TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

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OPTIMAL TRANSPORTATION PROTOCOL FOR HUMAN RETINAL PIGMENT EPITHELIUM CELL TRANSPORTATION ON BIOMATERIAL MEMBRANE

Master of Science Thesis

Examiner: Professor Minna Kellomäki and PhD Heli Skottman
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ABSTRACT

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Ophthalmology group of the Institute of Biosciences and Medical Technology (BioMediTech) is a research group concentrating on studying retinal pigment epithelium cell (RPE) and their possibilities in cure of degenerative eye diseases. In the near future Ophthalmology group will do animal tests in co-operation with an European partner. For that reason RPE cells will be sent from Tampere to Germany.

Tissue banks have a wide experience of tissue storage and delivery. Regea is a cell and tissue center, which co-operates intensively with BioMediTech and is one of the Finnish tissue banks. Cells and tissues are sent weekly from Regea inside borders of Finland. Transportations take only a few hours. Many cell types and tissues are transported either frozen or in hypothermal conditions. According to literature hypothermal conditions might not be the best choice for retinal pigment epithelium cells especially if transportation is over night.

Target of this thesis work was to formulate an optimal transportation protocol for human RPE cells on membrane material. Demonstrated transportations and cell analysing methods were tested. Experimental part of this thesis was done with real RPE cells. Because cells and membranes are expensive, experiments were done with a small amount of samples. By using reasonable experiments it was possible to find optimal transportation conditions without use of statistical methods.

Results show that after 24 hours transportation RPE cells transported in room temperature in Hibernate A medium survived best. Immunohistochemical methods gave information about RPE cells and were suitable for cell analysis. Measurements of pH and transepithelial resistance are not recommended because of their low level of information in this experiment. The role of mechanical stress was higher than expected. Laboratory environment did not mimic air transportation exactly so there might be some unexpected results in the real transportation.

TIIVISTELMÄ

TAMPEREEN TEKNILLINEN YLIOPISTO
Biomateriaalitekniikan koulutusohjelma
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Silmäryhmä on Tampereen yliopiston ja Tampereen teknillisen yliopiston yhteisen BioMediTech-instituutin tutkimusryhmä, joka keskittyy tutkimaan verkkokalvon pigmenttiepiteelin (RPE) soluja ja niiden mahdollisuuksia silmän rappeumasairauksien hoidossa. Lähitulevaisuudessa silmäryhmä tulee tekemään eläinkokeita eurooppalaisen yhteistyökumppanin kanssa. Tästä syystä RPE-soluja tullaan lähettämään Tampereelta Saksaan.

Kudospankeilla on paljon kokemusta kudosten säilyttämisestä ja jakelusta. Regea, joka toimii tiiviissä yhteistyössä BioMediTechin kanssa, on yksi suomalaisista solu- ja kudospankeista. Regealta lähetetään soluja ja kudoksia viikottain Suomen sisällä. Kuljetukset kestävät vain muutamia tunteja. Useita solutyyppejä ja kudoksia kuljetetaan jäädytettyinä tai hypotermisissä olosuhteissa. Kirjallisuuden mukaan hypotermiset olosuhteet eivät välttämättä sovi verkkokalvon pigmenttiepiteelin soluille etenkään silloin, kun kuljetus kestää lähes vuorokauden.

Tämän diplomityön tavoitteena oli muodostaa sopiva kuljetusprotokolla ihmisen RPE-soluille kalvomateriaalin päällä. Työ sisälsi koekuljetuksia ja myös solujen analysointitapoja testattiin. Koekuljetukset tehtiin oikeilla RPE-soluilla. Koska solut ja kalvot ovat melko kalliita, tehtiin koesarja vain pienellä näytemäärällä. Järkevällä koeasetelmalla oli mahdollista löytää optimaaliset kuljetusolosuhteet käyttämättä tilastollisia testejä.

Tulosten perusteella 24 tunnin kuljetuksesta selvisivät parhaiten huoneenlämmössä Hibernate A-liuoksessa pidetyt RPE-solut. Immunohistokemialliset menetelmät antoivat tietoa RPE-soluista ja sopivat soluanalyysiin. pH:n ja epiteeliresistanssin mittaamista sen sijaan ei voi suositella, koska ne eivät antaneet juurikaan hyödyllistä tietoa. Mekaanisen rasituksen merkitys oli odotettua suurempi. Laboratorio-olosuhteet eivät täysin vastanneet todellisia olosuhteita lentokuljetuksessa, joten oikea kuljetus voi paljastaa odottamattomia tuloksia.

PREFACE

This study was carried out in the Ophthalmology Group of BioMediTech which is a joint institute of Tampere University of Technology and University of Tampere. BioMediTech pioneers discoveries and innovations in life sciences.

First, I would like to thank Adjuct Professor Heli Skottman, the group leader of Opthalomology, for the opportunity to conduct my thesis in her group. I am grateful also for her contribution of working as the supervisor and examiner of this thesis. Second, I would like to thank Professor Minna Kellomäki who was the other examiner.

I owe my gratitude to the members of the Ophthalmology group. PhD Students Anni Sorkio and Alexandra Mikhailova had always time to answer my questions. Special thanks for PhD Kati Juuti-Uusitalo who took confocal microscope images for my thesis. I would also like to thank Outi Heikkilä, Hanna Koskenaho and Outi Melin for technical assistance in practical matters.

Finally, I would like to thank my family, especially my parents, and friends for encouragement during my studies and thesis process.

"By perseverance the snail reached the ark." - Charles H. Spurgeon.

Tampere, May 2014

Anna-Kaisa Pietiläinen

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ABBREVIATIONS

AMD	age-related macular degeneration
BF	bright field
BSA-PBS	bovine serum albumin-phosphate buffered saline
DAPI	4',6-diamino-2-phenylindole
DGR	dangerous goods
ECM	extra cellular matrix
EIS	electrical impedance spectrum
EPS	expanded polystyrene
ETHD-1	ethidium homidimer-1
hESC	human embryonic stem cell
hiPSC	human induced pluripotent stem cell
hMSC	human mesenchymal stem cell
IATA-DGR	International Air Transport Association Dangerous Goods Regulations
KO/SR	Knockout^{TM} Serum Replacemet
L/D	ratio of living cells compared to dead cells
PBS	phosphate buffered saline
PET	polyethylene terephtalate
PI	polyimide
P/S	Penicillin/Streptomyocin, antibiotic
REV/min	revolution per minute
RPE	retinal pigment epithelium
RT	room temperature
SEM	scanning electron microscope
STAP	stimulus-triggered acquisition of pluripotency
TER	transepithelial resistance
T^{in}	temperature inside the transportation box

1 INTRODUCTION

Sight is considered as human's most important sense. World Health Organization (WHO) estimates that there are 285 million people in the world who have visual impairment. 39 million are blind and 246 million have more or less low vision. Especially older people suffer from weak sight and it is estimated that over four fifth of blind persons are aged 50 and above. Major reason for that is degeneration of the retina and its retinal pigment epithelium (RPE) monolayer which causes age-related macular degeneration (AMD). When life expectancy grows, the number of AMD patients also increases in the developing countries. (World Health Organization 2013)

Regea is a cell and tissue center in Tampere which has a lot of experience of tissue transportation. They store and deliver for example bone tissue, tendons, corneal grafts and amniotic membrane grafts. (Regea 2014a). Also fertility clinics are experienced with national and international cell delivery and transportation. Most of tissues are transported hypothermally and some cells can be frozen. For some cell types also room temperature (RT) or body temperature are suitable as transport temperature.

The aim of this thesis was to formulate and test a transportation protocol for human RPE cell transportation. It is needed when Ophthalmology group of BioMediTech starts co-operation with European partner and animal tests with RPE cells will be done. If the protocol enables cells to survive the over night transportation, it might be possible to use the same guidelines in longer transportations. This would enable wider co-operation between international research groups.

RPE cells had been plated to the membranes around one month before transportation was demonstrated. Demonstrated transportations were based on the transportation facilities of transportation companies which were mapped in the beginning to this work. The test matrix combined then two possible transportation temperatures, three membrane materials and two mediums. When the most potential combinations had been found, an orbital shaker was added to simulate mechanical stress during transportation. Cells were analysed before and after transportation with several methods. Usefulness and informativity of each method was also evaluated. Transportation protocol was formed from the results.

This thesis consists of a theoretical and an experimental part. In the theoretical part the structure of the eye, retina and retinal pigment epithelium are introduced. Also the most common degenerative eye disease, age-related macular degeneration, is presented. Human stem cells and human embryonic stem cell-derived retinal pigment epithelium cells are presented shortly. Furthermore common biomaterials and cell analysis methods used in RPE cell studies are presented. In the end of the theoretical part there

are also explained the most significant requirements for transportation. The experimental part of this thesis presents materials and methods used in this experiment and three demonstrated transportations and their pre-trial review. Finally, the results of the demonstrated transportations are reported, discussed and concluded.

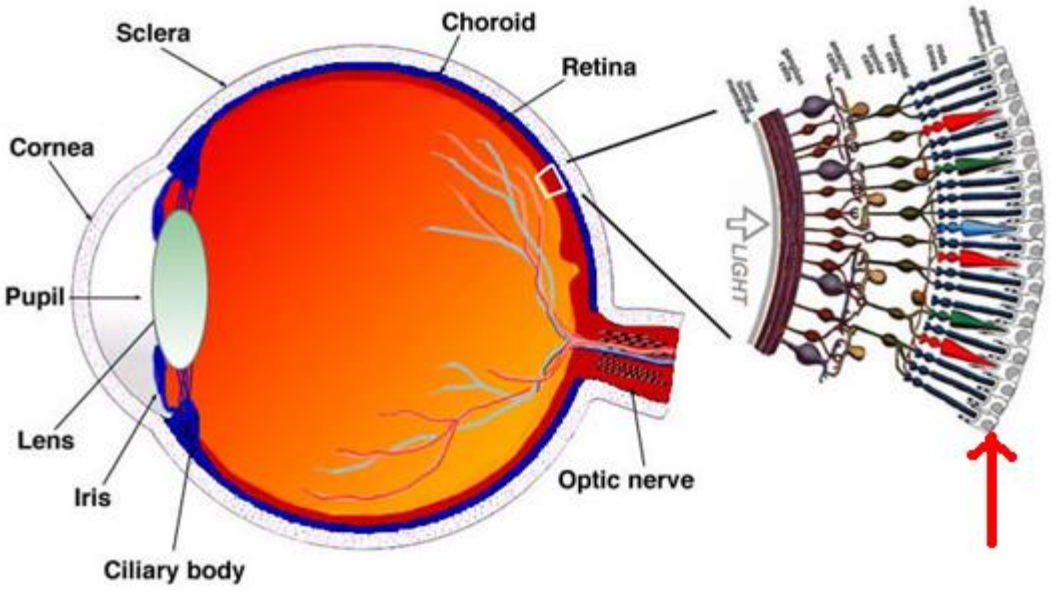
THEORETICAL PART

2 RETINA

Human forms with sight his understanding of the surrounding world. Sight gives more information than any other sense and approximately 70 percent of sensory cells are located in the eye. There is a lens system in the eye which is similar to camera. Light scattered from objects in surrounding fastens to retina in the back part of the eye where sensory cells are located. Information goes from retina to brain via optic nerve which is located in the back of the eyeball. Brain collects all information and turns them to subjective sight sensation. (Haug et al. 2007) Age-related macular degeneration is the most significant degenerative disorder in retina. The structure of the eye and the retina are presented in the next chapters. Also the retinal pigment epithelium and its functions and age-related macular degeneration are presented shortly.

2.1 Structure of the eye and retina

Globular eye balls are placed in cone like eye sockets. Ball diameter is around 2.5 cm and its' outerrmost layer is tough fibroelastic capsule called sclera. In the front of the eye sclera turns to transparent cornea. Cornea's function is to refract light in the eye. Together sclera and cornea form the external layer of the eye. The intermediate layer of the eye consists of anterior located iris and ciliary body and posterior choroid. Iris surrounds circle hole, pupil, in the middle of the eye. Iris is heavily pigmented and the colour of the eyes depends on it. Behind the pupil there is lens which is connected to the ciliary body with suspensory ligaments. The lens can change its form and enable refraction of the light into retina. Lens also divides the eye ball into anterior chamber and gel-like vitreous body. Blood vessels of the eye ball are fastened to choroid. Choroid includes a lot of pigmented cells which absorbs the light passing out of retina. The innermost layer of the eye, also called the sensory part of the eye, is the retina. Retina is approximately 0.5 mm thick. The light that comes into the eye goes to cells of macula which is a small spot near the centre of retina where optic nerve locates also. This around 2 x 1.5 mm diameter nerve carries sight information to brain. From the centre of the optic nerve radiate the major blood vessels of retina vascularising the retinal layers and neurons. (Haug et al 2007, Kolb et al 2005). The structure of the eye and schematic enlargement are presented in Figure 1.



***Figue 1**. The structure of the eye and a schematic enlargement of the retina. Macula (not marked) would be near the centre of retina under optic nerve. RPE cells are marked with red arrow. (Kolb et al 2005)*

All vertebrate have three layers of nerve cell bodies and two layers of synapses in their retina. The innermost layer consists of ganglion cells and displaced amacrine cells. In the middle there is the nuclear layer which consists of cell bodies of the bipolar, horizontal and amacrine cells. The outer layer contains cell bodies of the rods and cones. Between these cell layers happens synaptic contacts. This is a very simplified presentation of the structure of retina. (Kolb et al 2005)

The ganglion cells, which are the output neurons of the retina, lay innermost in the retina and are closest to the front of the eye and lens. In the Figure 1 they are presented as four dark grey spherical objects and marked with red arrow. The rod and the cones, also known as photo sensors, lay outermost in the retina against the pigment epithelium and choroid. Rods are sensitive to light and they activate in dim light. Cones enable to see colours and they need more light to activate. Rods are presented as blue sticks and cones as red, green and light blue tapers. When light comes into the eye it has to go through the retina before it activates the rods and cones. The absorption of photons by the visual pigment of the photoreceptors is turned first into biochemical form and then to electrical form which can stimulate all the succeeding neurons of the retina. Then the information is transmitted to the brain from electrically activated ganglion cells and sensation of visual image is formed. (Kolb et al 2005)

2.2 Retinal pigment epithelium

Retinal pigment epithelium (RPE) is located between blood supply of the choroid and the light sensitive outer segments of the photoreceptors. Interaction between the pig-

mented cells and the photoreceptors is essential for visual function. Already when an embryo starts to develop, the pigmented cells and the photoreceptor cells differentiation depends on each other. The RPE consist of hexagonally packed, tight-junction, single sheet of cells containing pigment granules. The RPE has the highest cell density in the human body. (Strauss 2011) In Figure 1 pigment epithelium is illustrated as light grey angular structure on the right side marked with red arrow. This cobblestone like structure can be seen when imaging perpendicular on the cell surface. It is shown also in Figure 2.

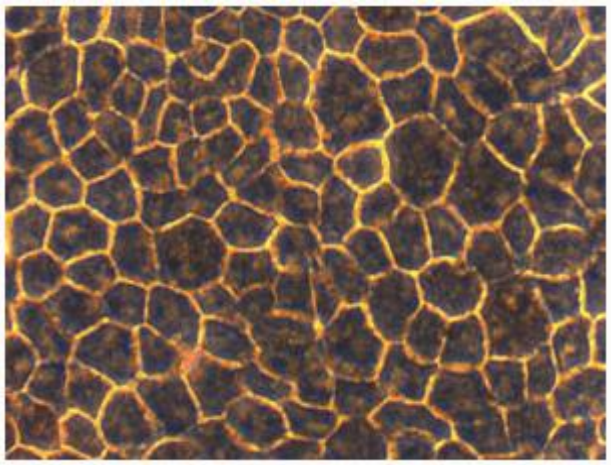


Figure 2. *Cultured human RPE cells. Picture was taken 2 weeks after isolation of RPE cells from the fetal eye tissue. (Vanderbilt University School of Medicine 2014)*

The RPE has several functions and light absorption is one of the best known. Sense of sight is a very complicated system in which the RPE has an eminent role. The RPE absorbs light with its heavily pigmented cells and covers the inner surface of the bulbus. It improves the optical systems' quality when scattered light is absorbed. The RPE protects retina against free radicals, photo-oxidative exposure and light energy which are caused by the light that comes into the eye. (Strauss 2011)

The RPE transports selectively nutrients and ions between photoreceptors and choriocapillaris. From the blood side to the retina go glucose, fatty acids and retinal which is a key molecule involved in vision. Photoreceptors use these nutrients in their essential metabolites. From the subretinal side to the blood side go water, ions and metabolic end products like lactic acids caused by the metabolic of photoreceptors, neurons and glial cells. (Strauss 2011)

With its basolateral membrane, the RPE is in contact with the specialized multilayered Bruch's membrane. Bruch's membrane represents an interaction matrix for the RPE cells with blood flow between RPE and choroid. It consists of RPE basement membrane, collagenous layer middle elastic layer, outer collagenous layer and the choroidal endothelial cell basement membrane. Together these structures forms a part of the blood-retina barrier system which is placed between the blood flow of the choroid and the outer segments of photoreceptors. Tight junctions between the RPE cells and very

selective transportation between the blood and the subretinal space guarantee isolation and high immune privilege. (Hogan and Alvarado 1967, Strauss 2011)

The RPE interacts closely with photoreceptors and cells on the blood side of the epithelium such as cells of immune system. The RPE is able to secretion of several growth factors and signalling molecules. Secretion is constant in a healthy eye and helps to maintain the structural integrity of the neighbour tissues. Secreted factors are, for example, fibroblast growth factors (FGF-1, FGF-2 and FGF-5), transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF). Among growth factors several other molecules like ATP, fas-ligand (fas-L) and members of the interleukin family are also secreted. There are even more factors activated if the retina or the RPE are exposed to pathological conditions like hypoxia or metabolic stress. (Strauss 2011)

Among functions mentioned earlier the RPE takes part also to visual cycle which is a complicated process where a photon is absorbed and several reactions to stereo-chemical change of 11-cis retinal into all-trans retinal. Also phagocytosis of photoreceptors in outer membranes is a function of the RPE. Photoreceptors are destroyed constantly due to photo-oxidative damage. (Strauss 2011)

2.3 Age-related macular degeneration

Age-related macular degeneration (AMD) is a progressive condition affecting older people. It is the primary cause of blindness in industrialized countries and globally on the third place after cataract and glaucoma. AMD causes damages in the area of central vision. Patients have difficulties to read normal size font without devices but often they can move without problems because there are no damages in the edges of the field of vision. There can be also night blindness and increased feeling of glare. (Retina ry 2013)

AMD causes damage to the macula. In some cases vision loss is so slow that patient does not notice it for a long time. Sometimes the disease progresses faster and patient can lose vision from one or both eyes in a relatively short time. AMD by itself does not lead to total blindness, but the loss of central vision can decrease patient's capability to do everyday activities. (National Eye Institute 2013)

AMD can be divided into two categories: dry and wet. In geographic atrophy, also called dry AMD, the light-sensitive cells in macula break down gradually. Damaged cells do not bring information to brain and the supporting tissue beneath the macula leading to vision loss. In neovascular AMD, also known as wet AMD, abnormal blood vessels grow under the retina. Fluid and blood can leak from vessels and macula can be swollen and damaged. Neovascular AMD advances often faster and is rougher than geographic atrophy. Both types can be expressed in the same eye. (National Eye Institute 2013)

There are around 10 000 blind and 70 000 other way visually impaired people in Finland. Numbers cover 1.5 % of all Finns. Occurrence is on around the same level as

in breast cancer. 40.4 % of all diagnoses were caused by AMD and number of cases is increasing. There are probably several thousands of patients who have not got diagnosis. (Ojamo 2013)

The main reason for AMD is ageing and occurrence increases among 60 years old and strongly among 70 years old people. Also smoking, genetic tendency, arterial hypertension, uv-light and non-balanced diet expose to AMD. Also low degree of pigmentation, in other words light coloured eyes, is a risk factor. Genotype might also affect. Good manners of living are the best way to prevent AMD. (World Health Organization 2014)

For a long time there was no medication for AMD. Dry AMD is still without treatment but during last decades new drugs have been developed for wet AMD. For the present medication and methods can stop degeneration or at least slow it but breakthrough to healing AMD has not been done. At the moment three types of treatments are given. Injections are used especially in neovascular AMD when there are often high levels of vascular endothelial growth factors (VEGF) secreted. Anti-VEGF injection therapy can block this overgrowth of blood vessels. Treatment need to be taken many times to get benefit. Photodynamic therapy is a bit more rare treatment. First verteporfin drug is injected into patient's vein in the arm. When drug has travelled into new blood vessels of retina, doctor shines a laser beam into patient's eye to activate the drug in abnormal vessels. Activated drug closes off new blood vessels and slows their growth. Third method is a laser surgery where a laser destroys abnormal blood vessels. Laser can be used if vessel growth is limited to compact area of the eye and laser can be easily targeted. (National Eye Institute 2013)

AMD has tried to be healed with cell transplantations. Mainly autologous RPE cells have been transplanted from good area or eye into the damaged one. Method improves reading vision and patient's quality of life but it does not heal the vision completely. (da Cruz et al. 2007) New treatments for both AMD types are looked all the time. There are studies regarding to for example oral medication, vaccination and eye drops but also with stem cells and biomaterials (Macular Degeneration Partnership 2014). Trials where RPE cells are combined with biomaterials will be done in the near future. For example pharmaceutical company Pfizer and University College in London have planned a study of implantation of hESC derived RPE cells as a treatment for AMD. They are not recruiting patients yet but in Republic of Korea recruitment for the same kind of study has been going on since fall 2012. (Clinical Trials 2014)

First clinical trials of phase I studies where RPE cells derived from human pluripotent stem cells were transplanted into the eye were done in the United States 2012. Researchers transplanted hESC-RPE cells into two patients' eyes. Both patients had age-related macular degeneration. Transplants improved patients' sight but not much. Probably this treatment method could work better if it would have been done earlier. According to the research groups results it is safe to transplant cells into retina. After four months control there were no hyperproliferation, tumorigenicity or ectopic tissue. Limiting factor of this method is that researchers injected RPE cells one by one into the

back part of the eye which might weaken cell escape and attachment. (Schwarz et al. 2012) For the present there have not been clinical trials in Finland with hESCs derived RPE cells (Regea 2014b).

So far one of the most promising trials has been made by Advanced Cell Technology. They started stem cell based treatment 2011 with 22 patients. Patients had either AMD or Stargardt's macular dystrophy. Stem cell derived RPE cells were transplanted beneath patient's retina. In May 2013 the company reported that one patient's vision had improved significantly. Earlier he was almost blind and after treatment he saw enough to drive a car. Advanced Cell Technology did not tell which disease the patient had. Also other patients have reported improvements in their sight even though the aim of the trial was to check if the stem cells are safe. (New Scientist 2013)

3 STEM CELLS

Pluripotent stem cells are cells which can be differentiated to any other cells. They can be produced in laboratory conditions and new methods are studied all the time. Stem cells are expected to be cure for degenerative disease like age-related macular disease and Parkinson's disease.

3.1 Human stem cells

Human stem cells are undifferentiated cells with a remarkable potential to develop many cell types. There are stem cells in several tissues of human body and these adult stem cells can be used in tissue regeneration or replacement. Drawback in the adult stem cells is that they have a limited differentiation potential. Instead stem cells of embryo have a really high differentiation potential. Use of these human embryonic stem cells (hESC) includes ethical questions which limits their use in research. Stem cell studies and the growth of that field of science have given hope that stem cells could be used in new treatments for degenerative conditions like diabetes, Parkinson's disease or age-related macular degeneration.

Another important stem cell resource is human induced pluripotent stem cells (hiPSCs). hiPSCs are adult cells that can be generated to pluripotent stem cells. They were found 2006 when Shinya Yamanaka isolated mice skin cells and reprogrammed them with four genes which are normally expressed in embryo. Cells turned to immature stem cells which can grow into all types of cells and are called hiPSCs. (Takahashi and Yamanaka 2006). 2012 Yamanaka won The Nobel Prize in Physiology and Medicine with Sir John B. Gurdon. The Nobel Prize was awarded for them "for the discovery that mature cells can be reprogrammed to become pluripotent" Gurdon had succeeded to clone frogs already 1962. (The Nobel Foundation 2014) Studies are not yet as far as with hESCs but also hiPSCs are expected to be a treatment for degenerative diseases and to be used to produce spare parts for human body. Compared to hESCs, hiPSCSs do not have as much ethical questions. (Regea 2014b)

hiPSCs are not yet in clinical trials but some successful animal tests have been done. Takebe et al. produced human liver tissue from hiPSCs. First liver blanks made of hiPSCs were grown on petri dishes and then transplanted into mice. Blanks developed to functioning liver with vascularisation. Also earlier researchers have succeeded in growing tissues from stem cells but this kind of three dimensional structures are challenging. It is still unsure if this kind of method would work in human body but it might be a promising approach. (Takebe et al. 2013)

3.2 Human embryonic stem cell-derived retinal pigment epithelial cells

Human embryonic stem cells (hESCs) are usually derived from a pre-implantation stage embryo. Cells are placed on a laboratory culture dish where they divide and spread. There is often a layer of fibroblasts of human or mice called feeder layer on the bottom of the dish to support stem cells and prevent that they do not differentiated unscheduled. Cells are kept in a cell culture medium which feeds cells. The process of generating a new sell line from embryonic stem cells is ineffective and lines are produced less frequently than embryos of pre-implantation are placed on the dish. If cells survive, divide and multiply well, they can be removed and plated into fresh culture dishes. The process of re-plating is repeated many times during the next months. Each cycle of re-plating is called passage. Researchers try to control the differentiation of stem cell line to produce targeted cells. (National Institutes of Health 2010)

Among other tissues and cell types hESCs can also be derived to RPE cells. Major of the studies in this field include animal based products like serums and feeders. If cells are planned to be implanted into human eye, they should be replaced with some other components. Risk of contamination or rejection is quite high in animal based products compared to human based products. There are some publications of successful derivations without animal based products like the one made by Vaajasaari et al. (2011). Group developed a protocol for a progressive differentiation of functional RPE like cell from hESCs and hIPSCs. Their results showed that putative hESC-RPE and hiPSC-RPE expressed genes and proteins which are characteristic for RPE cells. Conditions that the group defined can be used for further development of more efficient differentiation protocols.

Also the volume of the cells is a limiting factor. Cell culture is a very sensitive process and cell differentiation takes time. It is pretty common that cell acquisition is smaller than targeted but there are also opposite success. Researchers Outi Hovatta and Karl Tryggvason from Karolinska Institutet, Sweden, have experience of producing stem cells a lot. Over 400 kilograms of stem cells can be cultured from one embryonic stem cell in one month. In near future these cultured stem cells will be transplanted into patients who suffer from age related macular degeneration. (Rodin et al. 2014) Volume of cells compared to used time is exceptional and the method is not generally known.

Interest to use biomaterials in RPE cell culturing has been growing during the last years. Wide range of natural biomaterials has been tested as a cell culture substrate for RPE cells but researchers are not unanimous what material is the most suitable (Kurkela 2011). Biomaterial used with RPE cells is often in a form of membrane, thin film or 3D scaffold. Most of the studies concentrate on transplantation of mature RPE cell sheets (Julien et al. 2011, Harein et al.2010). In practise it means that RPE cells are cultured on a biomaterial membrane and then implanted together with the membrane into the retina. Trials are now in the beginning and expectations for this kind of method are high.

4 MEMBRANE MATERIALS

Biomaterials are materials that are used with living tissue. They can be used as prosthesis or in diagnostic or therapeutic use and they do not harm organism. Biomaterials can be divided in natural biomaterials and synthetic biomaterials. Natural biomaterials can be further divide into human and animal based biomaterials and other natural biomaterials.

4.1 Biomaterials generally

Biomaterials and products made of them are subjects which are used in contact with tissue, blood or tissue fluid. European Union and national legislation guide the use of biomaterials in European countries. There are demands to materials and products, their biocompatibility and physical, chemical and biological properties and sterility. The most essential standards are in EN ISO 10993 series which include standard of biocompatibility, clinical trial standard and risk analysis standard. (Valvira 2014) Biomaterials are either natural or synthetic materials which are meant to be used to cure or replace living tissue. Biomaterials can contain added factors like antibiotics or growth factors.

Biomaterials can be classified according to many classification systems. Origin of the biomaterial separates human, animal and other natural sources of biomaterials. Viability of biomaterial can vary from viable to non-viable even in the same product if some frozen grafts are combined with living cells. Classification from the juridical point of view is more complicated; legislation in European Union and Finland is updated slowly and there are products in the market without licensing. Biomaterials can be classified according to the use. Typical uses are treatments, prevention, diagnostics and modification of physiologic. Also the structure of the product and the level of processing can be classification methods. (Lääkelaitos 2003) Classifications are not unequivocal and can vary for example between European Union and the United States. Harmonization of classifications would promote development of tissue engineering.

When biomaterial is used in human body, it has to fulfil some basic requirements. Biocompatibility is very important. Material might be in corrosive environment and in contact with body fluids for many decades. Chemical composition and mechanical stability should not change. Material should have stable interfacial to be anchored to cells or tissue. Among these general criteria there are also some special requirements for ophthalmological applications. When biomaterial is used inside the eye, it should be thin, below some tenths of micrometer. Retina is quite fragile and thicker materials might harm it in implantation. (Kim et al. 2009) Material should also be flexible and stiff. A rolled membrane is possible to implant into the eye and it returns to its original

shape. If material has low moisture absorption, it will not swell up and shape stays constant. (Eurell et al. 2006, Lu L. et al. 2007, Lu, J.T. et al. 2007) Biomaterials used in eye are often non-degradable which means that chemical or physical properties of material do not change in biological conditions. Polymethylmethacrylate (PMMA) and silicone acrylate are examples of non-degradable biomaterials. These materials are used in contact lenses. Degradable materials like collagen and hyaluronic acid area instead degradable biomaterials. (Uusitalo 2004)

Biomaterials are widely used in ophthalmological applications. Contact lenses and implantable lenses are widely used. There are also implants for patients who suffer from destroyed cornea or glaucoma. Synthetic cornea implant made of hydrogel can suitable if patient has unexceptional high risk for rejection. Among these mainly transplantable devices there are also some new applications like drug releasing and tissue engineering applications. Drug release devices used in eye would help to allocate medication. Antibiotics or growth factors could be released controlled. Biomaterials can also be used in gene transplantation or as a cell substrate. These applications are not yet in everyday use but biomaterials seem to be very promising. (Uusitalo 2004)

4.2 Natural biomaterials

Natural biomaterials can be taken from human or animal bone, cartilage, lung or skin. They can be for example hyaluronic acid or collagens which are found from human body. Transplanted auto- and allografts like skin grafts can also be classified as biomaterials. Chitosan and silk protein are examples of commonly known biomaterials taken of animal origin.

Collagen materials are widely used in medicine and dentistry. Collagen's properties like biodegradation, biocompatibility and availability make it an interesting material. Being a protein collagen is difficult to sterilize without causing changes in its structure. The major advantage of collagens is that as natural components of ECM collagens can provide a natural substrate for cell attachment, proliferation and differentiation. (Parenteaus-Bareil et al. 2010) Lu et al. prepared a thin collagen film for RPE culture. Their film mimicked Bruch's membrane with its flow of nutrients and diffusion properties. RPE cells showed to form an epithelial phenotype and they were able to phagocytise photoreceptor outer segments. (Lu et al. 2006)

Chitosan is obtained by deacetylation of chitin, found in shellfish. Chitosan is biocompatible and it shows antibacterial and wound healing activities. Chitosan has been used in drug delivery applications in the studies of ophthalmology. Even though there are numerous studies done about the benefits of chitosan in delivering drugs onto the eye surface, there are not many reports about intraocular use of chitosan. Lai et al. have reported results of interaction between RPE cells and chitosan. Chitosan samples were treated either with genipin or glutaraldehyde. Research group found that ARPE-19 cells cultured in glutaraldehyde chitosan membranes had notable higher cytotoxicity and interleukin-6 levels compared to those ones which were not exposed to glutaraldehyde.

Genipin treated membranes were cytocompatible and could be a potential application as delivery carriers. They were also compatible with human RPE cells. (Lai et al. 2010)

Silk protein is collected from silk moth's chrysalis. Use of ultra-thin fibroin membrane made of the silk protein has been tested at the Queensland University of Technology, Australia. Researchers prepared membranes using highly polished casting table coated with cyclic olefin copolymer Topas® and 1:0.03 aqueous solution of fibroin and polyethylene oxide. Primary culture of RPE cells going to first passage was done on membranes with an average thickness of 3 µm and pore-like structures diameter 3-5 µm. Cells were grown six weeks in culture medium with 1 % fetal bovine serum. Comparative culture of ARPE-19 cells was done on 1.0 µm pore polyethylene (PET) membrane (Millipore). Results showed that the primary culture of RPE cells contained pigmented epithelial cells but also mesenchymal cells. In both cultures cells attached equally on membrane and later cells showed also a cobblestoned morphology. More studies to determine the degree of membrane permeability and RPE polarity are required. (Harkin et al. 2010)

Among materials mentioned above also several others have been used but there is not so much information available of comparative studies. One this kind of study, where biomaterial and synthetic substrates were used in parallel, was done in Opthalmology Group of REGEA 2011. Olli Kurkela showed in his study that natural materials collagens type I and type IV suited well for RPE cell substrate and cells showed good attachment and proliferation. Chitosan instead showed poor performance and Kurkela does not recommend it in future research. Xeno-free synthetic materials (poly(D,L-lactide) (96:4), poly(D,L-lactide-*co*-glycolic acid (75:25), poly(L-lactic acid-*co*-ε-caprolactone) (70:30)) demonstrated low potential as RPE cell substrate, poor cell survival and weak attachment. Despite that natural materials showed good results, they are problematic because of their ill-defined structure which might cause alterations in physiological conditions. Xeno-free synthetic materials are instead well-defined and might be safer to use in body. (Kurkela 2011)

4.3 Synthetic biomaterials

Synthetic biomaterials are mainly metals, ceramics or polymers. Also combinations of these materials, composites, can be used. Typical materials are hydroxy apatite, tricalcium phosphate and bioactive glass. Polylactide acid is an example of natural biomaterial which is processed to synthetic biomaterial polylactid. (Kellomäki & Paakinaho 2011) Case examples from literature show that biomaterials used with RPE cells are mainly synthetic polymers like polyethylene terephtalate and polyimide.

Polyethylene terephtalate (PET) is commonly used and trusted thermoplastic polyester. It is hard, stiff, strong and absorbs only very little water. It has good chemical resistance except alkalis which hydrolyse it. PET is very inert material and resistant to attack by micro-organisms which is an advantage especially in pharmaceutical and food industry packing. Crystallinity of PET varies from amorphous to high crystalline. Typi-

cal applications for PET are bottles, containers and packaging applications. (AZoM™ 2014a, PET Resin Association 2014)

In tissue engineering and medical applications PET is often used in 2D or 3D matrices. For example human mesenchymal stem cells have been cultured on PET scaffold with good success (Cao et al. 2010). PET is also used as a cell culture membrane. PET is normally transparent which is an advantage with phase contrast microscope used in cell cultures. This property is important especially in RPE cell culture because RPE cells pigment increases when getting older. (Savolainen et al. 2011)

Polyimide (PI) is a polymer which consists of imide monomers. This aliphatic polymer has great heat resistance and high wear resistance. Polyimide is quite expensive and it is not as widely used as PET. PI tolerates weak acids but not alkalis and hydrolysis. PI is used for example as a coating in electrical components and as a film in cable insulation and capacitors. It is biocompatible and flexible an can be turn to its original shape after implantation. (AZoM™ 2014b, Kim et al. 2004) Pores in PI allow oxygen and nutrients transport between the outer retina and choroid. PI is tolerated well in the subretinal space and living cells penetrated to the porous membranes might help mechanical anchoring of the implant. (Julien et al. 2011)These properties are essential in experiments of RPE cell transplantation.

Subrizi et al. have studied how polyimide membranes suit for pluripotent stem cell derived RPE cell substrate. They chose PI for a substrate material because it is clinically approved depending on its structure and its studied suitability to subretinal space. Researchers compared cell growth and attachment on uncoated PI membrane to coated membranes. Membranes coated with synthetic laminin peptide, heparin sulphate and hyaluronic acid were not much better than PI without coating. On the best coatings, laminins from human and mouse placenta, collagens type I and IV, CELLStart™ and MaxGel™, RPE cells pigmented well and showed cuboidal morphology. Group concluded that PI biomembrane is a suitable scaffold material for RPE tissue engineering. (Subrizi et al. 2012)

5 CELL ANALYSIS METHODS

Cells are analysed and characterized for several reasons. First it helps to prove original specie and original tissue. Second it enables to determine differentiation potential of cells and identification of cell lines. Third reason for cell characterisation is to rule out contaminations and genetic instability of cell lines. (Freshney 2005) This chapter presents shortly cell analysis methods that are typically used to estimate viability, purity and progress of RPE cells. Mainly used microscopes and immunohistochemical methods are explained shortly. Also two methods for analysis of electrical properties of a cell are presented.

5.1 Microscopes

Light microscope or compound microscope is the most commonly used microscope. It contains several lenses that magnify the image of a studied specimen. Light microscope can produce a multiple magnification but even more important is the resolution which is the ability to distinguish between two very closely positioned objects. Specimens for light microscopy are commonly fixed with solution containing formaldehyde or alcohol both denaturing most proteins and nucleic acids. Since cells include particles just a bit larger than the resolution of the light microscope (~0.2 µm), in theory all particles should be seen. In practice most cellular constituents are not coloured and they are hard to distinguish when they absorb about the same degree of visible light. For that reason specimens are stained to visualize the cell or tissue. (Lodish et al. 2000)

Fluorescence microscope is more versatile and powerful technique for localizing proteins. It is like a light microscope but the origin of the light in fluorescence microscope is ultra violet lamp. Specimen is stained with chemical to absorb one wavelength of light and to emit specific and longer wavelength. Rays of light are directed through a filter which permeates only specific wavelength of light. Parts, where light has been emitted can be seen strong coloured and they form a contrast to dark background. When two or three fluorescence dyes are used, multiple proteins can be localized within a cell. The resolution of fluorescence microscope is on the same level as in the light microscope. (Lodish et al. 2000)

Scanning electron microscope (SEM) uses electron beam instead of light. It allows viewing the surfaces of unsectioned specimens. The sample is fixed, dried and coated with a layer of a heavy metal like platinum by evaporation in a vacuum. An intense electron beam inside the microscope scans over the sample. Molecules in the specimen are excited and release secondary electrons that focus onto a scintillation detector. The resulting signal is then displayed on a cathode-ray tube. The scanning electron mi-

crograph has a three dimensional appearance because secondary electrons are scattered from the surface of the sample. The resolution of SEM is about 10 nm. (Lodish et al 2000)

Transmission electron microscopy (TEM) is similar to light microscopy by its functional principles. The major difference is that there are electromagnetic lenses instead of optical lenses. Lenses focus to high electron beam instead of visible light. Electron beam is directed through the specimen. When electrons are absorbed by atoms in air, the entire tube is kept between the electron source and the viewing screen which is maintained under an ultrahigh vacuum. In typical electron microscope electrons have the properties of a wave with only wavelength 0.005 nm which is also a theoretical resolution. Under optimal conditions resolution can be 0.10 nm which is about 2000 times better than the best resolution of light microscopes. (Lodish et al. 2000)

Confocal microscopy is a developed version of fluorescent microscope and it gives information in detail of cell layer. It focuses in a single plane and visualizes it as a sharp cross-sectional image. A laser beam moves rapidly to different spots in the sample in xy layer and images from these spots are recorded by a video camera. Spots are stored in a computer and then sample or pinhole is moved and laser can again scan one xy level. When these levels are added together, a composite 3D image is formed on a computer screen. (Lodish et al. 2000)

5.2 Immunohistochemistry

Immunohistochemistry is used in cell analysis when an antigen needs to be visualized. Specific reagents cause antigen-antibody interaction and antigens are visualized by a marker such as fluorescent dye or radioactive element. Immunohistochemistry staining identifies proteins, enzymes and tissue structures. There are several staining methods in use and the choice of suitable one should be based on parameters like degree of sensitivity. Immunohistochemistry is widely used in medical research laboratories and in clinical diagnostics. (IHC World 2014)

Immunohistochemically stained sample is observed with microscope. Commonly used stains are rhodamine and Texas red which emit red light, Cy3 which emits orange light and fluorescein which emits green light. These dyes can be chemically coupled to purified antibodies to be specific for almost any macromolecule. Coloured structures can be seen with the right filter in microscope. (Lodish et al. 2000)

Tissue sample has to be prepared before microscoping. First sample is fixed which means that sample is chemically treated to prevent decay. Aldehydes like paraformaldehyde are commonly used as a fixative. Small samples (< 5 mm thick) can be processed as whole mounts without sectioning. Results give 3D information and reconstruction is not needed like with sectioned samples. To make sure that antigens have stained sample completely, Triton X-100 or saponin is used to enhance penetration. Thicker samples have to be first deparaffined and then rehydrated before using the primary antibody. (IHC World 2014)

Sample can be stained directly or indirectly. Procedure of direct method is short and quick but it can be used only for one antibody at a time. Labeled antibody reacts with the antigen which is seen with a light microscope. For example trypan blue staining is done in direct method. In indirect methods an unlabeled primary antibody (first layer) reacts with tissue antigen and a labeled secondary antibody (second layer) reacts with primary antibody. Different antigens can be used to stain antibodies in the first layer when the second layer has once been stained. The second layer antibody can be labelled with a fluorescent dye rhodamine or Texas red. In addition to these two general methods there are also several developed methods like Avidin-Biotin Complex Method and Labeled SterptAvidin Biotin Method. (IHC World 2014)

Often it is useful to stain two or more antigens in one sample at the same time. This is possible with immunofluoresence method when using different fluorescent dyes. Three basic methods are used for multiple staining: parallel, sequential and adjacent. It is important to use suitable colour combination because improper combination can cause poor results and fail to demonstrate multiple antigens in the same sample. For example live/dead dyeing is an example of multiple labeling. In that method two kits are used to colour live cells to green and dead cells to red. (IHC World 2014)

5.3 Electrical properties of cells

Electrical properties of cells and tissues are a combination of insulators and conductors. Extra cellular matrix and cytoplasm are good conductors because ions have dissolved into them. Solid particles like macromolecules are more like insulators. These properties have an effect on how induced electric current flows in cells. (Jokela & Nyberg 2006). Measurement of transepithelial resistance and electrical impedance spectrum are examples of cell analysis type which is not so widely used.

Transepithelial resistance (TER) is measured to get information of cell monolayer health, homogeneity and confluence. High resistance means that cells are able to resist electric current that goes through them; they have good epithelia barrier properties and tight junctions between cells. If resistance is low cells or cell junctions might be harmed and there might be discontinuity on cell layer. TER measurement is quick, easy and inexpensive but it has some notable drawbacks. TER is often measured with handheld devices and when geometry between electrodes and the cell culture inserts vary between measurements, can results be inaccurate. (Millipore 2014, Onnela et al. 2011)

Electrical impedance spectrum (EIS) combines both TER and capacitive properties of cell layer. Different cell types and structures have different impedance responses. It has been speculated that EIS could be used to analyze and monitor cell differentiation and growth. Cells do not have to be fixed before measurement and they can be used after measurement. For the present EIS is not in as wide use as TER is. (Onnela et al. 2011)

6 REQUIREMENTS FOR TRANSPORTATION

Cells and tissues are not equal when their tolerances against changes in the surrounding environment are compared. It can be generalized that if tissue has complicated functions, it does not survive long time in storage or in transportation. A heart is an example of this kind of tissue or organism; a whole heart can be stored in hypothermal conditions only for 3-4 hours before transplantation but a part of it, a heart valve, can be stored in a tissue bank for multiple times. (SYKE ry 2009) Kidneys can survive after 24 hours transportation on ice (Barry et al. 1981) and egg cells can survive in nitrogen liquid from up to 20 years. (Riggs et al. 2010). Mentioned tissue examples give some idea about those factors that are crucial in tissue transportation; temperature, time and chemical environment. Those factors are presented in this chapter concentrating on RPE cells and their requirements.

6.1 Directives and statutes

Directives and statutes according to transportation of biological substances vary a lot between countries and areas. In European Union there are no customs inspections in country boarders which makes transportation quite easy for example between fertility clinics and sperm banks. In some countries like in the United States biological substances are observed more strictly and there are more agencies regulating shipping biological substances. (European Union 2014, Division of Research Safety 2014) For example customs inspections can take more than one day which increases notably transportation time and might be crucial for cells.

Most European countries including Finland and Germany have committed to comply with European Agreement concerning the international Carriage of Dangerous Goods by Road. Transportation company which carries dangerous goods has to take this agreement account but it does not affect significantly to the sender of the package. More important is to know statutes of air transportation. (Trafi 2014, UNECE 2014).

International Air Transport Association Dangerous Goods Regulations (IATA-DGR) which regulates international air transportation. According to IATA-DGR regulations transportation package needs to be marked with UN 3373 mark which is needed when transporting biological substances from Category B. Category B includes infectious substances which do not meet the criteria for inclusion in Category A, for example RPE cells. According to IATA Category A includes “An infectious substance which is transported in a form that, when exposure to it occurs, is capable of causing permanent disability, life-threatening or fatal disease in otherwise healthy humans or animals.”

(International Air Transport Association 2011 & 2013) Categories are presented in more detail in Table 1.

Table 1. *IATA-DGR categories for biological substances (International Air Transport Association 2011 & 2013)*

	Category A		**Category B**
Definition	Infectious substance affecting humans	Infectious substances affecting animals	Infectious substances which do not meet the criteria for inclusion in Category A
Marking	UN 2814	UN 2800	UN 3373
Examples	Ebola virus Poliovirus	African swine fever virus Foot and mouth disease virus	Food and water samples without a significant risk of infection Patient specimens when there is minimal likelihood of pathogens

IATA produces instructions for packing: the packaging has to be physically durable enough to tolerate shocks and vibration during transportation, loading or storage. Packaging has to be closed so well that content cannot issue even if there are changes in environments temperature, humidity or pressure. The packaging has to consist of three parts: primary packing protects sample and cannot break, be punctured or leak in normal conditions. Primary packing is packed inside a secondary packing which protects primary packing. Outer packing covers secondary packing and is marked with diamond-shaped square with marking "UN3373" with adjacent text "Biological Substance, Category B". An itemized list of content has to pack between the secondary and the outer packaging. (International Air Transport Association 2011 & 2013) An example of packing according to these directions is presented in Figure 3.

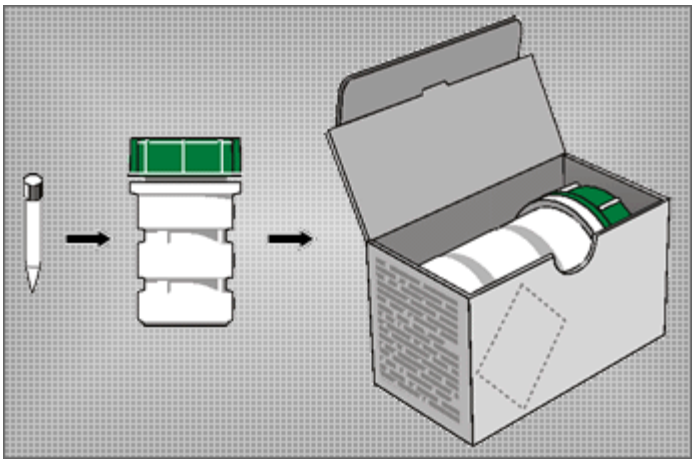


Figure 3. *Packaging for infectious substances according to IATA/DGR packing instruction. (Noax Sys AB 2013)*

In addition to instructions mentioned above there are also specific instructions for liquid substances which have to be taken into account in this thesis work. The primary packaging must be leak proof and volume at most 1 litre. Absorbent material like cotton wool or pulp paper has to be placed between the primary and the secondary packing to absorb the liquid substances if it releases. The primary or the secondary packaging has to be capable of defensive without leakage when the internal pressure is 95 kPa and temperature is between -40 °C and 55 °C. (International Air Transport Association 2013) Temperature scale is wide and even though packaging can survive, there are very few substances which would not damage if temperature changes dozens degrees.

The newest edition of IATA-Dangerous Goods Regulations is effective from 1 January to 31 December 2014. There are some changes between this 55th edition and earlier 54th edition but according to authors knowledge those changes do not touch transportation of category B substances.

6.2 Temperature

Cells and tissues are known to be stored and transported mainly in one of three temperatures: hypothermal (2...8 °C), room temperature (20 °C) and body temperature (37 °C). (Barry et al. 1981, Nichols et al. 2010) Also deep-frozen cells have been transported a lot during last decades for example from sperm banks to infertility treatment clinics. If cells are once frozen and melted like stored sex cells, a new cycle of freezing and melting might be critical for the cells. Freezing is justified if cells have to be stored or transported for long but in short transportations it causes too much stress for cells. (Riggs et al. 2010, Tiitinen and Hydén-Granskog 1998)

Cells are stored in laboratories mostly at body temperature which could be also possible for transportation when using wheel transportation. In airplane transportation body temperature would be difficult to organize. Heating or cooling gel elements lose their warmth quite soon and do not get energy continuously. If a transportation box would be electrically warmed and there would not be any breaks in current, then body

temperature might be possible. In practice it would work best in wheel transportation but not in an airplane. It is also notable that the risk for temperature changes inside a package is higher when there is difference between outdoor temperature and target temperature. This risk is naturally higher in winter than in summer. (Salonen 2013)

Hypothermal storage and transportation is common among tissue banks. Several tissues like kidneys and corneas are stored at 4 °C. It is important that temperature stays constant. If temperature lowers under 2 °C, water crystals might form inside cells and cell membrane might harm because of expansion. If temperature rises over 6 °C, there might be anaerobic metabolism which causes membrane ionic imbalance, edema and cytolysis. Also too slow metabolism caused by low temperature can cause cell damage and cell death. These disadvantages stand out especially when tissue or cells are stored long time and hypothermal storage is not recommended for long-term storage. Red blood cells are an exception. They can be stored at 4 °C for up to 42 days. (Acker 2006, Net at al. 2003)

Transportation at body temperature suits for some tissues better than for others. When grafts are often kept in hypothermal conditions, are stem cell sources like embryos stored in 37 °C. Also their transportation might be justifiable to do at body temperature. A preliminary study of long-distance transportation has been done at University of Puerto Rico. Rhesus monkey embryos produced in IVF were shipped in tubes from Puerto Rico to Wayne State University in Michigan as airmail. Earlier monkey embryos had been shipped from Puerto Rico to Michigan cryopreserved but now researchers were interested in embryo transportation in 37 °C. Reason for testing body temperature was that cryopreservation can damage embryos or affect their functional properties. Embryos were transported 2-4 days after insemination. Embryos were placed to 5 ml tubes with culture medium which had been pre-equilibrated with 5% CO_2 and 5 % O_2 in air and kept at 37.5 °C in incubator. Tubes were sealed with parafilm to avoid gas leakage. Then tubes were packed into the transportation incubator which kept temperature and gas conditions constant during whole 18 hours transportation. Of 11 embryos nine were in satisfactory condition and two embryos seemed to have intrinsic faults instead of caused by transportation. Researchers evaluated that this kind of transportation method could be used also in transportation between continents. (Nichols et al. 2010)

Transportation in body temperature can suit for embryos but not as well for RPE cells. A new study about optimized storage temperatures for RPE cells was published in September 2013. Pasovic et al. studied which temperature or temperatures are the most suitable for adult RPE cell line ARPE-19. Cells were cultured under controlled conditions and stored in Dulbecco's Modified Eagle's (DMEM) Serum buffered with HEPES (trypsin-EDTA, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Cells were stored seven days at nine temperatures (4 °C, 8 °C, 12 °C, 16 °C, 20 °C, 24 °C, 28 °C, 32 °C and 37 °C) and analysed then with microplate fluorometer, epifluoresence microscopy and scanning electron microscopy. Together findings showed that storage at 12 °C, 16 °C and 20 °C is optimal for maintenance of RPE cell viability, phenotype and morphology. (Pasovic et al. 2013)

As examples above show, transportation of cells or tissues can be done in many temperatures depending on tissue or cell type, transportation time and use after transportation. Stabile body temperature is often the most suitable temperature for cells but also the most challenging to organize. Transportation temperature is often a compromise between existent resources and cell viability.

6.3 Transportation medium

Among transportation temperature and used time transportation medium has a significant effect on cell viability after transportation. Transportation medium should have same kind of properties as culture medium where cells are normally stored. In BioMediTech Opthalmology laboratory RPE cells are cultured in retinal pigment epithelium medium (RPE DM-). It consists of Knockout™ D-MEM medium (Gibco, Invitrogen), added 15 % Knockout™ Serum Replacement (Gibco, Invitrogen), 2 mM Glutamax™-1 Supplement (Gibco, Invitrogen), 1 % MEM Non Essential Amino Acids (Lonza), 0,5 % Penicillin/Streptomyocin (Lonza) and 0.1 mM 2-mercaptoethanol (Gibco, Invitrogen). Fresh medium is changed three times a week. Medium can be stored at 4 °C for one week. (Savolainen et al. 2010, Vaajasaari et al. 2011)

Among DM- RPE cells might be cultured also in Hibernate A (Gibco). Hibernate A is a nutrient medium for the maintenance of adult neural cells, tissue and tissue slices. Neural tissue can be stored in Hibernate A up to one month at 2-8 °C. Neural cells are known to be highly demanding for storage medium and it can be presumed that if neurons survive well in Hibernate A, it suits also for RPE cells. There is also a sister product Hibernate E, which is meant for embryonic tissue. The only difference between these mediums is that Hibernate A has higher osmolality (280 mOsm/kg vs. 240 mOsm/kg) which suits for highly differentiated cells. RPE cells used in this study are differentiated so Hibernate A would be better choice of these two mediums. (Gibco 2010)

Hibernate A had been used widely in mice and rat tests and tissue storage. It is often used with B27 supplement (Gibco) which is a serum-free supplement. Kivell et al. (2001) used this combination when they storage 3 and 6 days old rat kits' brain. They found that storage in Hibernate A improves later cell culture and brains can be stored in that medium even three days. Also Vieira et al. have used Hibernate A and B27. They piloted a protocol which is linked to mice spiral ganglia neurons viability. Earlier they had used always some serum but now they succeeded to store neurons several days without any serum. (Vieira et al. 2007)

Gregory J. Brewer and his colleagues have done also experiments with Hibernate A and human neural tissue. In addition to B27 they used also 0.5 mM glutamine with Hibernate A and called this combination as Neuregen. 2001 they stored 1-3 mm thick samples cut in brain surgery operation in Neuregen at 4 °C for four hours which was the transportation time between operating theatre and culture laboratory. Brewer et al. 2001) In year 2007 they had same kind of samples which were transported overnight

in polystyrene tube at 4 °C. At destination samples were stored 1-3 days at 4 °C before cell culture. (Brewer et al. 2007) In both experiments cells kept their viability quite well.

6.4 Transportation time

Transportation time depends on the requirements of the tissue or cells, mainly target temperature and the source of heat or cold. Most transplantable organs are moved from the donor to the receiver in some hours. If transportation time is longer, viability can decrease and organ is not anymore transplantable. Some cell types like egg cells instead do not care about the transportation or storage time, if temperature and pH do not change much. They can be stored in liquid nitrogen even for several years.

Living cells that are transported for research subjects are less demanding than transplantable organs. For most of the cell types is true that if cells are already once melted, they cannot be transported frozen. In over 0 °C environment cells are more or less active and they need nutritious from environment. Cells are often stored in storage medium which is viable only for some days. If transportation takes longer, medium should be changed during the transportation.

Temperature and source of it is often more notable factor in transportation than medium viability. In some cases like in wheel transportation it is possible to control temperature inside transportation box electrically. If temperature is not kept constant with an electrical source, it often depends on the cold or the heat elements. Transportation time correlates with the elements' capability to emit or absorb heat. If transportation continues longer than planned, tissue might chill or warm too much and viability can decrease crucially. In case that there are some changing factors in a new transportation route, it might be reasonable to divide samples or tissues into two or more freights to make sure that at least some of the samples can survive. When transportation is well planned and transportation time can be kept in tolerance, this kind of secured system is not needed.

6.5 Mechanical stress in transportation

Cells will be stressed during transportation because of several types of simulations. There will be vibrations caused by roughness of the road and shock vibrations caused by lumps on the road in wheel transportation. There might be also some resonant vibrations in vehicle's boot caused by car or package. In airplane there are changes in air pressure and resonant vibration is also possible. Especially if Falcon tubes are packed horizontally there is also fluid slashing against inserts. It is difficult to know exactly what kind of mechanical stress is crucial to RPE cells. (Salonen 2013)

There are not so many studies made of damages in cells caused by mechanical stress. Some information can be found of corneas behaviour in transportation. It is not directly applicable but the field is studied widely because of international eye banks. In

University of Bern, Switzerland Halberstadt et al. studied impact of transportation to pigs' corneas. 320 corneas were stored in four different mediums 1, 3, 6 or 10 days. Transportation was simulated in laboratory after a short storage. Corneas were put in mediums on a laboratory shaker at 4 °C and accelerated from 0 to 100 km/h in 16 seconds and this was repeated five hours. Corneal endothelial cell density was defined before and after transportation. Pair of each cornea pair was used as a control. No significant cell loss was observed in any experimental group. Group made a conclusion that corneas can be routinely used after short-term storage without a re-evaluation. (Halberstadt et al. 2000)

Three years earlier researchers Wang and Hu had got opposite results from a bit different study. They divided 36 dog corneal buttons into two groups: in group I buttons were stored in Dexsol preservative medium at 4 °C. In group II buttons were put also in Dexsol and shaken in a shaking incubator 10 h at 4 °C at a speed of 5 rpm. After shaking buttons were stored in a fridge. Three buttons from each group were studied on days 0, 1, 3, 5, 7 and 9 with specular microscopy, scanning electrical microscopy (SEM) and alizarin red with trypan blue stain. There were no significant changes in group I but damages in group II were notable. Stormal edema was seen under SEM at day 5. Researchers concluded that vigorous shaking should be avoided during transport of corneal buttons. (Wang & Hu, 1997)

Those studies mentioned above were done with corneas and results cannot directly be utilized with RPE cells but studies point that probably cells are not as sensitive to all mechanical stresses. According to author's knowledge there are no studies of RPE cells and mechanical stress. That is why studies made with other cell types have to be reviewed a bit before discussing RPE cell transportation.

Nikolaev et al. (2010) have studied in effects of dynamic mechanical forces to cell suspensions in cold transportation. They found that effect of transportation vibrations is a crucial factor for cell viability in transportation. In three different tests they showed that human dermal fibroblast cells endured well vibration caused by 20 ms half sine shock pulses at 1 Hz but when vibration was random only 0.1 % of the initial cells survived. These experiments were done at 4 °C.

Same research group tested two years later how human mesenchymal stem cells (hMSCs) survive vibration. They showed that vibration made no effects to hMSCs suspension at 10 Hz vibration. At 25 Hz cells were sensitive to vibration and at 50 Hz there were only moderate effects. Vibration was continued 24 hours or 48 hours depending on the test group. The study showed also that cell viability decreases in cold storage especially after 48 hours. This result among those ones mentioned in section 6.1 encourages testing warm storage transportation side by side with cold storage transportation. (Nikolaev et al. 2012)

Four examples above show that a mechanical stress can be crucial or insignificant to cells depending on the sort of stress. It is hard to foretell what kind of acceleration, vibration or other type of stress cells will be exposed to during the transportation. When planning any kind of transportation for cells it is important to keep these results

in mind even though it would not be possible to demonstrate them in laboratory conditions.

EXPERIMENTAL PART

7 MATERIALS AND METHODS

The experimental part of this thesis work consisted of three demonstrated transportations, their preparation and pretesting and cell analysis before and after transportation. Used materials and methods are presented in this chapter.

7.1 Cells

hESC lines Regea 08/017 and Regea 08/023 are established in Regea Institute for Regenerative Medicine (known now as BioMediTech) in University of Tampere. Regea has an approval of the National Authority in Medicolegal Affairs Finland to study human embryos (Dnro1426/32/300/05). There is also statement of the Ethics Committee of Pirkanmaa Hospital District to derivate, characterize and differentiate hESC lines from surplus human embryos.

Pluripotent stem cell lines were adapted to culture on mitotically inactivated human foreskin fibroblast (hFF) feeder cells (CRL-2429). Undifferentiated stem cell were maintained in a culture medium consisting of Knock-Out Dulbecco's Modified Eagle Medium (KO-DMEM) and supplemented with 20 % Knock-Out Serum Replacement (KO-SR), 2 mM GlutaMax-I, 0.1 mM 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA, USA), 1 % non-essential amino acids (NEAA), 50 U/ml penicillin/streptomycin (both from Lonza Group Ltd, Basel, Switzerland) and 8 ng/ml human bFGF (PeproTech, Rocky Hill, NJ, USA). The culture medium was changed six times a week. Undifferentiated colonies were manually passaged using TrypLE Select (Invitrogen) onto fresh feeder cell layer at ten-day intervals.

Cells had been kept in RPE differentiation medium (DM-) since beginning of year 2013. Cells from cell line Regea 08/017 going to passage 35_9+3 had been plated into five wells of 24-well plate in April 2013. They were kept at 37 °C and fresh medium was changed three times per week. On October 2013 cells were plated again. Wells were washed twice with Phosphate Buffered Saline (PBS) to wash the medium away. 300 µl of Tryple Select (Gibco, Invitrogen) was added into each well and incubated 15 minutes at 37 °C. After that cell suspension was strained through 40 µm strainer and 7 ml of DM- was added. Cells were centrifuged with Heraus® Biofuge® Primo (Kendro, Germany) at 1500 rpm for five minutes. Then cells were resuspended into 1 ml of DM-. Cells were counted with Neubauer Improved hemocytometer and result was 3.25 x 10^6 cells/ml.

Cells were plated to polyethylene terephtalate (PET) membrane on Millicell Hanging Cell Culture inserts (Millipore) and to 24 µm thick polyimide (PI) membranes (it4ip CellCulture™) which were on CellCrown™24 (Scaffdex) inserts. 1700 µl of DM- was added to 210 µl of cell suspension. 100 µl of this dilution was added to eight PI and eight PET membranes which means more than 40 000 cells per insert. Rest cell suspen-

sion (799 µl) was added to 8 ml of DM- and this dilution was put on 16 wells of 24-well plate. Cell density of wells was around 152 000 cells per well. These inserts and cell well plate was kept in 37 °C and fed with fresh DM- three times per week until demonstrated transportation. From this cell line Regea 08/017 cells were used also for transportation medium testing but cells were in passage 60+5.

Later in October 2013 more cells were plated. There were no cells obtainable from the same cell line as earlier. The determining thing in cell chooses was the passage of the cells. From cell line Regea 08/023 there were cells going to passage 39_3+3 and they were plated according to the protocol explained earlier to three 7 µm thick PI membranes with CellCrown^{TM}48 and eight 24 µm thick PI membranes with CellCrown^{TM}24. All these cell treatments were done by laboratory technicians. They also took care of medium change three times per week before demonstrated transportations

7.2 Membranes and inserts

Two insert types and three membranes were used in this study. Thicker PI membrane is known to be suitable for RPE cell culture (Subrizi et al. 2012) and the target is to use thinner PI when cells are transplanted into rabbits. Polyethylene terephtalate is also a suitable material. Ophtalmology group has used it routinely and it is used as a comparable material. Dimensions and properties of membranes are summarized and compared in the end of this chapter.

7.2.1 Polyethylene terephtalate and Millicell® Insert

Polyethylene terephtalate (PET) membrane was used with Millicell Hanging Cell Culture Insert. Insert's unique structure with designed flanges suits for many cell types. Hanging insert enables that cells can access media from both sides of monolayer. According to manufacturer cell growth, structure and function mimic closely *in vivo* conditions. (Millipore^{TM} 2009a) Structure of Millicell Insert is presented in Figure 4.

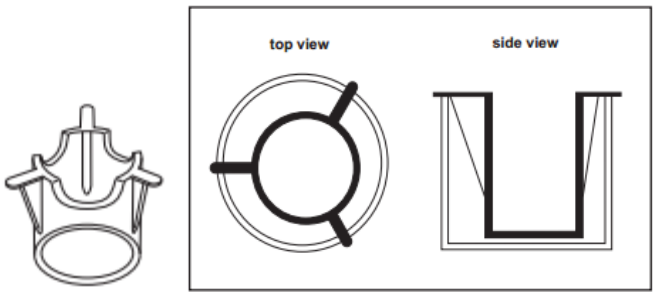


Figure 4. *Two scematic illustrations of Millicell Hanging Cell Culture Insert. (Modified from the source Millipore^{TM} 2009a)*

Membrane was placed into the insert industrially instead of hand work so membranes are identical. Because membrane is put into the insert beforehand, inserts are single-use. Membrane is 11 µm thick and colourless which is an advantage especially when imag-

ing with light microscope. Pore size is 1.0 µm. Figure 5 shows insert without cells or medium.

Figure 5. *Millicell 24 well Hanging Cell Culture Insert. (Merck Millipore 2014)*

Millicell inserts are made of polystyrene and they suit for 24 well plate. Even though the diameter of the bottom of the insert is only 6.5 mm, the barbs on the upper part of the insert come wider and the insert does not fit into a 15 ml Falcon tube.

7.2.2 Polyimide and CellCrown™

It4ip CellCulture™ polyimide membrane is used with Scaffdex CellCrown™ insert. It consists of ring part and body part which can be seen in Figure 6. Inserts are made of medical grade polycarbonate polymer. Manufacturer offers five different inserts and two of those, CellCrown™24 and CellCrown™48, are used in this work. Inserts are versatile. (Scaffdex, 2013)

Figure 6*. Scaffdex CellCrownTM[24] body part and ring part. (Scaffdex, 2013)*

Two thicknesses, 24 µm and 7 µm, of PI membrane were used. Most of the experiments in this work were done with thicker membrane because it is much cheaper than thinner one and suits for experimental use. Thinner membrane will be used when RPE cells are transplanted to rabbits during spring 2014. Thicker membrane is orange, thinner is more transparent. Pore size is 1.0 µm for both membranes. Thicker membrane is more difficult to photograph via microscope but otherwise there are not so many dif-

ferences between the two membranes. Thicker membranes inside Scaffdex CellCrown™ inserts are presented on 12-well plate in Figure 7.

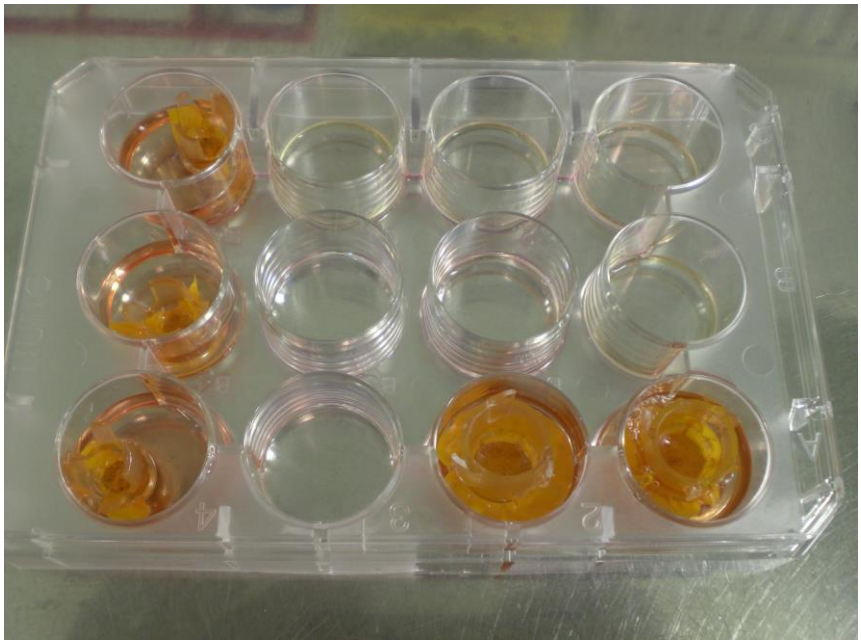


Figure 7. *On the left there are three Scaffdex CellCrown ™48 inserts (⌀ 7 mm). Two inserts on the right hand side are Scaffdex CellCrown™24 inserts (⌀ 10 mm). Number in the name of the insert stands for the size of well-plate where insert fits. Brown areas inside inserts are pigmented RPE cells.*

Figure 7 shows that PI membranes are not similarly inside inserts. Reason for that is that PI membrane is put one by one manually between the ring and the body of insert. Membrane is difficult to put smoothly between insert parts and there are more or less wrinkles on each membrane. For that reason it is a bit challenging to grow a constant cell laeyr compared to PET membranes which are all similarly inside the inserts.

7.2.3 Comparison of the properties membranes and inserts

All used membranes have the same pore and the pore density is also at the same grade. However those properties do not alone determine if cells are growing homogenously or not. When PI membrane is put into insert one by one by manually there are always some wrinkles which disturb the cell culture. For that reason thinner PI membrane is better because it is also more flexible which means probably less wrinkles. PET membranes' thickness is almost half of PI's thickness but because it is milled, it should have a smooth and constant surface. Table 2 summarizes the used membranes in this work and those properties which are most notable. Information is collected from manufacturers' website and from the packing of products used in this project.

Table 2. *Membranes and their properties.*

Membrane	Thickness (µm)	Pore size (µm)	Pore density (pores/cm^2)	Colour
PET	11	1.0	2 x 10^6	colourless
PI	24	1.0	2.27 x 10^7	orange
PI	7	1.0	2.27 x 10^7	light orange

Thicker PI membrane is used with CellCrown^{TM}24 insert which is larger than two other insert types. Thicker PI membrane has then the largest filtration area. Table 3 summarizes inserts used in this work and those properties which are most notable. Information is collected from manufacturers' website and from the packing of used products.

Table 3. *Inserts and their properties.*

Product Name	Insert material	Diameter (mm)	Filtration area (cm^2)	Sterility	Configuration
Millicell Cell Culture Insert	polystyrene	6.5	0.33	Sterile	24-well plate
CellCrown^{TM}24	polycarbonate	10.0	0.78	Non-sterile	24-well plate
CellCrown^{TM}48	polycarbonate	7.0	0.38	Non-sterile	48-well plate

Even though diameters of Millicell insert and smaller CellCrown are almost equal they are meant to be used with different cell well plate. Reason for that is the structure of Millicell inserts: barbs of the inserts upper part spread to large area and insert can hang over well of a 24-well plate. Both insert types are made of chemically inert material so there is no difference. Notable is that Millicell's insert is sterile but CellCrowns have to be sterilized before use.

7.3 Transportation company

In this project RPE cells have been planned to be carried from Tampere to Germany in an airplane. Transportation takes around 24 hours as an express delivery by air. Unusual circumstances like closed airport because of weather conditions might extend transportation time. Flight does not take many hours but wheel transportation from laboratory to

airport in Finland and from airport to laboratory with waiting between office hours increases total transportation time up to one day. Wheel transportation with car ferry over Baltic Sea is not possible because it requires a privet courier who takes care of the transportation box and transports it personally from door to door. Wheel transportation with a private courier would be more expensive than transportation in a normal air mail.

To find a suitable transportation company six companies were contacted by e-mail or by phone. Companies were Cool ID, World Courier, DHL, TNT, UPS and Biocair. All companies do international transportations. Companies were chosen according to recommendations of tissue coordinators of Regea Cell and Tissue Center. Also comments were asked from the European co-operator who will receive the RPE cells.

Companies were asked if they are able to transportation from Tampere to Germany, how long does it take and do they have facilities to do transportation at around 4 °C, in room temperature or in body temperature. All companies offer transportation at 4 °C. None of companies could guarantee that transportation at body temperature manages. Used heated or cooled gel elements might lose their heat capacity during an overnight transportation. Companies offered transportation by air; there were no comments about wheel transportation.

Costs of the transportation were also asked. Companies were not capable to give exact numbers beforehand because prize of air cargo varies every week. Some companies offered only transportation when other included also packaging into the prize. Prize was not a consequence element because transportation is needed only once, not repeatedly.

7.4 Medium test

According to Ophtalmology group's experience DM- is known to be a good culture medium for RPE cells. Cells are also transported in the same medium from Tampere to Helsinki on a wheel transportation. DM- has not been tested in an overnight transportation or in colder environment than 37 °C without constant CO_2 supply. For that reason it is good to take another medium for transportation experiment. Medium should be neutral and serum-free and suitable for demanding cell culture. Because other mediums have not been tested in Ophtalmology laboratory, the selection is based mainly on literature and it is Hibernate A.

Both mediums used in this project, DM- and Hibernate A, include phenol red which is a pH indicator. Disturbance in cell culture like overgrowth of contaminants causes often acidification of medium. It turns to yellow when pH is under 6.8. Instead alkaline by-products increase basicity and medium turns to pink when pH is over 7.4. Colour indicates when medium had to be change at the latest. Medium stays red as long as it is fine to be used.

Knockout^{TM} Serum Replacement (KO-SR) is used routinely with DM-. Hibernate is recommended to be used with B-27 Supplement. In this work it was replaced

with KO-SR to increase protein level and to make mediums similar. Also Penicillin/Streptomyocin was added to both mediums to minimize the risk of contamination. Content of used mediums are presented in Table 4.

Table 4. *Content of used cell culture mediums for 50 ml.*

Stock	**DM-**	**Hibernate A (ready to use)**
Cell culture medium	Knock-out DMEM 41,2 ml	Hibernate A (pure) 43,4 ml
Ko-SR	7,5 ml	6,5 ml
10 000 U/ml Pen-Strep	0,25 ml	0,26 ml
100 x GlutaMAX-I	0,5 ml	
100x NEAA	0,5 ml	
50 mM 2-Mercaptoethanol	0,1 ml	
Total	**50 ml**	**50 ml**

Hibernate A has not been used in BioMediTech Ophtalmology laboratory earlier so its suitability for RPE cells was tested before demonstrated transportations. Hibernate A is often used with B27 supplement (Gibco) which is a serum-free supplement developed for long-term culture of stem cells. There are also different versions of B27 supplement for special applications like electrophysiology studies and studies of insulin secretion or insulin receptors. In this experiment B27 was left out and replaced with KnockOut^{TM} Serum Replacement (Life Technologies) to increase protein level. It is used as much as in RPE DM- (15 %). Also antibiotics are added to prevent contamination as in DM-. Reason for leaving B27 is that it does not really add any benefit to Hibernate A in RPE cells in a short-term transportation. It is also good to keep Hibernate A and DM- as similar as possible to make it easier to compare mediums' influence to cells after transportation.

First 1.8 ml of KO-SR and 60 µl of P/S were diluted to 10 ml of Hibernate A to make it similar to DM-.Then cells were imaged with Nikon Eclipse TE-2000-S microscope (Nikon Instruments Inc.) and numbered according to medium and cell density. Numbering of wells is presented in Table 5. It was noticed that wells 1 and 2 have some area which did not had any cells. Wells 3 and 4 had more homogeneous cell layer and cells were lightly pigmented. After that cell culture medium (DM-) was taken away and fresh mediums (DM- and Hibernate A) were added. Mediums were put 2-3 ml to every well to make sure that all cells are engulfed. After that cell well plate was stored at 4 °C for 24 hours.

***Table 5**. Numbering of cell wells in the medium test.*

1. DM- + 60 000 cells/cm^2
2. Hibernate A + 60 000 cells/cm^2
3. DM- + 180 000 cells/cm^2
4. Hibernate A + 180 000 cells/cm^2

After cold storage wells were imaged again, now with Olympus IX 51 Fluoresence microscope (Olympus Europa Holding GMBH, Hamburg, Germany), it was noticed that cells in well 1 had been disengaged from each other and there were some dead cells floating in the medium. Also in well 2 there were some dead cells but cells in wells 3 and 4 looked viable. Wells 1 and 2 were chosen to be analyzed with live/dead method and wells 3 and 4 with phalloidin dyeing. Those analysing and dyeing methods are explained in more detail later in this thesis in chapters 6.5.3 and 6.5.5.

Cells in wells 1 and 2 were washed twice with PBS. Calcein AM and ethidium homidimer-1 (ETHD-1) were melted and spin in those eppendorfs where solutions had been frozen. 0.625 µl calcein-AM and 0.625 µl ETHD-1 were diluted into 5 ml PBS and mixed well. PBS was removed and solution was added over cells. Cells were incubated in dark 1 h at 4°C. After that wells were covered with circle cover glass (⌀19 mm) and imaged with Olympus IX 51 Fluorescence microscope. Green fluorescence light with wavelength 488 nm was used to detect live cells and red fluorescence light with wavelength 568 nm was used to detect dead cells.

Cells in wells 3 and 4 were washed three times with PBS on cell well plate and fixed in 4 % paraformaldehyde (PFA) for 10 minutes at RT. Then samples were washed three times with PBS and stored in 4 °C until imaged. After three days it was seen that in well 3 almost half of the well was empty and in well 4 there were no cells on the edges of the well. Instead there was a constant cell layer in the middle of the well.

Dyeing was continued and PBS was taken away from fixed cells and 0.1 % Triton® X-100 (4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol) (Sigma-Aldrich) was added. After 10 minutes cells were washed again three times. Then cells were incubated in 3 % bovine serum albumin-phosphate buffered saline (BSA-PBS) 1 h RT. During this blocking 1.66 µl of phalloidin was put into an eppendorf and vaporized in fume hood. After that phalloidin was diluted with 1 ml 0.5 % BSA-PBS and added to wells 3 and 4. Cells were incubated 15 minutes in RT in dark and after that washed three times with PBS. Vectashield Mounting with DAPI was added to cells and cover glasses were put over cells. Cells were imaged and phalloidin should be seen with light wavelength 568 nm (red fluorescence). Cell nuclei where DAPI should be seen when imaged with UV light.

7.5 Packing method

Inserts were packed into 50 ml Falcon tubes. Scaffdex inserts could also fit into 15 ml Falcon tube but this experiment was planned to be done with similar medium volumes to ensure that amount of medium does not have an effect on results. Both inserts had space to turn inside the tube. Tubes were packed into a polystyrene box with heated or cooled gel elements.

7.5.1 Transport box

Transportation box was covered with cardboard box. Outside measures of the transportation box were 41 x 29 x 32 cm. There were also needed markings “UN3373” and “Biological Substance, Category B” printed on the side of the box. On the gables of the box there were also arrows pointing which side is meant to be up. Transportation box is presented in Figure 8.

Figure 8. *The cardboard box with needed markings.*

Inside cardboard package is an expanded polystyrene (EPS) box measures of which are 34 x 20 x 22 cm. A possible referral is recommended to be taped over EPS box. EPS is insulating and hydrophobic material and it has good compression strength. These properties protect the product inside the box and keep the temperature quite stabile. Falcon tubes are put horizontally into transportation box inside EPS frame which is on the top of one gel element. EPS frame prevents tubes from moving and clatter. (Cool Id 2013b, Salonen 2013). Figure 9 presents the EPS frame over a gel element inside EPS box.

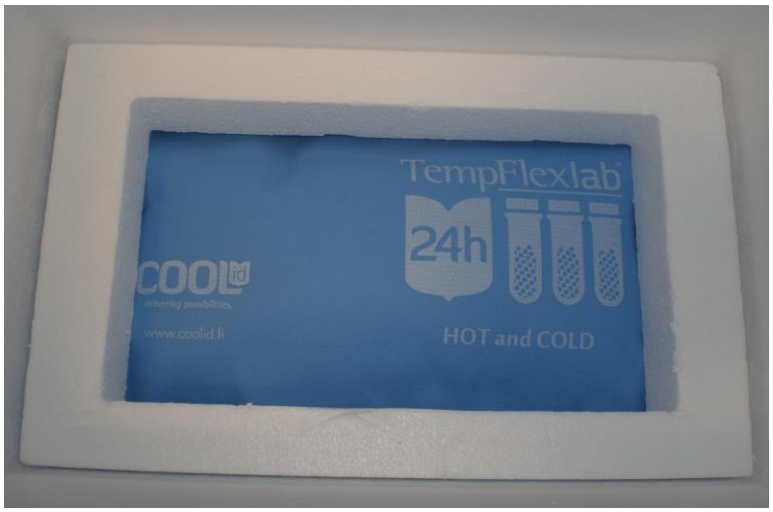


Figure 9. *The EPS frame and TempFlexLab gel element inside EPS box.*

Falcon tubes are wrapped in pulp paper which absorbs clatter and soak up transportation medium if it leaks. Tubes are packed into a plastic bag with glued seal which is necessary in airplane transportation. Volume of plastic bag is 1 litre which is according to IATA the maximum volume in air transportation.

7.5.2 Gel elements

TempFlexlab® gel elements are thermal storages for multiple uses. Gel elements include non-toxic and water-soluble gel which is packed into plastic bag. Bags are protected with elastic cladding. Cladding endures temperature from – 38 to 70 °C. Gel is chemically resistant also in – 80° and can be used several years. If elements are heated over 37 °C, the lifetime of elements decreases significantly. Gel elements are hard when frozen and soft when warm. They can be cooled in a fridge or a freezer and warmed in an incubator or a microwave oven. Frozen gel elements are hard and cannot be formed. Heated gel elements are soft and can be packed tightly around Falcon tubes so tubes will not clatter inside a package. (Cool ID 2013c, Salonen 2013)

7.6 Cell analysis before and after transportation

Cells and membranes can be studied with several methods. In this work the goal was to find analysing methods to be used not only in Tampere but also in Germany after transportation. Methods should be easy and quick. Tests should be reproducible and results should not depend on the operator. It would be good if results were unambiguous and comparable.

After the demonstrated transportation membranes were taken off inserts and cut into 4-5 pieces on PBS, one piece for each analysis or study. One piece of every sample was stored in DM- 24 hours in incubator which will happen also for real membranes in the laboratory of the co-operator. The goal was to let the cells rest before they are trans-

planted to rabbits. Next sections explain background of the used methods shortly. Also the practical realization is explained.

Even though a confocal microscope can be very informative tool for cell analyse, it did not have major role. When having large number of samples like in this work, a confocal microscope is not so useful. It has better accuracy than normal fluorescence microscope but it is slow to use and quite costly.

7.6.1 Light microscope

Light microscope was an important tool during this work and it was used before and after the transportation demonstration. Even though the resolution of light microscope is not very good, it was fine enough for the used cells. The aim was to observe morphology of RPE cells, pigmentation and cell layers homogeneity.

Cells were imaged before the transportation demonstration with Nikon Eclipse TE-2000-S microscope and after with Olympus IX 51 Fluorescence microscope. Reason for the change of microscope was that the further locates in the closed laboratory where cells are cultured and the latter in the open laboratory where the transportation demonstrations were done. A drawback with the Olympus microscope was that images taken with it were black-and-white instead of colour images.

7.6.2 pH

Mammalian cells survive best in pH 7.2-7.4 which is near the pH of blood. A drop in pH of the culture medium is often a sign of lactic acid which is a by-product of cellular metabolism. Lower pH can be unfavourable for the cell growth and lactic acid can be even toxic for the cells. Also carbon dioxide (CO_2) is formed in metabolic process. It can combine to hydrogen peroxide (H_2O_2) and produce carbonic acid and decrease the pH of the medium. If the cell culture medium has buffer capacity, small changes in pH are not immediately harmful for the cells. (Yang & Xiong 2012, Life Technologies 2014)

In this project pH was measured from medium used in the demonstrated transportation before and after transportation. Also pH of pure mediums without inserts was measured. Inserts were around half an hour in Falcon tubes before first measurement. After the transportation pH was measured before other tests. Temperature of the mediums was room temperature which varied from 20 to 22 °C. pH was measured with pH-Conductivity Meter (Denver Instrument, New York, USA). Meter was calibrated before every measurement.

7.6.3 Transepithelial resistance

Transepithelial resistance (TER) can be measured either from insert or clipping. Cells come to a room temperature before measurement. Measurement should be repeated couple of times because results might vary a lot depending on where the measurement is done.

In this work TER was measured with Millicell® ERS-2 System (Millipore). That system consists of the meter and the electrode which can be seen in Figure 10. The meter measures cell resistance (Ω) but it can be also used for voltage measurement (V). Before starting a TER measurement the functionality of the meter should be checked with a test electrode in case that a calibration is needed.

When TER is measured from insert it is put in a well with buffered based saline (PBS). The shorter tip is put inside an insert and the longer tip is put outside the insert in the well. Sensors are clamped lightly together to get a good contact between tips. The shorter tip should not contact cells because it might scratch cells and harm cell junctions. Electrode should be kept steady and at 90° angle to the plate insert to get trustable and reproducible results. This measurement technique is illustrated in Figure 10. (Millipore 2009b)

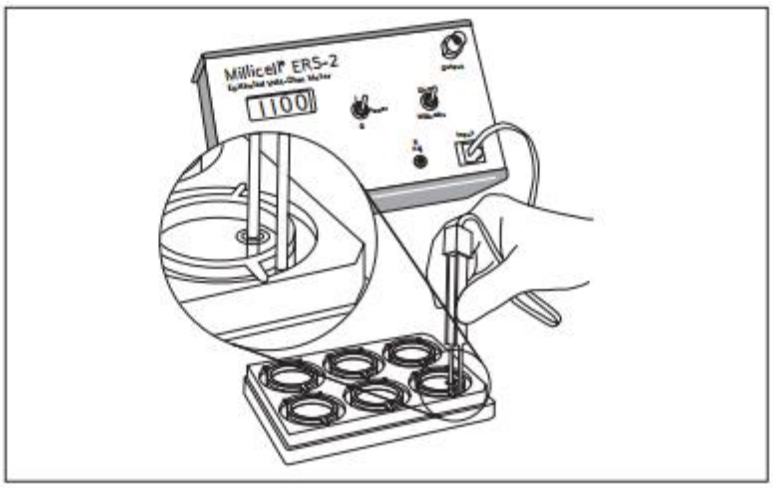


Figure 10. *The meter and the electrode used in TER measurement from an insert. (Millipore 2009b)*

TER can also be measured from clipping. Clipping is cut from the cell culture membrane used inside an insert. The device used in this method was developed by Ville Vuorinen. In his work he presents the test box which is meant to be used in fast in vitro frequency response measurement. This test box has been utilized in TER measurements at least in the Opthalmology group in Tampere University. The test box, manufactured in Tampere University of Technology work shop, is plastic and it utilizes the same sample sliders as the commercial ussing chambers of Physiological Instruments. These P2307 sliders are meant to hold small biopsies. The diameter of the circular aperture where sample is put is 2 mm and the area of aperture is 0,031 cm^2. (Vuorinen 2012, Physiologic Instruments 2014).

Clipping is washed with PBS and then put between two plastic sliders so that the aperture in the sliders is covered (aperture is marked with red arrow in Figure 11). Sliders are put together into the text box in a standing position sample part to the bottom. Around half of the sliders are inside and half outside of the box. The box is filled with

PBS and the tips are put around sliders so that electrodes cover pits on the sliders. Cell resistance is read from the meter.

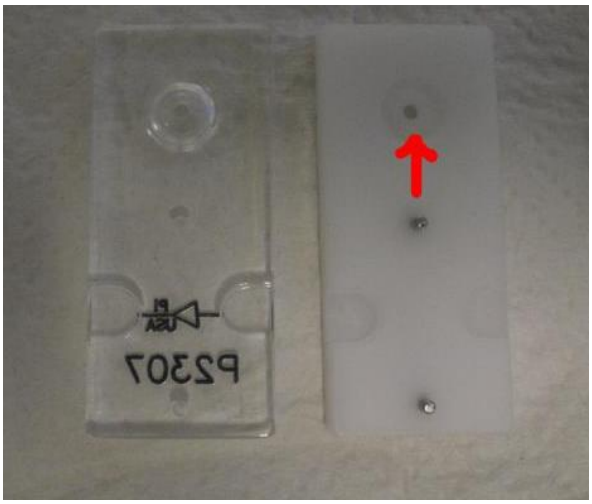


Figure 11*. Sliders are used when TER is measured from a clipping. The aperture which has to be covered with the cell culture membrane is marked with a red circle.*

Two main factors have to be taken into account before measurement results can be compared. The first one is the material of the cell culture membrane. Blank membrane is resistive even without cells. In some cases TER result of blank membrane can be in the same grade as for a cell cultured membrane. Even a small damage on a cell layer might cause a bad TER result. For that reason resistance of the blank membrane has to be measured and then subtracted from the samples' TER result. After that results are in a more comparable form.

Second notable factor is the measured area. It is known that the resistance is inversely proportional to the area of the tissue. In practice it means that the smaller the membrane, the higher the resistance. Results can be made into a comparable form using unit area resistance. The unit area resistance is acquired by multiplying the result from the meter (Ω) by the effective area of the membrane (cm^2). Unit area resistance is then expressed in Ωcm^2.

From those two factors two equations are obtained and those equations are used later when results are presented. It is recommended to use an average of two or more measurements.

$$R_{sample} - R_{blank} = R_{cell\ monolayer} \quad (1)$$

$$R_{corrected} = R_{cell\ monolayer} \times A_{effective} \quad (2)$$

After the calculations resistances are in a comparable form. As a comparison it can be mentioned that RPE cells from the same cell line (Regea 08/023) have got the following TER values: 6-10 Ωcm^2 (30) after plating on inserts, 145-188 cm^2 after (60) and 311 cm^2 (90). The numbers in brackets mean days after plating cells on inserts. In-

serts and the culturing method were a bit different than in this experiment but level of TER values is comparable. (Vaajasaari et al. 2011)

7.6.4 Trypan blue

The dye exclusion test is a quick and affordable method to study cell viability. It is based on information that viable cells do not absorb dyes such as trypan blue because of their intact cell membranes. Instead dead cells are more permeable and they absorb dye. After dyeing it is visually determined which cells are viable and without dye and which are non-viable with dye. (Storber 2001)

A cut sample, washed with PBS, was put onto a microscope slide and a drop of 0,4 % trypan blue solution {tetrasodium 3,3'-[(3,3'-dimethyl[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxynaphthalene-2,7-disulphonate]} (Sigma-Aldrich) was put over the sample. A cover slide was placed on the sample and the samples were imaged immediately with Olympus IX Fluorescence microscope.

7.6.5 Phalloidin

Phalloidin (tetramethylrhodamine B isothiocyanate) is toxin immunofluorescence made of mushroom *Amanita phalloides*. It can bind to filamentous actin in cells and make actin filaments strongly stabilized. It is known that phalloidin binds only to polymeric and oligomeric actin and not to monomeric actin. In histological applications phalloidin is used to label actin filaments. Vectashield MountTM with DAPI (4’,6-diamino-2-phenylindole) (Vector Laboratories, Inc., Burgingame, USA) is often used in phalloidin dyeing to accentuate cell nuclei visibility. (Sigma-Aldrich 2013)

In this work phalloidin staining was done according to the following protocol: cut samples were washed three times with PBS on a cell well plate and fixed in 4 % paraformaldehyde (PFA) for 10 minutes at RT. Then samples were washed three times with PBS and made permeable with 0.1 % Triton® X-100 (4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol) (Sigma-Aldrich). After 10 minutes samples were washed again three times. Then samples were incubated in 3 % bovine serum albumin-phosphate buffered saline (BSA-PBS) 1 h at RT. During this blocking a needed amount of phalloidin was put into an eppendorf and vaporized in fume hood. After that phalloidin was diluted with 0.5 % BSA-PBS. Used phalloidin dilutions are presented in Table 6.

Table 6. *Used phalloidin dilutions and their content.*

Dilution ratio	**Phalloidin**	**0.5 % BSA-PBS**
1:400	1.25 µl	500 µl
1:600	1.66 µl	1000 µl

Dilution was added over the samples. The samples were incubated 15 minutes at RT in dark and washed again three times with PBS to remove unbound phalloidin conjugate. Then samples were removed from the cell well plate onto a microscope slide and a drop of Vectashield MountTM with DAPI was added over the sample. Another slide was placed over the sample and the samples were stored at – 20 °C until they were imaged with Olympus IX 51 Fluorescence microscope. Cell nuclei where DAPI should be localized were imaged with UV light. Phalloidin in cells was imaged with a light wavelength of 568 nm.

7.6.6 Live/dead method

Live/dead analysis is a dyeing method based on the same idea as tryban blue. Cell-permeable dye colours viable cells and cell-impermeable dye colours dead and dying cells. These dyeings can be seen with fluorescence microscope. The live cell component calcein-AM indicates a good plasma membrane integrity and esterase activity. It is green fluorescent and can be seen with fluorescence microscope with light wavelength around 495-515 nm. The dead cell component ETHD-1 indicates a loss of plasma membrane and lowered esterase activity. It is red-fluorescent and can be seen with wavelength 495-635 nm. (Molecular Probes 2005)

The cut samples were washed twice with PBS. Calcein AM and ETHD-1 were melted and spinned in those eppendorfs where solutions had been frozen. 1.25 µl calcein-AM and 1.25 µl ETHD-1 were diluted into 10 ml PBS and mixed well. PBS was removed and the prepared solution was added over samples. Samples were incubated in dark 1 h at RT. After that samples were removed onto a microscope slide and covered with another. Samples were imaged immediately with Olympus IX Fluorescence microscope. For the green fluorescence a light wavelength of 488 nm was used and for the red fluorescence wavelength was 568 nm. Live and dead cells are counted from images with a utility program like ImageJ.

8 TRANSPORTATION DEMONSTRATION

The transportation demonstration was done in three stages to control the variables. The first transportation tested if there are any significant differences between polyethylene terephtalate (PET) and thicker polyimide (PI) membranes used with mediums Hibernate A and DM- at room temperature and at hypothermal conditions. If cells would be transported frozen, receiver should melt them and culture around two months before they could be transplanted. For that reason temperatures under 0 °C are not in line in this work. In the second transportation it was tested mainly what happens when an orbital shaker is added to cause physical strain. The third transportation was done to verify results with thicker (7 µm) and thinner (24 µm) PI membranes. Figure 12 summarizes how the transportation demonstration was carried out.

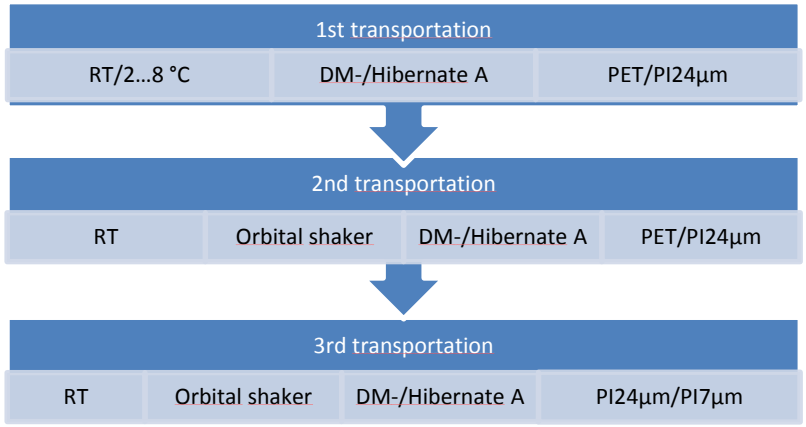


Figure 12. *Summary of transportation demonstration passage.*

Each of three transportations took 24 hours which is supposedly the same as in real transportation from Tampere to Germany. After a day and night travel cells will be stored in a culture medium in an incubator for one day and after that cells on membranes will be transplanted into laboratory rabbits. This storage after transportation is also simulated in these demonstrated transportations. All experiments were done in a general open laboratory in BioMediTech except light microscope imaging before transportation. It was done in the laboratory of Neuro group which is a closed laboratory.

8.1 The first transportation

PET and PI inserts were packed into 50 ml Falcon tubes. Falcon tubes were filled with a medium to ensure that the medium covers inserts despite tubes' position. Tubes were

packed into a plastic bag with pulp paper. The plastic bag was packed into a transportation box with a thermometer sensor. The nomination of the samples in series 1 and series 2 is shown in Table 7. T^{in} is temperature inside the transportation box. In this transportation all cells were from the hESC Regerea 08/017 and in passage p35_9 +3. In series 1 cells were plated on the membranes 39 days earlier and in series 2 40 days earlier.

Table 7*. The nomination of the samples in series 1 and series 2.*

Series 1 (T^{in} cold)	***Series 2 (T^{in} RT)***
A1: PET + Hibernate A	C1: PET + Hibernate A
A2: PET + DM-	C2: PET + DM-
B1: $PI_{24\mu m}$ + Hibernate A	D1: $PI_{24\mu m}$ + Hibernate A
B2: $PI_{24\mu m}$ + DM-	D2: $PI_{24\mu m}$ + DM-

Falcon tubes with inserts A1, A2, B1 and B2 (Series 1) were packed into transportation box with six TempFlexLab Cool and Heat elements. Two frozen TempFlexLab elements were packed on top of those. The transportation box was kept at room temperature for 24 hours. Target was to expose packaging to other temperature than there was inside the box and to control if gel elements can keep T^{in} stabile. Falcon tubes with inserts C1, C2, D1 and D2 (Series 2) were packed into the transportation box with eight TempFlexLab Cool and Heat elements taken from RT. Transportation box was kept in a cold-storage room for 24 hours to check if gel elements can keep T^{in} nearby room temperature.

After 24 hours exposure packages were taken down. Medium from each Falcon tube was taken for pH measurement. Inserts were taken out of Falcon tubes and PET membranes were cut out. PI membranes were extracted and extra membrane which had been outside of the body part of the insert was cut off. All membranes were cut into five pieces on PBS and characterized according to the methods explained earlier.

One piece of each sample was put into cell-well plate with DM-. After 24 hours storage in incubator pieces were analyzed with phalloidin. Phalloidin dilution was 1:600 for all samples. This after transportation test demonstrates what happens to cells after 24 hours rest in incubator before transplantation. Rest should normalize cells after transportation and decrease their stress. DM- will be used also in the European partner's laboratory so stress level should be quite realistic.

8.2 The second transportation

Based on the first transportation demonstration the transportation temperature was decided to be RT in the second transportation. Also mechanical stress simulation was included in the test setup to simulate movements during transportation. Simulation was done with an orbital shaker.

The transportation box was kept in a cold-storage room for 24 hours. Orbital shaker was used, the frequency of rotation was 90 revolutions per minute (REV/min). Eight TempFlexLab Cool and Heat- elements were taken again from room temperature and the packing method was the same as in series 2. The nomination of the samples in series 3 is shown in Table 8. Samples E3 and F3 were stored after the transportation demonstration 24 hours in incubator in DM- to demonstrate the rest after the transportation and after that cut and analysed. In this transportation all cells were again from the hESC line Regea 08/017 and in passage p35_9 +3 but they were plated on the membrane 47 days ago.

Table 8. *Nomination of samples in series 3.*

Series 3 (T^{in} RT)
E1: PET + Hibernate A
E2: PET + DM-
E3: PET + Hibernate A → 37 °C 24 h
F1: $PI_{24\mu m}$ + Hibernate A
F2: $P_{24\mu m}$ + DM-
F3: $PI_{24\mu m}$ + Hibernate A → 37 °C 24 h

After the demonstrated transportation packages were taken down and membranes were cut into pieces and analyzed as in series 1 and 2. TER was measured from two pieces and live/dead and phalloidin tests were done. At the same time phalloidin test was done for control pieces of E1, E2, F1 and F2. Phalloidin dilution was 1:400 because images taken of the samples of the first transportation were partly too pale.

8.3 The third transportation

During the second transportation at RT the temperature lowered 10.3 °C which was more than expected. For that reason in the third transportation test four gel elements were kept in 37 °C before packaging. Gel elements from RT were put first and then warmer elements. The transportation box was kept in a cold-storage room for 24 hours. The orbital shaker was again used with the frequency of 90 REV/min.

In series 4 there were two different PI membranes used. The nomination of samples in series 4 is shown in Table 9. Samples G3 and H3 were stored after the transportation demonstration 24 hours in incubator in DM- to demonstrate the rest after the transportation and after that cut and analysed. In this transportation all cells were from the hESC line Regea 08/023 and in passage p39_3 +3 and cells were plated on the membrane 32 days ago.

***Table 9**. The nomination of samples in series 4*

Series 4 (T^{in} RT)
G1: $PI_{7\mu m}$ + Hibernate A
G2: $PI_{7\mu m}$ + DM-
G3: $PI_{7\mu m}$ + Hibernate A → 37 °C 24 h
H1: $PI_{24\mu m}$ + Hibernate A
H2: $PI_{24\mu m}$ + DM-
H3: $PI_{24\mu m}$ + Hibernate A → 37 °C 24 h

After demonstrated transportation packages were taken down and membranes were cut into pieces and analyzed as in Series 3. Samples G3 and H3 were stored for 24 hours in 37 °C in DM- and after that cut into pieces. At the same time phalloidin test was done for control pieces of G1, G2, H1 and H2. Phalloidin dilution was 1:400 for all samples.

9 RESULTS

Cells were analysed with seven different methods. pH and TER measurements gave numeral results and the output of the other methods were images. In addition there were also calculated numbers of cells in live/dead method but they have to be observed together with images. Results are shown and analysed in this chapter. Measured TER values and numeral data of live/dead analysis are presented in more detail in Appendix A.

9.1 Selection of the transportation company

Six companies were contacted when a suitable transportation company was mapped. UPS did not answer to emails or phone calls. Biocair answered but they do not operate in Finland. Transportation time, transportation temperatures and transportation packaging were asked. When other companies offered a full service, DHL required that customer has a licence to transport dangerous goods. Answers are summarized into Table 10.

Table 10. *Summary of the transportation companies.*

Company	**Time**	**Transportation temperature**			**Other**
		2…8 °C	RT	37 °C	
Cool Id	Next day at the destination	Managed	Possible	Not possible	Packaging is included into the prize
World Courier	Next day at the destination	Managed	Possible	Not possible	Packaging is included into the prize
TNT	Next day at the destination	Managed	Not possible	Not possible	Packaging is included into the prize
DHL	Next day at the destination	Managed	Possible	Not possible	Customer should have own packaging

Two companies, Cool ID and World Courier, offered a room temperature transportation if temperature variation between 15…25 °C degree is acceptable. These two companies offered also a full service package including cool and heat elements, cladding and cardboard package with needed markings. Both companies could manage 24 hours transportation. Faster transportation is also possible but also much more expensive because a courier is needed. There is also a risk that transport time stretches is higher in express transportation than in normal transportation if something unexpected happens. Transportation protocol will be planned for over-night transportation but faster transportation is also fine. Both companies offer also a data logger service which gives information about transportation temperature. Unfortunately it is not possible to use in the real transportation. Data logger includes sensors sending radiofrequency which is forbidden to use in airplane.

When all claims had been gone through and companies' answers summarized, there was only one really potential candidate. A Finnish service branch company Cool ID answered best to transportation claims of this work. They are specialized to transport foodstuff and health care samples mainly in Europe but they have also transported biological samples to North America. This work was continued with materials and recommended methods of Cool ID. (Cool ID, 2013a)

Figures 13 and 14 show an example of temperature changes inside a Cool ID transportation box during over one day transportation in warm environment (21 °C, Fig. 13) and in cold environment (5 °C, Fig. 14). Gel elements of the cold transportation had been kept in freezer (two elements) and fridge (six elements). All eight elements that were used in warm transportation were kept at 25 °C before transportation. (Salonen, 2013)

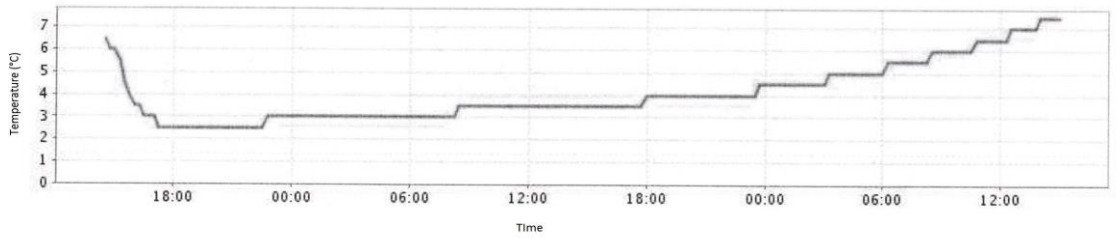


***Figure 13**. A graph of temperature changes inside a Cool ID transportation box during almost two days transportation at 21 °C environment. (Modified from the source Salonen 2013)*

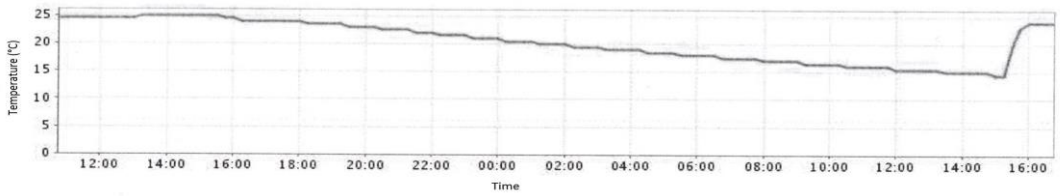


Figure 14*. A graph of temperature changes inside a Cool ID transportation box during over 24 hours transportation at 5 °C environment. (Modified from the source Salonen 2013)*

Graphs above show that there should be no risk of chilling or warming cells when temperature stays steady. Because it is not possible to say which temperature is more suitable for RPE cell transportation, transportation box and materials for transportation in both temperatures will be served. Then temperatures can be tested side-by-side.

9.2 Transportation medium

Two cell culture mediums, DM- and Hibernate A, were used tested parallel before the transportation demonstrations were done. Live/dead dyeing confirmed results which were seen just after cold storage: cells that had been in DM- were less viable as those which were stored in Hibernate A. Visual estimation of those cells along with phalloidin images showed that Hibernate A suits for RPE cells and does not harm them in a short term storage. Testing was done only in one condition with cell cultured on plated. For that reason Hibernate A was be used in parallel with DM- to collect more information if there are differences in used mediums when transportation conditions are tested using cells cultured on inserts.

9.2.1 Medium pH

pH was measured from both mediums as a control value before and after the transportation without any insert. Before the transportation Hibernate A's (0 days old) pH was 7.21 and DM-'s (5 days old) pH was 7.24. After transportation Hibernate A's was 7.34 and DM-'s was 7.49.

As speculated earlier the age of medium might have an effect on pH values. This seems to be true at least with Hibernate A. In series 1, 2 and 3 used Hibernate A was taken from the same production run. In series 3 Hibernate A was 13 days old and pH values were higher than in earlier series. In series 4 Hibernate A was made in the same day as demonstrated transportation but pH values were a bit higher than in series1 and 2. pH of fresh medium was not measured when Hibernate A was mixed first time in this project so exact pH – time reliance is not known. In the real transportation fresh medium will be used so there should not be a risk of high pH values because of the age of medium. Ages of mediums compared to pH results are presented in Table 11. pH results are calculated averages of all samples of one series before transportation.

***Table 11**. Ages of mediums compared to pH results in series 1 to 4. Ages are in days.*

Medium	***Series 1***		***Series 2***		***Series 3***		***Series 4***	
	Age	pH	Age	pH	Age	pH	Age	pH
Hibernate A	4	7.05	5	7.13	13	7.35	0	7.22
DM-	10	7.28	11	7.51	4	7.74	5	7.23

pH of DM- does not change as logically as Hibernate A. There is one exceptional change in pH between series 1 and 2 even though there is only one day difference in the age of medium. In series 3 pH values are very high but at the same time the change in pH during transportation is smaller than in other series. pH is most similar in series 1 and series 4 and there might be a correlation between the time when cells were plated on the membranes and pH of the medium. In series 1 cells were 39 days, in series 2 40 days, in series 3 42 days and in series 4 32 days old. In series 1 and 4 cells were younger than in other series and pH was also lower than in series 2 and 3. There are no exact instructions how long Hibernate A can be stored in a fridge when serum replacement and antibiotics have been added. With DM- the storage time had been kept under two weeks and it can be supposed that it is not more with Hibernate A. As pH measurements pointed there might be some correlation between the age of medium and pH values. For that reason it is better to use fresh medium.

Even though pH meter was calibrated before each series there is a possibility for a systematic human error. Also impurities or residues of DM- might impact on pH of Hibernate A. Most of the changes in pH were small and do not have significant meaning in planning of transportation. Changes in pH in medium without insert showed that change can be caused from some other reason than cell metabolism or unfastening of cells. It can be stated that pH is not an important tool when analysing cell well-being before and after transportation.

9.3 Confirmation of the temperature during the transportation

Temperature change during transportation was tested to make sure it changes as expected also in this work and in the used environments. A data logger would have given information in more detail but it was not at command. Changes in temperature were observed in a simple method with nine time points.

The transportation boxes were packed with thermometer sensors. As in the first transportation two frozen gel elements and five elements taken from a cold-storage room were packed into the transportation box which was kept at room temperature. The sixth element did not fit into the box because two frozen elements had changed their shape in a freezer because there was not enough space to lie smoothly horizontally.

A cold-storage room was used as a test environment in the second and in the third transportation. Temperature inside the box lowered more than expected. In the third transportation four gel elements were in an incubator only four hours and that was not enough to keep the temperature high. For that reason another temperature confirmation test was done with warmer elements. Four gel elements were kept overnight at 37 °C and then packed to the bottom. Three room temperature elements were packed over them. Temperature was measured in the beginning of experiment and eight times once in an hour after that.

Temperature changes can be seen from Figure 15. Nine time points, in other words eight hours covers only one third of the real transportation time but trend is similar as in Figure 14. Because the representative of the transportation company could not give exact information about gel elements, transportation circumstances and location of thermometer sensor available, graphs cannot be compared directly.

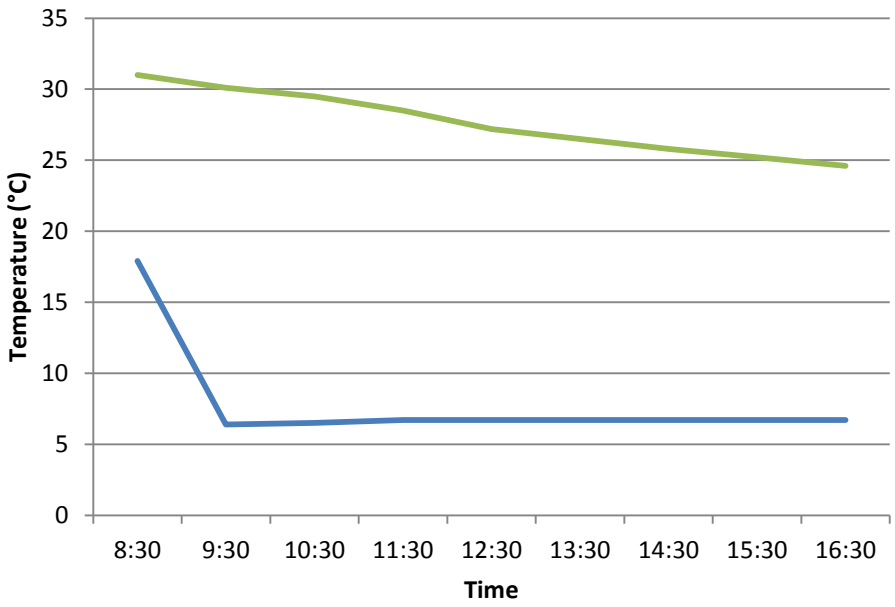


Figure 15. *Temperature changes during eight hours confirmation test. Green line indicates temperature changes inside the transportation box during transportation done at cold environment. Blue line indicates temperature changes inside the transportation box during transportation done at room temperature.*

Temperature in transportation box which was kept in cold-storage room was higher than in the third transportation. This can be explained with the packing order and shorter testing time. In the third transportation warmer elements were on the top, now they were under and thermometer sensor was in touch with warmer elements. For that reason measurements gave over 25 °C values but this would moderate if testing would have been continued up to 24 hours instead of eight hours.

If there is a need to transport cells longer than one day for example to another continent, it would be good to test temperature behaviour of gel elements again. It should

be tested how warm elements should be before transportation to keep temperature inside the transport box high enough and avoid that cells might cool. This testing was not included into this work.

9.4 The first transportation

The first transportation was done to map how the RPE cells and cell culture mediums react in different transportation temperatures. After transportation cells were analyzed with several methods. Results of the first transportation are itemized in the next sections.

9.4.1 pH changes

pH was measured from mediums at RT. Almost in all Hibernate A's pH was lower and closer to neutral than DM-' pH. Also changes in pH were smaller in Hibernate A than in DM-. Average pH of 12 measurements was 7.22 for Hibernate A both before and after the transportation and on an average Δ pH was 0.06. For DM- average pH of eight measurements was before transportation 7.43 and after 7.52 (average Δ pH 0.10). Average pH results of series 1 and 2 are collected in Table 12.

Table 12. *pH values for series 1 and 2.*

Sample	pH_i	pH_f	Δ pH
A1	7.03	7.10	0.07
A2	7.31	7.34	0.03
B1	7.06	7.09	0.03
B2	7.25	7.32	0.07
C1	7.11	7.24	0.13
C2	7.50	7.66	0.16
D1	7.14	7.21	0.07
D2	7.53	7.64	0.11

In both series pH increased a bit during transportation. Also the change in pH was smaller in Hibernate A. Generally changes in pH of Hibernate A were small (≤0.10). The only exception was sample C1 (PET + Hibernate A + RT) which pH change was 0.13 pH units. Interesting is the higher pH values of samples C2 (PET + DM- + RT) and D2 (PI + DM- + RT) compared to samples A2 (PET + DM- + 2...8°C) and B2 (PI + DM- + 2...8 °C). The difference in pH after transportation could be explained with different transport temperature but pH values of C2 and D2 were also quite high also before transportation. In the following tables pH_i means pH before transportation and pH_f means pH after transportation.

9.4.2 Resistance changes during transportation

Before comparing TER results with each other they should be calculated to get them into form of Ωcm^2. TER results are calculated from the measured ones with equations 1 and 2 (see section 7.6.3). Before calculation TER result of a blank membrane has to be measured. In this project it was measured from a clipping. Blank membranes gave next TER results: PET 803 Ω, $PI_{24\mu m}$ 655 Ω and $PI_{7\mu m}$ 677 Ω. Figure 16 illustrates TER values of blank membranes compared to measure of series 1 and 2. Measured TER values are presented in more detail in Appendix A.

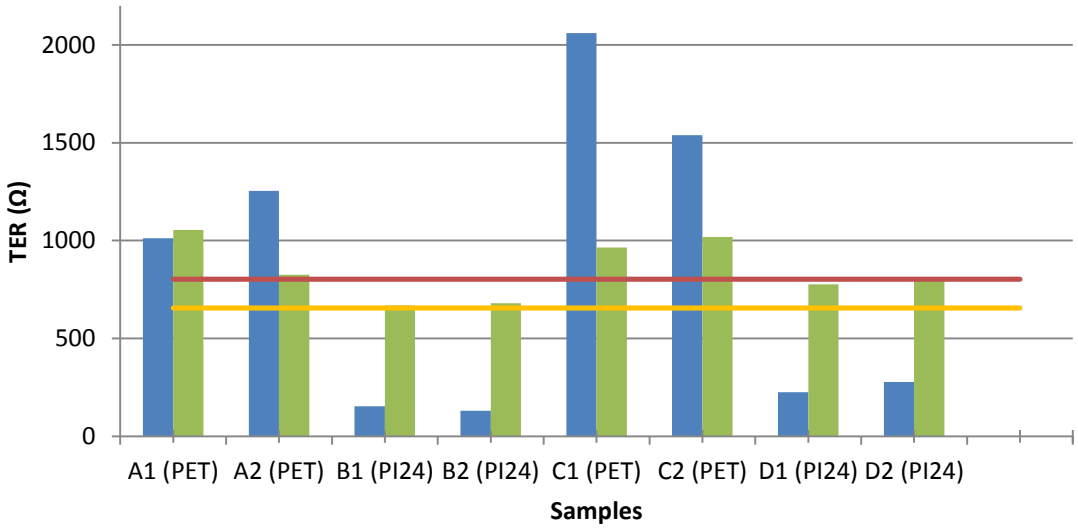


Figure 16. *Measured TER results of series 1 and 2. Blue columns represent TER values measured from inserts before transportation. Green columns present TER values measured from cutting after transportation. TER values, from which columns are drawn, are taken from one measurement or it is an average of two measurements. Red line stands for TER of PET membrane (803 Ω) and yellow line for TER of $PI_{24\mu m}$ (655 Ω). Membrane material of each sample (PET, $PI_{24\mu m}$) is written under the sample name to help reader to compare the materials. Resistances of blank PET and $PI_{24\mu m}$ membranes are marked with red and yellow lines.*

As can be seen from Figure 16 only blue columns of samples A1, A2, C1 and C2 are high enough to get over critical lines (red for PET, yellow for $PI_{24\mu m}$) before transportation. When TER is calculated for those four samples into comparable form with equations 1 and 2, results are following: A1 = 69 Ωcm^2, A2 = 467 Ωcm^2, C1 = 415 Ωcm^2 and C2 = 690 Ωcm^2. All four denumerable results are measured from PET membrane. Measured TER values of $PI_{24\mu m}$ were very low before transportation. This result correlates with taken light microscope images which showed several harmed areas in every sample.

After transportation TER was measured from cuttings. Difference between PET and $PI_{24\mu m}$ were not as significant as before transportation. Resistances of samples A1,

C1, C2, D1 and D2 got over the critical line after the transportation. Calculated TER results varied from 55 Ωcm^2 (C1) to 113 Ωcm^2 (D2).

9.4.3 Cell characterisations

In addition to pH and TER measurements the RPE cells were analysed with immunohistochemical methods. Results of trypan blue method were not very good. As mentioned in earlier, dead cells should absorb dye and seen as blue spots. Pigmentation of the RPE cells dominates the colouring of the image and blue colour is not well seen. Figure 17 represents examples of uninformative trypan blue staining in one PET sample and in one PI sample.

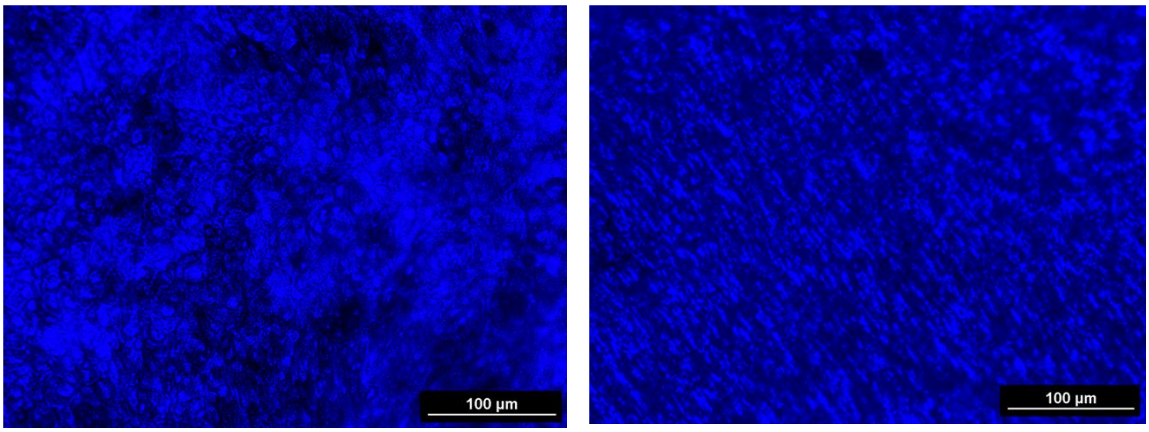


Figure 17. *Tryban blue stained samples C1 (PET) and D2 (thicker PI). Scalebar 100 µm.*

In sample C1 (PET) cell morphology can be somehow seen. Lighter areas do not have many cells and they are not pigmented yet. Darker areas illustrate pigmented cells. When blue colour had spread to all cells, dead cells cannot be specified. In sample D2 (thicker PI) problem is the same as in the bright field image of sample H3 (see Figure 16); orange colour gives too much background and cell specifying is difficult. Some conclusions can be made of the cell density but not about the dead cells.

Phalloidin was known to be a good method for RPE cell analysis. Actin filaments in phalloidin images and cell nuclei in DAPI images illustrate cell layer viability. In the most of the images it was seen that cell nuclei had settled themselves to the edges of the cutting. Actin filaments correlated quite well with the holes in the cell layers which were seen before transport. In series 1 and 2 phalloidin dilution was 1:600. Images showed that there are more stressed cells in those samples which were stored in cold in the transportation box.

From taken images the number of cells can be calculated with a utility program. In this work ImageJ was used to find the ratio of living and dead cells. That ratio indicates viability of cell layer. If the ratio is >1, there are more living cells than dead cells and when the ratio is <1, major of the cells is dead. Results of all samples are collected to Figure 21 to help comparison. Live/dead ratios of samples A1, A2, B1 and B2 are

missing from the graph. Reason for that was that the quality of the images was lower than in the images of the later samples. Cells analysed with live/dead method do not survive well in a freezer so they could not be imaged again later.

9.5 The second transportation

In the second transportation conditions were kept same as in the first transportation (RT) but an orbital shaker was added. Aim was to simulate mechanical stress which happens in transportation. Even though orbital shaker is only a rough generalization of all realistic stresses, it has a significant impact on results. Cell viability was lower in series 3 than in series 1 and 2 which was seen in images and TER results.

9.5.1 pH changes

In series 3pH was measured before and after transportation as earlier. Results are shown in Table 13. All pH values of both mediums were higher than in earlier series.

Table 13*. pH values for series 3.*

Sample	pH_i	pH_f	Δ pH
E1	7.36	7.28	- 0.08
E2	7.73	7.75	0.02
E3	7.36	7.26	-0.10
F1	7.35	7.24	-0.09
F2	7.74	7.71	-0.03
F3	7.34	7.25	-0.09

pH values decreased during transportation and surprisingly pH of DM- changed less than pH of Hibernate A. In other series DM- had more differentiation in pH values than Hibernate A. Supposedly this property depends on the age of medium. This subject is discussed in more detail in the end of this work.

9.5.2 Resistance changes during transportation

TER values of series 3 were worse than in series 1 and 2. It was because of mechanical stress caused by orbital shaker which damaged cell layer. Results from the TER measurements of this transportation are shown and analysed together with the results of the third transportation. Reason for that is that sample E3 is compared to two samples of the third transportation to find differences between membrane materials in the same environment. Measured TER values are also shown in Appendix A.

9.5.3 Cell characterisations

Trypan blue was left out from the second transportation. Samples of the second transportation were analysed with live/dead and phalloidin staining as in series 1 and 2. Imaged showed that cell layers in all samples had been damaged more than in the earlier series. The most probable reason was mechanical stress caused by the orbital shaker. Changes in the cell layer are shown best in images which are collected into the next section where sample E3 are compared to samples H3 and G3.

9.6 The third transportation

Aim of the third transportation was to compare thinner and thicker PI membranes. In this section images of sample G3 and H3 are compared to sample E3 which was used in the second transportation. Those samples were chosen for comparison because they went through the same transportation demonstration (24 hours at room temperature with an orbital shaker). After the transportation inserts were moved to DM- and kept 24 hours in incubator. After that they were analysed.

In every series all samples were imaged with light microscope before demonstrated transportation. Changes in cell layer were seen pretty well in PET samples and worse in PI samples. Images of samples E3, G3 and H3 are collected into Figure 18.

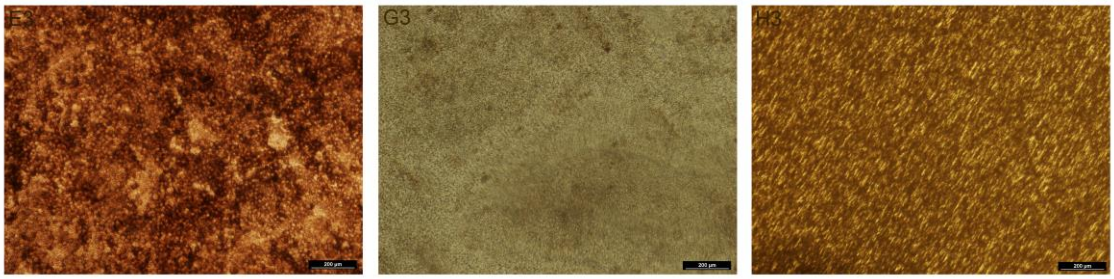


Figure 18. *Samples E3, G3 and H3 imaged with a light microscope before the demonstrated transportation. Scalebar 200 µm.*

In sample E3 cells were plated on the membrane 10 days earlier than in the other samples. The age had caused stronger pigmentation and also the cobblestone-like structure. Discontinuities of the cell layer are pretty well seen as lighter fuzzy areas. Sample G3 was less pigmented and the typical morphology of RPE cells had not yet been formed. Light orange background colour of the PI membrane was not a problem in this image compared to sample H3 where the morphology of RPE cells is not clearly seen and the membrane dominates the image. Light microscope was less informative for the thicker PI compared to other membranes

9.6.1 pH changes

pH changes in series 4 were quite similar as in series 1 and 2 as shown in Table 14. pH increased for two DM- samples 0.17 pH unit which was more than in earlier series. For Hibernate A samples change was just opposite; pH changes were smaller than in earlier series varying from 0.01 to 0.05 pH units.

Table 14*. pH values for Series 4.*

Sample	pH_i	pH_f	Δ pH
G1	7.19	7.23	0.04
G2	7.21	7.38	0.17
G3	7.23	7.26	0.03
H1	7.22	7.27	0.05
H2	7.24	7.41	0.17
H3	7.25	7.26	0.01

Cells in the samples were plated on the membrane 32 days ago in sample E3 and 42 days ago in samples G3 and H3. Ten days difference in the removing impairs comparison of the samples a bit. Cells at the same stage would have been easier to compare because morphology and pigmentation can change a lot during mentioned 10 daysFigure 18 represents bright field image (BF) taken of all the membrane types before the transportation. Images of the same samples after immunostainings are shown later in this chapter. Images were taken with 10 x magnification and they were representing the best areas of each insert. Cells were plated on the membrane 42 days ago in sample E3 and 32 days ago in samples G3 and H3.

9.6.2 Resistance changes during transportation

TER results were measured from insert and cutting as earlier. Figure 19 illustrates measured TER values of series 3 and 4 which are also seen in Appendix A. In Figure 19 Columns are drawn from one measurement value or an average of two measurements' values.

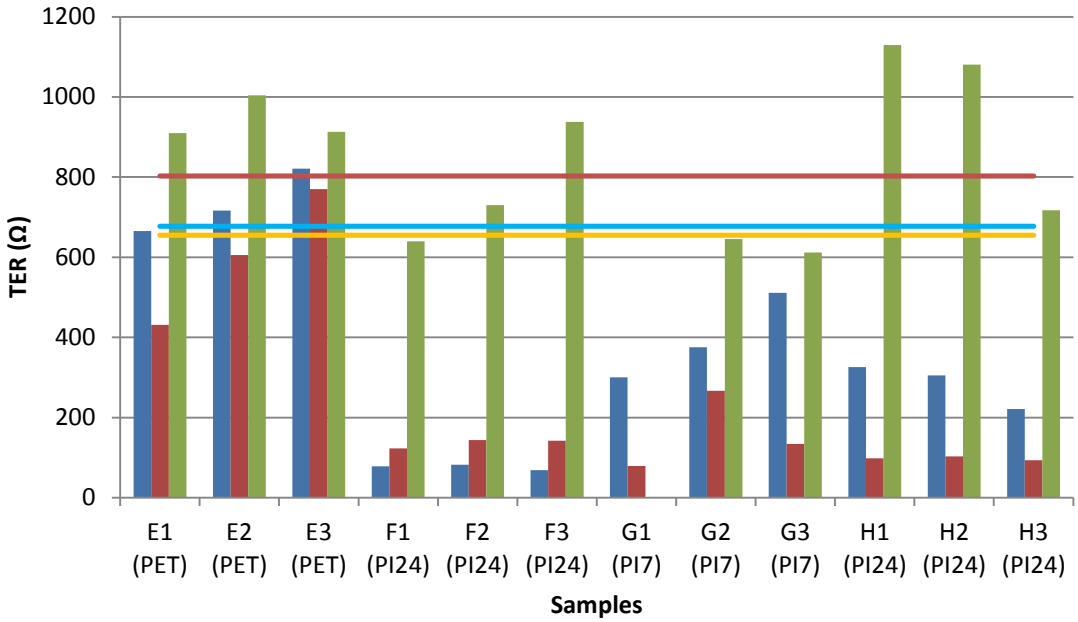


***Figure 19**. Measured TER results of series 3 and 4. Resistances of blank PET, $PI_{7\mu m}$ and $PI_{24\mu m}$ are marked with red, blue and yellow lines. Red and yellow lines represent the same things as in Figure 16. There is also turquoise line which stands for TER of $PI_{7\mu m}$ (677 Ω). Membrane material of each sample is written under the sample name. Blue column means TER measured from insert before transportation. Red column means TER measured from insert after transportation. Green column means TER measured from cutting after transportation. Sample G1's green column (measured 4776 Ω) is ignored. Exceptionally high result is caused by an air bubble or some other disturbance on the aperture instead of good transepithelial resistance.*

In series 3 and 4 TER_i (insert) results and TER_f (insert) were even worse than in series 1 and 2. There were no meaningful results to be calculated into comparable form. Instead TER_f (cutting) results seem to be positive when looking height of the green columns. As explained earlier, TER result of PET should be over red line and TER of $PI_{24\mu m}$ over yellow line. For $PI_{7\mu m}$ this critical line is the turquoise one. The most of green columns are over the critical line of each membrane material but when the effective area of aperture (0.031 cm^2) is taken into account; all results of samples E1, E2, E3, F2, F3, H1, H2 and H3 are under 15 Ωcm^2

9.6.3 Cell characterisations

Trypan blue was left out from series 4 as in series 3. Phalloidin images instead were taken of all samples after transportation. Figure 20 summarises phalloidin images of samples E3 (PET), G3 (thinner PI) and H3 (thicker PI) to help comparison of three materials. Phalloidin dilution was 1:400 for every sample. From each sample images were taken from three parts and those shown in Figure 20 are the best parts. There were some ruptures in each sample. Bright field (BF) image is taken without any filter; DAPI im-

ages were taken with UV light and phalloidin images with light wavelength 568 nm. All three images of each sample were taken from the same place to help comparison.

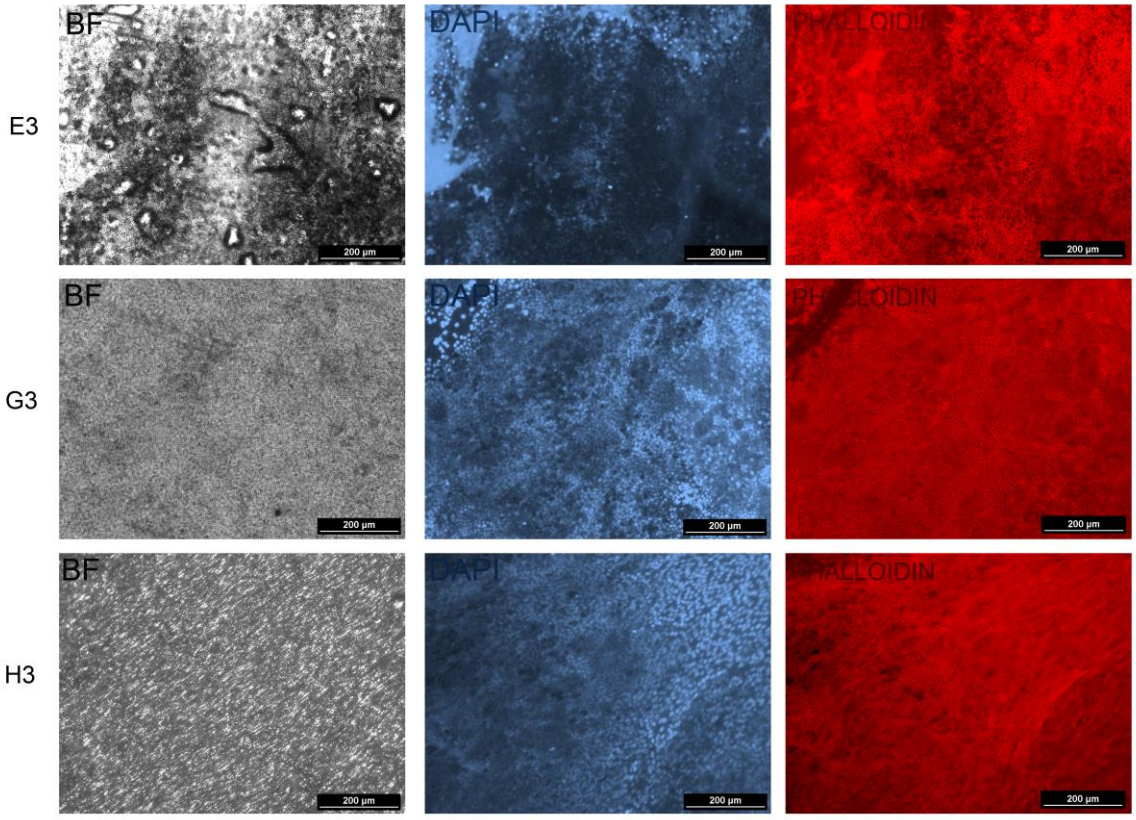


***Figure 20**. A summary figure of three samples after 24 hours transportation at RT. Bright field images are on the left column, DAPI images are in the middle and phalloidin images are in the right column. Scalebar 200 µm.*

BF image of sample E3 is not promising. There are discontinuities in cell layer and also air bubbles or some other impurities. DAPI image shows that cell nuclei are in the edges of the image. In the middle of the image there is a darker area which indicates pigmentation which can be seen also in the third image. Even though BF image looked bad, there are good areas with cobblestone structure. Apart from some empty areas cell layer looks quite smooth and viable.

Quality of the BF image of sample G3 is better than in the previous one. There are no significant impurities but there are some discontinuities which can be seen as fuzzy areas. Cell nuclei are quite evenly on the surface of the sample. There is one cell nuclei locus in the up left part of the image and it can be seen also in the phalloidin image. Probably it is a rupture in the cell layer and some nuclei were gathered to the edeges of it. Level of pigmentation is low and there is the cobblestone structure is not as clear as in sample E3. There are some areas of actin filaments but sample does not look very stressed.

BF image of sample H3 is again not very informative. There are some darker areas but now they are not indicating pigmentation. Cells in samples G3 and H3 were 10 days younger than in sample E3 and pigmentation had not formed much. In DAPI image can be seen high amount of cell nuclei, much more than in sample E3. Nuclei are gathered to the right and the same area can be seen also in phalloidin image. Cells in that area are stressed and there are a lot of stress fibers.

When sample E3, G3 and H3 are compared, it can said that sample G3 is the best. There are some fuzzy areas in the cell layer but they are not as worrying as the empty areas in sample E3 or amount of actin filaments in sample H3. The same trend can be seen images which were taken from two other parts of sample. It is still important to be conscious that there were only three parts where images were taken. Without more statistical analysis it cannot be said for sure that according to phalloidin staining thinner PI membrane seems to be the most suitable membrane material of the used ones

All samples were stained with live/dead method to analyse the number of viable cells compared to dead cells. Samples were then imaged with fluorescence microscope. Taken black and white images were coloured and collected to Figure 21. It shows a summary of earlier mentioned samples E3, G3 and H3 and images of their live and dead cells. Those two images are combined in the third image which is marked with LIVE/DEAD. Images were taken of three parts of each sample and the best shots are shown under.

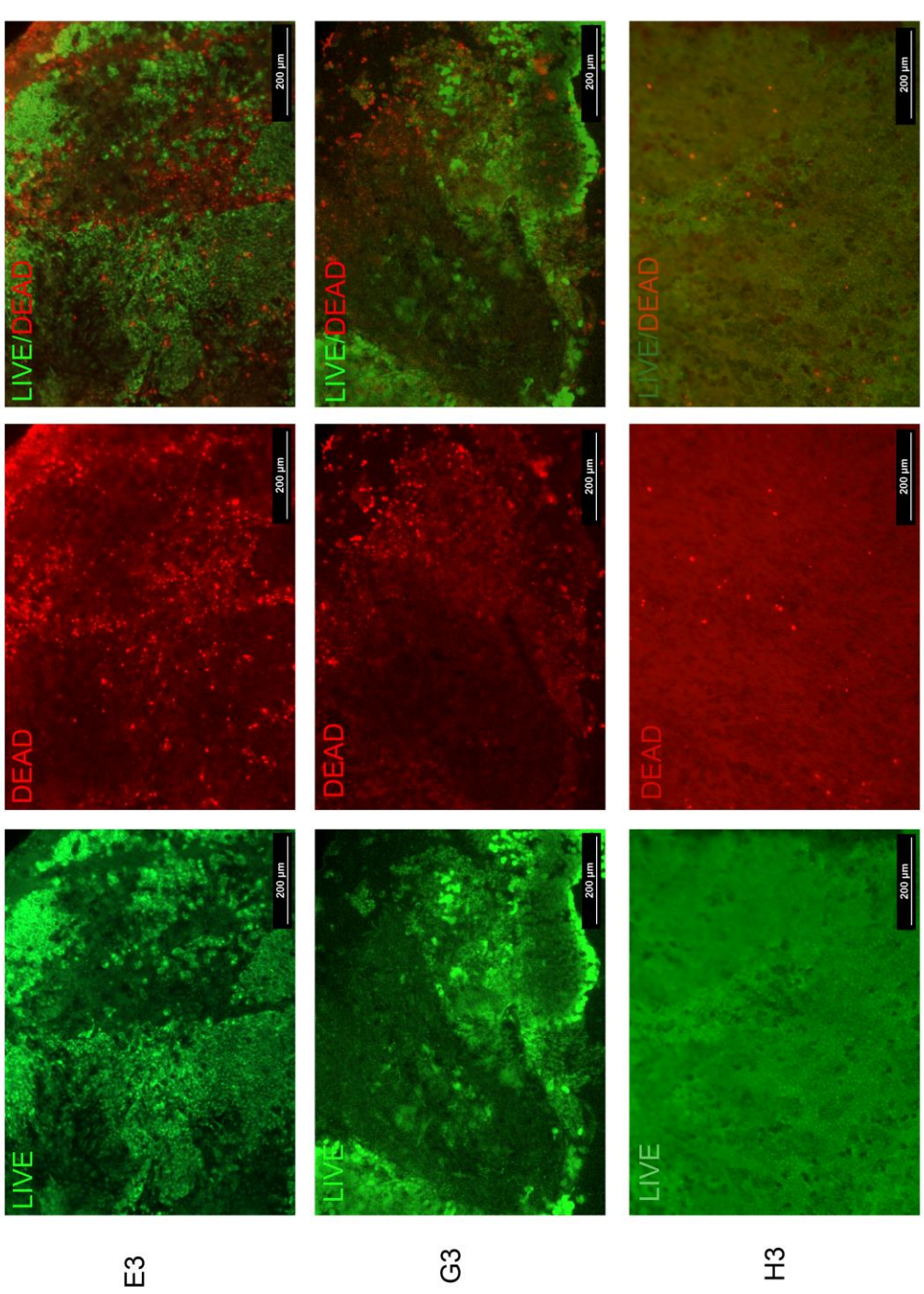


Figure 21. *A summary figure of three samples after 24 hours transportation at RT. Images of the living cells are shown in the left column and dead cells in the mi images are shown in the right column. Scalebar 200 µm.*

There were a lot of living cells in sample E3 (PET). The cell layer was not very constant and there were areas without any cells on the left but of three imaged areas this one was the most viable. Dead cells were spread more equable than living cells. In sample G3 (thinner PI) both living and dead cells were spread unevenly. There seemed to be less cells than in sample E3 but more than in sample H3 (thicker PI). The difference was not only in the number of the living and dead cells but the cells were not shown as well in sample H3 as in two other membranes. Sample H3 is totally coloured but contrasts are not seen well. Reason for that might be again the colour of the membrane; orange gives background in all three image types. Images taken of the other

thicker PI samples looked quite similar. Even though living cells are not shown as spots, the morphology of RPE cells can be seen. In the red fluorescence image the surface structure of the PI membrane is similar like in the bright field images of thicker PI.

Figure 22 illustrates ratios of living and dead cells, three ratios of each sample which means that samples were imaged from three places. Exact numbers are also shown in Appendix A. All images where taken with 10 x magnification and cells were calculated with the same accurance. Live/dead ratios of samples A1, A2, B1 and B2 are missing from the graph. Reason for that was that the quality of the images was lower than in the images of the later samples. Cells analysed with live/dead method do not survive well in a freezer so they could not be imaged again later.

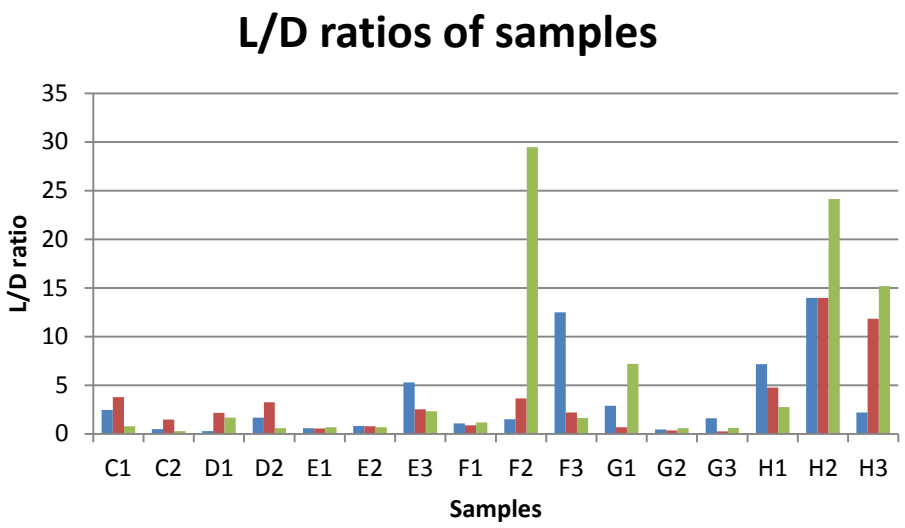


Figure 22. *Ratios of living cells compared to dead cells. Measured from three places of each sample. Blue columns indicates L/D ratio in area 1, red columns L/D ration in area 2 and green columns L/D ratio in area 3.*

As can be seen from the figure above, L/D ratios vary a lot even inside one sample. For example in samples F2 two L/D ratios are quite equal and the third one is multiple higher. The difference is not caused by the number of cells but the quality of the images. Sample F2 looked quite similar as sample H3 in Figure 21; sample was coloured but cells were not shown clearly. It causes a high number of both cell types, especially living ones, and can be seen as peaks in L/D ratios. According to Figure 21 sample E3 seems to have a bit more living cells compared to dead cells than in sample G3 and even more than in sample H3. Numbers in Table 15 show that calculated results differ from the estimations made of images.

Table 15. *Calculated numbers of living and dead cells*

Sample	Living cells	Dead cells	L/D ratio
E3	1431	271	5.28
G3	143	602	0.24
H3	1109	73	15.19

It can be seen from the table above that sample E3 there are a lot of living cells and the L/D ratio 5.28 is good. Low number of living cells in sample G3 is a bit surprising; it is only one tenth of living cells in sample G3. When there are multiple dead cells compared to living ones, the L/D ratio is only 0.28. Living cells of sample H3 is around the same level with sample E3 but there seems to be only few dead cells which causes very high L/D ratio 15.19. This is the second highest L/D ratio of all samples and does not correlate with the image as well as samples E3 and H3. The most probable reason is that when cells are calculated, some other particles than cells are calculated too. It could be eschewed if the particle size or other parameters used in ImageJ would be change but it changes also the comparability of the results.

Confocal microscope did not have a major role when planning how to analyse cells in this work but because there were resources to use it, some images were taken. Images can give more information about cells and their viability after transportation. These pictures were taken by PhD Kati Juuti-Uusitalo. Figure 23 represents sample G3 with phalloidin.

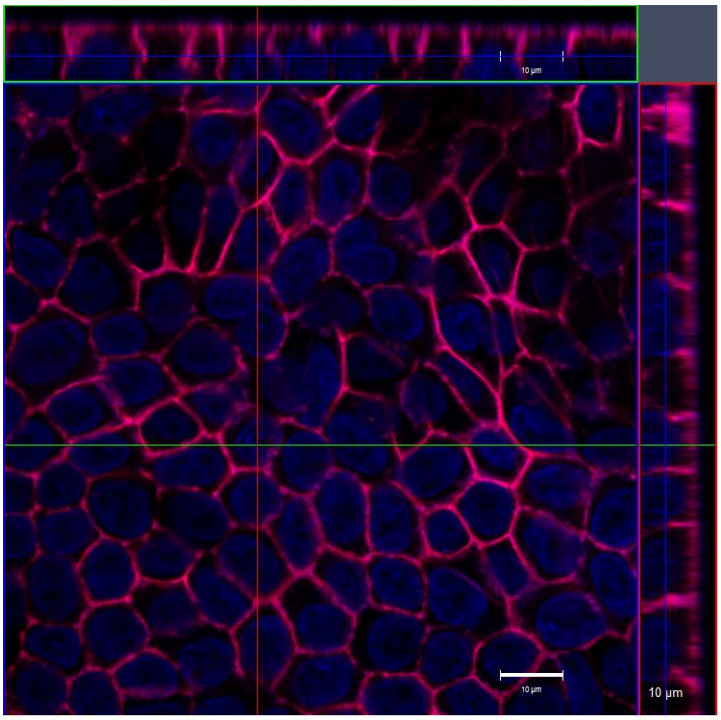


Figure 23*. Confocal microscope image of sample G3. Pink areas illustrate tight junctions and blue areas are cell nuclei. Pigmented areas are darker ones on the upper part of image. Image is taken from z direction. Areas on the right hand side of purple line and over turquoise line illustrate cell viewed from x and y directions. Scalebar is 10 µm.*

Also samples E3 and F3 were imaged to compare the used three materials. Unfortunately those samples had dried and images were not informative. Images were taken with LSM 700 confocal microscope (Carl Zeiss, Jena, Germany).

9.7 Summary of the cell analysis methods

All cell analysis methods mentioned in earlier have benefits and backwards. A summary of these properties is presented in Figure 24. Green colour indicates a positive (+) property, red colour is a negative (-) or unwanted property and blue colour is a neutral (0) property. Properties are graded from the perspective of this work. In some other work grading of properties can be totally different.

Method	Time	Cost	Informativity	Unequivocality
Light microscope	+	0	+	+
pH	+	0	-	+
TER	+	0	0	-
Trypan blue	+	+	-	-
Phalloidin	0	+	+	+
Live/dead	0	+	+	0
Confocal microscope	-	-	+	+

Figure 24. *A summary of the most important properties of used cell analysis methods. Positive properties are marked with +, negative properties with – and neutral ones with 0.*

Time that was used to each analysis is an important factor when thinking lab technician's or researcher's work. If there are a lot samples, analysis should not take long. If there are only a couple of samples, it might possible to analyse them in more detail. From used methods pH and TER measurements and trypan blue were the fastest ones. Also light microscope can be a quick method if samples are clear and easy to image. In this work thicker PI membrane was often difficult to image because orange colour gave quite much background and it was difficult to analyse cell viability. Phalloidin and live/dead are a bit slower methods but still analyse takes less than hours. Confocal microscope is the slowest one. Sample preparation and imaging took more than half an hour per sample. In some cases it might go faster but with used membranes and RPE cells it was a bit challenging.

Confocal microscope is bad also when thinking cost. Machine by itself is expensive and a person needs to be educated to use it. As mentioned above, imaging with confocal microscope is slope and time is money. pH and TER measurement and light microscope are graded as neutral. Reason for that is that even though machines and equipments of these methods cost a bit, their use does not require special experience or a lot of time. Trypan blue, phalloidin and live/dead are ranked for the highest. Their use does not require any other special equipment than a light microscope and some chemicals.

In this work pH and tryban blue were not very informative. pH changed without an insert in a medium and trypan blue did not work in pigmented cells. TER results could have been useful, if cell layers would not have damaged so much in transportation. Phalloidin, live/dead, light microscope and confocal microscope were all informative. Especially the last-mentioned could have given more in detail information, but it was not that important in this work.

Results should be unequivocal especially in case where lab technicians or researchers discuss about the same cells but parties are not physically in the same place. An example of unequivocal result is the number of pH measurement. If someone measures pH and it is 7.00, all colleagues understand that it is neutral. TER could also be understood as a quantitative study ad pH but its results are not very comparable because there is not enough comparison material. Results depend on also many factors and are not very unequivocal. Images taken with confocal microscope light microscope by itself or with phalloidin are quite unequivocal. Of course the quality of the images or their informativity might depend on a person, but interpretation should not vary as much as in some other methods. Tryban blue is graded to negative because in those part of cell layer where cell are not yet pigmented, it might indicate dead cells but not in pigmented cells. Live/dead is graded as neutral because ration of live and dead cells varies between the edges and the middle of the cutting.

10 DISCUSSION

Tissue banks store and deliver cells and tissues. Transportation of human tissue is done mainly in hypothermal conditions but human cells like gametes can be transported also frozen. In some cases once thawed cells cannot be frozen again and thus room temperature and body temperature might be better choices for transportation temperature than colder ones. In air transportation heated or cooled gel elements keep temperature on a acceptable level in a transportation box but their heat capacity is limited and might be too low in longer transportations. If cells could be transported safely, co-operation between research institutes could be more common.

Experimental part of this work included three demonstrated transportations which combined one PET membrane and two types of PI membranes, two mediums, two temperatures and also an orbital shaker. Resources were limited and test matrix made of components earlier did not cover all possible combinations. The first demonstrated transportation showed tentatively that room temperature is more suitable for RPE cells than hypothermal environment. The rest two transportations were done in room temperature and results showed that RPE cells' viability depends more on mechanical stress than chemical or thermal environment of cells.

The experimental part of this work supports earlier findings in the literature; cells survive quite well in room temperature transportation (Pasovic et al. 2013). When gel elements are warmed up to 37 °C, can temperature inside the transportation box be kept longer near to body temperature and cells do not stress about temperature changes. Heat capacity of gel elements is limited so it might be that temperature lowers under room temperature if transportation continues longer than 24 hours.

Hibernate A suits for transportation medium. Cell culture medium DM- is also a suitable option but it might be that lower pH of Hibernate A supports more cell viability. On the other hand mammalian cells should survive best in pH 7.2-7.4 (Yang & Xiong 2012) and almost all pH results stayed between those limits so difference between DM- and Hibernate A is not critical.

Thinner polyimide membrane kept cells quite viable. Attachment to membrane was on the same level as in PET membrane. Thicker PI membrane was the worse; cell layer did not stay unbroken in the demonstrated transportation and cell analysis was difficult when orange membrane gave coloured background for images. Even though earlier studies (Subrizi et al. 2012) showed that thicker PI is a suitable substrate material for RPE cells, according to this work its use cannot be recommended as much as thinner PI or PET.

It has to be kept in mind that in this experiment there were 7 PET membranes, 10 thicker PI membranes and 3 thinner PI membranes. High number of thicker PI samples makes the conclusion quite believable but three samples of thinner PI samples leave a thin doubt whether all thinner PI membranes can give such nice results or were three samples just better than an average. As mentioned earlier, results of this work are only suggestive and approximate and they could be confirmed with a higher amount of samples.

Trypan blue can be a good method for some other cells but not for pigmented RPE cells. This analysis method was left over from series 3 and 4 because of the low level of information in series 1 and 2. Despite the good sides of trypan blue like quickness, affordability and easiness, it cannot be recommended to be used in the co-operator's laboratory. Live/dead instead seems to be very useful method. Especially on PET and thinner PI membrane are very informative. Images of thicker PI are less informative and it could be justified to take some images with higher order magnification to see the cell surface in more detail. Calculated numbers of cells can give some additional information to support conclusions made of images but alone numbers have to be observed critically.

TER results measured from an insert were not very informative. Results were small and most of them could not be calculated into comparable form when the effect of membrane material and effective area are taken into account. Situation is a bit better with cuttings where TER results were more homogeneous in them. As explained in section earlier, low TER result is caused often of non-homogeneous cell layer. Probability for a hole in a cell layer is higher in a large area than in a small area. For CellCrown^{TM}'s those areas were 0.78 and 0.38 cm^2, for Millicell insert 0.33 cm^2 and only 0.031 cm^2 for cuttings.

In series 1 and 2 cells were plated on the membranes 39 to 40 days ago so they had been on the membranes longer than cells from the same cell line used in the study of Vaajasaari et al.2011. TER measurements were done 30, 60 and 90 days after removing cells and results showed that TER results increase when cells get older. Even though all calculated TER results of this experiment are higher than in that reference study, it cannot be said that cells in this work would have more tight junctions or a more homogeneous cell layer. Sampling is too small and when three of the eight samples had so low TER that they could not be calculated, average of eight measurements is not much better than in the study of Vaajasaari et al.

As a summary it can be said that TER measurement is not very useful tool to be used in the laboratory of the co-operator. It is known that TER results depend on the part of the insert or the membrane where TER was measured, measurer's experience and which TER meter was used. Even the same measurer can get changing results from the same insert not to mention that TER meter would change. Results are best comparable if they are taken by the same person with the same TER meter. With these arguments and results above it is justifiable to leave TER measurement out of transportation protocol.

Live/dead and phalloidin methods were as good as expected. Both methods were informative and suited for RPE cells; pigmentation was not a problem. Especially phalloidin staining is widely used in RPE studies (Pasovic et al. 2013, Savolainen et al. 2011) Most of the samples were coloured with phalloidin dilution 1:400 but also smaller might work if cells are not very pigmented.

Confocal microscope was used to get extra information about RPE cells on different membrane materials. Some important things were noticed when three samples were compared during imaging. First thing was that in sample G3 cell layer was only 12 µm thick when in samples E3 and F3 it was 24 µm. Probably the difference depends to the age of cells. In sample E3 and F3 cells were plated on the membranes 47 days ago and in sample G3 32 days ago. It is also possible that tight junctions had not yet formed as well in sample G3 than in other two samples. This supposition strengthens when looking at TER results and comparing three material types; thinner PI membrane had worse TER values measured of cutting. Sample G3 was also less pigmented than other two samples and also this is connected to age of cells.

10.1 Effect of the mechanical stress

It seems that mechanical stress has more effect on cell viability than temperature change or transportation medium. Orbital shaker has only one movement which makes inserts go around inside Falcon tubes and medium shaking against insert. Shaker does not simulate all stress what is caused during air and wheel transportation like pressure changes but it gives some idea how cells tolerate physical stress. Inspection with microscope showed that existent holes and discontinuities on cell layers enlarged and areas of stressed cells around those holes increased. Without orbital shaker results would have been more optimistic. It can be expected that those membranes that have a smooth and homogenous cell layer are after transportation are better to be transplanted into rabbits than those ones whose cell layer was already damaged before transportation.

If the mechanical stress caused by transportation would like to be known in more detail, it could have been tested with an acceleration transducer. A transducer can be send from Tampere to Germany and then the collected information can be transcribed. This causes extra cost for the planned transportation but might be a suitable tool if viability of the cells is in Germany lower than expected.

During this work an idea emerged whether the transportation box could be turned 90 degrees. Reason for that is that when Falcon tubes are horizontally and transportation box is moved, medium flows inside the tube and inserts move and turn. According to earlier tests this moving and fluid flow is more harmful for cells than temperature or the quality of the medium. In theory this turning could be possible but the answer of the transportation company was no. They justify their answer with the graphical appearance of the box which produces instructions how to handle it.

Medium flow might have been decreased with two changes. First one is the type of storage container. It might work to use flat cylinder instead of tube like a cell-well

added with a top. Diameter would not have to be much more than diameter of insert and volume of needed transportation medium would be only some millilitres. These cylinders might change their position inside the transportation bow but medium flow would be significantly smaller than in tubes. Other, more challenging solution to minimize medium flow against cells is to pack inserts in the same tube. Two or more inserts in one 50 ml tube might resist medium flow and stress per each inserts is smaller than if there is only one insert in each tube. Indeed a reliable way to stabilize inserts inside the tube is not known.

If the mechanical stress could be simulated better and the question of Falcon tubes in a standing position could be solved, it might be possible to use longer than 24 hours transportation. For example an estimated transportation time from Finland to the East Coast of the United States is around two days. In addition there is also customs and for a biological substance it might take one or two days before transportation can be continued. In the worse case cells arrive to customs just before weekend and cells have to wait almost a week before getting into an incubator. After couple of days the temperature inside a transport box is not the only problem; medium loses also its viability.
It is possible to test how long gel elements can emit heat like explained in earlier in this work. All eight gel elements had to be heated to body temperature to make sure that temperature stays high long enough. If temperature of the elements is higher than 37 °C, can temperature inside the transportation box rise to crucial.

10.2 Error sources

As every human made work, also this included several errors which might effect on the results. One significant source of cell stress was cutting of the membranes before analysis. PI membrane was first taken off between the ring part and the body part of the insert and then the extra part of membrane which did not have any cells was cut away with scalpel. For PET membrane extraction was simpler, the membrane was just cut away of the insert. Both membrane types were cut then into four or five pieces according to how many analyses were planned to be done. Especially thicker PI membrane was a bit tough to cut and scalpel had to twitch to separate membrane pieces. This twitching together with several grips of tweezers caused stress for cells. Stress was caused mainly to the edges of the cuttings but when a cutting is smaller than on average from five to ten square millimetres, there is not much unstressed area left on the cutting. That is the reason why it was challenging to image unharmed areas and the final results might show more unviable cells than will be in cells after a real transportation.

Human errors might have caused some changes in the results. Especially in the beginning of the experiment some practical matters were done in an illogical order and in a clumsy way. For example imaging with light microscope was slow in the beginning and samples had to wait at room temperature. In tryban blue staining samples should be imaged after 5 minutes but some of samples had to wait longer. Waiting does not effect to the fact that trypan blue does not suit well for pigmented RPE cells. Illogical work

order affected mostly to planning and timing but probably cells were not harmed and all experiments managed to be done.

The varying cell time on a membrane is one factor making the results less comparable. The used cells in this work were plated on the membranes 32, 39, 40 and 47 days ago. Practical matters like office time and reservation times of light microscope required a timetable for demonstrated transportation. Cells would have been ready for testing earlier but experiment could not be started earlier because ordering and shipment of transportation box took time. Timetable was also planned so that there was always three consecutive working days for transportation preparation and cell analysis. In the real transportation cells will be around the same age but more important than number of days is the degree of differentiation and pigmentation, morphology and viability. Timetable of the real transportation depends also on working days. Cells will be sent either on Monday or Tuesday; they are at the destination on Tuesday or Wednesday and will be transplanted on Wednesday or Thursday. Then researchers and lab technicians have one or two working days to control rabbits before a weekend.

The experimental part of this work was done in BioMediTech immunolaboratory which is practically an open laboratory. Instruments and equipments are not sterile and only a part of actions are done in a fume hood. Opthalmology laboratory is instead a closed laboratory which means that workers change special laboratory jacket, use headdress and long rubber gloves. Cleanness is not as good as in a real clean room but there are much less contaminants than in open laboratory. Some stress of cells might be caused by working in an open laboratory but risk of contamination can be minimized because before real transportation cells will be prepared and packed in the ophthalmology laboratory.

In this experiment cells were exposed to many temperature changes. First they were taken from + 37 °C to room temperature to be imaged and removed into Falcon tubes. Then Falcons were kept 24 hours in transportation box where temperature either lowered from room temperature under 10 °C or from 37 °C to 27 °C. After transportation cells were kept in room temperature before analysis and then either at 4 C° in storage or at 37 °C in incubator. Then again to room temperature and after imaging samples were stored in a freezer in case that some extra images are needed. In optimal situation cells stay nearby body temperature from Tampere laboratory to Germany laboratory. In Germany cells will be moved to incubators and they have one day to rest before membranes are transplanted into rabbits. If everything goes as planned there should not be significant stress in cells because of temperature changes.

11 CONCLUSIONS

In this experiment used RPE cells were similar to those which will be sent to Germany during year 2014. Cell culturing takes time and costs are higher with RPE cells compared to some other cells. For that reason the amount of cells plated on membrane was limited. From statistical point of view results of this experiment might not be so convincing but in general they give new information about RPE cells and mediums behaviour in different transportation circumstances.

Transportation company was chosen to be Cool ID. This Finnish company offers door to door service which includes the whole packaging. The company has experience of transportations in Europe. Transportations to North America are not so common and it is still a bit problematic how temperature inside the transportation box changes during two or three day's transportation and how cells survive.

Two mediums, DM- and Hibernate A were used parallel in this study. DM- is routinely used in RPE cell culture in the Ophthalmology group's laboratory. According to the results of this work there were no significant differences in cell viability. pH results were a bit higher in DM- than in Hibernate A which pH results were near to neutral. This difference is the main reason why Hibernate A is chosen to be the transportation medium.

Transportation temperature was chosen to be RT because of the literature and results of this work. Transportation in body temperature could have been the best but it was not an option because of the limited facilities of transportation companies. Cold transportation temperature might also be possible but then some degree changes in temperature could have more effect on metabolism of the cells than in RT. Gel elements keep temperature quite stabile inside the transportation box if transportation time stays under 24 hours. In longer transportations the heat capacity of the elements might be too low.

PET membranes are routinely used in the Ophthalmology group. This work supported earlier knowledge that PET is a suitable material for RPE cell culture. Cell layers were quite smooth and there were no dramatic changes in cell viability during transportation. Benefit of PET membrane is that membrane is put inside the insert industrially and membranes are equally smooth. PI membranes were instead put inside the inserts manually so there were more differences between membranes. Thinner PI suited also well for RPE cells. Changes in cell layer structure and cell viability were mainly caused by mechanical stress. Thinner PI membrane is easy to image because it is almost transparent. It should be also easy to transplant into rabbit's eye because it is so thin and flexible. Thicker PI was the worse; there were changes in cell layer even without me-

chanical stress and orange colour made imaging difficult. This membrane can be recommend to be used in laboratory conditions but not in animal tests.

When cell analysis methods are compared, it is clear that of immunohistochemical stainings live/dead and phalloidin were useful. Staining protocol is short and an experienced researcher or a laboratory technician can do it quickly. Trypan blue suits better for some other cell type but not for pigmented RPE cells. Measurement of pH is not very informative because results of pH before and after transportation differed even though there were no cells in the test medium. TER measurement can be left out because results depend on the measurement area and can vary a lot in different parts of an insert. Measurement from a clipping gives more trustable results but cutting harms membrane and it cannot be used after that.

Mechanical stress is a variable which seems to have more impact on cell viability than the other factors. The stress was not exactly mimicked in this work and in the real transportation it might be more critical to the cells. Results of this work showed that cell layers which were already non-uniform damaged more than smooth and uniform cell layers. It is recommended to send as high quality and uniform cell layers as possible. If mechanical stress could be minimized with different some solution like different packing method, cells might survive better.

As a conclusion it can be said that in a laboratory environment RPE cells tolerated transportation quite well. Cells should be viable enough to be transplanted to rabbits in Germany if they are transported according to transportation protocol (Appendix B). Notable is that all transportation conditions could not be simulated in laboratory and unexpected cell behaviour may turn up. If RPE cells survive well from transportation between Tampere and Germany, this protocol can be used from applicable parts in longer transportations also. It is probable that cells tolerate longer transportations as long as temperature does not lower too much and medium stays nutritious.

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APPENDIX A

Measured TER values and calculated live/dead numbers

Measured TER values

Sample	TER_i from insert (Ω) two measurements	TER average	TER_f from cutting (Ω)
A1	1033/991	1012	1055
A2	1256/1252	1254	825
B1	151/155	153	668
B2	140/120	130	680
C1	2010/2111	2061	964
C2	1590/1488	1539	1019
D1	246/206	226	776
D2	280/275	278	800

Sam-ple	TER_i from insert (Ω), two measurements	TER_i average	TER_f from inserts (Ω), two measurements	TER_f average	TER_f from cutting (Ω)
E1	450/880	665	401/460	431	910
E2	720/712	716	545/664	605	1004
E3	856/785	821	783/756	770	913
F1	90/66	78	125/120	123	640
F2	85/79	82	130/158	144	730
F3	68/70	69	143/140	142	938
G1	240/360	300	68/89	79	4776
G2	340/410	375	127/140	267	645
G3	462/560	511	128/140	134	596/635
H1	312/339	326	101/94	98	1129
H2	321/289	305	100/106	103	1081
H3	263/179	221	95/90	93	717/716

Calculated live/dead numbers

Sample	Live, place 1	Dead, place 1	L/D, place 1	Live, place 2	Dead, place 2	L/D, place 2	Live, place 3	Dead, place 3	L/D, place 3	L/D average
C1	86	35	2,4571	514	136	3,7794	498	633	0,7867	2,3411
C2	103	213	0,4836	157	106	1,4811	155	534	0,2903	0,7517
D1	33	112	0,2946	186	86	2,1628	594	354	1,6780	1,3785
D2	162	98	1,6531	149	46	3,2391	220	392	0,5612	1,8178
E1	563	963	0,5846	739	1384	0,5340	790	1148	0,6882	0,6022
E2	756	930	0,8129	951	1249	0,7614	345	521	0,6622	0,7455
E3	1431	271	5,2804	1569	624	2,5144	967	416	2,3245	3,3731
F1	513	481	1,0665	391	452	0,8650	486	411	1,1825	1,0380
F2	999	672	1,4866	814	223	3,6502	648	22	29,4545	11,5305
F3	499	40	12,4750	424	193	2,1969	237	146	1,6233	5,4317
G1	1975	687	2,8748	423	627	0,6746	1881	262	7,1794	3,5763
G2	419	968	0,4329	235	668	0,3518	156	265	0,5887	0,4578
G3	456	286	1,5944	143	602	0,2375	677	1094	0,6188	0,8169
H1	1805	252	7,1627	2169	456	4,7566	757	274	2,7628	4,8940
H2	755	54	13,9815	475	34	13,9706	1255	52	24,1346	17,3622
H3	966	444	2,1757	840	71	11,8310	1109	73	15,1918	9,7328