Visualising the Inter- and Extra-cellular Diffusion Pathways In Rodent Lenses

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Abstract

The lens is the largest avascular organ vertebrates. It functions to refract light onto the retina. In order to function appropriately, the lens must remain transparent and have a gradient refractive index to correct for spherical aberration. Intercellular communication in the lens is vital to supply nutrients deep into the lens and remove waste products. While the transfer of small solutes is known to be mediated by Gap Junction (GJ) proteins, more recent work has shown that an alternative and parallel pathway exists that permits the diffusion of large molecules. This Macro-molecular-Diffusion-Pathway (MDP) appears to develop at a distinct stage of lens fibre cell differentiation and is believed to be associated with the expression of the MP20 protein. MP20 protein is the second most abundant integral membrane protein specific to the vertebrate lens and previously has been suggested to be an adhesion protein important in zipping-up the extracellular space.

In this thesis, I present findings from experiments designed to probe the MDP and GJ mediated intercellular communication and to map the formation of MDP as a function of fibre cell differentiation using Two-Photon-Excitation-Flash-Photolysis (TPEFP) and caged 10 kDa-Fluorescein. This 10 kDa-Fluorescein is too big to diffuse through any GJ mediated intercellular communication. The TPEFP data were correlated with the mapping of MP20 protein expression through Immunohistochemistry in the mouse and the rat lenses. There is a species-specific differentiation-dependent membrane insertion of MP20 protein that occurs a lot earlier in the mouse lens (r/a 0.92) than in the rat lens (r/a 0.80). The uncaging of 10 kDa-Fluorescein results strongly suggests that MDP formation occurs in regions of membranous MP20 protein labelling shown by the movement of uncaged-10 kDa-Fluorescein. In the Lim2KO mouse model with absence of MP20 protein, the large 10 kDa-Fluorescein was restricted in the uncaged source cells, confirming past studies that MDP is no longer formed without presence of MP20 protein (Shi et al., 2009).

In the mature fibre cells of the lens, both GJs and the MDP mediate the intercellular diffusion pathways. Knock-out mouse models with absence of GJs (Cx46KO) or MDP (Lim2KO) were utilised to probe the two intercellular pathways separately, using TPEFP and caged-fluorescein and 10 kDa-Fluorescein. The data showed that the removal of Cx46 did not affect the formation of the MDP, which correlates well with a previous study (Cheng et al., 2008). Interestingly, the removal of Lim2 resulted in reduction of approximately 58% of extent of dye spread when
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compared to the wild-type mouse lens. It seems the presence of MP20 protein is vital to the GJ mediated intercellular diffusion.

Having established the importance of MP20 protein membrane insertion to the formation of MDP in the lens, I then turned my attention to the past findings which correlated the membrane insertion of MP20 protein with the zipping-up of the extracellular diffusion space. Findings from experiments using Confocal Reflectance Microscopy technique in conjunction with Gd-DTPA were first developed and optimised, and then used to investigate the relationship between the differentiation-dependent membrane insertion of MP20 protein and the zipping-up of the extracellular space in the mouse lens. It seems that the extracellular barrier to diffusion from the lens periphery occurs deeper (r/a 0.56) than the membrane insertion of MP20 protein (r/a 0.92) in the wild-type mouse lens. In lens without MP20 protein (Lim2KO), the extracellular space diffusion barrier was found to form at similar location to the wild-type lens, and it is apparent that zipping-up of the extracellular space is independent to the function of MP20 protein.

The significance of the overall findings is summarised and discussed at the very end.
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## Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>% v/v</td>
<td>Diluted volume (mL) in 100 mL of diluant volume</td>
</tr>
<tr>
<td>% w/v</td>
<td>Dry weight (g) in 100 mL volume</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celcius</td>
</tr>
<tr>
<td>1°Ab</td>
<td>Primary Antibody</td>
</tr>
<tr>
<td>10 kDa-Fluorescein</td>
<td>10 kDa-Dextran-Linked-Fluorescein</td>
</tr>
<tr>
<td>2°</td>
<td>Secondary</td>
</tr>
<tr>
<td>2°Ab</td>
<td>Secondary Antibody</td>
</tr>
<tr>
<td>3°</td>
<td>Tertiary</td>
</tr>
<tr>
<td>4°</td>
<td>Quaternary</td>
</tr>
<tr>
<td>AAH</td>
<td>Artificial Aqueous Humour</td>
</tr>
<tr>
<td>AICS</td>
<td>Artificial Intra-Cellular Solution</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APs</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosin Tri Phosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Ca⁺-ATPase</td>
<td>Calcium ATPase</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium Ion</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>Chloride Ion</td>
</tr>
<tr>
<td>cm</td>
<td>Centi-Metres ($10^{-2}$ m)</td>
</tr>
<tr>
<td>CMNB</td>
<td>Bis-(5-Carboxy-Methoxy-2-Nitro-Benzyl) ether (cage molecule)</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>Cx43</td>
<td>Connexin 43</td>
</tr>
<tr>
<td>Cx46</td>
<td>Connexin 46</td>
</tr>
<tr>
<td>Cx46⁻/⁻</td>
<td>Double Connexin 46 Knock-out</td>
</tr>
<tr>
<td>Cx46KO</td>
<td>Connexin 46 Knock-Out</td>
</tr>
<tr>
<td>Cx50</td>
<td>Connexin 50</td>
</tr>
<tr>
<td>Cx50⁻/⁻</td>
<td>Double Connexin 50 Knock-out</td>
</tr>
<tr>
<td>D</td>
<td>Dioptres</td>
</tr>
<tr>
<td>DF</td>
<td>Differentiating Fibre</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol Tetraacetic Acid</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>fs</td>
<td>Femto-Seconds ($10^{-15}$ s)</td>
</tr>
<tr>
<td>Gd-DTPA</td>
<td>Gadolinium Diethylene-TriaminePentaacetic-Acid</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GJ(s)</td>
<td>Gap Junction(s)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydro-Chloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)</td>
</tr>
<tr>
<td>IR</td>
<td>Infar-Red</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium Ion</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton (10⁻³ Dalton)</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Potassium Di-Hydrogen Phosphate</td>
</tr>
<tr>
<td>KI</td>
<td>Knock-In</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-Out</td>
</tr>
<tr>
<td>LASER</td>
<td>Light Amplification by Stimulated Emission of Radiation</td>
</tr>
<tr>
<td>Lim2</td>
<td>Lens Intrinsic Membrane protein-2</td>
</tr>
<tr>
<td>Lim2KO</td>
<td>Lim2 Knock-Out</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>M199</td>
<td>culture Medium 199</td>
</tr>
<tr>
<td>mA</td>
<td>Milli-Ampere (10⁻³ A)</td>
</tr>
<tr>
<td>MDP</td>
<td>Macro-molecular Diffusion Pathway</td>
</tr>
<tr>
<td>MF</td>
<td>Mature Fibre</td>
</tr>
<tr>
<td>mg.mL⁻¹</td>
<td>Milli-Gram per Milli-Litre</td>
</tr>
<tr>
<td>mg/year</td>
<td>Milli-Gram per Year</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>min(s)</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>mL</td>
<td>Milli-Litre (10⁻³ L)</td>
</tr>
<tr>
<td>mM</td>
<td>Milli-Molar (10⁻³ M)</td>
</tr>
<tr>
<td>mm</td>
<td>Milli-Metre (10⁻³ m)</td>
</tr>
<tr>
<td>MP20</td>
<td>Membrane Protein with molecular weight of 20 kDa</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>n</td>
<td>the size of the sample</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical Aperture</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium Ion</td>
</tr>
<tr>
<td>Na⁺-K⁺-ATPase</td>
<td>Sodium Potassium ATPase</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Di-Sodium Hydrogen Phosphate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>nm</td>
<td>Nano-Metres (10⁻⁹ m)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PMT</td>
<td>Photo-Multiplier Tube</td>
</tr>
<tr>
<td>r/a</td>
<td>Radial position from the lens centre. ( r ) is the distance from the centre of the lens to the region of interest; ( a ) is the total radius of the lens. ( r/a=1 ) is the lens periphery and ( r/a=0.5 ) is the lens core.</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium-Dodecyl</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium-Dodecyl-Sulphate-Polyacrylamide-Gel-Electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>Second(s)</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>StDev</td>
<td>Standard Deviation of the population</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TIF</td>
<td>Tagged Image File</td>
</tr>
<tr>
<td>TPE</td>
<td>Two-Photon Excitation</td>
</tr>
<tr>
<td>TPEFP</td>
<td>Two-Photon-Excitation-Flash-Photolysis</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris(hydroxymethyl)aminomethane Hydrochloric acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-Violet light</td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat Germ Agglutinin</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>μL</td>
<td>Micro-Litre (10⁻⁶ L)</td>
</tr>
<tr>
<td>μm</td>
<td>Micro-Metre (10⁻⁶ m)</td>
</tr>
<tr>
<td>μM</td>
<td>Micro-Molar (10⁻⁶ M)</td>
</tr>
<tr>
<td>μm.pixel⁻¹</td>
<td>Micro-Metre per Pixel</td>
</tr>
</tbody>
</table>
Chapter 1: Literature Review

1.1. Overview

“...there are currently 38 million blind persons in the world, and some 16 million are blind due to un-operated cataract...more than one billion people will be over 60 years old by 2025 and, as populations age, the burden of chronic diseases will increase.”

World Health Organisation statement- GENEVA, 6th September 2004

The ancient Greeks and Romans believed that the lens was the part of the eye responsible for the function of seeing. They thought that the lens was located in the centre of the globe and theorised that the optic nerves were hollow channels through which “visual spirits” travelled from the brain to meet visual lights from the outside world at the lens. The visual information would then flow back to the brain. The true position of the ocular lens was not illustrated until the 1600s by the Italian anatomist Fabricius ab Aquapendente, while the Swiss Physician Felix Plater was the first to hypothesise that the part of the eye responsible for visual perception was the retina and not the lens (Lovicu and Robinson, 2004, Zorab et al., 2006).

This realisation however, has not diminished the importance of the lens, since loss of lens transparency or cataract is the leading cause of blindness in the world today, affecting some 17.6 million people worldwide (Foster and Resnikoff, 2005). This is an alarming statistic since surgery to replace the cataractous lens with an artificial lens is an effective but costly procedure with relatively low risk (Baltussen et al., 2004). The incidence of senile cataract is increasing due to a growing elderly population (Foster, 2001). Hence the financial burden associated with the surgical treatment of cataract is escalating (Laidlaw and Harrad, 1993) and the demand for surgery outstrips available resources, especially in third world countries (Yorston, 2005). Alternative medical therapies to prevent or delay the onset of cataract and therefore reduce the need for surgical interventions are urgently needed. But first, a better understanding of how lens transparency is maintained in the normal lens is required. This thesis investigates the role of intercellular communication pathways in the maintenance of lens transparency and represents my contribution to our growing understanding of lens structure and function.
1.2. The Lens: A Component of the Optical Pathway

Vision is the most important primary sensory systems in humans. Without sight, our perception of the world would be very different. The eye transduces visual stimuli in the form of light into electrical signals that are interpreted by the brain. There are a number of tissues in the eye serving different functions to ensure the optimal light collection and formation of a focused image. This optical pathway consists of the cornea, aqueous humour, lens, vitreous humour and retina (Error! Reference source not found.).

Figure 1.1  Adapted schematic diagram showing the major tissues of the anterior eye (Zorab et al., 2006).  A: Sclera; B: Iris; C: Pupil; D: Cornea; E: Lens; F: Zonules. The light enters the eye via the cornea, passes through the pupil and the lens and is ultimately projected onto retina which contains the photoreceptors that transduce the light into an electrical signal.
The cornea and lens both function to focus the light onto the retina. The cornea acts as the primary refractive element, whereas the lens acts as a fine-focussing element (Fekrat and Weizer, 2006). This fine-focussing function of the lens is called accommodation and is achieved through the contraction and relaxation of the ciliary muscles. Encapsulating the lens is an elastic collagen capsule that is capable of changing lens shape during accommodation (Steinert et al., 2010). The outermost layer of the capsule (zonular lamella) acts as an anchor point for zonular fibres anteriorly and posteriorly to the equator of the lens (Error! Reference source not found.) (Zorab et al., 2006, Steinert et al., 2010). Zonular fibres arise from the basement membrane of the non-pigmented epithelium of the ciliary body and impose tension on the lens capsule. During accommodation the ciliary muscles contract, reducing radial tension on the anterior and posterior zonular fibres. Decreasing the tension on radial zonular fibres causes the lens to become more spherical and capable of near focussing (Danysh and Duncan, 2009, Steinert et al., 2010).

In the non-accommodative state, the lens contributes approximately 15-20 dioptres (D) of the total 60 D of convergent refractive power of the human eye. The remaining is contributed by the cornea. The lens grows continuously throughout life and with age the relative thickness of the cortex increases. At the same time the lens adopts an increasingly curved shape in the periphery, which increases the refractive power and decreases the refractive index gradient in the lens centre, causing a steepening of the refractive index profile in the lens periphery (Jones et al., 2005, Zorab et al., 2006). The changes in refractive index over time result in a reduction in the lens focal distance.
1.3. Lens Structure

Named after the genus of the *Fabaceae* family, the ocular lens is a unique biconvex organ that is located in the anterior segment of the eye. The layered lens cell structure can be likened to an onion, encapsulated by an elastic and transparent capsule which helps maintain lens shape (Error! Reference source not found.) (Mathias and Rae, 1985). The adult lens lacks a blood supply, and therefore depends solely on the aqueous humour for its metabolic supply and to carry off its wastes (Zorab et al., 2006). The lens grows throughout life and the cells neither die nor are shed. The size of the lens changes in a complex manner as the lens grows (Le and Musil, 2001).

![Figure 1.2](image.png)

**Figure 1.2** Schematic diagrams of lens structure (adapted from (Suzuki, 2010)). *A:* Axial view, the lens looks like an onion, composed of many layers of long fibre cells on top of one another. *B:* Equatorial section shows hexagonal fibre cells neatly arranged in columns.

New cells are regularly added to the lens as the time goes by, with the cells in the centre being as old as the individual (Bassnett, 1994, Shestopalov and Bassnett, 2000a, Bassnett and Winzenburger, 2003) (Error! Reference source not found.). The cortical fibre cell membrane domain can be divided into the broad and narrow sides. The two longer sides of the hexagonal cell are termed broadsides, whereas the four shorter sides of the hexagonal cell are called
narrow sides (Error! Reference source not found.). Fibre cells eventually lose all light-scattering organelles rendering the cells incapable of cellular division. Since these anucleate cells are by definition no longer "cells", they are referred to as fibres (Kuszak et al., 1984). The human lens is almost perfectly spherical in early foetal life (Malhotra, 2008). But by birth the sagittal profile is ellipsoidal as equatorial growth outstrips the growth in the sagittal plane, with the circumference of the lens being the greatest at its equator (Zorab et al., 2006).

The adult human lens is a biconvex structure, i.e. it is wider in diameter than it is thickness (anterior-posteriorly) (Steinert et al., 2010). Growth rate is not uniform throughout the human lifespan, and appears to be at maximal in foetal life. During foetal period, the total lens mass increases by approximately 180 mg/year late in gestation (lens mass is 90 mg at birth), but the growth rate decreases significantly after birth and is 1.3 mg/year between 10 and 90 years of age (Augusteyn, 2007). The estimated average lens density suggests that protein content must
remains relatively constant at around 33% of the wet weight over this age span (Malhotra, 2008). In the rat lens, growth rate is maximal between birth and postnatal day 26 day, and the wet weight increases by a factor of 23 (Brewitt and Clark, 1988). The growth rate is also not uniform and decreases with age (Donaldson and King, 1937). This process of embryonic development and fibre cell differentiation is reviewed below.

### 1.3.1. Embryonic Development

The development of the human lens is initiated around day 25 of gestation during early embryogenesis, when two lateral optic vesicles are formed from the diencephalon. A single layer of cuboidal cells are formed through vesicle enlargement opposite to the surface ectoderm. At approximately 27 days of gestation (9.5 days for mouse), the ectodermal cells thicken and become more columnar in shape, forming the lens placode (Error! Reference source not found. B). At about 29 days of gestation (10 days for mouse), the lens pit appears inferior to the centre of the lens placode and deepens through processes of cellular multiplication and invagination (Error! Reference source not found. C). Eventually the cells that connect the lens pit to the surface ectoderm constrict and disappear, leaving a hollow ball-like structure consisting of a single layer of cuboidal cells encapsulated within a basement membrane (lens capsule) that is referred to as the lens vesicle (Error! Reference source not found. D). At about 35 days of gestation (12 days for mouse), while the lens vesicle is forming, the optic vesicle invaginates forming the two-layered optic cup (Error! Reference source not found. E)

During early embryogenesis, the posterior epithelial cells of the lens vesicle elongate and fill the cavity of the lens vesicle (Error! Reference source not found. F). These primary fibre cells form the embryonic nucleus and are located in the core of the adult lens. The remaining epithelial cells covering the anterior surface of the lens vesicle remain unchanged and are now referred to as the mono-cuboidal epithelial cell layer (Zorab et al., 2006). The subsequent addition of fibre cells to the lens mass comes from cells that originate from the equatorial epithelium and are called the secondary fibre cells. They are laid down between 2 and 8 months of gestation in the human lens, and later constitute the fetal nucleus observed in the adult lens (Dahm, 2000). The lens capsule, which originally develops as a basement membrane, is maintained throughout life by the lens epithelium anteriorly, and by lens fibres posteriorly (Zorab et al., 2006).
The secondary lens fibres are formed through multiplication and elongation of the equatorial epithelium. The anterior aspect of the developing secondary lens fibre cells grows beneath the lens epithelium towards the anterior pole, while the posterior aspect grows towards the posterior pole underneath the lens capsule. Through this manner of multiplication and elongation, new lens fibre cells are continuously formed, new layer upon new layer (Zorab et al., 2006).

Figure 1.4 Embryonic lens development (adapted from (Lovicu and McAvoy, 2005)). A-F represents the different developmental stages of a rodent eye. A: Optic vesicle (blue) and surface ectoderm (yellow). B: The enlargement of the ectodermal cells forming the lens placode. C: Invagination of the lens pit and optic vesicles forming the lens vesicle and two-layered optic cup respectively. D: Closure of the lens pit and formation of the lens vesicle. E: The lens vesicle detaches and half of the cells (posterior half) elongate to form the primary fibre cells. F: Elongation of primary fibre cells (pf), filling up the lumen of the lens vesicle and making contact with the epithelium (ep).
1.3.2. **Fibre Cell Differentiation**

The lens cells are unique in that they are all derived from the same cell type, the mitotic epithelial cells of surface ectodermal origin (Kuszak et al., 1984). In the adult lens these epithelial cells consist of a single layer of cuboidal cells, located directly beneath the anterior lens capsule (Error! Reference source not found.), with their basal membrane facing the aqueous humour (Mathias and Rae, 1985). At the lens equator these epithelial cells proliferate and divide to give rise to differentiating secondary fibre cells (Menko, 2002). The process of fibre cell differentiation and elongation produces an inherent age gradient comprised of at least three distinct zones within the lens: an anterior epithelium layer, a cortex of Differentiating Fibre (DF) cells and a core of anucleate Mature Fibre (MF) cells (Error! Reference source not found.) (Mathias and Rae, 2004). The series of fibres are interlocked by a number of ball-and-socket-like membrane structures that minimise the extracellular space between each of the lens fibres (TenBroek et al., 1992, Blankenship et al., 2007).

The differentiating fibre cells at the equator elongate until the anterior and posterior ends of fibre cells from adjacent lens hemispheres meet, forming the anterior and posterior sutures, respectively. The interlocking of the ends of fibre cells at the sutures forms a growth shell or ring (Kuszak et al., 2004). In rodent lenses, these growth rings form an arrangement of inter-digitated Y-shaped sutures at both poles (Error! Reference source not found. C) that run through from the surface to the centre of the lens (Kuszak et al., 2006). While this Y-shaped suture pattern is repeated at both poles, the suture is twisted 180° to yield an inverted Y-pattern relative to the opposite poles.

Suture shape and structure is species-dependent (Error! Reference source not found.). Avian and reptilian sutures are formed by direct and opposing meridian-like fibre cells creating an ‘umbilical-like’ suture (Error! Reference source not found. A). Rabbits and amphibians have S-shaped fibre cells, creating a ‘line’ suture (Error! Reference source not found. B) (Kuszak et al., 2004). Rodent, feline, canine, porcine, ovine and bovine lenses are also formed from S-shaped fibre cells, forming a ‘Y-shaped’ suture (Error! Reference source not found. C) (Kuszak et al., 2004, Kuszak et al., 2006). In humans, the suture structure is ‘star-like’ and changes with age (Kuszak et al., 2004, Kuszak et al., 2006). In the young infantile lens, a ‘simple star-like’ suture (Error! Reference source not found. D) progressively changes into a ‘star-like’ (Error! Reference source not found. E) and then ‘complex-star-like’ (Error! Reference source not found. F).
suture in adolescent and adult human lenses, respectively (Kuszak et al., 2004). Suture structure has been implied to influence the ability of the lens to accommodate. Avian and reptilian lenses have sutures without branching, and they have powerful and sharp accommodative mechanisms that allow them to focus on near objects. In contrast, vertebrate lens accommodation is less powerful and more smooth (Kuszak et al., 2004). The difference could potentially be directly attributed to the shape and arrangement of the sutures.

Figure 1.5 Adapted schematic diagram showing the different fibre cell arrangement and suture structures in different species (Kuszak et al., 2004). A: Avian and reptilian lens. B: Rabbit and amphibian lens. C: Rodent, feline, canine, porcine, ovine and bovine lens. D: Infantile human lens. E: Adolescent human lens. F: Adult human lens.

The process of fibre cell elongation is accompanied by the degradation of all cytoplasmic organelles such as the nuclei, endoplasmic reticulum, Golgi and mitochondria in a process that resembles apoptosis (Dahm, 2000, Beebe et al., 2001, Menko, 2002, Bassnett and Winzenburger, 2003, Mathias and Rae, 2004). The loss of cytoplasmic organelles is necessary to maintain lens transparency, as these organelles differ in refractive indices (Error! Reference source not found.) and would cause light-scattering and in turn reduce overall optical clarity if they remain in the path of light (Mathias, 1985, Mathias and Rae, 1985). DF cells containing cellular
organelles are overlain by the iris, masking the light-scattering issue in this region (Bassnett, 2002).
Table 1.1: Adapted table showing the refractive index of cytoplasmic organelles (Bassnett, 2009).

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Refractive index</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lens extracellular fluid</td>
<td>1.35-1.36</td>
<td>Beauvoit et al., 1994</td>
</tr>
<tr>
<td>Lens cytoplasm</td>
<td>1.37</td>
<td>Brunsting and Mullaney, 1974</td>
</tr>
<tr>
<td>Nucleus</td>
<td>1.39</td>
<td>Brunsting and Mullaney, 1974</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.40</td>
<td>Wilson et al, 2007</td>
</tr>
<tr>
<td>Lysosome</td>
<td>1.60</td>
<td>Wilson and Foster, 1997</td>
</tr>
<tr>
<td>Lens membranes</td>
<td>1.42-1.47</td>
<td>Michael et al, 2003</td>
</tr>
<tr>
<td>Cornea</td>
<td>1.38</td>
<td>Ott, 2006</td>
</tr>
<tr>
<td>Aqueous humour</td>
<td>1.34</td>
<td>Ott, 2006</td>
</tr>
</tbody>
</table>

The extent of light-scattering by organelles depends on the degree of difference in the refractive index between the organelle and the surrounding environment (Bassnett, 2009). The fibre cell protein concentration is extremely high, with an index of refraction of approximately 1.4 which is significantly greater than that of the surrounding fluids (~1.34), enabling the lens to refract incident light.

While being optically advantageous, the loss of organelles means these MF cells are incapable of cell division and de novo protein synthesis, and also lack the metabolic functions previously carried out by the organelles. These MF cells contain very old protein that under normal circumstances, would be susceptible to denaturation with the potential to form protein aggregates and cause light-scattering (Mathias et al., 1997). This is not normally observed in the lens since the presence of α-crystallin in high concentration, which acts as a molecular chaperone capable of assisting refolding denatured lens proteins, prevents their aggregation (Horwitz, 1992, Rao et al., 1995). Indeed, lenses with disrupted α-crystallin genes develop a wide range of cataract phenotypes (Brady et al., 1997).

During differentiation, the expression of lens specific proteins such as the crystallins together with the loss of cellular organelles, creates concentric layers of fibre cells of increasingly advanced stages of differentiation from the periphery to the centre of the lens (Dahm, 2000). The crystallin concentration is the highest in the lens core, and gradually decreases by about 50% towards the surface (Slingsby and Miller, 1983). This produces a radial gradient of lens refractive indices, aiding lens function by correcting longitudinal spherical aberration (Artal and Guirao, 1998, Smith et al., 2001).
1.3.3. **Lens Function**

The primary function of the crystalline lens is to transmit incident light and focus that light onto the retina (Malhotra, 2008). This requires that the lens minimises light-scattering to promote transparency and generates a refractive index gradient to optimise its optical properties (Mathias et al., 1997). To avoid light-scattering, the lens lacks blood vessels or nerves, has an extracellular space smaller than the wavelength of light, loses light-scattering organelles in fibre cells along the visual axis (Error! Reference source not found.), and adopts a regular highly organised cellular architecture (Error! Reference source not found.).

In terms of its optical properties, the ocular lens is a convex optical element with a positive refractive power. Converging light rays pass through it onto a proximal focal point. If the lens had a uniform refractive index across its axis, not all light rays would converge on the same focal point, but would instead refract marginal rays more than the principal rays. This inherent spherical aberration needs to be corrected if a clear image is to be projected on the retina. To achieve such correction the lens generates a gradual and small incremental refractive index gradient from the periphery towards the core. In order to generate a refractive index high enough to refract light onto the retina, high concentrations of lens proteins, metabolites, ions and water need to be arranged and distributed amongst lens fibre cells in a highly sophisticated manner (Delaye and Tardieu, 1983, Jones et al., 2005). It is believed that this refractive index gradient is helped established by the over expression of crystallins, the water soluble proteins which make up 80% of total proteins in the lens (Derham and Harding, 1999, Jones and Pope, 2004). With advancing age, there is a gradual reduction in the solubility of lens crystallins which affects the refractive index and therefore the optical properties of the lens (Bloemendal et al., 2004).

Furthermore, the regular cellular architecture of the lens must be maintained since any disruption to the arrangement of fibre cell structure leads to lens opacification (Wolfe et al., 1985). A very small reduction in intracellular volume will expand the extracellular spaces between the lens fibres and cause the lens to become opaque (Mathias and Rae, 2004), hence the lens is not just a passive optical element. In order to preserve not only its overall transparency and also its optical properties, the lens needs to actively deliver nutrients, and control fluid and electrolyte balance in order to maintain crystallin solubility and cellular architecture, respectively (Zorab et al., 2006, Donaldson et al., 2009). Any perturbation of lens
homeostasis will therefore be manifested as changes in cellular hydration, and disturbance to the lens structure and macro-molecular organisation, and will eventually lead to cataract formation (Zorab et al., 2006).

Being avascular in nature, the ocular lens requires an unconventional system to deliver nutrients and remove wastes. Past studies have suggested an active transport system located in the epithelial cell layer accumulates metabolites from the aqueous humour which then move by simple diffusion into the underlying fibre cells through a network of Gap Junctions (GJ) (Goodenough, 1992). However, due to the relatively large size of the lens, passive diffusion alone is thought to be insufficient to deliver sufficient nutrients to MF cells (Baldo and Mathias, 1992). Instead it has been proposed that the lens operates an internal microcirculation system that delivers nutrients and, removes metabolic wastes faster than would occur via passive diffusion, while controlling the ionic homeostasis of the lens. This microcirculation system is believed to be central to the maintenance of the transparent and optical properties of the lens and is reviewed below.
1.3.4. Microcirculation System

It has been proposed that the lens operates a unique internal microcirculatory system to deliver nutrients, remove waste and maintain lens volume (Mathias et al., 1997, Donaldson et al., 2001, Mathias and Rae, 2004, Mathias et al., 2007). While this system is not universally accepted (Beebe and Truscott, 2010), evidence in its favour is accumulating (Donaldson et al., 2010). It is believed, that spatially distinct distributions of ion channels and transporters (Error! Reference source not found.) generate a circulating current that in turn, drives a fluid flux which delivers nutrients to the centre of the lens faster than would be predicted by passive diffusion alone. Briefly, the microcirculation system is primarily generated by a circulating flux of sodium ions (Na⁺), which is directed into the lens at the poles via the extracellular space. In the deeper lens Na⁺ diffuses across MF cell membranes and then flows towards the surface by an intercellular pathway mediated by GJ channels (Error! Reference source not found.). These GJs direct the Na⁺ fluxes towards the lens periphery where the Na⁺ is actively removed from the lens by sodium pumps (Na⁺-K⁺-ATPase) which are concentrated at the lens equator (Baldo and Mathias, 1992, Gao et al., 2000, Donaldson et al., 2001, Le and Musil, 2001, Candia and Zamudio, 2002, Tamiya et al., 2003, Mathias and Rae, 2004, Webb, 2004). In addition to sodium-pumps, these surface cells also contain potassium channels which by virtue of their connection to the underlying fibre cells set a negative resting membrane potential in the lens that is critical for the maintenance of lens volume (Baldo and Mathias, 1992, Donaldson et al., 2001, Mathias and Rae, 2004).

This circulating flux of Na⁺ has been proposed to drive a fluid flow that convects nutrients into the lens via the extracellular space, where they are taken up into DF and MF cells by a variety of amino acid and glucose transporters (Error! Reference source not found.) (Donaldson and Lim, 2008). While the circulating ionic fluxes have been measured experimentally (Donaldson et al., 2010), the fluid fluxes are theoretical and have yet to be measured or visualised directly. To address this knowledge gap, techniques that can visualise circulating fluxes in both the intercellular and extracellular compartments are required.
Figure 1.6  Spatial differences in the location of ion channels and transporters generate the lens circulation system. Schematic diagram showing the spatial locations of transport proteins in different regions of the lens (Donaldson et al., 2001). The inner fibre cells have an influx of Na⁺ and water (H₂O) which travels towards the surface cells through intercellular pathways mediated by GJ channels.
1.4. **Intercellular Communication**

In the current view of the lens circulation system, GJs mediated intercellular communication plays a central role. However, earlier electrophysiological and morphological studies suggested that the lens could be considered as one big cell. Initially, the finding of a uniform resting membrane potential across the lens was interpreted as degeneration of inner fibre cell membranes (Duncan, 1969), a view that was supported by electron microscope images that showed membrane fusions between fibre cells in the lens core (Kuszak et al., 1985). This was subsequently refuted by Mathias et al. who used signal-frequency domain impedance measurements to show that membrane resistance is very high in the lens, with the membrane resistance of inner MF cells being about three-fold higher than in DF cells (Rae et al., 1970, Rae, 1973, Eisenberg and Rae, 1976, Mathias et al., 1997). Instead, the uniform membrane voltage observed in the lens is due to an extensive network of GJ and not due to the degeneration of inner fibre cell membranes (Mathias et al., 1997). The recorded uniform voltage does not mean that all fibre cells are the same, but rather the cells are interconnected and the uniform voltage reflects the integrated properties of all of the cells, not just a single cell (Rae and Blankenship, 1973).

Indeed the lens is an abundant source of GJs, and consequently lens GJ have been extensively studied over the ensuing years due to their importance to overall lens structure and function (Goodenough, 1979, Goodenough, 1992, Goodenough et al., 1996). Against this background of intense study into lens GJ, the role of membrane fusion in lens function was virtually ignored. More recent studies by Bassnett et al. (Shestopalov and Bassnett, 2000a, Shestopalov and Bassnett, 2003, Shi et al., 2009) have refocused attention to the presence and role of membrane fusions in the lens. In a series of experiments designed to characterise Green-Fluorescent-Protein (GFP) expression in the lens, Bassnett et al. showed that in certain regions of the lens the large GFP (26.9 kDa) molecule could freely diffuse between neighbouring fibre cells. Thus it would appear that a low resistance Macro-molecular Diffusion Pathway (MDP) that links the cytoplasm of fibre cells exists in parallel with the higher resistance GJ mediated intercellular communication pathway in the lens. However, in the absence of methods with high temporal and spatial resolution, it is difficult to determine the relative contributions of these two pathways to overall intercellular communication in the lens. The contributions of these two pathways to the overall lens function are discussed below.
1.4.1. *Gap Junction Mediated Intercellular Communication in the Lens*

GJs are cell-to-cell channels that connect the cytoplasms of two adjacent cells (Kumar and Gilula, 1996, Shakespeare et al., 2009). By permitting the direct exchange of small metabolites ($M_r \leq 1.2$ kDa), ions, and second messengers between communicating cells, GJs play diverse roles in cellular signalling and growth regulation (Jiang and Goodenough, 1996, Kumar and Gilula, 1996). A complete, functional GJ is formed by two hemi-channels or connexons. Each cell supplies a connexon which dock together across the extracellular space to form a complete cell-to-cell channel *Error! Reference source not found.* (Kumar and Gilula, 1996, Shakespeare et al., 2009). GJ plaques are formed by a cluster or aggregate of multiple GJ channels in the plane of the membrane (Kumar and Gilula, 1996).

![Figure 1.7 Molecular structure of GJ channels.](image)

GJ channels can be made of identical connexons (homotypic) or two different connexons (heterotypic) *Error! Reference source not found.*. Connexons are in turn comprised of 6 connexin subunits, of which 20 different isoforms have been identified in humans (Jiang and Goodenough, 1996, Willecke et al., 2002, Prochnow and Dermietzel, 2008), while only 19 different isoforms have been identified in the mouse genome (Willecke et al., 2002). A connexon can be composed of either one (homomeric) or multiple (heteromeric) connexin isoforms *Error! Reference source not found.* (Kumar and Gilula, 1996). GJ properties are defined by their constituent connexin proteins. Each connexin consists of four transmembrane (M1-M4), two extracellular (E1 and E2) and three intracellular domains *Error! Reference source not found.*
not found.) (Kumar and Gilula, 1996, Willecke et al., 2002). The N- and C- termini represent two of the three intracellular domains. The two extracellular loops, E1 and E2, have been suggested to initiate connexon-connexon interaction or docking between neighbouring cells (Kumar and Gilula, 1996).

Figure 1.8 Connexin protein membrane topology. A connexin is comprised of four transmembrane (M1-M4) domains, two extracellular (E1 and E2) domains and three intracellular domains. The C- and N-termini both face the cytoplasmic side of the cell. Figure adapted from (Kumar and Gilula, 1996).

Three members of the connexin family, with different gating properties have been identified with distinct spatial and temporal expression patterns in the vertebrate lens; Cx43 (α1), Cx46 (α3) and Cx50 (α8) that are encoded by the Gja1, Gja3 and Gja8 genes, respectively (Goodenough, 1992). Together, these connexins provide a sophisticated regulatory network that coordinates lens growth and maintains lens transparency. Cx43 and Cx50 are expressed in the epithelium, while Cx46 and Cx50 are expressed at very high levels in fibre cells (Error! Reference source not found.) (Le and Musil, 2001, Shakespeare et al., 2009). Cx43 and Cx50 are located on the anterior surface between the epithelial cells. During differentiation from an epithelial cell into a fibre cell, Cx43 is dramatically down regulated, while Cx46 and Cx50 are up regulated (Musil et al., 1990, White et al., 1992, Gong et al., 1997). Through immunohistochemical staining with confocal scanning LASER microscopy (Gong et al., 1997), and immuno-gold labelling with electron microscopy (Benedetti et al., 2000), Cx46 and Cx50 proteins have been shown to be co-localise in the same junctional plaque, and Cx46 and Cx50 have been
suggested to form heteromeric connexons in lens fibre cells (Jiang and Goodenough, 1996). In the DF cells, Cx46 and Cx50 plaques are localised on the broadsides, but become more evenly dispersed around the plasma membrane in MF cells (Kistler, 1985, Jacobs et al., 2004). The lens connexins also undergo a differentiation-dependent truncation of their C-terminus at the DF/MF transition (Voorter et al., 1989, Kistler et al., 1990, Lin et al., 1997).

Figure 1.9  **Expression pattern of connexins in the lens.** Cx43 is located only in the epithelial cells. Cx46 and Cx50 are expressed in both differentiating and mature fibre cells with Cx50 also expressed in the epithelial cells and not functional in the mature fibres adapted from (Shakespeare et al., 2009).

A variety of transgenic mouse lines have been generated and interbred to investigate the individual roles that Cx46 and Cx50 play in the maintenance of lens transparency in vivo (Error! Reference source not found.). The ocular phenotypes of the homozygous Cx50 Knock-Out (KO) (Cx50/−) and Cx46KO (Cx46/−) mice generated through targeted gene disruption are distinct (Error! Reference source not found. B & C), indicating the two connexins have non-identical roles in lens homeostasis. Although Cx46 and Cx50 are independently regulated, when either connxin was knocked-out there was no up-regulation of the other connxin protein (Gong et al., 1998, Rong et al., 2002).
Cx50KO hindered lens growth which produced microphthalmia (Error! Reference source not found. and Error! Reference source not found. B) and abnormal lens fibre maturation (White et al., 1998, Rong et al., 2002, Gao et al., 2004), caused by a higher than normal membrane voltage affecting early lens development was also observed (Baldo et al., 2001). In contrast, knocking out Cx46 produced a normal sized lens with no apparent effect on lens growth but in the absence of Cx46 the lens developed a nuclear cataract (Error! Reference source not found. C) (Gong et al., 1998). With increasing age the nuclear cataract expands but the outer cortex maintains its transparency (Error! Reference source not found. C) suggested that intercellular communication is still maintained in the outer cortex, presumably by Cx50. Double KO of Cx46\textsuperscript{-/-} and Cx50\textsuperscript{-/-} exhibited a severe nuclear cataract with degenerated inner fibres and reduced lens size (Error! Reference source not found. D) (Xia et al., 2006). Knocked-in (KI) of Cx46 (Cx46\textsuperscript{+/+} \text{Cx50}\textsuperscript{ki/ki} or Cx46\textsuperscript{-/-} \text{Cx50}\textsuperscript{ki/ki}) produced a clear but smaller lens than the wild-type mouse lens, which highlights the importance of Cx46 in lens transparency but not lens growth (Gong et al., 2007).

![Genetic modification of gap junctional mediated intercellular communication in the mouse lens](image)

**Summary of the observed lens genotype, phenotype and conductance in the different KO and KI mice lenses.** Figure adapted from (Baldo et al., 2001, Martinez-Wittinghan et al., 2004, Gong et al., 2007).
To further characterise the individual contributions that Cx46 and Cx50 make to overall lens function, impedance analysis to calculate GJ conductance was applied to Cx50KO (Baldo et al., 2001) and Cx46KO (Gong et al., 1998) lenses. These experiments showed that coupling conductance in both transgenic animals was reduced by ~50% relative to the wild-type mouse lens, a result that suggesting both connexins make an equal contribution to intercellular communication between DF in the mouse lens (Error! Reference source not found.). In contrast, in the Cx50KO there was approximately an 80% reduction in coupling conductance in the MF, while the coupling conductance was abolished completely in Cx46KO (Error! Reference source not found.), indicating that Cx46 is the sole GJ channel that mediates intercellular communication in the lens core.
The role for Cx46 as the sole functional GJ channel in the core of the lens was confirmed by measurements of GJ conductance in Cx46KO lenses. In these lenses the ‘knocking’ of Cx46 into the Cx50 locus doubled not only the amount of Cx46 expressed in the lens centre but also GJ coupling in this region of the lens confirming that Cx46 mediates GJ intercellular communication in the lens core (Gao et al., 2004). Through various gap junctional KO and KI transgenic models, the importance of various GJ isoforms in mediating intercellular communication has been studied extensively. In contrast, the more recently discovered MDP and its role as an alternative intercellular communication pathway has not been as extensively studied. The identification of this pathway is reviewed below.
1.4.2. **Macro-molecular Diffusion Pathway**

Originally designed to visualise the three-dimensional structure of the primary lens fibre cells in the chick lens using GFP expression vector (Shestopalov and Bassnett, 2000b), the experiment unexpectedly also revealed a novel intercellular pathway that exists in parallel to that of the existing intercellular pathway mediated by GJ channels (Shestopalov and Bassnett, 2000a). Later, the group applied the same technique in a mouse lens expressing TgN(GFPU)5Nagy (Shestopalov and Bassnett, 2003, Shi et al., 2009). The expression of this large GFP molecule was initially isolated to distinct clusters of DF cells in the outer cortex of the lens. However, once the fibre cells reached a specific stage of fibre cell differentiation, GFP molecules uniformly spread amongst all fibre cells, indicating that fibre cells had formed a syncytium that allowed for the diffusion of large macro-molecules between adjacent fibre cells (*Error! Reference source not found.*). The existence of this MDP was further tested with microinjection of 10 kDa-Dextran into cortical fibre cells at different depths from the lens surface (Shestopalov and Bassnett, 2003) and into anucleated MF cells in the centre (Shi et al., 2009). The movement of the 10 kDa-Dextran dye was restricted in the peripheral nucleated differentiating fibre cells, where the mosaic expression pattern of GFP expression was evident, but the injected dye was able to spread to neighbouring cells in zones in which the MDP was deemed to have developed (*Error! Reference source not found.*).

![Identification of the macro-molecular diffusion pathway in the mouse lens.](image)

Figure 1.12  Identification of the macro-molecular diffusion pathway in the mouse lens. Equatorial image taken from a transgenic lens that expresses GFP and was micro-injected with 10 kDa Texas-Red-Dextran (red). GFP expression (green) is restricted to clusters of DF cells in the lens periphery but in the deeper lens a uniform spread of GFP to all cells occurred which signalled the formation of the MDP. Texas-Red-Dextran was injected at different depths and its subsequent spread monitored over the course of the next 10 hours. The spread of Texas-Red-Dextran only occurred in regions of uniform GFP expression that corresponded to the formation of the MDP. Scale bar = 25 μm. Adapted from (Shestopalov and Bassnett, 2003).

The origin and molecular mechanisms for the formation of this MDP are currently a mystery. Some have suggested the MDP formation occurs as a direct result of cell-to-cell fusion, but the
trigger for onset of these fusions remains unknown. To address this issue Bassnett et al. crossed the transgenic mouse expressing GFP with another transgenic mouse lacking the specific gene, *Lim2*, which encodes the Membrane Protein that has a molecular weight of 20 kDa (MP20). The lenses of the resultant F1 progeny exhibited no uniform spread of GFP in the inner lens (Error! Reference source not found.), but rather the mosaic pattern of GFP expression was retained throughout the whole lens, indicating that in the absence of MP20 protein the MDP failed to form (Shi et al., 2009).

![Figure 1.13](image)

**Figure 1.13 Involvement of MP20 protein in the formation of the MDP.** *Left panel* showing half of a wild-type mouse lens, the white arrow indicates the formation of the MDP (homogenous GFP expression pattern) approximately 100-200 μm in from the periphery. *Right panel* showing half of a *Lim2*KO mouse lens in which no MDP is formed since the mosaic expression pattern of GFP is observed throughout the whole lens. Scale bar: 250 μm. Figure adapted from (Shi et al., 2009).

MP20 is the second most abundant integral membrane protein in the vertebrate lens (Louis et al., 1985, Johnson, 1986, Mulders et al., 1988, Gonen et al., 2001, Grey et al., 2003) and it is a member of the PMP22/EMP gene family, a subfamily of the tetraspanins, a superfamily of integral membrane proteins (Van Itallie and Anderson, 2006, Cheng et al., 2008). Tetraspanins
have been implicated in different cellular processes including activation, proliferation, differentiation, migration, adhesion, and apoptosis (Gonen et al., 2001, Van Itallie and Anderson, 2006).

In the rat lens, MP20 protein undergoes a differentiation-dependent shift in its subcellular localisation from a cytoplasm vesicular storage pool to the plasma membrane, and this membrane insertion of MP20 protein in the rat lens occurs at the DF to MF transition where cell nuclei are lost (Error! Reference source not found.). This MP20 membrane insertion appears to coincide with the restriction of tracer molecule diffusion through the extracellular space (Grey et al., 2003). Since MP20 protein has also been shown to bind to the adhesion modulator Galectin-3 (Gonen et al., 2001), and both proteins move from a cytoplasmic location to a membrane location upon the loss of cell nuclei in the rat lens, it suggests that the MP20-Galectin-3 protein complex plays a role in fibre cell adhesion by modulating the size of the extracellular space in the rat lens to create a barrier to extracellular space diffusion (Grey et al., 2003). Thus by combining the data on the differentiation-dependent insertion of MP20 obtained in the rat (Grey et al., 2003) with the findings implicating MP20 protein in the formation of the MDP in transgenic mice (Shi et al., 2011), it is possible to speculate that the membrane insertion of MP20 protein triggers the formation of the MDP.
Differentiation-dependent membrane insertion of MP20 protein in rat lens coincides with the loss of cell nuclei. A: An overview of the MP20 protein expression pattern showing the boxes where the high power images shown in b-d are taken. B: In DF cells with cell nuclei MP20 is located in the cytoplasm. C: At the DF/MF transition where nuclei are lost, MP20 protein inserts into the membrane. D: In MF cells in the inner cortex the membranous MP20 protein pattern persists. Figure adapted from (Grey et al., 2003).
The precise nature of the MDP has not been identified. Some have suggested the MDP to be a fusion pore, formed through direct cell-to-cell membrane fusion (Shestopalov and Bassnett, 2000a, Shestopalov and Bassnett, 2003). In other cells, membrane fusion involves merging of the lipid bi-layer and formation of cytoplasm continuity, allowing intermixing of water-soluble substances bounded by the membranes (Error! Reference source not found.) (Blumenthal et al., 2003). Membrane fusion is a two part process. First, the membranes need to be brought into close proximity, and secondly the bilayer needs to destabilise (Jahn et al., 2003). The transition states are governed to minimise exposure of non-polar (hydrophobic) surfaces to water. While the fundamental processes involved in fusion appear common to most cellular systems, the specific molecular events are diverse and not well understood (Chen and Olson, 2005). It is interesting to speculate that in the lens the MP20-galectin-protein could act to pull the neighbouring membranes close enough for the fusion to occur (Grey et al., 2009). Interestingly, cell fusion only occurs in parts of the lens at a specific stage of fibre cell differentiation, and the fusion pores formed do not expand indefinitely (Shi et al., 2009).

Figure 1.15 Stages in the formation of a fusion pore between two cells. Figure adapted from (Ogle et al., 2005).

The above brief review of the MP20 protein (see Chapter 4 for a more details review) highlights two potential roles for MP20 in the lens. The membrane insertion of MP20 appears to play a role in the formation of MDP, since lenses lacking MP20 protein did not exhibit a MDP. Since it is likely that the MDP is a fusion pore, MP20 may also play a role in closing up the extracellular space creating the barrier to extracellular space diffusion observed in the rat lens upon MP20 membrane insertion. In this thesis, I propose to investigate the roles played by MP20 in mediating intercellular and extracellular ion and fluid fluxes in the lens by utilising state-of-the-art imaging modalities to characterise the MDP and the extracellular diffusion barrier in the rat lenses and in wild type and transgenic mouse lenses.
1.5. Thesis Objectives

As reviewed above intercellular and extracellular ion and fluid fluxes are critical to the maintenance of lens transparency. There seems to be two parallel intercellular communication pathways in the lens, one mediated by the GJs and the other by the MDP. Hence, a primary goal of my thesis is to investigate whether the differentiation-dependent membrane insertion of MP20 is responsible for the formation of the MPD. To achieve this I will pursue the following specific aims.

Chapter 3: Mapping the Diffusion Pathways in the Rat Lens
- To adapt previous TPEFP technique to distinguish the two parallel intercellular pathways (GJs and MDP) by using two different molecular weight caged-compounds.
- To optimise TPEFP technique for mapping the formation of the MDP to different stages of fibre cell differentiation in the rat.

Chapter 4: MP20 Protein Expression and the MDP in Mouse Lens
- To optimise the different MP20 antibodies in Western Blot and Immunohistochemistry.
- To validate the specificity of the MP20 antibodies in Western Blot and Immunohistochemistry using Lim2KO mouse model.
- To replicate previous MP20 protein Immunohistochemistry findings in rat and apply to the mouse lens.
- To map the formation of MDP as a function of the different stages of fibre cell differentiation in the mouse lens using TPEFP technique.

Chapter 5: Investigating Intercellular Communication in Knock-Out Mice
- To examine the role GJs play in the intercellular communication pathway in Lim2KO model using TPEFP technique.
- To investigate the role of the MDP mediated intercellular communication in Cx46 KO model using TPEFP technique.
A secondary goal is to determine whether MP20 contributes to the formation of a barrier to extracellular diffusion in the lens and to determine whether MP20 insertion initiates a closure of the extracellular space. Specific aims to be pursued include:

Chapter 6: Correlating MP20 Membrane Insertion and the Extracellular Barrier

- To develop a Confocal Reflectance Microscopy technique to investigate the nature of the extracellular barrier in the wild-type mouse lens using Gd-DTPA.
- To determine whether the MP20 protein correlates with the formation of the extracellular barrier Lim2KO lens with dye incubation experiment

By pursuing these two lines of investigation I hope to develop approaches that will enable me to visualise intercellular and extracellular fluxes in different regions of wild type and transgenic lenses. By providing new information on the MDP and by mapping the presence and extent of the extracellular space diffusion barrier I will be able to contribute to our knowledge of lens structure and function facilitating the development of novel anticitaract therapies.
2.1. General Laboratory Procedures

2.1.1. Laboratory Techniques and Safety

Extreme care was taken for all experiments to assure safety. Strict laboratory protocols were followed to ensure all experiments were executed under sterile conditions. Benches and dissection equipments were cleaned with 70% ethanol (EtOH) in MilliQ H₂O prior to commencing an experiment. All glass and plastic-ware were autoclaved at 15 psi for 15 min. Disposable latex gloves and a laboratory coat were worn at all times, gloves were changed frequently. Autoclaved water was used with PCR experiments to avoid any contaminations of foreign DNA.

To avoid eye damage when aligning the two-photon LASER, ambient lights were turned on, infrared-red safety goggles were worn, and at no time were the eyes directly aligned with the LASER. To protect against Ultra-Violet (UV) light exposure, an UV mask and laboratory coat were worn. When handling liquid nitrogen, appropriate gloves and mask wear were used.

All methods reported represent the final optimised experimental protocols.
2.1.2. Chemical Reagents and Experimental Solutions

Analytic grade reagents were obtained from Sigma (Sigma Chemical Company, St. Louis, MO) unless stated otherwise. General solution compositions are outlined in (Table 2.1).

Table 2.1 General solution composition.

<table>
<thead>
<tr>
<th>Artificial Aqueous Humour (AAH)</th>
<th>2.5 mM CaCl₂, 5 mM glucose 5 mM HEPES, 4.7 mM KCl, 149 mM NaCl, pH 7.4, osmolarity = 300 ± 3 mOsm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial Intra-Cellular Solution (AICS)</td>
<td>0.1 mM CaCl₂, 10 mM EGTA, 10 mM glucose, 5 mM HEPES, 130 mM KCl, 1 mM KH₂PO₄, 3 mM MgATP.</td>
</tr>
<tr>
<td>Phosphate Buffered Saline (PBS)</td>
<td>2.7 mM KCl, 10 mM KH₂PO₄, 137 mM NaCl, pH 7.4.</td>
</tr>
<tr>
<td>Tris Buffered Saline (TBS) [10x]</td>
<td>1.4 mM NaCl, 20 mM Tris-HCl, pH 7.4, adjusted with HCl.</td>
</tr>
</tbody>
</table>

The Artificial Aqueous Humour (AAH) and Artificial Intra-Cellular Solution (AICS) mimic the composition of the normal aqueous humour that surrounds the lens and the intracellular environment of the lens, respectively. The pH of AAH and AICS solutions were adjusted to pH 7.4 by adding appropriate amounts of either sodium hydroxide (NaOH) or hydrochloric acid (HCl) using a pH meter (ISFET pH Meter KS701, BAS, Tokyo, Japan). The osmolarity of the solutions were measured using an osmometer (Vapro; Wescor, Utah, USA) and were made up to 300 mOsm.kg⁻¹ with crystalline sucrose if required. AAH and PBS were stored at 4°C and AICS was stored in 1 mL aliquots at -20°C.
2.1.3. Animals

All animals were treated in accordance with the University of Auckland Animal Ethics Committee guidelines (R867) and The Association for Research in Vision and Ophthalmology Statement for the use of animals in ophthalmic and vision research. Animals were supplied by the Vernon Jansen Unit located in the Faculty of Medical and Health Sciences at the University of Auckland.

Wild-type C57Bl/6J mice ranging from 8-10 weeks of age, and 3 weeks old Wistar rats were used and no discrimination was made between male or female animals. Experimental protocols were optimised for use of weaner rats to standardise protocols in accordance to previous publications (Gonen et al., 2001).

C57Bl/6J Cx46KO mice were obtained from Associate-Professor Xiao-Hua Gong (University of California at Berkeley) whose group originally generated these KO mice (Gong et al., 1999). C57Bl/6J-albino/Lim2^Gt/Gt KO mice were obtained from Professor Steve Bassnett (Washington University at Missouri), which were originally generated by Professor Alan Shiels (Shiels et al., 2007). Breeding pairs were imported into New Zealand in accordance with The Ministry of Agriculture and Fishery regulations. Breeding colonies were established and maintained within the VJU in compliance with all appropriate regulatory guidelines.

2.1.4. Lens Dissection

Rats or mice were euthanised by carbon dioxide asphyxiation followed by cervical dislocation. Eyes were extracted shortly post-mortem by pressing scissors downwards on either side of the eye to release it from its socket, followed by cutting of the optic nerve to release the eye. Extracted eyes were then either placed in a dissecting chamber containing AAH (Table 2.1) pre-warmed to 37.5°C, or stored in an incubator until required for dissection. The dissecting chamber consisted of a small Petri dish filled with Sylgard® 184 silicone elastomer (Dow Corning, Midland, MI, USA), into which a small depression was moulded to immobilise the eye and facilitate dissection of the lens.
The extraction of lenses was performed under a variable-power binocular dissecting microscope (Nikon, Tokyo, Japan). On the posterior surface where the optic nerve exits the eyeball, a small pair of dissecting scissors (World Precision Instrument, Sarasota FL, USA) was inserted and the sclera was cut into four flaps exposing the posterior side of the lens (Figure 2.1). Cuts were made away from the optic nerve through the sclera and retina to produce four flaps in the posterior surface of the eye. Using forceps, two opposing flaps were gripped. One flap was held steadily while the other was carefully pulled away from it and down around the anterior surface of the eyeball to separate the lens from the eye. Any remnants of the ciliary body and retina were subsequently removed by dissection using a pair of fine surgical scissors. Lenses were then placed in either Petri dishes containing AAH or M199 for culturing (Section 2.7 Lens Culture), scintillation vials with fixative solution (Table 2.10) for future cryosectioning and immuno-labelling experiments, or placed into a cutting chamber to bisect the lens for functional experiments see (Section 2.6 Two-Photon-Excitation-Flash-Photolysis (TPEFP)).

Figure 2.1  **Lens dissection (adapted from Webb, 2006).**  
A: Side view of a rat eye showing the **Cornea (C)**, the **Lens (L)**, the **Sclera (Sc)** and **Optic Nerve (ON)**.  
B: The posterior view of the lens indicating the incisions to be made that originate from the ON.
2.2. Western Membrane Analysis (Western Blot)

2.2.1. Crude Lens Membrane Preparation

Crude membrane preparations were prepared from either whole or fractionated lenses. To fractionate the lens, it was first de-capsulated to separate the capsule and its attached epithelial cells (Epithelial Fraction). The remaining fibre cells were either processed directly (Total Fibre) or further fractionated using a pair of forceps. The outer layer of fibre cells was easily peeled off to produce an Outer Cortical Fraction. The inner cortical fibre cells (Inner Cortex Fraction) were then removed to reveal a hard mass that corresponded to the core of the lens (Core Fraction). All fractions were placed in a 1.5 mL Eppendorf tube containing 500 μL of homogenising buffer (Table 2.2). Tissues were homogenised in 500 μL of homogenising solution with a series of needles, ranging from 18 to 22 gauges. Typically around 8-10 mouse or rat lenses were pooled to produce these preparations.

<table>
<thead>
<tr>
<th>Table 2.2</th>
<th>Solutions used in crude membrane protein preparation.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Homogenising Solution</strong></td>
<td>5 mM EDTA, 5 mM EGTA, 1 x Protease Inhibitor Cocktail, 5 mM Tris-HCl (pH 8.0).</td>
</tr>
<tr>
<td><strong>Storage Solution</strong></td>
<td>2 mM EDTA, 2 mM EGTA, 100 mM NaCl, 1 x Protease Inhibitor Cocktail, 5 mM Tris-HCl (pH 8.0).</td>
</tr>
<tr>
<td><strong>25x Protease Inhibitor Cocktail</strong></td>
<td>Complete™, EDTA-free (Roche Diagnostics).</td>
</tr>
<tr>
<td><strong>Urea Stripping Solution</strong></td>
<td>5 mM EDTA, 5 mM EGTA, 5 mM Tris-HCl pH 9.5, 4 M Urea.</td>
</tr>
</tbody>
</table>

Crude membrane homogenates were spun at 12,000 rpm for 20 min in an Eppendorf 5402 Bench-top Refrigerated Centrifuge. The water soluble supernatant fraction containing cytoplasmic proteins was carefully removed and placed in a 1.5 mL Eppendorf tube, and stored at -20°C for further processing and analysis. The membrane pellet was re-suspended in approximately 500 μL of storage solution (Table 2.2), and subsequently washed twice more in storage solution with a 20 min spinning cycle in between. Following the last centrifugation step,
the pellet was re-suspended in a final volume of 50-300 μL of storage solution depending on the amount of protein extracted and were then stored at -20°C.

2.2.2. Stripped Membrane Preparations

To further purify membrane proteins, crude membranes were stripped of cytoplasmic and peripheral proteins using a Urea-alkaline stripping protocol (Kistler et al., 1993). Briefly, crude membranes were pelleted, re-suspended in 4 M Urea (Table 2.2) and then centrifuged at 12,000 rpm for 20 min. The resultant pellet was washed three times in 20 mM NaOH with a 20 min centrifugation at 12,000 rpm between each wash. The final pellet was re-suspended in approximately 50-100 μL storage solution at -20°C until required.

2.2.3. Protein Quantification

The concentration of membrane proteins was determined using a Bicinchoninic Acid (BSA) Protein Assay Reagent kit (Pierce biotechnology, Thermo Fisher Scientific, Inc., Rockford, U.S.A) (Table 2.3). A calibration curve was constructed using serially diluted BSA protein standards solutions of final concentration of 2.0 mg.mL⁻¹, 1.5 mg.mL⁻¹, 1.0 mg.mL⁻¹, 0.75 mg.mL⁻¹, 0.5 mg.mL⁻¹, 0.25 mg.mL⁻¹, 0.125 mg.mL⁻¹, 0.025 mg.mL⁻¹ and 0 mg.mL⁻¹ (water only as control).

Table 2.3 Solutions used in lens protein quantification and quality check.

<table>
<thead>
<tr>
<th>Bovine Serum Albumin (BSA) [Pierce, Rockford, IL, USA]</th>
<th>2 mg.mL⁻¹ in 0.9% saline, 0.05% sodium azide.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCA Reagent A [Pierce, Rockford, IL, USA]</td>
<td>Na₂CO₃; NaHCO₃, bicinchoninic acid, sodium tartrate in 0.1 M sodium hydroxide.</td>
</tr>
<tr>
<td>BCA Reagent B [Pierce, Rockford, IL, USA]</td>
<td>4% cupric sulphate.</td>
</tr>
</tbody>
</table>
Protein preparations were diluted (dilution factor 1:1, 1:2) and 10 μL of the diluted protein membrane and 10 μL of each standard solution placed in separate wells of a 96 well Elisa plate (Nunc, Denmark). Duplication of samples was made to account for possible human error and time delay during pipetting. 10 μL of Working Solution (Table 2.3) was added to each individual standard and sample well, and the plate was placed in a 37°C incubator for 30 min. Parafilm was used to cover the plate to minimise solution evaporation that could affect the final protein concentration. Light absorbance at 565 nm was read using a SpectraMax M2 microplate reader (Molecular Devices, MDS, Inc., USA), and the concentration of protein samples were determined.

2.2.4. SDS-PAGE Electrophoresis

Sodium-Dodecyl-Sulphate-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE) was used to size fractionate lens membrane proteins. A 15% poly-acrylamide gel (Table 2.4) was prepared to ensure separation of the small molecular mass MP20 protein (~20 kDa). Approximately 5 mL of separating gel was prepared and loaded into a Mini-PROTEAN® II Electrophoresis Cell Casting Apparatus (Bio-Rad, Hercules, CA, USA containing glass plates (10 x 8 cm) and 0.75 mm spacers (Amersham Life Science, Arlington Heights, IL, USA). A layer of 95% EtOH was added on top to eliminate bubble formation and separating gels left to set at room temperature for 20-30 min. EtOH was gently removed using filter paper.

A 4% stacking gel (Table 2.4) was layered on top of the separating gel and a 15-well comb was inserted and the stacking gels left at room temperature for 20-30 min to set. The Mini-PROTEAN® II system (Bio-Rad, Hercules, CA, USA) was then assembled for electrophoresis and the chamber was filled with 1 x SDS-PAGE buffer (Table 2.4). Once the stacking gel was set, the comb was removed and the gel was rinsed 3 x with 1 x SDS-PAGE running buffer to wash away excess polyacrylamide. Either crude or stripped membrane preparations (6 μg/well) were mixed 1:1 with the loading dye (Table 2.4) and added into the individual wells. 10 μL of BenchMark™ Pre-Stained protein ladder (Invitrogen™) was loaded into a separate well and used as a molecular weight marker. 10 μL of the loading dye was added to vacant lanes to ensure the lanes ran uniformly. Electrophoresis was performed at 170 V (50 mA) for approximately one hour using an Electrophoresis Power Supply (Amersham Pharmacia Biotech, Sweden) or until the loading dye ran off the bottom of the gel.
Table 2.4  Solutions used for SDS-PAGE Electrophoresis.

<table>
<thead>
<tr>
<th>Solution Type</th>
<th>Composition Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>30%/2.67 % Bis-Acrylamide</td>
<td>4.1 M acrylamide, 5.2 mM N,N-bis-methylene-acrylamide in MilliQ H₂O.</td>
</tr>
<tr>
<td>15% Acrylamide Separating Gel</td>
<td>0.1% w/v Ammonium persulphate, 14.1%/1.25% Bis-Acrylamide, 0.1% w/v SDS, 0.13% v/v N,N',N'-Tetramethylethylenediamine (TEMED), 0.4 M Tris-HCl, in MilliQ H₂O.</td>
</tr>
<tr>
<td>4% Acrylamide Stacking Gel</td>
<td>0.1% Ammonium persulphate, 3.9%/0.35% Bis-Acrylamide, 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.2% TEMED in MilliQ H₂O.</td>
</tr>
<tr>
<td>10 x SDS-PAGE Buffer</td>
<td>1.92 M glycine, 0.25 M Tris-HCl, 35 mM Sodium dodecyl phosphate (SDS) in MilliQ H₂O.</td>
</tr>
<tr>
<td>Protein Loading Dye</td>
<td>125 mM Tris (pH 6.8), 4% SDS; 10% v/v Glycerol, 4% v/v Bromophenol Blue, 5% v/v β-mercaptoethanol in MilliQ H₂O.</td>
</tr>
</tbody>
</table>

2.2.5. Electro-blotting Transfer

Following electrophoresis, nitrocellulose membranes (Amersham Biosciences, GE Healthcare) were pre-soaked in 100% methanol and left to equilibrate in transfer buffer (Table 2.5) for 10 min. The polyacrylamide gels were gently removed between the glass plates, soaked in transfer buffer and then placed on top of the nitrocellulose membrane. The gel and membrane were then inserted into an electrophoresis chamber (Mini-PREOTEAN®II, Bio-Rad Laboratories, Inc.) filled with transfer buffer. Protein transfer was performed at 50 V (170 mA) for 45 min.
To ensure successful protein transfer, nitro-cellulose membranes were stained with Ponceau Stain (Table 2.5) for 5 min at room temperature.

### 2.2.6. Detection of the Target Protein

Membranes were blocked in 5% milk (Table 2.6) for one hour at room temperature followed by 2 x rinses with MilliQ H₂O and 3 x 5 min washes in 1 x TBS + Tween with gentle rocking. From this step onwards, following each antibody application, the membrane was rinsed twice with MilliQ H₂O and 3 x 10 min in 1 x TBS + Tween.

<table>
<thead>
<tr>
<th>5% Milk Blocking Solution</th>
<th>5% w/v skim milk powder in 1 x TBS.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody Solution</td>
<td>1% w/v Bovine Serum Albumin in 1 x TBE + Tween® 20 (SERVA Electrophoresis), 2 mM EDTA.</td>
</tr>
</tbody>
</table>

Primary antibodies diluted in antibody solution (Table 2.12) were applied to membranes and left to incubate at 4°C overnight with gentle rocking.

Secondary antibodies, biotinylated anti-rabbit IgG raised in Donkey (GE healthcare UK Ltd., UK) or biotinylated anti-mouse IgG raised in Sheep (GE healthcare UK Ltd., UK) were diluted 1:10,000 in antibody solution and applied to the membrane for one hour at room temperature with agitation.
Tertiary antibodies, Avidin conjugated with horse radish peroxidase (GE healthcare UK Ltd., UK) were prepared by 1:20,000 dilution in antibody solution and applied to the membrane for 30 min at room temperature with gentle rocking.

Proteins were visualised using an ECL Plus™ chemilluminescence kit (Amersham Biosciences, GE Healthcare) according to the manufacturer’s instructions. Images were captured using a Fujifilm LAS-3000 scanner (Fujifilm Life Science, CT, U.S.A).

2.3. Genotyping

The Lim2\textsuperscript{Gt/Gt} knockout mice were genotyped to confirm homozygosity. Genotyping was performed based on the protocol from Shiels et al. (Shiels et al., 2007).

2.3.1. Isolation of DNA

Table 2.7 Reagents used to isolate DNA.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinase K Stock (QIAGEN)</td>
<td>20 mg.mL\textsuperscript{-1} in MilliQ H\textsubscript{2}O.</td>
</tr>
<tr>
<td>Lysis Buffer</td>
<td>5 mM EDTA, 200 mM NaCl, 2 mg.mL\textsuperscript{-1} Proteinase K, 0.1% SDS, 100 mM Tris pH 8.</td>
</tr>
</tbody>
</table>

Ear clippings from the mice were supplied by the VJU in separate 1.5 mL eppendorf tubes. 100 µL lysis buffer containing 0.1-0.2 mg.mL\textsuperscript{-1} Proteinase K was added to the tubes and left to digest overnight at 55°C. Samples were vortexed for 10 min followed by centrifugation at 12,000 rpm for 10 min. The supernatants were removed and diluted 1:4 using DNase-Free, MilliQ H\textsubscript{2}O. Samples were boiled for 5 min to inactivate Proteinase K activity and stored at -20°C until required.
### 2.3.2. Polymerase Chain Reaction

Table 2.8 Reagents used in the Polymerase Chain Reaction.

<table>
<thead>
<tr>
<th>Reagent Description</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Platinum® Taq reaction buffer (Invitrogen™)</td>
<td>600 mM Tris-SO(_2) (pH 8.9), 180 mM Ammonium Sulphate.</td>
</tr>
<tr>
<td>Platinum® Taq High Fidelity (Invitrogen™)</td>
<td>5 U.μL(^{-1}) Platinum® Taq in 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, stabilisers, and 50% (v/v) glycerol.</td>
</tr>
<tr>
<td>10 mM dNTP (Ray lab)</td>
<td>10 mM dATP, 10 mM dGTP, 10 mM dCTP and 10 mM dTTP.</td>
</tr>
<tr>
<td>Reaction mixture</td>
<td>1x Platinum® Taq PCR reaction buffer, 1.5 mM MgCl(_2), 2.0 mM dNTP, 1.0 μM forward and reverse primers, 1 μL DNA sample or 1 μL DMPC water (control) and 0.05 U.μL(^{-1}) of Platinum® Taq polymerase.</td>
</tr>
</tbody>
</table>

Reaction mixture was made to a final volume of 25 μL using DMPC water (Table 2.8). PCR reactions were run using an Eppendorf® Master Cycler Personal, with the reaction conditions optimised for primers sets used (Table 2.9). A negative control omitting the DNA template was included with every reaction.

Table 2.9 Showing the annealing temperatures for the different primer pairs.

<table>
<thead>
<tr>
<th>Primer Pairs</th>
<th>Annealing Temperature</th>
<th>Expected Product Size</th>
<th>Allele Fragment Specific to</th>
</tr>
</thead>
<tbody>
<tr>
<td>3F-4R</td>
<td>52.9°C</td>
<td>1.6 kb</td>
<td>Wild-type Mouse</td>
</tr>
<tr>
<td>3F-Rv3</td>
<td>54.1°C</td>
<td>1.2 kb</td>
<td>Lim2KO Mouse</td>
</tr>
</tbody>
</table>

The PCR reaction was run in a three step programme with initial 5 min incubation at 95°C to denature the DNA into single strand and activate the enzyme, followed by 30 cycles of denaturing, annealing and extension (1 min incubation at 95°C (denature), 1 min incubation at 54.1°C (annealing) and 1 min incubation at 72°C (extension) cycle).
2.3.3. Agarose Gel Electrophoresis

| 6x Loading Buffer/Dye | 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in MilliQ H₂O. |

PCR products were analysed in a 2% non-denaturing agarose gel containing 0.05% Ethidium Bromide (EtBr) alongside a 1Kb DNA Plus Ladder™ (GibcoBRL). Ultrapure agarose (GibcoBRL) was fully dissolved in 1x TBE with heat and was allowed to cool to room temperature before EtBr was added. EtBr intercalates between the DNA bases. Sample and loading buffer were in 1:1 ratio and loaded into the wells of the agarose gel.

Gels electrophoresis was run at 120 V for approximately 80 min in 1x TBE. Bands were visualised and recoded in a Gel Doc 2000 (Bio-Rad) Gel Imaging System.

2.4. Immunohistochemistry (IHC)

2.4.1. Fixation and Cryoprotection

Extracted lenses were fixed in 0.75% paraformaldehyde (Table 2.10) at room temperature for 24 hr using protocols developed in our laboratory (Jacobs et al., 2003). Lenses were then washed 3 x 10 min with PBS, and then cryoprotected by sequential incubation in 10% and then 20% sucrose solutions for 1 hr at room temperature. Finally, lenses were stored at 4°C in a 30% sucrose solution before cryosectioning.

Table 2.10 Solutions used in lens IHC.

<table>
<thead>
<tr>
<th>Fixative Solution</th>
<th>0.75% paraformaldehyde w/v in PBS, pH 7.4 adjusted with NaOH.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose Stock Solution</td>
<td>50% sucrose w/v in PBS.</td>
</tr>
</tbody>
</table>
2.4.2. Sectioning

Cryostat chucks were prepared by placing approximately 1 mL of Tissue Tek™ O.C.T compound on to the centre of the chuck and then frozen at -20°C overnight. The following day an additional 500 µL Tissue Tek™ O.C.T was layered on top and left to chill for half an hour at -20°C. Lenses were then mounted onto chucks in either an equatorial or axial orientation (Figure 2.2) before being encased in Tissue Tek™ O.C.T compound (Electron Microscopy Science, Fort Washington, PA) and then snap frozen in liquid nitrogen for 20 sec.

Lenses were sectioned at -21°C (chamber and objective temperature) on a cryostat (CM3050, Leica, Germany) using disposable blades (S-35; Feather Safety Razor Co., Japan). Sections from non-cultured (10-16 µm) and cultured lenses (20-40 µm) were collected onto poly-L-lysine coated microscope slides (Sail Brand). Sections were covered with PBS and then examined under a 40x light microscope to assess the quality and location of the section (Figure 2.2). The presence of a single layer epithelium and nucleated fibre cells in the lens cortex were used as indicator of an equatorial location. On the other hand, axial cryosections were collected that contained both anterior and posterior sutures with a visible bow region. Sections were then washed in PBS to remove excess OCT and were stored in a humidity box at 4°C to prevent drying of sections.
Figure 2.2  Schematic diagram showing equatorial and axial sectioning protocols (adapted from Bond, 1998).  
A: equatorial sectioning of the lens. The lower diagrams show representative sections from the lens epithelium, E1; lens sutures, E2/12; and from the equator where fibre cells are seen in cross-section, E7.  
B: axial sectioning of the lens. The lower diagrams represent sections from the fibre cell/epithelium interface, A1; from young fibre cells, A5; and through the centre of the lens, A8.
2.4.3. Immuno-labelling

To minimise non-specific background labelling, sections were incubated with blocking solution (Table 2.11) for 1 hr at room temperature and then washed in PBS for 3 x 5 min. Primary antibodies (1°Ab) were diluted in blocking solution (Table 2.12) and applied onto sections at 4°C overnight. Sections were then washed in PBS (3 x 5 min). Secondary antibodies (2°Ab) diluted 1:100 in blocking solution were applied for 2 hr at room temperature in the dark. Sections were washed in PBS (3 x 5 min) and then incubated in Wheat Germ Agglutinin* (WGA) conjugated to either Alexa 488 or Alexa 647 fluorophores (1:100 in PBS) for 30 min at room temperature to highlight cell morphology. Sections were washed and labelled with either 25 mM Propidium Iodide, 0.2 mM Hoechst or 4 μM DAPI (VECTASHIELD®, Vector Laboratories, Inc. Burlingame, CA) to highlight cell nuclei.

Table 2.11  Solutions used in immuno-labelling.

<table>
<thead>
<tr>
<th>Goat Blocking Solution</th>
<th>PBS containing 3% Bovine Serum Albumin (ICPbio, New Zealand) + 3 % Normal Goat Serum (Vector Laboratories, USA).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey Blocking Solution</td>
<td>PBS containing 3% Bovine Serum Albumin (ICPbio, New Zealand) + 3% Normal Donkey Serum (Vector Laboratories, USA).</td>
</tr>
<tr>
<td>Donkey Anti-Rabbit Antibody (1°Ab) Stock</td>
<td>2 mg.mL⁻¹ in MilliQ H₂O.</td>
</tr>
<tr>
<td>Horse Anti Rabbit Antibody (1°Ab) Stock</td>
<td>2 mg.mL⁻¹ in MilliQ H₂O.</td>
</tr>
</tbody>
</table>

Sections were washed and then mounted using an antifade mounting reagent (Citifluor AF1, Leicester, UK). Cover slips were carefully placed over the section and sealed with nail polish.

*Wheat Germ Agglutinin binds specifically to membrane proteins with N-acetylglucosamine-linked carbohydrates and acts as a general membrane label in the lens.
2.4.4. Primary Antibodies (1°Ab)

The panel of MP20 antibodies used in this study (Table 2.12) were obtained from the laboratories of Professor Charles Louis, University of Minnesota, St.Paul (Louis et al., 1989, TenBroek et al., 1992, Arneson and Louis, 1998), and from Professor Kevin Schey, Vanderbilt University, Tennessee, Nashville.

Table 2.12 Summary of the range of MP20 antibodies available in this project.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Name</th>
<th>Concentration IHC/ Western Blot</th>
<th>Antigen</th>
<th>Antibody Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Louis</td>
<td>2D10</td>
<td>1:100/1:1000</td>
<td>MP20 C-terminus synthetic peptide</td>
<td>Mouse</td>
</tr>
<tr>
<td></td>
<td>Purified</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TenBroek</td>
<td>1:100/1:4000</td>
<td>MP20 C-terminus synthetic peptide</td>
<td>Rabbit</td>
<td></td>
</tr>
<tr>
<td>AbP1</td>
<td>1:20/1:4000</td>
<td>MP20 1st Loop (26’ DHWMQY 31’)</td>
<td>Rabbit</td>
<td></td>
</tr>
<tr>
<td>AbP2</td>
<td>1:20/1:500</td>
<td>MP20 1st Loop (50’ KCYLQTESIAYWNATR 65’)</td>
<td>Rabbit</td>
<td></td>
</tr>
<tr>
<td>AbP3</td>
<td>1:20/1:4000</td>
<td>MP20 2nd Loop (84’ AQQTFTRLS 96’)</td>
<td>Rabbit</td>
<td></td>
</tr>
<tr>
<td>AbP4</td>
<td>1:20/1:4000</td>
<td>MP20 3rd Loop (127’ LGRRFGDWRFS 137’)</td>
<td>Rabbit</td>
<td></td>
</tr>
<tr>
<td>Schey</td>
<td>C-Term</td>
<td>1:100/1:1000</td>
<td>MP20 C-terminus synthetic peptide, human sequence (159’ CAYRMHECRLSTPR 173’)</td>
<td>Rabbit</td>
</tr>
<tr>
<td></td>
<td>Phos</td>
<td>1:100/1:1000</td>
<td>MP20 C-terminus, Phosphorylated form, human sequence (159’ CAYRMHECRLS(P)T(P)PR 173’)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Loop</td>
<td>---</td>
<td>MP20 2nd Loop, human sequence</td>
<td>Rabbit</td>
<td></td>
</tr>
</tbody>
</table>
2.4.5. Secondary Antibodies (2°Ab)

All 2°Ab used in this study were obtained from Molecular Probes Inc. (Molecular Probes Inc., USA). Goat anti-rabbit 2°Ab conjugated to either Alexa Fluor 488 or Alexa Fluor 568 was used at 1:100 diluted in blocking solution. Horse anti-rabbit 2°Ab linked to Alexa Fluor 568 was used at 1:100 in blocking solution. Various combinations of these 2°Ab were used for double- and triple-labelling for cell membrane, nuclei and MP20 protein as outlined in Table 2.13.

Table 2.13 Summary of different fluorophore combinations.

<table>
<thead>
<tr>
<th>Combination</th>
<th>WGA</th>
<th>MP20</th>
<th>Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Alexa Fluor 488</td>
<td>-</td>
<td>DAPI/Hoechst</td>
</tr>
<tr>
<td>B</td>
<td>Alexa Fluor 488</td>
<td>-</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>C</td>
<td>Alexa Fluor 647</td>
<td>Alexa Fluor 488</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>Alexa Fluor 647</td>
<td>Alexa Fluor 488</td>
<td>DAPI/Hoechst</td>
</tr>
<tr>
<td>F</td>
<td>Alexa Fluor 647</td>
<td>Alexa Fluor 568</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>Alexa Fluor 647</td>
<td>Alexa Fluor 568</td>
<td>DAPI/Hoechst</td>
</tr>
</tbody>
</table>

Combination A was applied to sections prepared for reflectance imaging (Section 2.5.2) and F & G were applied on mouse sections (Table 2.13). Remaining combinations were applied to rat sections using standard IHC protocols. Sections were imaged using a CLSM.
2.5. Imaging Acquisition - Microscope

Three different Confocal LASER Scanning Microscope (CLSM) all located in the Biomedical Imaging Research Unit at the University of Auckland were used during the course of this study. They included:

1. A Leica TCS SP2 4D CLSM (Leica Lasertechnik, Heidelberg, Germany) fitted with an Argon-krypton mixed gas LASER (488 nm) and an Argon-UV LASER (351 nm).
2. A Olympus FluoViewTM FV1000 CLSM (Olympus Corporation, Tokyo, Japan).
3. A LSM 710 Inverted CLSM (Zeiss, Jena, Germany).

The various objective lenses used to view specimens or to record images (Table 2.14) produced a resolution ranging from 1.66 μm.pixels⁻¹ to 0.195 μm.pixels⁻¹. Specific emission filters were used to separate signals from the different fluorophores used.

<table>
<thead>
<tr>
<th>Magnification</th>
<th>NA</th>
<th>Immersion</th>
<th>Type</th>
<th>Working Distance (µm)</th>
<th>XY Resolution (µm)</th>
<th>Z Objective Resolution (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x</td>
<td>0.45</td>
<td>dry</td>
<td>Plan-Apochromat</td>
<td>2000</td>
<td>470-850</td>
<td>1730-3110</td>
</tr>
<tr>
<td>20x</td>
<td>0.80</td>
<td>dry</td>
<td>Plan-Apochromat</td>
<td>550</td>
<td>270-480</td>
<td>500-980</td>
</tr>
<tr>
<td>40x</td>
<td>0.95</td>
<td>dry</td>
<td>Plan-Apochromat</td>
<td>250</td>
<td>220-400</td>
<td>390-700</td>
</tr>
<tr>
<td>40x</td>
<td>1.2</td>
<td>water</td>
<td>C-Apochromat</td>
<td>280</td>
<td>210-380</td>
<td>350-630</td>
</tr>
<tr>
<td>63x</td>
<td>1.40</td>
<td>oil</td>
<td>Plan-Apochromat</td>
<td>190</td>
<td>150-280</td>
<td>180-320</td>
</tr>
<tr>
<td>100x</td>
<td>1.40</td>
<td>oil</td>
<td>Plan-Apochromat</td>
<td>170</td>
<td>150-280</td>
<td>180-320</td>
</tr>
</tbody>
</table>
2.5.1. Imaging Protocol

Using a low power objective lens, appropriate areas of WGA-labelled section were identified under fluorescence microscopy. The integrity and quality of the labelling was examined before proceeding to image. A higher power objective lens was then used and the microscope switched to confocal mode.

To comprehensively map tissue morphology, antibody labelling and/or extracellular space dye distribution, image stacks were collected from the periphery to the core of the lens using line averaging of either 8 or 16. These image stacks allow the capture of antibody labelling fully and can eliminate affects such as tissue ripples. Overlapping stacks were collected with approximately 10% overlap between adjacent image stacks, resulting in a montage of antibody/dye labelling across the equatorial radius of the lens. Additionally, z-image stacks were taken to create maximum projection images to account for non-uniform antibody penetration in the specimen and also to aid elimination of tissue ripples.

2.5.2. Reflectance Imaging Mode

The reflectance imaging mode (Paddock, 2002) was used to determine the subcellular location of the MRI contrast agent Gd-DTPA (Vexler et al., 1994). Instead of collecting emission energy from an excited fluorophore, in this image modality, LASER light of a chosen wavelength (488 nm) illuminates the tissue, and light reflected back into the objective is collected. It appears the presence of Gd-DTPA increases the reflective properties of the lens allowing the penetration of Gd-DTPA into the lens to be determined.

Confocal Reflection Microscopy (CRM) is commonly used to image un-labelled specimen. Advantages of CRM are the requirement of relatively minimum specimen preparation and only instrument re-configuration is needed. CRM can also be combined with classical fluorescence techniques (Paddock, 2002).

However, the main disadvantage of CRM is the commonly observed artefact caused by the reflection from one or more of the optical element within the microscope in form of a bright spot (Figure 2.3). This artefact can be easily overcome by zooming in regions without artefact present or applying polarising filters to the instrument (Paddock, 2002).
2.5.3. Image Processing

Images were processed using ImageJ 1.37a (National Institute of Health, Bethesda, MD) and Adobe® Photoshop®CS version 8.0. In brief, images were loaded into ImageJ and channels were RGB combined. Tiling of stacks was done for individual stack layers in Photoshop®CS before combining back as a stack for analysis of individual stack layers or for z-projections.
Chapter 2: Methods

2.6. Two-Photon-Excitation-Flash-Photolysis (TPEFP)

2.6.1. Lens Preparation

Mice and rat lenses were extracted from the eye using standard dissection protocols (Section 2.1.4 Lens Dissection). A lens was then carefully placed into a custom made cutting chamber. A double-edged Teflon coated razorblade (ProSciTech, Australia) was snapped in half. One of the halves was used to bisect the lens equatorially, with a fresh razorblade being used for each lens. Both halves of a cut lens were carefully removed from the cutting chamber using forceps and/or a metal spatula and placed with the cut surface down into the Two-Photon-Excitation-Flash-Photolysis (TPEFP) recording chamber containing AICS (Table 2.1) pre-warmed to 37.5°C.

Cut lenses were incubated with 25 mM Propidium Iodide in AICS for 5 min to label the cell nuclei. The lens was then washed in AICS for 5 min before the chamber was filled with either 50 µL (rat) or 25 µL (mouse) AICS containing 1 mM of the caged fluorophores, CMNB-caged-fluorescein or 10 kDa-Dextran-linked-CMNB-caged-fluorescein (Molecular Probes Inc., USA). Lenses were incubated at 37.5°C for 30 min to allow sufficient fluorophore to be taken up into lens cells and for the cut surface to form a good seal with the cover-slip that formed the bottom of the recording chamber. TPEFP experiments were performed at room temperature (~20°C).

2.6.2. Lens Segmentation

Location in the lens was determined on the basis of radial position (r/a) from the lens centre. r is the distance from the centre of the lens to the region of interest, and a is the total radius of the lens. The outer cortex is defined as r/a between 1.00-0.80; inner cortex defined as 0.79-0.51; and anything deeper than 0.50 as the core (Sisley, 2007). The total radius of the lens was based on the average measurements of a specific age group of lens diameter taken from IHC sections, since epithelial cells and capsules were usually lost during lens cutting for functional experiments.
Table 2.15: Normalised distance (r/a) from lens centre with the corresponding depth in different rat and mouse.

<table>
<thead>
<tr>
<th>R/A</th>
<th>Distance from Lens Centre (µm)</th>
<th>Rat (~3 weeks)</th>
<th>Mouse (~8-10 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td></td>
<td>2400</td>
<td>1300</td>
</tr>
<tr>
<td>0.9</td>
<td></td>
<td>2160</td>
<td>1170</td>
</tr>
<tr>
<td>0.8</td>
<td></td>
<td>1920</td>
<td>1040</td>
</tr>
<tr>
<td>0.7</td>
<td></td>
<td>1680</td>
<td>910</td>
</tr>
<tr>
<td>0.6</td>
<td></td>
<td>1440</td>
<td>780</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>1200</td>
<td>650</td>
</tr>
<tr>
<td>0.4</td>
<td></td>
<td>960</td>
<td>520</td>
</tr>
<tr>
<td>0.3</td>
<td></td>
<td>720</td>
<td>390</td>
</tr>
<tr>
<td>0.2</td>
<td></td>
<td>480</td>
<td>260</td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td>240</td>
<td>130</td>
</tr>
<tr>
<td>0.0</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
2.6.3. Two-Photon Microscopy

A Mira 900 mode-lock Titanium:Sapphire (Ti:S) LASER (Coherent, Auburn, Ca, USA) pumped by two Verdi 532 nm solid-state pump LASERs (Coherent, Auburn, Ca, USA) was tuned to 750 nm with a pulse intensity of ~120 fs to provide the illumination for the Two-Photon Excitation (TPE) spot. The LASER beam was passed through a Pockels cell (model 350-50, Conoptis, Danbury, CT, USA) and directed by beam steering mirrors (Melles Griot, Carlsbad, CA, USA) into the side port of a customised inverted CLSM (Zeiss LSM 410, Jena, Germany), with compiling optics and dichroic mirrors in the light path (Figure 2.4). Two-photon illumination was continuous over the course of an experiment, but the TPE LASER spot was restricted to a stationary spot in the centre of field-of-view. The illumination of the TPE beam on the specimen was controlled by a mechanical shutter electronically operated by a Uniblitz SD-10 shutter drive/timer (Vincent Associates, USA). The continuously illuminated TPE spot was used to un cage fluorescein molecules until a maximal intensity and spread were reached. Uncaging volume was 0.5 µm in plane and 1.4 µm axially as previously determined (Soeller and Cannell, 1999).

The uncaging of fluorophore by the TPE beam was monitored using the 488 nm line of an Argon ion LASER (Uniphase, San Jose, California), with an emission band pass centred at 535 nm (HQ 535/50, bandwidth 50 nm) and measured by a Photo-Multiplier Tube (PMT) housed in the LSM 410. To ensure the TPE spot was in the same focal plane as the 488 nm light, axial alignment between the photolysis spot and the recording plane was obtained by adjustment of the Ti:S beam path by adjusting two mirrors (x- and y-) to correct for longitudinal chromatic aberration (Cannell et al., 2004). The TPE beam was tuned to 40 mW of excitation power by using a Power Meter (Coherent, Auburn, Ca, USA). For further details see Appendix A: Microscopy.
2.6.4. Experimental Protocols

To determine the radial position in a lens, lenses were first examined using bright-field illumination through a 10x objective lens. The centre of the half-cut lens was located and the x and y coordinates of the motorised stage set at [0,0]. To determine background fluorescence and to adjust image collection parameters, the objective was switched to a 40x water-immersion 1.2 NA lens. This objective has the highest working distance from the ranges of high magnification objective lenses available (Table 2.14).

Using the confocal mode and a 488 nm LASER focussed on the bathing medium away from lens surface, the brightness and contrast of the collected image was adjusted until a low level of background fluorescence was observed (Table 2.4 A). The TPE beam shutter was then opened to uncage the fluorophore in the bathing medium, and brightness and contrast adjusted so the centre of the uncaged spot was below maximum fluorescent intensity. The centre of the spot
was marked as a cross on the monitor, to enable accurate alignment of the spot and the middle of an individual cell. The TPE beam shutter was then closed and confocal imaging stopped.

Using transmitted bright-field mode, the surface of the cut-lens was brought into focus and the coordinates of the z-axis zeroed. The plane of focus in the z-axis was then moved some 20-50 µm into the tissue to visualise a suitable field of cells and the location coordinates (x, y, z) recorded. The cross marking of the centre of the TPE beam was positioned either in the centre of a fibre cell or between the broadside membranes of two adjoining fibre cells.

The imaging mode was then switched to confocal imaging to begin an experiment. Each experiment lasted for 84 sec. The first 2 sec were used to record background fluorescence, followed by 20 sec of uncaging by the TPE beam that was initiated and stopped by opening and closing of the mechanical shutter. Confocal scanning was continued after uncaging had stopped, so as to record the dissipation of the released fluorophore. During these experiments, images (256 x 256 pixels) were captured every 2 sec at a resolution of 0.2016 µm/pixels. Images were stored as 8-bit grey-scale images in Tagged Image File (TIF) format in the video memory rather than the computer’s hard drive to minimise the image writing time. The resultant time series were saved from the video memory to hard disk. In addition to these time series, single images of the nuclei labelling recorded using the 561 nm Helium+ LASER and bright-field images of the cellular morphology using 488 nm Argon+ LASER as transmitted light source were also captured at each location.

2.6.5. Data Analysis

Each time-series was imported as an Image Sequence into ImageJ 1.37a (National Institute of Health, Bethesda, MD, USA). Raw fluorescence pixel values were extracted using polygon selection to draw a hexagonal shape small enough to fit well inside the cell of interest to avoid sampling of the fluorescence signal from a neighbouring cell. A custom image analysis macro was written that extracted the mean signal intensity from the regions of interest in each image of the time series.

Extracted raw intensity values for individual cells were exported into Microsoft Excel (Microsoft Corporation, USA) and classified as either Primary (1°, the source cell where the fluorophore
uncaged was initiated), Secondary (2°, cells in direct contact with either the board or narrow-sides of a 1°-cell), Tertiary (3°, cells that exhibit fluorophore spread from a 2°-cell) or Quaternary (4°, cells that exhibit fluorophore spread from a 3°-cell). Fluorescein spread beyond 4°-cells was not extensive enough for measurements.

To facilitate comparison between data sets collected from the different species and fluorophores in different regions of the lens, extracted signal intensities were normalised to the maximum value reached in the 1° cell after the background fluorescence (value at t=0 before LASER shutter was opened) had been subtracted.

2.6.5.1. Image Filtering

Because only one background fluorescence image was obtained, it proved necessary to filter images to remove underlying random background noise (Figure 2.5 A) that could be mistaken as a signal artefact (Figure 2.5 B). To achieve this, the average background fluorescence pixel value was measured. The first image frame (background fluorescence) was duplicated and a Median Filter of 7 pixels radius (ImageJ “Process > Filter > Median > 7”) was applied (Figure 2.5 C). The filtered background fluorescence was then subtracted from each image frame (ImageJ “Process > Image Calculator > Subtract”) (Figure 2.5 D). The initial average background fluorescence pixel value was then added back to each image frame (ImageJ “Process > Math > Add”) (Figure 2.5 E). The filter size was chosen through trial and error. The final image was thresholded (Figure 2.5 F).

Maximum projections of images from the time series were preformed on the image sequences from TPEFP experiments to allow counting the number of cell spread by “eye” (ImageJ “Process > Stack> Projection> Maximum”). When two cells were uncaged simultaneously, there were more cells spread to but the number of cell spread did not double. The data from uncaging two-cells was not included in the data analysis unless state otherwise.

The number of cell spread was subdivided into either DF or MF zones to allow comparison between the two regions. Overall cell spread was the sum of number of cell spread from these two regions.
2.6.5.2. Rise-Time

To compare the rate of fluorescein uncaging between fluorescein and \textit{10 kDa}-Dextran-Linked-\textit{Fluorescein} (10 kDa-Fluorescein), the rise times of the two tracers in source cell were compared. The time taken to reach 67% of the normalised fluorescein intensity following uncaging in the $1^\circ$-cells and the time taken to reach 67% of the maximum normalised fluorescein intensity in the $2^e$-cells were recorded and compared.

2.6.6. Data Presentation

All tabulated data are presented as the Mean ± SEM (number of TPEFP experiments)
2.7. Lens Culture

Lenses were extracted from the eye (Section 2.1.4 Lens Dissection) and placed in a 37.5°C incubator with pre-warmed M199 (Table 2.16) for up to one hour (Figure 2.6 A). Lenses that turned opaque after 1 hr were discarded (Figure 2.6 B). Lenses were then incubated in 25 mM Gd-DPTA or 25 mM Texas Red-Dextran, extracellular markers for 2 hr. Control lenses were incubated in pre-warmed M199 only for the same period of time. Incubated lenses were then prepared for IHC (Section 2.4 Immunohistochemistry (IHC)) by placing in a final concentration of 0.75 % PFA supplemented with 25 mM Gd-DTPA or 25 mM Texas Red-Dextran.

**Table 2.16: Solutions used in lens culturing.**

<table>
<thead>
<tr>
<th>Medium 199 (M199)</th>
<th>With Earle’s Salts and Sodium Bicarbonate, pH 7.2-7.4, osmolarity 300 ± 5 mmol.kg⁻¹.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gadodiamide (Gd-DPTA) [Omniscan™, GE Healthcare]</td>
<td>0.5 M stock solution (stored at room temperature).</td>
</tr>
<tr>
<td>Texas Red-Dextran</td>
<td>25 mg.mL⁻¹ stock solution in MilliQ H₂O</td>
</tr>
</tbody>
</table>

M199 solution was made fresh the day prior to lens culturing and stored at 4°C. Gd-DPTA was stored at room temperature.
2.8. Statistical Analysis

The overall data was averaged and the standard error of the mean (SEM) calculated.

\[
SEM = \frac{StDev}{\sqrt{n}} \quad \text{where} \quad StDev = \sqrt{\frac{\sum(x-x)^2}{(n-1)}}
\]

StDev = the standard deviation of the population
n = the size of the sample

A 4 x 2 x 2 Factorial ANOVA was applied to the overall mean fluorophore spread and F-statistics calculated for a variety of null hypothesis to test the significant of the data.

A 4 x 2 Factorial ANOVA was applied to the mean source cell rise time and secondary cell rise time, and F-statistics calculated for a variety of null hypothesis to test the significant of the data.

For further details see Appendix C: Statistical Analysis.
Chapter 3: 

Mapping the Diffusion Pathways in the Rat Lens

A primary aim of my PhD project is to determine the relative contributions of GJs and the MDP to the overall intercellular communication in the rodent lens, and to determine whether the insertion of MP20 protein into the plasma membrane contributes to the formation of the MDP. Previous studies from our laboratory utilised the release of low-molecular weight caged-fluorescein to correlate the differentiation-dependent changes in the GJ localisation and the directionality of intercellular dye transfer in different regions in the rat lens (Jacobs et al., 2004). These studies did not, however, take into account the presence of the MDP (Shestopalov and Bassnett, 2000a, Shestopalov and Bassnett, 2003).

As the first step in achieving my aims, I have first developed the methods to distinguish these two intercellular communication pathways in the rat lens, based on the use of TPEFP and various caged-fluorescein compounds with different molecular weights. Using these methods, I was able to demonstrate for the first time that the rat lens also develops a MDP that exists in parallel to the GJ intercellular communication pathway.

In the mouse lens, the formation of the MDP has been shown to require the expression of MP20 protein (Bassnett, 2009). In the rat lens, the membrane insertion of MP20 protein coincides with both the transition zone from DF to MF cells marked by the loss of cell nuclei, and formation of diffusion barrier that restrict diffusion of tracer molecules via the extracellular space (Grey et al., 2003). Thus having developed methods to distinguish between GJ and the MDP mediated intercellular communication, I then used this approach to show that the formation of the MDP in the rat lens coincided with the loss of cell nuclei and presumably the membrane insertion of MP20 protein. The results obtained from the rat lens on the role of MP20 protein insertion and formation of MDP are used in later chapters to compare similar experiments performed in the wild-type and KO mice lenses to show the existence of species differences in the formation of the MDP.
3.1. The Use of TPEFP to Measure Intercellular Communication in the Rat Lens

The realisation that GJ mediated intercellular communication in the lens changes not only axially along the length of fibre cells (Baldo and Mathias, 1992), but also radially as fibre cells underwent differentiation, prompted the Molecular Vision Laboratory to develop a technique to measure intercellular dye transfer with high spatial and temporal resolution throughout the lens (Cannell et al., 2004, Jacobs et al., 2004). The technique developed utilised TPEFP to release caged-fluorescein loaded into fibre cells by cutting lenses through their equatorial axis. The release of fluorescein from its cage (masking compound) created a localised point source of dye within a single fibre cell located in a specific area of the lens. By monitoring the subsequent spread of the released dye to adjacent cells with confocal microscopy, the extent and pattern of dye movement could be compared between different areas of the lens. The advantages and disadvantages of this approach are first discussed before describing how the approach was modified to distinguish between intercellular communication via GJs and MDP.

3.1.1. Two-Photon Excitation Microscopy

While the theory of two-photon quantum transitions between atoms that underpins the development of two-photon microscopy (see Appendix A:) was first proposed by Maria Goeppert-Mayer in 1931 (Denk et al., 1990), the first commercial two-photon microscope did not become available until 1996 (Kaiser and Garrett, 1961, Zipfel et al., 2003). In contrast to a conventional single-photon CLSM whereby the excitation of fluorophores by a LASER occurs in a linear manner, excitation of fluorophores by TPE is non-linear and occurs only at the focal plane where two photons collide in quick succession (Zipfel et al., 2003, Svoboda and Yasuda, 2006). As the result, two-photon microscopy affords better optical resolution, localisation, and optical sectioning than standard one photon confocal microscopy. Furthermore, due to reduced scattering and absorption by endogenous chromophores, the longer wavelength of the Infra-Red (IR) two-photon pulse enables deeper tissue penetration than that obtained by conventional scanning microscopy. This ensures that out-of-focus effects do not contaminate the in-plane recording of fluorescence changes (Soeller et al., 2003). Although two-photon excitation limits photo-damage to the focal plane, the possibility of photo-bleaching and tissue damage still remains (Dakin and Li, 2006).
3.2. Caged-Compounds

Since TPE can be focused to a small focal volume (<1 μm³) of high IR intensity, it allows extremely localised photolysis of caged-compounds (Denk, 1994, Dakin et al., 2005). A classic caged-compound is a biologically relevant molecule rendered temporarily inactive by strategic attachment to a chemical group through a photoliabile bond (Gee et al., 1998). The molecule can be activated at anytime by breaking the bond with a pulse of intense light in the near UV (350-360 nm), or the IR range (720-780 nm) (Giovannardi et al., 1998).

Caged-substrate can be preloaded in either intra- or extra-cellular regions of a biological preparation without eliciting the substrate’s normal stimulus, and can then be activated at a very precise time, for a highly defined duration (Giovannardi et al., 1998). This is a major advantage over conventional methods as there is no time lag due to diffusion of substances into the preparation which, significantly minimises spatial and temporal inhomogeneities in substrate delivery (Giovannardi et al., 1998). Both the concentration and the spatial distribution of the caged-compound can be carefully managed and the temporal release can be varied over a range of first-order rate constants to achieve release from seconds to nanoseconds (Givens et al., 1998).

When combined with the spatial resolution provided by TPE photolytic, release of a caged-compound can be highly restricted to the three-dimensionally resolved source of the excitation (Soeller et al., 2003). With regard to monitoring intercellular communication, a readily available caged-compound commonly used is the GJ tracer fluorescein molecule (Figure 3.1 A).
In this regard, our laboratory has previously used fluorescein release induced by TPEFP to investigate regional differences in GJ channel mediated intercellular communication in the rat lens (Soeller et al., 2003, Cannell et al., 2004, Jacobs et al., 2004, Cannell et al., 2005). These studies showed that the spread of the released fluorescein is uni-directional and more pronounced through the broadside of the fibre cell membrane in the lens cortex, but becomes more isotropic in the deeper inner cortex. Observed changes in the pattern of dye spread were therefore able to be correlated with a change in the subcellular distribution of GJ plaques (Jacobs et al., 2004). However, these earlier measurements did not take into account the potential existence of the MDP in the rat lens. It is, nevertheless, possible to distinguish intercellular communication mediated by GJ channels and the MDP by utilising tracer molecules with high molecular weights that cannot permeate GJ channels, but are permeable through the MDP. In this chapter, I will introduce the high molecular weight tracer molecule, 10 kDa-Fluorescein (Figure 3.1 B), to investigate whether the MDP contributes to intercellular communication in the rat lens. This higher molecular weight reagent was unfortunately very expensive, so the TPEFP technique was first optimised in the rat lens using the lower molecular weight caged-fluorescein before introducing the larger 10 kDa-Fluorescein to characterise the MDP.

Figure 3.1 Molecular structures of the caged-fluorescein compounds used in this study. A: Bis-(5-Carboxy-Methoxy-2-Nitro-Benzyl) ether (CMNB)-caged-fluorescein. The fluorescein molecule highlighted in blue is bounded to the cage-compound CMNB on either side and has a molecular weight of 0.33 kDa. B: 10 kDa-Dextran linked to the caged-fluorescein creating a 10 kDa-Dextran-Linked-Caged-Fluorescein. Properties to consider when designing a caged-compound are: the efficiency of the photochemistry of the release reaction, by-products of the photo-release reaction need to be biologically benign, and the caged-compound need to be soluble in aqueous buffered medium. Factors to also taken into account are the difficulty of the synthesis process, the cost associated with production, and the instability of the cage compound to premature decomposition or to biochemically induced consumption prior to photolysis (Givens et al., 1998).
3.3. Optimisation of the TPEFP Technique in the Rat Lens

While a full description of the final methods adopted are provided in Chapter 2 (Section 2.4 Two-Photon-Excitation-Flash-Photolysis), a brief description of the key steps in the development and optimisation of the experimental protocols used to collect and analyse the data are presented here. To enable comparison to previous experiments conducted in the rat and mouse lenses, the same range of caged-compound concentrations were used as in previous studies (Jacobs et al., 2004, Sisley, 2007). To introduce the normally membrane impermeable caged-compounds into the rat lens, the lens was bisected through its equator (Figure 3.2). This procedure not only allowed caged-compounds to be taken up into the cut fibre cells, but also enabled imaging of the cut surface across the whole lens radius without any limitations imposed by the objective working distance. While the cutting of the lens is an invasive procedure, incubating cut lenses in AICS (Table 2.1), a solution that mimics the lens intracellular environment, minimises the effects on intercellular channels. In this cut lens preparation, the fibre cells were effectively depolarised, thereby short circuiting the micro-circulation system. However, junctional channels do not sense any change in junctional voltage and remain open in the cut lens (Jacobs et al., 2004).

3.3.1. The Cut Lens Preparation

The morphology of the cut lens is critical to the success of subsequent functional experiments. To assess the success of the bisection, the cut surface was first examined using transmitted light to access cell morphology (Figure 3.2). It proved impossible to bisect the lenses while retaining the capsule and the underlying lens epithelial and peripheral fibre cells attached to the capsule. Lenses were discarded if the cutting procedure resulted in the loss of substantial areas of the outer cortex, or if the cut was made at an angle that was not parallel to the lens equator, yielding non-hexagonal cellular cross-sectional profiles. Cut lenses that contained an appropriately intact outer cortex and exhibited a hexagonal cross-sectional cell profile, were then incubated in bathing medium in the presence of caged-compounds, and then transferred to the stage of the two-photon microscope. Different regions of the lens could then be viewed by simply utilising the motorised stage of the microscope to move the objective across the cut surface of a lens (Figure 3.2).
Figure 3.2  **Schematic diagram of experimental preparations.** Cut lenses were transferred to a recording chamber containing AICS with caged-compounds which was mounted on the stage of CLSM equipped with TPE. The surface of the lens placed face down on the cover slip allowing the microscope objective to be moved across the lens to visualise cellular morphology and TPEFP induced dye transfer in different regions of the lens. To quantify location in the lens, the distance moved by the objective is converted in a normalised scale of distance into the lens (r/a), where the lens periphery = 1 and the core = 0.
3.3.2. **Optimising the Uncaging Depth**

An example of the maximum projections (Figure 3.3, *middle panel*) and its associated surface plot (Figure 3.3, *right panels*) highlighting the pattern of dye spread observed following the uncaging of fluorescein is shown for a single region in the rat lens. For more details on the collection of maximum projections and surface plots, refer to Chapter 2: Section 2.6.5.1 Image Filtering. Releasing fluorescein in the bath at a focal plane just below the cut surface of the lens (Figure 3.3 A) resulted in a point source of fluorescein with its highest intensity at the release site and a symmetrical exponential decay away from the centre (Figure 3.3 B & C). If the point of release is moved slightly deeper into lens, the pattern of dye diffusion now produces very clear and distinctive cell outlines as the uncaged fluorescein spreads to adjacent cells (Figure 3.3 D-F). If the point of release is moved further in the z-direction into the lens, the extent of dye released and its subsequent spread falls as a function of distance in from the cut surface (Figure 3.3 G-L), indicating these deeper sites have less access to caged-fluorescein. The optimal depth for uncaging was found to be between approximately 15-50 μm away from the cut surface. All experiments were subsequently performed within this range.
Figure 3.3  Optimising the uncaging depth in the rat lens. Bright-field images to highlight the cellular morphology (left panel), and fluorescent images shown as a maximum projection (middle panel) and surface plots (right panel) of the spread of uncaged fluorescein in a cut lens preparation. The images were collected in the bath (A-C), at ~25 µm (D-F), ~65 µm (G-I) and ~105 µm (J-L) above the cut surface from the same position (r/a ~0.75) in the lens. Scale bars: 10 µm.
3.3.3. Optimising the Strength of TPEFP Uncaging

The strength of the TPE LASER was an important variable that had to be optimised to achieve a balance between maximising the uncaging of fluorescein and minimising photodynamic damage to fibre cells. At a TPE power of 40 mW, extensive dye spread was observed during uncaging (Figure 3.4 C), which rapidly dissipated once the uncaging ceased (Figure 3.4 D), indicating of successful uncaging of the dye. At this LASER power, no noticeable effects on cellular morphology were observed (Figure 3.4 B & E). However, when the LASER power was increased to 70 mW, the dye spread induced by the uncaging (Figure 3.4 F) did not dissipate upon termination of TPEFP (Figure 3.4 G), and rupturing of the fibre cell membrane was clearly visible as a “black hole” when viewed under bright-field microscopy (Figure 3.4 H). Dissipation of the uncaged molecule is dependent on the diffusion of the molecule away from the focal plane. The lack of dye dissipation with high LASER power could be due to the entrapment of uncaged dye following membrane rupture. To avoid such damage to fibre cells, the LASER power was kept below 50 mW during TPEFP experiments.
Figure 3.4  The effect of two-photon LASER power on dye release intensity and cellular morphology. Confocal and bright-field images from the same region of a lens showing fluorescein intensity (A, C, D, F, G) and cellular morphology (B, E, H) respectively.  (A-B) Prior to application of TPEFP, a homogenous background fluorescence is detected (A) and cellular morphology is normal (B). Fluorescein uncaged by TPEFP using a LASER power of 40 mW induced the spread of the tracer to adjacent cells (C) that rapidly dissipates following termination of TPEFP (D) and had no effect on cellular morphology (E).  (F-H) Fluorescein uncaged by TPEFP using a LASER power of 70 mW induced the spread of the tracer to adjacent cells (F) that did not dissipate following termination of TPEFP (G) and caused cellular damage and rupture (H). Scale bars: 10 μm.
3.3.4. Evaluating TPEFP Induced Dye Release in Different Regions of the Rat Lens

Having optimised the depth of focus and the strength of the LASER in the rat lens, these parameters were then kept constant while investigating the dye transfer patterns in different regions of the lens. Different regions of the lens were defined as a function of normalised distance from the lens centre (r/a). Uncaging of fluorescein in the outer cortex (DF, 1.00 < r/a < 0.80) (Figure 3.5 A) and inner cortex (MF, 0.79 < r/a < 0.60) (Figure 3.5 B) showed similar profiles of dye release, with the highest fluorescein intensity observed in the source cell and with an increase of fluorescein signal in neighbouring cells that depends on the direct connectivity to the source cell. The results correlated well with previous findings (Jacobs et al., 2004), which showed that the diffusion pattern becomes more isotropic as you move from peripheral DF (Figure 3.5 B) to deep MF cells (Figure 3.5 E), and correlates with the differentiation-dependent dispersion of Cx46 GJ plaques around the membrane of fibre cells (Jacobs et al., 2004).

It is apparent that the intensity of dye release in the source cell is dramatically reduced in the core (MF, r/a < 0.59) (Figure 3.5 H & I), suggesting that either TPEFP is less efficient in this area of the lens, or that the concentration of the caged-compound available for release is not as high as seen in the outer regions of the lens. In this regard, the concentration of crystallin proteins is known to be highest in the core, and as such a lower water to crystallin ratio may restrict the diffusion of the caged-fluorescein molecules through the cytoplasm of MF cells in these deeper regions of the lens. The reduced fluorescent signal observed in the core could therefore be a direct result of a reduced supply of uncaged compounds (Figure 3.5 C). The low efficiency of fluorescein released in the lens core along with the loss of peripheral fibre cells during lens bisection meant that the TPEFP technique could only be effectively used to study intercellular communication in the outer and inner cortical fibre cells of the lens (0.88 < r/a < 0.70). Despite this limitation, this r/a range still captures the transition of DF to MF cells (Grey et al., 2003).
Figure 3.5  Applying TPEFP to different regions of the lens. Bright-field images of fibre cell morphology (left panels), maximum projections of dye spread (middle panel) and surface plot profiles of fluorescein spread (right panel) obtained from the outer cortex (r/a ~0.85) (A-C), inner cortex (r/a ~0.75) (D-F), and core (r/a ~0/65) (G-I) of a cut rat lens. The intensity level of the released fluorescein although similar in the outer and inner cortex is significantly reduced in the core. Scale bars: 10 μm.
3.3.5. Analysis of the Time Course of Fluorescein Spread

Having optimised the TPEFP parameters, I then wanted to develop analysis protocols to visualise and quantify the extent of dye spread away from the point of uncaging to neighbouring cells. The images presented to date represent the maximum projections of all images collected over the time-course of the uncaging experiment. To visualise the temporal relationships between the initial uncaging in the source or primary (1°) cell and the subsequent spread to neighbouring cells, regions of interest were selected (see Chapter 2, 2.6.5 Data Analysis) within cells surrounding the 1°-cell and the increase in fluorescein intensity plotted as a function of time (Figure 3.6). All cells were colour coded and the cells directly in contact with a 1°-cell were defined as 2°-cells, while those in contact with a 2°-cell were deemed 3°-cells and so on.

Following the initiation of TPEFP, the signal intensity in the 1°-source cell exhibited a sharp rise, reaching a maximal intensity within 4 sec which was maintained until the LASER was turned-off (Figure 3.6, green). The amount of diffusion is an indicator of how well the cell is coupled near the imaging plane of the microscope. The increase in signal intensity in 2°-cells occurs at a much slower rate than seen in the 1°-cell, and represents the dye diffusion from the source cell to the neighbouring cell through intercellular pathways mediated by GJ channels and/or the MDP. At the highest point, signal intensity in the 2°-cell only reaches about 50% of the signal observed in the 1°-cell (Figure 3.6, blue). Similarly, fluorescein diffusion from 2°- to 3°-cells only occurred after an initial delay, and the intensity of the signal was only approximately 20% of the signal intensity recorded in 1°-cells (Figure 3.6, yellow).
Figure 3.6  Time-course of fluorescein uncaging and subsequent spread to adjacent cells. Data is extracted from the TPEFP experiment shown in the insert (B) that lasted 84 sec. A: Plot of the time-course of signal intensity normalised to the maximal fluorescein signal recorded in the 1°-cell. The duration of TPEFP is indicated by the red line above the graph. B: Cells colour coded in a maximum projection image corresponding to the colours of the signal fluorescein in graph A. The signal fluorescein intensity increased sharply when two-photon was activated in the 1°-cell (green) it remained high until the beam was turned-off. The blue cell has the greatest coupling with the 1°-cell indicating by the steep rise in signal intensity. The uncaged fluorescein would travel from the 1°-cell to 2°-cells (blues), and the fluorescein would in turn be spread to 3°-cells (yellow).

Following the closure of the mechanical shutter to terminate TPEFP, the fluorescein signal detected in all cells by the CLSM rapidly dissipated. While this dissipation of fluorescein could be attributed to photo-bleaching by the confocal LASER, this is unlikely as the amount of photo-bleaching that occurred during the period of data analysis was minimal as determined by monitoring the mean fluorescent signal of a small region (10 x 10 pixels) in the 4 corners of the field of view (data not shown). In the absence of significant photo-bleaching, and without continued uncaging of fluorescein, it is more likely that the observed dissipation of the signal represents the diffusion of fluorescein away from the focal plane.
The $2^\circ$-cell filling time is dictated by the properties of the mechanisms underlying intercellular communication between the $1^\circ$- and $2^\circ$-cell. The filling of $2^\circ$-cell occurs immediately upon uncaging, the fluorescein intensity in the $2^\circ$-cell never seemed to achieve saturation during the 20 sec duration of the uncaging period. During the 20 sec uncaging period, $2^\circ$-cells reached approximately 50% of the maximum normalised intensity of the $1^\circ$-cell and did not reach a plateau (Figure 3.6).

In order to observe more cells spread, there are two different approaches, 1. Increase the fluorescein uncaging rate by increasing the LASER power, or 2. Increase the period of uncaging. Increasing the TPE power would uncage fluorescein more rapidly. However, fluorescein also becomes inactivated faster through photo-bleaching, which could see less active fluorescein reaching further. The permanent destruction of fluorescein through photo-bleaching could yield free radical oxidative species that can react with the cells, an unwanted effect when doing live-cell imaging. Therefore, in an attempt to increase more cells spread, the period of uncaging was increased instead. Interestingly, extending the period of uncaging to 120 sec had no effect on the extent of dye diffusion in the $2^\circ$-cell (Figure 3.7 A). No further experiments were conducted to investigate the time taken to fill the $2^\circ$-cell to maximum fluorescent intensity as I was more interested in the extent of dye spread which, was not improved by extending the uncaging from 20 sec (Figure 3.7 B) to 100 sec (Figure 3.7 C). Since diffusion along the fibre cells out of the focal plane is faster than between the fibre cells (Cannell et al., 2004), this would ultimately limit the extent of cell spread observed between the fibre cells at an imaging plane (Figure 3.8).
Figure 3.7  Effect of increasing the duration of TPEFP on the extent of fluorescein spread to adjacent cells.  

**A:** Uncaging of fluorescein in the inner cortex (r/a ~0.75) of the rat lens for a total of 120 sec. Where two-photon beams were activated is marked by the yellow line above the graph. **White:** 1°-cell (source cell). **Red:** 2°-cell. **Green:** 3°-cell. **Blue:** 4°-cell. Fluorescent intensity in the 1°-cell remained maximal during 12-120 sec whereas the fluorescent intensity in the 2°-, 3°- and 4°-cells never reached maximal/plateau. 

**B:** Maximum projection image of fluorescein spread at t=24 sec (20 sec of uncaging duration) taken from time marked by (b) in A. 

**C:** Maximum projection image of fluorescein spread at t=104 sec (100 sec of uncaging duration) taken from time marked by (c) in A. Intensity is higher in C compared with B, the extra 80 sec of photolysis did not yield sufficiently more cell spread.

Scale bar: 10μm.
3.3.6. Distinguishing between GJ and MDP Mediated Intercellular Communication in the Rat Lens

Having optimised the parameters for TPEFP using caged-fluorescein, I then wanted to determine whether the same parameters could be used to uncage the 10 kDa-Fluorescein. The size of this 10 kDa-Fluorescein is above the molecular weight cut-off for GJ channels and its intercellular diffusion should therefore only be mediated by the MDP. Based on the work of Bassnett et al. conducted in the mouse lens (Shestopalov and Bassnett, 2003), if the MDP is also present in the rat lens, it will most likely manifest itself at a distinct stage of fibre cell differentiation. To test this assumption, rat lenses were incubated in 10 kDa-Fluorescein and TPEFP was performed in the DF cells in the outer cortex, and the resultant dye spread was compared to that observed in MF cells in the inner cortex.

In the DF cells, uncaging of 10 kDa-Fluorescein produced a clear increase in signal intensity in the 1\(^{\circ}\)-cell which did not spread to neighbouring cells (Figure 3.9 A). In contrast, the release of the smaller GJ permeable tracer fluorescein showed extensive spread between DF cells in a similar region of the lens (Figure 3.9 B), indicating that in the absence of the MDP the intercellular communication by GJ channels predominates. However, when TPEFP release of the 10 kDa-Fluorescein was performed in MF cells, extensive spread occurred (Figure 3.9 C) in a pattern very similar to that observed for the smaller fluorescein (Figure 3.9 D). This indicates that the MDP

Figure 3.8 Line scan in z-direction of uncaged fluorescein along the source fibre cell and the spread to the two \(2^{\circ}\)-cells at this plane. The uncaged fluorescein signal preferentially moves along the uncaged fibre cell represented by high fluorescent intensity, suggesting the diffusion is more rapid along than between fibre cells. The blue outlined arrow indicates the uncaged focal point. Scale Bar: 10 μm.
initially identified in the mouse lens also exists in the rat lens, but apparently only in MF cells in the inner cortex.

Figure 3.9 Identification of the MDP in the inner cortex of the rat lens. Maximum projection images of dye spread in the outer cortex with uncaged 10 kDa-Fluorescein (A) and fluorescein (B). Maximum projection image of dye spread in the inner cortex with uncaged 10 kDa-Fluorescein (C) and fluorescein (D). Plots of the time-course of normalised signal intensity recorded following TPEFP to uncaged 10 kDa-Fluorescein (E) and fluorescein (F) in the inner cortex of the rat lens ($r/a = \sim 0.75$). Inserts: maximum projection image of dye spread, each line in the graph corresponds to the same cell outline of same colour. Period when two-photon LASER was turned on is marked by the yellow line above the graph. Scale bar: 10 μm.
Not only did the extent of dye spread of the large and small fluorescein molecules looks similar in MF cells, the time-courses of the spread also appear near identical (Figure 3.9 E & F), with similar profiles for the initial rise, plateau and dissipation phase of the experiment. To analyse this further, the signal intensities obtained for the 1°-cells and selected 2°-cells from the experiments shown in Figure 3.9 E & F are overlaid in Figure 3.10 to facilitate a comparison between the two tracer molecules. As is apparent in Figure 3.10 A, the time-course of uncaging of the two molecules by TPEFP in the 1°-cell and the subsequent dissipation of the released molecules were identical. The time taken to reach 67% of maximum intensity in the 1°-cells showed no statistically significant difference between fluorescein and 10 kDa-Fluorescein \((p > 0.05)\) (Figure 3.10), while the total time taken to reach maximum fluorescent intensity was 12 sec for both molecules. Hence it was concluded that the photochemistry of the release process is not unduly influenced by the addition of the 10 kDa-Dextran molecule to fluorescein, and that TPEFP creates a similar point of source of fluorescein or 10 kDa-Fluorescein for subsequent diffusion to adjacent cells.
Figure 3.10  **Fluorescent intensity profile comparison** of uncaged fluorescein (blue) and 10 kDa-Fluorescein (red) in the inner cortex (r/a ~0.75) of the rat for 20 sec (A). The 1°-cell normalised fluorescent signal was used for comparison. Period when two-photon LASER was turned on is marked by the yellow line above the graph. Enlarged view of the fluorescent intensity profile of uncaged fluorescein and 10 kDa-Fluorescein (B). Statistical analysis (α = 0.05) suggested that there were no differences between uncaged fluorescein and 10 kDa-Fluorescein in the time taken to reach 67% of normalised fluorescent intensity in 1°-cell.
Due to its small size, the subsequent spread of fluorescein from the 1°-cells to an adjacent 2°-cell in the lens inner cortex will occur via both GJ channels and the MDP, whereas the spread of the larger 10 kDa-Fluorescein can only occur via diffusion through the MDP alone. We would also expect that the rate of diffusion of the 10 kDa-Fluorescein through the MDP to be slower than the diffusion of smaller fluorescein molecules. To investigate this, the time-course of fluorescent intensity profile in 2°-cells were compared between small and large tracer molecules (Figure 3.11). However, no statistically significant difference in the time taken to reach 67% of 2°-cell maximum intensity was observed. This suggests that either the MDP is a relatively low resistant pathway allowing the 10 kDa-Fluorescein molecule to diffuse as freely as fluorescein or that our technique does not have sufficient time resolution to detect small differences in diffusion time. These possibilities are re-examined in Chapter 5.

![Figure 3.11](image.png)

**Figure 3.11** Comparison of diffusion times for tracer molecules in the inner cortex (r/a ~0.75) of the rat lens. An example of a time-course of the changes in normalised signal intensity in representative 2°-cells taken from TPEFP experiments to release fluorescein (blue) and 10 kDa-fluorescein (red). The more rapid rise in signal intensity in the uncaged fluorescein in 2°-cells may indicate either the diffusion through both GJ and MDP is more rapid as there are two parallel pathways or the molecule itself without the extra 10 kDa-Dextran diffuses differently. Statistical analysis (α = 0.05) suggested that overall, there were no differences between uncaged fluorescein and 10 kDa-Fluorescein in the time taken to reach 67% of normalised fluorescent intensity in 2°-cells.
3.4. The Onset of the MDP Coincides With the Loss of Cell Nuclei in the Rat Lens

Having shown the existence of MDP in the MF but not in DF cells of the rat lens, I now wanted to map precisely where in the continuum of fibre cell differentiation the MDP develops. To achieve this, a marker of fibre cell differentiation which can provide an indication of the relative position of fibre cells in the cut lens preparation, and that is compatible with TPEFP is required. The loss of cell nuclei is a recognised marker of fibre cell differentiation that signals the transition between nucleated DF and anucleated MF cells. In the rat lens, the loss of nuclei (Figure 3.12 A) places the DF/MF transition at r/a of ~0.80. There are a number of commercially available dyes to stain nuclei, such as Propidium Iodide (PI) and Hoechst. PI was chosen since it can be excited by a 561 Helium” LASER and is therefore compatible with our experimental setup. By incubating the lenses in PI for 5 min (followed by a two washes in AICS to remove unbound PI), it proved possible to label the nuclei in cut lenses with minimal background labelling (Figure 3.12 C), thereby providing a reference point for the positional information relative to the state of fibre cell differentiation.
Figure 3.12  Use of cell nuclei as a marker of fibre cell differentiation and location in the cut lens preparation.  
A: Confocal image montage of an equatorial cryosection labelled with the membrane marker WGA (blue) and nuclei marker Hoechst (red).  B: Bright-field images taken from the periphery of a cut lens preparation showing the loss of the capsule, epithelium and peripheral fibre cells (yellow arrow).  C: Fluorescent image of PI labelling taken from the same area as shown in B and yellow arrow indicate the edge of the peripheral fibre cell same as in B. The confocal image montage has been used to cross-reference (red arrow, ~r/a 0.80) the image shown in C taken from a cut lens. Scale bars: 25 μm (A); 10 um (B).

Uncaging of 10 kDa-Fluorescein in DF cells containing cell nuclei resulted in dye restriction to the 1°-cell (  
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Appendix A: The Confocal LASER Scanning Microscopy Theory

Introduction

The invention of the double-focussing stage-scanning microscope (the confocal scanning microscope) in 1955 by Minsky revolutionised the imaging world (Minsky, 1988). This technique has since developed into the popular Confocal LASER Scanning Microscopy (CLSM) with the realisation of experimentally useful LASER about 40 years ago (Gratton, 1995). CLSM is a state-of-art imaging technique that has many advantages and some disadvantages over the different forms of conventional microscopy such as Fluorescent Microscopy (FM), Electron Microscopy (EM), and high-resolution Magnetic Resonance Imaging (MRI).

The lack of intrinsic contrast in biological specimens, especially transparent organs such as the ocular lens, poses a serious problem in biomedical imaging. Under the conventional transmitted Light Microscope (LM), the uniform opacity throughout the specimen allows similar levels of light to be detected from different structures, making their identification difficult. Also, large amounts of light scattering when imaging 3D specimens contribute blurring to the image, thus decreasing the image contrast (Halbhuber and Konig, 2003). The solution to this problem is labelling tissues with high-contrasting agents such as dyes, heavy metals and fluorochromes. Labelling tissues with fluorochromes and imaging with conventional wide-field FM suffers from the disadvantage of illumination of the whole sample resulting in inclusion of out-of-focus light (can be 90% of the collected fluorescence) into the otherwise in-focus image, resulting in a reduction in contrast in both axial and lateral direction (Wright et al., 1993, Cannell and Soeller, 1997, Conchello and Lichtman, 2005). While EM offers high resolution imaging with heavy metal staining, often the process of preparation damages the cells causing imaging artefacts from fixation and sectioning. MRI offers non-invasive, high resolution, 3D and functional imaging on the organ scale that no other microscopy offers, but MRI is less powerful than CLSM in terms of
resolution. CLSM has such high 3D resolution mainly because of its use of a pinhole, which gives it a shallow depth-of-field, allowing optical sectioning and eliminating out-of-focus blur.

Principles of CLSM

Light is an electro-magnetic wave made up of light particles (photons) propagating independent of each other (Urone, 2001). Light waves can either travel in-phase (constructive) or out-of-phase (deconstructive). Constructive (coherent) waves result in amplification of energy whereas deconstructive waves have cancelling affect and produces no colour (Urone, 2001). Light refraction is dependent on the speed of light which in terms is dependent on the wavelength and its frequency. The speed of light decreases with a highly refractive medium such as the glass slide and the degree of refraction various and is dependent on the medium. Constant speed of light is only achieved in vacuums.

The source of illumination for CLSM is LASER. LASER is an acronym that stands for “Light Amplification by Stimulated Emission of Radiation” (Gratton, 1995). LASER has several advantages over other sources of illumination: high brightness, high degree of monochromaticity, and small divergence. Conventional light sources are polychromatic, because light is made up of multiple wavelengths (λ). High brightness is equivalent to better signal, since the intensity of light is an indication of the amount of energy carried. The brighter the light, the more photons are being transmitted, which can generate more fluorescence from the specimen. Small divergence means the LASER beam cross-sectional area does not change dramatically along the beam, keeping intensity uniform. Intensity is inversely proportional to cross-sectional area and cross-sectional area goes up with largely diverging beams (Figure A.1).
The monochromatic property of LASER aids the imaging of fluorochromes by exciting only using the desired λ, meaning that only the fluorochrome of interest is excited. Mono-chromacity reduces the lateral chromatic aberration caused by the objective lens as a consequence of different λ focusing on different planes (axial chromatic aberration) (Keller, 1995). Usually longer λ sources of illumination are preferred in CLSM because with longer λ there are lesser chromatic aberrations, however shorter.

Disadvantages of using LASER are photo-bleaching and photo-toxicity, which are both major problems in both FM and CLSM (Conchello and Lichtman, 2005). Photo-bleaching is more problematic in FM than in CLSM as it is a wide-field modality, therefore exposing the whole field-of-view to bleaching at every time. CLSM minimises the area of bleaching only to the cone of scanning at the particular spot being scanned. Increasing the LASER power to obtain a better signal quality greatly increases sample bleaching, so a trade-off between signal and bleaching is required (Conchello and Lichtman, 2005). Photo-toxicity is the toxicity to living cells induced by high photon fluxes and produces highly reactive oxidative by-products which are problematic in live-cell imaging.

The exciting LASER passes through an excitation filter, gets reflected by the dichroic mirror towards the specimen and passes through the objective lens exciting the fluorochrome-labelled specimen (Figure A.2) (Stelzer, 1995). Upon excitation, fluorochromes absorb the energy and emit a lower energy and longer λ light as they return to their basal energy state (Figure A.3). The emitted λ is different from the excited λ and this difference allows their separation by the dichroic mirror. The emitted light is not reflected but passes through the dichroic mirror and emission filter, through the pinhole before reaching the detector (Figure A.2). The LASER scans
Appendix A: The Confocal LASER Scanning Microscopy Theory

the specimen in a raster system until the optical view of the specimen has been scanned. In conventional CLSM, one-photon light source is utilised. To acquire better optical resolution, localisation and sectioning a two-photon light source can be used.

Two-Photon Excitation (TPE) involves a very high local instantaneous intensity with temporal concentration of femtosecond pulsed LASER (Denk et al., 1990). The femtosecond pulse is produced by a colliding-pulse, mode-locked dye LASER and relies on two-photons interacting near simultaneously to produce a quadratic dependence of light intensity (Figure A.3) rather than the linear dependence observed in conventional scanning microscopy (Zipfel et al., 2003). By doubling the light intensity (TPE), the fluorescence production is quadrupled (Zipfel et al., 2003). This nonlinearity is advantageous as the scattering excitation photons are too diluted to cause significant fluorescence even in deep tissue environments, and consequently the excitation can therefore be limited to a small focal volume (Figure A.3) (Svoboda and Yasuda, 2006).

In conventional LM, the only components between the source of illumination and objective are lenses. In CLSM there are many additional components between the LASER and the objective: they are excitation filters, dichroic mirrors and scanning mirrors (Figure A.2). These additional components mean that the optical path becomes more complex and longer, which reduces the number of photons that get through (both excitation and emission pathways). Use of these components improves resolution and reduces chromatic aberrations, allowing 3D and 4D imaging.
Appendix A: The Confocal LASER Scanning Microscopy Theory

Figure A.2  Schematic representation of CLSM system. The different excitation and emission light are separated by the dichroic mirror, a vital component of the CLSM.

Figure A.3  Jablonski diagram illustrating the electronic states of a molecule upon excitation by single and double photons.
There are two filters: one filters the excitation beam and the other filters the emission light. The filters are examples of a band-pass filter (Figure A.4). Band-pass filters only allow $\lambda$’s between the cut-off $\lambda$’s to pass through. An improvement of the ordinary band-pass filter mounted on a rotating turret system is the Acousto-Optical Tunable Filters (AOTF) (Stelzer, 1995). AOTF is a solid-state electronically tunable band-pass filer relying on a birefringent crystal that modifies its diffraction properties upon interaction with an acoustic wave. The LASER $\lambda$ and intensity can be modulated by altering the acoustic wave frequency within microseconds, allowing rapid LASER line selection. AOTFs allow multi-labelled specimen to be scanned by one excitation wavelength and the following rapid LASER switching, by another. This reduces the amount of spectral cross-over which is usually the major problem in multi-wavelength excitation. Another benefit AOTF provides is the ability to modulate intensity beam to maintain strong fluorophores while accommodate weaker ones to generate uniform images (Stelzer, 1995).

![Figure A.4](image_url)  
An example of specific set-up in CLSM with LASER and filters $\lambda$ and mirror used. The excitation filter allow $\lambda$ between 465-495 nm to pass through (green light) and the emission filter allow $\lambda$ between 515-555 nm to pass through (red light). The filters do not perfectly filter according to the exact cut-off $\lambda$’s specified, thus it is important to have also have a monochromatic light source to control the excitation $\lambda$.

The dichroic mirror allows the reflection of specific shorter (excitation beam) $\lambda$ and the transmission of specific longer (emission beam) $\lambda$ (Carlsson et al., 1985). This component is very useful when imaging fluorochromes in both FM and CLSM. The shorter $\lambda$ is reflected by the mirror onto the specimen, the longer $\lambda$ is allowed to pass through the mirror onto the detector.
Appendix A: The Confocal LASER Scanning Microscopy Theory

This component prevents the detection of exciting λ resulting in excellent contrast because the only light detected originates from labelled structures. This mirror also removes the out-of-focus scatter caused by long coherent LASER, therefore preventing image blurring or interference (Gratton, 1995).

In CLSM, the objective lens is the focusing part of the system and is also the condenser of the system (Keller, 1995). The numerical aperture (NA) of the lens describes the angle from which light is collected; in CLSM it also describes the angle from which illuminating light is projected (Figure A.5). The distance between an objective and its focal point is its working distance. Large working distance allow optical sectioning with thicker specimen, but often the maximum thickness of the specimen would be smaller than the actual working distance. Objectives with high NA focus at a steeper angle than lower NA lenses, giving shorter and wider focal volume and thus are limited closer to the plane of focus.

![Illustration of angle from which light is collected (NA).](image)

**Figure A.5** Illustration of angle from which light is collected (NA). \( n = \) index of refraction (1.0 for air, 1.33 for pure water and up to 1.56 for oil). \( \Theta \) = the half-angle of the maximum cone of light that can enter or exit the lens. \( f \) = the light gathering ability of the lens. \( D \) = diameter of the entrance pupil of the objective lens.

Out-of-focus light (due to illumination from above and below the plane of interest in conventional LM and FM) contributes to a reduction in contrast and resolution (Cannell and Soeller, 1997). With FM, the amount of out-of-focus blur depends on the distribution of fluorochrome (Cannell and Soeller, 1997). The more the fluorochrome distribution is throughout the thickness of the specimen, the more out-of-focus blurring. The blurring in FM becomes a major issue when the cell thickness is more than 1 μm (Cannell and Soeller, 1997). CLSM overcomes this problem with the presence of a pinhole in front of the detector (Figure 2) (Stelzer, 1995). The light from above and below the focal point will be focused in front or behind the pinhole, and since it is not focused in the plane of the pinhole, only small amounts of...
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this light is detected (Stelzer, 1995). A smaller pinhole size confers better resolution but also means fewer signals are detected. Therefore an optimal pinhole size must be used to get the optimal resolution and signal at the same time.

The optimal pinhole size is roughly about the size of the full-width at half-maximum of the first Airy-disc peak; at this size only about 50% of the signal is lost (Hibbs, 2004). Smaller than this size, there is no effect other than reducing the signal. Pinholes maximise signal-to-background (STB) and signal-to-noise (STN) ratio, and eliminate background that is caused by light scattering of the specimen (Halbhuber and Konig, 2003).

Figure A.6  Showing a schematic of blocking of out-of-focus light with the presence of pinhole and the relative focus and out-of-focus airy discs.

There are two types of detector commonly used in CLSM: 1. Photo-Multiplier Tube (PMT) and 2. Charge-Couple Device (CCD). The photon detection systems in CLSM are based on direct photo-detection (converting photons into current) (Art, 1995).

PMTs are useful detectors when the signal is weak. PMTs are more sensitive and less noisy as well as having a faster response (Conchello and Lichtman, 2005). Disadvantages of using PMTs are their low efficiency detecting 10% or less of signal that passes through the pinhole. This low efficiency becomes a major issue when the scanning speed is increased. As the scanning speed increases, the number of photons detected per pixel will decrease to the extent that noise will limit the image quality. Since PMTs detect photons, they are unable to distinguish colour. So when doing multi-labelling imaging, either a succession of scans with different dichroic mirrors needs to be overlaid or the splitting of emitted light is required to distinguish different emission signals. PMTs are used for detecting photons with stage-scanning or xy-scanning system, and
CCDs or intensified CCDs are used with Nipkow disk scanning system (Hibbs, 2004). CCDs have higher efficiency than PMTs allowing rapid detection of photons, enabling rapid scanning near video rate. Often CCD cameras are cooled, making them more sensitive.

CLSM can scan the specimen through three different mechanisms: 1. by moving the specimen through the beam focal point (stage scanning); 2. by moving the scanners (beam scanning) or 3. by rotating a disk scanner (Stelzer, 1995). Stage scanning has the optical arrangement stationary and specimen scans through a single beam. Stage scanning offers the advantage of constant axial illumination, therefore avoiding off-axis aberrations, which is important in image processing, and is very photon efficient (Wright et al., 1993, Stelzer, 1995, Conchello and Lichtman, 2005). Disadvantages of this process are low scanning speed (10-150 lines/sec) and image distortion caused by movement of the specimen (Wright et al., 1993, Stelzer, 1995).

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\[ R = \frac{1.22\lambda}{2\text{NA}} \]

R is the radius of the Airy disk, which is the limit of resolution. An Airy disk is the 2D representation of the diffraction limit created by the objective lens and the medium the specimen is in (Figure A.1 A) and a point-spread function (PSF) is a 3D representation of the diffraction limit (Figure A.8) (Hibbs, 2004). 3D resolution in CLSM is determined by the intensity PSF. Lateral resolution (xy) and axial resolution (z) is determined by the width and height of PSF respectively (Sandison et al., 1995).

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Appendix A: The Confocal LASER Scanning Microscopy Theory

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![Figure A.8](image)  
**A**: 2D PSF of CLSM. **B**: 2D PSF of FM with the same NA as “A”. **C**: 3D PSF of FM. The large “x” shape of the FM cone are attributed by the out-of-focus light causing reduced lateral and axial resolution in comparison to the CLSM.

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Appendix B: Microscopy

XY Alignment

In case of an unlikely event the LASER beam path was projected to the eye, to ensure minimal damage to the eye, the ambient light was left on when aligning the two-photon spot with the recording plane. A mirror surface was focused through the 10x objective using transmitted optics and standard light source. Both the 488 nm and two-photon LASER attenuation was turned to maximal to ensure the lower power of light was used. 488 nm Argon+ LASER shutter was opened and the 488 nm spot was reflected by the mirror back out of the LSM410 rear aperture and the spot can be observed as a blue dot on a piece of paper. With the 488 nm LASER on continuous spot scanning mode, the mechanical shutter was then opened to illuminate the two-photon excitation (TPE) pathway.

To align the TPE beam to the illuminated spot in the centre of scan field, two beam steering mirrors were used. One mirror adjusted for the x-direction and the other for the y-direction (Figure B.1 I & F). The course knobs were used first to roughly get the two spots aligned at 4 different locations by placing a piece of tissue at each location:

1. Outlet of the Pockels Cell (Figure B.1 J).
2. Bottom steering mirror (Figure B.1 I).
3. Top steering mirror (Figure B.1 F).
4. Outlet of the side port to the microscope (Figure B.1 A).

The 488 nm was focused when aligning at each position. The fine knobs were then used to fine tune the two-spots (Figure B.1 E & G). When both the 488 nm and two-photon spots were deemed to overlap in all 4 locations, the two-photon controlled shutter was closed, 488 nm LASER was turned off and the mirror in the specimen holder was removed.
Laser attenuation for the 488 nm LASER was returned to the standard value. The two-photon beam was tuned to 40 mW of excitation power by using a Power Meter (Coherent, Auburn, CA, USA) by turning the LASER attenuation wheel.

Figure B.1 Schematic diagram of the mirrors used to align the two-photon excitation spot. A: spot directed to the specimen. A 488 nm stationary scanning spot was reflected back into the two-photon beam pathway as a reference for alignment. B: the modified side port of the microscope to provide a direct pathway for two-photon beam. C: the knobs used for z-alignment. D & H: course knobs used to move the mirrors. E & G: fine knobs used to move the mirrors. F & I: the beam steering mirrors, one can be adjusted in the x-direction and the other in the y-direction. J: two-photon beam coming from the Pockels cell (model 350-50, Conoptis, Danbury, CT, USA).
A slide containing 2 μm diameter green fluorescent beads or 1 μL of 1 mM caged-fluorescein in solution was then used to mark the two-photon spot on the computer monitor. Caged-fluorescein is used here for demonstration. The sea of caged-fluorescein was focused using the 10x objective lens and normal transmitted light. Under confocal imaging mode using the 488 nm LASER with continuous imaging at 0.7 s per frame, the two-photon beam shutter was opened and created a bright spot where fluorescein was released (Figure B.2 A). Pin-hole was adjusted to one-airy disc and the 488 nm Ar+ LASER attenuation, brightness and contrast were adjusted until the centre uncaged spot was just below saturation (Figure B.2 Rainbow Lookup Table). Continuous imaging mode was then stopped. 40x objective lens was changed to and the fluorescein was once again focused and a single 8 frames-average image was acquired (Figure B.2 B). The image was zoomed to get a final resolution of 4.960 pixels.μm-1. The centre of the two-photon spot was marked with a cross on the computer monitor to facilitate placing the TPE spot inside a single fibre cell cavity during experimentation.

Figure B.2 Two-Photon spot for x- and y- alignment. A: two-photon spot with no digital zoom to get 1.27 pixels.mm-1. B: two-photon spot digitally zoomed to get 4.960 pixels.μm-1. Scale bars: 10 μm. Right: Rainbow Lookup Table used to help clarify fluorescence intensity.
Z Alignment

A slide containing fluorescent beads was used for the z-alignment, to ensure the photolysis spot aligns with the 488 nm Ar+ LASER scanning focal plane. A z-stack was taken using the 488 nm Ar+ LASER in one channel of the confocal. A second z-stack was taken with the 488 nm LASER off, using the stationary TPE beam instead (Figure B.3 B). By displaying the channels together in RGB mode on the computer monitor it was possible to reveal whether the TPE spot overlapped with the excited bead. If not, the position of the lens at the side port (Figure B.1 C) was adjusted as appropriate until the 488 nm imaging plane is the same as the plane to which the TPE beam was focused (Figure B.3 A).

Figure B.3 Examples for two-photon spot z-alignment. A: z-stack in the red channel with the beads aligned in one plane and the two-photo beam activating one of the bead saturating in fluorescent intensity (white) while the 488 nm LASER was turned on. B: z-stack in the green channel, showing fluorescent intensity of a single bead. A-B). However, 10 kDa-Fluorescein spread from the source cell to 2°-cells was first observed immediately following loss of cell nuclei (Arneson ML, Louis CF (1998) Structural Arrangement of Lens Fiber Cell Plasma Membrane Protein MP20. Experimental Eye Research 66:495-509.


References


References


References


Appendix C: The Confocal LASER Scanning Microscopy Theory

Introduction

The invention of the double-focussing stage-scanning microscope (the confocal scanning microscope) in 1955 by Minsky revolutionised the imaging world (Minsky, 1988). This technique has since developed into the popular Confocal LASER Scanning Microscopy (CLSM) with the realisation of experimentally useful LASER about 40 years ago (Gratton, 1995). CLSM is a state-of-art imaging technique that has many advantages and some disadvantages over the different forms of conventional microscopy such as Fluorescent Microscopy (FM), Electron Microscopy (EM), and high-resolution Magnetic Resonance Imaging (MRI).

The lack of intrinsic contrast in biological specimens, especially transparent organs such as the ocular lens, poses a serious problem in biomedical imaging. Under the conventional transmitted Light Microscope (LM), the uniform opacity throughout the specimen allows similar levels of light to be detected from different structures, making their identification difficult. Also, large amounts of light scattering when imaging 3D specimens contribute blurring to the image, thus decreasing the image contrast (Halbhuber and Konig, 2003). The solution to this problem is labelling tissues with high-contrasting agents such as dyes, heavy metals and fluorochromes. Labelling tissues with fluorochromes and imaging with conventional wide-field FM suffers from the disadvantage of illumination of the whole sample resulting in inclusion of out-of-focus light (can be 90% of the collected fluorescence) into the otherwise in-focus image, resulting in a reduction in contrast in both axial and lateral direction (Wright et al., 1993, Cannell and Soeller, 1997, Conchello and Lichtman, 2005). While EM offers high resolution imaging with heavy metal staining, often the process of preparation damages the cells causing imaging artefacts from fixation and sectioning. MRI offers non-invasive, high resolution, 3D and functional imaging on the organ scale that no other microscopy offers, but MRI is less powerful than CLSM in terms of
Appendix A: The Confocal LASER Scanning Microscopy Theory

resolution. CLSM has such high 3D resolution mainly because of its use of a pinhole, which gives it a shallow depth-of-field, allowing optical sectioning and eliminating out-of-focus blur.

**Principles of CLSM**

Light is an electro-magnetic wave made up of light particles (photons) propagating independent of each other (Urone, 2001). Light waves can either travel in-phase (constructive) or out-of-phase (deconstructive). Constructive (coherent) waves result in amplification of energy whereas deconstructive waves have cancelling affect and produces no colour (Urone, 2001). Light refraction is dependent on the speed of light which in terms is dependent on the wavelength and its frequency. The speed of light decreases with a highly refractive medium such as the glass slide and the degree of refraction various and is dependent on the medium. Constant speed of light is only achieved in vacuums.

The source of illumination for CLSM is LASER. LASER is an acronym that stands for “Light Amplification by Stimulated Emission of Radiation” (Gratton, 1995). LASER has several advantages over other sources of illumination: high brightness, high degree of monochromaticity, and small divergence. Conventional light sources are polychromatic, because light is made up of multiple wavelengths (λ). High brightness is equivalent to better signal, since the intensity of light is an indication of the amount of energy carried. The brighter the light, the more photons are being transmitted, which can generate more fluorescence from the specimen. Small divergence means the LASER beam cross-sectional area does not change dramatically along the beam, keeping intensity uniform. Intensity is inversely proportional to cross-sectional area and cross-sectional area goes up with largely diverging beams (Figure A.1).
Appendix A: The Confocal LASER Scanning Microscopy Theory

The monochromatic property of LASER aids the imaging of fluorochromes by exciting only using the desired \( \lambda \), meaning that only the fluorochrome of interest is excited. Mono-chromaticity reduces the lateral chromatic aberration caused by the objective lens as a consequence of different \( \lambda \) focusing on different planes (axial chromatic aberration) (Keller, 1995). Usually longer \( \lambda \) sources of illumination are preferred in CLSM because with longer \( \lambda \) there are lesser chromatic aberrations, however shorter.

Disadvantages of using LASER are photo-bleaching and photo-toxicity, which are both major problems in both FM and CLSM (Conchello and Lichtman, 2005). Photo-bleaching is more problematic in FM than in CLSM as it is a wide-field modality, therefore exposing the whole field-of-view to bleaching at every time. CLSM minimises the area of bleaching only to the cone of scanning at the particular spot being scanned. Increasing the LASER power to obtain a better signal quality greatly increases sample bleaching, so a trade-off between signal and bleaching is required (Conchello and Lichtman, 2005). Photo-toxicity is the toxicity to living cells induced by high photon fluxes and produces highly reactive oxidative by-products which are problematic in live-cell imaging.

The exciting LASER passes through an excitation filter, gets reflected by the dichroic mirror towards the specimen and passes through the objective lens exciting the fluorochrome-labelled specimen (Figure A.2) (Stelzer, 1995). Upon excitation, fluorochromes absorb the energy and emit a lower energy and longer \( \lambda \) light as they return to their basal energy state (Figure A.3). The emitted \( \lambda \) is different from the excited \( \lambda \) and this difference allows their separation by the dichroic mirror. The emitted light is not reflected but passes through the dichroic mirror and emission filter, through the pinhole before reaching the detector (Figure A.2). The LASER scans

![Figure A.1](image_url)

Figure A.1  A: showing an image of illuminating cone. B: showing the area and the intensity of the relative cone. With increase in cross-sectional area of the light cone, there will be a decrease in the intensity. C: showing the 3D schematic illustration of the illumination cone like A and also showing the location of a focal volume.
the specimen in a raster system until the optical view of the specimen has been scanned. In conventional CLSM, one-photon light source is utilised. To acquire better optical resolution, localisation and sectioning a two-photon light source can be used.

Two-Photon Excitation (TPE) involves a very high local instantaneous intensity with temporal concentration of femtosecond pulsed LASER (Denk et al., 1990). The femtosecond pulse is produced by a colliding-pulse, mode-locked dye LASER and relies on two-photons interacting near simultaneously to produce a quadratic dependence of light intensity (Figure A.3) rather than the linear dependence observed in conventional scanning microscopy (Zipfel et al., 2003). By doubling the light intensity (TPE), the fluorescence production is quadrupled (Zipfel et al., 2003). This nonlinearity is advantageous as the scattering excitation photons are too diluted to cause significant fluorescence even in deep tissue environments, and consequently the excitation can therefore be limited to a small focal volume (Figure A.3) (Svoboda and Yasuda, 2006).

In conventional LM, the only components between the source of illumination and objective are lenses. In CLSM there are many additional components between the LASER and the objective: they are excitation filters, dichroic mirrors and scanning mirrors (Figure A.2). These additional components mean that the optical path becomes more complex and longer, which reduces the number of photons that get through (both excitation and emission pathways). Use of these components improves resolution and reduces chromatic aberrations, allowing 3D and 4D imaging.
Figure A.2  Schematic representation of CLSM system. The different excitation and emission light are separated by the dichroic mirror, a vital component of the CLSM.

Figure A.3  Jablonski diagram illustrating the electronic states of a molecule upon excitation by single and double photons.
Appendix A: The Confocal LASER Scanning Microscopy Theory

There are two filters: one filters the excitation beam and the other filters the emission light. The filters are examples of a band-pass filter (Figure A.4). Band-pass filters only allow λ’s between the cut-off λ’s to pass through. An improvement of the ordinary band-pass filter mounted on a rotating turret system is the Acousto-Optical Tunable Filters (AOTF) (Stelzer, 1995). AOTF is a solid-state electronically tunable band-pass filter relying on a birefringent crystal that modifies its diffraction properties upon interaction with an acoustic wave. The LASER λ and intensity can be modulated by altering the acoustic wave frequency within microseconds, allowing rapid LASER line selection. AOTFs allow multi-labelled specimen to be scanned by one excitation wavelength and the following rapid LASER switching, by another. This reduces the amount of spectral cross-over which is usually the major problem in multi-wavelength excitation. Another benefit AOTF provides is the ability to modulate intensity beam to maintain strong fluorophores while accommodate weaker ones to generate uniform images (Stelzer, 1995).

![Diagram of CLSM setup](https://example.com/diagram)

**Figure A.4** An example of specific set-up in CLSM with LASER and filters λ and mirror used. The excitation filter allow λ between 465-495 nm to pass through (green light) and the emission filter allow λ between 515-555 nm to pass through (red light). The filters do not perfectly filter according to the exact cut-off λ’s specified, thus it is important to have also have a monochromatic light source to control the excitation λ.

The dichroic mirror allows the reflection of specific shorter (excitation beam) λ and the transmission of specific longer (emission beam) λ (Carlsson et al., 1985). This component is very useful when imaging fluorochromes in both FM and CLSM. The shorter λ is reflected by the mirror onto the specimen, the longer λ is allowed to pass through the mirror onto the detector.
Appendix A: The Confocal LASER Scanning Microscopy Theory

This component prevents the detection of exciting $\lambda$ resulting in excellent contrast because the only light detected originates from labelled structures. This mirror also removes the out-of-focus scatter caused by long coherent LASER, therefore preventing image blurring or interference (Gratton, 1995).

In CLSM, the objective lens is the focusing part of the system and is also the condenser of the system (Keller, 1995). The numerical aperture (NA) of the lens describes the angle from which light is collected; in CLSM it also describes the angle from which illuminating light is projected (Figure A.5). The distance between an objective and its focal point is its working distance. Large working distance allow optical sectioning with thicker specimen, but often the maximum thickness of the specimen would be smaller than the actual working distance. Objectives with high NA focus at a steeper angle than lower NA lenses, giving shorter and wider focal volume and thus are limited closer to the plane of focus.

![Diagram of lens equation](image)

**Figure A.5** Illustration of angle from which light is collected (NA). $n$ = index of refraction (1.0 for air, 1.33 for pure water and up to 1.56 for oil), $\theta$ = the half-angle of the maximum cone of light that can enter or exit the lens. $f$ = the light gathering ability of the lens. $D$ = diameter of the entrance pupil of the objective lens.

Out-of-focus light (due to illumination from above and below the plane of interest in conventional LM and FM) contributes to a reduction in contrast and resolution (Cannell and Soeller, 1997). With FM, the amount of out-of-focus blur depends on the distribution of fluorochrome (Cannell and Soeller, 1997). The more the fluorochrome distribution is throughout the thickness of the specimen, the more out-of-focus blurring. The blurring in FM becomes a major issue when the cell thickness is more than 1 $\mu$m (Cannell and Soeller, 1997). CLSM overcomes this problem with the presence of a pinhole in front of the detector (Figure 2) (Stelzer, 1995). The light from above and below the focal point will be focused in front or behind the pinhole, and since it is not focused in the plane of the pinhole, only small amounts of
this light is detected (Stelzer, 1995). A smaller pinhole size confers better resolution but also means fewer signals are detected. Therefore an optimal pinhole size must be used to get the optimal resolution and signal at the same time.

The optimal pinhole size is roughly about the size of the full-width at half-maximum of the first Airy-disc peak; at this size only about 50% of the signal is lost (Hibbs, 2004). Smaller than this size, there is no effect other than reducing the signal. Pinholes maximise signal-to-background (STB) and signal-to-noise (STN) ratio, and eliminate background that is caused by light scattering of the specimen (Halbhuber and Konig, 2003).

Figure A.6  Showing a schematic of blocking of out-of-focus light with the presence of pinhole and the relative focus and out-of-focus airy discs.

There are two types of detector commonly used in CLSM: 1. Photo-Multiplier Tube (PMT) and 2. Charge-Couple Device (CCD). The photon detection systems in CLSM are based on direct photo-detection (converting photons into current) (Art, 1995).

PMTs are useful detectors when the signal is weak. PMTs are more sensitive and less noisy as well as having a faster response (Conchello and Lichtman, 2005). Disadvantages of using PMTs are their low efficiency detecting 10% or less of signal that passes through the pinhole. This low efficiency becomes a major issue when the scanning speed is increased. As the scanning speed increases, the number of photons detected per pixel will decrease to the extent that noise will limit the image quality. Since PMTs detect photons, they are unable to distinguish colour. So when doing multi-labelling imaging, either a succession of scans with different dichroic mirrors needs to be overlaid or the splitting of emitted light is required to distinguish different emission signals. PMTs are used for detecting photons with stage-scanning or xy-scanning system, and
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**Figure A.7**  Showing a schematic of the x-y system, including an artistic representation of raster scanning.

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![Figure A.8](image)

**Figure A.8** A: 2D PSF of CLSM. B: 2D PSF of FM with the same NA as “A”. C: 3D PSF of FM. The large “x” shape of the FM cone are attributed by the out-of-focus light causing reduced lateral and axial resolution in comparison to the CLSM.

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8. Outlet of the side port to the microscope (Figure B.1 A).

The 488 nm was focused when aligning at each position. The fine knobs were then used to fine tune the two-spots (Figure B.1 E & G). When both the 488 nm and two-photon spots were deemed to overlap in all 4 locations, the two-photon controlled shutter was closed, 488 nm LASER was turned off and the mirror in the specimen holder was removed.
Laser attenuation for the 488 nm LASER was returned to the standard value. The two-photon beam was tuned to 40 mW of excitation power by using a Power Meter (Coherent, Auburn, CA, USA) by turning the LASER attenuation wheel.

Figure B.1  Schematic diagram of the mirrors used to align the two-photon excitation spot. A: spot directed to the specimen. A 488 nm stationary scanning spot was reflected back into the two-photon beam pathway as a reference for alignment. B: the modified side port of the microscope to provide a direct pathway for two-photon beam. C: the knobs used for z-alignment. D & H: course knobs used to move the mirrors. E & G: fine knobs used to move the mirrors. F & I: the beam steering mirrors, one can be adjusted in the x-direction and the other in the y-direction. J: two-photon beam coming from the Pockels cell (model 350-50, Conoptis, Danbury, CT, USA).
A slide containing 2 μm diameter green fluorescent beads or 1 μL of 1 mM caged-fluorescein in solution was then used to mark the two-photon spot on the computer monitor. Caged-fluorescein is used here for demonstration. The sea of caged-fluorescein was focused using the 10x objective lens and normal transmitted light. Under confocal imaging mode using the 488 nm LASER with continuous imaging at 0.7 s per frame, the two-photon beam shutter was opened and created a bright spot where fluorescein was released (Figure B.2 A). Pin-hole was adjusted to one-airy disc and the 488 nm Ar+ LASER attenuation, brightness and contrast were adjusted until the centre uncaged spot was just below saturation (Figure B.2 Rainbow Lookup Table). Continuous imaging mode was then stopped. 40x objective lens was changed to and the fluorescein was once again focused and a single 8 frames-average image was acquired (Figure B.2 B). The image was zoomed 序 to get a final resolution of 4.960 pixels.μm-1. The centre of the two-photon spot was marked with a cross on the computer monitor to facilitate placing the TPE spot inside a single fibre cell cavity during experimentation.

![Two-Photon spot for x- and y- alignment. A: two-photon spot with no digital zoom to get 1.27 pixels.mm-1. B: two-photon spot digitally zoomed to get 4.960 pixels.μm-1. Scale bars: 10 μm. Right: Rainbow Lookup Table used to help clarify fluorescence intensity.](image)
Z Alignment

A slide containing fluorescent beads was used for the z-alignment, to ensure the photolysis spot aligns with the 488 nm Ar+ LASER scanning focal plane. A z-stack was taken using the 488 nm Ar+ LASER in one channel of the confocal. A second z-stack was taken with the 488 nm LASER off, using the stationary TPE beam instead (Figure B.3 B). By displaying the channels together in RGB mode on the computer monitor it was possible to reveal whether the TPE spot overlapped with the excited bead. If not, the position of the lens at the side port (Figure B.1 C) was adjusted as appropriate until the 488 nm imaging plane is the same as the plane to which the TPE beam was focused (Figure B.3 A).


References


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Appendix E: The Confocal LASER Scanning Microscopy Theory

Introduction

The invention of the double-focussing stage-scanning microscope (the confocal scanning microscope) in 1955 by Minsky revolutionised the imaging world (Minsky, 1988). This technique has since developed into the popular Confocal LASER Scanning Microscopy (CLSM) with the realisation of experimentally useful LASER about 40 years ago (Gratton, 1995). CLSM is a state-of-art imaging technique that has many advantages and some disadvantages over the different forms of conventional microscopy such as Fluorescent Microscopy (FM), Electron Microscopy (EM), and high-resolution Magnetic Resonance Imaging (MRI).

The lack of intrinsic contrast in biological specimens, especially transparent organs such as the ocular lens, poses a serious problem in biomedical imaging. Under the conventional transmitted Light Microscope (LM), the uniform opacity throughout the specimen allows similar levels of light to be detected from different structures, making their identification difficult. Also, large amounts of light scattering when imaging 3D specimens contribute blurring to the image, thus decreasing the image contrast (Halbhuber and Konig, 2003). The solution to this problem is labelling tissues with high-contrasting agents such as dyes, heavy metals and fluorochromes. Labelling tissues with fluorochromes and imaging with conventional wide-field FM suffers from the disadvantage of illumination of the whole sample resulting in inclusion of out-of-focus light (can be 90% of the collected fluorescence) into the otherwise in-focus image, resulting in a reduction in contrast in both axial and lateral direction (Wright et al., 1993, Cannell and Soeller, 1997, Conchello and Lichtman, 2005). While EM offers high resolution imaging with heavy metal staining, often the process of preparation damages the cells causing imaging artefacts from fixation and sectioning. MRI offers non-invasive, high resolution, 3D and functional imaging on the organ scale that no other microscopy offers, but MRI is less powerful than CLSM in terms of...
Appendix A: The Confocal LASER Scanning Microscopy Theory

resolution. CLSM has such high 3D resolution mainly because of its use of a pinhole, which gives it a shallow depth-of-field, allowing optical sectioning and eliminating out-of-focus blur.

Principles of CLSM

Light is an electro-magnetic wave made up of light particles (photons) propagating independent of each other (Urone, 2001). Light waves can either travel in-phase (constructive) or out-of-phase (deconstructive). Constructive (coherent) waves result in amplification of energy whereas deconstructive waves have cancelling affect and produces no colour (Urone, 2001). Light refraction is dependent on the speed of light which in terms is dependent on the wavelength and its frequency. The speed of light decreases with a highly refractive medium such as the glass slide and the degree of refraction various and is dependent on the medium. Constant speed of light is only achieved in vacuums.

The source of illumination for CLSM is LASER. LASER is an acronym that stands for “Light Amplification by Stimulated Emission of Radiation” (Gratton, 1995). LASER has several advantages over other sources of illumination: high brightness, high degree of monochromaticity, and small divergence. Conventional light sources are polychromatic, because light is made up of multiple wavelengths (\( \lambda \)). High brightness is equivalent to better signal, since the intensity of light is an indication of the amount of energy carried. The brighter the light, the more photons are being transmitted, which can generate more fluorescence from the specimen. Small divergence means the LASER beam cross-sectional area does not change dramatically along the beam, keeping intensity uniform. Intensity is inversely proportional to cross-sectional area and cross-sectional area goes up with largely diverging beams (Figure A.1).
Appendix A: The Confocal LASER Scanning Microscopy Theory

Figure A.1  
A: showing an image of illuminating cone. B: showing the area and the intensity of the relative cone. With increase in cross-sectional area of the light cone, there will be a decrease in the intensity. C: showing the 3D schematic illustration of the illumination cone like A and also showing the location of a focal volume.

The monochromatic property of LASER aids the imaging of fluorochromes by exciting only using the desired $\lambda$, meaning that only the fluorochrome of interest is excited. Mono-chromaticity reduces the lateral chromatic aberration caused by the objective lens as a consequence of different $\lambda$ focusing on different planes (axial chromatic aberration) (Keller, 1995). Usually longer $\lambda$ sources of illumination are preferred in CLSM because with longer $\lambda$ there are lesser chromatic aberrations, however shorter.

Disadvantages of using LASER are photo-bleaching and photo-toxicity, which are both major problems in both FM and CLSM (Conchello and Lichtman, 2005). Photo-bleaching is more problematic in FM than in CLSM as it is a wide-field modality, therefore exposing the whole field-of-view to bleaching at every time. CLSM minimises the area of bleaching only to the cone of scanning at the particular spot being scanned. Increasing the LASER power to obtain a better signal quality greatly increases sample bleaching, so a trade-off between signal and bleaching is required (Conchello and Lichtman, 2005). Photo-toxicity is the toxicity to living cells induced by high photon fluxes and produces highly reactive oxidative by-products which are problematic in live-cell imaging.

The exciting LASER passes through an excitation filter, gets reflected by the dichroic mirror towards the specimen and passes through the objective lens exciting the fluorochrome-labelled specimen (Figure A.2) (Stelzer, 1995). Upon excitation, fluorochromes absorb the energy and emit a lower energy and longer $\lambda$ light as they return to their basal energy state (Figure A.3). The emitted $\lambda$ is different from the excited $\lambda$ and this difference allows their separation by the dichroic mirror. The emitted light is not reflected but passes through the dichroic mirror and emission filter, through the pinhole before reaching the detector (Figure A.2). The LASER scans
the specimen in a raster system until the optical view of the specimen has been scanned. In conventional CLSM, one-photon light source is utilised. To acquire better optical resolution, localisation and sectioning a two-photon light source can be used.

*Two-Photon Excitation* (TPE) involves a very high local instantaneous intensity with temporal concentration of femtosecond pulsed LASER (Denk et al., 1990). The femtosecond pulse is produced by a colliding-pulse, mode-locked dye LASER and relies on two-photons interacting near simultaneously to produce a quadratic dependence of light intensity (Figure A.3) rather than the linear dependence observed in conventional scanning microscopy (Zipfel et al., 2003). By doubling the light intensity (TPE), the fluorescence production is quadrupled (Zipfel et al., 2003). This nonlinearity is advantageous as the scattering excitation photons are too diluted to cause significant fluorescence even in deep tissue environments, and consequently the excitation can therefore be limited to a small focal volume (Figure A.3) (Svoboda and Yasuda, 2006).

In conventional LM, the only components between the source of illumination and objective are lenses. In CLSM there are many additional components between the LASER and the objective: they are excitation filters, dichroic mirrors and scanning mirrors (Figure A.2). These additional components mean that the optical path becomes more complex and longer, which reduces the number of photons that get through (both excitation and emission pathways). Use of these components improves resolution and reduces chromatic aberrations, allowing 3D and 4D imaging.
Appendix A: The Confocal LASER Scanning Microscopy Theory

Figure A.2  Schematic representation of CLSM system. The different excitation and emission light are separated by the dichroic mirror, a vital component of the CLSM.

Figure A.3  Jablonski diagram illustrating the electronic states of a molecule upon excitation by single and double photons.
Appendix A: The Confocal LASER Scanning Microscopy Theory

There are two filters: one filters the excitation beam and the other filters the emission light. The filters are examples of a band-pass filter (Figure A.4). Band-pass filters only allow \( \lambda \)'s between the cut-off \( \lambda \)'s to pass through. An improvement of the ordinary band-pass filter mounted on a rotating turret system is the Acousto-Optical Tunable Filters (AOTF) (Stelzer, 1995). AOTF is a solid-state electronically tunable band-pass filter relying on a birefringent crystal that modifies its diffraction properties upon interaction with an acoustic wave. The LASER \( \lambda \) and intensity can be modulated by altering the acoustic wave frequency within microseconds, allowing rapid LASER line selection. AOTFs allow multi-labelled specimen to be scanned by one excitation wavelength and the following rapid LASER switching, by another. This reduces the amount of spectral cross-over which is usually the major problem in multi-wavelength excitation. Another benefit AOTF provides is the ability to modulate intensity beam to maintain strong fluorophores while accommodate weaker ones to generate uniform images (Stelzer, 1995).

![Figure A.4](image)

**Figure A.4** An example of specific set-up in CLSM with LASER and filters \( \lambda \) and mirror used. The excitation filter allow \( \lambda \) between 465-495 nm to pass through (green light) and the emission filter allow \( \lambda \) between 515-555 nm to pass through (red light). The filters do not perfectly filter according to the exact cut-off \( \lambda \)'s specified, thus it is important to have also have a monochromatic light source to control the excitation \( \lambda \).

The dichroic mirror allows the reflection of specific shorter (excitation beam) \( \lambda \) and the transmission of specific longer (emission beam) \( \lambda \) (Carlsson et al., 1985). This component is very useful when imaging fluorochromes in both FM and CLSM. The shorter \( \lambda \) is reflected by the mirror onto the specimen, the longer \( \lambda \) is allowed to pass through the mirror onto the detector.
This component prevents the detection of exciting λ resulting in excellent contrast because the only light detected originates from labelled structures. This mirror also removes the out-of-focus scatter caused by long coherent LASER, therefore preventing image blurring or interference (Gratton, 1995).

In CLSM, the objective lens is the focusing part of the system and is also the condenser of the system (Keller, 1995). The numerical aperture (NA) of the lens describes the angle from which light is collected; in CLSM it also describes the angle from which illuminating light is projected (Figure A.5). The distance between an objective and its focal point is its working distance. Large working distance allow optical sectioning with thicker specimen, but often the maximum thickness of the specimen would be smaller than the actual working distance. Objectives with high NA focus at a steeper angle than lower NA lenses, giving shorter and wider focal volume and thus are limited closer to the plane of focus.

![Illustration of angle from which light is collected (NA).](image)

Out-of-focus light (due to illumination from above and below the plane of interest in conventional LM and FM) contributes to a reduction in contrast and resolution (Cannell and Soeller, 1997). With FM, the amount of out-of-focus blur depends on the distribution of fluorochrome (Cannell and Soeller, 1997). The more the fluorochrome distribution is throughout the thickness of the specimen, the more out-of-focus blurring. The blurring in FM becomes a major issue when the cell thickness is more than 1 μm (Cannell and Soeller, 1997). CLSM overcomes this problem with the presence of a pinhole in front of the detector (Figure 2) (Stelzer, 1995). The light from above and below the focal point will be focused in front or behind the pinhole, and since it is not focused in the plane of the pinhole, only small amounts of
Appendix A: The Confocal LASER Scanning Microscopy Theory

this light is detected (Stelzer, 1995). A smaller pinhole size confers better resolution but also means fewer signals are detected. Therefore an optimal pinhole size must be used to get the optimal resolution and signal at the same time.

The optimal pinhole size is roughly about the size of the full-width at half-maximum of the first Airy-disc peak; at this size only about 50% of the signal is lost (Hibbs, 2004). Smaller than this size, there is no effect other than reducing the signal. Pinholes maximise signal-to-background (STB) and signal-to-noise (STN) ratio, and eliminate background that is caused by light scattering of the specimen (Halbhuber and Konig, 2003).

Figure A.6 Showing a schematic of blocking of out-of-focus light with the presence of pinhole and the relative focus and out-of-focus airy discs.

There are two types of detector commonly used in CLSM: 1. Photo-Multiplier Tube (PMT) and 2. Charge-Couple Device (CCD). The photon detection systems in CLSM are based on direct photo-detection (converting photons into current) (Art, 1995).

PMTs are useful detectors when the signal is weak. PMTs are more sensitive and less noisy as well as having a faster response (Conchello and Lichtman, 2005). Disadvantages of using PMTs are their low efficiency detecting 10% or less of signal that passes through the pinhole. This low efficiency becomes a major issue when the scanning speed is increased. As the scanning speed increases, the number of photons detected per pixel will decrease to the extent that noise will limit the image quality. Since PMTs detect photons, they are unable to distinguish colour. So when doing multi-labelling imaging, either a succession of scans with different dichroic mirrors needs to be overlaid or the splitting of emitted light is required to distinguish different emission signals. PMTs are used for detecting photons with stage-scanning or xy-scanning system, and
CCDs or intensified CCDs are used with Nipkow disk scanning system (Hibbs, 2004). CCDs have higher efficiency than PMTs allowing rapid detection of photons, enabling rapid scanning near video rate. Often CCD cameras are cooled, making them more sensitive.

Figure A.7  Showing a schematic of the x-y system, including a artistic representation of raster scanning.

CLSM can scan the specimen through three different mechanisms: 1. by moving the specimen through the beam focal point (stage scanning); 2. by moving the scanners (beam scanning) or 3. by rotating a disk scanner (Stelzer, 1995). Stage scanning has the optical arrangement stationary and specimen scans through a single beam. Stage scanning offers the advantage of constant axial illumination, therefore avoiding off-axis aberrations, which is important in image processing, and is very photon efficient (Wright et al., 1993, Stelzer, 1995, Conchello and Lichtman, 2005). Disadvantages of this process are low scanning speed (10-150 lines/sec) and image distortion caused by movement of the specimen (Wright et al., 1993, Stelzer, 1995).

Illumination-scanning systems utilise two oscillating mirrors to deflect the light beam onto the point-of-interest in the specimen, and deflect the returning emitted light on to the pinhole (Conchello and Lichtman, 2005). The light deflected onto the specimen is ‘scanning’ and emitted light deflecting to the detector is ‘de-scanning’. One mirror scans the horizontal direction (x-mirror) and one scans the vertical direction (y-mirror). Usually, the x-mirror scans the ‘fast-axis’ and the y-mirror scans the ‘slow-axis’. X-y scanning mirrors move the beam in an orthogonal direction in a raster scanning pattern (Figure 4), the change in the angle of the mirrors resulting in linear motion of the focal point in the specimen.
Appendix A: The Confocal LASER Scanning Microscopy Theory

The speed of scanning in illumination scanning systems is limited by the mechanical properties of the x-mirror as well as the dwell time (amount of time the illumination stays on a spot). Scanning too slow may contribute to limited information being captured when imaging fast changing signals (Conchello and Lichtman, 2005). Scanning too fast reduces the signal intensity, increasing the contribution of noise to the image and therefore decreases signal-to-noise ratio. The main drawback of CLSM is the scanning speed which is not as rapid as wide-field techniques: FM and EM (Conchello and Lichtman, 2005).

Resolution

The resolution is the smallest distance the system is able to distinguish between two adjacent points, and is given by the following equation:

\[ R = 1.22\lambda/2NA \]

R is the radius of the Airy disk, which is the limit of resolution. An Airy disk is the 2D representation of the diffraction limit created by the objective lens and the medium the specimen is in (Figure A.1 A) and a point-spread function (PSF) is a 3D representation of the diffraction limit (Figure A.8) (Hibbs, 2004). 3D resolution in CLSM is determined by the intensity PSF. Lateral resolution (xy) and axial resolution (z) is determined by the width and height of PSF respectively (Sandison et al., 1995).

The shortest visible \( \lambda \) of LM is 400 nm with maximum numerical aperture of 1.4 (with oil immersion lens): these are the two limiting factors of the resolution of LM (Art, 1995). The most commonly used \( \lambda \) in CLSM is 488 nm and the maximum NA is 1.4. The \( \lambda \) in EM is 0.004 nm and maximum NA is 0.02. If we calculate the resolution of LM using these theoretical values, we will overestimate it. With CLSM, the resolution is calculated to be around 0.21 \( \mu \)m and for EM it is calculated to be around 0.2 nm. The theoretical resolution for CLSM is identical in LM and FM. Practically this theoretical resolution is very hard to achieve by LM and FM, as LM uses light as a source of illumination and light is composed of different \( \lambda \). The main contribution to the poor resolution to both LM and FM is the inclusion of out-of-focus blur in the image. CLSM achieve this resolution value with the inclusion of a pinhole and using LASER illumination. Resolution of
Appendix A: The Confocal LASER Scanning Microscopy Theory

CLSM is improved by a factor of 1.4 (comparing with LM) just by having a pinhole (Figure A.8) (Halbhuber and Konig, 2003). Even the inclusion of pinhole cannot increase the resolution to below 0.2 µm (EM), and this is one of the disadvantages of CLSM compared to EM.

Figure A.8  
A: 2D PSF of CLSM. B: 2D PSF of FM with the same NA as “A”. C: 3D PSF of FM. The large “x” shape of the FM cone are attributed by the out-of-focus light causing reduced lateral and axial resolution in comparison to the CLSM.

Imaging Modes

CLSM allows imaging of both fixed and live specimens with single, double, and more λ illumination modes. Single λ uses one fluorochrome, double λ uses two fluorochromes and triple uses three fluorochromes. With multiple λ illumination modes, the chosen fluorochromes’ excitation λ must be relatively different to one another to ensure no simultaneous excitation of the fluorochromes. Multi-λ-illumination mode is a powerful imaging technique allowing multiple structures/proteins of interest to be labelled and imaged from one specimen. A lot of information can come out of multi-λ-illumination, for example, co-localisation of proteins or structures, and the site of structures in relation to other structures. EM also allows multi-label-illumination, but once again the quality is poorer compared with CLSM. MRI does not allow multi-labelling therefore the ability to contrast structures depends on the protein make-up of the structure of interest. CLSM differ from MRI in that it does functional imaging at a cellular level instead of at an organ level. Functional imaging is impossible in EM, in that it is can only image fixed specimens.

The ability to reconstruct a 3D structure from 2D images without physically sectioning the specimen is a key advantage of CLSM and MRI (Carlsson et al., 1985). The technique CLSM utilises is called optical sectioning, which is enabled by its small z-axis resolution. This technique
Appendix A: The Confocal LASER Scanning Microscopy Theory

allows the acquisition of images of thin slices by eliminating out-of-focus light in each image plane, and the thin slices can be computationally overlaid at the end to reconstruct a 3D image.

Reaching the Limit

CLSM has reached the peak of its form, in terms of resolution, being now limited by the diffraction limit of light. The objective lens performance has reached its theoretical limits (Keller, 1995). It is not the lens design that contributes to this limit but the wave nature of the light; as a consequence I believe that the limit of CLSM resolution has been reached.

Since the theoretical limits of objective lens has been reached, and further manipulation of light can be performed, the only way to advance imaging using CLSM is not by altering the microscope itself, but utilising special techniques to improve image quality, such as FRET (fluorescence resonance energy transfer), STED (stimulated emission depletion) and TIRF (total internal reflection fluorescence) (Reichert and Truskey, 1990, Jares-Erijman and Jovin, 2003, Simpson, 2006)
Appendix F: Microscopy

**XY Alignment**

In case of an unlikely event the LASER beam path was projected to the eye, to ensure minimal damage to the eye, the ambient light was left on when aligning the two-photon spot with the recording plane. A mirror surface was focused through the 10x objective using transmitted optics and standard light source. Both the 488 nm and two-photon LASER attenuation was turned to maximal to ensure the lower power of light was used. 488 nm Argon+ LASER shutter was opened and the 488 nm spot was reflected by the mirror back out of the LSM410 rear aperture and the spot can be observed as a blue dot on a piece of paper. With the 488 nm LASER on continuous spot scanning mode, the mechanical shutter was then opened to illuminate the two-photon excitation (TPE) pathway.

To align the TPE beam to the illuminated spot in the centre of scan field, two beam steering mirrors were used. One mirror adjusted for the x-direction and the other for the y-direction (Figure B.1 I & F). The course knobs were used first to roughly get the two spots aligned at 4 different locations by placing a piece of tissue at each location:

9. Outlet of the Pockels Cell (Figure B.1 J).
10. Bottom steering mirror (Figure B.1 I).
11. Top steering mirror (Figure B.1 F).
12. Outlet of the side port to the microscope (Figure B.1 A).

The 488 nm was focused when aligning at each position. The fine knobs were then used to fine tune the two-spots (Figure B.1 E & G). When both the 488 nm and two-photon spots were deemed to overlap in all 4 locations, the two-photon controlled shutter was closed, 488 nm LASER was turned off and the mirror in the specimen holder was removed.
Laser attenuation for the 488 nm LASER was returned to the standard value. The two-photon beam was tuned to 40 mW of excitation power by using a Power Meter (Coherent, Auburn, CA, USA) by turning the LASER attenuation wheel.

Figure B.1  Schematic diagram of the mirrors used to align the two-photon excitation spot. A: spot directed to the specimen. A 488 nm stationary scanning spot was reflected back into the two-photon beam pathway as a reference for alignment. B: the modified side port of the microscope to provide a direct pathway for two-photon beam. C: the knobs used for z-alignment. D & H: course knobs used to move the mirrors. E & G: fine knobs used to move the mirrors. F & I: the beam steering mirrors, one can be adjusted in the x-direction and the other in the y-direction. J: two-photon beam coming from the Pockels cell (model 350-50, Conoptis, Danbury, CT, USA).
A slide containing 2 μm diameter green fluorescent beads or 1 μL of 1 mM caged-fluorescein in solution was then used to mark the two-photon spot on the computer monitor. Caged-fluorescein is used here for demonstration. The sea of caged-fluorescein was focused using the 10x objective lens and normal transmitted light. Under confocal imaging mode using the 488 nm LASER with continuous imaging at 0.7 s per frame, the two-photon beam shutter was opened and created a bright spot where fluorescein was released (Figure B.2 A). Pin-hole was adjusted to one-airy disc and the 488 nm Ar+ LASER attenuation, brightness and contrast were adjusted until the centre uncaged spot was just below saturation (Figure B.2 Rainbow Lookup Table). Continuous imaging mode was then stopped. 40x objective lens was changed to and the fluorescein was once again focused and a single 8 frames-average image was acquired (Figure B.2 B). The image was zoomed 序 to get a final resolution of 4.960 pixels.μm-1. The centre of the two-photon spot was marked with a cross on the computer monitor to facilitate placing the TPE spot inside a single fibre cell cavity during experimentation.

![Figure B.2](image-url)  
**Figure B.2** Two-Photon spot for x- and y- alignment. A: two-photon spot with no digital zoom to get 1.27 pixels.mm-1. B: two-photon spot digitally zoomed to get 4.960 pixels.μm-1. Scale bars: 10 μm. Right: Rainbow Lookup Table used to help clarify fluorescence intensity.
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A slide containing fluorescent beads was used for the z-alignment, to ensure the photolysis spot aligns with the 488 nm Ar+ LASER scanning focal plane. A z-stack was taken using the 488 nm Ar+ LASER in one channel of the confocal. A second z-stack was taken with the 488 nm LASER off, using the stationary TPE beam instead (Figure B.3 B). By displaying the channels together in RGB mode on the computer monitor it was possible to reveal whether the TPE spot overlapped with the excited bead. If not, the position of the lens at the side port (Figure B.1 C) was adjusted as appropriate until the 488 nm imaging plane is the same as the plane to which the TPE beam was focused (Figure B.3 A).

![Figure B.3](image)

**Figure B.3** Examples for two-photon spot z-alignment. A: z-stack in the red channel with the beads aligned in one plane and the two-photo beam activating one of the bead saturating in fluorescent intensity (white) while the 488 nm LASER was turned on. B: z-stack in the green channel, showing fluorescent intensity of a single bead. D). The ability of the larger 10 kDa-Fluorescein molecules to move between adjacent fibre cells in the inner cortex shows that in the rat lens the formation of the MDP protein coincides with the transition of nucleated DF cells to anucleated MF cells and the membrane insertion of MP20 protein (Grey et al., 2003).
Figure F.1  Mapping the onset of MDP in the rat lens. Combined maximum projections of 10 kDa-Fluorescein release taken during a TPEFP experiment from a single cut rat lens showing 10 kDa-Fluorescein signal intensity (red), PI labelling of cell nuclei (blue), and transmitted light images of cellular morphology (green) taken at different regions of the lens. Scale bars = 10 µm.
Chapter 3: Mapping the Diffusion Pathways in the Rat Lens

3.5. Summary

In this chapter I have:

- Optimised the parameters required to perform TPEFP in the rat lens.
- Used two caged-compounds of different molecular weights to distinguish between GJ and MDP mediated intercellular communication pathways.
- Shown for the first time that the MDP exists in the rat lens.
- Shown that intercellular transfer of large molecules via the MDP occurs rapidly, indicating that it is probably a diffusion pathway.
- Developed methods to map the onset of the MDP and shown that in the rat lens this onset coincides with the loss of cell nuclei and the DF-MF transition.

In the DF cells, the restriction of the larger 10 kDa-Fluorescein to the source cell and the spread of the small fluorescein molecule, indicated that GJ mediated intercellular communication dominates in the outer cortex of the rat lens. While in MF cells in the inner cortex, both 10 kDa-Fluorescein and fluorescein were able to diffuse to neighbouring cells indicating that the MDP also contributes to intercellular communication in this region of the lens. Unfortunately, my TPEFP technique alone cannot distinguish the relative contributions made by MDP and GJ to overall intercellular communication in MF cells. Thus while there is strong evidence to suggest that functional GJs couple MF cells in the inner cortex (Mathias et al., 2010), my data is also consistent with the MDP forming the primary intercellular pathway linking cells in this region of the lens. These possibilities are directly tested in Chapter 5 by applying TPEFP to lenses from transgenic animals lacking Cx46 or MP20 protein.

The onset of MDP was mapped using TPEFP and showed the pathway formation in the rat lens in regions of DF to MF transition (~r/a 0.80), correlating well with the MP20 protein insertion (Figure F.2) (Grey et al., 2003). Based on GFP-expressed experiments (Shestopalov and Bassnett, 2003), the MDP seem to form earlier (~r/a 0.90) and did not coincide with the loss of nuclei in the mouse. From this, it seems that there is a species difference in the differentiation-dependent onset of MDP between the rat and the mouse, potentially accounted for by the difference in MP20 protein membrane insertions in the two species. As such, the expression patterns of MP20 protein will be examined at high resolution and compared between the rat and mouse lenses in the next chapter.
Figure F.2  Overview of the intercellular pathways in the rat lens.  

A: Schematic of an equatorial section of the lens.  

B: Schematic of close up of panel b in figure A. The insertion of MP20 protein occurs in the transition zone from DF to MF cells marked by the loss of the nuclei (Grey et al., 2003) and this zone coincides with the MDP in the rat lens.
Chapter 4: MP20 Protein Expression and the MDP in Mouse Lens

In the previous chapter, I utilised the TPEFP technique to not only show the existence of the MDP in the rat lens, but also that its differentiation-dependent onset coincides with the loss of cell nuclei. Since this loss also correlates with the recruitment of MP20 protein from the cytoplasmic pool into the plasma membrane of MF cells, my results from Chapter 3 are consistent with the hypothesis that the membrane insertion of MP20 protein is a key event in the formation of the MDP. However, in the mouse lens, Bassnett et al. (Shestopalov and Bassnett, 2003) have shown that the MDP develops before cell nuclei are lost, indicating that either MDP formation and membrane insertion of MP20 are unrelated or that MP20 insertion and MDP formation occurs at different stages of fibre cell differentiation in the two species. Unfortunately, the differentiation-dependent subcellular expression patterns of MP20 protein have not been extensively studied in the mouse lens.

In this chapter, I first used IHC to see whether the insertion of MP20 protein occurred earlier in the mouse lens, and if so, at what stage of fibre cell differentiation this occurred. These experiments are critically dependent on the quality of the antibodies used to label mouse sections. Several different anti-MP20 antibodies were therefore trialled and optimised in both Western Blot and IHC experiments using lenses from both wild-type and Lim2KO animals. My results showed that MP20 protein labelling in the mouse lens is initially predominately cytoplasmic in peripheral DF cells, and undergoes relocation to the plasma membrane at a distinct stage of fibre cell differentiation that occurs prior to the loss of cell nuclei. Having established the MP20 expression pattern, I then used TPEFP induced release of 10 kDa-Fluorescein to map the onset of the MDP in the mouse lens. My results showed that like the rat lens, the onset of the MDP coincides with the membrane insertion of MP20 protein in the mouse lens. However, MP20 protein insertion and MDP onset occur at distinctly different stages of fibre cell differentiation in the two different species of lens.
4.1. MP20 Protein Structure and Function

MP20 protein is the second most abundant intrinsic membrane protein in the vertebrate lens, and is encoded by the lens Intrinsic Membrane-2 (Lim2) gene (Louis et al., 1985, Johnson, 1986, Mulders et al., 1988, Gonen et al., 2001, Grey et al., 2003). Lim2 expression is lens specific and is not detected in liver, heart, muscle, spleen or kidney. Lim2 is highly conserved amongst several mammalian species, but appears to be absent in birds such as the chicken (Mulders et al., 1988). The MP20 protein has previously been known as MP17, MP18 and MP19 due to its migration on polyacrylamide gels. Based on its cDNA sequence, the protein has a molecular weight of 19.6 kDa and since determination of its sequencing, it has subsequently been referred to as MP20 (TenBroek et al., 1992). The identification of the Lim2 gene confirmed that the MP20 protein is a primary gene product that is unrelated to other lens membrane proteins, and not the result of any post-translational modification to other lens proteins (Mulders et al., 1988).

MP20 protein consists of 9.25% basic, 2.32% acidic, 52.01% non-polar and 36.41% polar amino acid residues, at neutral pH. Hydrophobic amino acids account for 57.21% of the residues (Gutekunst et al., 1990). Hydropathy plot analysis of the human Lim2 sequence predicted that the MP20 protein has four distinct transmembrane domains (M1-M4) (Figure 4.1) represented by amino acid residues 1-25, 67-88, 99-128, and 138-160 (Gutekunst et al., 1990). A longer first extracellular loop (E1), represents a splice variant, which was later identified to be specific to the human genome (Wistow et al., 2002). The first 21 amino acids of the protein appear to act as an ‘anchor peptide’, allowing the rest of the MP20 molecule to insert properly into the cell membrane (Chen et al., 2003). In the heritable cataractous mouse mutant To3 (total opacity of lens#3), a point mutation results in a switch from Valine (GTG) to Glycine (GGG) at amino acid position 15 in the first transmembrane domain (Figure 4.1) (Steele et al., 1997). This point mutation caused an inhibition of the trafficking of the mutant protein to the cell membrane, and resulted in MP20<sup>to3</sup> being accumulated in the endoplasmic reticulum (Steele et al., 1997). The highly cataractic phenotype of this MP20 mutant highlights the potential importance of MP20 membrane insertion in the maintenance of lens transparency (Chen et al., 2003).
MP20 protein is a substrate for cAMP-dependent kinase and calmodulin-binding proteins (Mulders et al., 1988). Sequence analysis has identified potential sites of phosphorylation by cAMP-dependent protein kinase, protein kinase C, cGMP-dependent kinase, and calmodulin-dependent protein kinase II in the C-terminal tail of MP20 protein, while tandem mass spectrometry has shown that Ser-170 and Thr-171 are phosphorylated (Ervin et al., 2005). However, results from ion chromatograms suggest that MP20 protein predominantly exists in its monophosphorylated form (Ervin et al., 2005). MP20 protein has been shown to bind Galectin-3, a well-known modulator of cell adhesion in other tissues (Gonen et al., 2001). Galectin-3 is a lectin binding protein (Gonen et al., 2000) and would be expected to bind to MP20 protein via a glycosylation site. MS/MS analysis of in-gel digestion of MP20 protein showed two novel glycosylation sites at Trp-43 and -61, indicating that this region of the protein is located in the...
extracellular space (Ervin et al., 2005). MP20 protein localisation seems to correlate with the change in localisation of galectin-3 from the cytoplasm to the cell membrane upon loss of cell nuclei in the rat lens (Gonen et al., 2001).

Each MP20 protein consists of four transmembrane (M1-M4), two extracellular (E1 and E2) and three intracellular domains (Figure 4.1). The topology of the MP20 protein is virtually identical to that adopted by the connexin family of proteins suggesting MP20 also plays a role in junction formation. However, based on the cDNA sequence analysis of the MP20 protein, the four putative transmembrane domains (Figure 4.1) do not exhibit the same characteristic amphipathic structure previously identified in the connexins (TenBroek et al., 1992). Furthermore, immuno-gold-negative stain electron microscopy experiments revealed that the MP20 protein is not a component of isolated GJs, and like another putative GJ protein, AQP0 protein, is localised predominately to non-junctional membranes (Voorter et al., 1989). IHC labelling of dissected fibre cell bundles has localised MP20 protein to ball-and-socket structures located at the corners of the narrow side of fibre cells in the inner cortex (TenBroek et al., 1992). This immuno-labelling data collected on isolated fibre cell bundles suggested that the MP20 protein distribution varies and depends on the developmental state of the lens fibre cells, becoming more uniformly distributed at later stages of fibre cell differentiation (TenBroek et al., 1992). More recently, Grey et al. have mapped the differentiation-dependent changes in the subcellular distribution of MP20 protein throughout the rat lens in equatorial and axial cryosections (Grey et al., 2003). This study showed that in DF cells MP20 protein is predominately cytoplasmic, but inserts into the membrane at the DF/MF transition which coincides with the loss of cell nuclei and the restriction of the extracellular space to the diffusion of tracer molecules (Grey et al., 2003).

The exact role of the MP20 protein in the lens remains unknown. The association with the ball-and-sockets (TenBroek et al., 1992), the restriction in extracellular space diffusion following membrane insertion (Grey et al., 2003), and the binding to adhesion modulator Galectin-3 (Gonen et al., 2001) suggest MP20 is an adhesive protein. Alternatively, more recent findings in the Lim2KO mouse, showed that in the absence of MP20 protein the MDP is not formed (Shi et al., 2009). This suggests a role for MP20 protein in mediating intercellular communication in the lens. Interestingly, in the Lim2KO lens, fibre cells are easier to dissociate (Shiels et al., 2007), suggesting that enhancing cell adhesion is an inherent feature of the role of MP20 protein in the formation of the MDP, or alternatively MP20 protein has multiple functional roles in different
regions of the lens. Before we can address these questions, the differentiation-dependent expression of MP20 protein in the mouse lens needs to be more fully characterised.

### 4.2. Characterisation and Optimisation of MP20 Antibodies

To ensure reliable and robust labelling of MP20 protein in the mouse lens, I experimented with a number of different antibodies that were raised in a variety of species. Seven of these antibodies were a gift from Professor Charles Louis (University of California, Riverside); while a further three were obtained from Professor Kevin Schey (Vanderbilt University, Tennessee, Nashville). The epitopes of these different MP20 proteins are listed (if known) in Table 4.1 and are shown on the membrane topology of MP20 protein in Figure 4.1.

#### Table 4.1 Summary of different MP20 antibodies and their respective epitopes

<table>
<thead>
<tr>
<th>Author</th>
<th>Target</th>
<th>Antibody</th>
<th>Target Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arneson (Arneson and Louis, 1998)</td>
<td>Bovine</td>
<td>AbP1</td>
<td>DHWMQYRLSGFAHEGLWR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AbP2</td>
<td>KCFLQTESIAYWNATR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AbP3</td>
<td>AQQSTFTRLSPRLS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AbP4</td>
<td>LGRRFGDWRFS</td>
</tr>
<tr>
<td>TenBroek (TenBroek et al., 1992)</td>
<td>Sheep</td>
<td>TenBroek</td>
<td>CRRLSTPR</td>
</tr>
<tr>
<td>Louis (Louis et al., 1989)</td>
<td>Bovine</td>
<td>2D10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2D10 Ascities</td>
<td></td>
</tr>
<tr>
<td>Schey</td>
<td>Human</td>
<td>C-Terminal</td>
<td>CAYRVHECRLSTPR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Loop</td>
<td>GHLYSRWRTLRLKEGKGAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-Terminal</td>
<td>CAYRVHECRLSpTPR</td>
</tr>
</tbody>
</table>
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The antibodies from the Schey group are designed against the human MP20 sequence and the Loop antibody (Table 4.1) detects a protein sequence (Wistow et al., 2002) that does not exist in mouse, rat, bovine and sheep proteins (Figure 4.2). This was confirmed through application of the Schey Loop antibody on mouse lens sections, where no signal was detected. No further experiments were performed with the Schey Loop antibody in mouse or rat lens.

CLUSTAL 2.0.11 multiple sequence alignment

<table>
<thead>
<tr>
<th></th>
<th>MYSFMGGGLFCAWVTTLVVAATDHWMQYRLSGFAHQGLWR</th>
<th>YCLGNKCFLQTESI-- 58</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Rat</td>
<td>MYSFMGGGLFCAWVTTLVVAATDHWMQYRLSGFAHQGLWR</td>
<td>YCLGNKCFLQTESI-- 58</td>
</tr>
<tr>
<td>Bovine</td>
<td>MYSFMGGGLFCAWVTTLVVAATDHWMQYRLSGFAHQGLWR</td>
<td>YCLGTKCYLQTESI-- 58</td>
</tr>
<tr>
<td>Sheep</td>
<td>MYSFMGGGLFCAXVTTLVVAATDHXM----------------- 29</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>MYSFMGGGLFCAWVTLLVVAATDHWMQYRLSGFAHQGLWR</td>
<td>YCLGNKCFLQTESI-- 58</td>
</tr>
</tbody>
</table>

Mouse                   |                  | AYWNATRAFMILSALCATSG 78 |
Rat                     |                  | AYWNATRAFMILSALCATSG 78 |
Bovine                  |                  | AYWNATRAFMILSALCATSG 78 |
Sheep                   |                  |                           |
Human                  | PPGQGPGRAWGKSADLGQAQGHLYSRWRTRLRLKEGKGATQAYWNATRAFMILSALCAISG | 120 |

Mouse                   | IIMGVLAFAQOSTFTRLSRPFSAGIMFFASLTFVLLALAIYTGVTVSFLGRFDWRF | 138 |
Rat                     | IIMGVLAFAQOSTFTRLSRPFSAGIMFFASLTFVLLALAIYTGVTVSFLGRFDWRF | 138 |
Bovine                  | IIMGIVAFQAQPFTFTLSRPFSAGIMFFASLTFVLLALAIYTGVTVSFLGRFDWRF | 138 |
Sheep                   |                           |                           |
Human                  | IIMGIMAFAHQPTFSRISRPFSAGIMFFSSTLFVVLALAIYTGVTVSFLGRFDWRF | 180 |

Mouse                   | SYILGWVALLMTFFAGIFYMCAYRMHECRLATPR | 173 |
Rat                     | SYILGWVALLMTFFAGIFYMCAYRMHECRLATPR | 173 |
Bovine                  | SYILGWVALLMTFFAGIFYMCAYRMHECRLATPR | 173 |
Sheep                   |                           |                           |
Human                  | SYILGWVAVLMFTFFAGIFYMCAYRVHECRLATPR | 215 |

Figure 4.2  Protein alignment of MP20 protein in mouse, rat, bovine, sheep and human.
4.2.1. Western Blotting

Antibody dilution optimisation was performed using crude membranes prepared from mice lenses, using initial dilutions reported in the literature (Louis et al., 1989, TenBroek et al., 1992). With the advancement and improvement in the sensitivity of the detection system utilised in this study (ECL Plus™ chemilluminescence kit), antibodies could often be used at lower dilutions than used previously. Figure 4.3 is an example of a dilution series performed using the TenBroek MP20 antibody.

Figure 4.3 Optimising of MP20 antibody labelling in Western blotting. A series of SDS-PAGE gels labelled with TenBroek MP20 Antibody at the stated dilutions. 1: 1:100. 2: 1:200. 3: 1:500. 4: 1:1000. 5: 1:2000. 6: 1:4000. Red bar represents Mw 20 kDa.

A summary of the dilutions for all eight MP20 antibodies are presented in Table 4.2 and the corresponding Western blots are shown in Figure 4.4. The TenBroek MP20 antibody was by far the most sensitive of all the antibodies trialled and is the most widely used antibody in the literature (TenBroek et al., 1992, Arneson and Louis, 1998, Grey et al., 2003). At the other extreme was the 2D10 which was raised in the mouse and, not surprisingly, did not work well on the mouse preparation. All MP20 antibodies exhibited 3 different bands. The higher molecular weight band at approximately 50 kDa is probably be the galectin-3-MP20 complex. Galectin-3 has a molecular weight of ~31 kDa and this band was eliminated in membrane preparations (see Figure 4.9) that were urea-washed to remove peripheral proteins (Gonen et al., 2000). The medium band is the full MP20 protein, while the lower band is the probably a truncation product.
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Table 4.2  Summary of MP20 antibody dilutions used for Western blotting. Most antibodies detected protein when used at 1:4000, but AbP3 antibody was not able to detect protein past 1:500 dilution. W = white/saturated signal.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>1:500</th>
<th>1:1000</th>
<th>1:2000</th>
<th>1:4000</th>
</tr>
</thead>
<tbody>
<tr>
<td>AbP1</td>
<td>W</td>
<td>W</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>AbP2</td>
<td>W</td>
<td>W</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>AbP3</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>AbP4</td>
<td>W</td>
<td>✓</td>
<td>✓✓</td>
<td>✓</td>
</tr>
<tr>
<td>TenBroek</td>
<td>W</td>
<td>W</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>2D10</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-Terminal</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-Terminal-Phos</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.4  Summary of Optimised Western blots of different MP20 antibodies. Left lane of the gel is the BenchMark™ Pre-Stained protein ladder, the red bar represent M_w 20 kDa. 1: TenBroek 1:4000 dilution. 2: Arneson AbP1 1:4000 dilution. 3: Arneson AbP2 1:2000 dilution. 4: AbP3 1:500 dilution. 5: AbP4 1:2000 dilution. 6: C-Terminal 1:1000 dilution. 7: C-Terminal-Phos 1:1000 dilution. TenBroek antibody gave the strongest signal with the highest dilution factor. The 2D10 antibody was raised in the mouse sample and did not perform well in protein preparation from the mouse lens. All the gels showed one or two strong bands approximately 20 kDa which correlates well with the MP20 protein staining patterns from past studies (Louis et al., 1989, TenBroek et al., 1992). Crude membrane preparations were used to optimise the dilution for the different MP20 antibodies and non-specific bands are observed. In urea washed protein preparations, the higher molecular weight bands were removed (Figure 4.9) suggesting that they represent Galectin-3 binding to MP20 protein.
4.2.2. Immunohistochemistry

The antibody dilution optimisation for IHC labelling was performed on whole rat lens serial sections to ensure that MP20 labelling patterns obtained using different antibodies were not due to biological variability. Due to the larger rat lens size, it was chosen over mouse, as a greater number of sections could be obtained in the zone of interest. Also when optimising the different antibodies for Western Blot in the previous section, it was noted that the 2D10 antibody raised against the mouse MP20 sequence did not perform well and would also give a lot of non-specific background if applied to mouse sections. Dilutions were initially prepared based on values previously used in other studies (Louis et al., 1989, TenBroek et al., 1992, Shiels et al., 2007). Figure 4.5 is an example of a dilution series performed using the TenBroek MP20 antibody in serial rat lens sections.

![Figure 4.5](image-url)

**Figure 4.5** Optimisation of MP20 IHC antibody labelling in rat lenses. Serial equatorial cryosections from a rat lens were labelled with the TenBroek MP20 Antibody used at different dilutions. A: 1:50; B: 1:100; C: 1:200; D: 1:500; E: 1:800. Scale bar = 500 μm.
TenBroek, 2D10, C-terminal and C-terminal phosphorylated MP20 antibodies were the most sensitive of the antibodies tested. Summarised dilutions for all eight MP20 antibodies are listed in Table 4.3, and the labelled sections are shown in Figure 4.6. The 2D10 antibody was not used in later studies, as it has been suggested to cross react with Cx50 (Louis, 2008), although no experiments were performed to verify this cross reactivity.

Most antibodies detected protein when used at 1:100, but the AbP1-4 antibody showed very weak staining. Louis’ 2D10 and Schey’s C-Terminal and C-Terminal-Phos antibodies performed well. The best and strongest labelling is by far the TenBroek antibody and which used for all subsequent MP20 protein labelling.

While optimising antibody labelling protocols is good scientific practice, it is still no guarantee that the antibody has high specificity (Rhodes and Trimmer, 2006) and is not producing some form of non-specific labelling. The best control against this is to utilise tissue from a transgenic animal, in which the gene of the protein of interest has been deleted (Saper and Sawchenko, 2003). In this regard we were fortunate to obtain a Lim2KO mouse in the final year of my project to further verify antibody labelling.
4.3. Use of the *Lim2* Knockout Mouse to Verify Antibody Specificity

Once the *Lim2*KO mouse colony was established, mice were genotyped to ensure that a pure line of animals was being used. The insertion of the gene trap vector into the *Lim2* gene (Figure 4.7 A) deletes part of the sequence, hence specific primer pairs (3F and Rv3) would amplify a shorter fragment (1.2 kb) in the *Lim2*Gt/Gt knockout mice (Figure 4.7 B), while the wild-type mice will have a larger fragment (1.6 kb) specific to primer 3F and 4R (Figure 4.7 B). If a mouse was only a heterozygous knockout, fragments specific to both primer 3F and 4R, and 3R and Rv3 would be present. Through genotyping, we were confident that the *Lim2*KO mice provided by the Bassnett laboratory did have the *Lim2* gene knocked out at the genetic level.

![Figure 4.7](image-url)

**Figure 4.7** Genotyping of the *Lim2*KO mouse. A: A schematic diagram showing the gene trap vector (VICTR48) and the site of insertion into the *Lim2* gene (approximately 500nt downstream from the end of exon3). B: DNA was extracted from tail clippings of transgenic and wild-type animals and PCR products amplified. Primers 3F and 4R are expected to amplify a 1.6 kb product specific to the *Lim2/+* allele, while the primers 3F and Rv3 should amplified a 1.2 kb product corresponding to the mutant allele specific to *Lim2*Gt/Gt (Shiels et al., 2007).
The Lim2KO lenses were extracted and examined under dark-field (Figure 4.8 A) and bright-field (Figure 4.8) microscopy to determine the extent of refractive error (Figure 4.8 B). In a healthy wild-type mouse, there is a uniform and gradual increment of lens refractive index throughout the lens. In the Lim2KO mouse there is a sudden change in refractive index at r/a ≈ 0.85 between the outer and inner cortex (Figure 4.8 A, red arrow), that compromises the optical properties of the lens as seen by the distortion of an EM grid placed beneath the lens (Figure 4.8 B). A small discrete nuclear pulverulent cataract is also observed in the centre of the lens (Figure 4.8 B, asterisk).

**Figure 4.8** Characterisation of the effect of deletion of Lim2 on the optical properties of the lens. Lenses were extracted from Lim2KO mice aged 8-10 weeks and imaged using dark-field (A) and bright-field microscopy (B). A: Dark-field microscopy reveals a distinct discontinuity at r/a ≈ 0.85 (red arrow) in the optical properties of the Lim2KO lens. B: Bright-field image of an EM hexagonal grid seen through the Lim2KO lens. The red arrow indicates the distinct zone where there is a sudden change in optical properties. The area below the asterisk indicates an area of nuclear cataract. Scale bar = 200 μm. EM Grid: 125 μm pitch, 100 μm hole and 25 μm bar.
4.3.1. *Western Blot*

The *Lim2KO* mouse is a good model to test the specificity of the antibodies raised for targeting the MP20 protein. Lens membranes were prepared from human, bovine, and both wild-type and *Lim2KO* mouse lenses, urea washed to remove peripheral proteins and Western Blotting performed. All the samples except for *Lim2KO* showed labelling around 20 kDa (Figure 4.9 A). To ensure that proteins were actually loaded into the well and the lack of signal in the *Lim2KO* lane was not an artefact, membranes were then stripped and re-labelled with an AQP0 antibody (courtesy of Professor Kevin Schey) at 1:1000 dilution (Figure 4.9 B). All lenses, including *Lim2KO* showed signal against the AQP0 antibody around 25 kDa.

![Figure 4.9](image)

**Figure 4.9** MP20 protein expression in different lens species. Western blotting of lens urea washed membrane preparations from wild-type mouse (Lanes 1); bovine (Lane 2); human (Lane 3); and *Lim2KO* mouse (Lane 4). A: The blot was probed with TenBroek MP20 antibody. B: The blot shown in A was stripped and re-probed with an antibody against AQP0. In both gels the red bar represents M_w 20 kDa on the ladder. Note that labelling at approximately 50 kDa observed in Western Blots performed using crude membrane preparation (Figure 4.4) is no longer present. Interestingly a band approximately 10 kDa is detected that is specific to the human lens, which could be due to either non-specific labelling or protein degradation. There is usually a long delay between the time of death and acquiring the human lens for crude membrane preparation, which could potentially contribute to protein degradation.

4.3.2. *Immunohistochemistry*

It is not uncommon for antibodies to perform well in Western Blotting experiments but lack specificity when used for IHC (Rhodes and Trimmer, 2006). To validate the specificity of the MP20 labelling obtained in the mouse lens sections, *Lim2KO* lens were labelled with the same MP20 antibody and a membrane marker WGA (Figure 4.10). The absence of labelling indicates that the MP20 antibody does not produce any non-specific labelling.
4.4. Differentiation-Dependent Insertion of MP20 Protein

Confident of the specificity of the MP20 antibodies, I now wanted to map the subcellular distribution of MP20 protein as a function of fibre cell differentiation in different regions of the mouse lens (Figure 4.11), to facilitate comparison with the rat lens (Figure 4.12). Consistent with labelling in the rat, the mouse lens showed a similar initial cytoplasmic MP20 labelling pattern in DF cells in the peripheral lens cortex (Figure 4.11 B), that was followed by a plasma membrane...
insertion (Figure 4.11 C) to yield extensive membrane labelling of MF cells deeper in the mouse lens (Figure 4.11 D). However, when compared to the rat, the switch from cytoplasmic to membranous labelling was located a lot closer to the lens surface (r/a ~0.98), and occurred in DF cells that contained cell nuclei (Figure 4.11 C). In the rat lens, membrane insertion of MP20 protein occurred at a greater depth into the lens (r/a ~0.80) (Figure 4.12 A), and coincided with a later stage of fibre cell differentiation that saw DF cells lose their cell nuclei and become MF cells (Figure 4.12 C). Thus my data suggested that the membrane insertion is a conserved feature of fibre cell differentiation, but the stage at which this occurs varies in different species. Whether MP20 protein insertion also coincides with the onset of the MDP in the mouse lens is investigated in the next section.

Figure 4.11  Differentiation-dependent changes in the subcellular distribution of MP20 protein in the mouse lens. A: An image montage showing an overview of an equatorial section through a wild-type mouse lens from the capsule to the lens core, showing the distribution of MP20 protein (red), membrane marker, WGA (green), and cell nuclei (blue). Boxes indicate the regions where the high power images (B, C, and D) were obtained. B: In peripheral nucleated DF cells MP20 protein labelling is predominately cytoplasmic. C: In a deeper region of the lens cortex a transition from cytoplasmic to membranous MP20 protein labelling is observed in DF cells that still contain nuclei. D: In MF cells that lack nuclei, strong regular membranous MP20 protein labelling is observed. Scale bars: 100 μm (A); 10 μm (B, C & D).
Figure 4.12  Differentiation-dependent changes in the subcellular distribution of MP20 protein in the rat lens.  
A: An image montage showing an overview of an equatorial section through a rat lens from the capsule to the lens core showing the distribution of MP20 protein (red), membrane marker, WGA (green), and cell nuclei (blue). Boxes indicate the regions where the high power images (B, C, and D) were obtained. B: In peripheral nucleated DF cells MP20 protein labelling is predominately cytoplasmic. C: At the DF/MF transition there is a shift from cytoplasmic to membranous MP20 protein labelling as cell nuclei degrade. D: In MF cells that lack nuclei strong regular membranous MP20 protein labelling is observed. Scale Bars: 100 μm (A); 10 μm (B, C & D).
4.5. Mapping the MDP formation in the mouse lens

4.5.1. Optimisation and Characterisation of TPEFP in the Mouse Lens

To date, the TPEFP data I have presented has been obtained in the rat lens. In this section, I first compare the intercellular diffusion of fluorescein released by TPEFP in the inner cortex of MF cells of the rat and wild-type mouse lens (Figure 4.13). In this region, since both GJs and the MDP contribute to intercellular communication, any potential differences in the uncaging properties and subsequent spread of the low molecular weight tracer that may exist between the two species can be assessed. It was apparent that relative to the rat lens (Figure 4.13 A), mouse fibre cells (Figure 4.13 D & G) have a narrower cross sectional profile. This morphological difference did not normally result in any obvious change in the fluorescein uncaging, the extent of dye spread, or the time-course of fluorescein spread in either the rat (Figure 4.13 B & C) or wild-type mouse (Figure 4.13 E & F) lens (Table 4.4). Thus it appears that the photochemistry of the release process of fluorescein is the same in both species. From this I can conclude that the uncaging profile of the fluorescein does not play a role in any differences observed when released in the two species. In the rat, both GJ and MDP mediated intercellular communication pathways exist in MF cells (r/a ~0.75) (Chapter 3), and similarly in the wild-type mouse (Shestopalov and Bassnett, 2003). The overall number of cells to which fluorescein spreads in all regions of the lens, appears to be different between the wild-type mouse and rat (Table 4.4). However, no significant differences were found when the cell spread was compared by regions (see Appendix C: for a full summary of statistical analysis performed).

Table 4.4 Summarised fluorescein spread properties between the wild-type rat and mouse.

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th>Wild-type Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rate of Uncaging (sec)</strong></td>
<td>2.47 ± 0.46 (37)</td>
<td>1.96 ± 0.11 (31)</td>
</tr>
<tr>
<td><strong>Mean Dye Spread (cell number)</strong></td>
<td>8.0 ± 0.9 (56)</td>
<td>5.2 ± 0.4 (32)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>DF</td>
</tr>
<tr>
<td></td>
<td>5.3 ± 0.6 (9)</td>
<td>5.0 ± 1.0 (9)</td>
</tr>
<tr>
<td></td>
<td>DF</td>
<td>MF</td>
</tr>
<tr>
<td></td>
<td>8.5 ± 1.0 (47)</td>
<td>5.2 ± 0.5 (23)</td>
</tr>
</tbody>
</table>
Figure 4.13  Optimising TPEFP parameters in the wild-type mouse lens. Comparison of TPEFP experiments to release fluorescein in MF cells (r/a ~0.75) in rat (A-C) and mouse (D-I) lenses showing cellular morphology (left panel), maximum projections (middle panel) and a graph of the time course of the change in normalised fluorescein signal intensity (right panel). In the mouse cell shown in G-I TPEFP uncaging occurs in two 1°-cells.
The narrower morphology of the inner cortex fibre cells in the wild-type mouse lens, meant that the TPE spot was not always restricted to only one cell, and on these occasions uncaging occurred in two cells simultaneously (Figure 4.13 G-I). While uncaging two 1°-cells provides a larger source of fluorescein for subsequent diffusion into 2°-cells, it did not result in an increase in the extent of fluorescein spread to other cells. The 2°-cells reached approximately 60% of the two 1°-source cells fluorescent intensity, as compared to approximately 50% in 2°-cells from single 1°-cells release (Figure 4.13 I). In the TPEFP experiments (Figure 4.13 E & H) that were undertaken in the inner cortex of the same mouse lens, a total of four cells were coupled to a single 1°-cell (Figure 4.13 E), whereas dye spread to five cells when the fluorescein was released via two 1° source cells (Figure 4.13 H). This finding further confirms that the extent of fluorescein diffusion between fibre cells is slower than the diffusion out of the focal plane along a fibre cell by an order of magnitude (Figure 3.8) (Cannell et al., 2004).

10 kDa-Fluorescein was introduced in Chapter 3 to map the onset of the MDP formation in the rat lens. This dye is too big to travel through GJ channels which have an upper molecular weight of 1.2 kDa (Gong et al., 1999). Therefore the 10 kDa-Fluorescein can only diffuse between cells via the MDP. Comparing the 10 kDa-Fluorescein releases in the rat and mouse lens, there were again no significant differences in the rate of uncaging (Table 4.5). Based on this, any difference observed between rat and mouse in 10 kDa-Fluorescein spread in other regions of the lens cannot be attributed to differences in the uncaging properties of the 10 kDa-Fluorescein.

### Table 4.5  Summarised 10 kDa-Fluorescein spread properties in wild-type rat and mouse.

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th>Wild-type Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of Uncaging (sec)</td>
<td>2.76 ± 0.5 (59)</td>
<td>2.53 ± 0.21 (44)</td>
</tr>
<tr>
<td>Mean Dye Spread (cell number)</td>
<td>1.2 ± 0.2 (62)</td>
<td>4.2 ± 0.3 (59)</td>
</tr>
</tbody>
</table>
4.6. Comparison of MDP Formation in the DF Cells of the Mouse and Rat lens

Having shown that the uncaging of fluorescein and 10 kDa-Fluorescein behaved the same in the inner cortex of the mouse and rat lens, I also wanted to compare the pattern of dye transfer in the DF cells of the outer cortex in the two species. The pattern of 10 kDa-Fluorescein spread obtained in the rat has been reproduced in Figure 4.14 to facilitate comparison with the mouse. In the mouse outer cortex, the uncaged 10 kDa-Fluorescein spreads freely to adjacent cells (Figure 4.14 A, Table 4.6), but is restricted in the rat source cell (Figure 4.14 B, Table 4.6), indicating that the MDP does not contribute to intercellular communication in the outer region of the rat lens. In contrast, in the MF cells, both the mouse (Figure 4.14 C, Table 4.6) and rat (Figure 4.14 D, Table 4.6) lens showed extensive 10 kDa-Fluorescein spread which suggests that the MDP contribute to intercellular communication in the majority of the fibres cells in the lens centre.

Table 4.6 Summarised 10 kDa-Fluorescein spread properties in wild-type rat and mouse in different regions of the lens.

<table>
<thead>
<tr>
<th>Mean Dye Spread (cell number)</th>
<th>Rat</th>
<th>Wild-type Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differentiating Fibre Cells</td>
<td>0.0 ± 0.0</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>Mature Fibre Cells</td>
<td>1.7 ± 0.3</td>
<td>4.3 ± 0.5</td>
</tr>
</tbody>
</table>
This difference in the differentiation-dependent onset of the MDP between species is also illustrated in Figure 4.15 where cell nuclei are used as markers of fibre cell differentiation to confirm relative measurements of distance into the lens. As presented previously for the rat lens (Chapter 3), no spread of 10 kDa-Fluorescein was observed in nucleated DF cells (Figure 4.15 A & B) until the cell nuclei had degraded, signalling the transition of DF cells to MF cells (Figure 4.15 C & D), an event that coincides with the membrane insertion of MP20 protein (Figure 4.12). In the mouse lens 10 kDa-Fluorescein spread was observed in all regions of the lens, including between nucleated DF cells (Figure 4.15 E & F). Equivalent regions (r/a 0.88 to 0.85) in the rat lens show no signs of MDP formation (Figure 4.15 A & B).
Unfortunately, due to the smaller size of the mouse lens, it was not possible to complete a full mapping of the onset of the MDP as it was technically difficult to retain the very outer layers of the mouse lens. Because of this constraint, only regions of r/a 0.88 and deeper could be obtained for TPEFP experiments to detect the onset of the MDP in these animals. GFP expression experiments indicate that the MDP forms at r/a ~0.92, (Shestopalov and Bassnett, 2003), which is similar to where I have shown MP20 protein undergoes membrane insertion (Figure 4.11). Hence, while I have not been able to perform TPEFP in the most peripheral DF cells of the mouse lens where one would expect the absence of a MDP, I have shown that the deeper DF cells located in a region of the lens that shows membrane labelling for MP20 protein also contains a functional MDP.
Figure 4.15  
Mapping of the differentiation-dependent formation of the MDP in the wild-type rat and mouse lens. TPEFP uncaging of 10 kDa-Fluorescein performed in the outer cortex and the inner cortex of a single cut rat (A-D) and mouse lens (E-H) to identify the stage of fibre cell differentiation at which the MDP forms. Images are RGB merged where, RED: fluorescent intensity; GREEN: transmitted light image of cellular morphology; and BLUE: nuclei marker, PI. Scale bars = 10 µm.
4.7. Summary

In this chapter I have:

- For the first time, fully characterised the MP20 protein expression in the mouse lens through IHC.
- Optimised the parameters required to perform TPEFP in the mouse lens.
- Confirmed previous findings using caged-10 kDa-Fluorescein and characterised the MDP in the mouse lens.

Taken together, my immuno-labelling results and TPEFP experiments in the mouse and rat lens all support the contention that insertion of MP20 protein into the plasma membrane is associated with the formation of the MDP, but that these events occur at different stages of fibre cell differentiation in the rat and mouse lens (Figure 4.16). Thus, it appears that the presence of MP20 protein is not only necessary for the formation of the MDP (Shi et al., 2009), but also that its insertion into the plasma membrane is required to initiate MDP formation. The initial cytoplasmic location of MP20 protein suggests that DF cells synthesise the MP20 protein and store it in the cytoplasm until a distinct stage of fibre cell differentiation; which differs in the rat and mouse lens, during which it is inserted into the plasma membrane allowing formation of the MDP. What the results do not show is the relative contributions that GJs and the MDP make to intercellular communication in the different regions of the lens. This is addressed in the next chapter by using transgenic animals to selectively remove the gap junction Cx46 and MP20 protein.
Figure 4.16  Overview of the intercellular pathways in the wild-type rat and mouse lens. A: Schematic of an equatorial section of the lens. B: Schematic of enlarged panel b in figure A. The translocation of MP20 protein from a vesicular storage pool to the plasma membrane is associated with the formation of the MDP in the rat (upper panel) and mouse (lower panel) lens, however the membrane insertion and MDP formation occur at different stages of fibre cell differentiation in the two species.
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By using TPEFP with caged-10 kDa-Fluorescein, the intercellular communication mediated by the MDP has been mapped in the rat (Chapter 3) and mouse (Chapter 4). It appears that a differentiation-dependent formation of the MDP is species-specific, albeit coincides with the transition of the MP20 protein from the cytoplasm into the membrane in both species (Chapter 4). Previously by using TPEFP with caged-fluorescein, intercellular communications were probed in different regions of the rat and mouse lens. Since this molecule is small enough to diffuse through both the GJ channels and the MDP, these two intercellular pathways could not be distinguished. In this chapter, I investigate the relative contributions made by the GJ and the MDP to the overall intercellular communication in the inner cortical DF cells of the mouse lens. To achieve this, I have performed TPEFP on lenses from Cx46<sup>−/−</sup> mice (Gong et al., 1998) to study the MDP in the absence of the GJ intercellular communication, and Lim2<sup>Gr/Gr</sup> mice (Shi et al., 2009) to characterise intercellular communication mediated by GJ channels in the absence of the MDP.

My results provide new insights into the relative contributions of GJ and MDP mediated intercellular communication, which allows their contributions to overall lens function to be discussed.
5.1. Intercellular Communication in Lenses Lacking Cx46

As outlined previously (Chapter 1), GJ coupling in DF cells is mediated by both Cx46 and Cx50, with each connexin isoform contributing up to half of the total intercellular conductance in this region of the lens (Figure 1.10) (Gong et al., 1998, Baldo et al., 2001). However, in the MF cells, in the inner cortex and core of the lens, it appears that Cx50 is rendered non-functional by the cleavage of its cytoplasmic tail at the DF/MF transition (Lin et al., 1998), hence GJ coupling is mediated solely by Cx46. These conclusions were obtained by performing impedance measurements in Cx46KO lenses which showed that the removal of Cx46 reduced the GJ conductance by half in DF cells (Figure 1.10) and completely abolished it in the MF cells (Figure 1.10) (Gong et al., 1998, Baldo et al., 2001). This loss of GJ coupling in the lens centre resulted in higher electrical resistance recorded between the MF cells, a finding that is not consistent with the presence of large low resistance membrane fusions that are believed to form the MDP. This suggests that either the MDP is not formed in the Cx46KO lenses, or the MDP does not form a low resistance intercellular diffusion pathway that links MF cells throughout the lens centre.

It was subsequently shown that crossing the Cx46KO mouse with a mouse line that expresses GFP, the MDP was still formed (Cheng et al., 2008). Hence, it appears that MDP mediated intercellular communication only couples fibre cells locally (Shi et al., 2009) and does not form a continuous intercellular pathway that links all the cells in the inner lens regions. Based on these findings from past studies, I would hypothesise that in the Cx46KO lenses, intercellular communication between DF cells should solely be mediated by Cx50 GJ channels; while in MF cells, cell-to-cell coupling will be mediated by the MDP only. To test these theories, I utilised TPEFP to release caged-fluorescein and 10 kDa-Fluorescein in different regions of Cx46KO lenses.

The pattern and extent of spread observed by uncaged fluorescein in the DF cells, was however similar in both wild-type and Cx46KO lenses (Figure 5.1). Since the MDP is also present in the mouse lens DF cells, any reduction in Cx46 coupling would have been masked by the presence of the MDP, hence no differences were observed between the wild-type and Cx46KO mouse lenses (Table 5.1).
Figure 5.1  Effect of removal of Cx46 on intercellular communication in the DF cells of the mouse lens. Representative maximum projection images obtained from wild-type (A) and Cx46KO (B) lenses showing uncaged fluorescein TPEFP experiments performed on DF cells (r/a=0.81). Scale Bar = 10 µm.

Table 5.1  Summarised uncaged fluorescein cell spread number in the differentiating fibre cells of wild-type and Cx46KO mouse.

<table>
<thead>
<tr>
<th>Differentiating Fibre Cells</th>
<th>Wild-type Mouse</th>
<th>Cx46KO Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Dye Spread (cell number)</td>
<td>4.0 ± 1.0 (9)</td>
<td>6.5 ± 1.2 (6)</td>
</tr>
</tbody>
</table>

TPEFP was then used to release the 10 kDa-Fluorescein dye in the MF cells located in the inner cortex of the Cx46KO lens (Figure 5.2). The pattern and extent of uncaged 10 kDa-Fluorescein spread in the wild-type (Figure 5.2 A) and Cx46KO (Figure 5.2 B) lenses was not significantly different (Table 5.2), suggesting that the MDP is not only present, but it is not affected by the removal of Cx46, confirming previous findings (Cheng et al., 2008).

Having confirmed the existence of the MDP in Cx46KO mouse, I then wanted to see if I could determine the contribution of Cx46 to the intercellular communication between MF cells. I subsequently compared the pattern and extent of uncaged 10 kDa-Fluorescein and fluorescein diffusion in the wild-type (Figure 5.2 A &C, respectively) and Cx46KO (Figure 5.2 B & D, respectively) lenses. My hypothesis was that the lower molecular weight fluorescein would exhibit a greater diffusion away from the 1°-cell in wild-type mouse, since it is able to travel through both GJ channels and the MDP. However, no significant differences in the extent of fluorescein release were observed between wild-type and Cx46KO lenses (Table 5.4). This
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indicates that in MF cells, the presence of the large membrane fusions thought to form the MDP dominates local intercellular diffusion between adjacent cells in both wild-type and Cx46KO lenses.

Figure 5.2 Effect of removal of Cx46 on intercellular communication in MF cells of the mouse lens. Representative maximum projects obtained from wild-type (A, C) and Cx46KO (B, D) mouse lenses showing TPEFP experiments performed on MF cells (r/a~0.75). A & B: 10 kDa-Fluorescein spread pattern in wild-type and Cx46KO lenses respectively. C & D: Fluorescein spread pattern in wild-type and Cx46KO lenses, respectively. Scale bar = 10 µm.
5.2. Intercellular Communication in Lenses Lacking MP20 Protein

Following the confirmation of the presence and functionality of MDP within Cx46KO lenses, I then wanted to examine the GJ mediated intercellular diffusion in the absence of the MDP. To achieve this, TPEFP was applied to lenses lacking MP20 protein due to the removal of the Lim2 gene, which has been shown to inhibit MDP formation (Figure 1.13) (Shi et al., 2009). Without the MDP, only Cx46 GJs should mediate the intercellular communication between inner cortical MF cells; while both Cx46 and Cx50 should mediate the intercellular communication in the outer cortical DF cells. To test these predictions, TPEFP experiments were performed in half cut Lim2KO lenses loaded with caged-compounds.

The Lim2KO lens bisection procedure, however, proved somewhat problematic. Lim2KO lenses were more difficult to cut and large areas of the outer cortical fibre cells were lost during the cutting process, this was consistent with a previous study that showed fibre cells dissociate more easily in the absence of the MP20 (Shiels et al., 2007). The loss of the outer cortex meant that it was impossible to compare GJ coupling between DF and MF cells using TPEFP in these lenses. Also, while performing TPEFP on cut Lim2KO lenses, it was noted that in the absence of MP20 protein, fibre cell membranes were more susceptible to damage from the two-photon LASER (Figure 5.3 C). I found that performing TPEFP in Lim2KO lenses caused a rupture of the membrane between neighbouring cells that resulted in the spread of 10 kDa-Fluorescein between MF cells (Figure 5.3 B). This unexpected result tended to suggest the continued presence of the MDP in the Lim2KO lenses. However, closer examination of this apparent spread to 2°-cells showed that the time taken to reach 67% of its maximum intensity in the 2°-cell in the Lim2KO was 14.58 sec, compared with 2.65 ± 0.23 sec for the wild-type mouse (Table 5.2.). This is too slow to be attributed by direct diffusion from the 1°-cell, and suggests that the observed 10 kDa-Fluorescein spread patterns were not due to the intercellular communication properties of the cell, but were a consequence of LASER induced membrane rupture within the Lim2KO lens.
Figure 5.3  Effect of uncaging with two-photon LASER of 40 mW input power on the membrane integrity of the Lim2KO mouse lens. A: Transmitted light image before uncaging experiment showing normal hexagonal cell morphology, the red arrow corresponds to the same arrow position as C, and also points towards the uncaged 1°-cell. B: Uncaged 10 kDa-Fluorescein maximum projection images showing dye “spread”. C: Transmitted light image showing the broadside of uncaged source cell membrane caved in following exposure to TPE. The red arrow indicates the collapsed membrane on one side. Note that the opposite membrane broadside also collapsed. Scale bar: 10 μm.

This inherent membrane fragility in the Lim2KO lenses necessitated a change in the uncaging protocol used in these lenses (Figure 5.4). To control for the inherent membrane fragility of Lim2KO lenses, the TPE LASER was deliberately positioned between two cells to induce uncaging in two 1°-cells thereby constraining any membrane damage to between the two 1°-cells. Relative to the standard single cell uncaging profile (Figure 5.4 A), the double cell uncaging (Figure 5.4 B) did not result in any transfer of 10 kDa-Fluorescein to adjacent 2°-cells. In contrast, applying the double cell uncaging protocol to wild-type mouse lenses produced substantial dye spread (Figure 5.4 C).
Figure 5.4  Optimisation of TPEFP protocols in Lim2KO lenses. A series of mages from a time-series taken at the times indicated showing the extent of 10 kDa-Fluorescein spread following TPEFP application to a Lim2KO (A & B) and a wild-type mouse lens (C). A: TPEFP was performed in a single cell from a Lim2KO lens. B: TPEFP was performed between two cells in a Lim2KO lens. C: TPEFP was performed between two cells in a wild-type mouse lens. At t=0 sec only a background fluorescence signal is observed as the TPE LASER is turned on. The LASER is subsequently turned off at t=24 sec and the signal dissipates over the next 84 sec.
All subsequent experiments in Lim2KO lenses were therefore conducted using the dual-cells uncaging protocol, and no intercellular diffusion of 10 kDa-Fluorescein in Lim2KO lenses was observed (Table 5.2).

Table 5.2 Summarised 10 kDa-Fluorescein spread properties in wild-type and Lim2KO mouse.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type Mouse</th>
<th>Lim2KO Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Dye Spread (cell number)</td>
<td>4.3 ± 0.4 (28)</td>
<td>0.0 ± 0.0 (18)</td>
</tr>
<tr>
<td>Rate of Uncaging (sec)</td>
<td>2.65 ± 0.23 (28)</td>
<td>3.33 ± 0.5 (18)</td>
</tr>
<tr>
<td>Rate of Diffusion (sec)</td>
<td>6.71 ± 0.35 (28)</td>
<td>-</td>
</tr>
</tbody>
</table>

Having verified that the absence of MDP in the MF cells within the Lim2KO lens, I then wanted to look at the diffusion properties of GJs within the MF cells that were previously indistinguishable using the uncaging of fluorescein with TPEFP in wild-type animals (Figure 5.5). Although fluorescein spread was observed between MF cells in Lim2KO lenses, indicating the presence of functional GJ channels, the extent of cell spread was significantly reduced by some 58% relative to wild-type mouse lenses (Figure 5.5 D).
Figure 5.5  Comparison of dye spread in the Lim2KO and wild-type MF cells (r/a~0.75).  A & B: Maximum projections of uncaged 10 kDa-fluorescein spread pattern in wild-type and Lim2KO mouse respectively.  C & D: Maximum projections of uncaged fluorescein spread pattern in wild-type and Lim2KO mouse respectively.  Scale Bar = 10 µm.
5.3. The Relative Contributions of GJ and MDP to Intercellular Diffusion

Having studied the GJ and MDP pathways by applying TPEFP to Lim2KO (GJ alone) and Cx46KO (MDP alone) lenses, I now wanted to compare the relative properties of the two pathways (see Appendix C: for a summary of statistical tests used). If I first consider the uncaging of 10 kDa-Fluorescein, no statistical significant differences were found in the time taken to reach 67% of normalised fluorescent intensity in 1°-cells across all experimental groups (rat and wild-type, Cx46KO and Lim2KO mouse) (Table 5.3), this suggest that TPEFP provides a similar and adequate supply of 10 kDa-Fluorescein for subsequent diffusion to 2°-cells across all groups of lenses. The subsequent spread of the uncaged 10 kDa-Fluorescein to other cells was also similar in both extent and time between control lens and Cx46KO lens, however 10 kDa-Fluorescein was significantly reduced in the Lim2KO lens, where absence of MP20 protein inhibited MDP formation (Table 5.3 & Figure 5.6).

Knocking-out the Cx46 protein had no statistically significant effect on the 10 kDa-Fluorescein diffusion in the Cx46KO mouse compared with wild-type mouse, indicating that MDP formation is normal and independent of Cx46 protein expression or the presence of functional GJ channels in the MF cells. This indicates that the presence of MDP is a conserved feature in rodent lenses.

Table 5.3 Summarised 10 kDa-Fluorescein spread properties in wild-type rat and mouse, and Cx46 and Lim2 KO mouse.

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th>Wild-type Mouse</th>
<th>Cx46KO Mouse</th>
<th>Lim2KO Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Dye Spread</td>
<td>2.3 ± 0.7 (33)</td>
<td>2.7 ± 0.2 (28)</td>
<td>2.5 ± 0.5 (6)</td>
<td>3.3 ± 0.5 (18)</td>
</tr>
<tr>
<td>Rate of Uncaging (sec)</td>
<td>1.71 ± 0.26 (39)*</td>
<td>4.33 ± 0.36 (38)</td>
<td>5.70 ± 0.77 (10)</td>
<td>0.00 ± 0.00 (26)*</td>
</tr>
<tr>
<td>Rate of Diffusion (sec)</td>
<td>6.66 ± 0.23 (33)</td>
<td>6.71 ± 0.35 (28)</td>
<td>7.87 ± 0.39 (6)</td>
<td>-</td>
</tr>
</tbody>
</table>

* Statistically significant (α = 0.05) when compared with the rest of the experimental groups.
Figure 5.6 Representative maximum projection images of uncaged tracer molecules in the MF (r/a~0.75) cells of the wild-type, Cx46KO and Lim2KO mouse lenses. Uncaged fluorescein (left panel) and uncaged 10 kDa-Fluorescein (right panel) in the wild-type (A & B), Cx46KO (C & D), and Lim2KO mouse lenses (E & F). Scale bar = 10 µm.
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TPEFP experiments performed with the smaller uncaged fluorescein also showed no statistical significant differences between experimental groups regarding the time taken to reach 67% of normalised fluorescent intensity in the 1°-cell within MF cells (Table 5.4 & Figure 5.6). This again suggests that the supply of fluorescein for subsequent diffusion from the source cell was the same across all experimental groups. No differences in the extent of released fluorescein spread were observed in the rat, and wild-type and Cx46KO mouse lenses, which are consistent with fluorescein being able to diffuse through both GJ channels and the MDP.

Unexpectedly, in the Lim2KO mouse, the removal of the MDP resulted in a statistically significant reduction in the number of cells coupled to the source cell (Table 5.4). This finding is consistent with a recent study in Lim2KO lenses that used impedance measurements to show GJ mediated intercellular conductance was reduced by approximately 55% (Shi et al., 2011). Unsure whether the reduction in conductance is based solely on the removal of the MDP, the group looked at the expression levels of Cx46 and Cx50. No change in Cx50 expression levels was observed, but a significant reduction in Cx46 abundance within the core of the Lim2KO lens was seen (Shi et al., 2011). The authors suggested that while Cx46 expression remains unchanged in the outer cortex, the presence of MDP could potentially aid the distribution or insertion of Cx46 deeper in the lens.

Table 5.4 Summarised fluorescein spread properties in wild-type rat and mouse, and Cx46 and Lim2 KO mouse.

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th>Wild-type Mouse</th>
<th>Cx46KO Mouse</th>
<th>Lim2KO Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Dye Spread (cell number)</td>
<td>2.6 ± 0.2 (34)</td>
<td>2.0 ± 0.2 (15)</td>
<td>2.2 ± 0.2 (13)</td>
<td>3.3 ± 0.3 (23)</td>
</tr>
<tr>
<td>Rate of Uncaging (sec)</td>
<td>8.47 ± 1.00 (56)</td>
<td>5.23 ± 0.47 (23)</td>
<td>5.26 ± 0.36 (41)</td>
<td>2.19 ± 0.26 (28)*</td>
</tr>
<tr>
<td>Rate of Diffusion (sec)</td>
<td>5.29 ± 0.31 (34)</td>
<td>5.62 ± 0.67 (15)</td>
<td>7.10 ± 0.67 (13)**</td>
<td>7.27 ± 1.08 (6)</td>
</tr>
</tbody>
</table>

* Statistically significant (α = 0.05) when compared with the rest of the experimental groups.

** Statistically significant (α = 0.05) when compared with the wild-type mouse.
Obviously, both MDP and GJ mediated pathways are important in maintaining intercellular diffusion in the lens. The diffusion rate of a molecule would be expected to be faster with the presence of two pathways, rather than with one pathway alone. With the removal of either GJs or the MDP, the transfer rate of fluorescein to 2°-cells is slower compared to the wild-type mouse (Table 5.4). Both GJ channels and MDP appear to act in parallel, and contributing equally to intercellular communication with no observed differences in the 2°-cells filling time rate between Cx46 and Lim2KO lenses.

The importance of Cx46 in mediating the intercellular communication is highlighted in the Cx46KO, where the time taken to fill the 2°-cell is significantly reduced in the Cx46KO mouse lens (Table 5.4). At a glance, it seems without MDP, the filling rate of the 2°-cells is also slower compared to the wild-type. Unfortunately, due to the small number of observations of fluorescein spread to 2°-cells obtained in the Lim2KO mouse, this result was not statistical significance. The reduced 2°-cells filling time in Cx46KO could be attributed by two things, firstly, diffusion through the GJ channel is faster than through the MDP; and secondly GJ channels are more abundant than the MDP.

As mentioned earlier, MDP has been suggested to couple the MF cells locally while GJ channels form a continues pathway that link all the MF cells together (Shi et al., 2009). Removing the Cx46 should restrict dye diffusion in MF cells to that modulated locally by MDP. This should be manifested as an overall reduction in the number of cells to which the diffusion would reach. However, the TPEFP technique developed in this thesis only examines the local diffusion of uncaged molecules and no differences were found. If there is an effect on intercellular communication on a global level through the removal of GJs, then the TPEFP technique would be unable to detect it. The global effect of GJ channels, nevertheless, has been shown through high impedance measurements with the complete abolishment of conductance in lens centre in Cx46KO mouse lenses (Gong et al., 1998, Baldo et al., 2001).
5.4. Summary

In this chapter I have:

- Used transgenic animals to show that the removal of Cx46 had no effect on either the formation of the MDP, or the extent of dye transfer mediated by it.
- Confirmed at the functional level, using novel high spatial and temporal resolution TPEFP technique that removal of MP20 protein inhibits MDP formation.
- Confirmed, using the same TPEFP technique that the MDP still forms in absence of Cx46 protein.
- In the absence of GJs the dye transfer via the MDP is rapid, suggesting that the MDP is a diffusive pathway.
- Visualised at the functional level, a reduction in GJ coupling with removal of MP20 protein.

It also appears, from my data that GJs and MDP mediate intercellular communication in parallel. Removal of either the GJs or the MDP in the DF cells caused a slower rate of filling of 2°-cells. Diffusion through the two pathways is indeed faster than diffusion through only one. It seems that the presence of MDP is fundamental to the expression of the Cx46 protein in deeper MF cells, since a significant reduction in the extent of dye spread was observed in the Lim2KO lens (Figure 5.7). Thus it appears that the MDP is a major contributor to local intercellular communication in the deeper regions of the lens.

Taken together, my data further confirms that MDP formation is dependent on the MP20 protein, and is consistent with the notion that MDP formation correlates with the membrane insertion of the MP20 protein. This transition zone from cytoplasmic to membrane MP20 protein has also previously been shown to coincide with the formation of an extracellular barrier in the rat (Grey et al., 2003). In the next chapter, I turn my focus from intercellular to extracellular pathways and investigate whether the species-specific differentiation-dependent insertion of the MP20 protein also coincides with the formation of a barrier to extracellular diffusion in the mouse lens.
Figure 5.7  
Summarised diagram of the uncaged fluorescein diffusion properties in the DF cells of various 
mouse types. In the Lim2KO mouse, there is a reduced filling time of 2°-cells and a decrease in 
extent of cellular spread by the uncaged fluorescein. While in the Cx46KO mouse, only a significant 
reduction of filling time of 2°-cells was observed with no change to extent of cellular spread.
Chapter 6: Correlating MP20 Membrane Insertion and the Extracellular Barrier

In the previous chapters, I concentrated on the relationship between the membrane insertion of MP20 protein and formation of the MDP. In this chapter, I turn my attention to the observations of Grey et al. (Grey et al., 2003), that showed in the rat lens the membrane insertion of MP20 protein, which occurs at the DF/MF transition when cell nuclei are lost, coincided with a restriction of the extracellular space (Grey et al., 2003). If we consider the MDP to represent a fusion of the cell membranes of adjacent cells, this implies that MP20 protein membrane insertion in addition to forming the MDP also narrows the extracellular space, so as to facilitate fusion formation between adjacent cells (Blumenthal et al., 2003). If this view is correct, like in the rat lens, the formation of the MDP should also cause a restriction of the extracellular space in the mouse lens. Since the MDP formation occurs at an earlier stage of fibre cell differentiation in the mouse lens, I would hypothesis that the extracellular diffusion barrier would also occur earlier in the mouse lens, prior to the loss of cell nuclei, if the membrane insertion of MP20 protein is indeed involved in the formation of this barrier.

Here, I have utilised CLSM to identify a change in membrane morphology that is present in wild-type, but not in Lim2KO lenses that could be responsible for the formation of the extracellular diffusion barrier. To test this, I have incubated wild-type and Lim2KO lenses in two different extracellular tracer molecules and monitored their penetration into the lens. These experiments demonstrated that a barrier to extracellular diffusion develops in the inner cortex of the mouse lens, but its formation neither coincides with MP20 insertion nor depends on the presence of MP20. While my data appear to rule out a major involvement of MP20 protein in the formation of the extracellular diffusion barrier, they highlight that the formation of a barrier to extracellular diffusion is a feature that needs to be incorporated into models of lens structure and function.
6.1. A Barrier to Extracellular Space Diffusion Exists in the Lens

By incubating rat lenses in 10 kDa Texas-Red-Dextran (\(M_w = 10\) kDa) and Lucifer Yellow (\(M_w = 456\) Da), Grey et al. (Grey et al., 2003) revealed a barrier to extracellular space diffusion that is formed some 400 \(\mu\)m (\(r/a \sim 0.80\)) in from the lens periphery (Figure 6.1 A). This apparent restriction of the diffusion of the extracellular tracer molecules coincided with the loss of cell nuclei and the membrane insertion of the MP20 protein (Figure 6.1 B). These observations suggest that the MP20 protein may, in addition to being involved in the formation of the MDP, also play a role in forming a barrier to the extracellular space diffusion of solutes.

Figure 6.1 Correlation of MP20 membrane insertion and the formation of a barrier to extracellular diffusion in the rat lens. Images of an equatorial section taken from a rat lens incubated in the extracellular space marker 10 kDa Texas-Red-Dextran (red) for 8 hr, and then labelled with an MP20 antibody (green). A: Overview of the extracellular tracer penetration in the lens and MP20 labelling pattern. B: Higher power view from the areas indicated by box b in panel A, showing the transition zone where MP20 protein becomes membranous and 10 kDa Texas-Red-Dextran diffusion via the extracellular space stops indicating the start of the outer boundary of the lens barrier to extracellular diffusion. Scale bars: 100 \(\mu\)m (A); 10 \(\mu\)m (B). Adapted from (Grey et al., 2003).

This barrier is not just restricted to the rat lens and also appears to be a feature of human lenses (Sweeney and Truscott, 1998, Lim et al., 2009). Incubating human lenses of different age in 10 kDa Texas-Red-Dextran also detected a barrier to extracellular diffusion, which was located 350 \(\mu\)m in from the lens periphery. While this is a similar distance to that observed in the rat lens, in human lenses the extracellular barrier formation occurred after the loss of fibre cell
nuclei (r/a~0.92), and this distance did not change as a function of lens age (Lim et al., 2009). Using MRI to detect water diffusion Truscott et al. (Sweeney and Truscott, 1998) identified an alternative diffusion barrier in human lenses that was located deeper into the tissue (r/a~0.69), and this distance again did not change with the age of the lens. The distinctly different locations of these two barriers to diffusion suggest they represent separate phenomena. An alternative explanation is that they represent an outer (r/a~0.92) and inner boundary (r/a~0.69) of the same diffusion barrier and with advancing age, while the relative positions of these boundaries stays constant, the width of the barrier increases as the lens grows. This barrier would effectively separate the outer and inner regions of the human lens, and therefore an alternative pathway to deliver nutrients to the lens centre that by passes this barrier would be required.

In the rat lens, the extracellular diffusion barrier observed by Grey et al. (Grey et al., 2003) would be equivalent to the outer boundary of the barrier identified in the human lens. To date, no inner boundary to the barrier has been identified in the rat lens. However, using an IHC method to map the regional distributions of cystine and glycine it has been shown that two distinct pools of the amino acids exist in the rat lens that appeared to be separated by a barrier, with an extracellular access pathway between the two pools being localised to the lens sutures (Lim et al., 2007). While it has been proposed that the formation of the outer boundary of this barrier is due to the membrane insertion of MP20 protein (Grey et al., 2003), this proposal has not been tested.

My findings that MP20 insertion in the rat and mouse lens occurs at different stages of fibre cell differentiation offers a system to test whether MP20 protein membrane insertion initiates the formation of the outer boundary of the extracellular diffusion barrier. If MP20 protein is involved in the formation of the barrier, the extracellular diffusion barrier would occur earlier in the wild-type mouse, and should be not formed in the Lim2KO lens. To test these predictions, I first tried to identify the area in the mouse lens where the barrier might be forming by comparing the membrane architecture of wild-type and Lim2KO lenses. Then I utilised two different extracellular tracers to locate the outer and inner boundaries of the barrier.
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6.2. Membrane Architecture

A previous study (Blankenship et al., 2007) that utilised scanning electron microscopy in 3-6 month old wild-type mouse lenses identified a sudden dramatic change in fibre cell membrane architecture (Figure 6.2). Using confocal microscopy to image mouse lens cryosections labelled with the membrane marker WGA, I was able to map these changes in membrane architecture. About 40 to 50 cell layers (~150 μm, r/a = 0.95) in from the peripheral of the mouse lens, there is an abrupt change in the arrangement of lens fibre cells, which is apparent initially as a loss of the parallel alignment of the fibre cell columns (Figure 6.3 A). This disorganisation continued for a further ~ 60 cells layer covering a zone some 150 to 350 μm from the periphery (r/a 0.95-0.87) (Figure 6.3 C). Having identified a transient zone of fibre cell column disorganisation, I then wanted to investigate whether membrane insertion of MP20 protein is involved in its formation and whether this zone represents formation of the barrier to extracellular diffusion detected previously in the rat and human lenses.

Figure 6.2 Differentiation-dependent changes in fibre cell arrangement in the mouse lens. 3D drawing illustrating wild-type fibre cells from the “irregular cell arrangement zone”, adapted from (Shi et al., 2011). The out of phased undulations of the fibre cells when view at different equatorial sections would be perceived as irregular cellular morphology.
With regard to the first question MP20 protein membrane insertion occurs at an earlier (r/a ~0.98) stage of fibre cell differentiation than the observed change in alignment of fibre cell columns (r/a ~0.95), suggesting that MP20 insertion is not involved in this change in fibre cell morphology. Nevertheless, a similar morphological analysis of fibre cell architecture reveals that this zone of irregular morphology is lost when MP20 protein is removed in the Lim2KO mouse (Figure 6.3 F). This confirms that the cell irregularities are not a sectioning artefact but related to the presence of MP20 protein. A similar result was recently obtained using scanning electron microscopy (Shi et al., 2011).

It is apparent that the presence of MP20 protein in the lens membrane may play an important role in the maintenance of this irregular architectural morphology, but the membrane insertion of MP20 does not appear to be required to initiate its formation. I wanted to use the species-specific difference in MP20 protein distribution between the rat and mouse lens to look further into the role of MP20 protein in this phenomenon. However, the change in fibre cell column arrangement appears to be species-specific to the mouse and was not detectable as an obvious change in fibre cell morphology in the rat lens with confocal microscopy (Figure 6.4).

To address the second question, whether the zone of irregular fibre cell columns represents the barrier to extracellular diffusion, I tried to correlate the penetration of extracellular space tracers to the change in fibre cell morphology detect in the mouse lens. To do this I utilised two extracellular tracers the conventional 10 kDa Texas-Red-Dextran and a novel lower molecular weight extracellular space marker Gd-DTPA, which I detected using a different imaging modality, Confocal Reflectance Microscopy (CRM). Before presenting my results, the development of the approach utilising Gd-DTPA and CRM is first introduced in the next section.
Figure 6.3  Irregular cellular architecture in the mouse lenses. Confocal images of an equatorial cryosection from wild type (A-D) and Lim2KO (E) lenses labelled with the membrane marker WGA. A: Image montage overview of the membrane structure of the wild-type mouse showing the start (red arrow) of a zone of irregular alignment of the fibre cell columns. B-D: Higher power images showing regular fibre cell column alignment in the outer cortex (B), the zone of irregular arrangement of fibre cell columns (C, blue arrow), and restoration of the regular alignment of fibre cell columns in the deeper lens (D). E: An image montage showing an overview of the membrane structure of Lim2KO lens showing no distortion of the fibre cell column order. Scale bars: 100 μm (A & F); 10 μm (B-E).
Figure 6.4  Regular cellular architecture in the rat lens. Confocal images of an equatorial cryosection from a rat lens labelled with the membrane marker WGA. A: Image montage showing an overview of regular cellular architecture. B-E: Higher power images of fibre cells at increasing depths into the lens show the alignment of fibre cell columns is maintained in the rat lens. Scale bars: 100 μm (A); 10 μm (B-E).
6.3. Use of Gd-DTPA and Confocal Reflectance Microscopy to map Barrier Formation

Gadolinium-Diethylene-Triamine-Pentaacetic-Acid (Gd-DTPA, MW = 0.57 kDa) is a chelated form of gadolinium that is used as a non-toxic Magnetic Resonance Imaging (MRI) contrast agent (Vexler et al., 1994). In currently unpublished studies performed by others in the Molecular Vision Lab, Gd-DTPA has been utilised as a tracer molecule to study extracellular solute fluxes in bovine lenses using T1-weighted MRI to visualise its penetration into whole lenses in vitro (Figure 6.4). Using this non-invasive imaging approach, a distinct barrier to Gd-DTPA penetration into the bovine lens was observed. An advantage of Gd-DTPA is that it is electron dense, and in preliminary studies conducted by others in our laboratory, we have shown in the bovine lens that it is fixable and can be detected in lens sections using CRM confirming the presence and absence of Gd-DTPA in the extracellular space in different regions of the lens (data not shown). I wanted to see if Gd-DTPA could also be used as tracer of extracellular diffusion into the mouse lens. To achieve this, I need to first optimise the CRM imaging parameters to ensure that the reflected light collected was due to the presence of Gd-DPTA and not due to random background reflections from the specimen and/or internal mechanics of the microscope.

Figure 6.4 Visualising a barrier to solute diffusion in the bovine lens. An axial T1-weighted MRI image of the penetration of the contrast agent Gd-DTPA into a bovine lens. The red arrow indicates the outer boundary of the extracellular barrier to diffusion (r/a~0.8) after 4.5 hr incubation with Gd-DTPA. The yellow arrow indicates the inner boundary of the extracellular diffusion barrier (r/a > 0.55). Unpublished image kindly provided by Dr. Ehsan Vaghefi. Scale bar: 3 mm.
CRM involves illumination with a chosen LASER wavelength and the collection of the total spectrum of light reflected back from the specimen. Since the lens is an optically transparent tissue, the amount of reflected light is usually relatively low, but is not zero. By incubating lenses in the electron dense compound Gd-DTPA, proposed that we would see an increase in the amount of reflected light in areas where Gd-DTPA is accumulated. To test this contention, equatorial sections taken from lenses that incubated in the presence of Gd-DPTA were imaged using CRM at different LASER powers (% of attenuation) (Figure 6.5). At the lowest attenuated LASER power settings (Figure 6.5 A-C), minimal reflection of the LASER light was observed and no obvious cellular structure could be determined. However, at higher attenuated LASER power settings (Figure 6.5 D-G), the higher energy excitation light produced greater reflection intensity that began to allow cellular morphology to be distinguished. Further increases in LASER power however, lead to increases in the background levels of reflected light and produced artefacts that obscured fibre cell morphology (Figure 6.5 G).
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Figure 6.5  **Effect of increasing LASER power on CRM imaging.** Images were collected from the outer cortex of an equatorial section taken from a mouse lens using the LSM 710 inverted CLSM with LASER powers settings of (number represents % of LASER attenuation) A: 99, B: 98, C: 97, D: 96, E: 95; F: 90, and G: 80. H: The same section labelled with the membrane marker WGA to show lens morphology. Scale bar: 100 μm.

To determine whether the reflections produced were in fact due to the presence of Gd-DTPA, lens sections incubated in the presence and absence of Gd-DTPA were examined by CRM using a constant LASER power setting (Figure 6.6). In the absence of Gd-DPTA, CRM produced artefactual reflections from the mounting medium and also the tissue itself, but no obvious morphological details were observed (Figure 6.6 A). In contrast the reflections observed in presence of Gd-DPTA were distinctly different and highlighted the membrane morphology of the lens (Figure 6.6 B).
Figure 6.6 Gd-DTPA highlights cellular morphology. CRM images of equatorial cryosections taken from lenses that were incubated in the absence (A) and presence (B) of Gd-DTPA for 1 hr using a LASER power setting of 4. The red arrow indicates the capsule. Scale bar: 100 μm.
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Have optimised CRM based imaging protocols to detect Gd-DTPA, I then used it to map the penetration of this extracellular tracer molecule in the wild-type mouse lens (Figure 6.7). A CRM overview image of half of wild-type mouse lens shows that the reflections presumably produced by Gd-DTPA, show that the molecule penetrated radially into the lens to a depth of around 500 μm, by 1 hour (Figure 6.7 A). Doubling the incubation time to 2 hr increased the signal intensity, but did not increase the extent of Gd-DTPA diffusion into the lens (Figure 6.7 B), indicating that the penetration of Gd-DTPA is not diffusion limited. This conclusion is supported by higher magnification images that allow lower levels of Gd-DTPA to be detected in the lens core (Figure 6.7 C). The presence of two areas of Gd-DTPA induced reflections separated by zone of Gd-DTPA exclusion (Figure 6.7 C, asterisks) effectively defines the outer and inner boundaries of a barrier to the extracellular diffusion of solutes.

The subcellular distribution of Gd-DTPA in the extracellular space is dramatically different on either side of the extracellular diffusion barrier. In the outer cortical region, Gd-DTPA reflections are restricted to the extracellular space on the narrow sides of fibre cells and manifested as three discrete points of signal intensity (Figure 6.7 E). By comparing the CRM images with those obtained for the membrane marker WGA, it is evident that these localised points of Gd-DTPA labelling align with the vertices of the hexagonal fibre cells and correlate with the position of the ball-and-socket inter-digitations previously shown using high resolution Scanning Electron Microscopy (Blankenship et al., 2007). Deeper into the lens in the area where the fibre cell column alignment becomes disorganised, the CRM signal increases in intensity (Figure 6.7 F), but becomes more diffuse suggesting that in addition to the presence of Gd-DTPA the irregular arrangement of the fibre cells may also contribute to the reflections produced in this area of the lens. Within the barrier region the images collected by CRM were not above background suggesting that in this area Gd-DTPA is excluded from the extracellular space (Figure 6.7 G).
Figure 6.7  Mapping the penetration of the extracellular tracer Gd-DTPA into the wild-type mouse lens. Equatorial sections from mouse lenses incubated in Gd-DTPA (red) and labelled with the membrane marker WGA (green). Overview of a half wild-type mouse lens incubated in Gd-DTPA for 1 hr (A) and 2 hr (B), with zoomed-in image in panel c. C: Zoomed in imagine of A, asterisks mark the boundaries of the diffusion barrier. D: Double label of C, with zoomed-in images in panels E-G. E: Zoomed-in image of the outer cortex showing the tri-point signal. F: Areas of irregular membrane morphology with strong Gd-DTPA reflectance signal. G: Area where the Gd-DTPA signal drops-off, meeting the extracellular space barrier. Scale bars: 500 μm (A & B); 100 μm (C & D); 10 μm (E-G).
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Having established the pattern of Gd-DTPA penetration into the mouse lens, I wanted to compare it to the pattern of MP20 protein labelling (Figure 6.8). Since MP20 membrane insertion occurs at an earlier stage of differentiation in the mouse (Figure 6.8 A, red arrow), if the outer boundary formation of the extracellular space diffusion barrier was due to MP20 protein insertion, the barrier should have formed at a distance of around 100 μm in from the periphery. However, the barrier formed much deeper into the lens at approximately 500 μm (~r/a 0.60) (Figure 6.8 B), suggesting that MP20 protein insertion and barrier formation are unrelated. To confirm this finding, Lim2KO mouse lenses were also incubated in Gd-DTPA and the penetration of the tracer into the lens visualised by CRM (Figure 6.8 C). In the absence of MP20 protein, the barrier (r/a ~0.60) still formed, suggesting that MP20 protein is not directly involved in the formation of the extracellular diffusion barrier. With the CRM images, there appears to be reduced signal intensity in the outer cortex relative to the deeper inner cortex (Figure 6.8 B & C)

To confirm the location of the outer boundary of extracellular diffusion barrier founded by the Gd-DTPA with CRM in the mouse, lenses were also incubated in Texas-Red-Dextran for 2 hr. Subsequent spread of this larger extracellular tracer into the lens was monitored by conventional CLSM (Figure 6.8 D), and showed the outer boundary of the extracellular diffusion barrier was found to also form some 500 μm into the lens. Thus my results showed that in the mouse lens, a barrier to extracellular diffusion is formed but is not related to the membrane insertion of the MP20 protein.
Figure 6.8  Mapping the onset of the outer boundary of the extracellular diffusion barrier in the mouse lenses. Equatorial sections from lenses incubated in the presence of extracellular space tracers Gd-DTPA (A-C) and 10 kDa Texas-Red-Dextran (D).  A: Section from a wild-type mouse lens labelled with an MP20 antibody showing the onset of MP20 membrane insertion (red arrow) and area of fibre column misalignment (white arrows).  B: CRM image of Gd-DTPA penetration in a section from a wild-type mouse.  C: CRM image of Gd-DTPA penetration in a section form a Lim2KO lens.  D: Standard CLSM image of 10 kDa Texas-Red-Dextran penetration in a section from an incubated wild-type mouse lens. Scales are in μm.
6.4. Summary

In this chapter I have:

- Developed a novel imaging technique that allows the extracellular space to be imaged in the lens.
- Identified a barrier to extracellular space diffusion in the wild-type mouse lens and showed that the formation of the barrier succeeded the MP20 protein membrane insertion.
- Confirmed the finding that knocking out the Lim2 gene removes the “zone of irregular fibre cell arrangement” in the mouse lens.
- Used the Lim2KO model to show for the first time that the extracellular diffusion barrier is independent to the presence of the MP20 protein.

Early studies had attributed the formation of the zipping-up of the extracellular space to the membrane insertion of the MP20 protein in the rat. It has been shown using real-time imaging technique (Chapter 3) that a MDP also forms in the same site as the extracellular barrier. However, through dye-incubation experiments with 10 kDa Texas-Red-Dextran and Gd-DTPA in the wild-type mouse and Gd-DTPA in the Lim2KO mouse, my results show that the formation of this barrier to extracellular diffusion barrier does not require the presence of MP20. These findings on the relative locations of MP20 insertion, MDP onset and the formation of the extracellular space diffusion barrier are summarised in Figure 6.9. This raises two questions: 1) What proteins are associated with the formation of this extracellular diffusion barrier?; and 2) What role does it serve in overall lens function? The techniques I have introduced will facilitate future experiments to address the former question, while the latter will be discussed in the next chapter.
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Figure 6.9  Summary of species differences in extracellular and intercellular diffusion pathways in the rat and mouse lens. A: Schematic of an equatorial section of the lens. B: Schematic of close up of panel b in figure A. The rat lens (upper panel) both the MDP and extracellular space diffusion barrier formation coincide with the loss of cell nuclei, and the membrane insertion of MP20 at the DF/MF transition suggesting the phenomenon are related. However, in the mouse lens (bottom panel) although MDP formation and MP20 insertion appear related, the formation of the barrier to extracellular space diffusion occurs at a much later stage of differentiation in the mouse lens.
Chapter 7: Discussion

In this thesis, I have used a variety of microscopy techniques to probe the structure and function of intercellular and extracellular diffusion pathways in different regions of rodent lenses to produce a map of these pathways in the rat and mouse lens (Figure 7.1).

My key findings include:

- The development of TPEFP protocols to distinguish between GJ and MDP mediated communication in different regions of rodent lenses.
- The first identification of the existence of the MDP in the rat lens and independent confirmation of the MDP in the mouse lens.
- Establishment that movement through the MDP is mediated by passive diffusion.
- Demonstration that MP20 membrane insertion is associated with the formation of the MDP in both the rat and mouse lens.
- Utilisation of transgenic animals to show that the MDP dominates local intercellular communication.
- Confirmation that deleting the Lim2 gene reduces Cx46 mediated intercellular communication in MF cells.
- Identification of a barrier to extracellular space diffusion the formation of which appears to be independent of the expression of MP20 protein.

In this chapter, I will discuss the significance of these findings and how they can be integrated into the lens circulation model to enhance our current understanding of how the structure and function of lens fibre cells contribute to the transparent and optical properties of the lens.
Figure 7.1  Intercellular and extracellular diffusion pathways in the rat and mouse lens.  A: Schematic of an equatorial section of the lens.  B: Schematic of close up of panel b in figure A.  MDP formation is initiated by membrane insertion of MP20 protein expression which occurs at different stages of fibre cell differentiation in the rat (top panel) and mouse (bottom panel) lens.  Once formed the MDP connects cells within discrete growth rings facilitating local diffusion of large proteins and macromolecules.  Adjacent growth rings are in turn coupled by GJ channels that form an intercellular communication pathway that connects MF cells in the lens centre with DF and epithelial cells in the lens periphery.  A barrier to diffusion via the extracellular space (yellow) forms at the transition zone between DF and MF cells in the rat, but occurs deeper in the mouse lens suggesting that the barrier formation is independent of MP20 protein insertion.
Chapter 7: Discussion

7.1. The MDP: Properties, Formation and Significance

In this project, I have utilised TPEFP to show that the formation of an intercellular communication pathway that is permeable to large macro-molecules is an integral part of lens fibre cell differentiation (Figure 7.1). The existence of such a pathway was originally thought to be formed by the degeneration of inner fibre cell membranes, and was first proposed to explain the uniform membrane potential observed in the lens (Duncan, 1969). This proposal was also supported by the observation of membrane fusions between fibre cells in the lens core (Kuszak et al., 1985). However, subsequent impedance measurements of the membrane properties of lens yielded high membrane resistances throughout the lens, indicating that the lens fibre cells could not have been connected by a network of low resistance membrane fusions (Rae et al., 1970, Rae, 1973, Eisenberg and Rae, 1976, Mathias et al., 1997). Instead it was proposed that an abundant network of GJs mediate intercellular diffusion between the different regions of the lens. The more recent identification of the MDP using transgenic mice expressing GFP (Shestopalov and Bassnett, 2003) has raised questions about the relative roles of GJs and the MDP, and prompted a re-investigation of the role membrane fusions play in mediating intercellular communication in the lens.

My results offer new insights into these questions. While Bassnett et al. (Shestopalov and Bassnett, 2003) have speculated that the MDP is formed by the fusion of neighbouring plasma membranes, their use of transgenic animals expressing GFP does not afford the spatial or temporal resolution to directly test this hypothesis. The use of TPEFP to create a point source of a large macro-molecule like 10 kDa-Fluorescein provides the necessary spatial and temporal resolution to observe the properties of the MDP in real-time. This analysis showed that the rate of intercellular dye transfer via GJ channels and MDP were not significantly different (Table 5.4), supporting the contention that the MDP consists of an aqueous pore that permits the free diffusion of large macromolecules between adjacent cells. The existence of such a large diffusive pathway is however, not consistent with the high membrane resistance recorded by impedance analysis in MF cells (Rae et al., 1970, Rae and Blankenship, 1973, Eisenberg and Rae, 1976, Mathias et al., 1997). It should however be noted, that the TPEFP technique is only capable of measuring local intercellular diffusion between no more than 4 to 6 cells. This is due to the rapid loss of released tracers from the focal plane due to high diffusion rates along the cytoplasm of fibre cells away from the focal plane (Figure 3.8). Thus, it is conceivable, that the
Chapter 7: Discussion

MDP is only a local low resistance pathway. This view is supported by experiments that utilised a transgenic animal in which GFP expression was turned on at day 29 of age through Tamoxifen injection (Shi et al., 2009). In these lenses, induction of GFP expression revealed distinct concentric rings of GFP expression that were approximately 10 cell layers thick (Figure 7.2 A). This pattern of GFP expression suggests that the MDP only mediates intercellular communication within each distinct growth ring. In this model, GJ channels form a continuous intercellular pathway for the movement of ions, water and small solutes from the lens core to outer cortex and serve to couple adjacent growth rings in which the local diffusion of large GJ impermeable macromolecules is mediated by the MDP (Figure 7.2 B). This model is consistent with impedance studies conducted in Cx46KO mice that showed that removal of Cx46 produced a complete loss of intercellular coupling as indicated by the recording of very high junctional resistances (Gong et al., 1998, Baldo et al., 2001), even though the MDP was still present in Cx46KO lenses (Cheng et al., 2008).

The existence of two parallel intercellular communication pathways with distinctly different permeability properties has important implications for the establishment of the refractive index gradient that contributes to the overall optical properties of the lens (Figure 7.2 B). Recently, our laboratory has implicated the lens circulation system in the establishment of this refractive index gradient through active maintenance of a low water content in the centre of the lens relative to the outer cortex (Vaghefi et al., 2011). The intercellular fluxes that are required to establish this water gradient are thought to be mediated by GJ channels that are permeable to ions, water and small solutes, but not large proteins (Gao et al., 2011). The ability of these GJ mediated water fluxes to create the local changes in protein concentration necessary to establish a gradient of refractive index, is in turn enhanced by the presence of the MDP. The local coupling of fibre cells by the MDP ensures that the intracellular protein concentration of the cells within an individual growth ring are the same, but allows the protein concentration in adjacent growth rings to be different. Thus the removal of ions and water via GJ channels will establish different protein concentrations in each individual growth ring, and will therefore actively establish the gradient of refractive index that improves the optical performance of the lens (Artal and Guirao, 1998).
Figure 7.2 Functional significance of parallel intercellular communication pathways in the lens.  A: The conditional expression of GFP by Tamoxifen reveals that the MDP mediates local diffusion within discrete growth rings. Image is adapted from (Shi et al., 2009).  B: A schematic representation of area indicated by box b, showing MDP mediated diffusion (yellow arrows) in three different growth rings that are interconnected by a GJ mediated diffusion pathways (red arrow). The discrete rings help create a protein concentration gradient that is highest in the lens centre (darker blue) and decreasing towards the lens periphery (lighter blue). This in turn, creates a gradual gradient of refractive index that enhances the optical properties of the lens. Scale bar: 250 μm.

If the refractive index gradient in the lens is in fact formed by distinct concentric growth rings of different protein concentration, then disruption of either the MDP or GJ channels should result in a change in the optical properties of the lens. This view is supported by examination of the cataract phenotypes produced in transgenic animals in which the Lim2 and Cx46 genes have been deleted (Figure 7.3). Lim2KO lenses are still relatively transparent, but exhibit a sudden step change in the refractive index in the region where MDP normally would have been formed.
Subsequent analysis of the optical properties of these lenses by ray tracing experiments showed that they were unable to correctly focus light suggesting that the loss of MDP affects the optical properties of the lens (Shiels et al., 2007). In contrast, Cx46KO lenses showed normal growth and development, but developed a severe nuclear cataract (Figure 7.3 C), which impacts on its transparent properties (Gong et al., 1997). This cataract phenotype has been attributed a loss of intercellular communication between the lens core and cortex that leads to an increase in [Ca\textsuperscript{2+}], in the nuclear region of the lens. This accumulation of [Ca\textsuperscript{2+}], in turn activates calcium-dependent protease that cleaves \(\gamma\)-crystallins promoting aggregation and the observed light scattering (Gong et al., 1999). Interestingly, a closer examination of the Cx46KO cataract phenotype reveals distinctive concentric white “rings” of light scattering of differing severity. Thus, it appears that in the absence of GJ coupling, the cataractic insult of [Ca\textsuperscript{2+}], accumulation is spread within an individual growth ring by the MDP to yield a pattern of concentric rings of opacity of differing severity. Taken together these observations tend to support my contention that GJ channels and the MDP mediate intercellular communication over different cellular ranges, but together interact to preserve the transparency and enhance of the optical properties of the lens.

I have also been able to confirm that MP20 expression is involved in the formation of the MDP by showing that the intercellular diffusion of 10 kDa-Fluorescein is abolished in Lim2KO lens. Interestingly, I found that GJ mediated coupling in the Lim2KO lenses is reduced by approximately 58% (Figure 5.6 & Table 5.4) a result that is consistent with recent impedance measurements performed on Lim2KO lenses (Shi et al., 2011) that revealed a 55% reduction in
GJ conductance. To determine what was responsible for this reduction intercellular coupling, these authors examined the relative expression levels of the GJs, Cx46 and Cx50, in Lim2KO lenses. While no change in Cx50 expression levels was detected, a significant reduction in Cx46 abundance in the core of the Lim2KO lens was observed (Shi et al., 2011). In contrast, the deletion of Cx46 had no apparent effect on the formation of the MDP in this present study (Figure 5.6 & Table 5.3) or a previous study (Cheng et al., 2008). These results show that although Cx46 does not affect MDP formation, the MDP can alter Cx46 expression in deeper fibre cells.

Perhaps the most novel finding from my thesis is the correlation of the formation of the MDP with the differentiation dependent insertion of MP20 into the membrane. This indicates that the formation of the MDP is a programmed event of fibre cell differentiation rather than some form of random degeneration of the fibre cell membranes. However, what role MP20 protein actually plays in the formation of the MDP remains unknown. My data on the rate of 10 kDa-Fluorescein transfer via the MDP suggests that the membrane insertion of MP20 triggers the formation of some sort of fusion between adjacent cells to form a cytoplasmic bridge or diffusion pore that can mediate the rapid intercellular diffusion of large molecules. How MP20 membrane insertion mediates the formation of this membrane fusion event is unknown, but based on the membrane fusion literature it probably involves interaction with other proteins (Blumenthal et al., 2003, Jahn et al., 2003). In this regard, the first extracellular loop of MP20 has a similar homology to members of the PMP-22/EMP/Claudin (Pfam00822) protein family (Maher et al., 2011), that are known to play a role in cellular adhesion. Furthermore, the extracellular loop of MP20 has been shown to interact with an adhesion modulator, Galectin-3 (Gonen et al., 2001). Hence, it is interesting to speculate that following the membrane insertion of MP20, the subsequent binding of Galectin-3 to the extracellular domains of MP20 could pull in the plasma membranes of adjacent cells close enough to induce membrane fusion (Grey et al., 2009). If this indeed is the case, it will be interesting to see whether galectin-3 protein also co-localises with the MP20 membrane insertion in the mouse lens to form the MDP.
7.2. The Barrier to Extracellular Diffusion

Using confocal microscopy to image the penetration of extracellular space tracers into the lens I have been able to visualise the outer and inner boundaries of a barrier to extracellular diffusion. Based on data from the rat that showed this barrier to extracellular diffusion coincided with the membrane insertion of MP20 I speculated that this boundary would occur earlier in the mouse lens if MP20 insertion was involved in the formation of the extracellular diffusion barrier. However, I found that the barrier formed deep into the inner cortex of the mouse lens at a much later stage of fibre cell differentiation (Figure 6.7) than seen in the rat (Figure 6.1). In addition, the extracellular diffusion barrier still formed in the Lim2KO lens, confirming that MP20 protein is not involved in the formation of this barrier.

This of course raises the question of what is driving the formation of the outer boundary of the extracellular diffusion barrier. One potential candidate is AQP0, a multi-functional protein that can act as both a water channel and an adhesion protein (Grey et al., 2009). Like MP20 protein, AQP0 undergoes an abrupt change in its membrane distribution in the rat lens at the DF/MF cell transition as cell nuclei are lost, which coincides with the formation of the extracellular diffusion barrier. It has been proposed that AQP0 undergoes post-translational modifications that signal a switch in function from a water channel to a junctional protein that restricts extracellular diffusion (Grey et al., 2009).

Using the lower molecular weight extracellular tracer Gd-DTPA I have also detected the inner boundary of the extracellular diffusion barrier (Figure 6.8). The presence of Gd-DTPA in the lens centre suggests that the delivery to this region is via a pathway that bypasses the extracellular diffusion barrier. I have assumed based on the work of others in our laboratory (Figure 7.4), that Gd-DTPA enters the lens centre via the sutures and then diffuses from the inside out until it reaches the inner boundary of the extracellular diffusion barrier. These observations suggest that the extracellular space inside this inner barrier boundary is open to the diffusion of solutes. In the rat lens, the cytoplasmic tail of AQP0 undergoes an abrupt truncation in this region of the lens (Grey et al., 2009). In future experiments, it would be interesting to co-localise AQP0 labelling with Gd-DTPA mapping of the extracellular diffusion barrier to determine whether AQP0 truncation and opening up of the extracellular space are related. While these experiments were out of the scope of my PhD project, I have developed the techniques to make them feasible to undertake.
7.3. New Information for the Microcirculation System

It is believed that lens transparency is maintained by an internal microcirculation system that delivers nutrients and removes waste products faster than would occur via passive diffusion alone (Mathias et al., 1997, Mathias et al., 2007). In this section, I integrate some of my findings into this model. The circulation system is thought to be generated by a circulating flux of Na\(^+\) ions that enter the lens via the extracellular space, and then crosses into the fibre cells before leaving the lens via an intercellular route mediated by GJ channels. My mapping of a barrier to extracellular space diffusion raises questions about the directionality of extracellular space ion fluxes in different regions of the lens. It could be that the barrier I observe has a size cut off that does not restrict the movement of ions. Alternatively, the barrier could also be tight to ion flow, and therefore effectively isolates the inner lens core from the outer cortex where ion and solute entry via the extracellular space is un-restricted. But at a certain depth zipping up of the extracellular space prevents further movement into the lens via this pathway. In this regard, it is envisaged that the sutures provide an alternative pathway that bypasses this extracellular space barrier providing a route to allow ions, fluids and solutes to be delivered to the lens core (Lim et al., 2009, Vaghefi et al., 2011). Within the lens core, it seems that the ability of Gd-DTPA to diffuse via the extracellular space is restored as Gd-DTPA is detected within the inner boundary of the barrier (Figure 7.4). Thus the roles of extracellular space diffusion barrier and the lens sutures in directing ionic and fluid fluxes in the lens need to be considered in future models of lens structure and function.
Figure 7.4  **Summary of ion and fluid fluxes in the lens.** In the outer cortex, ion and water fluxes (red arrows) can be mediated by passive diffusion but will only penetrate as far as the outer boundary of the barrier (dotted green lines). Directed fluxes driven by the circulation system (blue arrows) enter the lens at the poles and move circumferentially towards the equator in the outer cortex. Ion and fluid fluxes are also delivered to core via the sutures where they diffusion via the extracellular space towards the outside of the lens until they reach the inner boundary of the barrier (dotted green lines). Unpublished image kindly provided by Dr. Ehsan Vaghefi.
Similarly the relative roles of GJs and the MDP need to be incorporated into the lens circulation model. Of importance here is the growing realisation that the circulation system is not only involved in maintaining the transparent properties of the lens, but also its optical properties (Vaghefi et al, 2011). The refractive index gradient in the lens is vital for emmetropia (Smith et al., 2001) and to maintain a high refractive index in the lens centre, the water-to-protein-ratio must remain low (Babizhayev et al., 2002). Indeed, since elevated free water in lens centre has been found in cataractous lenses (Racz et al., 1983), such cataract-induced increase in free water can be mimicked in vitro by incubating lenses in high potassium solutions. This protocol depolarises the lens and inhibits the lens circulation system not only increasing the water content in the lens core, but also changing the refractive index (Vaghefi et al 2011). In the absence of a circulation the water gradient is abolished and the water-to-protein ratio in the different rings would become the same. But in the presence of the circulation system ions and water are actively removed from the lens centre and this establishes local differences in protein concentration which contribute to the refractive index gradient that is critical for establishing the optical properties of the lens.

In summary, the results of my thesis have increased our understanding of lens structure and function. While more work is required, I feel that I have contributed to the knowledge base that will one day lead to the development of non-invasive therapies to treat lens cataract.
References


References


References


Gao J, Sun X, Martinez-Wittinghan FJ, Gong X, White TW, Mathias RT (2004) Connections Between Connexins, Calcium, and Cataracts in the Lens Abbreviations used in this paper:


DF, differentiating fibers; Kl, knockin; KO, knockout; MF, mature fibers; WT, wild type. The Journal of General Physiology 124:289-300.


References


References


References


Appendix A: The Confocal LASER Scanning Microscopy Theory

Introduction

The invention of the double-focussing stage-scanning microscope (the confocal scanning microscope) in 1955 by Minsky revolutionised the imaging world (Minsky, 1988). This technique has since developed into the popular Confocal LASER Scanning Microscopy (CLSM) with the realisation of experimentally useful LASER about 40 years ago (Gratton, 1995). CLSM is a state-of-art imaging technique that has many advantages and some disadvantages over the different forms of conventional microscopy such as Fluorescent Microscopy (FM), Electron Microscopy (EM), and high-resolution Magnetic Resonance Imaging (MRI).

The lack of intrinsic contrast in biological specimens, especially transparent organs such as the ocular lens, poses a serious problem in biomedical imaging. Under the conventional transmitted Light Microscope (LM), the uniform opacity throughout the specimen allows similar levels of light to be detected from different structures, making their identification difficult. Also, large amounts of light scattering when imaging 3D specimens contribute blurring to the image, thus decreasing the image contrast (Halbhuber and Konig, 2003). The solution to this problem is labelling tissues with high-contrasting agents such as dyes, heavy metals and fluorochromes. Labelling tissues with fluorochromes and imaging with conventional wide-field FM suffers from the disadvantage of illumination of the whole sample resulting in inclusion of out-of-focus light (can be 90% of the collected fluorescence) into the otherwise in-focus image, resulting in a reduction in contrast in both axial and lateral direction (Wright et al., 1993, Cannell and Soeller, 1997, Conchello and Lichtman, 2005). While EM offers high resolution imaging with heavy metal staining, often the process of preparation damages the cells causing imaging artefacts from fixation and sectioning. MRI offers non-invasive, high resolution, 3D and functional imaging on the organ scale that no other microscopy offers, but MRI is less powerful than CLSM in terms of
resolution. CLSM has such high 3D resolution mainly because of its use of a pinhole, which gives it a shallow depth-of-field, allowing optical sectioning and eliminating out-of-focus blur.

**Principles of CLSM**

Light is an electro-magentic wave made up of light particles (photons) propagating independent of each other (Urone, 2001). Light waves can either travel in-phase (constructive) or out-of-phase (deconstructive). Constructive (coherent) waves result in amplification of energy whereas deconstructive waves have cancelling affect and produces no colour (Urone, 2001). Light refraction is dependent on the speed of light which in terms is dependent on the wavelength and its frequency. The speed of light decreases with a highly refractive medium such as the glass slide and the degree of refraction various and is dependent on the medium. Constant speed of light is only achieved in vacuums.

The source of illumination for CLSM is LASER. LASER is an acronym that stands for “Light Amplification by Stimulated Emission of Radiation” (Gratton, 1995). LASER has several advantages over other sources of illumination: high brightness, high degree of monochromaticity, and small divergence. Conventional light sources are polychromatic, because light is made up of multiple wavelengths (\(\lambda\)). High brightness is equivalent to better signal, since the intensity of light is an indication of the amount of energy carried. The brighter the light, the more photons are being transmitted, which can generate more fluorescence from the specimen. Small divergence means the LASER beam cross-sectional area does not change dramatically along the beam, keeping intensity uniform. Intensity is inversely proportional to cross-sectional area and cross-sectional area goes up with largely diverging beams (Figure A.1).
Appendix A: The Confocal LASER Scanning Microscopy Theory

Figure A.1  
A: showing an image of illuminating cone. B: showing the area and the intensity of the relative cone. With increase in cross-sectional area of the light cone, there will be a decrease in the intensity. C: showing the 3D schematic illustration of the illumination cone like A and also showing the location of a focal volume.

The monochromatic property of LASER aids the imaging of fluorochromes by exciting only using the desired \( \lambda \), meaning that only the fluorochrome of interest is excited. Mono-chromacity reduces the lateral chromatic aberration caused by the objective lens as a consequence of different \( \lambda \) focusing on different planes (axial chromatic aberration) (Keller, 1995). Usually longer \( \lambda \) sources of illumination are preferred in CLSM because with longer \( \lambda \) there are lesser chromatic aberrations, however shorter.

Disadvantages of using LASER are photo-bleaching and photo-toxicity, which are both major problems in both FM and CLSM (Conchello and Lichtman, 2005). Photo-bleaching is more problematic in FM than in CLSM as it is a wide-field modality, therefore exposing the whole field-of-view to bleaching at every time. CLSM minimises the area of bleaching only to the cone of scanning at the particular spot being scanned. Increasing the LASER power to obtain a better signal quality greatly increases sample bleaching, so a trade-off between signal and bleaching is required (Conchello and Lichtman, 2005). Photo-toxicity is the toxicity to living cells induced by high photon fluxes and produces highly reactive oxidative by-products which are problematic in live-cell imaging.

The exciting LASER passes through an excitation filter, gets reflected by the dichroic mirror towards the specimen and passes through the objective lens exciting the fluorochrome-labelled specimen (Figure A.2) (Stelzer, 1995). Upon excitation, fluorochromes absorb the energy and emit a lower energy and longer \( \lambda \) light as they return to their basal energy state (Figure A.3). The emitted \( \lambda \) is different from the excited \( \lambda \) and this difference allows their separation by the dichroic mirror. The emitted light is not reflected but passes through the dichroic mirror and emission filter, through the pinhole before reaching the detector (Figure A.2). The LASER scans
Appendix A: The Confocal LASER Scanning Microscopy Theory

the specimen in a raster system until the optical view of the specimen has been scanned. In conventional CLSM, one-photon light source is utilised. To acquire better optical resolution, localisation and sectioning a two-photon light source can be used.

Two-Photon Excitation (TPE) involves a very high local instantaneous intensity with temporal concentration of femtosecond pulsed LASER (Denk et al., 1990). The femtosecond pulse is produced by a colliding-pulse, mode-locked dye LASER and relies on two-photons interacting near simultaneously to produce a quadratic dependence of light intensity (Figure A.3) rather than the linear dependence observed in conventional scanning microscopy (Zipfel et al., 2003). By doubling the light intensity (TPE), the fluorescence production is quadrupled (Zipfel et al., 2003). This nonlinearity is advantageous as the scattering excitation photons are too diluted to cause significant fluorescence even in deep tissue environments, and consequently the excitation can therefore be limited to a small focal volume (Figure A.3) (Svoboda and Yasuda, 2006).

In conventional LM, the only components between the source of illumination and objective are lenses. In CLSM there are many additional components between the LASER and the objective: they are excitation filters, dichroic mirrors and scanning mirrors (Figure A.2). These additional components mean that the optical path becomes more complex and longer, which reduces the number of photons that get through (both excitation and emission pathways). Use of these components improves resolution and reduces chromatic aberrations, allowing 3D and 4D imaging.
Appendix A: The Confocal LASER Scanning Microscopy Theory

Figure A.2  Schematic representation of CLSM system. The different excitation and emission light are separated by the dichroic mirror, a vital component of the CLSM.

Figure A.3  Jablonski diagram illustrating the electronic states of a molecule upon excitation by single and double photons.
There are two filters: one filters the excitation beam and the other filters the emission light. The filters are examples of a band-pass filter (Figure A.4). Band-pass filters only allow λ’s between the cut-off λ’s to pass through. An improvement of the ordinary band-pass filter mounted on a rotating turret system is the Acousto-Optical Tunable Filters (AOTF) (Stelzer, 1995). AOTF is a solid-state electronically tunable band-pass filter relying on a birefringent crystal that modifies its diffraction properties upon interaction with an acoustic wave. The LASER λ and intensity can be modulated by altering the acoustic wave frequency within microseconds, allowing rapid LASER line selection. AOTFs allow multi-labelled specimen to be scanned by one excitation wavelength and the following rapid LASER switching, by another. This reduces the amount of spectral cross-over which is usually the major problem in multi-wavelength excitation. Another benefit AOTF provides is the ability to modulate intensity beam to maintain strong fluorophores while accommodate weaker ones to generate uniform images (Stelzer, 1995).

The dichroic mirror allows the reflection of specific shorter (excitation beam) λ and the transmission of specific longer (emission beam) λ (Carlsson et al., 1985). This component is very useful when imaging fluorochromes in both FM and CLSM. The shorter λ is reflected by the mirror onto the specimen, the longer λ is allowed to pass through the mirror onto the detector.
Appendix A: The Confocal LASER Scanning Microscopy Theory

This component prevents the detection of exciting λ resulting in excellent contrast because the only light detected originates from labelled structures. This mirror also removes the out-of-focus scatter caused by long coherent LASER, therefore preventing image blurring or interference (Gratton, 1995).

In CLSM, the objective lens is the focusing part of the system and is also the condenser of the system (Keller, 1995). The numerical aperture (NA) of the lens describes the angle from which light is collected; in CLSM it also describes the angle from which illuminating light is projected (Figure A.5). The distance between an objective and its focal point is its working distance. Large working distance allow optical sectioning with thicker specimen, but often the maximum thickness of the specimen would be smaller than the actual working distance. Objectives with high NA focus at a steeper angle than lower NA lenses, giving shorter and wider focal volume and thus are limited closer to the plane of focus.

\[
\text{NA} = n \sin \theta
\]

\(n\) = index of refraction (1.0 for air, 1.33 for pure water and up to 1.56 for oil). \(\theta\) = the half-angle of the maximum cone of light that can enter or exit the lens. \(f\) = the light gathering ability of the lens. \(D\) = diameter of the entrance pupil of the objective lens.

Out-of-focus light (due to illumination from above and below the plane of interest in conventional LM and FM) contributes to a reduction in contrast and resolution (Cannell and Soeller, 1997). With FM, the amount of out-of-focus blur depends on the distribution of fluorochrome (Cannell and Soeller, 1997). The more the fluorochrome distribution is throughout the thickness of the specimen, the more out-of-focus blurring. The blurring in FM becomes a major issue when the cell thickness is more than 1 μm (Cannell and Soeller, 1997). CLSM overcomes this problem with the presence of a pinhole in front of the detector (Figure 2) (Stelzer, 1995). The light from above and below the focal point will be focused in front or behind the pinhole, and since it is not focused in the plane of the pinhole, only small amounts of
this light is detected (Stelzer, 1995). A smaller pinhole size confers better resolution but also means fewer signals are detected. Therefore an optimal pinhole size must be used to get the optimal resolution and signal at the same time.

The optimal pinhole size is roughly about the size of the full-width at half-maximum of the first Airy-disc peak; at this size only about 50% of the signal is lost (Hibbs, 2004). Smaller than this size, there is no effect other than reducing the signal. Pinholes maximise signal-to-background (STB) and signal-to-noise (STN) ratio, and eliminate background that is caused by light scattering of the specimen (Halbhuber and Konig, 2003).

There are two types of detector commonly used in CLSM: 1. Photo-Multiplier Tube (PMT) and 2. Charge-Couple Device (CCD). The photon detection systems in CLSM are based on direct photo-detection (converting photons into current) (Art, 1995). PMTs are useful detectors when the signal is weak. PMTs are more sensitive and less noisy as well as having a faster response (Conchello and Lichtman, 2005). Disadvantages of using PMTs are their low efficiency detecting 10% or less of signal that passes through the pinhole. This low efficiency becomes a major issue when the scanning speed is increased. As the scanning speed increases, the number of photons detected per pixel will decrease to the extent that noise will limit the image quality. Since PMTs detect photons, they are unable to distinguish colour. So when doing multi-labelling imaging, either a succession of scans with different dichroic mirrors needs to be overlaid or the splitting of emitted light is required to distinguish different emission signals. PMTs are used for detecting photons with stage-scanning or xy-scanning system, and

Figure A.6 Showing a schematic of blocking of out-of-focus light with the presence of pinhole and the relative focus and out-of-focus airy discs.
CCDs or intensified CCDs are used with Nipkow disk scanning system (Hibbs, 2004). CCDs have higher efficiency than PMTs allowing rapid detection of photons, enabling rapid scanning near video rate. Often CCD cameras are cooled, making them more sensitive.

Figure A.7  Showing a schematic of the x-y system, including a artistic representation of raster scanning.

CLSM can scan the specimen through three different mechanisms: 1. by moving the specimen through the beam focal point (stage scanning); 2. by moving the scanners (beam scanning) or 3. by rotating a disk scanner (Stelzer, 1995). Stage scanning has the optical arrangement stationary and specimen scans through a single beam. Stage scanning offers the advantage of constant axial illumination, therefore avoiding off-axis aberrations, which is important in image processing, and is very photon efficient (Wright et al., 1993, Stelzer, 1995, Conchello and Lichtman, 2005). Disadvantages of this process are low scanning speed (10-150 lines/sec) and image distortion caused by movement of the specimen (Wright et al., 1993, Stelzer, 1995).

Illumination-scanning systems utilise two oscillating mirrors to deflect the light beam onto the point-of-interest in the specimen, and deflect the returning emitted light on to the pinhole (Conchello and Lichtman, 2005). The light deflected onto the specimen is ‘scanning’ and emitted light deflecting to the detector is ‘de-scanning’. One mirror scans the horizontal direction (x-mirror) and one scans the vertical direction (y-mirror). Usually, the x-mirror scans the ‘fast-axis’ and the y-mirror scans the ‘slow-axis’. X-y scanning mirrors move the beam in an orthogonal direction in a raster scanning pattern (Figure 4), the change in the angle of the mirrors resulting in linear motion of the focal point in the specimen.
The speed of scanning in illumination scanning systems is limited by the mechanical properties of the x-mirror as well as the dwell time (amount of time the illumination stays on a spot). Scanning too slow may contribute to limited information being captured when imaging fast changing signals (Conchello and Lichtman, 2005). Scanning too fast reduces the signal intensity, increasing the contribution of noise to the image and therefore decreases signal-to-noise ratio. The main drawback of CLSM is the scanning speed which is not as rapid as wide-field techniques: FM and EM (Conchello and Lichtman, 2005).

**Resolution**

The resolution is the smallest distance the system is able to distinguish between two adjacent points, and is given by the following equation:

\[
R = \frac{1.22\lambda}{2NA}
\]

R is the radius of the Airy disk, which is the limit of resolution. An Airy disk is the 2D representation of the diffraction limit created by the objective lens and the medium the specimen is in (Figure A.1 A) and a point-spread function (PSF) is a 3D representation of the diffraction limit (Figure A.8) (Hibbs, 2004). 3D resolution in CLSM is determined by the intensity PSF. Lateral resolution (xy) and axial resolution (z) is determined by the width and height of PSF respectively (Sandison et al., 1995).

The shortest visible λ of LM is 400 nm with maximum numerical aperture of 1.4 (with oil immersion lens): these are the two limiting factors of the resolution of LM (Art, 1995). The most commonly used λ in CLSM is 488 nm and the maximum NA is 1.4. The λ in EM is 0.004 nm and maximum NA is 0.02. If we calculate the resolution of LM using these theoretical values, we will overestimate it. With CLSM, the resolution is calculated to be around 0.21 μm and for EM it is calculated to be around 0.2 nm. The theoretical resolution for CLSM is identical in LM and FM. Practically this theoretical resolution is very hard to achieve by LM and FM, as LM uses light as a source of illumination and light is composed of different λ. The main contribution to the poor resolution to both LM and FM is the inclusion of out-of-focus blur in the image. CLSM achieve this resolution value with the inclusion of a pinhole and using LASER illumination. Resolution of
CLSM is improved by a factor of 1.4 (comparing with LM) just by having a pinhole (Figure A.8) (Halbhuber and Konig, 2003). Even the inclusion of pinhole cannot increase the resolution to below 0.2 µm (EM), and this is one of the disadvantages of CLSM compared to EM.

![Figure A.8](image)

**Figure A.8**  
A: 2D PSF of CLSM. B: 2D PSF of FM with the same NA as “A”. C: 3D PSF of FM. The large “x” shape of the FM cone are attributed by the out-of-focus light causing reduced lateral and axial resolution in comparison to the CLSM.

## Imaging Modes

CLSM allows imaging of both fixed and live specimens with single, double, and more λ illumination modes. Single λ uses one fluorochrome, double λ uses two fluorochromes and triple uses three fluorochromes. With multiple λ illumination modes, the chosen fluorochromes’ excitation λ must be relatively different to one another to ensure no simultaneous excitation of the fluorochromes. Multi-λ-illumination mode is a powerful imaging technique allowing multiple structures/proteins of interest to be labelled and imaged from one specimen. A lot of information can come out of multi-λ-illumination, for example, co-localisation of proteins or structures, and the site of structures in relation to other structures. EM also allows multi-label-illumination, but once again the quality is poorer compared with CLSM. MRI does not allow multi-labelling therefore the ability to contrast structures depends on the protein make-up of the structure of interest. CLSM differ from MRI in that it does functional imaging at a cellular level instead of at an organ level. Functional imaging is impossible in EM, in that it is can only image fixed specimens.

The ability to reconstruct a 3D structure from 2D images without physically sectioning the specimen is a key advantage of CLSM and MRI (Carlsson et al., 1985). The technique CLSM utilises is called optical sectioning, which is enabled by its small z-axis resolution. This technique
Appendix A: The Confocal LASER Scanning Microscopy Theory

allows the acquisition of images of thin slices by eliminating out-of-focus light in each image plane, and the thin slices can be computationally overlaid at the end to reconstruct a 3D image.

Reaching the Limit

CLSM has reached the peak of its form, in terms of resolution, being now limited by the diffraction limit of light. The objective lens performance has reached its theoretical limits (Keller, 1995). It is not the lens design that contributes to this limit but the wave nature of the light; as a consequence I believe that the limit of CLSM resolution has been reached.

Since the theoretical limits of objective lens has been reached, and further manipulation of light can be performed, the only way to advance imaging using CLSM is not by altering the microscope itself, but utilising special techniques to improve image quality, such as FRET (fluorescence resonance energy transfer), STED (stimulated emission depletion) and TIRF (total internal reflection fluorescence) (Reichert and Truskey, 1990, Jares-Erijman and Jovin, 2003, Simpson, 2006).
Appendix B: Microscopy

XY Alignment

In case of an unlikely event the LASER beam path was projected to the eye, the ambient light was left on when aligning the two-photon spot with the recording plane. A mirror surface was focused through the 10x objective using transmitted optics and standard light source. Both the 488 nm and two-photon LASER attenuation was turned to maximal to ensure the lower power of light was used. 488 nm Argon+ LASER shutter was opened and the 488 nm spot was reflected by the mirror back out of the LSM410 rear aperture and the spot can be observed as a blue dot on a piece of paper. With the 488 nm LASER on continuous spot scanning mode, the mechanical shutter was then opened to illuminate the two-photon excitation (TPE) pathway.

To align the TPE beam to the illuminated spot in the centre of scan field, two beam steering mirrors were used. One mirror adjusted for the x-direction and the other for the y-direction (Figure B.1 I & F). The course knobs were used first to roughly get the two spots aligned at 4 different locations by placing a piece of tissue at each location:

13. Outlet of the Pockels Cell (Figure B.1 J).
14. Bottom steering mirror (Figure B.1 I).
15. Top steering mirror (Figure B.1 F).
16. Outlet of the side port to the microscope (Figure B.1 A).

The 488 nm was focused when aligning at each position. The fine knobs were then used to fine tune the two-spots (Figure B.1 E & G). When both the 488 nm and two-photon spots were deemed to overlap in all 4 locations, the two-photon controlled shutter was closed, 488 nm LASER was turned off and the mirror in the specimen holder was removed.
Laser attenuation for the 488 nm LASER was returned to the standard value. The two-photon beam was tuned to 40 mW of excitation power by using a Power Meter (Coherent, Auburn, CA, USA) by turning the LASER attenuation wheel.

Figure B.1  Schematic diagram of the mirrors used to align the two-photon excitation spot. A: spot directed to the specimen. A 488 nm stationary scanning spot was reflected back into the two-photon beam pathway as a reference for alignment. B: the modified side port of the microscope to provide a direct pathway for two-photon beam. C: the knobs used for z-alignment. D & H: course knobs used to move the mirrors. E & G: fine knobs used to move the mirrors. F & I: the beam steering mirrors, one can be adjusted in the x-direction and the other in the y-direction. J: two-photon beam coming from the Pockels cell (model 350-50, Conoptis, Danbury, CT, USA).
Appendix B: *Microscopy*

A slide containing 2 μm diameter green fluorescent beads or 1 μL of 1 mM caged-fluorescein in solution was then used to mark the two-photon spot on the computer monitor. Caged-fluorescein is used here for demonstration. The sea of caged-fluorescein was focused using the 10x objective lens and normal transmitted light. Under confocal imaging mode using the 488 nm LASER with continuous imaging at 0.7 s per frame, the two-photon beam shutter was opened and created a bright spot where fluorescein was released (Figure B.2 A). Pin-hole was adjusted to one-airy disc and the 488 nm Ar⁺ LASER attenuation, brightness and contrast were adjusted until the centre uncaged spot was just below saturation (Figure B.2 Rainbow Lookup Table). Continuous imaging mode was then stopped. 40x objective lens was changed to and the fluorescein was once again focused and a single 8 frames-average image was acquired (Figure B.2 B). The image was zoomed to get a final resolution of 4.960 pixels.μm⁻¹. The centre of the two-photon spot was marked with a cross on the computer monitor to facilitate placing the TPE spot inside a single fibre cell cavity during experimentation.

![Two-Photon spot for x- and y- alignment.](image)

*Figure B.2* Two-Photon spot for x- and y- alignment. A: two-photon spot with no digital zoom to get 1.27 pixels.mm⁻¹. B: two-photon spot digitally zoomed to get 4.960 pixels.μm⁻¹. Scale bars: 10 μm. Right: Rainbow Lookup Table used to help clarify fluorescence intensity.
**Z Alignment**

A slide containing fluorescent beads was used for the z-alignment, to ensure the photolysis spot aligns with the 488 nm Ar⁺ LASER scanning focal plane. A z-stack was taken using the 488 nm Ar⁺ LASER in one channel of the confocal. A second z-stack was taken with the 488 nm LASER off, using the stationary TPE beam instead (Figure B.3 B). By displaying the channels together in RGB mode on the computer monitor it was possible to reveal whether the TPE spot overlapped with the excited bead. If not, the position of the lens at the side port (Figure B.1 C) was adjusted as appropriate until the 488 nm imaging plane is the same as the plane to which the TPE beam was focused (Figure B.3 A).

![Figure B.3](image)

**Figure B.3** Examples for two-photon spot z-alignment. A: z-stack in the red channel with the beads aligned in one plane and the two-photo beam activating one of the bead saturating in fluorescent intensity (white) while the 488 nm LASER was turned on. B: z-stack in the green channel, showing fluorescent intensity of a single bead.
Appendix C: Statistical Analysis

Spread of Fluorescein and 10 kDa-Dextran-Linked-Fluorescein (10 kDa-Fluroescein)

A 4 x 2 x 2 factorial ANOVA (Table C.2) was chosen as this analysis allows the examination of the interaction between the different factors, and used to analyse the difference between all the groups, agents and regions parameters summarised in Table C.1.

Table C.1  Class definition.

<table>
<thead>
<tr>
<th>Class</th>
<th>Level</th>
<th>Values</th>
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<td>Rat, Wild-type Mouse, Lim2KO Mouse, Cx46KO Mouse</td>
</tr>
<tr>
<td>Lens</td>
<td>6</td>
<td>1, 2, 3, 4, 5, 6</td>
</tr>
<tr>
<td>Region</td>
<td>2</td>
<td>DF (Differentiating Fibres), MF (Mature Fibres)</td>
</tr>
<tr>
<td>Agent</td>
<td>2</td>
<td>10 kDa (10 kDa-Dextran-Linked-Fluorescein), Fluorescein</td>
</tr>
<tr>
<td>Observations</td>
<td>47</td>
<td>1, 2, 3, 4, 5, 6...45, 46, 47</td>
</tr>
</tbody>
</table>

Table C.2  4 x 2 x 2 factorial ANOVA between all data groups.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Mean Square</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>2912.94</td>
<td>1</td>
<td>2912.94</td>
<td>269.11</td>
<td>0.0000</td>
</tr>
<tr>
<td>Group</td>
<td>379.52</td>
<td>3</td>
<td>126.51</td>
<td>11.69</td>
<td>0.0000</td>
</tr>
<tr>
<td>Agent</td>
<td>246.20</td>
<td>1</td>
<td>246.20</td>
<td>22.74</td>
<td>0.0000</td>
</tr>
<tr>
<td>Region</td>
<td>33.41</td>
<td>1</td>
<td>33.41</td>
<td>3.09</td>
<td>0.0798</td>
</tr>
<tr>
<td>Group *Agent</td>
<td>374.32</td>
<td>3</td>
<td>124.77</td>
<td>11.53</td>
<td>0.0000</td>
</tr>
<tr>
<td>Group *Region</td>
<td>55.33</td>
<td>3</td>
<td>18.44</td>
<td>1.70</td>
<td>0.1657</td>
</tr>
<tr>
<td>Agent*Region</td>
<td>1.10</td>
<td>1</td>
<td>1.10</td>
<td>0.10</td>
<td>0.7497</td>
</tr>
<tr>
<td>Group <em>Agent</em>Region</td>
<td>13.96</td>
<td>3</td>
<td>4.65</td>
<td>0.43</td>
<td>0.7317</td>
</tr>
<tr>
<td>Error</td>
<td>4178.27</td>
<td>386</td>
<td>10.82</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Post-hoc analysis was performed using contrast coefficients (Table C.3) for the different null hypothesis below.
Appendix C: Statistical Analysis

Null Hypothesis:

H₀ #1: There is no difference in 10 kDa-Fluorescein spread between Lim2KO mouse and the rat AND wild-type mouse AND Cx46KO mouse.

H₀ #2: There is no difference in fluorescein spread between Lim2KO mouse and the rat AND wild-type mouse AND Cx46KO mouse.

H₀ #3: There is no difference in fluorescein spread between Cx46KO mouse and the rat AND wild-type mouse.

H₀ #4: There is no difference in fluorescein and 10 kDa-Fluorescein spread between wild-type mouse and Cx46KO mouse.

H₀ #5: There is no difference between fluorescein and 10 kDa-Fluorescein spread in the rat.

H₀ #6: There is no difference between fluorescein and 10 kDa-Fluorescein spread between the rat and wild-type mouse.

H₀ #7: There is no difference between fluorescein and 10 kDa-Fluorescein spread in the wild-type mouse.

H₀ #8: There is no difference between wild-type rat and Lim2KO mouse overall 10 kDa-Fluorescein spread.

H₀ #9: There is no difference between in 10 kDa-Fluorescein spread between the outer cortex and inner cortex/core between rat and wild-type mouse.

H₀ #10: There is no difference between fluorescein and 10 kDa-Fluorescein spread in the outer cortex in the rat.

H₀ #11: There is no difference between fluorescein and 10 kDa-Fluorescein spread in the outer cortex in the wild-type mouse.

H₀ #12: There is no difference in 10 kDa-Fluorescein spread between wild-type mouse and Cx46 mouse.
Appendix C: Statistical Analysis

Table C.3  Summary of statistical significance for the different null hypothesis

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Mean Square</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1276.34</td>
<td>1</td>
<td>1276.34</td>
<td>842.02</td>
<td>0.0000</td>
</tr>
<tr>
<td>Group</td>
<td>60.96</td>
<td>3</td>
<td>20.32</td>
<td>13.41</td>
<td>0.0000</td>
</tr>
<tr>
<td>Agent</td>
<td>2.64</td>
<td>1</td>
<td>2.64</td>
<td>1.74</td>
<td>0.1877</td>
</tr>
<tr>
<td>Group*Agent</td>
<td>2.23</td>
<td>3</td>
<td>0.74</td>
<td>0.49</td>
<td>0.6888</td>
</tr>
<tr>
<td>Error</td>
<td>401.69</td>
<td>265</td>
<td>1.52</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Source Cell Rise-time

A 3 x 2 factorial ANOVA was chosen as this analysis allows the examination of the interaction between the different factors, and used to analyse the difference between all the groups and agents (Table C.4).

Table C.4  3 x 2 factorial ANOVA between all data groups.

Post-hoc analysis was performed using contrast coefficients (Table C.5) for the different null hypothesis below.
Appendix C: Statistical Analysis

Null Hypothesis:

H₀ #1: There is no difference in fluorescein rise-time in the source cell between the rat and wild-type mouse AND Lim2KO mouse AND Cx46KO mouse.

H₀ #2: There is no difference in 10 kDa-Fluorescein rise-time in the source cell between the rat and wild-type mouse AND Lim2KO mouse AND Cx46KO mouse.

H₀ #3: There is no difference between fluorescein and 10 kDa-Fluorescein rise-time in the source cell.

H₀ #4: There is no difference between fluorescein and 10 kDa-Fluorescein rise-time in the source cell between the rat and wild-type mouse AND Lim2KO mouse AND Cx46KO mouse.

Table C.5  Summary of statistical significance for the different null hypothesis

<table>
<thead>
<tr>
<th>Time to reach 67% Maximum Normalised Fluorescent Intensity in Source Cell</th>
<th>Rat</th>
<th>Wild-type Mouse</th>
<th>Lim2KO Mouse</th>
<th>Cx46KO Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong># Cells</strong></td>
<td>fluorescein</td>
<td>10kDa</td>
<td>fluorescein</td>
<td>10kDa</td>
</tr>
<tr>
<td>Mean (s)</td>
<td>2.47</td>
<td>2.76</td>
<td>1.96</td>
<td>2.53</td>
</tr>
<tr>
<td>SEM</td>
<td>0.46</td>
<td>0.53</td>
<td>0.11</td>
<td>0.21</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Contrast Coefficients</th>
<th>F Crit</th>
<th>Reject?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>-1</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>-1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>-1</td>
</tr>
</tbody>
</table>

- XXXV -
Primary Cell Rise-time in MF Cells

A 3 x 2 factorial ANOVA was chosen as this analysis allows the examination of the interaction between the different factors, and used to analyse the difference between all the species, and the agents (Table C.6).

Table C.6 One-way ANOVA between all data groups.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Mean Square</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1276.34</td>
<td>1</td>
<td>1276.34</td>
<td>842.02</td>
<td>0.0000</td>
</tr>
<tr>
<td>Group</td>
<td>60.96</td>
<td>3</td>
<td>20.32</td>
<td>13.41</td>
<td>0.0000</td>
</tr>
<tr>
<td>Agent</td>
<td>2.64</td>
<td>1</td>
<td>2.64</td>
<td>1.74</td>
<td>0.1877</td>
</tr>
<tr>
<td>Group*Agent</td>
<td>2.23</td>
<td>3</td>
<td>0.74</td>
<td>0.49</td>
<td>0.6888</td>
</tr>
<tr>
<td>Error</td>
<td>401.69</td>
<td>265</td>
<td>1.52</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Post-hoc analysis was performed using contrast coefficients (Table C.7) for the different null hypothesis below.

Null Hypothesis:

H₀ #1: There is no difference in fluorescein rise-time in the secondary cell between the wild-type and Cx46KO mouse.

H₀ #2: There is no difference in 10 kDa-Fluorescein rise-time in the secondary cell between the wild-type mouse and rat AND Cx46KO mouse.

H₀ #3: There is no difference in fluorescein rise-time in the secondary cell between the Lim2KO mouse and Cx46KO mouse.

Table C.7 Summary of statistical significance for the different null hypothesis
Appendix D: Conference Abstracts

Medical Science Congress (MedSciNZ), 2007, Queenstown, NZ. Oral Presentation

Probing Intracellular Communication in Rodent Lens Using Two-Photon Microscopy

JW Liu, A Sisley, M Cannell, C Soeller, PJ Donaldson. Department of Physiology, University of Auckland, n.liu@auckland.ac.nz

Intercellular communication between the fibre cells of the lens is known to be mediated by a network of gap junction (GJ) channels. While GJ’s are normally permeable to molecules with molecular weights <1200 Da, a recent study has shown that as differentiating fibre cells become incorporated into the lens core, an intercellular pathway that is permeable to molecules much greater than 1200 Da arises (Shestopalov and Bassnett, 2003). The lens specific protein MP20 has been implicated in the formation of this macromolecular pathway, since no transfer of large molecules was observed in MP20 knockout lenses (Shiels et al., 2007). In the rat lens MP20 is initially cytoplasmic but undergoes a differentiation-dependent insertion into the plasma membrane when fibre cell nuclei are lost (Grey et al., 2003). In the mouse lens the membrane insertion of MP20 occurs just prior to the loss of cell nuclei. To correlate MP20 insertion with the formation of the macro-molecular pathway we have utilised Two-Photon Excitation Flash Photolysis (TPEFP) (Jacobs et al.) to un cage either caged-fluorescein (MW ~350 kDa) or caged-fluorescein-dextran (MW ~10 kDa) in combination with confocal microscopy to monitor the extend of fluorophore spread in different regions of the mouse and rat lens. The initiation of fluorescein-dextran dye spread via the macro-molecular pathway was then correlated to MP20 insertion in both the rat and mouse lens. This correlation showed that the formation of the macro-molecular pathway in mice occurred before the loss of cell nuclei and coincided with the insertion of MP20. Similar studies are currently being conducted in the rat lens to determine whether the macromolecular pathway formation also correlates with MP20 insertion and nuclei degradation.

All of the studies described were approved by the University of Auckland Animal Ethics Committee. Supported by the HRC, the Marsden Fund and the University of Auckland Research Committee.
Cataract Satellite Meeting of the 18th International Conference for Eye Research and the 11th Conference of Chinese Cataract Society, Xi’an, China.

**Oral Presentation**

**THE USE OF TWO-PHOTON EXCITED FLASH PHOTOLYSIS TO STUDY THE CONTRIBUTIONS OF GAP JUNCTIONS AND MEMBRANE FUSIONS TO INTERCELLULAR COMMUNICATION IN RODENT LENSES.**

**JW Liu¹, A.M. Sisley¹, X Gong², M.B. Cannell¹, C. Soeller¹, P.J. Donaldson¹. ¹Department of Physiology, University of Auckland, Auckland, New Zealand; ²School of Optometry and Vision Science Program, University of California at Berkeley, Berkeley, CA, USA**

Regional differences in intercellular communication are apparent in the rodent lens. In the outer cortex of the lens intercellular communication appears to mediated by the gap junction proteins Cx46 and Cx50. However, a parallel intercellular communication pathway, apparently formed by membrane fusions that are permeable to large macromolecules that do not normally pass through gap junction channels, develops in the inner cortex. To differentiate between the these two pathways we have utilised Two-Photon Excited Flash Photolysis (TPEFP)² to study intercellular communication with high spatial resolution in different regions of the rodent lenses. Lenses were extracted from rats, wild-type (WT) mice or Cx46 knockout mice (Cx46KO)³. They were cut in half, and incubated in an intracellular solution that contained low (fluorescein) or high (fluorescein-dextran, 10,000MW) molecular weight caged compounds. Cut lenses were placed upon the stage of an inverted microscope and TPEFP used to create a cellular point source of fluorescent dye release. The spread of dye to adjacent cells was monitored using conventional confocal microscopy. Cage release was performed in different regions of the lens and correlated to markers of fibre cell differentiation to determine cellular position. In both rat and WT mouse lenses the extent of fluorescein spread was independent of location within the lens. In contrast, fluorescein-dextran spread following TPEFP was restricted in the outer cortex, but was extensive and similar to fluorescein spread in the inner cortex. In Cx46KO lenses fluorescein dye spread was reduced in the outer cortex relative to WT, but there was no difference in dye transfer between the low and high MW fluorophores in the inner cortex. Since Cx46KO animals only express functional Cx50 gap junction channels in the outer cortex, our results directly confirm that intercellular dye transfer in the outer cortex is mediated by gap junction channels. However, in the inner cortex a macromolecule-permeable pathway appears to operate in parallel to a Cx46 mediated pathway. The implications of our findings to current models of lens homeostasis will be discussed.

(Supported by the Marsden Fund, Health Research Council of New Zealand, and the University of Auckland Research Committee).
DIFFERENTIATION-DEPENDENT INSERTION OF MP20: DOES IT CORRELATE WITH THE FORMATION OF A MACRO-MOLECULAR DIFFUSION PATHWAY IN RODENT LENSES?

JW Liu, A.M. Sisley, P.J. Donaldson, M.B. Cannell, C. Soeller. Department of Physiology, School of Medical Sciences, University of Auckland, Auckland, New Zealand.

In the avascular lens intercellular communication is critically important for the maintenance of lens transparency. While the cell-to-cell transfer of ions and small molecules between lens fibre cells is known to be mediated by the gap junction proteins CX46 and CX50, more recent work has shown that a parallel intercellular communication pathway appears to develop in the inner cortex that is permeable to large macromolecules that do not normally pass through gap junction channels (Shestopalov and Bassnett). In the mouse lens the formation of this macromolecular diffusion pathway (MDP) is believed to be associated with the expression of MP20, since in mice that lacked the gene encoding for MP20, the MDP did not form\(^2\). In rat lenses we have shown that MP20 is initially located in the cytoplasm of differentiating fibre cells, but becomes abruptly inserted into the membranes of mature fibre cells following nuclei degradation\(^3\), suggesting that MP20 insertion may involved in the formation of the MDP. However, in the mouse lens it has been shown that MDP formation occurs before the loss of fibre cell nuclei. To further investigate the relationship between MP20 insertion and MDP formation we have utilised immunocytochemistry and Two-Photon Excited Flash Photolysis (TPEFP)\(^4\) to correlate MP20 insertion with the formation of the MDP in rodent lens. In the mouse lens high resolution mapping of MP20 expression also shows that MP20 undergoes a switch from cytoplasmic to membranous labelling, but unlike in the rat lens this change in subcellular location precedes the loss of cell nuclei. Application of TPEFP to half-cut mouse lens loaded with caged Fluorescein-dextran (MW = 10kDa), showed that follow uncaging significant transfer of Fluorescein-dextran, that is indicative of the formation of the MDP, only occurred after MP20 insertion. Similarly in the rat lens TPEFP-induced uncaging of Fluorescein-dextran showed that the MDP only formed after the insertion of MP20, which in this species occurred after cell nuclei had degraded. In summary we have used species specific differences in the differentiation dependent insertion of MP20 to show that it correlates with the formation of a macro-molecular diffusion pathway in both rat and mouse lenses. Whether MP20 forms the structure that mediates macro-molecule diffusion or is involved in the formation of pathway remains to be determined.

(Supported by the Marsden Fund, Health Research Council of New Zealand, and the University of Auckland Research Committee).
COMBIO, 2009, Christchurch, NZ. Oral Presentation

Differentiate dependent insertion of MP20: does it correlate with the formation of a macromolecular-diffusion-pathway?

Liu, J.W. ¹, Sisley, A.M. ², Cannell, M.B. ², Soeller, C. ², Donaldson, P.J. ¹ ¹Department of Optometry and Vision Science, and ²Department of Physiology, School of Medical Sciences, University of Auckland, Auckland, New Zealand.

Traditionally, intercellular communication in the avascular lens was thought to be mediated by gap junction (GJ) channels. More recently, an alternative and parallel intercellular communication pathway has been discovered, that develops in the inner-cortex which is permeable to large macro-molecules that do not normally pass through GJ channels. MP20 a putative lens specific adhesion protein has been implicated in the formation of this macromolecular-diffusion-pathway (MDP), since in MP20 knock-out mice the MDP did not form. In rat lenses we have shown that MP20 is initially located in the cytoplasm of differentiating fibre cells, and inserts into the membranes following nuclei degradation, suggesting that MP20 insertion may be involved in the formation of the MDP. In the mouse lens, MP20 insertion into the membrane precedes the loss of nuclei unlike in the rat lens. To further investigate the relationship between MP20 and MDP formation we have utilised immunocytochemistry and Two-Photon-Excitation-Flash-Photolysis (TPEFP) on half-cut rodent lens loaded with the GJ impermeable caged fluorescein-dextran (MW=10kDa) to correlate MP20 insertion with the formation of the MDP in rodent lens. By exploiting the species specific differences in the differentiation dependent insertion of MP20 detected by immunocytochemistry, and the TPEFP induced transfer of Fluorescein-dextran as a functional indicator of MDP formation, we have shown that formation of the MDP correlates with the insertion of MP20 in both rat and mouse lenses. Whether MP20 forms the structure that mediates macro-molecule-diffusion or is involved in the formation of pathway remains to be determined.
Involvement of MP20 in the formation of Macro-molecular Diffusion Pathway

Liu, J.W. 1, Sisley, A.M. 2, Cannell, M.B. 2, Soeller, C. 2, Donaldson, P.J. 3. 1Department of Optometry and Vision Science, and 2Department of Physiology, School of Medical Sciences, University of Auckland, Auckland, New Zealand.

Traditionally, intercellular communication in the avascular lens was thought to be mediated by gap junction (GJ) channels. More recently, an alternative and parallel intercellular communication pathway has been discovered, that develops in the inner-cortex permeable to large macro-molecules and do not normally pass through GJ channels (Shestopalov and Bassnett, 2003). MP20, a putative lens specific adhesion protein has been implicated in the formation of this macromolecular-diffusion-pathway (MDP), since in MP20 knock-out mice the MDP did not form (Shi et al., 2009). In the rat lenses, MP20 has been shown to be initially localised in the cytoplasm of differentiating fibre cells, and inserts into the membranes following nuclei degradation, suggesting that MP20 insertion may play a role in the formation of the MDP (Grey et al., 2003). In the mouse lens, MP20 insertion into the membrane precedes the loss of nuclei unlike in the rat lens. To further investigate the relationship between MP20 and MDP formation we have utilised immunocytochemistry and Two-Photon-Excitation-Flash-Photolysis (TPEFP) (Jacobs et al., 2004) on half-cut rodent lens loaded with the GJ impermeable caged fluorescein-dextran (MW=10kDa) to correlate MP20 insertion with the formation of the MDP in rodent lens. By exploiting the species specific differences in the differentiation dependent insertion of MP20 detected by immunocytochemistry, and the TPEFP induced transfer of Fluorescein-dextran as a functional indicator of MDP formation, we have shown that formation of the MDP correlates with the insertion of MP20 in both rat and mouse lenses. In the absence of MP20, caged fluorescein-dextran is restricted within the source cell unable to diffuse to the neighbouring fibre cells indicating the absence of the MDP. This is the first time real-time functional technique has been used to show the importance of MP20 in the formation of MDP.

(This work was supported by the Mardsen Fund of New Zealand and the Auckland Bioengineering Institute.)