GelMA mixture sticks to the vessel surface. However on glass slide setup crosslinked GelMA stick to the 3rd glass slide.

After crosslinking process, newly formed 3D structure is ready for static culture. If the encapsulation was done on the glass slide it has to be put inside a petri dish to provide a culture environment for 3D structure. With our structure inside the petri dish (on glass or on petri dish surface), we simply put culture medium enough to cover whole 3D structure and culture it inside an incubator.

Dynamic Culture

Dynamic culture is a completely different concept compared to the static culture (which is basically conventional culture of a bioengineered 3D structure) and they differ in many aspects. First of all, setup that we used for dynamic culture is the microfludic system that we designed before (Lacrimal Chip). This system was specifically designed for bioengineered 3D lacrimal gland culture. The encapsulation was done on tissue culture grade polystyrene base of the Lacrimal Chip (LC) by using appropriate photomasks. After respective crosslinking of the compartments, we put the base part

of the chip under static culture conditions for 1 day to reduce the stress on the cells/tissue parts that were encapsulated. After 1 day incubation on static conditions, we assembled the remaining parts of the LC and closed the system.

To culture this closed system, we connected the specific borosilicate micro tubing (Cole Parmer), which act as feeding and drain channels for the system. These pipes were connected to the system with an industrial grade epoxy glue (Dial). After the connections were completely dried, the system became ready for kinetic culturing. Ready-to-culture LC has to be connected to the syringe pump (Harvard Apparatus), which provided the continuous flow of culture medium to the system. Culture medium was put into 3ml or 5ml syringes (BD) for each chip (because LC has 3 inlets as mentioned before) and these syringes were connected to the respective inlets of the LC.

Figure 18: Connected syringe pump on top of a culture incubator.

Then 2 outlets of the LC were drained into 2 different 15 ml disposal falcons labeled as E (epithelial) and M (mesenhcymal). Whole system resided in an incubator during culture except the syringe pump.

Figure 19: Culturing LC inside an incubator.

Emptied syringes were filled; filled disposal falcons were emptied when needed until end of the planned culture time. All individual kinetic experiments were carried for a time range between 1 to 4 weeks.

3.6.6. Flow Cytometry Analysis and Sorting Experiments

To detect the epithelial and mesenchymal cell ratio from isolation experiments and sort them for encapsulation experiments we used flow cytometry technique. For analysis and sorting experiments we used Canto II Analyzer (BD) and Aria III Sorter (BD) respectively. In both analysis and sorting experiments we used CD90 antibody with PE (Abcam ab24904) to detect mesenchymal cells and EpCAM antibody with APC (Biolegend 118213) to detect epithelial cells. For both antibodies we used the respective IgG controls with APC (Biolegend 400511) and PE (Biolegend 400707).

Flow Cytometry Buffer

For both analysis and sorting experiments a special flow cytometry buffer (FCM buffer) is used. To prepare this buffer 0,25% Sodium Azide and 1% Bovine serum albumin (BSA) were dissolved in 1X PBS. This buffer can be stored in 4 °C and reused in the future.

3.6.6.1. Flow Cytometry Analysis Experiment Preparation

Before all steps 5 round bottom tubes (BD Falcon) were labeled as; Tube 1 – Unstained, Tube 2 – CD90 only, Tube 3 – EpCAM only, Tube 4 – IgG control, Tube 5 (the experiment tube) – CD90 + EpCAM. Collected cells were counted with hemocytometer and then fixated with 1% paraformaldehyde (PFA) for 20 minutes in room temperature. Cells were centrifuged at 600 g for 5 minutes and washed with 1X PBS and resuspended with DMEM/F-12, HEPES culture medium. Then 100.000 lacrimal gland cells were put inside each of the 5 tubes. 1 ml of FCM buffer added into each tube and all tubes were centrifuged at 600 g for 5 minutes. After centrifugation, supernatant was discarded and last drop in all tubes were preserved. Upon those drops, 1 µl of corresponding antibodies were added and tubes were incubated in dark at 4 °C for 30 minutes. After incubation, 1 ml of FCM buffer added into each tube and tubes were vortexed individually for 10-15 seconds. Then tubes were centrifuged at prior settings again and supernatant was discarded. After adding 250 µl of FCM buffer into each tube, they became ready for flow cytometry analysis experiment.

3.6.6.2. Flow Cytometry Sorting Experiment Preparation

Sorting experiments were done with the same protocol as analysis experiments except 2 major differences. First, on sorting experiments we did not fix the cells

because we need them alive after sorting. And second difference was after adding 100.000 cells into the first 4 tubes; we added all of our remaining cells into the tube 5 (the experiment tube) for maximum cell yield after sorting. At the end of the sorting experiments, we got 2 tubes from flow cytometry technician, one for CD90 positive (mesenchymal cells) and other for EpCAM positive (epithelial cells) for our encapsulation experiments.

All machine operating was done by IBG flow cytometry core facility technician.

3.6.6.3. Flow Cytometry Data Analyzing

Data gathered from flow cytometry experiments were analyzed on FlowJo software. Main populations were chosen from unstained tube data. Tube 2 (CD90 only) and tube 3 (EpCAM only) were used for single positive controls. Tube 4 (IgG control) acted as negative control. Data gained from tube 5 (the experiment tube) showed us the result of the experiments.

3.6.7. Scanning Electron Microscopy (SEM) Imaging of GelMA

5% GelMA solution in 1X PBS with 0.5% photoinitiator (PI) concentration was crosslinked as mentioned before. Then crosslinked GelMA was lyophilized before the SEM imaging. After that all procedures as well as SEM imaging of GelMA was carried by IBG Electron microscopy core facility operators under supervision of project researchers.

3.6.8. Fourier-Transform Infrared Spectroscopy (FTIR)

5% GelMA solution in 1X PBS with 0.5% PI concentration was crosslinked as mentioned before. Then crosslinked GelMA was lyophilized before the FTIR. Unmodified gelatin was used as control. FTIR measurements were taken at Manisa Celal Bayar University Materials Science Faculty.

3.7. Research Plan

	September 2016 – February 2017	**February 2017 – January 2018**	**January 2018 – January 2019**	**January 2019 – May 2019**
Literature Review	X	X	X	X
Experiment planning	X	X	X	
Collecting preliminary data	X	X		
Experiments and data collection		X	X	X
Data evaluation		X	X	X
Thesis writing				X

3.8. Evaluation of Data

Flow cyometry analysis was done on FlowJO v.10.4 software. FTIR results were calculated with PerkinElmer Spectrum Version 10.4.3 software.

3.9. Limitation of Research

In case of poor GelMA production due to synthesis errors, commercial Poly (ethylene glycol) diacrylate (PEGDA) biopolymer will be used as an alternative hydrogel. In case of having problems to obtain primer epithelial and mesenchymal cells, mechanically separated tissue parts will be used for encapsulation. In case of microfludic system manufacturing problems, more precise silicon based Polydimethylsiloxane (PDMS) microfluidic system will be used as replacement.

3.10. Ethic Committee Approval

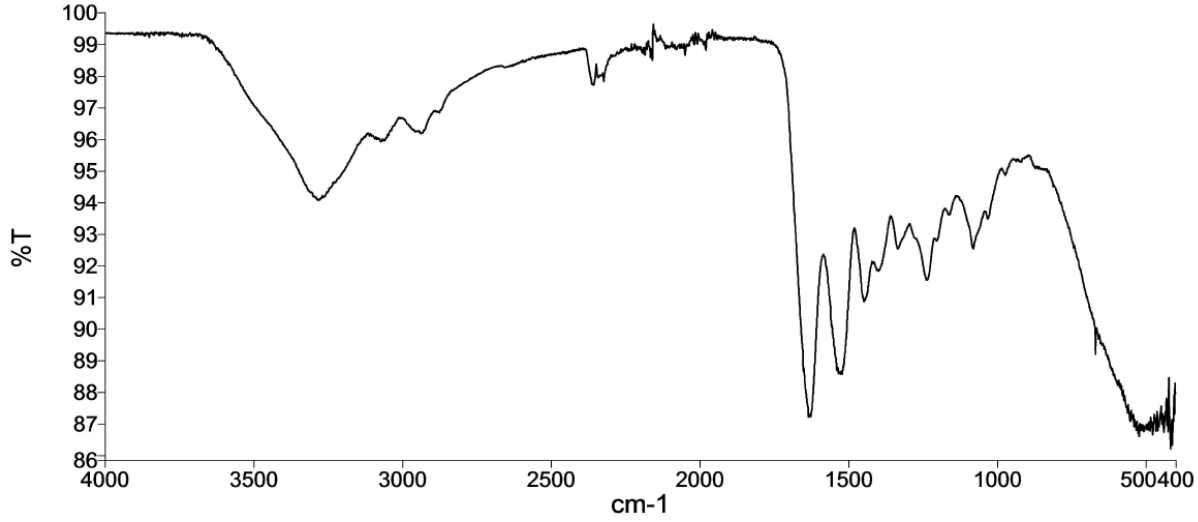
This thesis project has 16/2006 protocol numbered ethic committee approval from DEÜ IBG-HADYEK for animal experiments. Protocol was revised 2 times for extra animal addition.

4. RESULTS

4.1. GelMA Characterization Experiments

4.1.1. FTIR Results of GelMA Compared with Standard Bovine Gelatin

Standard Gelatin



Crosslinked GelMA

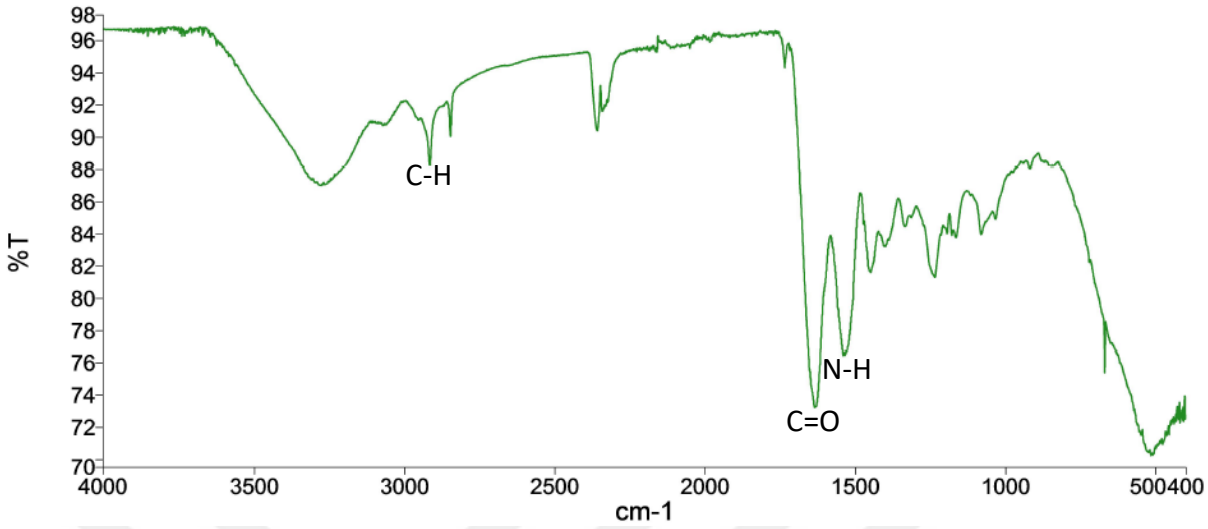


Figure 20: FTIR Comparison of Standard Gelatin and Corsslinked GelMA.

4.1.2. SEM Imaging of GelMA

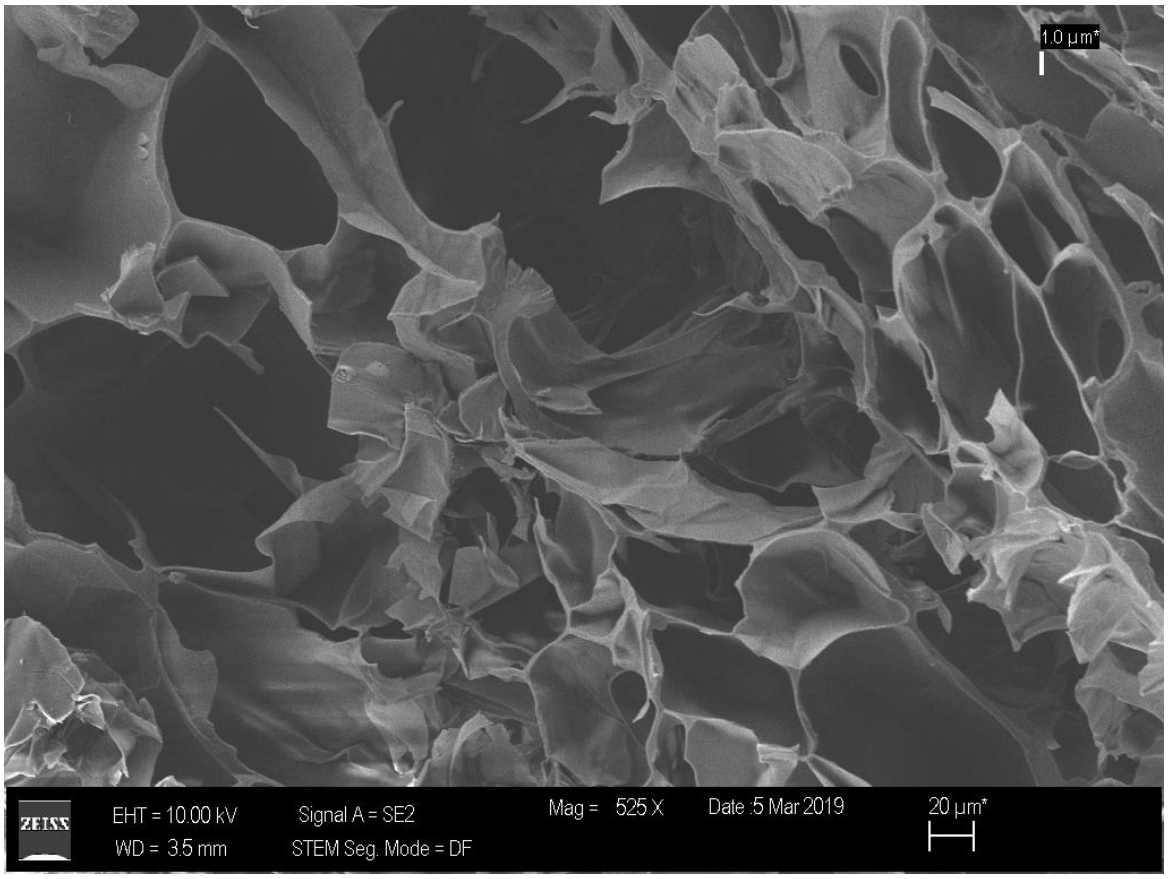


Figure 21: 5% GelMA under 4 power for 60 seconds.

4.2. Flow Cytometry Results

During all FACS experiments, epithelial cells were marked with EpCAM antibody with APC fluorescent conjugate dye whereas mesenchymal cells were marked with CD90 antibody with PE fluorescent conjugate dye. Q1 areas of the graphs represent epithelial population ratios whereas Q3 areas represents mesenchymal ratios per population on percentage.

4.2.1. Flow Cytometry Analysis Results

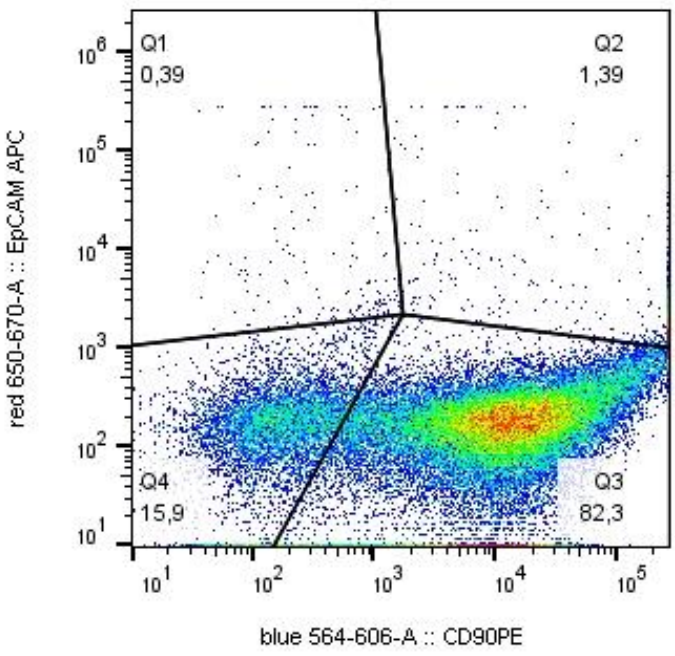


Figure 22: Cells after 4 days of culture on petri dish. Cells were first fixed than stained.

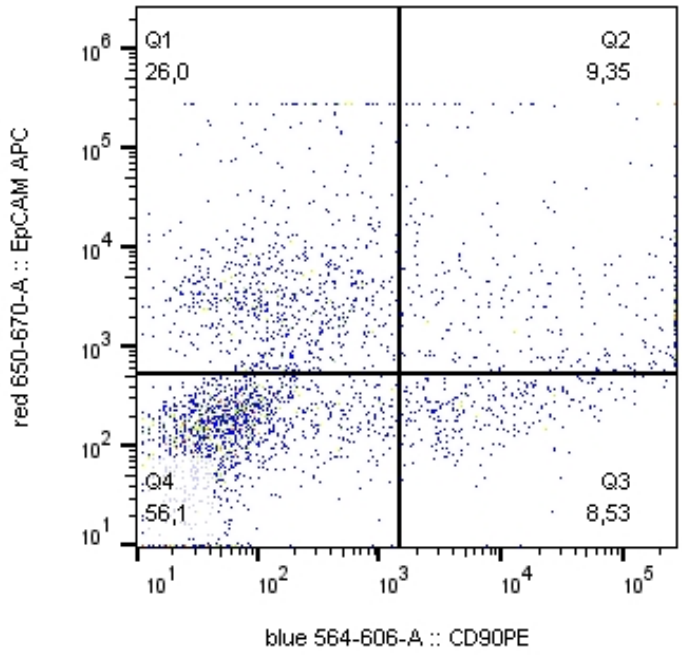


Figure 23: Cells that were frozen after isolation for 1 week. Then cells were thawed, fixed and stained.

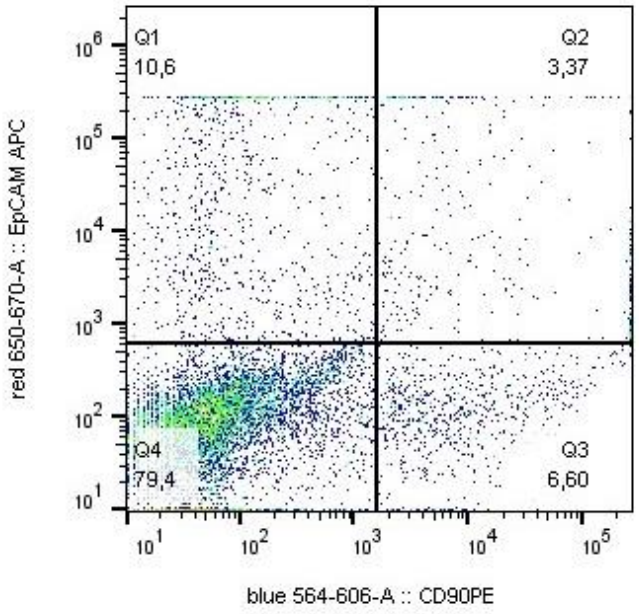


Figure 24: Cells were directly fixed and stained after isolation. 1st group of cells from same isolation.

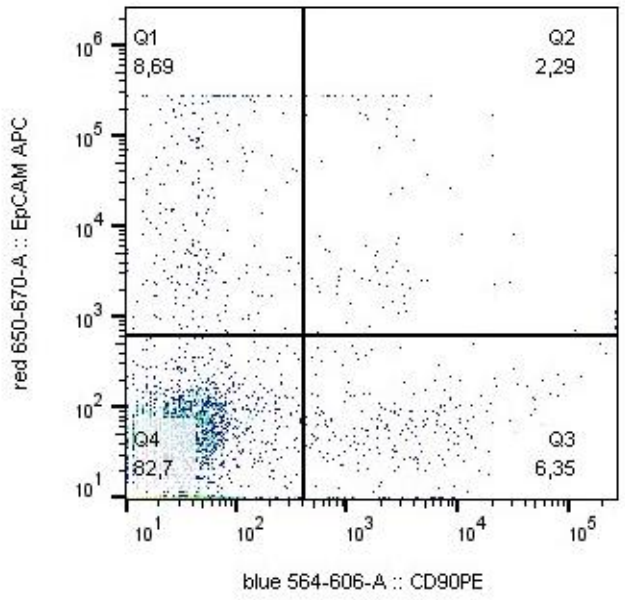


Figure 25: Cells were directly fixed and stained after isolation. 2nd group of cells from same isolation.

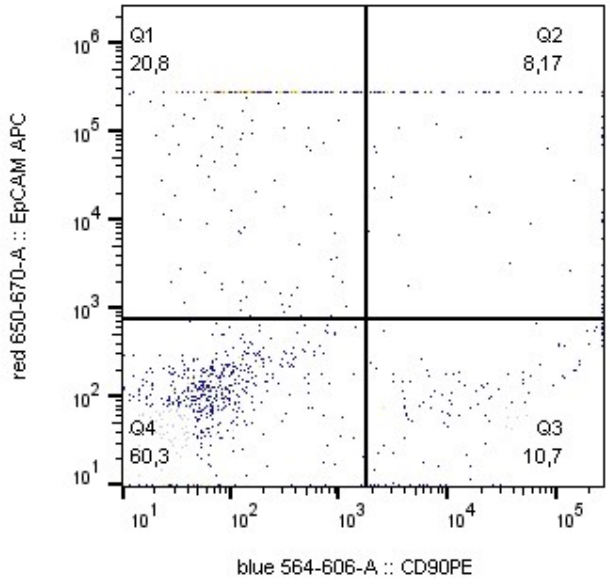


Figure 26: Tissue parts were incubated for 2 days in cell incubator. After 2 days cellss were isolated, fixed and stained.

4.3. Tissue Culture Imaging Results

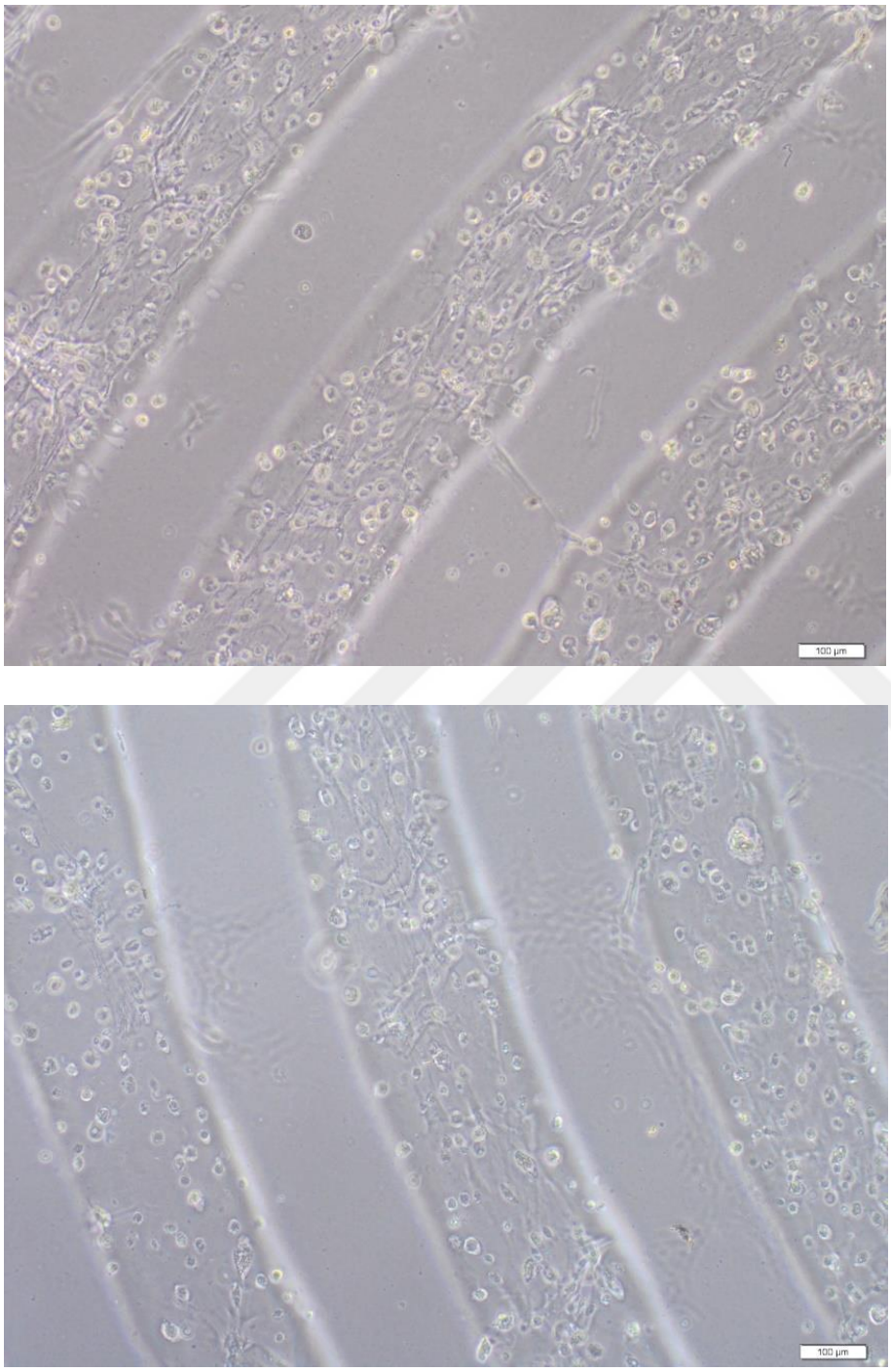


Figure 27: Encapsulation of mouse embryonic lacrimal gland cells done with ring pattern photomask. Day 5 after encapsulation. $5x10^6$ cells/ml. 10x magnification.

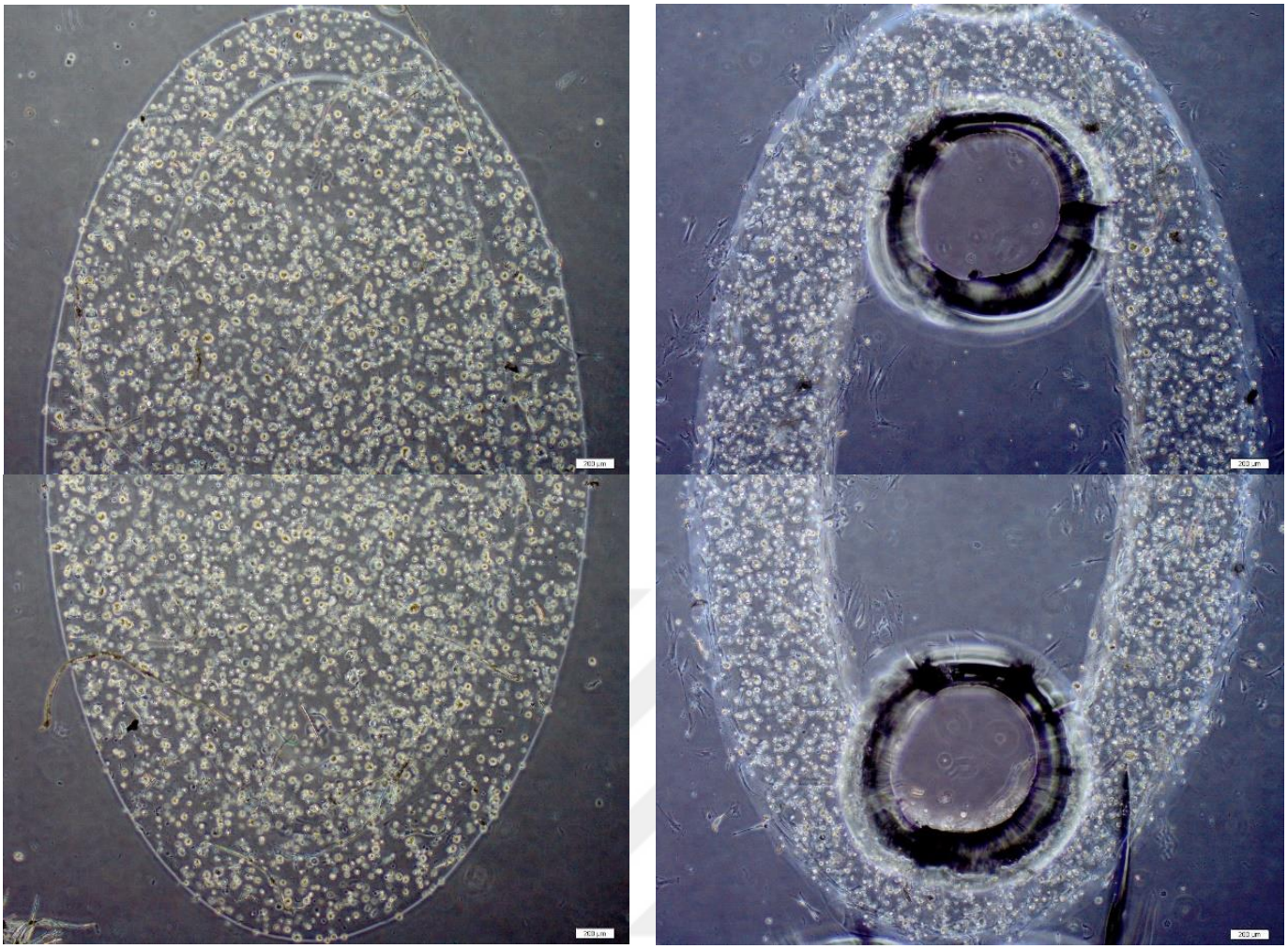


Figure 28: Mouse embryonic lacrimal gland cells encapsulation done in LC v5.0. LC version 5.0. Day 0 after encapsulation, before assembly. Both parts have $5x10^6$ cells/ml. 4x magnification

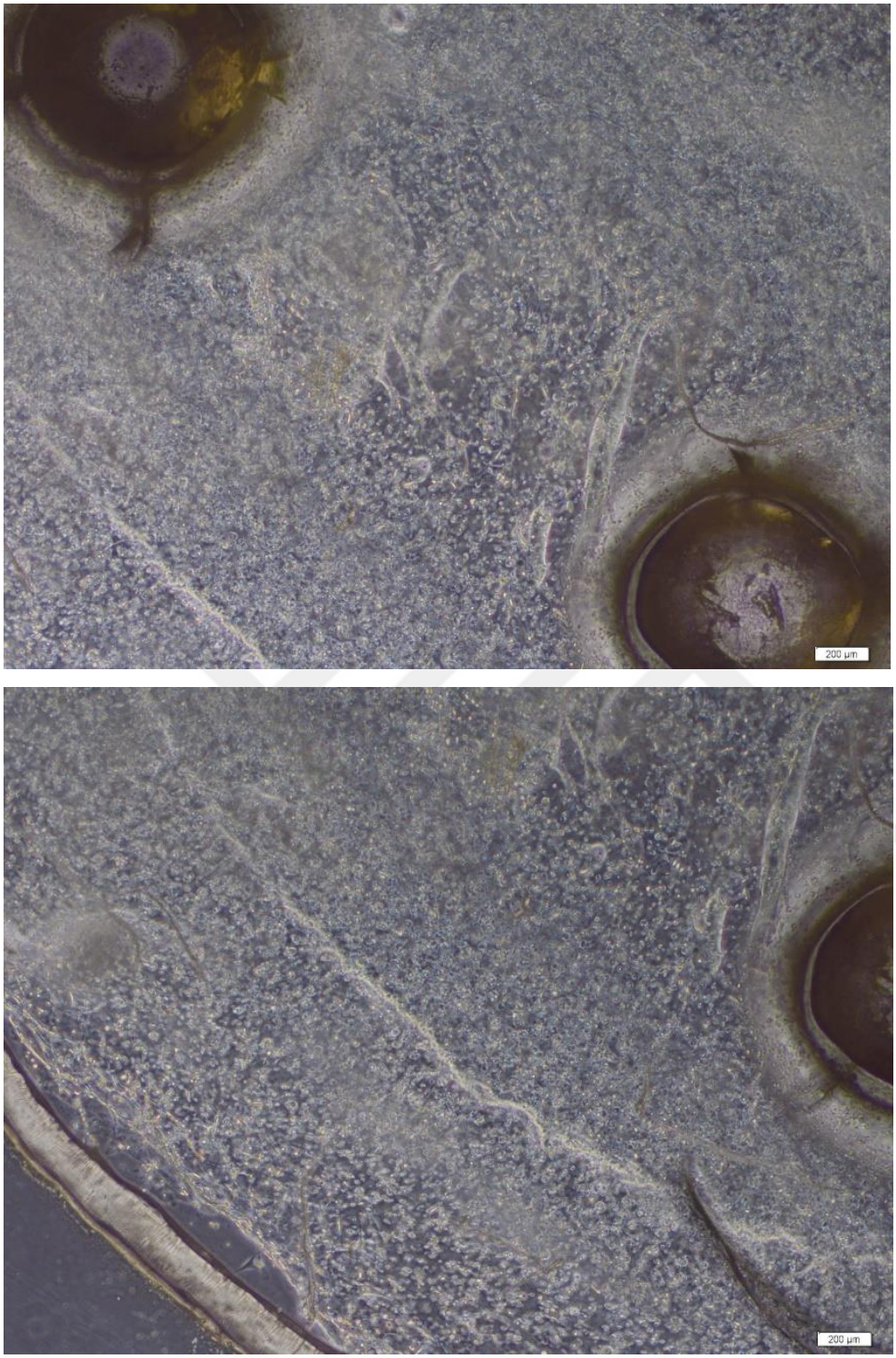


Figure 29: Assembled LC version 5.0. 10^7 cells/ml. Day 7 after encapsulation.

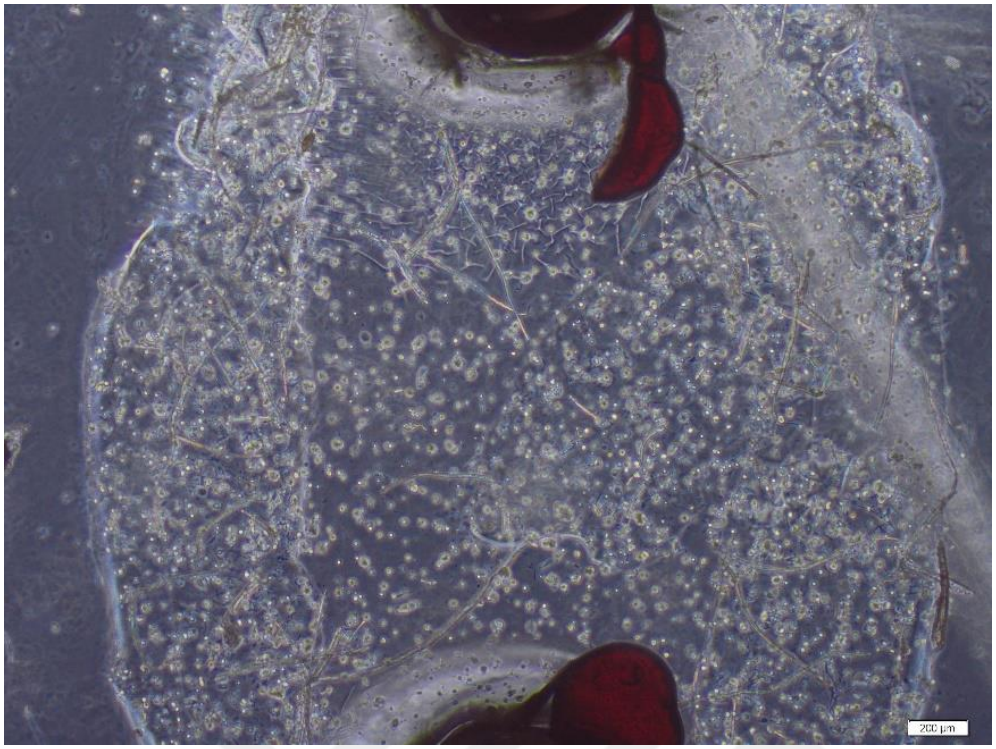


Figure 30: LC version 5.0. 10^7 cells/ml. Day 14 after encapsulation.

Figure 31: Bright field image of embryonic mouse lacrimal gland epithelial part structure. 4x magnification

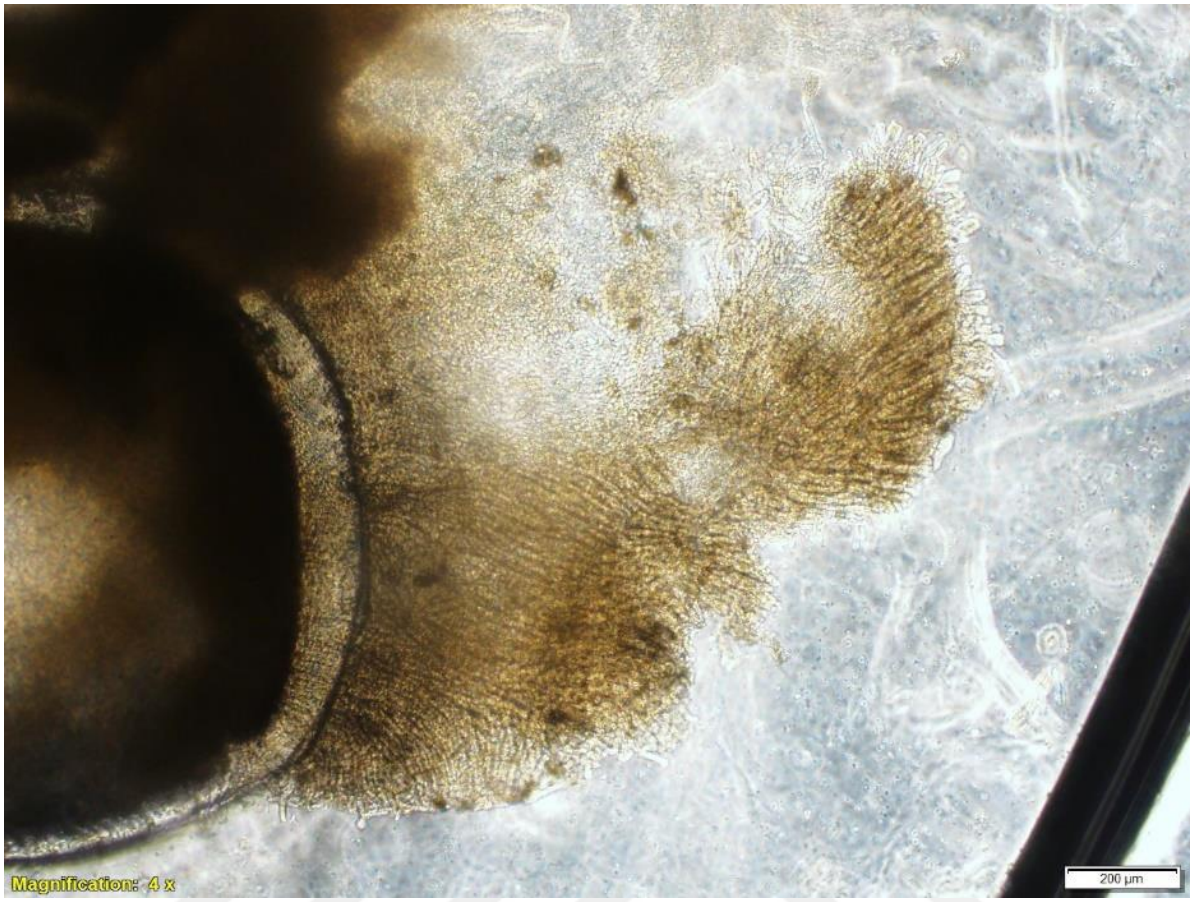


Figure 32: Encapsulated tissue parts were separated as epithelial and mesenchymal before encapsulation. Encapsulation has been done with respective to these parts. Day 7 after encapsulation. 4x magnification.

4.4. Histology Imaging Results

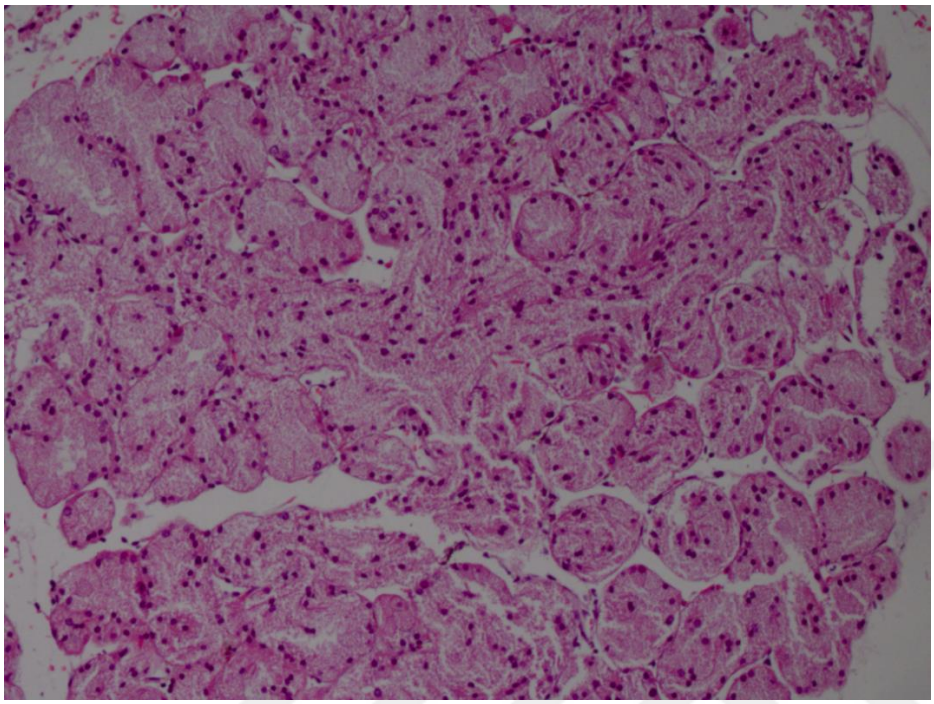


Figure 33: Adult mouse lacrimal gland H&E staining. 4% PFA. 10x magnification.

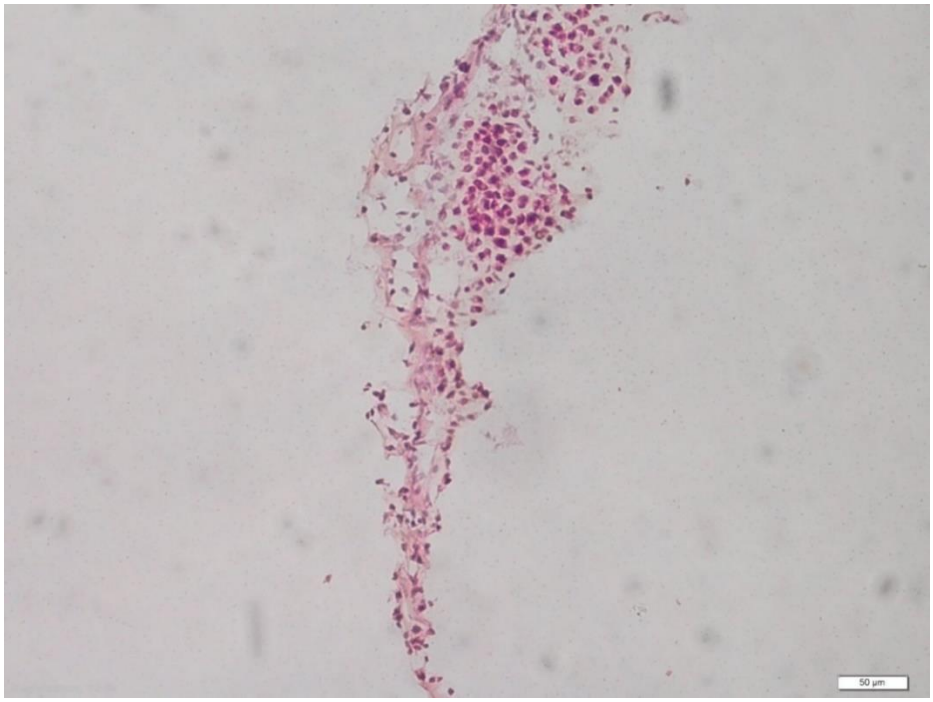


Figure 34: Embryonic mouse lacrimal gland H&E staining. 4% PFA. 10x magnification

4.5. Fluorescent Imaging Results

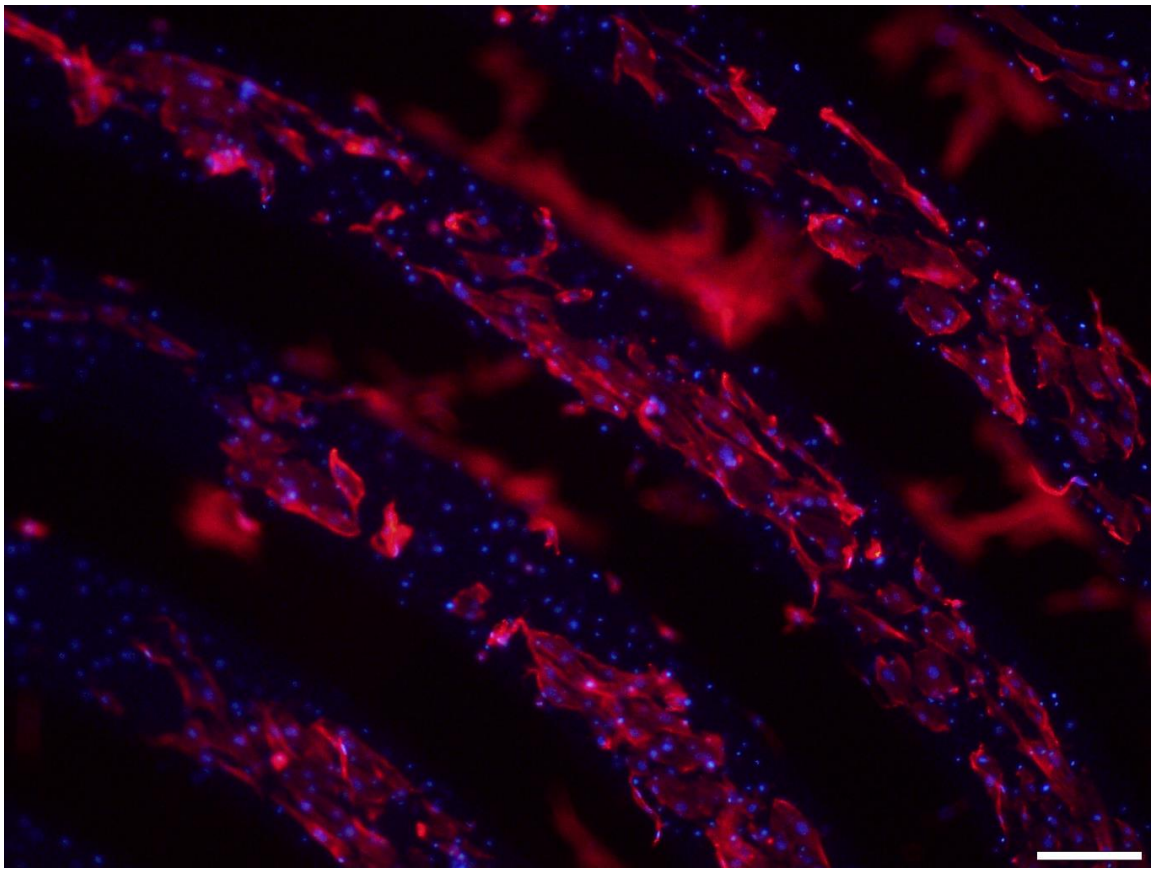


Figure 35: Encapsulation of mouse embryonic lacrimal gland cells done with ring pattern photomask. Red is phalloidin, blue is DAPI. 5m cells/ml. Day 10 after encapsulation. 4x magnification.

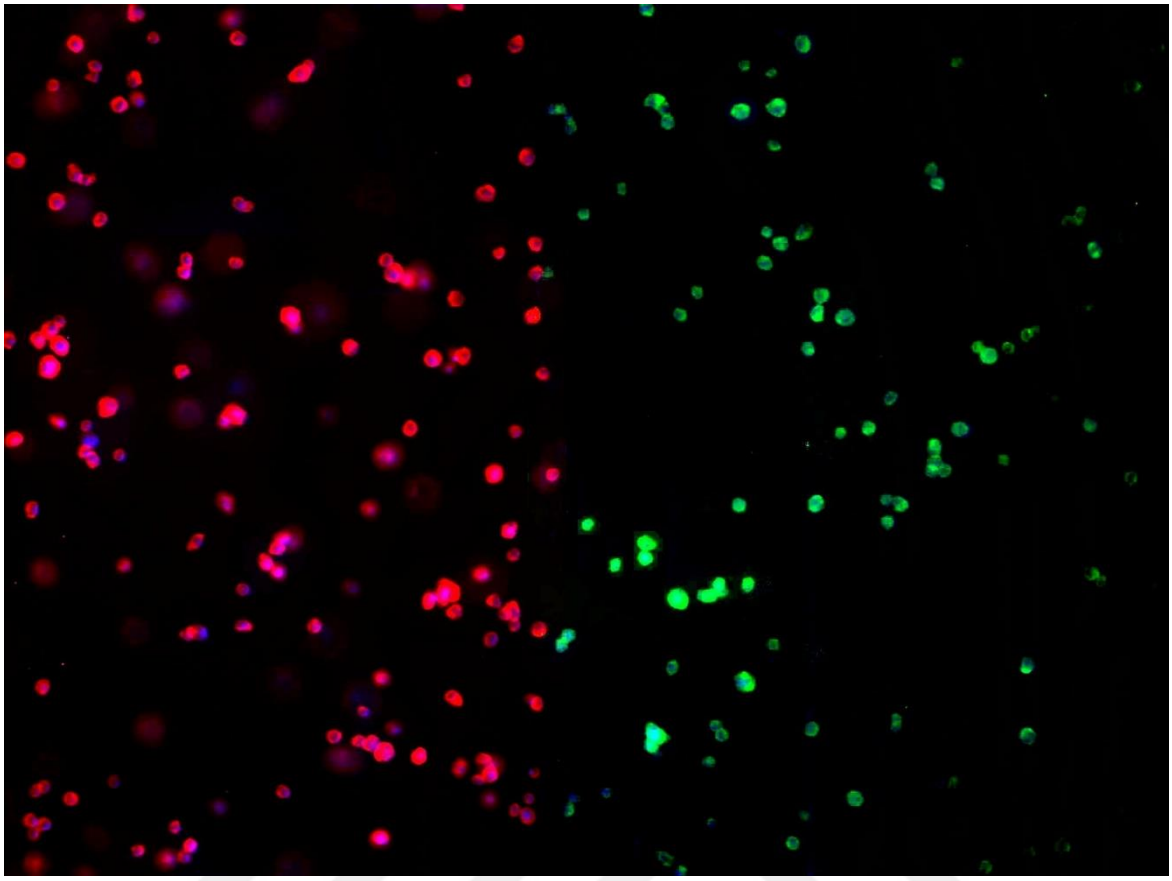


Figure 36: LC version 3.2. Day 0 after encapsulation. Sample NIH/3T3 Cells that encapsulated at the epithelial part were dyed with red phalloidin whereas cells at the mesenchymal part were dyed with green phalloidin. 5m cells/ml. DAPI is blue. 5m cells/ml. 10x magnification.

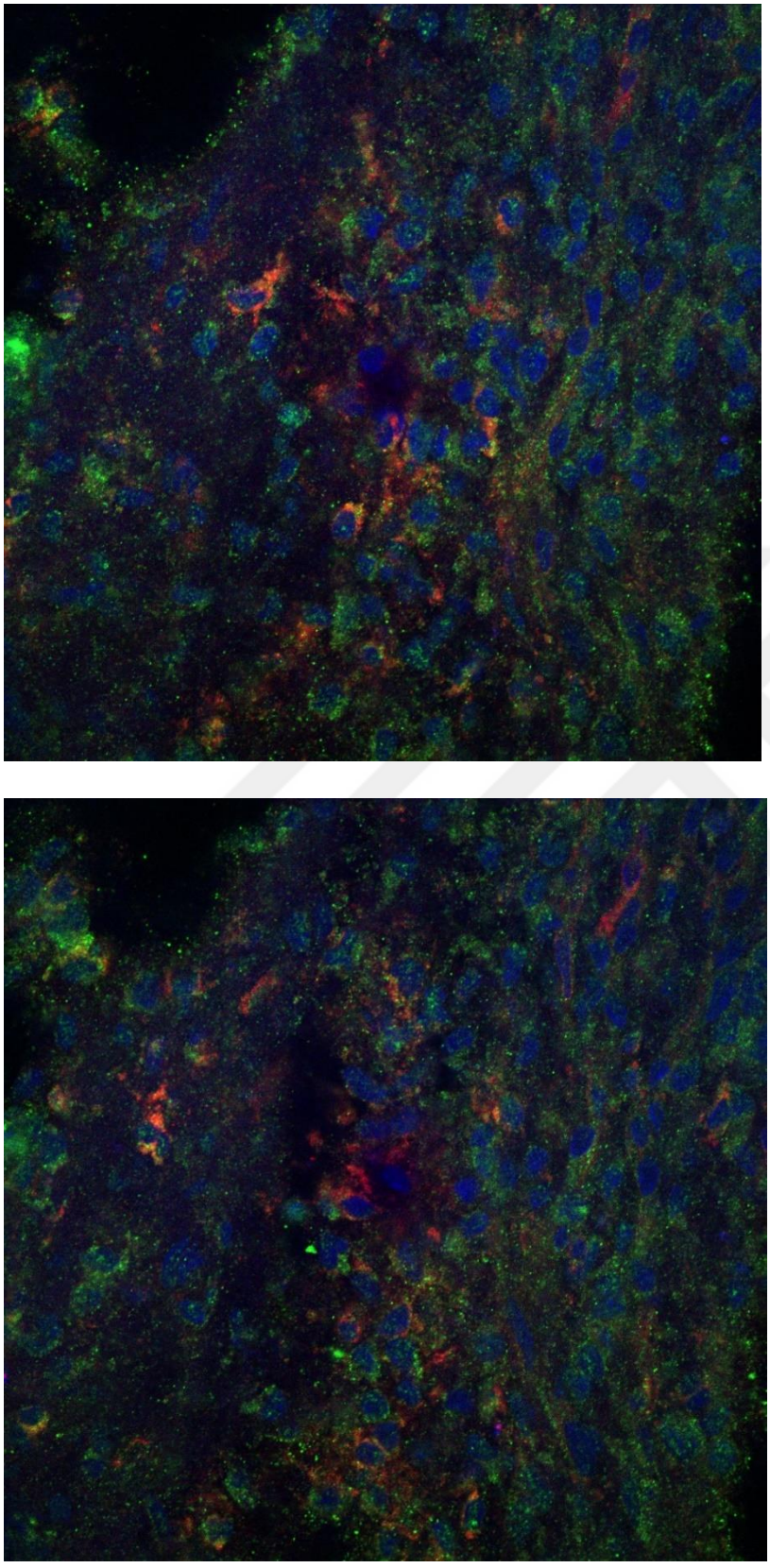


Figure 37: Different layers of native mouse embryonic lacrimal gland structure under confocal microscope. AQP5 is green, ZO-1 is red. DAPI is blue. 25x magnification.

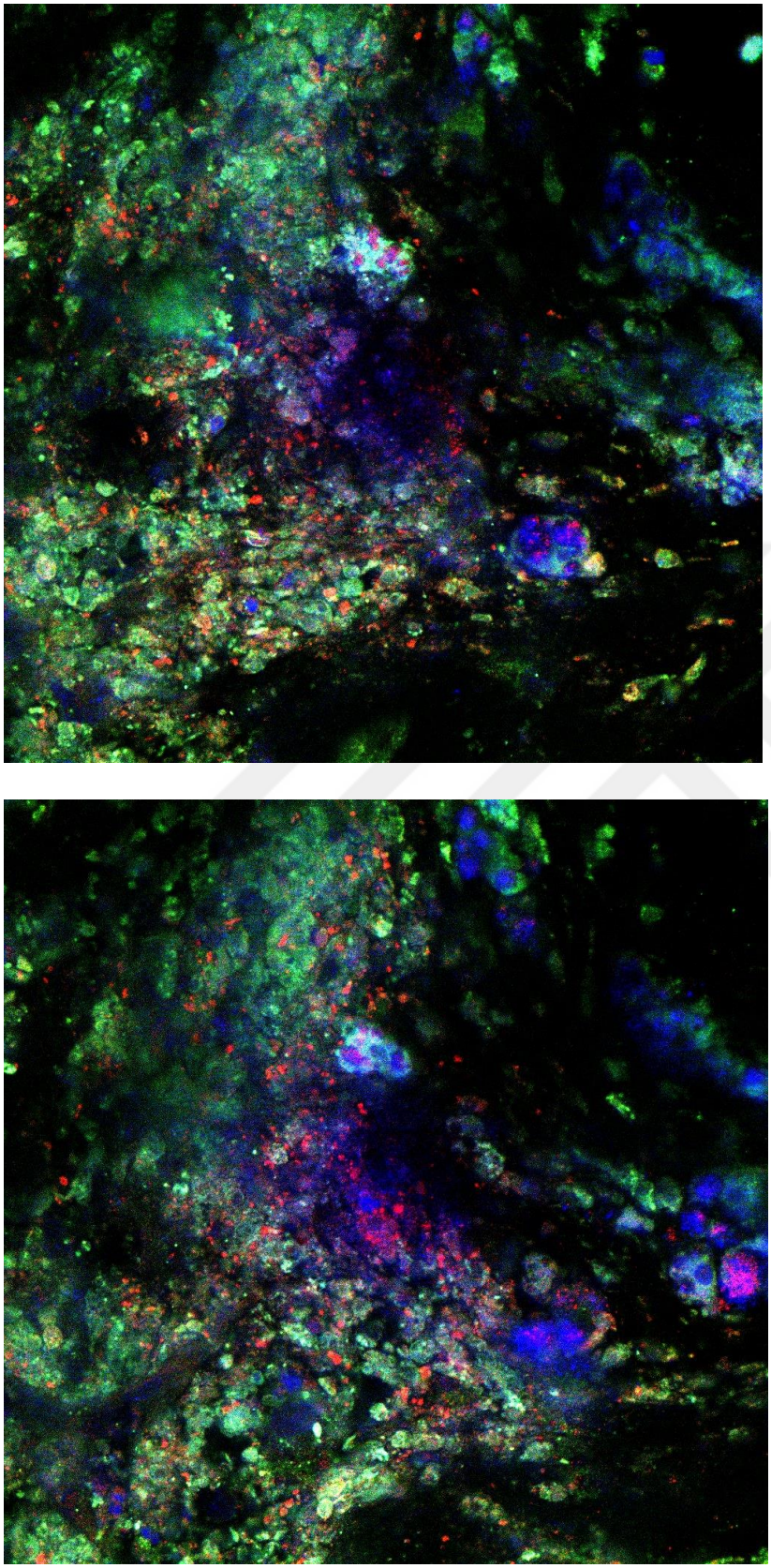


Figure 38: Different layers of mouse embryonic lacrimal gland tissue parts cultured for 2 weeks in LC version 5.4. AQP5 is green, ZO-1 is red. Blue DAPI. 25x magnification.

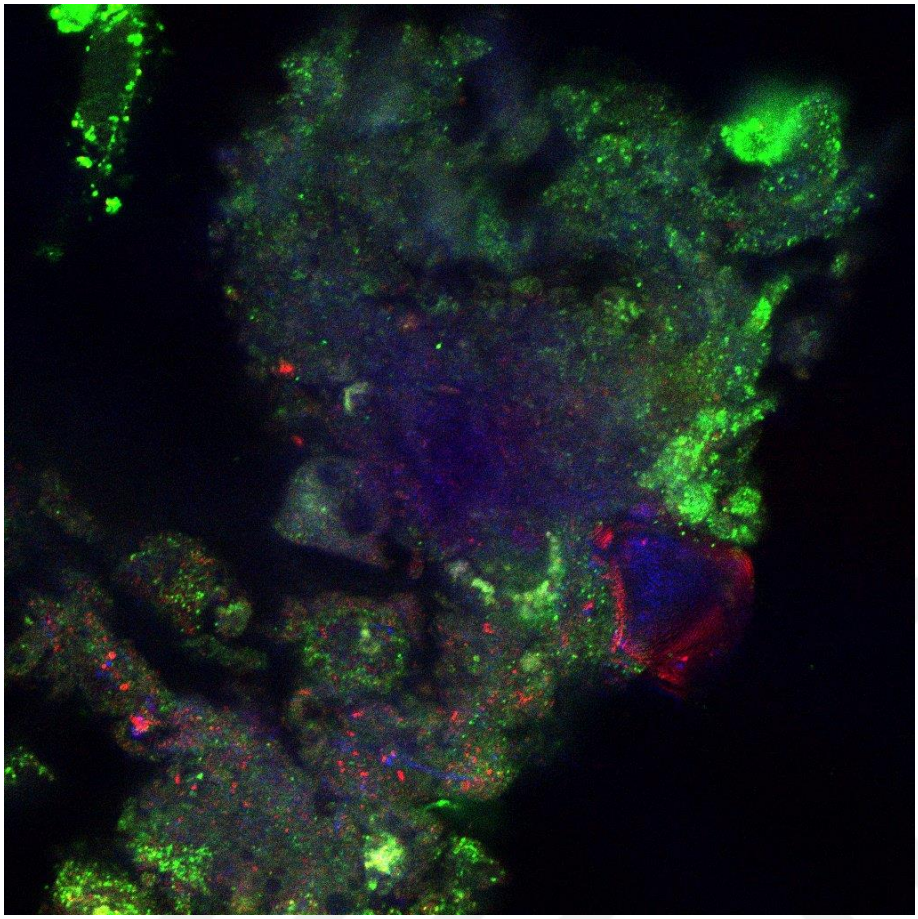


Figure 39: Mouse embryonic lacrimal gland tissue parts cultured for 2 weeks in LC version 5.4. AQP5 is green, ZO-1 is red. Blue DAPI. 63X magnification.

5. DISCUSSION

5.1. Optimization and Characterization of GelMA

5.1.1. GelMA Characteristics

There are different biomaterials that have different characteristics, which makes them useful for different situations as a scaffold for tissue culture platforms. This scaffold will be the environment of the culturing tissue so to successfully construct a 3D functional tissue the one needs to use right biomaterial as a scaffold. In our study

we chose Gelatin-Methacryloyl (GelMA) as biomaterial for our scaffold for the system. GelMA is actually a gelatin modified with methacrylic anhydride and it crosslinks (forms covalent bonds) with the presence of a water soluble photoinitiator under UV light exposure. It can be easily synthesized and relatively cheaper. Its porous structure allows the fluid flow between the cells that are encapsulated inside.

Gelatin-Methacryloyl (GelMA) is a biocompatible and biodegradable organic hydrogel. We synthesized,optimized and used our own GelMA throughout our studies. GelMA synthesis is a moderately long process and there are couple of important factors that need to be maintained carefully for the sake of GelMA end quality.

5.1.2. GelMA Synthesis Optimization

First of all temperature is very critical and it needs to be maintained around required values at all times from the very beginning. Stirring is also crucial during the addition of gelatin and methacrylate. Also during the addition of methacrylic anhydride, the process must be done very slowly, 1-2 drops each minute. This ensures the methacrylic anhydride to dissolve sufficiently. It is important to fill the dialysis bags quickly to prevent the effect of the temperature change. Daily change of the water that reaction takes places quickens the reaction time and also during the water changes temperature of the reaction needs to be maintained as best as possible. Finally when taking frozen GelMA to lyophilization the transfer must be done carefully without letting GelMA to melt to prevent boiling it during the lyophilization.

Also we found out that the amount of methacrylic anhydride added per g of gelatin heavily impacts the gelation quality of GelMA. Original method suggests that 0.6ml per g of gelatin is sufficient for a successful modification of gelation. However, in our most cases methacrylic anhydride modification of gelatin was insufficient due to common errors that occurred in reaction and dialysis steps like temperature maintaining, methacrylic anhydride addition frequency and water purity. To overcome this situation we increased the methacrylic anhydride amount added first to 0.8ml then

to 1.0ml per g of gelatin. Naturally, unreacted methacrylic anhydride is toxic for living cells and tissues, however the centrifuge step after the dialysis reaction removes the unreacted (excess) methacrylic anhydride eliminates this situation so we kept the amount of methacrylic anhydride per g of gelatin at 1.0ml at all times to be safe than sorry.

5.1.3. Photocrosslinking of GelMA

With the presence of a water soluble photoinitiator, GelMA becomes a photocrosslinkable biomaterial. With the help of UV exposure GelMA forms a covalently crosslinked hydrogel which acts as an extracellular matrix for the tissue culture applications. It is known that high UV exposure is lethal for the living cells due to DNA damage. This lethality depends on the exposure parameters as well as the target cell type. So these parameters like power, time and distance values must be in the safe range for the target-encapsulated cells/tissues to ensure their viability. For every batch of GelMA that synthesized, these values were tested both without and with cells respectively before using it for tissue culture applications. Due to standardization issues, not all batches had the exact same values for UV power and duration. However the values were never exceeded the viability limit (70%) of our cells/tissues.

5.1.4. Characterization of GelMA

5.1.4.1. FTIR Results

We conducted a FTIR experiment to confirm if our GelMA is actually a GelMA with its functional groups. Characteristic bonds can be seen as in peaks in figure 19. N-H group has peak between 3200-3400 cm^{-1}, C peak stretching between 2900-3000

cm^{-1}, and C=O (amide I) bonds formed between 1600-1650 cm^{-1} and 1230-1240 cm^{-1} peaks at N-H.

5.1.4.2. SEM Imaging of GelMA

We prepared a corsslinked GelMA solution without cells and then lyophilized it for SEM imaging to observe porous structure of our GelMA. Heterogenous porous structure can clearly be seen in figure 20.

5.2. Design of Microfludic System Lacrimal Chip

In this study we aimed to fabricate a specialized microfludic system, which is designed to support the artificial 3D lacrimal gland tissue inside of it. To support the artificial tissue, it needs to act as realistic as possible to the native tissue in terms of morphology and environment conditions. Originally the shape of a lacrimal gland is like a distorted oval so we wanted to go on from that shape in our system. As it was mentioned before functional lacrimal gland mainly consists of 2 major parts, an epithelial gland part which is surrounded by thin membrane like mesenchymal part (Figure 40). As it can be seen these parts are separate but together and this is the main idea behind our design. A system which allows the culture of 2 different parts with their corresponding 2 different flow routes together, in the same environment without any extra separators.

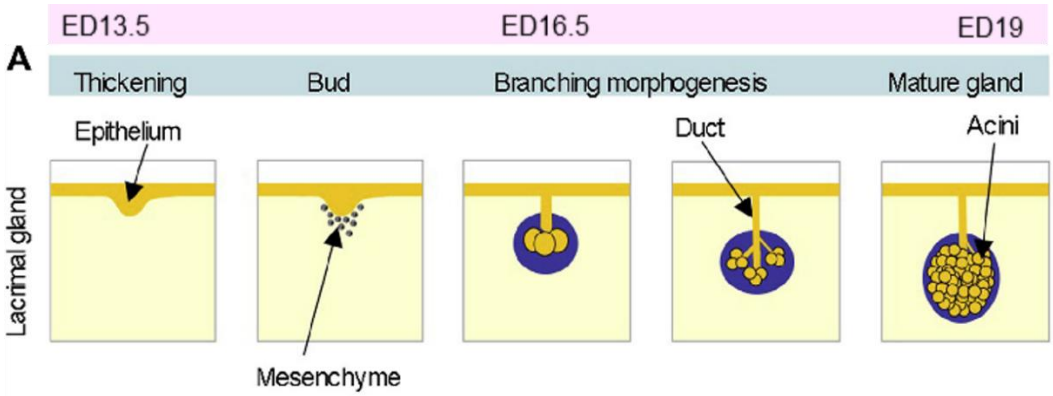


Figure 40: Embryonic development stage of mouse lacrimal gland. Blue part is surrounding mesenchymal part whereas yellow part is epithelial acini (glandular) part.(Hirayama, Tsubota, & Tsuji, 2015)

To create the optimum system we had to try couple of different approaches in terms of general design. After finding the optimum design concept then we needed to upgrade the system multiple times due to design and fabrication errors as well as visual improvement reasons.

Our microfludic system is built from Poly(methyl methacrylate) (PMMA), double sided adhesive (3M 486P) and medical grade polystyrene. These parts are cut with an industrial level laser cutter (Epilog Mini) and assembled together after bioprinting our artificial tissue inside. Then plastic microtubes are attached to the system with industrial grade epoxy adhesive. When epoxy adhesive is completely dried after 10-15 minutes our lacrimal chip becomes ready for the culturing system.

Design Version 1.0

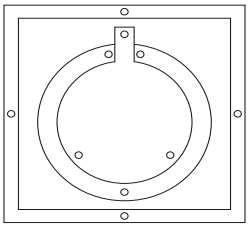
Design Version 1.1

Both versions 1.0 and 1.1 were never used for an application. They stayed as prototypes because these versions required a big bulk of biomaterial which was thick

in every dimension. This thickness is what we avoid while building our microfludic tissue culture systems.

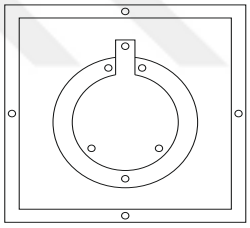
Design Version 2.0

Completely different design from the v1.0. This version is the ancestor of final version of the chip. System is based on the level difference between different compartments of artificial tissue inside the system. This level difference is gained by the thickness of double sided adhesive. Both sides of chip has inlets and otlets. These inlets and outlets located at the each side of the chip is feeding and draining only one compartment. This results non mixing simultaneous flow specific for each compartment inside the microfludic system.



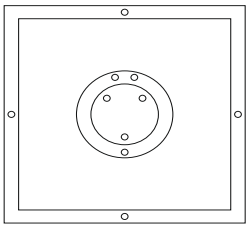
Design Version 2.1

Smaller version of v2.0 in terms of tissue size.



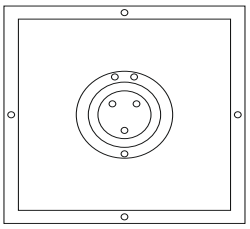
Design Version 3.0

This version has 2 major differences from v2.1. First one is removing the extension of epithelial part which gave hard time during the printing the tissue inside the chip. Second difference is much smaller size of total tissue than v2.1.



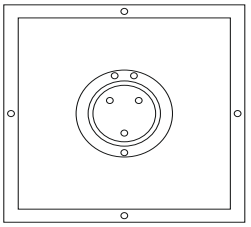
Design Version 3.1

This version changes the flow pattern of mesenchymal compartment. Previous versions had fluid flow above the tissue. This version has a separate channel next to the mesenchymal part. The reason behind this change is due to fluid leakage between compartments. Previous versions took advantage of level difference between compartments. However this level difference is not always perfect due to small gelation errors. Also pressure difference during the medium change sometimes distort this level difference so the flow pattern. To overcome this challenge in this version, mesenchymal part acts like a complete wall to separate channels more reliably. This change also opened way for having both inlets and outlets of all compartments on same side.



Design Version 3.2

This version thins down the mesenchymal part while increasing the epithelial parts total area. Reason of this change is due to diffusion rate rule. If mesenchymal part is wider than the diffusion rate limit, cells in the middle of mesenchymal part dies because of hypoxia and lack of resources. Increasing the epithelial parts area is solely for keeping the total area and volume of whole tissue.



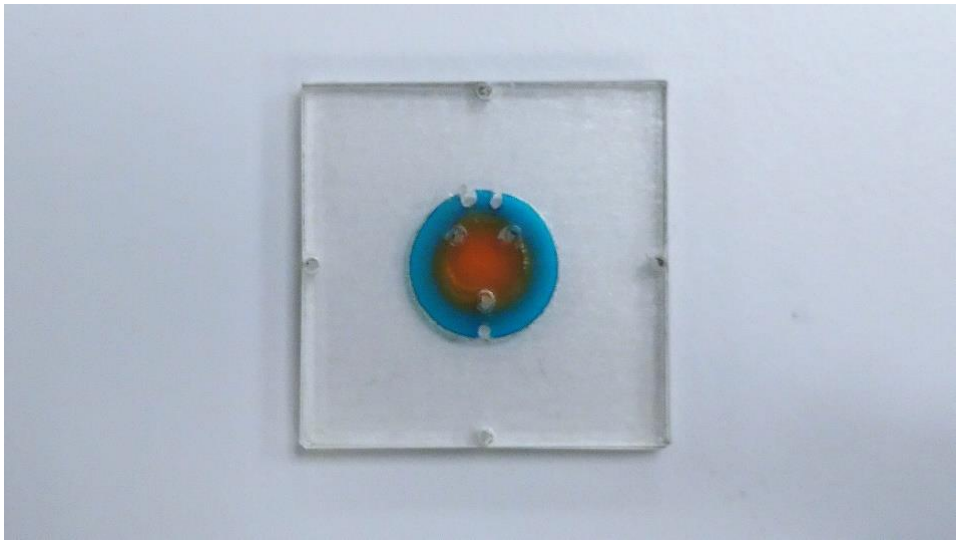
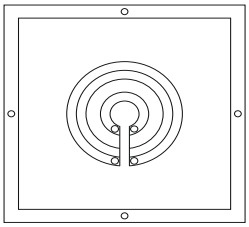


Figure 41: Version 3.2 tested with red (epithelial) and blue (mesenchymal) food dyes.

Design Version 4.0



An experimental version to try different approach. Instead of having a circle mesenchymal compartment around a round epithelial compartment, here we used two unfinished circles that touches each other on one side. On the other sides they have their specific flow channels. However the idea was not successfully applied because of gelation problems. Having both parts thin and circular made the process much harder to handle.

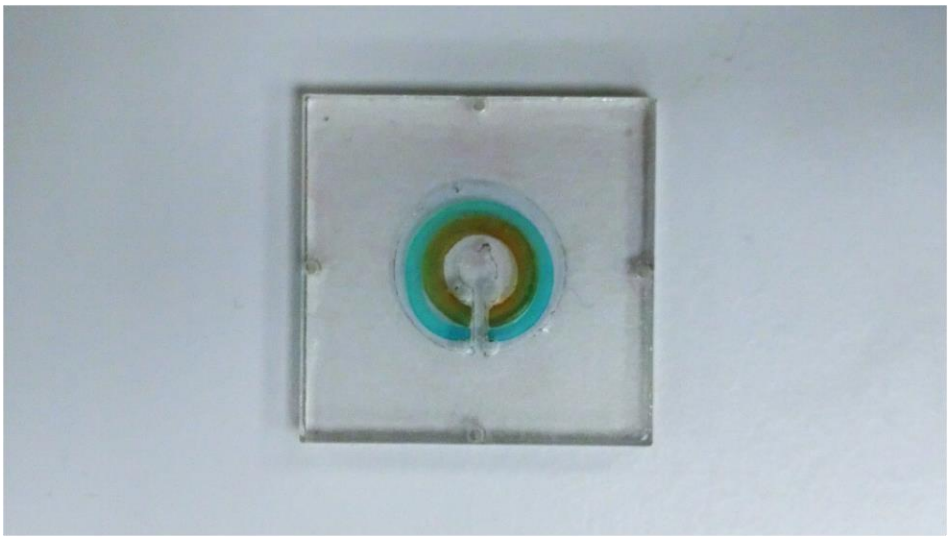
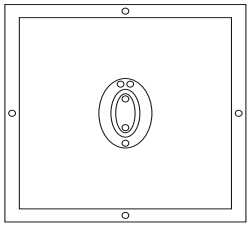


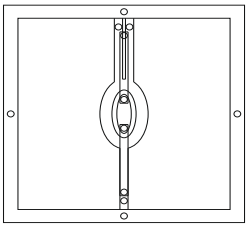
Figure 42: Version 4.0 tested with red (epithelial) and blue (mesenchymal) food dyes.

Design Version 5.0



This version is created after starting to use embryonic lacrimal gland cells. Because of the limited cell number we concluded on to make tissue compartment as smallest as possible. Also we made the structure as oval to make it look like more realistic as nature lacrimal gland tissue.

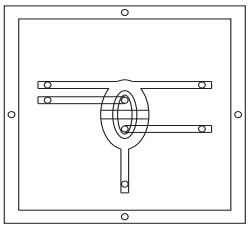
Design Version 5.1



Starting from this version, until last version v5.4 we tried to overcome the imaging challange occuring due to chip design. All inlets and outlets reside on the top of the tissue. So without detaching the inlets and

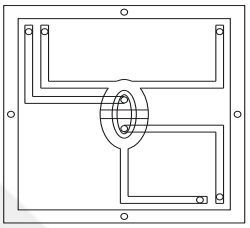
outlets it was impossible to image tissue inside the chip. So we extended the channels in y axis.

Design Version 5.2



Previous version had come fluid mixing problems because of the distance between channels being so close. To fix this we separated the channels in each side of the chip.

Design Version 5.3



This version only channels the inlet and outlet locations for aesthetic purposes. This naturally resulted in different channel patterns.

Design Version 5.4

V5.3 had inlets and outlets too close to the border of chip so this final version brings them a little inside to prevent leakage.

This is the final version of the Lacrimal Chip. For detailed working principle explanation and visual representation go to *3.6.2.3.* and figure 12.

5.3. Animal Experiments

To obtain our lacrimal gland cells we used both adult (BALB/c and NSG) and embryonic mice (BALB/c, NSG, C57BL/6) as tissue source. First we used random mice of varying ages. When we compared culture behaviors of cells from old and young mice we realized that Cells from younger mice tend to proliferate more rapidly compared to older ones. After this result we took one step further and decided to use cells from embryonic mice for maximum proliferation capacity.

Working with embryonic mice was much more harder in terms of handling of animals and excision of tissue. However the proliferation difference was notable. We seeded similar number of cells in the same sized culture plates and cells from embryonic mice became confluent nearly 3 times faster than cells that gained from the youngest adult mice. After seeing this we carried on our experiments with mouse embryonic lacrimal cells for better results.

5.3.1. Adult Mice Lacrimal Gland Tissue Isolation

First 4 isolation experiments was done for mastering the lacrimal gland tissue extraction and optimizing the enzymatic isolation protocol as well as determining the sufficient amount of lacrimal glands to gain adequate amount of cells for cell and tissue culture experiments. Following isolations were done for cell culture experiments, static tissue culture experiments and dynamic culture experiments respectively.

Lacrimal Gland Isolation 1 (LG ISO 1) 10.02.2017

2 mice, total 4 lacrimal glands. Isolated cells were seeded into 6 well culture plate for observation.

Lacrimal Gland Isolation 2 (LG ISO 2) 17.02.2017

3 mice, total of 5 lacrimal glands. Isolated cells were seeded into 4 wells of a 6 well culture plate for observation.

Lacrimal Gland Isolation 3 (LG ISO 3) 15.03.2017

2 mice, total of 4 lacrimal glands. Isolated cells were seeded into 4 wells of 6 well culture plate for observation. Contamination after 1 week.

Lacrimal Gland Isolation 4 (LG ISO 4) 15.12.2017

2 mice, 4 lacrimal glands. Isolated cells were seeded into 3 wells of 6 well culture plate to obtain enough cells for encapsulation experiment.

Lacriml Gland Isolation 5 (LG ISO 5) 20.12.2017

5 mice, 7 lacrimal glands. This time we decided to try explant culture method besides cell isolation. Cells were used for static encapsulation experiments.

Lacrimal Gland Isolation 6 (LG ISO 6) 28.12.2017

1 mouse, 2 lacrimal glands. Only explant culture was done with the tissues gained from this experiment due to low number of lacrimal glands. Cells were used for static encapsulation experiments.

Lacrimal Gland Isolation 7 (LG ISO 7) 18.01.2018

6 mice, 12 lacrimal glands. This was the experiment that we realized the age difference is an important factor on cell proliferation. We had 2 old (older than 9 months) and 4 young (approximately 2 weeks old) mice and we cultured them separately to see the difference. We did both explant culture and isolation. Cells were used for static encapsulation experiments.

Lacrimal Gland Isolation 8 (LG ISO 8) 09.02.2018

11 mice (5 young, 6 old), 20 lacrimal glands. Cell isolation and explant culture were done. Contamination after 2 weeks.

Lacrimal Gland Isolation 9 (LG ISO 9) 02.03.2018

5 mice (4 young, 1 old), 10 lacrimal glands. We stopped using the explant culture method starting from this experiment due to insufficient cell yield.

Lacrimal Gland Isolation 10 (LG ISO 10) 26.03.2018

6 mice (all young), 12 lacrimal glands. All cells isolated from this experiment was used for dynamic encapsulation experiments in lacrimal chip.

5.3.2. Embryonic Mice Lacrimal Gland Tissue Isolation

Lacrimal Gland Isolation Embryonic 1 (LG ISO E1) 14.04.2018 3 mice

The cells that were isolated in this experment was used for FACS analysis to see the population profile of the isolated cells and dynamic encapsulation experiments in lacrimal chip. Rest of the cells were frozen as stock for future cell and tissue culture experiments.

Lacrimal Gland Isolation Embryonic 2+3 (LG ISO E2+3) 26.05.18 / 27.05.18 3 mice

Here we treated the harvested lacrimal glands differently and sent to FACS for analysis to see how different treatments are going to affect the population profile.

Lacrimal Gland Isolation Embryonic 4 (LG ISO E4) 24.09.2018

Lacrimal Gland Isolation Embryonic 5 (LG ISO E5) 25.09.2018

Lacrimal Gland Isolation Embryonic 6 (LG ISO E6) 26.09.2018

Lacrimal Gland Isolation Embryonic 7 (LG ISO E7) 25.10.2018

Lacrimal Gland Isolation Embryonic 8 (LG ISO E8) 23.11.2018

Lacrimal Gland Isolation Embryonic 9 (LG ISO E9) 06.02.2019

Lacrimal Gland Isolation Embryonic 10 (LG ISO E10) 15.02.2019

Lacrimal Gland Isolation Embryonic 11 (LG ISO E11) 17.02.2019

All embryonic isolation experiments starting with 4th one are used as tissue part sources for LC culturing and imaging experiments.

5.4. Histological Experiments

To verify that we are dissection the lacrimal gland of adult and embryonic mice properly, we did histological sectioning on dissected parts. Both adult and embryonic lacrimal gland structures can easily be observed at figure 33 and 34. Circular glandular acini and long duct shapes can be spotted in both figures.

5.5. Cell Culture Experiments

In this project all cell culture applications were done to provide cell source for 3D encapsulation experiments. First we used sample NIH/3T3 cells, then we started to isolate our own cells from adult and eventually embryonic mouse lacrimal glands. When we analyzed our cultured cells population distribution as epithelial and mesenchymal at flow cytometry, we realized that mesenchymal cells overcome most of the population and epithelial cells were very low in numbers. After this point we decided to not to culture the isolated cells after isolating them. Instead we tried to separate them at flow cytometry immediately after isolating them.

5.6. Tissue Culture Experiments

Explant culture was done for couple of times to observe the explant culture outcome however results were not satisfying compared to cell culture so we decided to give up on explant culture.

Bio Engineered Tissue Culture experiments were the main part of this thesis. All experiments were conducted on static culture before kinetic culture to eliminate biomaterial, encapsulation and cell/tissue related errors. Prior to primer cell usage we first used sample NIH/3T3 cells on our trials. After verifying the parameters on sample cells we started to use our original cell source, mouse lacrimal gland cells. Majority of adult lacrimal gland cells were not used in our bio engineered tissue culture experiments due to contamination and insufficient cell yield. Cells that used were shown poor encapsulation results due to low cell density and poor proliferation capacity of older cells.

5.6.1. Static Culture

First we conducted static culture experiments to verify our hydrogels biocompatibility, encapsulation parameters safety on our cells. At Figure 27, mouse embryonic lacrimal gland cells that were encapsulated with ring pattern photomask were observed under bright field microscopy. On day 5 culture medium has changed the color (which means it is used) and morphological changes were seen on cells. These were clear signs of cell viability, which meant that our hydrogel, GelMA was biocompatible with our embryonic lacrimal gland cells as well as that our encapsulation parameters (UV power and duration during photocrosslinking) were in the safe range. On figure 35, same 3D construct was dyed with red phalloidin (actin filaments) and blue DAPI (nucleus) on day 10 to better visualize the cell residency in 3D construct.

5.6.2. Dynamic Culture

Following successful static culture, we moved to the kinetic culture, which is the ultimate goal of this thesis. First we showed that we can print (encapsulate) 2 different parts precisely next to each other successfully without using any extra culture equipment. On figure 36, sample NIH/3T3 cells were dyed with red (epithelial) and

green (mesenchymal) phalloidin as well as blue DAPI. With the help of corresponding photomasks, 2 different compartments were printed next to each other at cellular level precision.

After being successful with sample cells, we moved to our true cell source, mouse lacrimal gland cells. At Figure 28 and 29 day 0 and day 7 images of LC v5.0 were observed. Precision of encapsulation can be easily seen before and after the assembly. System was successfully cultured for 14 days until the GelMA structure started to lose its original shape due to biodegradability and distort the flow pattern, which can be seen at figure 30.

With verified working setup, our next step was encapsulating the separated epithelial and mesenchymal cells from mouse embryonic lacrimal gland. However due to flow cytometry related problems we could not be able to successfully sort epithelial and mesencmyal cells. So we concluded on encapsulating whole tissue parts, which were separated as epithelial and mesenchymal during dissection under stereo microscope. In Figure 31 epithelial part of mouse embryonic lacrimal gland can be seen with its compartments like ducts and acini. We encapsulated the parts in their corresponding parts. After successfully encapsulating and culturing tissue parts (Figure 32), we showed that our bioengineered lacrimal gland has its known functional cellular components like AQP5 (water channel protein) and ZO-1 (epithelial tight junction protein). At Figure 37 and 38 comparison between native lacrimal gland and 2 weeks kinetic cultured bioengineered lacrimal gland can be seen. AQP5 and ZO-1 levels of native lacrimal gland are nearly same as our bioengineered lacrimal gland. On figure 39 red ZO-1 can be seen around a cell, which is surrounded by secreted AQP5. Image sharpness difference between native and bioengineered parts occurred due to bioengineered construct being in GelMA (which affects refraction index) and also being thicker than native construct (which creates more background noise than native).

5.7. Flow Cytometry Experiments

For successful epithelial and mesehcnymal compartmentalization in our system, we had to separate epithelial and mesenchymal cells from our mixed population that was isolated from mouse embryonic lacrimal glands. First we wanted to see the population distribution of epithelial and mesenchymal cells that were cultured with conventional methods.

After 4 days mesenchymal cells dominated the population and epithelial cell numbers were very low in ratio (Figure 22). Then we wanted to check epithelial and mesenchymal ratios from uncultured mouse embryonic lacrimal gland cells. We analyzed populations under different conditions at Figure 23, 24, 25 and 26. All of those uncultured populations had higher epithelial cell concentrations as expected.

So after seeing this we concluded on doing direct flow cytometry sorting after isolation experiments. However all of the sorting experiments yielded dramatically low cell numbers for both epithelial and mesenchymal cells. We increased the embryo numbers and pooled multiple mothers but none of them fixed our issue. To prevent further waste of time and embryos, we decided to work with tissue parts instead of cells.

6. CONCLUSION AND FUTURE PERSPECTIVES

As conclusion, in this thesis project, we have successfully developed 3D bioengineering platform created for lacrimal gland tissue culturing. We were able to design a suitable microlfudic system and synthesize a biocompatible hydrogel for this purpose. We maintained the functionality of lacrimal gland tissue parts in our 3D culture platform for 2 weeks. However we failed to separate and culture epithelial and mesenchymal cell sorting issues. Tissue parts also proved to carry lacrimal gland properties. To further support this more functionality tests can be done to see how successful the approach was. Also maintaining a living tissue part was the first step of

aimed approach. Creating a bioengineered lacrimal gland from human stem cells for therapeutic reasons is a long way. System must be tested with differentiation and separate culturing properties. Also system might need some alterations for longer culture span.

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