An investigation into the use of Laser Speckle Interferometry for the analysis of corneal deformation with relation to biomechanics

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An Investigation into the use of Laser Speckle Interferometry for the Analysis of Corneal Deformation with Relation to Biomechanics

By
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M.Eng. (Hons)

A Doctoral Thesis submitted in partial requirement for the award of Doctor of Philosophy

Loughborough University

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...sometimes all you need
is a fresh pair of eyes...
Abstract

There has been widespread interest in corneal biomechanics over recent years, driven largely by the advancements in, and the popularity of refractive surgery techniques and subsequent concerns over their safety. Lately there has been interest into whether crosslinking, which is currently used for the treatment of keratoconus, could be developed as a minimally invasive technique to change the refractive power of the cornea by selectively changing the corneal biomechanics in specific regions to induce a shape change.

Successful application of this technique requires a detailed understanding of corneal biomechanics and so far, little is known about the biomechanics of this complex tissue. The current lack of understanding can be mostly attributed to the absence of a suitable measurement technique capable of examining the dynamic behaviour of the cornea under physiological loading conditions.

This thesis describes the development of a novel full-field, ex vivo, measurement method incorporating speckle interferometric techniques, to examine the biomechanics of the cornea before and after crosslinking in response to hydrostatic pressure fluctuations representative of those that occur in vivo during the cardiac cycle.

The eventual measurement system used for the experiments detailed in this thesis incorporated; an Electronic Speckle Pattern Interferometer (ESPI), a Lateral Shearing Interferometer (LSI) and a fringe projection shape measurement system. The combination of these systems enabled the 3-dimensional components of surface displacement and the 1st derivative of surface displacement to be determined in response to small pressure fluctuations up to 1 mmHg in magnitude. The use of both ESPI and LSI together also enabled the applicability of LSI for measurement of non-flat surfaces to be assessed, and limitations and error sources to be identified throughout this work.

To enable the measurement of corneal biomechanics, part of this thesis was concerned with the design of a bespoke loading rig. A chamber was designed that could accommodate tissue of both porcine and human origin. This chamber was linked to a hydraulic loading rig, whereby the cornea could be held at a baseline pressure representative of normal intraocular pressure and small pressure variations could be introduced by the automated vertical movement of the reservoir supplying the chamber.

Experiments were conducted on a range of non-biological samples with both flat and curved surface topography, and both uniform and non-uniform mechanical properties, to determine if
the measurement configuration was giving the expected measurement data and the loading rig was stable and repeatable.

Following experiments on non-biological samples, a range of experiments were conducted on porcine corneas to develop a suitable testing methodology and address some of the challenges associated with corneal measurement, including transparency and hydration instability. During these investigations, a suitable surface coating was identified to generate an adequate return signal from the corneal surface, while not interfering with the response. Alongside this, the natural variation in the response of the cornea was investigated over the total experimental time, and a range of data was presented on corneas before and after crosslinking, which confirmed the suitability of the measurement methods for the assessment of crosslinking.

Ultimately, a small sample size of six human corneas were investigated before and after crosslinking in specific topographic locations. From the experiments on human and porcine corneas, full-field maps of surface deformation have been presented, and a compliant region incorporating the peripheral and limbal areas has been identified as being fundamental to the response of the cornea to small pressure fluctuations. In addition to this, the regional effects of crosslinking in four different topographic locations on corneal biomechanics have been evaluated. From this, it has been demonstrated that crosslinking in specific regions in isolation can influence the way the cornea deforms to physiological-scale fluctuations in hydrostatic pressure and this could have implications for refractive correction.
Acknowledgments

Firstly, I would like to express my thanks to my supervisor Professor John Tyrer for giving me the opportunity to undertake this research project and for allowing me to carry out my work at Laser Optical Engineering during the final 2 years of my research, during which Loughborough University closed the optical labs. This leads me to express my sincerest gratitude to all the wonderful staff at Laser Optical Engineering for making me feel so welcome, letting me borrow equipment and lab space and helping me in any way and every way they could. I would especially like to thank John Jones for his excellent advice, expertise, patience and for always giving up his time to discuss and question my work. I learnt a great deal from our many discussions!

I am extremely appreciative of all the support and guidance given by Professor John Marshall throughout this project. His knowledge and expertise with respect to the cornea and refractive surgery was fundamental to deciding on the direction of research. Not only this, but he, Ann Patmore and his team at the Institute of Ophthalmology provided me with all the facilities, equipment and guidance required to carry out the crosslinking experiments. Working with him and his team was a pleasure and I thoroughly enjoyed learning so much about the cornea, general life and career development from his many years of experience.

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Finally, completion of this work would not have been possible without the support and encouragement of my family and Laurence, to whom I will forever be thankful.
Publications and presentations arising from this research

1. Journal Articles


2. Book Chapters


3. Conferences and presentations


A. Wilson, “The Use of Laser Interferometry for the Measurement of Corneal Biomechanics” - Invited Speaker, Cornea to Cortex, Cardiff University, UK. January 2016.
Claims of Originality

1. The development and application of a method incorporating; ESPI, LSI, shape measurement and video analysis, for the evaluation of corneal surface deformation in response to hydrostatic pressure variations equivalent to those that occur physiologically.

2. The development of a stable measurement rig capable of generating repeatable and reliable data using interferometric techniques from in-tact corneo-scleral sections.

3. Identification and verification of an impervious surface coating suitable for covering moist corneal tissue and generating an adequate return signal, while not influencing the overall response to loading.

4. The presentation of full-field data detailing the 3-D surface deformation of human and porcine corneas in response to small changes in hydrostatic pressure equivalent to those that occur physiologically during the cardiac cycle.

5. An analysis of the errors and limitations associated with measurement of the 3-D deformation of the cornea using both ESPI and LSI.

6. An evaluation of the effects of crosslinking, in specific topographic locations in isolation, on the response of individual corneas to changes in hydrostatic pressure equivalent in magnitude to those that occur physiologically during the cardiac cycle.

7. Demonstration of customised-crosslinking based on identification of areas of variation in biomechanical properties.
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List of abbreviations

AAC – Artificial Anterior Chamber
CBI – Corneal Biomechanical Index
CCD – Charged Coupled Device
CCT – Central Corneal Thickness
CMOS – Complementary Metal Oxide Semiconductor
DC – Direct Current
DIC – Digital Image Correlation
ESPI – Electronic Speckle Pattern Interferometry
ESPSI – Electronic Speckle Pattern Shearing Interferometry
HI – Holographic Interferometry
IOP – Intra-ocular pressure
IP – In-Plane
LASEK – Laser-Assisted Sub-Epithelium Keratomileusis
LASIK – Laser-Assisted In-Situ Keratomileusis
LSI – Lateral Shearing Interferometry
mmHg – millimetres of Mercury
N-T – Nasal - Temporal
OCT – Optical Coherence Tomography
OOP – Out-of-Plane
OPA – Ocular Pulse Amplitude
Pa – Pascal
PRK – Photorefractive Keratectomy
PTFE – Polytetrafluoroethylene
PZT – Piezoelectric Transducer
RK – Radial Keratotomy
RSI – Radial Shearing Interferometry
SBK – Sub-Bowman’s Keratomileusis
S-I – Superior - Inferior
SMILE – Small Incision Lenticule Extraction
WI – Wavefront Interferometry
X – Position with respect to the horizontal axis
XL – Crosslinking
Y – Position with respect to the vertical axis
2D – Two-dimensional
3D – Three-dimensional
Glossary of mathematical terms

d – distance between entrance pupil of camera and exit pupil of projector
D – diameter
E – Young’s modulus
$E_e$ – irradiance
$e_r$ – Relative error
$f_o$ – spatial frequency of projected fringes
$g$ – gravity
$h$ – height
$l$ – Illumination vector
$I$ – Intensity
$I_A$ – Total intensity of light received at the image plane with object in reference state
$I_B$ – Total intensity of light received at the image plane with object in deformed state
$I_o$ – Intensity of light scattered from object
$I_r$ – Intensity of light scattered from reference
$k$ – Sensitivity vector
$K$ – calibration constant
$l$ – vector of surface displacement
$L$ – Optical path length
$l_o$ – distance of camera from reference plane
$\delta$ – Observation vector
$P(x,y)$ – Point of interest with coordinates ($x,y$)
$P$ – pressure
$r$ – radius
$rc$ – radius of curvature
$t$ – thickness
$U_o$ – Complex light amplitude from object wavefront
$U_R$ – Complex light amplitude from reference wavefront
$u_o$ – Light amplitude from object
$u_R$ – Light amplitude from reference
$u$ – Horizontal in-plane displacement component
$v$ – Vertical in-plane displacement component
$w$ – Out-of-plane displacement component
$w_{apex}$ – Out-of-plane displacement component at the corneal apex
$x$ – Cartesian coordinate
$y$ – Cartesian coordinate
$z$ – Cartesian coordinate

$\delta x$ – Sheared distance between points with respect to the x-axis as viewed in the image plane

$\delta x_s$ – Sheared distance between points with respect to the object surface as viewed along the x-axis

$\delta y$ – Sheared distance between points with respect to the y-axis

$\varepsilon$ – Strain

$\theta_{xz}$ – Illumination angle with respect to the x-z plane

$\theta_{yz}$ – Illumination angle with respect to the y-z plane

$\theta_{Nx}$ – Angle normal to the surface of the object with respect to the x-axis

$\theta_{Ny}$ – Angle normal to the surface of the object with respect to the y-axis

$\lambda$ – wavelength

$\mu$ – shear ratio

$\nu$ – Poisson's ratio

$\pi$ – pi

$\rho$ – density

$\sigma$ – Stress

$\phi$ – Phase angle

$\phi_{PS}$ – Phase angle introduced due to phase stepping

$\frac{\partial l}{\partial x}$ – Rate of change of the vector of surface displacement with respect to the x-axis

$\frac{\partial l}{\partial y}$ – Rate of change of the vector of surface displacement with respect to the y-axis

$\frac{\partial u}{\partial x}$ – Rate of change of the horizontal in-plane displacement with respect to the x-axis

$\frac{\partial u}{\partial y}$ – Rate of change of the horizontal in-plane displacement with respect to the y-axis

$\frac{\partial v}{\partial x}$ – Rate of change of the vertical in-plane displacement with respect to the x-axis

$\frac{\partial v}{\partial y}$ – Rate of change of the vertical in-plane displacement with respect to the y-axis

$\frac{\partial w}{\partial r}$ – Rate of change of out-of-plane displacement radially from object centre

$\frac{\partial w}{\partial x}$ – Rate of change of out-of-plane displacement with respect to the x-axis

$\frac{\partial^2 w}{\partial x^2}$ – Second derivative of out-of-plane displacement with respect to the x-axis

$\frac{\partial w}{\partial y}$ – Rate of change of out-of-plane displacement with respect to the y-axis

$\frac{\partial^2 w}{\partial y^2}$ – Second derivative of out-of-plane displacement with respect to the y-axis

$\frac{\partial z}{\partial x}$ – Surface gradient with respect to the x-axis
\( \frac{\partial z}{\partial y} \) – Surface gradient with respect to the y-axis

\( \Delta h \) – Change in height

\( \Delta L \) – Change in optical path length

\( \Delta P \) – Change in pressure

\( \Delta \phi_A \) – Difference in phase angle between object and reference wavefronts for object in reference state

\( \Delta \phi_B \) – Difference in phase angle between object and reference wavefronts for object in deformed state

\( \Delta \phi_{def} \) – Difference in phase angle due to movement of object
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1. Introduction

1.1 Research Context

The cornea is the transparent window located at the front of the eye. It has a unique and complex structure which is responsible for protecting the inner eye from infection, resisting intra-ocular pressure (IOP) and focussing of light onto the retina. The cornea is responsible for over two-thirds of the focussing power of the eye and it is the specific shape of the cornea that governs its refractive power. Since the shape of the cornea is dependent upon its material properties, over recent years there has been a great deal of interest in gaining a deeper understanding of corneal biomechanics.

To date there remains a lack of understanding as to what the normal biomechanics of the cornea are and how these may change in diseased states or as a result of surgical intervention. Measurement of corneal biomechanics, both in vivo and ex vivo, presents many challenges due to the corneas heterogeneous structure and complex behaviour, and so far, a measurement technique capable of determining these properties with certainty has not been established.

The ability to measure corneal biomechanics has wide ranging implications and could impact the lives of many individuals worldwide through improving vision or preventing blindness. The potential benefits that could result from an increased understanding of corneal biomechanics include:

- Earlier diagnosis, increased understanding and better treatment of corneal diseases such as keratoconus.
- Reduction of the risks associated with current surgical procedures including; cataract surgery, corneal transplant surgery and refractive surgery.
- Improvement to current refractive surgery techniques and in the development of new less invasive alternatives.
- Improvement in refractive surgery outcomes.
- Better screening of corneas used for transplant.
- The development of synthetic alternatives to human donor tissue.
- Validation and improvement to current models of corneal biomechanics.

The overall impact of the aforementioned benefits is huge, considering that over 50 million people have undergone elective refractive surgery to date, with the World Health Organisation estimating 153 million people worldwide live with visual impairment due to refractive error \(^{(1)}\). In addition to this cataract surgery is one of the most commonly performed surgeries
worldwide, with approximately 4.3 million procedures carried out within EU countries alone in 2014 \(^\text{(2)}\), and corneal transplant is the most common form of transplant surgery with 184,576 procedures reported to have taken place worldwide in 2012 \(^\text{(3)}\).

1.2 Research questions

The focus of this research was the development of a technique capable of measuring the biomechanics of the cornea. At the start of this study the following research questions were established:

1. What are the benefits to an increased understanding of corneal biomechanics and what information is currently required to realise these benefits?
2. What is the current understanding of corneal biomechanics and how are corneal biomechanics currently evaluated?
3. What factors can affect the measurement of corneal biomechanics?
4. Could a technique be developed to measure corneal biomechanics and changes to biomechanics that may be introduced as a result of disease or surgical intervention?

1.3 Thesis structure

The work in this thesis is presented over nine chapters. A summary of the content of each of the eight chapters following this introduction is described here.

**Chapter 2** first describes the structure of the cornea and provides context to the research by exploring the importance of an understanding of corneal biomechanics and the many implications it would have. In this chapter, the current measurement techniques used to examine the biomechanics of the cornea are reviewed, along with their measurement capabilities and advantages and disadvantages. Following this, the reasons for selecting speckle interferometric measurement techniques to examine the cornea are detailed, and finally the primary research aims are outlined.

**Chapter 3** details the theory of laser speckle interferometric techniques, focusing specifically on electronic speckle pattern interferometry (ESPI) and lateral shearing interferometry (LSI), and explains how they can be implemented to obtain quantitative data regarding the deformation of a surface under loading. The second part of this chapter details the errors associated with quantitative measurement and the challenges associated specifically with measurement of a non-flat membrane under complex loading conditions.

**Chapter 4** is divided into two main parts. The first part describes the design of the measurement rig used throughout experiments on the cornea, detailing the development of
the artificial anterior chamber used to hold the corneal tissue, and the loading method used to replicate in vivo pressure fluctuations introduced during the cardiac cycle. The second part describes the iterative development of the measurement system which ultimately comprised out-of-plane ESPI and LSI measurement configurations alongside a shape measurement system. Following this, the data analysis procedures that were used to extract 3D information regarding surface deformation are detailed.

**Chapter 5** details the results from the experiments conducted on non-biological samples, and is divided into three main parts. This chapter begins by discussing the experiments that were conducted on simple flat objects that exhibited predictable behaviour, to check that the measurement set-up was giving the expected results and the loading rig was repeatable. This is followed by a discussion of the results from a range of experiments that were conducted on curved rubber samples that exhibited both non-uniform and uniform responses to loading. The purpose of these experiments was to test the measurement methods outlined in chapter 4, and to identify potential issues associated with the measurement of non-flat samples. In this section, the interpretation of the ESPI and LSI measurement data is also explained and examples of more complex fringe distributions are given.

The final part of chapter 5 details simulation crosslinking experiments that were conducted by stiffening certain areas of rubber samples, representative of the areas that would be crosslinked in the cornea experiments. This provided simple data with which the subsequent crosslinking data could be compared and demonstrated the ability of the measurement techniques for the assessment of regional changes in mechanical properties.

**Chapter 6** details the results from the experiments that were conducted on a large sample size of porcine corneas to develop a suitable testing methodology prior to the human cornea experiments. Within this first part of this chapter the response of the porcine cornea to hydrostatic pressure fluctuations is described in detail, and solutions to specific challenges associated with corneal measurement, namely transparency, hydration instability and inter-subject variability, are described. The first part of this chapter ends with a description of the methodology devised for the crosslinking experiments.

The second part of chapter 6 details the results of crosslinking experiments that were conducted on porcine corneas, which demonstrate the suitability of the measurement methods for the detection of biomechanical changes introduced by crosslinking.

**Chapter 7** presents the results from the experiments on human corneas. This chapter is divided into two main parts. In the first part the response of the corneas prior to crosslinking is
analysed in detail, while in the second part the changes to the response of the corneas before and after crosslinking in specific topographic locations is described.

Chapter 8 is a discussion of the research undertaken in this project and the main experimental findings. The first part of this chapter is an evaluation of the measurement methods used and a discussion of the main limitations of the experiments. The second part of chapter 8 describes the experimental outcomes and what they indicate with regards to the biomechanics of the cornea and the effects of crosslinking.

Chapter 9 details the main conclusions from the experiments conducted in this thesis and the current and potential implications of the work. This chapter ends with a short discussion on the potential for further work arising from this research.

Within this thesis pressure variations used in experiments have been expressed in millimetres of mercury (mmHg) and not Pascals (Pa). The reason for this was to maintain consistency with the terminology used within the ophthalmology sector and the current literature within this field.
2. Background Study

2.1 Introduction

During the background study research was conducted into the following areas:

2. Diseases and pathologies associated with biomechanical changes.
4. Current knowledge of biomechanics and the challenges associated measurement.
5. Current techniques used to quantify corneal biomechanics and their current capabilities.

The purpose of the background study was to gain a deeper understanding of corneal structure and its relationship to biomechanics, and to investigate areas where increased knowledge of biomechanics would have the greatest impact, to provide further context to the research. The research undertaken within the background study aimed to answer the first three research questions as outlined in chapter 1 so a suitable measurement technique could be identified.

2.2 Corneal structure in relation to biomechanics

Due to the availability of many different imaging technologies, including X-ray scattering and non-linear microscopy, there is now a great deal of information available on the structure of the cornea. A brief description of the structure of the cornea is given here.

A diagram of a transverse cross-section through a human eye is shown in Figure 2-1 (4). The cornea is the transparent collagenous structure situated at the front of the eye. The cornea is aspheric in shape being shorter in the vertical axis (~11 mm) than the horizontal (~12 mm) (5). It has non-uniform thickness being thinnest at the centre (~0.5 mm) and gradually increasing in thickness towards the periphery (~0.7 mm) (5). Due to this variation in thickness the radius of curvature \( r_c \) of the anterior (outer) surface differs from the posterior (inner) surface, with the anterior surface being approximately 7.7 mm and the posterior approximately 6.8 mm (5). Since the cornea is aspheric in shape the radius of curvature is only an approximation, in reality the curvature of the horizontal axis and vertical axis is slightly different, with the horizontal meridian tending to be flatter across the general population (6).
Structurally, the cornea is considered to have five main layers these are, from outermost to innermost; the epithelium, Bowman’s layer, the stroma, Descemet’s membrane and the endothelium. These layers are labelled in the image in Figure 2-2. Each of these layers has a unique function important to maintaining the overall functions of the cornea, which are; to maintain transparency, maintain visual acuity, resist intra-ocular pressure (IOP) and to protect against infection.
The epithelium accounts for approximately 10% of corneal thickness \(^7\) and is a layer of cells 5 - 7 cells thick. The epithelium cells are coated by the tear film which creates a smooth optical surface, hydrates and lubricates the underlying cells, and provides vital nutrients to the cornea, which is necessary due to the absence of blood supply. Due to the cellular structure of the epithelium it contributes very little to the overall biomechanics \(^8\). The cells of the epithelium are constantly being renewed, and if damaged or removed the epithelium is capable of regenerating within a few days.

The endothelium is a single layer of cells on the posterior side of the cornea and has no contribution physically to the overall biomechanics of the tissue. The cells of the endothelium are responsible for forming and maintaining the Descemet’s membrane, maintaining the hydration properties of the corneal stroma, and allowing the transfer of nutrients and metabolites to and from the aqueous humour \(^9\). Unlike the epithelium, the endothelium has only limited ability for regeneration. Since the endothelium is responsible for controlling the hydration of the stroma, and hydration is linked to biomechanics, absence or changes to the endothelium could result in changes to the biomechanical properties of the tissue.

The Descemet’s membrane is a thin collagenous basement membrane responsible for protecting the eye from injury and infection and maintaining the health of endothelial cells and clarity of the stroma. The type of collagen in the Descemet’s membrane is non-fibrous and it is thought to have low stiffness when compared to the stroma \(^10\), therefore the overall contribution to corneal biomechanics is thought to be small.

The Bowman’s layer is a thin collagenous layer of approximately 8 µm – 10 µm thickness \(^11\), situated between the epithelium and the stroma. The collagen in the Bowman’s layer has been described to exist as a fibrous network \(^7\) with random orientation. The contribution of the Bowman’s layer to corneal biomechanics is still poorly understood. In an extensometry study it was shown to have minimal effect on the response of corneal strips to tensile loading \(^12\). But studies of the corneal structure would suggest it plays a part in the maintenance of corneal curvature, as collagen fibres from the anterior stroma interweave with the collagen in the Bowman’s layer \(^13,14\). It is also highly resistant to swelling, even in conditions of hyperhydration, allowing the shape of the anterior surface of the cornea to be maintained.

The stroma makes up approximately 90% of the total corneal thickness \(^15\) and is the layer that is considered to be largely responsible for the overall biomechanical properties. The stroma contains lamellae consisting of parallel collagen fibres embedded in a proteoglycan rich matrix \(^7\). The organisation of collagen lamellae in the stroma varies both across the cornea and throughout the depth, with clear differences between the anterior and posterior stroma. It is the
collagen lamellae in the stroma, and their specific orientation that is thought to be responsible for the tensile and cohesive strength of the cornea.

The anterior most portion of the stroma is considered to have the highest tensile strength, this was demonstrated by a strip extensometry study in which strips of cornea were taken from different depths throughout the corneal stroma\(^\text{(10)}\). Results from this study showed the anterior 40% of the cornea was at least 50% stronger than the remaining posterior portion, this finding has since been replicated in other studies\(^\text{(14)}\). The high strength of this area is thought to be due to the distribution and orientation of collagen lamellae. A visual comparison obtained of the distribution and organisation of the collagen lamellae in the Bowman’s layer and the anterior and posterior stroma is shown in Figure 2-3.

The lamellae in the anterior stroma are thinner than those in the posterior stroma, and tend to run obliquely to the corneal surface. The orientation of the fibres is random and there is a high degree of interconnectivity in all directions, with fibres branching and fusing with other fibres both in the anterior stroma and the Bowman’s layer\(^\text{(14)}\), providing the cornea with high tensile strength in all directions.

It has recently been suggested that the majority of lamellae in the anterior stroma insert into Bowman’s layer, intertwine with fibres in the deeper stroma, then reinsert into the Bowman’s layer forming bow-spring like structures\(^\text{(18)}\), and in keratoconus there is a reduction in the number of these insertions and the amount of intertwining between lamellae contributing to the loss of strength and the loss of the ability to maintain corneal curvature seen in this disease\(^\text{(18)}\).
Approaching the mid-stroma, the level of interweaving and branching is gradually reduced, with a greater degree of interweaving maintained towards the peripheral regions \(^7\). In the posterior stroma the collagen lamellae are thicker and run from limbus to limbus \(^1\). In this region, the collagen orientation becomes more organised, with collagen fibres running parallel to the surface and the majority of collagen being orientated with respect to either the nasal-temporal (N-T) or superior-inferior (S-I) meridians as previously illustrated in a diagram by Boote, et al \(^1\), shown in Figure 2-4. The reason that the lamellae align in this way is thought to be to withstand the forces from the ocular rectus muscles \(^1\) by increasing the tensile strength of the cornea with respect to each of these directions.

![Figure 2-4](en-face-diagram-of-the-cornea-showing-the-preferential-lamellae-orientations-in-the-posterior-stroma.png)

*Figure 2-4 – En face diagram of the cornea showing the preferential lamellae orientations in the posterior stroma. Reproduced from \(^1\)*

The structure of the limbal region where the cornea joins the opaque sclera is currently an area of great interest as a number of studies have suggested this area is fundamental to the biomechanics of the cornea \(^2\). Approximately 1 mm into the sclera there is a ring of circumferentially aligned collagen referred to as the circum-corneal annulus \(^\text{a}2\). The specific collagen orientation in this area is thought to be fundamental to maintaining the curvature of the cornea by resisting the increased circumferential tension at the limbus that occurs due to the differing curvatures of the cornea and sclera. Despite the increase in circumferential strength in this region, a number of studies examining the biomechanics of the cornea across different regions have suggested that this area is axially weak \(^1\), and the relative axial weakness in this area allows it to compensate for small pressure fluctuations while the curvature of the central cornea is maintained.

In addition to the circum-corneal annulus, another area of interest biomechanically is the corneal periphery zone covering the 2.5 mm inside of the circum-corneal annulus, with several
studies suggesting that this area is mechanically weaker than other areas \(^{(21,22)}\). The structure of this area is complex with clear regional variations. Within this peripheral zone the axially aligned collagen lamellae transition to become circumferentially aligned, and there is also a greater degree of branching and interweaving \(^{(21)}\). This bending and branching of lamellae has been suggested to contribute to a reduction in meridional stiffness \(^{(21)}\). The size of this transition zone in which the collagen changes alignment, has been found to be different with respect to the N-T and S-I meridians \(^{(19)}\) suggesting the axes may respond differently under loading.

Another structural difference with regards to the peripheral cornea, is the presence of lamellae formed of fibres with a larger diameter than those in the central cornea. These lamellae are thought to originate in the sclera and enter the cornea in line with the locations of the extraocular muscles \(^{(7)}\) as previously illustrated in a diagram by Boote, et al \(^{(26)}\), shown in Figure 2-5. Their presence is likely one of the contributory factors to explain the gradual increase in the thickness of the cornea from the centre to periphery. These fibres, rather than running tangentially from limbus to limbus, have been reported to traverse across several layers terminating within the anterior stroma where they branch and fuse with other fibres \(^{(14)}\). Since these lamellae link the limbus with the anterior stroma they have been coined ‘anchoring lamellae’ \(^{(27)}\), and are thought to have a role in maintaining the structural integrity of the cornea and sclera \(^{(7)}\).

![Figure 2-5 - Theoretical model showing the orientation of anchoring lamellae in the cornea and adjacent sclera. Reproduced from \(^{(26)}\)](image)

Aside from the distribution and orientation of collagen in the stroma, both collagen crimp and the presence of elastic fibres are thought to play a role in the response of the cornea to small pressure changes such as those that occur during the cardiac cycle.
It has been well documented in previous studies that the response of the cornea is non-uniform and viscoelastic, exhibiting low stiffness at lower pressures and low strain rates and high stiffness under larger loads \(^{(20,28)}\). The actual contribution of collagen crimp to corneal biomechanics is not yet well understood, but it is likely that when the cornea is under pressures lower than approximately 12 mmHg, which represents the lower end of normal IOP (normal IOP is defined as lying within the range of 10 mmHg – 21 mmHg \(^{(29)}\)), that the collagen is slack and wavy. Then as the pressure is increased to values at or above normal IOP, a gradually increasing proportion of the collagen becomes straightened, accounting for the low stiffness of the cornea at low pressures \(^{(20)}\).

The presence of elastic fibres in the cornea was identified some time ago \(^{(30)}\), but recently, greater detail regarding the distribution of these elastic fibres has been determined. A recent study by Kamma-Lorger, et al \(^{(31)}\), identified the presence of elastic fibres that run parallel to the collagen fibres in the circum-corneal annulus. A further study by Lewis, et al \(^{(32)}\), identified that the elastic fibres within the cornea were most concentrated in the posterior cornea just above Descemet's membrane. In this study, the elastic fibres were found to originate in the posterior limbus where they formed elastic sheets or broad fibres, micro-fibrils were found to branch out from these sheets, extending into the peripheral posterior cornea mirroring the alignment of collagen. It is thought these elastic fibres may play a large part in governing the way that the cornea deforms and recovers under small stresses such as physiological variations in IOP and forces imposed by the extra-ocular muscles \(^{(31)}\).

In summary, it is clear from the evidence presented within the current literature that the cornea is highly complex and anisotropic, therefore it is difficult to make assumptions regarding the biomechanics of the cornea based on evaluation of the structure alone. Dynamic measurements of the cornea deforming in response to forces and pressure changes that occur in vivo is fundamental to gaining a clear understanding of the corneal biomechanical properties. Determination of these biomechanical properties is crucial to the understanding of corneal diseases and the risks associated with current refractive surgery procedures as discussed in the following section.

### 2.3 Corneal disease

Certain diseases and pathologies affecting the cornea have a strong association with changes and alterations in biomechanics, examples of diseases that result in a biomechanical weakening of the cornea include pellucid marginal degeneration and keratoconus, whereas other diseases and pathologies can contribute to corneal stiffening for example diabetes mellitus, glaucoma, and aging.
Out of the pathologies identified, keratoconus is one of the most common. Keratoconus usually occurs bilaterally (33), and is a non-inflammatory eye disease in which the normal collagen structure becomes disrupted resulting in progressive thinning of the cornea, eventually resulting in the formation of a cone-shaped bulge (34).

Keratoconus is often diagnosed when the patient is in their teens or as a young adult, but the severity and progression of the condition is variable and dependent on many different factors, some of which remain unknown. The incidence of the disease can be up to 1 in 1000 people, being more prevalent in people of Asian heritage (34). The disease is likely a result of both genetic pre-disposition and environmental factors such as eye-rubbing or pregnancy (35), it is also known to be associated with some other diseases including Down’s Syndrome and several diseases associated with altered collagen synthesis (35).

Treatment has generally been via contact lenses in the early stages, with around 20% of patients (33) requiring corneal transplant when the condition progresses and vision continues to deteriorate. More recently however, corneal collagen cross-linking, which is described in more detail in section 2.5, has been used to stiffen the cornea and prevent the progression of the disease (33) to the point where corneal transplant is required.

The availability of treatment options such as corneal cross-linking that can prevent the progression of the disease, means the requirement for tools that can enable early diagnosis, such as those that can detect minute changes in corneal biomechanics is increased, so that cross-linking can be used at a stage before any deterioration has occurred in vision. A tool that enables early diagnosis is also important to prevent people at risk from keratoconus from undergoing invasive refractive surgery which could accelerate the progression of the disease. In addition to this if there was a technique capable of measuring the biomechanics of the cornea across the entire surface, customised and more targeted treatments could be developed which may improve visual outcomes.

In addition to corneal diseases in which the symptomology includes changes to the normal biomechanics, other corneal diseases exist, such as Fuchs dystrophy, which eventually require the need for corneal transplant to prevent blindness. Corneal transplant surgery is the most commonly performed transplant surgery worldwide, with 184,576 procedures reported to have taken place across 116 countries in 2012 (3). Several different types of transplant surgery are available depending on the nature of the disease or damage that is present in the cornea of the individual patient. The most commonly used procedures are summarised in the following (36).
• Penetrating keratoplasty i.e. full thickness transplant – the central cornea is fully removed from the patient and replaced with that of a donor
• Deep Anterior lamellar keratoplasty i.e. partial thickness transplant – A portion of the patient’s outer cornea is removed and replaced with a portion of donor tissue from the same area.
• Endothelial keratoplasty – Replaces the Descemet’s membrane and endothelium in a patient’s cornea with that of a donor, this surgery is used to treat endothelial disorders.

As all these surgeries are invasive, they require incisions to be made in the cornea and for tissue to be removed and then replaced. This disruption to the structure of the cornea has a significant impact on biomechanics. Although a greater knowledge of biomechanics would not prevent the need for corneal transplant in many of these cases, it could assist in optimising the surgical procedures and in doing so minimise as far as possible the loss of structural integrity.

In addition to the optimisation of surgical procedures, greater knowledge of biomechanics is fundamental to the development of synthetic or tissue engineered alternatives to donor tissue. A worldwide shortage of donor tissue has been reported, with a recent survey suggesting only one cornea was available for every seventy required \(^{(3)}\). This shortfall is likely to increase over coming years due to the rise in people undergoing refractive surgery, which makes their corneas unsuitable for donation, combined with an increase in life expectancy raising the demand for tissue. In addition to this shortfall, immune rejection is an issue for several patients meaning donated tissue is unsuitable. Therefore, development of biomechanically viable alternatives to donor tissue is an important area of research that could be aided significantly by greater knowledge of biomechanics.

### 2.4 Refractive surgery techniques

By far the main driver of the interest in cornea biomechanics has been the revolution in corneal surgery that has occurred over the last 30 years, with over 50 million elective surgical procedures performed worldwide to date, in which lasers have been used to alter the shape of the cornea and change its refractive power.

Originally, prior to laser surgery, refractive changes were introduced via radial keratotomy (RK) a procedure where several radial incisions are introduced at specific positions around the edge of the cornea to induce a flattening of the tissue, correcting for myopia and astigmatism. RK is now rarely used due to common complications that are associated with the treatment such as over-correction, glare and night vision problems, along with long-term instability. In addition to the fact more precise, reproducible results can be achieved via laser surgery.
Photorefractive keratectomy (PRK) was the original laser eye surgery and is still used for some cases today, it can correct for myopia, hyperopia and astigmatism. The procedure involves removing and discarding the corneal epithelium and then ablating the surface of the underlying Bowman’s layer and anterior stroma using an excimer laser which removes very small amounts of tissue in a specific pattern to obtain the required shape change for vision correction. A soft contact lens then needs to be positioned over the cornea to protect the eye and reduce discomfort in the four to seven days following surgery, during which the epithelium grows back. Due to removal of the epithelium there can be significant discomfort during recovery from PRK and there is increased risk of infections and the development of haze, these factors led to the development of alternative surgeries including laser-assisted sub-epithelium keratomileusis (LASEK), and laser-assisted in-situ keratomileusis (LASIK) which aimed to avoid this problem.

LASEK is overall a similar procedure to PRK, however, in LASEK the epithelium is not fully removed but instead it is peeled back and then repositioned after surface ablation of the underlying tissue has been conducted. This can reduce discomfort or other complications such as haze post-surgery when compared with PRK, but the risk of discomfort is still greater than in LASIK and haze can still be an issue.

LASIK is the most popular form of refractive surgery today due to its ability to provide instant results and the fact there is minimal discomfort during healing. During LASIK, a flap is cut into the cornea using a microkeratome or a femtosecond laser, to a depth commonly between 100 µm – 160 µm, this flap is then lifted and the excimer laser is used to ablate the underlying surface to give the desired vision correction. After treatment, the flap is repositioned. No stitches are required to hold the flap in place, it is held in place by the osmotic gradient force created when the endothelial cells pump water out to the inner part of the eye, and over a few days the epithelium grows over the edges of the flap, and over a few weeks the flap becomes bonded to the underlying structure (37).

The advancement in various topographic measurement systems along with the development of new technologies such as the high resolution wavefront abberometer, means imperfections in the shape of the cornea can be identified with high resolution, and due to the high accuracy of the excimer and femtosecond lasers available today these imperfections can be treated with high precision meaning visual outcomes as good as, or better than, 20/20 vision can be achieved for the majority of patients.

There is much debate as to whether surface ablative techniques such as PRK or LASEK, or techniques that involve flap creation, namely, LASIK are better. There are patient specific considerations such as corneal thickness which can rule out certain options for an individual,
but they can all provide similar refractive outcomes, with patients generally preferring LASIK if there is an option due to the instant results and reduced discomfort post-surgery, meaning the patient can return immediately to their normal routine.

However, when considering the biomechanical implications of each of the techniques several different factors must be considered, and to date few studies have been conducted to examine the specific effects of the different types of treatment on the structural integrity of the cornea.

Generally, despite LASIK now being the most common form of refractive surgery performed, with over 28 million LASIK procedures performed up to 2009 (38), it is thought that surface ablative techniques are superior to LASIK when it comes to maintaining the biomechanical integrity of the cornea, as due to the absence of the flap, the number of collagen fibres severed during surface ablative techniques such as PRK and LASEK is 40% to 50% lower than in LASIK (39). It is also true that the incidence of post-refractive surgery ectasia is significantly higher after LASIK than PRK, with LASIK reportedly accounting for 96% of cases and PRK only 4% (40).

A concern common to all the techniques discussed, is that tissue is ablated from the Bowman’s layer and/or the most anterior portion of the stroma thereby reducing the strength of this area, which due to its highly-interwoven structure, has been suggested to contribute most significantly to the overall tensile and cohesive strength of the cornea, and be responsible for the overall maintenance of corneal curvature.

The creation of the corneal flap during LASIK is the area of greatest concern as a large amount of collagen fibres are severed throughout the depth of the anterior stroma. Post-surgery it has been found that the structural integrity of the flap region is not regained, with collagen fibres within this region showing disorganisation and atrophy (41), and the flap failing to reconnect properly with the stromal bed (42), meaning they no longer move in unison in response to pressure fluctuations.

Recently a compromise between surface ablation and LASIK called sub-Bowman’s keratomileusis (SBK) or thin-flap LASIK has been adopted by some surgeons. This is a procedure where the flap is created at a depth of only 80 µm – 90 µm (42). This thinner flap reduces the number of fibres severed during flap creation, keeping them confined mainly to the Bowman’s layer and the area just under this, allowing more of the fibres throughout the depth of the anterior stroma to remain in-tact. This reduction in damage to the anterior stroma during flap creation presumably reduces the magnitude of biomechanical weakening while still providing all the benefits of LASIK, including reduced recovery time. However, since the
anterior stroma is known to interweave with the collagen in the Bowman’s layer, and the
Bowman’s layer is known to contribute to the overall biomechanical strength, it is likely the
introduction of a flap, however thin, will still compromise the structural integrity of the cornea
to some degree. On top of this tissue ablation under the flap still introduces damage to the
anterior most portion of the stroma.

Due to the many risks associated with invasive surgeries, and a lack of knowledge regarding
the long-term effects, current interest has been in the development of minimally invasive or
non-invasive alternatives. Small Incision Lenticule Extraction (SMILE) is the latest
development in refractive surgery and is often referred to as keyhole refractive surgery. In
SMILE a femtosecond laser is used to cut an intra-stromal lenticule. The depth of stroma at
which the lenticule is cut is approximately 20% – 40% of the way down and therefore the most
anterior portion, considered to be most important biomechanically, remains intact (43). To
remove this lenticule a small 2 mm – 4 mm tunnel incision is made using the femtosecond
laser at the edge of the cornea on the superior-temporal side. The lenticule is then separated
and extracted by using specially designed tools that are inserted through the small tunnel
incision.

With SMILE because there is no flap, far fewer collagen fibres are severed than in LASIK and
therefore it is thought it will not carry the same risks with regards to post-surgical ectasia. Also,
due to the fact tissue is removed at a greater depth within the stroma when compared with
LASIK and PRK/LASEK, a greater proportion of the stiffer anterior stroma is left in-tact, and
this would be presumed to confer biomechanical benefits.

As SMILE is a relatively new procedure long-term follow up studies are currently not available,
and since no instrument has yet been developed that can establish the biomechanics of the
cornea in vivo with any certainty, the biomechanical implications of SMILE are not yet fully
understood and have so far mainly been predicted from models. Highlighting the requirement
for further investigation.

Overall, with regards to the biomechanical effects of refractive surgery, there remains a clear
lack of understanding, due to the absence of an instrument capable of reliable in vivo or ex
vivo assessment of the biomechanical changes that occur before and after surgery. Much of
the current data relies on the identification of complications that occur in patients several
months to several years after undergoing the procedures, or on models created based on data
collected ex vivo.
What is known, is that all the invasive procedures discussed will have some degree of a negative effect on corneal biomechanics, making a non-invasive procedure the most ideal treatment option. This has led to interest into whether corneal collagen crosslinking applied in specific topographical locations could be used to modify the shape, and therefore the refractive index of the cornea by changing the material properties of specific regions in isolation.

2.5 Collagen crosslinking

The formation of crosslinks in the corneal stroma is a procedure that occurs naturally throughout an individual’s lifetime and is thought to be responsible for the gradual stiffening of the cornea observed with age\(^{(44,45)}\). Crosslinking can be induced in the cornea above the levels that occur naturally by applying photosensitising agents such as vitamin B2 (riboflavin) to the cornea and then exposing it to a source of UVA-light. The free-radicals created when riboflavin is excited by the UVA-light leads to photodynamic crosslinking of the collagen tissue\(^{(46)}\). The nature of the crosslinks that form during corneal cross-linking remains unknown, with results from a study by Hayes et al\(^{(47)}\), indicating that it was likely crosslinking occurs within and between the molecules on the collagen fibril surface and with proteins in the surrounding protein network. The effects of crosslinking are depth dependent, with crosslinking generally reported to affect the anterior most 200 µm where the majority of the UVA light is absorbed\(^{(48)}\).

The clinical data presented so far within the literature indicates that the epithelium must be removed prior to crosslinking to introduce reliably significant changes to the stiffness of the cornea, with several studies reporting poor results with epithelium-on procedures to treat keratoconus\(^{(49,50)}\). This means the technique is currently invasive and a degree of recovery time is required, but since the epithelium does not contribute significantly to the biomechanics of the cornea the strength or integrity of the tissue is not compromised during the procedure.

The formation of the crosslinks has been shown to increase the resistance of the cornea to deformation under both hydrostatic loading and tensile loading. An increase in the Young’s modulus of corneo-scleral buttons subject to inflation testing after crosslinking by a factor of 4.3 has been reported in one study\(^{(51)}\) and a similar increase in Young’s modulus by a factor of 4.5 has been reported from a study that used strip extensometry testing\(^{(52)}\) and the same crosslinking protocol.

Aside from being used for the treatment of corneal ectasias, in recent years crosslinking has been used as an adjunct to LASIK in a procedure called LASIK-Xtra. In LASIK-Xtra, prior to repositioning the corneal flap over the ablated area, it is soaked with riboflavin for 90 seconds, after which the riboflavin is rinsed away\(^{(53)}\) and the flap is repositioned. The cornea is then
exposed to a UVA-light source to initiate photodynamic crosslinking in the area between the flap and the underlying stroma. The addition of crosslinking to the standard LASIK procedure has been reported to increase the stability of visual outcomes, especially in the case of hyperopia \(^{53,54}\), and is presumed to reduce the risk of post-LASIK ectasia, however long term follow up studies on large sample numbers have not yet been conducted.

Since the number of people with post-refractive surgery ectasia is increasing due to the popularity of LASIK, corneal crosslinking is becoming an important tool for the treatment of these complications and its application is likely to increase in the coming years. In addition to this, as crosslinking is a minimally invasive treatment that does not require any tissue to be removed from the cornea, if it can be used as a tool to reshape the cornea, it has the potential to be used as an alternative to refractive surgery in certain cases, making surgical vision correction available to a wider range of the population such as those with thinner corneas. It could also be used for touch-up treatments in people who may have previously undergone refractive surgery. In an interview in 2015 \(^{55}\), David Muller, CEO of Avedro Inc., estimated the potential market for refractive correction using crosslinking techniques to be greater than that of LASIK, at 8 million to 10 million cases a year.

Although crosslinking has been shown to be effective for preventing the progression of keratoconus \(^{48}\), and also in the improvement of outcomes of LASIK, so far only a few studies exist that demonstrate the efficacy of crosslinking as a stand-alone procedure to alter the shape of the normal cornea to induce a refractive correction. Kanellopoulos \(^{56}\) reported achieving myopic correction of approximately 1.5 diopters using high-fluence crosslinking and Hafez \(^{57}\) reported achieving astigmatic correction of 0.9 diopters.

To examine the effects of topographic cross-linking on the biomechanics of the cornea, a technique is required that can provide details of the biomechanical properties of the entire corneal surface, this information alongside the shape information could be used to establish how effective the technique would be for refractive correction, and how safe and effective the technique may be long term.
2.6 Current knowledge of corneal biomechanics and challenges associated with measurement

Due to the many reasons for the widespread interest in corneal biomechanics as outlined in the previous sections, a large volume of research has been conducted over many years with the desire to quantify the mechanical properties of the cornea. Several different techniques and methods have been employed for measurement, and a large range of different material parameters have been quantified including; Young’s modulus, shear modulus, longitudinal modulus, corneal resistance factor and dynamic modulus, amongst others.

Young’s modulus is the parameter which is most often quantified. Young’s modulus (E) is the measure of material elasticity and is defined as the ratio of stress (σ) to strain (ε), as shown in equation 2-1.

\[ E = \frac{\sigma}{\varepsilon} \]  

Equation 2-1

To establish Young’s modulus, it is necessary to quantify both stress and strain, stress is most often estimated as a function of the applied load, whereas strain is measured directly via a variety of different techniques. Measured values of corneal Young’s moduli have been reported to range from 0.01 MPa – 57 MPa \(^{(58)}\). This wide range of reported values highlights the complexities involved with the measurement of corneal biomechanics. Some of the variation in reported values of Young’s modulus can be accounted for by considering the following factors:

- The cornea exhibits non-linear behaviour.
- The cornea is viscoelastic and exhibits a degree of hysteresis.
- The structure of the cornea is heterogeneous resulting in regional variations in Young’s modulus.
- The cornea is anisotropic resulting in variations in Young’s modulus depending on the direction and nature of the applied load.

These factors and the findings from previous studies are discussed in more detail in this section.

2.6.1 Non-linear stiffness

Materials that exhibit a constant Young’s modulus are described as linearly elastic, whereas materials that exhibit a variable Young’s modulus are described as non-linear elastic. Previous studies conducted on the cornea have suggested that the response of the cornea is non-linear and that Young’s modulus varies depending on the magnitude of the applied stress \(^{(20,24,28)}\).
The results of several studies by Elsheikh, et al.\textsuperscript{(28,59)}, in which the displacement of the corneal apex (apical rise) of corneo-scleral buttons was measured in response to increasing hydrostatic pressure, indicated that the response of the cornea could be described in three main stages, these were:

1. **Linear elastic, low stiffness at pressures between $\sim 10$ mmHg – 21 mmHg.** These pressures represent normal physiological IOP. Over this pressure range, stress and strain increase approximately linearly. This is illustrated in the results from the study by Elshiekh, et al.\textsuperscript{(59)}, shown in Figure 2-6 where apical rise occurs at an approximately constant rate for pressures over this range. The rate at which apical rise increases is relatively high indicating low stiffness.

2. **Non-linear elastic, variable stiffness at pressures between $\sim 21$ mmHg – 60 mmHg.** These pressures are generally considered to be above normal IOP, but the IOP can be raised into this range for short periods of time due to natural processes such as blinking, eye rubbing or squinting, or for longer periods of time in certain conditions such as glaucoma. Over this range the relationship between stress and strain is not constant. This is illustrated in Figure 2-6 as apical rise does not occur at a constant rate. However, if this pressure range was divided into smaller sub-sections of approximately 10 mmHg the relationship of stress to strain could be approximated as linear.

3. **Linear elastic, high stiffness at pressures greater than $\sim 60$ mmHg.** These pressures are generally considered to be outside of the physiological range, although in rare instances IOP may be raised into this range for very short periods of time. In this range stress and strain are thought to increase approximately linearly resulting in a constant Young’s modulus. Young’s modulus in this range has been shown to be relatively high, this is demonstrated by the results in Figure 2-6, as the rate at which apical rise increases with increasing posterior pressure is relatively low.

Overall, it is clear from the data obtained in previous studies that the response of the cornea cannot be approximated as linear over a large pressure range and that variations in Young’s modulus occur between pressures of 12 mmHg – 60 mmHg. The reason for these variations is likely due to the structure of the cornea with the response being dominated by different structural components at each of these stages. The overall mechanism of the response remains unknown, but it is likely at low pressures the collagen fibres are not fully stretched resulting in lower stiffness, as the pressures gradually increases, increasing amounts of these
fibres become taut contributing to the variable stiffness stage and eventually all the fibres become taut contributing to the constant high stiffness seen at high pressures.

Figure 2-6 – Apical rise plotted with respect to posterior pressure demonstrating the non-linear response of the cornea, reproduced from (59).

The non-linear behaviour can partly account for the wide variation in reported Young’s moduli as identified at the beginning of this section. If reported Young’s moduli are considered only from studies that have examined the response of the cornea under low strain or at pressures close to the physiological range, then the reported values tend to fall between 0.01 MPa – 8.99 MPa as shown in Table 2-1 where the reported corneal Young’s moduli from various studies on human corneas using different measurement techniques are listed.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Measurement technique</th>
<th>Pressure range (mmHg)</th>
<th>in vivo/ex vivo</th>
<th>Young’s modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elsheikh, et al. (28)</td>
<td>Laser apical displacement tracking</td>
<td>10 - 20</td>
<td>Ex vivo</td>
<td>0.25 – 1.25 (estimated from plotted data)</td>
</tr>
<tr>
<td>Wollensak, et al. (52)</td>
<td>Strip Extensometry</td>
<td>N/A</td>
<td>Ex vivo</td>
<td>0.8 – 2.2 (4% - 8% strain)</td>
</tr>
<tr>
<td>Hjortdal (24)</td>
<td>Particle tracking</td>
<td>10 - 25</td>
<td>Ex vivo</td>
<td>6.21 – 8.99 (regionally dependent)</td>
</tr>
<tr>
<td>Lombardo, et al. (60)</td>
<td>Scheimpflug imaging of response to inflation testing</td>
<td>18 - 42</td>
<td>Ex vivo</td>
<td>2.28 +/- 0.87 (with epithelium)</td>
</tr>
<tr>
<td>Shih, et al. (61)</td>
<td>Scheimpflug imaging of response to air-puff</td>
<td>Physiological</td>
<td>In vivo</td>
<td>0.01 – 1.24</td>
</tr>
<tr>
<td>Knox-Cartwright, et al. (45)</td>
<td>Radial shearing interferometry</td>
<td>15 – 15.5</td>
<td>Ex vivo</td>
<td>0.27 – 0.52</td>
</tr>
<tr>
<td>Wang, et al. (62)</td>
<td>Ultrasound</td>
<td>Physiological</td>
<td>Ex vivo</td>
<td>5.3 +/- 1.1</td>
</tr>
</tbody>
</table>

Table 2-1 – List of studies that have examined the response of the human cornea over physiological pressure changes and their reported values of Young’s modulus.
The factors discussed, highlight that to understand the biomechanics of the cornea relevant to the in vivo case, experiments must be conducted over a physiological pressure range and that testing at pressures above this range may lead to an overestimation of corneal stiffness and changes to the way the cornea responds. Ideally it would be possible to examine the response of the cornea to pressure fluctuations it regularly experiences in vivo, such as small IOP fluctuations that occur during the cardiac cycle, as this would represent in vivo biomechanics.

Obtaining data over such a small pressure range requires a measurement method with high sensitivity. To illustrate this point, an estimation for the sensitivity required to measure strain in response to these small fluctuations in IOP was made using the range of reported Young’s moduli in Table 2-1. If it is assumed that the cornea is an isotropic thin-shelled hemispherical membrane of constant thickness and bending stiffness is ignored, then the apical rise ($w_{\text{apex}}$) can be described by equation 2-2 \(^{(63)}\):

$$w_{\text{apex}} = \frac{pr_c^2}{2Et} (1 - \nu)$$  \hspace{1cm} \text{Equation 2-2}

where; $p$ is the average increase in IOP that occurs during the cardiac cycle (400 Pa (3 mmHg \(^{(64)}\)), $r_c$ is the radius of curvature estimated half way between the anterior and posterior surface of the cornea (7.25 mm), $t$ is the average corneal thickness calculated as the average of central corneal thickness and peripheral corneal thickness (0.6 mm), $\nu$ is the Poisson’s ratio (0.49) \(^{(65)}\) and E is Young’s modulus.

To determine the worst-case scenario (corresponding to least movement), if Young’s modulus of the cornea is estimated as 2.28 MPa which is the highest non-regionally specific Young’s modulus reported from inflation based experiments in Table 2-1, then apical rise calculated from equation 2-2 in response to a 3 mmHg pressure increase would be 3.92 $\mu$m. Based on these predictions, along with the assumption that apical displacement would represent the maximum displacement and that any surrounding material i.e. sclera would remain rigid, the sensitivity of a measurement technique capable of assessing the deformation of the cornea in response to the pressure fluctuations that occur in response to the cardiac cycle would be a minimum of 1 $\mu$m and ideally lower, if sensitivity to deformation was required across the whole surface.

### 2.6.2 Viscoelasticity

The cornea is generally considered to exhibit viscoelastic behaviour \(^{(66)}\). If a purely elastic material is loaded and the load is subsequently removed the material will return immediately to its pre-loaded condition. However, the response of a viscoelastic material has both a viscous
and elastic component, if a viscoelastic material is loaded (within its elastic limits) and the load is subsequently removed the material will take time to return to its pre-loaded condition. This time delay is a result of the viscous component and describes the total energy lost during loading due to friction and molecular rearrangement. The total energy lost due to the viscous component can be determined through examining the loading and unloading behaviour of a material, the differences between the unloading and loading curves is termed hysteresis, a diagram of this is given in Figure 2-7.

![Diagrammatic representation of the hysteresis effect.](image)

The overall magnitude of the hysteresis is dependent on the strain rate, and may partly explain the results presented in a study by Elsheikh, et al, that showed variation in the response of the cornea at slow and fast pressure application rates \(^{(28)}\), with the corneas exhibiting lower stiffness during slower pressure application rates. Overall the viscoelastic properties of the cornea indicate the measured Young’s modulus may be influenced by the loading rate and the time of measurement, therefore the overall influence of these factors must be considered in the design of experiments and when interpreting results.

### 2.6.3 Heterogeneity and Anisotropy

As discussed in section 2.2 of this chapter, the structure of the cornea is heterogeneous, indicating that regional variations in material properties are likely. Despite this fact, at the commencement of this research very few studies had been conducted to examine the full-field biomechanics of the human cornea. Results from studies by Hjortdal \(^{(24)}\) and Shin, et al \(^{(25)}\), that tracked the movement of a small number of self-adherent particles positioned at different regions across the surface of the cornea, indicated regional variations in mechanical properties, however the results from these studies were conflicting, with Hjortdal suggesting the limbus was least resistant to axial strain and Shin, et, al suggesting the centre of the cornea...
was least resistant. A later study by Boyce, et al (20), that used digital image correlation to track the deformation of bovine corneas in response to increasing hydrostatic pressure, also showed regional variations in mechanical properties and echoed the findings of Hjortdal's study, that the limbal area was least resistant to axial strain.

The results from these studies, in addition to the structural aspects previously discussed, demonstrate that the mechanical properties of the cornea vary with respect to different regions and the definition of a single Young’s modulus may not be sufficient to describe the biomechanical behaviour of the cornea. Overall due to the limited number of studies, and the limited resolution of the current studies, the regional mechanical properties of different regions of the cornea remain poorly understood highlighting the need for a measurement technique capable of full-field assessment.

In addition to regional variations in the response of the cornea, the response of the cornea is likely to be affected by the nature and direction of the applied load. In vivo the cornea is subjected to several different forces, these include:

**IOP -** The cornea exists under a state of constant stress due to IOP. The normal range of IOP is reported to lie between 10 mmHg – 21 mmHg, as defined in section 2.2. Fluctuations in IOP by approximately 2 mmHg – 6 mmHg are common and occur due to diurnal variations, cardiac cycle, exercise, blinking, etc. Larger short term variations in IOP in excess of 90 mmHg (67) can also occur due to events such as eye rubbing or forced squinting. The force imposed by the IOP impacts the shape of the cornea which in turn governs the refractive power of the eye.

**Forces from the ocular rectus muscles** – The ocular rectus muscles insert into the sclera and are responsible for controlling the movement of the eye. Tension in these muscles exerts a force on sclera which in turn impacts the cornea.

**Forces from the eyelids during blinking** – The downward movement of the upper eyelids during blinking exerts compressive forces on the cornea and contributes to elevations in IOP.

**Forces due to accommodation** – The ciliary body and ciliary muscle are attached to the internal surface of the sclera and control accommodation, When the ciliary body contracts it imposes forces on the sclera, and in-turn the resultant movement of the sclera imposes forces on the cornea.
The structure of the cornea and its material properties have evolved to withstand these very specific forces, thus loading the cornea in ways that do not mimic these forces may give misleading results and be irrelevant to the understanding of in vivo behaviour.

2.7 A review of current measurement techniques

A review of the measurement techniques currently used for corneal biomechanical assessment was undertaken to gain an understanding of the current measurement capabilities and their limitations.

2.7.1 Ex vivo measurement techniques

To date, most of the data on the mechanical properties of the cornea has been collected ex vivo. Ex vivo testing has many advantages; the environment can be tightly controlled, testing can be destructive, it is easier to isolate specific variables and there are significantly less safety constraints. However, since it is generally the in vivo biomechanics of the cornea that are of interest ex vivo testing also presents some additional challenges and issues, such as, limited availability of human tissue, problems with maintaining the integrity and the properties of the tissue once removed from the body, and challenges with determining the relevance of the ex vivo data with respect to the in vivo case, or in replicating in vivo conditions as closely as possible.

Strip Extensometry

Ex vivo, strip extensometry is one of the most commonly used techniques to determine the biomechanical properties of corneal tissue due to its low cost and simplicity. Many of the current biomechanical models of the cornea are based on the data obtained via this technique. The testing method most commonly involves taking dissected strips of corneal tissue and subjecting them to uni-axial tensile testing while measuring the strain that occurs in response to the applied stress. Although the testing method can be useful to determine the comparative stiffness of different layers or meridians of the cornea with respect to loading in a specific direction, overall the results have very little relevance with respect to the in vivo properties of the cornea. The main issues with the technique are as follows:

- During preparation of the test strip the structure of the cornea is compromised with collagen fibres severed and the interactions between the various layers lost.
- If full thickness strips are used, the thickness of the strip is not constant and this has implications when interpreting the results.
- Since the cornea is curved, the anterior and posterior surfaces are different lengths and an initial flattening of the strip occurs during tensile testing. This flattening results in
initial higher tension on the shorter posterior side and compression of the anterior side

- The specific area from which the strip is taken, the clamping position and the direction of the applied load is likely to have significant effects on the measured value of Young’s modulus.
- The loads applied to the cornea are not representative of in vivo loads in either magnitude or direction.

In fact a study by Elsheikh, et al. (68), confirmed the issues with using strip extensometry data to predict the in vivo behaviour of the cornea when he conducted a test comparing the stiffness of corneas when subjected to inflation testing versus strip extensometry and found the measured stiffness to differ by 32%. In general, inflation testing of either whole globes or corneo-scleral buttons situated in an artificial anterior chamber (AAC) is considered a better option, as it more closely replicates in vivo conditions.

Several different measurement techniques have been used alongside inflation testing to determine corneal biomechanics, these include particle tracking and digital images correlation based techniques, optical coherence tomography based techniques, and laser based techniques.

**Laser based apical displacement tracking**

A number of studies have been conducted by Elsheikh, et al (28,59), where the movement of the corneal apex has been tracked in response to a change in hydrostatic pressure. The main advantage to this method is that the apical displacement can be tracked with high sensitivity so measurement is possible within the physiological range. However, as the movement is only measured at the apex no information is obtained regarding the specific way that the cornea is deforming, and therefore to obtain Young’s modulus from these measurements the cornea is generally assumed to be an isotropic, homogenous, thin-walled hemisphere. As discussed throughout previous sections, these assumptions are not valid and therefore this technique is not particularly useful for determining the overall biomechanics of the cornea, or for providing sufficient information whereby biomechanical abnormalities can be identified.

**Particle tracking and Digital Image correlation**

Tracking the movement of the whole surface of the cornea in response to a pressure change is a more suitable way to evaluate the biomechanics of the cornea over the two methods previously discussed, as it enables regional variations in mechanical properties to be evaluated. As previously identified, both Hjortdal (24) and Shin, et al (25), demonstrated the
importance of full-field evaluation in their studies where the movement of a small number of self-adherent particles positioned at different regions across the corneal surface were tracked in response to a pressure change.

Since the studies by Hjortdal and Shin, et al, significant improvements to digital image correlation techniques have occurred due to the availability of high resolution cameras and the advancements in digital image correlation software, enabling the movement of the corneal surface to be measured with higher sensitivity and greater resolution. This was demonstrated in a study by Boyce, et al, where the movement of many fine particles randomly distributed on the surface of a bovine cornea were tracked in response to pressure changes up to 240 mmHg.

In this study, Boyce, et al, reported a sensitivity of 5 \( \mu \)m \(^{20}\). This sensitivity was adequate to examine the deformation of the bovine corneal surface to pressure changes towards the upper end of those that occur physiologically, with measureable displacement occurring over steps of approximately 10 mmHg within the range of 27 mmHg to 60 mmHg.

Overall DIC techniques offer several significant advantages over point based measurement techniques as the movement of the whole surface can be captured over a measurement time of less than 1 second. However, the sensitivity limits of these technique may limit their ability to resolve subtle regional changes to corneal biomechanics that may occur due to disease or surgical intervention, while measuring over a physiological pressure range. The requirement for the presence of recognisable particles to be present on the corneal surface may limit the transferability of the technique to an in vivo situation. Also, since the technique is limited to giving surface information only, full biomechanical analysis of an in-tact cornea is not possible as information is not obtained throughout the depth.

_Holographic interferometry and laser speckle interferometry techniques_

Laser interferometry techniques including Holographic Interferometry (HI), Wavefront Interferometry (WI), Electronic Speckle Pattern Interferometry (ESPI) and Electronic Speckle Pattern Shearing Interferometry (ESPSI) have previously been used to examine the full-field biomechanics of the cornea, and are capable of the high, sub-micrometre sensitivities required to measure the response of the cornea to small pressure fluctuations, such as those that occur during the cardiac cycle.

Double exposure holography has been used on several occasions to examine the response of the cornea to small pressure fluctuations ex vivo. During double exposure holography, a coherent light source is divided into two parts and one part is used to illuminate the surface of
cornea and the other part is used as a reference beam, the wavefront reflected from the surface of the cornea is captured on holographic film where it is interfered with the wavefront from a reference beam. The resulting hologram contains information on the phase of light reflected from the surface of the object. If the cornea is then loaded in some manner and the process is repeated with the data captured on the same film, the superposition of the wavefronts captured during each of these exposures will result in interference fringes due to the different phase of the light reflected from the objects surface during each of the two loading states, these interference fringes correspond to displacement.

Early ex vivo studies using double-exposure HI demonstrated regional variations in the out-of-plane displacement of the cornea in response to a small pressure changes \(^{69-71}\) and showed how this response changed with the introduction of superficial radial incisions \(^{69,70}\). The pressure changes required in these studies to generate data were of the order of 0.01 mmHg \(^{69}\) to 3.50 mmHg \(^{70}\) and sensitivity was approximately one quarter of the wavelength of the illumination source at 158 nm \(^{70}\) allowing very fine detail to be picked up. The main problem with these studies was that the data was presented as interference patterns not as quantitative data, and without detailed knowledge of the measurement principles this data could not be interpreted.

Electronic Speckle Pattern Interferometry, which is described briefly here and in greater detail in chapter 3, is now often used as an alternative to holographic interferometry as processing of the speckle interferograms is simpler than numerical reconstruction of digital holograms \(^{72}\), enabling simple full-field quantitative analysis and the ability to view real-time results, with a total measurement time of a few milliseconds. In addition to this, the illumination angle can be manipulated during ESPI to obtain different components of displacement without changing the viewing direction which is not possible in HI.

ESPI is dependent on the speckle phenomenon. Speckle occurs when an optically rough surface (surface height variations greater than the wavelength of the illumination source) is illuminated with a coherent light source. If the light scattered from the surface of the object is combined with a reference beam in the image plane, the resulting speckle interference pattern contains information detailing the phase of light scattered from the object at that point in time. If this interference pattern is stored, and the object is subsequently deformed, a new speckle pattern will form, if this is digitally subtracted from the initial speckle pattern of the object in its undeformed state the resulting image will be an interference pattern representing the phase change that has occurred between the two object states. As with HI, the fringes contained within this interference pattern relate to displacement. If normal illumination is used the
resulting fringe distributions describe out-of-plane displacement and are equivalent to those obtained via holography.

Recently out-of-plane ESPI has been used in ex vivo testing by Jaycock, et al, to quantitatively examine the dynamic response of the sheep cornea to small changes in hydrostatic pressure\textsuperscript{(41)} where it was demonstrated that there was a weakening of the cornea after the introduction of a stromal flap. Unlike previous HI studies, the results presented by Jaycock, et al, were quantitative and presented in terms of displacement.

One of the main drawbacks to both HI and ESPI is that the measurement is restricted to a maximum displacement of approximately 10 µm - 20 µm over a measurement cycle, dependent upon the specific wavelength of the laser used, the illumination angle and the concentration of strain over a given area. However, the measurement range can be extended above this by capturing total deformation over a series of measurements immediately following one another while updating the reference image. An additional drawback, for ESPI especially, is the propensity for speckle decorrelation due to its high sensitivity to environmental disturbances, generally restricting its usage to a laboratory environment. Due to these reasons, there has been recent interest in ESPSI techniques, which are described briefly here and in greater detail in chapter 3.

The principles of ESPSI are similar to that of ESPI, however in ESPSI the object is used as its own reference. This is achieved by dividing the light scattered from the surface of the object into two parts, translating or transforming one of the parts with respect to the other, before interfering the two parts in the image plane. The specific way in which the two parts are transformed or translated with respect to one another determines the sensitivity of the device as the displacement of the object is compared at the interfered points in the image plane.

Due to this self-referencing feature, ESPSI is less sensitive to environmental disturbances and rigid body motion than ESPI, allowing it to be used as a measurement tool outside of laboratory environments. In addition to this, since the magnitude and direction of the applied shear determines the sensitivity there is more freedom to extend and optimise the range of sensitivity for a specific scenario.

So far the only reported application of ESPSI techniques to the measurement of corneal biomechanics has been in the studies of Knox-Cartwright, et al, where out-of-plane radial shearing interferometry was used to quantify the variation in corneal Young’s modulus with age\textsuperscript{(45)}, the stiffening effect of riboflavin and UVA collagen crosslinking\textsuperscript{(51)}, and the
biomechanical effects of variation in the angle of microkeratome incisions used to create stromal flaps (73).

There are several challenges to the use of laser interferometry techniques when considering corneal measurement, especially in vivo. Firstly, since the cornea is transparent the signal reflected from the surface is often poor and therefore a surface coating is generally required. Secondly, stability is hard to achieve due to tear film break up or head and eye movements, and these small unwanted movements can lead to loss or distortion of data. This second issue was evident in a study by Calkins, et al (74), where it was attempted to use double exposure holography in vivo to examine wound healing after corneal transplant by examining the response of the cornea to IOP fluctuations over a cardiac cycle, as the data was significantly distorted by tilt fringes.

**Optical Coherence Tomography based techniques**

In addition to the surface measurement techniques already discussed, recent developments in optical coherence tomography techniques have led to interest into whether they could be used to measure the dynamic behaviour of the cornea throughout the depth of the tissue. Ophthalmologists are familiar with OCT techniques as they are used as an imaging tool to provide high resolution cross-sectional images of the retina and optic nerve head (75) and also of the anterior segment of the eye (76).

OCT is well suited to measurement of semi-transparent biological materials. In classic time-domain-OCT a broadband light source is focussed onto the object of interest, this light is reflected from the object and interferes with light from a reference beam. Due to the low coherence of the light source, a signal is only produced when the optical path length of the light reflected from the object matches that of the reference beam (77), so by changing the length of the reference beam, the reflections from various layers of the sample are detected one depth at a time.

Several variations of conventional OCT are now available that offer advantages including better resolution and reduced scan time. In spectral-domain-OCT reflections through the entire depth of a sample can be obtained simultaneously, in this case the length of the reference beam is kept constant and the interference between the object and reference beams is detected as a spectrum which can then be resolved via Fourier transforms to give depth information (78). A further extension of this is swept-source-OCT where rather than using a spectrometer, the wavelength of the light source is tuned in rapid cycles (78). The advantages of swept-source OCT are high imaging speeds, improved resolution and the ability to measure a wider range of tissue depths (79).
Studies have been conducted using spectral-domain-OCT (80) and swept-source-OCT (81) in combination with digital image correlation to evaluate the 2D (80) and 3D (81) deformation of the cornea in response to a hydrostatic pressure change by tracking speckle motion. Phase-sensitive OCT has also been used to evaluate the propagation of an elastic wave through the depth of the cornea (82–84), and in vivo imaging of corneal deformation in response to an air-puff throughout the depth of the cornea has been achieved with spectral-OCT (85).

The main advantage of OCT based techniques over the techniques that have been previously discussed, is the ability to measure the 3D response of the cornea and hence all components of deformation in response to a pressure change. This enables biomechanical parameters to be calculated directly from the measured data without the need for assumptions to account for any absent components of deformation throughout the depth of the cornea, as is required with DIC and interferometry based methods. The sensitivity of OCT is also high at around 1 µm.

The main issue with current OCT-based methods with respect to 3D evaluation is that the scans are performed through a single cross-section at a time, with several scans required for 3D analysis and this can take several minutes to hours depending on desired resolution of the 3D data. Hence, as the stability of the cornea is hard to achieve over any given length of time, 3D assessment is limited to the laboratory until a method is identified whereby the total measurement time can be significantly reduced.

2.7.2 In vivo measurement techniques

An instrument capable of in vivo assessment of corneal biomechanics would be the ideal, as this would enable screening of corneas for biomechanical abnormalities and enable large volumes of in vivo data to be generated detailing the biomechanical effects of surgical procedures and associated risks, enabling the optimisation of current treatment approaches. In addition to this, due to the fact it is difficult to exactly replicate boundary conditions and maintain the hydration and structural qualities of the tissue post-mortem for ex vivo testing, it is not known whether data collected ex vivo closely mimics the in vivo response, and in vivo testing eliminates this problem and could provide validation for previous ex vivo methods.

Currently, evaluation of corneas for biomechanical abnormalities, such as those seen in keratoconus, is most commonly conducted using static measurement techniques that examine the topography of the cornea and changes that occur over long periods of time. In addition to this, suitability for corneal surgery is often decided based on static data such as corneal thickness, which, in reality, may not be as important as determining the strength of the tissue.
Due to the availability of a wide range of static measurement technologies including: the videokeratoscope, slit-lamp scanner, OCT pachymeter, ultrasound pachymeter, and Schiempflug camera, a wide variety of corneal shape parameters can now be accurately quantified including, anterior and posterior surface elevation and curvature, corneal thickness, and anterior chamber depth.

As the topography of the cornea is related to the biomechanics, variations in corneal topography away from the norm can be diagnostic of biomechanical abnormalities, but the static information alone does not provide enough data by which the biomechanical properties of the cornea can be determined. Also, it is likely biomechanical destabilisation of the cornea occurs before it manifests in a way that results in changes to normal topography or vision (86).

Ocular Response Analyser and Scheimpflug Tonometer

For the reasons discussed, there has been significant interest in the development of a tool capable of in vivo dynamic assessment. Currently only two in vivo devices that assess the dynamic response of the cornea are commercially available and these are the Ocular Response Analyser (ORA, Reichert Ophthalmic Instruments) and the Dynamic Scheimpflug Tonometer (Corvis ST, OCULUS Optikgeräte GmbH).

Both the ORA and the Corvis ST are non-contact tonometer's that measure the dynamic response of the cornea to an air puff, of duration between 25 milliseconds – 30 milliseconds, directed at the centre. The application of this air puff forces the cornea inwards, after which the force is removed and the cornea returns to its normal curvature. The ORA quantifies a parameter called corneal hysteresis (CH) by determining the pressure difference between inwards and outwards applanation (the point at which the centre of the cornea becomes flat), the larger the difference the greater the viscous component of the response. Corneal resistance factor (CRF) is another parameter measured by the ORA and is determined in the same way as CH except more weight is given to the inward motion as this is suggested to make the measured value independent of IOP (87).

In contrast to the ORA, which uses an electro-optical collimation detector to detect the movement of the corneal apex, Corvis ST uses high-speed imaging at 4330 frames/s to capture the deformation of the cornea, obtaining 140 frames over the 30 ms air-puff along a full cross-section, enabling the response to be fully resolved in both the time and spatial domain. This allows other parameters to be quantified, such as; applanation time, deformation amplitude, length of the flattened cornea, velocity of the cornea in response to the air puff, curvature at highest concavity and highest concavity time (88), among others.
Links between several of the parameters measured by the Corvis ST and specific diseases, such as keratoconus, have been suggested to exist, with Vinciguerra, et al \((89)\) recently defining the Corneal Biomechanical Index (CBI), which uses tomographic information in combination with specific dynamic parameters measured by the Corvis ST to diagnose keratoconus. Initial results have been promising with the study by Vinciguerra, et al \((89)\), reporting that the presence of keratoconus could be identified in over 90% of affected corneas, although it was not possible to stage the corneas in terms of disease severity.

There are several limitations of both the ORA and Corvis ST when it comes to quantifying useful biomechanical parameters of the cornea and changes to these parameters that occur in disease. For the ORA, the measured parameters including CH and CRF have found to be influenced by other factors including corneal thickness and IOP \((90,91)\), this is also true for the majority of the parameters measured by the Corvis ST and in many cases the exact details of how changes to measured parameters relate to disease or biomechanical abnormalities remains unknown with inconsistencies reported across studies \((35)\). The identification of the CBI appears to be a step in the right direction, however tests have so far only been successful in identifying established cases of keratoconus and it is not yet known if cases could be identified at the sub-clinical stage.

Ultimately, the information provided by the Corvis ST is not a measure of corneal biomechanics, as 3D spatial information is not available and the loading method used is only directed at the central cornea and forces the cornea outside its normal range of motion, hence the specific biomechanics of the full-surface cannot be determined. Instead what is measured are dynamic parameters that may be useful for the diagnosis of keratoconus. Outside of this, information is limited, for example, it could not be used to spatially map the presence of biomechanical abnormalities and this could prove to be problematic in cases where biomechanical abnormalities are regionally specific and means that the information obtained cannot be used to enable targeted and customised treatments.

**Ultrasound based techniques**

Several other techniques for in vivo assessment are currently in development. Tracking the propagation of shear waves using high speed ultra-sound is a technique that has been used on several organs including the liver \((92)\), to assess mechanical changes associated with disease. It works in a similar manner to OCT but instead of light, sound is used. Recently it was used on the cornea *in vivo* to assess mechanical anisotropy \((93)\). However the relationship of shear wave propagation to Young’s modulus is complex, as the shear wave speed depends on its propagation direction in relation to the fibre orientation \((93)\) which is known to be highly
variable throughout the cornea. In addition to fibre orientation, several other regionally variable material properties are known to influence shear wave propagation including hydration and density. For these reasons, it is difficult to establish how the measured parameters relate directly to corneal biomechanics, however it is possible that changes to shear wave propagation may occur in the presence of biomechanical abnormalities although this is yet to be investigated. So far, the reported values of Young’s moduli have been low when compared with other studies using alternative techniques, with Tanter, et al, reporting a Young’s modulus of 0.19MPa for porcine corneas (94). Another drawback to the technique is it requires contact with the cornea via a coupling fluid.

**Brillouin Microscopy**

Brillouin microscopy is a non-contact optical technique that has demonstrated potential for determining the 3D biomechanics of the cornea in vivo (95) with recent ex vivo studies also demonstrating that the technique can evaluate changes to the longitudinal modulus introduced by collagen crosslinking (96), and also identify the presence of keratoconus (97).

Brillouin light scattering is an inelastic form of light scattering that arises when monochromatic light incident on an object is scattered by the periodic fluctuations in density that occur as a result of thermally excited hyper-frequency soundwaves (98). This scattered light is shifted in frequency from the incident light by a specific amount dependent upon the interaction between incident light waves with the coherent longitudinal sound waves. Often in Brillouin optical microscopy a Fabry-Perot interferometer is used and the frequency shift is recorded via a high-resolution spectrometer.

The measured frequency shift is related to the complex longitudinal modulus of a material, also referred to as the Brillouin modulus. A direct relationship of Brillouin modulus to material stiffness or Young’s modulus does not exist and must be determined experimentally for individual tissues, this is because the Brillouin modulus is dependent on both density and refractive index, hence for materials with different densities or refractive indices a different relationship exists. This factor is problematic when using the technique to examine the cornea as these properties vary throughout the cornea, and are likely to change in disease states and after surgical procedures, making it difficult to determine if any change in biomechanics is independent of other factors.

Another issue with Brillouin Microscopy currently, is that data can only be obtained line by line, hence to obtain high resolution 3D-data, long scanning times in excess of 30 minutes are
required and this is not realistic for a clinical situation. Also signal-to-noise ratio is an issue due to elastic scattering.

**Dynamic videokeratoscopy**

For all the *in vivo* measurement techniques discussed so far, the measured data does not relate directly to corneal biomechanics as it is influenced by other factors, or requires certain assumptions to be made to relate it to the mechanical properties, hence there is still a lack of understanding as to how the cornea responds *in vivo* to normal pressure fluctuations.

With reference to this, most recently dynamic videokeratoscopy was attempted by Elsheikh, et al (21) to measure the topography changes in the cornea *in vivo* in response to an increase in IOP induced by applanation of the sclera using an ophthalmodynamometer. To achieve measurement across the whole cornea, including the important limbal zone, the measurement area of a normal videokeratoscope was increased by taking several images at slightly offset locations and correlating them post-measurement to produce a single larger topography map.

Via this method, it was possible to evaluate the full-field deformation of the corneal surface and show regional variations in the response. These regional variations were found to be similar to those that have been observed *ex vivo* (22) when digital image correlation has been used to assess whole globe inflation.

This testing method is a useful extension of an already well known static measurement technique, therefore ophthalmologists and clinicians already have good familiarity with the format of the data. However, due to the limited resolution of this technique when compared with HI, to generate a measurable change in shape, pressure had to be applied to the sclera to achieve an approximate doubling of normal IOP. The way this pressure was applied, via an ophthalmodynamometer pushing on the sclera, may have affected the response by inducing a change in the shape of the cornea different to that caused by a normal increase in IOP. In addition to this it is doubtful whether the resolution would be great enough to identify subclinical changes in biomechanics or biomechanical changes introduced by crosslinking. Also, since this technique measures surface deformation only further information would be required or assumptions would have to be made regarding any deformation throughout the depth of the cornea to quantify biomechanical parameters.

**Wavefront interferometry**

Dynamic corneal topography changes have also been assessed *in vivo* via the use of wavefront interferometry. In a study by Kasprzak, et al (99) a Twyman-Green interferometer was
used to measure the dynamic changes to corneal topography that occurred in response to the small changes in IOP that take place over the cardiac cycle. Measurement was achieved by examining the topography of the cornea with respect to a convex lens at 40 millisecond intervals, 1 second to 3 seconds after blinking. 40 millisecond intervals were chosen so the deformation of the cornea could be measured over a proportion of the total pressure change that occurred over one cardiac cycle while ensuring that total deformation did not exceed the maximum sensitivity of a few microns, and measurement was taken 1 second to 3 seconds after blinking to attempt to prevent irregularities in the tear film and tear film break-up from masking the desired deformation data. To determine the displacement that occurred over the measurement time topography maps taken over subsequent intervals were subtracted from one another. Data was obtained across the central 3 mm of the cornea only.

Some problems were experienced using this method due to low reflectivity, head and eye movements, and the break-up of the tear film. The main issue was that due to the presence of the tear film it was not possible to determine if the measured data represented the bulk movement of the surface, or if it was an artefact of changes to the tear film that occurred within the measurement time.

A summary of the measurement techniques discussed throughout this section is given in Table 2-2. The abbreviations D and ND refer to destructive and non-destructive, respectively, and indicates whether the cornea remains as part of the whole tissue/corneo-scleral button or is dissected into sections for testing.
<table>
<thead>
<tr>
<th>Measurement technique</th>
<th>Nature of tissue</th>
<th>Loading method</th>
<th>Full-field evaluation</th>
<th>Demonstrated capabilities for detecting biomechanical abnormalities or changes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strip extensometry</strong></td>
<td>ex vivo</td>
<td>Uniaxial</td>
<td>× - cornea dissected into strips</td>
<td>Yes – stiffness changes introduced by CXL have been measured⁵²</td>
</tr>
<tr>
<td></td>
<td>in vivo</td>
<td>tensile load</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Laser apical displacement tracking</strong></td>
<td>✓ (ND)</td>
<td>Hydrostatic</td>
<td>× - measurement at apex only</td>
<td>Yes – changes in corneal stiffness that occurs due to aging has been measured⁴⁴</td>
</tr>
<tr>
<td></td>
<td>Potentially - small movements may cause issues</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Digital image correlation</strong></td>
<td>✓ (ND)</td>
<td>Hydrostatic</td>
<td>✓ - full surface evaluation</td>
<td>Not yet demonstrated</td>
</tr>
<tr>
<td></td>
<td>Potentially - studies have so far not been reported</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Laser interferometry techniques</strong></td>
<td>✓ (ND)</td>
<td>Hydrostatic</td>
<td>✓ - full surface evaluation</td>
<td>Yes – Changes measured in response to an air puff using spectral-OCT⁸⁵</td>
</tr>
<tr>
<td></td>
<td>✓ - so far in vivo only been demonstrated with HI and WI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Optical coherence tomography</strong></td>
<td>✓ (ND)</td>
<td>Hydrostatic</td>
<td>✓ - 2D axial scans, 3D evaluation possible but successive scans required</td>
<td>Yes – Changes to CH and CRF are often seen in certain conditions but association is not yet clear</td>
</tr>
<tr>
<td></td>
<td>✓ - Only in some formats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ORA</strong></td>
<td>✓ (ND)</td>
<td>Air puff</td>
<td>× - centre only</td>
<td>Changes to measured parameters have been seen in certain conditions but association is not yet clear</td>
</tr>
<tr>
<td><strong>Corvis ST</strong></td>
<td>✓ (ND)</td>
<td>Air puff</td>
<td>× - 2D cross-sectional strip</td>
<td></td>
</tr>
<tr>
<td><strong>Ultrasound</strong></td>
<td>✓ (ND)</td>
<td>Shear wave</td>
<td>✓ - 3D evaluation possible</td>
<td>Yes - Changes to response have been measured after CXL¹⁰⁰</td>
</tr>
<tr>
<td><strong>Brillouin microscopy</strong></td>
<td>✓ (ND)</td>
<td>No pressure required</td>
<td>✓ - 3D evaluation possible except anterior most 70µm due to Fresnel reflection⁶⁵</td>
<td>Yes - Changes to response have been measured after CXL⁹⁶</td>
</tr>
<tr>
<td><strong>Dynamic videokeratoscopy</strong></td>
<td>✓ (ND)</td>
<td>IOP increase</td>
<td>✓ - full surface evaluation</td>
<td>Not yet demonstrated</td>
</tr>
</tbody>
</table>

Table 2-2 – Summary of measurement techniques that have been used to analyse corneal biomechanics.
2.8 Conclusions from background study

The research conducted during this background study highlighted that the biomechanics of the cornea are still not fully understood. Although a number of biomechanical parameters have been defined across studies, it remains to be determined how the cornea responds in vivo to IOP fluctuations and forces imposed by the intra-ocular muscles, or how these responses are modified in the event of disease or as a result of surgical intervention.

An understanding of corneal biomechanics is becoming increasingly important due to large numbers of people undergoing elective refractive surgery procedures and the concerns that these procedures potentially have a negative impact on biomechanics which could lead to problems such as ectasia in the long-term. In addition to this, new procedures, such as collagen crosslinking, have become available where it is possible to stiffen the cornea. Currently, little is known about the biomechanical impact of collagen crosslinking and how specific changes to biomechanics relate to changes in vision. If the effects could be quantified, its application could be optimised to improve visual outcomes in the treatment of keratoconus and ectasia and it may even have potential to be used as a minimally invasive alternative to refractive surgery in some cases, by changing the shape of the cornea through changing the stiffness of different regions in isolation.

Overall for the reasons outlined in this chapter, it was decided that the focus of this research would be on the development of a measurement technique to evaluate corneal biomechanics and the changes that can be introduced by collagen crosslinking.

Based on the results of previous studies it was evident that to measure the biomechanical properties of the cornea, and to truly understand how this translates in terms of in vivo behaviour, it is necessary to measure the response of the cornea to pressure changes that represent physiological pressure changes in both magnitude and direction. This requirement is due to the fact the cornea has evolved to deal with specific loads, and has been shown to exhibit non-linear stiffness, likely because of the fact different structural components of the tissue dominate the response at different pressures.

It was also identified that it is necessary to examine the response of the cornea in different regions, as the results from several previous studies, along with the structure of the cornea suggest that regional variations in mechanical properties exist. In addition to this, regional analysis is fundamental to understanding the specific implications of various surgical procedures and treatments such as collagen crosslinking and for the identification of biomechanical abnormalities.
Based on the information presented in this chapter, the essential and desirable capabilities of a measurement method to investigate corneal biomechanics with relevance to understanding \textit{in vivo} behaviour were summarised as follows:

**Essential:**
1. A technique capable of measuring the deformation response of the cornea to loads representative of those that occur physiologically.
2. A method whereby intact corneal tissue incorporating the limbal region can be evaluated.
3. A technique where by measured parameters relate directly to biomechanics.
4. A technique which enables regional variations in the mechanical response of the cornea to be evaluated, ideally in 3D but at least across the full surface.
5. A technique where the sensitivity is adequate to pick up subtle biomechanical abnormalities that are not evident through topographic examination.
6. A method where it is possible to evaluate individual corneas before and after introducing changes that may affect biomechanics i.e. CXL or surgical incisions.

**Desirable:**
1. A technique that has the potential for translation as a future clinical measurement tool.

Based on these requirements a measurement system specification was generated. This is shown in Table 2-3.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode of measurement</td>
<td>• Non-contact. • Non-destructive. • Full-field 3D load-displacement evaluation $\rightarrow$ minimum of full surface, ideally 3D.</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>• Minimum achievable displacement sensitivity &lt; 1 µm. • Low sensitivity to environmental noise.</td>
</tr>
<tr>
<td>Measurement range</td>
<td>• Ideal measurement range of approximately 0.5 µm - 10 µm</td>
</tr>
<tr>
<td>Spatial Resolution</td>
<td>• Imaging resolution of 0.1 mm or better.</td>
</tr>
<tr>
<td>Field of View</td>
<td>• Minimum circular region of diameter 20 mm.</td>
</tr>
<tr>
<td>Depth of Focus</td>
<td>• Minimum of 5 mm to account for corneal curvature.</td>
</tr>
<tr>
<td>Measurement Time</td>
<td>• Ideally &lt; 1 second for full-field measurement. • Ideally capture full-field data over a single loading cycle.</td>
</tr>
<tr>
<td>Repeatability</td>
<td>• High measurement repeatability, ideally +/- 10%.</td>
</tr>
<tr>
<td>Safety</td>
<td>• Eye safe. • Safe for use by untrained individual.</td>
</tr>
<tr>
<td>Data Format</td>
<td>• Data output in a format interpretable to clinicians and wider audience i.e. displacement data.</td>
</tr>
</tbody>
</table>

Table 2-3 – Specification for measurement technique to investigate corneal biomechanics.
With respect to these requirements as outlined in Table 2-3, the decision was made to further investigate the use of laser speckle interferometry techniques for corneal measurement. This is due to the fact laser speckle interferometry techniques have many of the features outlined in Table 2-3, including: the ability to carry out non-contact and non-destructive testing, high sensitivity, the ability to generate high resolution, spatially resolved surface data, the ability to provide real-time results and they also offer the convenience of being applicable to a wide variety of loading situations and object sizes. In addition to this previous ex vivo studies using ESPI and ESPSI have demonstrated the capability of the techniques to measure the response of the cornea to pressure changes within the physiological range, and to detect changes to biomechanics that occur due to age, refractive surgery and crosslinking.

The main drawback of laser speckle interferometric methods with respect to the requirements outlined in Table 2-3 is the fact it is not possible to evaluate deformation through the depth of the sample, but on the surface only, hence the Young’s modulus of the tissue cannot be calculated directly from the data. Further information is required regarding tissue deformation throughout the depth of the cornea to determine an accurate estimate for Young’s modulus from the measured data. However, since the anterior stroma is thought to contribute most significantly to corneal biomechanics and maintaining the overall shape of the cornea, investigating the deformation in this area is fundamental to understanding overall tissue biomechanics and the impact of treatments such as refractive surgery and corneal crosslinking which are thought to affect the biomechanics of this region.

2.9 Specific research aims and objectives

The primary aims of this study were as follows:

1. To develop a measurement technique using laser speckle interferometry capable of analysing full corneal surface deformation in response to loads representative of those that occur physiologically.
2. To design a suitable ex vivo loading rig to simulate pressure changes representative of those that occur physiologically.
3. To establish a suitable testing methodology to enable evaluation of the response of the corneal surface to loading and quantification of the changes to the response that occur after collagen crosslinking.
4. To investigate the sources of error associated with quantitative measurement.
5. To present the data in a format that is interpretable by a wide-ranging audience including clinicians, ophthalmologists, optometrists and engineers.
3. Speckle Interferometry – Applications, principles and associated errors

The purpose of this chapter is to introduce speckle interferometric measurement techniques. Research was conducted into speckle interferometry to evaluate the different techniques that are available, how they have previously been used, the measurement principles, and their respective capabilities and limitations. The purpose of this research was to identify a technique that would be most suitable for corneal measurement and to fulfil the aims as set out at the end of chapter 2.

3.1 Background

Electronic Speckle Pattern Interferometry (ESPI) and Electronic Speckle Pattern Shearing Interferometry (ESPSI), the latter of which is often referred to as shearography, are highly sensitive, non-destructive, full-field optical metrology techniques. As briefly introduced during chapter 2, ESPI techniques measure displacement by comparing the relative position of points on an object's surface before and after loading with that of a static reference, whereas ESPSI techniques can be used to measure changes to the relative displacement at two points on the surface of an object before and after loading.

Since their development, ESPI and shearography techniques have become popular measurement methods for non-destructive testing applications due to their whole-field, non-contact capabilities. Some of the main advantages of ESPI and ESPSI techniques include:

- Capable of resolving surface displacement or rate of displacement in 3D.
- Measurement can be achieved on objects across a whole range of sizes from a few millimetres to several metres.
- Measurement time is several milliseconds.
- Data can be viewed in real-time.
- Static, dynamic and transient loading conditions can be investigated and loading can be in many different formats i.e. thermal, mechanical, vibration.

Shearography measurement has some additional advantages over ESPI measurement. Since shearography is self-referencing, it is less sensitive to environmental noise and rigid body motion, enabling its usage within industrial environments; also, different types of sensitivity and ranges of sensitivity can be achieved using shearography via manipulating the magnitude and the way the shear is introduced between the two parts of the object wavefront.
Applications of ESPI and shearography

A large volume of literature is now available regarding the design and application of ESPI and shearography techniques and a wide variety of different optical configurations have been reported. Some of the main areas of application for ESPI and shearography techniques are introduced in this section along with some examples of how the techniques have been, or are being used across various industries, including the medical industry.

**Vibration analysis**

Speckle interferometric techniques are often used for vibration analysis. Vibration analysis has applications across many industries, it is useful during the design stage when creating components and structures so excitation of resonant frequencies in the working environment can be avoided and machine lifetime can be increased. It is also useful for evaluating the condition and efficiency of machinery, and predicting failure modes. ESPI and shearography are useful tools for the analysis of vibration modes of materials and structures as they can provide quantitative, full-field visualisation of the mode shapes\(^{101}\).

There are several examples of applications of ESPI and shearography in the automotive and aerospace industries in particular, where the techniques are used to examine different components of vehicles and aircrafts, with the aim to increase efficiency and reduce noise. For example, Farrant, et, al, reported the use of ESPI for vibration analysis of an engine\(^ {102}\), and Krupka, et al, used ESPI to examine break squeal, vibration of car body panels and catalytic converter fatigue\(^ {103}\), while Yang, et al reported the use of shearography to study the vibration of turbine blades\(^ {104}\).

**Thermal stress analysis**

Speckle interferometric techniques are also useful techniques for examining the thermal properties of materials. Often when materials are heated they expand, this expansion can be problematic in some instances if it results in significant deformation, this is especially true for composite materials or for parts composing several layers of different materials, where the relative expansions of the different component materials can vary, resulting in damage to the underlying structure. Using both ESPI and shearography it is possible to examine the thermal expansion of complex materials with high sensitivity in three-dimensions, and evaluate the stresses introduced due to expansion.

Some common applications include the examination of electronic components\(^ {105}\) including circuit boards and ball grid arrays. A recent application of ESPI within the medical field has
been the examination of the thermal expansion coefficients of teeth (106) and the stresses introduced due to the thermal expansion of different types of composite restorative filling materials (107).

Strain analysis

Strain analysis is a common application of speckle interferometry. It is useful to examine how structures respond to loading so potential failure modes can be predicted and designs can be optimised. However, sometimes it is difficult to predict how anisotropic or composite structures respond under loading. Hence, ESPI and shearography are useful techniques to measure displacement and strain on complex structures, as the deformation across the full area of interest can be measured simultaneously and with high resolution.

As biological materials and structures are often complex there has been recent interest in exploiting the strain analysis features of ESPI and shearography to determine the biomechanics of various body parts, by measuring their deformation in response to loads representative of those that occur in vivo. A number of studies on different body parts have been conducted. Tyrer, et al (108) used ESPI to examine the deformation of a human femur subjected to loads representative of those introduced by standing, and Petzing, et al (109), later followed this up by examining the impact of a prosthetic implant on the response. In addition to the studies on the femur and the studies on the cornea that were introduced in chapter 2, several studies have been conducted by different research groups using ESPI to examine the response of the human mandible to loading (110–112).

Defect Detection

The ability to detect defects in complex materials that cannot be identified by visual inspection is a useful feature of both ESPI and shearographic inspection. The presence of a defect or an area of non-homogeneity in a material will result in subtle changes to the material properties in the area of the defect, although evidence of the defect may not be visible from viewing the surface of the object, examples include delamination in composite materials and damage to honeycomb panels in sandwich structures. As these areas will respond slightly differently to loading they can be detected with high sensitivity and resolution via the use of ESPI and shearography techniques.

Generally, shearography is better suited to defect detection as it can highlight slight changes to the rate of displacement in specific areas and these subtle changes to the rate of displacement are sometimes lost when examining the overall displacement. Overall defect detection is the most popular industrial application of shearography and many applications
have been reported throughout the literature. It is worth noting that applications have generally been qualitative rather than quantitative.

Steinchen, et al (113), used shearography to examine composites used in the aerospace industry, identifying disbonds and microcracks in fibre-glass reinforced materials and honeycombed panels. The Royal National Lifeboat Institution use shearography to assess the quality of the composite material used within their lifeboats, with Cripps (114) stating that “…shearography offered the most effective means of resolving the manufacturing problems being experienced by the lifeboats.” Shearography is also used for the inspection of tyres (115) with several commercial instruments now available.

Some applications have also been reported in the medical industry. Sujatha and Murukeshan (116) used shearography for the inspection of tissue mimicking material (a mixture of latex free rubber and fibrous material) for defects. The same researchers also presented a shearographic endoscope for detection of abnormalities in the colon (117).

3.2 Measurement Principles

The principles of ESPI and shearographic measurement are the same, however the nature of the information obtained varies due to the type of reference employed. The common principles of measurement and data extraction are discussed here first, before the details of each of the individual techniques are described in the sections following this.

3.2.1 Speckle interference

ESPI and shearography are based around the phenomenon of speckle. Speckle occurs when a coherent light source is used to illuminate an optically rough surface, which can be defined as a surface where height variations exist that are greater than the wavelength of the illumination source. The light scattered from the objects surface has a random phase, its granular, "speckled” appearance is due to constructive and destructive interference of the scattered light waves viewed at a specific point by the eye.

Each speckle has a specific amplitude and phase that describes the surface of the illuminated object at a given point in time. If the object undergoes a displacement, the optical path length is changed in the areas where the surface has deformed, and this results in a change to the phase of light, resulting in changes to the intensity of the speckle pattern. Tracking the changes to speckle patterns which occur due to microscopic changes to an objects surface in response to a specific stimulus, is the basis for speckle interferometry measurement techniques.
To obtain useful information regarding the state of the object at a given time, light scattered from the object is captured by an imaging system and superimposed on a detector, commonly a charge-coupled device (CCD) or a complementary metal-oxide semiconductor (CMOS), with light scattered from a reference. In ESPI this reference wavefront remains static during loading, whereas in shearography the reference is generated from the wavefront reflected from the object and therefore moves during loading.

The total light received at the image plane is a sum of the light scattered from the object and the reference, and can be described by addition of the complex amplitudes as described by equations 3-1 and 3-2 for a point $P(x,y)$ in the image plane. Where $u_O(x,y)$ and $u_R(x,y)$ are the light amplitudes of the object and reference at point $P(x,y)$, and $\phi_O(x,y)$ and $\phi_R(x,y)$ are the phase angles of the object and reference at point $P(x,y)$ respectively.

$$U_O(x,y) = u_O(x,y)e^{i[\phi_O(x,y)]}$$  
Equation 3-1

$$U_R(x,y) = u_R(x,y)e^{i[\phi_R(x,y)]}$$  
Equation 3-2

The resulting light intensity at each point in the image plane is proportional to the square of the total light field, and can be described in terms of the intensity of the object ($I_o$) and reference ($I_r$) wavefronts and the phase difference between them via equation 3-3.

$$I = I_r + I_o + 2\sqrt{I_rI_o}\cos\Delta\phi$$  
Equation 3-3

Where,

$$\Delta\phi = \phi_R - \phi_O$$  
Equation 3-4

### 3.2.2 Subtraction Fringes

To obtain information regarding deformation, data must be captured when the object is in its initial reference/undeformed state and then again after it has deformed. If $I_A$ is the intensity distribution of the object in its reference state, and $I_B$ is the intensity distribution of the object in its deformed state, as described by equations 3-5 and 3-6, respectively.

$$I_A = I_r + I_o + 2\sqrt{I_rI_o}\cos\Delta\phi_A$$  
Equation 3-5
\[ I_B = I_r + I_o + 2\sqrt{I_r I_o} \cos \Delta \phi_B \]  

Equation 3-6

Where, the phase angle \( \Delta \phi_B \) is equivalent to the relative phase angle between the object and the reference with the object in its initial state (\( \Delta \phi_A \)), plus the phase change that has occurred as a result of deformation (\( \Delta \phi_{\text{def}} \)), as described by equation 3-7.

\[ \Delta \phi_B = \Delta \phi_A + \Delta \phi_{\text{def}} \]  

Equation 3-7

Then, if the intensity distribution recorded at the deformed state is digitally subtracted from the intensity distribution recorded at the undeformed state, the resulting intensity distribution can be represented mathematically by equation 3-8 and describes the deformation of the object that has occurred between the two states.

\[ I_A - I_B = 2\sqrt{I_r I_o} [\cos \Delta \phi_A - \cos \Delta \phi_B] \]  

Equation 3-8

Via the use of trigonometric identities this subtraction can be represented in a more useful format as shown in equation 3-9 \(^{(101)}\). When in this format it is evident that intensity \( I_A - I_B \) is a maximum (bright fringes) when \( \Delta \phi_{\text{def}} = (2n + 1)\pi \) and a minimum (black fringes) when \( \Delta \phi_{\text{def}} = 2n\pi \), where \( n \) is an integer.

\[ I_A - I_B = 4\sqrt{I_r I_o} \left[ \sin \left( \Delta \phi_A + \frac{1}{2} \Delta \phi_{\text{def}} \right) \sin \frac{1}{2} \Delta \phi_{\text{def}} \right] \]  

Equation 3-9

This resulting intensity distribution obtained via subtraction appears as a series of bright and dark interference fringes, that occur due to constructive and destructive interference and correspond to areas of equal phase change with respect to the sensitivity vector (\( \vec{k} \)) which is defined as the bisector of the angle between the illumination (\( \vec{i} \)) and observation (\( \vec{\hat{o}} \)) vectors \(^{(119)}\), this is shown diagrammatically in Figure 3-1.

![Diagram showing the sensitivity vector as a function of the illumination and observation vectors.](image1)

Figure 3-1 – Diagram showing the sensitivity vector as a function of the illumination and observation vectors.
In ESPI this phase change is proportional to displacement and in shearography this phase change is proportional to the relative displacement that has occurred between two points. Figure 3-2 shows a visual example of the bright and dark interference fringes obtained via the digital subtraction procedure \((I_A - I_B)\) described mathematically in equation 3-9. Where in this case, \(I_A\) is the intensity pattern captured using an ESPI configuration for a circular rubber sample clamped at the edges prior to loading and \(I_B\) is the intensity pattern captured after applying a small hydrostatic load. Since this subtraction process can be achieved digitally in real-time, it is possible to observe the development of the interference fringes during loading.

\[
I_A \quad I_B \quad I_A - I_B
\]

*Figure 3-2 – Visual representation of speckle subtraction procedure and resulting interference fringes.*

### 3.3 Data Extraction

#### 3.3.1 Phase Stepping

When the details of the optical measurement configuration are known, the distribution and quantity of interference fringes in the subtracted intensity distribution can give qualitative visual information as to how the object has deformed over the measurement time and for simple cases the magnitude of deformation can be estimated by counting fringes. However, to extract quantitative data and determine the magnitude and direction (positive or negative) of deformation, phase stepping can be implemented to extract the specific phase change that has occurred between the two object states.

**Temporal Phase stepping**

Phase stepping can be in the spatial or temporal domain. During temporal phase stepping several interferograms are captured in quick succession, with a known phase step introduced between the object beam and the reference beam in each case. This gives rise to several intensity distributions which can be described mathematically by equation 3-10 for \(n\) number of phase steps, where all components are equivalent except for the known phase step \((\phi_{PS})\).
Several different techniques can be used to introduce a temporal phase step. Some of the most commonly used techniques have previously been described by Creath (120), and are summarised in Figure 3-3. Methods include rotation of a half-wave plate, linear translation of a diffraction grating, tilting of a glass plate and linear translation of a mirror attached to a piezoelectric transducer (PZT). The latter of these is the most common technique employed, and involves mounting the mirror, directing either the signal reflected from the object or reference, to a PZT.

![Image of common methods](image)

*Figure 3-3 – Common methods employed to introduce a temporal phase step, as summarised by Creath. Reproduced from (120).*

Conventionally three or more phase steps are introduced. Since a larger number of phase steps requires more time, the decision regarding the number of phase steps to use is made based on the time over which the object and environment can be guaranteed to remain stable. Generally, if high stability of the object and measurement environment can be obtained, the number of phase steps is maximised as this reduces the sensitivity to errors in the phase step amount due to miscalibration.

The method of determining the phase change from the resultant data is dependent upon the number of interferograms captured. Table 3-1 gives a summary of the equations that can be used to determine the phase change from 3-step, 4-step and 5-step approaches and the magnitude of the phase step used in each case (121), several alternatives to these methods exist (122).
<table>
<thead>
<tr>
<th>No. of frames captured</th>
<th>Step size ($\phi_{PS}$)</th>
<th>Equation to determine phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>$\frac{2\pi}{3}$</td>
<td>$\Delta \phi = \tan^{-1}\sqrt{3} \frac{(I_3 - I_1)}{(2I_2 - I_1 - I_3)}$</td>
</tr>
<tr>
<td>4</td>
<td>$\frac{\pi}{2}$</td>
<td>$\Delta \phi = \tan^{-1} \frac{(I_4 - I_2)}{(I_4 - I_3)}$</td>
</tr>
<tr>
<td>5</td>
<td>$\frac{\pi}{2}$</td>
<td>$\Delta \phi = \tan^{-1} \frac{2(I_2 - I_4)}{(2I_3 - I_5 - I_1)}$</td>
</tr>
</tbody>
</table>

Table 3-1 – Summary of 3-step, 4-step, and 5-step approaches to phase stepping.

Spatial phase stepping

When using spatial phase shifting techniques, all the information is collected in one frame, this type of phase stepping is useful for the measurement of dynamic events where the object does not remain stable at the time of measurement. During spatial phase shifting the interferograms must be spatially shifted by a specific amount with respect to each other. This shift can be introduced via several different methods including: the use of rotational polarising components, diffraction gratings or computer generated diffractive optical elements (123). To image the data, several different cameras can be used or the phase stepped images can be recorded across different regions of the detector, however this results in reduced spatial resolution. A problem common to both the imaging techniques is that due to the variable imaging position slight variations in the sensitivity vector are introduced (123).

Spatial carrier method

The spatial carrier method is an alternative form of spatial phase stepping. In this method a phase step is introduced between adjacent pixels via the introduction of carrier fringes (124), these carrier fringes can be introduced by tilting one of the wavefronts between exposures (124). The requirement for the spatial carrier method is that the speckle size is a minimum of three pixels in the direction of tilt (125) so that the wavefront may be considered flat across the area over which the phase shift is introduced. Once obtained, Fast Fourier transform can be used to isolate and calculate the phase distribution (121), or similar algorithms can be used as for temporal phase stepping for each set of pixels in the direction of the carrier fringes.

The majority of interferometers described in the literature use temporal phase stepping, this is due to the fact for most situations temporal phase stepping has a number of advantages over spatial. With temporal phase stepping, greater spatial resolution can be achieved as the speckle size can be smaller (125), and since the speckle size is governed by the numerical aperture of the imaging system, this factor in turn leads to another advantage, because it allows the aperture to be opened wider, it enables adequate image intensity to be achieved under conditions of lower laser power or low levels of backscatter.
Overall, temporal phase shifting tends to give more accurate results with better spatial resolution and less noisy fringe patterns in conditions where the object remains relatively stable at the point of reference and after loading\(^\text{(125)}\). However, for situations where the object is undergoing constant dynamic displacements, or there is significant environmental noise, spatial phase shifting methods may offer a better solution as all the data is collected simultaneously and deformation does not occur over the time of data capture.

When considering the most suitable method of phase stepping for corneal measurement several factors must be considered. \textit{In vivo}, the cornea undergoes continuous deformations as a result of the dynamic pressure fluctuations that occur during the cardiac cycle, and in addition to this, movements are likely due to blinking and head and eye movements, therefore a spatial phase stepping technique would likely be required to enable instantaneous data capture to avoid movement over the measurement time. However, since it is also important to minimise the laser power to be within the maximum permissible exposure, spatial techniques may present problems due to the requirement for a small aperture. If only \textit{ex vivo} measurement is considered, temporal phase shifting is likely to be suitable as pressure changes can be timed and tightly controlled, and experiments can be conducted in laboratory conditions, minimising the influence of external disturbances.

### 3.3.2 Phase Unwrapping

The phase-stepped data is processed and the resulting outcome is a wrapped phase map of values between \(-\pi\) and \(\pi\) radians describing each fringe, the data is also filtered to remove high frequency noise\(^\text{(121)}\). After filtering, phase unwrapping is then used to remove the discontinuities that occur due to the asymptotic nature of the arctangent function\(^\text{(126)}\), resulting in a continuous phase map containing the total value of \(\Delta \phi_{\text{def}}\) at each pixel. A diagrammatic example of the unwrapping process is shown in Figure 3-4.

Many different algorithms for phase unwrapping have been reported. The simplest case of one-dimensional phase unwrapping involves scanning pixels along a given line of the wrapped image until a fringe boundary is reached, at this point the phase difference between adjacent pixels is approaching \(2\pi\) as shown in the line plot taken across a section of the wrapped data shown in Figure 3-4, hence these fringe boundaries can be identified via thresholding. Once identified, the phase jump can be removed by adding or subtracting \(2\pi\) from the phase from this point so it follows continuously from the previous pixel. This process is repeated for every line in the wrapped data to produce a two-dimensional unwrapped dataset.
Simple unwrapping methods, such as the one-dimensional method described, work well if the data is completely free of noise and the fringes are well defined. However, this is rarely the case, especially in speckle interferometry, where data quality is affected by issues such as; local variations in signal to noise ratio, discontinuities in wrapped phase maps and speckle decorrelation which contribute to path-dependent error propagation (127). For this reason, specific algorithms have been developed to minimise the effects of these issues and prevent error propagation caused by areas of low data quality.

A common method used in speckle interferometry is to implement a data-reliability weighting system, there are a number of variations on this method, but here the process developed by Arevalillo-Herráez, et al (128) is briefly described as this was the algorithm used in the experiments detailed within this thesis. In this instance, the value at each pixel in the wrapped data is compared with the value at pixels immediately adjacent, a weighting is then determined at the four edges of each pixel based upon the second difference of values between each pixel and its neighbours, where those with the least difference (likely to correspond to the areas of high quality data) are given the highest weighting so they are processed first. The unwrapping path is therefore determined based on unwrapping the highest quality pixels first and each pixel based on the edge which is deemed to have the highest reliability and hence the highest weighting. This method has demonstrated robustness for dealing with noisy and complex data sets containing discrete areas of low data quality (128) and has a relatively short data processing time of a few seconds for an image size of 1296 by 972 pixels and therefore it was selected as a suitable unwrapping procedure for the experiments detailed in this thesis.
3.4 ESPI

An example of a common ESPI configuration is given in Figure 3-5. Coherent light from a laser is expanded and collimated and directed to a beamsplitter (BS1) where part of the light is used to form a reference beam and the other part is used to form an object beam. The object beam is directed towards the surface of interest at an angle $\theta_{xz}$, light scattered from the surface is combined with light from the reference beam via a beamsplitter (BS2) and the combined light is imaged through a lens and an aperture onto a detector. An image is captured when the object is in its initial state and stored, then as the object is loaded, the intensity distributions received at the detector are digitally subtracted in real time from the reference image so the development of the interference fringes can be viewed in real time.

![Figure 3-5 – Typical ESPI configuration.](image)

3.4.1 Interpretation of ESPI data

The phase change in ESPI occurs due to the change in the optical path length from a point on the surface of an object in its initial state to the same point on the surface of the object in its deformed state. This is illustrated in Figure 3-6, where P represents a point on the surface of the object in its initial state with coordinates $x, y, z$ with respect to the position of the detector and $P'$ represents the same point on the surface of the object in its deformed state with coordinates $(x + u), (y + v), (z + w)$ with respect to the position of the detector, where $u, v, w$ are the components of horizontal in-plane, vertical in-plane and out-of-plane displacement with respect to the image plane.
If illumination is in the $xz$ plane as shown in Figure 3-6, the optical path length at point $P$ for the object in its initial state can be described by $(SPD)$, where $S$ and $D$ represent the position of the source and the detector respectively. The optical path length for the same point after the object has deformed can be described by $(SP'D)$, hence the difference in the optical path length ($\Delta L$) can be represented by equation 3-11, where $\theta_{xz}$ is the angle between the illumination and observation directions.

$$\Delta L = u \sin \theta_{xz} + w(1 + \cos \theta_{xz})$$  \hspace{1cm} \text{Equation 3-11}$$

The resulting phase change can therefore be represented by equation 3-12, and is a function of the displacement in the horizontal in-plane and out-of-plane directions and the wavelength ($\lambda$) of the illumination source $^{(101)}$.

$$\Delta \phi_{def} = \frac{2\pi}{\lambda} [u \sin \theta_{xz} + w(1 + \cos \theta_{xz})]$$  \hspace{1cm} \text{Equation 3-12}$$

If illumination is in the $yz$ plane then the change in optical path length is measured with respect to the vertical in-plane and out-of-plane directions, and the resulting phase change is a function of the vertical in-plane and out-of-plane displacement and the wavelength of the illumination source as shown in equation 3-13.

$$\Delta \phi_{def} = \frac{2\pi}{\lambda} [v \sin \theta_{yz} + w(1 + \cos \theta_{yz})]$$  \hspace{1cm} \text{Equation 3-13}$$
If the direction of illumination is at an angle with respect to the viewing plane the total measured phase change contains contributions from both in-plane and out-of-plane components of displacement. To determine the deformation that has occurred in each of these planes the displacement components must be separated, this can be achieved via manipulation of the optical set-up.

**Out-of-plane sensitivity**

If the illumination is set normal to the objects surface ($\theta = 0^\circ$) then there is no sensitivity to in-plane deformation as $\sin(0^\circ) = 0$. The resulting phase change is a function of $\lambda$ and $w$ only, as described by equation 3-14. In this case, each interference fringe in the subtracted intensity distribution represents a total out-of-plane displacement ($w$) of $\frac{\lambda}{2}$, an example is shown in Figure 3-7, where the response of a circular plate to a point load at the centre has been measured using an out-of-plane ESPI configuration where the wavelength of the illumination source was 532 nm.

$$\Delta \phi_{def} = \frac{4\pi}{\lambda} w$$  \hspace{1cm} \text{Equation 3-14}

*Figure 3-7 – Diagram demonstrating how fringes relate to out-of-plane displacement when illumination is set normal to the viewing plane.*
**In-plane sensitivity**

To determine the in-plane component, the optical configuration can be designed so the components of in-plane displacement can be isolated, this can be achieved in a number of ways, commonly the object is illuminated from equal and opposite angles simultaneously \(^{101}\) as shown diagrammatically in Figure 3-8.

![In-plane ESPI configuration diagram](image)

*Figure 3-8 – Schematic of an in-plane ESPI configuration using simultaneous illumination from equal and opposite angles.*

In this case, the change in the optical path length with respect to each of the illumination sources \(S_1\) and \(S_2\) can be described by equations 3-15 and 3-16 respectively:

\[
\Delta L_1 = u \sin \theta_{xz} + w(1 + \cos \theta_{xz}) \quad \text{Equation 3-15}
\]
\[
\Delta L_2 = u \sin -\theta_{xz} + w(1 + \cos -\theta_{xz}) \quad \text{Equation 3-16}
\]

The resulting change in the optical path length is a function of both, such that the resulting phase change can be described by equation 3-17 \(^{101}\).

\[
\Delta \phi_{def} = \frac{2\pi}{\lambda} (\Delta L_1 - \Delta L_2) \quad \text{Equation 3-17}
\]

Due to the fact illumination is from equal and opposite angles \(\cos (-\theta) = \cos(\theta)\) and \(\sin(-\theta) = -\sin(\theta)\). The out-of-plane components cancel each other out and the resulting phase change is due to horizontal in-plane displacement only, as described in equation 3-18.
The same procedure can be carried out with equal and opposite illumination in the $yz$ plane to achieve sensitivity to vertical in-plane displacement. In this instance, the phase change would be described by equation 3-19.

\[
\Delta \phi_{def} = \frac{4\pi}{\lambda} u \sin \theta_{xz}
\]

\text{Equation 3-18}

\[
\Delta \phi_{def} = \frac{4\pi}{\lambda} v \sin \theta_{yz}
\]

\text{Equation 3-19}

A second option to isolate the in-plane component is to illuminate the object with equal and opposite angles individually while interfering the signal from the object with that from a reference beam, Wang, et al, described an interferometer based on this principle that used four separate illumination angles to obtain three-dimensional sensitivity\(^{(129)}\).

In this case two sets of data must be captured, either in quick succession or over two measurement cycles and the data must be subtracted to obtain the in-plane component. The in-plane fringes cannot be viewed directly during measurement and high stability of the object and environment is required so the data captured to each point in time represents the same movement. The main advantage to this method is that it provides the ability to simultaneously measure the out-of-plane deformation, as addition of the data recorded at each of the illumination angles isolates the out-of-plane component and this grants the ability to reduce the sensitivity to the out-of-plane component, which can be beneficial in some situations.

3.5 Shearography

As previously discussed, in shearography the object is used as its own reference, this is achieved by splitting the signal from the object into two equal parts, prior to it reaching the detector, and transforming one part of the signal in a specific way so two points on the objects surface separated by an arbitrary distance interfere at one position on the detector. The intensity distribution of the object in its initial state is therefore governed by the optical path difference of the light scattered from each of the interfered positions of the objects surface.

Unlike in ESPI where the reference remains static, in shearography, when the object deforms both the contributing light fields at each point on the detector can undergo a phase change due to a change in the optical path length and therefore the resulting interferogram obtained via subtraction describes the relative displacement that has occurred between each of the interfered points.
### 3.5.1 Types of shearing interferometry

The way in which the signal from the object is sheared prior to reaching the detector governs the overall direction and magnitude of sensitivity of the device. A summary of some of the different ways the two parts of the object wavefront can be sheared with respect to one another are given in Table 3-2 \(^{(130)}\).

<table>
<thead>
<tr>
<th>Type of shear</th>
<th>Method of application</th>
<th>Sensitivity</th>
<th>Diagram</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horizontal lateral</td>
<td>Laterally shift one part of the wavefront with respect to the other in the x-axis</td>
<td>Relative displacement between two points separated by a distance (\delta x) in the x-axis</td>
<td><img src="dx" alt="Diagram" /></td>
</tr>
<tr>
<td>Vertical lateral</td>
<td>Laterally shift one part of the wavefront with respect to the other in the y-axis</td>
<td>Relative displacement between two points separated by a distance (\delta y) in the y-axis</td>
<td><img src="dy" alt="Diagram" /></td>
</tr>
<tr>
<td>Radial</td>
<td>Expand or shrink one wavefront with respect to the other about the centre point</td>
<td>Relative displacement between points separated by a distance ((r - \mu r)) radially from the centre. Where (r) is radial distance from the centre and (\mu) is the expansion of one wavefront with respect to the other</td>
<td><img src="dr" alt="Diagram" /></td>
</tr>
</tbody>
</table>

**Table 3-2 – Summary of different types of shear.**

**Radial shearing interferometry**

Within the literature there is currently only one research group reporting the use of speckle shearographic methods to examine the corneal biomechanics. In this work Knox-Cartwright, et al, used out-of-plane radial shearing interferometry (RSI) to examine the biomechanics of the cornea and quantify age related stiffening \(^{(45)}\), the effects of different cutting depths and profiles on corneal strain distribution \(^{(73)}\) and the stiffening effect of cross-linking \(^{(51)}\).

With out-of-plane RSI, shear is introduced by expanding or shrinking one of the wavefronts with respect to the other. The measured phase change is related to \(\frac{\partial w}{\partial r}\) which approximates a
change in the surface steepness measured radially from the centre. The relationship of $\frac{\partial w}{\partial r}$ to the measured phase change ($\Delta \phi_{def}$) can be described mathematically by equation 3-20 \(^{(45)}\),

Where $r$ is the radial distance from the shear centre and $\mu$ is the shear ratio.

$$\frac{\partial w}{\partial r} = \frac{\lambda \cdot \Delta \phi_{def}}{4\pi (r - \mu r)}$$

Equation 3-20

It is assumed that radial shearography was used by Knox-Cartwright, et al, because the cornea would be expected to deform in all directions in response to a change in hydrostatic pressure, and by using radial shearing interferometry it would be possible to evaluate the change in surface steepness in all radial directions from the data obtained during one measurement cycle. However, there are several disadvantages to the RSI method in comparison to lateral shearing interferometry, these include:

- **Zero sensitivity at the centre** - Since the wavefront is expanded about the centre of the object, the magnitude of shear at the centre is zero resulting in zero sensitivity. This is not a problem when examining an object that deforms isotopically in all directions as the rate of change of the out-of-plane displacement would be zero, but this cannot be presumed to be the case for the cornea which is anisotropic.

- **Variable sensitivity across the object** – The magnitude of shear increases from zero at the centre to maximum at the edge. This results in minimal sensitivity towards the central areas when compared with the edges, this effect is further exacerbated when measuring a curved object such as the cornea as the slope of the surface towards the outer areas effectively increases the shear distance between points even more. This effect could lead to a mismatch in the pressure changes required to generate high quality data at the edges and towards the central areas and issues with the interpretation of interferograms. Also as displacement is only measured in the radial direction the full deformation of the surface cannot be resolved with respect to all directions.

- **Complex interferograms** – The non-uniformity in the magnitude and direction of sensitivity across the object makes the interferograms difficult to interpret directly. Therefore, the raw data requires significant further processing before it can be of any use. The lack of sensitivity at the centre also means the data cannot be fully integrated to obtain the corresponding displacement derivative.
Lateral Shearing Interferometry

Unlike radial shearing interferometry, lateral shearing interferometry (LSI) can provide uniform sensitivity with respect to the direction of shear. The direction of sensitivity can easily be manipulated in LSI by changing the direction of shear or the orientation of the test piece with respect to the imaging system, therefore by using LSI with the shear applied both in the horizontal and vertical axis sequentially it is possible to obtain all the displacement derivative components that describe the deformation of the surface during loading.

Lateral shear can be introduced via several different methods, with a large variety of optical configurations described within the literature. Commonly a Michelson type arrangement is used, an example of which is shown diagrammatically in Figure 3-9. In this arrangement, the object wavefront is split 50/50 by a beam splitter, with one half of the wavefront reflected from a plane mirror (M4), and the other part of the wavefront reflected from a mirror (M5) tilted at a slight angle to introduced the required amount of shear.

![Figure 3-9 – Schematic of a Michelson lateral shearing interferometer configuration.](image)

The Michelson arrangement is popular due to the fact it is simple to implement, the shear magnitude can be easily adjusted, and temporal phase stepping can be conveniently incorporated by attaching one of the mirrors directing one part of the object wavefront to a PZT. However, there are some drawbacks to the Michelson interferometer, it is not very light efficient
as 50% of the light scattered from the object is lost due to the dual pass through the beamsplitter \(^{(121)}\), and since the shear is introduced via rotation of the mirror there is a slight variation in the magnitude of shear introduced across the object, which can be significant if the shear magnitude and the imaged area are large.

Other methods of introducing shear described in the literature include the use of parallel plates, where part of the wavefront is reflected from the front surface and part of the wavefront is reflected from the back surface of the plate \(^{(130)}\). A variation on this is to use a pair of glass plates with partially reflective coatings separated by an air gap, as described by Mihaylova, et al \(^{(131)}\), as the size of the air gap can be adjusted to control the amount of shear.

Another common technique is to use birefringent materials, examples include the Wollaston prism or Savart plate \(^{(132)}\). In this case, the incoming, linearly polarised, light is separated into two orthogonally polarised wavefronts, these wavefronts take slightly different paths through the material due to the variation in the effective refractive index at each of the polarisation states, and hence a lateral shear is introduced.

Another option is to use diffraction gratings that transmit only zeroth and first order beams. Zhao and Chung \(^{(133)}\) presented an interesting interferometer, where a spatial light modulator was used to display a grating that introduced the shear. The benefit to their configuration was that phase shifting could be introduced by shifting the grating between frames, as this was electronically controlled, the common errors associated with phase shifting techniques that involve moving parts, including miscalibration and thermal drift \(^{(121)}\), were avoided.

3.5.2 Interpretation of LSI data

The phase change that occurs in LSI is a result of the relative difference in the change in optical path lengths that have occurred at two points on the surface of an object separated by an arbitrary shear distance. With respect to the illustration shown in Figure 3-10, if points \(P_1\) and \(P_2\) separated by the shear distance \(\delta x\) are illuminated at an angle \(\theta_{xz}\) and interfere at one point on the detector before and after deformation, the total phase change can be described by equation 3-21, where \(\Delta L_1\) and \(\Delta L_2\) are the change in the optical path lengths at points \(P_1\) and \(P_2\) respectively.

\[
\Delta \phi_{def} = \frac{2\pi}{\lambda} (\Delta L_2 - \Delta L_1)
\]

\[
= \frac{2\pi}{\lambda} [(u_2 - u_1) \sin \theta_{xz} + (w_2 - w_1)(1 + \cos \theta_{xz})]
\]

Equation 3-21
Hence, the phase change can be described as a function of the rate of change in the displacement components per unit length, multiplied by the shear magnitude in the direction of shear as shown in equation 3-22

\[
\Delta \phi_{def} = \frac{2\pi}{\lambda} \left[ \frac{\partial u}{\partial x} \delta x \sin \theta_{xz} + \frac{\partial w}{\partial x} \delta x (1 + \cos \theta_{xz}) \right]
\]

Equation 3-22

If shear is applied in the vertical axis and illumination remains in the \(xz\) plane, the phase change can be described by equation 3-23.

\[
\Delta \phi_{def} = \frac{2\pi}{\lambda} \left[ \frac{\partial u}{\partial y} \delta y \sin \theta_{xz} + \frac{\partial w}{\partial y} \delta y (1 + \cos \theta_{xz}) \right]
\]

Equation 3-23

\[\Delta \phi_{def} = \frac{2\pi}{\lambda} \left[ \frac{\partial u}{\partial y} \delta y \sin \theta_{yz} + \frac{\partial w}{\partial y} \delta y (1 + \cos \theta_{yz}) \right]
\]

Equation 3-24

\[\Delta \phi_{def} = \frac{2\pi}{\lambda} \left[ \frac{\partial v}{\partial x} \delta x \sin \theta_{yz} + \frac{\partial w}{\partial x} \delta x (1 + \cos \theta_{yz}) \right]
\]

Equation 3-25

Figure 3-10 – Diagram showing the deformation of points \(P_1\) and \(P_2\) separated by shear distance \(\delta x\).

If illumination is in the \(yz\) plane, the resulting phase change for the cases of horizontal and vertical shear can be represented by equations 3-24 and 3-25 respectively.
For the case of a flat object where the surface is positioned normal to the viewing direction the in-plane components $\frac{\partial u}{\partial x}$ and $\frac{\partial v}{\partial y}$ relate to the strain components $\varepsilon_{xx}$ and $\varepsilon_{yy}$ in the $x$ and $y$ directions respectively. The out-of-plane components $\frac{\partial w}{\partial x}$ and $\frac{\partial w}{\partial y}$ relate to changes to the slope of the surface in the $x$ and $y$ directions respectively, and their corresponding derivatives $\frac{\partial^2 w}{\partial x^2}$ and $\frac{\partial^2 w}{\partial y^2}$ relate to flexural strain. Finally, $\frac{\partial v}{\partial x}$ and $\frac{\partial u}{\partial y}$ relate to rotational aspects of deformation and addition of these components gives the in-plane shear strain $\gamma_{xy}$ at a given point. Therefore, it is possible to define all the components of surface strain via LSI.

To obtain information regarding the magnitude of each of the displacement derivative components they must be isolated. This can be achieved in a similar manner to that detailed for the ESPI case.

**Out-of-plane LSI**

To isolate the derivatives of out-of-plane displacement, normal illumination and observation can be used, in this case the phase change can be described by equations 3-26 and 3-27 for horizontal and vertical shear respectively.

$$\Delta \phi_{def} = \frac{4\pi}{\lambda} \frac{\partial w}{\partial x} \delta x$$  
Equation 3-26

$$\Delta \phi_{def} = \frac{4\pi}{\lambda} \frac{\partial w}{\partial y} \delta y$$  
Equation 3-27

A comparison of the data that is obtained when using out-of-plane ESPI and out-of-plane horizontal LSI to measure the deformation of a flat circular rubber sample clamped around the edges and subjected to an increase in hydrostatic pressure is given in Table 3-3.

A further example is given in Table 3-4, to demonstrate the advantages of shearography when the aim is to identify defects. In this table, the ESPI and horizontal LSI wrapped and unwrapped data recorded on a piece of rubber with a superficial cut running from top-right to bottom-left as it responded to an increase in hydrostatic pressure is shown. As can be seen from viewing the data in Table 3-4 the position of the superficial cut is not as easily identified from the ESPI data as it is in the LSI data where it introduces a clear distortion to the wrapped fringes and is more obvious on the unwrapped phase map.
<table>
<thead>
<tr>
<th>Measurement method</th>
<th>Wrapped data</th>
<th>Line plot of displacement component relating to fringe distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESPI</td>
<td><img src="image1" alt="Wrapped fringes" /></td>
<td><img src="image2" alt="Line plot" /></td>
</tr>
<tr>
<td>LSI (Horizontal shear)</td>
<td><img src="image3" alt="Wrapped fringes" /></td>
<td><img src="image4" alt="Line plot" /></td>
</tr>
</tbody>
</table>

Table 3-3 – Comparison of the data obtained when measuring the deformation of a flat rubber sample with fixed boundaries under an increase in hydrostatic pressure using ESPI and LSI.

<table>
<thead>
<tr>
<th>Wrapped fringes</th>
<th>Unwrapped phase map</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image5" alt="Wrapped fringes" /></td>
<td><img src="image6" alt="Unwrapped phase map" /></td>
</tr>
<tr>
<td><img src="image7" alt="Wrapped fringes" /></td>
<td><img src="image8" alt="Unwrapped phase map" /></td>
</tr>
</tbody>
</table>

Table 3-4 – Comparison of ESPI and horizontal LSI data obtained when measuring the response of a flat rubber sample with a superficial cut introduced diagonally from top right to bottom left to an increase in hydrostatic pressure.
**In-plane LSI**

In the case of LSI, it is not possible to isolate the in-plane components while illuminating the surface at equal and opposite angles simultaneously, as previously described for ESPI, as the superposition of the ESPI fringes that form due to the illumination from two beams results in the shearing fringes being of poor contrast and generally unresolvable\(^{(121)}\). Instead, the most commonly used method to determine the in-plane component for LSI is to record data in quick succession or over two measurement cycles while illuminating at equal and opposite illumination angles in turn. Then isolating the in-plane and out-of-plane components of deformation through subtraction of the resulting phase data. The phase change obtained via subtraction of the data obtained in the two interferograms can be described by equations 3-28 and 3-29 for illumination in the \(xz\) plane with horizontal and vertical shear respectively, and equations 3-30 and 3-31 for illumination in the \(yz\) plane with horizontal and vertical shear respectively.

\[
\Delta \phi_{def} = \frac{4\pi}{\lambda} \frac{\partial u}{\partial x} \delta x \sin \theta_{xz} \quad \text{Equation 3-28}
\]

\[
\Delta \phi_{def} = \frac{4\pi}{\lambda} \frac{\partial u}{\partial y} \delta y \sin \theta_{xz} \quad \text{Equation 3-29}
\]

\[
\Delta \phi_{def} = \frac{4\pi}{\lambda} \frac{\partial v}{\partial x} \delta x \sin \theta_{yz} \quad \text{Equation 3-30}
\]

\[
\Delta \phi_{def} = \frac{4\pi}{\lambda} \frac{\partial v}{\partial y} \delta y \sin \theta_{yz} \quad \text{Equation 3-31}
\]

The advantages to this method are that the out-of-plane components can be obtained simultaneously via addition of the data obtained across the two measurement cycles, and since the angle of illumination can be altered this method grants the ability to alter the sensitivity to both the in-plane and out-of-plane deformation components. The disadvantages to this method are that in-plane fringes cannot be viewed directly, and the object must either be stable for some time at each load condition, or the response to loading must be repeatable.

Several other methods for three-dimensional shearography have been proposed in literature. Groves, et al, described a system where the deformation of the object was recorded while illuminating from three different illumination angles sequentially\(^{(135)}\), as the illumination angles did not lie in either the \(xz\) or \(yz\) planes, the resulting phase change was due to movement in all three planes and the individual components could be determined by analysing the results recorded at each of the illumination angles. Anisimov and Groves\(^{(136)}\), and Goto and Groves
described systems that used one illumination direction with three and four separate interferometers respectively, positioned at different locations with respect to the objects surface. This method is particularly prone to errors as complex matrix transformations are required to determine the displacement derivative components.

Overall, shearography offers several advantages over ESPI; it is often better for highlighting defects, offers the opportunity for measurement in an industrial environment, and gives greater flexibility and control over the measurement sensitivity. Despite these advantages, interpretation of the shearography data is less intuitive than for ESPI, it is therefore useful to obtain information regarding both the displacement and the rate of change of displacement. Displacement can either be quantified from data obtained using ESPI or it can be quantified from the LSI data via integration with respect to the direction of applied shear, the latter is more convenient for a wider variety of measurement situations due to the greater stability of LSI methods however, when considering accuracy of measurement, LSI has some drawbacks when compared to ESPI.

In the case of ESPI, for the ideal case where it is assumed the measured phase data represents the phase change that has occurred due to change in optical path length of light scattered from the object, the accuracy of calculated displacement depends upon the accuracy by which the wavelength of the illumination source and the angle of illumination can be defined. However, in the equivalent case when using LSI, the accuracy of the calculated displacement gradient depends not only upon the accuracy by which the wavelength of the illumination source and the angle of illumination can be defined, but also on the magnitude of shear amount, the accuracy of the quantification of the shear magnitude and the nature of deformation, which are discussed in detail in the following section. Overall these factors mean that for LSI, the measured displacement derivatives are only an approximation of the true displacement derivatives, even for the ideal case, and the accuracy of this approximation varies dependent upon the aforementioned factors.

The following section discusses the main sources of error associated with the use of ESPI and LSI, some of them are common to both methods and others are a concern in LSI only. The latter part of this section discusses the challenges associated with measurement of non-flat surfaces and the potential for errors in interpretation of the measured data.
3.6 Errors Associated with Quantitative Analysis

Errors can come from a variety of sources when using speckle interferometry techniques, for example; phase stepping calibration errors, misalignment errors, image distortion errors, speckle decorrelation effects, external disturbances, rigid body motion or test object instability, shearing errors, errors due to the illumination source, and errors introduced via the filtering and unwrapping algorithms. Some of these errors are easy to control and quantify whereas others can be more challenging to control and somewhat unavoidable.

The main error sources related to interferometry and described in the literature have been summarised in Table 3-5. The causes of the specific errors are described along with a summary of their effect on the measured results. Methods that can be used to overcome these errors have been listed and the error sources have been shaded as green, orange or red.

Green error sources relate to those that are easy to identify as they result in speckle decorrelation or obvious disruption to the measured data and they can be eliminated or minimised without changes to the optical configuration. The orange colour relates to error sources that are easy to identify but require changes to the optical configuration to eliminate and minimize, or they are more difficult to identify but relatively easy to check for and eliminate or minimise. Whereas the error sources coloured red can be difficult to quantify as there are several contributing factors, and addressing these sources can require more complex analysis or methods. Some of the more significant errors referred to in the table and the specific techniques that can be employed to overcome them, minimise them or assess their influence have been described in more detail within this section.
<table>
<thead>
<tr>
<th>Error source</th>
<th>Causes</th>
<th>Effects and magnitude of error</th>
<th>Potential methods to eliminate or minimise errors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental disturbance</td>
<td>Temperature variations, external vibrations.</td>
<td>In ESPI fringes are introduced that are unrelated to loading. Can cause decorrelation and errors in measured phase. ESPI is relatively resilient as object is used as its own reference.</td>
<td>Minimise external disturbances. ESPI work can be done in laboratory environment. Filtering and averaging can be used in some cases. If work is outside of a laboratory ESPI can be used. Pulsed laser systems can be used to reduce noise.</td>
<td></td>
</tr>
<tr>
<td>Rigid body motion</td>
<td>Instability of the test object, tilting during testing.</td>
<td>Tilt fringes. Decorrelation if motion is greater than speckle size.</td>
<td>Ensure rig is designed to hold object in a stable position. Use speckle tracking to follow object motion. Regularly update the reference image.</td>
<td>(138)</td>
</tr>
<tr>
<td>Excess motion</td>
<td>Too much motion of the object under loading.</td>
<td>Fringes become unresolvable. Quantitative evaluation not possible.</td>
<td>Choose appropriate load to give clear fringes. Reduce shear amount in ESPI to decrease sensitivity. Illuminate at an angle if possible. Update reference image.</td>
<td></td>
</tr>
<tr>
<td>Phase shifting errors</td>
<td>Miscalibration of the PZT. Nonlinear movement of the PZT. Errors in the phase stepping algorithms.</td>
<td>Incorrect phase value. If error is significant then unwrapped phase map will not be smooth. With proper calibration error is relatively insignificant.</td>
<td>Use of a higher number of phase steps.Use of modified algorithms to reduce effects of miscalibration. Generate phase shift electronically to eliminate errors.</td>
<td>(122,139)</td>
</tr>
<tr>
<td>Unwrapping errors</td>
<td>Low quality data, lack of robustness of unwrapping algorithm.</td>
<td>Incorrect phase value, not equivalent to phase change introduced due to object deformation.</td>
<td>Disregard low quality data for quantitative analysis, use a robust unwrapping algorithm.</td>
<td></td>
</tr>
<tr>
<td>Beam divergence</td>
<td>Use of a divergent Illumination source.</td>
<td>Overall error contribution increases if distance from the source to the surface of the object is small the size of the inspected area is large, and the angle of illumination approaches 45°.</td>
<td>To minimize, increase the distance between source and object (only for small inspection areas). Error source can be eliminated by using collimated illumination.</td>
<td>(140)</td>
</tr>
<tr>
<td>Misalignment of optical set up</td>
<td>Misalignment of beam, object and camera. Angle discrepancy in components.</td>
<td>May not be possible to separate in-plane and out-of-plane components of phase change. Distortion of data.</td>
<td>Precision machine components into correct alignment if possible. Limit number of moveable parts.</td>
<td></td>
</tr>
<tr>
<td>Shear amount</td>
<td>Shear amount effects accuracy. Shear amount not accurately quantified. Shear amount varies across image field.</td>
<td>Error is reduced if shear amount is small. Magnitude of relative error is dependent upon ( \frac{\Delta \phi}{\Delta x} ). If shear is too small fringe quality is lost. Variance of the shear field across the object can cause distortion of the data.</td>
<td>Minimise errors by determining the smallest amount of shear possible to generate good data at the correct sensitivity. Accurately quantify the shear magnitude across image space using DIC or speckle photography.</td>
<td>(141,142)</td>
</tr>
<tr>
<td>Non-flat object geometry</td>
<td>Variance in illumination intensity across object. Distortion of speckle shape. Sensitivity direction mismatch with surface.</td>
<td>Distortion of data. In-plane and out-of-plane measurements relate to the image plane not to the objects surface. Distortion of speckle shape can introduce a degree of error that is difficult to quantify.</td>
<td>Optical methods can be used to determine shape of object surface. Post processing methods can be employed to account for the effects of the non-flat geometry on data.</td>
<td>(137,140)</td>
</tr>
</tbody>
</table>

Table 3-5 – Summary of the main error sources associated with speckle interferometric measurement.
3.6.1 Fringes unrelated to applied loading and speckle decorrelation effects

The first three factors listed in Table 3-5 all lead to the formation of interference fringes unrelated to those of interest, or speckle decorrelation. These effects can usually be noticed in the wrapped image. For example, if there is tilt or rigid body motion, a series of regularly spaced or parallel fringes will form. If there are thermal effects, transient fringes unrelated to loading will be present in the live difference image. In the case of speckle decorrelation there is loss of definition in the fringes as shown in Table 3-6, in this case the speckle decorrelation has occurred due to excess motion in the direction of interest which has resulted in the fringe spacing becoming too small.

If speckle decorrelation is present, the measured phase change is no longer accurate and therefore quantitative analysis is no longer possible. Although quantitative analysis is not possible in the presence of speckle decorrelation, the data can sometimes still be visually useful, as the decorrelated areas may indicate a region that is unstable or moving excessively. Because speckle decorrelation may not occur across the entire object at once if there are large stiffness variations, decorrelated areas can be masked prior to unwrapping and the areas where decorrelation is not present can be analysed separately.

<table>
<thead>
<tr>
<th>Set Displacement</th>
<th>Wrapped</th>
<th>Unwrapped</th>
<th>Displacement plotted along central cross section</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1 µm</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>&gt; 3.5 µm</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Table 3-6 – Comparison of the data obtained from a 20 mm diameter flat metal plate with fixed boundaries point loaded at the centre to achieve maximum displacements of < 1 µm and > 3.5 µm.

These sources of error are relatively easy to control in a laboratory environment and have therefore been classified as ‘green’ error sources as the experimental work within this thesis.
was all ex vivo in nature. However, when considering in vivo measurement, minimising these sources of error becomes significantly more challenging and the overall effect of these errors, if they are present, is large as the data becomes unquantifiable.

3.6.2 Phase shifting errors

If a PZT is used for phase shifting, errors can be introduced if the PZT is miscalibrated or as a result of thermal drift. Significant errors in the movement of the PZT can be recognised from the visual data. The effect has been simulated in Figure 3-11. If the PZT does not move by the required amount the wrapped fringes do not have a smooth transition from black to grey to white and the unwrapped data has a stepped appearance with visible lines present on close inspection, hence these errors can often be recognised if they occur. The sensitivity to errors caused by the movement of the PZT can be minimised by using a larger number of phase steps. Errors in movement can also be reduced by using closed-loop control of the PZT, or to eliminate movement errors, a phase stepping method can be chosen that does not involve the use of moving components.

Figure 3-11 – Simulated data showing the effects of errors in the movement of the PZT introducing the phase step on the wrapped and unwrapped data plots.

3.6.3 Beam divergence and misalignment errors

Errors due to beam divergence and small misalignments in the optical configuration cannot be recognised from viewing the phase data as the obvious appearance of the fringes is not altered, the resultant effect is due to a change in the sensitivity vector, this is shown diagrammatically in Figure 3-12 for the case of beam divergence. The result of this effect is that the direction of
sensitivity will vary across the object, and the value of phase change at each point will not accurately represent the phase change in the desired measurement direction.

![Diagram demonstrating the change in sensitivity vector due to divergent illumination.](image)

The magnitude and effect of the errors introduced by beam divergence for the lateral shearing interferometry case have been evaluated by Abdullah (140), who showed that the relative error could be up to 10% for out-of-plane measurement and up to 40% for the in-plane case. He described the total relative error to be a function of illumination angle, curvature of the illumination beam, speckle size, shear amount and load position.

Dual beam interferometry which is often utilised to obtain the in-plane component of deformation is more prone to errors introduced due to beam divergence or misalignment, as the combined error in out-of-plane and in-plane data as a result of beam divergence increases as the illumination angle approaches 45°, and misalignment of the two illumination sources with respect to each other and the imaging direction means the various in-plane and out-of-plane components will not be completely separated, hence the calculated displacement will be inaccurate.

Overall the effects of beam divergence can be minimised by ensuring the object is positioned as far from the source as possible so the beam diameter at the point of inspection is large compared to the length of the object being inspected. If high measurement accuracy is required, the best option is to remove this error by using collimated illumination which results in a plane wavefront and thus a uniform sensitivity direction. Collimation may or may not be feasible depending on several factors, including; the overall size of the desired measurement area, the numerical aperture of the laser, the laser coherence length, the area available for the optical set-up and the collimated beam size restriction imposed by the size of commercially available collimation optics.
3.6.4 Shear amount

*Effect of shear amount on accuracy*

In shearing interferometry as the rate of change in displacement is averaged over the distance between two interfering points, the accuracy of shearography as a measurement technique to determine displacement derivatives is only high if the shear amount is small, however with very small values of shear, the neighbouring points in the image field can become too close, resulting in poor fringe contrast and unresolvable fringes, along with a reduction in sensitivity. Therefore, a balance must be obtained.

The phase change is a measure of the difference (in a chosen plane) of displacement at two interfering points separated by the shear distance ($\delta x$ or $\delta y$) and represents an approximation of the partial first derivative of displacement in that plane. If the second derivative of displacement ($\frac{\partial^2 w}{\partial x^2}$ - flexural strain for the out-of-plane case) that occurs during measurement is relatively high compared to $\frac{\partial w}{\partial x}$, and the shear distance is also large, a greater error is introduced into the measurement. A diagram demonstrating this effect is given in Figure 3-13.

Referring to Figure 3-13, if the shear distance is approximately 100 pixels, point 1 and point 2 will interfere, the out-of-plane displacement at each of these points is approximately the same, therefore, the rate of change of displacement ($\frac{\partial w}{\partial x}$) will be zero, when in reality, this is not the case. Therefore, if the displacement is obtained by integrating the measured value of $\frac{\partial w}{\partial x}$, $w$ will be underestimated slightly.

![Figure 3-13](image-url)  
*Figure 3-13 – Out-of-plane displacement plot to demonstrate the errors that could be introduced due to shear magnitude in areas where the bending strain is high.*
Steinchen et al \(^{(141)}\) investigated this source of error and developed an equation from which its influence can be approximated, the equation relating to the out-of-plane case with shear applied in the x-axis is given by equation 3-32, where \(e_r\) is the relative error, \(\delta x\) is the shear amount and \(M\) can be described by equation 3-33.

\[
e_r = \frac{\delta x}{M + \delta x} \quad \text{Equation 3-32}
\]

\[
M = 2 \left( \frac{\partial w}{\partial x} \right) \left( \frac{\partial^2 w}{\partial x^2} \right)^{-1} \quad \text{Equation 3-33}
\]

Hence, the relative error increases if \(\frac{\partial^2 w}{\partial x^2}\) is large compared to \(\frac{\partial w}{\partial x}\). The equivalent relationship exists for the horizontal and vertical in-plane case. The most effective way to reduce this error is to keep the shear magnitude as small as possible while still maintaining fringe quality, while being aware that in areas where the rate of change of the displacement derivative is high, the accuracy of the data may be compromised.

**Errors in determining the magnitude of applied shear**

Errors can be introduced if the amount of applied shear is not accurately measured. Often the shear amount is measured using a calibration piece that can be placed into the imaged area and has obvious points of interest that can be picked out from the live sheared image. However, depending upon the specific optical set-up used, a constant value of shear cannot be assumed across the whole image. This is true for the typical Michelson arrangement that uses a tilted mirror to introduce the shear.

Due to reflection from a tilted mirror, the sheared portion of the wavefront is not purely translated but the translation of the image is achieved via rotation. This rotation means that the shear distance across the object will not be constant \(^{(143)}\), as the size of the object reflected from the mirror introducing shear will not be equal in size to the object reflected from the planar mirror in the image plane. This effect will be exaggerated as the shear and hence the shear angle increases.

For maximum accuracy, the shear amount must be quantified at each point across the whole image. This can be achieved by capturing live images of the object reflected from each of the mirrors in the shearing cube individually, and using digital image correlation or speckle photography \(^{(138)}\) to compare the absolute position of the object in each image. For a cruder
analysis, several marks can be put onto the test piece across the image field and the shear can be evaluated at each of these points, interpolating in between. Once the absolute value of shear is determined a matrix of shear values can be created.

Generally, if the shear is small and the length of the test object is small, the variance in the magnitude of shear can be considered to be negligible when compared with other sources of possible error.

3.6.5 Effects due to non-flat surface geometry

By far the biggest possible source of significant errors and added complexities when interpreting the data is bought about by the initial geometry of the test object being non-flat. Much of the literature to date detailing quantitative analysis, especially that concerning in-plane analysis is on flat objects under non-complex loading conditions i.e. force applied in one plane. For the cases where 3D-analysis has been conducted on objects with higher complexity, a thorough analysis of the errors associated with these measurements is generally not undertaken and the measurements are rarely validated against other techniques.

A few commercial instruments are available that use ESPI to measure 3D deformations in complex objects, such as the 3D ESPI System (Q300) by Dantek Dynamics (144) however, there is little information on the measurement errors. Significant errors are likely, considering the complexities involved with applying a generalised analysis method to a range of complex surfaces undergoing complex deformations, in addition to the fact a divergent illumination source is often used.

Overall, from examining the current literature it is not possible to conclude how accurate speckle interferometric techniques are when applying the measurement principles to curved or non-uniform surfaces. Several phenomena which may affect the measured value of phase change have been highlighted in other studies such as the distortion of speckle shape (140) and therefore more work is needed in this area to evaluate accuracy.

In terms of analysis, when considering the use of ESPI and shearography with regards to the cornea, to date, little attention has been paid to the implications of the initial object curvature on the interpretation of measured data. In previous work by Jaycock (145), the cornea was assumed to be a hemispherical, isotropic membrane exhibiting uniform deformation and only the displacement at the apex was used in the calculations for Young’s modulus, negating the advantages of using a full-field measurement technique in the first place. The same method to determine Young’s modulus was utilised by Knox-Cartwright et al (45) by integrating the data
they obtained via radial shearing interferometry, despite the fact no data was measured at the apex due to the lack of sensitivity in this area.

For analysis of a non-flat object the following factors must be considered:

1. The distance between points on the objects surface is not equal with respect to the surface of the object when viewed in the image plane.
2. The deformation will have an in-plane and out-of-plane component and this is measured with respect to the image plane, not the objects surface.

**Effects due to curved object being viewed as flat object in the image plane**

The first factor affects both ESPI and LSI measurement and the resulting interferograms. For the ESPI case the interference fringes will appear more closely packed if the area of the object being studied is at an angle to the image plane rather than normal to the image plane. This may give a false impression that the rate of change of displacement is high in these regions.

When considering shearing interferometry, the curvature of the test piece introduces variations in the actual distance between sheared points with respect the object surface. This effect is demonstrated in the diagram in Figure 3-14. The lines extending from the image plane to the surface represent the approximately constant value of applied shear introduced via the optical configuration. The distance, with respect to the surface of the object, between the two sheared points at the outer area, labelled O, is significantly greater than the distance between two sheared points at the central area, labelled C, where the surface is approximately flat. Therefore, since the displacement will be compared at two points spaced further apart in the curved areas, the rate of change of displacement will be overestimated per unit of actual length with respect to the objects surface. Similar to the ESPI case, the appearance of the interferogram may give the false impression of a high rate of change of displacement in these areas.

![Diagram](image)

Figure 3-14 – Diagram demonstrating how the presence of an initial object curvature results in variation in the magnitude of the sheared distance between points with respect to the objects surface.
A correction factor can be applied to account for this effect if the shape of the surface being examined is known. Through determining the actual elevation of the surface at each point a 3D surface map can be generated. This elevation data can then be differentiated with respect to the axis of applied shear to obtain the gradient of the surface in this direction. This information can then be used to approximate the distance between two sheared points with respect to the objects surface \((\delta x_s)\) via equation 3-34 \(^{(135)}\).

\[
\delta x_s = \delta x \sqrt{\left(\frac{\partial z}{\partial x}\right)^2 + 1^2} \quad \text{Equation 3-34}
\]

Where \(\delta x\) is the magnitude of applied shear, and \(\frac{\partial z}{\partial x}\) is the gradient of surface elevation with respect to the direction of shear. When using this equation to approximate the distance between sheared points with respect to the object surface, it is assumed that the surface gradient does not change significantly between the two sheared points and therefore the distance between them can be approximated by a straight line, as shown in Figure 3-14.

The value of \(\delta x_s\) calculated for each point in the image can be input into the equations to calculate the in-plane and out-of-plane displacement derivatives in the place of \(\delta x\), as shown for the out-of-plane case in equation 3-35, to determine the rate of change of displacement per unit length between two interfered points with respect to the objects surface. The equivalent correction can be applied for vertical shear using the gradient of surface elevation with respect the vertical axis \(\frac{\partial z}{\partial y}\).

\[
\frac{dw}{dx} = \Delta \phi_{def} \left( \frac{\lambda}{4\pi\delta x_s} \right) \quad \text{Equation 3-35}
\]

**Effects due to 3D deformation**

Analysis of simple objects under simple loading conditions is relatively straightforward using ESPI or LSI techniques. For example, to analyse the material properties of an initially flat membrane with fixed boundary conditions subject to a hydrostatic load, either ESPI or LSI with out-of-plane sensitivity could be used. Even though there would be a small amount of in-plane strain, it is not necessary to measure this directly as the in-plane lengthening of the specimen could be determined from the second derivative of the out-of-plane LSI data \(^{(146)}\). Similarly, if a flat specimen was subject to tensile loading, only in-plane analysis would be required to determine the specific strain components.
For a curved membrane under hydrostatic loading the analysis becomes more complex as the deformation across most of the surface has both out-of-plane and in-plane components of displacement with respect to the viewing direction, and the contribution of each of these components to overall deformation varies with surface position.

Figure 3-15 shows a diagram of a curved object deforming under hydrostatic pressure with an example of what the measured out-of-plane data depicts at two different points around the curved surface. At the centre point the measured out-of-plane component likely represents the full surface displacement, whereas towards the outside, the measured out-of-plane component of displacement only represents a portion of the total displacement of a point between two loading states.

Hence, due to the presence of curvature and multidirectional deformation, to determine the material properties of the test piece and understand the biomechanics, both shape information and 3D analysis is required to determine both the out-of-plane and in-plane components of deformation at all points on the objects surface.

Consideration of this effect is especially relevant when using shearing interferometry techniques. When analysing a flat object, the rate of change of out-of-plane displacement represents a change in surface slope, with its derivative representing flexural strain, and the rate of change of in-plane displacement represents in-plane strain. The same cannot be assumed to be true for the case of a curved object.

When using shearing interferometry, the deformation at different positions across the surface is compared. For the case of a curved object, the relative contributions of the in-plane and out-of-plane components of deformation may vary at different positions and this can lead to distortion of the data.
An example is given in Figure 3-16. Points $P_1$ and $P_2$ interfere on the detector, when deformation occurs point $P_1$ goes to point $P_1'$ and point $P_2$ goes to point $P_2'$. For the out-of-plane case, the measured rate of change of out-of-plane displacement would be $w_1 - w_2$, however this does not describe the slope change of the surface. In this case, the $u_1 - u_2$ component would also be required along with knowledge of the original gradient of the surface between $P_1$ and $P_2$ to determine the gradient change.

![Figure 3-16 – Diagram showing the directionally different deformation of points $P_1$ and $P_2$ which are imaged at one point on the detector.](image)

However, if it can be assumed that overlapping points in the sheared image, deform in a directionally similar manner, as shown in the example shown in Figure 3-17. Then $\frac{u_1}{w_1} \propto \frac{u_2}{w_2}$, and in this case, the gradient change of the surface between $P_1$ and $P_2$ is equivalent to the difference in length of $l_1$ and $l_2$ and this value of $\frac{\partial l}{\partial x}$ could be calculated if $\frac{\partial w}{\partial x}$ and $\frac{\partial u}{\partial x}$ were known.

![Figure 3-17 – Diagram showing the directionally similar deformation of points $P_1$ and $P_2$ which are imaged at one point on the detector.](image)

Overall, the validity of the assumption that deformation occurs in a directionally similar manner would be increased if the radius of curvature of the test piece was relatively large, the shear was small, or the deformation occurred predominantly in one direction.
3.7 Conclusions

From the research into different speckle interferometric methods it was identified that LSI offered the best potential when compared to ESPI and other shearographic techniques such as RSI for fulfilling the aims as outlined at the end of chapter 2, this was because LSI measurement offered the following capabilities:

- High sensitivity measurement enabling testing to be conducted over a physiological range.
- Full-field assessment enabling deformation to be monitored across different regions of the cornea.
- The ability to resolve the rate of displacement of the surface in 3D.
- The ability to obtain displacement data in 3D via integration.
- High sensitivity to defects on non-homogeneities.
- Greater control over sensitivity and greater range of sensitivity than ESPI.
- Less sensitive to environmental disturbances or rigid body motion than ESPI, hence potential for development into an *in vivo* measurement tool.

Despite the many advantages of the techniques, it was clear from the error analysis there are several added complexities when using laser speckle interferometry techniques for the quantitative analysis of curved objects, and that this is especially true for the case of LSI. For the full analysis of a curved object such as the cornea both the in-plane and out-of-plane components of deformation are required and so far, this has not been achieved in the previous studies that have examined the cornea using similar techniques. The mismatch in the coordinate systems of the object surface and the image plane can introduce difficulties with respect to the interpretation of the raw data in the format of fringe distributions, therefore, the in-plane and out-of-plane data must be combined to establish how a 3D object deforms in response to a multi-directional load.
4. Method Development and Design of Experimental Rig

4.1 Introduction

It was decided that to determine the capabilities of LSI as a measurement method to evaluate corneal biomechanics, *ex vivo* testing would be conducted. This testing would involve using LSI to measure the response of corneas to pressure changes representative of those that occur *in vivo*. It would also involve using LSI to measure changes to the response that could be introduced by means of collagen crosslinking in specific topographic locations, to determine if LSI could be used to detect and evaluate localised changes to biomechanics. This testing would first be conducted on porcine corneas, where the purpose of experiments would be to optimise the measurement technique and establish an effective testing methodology suitable for human cornea testing.

Prior to the commencement of any testing, a suitable optical configuration had to be designed to enable measurement, and a suitable experimental rig had to be designed to simulate *in vivo* pressure variations. This chapter first describes the design of the experimental rig and then the development of the optical measurement technique.

4.2 Experimental rig design

As discussed in chapter 2, when *in vivo*, the cornea is subjected to several different forces, including; Intra-ocular pressure forces, forces from the ocular rectus muscles, forces from the eyelids during blinking, and forces at the limbus due to accommodation. Due to the complexities involved with recreating the latter three forces *ex vivo*; and since IOP is thought to play a significant part in governing the overall shape of the cornea and its refractive power, most biomechanics studies of the cornea measure the biomechanics of the cornea in response to IOP fluctuations, and this was also the approach taken in this study.

To measure the response of the cornea to IOP it was necessary to design an experimental rig were the cornea could be hydrostatically loaded to simulate normal IOP conditions and fluctuations. At the beginning of the development stage the requirements for the experimental test rig were defined, as a rig where it was possible to:

1. Measure both human and porcine corneas.
2. Measure the response of the whole cornea without need for the tissue to be dissected.
3. Replicate *in vivo* conditions as closely as possible.
4. Enable very small hydrostatic pressure changes to be applied.
5. Achieve highly repeatable loading.
6. Achieve high stability during testing.
4.2.1 Cornea mounting methods

The initial stage of the experimental rig design was concerned with determining a suitable mounting method for the cornea. To do this, the different techniques that had been used for corneal mounting in previous studies, where the response of the cornea had been measured in response to inflation testing, were evaluated and their specific advantages and disadvantages were considered.

Inflation testing of the cornea is generally either conducted as whole globe inflation testing, or by inflation testing of corneo-scleral buttons (circular tissue sections removed from the whole globe incorporating the whole cornea with approximately 1 mm – 3 mm of scleral tissue around the circumference) mounted in a specially designed chamber. In whole globe inflation testing, the whole eye remains intact and so this method is generally considered to more closely replicate in vivo conditions, as there is no damage to the structure of the eye and the sclera can contribute to the overall biomechanical response as it would in vivo.

During whole globe inflation testing the eye must be supported in some way to hold it in a stable position for measurement, this can be difficult as the tissue is not rigid, and care must be taken to ensure the mounting does not result in stresses on the tissue that would not be experienced in vivo. Several methods for supporting the whole globe have been reported across studies. Smolek (70), Kasprzak, et al (69), and Jaycock, et al (41), used cup designs, where the cornea was positioned in a cup designed to a specific size to gently support the edge of the sclera around the base while the cornea was viewed from above. Kling et al (147), used an alternative design and held the eye in a horizontal orientation as if in the head of a person sitting up, in this case they eye was held in position via a rigid ring around the sclera approximately half way down the globe. The main problem with the cup or ring method is that if the whole globe inflates during testing due to an increase in intra-ocular pressure the sides of the sclera will be prevented from deforming due to contact with the rigid cup or ring, and this may affect the deformation of the cornea in a way that would not naturally occur.

Recently, an alternative design was suggested by Whitford, et al (22), who suspended the eye globe within a low-stiffness, clear gelatine matrix, which was suggested to represent in vivo conditions more closely and enabled all parts of the globe to be monitored at once. The main challenge when using this method is that the whole globe must be monitored during testing so the relative movement of the cornea can be isolated.

Another factor that requires careful consideration when testing whole globes is the way the pressure in the ocular chamber is increased. Generally, this is achieved by means of a cannula.
inserted into the eye globe at a specific point. The point of insertion varies between studies with some opting to insert the cannula at the back of the eye \cite{70,147}, around the position of the optic nerve, and others opting to insert it directly into the cornea \cite{41}. When inserting the cannula into the back of the eye it can be difficult to determine if any air has been introduced into the globe and leakages are often a problem. When inserting the cannula directly into the cornea, small damage to the cornea can occur and this may affect its response to pressure changes.

In theory, testing of whole eye globes is optimal when attempting to determine the biomechanical properties of the cornea that most closely represent those of an *in vivo* case, however, in practice several problems can be encountered including mounting, and pressure application, distribution and monitoring. Also, if the aim is primarily to determine the mechanical properties of the cornea and not the whole eye, the data may be somewhat influenced by any deformation that occurs in the sclera and therefore there is added complexity to the analysis. Finally, another factor that is important to consider with regards to *ex vivo* work is that human donor tissue is often provided as it would be for transplant, which is in the form of corneo-scleral buttons and full globes can often be difficult to acquire.

For these reasons, many now opt to mount corneo-scleral buttons into specially designed artificial anterior chambers. This method has several advantages over whole globe testing, these include:

- Easy to prevent leaks.
- Repeatable and well-defined boundary conditions.
- Accurate control and monitoring of pressure changes.
- The ability to isolate the cornea from the rest of the globe, simplifying analysis.
- Opportunity to buy standard artificial anterior chambers as used for tissue preparation in corneal transplant surgery, negating the need to manufacture a custom chamber, while providing a repeatable method that can be replicated in other studies to produce directly comparative results.

In contrast, there are several disadvantages to this method. By removing the corneo-scleral buttons from the eye the overall structure of the tissue is disrupted and it is no longer supported naturally, this introduces two problems. Firstly, the boundary conditions will change and this may affect the overall behaviour of the tissue. Secondly, if the cornea is removed sometime in advance of testing and left unsupported, some relaxation of the collagen fibres may occur due to the fact they are no longer held in tension as part of the intact pressurised globe, therefore as it resumes its natural shape during testing there may be a period of stress relaxation.
A further factor to consider is that, although the sclera is reported as having greater stiffness than the cornea (148) it cannot be considered rigid, hence if the scleral portion of the globe is replaced with a rigid vessel, as in the case of testing corneo-scleral buttons mounted in a chamber, any deformation that the sclera would have undergone to compensate for increases in IOP will not occur. Therefore, the effective force on the cornea in response to an equivalent increase in IOP will be greater in the presence of a rigid chamber than it would be if it remained as part of a whole globe. Since it is not known how much the sclera deforms in response to increases in IOP it is difficult to take account of this in the analysis when testing the corneas mounted in this way.

The specific design of the artificial anterior chamber and the position of clamping, be it on the cornea or on the sclera, may affect the response to loading. Understanding of the corneo-scleral boundary conditions is an important area for which the data is currently lacking. In most studies the boundary is assumed as fixed as this simplifies the analysis, however, this is unlikely to be an accurate representation of in vivo conditions. A number of finite element studies have explored alternatives (149) including roller or spring supports but data is lacking to validate these options. Clamping as far from the corneo-scleral boundary as is possible is perhaps the best option as it allows analysis of this area and minimises the influence of the fixed boundary.

After weighing up the advantages and disadvantages of both whole globe testing and inflation testing of corneo-scleral buttons, a decision was made to proceed with inflation testing of corneo-scleral buttons. It was concluded that by using this method the pressure applied to the cornea could be more accurately controlled and monitored, it would provide greater stability of the cornea during testing, the repeatability of measurement would be greater as the variance in the biomechanical properties of the sclera from eye to eye would not have to be considered, and it would simplify the mathematical analysis.

4.2.2 Chamber Design

Initial chamber

Initially a Barron Artificial Anterior Chamber (Katena products, Inc., NJ, USA) was selected, as shown in Figure 4-1. The Barron chamber is a disposable chamber designed for human tissue sections between 14 mm – 18 mm in diameter. The opening at the top of the Barron tissue retainer is 12.5 mm providing a large enough diameter to enable scleral clamping when using human corneas. This chamber was selected as it had been used in previous studies by Knox-Cartwright, et al (45,51) and it was desired to maintain consistency with these studies to enable data comparison.
With this device, clamping is achieved by securing the tissue retainer onto the cornea by rotation of a locking ring around a lip section on the base which can be seen in Figure 4-1. Pressurisation of the cornea, once it is in the chamber, is achieved by filling the chamber with a solution via one of the silicone tubes which inserts into the base of the chamber.

Several issues were experienced when using this chamber. The chamber was found not to be compatible for use with porcine corneas, as the locking mechanism was limited to securely clamping tissue within the 0.4 mm – 0.7 mm range, and the 12.5 mm opening at the top of the chamber was not sufficient to clamp outside of the cornea on porcine corneas due to their larger diameter.

Also, the silicone pipes supplying the chamber are thin with a low stiffness, and it was found when increasing the hydrostatic pressure in the chamber, expansion of the flexible pipe occurred, this was confirmed by viewing a section of pipe with the shearing interferometer during loading. Fringes appeared on the pipes prior to their appearance on the cornea suggesting the silicone pipes had a lower modulus of elasticity than the cornea. Bending strain in the locking ring was also evident, suggesting the material that the chamber was made from was not of suitable strength to provide stable clamping.

Overall it was concluded that the Barron chamber had the following limitations that made it unsuitable for testing:

1. Limited to clamping human corneal tissue with a maximum thickness of 0.7 mm.
2. The base to the chamber was of a push-fit design and was prone to leaking.
3. Low stiffness pipes supplying the chamber absorbed pressure fluctuations.
4. Plastic locking ring was prone to bending.

As a result, a custom chamber was designed for use in the experiments detailed in this thesis.

**Chamber used during experiments**

At the beginning of the design stage the specific requirements for a suitable chamber were defined as a chamber where it was possible to:
1. Accommodate tissue of different thicknesses ranging from 0.5 mm to 1.5 mm.
2. Hydrostatically load corneo-scleral sections at pressures ranging from to 10 mmHg (1.3 kPa) to 60 mmHg (8 kPa).
3. Provide leak-proof clamping up to pressures of 60 mmHg.
4. Ensure high stability of corneal tissue over experimental time of up to 1 hour per cornea.
5. Provide repeatable clamping across specimens.
6. Prevent the introduction or enable the removal of any trapped air that may be introduced during filling.
7. Connect to a supply tank of fluid.

Based on these requirements a design specification was outlined prior to development of the custom chamber as detailed in Table 4-1.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chamber features</td>
<td>- 1 inlet and 1 outlet port</td>
</tr>
<tr>
<td>Dimensions</td>
<td>- Minimum tissue aperture of 11 mm.</td>
</tr>
<tr>
<td></td>
<td>- Accommodate tissue sections up to 19 mm total diameter.</td>
</tr>
<tr>
<td>Clamping Mechanism</td>
<td>• Leak-proof at pressures up to 60 mmHg</td>
</tr>
<tr>
<td></td>
<td>• Adjustable clamping distance of 0.5 mm to 1.5 mm</td>
</tr>
<tr>
<td></td>
<td>• Clamping diameter of 14 mm to 16 mm for porcine corneas and 12 mm to 14 mm</td>
</tr>
<tr>
<td></td>
<td>• for human corneas to enable clamping approximately 1 mm to 2 mm from edge</td>
</tr>
<tr>
<td></td>
<td>• Clamp angle of between 30° to 45° degrees to accommodate scleral curvature.</td>
</tr>
<tr>
<td></td>
<td>• Clamp contact area between 2 mm to 4 mm.</td>
</tr>
<tr>
<td></td>
<td>• Slip-proof.</td>
</tr>
<tr>
<td></td>
<td>• Provide fixed boundary.</td>
</tr>
<tr>
<td>Chamber Materials</td>
<td>• Waterproof.</td>
</tr>
<tr>
<td></td>
<td>• Easily cleanable.</td>
</tr>
<tr>
<td></td>
<td>• Resistant to alcohol based cleaning chemicals.</td>
</tr>
<tr>
<td></td>
<td>• High bending stiffness.</td>
</tr>
<tr>
<td>Pipes</td>
<td>• Waterproof.</td>
</tr>
<tr>
<td></td>
<td>• Resistant to deformation under pressures up to 60 mmHg.</td>
</tr>
<tr>
<td></td>
<td>• Flexible to enable controlled movement of loading mechanism and chamber if</td>
</tr>
<tr>
<td></td>
<td>required.</td>
</tr>
<tr>
<td>Connectors</td>
<td>• Waterproof.</td>
</tr>
<tr>
<td></td>
<td>• Resistant to deformation under pressures up to 60 mmHg.</td>
</tr>
<tr>
<td></td>
<td>• Rigid to enable high stability of chamber.</td>
</tr>
</tbody>
</table>

Table 4-1 – Chamber specification.

A titanium artificial anterior chamber (Akriti Oculoplasty Logistics, India) was purchased, similar to the chamber shown in Figure 4-2. Although a titanium chamber was ordered, when it arrived it appeared to be made of an alternative metal, but this was not an issue as it still had the desired design features. The reason for selecting this chamber was two-fold. Firstly, it had similar features to many of the standard chambers currently used for corneal preparation in
transplant surgery including the Barron chamber that had been used in previous studies\textsuperscript{(45,51)} meaning it would be simple for future studies to replicate the specific clamping mechanism to enable data comparison. Secondly, it addressed the main limitations of the Barron chamber, as in this case the locking ring could be screwed to the base via a thread which provided a more secure and adjustable locking mechanism, meaning tissues of different thicknesses could be accommodated. Also, since the chamber was made of metal and the locking ring clamped evenly all the way around, bending of the locking ring was not an issue when clamping tissue.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure2.png}
\caption{Titanium Artificial Anterior Chamber the same design as the ones used during experiments.}
\end{figure}

The top area of the base of the chamber and the inside of the tissue retainer/lid in between which the scleral tissue was clamped was chamfered to mimic the curvature of the sclera, and stepped slightly to enable gripping to prevent any tissue slippage. The aperture of the tissue retainer was 12.5 mm. A diagram representing the clamping position on the human cornea is given in Figure 4-3.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure3.png}
\caption{Diagram of a cross-section of a human corneo-scleral button clamped in the AAC showing approximate position of clamping.}
\end{figure}

The chamber was modified by machining through the top to create an open cavity and also through the base onto which a custom designed extension was attached, this is shown in the image in Figure 4-4. Two holes were made in either side of this chamber to provide an inlet and an outlet into which two valves were attached either side via a rigid metal connector to enable control of fluid flow into and out of the chamber. Tubes connecting a container that would hold the fluid used to pressurise the cornea were attached to the valve at the inlet. The
Polyvinyl chloride tubes were flexible enough to allow movement of the container but would not deform due to the pressure increases used in testing.

A stand for the chamber was designed, this is shown in Figure 4-5. This stand was designed to fit the bottom of the chamber which could then be secured into position via a locking screw. A rotation feature was also introduced, this rotation feature involved a spring-loaded ball-bearing locator mechanism, where a ball-bearing could drop into indents positioned at 90° locations to hold the stand stable in a specific orientation. The design of this stand is shown in Figure 4-5.

The reason for introducing this feature was to allow for the introduction of shear in both the horizontal and vertical axes when using LSI, to enable sensitivity to the displacement derivatives with respect to each of these directions. There are two ways this can be achieved, the first is to change the direction of shear by 90° by reorienting the shearing element, the second is to change the orientation of test piece by rotating the test piece through 90°.
It was decided to go with the latter of these two options because changing the direction of shear to be exactly equal in both the horizontal and vertical directions would require calibration and this would take time during testing, whereas, rotating the test piece is quick and simple and allows exactly equal sensitivity to be achieved in these axes without having to adjust the optical set-up which could introduce measurement errors.

It was realised, during the course of experiments on porcine corneas, that slight modifications were required to be made to the chamber so it could accommodate porcine corneas while clamping onto the scleral region only. For this reason, a second identical chamber was purchased for use with porcine corneas and the same modifications were made. However, with this chamber, the aperture of the chamber and of the tissue retainer were machined out to 15.7 mm, as it was found that an aperture of 15.7 mm was adequate to clamp onto the scleral region on the majority of porcine corneas, while still providing an adequate clamping region of 3.3 mm between the top of the chamber and the lid. This modification meant the porcine corneas were clamped at a similar location to human corneas when positioned in the original chamber.

One issue that was evident with the porcine corneas was that the irregular, almost tear drop shape of the circumference of the porcine cornea resulted in uneven clamping i.e. the clamping position with respect the cornea varied around the circumference, this is shown in Figure 4-6, which shows a typical porcine cornea clamped in the modified AAC.

As the position of the boundary could impact the deformation of the cornea under loading it is possible the un-even clamping result in irregularities with respect to normal behaviour. To negate this concern, it would have been necessary to create two elliptical clamps that approximated the shape of the corneal circumference for left and right eyes. However, since the focus of this research was not to determine the biomechanics of the porcine corneas this modification was not seen to be necessary, as the main purpose of testing on porcine corneas was to optimise the measurement technique for human cornea testing. Since the
circumference of the human cornea is more circular, the same issues were not experienced with human corneas.

Mounting the cornea into the chamber

Positioning of the cornea into the optical chamber was achieved by aligning the cornea based on the position of the extraocular muscles while attempting to centralise the cornea within the chamber to achieve clamping at an approximately equal distance to the cornea all around the circumference.

To ensure the cornea was not deformed during clamping, the cornea was laid over the base and air pressure was used to inflate the cornea to approximately its natural curvature, the tissue retainer was then positioned on top and secured in place using the locking ring. In some cases, there were a few issues when putting the tissue retainer over the cornea during the porcine cornea experiments as it could pull along certain sections, a photographic example is given in Figure 4-7. This problem was overcome by cutting the corneo-scleral buttons to the correct size of between 17 mm -19 mm diameter, positioning the cornea centrally and using air to inflate the cornea to its natural shape prior to positioning the tissue retainer.

![Image showing poor positioning of a porcine cornea in the modified AAC.](image)

Overall, the chambers provided a stable and leak proof means of clamping and hydrostatically loading both human and porcine tissue, and the rotation feature included in the base enabled the orientation of the cornea with respect to the imaging system to be easily and quickly changed during experiments.

4.2.3 Hydraulic loading system

The following stage of the experimental rig design was concerned with determining a suitable loading method. Since it was of interest to understand the biomechanics of the cornea in
response to \textit{in vivo} pressure fluctuations, and to determine if LSI would be capable of measurement under physiological pressure changes, it was required to develop a loading technique that could simulate these \textit{in vivo} pressure fluctuations in magnitude, while providing high loading repeatability and adequate stability for measurement.

\textit{In vivo}, the cornea is constantly subjected to IOP. As previously defined in chapter 2, normal IOP is considered to lie within the range of 10 mmHg – 21 mmHg \cite{29}, with an individual's IOP fluctuating by several mmHg within this range over a given period of time, as previously discussed in section 2.6.1 of chapter 2. The cardiac cycle is known to cause rhythmic fluctuations in IOP, the magnitude of these fluctuations is often referred to as the ocular pulse amplitude (OPA) and has been reported to lie between 0.9 mmHg - 7.2 mmHg \cite{64}, with medians of 3 mmHg \cite{64} and 2 mmHg \cite{152} for healthy eyes. Hence, it was desired to have a loading technique where the cornea could be subjected to a baseline pressure at all times while small variations in pressure, within a 3 mmHg range, could be introduced.

The specific size of the pressure variation over which measurement could be achieved was dependent on the sensitivity range of the measurement technique and the stiffness of the cornea, and at this stage these factors were not known explicitly. In a study by Jaycock, et al \cite{41}, where the response of sheep corneas to hydrostatic pressure changes were measured using ESPI, a pressure change of only 0.15 mmHg was required to generate data. In studies by Knox-Cartwright, et al \cite{45,51}, that used radial shearing interferometry to examine the response of human corneas to hydrostatic loading, the response was measured over a 0.50 mmHg pressure change.

Thus, to achieve pressure changes of a similar magnitude to those that occur during the cardiac cycle, and to ensure measurement could be achieved within the sensitivity range of LSI, a loading rig was designed where the cornea could be held under a constant baseline pressure representative of normal IOP, from which the pressure could be raised and lowered by up to 3 mmHg in small steps of less than 0.05 mmHg if required.

A diagram of the loading rig is shown in Figure 4-8. Pressurisation was achieved by mounting a reservoir of fluid connected to the artificial anterior chamber via flexible tubing at a specific height above the cornea to simulate normal IOP. A baseline IOP of 16.50 mmHg was selected as this lay within the 10 mmHg - 21 mmHg normal IOP range and is in agreement with values measured across a number of studies using various measurement techniques \cite{153,154}.

The fluid used in the experiments detailed in this thesis was Phosphate Buffered Saline (PBS) solution (PBS, Sigma-Aldrich, UK, ρ = 0.995 g/ml at 25°C), the reason for this is because PBS
solution maintains the hydration properties of the cornea better than water and hence overall tissue swelling is less over the total time spent in contact with the fluid (155). Alternatives with even better hydration balancing properties are available, such as balanced salt solutions, however PBS was readily available and comparatively inexpensive compared to other solutions so this made it the most suitable option for the large number of experiments as detailed in this thesis.

The relationship of the hydrostatic pressure (P) in mmHg to the height of the tank in metres, is given by equation 4-1.

\[ P = 0.0075 \rho gh \]  

Equation 4-1

Where \( \rho \) (kg/m\(^3\)) is the density of the fluid, g (9.81 m/s\(^2\)) is gravity and h (m) is the height difference between the cornea and the maximum height of the fluid. Hence, it was determined that to achieve a pressure of 16.50 mmHg it was necessary to position the tank at a level where the maximum height of the fluid was 225 mm above the height of the corneal surface, which was taken as the height midway between the height of the apex and the height at which the cornea meets the sclera.

![Diagram of loading rig](image)
During loading rig calibration to validate that the initial pressure in the chamber was 16.50 mmHg when the maximum height of the PBS solution was set at the measured distance of 225 mm above the surface of the cornea a digital manometer (Digitron 2021P Pressure Meter, British Rototherm Co. Ltd., Port Talbot, UK) was attached at outlet 1 and positioned with the sensor at the same height as the cornea. The read-out from the digital manometer was 16.50 mmHg (sensor accuracy +/- 0.01 mmHg) confirming that the measured height of the PBS solution above the surface of the cornea was correct and that this height corresponded to a pressure of 16.50 mmHg.

Pressure variation was achieved by changing the height of the reservoir with respect to the cornea, this was facilitated by securing the reservoir to a vertical movement stage from which the height of the reservoir could be controlled via a rotatory mechanism. The height change of the reservoir was quantified to an accuracy of 0.005 mm via the use of a digital dial gauge (SPI 14-797-5, Swiss Precision Instruments, Inc. CA, USA), mounted so the dial was incident on top of the reservoir as shown in the diagram in Figure 4-8. Prior to measurement the digital dial gauge was set to zero, then after measurement the total movement was recorded from the gauge and the resulting pressure change (ΔP) was calculated from equation 4-2. Where Δh(m) is the height difference between the initial height of the tank at the start of measurement to the final height of the reservoir, as displayed by the dial gauge.

\[
\Delta P = 0.0075 \rho g \Delta h
\]

Equation 4-2

It was decided to automate the process of pressure variation to gain greater repeatability for both the total pressure change and the loading time. Automation also removed the need to touch the test rig during measurement which could contribute to noise in the data. To achieve this an adaptor was made to attach a stepper motor (Series FMJ7301, Cliff Electronics, Surrey, UK) to the top of the screw that adjusted the height of the reservoir attached to the vertical movement stage. The stepper motor was driven via a motor (SE2 Samotronic 101, Saia Burgess, Murten, CH) which was digitally controlled via a subroutine that was added to the measurement control software via which the rotation direction and rotation time of the stepper motor could be set, and hence the vertical movement of the reservoir could be controlled.

To quantify the change in height that occurred during a set movement time, so a movement guide could be produced and the repeatability of the loading system could be determined, calibration was carried out by setting the movement time at one second intervals and recording the vertical distance moved via the digital dial gauge. Measurements were repeated 10 times at each interval for a set movement time of up to 18 seconds. The results of this calibration are plotted in Figure 4-9. The repeatability of the movement recorded for a set time was found to
be high (0.0069 mm 95% Confidence Interval) which equates to a repeatability in pressure variation of +/- 0.0005 mmHg (0.07 Pa).

![Graph showing the relationship between movement time set (msec) and vertical distance moved (mm). The equation y = 0.0008x - 0.23 is shown on the graph.](image)

*Figure 4-9 – Vertical movement of the reservoir as a function of set movement time.*

The relationship of movement time to vertical movement over the measurement range was found to be linear. The y-intercept was found to be at -0.23 mm as opposed to zero as would have been expected, the reason for this was because a degree of backlash occurred due to the fit of the connector connecting the micrometre screw on the movement stage to the motor, resulting in a small initial delay to movement. However, this delay was found to be consistent across measurements and therefore did not affect the linearity or repeatability of results.

The results of the calibration were used to create a movement guide as shown in Table 4-2, from which the movement time for a desired pressure increase in either Pa or mmHg could be determined. This conversion chart was used to set the desired movement but was not used to determine the absolute pressure change during testing as a higher accuracy (+/-0.005 mm) could be achieved by recording the vertical height change from the digital vertical gauge.
<table>
<thead>
<tr>
<th>Pressure (mmHg)</th>
<th>Pressure (Pa)</th>
<th>Time (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>6.67</td>
<td>855</td>
</tr>
<tr>
<td>0.10</td>
<td>13.33</td>
<td>1709</td>
</tr>
<tr>
<td>0.15</td>
<td>20.00</td>
<td>2564</td>
</tr>
<tr>
<td>0.20</td>
<td>26.66</td>
<td>3418</td>
</tr>
<tr>
<td>0.25</td>
<td>33.33</td>
<td>4273</td>
</tr>
<tr>
<td>0.30</td>
<td>40.00</td>
<td>5127</td>
</tr>
<tr>
<td>0.35</td>
<td>46.66</td>
<td>5982</td>
</tr>
<tr>
<td>0.40</td>
<td>53.33</td>
<td>6836</td>
</tr>
<tr>
<td>0.45</td>
<td>60.00</td>
<td>7691</td>
</tr>
<tr>
<td>0.50</td>
<td>66.66</td>
<td>8545</td>
</tr>
<tr>
<td>0.55</td>
<td>73.33</td>
<td>9400</td>
</tr>
<tr>
<td>0.60</td>
<td>79.99</td>
<td>10255</td>
</tr>
<tr>
<td>0.65</td>
<td>86.66</td>
<td>11109</td>
</tr>
<tr>
<td>0.70</td>
<td>93.33</td>
<td>11964</td>
</tr>
<tr>
<td>0.75</td>
<td>99.99</td>
<td>12818</td>
</tr>
<tr>
<td>0.80</td>
<td>106.66</td>
<td>13673</td>
</tr>
<tr>
<td>0.85</td>
<td>113.32</td>
<td>14527</td>
</tr>
<tr>
<td>0.90</td>
<td>119.99</td>
<td>15382</td>
</tr>
<tr>
<td>0.95</td>
<td>126.66</td>
<td>16236</td>
</tr>
<tr>
<td>1.00</td>
<td>133.32</td>
<td>17091</td>
</tr>
</tbody>
</table>

Table 4.2 – Vertical movement guide for reservoir showing movement time required to achieve a pressure change up to 1.00 mmHg in steps of 0.05 mmHg.

In summary, the loading rig described enabled the cornea to be held under constant pressure representative of normal IOP, while small variations in pressure within a range of 0.05 mmHg - 3 mmHg could be introduced, as desired. The main drawback to the loading rig design with respect to simulating *in vivo* conditions was that the rate at which the pressure could be varied was limited to 0.058 mmHg per second, which is considerably lower than the *in vivo* loading rate where the pressure can change by several mmHg in less than a second.

As discussed in section 2.6.2 of chapter 2, the response of the cornea is thought to be dependent upon loading rate. However, since all experiments were being conducted within a very small pressure range where the behaviour of the cornea is considered to be predominantly elastic, it was thought any differences in the response as a result of the loading rate within this range of pressures would be small. The slow loading rate was advantageous in some respects, as it allowed the development of interference fringes to be viewed during loading and this often helped to visualise how the cornea was deforming.
4.3 Development of the Optical Measurement System

The main functional requirements for the optical measurement system are outlined in detail in section 2.8 and a general specification for a measurement method to fulfil these requirements is given in Table 2-3. Since it had been identified that speckle interferometry techniques would be used and the response of the cornea would be investigated in response to variations in hydrostatic pressure representative of the variations in IOP that occur during the cardiac cycle, both before and after cross-linking, the specific required capabilities for the measurement technique were as follows:

1. A technique whereby full 3D surface deformation of corneo-scleral section mounted and loaded as described in section 4.2 could be evaluated in response to hydrostatic pressure variations of less than 3 mmHg.
2. A technique whereby the load-displacement response of the corneal surface could be evaluated and compared before and after cross-linking.

The development of the optical measurement method took place iteratively with several different optical configurations investigated prior to arriving at the configuration used during the experiments detailed in this thesis. During optical measurement system development, to evaluate the suitability of the different optical configurations investigated, experiments were carried out using both non-biological samples and porcine corneas clamped and loaded in the experimental rig that was described in the previous section. This section discusses the iterative development of the final measurement system.

4.3.1 Combined in-plane and out-of-plane LSI system using dual-beam illumination

It was identified in section 3.6.5 of chapter 3, that to analyse the response of the cornea to a hydrostatic pressure change it is necessary to measure both the in-plane and out-of-plane components of deformation with respect to the image plane. This is necessary due to the curvature of the cornea, which means the total deformation across different areas of the surface has varying contributions of deformation with respect to each of these directions.

As detailed in section 3.5.2 of chapter 3, it is possible to obtain sensitivity to both in-plane and out-of-plane deformation using LSI, if two data sets are captured while illuminating the surface of the object from equal and opposite angles in turn. The data recorded when illuminating at the two illumination angles can then be subtracted to isolate the in-plane component, and summed to isolate the out-of-plane component. Hence, an initial LSI configuration was designed based on this dual beam illumination technique.
Optical configuration

A diagram of the set-up is given in Figure 4-10. The illumination source used was a diode pumped single-mode solid-state laser at a wavelength of 532nm with a Gaussian beam (06-DPL, Cobolt AB, Solna, SE). This laser was expanded and collimated to a diameter of 25 mm which was sufficient to illuminate the full sample when clamped in the chamber. Collimated illumination was used, as opposed to divergent illumination, as it was desired to minimise any variability in the sensitivity vector, that may result in errors or further complication with respect to the interpretation of the measured data.

The illumination beam was passed through a 50/50 beam splitter (BS1) which split the beam into two parts with equal intensity. The two parts of the beam were then reflected from mirrors M1 or M2 respectively, to enable illumination at equal and opposite angles with respect to the viewing direction. Since illumination was desired from each side individually shutters S1 and S2 were used.
S2 were positioned so that illumination could be enabled or blocked as desired from each side individually.

Shearing was achieved using a Michelson arrangement as shown in Figure 4-10. A Michelson configuration was used as opposed to other shearing methods, as it granted the ability to easily control the magnitude and direction of applied shear. With this arrangement, the signal from the object was divided into two parts with equal intensity via a 50/50 beam splitter (BS2), with one part reflected from a plane mirror (MP) and the other reflected from a mirror at a specific angle to introduce the desired amount of shear (MS), the angle of this mirror could be adjusted as desired via positioning screws attached to the mirror mount. The wavefronts reflected from each of these mirrors were then combined in the image plane to produce an interferogram. Imaging was via a CMOS camera (CMOS Aptina MT9P031, Basler AG, Ahrensburg, DE) with a selected resolution of 1296 pixels by 972 pixels. Imaging was through a 12.5 mm – 75 mm zoom lens (C31204, Pentax, Tokyo, JP) mounted to the camera.

To enable the acquisition of quantitative data temporal phase stepping was implemented. Temporal phase stepping was used as opposed to a spatial method for several reasons:

- It could be conveniently incorporated into the optical set-up.
- It granted greater spatial resolutions than spatial methods.
- Spatial methods were less desirable as they impose restrictions with regards to the speckle size and hence the aperture size.
- Since all testing at this stage would be ex vivo, stability could be controlled and therefore wasn’t an obstacle to the use of temporal phase stepping.

Temporal phase stepping was achieved via attaching the mirror MP to a PZT (P840.1, Physik Instrumente, Karlsruhe, DE). A five-step phase stepping procedure using a step size of \( \frac{\pi}{2} \), as was previously detailed in Table 3-1, was implemented. This five-step procedure was selected in-order to reduce the effects of any potential errors that could be introduced via inaccurate movement of the PZT. This resulted in a total data capture time of 633 milliseconds.

Data acquisition and processing was controlled via bespoke software which was created in LabVIEW in partnership with Laser Optical Engineering (LOE, Leicestershire, UK). This measurement software provided a user interface from which imaging parameters could be set, and loading and data capture could be controlled. The measurement software was designed to provide the following capabilities:

- Manual control of the image exposure levels.
- The ability to mask around square or elliptical regions of interest in the imaged area.
- Manual control of loading time and direction.
- Automatic phase stepping control.
- Manual or automatic control of data capture.
- Automatic generation and storage of wrapped and unwrapped phase maps and data files on data capture.
- The ability to capture and save live images and live interference fringes.
- The ability to view real time data

For phase unwrapping the algorithm developed by Arevalillo-Herráez, et al (128) and described in section 3.3.2 was used.

**Measurement procedure**

The measurement procedure was as follows:

1. The sample was set under an initial pressure of 16.50 mmHg
2. While illuminating with one beam reference data was recorded with the sample at a pressure of 16.50 mmHg
3. A movement time of 8.454 seconds was set for the stepper motor driving the vertical movement stage to obtain a pressure increase of 0.50 mmHg.
4. Loading was initiated and the pressure was increased by 0.50 mmHg.
5. One second after the end of loading phase stepped data was captured with the sample at a pressure of 17.00 mmHg, this data was digitally subtracted from the reference data recorded at step 2.
6. A wrapped data file was generated and automatically unwrapped and saved.
7. Load was removed.
8. Process was repeated exactly as detailed with illumination from the opposite angle.

The phase data obtained while illuminating at equal and opposite angles over an equivalent pressure change was then imported into MATLAB (Math Works Inc., MA, USA) for processing. Using MATLAB, the 1296 by 972 element image matrices detailing the phase data recorded at equal and opposite illumination angles were summed to isolate the out-of-plane component for each pixel in the image, and subtracted to isolate the in-plane component, as previously described in section 3.5.2.

**Results**

Some issues were encountered when trying to use the dual beam configuration for corneal analysis. Illuminating the cornea at an angle was found to be problematic as the curvature of the cornea meant there were differences in the intensity of illumination across the surface, with shading occurring on the side of the cornea facing away from the illumination source. This is
demonstrated in Figure 4-11 for a curved rubber section of diameter 20 mm that was cut from the fingertip of a cotton lined rubber glove (Marigold Large Kitchen Gloves, Freudenberg, Weinheim, DE) and mounted in the AAC. This sample was selected as it had similar curvature to the porcine cornea when set under a pressure of 16.50 mmHg in the AAC. The left image shows the sample when illuminated at an angle normal to the centre of the surface, and the right image shows the same sample when illuminated at an angle of approximately 30°. The diameter of the interfered area on the corneal surface is labelled D and the diameter of the shaded area that occurred when illuminated at an angle is labelled d_s. The absence of adequate image intensity in this area resulted in poor fringe contrast in this region and a lack of data, as demonstrated in Figure 4-12. Since data was required when illuminating from each side to resolve the in-plane and out-of-plane components, shading affected the quality of data at both sides of the test surface, and this resulted in data across approximately 31% of the surface being unresolvable after combining the two data sets. Since it was expected that the in-plane component would be most significant towards these boundary regions the absence of data in these regions was not ideal.

Figure 4-11 – Live image of a rubber sample with a similar curvature to the porcine cornea when illuminated with the laser at different illumination angles.

Figure 4-12 – Image of the live interference fringes viewed on a rubber sample with similar curvature to the porcine cornea subjected to an increase in hydrostatic pressure. With normal illumination (left), with illumination at an angle of approximately 30° to the normal (right).
Reducing the illumination angle as far as possible was considered, however, to minimise shading it would have been necessary to illuminate at an angle of less than 20° with respect to the normal, at this angle the relative sensitivity to the rate of in-plane displacement with respect to the rate of out-of-plane displacement would have been low.

Based on a study by Boyce, et al (20), where the in-plane and out-of-plane displacement components had been measured on bovine corneas in response to an increase in hydrostatic pressure, it was expected the in-plane component would be significantly lower than the out-of-plane component, as in their study the maximum in-plane displacement was measured at approximately a third of the maximum out-of-plane displacement for a given pressure change. Therefore, this mismatch in sensitivities at shallow illumination angles would likely mean the pressure required to generate adequate stability to in-plane motion would result in speckle decorrelation due to excess movement out-of-plane.

Finally, it was considered that since the human cornea is flatter than the porcine cornea, shading would be less of an issue. However, tests on a silicone rubber sample that was manufactured from liquid silicone (Unibond white sealant, Henkel AG & Co, Düsseldorf, DE) set in between contact lens mould so that it was of a similar size and topography to the human cornea demonstrated that at an illumination angle of 30°, shading would still affect an area covering 26% of the surface of the cornea. An image showing the shading that was present on the rubber sample with similar curvature to the human cornea is shown in Figure 4-13.

![Normal illumination vs. 30° illumination](image)

*Figure 4-13 – Live images showing sample with similar curvature to the human cornea when illuminated with the laser at different illumination angles.*

Ultimately, it was decided that the dual-beam method was not suitable for measurement of the cornea as the illumination issues meant that full-field evaluation could not be achieved via this method. Alongside the issues with illumination, there were also other challenges associated with the implementation of this method. Since this method required two data sets to be obtained
at different times while illuminating at each angle in turn, noise was a significant issue after subtracting the two data sets.

In the experiments detailed, measurements were recorded over two separate loading cycles, and therefore any slight differences that occurred in the response, contributed to noise when calculating the in-plane component. It was considered that measurements could be taken in quick succession rather than over repeat loading cycles, however this required high stability of the cornea at each of the loading states, and the cornea was found to continue deforming slightly for some time after loading.

4.3.2 Out-of-plane configuration

*Method to determine the in-plane component using out-of-plane data*

It was eventually decided to focus on the development of an out-of-plane measurement configuration using normal illumination, and to use this optical configuration in combination with other techniques to analyse the 3D deformation of the corneal surface in response to a change in hydrostatic pressure.

The first option that was considered was to measure the out-of-plane response of the test piece over several measurement cycles with the surface in different orthogonal orientations with respect to the imaging direction while using normal illumination, as shown in the diagram in Figure 4-14. However, it was concluded that it would not be possible to generate full-field information via this method as it was not possible to achieve uniform illumination or the depth of focus to measure across half the cornea when in held in the vertical orientation. Also, differences in the gradient of the surface with respect to the image plane would cause sensitivity issues.

![Diagram showing a proposed method of determining different components of deformation, by changing the orientation of the cornea with respect to imaging and illumination directions.](image)

*Figure 4-14 – Diagram showing a proposed method of determining different components of deformation, by changing the orientation of the cornea with respect to imaging and illumination directions.*
Finally, it was decided to measure the out-of-plane deformation of the cornea with the chamber positioned normal to the imaging and illumination directions only, as shown by the central orientation in Figure 4-14, and to make an estimate for the in-plane component via the use of one of the following methods:

1. Approximate the cornea as a pressure vessel with uniform thickness and equal stiffness with respect to the axial and circumferential planes. Hence, assume that deformation occurs radially with respect to the surface normal and make an estimate for the in-plane component of displacement using out-of-plane data and surface gradient data.

2. Video record the deformation of the cornea to larger pressure changes across the central cross-section and then analyse the video data to determine the angle at which the surface deforms at several points across the cross-section. In this case, assume that the angle of deformation remains the same at higher loads and use the angle of deformation predicted from the video data along with the measured out-of-plane component and surface profile information to make an estimate for the corresponding in-plane component.

The benefit of these methods is that relatively even intensity of illumination could be achieved across the full surface of the cornea, and only one measurement was required with respect to each direction of shear (horizontal or vertical) to generate full-field 3D information when used in combination with the shape or video-recorded data.

**Out-of-plane LSI configuration**

Initially an out-of-plane LSI system was designed based on the common Michelson configuration as described previously in Figure 4-10, but with normal illumination. Initial testing was conducted on porcine corneas using this LSI configuration, however the data obtained during these initial experiments showed an unfamiliar distribution, with high concentration of fringes forming close to the boundary areas. An example of the wrapped data obtained when measuring the response of a porcine cornea to a 0.50 mmHg increase in hydrostatic pressure is shown in Figure 4-15.

![Shear with respect to N-T axis](image1)

![Shear with respect to S-I axis](image2)

Figure 4-15 – Wrapped fringes obtained via LSI for a porcine cornea subjected to a 0.50 mmHg increase in hydrostatic pressure. Cornea in initial orientation (left), same cornea after rotating through 90° (right).
Since the data was difficult to interpret in this format, the data was imported into MATLAB where the phase data was processed to determine out-of-plane displacement. This was achieved via the following procedure:

1. The rate of change of out-of-plane displacement ($\frac{\partial w}{\partial x}$ (horizontal shear) or $\frac{\partial w}{\partial y}$ (vertical shear)) between interfered points in the image was calculated from the phase data using the relationship between phase change and $\frac{\partial w}{\partial x}$ and $\frac{\partial w}{\partial y}$ given in equations 3-26 and 3-27 respectively.

2. The rate of change of out-of-plane displacement data was then integrated along each line of the image with respect to the direction of applied shear using the cumulative trapezoid method to obtain a full-field plot of out-of-plane displacement.

However, several problems were experienced with the integration of data obtained across the range of samples tested, as the closeness of the fringes that formed towards the boundary regions often resulted in a loss of fringe resolution in specific areas and due to the cumulative nature of integration this resulted in the propagation of errors from these regions preventing accurate integration of the data.

There were concerns at this stage that the distribution of fringes, especially in the boundary regions, were a result of effects introduced due to the curvature of the test piece, and that these effects may prevent the LSI technique from being used to evaluate the response of the cornea successfully.

**Combined out-of-plane ESPI and LSI configuration**

To address concerns held about the performance of the shearography system, it was decided to redesign the optical configuration to include an ESPI system alongside the existing out-of-plane LSI configuration. The addition of the ESPI system would allow direct analysis of the displacement component, and this would enable direct comparisons to be made between the ESPI and LSI data to validate the success of the LSI measurement technique.

To enable this data comparison, it was desired to have a system where both ESPI and LSI measurement could be obtained at the same time or in quick succession without the need to move the test piece or any other components in the optical configuration. Based on this requirement, an optical configuration was designed where measurement could be shifted from ESPI measurement to shearographic measurement without having to move any parts. A diagram of the rig for both ESPI and shearography conditions is given in Figure 4-16.
Figure 4-16 – Combined ESPI and LSI interferometer configuration. (a) Configuration when in ESPI mode, (b) configuration when in LSI mode.
With the configuration as shown in Figure 4-16, it was not possible to take both ESPI and LSI measurements simultaneously, so the measurements were performed over separate loading cycles, this was enabled by positioning two moveable shutters (S1 and S2) to block specific arms of the beam. Figure 4-16(a) shows the configuration as it was for ESPI measurement. In this case, the moveable shutter S1 was opened to allow combination of the object beam with a phase stepped reference beam, and the moveable shutter at S2 was closed to prevent shearing of the two beams. The piezo attached to the plane mirror adjacent to the shearing cube (PZT 2) was disabled and the piezo attached to the reference beam mirror (PZT 1) was enabled.

Conversely for shearographic measurement the configuration was as shown in Figure 4-16(b). In this case, the moveable shutter at S1 was closed and the moveable shutter at S2 was opened, this removed the reference beam and enabled the object beam to be interfered with a transformed copy of itself. The PZT (PZT 2) attached to the plane mirror adjacent to the shearing cube (MP) was enabled so phase stepping could be achieved.

It was identified that simultaneous measurement via ESPI and LSI was possible using a similar configuration if two lasers of different wavelengths were used to illuminate the object simultaneously. In this case two notch filters specific to the different wavelengths of illumination could be positioned in place of the shutters so one of the beams followed the ESPI path, shown in Figure 4-16(a) and the other the LSI path, as shown in Figure 4-16(b). A dichroic beam splitter would then be required in front of the camera position as shown in Figure 4-16 to separate the two wavefronts and direct them to two identical cameras. However, this option was not chosen as it would have been significantly more expensive than the method that was employed and could not be afforded by the research budget. For the method shown in Figure 4-16 the only additional equipment required on top of that used in the original shearography set-up shown in Figure 4-10 was an additional PZT to enable phase stepping of the reference beam in the ESPI set-up.

All equipment used was identical to the equipment described in section 4.3.1 for the set-up shown in Figure 4-10, the additional PZT was the same model as the PZT used in the original set-up. The LabVIEW software used to control loading and data capture, described in section 4.3.1, was modified to enable activation and deactivation of PZT 1 and PZT 2 depending on the desired measurement mode.

It is noted that although care was taken to align all the components in the rig, due to the experimental nature of the rig components were not precision machined into position, and therefore it is possible small misalignments were present. Since the position of components
within the configuration remained static throughout all experiments, any errors introduced due to these misalignments remained consistent throughout testing.

**Laser safety requirements**

Eye safety was considered at an early stage in development, this was for two main reasons; firstly, it was desired to create an ex vivo measurement device that could be used without the need for personal safety equipment. Secondly it was of interest to determine if data could be obtained within the maximum permissible exposure limits, as this would have implications with regards to the potential for in vivo measurement.

To determine the safety of the laser with respect to the eye consideration was made into how the eye deals with exposure to a light source. Light enters the eye via the pupil and it is focussed by the cornea and the lens onto the retina as shown by the diagram in Figure 4-17 (156). The retina contains photoreceptor cells (rods and cones) which convert the optical image into electric signals, which are sent via the optic nerve to the brain. The most sensitive part of the retina is the fovea centralis, which is a central pit in the retina formed of densely packed cone cells. The fovea is the area responsible for sharp central vision and therefore damage to this area causes vision loss.

![Diagram showing how light enters the eye and is focussed onto the retina. Reproduced from (156).](image)

The wavelengths of light that are hazardous to the retina lie in the $\lambda = 400$ nm – 1400 nm region as these wavelengths are not absorbed at the cornea or lens (156). Because the light is focussed to a small point on the retina, irradiance ($E_o$) which is calculated by dividing the laser power by the area onto which it is focused, is very high at this point.

Maximum permissible exposure (MPE) levels are set at the level where the risk of damage from exposure is negligible. The MPE value assumes that the pupil is fully dilated at 7 mm and therefore represents a worst case scenario (157). The MPE versus exposure time according to IEC 60825 is plotted for lasers of various wavelengths in Figure 4-18 (158). The MPE for a laser
between \( \lambda = 400 \text{ nm} - 700 \text{ nm} \) and for an exposure time of \( > 10 \text{ seconds} \) is \( 0.001 \text{ W/cm}^2 \) or 1 mW/cm².

The laser beam that was used in the experimental system was a green laser \( (\lambda = 532 \text{ nm}) \) with a power of 100 mW measured using a power meter (PowerMax PS10 Sensor connected to LabMax-TOP, Coherent, CA, USA), therefore this beam was dangerous to view directly. To ensure the beam was safe for viewing, it was passed through a filter to reduce the power to a maximum of 4.9 mW and it was expanded and collimated to a spot size of 25 mm meaning the irradiance at the cornea was 0.000998 W/cm², as shown in the calculation in equation 4-3.

\[
E_e = \frac{0.0049}{\pi \cdot (1.25)^2} = 0.000998 \text{ W/cm}^2
\]

Equation 4-3

Therefore, at the cornea the irradiance of \( < 0.001 \text{ W/cm}^2 \) was within the MPE for the exposure time of greater than 10 seconds that was required during testing and is suitable for deliberate viewing. Sufficient fringe contrast was found to be observed down to 0.0005 W/cm² during experiments suggesting that an in vivo instrument could be designed well within the MPE limits.

### 4.3.3 Shape measurement configuration

To enable 3D analysis, it was necessary to measure the shape of the object, and for this an LCD fringe projection technique was used. The reason an LCD fringe projection technique was used over alternative shape measurement methods, was due to the fact the fringe projection technique could:
1. Be easily incorporated as part of the existing optical set-up without the need to move any parts.
2. Be used to generate a phase map detailing surface elevation for the object in the same position as it would be during ESPI and shearography measurement, making data combining during post processing simple and convenient.

A schematic of the shape measurement configuration is given in Figure 4-19. A projector (ASK C5 compact projector, resolution 1024x768) was connected to a computer then positioned to illuminate the test piece at an angle, and focussed onto the surface. The shutters at S2 and S3 were closed to block laser illumination and prevent shearing respectively. A bespoke shape measurement programme written in LabVIEW in partnership with Laser Optical Engineering (Shape Dect, LOE Ltd, Leicestershire, UK) was used to digitally generate sets of sinusoidal vertical fringes, provide phase stepping and capture data. Phase stepping was controlled by digitally shifting the projected fringes by $\frac{\pi}{2}$ radians, over a five-step process as was used for the ESPI and shear configurations as this enabled the same phase wrapping and unwrapping algorithms to be utilised.

The measurement process is summarised diagrammatically in Figure 4-20. It first involved measuring a flat reference object the same height as the top of the AAC and positioned in the same place, the data from this flat reference was unwrapped and stored as a reference. The AAC was then returned to its measurement position and the measurement process was
repeated, the data from this measurement cycle was then unwrapped and subtracted from the reference file. This subtraction process generated a phase data file in which the phase change was proportional to surface elevation and this file was saved.

![Diagram](image)

*Figure 4-20 – Visual summary of shape measurement procedure using the fringe projection technique.*

Gorthi et al (159) stated that for an off-axis optical set-up the measured phase change ($\Delta \phi_{(x,y)}$) can be related to the surface elevation at a specific point on the surface ($z_{(x,y)}$) via equation 4-4.

$$z_{(x,y)} = \frac{l_0 \Delta \phi_{(x,y)}}{\Delta \phi_{(x,y)} + 2\pi f_o d}$$

**Equation 4-4**

Where $l_0$ is the distance of the camera from the reference plane, $d$ is the distance between the entrance pupil of the camera and the exit pupil of the projector and $f_o$ is the spatial frequency of the projected fringe pattern. If $l_0$ is large compared to $h$ then the relationship can be approximated as linear and equation 4-4 can be reduced to equation 4-5 (159).

$$z_{(x,y)} = \frac{l_0 \Delta \phi_{(x,y)}}{2\pi f_o d}$$

**Equation 4-5**

Due to the fact the parameters $l_0$ and $d$ can be difficult to accurately quantify and that $f_o$ varies over the image plane due to the divergent nature of the projector, the simplest way to determine the relationship of the phase change to the surface elevation is via calibration. Since the
relationship can be presumed as linear, the relationship of $z_{(x,y)}$ to $\Delta \phi_{(x,y)}$ can be represented by equation 4-6 \(^{(159)}\), where $K_{(x,y)}$ is the calibration constant.

$$z_{(x,y)} = K_{(x,y)} \Delta \phi_{(x,y)}$$

Equation 4-6

Calibration was achieved by measuring the phase change between gauge blocks of different heights positioned in the imaging area, data was recorded 10 times for each gauge block height. Calibration data was processed in MATLAB via importing the data files obtained via the shape measurement software. The calibration constant was determined by measuring the phase difference between the gauge blocks of different heights and the reference surface, by taking the average phase change across the whole gauge block area for each repeated measurement cycle, the data from this calibration is plotted in Figure 4-21. As expected the relationship of the measured phase change was found to be linear. From this, the calibration constant ($K_{(x,y)}$) was determined as 0.9654 mm/rad.

The standard deviation in the measured phase change averaged over the area of the gauge block for a given elevation across repeated measurement cycles was found to be +/- 0.04 radians corresponding to a surface elevation measurement repeatability of 0.04 mm. However, due to image noise, the variability in the measured phase change across a given area of uniform height was +/- 0.21 radians, hence, the uncertainty in the surface elevation for any individual point was found to be +/- 0.20 mm.

![Figure 4-21 – Plot showing the relationship of surface elevation (mm) to measured phase change (radians).](image)

\[ y = 0.9654x \]
Once the calibration constant had been determined it was then used as detailed in equation 4-6, to convert the phase map into a map of surface elevation. An example of the elevation data plotted as a 3D plot is shown in Figure 4-22, for a simulation cornea positioned in the AAC.

![Figure 4-22 – 3D plot of surface elevation for a simulation cornea positioned in the AAC.](image)

Overall, the combination of the shape measurement system, and the interchangeable ESPI and shearography configurations meant that shape information, out-of-plane displacement data and it’s 1st derivative could all be obtained in quick succession without the need to move the object or make significant adjustments to the set-up. The way in which these data sets were used during the processing of results is detailed in the following section.

### 4.4 Data Analysis and Processing

The procedure used to estimate the in-plane components of deformation while assuming displacement occurred normal to the surface involved two main steps, these were:

1. Processing of the elevation data to determine the gradient of the surface at all points across the imaged area.
2. Using the surface gradient data in combination with the out-of-plane ESPI data to estimate in-plane displacement.

Each of these steps is described in detail in the following sections.

#### 4.4.1 Shape data processing

Firstly, the surface elevation data was processed to determine the gradient of the surface at each pixel. So that the elevation data could be successfully differentiated to obtain the surface gradient, smoothing was required to remove some of the noise. The noise was present due to the limitations imposed by the resolution of the projector which resulted in visible grid lines in the background of the projected fringes as shown in the image in Figure 4-23.
To reduce the noise and smooth the surface profile, a Gaussian filter was applied in MATLAB. The degree of smoothing was altered via changing the standard deviation of the Gaussian distribution ($\sigma$), a $\sigma$ value of 10 was found to be the minimum value for which an adequately smooth surface profile could be obtained that enabled differentiation.

It was found that errors could be introduced as a result of smoothing in areas where the gradient of the surface changed significantly from one area to the next, i.e. at the edge of the sample adjacent to the chamber. An example of the effects of data smoothing on the profile of a porcine cornea surface are shown for a central cross-section in Figure 4-24. It is evident from this example, that towards the edge area on the left of the image where the gradient of the surface changes significantly over a short distance the angle of the surface will be underestimated.

![Figure 4-23 – Image of projected fringes demonstrating the presence of visible gridlines.](image)

![Figure 4-24 – Plot showing the effects of smoothing on the measured phase change (radians) proportional to surface elevation along the central horizontal axis of a porcine cornea.](image)
After the image had been smoothed, the calibration constant that was determined from the plot in Figure 4-21, was used to convert the measured phase change into surface elevation. The surface elevation data was then differentiated with respect to both the x and y axes to obtain the respective surface gradient terms $\frac{\partial z}{\partial x}$ and $\frac{\partial z}{\partial y}$ in each of these directions over the whole surface area. The corresponding normal to surface angle ($\theta_N$) was then calculated at each pixel for the x and y directions respectively using equations 4-6 and 4-7.

\[
\theta_{Nx} = \tan^{-1} \frac{\partial z}{\partial x} \quad \text{Equation 4-6}
\]

\[
\theta_{Ny} = \tan^{-1} \frac{\partial z}{\partial y} \quad \text{Equation 4-7}
\]

### 4.4.2 Estimation of in-plane ESPI component

**Assuming deformation normal to the surface**

By assuming deformation occurred normal to the surface, the out-of-plane ESPI data and the surface elevation data could be used to estimate the corresponding in-plane components ($u$ and $v$) with respect to the x and y axes via equations 4-8 and 4-9 respectively.

\[
u = w \tan \theta_{Ny} \quad \text{Equation 4-9}
\]

**Using video data**

To obtain a more realistic estimation for the relationship between out-of-plane and in-plane deformation of the corneal surface in response to hydrostatic pressure variations, cross-sectional frame-subtracted video analysis was used. Video analysis was carried out via the following procedure:

1. The cornea mounted in the AAC was imaged from the side with the camera (piA1000-48-gm, Basler AG, Ahrensburg, DE) focussed on the central cross-section with respect to either the N-T or S-I axis.
2. Frame subtracted video data was captured of the cornea deforming in response to a pressure increase of greater than 90 mmHg (enough to cause a visible surface displacement).

The angle of surface deformation was then estimated by tracking the movement of several recognisable points on the corneal surface over a pressure change via software developed in...
LabVIEW. The resulting angle data was then substituted for the normal angle data in equations 4-8 and 4-9 to estimate the in-plane component based on the out-of-plane data along the horizontal and vertical central horizontal cross-sections respectively.

### 4.5 Chapter Summary

This chapter described the design of both the experimental rig, which incorporated a custom designed chamber and a hydraulic loading set-up; and the design of the optical measurement set-up, which incorporated an ESPI system, an LSI system and a LCD fringe projection system. A summary of the main outcomes of this chapter is given here.

The custom chambers that were manufactured offered several advantages compared to similar chambers used in previous interferometry studies on the cornea\(^{(45,51)}\), these were:

1. A leak-proof tissue clamping mechanism that could incorporate tissues of different thicknesses from approximately 0.1 mm to 3 mm, enabling both human and porcine corneas to be clamped using the same chamber.
2. Two clamping diameters of 12.5 mm and 15.7 mm, enabling human and porcine corneas to be clamped on the scleral region.
3. High stiffness which prevented any bending of the locking mechanism during clamping.
4. A rotatable metal positioning stand to enable positioning of the cornea at 90° enabling LSI data to be obtained with shear applied with respect to different corneal axes without the need to change the angle of the shearing mirror.
5. The rigid connectors and flexible PVC tubes that linked the chamber to the fluid supply were resistant to deformation over a 60 mmHg pressure range.

The design of the hydrostatic loading rig enabled the corneal specimens to be held under pressure representative of normal IOP while pressure changes of up to 3 mmHg could be introduced as desired. The automation of the hydrostatic loading rig meant the following could be achieved:

1. Repeatability in the positioning of the reservoir over a set movement time was +/- 0.0069 mm corresponding to a repeatability in pressure variation of +/- 0.0005 mmHg.
2. Uniform loading rate of +/- 0.058 mmHg/second across all experiments.
3. The ability to load the sample without the need to touch the set-up, thus preventing the introduction of noise in the data that could occur due to movement of the loading rig.

In addition to this, data capture was automated so the time between the end of loading and the time of data capture could be standardised across all experiments to ensure there was no variation in measurements due to a time dependent response. All these factors were important to enable the comparison of data recorded over different loading cycles.
The novel optical measurement set-up described in this chapter combining an out-of-plane ESPI system, an out-of-plane LSI system and a fringe projection based shape measurement system enabled:

1. Full surface measurement of the out-of-plane displacement of the corneal surface with a sensitivity of less than 0.27 µm, using ESPI.
2. Direct full surface measurement of the rate of change of the out-of-plane displacement of the corneal surface using LSI.
3. The ability to generate a map of corneal surface elevation and hence account for any effects on the measured data introduced due to the non-flat nature of the cornea.
4. The ability to directly compare data obtained via ESPI and LSI on the same specimen and hence evaluate the sources of error that may contribute to inaccuracies in the approximation of the rate of change of out-of-plane displacement measured via LSI.
5. The ability to estimate the in-plane component of displacement using the out-of-plane data and shape data and hence approximate 3D surface deformation.

The overall method described offered significant improvements over the methods used in previous interferometry studies on the cornea, where only the out-of-plane components of deformation were analysed and the effects of the corneal curvature were not considered. Also, in previous shearography studies RSI was used and hence the full-field movement of the corneal surface was not analysed with respect to all directions or regions.

The ability to compare ESPI and LSI data measured on the same specimen is a particularly important feature as there remain several unknowns when it comes to the use of LSI to measure objects with non-flat geometry under complex loading conditions. These factors require evaluation if LSI is to be used as a standalone measurement tool for corneal analysis.

Following the method development stage, several further experiments were conducted prior to experiments on human corneas. The first set of experiments were performed on non-biological samples and are detailed in the following chapter. The purpose of these experiments was to validate that the measurement system was performing as expected, determine the repeatability of the measurement rig, to investigate the effects of curvature on the measured data, to determine if the measurement techniques could detect topographical changes to stiffness and to generate data with which the cornea data could be compared. The second set of experiments were performed on porcine corneas. These are detailed in chapter 6. The purpose of these experiments was to optimise the measurement technique for human cornea experiments, to identify any issues with regards to corneal measurement, to determine if the techniques could be used to measure the changes introduced by topographic cross-linking and to generate a suitable corneal testing methodology.
5. Experiments of non-biological samples

5.1 Introduction

A range of experiments were carried out on non-biological samples. The first set of experiments on non-biological samples were conducted on simple flat samples and are detailed in the following section. The aims of these experiments were as follows:

- To determine the working parameters of the optical set-up.
- To check that measurements on simple objects were in-line with what was expected.
- To determine the repeatability of the measurement system.

The set of experiments following the initial experiments on simple flat surfaces, were concerned with investigating if an initial curvature of the test surface introduced obvious distortions to the measured results, the results from these experiments are described in section 5-3. Alongside investigating the effects of curvature, the aims of these experiments were:

- To provide examples of complex fringe distributions for both the ESPI and LSI case.
- To trial the full measurement technique incorporating the shape measurement system for evaluation of the in-plane component.

The final set of experiments on non-biological samples are detailed in section 5-4. These experiments were focussed on both, determining whether the ESPI and LSI techniques described could detect stiffness changes introduced in different topographical locations; and generating simple data with which the cross-linked corneal data could be compared.

Except for one set of experiments that was conducted using a specifically designed test piece, all experiments on non-biological samples were carried out on samples mounted in the AAC and loaded hydrostatically as described in section 4.2.3, this was to ensure all aspects of testing replicated as closely as possible the methods used to examine corneas. The specific magnitude of pressure variations used during each investigation were modified based upon the properties of the samples being tested and the acquisition of an adequate number of fringes and are detailed for individual experiments where necessary.

Descriptions of each of the samples that were used in the non-biological experiments detailed in this chapter are given in Table 5-1. All of the samples were cut into circular sections of 18 mm diameter and made of waterproof rubber based material, with thicknesses of between 0.5 mm and 2.0 mm, so that they could be hydrostatically loaded in the AAC and would deform under pressure variations of 3 mmHg or less.
<table>
<thead>
<tr>
<th>Sample ref no.</th>
<th>Description</th>
<th>Thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Uniform, flat samples cut from the sleeve of a rubber glove (Marigold Large Kitchen Gloves, Freudenberg, Weinheim, DE).</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>Uniform, curved silicone samples ((r_c = 15 \text{ mm})) cut from hemispherical chocolate moulds (Mini Semisphere Mould, Silikomart, Venezia, IT)</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>Non-uniform curved samples cut from the fingertip (thumb) of the same rubber glove as used in sample no. 1.</td>
<td>0.5 – 1.0</td>
</tr>
<tr>
<td>4</td>
<td>Non-uniform rubber samples manufactured using liquid silicone (Unibond white sealant, Henkel AG &amp; Co, Düsseldorf, DE) set between contact lens moulds to have similar curvature to the human cornea</td>
<td>0.4 – 2.0</td>
</tr>
</tbody>
</table>

Table 5-1 – Description of samples used for non-biological experiments.

5.2 Experiments to examine measurement rig capabilities and limitations

5.2.1 Determining suitable range of shear magnitudes for experiments

Initial experiments were conducted using the LSI method to determine the range of shear that could be introduced. As previously discussed in section 3.6.4 of chapter 3, it is advantageous to keep the magnitude of shear small as this results in greater accuracy in the approximation of the first derivatives of displacement, however, at the same time, fringe quality must be maintained and the sensitivity must be adequate to detect deformation under small pressure changes.

As it was not yet known what the ideal range of sensitivity was for corneal testing, experiments were conducted on non-biological samples to determine the minimum possible value of shear that could be used while still maintaining fringe quality. To do this, the response of a range of different samples were recorded using the LSI technique, while tilting the mirror used to apply the shear by different amounts between successive loading cycles to achieve a shear magnitude of 20 pixels to 100 pixels in 5 pixel increments.

From these experiments, it was found that the fringe quality was poor below shear magnitudes of 50 pixels, equivalent to an actual distance of 1.04 mm. Fringe quality was adequate at 50 pixels but continued to improve slightly over the range of 50 pixels to 65 pixels. This is demonstrated in Table 5-2, which shows the wrapped phase data recorded when using shear magnitudes of 30 pixels (0.625 mm), 50 pixels (1.04 mm) and 65 pixels (1.35 mm) and
recording the response of a silicone rubber sample with similar curvature to the human cornea (ref. 1, Table 5-1) deforming in response to a 0.50 mmHg pressure increase.

<table>
<thead>
<tr>
<th>Shear magnitude (pixels)</th>
<th>30</th>
<th>50</th>
<th>65</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wrapped fringes</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Table 5-2 – Deformation of a rubber sample subjected to hydrostatic loading measured using LSI with different magnitudes of shear applied with respect to the horizontal axis.

Based on these results it was decided to use a shear magnitude of greater than 50 pixels for all experiments. The exact amount of shear that would be used in the corneal experiments would be determined based upon the required sensitivity to detect deformation within the physiological range of pressure changes, while minimising the shear as far as possible to maintain high accuracy.

5.2.2 Experiments to check optical measurement configuration

**ESPI Configuration**

A set of experiments were conducted as a ‘sanity check’ to determine if the set-up was giving the expected measurement values. For these experiments a test piece was designed where a predictable displacement could be introduced, a drawing of this test piece is shown in Figure 5-1. The test piece comprised of a 1 mm thick aluminium plate that was clamped around a 20 mm circular section. A 6 mm diameter ball bearing was positioned between the centre of the plate and a piezo actuator (PA8-12, Piezosystems jena GmbH, DE), this enabled a point load to be applied to the centre of the plate to deform it by a specific amount. The movement of the piezo used to introduce the displacement of the plate was determined via interpretation of the voltage vs displacement curve supplied by the piezo actuator manufacturer, this is included in appendix A.

The following procedure was used during experiments to check the ESPI system. Firstly, to ensure the ball bearing was in contact with the test plate prior to measurement a pre-load was applied until deformation fringes were visible on the live difference image. Once contact had been established the difference image was refreshed and the reference data was captured. The piezo was then set to introduce a displacement of 1 µm to the centre of the metal plate,
by supplying the voltage determined from the voltage-displacement curve. Data was captured at the end of loading. This process was repeated over ten cycles with the optical system in the ESPI configuration so the repeatability of loading could be assessed. The results were processed to convert the measured phase change to displacement. The averaged results recorded over the ten loading cycles detailing the out-of-plane displacement are plotted in Figure 5-2.

![Diagram of test piece](image)

*Figure 5-1 – Drawing of the test piece used for ‘sanity check’ experiments of the ESPI configuration.*

![Graph of out-of-plane displacement](image)

*Figure 5-2 – Plot of the average out-of-plane displacement (w) of the surface of the metal plate loaded via a piezo actuator to introduce a displacement of 1 µm measured via ESPI over 10 repeated loading cycles.*

The average peak value of measured out-of-plane displacement obtained via the ESPI technique was 0.99 µm (SD +/- 0.01 µm) which was close to the expected displacement of 1
µm estimated from the piezo voltage-displacement curve, suggesting that the ESPI configuration was performing as expected.

The results from the experiments with the test piece shown in Figure 5-1 do not indicate the accuracy of the optical set up, as the magnitude of the piezo displacement was not confirmed using an alternative technique, and interpretation of the piezo voltage displacement curve supplied by the manufacturer could only be achieved to an accuracy of +/- 10%. The tests were performed as an initial check to see if the technique was giving the expected results. To obtain detailed information about the absolute accuracy of the system, the results obtained here would have to be compared with those obtained using the same test piece with a previously calibrated interferometer or another very high sensitivity measurement technique, or further tests would be required using a calibrated test piece where a known displacement could be introduced.

**LSI configuration**

To check that the LSI configuration was also giving the expected results, the data obtained via shearography could be compared with differentiated ESPI data so long as the measurement procedure was repeated exactly for each case. This was performed across a range of samples during testing.

To numerically compare the shear results with the ESPI results, the ESPI data was differentiated to give \( \frac{\partial w}{\partial x} \), and \( \frac{\partial w}{\partial x} \) was calculated from the LSI phase data using equation 5-1.

\[
\frac{\partial w}{\partial x} = \frac{\lambda}{4\pi \delta x} \Delta \phi_{def}
\]

Equation 5-1

An example of the out-of-plane displacement derivative data obtained from the differentiated ESPI data and from the LSI data for a flat rubber sample (ref. 1, Table 5-1), responding to a 0.20 mmHg increase in hydrostatic pressure, is shown in Figure 5-3. Data was compared when using a shear of 52 pixels (1.08 mm) and when using a shear of 95 pixels (1.98 mm) to reflect the range of shear values used during the experiments detailed in this thesis.

Overall, it was found that there was good agreement between the LSI data and the differentiated ESPI data for both magnitudes of shear used, with less than 1% difference in the measured values of maximum rate of change of displacement. This confirmed that the LSI system was giving the expected data relative to the ESPI system.
Figure 5.3 – Plot comparing $\frac{\partial w}{\partial x}$ obtained via differentiation of ESPI data and via LSI with shear magnitudes of 52 pixels and 95 pixels along the central axis of a flat rubber sample subjected to a 0.20 mmHg increase in pressure.

However, although the agreement between the ESPI and LSI data proved to be good for the case of a uniform flat rubber sample across the range of shear values used, this did not confirm that the LSI data would be this accurate for all samples tested due to the fact the accuracy of the LSI approximation is dependent upon how an object deforms, and the magnitude of the second derivative of out-of-plane displacement as discussed in section 3.6.4 of chapter 3. Therefore, specific analysis would be required on individual samples to determine the accuracy of the approximation of $\frac{\partial w}{\partial x}$ or $\frac{\partial w}{\partial y}$ measured via the LSI technique.

The data was compared via differentiating the ESPI data rather than integrating the LSI data, due to the fact the integration process is cumulative, so any errors present at any point along the line of integration are propagated along the entire integrated area. An example of the ESPI data and the integrated LSI data (shear 52 pixels) compared for a central cross-section across a flat rubber sample subjected to a pressure increase of 0.20 mmHg is given in Figure 5-4. It was found that good agreement between the ESPI data and integrated LSI data could be achieved from the data obtained when measuring the response of a flat rubber sample at low values of shear, however this was due to the fact the fringe quality obtained during measurement was high and the 2nd derivative of out-of-plane displacement was low across the sample.
In addition to any errors that could be introduced into the LSI data due to increases in the 2nd derivative of out-of-plane displacement, the calculation of \( \frac{\partial w}{\partial x} \) or \( \frac{\partial w}{\partial y} \) was also prone to errors if there were inaccuracies in the quantification of the applied shear. It was found that relatively small errors in the measurement of the applied shear could result in small changes to the calculated values of \( \frac{\partial w}{\partial x} \) or \( \frac{\partial w}{\partial y} \), and if integrated these small changes could result in significant changes to the predicted displacement due to the cumulative effect, an example is shown in Figure 5-5, where the input value of shear magnitude was changed from 50 pixels to 56 pixels in 2 pixel increments. Actual shear magnitude was 52 pixels. An error in the shear magnitude of 6 pixels in this case resulted in a 12% error in the calculated value of peak displacement.

Figure 5-4 – Plot comparing the out-of-plane displacement component calculated from the data obtained via ESPI and LSI along the central axis of a flat rubber sample subjected to a hydrostatic pressure increase of 0.20mmHg.

Figure 5-5 – Plot demonstrating the effects of inaccuracies in the quantification of applied shear on the measured value of out-of-plane displacement when obtained from the integrated LSI data.
In addition to these errors which affected the accuracy of the approximation of \( \frac{\partial w}{\partial x} \), further errors could be introduced specifically during integration due to inaccuracies in masking. Masking involves isolating a specific region of interest in an image so that all values outside of this area are set to zero. Using the data capture software described in section 4.3.1 it was possible to apply a mask before capturing data so data was only captured in the region of interest, a mask could also be applied during post-processing to isolate a specific area of interest. With respect to integration inaccurate masking could result in the inclusion of data from the outside of the measurement area, or the discounting of data from inside of the measurement area. These errors would not affect the accuracy of the approximation for \( \frac{\partial w}{\partial x} \) or \( \frac{\partial w}{\partial y} \) at a given point unless immediately adjacent to the mask, but would prevent the accurate determination of \( w \) from the integrated LSI data.

5.2.3 Loading rig repeatability

To determine the repeatability of measurement that could be achieved with the specific set-up and loading regime described, in an ideal situation, experiments were conducted on a flat rubber sample (ref. 1, Table 5-1) positioned within the artificial anterior chamber. A flat rubber sample was used as opposed to a cornea when examining the repeatability of the measurement set-up, as a flat rubber sample could be expected to behave consistently over repeated loading cycles, whereas this could not be guaranteed with a cornea.

To determine the repeatability that could be achieved over repeated loading cycles, the response of the rubber sample was measured with the set-up in the LSI configuration while the rubber sample was subjected to ten repeated loading and unloading cycles where the pressure was increased from 16.50 mmHg by 0.15 mmHg. Measurement was recorded one second after the maximum pressure was reached, after which the load was removed to return the sample to baseline pressure and the process was repeated 10 times. The data obtained was then processed in MATLAB to determine the standard deviation in the measured response over the 10 cycles.

Figure 5-6 shows the average measured phase change that occurred in response to loading across a central horizontal cross-section of the rubber sample, with the dashed lines corresponding to the standard deviation in the response. The magnitude of standard deviation shown in Figure 5-6 for the central horizontal cross-section was typical of all cross sections taken across the sample. Overall the repeatability of measurement was found to be high with an average standard deviation in the measured phase change of +/- 0.3187 radians, with a
percentage standard deviation of +/- 2.0% with respect to the maximum measured phase change.

![Figure 5-6](image.png)

*Figure 5-6 – Plot showing the average phase change and the standard deviation in the phase change recorded along the central axis of a rubber sample over 10 repeated loading cycles.*

During experiments on corneas it was required to move the chamber out of the measurement area after an initial set of measurements so cross-linking could be carried out. The chamber was then repositioned in the test area and a second set of measurements were recorded to examine the effects of cross-linking. Hence, it was of interest to determine if this movement of the chamber had any effect on the repeatability of the data.

To move the chamber, both the chamber and chamber stand were moved out of the measurement area via sliding of the base plate positioned on the optical table. The chamber and stand were moved as a whole, as opposed to just the chamber individually to prevent any large pressure changes in-between sets of experiments and to minimise any disturbances to the test piece.

To investigate the effects of moving the chamber in and out of the measurement area on the repeatability of the data, experiments were carried out where the response of a flat rubber sample was measured using LSI over 10 repeated loading cycles where the hydrostatic pressure was increased by 0.15 mmHg, with the chamber removed from the measurement area and repositioned between loading cycles. The results of these experiments are shown in Figure 5-7, which shows the average measured phase change obtained over the 10 measurement cycles for a central horizontal cross-section of the rubber sample, with the dashed lines representing the standard deviation in the measured response.
The percentage standard deviation with respect to the area of maximum measured phase change in the experiments where the chamber was moved between each repeated loading cycle was approximately +/- 2.6%. The increase in the variability of the response compared to the case where the chamber was not moved in between loading cycles, was likely a result of small misalignments introduced between each repeated measurement due to inaccuracies in the repositioning of the chamber. The alignment was based on the position of holes in the base plate which lined up with holes in the optical table through which screws were inserted to secure it into position.

Overall these experiments demonstrated that it was possible to achieve highly repeatable measurement over repeated loading cycles confirming that the repeatability of loading and measurement was high. This level of repeatability meant it was possible to compare the response of samples measured over separate loading cycles and be confident that any changes above the levels demonstrated here were due to changes in the response of the sample.

5.3 Experiments on samples with non-flat geometry

During initial testing on porcine corneas using both ESPI and LSI the interference fringes in the wrapped data showed an unfamiliar distribution. An example of the typical ESPI and LSI
Fringe distributions obtained from a porcine cornea subjected to a 0.50 mmHg increase in hydrostatic pressure are shown in Figure 5-8.

![ESPI, Horizontal LSI, Vertical LSI](image)

*Figure 5-8 – Examples of the typical fringe distributions observed when measuring the response of a porcine cornea to a 0.50 mmHg increase in hydrostatic pressure.*

The observed fringe distributions did not reflect the expected response, which was that the measured out-of-plane displacement would increase from zero at the fixed boundary to a maximum around the central area, furthest from the boundary. Hence, the ESPI fringes were expected to show a circular or elliptical distribution, and the LSI fringes were expected to form approximately semi-circular fringes either side of the centre line with respect to the direction of shear.

Since there was a lack of data available in the literature describing the full field response of the porcine cornea to small pressure changes, there was limited data with which the results could be compared to determine if this measured response was normal. There was also a lack of validated data reporting the use of ESPI and LSI techniques to measure displacement on non-flat objects. Overall, this lead to concerns that the observed fringe distributions were a result of distortions that could be introduced when trying to apply the measurement techniques to complex curved objects that deformed in more than one plane, or a result of the interaction of the curved sample with the edge of the chamber during loading.

To investigate this, experiments were carried out on non-biological samples with non-flat surface geometry prior to further testing on porcine corneas to gain a greater understanding of the effects of an initial object curvature. Two main sets of experiments were conducted on objects with non-flat surface geometry positioned in the AAC, these were:

1. Experiments on a homogenous curved membrane expected to exhibit uniform behaviour.
2. Experiments on non-homogeneous samples with non-flat surface geometry expected to exhibit non-uniform behaviour.

The results of these experiments are detailed in the following section.
5.3.1 Experiments with uniform curved rubber sample

In the initial set of experiments on objects with non-flat geometry, the measured response of a uniform curved silicone sample (ref. 2, Table 5-1) to a hydrostatic pressure increase was measured using ESPI and LSI and compared with that of a flat rubber sample (ref. 1, Table 5-1). The aim of these experiments was to identify any visual differences in the ESPI and LSI data between the two samples, and to determine whether the data measured on the curved rubber sample showed the expected distribution.

Method

The curved sample was positioned within the AAC and set under an initial pre-load of 16.50 mmHg. The pressure was increased by 1 mmHg during each measurement cycle, as this pressure change was found to generate an adequate number of fringes for analysis. To generate data to compare with the curved rubber sample, similar experiments were conducted on the flat rubber sample. In this case, the sample was set under the same pre-load of 16.50 mmHg but pressure was increased by only 0.20 mmHg over a loading cycle, this was due to the fact the material properties of the flat sample and the curved sample were not the same and it was desired to generate a similar number of fringes so their distribution could be compared. Finally, to provide some visual data with which the ESPI and LSI could be compared for the case of the curved rubber sample, a cross-sectional frame subtracted video was recorded while subjecting the membrane to an increase in hydrostatic pressure substantial enough to cause the membrane to visibly deform.

Results and Analysis

The wrapped and unwrapped data obtained during measurement of both the flat and curved samples is shown in Table 5-3 and Table 5-4 for ESPI and LSI respectively. Except for slight difference in fringe numbers, there appeared to be no obvious visual differences in the distribution of the ESPI and LSI interferograms obtained from the flat rubber sample and the curved rubber sample, suggesting that the presence of a small initial curvature did not result in obvious visual changes to the appearance of the data.

The ESPI fringes visible in the wrapped data for both the flat and curved samples in Table 5-3 appeared as approximately circular fringes forming around the centre of the object. The unwrapped phase maps were as expected, indicating zero displacement at the edge (black) where the fixed boundary was imposed and peak displacement at the centre point furthest from the boundary (white).
Table 5-3 – Comparison of the response of a uniform flat sample and a uniform curved sample to an increase in hydrostatic pressure, measured via ESPI.

<table>
<thead>
<tr>
<th>Flat</th>
<th>Curved</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wrapped ESPI</strong></td>
<td><img src="image1" alt="Flat ESPI" /> <img src="image2" alt="Curved ESPI" /></td>
</tr>
<tr>
<td><strong>Unwrapped ESPI</strong></td>
<td><img src="image3" alt="Flat ESPI" /> <img src="image4" alt="Curved ESPI" /></td>
</tr>
</tbody>
</table>

Table 5-4 – Comparison of the response of a uniform flat sample and a uniform curved sample to an increase in hydrostatic pressure, measured via LSI (shear with respect to horizontal axis).

<table>
<thead>
<tr>
<th>Flat</th>
<th>Curved</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wrapped LSI</strong></td>
<td><img src="image5" alt="Flat LSI" /> <img src="image6" alt="Curved LSI" /></td>
</tr>
<tr>
<td><strong>Unwrapped LSI</strong></td>
<td><img src="image7" alt="Flat LSI" /> <img src="image8" alt="Curved LSI" /></td>
</tr>
</tbody>
</table>

The corresponding LSI fringes visible in the wrapped data in Table 5-4 were approximately semi-circular in appearance and distributed approximately symmetrically either side of the object centre for each case, with the unwrapped phase map indicating a gradient change to the surface in opposing directions either side of the centre, as expected. These results confirmed that the initial curvature of the test surface could account for the fringe distributions seen when examining porcine corneas.
To check that the ESPI data was showing the expected distribution of displacement for the curved sample, a frame-subtracted video was recorded of the central cross-section of the sample deforming under a larger change in hydrostatic pressure to enable a visual comparison. A screenshot from this video is given in Figure 5-9, the lighter section in the screenshot is the area over which the surface moved from its initial to its final position under the pressure change. The measured out-of-plane displacement of the sample in response to a 1 mmHg increase in hydrostatic pressure recorded via ESPI of the central horizontal cross-section is also given in Figure 5-9 for comparison.

The appearance of the ESPI data appeared to be in agreement with the profile of deformation shown in the video screenshot. Both sets of data showed maximum displacement at approximately the central region. In a region either side of the centre the out-of-plane displacement decreased very gradually. Approximately half-way between the centre and the boundary on either side the out-of-plane displacement started to decrease more rapidly, exaggerated by the fact there was a gradually increasing in-plane component to the overall response, and at the boundary the displacement returned to zero.
To examine whether there were specific distortions introduced into the LSI data relative to the ESPI data, $\frac{\partial w}{\partial x}$ that was calculated from the differentiated ESPI data was compared with $\frac{\partial w}{\partial x}$ calculated directly from the measured LSI data. Figure 5-10 shows a comparison of these two data sets across a central horizontal cross-section of the curved silicone sample. It was found that the agreement between the differentiated ESPI and LSI data was similar to that shown previously in Figure 5-3 for an initially flat rubber sample. Suggesting the presence of a small initial curvature did not introduced obvious distortions with respect to the LSI data.

Figure 5-10 – Comparison of $\frac{\partial w}{\partial x}$ calculated from the differentiated ESPI data and from the LSI data recorded across a central cross-section of the curved silicone sample

**Conclusions**

Although the curvature of the curved silicone sample was less steep than that of human and porcine corneas which have been reported to have radii of curvature of 8.45 mm \(^{(160)}\) and 7.79 mm \(^{(161)}\) respectively. The absence of any obvious distortion to the measured data demonstrated that the data was in line with what had been expected and the curved sample was not interacting with the edge of the chamber during loading. Based on this, the appearance of the fringe distributions observed when initially examining porcine corneas could not be accounted for by distortion introduced by the initial object curvature.

In addition to this, the measured results from the curved silicone sample appeared to reflect the profile of out-of-plane displacement recorded during video analysis, suggesting the results observed on porcine corneas likely reflected the deformation of porcine corneas in terms of the out-of-plane displacement component in response to a hydrostatic pressure increase.
5.3.2 Experiments with curved samples exhibiting non-uniform behaviour

Experiments were conducted on a range of objects expected to exhibit non-uniform behaviour due to their non-homogenous nature. The specific aims of these experiments were as follows:

- To examine samples with a similar or steeper curvature than the cornea using ESPI and LSI to establish if the measured behaviour was as expected based on comparisons with data recorded via video analysis.
- To provide examples of more complex fringe distributions and to evaluate what the measured data indicates.
- To determine if areas of defects could be clearly recognised from the ESPI and LSI data.
- To trial the method described in chapter 4 to determine the in-plane component of deformation using the shape data and the measured out-of-plane data while assuming deformation normal to the surface.

The non-uniform curved samples used during testing included:

1. A selection of finger tips from rubber gloves (ref. 3, Table 5-1) – these samples were chosen as they had a relatively steep curvature when compared to both the porcine corneas and human corneas and fit well into the AAC. The dip-moulding manufacturing processes used to make the gloves and the specific texturing also meant that they were non-uniform.

2. Curved white silicone samples (ref. 4, Table 5-1) – these samples were made using contact lens moulds so they had a similar curvature to the human cornea but non-uniform thickness. They were thicker in the domed section (~2 mm) and thinner around the base of the dome at the point where the surface went from curved to flat (~1 mm). On each of the samples there was a particularly thin area (~0.5 mm) on one side at the base of the dome which was expected to be weaker than the surrounding areas.

The same testing methodology as described for the uniform curved sample was used. In this case, the shape of the samples was measured using the shape measurement technique as described in section 4.3.3 of chapter 4 before the ESPI and LSI measurements were conducted.

**Interpretation of ESPI and LSI data on non-uniform curved sample**

The raw data in the form of wrapped and unwrapped phase plots obtained via both ESPI and LSI for a rubber glove tip deforming due to an increase in hydrostatic pressure are shown in Table 5-5.
Table 5-5 – Wrapped and unwrapped data recorded via ESPI and LSI of the response of a rubber glove tip to an increase in hydrostatic pressure.

For the glove tip example, the ESPI fringes showed a non-uniform distribution, being more concentrated towards the bottom of the sample, indicating that the greatest out of plane displacement occurred in this region. They were not circular in appearance as the object was not deforming by equal magnitudes in the horizontal and vertical axis. A 3D plot of the measured out-of-plane displacement component \((w)\) calculated for the ESPI data is shown in Figure 5-11, to highlight these features.

Figure 5-11 – Plot of out-of-plane displacement component \((w)\) calculated from the ESPI data recorded for a glove tip deforming due to an increase in hydrostatic pressure.
For the uniform samples shown previously, the LSI fringes with respect to the horizontal and vertical sensitivity directions have been identical in appearance and distribution but rotated through 90° with respect to one another, indicating an equal rate of change for the out-of-plane displacement component in each of these directions. For the case of the glove tip, the LSI fringe distributions for the horizontal and vertical sensitivity were non-similar due to the rate of change of the out-of-plane displacement component varying with respect to each of these directions.

The areas on the ESPI wrapped data images where the fringes are concentrated, indicate the areas with the greatest rate of change for the out-of-plane displacement component. These are the areas in the LSI wrapped data images that the fringes are centred around. A detailed explanation of the LSI fringe distribution for horizontal and vertical sensitivity is given in relation to the numbered areas in both the wrapped data images and the plots through a central cross-section in the x and y axes shown in Figure 5-12.

![Figure 5-12](image)

**Figure 5-12** – Deformation of a rubber glove tip in response to an increase in hydrostatic pressure measured via LSI. a) wrapped data recorded via LSI with shear applied with respect to the horizontal axis, b) line plot of $\frac{\partial w}{\partial x}$ along section A-A, c) wrapped data recorded via LSI with shear applied with respect to the horizontal axis but with the object rotated through 90°, d) line plot of $\frac{\partial w}{\partial y}$ along section B-B.

**Horizontal case:**

1. In this area, there is a steep increase in the magnitude of $w$ over a short distance in the $x$ direction with respect to the direction of shear, hence maximum $\frac{\partial w}{\partial x}$. For an initially flat object this would indicate steepening of the gradient of the surface in this area, however
this may not be the case for an initially curved object due to the variable contributions of the out-of-plane and in-plane displacement components describing total movement.

2. The magnitude of $w$ stops increasing for neighbouring points along the $x$ axis, and starts to decrease slightly resulting in a negative $\frac{\partial w}{\partial x}$. Since the magnitude of $w$ is not changing rapidly over the $x$ distance the fringes are not concentrated across this area. This indicates a slight flattening of the initial curvature of the object.

3. The magnitude of $w$ is rapidly decreasing for neighbouring points along the $x$ axis and returns to zero at the boundary hence a high fringe concentration and negative $\frac{\partial w}{\partial x}$.

**Vertical case:**

1. In this area, there is a steep increase in the magnitude of $w$ over a short distance with respect to direction of shear in the $y$-axis hence maximum $\frac{\partial w}{\partial y}$ and a high fringe concentration.

2. The magnitude of $w$ decreases over neighbouring points in the $y$-axis, resulting in a slightly negative $\frac{\partial w}{\partial y}$.

3. The magnitude of $w$ increases very slightly over neighbouring points in the $y$-axis, resulting in a slightly positive $\frac{\partial w}{\partial y}$.

4. The magnitude of $w$ decreases slightly over neighbouring points in the $y$-axis back to zero at the boundary, and hence a slight negative $\frac{\partial w}{\partial y}$.

**Comparison of ESPI and LSI data with recorded deformation**

The deformation of a curved silicone sample that was created using contact lens moulds to have a similar curvature to the cornea (ref. 4, Table 5-1), was examined using ESPI and LSI and also via video analysis, to assess whether the ESPI and LSI data for the non-uniform curved silicone samples was as expected.

Table 5-6 shows the raw data in the form of wrapped and unwrapped phase plots obtained via both ESPI and LSI for a silicone sample deforming due to a 0.20 mmHg increase in hydrostatic pressure. For the silicone sample, the fringes shown in the wrapped ESPI data were elliptical and concentrated towards the left side, indicating that out-of-plane displacement was greatest in this area relative to other areas. The non-circular nature of the fringes indicated that the rate of change of the out-of-plane displacement component was different across the $x$ and $y$ axes, and this was confirmed by the slightly different distribution of fringes observed for the LSI data when the sample was measured in the horizontal and vertical orientation. This distribution was as expected based on initial examination of the material, as the material was thinnest in the
area on the left side where the curved dome met the flat base, and this corresponded to the region of highest fringe concentration.

<table>
<thead>
<tr>
<th></th>
<th>ESPI</th>
<th>LSI – horizontal orientation</th>
<th>LSI – vertical orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wrapped</td>
<td><img src="image1" alt="Wrapped ESPI" /></td>
<td><img src="image2" alt="LSI horizontal orientation" /></td>
<td><img src="image3" alt="LSI vertical orientation" /></td>
</tr>
<tr>
<td>Unwrapped</td>
<td><img src="image4" alt="Unwrapped ESPI" /></td>
<td><img src="image5" alt="Unwrapped LSI horizontal orientation" /></td>
<td><img src="image6" alt="Unwrapped LSI vertical orientation" /></td>
</tr>
</tbody>
</table>

Table 5-6 – Wrapped and unwrapped data recorded via ESPI and LSI of the response of a silicone sample with a similar curvature to the human cornea to a 0.20 mmHg increase in hydrostatic pressure.

To generate data with which the measured response could be compared, the deformation of the silicone sample was video recorded along the cross-section highlighted in Figure 5-13(a) in response to a relatively large change in pressure. A screenshot from this frame subtracted video recording is shown in Figure 5-13(b) where the lighter area represents the area over which the surface moved during the pressure increase.

Figure 5-13 – a) Position of cross-section across which deformation was video recorded shown relative to the wrapped ESPI data, b) screenshot taken from the video recording of the deformation of the silicone sample in response to an increase in hydrostatic pressure.

The screenshot from the cross-sectional video analysis confirmed that the object deformed by a larger amount on the left side when compared to the right. Because the screenshot detailed the 2D deformation, both in-plane and out-of-plane, and there was a lack of recognisable points on the surface that could be tracked from low to high pressure, a detailed comparative analysis of the measured out-of-plane component and that estimated from the screenshot was not possible but a general visual comparison could be carried out.
A plot of the measured out-of-plane displacement component, and the measured rate of change of the out-of-plane displacement component calculated directly from the ESPI and LSI data respectively, for approximately the same cross section as detailed in the video analysis are shown in Figure 5-14. The numbered areas relate equivalent points of interest in each of the data sets.

In the ESPI plot shown in Figure 5-14(a) area 1 corresponds to the region where \( w \) is increasing significantly over a short distance in \( x \), the corresponding area is labelled in the LSI plot shown in Figure 5-14(b) and is the area where \( \frac{\partial w}{\partial x} \) is a maximum. The equivalent area is highlighted in the cross-sectional image in Figure 5-14(c) and can be seen to be the region where the gradient of the surface increases the most after increasing the hydrostatic pressure. Although the observed gradient change in the image is a product of both in-plane and out-of-plane deformation, it is reasonable to assume that this is the area over which \( w \) is increasing at the greatest rate with respect to \( x \).

The position of area 2 in the ESPI plot is the area over which \( w \) remains relatively constant with respect to \( x \) over a short distance, hence the equivalent area in the LSI plot is the region...
where $\frac{\partial w}{\partial x}$ is close to zero. For the equivalent area in the cross-sectional image, the position of the surface changes but the gradient of the surface does not appear to change by a noticeable amount before and after increasing hydrostatic pressure. As the angle of the surface with respect to the image plane does not change by a large amount over this specific area, the contribution of the out-of-plane component to overall displacement could be assumed to proportionally constant, therefore the lack of surface gradient change would indicate a constant $w$ with respect to $x$, which agrees with measured ESPI and LSI data.

Area 3 in the ESPI plot is the area where $w$ is gradually decreasing with respect to $x$, this corresponds to the area in the LSI plot where $\frac{\partial w}{\partial x}$ is slightly negative. A gradual decrease in overall displacement is evident in the corresponding area in the cross-sectional image. Across most of this area the measured value of $w$ is likely to be approximately equivalent to the out-of-plane displacement with respect to the surface normal, as the surface is not at a steep angle with respect to the image plane, any in-plane contribution to total displacement could be assumed to be relatively minimal.

Towards the right-hand side of area 3 the gradient of the surface becomes relatively steep. In this area, it is likely the decrease in $w$ will be greater than the decrease in overall out-of-plane displacement with respect to the surface normal, as the in-plane component could be assumed to have a gradually increasing contribution to overall displacement.

In the ESPI plot, area 4 represents the area where there is a steep decrease in $w$ over a short distance in $x$ and therefore the maximum negative $\frac{\partial w}{\partial x}$ which is shown in the equivalent area in the LSI plot. On the cross-sectional image $w$ decreases back to zero in this area but there appears to be a step half way along over which the amount of displacement decreases almost instantaneously.

Overall despite the curvature of the sample the ESPI and LSI results appear to be in visual agreement with the data recorded using video analysis, again confirming that the unfamiliar fringe distributions that had been observed on porcine corneas likely reflected the out-of-plane deformation of the cornea in response to loading.
Estimation of in-plane component assuming deformation normal to the surface

3D analysis of surface deformation was conducted on the silicone sample with similar curvature to the human cornea. To determine an initial approximation for the in-plane component of displacement the following assumptions were made:

1. The sample was approximated as a pressure vessel of uniform thickness and hence the angle of the inner surface was considered equivalent to the outer surface.
2. For a given point the axial and circumferential stiffness of the sample was equivalent, hence deformation under hydrostatic loading occurred normal to the surface (radial expansion).

Based on these assumptions an estimation for the in-plane component of displacement and the radial component of displacement could be estimated using the measured out-of-plane component data, alongside the elevation data as detailed in section 4.4 of chapter 4. Obviously due to the known variation in thickness of the sample the first assumption outlined above was not valid, however the purpose of this analysis was to generate an initial rough approximation for in-plane displacement which could then be compared with the cross-sectional video data.

Figure 5-15 shows a plot of \( w \) calculated directly from the ESPI data, the calculated in-plane component of displacement with respect to the image plane (\( u \)) and the calculated out-of-plane component with respect to the surface normal (radial displacement), for a central horizontal cross-section of the silicone sample (ref. 4, Table 5-1).

![Figure 5-15 – Plot showing out-of-plane displacement, approximated in-plane displacement and approximated out-of-plane displacement normal to the surface of the object along the central horizontal axis of the silicone sample subjected to an increase in hydrostatic pressure.](image-url)
Using the out-of-plane data and the calculated in-plane data, a vector plot was produced as shown in Figure 5-16 to demonstrate the overall profile of deformation with respect to the objects surface predicted based on the assumption of radial expansion. The length of the arrows in the vector plot and the size of the arrow heads, plotted onto the original surface profile, represent the relative magnitude of displacement at a given point compared to other points and were generated by scaling up the measured out-of-plane displacement data and the estimated in-plane displacement data by a factor of 500.

![Vector plot demonstrating the profile of displacement with respect to the surface of the silicone sample along the central horizontal axis obtained when assuming that deformation occurs normal to the surface.](image)

With respect to approximated data shown in the vector plot shown in Figure 5-16, across the central areas the out-of-plane component of displacement was predicted to dominate the response with little in-plane contribution, due to the angle of the objects surface with respect to the image plane. However, towards the edges of the test piece where the gradient of the surface with respect to the image plane was greater, the in-plane component was predicted to have a greater contribution to total deformation, most significantly for the steeper right side edge of the test piece. The vector plot was visually compared with the results obtained during the cross-sectional video analysis, as shown in Figure 5-13 (b), to examine the validity of the assumption that deformation occurred normal to the surface.

From comparing the profile of displacement in the vector plot with the cross-sectional video analysis, it appeared that the assumption that deformation occurred normal to the surface did not reflect the actual results in all areas. On the screenshot from the video analysis, shown in Figure 5-13 (b), it appeared that the left side of the white silicone sample was more prone to out-of-plane displacement over in-plane as it did not appear to displace in-plane as significantly as predicted when assuming normal deformation. For the central area of the test piece there appeared to be good agreement between the predicted results and the appearance of the deformation from the screenshot, which was expected due to the non-steep angle of the surface with respect to the image plane in this area.
The presence of the thinner section of material at the point where the curvature of the test sample changed from the flat base to the dome section likely contributed to the fact the object was more prone to out-of-plane deformation in this area. Overall, despite the fact only the out-of-plane deformation component could be quantified with confidence, the out-of-plane data alone was sufficient to identify the presence of the defect, even though its position was in the area where the curvature of the object was significant.

**Distortion of rate-of-change of out-of-plane displacement component due to curvature**

To demonstrate the effects of the variable shear magnitude with respect to sheared points on the objects surface, introduced due to the initial curvature of the sample, on the calculated rate of change of the out-of-plane displacement component a shear correction factor was applied. This correction factor was applied to adjust the shear magnitude with respect to position on the object surface to reflect the actual spacing between points with respect the surface of the object in the curved areas.

Figure 5-17 shows the value of $\frac{\partial w}{\partial x}$ calculated from the LSI data without taking into account object curvature and hence assuming a constant shear magnitude (blue line). This is compared with the calculated value of $\frac{\partial w}{\partial x}$ for the same cross section after applying a correction factor to take account of the variable shear distance between interfered points due to the object curvature (red line), in this case $x$ corresponds to the distance along the surface of the object.

![Figure 5-17](image_url)

*Figure 5-17 – Comparison of $\frac{\partial w}{\partial x}$ calculated from the LSI data using a constant shear with $\frac{\partial w}{\partial x}$ calculated when taking into account the variable sheared distance between points with respect to the objects surface.*
After taking account of the variable shear distance due to the curvature of the object, the magnitude of $\frac{\partial w}{\partial x}$ (x corresponding to distance along object surface) was reduced by up to 14.7% in the curved areas compared to $\frac{\partial w}{\partial x}$ (x corresponding to distance across the image plane), indicating the rate of change of out-of-plane displacement in these regions was overestimated in the LSI data if considered per unit length with respect to the surface of the object.

**Comparison of LSI data with ESPI data for curved sample**

To determine if there was good agreement between the ESPI data and the LSI data recorded when measuring the response of the silicone test piece to an equivalent pressure increase, $\frac{\partial w}{\partial x}$ calculated from the LSI data was compared with $\frac{\partial w}{\partial x}$ obtained via differentiation of the ESPI data. The data obtained via each method is plotted for an equivalent horizontal cross-section in Figure 5-18.

![Figure 5-18](image_url)

*Figure 5-18 – Comparison of $\frac{\partial w}{\partial x}$ calculated via differentiation of the ESPI data along the central horizontal x-axis, with the same component calculated directly from the horizontal LSI data.*

From the comparison shown in Figure 5-18, it was clear that there were differences between the differentiated ESPI data and LSI data for the silicone sample, specifically at the left side and right side at the positions labelled A and B respectively. The observed differences were a result of the fact the rate of change of $\frac{\partial w}{\partial x}$ was high in these areas and this is known to lead to reduced accuracy in the approximation of $\frac{\partial w}{\partial x}$ obtained via LSI, for the reasons previously
identified in section 3.6.4 of chapter 3. Hence, $\frac{\partial w}{\partial x}$ calculated from the LSI data was not accurate at positions A or B.

### 5.4 Crosslinking Simulation Experiments

The final set of experiments that were carried out on non-biological samples were conducted to examine the effects stiffening in certain locations had on the deformation of the sample in response to a hydrostatic pressure change, and how these changes affected the distribution of the out-of-plane displacement component and its first derivative as measured by ESPI and LSI respectively. The main purpose of these experiments was to generate some simple data with which the cross-linking results from corneas could be compared. Experiments were conducted on a homogenous rubber sample (ref. 1, Table 5-1) to provide reference data describing the simplest case.

In the experiments, to increase the stiffness of a specific areas adhesive tape was used. Tape was used as it was more resistant to stretching than the rubber, and could be used to increase the resistance of the rubber sample to deformation in specific areas. The adhesive tape was cut into shapes to simulate the topographic patterns that would be used in the crosslinking experiments, these are shown diagrammatically in the first column of Table 5-7.

The experimental procedure first involved measuring the response of a control sample positioned in the artificial anterior chamber held under a baseline pressure of 16.50 mmHg to a change in hydrostatic pressure, in the same way as has been described for previous experiments. After this control testing, the sample was then removed from the chamber and adhesive tape was adhered to the underside of the sample to increase the stiffness of a specific area. The sample was then repositioned in the chamber and set under the same baseline pressure, the testing was then repeated as for the control. This process was repeated to obtain data for the sample after applying the adhesive tape in each of the locations shown in Table 5-7.

An example of the appearance of the wrapped and unwrapped data obtained during simulation crosslinking for the case of a homogenous sample are given in Table 5-7. These results were not necessarily obtained under the same pressure change but have been selected to best highlight the changes to fringe distribution and appearance.
Table 5-7 – Summary of the changes to the wrapped and unwrapped phase data that occurred due to stiffening in different regions.
The data along the central horizontal cross-section was plotted for the ESPI and LSI results obtained under an equivalent change in hydrostatic pressure to demonstrate the effects of the topographic stiffening on the profile and magnitude of deformation and the change of surface gradient, these cross-sections are plotted in Figure 5-19 to Figure 5-22. With Figure 5-19 and Figure 5-20 comparing the effects of central and outer stiffening on the ESPI and horizontal LSI data respectively, and Figure 5-21 and Figure 5-22 comparing the effects of stiffening along a vertical and horizontal strip for the ESPI and horizontal LSI data respectively.

**Figure 5-19** – Plot of out-of-plane displacement along the central horizontal axis of a rubber sample before stiffening, after stiffening in a central 3 mm circular region and after stiffening around a central 3 mm circular region, for an equivalent increase in hydrostatic pressure.

**Figure 5-20** – Plot of the phase change proportional to the rate-of-change in the out-of-plane displacement measured via LSI along the central horizontal axis of a rubber sample before stiffening, after stiffening in a central 3 mm circular region and after stiffening around a central 3 mm circular region, for an equivalent increase in hydrostatic pressure.
Figure 5-21 – Plot of out-of-plane displacement along the central horizontal axis of a rubber sample before stiffening, after stiffening down a 3 mm strip in the y-axis and after stiffening along a 3 mm strip across the x-axis, for an equivalent increase in hydrostatic pressure.

Figure 5-22 – Plot of the phase change proportional to the rate-of-change in the out-of-plane displacement component measured via LSI along the central horizontal axis of a rubber sample before stiffening, after stiffening down a 3 mm strip in the y-axis and after stiffening along a 3 mm strip across the x-axis, for an equivalent increase in hydrostatic pressure.

For the uniform rubber sample, the addition of the adhesive tape to specific topographic areas had a clear effect on the deformation of the sample under increased hydrostatic pressure, and this was evident from the distribution of the wrapped fringes, and the appearance of the unwrapped data. The findings for each of the conditions are summarised here:
1. **Central stiffening** – The addition of adhesive tape to the central area was expected to result in increased resistance to displacement and a reduction in bending strain in this area. These effects were reflected in the distribution of the out-of-plane displacement data shown in Table 5-7 as the circumference of the central fringe was increased in diameter when compared with the control. Only subtle changes were seen to the distribution of fringes in the LSI data where the fringe spacing was increased slightly in the central region, resulting in a subtle bending of the fringes in this area.

   More detailed evidence of the effects could be seen from examining the cross-sectional plots showing the response over the same pressure increase. When compared with the control sample, the peak displacement was reduced for the centrally stiffened sample subjected to the same pressure change, as shown in Figure 5-19. The gradient of the curve describing $\frac{\partial w}{\partial x}$, shown in Figure 5-20 was also reduced, especially across the central region when compared with the control indicating a reduction in bending strain $\frac{\partial^2 w}{\partial x^2}$.

2. **Outer stiffening** – The addition of adhesive tape around the circumference of a central 3 mm area was expected to result in an increase in the resistance of this area to displacement and relatively greater displacement in the central region that was not covered by the adhesive tape. These effects were evident in the ESPI and LSI fringe distributions shown in Table 5-7. For the ESPI case there were no fringes observed towards the edges of the object, with the fringes concentrated to the central region. For the LSI case the semi-circular fringes formed closer to the central region when compared with the control.

   From examining the cross-sectional plots, it was clear that the magnitude of out-of-plane displacement (Figure 5-19) had been significantly reduced overall when compared with the control case and that the majority of out-of-plane displacement occurred within a smaller central region. From the LSI plot (Figure 5-20) it was clear the gradient of the curve describing $\frac{\partial w}{\partial x}$ was reduced compared to the control in the outer regions, but similar across the central regions, indicating a localised reduction in $\frac{\partial^2 w}{\partial x^2}$ towards the edges where the tape was present.

3. **Horizontal strip** – the addition of adhesive tape along a central strip parallel to the horizontal axis was expected to result in a reduction in displacement across this axis and a localised reduction in displacement at the centre of the vertical axis. These effects were identifiable from the fringe distributions shown in Table 5-7. For the ESPI
case, the fringes became elliptical in distribution, being longer in the vertical axis than the horizontal, this occurred because bending was reduced specifically across the central area in the vertical axis and reduced across the entire length of the object in the horizontal axis. This effect was easier to identify when viewing the LSI data as a lack of fringes were evident in the central region where the adhesive strip was when the gradient of displacement was examined with respect to the $y$-axis.

The effects were evident from viewing the cross-sectional plots taken along a horizontal cross-section. The overall magnitude of out-of-plane displacement along the horizontal cross-section in-line with the strip was reduced compared with the control as shown in Figure 5-21, and the gradient of $\frac{\partial w}{\partial x}$ was reduced in comparison to the control indicating lower bending strain. If a cross-section had been taken down the vertical axis, the profile of displacement would have been like that shown for the case of a vertically positioned adhesive strip, discussed next.

4. **Vertical strip** – The addition of adhesive tape along a central strip parallel to the vertical axis was expected to result in similar effects to the case of the horizontal strip but with the data rotated through $90^\circ$. These effects were confirmed when viewing the distribution of the ESPI and LSI fringes shown in Table 5-7.

When examined through a central horizontal cross-section in the plots in Figure 5-21 and Figure 5-22. The presence of a vertical strip resulted in a reduction in the overall magnitude of the out-of-plane displacement component, specifically in the central region where the decrease in out-of-plane displacement was most significant, with out-of-plane displacement either side of this being relatively higher. The gradient of $\frac{\partial w}{\partial x}$ in this central region, shown in Figure 5-22, approaches zero, indicating that there is little bending strain in this region specifically, outside of this region the gradient of $\frac{\partial w}{\partial x}$ is increased compared to the control in some areas indicating the presence of the stiffened strip along the axis at $90^\circ$ increases bending strain in the non-stiffened axis outside of the stiffened regions.
5.5 Summary of findings from experiments on non-biological samples

This chapter described the experiments that were conducted on non-biological samples to determine the working parameters of the optical set-up and measurement rig, to and validate that the measured data was as expected. In addition to this a selection of complex fringe patterns were presented to aid understanding with regards to data interpretation. Finally, fringe patterns and data were presented where a sample was selectively stiffened in the specific areas where the corneas would be crosslinked to provide comparative data representative of the simplest case. A summary of the main findings from these experiments is given here.

With respect to the optical set-up and the measurement rig:

- It was established the minimum shear value required to ensure high quality fringes, using the LSI rig as described in section 4.3.2, was 1.04 mm (50 pixels) and that fringe quality continued to improve slightly up to shears of 1.35 mm (65 pixels).
- It was validated that the ESPI system was giving the expected measurement values as the average measured maximum deformation of a piezo-loaded aluminium plate, set to deform by approximately 1 µm (based on piezo voltage-displacement data), was 0.99 µm (+/- 0.01 µm) when measured using the out-of-plane ESPI set-up as described in section 4.3.2.
- When measuring the response of a rubber sample subjected to repeated loading cycles using LSI, it was demonstrated that a measurement repeatability of +/- 0.32 radians could be achieved, equivalent to +/- 2.0% of the maximum measured phase change. This confirmed that the stability of the loading rig, the loading repeatability and the measurement repeatability was high. If the chamber was moved from the measurement area between loading cycles the measurement repeatability dropped to 2.6% of the maximum measured phase change, most likely due to small errors in repositioning.

A variety of objects with different initial curvatures were analysed, from these experiments it was found that:

- The presence of a small initial curvature ($r_c = 15$mm) did not introduce obvious distortions to the distribution of ESPI and LSI fringes. In addition to this the ESPI and LSI data obtained on objects with a non-flat surface geometry subject to changes in hydrostatic pressure appeared to be in good visual agreement with data obtained via video analysis of the object responding to larger changes in hydrostatic pressure. Hence the curvature of the cornea was not responsible for the initial fringe distributions observed during porcine cornea testing.
- For an object with similar curvature to the human cornea, $\frac{dw}{dx}$ calculated from the LSI data using a constant shear (i.e. assuming interfered points were spaced equally with
respect to the objects surface) was up to 14.7% greater in some areas than \( \frac{\partial w}{\partial x} \) calculated when compensating for the variable shear distance between points with respect to the objects surface.

Throughout this chapter ESPI data and LSI data obtained for the same samples in response to equivalent loads was compared. The following conclusions were drawn from these experiments:

- For numerical comparison of the ESPI and LSI data it was better to compare the rate of out-of-plane displacement obtained from the LSI data and the differentiated ESPI data, than to compare the displacement component obtained from the ESPI data and the integrated LSI data. This was because, the integrated LSI data was prone to significant errors, as the cumulative nature of the integration procedure meant any errors present at any point were propagated and accumulated across a given line.

- There was good agreement between the rate of change of out-of-plane displacement obtained from the differentiated ESPI data and the LSI data across a range of shear magnitudes (52 pixels to 95 pixels) for the case of a flat rubber sample, with only a 1% difference in the maximum measured rate of change of out-of-plane displacement. Similar agreement in data was found for the case of the uniform curved rubber sample suggesting that the presence of a small initial curvature did not directly affect the accuracy of the approximation of \( \frac{\partial w}{\partial x} \) obtained via LSI.

- If the rate of change of \( \frac{\partial w}{\partial x} \) was high this affected the accuracy of the approximation of \( \frac{\partial w}{\partial x} \) obtained via LSI.

With regards to the simulation crosslinking experiments it was established that both ESPI and LSI could be used to detect regional stiffness changes. In terms of data presentation, it was found that evaluation of the data was often easier when the data was presented in the format of full surface displacement plots or as line plots through a specific region of interest. The latter was particularly effective for highlighting specific changes such as the effects of localised stiffening and for analysing the displacement derivative data, by reducing the overall complexity of the data making it more interpretable to a wide-ranging audience.

Overall the tests on non-biological samples provided a basis for further testing on corneas by validating that the measurement configuration was giving the expected results and establishing confidence in the fact the data obtained represented the response of the sample and was not an artefact of distortion due to curvature. In addition to this, the experiments demonstrated it was possible to achieve repeatable loading and measurement and highlighted some instances when the LSI data may be prone to errors.
6. Porcine Cornea Experiments

6.1 Introduction

The experiments on non-biological curved samples had successfully demonstrated that the loading rig and measurement methods were repeatable, and that the ESPI and LSI techniques could be used to evaluate the out-of-plane displacement component and its first derivative on curved surfaces. Several unique challenges are introduced when trying to test biological tissue, therefore to determine if the measurement technique could be used successfully for corneal evaluation and to establish an effective testing methodology, a variety of experiments on corneas were required prior to the main experiments, the aims of these experiments were:

1. To establish a suitable testing methodology for corneal tissue.
2. To address corneal specific issues with respect to measurement i.e. transparency and hydration instability.
3. To determine the range of pressure changes over which high quality data could be obtained when using ESPI and LSI.
4. To investigate the repeatability of the response, and evaluate the linearity of the response over a physiological pressure range.
5. To examine the efficacy of ESPI and LSI for corneal measurement.
6. To determine a methodology for the cross-linking experiments and gain experience of using the cross-linking technique.
7. To determine if ESPI and LSI could be used to detect the biomechanical changes to the cornea introduced by crosslinking.
8. To determine if ESPI and LSI could be used to identify areas of biomechanical abnormality.

6.1.1 Tissue selection

Due to the shortage of human donor corneas available, all the proof of concept work in this thesis was conducted on porcine corneas. There were several reasons why porcine tissue was chosen as a substitute for human tissue. One of the main reasons was that porcine corneas were relatively easy to acquire in adequate sample sizes as many pigs are slaughtered regularly across the United Kingdom to supply the meat industry. Since the eyes are not consumed they are routinely disposed of as waste, so by utilising these eyes it ensured animals were not slaughtered for the sole reason of these experiments.

An additional reason was that several other studies have used porcine corneas and therefore some comparative data was available. From these studies porcine corneas have been shown to be similar to human corneas in terms of stress-strain behaviour \( ^{52,59,162} \). In a strip
extensometry study by Elsheikh et al \(^{(59)}\), porcine corneas were shown to exhibit slightly lower overall stiffness than human corneas, but with a similar pattern of anisotropy i.e. both human and porcine corneas were found to have greater stiffness in the superior-inferior (S-I) axis when compared with the nasal-temporal (N-T) axis (20\% human, 4\% porcine), and strips from both these axes were shown to exhibit greater stiffness than strips taken along the diagonal axis with the S-I axis showing 76\% greater stiffness than the diagonal axis in porcine corneas compared to 49\% greater stiffness in human corneas \(^{(59)}\).

Many studies have also suggested the structure of the porcine cornea is comparable to that of a human cornea, and they have even been considered for xenotransplantation \(^{(163)}\). The main differences between human and porcine corneas are:

- **Thickness** – Porcine corneas are thicker than human corneas. Central corneal thickness (CCT) values for porcine corneas are commonly reported with averages between 900 µm to 1000 µm \(^{(59,164,165)}\), compared to 534 µm \(^{(166)}\) for human tissue.
- **Shape** – Porcine corneas are more elliptical than human corneas, being significantly longer in the nasal-temporal axis than the superior inferior (~16\% longer in porcine corneas vs ~8\% longer in human corneas \(^{(5)}\)).
- **Size** – Porcine corneas are bigger than human corneas, reported size varies significantly between studies, with an average horizontal and vertical corneal diameter of 14.3 mm and 12.0 mm respectively, reported from a study in Spain \(^{(160)}\) and corresponding values of 16.6 mm and 14.0 mm from a study in the US \(^{(164)}\), in both cases this is significantly larger than the human cornea at approximately 12.0 mm and 11.0 mm respectively \(^{(5)}\).
- **Bowman’s layer** – Some studies on porcine corneas have reported the absence of a distinguishable Bowman’s layer \(^{(167)}\), although others have not \(^{(163)}\).
- **Epithelium thickness** – the epithelium is significantly thicker in the porcine cornea consisting of 7 to 9 layers of cells as opposed to 5 in the human cornea.

Overall, porcine corneas were concluded to be suitably similar to human corneas to meet the main aims of the experiments which are outlined at the beginning of the chapter.

### 6.1.2 Tissue acquisition, storage and preparation

Prior to experimentation pig’s eyes were sourced from a local abattoir (Joseph Morris Butchers, South Kilworth, UK), the pigs were of Gloucestershire Old Spot breed and were on average 7 months old when slaughtered with an average weight of 75 kg. Enucleation took place within 12 hours of slaughter, and all eyes were kept in a moist environment, refrigerated at 4°C and used within three days of slaughter.
In UK slaughterhouses, it is standard practice to scald the pigs after slaughter by either steaming them or dipping them into a tank at a temperature between 60°C - 70°C to remove hair and surface pathogens. This practice can affect the corneas, as exposure to these temperatures could potentially cause partial cooking which would subsequently result in changes to the biomechanics.

Requests were made at several abattoirs to ascertain whether the eyes could be removed prior to steaming or dipping, unfortunately, this was not possible due to time and space constraints, the number of staff available, and the regulations in place with regards to where certain processes could be carried out on the premises. Ultimately a small-scale meat supplier was chosen that used chemical-free dipping rather than steaming to minimise the effects of these treatments on the eyes. Once the eyes were received only eyes with clear corneas with no visible signs of damage were selected for experiments.

Although it was not possible to obtain eyes from pigs that had not been dipped, it was concluded that any small, non-visible changes that may have been introduced to the cornea during the dipping and extraction procedures would not prevent the initial project aims from being fulfilled. This was because the main purpose behind the experiments on porcine corneas was to determine if ESPI and LSI could be used for corneal testing and to develop a suitable corneal testing methodology before experimentation on human tissue. It was not to evaluate the biomechanics of the porcine corneas.

As the tissue came in the form of whole eye globes, some preparation was required prior to testing. All porcine cornea experiments were conducted on corneo-scleral sections clamped in the modified AAC with the 15.7 mm aperture as described in section 4.2.2 of chapter 4. All preparation was carried out immediately before experimentation using the following procedure.

1. **Epithelium removal** – Prior to all experimentation, the epithelium was removed, this was achieved by gently dragging a blunt scalpel across the surface of the cornea. There were several reasons for epithelium removal:
   - It has previously been shown to have a negligible contribution to biomechanics\(^8\).
   - It was often not preserved in perfect condition during enucleation.
   - After removal from the living host the epithelial cells degenerated and often became taught, and when this occurred their presence was found to influence the deformation of the cornea.
   - The epithelium had to be removed prior to crosslinking, and it was desired to isolate the changes that occurred during cross-linking from any changes that may have occurred due to epithelial removal.
2. **Dissection of corneo-scleral section** – Prior to dissection, the positions of the extra-ocular muscles (if present) were marked to assist with orientating the cornea in the AAC. A 17 mm to 18 mm section, with the cornea at the centre, was then cut from the globe using surgical scissors and the iris was removed from the back of the sclera using tweezers. The corneo-scleral section was then washed with a small amount of PBS solution to remove any remanence of the iris or epithelium.

3. **Positioning and clamping into AAC** – The cornea was orientated in the AAC based on the position of the extra-ocular muscles. Some of the eye globes had been trimmed prior to receiving and in this case the position of the extra-ocular muscles was ambiguous, so orientation was achieved based on the shape of the cornea. The cornea was initially laid over the base and air was used to inflate the cornea slightly to ensure the clamping did not influence its initial shape as previously described in section 4.2.2 of chapter 4. The cornea was then clamped by positioning the tissue retainer over the top of the corneo-scleral section and securing it into place via the locking ring.

4. **Chamber filling** – After clamping the cornea into the AAC the chamber was filled with PBS solution. To ensure no air became trapped in the chamber, the chamber was filled upside down. At the end of filling the outlet was blocked with a syringe, and solution was drawn out of the outlet gently via this syringe to ensure no air bubbles were present in the system.

5. **Pressurisation** – All corneas were set under an initial baseline pressure by mounting the reservoir of PBS solution connected to the AAC above the height of the cornea. A baseline pressure of 16.50 mmHg was chosen for all experiments, as this was also within the range of normal IOP reported for porcine corneas.  

6.1.3 **Overview of Experiments on porcine corneas**

The following sets of experiments were conducted on porcine corneas to address the experimental aims as outlined at the beginning of this chapter.

1. Experiments to determine a suitable method that could be used to obtain an adequate return signal from the surface of the cornea.
2. Experiments to determine the pressure range over which adequate data could be obtained while using ESPI and LSI.
3. Experiments on a large number of samples to assess the variability of the response between samples.
4. Experiments assessing the repeatability in the response of individual corneas.
5. Experiments to determine the linearity of the response over a physiological pressure range.

6. Experiments to determine whether ESPI and LSI could be used to detect the changes to the biomechanics of the cornea introduced by crosslinking in select topographic locations.

7. Experiments to determine if ESPI and LSI could be used to identify areas of biomechanical abnormality.

These specific experiments are discussed throughout this chapter, with the results from these investigations ultimately used to generate an effective testing methodology for human corneas.

6.2 Experiments to determine corneal testing methodology

6.2.1 Achieving an adequate return signal from the corneal surface

Obtaining an adequate signal to noise ratio in interferometry relies on the formation of a speckle pattern and the test surface generating enough back scattered light. The cornea is designed to have both a smooth optical surface, a wet surface, and to transmit light that lies in the $\lambda = 400\text{ nm} – 1400\text{ nm}$ range, making it far from ideal for speckle interferometric testing.

In previous studies using interferometric methods to measure corneal biomechanics, these issues were addressed by applying some external coating to the surface of the cornea. Smolek (70) used a thin layer of white paint pigment powder, Kasprzak (69), et al, used talcum powder, Jaycock (41) used a thin layer of white solvent based developer spray, while Knox-Cartwright, et al (45,51), used a layer of PTFE tape which they positioned on the top of the cornea.

There are several requirements when considering a coating for the cornea:

- The coating must generate an adequate signal from the corneal surface.
- The coating must follow the movement of the corneal surface.
- The coating must not interfere with the movement of the corneal surface or change the biomechanics in anyway.
- The coating must have a minimal effect on the hydration properties of the cornea as this can affect the biomechanics.
- The coating must be stable during the time needed for measurement.

Other methods besides external coatings have been considered such as manipulating the wavelength of the illumination source. Jaycock (145), suggested the use of an illumination source with a wavelength approaching the UV end of the spectrum. However, this method does not get around issues regarding the smoothness of the surface and hydration instability. It would
also potentially introduce issues with regards to examining the effects of crosslinking, as UV is used to initiate crosslinking. Overall it was concluded that establishing an effective external coating method would best option for ensuring an adequate return signal from the corneal surface.

To investigate the suitability and overall effects of different coatings, experiments were carried out on both porcine corneas and non-biological samples. Several different coatings were first investigated to determine if they could generate an adequate signal from the corneal surface, then if an adequate signal could be obtained, the effects of the coatings on the overall response were investigated.

**Ophthalmic emulsions and gels**

Initially, several commercially available ophthalmic products were examined, including emulsions and gel ointments, as it was of interest to obtain a solution that may have been easily transferable to an *in vivo* measurement situation and would have maintained the hydration properties of the cornea. However, it was found that the emulsions and gels did not generate enough backscattered light from the surface of the cornea alone, they also tended to be unstable and move independently of the corneal surface.

Eventually, the consideration of applicability of the coating method to an *in vivo* situation was halted as a simple solution could not be identified. Since the potential for *in vivo* measurement had yet to be confirmed in the presence of many other challenges, it was not logical to spend significant time focusing on this specific aspect prior to conducting any *ex vivo* experiments, hence focus was moved to obtaining a suitable coating for *ex vivo* measurement. Several different types of coating were tested including powder based and film based coatings, the results from these tests are discussed throughout this section.

**Powder based coatings**

Coating a surface with self-adherent particles is a technique commonly used in digital image correlation and it has been used previously on the cornea to determine displacements (24). A suitable coating is generally considered as one that is particle based and therefore has no measureable stiffness, one that adheres to the test surface and deforms with it, and one that has negligible weight so as not to affect the way the object deforms.

In interferometry, it is common practice to coat surfaces with a fine powder to increase the signal to noise ratio so good fringe contrast can be attained. In many interferometry applications, solvent based sprays are used due to ease of application and the ability to
establish a thin coating. However, for the cornea, solvent base sprays are not ideal, as the solvents may damage the surface of the cornea, and the solubility of the powder may prevent it from drying to form a stable surface on the wet surface of the cornea.

Several insoluble white powders were trialed to determine if they could generate an adequate signal from the corneal surface and form a thin stable layer. From these investigations Sphericel 110P8 fused borosilicate hollow glass microspheres (Potters Industries LLC, PA, USA) were identified as a potentially suitable coating as they generated an adequate signal from the corneal surface, were light, non-soluble, and adhered well to the surface of the cornea while being relatively easy to apply in a thin layer using a fine sieve. They were also easy to wash away after testing and did not leave any residue that may have interfered with crosslinking.

To evaluate the effects of the powder coating on the response of the cornea to hydrostatic loading, experiments were conducted. These experiments involved measuring the response of a rubber test piece (ref.1, Table 5-1) clamped in the AAC and subject to a hydrostatic pressure increase before and after coating with the selected powder. The reason the experiments were conducted on a rubber test piece as opposed to the cornea was because no data could be obtained from the cornea in absence of a coating. The experimental procedure was as follows:

1. The uncoated silicone sample was mounted in the chamber and set under an initial baseline pressure of 16.50 mmHg.
2. LSI was used to evaluate the response of the uncoated silicone sample. Reference data was captured at the baseline pressure, the hydrostatic pressure was then increased by 0.15 mmHg and data was captured. After data had been captured the load was removed. The chamber was then removed from the measurement area and then positioned back into the measurement area, and the measurement procedure was repeated exactly as before. This whole process was repeated a total of 10 times.
3. The sample was then coated with the selected powder, and the measurement process that had been used to test the un-coated sample was repeated. However, in this case when the chamber was removed from the measurement area the coating was removed and re-applied before returning to the measurement area, this was to evaluate the effects of removing and reapplying the coating on the repeatability of the response.

From these experiments, it was found that the presence of the powder coating had no obvious visually recognisable effect on the response to loading, as it was not possible to identify clear differences in the wrapped phase plots obtained over each round of testing, an example of two
of the wrapped phase plots obtained during tests before and after applying the powder coating are shown in Figure 6-1.

![Control and Powder coating interferograms](image)

*Figure 6-1 – Wrapped interferograms obtained when measuring the response of a rubber sample to a hydrostatic pressure change of 0.15 mmHg using LSI, before and after coating with a layer of the selected powder.*

When the effects of the powder coating on the measured phase change were examined in detail, it was found that the presence of the powder coating had a negligible effect, this is demonstrated in Figure 6-2, where the phase change measured via LSI has been plotted along a central horizontal cross section for the sample responding to the same magnitude of pressure variation prior to coating and after coating with the selected powder.

![Phase change plot](image)

*Figure 6-2 – Plot comparing the phase change, measured via LSI, along the central horizontal axis of a sample in response to the same pressure change before and after coating the surface with a layer of the selected powder.*

In Figure 6-2 the lines showing average measured phase change before and after coating are almost overlaid with one another, with the difference in the average phase change before and after coating found to be +/- 2.1% of the maximum measured phase change, which is within the measured standard deviation of +/- 2.6% of the maximum measured phase change.
previously demonstrated in chapter 5 in Figure 5-7 for repeated experiments on a single sample.

The removal and reapplication of the powder appeared to have no effect on the repeatability as the magnitude of the standard deviation over 10 repeated measurements during the control testing was comparable to the magnitude of the standard deviation over 10 repeated measurements during which the coating was removed and reapplied. This standard deviation in the response across a typical cross-section is shown in Figure 6-2 for each case, where the dashed lines correspond to standard deviation.

*Film based coatings*

An analysis of film based coatings, namely PTFE tape was also conducted, this was to establish whether they could provide a better or equally effective coating option as the powder, and to evaluate any effects they may have had on results in previous studies that have used this option (45,51,73).

There were initial concerns with regards to using film based coatings such as PTFE tape. PTFE tape itself is a material with measurable stiffness, and so if it is clamped into the chamber covering the material to be tested it could be assumed to contribute to the measured stiffness. It is also a material that exhibits non-linear behaviour. Initially under low strain it has a low Young’s modulus as the polymer chains are not fully extended, as it is strained to the point where the polymer chains are fully extended Young’s modulus increases. Depending on how it is initially stretched to cover the test piece the strength may differ along different axes making it difficult to analyse the overall contribution, and making it almost impossible to take account of when analysing data.

A comparison test was conducted using both ESPI and LSI to compare the response of a porcine cornea in the test chamber, before and after covering with PTFE tape. The reason a comparison test was conducted on a porcine cornea and not a flat rubber test object, as was used for the powder coatings, was because the addition of PTFE tape would have a different affect when used to coat a flat membrane than it would when used to coat a more complex object such as the cornea, and it was of interest to examine its effect on the cornea.

To examine the effect of a PTFE tape coating, a porcine corneo-scleral section was clamped in the chamber and coated with a thin layer of the Sphericel 110P8 fused borosilicate hollow glass microspheres, as these had previously been demonstrated to have a negligible effect on the response of a sample to hydrostatic loading (Figure 6-2).
The cornea was set under an initial hydrostatic pressure of 16.50 mmHg where it was left to settle for 10 minutes prior to loading. The porcine cornea was then loaded by increasing the hydrostatic pressure in the chamber by magnitudes between 0.25 mmHg – 1.00 mmHg over a series of measurements. At the end of testing the cornea was removed from the chamber, the powder coating was washed away with PBS solution, and the cornea was then covered with a thin layer of PTFE tape and repositioned in the chamber. The same procedure as detailed above was then repeated with the cornea covered with a layer of PTFE tape.

An example of the wrapped data recorded using ESPI and LSI on the same cornea before and after the addition of the PTFE tape, is shown in Table 6-1. Clear differences were identifiable in the distribution and number of fringes in the wrapped data for each case, suggesting the presence of the PTFE tape introduced differences in both the nature and magnitude of deformation of the sample in response to a hydrostatic pressure increase.

<table>
<thead>
<tr>
<th></th>
<th>ESPI</th>
<th>LSI (vertical sensitivity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Powder coating</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>PTFE tape coating</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>

*Table 6-1 – A comparison of the wrapped interferograms obtained when using ESPI and LSI to measure the response of the same cornea to a 0.50 mmHg increase in hydrostatic pressure when coated with powder and when coated with PTFE tape.*

Figure 6-3 shows a comparison of the phase change measured via ESPI across the same horizontal cross-section of the cornea when coated with powder and when coated with PTFE tape. Both measurements were taken for a pressure variation of 0.50 mmHg above the baseline pressure of 16.50 mmHg.

Interestingly, it was identified that the cornea with the PTFE coating exhibited greater out-of-plane displacement than the powder coated cornea in response to the same pressure increase. This was contrary to what was initially expected as it was thought the combined stiffness of the cornea and the PTFE tape would increase the resistance of the sample to deformation. However, the explanation for the observed behaviour was that the introduction of the PTFE
tape had prevented the cornea from reaching its natural curvature under the baseline pressure and therefore the structure was not under its initial natural tension, thus when subjected to loading it displaced by a greater amount and the response was irregular.

Figure 6-3 – Plot comparing the phase change measured via ESPI along the central horizontal axis of a cornea subjected to a 0.50 mmHg increase in hydrostatic pressure when coated with powder vs PTFE tape

In summary, it was concluded that PTFE tape and other film based coatings should be avoided due to the fact, as a stand-alone material, they exhibit a measurable stiffness and their presence alters the behaviour of an object in response to loading. In addition to this, as the PTFE does not adhere to the corneal surface but just covers it, slippage between the cornea and PTFE tape could occur hence the movement of the tape may not reflect the movement of the surface.

Powder based coatings that adhere well to the object surface are more suitable option due to the fact they have no measurable stiffness, and as demonstrated by the results in this section, they follow, but do not restrict, the motion of the object surface. Thus, for all the experiments on corneas throughout this thesis Spherical 110P8 fused borosilicate hollow glass microspheres were used to coat the surface of the corneas so an adequate return signal could be obtained.

Coating was achieved via dispersing the powder onto the surface of the cornea using a fine sieve. Any excess loose particles on the surface of the cornea were removed after application via blowing compressed air across the surface of the cornea to ensure all remaining particles were adhered to the corneal surface.
6.2.2 Establishing measurement range

Initially during the experiments on porcine corneas, the response of each of the corneas was measured over a range of pressures variations in 0.05 mmHg steps so an effective range of pressure changes over which data could be obtained using ESPI and LSI could be established.

The range of pressure variations that could be introduced during one measurement cycle was limited by the measurement range of the ESPI and LSI techniques. For the ESPI case, in the instance of normal illumination the sensitivity range is governed by the wavelength of the illumination source as each fringe represents a displacement of $\frac{\lambda}{2}$. Hence, the range of pressure variation that could be achieved was limited by the number of fringes that could be resolved across a given area. For the LSI case, there is a little more freedom in the sensitivity range as the magnitude of the shear can be adjusted to alter the sensitivity range, however this can affect the accuracy as previously described in chapter 3.

To establish the specific range of pressure variations over which data could be obtained, experiments were first carried out where the response of the porcine corneas to a hydrostatic pressure increase above the baseline pressure was measured using ESPI. ESPI testing was conducted first as it was not possible to alter the sensitivity range over which ESPI results could be obtained, whereas the sensitivity range of the LSI could then be tuned slightly to obtain measurements over the same range.

A range of 20 corneal samples were examined. For each cornea, pressures increases from 0.05 mmHg to 2.00 mmHg were tested in increments of 0.05 mmHg. From these experiments, it was found that data quality remained adequate across the majority of the corneal surface for pressure changes of up to 1.00 mmHg for most samples (15/20), with a few losing quality before this point (3/20), and a few maintaining quality up to higher pressures (2/20).

Since the fringe density was consistently higher in a 2 mm boundary region adjacent to the edge of the chamber relative to other areas, the quality of data in these regions tended to deteriorate at lower pressure variations than data in the regions inside of this area. The can be seen in Figure 6-4 where the wrapped and unwrapped phase maps obtained for measurements over pressure variations ranging from 0.25 mmHg to 1.00 mmHg are shown.
With respect to the images shown in Figure 6-4, for pressure variations of 0.75 mmHg and above there is a loss in the definition of some of the fringes towards the boundary regions in the wrapped data, this manifests in the unwrapped data as an area where there is a sharp, non-gradual and inconsistent change in the grey level which results due to the fact the phase cannot be accurately resolved in these areas. Hence, in experiments where full-field quantitative data was desired, testing over a lower range of pressure variations from 0.25 mmHg to 0.50 mmHg was optimum as good fringe quality was better maintained across all areas.

While the ability to quantitatively analyse the data over the whole cornea was generally lost over higher pressure variations, the higher fringe density in the regions inside of this often helped to visually highlight the patterns of deformation and enabled subtle details to be picked out. Overall this led to the decision to test the corneas over a range of pressure changes of 0.25 mmHg, 0.50 mmHg, 0.75 mmHg and 1.00 mmHg.

Experiments were conducted using LSI to determine the shear magnitudes over which high quality LSI data could be obtained over the same range as the ESPI data. For these experiments the shear was initially set at the lowest value possible, which was 50 pixels (1.04 mm) as discussed in section 5.2.1 of chapter 5, and the same loads were applied to the corneas to determine if data could be obtained in the same range.

It was found that even at the minimum sensitivity, fringes rapidly formed at the boundary areas for pressure variations up to 0.50 mmHg. Overall shear was set at 65 pixels (1.35 mm) for the porcine cornea experiments, as the fringe quality was generally found to be high at this shear
level for pressure variations between 0.25 mmHg and 0.50 mmHg. During some experiments the shear was altered slightly to optimise the sensitivity for a specific cornea and in a few experiments the shear was increased up to 100 pixels (2.08 mm) when greater sensitivity was desired to examine more central areas. The shear magnitude remained constant throughout all measurements on a given cornea.

6.3 Results of experiments on untreated porcine corneas

Measurements were recorded using both ESPI and LSI on a sample size of 44 corneas, which were subject to pressure variations of between 0.25 mmHg to 1.00 mmHg above a baseline pressure of 16.50 mmHg. The purpose of these experiments was to investigate the following:

- How the corneas respond to hydrostatic pressure variations.
- The variability in the response across a range of samples.
- Any potential issues with respect to ESPI or LSI measurement on corneas.
- The repeatability of the response across repeated loading cycles.
- The linearity of the response over a physiological range of pressures.

6.3.1 Variability across the range of corneas

After testing a range of samples, the data was visually inspected. The visual appearance of the ESPI fringe distributions in the wrapped data obtained indicated that there was significant variability in the way the corneas deformed to a hydrostatic pressure increase across the range of samples examined. However, it was found that the data could be divided into three main groups based on observed similarities in the distribution of the ESPI fringes. These were as follows:

1. Sets of fringes forming at the top and bottom of the vertical axis. Shown in the three examples in column 1 in Figure 6-5.

2. Set of fringes forming towards the left and right sides of the horizontal axis. Shown in the three examples in column 2 in Figure 6-5.

3. ESPI fringes with an approximately elliptical distribution forming either around the centre or off towards one side. Shown in the three in column 3 in Figure 6-5.
Figure 6-5 – Examples of the common fringe distributions observed in the ESPI data recorded on porcine corneas subjected to hydrostatic pressure increases ranging between 0.25 mmHg to 1.00 mmHg above baseline pressure.

The most common distribution seen across the samples examined was distribution 1, with sets of fringes concentrating towards the top and the bottom of the vertical axis, with 25/44 (57%) samples showing a similar distribution to this. The next most common distribution was distribution 3 with 13/44 (30%) of the corneas tested showing a similar distribution to this, for these samples the fringes often formed in an area off-centre with very few samples showing centred elliptical fringes. For the remaining 6 (13%) samples the distribution was similar to that described for distribution 2, with fringes concentrated towards the left and right sides of the horizontal axis.

Although the distribution of the fringes could be grouped into three main groups there was still a large degree of variation observed between samples in each of these groups, in terms of fringe morphology and fringe number. For example, for corneas showing a similar fringe distribution to those labelled 1 or 2, some corneas showed relatively equal fringe numbers at the superior and inferior or the nasal and temporal locations, whereas in others, the fringes were more concentrated at one side specifically. For corneas showing a similar distribution to that labelled 3, the position on the cornea that the fringes were centred around varied, with some being closer to the central sections and some more out towards the periphery.
6.3.2 Analysis of ESPI measurement data

To demonstrate what each of the ESPI fringe distributions described in the section 6.3.1 indicated in terms of the measured out-of-plane displacement component, a 3D surface plot of the out-of-plane displacement component calculated from the ESPI data is given in Table 6-2. One example is shown for each of the 3 common fringe distributions identified.

<table>
<thead>
<tr>
<th>Distribution</th>
<th>Surface plot of $w$ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image" alt="Surface plot 1" /></td>
</tr>
<tr>
<td>2</td>
<td><img src="image" alt="Surface plot 2" /></td>
</tr>
<tr>
<td>3</td>
<td><img src="image" alt="Surface plot 3" /></td>
</tr>
</tbody>
</table>

Table 6-2 – Examples of the common distributions of the out-of-plane displacement component recorded on porcine corneas responding to small changes in hydrostatic pressure. Numbered distributions relate to the fringe distributions previously identified in figure 6-5.
The plots showed that for distribution 1, the measured out-of-plane displacement component was greatest towards the top and bottom of the S-I axis (Y-axis), and for distribution 2 it was greatest towards either side of the N-T axis (X-axis). For distribution 3, the measured out-of-plane displacement component peaked in a single specific area, with the position of this area varying between samples in this group.

As previously identified, the majority of the corneas tested showed a similar pattern of out-of-plane displacement to that shown in distribution 1, therefore this displacement distribution has been analysed in greater detail. Figure 6-6 shows the profile of the out-of-plane displacement component (w) across the full surface, along a cross-section across the centre of the N-T axis (A-A), and also along a cross-section down the centre of the S-I axis (B-B), for a cornea subjected to a 0.50 mmHg increase in hydrostatic pressure.

Figure 6-6 – Measured out-of-plane displacement component recorded on a porcine cornea in response to a 0.50 mmHg increase in hydrostatic pressure.

For the N-T axis (section A-A) the magnitude of the out-of-plane displacement component increased most rapidly at the edges, then continued to increase, but more gradually towards the centre. For the S-I axis (section B-B) the magnitude of the out-of-plane displacement component again increased most rapidly at the edges, however, for this axis, out-of-plane displacement peaked close to the edge and then decreased slightly towards the centre.
To visualise what these results may indicate in terms of the deformation of the corneal surface, an approximation for the in-plane components of displacement with respect to the x and y axes were made by using the measured out-of-plane data obtained via ESPI and the surface elevation data and by assuming that displacement occurred at an angle normal to the surface, as originally described in section 4.3.2.

The measured out-of-plane, and calculated horizontal in-plane components of displacement are plotted for a cross-section taken along the centre of the N-T axis in Figure 6-7, along with the magnitude of the total displacement normal to the surface (radial displacement) due to the combined out-of-plane and in-plane contributions.

For the N-T axis, shown in Figure 6-7, based on the measured out-of-plane data and the assumption that displacement occurred normal to the surface, the total magnitude of displacement with respect to the surface normal is predicted to increase most rapidly in the 2 mm area on the left side of the sample adjacent to the boundary. The magnitude of the total displacement is then expected to remain relatively consistent across the central 8 mm and then gradually decrease towards the right side of the sample.

To assist with visualisation of the predicted response, the data shown in Figure 6-7 was used to produce a vector plot, where the predicted horizontal in-plane and measured out-of-plane displacement components were scaled up by approximately 500 times and plotted as vectors onto the original surface profile of the sample. This is shown in Figure 6-8. Where the size of the arrows relative to one another is proportional to the scaled-up movement at a given point compared to other points.
Figure 6-8 – Vector plot to demonstrate the approximated profile of displacement of the surface of the porcine cornea along the central N-T axis in response to an increase in hydrostatic pressure.

The equivalent line plot and vector plot for the S-I axis are shown in Figure 6-9 and Figure 6-10 respectively. In this case, the measured out-of-plane and approximated vertical in-plane components of displacement are shown along with the approximated magnitude of the total displacement normal to the surface (radial displacement).

Figure 6-9 – Plot showing out-of-plane displacement, approximated vertical in-plane displacement (v), and approximated out-of-plane displacement with respect to the surface normal down the central S-I axis of a porcine cornea subjected to a 0.50 mmHg increase in hydrostatic pressure.

Figure 6-10 – Vector plot to demonstrate the approximated profile of displacement of the surface of the porcine cornea down the central S-I axis in response to an increase in hydrostatic pressure.
For the S-I axis, based on the measured out-of-plane displacement and the approximated vertical in-plane displacement, the total magnitude of displacement with respect to the surface normal was expected to increase rapidly in the 1 mm regions closest to the boundary on either side of the sample, peaking just inside of this. Displacement was then expected to decrease gradually towards the central area. When plotted onto the measured surface profile of the sample, the results seemed to indicate that the surface of the cornea would be expected to bulge out towards the edges, and that this would account for the pressure increase, contributing to a lower total displacement in the central regions.

A frame subtracted video of the N-T and S-I axes of a cornea that had responded in a similar manner to the cornea discussed throughout the analysis was recorded. The cornea was filmed responding to a larger variation in hydrostatic pressure than used in the interferometric testing so a visible movement occurred. This data was visually compared with the predicted profile of deformation to see if the two sets of data showed similar features.

A screen-shot of the video data taken along the N-T axis is shown in Figure 6-11. The light area in the screenshot represents the area over which the surface moved during loading and the labelled sections represent specific regions of interest. As the surface of the cornea was rough due to the presence of the surface coating, several recognisable areas on the surface could be examined before and after loading. This allowed rough estimates to be made regarding the out-of-plane and in-plane contributions to total displacement.

![Figure 6-11](image)

*Figure 6-11 – Screenshot taken from a frame subtracted video of the surface of the cornea deforming due to a hydrostatic pressure increase. Cornea was positioned so the camera was focused on the centre of the N-T axis.*

With respect to Figure 6-11, in region A there was a rapid increase in the magnitude of total surface displacement immediately inside of the fixed boundary, and the total magnitude of deformation in this area appeared to be high relative to other areas, with an apparent significant in-plane contribution to the overall response. These observations appeared to be in good agreement with the predicted 2D profile of displacement shown in the vector plot in Figure 6-8.

However, the surface profile of the cornea shown in the video data highlighted that errors were likely at the edge regions in the predicted data. This was due to the fact the gradient of the
surface changed rapidly over a short distance and this can contribute to errors in the surface elevation data, as previously discussed in section 4.4.1 of chapter 4.

Across region B, the total magnitude of the surface displacement appeared to remain relatively even with the majority of displacement appearing to occur out-of-plane. This observation was also in agreement with the data shown over approximately the same area in the vector plot in Figure 6-8. Finally, in region C the total magnitude of the surface displacement decreased gradually towards the boundary, and again, an in-plane contribution to the total response was evident. This was also in agreement with the profile of the response shown in the vector plot, although for similar reasons as discussed for region A the prediction of the in-plane contribution at the edge of this reason was subject to errors.

A screen-shot of the video data taken along the S-I axis is shown in Figure 6-12. In region A, the magnitude of the total surface deformation increased rapidly at the boundary, with an apparent significant in-plane contribution to the overall response. This was similar to the response shown in the vector plot in Figure 6-10, taking into account the errors that were likely in the calculated in-plane component in the region immediately adjacent to the boundary.

Across region B, the overall magnitude of surface displacement gradually decreased towards the central region and across region C gradually increased again from the centre approaching the right boundary, and this too was in agreement with the predicated data. Finally, in region D the total surface displacement was relatively high as a result of both the in-plane and out-of-plane contributions.

Overall, it was found that the predicted profiles of surface displacement, generated from assuming displacement normal to the surface, showed similar features to those shown in the screenshots taken from the video analysis data, demonstrating that they could provide a useful initial approximation for how the corneal surface deformed due to an increase in hydrostatic pressure.

![Figure 6-12 – Screenshot taken from a frame subtracted video of the surface of the cornea deforming due to a hydrostatic pressure increase. Cornea was positioned so the camera was focussed on the centre of the S-I axis](image-url)
6.3.3 Analysis of LSI measurement data

As shown by the ESPI data in Figure 6-5, for the porcine corneas the greatest rate of out-of-plane deformation was generally concentrated towards the boundary areas, in the 2 mm region adjacent to the edge of the chamber, hence it was expected that the fringes in the LSI data would be concentrated here. Figure 6-13 shows the LSI fringe distributions recorded for a typical porcine cornea over a pressure change of 0.50 mmHg and 0.75 mmHg with sensitivity to the rate of change in out-of-plane displacement with respect to both x and y axes, labelled as the N-T and S-I axes respectively in Figure 6-13. An example of the ESPI data from the same cornea is presented alongside for reference.

![Figure 6-13](image)

Figure 6-13 – Wrapped LSI data recorded on a porcine cornea over pressure increases of 0.50 mmHg and 0.75 mmHg with shear applied with respect to the N-T and S-I axes. Corresponding ESPI data is shown for reference.

The LSI data showed the expected distribution based on the features as previously described for the ESPI data. Figure 6-14 shows a plot of the phase change proportional to the rate-of-change of displacement between sheared points along the N-T axis that occurred due to a 0.50 mmHg increase in hydrostatic pressure.

![Figure 6-14](image)

Figure 6-14 – Phase change plotted along section A-A, proportional to the change in displacement between sheared points along the N-T axis, recorded using LSI for a cornea subjected to a 0.50 mmHg increase in hydrostatic pressure.
It was noted that for the N-T axis, the fringes formed very close to the boundary regions, within approximately 1 mm of the edge of the chamber, and were irregular in shape. Inside of this 1 mm region their tended to be a general lack of fringes over the range of pressures tested. Analysis of the phase change taken through a central cross-section, showed that the rate of change in out-of-plane displacement between sheared points along the x-axis was highest at the left of the sample indicating a steepening in the surface gradient in response to an increase in hydrostatic pressure in this area. It was close to zero across the central regions indicating a minimal change to the curvature of the surface across these regions, and it was lowest at the right side of the sample indicating a steepening of the surface gradient in response to an increase in hydrostatic pressure in this region.

Figure 6-15 shows a plot of the phase change proportional to the rate-of-change of displacement between sheared points along the S-I axis that occurred due to a 0.50 mmHg increase in hydrostatic pressure.

![Figure 6-15](image)

*Figure 6-15 – Phase change plotted along section B-B, proportional to the change in displacement between sheared points along the S-I axis, recorded using LSI for a cornea subjected to a 0.50 mmHg increase in hydrostatic pressure.*

For the S-I axis, although the concentration of fringes at the edges was still highest, more widely spaced fringes would form at location just inside of the 2 mm boundary region. Analysis of the phase change plotted for section B-B in Figure 6-15 showed that the rate of change in displacement between sheared points in the y-axis was highest at the left side indicating a steepening of the surface gradient in this area. The phase change then became negative between the boundary region and then positive on the other side of the centre, and this indicated a flattening of the curvature of the cornea across this region due to an increase in hydrostatic pressure. Finally, the phase change became negative in the boundary region.
indicating a steepening of corneal curvature in this region due to increasing hydrostatic pressure.

The general profile of the phase change measured from the LSI data was in agreement with what had been shown by both the ESPI data and the video analysis data in the previous sub-section. However, the LSI data was prone to errors with respect to the approximation of the 1st derivative of the out-of-plane displacement component, this was due to the following reasons:

- High fringe density at the boundary regions.
- Curvature of the cornea in the boundary regions.

The high fringe density contributed to two main problems with respect to quantitative analysis. Firstly, the high density and close spacing of fringes that formed in this region meant that this area was prone to speckle decorrelation issues over relatively small pressure variations, hence it was difficult to generate high quality, repeatable data in these regions, unless data was captured at very low loads and the overall fringe number was reduced to approximately two fringes.

Secondly, the rapid change in the rate of change of out-of-plane displacement within these regions results in errors in the approximation of the 1st derivative of the out-of-plane displacement components $\frac{\partial w}{\partial x}$ and $\frac{\partial w}{\partial y}$, as previously demonstrated for the silicone sample in Figure 5-18 in chapter 5. Therefore, the data in these regions could not be considered to be quantitatively accurate. The speckle decorrelation effects and the quantitative errors combined, meant that integration of the data to obtain the displacement component was numerically inaccurate and prone to distortions, hence interpretation of the data was often difficult in absence of the corresponding ESPI data.

The curvature of the cornea contributed to the high fringe density at the boundaries, as it meant the sheared distance between points on the objects surface was increased in these regions. This is demonstrated in Figure 6-16, which shows a plot of the shear-multiplication factor required to determine the actual sheared distance between points with respect to the surface of the object along the S-I axis.

From the data plotted in Figure 6-16, it was evident that the effects of the initial curvature of the surface on the sheared distance between points was significant, with an increased spacing by up to 71% approaching the boundary regions in some areas, hence the sensitivity would have been increased in these regions by the equivalent amount. The variation in the shear distance could be accounted for during processing of the results, as previously demonstrated for the non-biological sample in Figure 5-17. However, this would not directly address the
measurement issues associated with variable sensitivity, or the errors due to the high rate of change of the out-of-plane displacement derivatives, hence the data was still prone to errors.

![Figure 6-16 – Plot of the shear multiplication factor required to take account of the variable sheared distance between interfered points with respect to the objects surface.](image)

The curvature of the cornea also meant that the measured LSI data in the boundary regions did not directly relate to the gradient change of the corneal surface in response to an increase in the hydrostatic pressure, due to the fact the overall gradient change is a function of both the out-of-plane and in-plane components of displacement, hence the measured data could only provide an indication. An estimate for the gradient change could have been made from the measured LSI data and the surface elevations data by assuming that sheared points deformed in a directionally similar manner, however this was unlikely to be valid at the boundary regions and the data was already subject to errors for the previous reasons identified.

In summary, it was concluded that the LSI data could be used to quantitatively analyse the response of the cornea in the region inside of the 2 mm area adjacent to the edge of the chamber, as the magnitude of the second derivative of out-of-plane displacement was generally small in this area, fringe quality was high and deformation appeared to occur predominantly out-of-plane.

Within the 2 mm region adjacent to the boundary the ability to quantitatively analysis the data was lost due to the curvature of the cornea, the reduction in data quality, the larger contribution of the in-plane component to total deformation, and the fact that the second derivative of out-of-plane displacement was often high in this region compared to the first derivative out out-of-plane displacement.
Since the ability to quantitatively analyse the LSI data was only lost in the 2 mm region adjacent to the boundary, LSI could still be used for the quantitative analysis of the cornea across the central regions, ergo the effects of cross-linking could be analysed as cross-linking was performed in the areas inside of this region.

6.3.4 Repeatability of the response over repeated loading cycles

The repeatability analysis on the cornea was conducted to determine if the response of the cornea was repeatable over several measurement cycles. The repeatability of the loading technique and optical measurement configuration had previously been defined using non-biological samples that exhibited a repeatable response to loading, as described in section 5.2.3 of chapter 5. Therefore, any change in repeatability observed during these experiments over the previous experiments could be concluded to be due the behaviour of the measured sample.

To investigate the repeatability of the response of the porcine cornea, six corneo-scleral samples were subjected to 10 repeated loading and unloading cycles for hydrostatic pressure increases of 0.25 mmHg and 0.50 mmHg above baseline. As with previous repeatability experiments, the response of the cornea to loading was recorded using LSI by capturing reference data at the initial baseline pressure and then again at the end of loading.

For repeated loading cycles on the same cornea it was found that the visual appearance of the wrapped data was consistent, suggesting the response of the cornea was repeatable. An example of the wrapped LSI interferograms obtained over 10 repeated loading on one of the corneas tested is shown in Table 6-3. These results were typical of other corneas tested in terms of visual repeatability.

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**Table 6-3** – Wrapped LSI data recorded over 10 repeated loading cycles where the cornea was subjected to a pressure increase of 0.50 mmHg above baseline pressure.
The repeatability in the visual appearance of the interferograms was found to provide useful visual check for repeatability during testing, because the morphology of the fringe pattern was only found to change during loading in the presence of damage or leakages. This occurred for two samples during testing, and as a visually repeatable response could not be achieved the results from these specific samples were discounted.

The repeatability of the measured response of the corneas when subjected to a pressure increase of 0.50 mmHg was analysed in more detail by assessing the standard deviation in the measured response of a cornea over the 10 loading cycles. A plot of the average measured phase change (+/- standard deviation) obtained via LSI, across a central horizontal cross-section for the same cornea as shown in Table 6-3 is given in Figure 6-17.

Over a pressure variation of 0.50 mmHg, the average standard deviation in the measured response over 10 loading cycles inside the 2 mm boundary regions was +/- 0.47 radians for the cornea shown in Table 6-3. This was increased over the average standard deviation of +/- 0.32 radians measured previously across the rubber sample shown in Figure 5-6, however, some increase in variability had been expected due to the biological nature of the sample.

Within the 2 mm boundary regions the standard deviation in the measured phase change increases significantly, as can be seen in Figure 6-17. The large variation seen in these regions was likely due to the localised reduction in fringe quality, which is evident in the images of the same cornea shown in Table 6-3. This loss of fringe quality prevents the phase change from being accurately resolved and hence there can be significant variation in the measured phase change between repeated measurements. However, it is also possible that there was an
increase variability in the response in these regions relative to the central regions but this could not be quantified with confidence due to the quality of data obtained in these areas. Related to the previous point, it was found that the variability in the measured response across different corneas varied, over a range to approximately +/- 0.32 radians to +/- 0.68 radians with better repeatability observed over smaller pressure variations where fringe quality was better.

6.3.5 Linearity Experiments

Natural variations in IOP can be significantly greater than the pressure changes of up to 1.00 mmHg that lay within the sensitivity range of the ESPI and LSI configurations used for the experiments in this thesis, hence it was of interest to examine if the response of the cornea remained similar over a slightly larger range of pressure changes. The purpose of these experiments was to evaluate whether the data obtained at pressure variations with magnitudes between 0.25 mmHg to 1.00 mmHg may be scalable over a larger pressure range, or whether a method would be required where data would be summed from measurements taken over a number of small steps to total a larger pressure range.

Studies were conducted on six porcine corneas where the hydrostatic pressure was increased by a total of 3.00 mmHg in incremental steps of either 0.25 mmHg or 0.50 mmHg. A 3.00 mmHg range was used as this represented the average variation in IOP that has been reported to occur during the cardiac cycle \(^{(64)}\). However, in reality, in vivo, it is reasonable to assume that some of the pressure fluctuation will be absorbed by the sclera.

An example of the wrapped ESPI data obtained over 12 incremental loading steps of 0.25 mmHg from an initial baseline pressure of 16.50 mmHg is shown for one of the corneas examined in Table 6-4, where the numbers above the wrapped data images correspond to the total pressure in mmHg.

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<td>18.75</td>
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Table 6-4 – Wrapped ESPI data recorded on a porcine cornea over 10 incremental loading cycles where the hydrostatic pressure was increased by 0.25 mmHg at each increment.
In general, it was found that, for all the corneas examined the overall morphology and number of the fringes that formed over each incremental loading step was similar. However, it was noticed that, over the first loading increment from 16.50 mmHg to 16.75 mmHg the number and size of fringes was slightly less than for following increments and this was true for all corneas tested, indicating less out of plane displacement relative to the following loading increments. This result was thought to be due to the fact the cornea was initially still, because it had been sitting at the baseline pressure for 10 minutes prior to the first loading, whereas for following increments the cornea was likely to be still moving slightly due to the previous loading.

The variability in the response of the corneas over incremental loading measured using LSI was examined to see if it was similar to that seen for corneas tested over repeated loading cycles. Figure 6-18 shows the plots of the average measured phase change (+/- standard deviation) recorded by LSI for a cornea subjected to 12 incremental loading steps of 0.25 mmHg.

The variability in the response of the cornea subjected to incremental loading appeared to be on a similar scale to the variability that had been observed for corneas when subjected to a repeated load from the same baseline pressure as previously shown in Figure 6-17, however, due to each cornea exhibiting slightly different repeatability it was difficult to compare the results obtained from different corneas.

To analyse the variability of the response to incremental loading in greater depth the standard deviation of the phase change recorded via LSI for a cornea subjected to 5 repeated loading
cycles of 0.50 mmHg, was compared with the standard deviation of the response of the same cornea subjected to incremental loading in steps of 0.50 mmHg over 5 measurement cycles. The boundary areas were excluded from the calculation as any decorrelation could misrepresent the repeatability of the response.

The average standard deviation of the measured phase change recorded across the test surface for the cornea subject to repeated loadings from the same baseline pressure was +/- 0.326 radians, whereas for the cornea subjected to incremental loading the average standard deviation in the measured response for the same surface area was +/- 0.345 radians, indicating that there was a slight increase in the variability of the response for the incrementally loaded case.

If the initial measurement was not considered in the variability analysis for the reason previously discussed, and data was used from the five measurements following this, the average standard deviation in the measured phase change was +/- 0.332 radians for the incrementally loaded case. This was similar to the repeatability of +/- 0.326 radians achieved across repeated loading cycles, suggesting the response of the cornea remained similar over a slightly larger range of pressure changes, up to a maximum pressure of 19.50 mmHg.

In summary, the corneas examined using ESPI and LSI were shown to behave in an approximately linear manner across a pressure range of 3.00 mmHg above the baseline pressure of 16.50 mmHg. These results echo the findings of previous studies \(^{20,28}\), where the behaviour of the cornea has been found to be approximately linear over a physiological range of pressures. Therefore, the data measured at lower pressure changes should be scalable over a physiological range of pressures from 16.50 mmHg to 19.50 mmHg.

6.4 Crosslinking experiments

6.4.1 Methodology

Based on the results of the previous experiments, a testing methodology was developed to investigate corneal biomechanics in response to a hydrostatic pressure changes and the changes that could be introduced to this response by crosslinking. The results of the previous experiments had highlighted several factors that were considered in the development of an appropriate testing methodology, these were as follows:

- The pressure variations that could be introduced while maintaining data quality were limited to a maximum of 1.00 mmHg, with higher quality data, with greater repeatability generally achieved over lower pressure variations of 0.25 mmHg to 0.50 mmHg.
• There was significant variability observed in the response across different corneas. Hence, to assess the effects of crosslinking it would be required to use each cornea as its own control, by comparing the same cornea before and after crosslinking so variability in the normal response could be accounted for.

• The response of the corneas was found to be visually repeatable, with a lack of repeatability in the morphology of the fringe distributions obtained over repeated loading cycles or large differences in the number of fringes generally indicating the presence of damage or leakages.

• The response was found to be approximately linear at pressures between 16.50 mmHg to 19.50 mmHg.

Based on these findings the following testing methodology was developed for the main experiments:

1. **Corneal preparation** – this differed between human and porcine corneas and is described for each case at the beginning of each of the respective chapters.

2. **Mounting and pressurisation** – The mounting and pressurisation procedure was described at the beginning of this chapter in section 6.1.2. All corneas were initially held at a baseline pressure of 16.50 mmHg by mounting the reservoir so the maximum height of the PBS solution was 225 mm above the height of the corneal surface. After mounting the cornea was left to sit at the baseline pressure for 10 minutes so the surface was stable prior to the first measurement.

3. **Coating** – Each cornea was checked for any visual defects prior to coating and a live image of the uncoated cornea was captured with the camera. Once under the required pressure a thin layer of Sphericel 110P8 fused borosilicate hollow glass microspheres were applied by using a fine micro sieve to disperse them across the surface. Any excess powder that had not adhered to the corneal surface was removed by using compressed air to blow away any excess. The quality of the coating was checked and if it was inadequate the coating was washed away and the procedure was repeated until an adequate, suitably even coating was obtained.

4. **Shape measurement** – The chamber was positioned in the desired orientation for testing and clamped to ensure stability. The fringe projection technique as detailed in section 4.3.3 of chapter 4, was carried out to obtain a phase map of the surface elevation of the cornea.
5. **Interferometric testing** – The response of the cornea was examined using both ESPI and LSI over pressure increases of 0.25 mmHg, 0.50 mmHg, 0.75 mmHg and 1.00 mmHg from the baseline pressure of 16.50 mmHg. Three repeated measurements were taken for each of these pressure variations with the optical set up in the ESPI and LSI configurations in turn. Measurements were first carried out with the cornea orientated so the shear was applied with respect points in the direction of the N-T axis, and half way through measurement the chamber was rotated 90º so shear was with respect to points in the direction of the S-I axis. This resulted in a total number of measurements at each load increment of 12, and a total number of measurements during each round of testing of 48.

The reason repeated measurements were limited to three was because it was desired to look at the response of the cornea over a range of pressure variations, but it was also desired to limit the overall testing time so that any changes to the cornea that may occur over this time, such as hydration changes, creep etc could be minimised. Three measurements gave the ability to visually assess if the cornea was behaving in a repeatable manner so any problems with leakage or instability could be identified and the results could be discounted.

Processing of the wrapped and unwrapped data was all carried out automatically after each measurement had been taken. This was so the wrapped and unwrapped data could be viewed immediately after each measurement to check for issues or to evaluate the response. In the future, these processes could be moved offline to save time.

6. **Washing** – After the first round of interferometric testing the chamber was removed from the measurement area via sliding the of the baseplate so crosslinking could be conducted. Prior to crosslinking the coating of the cornea was removed by washing the surface of the cornea with PBS solution.

7. **Riboflavin application** – A riboflavin solution consisting of 0.22% riboflavin, saline, isotonic (VibeX-Xtra, Avedro Inc., MA, USA) was applied to the surface of the cornea, full coverage was obtained and the cornea was then left to soak for 10 minutes, with riboflavin reapplied to the central area at 5 minutes. This process was carried out in the dark to prevent any crosslinking that may have occurred due to ambient lighting. At the end of the soaking time any excess riboflavin was washed away.

8. **Crosslinking** – A specific crosslinking mask was created for each cornea to allow certain topographic locations to be crosslinked while the remaining surface was
protected. These masks were created by cutting out sections of 0.1 mm thick aluminium foil (Kitchen foil, Sainsburys, London, UK) to a specific size to cover and prevent UV illumination of the areas on the surface of the cornea where it was desired to have no crosslinking. The specific design of the mask used is detailed for each of the results. The masks were positioned over the surface of the cornea and folded around the sides of the chamber to prevent any movement. A crosslinking device with a UVA light source was used for crosslinking (KXL, Avedro, Inc., MA, USA). The power and total exposure time selected was generally 15 mW/cm² for 8 minutes delivering a total energy of 7.2 J/cm². Any variations from this are detailed in the results.

The reason for selecting these initial crosslinking parameters was because 7.2 J/cm² was the maximum energy that was possible during one treatment using the treatment cards supplied with the KXL crosslinking device, and initially it was desired to maximise any effects of crosslinking to determine whether a clear change could be identified in the response of the cornea measured via ESPI and LSI before and after crosslinking.

9. **Re-coating** – After the cornea had been crosslinked the surface was re-coated with a thin layer of Sphericel 110P8 fused borosilicate hollow glass microspheres as detailed in step 3. The chamber was then repositioned into the measurement area.

10. **Repeat interferometric testing** – The interferometric testing was repeated exactly as outlined in step 5 to measure the response of the sample after crosslinking. The shape measurement was not repeated as due to the sensitivity of the shape measurement system any changes to the shape of the cornea due to crosslinking would have been unlikely to have been detected.

### 6.4.2 Repeatability over total experimental time

To establish whether any changes introduced during crosslinking were a result of the crosslinking procedure alone, and not due to natural variations that occurred over the testing time, as a result of factors such as creep or hydration instability, changes to the response of the cornea over the total testing time were examined.

To determine the effects of the full testing procedure on the response of the corneas, a cornea was subjected to the full experimental testing procedure as outlined in steps 1 to 5 of the testing methodology, however for a pressure variation of 0.50 mmHg, 10 repeated measurements were taken for the LSI case instead of 3. After this, the chamber was removed from the measurement area, but instead of crosslinking, the coating was washed away from the surface.
of the cornea using the PBS solution and the cornea was left to sit at the baseline pressure for 20 minutes, which was the approximate time needed for the soaking and crosslinking procedure. A fresh coating was then applied to the cornea and it was subjected to another full loading cycle. The results were compared for a pressure change of 0.50 mmHg.

An example of two of the wrapped interferograms, one of which was recorded over the 1st measurement cycle and the other of which was recorded over the 2nd measurement cycle are shown in Figure 6-19. It was found that the overall morphology of the wrapped interferograms recorded over the two cycles did not change significantly, with the distribution of the fringes remaining similar inside of the 2 mm boundary regions, although the size of the fringes varied slightly.

![1st round and 2nd round](image)

Figure 6-19 – Wrapped LSI data obtained for a porcine cornea subjected to a 0.5mmHg increase in hydrostatic pressure over two rounds of testing.

On closer inspection of the data, it appeared that there were differences in the magnitude of the response that occurred over the experimental time that could not be accounted for by the variability of the response that occurred over repeated loading cycles during each round of testing. These changes are demonstrated in the plot in Figure 6-20, which compares the average measured phase change and the standard deviation in the measured phase change across a central horizontal cross-section of the cornea recorded over the 1st and 2nd rounds of testing. A simplified plot excluding the boundary regions and the standard deviation lines is also given in Figure 6-21 to highlight the difference in the average response along the same cross-section.

When examining the change in the response that occurred over the two loading cycles shown in Figure 6-21, it was evident that the profile of the response showed similar features, however, during the second loading cycle the magnitude of the measured phase change, was reduced in some areas when compared with the first loading cycle.
Figure 6-20 – Plot comparing the average phase change measured via LSI across a central horizontal cross-section of a porcine cornea subjected to a hydrostatic pressure increase of 0.50 mmHg during the 1st round of testing and during the 2nd round of testing.

Figure 6-21 – Average phase change measured via LSI across the same cross section as shown in figure 6-20.

There are several factors that could account for the change in the response over the experimental time, including hydration changes or creep, therefore it is not possible to isolate a specific variable that resulted in these changes. Overall, it could be concluded that small changes to the magnitude response occurred over the experimental time, but the overall nature of the response remained similar. Hence the overall profile of the LSI curve taken along a specific cross-section, and the morphology of the LSI fringe distributions visible in the wrapped data would be expected to remain similar in the absence of any changes introduced by crosslinking.
6.4.3 Results of crosslinking experiments

Crosslinking was conducted on porcine corneas in various topographical locations to assess the ability of both the ESPI and LSI measurement techniques to detect any changes introduced by crosslinking, and to show the effect of these changes across the entire corneal surface. The purpose of these experiments on porcine corneas was to assess the viability of ESPI and LSI for measuring the changes introduced by crosslinking, prior to human cornea experimentation, and to provide a set of results with which results from human corneas could be compared.

Crosslinking was conducted in four specific topographic locations including a central 3 mm circular region, around the outside of a central 3 mm circular region, along a 3 mm strip across the centre of the N-T axis, and in a 3 mm strip down the centre of the S-I axis, matching the locations previously shown in Table 5-7 of Chapter 5 during the simulation experiments on non-biological samples. Towards the end of porcine cornea testing a few other crosslinking locations were attempted and these are detailed later for specific results. Most experiments were conducted using the methodology outlined in section 6.4.1, any variations from this are detailed for individual results.

A single example is given for the results obtained when crosslinking a cornea at each of the four topographic locations described. All the results that have been presented detail the response of a porcine cornea to a pressure increase of 0.50 mmHg above baseline pressure of 16.50 mmHg. As with all previous data described throughout this thesis the X-axis corresponds to the N-T axis and the Y-axis corresponds to the S-I axis.

Central 3 mm circle

A foil mask with a 3 mm circular disc cut out from the centre was made and positioned over the surface of the cornea after it had been soaked in riboflavin for 10 minutes. Crosslinking was then conducted as described in step 8 of the methodology outlined in section 6.4.1 with only the central 3 mm region of the cornea exposed to UVA light.

Based on the data gathered during testing on non-biological samples, it was expected that stiffening the cornea in a central 3 mm circular region would result in a reduction of the out-of-plane displacement within this central region, along with a reduction in both the first and second derivative of out-of-plane displacement when compared with the pre-crosslinking experiments under equivalent pressure variations.

Surface plots of the out-of-plane displacement component \(w\) calculated from the ESPI data recorded on a cornea responding to a pressure increase of 0.50 mmHg, before and after
crosslinking in an approximately central 3 mm area are shown in Table 6-5. The corresponding line plots of out-of-plane displacement along the centre of the cornea with respect to both the X (N-T) and Y (S-I) axes are shown in Figure 6-22 (a) and (b) respectively. The region of crosslinking has been highlighted on the plots.

<table>
<thead>
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<th>Pre-crosslinking w (mm)</th>
<th>Post crosslinking w (mm)</th>
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<td><img src="image1" alt="Surface plots before crosslinking" /></td>
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**Table 6-5** – Surface plots showing the out-of-plane displacement of the corneal surface in response to a 0.50 mmHg increase in hydrostatic pressure before and after crosslinking in an approximately central 3 mm region.

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**Figure 6-22** – Line plots of the out-of-plane displacement in response to a 0.50 mmHg increase in hydrostatic pressure before and after crosslinking in an approximately central 3 mm region. a) along central N-T axis, b) down the central S-I axis.

From viewing the data shown in Table 6-5, it was evident that there was a change to the magnitude of the measured out-of-plane displacement that occurred in response to a 0.50
mmHg increase in hydrostatic pressure before and after crosslinking. Interestingly, the appearance of the data indicated that the changes were not limited to the central 3 mm area in isolation, as there were visible reductions in surrounding regions, particularly towards the top of the cornea.

The changes to the response along the centre of the N-T and S-I axes were analysed in more detail using the line-plots in Figure 6-22 (a) and (b) respectively. Along the N-T axis, prior to crosslinking, the magnitude of out-of-plane displacement peaked in the central area, whereas after crosslinking, while the magnitude of the response remained similar at the boundaries, it decreased in magnitude slightly towards the centre, specifically in the region of crosslinking. The overall profile of out-of-plane displacement along the N-T axis after crosslinking indicated a flattening of the curvature with respect to this axis.

The changes to the response along the N-T axis after crosslinking were also evident from the LSI data, from which the calculated value of $\frac{\partial \nu}{\partial x}$ has been plotted along the same cross section in Figure 6-23. The gradient of displacement goes slightly negative and then positive again between (X = 10 mm to 14 mm) indicating a dip in the magnitude of the out-of-plane displacement at this point. For the LSI data, the apparent shift in position with respect to the displacement data shown in Figure 6-22 (a) is due to the applied shear, as the displacement at a given reference point is compared with a point 65 pixels (1.35 mm) ahead, hence the gradient at a given point in the LSI data relates to the gradient between that point and a point 1.35 mm in front with respect to the ESPI data.

With respect to the S-I axis, from Figure 6-22 (b) it was evident that the out-of-plane displacement was also reduced in the central area of this axis after crosslinking, however, the
most significant reduction was seen towards the top of this axis from Y = 11 to Y = 15. The reason a relatively large reduction in out-of-plane displacement was seen in this area is unknown since this area was not exposed directly to the UVA and therefore not crosslinked. However, since the cornea has a fibrous and complex structure, it is possible that crosslinking in any given area could also result in reductions in displacement in surrounding areas.

**Outer ring**

To enable crosslinking around an outer ring a 3 mm diameter circular foil disk was cut out and positioned approximately centrally on the surface of the cornea, after it had been soaked in riboflavin, to prevent this area from being exposed to the UVA source.

Based on the data from non-biological samples detailed in section 5.4 of chapter 5, the expected effects of crosslinking around a masked central 3 mm region were that there would be an overall reduction in the out-of-plane deformation across the sample in response to a pressure increase, and that the relative displacement of the non-crosslinked central 3 mm region would be the same, or slightly increased over that of the control.

The surface plots of the out-of-plane displacement component (w) calculated from the ESPI data recorded for a cornea deforming in response to a 0.50 mmHg pressure increase before and after crosslinking around a masked central 3 mm area are shown in Table 6-6. Line plots of the out-of-plane displacement component taken along the centre of the cornea with respect to both the X (N-T) and Y (S-I) axes are shown in Figure 6-24 (a) and (b) respectively.

The ESPI results obtained during porcine cornea testing when crosslinking in this specific topographic location generally didn’t show similar changes in the out-of-plane displacement component to those that had been expected based on the simulation data obtained from experiments on non-biological samples.

For the porcine cornea, it was found that around the outer boundary areas there was a reduction in the magnitude of out-of-plane displacement in some areas, specifically towards the left side of the sample, but an increase in others. Along the centre of the N-T axis, shown in line plot in Figure 6-24(a), the most significant reduction in out-of-plane displacement was seen in the area on the left-side that had shown relatively high out-of-plane displacement during control testing, this corresponded to an area of crosslinking, so appeared to show what was expected. However, in the crosslinked region to the right of the centre the out-of-plane displacement actually increased after crosslinking, resulting in the magnitude of out-of-plane displacement becoming more even across this axis when compared to the pre-crosslinking case where displacement was significantly greater on the left side of the sample.
Table 6-6 – Surface plots showing the out-of-plane displacement of the corneal surface in response to a 0.50 mmHg increase in hydrostatic pressure before and after crosslinking around a masked central 3 mm circular region.

![Surface plots showing the out-of-plane displacement of the corneal surface](image)

<table>
<thead>
<tr>
<th>Pre-crosslinking w (mm)</th>
<th>Post crosslinking w (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Surface plot" /></td>
<td><img src="image" alt="Surface plot" /></td>
</tr>
</tbody>
</table>

Figure 6-24 – Line plots of the out-of-plane displacement in response to a pressure increase of 0.50 mmHg before and after crosslinking around a masked central 3 mm circular region. a) along central N-T axis, b) down central S-I axis.

With regards to the centre of the S-I axis, shown in Figure 6-24(b), the reduction in out-of-plane displacement was most significant at the edges of the sample, but again there was an increase in out-of-plane displacement after crosslinking just above the centre of the sample as viewed in Table 6-6. Overall, after crosslinking the magnitude of the response was slightly more even from the top to the bottom of the sample when compared with the pre-crosslinking data.
Interestingly, in the central 3 mm area where the crosslinking was absent, there did not appear to be a significant increase in out-of-plane displacement relative to surrounding crosslinked areas, and the position of the crosslinked area was not obvious from the plots. Several different explanations were considered for why the response changed in the specific way shown in Table 6-6 and Figure 6-24, these were:

- The diameter of the UVA beam was 9 mm as it was designed at a specific size to treat human corneas, therefore, it did not cover the entirety of the porcine cornea as highlighted in Figure 6-25. This resulted in the outer areas not being subjected to the UVA source and therefore the crosslinking was confined to the central 9 mm area minus the most central 3 mm area.
- The steeper curvature of the porcine cornea compared to the human cornea could have contributed to variable UVA power across the corneal surface with lower power in curved regions further from the source.
- The central area is resistant to changes in curvature and therefore the lack of crosslinking in this area did not necessarily lead to a significant degree of change in the rate of change of the out-of-plane displacement component compared to surrounding areas despite the edge areas having increased stiffness relative to the control case.

![Image of a porcine cornea undergoing crosslinking around an approximately central 3mm circle](image)

To further examine the effects of crosslinking around the outer areas, the same cornea was exposed to a second round of crosslinking, however on this occasion the desired crosslinking area was divided into four separate sections and the UVA source was focussed on each section individually to ensure full coverage. The total energy from the UVA source to which the cornea was exposed was increased to 21.6 J/cm² to maximise the level of crosslinking possible across all areas, to ensure that the area that had been exposed to the UVA source during the previous round of crosslinking were not likely to be crosslinked more than other areas.
Surface plots of out-of-plane displacement calculated from the ESPI data recorded for the cornea responding to a 0.50 mmHg increase in hydrostatic pressure after the 2nd round of crosslinking are shown in Figure 6-26.

![Surface plots](image)

*Figure 6-26 – Surface plots showing the out-of-plane displacement of the corneal surface in response to a 0.50 mmHg increase in hydrostatic pressure after a 2nd round of crosslinking around a masked central 3mm region.*

The second round of crosslinking on the same cornea, resulted in a change to the magnitude and distribution of the out-of-plane displacement component over that of the first round of crosslinking. The overall distribution of out-of-plane displacement was more like the distribution shown pre-crosslinking, however, the magnitude of out-of-plane displacement was reduced for the same pressure change. The greatest rate of change in out-of-plane displacement remained at the outer edges despite the presumed large increase in the number of crosslinks in this area. There was a very slight increase in the out-of-plane displacement component relative to immediately adjacent areas in the very centre of the cornea (highlighted by A in Figure 6-26 and Figure 6-27(a) and (b)) and this corresponded to position of the area that was masked during UVA exposure.

![Line plots](image)

*Figure 6-27 – Line plots of out-of-plane displacement in response to a 0.50 mmHg pressure increase before crosslinking and after a 2nd round of crosslinking around a masked central 3 mm region. a) along central N-T axis, b) down central S-I axis.*
This area was found to be highlighted better in the LSI data as shown in Figure 6-28, where there was a clear peak in the value of $\frac{\partial w}{\partial x}$ relative to adjacent areas, indicating a slight steepening of the gradient of the surface in this region.

![Figure 6-28](image_url)

Figure 6-28 – Line plot of $\frac{\partial w}{\partial x}$ calculated from LSI data, along central N-T axis of a cornea responding to a 0.50 mmHg increase in pressure before crosslinking and after a 2nd round of crosslinking around a masked central 3 mm circle.

Overall it appeared that only very small changes could be made to $\frac{\partial w}{\partial x}$ in the central 3 mm area despite crosslinking the surrounding areas with high energy. These results suggested that, for the porcine corneas tested, the central area even in the absence of crosslinking was relatively resistant to changes in $\frac{\partial w}{\partial x}$ and $\frac{\partial w}{\partial y}$.

**Horizontal strip**

To enable crosslinking along a central horizontal strip, a foil mask was prepared with a 3 mm wide strip cut out along the centre, so when positioned on the cornea the full surface was covered except for a 3 mm wide section along the full length of the N-T axis. Although a 3 mm wide section was exposed along the full length of the N-T axis, as with the previous example discussed, due to the size of the beam the crosslinked area did not extend to the edges of the cornea but was limited to the central 9 mm region only.

Surface plots of the out-of-plane displacement component ($w$) calculated from the ESPI data of a cornea responding to a 0.50 mmHg increase in hydrostatic pressure before and after crosslinking along a horizontal 3 mm wide strip along the N-T axis are shown in Table 6-7.
plots of the out-of-plane displacement component taken along the centre of the cornea with respect to both the X (N-T) and Y (S-I) axes are shown in Figure 6-29 (a) and (b) respectively.

<table>
<thead>
<tr>
<th>Pre crosslinking w (mm)</th>
<th>Post crosslinking w (mm)</th>
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</thead>
<tbody>
<tr>
<td><img src="image1" alt="Pre crosslinking" /></td>
<td><img src="image2" alt="Post crosslinking" /></td>
</tr>
</tbody>
</table>

Table 6-7 – Surface plots showing the out-of-plane displacement of the corneal surface in response to a 0.50 mmHg hydrostatic pressure increase before and after crosslinking along a 3 mm strip along the central N-T axis.

![Line plots](image3)

Figure 6-29 – Line plots showing the out-of-plane displacement in response to a 0.50 mmHg pressure increase before and after crosslinking along a 3 mm strip along the central N-T axis. a) along central N-T axis, b) down the central S-I axis.

Based on the results of simulation testing with non-biological samples it was expected that crosslinking along a 3 mm wide strip along the centre of the N-T axis would result in a reduction in out-of-plane displacement and the 1st and 2nd derivatives of out-of-plane displacement along the crosslinked axis. With respect to the vertical axis (S-I axis), it was expected that the out-of-plane displacement component would remain similar to the pre-crosslinking case in the regions...
either side of the 3 mm wide central area in which crosslinking was focussed, but be reduced slightly in the 3 mm crosslinked region.

However, unlike for the non-biological sample, for the cornea shown in Table 6-7, the introduction of crosslinking along the N-T axis appeared to result in an overall reduction in the out-of-plane displacement component in all areas. Along the crosslinked region specifically, highlighted in the line plot in Figure 6-29 (a), the peak in the magnitude of the out-of-plane displacement that had been evident at X = 15 mm prior to crosslinking was not evident after crosslinking and the magnitude of response along this axis became more even in magnitude. Although, the magnitude of the out-of-plane displacement component did peak slightly at the right boundary (X = 21 mm) after crosslinking, but it was thought that this may have occurred because the crosslinking did not extend to this area due to the diameter of the UVA beam.

These changes to the response along the central N-T axis before and after crosslinking were also evident from examining $\frac{\partial w}{\partial x}$ calculated from the LSI data, as shown in Figure 6-30. This data was captured at a slightly lower pressure change of 0.25 mmHg to avoid speckle decorrelation issues. From Figure 6-30, it can be seen that after crosslinking the magnitude of $\frac{\partial w}{\partial x}$ remained close to zero along the crosslinked area, apart from a slight rise in the area approaching the boundary region, whereas prior to crosslinking a peak was evident near the centre.

![Figure 6-30](image)

*Figure 6-30 – Line plot of $\frac{\partial w}{\partial x}$ measured via LSI data along the central N-T axis of a cornea over a 0.25 mmHg increase in hydrostatic pressure before and after crosslinking along the central 9 mm of this axis.*

With respect to the S-I axis, shown in Figure 6-29 (b), although the magnitude of the response was decreased in all areas after crosslinking, the overall profile of out-of-plane displacement...
remained similar, with peaks in the magnitude of out-of-plane displacement occurring at the top and bottom of this axis and a dip in the central area, corresponding to the region of crosslinking. Although, the overall profile of the response did not change much with respect to this axis, the response after crosslinking was in-line with what had been expected from the simulation experiments, as the magnitude of out-of-plane displacement component was reduced in the crosslinked region relative to surrounding areas.

**Vertical strip**

To limit crosslinking to a 3 mm wide strip down the centre of the S-I axis a foil mask was created, similar to the one described for the previous example, but positioned on the cornea so the exposed area was along the centre of the S-I axis while the rest of the surface was covered. As with the previous example due to the beam size the length of the area exposed to the UVA source was 9 mm, however as the S-I axis was slightly shorter than the N-T axis, a larger proportion of the cornea along this axis was exposed to the UVA source.

Surface plots of the out-of-plane displacement component ($w$), calculated from the ESPI data, of a cornea responding to a 0.50 mmHg increase in hydrostatic pressure before and after crosslinking down a central 3 mm wide strip down the S-I axis are shown in Table 6-8. Line plots of the out-of-plane displacement component taken along the centre of the cornea with respect to both the X (N-T) and Y (S-I) axes are shown in Figure 6-31 (a) and (b) respectively.

Based on the simulation crosslinking data on non-biological samples it was expected that crosslinking in this region would result in an overall reduction in the magnitude of the out-of-plane displacement along the crosslinked axis, along with a reduction in the $1^{st}$ and $2^{nd}$ derivatives of out-of-plane displacement. As with the previous example, in the orthogonal axis it was expected that there would be a dip in the magnitude of the out-of-plane displacement component relative to adjacent regions where the 3 mm wide crosslinked area crossed this axis.

Overall, the results generally quite fit well with what had been expected based on the simulation data. From the results shown in Table 6-8 there appeared to be a significant reduction in out-of-plane displacement down the central S-I axis where the out-of-plane displacement had been greatest prior to crosslinking. When this axis was examined in more detail using the data in the line plot shown in Figure 6-31 (b), it was evident that down the region of crosslinking the magnitude of out-of-plane displacement became more even, with a large decrease occurring towards the top of the sample (as viewed in Table 6-8), which had initially shown relatively large displacement prior to crosslinking. As with the previous example, after crosslinking, towards the edges of this axis ($Y = 6$ mm and $Y = 15$ mm) there was a slight increase in out-
of-plane displacement relative to the areas inside of this, presumably, this occurred as these areas were not crosslinked as they had not been exposed to the UV source due to the 9 mm beam diameter.

<table>
<thead>
<tr>
<th>Pre crosslinking w (mm)</th>
<th>Post crosslinking w (mm)</th>
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<tr>
<td><img src="image1" alt="Pre crosslinking" /></td>
<td><img src="image2" alt="Post crosslinking" /></td>
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*Table 6-8 – Surface plots showing the out-of-plane displacement of the corneal surface in response to a 0.50 mmHg increase in hydrostatic pressure before and after crosslinking down a 3 mm wide strip along the central S-I axis.*

![Line plots](image3)  

*Figure 6-31 – Line plots showing the out-of-plane displacement in response to a 0.50 mmHg pressure increase before and after crosslinking down a 3 mm wide strip down the central S-I axis. a) along central N-T axis, b) down central S-I axis.*

With respect to the central N-T axis, as shown in Figure 6-31 (a), the magnitude of the response remained similar at the edges of the sample before and after crosslinking, however after
crosslinking, the magnitude of the out-of-plane displacement decreased from the edges to the centre of the sample, this was in-line with what had been expected due to the central 3 mm region of crosslinking along this axis. This decrease in the magnitude of out-of-plane displacement in the central area relative to adjacent areas after crosslinking was highlighted in the horizontal LSI data across the same central N-T cross-section, shown in Figure 6-32, where $\frac{\partial w}{\partial x}$ went from slightly negative to positive in this region.

![Figure 6-32](image)

*Figure 6-32 – Line plot of $\frac{\partial w}{\partial x}$ measured via LSI, along the central N-T axis of a cornea over a 0.50 mmHg increase in hydrostatic pressure before and after crosslinking down a 3 mm wide strip down the central S-I axis.*

### 6.4.4 Demonstration of customised crosslinking based on identification of areas of abnormality

Ultimately, if the measurement technique was to be further developed to be used *in vivo*, the aim would be to use the technique to identify weak areas, or areas of abnormality on individual corneas so specific treatment protocols could be designed to restore them back to a normal state, or to prevent any further progression of abnormalities. The measurement technique could then be used post-intervention to determine the success of various treatments.

To demonstrate the capabilities of the techniques with regards to this aim, towards the end of the porcine cornea experiments, attempts were made to identify corneas that showed an abnormal or unbalanced fringe distribution when compared to the majority of corneas tested. Once identified, the aim was to then to crosslink these corneas in a specific area based on the initial fringe distribution, attempting to make the displacement profile more uniform.
During these initial experiments, the judgement on where to treat the cornea was based on fringe distribution and the appearance of the unwrapped phase plots alone, as the results were not fully processed in-between measurement cycles.

An example is shown for a porcine cornea which initially showed increased out-of-plane displacement on the right side compared to the left. This increased deformation was evident from both the ESPI data and the LSI data as there was a high fringe concentration on the right side of the interferograms relative to the left, these fringe distributions are shown in Table 6-9.

<table>
<thead>
<tr>
<th>Wrapped data pre-crosslinking</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESPI</td>
</tr>
<tr>
<td>![ESPI image]</td>
</tr>
<tr>
<td>LSI</td>
</tr>
<tr>
<td>![LSI image]</td>
</tr>
</tbody>
</table>

Table 6-9 – Wrapped ESPI and LSI data recorded on a porcine cornea subjected to a 0.50 mmHg increase in hydrostatic pressure prior to crosslinking.

A surface plot and a line plot taken along the centre of the N-T axis, of the out-of-plane displacement component that were generated from the ESPI data shown in Table 6-9, post-experimentation are given in Figure 6-33 (a) and (b) respectively. These plots confirm that the cornea was displacing more on the right side as opposed to the left, as had already been deduced from the fringe distributions during experimentation.

![Surface plot](image1)

![Line plot](image2)

Figure 6-33 – a) Surface plot of out-of-plane displacement recorded via ESPI on a porcine cornea subjected to a 0.50 mmHg increase in hydrostatic pressure. b) Corresponding line plot along central N-T axis.
Based on the initial fringe distributions, the decision was made to crosslink the right side of the cornea while masking the left. To achieve this a semi-circular foil mask was made and positioned to protect the left half of the cornea from the UVA source. The standard crosslinking procedure as detailed in the methodology was used, delivering a total energy of 7.2 J/cm$^2$ to the crosslinked area. The measurement procedure was then repeated post crosslinking. The ESPI and LSI fringe distributions recorded post-crosslinking for a pressure change of 0.50 mmHg are shown in Table 6-10.

<table>
<thead>
<tr>
<th>Wrapped data post-crosslinking</th>
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</thead>
<tbody>
<tr>
<td>ESPI</td>
</tr>
<tr>
<td>LSI</td>
</tr>
</tbody>
</table>

![Wrapped ESPI and LSI data recorded on the same porcine cornea as shown in table 6-9, subjected to a 0.50 mmHg increase in hydrostatic pressure after crosslinking the right side.](image)

Table 6-10 – Wrapped ESPI and LSI data recorded on the same porcine cornea as shown in table 6-9, subjected to a 0.50 mmHg increase in hydrostatic pressure after crosslinking the right side.

Post-crosslinking the number of fringes generated under the same pressure change of 0.50 mmHg was reduced on the right side for both the ESPI and LSI case, and the number of fringes appeared more even at each side for the ESPI case. When examining the results in more detail post-experimentation, it was clear that the application of crosslinking to the specific area identified in preliminary testing had had the desired effect, as the magnitude of the out-of-plane displacement component had become more even across the entire cornea, as evident from the surface plot of the out-of-plane displacement component shown in Figure 6-34(a), and the line plot taken across the central N-T axis, shown in Figure 6-34 (b).

![Figure 6-34 – a) Surface plot of out-of-plane displacement recorded via ESPI on a porcine cornea subjected to a 0.50 mmHg increase in hydrostatic pressure after crosslinking the right side only. b) Line plot comparing the out-of-plane displacement of the corneal surface along the central N-T axis before and after crosslinking.](image)
This experiment demonstrated that regions of abnormality could be identified directly from the ESPI and LSI plots. However, the ability to identify regions of abnormality relied on the fact many samples had been examined prior to this experiment, as these tests established the parameters of what could be considered a normal response.

6.4.5 Further observations from crosslinking experiments

Use of larger pressure variations

As demonstrated in the previous section, one of the things that was noticed during testing of porcine corneas was that a lot of information could be derived from examining the ESPI and LSI fringe distributions even before performing any quantitative analysis. In addition to this it was found that measurements taken over greater pressure increases (exceeding 1.00 mmHg) sometimes helped to highlight the specific effects of crosslinking or abnormalities prior to data processing. Some examples are provided in this section.

An example of the wrapped ESPI data obtained on a cornea that was crosslinked in the shape of a bow-tie along the centre of the N-T axis, in response to both a 0.50 mmHg hydrostatic pressure increase, and also in response to a 1.50 mmHg hydrostatic pressure increase, is shown in Figure 6-35.

![Wrapped ESPI data recorded on a crosslinked porcine cornea in response to a hydrostatic pressure increase of 0.50 mmHg (left), and 1.50 mmHg (centre). Image on the right shows position of crosslinked bowtie.](image)

The fringes in the wrapped image generated for a smaller pressure increase of 0.50 mmHg are of higher quality, but the fringes in the wrapped image generated for a larger pressure increase of 1.50 mmHg visually highlight the position of the crosslinked bow tie area. The absence of fringes in the area of crosslinking, even under higher pressure changes where the fringe number in surrounding areas was relatively high, helps to highlight that this area may be biomechanically different.

Using higher loads (greater than 1.00 mmHg) was found to be especially useful for the LSI case. As it was generally not possible to obtain many fringes across the central area while trying to maintain fringe quality across the whole sample, hence it was often difficult to interpret
the changes that occurred during crosslinking directly from the data. However, if the data quality at the boundary areas was ignored, the use of higher loads enabled more fringes to be generated across the more resistant central area.

Generating a higher number of fringes across the central area assisted in highlighting changes and defects across this area prior to processing of the results. An example of the wrapped and unwrapped data obtained via LSI over different pressure changes for a porcine cornea after crosslinking in a central 3 mm circle is given in Table 6-11 to illustrate this point. The red circle on the wrapped images indicates the approximate area of crosslinking.

<table>
<thead>
<tr>
<th>Pressure increase (mmHg)</th>
<th>Wrapped data</th>
<th>Unwrapped data</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td><img src="image" alt="Wrapped data 0.50" /></td>
<td><img src="image" alt="Unwrapped data 0.50" /></td>
</tr>
<tr>
<td>1.50</td>
<td><img src="image" alt="Wrapped data 1.50" /></td>
<td><img src="image" alt="Unwrapped data 1.50" /></td>
</tr>
</tbody>
</table>

Table 6-11 – Comparison of wrapped and unwrapped LSI data obtained on a porcine cornea in response to a hydrostatic pressure increase of 0.50 mmHg and 1.50 mmHg. Red circle indicates area of crosslinking.

As can be seen from the results in Table 6-11, the crosslinked area is visibly more noticeable, in the unwrapped image for the case where the pressure increase was greater. It was also noted that using LSI at higher pressure increases sometimes helped to visually highlight certain effects better than using ESPI alone. An example of the ESPI results from the same cornea as shown in Table 6-11 are shown in Figure 6-36.

![Figure 6-36 – Wrapped and unwrapped ESPI data recorded over a hydrostatic pressure increase of 0.50 mmHg on the same cornea as shown in table 6-11.](image)
Although the crosslinked area is also evident in the ESPI data, it does not stand out as much as for the LSI case. Based on these results it was concluded that in some situations, where it is desired to highlight changes or abnormalities, it may be more effective to record LSI data at higher loads, while masking out the edge areas.

**Interpretation and integration of LSI data**

Although the presence and shape of defects or changes could often be identified in the wrapped and unwrapped forms of the LSI data, it was found that Interpretation of the LSI data was not intuitive. To enable interpretation, it was often found to be most useful to examine specific cross-sections across areas of interest, especially when examining the effects of crosslinking as this helped to simplify the data.

It was also found to be useful to compare the LSI data with the displacement data obtained via ESPI to aid interpretation. However, there are situations where ESPI measurement would not be achievable, such as outside the laboratory environment or if considering in vivo measurement, thus if displacement data was required it would have to be obtained via integration of the LSI data.

Problems were experienced with integration of the LSI data on porcine corneas due to the issues with inaccuracies, and loss of data quality in the data at the boundary regions. This meant the integrated LSI data could not provide a numerically accurate measurement of the out-of-plane displacement component across the full sample. However, the LSI data could be integrated to provide a visual reference to help with interpretation of the LSI plots. An example of integrated LSI data recorded on a porcine cornea over a hydrostatic pressure increase of 0.25 mmHg before and after crosslinking down a 9 mm long, 3 mm wide strip down the centre of the S-I axis, is shown in Figure 6-37.

![Figure 6-37](image-url) – Integrated LSI data plotted along the central N-T axis of a cornea responding to a hydrostatic pressure increase of 0.25 mmHg before and after crosslinking a 3 mm wide central vertical strip.
Although numerically inaccurate, the integrated LSI data shown in Figure 6-37 could demonstrate the effect of changes to the profile of out-of-plane displacement that were introduced by crosslinking, as a dip in the magnitude of out-of-plane displacement component could be identified in the centre of the cross section after crosslinking, and this was similar to the response shown in the ESPI data previously detailed for crosslinking in the same topographic location as shown in Figure 6-31(a) in section 6.4.3.

**Reverse loading direction**

Throughout testing, the response of the cornea was only measured for increasing hydrostatic pressure. An observation that was made towards the end of experimentation, was that sometimes, when removing the load to return to baseline pressure the cornea responded slightly differently to decreasing pressure than it did to increasing pressure. This was found to be especially true after crosslinking. An example is given in Table 6-12, where the wrapped and unwrapped data is compared over each loading direction for a cornea that had been crosslinked around the outside of a central 3 mm circular area.

<table>
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</tr>
<tr>
<td>Negative</td>
<td><img src="image3" alt="Wrapped data" /></td>
<td><img src="image4" alt="Unwrapped data" /></td>
</tr>
</tbody>
</table>

*Table 6-12 – Comparison of wrapped and unwrapped ESPI data recorded on a cornea in response to a 0.50 mmHg increase in hydrostatic pressure and a 0.50 mmHg decrease in hydrostatic pressure.*

The difference between the crosslinked and non-crosslinked areas was more evident when the loading direction was reversed, as the central area that had not been crosslinked was clearly less resistant to out-of-plane displacement in this direction than the outside crosslinked regions. The exact reasons why this occurred are unknown, however it is likely due to hysteresis as first discussed in section 2.6.2 of Chapter 2. In summary, because the cornea is viscoelastic, when it is loaded and the load is removed it takes time to return to its original state. It is likely different regions exhibit a different hysteresis response, hence the different morphology of the fringe distributions observed during loading and unloading. Also, based on the data shown in Table 6-12, it is also likely that crosslinking changes the hysteresis response...
by changing the viscoelastic properties of the cornea. Initially it was thought that under small pressure variations that any hysteresis would be minimal, however, these results indicate that this may not be the case, so to examine the full dynamic behaviour of the cornea before and after crosslinking analysis in both loading directions is required.

6.5 Summary and conclusions from porcine cornea experiments

This chapter described the experiments on porcine corneas that were carried out to establish; a suitable ex vivo experimental methodology for human cornea experimentation, to investigate any changes to the tissue that may occur over the experimental time, to establish a methodology for crosslinking experiments and to validate that the ESPI and LSI techniques were capable of detecting the changes introduced due to crosslinking. The following is a summary of the main outcomes from the porcine cornea experiments.

The transparency of the corneal surface posed a challenge with regards to achieving an adequate signal from the surface. To address this problem several experiments were conducted to identify a suitable surface coating. The following conclusions were drawn from these experiments:

- Sphericel 110P8 fused borosilicate hollow glass microspheres (Potters Industries LLC, PA, USA) distributed as a thin layer on the surface of the cornea provided a suitable stable and impervious coating, from which an adequate signal could be obtained while not affecting the deformation of the cornea.
- Whilst it was demonstrated that the powder based coatings had no effect on the response of the cornea to pressure variations it was demonstrated that coating with a layer of PTFE tape over the corneal surface, as has been used in some previous studies\(^{(45)(51)}\), did.

These experiments were necessary to validate that what was being measured was the deformation of the corneal surface and not movement of the surface coating. Interestingly, this work was quite novel, as although many studies on the cornea have used surface coatings, few have reported evaluating the effects of the surface coating on the measured response and none have provided data to back up any statements.

From the experiments conducted to establish a suitable measurement range and optimise the technique for corneal testing the following conclusions were drawn:

- The total magnitude of the pressure variation over one measurement cycle was limited to around 1.00 mmHg, for both ESPI and LSI, as variations greater than this resulted in a loss of fringe quality over a significant proportion of the cornea. Although, this was
less than the average pressure change of 3 mmHg reported to occur over the ocular pulse cycle, this was not considered to be an issue as experiments demonstrated the cornea behaves approximately linearly over a 3 mmHg pressure range. In addition to this, in vivo, a proportion of the 3 mmHg pressure variation would likely be accommodated by a degree of scleral deformation.

- Overall, data quality for both the ESPI and LSI case was better for pressure variations less than 0.50 mmHg.
- Although quantitative analysis was not possible for larger pressure variations sometimes the higher fringe density helped to highlight certain details in the wrapped and unwrapped data that were not as obvious at lower pressures.
- For the LSI case, both the curvature of the cornea and the way the cornea deformed meant that the LSI data obtained over the 2 mm of the cornea adjacent to the edge of the chamber was prone to inaccuracies and lacked repeatability.
- It was demonstrated towards the end of experimentation that the response of the cornea varied with respect to the direction of pressure variation suggesting a degree of hysteresis and the need to examine the response to both loading and unloading.

Over the course of experiments the responses of over 44 porcine corneas to the same loading regime were analysed, this large sample size relative to many other ex vivo corneal studies enabled the variability of the response across different corneas to be analysed and the following conclusion to be made:

- There is large variation in the response of different corneas to changes in hydrostatic pressure, although the overall nature of the response across samples examined could generally be grouped into one of three common categories.
- Over half of the corneas tested were found to deform in a similar manner, with greatest out-of-plane displacement occurring at the top and bottom of the S-I axis, with the greatest rate-of-change of out-of-plane displacement occurring at the boundary regions. The nature of this response suggested that the limbal/peripheral regions of the cornea were more compliant than the central regions and deformed to accommodate pressure variations, minimising displacement and curvature change in the central regions.

Overall, due to the variability in the response observed across the different corneas examined a testing methodology was developed where the effects of crosslinking could be examined while using each cornea as its own control.

Experiments to investigate the repeatability of the response of the cornea over repeated loading cycles and any changes that occurred over the experimental time confirmed that:
• The response of individual corneas was found to be repeatable over repeated loading cycles, with the average standard deviation in the phase change measured via LSI to be +/- 0.47 radians.

• Over the total experimental time of 1 hour, small changes to the magnitude of the response of the cornea to pressure variations occurred that were larger than the expected variability observed over repeated loading cycles. However, the distribution of fringes and the visual appearance of the interferograms remained the same and the distribution of LSI data across a given cross-section remained similar.

These experiments enabled the results of the crosslinking experiments to be analysed while taking into account any changes that could be considered to be due to natural variability in the response over repeated loading cycles or a result of the experimental procedures used.

The crosslinking experiments on porcine corneas, are, so far, the only experiments to show the regional effects of crosslinking to the dynamic response of the cornea to pressure variations across the entire corneal surface, these experiments demonstrated that:

• Both the ESPI and LSI techniques were sensitive enough to pick up changes that occurred to the response of the cornea due to the crosslinking procedure as outlined in section 6.4.1.

• Crosslinking introduces measureable changes to the response of the cornea and these measured changes could not have been concluded to be due to changes that occurred to the tissue over the course of measurement, as different effects were observed when crosslinking across different topographic locations, and the changes were above those seen when investigating any changes that occurred over the experimental time in the absence of crosslinking.

• The changes to the response introduced due to crosslinking were not always as expected based on the data obtained from simulation testing on simple homogenous non-biological samples. The effects of crosslinking were also found to not be isolated to the region of crosslinking only.

• After testing on a range of samples and establishing what a normal response should be in terms of ESPI and LSI fringe distribution, it was possible to identify areas of biomechanical abnormality directly from the wrapped and unwrapped data plots. It was then possible to design a custom crosslinking protocol to address these areas of abnormality and make the response more uniform.
7. Human Cornea Experiments

7.1 Introduction

Through previous testing on both non-biological samples and porcine corneas, the capabilities and limitations of the ESPI and LSI techniques with respect to corneal measurement had been investigated, and a specific methodology had been established to examine human tissue.

7.1.1 Aims of human cornea experiments

The aims of human cornea testing were:

1. To investigate the surface deformation of human corneas with full-field resolution under pressure changes representative, and not exceeding those experienced physiologically.
2. To investigate the effects of crosslinking at specific topographic locations on the measured response to a pressure change.
3. To present the results in a format that is interpretable by a wide-ranging audience including ophthalmologists, clinicians, scientists and engineers.

7.1.2 Ethics statement and Tissue storage and handling

Six human corneas were obtained in the form of corneo-scleral button pairs from three donors. This tissue was provided by Moorfields Biobank and supported by NIHR funding. Research ethical approval was obtained from The Moorfields Biobank Internal Ethics Committee. The human corneas obtained were corneas that had been stored for longer than the 4 week to 8 week storage time over which endothelial cells are still viable and present in suitable numbers, hence the corneas were no longer considered suitable for transplantation \(^{(169)}\), but they were otherwise suitable for testing.

Post-collection from the donor, the corneas had been stored individually by suspending via a line of surgical thread in an airtight bottle of organ donor culture containing 80 ml Eagle’s minimum essential medium with HEPES buffer, 26 mmol/l NaHCO\(_3\), 2% fetal bovine serum, 2 mmol/l L-glutamine, penicillin, streptomycin and amphotericin B \(^{(169)}\). Once received, the corneas remained in their containers and were refrigerated at 4\(^\circ\)C until they were required for testing.

One hour prior to testing the corneas were removed from the refrigerator and transferred into a petri dish with 30 ml of the storage solution to allow them to come up to the room temperature of 20\(^\circ\)C. An initial visual examination of the corneas was made to check for any obvious
abnormalities. The surgical thread was then removed after marking the point of insertion with a waterproof marker, photographs of the specimens were taken for reference.

For testing, the corneas were positioned in the AAC in an orientation based on the N-T and S-I meridians. As with all previous data presented throughout the thesis, with respect to imaging orientation, the N-T axis is parallel to the X-axis and the S-I axis is parallel to the Y-axis as labelled in the images presented. Since all loose adnexa had been removed prior to storage, the position of the specific meridians could not be made based upon the position of the ocular muscle insertions, as they had been for the porcine cornea experiments. Identification of the meridians was made based on the presence of slight scleral overgrowth at the top and bottom of the S-I axis and the slight difference in length of the two axes, with the assumption that the S-I axis was shorter than the N-T when viewed en face\(^{(170)}\). Due to the similarity between the length of the axes, errors in positioning could not be ruled out with certainty, and it was not possible to identify the specific poles of each axes and therefore the poles of each axis remained ambiguous.

7.2 Experimental methodology for human cornea experiments

Based on the results of previous experiments on porcine corneas, it was decided to test the human corneal tissue in the AAC using both the large aperture tissue retainer (15.7 mm) that had been machined for testing with porcine corneas, and the original smaller aperture lid that came with the chamber (12.5 mm).

The reasons for conducting experiments while using both tissue retainers was because the previous experiments on porcine corneas had shown that the limbal area where the cornea meets the sclera was integral to the overall response to hydrostatic pressure variations, as it appeared to be the area that was least resistant to deformation. Due to this finding, it was desired to investigate this area on human corneas. Because the larger aperture tissue retainer clamped further away from the cornea, it enabled this limbal area to be investigated while reducing the influence from the fixed boundary constraints, as there was an approximately 2 mm region of sclera between the edge of the cornea and the edge of the chamber.

Whereas, when conducting experiments with the original aperture tissue retainer the response of the cornea could be isolated from the surrounding sclera, allowing for simpler analysis of the cornea only. Also, clamping with this tissue retainer more closely resembled the clamping conditions used during previous corneal experiments and in other studies, allowing for comparative analysis.
An example of the clamping position with respect the human cornea for each of the tissue retainers is given in the images in Figure 7-1. Tests with the large aperture tissue retainer were performed first to prevent any damage introduced via clamping from affecting the response.

![Image](image.png)

*Figure 7-1 – Human corneo-scleral section when positioned in the AAC with the large 15.7 mm aperture tissue retainer (left) and with the original 12.5 mm aperture tissue retainer (right).*

Once positioned within the chamber prior to applying the surface coating any remaining epithelium was removed from the cornea by gently dragging a blunt scalpel over the surface. Each of the human corneas was subjected to three rounds of interferometric testing. During the first round the response of the cornea was measured while clamping with the 15.7 mm tissue retainer. During the 2nd round the response of the cornea was measured while clamping with the original tissue retainer and during the 3rd round the response of the cornea was measured after crosslinking.

The testing procedure used during human cornea experiments was identical to the methodology outlined in chapter 6 in section 6.4.1, with the response of the cornea measured in response to hydrostatic pressure increases of 0.25 mmHg, 0.50 mmHg, 0.75 mmHg and 1.00 mmHg, above a baseline pressure of 16.50 mmHg.

The riboflavin used to soak the corneas in all experiments was VibeX-Xtra (Avedro Inc., MA, USA) and all crosslinking was conducted using KXL (Avedro Inc., MA, USA). The crosslinking parameters and crosslinking procedure remained the same as for the porcine cornea experiments outlined in section 6.4.1. The reasons for selecting the crosslinking parameters of 15m W/cm² for 8 mins delivering a total energy of 7.2 J/cm², was because the aim was to demonstrate how the topographical positioning of crosslinking affected the response, therefore it was desired to maximise the total energy delivered to the cornea. This energy level and exposure time had previously shown measurable changes during porcine cornea testing, as discussed in section 6.4.3 of chapter 6.
Several factors that had been discussed for the porcine cornea experiments were not investigated during human cornea testing. Since the focus for the human cornea experiments was on obtaining high quality data to enable quantitative analysis, the response of the corneas was not investigated for pressure variations over 1.00 mmHg. It was also of interest to determine for human corneas if the LSI data could be successfully integrated to provide an approximation for the out-of-plane displacement component, and for this data across the full sample needed to be resolvable.

The response of human corneas was investigated only to increasing hydrostatic pressure. This was because the experiments on porcine corneas that had shown differences in the response of corneas to loading and unloading cycles, as detailed in section 6.4.5 of chapter 6, were carried out after human cornea testing during further investigations on porcine corneas. However, this would be an area of interest for any further work.

7.3 Observations prior to loading

7.3.1 Tissue appearance

When examining the corneas prior to loading it was evident that some changes had occurred to the corneas during the storage period. The appearance of the front and back surface of one of the sample corneas immediately after removal from storage is shown in Figure 7-2.

![Figure 7-2 – Images showing the front (left) and back (right) surface of the cornea immediately after removal from the storage medium showing the presence of ’Descemet’s folds’ due to stromal swelling.](image)

This wrinkled appearance was a result of the fact the cornea had been in the storage medium for a period of time prior to testing which had led to swelling, this is a well-documented side-effect of storage in organ culture (171). As the cornea has a non-homogenous structure with several distinct layers with different properties, some layers can swell more than other resulting
in the wrinkled appearance, sometimes referred to as ‘Descemet’s folds’. The wrinkling is confined to the back surface of the cornea as the highly-interwoven structure of the anterior surface prevents it from swelling. Often a solution containing 15% Dextran is used to reduce swelling and restore the corneas to physiological thickness (172) after removal from storage medium, however Dextran was not used in these experiments based on collaborator advice, as it was desired to minimise changes that may occur over the experimental time during which the cornea would be exposed to PBS solution.

It was observed that once the cornea had been situated in the chamber and been left to rest at the baseline pressure for 10 minutes the wrinkling was not as obvious, but still evident on close inspection. Since tissue hydration contributes to biomechanics, it is possible any changes to hydration that occurred during the storage time may have affected the magnitude of the response to pressure changes. In addition to this, as the thickness of the cornea was increased the penetration depth of any crosslinking would have been reduced in the presence of swelling potentially reducing any stiffening effects.

In the literature, it has been reported that stromal swelling increases the interfibrillary distances in the posterior stroma, but the diameter or quality of the collagen fibres remains unaffected (171). Based on this, and the fact that the more resistant anterior stroma is thought to play the largest role in governing the overall biomechanics of the cornea, as discussed in section 2.2 of chapter 2, it was assumed that, for the swollen corneas, the magnitude of deformation may have differed slightly from fresh tissue but the mode of deformation would be likely to remain the same. With regards to crosslinking, since crosslinking has previously been demonstrated to only increase the stiffness of the anterior stroma while having no effect on the stiffness of the posterior stroma (173,174), swelling in the posterior stroma should be unlikely to effect the results. Obviously future testing with fresh corneas would be required to validate these assumptions.

When clamping the corneas in the chamber with the large aperture tissue retainer, it was noted that the hole in the sclera into which the surgical thread had been inserted was not fully covered for all the samples. Since the surgical thread was inserted at an angle it was hoped the holes may be self-sealing under pressure, however, this was found not to be the case for some of the samples. Putting a small dot of surgical-glue over the hole to prevent leakage was considered, however this would have likely influenced the response of the cornea so it was avoided. Instead the position of the hole was noted and the samples were monitored closely during testing time for any leakage. As the small chamber tissue retainer covered the position of the hole on all samples this was used to test each sample before and after crosslinking, to ensure the response was not influenced by the presence of the hole or any leaking.
7.3.2 Observations during settling time

After setting the corneas under the initial pressure of 16.50 mmHg, some interesting observations were made regarding the ESPI fringe patterns that formed during the settling time. An example of the fringes that were observed during this time despite no change in hydrostatic pressure, for a cornea in the AAC clamped with the large tissue retainer are shown in Figure 7-3.

From the wrapped data shown in Figure 7-3, it could be seen that a circular fringe appeared to form approximately around the outer edge of the cornea. Several almost triangularly shaped fringes also formed at locations on the sclera around the edge of this circle. This was despite the pressure remaining constant and no leaks being obvious for the specific cornea shown in Figure 7-3.

The reason for the observed fringe distributions was unknown, and it had not been something that had previously been encountered during porcine cornea testing. At first, it was thought that the effects may be due to very small pressure variations due to unnoticeable leaking from the hole in the sclera, however, no wetting of the surface coating was observed over the testing time, and it was found that during the initial settling time the fringe formation slowed and eventually the cornea became stable, making this unlikely.

It was considered that the observed effects could be a result of the cornea being in storage for some time. The cornea is adept to being under a constant state of stress, therefore when removed from a living host and from the remaining outer globe the fibres are no longer under stress and become relaxed. When returned to a stressed state, as in the chamber, it is likely that a degree of stress relaxation will occur initially, as the cornea returns to its natural state. The same phenomenon was not obvious during the porcine cornea experiments, likely due to the fact the corneas were removed from the fresh globe immediately prior to testing and therefore had been maintained under tension, albeit reduced from that experienced in vivo.
7.4 Pre-crosslinking results

7.4.1 Results from experiments when clamping with the large aperture lid

When clamping with the larger tissue retainer, a wide variation in the response of the corneas to pressure changes were observed across the range of corneas tested. For some samples leaking was evident which meant it was difficult to establish stability, and evidence of damage could be seen in the ESPI fringes. This damage manifested as an increased number of fringes at the position where the surgical thread had been inserted, an example is shown in Figure 7-4 for two samples where this was the case. Although these measurements did not provide information regarding the biomechanics of the cornea, they did demonstrate the sensitivity of the technique for detecting small areas of damage.

Figure 7-4 – ESPI fringes observed on two corneas in response to a 0.50 mmHg hydrostatic pressure, demonstrating evidence of damage in the region where the surgical thread had been inserted.

For the samples where leaking was not evident, and the hole on the anterior side of the sclera was covered by the tissue retainer, the fringes were generally concentrated to the scleral region on the outside of the measurement area, in-line with what had been expected based on the results previously observed on porcine corneas. The fringe formation at the edges was not regular, with sets of fringes extending from the outer edges towards the central areas of the cornea in locations in-between the S-I and N-T meridians. This is shown for two examples in Figure 7-5.

Figure 7-5 – Wrapped ESPI data from two corneas in response to a 0.50 mmHg increase in hydrostatic pressure, when clamped with the 15.7 mm aperture tissue retainer.

When LSI was used to measure the response of the corneas, the fringes in the wrapped data were found to be concentrated to the outside of the sample with few fringes forming across the
central area, similar to the results that had previously been observed on porcine corneas. An example of the wrapped and unwrapped LSI data recorded on a cornea in response to a 0.50 mmHg pressure change is shown in Table 7-1. An example is shown with shear applied with respect to both the horizontal and vertical axis of the cornea.

<table>
<thead>
<tr>
<th>Sensitivity Direction</th>
<th>Wrapped fringes</th>
<th>Unwrapped phase plot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horizontal</td>
<td><img src="image1.png" alt="Wrapped fringes" /></td>
<td><img src="image2.png" alt="Unwrapped phase plot" /></td>
</tr>
<tr>
<td>Vertical</td>
<td><img src="image3.png" alt="Wrapped fringes" /></td>
<td><img src="image4.png" alt="Unwrapped phase plot" /></td>
</tr>
</tbody>
</table>

*Table 7-1 – Wrapped and unwrapped LSI data with shear applied with respect to both the horizontal and vertical axis recorded for a cornea in response to a 0.50 mmHg pressure change.*

These fringe distributions indicated that the measured values of $\frac{\partial w}{\partial x}$ and $\frac{\partial w}{\partial y}$ were greatest in the scleral region at the edge of the cornea, and that this area was least resistant to out-of-plane deformation relative to the areas inside of this.

One further observation that was made from the LSI data was that the LSI phase plots appeared to have a random wrinkled appearance across the central area. This was not observed on the porcine corneas and is possibly an artefact of the swelling that took place during the storage time, with this resulting in small changes to the response across this region.

As the large aperture lid did not clamp inside of the area of damage on the human corneas, the tissue was structurally compromised, therefore conclusions regarding the biomechanics could not be drawn from the results obtained, so a full analysis was not carried out. All further work was carried out in the chamber with the original tissue retainer.

### 7.4.2 Results from experiments with the cornea in the original AAC

Table 7-2 shows the ESPI data recorded across each of the six human cornea samples in response to a pressure change of 0.50 mmHg above baseline pressure. The data is presented
in the format of wrapped fringes and surface plots of the out-of-plane displacement component that was calculated from the ESPI data. For reference, the three donor pairs were corneas 1 and 2, corneas 3 and 4, and corneas 5 and 6.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>ESPI wrapped data</th>
<th>Surface plots of w (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>2</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>3</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>4</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
</tbody>
</table>

(Table is continued on the following page)
The ESPI fringe formation across all the individual samples examined was found to be consistent and visually repeatable over repeated loading cycles, indicating the sample were responding in a repeatable manner to hydrostatic pressure variations and that leakage or instability was not an issue. To demonstrate the visual repeatability of the ESPI fringe distributions obtained an example of the wrapped ESPI data recorded over three repeated loading cycles for cornea 3 is shown in Table 7-3.

Table 7-2 – Wrapped ESPI data and plots of out-of-plane displacement calculated from the ESPI data obtained across the six human corneal samples clamped in the original AAC responding to a hydrostatic pressure increase of 0.50 mmHg from 16.50 mmHg to 17.50 mmHg.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>ESPI wrapped data</th>
<th>Surface plots of w (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>6</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
</tbody>
</table>

The response of the corneas also appeared to be relatively linear across the measurement range as the fringe number gradually increased while showing the same distribution, an
example is given for cornea 3 in Table 7-4. This had been expected based on the previous results from linearity experiments on porcine corneas, detailed in section 6.3.5 of chapter 6.

<table>
<thead>
<tr>
<th>Changes to ESPI fringes over pressure range</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta P = +0.25 \text{ mmHg}$</td>
</tr>
<tr>
<td><img src="image1.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Table 7-4 – Wrapped data recorded on cornea 3 in response to hydrostatic pressure increases of 0.25 mmHg, 0.50 mmHg, 0.75 mmHg and 1.00 mmHg.

The LSI data obtained from human corneas was also found to be visually repeatable across repeated loading cycles, however, there were similar problems with fringe quality to those that were experienced during porcine cornea testing. This was because, fringes tended to form close together at the edge of the sample adjacent to the edge of the chamber and they rapidly decorrelated with increasing loads. An example is given in Table 7-5 for cornea 2. From the data in Table 7-5, it can be seen that for pressure increases over 0.50 mmHg for this cornea the fringes at the boundaries become unresolvable.

<table>
<thead>
<tr>
<th>Changes to LSI fringes over pressure range</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta P = +0.25 \text{ mmHg}$</td>
</tr>
<tr>
<td><img src="image1.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Table 7-5 – Wrapped LSI data recorded on cornea 2 in response to hydrostatic pressure increases of 0.25 mmHg, 0.50 mmHg, 0.75 mmHg and 1.00 mmHg.

Although it had been found to be difficult to obtain LSI data absent of any speckle decorrelation in the areas adjacent to the edge of the chamber during porcine cornea testing, during the human corneas experiments it was found that LSI data without any speckle decorrelation could be obtained on most of the corneas under very small pressure changes of 0.25 mmHg. However, the total number of fringes was limited to approximately two.

Although the overall fringe number was limited at this pressure range, it was found there were a number of advantages to measuring the response of the cornea to pressure variations of this magnitude during experiments. Firstly, it was found that good repeatability could be achieved
over repeated measurement cycles at these pressure variations even at the boundary areas. This is demonstrated in Figure 7-6 where the phase change across the central N-T axis of cornea 3 measured using LSI over 3 repeated loading cycles where pressure was increased by 0.25 mmHg is plotted.

![Figure 7-6](image_url)  
*Figure 7-6 – Plot showing the phase change measured along the central N-T axis of cornea 3 over three repeated loading cycles where the hydrostatic pressure was increased by 0.25 mmHg*

Secondly, if the LSI data was compared with the corresponding differentiated ESPI data recorded over the same pressure increase of 0.25 mmHg, the results were generally found to be in good agreement, although small differences were still present due to the limitations of the LSI technique as discussed in section 3.6.4 of chapter 3. An example is shown in Figure 7-7 where $\frac{\partial w}{\partial x}$ calculated directly from the LSI data is compared with $\frac{\partial w}{\partial x}$ calculated from differentiated ESPI data obtained over the same pressure increase of 0.25 mmHg along an equivalent central horizontal cross section.

![Figure 7-7](image_url)  
*Figure 7-7 – Plot comparing $\frac{\partial w}{\partial x}$ calculated from the LSI data with $\frac{\partial w}{\partial x}$ calculated via differentiation of the out-of-plane displacement component calculated from the ESPI data along the central N-T axis of a cornea responding to a 0.25 mmHg increase in hydrostatic pressure.*
However, despite these advantages in terms of data accuracy, there were concerns that at such low-pressure changes the sensitivity to abnormalities, or changes to the central regions that could be introduced by crosslinking would be limited. Hence evaluation of the data obtained across a range of pressure variations was still carried out.

7.4.3 Analysis of the measured response pre-crosslinking

The data recorded across the six cornea samples, as shown in Table 7-2, was analysed in detail to gain an understanding of how the corneas responded to hydrostatic pressure increases. Four out of six of the samples (Sample no.’s 1,2,3,4) showed a similar out-of-plane response profile, this response profile had several identifiable features including:

- The greatest rate of change of the out-of-plane displacement component, was observed within the 1 mm boundary region where the cornea joined the sclera. This observation was confirmed by the LSI results shown in Table 7-6, where the formation of fringes was confined mostly to the outside areas, for both the horizontal and vertical case.

- The ESPI fringes were not circular in distribution indicating that the horizontal and vertical axes were deforming differently in the response to the pressure change. This was confirmed by the distribution of the LSI fringes obtained for horizontal and vertical sensitivity, as the distribution of fringes was different for each sensitivity direction. An example is given in Table 7-6 which shows the LSI fringe distributions obtained from cornea 3 with respect to each sensitivity direction.

<table>
<thead>
<tr>
<th>Sensitivity Direction</th>
<th>Wrapped fringes</th>
<th>Unwrapped phase plot</th>
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</thead>
<tbody>
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<td>Horizontal</td>
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</tr>
<tr>
<td>Vertical</td>
<td><img src="image3" alt="Wrapped fringes" /></td>
<td><img src="image4" alt="Unwrapped phase plot" /></td>
</tr>
</tbody>
</table>

Table 7-6 – Wrapped and unwrapped LSI data with shear applied with respect to both the horizontal and vertical axis recorded for cornea 3 in response to a 0.50 mmHg pressure change.
• Across the X-axis (corresponding to the N-T axis with respect to corneal orientation), the magnitude of the out-of-plane displacement component remained relatively even and had a low rate of change inside of a 1 mm boundary, this was confirmed by the horizontal LSI data shown in Table 7-6, as fringes were generally absent in the area inside of the 1 mm boundary. This indicated minimal curvature change across the central regions of the cornea in response to increasing hydrostatic pressure. The magnitude of the out-of-plane displacement component was also asymmetric in this axis across all of the samples examined, with slightly higher displacement observed at one side over the other, with peak out-of-plane displacement usually occurring off-centre. A line plot of the out-of-plane displacement component across a central horizontal cross section of cornea 3 when responding to a 0.50 mmHg hydrostatic pressure increase is shown in Figure 7-8, demonstrating these features.

Figure 7-8 – Surface plot and line plot along section A-A of the out-of-plane displacement calculated from the ESPI data recorded on cornea 3 over a pressure increase of 0.50 mmHg.

• Down the Y-axis (corresponding to the S-I axis with respect to corneal orientation) the rate of change of the out-of-plane displacement was also maximum at the boundary regions, however in this axis the magnitude of the out-of-plane displacement component continued to increase gradually inside of this towards the central ~ 3 mm area, with peak displacement generally occurring slightly off centre, and with the rate of change of displacement being steeper at one side of the axis over the other. These effects are evident from the vertical LSI data, as shown in Table 7-6, as the fringe formation extends across the central regions of the cornea indicating a change in the magnitude of out-of-plane displacement across this region. They are also evident in the line plot in Figure 7-9, which shows the out-of-plane displacement component down a
central vertical cross section of cornea 3 when responding to a 0.50 mmHg increase in hydrostatic pressure.

For the two remaining samples, the response to increasing hydrostatic pressure was different. Sample 6 showed a large amount of out-of-plane displacement localised to a specific area on the left side. This was thought to occur because the hole for the surgical thread had been inserted immediately adjacent to the cornea on the posterior side, this is shown in the image in Figure 7-10, and so even though the small aperture tissue retainer covered the hole on the anterior side, preventing any leaking, the presence of this damage to the specific region resulted in a change to the normal response.

Figure 7-9 – Surface plot and line plot along section B-B of the out-of-plane displacement calculated from the ESPI data recorded on cornea 3 over a pressure increase of 0.50 mmHg.

Figure 7-10 – Image of cornea 6 showing the proximity of the surgical thread used to suspend the cornea in the storage solution to the edge of the cornea.
The change in response and evidence of damage was detectable from both the ESPI and LSI fringe distributions due to the increased deformation in one area relative to the others. An example of the LSI fringe distribution for this case is shown alongside the corresponding unwrapped phase map in Figure 7-11. With the area of damage evident from the increased number of fringes in this region specifically.

![Area of damage](image)

*Figure 7-11 – Wrapped and unwrapped LSI data recorded for cornea 6 over a pressure increase of 0.25 mmHg.*

For sample 5, the fringe distribution was not overly dis-similar to the other samples, but showed more circularly distributed fringes, indicating that the horizontal and vertical axis were deforming more similarly. The fringes were still more concentrated to the outside areas but the rate of change of the out-of-plane displacement component to the left of the N-T axis was more gradual than seen for samples no.’s 1,2,3 and 4. Although it is difficult to make conclusions based on the results from a small sample size, it is possible that cornea 5 had a weak area in a region near to the centre allowing it to deform more in this region relative to other regions when compared to other samples.

The overall variability in the magnitude of the response across the corneal samples examined in response to a given pressure change was difficult to define due to the variation in the response across different areas. If the magnitude of the out-of-plane displacement component was considered at the centre of the cornea, where the measured out-of-plane component could be considered equal to the total displacement, the magnitude of measured out-of-plane displacement for a pressure increase of 0.50 mmHg ranged from 0.56 µm to 0.88 µm across samples with a median of 0.82 µm. Excluding sample 6 where obvious damage was present due to the position of the hole.

A central corneal displacement of 0.82 µm over a pressure change of 0.50 mmHg equates to a central corneal displacement of between 1.48 µm – 11.8 µm, assuming linearity of the response, for the normal range of reported ocular pulse amplitudes of 0.9 mmHg – 7.2 mmHg. This magnitude of central corneal displacement per mmHg rise in IOP is comparable to that shown in data presented over the pressure range of 15 mmHg – 20 mmHg in a recent study by Whitford, et al (22), where the response of a whole human eye globe was measured to increases in hydrostatic pressure up to 60 mmHg. It is also similar to the displacement of 1.30
µm per 1 mmHg predicted in section 2.6.1, that was based on using Equation 2-2 and the Young’s modulus values presented in Table 2-1 that were obtained from previous studies that had tested the response of the cornea over a physiological pressure range.

3D analysis

The results detailed up to this point have described the measured out-of-plane displacement component only, and as discussed throughout previous chapters, as the cornea is a 3D object, the horizontal and vertical in-plane components of deformation must be considered to establish how the cornea is responding to a pressure increase.

For the human corneas, the same method as detailed throughout previous chapters was used to initially estimate the in-plane components of deformation, using the surface elevation data and the assumption that deformation occurred normal to the surface.

During the human cornea testing it was found that the lesser overall elevation of the corneal surface in comparison to porcine corneas, and the more gradual changes in surface gradient gave advantages with respect to both shape measurement and interferometric testing. For shape measurement, less problems were experienced with regards to errors in the surface elevation data, as shading was less of an issue. Also, inaccuracies introduced into the surface elevation data during smoothing were reduced due to the non-steep changes in surface gradient. With respect to interferometric analysis, the reduced steepness in the gradient of the surface towards the boundary regions resulted in less variation in the sheared distance between points when using LSI, resulting in less significant variations in sensitivity.

An example 3D analysis is detailed for cornea 1 to illustrate how the measured data would be expected to relate to 3D surface deformation based on the assumption that displacement occurs radially. Cornea 1 was selected as an example as cornea samples 1, 2, 3 and 4 all showed similarities in the overall profile of deformation in response to increases in hydrostatic pressure.

Figure 7-12 (a) shows a full-field plot of the out-of-plane displacement component (w) calculated from the ESPI data recorded on cornea 1 over a hydrostatic pressure increase of 0.50 mmHg. A full-field plot of the horizontal in-plane component (u), that was estimated based on the assumption of radial deformation, is shown in Figure 7-12 (b), and a line plot of the magnitude of the out-of-plane displacement component, the horizontal in-plane displacement component and the estimated overall magnitude of displacement with respect to the surface.
normal (radial displacement) due to a combination of out-of-plane and in-plane deformation across a central horizontal cross-section is shown in Figure 7-12 (c).

![Figures](image)

**Figure 7-12** – a) Surface plot of out-of-plane displacement component \( w \) calculated from the ESPI data recorded on cornea 1 over a hydrostatic pressure increase of 0.50 mmHg. b) Surface plot of the approximated horizontal in-plane component \( u \). c) Line plot of \( w, u, \) and approximated out-of-plane displacement with respect to the surface normal across section A-A.

A vector plot to illustrate the surface deformation across the central N-T axis, based on the assumptions as previously described, was produced by scaling up the measured out-of-plane data and approximated horizontal in-plane data shown in Figure 7-12 by a factor of approximately 500, this is shown in Figure 7-13.

In addition to the above, a frame subtracted video of the cornea deforming to a relatively larger increase in hydrostatic pressure was also recorded to enable comparison with the predicted data. A screenshot from this video analysis is shown adjacent to the vector plot in Figure 7-14, for the central N-T cross-section.
Figure 7-13 – Vector plot demonstrating the predicted profile of displacement along the central N-T axis in response to a hydrostatic pressure increase.

Figure 7-14 – Screenshot from frame-subtracted video analysis data showing the deformation of the surface along the central N-T axis due to an increase in hydrostatic pressure.

The measured and estimated data as shown in Figure 7-12, suggested that across the N-T axis the greatest rate of change of displacement would be within the 1 mm boundary regions adjacent to the chamber, and the total surface displacement would be similar in magnitude across the majority of the surface of the cornea but slightly higher at the right side compared to the left. Overall, the in-plane contribution was expected to be small, relative to the out-of-plane contribution, as the angle of the surface never exceeded 45º with respect to the viewing plane.

The surface profile change predicted in the vector plot was found to show several similarities to the deformation of the cornea to a larger change in hydrostatic pressure shown in the screenshot from the video analysis. Equivalent areas of interest have been labelled in the vector plot in Figure 7-13 and the screenshot from the video analysis in Figure 7-14.

Between the positions labelled 1 and 2 in both the vector plot and the screenshot the magnitude of the total surface displacement appears to gradually increase. Between the positions labelled 2 and 3 the total magnitude of surface displacement in the screenshot from the video analysis appears to remain relatively constant which is in-line with the predicted data shown in the vector plot. Finally, after position 3 the magnitude of total surface displacement in the screenshot starts to gradually decrease, before decreasing sharply at the boundary, which is again similar to the predicted response shown in the vector plot.
Similar analysis was conducted with respect to the Y-axis (S-I axis) to determine the vertical in-plane component of displacement. Figure 7-15 (a) shows a full-field plot of the out-of-plane displacement component (w) calculated from the ESPI data recorded on cornea 1 over a 0.50 mmHg increase in hydrostatic pressure. A full-field plot of the vertical in-plane component (v), that was estimated based on the assumption of radial expansion, is shown in Figure 7-15 (b), and a line plot of the magnitude of the out-of-plane displacement component, the vertical in-plane displacement component and the estimated overall magnitude of displacement with respect to the surface normal (radial displacement) due to a combination of out-of-plane and in-plane deformation across a central horizontal cross-section is shown in Figure 7-15 (c).

Figure 7-15 – a) Surface plot of out-of-plane displacement component (w) calculated from the ESPI data recorded on cornea 1 over a hydrostatic pressure increase of 0.50 mmHg. b) Surface plot of the approximated vertical in-plane displacement component (v). c) Line plot of w, v and approximated out-of-plane displacement with respect to the surface normal down section B-B.

The corresponding vector plot illustrating the profile of surface deformation along the central S-I axis, produced by scaling up the measured out-of-plane and estimated vertical in-plane data plotted in Figure 7-15 is shown in Figure 7-16. In addition to this the screenshot from the
frame subtracted video of the central S-I axis of a cornea deforming to a larger increase in hydrostatic pressure is shown in Figure 7-17.

![Vector plot demonstrating the predicted profile of displacement down the central S-I axis in response to a hydrostatic pressure increase.](image1)

![Screenshot from frame-subtracted video analysis data showing the deformation of the surface down the central S-I axis due to an increase in hydrostatic pressure.](image2)

The measured out-of-plane data and the estimated in-plane data shown in Figure 7-15 suggested that along the S-I axis the greatest rate of change of displacement would be on the left side of the cornea, as plotted in Figure 7-16, in the 1 mm adjacent to the chamber. The magnitude of the surface displacement would then be expected to remain relatively even in magnitude up to approximately the centre of the cornea. In contrast, for the right side of the cornea the increase in surface displacement towards the centre would be expected to be more gradual.

Again, the predicted displacement profile shown in the vector plot showed similarities with the displacement profile shown in the screenshot of the cornea deforming to a larger increase in hydrostatic pressure. In the screenshot from the video data, shown in Figure 7-17, between positioned labelled 1 and 2 on the right side of the cornea, the magnitude of surface displacement gradually increases towards the centre, in a similar manner to the same region shown in the vector plot. The magnitude of surface displacement then appears to remain approximately even between positions 2 and 3 with displacement then decreasing sharply after position 3, returning to zero at the fixed boundary, which is in good agreement with the corresponding areas highlighted in the vector plot.
Although the assumption of deformation normal to the surface appeared to approximate the profile of deformation relatively well and provide a good first approximation for 3D deformation, to further develop this analysis and investigate in further detail the relationship between in-plane and out-of-plane deformation, the cross-sectional video data recorded for the cornea deforming under a larger pressure change was examined more closely to estimate the angle of deformation at different points across the surface of the cornea.

Cross-sectional videos were recorded while focussing on the S-I and N-T axis in turn, three videos were taken of each axis while undergoing three repeated loading cycles. The angle of deformation was estimated from these videos by tracking the movement of several notable points on the objects surface, distinguishable due to the non-smooth surface provided by the powder coating. Figure 7-18 shows an example of this analysis that was completed using software developed in LabVIEW. The red lines correspond to the angle normal to the surface and the green lines correspond to the angle of deformation based on tracking the movement of recognisable points.

![Figure 7-18 – Screenshot of cornea deforming to an increase in hydrostatic pressure, the red lines correspond to the angle normal to the surface, the green lines correspond to the actual angle of deformation at specific points.](image)

The measured angles recorded across the surface on each of the videos were averaged and a data file was produced containing a matrix that detailed the estimated corrected angle of deformation for each point on the cornea. This corrected angle was then used to estimate the in-plane component of deformation in the same way the normal angle had been used in previous examples.

Overall it was found, when examining the screenshots from the video analysis, that during loading, the surface deformation did not appear to occur normal to the surface but that the surface tended to deform in a way that favoured out-of-plane motion over in-plane, as shown by the green lines in Figure 7-18 which track the movement of a point on the surface before and after loading.
Figure 7-19, is a plot comparing the horizontal in-plane component of displacement calculated when assuming deformation occurs normal to the surface, and the horizontal in-plane component after considering the angle of deformation estimated from the video data. Figure 7-20 shows the effect of applying the angle correction to the outcome of the vector plot, and the predicted overall profile of deformation.

![Image](image_url_placeholder)

**Figure 7-19 – Plot comparing the horizontal in-plane component approximated when assuming deformation normal to the surface and when approximated based on the video analysis data, across the central N-T axis of a cornea in response to a pressure increase of 0.50 mmHg.**

![Image](image_url_placeholder)

**Figure 7-20 – Vector plot comparing the predicted profile of deformation along the central N-T axis when assuming deformation normal to the surface vs when using the angle of deformation from the video data.**

When using the modified angle, the overall magnitude of the in-plane deformation component was reduced significantly at the edge areas, relative to the in-plane component when assuming that deformation occurred normal to the surface. Overall, it appeared, from the data obtained via video analysis that the in-plane component maintained a relatively constant value either side of the central areas, where the deformation was purely out-of-plane. Overall, since the angle of the surface of the cornea at all positions with respect to the viewing direction was less than 45º and the deformation of the cornea tended to occur to a greater extent out-of-plane,
the out-of-plane component dominated the response, and therefore the plots obtained directly from measurement with the ESPI and LSI techniques, depicting the out-of-plane component alone provided a good indication of the overall profile of surface deformation.

Due to the fact the overall contribution of in-plane deformation appeared to be small across the human corneas, and the angle at which the surface deformed did not appear to change significantly over points separated by a shear distance of 50 pixels to 65 pixels (1.04 mm to 1.35 mm) that was used in experiments, it could be assumed that sheared points were deforming in a directionally similar manner. Also, since the deformation was mainly out-of-plane \( \frac{dw}{dx} \) or \( \frac{dw}{dy} \) calculated directly from the measured LSI data could be considered to roughly approximate the surface gradient change.

However, there was still distortion present due to the increased spacing between interfered points in these curved areas. If this distortion due to the shape of the cornea was taken into account the rate of change of the out-of-plane displacement component per unit length with respect to the object surface was reduced by up to 15.2% in areas where the curvature was most significant, compared to the measured value of \( \frac{dw}{dx} \) which represents the rate of change in out-of-plane displacement with respect to the X-axis in the image plane. The overall effect of this reduction on the profile of \( \frac{dw}{dx} \) is shown across a central horizontal cross section of a cornea in Figure 7-21.

![Figure 7-21](image-url)

*Figure 7-21 – Line plot showing the reduction in the rate-of-change in the out-of-plane displacement component if the increase in the shear distance between points with respect to the object's surface is considered.*

Overall, in addition to the other evidence provided by the video analysis, the data presented in Figure 7-21 demonstrated that the increased rate of change of the out-of-plane displacement
components towards the boundary regions of the cornea relative to other regions was not an artefact of distortion introduced due to the curvature of the cornea, but was in fact due to the increased compliance of this region compared to other regions in response to pressure variations.

This finding, of increased compliance in terms of out-of-plane displacement in the outer and limbal regions of the cornea is in agreement with previous data presented for bovine corneas by Boyce, et al (20), and also with recent data, gathered at the same time as the data presented in this thesis, by Whitford, et al (22) and by Elsheikh, et al (21) on a small number of human corneas where it was identified that the meridional stiffness of the cornea appeared to be reduced in the peripheral regions when compared to the more central regions. In addition to this, the finding that deformation at the limbus tended to occur predominantly out-of-plane compared to what was predicted when assuming deformation normal to the surface was also in agreement with the data from Boyce, et al (20) who suggested the circumferential alignment of limbal fibrils made them more compliant along the radial axis, and also with data from Whitford, et al (22) and by Elsheikh, et al (21) where it was suggested that the circumferential modulus of the limbal region was high, hence preventing significant in-plane motion in this region.

Overall, the identification of significant regional variations in corneal biomechanics and the demonstration of the effect of these regional variations on the surface deformation in response to pressure variations is of high importance, as it could have significant implications across a range of corneal surgeries with respect to identifying areas where it is best to make incisions in order to best preserve the biomechanics of the cornea. It could also have significant importance with respect to planning targeted treatments such as topographic crosslinking as the outcomes may differ from those predicted when assuming the biomechanics of the cornea are homogenous. The effect of the regional variations in biomechanics on the outcomes of crosslinking in different topographic regions are investigated in the following sub-section which details the results of the crosslinking experiments conducted on human corneas.
7.5 Crosslinking Results

Five of the six corneal samples were crosslinked in the same topographical locations that had been previously demonstrated throughout the testing on non-biological samples and on porcine corneas. Each cornea was used as its own reference and the specific topographic location to be crosslinked was chosen at random and not based on the initial response observed during the first round of testing. For one of the cornea pairs the crosslinking was performed in the same topographical location, to examine whether the response to crosslinking was similar for two different corneas.

The response before and after crosslinking was examined predominantly in terms of the out-of-plane displacement component because, as previously demonstrated within this chapter, the out-of-plane deformation contributes most significantly to the overall response, and this is the component that was measured directly. Estimations of the in-plane component have been made for some of the samples to produce 2D scaled-up vector plots to demonstrate how the profile of displacement may have changed before and after crosslinking.

7.5.1 Central 3 mm circle

Human cornea samples 1 and 2 were both crosslinked in an approximately central 3 mm diameter circular region. The region of crosslinking with respect to the corneal surface is shown diagrammatically in Figure 7-22 for clarity, where the yellow area corresponds to the region that was exposed to the UVA light source.

![Figure 7-22 – Image highlighting the position of crosslinking with respect to the corneal surface. Yellow area corresponds to region of crosslinking.](image_url)

Surface plots of out-of-plane displacement calculated from the ESPI data recorded on cornea 1 when responding to a 0.25 mmHg increase in hydrostatic pressure before and after crosslinking in a 3 mm diameter circular area are shown in Table 7-7. The corresponding line plots comparing the out-of-plane displacement along the central N-T axis (X-axis) and central
S-I axis (Y-axis) of cornea 1 before and after crosslinking are shown in Figure 7-23 (a) and (b) respectively. The approximate regions of crosslinking are highlighted on the plots. The crosslinking results for cornea 1 were examined over a pressure increase of 0.25 mmHg as opposed to 0.50 mmHg, as is shown for other corneas, as the data quality was better at the boundary regions and it of interest to identify if crosslinking could be detected over pressure variations of this magnitude.

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Table 7-7 – Surface plots showing the out-of-plane displacement of the surface of cornea 1 over a 0.25 mmHg increase in hydrostatic pressure before and after crosslinking in a 3 mm diameter circular region at the centre of the cornea.

![Figure 7-23](image5) – Line plots comparing the out-of-plane displacement of the surface of cornea 1 over a 0.25 mmHg pressure increase before and after crosslinking in a 3 mm circular region at the centre of the cornea. a) along central N-T axis, b) down central S-I axis.
Surface plots of the out-of-plane displacement calculated from the ESPI data recorded on cornea 2 when responding to a 0.50 mmHg increase in hydrostatic pressure before and after crosslinking in the same region as detailed for cornea 1 are shown in Table 7-8. The corresponding line plots comparing the out-of-plane displacement along both the central N-T axis and central S-I axis of cornea 2 before and after crosslinking are shown in Figure 7-24 (a) and (b) respectively.

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Table 7-8 – Surface plots showing the out-of-plane displacement of the surface of cornea 2 over a 0.50 mmHg increase in hydrostatic pressure before and after crosslinking in a 3 mm diameter circular region at the centre of the cornea.

![Line plot](image5) ![Line plot](image6)

Figure 7-24 – Line plots comparing the out-of-plane displacement of the surface of cornea 2 over a 0.50 mmHg pressure increase before and after crosslinking in a 3 mm diameter circular region at the centre of the cornea. a) along central N-T axis, b) down central S-I axis.
For both the corneas subjected to crosslinking in a 3 mm circular region at the centre there was a reduction in the magnitude of out-of-plane displacement in this region post-crosslinking compared to pre-crosslinking, in response to the same hydrostatic pressure increase. After crosslinking the reduction in the magnitude of out-of-plane displacement compared to the pre-crosslinked response for an equivalent pressure change, was the same for both corneas at 16.2% within the 3 mm region exposed to the UVA source.

Interestingly, the magnitude of out-of-plane displacement in the areas that were not crosslinked was sometimes greater than the measured out-of-plane displacement prior to crosslinking. This effect, was again evident on both corneas that were crosslinked in this region. The areas of increased displacement were generally towards the boundary areas, with the overall displacement profile (out-of-plane displacement greater at the boundaries than the centre) indicating an increase in the radius of curvature of the cornea (flattening of the surface) in response to an increase in hydrostatic pressure after crosslinking.

The dip in the magnitude of the out-of-plane displacement component compared to adjacent regions, that occurred as a result of crosslinking could also be identified from the LSI data. Figure 7-25 (a) shows $\frac{\partial w}{\partial x}$ calculated from the LSI data (shear applied with respect to the X-axis), along the central N-T axis (X-axis) of cornea 1 in response to a 0.25 mmHg pressure increase before and after crosslinking. Figure 7-25 (b) shows $\frac{\partial w}{\partial y}$ calculated from the LSI data (shear applied with respect to the Y-axis), along the central S-I for cornea 1 in response to a pressure change of 0.25 mmHg before and after crosslinking.

![Figure 7-25](image-url)

*Figure 7-25 – a) Line plot comparing $\frac{\partial w}{\partial x}$, measured via LSI, along the central N-T axis of cornea 1 before and after crosslinking in a central 3 mm diameter circular area. b) Line plot comparing $\frac{\partial w}{\partial y}$, measured via LSI, down the central S-I axis before and after crosslinking in a central 3 mm diameter circular area.*
Changes to the LSI data were relatively subtle due to the fact the comparison was made for a very small pressure change of 0.25 mmHg, but the reduction in the magnitude of the out-of-plane displacement component in the central crosslinked region relative to other regions could be identified from the fact that the measured values of $\frac{dw}{dx}$ and $\frac{dw}{dy}$ went from slightly negative to slightly positive in the region of crosslinking and this was not present in the original response. The changes were more evident when shear was applied with respect to points in the y-axis.

The overall changes to the profile of the out-of-plane displacement component were similar to those that had been seen when crosslinking in the same location on porcine corneas (chapter 6, section 6.4.3). A possible explanation for why the response changed in the manner detailed, is that crosslinking in the central region led to an increase in the stiffness of the cornea in this region and greater resistance to deformation, and this could have in turn resulted in greater deformation in the surrounding areas, especially in peripheral/limbal regions which have been identified as being more compliant, to accommodate the pressure increase. Overall this effect would lead to an overall flattening of the corneal profile when under pressure.

7.5.2 Outer ring

Human cornea sample 3 was crosslinked over a total diameter of 9 mm around a masked 3 mm diameter circular area at the centre of the cornea. The specific region of crosslinking with respect to the corneal surface is shown diagrammatically in Figure 7-26, where the yellow area corresponds to the region that was exposed to the UVA source.

![Figure 7-26](image)

*Figure 7-26 – Image highlighting the position of crosslinking with respect to the corneal surface. Yellow area corresponds to region of crosslinking.*

Surface plots of out-of-plane displacement calculated from the ESPI data recorded on cornea 3 when responding to a 0.50 mmHg increase in hydrostatic pressure before and after undergoing crosslinking in a 9 mm diameter area around a central 3 mm diameter masked area are shown in Table 7-9. The corresponding line plots comparing the magnitude of out-of-plane displacement along both the central N-T axis (X-axis) and central S-I axis (Y-axis) of cornea 3 before and after crosslinking are shown in Figure 7-27 (a) and (b) respectively.
After crosslinking in the location shown in Figure 7-26, the magnitude of the out-of-plane displacement component was decreased substantially across the whole surface of the cornea compared to the out-of-plane displacement that had been measured over the same hydrostatic pressure increase pre-crosslinking. Across the N-T axis, shown in Figure 7-27(a) the magnitude of the response was reduced most significantly towards the boundary areas. Down
the S-I axis, shown in Figure 7-27 (b), the response was reduced more substantially at one side than the other corresponding to the top of the cornea as viewed in Table 7-9. The changes to the magnitude of the response were also evident from the LSI data as significantly fewer fringes formed during loading for the same pressure change. The measured components of $\frac{\partial w}{\partial x}$ and $\frac{\partial w}{\partial y}$ were found to be significantly reduced at the boundary areas when examining the data obtained via LSI over a hydrostatic pressure change of 0.25 mmHg where no decorrelation was present. This is highlighted in Figure 7-28, where $\frac{\partial w}{\partial x}$ is compared for a hydrostatic pressure increase of 0.25 mmHg along the central N-T axis of cornea 3 before and after crosslinking.

Specific differences in the response of the 3 mm central area that had not been exposed to the UVA source were not obvious from viewing either the ESPI and LSI data, suggesting that the absence of crosslinking in this area did not seem to cause it to deform by a relatively larger amount compared to the surrounding crosslinked areas, as had been seen in the previous experiments on non-biological samples.

On close examination of the data taken along the central cross-section of the N-T axis it was evident that there was a slightly lesser reduction in the magnitude of out-of-plane displacement after crosslinking, across the central 6 mm of this axis (from 13 mm to 19 mm with reference to the X-axis in the plot shown in Figure 7-27(a)). This was further highlighted by calculating the percentage reduction in out-of-plane displacement along the central horizontal cross section as shown in the plot in Figure 7-29. An approximately 50% reduction was seen in displacement in the central area, compared to a 55% - 80% reduction in areas surrounding this.
Interestingly, a similar resistance to substantial increases in out-of-plane displacement in the central area relative to surrounding areas was also seen when examining porcine corneas crosslinked over a similar region, as discussed in chapter 6, section 6.4.3, where the region that was not crosslinked only became evident after very high energy crosslinking of the surrounding regions. The observed resistance to significant curvature change in the central area in response to a pressure change further supports previous observations that have indicated that this area shows higher resistance to deformation and curvature change relative to the peripheral and limbal regions of the cornea.

7.5.3 Horizontal strip

Human cornea sample 6 was crosslinked along a 9 mm long, 3 mm wide strip positioned along the centre of the N-T axis. The specific region of crosslinking with respect to the corneal surface is highlighted in Figure 7-30.
As discussed previously, cornea 6 did not behave as expected during the control testing due to the damage that was present on the posterior side of the sample where the surgical thread had been inserted in close proximity to the edge of the cornea. However, as only a small number of human corneas had been tested prior to testing cornea 6, there was only limited knowledge of the expected response, so the damage present in cornea 6 was not diagnosed from the fringe distribution at the time of testing and therefore it was still used for crosslinking.

Surface plots of the out-of-plane displacement component calculated from the ESPI data recorded on cornea 6 when responding to a 0.50 mmHg increase in hydrostatic pressure before and after crosslinking along a 9 mm long, 3 mm wide strip along the centre of the N-T axis are shown in Table 7-10.

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Table 7-10 – Surface plots showing the out-of-plane displacement of the surface of cornea 6 over a 0.50 mmHg increase in hydrostatic pressure before and after crosslinking in a 9 mm long, 3 mm wide strip along the centre of the N-T axis.

Despite the area of damage, the crosslinking of a 3 mm strip along the N-T axis resulted in a change in the response of the cornea, before and after crosslinking when measured over the same increase in hydrostatic pressure. However, since this cornea was damaged it is unlikely that the changes that were observed would be representative of the changes that would occur in a normal cornea crosslinked in the same topographical location.
Interestingly, the crosslinked area did cover the area of damage and crosslinking in this area did appear to reduce the effects of the damage present as the magnitude of the out-of-plane displacement component was reduced by over 50% in this area after crosslinking. This is demonstrated in the line plot in Figure 7-31, which shows the out-of-plane displacement before and after crosslinking along the central horizontal cross-section of cornea 6.

![Line plot comparing the out-of-plane displacement measured along the central N-T axis of cornea 6 over a 0.50 mmHg increase in hydrostatic pressure before and after crosslinking along this area.](image)

Overall, after crosslinking the magnitude of the out-of-plane displacement became more even across the treated axis, and despite the initial area of damage, this effect was similar to the results previously been demonstrated in porcine corneas when crosslinking in the same region. However, since this cornea was damaged the results could not be considered to be representative of a normal cornea so were not investigated any further.

### 7.5.4 Vertical strip

Human cornea sample 5 was crosslinked in a 9 mm long, 3 mm wide region down the centre of the S-I axis. This specific region of crosslinking with respect to the corneal surface is shown in Figure 7-32.

As discussed previously in this chapter, cornea 5 showed a slightly different distribution of out-of-plane displacement when compared to the majority of samples tested. However, due to the small sample size examined it was not known whether this distribution was normal or abnormal. Since there were no visible signs of any differences or damage to the cornea, and
the response of the cornea could be compared with itself before and after treatment, it was still included in the crosslinking experiments.

Figure 7-32 – Image highlighting the position of crosslinking with respect to the corneal surface. Yellow area corresponds to region of crosslinking.

Surface plots of the out-of-plane displacement component calculated from the ESPI data recorded on cornea 5 when responding to a pressure increase of 0.50 mmHg before and after crosslinking in a 9 mm long, 3 mm wide region down the centre of the S-I axis are shown in Table 7-11.

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Table 7-11 – Surface plots showing the out-of-plane displacement of the surface of cornea 5 over a 0.50 mmHg increase in hydrostatic pressure before and after crosslinking in a 9 mm long, 3 mm wide strip down the centre of the S-I axis.
The corresponding line plots comparing the out-of-plane displacement along both the central N-T (X-axis) and central S-I axis (Y-axis) of cornea 5 before and after crosslinking in the area shown in Figure 7-32 are shown in Figure 7-33 (a) and (b) respectively.

Figure 7-33 – Line plots comparing the out-of-plane displacement of the surface of cornea 5 over a 0.50 mmHg pressure increase before and after crosslinking in a 9 mm long, 3 mm wide strip down the centre of the S-I axis. a) along the central N-T axis, b) down the central S-I axis.

A comparison of the results obtained before and after crosslinking, indicated that crosslinking along a 9 mm long, 3 mm wide strip down the centre of the S-I axis increased the resistance of the cornea in this area to out-of-plane displacement in response to a pressure change. The way the cornea deformed in response to the pressure change changed significantly after crosslinking and this was evident in the surface plots and further highlighted by the line plots taken along the centre of the N-T and S-I axes as shown in Figure 7-33.

Along the N-T axis, shown in Figure 7-33 (a), the magnitude of out-of-plane displacement was decreased by approximately 60% in the central area in the region where the crosslinked area crossed this axis. Interestingly, reductions were also seen at the edge areas especially at the left side of the sample. These changes were also evident from the LSI data. A line plot of $\frac{\partial w}{\partial x}$ calculated from the LSI data over a pressure change of 0.25 mmHg from the central horizontal cross-section is shown in Figure 7-34.

From the LSI data, the decrease in the magnitude of the out-of-plane displacement component in the crosslinked area relative to surrounding regions was evident due to the change in the sign of the $\frac{\partial w}{\partial x}$ in this region. This Indicated a flattening of this region in response to increasing hydrostatic pressure, as opposed to a slight steepening that was evident prior to crosslinking.
After crosslinking, the magnitude of the out-of-plane response to an equivalent pressure increase was significantly reduced, by over 70% in some areas along the S-I axis, as shown in Figure 7-33 (b). Also, the magnitude of the out-of-plane displacement component did not vary by much across this axis inside of the 1 mm boundary regions adjacent to the edge of the chamber, whereas prior to crosslinking the magnitude of out-of-plane displacement continued to gradually increased towards the centre. These changes were also evident from the LSI data. A plot of $\frac{\partial w}{\partial y}$ calculated from the LSI data is shown in Figure 7-35 for the central vertical cross-section.
The data shown in Figure 7-35 highlights that after crosslinking the magnitude of the out-of-plane displacement component along the region of crosslinking was close to zero inside of the boundary regions, indicating this area was resistant to curvature change under increasing hydrostatic pressure.

Once again, the changes introduced by crosslinking in this specific topographic location on the human cornea were similar to the changes that had been seen when crosslinking in the same topographic location on the porcine cornea.

To assist with visualisation of how the measured data related to deformation of the corneal surface and how this response was affected after the introduction of crosslinking in this specific location, the in-plane component was estimated by assuming that displacement occurred normal to the surface and the data was scaled up to produce vector plots to show how the profile of the cornea would be expected to change in response to a pressure increase. An example is shown in Figure 7-36 for a central cross-section taken along the N-T axis before and after crosslinking. As with previous vector plots the length of the arrows and the size of the arrowheads relative to each other is proportional to relative displacement.

Based on the data presented in the vector plots, after crosslinking the profile of the cornea would be expected to become flatter in response to increases in hydrostatic pressure, due to the increased displacement at the sides relative to the centre.

**Figure 7-36 – Vector plots demonstrating how the profile of the cornea would be expected to change along the central N-T axis in response to an increase in hydrostatic pressure, before (top) and after (bottom) crosslinking in a 9 mm long, 3 mm wide strip down the S-I axis.**
### 7.5.5 Integration of LSI data to show changes introduced by crosslinking

Since the LSI technique has greater stability and therefore has greater potential than ESPI to be used outside a laboratory environment as a commercial instrument, it was of interest to see if the LSI data from human corneas could be integrated to provide a visual representation of the out-of-plane displacement component, as it was found the data was easier to interpret in this format.

As described in previous chapters, due to the limitations of the technique and the additional challenges introduced due to the way the cornea deformed, along with some of the issues associated with integration, it was not possible to reliably generate numerically accurate data from the LSI measurement technique across all the samples tested. Since numerical accuracy of the LSI data could not be guaranteed, the aim of the integration at this stage was to provide a plot that resembled the ESPI data and that could demonstrate changes to the distribution of the out-of-plane displacement component before and after crosslinking.

Four examples of out-of-plane displacement maps generated directly from ESPI data are compared with the equivalent out-of-plane displacement maps generated from the integrated LSI data in Table 7-12 and Table 7-13, for data obtained on corneas 1 and 5 respectively.

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<tr>
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<th>Pre-crosslinking</th>
<th>Post-crosslinking</th>
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<tr>
<td><strong>ESPI</strong></td>
<td><img src="image" alt="ESPI Pre-crosslinking" /></td>
<td><img src="image" alt="ESPI Post-crosslinking" /></td>
</tr>
<tr>
<td><strong>Integrated LSI</strong></td>
<td><img src="image" alt="Integrated LSI Pre-crosslinking" /></td>
<td><img src="image" alt="Integrated LSI Post-crosslinking" /></td>
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Table 7-12 – Comparison of the visual appearance of full-field out-of-plane displacement maps obtained from the ESPI data or via integration of the LSI data for cornea 1 in response to a pressure increase of 0.25 mmHg before and after crosslinking.
## Table 7-13 – Comparison of the visual appearance of full-field out-of-plane displacement maps obtained from the ESPI data and via integration of the LSI data for cornea 5 before and after crosslinking in response to a pressure increase of 0.25 mmHg.

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<tr>
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<th>Pre-crosslinking</th>
<th>Post-crosslinking</th>
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<tbody>
<tr>
<td><strong>ESPI</strong></td>
<td><img src="Image1" alt="Pre-Crosslinking" /></td>
<td><img src="Image2" alt="Post-Crosslinking" /></td>
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<tr>
<td><strong>Integrated LSI</strong></td>
<td><img src="Image3" alt="Pre-Crosslinking" /></td>
<td><img src="Image4" alt="Post-Crosslinking" /></td>
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As is evident from the appearance of the plots in Table 7-12 and Table 7-13 the success of the integration was variable across samples, with the integrated LSI plots showing similar features to the ESPI plots but sometimes appearing slightly distorted. This distortion was generally introduced because of the presence of small areas of erroneous data at the edge of the sample adjacent to the chamber, or due to errors introduced due to small inaccuracies in the masking of the measurement area.

The integration appeared to more successfully identify the changes introduced due to crosslinking at the boundary regions, and some of the detail across the central regions was lost. This is likely due to the comparative lack of fringes across the central area meaning the sensitivity in this areas was not adequate enough to pick up very small details at lower pressure changes.

Although there were challenges associated with the integration of the LSI data, the integration of the LSI data was successful in demonstrating visible changes to the response of the cornea that occurred before and after crosslinking, and the changes shown were in good agreement with those shown by the ESPI data. Overall, further development of the LSI system and integration procedures to obtain more reliable data with higher accuracy is an area which requires further work.
7.6 Summary and conclusions from human cornea experiments

This chapter described the results from experiments conducted on six human corneal samples. The main outcomes from these experiments are summarised here.

Experiments were conducted where the response of the corneas was measured over pressure increases of 0.25 mmHg, 0.50 mmHg, 0.75 mmHg and 1.00 mmHg while clamping in the chamber with both the large aperture tissue clamp (15.7 mm) and the small aperture tissue clamp (12.5 mm). Unfortunately, the tissue was not compatible with the larger aperture tissue clamp due to the fact there was a hole in the sclera where the surgical thread had been inserted to keep them suspended in the organ culture and this was not covered by the clamp. Although these experiments could not give information regarding the normal biomechanics of the cornea they clearly demonstrated the benefits of the ESPI and LSI techniques for identifying small areas of damage.

Since the 12.5 mm aperture tissue clamp clamped inside of the hole on most of the corneas it did not affect the response to loading. During these experiments, it was identified that four out of 6 of the corneas showed similar features in their response to pressure variations these were:

1. The rate of change of out-of-plane displacement was greatest in the 1 mm region adjacent to the clamp, suggesting that this region was more compliant than other regions.
2. The magnitude of out-of-plane displacement remained relatively even across the central areas especially with respect to the N-T axis suggesting that there was little change in the curvature of the cornea across these regions in response to pressure variations.
3. The response was non-symmetrical with peak out-of-plane displacement occurring off-centre, with one side showing more compliance than the other.
4. The average magnitude of the out-of-plane displacement of the central cornea was 1.64 µm/mmHg.

Interestingly, the observations regarding the distribution of the response from the human cornea experiments showed similarities with previous observations from the porcine cornea experiments where the sample size was significantly larger. These experiments demonstrated that there is significant non-symmetric regional variation in the mechanical properties of the cornea and the specific mechanics of the limbal region are fundamental to the overall response of the human cornea to small pressure fluctuations, backing up the findings of a previous study on bovine corneas\(^{(20)}\).

In previous interferometry studies on the cornea\(^{(41,45,51,70,73,145)}\), only the out-of-plane response has been considered and the effects of corneal curvature on the data has not been taken into
account. During the experiments detailed in this chapter 3D analysis of the response was conducted, first by assuming that deformation occurred normal to the surface and then by examining the angle of deformation in response to a larger pressure change, from this analysis it was concluded:

1. The results obtained when assuming deformation occurred normal to the surface showed a similar profile of displacement to that observed in the videos of the cornea responding to larger increases in hydrostatic pressure and so were concluded to provide a good initial approximation for 3D surface displacement.
2. As the angle of the corneal surface did not exceed 45º with respect to the image plane when assuming deformation normal to the surface the out-of-plane component of the response was always more significant than the in-plane component.
3. From analysing the video data it was found that the deformation of the cornea in response to hydrostatic pressure increases tended to occur to a greater extent out-of-plane than was predicted when assuming deformation normal to the surface. Suggesting higher circumferential stiffness relative to meridional stiffness. This meant the out-of-plane displacement data alone provided a relatively good approximation for overall surface displacement as the in-plane component was comparatively small.
4. For the LSI data, if the variation in shear distance between interfered points introduced due to the curvature of the corneal surface was considered the rate of change of out-of-plane displacement was reduced by up to 15.2% in some regions when compared with the rate of change of out-of-plane displacement when assuming constant shear.

Experiments were conducted where the changes to the response of individual corneas to hydrostatic pressure variations were investigated before and after crosslinking in specific topographic regions in isolation. This is the first time the effects of crosslinking in specific regions in isolation has been investigated on human corneas. From these experiments it was found that:

1. ESPI and LSI had adequate sensitivity to detect changes to the response that occurred as a result of crosslinking.
2. Crosslinking results in changes to the response of a cornea to hydrostatic pressure variations and these changes are not isolated to the region of crosslinking in isolation.
3. Crosslinking with a total energy of 7.2 J/cm² (UVA intensity of 15 mW/cm², 8 minutes exposure time) in the central 3 mm diameter region in isolation, resulted in 16.2% decrease in the magnitude of out-of-plane displacement in the region of crosslinking for the two corneas tested, however in the regions outside of the crosslinked area the out-of-plane displacement increased.
4. Crosslinking around the outer regions while masking the central regions significantly reduced the magnitude of out-of-plane displacement in response to pressure variations.
by up to 80% in the outer regions. However, absence of crosslinking in the central regions did not appear to result in large increases in displacement in this region relative to adjacent crosslinked regions.

5. Crosslinking down the centre of the S-I axis resulted in a significant reduction in out-of-plane displacement, especially along the axis of crosslinking, where it reduced the rate of change of out-of-plane displacement to close to zero.

Overall, the crosslinking results showed some similarities with the simulation results obtained from non-biological samples, however it was evident that the complex structure of the cornea contributed to a number of effects that were not expected based on the simulation data obtained from a simple homogenous sample. In general, the results of crosslinking on human corneas in different locations showed similar effects to those that had been seen on porcine corneas.

On several occasions throughout testing, the ESPI data was compared with the LSI data. With respect to the accuracy of the LSI data on human corneas it was found that:

1. For small pressure variations of 0.25 mmHg the LSI data was in good agreement with the differentiated ESPI data even at the boundary regions with a difference of approximately 7% in the maximum \( \frac{\partial w}{\partial x} \) obtained using each of the techniques (Figure 7-7), however the fringe number for the LSI data for this pressure change was reduced to approximately 2 to 3.

2. Due to the way the cornea deformed, with a high rate of change of out-of-plane displacement in the 1 mm region next to the clamp and then a relatively low rate of change of out-of-plane displacement across the surface, the accuracy of the LSI data in the 1 mm adjacent to the chamber was reduced relative to other regions.

3. During LSI measurement the quality of the fringes deteriorated significantly at pressure variations over 0.50 mmHg in magnitude. Poor quality fringes prevented accurate phase unwrapping and hence the data obtained via LSI at pressure variations above 0.50 mmHg could not be quantitatively analysed.

4. Accurate integration of the LSI data to obtain the displacement component was prone to errors both due to the limitations of the LSI technique and areas of poor fringe quality, for this reason it was not possible to use the LSI technique with confidence to quantify displacement. However, for pressure variations up to 0.25 mmHg the visual appearance of the LSI data showed good agreement with the ESPI data in most cases.

The results detailed in this chapter and the specific implications of these findings are discussed in greater detail and compared with the results from other studies in the following discussion chapter. In addition to this, an overview of the experimental methods used and the limitations of these methods are discussed in further detail.
8. Final Discussion

The work in this thesis has presented a method for the *ex vivo* analysis of corneal surface displacement in response to hydrostatic pressure variations using speckle interferometric methods, with the overall aim of increasing understanding of corneal biomechanics. Within this work, a measurement system has been designed, potential measurement errors and limitations have been explored, and results have been presented on a range of samples including non-biological materials, porcine corneas and human corneas. The purpose of this chapter is to provide an overall discussion of the work undertaken within this research project and to build on the discussions given throughout the individual design and results chapters.

8.1 Evaluation of measurement methods

The motivation for the work undertaken in this thesis was to increase the current understanding of corneal biomechanics and how these mechanical properties relate to *in vivo* behaviour. This was because an increased knowledge of biomechanics has been identified as having wide ranging implications across many areas of ophthalmology, and being fundamental to understanding the effects of current refractive surgery procedures, and in the development of new safer alternatives.

The main aim of this research was to investigate the use of speckle interferometric techniques, namely LSI, for the measurement of corneal biomechanics. Speckle interferometric techniques were identified as potentially suitable measurement tools for this task due to their high sensitivity, full-field 3D surface measurement capabilities and non-destructive nature, which granted the ability to investigate the response of the cornea across the whole surface in response to physiological scale pressure variations. LSI was of particular interest due to its enhanced stability in comparison with ESPI allowing it to be used outside of a laboratory environment, which may enable it to be developed, in the future, as an *in vivo* measurement tool.

At the commencement of this project several specific challenges were identified with respect to corneal measurement using speckle interferometric techniques, these included; non-flat geometry, transparency, smoothness and biological factors such as hydration and stability. The early part of this work focussed on developing solutions to these challenges or determining their overall influence on the results, an overview of this work and the associated limitations is given in the following sections.
8.1.1 Loading rig and experimental methodology

Prior to the experiments, the initial work in this thesis was focussed on the development of a loading rig that could hold the cornea in a stable position for measurement, simulate in vivo conditions and provide highly repeatable loading.

The eventual loading rig, as described in detail in chapter 4, consisted of a metal artificial anterior chamber into which both porcine or human corneo-scleral sections could be clamped and loaded hydrostatically using a reservoir of PBS solution attached to the AAC at a specific height above the surface of the cornea. Small pressure variations were applied via vertical movement of the reservoir with respect to the surface of the cornea, this process was automated to ensure high repeatability.

Significant effort was put into the development of the AAC. This was due to the fact early experiments with a plastic Barron AAC, as shown in Figure 4-1 of chapter 4, had been unsuccessful as it had not been possible to achieve adequate stability of the tissue, and the chamber had not been capable of accommodating porcine corneas due to their increased size and thickness. The eventual metal chamber and rigid connectors granted high stability during measurement, and the threaded locking mechanism of the tissue retainer enabled tissues of different thickness to be clamped securely. The addition of a second tissue retainer with a larger aperture ensured that both human tissue and porcine tissue could be clamped on the sclera so the movement of the cornea was unrestricted.

With respect to the repeatability of the loading rig, it was demonstrated during experiments detailed in section 4.2.3 of chapter 4 that the repeatability of the vertical movement stage was +/- 6.9 µm. Hence the repeatability of the pressure variation achieved over repeated loading cycles was +/- 0.0005 mmHg which was equivalent to a variability of 0.1% with respect to the average pressure variation of 0.50 mmHg used across cornea experiments. Later during experiments on non-biological samples detailed in section 5.2 of chapter 5, it was confirmed that the measurement rig provided adequate stability for measurement using both ESPI and LSI, as measurement on a rubber sample was found to be repeatable to within 2.0% of the maximum measured phase change across repeated loading cycles, as shown in Figure 5-6 of chapter 5.

Overall the high stability of the AAC and the repeatability of the loading rig was fundamental to the success of the experiments detailed in this thesis, as it enabled the response of corneas to be compared before and after crosslinking and with other corneas with confidence that the
loading and clamping conditions remained the same across all experiments and therefore did not contribute to any changes to the response.

A second area of focus prior to experiments on corneal tissue was the development of method to generate an adequate return signal from the corneal surface. This was a key area of interest, because in previous studies (41,45,51,73) where interferometric techniques had been used to investigate the biomechanics of the cornea, data had not been presented to validate that the surface coating used had no effect on the measured response of the cornea to pressure variations and hence there was doubt over the validity of the data presented in these studies.

Experiments into a range of potential surface coatings were conducted during this research, and from these experiments an inert coating was identified (Sphericel 110P8 fused borosilicate hollow glass microspheres). This coating adhered well to the surface of the cornea, formed a stable layer on the moist surface, had negligible impact on the deformation of a sample in response to hydrostatic loading and could be easily removed and reapplied without resulting in changes to the response. This was demonstrated in Figure 6-2 in chapter 6, where the response of a rubber sample measured via LSI was compared before coating and then after coating in the selected powder over 10 cycles where the coating was removed and reapplied between each cycle. From this experiment, it was shown that the difference in the maximum measured phase between coated and uncoated samples was +/- 2.1% which was within the standard deviation of +/- 2.6% previously measured for an uncoated sample subjected to repeated loading cycles. Removal and reapplication of the coating was found to have no effect on the magnitude of standard deviation.

Overall, the application of the coating to the surface of the cornea ensured a good return signal, and provided a stable surface from which measurement could be achieved. The main drawback to the coating method chosen was that it would not be transferable to an in vivo measurement situation. Therefore, research into a suitable in vivo coating would be an area for future research if in vivo measurement was desired.

A further outcome of the experiments into surface coatings was that it was demonstrated in Figure 6-3 that applying a thin strip of PTFE tape over the cornea significantly changed its response to hydrostatic pressure variations. This was an important finding as previous studies have used this method (45,51) and stated that the presence of a stretched layer of PTFE tape had a negligible effect on the response of the cornea to hydrostatic pressure variations when compared to a surface coating of aerosol spray (45). Since this was not the finding in this study, it draws into question the validity of the data presented in these previous studies.
8.1.2 Limitations of measurement rig and experimental methodology

Overall, there were several limitations to the measurement rig and experimental methodology employed with respect to replicating *in vivo* conditions these were:

1. The fixed boundary conditions imposed by the AAC and absence of the full eye globe.
2. The speed of loading.
3. Tissue quality and changes to the properties of the corneal tissue over the total measurement time.

**Boundary Constraints**

The decision to clamp the corneas in an AAC rather than testing the whole globes was because while using the chamber, it was easier to achieve high stability, it provided well defined and repeatable boundary constraints, and it simplified analysis as variations in scleral properties did not have to be considered. Another important factor on which this decision was based was that human tissue was commonly provided in the form of corneo-scleral sections, as they would be for transplant, hence a method was required that could accommodate tissue in this format. There were two main concerns when using this method when considering the relevance of the results to predicting *in vivo* behaviour, these were:

1. The imposition of the fixed boundary adjacent to the cornea would change the nature of the response in the regions adjacent to the boundary constraints.
2. The rigid chamber would not deform at all in response to pressure variations as the sclera would be expected to *in vivo*.

With reference to the first point, during experiments on porcine corneas, it became evident that the region at which the cornea joined with the sclera appeared to respond differently to pressure fluctuations relative to other regions of the cornea, and since this area was adjacent to the fixed boundary there were concerns that the imposition of the fixed boundary was contributing to the observed effects. However, it was confirmed via experiments on non-biological samples with similar or greater curvature to the cornea in sections 5.2 and 5.3 of chapter 5, that the boundary constraints alone were not responsible for the observed effects. This could be concluded as similar responses were not observed across all samples examined, and there was no evidence that the boundary constraints resulted in irregularities in the response of uniform rubber samples, as shown in Table 5-3 and Table 5-4 of chapter 5.

However, it is possible that the fixed boundaries imposed on the sclera within 1 mm – 2 mm of the cornea resulted in changes to the response of the sample within this region over what would have been seen if the cornea was tested as part of a whole globe or if the boundaries had been located further from this area. An attempt was made to investigate this during human
cornea testing by using the larger aperture tissue retainer that had been designed for porcine corneas to clamp the human corneas. Unfortunately, due to holes that had been made in the sclera’s of the human tissue sections, damage to the sample could not be ruled out and leakage was an issue, therefore the results from these investigations were not conclusive.

An initial evaluation regarding the possible impact of the boundary conditions on the response of the corneas in these regions has been made here by examining and comparing the data obtained on porcine corneas and human corneas during this study, and also by comparing the measured data with data obtained in previous studies that had used alternative clamping mechanisms or whole globe testing, this is detailed in the following discussion.

During the porcine cornea testing, due to the elliptical shape of the cornea and the round shape of the tissue retainer, the position of clamping with respect to the edge of the cornea was different for the N-T and S-I meridians. This is illustrated in the image in Figure 8-1 which shows a typical porcine cornea situated in the AAC. With respect to the N-T meridian the clamping was approximately 1 mm from the edge of the cornea, which was approximately equivalent to the clamping position with respect to the edge of the human cornea when using the small (12.5 mm) aperture lid. With respect to the S-I axis the clamping position was slightly further from the edge of the cornea so there was approximately 2 mm – 3 mm of sclera between the cornea and the fixed boundary.

During the porcine cornea experiments, the behaviour of the cornea with respect to each of these axes was found to vary significantly, as described in detail in section 6.3.2 of chapter 6. Deformation of the cornea in response to pressure increases was found to be large relative to other regions at the top and bottom of the S-I axis, corresponding to the position where the cornea joined the sclera, and from the video analysis, shown in Figure 6-12, there appeared
to be a significant in-plane component to the response at the corneal periphery. Whereas across the N-T axis on the magnitude of deformation across the majority of the surface remained relatively even inside of the boundary regions. Hence, there were concerns that the different behaviour observed across these axes was an artefact of clamping and the close proximity of the clamp to the edge of the cornea with respect to the N-T axis prevented the boundary areas from deforming naturally.

Interestingly, in a holography study by Smolek (70) where the response of human corneas in the form of whole globes was examined in response to IOP fluctuations of a similar magnitude using double exposure holography, the observed fringe distributions were similar to those that were observed during porcine cornea testing, only rotated through 90°. This is illustrated in Figure 8-2 which shows the results from Smolek’s study alongside a typical ESPI fringe distribution obtained during the experiments on porcine corneas conducted during this thesis.

![Figure 8-2](image)

*Figure 8-2 – Comparison of a double exposure hologram obtained in a study by Smolek showing the fringes that formed due to an increase in IOP of 3.50 mmHg (left) [Reproduced from (70)], with the ESPI fringes obtained on a typical porcine cornea due to a hydrostatic pressure increase of 0.50 mmHg (right)*

Although, the clamping mechanism was different in Smolek’s experiments, that used whole globes, similarities were seen in the distribution of the fringes, albeit with respect to orthogonal axes. This suggested that the observed effects on porcine corneas, which indicated a flattening of the corneal curvature with respect to the S-I axis in response to increasing hydrostatic pressure, were not likely to be entirely an artefact of irregularities in the clamping location.

Differences were seen in the response of the human corneas that were examined in this thesis when compared with the porcine corneas and also with the results of Smolek’s previous study on human tissue. Although the human corneas examined still exhibited lower resistance to deformation at the boundary regions and an overall flattening of corneal curvature in response to a pressure increase, the overall contribution of any in-plane deformation at the boundary regions, when viewed in the cross-sectional video data as shown in Figure 7-14 and Figure 7-17, appeared to be small, with the samples showing a tendency to deform to a greater extent
out-of-plane than would have been predicted when assuming deformation normal to the surface.

The way in which the surface deformed could have either been due to the properties of the human cornea, or it could have been that the location of the fixed boundary imposed on the human corneas contributed to an overall reduction in in-plane deformation. The locations of the fixed boundary regions with respect to the cornea have been shown diagrammatically in Figure 8-3, for the case of the human cornea clamped in the chamber by the original 12.5 mm tissue retainer, and for the case of the porcine cornea, at either end of the S-I axis, when clamped in the chamber with the larger 15.7 mm tissue retainer.

As can be seen from the diagrams, the location of the boundary constraints with respect to the edge of the porcine cornea imposes less restrictions to in-plane motion at the edge of the cornea in response to hydrostatic pressure, whereas for the human case, the close proximity of the boundaries to the cornea may have influenced the overall direction of deformation slightly in these regions.

Overall, the influence of the boundary constraints on the measured response at the peripheral and limbal regions of the cornea remains to be determined, and to achieve this, experiments would be required, ideally on whole globes, or on corneo-scleral sections where the location of the fixed boundary is positioned further from the edge of the cornea. However, based on the results from the experiments on non-biological samples it can be concluded that the clamping mechanism used was not responsible for the increased deformation at the region where the cornea joined the sclera and that this region had lower resistance to deformation relative to other areas.

The second concern when using the AAC, was the absence of the whole eye globe and its replacement with a rigid chamber. In vivo, because the sclera is not rigid it would be expected to undergo some deformation in response to hydrostatic pressure variations, hence the deformation of the corneal surface alone would not accommodate the full pressure variation.
as it does when situated in the AAC. Studies conducted on whole globes of human and porcine origin \cite{22,148} have confirmed that the sclera does deform under increases in hydrostatic pressure, with several studies demonstrating that the posterior sclera is the most compliant region of the sclera \cite{22,175}, especially around the location of the optic nerve head \cite{176,177}.

It is difficult to determine what the overall effect of scleral deformation on the response of the cornea would be, as very few studies have been conducted where the response of the whole eye globe to pressure variations has been measured due to the difficulties associated with obtaining human tissue in this form. However, to make an estimate for how the absence of the sclera may have affected the magnitude of the response of the corneas to pressure variations, the results of the human cornea experiments in this thesis were compared with the results presented in the holography study by Smolek \cite{70} where whole human eye globes were used. Comparison was made by comparing the fringe numbers obtained over a given pressure increase, only a rough approximation could be made due to the variable distribution of fringes.

From this comparison, it was roughly approximated that the fringe number obtained over a pressure change of 0.50 mmHg for the corneas in the AAC was roughly similar to the fringe number obtained on corneas that remained as part of a whole globe in Smolek’s experiments \cite{70} over a pressure change of 2.50 mmHg. This suggested that to replicate the results obtained for a pressure increase of 0.50 mmHg in the experiments in this thesis it would be necessary to increase IOP by 2.50 mmHg \textit{in vivo}, this would be ideal as 2.50 mmHg is within the range of IOP changes that occur over the cardiac cycle \cite{64}. However, further studies with whole eye globes would be required to determine the validity of this approximation.

\textbf{Speed of loading}

During the experiments, the speed with which the hydrostatic pressure was increased was 0.058 mmHg/s which is much slower than the rate at which fluctuations in IOP occur \textit{in vivo}, where the pressure can change by several mmHg over less than a second. This slower loading regime was chosen as it allowed the fringe formation to be viewed during loading.

A study by Elsheikh ad Pye \cite{28}, has previously demonstrated that the response of the cornea is time dependent, with greater deformation occurring when slower loading rates are used. As the total magnitude of the pressure increase was small, it was expected that any differences in the response due to the loading rate would be minimal. However, to confirm this it would be necessary to update the design of the loading rig to allow pressure to be applied at a similar rate to the \textit{in vivo} case.
There were three main considerations that were made with regards to the tissue used during experiments and the impact on the measured results and the validity of the conclusions that could be drawn from experiments, these were:

1. The availability of tissue
2. The quality of the tissue prior to experimentation
3. Changes to the tissue that occurred during experimentation

Because the majority of donated human corneas are reserved for transplant it was difficult to obtain human corneas in adequate sample numbers, and ultimately this limited the number of human corneas to be examined during this research to six. Despite the fact only six human samples were examined, similarities in behaviour across the samples were identified as described in section 7.4 of chapter 7. However, to make solid statements regarding the biomechanics of the human cornea, and to confirm the validity of the initial findings from these experiments, further investigations would be required on a larger sample size.

Due to the limited availability of human tissue, two types of tissue were used during the experiments in this thesis. Tissue of porcine origin was used for all preliminary experiments and human tissue was used for the main experiments.

As discussed in section 6.1.2 of chapter 6, the porcine cornea tissue used during experiments was obtained from pigs that had been subjected to scalding post-slaughter. Although only corneas with no visual signs of damage or abnormality were used during the experiments, the overall influence of the scalding procedure on the tissue remained unknown, and ultimately this impacted the validity of the conclusions that could be drawn from the porcine cornea experiments regarding corneal biomechanics.

The human cornea tissue that was used during the experiments, detailed in chapter 7, was transplant tissue that had been in storage in excess of the four-week to eight-week period over which it is considered to be viable for transplant. The way the tissue had been stored prior to the human cornea experiments had also led to some changes to the hydration properties of the tissue and this had manifested as swelling of the posterior stroma leading to visible wrinkling.

As identified in section 7.3.1 of chapter 7, changes to the hydration properties of the tissue could potentially contribute to changes to the biomechanics, hence the biomechanics of the posterior stroma could have changed due to the presence of swelling. Since the anterior stroma was resistant to hydration changes and is thought to be the area to contribute most
significantly to the bulk mechanical properties of the material, it was presumed changes to the overall biomechanics would be small. However, to determine the overall influence of any changes, further experiments would be required on fresh human tissue.

A final area with respect to the tissue properties that was considered during this research was the changes to the properties of the tissue that occurred over the total testing time. As the anterior surface of the cornea was coated during experiments it could not be exposed to a moist environment and the back surface of the cornea was exposed to PBS solution which could contribute to swelling of the tissue, hence it was assumed that the hydration properties of the tissue would gradually change over the total experimental time of approximately one hour. Changes to the response of the tissue over the testing time were investigated on porcine corneas, as detailed in section 6.4.2 of chapter 6 and it was confirmed that small changes to the magnitude of the response occurred over the total testing time, but the overall mode of deformation remained similar, as shown in Figure 6-20 and Figure 6-21. Although the changes to the response could not be confirmed to be a result of hydration changes in isolation, this experiment provided a necessary control from which the effects of crosslinking could be analysed. It was identified that, in future experiments using similar methods, it would be useful to track changes to the thickness of the cornea to monitor any hydration changes and assess their potential effects.

In these initial experiments the total experimental time for each cornea was approximately one hour as it was desired to examine the response over several loads of different magnitudes and to carry out several repeated measurements. For future experiments the influence of changes to the tissue hydration properties or other factors over the experimental time could be reduced by reducing the number of measurements required on each cornea, and the time for each measurement cycle, by speeding up loading, and moving the wrapping and unwrapping procedures offline.

8.1.3 Measurement Techniques

It was realised at an early stage in the project that the non-flat geometry of the cornea would introduce several measurement challenges especially when considering shearographic evaluation. These challenges were identified in section 3.6 of chapter 3, where it was concluded that in order to obtain complete information regarding the response of the cornea to a pressure changes, both the in-plane and out-of-plane components of displacement had to be defined with respect to the viewing direction. Although several studies had previously used speckle interferometric methods, including ESPI (41) and RSI (45,51,73), to examine the biomechanics of the cornea these studies had only considered the out-of-plane component of
deformation and thus not fully defined the deformation of the surface in response to pressure variations.

For shearography, the variable contribution of the in-plane and out-of-plane components over the surface was the most significant issue with regards to quantitative assessment and interpretation of the interferograms. When evaluating an initially flat object, the data describing the 1st derivative of out-of-plane displacement relates approximately to surface gradient change, whereas, for a curved object the surface gradient change is a function of both in-plane and out-of-plane displacement, and these components have variable contributions across different regions of the surface.

However, during the experiments in this thesis it was thought that if the shear amount remained small interfering points on the surface would be likely to deform in a directionally similar manner, hence the various components describing the first derivative of displacement with respect to the objects surface could be estimated so long as the in-plane and out-of-plane components could be defined with respect to the image plane.

Initially, attempts were made to implement a dual-beam shearography system that could analyse the in-plane and out-of-plane components of displacement simultaneously, however after several unsuccessful experiments it was concluded, in section 4.3.1 of chapter 4, that this system would not be suitable due to the fact the illumination angle was limited to less than 20° from the normal otherwise it was not possible to achieve adequate illumination across the entire object. This limited the sensitivity to in-plane deformation relative to out-of-plane deformation, therefore combined measurement could not be achieved.

Considerations into alternative systems for combined in-plane and out-of-plane analysis were made at this stage, however it became evident that implementation of such a system would be difficult due to the variable sensitivities that would likely be required to in-plane and out-of-plane deformation. Later when testing human corneas, it became evident that the in-plane component had only a relatively small contribution to the overall response meaning a combined system would have not been viable, as the instrument would not have been sensitive enough to in-plane motion at the limited pressure changes at which out-of-plane data could be successfully recorded.

The eventual solution was the implementation of a pure out-of-plane measurement system, at first an LSI system was designed, but shortly after this an ESPI system was integrated to aid with the interpretation of the LSI data, and to evaluate the concerns held over the potential introduction of distortion or errors in the LSI data for the reasons discussed in chapter 3. Since
only the out-of-plane component could be determined via interferometry an alternative method was required to assess the in-plane component, this was addressed via the introduction of a shape measurement system.

The information on surface elevation obtained via the shape measurement system was used in combination with the out-of-plane data to make an estimate of the in-plane component based on the assumption that deformation occurred normal to the surface. However, since there were doubts over the validity of this assumption for complex objects such as the cornea, video analysis was also used to examine the deformation of a central cross-section. This enabled this data to be visually compared with the predicted data and to be used to implement an angle correction, as demonstrated for the human corneas in section 7.4.3 of chapter 7.

8.1.4 Limitations of measurement techniques

**Out-of-plane data**

Initial experiments on a loading plate where the out-of-plane displacement had been set to 1 µm, as detailed in section 5.2.2 of chapter 5, demonstrated that the ESPI configuration was giving the expected measurement values. Subsequent testing on a range simple samples confirmed that the LSI was also giving the expected measurement values, as the differentiated ESPI data and the LSI data was found to be approximately equivalent as demonstrated in Figure 5-3 in chapter 5, across a range of shear magnitudes. Overall, any errors present with respect to ESPI measurement were consistent throughout testing so did not affect the comparison of data before and after crosslinking. On the other-hand some of the measurement errors associated with the LSI data were dependent on the way the object deformed, so they could not be guaranteed to remain consistent before and after crosslinking.

The main source of error during LSI measurement was introduced due to the way the object deformed, with the greatest errors possible in areas where the rate of change of the first derivative of out-of-plane displacement was high. This was demonstrated on non-biological samples in chapter 5 and highlighted in Figure 5-18 where differences were evident between the differentiated ESPI data and the LSI data in the regions where the rate of change of the 1st derivative of out-of-plane displacement was high. This source of error was found to be problematic during corneal measurement, especially for the case of porcine corneas, due to the rapid increase in the rate of displacement at the edges, compared to minimal changes in the rate of displacement across the central areas, and the increase in sensitivity due to the curvature of the cornea in these regions. This effect meant that for porcine corneas the LSI data could only be quantitatively analysed over the central 10 mm area.
This effect seemed to be less of an issue during human corneal measurement if the magnitude of the pressure increase was small (between 0.25 mmHg to 0.50 mmHg), as good agreement could be obtained between the differentiated ESPI data and the LSI data across the sample. However, to determine if reliably good data could be obtained consistently within these boundary regions on human corneas it would be necessary to test a much larger number of human cornea samples.

Another issue that was experienced when using both ESPI and LSI for corneal measurement, was a loss of resolution in the fringes towards the boundary regions due to the high fringe density in this region. For the ESPI case this could be avoided for most human corneas up to pressure changes of 0.50 mmHg, above which the fringe spacing in this region often became too small. For the LSI case, data quality was difficult to maintain at the boundary regions, except for very low pressure changes and this introduced challenges when trying to optimise the technique for full-field evaluation.

Ultimately, the differences in behaviour across different regions led to concerns that, due to lack of data across the central regions, it would not be possible to pick up changes to the response that occurred across the central areas after crosslinking while maintaining data quality across the whole measurement area. However, during human cornea testing it was found that although very few fringes formed across the central areas, the introduction of crosslinking at specific topographic locations did introduce changes to the value of the measured phase change across these regions as demonstrated in the LSI line plots included throughout section 7.5 of chapter 7.

Integration of the LSI data taken over the smallest pressure increase of 0.25 mmHg, as detailed in section 7.5.5 of chapter 7, confirmed the sensitivity was adequate to detect phase changes in these areas despite the lack of full fringe formation, as the distribution of the integrated LSI data before and after crosslinking showed evidence of the changes introduced during crosslinking.

From later experiments on porcine corneas, as detailed in section 6.4.5 of chapter 6, it was found that if fringe quality at the edge areas was ignored and pressure was increased by larger amounts, a greater number of fringes could be generated across the central regions and this often helped to highlight the effects of crosslinking, as demonstrated in Table 6-11. The outcomes from these experiments led to the following conclusions:

- If it is desired to quantitatively evaluate the 1st derivative of out-of-plane displacement across the whole sample then data should be recorded for very small pressure
changes at which speckle decorrelation can be avoided and good fringe resolution can be maintained in all areas.

- If it is desired to examine the cornea for the presence of abnormalities across the central regions, or if it is desired to examine the changes that occur across these regions due to crosslinking, then it would be most effective to mask the areas at the edges and to evaluate the response over larger pressure increases as this may help to highlight any changes or abnormalities.

A combination of the two approaches may be best to extract the most information, and could easily be achieved by obtaining data over a range of pressure variations of different magnitudes. A further factor to take into consideration regarding the boundary effects is that they may have been exaggerated due to the fixed boundary conditions imposed, therefore it is possible improvements in data quality and accuracy in these regions would be possible if clamping was further from the cornea or if whole globes were examined, however this has yet to be confirmed.

**Integration of LSI data**

As identified on numerous occasions throughout this thesis, the integration of the LSI data to determine the out-of-plane displacement component was prone to errors due to the cumulative nature of the trapezoidal integration procedure used and the presence of regions of low data quality. Alongside this, the approximation for the 1\textsuperscript{st} derivative of out-of-plane displacement obtained via LSI was inaccurate in regions where the 2\textsuperscript{nd} derivative of out-of-plane displacement was large compared to the 1\textsuperscript{st} derivative of out-of-plane displacement as described in section 3.6.4.

To obtain accurate quantitative information regarding the out-of-plane displacement across the full sample in response to a pressure change it is necessary that the approximation for the 1\textsuperscript{st} derivative of out-of-plane displacement obtained via LSI is accurate across the full data set and so far this has been difficult to achieve. However, across the central cornea where data quality was high and the approximation provided by the LSI data matched well with that of the ESPI data, it would be possible to produce a map of relative displacement showing the pattern of deformation across a region of interest, this approach may be useful in several scenarios, for example if it was desired to evaluate the effects of crosslinking across the central regions or if it was required to help with interpretation of the LSI data without the need for accurate quantitative information.
**Estimation of in-plane component**

Interpretation of the measured data at the boundary regions and how it related to the deformation of the cornea was not possible without considering the in-plane component to the response.

The estimation of the in-plane component using the techniques described was subject to several errors. Firstly, due to the resolution of the projector that was used to project fringes onto the surface of the cornea and the subsequent magnification of the corneal image, the measured shape data was noisy and required smoothing prior to integration. This smoothing resulted in an underestimation of the gradient of the surface in areas where the angle of the surface changed rapidly. This issue was found to be problematic during the porcine cornea experiments at the edge of the cornea adjacent to the chamber, but reduced in human cornea experiments due to the more gradual changes in surface gradient. Overall in the human cornea experiments, the errors introduced to the measured surface elevation data due to smoothing were up to a maximum of 13% at the boundary regions.

Secondly, the estimation of deformation occurring normal to the surface did not appear to be valid for corneal samples and the estimation of the angle of deformation from the video analysis, an example of which is shown in Figure 7-18, was only conducted at a few well-spaced points along the central horizontal and vertical cross-sections on a limited number of samples. In addition to this, the video analysis was conducted at relatively larger pressure changes than were used during the interferometric analysis, and it is not known whether the ratio of in-plane to out-of-plane deformation would be altered at these pressures.

Overall, several improvements could be made to the method used to estimate the in-plane component to obtain greater accuracy. A high-resolution projector could be employed to reduce the noise levels in the shape data which would in turn reduce the level of smoothing required. Alternatively, the fringes could be generated via the use of a grating, a grating based measurement system to evaluate corneal topography and elevation has previously been reported to be capable of defining corneal elevation with an accuracy of 0.015 mm in the central 10 mm and 0.031 mm around the periphery \(^{178}\).

Alongside improvements to the shape measurement method, accuracy of the estimation for the in-plane component could be improved if the angle of deformation could be estimated with greater accuracy. This could be achieved if high speed, high resolution video recording could be combined with digital image correlation techniques enabling the movement of the corneal surface along several cross-sections to be tracked with high precision over a pressure change.
8.2 Experimental outcomes

From the data obtained during this thesis full-field plots describing the 3D response of the surface of the human cornea to hydrostatic pressure changes, representative in magnitude to those that occur physiologically during the cardiac cycle have been generated, and so far, this is the first study to present such data. Not only this but ESPI and LSI have been demonstrated to be useful measurement tools, with adequate sensitivity to identify areas of subtle biomechanical abnormalities and assess the effects of topographic crosslinking.

8.2.1 Data Presentation

The data in this thesis has been presented mainly in terms of displacement and sometimes in terms of the first derivative of displacement, there were several reasons for choosing to present data in this format, these were:

1. Displacement data is easily interpretable to a wide-ranging audience.
2. By evaluating displacement and the 1st derivative of displacement it is possible to evaluate regions where strain is most significant during loading and identify regional differences in the response.
3. Using displacement data it is possible to demonstrate how the shape of the corneal surface changes under loading, this is important as the shape of the cornea governs its refractive properties.

As discussed in section 2.6 of chapter 2, many previous studies have focussed on determining the corneal Young’s modulus, with most studies quantifying a single Young’s modulus for the cornea. In many studies (28, 45, 51), this value of Young’s modulus has been determined by measuring the displacement at the corneal apex in response to hydrostatic pressure variations and assuming the cornea behaves as a homogenous, isotropic, hemispherical membrane of uniform thickness. As demonstrated in this study, this assumption is not valid, because in addition to the variable thickness and aspherical nature of the cornea there are significant regional variations in its response to loading, therefore defining the biomechanics of the cornea in this way gives us little information as to how the cornea deforms in vivo and cannot be used for useful applications, such as generating predictive treatment models for corneal crosslinking or reducing the risks and optimising the outcomes of refractive surgery procedures.

Definition of different Young’s moduli with respect to different regions of the cornea would be more realistic. An approximation for Young’s modulus at each point on the corneal surface could be made by determining strain with respect to the meridional and circumferential directions using the full-field out-of-plane data obtained via ESPI and LSI and the estimated in-plane data. Then if the magnitude of the pressure change and the thickness of the cornea was
also known, stress could be approximated and this could be used to estimate Young’s modulus using the methods previously described in detail by Anderson, et al \cite{63}, where it is assumed that the cornea can be approximated as a thin-walled hemispherical pressure vessel and the thickness remains constant during loading i.e. the cornea is incompressible. However, in reality, it has been demonstrated that the cornea is not incompressible \cite{81}, therefore to obtain an accurate estimation of Young’s modulus the deformation that occurs throughout the depth of the cornea in response to pressure variations must also be taken into account.

Ultimately the data in this study was not used to approximate corneal Young’s modulus as the thickness of the corneas was not recorded at the time of experiment. Also, focus was put on presenting data in a format that would be most useful to people who may use it, such as clinicians, and since Young’s modulus data is often used to determine how the corneal surface deforms in response to a given pressure and how this response may change in the presence of biomechanical changes it seemed logical to present the information in this format rather than as Young’s modulus.

Generally, when discussing the crosslinking results, the data was presented in terms of the out-of-plane displacement component and the first derivative of out-of-plane displacement with 3D deformation information not detailed for all samples. The reason for this was because for the human corneas examined, the out-of-plane component of deformation was found to dominate the response, as shown in Figure 7-20, with in-plane deformation being relatively small in comparison. This meant the out-of-plane displacement data alone provided a relatively good approximation for the magnitude of total surface displacement and the LSI data provided a good approximation of surface gradient change, especially across the central regions, which was useful for identifying regions where there may have been a refractive change.

On several occasions throughout the results chapters the measured data was presented in the format of the wrapped data plots describing the out-of-plane displacement and its 1st derivative as a series of fringes. The reason for including data in this format was because the distributions of fringes in the interferograms often helped to highlight subtle features, or changes to the response, that were not always as obvious when viewing the full-field unwrapped data. The advantage to the wrapped data is that the total displacement or 1st derivative of displacement is divided over several fringes over which the greyscale is divided providing high resolution visual data.

Overall, with a general understanding of what the fringe distributions indicated, quick visual analysis of the data could be carried out to check for certain features or irregularities. An example was provided at the end of chapter 6 in section 6.4.4, where irregularities in the
response of a porcine cornea were recognised directly from the appearance of the wrapped data, and from this data alone an appropriate region for crosslinking was identified.

8.2.2 Pre-crosslinking results

As described in chapter 7, the key findings that were made when examining the human corneas prior to crosslinking were as follows:

1. The rate of change of out-of-plane displacement was a maximum at the edge of the sample at the position where the cornea joined the sclera. Indicating that this region of the tissue is more compliant than other regions in response to pressure fluctuations.
2. The rate of change of out-of-plane displacement was lowest across the central areas with the N-T axis being more resistant to changes in $\frac{\partial w}{\partial x}$ across the centre than the S-I axis was to changes in $\frac{\partial w}{\partial y}$. Indicating that the N-T axis was particularly resistant to changes in curvature across the central regions in response to pressure fluctuations.
3. The profile of deformation was not symmetrical with peak displacement generally off-centre, and one side of the cornea generally appearing more resistant to out-of-plane deformation than the other for both axes.

The results from this study, on a small number of samples were compared with the results of previous studies to see if similarities could be identified, as detailed in the following discussion. However, when reviewing the results of previous studies, it was evident that there has so far been a lack of consistency in the findings.

Several studies have reported similar observations to this study with the limbal/peripheral areas of the cornea showing lower resistance to deformation in response to IOP fluctuations and the central areas showing high resistance to curvature change. The results of an early study by Hjortdal (24), where the response of the cornea to large IOP increases was measured by tracking the movement of several well-spaced particles on the surface of the cornea, concluded that the limbus had the lowest meridional stiffness but the highest circumferential stiffness, and the peripheral regions of the cornea had both low meridional and circumferential stiffness.

The double-exposure holography study by Smolek (70) on intact human eye globes, as discussed at the beginning of this chapter, also showed evidence of an area of reduced stiffness at the peripheral/limbal regions of the cornea as fringe formation was concentrated in these regions specifically. A study by Boyce et al (20) on bovine cornea-scleral buttons using DIC, concluded that “…the central cornea deforms very little over a physiologic pressure range while most deformation is accommodated in the peripheral/limbal cornea”.

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In addition to this, studies that have tracked the shape of the central cornea have suggested that only minimal changes occur to the curvature of the cornea with increasing IOP. Pierscionek, et al (148) found only minimal changes in the curvature of the cornea during an increase of IOP from 15 mmHg – 50 mmHg, and a further study only found minimal changes to corneal shape when IOP was increased up to 443% above normal (179).

Several recent studies that have been conducted at the same time over which this research has taken place have also demonstrated similar findings regarding lower resistance to deformation in the peripheral regions. An in vivo study by Elsheikh et al (21), where the shape of the cornea was compared using a videokeratoscope before and after IOP was doubled, found a stiffness reduction of 47.3% in a 2.5 mm peripheral region bounded by the limbus when compared with the centre. This finding was later followed up by the results of an ex-vivo study on a single intact human eye globe (22), where the deformation of the globe was measured using DIC, and similar behaviour was reportedly observed.

However, contrary to the previous studies discussed, several studies have presented results that suggest the centre of the cornea is not resistant to deformation or curvature change. An early study by Shin (25), that tracked the movement of several particles positioned in different regions across the surface of the cornea, concluded the centre of the cornea had the lowest stiffness relative to other regions.

Notably a study by Jaycock at al (41) that used a similar ESPI technique to the technique described in this study to investigate sheep corneas, demonstrated a steepening of the curvature of the cornea in the central region under an increase in IOP. From this study, there was no evidence from the fringe distributions of a lower stiffness region around the limbus/periphery.

An earlier holographic interferometry study by Kasprzak et al (69) on bovine corneas also showed that the curvature of the central cornea changed during an increase in IOP, although there was some evidence in the holograms presented of a higher fringe concentration towards the edges. However, the baseline pressure used in this study, was towards the lower end of normal IOP at 10 mmHg, and this likely influenced the results, as the study by Boyce et al (20), demonstrated that the mode of deformation differed at pressures lower than normal IOP from that seen for pressures within the range of normal IOP.

In summary, based on the findings of this study and the other studies described there are two scenarios that could describe the response of the central cornea to pressure fluctuations, these are:
a) The cornea undergoes changes in curvature across the central optical zone in response to normal changes in IOP, as concluded in the studies by Shin, et al (25), Jaycock (41), et al, and Kasprzak, et al (69). This scenario is illustrated in Figure 8-4 (a).

b) The cornea is highly resistant to changes to curvature across the central optical zone under normal variations in IOP, due to the specific structure that enables pressure variations to be accommodated for mainly in the peripheral and/or limbal regions, as found in this study and suggested by the results of several other studies (20,148,179). This scenario is illustrated in Figure 8-4 (b).

![Figure 8-4](image)

*Figure 8-4 – Illustrations showing two possible ways the central cornea deforms in response to IOP fluctuations.*

It is known that the cornea provides most of the focusing power of the eye and it is the specific shape and curvature of the cornea, and its position relative to the retina, that facilitates this. Therefore, based on the two scenarios described, there are two explanations to account for the fact that no noticeable changes occur to vision during variations in IOP that occur naturally throughout the day:

a) In scenario (a) there is both a refractive change due to the change in the shape of the cornea, and a focal change due to the change in the position of the cornea with respect to the retina. However, the vision changes introduced by these factors are suppressed by cortical processes.
b) In scenario (b) the refractive power of the cornea remains the same and only the position of the cornea with respect to the retina is changed, hence the brain only must account for changes to focus.

There are several structural elements that would support scenario (b), that the curvature in the central optical zone is maintained and deformation mainly occurs towards the peripheral and limbal regions of the cornea in response to a change in IOP within the physiological range. The structure of the cornea has previously been discussed in detail in section 2.2 of chapter 2, therefore only the specific features that are thought to contribute specifically to the observed behaviour are summarised in the following:

- The anterior stroma has a structure consisting of highly intertwined and randomly orientated lamellae, that interweave with fibres in the Bowman's layer (7) providing this layer with high stiffness and high resistance to curvature change.

- The net orientation of collagen fibres changes at the periphery approximately 1.5 mm from the limbus (23) due to lamellae bifurcation where the collagen fibres branch out and adjoin with circumferentially aligned fibres in the ‘circum-corneal annulus’ (180) located approximately 1 mm into the sclera (23). Elsheikh’s research group (21,22) suggested that this bifurcation of lamellae that occurs in the peripheral cornea along with a degree of interweaving and braiding to form new lamellae results in a material stiffness reduction in what they termed the ‘transition zone’. Whereas, Boyce et al (20) suggested the circumferential arrangement of fibres at the limbus resulted in a reduction meridional stiffness in this area which accounted for the observed behaviour.

- Recent studies have described both the presence and distribution of elastic fibres in the cornea (31,32). Both of the referenced studies suggested that elastic fibres are most concentrated in the peripheral cornea on the posterior side where they run in parallel to the circumferentially orientated collagen fibres in this region. The elastic fibres are thought to originate in the limbus where they form sheets from which narrower fibres extend into the cornea. Interestingly, the elastin content of these fibres has been shown to decrease towards the centre of the cornea (181). It is thought that the presence of these elastic fibres plays an important role in the response and the recovery of the cornea to small pressure changes and in the maintenance of the change in curvature between the cornea and sclera.
With respect to the results of this study from testing on both human and porcine corneas, and the structural aspects described, two possible mechanisms for how the corneas deforms in response to in vivo pressure fluctuations have been suggested.

1. The first is that the low relative stiffness of the limbal/peripheral regions allows the cornea to bulge outwards in these regions resulting a flattening of corneal curvature in response to pressure fluctuations, as seen for the S-I axis in porcine corneas. This deformation at the periphery accounts for the pressure increase and minimises both the out-of-plane movement and curvature change of the central optical zone. This is illustrated in Figure 8-5.

2. The second is that the orientation of the fibres in the limbal region contributes to a reduction in the meridional stiffness in this region facilitating mainly out-of-plane movement in this area in response to pressure fluctuations, as seen in the human cornea experiments when using the small aperture chamber. This response at the limbus provides a damping affect and results in only out-of-plane translation of the central optical zone, while curvature across the majority of the cornea is maintained. This is illustrated in Figure 8-6.

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**Figure 8-5** – Illustration showing a mechanism for the way the cornea may deform due to IOP fluctuations.

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**Figure 8-6** – Illustration showing an alternative mechanism for the way the cornea may deform due to IOP fluctuations.
It is possible that both mechanisms may contribute, however further work would be required to determine the influence of the boundary conditions before conclusions could be drawn.

In addition to the identification of a lower stiffness region at the limbus/periphery, more subtle differences were noted in the response across other areas. For example, differences were seen in the responses of the two preferential axes and this is not something that has been described in detail in previous studies. Although differences were evident from results presented in the studies by both Smolek (70) and Boyce, et al (20). While Elsheikh, et al (165) previously reported that the vertical meridian was stiffer than the horizontal meridian when testing via strip extensometry.

The results from the majority of the corneas tested in this study showed that for the N-T axis the magnitude of the out-of-plane displacement changed very little inside of the 1 mm boundary region, indicating minimal curvature change. Whereas, for the S-I axis the magnitude of out-of-plane displacement continued to change gradually towards the centre indicating this axis may be slightly less resistant to changes in curvature. The reason for this difference is unknown but structural differences between the two axes have been described within the literature.

One visually recognisable difference is the extension of the limbal tissue onto the cornea for the S-I axis (170) making this axis appear shorter than the N-T. In addition to this Newton and Meek (23) described differences in the way that the collagen transitioned from being axially aligned with respect to each meridian to then being circumferentially aligned at the circum-corneal annulus. They described the N-T axis as having a 1.5 mm transition zone in which the collagen gradually became circumferentially aligned via bifurcation, however, they reported the same was not visible for the S-I axis with the collagen described as remaining axially aligned right up until the limbus. Boote et al (26) also identified that the structure of the circum-corneal annulus “…appeared asymmetrical – It’s width and degree of fibril alignment varying with circumferential position.”

In addition to the differences in the observed responses of the preferential axes with respect to one another, differences were noted in the responses across each of these axes, as the response to a pressure increase was not symmetrical about either the horizontal or vertical centre line. Generally greater deformation occurred towards one side of each axis and this was reflected in the screen shots from the video analysis as shown in figures 7-14 and 7-17. Unfortunately, as previously stated, it was not possible to identify the poles of the specific axes and therefore the differences can only be discussed as one side with respect to the other and not with reference to specific positions.
It is possible that this variation in the magnitude of displacement across the axes was due to natural biomechanical variation. Evidence exists that demonstrates the collagen orientation in left and right eyes is structurally distinct \cite{26}, and this supports the fact that biomechanical behaviour may not be axis-symmetrical.

Since the cornea is designed to deal with different forces at different positions i.e. forces from the ocular muscles and forces imposed from the eyelids during blinking, it seems logical that the biomechanical properties may vary across the surface. However, it is clear from the structural analysis that has been carried out in previous studies \cite{26} that the structure of individual corneas is highly variable and therefore testing on a larger range of samples, that have not been structurally compromised close to the measurement area would be required before any conclusions regarding a ‘normal’ response could be made.

8.2.3 Crosslinking results

Due to the non-destructive nature of the measurement methods described throughout this thesis it was possible to analyse how topographic crosslinking changed the response of an individual cornea to hydrostatic pressure variations. The ability to evaluate changes to the response of individual corneas was fundamental to understanding the effects of crosslinking, this was because there were significant variations in the way corneas responded to hydrostatic pressure fluctuations, as demonstrated throughout the human and porcine cornea experiments. Therefore, it was necessary to compare corneas with themselves so pre-existing regional variations in mechanical properties prior to crosslinking could be considered.

It was demonstrated during the crosslinking experiments that changes to the out-of-plane displacement and 1st derivative of out-of-plane displacement occurred in response to the same pressure change before and after crosslinking with a total energy of 7.2 J/cm$^2$ (15 mW/cm$^2$, 8 minute exposure). These changes could be detected by ESPI and LSI and were different to those that could be expected to occur over the experimental time. The specific changes that occurred to the response of the corneas after crosslinking varied depending on the region that was crosslinked, with similar changes seen across the human and porcine cornea samples examined. A summary of the changes that occurred after crosslinking in each of these topographic locations of human corneas is given in the following:

- **Central 3 mm circle** – Crosslinking in this region resulted in a reduction of the out-of-plane displacement component at the centre of the cornea by approximately 16.2% in human corneas, and an increase in the out-of-plane displacement towards the
periphery. Indicating that crosslinking in this area would result in a flattening of the corneal profile.

- **9 mm circle around a masked 3 mm central region** – Crosslinking in this region resulted in a large reduction in the magnitude of the out-of-plane displacement component by up to 80% towards the peripheral regions. There was no clear evidence of any steepening of the non-crosslinked central area relative to adjacent areas, as there had been during the simulation crosslinking experiments detailed in section 5.4 of chapter 5.

- **3 mm wide strip along central horizontal axis** – Crosslinking in this region for the specific sample tested resulted in a reduction in the magnitude of out-of-plane displacement along the axis of crosslinking, with the overall magnitude of out-of-plane displacement becoming similar across the central region of the cornea along this axis after crosslinking indicating a reduction in bending strain. However, since the sample that was crosslinked in this region was damaged the observed effects may not be applicable to other samples.

- **3 mm wide strip down central vertical axis** – Crosslinking in this location resulted in a large decrease in the magnitude of out-of-plane displacement across the cornea especially along the region of crosslinking, with the rate of change of out-of-plane displacement with respect to this axis becoming close to zero in this region after crosslinking, indicating very little bending strain. With respect to the horizontal axis there was a greater reduction in out-of-plane displacement in the central region relative to adjacent areas which coincided with the region of crosslinking, with the overall profile of displacement indicating a flattening of the corneal profile with respect to this axis similar to what had been seen when crosslinking in the central 3 mm area.

Overall, the results of crosslinking backed up some of the previous observations that were made regarding corneal biomechanics prior to crosslinking. For example, crosslinking in the central 3 mm area was found to result in less significant changes to the magnitude of the out-of-plane displacement component than in instances where regions closer to the peripheries were crosslinked, and this supports the previous observation that pressure fluctuations are generally accommodated in the peripheral/limbal regions. Whereas, crosslinking around the outside of a central 3 mm area resulted in large changes to the magnitude of out-of-plane displacement in response to pressure fluctuations, but did not result in significant steepening of the central 3 mm relative to adjacent areas, confirming this area appears to be highly resistant to curvature change.
From the results of this initial study it would seem that crosslinking in specific locations in isolation to change the mechanical properties of specific regions could be used to introduce refractive changes by introducing small changes to the shape of the cornea when subjected to IOP. What is evident from this study, is that these changes may be different to those expected to occur when modelling the cornea as a homogeneous, isotropic hemisphere. Therefore, for successful application of this type of treatment further research would be required using methods, such as those described in this thesis, that are capable of analysing the changes that occur to individual corneas.

Overall, the results of this initial study are promising and demonstrate the potential for the use of customised crosslinking treatments to improve visual outcomes in the treatment of keratoconus and ectasia over what is currently achieved when crosslinking the whole corneal area. The results also suggest, with a better understanding, it may be possible to use customised crosslinking to correct refractive errors, potentially offering a minimally invasive alternative to current refractive surgery procedures for some cases.

However, there were several limitations to the crosslinking experiments conducted in this thesis. Firstly, the sample size was small so only one human cornea was analysed for crosslinking in each of the selected locations, except for the central 3 mm locations where two corneas were examined. In addition to this, since this is the first study to examine the effects of topographic crosslinking there is a lack of data with which the results can be compared to determine if the findings were in-line with what was expected. Hence further testing is required to determine the validity of these findings.

Secondly, it was not quantified what level of crosslinking was achieved due to the crosslinking procedure outlined in section 6.4.1 of chapter 6, so it is not known what level of crosslinking introduced the observed changes. The effects of soaking the cornea in riboflavin alone without crosslinking were also not quantified during experiments due to the limited availability of riboflavin. Since the riboflavin selected (VibeX-Xtra, Avedro Inc., MA, USA) did not contain dextran it was assumed that it did not result in large changes to the hydration state of the cornea that may have affected biomechanics. Also, the riboflavin alone could not have accounted for the effects seen across the corneas tested, as different effects were seen after crosslinking in different locations, and for each case the surface of the cornea was fully soaked in riboflavin.
9. Conclusions and Further work

At the commencement of this research project it was identified that, to-date, there is a lack of understanding and agreement within the ophthalmic community regarding the biomechanics of the cornea due to the failure of current measurement techniques, used for the assessment of biomechanics, to provide reliable data relevant to the in vivo case. Detailed knowledge of corneal biomechanics and the ability to measure biomechanical properties has become increasingly important over recent years due to the introduction and popularity of refractive surgery procedures and subsequent concerns over their safety due to their presumed impact on corneal biomechanics. In addition to this, procedures are now being introduced for the treatment of ectasia, where it is possible to specifically alter the biomechanics of the tissue by initiating collagen crosslinking and to understand the potential of these treatments and to optimise their application, it is important to be able to quantify their effects. The focus of this research was therefore on addressing the question:

Could a technique be developed to measure corneal biomechanics and changes to biomechanics that may be introduced as a result of disease or surgical intervention?

It was chosen to investigate the use of speckle interferometric measurement techniques, specifically LSI, as measurement tools to evaluate corneal biomechanics as they were concluded to best fit with the specification for a suitable measurement method as outlined in Table 2-3, when compared with the other methods reviewed in chapter 2.

Ultimately, the outcomes of this research spanned three main areas, these were:

1. The development and evaluation of a measurement technique for corneal biomechanical assessment.
2. The development and implementation of a suitable ex vivo experimental methodology.
3. An analysis of corneal surface deformation in response to hydrostatic pressure variations representative in magnitude of those that occur physiologically and the changes that can be introduced due to crosslinking in specific regions in isolation.

The main novel elements of the research presented in this thesis have been summarised as follows:

1. The development and application of a method incorporating; ESPI, LSI, shape measurement and video analysis, for the evaluation of corneal surface deformation in response to hydrostatic pressure variations equivalent to those that occur physiologically.
2. The development of a stable measurement rig capable of generating repeatable and reliable data using interferometric techniques from in-tact corneal-scleral sections.

3. Identification and verification of an impervious surface coating suitable for covering moist corneal tissue and generating an adequate return signal, while not influencing the overall response to loading.

4. The presentation of full-field data detailing the 3D surface deformation of human and porcine corneas in response to small changes in hydrostatic pressure equivalent to those that occur physiologically during the cardiac cycle.

5. An analysis of the errors and limitations associated with measurement of the 3D deformation of the cornea using both ESPI and LSI.

6. An evaluation of the effects of crosslinking, in specific topographic locations in isolation, on the response of individual corneas to changes in hydrostatic pressure equivalent in magnitude to those that occur physiologically during the cardiac cycle.

7. Demonstration of customised-crosslinking based on identification of areas of variation in biomechanical properties.

This chapter details the main conclusions from the research and the overall contribution to knowledge in the context of the limitations of the measurement methods used. This is followed by a discussion of the potential for further work that has arisen from this research.

9.1 Conclusions

9.1.1 Suitability of the measurement methods for the evaluation of corneal biomechanics

*Optical measurement methods*

As defined at the end of chapter 2, the aim was to develop an optical measurement system using laser speckle interferometry that was capable of analysing full corneal surface deformation in response to loads representative of those that occur physiologically. A summary of the optical measurement system and its capabilities, related to the original specification for a method for corneal evaluation first outlined in Table 2-3, is given in Table 9-1.
<table>
<thead>
<tr>
<th>Feature</th>
<th>Capabilities</th>
</tr>
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</table>
| Mode of measurement         | • Non-contact.  
• Non-destructive.  
• Full-field surface deformation in response to hydrostatic loading measured in terms of out-of-plane displacement and 1\textsuperscript{st} derivative. |
| Sensitivity                 | • Minimum displacement sensitivity of < 1 fringe (0.266 \textmu m).  |
| Measurement range           | • 0.266 \textmu m to approximately 2 \textmu m (depending on fringe distribution).  |
| Spatial Resolution          | • Imaging resolution 0.02 mm/pixel  |
| Field of View               | • 25 mm diameter circle  |
| Depth of Focus              | • 6 mm  |
| Measurement Time            | • 633 ms (full surface measurement with ESPI/LSI)  
• 5 seconds (shape measurement)  |
| Repeatability               | • Repeatability of phase measurement not known independent of overall repeatability.  
• Dependent on data quality.  
• ESPI, for case of flat metal plate loaded with piezo SD = +/- 0.274 radians (0.016 \textmu m)  
• LSI, for case of sample in chamber used for experiments SD = +/- 0.3187 radians  |
| Safety                      | • $E_s = 0.998$ mW/cm\textsuperscript{2}  
• Safe for use by untrained individual.  |
| Data Format                 | • Whole-field out-of-plane displacement  
• Whole-field 1\textsuperscript{st} derivative of out-of-plane displacement  
• Surface elevation.  |

Table 9-1 – Optical measurement system features.

Through the experiments detailed in chapters 4, 5, 6 and 7 the capabilities of the system with respect to corneal evaluation were determined. They are summarised here:

- The system was not capable of achieving measurement directly from the surface of the cornea due its transparency, however an impervious surface coating was identified Sphericel 110P8 fused borosilicate hollow glass microspheres (Potters Industries LLC, PA, USA) that generated adequate back scatter. Experiments detailed in section 6.2.1 of chapter 6 validated that this coating moved with the corneal surface while having negligible impact on the movement allowing the normal movement of the surface to be evaluated.

- The system was capable of non-destructive evaluation, enabling the same cornea to be evaluated before and after crosslinking.

- The sensitivity range over which data could be obtained using ESPI and LSI corresponded to a hydrostatic pressure variation of between 0.25 mmHg to 1 mmHg for the cornea when situated in the chamber, and based on a previous study\textsuperscript{70} on whole globes this should correspond to an IOP change of between 1.25 mmHg to 5 mmHg which is equivalent to the IOP change reported to occur during the cardiac cycle.

- Using the out-of-plane data, shape elevation data and the assumption that deformation occurred normal to the surface it was possible to approximate full-field 3D surface...
deformation of human corneas in response to physiological pressure variations. When
the profile of the response predicted using this method was compared with the frame
subtracted video data showing the profile of the response of a cornea deforming to a
larger pressure change it was found to provide a good initial approximation.

- When the frame subtracted video data was analysed in greater detail for human
corneas it was evident that the surface tended to deform to a greater extent out-of-
plane with any in-plane motion appearing to be comparatively small, therefore the out-
of-plane data alone provided a good visual representation of the overall response.
- It was demonstrated during chapters 5,6 and 7 that both ESPI and LSI were effective
for identifying areas of weakness or non-homogeneity in the response of samples to
loading.
- It was demonstrated during chapters 6 and 7 that ESPI and LSI had adequate
sensitivity to detect changes to the response of corneas that could be introduced by
crosslinking and so far this is the first techniques shown to be capable of evaluating the
regional effects of crosslinking under physiological pressure variations.
- It was identified that if the thickness of the cornea was also measured the out-of-plane
and in-plane data obtained using the methods described could be used to estimate a
regionally specific Young’s modulus (if it is assumed the cornea is incompressible).

There were two main drawbacks to the system that was designed with respect to the ultimate
aim which was to evaluate corneal biomechanics, these were:

1. Only surface deformation could be measured, therefore the deformation response
through the tissue was not quantified, hence corneal Young’s modulus could not be
accurately defined.
2. Only the out-of-plane component of displacement and its 1st derivative were measured
directly, with the corresponding in-plane components estimated by assuming that
deformation occurred normal to the surface, or by estimating the angle of deformation
based on cross-sectional video data recorded along the N-T and S-I axes for higher
pressure changes. Hence, where 3D surface deformation is detailed it can only be
considered to be an approximation.

To address the first issue, another technique capable of evaluation through the thickness of
the sample would be required. OCT based techniques \(^{(81,182)}\) have shown good potential for
full-thickness evaluation albeit with a slightly reduced sensitivity when compared with
interferometry of approximately 1 µm and the requirement to take several scans to build up full
3D information, hence a longer measurement time. It is possible that a combination of these
techniques could be used to obtain a ‘best of both worlds’ approach. For example speckle
interferometry could be used to obtain high resolution data regarding full surface deformation
in less than a second, while OCT could be used to obtain data regarding the deformation
through the thickness of the sample along a few representative cross-sections negating the need for hundreds of scans and a long measurement time to build a 3D picture.

To address the second issue, a similar approach could be used by combining the advantages of different techniques. For example a combination of OCT and digital volume correlation has previously been used to obtain information regarding the 3D motion of a porcine cornea\textsuperscript{(81)} in response to pressure changes of 3.75 mmHg, the drawback to this method was that data had to be obtained line by line and hence a long measurement time was needed and meaning it would not be feasible as a clinical measurement tool. However, if this technique could be used to test a large number of corneas \textit{ex vivo} it may be possible to determine what the normal relationship of in-plane an out-of-plane motion is for most corneas, so if only the out-of-plane data was measured going forward it would be possible to make a good evidence based approximation for in-plane motion. Another option, similar to the video analysis procedure described in this thesis, would be to use the Scheimpflug camera to track corneal deformation along cross-sections in response to inflation testing and track the in-plane and out-of-plane movement using DIC to determine the expected angle of deformation with better resolution.

Overall, although further information would be required to accurately define Young's modulus the information that can be obtained using the techniques described offers significant advantages over current methods used to determine the biomechanics of the cornea, which rely on measuring deformation at the corneal apex only, to predict a single Young’s modulus for the tissue based on the assumption that the cornea behaves as an isotropic, homogenous, hemispherical membrane.

The high sensitivity of the measurement techniques and their ability to assess subtle regional differences in out-of-plane displacement and its 1\textsuperscript{st} derivative with high spatial resolution, makes them particularly effective for identifying differences in the way different regions of the corneal surface respond to pressure variations, this is highly important with regards to understanding how the biomechanics of the cornea correspond to its \textit{in vivo} behaviour and factors that may influence this behaviour. The level of detail regarding regional differences in the response of the cornea that could be achieved using the measurement system presented, as demonstrated in chapters 6 and 7, was significantly better than what has been achieved using alternative methods. Based on this, there are several areas where the technique that has been developed could have important applications:

- It could be used to better understand conditions associated with biomechanical changes such as keratoconus, by evaluating how the biomechanics of specific regions of the cornea are affected in a keratoconic cornea. This information could then be used to make informed decisions on the best treatment approaches to adopt.
• It could be used to better understand the biomechanical impact of current refractive surgery procedures, cataract surgery procedures and corneal transplant surgery procedures and in doing so enable safer techniques to be developed.
• It could be used to quantify the regional biomechanical effects of topographic crosslinking enabling predictive treatment models to be generated which could potentially improve visual outcomes in the treatment of keratoconus and ectasia and also enable the use of crosslinking for refractive correction.

**Testing procedure**

It was confirmed during experiments detailed in chapter 5, that the combination of the rigid metal chamber and the automated hydrostatic loading system was successful in enabling repeatable data to be captured over repeated loaded cycles on non-biological samples. For a rubber sample clamped in the chamber the average standard deviation in phase measured using LSI was +/- 0.32 radians. This repeatability could be attributed to several design features:

1. Rigid metal chamber and metal connectors that were stable over the pressure range of 16.50 mmHg to 19.50 mmHg used in experiments.
2. Threaded tissue locking mechanism capable of ensuring leak-proof clamping on samples of thicknesses between 0.1 mm to 3.0 mm.
3. Highly repeatable automated loading. Repeatability in the vertical movement of the tank connected to the chamber was +/- 0.0069 mm, corresponding to a repeatability in pressure variation of +/- 0.0005 mmHg.
4. Uniform loading speed of 0.058 mmHg/s.
5. Uniform timing of data capture at 1 second after the end of loading, ensuring even loading/data capture time which was important due to the viscoelastic nature of the cornea.

Ultimately, the high level of repeatability that could be achieved enabled ESPI and LSI data obtained over different loading cycles to be compared, the repeatability in the response of the cornea to be examined and changes to the response of the cornea after crosslinking to be analysed.

Factors unrelated to the loading rig that were found to affect measurement repeatability were:

1. **Data quality** – if the quality of fringes in the wrapped interferograms were poor the phase change could not be accurately resolved and the repeatability of measured data was poor. This was found to be a problem during corneal testing, especially with LSI, due to the closeness of fringes that formed in the boundary regions, for this reason data repeatability was better for lower pressure variations of 0.50 mmHg or less.
2. **Nature of sample** – The repeatability of the response of porcine corneas was lower than that of rubber samples with an average standard deviation of +/- 0.47 radians.
recorded for a porcine cornea subjected to 10 repeated loading cycles compared to +/- 0.32 radians for the rubber sample. The average standard deviation in the response across different corneas also varied slightly between +/- 0.32 radians to +/- 0.68 radians. However, it was not possible to determine if this was due to differences in data quality or differences in the repeatability of the response.

3. **Changes to the tissue over the experimental time** – Experiments detailed in section 6.4.2 of chapter 6 demonstrated that small changes to the magnitude of the response of the tissue occurred over the total testing time of 1 hour, however the overall nature of the response remained the same. These could have been due to a number of reasons such as hydration changes or creep. For future tests these changes could be minimised by significantly reducing the experimental time by conducting less repeated measurements and moving data processing offline.

With respect to the aim of determining corneal biomechanics relevant to the in vivo case, it was identified in the previous chapter that the testing procedures used had several drawbacks these were:

1. **Fixed boundary conditions** – The test rig enabled the response of corneo-scleral sections across a diameter of either 12.5 mm or 15.7 mm to be examined, for the corneas this corresponded to the analysis of the full corneal area and approximately 1 mm to 3 mm (depending on the size of the cornea) of adjacent sclera incorporating the limbal region. The effect of the imposition of fixed boundaries at the edge of the sclera was not known and it could have influenced the response of the cornea in the areas immediately adjacent to the clamp.

2. **Absence of the whole globe** – The AAC was rigid whereas in vivo the deformation of the sclera would accommodate in part for any increase in IOP. Therefore, the response of a cornea to a given pressure increase in the chamber could not be expected to be equivalent to the response of the cornea to the equivalent increase in IOP.

3. **Speed of loading** – changes in IOP of several mmHg can occur in less than a second in vivo whereas the loading rate was limited to 0.058 mmHg in experiments. Based on previous data \(^{(28)}\) it would be expected that at faster loading rates the cornea would deform by a lesser amount for a given load.

4. **PBS solution** – PBS solution was used to pressurise all corneas and this could have resulted in gradual swelling of the tissue over the experimental time, which in turn could have affected the overall biomechanics.

In conclusion, it was identified that several improvements were required to the loading set-up and testing procedure to determine the effects of the above factors and obtain data that would be more relevant to the in vivo case, these were:
1. Development of a rig to accommodate whole globes in a way representative of how they are supported in vivo.
2. Development of a method to achieve pulsatile loading at a rate of approximately 10 mmHg/second.
3. The use of balanced salt solution over PBS solution to reduce swelling.

Going forward a mounting method similar to the one described by Whitford, et al (22) could be employed where the cornea was mounted in a low stiffness gelatine matrix enabling the whole globe to be evaluated. Although us of such a method would also require modification of the measurement technique to track the movement of the whole globe.

9.1.2 The effectiveness of LSI as a stand-alone technique

As identified at the beginning of this research project, the ultimate aim would be to have a system capable of in vivo evaluation as this would enable; early diagnosis of disease, evaluation of potential risk factors that may make an individual ineligible for various refractive surgeries, and the development of customised treatment plans for the treatment of disease or to enhance vision. The potential to develop a laser speckle interferometry system as an in vivo measurement tool would be likely to rely on the use of LSI as a stand-alone technique, as ESPI would be unlikely to be a viable option due to its sensitivity to external disturbances.

Prior to study, although many studies could be found where LSI had been used for applications such as defect detection, very few studies had reported the use of LSI for the quantitative analysis of complex objects under complex loading conditions and therefore little was known about the potential for measurement errors and the challenges associated with measurement. A significant advantage to the combined optical set-up developed for this study was that LSI data could be directly compared with ESPI data, from this research the following conclusions were drawn regarding the use of LSI for corneal evaluation and the challenges associated with quantitative measurement:

- LSI provides an approximation for the 1\textsuperscript{st} derivative of displacement with respect to the direction of shear by comparing the relative displacement at two points separated by a specific distance. The accuracy of this approximation is dependent on the following;
  - The distance between sheared points. Accuracy increases as the shear distance between points becomes shorter, until the point at which the shear distance becomes too small and the fringes are too fine to be accurately resolved. For the set-up detailed in this thesis the minimum shear distance was 50 pixels (1.04 mm).
  - The way the sample deforms. If the second derivative of displacement is high compared to the first derivative (high bending strain) the accuracy of the approximation is reduced.
During testing on both flat and curved rubber objects, detailed in chapter 5, it was demonstrated that there was less than 1% difference in the maximum value of $\frac{\partial w}{\partial x}$ calculated from differentiation of the ESPI data and from the LSI data when using shear magnitudes of 52 pixels and 95 pixels. However, the high rate of change of $\frac{\partial w}{\partial x}$ over a small distance was found to be an issue during corneal measurement, especially for porcine corneas in the 1 mm to 2 mm area closest to the clamp. This meant that the approximation for the 1st derivative of out-of-plane displacement was inaccurate in these regions and therefore the data could not be quantitatively analysed.

When using LSI to measure curved surfaces the shear distance between points with respect to the surface of the object increases as the gradient of the surface with respect to the image plane increases and this results in variable sensitivity across the object. For human corneas this effect was found to result in an increase in sensitivity by around 15% towards the edges of the cornea. For porcine corneas the sensitivity was increased by up to 71% in the steepest regions.

It was demonstrated that this increase in sensitivity can be accounted for in post-processing by measuring the gradient of the surface and then using this to calculate the distance between points with respect to the surface of the object. However, the variation in sensitivity can contribute to issues with regards to obtaining full-field data if the mismatch in sensitivity is large, this issue is further exaggerated for corneas due to the fact the 1st derivative of out-of-plane displacement was relatively higher in the curved areas and this led to problems with maintaining high fringe quality in these regions. Overall this meant the repeatability of data in these regions was poor.

Direct interpretation of the fringe distributions obtained when using LSI to examine the deformation of a curved object is not intuitive. For a simple object such as a metal plate loaded with a point load to the centre the fringes describing the 1st derivative of out-of-plane displacement relates to the surface gradient change in response to loading, however for a complex object deforming in 3D, such as the cornea, the surface gradient change is a function of both out-of-plane and in-plane motion therefore the fringes cannot be considered to relate directly to surface gradient change.

Overall, for the human corneas because the contribution of any in-plane displacement was found to be small the 1st derivative of out-of-plane displacement was considered to approximate surface gradient change especially across the central regions.
With regards to data interpretation it is useful to have displacement information. LSI data can be integrated with respect to the direction of shear to obtain the equivalent displacement component. However, during experiments it was found that this process was subject to several errors that could arise for the following reasons:
- Inaccuracies in the approximation of the 1st derivative of out-of-plane displacement due to the reasons described above.
- Inaccuracies in measured phase due to speckle decorrelation and areas of unresolvable data.
- Errors in the quantification of applied shear.

Due to the issues with data accuracy in the 1 mm to 2 mm region adjacent to the chamber during corneal testing it was not possible to quantify out-of-plane displacement via integration of the LSI data with confidence for all corneas tested. However, a good visual representation of the profile of displacement and the changes to the profile of displacement for a given cornea after crosslinking was achievable when integrating the LSI data obtained on human corneas over pressure increases of 0.25 mmHg. Also, as the accuracy of the approximation of the 1st derivative of displacement was found to be comparable to the differentiated ESPI data in the regions inside of the 1 mm to 2 mm boundary regions. Therefore, the LSI data could be integrated across these regions to examine relative displacement, this could be useful for examining the effects of crosslinking across the central regions.

In conclusion, there are several areas that would have to be addressed if it was desired to use LSI as a stand-alone quantitative measurement tool for the analysis of corneal biomechanics as the deformation at the boundary regions introduces challenges with respect to achieving high quality, accurate data. It is possible the fixed boundary adjacent to this region contributes to the observed effects and that there would be less problems if testing whole globes, but this remains to be seen.

However, even in the absence of quantitative assessment, the distribution of the wrapped fringes in the interferograms could provide information that could be useful for diagnosing the presence of areas of biomechanical abnormality or identifying patients that may be ineligible for refractive surgery if LSI was developed as an *in vivo* measurement tool. The use of LSI in this way would rely on testing enough corneas to determine what a ‘normal’ response would look like and then training clinicians how to interpret variations from this. This type of analysis was successfully demonstrated on a porcine cornea at the end of chapter 6, where customised crosslinking was conducted based on the distribution of fringes observed during control testing.
If successful, this type of analysis would offer significant improvement over the current clinical biomechanical assessment techniques which are based on measuring the response of the cornea to an air puff, as it would provide spatially resolved information across the full surface of the cornea potentially enabling targeted and customised treatment. Also, the high sensitivity may mean biomechanical abnormalities could be identified earlier.

9.1.3 Obstacles to the development of a clinical measurement tool

Although LSI could be a useful tool for clinical assessment of corneas for the reasons stated in the previous section, there remain several significant challenges to the development of LSI as a clinical measurement tool, these include:

1. **Generating an adequate return signal from the surface of the cornea** – It was confirmed in this study that it was not possible to obtain an adequate signal directly from the surface of the cornea when using interferometric methods. The coating method used in this study to address this issue would not be transferable to an *in vivo* situation, hence research would be required into alternative methods to achieve an adequate return signal from the cornea that would also be eye safe.

2. **Stability** – All experiments during this thesis were conducted on an optical table with the cornea clamped in a highly stable chamber. *In vivo* stability of the cornea during measurement would be affected by movements of the head, movement of the eye, blinking, hydration changes and tear film break-up, therefore measures would be required to control these movements and account for their potential affects.

3. **Speed of measurement** – During this study, it was possible to control the frequency and magnitude of loading, whereas *in vivo* the IOP is constantly changing as a result of the pressure changes introduced during the cardiac cycle. Because the pressure change over a cardiac cycle can be greater than the pressure changes used during these experiments, the measurement would potentially have to be timed so data could be captured for a fraction of the total pressure change. Also, because the cornea would not remain stable at the time of measurement it would not be possible to use temporal phase stepping techniques, as have been employed in these experiments, therefore alternative methods to extract quantitative information from the data would be required.

9.1.4 Experimental findings and implications

**Corneal biomechanics**

During this research the responses of 44 porcine corneas and 6 human corneas were measured to hydrostatic pressure variations as detailed in chapter 6 and 7. From these
experiments several important features in the response of the cornea to pressure changes were identified and the following conclusions were made:

- There is an area of biomechanical importance in the region where the cornea meets the sclera. This area shows lower resistance to deformation under hydrostatic pressure variations and appears to play a role in accommodating for small fluctuations in IOP.
- The central regions of the cornea appear to be highly resistant to changes in curvature and this appears to be facilitated by deformation at the boundary regions.
- The response of the cornea is complex and non-symmetrical and regional variations in mechanical properties exist.
- The response of the cornea appears to be linear over a physiological range of pressure variations.

The area of increased compliance at the limbal/peripheral region was evident on all of the human corneas tested and on the majority of the 44 porcine corneas that were tested. In addition to this it was identified that several previous studies have reported comparable findings. Overall, these features require further investigation on a larger number of human corneas in-order to validate these initial findings.

The findings regarding the region of high compliance at the limbus is particularly important as incisions are often made in this area during surgical procedures such as cataract surgery and refractive surgery. Gaining an understanding of how these incisions affect this area is important to guarantee the long-term safety of these treatments. It is evident that current treatment models require updating to take account of the unique biomechanics of this area as, from the results of this study, it is evident that the current assumptions that are made based on assuming the cornea behaves as an isotropic homogenous membrane are not valid. In addition to this, since this zone appears to be fundamental to maintaining the shape of the central region of the cornea, manipulation of the mechanics of this zone by either crosslinking or surgery could potentially offer an opportunity for a new way to achieve refractive correction.

**Crosslinking**

This was the first study to demonstrate the effects of crosslinking in specific regional locations. This was possible due to the following capabilities of the measurement methods used:

- Non-destructive measurement capabilities so each individual cornea could be measured before and after crosslinking which was important due to the high inter-subject variation between samples.
- High sensitivity and high resolution which enabled subtle variations in the response to be detected with high spatial resolution across the surface of the cornea.
Robust experimental methodology where high loading and measurement repeatability could be achieved.

The main conclusions from the crosslinking experiments were as follows:

- Crosslinking with an energy of 7.2 J/cm$^2$ (15 mW/cm$^2$, 8 minute exposure) introduced measureable changes to the response of the cornea to variations in hydrostatic pressure and these changes varied depending upon the location of crosslinking.
- The porcine corneas tested and the human corneas tested showed similarities in the way the response of the cornea changed after crosslinking in each of the locations investigated when compared with the original response. However, changes were not always isolated to the region of crosslinking and the changes seen showed some differences to those that had been predicted based on experiments on the uniform, homogenous rubber sample detailed in chapter 5, indicating that this model is too simple to represent the complexity of corneal structure and biomechanics.
- The initial results of the experiments conducted for each crosslinking location would suggest the following:
  - Crosslinking in a 3 mm diameter central region could result in a flattening of corneal curvature and a reduction in central displacement in response to a pressure change of 16.2%.
  - Crosslinking the 9 mm diameter region around a 3 mm diameter central region could increase the resistance of the cornea to out-of-plane deformation by up to 80% in some regions but would be unlikely to significantly change the curvature of the cornea across the central non-crosslinked regions, although small changes may occur.
  - Crosslinking down the centre of either the N-T or S-I axes could increase the resistance of the cornea to out-of-plane deformation and increase the resistance of the crosslinked axis to curvature change in response to hydrostatic pressure variations while resulting in a flattening of corneal curvature in the axis orthogonal to the crosslinked strip.

However due to the limited number of corneas examined so far and the condition of the corneas tested more samples would require testing to validate these findings.

Overall, the initial findings would suggest that it should be possible to introduce changes to the shape of the cornea through crosslinking in specific locations, however the nature of these changes may be different than those predicted if assuming the cornea behaves as a homogenous isotropic membrane. These findings are highly significant as they demonstrate that crosslinking may have the potential to be used as a method to change the refractive properties of the cornea and this could have applications both in delivering customised
treatments for corneal diseases such as keratoconus and also in addressing astigmatism and other refractive errors. The measurement methods used in this research study have, so far, been the only methods demonstrated to be capable of evaluating these changes and therefore further experiments using these techniques could be important for the further development and optimisation of crosslinking treatments.

9.2 Further work

The broad, novel and multidisciplinary nature of the work presented in this thesis meant the research path was chosen as the one that could best fulfil the research aims in the allocated project time. As a result, there remain a number of areas that require further research to fully address the original challenges discussed and questions posed at the beginning of this thesis and there is significant scope to take this research further forward with regards to metrological bioengineering and clinical perspectives. Areas where further work would be beneficial have been identified on numerous occasions throughout this thesis, the main areas are summarised in this section.

9.2.1 Metrological aspects

**Accuracy and traceability**

The ESPI and LSI configurations described in this thesis were not calibrated with respect to a known reference and therefore the accuracy of the measurements remains unknown. To quantify this, experiments could be conducted using a calibrated reference artefact where known displacements could be introduced, or measurement data for a given sample could be compared to equivalent data achieved on a previously calibrated interferometer.

**Error analysis**

Error analysis of interferometric measurement techniques is a complex field that remains under-researched, especially with regards to the use of LSI for the quantitative measurement of curved or complex objects under multi-directional loading states. Attempts were made in this thesis to identify and quantify the main the sources of measurement error with respect to LSI by comparing the data to ESPI data, but it was found that the magnitude of error was case dependent and dependent upon several factors.

Overall, the combined ESPI and LSI system that was developed provided a useful method of comparing data. This system could be further developed specifically for LSI error analysis by incorporating a calibrated ESPI system and also, depending on the available budget, the system could be developed to enable ESPI and LSI measurement to be achieved simultaneously using the methods described in chapter 4. Using such a system, further
research could be carried out on simple objects with similar geometry to the cornea and known modes of displacement, this would increase understanding of the errors associated with LSI measurement and enable quantification and modelling of their effects, which would in turn enable predictions to be made for the corneal case. In the long-term this work is necessary if LSI is to be used as a quantitative measurement tool as currently the measurement uncertainty is too great.

9.2.2 Bioengineering aspects

**Modelling of corneal biomechanics**

Ultimately the aim would be to produce a model of corneal biomechanics as this would enable:

- An increased understanding of how the specific biomechanical properties relate to *in vivo* behaviour.
- The ability to model biomechanical changes that occur in disease or a result of surgical intervention.
- The ability to predict the effects of surgical procedures and optimise them to reduce risk and improve visual outcomes.
- The ability to use the information to further the development of synthetic alternatives to human donor tissue.

To realise this aim, further research is required into the following areas to validate the findings and address some of the limitations of the studies in this thesis, and to also address the remaining gaps in knowledge.

1. **Understanding of in-plane response** — Throughout this research, approximations were made for the in-plane component of displacement based on the assumption that deformation occurred normal to the surface. However, from the frame subtracted video data of a cornea deforming to a hydrostatic pressure increase, presented in section 7.4.3, it was identified that this assumption is unlikely to be valid for the cornea due to the regional differences in meridional and circumferential stiffness. To gain a more thorough understanding of the in-plane component of the response, the movement of the corneal surface in response to loading could be recorded along different cross-sections using a Scheimpflug camera and DIC could be used determine the direction of movement in different regions, this could be carried out on a larger number of corneas than was possible in this study to determine if there is a general trend across corneas. Alternatively, other measurement techniques could be employed such as OCT and digital volume correlation where it is possible to evaluate in-plane motion, as described in the previous section.
2. **Understanding of the depth dependent response** – Using the interferometric techniques described it was only possible to measure surface deformation and since deformation through the thickness of the sample will contribute to biomechanics it must be investigated to enable the development of an accurate biomechanical model. This could be achieved ex vivo using alternative techniques capable of imaging through the depth of the sample such as OCT.

3. **Clamping effects** – To determine the relevance of the measured data to the *in vivo* case further research is required into the effects of the clamping the cornea into a rigid metal chamber. To achieve this, ideally a method would be developed whereby the cornea could remain as part of a whole globe and be supported in some way that is representative of *in vivo* conditions. However, this would also require further development of the measurement technique to monitor the movement of the whole globe.

4. **Loading speed** – The loading speed used in experiments was not representative of the *in vivo* case where the IOP can change by several mmHg in less than a second and this could have affected the magnitude of the corneal response to pressure variations. The effects of loading speed require further investigation to determine the relevance of the data from this study to the *in vivo* loading case. To achieve data more representative of the *in vivo* case in future experiments, the loading rig could be redesigned to enable loading at a rate of several mmHg/second.

5. **Hysteresis response** – It was demonstrated during porcine cornea testing in section 6.4.5 that the way the cornea responded to increasing pressure and decreasing pressure was different, suggesting that even at low loads a degree of hysteresis was evident in the response. As the overall profile of the response was different for each loading direction it also appeared that the magnitude of hysteresis may be different across different areas. This hysteresis effect requires further investigation if a complete biomechanical model is to be developed, this could be achieved by examining the response of the cornea to both increasing and decreasing pressures.

6. **Tissue quality/sample size** – Ultimately it was only possible to evaluate six human cornea samples during the course of this study and these samples were swollen due to having been stored in organ culture and this could have affected the biomechanics. To generate a representative model of corneal biomechanics or surface deformation in response to pressure changes it would be necessary to test large numbers of fresh human corneas. Ideally, testing of at least several hundred human corneas would be possible as inter-subject variability is likely to be significant due to the biological nature
of the samples. However, to achieve this it is likely a device and testing methodology would have to be developed whereby the corneal tissue could be tested before it was used for transplant as opposed to instead of being used for transplant as it was in these experiments.

Overall, there is significant opportunity to further develop the techniques and testing procedures used in this research to obtain information more relevant to the in vivo case. In addition to this, there potential to combine the advantages of different imaging techniques to achieve the ultimate aim which would be a comprehensive biomechanical model of the human cornea.

9.2.3 Clinical aspects

Many important clinical questions remain unanswered, including:

1. How does keratoconus affect the full-field biomechanics of the cornea? Are biomechanical changes isolated to the region of the cone?
2. What is the biomechanical impact of refractive surgery? Which procedures (PRK, LASEK, LASIK, SMILE, etc.) carry the least risk? Can these procedures be optimised even further?
3. Are current procedures used for cataract surgery or corneal transplant optimal? Could they be improved to reduce risk and improve visual outcomes?
4. What are the biomechanical effects of crosslinking? Could crosslinking specific regions in isolation be used to achieve refractive correction?

There is potential to conduct investigations, using the techniques described in this thesis, to help to answer some of these questions. For example, if keratoconic tissue could be obtained for research, in addition to larger number of normal corneas it would be possible to map how the response of a keratoconic cornea differs from that of a normal cornea and determine whether changes are isolated to the region of the cone, which could ultimately justify the use of customised crosslinking for the treatment of keratoconus.

To investigate the impact of surgical procedures, the responses of corneas to hydrostatic pressure variations could be measured before and after introducing surgical incisions enabling the regional effects on biomechanics to be assessed. Different variations on techniques could be attempted and evaluated without concerns about causing harm to patients enabling techniques to be optimised to preserve the normal biomechanics of the cornea as far as possible. In addition to providing useful information regarding the safety of surgical procedures, the ability to evaluate the changes that occur to biomechanics after corneal surgery could
provide information regarding the variability in outcomes that occur due to human factors and ultimately provide a useful training tool for ophthalmic surgeons to optimise their technique.

With respect to the understanding and optimisation of crosslinking treatments, a large amount of work still remains. This study demonstrated that ESPI and LSI could be used to evaluate changes to the response of corneas to hydrostatic pressure variations that occurred after crosslinking, but due to the lack of tissue and time constraints it was not possible to examine crosslinking in the detail required to make recommendations regarding how the treatments should be used. To optimise the procedures and ultimately generate data that could support and enable the use of customised crosslinking further experiments would be required, including:

1. Investigations into how variable parameters affect changes to the response of corneas after crosslinking, including; UVA power, exposure time, riboflavin soak time, type of riboflavin solution, etc.
2. Investigations into the effectiveness of epithelium-on and epithelium-off procedures.
3. Investigations to establish the effects of crosslinking in different regions.
4. Investigations to determine how changes to the responses of corneas to hydrostatic pressure variations after crosslinking correlates with refractive changes.

If these studies could be carried out it may be possible to generate models of predicted treatment outcomes based on different combinations of the parameters detailed, ultimately making customised treatment a possibility.

**Development of a clinical device**

As identified at the beginning of this thesis, there is currently a requirement for a clinical measurement tool capable of biomechanical assessment to enable the following:

1. Early diagnosis of keratoconus.
2. Evaluation of risk associated with undergoing refractive surgery.
3. Individualised and customised treatment of disease or refractive error.

Although the data obtained from the measurement methods detailed within this thesis does not give enough information alone to determine specific biomechanical parameters such as Young’s modulus, the information that can be obtained could be sufficient to meet the clinical needs outlined above and offers significant improvements over the methods currently used for biomechanical assessment. Therefore, further research into the development of a clinical measurement tool based on the methods described in this thesis is justified.
As described in the previous section there are many obstacles to clinical translation of the technology, including; generating an adequate signal from the corneal surface and obtaining adequate stability for measurement, therefore significant further research is required in each of these areas to address the current challenges.
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Appendix A

**Piezosystem Jena**

Order number: 430132  
Actuator type: PA8-12  
Serial number: 71118

Nominal capacity: 0.8 μF  
Maximum hysteresis: 1.35 μm

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