TAMPERE UNIVERSITY OF TECHNOLOGY

LEENA LEHTONEN

DEVELOPMENT OF ERG MEASUREMENT SETUP FOR ISOLATED MOUSE RETINAS AND HESC -DERIVED RPE CELLS

Master of Science Thesis

Examiners: Professor Jari Hyttinen, PhD Heli Skottman, PhD Soile Nymark
Examiners and topic approved in the Computing and Electrical Engineering Faculty Council meeting on 9th Sep 2009

ABSTRACT

TAMPERE UNIVERSITY OF TEHCNOLOGY
Master's Degree Programme in Electrical Engineering
LEHTONEN, LEENA: **Development of ERG Measurement Setup for Isolated Mouse Retinas and hESC -Derived RPE Cells**
Master of Science Thesis, 64 pages
June 2010
Major: Biomedical Engineering
Examiners: Professor Jari Hyttinen, PhD Heli Skottman, PhD Soile Nymark
Keywords: ERG, retina, hESC, RPE, MEA,

This thesis studies a new possible method for testing the functionality of human embryonic stem cells (hESC) that are differentiated into retinal pigment epithelium (RPE) cells by using electroretinography (ERG). RPE is a cell layer that is situated behind the neurosensory retina at the back of the eye. The main function of the RPE is to support the light sensitive photoreceptor cells of the retina. ERG is a commonly used method for evaluating the functionality of the retina electrophysiologically. In ERG, a light stimulus is given to the retina and an electrical response to the stimulus is recorded. RPE functionality can be seen in the ERG signal in different ways: 1) the presence of the c-wave, which is generated in the RPE, 2) increased amplitude of the b-wave when recovering from light adaptation and 3) ability to record light responses longer than without the RPE, because a functional RPE helps the retina to retain its viability.

A literature review is done to evaluate the theoretical possibilities for developing a functionality test for hESC-derived RPE cells that is based on ERG. The idea is to bring a mouse retina in contact with a layer of hESC-derived RPE cells and record the effect of the RPE with ERG. Based on the review, no such test has been done before. However, it has been studied that RPE cell layer forms contacts with the retina even after the two have been detached and then reattached, and this can be registered with ERG measurement. This indicates that the hESC-derived RPE cells could behave similarly.

A measurement setup is developed for measuring light responses first from isolated mouse retinas alone and later from mouse retinas together with hESC-derived RPE cell layers. The ERG measurements are done with a microelectrode array (MEA) system (Multi Channel Systems, MCS GmbH, Germany). The development of the system included designing a light stimulator, finding a suitable way for performing the tissue preparation and the measurements in darkness as much as possible and constructing a functional method for a short term culture for retina-RPE complexes.

The functionality of the measurement setup developed is evaluated by the recorded responses. The recorded light responses of the isolated mouse retinas were good even though an undesired artefact caused by the light stimulator is present in the signals during stimulation. In the measurements from retina-RPE complexes the light stimulator was replaced with a monochromator so that the artefact is not present in the responses. Even though responses to light could be recorded, the stimulus intensity appeared to be too small to gain good responses.

The measurement setup developed was found to be functional. Based on these measurements the functionality of the hESC-derived RPE cells could not yet be evaluated, and further development of the setup especially with the light stimulation is still needed. However, the results were promising and this kind of an approach for testing the functionality of the hESC-derived RPE cells might work also in practice.

TIIVISTELMÄ

TAMPEREEN TEKNILLINEN YLIOPISTO
Sähkötekniikan koulutusohjelma
LEHTONEN, LEENA: ERG-mittausjärjestelmän kehittäminen hiiren verkkokalvoleikkeille ja alkion kantasoluista erilaistetuille RPE-soluille
Diplomityö, 64 sivua
Kesäkuu 2010
Pääaine: Lääketieteellinen tekniikka
Tarkastajat: Professori Jari Hyttinen, FT Heli Skottman, TT Soile Nymark
Avainsanat: ERG, verkkokalvo, hESC, RPE, MEA

Tässä diplomityössä tutkitaan uutta mahdollista menetelmää ihmisalkion kantasoluista erilaistettujen verkkokalvon (retinan) pigmenttiepiteelisolujen toiminnallisuuden testaamiseen käyttämällä elektroretinografiaa (ERG). Retinan pigmenttiepiteeli (RPE) on solukerros, joka sijaitsee silmän pohjassa, verkkokalvon alimpana kerroksena. RPE:n päätehtävä on toimia verkkokalvon valoa aistivien fotoreseptorisolujen tukisoluina muun muassa tuomalla näille happea ja ravinteita. ERG on yleisesti käytössä oleva sähköfysiologinen mittausmenetelmä, jonka avulla voidaan arvioida verkkokalvon toiminnallisuutta. ERG-mittaus tehdään antamalla verkkokalvolle valostimulus, jonka tuottama sähköinen signaali tallennetaan. Saatu vaste kuvaa hyvin verkkokalvon solujen toiminnallisuutta, erityisesti ulompien solukerrosten osalta. RPE:n toiminnallisuus voidaan nähdä ERG-mittauksessa erilaisin tavoin: 1) RPE:n aikaansaaman c-aallon läsnäolona, 2) b-aallon kasvavana amplitudina, kun verkkokalvo toipuu valoadaptaatiosta ja 3) onnistuneina valovasteiden mittauksina pidempään kuin ilman RPE:ä, koska toimiva RPE auttaa verkkokalvoa pysymään elinkelpoisena.

Työssä tehdään kirjallisuuskatsaus, jonka avulla pyritään tutkimaan alkion kantasoluista erilaistettujen RPE-solujen toiminnallisuustestin teoreettisia mahdollisuuksia, kun toiminnallisuustesti perustuu ERG-mittauksiin. Tutkittavan toiminnallisuustestin ideana on tuoda hiiren verkkokalvoleike erilaistetun RPE-solukerroksen yhteyteen ja rekisteröidä toiminnallisen RPE:n aikaansaama vaikutus ERG-mittauksessa. Tämankaltaisen testin käyttämistä alkion kantasoluista erilaistettujen RPE-solujen toiminnallisuuden todentamiseen ei kirjallisuudessa ole raportoitu. Kirjallisuuskatsauksen perusteella voidaan todeta, että RPE muodostaa yhteyksiä verkkokalvon kanssa jopa sen jälkeen kun nämä on erotettu toisistaan ja tuotu jälleen yhteen. Näiden uudelleen muodostettujen yhteyksien vaikutus on voitu rekisteröidä ERG-mittausta hyödyntäen. Näin ollen voidaan ajatella, että alkion kantasoluista erilaistetut RPE-solut toimisivat samoin, mikä voitaisiin havaita ERG-mittauksen avulla.

Alkion kantasoluista erilaistettujen RPE-solujen toiminnallisuuden havaitseminen ERG:a käyttäen edellyttää näiden kahden kudoksen välille muodostuvaa toiminnallista yhteyttä. Tutkimusten mukaan tällaisen yhteyden muodostuminen irrotetuilla ja uudelleen liitetyillä verkkokalvolla ja RPE:llä alkaa välittömästi, kun kudokset saatetaan kosketuksiin toistensa kanssa. Yhteyden kehittyminen kestää kuitenkin useita tunteja, ja mahdollisesti pidempäänkin. Yhteyden kehittymistä oli tutkimuksessa seurattu kymmenen tunnin ajan. Erilaistettujen RPE-solujen ja verkkokalvon välisen yhteyden muodostuminen ja sen havaitseminen ERG-mittauksella edellyttää näin ollen kudosten lyhytaikaista viljelyä siten, että kudosten kontakti toisiinsa säilyy muuttumattomana. Verkkokalvon viljely on tutkimusten perusteella melko haastavaa erityisesti, jos viljellään aikuisen eläimen verkkokalvoa. Tämä johtuu verkkokalvon erityisen voimakkaasta ai-

neenvaihdunnasta. Viljelymenetelmiä, joissa aikuisen eläimen verkkokalvo on säilynyt elinkelpoisena useita vuorokausia, on kuitenkin olemassa.

Työn käytännön osa sisältää mittausjärjestelmän kehittämisen valovasteiden rekisteröimiseen. Ensin järjestelmää kehitettiin siten, että ERG-vaste voitiin mitata hiiren eristetystä verkkokalvoleikkeestä. Mittausten onnistumiseksi suunniteltiin valostimulaattori, jolla mitattavalle verkkokalvolle annettiin valoärsykkeitä. Jotta verkkokalvon kyky tuottaa vaste valoimpulssiin säilyisi mahdollisimman hyvänä, verkkokalvon preparointi ja mittaukset tuli suorittaa pimeässä, tai mikäli se ei ollut mahdollista, käyttäen himmeää punaista valoa. Hiiren näköaistinsolut absorboivat huonosti punaisen valon aallonpituuksia, minkä tähden sitä voitiin käyttää.

Kun valovasteita eristetystä verkkokalvoleikkeestä saatiin onnistuneesti mitattua, järjestelmää kehitettiin edelleen, jotta valovasteiden mittaaminen olisi mahdollista verkkokalvoleikkeestä myös yhdessä alkion kantasoluista erilaistetun RPE-solukerroksen kanssa. Tässä vaiheessa kehitettiin menetelmä lyhytkestoiselle verkkokalvon ja RPE-solukerroksen yhteisviljelylle. Menetelmällä viljeltiin verkkokalvoa ja RPE-solukerrosta muutamien tuntien ajan, pisimmillään noin neljä tuntia. Viljelyn jälkeen suoritetuissa mittauksissa voitiin havaita valovasteita.

Kaikki työssä suoritetut ERG-mittaukset tehtiin mikroelektrodimatriisilaitteistolla (microelectrode array, MEA) (Multi Channel Systems, MCS GmbH, Saksa). Laitteiston peruskomponentti on lasimalja, jonka pohjalle on sijoitettu halkaisijaltaan muutaman sadan mikrometrin kokoinen elektrodimatriisi. Mitattava verkkokalvoleike sijoitetaan tämän matriisin päälle, jolloin elektrodit mittaavat kudoksen sähköistä toiminnallisuutta. Laitteistoon kuuluu maljan lisäksi muun muassa vahvistin ja stimulusgeneraattori sekä ohjelmistot kaikkien laitteiston komponenttien hallintaan ja mitatun tiedon tallentamiseen.

Kehitetyn mittausjärjestelmän toiminnallisuutta arvioidaan työssä mittaustulosten perusteella. Hiiren eristetystä verkkokalvoleikkeestä tehdyissä mittauksissa saatiin hyviä tuloksia, joista oli havaittavissa ERG-signaalille tyypillinen käyrämuoto. Tallennetuissa vasteissa oli kuitenkin näkyvissä valostimulaattorin aiheuttama häiriösignaali. Kyseinen häiriö esiintyi stimuluksen aikana kaikissa mittauksissa, joissa suunniteltua valostimulaattoria käytettiin. Myöhemmissä mittauksissa, joissa RPE-solukerros oli mukana, käytettiin valostimulaattorin sijaan monokromaattoria, jotta stimulaattorin aiheuttamalta häiriösignaalilta voitiin välttyä. Nyt stimuluksen aikaista häiriötä ei näkynyt mitatuissa vasteissa, mutta vasteiden amplitudit olivat merkittävästi pienempiä kuin valostimulaattoria käytettäessä. Tämä johtuu luultavimmin siitä, että monokromaattorin tuottaman valon maksimi-intensiteetti ei ole riittävä, jotta hyviä valovasteita voitaisiin mitata. Tähän viittaa myös se, että mitattujen vasteiden käyrämuoto on samankaltainen kuin kirjallisuudessa esitetyissä tyypillisissä hiiren ERG-vasteissa, kun valostimuluksella on matala intensiteetti.

Mittausjärjestelmä, joka työssä kehitettiin, todettiin toimivaksi. Mitattujen valovasteiden perusteella ei vielä ole mahdollista arvioida alkion kantasoluista erilaistettujen RPE-solujen toiminnallisuutta. Jotta tämä olisi mahdollista, järjestelmää tulee kehittää edelleen. Erityisesti toimivan ratkaisun löytäminen verkkokalvon stimuloimiseen valolla on tarpeen. Mittauksissa käytettävä valostimulaattori ei saisi tuottaa häiriötä mitattuun signaaliin, ja lisäksi sillä tulisi voida tuottaa valoimpulsseja hyvin laajalla intensiteettialueella. Eri intensiteettien käyttö mittauksissa mahdollistaa sekä erittäin pienten valovasteiden että saturoituneiden valovasteiden havaitsemisen. Muita työssä havaittuja kehitystarpeita ovat käytännöllisen perfuusiojärjestelmän kehittäminen pitkäkestoisissa mittauksissa käytettäväksi, mahdollisuus mediumin sekoittamiseen verkkokalvon ja

RPE-solukon yhteisviljelyssä sekä paremmin toimivan menetelmän kehittäminen verkkokalvon ja RPE-solukerroksen kontaktin säilymiseen.

Mittauksissa saadut tulokset vaikuttavat lupaavilta, ja rohkaisevat jatkamaan tähän lähestymistapaan perustuvan toiminnallisuustestin kehittämistä. On hyvinkin mahdollista, että ihmisalkion kantasoluista erilaistettujen RPE-solujen toiminnallisuus voitaisiin todeta sähköfysiologisesti ERG:a hyödyntäen.

PREFACE

This thesis was done in and funded by the Department of Biomedical Engineering of Tampere University of Technology and Regea Institute for Regenerative Medicine, a joint institute under the administration of the University of Tampere.

I would like to thank my supervisor Jari Hyttinen for his important insights throughout the process and his understanding and encouragement even in difficult situations. I am also deeply grateful to Heli Skottman and Tanja Ilmarinen for their help and interest in the thesis. I thank Soile Nymark for her help in the practical work and sharing her experience in the field.

I would also like to express my gratitude to Niina Onnela who has been a great help to me by being responsible for data processing and also as a working partner during the measurement days. I thank Elina Konsén for her help, availability and knowledge especially with media but also with other practical issues. Other people, who have been essential for this thesis to be done and whom I would also like to thank, are Hannele Uusitalo-Järvinen, Hannu Uusitalo, Atte Kekonen, Nikolai Beev and all the animal attendants at the University of Tampere.

My husband Jyri has been a great and supporting spouse throughout the process and him I thank especially for his patience and love for me. Our son Valo has been the light in my life even when things were hard, thank you for that. They have given me the strength to carry on.

My final expression of gratitude is to God, my Heavenly Father, who is the source of my life and gives me purpose to do the things that I do. He has been the ultimate inspiration to me throughout this process. To Him I am eternally grateful for everything, including this thesis.

June 2nd 2010, Tampere

Leena Lehtonen

CONTENTS

ABBREVIATIONS, TERMS AND DEFINITIONS

µERG	See MicroERG.
AC-coupled	An electrical circuit is that passes only AC signals and blocks all DC signals. Ac-coupling is done in amplifiers to remove differences in the input potential levels. As a drawback, the low frequency signals are lost. See also Dc-coupled.
ADC	Analog-to-digital conversion or converter. An electronic device that converts an input analog voltage (or current) to a digital number proportional to the magnitude of the voltage or current.
Aliasing	Phenomenon that happens when a signal sampling rate is too low compared to the signal frequency and because of that the reconstruction of the signal from the samples will be different from the original signal.
CMRR	Common-mode rejection ratio. A measure that describes the device's ability to reject input signals common to the inputs. Defined as the ratio of powers, measured in positive decibels.
Conjunctiva	Clear mucous membrane in the eye that covers the sclera and lines the inside of the eyelids.
DC-coupled	An electrical circuit that passes the DC component together with the AC signal. This way no low frequency signals are lost, but also erroneous DC input variation can be seen in the output. See also AC-coupled.
Decapitation	Separation of the head from body.
Depolarization	Change in a membrane potential that makes it more positive, or less negative. May result in an action potential. See also Hyperpolarization.
Differential amplifier	Electronic amplifier that multiplies the difference between two inputs by the gain of the amplifier. See also Single-ended amplifier.
Dipole	Separation of positive and negative electric charges.
DTL fibre electrode	Electrode-type, that Dawson, Trick & Litzkow (DTL) discovered in 1979, for recording retinal potentials from the cornea. Properties of DTL fibre are flexibility, low electrical resistance, inexpensiveness and disposability.
Electroretinography	Method for measuring the electrical responses of the retina to light stimulation.
Enucleation	Removal of the eye.
ERG	See Electroretinography.

ERP	Early receptor potential. Voltage arising across the eye from a charge displacement within photoreceptor pigment, in response to an intense flash of light. Happens within the first few milliseconds after the light onset.
Extracellular	Something outside the cell.
Eyecup	Cup-shaped structure that is left when the anterior part of the eye is removed along the ora serrata together with the lens and vitreous inside the eye.
Faraday cage	Enclosure formed by (a mesh of) conducting material. Blocks out external static electric fields.
HESC	Human embryonic stem cell.
HexaMEA	MEA with a hexagonal electrode matrix. See also MEA.
Hyperpolarization	Change in a membrane potential that makes it more negative, or less positive. Inhibits the rise of an action potential. See also Depolarization.
In vitro	Latin: within the glass. Something is performed in a controlled environment, such as in a test tube or Petri dish, not in a living organism. See also In vivo.
In vivo	Latin: within the living. Something is performed using a whole, living organism as opposed to a partial or dead organism, or in vitro environment. See also In vitro.
Lid-hook electrode	Electrode-type for recording retinal potentials from the cornea. The electrode has a shape of a lid hook and it is inserted in the lower fornix when conducting the measurement. Can be made of many materials.
MEA	Microelectrode array. A dish whose bottom has an array of micro-sized electrodes. Used for extracellular recordings from biological tissues/cells.
Medium	Liquid or gel designed to support the growth of microorganisms or cells when in (contact with) the medium.
Metabolism	Set of chemical reactions in living organisms that maintain life.
MicroERG	ERG measurement performed in a micro-scale. Can be done with microelectrodes from a small proportion of a retina instead of measuring the ERG from a whole eye.
Neonatal	Of or pertaining to the period of time immediately following birth. See also Postnatal.
Noise	In electrical recordings, unwanted data from a different source than the signal, that should not be recorded with the signal.
OP	See Oscillatory potential.
Oscillatory potential	Variable voltage in the B-wave of the electroretinogram of

	the dark-adapted eye.
Patch clamp	Laboratory technique used in electrophysiology that allows the study of single or multiple ion channels in cells.
PDMS	Polydimethylsiloxane. A material that belongs to a group of polymeric organosilicon compounds (silicones).
Perfusion	Act of pouring liquid over or through an organ or tissue.
Phagocytosis	Cellular process of engulfing solid particles by the cell membrane.
Photopigment bleaching	Phenomenon that reduces the effective pigment concentration of the photoreceptor, decreases the effective pigment density, and makes the spectral sensitivity narrower.
Postnatal	Period beginning immediately after the birth of a child and extending for some weeks. See also Neonatal.
PTFE	Polytetrafluoroethylene. Synthetic fluoropolymer of tetrafluoroethylene which is better known as Teflon.
Retina	Light sensitive tissue lining the inner surface of the eye.
RPE	Retinal pigment epithelium. Pigmented cell layer just outside the neurosensory retina that nourishes retinal visual cells, and is firmly attached to the underlying choroid.
Single-ended amplifier	Electronic amplifier, which amplifies a single input signal. See also Differential amplifier.
Synapse	Structure that permits a neuron to pass an electrical or chemical signal to another cell.

1. INTRODUCTION

In this thesis a new approach for evaluating the functionality of retinal pigment epithelial (RPE) cells derived from human embryonic stem cells (hESC) by using electroretinography (ERG) measurement is studied and a measurement setup for such test developed. RPE is a cell layer that is situated behind the neurosensory retina at the back of the eye. The main function of the RPE is to support the photoreceptor cells of the retina in several ways, including phagocytosis of the photoreceptor outer segments and transportation of oxygen and water-soluble nutrients to the subretinal space [Kaufman & Alm 2003].

The differentiation of hESC to RPE cells has been studied and methods have been developed over the past few years. The differentiation into RPE cells has been successful, but improvement of methods still needs more work. Also, methods for evaluating the functionality of the differentiated cells in vitro have been limited, and typically, electrical measurements have not been used for this purpose.

The functionality of the retina is traditionally tested with electrophysiological measurements, and ERG is one of them. With ERG, the potential difference across the retinal tissue is measured and a signal that represents well the functionality of the retina is obtained. ERG can be measured from both living objects and isolated retinas and the presence of a functional RPE can be seen in the ERG in different ways. The decision of using ERG as a basic measurement for developing a new functionality test for hESC-derived RPE cells was based on these qualities of ERG.

Up to the present, no publications of functionality tests where ERG is measured from isolated retinas together with hESC-derived RPE cells exist. Thus a test like this could bring new important information on the functionality of hESC-derived RPE cells. Measuring ERG from an isolated retina with a microelectrode array (MEA) system is not very complicated and instructions for performing the measurement are available from a MEA manufacturer [Multi Channel Systems 2005]. Culturing the isolated retinas is more demanding and new studies with better culturing techniques for prolonged measurements have rather recently been published [Kretz et al. 2004; Koizumi et al. 2007; Kobuch et al. 2008; Johnson & Martin, 2008; Kaempf et al. 2008]. Some publications from retinal detachment and reattachment, where the RPE is first removed from the retinal surface and then returned to its original position, exist, and they show results from successful electrophysiological measurements of the reattached retinas [Monaim et al. 2005; Kaempf et al. 2008]. One quite recent study also used a technique where hESC-derived RPE cells were brought in contact with an isolated retina and phagocytosis of the photoreceptor outer segments was observed [Carr et al. 2009]. Results of these

studies show that the approach which has been taken in this thesis to RPE functionality test might be possible and should be studied carefully.

This work started at a point where a measurement system for isolated retinas stimulated with electrical stimuli was already constructed. The system included a full MEA measurement system, perfusion, oxygenation of the medium and a preparation technique for the retinas. The practical part of this thesis includes the development of this existing measurement system to such that light responses from isolated retinas as well as retinas together with the hESC-derived RPE cells can be measured. In order to develop the setup to such that measuring the light response of the retina could be possible, several adjustments needed to be made and tested for the system. These included performing the preparation in darkness under dim red light, performing the measurements in darkness and testing a new medium that can be used with the cells. Further development of the setup for performing measurements with the hESC-derived RPE cells included a suitable method for a short-term culture of few hours for retinas attached to the RPE cell layers and a carrier that facilitates the moving of the retina-RPE without damaging the possible contacts between the two tissue layers. Naturally also performing several measurements and trials, first with the isolated retinas alone and then with retinas together with the hESC-derived RPE cells, was needed to find working methods. Planning and determining properties of a light stimulator that could be used for stimulating the retinas was also done even though the actual construction of the stimulator was done elsewhere.

Results from ERG measurements done with the developed setup are included in the thesis to show that light responses from both isolated retinas and retinas together with the hESC-derived RPE cells could be measured with the setup. With the results the functionality of the setup for recording light responses can be shown, but the functionality of the hESC-derived RPE cells could not be proven in the measurements that were done in the course of this thesis.

2. THEORETICAL BACKGROUND

This chapter covers the theory related to the ERG measurements. The anatomy, physiology and electrophysiology of the retina are briefly discussed while the theory of the ERG is presented in more detail. Some more detailed sections about RPE and mouse eye ERG exist due to their significance to this specific study. Sections about retinal culture and retinal detachment and reattachment are included because they are closely related to the methods and purpose of this study.

2.1. Anatomy and Physiology of the Retina

The anatomy and physiology of the retina are very similar in all vertebrates. Thus most of the properties of the human retina that are presented here can also be applied to mouse retinas that are used in the measurements done in this thesis.

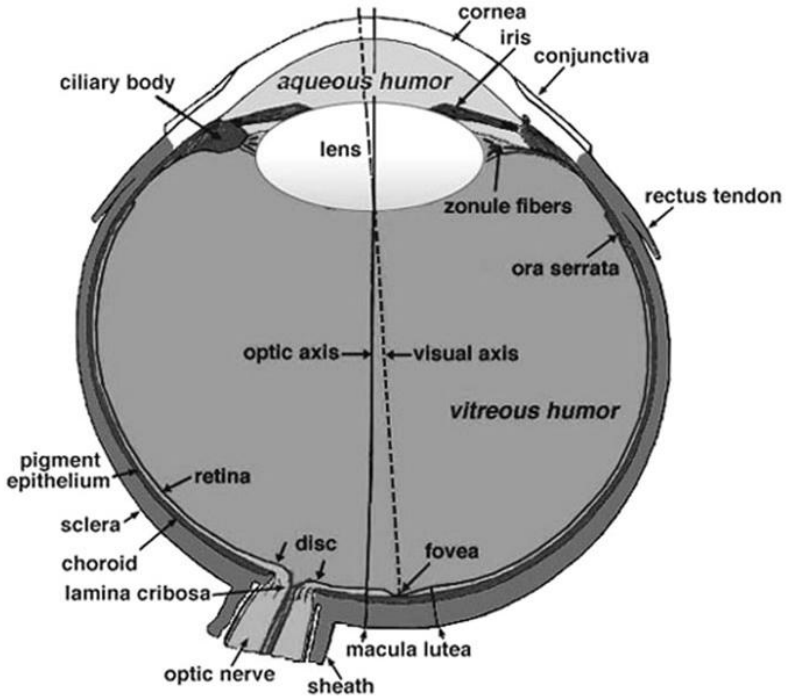

Figure 2.1. *Horizontal cross-section of the eye. [Webvision 2009]*

Retina rests at the back of the eye between vitreous humor and choroid (Figure 2.1.). It lines the inside of the eyeball back from ora serrata. Retina is a thin and delicate structure that contains several cell-size structures within itself. When light hits the retina a visual sensation is first formed and then processed in the retina by collaboration of millions of cells and several different cell types. After the processing the information is transmitted to the brain to be further processed and interpreted there. Parts of the com-

plex processing that the retina does to the visual information are still not understood. [Kaufman & Alm 2003]

Retina is a multi-layered structure that comprises six types of neurons: photoreceptor cells, horizontal cells, bipolar cells, amacrine cells, interplexiform cells, and ganglion cells. Radial glial cells that support the neurons within the retina are called Müller cells. Photoreceptor cells react to light and thus they are essential for the visual sensation to be formed. There are two main types of photoreceptor cells, rods and cones. In human eye, most photoreceptor cells, approximately 95 % are rods that are more sensitive to light than cones and give the ability to see in dim light. The remaining 5 % of photoreceptors are cone cells. In human retina there are three types of cones that are most sensitive to different regions of wavelengths of light and thus make it possible to distinguish between colours. Figure 2.2. shows a simplified diagram of retinal structure with the major cell types of retina included. [Kaufman & Alm 2003]

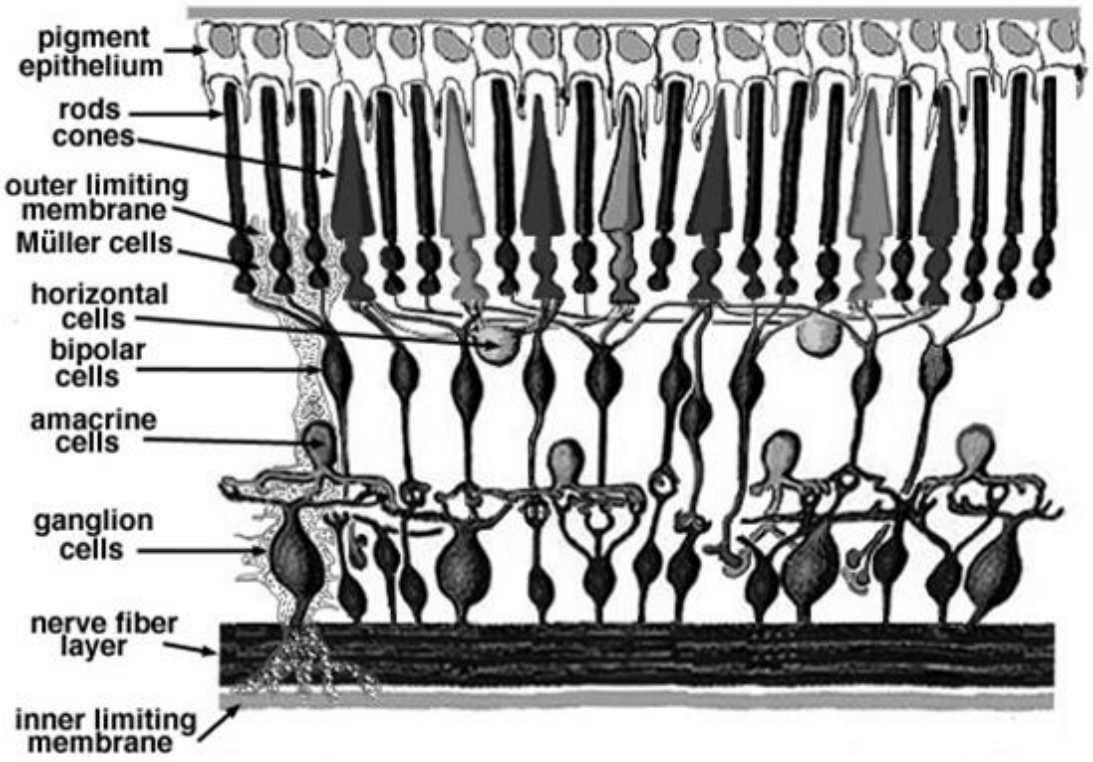

Figure 2.2. *A simplified diagram of the cellular structure of the retina. [Webvision 2009]*

Cell distribution in human retina is not homogeneous. Central retina is significantly thicker than peripheral retina. Rod photoreceptor density increases nearly linearly towards central retina reaching the peak density approximately at a 5 mm (~20 degrees) distance from the fovea. Fovea is the site in the middle of macula lutea (see Figure 2.1.) where highest visual acuity is achieved. In the region of macula lutea the spatial density of rod photoreceptors radically decreases reaching zero at fovea. Thus fovea only con-

tains cone receptors. The cone photoreceptor density is rather stable throughout the retina but peaks at fovea where it reaches about 150 000 per/mm^2. [Kaufman & Alm 2003]

Optic disk is the area where ganglion cell axons form the optic nerve and penetrate the retina. The area contains no photoreceptor cells at all and thus causes a blind spot to the field of vision. The blind spots in the two eyes compensate for each other so that when watching with both eyes no blind spots remain since one eye covers the area where the other eye has the blind spot. [Kaufman & Alm 2003]

When light hits the retina a process called phototransduction takes place in the light-sensitive photoreceptor cells. In phototransduction a light-sensitive molecule in the outer segment of a photoreceptor cell absorbs a photon which causes molecular changes within the cell. As a result of these molecular changes the photoreceptor hyperpolarizes. The hyperpolarization of photoreceptors causes the other cells of the retina to depolarize and hyperpolarize according to the specific cell type and/or their way of reacting to a specific kind of signal they receive. The information transportation process is complex and partly unclear. The modification of the signal happens at every synaptic level. In the simplest case the information is first transmitted from the photoreceptors to bipolar cells and then on to the ganglion cells. The signal often takes a more complex path than this simplest one. The retinal network makes it possible to process different stimuli in parallel and thus create many different data from the photoreceptor responses that can be sent to the brain via parallel channels. The most important of these parallel processing channels are the ON and OFF channels. These detect the light onset and offset. Other signalling systems exist for example for resolution, illumination changes and slow motion in certain directions. Also an inhibitory feedback system by horizontal cells to the photoreceptors exists. Even though the visual information processing begins in the retina, in mammals much of the processing takes place in the cortex. [Kaufman & Alm 2003]

RPE lies between the neural retina and Bruch's membrane (Figure 2.2). It is a monolayer of RPE cells that have a cuboidal shape. The RPE cells are joined together by junctional complexes with tight junctions. These junctions divide the epithelium into two halves: the apical half facing the retina and the basal half facing the choroid. The retinal side of the RPE has microvilli that surround the retinal rod outer segments. The cone outer segments are surrounded by multilamellar specializations or the RPE. On the choroidal side the RPE rests on Bruch's membrane which is a thin, elastic membrane separating the RPE from choriocapillaries. Both photoreceptors and choriocapillaries are dependent on the presence of a functional RPE. The main RPE functions are to support the photoreceptors by participating in the renewal of the photoreceptor outer segments (phagocytosis), regeneration of visual pigments, epithelial transport of oxygen and nutrients, and barrier function. Other, less studied functions are the absorption of stray light, the scavenging of free radicals and drug detoxification. [Kaufman & Alm 2003]

2.2. Electrophysiological Phenomena in the Retina

The retina may be seen as a dipole as the most simple electric equivalent. A standing potential is generated across the retina, and it changes when the retina is in use. Retina is an electrically active tissue that has several different kinds of cells to create the visual information for brain. The electrophysiology of the retina can be evaluated as a whole by measuring the standing potential across the entire retina or regionally by measuring single cells or cell groups.

2.2.1. Electroretinography

A measurement of an electrical retinal response to a light stimulus is called an electroretinogram (ERG). It represents the potential difference across the retina which is generated by the sum of all radial current changes caused by different retinal structures. The radial current changes originate in different levels of the retina, although ERG represents most prominently the activity of retinal photoreceptors and bipolar cells. For this reason, normal ERG's are measured from patients suffering from inner cell layer or optic nerve diseases. [Kaufman & Alm 2003]

Several factors affect the ERG measurement result. Naturally, the light stimulus and its properties have great influence on the results as it causes the whole response. Other factors that affect the response are the adaptation state and health of the retina under measurement. ERG also has variations between species.

ERG Components

ERG has three major components, a-, b- and c-waves. In addition to these, several minor ERG components have been defined. The basic ERG waveform includes a biphasic curve demonstrated in Figure 2.3. The first part of the curve is called the a-wave which is corneal negative, followed by the b-wave which is corneal positive and usually larger in amplitude than a-wave. C-wave is a corneal positive slow wave that appears after b-wave on the ERG. The basic waveform can be obtained from a healthy object by using full-field stimulation where the eye is stimulated with a bright light flash. [Webvision 2009] The frequency range of the ERG is 0.1 – 300 Hz and the amplitude range is 10 nV to 1000 µV [Heckenlively & Arden 2006].

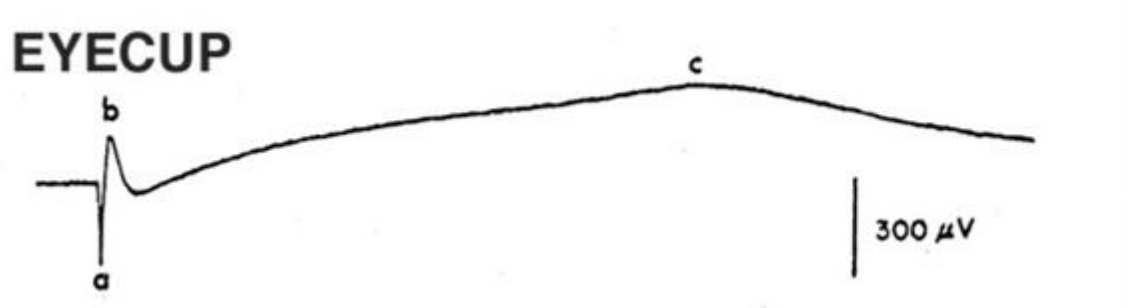

Figure 2.3. *A basic ERG waveform with a, b and c-waves. [Webvision 2009]*

A-wave is formed by an extracellular radial current in the photoreceptor layer. Light absorption in photoreceptors causes reduction of the so called dark current in them which affects the radial current and is seen as the a-wave of the ERG. [Webvision 2009]

B-wave has many properties and great value in clinical and experimental analysis of retinal functionality. The origin of the b-wave is not as simply explained as most other waves of the ERG since more than one cell type contribute to the formation of the wave. The first studies on the origin of the b-wave implied that Müller cells would be the main source. However, later studies have shown that the biggest contributors to the b-wave are ON-center bipolar cells. In addition, amacrine cells affect the amplitude and kinetics of the b-wave through a negative feedback. [Webvision 2009]

C-wave originates in the pigment epithelium layer. The photoreceptor activity under illumination causes a decrease in extracellular potassium ion concentration. This change of concentration is seen as an increase in the standing potential of the eye, which is actually trans-epithelial potential (potential across the RPE), and the c-wave of the ERG. [Webvision 2009] When ERG is measured from neurosensory retina alone, the c-wave is lost because RPE is not present. Especially with mammals the pigment epithelium layer is very difficult to remove along with the retina to be measured together and for the c-wave to be seen in the measured response.

The minor components of the ERG are more specific and sometimes require special kind of stimulus to appear. The early receptor potential (ERP) appears in the ERG immediately after the stimulus onset. It has a biphasic waveform whose amplitude depends directly on the stimulus intensity. In humans, ERP ends within 1.5 ms and is followed by the a-wave. ERP is generated in the photoreceptors. [Webvision 2009]

Oscillatory potentials may be seen in the rising phase of the b-wave when a bright light stimulus is used. They are rapid, oscillating waves whose frequency is in the range of 100 – 150 Hz and they are easy to distinguish from major ERG components by an additional bandpass filter. OP's are likely to originate from the inner plexiform layer (a retinal layer where ganglion cells make contact with bipolar and amacrine cells). [Webvision 2009]

If the stimulus duration is prolonged (>100 ms), a d-wave shows at the off-phase of the stimulus. It is a positive wave whose shape and frequency are rather similar to those of b-wave. With short stimulus duration the d-wave tends to blend with the b-wave and cannot be seen separately. OFF-center bipolar cells generate the d-wave. [Webvision 2009]

Scotopic threshold response (STR) may be recorded when a very dim light stimulus is given to a dark-adapted retina. It is a slow corneal negative potential, and for this reason may sometimes be misinterpreted as the a-wave even though its frequency is lower. The STR is produced by the Müller cells. [Webvision 2009]

The last of ERG components presented here is the m-wave which also originates from the Müller cells. It is a negative potential change that can be detected in a light-adapted state at stimulus onset and offset. The frequency of m-waves is higher than that of STR. [Webvision 2009]

History of ERG Measurements

The history of ERG measurements begins in 1865, sixteen years after the standing potential of the eye was discovered, when Holmgren noticed that frog eye gave an electrical response to light stimulus. A few years later, after continuing with the measurements he became convinced that the source of the response was the retina. [Heckenlively & Arden 2006]

Nearly the same time, in 1873, ERG was discovered independently by Dewar and McKendrick. In 1877 Dewar was the first to measure successfully human ERG and to show that measurements could be done from an intact eye. The development of the string galvanometer in the beginning of the 20th century gave way to the more accurate recordings of the ERG. Based on these recordings Einthoven and Jolly named the portions of typical ERG signal with letters from a to d that are still in use today. [Heckenlively & Arden 2006]

Ragnar Granit studied the ERG extensively from 1933 to 1947 and he ended up with an analysis that is still in use today. He used ether anaesthesia to discover the components of ERG. He found three potentials that he named PI, PII and PIII according to their disappearing order when anesthesia was deepened. The components of Granit's ERG-research are presented in Figure 2.4. [Heckenlively & Arden 2006]

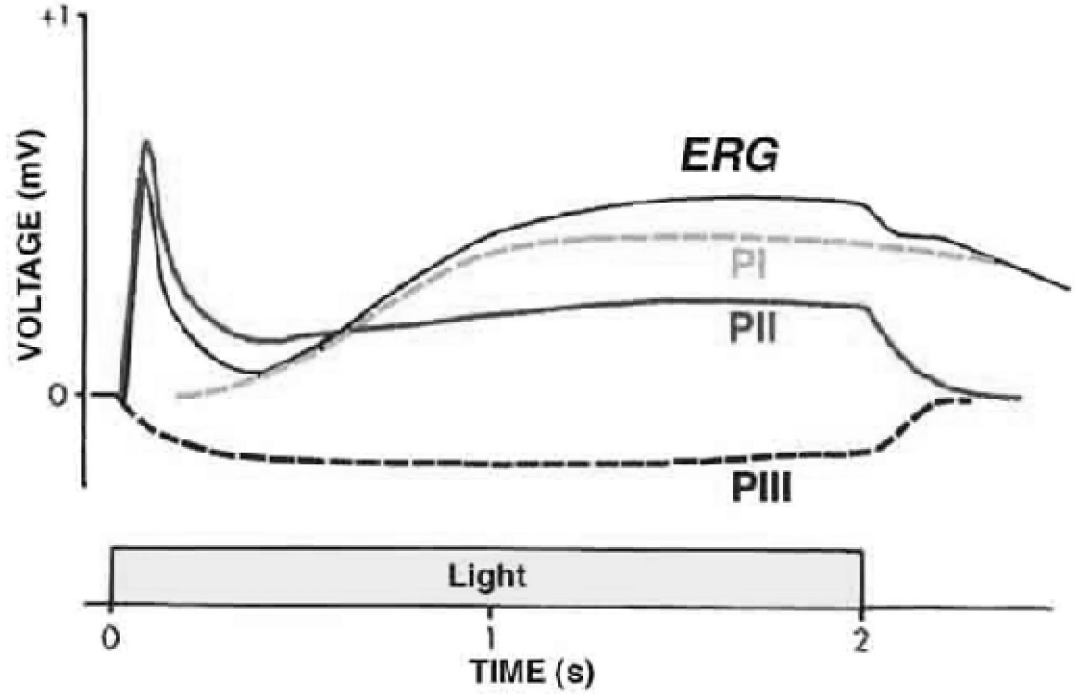

Figure 2.4 *The components of the ERG observed by Ragnar Granit. [Webvision 2009]*

Granit's analysis suggests that the corneal negative PIII that develops rapidly after the beginning of the light stimuli is the source of the a-wave. Corneal positive b-wave is the sum of PIII and much larger corneal positive PII which develops little after PIII. B-wave ends as PII decreases. The c-wave is formed as the result of the increasing potential of PI when PII and PIII remain rather stable. At the end of the light stimuli a posi-

tive d-wave can be seen. Corneal negative PIII produces this wave when it returns to zero potential. [Heckenlively & Arden 2006]

ERG studies continued with cell-level research. Noell used sodium azide, iodoacetate and sodium iodate to study their effect on ERG on a cellular level. He was able to make some conclusions about the origin of major ERG waves. The development of microelectrodes made it possible to conduct more profound cell-level research. With this method Brown and his group found several minor ERG components, most importantly the early receptor potential (ERP). [Heckenlively & Arden 2006]

In 1941 a corneal electrode for human use was introduced by Riggs. This led to further ERG studies with humans and to clinical ERG. Other factors that contributed to the development of clinical practice were a better understanding of the major components of the ERG and technical improvement of recording devices. Clinical ERG has evolved through many researchers' work to become the powerful tool for diagnosing retinal diseases that it is today. [Heckenlively & Arden 2006]

2.2.2. Intracellular Responses

Potentials of retinal cells can be recorded individually by using microelectrodes that have access to the vicinity of the cell. Different cells in the retina react differently when a light stimulus is applied to the retina. Even cells within the same cell group sometimes have different responses. Some of the bipolar cells depolarize and some hyperpolarize. Some of them are rod-dominated and some cone-dominated. That results already four different kind of typical responses within one cell group. The photoreceptor cells are the only cell group that reacts directly to the light stimulus. The responses of the other cell groups are reactions to the responses of the photoreceptors or other cells before them in the course of light information processing in the retina. In Figure 2.5. major cell types of the retina are represented with their voltage responses when the retina that they are a part of is given a light stimulus. These recordings are from a tiger salamander, but they can be generalized also to humans. [Kaufman & Alm 2003]

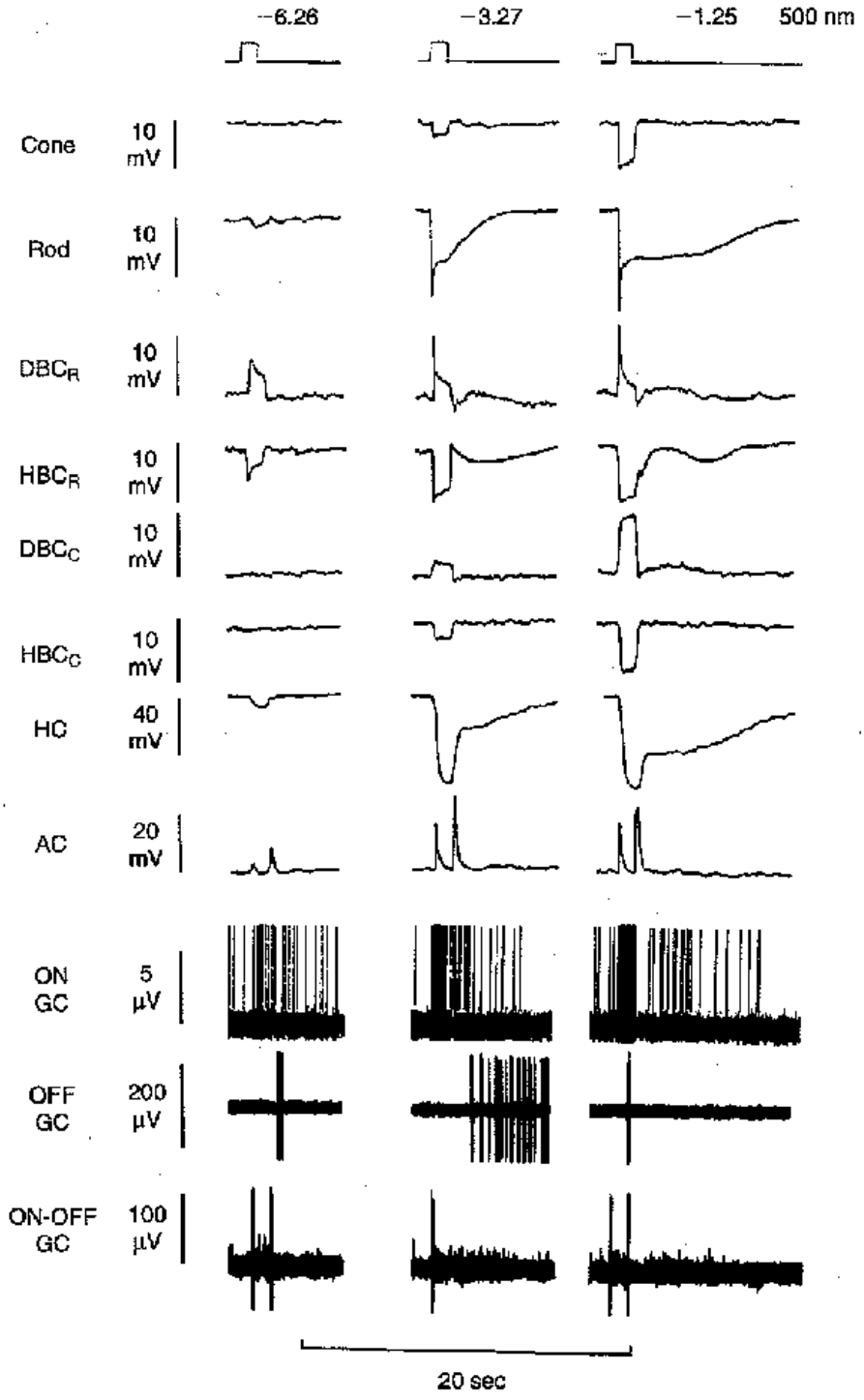

Figure 2.5. *Intracellular light responses of the major types of neurons in the tiger salamander retina. The stimulus light is 500 nm at dim, moderate and high intensities (demonstrated by the light attenuation factors above the image). The cells from top down: cone photoreceptors, rod photoreceptors, depolarizing bipolar cell (rod-dominated), hyperpolarizing bipolar cell (rod-dominated), depolarizing bipolar cell (cone-dominated), hyperpolarizing bipolar cell (cone-dominated), horizontal cell, ON-*

OFF amacrine cell, ON-center ganglion cell, OFF-center ganglion cell and ON-OFF ganglion cell. [Kaufman & Alm 2003, p. 423]

2.2.3. RPE Responses

The light-induced responses of the RPE are based on changes in the subretinal space to which RPE responses. These subretinal changes take place due to light onset (in dark-adapted retinas) when the photoreceptors react to light. As a consequence, the K^+ concentration decreases, pH increases and lactate concentration decreases in the subretinal space. Also the volume of the subretinal space increases. Some of the RPE responses to these changes can be electrically recorded. [Kaufman & Alm 2003]

The first of the recordable responses of the RPE is the c-wave of the ERG. A hyperpolarization across the membrane potential of the RPE retinal membrane occurs at the decrease of the K^+ concentration in the subretinal space. As a consequence, the cornea-positive transepithelial potential across the RPE increases, which is seen as the c-wave in the ERG. [Kaufman & Alm 2003]

The lowered concentration of K^+ causes decline in the rate of ion transportation, including Cl^- -ion transportation, across the RPE retinal membrane. As a consequence to that, the efflux of the intracellular Cl- reduces causing a hyperpolarization of the membrane potential across the predominantly Cl^- permeable choroidal membrane of the RPE. This results in reduced transepithelial potential across the RPE that can be seen in the ERG to terminate the c-wave. The waveform is called the fast oscillation (FO). FO develops within 1 to 2 minutes after light onset and is too slow to be recorded with standard ERG. Recording can be done with DC-coupled ERG. [Kaufman & Alm 2003]

A third recordable RPE response occurs minutes after light onset when Cl^- permeability increases in the choroidal membrane. This produces depolarization of the membrane potential as a counteract to the above-described hyperpolarization which took place after the decrease in the Cl^- permeability of the choroidal membrane. Now, a corneal positive light peak that can be recorded with DC-ERG or electrooculography is generated. [Kaufman & Alm 2003]

C-waves and FO's have been reproduced in vitro in the absence of the neuroretina by a reduction of K+ concentration in the subretinal space. Light peaks are induced by a substance that is not yet identified and thus light peaks are not reproducible in vitro in the absence of the neuroretina. [Kaufman & Alm 2003]

All of the above-mentioned RPE responses are lost when measuring the ERG from the isolated retina alone without the presence of the RPE. The lacking of the RPE layer has not been reported to affect the retinal responses. So not having the RPE in the measurement does not affect the ERG in other ways besides losing the RPE responses. The retinal recovery from an intense light exposure that does photopigment bleaching, however, is impossible without the presence of a functional RPE since regeneration of the pigments requires RPE. Thus also dark adaptation is impossible to study without the RPE. [Newman & Bartosch 1999]

2.3. ERG Measurement

ERG is a powerful diagnostic tool for evaluating the functionality of the retina. ERG measurements can be done from living objects, but also from enucleated eyes, eyecups and isolated retinas. The clinical measurements have their own standard methods to ensure the comparability of measurements done at different locations with different equipment and by different people. For research ERG these guidelines are often too limiting and great variety in measurement setups and methods occur. Here the clinical ERG measurement standards are briefly discussed and a general overview of the basic function of the measurement system with its components is presented. MicroERG recordings done with a microelectrode array setup similar to the one used in the measurements of this thesis are presented. These measurements represent recording the ERG from isolated retinas. Finally, the specialities in measuring the ERG from the mouse eye are covered in a separate section because of the significance to this study.

2.3.1. Clinical Measurement of the ERG

According to the ERG standard, clinical ERG is recommended to be measured on the cornea surface with contact lens electrode and the reference electrode being placed in contact with the conjunctiva. This kind of measurement setup gives the most stable results in clinical measurements. The ERG standard measurement responses are presented in Figure 2.6. [Marmor et al. 2004]

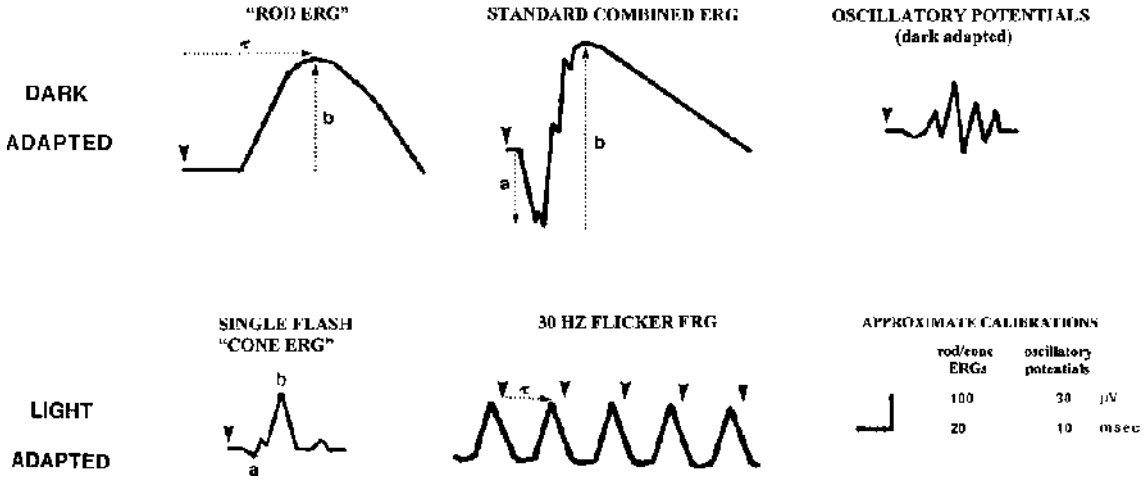

Figure 2.6. *ERG standard responses: dark-adapted rod response, dark-adapted maximal response, oscillatory response, light-adapted cone response and flicker cone response. [Marmor et al. 2004]*

In the standard there are five different kinds of light stimuli of white light that can be applied in measuring the ERG. Each of the stimulus types gives a different kind of response according to the function of retina that is stimulated. The standard defines five basic waveforms: dark-adapted rod response, dark-adapted maximal response, oscillatory response, light-adapted cone response and flicker cone response. [Marmor et al. 2004]

2.3.2. Components of an ERG Recording System

A measurement system for ERG comprises of several components. Figure 2.7. shows a general overview of the equipment used in a clinical ERG. The patient gets a stimulus from the lamp. The response is recorded with electrodes that lead to amplifiers. The data gets processed as it is filtered and converted to digital form. The digital data is brought to a computer where it is stored and shown through a display. Finally, a control program is used to operate the whole system, including the stimulus control. The system components are practically identical in a setup for non-clinical ERG measurements even though the patient is replaced with an isolated eye or retina. [Heckenlively & Arden 2006]

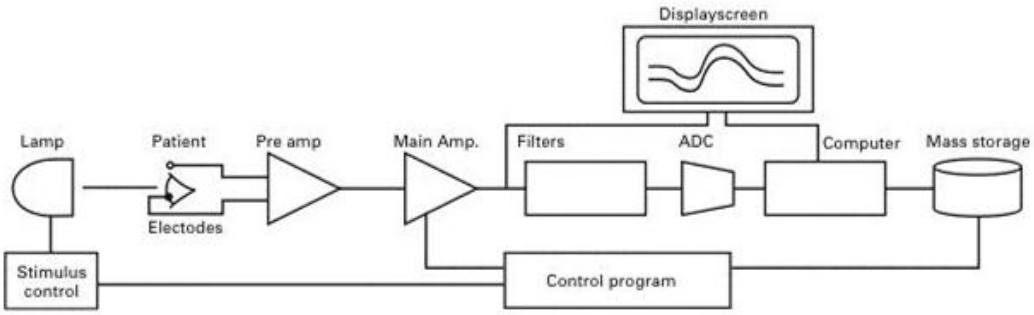

Figure 2.7. *Components of a measurement system for clinical ERG. [Heckenlively & Arden 2006]*

The stimulator is the first part of the system. Often it is chosen so that it may be synchronized with the data acquisition components. The stimulating lamp needs to be correctly calibrated, and the calibration needs to be repeated regularly. This is essential since the measured responses vary significantly as the stimulus is altered and thus the measurements are not comparable. [Heckenlively & Arden 2006]

The lamps for ERG stimulation can be of many different kinds. A major division is between unstructured and spatially structured stimulators. The latter ones can display a pattern while the first ones just display a light. The spatially structured stimulators can be for example different kind of TV or computer monitors like LCD displays or plasma displays. The unstructured stimulators are some kind of lamps. The most common unstructured stimulator is the Ganzfeld stimulator that has a bowl where a xenon flash lamp is used to generate the stimulus. Light emitting diodes (LEDs) have developed rapidly over the last decade and now they offer a reasonable option to the xenon lamps as the light source. Their benefits are low cost, small size, low driving currents and voltages, wide range of intensities and possible waveforms, simple electronics and good sustainability in extended use. [Heckenlively & Arden 2006]

There are a great variable of electrodes for ERG recording but the choice between them should be done carefully since poor electrodes or their poor handling can cause severe noise in the measurement. Most commonly used in the clinical setup are the contact lens electrodes because of their good signal-to-noise ratio, durability, and consistency of the recorded ERG's. The lid-hook electrodes and the DTL (Dawson, Trick,

Litzkow) fibre electrodes are less common but still widely used. The lid-hook electrodes' benefits are good patient-acceptance, ease of use, unaltered optical quality and good recording results. DTL fibre electrodes also have good patient-acceptance and ease of use as well as unaltered optical quality but they are also disposable and no sterilization is needed. Sometimes, especially with young children, skin electrodes are used. Their benefits are patient comfort and ease of use. The skin electrodes also need no sterilization. [Heckenlively & Arden 2006]

The ERG data has small amplitude and thus it needs to be amplified. The amplifiers used for ERG measurements usually need to have a gain of 1000 to 10 000. Measuring from a patient makes it a necessity to use a differential amplifier instead of a single-ended one because the noise sources produce much greater signals than the one that is actually measured. The differential amplifier discards the common mode signals and thus improves the quality of the measured responses. The modern amplifiers have good common mode rejection ratio (CMRR) that is > 100 dB for ideal inputs. In practice, however, poor electrode contacts may drop this significantly. Many amplifiers are AC-coupled to prevent small offset voltage differences that often exist between electrodes from being amplified. This causes problems when measuring the slow components of the ERG. Often the c-wave is already too slow to the AC-coupled amplifier and is rejected. [Heckenlively & Arden 2006]

Filtering the signal is a common way to reduce the noise from the measured data. In ERG the frequency range of interest is usually between 0.1 and 300 Hz and any data beyond this range can be filtered. The filters are not ideal and some compromises need to be done. The most suitable filter for electrophysiological signals would be the Bessel filter that has minimal phase shift with frequency even though the amplitude versus frequency profile is not as good as with other options. The cut-off level of the filter often refers to the point where the signal reduction is -3 dB which means reduction of 70 % from the unfiltered data. If the range of interest is desired to be kept practically unfiltered, the range needs to be stretched so that the filtering that happens already before reaching the cut-off level is taken into account. With correct filtering, the signal-to-noise ratio of the measurement can be improved. [Heckenlively & Arden 2006]

The next stage is to convert the signal to a digital form. This is done by an analog-to-digital converter (ADC). The ADC is characterised by the voltage resolution and the conversion rate. The voltage resolution is defined by the available bits. The minimum requirement should be at least 12 bits that gives 4096 voltage levels. The levels are divided to the whole input range and the range of the actual response normally has fewer levels than the maximum resolution. The conversion rate is the maximum throughput of the ADC. The maximum signal frequency that can be converted is sometimes quoted as the data throughput divided by 2. In practise frequencies that high would not be converted correctly. If too high frequencies are being converted aliasing might occur. For multiple channel systems also sample and hold amplifier is needed for a simultaneous sampling to prevent slewing of the outputs. [Heckenlively & Arden 2006]

After conversion the digital signal still often needs some processing. Signal extraction and artefact rejection are two possible ways for improving the signal quality. The most common way for signal extraction is averaging. The stimulus is repeated synchronously and the responses can be averaged based on the synchronous timing. Since the noise is considered to be random, it is deleted in the averaging while the response remains visible. The improvement to the signal quality depends on the number of the repetitions that can be used for the averaging. If the signal has some artefacts, they should be removed before averaging or the result is not as good as desired. The best way to remove artefacts is to set limits to the signal amplitude and if they are crossed, reject the entire sweep. Fourier analysis method can also be used for signal extraction but it is more complicated and loses information about the signal waveforms. The power and phase at a particular frequency can be analyzed. The method has pitfalls if some unexpected changes happen in the stimulus, patient or the technology. [Heckenlively & Arden 2006]

The control software is the heart of the whole recording system. Two main approaches for it exist. One gives the user the possibility to alter all the variables for the stimulus and the data acquisition as he desires and the other one gives just the minimal possibilities to vary parameters while the program runs through a detailed set of protocols. The first one is ideal for research purposes and the second one is more for the clinical routine use. The recorded data should be stored with the metadata that contains the information about the measurements like dates, stimulus parameters, recording settings, etc. [Heckenlively & Arden 2006]

2.3.3. Special Features of an ERG Setup for Isolated Retinas

In addition to the ERG measurement from a living object ERG can be measured from an isolated eye, eyecup or retina. These methods are applied in eye research with animal models when for some reason living objects cannot be used. Using isolated retinas or eyecups also makes it possible to apply research methods that cannot be used with whole eyes or living objects such as application and washing of drugs, and studying single cells or cell groups. That makes it beneficial to use isolated retinas or eyecups compared to using living objects for research purposes in several occasions.

When measuring ERG from an isolated retina, some features need special attention. Retina is a delicate and fragile tissue that is highly metabolic. For successful ERG measurements, retina needs to be carefully prepared and placed to a suitable medium. Sufficient oxygenation and perfusion are especially important. The retina is flattened on measurement electrodes usually with ganglion cell side facing the electrodes because the photoreceptor side damages easily. A reference electrode is brought to the opposite side of the retina so that it is in contact with the medium. Now the potential differences across the tissue can be measured. If micro scale electrodes are used, local ERG responses can be measured. Single cell responses can be recorded with patch clamp techniques.

With well chosen methods it is possible to culture the retina for some days and sustain the retinal ability to response electrically to light stimuli [Koizumi et al. 2007]. However, the best ERG responses are obtained when measurement takes place within the first hours after preparation and often retina loses its viability within a day after the preparation.

Since the measurement from an isolated retina is done with light stimuli, light from no other light sources should reach the retina. For this reason, the animals used in the measurements have often been dark-adapted for at least three hours prior to the preparation, ERG is measured in darkness and even tissue preparation is often done under dim red light to prevent photoreceptor bleaching. [see Green & Kapousta-Bruneau 1999; Chen et al. 2004; Koizumi et al. 2007]

2.3.4. Microelectrode Array Recordings

Modern microelectronics has made it possible to manufacture electrodes in micrometer scale. With an electrode array of microelectrodes it is possible to measure ERG simultaneously from several locations of an isolated retina. This enables drug testing and continuous monitoring of the retinal state for several hours. Different drugs may be delivered to the retina through superfusion and if their effects are reversible, many tests can be performed with one retina which gives great benefits for testing purposes.

Here ERG recordings done with one commercially available microelectrode array (MEA) measurement system are presented. The system is similar to the one used in the measurements done in this thesis. The MEA has 60 flat electrodes on the bottom of a dish and an isolated retina is placed on them for the measurement. The system with its components is presented in detail in chapter 3.1.1.

Several publications where retinal signals have been studied with the MEA system have been published up to date. The general ERG of different species has been studied widely as well as the ganglion cells' spike train responses, but also the electrical stimulation of the retina and spontaneous activity of the fetal retina have been studied with the MEA system. [MEA Homepage 2009] The ganglion cell activity, which is not easily seen in the standard ERG, can be studied with the microERG recorded with the MEA because the measuring electrodes are situated right next to the ganglion cells. Here two basic ERG studies with different approaches that were conducted with the MEA are introduced.

A basic measurement for both chick ERG and ganglion cell spikes has been reported in [Stett et al. 2003]. For recording the microERG a retinal segment with the RPE attached was prepared and recorded ganglion cell side down on a MEA with the stimulating light coming from below. The bandwidth for measuring the ERG waves was filtered to the range of 0.5 Hz to 100 Hz. The same setup is suitable for ganglion cell spike measurement with a pass-band from 200 Hz to 2.8 kHz. The tissue orientation and measurement results are presented in Figure 2.8. [Stett et al. 2003]

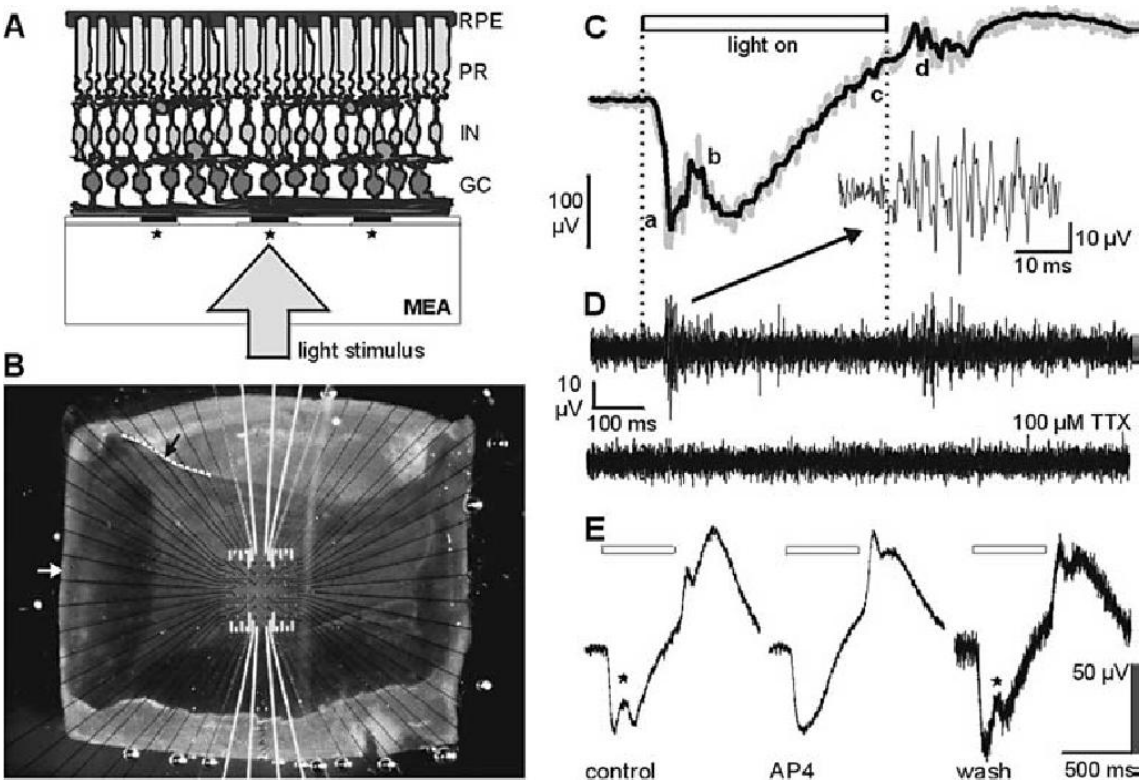

Figure 2.8. *Measuring ERG from a chicken retina. (A) Orientation of the retina on a MEA. (B) View through a MEA where the border of the RPE tissue is seen. (C) Micro-ERG of a chicken retina with a-, b-, c- and d-waves. (D) Spike activity. (E) Drug action on a microERG. [Stett et al. 2003]*

In Figure 2.9. local ERGs from a similar measurement as above are presented with responses from 60 electrodes. There is variation in signal quality between electrodes and good signal-to-noise ratio is only achieved in the central parts of the retina-RPE complex. In the figure the effect of the RPE can be clearly seen as the appearance of the c-wave in the ERG in the shadowed area where the RPE is firmly attached to the retina. [Guenther at al. 2006]

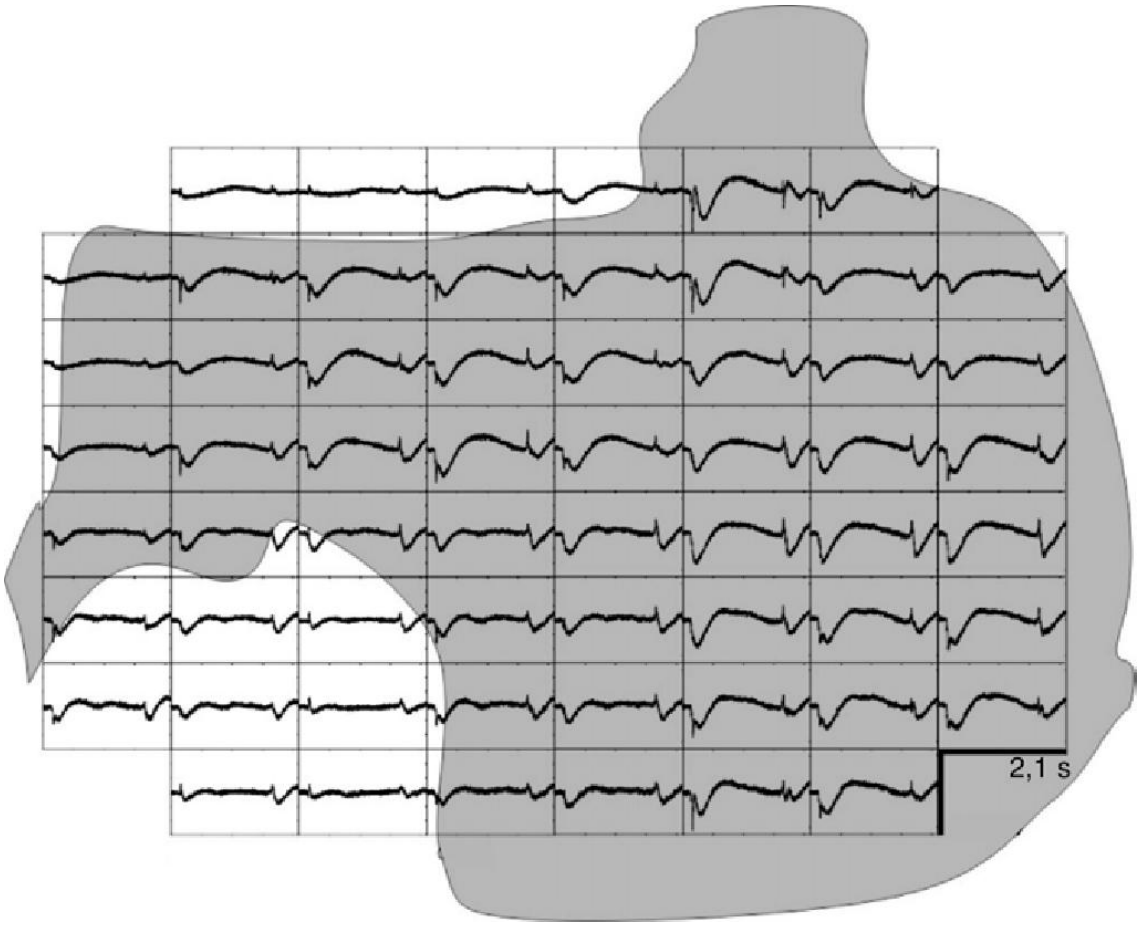

Figure 2.9 *Local ERGs recorded on a microelectrode array with 60 electrodes. On shaded areas retina is attached to RPE and c-wave is present in ERG. [Guenther at al. 2006]*

One significant benefit of the MEA system is the possibility of studying ganglion cell responses simultaneously from multiple locations in the retina. Without the electrode array ganglion cell responses could only be recorded with single cell recordings that record responses from individual cells. Another benefit is the ability to study the effects of different drugs and toxicity to the retinal or ganglion cell responses. Rosolen et al. [2007] have studied ganglion cell responses and their changes, when some drug with well-known effects on the synaptic activity was applied. Figure 2.10. shows spontaneous retinal ganglion cell responses (A), responses elicited with a light stimulus (B), a single cell ON-OFF response (C) and template-analyzed spikes (D). The study shows that the retinal function retains similar characteristics as an explant in in vitro MEA measurement as it does in vivo and therefore the MEA measurement system gives valuable possibilities in studying retinal signalling. [Rosolen et al. 2007]

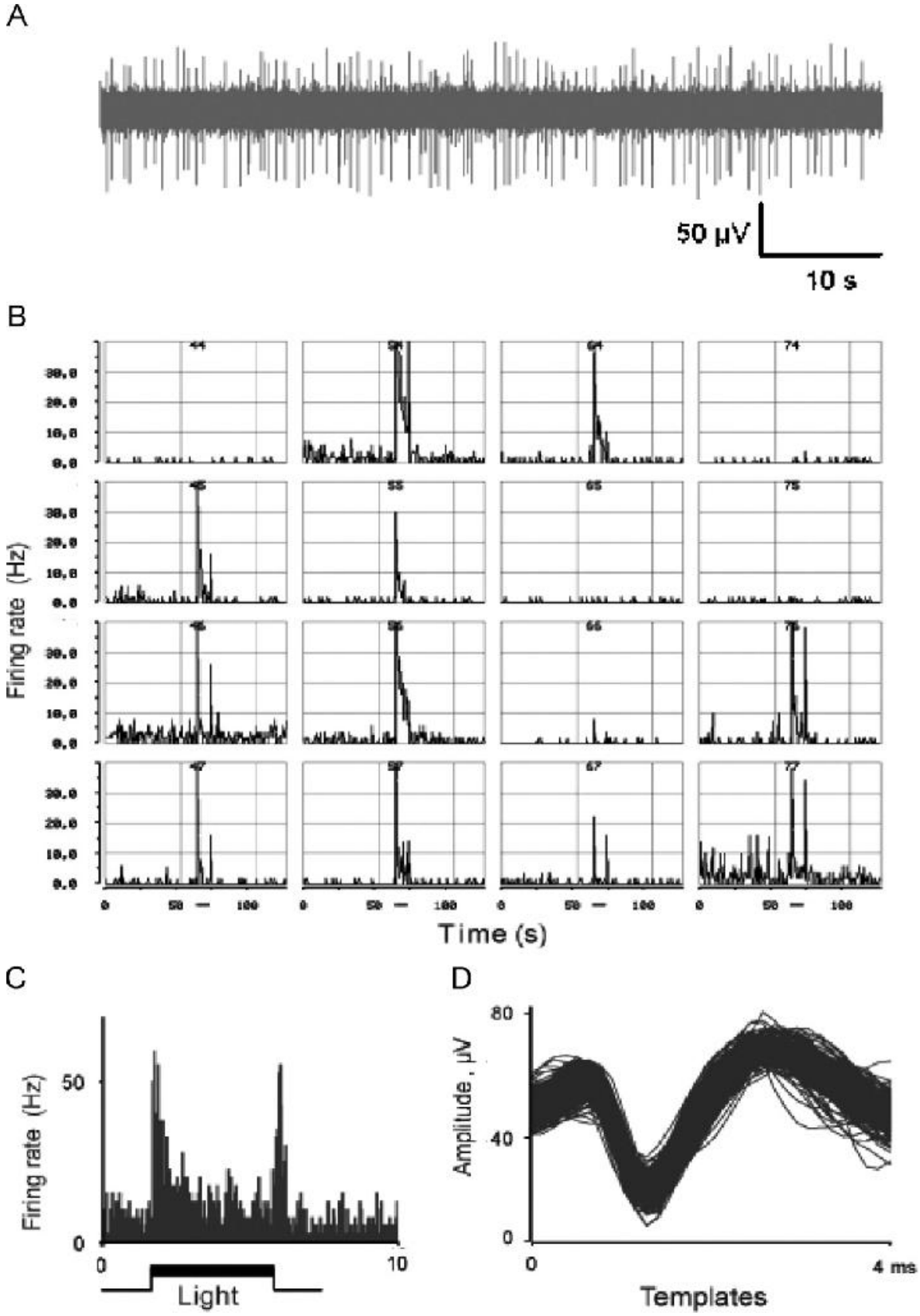

Figure 2.10. *Visual responses of retinal ganglion cells to light stimulation. (A) Spontaneous activity. (B) Simultaneous recording of response to a light stimulus from 16 different channels. (C) Typical ON-OFF response after subtraction of (D). [Rosolen et al. 2007]*

2.3.5. ERG Measurements from Mouse Eye

Mice are widely used in eye research for many reasons. Perhaps the most important reason is the ease of their genetic manipulation and the possibilities it creates. Mice eyes are therefore widely studied and a lot of information is available. Many similarities exist between mouse and human eyes but also some differences. These major differences are discussed here briefly.

Mouse eye is significantly smaller from that of human. This is important when considering the equipment that is used for ERG measurements. Mouse is naturally an animal that is active at night. For this reason, a mouse eye is more sensitive to light than a human eye, and it contains almost exclusively rods as photoreceptors in the retina. It is estimated that only about 3 % of the mouse retinal photoreceptors are cones [Nusinowitz et al. 2002] compared to approximately 5 % in human retinas [Kaufman, Alm 2003]. And while in the human eyes the cones are focused in the area of macula lutea reaching the peak density at fovea in mouse eye the cones have no area of higher density but is steadily about 3 % in all areas of the retina. [Nusinowitz et al. 2002]

Compared to human cones that have three photopigments, mouse cones only have two: one peaking near 350 nm and the other near 510 nm. Mouse rods spectral sensitivity peaks at approximately 510 nm. [Nusinowitz et al. 2002]

Typical mouse ERG response to a bright light flash includes the a-wave and the b-wave, but for technical difficulties in recording the c-wave from isolated retinas it has not been widely used in mouse ERG studies. OP's are typically seen at the rising edge of the b-wave. In Figure 2.11. separated mouse rod and cone responses to light stimuli with different intensities are shown together with a plot of response amplitude vs. stimulus intensity. [Nusinowitz et al. 2002]

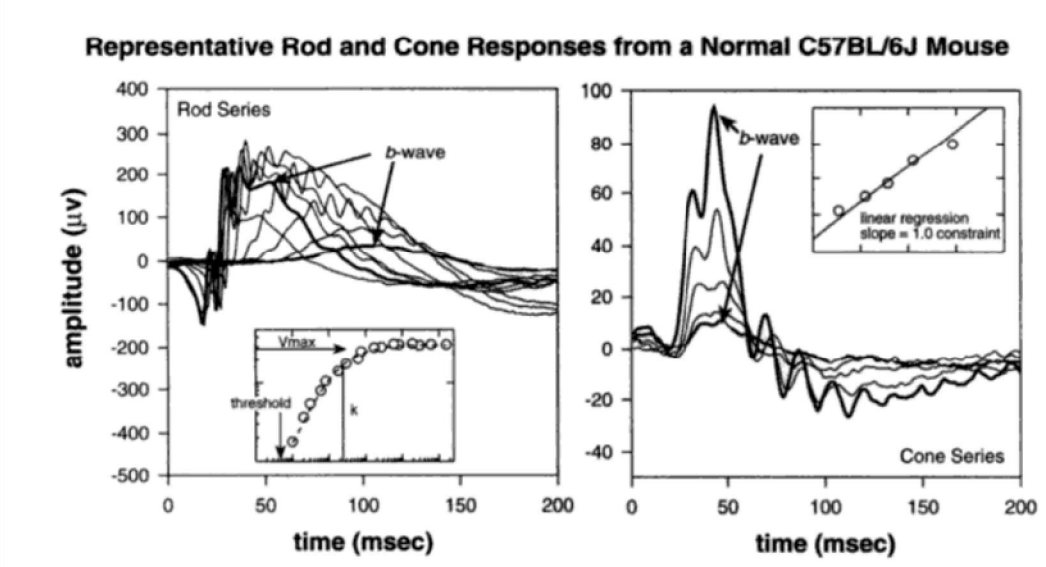

Figure 2.11. *Rod (left) and cone ERGs of a normal mouse, responses to light with increasing intensities. [Nusinowitz et al. 2002]*

2.4. Retinal Detachment and Reattachment

One of the basic ideas behind this study is to make cultured RPE cells to work together with an isolated retina. This involves attaching the two separate tissues together and observing whether co-operation occurs or not. A somewhat similar situation is present when retina is first detached from the pigment epithelium and then reattached back to its original position. This detachment and reattachment has been studied to some extent and a summary of the results from these studies is presented here.

The structure of the retina is naturally such that RPE at the bottom of the retina lies firmly on Burch's membrane. The photoreceptors which form the next layer are, especially in some animals, very loosely attached to the RPE. This property causes the natural retinal structure to break very easily during the preparation of retinal explants since the RPE stays attached to the eyecup while the rest of the retina detaches from the RPE. Often retinal explants are purposely prepared without the RPE, but then a natural connection which exists in vivo is lost. This detaching of retina from the RPE is called retinal detachment. It may also occur in vivo and cause problems in sight.

The intuitive way to cure retinal detachment is to reattach the two retinal layers. Some in vitro studies have been made to study whether it is possible to reattach a detached retina and regain the functionality of the retina together with the RPE. Results of a study by Monaim et al. [2005] have shown that when both detachment and reattachment are carefully made, retinal functionality recovers quite well in a toad retina. The electrical functionality was studied with ERG. These measurements showed that after 10 h the amplitudes of the b- and c-waves were only partially recovered. The sensitivity of the b- and c-waves recovered fully in 10 h even though it was significantly reduced right after detachment and reattachment. [Monaim et al. 2005]

In light microscopy the development of renewed contact between retina and RPE can be seen as the disappearance of the space between the two tissues as time passes and as the reduction in the number of broken-off photoreceptor outer segment pieces. The process of retinal reattachment is demonstrated in Figure 2.12. where light microscopy images at different times after retinal reattachment are presented. [Monaim et al. 2005]

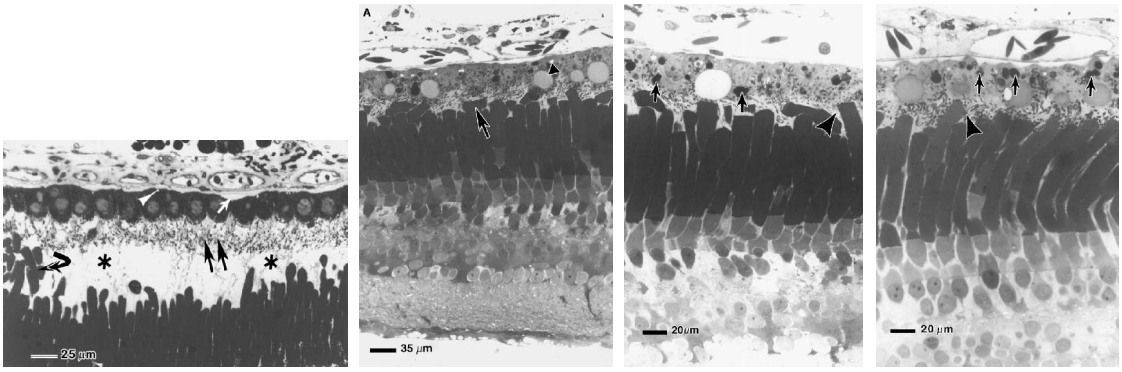

Figure 2.12. *Light microscopy images of progress in reattaching the retina with the RPE after detachment. Images are from timepoints 30 minutes (left), 2.5 hours, 5 hours and 10 hours after the reattachment. Disappearing of the space between retina and*

RPE as well as decline in the number of broken-off photoreceptor outer segments can be clearly seen as time has passed. [Monaim et al. 2005]

A review study on retinal detachment and its modelling has been done by Fisher et al. [2005] where the reattachment of the retina with the RPE is briefly discussed. The photoreceptor outer segments re-growth after reattachment has been known for decades but studies for completely unveiling the mechanisms still remain to be done. Photoreceptors have an ability to constantly add new outer segment material and they do so whenever the circumstances are promising. Based on studies [Lewis et al. 1991, according to Fisher et al. 2005] rods continue to transport radiolabeled proteins to the remaining outer segments after the detachment. No comparable studies on the protein transport of the cones exist but there is reason to believe that cones react differently from rods when detachment takes place. The membrane renewal sequence is similar in rods and cones, but experimental data suggests that protein production is different in the two after detachment. This indicates that after detachment the membrane renewal in cones may be adjusted. In terms of cell death during detachment and recovery of sight after reattachment this would mean that rods are more vulnerable to apoptotic cell death during detachment but when reattached the recovery is fast. With cones the situation would be the opposite, more cones would survive the detachment period but the recovery of the sight is more gradual. However, this hypothesis has not yet been systematically studied. [Fisher et al. 2005]

One situation when reattachment of RPE and the retina occurs is the transplantation of the RPE. Based on studies about RPE transplantation and its effects on retinal diseases RPE transplantation appears to cause photoreceptor rescue by two different means: 1) the direct contact between the photoreceptors and the RPE and 2) the indirect influence that the RPE has to the photoreceptors through the components it releases such as ciliary neurotrophic factor, glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor and basic fibroblast growth factor. How these two parts affect the rescue as a whole as well as the mechanism itself behind the photoreceptor rescue is still unknown. [da Cruz et al. 2007]

2.5. Retinal Explant Culture

For performing measurements on the same retina on consecutive days it is necessary to keep the retina viable for an extended period of time. This is done by culturing the retina. The difficulty in retinal culture is the high metabolism of the tissue. There are different methods that have been utilized in order to maintain the retinal viability for as long as possible or necessary. Two main approaches exist: explant and slice culture. For the purposes of this study we focus on the explant cultures where the whole retina or a rather large part of it is cultured. In slice cultures the retina is first cut in very narrow slices which are then cultured [Kretz et al. 2004].

Many retinal culture studies are done with neonatal or early postnatal retinas of species that do not have fully developed photoreceptors and thus have lower metabolism.

The viability of these retinas can be remained up to several weeks based on the parameters set by the researchers [Mosinger Ogilvie et al. 1999], and due to the lower metabolism this is simpler than with adult retinas. Only one study that reports a culturing method for a whole retinal explant of adult mammalian retinas where viability is evaluated by electrophysiological measurements has been published up to date [Koizumi et al. 2007]. Some other studies, too, even though not quite comparable, do give important information on the optimal culturing conditions of the retina.

Koizumi et al. [2007] have presented a method for culturing physiologically functional adult mammalian retinas for up to six days. Isolated retinas from adult rabbits were cultured in Ames medium and two different culture chamber designs were tested one of which was found easier to use. Figure 2.13. shows the incubation chamber that was utilised. Electrophysiological recordings were done from ganglion cells by whole-cell patch-clamp and light responses were measured. Multielectrode array recordings of ganglion cell activity were also done for some retinas after two or three days of incubation. [Koizumi et al. 2007]

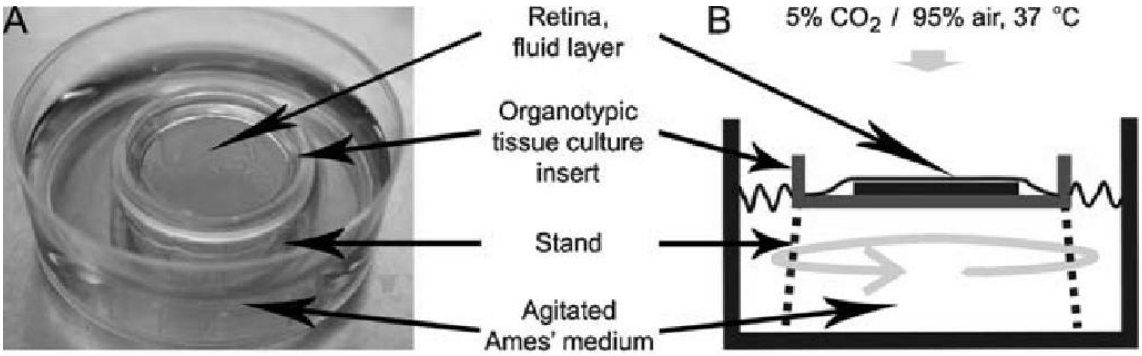

Figure 2.13. *A Photograph and B schematic diagram of an incubation chamber used for culturing adult mammalian retinas for up to six days. [Koizumi et al. 2007]*

In retinal culturing conditions it is mentioned that daily exchanging of the medium as well as (constant) stirring of the medium were required to maintain the retinal viability. The stirring was done with either a rocking platform or a magnetic stirrer. In electrophysiological recordings evident light responses were recorded with patch-clamp up to four days in culture. Only three out of six cells gave a positive light response after six days in culture. A slight difference is mentioned to have been observed between incubated and fresh retinas in the multielectrode array recordings after two or three days of culture. Fresh retinas had somewhat higher firing rates, more precise response timing and smaller receptive fields. These differences were delicate and none of them was statistically significant by itself. System limitations mentioned are cutting the optic nerve which leads to degeneration of ganglion cells, and separating the retina from the underlying RPE which limits the usage of bright light for stimulation and makes the photoreceptors more vulnerable to mechanical damage. [Koizumi et al. 2007]

Kaempf et al. [2008] have compared porcine retina-RPE cultures with retina alone cultures and freshly isolated retinas. The results suggest that having the RPE-choroid in

contact with the retina when culturing for three days preserves the photoreceptors better than in the cultures of retina alone. [Kaempf et al. 2008]

Kobuch et al. [2008] compared static and perfusion cultures with adult porcine retina-RPE-choroid tissue complexes. Perfusion culture was found superior as it was possible to keep the tissues morphologically intact for at least ten days when the tissue in the static culture had lost its structure already after four days. [Kobuch et al. 2008]

There are other factors that are mentioned in the studies related to this topic that need some consideration, as well. These methods might have a positive effect in culturing the retina successfully for longer periods of time. The methods are: 1) using sterile or semi-sterile preparation and tissue culture that is obtained by using sterile tools in preparation [Kretz et al. 2004, Kaempf et al. 2008; Xin et al. 2007], 2) using a laminar flow hood [Kretz et al. 2004; Xin et al. 2007], 3) sterilization of the tissue or the eye [Kretz et al 2004; Kaempf et al. 2008], 4) short post-mortem time with a closed culture system [Kobuch et al. 2008; Kaempf et al. 2008] and 5) using antibiotics [Kaempf et al. 2008; Kobuch et al. 2008; Koizumi et al. 2007; Kretz et al. 2004; Xin et al. 2007]. The species used also affects the time for which the culture remains good [Koizumi et al. 2007; Werner et al. 2008]. Furthermore, to obtain a good contact between the retina and the carrier, some apply a gentle suction [Koizumi et al. 2007; Kretz et al. 2004].

3. MATERIALS AND METHODS

The practical part of the study consists of the development of the ERG measurement setup for freshly isolated mouse retinas so that light responses could be measured reliably and repeatedly with the system. Further evolution that included measuring light responses from mouse retinas attached to hESC-derived RPE cells has also been done. The progress of the setup was done gradually one step at a time that was needed for the ERG measurements to be done. At different phases of the process trial measurements were performed to see the effect of the changes to the measured responses.

The measurement system developed and its components are described in this chapter in detail. Here specific methods and material are seen as components of the system because they are essential for the measurement to be successful. Finally, the course of the measurement in practice is introduced.

3.1. Components of the Measurement Setup

At the beginning of the work the system setup was done for electrical stimulation and measurements for the mouse retina. Many parts of the measurement setup, like the MEA system and oxygenation, remained the same throughout the development process. Nevertheless, many things, such as perfusion, also needed to be re-evaluated and modified for these measurements. Some aspects, like the light stimulator, were completely new and had to be determined, integrated and tested for the setup.

Here each component of the measurement setup is described. If seen as relevant information, also the process how the component was chosen/developed is included.

3.1.1. MEA System

The microelectrode array (MEA) system that includes the basic measurement equipment needed for the in vitro ERG measurements is manufactured by Multi Channel Systems (MCS GmbH, Germany). It consists of the MEA, preamplifier with a heater, stimulus generator, perfusion cannula, temperature controller and software for determining stimulus parameters and recording the data of the measurement. In Figure 3.1. the parts of the MEA system are presented. The system is developed for performing electrophysiological extracellular recordings with multiple channels from cells or acute slices in general and it is suitable for ERG recordings from an isolated retina. The system properties depend on the properties of the individual components that can be chosen from a variety of options. Here, a system that corresponds with the system used in the measurements of this study is described.

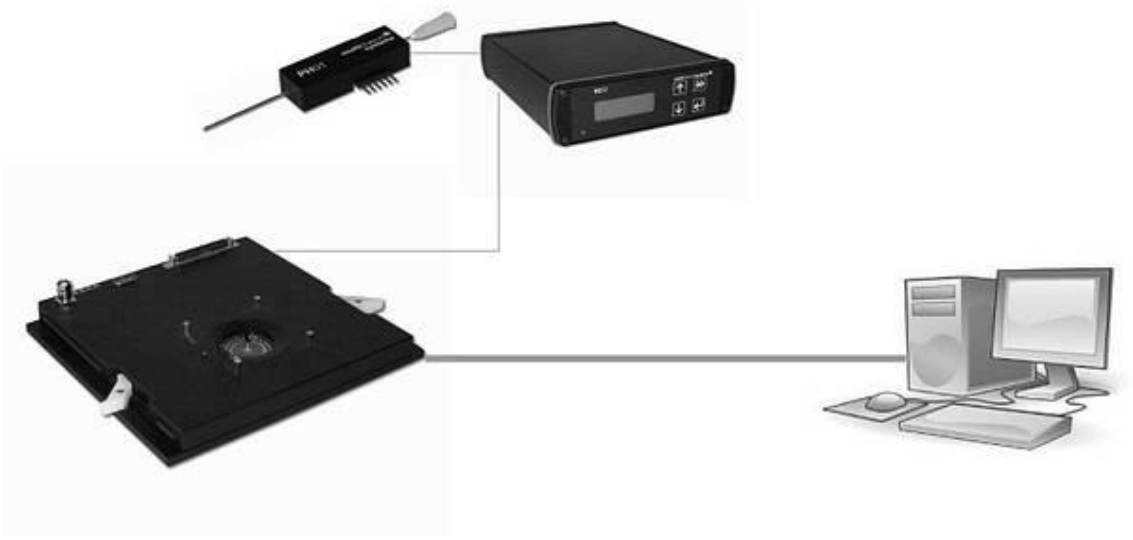

Figure 3.1. *The components of a standard MEA system. [Scientifica Image Library 2010]*

The MEA system preamplifier used in the measurements (MEA1060-Inv-BC-Standard) has a gain of 1100 and a bandwidth from 1 Hz to 8 kHz. It is designed for inverted microscopes and has a blanking circuit for preventing amplifier saturation during electrical stimulation through MEA electrodes. The preamplifier has an integrated heating element and temperature sensor. [MEA Amplifier Manual 2008]

The preamplifier heating element is controlled with a temperature controller that has two output channels if two elements' temperatures need to be controlled independently. The control range of the controller is from ambient temperature (minimum 5 °C) to 95 °C. The first output channel is generally used for controlling the preamplifier heating element and the second output channel is suitable for operating the perfusion cannula (PH01) temperature that heats the medium entering the MEA-dish. [MEA Temperature Controller Manual 2007]

The ADC of the MEA system is done with MC_Card that has a data resolution of 14 bits and a conversion rate of 12.8 MB/s. The maximum sampling frequency is 50 kHz for each channel. The card has 64 analog and 16 digital input channels, and 16 digital output channels. [MEA System Manual 2006]

The system's stimulus generator (STG2004) has four analog output channels and four digital channels for synchronization with following and preceding devices. The output voltage range is from -8 V to 8 V at max +/-20 mA and output current range is from -0.8 mA to 0.8 mA at max 120 V compliance voltage. The output signal can be freely programmed from rectangular, ramp and sinusoidal waveforms. The resolution of the generator is 13 bits and maximum frequency 25 kHz. The generator is controlled through software. [MEA Stimulus Generator Manual 2007]

Microelectrode Arrays

There are different MEAs that have different electrode sizes and spacing. Standard MEAs have 8 x 8 microelectrode matrix where the corner electrodes are missing. This results in having a matrix of 60 equally sized electrodes at equal distances from each other. The round electrodes can have either 10 µm or 30 µm diameter and the interelectrode spacing can be 100 µm, 200 µm or 500 µm. [MEA Manual 2005]

HexaMEA is specially designed for measurements from retinal explant and is used in the measurements of this thesis. HeaxaMEA has 60 electrodes that form a hexagonal figure. The electrodes in the center are smallest with a diameter of 10 µm and spacing of 30 µm. The electrode spacing increases first to 60 µm and then to 90 µm as the distance of the electrodes from the center of the hexagon increases. Electrode sizes also increase first to 20 µm and finally to 30 µm at the outermost layer. The electrode sizes as well as their spacing and positions are demonstrated in Figure 3.2. [MEA Manual 2005]

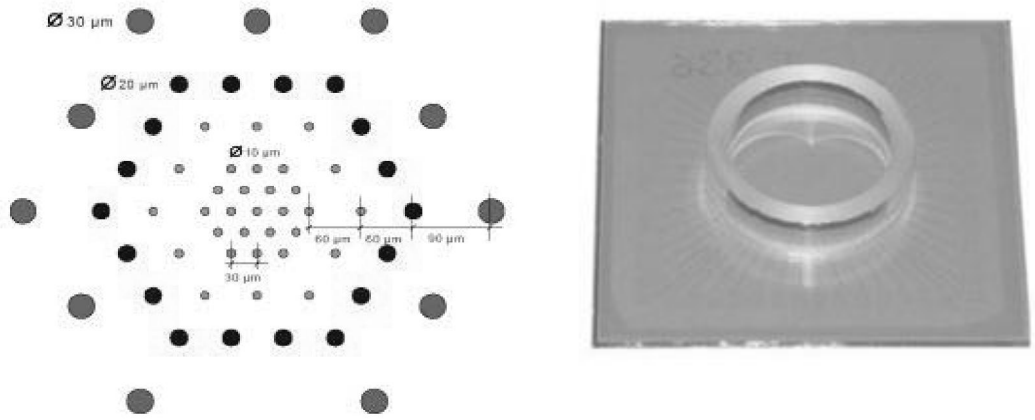

Figure 3.2. *HexaMEA layout [MEA Manual 2005] and image of a HexaMEA [MCS Homepage 2009]. HexaMEA is completely transparent, which is not apparent in the image due to the background colour.*

HexaMEAs have flat, round electrodes made of titanium nitride and transparent wires and contact pads made of indium tin oxide. Electrical insulation is achieved with silicon nitride. The electrodes are placed on a glass substrate and a glass ring is placed around the electrode area so that a dish is formed. This enables the perfusion that is necessary for the survival of retinal explants. The inner diameter of the glass ring is 19 mm and outer diameter 24 mm. The size of the glass MEA substrate is 49 mm x 49 mm x 1 mm (L x W x H). The electrodes in the middle of the MEA are situated in an area with 540 µm diameter. An image of a HexaMEA is displayed in Figure 3.2. [MEA Manual 2005]

Coating of the MEA

In the previous measurement setup the MEA dishes for the retinal measurements were coated with a cellulose nitrate coating before placing the explant on the MEA. The reasons for using such coating in the measurements are that the coating is supposed to im-

prove the electrode-tissue interface and this specific coating was recommended for recordings from retinal tissues in the MEA application note [Multi Channel Systems 2005]. A poor electrode-tissue interface was found to be a significant source of noise and a major factor in worsening the quality of the recordings. Also, if poor recordings were obtained, virtually with no exception the reason could be found from poor contact between the tissue and the electrodes.

Even then, the contact did not seem to be any better with the coating compared to if there was no coating at all. In every case, an external weight was needed to keep the tissue firmly on the bottom of the MEA dish. The cellulose nitrate coating is also a little tricky when cleaning the MEA dishes because it has to be removed with methanol. If some leftover tissue is still attached on the bottom of the MEA dish, the methanol fixes the remaining tissue firmly to its location and thus damages the MEA dish permanently. In a manual for MEAs it is also stated that for most acute slices, such as the retina, no coating is needed [MEA Manual 2005]. Encouraged by these facts, measurements were tried out without the coating and because no difference compared with previous recordings was observed, a decision of abandoning the usage of coating was done.

MEA Software and Stimulation Parameters

Light stimulation of the measurements was controlled through the stimulus generator of the MEA system which in turn was operated with software (MC_Stimulus II version 3.0.1). Stimulus generator was used either for giving the driving voltage of 1.5 V to the LED stimulator or sending a digital signal to the monochromator. The duration of the impulses and pauses between them as well as number of repeats were easily altered with the controlling software depending on the desired stimulus parameters. A trigger signal from the generator was sent simultaneously with every stimulus to be recorded together with the electrode data in the recording software (MC_Rack version 3.7.0). This facilitated data analysis.

Also the preamplifier was operated through software (MEA_Select version 1.1.0). All the software was provided by Multi Channel Systems through their website. In Figure 3.3. a view on a computer screen with the above mentioned programs running during a measurement is shown.

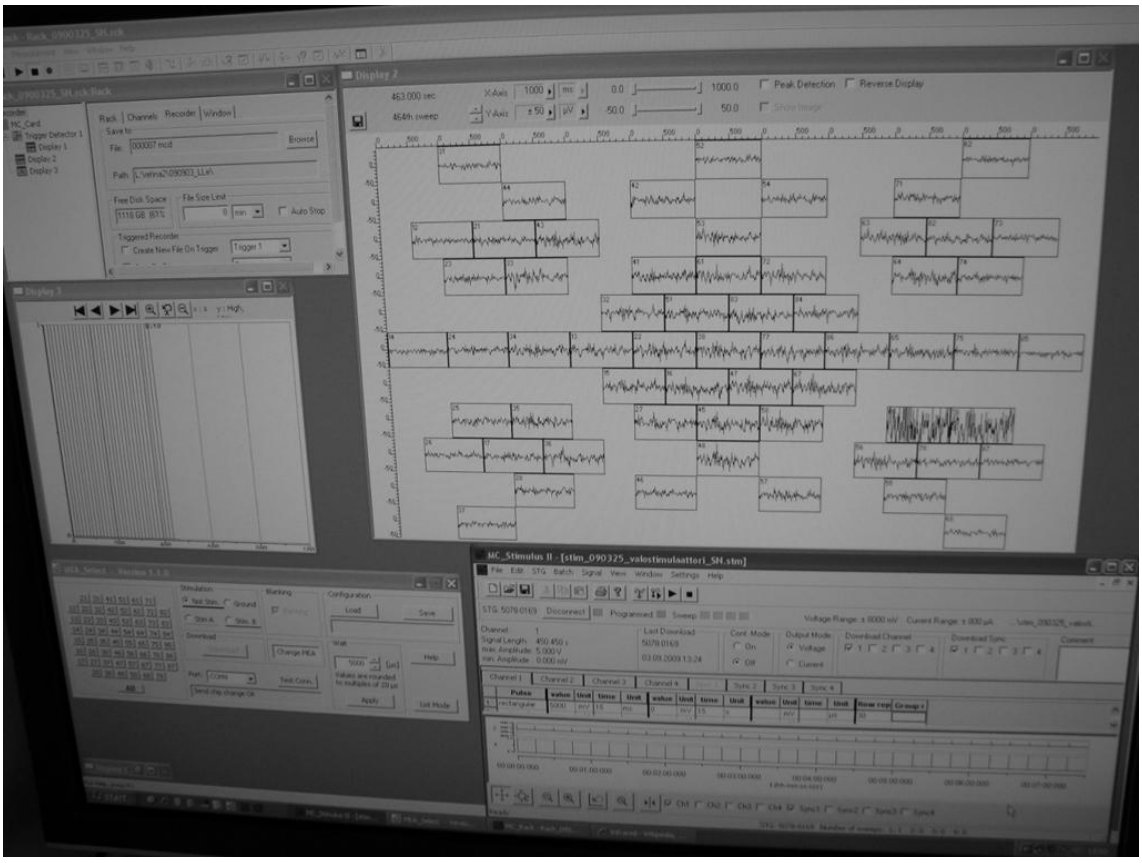

Figure 3.3. *Measurement control programs running on a computer screen. Left bottom window controls the preamplifier (MEA_Select), right bottom window is the signal generator control (MC_Stimulus II) and other windows belong to the recording program (MC_Rack).*

A few different kinds of stimuli and their effects on the recorded data were tested. The most common stimulus that was used was a short, rectangular pulse of 15 ms with rather long inter-stimulus intervals of 30 s. This short stimulus time is in line with stimuli used in previous similar studies [Green & Kapousta-Bruneau 1999; Heikkinen et al. 2008; Newman & Bartosch 1999].

A longer stimulus was used during one measurement. The stimulus duration was set to one second while the inter-stimulus intervals were kept at 30 seconds. This is in line with the longer stimuli used in previous studies [Multi Channel Systems 2005; Stett et al. 2003]. The longer stimulus duration makes it possible to separate the ON- and OFF-responses in the ERG at stimulus onset and offset and to record the c-wave if RPE is present.

We also used a one-minute continuous stimulus to see if the responses would be affected by the prolonged exposure to light. This kind of stimulus is used if rod bleaching is intended or one desires to study light and dark adaptation of the retina. This is in line with a stimulus used in [Newman & Bartosch 1999].

Most stimuli were repeated for 30 times for each measurement, some were repeated for 15 times, for averaging and thus improving the signal quality. Only the prolonged stimuli of one minute were done just once each time, but in that case the measurement

following the prolonged stimuli was of more interest than the response to the prolonged stimulus itself.

3.1.2. Light Stimulator

Since the previously set up system included no stimulation of the retinas with light, the stimulator had to be developed from the beginning. The light stimulus has great effect on the measured response which needed to be kept in mind while designing the stimulator. The important light stimulus properties, which all have different impact on ERG, are light intensity, colour of the light, stimulus duration and interval between two stimuli [Webvision 2009].

Two stimulators were used in the measurements. The first of them was a LED stimulator that was specially designed and constructed for these measurements. Another stimulator, a commercially available monochromator, was used in measurements done at a later time. The monochromator had not yet been acquired during the earlier measurements.

LED stimulator

The stimulator evolution started by determining its desired properties. The requirements for the stimulator were 1) sufficient intensity of light, 2) rather small size due to the restrictions of the measurement site, 3) possibility to control the stimulation duration and intervals with the stimulus generator of the MEA system, 4) possibility to vary the stimulus intensity and 5) use of a wavelength that mouse retina is sensitive to. As a reference a publication that reported properties of a full field light stimulator for similar purposes as ours was used [Herrmann et al. 2008]. Also the casing of the stimulator was designed to such that it fits to the measurement site. At a later time the stimulator was fixed to work with batteries to minimize the distortion in the measurement.

The actual construction of the stimulator was done elsewhere based on these requirements. The stimulator has a powerful LED light with a wavelength of 490 – 520 nm. This wavelength fits well to the spectral sensitivity of the photoreceptors in the mouse eye. Mouse rods are most sensitive to wavelengths of 500-510 nm and cones of type M are most sensitive to wavelengths of approximately 510 nm. S-type cones of the mouse eye are most sensitive to wavelengths of approximately 360 nm but since only one wavelength was chosen, this was ignored. The maximum and minimum intensities of the stimulator were measured after it was constructed and a minimum intensity of 4 000 lx and a maximum intensity of 25 000 lx were obtained. This intensity level was high enough for our purposes.

The stimulator was found functional, but unreliable, which caused problems when measurements were conducted. For this reason the number of measurements that were done for this thesis was reduced, but a sufficient number could still be achieved. The stimulator was later on further developed in order to solve the problems with reliability. After redesign, the stimulator was reliable, but it still caused distortion in the measured

signal while stimulating. Thus the stimulator was replaced with the monochromator in the measurement setup at least until the problem with the artefact is solved.

Monochromator

A commercially available monochtomator (Polychrome V, TILL Photonics GmbH, Germany) was acquired to the measurement laboratory during the development of the setup. The monochromator can be adjusted to work as a light stimulator for the purposes of the ERG measurements. A desired wavelength can be chosen for the stimulating light and the inter-stimulus time when no stimulus is desired, can be handled either by using a resting wavelength from the ends of the wavelength range (320 nm to 680 nm [Polychrome V data sheet 2009]) or by using a physical shutter.

In the measurements a wavelength of 510 nm was used for the stimulus and a resting wavelength from the high end of the range was applied during the inter-stimulus times. The intensities obtained when stimulating with the monochromator were not measured, but adjustment of the light intensity was possible. A maximum intensity was used with a wavelength bandwith of 15 nm. Narrowing the bandwidth would have caused reduction in the intensity of the stimulus which is why the maximum bandwidth was chosen. This had no significant change when comparing with the LED stimulator wavelength bandwidth.

The monochromator was operated through software obtained from TILL Photonics. The desired properties for the light stimuli were fed to the software with the information that the trigger signal for the monochromator comes from an external source. A digital signal from the MEA stimulus generator was brought to the monochromator to act as a trigger for the stimulus. This way the synchronization of the stimuli and data could be done in a similar manner as when using the LED stimulator.

3.1.3. Media

Two different media were used in the measurements. The first medium, Ames HEPES, was also used in the earlier setup and the other, RPE DM-, was added to the measurement setup to be used with the hESC-derived RPE cells.

The preparation of the Ames medium was done by the following method: Ames (A1420-10x1 l, Sigma) was buffered with HEPES (Fluka 54461) departing from the manufacturer's instructions so that the medium could be oxygenated with 100 % oxygen instead of carbogen (95 % oxygen, 5 % carbon dioxide). HEPES buffered Ames medium has also been used in previous similar measurements [Syed et al. 2004; Shyu et al. 2006]. The Ames medium is prepared by dissolving 8.8 g of Ames medium powder together with 4.77 g of HEPES into water. The pH of the solution is adjusted to 7.4 with NaOH so that the total volume of the solution is one litre. The medium may be preserved in the refrigerator for approximately two weeks.

The measurements had previously been done in Ames medium. When developing the setup for making measurements from retinas and RPE cells together it was necessary to consider the effects of different media to the recorded responses. The RPE cells

have been cultured in RPE DM- medium (containing 20 % Knock-Out Serum Replacement (KO-SR), 2 mM Glutamax, 0.1 mM 2-mercaptoethanol, (all precedings from Invitrogen, Carlsbad, CA) 1 % MEM Eagle non-essential amino acids, 50 U/ml Penicilin/Streptomycin (both from Cambrex Bio Science, Walkersville, MD) and the cells are quite sensitive to changes in the medium. Therefore it was reasoned to make trial measurements of the plain retinas with the RPE DM- medium. When no effects on retinal responses were observed when compared to the measurements done with Ames medium, using the RPE medium in the recordings was found suitable.

3.1.4. Perfusion

Developing the perfusion for the measurements proved to be a challenging task. The two-channel peristaltic pump (Masterflex, C/L, Cole Parmer Instrument Co, Model 77120-42) that had been used also previously was used for bringing fresh medium through one channel and for leading the old medium away from the MEA through the other channel at a rate of 1 ml/min. The flow in the two channels, however, was found not to be exactly equal which resulted in either overflow of the medium or drying out. The problem of overflow could be solved with using a wider tube in the outgoing channel that had enough capacity to carry out all medium that was brought in through the other channel. For solving the problem of drying out the outgoing needle needed to be fixed at a sufficient height. The medium surface could be kept stable at that height because the wider tube makes sure the surface drops to the same level as the needle if the medium surface level for some reason gets any higher. If the level gets lower, the outgoing flow would cease until enough medium is brought in to reach the level of the outgoing needle.

Using a polydimethylsiloxane (PDMS) ring for keeping the needles at good locations was tried but both the material and structure of the ring did not prove to be useful for the purpose. Little pieces of PDMS would break off and block the needles and thus prevent the whole perfusion. Also the hole for the needle in the PDMS ring would act as a continuation for the needle and cause continuous outflow until the bottom of the MEA dish is reached.

A stand made of polytetrafluoroethylene (PTFE) was a second trial for obtaining a steady flow of medium. The stand was placed on top of the MEA dish so that the circular edge of the stand was tightly around the MEA glass ring and the needles that were placed through small holes in the stand were just inside the MEA glass ring. This system was functional but not as practical as desired. Nevertheless, when perfusion was used, the stand was used for keeping the needles still.

A final solution to the perfusion problem arose in the measurement when RPE DM-medium was tested. In the measurements of the isolated retinas the recording time of a single retina was so short that there was no use in turning the perfusion on because by the time the fresh medium would reach the retina the recording would practically be over. So perfusion was not used and the medium in the MEA dish was evaluated to give sufficient nutrition and oxygenation to the retina.

With the measurements of the retinas and RPE cells together the recording times were significantly longer, but responses could still be measured even though no perfusion was applied. However, the medium was changed manually between measurements to improve nutrition of the tissues.

3.1.5. Oxygenation and Heating of the Media

Medical oxygen was used for the Ames HEPES medium oxygenation. The oxygen was brought through tubing to long hypodermic needles that penetrated the medium container lid. Medium was oxygenated for at least half an hour before it was used at a rate of 0.5 – 1.0 litres per minute. The sufficient rate of oxygenation was evaluated visually based on the oxygen bubbling in the medium. The oxygenation setup can be seen in Figure 3.4. RPE DM- medium was not oxygenated, but used in a similar way than it is in the RPE cell cultures to ensure similar conditions in the retina-alone measurements as would be in the retina-RPE measurements. Both media were heated in a water bath shown in Figure 3.4. to 37 °C before use.

Figure 3.4. *Heating Ames medium to 37 °C and oxygenating it. The lid of the water bath is removed for photographing purposes.*

3.1.6. Darkening of the Preparation and Measurement Sites

In order to record light responses from the retina it is essential to control the amount of light that reaches the retina. For this reason the measurements needed to be done in darkness. Another reason for this is that when the retina is separated from the RPE and

is exposed to bright light, photopigment bleaching occurs, and the retinal responses weaken. Without the RPE rod photoreceptors cannot recover from an exposure to bright light. To maintain rod responses at a maximal level after separating the retina from the RPE the retina is kept in darkness as much as possible.

To maximize the light responses in the recordings the retina needed to be protected from light the whole time after the mouse eye was enucleated. Even longer dark adaptation would have been beneficial, but not quite possible in the current measurement location. The enucleated eye was brought in a cool box to the preparation site. Protecting the retina from light during preparation meant in practice that the preparation of the tissue should have been done in darkness. Because this was not possible the light used for the preparation needed to be modified. Red light is absorbed poorly in mouse photoreceptors so the fiber optic illuminator lamps were covered with red foil. It is possible that all white light was not blocked with this method but for our purposes it proved to be sufficient. The preparation room was darkened in a similar manner as the measurement room described in the next paragraph so that no other light sources were used besides the red light. After the preparation the retina on a MEA dish was put in a cool box for transporting to the measurement room.

The measurement room was darkened with blackout curtains that blocked the sunlight completely when the edges were taped on the wall. The MEA dish was brought into the darkened measurement room inside a cool box that protected the retina from any additional light during transport. When moving the MEA dish from the cool box to the amplifier lamps, computer screens and other significant light sources were turned off. Red LED light was used for sufficient seeing during the placement. After the stimulator box was placed on top of the retinal sample and protected the retina from light computer screens were turned back on because it was necessary for controlling the measurements. The stimulator box was also used to protect the retina-RPE complex from the computer screen light even though the stimulation was given from below with the monochromator.

3.1.7. Membrane Carrier

Membrane carriers are used in the measurements to ease the transport of the retinal tissue from one location to another. After preparation, the retina is placed on a carrier and flattened there so that it can be handled easily without damaging the tissue by additional touching. Membrane carriers used in the measurements were made from black membranes (Filter type 0,45 µm, Black HABP, 25 mm, cat no HABP02500, Millipore) by cutting a circle shaped membrane to six pieces and making a ⌀2 mm hole in the middle of every piece with a leather punch tool.

The membrane carrier used in the previous setup had three holes with diameters of 1.2 mm. For these measurements the hole size was enlarged to have a diameter of 2 mm for two reasons. Firstly, this larger hole could be done with a punch tool instead of the previously used needles since a standard leather punch tool only makes holes with di-

ameters of 2 mm or more. Making the holes with needles was not simple because the membrane tends to tear. Tearing is prevented when a punch tool is used. Another motivation for a larger hole was to improve the positioning of the retinal sample on the MEA dish. The carrier with a larger hole is easier to place on the dish so that the hole fits the area of the electrodes completely. In these measurements the light stimulus came to the retina from above which means that the light enters the retina only in the area of the hole. If this area is not on the electrodes no responses can be measured.

3.1.8. Retina-RPE Culture

For culturing the retinas when brought in contact with the hESC-derived RPE cell layer a culturing setup was developed. A similar approach as described by Koizumi et al. [2007] was chosen. This comprised of a stand for the tissues that was permeable to the medium and a dish with medium where the stand could be placed. Departing from the publication no stirring of the medium could be implemented.

A stand was constructed by using 50 ml tubes and their screw caps together with a piece of mesh fabric (100% Polyester). The mesh was tightened around the tube and elevated so that medium could be brought to the mesh level from below. A photograph of a stand is presented in Figure 3.5. A standard 10 cm Petri dish was used for the medium and the stand and 50 ml of medium was found to be sufficient amount for the surface level to reach the mesh.

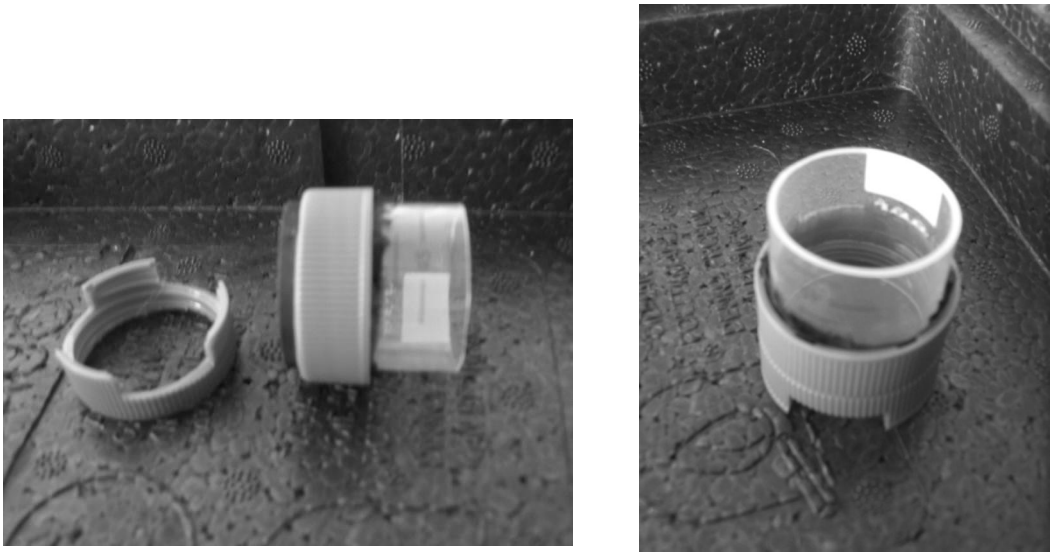

Figure 3.5. *A stand for the retina-RPE culture. (Left) The pieces of the stand with the mesh fabric tigtened to its position. (Right) The stand as used in the culture.*

The stand with the retina-RPE sample on the mesh was placed in the Petri dish with the medium. The whole complex was put in an incubator with the petri dish lid on top to prevent the evaporation of the medium.

3.2. Course of the ERG Measurements

The ERG measurement should be repeated in a similar manner every time for consistency. Here the practice used in these measurements is introduced in such detail that the measurements could be repeated with the given information. A single measurement set was done during one day and here the course of a measurement day is introduced chronologically to remain the natural course of events.

First the details of a measurement from an isolated mouse retina are described. At the end of the chapter is a separate section that describes the specialities of a measurement from retina together with the hESC-derived RPE cells. The course of that measurement is mostly identical to the isolated mouse retina measurement and only parts that are different are described.

3.2.1. Measurement of Isolated Mouse Retinas

Preparing for the measurement

Before the actual measurement could be started, or even the retinas prepared, the setup was prepared for each measurement phase as much as possible. The first thing to be prepared was to ensure that sufficient amount of both media were prepared and to place them in the water bath to be heated to 37 °C and while heated also the oxygenation for the Ames medium took place.

Figure 3.6. *The cool box with hot/cold packs and a container with Ames medium where enucleated eyes were transported from enucleation site to preparation room. The cool*

box was used to remain the 37 °C temperature of the hot/cold packs and the medium and to keep the eyes in darkness.

Also a few hot/cold packs were heated to 37 °C before putting them to a cool box with a small container of heated and oxygenated Ames medium. These packs were used to keep the medium temperature stable during the transportation of the eyes. The transportation setup is demonstrated in Figure 3.6. It was found helpful to put the Ames medium in few separate containers to ease the access to a fresh medium when needed.

The preparation room and the measurement room were darkened with blackout curtains whose edges were taped to achieve a complete black out. Also additional light sources, like control lights of different equipment, were covered.

70 % ethanol was used to sterilize the materials before use. The preparation tools, the PDMS rings and the Petri-dishes were sterilized and thoroughly rinsed to remove any excess ethanol. The preparation site was prepared by placing a dissecting microscope and a fiber optic illuminator to desired positions and covering the optic fiber ends with red foil to get red light for the dissection. The preparation tools and other material were placed next to the microscope so that they were ready when needed. The MEA-dishes were taken from Milli-Q water where they were stored and allowed to air dry by the microscope. In Figure 3.7. a photograph of a preparation site is presented.

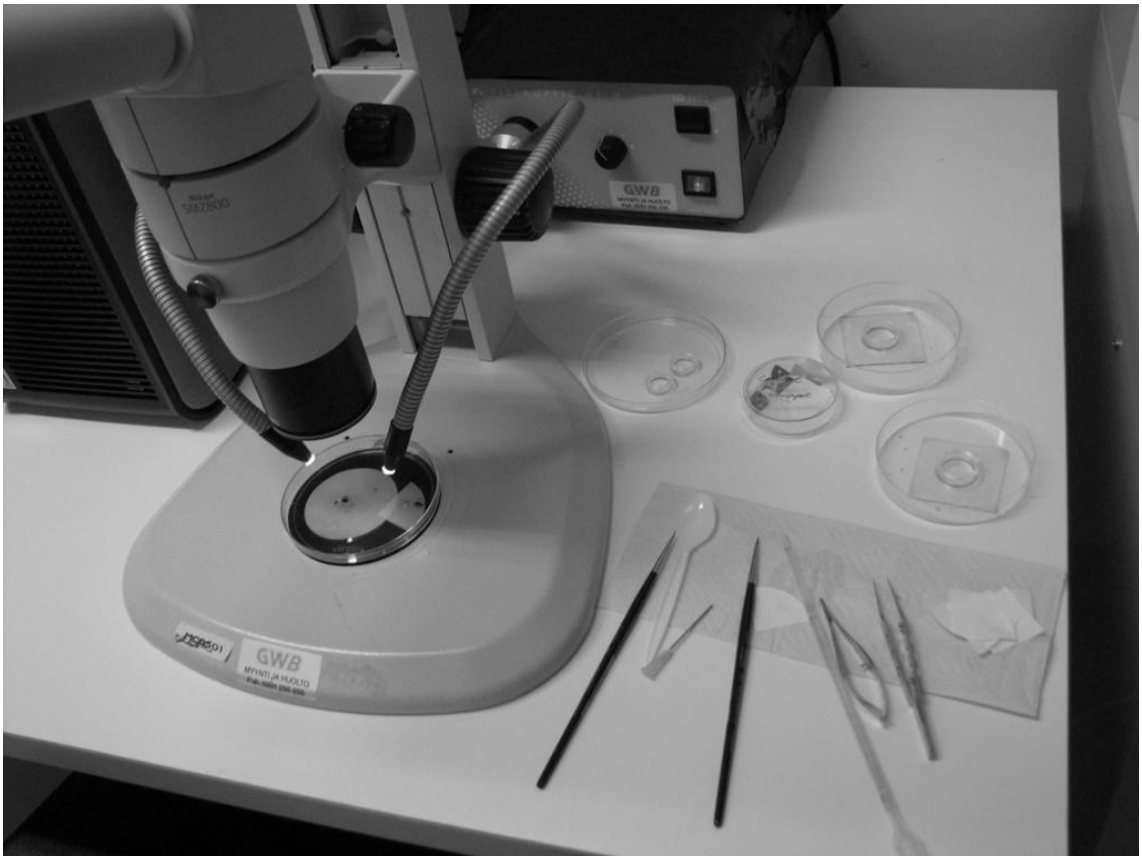

Figure 3.7. *Preparation site. Preparation tools are placed on the right side of the dissecting microscope. MEAs, carriers and PDMS rings are on Petri dishes behind the tools.*

The measurement site also needed some preparations. The light stimulator was connected and tested with the stimulus file. The connections of the MEA system were checked to be as desired and naturally changed if they were not. If perfusion was used it was set up ready and tested with water that the liquid was running as it should.

Mouse Decapitation and Enucleation of the Eyes

Mice were obtained as a kind gift from Dr. Hannele Uusitalo-Järvinen at University of Tampere. All of the mice used in the measurements were less than six months old. A mouse was picked up from the animal laboratory anesthetized (with Domitor + Ketalar). It was taken to a fume hood where it was decapitated on an opened waste bag and some paper hand towels by cutting the neck with firm scissors. The site is demonstrated in Figure 3.8.

Figure 3.8. *The enucleation site. The actual enucleation was performed on the paper towels in the plastic bag. Tools were placed on the side for easy access.*

Teethed forceps were used to hold the mouse head and skin still while curved scissors on the other hand were used first to cut the whisker and then the upper and lower eyelids or the mouse to improve the access to the eye. The connective tissue around the eye was cut break to get the eye attached only at the back with the optic nerve. Finally the eye could be lifted from the orbit with the forceps so that cutting the optic nerve became possible. The enucleated eye was placed in an oxygenated Ames medium. Then the enucleation was repeated to the other eye. The enucleation of the two eyes took

about one minute. The eyes were transported in complete darkness to the preparation site. The transportation took approximately five minutes.

Preparation of the retina

The preparation was performed under dim red light to minimize photopigment bleaching of retina. The eye was picked up with a spoon and placed in a Petri-dish (10 cm) on a filter paper (Schleicher & Schuell, 5893 Blue ribbon ashless filter paper circles, 55 mm) that was soaked with Ames medium. The eye was then perforated with a hypodermic needle (16 gauge) and cut open with very fine Vannas-style spring scissors (Fine Science Tools GmbH, 15000-08, cutting edge 2.5 mm) along the ora serrata. After removing the lens, the retina was gently detached from the underlying pigment epithelium layer with very fine brushes (sizes 5/0 and 2/0, materials 100% nylon and weasel hair). The retina was placed on a membrane carrier photoreceptor side down and brushes were used to unfold the retina gently to cover the hole punched in the carrier as well as possible.

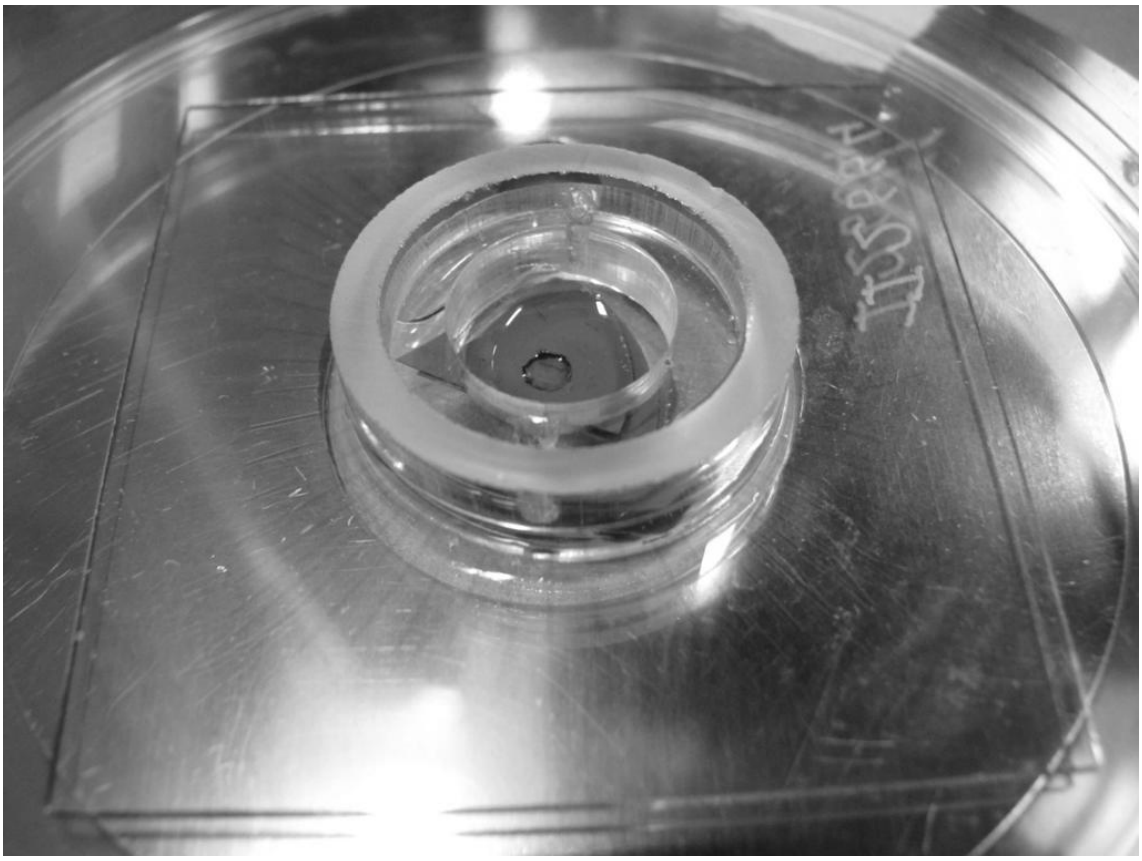

Figure 3.9. *Retinal explant placed on a MEA with a carrier and weighed down with a PDMS ring.*

The retina with the carrier was placed on a MEA ganglion cell side down so that the hole of the carrier was situated on top of the MEA electrodes and thus the area of the electrodes was covered with the retina alone. A ring made of PDMS was placed on top of the carrier to keep the retina firmly at the right location on the bottom of the MEA dish. Finally, the MEA-dish was either filled with oxygenated Ames medium or the

bottom of the MEA dish was filled with RPE DM- medium. A readily prepared and placed retinal explant on a MEA dish is presented in Figure 3.9. In Figure 3.10. the preparing of the retinal sample for the measurement is demonstrated as a diagram. In Figure 3.11. a photograph of a MEA dish with a retinal explant placed on the MEA preamplifier is presented.

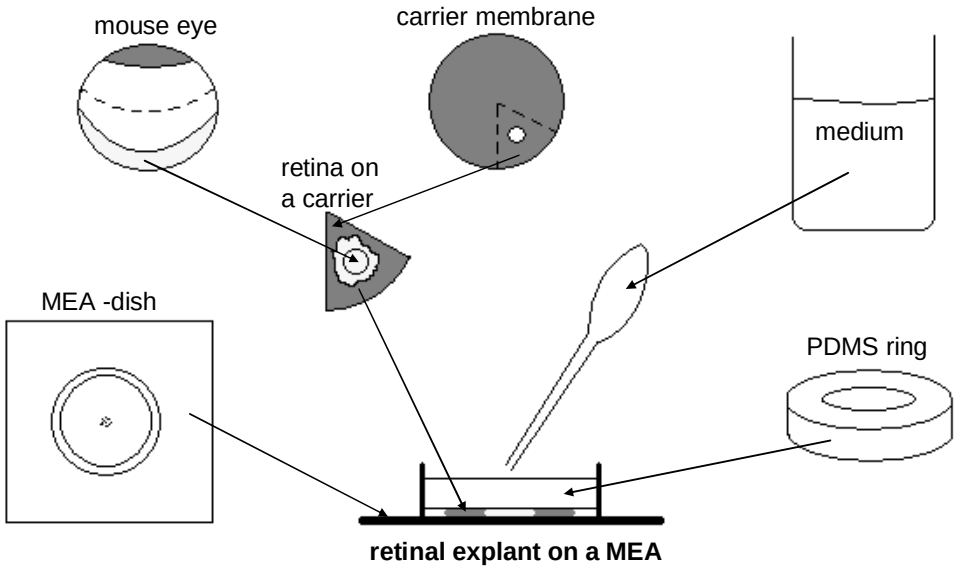

Figure 3.10. *Preparation of the retina for ERG measurements. First retina is removed from the mouse eye and unfolded on a carrier that has a punched hole in it. Retina with the carrier is placed at the bottom of a MEA-dish and a PDMS ring is placed on top of the carrier to keep the retina firmly attached to the electrodes. Finally, medium is brought to the MEA-dish.*

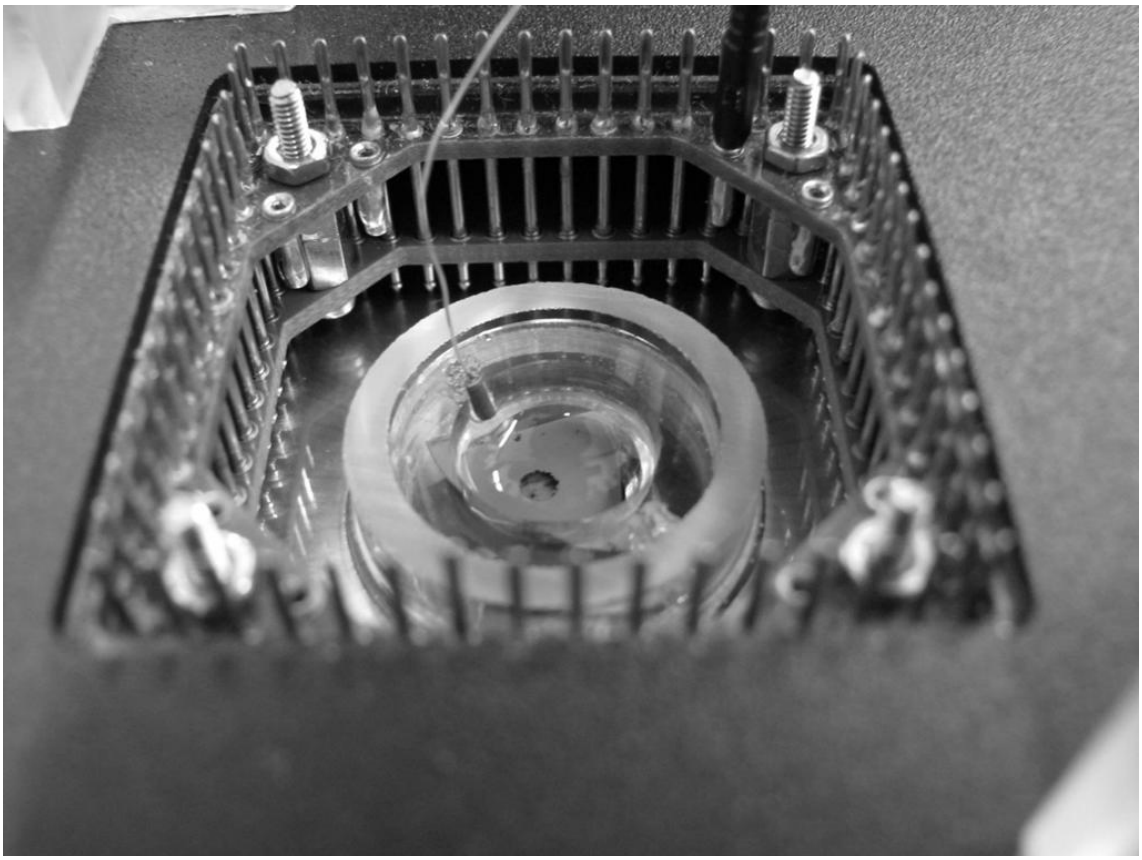

Figure 3.11. *MEA dish with a retinal explant placed on the preamplifier as it is during measurements. The grounding electrode comes in contact with the medium from above.*

ERG Measurements

The MEA-dish with the retina was transferred in darkness to the darkened measurement room few meters away from the preparation site. The positioning of the MEA dish with the retina on to the amplifier was done by using red light for sufficient seeing. The light stimulator was placed on top of the amplifier and the MEA dish, and before starting the measurements a few minutes time is given to the retina to settle after the moving. If perfusion is applied when using Ames medium, the settling time needs to be some minutes longer after the perfusion start, due to the medium flow.

The measurement equipment used was described earlier in section 3.1.1. The HexaMEA with the explant was placed on the amplifier. Depending on the medium that is used with the specific retina, perfusion may or may not be used. The whole MEA system is inside a Faraday cage to minimize the noise from the surrounding electricity. In Figure 3.12. is a photograph of the preamplifier and the light stimulator box next to it.

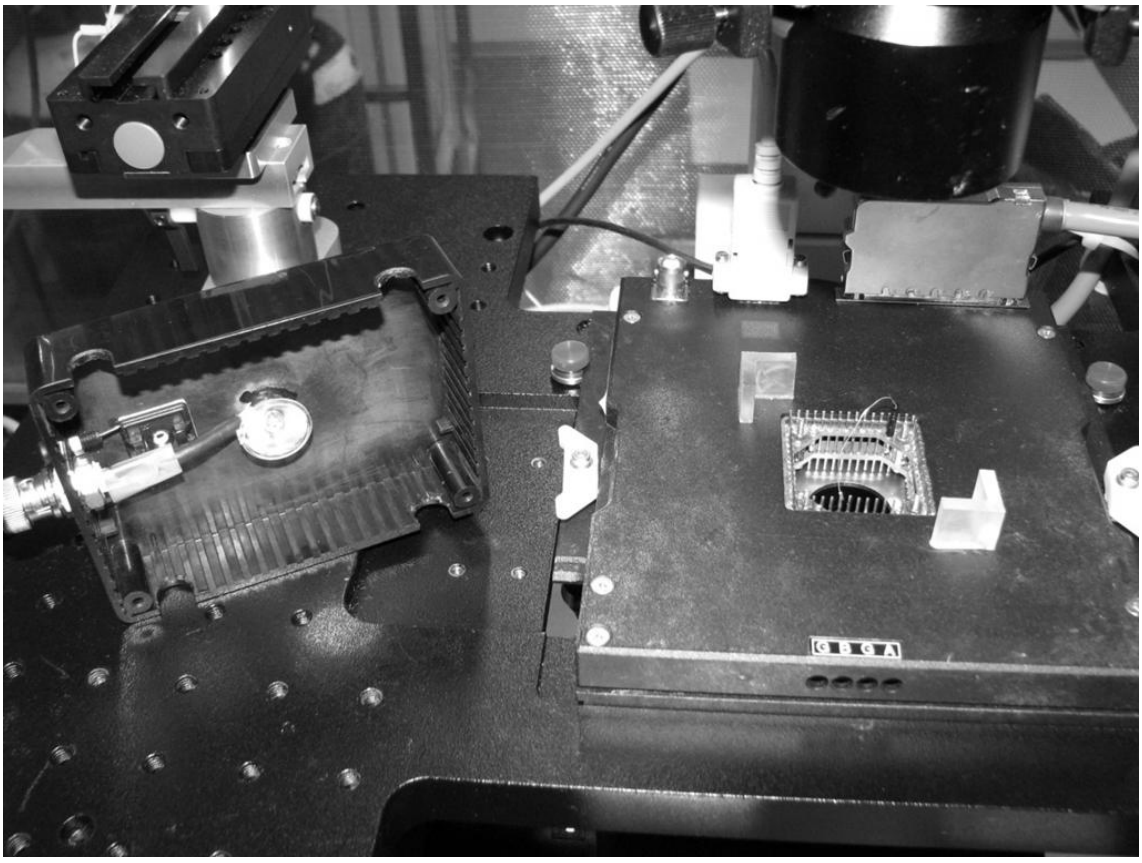

Figure 3.12. *MEA preamplifier and light stimulator box. When performing measurements the light stimulator box is placed on the preamplifier so that it covers the MEA area completely.*

During the measurements done with Ames medium perfusion was applied to the measured tissue. Oxygenated 37 °C medium was brought to the MEA-dish through tubing with a two-channel peristaltic pump. The flow rate was 1 ml/min. The perfusion system was previously described in section 3.1.4.

With Ames HEPES medium perfusion helped to remain the tissue viability for longer time periods when fresh oxygenated medium is constantly brought to the tissue. Perfusion was brought to MEA through the perfusion cannula of the MEA system that heats the medium to 37 °C right before entering the dish. Also the amplifier is heated to 37 °C. Both heating elements were being controlled by the temperature controller.

Two different intensities were used for the stimulating light, maximum intensity and minimum intensity that could be obtained with the stimulator. These correspond to intensities of 25 000 lx and 4 000 lx. The different stimulus types that were used for stimulation were previously described in a section titled 'MEA Software and Stimulation Parameters' under section 3.1.1. A typical measurement took about 1 to 2 hours of time.

After the measurements all washable material was rinsed under running water and/or MilliQ –water and left to air-dry. The retinal samples from the MEA dishes were removed with forceps and the dishes were rinsed a few times and then stored in MilliQ

–water. 70 % ethanol was used for cleaning up surfaces and equipment if needed. Everything was put ready in a suitable place for the next measurement.

3.2.2. Measurement of Retina and RPE Cells

The measurement protocol for measuring retinas and RPE cells together is a modification of the previously described measurement for isolated retinas alone. The most significant difference between these two measurement types is the need for a short term culture for giving the retina and RPE cell layer a possibility to form contacts. Here the alterations in the course of the measurement compared to the retina alone measurement are briefly introduced.

Preparation

The preparation of the retina was done in a similar manner as previously described. The hESC-derived RPE cell layer on a collagen insert (BD Biocoat collagen IV inserts (BD Biosciences)) was placed on the membrane carrier cell side facing up so that it covered the hole on the carrier entirely. The retina was then placed and unfolded on the RPE cells photoreceptor side down so the tissues are in natural positions in relation to each other. To help the two tissues to have steady positioning, another carrier was placed on the retina so that the holes of the two carriers were on top of each others. So the retina and the RPE cell layer were between the two carriers in a sandwich-like structure.

A similar sandwich was done also for retinas alone that were used as a reference for the measurement. The only difference was that the RPE cell layer on collagen was left out from the sandwich. The preparations were done in such a manner that the reference retina was from the other eye of the same mouse as in the retina-RPE sample. This was considered to improve the comparability of the two samples.

Tissue Culture

After the preparation the sandwich was placed on a net of a stand described in section 3.1.8 in a Petri dish and warm RPE DM- medium was added to the dish so that its surface level reached the net of the stand. The dish with the stand was placed in an incubator for some (up to four) hours depending on the course of the preparation and measurement of other samples. However, the retina-RPE sandwiches and their reference samples were kept in the incubator for the same time so that their responses could be comparable.

After the sandwich had been placed on a MEA for a longer period of measurements, it was no longer replaced on the culture stand after the current measurement set was over, but left on the bottom of the MEA dish with sufficient amount of fresh medium and placed in an incubator to be measured again after more time had passed. This was done because the moving of the sandwich especially from MEA to the stand was still difficult to do so that the cell layers did not move, too.

Measurement

The measurement of the retinas together with the RPE cells was done so that light and dark adaptation could possibly be seen in the results. Similar light stimuli were used as for the retinas measured alone, but the source of the light was the monochromator and the light stimulus came from below. The sample was placed on the MEA ganglion cell side down so that the light stimulus came from a natural direction.

The samples were measured (ten repeats of 15 ms stimuli) the first time right after the preparation before they were put in an incubator. After this initial measurement the sandwich was moved back on the stand and then left to the short term culture.

After culturing, a longer measurement set took place. First, responses right after culturing were measured after which the sample was exposed to bright light for two minutes. Then, light responses were measured every five minutes for a period of about forty-five minutes, to see if dark adaptation occurred. After this measurement, the medium was replaced and the sample put back in an incubator, this time in a MEA dish. The measurement was repeated without the exposure to light when other samples were moved on and off the MEA preamplifier. This made it possible to observe if changes in the responses occurred at a later time than the first forty-five minutes.

4. RESULTS AND DISCUSSION

The measurements for testing the setup for recording light responses from isolated retinas were done during spring and summer 2009. The measurements that included also hESC-derived RPE cells were performed during spring 2010. The development and testing of the entire setup included altogether approximately twenty measurement days.

The final setup for the isolated retina measurements was tested with four separate measurements. Results from three of them are introduced and discussed in this chapter and they cover the different variations of measurements that were performed to isolated retinas. The measurement setup for retinas and RPE cells still needs more work, but light responses have already been recorded. Results of one measurement where light responses from a retina-RPE compex were obtained, are presented. Also the measurement setup is evaluated based on experiences from the measurements and the measurement results.

4.1. Overview of the Measured Data

The data recorded during the measurements is presented as an overview with representative captures from the results. The measurement results include responses from 60 channels for each stimulus and presenting only an overview of them suits well to the purpose of evaluating the functionality of the measurement system. First, responses recorded from isolated retinas alone are presented and then results from one measurement with retina-RPE complexes.

4.1.1. Responses from ERG Measurements of Isolated Mouse Retinas

Different aspects of the isolated retina recordings were followed in order to find out the effect of variations in the measurements. Firstly, and most importantly, obtaining the ERG response itself was the main goal. Once that was obtained, variations in medium, stimulus intensity, stimulus duration and the effect of a prolonged exposure to light were applied and their effects in the measured response recorded. No extensive testing of these factors was conducted but rather trials to have some understanding of the response in different situations. Here the an overview of the results from these measurements are presented.

ERG Response

The main goal for the work was to be able to measure a basic ERG with the setup developed. This goal was reached. Here measurement results recorded on July 15th 2009

are used as a representative to show a basic ERG curve recorded with the setup described in this thesis.

In Figure 4.1. a recorded response from the measurement is presented. 30 responses from several channels of the same measurement are averaged and brought to the same image so that comparing the responses from different channels is simpler. The averaging has been done in order to remove the 50 Hz noise from the curves. The stimulus duration has been set to 15 ms and maximum stimulus intensity of approximately 25 000 lux is applied. In many of the channels a similar waveform can be seen as in Figure 2.11. where typical mouse rod/cone ERG responses were presented. Differences in responses between channels can be caused by many factors. One obvious reason for some channels to have a positive response and some to have a negative one would be folding of the retina in some locations that could easily happen during preparation and handling of the retina when placing it on the MEA. When orientation of the retina changes (ganglion side up vs. down) the direction of the b-wave changes likewise from positive to negative. However, a typical ERG curve is obtained with this measurement, which shows that the measurement setup is functional.

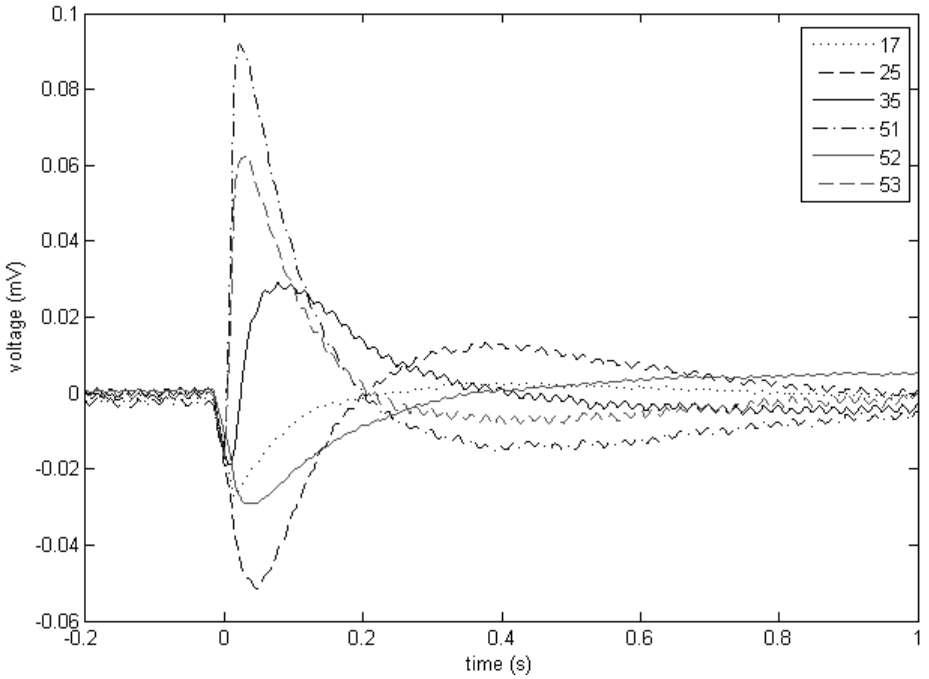

Figure 4.1. *Averaged ERG responses from 30 responses measured from a retinal explant (June 15th 2009). Different curves represent different channels, the channel numbers are being marked in the top right corner.*

In Figures 4.2. and 4.3. responses of single channels (51 and 35) of the same measurement are presented both averaged from 30 stimuli and unprocessed from a single stimulus. In the responses of single sweeps the time of applying the stimulus (at approximately 59.3 seconds) can be seen as a dramatic drop in the noise level. The actual positive wave (b-wave) response can be seen after the 15 ms stimulus has ended. This reduction of noise level and recording a stable voltage level during stimulation is stimulus artefact and it was constantly seen in every channel and all of the measurements that

were done with the LED stimulator. Thus reliable information of ERG response while stimulation takes place is impossible to gain as long as this kind of artefact is present.

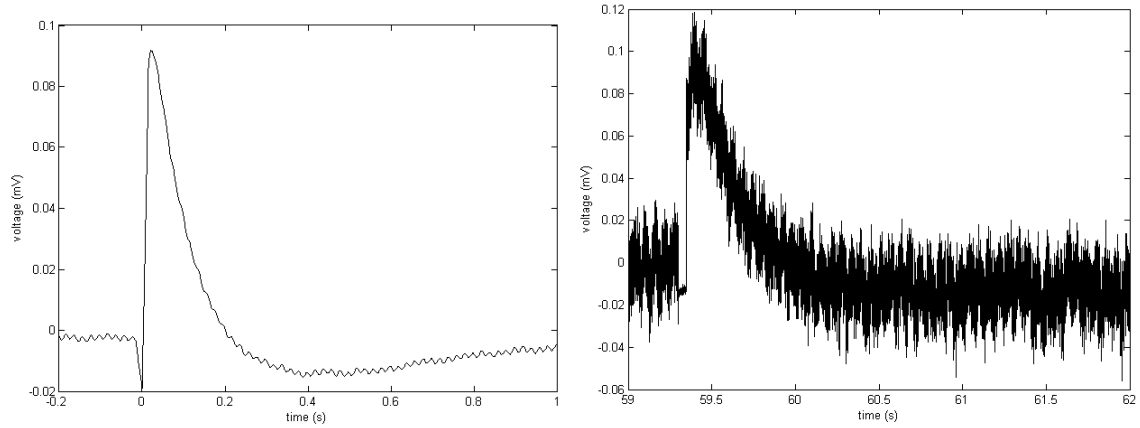

Figure 4.2. *ERG response from channel 51. Averaged curve from 30 responses of the same channel (left) and a response to a single stimulus unprocessed (right). Differences in the scales should be noticed.*

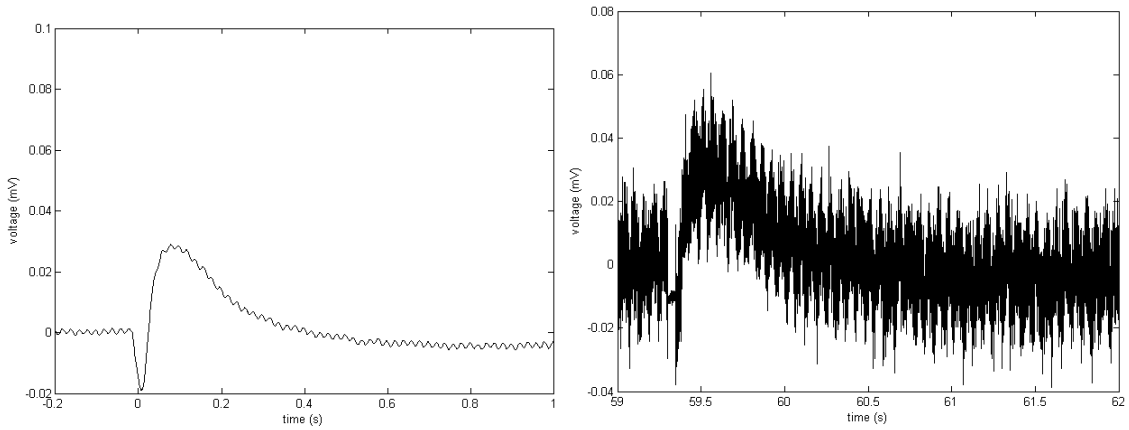

Figure 4.3. *ERG response from channel 35. Averaged curve from 30 responses of the same channel (left) and a response to a single stimulus unprocessed (right). Differences in the scales should be noticed.*

Some remarks from these responses of the two channels could be done. The amplitudes of the responses are quite different from each other (the scales in the averaged images are the same). This may be due to variations in the tissue-electrode interphase, folding of the tissue, tissue damage in some location or just natural variation in different parts of the retina. When comparing to the amplitudes of Figure 2.11. the amplitudes are rather small. This could be an indication of measuring just cone responses which could be caused by bleaching of the rods during preparation in spite of the effort to spare them. The smaller amplitudes can also be caused by some of the reasons listed above to explain the possibilities why the variation in amplitudes is so great. The duration of the response seems to be approximately 200 ms, which is a quite long response in comparison with the duration of the responses in Figure 2.11. The long lasting responses rather

indicate having also rods contributing to the response than measuring just cones. Increasing the number of measurements, so that more data could be used in processing and comparing, would help to evaluate better the origin of these responses.

The stimulus artefact is present in both channels but the waveform right after the artefact ends is somewhat different. In channel 51 the response has already reached a rather high level of the b-wave and the presence of a negative peak (a-wave) before that is impossible to evaluate. In channel 35, however, the b-wave is just about to begin at the end of the stimulus artefact, and the voltage level right at the end of the artefact seems to be lower than before the stimulus which would implicate the presence of an a-wave even though the wave cannot be seen.

Effect of the Medium on the Response

When ERG could be done with the setup, a desire to alter some measurement parameters and see their effect on the recorded responses arose. All of the measurements where some measurement parameters were changed were done together with measurements of the normal case. This way the results can be compared and the effect of the single change evaluated. The parameters that were changed were medium, stimulus intensity, stimulus duration and prolonged exposure to light.

In order to prepare for the measurements done together with RPE cells the effect of the RPE DM- medium in comparison with the Ames HEPES medium was tested. This measurement took place on July 6th 2009 and the results are presented here. The medium was changed between stimulus sets and a similar set of stimuli was then applied to the same retina in different medium. The medium was then changed again to be sure that the responses remained similar to the first recording. A comparison of responses measured with these media is presented in Figures 4.4. and 4.5. Based on the results shown here the medium seems not to have any effect on the measured responses. With both media a similar negative peak – positive wave response is obtained. The usage of just RPE DM- as the medium has not caused any problems in obtaining the ERG responses in later recordings, the responses in Figure 4.1. were also recorded in RPE DM- medium. The effect of the stimulus artefact discussed earlier can be seen in these recordings, too. The negative peak is probably entirely caused by the artefact. The stimulation time is marked in the images above the time scale.

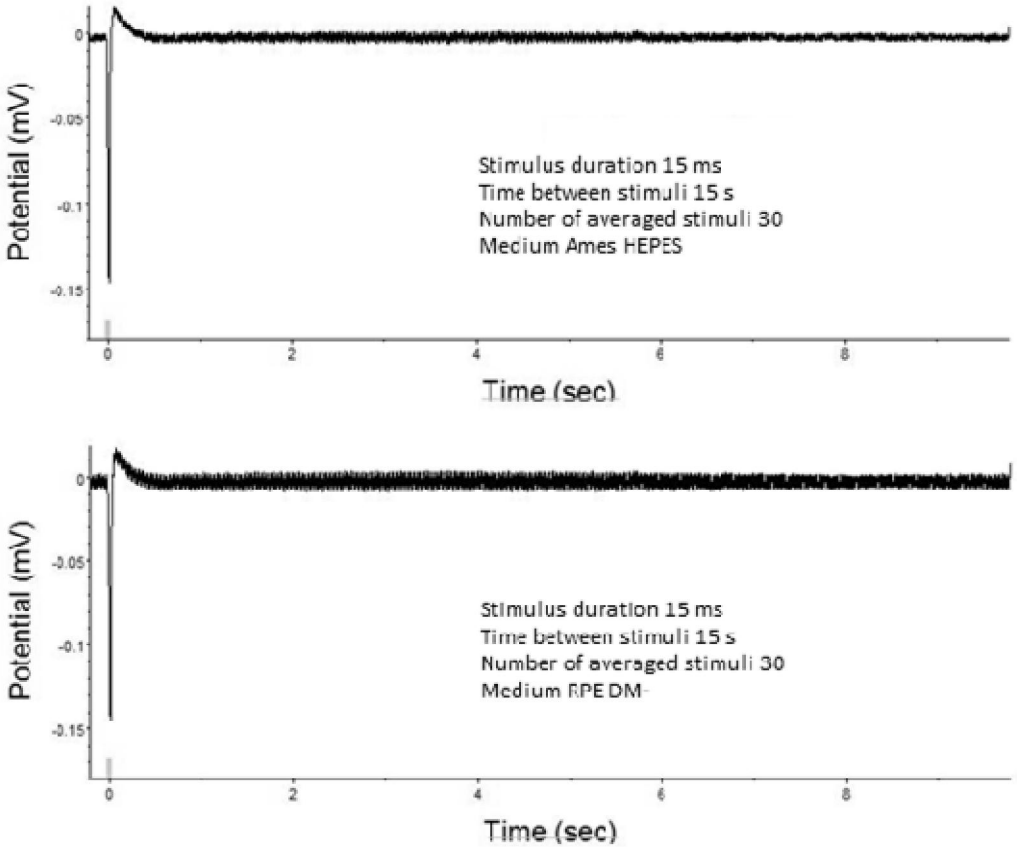

Figure 4.4. *Comparison between two media. Above an averaged response of 30 stimuli on a single channel when Ames HEPES was used, below a similar curve from the same channel obtained with RPE DM- as medium.*

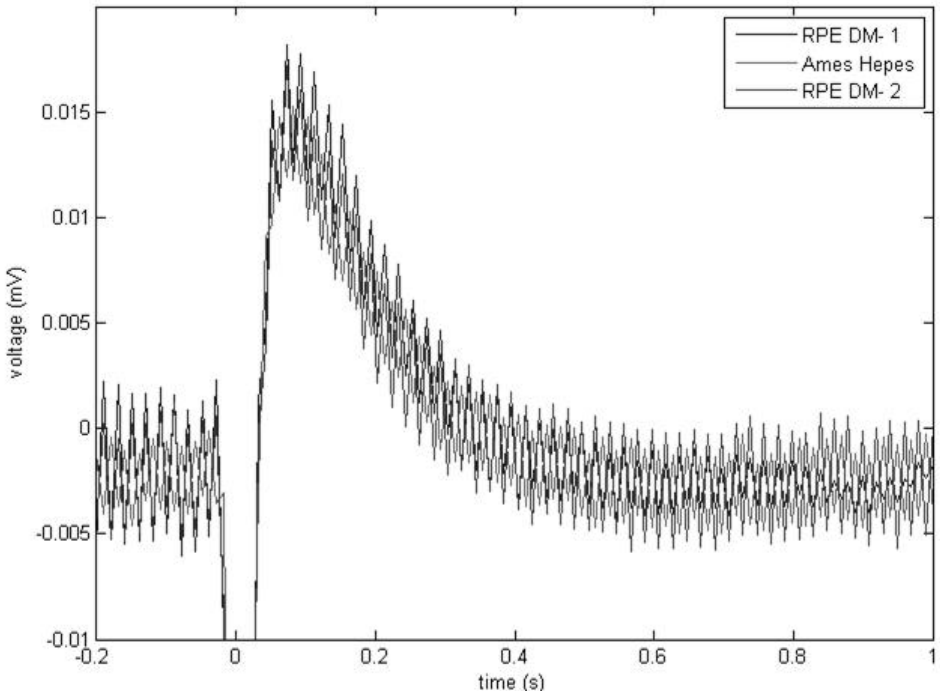

Figure 4.5. *Three averaged curves compared and zoomed. The responses of a single channel from measurements done with different media are placed in same image to help*

comparison. Altering the medium seems to have no effect on the response. The negative peak is cut off from the image because it is caused by stimulus artefact.

Effect of the Light Intensity on the Response

The effect of changes in light intensity was measured on June 10th 2009 by using maximum and minimum intensities of the stimulator with the same retina. The responses of maximum intensity vs. minimum intensity are compared in Figure 4.6. In this measurement the only difference caused by the variation in the light intensity seems to be a minor positive peak during the light stimulus when using the maximum intensity. With minimum intensity this peak is absent. The amplitudes in the negative peaks are similar as is the waveform otherwise, too. However, a positive wave after the negative peak which is typical in ERG is absent in both cases. This might suggest that the retina used in the measurement was not responding well to the stimulation. The negative peak seen in the image can be entirely generated by the stimulus artefact. In that case no conclusions on the effect of stimulus intensity change can be made based on these measurements, but this should be tested again.

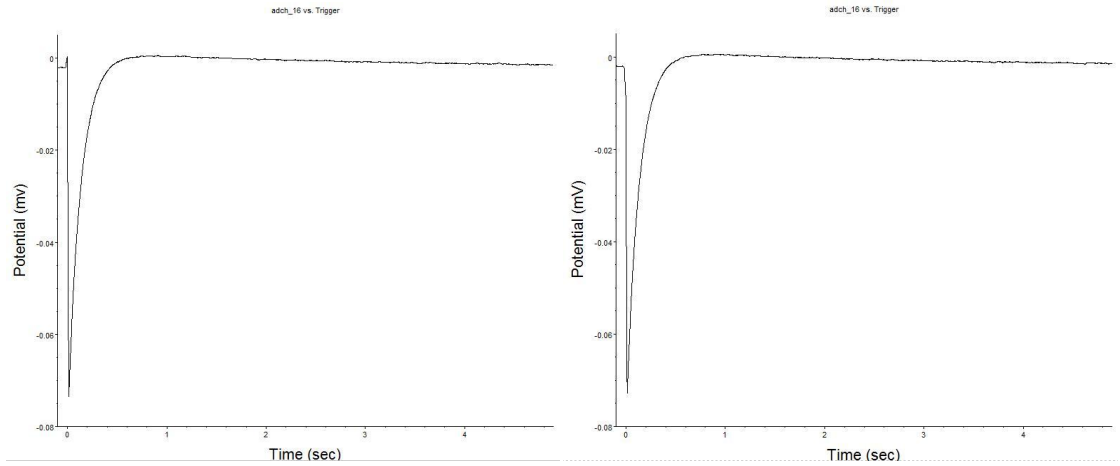

Figure 4.6. *Responses obtained when using maximum intensity stimulus (left) compared to minimum stimulus intensity (right). The entire response may be caused by a stimulus artefact.*

Effect of the Stimulus Duration on the Response

The effect of the duration of the light stimulus was also tested during the measurement done on June 10th 2009. The variation in stimulus duration was from one second to 15 ms and the stimulus intensity in both measurements was the minimum intensity of 4500 lux. Responses from these measurements are presented in Figure 4.7. The stimulus timing and duration is marked under or above the time scale. The longer stimulus duration makes the negative peak to widen according to the duration of the stimulus. The response returns to the ground level rather rapidly after the stimulus offset. The longer stimulus causes the return phase to take a positive wave before returning to the zero-level while this positive wave is absent when using the shorter stimulus. These responses have no clear positive wave after the negative peak that would be typical to an ERG, as was the case when comparing the effect of stimulus intensity. Also here the

negative peaks could be just artefact response. The measurements were done with the same retinas as the stimulus intensity measurement. These facts allow no conclusions to be done based on these measurement results since their credibility is highly questionable. A new measurement would be needed.

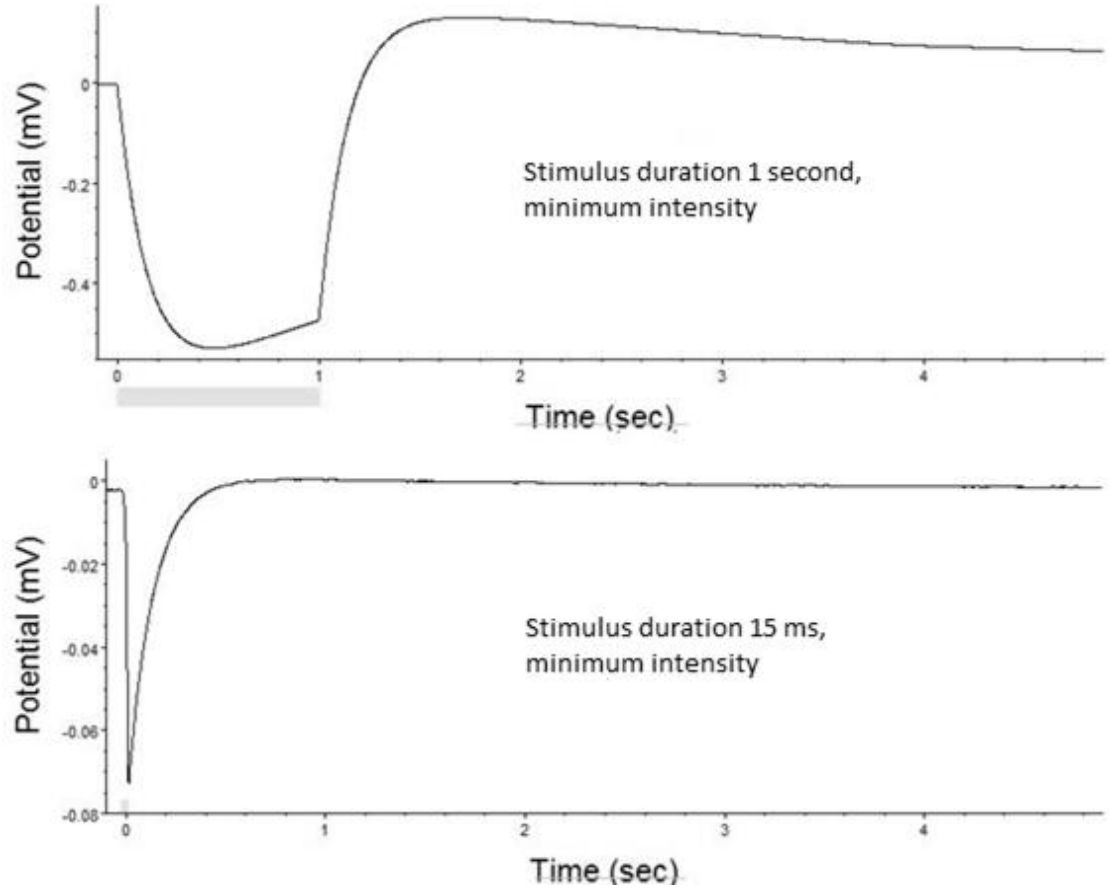

Figure 4.7. *Comparison of responses obtained with different durations of the stimulus. Responses from measurements with stimulus duration of one second (above) and 15 ms (below). The responses may be caused by stimulus artefact in which case no retinal response is seen.*

Effect of a Prolonged Exposure to Light on the Response

The same retinas that were used for measuring the effects of stimulus intensity and duration were also used to measure whether a prolonged exposure to light affects the response on June 10th 2009. With this kind of measurement, if there is difference in the responses, the contribution of rods could be seen in the responses before the light exposure and after the exposure only cone responses should be recorded due to bleaching of rod pigment. The responses measured before and after a prolonged exposure of the tissue to light are presented in Figure 4.8. No other variations between the measurements exist. The duration of the light exposure used in the measurement was two minutes in phases of 20 s with 5 s pauses between the phases. The pausing was used since the capacity of the light stimulator electronics to handle a light stimulus of several seconds was not known. This pausing should not have caused any effects on the reaction of the retinal cells to the exposure. In the responses recorded no differences between the

measurements before and after light exposure was observed. Typically this would imply that only cone responses were recorded both before and after exposure, but as with the other measurements done with these same retinas, also here the typical positive waveform is not present and the response could be just artefact. So no conclusions on the responding cell types based on this measurement alone should be done. Repetition of the measurement would be needed.

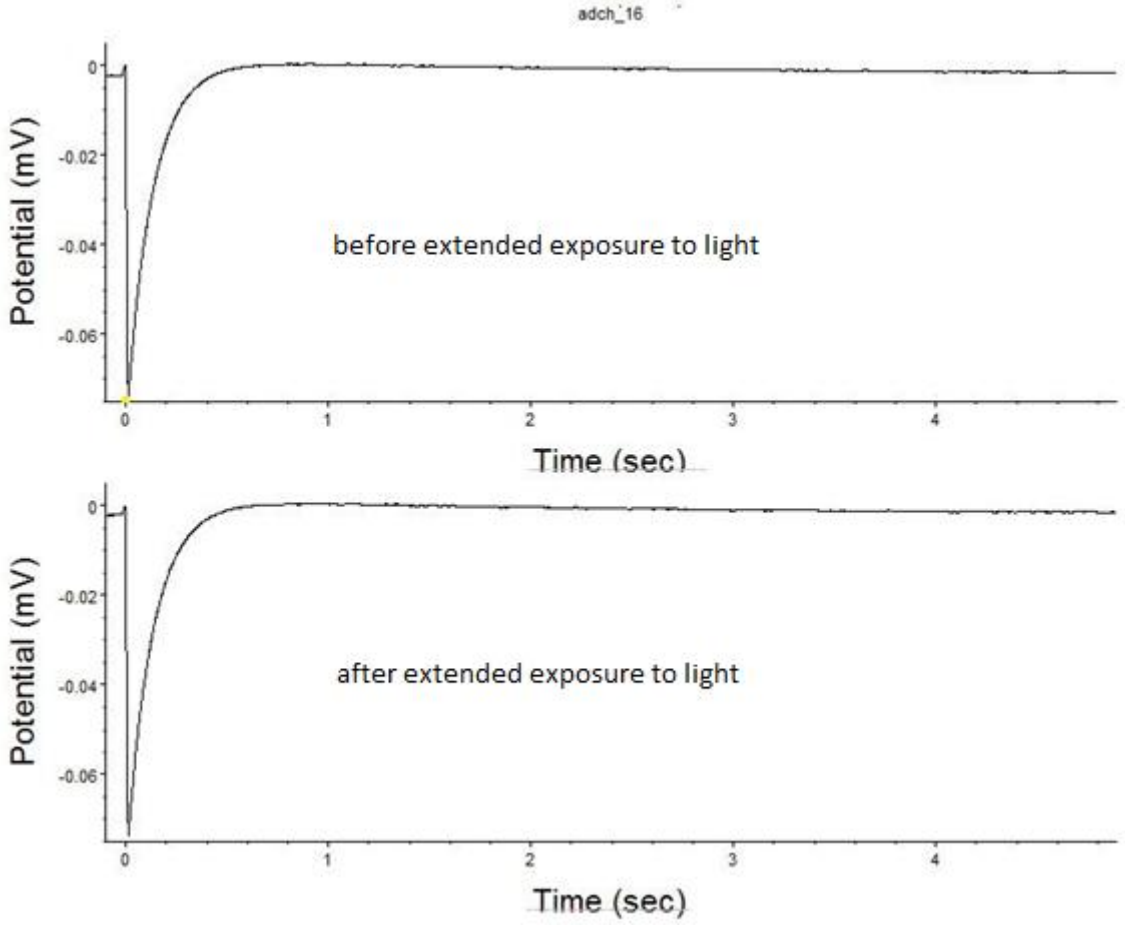

Figure 4.8. *The effect of a prolonged light exposure to the ERG response. Comparison of responses before (above) and after (below) exposure.*

4.1.2. Responses from ERG Measurements of Retinas and RPE Cells

Here an overview of the responses recorded on April 15th 2010 is presented. The measurement was done to two pairs of retina alone – retina-RPE tissues. One of these pairs is used here to demonstrate the results of this measurement. Unlike in the previously described measurements, now the light stimulus was generated with the monochromator and brought to the explant from below. The results show that light responses can be measured from both isolated retinas and retinas with the hESC-derived RPE cell layer. Even though RPE cell functionality cannot be proven based on these measurements, the results are promising.

The responses presented here are measured right after the preparation before the tissues were placed in an incubator for the short term culture and right after the culture of

4 hours. In Figures 4.9. and 4.10. averaged responses of isolated retina measurements are presented. The responses measured before and after culturing are placed in the same images to facilitate comparison. Somewhat surprisingly, the responses recorded after the short term culture are larger than the responses measured right after preparation, which might indicate that some light adaptation occurred during the preparation. The negative waveform can be explained by the orientation of the retina. In Figure 4.9. responses from all channels are shown and the best response of channel 67 is seen separately in Figure 4.10.

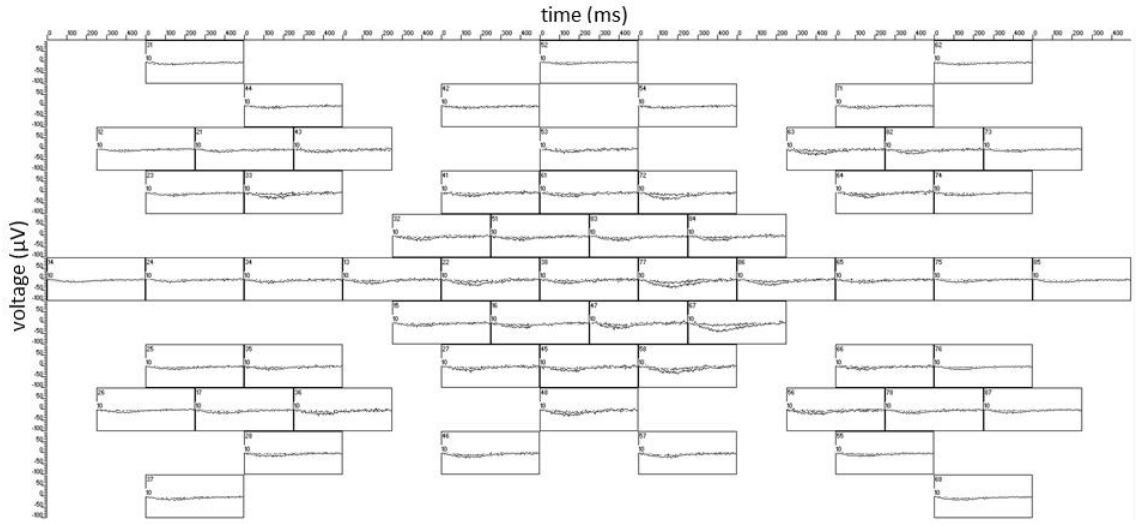

Figure 4.9. *Averaged responses of isolated retina to 10 stimuli (all channels). Responses were recorded right after the preparation (nearly flat responses) and after 4 hours of culture (negative curves).*

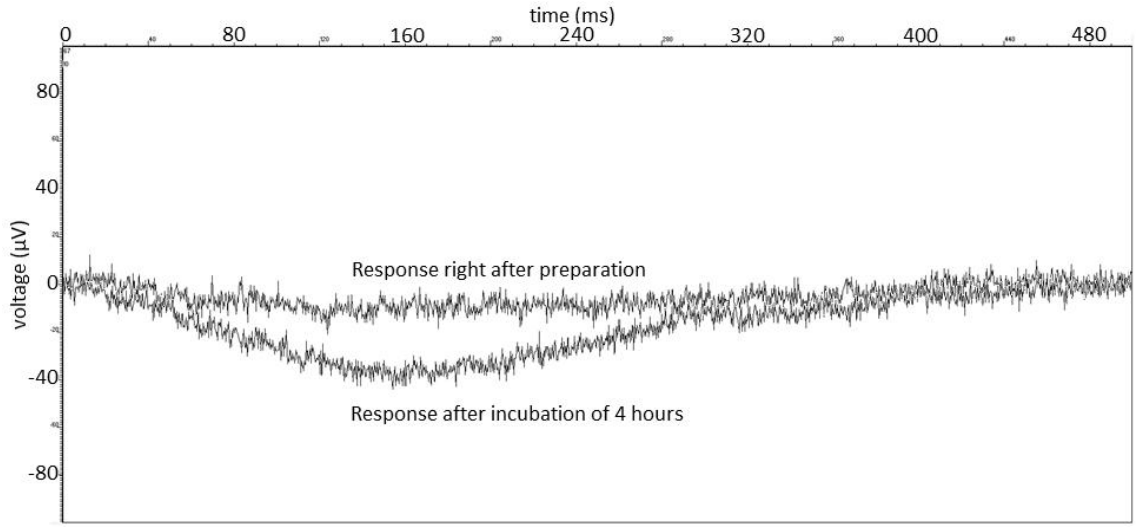

Figure 4.10. *A single channel averaged response of isolated retina to 10 stimuli (channel 67). The response was recorded right after the preparation and after 4 hours of culture (marked in the image).*

Figures 4.11. and 4.12. present similar responses recorded from retina-RPE complex. First responses from all channels are shown and then the best channel response,

which in this case is channel 15. Again the better responses were obtained after the culture. In these figures the responses recorded from retina-RPE complexes appear to have somewhat greater amplitudes than the responses of isolated retinas alone. This could indicate that the retina with the RPE has had some benefit in adapting to darkness compared to the retina which was cultured alone. However, the differences are so slight that no such conclusion can be made based on this single measurement.

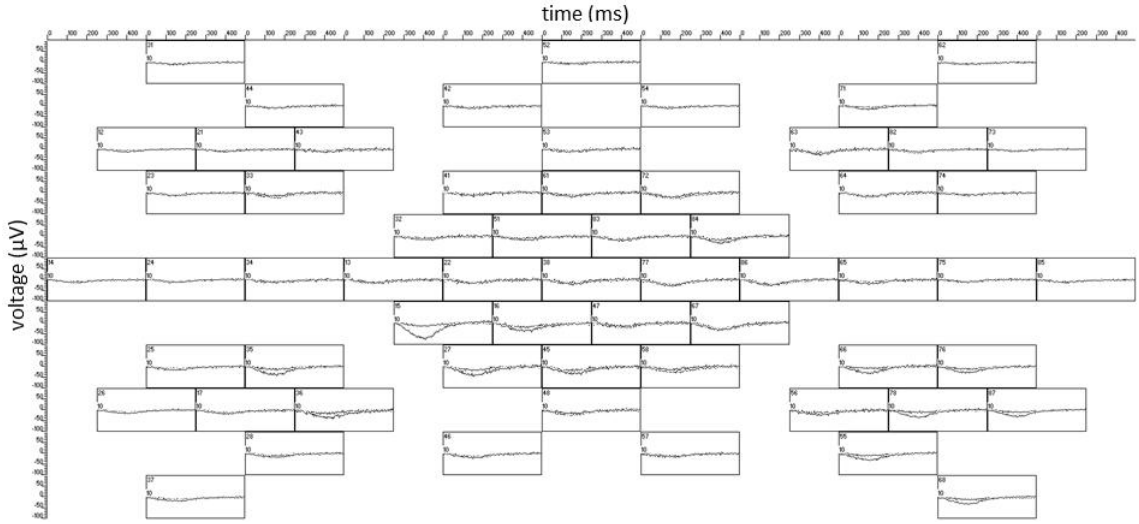

Figure 4.11. *Averaged responses of retina-RPE complex to 10 stimuli (all channels). Responses were recorded right after the preparation (nearly flat responses) and after 4 hours of culture (negative curves).*

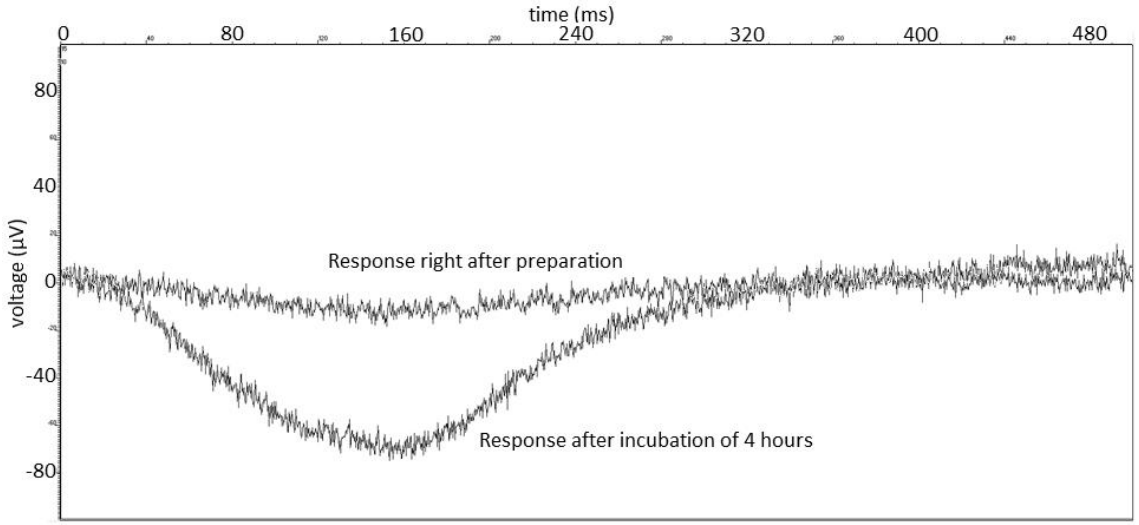

Figure 4.12. *A single channel averaged response of retina-RPE complex to 10 stimuli (channel 15). The response was recorded right after the preparation and after 4 hours of culture (marked in the image).*

The amplitudes that were recorded in these measurements are rather small. This is most likely due to the usage of the monochromator as the light source. The maximum intensity of the monochromator is not known, but it is clearly less than the intensities of the LED stimulator. This was easily observed with the human eye. Also the waveform

recorded is similar with the responses to low intensities in Figure 2.11. but has opposite polarity due to orientation of the retina. This would explain the lack of a-wave and the quite slow b-wave in the responses.

4.2. Evaluation of the Measurement Setup

The goal of the measurement setup development was to discover such a method that would be simple enough to be repeatable and yet delicate enough to ensure the viability of the retinal tissue. Based on the recorded responses the measurement setup developed for the ERG measurements was found functional. The functionality of the measurement setup can be evaluated by comparing the responses recorded with other ERG-recordings done previously. However, due to technical problems with the light stimulator the measurements could not be repeated as many times as wished, but sufficient number of measurements could be performed in order to discover the method to be functional.

4.2.1. Components of the Setup

Here the different components of the measurement setup are evaluated. Their functionality and suitability to the measurement is evaluated briefly and focus is given to properties that are not found either suitable or practical.

MEA System

Using the full MEA system in the measurements was found a good choice. Many basic features that were needed were comprised in the system. Also the recording and processing the recorded data were found easy to use and practical. The only drawback the system has is that at its current state the MEA-dishes used are not suitable to be used while culturing the retina and the RPE. This causes moving of the tissues to and from the MEA and makes it more difficult to maintain their possible contacts.

Light Stimulator

The LED stimulator had problems first with the 50 Hz noise and unreliability. When both of these problems were solved, another problem with the stimulus artefact seen in Figures 4.2. and 4.3. occurred. While the stimulus artefact still exists, the LED stimulator cannot be recommended to be used in the measurements. However, other properties of the stimulator were found good and it was easy to use. If the problem with the artefact the stimulator produces could be fixed, it would be a good choice for stimulation.

The monochromator was reliable and produced no artefacts. However, the stimulus intensity that the monochromator could produce was not as high as desired, when evaluated visually by looking at the light impulse. The ability to vary the light intensities on a wide scale would be very important for more detailed measurements. Another slight drawback is the use of the resting wavelength or the physical shutter when not stimulating. Using the resting wavelength still sends some light to the retina and using the phys-

ical shutter causes delay in the stimulus end. Turning the light source off would be a preferred solution.

Media

Both media were found good and suitable for the measurements. The only negative issue when using RPE DM- medium was that it was not oxygenated. If the RPE DM-medium could be oxygenated when a suitable buffering is used, it would improve the situation. However, this appears to be a minor problem that might not need to be addressed.

Perfusion

The problems with the perfusion have not been solved. The choice of not using perfusion was suitable for short measurements, but for extended measurements it would be preferable to use perfusion.

The PTFE stands that were used in some of the measurements were functional but not practical. With further developing they could probably be used when longer measurements take place.

Oxygenation and Heating of the Media

Both oxygenation and heating of the media were done in a manner that was both suitable and practical. If improvement of these methods would be desired, a method for keeping the media warm on the table during preparation and/or measurements could be useful.

Darkening of the Preparation and Measurement Sites

After trials of darkening the rooms with black rubbish sacks the blackout curtains worked really well for achieving a complete blackout. In practice it would have been better if the curtains could have been placed on the windows by simpler means than taping, but as for now, the practice is sufficiently good.

Membrane Carrier

The enlarged hole of the carrier was found to be good especially since the retinas needed to be placed on the MEAs under dim red light. With a hole big enough it was quite easy to position the carrier hole right on the electrode area of the MEA.

Retina-RPE Culture

The stand for the retina-RPE –complexes was functional and easy to use in the measurements. The essential question with the culture system is the stirring of the medium that would help the transport of the nutrients from the medium to the tissues. To find a suitable method for this would help to lengthen the culturing time.

The sandwich carrier that was used with retina-RPE complexes was found not to be a sufficient way for ensuring the two tissue layers to remain in their positions in relation to each other. However, using two carriers instead of one improved the situation signifi-

cantly after the first trials which were done with one carrier only. A frame of some kind that would fix the tissue layers in between could be designed to improve the situation.

4.2.2. Methods Used in the Measurement

The measurement protocol described in this thesis is rather complicated and includes many phases. Due to the complexity, human errors are quite common and they can sometimes be crucial to the success of the measurement. Human errors in the measurements that were done during the development of the setup included forgetting essential or important parts of the protocol, running out of a needed material, not testing the functionality of the technical devices and making mistakes while preparing or handling the tissues. Technical problems with the devices also caused problems, but their effect could have been minimized if they were tested before the actual measurement.

An important part of the development of the measurements was to simplify the protocol as much as possible. When the number of the components needed could be reduced, like dropping the perfusion from the measurement, the proportion of successful measurements from all increased. All phases that were not seen compulsory were cut off from the protocol and only essential phases were left to the current method. In addition to increasing the number of successful measurements the simplified protocol made it possible for one person to perform the measurements alone successfully as before two persons were required.

At its current state most of the methods used in the measurement are functional and practical. Nevertheless, measurements done with the system successfully need practice and experience so that some routine is gained. Even then, using a check list to be sure that everything has been done as supposed may be recommended.

There still remain aspects in the measurement protocol that are not optimal, the most significant of which being the need of different locations for enucleation, preparation and measuring, but these aspects could not be changed in the current location for the measurements. The system development had to be done on the terms of the location and thus some compromises had to be done. This far these compromises do not seem to cause problems in recording the light responses, but it seems obvious that the setup would be better than it currently is without the need for such many transportation phases.

5. CONCLUSIONS

The purpose of this thesis was to evaluate how ERG measurement could be used for testing the functionality of hESC-derived RPE cells and to develop an ERG measurement setup for recording light responses from mouse retinal explants alone and together with hESC-derived RPE cells. The evaluation of the suitability of ERG to the functionality test has been done by reviewing literature and recent publications. The development of the setup can be assessed based on the recordings that were done. The findings in the literature review and measurements are collected in this chapter and conclusions based on them are presented. Also suggestions for further research and next steps are proposed.

Measuring ERG from a retinal explant is delicate work. Retina is fragile and especially the photoreceptors damage easily. Using mouse retinas in the ERG measurements brings additional challenge because of the small size of the eye and the retina. The mouse retina is loosely attached in the bottom of the eye. Therefore it can be removed with minimal damage to the tissue and successful recordings can be done. In order to measure both rod and cone responses the tissue needs to be processed in darkness or using only dim red light. Dark adapting the mouse for at least three hours before the preparation would be preferable.

For ERG recordings from retina and RPE together, some successful publications exist, where the RPE has been prepared together with the retina [Stett et al. 2003; Monaim et al. 2005]. No studies where ERG is measured from retina together with hESC-derived RPE cells have been published. RPE is very tightly attached to its location in the eye and removing it requires other methods than preparing the retina alone. The species used also has great effect on the possibility of preparing the RPE successfully. The published ERG measurements with retina and RPE together have been done with chick [Stett et al. 2003] and toad [Monaim et al. 2005] retinas. Even after extensive searching, no publications on removing rodent RPE for measurements could be found. This implies that for the hESC-derived RPE cell functionality test mouse retina together with mouse RPE could not be used as a reference.

The few retinal reattachment studies that have been published show that retinal recovery after detachment and reattachment with the RPE in vitro occurs [Monaim et al. 2005; Fisher et al. 2005; da Cruz et al. 2007]. The results of these studies are promising when thinking of testing the hESC-derived RPE cell behavior with retinal explants. The recovery after reattachment begins instantly, but continues for at least several hours. This means that for the RPE functionality test the contact of the RPE with the retina

should be secured for hours and this contact should not be damaged during measurements.

Due to the high metabolism of the retina, it is hard to culture it successfully. Especially adult retinas tend to die easily when culturing. In order to remain the viability of the retina for long enough so that attachment with the RPE could occur, special care in preparation and culturing conditions should be taken. Especially oxygenation and nutrition need to be taken care of. Other factors that might help to keep the retina viable is improving sterility in different ways, using antibiotics and choosing carefully the species used [Kretz et al. 2004; Xin et al. 2007; Koizumi et al. 2007; Kobuch et al. 2008; Kaempf et al. 2008; Werner et al. 2008].

Based on the above referenced studies it seems that technically the ERG measurement would be suitable for testing the functionality of RPE cells. The measurement setup that was developed in this thesis could be used in a functionality test for the hESC-derived RPE cells with some further development. This naturally requires that the human-derived RPE cells have an ability to co-operate with animal tissue. It is one of the questions that could not be answered in this thesis and needs further studying.

The ERG measurement setup that was developed in this thesis proved to be functional and light responses could be recorded. Due to the small number of repeated measurements it is hard to evaluate the reliability of the system to give good ERG responses. The presented results from one measurement seemed to have no real ERG responses but only artefact. This shows that the system is still vulnerable to errors. The possibility of human errors can probably never be completely excluded from the setup, but it can be decreased by experience and choosing simplest possible methods.

The ERG responses recorded from isolated retinas were good. Also the responses recorded with different media had the components of an ERG and using the RPE DM-medium instead of the Ames medium was found suitable. However, the measurements done to evaluate the effect of light intensity, stimulus duration and exposure to prolonged light should be repeated since those could not be assessed based on these measurements. Gaining insight in these aspects could also be used when considering different possibilities for proving the RPE functionality. For example the recovery of the retina from light adaptation (prolonged exposure to light) requires the presence of a functional RPE [Newman & Bartosch 1999].

With the measurement setup light responses from retinal explants together with RPE cells could be recorded. Even though the functionality of the RPE cells could not be evaluated based on this single measurement the recording results look promising when thinking of proving RPE cell functionality in future.

There are some aspects in the current measurement setup that need to be changed before better results should be expected. Most importantly, the problems with the light stimulation have to be solved. Currently, the artefact produced by the LED stimulator makes it impossible to record responses during stimulation and when using the monochromator, the maximum intensity is too small to gain good light responses. Also, even though tuning the light intensity is possible in both of these stimulators the intensity

range in neither one of them is sufficiently wide. With a sufficient intensity range both very small light responses and saturated responses could be generated.

Apart from the problems with the light stimulation, some other issues also need to be considered when developing the system from now on. If longer periods of time are used with a single measurement, it would be beneficial to use perfusion during measurements. That may need further developing since the method used in these measurements was functional, but not very practical. The culture of the retinas with the RPE cell layers would most likely be better if the medium below the culturing stand could be stirred constantly. Also, an alternate solution for the current carrier sandwich structure could help to maintain the retinal and RPE tissue contact.

The possibility of developing an ERG measurement setup for a functionality test for hESC-derived RPE cells by bringing the cells in contact with an isolated retina has been studied. According to the related publications, there is reason to believe that it could be realized. Using this kind of setup for testing the hESC-derived RPE cells electrophysiologically has not been reported in the literature. If the method proves to be functional, it brings new insight on the co-operation of the hESC-derived RPE cells and the retina.

A measurement setup was developed successfully so that light responses from isolated retinas alone and together with hESC-derived RPE cell layer could be recorded. Some areas of the current setup still need more work before the system can be seen as fully satisfying. After these few problems are solved the system can be used to see whether this approach to study the functionality of hESC-derived RPE cells is possible in practice or not. The challenge still ahead is interesting and in my opinion well worth taking.

REFERENCES

Carr, A.J., Vugler, A., Lawrence, J., Chen, L.L., Ahmado, A., Chen, F.K., Semo, M., Gias, C., da Cruz, L., Moore, H.D., Walsh, J. & Coffey, P.J. 2009. Molecular characterization and functional analysis of phagocytosis by human embryonic stem cell-derived RPE cells using a novel human retinal assay. Molecular Vision 2009, 15, pp. 283-295.

Chen, A., Zhou, Y., Gong, H. & Liang, P. 2004. Firing rates and dynamic correlated activities of ganglion cells both contribute to retinal information processing. Brain Research 1017 (2004), pp. 13– 20.

da Cruz, L., Chen, F.K., Ahmado, A., Greenwood, J. & Coffey, P. 2007. RPE transplantation and its role in retinal disease. Progress in Retinal and Eye Research 26 (2007), pp. 598–635.

Fisher, S.K.,Lewis, G.P., Linberg, K.A. & Verardo, M.R. 2005. Cellular remodeling in mammalian retina: results from studies of experimental retinal detachment. Progress in Retinal and Eye Research 24 (2005), pp. 395–431.

Green, D.G. & Kapousta-Bruneau, N.V. 1999. Electrophysiological properties of a new isolated rat retina preparation. Vision Research 39 (1999), pp. 2165–2177.

Guenther, E., Herrmann, T. & Stett, A. 2006. The Retinasensor: An In Vitro Tool to Study Drug Effects on Retinal Signaling, pp. 321-331. Taketani, M. & Baudry, M. Advances in Network Electrophysiology Using Multi-Electrode Arrays. Springer, Singapore 2006.

Heckenlively, J.R. & Arden, G.B. 2006. Principles and Practice of Clinical Electrophysiology of Vision, 2nd ed. MIT Press, USA 2006. 999 p.

Heikkinen, H., Nymark, S. & Koskelainen, A. 2008. Mouse cone photoresponses obtained with electroretinogram from the isolated retina. Vision Research 48 (2008), pp. 264–272.

Herrmann, T., Krause, T., Gerhadt, M., Hesse, M., Boven, K-H. & Stett, A. 2008. LED-based illumination system for the MEA60 system for full field stimulation of explanted retinas. Conf Proc of the 6th International Meeting on Substrate-Integrated Microelectrodes, Reutlingen, Germany, 2008. pp. 199-200.

Johnson, T.V. & Martin, K.R. 2008. Development and Characterization of an Adult Retinal Explant Organotypic Tissue Culture System as an In Vitro Intraocular Stem Cell

Transplantation Model. Investigative Ophthalmology & Visual Science, August 2008, Vol. 49, No. 8, pp. 3503-3512.

Kaempf, S., Walter, P., Salz, A.K. & Thumann, G. 2008. Novel organotypic culture model of adult mammalian neurosensory retina in co-culture with retinal pigment epithelium. Journal of Neuroscience Methods 173 (2008), pp. 47–58.

Kaufman, P.L. & Alm, A. 2003. Adler's Physiology of the Eye – Clinical Application, 10th ed. Mosby, USA 2003. 876 p.

Kobuch, K., Herrmann, W.A., Framme, C., Sachs, H.G., Gabel, V.P. & Hillenkamp, J. 2008. Maintenance of adult porcine retina and retinal pigment epithelium in perfusion culture: Characterisation of an organotypic in vitro model. Experimental Eye Research 86 (2008), pp. 661-668.

Koizumi, A., Zeck, G., Ben, Y., Masland, R.H. & Jakobs, T.C. 2007. Organotypic Culture of Physiologically Functional Adult Mammalian Retinas. PLoS
ONE [on-line journal] 2(2): e221. Published 21 Feb 2007, 8 p.

Kretz, A., Hermening, S.H. & Isenmann, S. 2004. A novel primary culture technique for adult retina allows for evaluation of CNS axon regeneration in rodents. Journal of Neuroscience Methods 136 (2004), pp. 207–219.

Lewis et al. 1991. Lewis, G.P., Erickson, P.A., Anderson, D.H., Fisher, S.K., 1991. Opsin distribution and protein incorporation in photoreceptors after experimental retinal detachment. Exp. Eye Res. 53, 629–640.

Marmor, M.F., Holder, G.E., Seeliger, M.W. & Yamamoto, S. 2004. Standard for clinical electroretinography (2004 update). Documenta Ophthalmologica 108, pp. 107–114.

MCS Homepage 2009. Multi Channel Systems MCS GmbH Homepage. Last update: 2010-05-19, [cited 20.5.2010]. Available online: http://www.multichannelsystems.com/

MEA Amplifier Manual 2008. Multi Channel Systems. MEA Amplifier with Blanking Circuit for Inverse Microscopes. Printed: 2008-12-11. 56 p. Available online: http://www.multichannelsystems.com/fileadmin/user_upload/Manuals/MEA1060-Inv-BC_Manual.pdf.

MEA Manual 2005. Multi Channel Systems. Microelectrode Array (MEA) User Manual. Printed: 2005-11-04. 45 p. Available online: http://www.multichannelsystems.com/fileadmin/user_upload/Manuals/MEA_Manual.p

df.

MEA Stimulus Generator Manual 2007. Multi Channel Systems. Stimulus Generator 2000 Series User Manual. Printed: 2007-06-20. 84 p. Available online: http://www.multichannelsystems.com/fileadmin/user_upload/Manuals/STG2000_Manual.pdf.

MEA System Manual 2006. Multi Channel Systems. MEA System User Manual. Printed: 2006-05-11. 28 p. Available online: http://www.multichannelsystems.com/fileadmin/user_upload/Manuals/MEA-System_Manual.pdf.

MEA Temperature Controller Manual 2007. Multi Channel Systems. Temperature Controller TC01/02 User Manual. Printed: 2007-10-23. 13 p. Available online: http://www.multichannelsystems.com/fileadmin/user_upload/Manuals/TCX_User_Manual.pdf.

Monaim, M.A., Suleiman, J.H. & Ashraf, M. 2005. Morphological Recovery in the Reattached Retina of the Toad Bufo marinus: A New Experimental Model of Retinal Detachment. Archives of Medical Research 36 (2005), pp. 107–112.

Mosinger Ogilvie, J., Speck, J.D., Lett, J.M. & Fleming, T.T. 1999. A reliable method for organ culture of neonatal mouse retina with long-term survival. Journal of Neuroscience Methods 87 (1999), pp. 57–65.

Multi Channel Systems 2005. MEA Application Note: Retina Recordings (Micro Electroretinograms) from Rattus norvegicus. Multi Channel Systems MCS GmbH 2005-07-28. 17 p. [cited 16.5.2010]. Available online: http://www.multichannelsystems.com/fileadmin/user_upload/Application_Notes_Tutorials/MEA-Applications_Retina.pdf.

Newman, E.A. & Bartosch, R. 1999. An eyecup preparation for the rat and mouse. Journal of Neuroscience Methods 93 (1999), pp. 169–175.

Nusinowitz, S., Ridder, W.H. & Heckenlively, J.R. 2002. Electrophysiological Testing of the Mouse Visual System, pp. 320-343. Smith, R.S., John, S.W.M., Nishina, P.M. & Sundberg, J.P. Systematic Evaluation of the Mouse Eye: Anatomy, Pathology, and Biomethods. CRC Press, USA 2002.

Polychrome V data sheet 2009. TILL Photonics GmbH. TILL Polychrome V – Fast Monochromator for Fluorescence Microscopy, 2009. 2 p. [cited 31.5.2010]. Available online: http://i-mic.eu/Support/files/Polychrome%20V_data%20sheet.pdf.

Rosolen, S.G., Kolomiets, B., Varela, O. & Picaud, S. 2007. Retinal electrophysiology for toxicology studies: Applications and limits of ERG in animals and ex vivo recordings. Experimental and Toxicologic Pathology 60 (2008), pp. 17–32.

Scientifica Image Library 2010. Image of MEA Basic System [cited 17.5.2010]. Available online: http://www.scientifica.uk.com/imagelibrary/MEA-60%20Basic%20Systems.jpg.

Shyu, J., Maia, M., Weiland, J.D., O'Hearn, T., Chen, S., Margalit, E., Suzuki, S. & Humayun, M.S. 2006. IEEE Transactions on Neural Systems and Rehabilitation Engineering, vol. 14, No. 3, September 2006, 290-298.

Stett, A., Egert, U., Guenther, E., Hofmann, F., Meyer, T., Nisch, W. & Haemmerle, H. 2003. Biological application of microelectrode arrays in drug discovery and basic research. Analytical and bioanalytical chemistry (2003) 377, pp. 486–495.

Syed, M.M., Lee, S., He, S. & Zhou, Z.J. 2004. Spontaneous Waves in the Ventricular Zone of Developing Mammalian Retina. Journal of Neurophysiology 91 (2004), pp. 1999–2009.

Webvision 2009. Kolb, H., Fernandez, E. & Nelson, R. Webvision – The Organization of the Retina and Visual System. John Moran Eye Center, University of Utah. [cited 7.5.2009]. Available online: http://webvision.med.utah.edu/.

Werner, B., Cook, P.B. & Passaglia, C.L. 2008. Whole-cell Recordings of Light Evoked Excitatory Synaptic Currents in the Retinal Slice. [Video Article] JoVE. 17. Published 02.07.2008. Available online: http://www.jove.com/index/details.stp?id=771.

Xin, H., Yannazzo, J.S., Duncan, R.S., Gregg, E.V., Singh, M. & Koulen, P. 2007. A novel organotypic culture model of the postnatal mouse retina allows the study of glutamate-mediated excitotoxicity. Journal of Neuroscience Methods 159 (2007), pp. 35–42.