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<th><strong>Title</strong></th>
<th>Diffusion of oxygen and riboflavin during corneal cross-Linking (CXL)</th>
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<tr>
<td><strong>Authors(s)</strong></td>
<td>McQuaid, Rebecca Marian</td>
</tr>
<tr>
<td><strong>Publication date</strong></td>
<td>2017</td>
</tr>
<tr>
<td><strong>Publisher</strong></td>
<td>University College Dublin. School of Physics</td>
</tr>
<tr>
<td><strong>Link to online version</strong></td>
<td><a href="http://dissertations.umi.com/ucd:10155">http://dissertations.umi.com/ucd:10155</a></td>
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Diffusion of Oxygen and Riboflavin during Corneal Cross-Linking (CXL)

Rebecca Marian McQuaid
05432081

Thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

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April 27th 2017
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Abstract

Corneal Cross-Linking (CXL) is a one hour therapeutic treatment involving epithelial removal, application of riboflavin drops for 30 min. followed by UV light exposure to the cornea for the treatment of keratoconus, ectasia and infection. The photochemical reaction mechanism during CXL effectively halts the progression of corneal disease. The three main components of successful CXL are 1) the diffusion of riboflavin, 2) the presence of molecular oxygen (O₂) and the formation of reactive oxygen species, and 3) UV interactions during CXL which results in the biomechanical strengthening in the corneal stroma, thus halting disease progression. Recent applications such as reduced treatment time, epithelial by-passing and the combination of advanced CXL protocols as an alternative to refractive procedures have been investigated. The purposes of this study are to 1) investigate the diffusion of riboflavin using intra-stromal channels in order to determine the effective diffusion coefficients as compared to traditional axial diffusion with epithelium on or off, and 2) investigate O₂ behaviours during CXL in order to better understand Type I and II photochemical reactions using an established luminescence quenching technique.

Measurements of riboflavin diffusion using intra-stromal channels were created by means of a mechanical stromal instrument in whole-mounted post-mortem porcine eyes. The use of fluorescent imaging along with numerical modelling allows determination of effective diffusion coefficients under different conditions. Time-Correlated Single Photon Counting Phosphorescence Lifetime Imaging (TCSPC-PLIM) is an innovative and complex method for fluorescence and phosphorescence lifetime measurements. Phosphorescence sensitive O₂ based probes in soluble form have the ability to detect O₂ concentrations repeatedly and non-invasively in living biological tissue. This study investigated the use of O₂ sensitive phosphorescent probes using three models: (i) O₂-sensitive nanoparticles in soluble solution of riboflavin without collagen, (ii) collagen type-I gel with O₂-sensitive nanoparticles and riboflavin, and (iii) porcine eyes stained with O₂-sensitive nanoparticles and riboflavin. Several O₂-sensitive nanoparticle probes were evaluated. One probe was chosen (SII-
A), displaying sufficient brightness, photostability and efficient in-depth staining. Results showed O₂ behaviour after UV-induced CXL in all samples measured, concluding TCSPC-PLIM to be a novel, effective method in measuring CXL.
Declaration of authorship

I, Rebecca Marian McQuaid, declare that this thesis titled, 'Diffusion of oxygen and riboflavin during corneal cross-linking (CXL)' and the work presented in it are my own. I confirm that:

- This work was done entirely while in candidature for a research degree at this University.
- Where any part of this thesis has previously been submitted for a degree or any other qualifications at this University or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, this is always attributed.
- I have acknowledged all main sources of help.
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

Signed: 

Date: 
Thesis structure/Collaborations

**Chapter 1** addresses the physiological background of the eye, with special emphasis on the corneal structure. The role of oxygen in the eye is also discussed, and ophthalmic disease associated with oxidative stress. Finally, the author discusses corneal disease and describes treatment modalities for this disease.

**Chapter 2** introduces Corneal Cross-Linking (CXL) for the treatment of keratoconus, ectasia and bacterial infection. The history of CXL is briefly discussed, followed by the components that allow the safe application of CXL in the human eye (e.g. riboflavin, UV intensity). Finally, the use of CXL and alternative treatments such as refractive correction are briefly introduced.

**Chapter 3** discusses the photochemical reaction mechanisms during CXL including riboflavin excitation, type I and type II mechanisms and the relationship between and oxygen when the cornea is exposed to UV light.

**Chapter 4** gives experimental results for a riboflavin diffusion model done in-vitro with porcine corneas and with mathematical modelling. Prof. M. Mrochen assisted in the experimental set-up reviewed the manuscript for publication. Dr. B. Vohnsen performed Comsol™ simulations and reviewed the manuscript for publication. The author of this thesis (i) prepared samples for imaging (ii) performed cross-linking and diffusion measurements (iii) processed the data and analysed the results and (iv) prepared the manuscript in collaboration with Prof. Mrochen and Dr. Vohnsen.

**Chapters 5-8** introduce a non-invasive method of measuring oxygen concentrations in the cornea before and after corneal cross-linking, in order to investigate the role of Type I and Type II photochemical reactions. The experiments were performed in the School of Biochemistry and Cell Biology, UCC, Ireland in collaboration with Dr. Ruslan Dmitriev and Prof. Dmitri Papkovsky. Dr. Dmitriev controlled the PLIM system and assisted in preparation of O₂ samples. The O₂ probes were prepared by Prof S.Borisov (Graz University of Technology, Austria) in collaboration with UCC. Calibration of probe was performed by Dr. James Jenkins. Dr. B. Vohnsen performed Comsol™ simulations and reviewed the manuscript in preparation for publication. The author of this thesis (i) prepared samples for imaging (ii) performed cross-linking measurements (iii)
processed the data and analysed the results and (iv) prepared the thesis in collaboration with UCC and UCD.
Acknowledgements

So, here I am after 3.5 years. What an experience. I would first like to thank my principal supervisor Brian for his incredible support, guidance and encouragement throughout my MSc and PhD journey. I truly appreciate it.

I would like to thank Dr. Ruslan Dmitriev and Dr. Dmitri Papkovsky in UCC for agreeing to this amazing collaboration. When I approached them with the idea to apply their oxygen methods to the cornea, I thought it would take 3-6 months maximum, not 2 years! It has been a great experience and I really appreciate their patience, guidance and enthusiasm, especially when I needed it the most. I would also like to thank James and Neil from the Biophysics group; it was really nice to spend time with you guys.

The AOI Group past and present- lovely Denise, Ben, JiaJun, Sara, Atik, Vyas, Alessandra, Salihah and Najnin- you are a great group to be part of- thank you! I had so much fun with you. I would also like to acknowledge the UCD School of Physics staff, admin (John & Sarah), and management (Bairbre Fox). You have all been so kind since the day I began my studies. There was never a question I could not ask! Also thanks to UCD Seed Funding for giving me the opportunity to attend International conferences, and Arwa Bazaid for kindly helping me to culture the collagen type-I.

I would like to thank Marc Friedman, Sabine Kling and Silvia Schumacher for scientific advice and support. I learned the only way to expand your scientific knowledge is to ask, engage and discuss. Thank you for this.

I want to say an enormous thank you to Michael Mrochen for taking the risk to fund my project with the Irish Research Council EPSPG/2013/598. Thank you for being a terrific mentor, inspiration and friend throughout my MSc and PhD.

I would also like to give a heartfelt thank you to the Wellington Eye Clinic. Dr. Arthur Cummings hired me in 2009 as an Ophthalmic technician/research assistant. I never thought for one moment that I would go on to do a PhD. It was Arthur’s confidence in me that pushed me to achieve great things. *Thank you*. I also want to thank nurse manager Lisa (for putting up with me throughout the years!), Clinic manager Ed, and the Welly girls: Maria, Celine, TT, Amo, Isobel, Liz, Rachel, Carol & all of the Stephs. Thank you guys.

To my mum, dad, Gill, Billy, my little niece Megan and my closest friend Jen, thank you for believing in me and supporting me every day. Without you, I am nothing.
Peer reviewed articles


McQuaid R, Dmitriev RI, Papkovsky DB, Borisov S, Mrochen M, Vohnsen B. In-Vitro Estimation of O2 Concentrations during Corneal Cross-Linking (CXL) for Porcine Corneas and Collagen Type-I Gels. (Manuscript in preparation)

Book chapter contributions


Oral Conference presentations

Rebecca McQuaid, Michael Mrochen, Brian Vohnsen. "Rate of riboflavin diffusion for corneal cross-linking". Photonics Ireland, Cork, September 2015.


**Poster Conference presentations**


Rebecca McQuaid, JiaJun Li, Michael Mrochen, Brian Vohnsen. "Lateral diffusion coefficient in CXL using corneal channels”. ARVO May 2014, Orlando Fl.

Rebecca McQuaid, Michael Mrochen, Brian Vohnsen. "Rate of riboflavin diffusion prior to corneal cross-linking (CXL) under the influence of corneal channels”. 10th International Congress of Corneal Cross-Linking. Zurich, Switzerland, December 5-6th 2014.


Rebecca McQuaid, Michael Mrochen, Ruslan Dmitriev, Dmitri Papkovsky, Brian Vohnsen. "Monitoring of O₂ concentrations during CXL by Phosphorescence Lifetime Imaging Microscopy (PLIM)”. ARVO May 2016, Seattle WA.

Rebecca McQuaid, Michael Mrochen, Ruslan Dmitriev, Dmitri Papkovsky, Brian Vohnsen. "In-Vitro Estimation of O₂ Concentrations during Corneal Cross-Linking (CXL) for Porcine Corneas and Collagen Type-I Gels”. ARVO May 2017, Baltimore MA.
Abbreviations

O₂ (Oxygen)
FS (Femtosecond)
LASIK (Laser Assisted Intrastromal Keratomileusis)
ROS (Reactive oxygen species)
HIF (Hypoxia Inducible Factor)
CNV (Choroidal neovascularization)
HBO (Hyperbaric oxygen therapy)
KC (Keratoconus)
KG (Keratoglobus)
PMD (Pellucid Marginal Degeneration)
CCT (Central corneal thickness)
CL (Contact lens)
CXL (Corneal Cross-Linking)
KP (Penetrating Keratoplasty)
RGP (Rigid Gas Permeable Lenses)
ICRS (Intrastromal ring segments)
UV (Ultraviolet)
SHG (Second-Harmonic Generation)
Trans-epi (Trans-epithelium)
BAC (Benzalkonium chloride)
PiXL (Photorefractive intrastromal cross-linking)
D (Diopters)
SMILE (Small Incision Lenticule Extraction)
BSCVA (Best Spectacle-Corrected Visual Acuity)
AP (Athens Protocol)
PRK (Photorefractive Keratectomy)
PACK (Photo-activated Chromophore for Keratitis-Collagen Cross-Linking)
NCT (Non-contact tonometry)
CH (Corneal hysteresis)
CRF (Corneal resistance factor)
IOP (Intra-ocular pressure)
ORA (Ocular response analyser)
CCD (Charge Coupled Device)
OCT (Ocular Coherence Tomography)
BSS (Balanced Salt Solution)
FWHM (Full Width at Half Maximum)
FEM (Finite Element Modelling)
PO₂ (Oxygen partial pressure)
NO (nitric oxide)
TM (Trabecular meshwork)
TCSPC-PLIM (Time-Correlated Single Photon Counting Phosphorescence Lifetime Imaging)
NP (Nanoparticle)
IRF (Instrument Response Function)
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Figure 7.10. Control results for 1) without substrate (i.e channels), 2) Collagen type-I, and 3) Porcine cornea. Little to no change in O$_2$ concentration after UV can be seen in the absence of riboflavin. Colour images display phosphorescence decay changes (seen in scale bar), approximate ROI are indicated within the red boxes. Scale bar 500 µm.

Figure 7.11. Solution based channels displaying O$_2$ changes before and after UV-corneal cross-linking. (A) displays phosphorescence decays (µs), (B) is the converted O$_2$ concentration (µM). A-F describes the stages of imaging. Phosphorescence decay of SII-A ranges from 10 (200 µM)-18 µs (0 µM).
Figure 7.12. Colour images of lifetimes for one channel. Approximate regions of interest (ROI) during analysis are indicated within the red arrows. Phosphorescence decay of SII-A ranges from 10 (200 µM)-18 µs (0 µM). Scale bar 500 µm.

Figure 7.13. Distribution histogram of one solution-based micro-chamber channel. Changes in phosphorescence decays can be seen after UV exposure, followed by a dramatic increase in decays after sulphite addition.

Figure 7.14. Boxplots of 10 collagen samples displaying O₂ changes before and CXL (above). Phosphorescence decays (µs) are shown on the left, converted O₂ concentration (µM) (right). Phosphorescence decay of SII-A ranges from 10 (200 µM)-18 µs (0 µM).

Figure 7.15. Colour images of one collagen sample with approximate ROI indicated within the red boxes. Phosphorescence decay of SII-A ranges from 10 (200 µM)-18 µs (0 µM). Scale bar 500 µm.

Figure 7.16. Average distribution histogram of one collagen type-I sample.

Figure 7.17. Boxplots of 4 porcine eyes displaying O₂ changes before and CXL (above). Phosphorescence decays (µs) are shown on the left, converted O₂ concentration (µM) (right).

Figure 7.18. Colour images of one eye with ROI indicated within the red boxes. Phosphorescence decay of SII-A ranges from 10 (200 µM)-18 µs (0 µM). Little to no change can be observed after sulphite addition. Scale bar 500 µm.

Figure 7.19. Average distribution histogram of one porcine cornea.

Figure 7.20. Diffusion model estimating O₂ throughout the cornea. 0.1mm represents the experimental results in this study.

Figure 7.21. Diffusion model based on experimental findings in this study. O₂ is fully depleted upon illumination, followed by replenishment over time (t=0-200 sec)
Chapter 1: The Human eye, function, structure

This chapter will focus on the function and components of the eye, limitations of oxygen (O₂) in the eye and diseases that can occur in the anterior segment.

The eyes play a key role in forming images and transmitting visual sensations to the brain. Each eye is divided into three main components: The anterior chamber, posterior chamber and the vitreous chamber. The anterior chamber lies between the cornea and iris and it is comprised of the aqueous humour which contributes to the approximately hemispherical shape of the cornea. The posterior chamber contains the iris and it plays an important function in the size of its aperture (i.e. pupil expansion and reduction). The ciliary body is comprised of aqueous fluid, and the lens which is responsible for accommodation (the eye’s ability to focus on near or far objects). The vitreous chamber is comprised of the vitreous humour, which is transparent and lies between the lens and retina. The cornea and lens focus incoming light onto the retina at the back of the eye which allows photoreceptors to absorb and convert images into chemical and electrical signals. Images are then transmitted by the optic nerve into the visual cortex for further processing and final acuity of the visual information (Fig 1.1).
1.1 The cornea

The cornea is the outermost layer of the eye and it is comprised of highly organised collagen fibrils, surrounded by a proteoglycan rich extrafibrillar environment. The tightly packed and highly rigid collagen fibrils determine corneal shape and curvature and therefore its refractive power. Due to its high transparency, it enables transmission of up to 90% of light at visible wavelengths (450nm to 600nm and even up to 98% for wavelengths from 600-1000nm). The surface of the cornea is convex and aspheric, measuring 11 to 12 mm in the horizontal diameter, 9 to 11 mm in the vertical diameter with an average central thickness of 520 µm to 670 µm peripherally. Corneal curvature is steepest in the centre and flatter in the periphery with asphericity of $Q = -0.3$. Average values for the radius of curvature for the anterior surface is 7.8 mm and 6.5 mm for the posterior surface. The aqueous humor in the anterior chamber of the eye provides the cornea with nutrients, whilst a layer of tear-film on the exterior surface of the cornea ensures lubrication and maintains a smooth surface.

1.2 Layers of the cornea

Each layer of the cornea represents a biological function while ensuring transparency and proper optical functioning. The main layers are shown in Fig. 1.2 A-E and are described below.

![Histology of the cornea indicating the main five layers](image)
1.2.1 Epithelium

The epithelium is the outermost layer of cornea and mainly controls hydration from the anterior side through the prevention of liquids from entering the stroma. Epithelial thickness is approx 50 µm, consisting of a single layer of basel cells with 4-5 layers of squamish cells\(^6\). The tear film (~3µm) lies over the epithelium and can recover from abrasion due to stem cells located in the limbus\(^6\).

1.2.2 Bowman’s layer

The Bowman’s layer is a nonregenerating, acellular layer located between the epithelial basement membrane and anterior corneal stroma. It is comprised of densely packed collagen fibrils 8-14 µm thick\(^7\). Collagen type-I and III fibrils are present in both the Bowman’s layer and stroma, however fibril diameter is slightly smaller (20-30 nm) when compared to the stroma (22-35 nm)(Fig. 1.2 B).

1.2.3 Stroma

The stroma is located beneath Bowman’s layer (Fig. 1.2 C). It is the main layer representing up to ~90% of the total corneal thickness. Collagen type-I is the most abundant protein in the stroma, it makes up of approximately 68% dry weight\(^8\). The stroma contains 78% water and 16% collagen (mainly type-I, however type III, V and VI are also present). The collagen contained in the cornea maintains elasticity and strength which determines most biomechanical properties of the cornea in this layer. It also maintains its shape and transparency. The extracellular matrix, nerve fibres and keratocytes are also located in the stroma along with collagen fibres which form lamellae of about 1.5 – 2.5 µm thickness. Approximately 200-300 lamellae layers stretch from limbus to limbus, creating the spherical shape of the cornea\(^9\). Glycosaminoglycans fill the extracellular space between collagen fibres that form proteoglycans, a protein normally present in connective tissue. The role of glycosaminoglycans subsidizes corneal hydration, as they have the ability to absorb large amounts of water\(^10\)(Fig. 1.2 A).
1.2.4 Collagen lamellae

Collagen is laid down in fibrils which are arranged in bundles or lamellae in the corneal stroma. These collagen fibrils are bounded by a matrix of proteoglycans, which can be described as the packing substance existing between cells in an organism. These lamellae run parallel in the stroma. Bowman’s layer contains densely packed collagen fibrils which become part of the anterior stroma lamellae, determining the shape of the cornea (Fig. 1.3). Most of the lamellae are found in the corneal stroma. The anterior and posterior lamellae are morphologically different to one another. The posterior lamellae are wider (100-200 µm) and thicker (1.0-2.5 µm), whilst the anterior lamellae are shorter (0.5-30 µm) and narrower (0.2-1.2 µm). Collagen fibrils are seen to anchor the cornea to the sclera, providing structural support and rigidity.

Fig. 1.3: Lamellae split in both anterior and posterior directions, as well as parallel to the corneal surface. Fibres cross over or interweave between branches of lamellae. Large angles are created with lamellae adjacent to each other (indicated by large arrow). Near to the front of the stroma, lamellae are seen to split and interweave (indicated by arrow). Scale bar =10 µm.

1.2.5 Descemet’s membrane

Descemet’s membrane is located in the posterior segment (Fig. 1.2 D). It contains collagen type-IV, laminin and fibronectin. Collagen type-IV is 15-20 nm thick and is mechanically weaker than fibrilar collagen type-I. The thickness of the membrane is 5-10 µm and its function is to prevent aqueous humour from diffusing into the stroma.
Endothelial cells lie tightly packed below Descemet’s membrane. It does not regenerate after rupture.  

1.2.6 Endothelium  

The endothelium is the innermost layer of the cornea (Fig. 1.2 E). Endothelial cells are essential to keep the cornea clear by means of pumping excess fluid out of the stroma to maintain hydration. Without this pumping function, the stroma will swell, become opaque and obstruct vision. Endothelial cells are polygonal, with an approximate diameter of 20 µm and thickness of 5 µm.  

1.3 Refractive power of the cornea  

Refractive or optical power is the ability of light rays to bend as they pass through the eye. The cornea is the core refractive element of the eye (40-43 Diopters) due to its convex hemispherical shape. The curvature of the anterior cornea contributes to about 90% of the corneal refractive power and about 65% of the absolute refractive power of the eye. The refractive index is larger in the anterior (1.376) segment compared to the posterior cornea (1.336). Due to the corneas significant contribution to the refractive power of the eye, any corneal irregularities can reduce visual acuity, image contrast and resolution.  

1.4 Refractive error and correction  

Refractive aberrations or irregularities in the cornea or eye can create problems for vision. Myopia (or short-sightedness) is the most common type of refractive error, affecting an estimated 2.5 billion people and thus about 35% worldwide. It occurs when light and images are focused in front of the retina rather than directly on its surface, causing a spherically defocused image. Correction for myopia can be through refractive surgery or with glasses or contact lenses adding negative power.  

Hyperopia (or long-sightedness) affects approximately 10% of the global population. It is caused by light focusing behind the retina rather than directly on its surface, resulting in a spherically defocused image. Correction for hyperopia can be through
refractive surgery or with glasses or contact lenses adding positive power. Astigmatism is present when light falls both in front and behind the retina along two different meridians, resulting in a distorted image. This occurs when the ocular refraction is different along the horizontal and vertical axes. A certain range of astigmatism can be treated through refractive surgery, or with specialized corrective lenses.

LASIK (Laser Assisted Intrastromal Keratomileusis) is a type of refractive surgery for the correction of myopia, hyperopia and astigmatism. This is achieved through the creation of a flap by a femtosecond (fs) laser or keratome in the cornea, and then using another laser to ablate the stroma and therefore change its refractive power. Customised laser eye surgery based on wavefront-guided aberrations can be achieved through the use of clinical diagnostic equipment which ultimately enhances vision (such as glare) post operatively.

1.5 Oxygen in the eye

In order to maintain health of the eye, a required amount of oxygen (O₂) is needed. Most of the anterior segment (cornea) obtains O₂ through atmospheric conditions whereas the posterior eye and retina receives O₂ via blood flow.

1.6 Molecular Oxygen (O₂)

Molecular oxygen is of utmost importance for biological systems and living organisms. It is vital for gene expression¹⁵,¹⁶, the regulation of metabolism via cellular respiration¹⁷, and photochemical processes¹⁸. The O₂ capacity for air is approximately 50 times higher than its solubility in water¹⁵. O₂ diffuses fast across cell membranes, tissues and solid matter, which can be a challenge when measuring O₂ concentrations in animal or cell samples. As a result of such a high diffusion rate, changes in ambient air can greatly affect O₂ concentrations and alter conditions in laboratory experiments. With moderate solubility in aqueous solutions and under atmospheric conditions, O₂ is dissolved under concentrations of 219 µM at 35°C and is strongly influenced by temperature and salinity. The normal atmospheric concentration of O₂ is 20.86%¹⁹,²⁰.
1.7 Diffusion of oxygen (O₂) in the eye

The cornea relies on atmospheric O₂ (approx. 155 mm Hg) and nutrients supplied by the tear film to maintain stability due to the lack of blood vessels in its avascular structure. The epi- and endothelium have a lower diffusivity than the stroma due to their protective functions. Upon closure of the eyelid, the cornea maintains O₂ uptake through blood vessels in the ciliary vasculature, shown in Fig. 1.4 (61.5 mm Hg).

Diffusion of O₂ in the cornea was established in the early 1970s. Improved scientific knowledge led to a two-dimensional model to determine O₂ diffusion in contact lenses. Larrea and Buchler reported an oxygen diffusion coefficient of $2.81 \times 10^{-3} \text{ mm}^2/\text{s}$ for the human corneal stroma. Earlier studies reported values in-vivo close to $7 \times 10^{-6} \text{ cm}^2/\text{s}$. Kamaev et al. reported $4 \times 10^{-6} \text{ cm}^2/\text{s}$ at 25° and $6 \times 10^{-6} \text{ cm}^2/\text{s}$ at 35°. The fluctuating data suggests corneal hydration and temperature play a role as well as individual differences to measured diffusivity in-vivo or ex-vivo.
1.8 Problem of O₂ in the eye

Excessive or reduced O₂ in the eye can contribute to the pathogenesis in many sight threatening ocular diseases. Oxidative stress is a common cause of abnormalities caused by elevated or reduced oxygen concentrations and can be described as the disruption of cellular balance due to elevated levels of Reactive Oxygen Species (ROS) production. ROS are created when radical molecular oxygen is exposed to specific conditions such as ionizing radiation\textsuperscript{32,33}, exposure to UV light\textsuperscript{34,35} or as a product of the respiratory chain in mitochondria, photochemical and enzymatic reactions\textsuperscript{36}. ROS consist of superoxide anion (\(O_2^-\), half-life 1-15 minutes\textsuperscript{37}, hydroxyl reaction (\(OH^-\), half-life \(10^{-9}\) sec\textsuperscript{38}, hydrogen peroxide (\(H_2O_2\), half-life of 8 hours in freshwater\textsuperscript{39} and singlet oxygen (\(^1O_2\), half-life \(~3.5\ \mu s\)\textsuperscript{40}.

1.8.1 Oxidative stress and ocular disease

Oxygen is responsible for the regulation of the hypoxia-inducible factor (HIF-1\(\alpha\)), a process of gene expression that responds to decreases in O₂ in the cellular environment, or hypoxia, which is defined as oxygen concentrations that are below normal conditions. When cells in the retina become hypoxic, HIF-1\(\alpha\) can accumulate and release a large number of genes which leads to the formation of oxygen-dependent diseases such as diabetic retinopathy\textsuperscript{41} and glaucoma\textsuperscript{42}. In the retina, high O₂ tension and high levels of polyunsaturated fatty acids in the photoreceptor outer segments enhance vulnerability to oxidative damage. Choroidal neovascularization (CNV), a main component of age-related macular degeneration is also contributory to oxidative stress\textsuperscript{43}.

In a study conducted in 1984, Palmquist et al\textsuperscript{44} found nuclear lens opacities (or cataracts) formed in patients after prolonged exposure to hyperbaric oxygen therapy (HBO- the medical use of oxygen at an ambient pressure higher than atmospheric levels). Further studies concluded that cataracts are formed mainly from oxidative stress, particularly ROS contributor \(H_2O_2\)\textsuperscript{45}. Post vitrectomy subjects are also prone to cataract formation due to the high levels of oxygen infused intra-operatively\textsuperscript{46}. In the cornea, prolonged use of contact lenses results in the formation of neovascularization
and corneal edema, caused by chronic hypoxia from the inability of atmospheric O₂ to flow freely through the lenses covering the entire cornea⁴⁷. Hypoxia in the cornea has also been connected to alterations in corneal hydration⁴⁸.

1.9 Corneal disease

Refractive error is common in the population¹³. However, certain corneal diseases can cause further refractive complications and light scattering that cannot be corrected with glasses, contact lenses or refractive correction.

1.9.1 Corneal ectasia

Corneal ectasia can be classified into three primary disease types: keratoconus (KC), pellucid marginal degeneration (PMD), or keratoglobus KG (not discussed). Corneal ectasia occurs due to chronic biomechanical failure, leading to thinning and protrusion of the cornea⁴⁹. Due to the biomechanical failure, the condition causes astigmatism and irregularities in the form of higher order aberrations. Ectasia is progressive, often occurring after trauma or refractive procedures such as LASIK⁵⁰.

1.9.2 Pellucid marginal degeneration (PMD)

PMD is a rare, bilateral, asymmetric, noninflammatory, progressive, ectatic disease of the cornea⁵¹. It shows as peripheral corneal thinning, whereas central corneal thickness (CCT) is usually unaffected. PMD is less prevalent than keratoconus, but occurs more than keratoglobus⁵¹-⁵³. PMD predominantly affects males between the second and fifth decade⁵⁴. Treatment can include spectacle or contact lens (CL) fitting, corneal cross-linking⁵⁵, lamellar keratoplasty⁵⁶ or intrastromal ring segments⁵⁷,⁵⁸. PMD can easily be misdiagnosed as keratoconus in its later stages due to the cone-like structure in the corneal periphery and irregular astigmatism. Therefore, corneal topography or other diagnostic devices such as Corvis ST is essential for proper diagnosis⁵⁹.
1.9.3 Infectious keratitis

Infectious keratitis is also known as a corneal ulcer which can be caused by many factors such as virus, fungi, mycobacteria and protozoa. Contact lens wear is the primary cause of infection\textsuperscript{60-62}. Treatment can be topical antibiotic steroids, or in recent studies, therapeutic corneal cross-linking (PACK-CXL), described in the following chapter.

1.10 Keratoconus

Keratoconus (KC) is the most common form of ectasia. The term keratoconus, originating from the Greek term (kerato: Cornea; konos: cone) meaning a cone shaped steepening of the cornea. Keratoconus is a progressive, non-inflammatory disorder occurring in the anterior cornea that results in structural weakening of collagen fibres which ultimately leads to corneal thinning and a loss of rigidity (conical bulging). A typical example of a cornea affected by keratoconus compared to a normal structure is displayed in Fig.1.5. It can occur unilaterally, generally due to eye rubbing, but it mostly found bilaterally.

![Fig. 1.5. Schematic diagram of collagen lamellae (A) Normal structure, and (B, C) structure in patients with mild to severe keratoconus\textsuperscript{63}.](image-url)
1.10.1 Keratoconus Incidence and Prevalence

Keratoconus typically occurs at puberty early teens or mid-twenties. It can also form in later stages of life as a result of laser eye surgery (ectasia), or corneal injury. Environmental incidence of keratoconus such as eye rubbing, ocular inflammation (atrophy), and allergies prompted by pollen, dust, and animal fur have been observed. Patients with Down Syndrome have also been linked to KC. Studies have also shown changes of oestrogen, cortisol and thyroxin levels throughout pregnancy can contribute to weakening of corneal biomechanics and thus activates keratoconus or post-LASIK ectasia.

The prevalence of keratoconus is primarily known to affect every 1 in 2000 people. Over time, factors such as geographical location, ethnic background and diagnostic criteria were taken into consideration, resulting in varied populations in each country. Population studies have shown a high keratoconus prevalence of 2.3% in Central India, and 2.5% in Iran. Authors postulated a possible reason for a high prevalence is the hot, sunny climates in which ultraviolet light may have a role to play. Cooler climates with less sunshine such as Russia, Finland, Denmark, Japan and the Netherlands have shown a lower prevalence in comparison. Differences in ethnic backgrounds also contribute to the prevalence of keratoconus. Studies have found a greater presence of KC in Asian subjects when compared to white Caucasians. The authors postulated the high prevalence in Asian communities could be due to consanguinity, a factor typically associated with the hereditary of a population or nation. To the author’s knowledge, there is no ongoing population study of keratoconus prevalence in Ireland. However, the Light for Sight Foundation in Zurich, Switzerland has created a worldwide study in order to gain more up to date knowledge of KC prevalence in current populations.

1.10.2 Clinical observations

Physical eye examination (Snellen chart, refraction and slip-lamp examination) and the use of analytical equipment such as corneal Topography aid keratoconus diagnosis. Diagnostic signs of keratoconus are increased astigmatism, corneal
thinning, and presence of scarring or Vogts striae. Keratoconus can be classified in four stages according to the Amsler-Krumeich classification system, shown in Table 1\textsuperscript{93-95}. This enables practitioners to diagnose keratoconus by following the guidelines.

Table 1. Amsler-Krumeich classification system displaying Stage I-IV of progressive keratoconus.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Clinical observations:</th>
</tr>
</thead>
</table>
| I     | • Eccentric Steepening  
       | • Myopia and Astigmatism <5.00 D  
       | • Mean central Keratometry (K) readings <48.00 Diopters |
| II    | • Myopia and Astigmatism from 5.00-8.00 Diopters  
       | • Mean central K readings <53.00 Diopters  
       | • Absence of scarring  
       | • Minimum corneal thickness <400µm |
| III   | • Myopia and Astigmatism from 8-10 Diopters  
       | • Mean central K readings >53.00 Diopters  
       | • Absence of scarring  
       | • Minimum corneal thickness 300-400µm |
| IV    | • Refraction not measurable  
       | • Mean central K readings >55.00 Diopters  
       | • Central corneal scarring  
       | • Minimum corneal thickness 200µm |

In 2012, Belin et al\textsuperscript{96} classified KC into five categories which allow clinicians to follow progression and symptoms of the disease. Based on the classifications mentioned, the clinician can decide which step of treatment to pursue.

I. Symptomatic KC: Irregular astigmatism is present due to changes on the anterior corneal surface, resulting in a loss of visual function.

II. Asymptomatic KC: Changes in corneal thickness and regular astigmatism is present. The anterior corneal surface shows steepening (keratometry) with
minor vision loss (e.g. contrast sensitivity, higher-order aberrations) may be present, but not significant enough to cause symptoms.

III. Progressive or non-progressive disease is defined by the increasing loss of visual function, advancing ectatic change or further corneal thinning. Diagnostic equipment such as tomography, topography or pachymetry measurement is the best way to determine these parameters.

IV. KC suspects (KCS): This category can be defined as a patient who does not display obvious disease, but have either a strong family history of ectatic disease or presents with one or more known associated parameters (i.e. corneal thickness, anterior and posterior elevation, biomechanical change) that are outside the normal range but do not meet the criteria of clinical disease\textsuperscript{96}.

1.11 Keratoconus treatments

Clinical observations and classification of keratoconus have been established. The treatment of keratoconus has been widely discussed in terms of which method is the most effective in halting the progression of keratoconus. Treatment for keratoconus is discussed below. Chapter Two will focus on therapeutic corneal cross-linking (CXL).

1.11.1 Penetrating Keratoplasty (PK)

Penetrating Keratoplasty (PK), otherwise known as a corneal transplant, is the longstanding method of treating keratoconus. It involves the replacement of the diseased tissue with a donor corneal graft which restores vision. Due to economic costs associated with transplants, biocompatibility, and reduced availability of donor corneas, PK is not the foremost option for keratoconus treatment. However, if the keratoconus is too advanced and continuously worsening, PK may be required.

1.11.2 Rigid Gas permeable lenses (RGPs)

The use of rigid gas permeable lenses (RGPs) can be used as an alternative to soft contact lenses to restore normal visual acuity. Due to apical protrusion that occurs in keratoconus, soft contact lenses can only be worn in the early stages of KC. RGPs have a double effect of flattening the cone due to its hard material, therefore achieving
adequate vision. However, advanced KC patients are unable to be fitted to RGPs due to advanced corneal steepening, therefore treatment is required.

1.11.3 Intrastromal Corneal Ring Segments (ICRS)

Intrastromal corneal ring segments (ICRS) are predominantly used to flatten corneal curvature as a result of keratoconus, and effectively increase biomechanical rigidity by increasing corneal stability. ICRS can also be used to correct myopia and high astigmatism. The procedure involves a 2 mm radial incision (Fig. 1.6) corneal pocket at 70-80% corneal thickness using a keraring or femtosecond laser, followed by insertion of the ring segments in the corneal stroma. Therapeutic Ultra-Violet (UV) Corneal cross-linking (CXL) can also be achieved after ICRS insertion to “lock in” the effect of corneal flattening.

In summary, the cornea is comprised of mostly collagen type-I protein, which determines its biomechanical properties, shape and transparency. The measurement of oxygen in the eye is important to better understand the role of oxidative stress and ocular disease. Refractive error such as myopia and hyperopia is prevalent worldwide, and contact lenses, glasses or refractive treatments are required for correction. Some refractive errors such as ectasia and keratoconus cannot be effectively corrected and may require further treatment.
Chapter 2: Clinical applications of Corneal Cross-Linking (CXL)

Corneal cross-linking (CXL) has become an established surgical procedure since its clinical introduction in 2003. The intention of CXL is to increase the biomechanical strength of the cornea in order to stop the weakening of collagen packing which leads to ectasia. The most common use of CXL is to inhibit the progression of keratoconus\textsuperscript{98}, corneal infection\textsuperscript{99} and post LASIK-ectasia\textsuperscript{100}. This chapter will summarise the components that makes CXL successful, along with current treatment modalities that have advanced CXL since its introduction.

2.1 History of CXL

Collagen corneal cross-linking was first investigated experimentally (1993) in the University of Dresden by Spoerl et al\textsuperscript{101}. The aim of their study was to find a treatment that could stabilise biomechanical weakening of the cornea resulting in keratectasia. It is known that cross-linking occurs naturally over time by the enzyme lysyl oxidase\textsuperscript{102}. Initial experiments explored cross-linking agents using porcine eyes treated with riboflavin and UV light of different intensities and treatment times, chemical cross-linking agents glutaraldehyde and Karnovsky’s solution, and aldehyde sugars (ribose, methylglyoxal, glucose and glyceraldehyde) of varying concentrations. Results revealed significant stiffness with stress-strain measurements in glutaraldehyde and Karnovsky’s solution and in riboflavin/UV interactions\textsuperscript{103}. This finding began exciting developments of the possibility of applying CXL to an ectatic cornea. Further studies examined the appropriate intensity of UV needed for efficient CXL\textsuperscript{104}, riboflavin wavelength\textsuperscript{105}, absorption\textsuperscript{106}, treatment time\textsuperscript{106}, and endothelial cell studies to monitor possible damage from CXL\textsuperscript{107-109}. The treatment was also tested on human corneas to ensure safety to endothelial cells\textsuperscript{117}. The first keratoconus patient was treated in 1998, followed by a nonrandomised clinical pilot study in 23 eyes with moderate or advanced keratoconus\textsuperscript{98,118}. Results from this study showed treatment with riboflavin-induced UV-cross-linking successfully halted the progression of keratoconus in all eyes and
caused disease regression in 70% of the cases. Following this, the Dresden protocol was introduced.

### 2.2 CXL Procedure (Dresden protocol)

The standard ‘Dresden protocol’ is the conventional method of the CXL procedure which involves epithelial removal, followed by drops of isotonic riboflavin (vitamin B2) with 20% dextran on the cornea for 30 min. Once sufficient diffusion of riboflavin has taken place in the anterior chamber (witnessed as a blue band on a slit lamp), the cornea is exposed to 3mW/cm² ultra-violet (UVA) for 30 min. Multiple clinical studies have been reported in the past 15 years using the Dresden protocol in Europe and around the world. In 2016, the Dresden protocol was approved by the FDA in the United States.

### 2.3 Photosensitizers

A photosensitizer can be described as a molecule that creates a chemical change in another molecule via a photochemical process. Photosensitizers can react with living cells or tissues, or can occur in pure chemical systems. In this study, the photosensitizer riboflavin has been chosen because of its hydrophilic properties and ability to diffuse with ease into corneal layers over a short period of time, and effectively react when triggered by UV light.

#### 2.3.1 Riboflavin (vitamin B2) as a photosensitizer

The photosensitizer riboflavin phosphate is a highly absorbing, hydrophilic molecule, playing a key role in the photochemical CXL process through the absorption of UV photons. The excited riboflavin molecules have the ability to transfer the energy to surrounding reaction partners such as oxygen or other molecules. This triggers the formation of molecular crosslinks that lead to stabilization of corneal curvature. Thus, the activated riboflavin needs O₂ in order to create the oxygen radicals that form covalent crosslinks throughout the corneal stroma. Apart from its active role in CXL, riboflavin provides a safety barrier that hinders UV exposure of the posterior parts of
the eye and thereby retinal damage\textsuperscript{110}. Therefore, the corneal epithelium must be removed for sufficient riboflavin to diffuse across the stroma before treatment.

### 2.3.2 Riboflavin Absorption Spectrum

Riboflavin has several absorption maxima in the UV range (270, 366, 445 nm), shown in Fig. 2.1. At 270 nm, there is a high absorption by DNA resulting in tissue damage by photo-conjunctivitis and photo-keratitis. At 445 nm, photo-chemical damage in the retinal pigment epithelium (RPE) may occur due to high intensity in the visible spectrum. However, using a wavelength of 366 nm is considered the safest and most efficient choice for CXL as unwanted side effects in the cornea or retina are minimized\textsuperscript{111}.

![Fig. 2.1: Absorption and fluorescence emission spectra of diluted Hypotonic 0.5% riboflavin (0.01%) T=20°C (normalized).](image)

The UV light distribution within the tissue is responsible for riboflavin photo-activation through the photosensitization process. Thus, riboflavin needs $O_2$ to create oxygen radicals to maximise the CXL effect within the cornea. Thus, the diffusion of riboflavin, the absorbed number of photons (energy dose) of UV light and concentration of $O_2$ within the tissue are of relevance to optimize the CXL photochemical effect. This will be discussed in more detail in Chapter 3.
### 2.3.3 Photochemical reactions in riboflavin

During CXL, riboflavin is the primary acceptor of the UV radiation and serves as a photo initiator of the active forms of oxygen known as 1) Singlet $O_2$ and 2) superoxide anion. The UV light triggers a photochemical reaction in riboflavin when absorption occurs in the cornea stroma. This alters the riboflavin molecules to an excited state, which can then transfer electrons, hydrogen atoms or excitation of energy to other molecules\(^{31}\).

The excitement of Riboflavin molecules to singlet and triplet states when exposed to light; can be described as:

$$ R_f \rightarrow R_f^1, I_{\text{abs}} \quad (1) $$

$$ R_f^1 \rightarrow R_f, \quad (2) $$

$$ R_f^1 \rightarrow R_f^3, \quad (3) $$

$$ R_f^3 \rightarrow RF, \quad (4) $$

Here, $R_f$ represents riboflavin in the ground state, $R_f^1$ is the excited riboflavin molecules in the singlet state, $R_f^3$ is the excited triplet state. $I_{\text{abs}}$ is the absorbed light intensity (UV)\(^{112}\). Therefore, riboflavin excitation can be explained as follows:

![Energy diagram of riboflavin when exposed to UV light.](image)

1) The excited riboflavin molecule converts from ground state ($S_0$) to excited singlet state ($S_1, S_2$) due to the absorption of a photon through exposure to UV light.

2) Excited singlet state riboflavin returns to ground state.

3) Excited singlet state riboflavin converts to excited triplet state ($T_1$) through intersystem crossing.

4) Finally, excited triplet state riboflavin returns to ground state ($S_0$) and the process starts again.
1) The excited riboflavin molecule converts from ground state to excited singlet state due to the absorption of a photon by exposure to UV light.

2) Excited singlet state riboflavin returns to ground state.

3) Excited singlet state riboflavin converts to excited triplet state through intersystem crossing.

4) Finally, excited triplet state riboflavin returns to ground state and the process starts again.

Upon returning to the ground state, the process can start again. This is why CXL by pulsing (UV-on/UV-off technique) could be more beneficial to treatment outcome due to the regeneration of excited triplet states during each break from UV.

2.3.4 Reduced riboflavin

Once a cornea saturated with riboflavin is exposed to UV, the excited state riboflavin and reduced riboflavin compete with oxygen, leading to a rapid consumption of oxygen in the cornea (anaerobic condition). The concentration of oxygen is important for this process. The reduced riboflavin does not act as a photosensitizer and no longer absorbs UV light as it is degraded to lumiflavin and lumichrome. Increasing the intensity of UV light will therefore not create more activated riboflavin, once the molecules are in the reduced state. Reduced riboflavin endures an oxidation reaction involving the generation of free radicals that can induce radical polymerization. After several minutes, oxygen will replenish itself and the process will begin again. The integrated role of photochemical degradation of riboflavin is a function of light, intensity and exposure time.

2.4 Role of UV-A light during CXL

The efficient delivery of UV light to the riboflavin in the stroma and the availability of oxygen are crucial for CXL. Without the combination of the three factors, CXL will
display no effect on corneal biomechanics. When CXL was originally introduced all experimental work was based on low intensity UV light dose. The intensity was selected to be in a safe dose range to avoid possible UV light damage of underlying tissues structures such as the lens or the retina. The original Dresden protocol used a total radiant exposure of 5.4 J/cm² with an intensity of 3 mW/cm² for an exposure time of 30 min⁹⁸.

These chosen parameters allowed 3 important factors to happen during the CXL procedure:

a) The cornea contains sufficient Riboflavin molecules after an initial application and 30 min diffusion.

b) The relative low intensity (number of photons per time) allows the reaction mechanisms to activate riboflavin and O₂ radicals as well as to create reduced riboflavin and reactive oxygen species.

c) During the CXL process, there is enough time for O₂ to diffuse from the anterior cornea into the deeper stroma and, thus, there is enough reactive oxygen available. This additional oxygen is crucial for the efficacy of the process and consequently drives the clinical outcomes.

2.4.1 Difference between energy dose and intensity

A series of in-vitro investigations on human and porcine corneas examined the best treatment parameters for CXL, such as riboflavin concentration, intensity, wavelength of UV light and duration of treatment. To avoid damage to the endothelium caused by UV, effective CXL should only occur in the first 200-250 µm of the corneal stroma¹⁰³. Using a wavelength of 360 -370 nm, and an intensity of 3 mW/cm² and 5.4 J/cm² ensures that the corneal exposure to UV light is below harmful levels. Wollensak et al¹⁰⁷,¹¹⁶ determined the damage threshold at the endothelium to be 0.36 mW/cm² (0.65 J/cm²); however this intensity may cause damage if corneal thickness is below 400 µm¹⁰⁵. By using riboflavin with a concentration of 0.1% and UV irradiance of 3 mW/cm², a large reduction of UV light by 95% is present at the surface of the cornea, resulting in an irradiance of 0.15mW/cm² at the endothelium, which is safe under the cytotoxic threshold of 0.36mW/cm⁹⁸.
2.7 Current CXL treatment protocols

Current treatment protocols for CXL have been adapted from the original Dresden Protocol, which is the established ‘gold standard’ in cross-linking. Newly modified protocols have been introduced which may have equally effective outcomes. These are discussed in the following section.

2.7.1 Higher intensity CXL

As a total treatment time of about 1 hour (30 min for riboflavin diffusion and 30 min UV exposure) for a standard Dresden protocol had become an issue for many surgeons around the globe, attempts were made to reduce the total treatment time or at least the required illumination time. A shorter procedure time would be beneficial for both patient and surgeon. If illumination time is reduced, patient discomfort will decrease. Schumacher et al\textsuperscript{119} investigated the biomechanical stability of porcine corneas when exposed to UV intensities ranging from 3 mW/cm\textsuperscript{2} to 90 mW/cm\textsuperscript{2} while maintaining the same energy dose as the Dresden protocol. This laboratory study found the equivalent outcome with higher intensity CXL (30mW/cm\textsuperscript{2} for 3 min), to that of standard treatment (3mW/cm\textsuperscript{2} for 30 min). However, there was no statistical significance in biomechanical stiffening for higher intensities in the range of 45-90 mW/cm\textsuperscript{2} and corresponding shorter treatment times\textsuperscript{120}. This suggested that the efficacy of the cross-linking treatment is time dependent, if the energy dose is kept constant. An explanation for this can be given by the O\textsubscript{2} consumption during the photochemical reaction creating an anaerobic environment within the stroma. Results correlated with a study by McQuaid et al\textsuperscript{121}, found reduced fibrilar structure in porcine corneas at depth of 160-200 µm after high intensity CXL (30-100 mW/cm\textsuperscript{2}) using second harmonic generation (SHG) imaging. Hammer et al\textsuperscript{122} investigated biomechanical changes in porcine eyes of different UV intensities between 3-18 mW/cm\textsuperscript{2}, and found a decreased stiffening effect with high irradiance/short irradiation treatment times of 9 mW/cm\textsuperscript{2} and 18 mW/cm\textsuperscript{2}. However, Krueger and Spoerl\textsuperscript{123} found no significant difference in biomechanical stiffening in standard versus high intensity irradiance (i.e. 2 mW/cm\textsuperscript{2} for 45 min; 3 mW/cm\textsuperscript{2} for 30 min; 10 mW/cm\textsuperscript{2} for 9 min; and, 15 mW/cm\textsuperscript{2} for 6 min).
In clinical studies, higher intensity CXL has also been investigated. Cummings et al\textsuperscript{124} found no significant difference between standard (66 eyes: 3 mW/cm\textsuperscript{2} for 30 min) and high intensity (9 mW/cm\textsuperscript{2} for 10 min) CXL in 36 eyes. Other clinical studies have reported similar to Cummings et al for higher intensity CXL (9mW/cm\textsuperscript{2} or 30mW/cm\textsuperscript{2})\textsuperscript{125-127}. In terms of safety, higher intensity CXL can be successful once it applies to the Bunsen-Roscoe Law of Reciprocity which states “One achieves the same photochemical effect with a reduced irradiation time and increased radiation intensity as long as the total dose remains the same”\textsuperscript{128}.

Based on a theoretical model by Kamaev et al\textsuperscript{31}, pulsed accelerated CXL have also been investigated\textsuperscript{129}. By switching the UV source off for a ‘rest’ period, it allows for restoration of oxygen levels that are otherwise depleted during UV irradiation. However, this prolongs total treatment time.

**Bunsen-Roscoe law of reciprocity**

The Bunsen-Roscoe law of reciprocity\textsuperscript{128} states the radiant exposure should remain constant if the delivered energy dose remains contact. The absorbed UV energy and its biological effect is proportional to the total energy dose delivered to the tissue. In standard CXL (3 mW/cm\textsuperscript{2} for 30 min), the radiant exposure of 5.4 J/cm\textsuperscript{2} allow for biomechanical stiffening of the cornea following treatment. Studies have shown failure of the Bunsen-Roscoe law of reciprocity for short illumination time and high intensities over 45 mW/cm\textsuperscript{2}\textsuperscript{120,130}. However, according to “equal-dose” principle 10 mW/cm\textsuperscript{2} for 9 min, 30 mW/cm\textsuperscript{2} for 3 min, 18 mW/cm\textsuperscript{2} for 5 min, 45 mW/cm\textsuperscript{2} for 2 min at constant energy dose of 5.4 J/cm\textsuperscript{2} produce the same stiffening effect in the cornea as the standard 3 mW/cm\textsuperscript{2} for 30 min (the Dresden protocol)\textsuperscript{131}.

**2.7.2 Trans-epithelium CXL**

Trans-epithelium (Trans-epi) CXL reduces patient discomfort during and after the procedure by avoiding corneal epithelial removal, compared to the Dresden Protocol which requires de-epithelization. However, riboflavin is a hydrophilic molecule which cannot penetrate the tight junctions of the epithelium as effectively as it would when
the epithelium is removed. There are many advantages with Trans-epi CXL such as less pain and a faster visual recovery. However, it is known that Trans-epi CXL is not as effective as standard epithelium-off CXL. A clinical study using 26 eyes measured the effect of Trans-epi CXL with the addition of a new riboflavin formulation benzalkonium chloride (BAC) with a UV intensity of 9mW/cm² for 10min. Follow up of 12 months post-op concluded Trans-epi did not effectively halt keratoconus progression, and epithelium damage was reported. Baiocchi et al reported riboflavin delivery in intra-stromal methods is only effective after epithelial removal. Nonetheless, the Trans-epi technique can be useful for treating thin corneas and patients susceptible of developing keratoconus.

Alternative methods to by-pass the epithelium without removal have also been investigated such as Iontophoresis assisted CXL. This consists of the application of an electric current to disrupt epithelial cells and allow for sufficient riboflavin penetration. Studies are ongoing and have seen positive results so far.

2.7.3 Intra-stromal channels

Intra-stromal channels or pockets have shown to be an effective method of by-passing the epithelium for efficient riboflavin delivery. A clinical study investigated femtosecond laser assisted intra-stromal delivery of riboflavin in 10 eyes with early keratoconus. The study reported no adverse events, reduced post-operative pain or negative biomechanical effect in the treated corneas. Seiler et al investigated the stress-strain relationship and UV absorption in channels created by a femtosecond laser and reported the same biomechanical stiffening effect of the corneas after stromal creation to be similar to those found in the Dresden protocol (stiffness increase of 82% vs 77%). However, Wollensak et al found only 50% increases in biomechanical stiffening in porcine corneas after Intra-stromal channel and CXL, raising concerns on the lack of riboflavin in the anterior cornea. Thus suggesting further investigative studies are required before applying this method in-vivo.
2.8 CXL and refractive correction

The standard Dresden protocol is established. It is known to be safe, reliable and it halts the progression of keratoconus and ectasia. Recent studies have combined CXL with refractive procedures in order to correct low myopia or hyperopia.

2.8.1 PiXL

Photorefractive intrastromal cross-linking (PiXL) has the ability to correct myopia, hyperopia or astigmatism of small refractive errors up to 3 Diopters (D) without the need for laser ablation or stromal flap creation. The system for PiXL uses individual treatment profiles, with the ability to customize the shape of each individual case by selecting a region of the cornea for treatment instead of irradiating 8mm of the cornea. Clinical results reported an average improvement of 2.3 D using high fluence, transepithelial PiXL.141-142 Future application using PiXL could eventually treat early to moderate stages of keratoconus, with potential to restore vision lost to progression of the disease.

2.8.2 SMILE and CXL

The standard SMILE procedure (Small Incision Lenticule Extraction) is safe and effective for the treatment of myopia.143-144 The treatment involves an incision inside the cornea using a femtosecond laser, followed by a smaller incision creating a lenticule like channel. SMILE-Xtra is combined with CXL. In SMILE-Xtra, riboflavin can be injected or instilled in the small channel and CXL treatment can follow. Clinical studies145 evaluated both standard146 and high intensity147 CXL using SMILE. The studies revealed mixed findings such as no significant improvement in best spectacle-corrected visual acuity (BSCVA) in the former, and a reduction of corneal keratometry in the latter.

2.8.3 PRK with CXL: The Athens protocol

The Athens Protocol (AP) is a combination of photorefractive keratectomy (PRK) and CXL. The epithelium is removed by laser ablation (50 µm), and the stroma is partially ablated using a topography-guided laser excimer. Drops of riboflavin are applied to
the cornea followed by high fluence UV (10 mW/cm²) for 10 minutes. The reasoning behind stromal ablation of the anterior cornea before treatment is to normalize the area of cone steepening, which helps to improve post-operative visual restoration, corneal keratometry and refraction. Studies indicate long-term stability using this treatment.

2.8.4 PACK-CXL

Photo-activated Chromophore for Keratitis-Collagen Cross-Linking (PACK-CXL) was first named by an audience vote in 2013 at the CXL congress in Dublin, Ireland. It is designed to treat corneal infection. PACK-CXL is based on the Dresden protocol, using riboflavin as the chromophore which has molecular and macroscopic effects on the infection. Molecular effects include cross-links in nucleic acids in RNA/DNA which is activated by the UV light. Macroscopic effects include increases resistance to enzyme digestion and reduced post-operative pain. PACK-CXL can be performed on a slit-lamp and can be suited to any ophthalmologist instead of a specialized corneal surgeon. Various studies have published successful treatment for bacterial and fungal keratitis using PACK-CXL.

In summary, corneal cross-linking (CXL) is an established surgical procedure for the treatment of keratoconus, infection, and post-refractive ectasia. The conventional ‘Dresden Protocol’ is by far the most effective, although many other treatment protocols have been introduced including CXL integrated with refractive procedures. CXL is ultimately limited by the degree of progression in the affected cornea. If the disease progression and advanced and continuously worsening, corneal transplantation may be required.

Better understanding of the CXL process is vital in terms of planning better treatment outcomes. The concentration of riboflavin plays an important role and a standardised method needs to be applied for efficient diffusion in the cornea before UV exposure. The role of oxygen is also important for optimization of CXL due to the photochemical Type I and Type II mechanisms that triggers riboflavin when exposed to UV light. This will be discussed further in the following chapter.
Chapter 3: Principles of photochemical reactions and photochemistry

Efficient corneal cross-linking (CXL) comprises three key ingredients: energy dose of the applied ultraviolet (UV) light, concentration or composition of riboflavin (vitamin B2), and O₂. The combination of UV light and riboflavin which triggers the polymerization (cross-linking) process, but requires available O₂ to be effective. This chapter will explore the three components involved in successful CXL and their corresponding photochemical reactions.

3.1 General diffusion

Diffusion can be described as the movement of a substance down a concentration gradient. Upon application of riboflavin to a de-epithelized cornea, diffusion occurs over a time period of 30 min.

3.2 Molecular diffusion of riboflavin during CXL

Riboflavin diffusion is calculated using Fick’s 2nd law in 3-D:

$$\frac{\partial \zeta(x, y, z; t)}{\partial t} = D \nabla^2 \zeta(x, y, z; t)$$ (5)

Where $\zeta(x, y, z; t)$ is the riboflavin concentration at any point across the corneal model at time $t$, and $D$ is the characteristic diffusion constant. Axial corneal diffusion for common epi-off CXL has been found to equal $D = 6.5 \times 10^{-5}\text{ mm}^2/\text{sec}$, with a typical diffusion time of 30 min. Diffusion of riboflavin plays a key role in the CXL procedure. The standard CXL protocol consists of riboflavin (vitamin B2) applied to a de-epithelized cornea for 30 min. to facilitate molecular diffusion into the stroma to a depth of ~200 µm, followed by exposure to UV (365nm) at 3 mW/cm² for an additional 30 min. Riboflavin plays a key role in the CXL process as it triggers the formation of molecular crosslinks that halt the progression of the condition and lead
to stabilization of corneal curvature. Apart from its active role in CXL, riboflavin acts as a safety barrier that hinders UV exposure of the posterior parts of the eye\textsuperscript{110}. Because of the tight junctions between epithelial cells, riboflavin cannot penetrate fully unless the epithelium is removed prior to application\textsuperscript{133}. A clinical limitation of the standard treatment is epithelium removal leading to pain for the patient and a potential higher clinical risk after the treatment during the phase of epithelium closure\textsuperscript{157}.

The introduction of new riboflavin solutions and stromal delivery prior to CXL such as trans-epithelium\textsuperscript{158}, Iontophoresis\textsuperscript{136,159} and femtosecond (FS) laser channels\textsuperscript{139-140} have been investigated. Studies have examined the UV absorption coefficient of riboflavin and found a linear correlation for concentrations up to 0-0.5\%\textsuperscript{105,160}. This suggests that changes in the method to administer at the corneal surface are linked to the effectiveness of different riboflavin concentrations and the reservoir of riboflavin available at the corneal surface (e.g. layer thickness and dropping intervals)\textsuperscript{111}. Thus, standardization to the clinical dropping scheme related to the application of riboflavin is important to achieve consistency in the treatment outcome as well as in optimizing the procedure itself.

<table>
<thead>
<tr>
<th>Type of Riboflavin</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotonic (riboflavin-5-phosphate 0.1% with 20% dextran T-500).</td>
<td>Standard use for Dresden Protocol</td>
</tr>
<tr>
<td>Isotonic (riboflavin-5-phosphate 0.1% with 1.1% Hydroxypropyl Methylcellulose, HPMC).</td>
<td>HPMC lower absorption coefficient, a thinner meniscus</td>
</tr>
<tr>
<td>Hypotonic (riboflavin-5-phosphate 0.5% without dextran T-500).</td>
<td>Swells thin corneas to meet minimum 400\µm thickness.</td>
</tr>
<tr>
<td>TE Trans-epi (riboflavin-5-phosphate 0.25% with 1.2% Hydroxypropyl Methylcellulose (HPMC) &amp; 0.01% Benzalkonium chloride (BAC).</td>
<td>Does not require epithelial removal. BAC loosens tight junctions of the epithelium which allows diffusion of riboflavin before CXL.</td>
</tr>
</tbody>
</table>

Table 1: Table of riboflavin formulations and use.
3.3 Oxygen and CXL

The role of oxygen (O\textsubscript{2}) has been underestimated in the past, but must be taken into account during the CXL process, especially when developing new treatment protocols. By applying riboflavin drops to the surface of the cornea, atmospheric O\textsubscript{2} is depleted during UV illumination and reacts with riboflavin due to photochemical Type I and II mechanisms. This will be discussed in more detail in section 3.6.

3.4 Light distribution in tissue

Light entering the cornea is affected by the following processes\textsuperscript{1}:

- Refraction and reflection at each structural interface, mostly the cornea due to refractive index differences.
- Scattering within the ocular media. Scattering increases in eyes affected by diseases such as cataract.
- Absorption and then either re-emitted at other (longer) wavelengths, which is known as fluorescence, or light is absorbed and converted to other forms of energy (mainly heat).

3.4.1 Lambert-Beer’s law

The distribution of light intensity within the cornea \(I(z, t)\) is derived from Lambert-Beer’s law with the absence of light scattering in tissue:

\[
I(z, t) = I_0(t) \exp(-\mu z)
\]  
(6)

where \(I_0\) is the intensity of the incident light, \(I\) is the transmitted intensity at distance \(z\) and \(\mu\), the total absorption coefficient. Schumacher et al\textsuperscript{111} derived an equation based on the Lambert-beer’s law including concentration variations as:

\[
I(z, t) = I_0(t) \exp(-\int_0^z \varepsilon(x, t) dx) \exp(-\mu z)
\]  
(7)

where \(\mu\) is the total absorption split into the absorption by the corneal stroma, and riboflavin absorption \(\mu_p\), \(\mu = \mu_c + \mu_p\). The riboflavin absorption depends on the molar
extinction coefficient \( \varepsilon \), and the concentration \( \zeta(z,t) \). The absorption coefficient depends linearly on the riboflavin concentration of the applied solution.\(^{111}\)

### 3.5 Photochemical reaction mechanisms during CXL

Corneal cross-linking (CXL) is comprised of three important photochemical processes: energy dose (absorbed number of photons) of the applied UV light, concentration (how much riboflavin is needed) or composition (formulations) of riboflavin (vitamin B2), and the role of oxygen (O\(_2\) concentration within the tissue).

#### 3.5.1 Principle

A photochemical reaction can be described as a chemical reaction created by the absorption of energy in the form of light. Photochemical reactions take place in many circumstances:

1. Photosynthesis in plants (conversion of energy from sunlight into stored chemical energy resulting in molecular O\(_2\)).\(^{161}\)
2. A photochemical reaction in the retina takes place when rhodopsin in the photoreceptor cell isomerizes after absorbing light, enabling us to see.\(^{162}\)
3. Vitamin D formed in the skin of mammals when exposed to light.\(^{163}\)
4. Likewise, photography and xerography are based on photochemical processes.

Along with photochemical reactions occurring during CXL, photomechanical and photo-thermal effects also need to be taken into account. A photomechanical effect is the change in mechanical properties of a biological material when exposed to light (e.g. biomechanical stiffness in the cornea after CXL, proven by ex-vivo stress-strain measurements).\(^ {164}\) In laser eye surgery, a photo-thermal effect occurs due to the removal of corneal tissue using ablation with a high powered laser instead of a scalpel. It is produced by photoexcitation of a material, resulting in the production of thermal energy (or heat).\(^ {165}\).
3.5.2 First law of photochemistry

The first law of photochemistry (or the Grotthuss-Draper law) states in order for a photochemical reaction to take place, light must be absorbed. When a molecule in ground state (S₀) absorbs light, one electron is excited to a higher level or state. In the case of photochemical reactions during CXL, UV light delivers the activation energy for riboflavin excitation. The absorption of a photon of light by a reactant molecule may also permit a reaction to occur.

3.5.3 Photosensitization process: Type I and Type II

Recent studies have established that the oxygen concentration plays a role in the CXL effect³¹,¹⁶⁶, due to the photochemical properties displayed during type I and type II reactions. Type I (anaerobic) and Type II (aerobic) reactions can be described as the two major photochemical pathways for riboflavin photosensitization. Type I reaction is dominant at low oxygen concentrations. In this reaction, the photosensitizer (riboflavin) interacts with the surrounding molecules by hydrogen transfer resulting in the formation of crosslinks by oxygen (O₂) molecules and hydrogen peroxide (H₂O₂), resulting in a depletion of molecular O₂. During photosensitization, a photon is absorbed which excites the sensitizer to a higher energy state. The excited sensitizer undertakes internal conversion which can occur in two types of reaction: I or II, resulting in a chemical modification within the cornea. The pathways are described as:

1) Direct (or Type I): The light activated photosensitizer interacts directly with the surrounding molecules by hydrogen abstraction (removal of an atom or group from a molecule by a radical) to form covalent cross-links which produces free O₂ molecules and hydrogen peroxide. In type I reaction, a one electron transfer reaction produces a radical or radical ion in both sensitizer and substrate when the excited sensitizer reacts directly with the substrate. The substrate then donates an electron to the sensitizer, resulting in a substrate radical cation, and a sensitizer radical anion. When O₂ is present, both processes can react further to produce oxygenated products. This can lead to a loss of the sensitizer because of its conversion to an oxidized molecule (see reduced riboflavin: section 2.3.4). Another pathway in Type I can result in the
creation of a superoxide radical anion, resulting in the sensitizer returning to its original state.

2) **Indirect (or type II):** The light activated photosensitizer interacts with the ground state oxygen, producing reactive singlet oxygen to oxidize the surrounding molecule. In type II reactions, the excited sensitizer transfers its energy to ground-state molecular/triplet oxygen \((^3\text{O}_2)\), creating excited state singlet oxygen \((^1\text{O}_2)\), resulting in the regeneration of the ground state sensitizer. In this case, the sensitizer is not consumed in the reaction. Singlet oxygen becomes an oxidized product. \(\text{O}_2\) depletion during CXL suggests a combination of Type I and Type II mechanisms.

### 3.6 Reaction mechanisms during CXL: Type I and Type II

The above explanation of type I and Type II reactions can be described using the following equations:

The role of Type I and Type II CXL reaction mechanisms are displayed in Fig. 3.1.

![Fig. 3.1. Role of Type I and Type II photochemical reactions during UV cross-linking. Illustration adapted from Raiskup and Spoerl.](image-url)
Type I:

\[ Rf_3^+ + SH \rightarrow Rf^- + RfH + S \]  
\[ 2RfH \rightarrow Rf + RfH_2 \]  
\[ RfH_2 + O_2 \rightarrow Rf_{ox} + H_2O_2 \]

Type II:

\[ Rf_3^+ + O_2 \rightarrow Rf + O_2 \]  
\[ SH + O_2 \rightarrow S^{ox} \]

In Type II reaction (equation 11,12), riboflavin in its excited triplet state reacts with oxygen to form singlet oxygen (\(^1O_2\)). The singlet oxygen causes the substrate to become oxidised \(S^{ox}\), which can be described as the oxidised form of the substrate (cornea). During UV irradiation, the excited sensitizer (riboflavin) reacts with the substrate (cornea). Singlet oxygen (or free oxygen radical) is converted to hydrogen peroxide and thereby cause depletion of \(O_2\).

3.6.1 Previous literature on the measurement of \(O_2\) during CXL

Research has established that the presence of oxygen is necessary for efficient cross-linking to occur. Richoz et al\(^{166}\) found that the biomechanical effect of CXL is oxygen dependent by measuring stress-strain of porcine corneas under low and normal \(O_2\) saturation. Krueger et al\(^{168}\) and Herekar\(^{113}\) observed depletion up to 50% of molecular \(O_2\) at 245 sec at 3 mW/cm\(^2\) and 46 sec at 16 mW/cm\(^2\) after CXL, showing a linear association with UVA irradiance.
Kamaev et al\textsuperscript{31} found that riboflavin and UV-induced cross-linking involving \( O_2 \) photochemical type II mechanism occurs in the collagen corneal matrix through the generation of reactive oxygen species (ROS), particularly singlet molecular oxygen. In their experimentation, \( O_2 \) depletion to 0\% concentration was observed after 10-15 secs, suggesting a predominantly Type I mechanism. They also suggest that halfway through illumination, the \( O_2 \) concentration gradually replenishes to a concentration where Type II mechanisms begin to play an additional role. This study used sodium azide to quench riboflavin tripletts, a primary cross-linking reactant.

McCall et al\textsuperscript{169} investigated the involvement of singlet \( O_2 \) in CXL and also the role of carbonyl or free amino groups were involved in forming functional crosslinks. The study proved singlet oxygen was involved in CXL and was stopped by the presence of sodium azide, a substance known to stop reactions that require singlet \( O_2 \). Also, singlet \( O_2 \) was greatly stimulated by the presence of \( D_2O \), which prolongs the half-life of singlet \( O_2 \). The study also showed free carbonyl groups are present and essential for CXL process. Carbonyl groups are free aldehyde and ketone groups- likely maintained by steady state action of lysyl oxidase, produced in keratocytes and the endothelium.

Vasilios et al\textsuperscript{170} observed that the addition of \( O_2 \) during high intensity CXL (30 mW/cm\textsuperscript{2}) did not increase the CXL effect when elasticity was measured through AFM. Moreover, results of high intensity CXL (30 mW/cm\textsuperscript{2}) and groups with enriched \( O_2 \) high intensity showed similar changes in corneal elasticity when compared to the conventional Dresden protocol.

### 3.7 Tissue property changes after CXL

Corneal biomechanics is of utmost importance in observing weakening or thinning of corneal structures caused by keratoconus or ectatic diseases. Experimental studies have shown that CXL has a depth dependent, stiffening effect on the biomechanics of the cornea ex-vivo of up to 70\%\textsuperscript{101,171,172}, corroborating with clinical reports of reduced regression or halting the progression of keratoconus\textsuperscript{98,173,174}. Kohlhaas et al\textsuperscript{171} found the greatest depth at which stiffening occurs within the anterior 200 µm of the cornea and only 20\% effect in the next 200µm. In terms of histological changes to the cornea, an increase in corneal fibril diameter post treatment has been reported\textsuperscript{175}, and aptosis
of corneal cells indicated by a demarcation line up to 300 µm specifies the areas of cross-linked tissue\textsuperscript{176}.

Techniques used to measure effective corneal biomechanical stiffening ex-vivo include tensile testing of corneal strips post treatment\textsuperscript{98,171}, Brillouin microscopy\textsuperscript{177,178}, shear wave elastometry\textsuperscript{179}, ultrasound and ultrasound speckle tracking\textsuperscript{180,181}. Recent in-vivo measurements of biomechanical reactions after CXL have made it possible to gain more knowledge of tensile and histological changes in the cornea. Two commercially available diagnostic devices are 1) Ocular response analyser (ORA), and 2) Corvis ST. The ocular response analyser requires an air-puff to the cornea similar to non-contact tonometry (NCT), producing values of corneal hysteresis (CH) and corneal resistance factor (CRF)\textsuperscript{182}. Corneal hysteresis (CH) measures the viscoelastic properties of the cornea measured by the inward (air-puff) and outward applanation pressures measured by the ORA\textsuperscript{183}. Corneal resistance factor (CRF) can be described as the resistance that is relatively independent of intra-ocular pressures (IOP). Besides measurement of corneas post CXL\textsuperscript{184}, the ocular response analyser is also useful in diagnosis and prognosis after refractive surgery\textsuperscript{185}. The Corvis ST has a similar air puff deformation to the cornea upon applanation, however the Corvis can also obtain Scheimpflug images of the cornea, resulting in direct visualization of corneal deformation\textsuperscript{186,187}.

In summary, the diffusion of riboflavin, the absorbed number of photons (energy dose) of UV light; and concentration of O\textsubscript{2} within the tissue are of relevance to optimize the CXL effect. Efficient delivery of UV light to the diffused riboflavin in the cornea along with the availability of O\textsubscript{2} is essential for CXL. Without the combination of all three factors (riboflavin, O\textsubscript{2}, and UV), there will be no biomechanical effect on the cornea. The most important aspect of CXL is to have intensity in a safe range to avoid UV damage to posterior structures such as the lens and retina. In this study, we will investigate Type I and Type II photochemical reactions in the cornea before and after CXL using a method involving O\textsubscript{2}-sensitive nanoparticles that has not been implemented before.
Chapter 4: Measurement of the rate of riboflavin diffusion from intra-stromal channels prior to corneal cross-linking (CXL)

4.1 Introduction

In this chapter, measurements of riboflavin diffusion using intra-stromal channels created by means of a mechanical stromal instrument (commonly used for the insertion of intrastromal corneal rings. Suarez Spreader, Fig.4.1) in whole-mounted post-mortem porcine eyes will be described. The use of fluorescent imaging along with numerical modelling allows determination of effective diffusion coefficients under different conditions. The use of corneal channels could potentially allow surgeons to apply the riboflavin into the stroma without epithelium removal if proven equally effective in terms of surgical outcome and biomechanical stability.

4.2 Methods

4.2.1 Experimental setup

Fig. 4.1: Suarez Spreader used to mechanically create the channel and example of fluorescence obtained.

Example of riboflavin fluorescence in corneal channel
The rate of diffusion was monitored using an optical setup with a CCD camera (Thorlabs Inc, DCC 1240C). A bandpass filter (central wavelength 550nm and bandpass 40nm) was attached to the camera to visualize riboflavin fluorescence when exposed to UV (wavelength 365nm ± 10 nm). The layout of the system is shown schematically in Fig. 4.2.

![Schematic diagram of the system](image)

**Fig. 4.2: Schematic diagram of the system: CCD: Charge-coupled colour camera, Bandpass filter, f1 and f2 achromatic lenses, M - Mirror, UV-A radiation source at 365 nm, Ultra-violet light wavelength.**

### 4.2.2 Preparation of eyes

Enucleated porcine eyes were obtained 2 -3 hours post-mortem from the local abattoir. The eyes were stored at 4ºC and used within 6 hours. All corneas were found to be clear, with no presence of corneal scarring or opacities. Balanced salt solution (BSS) was injected through the optic nerve to enhance and maintain the natural globe shape, and the eye was placed in a holder with the cornea facing upwards. The epithelium was removed by loosening post mortem using an epi-hook as it otherwise would increase unwanted light scattering and decrease the optical quality of fluorescent images. A small incision was created in the cornea to allow for the Suarez Spreader (Mediphacos Ltd, Brazil) to be inserted. The mechanical channel has a nominal diameter of 5.7 mm. The incision channel was created in the upper half of the stroma ideally at 200µm depth. Riboflavin (0.2ml) was injected into the channel.
using a lacrimel cannula (Visitec®). The injection was performed until the channel was entirely filled by the riboflavin solution at which point no further riboflavin re-injection was applied during the imaging sequence in order to mimic a clinical situation and avoid unintended movement of the eye. Each experiment was repeated multiple times with similar diffusion results found. Porcine eyes were used, rather than human donor eyes as these can be easily obtained and do not require special safety protocols. Moreover, their size and properties are highly similar to those of human eyes.

4.2.3 Imaging method

The cornea was illuminated with UV light at 365nm wavelength (UV-X prototype, IROC Innocross, Zurich Switzerland) under 6 mW/cm² in order to excite the fluorescence of the riboflavin in the channel. An image was taken immediately (approx. 2 min. after having injected riboflavin into the channel) to measure a baseline of riboflavin in the corneal channel and repeated every 10 min. for a total of 30 min.

4.2.4 Riboflavin formulations

Six riboflavin formulations (Medio-Haus-Medizinprodukte GmbH, Germany) were tested in this study:

A. Mediocross®-R Isotonic (riboflavin-5-phosphate 0.23% without dextran T-500).
B. Mediocross®-D Isotonic (riboflavin-5-phosphate 0.1% with 20% dextran T-500).
C. Mediocross®-M Isotonic (riboflavin-5-phosphate 0.1% with 1.1% Hydroxypropyl Methycellulose, HPMC).
D. Mediocross®-H Hypotonic (riboflavin-5-phosphate 0.1% without dextran T-500).
E. Streuli Hypotonic (riboflavin-5-phosphate 0.5% without dextran T-500).
F. Mediocross®-TE Trans-epi (riboflavin-5-phosphate 0.25% with 1.2% Hydroxypropyl Methycellulose (HPMC) & 0.01% Benzalkonium chloride (BAC).
4.2.5 Image processing

Fig. 4.3 shows fluorescent images comparing diffusion results for hypotonic and isotonic solutions with two different concentrations. A difference in the diffusion rate is clearly seen. Using Matlab™ software (Mathworks Inc, R2013b), 100 radial cuts were made in the recorded images covering a 180º arc (left side of images in Fig. 4.3) whereby the typical Full Width at Half Maximum (FWHM) of the riboflavin distribution at each instant was determined. This averaging of the results allows for more reliability of diffusion measurements.

![Fluorescent images comparing diffusion results for hypotonic and isotonic solutions](image)

**Fig. 4.3**: Experimental results of diffusion over time under the influence of corneal channels in porcine eyes using two different concentrations of hypotonic and isotonic riboflavin.

4.2.6 OCT analysis

Ocular Coherence Tomography (OCT-2000, Topcon, UK) was performed in 3-D on one eye to estimate the approximate channel depth in the stroma, and to confirm that channels could be successfully made by the procedure. The average porcine corneal thickness is 800-1200µm, which is thicker than the corresponding parameter for human eyes\(^{188}\). Fig. 4.4 shows OCT images of the created channel. Image analysis shows the channel almost closed pre-riboflavin injection (80-81µm thickness), and increasing in thickness once riboflavin has been injected into the channel (146-148µm).
4.2.7 Diffusion model

An isotropic corneal stroma model was made to solve Fick’s 2\textsuperscript{nd} law of diffusion using COMSOL\textsuperscript{TM} (COMSOL Inc) with the aim of analysing the effectiveness of diffusion of riboflavin from an intra-stromal channel as a function of time for different diffusion constants and boundary conditions. The diffusion is calculated using Fick’s 2\textsuperscript{nd} law in 3-D can be seen in equation 13:

\[
\frac{\partial \zeta(x, y, z; t)}{\partial t} = D \nabla^2 \zeta(x, y, z; t)
\]

where \( \zeta(x, y, z; t) \) is the riboflavin concentration at any point across the corneal model at time \( t \), and \( D \) is the characteristic diffusion constant which, in the case of axial-corneal diffusion for common epi-off CXL has been found to equal \( D = 6.5 \times 10^{-5} \text{ mm}^2 / \text{sec} \) in previous studies\textsuperscript{110,111,189}. The rotationally-symmetric corneal model has a diameter of 12 mm and a thickness of 1.0 mm and the surgical incision (applied halfway into the stroma) has a 5.7 mm diameter with a rectangular 0.6 mm \( \times \) 0.2 mm cross section. Analysis was implemented with a finite amount of riboflavin applied to the channel as initial condition (with no riboflavin present elsewhere at Time \( t=0 \)) and then diffused over time into the stroma. This is the case used in the experiments and would be preferred in a clinical setting. Fig.4.5 shows the simulated case of a constant supply of riboflavin applied to the channel wherefrom it spreads.
Note that a sector has been removed for visualization of the diffusion inside of the rotationally-symmetric stroma and channel. Related radial averages for the diffusion over time are shown in Fig. 4.6. The numerical analysis used the standard diffusion coefficient (or multiples thereof) of riboflavin seen in previous work\textsuperscript{110,111,189}, and calculated the best fit to the experimental results.

Fig. 4.5: Stromal model showing riboflavin distribution as a function of time (horizontal) for three different diffusion rates (vertical) equal to $D_0$, $5D_0$ and $10D_0$. In the model, a finite supply of riboflavin is assumed in the channel at $T=0$. All subimages have been normalized individually to ease visualization at increasing time.
Fig. 4.6: Radial averages of the calculated riboflavin distribution at increasing time for diffusion where each plot has been normalized.

### 4.3 Experimental results

The outcome of the experimental analysis is seen in Fig. 4.7 showing an increase in FWHM of the riboflavin between T=0 min. and T=30 min for all of the tested formulations. Over the 30 min interval, isotonic 0.23% (Fig.4.7a), Dextran (Fig.4.7b), and HPMC (Fig.4.7c), the estimated FWHM increased by 31%, 25% and 31% respectively. For hypotonic 0.1% (Fig.4.7d), 0.5% (Fig.4.7e) and TE riboflavin (Fig.4.7f), the FWHM increased by 44%, 45% and 42% respectively from T=0 to T=30min.

Both isotonic solutions containing 0.1% riboflavin shown in Fig 4.7b and 4.7c have a similar diffusion rate seen to spread out of the channel as the normalized intensity at R=0 shows 0.2 (Dextran), and 0.3 (HPMC) at T=30 min., fitting best to the theoretical model of $5D_0 = 32.5 \times 10^{-5}$ mm$^2$/sec (Fig.4.6b). A higher rate of diffusion over 30 min. can be seen in isotonic 0.23% (Fig. 4.7a) which peaks at 0.4 for R=0. The channel
acts as a chamber for riboflavin absorption which is highlighted more in hypotonic when compared to the isotonic solution. Results show that hypotonic solutions and TE (Fig. 4.7d, 4.7e, and 4.7f) have a higher diffusion coefficient of close to \(10D_0 = 65.0 \times 10^{-5} \text{ mm}^2 / \text{sec}\) which is an order of magnitude higher than in standard axial diffusion into the stroma via corneal drops. At T=30min. both hypotonic solutions (0.1%, 0.5%) show a wider spread than for isotonic 0.1%.

Fig. 4.7: Experimental results showing radial averages of fluorescent images of the corneal stroma following injection of riboflavin showing the resulting distribution in time increments of 10 min. from 0 to 30 min. using 6 different riboflavin solutions where each plot has been normalized.
4.4 Discussion

The objective of the study was to investigate diffusion in the corneal stroma through mechanically created stromal channels for riboflavin delivery. Six solutions of riboflavin with varying concentrations were analysed under fluorescence to observe diffusion over time.

Two isotonic solutions containing 0.1% riboflavin display a similar diffusion rate, correlating with the numerical model having a diffusion constant of $5D_0 = 32.5 \times 10^{-5} \text{ mm}^2 / \text{sec}$, i.e five times higher than the axial diffusion rate\textsuperscript{105,111,189}.

Furthermore, the results show that hypotonic solutions and TE have a higher diffusion coefficient approaching $10D_0 = 65.0 \times 10^{-5} \text{ mm}^2 / \text{sec}$, demonstrating an order of magnitude increase compared to the diffusion coefficient found for standard CXL.

Numerical and experimental results show that each riboflavin concentration has a higher diffusion rate when applied to the channel than if applied to the front surface of the corneal stroma. It is expected there is a gradient of the diffusion constant across the cornea due to density differences in the anterior and posterior cornea\textsuperscript{190}. Also, a difference in diffusion structure based on each riboflavin formulation can be seen. Other boundary conditions have been examined in COMSOL™ such as a continuous supply of riboflavin over time to the channel. However, this has no significant impact on the predicted results (except that the highest concentration coincides at all times with the location of the channel) and highly similar diffusion coefficients were found.

Experimental results show a variation in structure based on each riboflavin formulation tested. 0.01% Benzalkonium Chloride 0.01% (BAC) is commonly used for trans-epithelial (TE) CXL\textsuperscript{154,191}. Its function is to loosen the tight junctions between epithelial cells without the need for removal in order for the hydrophilic riboflavin molecule to pass into the corneal stroma before exposure to UV, reducing pain and discomfort for the patient\textsuperscript{192}. Using TE riboflavin via a corneal channel could increase diffusion into the stroma as seen in Fig. 3.7f by the 42% increase in FWHM between T=0 min and T=30 min.
Previous studies found a diffusion coefficient $D_0 = 6.5 \times 10^{-5} \text{ mm}^2 / \text{ sec}$ for 0.1% riboflavin with 20% dextran$^{193}$. Our findings show that the rate of diffusion from an intrastromal channel is approximately 5× higher. Experimental results indicate a 25% FWHM increase over 30 min. This is likely due to dextran being of high viscosity, preventing the riboflavin from spreading out of the channel at a fast rate. Schmidinger et al$^{193}$ found riboflavin phosphate solution (HPMC) to have a lower absorption coefficient, a thinner meniscus and a viscosity dissimilar to isoosmolar riboflavin with 20% dextran. A difference in diffusion rates between dextran and HPMC is clearly seen as the FWHM increases by 42% for HPMC between 0 and 30 min.

Hypooosmolar riboflavin was introduced to the CXL procedure in order to swell the cornea before exposure to UVA for eyes with thin corneas$^{134}$ for safety of posterior structures of the eye. As the hypotonic is injected into the channel, this may swell the stroma and therefore increase the space between lamellae enhancing the transverse diffusion process. Also, a high concentration of hypooosmolar 0.5% increases the availability of riboflavin molecules. When comparing the FWHM increase seen in Fig. 4.7, TE & hypotonic results correspond to this stromal swelling, allowing for a reasonably large spread of riboflavin over 30 min. Fig. 4.7e shows a diverse distribution of diffusion compared to other formulations, perhaps due to the high concentration of riboflavin.

Epithelial-on$^{158,192}$ or trans-stromal disruptors$^{136,159}$, without the need for epithelial removal would be preferable for both patient and surgeon in a clinical setting. However, reducing application time may be patient dependent with isotonic displaying a lower diffusion rate then hypotonic. Future studies using fluorophotometry$^{189}$ or optoacoustic methods$^{194}$ in order to learn more about the diffusion process whilst monitoring corneal depth in the lateral and axial dimensions of the stroma would be preferable.

The channels created in this study have demonstrated that riboflavin can be efficiently delivered into the stroma as a method of bypassing the epithelium. However, we do not know the biomechanical effect of CXL using stromal channels in terms of efficacy. Although IOP was not monitored throughout the experiment, this could have an impact on the rate of diffusion. McQuaid et al$^{121}$ found that high IOPs push collagen
fibrils apart, which could increase the rate of diffusion by allowing the riboflavin to pass through with ease. Porcine eyes were used in conditions that emulate in-vivo situations by injecting saline via the optic nerve to avoid globe deflation. Future studies should take hydration through inflation into account.

During CXL, riboflavin and UV trigger the creation of cross-links, resulting in biomechanical strengthening\textsuperscript{101-104}. By applying drops to the surface of the cornea using the Dresden protocol, atmospheric O\textsubscript{2} is taken up by the UV light and reacts with the riboflavin\textsuperscript{204}. When cross-linking through a stromal channel, the O\textsubscript{2} concentration available would be lower than on the surface of the cornea and therefore may reduce the CXL effect. Future biomechanical measurements and the use of Finite Element Modelling (FEM) of the CXL effect could examine this in more detail.

In summary, the instillation of riboflavin through mechanically created channels has been shown. The diffusion rate shows dependency on riboflavin formulations when compared to literature of standard diffusion dropping intervals. Results found a fast lateral diffusion when injected into a corneal channel. This study shows that numerical modelling of the diffusion process based on experimental observations allows optimization of the channel structure. Future work on the role of oxygen and photon distribution in combination with biomechanical simulation is required. Finally, it should be stressed that even when the diffusion rate is higher from a channel than when riboflavin is applied via drops, it remains to be studied how efficiently it will promote the formation of cross-links during CXL. The latter can only be answered through mechanical stress-strain analysis.
Chapter 5: Oxygen diffusion during CXL using phosphorescent \(O_2\) sensitive probes

The measurement of oxygen in the eye is crucial to monitor and understand ocular disease progression. This chapter will focus on methods of measuring oxygen \((O_2)\) in the eye and previous studies investigating oxygen diffusion during corneal cross-linking (CXL). A method involving luminescence quenching for measuring molecular \(O_2\) in the eye before and after CXL will be introduced.

5.1 ‘Traditional’ methods of measuring \(O_2\) in the eye

5.1.1 Clark electrode system (Flexible licox polarographic \(O_2\) electrodes)

Also known as polarography electrodes, the Clark electrode system was first reported in 1956 as a method to measure oxygen during cardiac surgery\(^{205,206}\). \(O_2\) is measured when a cathode and anode immersed in an electrolyte solution comes into contact with \(O_2\) in the form of a current, which is obtained and measured. The sensors then consume oxygen by electrochemical reduction (rate of oxidation=rate of reduction). The measured current correlates linearly with the oxygen partial pressure (\(P_{O_2}\)). The electrode varies with temperature which is measured by a thermocouple in the probe, and takes over 3 min to obtain an accurate value. The net reaction can be described as:

\[
O_2 + 4e^- + 2H_2O \rightarrow 4OH^- 
\]  

(14)

Studies involving Clark electrodes include in-vitro measurement observing nitric oxide (NO) in the trabecular meshwork (TM) in bovine eyes\(^{207}\), and in-vivo measurement of \(O_2\) distribution in the vitreous chamber using a commercial electrode in patients undergoing vitrectomy intra-operatively\(^{208}\). Limitations of the Clark electrode are the measurement of relative \(O_2\) levels rather than absolute intracellular \(O_2\) concentrations, and consumption of \(O_2\) which can be harmful to small or \(O_2\) deficient samples\(^{209}\).
5.1.2 Fibre optic sensors

![Fibre optic sensors](image)

Fig. 5.1: Example of fibre-optic sensors for pO₂ detection. *Source: pyro-science.com*

Fibre optic probes or sensors come with various sized needle sensors that has the ability to penetrate and access small cells or larger tissue (Fig. 5.1). Fibre-optic sensors detect PO₂ values through the transmission of short pulses from a light emitting diode along the sensor to excite a platinum-based fluorophore situated at the sensor tip. The light emitting diode produces fluorescent light which quenches O₂ molecules that are detected by the instrument. The lifetime of the emitted fluorescence is inversely proportional to the concentration of dissolved O₂, therefore measuring PO₂ values. Unlike the Clark polarographic electrode, this application does not consume O₂ nor is it temperature sensitive; however penetration across cells or tissue can cause damage. The response time for a measurement is approximately 30 seconds. Increased interest in the use of fibre-optic sensing probes for the measurement of intra-ocular oxygen including O₂ concentration in the retina, lens and vitreous body have been adapted in-vivo and ex-vivo²¹⁰-²¹⁶.

5.1.3 Electron paramagnetic resonance (EPR)

Constantin et al²¹⁷ used EPR to detect free O₂ radical formed during UV and riboflavin induced cross-linking. The study identified short-lived hydroxyl radicals formed during UV interactions. EPR is mainly used for tumour grafts or tissue cells. To date, no studies using EPR in the eye have been recorded.
Sensors for $O_2$ detection have many advantages for biological and ophthalmology applications. An important question to consider is how invasive previous methods are; and what potential applications can be introduced clinically to improve reliability and safety.

5.2 Phosphorescence lifetime imaging microscopy (PLIM)

In-vivo methods of measuring $O_2$ such as fibre optical micro-sensors and the Clark electrode have proven to be a sufficient method to measure $O_2$ values with a quick response time of 30 sec to 3 mins. However, a method that requires the insertion of a needle into the ocular cavity or the anterior segment is highly invasive. This study introduces a minimally invasive method of measuring $O_2$ in the cornea. As mentioned previously, $O_2$ can have a fast diffusion rate across tissue which can alter results when measuring $O_2$ concentrations. Taking this into account, it is important to have an experimental setup with conditions suitable for the most accurate readings such as a temperature controlled environment, and the ability to measure across a sample without causing damage to the tissue or structure. Time-Correlated Single Photon Counting Phosphorescence Lifetime Imaging (TCSPC-PLIM) using phosphorescence based probes in soluble form have the ability to detect $O_2$ concentrations repeatedly and non-invasively in living biological tissue. In this case, the TCSPC-PLIM method will be applied to the cornea.

5.2.1 TCSPC-PLIM

TCSPC-PLIM (Becker & Hickl, Picoquant) is an innovative and complex method for fluorescence and phosphorescence lifetime measurements in the time domain. Optical signals pass through a dichroic mirror and are therefore detected by a single photon sensitive detector, which is connected to Time Correlated Single Photon Counter software (TCSPC). The TCSPC software calculates the phosphorescence lifetime based on the time it takes the sample to be excited by the pulsed laser and the arrival of the signal at the detector. By recording the arrival times of the photons emitted, a decay curve can be drawn based on the Phosphorescence lifetimes. Photon counting detectors have the ability to analyse one pixel of a sample at a time at a fast pace,
providing high detection sensitivity and high accuracy of lifetime determination. Luminescence lifetime measurements can be obtained in the frequency domain or time domain\textsuperscript{218}. The frequency domain is based on excitation of the sample with intensity-modulated light (sine or square wave), followed by measurement of the delay of the emission signal of the probe (i.e phase shift). Time-domain systems use short pulse excitation and the analysis of the decay of the emission signal. Time-domain detection enables a reduction of scattering and autofluorescence, leading to an increase in the signal-to-noise (S:N) ratio and a better image contrast\textsuperscript{218}. The phosphorescence quenching method is a direct, non-invasive, nonchemical measurement of the physiological range of O\textsubscript{2} which is dependent on the dye/sensor/probe (0-200 µM, equal to 0-21\% atmospheric O\textsubscript{2}). Fast time acquisition, high resolution of lifetime measurement, General ease of use, the potential ability to map O\textsubscript{2} concentrations in the cornea, and minimal invasiveness makes TCSPC-PLIM superior to the Clark Electrode and fiber-optic O\textsubscript{2} sensors.

### 5.2.2 Fluorescence, phosphorescence and luminescence quenching

Luminescence can be defined as the emission of light without heat. The excitation and emission of a molecule by ultraviolet or visible light photons is known as photoluminescence. Photoluminescence is divided into two categories: fluorescence and phosphorescence\textsuperscript{219}. Fluorescence and phosphorescence start from the absorption of a photon released from ground state (Fig. 5.2)(A) to the excited singlet states S\textsubscript{1} and S\textsubscript{2} (excitation 10\textsuperscript{-15} sec) shown in (B). Once the molecule is in the excited state vibrational level, it will slowly relax to the lowest vibrational level of the first excited state by internal conversion (IC) shown in (C) (10\textsuperscript{-4}-10\textsuperscript{-9} sec). From this level (10\textsuperscript{-14}-10\textsuperscript{-11} sec), the electron will perform, depending on the selected process, fluorescence or phosphorescence emission. In fluorescence emission, the molecule will return to ground state (10\textsuperscript{-9}-10\textsuperscript{-7} sec) following internal conversion in the first excited state. Fluorescence lifetime imaging microscopy (FLIM) is determined by this principle. The fluorescence lifetime (\(\tau\)) is the average time a fluorophore stays in the excited state after excitation\textsuperscript{221-223}(in the ns range)\textsuperscript{224}. Fluorescence emission is short-lived and discontinues once the excitation source is removed.
Phosphorescence Lifetime Imaging Microscopy (PLIM) is similar to FLIM but uses phosphorescent O$_2$ sensitive dyes. In phosphorescence emission, Intersystem crossing occurs (ISC) (seen in (D)) to the excited triplet state $T_1$, followed by internal conversion to the lowest vibrational of the triplet excited state (E). Finally, excited triplet state $T_1$ returns to the ground state $S_0$ ($10^{-3}$-10$^2$ sec) through radiative transition in the form of phosphorescence emission (F). Unlike fluorescence, phosphorescence provides extended, longer lifetimes in the microsecond (µs) range. Both fluorescence and phosphorescence exhibit Stokes Shift, which occurs as the excited molecule returns to ground state; it emits light at a longer wavelength and lower energy compared to the absorbed light. This phenomenon is more pronounced for phosphorescence emission since it occurs from a lower energy state.

Fig. 5.2 displays the oxygen states after phosphorescence quenching in their first (or singlet) state ($^1\Delta_g$), second ($^1\Sigma_g^+$) and triplet excited states ($^3\Sigma_g^-$). Some excited dye molecules endure quenching through collisional interaction with molecular O$_2$, reducing the production and lifetime of phosphorescence in a concentration-
dependent manner\textsuperscript{225}. The reaction by which O\textsubscript{2} quenches luminescence is not entirely understood. However, it has been suggested that the paramagnetic O\textsubscript{2} causes the luminophore to convert to the excited triplet state (\textsuperscript{3}Σ\textscript{g}⁻) through intersystem crossing. Molecular O\textsubscript{2} changes to the first (\textsuperscript{1}Δ\textscript{g}), or the second excited state (\textsuperscript{1}Σ\textscript{g}⁺), thus finally returning to the ground state\textsuperscript{226,227}. The main molecule of the quenching process is reactive singlet oxygen (\textsuperscript{1}O\textsubscript{2}) which has a relatively short lifespan and can quickly deactivate back to the ground state O\textsubscript{2} following excitation.

\textbf{5.2.3 Luminescence Quenching}

Most O\textsubscript{2} sensitive probes have properties designed to quench the luminescence (either fluorescence or phosphorescence) in order to detect O\textsubscript{2}. Luminescence quenching involves the excitation of a phosphor and returns to ground state either by the emission of light (phosphorescence) or by transferring energy to other molecules (or quenchers in the environment). Luminescence quenching occurs after the dynamic collision of molecular triplet state oxygen and the excited electronic state from the phosphor by energy transfer. This is mostly accompanied by the formation of singlet oxygen (\textsuperscript{1}O\textsubscript{2}). Luminescence quenching is fully reversible, and does not have an effect on the absorption spectra. Phosphorescence quenching displays a reduction of intensity and decay time, which can be converted to O\textsubscript{2} concentrations using the Stern-Volmer equation\textsuperscript{228}. The measurement of the intensity is another method of monitoring photoluminescence. However, intensity signals can be altered by factors such as photo bleaching of the O\textsubscript{2} probe, probe concentration, autofluorescence and light scattering by the sample, which can lead to improper readings\textsuperscript{229}. Lifetime measurements are not affected by light exposure or photo bleaching, thus proving the most reliable method of conveying PLIM results.

\textbf{5.3 Phosphorescent O\textsubscript{2} sensitive probes}

In recent times, a large group of phosphorescent probes have been developed which allow extra-, peri- and intracellular measurements of O\textsubscript{2}. Each probe differs in its compatibility with cell and tissue models, imaging modalities and analytical tasks. Various in-vitro, ex-vivo and in-vivo models using these probes have allowed for better
understanding of their requirements for efficient O$_2$ imaging, such as probe distribution in the sample, stability of calibration and sensitivity to environmental factors$^{230}$. This section will briefly introduce O$_2$ sensing probes, their characteristics and challenges faced when applying this method to the eye. Phosphorescence based O$_2$ sensitive probes come in soluble form. Probes can be macromolecular, micro- or nano-particle.

5.3.1 Phosphorescent O$_2$ dyes, sensors, probes (luminescent molecular probes)

Phosphorescent O$_2$ dyes are the most popular and widely used O$_2$ probes and are based on the measurement of photoluminescence, particularly phosphorescent signals in relation to O$_2$ quenching. The O$_2$ sensitive materials contained in the dye molecules are based on indicator dyes with lifetimes in the microsecond (µs) range$^{227,231}$. Pt(II) and Pd(II) porphyrins are the most popular indicator dyes due their bio-compatibility, availability and cost. Pt-porphyrins are best adapted for observing O$_2$ from 0-200 µM physiological range, while Pd-porphyrins are more beneficial on the lower < 50 µM O$_2$ range$^{229}$. Pt-porphyrins have shorter emission lifetimes, allowing quicker acquisition times and would be preferred over dyes containing Pd-porphyrins$^{226}$. Other popular dyes include PdTCPP, PtTFPP and PtCP. PtTFPP has high photo-stability and brightness, which would be the favoured dye for high resolution microscopy. The use of Pt-benzophosphyrins are preferable in more complicated structures such as tissue due to their ability to emit in the red and near infrared spectrum (600-900 nm).

5.3.2 Conjugated polymers

Conjugated polymer nanoparticles are decidedly compatible with high resolution O$_2$ imaging, allowing for measurement of both phosphorescent lifetime and ratiometric intensity in the visible or near infra-red spectral ranges due to their charged groups. They comprise a conjugated polymer matrix which is copolymerised with the indicator O$_2$ sensitive dye. Conjugated polymer nanoparticles have improved depth penetration, cell staining and brightness when compared to other probes$^{232}$. The polymers are made up of a polyfluorene/ poly (fluorine-alt-benzothiadiazole) backbone antenna, which emits in red and infrared based on a Pt(II)-porphyrin. Copolymer nanoparticles can be prepared with a variety of charged groups, described as negative, positive or
zwitterionic which are responsible for efficient intracellular staining and deeper penetration within samples. In this case, zwitterionic charged groups display the best staining and penetration\textsuperscript{232}.

Nanoparticle-based (NP) fluorescent probes of \( \text{O}_2 \) usually comprise an \( \text{O}_2 \)-sensitive polymeric matrix in which the indicator dye is chemically adapted or surrounded by a supramolecular or nanoparticle (NP) structure. Nanoparticle probes have a typical size of 30-200 nm. They have the ability to penetrate cells or tissue and can remain stable for physiological and imaging experiments. NP Probes have 5-10 times higher brightness and improved performance for \( \text{O}_2 \) imaging. NP probes known as SII-(0.2+,SI-0.1+/0.1-,SI-0.2+/0.2-)\textsuperscript{232} allow ratiometric intensity and phosphorescent lifetime based \( \text{O}_2 \) detection, under one and two photon excitation, in the visible and near-infrared spectral ranges\textsuperscript{233}.

In this study, the dominant \( \text{O}_2 \) probe was SII polyfluorene 2,1,3-benzothiadiazole. The size of the SII probe is approximately 50 nm, which is relatively small for intracellular measurements. Each \( \text{O}_2 \) probe has different spectral characteristics and cannot be designated as a “one for all group”, however previous studies have shown SII-copolymers offers fast and efficient staining and deep penetration\textsuperscript{232}.

### 5.4 \( \text{O}_2 \) Probe Calibration

A benefit of PLIM using TCSPC is the need to calibrate each \( \text{O}_2 \) probe only once, offering stable readings of average phosphorescent lifetime decays, and intensity ratios. All \( \text{O}_2 \) probes vary in their average lifetime decays and intensities, as each serve different functions in imaging microscopy (ie cells, tissue, membranes of varying thickness, excitation etc).

The calibration process should replicate physiological conditions for planned experimentation. The \( \text{O}_2 \) probe is measured at several \( \text{O}_2 \) concentrations such as atmospheric levels (21\% \( \text{O}_2 \)) and hypoxia (0\% \( \text{O}_2 \)), manipulated by deoxygenating agents. Stern-Volmer typically predicts a linear relationship from dynamic collisional quenching method of the lifetime and intensity values of the probe within a limited range (Fig 5.3). The real Stern-Volmer exhibits a non-linear function due to static
quenching, which can occur in some O₂ sensitive materials due to the formation of a stable complex with another molecule, resulting in a reduction of the fluorophore since the quencher is essentially reducing the number of fluorophores which can emit. The resulting averaging signals of the phosphorescence intensity (I), lifetime decay (τ) relate to the O₂ concentration reflected by the Stern-Volmer equation, described as:

\[
\frac{I_0}{I} = 1 + k_{sv}[O_2] \quad \text{or} \quad \frac{\tau_0}{\tau} = 1 + K_{sv}[O_2]
\]

(15)

\(I_0\) and \(\tau_0\) represent the intensity and lifetime values at zero O₂. The quenching constant \(K_{sv}\) is the Stern-Volmer constant which includes sensitivity of the O₂ material and is also a function of \(\tau_0\). Finally, \([O_2]\) is the concentration of molecular O₂ in the sample, which can be converted to µM by inserting the averaged lifetime decays into the Stern-Volmer equation.

Fig 5.3. Example of a Stern Volmer relationship to lifetimes and intensity. The Y axis (left) shows intensity and the ideal versus real Stern-Volmer plot is shown.

5.5 Benefits of TCSPC-PLIM

Obtaining the best resolution for real-time PLIM imaging is of the utmost importance. High resolution can be achieved through compatibility with intense red lasers leading to two-photon processes, and single channel recording (contrasting with intensity-
based ratio measurements). Phosphorescent dyes provide mono-exponential decay, which is more candid than FLIM and fluorescent dyes (which have multiple-exponential decays).

Confocal microscopy consists of a scanning laser beam that is focussed onto the sample for imaging, with an adjustable pinhole placed in front of the detector. The pinhole prevents out-of-focus light from reaching the detector, which results in increased contrast and optical resolution and, most importantly, optical sectioning capability. However, confocal microscopy has limitations such as photo-damage, optical scattering, photo-bleaching, and limited depth resolution. Two-photon microscopy provides deeper tissue penetration and lower photo-toxicity at sub-micron resolution\textsuperscript{235}. During the two-photon process, a molecule absorbs two photons simultaneously whose individual energy is only half of the energy needed to excite the molecule. Two-photon microscopy typically requires a laser scanning microscope equipped with a near infra-red (NIR) ultrafast pulsed laser. Therefore, the difference between a confocal and two-photon microscope is the excitation light source and the fluorescence detection unit. Phosphorescence quenching of \( \text{O}_2 \) probes have been investigated using high resolution in the cerebral vasculature\textsuperscript{236}.

### 5.6 Previous work using TCSPC-PLIM

\( \text{O}_2 \) imaging using the luminescence quenching technique is not a new subject area. A multitude of peer-reviewed literature can be found on topics such as marine biology\textsuperscript{237}, oncology\textsuperscript{238}, cardiology\textsuperscript{239}, cell metabolism\textsuperscript{240} etc.

Studies using PLIM in the eye, particularly the retina have been conducted in animal models in-vivo. Shonat et al\textsuperscript{241-242} investigated \( \text{O}_2 \) values in the optic nerve head of cats, and obtained 2D intravascular \( \text{O}_2 \) maps of the retina and optic nerve. The group used a porphyrin based \( \text{O}_2 \) sensing probe (PtTFPP) which was injected into the blood stream. Other animal models such as pigs\textsuperscript{243}, mouse and monkey have also been studied using PLIM. In the mouse model, the \( \text{O}_2 \) phosphorescence probe Oxyphor G2 (polyglutamic Pd tetrabenzoporphyirin dendrimer) was injected through the tail vein whilst the animal was under anaesthesia. The phosphor dissolved through the vascular system enabling \( \text{O}_2 \) distribution maps in the retina within 3 min\textsuperscript{244-245}.  

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55
Anterior segment studies compared previous mathematical models of oxygen consumption in rabbit corneas to the phosphorescence quenching method. The group also measured effect of $O_2$ when wearing rigid gas permeable contact lenses (RGPs)\textsuperscript{246-247.}

Based on previous literature, phosphorescence quenching of $O_2$ sensitive probes has been successfully applied to the cornea of rabbit eyes. Factors that must be considered in order to measure corneal oxygenation pre- and post- CXL treatment include the achievement of good depth penetration of the probe in the cornea (or other sample models), stability of atmospheric $O_2$ under treatment conditions (e.g. use of humidified chamber of 37°C during imaging) and the use of the correct excitation and emission wavelengths to avoid photo bleaching of riboflavin during imaging. Finally, repeatability of results is essential for future application in the cornea.

In summary, previous methods of measuring $O_2$ in the eye discussed in this chapter include invasive fibre-optic needle sensors and the Clark electrode method. Fibre-optic needle sensors have been used under anaesthesia, in-vivo and ex-vivo animal models. A minimally invasive, safer technique would be beneficial for both patient and surgeon for future human in-vivo use. Time-Correlated Single Photon Counting Phosphorescence Lifetime Imaging (TCSPC-PLIM) is an innovative and complex method for fluorescence and phosphorescence lifetime measurements due to luminescence $O_2$ quenching. TCSPC-PLIM can measure molecular oxygen ($O_2$) in an X-Y scan, potentially with 3D, if the $O_2$ probe is available. This technique is minimally invasive, involving drops of a soluble phosphorescent sensitive $O_2$ probe onto the eye following by imaging. This study aims to determine the most suitable $O_2$ sensitive phosphorescence probe to image the cornea before and after CXL, in order better understand the photochemical processes occurring during CXL. To date, CXL $O_2$ analysis has not been reported using the TSCPC-PLIM technique.
Chapter 6: Oxygen Sensing Methods

This chapter reports on the use of O₂ sensitive phosphorescent probes to determine the presence and behaviour of O₂ before and after CXL.

6.1.1 Study Design

This study investigates the use of O₂ sensitive phosphorescent probes using three models: (i) O₂-sensitive nanoparticles in a soluble solution of riboflavin without collagen, (ii) collagen type-I gel with O₂-sensitive nanoparticles and riboflavin, and (iii) porcine eyes stained with O₂-sensitive nanoparticles and riboflavin.

6.1.2 Experimental Setup

Fig. 6.1 illustrates the standard TCSPC-PLIM hardware (Becker& Hickl GmbH), compatible with conventional laser-scanning microscopes as well as with multi-photon excitation systems based on near-infrared femtosecond lasers²⁴⁸. The one-photon confocal PLIM system used consists of an upright microscope, Axio Examiner Z1 with
a motorized Z-stage and temperature-control (Carl Zeiss), to which a DSC-120 confocal scanner, TCSPC hardware and detectors (Becker&Hickl) are attached\textsuperscript{249}. The scanner has two optical channels for excitation, connected to a 6 W picosecond supercontinuum laser model SC400-4 (Fianium, UK), which covers a broad range of excitation wavelengths 400 – 650 nm, and a 1 mW 405 nm picosecond diode laser (Becker& Hickl). The latter has a higher energy output at the Soret band (390-410 nm) and is more efficient in exciting Pt-porphyrin luminescences. The Fianium laser is used for excitation of Pd- and Pt-benzoporphyrins (these have strong bands at around 440-470 nm and 614-630 nm). The power of excitation is regulated manually by the aperture and neutral density filter(s) in the DSC-120 unit. The two emission channels of the DSC-120 scanner are connected to two high-speed, high-sensitivity photon-counting detectors (R10467U-40/50, Hamamatsu Photonics K.K.), which operate in the visible - red (400 – 700 nm) and red - near-infrared (600 – 900 nm) spectral regions. Emission is collected through a combination of relevant longpass and bandpass filters (Fig 6.2).

6.1.3. Data analysis and image processing

Fig 6.2. The confocal TCSPC-PLIM system for O\textsubscript{2} imaging. A: upright microscope Axio Examiner Z1 with motorized heat stage (Zeiss) and Nikon 5100 digital camera for transmission light images (not shown). B: microscope and temperature controllers. C: picosecond 405 nm laser. D: DCS-120 confocal scanner attached microscope, detectors are connected from the back of the scanner (not shown). E: TCSPC and laser synchronization hardware. F: Enclosed humidified chamber which is controlled by B.
By applying the \( \text{O}_2 \) calibration function, lifetime images can be converted into \( \text{O}_2 \) concentration images and used for graphical or numerical representation. Fig. 6.3 shows an example of SPC Image software displaying the intensity (A) and PLIM (B). The distribution histogram of the phosphorescence decays across the sample. The decay matrix was calculated and presented as pseudo-colour image (range 5000-25,000 ns) (D) shows IRF mono-exponential fitting of the phosphorescence decay.

Fig. 6.3. Example of phosphorescence decay fitting of each sample obtained. (A, B) are the intensity and PLIM images, where nanoparticles can be seen. (C) represents the distribution histogram of the phosphorescence decays across the sample. The decay matrix was calculated and presented as pseudo-colour image (range 5000-25,000 ns) (D) shows IRF mono-exponential fitting of the phosphorescence decay.

By applying the \( \text{O}_2 \) calibration function, lifetime images can be converted into \( \text{O}_2 \) concentration images and used for graphical or numerical representation. Fig. 6.3 shows an example of SPC Image software displaying the intensity (A) and PLIM (B). The distribution histogram of the phosphorescence lifetime decay is calculated as a decay matrix and visualized as a pseudo-colour image across the sample (C), and (D) represents the Instrument Response Function (IRF) of intensity, exponential fitting of the lifetime decays and the laser signal. Chi-squared \( (X^2) \) indicates accuracy of the fitting. Four sections of each calculated decay matrix image were chosen as a region of interest (ROI), averaged and standard deviations were calculated in order to verify phosphorescence lifetimes across the whole image. The following calibration was produced: \[ [\text{O}_2, \mu\text{M}] = \frac{3450}{\tau - 145}, \] where \( \tau \) is in \( \mu\text{s} \). To ensure consistency of results, all experiments were performed in a number of samples i.e. 6 channels, 10
collagen type-I, 4 porcine eyes. As before, porcine eyes were used rather than human donor eyes as these can be easily obtained and do not require special safety protocols. Moreover, their size and properties are highly similar to those of human eyes.

6.1.4. Choice of O\textsubscript{2} Probe: diffusion ability

The first set of experiments investigated various O\textsubscript{2} probes to ensure suitability for this study. Porcine corneas are thicker than human corneas\textsuperscript{188} which can be difficult for deep penetration using a one-photon laser. Taking this into account, the chosen probe should have efficient, quick and deep staining. Preliminary measurements tested 9 phosphorescent based O\textsubscript{2} probes of varying properties to investigate the most suitable O\textsubscript{2} probe for application to the eye. Their properties are summarized in Table 2.

6.1.5 Method

Porcine globes were stained with individual O\textsubscript{2} probes for 30 min. after epithelial removal (concentrations shown in Table 2). For imaging, 5 optical sections were measured (0-200 µm) in steps of 50 µm. 256 x 256 pixel scanning time in PLIM mode usually takes 1-2 min for a 2-D section. The excitation/emissions for each probe are shown in Table 2.

Table 2. Phosphorescent characteristics for dyes used in preliminary O\textsubscript{2} sensing experimentation. Dyes in (*) were also imaged but are not shown in results due to poor compatibilities in the cornea. ‘SII-A’ is a zwitter-ionic copolymer nanoparticle, prepared and provided by Prof S.Borisov (Graz University of Technology, Austria).

<table>
<thead>
<tr>
<th></th>
<th>Properties/description</th>
<th>Excitation nm</th>
<th>Emission nm</th>
<th>Concentration used</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SI-0.2+</strong></td>
<td>Conjugated polymer, cationic red</td>
<td>405</td>
<td>635-675</td>
<td>20 µg/ml</td>
<td>232</td>
</tr>
<tr>
<td><strong>SI-0.1+/0.1-</strong></td>
<td>Conjugated polymer, mixed charge, cationic red</td>
<td>405</td>
<td>635-675</td>
<td>20 µg/ml</td>
<td>232</td>
</tr>
<tr>
<td></td>
<td>Properties/description</td>
<td>Excitation nm</td>
<td>Emission nm</td>
<td>Concentration used</td>
<td>Reference</td>
</tr>
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<td>------------------------------------------------------</td>
<td>---------------</td>
<td>-------------</td>
<td>--------------------</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>SI-0.2+/0.2-</strong></td>
<td>Visible light infrared, cationic red</td>
<td>405</td>
<td>635-675</td>
<td>20 µg/ml</td>
<td>232</td>
</tr>
<tr>
<td><strong>SI-0.05+/0.15-</strong></td>
<td>Visible light infrared, cationic red</td>
<td>405</td>
<td>635-675</td>
<td>20 µg/ml</td>
<td>232</td>
</tr>
<tr>
<td><strong>PA2 (PtTFPP)</strong></td>
<td>Anionic, visible light emitting cationic red</td>
<td>405</td>
<td>635-675</td>
<td>25 µg/ml</td>
<td>253</td>
</tr>
<tr>
<td><strong>MM2 (PtTFPP)</strong></td>
<td>Anionic, visible light emitting cationic red</td>
<td>405</td>
<td>635-675</td>
<td>25 µg/ml</td>
<td>254</td>
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<tr>
<td><strong>PEPP3 (PtCP)</strong></td>
<td>Peptide based (peptide porphyrin conjugate 0)</td>
<td>405</td>
<td>635-675</td>
<td>10 µM</td>
<td>255</td>
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<tr>
<td><strong>PEPP0 (PtCP)</strong></td>
<td>peptide based (peptide porphyrin conjugate 0)</td>
<td>405</td>
<td>635-675</td>
<td>10 µM</td>
<td>255</td>
</tr>
<tr>
<td><strong>SII-0.2+</strong></td>
<td>Conjugated polymer, infrared, cationic</td>
<td>405/488</td>
<td>760-810</td>
<td>100 µg/ml</td>
<td>232</td>
</tr>
<tr>
<td><strong>SII-0.2-</strong></td>
<td>Conjugated polymer, infrared, anionic</td>
<td>405/488</td>
<td>760-810</td>
<td>100 µg/ml</td>
<td>232</td>
</tr>
<tr>
<td><strong>Pt-Glc</strong></td>
<td>Small molecule conjugate, neutral</td>
<td>405</td>
<td>635-675</td>
<td>5 µM</td>
<td>250</td>
</tr>
<tr>
<td><strong>Oxyphor G2</strong></td>
<td>Anionic, red-emitting, dendrimer-based</td>
<td>440/632</td>
<td>760-810</td>
<td>1 mM</td>
<td>251</td>
</tr>
<tr>
<td>Properties/description</td>
<td>Excitation nm</td>
<td>Emission nm</td>
<td>Concentration used</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>--------------</td>
<td>-------------</td>
<td>--------------------</td>
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<tr>
<td><strong>IR-GLC</strong></td>
<td>405-570</td>
<td>733</td>
<td>5 µM</td>
<td>252</td>
<td></td>
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<tr>
<td>Small molecule conjugate, neutral, near infra-red</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SII-A</strong></td>
<td>405/488</td>
<td>760-810</td>
<td>100 µg/ml</td>
<td>Not published</td>
<td></td>
</tr>
<tr>
<td>Conjugated polymer, red and infrared, zwitter-ionic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 6.1.6 Sulphite-aided depletion of O₂

The use of a deoxygenating sulphite agent in this study was beneficial to observe levels of oxygen depletion, since it was not feasible to have a controlled chamber with accurate control of O₂.

Sodium sulphite 50 mg/ml (Sigma Aldrich®) was mixed with 50 mg/ml buffering agent potassium dihydrogen phosphate (\( KH₂PO₄ \)) to create a deoxygenating agent capable of depleting O₂ concentrations.

\[
Na₂SO₃ + O₂ → Na₂SO₄
\]  \hspace{1cm} \text{(16)}

Equation 16 demonstrates the sulphite reacting with O₂ to form sulphate, depleting molecular O₂.

### 6.2. SII-A nanoparticles dilution

The working concentration of SII-A was 100 µg/ml, diluted either in PBS or 0.1% riboflavin. Streuli Hypotonic (riboflavin-5-phosphate 0.5% without dextran T-500) was diluted to 0.1%. Preliminary testing found that higher concentrations of riboflavin (<0.5%) showed higher lifetime decays, possibly due to spectral interference. For control measurements, 200 µl PBS was mixed with 10 µl SII-A.
6.2.1 Imaging setup

Table 3 describes the properties and laser settings used for each experimental model. Decreasing pixel resolution to 4 times less than 256 x 256 (e.g. 64 x 64) allows for better signals and shortened acquisition time (from 2 min to 30 sec). For porcine corneas, excitation was done at 405 nm wavelength where laser power was highest. Thus, scan time was reduced to 20 sec due to high signals and a bright contrast in the cornea.

6.2.2 UV Source and depth of imaging

For cross-linking, a CE approved, UV-X™ prototype (IROC Science AG) lamp was used for UV exposure. 7 mW/cm² for 7 min (wavelength 365 nm) was the chosen intensity for all samples due to reduced exposure time (allowing for multiple samples to be imaged consecutively). Once the UV was switched off, samples were placed on the stage and imaged within 10 sec. A fixed imaging depth of 100 µm was chosen for comparison of results to previous experimental studies.

Table 3. Characteristics used when characterizing each model sample.

<table>
<thead>
<tr>
<th></th>
<th>Solution based Micro-chamber ‘channels’</th>
<th>Collagen type-I gel</th>
<th>Porcine Eyes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pixel size/scale bar</td>
<td>64 x 64 (500 µm)</td>
<td>64 x64 (500 µm)</td>
<td>64 x64 (500 µm)</td>
</tr>
<tr>
<td>Excitation/Emission</td>
<td>488 /760 nm</td>
<td>488 /760 nm</td>
<td>405 /760 nm</td>
</tr>
<tr>
<td>Objective</td>
<td>5x, NA 0.25</td>
<td>5x, NA 0.25</td>
<td>5x, NA 0.25</td>
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<tr>
<td>Scan time</td>
<td>40 sec</td>
<td>40 sec</td>
<td>20 sec</td>
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<tr>
<td>Longpass</td>
<td>665 nm</td>
<td>665 nm</td>
<td>665 nm</td>
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<tr>
<td>Emission Bandpass</td>
<td>760-810</td>
<td>760-810</td>
<td>760-810</td>
</tr>
<tr>
<td>Z-scan depth</td>
<td>N/A</td>
<td>100 µm</td>
<td>100 µm</td>
</tr>
</tbody>
</table>

6.2.3 O2 Diffusion Model

An axial diffusion model of O₂ was made based on Ficks 2nd law of diffusion using COMSOL™ (COMSOL Inc), with the aim of analysing the effectiveness of diffusion of oxygen in porcine cornea as a function of time for different diffusion constants and boundary conditions. The numerical analysis used the standard diffusion coefficient of
oxygen in the corneal stroma seen in previous work with $D = 2.81 \times 10^{-3} \text{ mm}^2/\text{s}$ and compared to experimental results.

### 6.3 Solution based sample (Micro-channels)

In this study, 6 channels and 1 control were imaged before and after corneal cross-linking to determine reactions without substrates such as collagen type-I and pig corneas. Due to high variability of sample age from animals, a micro-chamber with channels was chosen as a suitable control in order to investigate the role of O$_2$ nanoparticles and riboflavin-UV interactions in the absence of other structures that could confound the analysis. The µ-Slide VI$_0^\circ$ (shown in Fig 6.4), is typically used for immunofluorescence assays due to their ability for homogenous distribution over the channels surface, enabling change of O$_2$ concentration using deoxygenating sulphite.

![Image of micro-chamber](image)

**Fig. 6.4.** Example of Multichannel micro-chamber (µ-Slide VI$_0^\circ$·4 ibidi®). The image shows (indicated by yellow) riboflavin and SII-A O$_2$ probe homogenously distributed across the channel.

#### 6.3.1 Method

SII-A (100 µg/ml in PBS) was added into the channels using a pipette. Hypotonic riboflavin 0.1% was added to the sample and imaged. The channel was then illuminated (7 mW/cm² for 7 min) and imaged twice consecutively (UV1, UV2) within 10 sec post illumination. To restore O$_2$ levels, the channel was equilibrated for 20 min and imaged again. Finally, the sample was deoxygenated using Sodium sulphite and imaged again. In the control experiment, no riboflavin was added to the channel.

### 6.4 Collagen type-I gel

Collagen type-I is the most abundant protein in the cornea and is a suitable model for corneal studies as an alternative to ex-vivo tissue. Nutragen® Bovine Collagen type-I
gel solution (3 mg/ml) was used in this experiment to confirm results from others models in this study. In this study, 10 corneal ‘buttons’ and 1 control sample were imaged before and after corneal cross-linking.

6.4.1 Collagen type-I Gel Preparation

Nutragen® Bovine Collagen type-I (Advanced Biomatrix) was placed in a beaker (4680 µl), pH 2.0 and mixed with 432 µl PBS (10X) pH 7.4. The pH was adjusted using a pH meter to 7.2 by using sterile 0.1 M of Sodium Hydroxide (NaOH). The solution was pipetted into individual rings of average thickness (1 mm) (see Fig. 6.6).

![Image]

**Fig. 6.5.** Transmission light image of collagen type I gel and O<sub>2</sub> nanoparticles. Interweaving fibril structure can be seen. Nanoparticles are distributed across the sample and are indicated by red arrows.

To form the gel, samples were incubated at 37° for 3-24 hours. Once the solution had compacted into a solid gel, the samples, which resembled corneal buttons were left at room temperature until imaging commenced. Mean central corneal thickness (CCT) was measured using an Ultrasound pachymeter (Sonomed Micropach Model 200p+) after imaging. Collagen type-I gels are considered to be a good tissue equivalent for pig or humans corneas, providing the same fibril structures that are found in the eye. Fig 6.5 displays a transmission light image of fibrilar structures and presence of O<sub>2</sub> nanoparticles in Collagen type-I gel.
6.4.2 Method

SII-A (100 µg/ml in PBS) was added to the collagen gel ‘buttons’ using pipette and imaged (Fig 6.6). Hypotonic riboflavin 0.1% was added to the sample and imaged. The collagen was then illuminated (7 mW/cm² for 7 min) and imaged for approx. four minutes in 8 cycles (UV1- UV8) within 10 sec post illumination. To restore O₂ levels, collagen buttons was equilibrated for 10 min and imaged again. Finally, the sample was deoxygenated using Sodium sulphite and imaged again. In the control experiment, no riboflavin was added to the collagen samples.

6.5 Eyes

The final part of the study investigated the use of O₂ phosphorescent sensitive probes using PLIM and applied it to porcine eyes and compared to results from the other models (i.e. solution-based channels and collagen type-I gel). In this study, 4 porcine corneas and 1 control eye were imaged before and after corneal cross-linking.
6.5.1 Porcine eye preparation

Enucleated porcine eyes from 5- to 6-month old animals were obtained 2 to 3 hours post-mortem and stored at 4°C until used within 12-24 hours (eyes were kindly provided by an abattoir). All treated corneas were found to be clear, with no presence of corneal scarring or opacities. The epithelium was removed using an epi-hook, and the eyes were mounted in a holder. A ring was placed on the cornea to ensure efficient diffusion of solutions.

6.5.2 Method

SII-A (100 µg/ml in PBS) was added to the cornea using a pipette and imaged after 30 min. Hypotonic riboflavin 0.1% was added to the sample and imaged after 1 hour. The eyes were then illuminated (7 mW/cm² for 7 min) and imaged for approx. two minutes in 8 cycles (UV1- UV8) within 10 sec post illumination. To restore O₂ levels, eyes was equilibrated for 10 min and imaged again. Finally, the sample was deoxygenated using Sodium sulphite for 1 hour and imaged again. In the control experiment, no riboflavin was added to the eye.
Chapter 7: Experimental results using O₂-sensitive probes using PLIM

The aim of the study is to investigate the feasibility of measuring O₂ concentrations in the cornea during corneal cross-linking and to analyse O₂ diffusion in porcine corneas during CXL. Phosphorescent O₂-sensitive nanoparticle probes and TCSPC-PLIM counting software are used as a novel method to measure O₂ in the eye before and after CXL. A total of 9 O₂ probes of varying phosphorescent conjugated polymers were investigated for suitability when imaging in the eye.

7.1 Probe signal investigation

In order to apply phosphorescent O₂ sensitive probes to the eye, it is important first to measure available probes and analyse the resulting penetration depth. If phosphorescent based O₂ probes can eventually be made suitable for in-vivo use, it is important to determine which O₂ probe would be most effective and allow deep staining.

7.1.1 Decay Fitting

Fig. 7.1 shows an example of exponential decay fitting of the intensity of photons per pixel (blue dots). The instrument response function, IRF, has a small influence on the true phosphorescence decay profile as shown by the signal from the laser pulse directly detected by the system (black triangle). Fig. 7.1 also shows an example of the decay fit (red), a mono-exponential fitting obtained by adjusting a single exponential function to the temporal decay of the intensity values.
7.1.2 Efficacy of staining of cornea with O₂ probes

The first set of results investigated in-depth penetration of nine sets O₂ sensitive probes in porcine whole globes. Each cornea was stained for 30 min before imaging with individual probes (Fig 7.2). Results in Fig 7.2 display nanoparticle structures in porcine corneas ranging from 0 - 200 µm. All probes show reasonable depth penetration in the first 100 µm into the corneal stroma, with the exception of PA2 (PtTFPP) which shows little to no nanoparticle structure throughout. This could be due to staining time (30 min) or its spectral ability to emit in visible light only. During imaging, SI-0.1+/0.2- and SII-0.2+, and zwitter-ionic SII-A reveal high signals throughout the cornea, and show significant depth penetration up to 200 µm, possibly due to their infrared, zwitter-ionic characteristics.
Fig 7.3. show intensity profiles corresponding for probes (SI-0.5+/0.15-(A), and PtTFPP probes (B,C)), seen in Fig 7.2. The background (BG) count is the lowest photon count per pixel in each sample (or depth). Nanoparticle visualization and intensity error for SII-A show consistent staining (and stable intensities) throughout depths 0-200 µm in the cornea, whereas O₂ probes PtTFPP, SI-0.1+/0.1- show contrary staining. High errors of standard deviation are visible in the intensities due to their poor ability to work in the cornea, possibly due to the excitation wavelength (405 nm).
and spectral properties of the probes. The SPCImage software produces the intensity image in the form of values (photons per pixel of image), and as a jpeg (Fig. 7.2). Results in Fig. 7.3 show for each depth, the average and standard deviation of four sub-regions is calculated. This is also performed when determining phosphorescence decays. Fig 7.3 (A,B,C) shows non-normalized intensities of photon counts per pixel. High standard deviations and uncertain values can be seen throughout depths 0-200 µm. Fig 7.3 (D,E) reveals both normalized and non-normalized intensities of SII-A from 0-200 µm, showing low standard deviations (i.e. better fits) and stable staining throughout. Of the 9 O₂ probes imaged, Fig 7.2 and Fig 7.3 conclude SII-A as the most suitable probe due to sufficient staining from 0 -200 µm (Fig. 7.2), low error standard deviation when determining the intensity (Fig. 7.3), and its spectral characteristics of red and infrared ranges in the spectrum. This could reduce chances of riboflavin interference during imaging. This probe will be described as ‘SII-A’ in the following results.
The comparison of spectra between SII-A and riboflavin confirmed that riboflavin shows strong yellow fluorescence and broad excitation, which interferes with most of the tested $O_2$ probes. Infra-red emitting SII-A show the best properties.

Fig. 7.3 Intensity of photons per pixel investigation for $O_2$ probes when selecting the most suitable for imaging in the cornea. SI-0.5+/0.15-(A), and PtTFPP probes (B,C) display high standard deviations and weak staining ability in porcine cornea throughout. (D,E) shows in depth staining throughout the porcine cornea (Normalized versus original intensity values) 0-200 µm.
Fig. 7.4. Normalized absorption/excitation and emission spectra of SII-A and riboflavin (left). SII-A peaks at approximately 450 and 620 nm. Riboflavin excitation peaks at 365 nm and 450 nm approximately. SII-A excitation and emission (right) is sensitive to deoxygenating sulphite agent. (1µg/ml SII-A, 0.01% Rf), T=20°C).

Fig. 7.4 (left) shows spectral overlap between the excitation and emission of SII-A and riboflavin. Absorption spectra for riboflavin and SII-A were recorded on a 8453 UV-vis diode-array spectrophotometer (Agilent) and luminescence spectra on a LS50B luminescence spectrometer (PerkinElmer). Hypotonic 0.5% riboflavin was diluted to 0.01% in a cuvette. The cuvette was placed in the spectrometer and the absorption spectrum was observed. SII-A (100 µg/ml) was also placed in a cuvette and spectrum was observed.

SII-A excitation peaks at ~450 nm or 620 nm approximately, whereas riboflavin is excited at 365 nm or 450 nm. Fig. 7.4 also displays O₂ sensitivity when deoxygenating agent sodium sulphite is added. At concentrations of 0.1% riboflavin is very bright. Previous O₂ probes (seen in Figure 7.2) are excited at 405 nm. In this study SII-A was excited using the Fianium laser (488 nm), with the exception of 405 nm excitation for porcine corneas. In this case, autofluorescence is always expected at the beginning of scanning, as it has shorter lifetimes.

### 7.1.3 Effect of UV illumination on SII-A

To test for photo bleaching of the probe when exposed to UV, a cuvette with SII-A solution was illuminated with 7 mW/cm² UV for 7 minutes and placed in the spectrometer. The resulting curves show the SII-A excitation peak between 550-770 nm before and after UV exposure.
At 620 nm, spectral absorption values were taken before and after UVA. A difference of 7% between the absorption values were found, suggesting UV has little effect on SII-A nanoparticles. The differences are displayed in Fig. 7.5 (right).

7.1.4 Choosing the optimal excitation wavelength for SII-A in PLIM

The most suitable excitation wavelength using SII-A was investigated. Fig 7.6 displays intensity values of SII-A comparing channels Fianium 488 nm and 632 nm in porcine corneas at a fixed depth of 50 µm. Each scan took 2 min. (256 x 256 pixels), and were measured from T=2 min to T=10 min. Fig. 7.6 (A) shows decreased intensity over time at 632 nm when compared to 488 nm. Fig. 7.6 (B) displays confocal images of 488 nm compared to 632 nm. A visual difference can be seen in the quality and contrast. Due to the results shown, Fianium 488 nm was the preferred wavelength during imaging for solution based channels and collagen type-I.

Fig. 7.5. Normalized SII-A absorption spectra with and without UV seen at 550-770 nm excitation peaks.
7.1.5 \( \text{O}_2 \) concentration and phosphorescence decay time

Fig. 7.7 displays the relationship between the \( \text{O}_2 \) concentration and the decay times for SII-A based on the phosphorescence lifetimes obtained during calibration. The higher the lifetimes, the lower the \( \text{O}_2 \) concentrations as the phosphorescence decay is
inversely proportional to the concentration of molecular O\textsubscript{2}. The lifetimes increased due to the addition of sulphite (18 µs), whereas atmospheric O\textsubscript{2} concentrations (200 µM) show a phosphorescence decay time of 10 µs. The following results will display both phosphorescence decay times and the converted O\textsubscript{2} concentration.

![Phosphorescence decay graph](image)

**Fig.7.7.** Phosphorescence decay is inversely proportion to molecular O\textsubscript{2} concentrations. The higher the lifetimes, the lower the O\textsubscript{2} concentrations and vice-versa.

### 7.1.6 Depth profile of O\textsubscript{2} distribution in the porcine cornea

6 porcine corneas were imaged to obtain a depth profile of O\textsubscript{2} distribution. Fig. 7.8 shows phosphorescence decay times and converted O\textsubscript{2} concentrations using SII-A with six porcine corneas from 0-250 µm. Results show a reduction in O\textsubscript{2} with increasing depth. The region of interest (ROI) is chosen by averaging four sections of the image (64 x 64 pixels). Fig 7.9 shows the depth profile of porcine corneas from 0-250 µm. At 0 µm, four ROI are indicated (red boxes). Approximate ROI was estimated to range from 50-250 µm.
In order to confirm the occurrence of a photochemical reaction between riboflavin and UV detected by PLIM, a control, without riboflavin added, of each sample model was imaged (solution based, collagen type-I and porcine corneas). SII-A (100 µg/ml in PBS) was added followed by UV exposure (7mW/cm² for 7 min). The sample was then imaged in a series of 8 cycles ranging from 20 sec to 3 min post UV (Table 4). Finally, the sulphite was added to deplete O₂ concentrations. For all controls, results show little to no change in O₂ concentrations before and after UV exposure (Fig.7.10). This confirms that the presence of riboflavin is essential for photochemical O₂ reactions to occur in the cornea when exposed to UV.

**7.1.7 Control measurements**

![Fig. 7.8. Depth profile 0-250 µm of 6 porcine corneas with PBS and SII-A. Measurements were recorded sequentially from 0-250 for ~4 min (20 sec per section).](image)

![Fig. 7.9. Colour images of lifetimes for porcine corneal depth profile. Approximate regions of interest (ROI) during analysis are indicated within the red boxes. The same ROI were calculated throughout 50-250 µm. Phosphorescence decay of SII-A ranges from 10 (200 µM) - 18 µs (0 µM). Scale bar 500 µm.](image)
Fig. 7.10. Control results for 1) without substrate (i.e channels), 2) Collagen type-I, and 3) Porcine cornea. Little to no change in $O_2$ concentration after UV can be seen in the absence of riboflavin. Colour images display phosphorescence decay changes (seen in scale colour bar), approximate ROI are indicated within the red boxes. Image scale bar 500 µm.
Table 4. Imaging steps taken for measurements in 2) Control UV interactions using collagen Gel and, 3) Control UV interactions using porcine corneas

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<td>UV7 (4 min 40 sec)</td>
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<td>UV8 5 (min 20 sec)</td>
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7.2 Investigation of oxygen depletion during UV CXL

Results shown above demonstrate the SII-A probe may be the most suitable for O\textsubscript{2} determination in the cornea. The next step in this study is to investigate O\textsubscript{2} concentrations before and after CXL.

7.2.1 Solution based sample (Micro-channels)

A solution based micro-chamber comprising six channels was chosen as an alternative model in order to investigate the role of O\textsubscript{2} nanoparticles and riboflavin-UV interactions. Fig 7.12 displays the colour images of phosphorescence decays for one channel. The red boxes represent the regions of interest (ROI) when calculating decays. The changes in lifetime can be observed visually and experimentally by observing lifetime changes on the colour scale bar.

Fig. 7.11 shows box plots of six solution based micro-chamber channels imaged before and after CXL. SII-A (100 µg/ml in PBS) was added into the channels using pipette and imaged Fig. 7.11 (A). Hypotonic riboflavin 0.1% was added to the sample and
imaged (B). Results show little to no change in atmospheric O$_2$ concentrations (~200 µM) between the addition of riboflavin 0.1% and baseline measurements of SII-A probe in PBS (Fig. 7.11 A,B). Within 10 sec post illumination of the channel (7 mW/cm$^2$ for 7 min), O$_2$ concentrations deplete to <150 µM. Results show O$_2$ concentrations return to baseline after 20 min (E), followed by depletion of O$_2$ concentration (50 µM) upon sulphite addition. Results show the photochemical reaction mechanism that occurs between photosensitizer riboflavin and UV light.

![Fig.7.11](image)

PBS+ SII-A (A)  
SII-A + Ribo (B)  
UV1 (C)  
UV2 (D)  
20 min post UV (E)  
Sulphite (F)

**Fig.7.11.** Solution based channels displaying O$_2$ changes before and after UV-corneal cross-linking. (A) displays phosphorescence decays (µs), (B) is the converted O$_2$ concentration (µM). A-F describes the stages of imaging. Phosphorescence decay of SII-A ranges from 10 (200 µM)-18 µs (0 µM).

![Fig 7.12](image)

**Fig 7.12.** Colour images of lifetimes for one channel. Approximate regions of interest (ROI) during analysis are indicated within the red boxes. Phosphorescence decay of SII-A ranges from 10 (200 µM)-18 µs (0 µM). Scale bar 500 µm.
### 7.2.2 Distribution histogram of solution based Micro-chamber channel

Distribution histograms have the ability to show the photon distribution over distance along the scan. Displaying the distribution histogram is an efficient tool to determine decay parameters in selected regions-of-interest (ROI), and to determine the decay parameters of different samples. Fig.7.13 shows the changes in the distribution profile due to changes in phosphorescence decays (UV1, UV2). Upon sulphite addition, the distribution changes due to the large change in phosphorescence decays (decays change drastically).

![Distribution histogram of one solution-based micro-chamber channel.](image)

**Fig 7.13.** Distribution histogram of one solution-based micro-chamber channel. Changes in phosphorescence decays can be seen after UV exposure, followed by a dramatic increase in decays after sulphite addition.

### 7.3 Collagen type-I gel

Collagen type-I was chosen to observe $O_2$ depletion at a fixed depth using a sample suitable as a corneal eye model. A fixed depth of 100 µm was chosen for experiments due to the observation of $O_2$ depletion at this depth in previous literature$^{31}$. 
7.3.1 Collagen type-I Results

Fig 7.14 displays box plot results of 10 collagen type-I samples (average CCT 600-700 µm) before and after UV exposure. The sample were first imaged with SII-A (100 µg/ml in PBS), followed by the addition of hypotonic 0.1% riboflavin (Fig 7.14 A,B). The samples were then exposed to UV (7 mW/cm² for 7 min), and imaged within 10 seconds post UV involving 8 cycles between 40 sec and 5 min. Results reveal a slight change in O₂ concentrations after riboflavin addition (15 µM approx.). After UV exposure, O₂ levels deplete to approx. 150 µM (Fig. 7.14 C), followed by an increase of O₂ over 5 min. O₂ levels equilibrate to baseline measurements 10 minutes post UV exposure. Finally, O₂ levels drop significantly after addition of O₂ deoxygenating agent (below 50 µM). Fig 7.15 displays the colour images of phosphorescence decay for one channel. The red boxes represent the approximate regions of interest (ROI) when calculating decays. The changes in lifetime can be observed visually and experimentally by observing changes to the scale bar.

| A | PBS+SII-A |
| B | Rf+SII-A |
| C | UV1 (40 sec) |
| D | UV2 (1 min 20 sec) |
| E | UV3 (2 min) |
| F | UV4 (2 min 40 sec) |
| G | UV5 (3 min 20 sec) |

Fig.7.14. Boxplots of 10 collagen samples displaying O₂ changes before and CXL (above). Phosphorescence decays (µs) are shown on the left, converted O₂ concentration (µM) (right). Phosphorescence decay of SII-A ranges from 10 (200 µM)-18 µs (0 µM).
**7.3.2 Distribution histogram for collagen type I-based experiment**

Fig. 7.15 changes to phosphorescence decays based on the method of imaging (UV 1, 3, 5, 7). The change in decays after UV confirms O₂ fluctuations in the collagen sample after CXL, confirming collagen type-I gels is a suitable model when comparing photochemical reactions in collagen type-I gel to those in a porcine cornea. Upon sulphite addition, the distribution changes due to the large change in phosphorescence decays (lifetimes change dramatically).
The use of collagen type-I gels in this study reveals efficient staining, fast acquisition scanning time and good resolution for PLIM. A change in O₂ concentrations were observed after UV and riboflavin induced cross-linking. The next step is to apply this method to ex-vivo porcine globes.

7.4 Study of O₂ diffusion during CXL in porcine corneas

Results seen in solution based and Collagen type-I gel matrix confirm the PLIM method is compatible when used to monitor O₂ concentrations before and after UV cross-linking. Finally, the PLIM method will be applied to porcine globes (corneas) and the results will be discussed. A wavelength of 488 nm was used to obtain PLIM in solution-based micro-channels and collagen Type I. Here, a 405 nm laser was used to ensure higher signals.
7.4.1 Corneal CXL results

Fig. 7.17. Boxplots of 4 porcine eyes displaying O$_2$ changes before and CXL (above). Phosphorescence decays (μs) are shown on the left, converted O$_2$ concentration (μM) (right).

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Fig 7.17 displays box plot results of 4 porcine corneas before and after UV exposure. The sample were first imaged with SII-A (100 μg/ml in PBS), followed by the addition of hypotonic 0.1% riboflavin (Fig 7.17 A,B). The eyes were then exposed to UV (7 mW/cm$^2$ for 7mins), and imaged within 10 seconds post UV involving 8 cycles between 20 sec and 3 min. Results reveal a slight change in O$_2$ concentrations after riboflavin addition (15 μM approx.). After UV exposure, O$_2$ levels deplete to approx. 150 μM (Fig. 7.17. C), followed by an increase of O$_2$ concentrations over 3 min. O$_2$ levels equilibrate close to baseline measurements 10 minutes post UV exposure (Fig 7.17.K). Drops of deoxygenating sulphite were added to the eyes every 10 min for 1 hour before
imaging. Interestingly, O₂ levels did not drop as significantly in the eye when compared to solution-based Micro-chamber channels or collagen type I (Fig 7.17. L). The reason for this is unknown. Fig 7.18 displays the colour images of phosphorescence decay for one eye. Approximate ROI are represented within the red boxes when calculating decays. The images in Fig 7.18 confirm little change in decays after the addition of sulphite.

Fig. 7.18. Colour images of one eye with ROI indicated within the red boxes. Phosphorescence decay of SII-A ranges from 10 (200 µM)-18 µs (0 µM). Little to no change can be observed after sulphite addition. Scale bar 500 µm.

7.4.2 Distribution histogram porcine cornea

Fig. 7.19. Average distribution histogram of one porcine cornea.

Fig. 7.19 shows changes in distribution profile post UV illumination throughout sample imaging (UV 1,3,5,7). Upon sulphite addition, the histogram shows little change to phosphorescence decays in the porcine cornea.
7.5 \( \text{O}_2 \) Diffusion Model

A diffusion model of oxygen was studied with COMSOL™ software. The diffusion of \( \text{O}_2 \) over a 0-200 sec interval was calculated and predicted throughout the corneal stroma (0-1 mm). Also, time versus depth (fixed at 100 \( \mu \text{m} \)) plot of oxygen is observed. The amount of oxygen available after depletion to 0% concentration was also calculated. The oxygen enters from the atmosphere at one side only, similar to the whole eye experiments. \( D = 2.81 \times 10^{-3} \text{ mm}^2/\text{s} \) was used\(^\text{22} \).

![O₂ Diffusion Rate Porcine Cornea](image)

Fig. 7.20. Diffusion model estimating \( \text{O}_2 \) throughout the cornea. 0.1mm represents the experimental results in this study.

Fig. 7.20 demonstrates the behaviour of \( \text{O}_2 \) post CXL from 0-1 mm for \( t= 0 \)-200 sec. We presume \( t=0 \) corresponds to total depletion of \( \text{O}_2 \) occurring during UV exposure (Type II reactions), suggesting longer recovery of \( \text{O}_2 \) at larger depths.
Fig. 7.21. Diffusion model based on experimental findings in this study. O$_2$ is fully depleted upon illumination, followed by replenishment over time (t=0-200 sec).

Fig. 7.21 shows the calculated diffusion of oxygen over time (t=0-200 sec) at a stromal depth of 100 µm. Based on porcine experimental results, the O$_2$ level return to atmospheric concentrations ~180 sec post UV exposure, which is shown here. A change in corneal stiffness after CXL may alter the effective diffusion coefficient and decrease the speed of O$_2$ recovery.

In summary, results have tested various O$_2$ probes for suitability for measuring molecular O$_2$ before and after CXL. Once the most suitable O$_2$ probe was chosen, SIIA showed good diffusion ability and strong signals with little interference from UV exposure. The 3 models revealed depletion of O$_2$ when exposed to UV and riboflavin induced CXL. We observed O$_2$ depletion followed by equilibration of concentrations returning to each sample model over time.
Chapter 8: Discussion

The objective of the study was to investigate a minimally invasive method of measuring molecular oxygen (O$_2$) diffusion in the cornea before and after UV-induced corneal cross-linking (CXL). To ensure repeatability, (i) O$_2$-sensitive nanoparticles in soluble solution of riboflavin without collagen, (ii) collagen type-I gel with O$_2$-sensitive nanoparticles and riboflavin, and (iii) porcine eyes stained with O$_2$-sensitive nanoparticles and riboflavin were used to mimic in-vivo capabilities and better understand the photochemical processes occurring during CXL. A number of available O$_2$ probes (small molecule and nanoparticle based) were compared in order to identify the most suitable for staining in porcine corneas. The ability to diffuse through the cornea, spectral cross-talk with riboflavin and photo-stability upon UV-light exposure were considered and SII-A copolymer nanoparticles were chosen as the most suitable for our experiments. Porcine whole globes were the chosen model in this study as they can be easily obtained and do not require safety protocols. Porcine and human eyes are biologically similar; however porcine corneas can be twice as thick, and do not have a Bowman’s layer$^{259}$. These differences should not have influence the relevance in the results obtained$^{260}$.

The use of TCSPC-PLIM is an efficient method to determine O$_2$ concentrations in the cornea. It is minimally invasive, with the ability to obtain O$_2$ behaviours in real-time. This study investigated novel O$_2$ sensitive conjugated polymer nanoparticles similar to previously tested O$_2$ nanoparticles$^{232,250,251,254,255,258}$. Compared to other methods, such as the Clark electrode, PLIM requires no mechanical interaction, and does not consume oxygen. Limitations of the Clark electrode are the measurement of relative O$_2$ levels rather than absolute intracellular O$_2$ concentrations, and consumption of O$_2$ which can be harmful to small or O$_2$ deficient samples$^{209}$. Penetration of cells using fiber-optic sensors can cause damage, proving invasive.

The chosen probe (SII-A) is compatible with one- and two- photon excitation along with both intensity and lifetime based imaging modes$^{232}$, and has not been used in any peer-reviewed journals to date. SII-A showed good staining in depth, no photo-
damage from UV, sufficient sensitivity to O_2, and capability of measuring across the physiological range (0-200 µM O_2). The SII-A probe is a conjugated polymer made up of a polyfluorene/ poly (fluorine-alt-benzothiadiazole) backbone antenna, which emits in red and infrared based on a Pt(II)-porphyrin, allowing for longer wavelengths of 600-900 nm. SII-A carries a neutral (zwitter-ionic) charge, which could explain efficient staining and penetration. Riboflavin is a highly reactive photosensitizer when exposed to UV light. In this study, the absorption spectra of the chosen SII-A probe was compared to riboflavin in order to investigate any interruptions such as phosphorescence, fluorescence quenching or spectral cross-talk. Riboflavin showed strong orange fluorescence and broad excitation, which interfered with most of the tested O_2 probes except for SII-A. Results show a relatively small change in phosphorescence decays after riboflavin addition (Fig.7.14 (B), 7.17(B)), suggesting quenching of nanoparticles fluorescence by riboflavin. This is short-lived and can be detected in the initial stages of imaging. However, this quenching is not enough to alter the lifetimes drastically. The compatibility of SII-A probe to UV was also observed (Fig.7.5). SII-A absorption curve peaks at 620 nm, if photo-bleaching from UV occurred, the peak would be significantly altered. Phosphorescence lifetime measurements are not affected by light exposure or photo bleaching (compared to intensity based measurements), thus proving the most reliable method of conveying PLIM results.

Non-substrate, solution-based channels (Fig.6.4) typically used for immunofluorescence assays were chosen as the first experimental model in assessing photochemical reactions occurring during CXL due to their ability to observe homogenous O_2 nanoparticle distribution (Fig. 7.11). Results showed O_2 depletion from atmospheric levels of 200 µM of approximately 25% (140 µM) within 40 seconds after UV exposure. After a rest period of 20 minutes post UV to allow the sample to equilibrate, O_2 concentrations in the channels returned to atmospheric concentrations (200 µM). The use of non-substrate, solution-based channels was an efficient method to begin investigative measurements in order to validate the O_2 results obtained using Collagen type-I gels and porcine corneas.
In collagen type-I samples and porcine corneas, a fixed depth of 100 µm was chosen based on previous scientific observation of O₂ depletion after CXL in 130µm corneal flaps. The findings for collagen type-I and porcine corneas revealed the same proportion of O₂ depletion observed in the solution-based channels (~25%) post UV exposure. We observed a gradual increase in O₂ concentrations to atmospheric levels over 10 min post illumination. This confirms calibration accuracy of SII-A and repeatability of experiments. Deoxygenating sulphite was added to all models after O₂ levels were restored in order to observe O₂ depletion as an alternative to a hyperbaric or nitrogen induced chamber typically used for O₂ studies. Further O₂ depletion was observed in solution-based channels and collagen samples upon sulphite addition; however, it was not effective in porcine corneas. Finally, a control with no riboflavin for each model was investigated in order to verify our results (Fig. 7.10). Slight changes in O₂ concentrations after UV were observed, which was expected as riboflavin plays a dominant role in CXL photochemical reactions.

Richoz et al. found that the biomechanical effect of CXL is oxygen dependent by measuring stress-strain of porcine corneas under low and normal oxygen saturation. Krueger et al. observed depletion by up to 50% in O₂ at 245 sec at 3 mW/cm² and 46 sec at 16 mW/cm² after CXL, showing a linear relationship to UVA irradiance. Kamaev et al. found that O₂ depleted to 0% concentration after 10-15 secs. They also suggest halfway through illumination, the O₂ concentration gradually replenishes to a concentration where Type II mechanisms begin to play an additional role. This study used sodium azide to quench riboflavin triplets, a primary cross-linking reactant. McCall et al. investigated the involvement of singlet O₂ in CXL and also the role of carbonyl or free amino groups were involved in forming functional crosslinks. The study proved singlet oxygen (¹O₂) was involved in CXL and was stopped by the presence of sodium azide, a substance known to stop reactions that require singlet O₂. Singlet O₂ was greatly stimulated by the presence of D₂O, which prolongs the half-life of singlet O₂. The study also showed that free carbonyl groups are present and essential for the CXL process. Vasilios et al. observed the addition of O₂ during high intensity CXL did not increase the CXL effect when elasticity was measured through Atomic Force Microscopy (AFM). Moreover, results of high intensity CXL (30 mW/cm²)
and groups with enriched O$_2$ high intensity showed similar changes in corneal elasticity when compared to the conventional Dresden protocol.

In this study, molecular O$_2$ was measured within 10 sec post illumination and with a scan time between 20-40 sec. Results show 25% decrease of O$_2$ in all samples and a gradual replenishment in O$_2$ concentrations over time (2-5 min). The photochemical reaction during CXL comprises of Type I (aerobic) and Type II (anaerobic) mechanisms. In Type II reactions, reduced riboflavin is formed and all O$_2$ is consumed in the cornea within 10-15 sec during UV illumination. The reactive oxygen forms hydrogen peroxide (H$_2$O$_2$) and free radicals, which enable the formation of cross-links. Halfway through illumination, the O$_2$ concentration gradually replenishes itself through Type I reactions from atmospheric O$_2$ molecules diffusing into the cornea. Thus, our results observed Type I mechanisms in which the O$_2$ is gradually returning to the cornea following Type II anaerobic reaction. It was not possible to observe Type II mechanism as it can only be seen during UV illumination. Preliminary testing in this study had an image acquisition time of 2 min per scan, in which little to no changes in O$_2$ concentrations were observed (results not shown). It is now clear we were missing the Type I reaction of the O$_2$ replenishment from a prolonged scanning time. Reducing scanning time further or enabling imaging during illumination would allow for us to visualize the Type II reaction. A mathematical model observing the diffusion of oxygen based on experimental results was made using $D = 2.81 \times 10^{-3} \text{ mm}^2/\text{s}$

Fig 7.20 shows O$_2$ concentrations further depleted in deeper regions of the cornea over time. Fig 7.21 shows fast O$_2$ replenishment from the atmosphere over time (0-250 sec), thus agreeing with our experimental findings. A fixed depth of 100 µm used in this study in comparison to previous studies of oxygen measurements using a fiber-optic probe in corneal flaps at a depth 130µm$^{31}$. This depth is relatively shallow as porcine corneas are thicker than those of humans$^{226}$. However, studies have shown the most prominent biomechanical stiffening occurs after CXL in the anterior 200 µm$^{171}$. This finding was beneficial to this study as it was a safe depth to monitor in terms of obtaining high signals using one-photon PLIM. Two-photon PLIM would have the ability to image deeper into the cornea (>250µm), which was not feasible for this study.
This study used a phosphorescent $O_2$ probe suitable for determining $O_2$ concentrations before and after CXL. However, there are limitations to this method. 1) UV exposure cannot occur during scanning as it affects the TSCPC-PLIM detector. If this could be bypassed, a UV ring can be put on the objective to observe $O_2$ depletion (Type II) in real time. Preliminary experiments illuminated samples on the stage using the UV off-axis (not homogenously distributed), in which little to no changes in $O_2$ concentrations were observed. 2) The deoxygenating sulphite agent was beneficial to observe levels of $O_2$ depletion, since it was not feasible to have a controlled chamber with accurate control of $O_2$ concentrations. We observed $O_2$ depletion in solution-based channels and Collagen type-I. Interestingly, $O_2$ concentrations did not deplete as drastically in porcine corneas. This was observed early in the study; therefore drops of sulphite were applied porcine corneas for 1 hour before imaging to ensure sufficient diffusion, with the addition of drops every 5-10 min throughout. The reason for the failure of sulphite to deplete $O_2$ in the cornea is unknown, although it may be that the sodium sulphite and buffering agent may not have had high diffusion rates compatible with a porcine cornea. 3) Samples were imaged with SII-A first, removed from the scanning stage and riboflavin was added, and so on. This means although there was estimated regions of scanning, the same area may not have been imaged every time. However, in the cycles post illumination (e.g UV1-8), the same region was imaged. This could be improved by our first limitation- of the ability to scan whilst illuminating the sample. The TCSPC-PLIM confocal microscope is in a temperature controlled humidified enclosed chamber, which could prove beneficial, as the $O_2$ molecule is also temperature sensitive.

The eventual introduction of $O_2$ sensitive phosphorescence probes in-vivo would be highly beneficial due to being minimally invasive (by only needing to apply nanoparticle solution to the cornea), general ease of use, and the ability to observe molecular $O_2$ behaviours with long or short image acquisition times. Apart from applying PLIM in-vivo to monitor $O_2$ concentrations during CXL treatment, phosphorescence $O_2$ probes could be a useful tool for corneal clinical diagnostics such as corneal neovascularisation from excessive contact lenses use\textsuperscript{47} and monitoring of $O_2$ elevations during cataract surgery\textsuperscript{44}. Future observation of $O_2$ in deeper ocular structures could be applied using
the same principal as Fluorescein Angiography in diseases such as diabetic retinopathy\textsuperscript{41} and glaucoma\textsuperscript{42}, and Age-Related Macular Degeneration\textsuperscript{43}.

From a laboratory perspective, O\textsubscript{2} probes are typically tested for toxicity through the analysis of total cellular ATP and extracellular acidification. However, future work would need to determine probe toxicity in the cornea for eventual future human use. Many animal studies using PLIM have been investigated, postulating minimal toxicity. Future work should also focus on stress-strain measurements to determine how effective CXL is based on type I O\textsubscript{2} concentrations found in this study.
**Conclusion**

Corneal cross-linking (CXL) is comprised of three important photochemical processes: energy dose (absorbed number of photons) of the applied UV light, concentration (how much riboflavin is needed) or composition (formulations) of riboflavin, and the role of oxygen (O₂ concentration within the tissue). In this study, the latter principal drivers of the photochemical reaction mechanisms occurring during CXL were investigated. The findings conclude that:

1) Riboflavin has a faster stromal diffusion when injected into a corneal channel than when applied as drops to the anterior corneal surface. Six riboflavin formulations using intra-stromal channels were studied to determine the effective diffusion coefficients as compared to traditional axial diffusion with epithelium on or off. All riboflavin formulations diffused across the channel laterally in a concentration and formulation dependent manner. Future work on mechanical stress-strain analysis should be explored in order to determine cross-linking efficiency in the anterior region above the intra-stromal channel.

2) The role of molecular O₂ is conducive to photochemical type I mechanisms, where the O₂ has been depleted due to Type II reactions during UV illumination, and is gradually replenishing itself over time due to atmospheric O₂ molecules entering the cornea. The use of minimally invasive, TCSPC-PLIM using O₂ sensitive phosphorescence probes has been shown. SII-A, an unpublished and conjugated, zwitter-ionic polymer, revealed efficient staining depth, high signals and good resolution when imaging the cornea before and after CXL. The use of phosphorescent O₂ probes allows for efficient and a minimally-invasive determination of O₂ concentrations prior to and during CXL. Results indicate that collagen type-I gels is an efficient model for measurements of O₂ during CXL due to restrictions in hydration control when using ex-vivo tissue samples. 2D and 3D maps of O₂ concentrations during CXL will enable us to better understand the role of oxygen during CXL.

Future work on the relationship with high intensity CXL and O₂ using mechanical stress-strain analysis should be explored, along with the use of TCSPC-PLIM during UV irradiation in order to visualize the role of molecular O₂ during Type II
photochemical reactions during CXL. Future work will also focus on the suitability of the $O_2$ PLIM method for in-vivo use along with increased imaging depth profiles beyond 100 µm for porcine and collagen type-I samples.
References


67. Lindsay RG, Bruce AS, Gutteridge IF. Keratoconus associated with continued eye rubbing due to punctual agenesis. Cornea. 2009; 4:567-569.


115. Deherre LJ, Brown VS. Arch Biochem and Biophys. 1944; 5: 181


259. Jay L, Brocas, Singh K, Ozaki T. Determination of porcine corneal layers with high spatial resolution by simultaneous second and third harmonic generation