The human retina is the key element for light detection and plays a pivotal role in our visual system. Retinal damages and diseases significantly hamper the daily life of affected persons, often resulting in severe vision impairments up to blindness. However, no cure or treatment is available for most diseases, although extensive research has been conducted in this field for many years. The main reason for that might be the lack of appropriate model systems replicating the human retina's physiological in vivo architecture and functionality. In recent years, two advanced technologies have emerged as more precise models of the human retina: human retinal organoids (ROs) and Organ-on-Chip (OoC) systems, both representing promising advantages, but still showing limitations for the emulation of a functional retina model. This thesis presents a novel Retina-on-Chip (RoC) by combining ROs and OoC technology to better replicate the human retina. The RoC includes a bottom channel for nutrient flow, a porous membrane on which a monolayer of retinal pigment epithelium is placed, and an RO on top. The initial RoC version proves functionality, but shows restrictions for drug testing, as it is made from a high-absorption material. To address this, the work focuses on fabricating chips from low-absorption materials. Apart from this, several approaches are presented to mimic the physiological tissue environment more closely: The design-adaption to generate a reproducible subretinal space or the integration of biologically inspired membranes. Furthermore, a light housing is presented, that aims to simulate in vivo light conditions. All these methods shall allow flexible module combination to focus on different research questions and can be adapted to their needs. Overall, the new RoC platforms offer versatile possibilities for monitoring events in the retinal tissue in an *in vitro* system, hopefully leading to the reduction in the use of animal models in the long term and providing a relevant tool for testing the efficacy of therapeutics.

Development and implementation of next-generation Retina-on-Chip platforms Johanna Chuchuy



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Next-generation Retina-on-Chip platforms



vorgelegt an der Universität Stuttgart

Development and implementation of next-generation Retina-on-Chip platforms

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Vorgelegt von

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ISBN 978-3-946412-16-8 (D 93 – Dissertation, Universität Stuttgart) "Animal models are wrong more often than they are right" (Don Ingber)

Abstract

The human retina is the key element for light detection and plays a pivotal role in our visual system. Retinal damages and diseases significantly hamper the daily life of affected persons, often resulting in severe vision impairments up to blindness. However, no cure or treatment is available for most diseases, although extensive research has been conducted in this field for many years. The main reason for that might be the lack of appropriate model systems replicating the human retina's physiological *in vivo* architecture and functionality.

In the last decade, new technologies have emerged that can be used as more accurate models to represent the human retina: 1) On the one hand, protocols have been developed to generate human retinal organoids (ROs) based on human induced pluripotent stem cell (hiPSC) technology. ROs can be considered as an attractive potential alternative because all retinal cell types are arranged in a self-assembled sphere-shaped 3D structure, surrounded by photoreceptors. Although these ROs map well the human retina, they still lack the retinal pigment epithelium (RPE) - a monolayer that plays a crucial role for the preservation of a functional retina. Moreover, they are usually cultured statically in cell culture dishes, and lack a continuous exchange of nutrients and metabolic waste products, as given by the blood circulation in the human body. 2) Concurrent with RO development, Organ-on-Chip (OoC) systems - also called microphysiological systems - arose as a powerful alternative to conventional cell culture and animal models. The microfluidic chips house a microphysiological tissue chamber for the smallest functional units of the tissues under investigation. In addition, the tissue chambers are designed according to their physiological environment, so usually the chambers are separated from fluid flow by a vascular-like barrier. However, these systems are mainly cultured with few different cell types up to now, and the replication of a functional human retina (with up to seven cell types) was not possible in these systems so far.

In this thesis, as a concept, the combination of the two advanced technologies is presented, allowing the development of a physiological Retina-on-Chip (RoC). The chip comprises a bottom channel and four tissue compartments separated by a porous synthetic membrane. A confluent monolayer of RPE is cultured on the membrane and a RO is placed on top, enabling the recreation of the full retinal tissue. At the same time, continuous tissue nutrition given by the media flow in the channel below can be guaranteed.

The first chip generation is based on a material that highly absorbs small hydrophobic molecules. Therefore, it can be only used with restrictions for drug

or compound testing. To overcome this limitation, one focus of the work is laid on the chip fabrication from alternative low-absorption materials. A manufacturing process is presented for this purpose, with the integration of a flat membrane being the greatest challenge. A method with supporting structures is established, which allows the integration of a flat membrane without deflection. The efficacy of the method is analyzed by visually checking the membrane flatness. Further, the tightness of the chip is characterized and the lower absorption is demonstrated compared to the first chip version.

In addition, several approaches for chip platforms are established, which can increase the general physiological relevance of the RoC: First, a subretinal space is integrated on-chip, which has dimensions similar to the space in the human eye. For the fabrication, the chip design and chip assembly is slightly modified. A thin spacer layer is placed above the membrane and additionally covered by a coarse, electrospun mesh. This change results in the RO with a diameter of 500-800 µm being held in place on the mesh. At the same time, singularized RPE cells (several microns in diameter) still can pass the coarse mesh reaching the membrane. Therefore, a defined distance between RO and RPE can be realized using this mesh. The distance is measured to confirm the reproducible generation of the space and a robust method.

Besides, the physiological character can be further increased by replacing the synthetic membrane with biologically inspired membranes. Therefore, three types of membranes are chosen and methods for on-chip integration are established. All types of membranes are critically examined. The advantages and drawbacks concerning biological relevance and chip fabrication speed and manual handling are summarized. Overall, it can be argued that the biological relevance might increase using these membranes but it might also slow down the chip fabrication time.

Apart from the physiological improvements on-chip, adding a light source can also improve the chip ambient towards *in vivo*-like conditions. With a light source, the beneficial but also harmful effects of light can be simulated. For this reason, a light housing setup is developed, where it is crucial that no heat is produced by illuminating the chip. For the examination the temperature profile is plotted against time. It demonstrates a constant light exposure without heating the tissue due to light energy. Furthermore, the applicability of the device for RoCs is presented by measuring the harmful impact of high energy light on the retina given by a reduced cell activity.

Finally, when complex, thick tissues are integrated into OoCs, such as the retina, images can only be generated from the bottom tissue layer and the user is limited to extracting information from those tissue layers. For this reason, a new chip fabrication approach is presented in which the tissue chamber is placed close to the chip's edge. Several coordinated treatment steps of the laser-cut edge can create a transparent side window for the tissue chamber. In the next step, the chip can be rotated by 90°, allowing fast acquisition of images from the side without requiring time-consuming z-stack generation. This rapid imaging technique can be performed with live cells, as they are only removed from the incubator for a few minutes, and the cultures can then be continued without harming the tissue. The chip fabrication is presented and imaging accuracy is controlled by comparing images of spheres with a defined diameter from below (conventional imaging) and from the side. Exemplary images from ROs demonstrate the applicability for tissue imaging.

In summary, several platforms are presented in this thesis, allowing the end user to select between platforms depending on the biological question to be answered. In case profound biological experiments should be conducted, the physiological character of the chips can be increased. On the other hand, if experiments are to be carried out with high throughput, the chip production method for chips with low absorption character enables a quick production of robust chips. Overall, the new RoC platforms offer versatile possibilities for monitoring events in the retinal tissue in an *in vitro* system, hopefully leading to the reduction in the use of animal models in the long term and providing a relevant tool for testing the efficacy of therapeutics.

Deutsche Zusammenfassung

Die menschliche Netzhaut (Retina) stellt das Schlüsselemenet zur Erkennung von Licht dar und spielt daher eine zentrale Rolle für das Sehvermögen. Im Falle von Netzhautschäden und -erkrankungen wird der Alltag betroffener Personen erheblich beeinträchtigt, da sich die Schäden/Erkrankungen oft zu schweren Sehbehinderungen entwickeln und bis hin zur vollständigen Erblindung fortschreiten können. Aktuell gibt es für die meisten Krankheiten jedoch noch keine Heilung und nur begrenzt Behandlungstherapien, obwohl seit vielen Jahren umfangreiche Forschung in diesem Bereich betrieben wird. Grund hierfür könnte das Fehlen geeigneter Testsysteme sein, welche die physiologische *in vivo*-Architektur und -Funktionalität der menschlichen Netzhaut nachbilden.

Im letzten Jahrzehnt haben sich allerdings neue Technologien etabliert, die vielversprechende Alternativen zu klassischen in vitro Systemen darstellen und welche die humane Retina genauer abbilden können: 1) Einerseits wurden Protokolle zur Generierung von Retina Organoiden (ROs) entwickelt, welche auf der Verwendung von humanen induzierten pluripotenten Stammzellen (hiPSC) basieren. ROs sind ballförmige 3D-Strukturen, die aus den relevanten Netzhaut-Zelltypen bestehen und dabei in ihrer natürlichen Struktur angeordnet und schließlich von Photorezeptoren ummantelt sind. Aufgrund ihrer Gewebe-Komplexität und der physiologischen Zell-Anordnung sind sie eine attraktive, potenzielle Alternative zu klassischen Zellkulturmodellen. Obwohl diese Organoide vielversprechend sind, da sie die humane Retina gut abbilden, fehlt den Modellen immernoch das retinale Pigmentepithel (RPE), welches wichtige Aufgaben zur Erhaltung einer funktionalen Retina übernimmt. Außerdem werden die Organoide normalerweise statisch in Zellkulturschalen kultiviert. Da der Medienwechsel dort nur alle paar Tage erfolgt, können Nährstoffe und Abfallprodukte nicht kontinuierlich zugeführt und abgetragen werden, wie im menschlichen Körper durch den Blutkreislauf. 2) Zeitgleich zur RO-Entwicklung haben sich ebenso Organ-on-Chip (OoC)-Systeme – auch mikrophysiologische Systeme genannt – als potente Alternative zu herkömmlichen Zellkultur- und Tiermodellen etabliert. Die mikrofluidischen Chips beherbergen normalerweise eine Gewebekammer, in welcher die kleinsten funktionalen Einheiten der zu untersuchenden Gewebe aufgebaut werden. Des Weiteren werden die Gewebekammern so gestaltet, dass sie die physiologische Mikro-Umgebung der jeweiligen Gewebe nachstellen, und so werden sie zum Beispiel meist durch eine gefäßähnliche Barriere vom Flüssigkeitsfluss getrennt. Bislang werden diese Systeme jedoch hauptsächlich mit Co-Kulturen besiedelt, was für die Nachbildung einer funktionalen humanen Retina (bestehend aus bis

zu sieben Haupt-Zelltypen) nicht ausreichend ist.

In dieser Arbeit wird als Konzept eine Kombination der beiden fortschrittlichen Technologien vorgestellt, was es ermöglicht hat einen physiologischen Retinaon-Chip (RoC) zu entwickeln. Der Chip besteht aus einem unteren Kanal und vier Gewebekammern, die durch eine poröse, synthetische Membran getrennt sind. In den Gewebekammern werden zunächst konfluente Lagen aus RPE auf der Membran kultiviert und anschließend ein RO pro Gewebekammer platziert. Dadurch lässt sich das vollständige Retina-Gewebe in einer physiologischen Umgebung abbilden. Zeitgleich wird eine kontinuierliche Gewebe-Ernährung durch den Medienfluss im darunter liegenden Kanal garantiert.

Die erste Chip-Generation basiert auf einem Material, welches die Eigenschaft hat kleine hydrophobe Moleküle zu absorbieren und ist daher nur bedingt geeignet für Medikamenten-tests. Daher liegt ein Fokus der Arbeit auf der Chip-Herstellung aus alternativen Materialien mit geringerer Absorption. Hierfür wird ein Herstellungsprozess vorgestellt, wobei auf die unterschiedlichen Herausforderung eingegangen wird. Zum Beipiel wird eine Methode etabliert, welche die Integration einer flachen Membran ohne Verformung mithilfe von Stützstrukturen ermöglicht. Die Ebenheit der Membran wird im Anschluss optisch überprüft. Die Dichtheit des Chips wird charakterisiert und die geringere Absorption wird im Vergleich zur ersten Chip-Generation nachgewiesen.

Darüber hinaus werden mehrere Ansätze für unterschiedliche Chip-Plattformen vorgestellt, welche die allgemeine physiologische Relevanz des RoCs erhöhen können: Zunächst wird ein subretinaler Abstand in den Chip integriert, welcher ähnliche Dimensionen wie im menschlichen Auge aufweist. Zur Herstellung wird das Design und der Chip-Zusammenbau leicht abgeändert. Es wird eine dünne Distanzschicht oberhalb der Membran platziert und mit einem groben, elektrogesponnenen Netz bedeckt. Diese Änderung führt dazu, dass der RO mit einem Durchmesser von 500 - 800 µm auf dem Netz an Ort und Stelle gehalten wird, die RPE Zellen (wenige µm im Durchmesser) jedoch noch durch das grobe Netz auf die Membranoberfläche gelangen. Gleichzeitig wird dadurch ein definierter Abstand zwischen RO und RPE ermöglicht. Der Abstand wird gemessen, um die reproduzierbare Erzeugung des Abstands und damit einhergehend eine robuste Methode zu bestätigen.

Eine weitere Möglichkeit zur Steigerung des physiologischen Charakters kann durch den Austausch der synthetischen Membran durch eine biologisch inspirierte Membran erfolgen. Es werden drei Arten von bioinspirierten Membranen ausgewählt und Methoden für die On-Chip-Integration etabliert. Alle Arten von Membranen werden kritisch untersucht und Vor- und Nachteile hinsichtlich biologischer Relevanz und Chip-Herstellungsgeschwindigkeit, bzw. manueller Handhabung aufgelistet. Insgesamt kann festgehalten werden, dass die Verwendung solcher Membranen die biologische Relevanz erhöht, aber auch die Chip-Herstellungszeit verlangsamen könnte.

Abgesehen von den physiologischen Verbesserungen im Chip selbst, kann auch die Chip-Umgebung durch das Hinzufügen einer Lichtquelle in Richtung *in vivo*-ähnlicher Bedingungen verbessert werden. Mit einer Lichtquelle können die wohltuenden, aber auch schädlichen Wirkungen des Lichts auf die Retina

untersucht werden. Es wird ein Aufbau für ein Licht-Gehäuse vorgestellt mit dem Fokus die erzeugte Wärme der Lichtquelle zu kontrollieren. Mittels eines Sensors wird der Temperaturverlauf über die Zeit aufgezeichnet und eine konstante Licht-Zufuhr auf das Gewebe demonstriert ohne dieses zu erhitzen. Darüber hinaus wird die Anwendbarkeit des Geräts für die RoC-Systeme demonstriert, indem der schädliche Einfluss hoher Lichtintensitäten auf die Retina nachgestellt wird. Schließlich wird noch ein weiteres Thema fokussiert: Bildgebende Methoden sind derzeit der Goldstandard für die Gewebecharakterisierung auf dem Chip. Wenn jedoch komplexe, dicke Gewebe wie die Netzhaut in OoCs integriert werden, können Bilder nur von der unteren Gewebeschicht erzeugt werden und der Anwender ist darauf beschränkt, ausreichende Informationen aus jenen Gewebeschichten zu gewinnen. Aus diesem Grund wird ein neuer Chip-Herstellungsansatz vorgestellt, bei dem die Gewebekammer nahe an die Chipkante platziert wird. Durch mehrere abgestimmte Behandlungsschritte des lasergeschnittenen Randes kann ein transparentes Seitenfenster für die Gewebekammer erzeugt werden. Im nächsten Schritt kann der Chip um 90° gedreht werden, wodurch Bilder der Gewebe von der Seite schnell aufgenommen werden können, ohne dass zeitaufwändige Bilder in Form von Z-Stapeln generiert werden müssen. Diese schnelle Bildgebungstechnik hat den Vorteil, dass sie an lebenden Zellen durchgeführt werden kann, da diese nur für wenige Minuten aus dem Inkubator genommen werden und im Anschluss deren Kultur fortgesetzt werden kann. In der Arbeit wird die Chipherstellung vorgestellt und die Bildgenauigkeit wird verglichen, indem Bilder von Kugeln mit einem definierten Durchmesser von unten (konventionelle Bildgebung) und von der Seite aufgenommen werden. Schließlich zeigen beispielhafte Bilder von ROs die Anwendbarkeit für die Gewebebildgebung.

Zusammenfassend werden in dieser Arbeit mehrere Plattformen vorgestellt, die es dem Nutzer ermöglichen, je nach biologischer Fragestellung zwischen den Plattformen zu wählen. Sollten profunde biologische Experimente durchgeführt werden, kann der physiologische Charakter der Chips erhöht werden. Wenn hingegen Experimente mit hohem Durchsatz durchgeführt werden sollen, ermöglicht die Chipherstellungsmethode von Chips mit niedrigem Absorptionscharakter eine schnelle Möglichkeit robuste Chips zu produzieren. Insgesamt bieten die neuen RoC-Plattformen vielseitige Optionen zur Überwachung von Ereignissen im Netzhautgewebe in einem *in vitro*-System. Die entwickelten RoC Platformen stellen ein relevantes Werkzeug für die Wirksamkeitsprüfung von Therapeutika dar, was langfristig hoffentlich zu einer Reduzierung des Einsatzes von Tiermodellen führt.

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Abbreviations

AAV	adeno-associated viral vector
AMD	age-related macular degeneration
ATP	a denos intriphos phate
BRB	blood-retina-barrier
BSA	bovine serum albumin
CCC	Collagen cell carrier
cGMP	cyclic guanosine monophosphate
CO_2	carbon dioxide
COC	cyclic olefin copolymer
$\mathbf{C}\mathbf{Q}$	Chloroquine
DNA	deoxyribonucleic acid
DR	diabetic retinopathy
EC	endothelial cell
ECGM	endothelial cell growth medium
ECM	extracellular matrix
ESM	electrospun membrane
EtOH	Ethanol
EUROoCS	European Organ-on-Chip Society
FBS	fetal bovine serum
FEPM	tetrafluoroethylene-propylene
FDA	fluorescein diacetate
FDM	fused deposition modeling
GDP	guanosine diphosphate
$\mathbf{G}\mathbf{M}$	gelatin methacryloyl
GTP	guanosine triphosphate
hESC	human embryonic stem cell
HFIP	1, 1, 1, 3, 3, 3-Hexafluoro-2-propanol

hiPSC	human induced pluripotent stem cell
HUVEC	human umbilical vein endothelial cell
IPA	isopropanol
LED	light emitting diode
$\log P$	n-octanol/water partition coefficient
LSM	laser scanning microscopy
MIP	maximum intensity projection
\mathbf{mvEC}	micro-vascular endothelial cell
NEAA	MEM Non Essential Amino Acids
NSR	neurosensory retina
OCT	optical coherence tomography
OS	outer segments
OoC	Organ-on-Chip
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS-	Phosphate buffered saline without calcium and magnesium
PC	polycarbonate
PCR	polymerase chain reaction
PET	polyethylene terephthalate
PDE6	phosphodiesterase 6
PDMS	polydimethylsiloxane
PFA	paraformaldehyde
PI	propidium iodide
PLA	polylactic acid
POMaC	poly(octamethylene maleate (anhydride) citrate)
PMMA	polymethyl methacrylate
PS	polystyrene
PU	polyurethane
Ra	average roughness
RGB	red-green-blue
ROI	region of interest
RPE	retinal pigment epithelium
RT	room temperature
RO	retinal organoid

RoC	Retina-on-Chip
RP	retinitis pigmentosa
SEBS	styrene-ethylene-butylene-styrene
SEM	scanning electron microscopy
SLA	stereolithography
TEER	transepithelial electrical resistance
T_{g}	glass transition temperature
TPE	Thermoplastic elastomer
UV	ultraviolet
VEGF	vascular endothelial growth factor

Introduction

Vision loss can severely impact affected persons in their daily lives. Leading causes of blindness are retinal diseases [1], whereby age-related macular degeneration (AMD) and diabetic retinopathy (DR) are ranked among the most common forms of diseases in the elderly population or when being affected with diabetes, respectively [2]. AMD, for instance, affects one in eight people over 60 years in developed countries. However, currently, for retinal diseases no cure and only limited treatment exists [3]. The reason, therefore, might be the lack of appropriate model systems - that can be used to study the disease in a first step and later develop treatment options. Currently, animal models still are the gold standard for drug development. However, there are many differences in the architecture and physiology of the human retina compared to the retina in animals. For example, in mammals it is not possible to investigate the cone-rich fovea and macula region, which is responsible for high acuity vision [4].

As animal models do not provide sufficient accuracy to the human retina and also due to ethical concerns, the focus should be shifted towards human-based model systems for the research on retinal diseases. In recent years complex 3D tissue model systems, such as retinal organoids (ROs), were developed and investigated. These organoids highlight the ability to generate the complex tissue architecture of the retina in a structured, *in vivo* like order, based on human cell sources [5]. However, they still lack functional tissue nutrition as provided by the blood flow. One approach to overcome this limitation can be Organ-on-Chip (OoC) (also called microphysiological system) technology. OoCs are microfluidic systems, usually comprising a tissue and a media channel for dynamic fluid flow, separated by a porous membrane. The systems aim for the *in vitro* integration of the minimal functional unit of an organ for different purposes: At first glance, the systems should serve as models for research studies and preclinical drug/compound testing. Looking ahead, they can also be a promising tool in terms of personalized medicine. OoCs have gained a lot of interest in recent years and several research groups have taken afford of extensive development. A few spin-off companies have been founded from mainly academic research groups, such as Mimetas, TissUse, InSphero, or Emulate [6]. The latter highlighted the benefit of OoCs compared to animal models in a publication from 2022 [7], where 870 liver chips were analyzed to predict drug-induced liver injury. Therefore, 27 drugs were tested that either have hepato-toxic effects or no effects. Results showed a specificity of 100%. In conclusion, the chips outperform conventional model systems offering an advantage for drug discovery [7]. Another big step was made

in December 2022, when the legislation concerning the need of animal models for the market introduction of new medicine was changed by President Joe Biden. The new legislation now states that the U.S. Food and Drug Administration can approve drugs without the requirement of animal models but with the use of alternative models, such as computer modeling or OoCs [8].

All this underlines the importance of microphysiological *in vitro* systems. Furthermore, looking at current model systems available for retinal drug development, it becomes clear that the time has come to develop a microphysiological Retina-on-Chip (RoC) system that can display retinal function and which can be used to study diseases.

This thesis introduces a new approach to emulating of the retina in OoC platforms. Focus is laid on the following topics:

- Proof-of-Concept chip development showing tissue functionality on-chip.
- Chip development and fabrication with advanced low-absorption materials.
- Increase of physiological relevance by integrating bioinspired membranes and adding a light source to the chip environment.
- The improvement of imaging capacities of OoCs.

Chapter 1

State of the art & theoretical background

1.1 The role of the human retina in the body

To develop a microphysiological retina system, it is crucial in the first step to understand the basic structure and function of the retina. Vision is one of the most important senses for humans. Within the human eye, light is bundled and focused on the retina, where the visual information from the light starts to be processed [9]. Therefore, the retina is the essential component for vision, where the signaling cascade starts and photon signals are transferred into electrochemical signals and transmitted via the optic nerve to the brain. Finally, in the brain the electric signals are converted into images.

1.1.1 Anatomy and physiology of the retina

The human eye can be divided into anterior and posterior structures, whereby the neural retina and the retinal pigment epithelium (RPE) are components of the posterior segment. The retina can be described as the innermost layer of the eye [13]. It is a complex tissue comprising five main classes of interconnected neuronal cells (Figure 1.1). Three major cell types are vertically connected: **ganglion cells, bipolar cells, photoreceptors** and two additional cell types interconnect the tissue horizontally. The **horizontal cells** - as their name already reveals - horizontally interconnect photoreceptors with bipolar cells. The area where the synapses of the horizontal cells are interconnected with the photoreceptors is also called the outer plexiform layer. Similarly, an inner plexiform layer exists, where **amacrine cells** interconnect bipolar cells and ganglion cells. Besides, Müller glial cells are situated in the neurosensory retina, contributing to the balance of metabolism and homeostasis [14].

Photoreceptors and their signaling cascade After arriving at the retina, light first has to pass the entire retinal tissue until the first reaction to light starts in the back of the retina - where the photoreceptors are situated. The photore-



Figure 1.1 Anatomy of the human retina. Left: Overview, showing the light path in the eyeball reaching the retina. Right: Detailed view of the retinal structure with its 7 main cell types. Inspired by [10], [11], and [12].

ceptors themselves can be broadly classified into rod and cone photoreceptors, whereby only 5 % of the photoreceptors are cone cells. These are predominantly concentrated at one region in the retina, called the fovea, and are responsible for daylight, high acuity color vision [15, 16]. The cones are less sensitive to light than rods and have a faster response to light, as they process bright signals and, therefore, can recognize fast light variations [16, 17]. They can be further distinguished by being sensitive to different wavelengths of visible light, such as mid and long-wavelength light (green and red) and short-wavelength light (blue) [17]. Rod photoreceptors, on the other hand, are responsible for dim-light vision [14].

Concerning the structure of the photoreceptors, they have a unique elongated shape. They can be categorized into five main components: outer segments (OS), which in the case of rods comprise highly packed disk membranes enclosed by a plasma membrane. The plasma membrane contains ion channels open in a dark environment, constantly releasing the neurotransmitter glutamate. Next to the OS are connecting cilia, inner segments with mitochondria and endoplasmatic reticulum, nuclear region, and the synaptic region responsible for transporting the electrochemical signal to the adjacent cells [15]. Therefore, the photoreceptors play a pivotal role in detecting and transporting light information. In detail, to gain the electrochemical signal from the light photons, the light photons will be converted in the photoreceptor OS - this event is called **phototransduction** [9]. In the membrane disks photopigments are located, which comprise an apoprotein (opsin) and a chromophore molecule (11-cis-retinal in the case of rods, which is a chromophore in the dark state). Once light reacts with the retinal, it becomes light-isomerized converting from 11-cis to an activated form of rhodopsin, called all-trans-retinal [18].

As a result, all-*trans*-retinal uncouples from the opsin due to low binding affinity [9] and temporarily binds to the G-protein transducin by activating the transformation of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) [18]. The activation of the G-protein containing α , β , and γ subunits allows the separation of the α subunit bound to GTP from the β and γ subunits. The GTP α then can activate the enzyme phosphodiesterase 6 (PDE6). This further releases the hydrolysis of cyclic guanosine monophosphate (cGMP), leading to a reduction in the cGMP level, which then causes the closure of cation permeable channels in the plasma membrane of the OS [9]. As a consequence, the influx of Na^+ and Ca^{2+} will be blocked and hyperpolarization in the cells occurs as long as the light continues shining on them, which eventually inhibits the release of the neurotransmitter glutamate in the synaptic region of the photoreceptors [15]. In this region, both rods and cones directly connect with the bipolar cells. These cell types are responsible to transport the nerve signals towards the ganglion cells. Generally, bipolar cells can be divided into two classes of bipolar cells, cone, and rod bipolar cells. Further, depending on the signal information they can be distinguished into ON and OFF bipolar cells, whereby ON bipolar cells depolarize in response to light (rod bipolar cells) and can detect light areas on dark backgrounds and OFF cells hyperpolarize, detecting dark areas on light backgrounds [16]. Cone bipolar cells can be either ON or OFF, in contrast to rod bipolar cells [14]. The synaptic information is then further modulated by horizontal cells.

As a next signal transmission, the bipolar cells inform the retinal ganglion cells, whereby amacrine cells can provide additional information, further defining the information that already has been modulated by the horizontal cells [16]. In the last step, the ganglion cells bundle to an optic nerve and transmit the visual information to the brain.

Retinal pigment epithelium The RPE is situated directly beneath the neural retina, adjacent to the photoreceptors, as a tightly packed monolayer of hexagonally shaped cuboidal epithelial cells. Although seeming rather to be relatively simple at a first glance in contrast to the the neurosensory retina, the RPE plays a pivotal role in the preservation of retinal functionality [19]. As its name already reveals, the tissue contains pigments responsible for the absorption of scattered light, preventing light deflection and also damage from too high light energy levels [16, 19, 20]. The pigmentation in the RPE exists due to the melanin that is synthesized and housed in the melanosomes of the RPE [19]. Besides light absorption, pigmentation prevents photo-oxidation, whereby different pigments protect against wavelengths [21].

Apart from light protection, the RPE acts as a barrier layer responsible for the transepithelial transport of nutrients and metabolites. One of its concrete functions is the transport of water and ions from the subretinal space to the choroid to ensure a functional interplay of photoreceptors with the RPE. Water is thereby produced due to the metabolic turnover in neurons and photoreceptors [21]. Further, the RPE monolayer is polarized, containing apical and basal sides. The apical surface is covered with microvilli that envelop the OS of the photoreceptors enabling direct communication [19]. Moreover, the apical tight junctions of RPE contribute to maintaining polarity by forming a barrier limiting diffusion between apical and lateral membrane domains. On the basolateral side, RPE sits on a specialized basement membrane, called Bruch's membrane, that separates the vascularized choroid from the RPE. The RPE is responsible for transporting vascular endothelial growth factor (VEGF)- α to the choroid layer below. The VEGF- α is a crucial survival factor for endothelial cells, modulating vascular permeability and angiogenesis [19].

During phototransduction, the chromophore 11-*cis*-retinal in the photoreceptors is isomerized into all-*trans*-retinal and has to be transformed back into its original conformation after light exposure. Part of the re-isomerization is performed by the RPE. This metabolic process that enables visual pigment regeneration after light exposure is also called the visual cycle [21]. In the photoreceptors, all-*trans*retinal is transformed to all-*trans*-retinol through an enzyme-mediated reduction and transported to the RPE, where it is transformed back into 11-*cis*-retinal to be delivered back to the photoreceptors [19]. This event highlights the close and important interplay between photoreceptors and adjacent RPE layer for a functional vision [21].

Another crucial function of the RPE is the phagocytosis of photoreceptor OS. Light exposure produces photo-oxidative products in the photoreceptors, which can have toxic effects if accumulated. With the daily renewal of OS tips, the photoreceptors can circumvent an accumulation of photo-oxidative products. Furthermore, the fast renewal of OS tips enables the preservation of high photosensitivity. The RPE phagocytoses the waste products in form of shed OS in the form of engulfment and degradation of the material [22]. One single RPE cell can be in contact with around 30 photoreceptor cells, and up to 10% of OS are internalized daily by the RPE [11]. The ingested OS are encapsulated by the phagosomes within the RPE cell and degradation takes place in several steps: Reaction with an early endosome, late endosome, and finally fusion with a lysosome to be fully enzymatically degraded [23].

Bruch's membrane As already mentioned, the RPE is adjacent to the Bruch's membrane on the basolateral side. The Bruch's membrane is an extracellular matrix (ECM) composed of five stacked layers [24] with a thickness of around $2-4 \mu m$ [25]. The five layers consist of the RPE basement membrane, the inner collagenous layer, the elastin layer, the outer collagenous layer, and lastly, the choroicapillaris basement membrane [26]. The full membrane generally has two main functions: On the one hand, it separates the RPE from the vascularenriched choroid layer. On the other hand, it serves as a substrate for the RPE cells' adhesion. Due to the differences in intraocular pressure, the membrane is stretched reversibly and therefore it requires elasticity [26]. The human Bruch's membrane has a Youngs Modulus around 7-19 MPa [27]. Besides the structural role, Bruch's membrane also fulfills transport functions. For a continuous supply of the retina, oxygen, electrolytes, nutrients and cytokines are transported from the choroid towards the RPE and the photoreceptors. At the same time, waste products are transported back from the retina side, as well as the excessive water from the subretinal space [25]. These different aspects demonstrate that all cell types in the retina and choroid interact closely, and tissue function stems from a

synergistic interplay of all individual components.

1.1.2 Retinal diseases affecting the outer retina

In general, most retinal diseases disturb photoreceptor function directly or indirectly [14]. A variety of diseases affect the retina, whereby AMD, DR and retinitis pigmentosa (RP) are thereby the most frequent causes of visual impairment, leading to blindness [20].

Age-related macular degeneration The macula is situated in the back of the eye and comprises the fovea with its highly packed cone photoreceptors. The degeneration of this tissue area due to the loss of RPE and photoreceptors leads to a loss of vision acuity, thereby reducing life quality of affected persons [28, 29]. Especially in developed countries, AMD is a major cause of vision loss [30, 31]. In an early stage, AMD evolves due to an accumulation of undigested waste material caused by oxidative damage, for instance [32]. This leads to a drusen formation, that usually lies in accumulated debris between RPE and Bruch's membrane, accompanied by a reduction of the function and later loss of RPE [29]. In an advanced stage, AMD can be distinguished into two different forms of AMD: wet (neovascular/exudative) AMD and dry (atrophic) AMD, which is the predominant form of the disease [29, 33]. In dry AMD, the RPE declines, which in turn leads to the degeneration of photoreceptors. In wet AMD, an excessive angiogenic sprout formation in the choroid forms can disrupt the RPE layer. In this way, the RPE - photoreceptors interaction is disrupted [31, 33]. Currently there are no treatment options available for dry AMD. A rather invasive solution could be transplantation therapies, which are currently in clinical trials [29]. For wet AMD, intravitreal injection of drugs suppressing VEGF can be used to retard/stop this form of degeneration [30]. In general, AMD is a mulitactorial disease, whereby age is the most critical risk factor. Besides, genetic variations can support the pathogenesis, smoking, or low dietary intake of antioxidants [31, 32].

Diabetic Retinopathy Diabetes mellitus has become a major societal problem with increasing prevalence [34]. Often, the disease is accompanied by hyperglycemia. Further progression to chronic hyperglycemia can also lead to DR, a disease that causes microvascular abnormalities and retinal neurodegeneration. In an advanced state, DR can result in central vision loss [33]. In the early stage, the **non-proliferative DR** is characterized by a loss of pericytes in the choroid, which comes with forming acellular capillaries and hence an increase of vascular permeability. Thereby, the functionality of the inner blood-retina-barrier cannot be guaranteed anymore. However, at this stage the disease does not show any symptoms to the patient. At the advanced stage - the **proliferative DR** new blood vessels are formed, which disrupt the barrier of the RPE, similar to AMD, and proper retina nutrition cannot be provided anymore [33, 35]. DR has multifactorial causes and its pathophysiology is not fully understood yet, not least of all due to the lack of human-relevant *in vitro* models [33]. **Retinitis pigmentosa** Besides these multifactorial retinal diseases inherited diseases, such as RP, that come along with the dystrophy of rod and cone photoreceptors are of major interest. One of the first symptoms is night blindness and progressive loss of peripheral vision during the day. The pathogenesis of the disease starts with pigment deposits mainly in the peripheral retina. In the first stage, rods start degenerating, followed by the loss of cones. This also explains the symptoms starting with night blindness [36]. So far, the therapeutic approach for this disease is limited to taking up vitamin A, and/or vitamin E (vitamonotherapy) or sunlight protection to slow down the degeneration [36, 37].

1.2 Retina model systems for biomedical research and drug development

As explained in 1.1.2, many retinal diseases are not fully understood yet; for most, there exists no cure and just limited treatments. Therefore, the need for new model systems for drug research becomes obvious. Besides conventional model systems, research is now focusing on advanced 3D model systems highlighting more complex tissues. Traditional and advanced model systems will be presented in this section to provide an overview of model systems currently existing in the ophthalmic field and to highlight advantages and limitations.

This section is based on a review of the different model systems published previously by Haderspeck^{*}, **Chuchuy^{*}** *et al.* [13].

1.2.1 Conventional model systems

To overcome the lack of understanding of retinal pathologies, animals are commonly employed. However, it should be considered that animals have different physiology; hence, they do not fully emulate human diseases. Further, there is societal pressure to reduce animal models as much as possible (3R principle) [32]. In general, model systems are categorized into three main groups: *in vivo*, *in vitro*, and *ex vivo* model systems. Apart from the three main model systems, mathematical and computational models are available, nicely summarized by Roberts *et al.* [38].

In vivo animal models One approach for the investigation of the retina is the use of animal models. The models can give a first insight into the potential success of new therapeutic approaches [39]. However, to gain appropriate information from an *in vivo* experiment, the animal model has to be chosen carefully to enable a translation from the animal to a human. This does not mean that the model has to replicate the human in its entirety, but it has to replicate the tissues of interest and mechanisms [39]. One of the most commonly used (disease) models in the ophthalmic field is the mouse, wild-type mice, or gen-manipulated ones, which can evolve certain diseases or degeneration [32, 39]. Besides that, light can also be applied to wild-type mice to induce retinal degeneration or photoreceptor death [39, 40]. Animal models are favored especially in terms of

their physiological complexity, involving inter-organ and tissue-tissue crosstalks, as well as a functional blood circulation that transports different compounds such as hormones or immune cells [20]. Nevertheless, mice do not exhibit a macula and mainly contain rod photoreceptors [39]. Almost all mammalian species show significant retinal differences compared to humans with exception of higher primates. Therefore it must be considered, that - even if drugs are successful in animal models - the translation of drugs to humans still can fail [32]. Also, alternative approaches should be considered before using animal models for ethical reasons.

Ex vivo explant cultures One alternative to animal models can be (human) retinal tissue explants, which are a promising model for translational research comprising the complex human intercellular interactions close to *in vivo* conditions (as reviewed in [41]). Usually, post-mortem human donor eye cups are used for this purpose. In less common cases, diseased human retinal tissues can also be obtained from living donors due to surgical extraction. As the tissue is received from an affected patient, it can give a conclusion about retinal pathologies - one standalone advantage with consideration that the full medical history is documented and can provide additional useful information. Further, retinal disorders often develop in aged persons; therefore, it is an evident advantage to use post-mortem explants gained from donors of advanced years, in contrast to young animal models [41]. However, the main limitation of using these models is their restricted availability. Research laboratories need to be located close to eye banks or hospitals (if using tissues from live donors). As availability cannot be predicted, experiments have to be adapted in case of low availability [41].

In vitro cultures The simplest approach for fundamental retinal research might be cell culture. In conventional *in vitro* cultures, single-cell types are cultured in a dish to examine biological questions concerning the retina. The advantages compared to the above mentioned model systems are their high reproducibility, reduced costs, simple handling, high throughput of results, and rapid readouts [13, 32].

Generally, two types of cell sources are used: Cell lines and primary cells. **Cell lines** were immortalized either with the help of chemicals or viruses or were established from human tumor cells [42, 43]. This cell source offers opportunities such as cost-effectiveness and an easy use. Further, cells usually can be cultured up to high passages, as they do not stop proliferation [44]. On the other hand, they are genetically manipulated; therefore, genotypic and phenotypic variation might occur and the cells can become tumorigenic [42, 44]. One major limitation is the cross-contamination of cell lines with other cell types. Consequently, in 2010 a list of 360 cell lines from cross-contaminated cells was published to provide an overview for researchers [45]. For this reason, cell lines should be verified for their correct phenotype before performing experiments [32]. **Primary cells** can be used as an alternative, comprising physiological characteristics closer to the human tissue than immortalized cell lines, as they are isolated from a (healthy) human tissue. Apparently, by using isolated tissues, its access can be limited, and inter-donor variation can occur [46]. Apart from this, the cells only have a restricted cell division; therefore, if they proliferate at all, usage beyond the third or fourth passage is not recommended, as the cells then tend to lose their cell characteristics [42].

1.2.2 Next generation model systems

In recent years the development of advanced biological *in vitro* model systems has shown great progress. With the introduction of stem cell technology new opportunities arose, which can be summarized as the following:

Adherent stem cell technology An advanced alternative to conventional *in vitro* models can be stem cell-based *in vitro* model systems. Stem cell research has been widely focused on retinal tissue transplantation or repair. In retinal research, most stem cells are generated from either human embryos or reprogrammed adult somatic cells [32]. Human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) are pluripotent stem cells - meaning that they have the potency to self-renew and to differentiate into all cell types of a developed organism [5]. However, hESCs are obtained from embryos after *in vitro* fertilization; hence, ethical issues and low availability are involved when using these cell types.

In contrast, hiPSCs offer an elegant and easily accessible alternative, as these cells can be generated from almost any somatic cell, such as blood cells, fibroblasts, keratinocytes or muscle cells [5, 32]. Apart from the advantages such as easy and unethical availability, tissues from individuals can be generated, a powerful tool for (stem) cell replacement therapies or disease modeling. With conventional monolayer differentiation several different retinal cell types can be generated, but they are still restricted to two-dimensionality and do not comprise the full complexity of the retinal tissue architecture [5]. To enable increased tissue complexity, 3D 'mini-organs', so-called organoids, can be generated with the knowledge gained from stem cell research.

3D retinal organoids Nowadays, organoid technology has enabled the opportunity to investigate the retina's complex and highly organized tissue architecture. In brief, organoids are self-organizing, multicellular 3D tissues derived from stem cells, that exhibit similar functions and morphology as human tissues and organs [5, 47]. For RO generation, currently two protocols are typically applied (reviewed in [5]): In 2012, Nakano *et al.* published a protocol for the development of ROs generated from embryoid bodies using hESCs [48]. Later, in 2014, Zhong *et al.* achieved organoid formation from adherent hiPSC culture, where selected detachment of retinal fields yielded the formation of 3D structures [49]. This group also was the first to show successful maturation of OS formation and light response from the retinal organoids [5]. ROs comprise the full range of cells from the neural retina and can be generated from human cell origin, offering several advantages compared to animal models or 2D cell cultures. However, the ROs still are missing RPE in a physiological arrangement, which is essential for the healthy maintenance of photoreceptors. Compared to animal models, all models discussed so far lack physiological nutrition and metabolic exchange through continuous blood circulation in humans (or animals). Advanced model systems which overcome these limitations can be generated via OoC technology.

1.3 Organ-on-Chips

OoCs are microfluidic devices that incorporate cell chambers and can be continuously perfused. These systems aim to recreate minimal functional units of organs of interest instead of whole organs [50]. In a simple setup, the systems contain a tissue chamber connected to a perfused microchannel. Besides individualized tissue arrangement within the chambers, physiological relevance can be increased by adding controlled physical forces to the tissues, such as a cyclic strain or mechanical compression [50]. Further, the device enables the characterization of cultured tissue via several assays, for example real-time monitoring of the cells on-chip, terminal analyses, and off-chip assays [51].

A general definition has been provided by the European Organ-on-Chip Society (EUROoCS), describing an OoC as "a fit-for-purpose microfluidic device, containing living engineered organ substructures in a controlled microenvironment. that recapitulates one or more aspects of the organ's dynamics, functionality and (patho-)physiological response in vivo under real-time monitoring" [52]. Huh et a al. published one of the first systems, a lung-on-a-chip, in 2010 [53]. With a flexible membrane between two channels and two vacuum chambers on each side, it was possible to stretch the membrane, emulating the breathing movements in the lung. With that model, the group could highlight the need for a physiological environment for the cells, as cells displayed morphological and functional differences compared to being placed on a static membrane. Since that, the field of OoC has grown tremendously, and nowadays, the first proof-of-concept chips were even translated into industry [51]. The fast growth of OoC in this field shows the demand for new model systems. As a first step for the development of each OoC, it is not only required to know the biological background of the respective tissue but also to consider the different fabrication methods. Therefore, the following sections will summarize the key aspects of chip fabrication.

1.3.1 Material selection and chip fabrication methods

Usually, the development of an OoC system starts with the chip design and chip fabrication. Therefore, it is essential to choose an appropriate material depending on different criteria based on the targeted future applications, such as the type of experiments or scalability. This subsection shall give an overview of existing possibilities for the use of materials for chip fabrication, focusing on their advantages and drawbacks (also reviewed in [54, 55]).

Elastomers To date, the gold standard for OoC fabrication is the use of a polymer called polydimethylsiloxane (PDMS) as the main material. It has

several advantages, such as elasticity, optical transparency, biocompatibility, and oxygen permeability, and it is relatively low-cost [54]. OoCs made of PDMS usually are produced by soft lithography. The term 'chip' thereby originates from the fabrication method, where a modified form of photolithographic etching on a silicon wafer is applied to produce thin features (mainly channels and chambers) in an nm to µm range. Initially, photolithographic etching was used as a method applied for the manufacture of computer microchips [50]. In the case of OoC production, a silicon wafer is first covered with a photoresist, on which a photomask is placed, containing the lateral dimensions of the desired structures. When applying ultraviolet light on the wafer, covered by the mask, usually local cross-linking occurs, and the structures remain on the wafer after washing it and removing excessive photoresist. The negative structures can be obtained by pouring PDMS onto the wafer [50]. Inlets to access the channels can be punched through the material using a biopsy punch. Finally, a membrane can be integrated between multiple structured PDMS slabs and bonded tightly together using oxygen plasma. Although PDMS is used in the majority of OoC devices, it comes with unselective absorption of small (hydrophobic) molecules due to its highly lipophilic character [52, 56]. This leads to the conclusion that for developing a first concept or preliminary studies, PDMS-based OoCs can be the material of choice. In contrast, alternative materials for the chip should be considered when drug experiments should be performed.

Besides PDMS, there are a couple of alternative elastomers available that have been used already for OoC systems: Polyester elastomers, tetrafluoroethylenepropylene (FEPM) elastomers, and poly(octamethylene maleate (anhydride) citrate) (POMaC) polymers can offer alternatives with lower absorption character. They have gained attraction due to their ease of fabrication and low costs [54, 55]. Apart from this, surface treatments can reduce absorption but involve an additional fabrication step increasing the fabrication time. Therefore, scaling up the process might be challenging.

Thermoplastics When materials of high resistance towards molecule absorption are needed, OoC developers usually consider thermoplastics. This class of materials is characterized by optical transparency, low water absorption, biocompatibility, low-cost, and rigidity [55]. Common thermoplastics used in the field of OoC are polymethyl methacrylate (PMMA), cyclic olefin copolymer (COC), polystyrene (PS), and polycarbonate (PC) [54, 55]. The rigid thermoplastics soften above the glass transition temperature (T_g). They can be fully reshaped without losing chemical stability [57]. Furthermore, high temperature and pressure can be applied, offering various structuring and fabrication techniques, including replication methods, such as hot embossing, injection molding, or thermoforming. At the same time, this enables mass fabrication on a large scale [54, 57]. Another way for microstructuring is lasercutting and mechanical milling. Once the channels and chambers are structured into the material, a final bonding with a capping layer is required to close the open channels. For bonding, three main options are frequently used:

1) During **thermal fusion bonding** materials are heated to a temperature in the region of the T_g and pressed together. With both pressure and temperature, the material partially melts up, so that interdiffusion of polymer chains between the surfaces from both or more materials can be achieved. In the best case, with this method, the final chip contains the original cohesive strength [57]. This bonding method should be used, when the same materials or materials with similar T_g are selected [57]. The challenge when using thermal fusion bonding can be channel deformation when temperature, pressure, and time are not coordinated precisely. Furthermore, even if commercially available materials are used, the parameters might need to be adjusted again depending on the polymer batch [57].

2) The **solvent bonding** method relies on the surface treatment of thermoplastics with an appropriate solvent (e.g., ethanol or isopropanol) that initiates an increase of the polymer chain mobility on the surface of the materials. As a result, the bonded parts are tightly welded together after the evaporation of the solvent [58]. Initially, the process might appear simple, but generating a tight chip bonding without a clogging of the channels due to residual solvent inside can be a big challenge [58]. The remaining solvent can also cause toxic reactions to the tissue that shall be cultured later in the chip.

3) Finally, the chip parts can also be bonded using adhesives between the layers (adhesive bonding). Thereby, different types of glues or epoxies can be added as an additional layer between the materials. As the adhesive remains after bonding, it is accompanied by the term 'indirect bonding' [57]. Considering that, special attention has to be paid on the biocompatibility of the glue [58].

To summarize, thermoplastic materials should be used, when low absorption properties and a large number of relatively inexpensive chips are needed. However, one has to be aware that the material is less gas permeable, which might be problematic for cell survival depending on the chip design. Furthermore, bonding these materials involves complex techniques, and the definition of parameters might vary from material charges [59].

Thermoplastic elastomers Thermoplastic elastomers (TPEs) can be chosen as promising alternatives when the properties of both elastomers and thermoplastics are required. Usually, this class of materials is biocompatible, transparent, and elastic. TPEs are commercially available as extruded sheets, providing an advantage over PDMS, as the pre-compounding step can be skipped [60]. With the features of both material classes, TPE can be processed similarly to thermoplastics (processing via hot embossing, injection molding). Still, at the same time, the material provides the properties of vulcanized rubber with a high elasticity [60, 61]. The TPE is based on organized block copolymers generally arranged in an 'A-B-A structure', whereby A stands for thermoplastic block copolymer and B is the elastomeric block copolymer [61]. A range of different TPE types exists, such as styrene block polymers, co-polyesters, polyamide/elastomer copolymers, and polyurethane/elastomer block copolymers [62]. Noteworthy in the field of microfluidics is styrene-ethylene-butylene-styrene (SEBS), a sub-type of TPE, that is a combination of PS domains in the elastomeric ethylene-butylene phase [58, 60, 63]. Especially with a focus on drug absorption, oil-free SEBS should be chosen [63]. In addition to the simple processing of TPE itself, it is notable that is has a sticky, self-adhesive surface, that enables fast and easy bonding to other materials or another TPE layer [58]. Overall, the properties of TPEs make it an attractive material for developing of OoC systems.

1.3.2 Membranes

In most OoCs, a porous membrane is integrated as a tissue substrate and to separate media from tissue channels. Thereby, it aims to mimic the vascular-like barrier or ECM. It is often covered with endothelial cells (ECs) from the side where media flow is applied to increase the physiological relevance [64]. One of the main functions of the porous membrane is the separation of transport processes, which means that convective fluid flow remains in the perfused channel. Still, at the same time, the membrane enables diffusive transport of nutrients and metabolites [65].

In OoCs, most membranes are made of PDMS, PC, and polyethylene terephthalate (PET) [64]. The choice of the membrane mostly depends on practical reasons rather than physiology. PDMS is used if stretchable membranes are needed as it features high transparency and elasticity, resisting deformations up to many cycles. Due to its hydrophobic character, the membrane is not ideal for cell adhesion, but coating the membrane with proteins can improve cell adhesion. Usually, this type of membrane is fabricated in-house, allowing for flexible customization of the membrane's mechanical properties [64]. Besides PDMS, PET and PC offer alternative transparent, inert and non - biodegradable membranes. They are commercially available and integrated with several OoCs. However, these membranes are very stiff and cannot be used for mechanical stimulation, such as stretching. Further, they also need to be treated for improved cell adhesion. In general, almost all employed membranes consist of restricted planar 2D surfaces, which barely emulate the 3D structure of the native ECM [64]. Alternatives with increased physiological character can be membranes from natural biomaterials. Vitrified collagen membranes [66] or stretchable collagen-elastin membranes [67] have, for instance, already been successfully integrated in OoC devices. They have the advantage that with collagen being a major component of the native ECM, they can better mimic the physiological microenvironment and that cells are adhering and growing on these materials without the need of any additional coating. Furthermore, hydrogels can also be integrated as a kind of membrane, which vary from protein-based hydrogels, such as collagen, gelatin, fibrin, or hyaluronic acid, to polysaccharide-based hydrogels comprising chitosan or alginate. In this field, the OrganoPlate[®] from the company Mimetas (https://www.mimetas.com/en/home/) is a pioneer: A hydrogel barrier is added, for instance, in the middle channel of three overall channels, and by this, a separation of the external channels from each other can be created. Two ledges on the bottom edges of the middle channel thereby prevent pouring of the hydrogel into the adjacent channels. The system allows for the development of very different model systems using various arrangements of the hydrogel. Nonetheless, one must be aware that membranes based on biomaterials stem from natural sources, meaning that batch-to-batch variations can occur. As a consequence, results can significantly differ. Moreover, sterilization can be challenging as well as membrane integration due to potential protein denaturation when using chemicals or high temperatures [68]. For higher reproducibility, synthetic hydrogels could be applied, but in this case, cell adhesion sites are missing, and additional treatments are needed before cells can be integrated. Besides hydrogels, electrospun membranes can be integrated to overcome the limitations of commonly used membranes. Electrospun membranes are thin 3D scaffolds formed by several randomly overlaying micro- and nanofibers [69]. This kind of 3D arrangement results in a high surface-to-volume ratio, emulating the characteristics of the native ECM [70]. The fabrication technique allows to tailor the properties of the membrane by being able to adjust fiber diameter, porosity, mechanical strength, and thickness. Moreover, the characteristics of the membrane can be adjusted by choosing from a variety of polymers ranging from synthetic to biological materials or even by creating a combination of both. Due to these advantages, electrospun membranes have already been widely explored for transplantation and regenerative medicine, even as substrates for retinal transplantation [71–73]. However, in the field of OoC technology so far, only a few chips have been presented that contain this type of membrane. The reason, therefore, might be the difficulties arising with the membrane integration in a tight way. The few approaches that show the successful integration of electrospun membranes into chips are simply clamping them between PDMS modules [73]. In sum, the demand exists to increase the physiological character of chip membranes to closer emulate the characteristics of the native ECM. This can be realized by using alternative membranes. Nevertheless, one always has to be aware of the advantages and drawbacks of each type of membrane, and depending on the main research focus of the chip, compromises have to be made - similar to the chip material itself.

1.3.3 Organ-on-Chips emulating the retina

OoCs offer a great potential alternative to currently existing animal models in the ophthalmic field. As the chips enable the integration of multilayered tissues and provide vasculature-like perfusion they are an appropriate platform for the generation of a multi-cellular retinal tissue that is completed by the barrier formed by RPE and endothelial cells, also called the blood-retina barrier [74].

OoCs emulating the blood-retina-barrier Considering that most degeneration of retinal diseases starts in the region of the blood-retina-barrier (BRB), it is a logical consequence, that researchers are putting emphasis, especially on the development of a system that includes at least the RPE layer and the EC layer. In 2014, one of the first microfluidic systems, co-culturing human RPE and EC lines, was presented at the microTAS conference [75]. To build up the model, the authors fabricated a two-channel chip separated by a porous membrane - both made from PDMS. On the upper side of the membrane, they seeded ARPE-19,
an RPE cell line, and on the bottom side human umbilical vein endothelial cells (HUVECs). The group characterized the tissue by measuring higher VEGF levels on the basolateral side (HUVEC compartment), highlighting the physiological behavior of the cells, as this event can also be found in the native BRB. Further, they showed that a cell migration of HUVECs through the pores of the membranes with a diameter of 10 µm can be driven with the flow of VEGF-rich medium of the adjacent side of the HUVEC compartment. In 2017, the same group published a paper on this first model with the goal to investigate AMD pathology [76]. Since in AMD, hypoxia leads to an increased VEGF production of RPE, hypoxic conditions were initiated by exposing the chip to cobalt chloride (CoCl₂). The treatment with CoCl₂ caused a lowered glucose level, leading to an increase in VEGF secretion compared to the control. As further consequence, an alteration in HUVEC directional growth was observed [76].

Later, another model system that specifically focuses on the investigation of angiogenic sprouting of ECs was developed by Chung et al. [77]. Their overall goal was to model wet-AMD on a chip by integrating an RPE layer and a choroid consisting of endothelial blood vessel sprouts. To enable the emulation of the angiogenic sprouting without the obstacle of a conventional membrane, the chip was designed with two central and two lateral channels separated by an array of microposts: The lateral channels mainly served as media supply, and in one of the central channels, a mixture of ECs (HUVECs) and fibrin gel was added, emulating the choroid. The blood vessels formed with the help of fibroblasts that were injected into the lateral channels. The second central channel designed with a width of 200 µm, was only filled with fibrin gel to form an ECM-like layer that should imitate the Bruch's membrane (gap channel). On the lateral channel touching the gap channel, RPE cells (ARPE-19 cells) were seeded, adhered to the fibrin surface, and formed a monolayer. In contrast to the previous model system, a hydrogel was used to replace a PDMS membrane in this case. This enables the angiogenic growth towards the RPE and RPE rupture similar to in AMD, which would not be possible through a conventional membrane allowing direct contact of ECs and epithelial cells only through defined pores. The model was characterized concerning barrier integrity as this feature is a key aspect for a functional healthy BRB. The diseased state could be evoked again by an increase of VEGF in the medium perfused on the side of the RPE. This resulted in a vessel expansion and finally a rupture of RPE evolved by the blood vessel sprouts. Lastly, successful treatment of a diseased system could be demonstrated with the application of bevacizumab, a drug that can be used for the treatment of wet AMD [77].

In the same year, another BRB model was presented by Yeste *et al.* [78]. The model system consists of five central cell compartments and two lateral compartments arranged in parallel with the aim to simplify imaging. To enable cell-cell interaction and paracrine signaling, the compartments are connected with each other via a grid of microgrooves on the bottom layer. In addition, the bottom layer of the chip contains integrated electrodes that can record transepithelial electrical resistance (TEER). In the three central channels, a human neuroblastoma cell line was seeded. In the two neighbouring channels human retinal ECs and

in APRE-19 cells were seeded. TEER measurements, together with permeability assays, confirm the barrier integrity of endothelial and epithelial cells [78]. This model approach can allow multicellular interconnections and the overall system development can be interesting from an engineering perspective. However, it has to be stated that the model itself does not emulate the physiological structure of the BRB.

In 2021, Arik et al. developed another BRB-chip focusing on including clinically relevant read-outs for the translation of experimental results to the pathophysiology of patients [79]. For the chip development, they created an open-top compartment sitting on a straight, rectangular channel - both parts were made from PDMS and separated by a porous polyester membrane. In contrast to the earlier presented models, the channel featured a tubular geometry by filling up the channel with collagen I hydrogel while having placed a centered needle that was later removed. This method, called subtractive micropatterning, enabled the formation of a microvessel by seeding ECs (HUVECs) into this channel. The cells attached easily to the walls due to the collagen content and formed a confluent tube. Furthermore, the vessel diameter could be adapted using needles with different diameters. On the other side - in the upper compartment, RPE (ARPE-19 cell line) was seeded. To validate the chip as a potential system for clinical research, they exposed the model to different concentrations of H_2O_2 to cause oxidative stress. To investigate whether leakage occurred, the group added fluorescence labeled 40 kDa dextran to the media and could see an effect on the diffusion of the dye, depending on the applied dose of H_2O_2 . Further, they tested optical coherence tomography (OCT) for the chips, a non-invasive medical imaging method used for AMD patients to assess the retinal tissue layer and disease course. In the experiment, it was possible to visualize the hydrogel itself (without cells) and also the tissue structure as well as structural defects on the tissue of the treated chips. These readout methods could be an interesting tool for the direct comparison of *in vitro* and *in vivo* tissue [79]. However, conventional imaging methods of the round channel structure require a larger effort, as time-consuming z-stacks are needed.

Lee *et al.* presented another model in 2022, featuring two main improvements over the previous models [80]: First, they highlight their achievement of a distance between RPE and choroid analogously to *in vivo* dimensions (less than 100 µm). Therefore, they only used hydrogel without any external support structures as barrier. In contrast to the previous publications, they were able to generate a smaller hydrogel layer between RPE and choroid by using a chip with a three-channel design. In the central channel, a mixture of fibrin and HUVECs was added. After hydrogel gelation, RPE cells (ARPE-19) were seeded via one side channel and allowed to attach. By seeding optimization, they adjusted the HUVEC cell number and hydrogel density in the central channel which resulted in the reduced distance between HUVECs and RPE. Second, they highlight the creation of a fully vascularized choroid with a perfusable lumen, again closely resembling the *in vivo* architecture. They characterized the tissue morphology and barrier integrity and finally induced an upregulated vascularization by culturing the chip under hypoxic conditions without the addition of any cytokines. This is the same method used to provoke choroidal neovascularization in animal models and hence could be an appropriate model system for disease investigations [80]. Finally, another complex immunocompetent Choroid-on-Chip was presented by Cipriano et al. [81]. This system differs from the previously presented model systems in some aspects: It is the only system that integrates immune cells that are circulating during the perfusion. Furthermore, the system also included another stromal cell types, namely melanocytes, which are present in the choroid around the blood vessels. Another unique aspect of the system is that it doesn't use cell lines - the model is based on either hiPSCs (RPE) or human primary cells (micro-vascular endothelial cells (mvECs), freshly isolated peripheral blood mononuclear cells (PBMCs), primary melanocytes), which can better resemble in vivo physiology than cell lines (see 1.2.1). Concerning the chip design, it has three overlaying channels that are separated by two porous PET membranes. For the fabrication, PDMS was used as bulk material. In the top channel, a confluent layer of RPE was generated, and below, in the central channel, an EC-layer was tightly formed on both sides of the membrane. The bottom channel served for the culture of melanocytes. With the perfusion of immune cells through the EC compartment, it was possible to emulate immune cell recruitment into the melanocyte compartment and monitor cytokine release. Finally, the applicability of the chip for drug testing was demonstrated by exposing it to Cyclosporine A, a well-known immunosuppressor [81].

OoCs emulating the neural retina The disruption of the RPE barrier is one of the events happening in many diseases. Still, the actual symptoms typically stem from the degeneration of photoreceptors, a big reason why these cell types should not be neglected in retinal OoC development. Furthermore, congenital diseases can start with the loss of photoreceptors. Hence, for studying these diseases the integration of the neural retina is pivotal. However, the physiological emulation of the neural retina on-chip is certainly more challenging than developing a BRB model, as the neural retinal tissue comprises multicellular structures interconnected horizontally and vertically. Therefore, solely few researchers have faced the challenge of developing such a system. Su et al. developed a system to study retinal synaptic regeneration [82]. Retinal precursor cells were embedded in a microfluidic PDMS chip that comprises two parallel channels interconnected with an array of microchannels. The two main channels were used for culturing two independent cell populations derived from postnatal mouse retina and seeded separately into the channels. After three days, the formation of axons through the microchannels could be observed, which grew from one population towards the other and developed a network of physiologically functional orientated synapses. For the characterization of the platform, the group developed an automated imaging method to quantify the kinetics of cell regeneration using inhibitory and excitatory molecules [82].

Another research group introduced a so-called μ Retina - a microfluidic PDMS chip that should replicate either human or mouse retina [83]. Again, mouse retinal progenitor cells were used. For the chip, the authors chose an arch-

shaped design as a tissue chamber with the consideration of human and mouse retinal dimensions and two reservoirs connected via several channels to the tissue chamber. The main focus of the paper was the investigation of cell migration behavior as a model for transplant therapies for patients affected by retinal diseases [83].

To generate a complex multicellular retina model, Dodson *et al.* presented a very different approach [84]: The group aimed for the controlled and localized analysis of tissue slices from mouse explants. With this approach, it was possible to integrate the whole mouse retina, including microglia. To enable imaging, the explant was flattened using specially designed suction channels. Furthermore, by having 12 independent channels reaching different areas of the tissue, it allowed to address each area individually with drugs or signaling molecules. Through theses channels, the tissue was further nourished, which kept the tissue slide viable up to five days. The applicability of the platform was tested by stimulating it with lipopolysaccharide to mimic inflammation, and activation of microglia could be investigated [84].

Overall, this summary clearly highlights that most chips in the ophthalmic field are based on PDMS as the main material and only few publications include cell types other than cell lines. Taken together, the models seem to serve as the first proof-of-concept, where using PDMS as chip material and cell types easy to handle makes sense. At the same time, it also shows that in this field, OoC research is still in its infancy and focus should be laid on the integration of more relevant hiPSC lines from a physiological point of view and the fabrication of chips with alternative materials. Finally, the integration of a fully functional retina comprising all relevant retinal cell types makes the development of an OoC quite challenging and alternative approaches might be needed, such as the combination of 3D *in vitro* model systems and OoC technology.

Chapter 2 Materials & methods

The following chapter provides all information about materials and methods used in this thesis to develop the different chips and characterize those from a biological perspective as well as from a technical point of view.

2.1 Fabrication methods

The chip fabrication methods start with the first chip generation made from PDMS to highlight the integration and functionality of a complex retina on-chip. Later, the focus was laid on alternative fabrication methods and the increase of relevance of the system with the inclusion of a light source. The materials used for chip fabrication are listed in Table 2.1. All fabrication methods are explained in the following subsections:

2.1.1 Polydimethylsiloxane (PDMS) based chip fabrication

The fabrication of the first chip generation is based on PDMS (**PDMS-chip**) and has previously been published in Achberger *et al.* ([85, 86]). Two chip versions have been designed, slightly varying in the height of the top well layer (see Figure 2.1). For the design, the software AutoCAD was used. The channel was designed with a width of 400 μ m, being expanded in the region of the wells to cover the diameter of 2 mm from the tissue compartments. The channel connects the tissue chambers in a row and contains no division. Further, the channels were designed with a height of 100 μ m.

Microstructuring using soft lithography For the PDMS chip fabrication, usually any negative master mold is required. In the case of this RoC generation, wafers or 3D-printed molds have been used. For the bottom layer in both versions a wafer was microstructured containing negative channel structures (media layer, see Figure 2.1). This process using photolithography will be described exemplary in detail for the generation of the media layer master mold: SU8-50 photoresist (MicroChen, USA) was poured onto a 4" silicon wafer and spin-coated to distribute the photoresist to a height of 100 µm homogeneously. A patterned mask containing the channel structures was added on top and allowed localized



Figure 2.1 First chip generation RoC design. a) Drawing from the first version, based on the publication from 2019 ([85]). b) Model from the second version with an elevated top layer including a reservoir for additional media supply (published in 2021 ([86]).

exposure only in the region of the channel geometry. Wafer and mask were placed below an ultraviolet (UV) lamp to evoke a partial curing of photoresist only in the channel region. Parameters were used according to the manufacturer's data sheet. The structures then were developed in SU-8 developer (Microresist Technology GmbH, Germany) for 6 min. Next, the wafer was rinsed with isopropanol (IPA) and blow dried with a nitrogen gun. To enable an easy removal of PDMS molds after curing it on the wafer, the wafer was also silanized before its use with chlorotrimethylsilane (Sigma-Aldrich, Germany).

The master mold for the top/well layer of the first version was generated similarly to the media layer master mold, but in two steps. A base layer of 25 µm height served as a membrane insert and was generated from SU8-50. After exposure to UV light, another photoresist was added onto the first (SU8-3025) to generate the mark for the tissue channels in- and outlets. For this purpose, a height of 40 µm was chosen. It has to be mentioned that the membrane insert was used for the very first experiments. Still, observations revealed that the insert was not necessarily needed for a tight membrane integration. For this reason, the well layer master mold fabrication was reduced in the future to a one-step wafer fabrication containing only the structures for the wells and marks for the locations where in- and outlets should be punched.

For PDMS replica molding, Sylgard 184 PDMS (Dow Corning, USA) was prepared at a ratio of 10:1 prepolymer to curing agent, as proposed by the manufacturer and poured onto the wafers. The mixed polymer was degassed before being added to the wafers. Then, it was cured overnight at 60°C in a convection oven and could be peeled off the next day. Initially, the channel layer was generated using exclusion molding: With this method, the channels have open structures from two sides and must be bonded to a glass slide to seal the channels, guaranteeing a high imaging quality through the glass slide. However, usually exclusion molding can cause problems, which is apparent because the layer consists of a slab with a thickness of less than 100 µm and open fragile structures. Often, either rupture or partially closed channels were occurring. Therefore, later slightly more PDMS was added to cover the channels and generate a sealed channel structure. Using still a small amount of PDMS it is possible to have a very low distance to the channel, similar as with the glass slide and the tricky exclusion molding process can be skipped. In this case the chip is fully made of PDMS except for the membrane. For the well layer, 25 g of PDMS was poured on the wafer to generate a layer thickness of around 2 mm. Additionally, for the well layer, four chambers were punched into the material along the marks with a conventional biopsy puncher of 2 mm diameter. Apart from this, in- and outlets were also punched with a biopsy puncher of 0.75 mm to access the media layer. In contrast to that, the master mold of the well layer of second version of the PDMS RoC consisted of a 3D-printed mold that was purchased from a 3D-printing company (Protolabs) with a smooth surface roughness. This allows the formation of the tissue chambers without the need of punching and in this case only in- and outlets needed to be punched out.

Chip bonding using plasma The overall PDMS chip consists of media, and well layer made from PDMS, and a porous membrane, that separates channels from tissue compartments. Therefore, a track-etched PET membrane (Sabeu GmbH, Germany) with a pore diameter of 3 µm and thickness of 10-20 µm was functionalized using bis-[3-trimethoxysilypropyl]amine (Sigma-Aldrich, Germany) to generate bonding sides on the membrane. Prior to the chip assembly both PDMS layers were cleaned with IPA and scotch tape to remove remaining dust particles on the layers. Then, the layers as well as the membrane were activated with oxygen plasma at 50 W for 30 s (Zepto, Diener). The membrane was placed into the membrane insert, and then, both PDMS layers were aligned and bonded together. To stabilize the bonding strength, chips were placed in the oven overnight at 60°C.

2.1.2 Thermoplastic based chip fabrication

Starting from the second chip generation, every chip was produced with components of PMMA and the majority of the chip versions also contained a media channel made from TPE. Due to the usage of alternative materials, different chip fabrication methods were applied. For the second chip generation, also two variations were developed: A simplified chip, being similar to the PDMS-chip in its design with slight modifications (now called **TPE-chip**) and a more complex chip with an integrated mesh (now called **Mesh-chip**). The chips were designed using the software CorelCAD (CorelDraw, Corel Corporation, Canada) for 2D sketches and SolidWorks for 3D designs. The outer dimensions of this chip are 34 mm x 22 mm.

Microstructuring using lasercutting Starting with microstructuring, the main method used in this thesis for structuring thermoplastic materials was carbon dioxide (CO_2) lasercutting (ULTRA R5000, Universal Laser Systems). Within the lasercutting platform, two laser sources were integrated, enabling

different powers (40W for laser 1, 30W for laser 2 and 70W by turning on both). For microstructuring, mainly the lower power was sufficient to generate fine structures without excessive power load. Depending on material thickness and supplier, several parameters were optimized for lasercutting and summarized (see Table 2.2). The two TPE-chip top layers were fabricated from PMMA and lasercut: The lower part was structured from 1.5 mm PMMA and contained four cylindrical chambers for the organoid culture of 1.6 mm diameter. Further, two holes were cut as in- and outlet with a diameter of 3.0 mm in this layer, that allow access to the channel. Above, 2 mm thick PMMA was structured with four oval chambers used as media reservoir and in accordance to the second version of the PDMS-based chip. Again, in- and outlets were cut in form of round holes of a diameter of 1.4 mm. In addition to the PMMA parts, the PC membrane was lasercut into rectangles with dimensions of 6 mm x 21 mm, as the process was faster and more accurate than cutting them manually with a scissor. Apart from these layers, two additional materials were laser structured and exclusively used for the Mesh-chip: 50 µm thick PMMA was structured fitting

exclusively used for the Mesh-chip: 50 μ m thick PMMA was structured fitting to the design of the well layer, as well as 750 μ m thick TPE, which served as adhesive layer between the 50 μ m thick PMMA layer and the 1.5 mm thick well layer and also contained the same structures as the PMMA well layer.

Microstructuring using hot embossing For structuring the channel layers, a previously presented method by Schneider *et al.* with slight adaptions was applied [58]. Hot embossing usually uses a master mold, which contains the negative structures of the actual design for the chip and will then be pressed into another material at high temperatures (near above the T_g . In this case, four stereolithography (SLA) 3D-printed molds ordered from a company (Protolabs, Germany) were used as master molds. The parts consist of PC-like translucent material (Accura5530), where a natural surface finish was chosen and "smart best surface finish" was set to realize a very smooth surface. (The manufacturer states on his website, that a resolution of 0.07 mm in XY-drawing plane and 0.2 mm in Z-direction can be reached.) The channels consist of a width of 600 µm and are widening in the area of the wells to cover the diameter of the wells (1.6 mm). The channels have a height of 150 µm and are arranged in parallel (Figure 2.2 a, b).

The material of choice for hot embossing is a 750 µm thick TPE-layer, which contains a PMMA below for an enhanced transparency. Furthermore, it simplifies the handling, as TPE usually has a sticky character and cannot be processed easily when used alone. These findings were already published by Schneider *et al.* ([58]) and adopted to this chip fabrication process. Custom-made TPE foils were used for the chip fabrication, extruded previously from commercial SEBS pellets from an external service provider (Fraunhofer Institute for Process Engineering and Packaging, IVV). A relatively big square of PMMA and TPE (12 cm x 12 cm) was laminated by removing the protective foil of one side of the PMMA and pressing both layers together using a lamination roller for a homogeneous pressure distribution. Care was taken to avoid any air bubble

encapsulation. Then, the parts were cut into slightly bigger sizes than the original 3D-printed master molds and attached to the molds with the TPE facing to the molds, respectively (Figure 2.2 c). Next, mould release agent (11450400, WEICON, Germany) was sprayed onto a round polished stainless steel plate and the prepared parts were placed on the plate - whereby the PMMA layer was in direct contact with the plate. With the addition of the mould release agent a reversible removal of the parts was achieved in the end (Figure 2.2 d). The whole set-up was placed in the hotpress (LabEcon100, Fontijne Presses b.v., Netherlands). In advance, the hotpress was heated up to 100°C and then the parts were inserted and structured using a total force of 8 kN for 10 min. Afterwards, the parts were fully structured and cut into the chips' size (Figure 2.2 e). This produced layer will be named the TPE bottom layer in the following and it was used as part for both chip versions - the TPE-chip, and the Mesh-chip.

PDMS support structure for TPE-chip top layer bonding and membrane bonding For the bonding of the TPE-chip top layers, a PDMS support structure was used containing the negative structures of the top layers: The PDMS support structure was fabricated using a custom-designed 3D-printed mold, designed with the software *SolidWorks*. The design was printed using a SLA printer (Form3, Formlabs, Germany) and Clear resin (RS-F2-GPCL-04) from the same company, that allows a relatively high resolution with layer thicknesses starting from 25 µm. The mold is designed with the negative structures of the PMMA parts and has a border on three sides defining the chips' edges. On this border, a glass slide can be fixed using two foldback clips, so that a pocket is created where PDMS can be poured easily (see Figure 2.3 a). PDMS was prepared, as for the PDMS chip generation. For an easier handling PDMS was first filled into a syringe and then added into the pocket to avoid uncontrolled overflow. The filled forms were degassed, cured overnight at 60°C, and removed from the 3D-printed mold by removing the glass slide.

Usually, eight top layers were bonded at the same time. Therefore, PDMS support structures were placed on a stainless steel plate to simplify the handling (Figure 2.3 b). To ensure a strong bonding, the PMMA parts were cleaned using IPA and a non-fluffy wipe and then placed on top of the PDMS support structures. By this, the two PMMA layers were already aligned. At the same time, the PDMS support was used to bond the membrane tightly onto the PMMA layers and prevent deformation of the PC membrane. The membrane was placed onto the PMMA covering the holes of the chambers and fixed with a tiny drop of IPA in case it was still moving (Figure 2.3 c). After evaporation of the solvent the process was continued. On top of the prepared layers another stainless steel plate was placed and in between a PET foil was added, being in direct contact with the PMMA layers. The PET foil ensures easy removal from the plate as it is coated on one side. The whole setup was inserted into the hotpress, previously preheated to 130°C, and the actual bonding was also performed at 130°C with a pressure of 3.5 kN for 10 min, and cooled down to 40°C, before the parts were



Figure 2.2 Chip fabrication steps - Hot embossing of the RoC channel structures. a) Design of the 3D-printed master mold. b) Cleaned 3D-printed mold. c) Assembly of 3D-printed mold with TPE and PMMA layer. d) Assembly of four prepared parts for hot embossing. e) Fully molded TPE layer with PMMA below.

removed. The pressure applied by the hot press was further equilibrated using the support structures made from PDMS. PDMS contains flexibility and casted PMMA layers usually slightly differ in its height (up to $\pm 20\%$) depending on the charge and laser cutting location. With the combination of both, the differences of the individual PMMA layer heights can be balanced.



Figure 2.3 Thermal bonding of PMMA top layers using PDMS support structure. a) Design and image of the 3D-printed mold for the generation of the support part. b) PDMS supports were arranged on a steel plate. c) The two PMMA top layers were placed on top and the membrane was added, covering the wells.

Fabrication of PDMS connectors Once the top layers were produced, the holes from the in- and outlets were sealed from the top (reservoir layer) using PCR tape cut into size. Then, the parts were transferred into a petri dish and placed upside down, so that the sealed part faced the dish. Previously prepared PDMS was added into a 5 mL syringe with Luer lock adapter, to which a needle with 0.7 mm diameter was connected (Luer lock prevents that the needle is pushed out due to the applied pressure). Then, in- and outlets were filled with PDMS thoroughly to avoid air bubbles. Excessive polymer was removed with a wipe and the parts were placed in the oven at 60°C for at least 4 h. Finally, the tape was detached with care to avoid removal of the PDMS inserts themselves and punched out from the smaller diameter in the direction of the bigger one. By this, the polymer stayed inside the PMMA and could not escape, when the puncher was removed. Alternatively, other 3D-printed connectors were also tested to replace this PDMS-based procedure to increase the reproducibility and simplify the process. Therefore, parts with the dimensions of the holes cut for inand outlets with a hole of a diameter of 0.7 mm were printed using the Form3 SLA printer and Elastic 50A resin (RS-F2-ELCL-01, Formlabs).

Mesh-chip top layer chip fabrication and membrane bonding The fabrication of the adapted version of the TPE-chip was produced in a very

similar way: The PMMA top layers were also bonded using PDMS support structures, but in this case the membrane was not added to the top layers. PDMS connectors were also integrated identically as to the TPE-chip. Then, another lasercut 750 µm thick TPE layer containing the structure of the holes for the wells and in- and outlets was placed below the well layer and clamped together between two glass slides using 4 foldback clamps, and attached in the oven at 60°C for around 1-2 h. During this fabrication step, the open side of the TPE was covered with a protection foil (provided from the original TPE sheets) to prevent adhesion to the glass slide as well as for dust protection. In a next step, a very coarse mesh was electrospun onto the TPE, using a EC-CLI apparatus from IME medical electrospinning. First, a solution of 8 wt% (w/v) polyurethane (PU) in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was prepared over- night with the help of a magnetic stirrer. The solution was transferred into a 1 mL syringe with a needle of 0.4 mm diameter. The syringe was inserted into a pump, where a flow rate of 1.5mL/h was set. The distance between needle and collector was set to 18 cm and the electrospinning process occurred at 23°C and 30% humidity. For an aligned fiber orientation, the protective foil was removed and the chip part was covered with aluminum foil from two sides and then fixed to the collector being in contact with the reservoir layer of the chip, hence facing the sticky TPE side towards the needle tip. The spinning process took place at a voltage of +16/-0.2 kV at a short spinning period of only 40 sec to generate a coarse mesh, through which singularized cells still can be seeded. The chip parts were removed and could be stored in a petri dish until further use. Next, the PC membrane was thermal fusion bonded on the lower side of the 50 µm thick PMMA layer, again with a PDMS support structure, but this time, the well height was significantly reduced to the layer height of the material with 50 µm. As a lower pressure was required for this bonding step, the parts (PDMS $support + 50 \ \mu m$ thick PMMA layer + membrane) were again attached between two glass slides using two small foldback clamps and placed in the oven at 130°C for 15 min. When the 50 µm thick layer was removed from the oven, the top layers prepared with the mesh were bonded to this layer simply by manually pressing both parts together and could be used for the final chip assembly.

Full second generation chip assembly To conclude, the TPE bottom layers as well as the prepared top layers were aligned together and due to the sticky character of the TPE already a weak bonding was realized. Both layers were placed in between two glass slides and fixed with four small foldback clips to enhance bonding strength. Then, the complete chips were placed in a convection oven at 60°C overnight, removed the next day and stored until use.

Fabrication of a microscopy holder for chip culture To enable an easy handling during chip culture, a chip holder was designed to fit into the microscopy stage. The dimensions were taken from conventional microscopy holders and the holder was designed with SolidWorks. Six chips are fitting on one holder. Apart from chip inserts, also cylinders, in which Eppendorf tubes can be placed

as reservoirs were added. Furthermore, the tubing can be reversibly fixed with some support, reducing the unintended tension or pressure to the chip and tissue. The microscopy holder was 3D-printed with a fused deposition modeling (FDM) printer (Prusa i3 MK3S+, Prusa Research a.s., Czech Republic) using 1.75 mm thick PLA filament (2178410, Conrad, Germany). To ensure that the chips are not moving during culture (which might be important for automatic imaging), additional lids have been designed, that seal the chips from the top. These lids also contain an insert, in which a transparent, flexible PDMS slab was inserted that was slightly protruding. Thereby the well keeps being closed tightly, while allowing gas exchange. The lids were printed with the SLA printer and Clear resin, as this material was stiffer than PLA, which started to deform over time. To fix the lid to the holder in a tight, but reversible way, in total four magnets (Q-10-03-02-HN, Webcraft GmbH, Germany) with an adhesive force of around 6.86 N were added to each chip chamber - two on the bottom side of the holder and two on top of the lid.

2.1.3 Chip fabrication integrating bioinspired membranes

The third chip generation is reffered to bioinspired membranes. Three types of chips/membranes were presented: 1) a chip incorporating electrospun membranes (**ESM-chip**), 2) a chip incorportating a commercially available collagen membrane (Collagen cell carrier (CCC)), and 3) a chip incorporating a Bruch's membrane. Main focus was laid on the ESM-chip. All chips were designed using the software CorelCAD and CorelDraw 2018 and consist of a simple layer structure with two overlapping channels.

ESM fabrication For the ESM-chip, as chip bulk material fully PMMA was chosen. The chip consists of four PMMA sheets, where the three bottom layers have a height of 175 µm and the top layer has a thickness of 3 mm. The thick top layer has been chosen to enable easy access to the chip through in- and outlets with a diameter of 1.36 mm that exactly fits the Tygon tubing used for chip connection. In the case of the ESM-chip materials structuring, a 10 W CO₂ lasercutter was applied (Universal Laser Systems, VLS 2.30), hence slightly different parameters were chosen compared to the previous presented chip (see table 2.3). All PMMA parts were lasercut and cleaned with IPA until further use. Prior chip assembly, the membrane was spun onto the bottom side of the 175 µm top channel layer. Two solutions were used for the membrane fabrication: 1) 12% w/v of pure PLA or 2) a 12% w/v blend of PLA with GM_2 (90:10% w/w) dissolved in HFIP overnight with a magnetic stirrer. Again, the electrospinner from IME medical was used for the membrane fabrication using the same setup as for the Mesh-chip with slight variations: The spinning duration of 2.5 min was applied to generate a tight stack of fibers instead of a coarse mesh. Further, different voltages were applied: -16/-0.5 kV was set for PLA, and -18/-4kV was set for the $PLA-GM_2$ solution. The thin PMMA layer was attached to a roughly 2 mm thick PDMS slab for the spinning process. Then, both were wrapped into aluminum foil, whereby major aluminum material was arranged on the two

shorter opposite sides. The prepared PMMA layer was fixed on the collector, and fibers were spun onto the material. Once removed from the collector, the surrounding fiber mat was cut with a scalpel wetted in HFIP. To ensure a complete evaporation of the solvent, the chip parts were placed in the fume hood overnight. To minimize the membrane size to a needed minimum, the membrane was welded into the PMMA using the lasercutter (by applying the parameters listed in Table 2.3 twice). Excessive material was later removed with a wipe wetted with IPA.

ESM chip assembly For the chip assembly, all layers were cleaned with IPA and aligned. To guarantee precise alignment during bonding, two opposite sides were additionally fixed with a thin stripe of scotch tape. The assembled chips were sandwiched in between two glass slides and clamped with two small foldback clips on each of the two long sides. This setup was placed for 15 min in a convection oven at 130°C. Later, the chips were removed and cooled down to room temperature (RT) before the clamps and glass slides were removed.

Bruch's membrane extraction For the Bruch's membrane integration, pig eves donated from a slaughterhouse were used. The membrane was extracted by following the protocol from McHarg *et al.* [88] (Figure 2.4). In brief, the eye was placed in a petri dish, and with a disposable scalpel, a first cut was set surrounding the cornea. The eye could be opened with four incisions to flatten the eyeball. Then, the lens, vitreous humor, and neural sensory retina were detached from the RPE using two tweezers. Next, using a cell scraper, the Bruch's membrane could be gently cleaned from RPE with of some sterile water. Later, the membrane was peeled off together with the choroid with the help of two tweezers and a cell scraper. When this part was removed from the eyeball, the other side of the Bruch's membrane was cleaned with the cell scraper and fresh water. Being placed in a fluid, the membrane could be unfolded. Then, a conventional PET foil was cut into a square with the dimensions of a microscopy slide (75 by 26 mm) and was placed below the membrane. The foil was lifted together with the membrane on top and thereby kept in a flat position. To avoid unfolding, it was covered with a second foil. The membrane was dried in this setup at RT before further use. Still covered by the two foils, the membrane was cut into size with scissors. Then, one foil was removed and the membrane was placed onto one chip side, as explained in the following:

CCC and Bruch's membrane on-chip integration For the integration of CCCs and Bruch's membranes into the chip, two different approaches were pursued: To test the versatile applicability of the membrane into different chips, the chips were either fabricated from pure PMMA, similar to the ESM chip fabrication, but with only one channel layer and above a 3 mm layer with additional well layers to fit the original PDMS chip design. Apart from this, also the TPE-chip version was tested as model for CCC and Bruch's membrane integration. Again, instead of adding a membrane below the top layers, an additional



Figure 2.4 Removal of the Bruch's membrane from a pigs' eye. a) Eye without cornea and lens, highlighting the layering of the innermost eyeball. b) four incisions enable flattening of the eyeball. c) With the help of two tweezers, the vitreous body and neurosensory retina (NSR) can be removed. d) a cell scraper can help removing the RPE from the membrane. e) The membrane and choroid can be peeled off and f) choroid can be removed similarily to RPE using a cell scraper. Adapted from [88], CC BY-NC-ND 3.0.

750 µm TPE layer was attached below the PMMA top layers. The Bruch's membrane could be integrated by direct bonding on this layer. CCC membranes (500042928, Viscofan Bioengineering) were also cut into square dimensions of 6 mm x 21 mm and integrated in between the two TPE layers - channel and well layer. With the direct contact of both TPE layers, a strong bonding at relatively low temperatures could be realized. This is especially crucial, as collagen will be denaturated by applying high temperatures above body temperature. Therefore, bonding was performed overnight at 37°C in an oven for this chip fabrication method.

2.1.4 Side-view-chip fabrication

The **Side-view-chip** was produced similar to the TPE-Chip with special attention to the side edge treatment for improved transparency. The chip is designed similar to the original RoC platform with a well and reservoir, but simplified containing only one chamber. Well and reservoir layers were produced from 1.5 mm PMMA, whereby the well layer was arranged in a square of 1.2 mm x 1.2 mm to enable easy access through the side window without distortion. Further, the distance between well layer to outer edge was designed with a distance of 1.1 mm, thereby creating a window of approximately 1.1 mm thickness. The access for in- and outlets was designed with a diameter of 1.34 mm to fit to the size of the Tygon tubing. Again, a PC membrane separated the PMMA top layers from the TPE channel layer.

Channel layer fabrication The media channel was hot embossed as already explained, but structured with an epoxy mold (instead of a 3D-printed part), produced according to the protocol from Schneider *et al.* [58]. By this, five channel structures were arranged on one master stamp. Using a bigger stamp, $12 \times 12 \text{ cm}$ square pieces of PMMA and TPE were prepared and hot embossing took place at 120° C with a pressure of 8 kN (see [58]). The press was cooled down to 40° C and the parts could be removed and cut into five individual parts.

Top layer fabrication The top layers were structured again in a first step using the ULTRA R5000 lasercutter (see Table 2.4). For the top PMMA layers (1 and 1.5 mm) the sheets were elevated 5 mm up from the table to prevent the risk of unintended laser reflection from the cutting table back into the material [89]. Without the elevation of the PMMA sheet, heat reflection damages could be regularly seen, and as a consequence, the damage would significantly complicate the treatment to reach a transparent side. Furthermore, the membrane was structured with the lasercutter.

To treat the top layers, a frame was designed with the negative structures of the PMMA top layers. The frame consists of two parts, that can be reversibly mounted with two screws. The main part consists of the negative structures of the chip, except for the side wall that is close to the well and forms the window to the well. This side wall forms the second part that can be fixed tightly to the chip with the screws. It is important that pressure can be applied in this region. Therefore, the distance of the side walls of the main part were designed with a reduced distance of 0.95 mm, meaning that when fixing the chip layers in the frame, there will be a gap between both metal parts, but a tight attachment of the chip to all edges. The frame was milled from the metal AnZnMgCu1.5 by an external company (Spanflug Technologies GmbH) with an average surface roughness of Ra 0.4. To enhance the surface quality, two additional flexible PET foils (3M, Germany) were added to the critical surfaces: 1) the inner well edge, forming one edge of the side window, and 2) the outer side edge, forming the second side of the window. The foil for the side edge consists of a simple square geometry of 29.2 mm x 5.4 mm and the foil to be inserted between pillar and well was designed with a stack of two squares, arranged on top. The bigger one, consists of 2.4 mm width and a height of 1.5 mm (to fit to the reservoir well) and the second one consists of a width of 1.3 mm and 1 mm height, which is centrally placed on top and fits to the geometry of the well layer. Both layers were lasercut into size. The foil for the well insertion was cut with an additional line between the squares that was only laser engraved and did not cut through the material, leading to a controlled bending of the material at the region of the interface of both top layers. The lasercut foils were dipped in IPA and could be temporarily attached to the pillars. The PET foil is coated on one side, thereby

the coated surface always should be in contact with the PMMA material and the other side should be touching the frame.

For the bonding of the top layers, the reservoir layer was first placed into the frame, followed by the well layer. Then, the frame was mounted together with the side wall. Finally, the membrane was added on top, covering the well. Usually, six parts were prepared and bonded simultaneously, and placed on a metal plate. The parts were covered with another 3M foil to keep the surface smooth. An additional silicon layer of 1.5 mm thickness was placed above to compensate for slight differences in the height of the individual parts. This can happen as the purchased PMMA layers were casted. The six layers were hot pressed at 130°C for 10 min with an applied pressure of 3.1 kN. The bonded layers could be removed, when the press was cooled down to 40°C and the outer side edge was further treated with acrylic polishing paste (Acrylglasprofi, Germany). The paste was added on a wipe on a table and the chip was dipped into the paste being moved back and forth for around one minute. Then, the chips were cleaned from the paste with lukewarm water, IPA and a nitrogen gun.

Final chip bonding For the final chip bonding, the top layers were aligned to the channel layers and attached. Again, six chips were bonded in parallel on a metal plate. The chips were covered with a 1.5 mm thick silicon sheet and bonded with 3.1 kN for 10 min at a temperature of 60°C in the hotpress. Once the temperature was reduced to 30°C the chips could be removed and were ready for tissue culture.

2.1.5 Fabrication of a light exposure setup

For the development of a light exposure setup an Arduino (Arduino UNO, Arduino) was chosen to set the light exposure intensity and light color in a flexible way. Therefore, a 4x4 red-green-blue (RGB) light emitting diode (LED) WS2812 array (RBS11097, anzado GmbH, Germany) has been integrated as light source. Furthermore, with a DHT22 temperature and humidity sensor (DEBO DHT 22, reichelt elektronik GmbH & Co. KG, Germany) the chip environment could be recorded constantly. To enable a heat transfer away from the chip and keep the temperature constant, a heat sink was directly bonded to the LED module using heat-conducting paste (LEITPASTE 5ML, reichelt elektronik GmbH & Co. KG, Germany). Above, a TEC1-12706 peltier module (189115 - 62, Conrad Electronic SE, Germany) was attached, followed by a second heat sink (ICK PGA 11X11), that was also bonded with heat-conducting paste on top of the peltier module. In addition, a fan (978-9G0912G102, Mouser Electronics, Inc., Germany) was fixed above this assembly to transport the heat away from the chip. The whole setup was placed into a specific incubator called ALS Incubator FlowBoxTM (ALS Automated Lab Solutions GmbH, Germany) that also exchanges air and regulates the temperature.

According to the data sheets, the individual components were solded to the Arduino for the assembly of the whole setup. The DHT22 sensor was connected to Pin2 and the LED was operated via Pin5. While sensor and LED worked with

the supply energy from the Arduino itself (5V energy), the fan and peltier were externally supplied with a load of 12V. Furthermore, these components were connected to a thermostat (DEBO XH-W1209 T, reichelt elektronik GmbH & Co. KG, Germany), that automatically regulates the temperature at a constant level. The sensor was placed below the chip, together with the DHT22 sensor. When connected to the laptop, the serial monitor from the Arduino software (Arduino IDE 1.8.13) allowed to record the environmental conditions during an experiment. For the control with the Arduino a code was generated and applied that can be easily adapted (see Appendix).

Material	Product reference	Manufacturer	
PDMS SLYGARD 184 kit	1673921	Dow Corning, USA	
PMMA (3 mm)	0133466	Modulor, Germany	
PMMA (2 mm)	0133420	Modulor, Germany	
PMMA (1.5 mm)	0279836	Modulor, Germany	
PMMA (1 mm)	301-30814	Architekturbedarf, Ger- many	
PMMA (175 μm)	PLEXIGLAS Resist, Clear 99524 GT	Evonik, Germany	
PMMA (50 µm)	5509-0005-36	Schlösser GmbH & Co. KG	
PC membrane (22 μ m)	1000M25/610M303	it4ip S.A., Belgium	
TPE (750 μ m)	Mediprene OF400M (HEXPOL TPE AB)	Custom-made	
PET foil	Scotchpak™ 1022 Flu- oropolymer Coated Polyester Film − − − − − − − − − − − − − − − − − − −	3M, Germany	
Sealing film, polymerase chain reaction (PCR) Polypropylene	EN83.1	Carl Roth, Germany	
Polyurethane (PU)	81367	Sigma-Aldrich, Germany	
1,1,1,3,3,3-Hexafluoro-2- propanol (HFIP)	105228	Sigma-Aldrich, Germany	
РММА (175 µm)	PLEXIGLAS Resist, 99524 GT	Evonik, Germany	
polylactic acid (PLA)	81273	Sigma-Aldrich, Germany	
gelatin methacry- loyl (GM) ₂)	custom-made	See protocol by Hoch <i>et al.</i> [87]	
Small foldback clips	2141999	Jakob Maul GmbH	
Tygon tubing	VERNAAD04103	VWR international GmbH	
Silicon (1.5 mm)	Elastomer sheet VMQ 50 Shore A transparent	APSOparts AG, Ger- many	
Collagen Cell Carrier (CCC))	76518-078	VWR International GmbH	

Table 2.1 List of materials & consumables used for chip fabrication

Material	Thickness [mm]	Laser power [Watt]	Lasercutting parameters		neters
			$\operatorname{Power}[\%]$	speed $[\%]$	PPI
PMMA	2	30	80	1.5	200
PMMA	1.5	30	80	1.5	200
PET foil $(3M)$	0.1	30	6	10	500
PC membrane	0.02	30	6	10	500
TPE	0.75	90			

Table 2.2 Materials and laserparameters of the chips' second generation- ULTRA R5000 Laser-

Table 2.3 Materials and lasercutting parameters of the electrospun membrane(ESM) chip fabrication - VLS2.30 Lasercutter

Material	Thickness [mm]	Laser power [Watt]	Lasercutting parameters		neters
			$\operatorname{Power}[\%]$	speed $[\%]$	PPI
PMMA	3	10	100	2	1000
PMMA	0.175	10	10	10	1000
PMMA weld-	_	10	2	10	1000
ing (2x)					

Table 2.4 Materials and laserparameters of the Side-view-chip - ULTRAR5000 LaserLaser

Material	Thickness [mm]	Laser power [Watt]	Lasercutting parameters		neters
			$\operatorname{Power}[\%]$	speed $[\%]$	PPI
PMMA	1.5	30	90	1.5	500
PMMA	1	30	90	1.5	500
PC membrane	0.02	30	6	10	500
PET foil $(3M)$	0.1	30	13	10	500
PET foil $(3M)$	0.1	30	6	10	500
laser engraving					

2.2 Cell culture and on-chip integration

Different types of cells were integrated to fulfill various tasks: For the technical chip characterizations, the tissues in the chips could be labeled with fluorescent markers, which enabled to visualize the chip environments with imaging methods. The biological chip characterization focus of the tissue analysis was laid on their morphology and viability to show the system's applicability for cell culture experiments.

All hiPSC cell lines were kindly provided from collaborators of the Institute of Neuroanatomy & Developmental Biology (INDB), Eberhard Karls University Tuebingen, where RO and RPE were produced from hair of healthy persons as described in Linta *et al.* [90]. Furthermore, all procedures were in accordance by the Helsinki convention and according to the permission by the Ethical Committee of the Eberhard Karls University Tuebingen (Nr. 678/2017BO2 for hiPSC derived retinal pigmented epithelial cells and Nr. 495/2018-BO02 for the isolation of PBMCs from whole blood)). Human skin-derived mvECs were obtained from human skin removed during plastic surgeries by Dr. Ulrich E. Ziegler (Klinik Charlottenhaus, Stuttgart, Germany), approved by the local medical ethics committee. The patients gave an informed consent according to the permission of the "Landesärztekammer Baden-Württemberg" (IRB#: F-2012-078; for normal skin from elective surgeries). For the mvEC-related experiments cells from one donor have been used (abdomen skin tissue of a 53-year old female; body-mass-index of 29).

All media components used for tissue culture are listed in Table 2.5. This section shall give an overview of the different tissue sources used in this thesis with an explanation of how the tissue can be maintained in conventional cell culture and how the tissue can be integrated into the chips. For the majority of chip generations, mainly RPE (and ROs) were integrated, with exception of the third generation (the ESM-chip), where in addition to RPE also mvECs and T-cells were integrated to test the suitability of the membrane surface on different cell types.

Reagent	Stock con-	Used con-	Product ref-	Supplier
	centration	centration	erence	
Corning Matrigel	$100 \ \%$		354277	Corning Life
hESC-Qualified				Sciences
Matrix				
TrypLE Express	$100 \ \%$		12604013	ThermoFisher
Enzyme $(1X)$				Scientific Inc.
PeproGrow hESC	100 %			PeproTech,
embryonic stem cell				Hamburg,
media				Germany
Blebbistatin	$100 \ \%$		203391	Sigma Aldrich

Table 2.5 List of cell culture components

Sodium selenite	99 %	24 nM	214485	Sigma Aldrich
Progesterone	00 /0	16 nM	P8783	Sigma Aldrich
Human holotrans-		$\frac{10 \text{ m/r}}{80 \text{ µg/mL}}$	10100	Serologicals
ferrin		00 /28/		201010810415
Human recombi-		$20 \ \mu g/mL$	91077C	Sigma Aldrich
nant insulin				
Putrescin		88 µM	51799	Sigma Aldrich
Retinoic acid		$1 \mu M$	R2625	Sigma Aldrich
Corning Matrigel			354230	Corning Life
Growth Factor Re-				Sciences
duced				
DMEM, high glu-	100 %	*	41965039	ThermoFisher
cose				Scientific Inc.
DMEM/F12 + Glu-	100 %	*	10565018	ThermoFisher
tamax				Scientific Inc.
B27 w/o VitA	100 %	2 %	12587-010	ThermoFisher
,				Scientific Inc.
Antibiotic-	100 %	1 %	15240-062	ThermoFisher
Antimycotic,				Scientific Inc.
100X				
MEM Non Essen-	100 %	1 %	11140-050	ThermoFisher
tial Amino Acids				Scientific Inc.
(NEAA) (100X))				
fetal bovine serum	100 %	10 %	10500-064	ThermoFisher
(FBS)				Scientific Inc.
Taurin	100 mM	1:1000	4271	Carl Roth
EGF (human re-	$10 \ \mu g/mL$	20 ng/mL	GFH26-100	Cell Guidance
$\operatorname{combinant})$				Systems LLC
FGF2	$25 \ \mu { m g/mL}$	20 ng/mL	GFH146-50	Cell Guidance
				Systems LLC
Heparin	2 mg/mL	$2 \ \mu { m g/mL}$	H3149	Sigma Aldrich
ROCK-inhibitor Y-	10 mM	$10 \ \mu M$	S1049	Selleck Chem
27632				
Poly-l-Ornithin			P4957	Sigma Aldrich
Laminin	$500 \ \mu { m g/mL}$	$50 \ \mu g/mL$	11243217001	Roche
Accumax	Ready to us	e, no dilu-	A7089-	Sigma Aldrich
	tion		100ML	
Hystem-C hydrogel	Use hydroge	l according	HYSC020-	Sigma Aldrich
	protocol from	n supplier	1KT	
Dispase	100%	2 U/mL in	350500	Serva Elec-
		PBS-		trophoresis
Trypsin	100%	0.05%	59418	Sigma Aldrich
EDTA	100%	100%	15040033	ThermoFisher
				Scientific Inc.

endothelial	100%	100%	C-22010	PromoCell
cell growth				
medium (ECGM)				
Gentamicin	100%	1%	15710049	ThermoFisher
				Scientific Inc.
Penicillin/strepto-	100%	1%	15140-122	ThermoFisher
mycin (pen-strep)				Scientific Inc.
Biocoll, isotonic,	100%	100%	L6115-BC	Merck KGaA
$1.077 \mathrm{g/mL}$				
bovine serum albu-	100%	0.1%	CP77.1	Carl Roth
min (BSA) / Al-				
bumin fraction V,				
very low in endo-				
toxin				
X-VIVO15 medium	100%	100%	BE02-060	Biozym
TransAct	100%	100%	130-111-160	Milteny Biotec
Deep Red Dye		$1 \ \mu M$	C34565	ThermoFisher
				Scientific Inc.

*according to media formulation

2.2.1 Chip preparation - sterilization methods

All chips were sterilized prior to cell seeding to enable a sterile cell culture. For the first (PDMS) generation, all chips were sterilized using oxygen plasma for 3-5 min at 50 Watt and filled with Phosphate buffered saline without calcium and magnesium (PBS-) in a sterile environment. Apart from sterilization, the plasma treatment also changed the hydrophobic surface character into temporarily hydrophilic and simplified the following filling step of the channels with Phosphate buffered saline (PBS). At the same time it reduces the amount of air bubbles inside the channels. For the later generations, first, the surfaces of the chips were cleaned with 70% Ethanol (EtOH) under the sterile bench and also the channels were flushed with 70% EtOH using a pipette tip or a syringe. It was ensured that the channels were filled and flushed twice with PBS- to ensure that no EtOH remains inside the chips. The majority of the PBS was aspirated from top of the chips and PBS- inside the channels could be replaced with medium by filling them, followed by coating substances and/or tissues. For the chips with open wells, the tissue could be integrated very simply from top, similar to conventional wellplate tissue culture. For the ESM chip, the tissue was injected into the channels, due to the different chip design and will be explained in the mvECs subsection.

2.2.2 Culture of hiPSC-derived RPE and ROs and on-chip tissue generation

For the hiPSC RO differentiation a protocol from Zhong *et al.* [49] was applied, with slight modifications. Details about the differentiation of RO and RPE can be found in the publication from Achberger *et al.* [85]. RPE cells were integrated in all chip versions presented in this thesis, and ROs were also integrated into most of the chip versions (except for the third generation integrating bioinspired membranes).

In brief, the hiPSC lines generated from the keratinocytes of human hair roots picked from healthy donors while following the protocol from Linta *et al.* [90]. Then, hiPSCs were tested for stem cell markers and its potential for germlayer differentiation. Cells were seeded on Matrigel-coated 6-well plates to form colonies and were supplied with FDTA medium [91]. On a regular base, cells were passaged with Dispase (once a week). Manual scraping with a cell scraper was applied toe remove differentiated cell colonies.

The RO generation was realized from embryoid bodies. Those were produced using a concentration of 2.88×10^6 hiPSCs that were removed from wellplates and singluarized with TrypLE on day 0. PeproGrow was added to the cells to stop the reaction from TryPLE, the cells were centrifuged and medium was replaced by fresh PeproGrow in addition with 1 % Antibiotic-Antimycotic, 10 μ M ROCK-inhibitor and 10 μ M blebbistatin. The cells were resuspended and transferred to v-shaped-96 well plates at a concentration of 100 μ L/well. By centrifugation, cell aggregations could be formed. 80% of the medium was changed on day 1 with N2 medium (DMEM/F12 (1:1)+Glutamax supplement, 24 nM sodium selenite, 16 nM progesterone, 80 mg/ml human holotransferrin, 20 mg/ml human recombinant insulin, 88 mM putrescin, 1x NEAA, 1x antibioticsantimycotics). On day 4, medium change was repeated. Three days later, 32 embryoid bodies were transferred into one 6-well plate coated with Growth-Factor-Reduced Matrigel. Since then, daily media change with N2 medium happened until day 16. From that day on, medium was changed to RPE media (see Table 2.7, left) and media was changed 2-3 times per week since then. On day 24, it was possible to detach selectively eye fields with the help of 10 μ L pipette tips and collecting them in 10cm petri dishes, supplementing them with RPE media and 10 μ M ROCK-inhibitor for one day. After their formation, ROs could be taken from the dish and occasionally attached non-retinal tissue could be removed with microscissors. From day 40, medium was changed to RO media (see Table 2.8). Furthermore, from day 70-100, 1 mM retinoic acid was added to RO medium. Then, the amount of retinoic acid was reduced to half of it from day 100-190 and finally again completely removed.

Coming along with the ROs differentiation, also RPE could be produced as a byproduct from the ROs (in accordance to [49, 92] with slight adaptions). Therefore, pigmented areas on the RO were collected in reaction tubes, once removed with a microscissor. They were first washed with PBS- and then, the tissue was singularized with Accumax for 90 min and the tissue was resuspended three times during this incubation. The reaction was stopped with RPE media, supplemented with 10% FBS. Cells were centrifuged and plated into wellplates that were treated in advance to improve tissue adhesion: First, the wells were covered with 0.01% Poly-l-Ornithin for 30 min at RT and then incubated with 20 μ g/mL laminin for 4 hours. The cells were cultured for one day on these plates with RPE⁺⁺ medium (see 2.7, right) and when the cells formed a confluent tissue layer, medium was again changed to conventional RPE media.

RPE culture RPE usually was delivered from the collaborators of the Institute of Neuroanatomy in form of confluent monolayers on 24-well plates. For conventional cell culture maintenance, medium was replaced almost daily (6 days a week) with the belonging medium (listed in Table 2.7). For the tissue transfer from the well and **on-chip seeding**, cells were detached by aspirating the media, washing with PBS- and then placing 300 μ L of Accumax on the tissue, dissociating the cells into singularized ones. The tissue was incubated with Accumax for 20-30 min in an incubator and in case they were not fully detached yet, the solution on the tissue was resuspended several times. The reaction of Accumax was stopped by adding the same amount (300 μ L) of RPE⁺⁺ medium on top. RPE⁺⁺ medium always had to be prepared freshly as the supplements in the medium are not stable for more than 48 h. When the reaction was stopped, the individual cells were aspirated from the well and passed through a cell strainer with a mesh size of 40 μ m to ensure no cell clumps were transferred into a 15 mL falcon tube. Then, the cells were centrifuged at 800 rpm for 3 min. The fluid was removed from the cell pellet and replaced with RPE^{++} medium (Table 2.7). Cell concentration was adjusted to a final concentration of 2.5×10^6 cells/mL. Depending on the chip generation and membrane/tissue surface, different volumes were seeded (Table 2.6). Before RPE seeding, the according membranes were coated with laminin at a concentration of 50 μ g/mL in DMEM/F12 for two hours in the incubator. Once, cells were seeded, RPE⁺⁺ medium was added for two days with replacement of fresh medium after 24 h and later nourished with the usual RPE medium for daily culture - either statically or continuously by connecting them to the pump.

RPE was also integrated into the ESM-chip; for this purpose, the cells were integrated the same way as the mvECs (see 2.2.4).

Chip generation	Cell concentration	Used volume
1. chip generation (PDMS-chip)	2.66 Mio cells/mL	$4.5 \ \mu L$
2. chip generation (TPE-chip & Mesh-chip)	2.66 Mio cells/mL	$2.9 \ \mu L$
3. chip generation (ESM-chip)	1.0 Mio cells/mL	$100 \ \mu L$
4. chip generation (Side-view-chip)	2.5 Mio cells/mL	$3.0 \ \mu L$

Table 2.6 RPE seeding - depending on chip generation

Supplements	RPE formulation - For daily culture of a conflu- ent tissue	RPE ⁺⁺ medium formu lation - For cell seeding (first 24hs)	
_	Total volume = 20 mL	Total volume = 20 mL	
$\overline{\text{DMEM/F12} + \text{DMEM,}}$ high glucose mix (50%/50%)	19.2 mL	17.04 mL	
B27 w/o Vit. A	$400 \ \mu L$	$400 \ \mu L$	
NEAA	$200 \ \mu L$	$200 \ \mu L$	
Anti-Anti	$200 \ \mu L$	$200 \ \mu L$	
FBS		2 mL	
EGF		$40 \ \mu L$	
FGF2		$16 \ \mu L$	
Heparin		$20 \ \mu L$	
ROCK-inhibitor		$20 \ \mu L$	

 Table 2.7 RPE medium formulation

RO culture When delivered from the collaborators, around 10-15 ROs were cultured on one uncoated 6-well with 3 mL of medium until on-chip integration, whereby 2/3 of the media was replaced with fresh media (see Table 2.8) three times per week. The medium could be stored up to two weeks before fresh medium had to be prepared (due to the restricted stability of media components). The ROs usually consisted of a diameter of 500-800 μ m, which enabled handling without the need of an additional microscopy. For the removal and **on-chip** placing of an RO, a big pipette tip (1 mL max. volume) could be used with an aspiration volume set to around 150 μ L. Then, the tip was placed close to one RO, and medium and RO were aspirated. The pipette was placed vertically for a precise positioning so that the RO could move towards the tip due to gravitational forces. When it reached the tip, the tip could be dipped into another medium (e.g., into a pre-filled well of a chip) and by this, the RO moved further downwards and was transferred. For on-chip culture, most of the medium surrounding the RO in a chip well was removed and filled up with 10 μL of hystem-c hydrogel. For further positioning, the organoid could be moved with a sharp needle tip (0.4 mm diameter) to be placed centrally in one well. This can prevent the disintegration of the organoid when touching the well wall.

In the case of the fourth generation, where the Side-view-chip was characterized, the ROs was cut into half using a microscissor. Tissue integration on-chip was performed similarly as described above, with a special focus on the orientation of the RO so that the cut side is facing upwards. Therefore, the chip was tilted to the side towards the transparent window, and the position of the RO could be controlled using a fluorescent microscopy (EVOS FL Imaging System, ThermoFisher Scientific).

Supplements	Total volume = 20 mL
$\overline{\text{DMEM}/\text{F12} + \text{DMEM}, \text{high glucose mix } (50\%/50\%)}$	17.0 mL
B27 w/o Vit. A	$400 \ \mu L$
NEAA	$200 \ \mu L$
Anti-Anti	$200 \ \mu L$
FBS	2 mL
Taurine	$20 \ \mu L$

Table 2.8 RO medium formulation

2.2.3 Chip sealing and chip perfusion

Once all cell types were successfully integrated into the chips, the wells were filled up with medium to supply the tissue from above. It was checked that the medium was not completely filling up and there was still a small air gap to the chips' surface. Then, the top surface of the chip was carefully cleaned with 70% EtOH and sealed with a transparent, sterile adhesive tape, that was cut into size to cover all wells with one stripe, but leaves the in- and outlet accessible - in the case of the PDMS version a simple PCR tape was used (optical adhesive covers, 4311971, ThermoFischer Scientific Inc.) - in the case of the later generations, a gas permeable tape (Breathe-easy, Z380059-1PAK, Sigma Aldrich) was used. The TPE- and Mesh-chips were placed into a 3D-printed microscopy holder, in which six chips fit. Then, a lid with integrated PDMS slab was magnetically connected to the holder, to hold the chips in place and support the sealing of the chip with the tape.

The chip connection usually contained the following setup: a syringe in a 12-channel syringe pump (LA-190, 106720190, Landgraf Laborsysteme HLL GmbH) was connected to a tubing and the tubing was connected to the chip, so that medium could be transported through the chip reaching the outlet. The outlet was connected to another tubing, transporting the wasted medium to an outlet container, such as an Eppendorf tube (1.5 or 2 mL). Being more concrete: The flexible plastic tubing with an inner diameter of 0.51 mm (Tygon, VERNAAD04103, VWR International) was connected to the metal part from a G23 needle (Sterican, 4657640, B. Braun). In advance, the plastic parts could be removed from the metal by placing it in EtOH overnight, which leaded to dissolve the adhesive from it. By this, the chips could be connected to the tube. While the other side of the outlet tubing was placed into a tube (Eppendorf) with a punched hole, the inlet tubing was connected to a 2 mL, 5 mL, 10 mL or 20 mL luer-lock syringes from B. Braun with a G23 needle. Syringe size was chosen depending on the experiment duration. Inlet tubing was cut into relatively long sizes (around 10 cm), so that all chips could be connected with the tubings to

one pump without risking any tensions of the tubing. Medium could be filled up in the syringes before connection, and excessive air bubbles needed to be removed. For this, the syringe cylinder could be pulled more, increasing the air bubble first, then removing it by moving the syringe tip upwards and carefully pushing the cylinder again into the syringe. The inlet tubing was always entirely filled before it was connected to the chip, and the direct connection took place by adding a drop from the tip to the filled channel. Ideally, using this approach, air bubbles in the channel should be avoided. The outlet tubing was connected without pre-filling. The syringe pump was set to *push-mode* using this setup. Apart from this setup, another approach was used, when substances were incorporated during a short period and directly should be added to the channel without the need to pass the long inlet-tubing in the beginning, used in the previous setup. A 20 mL syringe was disassembled from its cylinder and cut along the 2 mL line with scissors. The syringes were again connected to the G23 needle and could be autoclaved before use. Furthermore, 3D-printed lids were produced with the SLA printer to enable a closure of the syringes and a sterile handling. High Temp resin was chosen so the lids could be autoclaved and reused. The outlet in this case was connected to a long tubing pre-filled with medium, identically to the inlet tubing in the other approach. On the other inlet, the adapted, empty syringe was placed. The syringe in the pump was also pre-filled and contained a small amount of medium (around 200 μ L). It was set to push-mode until medium could be seen in the open-cut syringes. Then, the syringe was filled with the according medium and closed with the lid. Finally, the pump mode was changed to *pull-mode* for the duration of the experiment.

2.2.4 Cell and chip culture of the third chip generation with bioinspired membranes featuring additional cell types

As already mentioned, the chips usually contained open wells and tissue injection could be performed in a simple manner. As focus was laid on the system's applicability for retina-specific cell types, mainly RPE and RO were injected into the chips. Only for the third generation also the aspect of a usage for a wide range of different cell types, tissues and objectives was of interest. Therefore, additional cell types were integrated into this chip generation. Details about the procedure are published [73].

mvECs isolation and culture For the cell isolation, the subdermal layer was removed, then the cutis was cut into 1-2 mm wide pieces and stored in a dispase solution overnight at 4°C. Next, the epidermis could be removed from the pieces and the dermis strips were stored in a solution of 0.05% trypsin in EDTA for 40 minutes at 37°C for digestion. Digestion was stopped with the addition of 1% FBS to the mixture of trypsin in EDTA. With a cell scraper, mvECs could be detached from the dermis and transferred to ECGM supplemented with 1% of gentamicin. The cells were singularized twice, using cell strainers with mesh sizes of 100 and 70 μ m and then centrifuged at 209 g for 5 min. Medium was removed and cells were resuspended with fresh ECGM and seeded into T175

culture flasks at a cell density of $4-5 \times 10^4$ cells/cm². Additionally, to keep the mvECs pure and detach remaining fibroblasts, the cells were treated with trypsin for 6-10 min every second day the first week.

For on-chip culture mvECs were detached with 0.05% trypsin in EDTA for 3-5 min at 37°C. Similar to the previous presented procedure, the reaction was stopped by adding 1% of FBS to the medium. Cells were transferred to a falcon and centrifuged at 209 g for 5 min. For on-chip culture the cells were cultured in ECGM medium supplemented with 1% pen-strep instead of gentamicin. To enhance the cell adhesion on PLA-membranes, the membrane was treated with 0.01% Poly-L-ortnithine for 30 min at RT and washed with PBS. Then, the membranes were coated with laminin - as explained - by filling the channels at a concentration of 50 μ g/mL in DMEM/F12 and incubation of 2 h in the incubator. As the seeding procedure for this chip generation (ESM-chip) varies due to the different design it will be briefly explained: For the injection of mvECs (but als RPE), the cells were adjusted to a final concentration of 1×10^6 cells/mL in the belonging medium. As the chip consists of four in- and outlets, the in- and outlet of the bottom channel and the outlet of the top channel were connected to an empty pipette tip. Next, a pipette tip was placed into the last empty port, filled with 100 μ L of cell suspension. Due to the imbalance of the medium height in all pipettes, it took around 2 h until the empty pipette tips were filled up and reached the same level being equilibrated. Each pipette tip was filled up to 100 μ L/pipette tip, enabling adequate cell nutrition. At the same time, the chips were moved as minimal as needed, thereby enabling a strong adhesion of the cells on the membrane.

T-cell isolation T-cells were also integrated into the ESM-chip to test the applicability of these membranes in terms of immune cell diffusion. Therefore, human primary PBMCs were isolated from whole blood within one hour after donation using Biocoll and standard density centrifugation following the instructions from the manufacturer. After successful isolation, the cells were marked with a cell tracker (Deep Red) for 45 min at a concentration of 1 μ M in x-vivo medium in the incubator. The cells were cultured in a volume of 1 mL at a density of 0.5 Mio cells/mL for the plate control and chip experiments. To activate the cells, 10 μ L of TransAct was added to the cell suspension (0.5 Mio cells/mL).

On-chip culture was performed differently in this case, as immune cells are circulating constantly and migrating in the human body and do not adhere on one specific location. To stimulate the migration, the bottom channel was first filled with hydrogel (Hystem-C) in which a chemoattractant CCL19 and Lipopolysaccharide was mixed. For the injection, in- and outlets of the top channels were sealed with sterile tape, then a pipette tip was filled with 20 μ L of the prepared hydrogel, inserted in the inlet and the gel was pushed through the channel until it reached the outlet. Next, the pipette tip was removed and the chip was incubated for 15 min to allow for the hydrogel to cross-link. The chip was removed again and the top channel was briefly flushed with PBS- to

control it was not clogged. A filter pipette tip with 150 μ L of PBMCs suspension was placed to the outlet, while the inlet was connected to a tubing and syringe. Using a syringe pump in pull-mode with 5 μ L/h, a controlled perfusion rate could be set. The perfusion was conducted for 17 h and then the cells in the chip were examined.

2.2.5 Visualization of the tissue morphology on-chip

In each developed chip generation, the visualization of the tissue played a major readout method. Several factors thereby have to be controlled: When integrating the organoid, RPE has to stay functional without disruptions, RO has to be placed above the RPE and it has to be examined, that the segments are not shed after the placement on-chip. Therefore, live-cell imaging was often performed, which was conducted by labeling the cells with different methods, that are explained in this subsection.

Live cell labeling ROs were labeled with PNA-lectin 568 or 647 (see Table 2.9) to visualize the OS and/or the overall surface of the RO. Therefore, the ROs were picked in the according medium volume and transferred to a reaction tube. The labeling dye was added to a concentration of 20 μ g/mL and the ROs were incubated in this mixture for 30 min. They were transferred to another tube and washed with medium four times to avoid remaining unspecific fluorescence in the medium. Next, the ROs could be placed on the chip. ROs were generally labeled with PNA-lectin for several experiments, such as segment labeling in the first chip generation or the fourth chip generation to control the imaging accuracy through the side window. RPE was labeled in the experiments using fluorescent cell trackers were applied to the RPE for live-cell imaging. Depending on the experiment green or deep red dye was chosen and added in the according concentration to the standard medium (see Table 2.9). Incubation took place for 45 min, then the medium was replaced by fresh culture medium. The dyes usually are visible for 3-5 days and this labeling method was used for several experiments, which will be mentioned below in the individual experiments. Viability staining could also be performed using Calcein AM. Calcein was diluted 1:500 in medium and incubated on the tissue for 30 min, releasing a green fluorescent signal on viable cells. This dye can be used as alternative to FDA and stains viable cells. Besides, the cells were labeled with HOECHST33342. This staining can be used on living cells and no fixation is needed, but the cells cannot be cultured for a long period afterwards, as it contains toxic components. For HOECHST staining, usually a dilution of 1:250 was prepared in fresh medium and added to the tissue for 30-60 min. Later, the staining solution was removed and replaced by fresh medium.

Immunohistochemical stainings Imaging based endpoint analysis can be performed via immunohistochemical staining. With this method, tissue-specific expression can be detected. This method was applied to the PDMS generation and the ESM-chip (third chip generation). In general, tissues must be fixed in a

first step for immunohistochemical stainings.

For in situ chip staining, 4% paraformaldehyde (PFA) was added to the chips for 15-20 min. For the RoCs, in advance the foil was removed from the chip and PFA could be added on top of the chip. Furthermore, the channels were flushed with PBS and then incubated with PFA using a pipette tip. A blocking solution 5 or 10% normal donkey serum was mixed with 0.2% Triton-X for permeabilization and placed twice for 1 h in the RoCs. Next, primary antibodies were added to the blocking solution and incubated in the chips for 1-2 days, stored in a fridge at 4°C. The chips were washed with PBS three times for 2 h. Then, the secondary antibody were prepared in blocking solution and again stored in the chips overnight at 4°C. Lastly, HOECHST was added at a concentration of 1:2000 for 10 min to the tissue. In between the individual steps always the PBS washing procedure was applied.

Reagent	Stock con-	Use concen-	Product ref-	supplier
	centration	tration	erence	
Roti-Histofix	4 %	4%	P087	Carl Roth
BSA	100%	varying	A9418	Sigma- Aldrich
Triton X-100	100%	0.1 %	28314	ThermoFisher Sci- entific Inc.
$\begin{array}{c} \text{CellTracker}^{\text{TM}} \text{ Deep} \\ \text{Red} \end{array}$	$1 \mathrm{mM}$	$1 \ \mu M$	C34565	ThermoFisher Sci- entific Inc.
Lectin PNA From Arachis hypogaea (peanut), Alexa Fluor TM 568	1 mg	$20 \ \mu {\rm g/mL}$	L32458	ThermoFisher Sci- entific Inc.
Lectin PNA From Arachis hypogaea (peanut), Alexa Fluor TM 647	1 mg	$20 \ \mu g/mL$	L32460	ThermoFisher Sci- entific Inc.
$\begin{array}{c} CellTracker^{TM} \\ Green \ CMFDA \end{array}$	10 mM	$15 \ \mu M$	C7025	ThermoFisher Sci- entific Inc.
Calcein AM	1 mg/mL	0.002 mg/mL	C3099	ThermoFisher Sci- entific Inc.
HOECHST33342	1 mg/mL	1:250	62249	ThermoFisher Sci- entific Inc.
CellTiter-Glo® 3D Cell Viability As- say	100 %	1:2	G9681	Promega
CellTox TM Green Cytotoxicity Assay	100%	1:5000	G8741	Promega
fluorescein diac- etate (FDA)	1 mg/mL in acetone	3:100	F7378	Sigma-Aldrich
propidium iodide (PI)	1 mg/mL in PBS—	8:100	P4170	Sigma-Aldrich
CD31-APC conjugated antibody	100%	1:50	REA730	Miltenyi Biotec

Table $\mathbf{2.9}$ List of cell labeling and staining components

2.3 Chip characterization methods for the PDMS-chip

Different chip characterization methods were explored to investigate the physiological tissue behavior on the chips' first generation and determine whether the chip can be used as a model for drug testing or gene therapy. In this section, all the characterization methods used for the RoC PDMS-chip are listed in the following and can be found in the publication from Achberger *et al.* [85, 86].

2.3.1 Testing the chip applicability for drug testing and gene therapy

One of the main applications for the RoC can be using the systems for drug testings to have a higher accuracy with human tissues than e.g. with animal cell-based models. The system's possibilities concerning these needs were examined in the first generation, and readout methods were defined.

Drug treatment with Gentamicin and Chloroquine (CQ) Tissue was integrated on-chip as described and treated with two individual drugs to show the applicability of the chips for drug testing. Experiments were performed and evaluated by Dr. Kevin Achberger. CQ was added in two concentrations (20 and 80 μ g/mL) to the medium for 3 days at a constant flow rate of 30 μ L/h. After 3 days, the tissue on-chip was stained with HOECHST and 3 μ M PI. The chips were washed two times with PBS, imaged and then fixed with 4% PFA for immunostaining of LAMP2. Besides CQ, the chips were also treated with Gentamicin. Gentamicin was supplemented to the medium with 0.5 and 2.5 mg/mL for 6 days for this experiment. Apart from this, control chips without any drug addition were included to the experiments. After 6 days, the chips were stained for PI and imaged. Intensities were measured with ImageJ by selecting the region of interest (ROI) and subtracting background values from images taken before the tissue was labeled with 3μ M PI.

Gene therapy using adeno-associated viral vectors (AAVs) The gene therapy experiments on the PDMS-based RoC were already published. Details can be found in the publication from Achberger et al. from 2021 [86]. First, AAV2, AAV8, AAV9 and AAV2.7m8 [93] were used with ss expression cassettes ss-CMV-eGFP-SV40poly(A), ss-CMV-anti-FITC, or sc-CMV-eGFP-SV40poly (A) [94]. All vectors were prepared as described in Strobel *et al.* [95] and transfected as again described earlier [96]. AAV was purified via polyethylene glycol precipitation, iodixanol gradient, ultrafiltration. The sterile filtration was performed following the protocol from Strobel et al. [95]. On-chip tissue integration was performed similarly but RPE was cultured statically for 2 weeks instead of two days before it was connected to the pump to ensure a proper barrier integrity. Furthermore, the chip well contained an increased well, in which 27 μ L can fit. Viral transduction occurred by adding the AAVs to the well above the hydrogel. In a first experiment designed and evaluated by Dr. Kevin Achberger barrier integrity was examined. RPE was seeded, then hyrogel was

added on top and a finally the well was filled up with a defined amount of viral vectors. In this experiment, RO was excluded, as only the barrier integrity given by RPE was of interest. Two conditions were included: chips with RPE and without hydrogel and chips with RPE and hydrogel, to investigate whether the hydrogel influences the barrier integrity. The top compartments of the wells of the chips were filled up with a concentration of 1E+10 vector genomes scAAV2. sealed and connected to the pump for 6 days. In addition, control chips without AAVs were included. After the first 24 h, the AAV dose, that passed the RPE barrier was measured from the waste medium in the effluents (n = 3-4). Again, after 6 days of perfusion, effluents were collected from the last 24 h and measured. The experiment was stopped and the supernatants (n = 5, taken from wells)of 3-4 chips) from the reservoir wells were removed, analyzed, and compared with the values received from the effluents. The AAV titers were measured by digital droplet PCR with a CX200 system (Bio-Rad, USA). The deoxyribonucleic acid (DNA) was prepared with ViralXpress (Merck Millipore, USA) and droplets were developed with a Droplet Generator (Bio-Rad, USA). Denaturation took place using the X50s PCR Mastercycler (Eppendorf, Germany) with a first treatment at 95°C for 10 min, and was then continued with 40 cycles of 30 s at 95°C and 1min annealing at 60°C. The analysis of the AAV concentration was performed with the Droplet Reader and Quanta-Soft software (Bio-Rad, USA). Apart from this, the chip's capability for long-term monitoring of AAV transgene production was demonstrated by Dr. Madalena Cipriano. Therefore, three different eGFP-labeled AAVs (ssAAV8, ssAAV2, ssAAV2.7m8) were added in a concentration of 1×10^{10} virus genomes per well. One chip per condition was prepared and three wells were imaged over time. Fluorescent images were taken after 3, 7, 14 and 21 days with a fluorescent microscopy (Leica DMi8) and mean fluorescence intensity was analyzed using ImageJ and plotted using GraphPad Prism.

2.4 Characterization methods - second chip generation

The fabrication of the second chip generation is produced by different methods. The results were examined in detail to define whether the chips are appropriate for on-chip culture. The following subsections describe all the procedures for the experiments in this context.

2.4.1 Characterization of membrane flatness

To underline the need for a membrane support during chip fabrication, two chips were produced: One with and one without supporting structure. Next, RPE was integrated into these chips. After one day, the tissue was confluent and labeled with Calcein AM and HOECHST33342 for 30 min. Then, the tissue was washed and imaged with a microscopy that enables z-stack imaging (Apotome1, Carl Zeiss, Germany). Finally, the images were processed using the software Zen 3.4

(blue edition).

2.4.2 Material toxicity of connectors

3D-printed connectors were tested as alternative chip adapters to the tubing. As the supplier provided no material information about biocompatibilit, an experiment was conducted that should give information about whether the material (Elastic 50A resin) can be integrated into the chip without showing any side effects from the material itself. In total, 9 chips were included in the experiment: 3 chips with a PDMS connector - serving as negative control, 3 chips with PDMS connector and perfused with a drug (CQ) - serving as positive control and 3 chips containing the 3D-printed connectors. RPE was integrated into each well of the chip and in addition, three ROs (250 days old) per chip were added. During chip perfusion, 80 μ g/mL of CQ was added to the medium of the chips serving as positive control and a flow rate of 40 μ L/h was adjusted to the pump. The experiment was performed for 7 days. After 1, 4 and 7 days one chip of each condition was analyzed for its adenosintriphosphate (ATP) amount using **Celltiter Glo 3D assay**. ATP displays the cell activity and a high value indicates a high cell functionality. For this, the foils were removed from the chips, 1 mL of PBS- was added on top and ROs were aspirated from the wells in 100 μ L PBS- and transferred into reaction tubes. RPE was removed from the membrane by disassembling the bottom TPE layer from the PMMA part and peeling off the membrane with scissors and cutting it into 4 individual parts at the region of each well (later, this procedure was replaced by punching each well out together with the membrane using a 1.3 mm thick punch). The membrane with RPE was also placed into a 48-wellplate covered with 100 μ L of PBS. Then, 50 μ L of CellTiter Glo 3D substance was added on top to start the lysis and reaction with the cells. In addition, a standard curve for ATP was generated with rATP (10 mM, 0.5 mL, P1132, Promega). The prepared components were shaken at 1000 rpm for 30 min at room temperature using a thermomixer and by always covering the tissue to protect them from light. for the luminescence measurement, 20 μ L of the substances with lysed cells were transferred to an opaque 384-wellplate and luminescence was measured with an integration time of 1 second/well using a microplate reader (Tristar 5, 69185-55, Berthold Technologies GmbH & Co.KG). Data was plotted using GraphPad Prism 9 software (GraphPad Software, San Diego, USA).

2.4.3 Analysis of chip applicability concerning tightness independent of their flow rates

Normally, once the chip is connected to flow, the perfusion can be applied for up to several weeks. Therefore, four TPE-chips from the second chip generation and also PMMA-chips from earlier attempts were connected in a microscopy holder and covered with the according lid. Then, the chips were perfused with PBS- instead of medium, as no tissue was integrated in these test systems. The chips were placed into the incubator during the experiment to imitate real
conditions. On the outlet, the tubings were placed into waste tubes from the supplier *Eppendorf*, that were weighted daily with a conventional laboratory high resolution balance. To ensure, that every chip starts with the whole outlet tubing filled, only after droplet formation on all outlet tubing tips, the tubing was inserted into the tubes. Additionally, two syringes were connected with a tubing that was directly entered into additional tubes. These values served as control, as thereby leakage is excluded. The value of all empty tubes was also measured before the experiment was started to ensure that no variance occurs. The flow rate was set to 30 μ L/h, as a flow rate of either 20 or 40 μ L/h was usually chosen for chip perfusion. After 3 days, the flow rate was ramped up to 60 μ L/h for another 3 days. Finally, the flow rates for this tissue model, but should highlight the chip integrity. After 8 days the experiment was finished. The data was analyzed and plotted using the software with GraphPad Prism 9.

2.4.4 Characterization of chip tightness and chip materials' absorption - TPE-chip and ESM-chip

For OoCs, it is crucial that the overall chip must not be leaking, so no important media components can disappear. Apart from this, it is also especially pivotal for drug studies that the chip does not absorb significant amounts of hydrophobic molecules - otherwise it can be used only for a restricted amount of drugs, depending on their logP value. For this reason, two chip versions have been tested for their absorption behavior when using Rhodamine B: the TPE-chip and the ESM-chip. Additionally, the results from the absorption with rhodamine B were further supported with additional experiments using RPE and a known hydrophobic drug in the TPE-chip.

Chip tightness and chip absorption using Rhodamine B For the experiment with Rhodamine B, first control chips from PDMS with the same dimensions of each chip generation were produced, using the methods presented in the chip fabrications' section. For the TPE-chip, 3D-printed negative master molds were designed for the top layers and printed with the Formlabs SLA printer using Clear resin. The top layers were molded similarly to the PDMS support structure for the TPE-chip (presented in 2.1.2). For the bottom layers, the 3D-printed molds from Protolabs could directly serve as negative molds, which were dipped into a petri dish in which in advance a coated PET foil was placed and then the whole surface was covered with PDMS. Thereby, a relatively thin bottom channel layer could be created. For the ESM-chip, negative PDMS master molds from the lasercut PMMA layers were produced, that later were coated with chlorotrimethylsilane (386529, Sigma-Aldrich) to enable a removal of poured PDMS from the previous fabricated PDMS master molds (see also [73] for detailed explanation). In between the two PDMS layers a PET membrane was integrated and bonded as in the first chip generation using physical Plasma. For the experiment, a base solution was prepared in distilled water at a concentration of 5 mg/mL of the fluorescent dye Rhodamine B (83689, Sigma-Aldrich). Prior to the actual experiment, the solution was further diluted to 1:100 in PBS, following the protocol from Virumbrales-Muñoz *et al.* [97]. Depending on the chip type two different approaches were pursued:

The TPE-chips and the according control chips were incubated with empty pipette tips in the outlets, followed by placing pipette tips with 50 μ L of the prepared solution to the inlet. Every 15 minutes images were taken over a period of 13 h. At the same time, incubation was turned on at an inverted fluorescence apotome microscopy (431007-9904-000, Zeiss, Germany), which allows to closely emulate the conditions of a chip in the incubator. For the analysis, the software ImageJ 1.52p (National Institute of Health, Bethesda, MD) was used. The mean gray intensity was plotted along a line drawn orthogonal to the channel direction. All images were analyzed, but to enable a clear graph only the first and last time point (0 h and 13 h) were plotted as an overview, which shows the channel profile over the intensity. The graph was created using GraphPad Prism. Apart from this, the chip integrity could be proven from images of the chips taken after a washing step with PBS- (by flushing the channel for at least 10 times with $50 \ \mu L$ PBS- each time). Thereby remaining rhodomine dye can be visualized if it has been diffused into the material itself. In contrast, no remaining dye can be found in case it was located only inside the channel without diffusion.

For th ESM-chip, a slightly varying analysis method was chosen, where empty pipette tips on one side and filled pipette tips on the other side were placed in the same manner as the chips for static incubation with the dye. Once the channels were filled with dye, for two hours images every 10 minutes were taken with an inverted fluorescence microscopy (Leica DMi8, LEICA Microsystems GmbH, Germany) at a 10 x magnification. The image incorporated the overlapping region of both channels and also the border to the bulk PMMA material. For the evaluation, ImageJ was used and the mean gray intensity was analyzed from a defined square that was covering 50% of the channel and 50% of the bonded region of the individual layers outside of the channel. The background value of the pure PMMA material was subtracted from the individual measurements and the mean intensity over time was represented in a graph using Origin 2019 (OriginLab).

Investigation of absorption behavior using drug testing To examine the efficacy of drug testing depending on the chip material, three TPE-chips and three PDMS chips (controls) with the same dimensions were produced. RPE was integrated to the chips and after 2 days of static culture, chips were connected to the pump with a flow rate of 20 μ L/h using pull mode and cut syringe tips as medium reservoir. For the experiment, 0.5 μ M of tamoxifen was added to the medium of all chip conditions to evoke a toxic effect. In addition, CellTox Green was added at a concentration of 1:5000 to the medium. This labels nonviable cells with a disrupted membrane that lets the dye pass, leading to a reaction by activating the green fluorescent signal. Images were taken at the beginning of the experiment and after 20 hs of perfusion. The green signals were counted using the software ImageJ and the values of green signals after one day were divided through the values from the images taken at the start of the experiment. Graphs were plotted using GraphPad Prism. Besides image analysis, CellTiter Glo 3D assay was included in the experiment, and conducted as already described in 2.4.2. Tissue was removed with the membrane using a 1.3 mm thick punch and ATP was measured with a plate reader. Data was outlined with GraphPad Prism software and unpaired t-test was performed (n=12).

2.4.5 Distance measurement

For the distance measurement, three Mesh-chips were produced with the PMMA top layers and a mesh - but instead of adding the bottom layer, only the thin distance layer (50 μ m thick PMMA) was bonded below, followed by a sterile, adhesive foil covering the bottom layer and replacing the location of the membrane. This setup enabled an increased transparency through the chip and clear imaging of all cell types. For the experiment, RPE was seeded on the foil and after one day, an organoid (age: 80-150 days) was placed on top and fixed with hydrogel. Both tissues were labeled using HOECHST33342 in a concentration of 1:250 for 30 min and washed carefully with fresh medium. Then a foil was added on top of the well to seal the chip and z-stack imaging was performed on two wells per chip (n=6) with 10x magnification and z-resolution of 3 μ m/stack using the Apotome1 microscopy (Figure 2.5 a). The images were analyzed using the software ImageJ. First, z-axis profile was plotted. Thereby, two elevations (maxima) can usually be found and three minimal peaks indicated the background signal. For analysis, the mean value of the three minima was subtracted from the raw values.

For the analysis, it was clear that the first maximum indicates the location of the RPE at its peak. The second elevation indicates the location of the RO. As its structure is formed as a round ball and the overall area is surrounded by HOECHST, it is not fully clear where the distance can be measured and therefore, two theories were investigated: 1) The distance between first and second peak was analyzed, as the maximum area covered of the RO with dye should display the area with the major contact to the mesh (Figure 2.5 b, left). 2) The elevation, where the intensity of the second peak starts to be increased was plotted using a balance line, and where the balance line cuts with the x-axis was defined as the start of the RO location (Figure 2.5 b, right). The values from the z-profiles and peaks were obtained in excel and balance line was calculated, then graphs were plotted using GraphPad Prism.



Figure 2.5 Analysis of distance between RPE and RO. a) Representative 3D image of labeled tissue with Hoechst and orthogonal view of cell types, already showing the distance. Scale bar: 100 μ m. b) Graph obtained with ImageJ software showing the peaks in the z-axis profile (left) and evaluation method with balance line (right).

2.5 Characterization methods of the third chip generation - bioinspired membranes

The whole characterization methods of the membranes aimed for the investigation of its structural composition and the suitability of the membranes as tissue substrate for culture. Therefore, the next characterization methods are focusing on this purpose. The ESM was characterized with special attention and methods were defined for a proper characterization, that can be also applied for the other types of membranes. Data was already published and can be found elsewhere [73]. The main focus of the other membranes - collagen membrane and Bruch's membrane - was laid on its applicability for on-chip integration, and only preliminary chip characterizations with cells were performed.

2.5.1 Optical investigations of the electrospun membrane texture

The morphology of the ESM could be observed in a first step via brightfield imaging. Besides that, scanning electron microscopy (SEM) (ZEISS Auriga 40) images were generated to obtain a closer insight into membrane texture and fiber

morphology. SEM images were taken from the membrane and the region, where the membrane was welded with the lasercutter into the PMMA sheet. The **fiber diameter** was analyzed manually by measuring the diameter of more than 40 fibers with the software ImageJ. From these results, mean diameter and standard deviation was calculated. An approximation for the mean **membrane pore size** could be gained with ImageJ using tresholding and particle analysis of three images per membrane.

2.5.2 Imaging of tissue viability and morphology on the individual membranes

All three types of membranes were characterized concerning its functionality for tissue culture using several methods, that are explained in the following:

Cell tracker based live-cell imaging Especially for the Bruchs' membrane that was extracted from an animal it was important so prove, whether human based cells can be grow on it. Therefore, RPE cells have been labeled in a well plate using a fluorescent cell tracker (Green CMFDA). The cells were detached and transferred into a TPE-chips that integrated a Bruch's membrane and a control with a conventional PC membrane was added as well. The fluorescent signal of the singularized cells on the membrane was checked directly after seeding and again after two days, when the tissue was confluently attached to the membrane with an inverted fluorescent microscopy (Leica Microsystems CMS GmbH, DMi8).

Live-dead staining The chips with integrated ESM were made of full PMMA. For this reason, tissue viability was examined to see if the tissue is supplied with enough oxygen during static culture. For this, an imaging based live-dead staining was applied to the tissue. After static culture, one chip was always examined with this staining to ensure the cells were intact before the other chips were connected to continuous perfusion. For staining preparation, FDA and PI was mixed to PBS- in a ratio of 3:8:100. The solution was added to the upper channel inlet with a pipette tip, filled up with 20 μ L and the staining solution expanded inside the channel due to hydrostatic forces. The chips were incubated for 15 min and washed with PBS prior imaging. Using a laser scanning microscopy (LSM) (Zeiss LSM 710, Carl Zeiss), z-stack images along the membrane height were generated and images were illustrated as maximum intensity projections (MIPs) using ImageJ. In addition, long-term cultured ESM-chips with integrated RPE were also labeled with live-dead staining to control the viability of the tissue in the chips after a longer culture period. Therefore, the same procedure for staining was applied.

Immunostaining Tissue morphology and phenotype can be investigated with different staining solutions, such as tissue-specific antibodies. For the mvECs on the ESM, anti-CD31 antibody was chosen, that stains the intracellular junctions of endothelial cells. After 7 days of on-chip culture, the chips were washed with

PBS- and similar to the live-dead staining, a pipette tip filled up with 20 μ L of the prepared staining solution with CD31-APC conjugated antibody (1:50 in PBS- + 0.5 % BSA) was added to the chip. After the incubation period of 15 min at RT, the chip was washed twice with PBS- and fixed with 4% of Roti-Histofix for 10 min, similar to the procedure before, using pipette tips with a volume of 20 μ L of the specific substance. Then, the chips were washed again with PBSand stored in PBS until imaging. ESM-Chips containing RPE were fixed with 4% Roti-Histofix on day 4 for 20 min at RT. For blocking and permeabilization a mixture of 3% BSA and 0.1% Triton X-100 was added to the chips for one hour. The chips were washed with acPBS- and stained with DAPI (1:500) and phalloidin (1:50) in PBS- to visualize the cell nuclei and actin filaments. After an incubation of 1 h, the chips were washed with PBS- and covered with it until imaging.

Similarly, collagen membranes of chips resembling the RoC design were seeded with RPE from the top wells and cultured statically. The staining was performed similar to the ESM-chip, but instead of flushing the staining dye through the channel labeling was performed from above by directly adding the media through the well. In a first experiment, one chip was statically cultured with RPE and stained for phalloidin and dapi after 3 days. A long-term culture was conducted with 4 wells per condition: collagen membrane without coating of laminin and collagen membrane with laminin coating was examined. At the same time, normal cell density was seeded and only 3/4 of the normal cell amount was seeded. Additionally, the cells were seeded on conventional PC membrane with the same conditions to enable a comparison. After 15 days of static on-chip culture, all wells were stained for phalloidin and DAPI with the same staining protocol as for the ESM-chips. For imaging of all immunostainings, the LSM was used and z-stacks were acquired, that were processed to MIPs using ImageJ. For the evaluation of cell alignment on the ESMs, the images have been analyzed using the plugin Orientation from the software Imagej.

Immune cell membrane diffusion For the evaluation, whether the cells were migrated through the membrane, the chips were fixed after the experiment and stained for DAPI. For the evaluation, z-stack images were acquired with the LSM and plotted with the ZEN Black software (version 2.1).

2.6 Chip characterization of the Side-view-chip

In the fourth chip generation, main focus was to clarify the imaging accuracy through the side window. The quality was examined with a technical focus first, and then tissue was integrated to prove the imaging possibilities for tissues on-chip. All methods for the validation are listed in the following:

2.6.1 Measurement of surface roughness

Surface roughness can be measured as a first indicator for a smooth surface that can be an indicator for clear imaging. The individual steps of the side edge treatment were characterized and compared using the following three conditions: 1) surface roughness after laser cutting, 2) surface roughness that can be generated only with the frame, 3) surface roughness after complete treatment.

Both sides of the side window were investigated. To enable a measurement of the inner well wall, the chip was cut through the center of the well along a parallel line to the side edge with the laser cutter, allowing access to the inner window's area. For this purpose, the laser cutting parameters were set to 100% power, 2% speed, and 700 PPI. The surface roughness was measured with a Dektak XT profilometer, a diamond stylus and the data was obtained from the software from the profilometer. For the outer side edge a lateral length of 2000 μ m could be measured, for the opposite side of the window, the measured length was reduced to 100 μ m. A minimum of 5 chips was measured for each condition and the data was plotted using GraphPad Prism software.

2.6.2 Imaging accuracy through the side window

For the investigation of imaging accuracy, images were generated from fluorescent spheres with a diameter of around 500 μ m, which should resemble the dimensions of the organoid. Images were taken from the bottom and the side view with an inverted fluorescent microscopy (Zeiss Axio Oberserver 7) at a 10x-magnification from the same sphere. The experiment was performed on 10 different chips to receive a statement about reproducibility. In addition, the same sphere was also imaged in a petri dish as control. With the software ImageJ, the shape of the sphere could be analyzed from the generated images by manually drawing a line along the border of the sphere. Different readouts could be set with the software from the marked location, such as circularity and roundness. The results from bottom images and side view images were compared with each other using unpaired t-test and plotted using the software GraphPad Prism.

2.6.3 Imaging quality of integrated retinal tissue

RPE and RO have been integrated into the chips to prove the systems' capability for tissue analyses. RPE was seeded on the membrane, and once it was confluent, RO was added on top. ROs (age around 250 days, comprising clearly segments) were labeled with HOECHST and PNA Lectin 568. Two types were integrated: Full organoids (conventional method) and organoids, that were cut into half after labeling. By this, it could be highlighted, that from the bottom view the images looked similar, and from the side, the difference could be clearly seen. Fluorescent and brightfield images were taken with the Apotome microscopy from both sides, always using several close stacks at the region of interest. Maximum intensity projections were generated to visualize the recorded images. Images were processed with the software ImageJ.

2.7 Characterization of the light exposure setup

For the light exposure setup, the device was built as explained above (2.1.5). Different tests were performed to characterize the device's suitability for on-chip tests in an incubator.

2.7.1 Intensity profile and temperature profile

For the intensity measurements a lux meter (P 5165 Lux-Meter, PeakTech®) was placed at around 5 cm below the chip. Measurements were performed at the dark and by adjusting the brightness settings, continuous intensities could be recorded. The measurements was performed for white light, which was applied in the first experiments.

Furthermore, influence of the cooling elements could be shown by measuring the temperature profile with the DHT22 sensor with two light setup version: For the test without cooling elements, the setup only contained RGB-LED and one heat sink on top, that was fixed. The setup was placed into the incubator (ALS Incubator FlowBoxTM, Automated Lab Solutions GmbH, Germany) and measurements were taken for two hours every ten seconds. Then the same experiment was repeated with a complete light device for two hours and the resulting values were plotted with GraphPad Prism.

2.7.2 Investigations of the effect of high energy light to tissue viability

As a proof-of-concept, 8 chips were seeded with RPE and three ROs/chip (age: around 250 days). Four different conditions were included for the investigation: Two chips served as control, so they were not treated at all (condition 1). Two chips were exposed for two hours at around 5000 lx, while always controlling that the chip temperature stayed at 37°C (condition 2). These chips were connected to the pump for two days. In addition, to increase the effect of light to the tissue, four chips were perfused with 80 μ g/mL chloroquine in the media from the beginning. To ensure, that the drug was affecting the tissue, these chips were perfused for one additional day as earlier it was shown that effects can be detected after this period of perfusion (see PDMS version - 1st chip generation). Again, two chips were exposed to light for two hours before the tissue was analyzed (condition 3). Finally, to compare the values, two chips were only exposed to the drug without any light exposure (condition 4). For the analysis, ATP was measured from ROs as explained in 2.4.2 including an ATP standard curve. For the analysis of the raw data, excel was used and the values were plotted with GraphPad Prism.

Chapter 3

First chip generation -Polymethylsiloxane (PDMS) based Retina-on-Chip (RoC) system

Results from this chapter including concept, tissue characterization and drug testing have been published as

Achberger, K.; Probst, C.; Haderspeck, J.; Bolz, S.; Rogal, J.; Chuchuy, J.; Nikolova, M.; Cora, V.; Antkowiak, L.; Haq, W.; Shen, N.; Schenke-Layland, K.; Ueffing, M.; Liebau, S.; Loskill, P. Merging Organoid and Organ-on-a-Chip Technology to Generate Complex Multi-Layer Tissue Models in a Human Retinaon-a-Chip Platform. eLife 2019, 8, e46188 [85].

Furthermore, a chip version used for AAV based gene therapy testing has been published as

Achberger K, Cipriano M, Düchs MJ, Schön C, Michelfelder S, Stierstorfer B, Lamla T, Kauschke SG, **Chuchuy J**, Roosz J, Mesch L, Cora V, Pars S, Pashkovskaia N, Corti S, Hartmann SM, Kleger A, Kreuz S, Maier U, Liebau S, Loskill P. *Human stem cell-based retina on chip as new translational model for validation of AAV retinal gene therapy vectors.* Stem Cell Reports. 2021 Sep 14;16(9):2242-2256. doi: 10.1016/j.stemcr.2021.08.008. PMID: 34525384; PMCID: PMC8452599 [86].

3.1 Concept

The first RoC generation was based on a PDMS microfluidic module that comprises four open tissue chambers on the upper side of the chip (Figure 3.1 a). By having multiple chambers on one chip, the throughput for tissue analysis can be increased. Each chamber is separated by a porous membrane from the media channel below the chambers and the chambers are nourished through one straight media channel connecting the four chambers in a row. The membrane plays a crucial role as it enables tissue nutrition and at the same time protection from the shear forces of the perfusing medium. By that, it takes on two crucial functions of the endothelial barrier. The **PDMS-chip** fabrication was performed using soft lithography and replica molding, an established method in the field of OoC already presented earlier by other groups (see [50]). To integrate the physiological, complex architecture of retinal tissue into the developed RoC, ROs served as building blocks. The tissue was thereby generated in a two step approach: First, RPE cells were inserted into the system and attached to the membrane, forming a confluent tissue layer after 24 h. In a second step, one RO per chamber was placed on top of the monolayer. To avoid disintegration of the RO and to provide an *in vivo* like ECM at the same time, a hyaluronic hydrogel was added to the chambers (Figure 3.1 b). Once the hydrogel was cross-linked, it also helped to fix the organoid in a permanent position into the chamber. Next, the chip was sealed with sterile tape before perfusion of the chip was started using syringe pumps.



Figure 3.1 Micofluidic RoC. a) Image of the chip (left) and schematic of cross section of one chamber with tissue assembly. b) Seeding procedure of RPE (left) and whole retinal tissue (right). Adapted from [85], CC BY 4.0.

3.2 Investigation of retinal tissue key characteristics and drug testing of the system

The applicability of the microfluidic system for generating a physiological retinal *in vitro* tissue was investigated by Dr. Kevin Achberger. In a first step, he presented an increase in OS growth of the RO when culturing the tissue with RPE in the chip instead of conventional culture in a dish. To highlight biological studies that can be performed with the RoC, he further analyzed the behavior

of OSs during chip culture: In humans, daily renewal of the OSs occurs in the retina, as the tissue is exposed daily to high light energy levels, which stresses the segments. Once damaged, the segments are shed from the photoreceptors and phagocytosed by the RPE. Besides other important functions for the preservation of retinal functionality, phagocytosis of outer segments is crucial to maintaining human vision [22]. For this reason, it is important that the phagocytosis process also takes place in the *in vitro* model to maintain the tissue functionality over a long culture period. By labeling the OSs with a specific marker, Dr. Kevin Achberger was able to record the phygocytosis of shed OSs by the RPE (see also Achberger *et al.* for further details [85]). To demonstrate the chips' suitability for drug testing or toxicity studies, two drug experiments were performed, where chips were exposed to the two drugs CQ and Gentamicin, which both are known to have retinopathic side effects: The primary purpose of the drug CQ aims for the prevention and treatment of malaria and amebiasis, but it can also have adverse effects on the retina. The drug binds to the melanin in the RPE, leading to a decrease in lysosomal activity. The shed OSs cannot be fully digested by the RPE anymore. As a consequence, the RPE starts to degenerate, followed by an irreversible loss of photoreceptors [98]. Gentamicin, which belongs to the class of aminogly cosides, is a drug usually prescribed for the treatment of endophthalmitis. However, local administration can also cause retinal toxicity, potentially leading to vision loss. Nonetheless, the clinical development of the disease is not fully understood yet [99].

The RoC might be useful for further drug analyses affecting the retinal tissue. Therefore, Dr. Kevin Achberger exposed the chips to different concentrations of both drugs and showed cell death involved by high dosages as well as dilation of lysosomes in the RPE, which is a preliminary phase of its dysfunction. Also interesting, he showed the protective effect of RPE for the photoreceptors comparing chips with and without RPE [85].

3.3 Use of the RoC for the validation of Adeno-associated viral vector (AAV)-based gene therapy

As presented in 3.2, the first preliminary drug testings showed promising results. Therefore, the developed RoC platform was also used for gene therapy studies. In the ophthalmic field, different administration routes for the application of drug therapies can be chosen. So far, the previous presented drug experiments allowed the emulation of systemic drug administration in the eye, which means that the drug was applied similarly to administration via the blood flow. In contrast to that, in the case of ophthalmic gene therapy the AAV vectors are usually injected through the eye via intravitreal or subretinal applications (Figure 3.2 a) [100]. Therefore, the chip design was slightly adapted by adding a reservoir well on top of each tissue chamber. This adjustment increased the chamber to an extra volume of 27 μ L on top of the tissue chamber, which simplified the addition of the genes from top. At the same time, it enabled the administration of genes similar

to the *in vivo* subretinal injection. Furthermore, it enables to keep the viral vectors in the upper tissue chamber, while still being able to continuously nourish the tissue through the vascular-like channel (Figure 3.2 b). The fabrication procedure was adapted to realize this chip, and the top layers were produced in the following using 3D-printed molds instead of wafers.

For gene therapy one option can be the application of AAVs. AAVs are engineered protein shells in which single-stranded DNA genomes are encapsulated, that can be delivered to target cells by traversing its cell membrane. Therefore, the DNA can reach the nucleus where it settles [101]. To investigate, how accurate the microphysiological RoCs to serve as appropriate *in vitro* model, a cooperation project with *Boeringer Ingelheim Pharma GmbH & Co, BI* was established. Thereby, experiments with AAVs were performed (AAVs were produced and delivered from *BI*) comparisons of the effects of the AAVs on animals and chips, as well as ROs in dish culture were performed. Results from this are already published and can be found in Achberger at al. [86].

In contrast to *in vivo* models, the microfluidic RoC system showed the advantage that the tissue can be continuously examined over a long period. Therefore, a long-term experiment over 21 days with three different incubated AAVs was performed by Dr. Madalena Cipriano. The applied AAVs were labeled with a GFP-promoter and depending on the dose and vector, different intensities could be observed indicating the efficacy of the AAVs. Over the period of 21 days, a constant increase of the fluorescent signal could be detected in all different AAVs. Differences in the efficacy of the three vectors were clearly visible after 21 days, which was not apparent after only three days (Figure 3.2 d). Overall, it can be emphasized, that the improved RoC system with increased top layer enables a stand-alone possibility to continuously record transgene production in the tissue over an extended period [86].



Figure 3.2 Variables of drug injection routes and emulation on-chip. a) Presentation of two important injection routes in the eye. b) Chip design with increased chamber by addition of a reservoir compartment and schematic, how the injection routes can be emulated on-chip. c) Analysis of AAV amount in chip effluents and supernatants to define whether hydrogel impacts results. d) Analysis of AAV expression over a period of 21 days in the RoC. c, d) were taken from [86], CC BY 4.0.

3.4 Discussion

The field of OoCs has gained increasing interest in recent years, due to the possibilities that these systems offer concerning tissue architecture, mechanical stimulation and physiological tissue nutrition via a vasculature-like perfusion. Nevertheless, in the ophthalmic field existing model systems are still quite limited, especially for retina models [102]. Reason for this can be the complex, stratified retinal tissue architecture, where cells are connected in a horizontally and vertically manner being comprised of more than seven different cell types. All these aspects evolved complex challenges for developing a physiologically relevant RoC system. It was possible to overcome the challenge by integrating advanced tissue models in an engineered microphysiological system. The developed RoC design consists of open micro-wells which allow the integration of all cell types and closure of the chip after tissue injection. By that, tissue culture can be

performed in an easy way. With the application of drugs through the channel layer, systemic drug delivery could be emulated - a common method of drug application in the ophthalmic field. Furthermore, the change of chip design with increased well height enabled the emulation of another drug/compound administration - the subretinal injection. With the increase of the well, drugs can be easily applied from the top and settle down in the space between RPE and RO. At the same time this adaption of the chip design simplified the chip handling. Moreover, intravitreal injection could be also realized in the system. Due to these options, the RoC offers a flexible platform for compound testing, making the system attractive especially for pharmaceutical companies.

However, from a technical point-of-view, the usage of PDMS as material for chip fabrication can also lead to restricted possibilities for drug testing. PDMS is known to absorb small hydrophobic molecules, so when using a microfluidic chip based on PDMS for a drug experiment the n-octanol/water partition coefficient (logP) usually has to be considered. The logP value gives information about the lipophilicity/hydrophobicity and in case of low/negative values the substance can be stated as hydrophilic. For a wide range of existing drugs experimental values, but also theoretically calculated values can be found in open, available databases (see https://go.drugbank.com/drugs/). For example, Gentamic has a logP value of -3.1, which is stated to be hydrophilic. This is in accordance with the toxic effect, that was recorded when applying different concentrations of Gentamicin to the PDMS-based RoC. In contrast, another substance, Tamoxifen, with a high logP (logP of around +6.0) and hydrophobic character was also applied to the system in a preliminary experiment (data not shown). However, it did not induce toxicity, even though the drug is reported to be involved in eye diseases in humans [103, 104]. This underlines the impact of drug properties on the experiment's outcome, when using PDMS-based systems. Especially considering that the developed RoC system should be applied for drug and compound testing in the future to reduce animal models and increase physiological relevance, the choice of material for the chip housing should be turned to alternative materials with low absorption behavior. This could guarantee increased system flexibility for a wide range of compound testings.

Chapter 4

Second chip generation -Thermoplastic based RoC systems

In the field of OoC, the most commonly used material for chip fabrication is PDMS, which indeed provides numerous advantages especially for fast prototyping and conceptual phases. For the RoC, PDMS was used to demonstrate general feasibility of the concept and tissue functionality. Nevertheless, the main drawback of PDMS is the absorption of small hydrophobic molecules which limits applicability of the system, especially for drug testing purposes. Therefore, a second chip generation with focus on alternative materials with reduced absorption character was developed.

4.1 Concept & chip fabrication

To translate the first generation PDMS-chip into a system made of alternative materials, the chip design was kept constant with one slight adaption: the channel was changed from one straight channel to a parallel channel system, distributing the medium equally to four individual channels and therefore providing balanced nutrition of all chambers (Figure 4.1 a). In contrast, one single channel connecting each chamber in a row generates the artifact, e.g., the fourth chamber is exposed to less nutrients or lower drug concentration than the previous chambers.

As main chip material, PMMA sheets with different heights were chosen as they feature excellent optical characteristics and low absorption properties. Moreover, PMMA can be easily structured via a CO₂ lasercutter and thermal fusion bonded, resulting in a tight, irreversible bonding with the need of only temperature and pressure [62]. To recreate the established design with PMMA sheets, the tissue wells were divided into well and reservoir layers using two PMMA plates featuring different thicknesses (see Figure 4.1 b). Lasercutting as structuring method was applied for the top layers. For the bottom layer featuring the parallel channels, lasercutting proved to be challenging as the channel could not be cut from one



Figure 4.1 Concept of a the second RoC generation based on alternative materials to PDMS. a) Image of the chip with parallel channels filled with watercolor. Cross section of the tissue chamber and 3D-reconstruction of an image from the tissues (RO - red, PNA lectin Alexa Fluor 568; PDMS - purple, PNA lectin Alexa Fluor 647). b) Chip design with four consecutive fabrication steps: 1) The channel layers were fabricated by hot embossing. 2) The laserstructured top layers and membrane were bonded by thermal fusion bonding. 3) Flexible connectors were integrated into the top layers. 4) Previously prepared top and channel layers were aligned and finally pressed together using thermal bonding.

single piece. If this were done, the central parts would completely come off. To circumvent this, the bottom layer was divided onto two layers with a small overlaying area, whereby the channels split up and passed the wells in the first layer and on the second layer the four ending points of the channels were bundled again to one channel towards the outlet. For this approach, two 175 μ m thick PMMA sheets were laser structured. When overlaying all parts, one connected channel system could be realized, and a reproducible bonding process of the whole chip implemented (**PMMA-chip**). Although this approach resulted in the formation of a parallel channel layer, the design was prone for the creation of air bubbles especially in the overlapping channel area - depending on the accuracy of the alignment of both layers. Consequently, the fabrication approach was adapted, and hot embossing was applied. Hot embossing of PMMA is in principle possible. The subsequent thermal fusion bonding, however, showed to be non-robust leading to either leaking or collapsed channels. One potential reason is that a combination of comparatively thick materials from the top

compartments with the thin bottom layer might not be optimal, as the top layers would need a longer temperature exposure for melting up, respectively, compared to the thin channel layer. For this reason, TPE was chosen as base material for the lower channel layer. TPE has recently gained rising interest for the fabrication of microfluidics, since it features reduced absorption compared to PDMS and simultaneously enables hot embossing, self-sealing and bonding at low temperatures [60]. The material has a restricted light transmission (around 80% of transmittance compared to PDMS), which can be improved by adding a transparent thermoplastic material below, for instance PC or COC. This might reduce the surface roughness resulting in a higher transmittance than the pure material [58]. Instead of the previously mentioned materials that result in a very tight bonding, combining PMMA with TPE generates a tight but also reversible bonding. This provides the big advantage that the bottom layer can be removed in case high-resolution imaging is required and a reduced distance to the tissue is needed. The chip developed by this fabrication method will be called **TPE-chip** in the following. Overall, the implemented chip fabrication can be divided into four main steps (Figure 4.1 b):

Step 1) Hot embossing of the channel layers Here, a process in accordance to the protocol from Schneider *et al.* [58] with slight modifications was implemented: Instead of a custom-made epoxy mold for the channel structure molds 3D printed molds were used. Bonding parameters were optimized for the specific chip height and surface. Structures were embossed into TPE with a 175 μ m PMMA underneath. Using a hotpress for this structuring step, defined parameters (such as constant temperature and controlled pressure) could be defined, and reproducible structures were generated. Moreover, several parts could be structured at the same time. At this point, six parts were produced at once, but fabrication throughput could in principle be increased until the entire area of the plate in the hotpress is completely covered.

Step 2) Thermal fusion bonding of the top layers and the membrane Thermal fusion bonding of membranes encompasses a major challenge in the field of OoC, as the membrane tends to be deformed during the application of high temperature (near above the T_g) and pressure. Without any supporting structure, this would also occur during the fabrication of the thermoplastic-based RoC, since it comprises relatively big chamber dimensions (1.6 mm diameter) required to integrate the ROs (Figure 4.2 a). Therefore, a tailored bonding method was developed using support structures made from PDMS. The supporting structures contain the negative features of the PMMA top layers, so that first the reservoir layers can be placed onto the molds - followed by the well layers - also ensuring precise alignment of the layers. Further, the pillars inserted into the chambers are designed slightly higher than the PMMA (>5 μ m). This ensures, that the PDMS will be attached to the membrane placed on top and prevents deformation downwards. As the molds are flexible, the pillars are slightly pressed down during the bonding with the hotpress by the upper plate. Thereby, they are equilibrated



Figure 4.2 Evaluation of membrane flatness depending on the bonding method. Bonding a) without and b) with supporting structure was performed. Tissue wells were seeded with RPE and labeled with Calcein AM (green) and HOECHST (blue). Orthogonal view shows membrane curvature/flatness. Scale bars: 50 μ m.

to the height of the PMMA. Having the metal plate from the hotpress on one side and the PDMS on the other side, the membrane is stabilized in a flat position during the thermal bonding resulting in a flat membrane (Figure 4.2 b). As the final TPE bonding (see Step 4) requires only a heat application (60°C) way below the T_g of PMMA (10-122°C) or PC (145-148°C) [57] no further deformation will occur. To visualize the impact of the developed supporting structure approach, RPE cells were cultured for two days on the membranes until they reached confluence and were labeled with DAPI (cell nucleus) and Calcein AM (a dye that can enter the membrane of viable cells and reacts there, so that the originally non-fluorescent dye will be activated by enzymes within the cell and transformed into a green fluorescent dye). Comparing chips fabricated with and without supporting structures, a clear difference in membrane flatness can be seen, highlighting the necessity of including this fabrication step (Figure 4.2).

Step 3) PDMS molding to generate flexible connectors The following fabrication step focuses on integrating connection ports in the chip for the tubings. In the case of PDMS-chips, tiny holes can be punched into the upper layers, connecting the channels and the external environment. As the chip material itself is flexible and provides sealing, a hollow rigid metal needle connected to



Figure 4.3 Tight chip connection with connectors. a) Fabrication steps for the insertion of tight PDMS connectors. b) Alternative 3D printed connectors. c) Evaluation of 3D printed connector toxicity via analyzing ATP.

a tubing (or the tubing directly) can be inserted to the chip. In the case of the thermoplastic top layers, a different solution for the connection ports is required since the thermoplastic material is rigid. Here, a tailored approach was developed based on micro-PDMS connectors integrated into the top layer, that allowed tubing connection similar to PDMS-chips. The PDMS connectors were directly formed from liquid PDMS and cured the in- and outlet holes in previously bonded PMMA top layers. The direct molding of the PDMS has the advantage, that possible slight deformations of the holes that can arise during thermal fusion bonding do not impact the tightness of the later integrated chip connectors. For the generation of these connectors the bigger holes of the inand outlets were sealed with a tape and PDMS could be filled up into the holes using a syringe with a needle (see Figure 4.3 a, Step 1). To access the channels through the PDMS connector a hole was punched after curing. Two different hole diameters enabled punching the holes centrally into the PDMS from the top side, featuring the smaller diameter. Moreover, when removing the punch, the PDMS was not pulled out of the chip, as it was blocked (Figure 4.3 a, Step 2). Overall, the integration of these connectors enabled a simple chip culture, being versatile to connect not only tubings to the chip but also pipette tips (Figure 4.3 a, Step 3). This might be of interest in case an endothelial layer needs to be integrated in the lower channel, requiring the addition of a defined cell concentration/volume via pipette tips.

Further, tests with 3D-printed connectors from an elastic resin produced with a SLA printer (Form3) demonstrated that this could also be a promising approach, especially regarding scaling up the chip fabrication. Even though the 3D-printed connectors were successfully integrated into the chips, release of compounds from this material that can have a toxic side effect had to be tested. Therefore, an experiment with three conditions was performed: Chips with PDMS connectors (negative control), chips with connectors printed from elastic resin, and chips with PDMS connectors, treated with CQ (positive control). Three chips were cultured for each condition, and ATP amount of the tissues from one of each chip was tested at three time points over seven days. No obvious toxicity could be observed. Nevertheless, for all subsequent experiments PDMS connectors were used to guarantee biocompatibility.

Final step 4) - Chip assembly and bonding For the final chip assembly, the hot embossed channel layer was aligned to the top layer with integrated PDMS connectors and pressed together. The attachment of both parts took place at a low temperature of 60° C by applying pressure to the chips placed between two glass slides using four clamps per chip for at least 4 hours in a convection oven. This resulted in a tight bonding that was further characterized in the following.

4.2 Chip sealing and connection

Besides the chip fabrication and tight chip connectors it is as essential that the chips with open top wells can be sealed properly. Therefore, a chip holder has been constructed, that contained 6 chip inserts. The chip was first sealed with a thin gas-permeable foil. Then, a 3D printed lid was placed onto the chips with two responsibilities: First, ensuring a tight sealing of the wells and second, fixing the chips in position of the holders, which shall simplify the handling. This was especially advantageous when imaging was performed, and the chips were analyzed several times. Then, only once 24 positions of the 6 chips had to be defined and saved in the microscopy software and the settings could be used for the experiment during several days or even used for future experiments by only checking the positions and re-adjusting them if needed (Figure 4.4 a-c). The tight fixation also avoided unintended chip displacement during the motion of the microscope, when changing positions. The lid was fixed reversibly with the microscopy holder using magnetic connections. A flexible PDMS layer was integrated into the lid to enable a tight sealing of the lid with the chip. It was slightly extended, so the pressure from the magnets ensured a pressure against the chips. Finally, the lid was designed with open holes in the region of the wells, so that light coming from the microscope still can pass the wells and light microscopy imaging can be performed. Overall, this setup enabled a faster

experiment performance and simplified the careful handling with chip culture. Once sealed, the chips usually were connected to a pump. As mentioned in 4.2, the chip bonding seemed to be tight by eye. Nevertheless, the combination of PMMA and TPE leads to a reversible bonding, weaker than thermal fusion bonding. This was an intended feature from the chip as the removal of the channel layer can enable simplified tissue analysis due to an easier access to the tissue construct. Nonetheless, the chip has to withstand the pressure given by the flow rates applied on the system. Therefore, the tightness of the whole setup was tested daily for 8 days with increasing flow rates up to 90 μ L/h, which was a flow rate way higher than usually set in the RoC (20 μ L/h or 40 μ L/h). No obvious weight reduction could be recorded by comparing the weights of the effluents with control effluents, where fluid was directly collected in a tube without passing the chips. Moreover, the full PMMA-chips developed as preliminary version were included in this test setup, as those were completely bonded by thermal fusion bonding method. For this reason, they could serve as second control with a tight, irreversible bonding of all layers. Comparing the results from the three conditions, no clear difference could be observed over the full period. Also, after dismantling the setup no obvious leakage could be observed and the foil on top of the wells still was sticking tightly onto them. Summarized, up to the flow rate of 90 μ L/h the chip perfusion seemed to be successful and chip tightness did not show a difference, when comparing full bonded PMMA-chips with TPE-chips over a period of 8 days of perfusion in the incubator (Figure 4.4 d).



Figure 4.4 Tight chip sealing. a) Concept of the sealing from open top wells using magnets and a lid. b) Image of a holder with 6 chip compartments and integrated chips closed by lids that seal and fix the chips. c) Image of the chip holder. d) Evaluation of the chip tightness over time.

4.3 Characterization of small molecule absorption

As stated in the beginning of this chapter, the main advantage of this chip generation compared to the previously presented PDMS-chip should lay in its low absorption character. The analysis of this material property can be performed by filling up the chips with a fluorescent dye with known hydrophobicity, such as Rhodamine B (log P = 2.4; [63]). Readout methods with this dye have been applied previously in several research groups to test the absorption character of materials in comparison to PDMS [97, 105]. Furthermore, it has been shown earlier that the TPE material does not absorb the dye significantly when hot embossed [58]. Still, this publication focused on the bonding with other materials than PMMA. Furthermore, in this paper slightly different parameters were applied on the material for hot embossing, that can have a different effect on its properties. For this reason, a similar experiment was performed to not only show the low absorption, but also to prove the bonding quality that was assumed to be tight in the previous experiment with the weight of effluents. For the experiment Rhodamine dye was incubated in two chip types - the TPE-chip and a PDMS-chip that was produced with the same dimensions as the TPE-chip and then the course of the dye was recorded over time with the microscopy by



Figure 4.5 The absorption behavior after channel incubation with Rhodamine B. a) Left: Intensity measurement along the channel profile of the developed TPE-chip, comparing time point 0, where the dye was just added and after 13 h of incubation. Right: Representative microscopic images from the channel region of the TPE-chip at the two time points, and additionally after washing out the dye from the channel. b) Left: Intensity measurement along the channel profile of a PDMS-chip, again comparing time point 0, and after 13 h. Right: Representative microscopic images from the channel the two time points and additionally after washing at two time points and in addition after washing out the dye from the channel.

constantly taking images over a period of 13 h. Finally, the chip was washed with PBS.

For analysis, the intensity profile was compared vertically to the channel direction to examine the region of the channel edges, which - as logical consequence - are assumed to be the most critical interface for leakage or diffusion into the material. For a clear overview, only the first and the last time point were compared in a graph highlighting that the curve generated by the TPE-chip stayed at a constant level (Figure 4.5 a). The images included the interface between channel and overlapping materials. The intensity curve also stayed constant at this region (the left and right side of the graph), confirming a tight bonding between TPE and PMMA. Furthermore, the channel itself also kept being at a same intensity, which indicates that also the TPE material itself stays impermeable for this dye. In comparison, the PDMS-chip shows the opposite results (Figure 4.5 b). Already at the beginning, the dye starts to diffuse relatively fast into a small area along the channel edges. This can be seen by looking at the intensity curve generated from the images. Those have a further inclined increase in intensity towards the channel region and by considering the channel width, it confirms the presumed diffusion into the material. After 13 h, it can be seen, that the course of the curve completely changed. Most fluorescent dye has been accumulated at the channel edges. It is diffused along both sides of the channels with a width of around 500 μ m on each side - having expanded almost as wide as the channel width itself. Further, this effect could be even seen by eye, when looking at the chip at the end of the experiment. One could argue that this might also be due to leakage, but plasma bonding of the PDMS-chips usually led to irreversible bonding. Moreover, after washing out the dye from the chip, residuals remained between the layers, further suggesting a diffusion. Apart from this, in previous experiments the absorption behavior in PDMS by that dye has been proven as well [97, 105].

With this experiment, it was possible to confirm that the channel layers of the TPE-chip are bonded strong enough to the top PMMA layers. In addition, it could be highlighted that the channel material is appropriate for drug/compound testing. This is in consistence with earlier publications [63]. However, it should be considered, that only one well characterized hydrophobic dye was tested in this experiment with one logP value, so for the future it might be useful, to always include controls of planar monolayers of RPE in a conventional well plate setup to ensure, that the material does not show an effect. If very important experiments with hydrophobic drugs are performed (such as to transfer in vitro to in vivo experiments) it should be reflected whether liquid chromatography tandem mass spectrometry measurements can be included, whereby medium is taken from the inlet and the outlet of a blank chip and the amount of measurable compounds from both sides are compared proving a material inertness for a specific drug. This method is already implemented in combination with simulations by the group from Don Ingber, who developed one of the first relevant OoCs and can be used as reference [106]. However, the method is time-consuming and should be applied only when needed.

Apart from the channel characterization, it is obvious that the PDMS-connectors still are critical regarding molecule absorption (see chip images in Figure 4.5). However, the absorption is restricted to a defined small absorptive area and limited to a maximum by the borders given from the PMMA holes. Nevertheless, for the future, it should be considered to further reduce the diameters of the cut hole of the PMMA, which would be a simple and fast approach to reduce the absorptive area further. Another approach could be the change of connector types to biocompatible 3D-printed ones (as discussed in 4.2), which could also have reduced absorption properties. Summarized, comparing both types of chips it can be seen that after washing out the channels, Rhodamine dye is mainly removed from the presented TPE-chip except for the connection sides, and overall, it shows an advantage compared to the first chip generation concerning absorption character.



Figure 4.6 Drug testing experiment using a highly hydrophobic drug (Tamoxifen) with pathogenic side effects. a) Image of the experimental setup - highlighting the flow direction to keep the compound relatively close to the tissue and avoid unintended drug absorption by other components such as tubing. b) Increase of the cell death in PDMS-chips and TPE-chips (relative to the beginning (d0)) after drug exposure by comparing the mean intensity from CellTox - a fluorescent substance that stains dying cells. c) Exemplary image highlighting the advantage of chip part removal - punching out of RPE tissue from PC membrane was simplified enabling tissue analysis. d) Low values from ATP measurement from treated tissue indicate lower cell activity than high values.

4.4 Hydrophobic drug application

As the material inertness was tested from a technical point of view, the system's applicability for a hydrophobic drug should also be tested by performing a biological experiment. Therefore, tamoxifen was chosen as appropriate drug with a logP value of 5.96/6.35 (taken from https://go.drugbank.com/drugs/DB00675, 11:00, 18.03.2023). To be able to compare the effect of tamoxifen depending on the chips' material for the experiment, RPE was cultured in three PDMS-chips and three TPE-chips with the same dimensions for one week statically to ensure the formation of a confluent monolayer before the chips were connected. To perform an experiment with a hydrophobic drug, it must also be considered that the tubing itself can absorb the drug and preliminary testings of the perfusion with Rhodamine B using tubing in an experiment confirmed this (data not shown). Therefore, cut syringe tips were connected to one side of the chips and pulled the medium from that side through the chips. This paves the way to reduce the contact of the hydrophobic drug in the medium to other absorptive material before it enters the chip (Figure 4.6 a). The syringes were then covered by a 3D-printed and autoclavable lid, so that the experiment can be performed under sterile conditions. During this experiment, a defined amount of CellTox Green was administered to the chips to elucidate damaged or dead cells. This assay can be performed on living cells and enables to visualize damaged cells, as it can bond to the DNA, if the cell membrane is not intact anymore. Therefore, it enters the membrane, releasing a green fluorescent signal. Apart from that, it does not affect viable cells. As a result, it can be claimed that an increasing fluorescent signal leads to an increasing cell death. At the start of the perfusion, the chips were only perfused with medium containing CellTox green and images were taken (d0). Afterwards, medium supplied with Tamoxifen was added and the chips were perfused overnight. Finally, the chips were perfused again with medium containing CellTox and images were taken again (d1).

Comparing the mean intensity of the whole wells relative to an image taken before the addition of the drug, a stronger effect of the drug in the new chip generation was observed (Figure 4.6 b). The mean intensity was plotted relative to the value given by d0 and it was found out that the values from the TPE-chips resulted in a value of 1.1 (\pm 0.18), whereas the PDMS-chips showed a slight decrease with a value of 0.9 (\pm 0.05). Overall, the experiment showed that the values of the PDMS-chips showed no cell death. To confirm the result, the ATP amount from the tissues in these chips was also measured. A high ATP value usually indicates a high cell viability. For this assay, the cells must be lysed to reach the internal ATP. To access reproducible amounts of the tissue from the chip, the bottom and top layers of the chip were disassembled. As the bonding of the top layers with the membrane results in a highly strong bonding, the membrane was still fully attached to the PMMA layer and could be easily punched out from the wells using a punch with a diameter of 1.3 mm, The tissue on the membrane was transferred into a 24-wellplate and could be lysed there. Comparing the ATP amount from both chip types after the drug treatment again a higher cell death was observable in the TPE-chip version, which comes along with previous results from cell viability tests with CellTox green. The ATP value in this chip was at 0.39 μ M (± 0.13 μ M) in contrast to the values received from a PDMS based chip with a value of 0.68 μ M (± 0.26 μ M), containing a cell activity that was around 75% higher. Overall, the results gained from this experiment lead to the assumption that even though the PMMA-chip's connection sides were still made from PDMS, a significant dose reached the cells, resulting in increased cell death.

4.5 Generation of a controlled distance between 2D and 3D tissues



Figure 4.7 Generation of a controlled distance between RPE and RO - Meshchip. a) The concept of the distance generation with a defined spacer and a coarse mesh that holds the organoid. b) Microscopic image of a chamber with integrated electrospun fiber. c) Chip design adaption from the original TPE-chip (4.1) with increased complexity. d) Evaluation of minimal and peak-to-peak distance between RPE and RO.

As the material seemed a promising alternative to the first chip generation, a system with increased relevance was developed by generating a reproducible distance between RPE and RO. This region is called subretinal space and could be already realized in the first chip generation by adding hydrogel that filled up a small space between both tissues. However, reproducibility of the space was limited at this point depending on the handling (e.g. speed of inserting the hydrogel, which could lead to lifting the RO to a higher level than wanted). This distance plays an important role, for instance for the addition of drugs via the subretinal injection route. Moreover, it is important for OS growth. In healthy humans, the average outer physiological OS length is around 52-53 μ m depending on sex and age [107]. For this reason, another version of the TPE-chip was engineered - here called **Mesh-chip**, that allows to generate this distance and accompanied by that, further a broader application of the chip for biological studies - also focusing of the visual cycle - can be performed.

The conceptual idea of this chip version was to lift the RO with the integration of an additional distance layer on top of the membrane, on which a course mesh is integrated that can hold the RO in position (Figure 4.7 a, b). The mesh thereby must be coarse enough to let the singularized RPE cells pass through the mesh to reach the membrane below and form a confluent layer. At the same time it has to be strong enough to hold the RO and it should still be enable that OS can grow through the mesh. To realize this concept, the chip design and fabrication was slightly changed and additional layers were integrated to the system (Figure 4.7 c): Below the top layers of PMMA an additional TPE layer was attached on which a coarse mesh could be generated by electrospinning thin PU fibers. It was possible to generate a coarse mesh of straight fibers mainly orientated in one direction using a very low spinning time. To generate a distance between membrane and mesh another PMMA layer of around 50 μ m was integrated into the chip above the membrane. The membrane and the thin distance layer were bonded via thermal fusion bonding, and placed between both TPE layers. The preservation of the height given by the distance layer was briefly checked with a profilometer, which revealed that the thermal fusion bonding of both layers did not affect the material thickness. Final bonding was performed as described above for the TPE-chip. Interestingly, when some fluid was integrated into the system the mesh was barely visible, so almost no imaging restrictions occurred with the mesh being incorporated in the chip. To analyze if the mesh keeps being intact also during the integration of the tissue (RPE and later RO), and also to ensure that the RO does not lead to a sagging of the mesh or being lifted by the addition of hydrogel, the tissue was integrated and labeled with HOECHST to visualize the cell nuclei of both tissues (RPE and RO). Then, two methods were implemented for the definition of the distance. In a first step, the peak-to-peak distances of the intensities were analyzed, where the maximal intensity of RPE revealed the position of RPE and the maximal intensity of RO was interpreted as the biggest area of the surface of the sphere from the organoid. This method revealed a mean distance of 52.3 μ m (± 7.7 μ m), which coincided with the material thickness. As the maximal intensity given by the RO signal might be located slightly above the area where the tip of the

RO is positioned, the minimal distance from RPE to RO was also calculated. Therefore, the distance starting from the intensity peak of the RPE until the area, where the intensity starts to increase again was measured and defined as minimal distance. This resulted in data with narrower results of the distance being in a range of around 25 - 29 μ m (mean = 26.8 μ m, ± 2.3 μ m) (Figure 4.7 d). For this experiment ROs were chosen at a very young age (80-120 days), which usually do not comprise OSs. Furthermore, HOECHST stains the cell nuclei, which are located at the region of the inner segments in the case of segments. Therefore, the outer segments stay un-labeled. Furthermore, in a preliminary experiment also ROs labeled with PNA Lectin (labeling the outer segments) at an age of around 300 days were integrated. The experiments revealed, that the segments were passing the mesh and were in contact with RPE without being shed (data not shown). This can be especially of interest, when the growth should be further investigated.

Overall, it can be concluded that a defined distance inside the chip was successfully realized, and a method was implemented to measure the distance with two different focuses. Depending on the biological focus the integration of a mesh can be generated but should omitted in case it is not needed, as in increases time for the chip fabrication. To highlight the relevance of the mesh further biological studies might be useful with focus on subretinal events.

4.6 Discussion

Chip design and fabrication The chip was designed with four parallel channels instead of using one straight channel as done in the first chip generation. This allows a more accurate substance distribution between the individual chambers. However, it must be considered that very careful handling is required to avoid any bubble formation in the channels, that will occur more likely due to channel separation. The feasibility was shown in this chapter, nonetheless it should be decided wisely whether the accuracy is needed, or robustness is more relevant. In case the second one is more relevant, a straight channel should be chosen or at least a channel, that splits up only once and then connects two to four chambers in a row. Concerning material bonding, it was possible to generate a tight but reversible bonding between channel layer and well layer. This paves the way for more possibilities concerning endpoint tissue characterization, where cells need to be extracted from the chip. At the same time, a sufficient chip tightness for permanent perfusion was controlled and was stable for 8 days. If a stronger bonding is desired, the material could be treated with plasma before bonding. This should enhance bonding strength, as shown earlier, see [108]. Apart from this, UV-assisted bonding could be performed, which again can strengthen the bonding [62].

Chip connectors The second RoC generation was based on mainly low absorption materials. Only the connectors were still made from PDMS to enable an easy connection. As seen from tests with a hydrophobic substance, the PDMS

material from the connectors still takes up some amount of the substance, which might influence drug experiments, depending on the absorption character of the respective drug. However, from an experiment with Tamoxifen - a known hydrophobic drug - differences in viability could be recorded, when comparing PDMS-chips with TPE-chips. This indicates that the chip brings an advantage compared to the earlier developed PDMS-chip in terms of substance/drug testing. Nevertheless, if a specific amount is tested, one should be aware, that only some of the concentration might reach the tissue and a small amount still can be absorbed. In case this plays a relevant role, there exists either the option to adapt the design of the PDMS connector, or to test the absorption character from the substance in the chip. Furthermore, research on reduced drug absorption by design changes has already published, which could be also used as base [109]. As alternative, the PDMS connector could be fabricated from another material, such as the 3D-printed prototypes, for instance. An alternative low absorption material could be chosen to increase relevance, such as TPE. At the same time, changing the connectors' fabrication to another method, such as 3D-printing can also increase the chip fabrication throughput. Nonetheless, the presented PDMS connectors have the advantage of individually fitting to their belonging in- and outlets, as they are fabricated and integrated after top layer bonding. By this, a perfect fitting of the connectors can be guaranteed. If this type of PDMS connector should be used also in the future, coatings might be an option to reduce the absorption. There are several publications that already developed different coating techniques, such as TiO_2 or glass coating to give some examples [110]. Finally, as a completely different approach, besides the presented options another type of connector could be included in accordance to the connector concept presented from van Swaay [111]. This connector has three openings to not only connect the chip to the tubing but also to introduce samples to the chip which might be interesting especially for drug testing. Furthermore, with the third opening, pressure fluctuations during tubing insertion could be reduced, which could be of special interest when endothelial cells are integrated in the channel directly connected to flow.

Scalability Especially for a high throughput a robust chip fabrication method it is required, where chips can be scaled up easily. This is particularly interesting, when the chip should be applied on an industrial scale for drug or compound testing. For the TPE-chips, methods were implemented, that are relatively fast and still can be scaled up easily: The fabrication of TPE channel layers and the top layers of PMMA including membrane bonding were already produced in a high quantity using the hotpress and the processes are time-effective. Furthermore, a tight but robust bonding of two thick PMMA materials and the PC membrane was achieved in one step without membrane deformation by placing the PDMS support structures below. Nonetheless, the insertion of PDMS connectors involves several manual handling steps and overnight curing, which therefore needs to be planned with time. For the purpose of fabrication establishment it was adequate, as the invested time on the other hand guaranteed a tight connection from the chip to the tubing. Looking to the future, it might be interesting to replace the connectors - also in terms of the absorption characteristics from PDMS. Overall, the entire process worked in a reproducible manner, and can be further scaled up. Yet, some slight deformation of the surrounding chip edges sometimes was recorded, which was unproblematic. Still, it can be easily avoided by adding a frame surrounding the outer edges of the chip.

In contrast, the Mesh-chip production is still accompanied by manual chip part wrapping and electrospinning of the coarse mesh on individual chip parts. Although one spinning period takes less than one min, the process still has to be performed one-by-one for each part, accumulating much time to prepare several chips. Consequently, regarding high throughput, the mesh should be replaced by another type with a coarse pore size. Ideally, this mesh would be biodegradable, so the imaging is not restricted. Apart from the mesh generation, the fabrication method involving electrospinning did not reveal significant differences in comparison of both chip types (TPE-chips and Mesh-chips) - still being fast and robust. Furthermore, the established readout method for subretinal space characterization allows an easy adaption and comparison of alternative mesh-materials for the chip, which could speed up the translation, in case, another mesh integration method might be needed. Overall, the development RoC versions with alternative materials can be seen as promising approach towards scalability with slight adaption.

Mesh integration Having a closer look at the development of the controlled subretinal space, it can be highlighted that to my knowledge this is the first time that a defined, robust, and reproducible distance with dimensions similar to humans could be realized *in vitro*. This setup could elucidate new insights, especially for biological events in the region between RPE and OSs. Previously, in the PDMS version it was already possible to create a tiny distance between RPE and RO by the insertion of the hydrogel, that slightly lifted the RO a few micrometers. Nonetheless, the distance was not controllable in such a way as with the integration of a mesh. Furthermore, the OS growth was limited to several micrometer by the hydrogel, hence very old and matured ROs had to be integrated, already containing long OSs. With the mesh holding the young ROs in place, the segment growth could be recorded from the developmental state. Furthermore, it could be beneficial for having a closer look on interacting events of these tissues, such as in the visual cycle. It has to be critically considered that with the mesh being located around 35 μ m above the RPE, the RO is situated even farther away from the bottom than in the first chip generation. This setup can become challenging, when conventional imaging with a restricted focus depth is used as investigation method. One possibility for high quality imaging can be removing the bottom channel layer - at least for endpoint analysis. However, having the additional fragile PMMA distance layer between both TPE layers, the removal cannot be as controlled as with the simplified design (see 4.1: TPE-chip), which might be due to different adhesion characteristics and stiffness's of the thin layer compared with the thick and rigid PMMA top layers. As image-based

read-out methods are yet the gold standard for the characterization of OoCs, imaging from the side might be a possible way for easy tissue access. An approach was developed for this, which will be presented later in the side-view chapter (see 6). Looking ahead, it could be an auspicious way to combine both.

Chapter 5

Third chip generation -Chips with integrated bioinspired membranes

To increase physiological relevance, further efforts were needed to integrate alternative membranes for RPE support. In a healthy human eye, the retina is nourished through the blood-retina barrier by the choroid, which is laying below the RPE in form of a choroidal vasculature and separated by a fibrous acellular layer called Bruch's membrane [112]. To study retinal diseases, such as AMD, these two components become pivotal for *in vitro* studies of the pathogeneses. Therefore, it would be essential to integrate 1) an endothelial vasculature and 2) a membrane with improved physiological character, that resembles the architecture of the native environment. Concerning 1) - the choroidal integration on-chip - work has been already presented from Cipriano et al. [81] and the protocol could be adapted to include the choroid to the presented RoC and in addition to that develop a model with increased complexity and biological relevance. Nevertheless, for 2) materials are restricted to flat, porous polymer membranes in the mayority of OoCs. This is likely for practical reasons, such as their commercial availability [64]. Especially for OoCs made from other materials than PDMS, only few approaches exist where alternative membranes were integrated [73]. As a physiological membrane can be crucial also for other types of tissues, it is important to look further into alternative membranes that can be integrated into OoCs, that can better emulate the physiological architecture of membranes in the human body, including their 3D-character in the native ECM. For this reason, in this chapter, focus is laid on the integration approach and characterization of three different types of membranes. The section explaining ESM-integration is already published as Chuchuy J, Rogal J, Ngo T, Stadelmann K, Antkowiak L, Achberger K, Liebau S, Schenke-Layland K, Loskill P. Integration of Electrospun Membranes into Low-Absorption Thermoplastic Organ-on-Chip. ACS Biomater Sci Eng. 2021 Jul 12;7(7) :3006-3017. doi: 10.1021/acsbiomaterials.0c01062. Epub 2021 Feb 16. PMID: 33591723 [73].



Figure 5.1 Chip concept and chip fabrication. a) Left: Image of the ESM-chip filled with watercolor. Center: Microscopic brightfield image, , showing channels covered by a membrane. Scale bar: 500 μ m. Right: Confocal image taken from the autofluorescent PLA-GM2 fibers (DAPI). Scale bar: 50 μ m. b) Chip concept and composition of individual chip layers. c) Setup for generating ESMs featuring the additional non-conductive PDMS on the collector with a distance "d" to realize a flat membrane. d) Overview of consecutive fabrication steps for membrane generation: 1) Wrapping of chip part with PDMS slab below into aluminum foil and fixation with stripes. Aluminum foil on both sides should lead to directed fiber orientation. 2) Fiber mat on chip part. 3) Removal of aluminum from assembly. 4) Chip layer without PDMS slab. 5) Laser welded line surrounding channel structure. 6) Outcome of membrane. e) Laserwelding of membrane realized with a CO2 lasercutter: e1) PLA membrane. e2) PLA-GM2 membrane. Scale bars: 100 μ m. Reprinted and adapted with permission from [73]. Copyright 2021 American Chemical Society.

5.1 Electrospun membrane chip (ESM-chip) -Concept and fabrication

To develop an integration approach of alternative membranes into OoCs based on thermoplastic materials, simple chip was designed consisting of two straight, overlaying channels separated by the membrane (**ESM-chip**, Figure 5.1 a, b). The chip design was chosen to demonstrate the concept of membrane integration into low-absorption materials. It can be further adapted in a simple way to other chip designs for the integration of any relevant tissue. As main material for the chip fabrication, PMMA layers with different heights were chosen and channel layers, as well as in- and outlet-ports were laser-structured. Besides that, very thin membranes were produced (approximately 20 μ m) with an electrospinner. The following process was established to realize a robust integration on-chip without destroying the fragile membrane: Instead of spinning the membranes onto different substrates, direct spinning onto the bottom side of the top channel layer was applied (Figure 5.1 b). The layer was placed onto a 2 mm thick square PDMS slab, which served as reversible supporting material of the thin channel layer (175 μ m thick PMMA). At the same time and even more essential, this PDMS block was consisting of a non-conductive material. It reduced the locally focused conductivity into the channel structures (Figure 5.1 c). In the case of electrospinning, fibers usually are directed from the needle tip towards a conductive collector using an electric field. For this reason, the channel layer attached directly on the collector would lead to a slight reduction of conductivity at the region, where there is material and the fibers would be located mainly where they can reach the collector in a direct way - in that specific case, where the channels are structured and let the fibers access the collector without any barrier. In a preliminary approach, this method was attempted, but no flat membrane with homogeneous fiber distribution could be produced. However, generating a homogeneous fiber mat on the channel layer was possible with the PDMS support. To achieve a unidirectional fiber orientation in flow direction, aluminum foil was placed on the left and right side of the chip layer (Figure 5.1 d1). To reduce the spinning time to a needed minimum and to avoid excessive material consumption, the chip was surrounded with a non-conductive tape along its edges and placed on the collector (Figure 5.1 d2). Then, after electrospinning, the parts were removed together with the surrounding tape. The fibers were cut along the border of the PMMA and aluminum foil using a scalpel moistened with HFIP. This also provoked a membrane fusion to the PMMA and simplified the removal of the aluminum foil and PDMS slab (Figure 5.1 d3, 4). As the membranes should be integrated into the chips tightly, the membrane should not cover too much area of the chip layer, so that a maximum area of the PMMA layers were in contact with each other and hence ensuring a tight bonding. To reduce the size of the membrane, the membranes were laser engraved by surrounding the channel structure using the CO_2 lasercutter. With this approach, the fibers were melted into the PMMA material. By that, the external fibers could be removed with a wipe without moving or even destroying the fragile
membrane on the layer (Figure 5.1 d5, 6). To highlight the applicability of this fabrication process, two different kinds of membranes were integrated and found that the fibers seemed to influence the welded structure morphology (Figure 5.1) e). Nevertheless, in both cases the membranes could be handled similarly and overall, no significant differences in the handling could be observed. In a last step, all layers were aligned in a stack and thermally bonded (Figure 5.1 b). Two different membrane types were integrated, PLA and PLA- GM2. One membrane was fully synthetic and produced from the synthetic polymer PLA, as the material shows biocompatibility and is a common material for the fabrication of electrospun scaffolds, and on which endothelial cell viability has been demonstrated earlier [113]. It has been even approved by the US Food and Drug Administration for biomedical purposes, and can be found e.g. as manufacturing material for stents [114]. With its high melting point of 180°C [115], PLA is an appropriate membrane material to be used together with the thermal bonding of the chip. Besides its advantages, PLA is a hydrophobic material, on which cells usually tend to barely adhere due to a lack of cell recognition sites [116]. Several methods exist to reduce the hydrophobic character of synthetic polymers with different subsequent treatments, such as plasma treatment or surface coating [116]. Another method for adding cell recognition sites is the so called "bulk blending", where a natural polymer is mixed with a synthetic polymer. As this method also is advantageous in terms of time-reduction compared to subsequent treatments, a membrane consisting of a blend from PLA and the biopolymer GM was produced (PLA-GM2). The human ECM consists of different proteins, such as collagen, laminin anf fibronectin. Gelatin is a denaturated form of collagen with a high protein content (85-92%) and contains a similar structure as collagen, including binding moieties for cell attachment [117]. Due to the denaturation process at high temperatures, it is assumed to be a suitable membrane material that can withstand the high temperatures applied during the chip bonding process. GM has been chosen instead of conventional gelatin, as in an earlier work comparison of electrospun fiber mats made from GM with fiber mats from conventional gelatin revealed a higher cell viability on GM-membranes [118]. Usually, the application of GM involves a cross-linking, but it has also been shown that higher cell density can be obtained from blends with uncross-linked GM [119]. Therefore, no crosslinking was planned and a GM with two-fold methacrylic anhydride was used. Otherwise, in case of subsequent crosslinking a higher amount of methacrylic anhydride should be chosen [119].

5.1.1 Technical characterization

Membrane characterization The membrane morphology and possible changes arising from the thermal fusion bonding were analyzed by microscopic and SEM imaging. The brightfield images elucidated that the membranes still cover the whole channel area after the bonding process and do not show any ruptured areas. Further, a white border around the channel area had become visible, suggesting a tight sealing of the PMMA layers (Figure 5.2 a&b, 1-4). Having a closer look on the fiber structure via SEM imaging, the electrospinning process



Figure 5.2 Membrane characterization. a) Representative images of the PLA membrane before and after the application of thermal fusion bonding. b) Representative images of the PLA-GM2 membrane before and after the application of thermal fusion bonding. 1, 2) Photography comparing the membranes before 1) and after 2) bonding. 3, 4) Brightfield microscopy of the membranes before 3) and 4) after bonding. 5, 6) SEM images of the morphology of the membranes before 5) and after 6) temperature treatment. 7, 8) Changes in morphology and contact angle of the PLA membranes due to coating: 7) Before coating, featuring high hydrophobicity. 8) After coating, featuring low hydrophobicity. Microscopy images - scale bars: 500 μ m (3-4). SEM images - scale bars: 20 μ m (5-8). Reprinted with permission from [73]. Copyright 2021 American Chemical Society.

itself created membranes consisting of straight, stretched fibers with a main unidirectional orientation along the channel, which is more prominent in the case of the PLA-GM2 membrane, but can also be recognized in the PLA membrane before bonding. In contrast, especially on the PLA membrane clear effects can be seen after the bonding process: The fibers appear curled instead of stretched, which implies a wrinkled membrane. However, no clear difference could be observed from the SEM images of the PLA-GM2 membranes after bonding (Figure 5.2 a&b, 5-6). To receive further information from the images, the fiber diameter was measured. A similar mean fiber diameter of $0.6 \pm 0.2 \ \mu m$ (PLA) and $0.6 \pm 0.3 \ \mu m$ (PLA-GM2) was analyzed from both membranes after heat

and pressure application. Prior treatment, the analysis revealed a larger fiber diameter of the membrane of $3 \pm 1 \ \mu m$ and $2.1 \pm 0.8 \ \mu m$ for PLA and PLA-GM2. This is likely because the bonding above the glass transition temperature allows the membranes to deform slightly, reducing their fiber diameter. Even though no apparent differences can be seen on the PLA-GM2 membrane from the images, a reduction of the fiber diameter might conclude some morphological changes resulting in a slightly sagged membrane (Figure 5.2 a&b, 5-6). Another important indicator for the membranes is its pore size, as the fibers must be spun dense enough to give sufficient support to culture a dense cell tissue on top of the membrane. Therefore, the pore sizes were analyzed from the images and a mean pore size of 5 μ m for the PLA membranes could be measured. The analyzed pore size is in a similar range as commercially used membrane in the RoC (3 μ m for PC or 3 μ m for PET membranes).

With focus on vascularization from one side, the membrane has to be dense enough to capture the cells on the membrane. ECs have an average diameter of 13-14 μ m [120] in suspension, so the membrane seems to be dense enough for this purpose. Like the EC size, RPE has a center-to-center spacing of 14-15 μ m, which was analyzed by another group from excised retinas [121]. In relation to this information, it should be feasible to seed tissues on the ESMs. Nevertheless, it has to be mentioned that the calculation of the pore sizes was analyzed from the images of stacked fiber mats, which do not show conventional circular pores and thereby the results are not directly comparable with a conventional flat, porous membrane, such as the PET membrane. The method was only used to get an orientation, whether the fiber mat seems to be dense enough. Apart from that, coating of the hydrophobic PLA membrane can be applied to reduce the hydrophobic character on the one hand, and simultaneously, the surface becomes more homogeneous, as the pores are covered with the coating substance (Figure 5.2 a, 7-8).

Chip sealing and absorption behavior After a successful membrane integration, the tight sealing of the chip needed to be proved, which - especially in the case of the combination of ESMs with thermoplastic systems - can be challenging [122]. For this reason, the experiment has been performed again, even tough it has already been performed in a similar war for the TPE-chips. Again, by perfusing the microfluidic systems with the hydrophobic dye Rhodamine B, not only the system's tightness could be analyzed but also the absorption behavior of the bulk material PMMA. As reference, a PDMS-chip with the same dimensions was included to the experiment. After two hours of incubation with the fluorescent dye, a clear difference between PDMS and PMMA-chip was measured. To measure an increase or possible leakage into the bulk material, a ROI was defined covering 50% of the channel and 50% the area next to the channel, where the bonding should have worked. After two hours an increase from the original value of 50% to 80% was measured in the PDMS-chip. Still, in the ESM-chips, the intensity kept constant, independent of the type of the membrane (Figure 5.3).



Figure 5.3 ESM-chips characterization on absorption and chip tightness in comparison to a PDMS-chip. a) Representative fluorescent images taken at the beginning and the end of an experiment. Scale bars: 100 μ m. b) Graph showing the mean ROI intensity over a period of 120 min. Mean values and standard deviation were derived by measuring the intensity over a defined square area that covered 50% of the channel and half of it covered the bordering area of the channel. For this reason, the graph starts at 50%, equivalent to no diffusion. Reprinted with permission from [73]. Copyright 2021 American Chemical Society.

The results provide insights about a tight sealing of the chip, and at the same time it highlights the low absorptive material characteristics of the bulk material PMMA. For this reason, the chip fabrication method could be applied in terms of chips needed for drug testing while having integrated an intact physiological membrane that resembles the ECM's architecture.

5.1.2 Biological characterization

One of the membrane's main purposes is the provision of a tissue substrate for cells to be successfully cultured. For this reason, tissue viability of the two main cell types sitting on the Bruch's membrane was evaluated by integrating human skin-derived primary mvECs and the already presented hiPSC-derived RPE. As the dense stack of fibers limits the transparency, imaging via brightfield microscopy can become challenging. Nonetheless, using fluorescent microscopy, these limitations can be overcome. Therefore, live/dead assay was performed, whereby viable cells were fluorescently labeled with FDA in green and dead cell nuclei appeared in red using PI. The evaluation of the viability was of special interest as one critical consideration can be low oxygen permeability when using PMMA as chip material [5.8 cm³.mm/(m².day.atm)] [54]. This might reduce cell survival especially during static culture, where continuous oxygen supply cannot be guaranteed through perfusion with fresh media. However, after two days of static culture, predominantly dense and viable tissue layers were visible with only a few dead cells in all culture conditions, independently of cell type or



Figure 5.4 On-chip tissue viability. Cells were stained with FDA (green, viable cells) and PI (red, dead cells), white dotted line highlights overlapping channel area; cells outside of this area indicate channel misalignment. a) mvECs on PLA membrane. b) mvECs on PLA-GM2 membrane. c) RPE on PLA membrane. d) RPE on PLA-GM2 membrane. Scale bars: 100 μ m. Reprinted and adapted with permission from [73]. Copyright 2021 American Chemical Society.

membrane (Figure 5.4). Taken together, the results from this experiment suggest that the material does not restrict oxygen supply during static culture over a period of two days for the tested tissues.

In general, ECs are situated at various locations in the human body covering the inner walls of blood vessels. Therefore, they are exposed to direct shear forces from the blood flow. In contrast, cell types, such as epithelial cells, are usually protected from direct shear forces. Using these two cell types, it was also possible to investigate the two culturing setups (Figure 5.5 a, b, left). Especially for the ECs, it is important to slowly ramp up the flow rate to avoid cell detachment because of direct shear forces. Therefore, three ramping steps were applied, before a final flow rate of 20 μ L/h was set. After 7 days of on-chip culture, immunofluorescence staining was applied on the tissue using PECAM-1 (CD31,



Figure 5.5 Immunostaining of tissues cultured with perfusion on different membranes. a) Left: Schematic showing culture setup for mvECs with flow on side of tissue. Right: mvECs on PLA and PLA-GM2 membrane, stained with CD31 (red) and DAPI (blue). b) Schematic showing culture setup for RPE with protection from flow. Right: RPE on PLA and PLA-GM2 membrane, stained with Phalloidin (green) and DAPI (blue). Scale bars: 50 μ m. Reprinted and adapted with permission from [73]. Copyright 2021 American Chemical Society.

red), expressed on the surface of ECs. The signal was present in the channels on both types of membranes, showing the maintenance of the functional tissue during perfusion, and also confirming the phenotype of the cells (Figure 5.5 a). In the second culturing setup, perfusion of the epithelial cells was applied on the opposite channel, which reduces the risk of cell detachment resulting from direct shear forces. For this reason, no ramping up of the media flow rate was needed and the channels were directly perfused with a flow rate of 20 μ L/h after two days of static culture. After four additional days of culture, immunofluorescence staining was performed by using phalloidin - a substance, that labels the F-actin in epithelial cells. Again, tissue preservation could be observed (Figure 5.5 b). In addition, live/dead staining of RPE tissue cultured for 14 days on-chip confirmed the applicability of the chip for long-term experiments (Figure 5.6 a).

Apart from tissue viability, it could be also noted, that the cells adhere mainly along the fiber direction, which can be seen especially when low cell density



Figure 5.6 Long-term tissue viability and morphology on ESMs. a) Long-term RPE tissue culture on-chip. b) Representative images highlighting influence of fiber morphology to EC alignment at low density seeding. c) Distribution of tissue orientation with a predominant alignment in fiber direction (0°). Reprinted and adapted with permission from [73]. Copyright 2021 American Chemical Society.

is seeded (Figure 5.6 b). This is of special interest for ECs, that also tend to be aligned along the flow direction in physiological environments and could be engineered by the fiber direction. As with higher cell density cell orientation was not obvious at a first glance, it could be found out with the help of image analysis that in all cases the cells tend to align along the channel direction (Figure 5.7 a). This corresponds to a previous publication that has already presented a possible guidance of ECs using fibers [123]. To confirm that this effect comes from the fibers, analysis was also performed from images taken from conventional RPE culture, where no tendency towards one direction could be seen. Overall, the direction of tissues underlines how the membranes can be beneficial, for instance, in developing a physiological model where endothelial lining along the flow can be emulated.

In an advanced on-chip model even perfusion including immune cells should be enabled [81]. Therefore, it is pivotal that perfused immune cells in the media flow can migrate through the membrane and reach the tissue on the other side. To test the competence of the membranes for this purpose, an experiment including immune cells was implemented: the bottom channel was filled with hydrogel, whereas the other channel was perfused for 17 hours with media containing PBMCs that were isolated from fresh humane whole blood and activated with anti-CD3/CD28 antigens. After staining the chips with DAPI through the top channel, cell nuclei could be found in the bottom channels of the chips with PLA and PLA-GM2 membrane (Figure 5.7 a, b). Overall, the finding with cells confirms the versatility of the membranes to be used for flow-protected, shear-force guided and immune cells. Both membranes worked in terms of tissue culture without apparent differences in cell migration and attachment. Nonetheless, it has to be noted, that the PLA membrane had to be coated prior usage to add cell recognition sides and to avoid the risk of low cell attachment. This involves an additional preparation step of the chip, increasing the whole preparation time, compared to the PLA-GM2 membrane. In addition, coating of the membrane cannot be selectively performed only in the areas of the membrane, and therefore the whole channel is coated after the process. Thereby - apart from cell attachment on the membrane - unspecific cell attachment on the surrounding channel walls can happen, which might reduce the accuracy of the emulation of certain tissues. In contrast, PLA-GM2 membranes already provide cell recognition sides with the gelatin-content in the membrane. Thereby, localized tissue culture can be enabled. Furthermore, using these membranes can facilitate the cell seeding process in terms of time.



Figure 5.7 Confocal images from immune cell migration through the membranes, stained with DAPI (blue). a) Immune cell distribution in the chip with PLA-GM2 membrane. b) Immune cell distribution in the chip with PLA membrane. Reprinted and adapted with permission from [73]. Copyright 2021 American Chemical Society.

5.2 Integration of collagen membranes

As described in 5.1, synthetic membranes can be combined with proteins such as the denatured form of collagen. To go further, fully natural membranes can be a promising alternative for integrating OoCs. In that case, collagen membranes might be considered, as collagen is the the most substantial protein within the ECM and covers up to 30% of the total protein mass [124]. There have been publications where custom made vitrified collagen membranes were produced and integrated into PDMS-based OoCs [66, 125]. Apart from custom membrane fabrication collagen membranes can also be purchased. Several commercially available membranes already exist on the market, as they are widely applied in the dental sector to guide tissue generation such as for regeneration of periodontal defects or bone regeneration [126].

In this experiment a commercially available **Collagen cell carrier (CCC)** membrane from Viscofan Bioengineering was integrated into a PMMA-based chip by thermal fusion bonding, like the fabrication process for the ESM-chip. The membrane was chosen due to its small thickness of around 20 μ m. The chip design was slightly adapted to resemble the developed RoC model more precisely. Instead of a top channel layer, the new version consists of 4 chambers in the top layer and one channel layer below (similar to the first chip generation). To test whether the fabrication process including the new version of membrane also



Figure 5.8 Chips integrated with a CCC. a) Image taken from a chip flushed with a blue dye (left) and with incubated dye over 5 days (center). Brightfield image of the membrane. Scale bar: 250 μ m. b) RPE cultured for 3 days statically on a CCC and stained for phalloidin (red) and DAPI (blue). Scale bars: 250 μ m (left), 50 μ m (center, right). c) Long-term culture on the CCC and a conventional PC membrane for 15 days. Tissue was stained again with phalloidin (red) and DAPI (blue). Scale bars: 150 μ m.

resulted in a tight chip, the microfluidic system was flushed with a colored blue dye. A dye diffusion into the whole membrane could be observed (Figure 5.8 a, left). This illustrates that the chip itself is bonded tightly at the region where PMMA faces PMMA on the other side, and the membrane is fixed in between but still seems intact, independent from the direct pressure, that took place onto the membrane from two sides. To reduce the diffusion beyond the channel, the membrane size could be reduced or even separated into four independent membranes in case where a chip experiment is performed with four individual wells/conditions on one chip. Interestingly, after chip fabrication a predominant flat membrane integration could be noted (Figure 5.8 a, right). This underlines the hypothesis that the thermal bonding step did not significantly affect the membranes.

To further examine the CCC as appropriate membrane for the integration in a RoC, RPE has been seeded on the membrane. An initial test revealed that the cells formed a confluent tissue layer on the membrane within three days of static culture. Using phalloidin (red), a clear side-by-side cell arrangement can be visualized (Figure 5.8 b). What stands out from these images is, that despite the appearance of a flat membrane - the tissue seems to have formed in a slightly wavy way. Reason for that might be the topological structure in form of collagen fibers on the membrane.

Further experiments revealed that the tissue culture on this membrane can be quite challenging, as after several days the tissue on the membrane starts to detach. Therefore, investigations concerning the temperature/pressure treatment have been undertaken, but no differences in both conditions could be found (data not shown). It also did not make a difference, if chips were cultured statically or with flow. To investigate, whether cell number or coating can be an influencing factor on cell adhesion, an additional experiment was performed, where cells were cultured on both, CCC and a conventional PC membrane. Results from this experiment clarify, that appropriate conditions for long-term tissue culture on these membranes have yet to be found for RPE. Reason for this might be, that the cell number has to be further modified, as successful culture on these substrates have been shown earlier, whereby seeding density played a critical role [127, 128]. Looking ahead, closer biological studies would be needed to fully define whether the membrane can be a suitable replacement. At least from the technical point of view, the membrane integration was successful.

5.3 Integration of decellularized Bruch's membranes

The use of decellularized Bruch's membranes from animals, like pigs offers once again new possibilities. One big advantage from using these membranes is that the decellularization induces the removal of animal tissue. Still, the membrane's mechanical, biological and structural properties can be maintained [129]. Released from the animal tissue, the membranes can be seeded with human cells and a human model can be built up. This has been shown already earlier by the Maqueda group [129]. Apart from that, pig eyes are usually a waste product from slaughterhouses, so accessibility is easily enabled. Due to the advantages given by this biological membrane, an integration approach into OoCs was explored. In collaboration with the Clark Lab from the Institute of Ophthalmic Research in Tuebingen Bruch's membranes were extracted from pig eyes based on previously



Figure 5.9 Integration of a biological Bruch's membrane into the chip. a) Membrane extraction and insertion into the chip. 1) First incision to cut around the cornea. 2) Cut eye with pigmented RPE-choroid facing up (dark area). 3) Placement of decellularized membrane between two PET layers. 4) Cutting the membrane into size while sandwiched between the layers. 5) Membrane placed on PMMA layer. 6) Complete membrane integration on-chip. b) Comparison of RPE seeding and attachment on PC membrane (left) and pigs' Bruch's membrane (right). RPE cells labeled with green CMFDA and images were taken on d0 and d2 on the membrane. Scale bars: 250 μ m.

established protocols (Figure 5.9 a1, 2) [88]. Once the membrane was extracted, it had to be flattened. As the membrane contracts, as soon as it is removed from liquid, the membrane was first flattened in a big drop of PBS and then removed by sliding a piece of overhead foil underneath the membrane before lifting it.

Using this approach, it was possible to keep the membrane flat. As the membrane still was wetted with some liquid, another foil was placed on top of the membrane to remove the excessive fluid and let the membrane dry (Figure 5.9 a3). This also gives the advantage, that the fragile membrane can be cut in size, as long as it is sandwiched between the two foils, that are giving support (Figure 5.9 a4). Usually, one foil can be removed easily. The membrane sticks only to one foil. The membrane was positioned with the foil on the chip to integrate the membrane into the chip. Then, the foil was slowly bent at the edges of the membrane, which evolved a detachment of the membrane. In case it did not completely detach. tweezers could be used as help, whereby care had to be taken not to rupture the membrane with their tips. Alternatively, a small amount of PBS could be applied, which should not completely wet the membrane again and only help to disassemble the foil from the membrane. Then, the chip could be fully assembled and with a short thermal bonding step a tight integration of the membrane could be realized (Figure 5.9 a5, 6). To investigate the suitability of the membrane for on-chip tissue culture, preliminary biological investigations were performed by seeding RPE on the membrane. The cells were labeled with green fluorescent cell tracker to be able to see whether cells are sitting on the membrane. As control the conventional PC membrane was used, on which successful tissue formation was demonstrated earlier. Compared to this membrane, the porcine membrane shows similar cells sitting on the membrane, whereby the membrane topology again seems slightly curled. Furthermore, brightfield imaging might be challenging with the Bruch's membrane still containing residuals of pigments. Summarized, the integration approach was successful from a technical point-ofview. Nevertheless, further biological experiments would be necessary to evaluate membrane characteristics after the temperature application and tissue formation over a longer culturing period.

5.4 Discussion

Three different types of membranes were presented, starting from mainly synthetic membranes (PLA-, PLA-GM2 membranes) that only contain the resemblance of the native ECM due to the architecture of 3D stacked fibers by the fabrication method of electrospinning. Next, biological membranes, that fully contain proteins existing in the native ECM can be integrated, and even one step further, actual Bruch's membranes can be extracted from animal eyes and integrated, that contain all relevant structures and mechanical properties close to the Bruch's membrane from the human eye.

Handling & scale-up Comparing all types of membranes, even though the last presented type of membrane initially seems to be quite promising due to its beneficial characteristics it will be challenging to scale up the process, as the extraction of the thin and fragile membrane (2-4 μ m [25]) is quite challenging. Therefore, one membrane extraction can last around 30 - 60 min. Further, integrating material from different donors can increase inter-donor variability,

hence increasing experimental variability. In terms of scaling up, the collagen membrane would be the most promising material, as the membranes can be commercially acquired in a big amount and do not need to be greatly prepared. However, using this membrane, no long-term culture was achieved so far; therefore, the membrane would have to be further explored. At least on the manufacturer's website (https://viscofan-bioengineering.com) it is stated, that bovine RPE has been cultured already on the CCC. If the membrane should be further investigated, it would be beneficial to contact the company for further advice. The PLA-GM2 membrane might be a compromise between the two other membranes. because it is not commercially available, but can be produced relatively fast (3 min spinning time) compared to the Bruch's membrane extraction. As it contains only a small amount of gelatin it is also quite cheap in comparison to the collagen membranes. Most importantly, long-term tissue culture has been succeeded on these membranes. Looking ahead, for the conversion to this type of membrane, it might be useful to examine further the mechanical characteristics, such as elasticity which have not considered so far.

Chip fabrication Concerning the chip fabrication it was possible to integrate all types of membranes, and for Collagen and Bruch's membranes no significant differences before and after the membrane integration could be observed. In contrast, the ESMs have slightly deformed due to the temperature application, which might impede cell culture, but also imaging. For this reason, all membranes have been integrated in a preliminary test into a chip featuring a TPE bottom channel and an additional TPE layer below top layer (similarly to the presented version 4.1). This has the great advantage compared to the presented method, that no further temperature application on the membranes is needed after the chip fabrication, due to the sticky character from the TPE itself. It has been also successfully tested, whether the ESMs can be laser engraved into the TPE. As outcome, the generation of four individual membranes can be realized covering only the individual chambers separated from each other. Overall, the whole process was transferable without any significant adaptions. This might be the most obvious way to continue for future applications, as it does not require the characterization of the membrane deflection in terms of the influence from the temperature during chip fabrication when bonded at temperatures around 50°C.

Imaging One critical aspect that arose for all alternative membranes is the fact, that imaging became quite challenging, especially for brightfield microscopy. The ESM appeared opaque from brightfield imaging and only fluorescent microscopy could be applied for tissue imaging. However, cells could be barely recorded from all the presented membranes using brightfield microscopy - even though collagen membranes for instance seemed to be mainly translucent. The surface structures on the membranes given by the ECM/Collagen fibers thus may play a significant role. Consequently, only fluorescent imaging can be performed using these membranes. Ideally, confocal microscopy would be applied, where via z-stacks information from different height levels can be gained. Overall, using these

types of membranes gives great advantage in resembling the native structure of the ECM and thereby increases the relevance of the model. Nevertheless, it has to be carefully considered for which purpose the chip is needed. If the membrane structure plays a relevant role for the model system, it can be reasonable enough to increase the complexity of the chip fabrication process. In case of the development of a disease model, such as AMD for instance, the membrane plays a crucial role and especially the ESM could be beneficial, as the formation of an excessive vascular networks leads to a growth of blood vessels towards the RPE and finally they disrupt the RPE monolayer. To emulate this event, the vessels have to pass the membrane, which might be feasible only in a very restricted way regarding a rigid membrane, such as the PC membrane.

In summary, the chapter presents different approaches for membrane integration on-chip, which all could be generated and integrated using similar approaches. Depending on the purpose of the chip, the usage of these membranes might be an interesting alternative to conventional membranes.

Chapter 6

Fourth chip generation -Chip enabling optical accessibility of the tissue from two sides

Although several read-out methods exist in the field of OoC, such as effluent analysis via colorimetric or luminescence assay, or sensor integration, imaging still is the gold standard for tissue analyses [51, 130]. Especially in the case of the Retina-chip, where complex 3D tissues are cultured, it would be beneficial to access the tissue quickly without the need for expensive, time-consuming microscopy techniques, as given by confocal imaging. Mainly terminal analyses can be performed or a microscopy with integrated incubator must be chosen for live-cell tissue analysis.

For this reason, a chip was developed that can be rotated by 90° to enable imaging of the tissue chamber from two sides, called **Side-view-chip**. Results from this chapter were conducted together with B.Sc. student Dominic Baum and M. Sc. student Dominick Ntamag in the framework of their thesis supervised by me.

6.1 Fabrication of a transparent side

Concept To access the tissue in the chip via imaging from two sides, two main specifications have to be considered: 1) It is pivotal, that the tissue chamber is placed in close distance to the chips' edge and 2) the chamber should be designed in form of a rectangle to be able to realize an image from the side without any image distortions. With consideration of the previous presented points, a microphysiological system was designed, which on the one hand enables imaging through the bottom view in a conventional manner (Figure 6.1 a left). On the other hand - by having the chamber close to the side edge - the chip can be rotated by 90° and the orthogonal view from the chamber can be captured

Chapter 6 Fourth chip generation - Chip enabling optical accessibility of the tissue from two sides



Figure 6.1 Concept of the microfluidic chip platform. a) Presentation of the chip setup for microscopic imaging, accessing the tissue from the bottom (bottom view) and the side of a rotated chip (side view). b) Representative image taken from the lateral side of a chip, where a RO can be seen by eye.

(Figure 6.1 a right). Similar to the previous presented chip design (4.1) TPE was used as channel layer and two PMMA top layers as tissue layer, which include the window to the tissue chamber (Figure 6.2 a, b). PMMA for the tissue well fabrication has been chosen, as with this material highly transparent sides can be processed with an appropriate surface treatment, that allow to see the tissue even with the naked eye through the side (Figure 6.1 b). The design in this case again solely serves as proof-of-concept containing only one chamber but can be easily scaled up to several tissue chambers in a line.

Chip design and fabrication To realize the fabrication of the Side-view-chip, the design was arranged in form of two PMMA top layers, a PC membrane below and a bottom hot embossed TPE channel layer, that shall close the system (Figure 6.2 a). To create a side window, the dimensions had to be chosen as a compromise between thin window close to the tissue and having a wall that will not break easily during the bonding process. Therefore, a side window thickness of 1.1 mm was defined (Figure 6.2 b).

To produce a smooth side edge, several fabrication steps were involved. First, the plain PMMA material was initially structured using a CO2 lasercutter, leading to accurate structures on a macroscopic level. Yet, it is known that with this structure method, a surface roughness around several hundred micrometers can usually be realized [131, 132]. For this reason, the top layers were further treated: They were inserted into a custom-made metal frame containing the PMMA layers' negative structures. Moreover, the frame is divided into two parts with a removable side wall, that can be fixed with two screws to the main part (see Figure 6.2 c1). When assembling the top layers in the frame and fixing them with the screws, tight pressure to the critical areas was enabled. The frame was ordered from an external company, that unfortunately could only provide the fabrication of the frame with a minimal surface roughness of average roughness (Ra) 0.4 led to additional surface treatment for the inside and outer side edge from the PMMA sheets for a transparent view. Hence, two thin and



Figure 6.2 Chip design and fabrication of a transparent side. a) Overview of the chip composition. b) Detailed chip design including dimensions. c) Steps for realizing a transparent window: 1) Top layers were placed into a custom-designed frame consisting of the negative structures. 2) Thin, flexible layers were inserted between top layers and frame on critical surface areas for improved surface smoothness. 3) Six chips were prepared in parallel and bonded with the membrane using a hotpress. 4) Microscopic images, highlighting the effect of final polishing on surface roughness in comparison to an edge produced by lasercutting. d) Comparison of two windows, left: produced using only the frame and with complete treatment including the polishing paste. Scale bars: 100 μ m (c, 4, d).

flexible PET layers were inserted to the critical areas to obtain a smooth side edge: A straight, angular piece was placed on the external side edge of the chip, and in case of the inside wall, a tiny piece of the same flexible layer was attached to that side of the pillar in the frame, that is in contact with the appropriate inner side wall (Figure 6.2 c2). Once the PMMA layers were assembled with the flexible layers in the frame and fixed, the membrane was placed on top of the layers and the whole assembly could be thermal fusion bonded (Figure 6.2 c3). By applying a temperature above the T_g , it was possible to bond the two top

layers and the membrane tightly. At the same time, being above the T_g , the material melted and became deformable, leading to an adoption of the frame's geometry, including the flexible layers.

Lastly, once the parts were removed, the treatment of the side edges could be finished with a manual polishing step, which smoothed the surface and can be performed unproblematic for the outer side edge. Overall, the manual polishing is a method to reach high transparency (see Figure 6.2 c4), but in the case of the inner side wall it cannot be applied in an easy and reproducible way. Further it has to be taken into consideration that this step involves manual handling, which means it can vary from individual persons, when performing this treatment step. After the full treatment of the top layers, the chip was assembled and bonded as previously described (see 4.1). The process seemed to lead to a tight bonding again, but it should be critically reflected whether the thin contact on the side might be sufficient for long-term perfusion of the built chips. Therefore, it should be considered to include an UV-assisted bonding in the fabrication according to the publication from Busek *et al.* [62], where bonding strength between PMMA-TPE could be successfully proven.

To examine the positive effect of the side edge treatment with the additional flexible layers, a comparison of a chip built up only with the frame without foil or polishing paste, and a fully treated chip revealed a clear difference when inserting a fluorescent bead. A clear round bead can be imaged with the full treatment, whereas for the chip fabricated only with the frame, no sharp border from the bead could be focused (Figure 6.2 d).

6.2 Technical characterization

As seen from the image 6.2, the accessibility of tissues through the side window seems promising. Further investigations were performed to analyze the surface roughness of both walls on the one hand and to compare the treatment of both sides. On the other hand, imaging accuracy was analyzed, to prove that in a further experiment with tissue culture, the actual tissue can be represented through the side window without any distortion.

Surface roughness To characterize the roughness of both sides of the so called "window", the top chip layers were lasercut after treatment along the center of the chamber in parallel to the outer edge. With this, the walls can be easily accessed from both sides.

To examine the influence of the individual side-edge fabrication steps, roughness measurement was taken from 1) the edges resulting from the lasercutting step, then from 2) the edges that were generated by using only the frame, and finally, surface roughness from the 3) ultimately full treated side edge was measured. At a first glance, it can be already seen that each treatment step led to a reduction of the surface roughness (Figure 6.3 a). Having a closer look on the roughness values, the lasercutting step produced an Ra of 0.6 μ m (± 0.14 μ m, n=9) for the outer side edge and value of around Ra = 1.1 μ m (± 0.27 μ m, n=9) for the



Figure 6.3 Technical characterization of the imaging accuracy from the side view. a) Comparison of side edge and inner well roughness depending on treatment method: Analysis was performed on lasercut edges, a treated edge pressed only with the frame, and the edges from a completely treated chip. b) Overview of roundness and circularity difference, taken from [133], CC BY 4.0. c) Analysis of roundness and circularity from the same sphere, imaged in 10 different chips from bottom and side. Dotted line indicates sphere, that was imaged in a petri dish as control.

inner well wall. The bigger surface roughness from the inner well wall can be attributed to a very short straight cut, bordered from curved edges. Nonetheless, the values are in consistency with other publications, where focus was laid on the optimization of cutting parameters using a CO2 lasercutter, By that, surface roughness with minimal values of several hundred nanometers up to a few micrometers could be obtained after lasercutting optimization [131, 132]. For the side and inner well edge created by only pressing the frame on the walls, an average Ra of 0.2 μ m (± 0.16 μ m, n=6) for the outer edge and Ra = 0.2 μ m (± 0.03 μ m, n=5) for the inner well could be realized. Interestingly, the surface values are slightly smoother than the original value from the metal frame, which is supposed to have a Ra of 0.4 μ m. This can indicate that the melting up of the material does not lead to a full adoption to the frame, which might be beneficial in this case. However, especially in the case of the side edge, a high variability in the roughness values can be noted, as the standard deviation with a value of

0.16 μ m is almost as high as the surface value itself. Consequently, the values do not show a clear trend (in particular the side edge values). Nonetheless, the results generally indicate a clear improvement of surface treatment compared to the lasercutting structuring.

Finally, the fully treated chips result in a side edge with Ra of 0.02 $\mu m \pm 0.01 \mu m$ (n=10) and the inner well wall roughness was reduced to Ra = 0.05 μ m (± 6 nm, n = 10). As observed in all measurements, the values for the inner well wall were slightly bigger, most likely due to the increased complex geometry, compared to the straight outer side wall. Interestingly, for the flexible layer itself a mean surface roughness of Ra = 0.02 μ m (± 3 nm, n=6) could be measured from different locations within the foil, consistent with the roughness of the outer side edge that was additionally treated with polishing paste. This leads to the assumption that no full adoption of the surface roughness occurred here, yet a very smooth surface could be generated. It is also important to mention, that although the final polishing step involved a manual handling, it seemed possible to perform this treatment in a predominantly reproducible manner, as the roughness of 0.02 μ m only had a low standard deviation of \pm 6 nm. Comparing these values with a glass slide with an average surface roughness of $Ra = 0.04 \ \mu m$ or a silicon wafer with 0.07 μm [134], the gained results from this experiment highlight an adequate surface roughness for imaging. One of the biggest advantages using glass in the field of OoC is the excellent optical transparency of the material [55], and at least concerning the surface roughness similar roughness values can be realized with the presented treatment. It has to be considered, that measurement was only performed in one direction along the edge, so information about vertical topography might be missing.

Imaging accuracy Apart from the surface roughness, the chip must be investigated concerning imaging accuracy. To analyze, whether a curvature or other effects reducing the precision of images, a round fluorescent bead has been analyzed concerning roundness and circularity outside of the chip and later integrated into the chip to see, if the same information can be gained from imaging through the membrane from the bottom, and most importantly, through the side window. The analysis of the roundness demonstrates that the average roundness values (R) were almost the same in bottom and side view, measuring a value of $R = 0.96 (\pm 0.02; n=10)$ for the side view and a marginal different value of $R = 0.96 \ (\pm 0.03; n=10)$ for the bottom view. Similarly, a roundness value of R = 0.98 from the bead in the petri dish (n=1) could be measured. All in all, the roundness of the sphere still seems to be given and no distortion in form of an oval shape could be found, indicating that the wall contains two flat and parallel walls, and no significant deflection could be detected. Concerning the measurement method, it should be critically reflected that the analysis of the border from the bead was performed by manually drawing a line around the circle. This method could be improved using alternative analyzing methods such as threshold analysis via imageJ. However, this readout can only be performed when analyzing beads not located next to a border, as this border might restrict

that the treshold only includes the bead an also considers this area for automated analysis. For this reason, in this experiment manually drawing was chosen.

As roundness only takes into account whether the overall circle is round or oval, the circularity can be analyzed: The circularity (C) also considers, if the border of the sphere arises from a straight line from a curled line. Again, the circularity from bottom (C = $0.93,\pm 0.01$, n=10) resulted in slightly bigger values compared to the side view (C = $0.91, \pm 0.02$; n=10), which could be an indication for lower accuracy. Nonetheless, the control in the dish (n=1) revealed an even lower value of 0.89 (Figure 6.3 b). This leads to the conclusion, that the main imaging accuracy is given within the range of the values. However, it has to be considered again, that the analyzed data was generated by manually tracing the borders of the circles, hence some detailed information might be missing. Nonetheless, conspicuous effects of imaging distortions would have been disclosed with the presented method.

6.3 Tissue visualization

To test the system with respect to imaging capacity of the actual tissue, full or cut ROs were integrated and imaged from the bottom as well as from the side (Figure 6.4 a). The cut version has been chosen, as this would offer several new possibilities, such as for drug testing (intravitreal injection routes could be enabled) or for the development of an outer blood retinal barrier or an optic nerve on the cut side. Attempts on this were explored earlier, yet it was critical to position the RO in the right position in the well, and it was very difficult to check the right orientation - meaning the cut side facing upwards.

For further investigation the following experiment was performed: RPE was integrated, previously labeled with celltracker to ensure that cells are grown on the membrane. After two days of culture, the membrane was fully covered with RPE and the RO could be placed on top. To see the RO clearly, it was labeled with HOECHST (blue) to mark the cell nuclei and also with PNA lectin 568 (red) to highlight the OSs around the RO. Then, the RO was inserted in the chip and the tissue well was filled up with hydrogel, which resembles the physiological environment for the tissue but also gives the advantage, that it holds the tissue in place even by rotating the chip.

When images were taken from the bottom, both - full and cut-opened ROs looked similar and could not be clearly distinguished, as imaging through the pigmented RPE and then the dense tissue of the RO usually restricts the imaging to few hundred micrometers and the RO can only partially be imaged from the bottom up to few hundred micrometers. Consequently, with the mean RO size of 500 - 800 μ m the imaging capacity does not even allow reaching the cut area and from the bottom both spheres looked similar. Only the change in the round spherical shape from the cut organoid into an oval shape, indicated that the RO was cut, still not uncovering whether the cut side is facing downwards or upwards (Figure 6.4 b&c left). Moreover, the RO shape change resulted from the cut, that created a compression of the tissue. The cut was set shortly



Chapter 6 Fourth chip generation - Chip enabling optical accessibility of the tissue from two sides

Figure 6.4 Tissue visualization on-chip. a) Concept for comparing bottom and side view from two types of ROs. Representative images taken from both positions and both types of RO: b) Cut RO and c) full RO was labeled with PNA lectin (red) and HOECHST (blue) prior seeding, and images highlight that from bottom view both images look similar and only from the side it can be distinguished whether the RO is full or cut. Scale bars: 250 μ m.

before RO was placed in the chip. As the deformation is reversible and the RO formed into its original shape after a few hours the shape might not be a meaningful indicator. Furthermore, every RO is individual in its shape and the do not generally comprise a perfect round sphere. In contrast, when rotating the chip, images clearly reveal the difference between the cut version and the full RO (Figure 6.4 b&c right). The Side-view-chip can also be beneficial in correctly positioning of the cut RO inside the chip, as subsequent moving of the RO directly after hydrogel insertion was easily manageable using either light or fluorescent microscopy and a needle while having the chip in its tilted position. This also indicates, that the hypothesis of an automatic positioning of the cut RO in its right direction after insertion could not be confirmed. This might be user-dependent, but it should be considered for future experiments with cut ROs, especially when not using this chip version including the side "window" and a control can be possible only after the experiment using confocal imaging.

6.4 Discussion

The application of this chip version can be a promising approach for further OoC experiments, where complex tissues are integrated and (fast/live-cell) imaging was a limitation factor so far. The system offers a wide range of applications for other complex cell types/tissue than for the presented cut and full RO. So far the chip has also been successfully tested for other applications, such as for immune cell integration embedded in a 3D hydrogel, highlighting that resolution is also given on a single cell level. Furthermore, the migration of two fused organoids was recorded using live-cell imaging (data not shown). Therefore, the system seems to be a powerful tool concerning the investigation of different tissue types. Besides the presented advantages, it must be carefully considered, that the membrane integration on the chip still can be challenging, as the PMMA sheets sometimes can have a relatively high tolerance in height (up to 10 %) due to their casted fabrication process. As a result, it can happen, that the material is not fully reaching the height of the frame itself. When the membrane is attached on top of the tiny, rigid pillar, the membrane will be pressed to the PMMA, but an imprint of the pillar will be left. To minimize the variation in the materials' height, extruded PMMA sheets could be used, as they should be very accurate in its height. Another approach could be to cast the whole top part directly from PMMA using some acrylic resin, and thereby even further reducing the treatment steps, as no cutting would be needed and depending on the surface of the master mould, even no thermal pressing would be needed for the surface treatment anymore. Apart from the presented alternatives, a frame with smoother surface roughness would create a more robust process and save some time. A system was developed with main focus on the treatment of the side, but so far little attention was paid on the tightness of the chip. It can be discussed that the chip tightness of the TPE-PMMA complex was presented earlier in another chip design (see 4.2). However, as the contact area of both materials in the window region is restricted to a width of only about 1 mm, additional surface treatment of the materials in form of UV treatment [62] or plasma treatment [108] could be performed to improve the bonding strength.

Concerning the innovation of the setup, in the field of OoC only one research group presented a similar approach so far, sandwiching a membrane between two rectangular open-cut glass capillaries [135]. Although the approach seems interesting, the system is restricted to one straight channel focusing on the analysis of barrier tissues. Due to the usage of glass capillaries, the chip design cannot be changed in its geometry. In contrast, by using this approach with PMMA as main material and several post-processing steps, the design of the chip can be individually adapted. Alternatively, only expensive 3D-printing can be used for complex glass geometries. Summarized, especially in terms of costs and scale-up, the presented model can be an outstanding system for new investigation approaches, such as live-cell migration analyses of complex 3D-systems, which cannot be easily imaged through the bottom side. Also with regard to the presented Mesh-chip version where an additional mesh is integrated to hold the RO in a defined distance from the RPE (see 4.5), the side window could significantly facilitate imaging.

Chapter 7 Chip illumination unit for controlled light exposure

The previous chapters focused on bioengineering the microenvironment of the tissue in the RoC system. Having a look on the ambient influences on specifically the retinal tissue, light plays a crucial role. The human retina is exposed daily to a high light energy, which significantly influences physiological processes, such as the phototransduction or the functional visual cycle [15] (see also 1.1). Even the phagocytosis of outer segments of the retina from the RPE is influenced. All these processes underline the need of a light component to emulate a complex physiological retina-model. Besides physiological operations, light can also have several phototoxic damaging effects to the retina [136]. However, most findings are based on animal models, which do not accurately resemble the human tissue architecture, and further it is ethically controversial. Furthermore, light and specifically photochemical damage plays an important role in mechanisms of aging [137]. This can be of particular interest, as the tissues integrated in the RoC system are developed from hiPSCs, hence they still are in a relatively immature state.

Hence, the focus of this chapter is the development of a light housing specifically tailored for the RoC. The focus was laid on the compliance of the technical requirements to be used inside an incubator without heating the tissue or even the whole incubator.

7.1 Light exposure setup

For the development of the electronic light device, the main challenge was that the device had to be placed in an incubator. Specifically, this meant, that the device is exposed to a high humidity (up to 95%), which might lead to corrosion of the metal parts of the electronics. In addition, light generation usually is accompanied by heat, so thermal control must be assured. There exist different light sources and depending on the wavelength they can be either visible (400 nm - 760 nm), in the infrared (760-10000 nm) or in the UV spectrum (100 - 400 nm), whereby visible and infrared light belongs to retinal hazard region [138]. To



Figure 7.1 Light exposure of RoCs. a) Intensity profile of white LEDs in dependence of brightness settings. b) Image of the light housing for the chips using white light. c) Cross section of the setup of the light housing, when exposing chips. d) Comparison of the temperature profile of chips exposed to white LEDs with and without cooling elements. e) ATP activity of tissue exposed to different conditions: normal culture conditions, light exposed tissues (2h), drug treated tissues, drug treated chips that were exposed to light in addition (2h).

start with a prototype, visible white light was chosen as an array of RGB-LEDs. Using these LEDs not only white light can be applied, but also red, green or blue light can be turned on, which allows testing other wavelengths of visible light, which is also known to induce light toxicities, and gives flexibility to the device. LEDs have been characterized concerning their intensity, when white light was adjusted, to determine the intensity range in which they can be applied.

It could be found, that they fulfill the requirements to induce some effects to the retina, as they can cover a range of 0 - 4500 lux, when sitting around 5 cm above the tissue (Figure 7.1 a). From literature, there is little report about reaction from ROs to light, yet one publication revealed that ROs could respond to white light when exposed at 217 W/cm2 [139]. If the value is converted to *lux*, estimating that the lightest light takes place at 555 nm, the presented value would correspond to 1483 lux. Several research groups have exposed either animals ([140, 141]) or *in vitro* RPE ([142, 143]) to diffuse white light with an intensity of 2500 lux. For this reason, the LEDs were integrated into the light device. Intensity as well as wavelengths could be individually adjusted using a microcontroller (Arduino).

7.2 Tempterature control

Most critical during the use of the device appeared to be the heating of the device and hence, of the chip. There needed to be more than a simple cooling element to reduce the heating during light exposure. For this reason, a peltier module was mounted directly on the light sources, which uses electric power to cool down one side and creates heat on the other. A cooling element was placed on top and a fan was mounted on top to transport the created heat on the adjacent side away from the chip. To control the temperature on the chip, a thermostat was also added, connected to the peltier module and the fan. Thereby, the thermostat can adjust the power, in case the chip is heating too much (or not enough) and uncontrolled heating of the chip can be prevented. Hence, thermal damage from increased temperature could be avoided (Figure 7.1 b, c). However, all these components cannot transport away the produced heat in a closed and small incubator. Furthermore, the high humidity in the incubator is critical due to possible corrosion of the electrical parts. Therefore, the device was tested in an innovative incubator (ALS Incubator FlowBoxTM), instead of a conventional incubator. The incubator can also be used as sterile bench and therefore, it consists of a bigger incubation area so that the heat can be further distributed. Its maximum possible humidity can be only set to 65 %. Therefore, no corrosion of the electronics in the incubator could be detected for two days. In addition, the incubator is actively controlling the temperature in the incubator and helps to transport developed heat out of the incubator.

A temperature sensor below the chip was also installed to measure the chip's temperature. By that, during an experiment, temperature and humidity can be recorded with the arduino, and in case an experiment fails, it can be investigated, if these parameters influenced the experiment. To see, whether the complex setup with peltier module and fan is needed, the temperature profile of a chip exposed to the maximum intensity of white light was measured. First, peltier module and fan were disconnected. This elucidated, that the temperature below the chip was increased up to 40°C, while the incubator worked hard to transport the produced heat away, often adjusting the temperature. This indicated that the

temperature did not further increase because the incubator itself kept being able to control the environment inside the box constantly at 37°C. When considering that temperature above 38°C in the human body is referred to fever and can cause irreversible damage at a temperature of 40°C, the light device without additional cooling elements cannot be used as light source for the retina system. In comparison, using the device including all presented cooling components, it was possible to achieve a constant temperature to the chip about 37°C, which was measured constantly for about two hours (Figure 7.1 d). From the measurements taken, the setup seems to be appropriate to be used for cell culture experiments.

7.3 Biological proof-of-concept

As proof-of-concept, it was tested, if retinal light toxicity in the RoC can be provoked by applying high energy light from the device. As for the susceptibility of ROs to light damage only limited data is available ([139]), a condition was included featuring simulataneous light and drug (CQ) response. An ATP assay was performed to evaluate the effects of light or drug on the cells. For this assay, the ROs were removed from the chips after the treatment and lysed to extract the ATP content from the tissue. To access the most reliable ATP content, the chips were not imaged or investigated in another form that could effect the ATP. The data was analyzed relative to the untreated chip (mean = 1, standard error of the mean (SEM) = 0.16, and a trend could be detected, showing a lower ATP value of light exposed ROs (mean = 0.8, SEM = 0.12), which indicates lower cell activity. Interestingly, the exposure to CQ did not show a predominant effect on ATP reduction (mean = 0.9, SEM = 0.25), yet compared to the untreated control, a marginal lower ATP value could be analyzed. Concerning the results, the effect of light has a bigger impact on the cells than CQ. This can be explained, as this drug predominantly affects the RPE. This can conclude the low influence of cell activity in the RO. As expected, the exposure to a combination of drug and light, showed the biggest influence on the cells (mean = 0.7, SEM = 0.14). To assess, whether the effect comes from light and not from heating, the parameters were recorded by the arduino over the time of exposure and no increase in heat could be detected. Overall, this experiment shows, that the light device can be an appropriate system to elucidate the tissue functionality concerning different light conditions.

7.4 Discussion

An illumination holder tailored for the RoC was developed with consideration of the conditions inside an incubator. The device enables the exposure of RoCs in a very versatile way, being able to set several wavelengths (red, green, blue, white light), and adjusting the intensities. Therefore, it offers the possibilities to either apply physiological conditions or imitate the day-night rhythm, hence, controlling the opening and closure of ion channels in the OSs of the retina. Again, this might lead to improved segment growth as the function keeps active. Furthermore, it can be also used to initiate harmful conditions, as confirmed by a preliminary experiment. Up to now, the settings were controlled and adjusted via the Arduino platform using a laptop. For future research, it might be more user-friendly to integrate a display to the system, where settings can be adjusted and controlled simply with some bottoms. This might be especially interesting, as the OoC field is marked by interdisciplinary nature and researchers with different backgrounds should be able to use the light system. It has to be critically regarded, that the experiments can only be performed in a special incubator with a maximum humidity of 65 %, which can raise evaporation. Since chip experiments are usually performed in a sealed chip, nourished continuously by fresh media evaporation is a much smaller issue than in classical well culture. However, it should be considered, that for effluent analysis, waste containers for effluents should be sealed relatively tight due to increased evaporation. What has also been experienced from the handling with the chips, when placing the light device on top, was that the tubing had to be carefully positioned to not move the inserts, thereby blocking the flow or even destroying the chip. Therefore, using a bent needle to reduce its height and connect tubing to the chip was beneficial or even crucial.

The presented light device can already be used as a prototype, but optimizations might still be considered for being used o a big scale,. It should be highlighted that in a first experiment, it was possible to confirm the influence of light already. This can be very promising regarding reducing animal experiments and testing light conditions in an appropriate *in vitro* system, such as the RoC, where all important retina cell types are based on cells with human background and can be exposed to light.

Summary & outlook

OoCs are microfluidic systems that enable advanced in vitro tissue integration based on human cell sources. Having the possibility to nourish the cells via a perfusion system continuously, the technology gained much interest as alternative models for drug testing. However, in the field of ophthalmology, no OoCs had been developed that replicate the complex structure and function of the retina. Together with experts in biology, it was possible to realize a biologically relevant RoC model platform with high physiological character and applicability for drug testing. Furthermore, in this thesis, several different chip versions were presented and a multitude of different fabrication processes were established. The goal of the different approaches presented here should be the provision of a toolset that can be used and easily adapted. Depending on the question that shall be answered, a certain chip generation can be chosen, or generations can be even combined. The principle concept of the RoC consists of four open chambers, similar to transwells, but with a bottom channel below for continuous fluid flow (PDMS-chip, see 3). This enables user-friendly integration of the complex retinal tissue consisting of a RPE monolayer directly on the membrane and a RO on top embedded in a hydrogel. With the increased well layer height, three different drug injection routes can be recreated on-chip: systemic administration (application of a substance through the blood flow), intravitreal and subretinal injection route. Subretinal injection can be realized by entering a drug/compound from the top compartment, letting the substance settle in the space between RPE and neural retina (represented by the RO), which is of special interest for gene therapy. As future direction, even intravitreal injection could be imitated using a needle and injecting substances directly into the RO. However, this might not be as user-friendly as the other options. Overall, the presented concepts offer versatile administrations of drugs/compounds, including the possibility for gene therapy testings, where other than systemic administrations are favorable for the retina. Besides, the biological relevance of this model was investigated by colleagues who showed an increased segment growth in the chip compared to conventional model systems and recorded a key aspect of the retina - the phagocytosis of shed OSs from the photoreceptors, which is a daily physiological event in the human eye. As the chip material based on PDMS has limitations, when it comes to drug testing due to its absorption character, the chip was recreated based on low-absorption materials (TPE-chip, see 4). The established fabrication techniques were presented, and absorption character was investigated, showing a reduced absorption in comparison to the PDMS-chip. Furthermore, to enhance the physiological environment of the chip, a method for the generation of a defined subretinal space was established (Mesh-chip): A reproducible distance between RPE and RO could be realized by integrating an additional thin PMMA layer on top of the membrane and a coarse mesh above. Distance measurements proved, that the space can be generated reproducibly with only low variations between individual chips. This offers further possibilities for future investigations of biological events in the subretinal space, such as the visual cycle. In case the RPE-choroid interface should be investigated, the synthetic, rigid PC membrane can lead to restrictions, as diseases, such as wet-AMD, affecting this region might not be possible to be emulated. Therefore, methods for the integration of different biologically inspired membranes were established in low-absorption chips (see 5). The integration of the following three types of membranes was presented: Electrospun membranes, collagen membranes and extracted Bruch's membranes from pig eyes. The feasibility of the integration on-chip was proven from a technical point of view, offering versatile opportunities to increase the physiological character of the membrane in case it is needed. However, depending on the research question, it has to be weighed whether these types of membranes are needed, as this can elongate the chip fabrication time. Further biological experiments should be performed to gain insights, which membrane might be the most suitable for long-term culture. Apart from this, another chip concept was presented allowing imaging from the side (Side-View-chip, see 6). For OoCs, imaging techniques are often applied as read-out methods to characterize the tissue. However, for complex, thick tissues, such as the retina, usually only the bottom layers can be recorded, as the thick tissue does not allow the light to pass through the entire tissue and focus depth is also restricted. For this reason, a side window was fabricated next to the chamber, allowing the chip to turn by 90° and image from the side. The fabrication technique was presented, and imaging accuracy was evaluated. Finally, exemplary images from ROs were taken. It was shown that images from at least two sides can be acquired in a fast way, which paves the way to gain further information about the tissue.

Finally, a light housing was set up to generate a physiological environment for the retinal tissue (see 7). Light plays two major roles for the retina: It influences its functionality (circadian rhythm, light tansduction from OSs), but it is also responsible for diseases and eye disease exacerbation. To cause effects with the light housing only by light, but not by heat, it was crucial to avoid additional heating from the light. Using a special incubator with air flow and cooling elements on top of the light-source, generating a constant temperature profile without heating of the tissue was possible. Furthermore, the efficacy of the light housing on tissue could be demonstrated, as its application at high intensities evoked a reduction of ATP activity on RO tissue. Summarized, a toolkit of different platforms are provided in this thesis, and with the opportunity to combine some, such as the TPE-chip and the light housing for drug testings or the TPE-chip and a biologically inspired membrane for disease modeling a variety of platforms can be generated to address different questions in the retina biology. Human OoCs have demonstrated to emulate human pathophysiology and clinical drug responses more accurately than animal models [144]. This can be confirmed by the fact, that currently for drug testing, 9 from 10 drugs tend to fail in human trials - even after testing them in *in vivo* models provided by animals [8]. Based on these findings, OoCs should be integrated in the apeutic studies to reduce animal models and costs, as well as time. The system provides an attractive platform for future applications, especially with respect to the cell sources in this chip, which are produced by hiPSC technology from human donors. Having the possibility to generate tissue from any donor, donors worldwide can be included in studies, hence allowing for the inclusion of subgroups/minorities, such as female cell sources, which are currently neglected in clinical trials. Finally, it paves the way for personalized medicine, as patient-derived cells can be engineered for individual therapeutic development, which can be of special interest for patients with rare genetic disorders [144]. Alternatively, the most effective treatment can be discovered for a patient before treatment, enabling the treatment's maximum efficacy.

Looking ahead to the future of OoC applications, by the end of last year, in the USA, the law has changed concerning the regulatory for drug testing and from now on, also other models than animal models, can be used to validate a new drug [8]. For this reason, there is hope that the OoC technology will be applied soon as state of the art and as animal-free testing platform, thereby reducing the total amount of animal models.

Appendix: Arduino Code for light exposure setup

```
1 #include <SD.h>
  #include <Adafruit_NeoPixel.h>
3 #include "DHT.h"
  #include <Adafruit_Sensor.h>
5 #include <Wire.h>
_7 #define DHTPIN 2 //Connection of temperature and humidity sensor (
      DHT22) to PIN2
  #define LEDPIN 5 // Connection of LED to PIN5
  const int ledPin = 5;
9
  #define DHTTYPE DHT22
  Adafruit_NeoPixel strip = Adafruit_NeoPixel (16, ledPin, NEO_GRB +
      NEO_KHZ800);
  DHT dht (DHTPIN, DHTTYPE);
13
  void setup() {
    strip.begin();
15
    strip.setBrightness(100);
17
    strip.show();
    dht.begin();
    delay(1000);
19
     Serial.begin(9600);
21 }
23 void loop() {
    // put your main code here, to run repeatedly:
25
     \{ int n = 0; \}
       for (n = 0; n \le 15; n++)
27
       {
       strip.setPixelColor(n, 255, 255, 255); //255, 0 0 = red; 0, 255, 0 = green; 0, 0, 255 = blue; 255, 255, 255 = white
29
       }
       strip.show();
31
33
       delay(10000); //2seconds (=2000) Waiting period until first
      measurement
35
```
```
float h = dht.readHumidity(); //Read humidity and save as
      ""humidity
      float t = dht.readTemperature(); //Read temperature and save as
37
      ,"temperature
      // Check, if real number was read. If NaN (not a number) was
      returned back, the following error appears.
      if (isnan(t) || isnan(h))
39
      {
         Serial.println("DHT22 was not able to be read");
41
      }
      {\rm else}
43
      {
         Serial.print("humidity: ");
45
         Serial.print(h);
         Serial.print(" \%\t");
47
         Serial.print("temperature: ");
         Serial.print(t);
49
         Serial.println("C");
         delay(60000);// delay 1 min
51
      }
    }
53
```

Bibliography

- Gupta, M. P. et al. Retinal anatomy and pathology. Developments in Ophthalmology 55, 7–17 (2015).
- [2] Sahoo, N. K. *et al.* Impact of age-related macular degeneration on diabetic retinopathy: An electronic health record based big data analysis from a tertiary eye centre in South India. eng. *Indian journal of ophthalmology* 69, 3184–3188 (2021).
- [3] Vyawahare, H. & Shinde, P. Age-Related Macular Degeneration: Epidemiology, Pathophysiology, Diagnosis, and Treatment. *Cureus* 14, 7–12 (2022).
- [4] Eldred, K. C. & Reh, T. A. Human retinal model systems: Strengths, weaknesses, and future directions. *Developmental Biology* 480, 114–122 (2021).
- [5] Achberger, K. et al. Stem cell-based retina models. Advanced Drug Delivery Reviews 140, 33–50 (2019).
- [6] Marx, U. et al. Biology-inspired microphysiological systems to advance patient benefit and animal welfare in drug development. Altex 37, 365–394 (2020).
- [7] Ewart, L. et al. Performance assessment and economic analysis of a human Liver-Chip for predictive toxicology. Communications Medicine 2, 1–16 (2022).
- [8] Wadman, M. FDA no longer has to require animal testing for new drugs. Science 379, 127–128 (2023).
- [9] Prasad, S. & Galetta, S. L. in *Neuro-ophthalmology* (eds Kennard, C. & Leigh, R. J.) 3–19 (Elsevier, 2011).
- [10] Grigoryan, E. N. Self-Organization of the Retina during Eye Development, Retinal Regeneration In Vivo, and in Retinal 3D Organoids In Vitro. *Biomedicines* 10 (2022).
- [11] Kwon, W. & Freeman, S. A. Phagocytosis by the Retinal Pigment Epithelium: Recognition, Resolution, Recycling. Frontiers in Immunology 11, 1–15 (2020).
- [12] Cholkar, K. et al. in Ocular Transporters and Receptors (ed Mitra, A. K.) 1–36 (Woodhead Publishing, 2013).

- [13] Haderspeck, J. C. et al. Organ-on-a-chip technologies that can transform ophthalmic drug discovery and disease modeling. Expert Opinion on Drug Discovery 14, 47–57 (2019).
- [14] Hoon, M. et al. Functional architecture of the retina: Development and disease. Progress in Retinal and Eye Research 42, 44–84 (2014).
- [15] Molday, R. S. & Moritz, O. L. Photoreceptors at a glance. Journal of Cell Science 128, 4039–4045 (2015).
- [16] Kolb, H. How the Retina Works. (2003).
- [17] Gaudana, R. et al. Ocular Drug Delivery. The AAPS Journal 12, 348–360 (2010).
- Shichida, Y. & Matsuyama, T. Evolution of opsins and phototransduction. *Philosophical Transactions of the Royal Society B: Biological Sciences* **364**, 2881–2895 (2009).
- [19] Golden. Retinal Pigment Epithelium in Health and Disease. Retinal Pigment Epithelium in Health and Disease 63, 1–18 (2020).
- [20] Alfonsetti, M. et al. Looking for In Vitro Models for Retinal Diseases. 1–20 (2021).
- [21] Strauss, O. The retinal pigment epithelium in visual function. *Physiological Reviews* 85, 845–881 (2005).
- [22] Kevany, B. M. & Palczewski, K. Phagocytosis of retinal rod and cone photoreceptors. *Physiology* 25, 8–15 (2010).
- [23] Lakkaraju, A. *et al.* The cell biology of the retinal pigment epithelium. 1–88 (2022).
- [24] HOGAN, M. J. Ultrastructure of the choroid. Its role in the pathogenesis of chorioretinal disease. eng. Transactions of the Pacific Coast Oto-Ophthalmological Society annual meeting 42, 61–87 (1961).
- [25] Curcio, C. A. & Johnson, M. Structure, Function, and Pathology of Bruch's Membrane in (2012).
- [26] Booij, J. C. et al. The dynamic nature of Bruch's membrane. Progress in Retinal and Eye Research 29, 1–18 (2010).
- [27] Chan, W. H., Hussain, A. A. & Marshall, J. Youngs Modulus of Bruchs Membrane: Implications for AMD. *Investigative Ophthalmology & Visual Science* 48, 2187 (2007).
- [28] Somasundaran, S. et al. Retinal pigment epithelium and age-related macular degeneration: A review of major disease mechanisms. Clinical and Experimental Ophthalmology 48, 1043–1056 (2020).
- [29] Forest, D. L., Johnson, L. V. & Clegg, D. O. Cellular models and therapies for age-related macular degeneration. 421–427 (2015).
- [30] Lim, L. S. et al. Age-related macular degeneration. The Lancet 379, 1728–1738 (2012).

- [31] Mitchell, P. *et al.* Age-related macular degeneration. *The Lancet* **392**, 1147–1159 (2018).
- [32] Schnichels, S. *et al.* Retina in a dish: Cell cultures, retinal explants and animal models for common diseases of the retina. *Progress in Retinal and Eye Research* **81** (2021).
- [33] Molins, B., Mesquida, M. & Adan, A. WITHDRAWN: Bioengineering approaches for modelling retinal pathologies of the outer blood-retinal barrier. *Progress in Retinal and Eye Research*, 101097 (2022).
- [34] Mrugacz, M., Bryl, A. & Zorena, K. Retinal vascular endothelial cell dysfunction and neuroretinal degeneration in diabetic patients. *Journal* of Clinical Medicine 10, 1–18 (2021).
- [35] Gui, F. et al. Endothelial Dysfunction in Diabetic Retinopathy. Frontiers in Endocrinology 11, 1–16 (2020).
- [36] Hamel, C. Retinitis pigmentosa. Orphanet Journal of Rare Diseases 1, 1–12 (2006).
- [37] Hartong, D., Berson, E. & Dryja, T. Retinitis pigmentosa Prevalence and inheritance patterns. *Lancet* 368, 1795–1809 (2006).
- [38] Roberts, P. A. et al. Mathematical and computational models of the retina in health, development and disease. Progress in Retinal and Eye Research 53, 48–69 (2016).
- [39] Zeiss, C. J. Translational models of ocular disease. Veterinary Ophthalmology 16, 15–33 (2013).
- [40] B. Turgut, F. C. K. Experimental Animal Models for Retinal and Choroidal Diseases. Advances in Ophthalmology & Visual System 7, 360–368 (2017).
- [41] Murali, A. et al. Retinal explant culture: A platform to investigate human neuro-retina. Clinical and Experimental Ophthalmology 47, 274–285 (2019).
- [42] Shafaie, S. et al. In Vitro Cell Models for Ophthalmic Drug Development Applications. BioResearch open access 5, 94–108 (2016).
- [43] MacLeod, R. A. et al. Widespread intraspecies cross-contamination of human tumor cell lines arising at source. International Journal of Cancer 83, 555–563 (1999).
- [44] Kaur, G. & Dufour, J. M. Cell lines Valuable tools or useless artifacts. landesbioscience 2, 1–5 (2012).
- [45] Capes-Davis, A. *et al.* Check your cultures! A list of cross-contaminated or misidentified cell lines. *International Journal of Cancer* **127**, 1–8 (2010).
- [46] PromoCell. Human primary cells and immortal cell lines: differences and advantages. *Blog, In the lab, https://promocell.com/ (2019).*
- [47] Rossi, G., Manfrin, A. & Lutolf, M. P. Progress and potential in organoid research. *Nature Reviews Genetics* 19, 671–687 (2018).

- [48] Nakano, T. *et al.* Self-formation of optic cups and storable stratified neural retina from human ESCs. *Cell Stem Cell* **10**, 771–785 (2012).
- [49] Zhong, X. et al. Generation of three-dimensional retinal tissue with functional photoreceptors from human iPSCs. Nature Communications 5, 1–14 (2014).
- [50] Bhatia, S. N. & Ingber, D. E. Microfluidic organs-on-chips. Nature Biotechnology 32, 760–772 (2014).
- [51] Junaid, A. et al. An end-user perspective on Organ-on-a-Chip: Assays and usability aspects. Current Opinion in Biomedical Engineering 1, 15–22 (2017).
- [52] Mastrangeli, M., Millet, S. & van den Eijnden-Van Raaij, J. Organ-on-chip in development: Towards a roadmap for organs-on-chip. *Altex* 36, 650–668 (2019).
- [53] Dongeun Huh Benjamin D. Matthews, A. M. M. M.-Z. H. Y. H. D. E. I. Reconstituting Organ-Level Lung. Science 328, 1662–1669 (2010).
- [54] Campbell, S. B. et al. Beyond Polydimethylsiloxane: Alternative Materials for Fabrication of Organ-on-a-Chip Devices and Microphysiological Systems. ACS Biomaterials Science and Engineering 7, 2880–2899 (2021).
- [55] Nahak, B. K. et al. Advances in Organ-on-a-Chip Materials and Devices. ACS applied bio materials (2022).
- [56] Van Meer, B. J. et al. Small molecule absorption by PDMS in the context of drug response bioassays. Biochemical and Biophysical Research Communications 482, 323–328 (2017).
- [57] Tsao, C. W. & DeVoe, D. L. Bonding of thermoplastic polymer microfluidics. *Microfluidics and Nanofluidics* 6, 1–16 (2009).
- [58] Schneider, S. *et al.* Facile patterning of thermoplastic elastomers and robust bonding to glass and thermoplastics for microfluidic cell culture and organ-on-chip. *Micromachines* **12** (2021).
- [59] Gonçalves, I. M. *et al.* Recent trends of biomaterials and biosensors for organ-on-chip platforms. *Bioprinting* **26** (2022).
- [60] Roy, E., Galas, J. C. & Veres, T. Thermoplastic elastomers for microfluidics: Towards a high-throughput fabrication method of multilayered microfluidic devices. *Lab on a Chip* **11**, 3193–3196 (2011).
- [61] Holden, G. Thermoplastic Elastomers. Applied Plastics Engineering Handbook: Processing and Materials 26, 37–57 (1969).
- [62] Busek, M. et al. Thermoplastic elastomer (Tpe)-poly(methyl methacrylate) (pmma) hybrid devices for active pumping pdms-free organ-on-a-chip systems. Biosensors 11 (2021).
- [63] Domansky, K. et al. SEBS elastomers for fabrication of microfluidic devices with reduced drug absorption by injection molding and extrusion. *Microfluidics and Nanofluidics* 21, 1–12 (2017).

- [64] Pasman, T. *et al.* Flat and microstructured polymeric membranes in organs-on-chips. *Journal of the Royal Society Interface* **15** (2018).
- [65] Schneider, S. *et al.* Membrane integration into PDMS-free microfluidic platforms for organ-on-chip and analytical chemistry applications. *Lab on a Chip* **21**, 1866–1885 (2021).
- [66] Arlk, Y. B. et al. Collagen i based enzymatically degradable membranes for organ-on-a-chip barrier models. ACS Biomaterials Science and Engineering 7, 2998–3005 (2021).
- [67] Zamprogno, P. *et al.* Second-generation lung-on-a-chip with an array of stretchable alveoli made with a biological membrane. *Communications Biology* **4**, 1–10 (2021).
- [68] Ahadian, S. et al. Organ-On-A-Chip Platforms: A Convergence of Advanced Materials, Cells, and Microscale Technologies. Advanced Healthcare Materials 7 (2018).
- [69] Wang, X. & Hsiao, B. S. Electrospun nanofiber membranes. Current Opinion in Chemical Engineering 12, 62–81 (2016).
- [70] Khorshidi, S. et al. A review of key challenges of electrospun scaffolds for tissue-engineering applications. Journal of Tissue Engineering and Regenerative Medicine 10, 715–738 (2016).
- [71] Chan, S. Y. et al. Electrospun pectin-polyhydroxybutyrate nanofibers for retinal tissue engineering. ACS Omega 2, 8959–8968 (2017).
- [72] Xiang, P. et al. A novel Bruch's membrane-mimetic electrospun substrate scaffold for human retinal pigment epithelium cells. Biomaterials 35, 9777–9788 (2014).
- [73] Chuchuy, J. et al. Integration of electrospun membranes into lowabsorption thermoplastic organ-on-chip. ACS Biomaterials Science and Engineering 7, 3006–3017 (2021).
- [74] Manafi, N. et al. Organoids and organ chips in ophthalmology. Ocular Surface 19, 1–15 (2021).
- [75] Kaji, H. et al. Characterization of Retinal Pigment Epithelial Cells and Endothelial Cells Within a Microfluidic Device. 742–744 (2014).
- [76] Chen, L. J. et al. Microfluidic co-cultures of retinal pigment epithelial cells and vascular endothelial cells to investigate choroidal angiogenesis. *Scientific Reports* 7, 1–9 (2017).
- [77] Chung, M. et al. Wet-AMD on a Chip: Modeling Outer Blood-Retinal Barrier In Vitro. Advanced Healthcare Materials 7, 1–7 (2018).
- [78] Yeste, J. *et al.* A compartmentalized microfluidic chip with crisscross microgrooves and electrophysiological electrodes for modeling the blood-retinal barrier. *Lab on a Chip* **18**, 95–105 (2018).
- [79] Arik, Y. B. *et al.* Microfluidic organ-on-a-chip model of the outer bloodretinal barrier with clinically relevant read-outs for tissue permeability and vascular structure. *Lab on a Chip* **21**, 272–283 (2021).

- [80] Lee, S., Kim, S. & Jeon, J. S. Microfluidic outer blood-retinal barrier model for inducing wet age-related macular degeneration by hypoxic stress. *Lab on a Chip*, 4359–4368 (2022).
- [81] Cipriano, M. et al. Human immunocompetent choroid-on-chip: a novel tool for studying ocular effects of biological drugs. Communications Biology 5, 1–13 (2022).
- [82] Su, P. J. et al. Retinal synaptic regeneration via microfluidic guiding channels. Scientific Reports 5, 1–7 (2015).
- [83] Mishra, S. *et al.* A model microfluidics-based system for the human and mouse retina. *Biomedical Microdevices* **17** (2015).
- [84] Dodson, K. H. et al. Retina-on-a-chip: a microfluidic platform for point access signaling studies. Biomedical Microdevices 17, 1–10 (2015).
- [85] Achberger, K. et al. Merging organoid and organ-on-a-chip technology to generate complex multi-layer tissue models in a human retina-on-a-chip platform. eLife 8, 1–26 (2019).
- [86] Achberger, K. et al. Human stem cell-based retina on chip as new translational model for validation of AAV retinal gene therapy vectors. Stem Cell Reports 16, 2242–2256 (2021).
- [87] Hoch, E. et al. Chemical tailoring of gelatin to adjust its chemical and physical properties for functional bioprinting. Journal of Materials Chemistry B 1, 5675–5685 (2013).
- [88] McHarg, S. et al. Enrichment of Bruch's membrane from human donor eyes. Journal of Visualized Experiments 2015, 1–7 (2015).
- [89] Schindhelm, D., Weber, R. & Graf, T. Spatial analysis of the back reflected laser light during CO 2 -laser cutting of metal sheets. *Journal* of Laser Applications 25, 012001 (2013).
- [90] Linta, L. et al. Rat Embryonic Fibroblasts Improve Reprogramming of Human Keratinocytes into Induced Pluripotent Stem Cells. Stem Cells and Development 21. PMID: 21699413, 965–976 (2012).
- [91] Frank, S. et al. Small molecule-assisted, line-independent maintenance of human pluripotent stem cells in defined conditions. PLoS ONE 7, e41958 (2012).
- [92] Ohlemacher, S. K. et al. Generation of highly enriched populations of optic vesicle-like retinal cells from human pluripotent stem cells. Current Protocols in Stem Cell Biology 2015, 1h.8.1–1h.8.20 (2015).
- [93] Dalkara, D. et al. In vivo-directed evolution of a new adeno-associated virus for therapeutic outer retinal gene delivery from the vitreous. eng. Science translational medicine 5, 189ra76 (2013).
- [94] Strobel, B. et al. A Small-Molecule-Responsive Riboswitch Enables Conditional Induction of Viral Vector-Mediated Gene Expression in Mice. ACS Synthetic Biology 9, 1292–1305 (2020).

- [95] Strobel, B. et al. Standardized, Scalable, and Timely Flexible Adeno-Associated Virus Vector Production Using Frozen High-Density HEK-293 Cell Stocks and CELLdiscs. Human Gene Therapy Methods 30, 23–33 (2019).
- [96] Strobel, B. et al. Comparative Analysis of Cesium Chloride- and Iodixanol-Based Purification of Recombinant Adeno-Associated Viral Vectors for Preclinical Applications. Human Gene Therapy Methods 26, 147–157 (2015).
- [97] Virumbrales-Muñoz, M. *et al.* Development of a microfluidic array to study drug response in breast cancer. *Molecules* **24**, 1–12 (2019).
- [98] Stokkermans, T. J., Goyal, A. & Trichonas, G. Chloroquine And Hydroxychloroquine Toxicity [Updated 2022 Sep 25]. StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing (2022).
- [99] Hancock, H. A. et al. Acute aminoglycoside retinal toxicity in vivo and in vitro. Investigative Ophthalmology and Visual Science 46, 4804–4808 (2005).
- [100] Retinal gene therapy: Surgical vector delivery in the translation to clinical trials. *Frontiers in Neuroscience* **11**, 1–7 (2017).
- [101] Naso, M. F. et al. Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. BioDrugs 31, 317–334 (2017).
- [102] Haderspeck, J. C. & Brenz, D. Development of a three-dimensional microphysiological Retina-on-a-Chip system. (2019).
- [103] Kim, L. A. et al. Tamoxifen toxicity in cultured retinal pigment epithelial cells is mediated by concurrent regulated cell death mechanisms. Investigative Ophthalmology and Visual Science 55, 4747–4758 (2014).
- [104] Nayfield, B. S. G. & Gorin, M. B. Tamoxifen-Associated Eye Disease: A Review. 14, 1018–1026 (2017).
- [105] Roman, G. T. *et al.* Sol-gel modified poly(dimethylsiloxane) microfluidic devices with high electroosmotic mobilities and hydrophilic channel wall characteristics. *Analytical Chemistry* **77**, 1414–1422 (2005).
- [106] Grant, J. et al. Simulating drug concentrations in PDMS microfluidic organ chips. Lab on a Chip 21, 3509–3519 (2021).
- [107] Maden, G. *et al.* The Distribution of the Photoreceptor Outer Segment Length in a Healthy Population. **2017** (2017).
- [108] Yu, S. et al. Thermal bonding of thermoplastic elastomer film to PMMA for microfluidic applications. Surface and Coatings Technology 320, 437– 440 (2017).
- [109] Shirure, V. S. & George, S. C. Design considerations to minimize the impact of drug absorption in polymer-based organ-on-a-chip platforms. *Lab on a Chip* 17, 681–690 (2017).

- [110] Wang, J. D. et al. Quantitative analysis of molecular absorption into PDMS microfluidic channels. Annals of Biomedical Engineering 40, 1862– 1873 (2012).
- [111] Van Swaay, D. et al. A chip-to-world connector with a built-in reservoir for simple small-volume sample injection. Lab on a Chip 14, 178–181 (2014).
- [112] Murphy, A. R. et al. Bio-inspired human in vitro outer retinal models: Bruch's membrane and its cellular interactions. Acta Biomaterialia 104, 1–16 (2020).
- [113] Xu, C. et al. Electrospun nanofiber fabrication as synthetic extracellular matrix and its potential for vascular tissue engineering. *Tissue Engineering* 10, 1160–1168 (2004).
- [114] Varshey, S. K. et al. Polyanhydride polymers and their uses in biomedical devices U.S. Patent 2007/0225472 A1.
- [115] Ahmed, J. & Varshney, S. K. Polylactides-chemistry, properties and green packaging technology: A review. *International Journal of Food Properties* 14, 37–58 (2011).
- [116] Oh, S. H. & Lee, J. H. Hydrophilization of synthetic biodegradable polymer scaffolds for improved cell/tissue compatibility. *Biomedical Materials* (*Bristol*) 8 (2013).
- Bello, A. B. et al. Engineering and functionalization of gelatin biomaterials: From cell culture to medical applications. Tissue Engineering - Part B: Reviews 26, 164–180 (2020).
- [118] Sun, X. et al. Electrospun Photocrosslinkable Hydrogel Fibrous Scaffolds for Rapid In Vivo Vascularized Skin Flap Regeneration. Advanced Functional Materials 27, 1–12 (2017).
- [119] Lobo, A. O. et al. Electrospun nanofiber blend with improved mechanical and biological performance. International Journal of Nanomedicine 13, 7891–7903 (2018).
- [120] Wagner, W. H. et al. Differences in morphology, growth rate, and protein synthesis between cultured arterial and venous endothelial cells. *Journal* of Vascular Surgery 8, 509–519 (1988).
- [121] Roorda, A., Zhang, Y. & Duncan, J. L. High-resolution in vivo imaging of the RPE mosaic in eyes with retinal disease. *Investigative Ophthalmology* and Visual Science 48, 2297–2303 (2007).
- [122] Moghadas, H. *et al.* A high-performance polydimethylsiloxane electrospun membrane for cell culture in lab-on-a-chip. *Biomicrofluidics* **12** (2018).
- [123] Ma, Z. et al. Grafting of Gelatin on Electrospun Poly(caprolactone) Nanofibers to Improve Endothelial Cell Spreading and Proliferation and to Control Cell Orientation. *Tissue Engineering* 11 (2005).
- [124] Frantz, C., Stewart, K. M. & Weaver, V. M. The extracellular matrix at a glance. *Journal of Cell Science* 123, 4195–4200 (2010).

- [125] Lee, J. S. et al. Placenta-on-A-chip: A novel platform to study the biology of the human placenta. Journal of Maternal-Fetal and Neonatal Medicine 29, 1046–1054 (2016).
- [126] Bunyaratavej, P. & Wang, H.-L. Collagen Membranes: A Review. Journal of Periodontology 72, 215–229 (2001).
- [127] Herrmann, F. E. et al. In vitro biological and mechanical evaluation of various scaffold materials for myocardial tissue engineering. Journal of Biomedical Materials Research - Part A 102, 958–966 (2014).
- [128] Aufderklamm, S. *et al.* Collagen cell carriers seeded with human urothelial cells for urethral reconstructive surgery: first results in a xenograft minipig model. *World Journal of Urology* **35**, 1125–1132 (2017).
- [129] Maqueda, M. *et al.* Repopulation of decellularized retinas with hiPSCderived retinal pigment epithelial and ocular progenitor cells shows cell engraftment, organization and differentiation. *Biomaterials* **276** (2021).
- [130] Peel, S. & Jackman, M. Imaging microphysiological systems: A review. American Journal of Physiology - Cell Physiology 320, C669–C680 (2021).
- [131] Davim, J. P. et al. Evaluation of cutting quality of PMMA using CO2 lasers. International Journal of Advanced Manufacturing Technology 35, 875–879 (2008).
- [132] Huang, Y. et al. Surface roughness analysis and improvement of PMMAbased microfluidic chip chambers by CO 2 laser cutting. Applied Surface Science 256, 1675–1678 (2010).
- [133] Takashimizu, Y. & Iiyoshi, M. New parameter of roundness R: circularity corrected by aspect ratio. *Progress in Earth and Planetary Science* 3 (2016).
- [134] Wang, J., Wang, C.-f. & Chen, S. performance in light enhancement of light-emitting diodes †. (2012).
- [135] Sakai, K. et al. Membrane-integrated glass chip for two-directional observation of epithelial cells. Sensors and Actuators, B: Chemical 326, 128861 (2021).
- [136] Glickman, R. D. Phototoxicity to the retina: Mechanisms of damage. International Journal of Toxicology 21, 473–490 (2002).
- [137] Van Norren, D. & Vos, J. J. Light damage to the retina: An historical approach. *Eye (Basingstoke)* **30**, 169–172 (2016).
- [138] Youssef, P. N., Sheibani, N. & Albert, D. M. Retinal light toxicity. *Eye* 25, 1–14 (2011).
- [139] Hallam, D. et al. Human-Induced Pluripotent Stem Cells Generate Light Responsive Retinal Organoids with Variable and Nutrient-Dependent Efficiency. Stem Cells 36, 1535–1551 (2018).
- [140] Kuse, Y. et al. CCR3 is associated with the death of a photoreceptor cell-line induced by light exposure. Frontiers in Pharmacology 8, 1–8 (2017).

- [141] Zernii, E. Y. et al. Light-induced thiol oxidation of recoverin affects rhodopsin desensitization. Frontiers in Molecular Neuroscience 11, 1–19 (2019).
- [142] Tsuruma, K. et al. SEMA4A mutations lead to susceptibility to light irradiation, oxidative stress, and ER stress in retinal pigment epithelial cells. *Investigative Ophthalmology and Visual Science* 53, 6729–6737 (2012).
- [143] Liu, Y. et al. Blueberry anthocyanins: Protection against ageing and light-induced damage in retinal pigment epithelial cells. British Journal of Nutrition 108, 16–27 (2012).
- [144] Ingber, D. E. Human organs-on-chips for disease modelling, drug development and personalized medicine. *Nature Reviews Genetics* 23, 467–491 (2022).

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