

The Ovine Lens Cytoskeleton

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The lens of the eye is a vital tissue in the visual system, responsible for the collection and focusing of light on to the retina. Comprised of epithelial cells at differing stages of differentiation, the transparency of the lens is dependent on the highly ordered crystalline structure of lens proteins. The lens consists of several proteins including crystallins (α , β , γ) that make up 90% of the soluble protein, and the lens cytoskeletal proteins. Cytoskeletal proteins contribute only a fraction of the total lens protein, but are thought to play an important role in the establishment and maintenance of transparency. Calpain-induced degradation of these proteins may be involved in the development of cataracts. This has been an area of research at Lincoln University where a flock of sheep genetically predisposed to cataract maintained as a cataract development model.

The aim of this research was to investigate the distribution of cytoskeletal proteins in the lens, and to examine the effects of calpain proteolysis on these proteins, with the goal of establishing the role of the lens cytoskeletal proteins in the ovine cataract model. A combination of techniques was used including immunohistochemistry, which required the development of a specific protocol for ovine lenses. Cytoskeletal proteins were identified using immunohistochemistry in lens tissue sections and exhibited characteristic distributions. Actin displayed preferential distribution in the short sides of the fibre cells in the cortex of the lens but was absent in the lens nucleus, while spectrin in the cortex and nucleus was associated with the fibre cell membrane. Filensin was observed in the outer cortex of lens sections associated with the fibre cell membrane and cytoplasm, although the pattern of localisation was indistinct due to the abundance of filensin breakdown products. Vimentin displayed membrane and cytoplasmic association in the outer cortex that diminished toward the lens nucleus, with membrane associated vimentin only persisting in the deeper regions of the cortex and nucleus.

Additionally, the effect of novel calpain inhibitors (*Cat0059* and *Cat811*) in preventing proteolysis of lens cytoskeletal protein was investigated and compared with calpain inhibitors developed elsewhere (*SJA6017*). The inhibitors were tested at between 10 and 0.1 μM (100 nM). All inhibitors were effective at 10 μM . *SJA6017* provided significant protection to vimentin at 1 μM . *Cat0059* was found to protect spectrin and filensin at 1 μM , but not vimentin, while inhibitor *Cat811* was found to protect spectrin only. *SJA6017* added to assays at 100 nM offered significant protection to spectrin, and *Cat0059* was found to protect filensin and spectrin to a significant degree at 100 nM, indicating the novel inhibitors were comparable to those developed elsewhere in terms of their effectiveness.

Taken together, the evidence presented in this thesis shows the cytoskeletal proteins as crucial elements in the lens. Their pervasive presence coupled with evidence that lens cytoskeletal proteins are sensitive to calpain-induced proteolysis that is inhibited with novel calpain inhibitors suggests that the lens cytoskeletal proteins may be useful targets in cataract prevention for future research.

Keywords: Lens, immunohistochemistry, cytoskeleton, actin, spectrin, vimentin, filensin, fluorescence, microscopy, calpain, proteolysis, inhibitor, cataract.

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ABBREVIATIONS

ATA	aurintricarboxylic acid
AQP-	aquaporin
BCA	bicinchoninic acid
Bfsp2	human CP49 gene
CP49	phakinin
CP115	filensin
Cx-	connexion
DTT	dithiothreitol
E64	trans-epoxysuccinyl-L-leucylamido-4-guanidino-butane
F-actin	filamentous actin
FITC-lectin	fluorescein isothiocyanate conjugated lectin
<i>g</i>	gravity
G-actin	globular actin
IgG	immunoglobulin G
IHC	immunohistochemistry
LBCF	lens based cell free

Lectin	<i>Triticum vulgare</i> lectin
LDS	lithium dodecyl sulphate
Lp-	lens specific calpain isoforms
MDa	mega Dalton
MeOH	methanol
MIP	major intrinsic protein
NFDM	non-fat dairy milk
O.C.T.	optimal cutting temperature
O.D.	optical density
PBS	phosphate-buffered saline
PI	propidium iodide
PFA	paraformaldehyde
R ²	coefficient of determination
SJA6017	<i>N</i> -[4-fluorophenylsulfonyl]-L-valyl-L-leucinal
TBS	tris-buffered saline
TTBS	tween-20/tris-buffered saline
Tris-HCl	tris-hydrochloride

TRITC	tetramethylrhodamine isothiocyanate
TRITC-lectin	tetramethylrhodamine isothiocyanate conjugated lectin
Tween-20	polyoxyethylene (20)sorbitan monolaurate
USF	urea soluble fraction
v/v	volume per volume
w/v	weight per volume

SECTION 1 – INTRODUCTION AND LITERATURE REVIEW

1.1 The Lens

Lens Function

The lens is a transparent tissue found in the eye. The primary function of the vertebrate lens is to collect and concentrate electromagnetic radiation between 400-700 nm (visible light) on the retina of the eye, providing increased sensitivity and allows for information contained by that light to be spatially resolved (Fernald, 2006; Lou, 2003; Roberts, 2001).

Lens Structure

The vertebrate lens is comprised principally of epithelial cells at varying stages of proliferation and differentiation surrounded by a thick basement membrane, or capsule, of extracellular matrix material (Rao & Maddala, 2006; Straub *et al.*, 2003). The outer zone is made up of metabolically active, structurally unspecialized cuboidal epithelial cells that cover the anterior periphery of the lens, and is present in both the embryonic and mature lens. These epithelial cells secrete the material that makes up the capsule, which is formed by the apposition of multiple layers of basal lamina composed mainly of collagen type IV (Barraquer *et al.*, 2006). The lens epithelial cells migrate from the anterior proliferative zone toward the equatorial region of the lens, known as the bow region, where they form a second discernable zone, exiting the cell cycle and commencing differentiation into elongated fibre cells. This transitive zone is the site where organelles are lost from the fibre cells. Finally, aged quiescent fibre cells reside in the core or nucleus of the lens, which is discernable at a distance 10–20% of the radius into the lens (see *fig. 1.1*; Fernald, 2006; Weber & Menko, 2006; Kyselova *et al.*, 2004; Gao *et al.*, 2004; Mathias & Rae, 2004; Shestopalov & Bassnett, 2003; Menko, 2002; Beebe *et al.*, 2001; Quinlan *et al.*, 1999; Clark *et al.*, 1999).

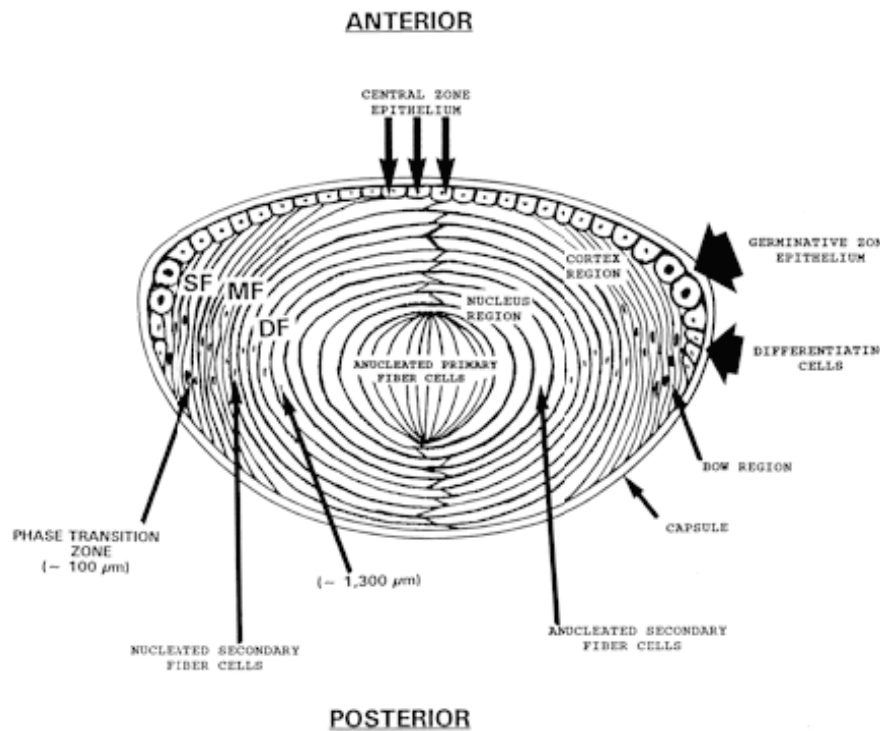


Figure 1.1 Schematic of a mammalian lens, illustrating salient points of lens structure.

Beneath the surrounding collagenous capsule, nucleated epithelial cells are seen to cover the anterior face of the lens extending back to the equatorial bow region, where they begin differentiation and elongation into fibre cells (**SF**; superficial fibres). As new fibre cells are laid over pre-existing cells (**MF**; middle fibres) the lens becomes a compacted mass of fibre cells, and the MF contributes 80-90% of the lens diameter (Gao *et al.*, 2004). The gradual loss of nuclei from the fibre cells as they track inward toward the nucleus of the lens (**DF**; deep fibres) indicates the loss of organelles from the lens fibre cell as they differentiate. Diagram from Gagna *et al.*, (1997).

The fibre cells found in the cortex of the lens elongate 50- to 100-fold, potentially reaching up to 1 cm in length when fully differentiated in an adult human lens, and are capable of elongation rates of 150 $\mu\text{m}/\text{day}$ in some species (Bassnett, 2005). Differentiation of fibre cells from lens epithelial cells involves major structural changes and partial fusion of adjacent fibre cells, as well as changes in gene expression and the expression of differentiation-specific proteins such as the crystallins, water channel and gap junction proteins, and beaded filament proteins (Rao & Maddala, 2006; Beebe *et al.*, 2001; Blankenship *et al.*, 2001). This process involves a subset of apoptotic-related proteases including the caspases (reviewed by Bassnett, 2002). Fully differentiated fibre cells lose the capability to synthesize new proteins and maintain metabolic processes, owing to the progressive loss of organelles during the differentiation process. The loss of organelles is thought to facilitate a high degree of transparency in the lens by removal of potential light-scattering mitochondria and nuclei from fibre cells that make up the bulk of the lens (Donaldson *et al.*, 2004; Frederikse *et al.*, 2004;

reviewed by Basnett, 2002; True & Carroll 2002; Moffat *et al.*, 1999; Sandilands *et al.*, 1995; Sandilands *et al.*, 1995a; Spector, 1995).

The process of epithelial cell differentiation occurs throughout life, with fibres being added the periphery of the lens fibre mass over the pre-existing fibre cells, resulting in the lens constantly increasing in size and early fibre cells progressively becoming internalised toward the lens nucleus (True & Carroll, 2002; Ireland *et al.*, 2000). Importantly, there is no turnover of lens cells, such that the fibre cells residing in the nucleus of the lens have been there the entire life of a particular organism, and thus the lens will contain cells that were present in the embryonic lens right through to adulthood (Shestopalov & Bassnett, 2003; Moffat *et al.*, 1999; Spector, 1995). As essentially all stages of lens fibre cell differentiation are present in a lens at any time, the spatial layout of fibre cells from the lens periphery to the centre thus represents a temporal profile of fibre cell differentiation (Perng & Quinlan, 2005; Donaldson *et al.*, 2004). Radial cell columns can be seen in equatorial lens sections that emanate from deep in the lens to the surface of the fibre mass. An elongating fibre cell in one of these columns becomes flattened between the cells that differentiated just before and after it. This results in the appearance of lens fibre cell in cross section having two longer sides, where it adheres to the cells in its radial column, and four shorter sides (“short sides/edges”) where the fibre cell makes contact with those in adjacent columns. These cell-cell associations cause the cross sections of fibres to approximate to flattened hexagons, and this precise arrangement may be important for lens transparency, as disruption is symptomatic in cataract patients (Beebe *et al.*, 2001).

The lens is an avascular tissue, and as such, the inner lens tissue is reliant upon the metabolically active epithelial cells for delivery of nutrients and other molecules, as well as removal of waste. The transport of molecules through the lens is carried out via a poorly understood gap junction-based internal microcirculation system that creates a functional syncytium, linking the fibre cells such that cytoplasmic continuity is maintained across the mass of cells that make up the lens (Donaldson *et al.*, 2004; Mathias & Rae, 2004; Baruch *et al.*, 2001; Moffat *et al.*, 1999).

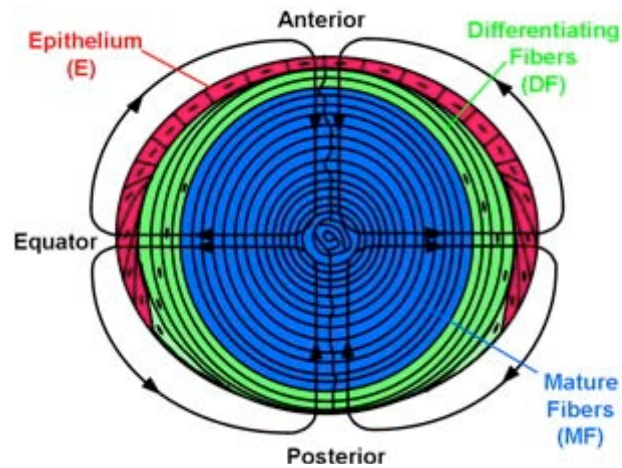


Figure 1.2 *Internal Circulation System in the Lens.*

Axial presentation of a lens indicating the direction that fluid and ions in the internal microcirculation of the lens follows (flow direction indicated by the arrows), emerging from the lens nucleus at the equator, out to the periphery. Fluid and ions then traverse the outer regions of the lens around to the anterior and posterior regions of the lens, where they then proceed to flow through the cortex back toward the nucleus. Diagram from Gao *et al.* (2004).

The lens is particularly vulnerable to insult due to its lack of organelles in mature fibre cells, as there is no mechanism for replacing or repairing damaged proteins and membranes in the interior of the lens. Consequently, any damage that is incurred by the lens will tend to be cumulative due to the lack of turnover of tissue in the lens (Bloemendal *et al.*, 2004). Additionally, the absence of turnover in the fibre cells allows a great opportunity for posttranslational modification of lens proteins to occur that may interfere with the passage of light through the lens (Ponce *et al.*, 2006; Kyselova *et al.*, 2004).

1.2 Components of the Lens

The Lenticular Cytoskeleton

The cytoskeleton is essential for the normal growth, maturation, differentiation, integrity, and function of cells in the human eye. In common with other cells, lens fibre cells possess a cytoskeletal system that is comprised of microfilaments, microtubules and intermediate filaments (Bozanic *et al.*, 2006). Lens cytoskeletal proteins are present at much lower levels than the crystallins that make up the bulk in the lens. Cytoskeletal proteins make up around 2-4% of lens proteins, though the level of cytoskeletal proteins found in a lens cell is much higher than that found in other cell types (Clark *et al.*, 1999). An assortment of cytoskeletal

proteins is found in the lens, with actin and vimentin being among the most abundant. Other cytoskeletal proteins involved in modulating the assembly, function and stability of the actin membrane skeleton such as spectrin, band 3 protein, band 4.1 protein, band 4.9 protein, myosin, tropomyosin, tropomodulin, caldesmon, α -actinin, ankyrin, ezrin and talin have also been identified in the lens. Alongside these actin associated proteins, filamentous proteins, filensin, CP49 (phakinin), beaded filaments, glial fibrillary acidic protein, and microtubule proteins including tubulin have been isolated in the lens (Guest *et al.*, 2006; Reed *et al.*, 2003; Beebe *et al.*, 2001; Blankenship *et al.*, 2001; Padgaonkar *et al.*, 1999; Matsushima *et al.*, 1997).

Cytoskeletal proteins in the lens account for only a small proportion of total protein, but they are thought to play a significant role in the maintenance of lens transparency, particularly in the stabilisation of the fibre cell during and after differentiation when the fibre cell undergoes massive changes in its dimensions (Perng & Quinlan, 2005; Menko, 2002). Additionally, cytoskeletal proteins may play an important role in the facilitation of the chaperone function of α -crystallin in the lens, which is thought to be crucial in maintaining optical clarity in the lens (Guest *et al.*, 2006; Graw, 2004; Menko, 2002). Finally, cytoskeletal proteins have been found to be associated with the formation of interlocking junction domains within the lens (Zhou & Lo, 2003).

Spectrin

The spectrin-actin membrane skeleton is expressed in all cells throughout the body, and is important for cellular shape, membrane stability, deformability, polarity, adhesion, as well as the formation of discrete membrane sub-domains and in the Golgi where it may be involved in sorting events (Beck, 2005; Thomas, 2001; Woo *et al.*, 2000). Spectrin has been extensively characterised in the erythrocyte, where it is found in a filamentous network with actin, protein 4.1 and ankyrin that forms direct and indirect connections to the membrane and is crucial for maintaining red blood cell shape and elasticity (Czogalla & Sikorski, 2005).

Structurally, spectrin is a tetramer comprised of antiparallel heteromers (α and β) of 280 and 247-460 kDa that are cross-linked by actin (reviewed by Czogalla & Sikorski, 2005; Rotter *et al.*, 2004; Thomas, 2001). Spectrin in the lens is known variously as non-erythroid

spectrin, α -spectrin, α II-spectrin or fodrin. It is similar to erythroid spectrin in terms of immunochemical cross reactivity, tetramer formation, and binding of actin, protein 4.1 and ankyrin, although it differs in some other qualitative characteristics (Winkelmann & Forget, 1993). Isolation of spectrin from lens homogenates via electrophoresis has revealed it is found primarily as 235-280 kDa bands (Robertson *et al.*, 2005; Tamada *et al.*, 2000; Fukiage *et al.*, 1997; Matsushima *et al.*, 1997). During the course of normal lens maturation, spectrin is proteolytically processed by a range of proteases to fragments ranging in mass from 60 to 160 kDa, with a highly sensitive cleavage site producing a 150 kDa band that is important in changes of cell shape (Lee *et al.*, 2001; Lee *et al.*, 2000; Fukiage *et al.*, 1997). The proteolytic activity of calpain on spectrin in the lens is known to produce fragments 145 and 150 kDa, while the action of caspase 3 is known to produce fragments of 120, 145 and 150 kDa during apoptosis, a process in which spectrin is a major substrate (Czogalla & Sikorski 2005; Robertson *et al.*, 2005; Rotter *et al.*, 2004; Lee *et al.*, 2001). Interestingly, Sjögren's syndrome, which is an organ specific autoimmune disease that affects around 4 million Americans and occurs mainly in middle-aged women, is the result of the production of autoantibodies to cleaved fragments of non-erythroid spectrin (Williams *et al.*, 2003).

Actin

As noted above, the spectrin-actin membrane cytoskeleton is an important component nearly all higher eukaryotic cell types, and actin has been purified and characterised in the lens fibres of several species (Fischer & Fowler, 2003; Lee *et al.*, 2000; Lo *et al.*, 1997). As well as being one of the major cytoskeletal proteins in the lens, actin participates in and regulates a host of events associated with fibre cell differentiation including the withdrawal of the lens epithelial cell from the cell cycle, and possibly plays a role in lens accommodation via the stabilisation of epithelium under tension (Rao & Maddala, 2006; Kivela & Uusitalo, 1998).

Actin exists in the lens both as filamentous (F-actin) and monomeric, globular actin (G-actin), a polypeptide of 47 kDa. F-actin assembles into two kinds of structural forms known as bundles and networks. The ratio between the amount of F-actin and G-actin in the lens is dependent on the stage of differentiation, with F-actin increasing as the fibre cells elongate (Rao & Maddala, 2006; Lee *et al.*, 2000). The polymerisation of G-actin into F-actin, and vice-versa, is controlled by actin modulating proteins such as gelsolin and tropomodulin, which can bind the ends of the actin molecules, or in the case of gelsolin, sever actin

filaments, thus controlling the dynamics and organisation of the filaments (Archer *et al.*, 2005; Fischer & Fowler, 2003).

The spectrin-actin membrane cytoskeleton is an important system found in cells throughout the body, and again actin, like spectrin, is found throughout the lens with its distribution altering with cell differentiation and morphological changes. F-actin in the lens epithelial cells is organised in unique, multilateral collections of stress fibres that lie beneath the apical membrane, as well as in adherens belts that are associated with cell-cell contacts and are typical of epithelial cell types (Tepass, 2002). Contrastingly, lens fibre cells contain prominent bundles of F-actin that are aligned along the vertices of the hexagonal fibre cells, and there is additionally a continuous F-actin network that underlies the entire plasma membrane of these cells (Fischer *et al.*, 2000; Lo *et al.*, 1997). Actin has been detected in the lens nucleus in some species (Lee *et al.*, 2000), where it has been found to remain associated with the nucleus fibre cell membrane. The persistence of the spectrin-actin system following organelle removal is one significant difference between the highly similar processes of lens fibre cell differentiation and apoptosis, and it has been suggested that cortical actin fibres play a central role in protecting differentiating lens fibre cells from apoptosis during and following organelle removal (Weber & Menko, 2006; Bassnett, 2002).

Tubulin

Tubulin is present in the lens as the protofilaments that form the microtubule system. Microtubules are long tubes (up to 50 nm) with a diameter 25 nm that provide mechanical stability and tracks for molecular transport by motor proteins from the kinesin and dynein families, as well as being involved in cellular events such as chromosome sorting, controlling cell polarity, and organelle localization in a large variety of cell types (reviewed by Karsenti *et al.*, 2006; Murray & Wolkoff, 2003). Tubulin itself is formed from dimers of α - and β -tubulin, each of which are approximately 55 kDa (Padgaonkar *et al.*, 1999).

Very little work appears to have been completed regarding the localisation of tubulin within the lens. Padgaonkar and co-workers (1999) followed the distribution of a selection of cytoskeletal proteins in Guinea Pig lenses that had been treated with hyperbaric oxygen, and reported the presence of tubulin in the lens nucleus. Matsushima and co-workers (1997), as well as Clark and co-workers (1999) followed the loss of cytoskeletal proteins that is seen in

the rat lens selenite cataract model, and reported tubulin was present in the lens cortex and the nucleus at reduced levels by SDS-PAGE and western blots.

Intermediate Filaments

Intermediate filament proteins are referred to as such on the basis of their average diameter (10 nm) is intermediate to that of microfilaments (characteristically 5-8 nm in diameter) and the microtubules (typically 20-25 nm in diameter; Strelkov *et al.*, 2003; Pitz & Moll, 2002). Intermediate filament proteins range in molecular weight from 45 to 150 kDa, and share a common structure based on the presence of non- α -helical N- and C-terminal domain ends and a central α -helical rod domain that is well conserved in size and predicted secondary structure (Alizadeh *et al.*, 2002). The intermediate filament system is a large and varied protein group with up to 70 known genes (DePianto & Coulombe, 2004). The intermediate filaments are thought to be involved in numerous processes, such as cell division, motility and plasticity, as well as being responsible in large part for the mechanical integrity of the cell with the other cytoskeletal systems (reviewed by Strelkov *et al.*, 2003). Six classes of intermediate filaments have been defined: class I, the acidic keratins; class II, the basic keratins; class III, desmin, glial fibrillary acidic protein, peripherin, and vimentin; class IV, the neurofilament proteins; class V, the nuclear lamins; class VI, nestin; and the lens-specific orphan intermediate filaments CP49 and filensin (Bozniac *et al.*, 2006; DePianto & Coulombe, 2004). A distinctive attribute of intermediate filament proteins is their insolubility in conditions that readily solubilise microtubules and microfilaments, which is useful property when it comes to the isolation of intermediate filaments including vimentin (Helfand *et al.*, 2003).

The lens is remarkable in its use of multiple iterations of intermediate filaments proteins. Throughout its development, the lens is known to express type I and type II cytokeratins early in lens development, followed more or less by the emergence of type III intermediate filaments (vimentin), which are present mainly in the outer epithelial cells of the lens. Lens specific beaded filaments comprised of filensin and CP49 are the last of the intermediate filaments to appear in the lens, but are also the longest surviving of this group of cytoskeletal elements (Blankenship *et al.*, 2001; Lee *et al.*, 2000; Sandilands *et al.*, 1995; Quinlan *et al.*, 1996).

Vimentin

The first intermediate filament to be identified in the lens was vimentin, which is a type III intermediate filament protein of 56 kDa predominantly expressed in cells of mesenchymal origin (Perng & Quinlan, 2005; Colucci-Gyon *et al.*, 1994).

Vimentin is known to exist in cells in a network that extends out from the cell centre in a radial organisation (Clarke & Allan, 2002). The specific role of vimentin in the lens is not well understood, and knockout studies have found that vimentin deletion does not have an obvious phenotype (Blankenship *et al.*, 2001; Colucci-Gyon *et al.*, 1994). Vimentin is found in the lens in epithelial cells and early differentiating fibre cells, but is absent from mature lens fibre cells, with its disappearance defining a specific stage of differentiation (Quinlan *et al.*, 1996). Mature lens fibres and mammalian erythrocytes share a common feature that they are devoid of vimentin filaments as well as nuclei, while lens epithelial cells and avian erythrocytes are nucleated and contain vimentin. This has led some to suggest vimentin actively contributes to the retention of the nucleus in the cell; however there is evidence that vimentin is lost from the lens fibre cell after the completion of nuclear loss (Sandilands *et al.*, 1995).

Filensin, CP49 and Beaded Filaments

Filensin (115 kDa), also known as CP95/CP115, and CP49 (~49 kDa), also known as phakosin/phakinin, are lens specific intermediate filaments that are found at all stages of lens fibre cell differentiation (Lee *et al.*, 2000; Sandilands *et al.*, 1995; Quinlan *et al.*, 1996). Filensin and CP49 combine with α -crystallins to form the unique lens filament known as the beaded filament, whose function in the lens is unknown, but which is thought to be essential for normal lens function as deduced from knock-out studies and intermediate filament function in other physiological systems (Sandilands *et al.*, 2004; Alizadeh *et al.*, 2002; Blankenship *et al.*, 2001; Ireland *et al.*, 2000; Quinlan *et al.*, 1996). The beaded filament structures of the lens fibre cell were discovered by Maisel and Perry in 1972, and have since been described in all vertebrate lenses, as well as in non-vertebrates such as the squid (Perng & Quinlan, 2005; Quinlan *et al.*, 1996).

Structure and Assembly Characteristics of the Beaded Filament

The organization of CP49 and filensin into the beaded filament in fibre cells is the only known example of intermediate filament proteins existing in something other than the usual 10nm filaments (Hess *et al.*, 1998). *In vivo*, beaded filaments are found as 5-6nm filaments regularly decorated with 15-20 nm beads that have a regularly spaced pattern and a 19-21nm axial repeat (Georgatos *et al.*, 1997). CP49 is thought to provide the filament core of the beaded filament, with filensin contributing to the peripheral regions (Sandilands *et al.*, 2004). *In vitro*, filensin and CP49 co-assemble into intermediate filaments of 10 nm when mixed in a 1:3 molar ratio that appear similar to classical smooth-surface intermediate filaments (Perng & Quinlan, 2005; Georgatos *et al.*, 1997; Goulielmos *et al.*, 1996). Also, transfection of filensin and CP49 into non-lens cells results in co-assembly of non-beaded filamentous structures (Goulielmos *et al.*, 1996).

Usually in the intermediate filament protein family there is a high degree of sequence identity between species (generally >85%), however between bovine and chicken filensin there is only a 62% level of overall primary sequence identity (Hess *et al.*, 1998). This may be in part due to the fact that different species differ in their ability and requirement to accommodate the lens for focusing, thus requiring different properties in the lens fibre cell intermediate filaments to achieve deformation as the lens is focused in a particular species (Perng & Quinlan 2005; Georgatos *et al.*, 1997). For example, the mouse lens is completely spherical and hardly accommodating, whereas the lens of a bird or human is biconvex and highly accommodating (Georgatos *et al.*, 1997).

Functions and Roles in the Lens of the beaded filament

The function of the beaded filament in the lens is poorly understood, but may be involved in stabilization and/or organization of the fibre cell membranes, and be an important structural element in the maintenance of long-term lens transparency (Ireland *et al.*, 2000; Quinlan *et al.*, 1999). A deletion mutation in the human CP49 gene (*Bfsp2*) that causes disruption in coiled-coil formation has been found in a family exhibiting an autosomal-dominant hereditary cataract (Jakobs *et al.*, 2000). This was the first discovery of a mutation in a cytoskeletal protein, and importantly non-crystallin protein, that results in cataract formation. An amino acid substitution mutation *Bfsp2* gene has also been identified in a juvenile-onset cataract, distinguished by initial lens clarity at birth followed by gradual development of opacity in the second and third decades of life (Conley *et al.*, 2000). Characterization of a naturally

occurring mutation in the *Bfsp2* gene in mice (mouse 129 strains) found that a truncated protein product is formed that destabilizes filensin and changes the morphology of the lens fibre cytoskeleton (Sandilands *et al.*, 2004).

Membrane Associated Proteins

A network of gap junctions links the fibre cells in the lens, and allows for the direct coupling between the cytoplasm of neighbouring cells (Zampighi *et al.*, 2005). The resting voltage of the lens is essentially the same wherever it is recorded, and this fact lead early lens researchers to conclude the lens was akin to a large cell, when actually the homogenous voltage witnessed was due to the intercellular connections afforded by the gap junction network (Mathias & Rae, 2004; Shestopalov & Bassnett, 2003). The gap junction-based internal microcirculation system that exists in the lens is responsible for circulating nutrients into and removal of waste from the lens nucleus. In addition to gap junctions comprised of members of the connexin family, in the lens fibre cell membrane also contains various ion channels, Na/K pump proteins, as well as the aquaporin family of water channels.

Hexameric structures of connexin molecules (connexons) interact with connexons in neighbouring cells to form the channel structure of gap junctions (Baruch *et al.*, 2001). Lens fibre cells express two connexin isoforms, Cx46 and Cx50, while the outer epithelial cells express Cx43 and Cx50 (Zhang & Qi, 2005; reviewed by Mathias & Rae, 2004). (Two nomenclatures for connexin types are commonly used, one using lowercase Greek letters that is based on closer similarity to archetypes, e.g., $\alpha 1$ and $\beta 1$, and the other using the molecular weight deduced from cDNA clones e.g. Cx46 and Cx50; Hopperstand *et al.*, 2000). Cx43 is removed from the lens epithelial cells via internalisation as well as proteosome degradation, while Cx46 and Cx50 are modified post-translation and remain in the lens nucleus (Mathias & Rae, 2004). Cx46 and Cx50 are found in high concentrations near the lens equator where they are thought to direct the outward component of the circulating current found in the lens. Interestingly, Cx50 is proteolytically processed by calpain deep within the lens, whereby its cytoplasmic tail region is cleaved in order to maintain cell coupling in acidic conditions that would normally result in the channel closing (Donaldson *et al.*, 2004).

Aquaporins in mammals comprise a family of more than 10 integral membrane proteins found in a variety of tissues where they facilitate bidirectional osmotic water transport, as well as having the ability to transport glycerol and other small solutes (Ruiz-Ederra & Verkman,

2006). The most common aquaporin in the lens fibre cell membrane is AQP0, previously known as major intrinsic protein (MIP), which is found expressly in differentiated fibre cells (Lindsey-Rose *et al.*, 2006; Ma *et al.*, 2005; Mathias & Rae, 2004). AQP0 acts as an adhesion molecule as well as a membrane water channel, and cleavage of AQP0 in the lens nucleus is thought to trigger this change of function (Gonen *et al.*, 2005; Ma *et al.*, 2005; Mathias & Rae, 2004; Gonen *et al.*, 2004). Mutations and deficiency of AQP0 cause wide ranging disorganisation in the lens fibres and result in cataracts, suggesting a structural role for these proteins also (Lindsey-Rose *et al.*, 2006). The other common aquaporin in the lens is AQP1, which is found in lens epithelial cells only. Currently, the role of AQP1 in the lens epithelium is unknown (Ruiz-Ederra & Verkman, 2006).

Crystallins

During differentiation there is a marked increase in the protein concentration of the fibre cells, which can reach 450 mg/ml (Reed *et al.*, 2003). The protein concentration in the lens is primarily due to the presence of water-soluble crystallins. The crystallins contribute between 30 and 40% of the total lens mass, and around 90% of the water-soluble protein in the lens (Ponce *et al.*, 2006; Kyselova *et al.*, 2004; Bhat, 2004; Horwitz, 2003; True & Carroll, 2002; Ueda *et al.*, 2002; Piatigorsky, 1998). Crystallins are particularly long lived owing to the nature of lens growth, and are relied upon to provide transparency, maintain viscosity and preserve the optimal refractive index in the lens. These qualities are primarily achieved by their tight, highly ordered packing within the fibre cells, and interactions between themselves, other lens proteins such as cytoskeletal proteins, and membranes (Sun & MacRae, 2005; Fernald, 2006; True & Carroll, 2002; Moffat *et al.*, 1999; Spector, 1995).

Crystallins were first described as the structural proteins of the vertebrate lens in the 1890s by Mörner, and were so named owing to their presence in the crystalline lens (Augusteyn, 2004; Graw, 1997). The main groups of crystallins found in the lens are the α and β/γ crystallins (Bhat, 2004; Horwitz, 2003). These crystallins are sometimes referred to as the ubiquitous crystallins, as they are the most common crystallins in the lens, particularly in mammals, and α and β are found in all vertebrate lenses. It was originally thought that the crystallins had evolved uniquely to function in the lens, but crystallins have since been found to be expressed in the heart, brain, and other tissues of the eye (Fernald, 2006).

α Crystallin

The α crystallins represent the major class of water-soluble proteins in the lens, and are found in all vertebrate lenses. α crystallin-type proteins comprise a large protein family that is distributed widely across species ranging from plants and bacteria to human (Franck *et al.*, 2004; Narberhaus, 2002). In the lens α crystallin is found in limited amounts in the lens epithelial cells, but its synthesis is strongly up-regulated upon differentiation into fibre cells, where they are the major protein constituent (Bloemendal *et al.*, 2004). In the fibre cells it is found as a large polydisperse multimeric assembly with a molecular weight ranging anywhere from 300 to 1200 kDa, averaging 800 kDa, and can be found in lens extracts in aggregates with molecular masses in excess of 1 MDa (Biswas & Das, 2004; Derham & Harding, 1999).

The α crystallin complex is composed of two related subunit proteins, α A- and α B crystallin of approximately 19.9 and 20.1 kDa, respectively. The α A subunit is essentially a lens specific crystallin, while α B crystallin is considered to be a ubiquitous protein particularly abundant in the heart, brain and muscle (Bloemendal *et al.*, 2004; Horwitz, 2003; Piatigorsky, 1998; Graw, 1997). The α A and B subunits exist in the mammalian lens at an approximate ratio of 3:1 respectively, although this ratio is variable depending on the age of the lens (Bloemendal *et al.*, 2004). Interestingly there appears to be no specific requirement for either subunit in the formation of a stable α crystallin multimer protein (Bloemendal *et al.*, 2004; Horwitz, 2003; Graw, 1997). The basis of the observed variation in the mass of α crystallin aggregates is not fully understood, but appears to depend on the source of the α crystallin, the purification protocols employed, and the presence of age-related post-translational modifications (Kumar *et al.*, 2005; Augusteyn, 2004).

In recent times, α crystallin has been subject of intense investigation of its structure and function, particularly in light of the fact that it is a key member of the small heat shock protein family, a large family of proteins that display chaperone-like activity. These proteins can bind denatured proteins to maintain their solubility and prevent binding and aggregation with other proteins, as well as conferring enhanced stress-resistance on cells (Sun & MacRae, 2005; Augusteyn, 2004). The structurally divergent small heat shock proteins are defined by the presence of the “ α crystallin” domain, a sequence of 80 to 100 amino acid residues located toward the C-terminal that is moderately-to-highly conserved between species (Sun &

MacRae, 2005). In the lens, α crystallin is thought to assist in the folding and stability of lens proteins, ensuring the solubility and prevention of aggregation of other proteins in the lens through an ATP-mediated process that is currently not well understood and may involve hydrophobic surface interactions between the chaperone and its target (Biswas & Das, 2004; Franck *et al.*, 2004). α crystallin has been shown to inhibit the precipitation of β and γ crystallin *in vitro*, in addition to various other proteins (Augusteyn, 2004). In the lens it is thought that the other crystallins are the major target of α crystallin chaperone function, based on extracts of 50-65 year old lenses in which the “water insoluble” fraction contained mostly α crystallin, along with some γ and β crystallins (Hanson *et al.*, 2000). Other targets in the lens that may require assistance in maintaining solubility over the long lifespan of the lens includes cytoskeletal proteins, and “housekeeping” enzymes such as glyceraldehyde-3-phosphate dehydrogenase and enolase (Horwitz, 2003).

Aggregation of proteins in the lens occurs as a result of aging when proteins unfold and denature. As there is no protein turnover or diffusion in the lens (Bloemendal *et al.*, 2004), the chaperone activity of the α crystallins may be responsible for the long-term maintenance of lens clarity through the prevention of protein aggregation that can cause light scattering and eventually cataract, a role that is in addition to its structural function. At around 40 years of age, it is thought that all the α crystallin in the nucleus of the lens are bound to denatured proteins to maintain transparency, and it is around this time that physical changes begin to noticeably affect the lens (Bhat, 2004; Truscott, 2004; Horwitz, 2003).

Further support the hypothesis that α crystallins are largely responsible for the maintenance of lens clarity through chaperone function is that owing to its abundance in the lens α crystallin could bind all of the other proteins in the lens, assuming a 1:1 stoichiometry of α -crystallin to other protein. This would prevent aggregation and insolubilization of these proteins that would otherwise to light scatter and cataract (Augusteyn, 2004). Findings that the α crystallins can also inhibit caspase function, are anti-apoptotic regulators and have auto-kinase activity indicate other general physiological functions (Zhang *et al.*, 2005; Xi *et al.*, 2003).

β and γ Crystallin

The β and γ crystallin polypeptides are recognized as members of a related β/γ super family of proteins based on amino acid sequence homology and crystallographic studies. Perhaps the most prominent feature of the β/γ crystallin family is the presence of a distinctive “Greek key” structural motif (Bhat, 2004; Branden & Tooze, 1999; Piatigorsky, 1998; Graw, 1997). The β/γ crystallins differ from the α crystallins in that they are a relatively large, multi-gene family, and no non-refractive functions have yet been found for either β or γ crystallin (Piatigorsky, 1998). In addition, both β and γ crystallin are lens fibre cell specific, although there have been reports of low-level expression in the retina, brain and testis of mice, chicken and other species (Bloemendal *et al.*, 2004; Xi *et al.*, 2003).

β crystallins are found in the lens as oligomers of up to 200 kDa, with the constitutive monomers having molecular weights of between 22 and 28 kDa. The β crystallin family is composed of acidic (β A1- β A4) and basic (β B1- β B3) polypeptide members (Bloemendal *et al.*, 2004). γ crystallin has a molecular weight of 20 kDa, but in contrast to oligomeric β crystallin, γ crystallin is monomeric (Giancola *et al.*, 2004). There are seven members in the γ crystallin family, γ A, γ B, γ C, γ D, γ E, γ F, and γ S, all of which are around 20 kDa (Bloemendal *et al.*, 2004). There also is evidence for another γ crystallin in some mammals known as γ N (to denote that it is a New γ crystallin; Wistow *et al.*, 2005). γ N crystallin is a recently discovered crystallin with genes found in rats, mice, chicken, humans and chimps. γ N crystallin has features similar to both γ and β crystallin and could be described as an evolutionary bridge or “missing link” between the two related γ and β crystallin proteins (Wistow *et al.*, 2005).

1.3 Calpain and the Lens

The calpain enzymes are a family of calcium-activated cysteine proteases of considerable interest because of their implication in numerous physiological and pathological events (Cuerrier *et al.*, 2007; Sanders & Donkor, 2006). Their association with the development of opacity in the lens is of particular significance (reviewed by Zatz & Starling, 2005; Goll *et al.*, 2003; Reed *et al.*, 2003). The principle calpain isoform in the lens is calpain II/m-calpain, but other calpain isoforms are also present, including calpain I/ μ -calpain, Lp82, Lp85 and calpain

10 (Reed *et al.*, 2003). Calpain I and II are ubiquitous, and Lp82, a lens specific splice variant of calpain 3, is present in several animals including sheep (Robertson *et al.*, 2005) and rodents, but not in human lens (reviewed by Goll *et al.*, 2003). Calpain II has been immunologically detected in rat lens during early development and persists to maturity. Lp82, while being detected earlier in development than calpain II, has been found to be present at a markedly decreased level in mature lenses (Reed *et al.*, 2003).

Many proteins are partially hydrolysed by the action of calpain, however no natural specific substrates have been identified (Chicharro *et al.*, 2006). Calpain may be involved in basic cellular functions, such as differentiation, cell cycle, signal transduction and apoptosis, along with its possible role in pathological conditions (Reed *et al.*, 2003). There is accumulating evidence that calpain is implicated in cataractogenesis (Biswas *et al.*, 2005; Robertson *et al.*, 2005; Sanderson *et al.*, 1996; Azuma *et al.*, 1995; David & Shearer, 1986, 1984). It is thought that calpain, particularly calpain II, is responsible for the formation of opacities in the lens via the calcium-activated degradation of α and β crystallins that leads to their insolubilization, resulting in the scattering of light in the lens (Zatz & Starling, 2005; Matsushima *et al.*, 1997). This is backed up by assays of calpain where it has been found to proteolyze crystallins *in vitro*, and through the use of calpain inhibitors that can prevent the progression of cataract in rodent lenses (Biswas *et al.*, 2005).

Cataract is the leading cause of blindness worldwide, and it is thought between 17 and 19 million people are affected by cataract, accounting for 43% of global blindness (WHO estimate), while an estimated 28,000 new cases are reported each day (Kelly *et al.*, 2005; Kyselova *et al.*, 2004; Matsui *et al.*, 2003). The associated economic burden of cataracts amounts to around \$USD5-6 billion annually, with surgery costs over \$3 billion in the US alone, where 1.3 million cataract operations are performed annually (Ruiz-Ederra & Verkman, 2006; Kyselova *et al.*, 2004; Taylor & Hobbs 2001). A wide variety of insults to the lens can result in a rise in calcium levels, with intracellular concentrations reaching up to 1 mM (reviewed by Goll *et al.*, 2003; and Duncan *et al.*, 1994), sufficient to activate calpain II (calpain II is capable of being activated by 400 μ M calcium; Zatz & Starling, 2005; Goll *et al.*, 2003). Aging and diabetes are the primary factors associated with the majority of cataract cases, with smoking, low antioxidant intake, gout, drugs, metabolic disturbances, family history and congenital disorders as additional causative factors (Biswas *et al.*, 2005; Graw, 2004). The aging process naturally results in the accumulation of light-scattering opacities, at

first slowly, but accelerating around middle age and especially in older age, such that at around 80 years old, more than 50% of people in the USA are affected with cataracts (Mares, 2004). Aging is generally associated with compromised lens function and a decrease in reserves of antioxidants such as GSH (reduced glutathione) as well antioxidant enzyme activity (Kyselova *et al.*, 2004). These cataracts are often referred to as age-related cataracts, which distinguishes them from cataracts that can develop due to other complications, such as congenital, metabolic disorders (e.g. diabetes-induced cataract) and trauma (Taylor & Hobbs, 2001).

Calpain Activation & Regulation

The temporal and spatial regulation of calpain activity is essential because calpain is an abundant cytoplasmic protease, capable of cleaving many signalling and structural proteins (Perrin & Huttenlocher, 2002). For instance, it has been estimated that if all of the calpain residing in muscle were proteolytically activated simultaneously, the entire complement of Z-disks in skeletal muscle would be destroyed in less than five minutes (Goll *et al.*, 2003).

In inactive calpain the two subdomains of domain II (see *fig. 1.3*), in which the active site is located, are arranged with a gap of approximately 10 Å separating cysteine 105L from histidine 262L and asparagine 286L (the catalytic triad residues). This gap renders calpain unable to bind peptide substrates and therefore catalytically inactive (Strobl *et al.*, 2000). In this state, it resides in the cytosol, however upon increased intracellular calcium concentration and binding, and translocation to the membrane, the gap is reduced to approximately 3.7 Å, thereby allowing the catalytic triad to be completed and the calpain molecule activated.

This mechanism was elucidated from observations of numerous cysteine proteases (particularly the catalytic cysteine and histidine residues), of which calpain is a family member, as well as studies on calpain I and calpain II (Suzuki *et al.*, 2004; Jia *et al.*, 2002; Goll *et al.*, 2003). While the properties of calpain have been extensively characterised, the role of calcium in the activation of the proteolytic activity of calpain is less well understood. The structural changes and effects of calcium binding has been a much debated topic over the years (Jia *et al.*, 2002; Dutt *et al.*, 2000).

Difficulty in studying the conformational changes and sites of calcium occupation occurring upon calcium binding arises due to the inability to obtain crystals of this form of calpain, as activation of calpain triggers aggregation, precipitation, autolysis and degradation (Dutt *et al.*, 2000). Thus structural information and the effects of calcium activation have been made from inactive, truncated and mutated forms of calpain, and the details of the required conformational changes are speculative (Jia *et al.*, 2002; Reverter *et al.*, 2001a). It is thought that binding of calcium to the EF-hands in the IV-VI domains should not change its overall structure (Reverter *et al.*, 2001). This is because both the calcium-bound and unbound form of the EF-hand structure is known (there is a large amount of literature about it due to their presence in other proteins; Dutt *et al.*, 2002), and binding produces only a small change in their conformation indicating that it may not provide the required shifting of the domain II subdomains to bring the active site residues together (Strobl *et al.*, 2000; Goll *et al.*, 2003). This is backed up by the fact that calpain in which all EF-hands have been mutated (and therefore, assumingly inactive/incapable of binding calcium) is still activated upon calcium binding at high enough concentrations (Dutt *et al.*, 2000). For these reasons, it is believed that the calmodulin-like domains are possibly more structural than explicitly regulatory with regards to calcium binding (Reverter *et al.*, 2001). Also, it suggests calcium binding sites must exist on calpain aside from the EF-structures (Goll *et al.*, 2003).

While the function of domain III has been difficult to determine, owing to its lack of sequence homology to any known protein (Hosfield *et al.*, 1999), its central location and proximity to the catalytic domain suggests it may be important in the activation of calpain. Notably the acidic loop, with its many negatively charged side chains seems to be of particular interest in calpain activation (Reverter *et al.*, 2001). The acidic loop is in direct contact with domain IIb, which possess a number of basic (lysine) side chains that are attracted to the negative charges of the acid loop, which is highly conserved in the calpain family (Goll *et al.*, 2003). Binding of calcium to the acidic loop could reduce or alleviate the negative potential of the acidic loop, thus decreasing the electrostatic interaction between it and domain IIb (Reverter *et al.*, 2001a). To this end, it has been found that the acidic loop of calpain B (*Drosophila*) does indeed bind calcium (Alexa *et al.*, 2004). This would then allow fusion of the catalytic domain, as domain IIb would be free to move toward IIa, which is clamped down via many polar contacts to domain III, as well as being held by the N-terminal α -helix of domain I (see *fig. 1.3*; Alexa *et al.*, 2004).

This mechanism, termed the “electrostatic switch mechanism” could also account for the differences in calcium between calpain I and calpain II, as calpain II has less acidic residues in its acidic loop, and may consequently require less calcium reduce the electrostatic attraction of domain IIb to III (Strobl *et al.*, 2000). Other investigations where lysine residues of domain IIb were removed via point mutations significantly reduced the calcium requirement of activation, and replacement of two acidic residues in the acidic loop caused a large decrease in the half-maximal calcium requirement of rat calpain II (250 μ M down to 7 μ M; Alexa *et al.*, 2004). However, studies that utilized expressed domain IIa/IIb from calpain I have shown that they can bind one calcium atom, which induces a conformational change that brings together the catalytic triad, indicating that at least some of the activation switch of calpain resides in the catalytic domain itself (Goll *et al.*, 2003; Moldoveanu *et al.*, 2002).

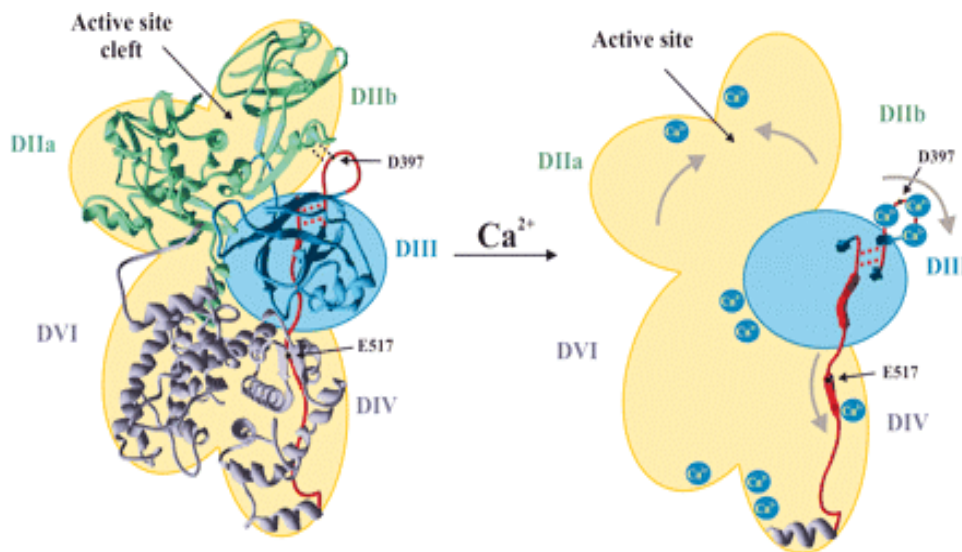


Figure 1.3 Proposed activation mechanism of calpain by calcium

Depicted are the crucial local conformational changes and sub domain movements (the latter indicated by grey arrows in the structure to the right), which occur upon calcium binding leading to calpain activation. Domain III (in blue), binds sub domain IIb via electrostatic interaction between acidic and basic residues in each of the respective regions (shown as two dotted lines near D397). Binding of calcium to the acidic residues disrupts this attraction, and binding in domain IV also results in conformational changes that are transduced to domain III resulting to its pulling down (i.e. as depicted), allowing domain IIb to move toward IIa and complete activation of calpain. The domain structures that comprise calpain are labelled as in the text (Adapted from Alexa *et al.*, 2004).

Calpain Inhibitors

Calpastatin is an endogenous calpain regulatory protein found in the body, and is the only specific calpain inhibitor that has been identified (Biswas *et al.*, 2005; Goll *et al.*, 2003; Donkor, 2000). Calpastatin has a large mass (120 kDa) and consequent low membrane permeability, and thus is thought to be of little therapeutic use (Biswas *et al.*, 2005; Moldoveanu *et al.*, 2004; Todd *et al.*, 2003). The absolute selectivity of calpastatin has raised interest in developing synthetic calpain inhibitors, and more than 50 endogenous and exogenous/synthetic calpain inhibitors have been described (Zatz & Starling, 2005). These inhibitors may be subdivided into 4 classes, the largest and perhaps best understood of which are the peptidyl aldehydes, such as leupeptin, which was originally isolated from *Streptomyces* species (Carragher, 2006; Donkor, 2000). Leupeptin and other peptidyl aldehyde inhibitors inhibit calpain by way of the aldehyde functional group reversibly binding the active site cysteine, which it does so in a calcium dependent manner (Biswas *et al.*, 2004; Fukiage *et al.*, 1997).

The second distinct sub-group of calpain inhibitors are the peptidyl epoxides, which includes the irreversible inhibitors E64 (trans-epoxysuccinyl-L-leucylamido-4-guanidino-butane) and E64c. E64 and its peptidyl epoxide derivatives covalently bind to the thiol group of the active site cysteine residue, thereby blocking calpain proteolytic activity (Biswas *et al.*, 2005; Moldoveanu *et al.*, 2004).

The third sub-class of calpain inhibitors is the peptidyl α ketoamides, which are third generation reversible inhibitors that exhibit improved cellular permeability and solubility than peptidyl epoxides and aldehydes, in addition to circumventing the metabolic instability of the aldehydes *in vivo* (Carragher, 2006).

It is important to note is that several of the inhibitors mentioned so far are not specific for calpain. Other cysteine proteases such as plasmin, trypsin and papain, as well as the proteasome complex, may be affected by these calpain inhibitors, which potentially could cause side effects in physiological systems were they to be used in a therapeutic setting (Carragher, 2006; Moldoveanu *et al.*, 2004; Donkor, 2000).

The final sub-class of calpain inhibitors are the non-peptide inhibitors, such as ATA and quinolinecarboxamide. These inhibitors are reversible and non-competitive fourth generation

inhibitors that have been developed and display greater specificity for calpain over other cysteine proteases (Carragher, 2006).

Calpain Inhibitors as Anticataract Agents in Animal Models

Several calpain inhibitors have been tested as anticataract agents in various animal models, including rats, sheep and pigs, both in organ culture as well as *in vivo* (e.g. Robertson *et al.*, 2005; Biswas *et al.*, 2004; Sanderson *et al.*, 1996; Lampi *et al.*, 1992; Shearer *et al.*, 1991; Azuma *et al.*, 1991). Success in these trials has been limited, particularly due to low water solubility of the compounds coupled with low membrane permeability for first generation inhibitors including E64 (Biswas *et al.*, 2005). E64 has also been found to offer poor protection from proteolysis for lens cytoskeletal proteins such as vimentin and spectrin that are important proteins for lens function as well as prime substrates for calpain degradation (Guest *et al.*, 2006; Biswas *et al.*, 2005; Sanderson *et al.*, 1996). Additionally, it has been found for some inhibitors such as E64 that calpain inhibition *in vivo* is less potent than *in vitro* lens culture system, and some peptidyl aldehyde inhibitors have been found to exhibit lens toxicity (Biswas *et al.*, 2005; Azuma *et al.*, 1992).

Fukiage *et al.* (1997) synthesized a peptidyl aldehyde calpain inhibitor, *SJA6017* (*N*-[4-fluorophenylsulfonyl]-L-valyl-L-leucinal), in an attempt to ameliorate the shortcomings of these other calpain inhibitors, particularly in terms of cell penetration and calpain inhibition. The efficacy of *SJA6017* to inhibit calpain and its affect on calcium ionophore-induced cataracts in cultured rat lenses was investigated, and it was found that *SJA6017* reversibly bound to and strongly inhibited calpain, while reducing nuclear opacity and reducing proteolysis of lens proteins. Comparative studies of a range of calpain inhibitors in rodent lenses also found that *SJA6017* displayed superior efficacy as an anticataract agent (Biswas *et al.*, 2005).

With regards to non-rodent lenses, similar results have been found in an *in vitro* pig lens culture system, where *SJA6017* considerably reduced the progress of induced cataract, (Biswas *et al.*, 2004), while Robertson *et al.* (2005) found that in sheep that developed hereditary cataracts opacification was temporarily delayed following treatment with *SJA6017*.

1.4 The Lens Cytoskeleton and Cataract

While the majority of research into cataracts tends to focus on calpain-induced degradation of α and β crystallins, no typical calpain cleavage sites have been detected from crystallins in aged human lenses (Nakajima *et al.*, 2006; Ma *et al.*, 2005). This suggests that for calpain to play a role in human cataract, proteolysis of proteins other than crystallin must be important, either additionally or principally. The lens cytoskeleton comprises a small proportion of the total lens protein; however it is thought that it plays an important role in the development and maintenance of transparency in the lens (Guest *et al.*, 2006; Perng & Quinlan, 2005; Menko, 2002; Quinlan *et al.*, 1999). Actin, vimentin, spectrin and filensin have all been found to be substrates or potential substrates for calpain proteolysis, which may fit in with the currently accepted model for some types of cataract formation where calpain has been implicated (Guest *et al.*, 2006; Perng & Quinlan, 2005; Sanderson *et al.*, 2004; Reed *et al.*, 2003; Lee *et al.*, 2001; Quinlan *et al.*, 1999; Matsushima *et al.*, 1997; Marcantonio 1992; Marcantonio & Duncan, 1991). A number of diseases have been linked to mutations and disruptions of cytoskeletal proteins, and breakdown of lens cytoskeletal proteins may be a major factor in cataract formation (Sandilands *et al.*, 2004; Lee *et al.*, 2001; Quinlan *et al.*, 1999; Matsushima *et al.*, 1997).

Sanderson *et al.* (2000) reported that loss of the cytoskeletal proteins, spectrin, vimentin, and filensin, seen during incubation of rat and bovine lenses with a calcium ionophore was prevented on incubation in a calcium-free medium and reduced by calpain inhibitors. Matsushima *et al.* (1997) reported similar results in rats with selenite-induced cataract, and that cytoskeletal proteins were among the earliest proteins degraded in this model indicates that their breakdown may play a significant role during the early stages of cataract formation. Inhibition of cytoskeletal breakdown with calpain inhibitors has been associated with a reduction in ionomycin-induced lens damage (Shearer *et al.*, 1997). As mentioned above, mutations in the beaded filament gene *Bfsp2*, the gene for the lens specific intermediate filament protein CP49, have been associated with inherited and juvenile-onset cataracts in humans (Hejtmancik & Kantorow, 2004; Conley *et al.*, 2000; Jakobs *et al.*, 2000). Additionally, changes in cytoskeletal proteins have been detected in congenital and childhood cataracts in humans, suggestive of a role in cataract etiology (Guest *et al.*, 2006; Matsushima *et al.*, 2000).

At Lincoln University, a flock of sheep (Coopworth-Romney cross) are maintained that naturally develop a hereditary cataract. The ovine cataract is cortical in nature and emerges at 1-2 months of age, developing bilaterally over approximately 10 months (Lee, 2006; Robertson *et al.*, 2005; Brooks *et al.*, 1982; Brooks *et al.*, 1982-1983). A sheep based model for human cataracts may present a better alternative to currently available models such as rodent lenses (Robertson, 2003). The ovine lens is large, and the bi-convex lens shape more closely resembles that of a human. Additionally, the ovine lens is an accommodating lens, as is the human lens, and as such may share more commonality than the spherical and poorly accommodating rodent lens (Lee, 2006; Lo, 1988). The absence of alternative natural large animal cataract models makes the ovine cataract a convenient model for such studies and reproducibility of the cataract enhances the claim for an ovine model (Robertson *et al.*, 2005).

Among the aims of researching the ovine cataract is to further the understanding cataract progression in other species, including humans. Additionally, inhibition of the ovine cataract has been used as a platform in the development of potential cataract treatments that are based on the inhibition of calpain proteolysis. Further investigation into the nature of the ovine cataract will clarify its usefulness as a model for human cataracts

1.5 The Use of Immunohistochemistry to Study the Lens

Principles of Immunohistochemistry

Immunohistochemistry, the process of localizing and identifying proteins in tissue sections by exploiting the principle of specific antibody binding to antigens in biological tissues (Mao *et al.*, 1999) coupled with fluorescent or enzymatic markers, is a sensitive technique that offers a high degree of resolution. Immunohistochemistry is sometimes referred to interchangeably with immunocytochemistry (Bratthauer, 1999). The distinction between the two that is that the latter term is more suited to describe the general technique of visualising protein using labelled antibodies when it is applied to biological samples collected in some way other than via histology, such as for example from cell culture.

The first step in immunohistochemistry is fixation of tissue, as it is essentially impossible to perform immunohistochemical microscopic studies with living specimens (Melan, 1999). Fixation is required to preserve the cellular features, prevent tissue autolysis, and prevent or reduce the movement of labile proteins and carbohydrates (Taylor & Levenson, 2006). Tissue

may be fixed by chemical or physical (freezing) processes. The most common chemical fixative is formaldehyde, usually made from its solid hydrated polymeric form paraformaldehyde (PFA) dissolved in phosphate-buffered saline (PBS) solution (Taylor & Levenson, 2006; Montero, 2003; Melan, 1999).

Formaldehyde is favoured because of its high degree of tissue penetration, and its preservation of morphological detail and maintenance of immunoreactivity, especially when compared to other fixatives such as glutaraldehyde or solvent-based fixatives (e.g. acetone/methanol; Bratthauer, 1999; Melan, 1999). For example rapid fixation (1-3 hours) of rodent lenses in concentrated PFA solutions has been found to offer excellent tissue preservation, but at the expense of antigen preservation (Jacobs *et al.*, 2003). Qualities associated with the use of formaldehyde fixatives are ease of storage, low cost, availability, and its long history as a primary fixative in histology and the resultant familiarity which that brings (Taylor & Levenson, 2006; Berod *et al.*, 1981).

Tissue is sectioned after fixation. In order to cut the thin (<20 µm) sections required for immunohistochemistry, tissue must be embedded in a support medium, and this is achieved by either freezing or embedding it in paraffin wax. The latter technique requires dehydration as paraffin is not miscible with water present in cells. This is achieved by exposing the tissue to a graded alcohol series containing increasing proportions of alcohol and decreasing amounts of water. Paraffin is not miscible with ethanol however, and xylene likewise not with water; therefore the dehydrated tissue must next be infiltrated with xylene prior to embedding in molten paraffin. The paraffin is then cooled and solidified prior to precision microtome sectioning of the tissue. Removal of paraffin is required before the sections may be stained, and this is achieved by clearing in xylene, followed by rehydration (Bratthauer, 1999).

Sectioning frozen tissue, also known as cryosectioning, involves freezing the tissue in liquid nitrogen, or less commonly on dry ice or in super-chilled alcohol, embedded in a frozen medium sectioning medium such as *Tissue-Tek*[®] O.C.T. (for *Optimal Cutting Temperature*) compound (Bratthauer, 1999a). Preparing tissue for cryosectioning often requires a cryopreservation step, which involves exposing the tissue to PBS containing gradually increasing amounts of cryoprotectant, such as sucrose. Cryopreservation assists in the prevention of cellular damage induced by the stresses of freezing, possibly by affecting the

formation of ice crystals, although this process is poorly understood (Karlsson & Toner, 1996).

Paraffin processing is the favoured processing technique in routine pathological diagnostic laboratories, where immunohistochemistry is used for diagnosis of diseases with more specificity such as cancer (Niki *et al.*, 2004). Paraffin processing is favoured as it provides superior preservation of tissue morphology, and is conducive to retrospective studies for potentially several decades owing to the apparent stability of proteins in wax block (Walker, 2006; Krenacs *et al.*, 1999). A degree of antigenicity may be compromised during fixation and processing of tissue, as the action of the chemical fixative causes cross-linking of proteins, thereby altering their properties. Additionally, the other chemicals and physical extremes that the tissue is exposed to during the embedding process, including heat from molten paraffin, may alter the immunological properties of an antigen (Taylor & Levenson, 2006; Bratthauer, 1999; Krenacs *et al.*, 1999; Melan, 1999). Sections obtained from tissue that has been frozen are considered to have superior antigen preservation, by virtue of the absence of chemical modification of the target tissue that fixatives introduce. Although frozen sections can suffer from inferior morphological preservation that may be attributable to the stresses of freezing and thawing, this method of immunohistochemical analysis is considered to be the closest to *in vivo* conditions (Bratthauer, 1999a). Additionally, it offers the benefit of being relatively quick and comparatively simple process.

Immunohistochemistry in the Lens

Myriad techniques have been used in the processing of lenses from other species for immunohistochemistry. Rodent lenses have been processed following two general strategies; *a*) fixation followed by sectioning either from paraffin blocks (e.g. Blankenship *et al.*, 2001) or from frozen blocks (e.g. Jacobs *et al.*, 2003; Grey *et al.*, 2003), or *b*) sectioning from fresh frozen tissue blocks (i.e. no prior fixation), followed by fixation after sections were obtained (e.g. Alizadeh *et al.*, 2003; Reed *et al.*, 2003; Reed *et al.*, 1999; Kistler *et al.*, 1985). There is an interesting lack of concurrence among the research on rodent lenses regarding the strength of fixatives made using PFA as the primary agent of fixation. Values can be found in the literature ranging from 0.75% PFA in Jacobs *et al.* (2003) and Grey *et al.* (2003), to 4% for Blankenship. Chicken lenses may be fixed prior to sectioning in 4% PFA (Beebe *et al.*, 2001), while embryonic chicken lenses require a more gentle fixation solution containing 1% PFA

prior to cryosectioning (Lee *et al.*, 2000). Lee *et al.* (2000) also sectioned adult lenses, but these were sectioned unfixed, and were fixed post sectioning in 1% PFA.

Immunohistochemical studies on bovine lenses (e.g. Girao *et al.*, 2005), especially the type of fixation method applied to these larger lenses, are particularly useful for formulating an immunohistochemical protocol for the ovine lens, as they share more features with the ovine lens than rodent lenses. In addition to similar proportions, bovine lenses are soft and are thought to be accommodating lenses (Augusteyn & Stevens, 1998), whereas rodent lenses are hard and are known to be hardly accommodating (Georgatos *et al.*, 1997). Finally, bovine lenses offer the advantage of having been reasonably well studied with regards to fixation and sectioning compared to ovine lenses.

SECTION 2 – EXPERIMENTAL RATIONAL

2.1 Aim of Current Investigation

The aim of this research project was to investigate the distribution of cytoskeletal proteins in the lens, and to examine the effects of calpain proteolysis and cataract on these proteins, with the goal of establishing the role of the cytoskeleton in the ovine cataract model.

An immunohistochemical approach will be used to achieve these objectives that requires the development and optimisation of a protocol that was specific for the ovine lens.

Proteolysis of cytoskeletal proteins with calpain and in the presence of inhibitors also will be investigated to evaluate the roll of calpain in the ovine cataract model.

2.2 Hypothesis

It is proposed that proteolysis the of cytoskeleton in the ovine lens by calpain will result in breakdown of proteins similar to that seen in cataract, indicative of a role for this protease in cataractogenesis through the proteolysis of the lens cytoskeleton. (i) Inhibition of calpain with novel inhibitors will result in the absence of proteolytic products associated with calpain.

Additionally, (ii) immunohistochemical localisation of cytoskeletal proteins in the ovine lens will reveal a characteristic distribution for that selected protein and will be distributed in the lens following a pattern similar to that seen in other species.

2.3 Outline of Sections

A variety of techniques have been utilised in order to investigate the ovine lens cytoskeleton and test the hypothesis that calpain drives proteolysis of the cytoskeleton in the lens, including immunohisotochemistry and a lens based cell free assay of exogenous calpain and lens cytoskeletal extracts. The efficacy of novel calpain inhibitors in the lens based cell free

assay also was investigated. There are 3 major experimental sections that comprise this investigation, in addition to a concluding section.

Section 3 Optimisation of a Method for the Immunohistochemical Processing of the Ovine Lens

In this section, a method for processing the ovine lens for immunohistochemistry was investigated, as no method could be identified in the literature that was specific for the ovine lens. This required evaluation of common methods used for rodents and other large mammalian lenses, and the adoption and optimisation of useful elements therein for use in an immunohistochemical investigation of the ovine lens (see Section 5).

Section 4 The Effect of Calpain on Lens Cytoskeletal Proteins

A method for investigating the effect of exogenous calpain on lens cytoskeletal proteins was adapted, with the aim of establishing a role for the proteolysis of these proteins by calpain in the ovine lens. Lens cytoskeletal extracts were incubated in the presence of calpain, calcium, and one of a selection of calpain inhibitors, including two that have been developed as part of an ongoing research project investigating calpain inhibitors as a treatment for cataracts. Novel calpain inhibitors were included to examine their potential to inhibit cytoskeletal proteolysis, while observing the ability of exogenous calpain to proteolyse lens cytoskeletal proteins and comparing to what has been observed in cataract.

Section 5 Immunohistochemical Localisation of Cytoskeletal Proteins in the Ovine Lens

An immunohistochemical survey of the ovine lens cytoskeleton was completed in order to examine its structure and compare with what is observed in other species.

Section 6 Conclusions and Future Directions

This final section emphasises the experimental evidence garnered from the preceding experimental sections in relation to the original hypothesis, as well as covering possible future research opportunities that may follow from the work completed here.

SECTION 3 – OPTIMISATION OF A PROTOCOL FOR PROCESSING OVINE LENSES FOR IMMUNOHISTOCHEMISTRY

3.1 Introduction

Methods for the processing of lenses for immunohistochemistry – that being the duration and strength of fixation, as well as appropriate sectioning procedure specific to the tissue – are numerous for species such as rat, mice and bovine (e.g. Girao *et al.*, 2005; Alizadeh *et al.*, 2003; Jacobs *et al.*, 2003; Reed *et al.*, 2001), however no specifically optimised method for immunohistochemical processing of the ovine lens is to be had in the literature. As a result, the aim of the initial experiments was to optimise a method for the processing of ovine lenses for immunohistochemistry, with the aim of carrying out an immunohistochemical investigation of the ovine lens cytoskeleton using these techniques.

Four main variables to be considered when optimising a immunohistochemical method are *i)* preservation of tissue morphology, *ii)* immobilization of the antigen, *iii)* preservation of antigen immunoreactivity, and *iv)* adequate penetrability of tissue to the immunochemical reagents (Berod *et al.*, 1981).

Three general procedures were investigated in order produce an optimised method for sectioning and immunohistochemistry ovine lenses, as follows (see **Table 3.1** for further detail):

1. PFA fixed paraffin-embedded sections
2. Fresh frozen lenses with fixation post cryosectioning
3. PFA fixation, cryoprotection, cryosectioning

As outlined above, four main variables will be considered when optimising the method for processing lenses for immunohistochemistry, and the ideal method will be that which satisfies these criteria to an acceptable degree. Additionally, reproducibility and overall time to complete the procedure will be taken into account when determining which of the general procedures will be adopted for further use. The method will be optimised for normal lenses and applied to cataract affected lenses also.

3.2 Methods

Table 3.1 Summary of Fixation and Sectioning Protocols Attempted with Ovine Lenses

General Procedure	Fixation	Pre-Embed Tx	Embedding	Sectioning	Post Sectioning Tx
PFA/paraffin-embed ¹	4% PFA, several hours*	Dehydrate/clear (EtOH/xylene)	Paraffin	6 µm serial, equatorial, affixed to slides	Clear/rehydrate (xylene/ water)
Fresh frozen, fix post section ^{2,3,4,5}	10 min acetone: MeOH ¹ fixative (post sectioning)	N/A	O.C.T. ² , frozen 25 sec liquid N ₂	15-20 µm* equatorial cryo- (-16 °C), affixed to slides	Air-dry 5 min, fix 10 minutes (acetone: MeOH)
PFA/ cryoprotect & cyrosection ^{6,7,8}	4% PFA, several hours*	Cryoprotect (10-30% sucrose solution)	O.C.T. ¹ , frozen 25 sec liquid N ₂	15-20 µm* equatorial cryo- (-16 °C), affixed to slides	Brief air-dry (<2 min), PBS wash to remove O.C.T.

¹Blankenship *et al.* (2001), ²Girao *et al.* (2005); ³Alizadeh *et al.* (2003); ⁴Reed *et al.* (2001); ⁵Ma *et al.* (2001); ⁶Jacobs *et al.* (2004); ⁷Grey *et al.* (2003); ⁸Jacobs *et al.* (2003).

* Precise duration to be determined experimentally.

Cryo-, cryosectioning; EtOH, Ethanol; MeOH, Methanol; N/A, non-applicable; O.C.T., *Optimal Cutting Temperature* (manufacturer name for embedding compound used for cryosectioning); Tx, Treatment. Shaded blocks indicate where methods coincide.

3.3 Experiment 1 – PFA fixed paraffin-embedded sections

The aim of this set of experiments was to investigate processing ovine lenses for immunohistochemistry by fixation in paraformaldehyde, followed by embedding in paraffin and sectioning, which is the most common technique employed for immunohistochemistry (Krenacs *et al.*, 1999).

3.3.1 Methods

Sample Collection

Whole eyes were obtained post mortem from sheep (<12 months old) of unknown breed from a local abattoir, and the lenses removed using a posterior approach (Robertson *et al.*, 2005).

Dissected lenses were placed in a fixative containing 4% w/v PFA in PBS (pH 7.3; PBS prepared from tablets; Oxoid, Hampshire, England). Fixative was made in batches from PFA powder dissolved in hot PBS containing 0.01% 10 M NaOH, filtered, cooled and stored at -25 °C in 50 mL aliquots. Lenses were fixed either whole or cut in half along the equatorial or longitudinal axis. Fixation was carried out at room temperature for varying lengths of time between 4 and 120 hours, increased initially by 4 hours at a time, then by 8 hours, so as to determine the optimum fixation duration. Two lenses at a time were fixed for each period.

Two-stage fixation was also investigated, where lenses were briefly fixed for between 4 and 8 hours, then removed from fixative and cut in half or quarter with a sharp scalpel blade, then returned to fixative for the remaining time.

Following fixation, lenses were washed in PBS then transferred to a 70% v/v ethanol in PBS solution, then sent to a histopathology laboratory for routine processing involving dehydration and embedding in paraffin, and serial equatorial sectioning to 6 µm thick sections (Bratthauer, 1999). Several slide types and coatings were also investigated so as to find the best in terms of section adherence for slide mounting of sections. Prior to staining, paraffin sections required clearing of the wax through several washes with xylene, followed by rehydration through a decreasing alcohol series (100% ethanol, 95%, 90%, 70%, 30%, 10%, pure water, 2 x 2 min washes in each).

Histology

Triticum vulgaris lectin conjugated with the fluorescent labels fluorescein isothiocyanate (FITC; 495 nm absorption, 520 nm emission) and tetramethylrhodamine isothiocyanate (TRITC; 547 nm absorption, 572 nm emission; referred to hereafter as FITC-lectin and TRITC-lectin respectively; purchased from Sigma, Missouri, USA) was used as a general marker of membranes throughout lens sections by way of agglutination of glycosylated proteins of the cell membrane (e.g., Jacobs *et al.*, 2004; Jacobs *et al.*, 2003; Bond *et al.*, 1996; Kistler *et al.*, 1986). Propidium iodide (PI; Molecular Probes, USA) which produces red fluorescence, has an absorption maximum of 535 nm and emission of 617 nm (Molecular Probes Product Information) and was used to visualize cell nuclei through binding DNA as an intercalating agent (e.g. Jacobs *et al.*, 2004).

Staining Procedure

Slide mounted sections were blocked using a 5 mg/ml bovine serum albumin (BSA) solution in PBS (blocking solution) for 2 hours at room temperature, or overnight at 4 °C. Blocking was required to protect charged sites on the tissue that may result in non-specific staining due to electrostatic attraction of these sites to any stain used. Blocking typically involves incubating sections in a proteinaceous solution that contains animal serum in buffer (Bratthauer, 1999). Depending on the type of signal being used for localisation, other types of blocking may be required to block endogenous enzymes or fluorochromes, endogenous biotin, or endogenous proteins that may cross-react with the secondary reagents being used to detect target molecules and result in false-positives.

FITC- or TRITC-conjugated lectin was diluted from a 1 mg/ml stock solution to 20 µg/mL, and then to 5 µg/mL, in blocking solution, and incubated with sections for 2 hours at room temperature in the dark. PI nuclear stain was diluted from a 1 mg/ml stock solution to 500 nM, also in blocking solution. De-staining was achieved by aspirating staining solution, followed by 3 times 15 minute washes in PBS (250 µL), aspirated, and dried further from around the sections using paper towels. De-staining was carried out in a bath containing 200 mL of PBS for 3 washes 5 minutes each.

Approximately 180 µL of blocking solution containing the diluted staining reagents was applied to the slides and fitted with cover slip incubation wells (CoverWell™ chamber gaskets, Molecular Probes, USA – 40 mm by 22 mm by 0.2 mm length by width by depth) to prevent evaporation and drying of the section. Staining was carried out at room temperature for an hour in the dark, or alternatively overnight at 4 °C in the dark. To prevent sections from drying out, sections were placed in a covered container with moistened paper towels during incubation (Reed *et al.*, 2001). Prior to viewing, lens sections were mounted in a single drop of anti-fade fluorescent mounting medium (DakoCytomation, USA) and cover slipped.

Morphological Assessment & Imaging

Sections were viewed using a Leica DMIRB microscope (Leica Microsystems, Nussloch, Germany) equipped with a mercury lamp plus filters capable of filtering 615 nm, 572, and 520 nm for PI/TRITC, and FITC fluorophores, respectively. Images were acquired using a

SPOT[®] RT digital camera (Diagnostic Instruments Inc., Michigan, USA) operated with *SPOT*[®] Advanced for Windows[™] software version 4.0.9.

Image enhancement (balancing image contrast and brightness, and cropping) and annotation was carried out using *Microsoft Photo Editor 2002*[™] (Microsoft, Seattle, USA), *iPhoto*[™] 6.0.5 (Apple, Cupertino, USA) and *Adobe*[®] *Photoshop CS*[®] 8 and *Adobe*[®] *ImageReady*[®] CS 8 (Adobe Systems Inc., San Jose, California). Section morphology was assessed initially from references in the literature of similar types of research.

3.3.2 Results

Fixation was initially carried out using lenses either whole or cut in halves or quarters along the equatorial or longitudinal axis, so as to allow better fixative penetration. It was found that there was little advantage gained from fixing lenses that had been halved or quartered. The cutting itself caused extensive damage and this approach was abandoned. Two-stage fixation where partial fixation of the cortex was achieved prior to halving was also investigated. This method was also abandoned due to the deleterious effects of cutting the lens in half. The comments below are based on whole lens fixation.

The initial fixation for 4 hours resulted in incompletely fixed lenses, in which only the cells at the periphery of the lens were fixed, while the inner regions of the lens would section smoothly but would not adhere to adhesive coated slides. As a result of this, large parts of the lens would be lost from further analysis as they fell out during rehydration or later steps. Fixation duration was increased in increments of between 8 and 12 hours, up to 120 hours, and it was found that around 96 hours fixation produced the most completely intact sections when cutting, and increasing fixation further was not of any obvious benefit. While sectioning of lenses fixed for 96 hours was for the most part successful, occasional sections were incomplete, although this may have been due to sectioning technique rather than inadequate fixation.

Lenses fixed for 96 hours were found to display a good degree of morphological preservation, with the characteristic hexagonal pattern of fibre cells visible across lens sections stained with FITC-lectin (*fig. 3.3.1*, page 35) and minimal perceptible cell damage. The FITC signal for paraffin sections was found to be much weaker in cells located deeper in

the lens section (*fig. 3.3.1, b*), which was possibly related to fixative penetration problems mentioned above. The lens nucleus of sections fixed for this duration was generally found to be intact, but sections would still not section smoothly across the entirety of the lens consistently and reliably – some days it would work, and others it would not – and the time it took to process lenses in this manner meant that it was not a favourable method.

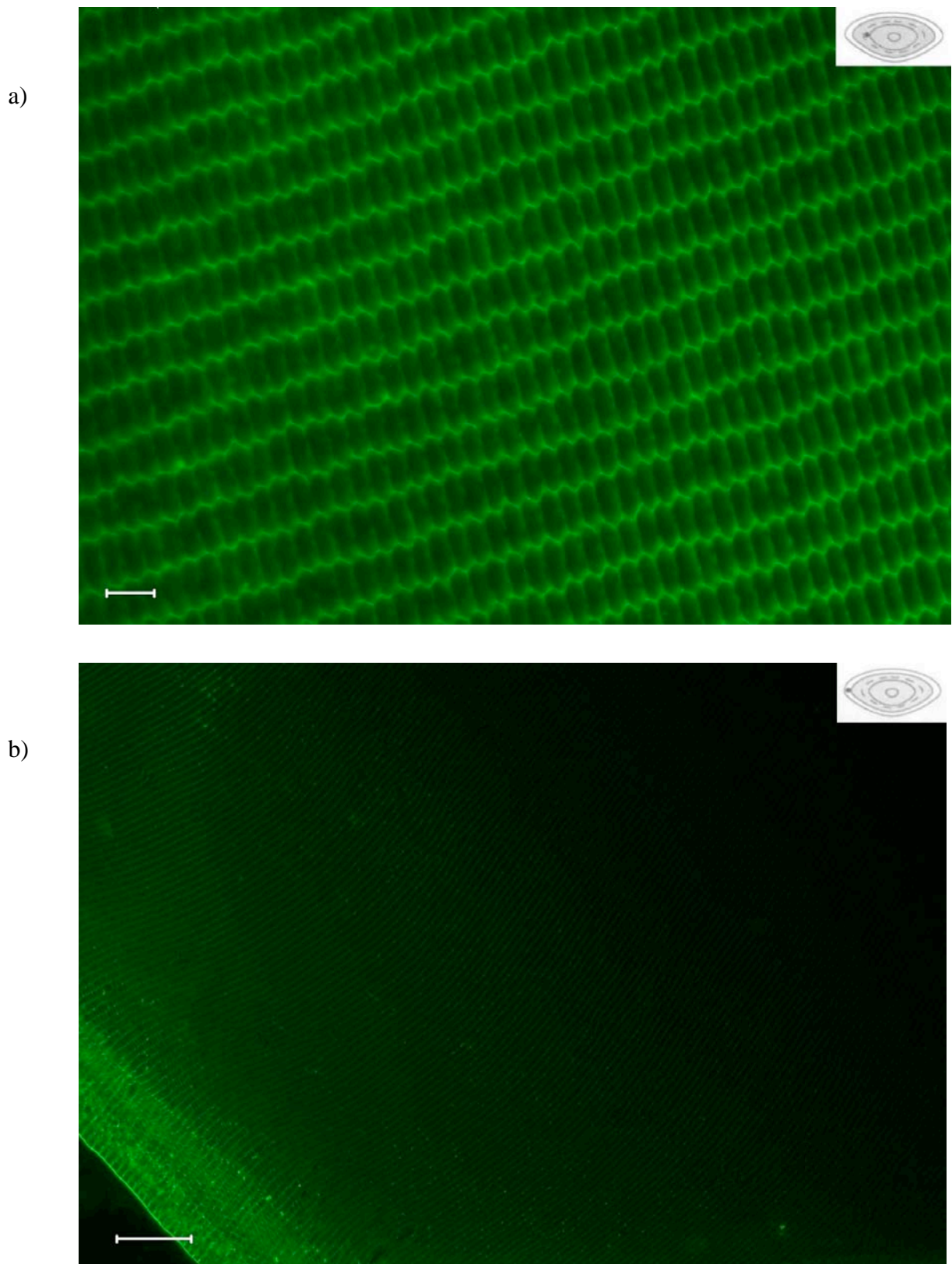


Figure 3.3.1 *Paraffin Embedded Lenses*

Cortical lens fibre cells in lenses obtained from sheep less than 12 months old that underwent paraffin processing were seen to remain intact across large portions of the lens tissue (a, 600x, b, 100x). Inconsistent FITC-lectin staining of fibre cell membranes can be seen with decreased fibre cell staining deeper in the lens (b). *Inset*: schematic illustrating approximate location within the lens where the picture was taken (after Taylor *et al.*, 1997). Scale bars: 10 μm for a, 100 μm for b).

3.4 Experiment 2 – Fresh Frozen Lenses with Fixation Post Cryosectioning

The aim of these experiments was to investigate sectioning lenses that had been frozen immediately following dissection, then sectioned and sections subsequently fixed (after Girao *et al.*, 2005; Alizadeh *et al.*, 2003; Reed *et al.*, 2003; Reed *et al.*, 1999).

3.4.1 Methods

Following an adapted version of the method devised by Reed *et al.* (2001), dissected intact lenses were immediately mounted in *Gurr*[®] O.C.T. compound (BDH, Poole, UK) at 4°C on pre-chilled cryosectioning chucks. Lenses were then frozen in embedding media in liquid nitrogen. Lenses were stored at -80 °C, or transferred to a pre-chilled *Leica* CM1100 cryostat equipped with a rotary microtome (Leica Microsystems, Nussloch, Germany) and disposable cryosectioning blades (S-35; Feather Safety Razor Co., Osaka, Japan) for sectioning. Equatorial sections were cut 15-20 µm thick at between -20 and -16 °C, and mounted on microscope slides coated with poly-L-lysine (Sigma-Aldrich, St. Louis, USA). Sections were air dried for a 5 minutes, then stored at -80 °C prior to fixing in pre-chilled (-20 °C) 1:1 acetone:methanol fixative for 10 minutes, then dried at room temperature for 2 minutes prior to rehydration in PBS and proceeding on to staining as for PFA fixed paraffin-embedded sections (see *Staining Procedure*, **Methods 3.3.** above). Sections were viewed as for paraffin-embedded sections (see *Morphological Assessment & Imaging*, **Methods 3.3.1.** above).

3.4.2 Results

Sections from lenses that were frozen fresh were found to lack the higher-level morphological preservation seen in sections obtained from lenses fixed prior to sectioning (i.e. the other two methods used in this investigation). Cells located at the periphery of the lens did not survive freezing intact, and were seen following FITC staining to have degraded substantially compared to that seen in the other sectioning methods use in this investigation (i.e. in sections shown in *figs. 3.3.1* and *3.5.1*), as well as in the literature (e.g. Girao *et al.*, 2005; Alizadeh *et al.*, 2003; Reed *et al.*, 2001). Fibre cells at the lens periphery were seen to come away from the surrounding lens capsule (*fig. 3.4.1, a*), and fibre cells further in from the periphery had the appearance as if they had been torn apart from adjacent cells.

Fresh frozen lenses tended not to section smoothly, resulting in sections of inconsistent thickness that hindered high magnification viewing (see *figs. 3.4.1, b, c*). Additionally, some sections took on a distinctly chattered appearance (*fig. 3.4.1, b*) following sectioning that was quite inexplicable but again made visualisation of cellular morphology difficult. One advantage offered by this method was that, while cells at the edges of the lens were damaged by direct plunging into liquid nitrogen, the lens nucleus (*fig. 3.4.1, d*) was better preserved than in the other methods that required fixation prior to sectioning.

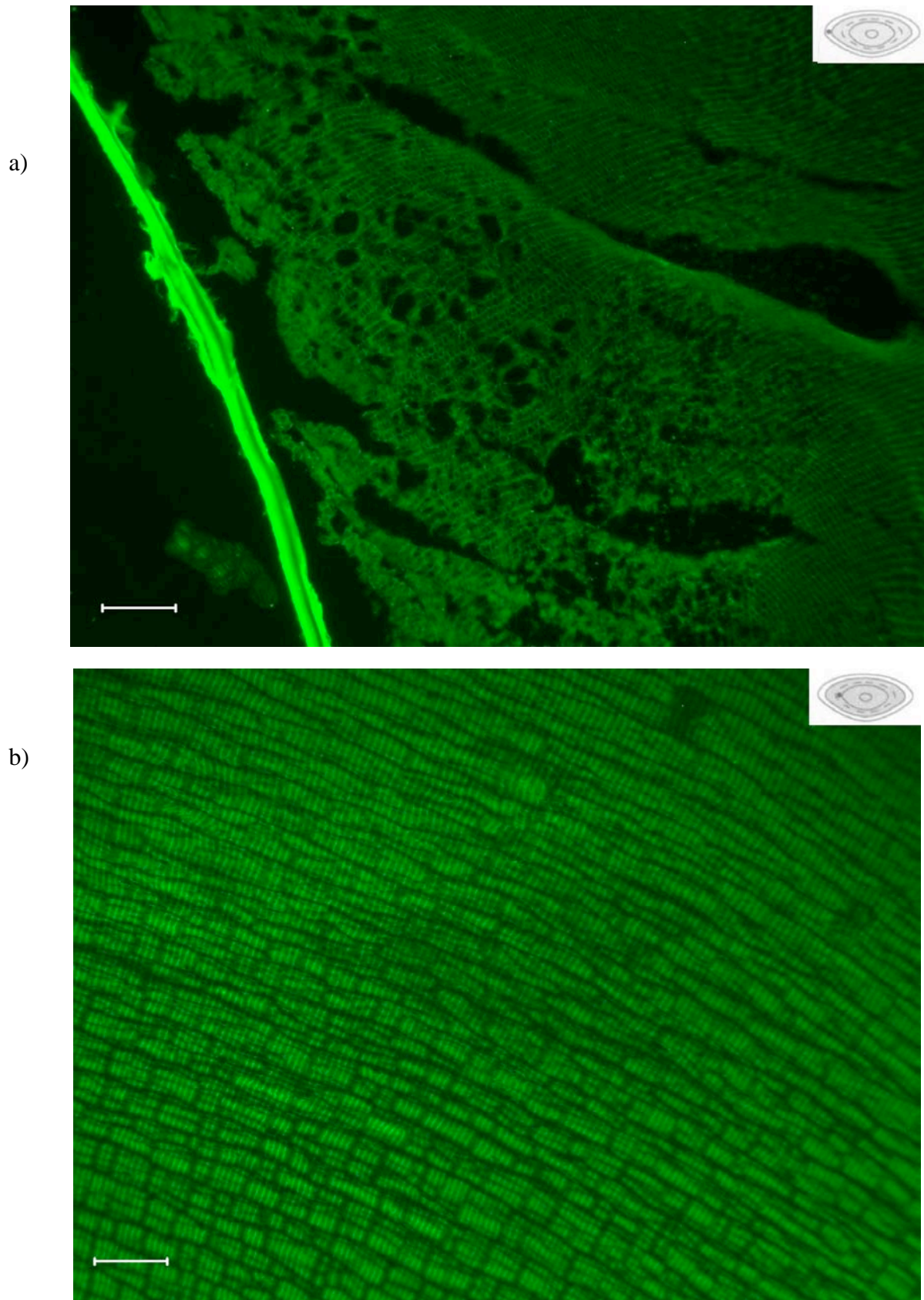


Figure 3.4.1 *Fresh Frozen Lenses*

a) Separation of capsule and cortical lens tissue in fresh frozen lens sections from sheep <12 months of age; b) Chattering pattern observed occasionally in fresh frozen lenses. *Inset* schematic illustrating approximate location within the lens where the picture was taken (after Taylor *et al.*, 1997). Both pictures are at 100x magnification and Scale bars are 100 μm .

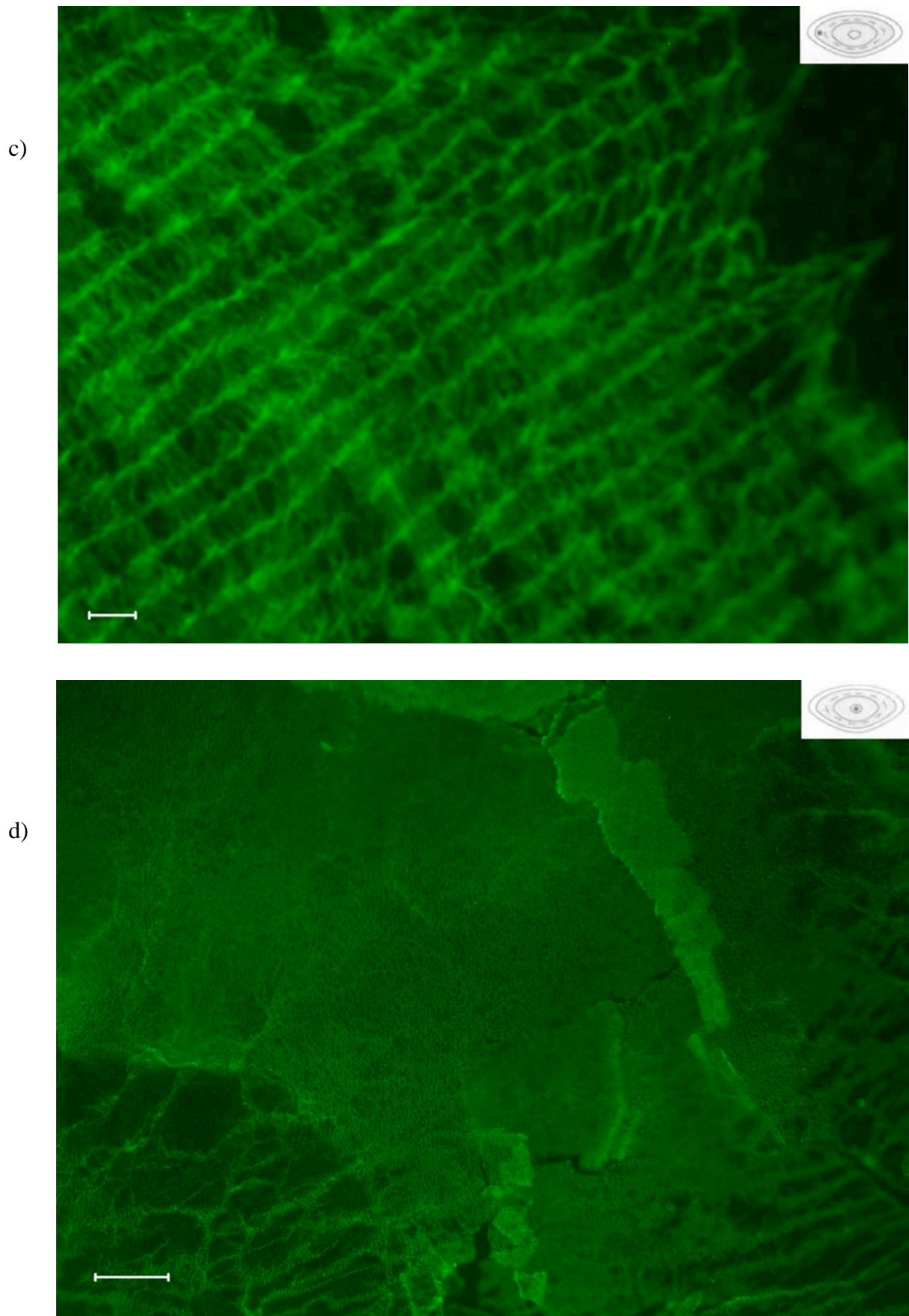


Figure 3.4.1 *Fresh Frozen Lenses*

Low quality morphological preservation of cortex fibre cells seen at high 400x magnification in normal lenses; d) largely intact core section illustrative of level of preservation of this part of the lens when processed using the fresh frozen method compared with alternative methods (100x magnification). *Inset* as above. Scale bars: c, 10 μm ; d, 100 μm .

3.5 Experiment 3 – PFA Fixation-Cryoprotection-Cryosectioning

The aim of these experiments was to investigate the effectiveness of fixation in paraformaldehyde fixative followed by cryoprotection in sucrose solution and cryosectioning for immunohistochemistry (after Grey *et al.*, 2003; Jacobs *et al.*, 2003).

3.5.1 Methods

Sample Collection and Tissue Processing

Eyes were obtained and lenses removed as for the other experiments. Intact lenses were fixed in 4% PFA at room temperature for between 4 and 120 hours to determine optimum fixation time.

Lenses were equilibrated in 10% w/v sucrose cryoprotectant solution (Grey *et al.*, 2003; Jacobs *et al.*, 2003) at room temperature for between 4 and 8 hours, followed by 20% and finally 30% w/v sucrose overnight (all in PBS) prior to cryosectioning. Fixed and cryoprotected lenses were mounted in O.C.T. compound at 4 °C on pre-chilled chucks, and then were immersed in liquid nitrogen for 25 seconds and stored at -80 °C until required for sectioning, or alternatively tissue blocks were stored on dry ice and immediately transferred to a cryostat pre-chilled to approximately -20 °C.

Sections (16 µm) obtained as for fresh fixed lenses (see **3.4.1 Methods**, above) were collected on to HistoBond[®] adhesion microscope slides (Marienfeld GmbH & Co., Germany) factory coated with adhesive, and briefly air dried. Sections were rehydrated and cleared of embedding media with approximately 200 µL PBS, and then covered using CoverWell[™] chamber gaskets (Molecular Probes, USA) so as to prevent evaporation and drying of the section between collection and subsequent steps.

All staining was carried out as for the paraffin embedded sections (see section *Staining Procedure*, **3.3.1 Methods**, above). Incubation of tissue in the presence of stains was adjusted due to the delicate nature of these sections in that it was carried out uncovered in a humid chamber (Jacobs *et al.*, 2003; Reed *et al.*, 2000). Section viewing and morphological assessment was as for paraffin embedded sections.

3.5.2 Results

As for the PFA/paraffin lenses, the length of time of fixation for which lenses that were to be sectioned frozen was altered as necessary to improve the level of penetration and the resulting degree of tissue fixation. It was found 120 hours in 4% PFA fixative provided adequate fixation, however as with the paraffin embedded lenses the nucleus region of the lens would not always section consistently. This resulted in sections that lacked a large portion of the nucleus region of the lens.

Tissue morphology was maintained to a reasonable degree in PFA fixed cryosections. Fibre cells near the periphery of the lens (page 42, *fig. 3.5.1, a-c*) were protected from freezing damage due to the cryoprotection step, and did not display damage seen in lenses frozen from fresh (compare with *fig. 3.4.1*). Across the cortex, cells maintained their expected morphology and did not suffer tearing or apparent shrinkage as experienced in sections acquired using the other methods attempted (i.e. fresh frozen or paraffin sections). Good preservation of the lens nucleus (*fig. 3.5.1, d, e*) was also possible when processing lenses using the 96-hour PFA fix prior to sectioning, although as mentioned above, this was not consistently achieved.

The cryoprotection step used in this procedure, in contrast to fixation, did not require optimisation from what was originally set out in the protocol. In occasional sections fibre cell structure appeared shrunken/disfigured (e.g. *fig. 3.5.1, c*), possibly due to the fixation and cryosectioning steps that could potentially cause cells to lose rigidity and collapse through fluid exchange.

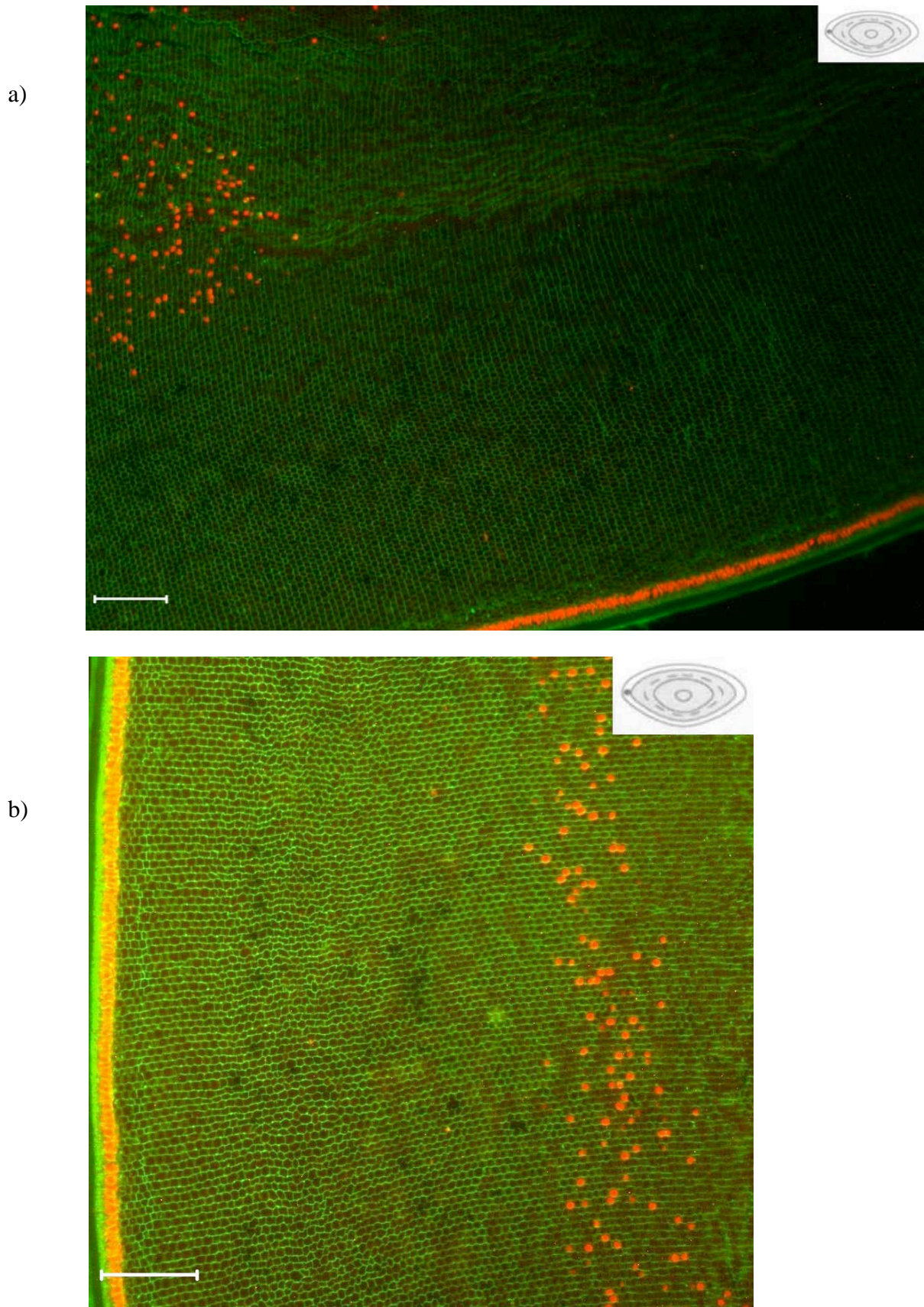


Figure 3.5.1 PFA-Cryoprotect-Cryosection Lenses

Low magnification images showing intact cortex of PFA fixed cryosections obtained from lenses of sheep aged <12 months (a and b). Green signal, FITC, Red signal, PI. *Inset* schematic illustrating approximate location within the lens where the picture was taken (after Taylor *et al.*, 1997). Scale bars: 100 μ m. *Nb.* sections were obtained across the equatorial plane, while inset schematic shows axial view of lens.

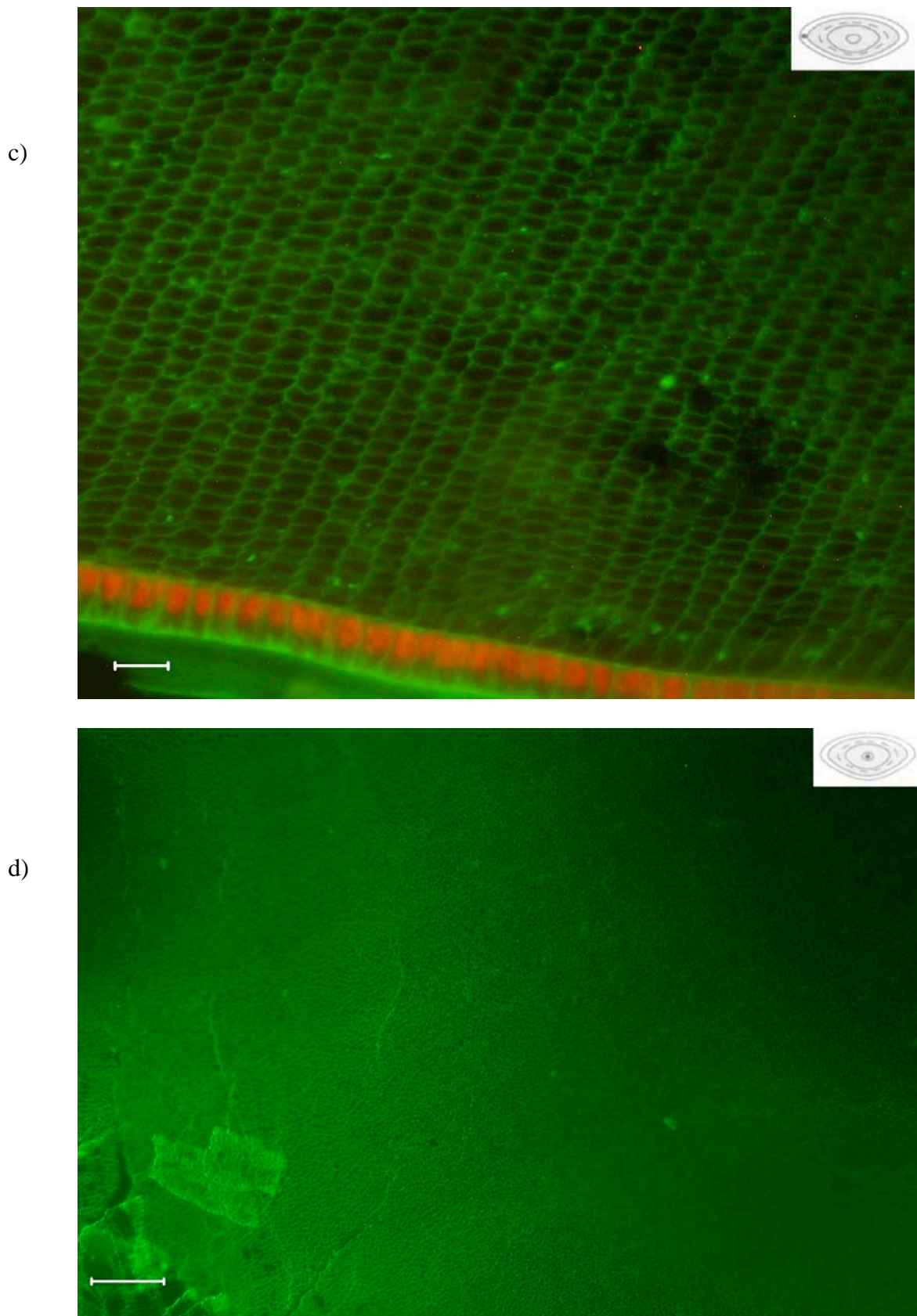


Figure 3.5.1 PFA-Cryoprotect-Cryosection Lenses

Fig. 3.5.1 (c) shows a 400x magnification of areas covered in *fig. 3.5.1* (a) and (b). Intact cortical fibre cells with characteristic hexagonal pattern were visible. *Fig. 3.5.1* (d) is a 100x magnification of lens nucleus. The intact nature of the compressed fibre cells was seen across a relatively large area of nucleus in this section was indicative of a high level of fixation in the nucleus when processed using the PFA-cryoprotect-cryosection method. Scale bars: c, 10 μm ; d, 100 μm . Green signal, FITC, Red signal, PI. *Inset* as above.

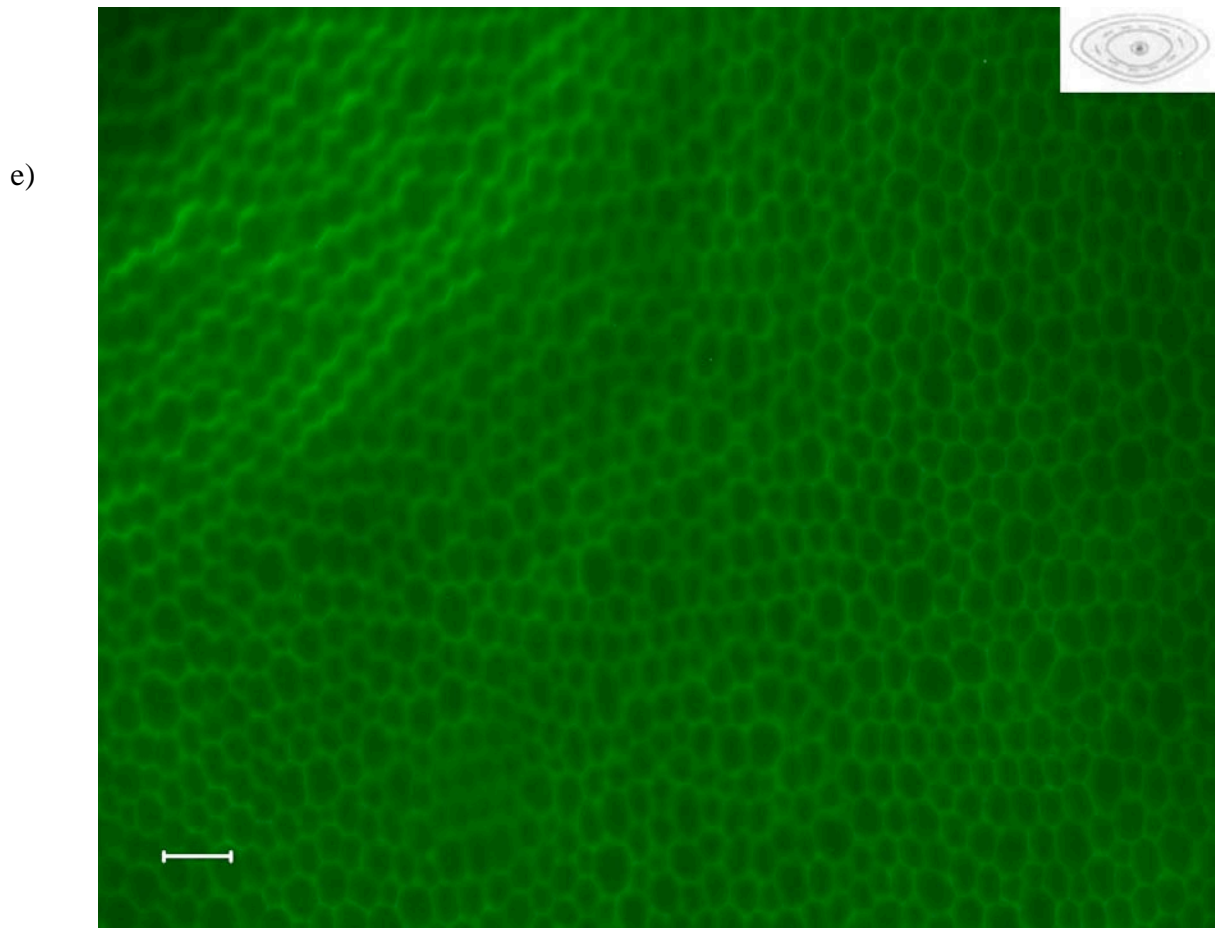


Figure 3.5.1 *PFA-frozen Sections*

High (630x) magnification of intact lens nucleus shown in *fig. 3.5.1* (e), showing compressed fibre cells found in this region of the lens. Good preservation of this area that was difficult to obtain in other lens processing methods was possible in PFA-cryosections. *Inset* as above. Scale bar 10 μm .

3.6 Experiment 4 – Cataractous Lenses

The aim of these experiments was to investigate the applicability of the PFA fix/cryoprotection based cryosectioning method to cataractous lenses, for future study of cataract lens with immunohistochemistry.

3.6.1 Methods

Sample Collection & Tissue Processing

Eyes were obtained from an experimental flock of similarly aged (<12 months) sheep that were genetically predisposed to develop cataract (Robertson *et al.*, 2005; Brooks *et al.*, 1982). A range of cataract damaged lenses were investigated. Cataract damage was scored on a scale

of 1-6 following the procedure of Robertson *et al.* (2005) by a veterinary ophthalmologist using a slit lamp (Kowa, SL/5, Japan) and ophthalmoscope (Vista Diagnostic Instruments, Kellar, UK). Cataract lenses that scored 2 or less (“early stage cataract”) were dissected from the eye as for normal lenses using a posterior approach. Lenses that scored over 2 (“late stage cataract”) were extremely delicate, and could not be removed from the eye in the same way as normal lenses or early cataract lenses and fixed as a completely dissected lens. Late stage cataract lenses required a modified fixation procedure in which the back of the eye behind the lens was removed, along with vitreous humour, and eye tissue was carefully trimmed from around the lens but the lens fixed while still attached to suspensory ligaments and the cornea and iris intact. Lenses were fixed in 4% PFA at room temperature for 120 hours to determine optimum fixation time, after which they were processed for cryosectioning as for lenses in **3.5.1 Methods**, above, and sections and staining were carried out in the same manner as above. Lenses that were fixed with surrounding eye tissue were trimmed further, and the cornea and iris material carefully removed post fixation prior to further processing (i.e. cryopreservation and embedding in O.C.T.).

3.6.2 Results

Fixation for 120 hours was found to provide adequate preservation of cataract lenses. This was the upper end of which useful fixation could be had in normal lenses, and as cataract lenses were at a premium the decision was made to not sacrifice several of these lenses in determining an optimum fixation.

Cataract damage in lenses scored at 2 or less was visible as isolated perturbations (as opposed to widespread damage) in the otherwise uniform hexagonal structure typical of lenses of normal animals (*fig. 3.6.1 a-c*). Damage to the fibre cells was generally present in these lenses at a distance of approximately 600 μm from the surface of the lens, wherein this 600 μm zone fibre cells were present that appeared normal (see arrows, *fig. 3.6.1 a and b*). The fibre cell membranes of cataract lenses did not take up the FITC-lectin stain as well as in normal lenses, possibly due to degradation related to the cataract condition, and consequently were found to produce much dimmer images when visualised. Interestingly this problem was not as apparent with the TRITC-lectin stain (see *fig. 3.6.1 a and c, versus d*).

“Adequate” fixation in the case of late stage cataract lenses was difficult to define, as the amount of damage to these lenses was often such that there was not much lens tissue left that could be fixed. As such, late stage cataract lenses were fixed for 120 hours, cryoprotected and sectioned. Sections collected from late stage cataracts tended to be full of ice that rapidly melted and washed away a lot of tissue following collection, resulting in loss of most of the tissue. The nucleus of late stage cataract lenses tended to section with less of the problems encountered in normal lenses, and indeed was typically the only remaining intact part of the lens in late stage cataracts.

Very weak punctuate staining interrupted irregularly by areas of intense staining was observed in sections obtained from late stage cataract lenses, and revealed a highly disordered state of the fibre cell membranes. The characteristic hexagonal shape of fibre cells was absent, and the large areas of atrophied cells observed in earlier cataracts (arrow-heads, *fig. 3.6.1 c* and *d*) appeared to have become more widespread at depth in the cortex, and some fibre cells dissociated into the milieu of liquefied interior of the lens. The sub-capsular area of unaffected cells seen in earlier stage cataracts (*fig. 3.6.1, a* and *b*) extended only approximately 100 μm (*fig. 3.6.2 a*), compared to about 600 μm in early cataracts. The fibre cells within this 100 μm boundary were not as well defined in terms of the cell morphology as those in the early cataract, and were possibly shrunken.

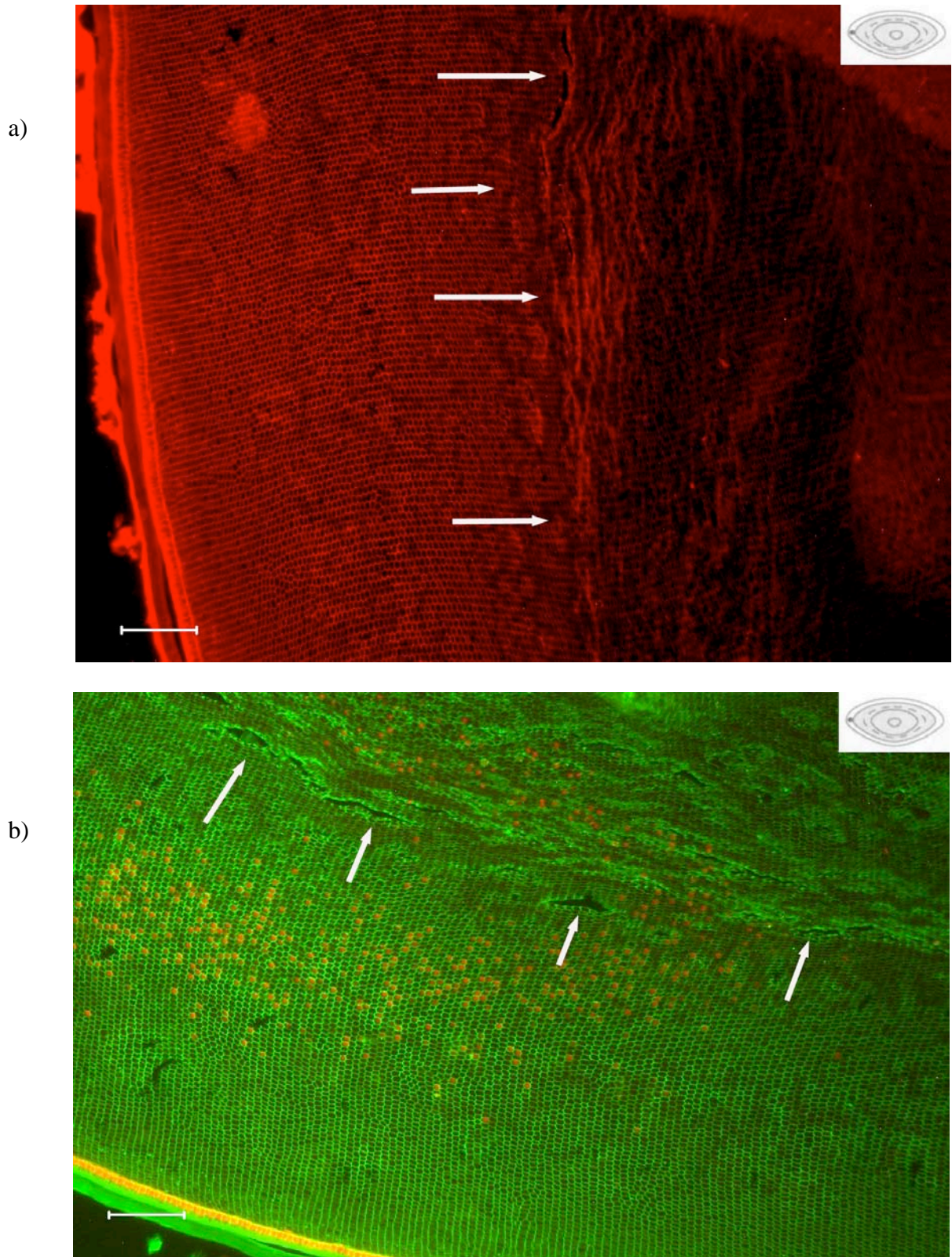


Figure 3.6.1 *Cataractous Lenses*

The above images (a, TRITC and b, FITC stain + PI stained cell nuclei) show sections obtained from lenses that scored 1 on the scale of cataract damage developed by Robertson *et al.* (2005). Damage to the lens was similar to that encountered in poorly fixed lenses, however it was characterized by a continuous area of essentially normal lens morphology directly beneath the lens capsule/surface, followed by distinct degeneration of the regular lens structure around 600 μm in from the edge of the lens (as indicated by the white arrows). Scale bars are 100 μm . *Inset* schematic illustrating approximate location within the lens where the picture was taken (after Taylor *et al.*, 1997).

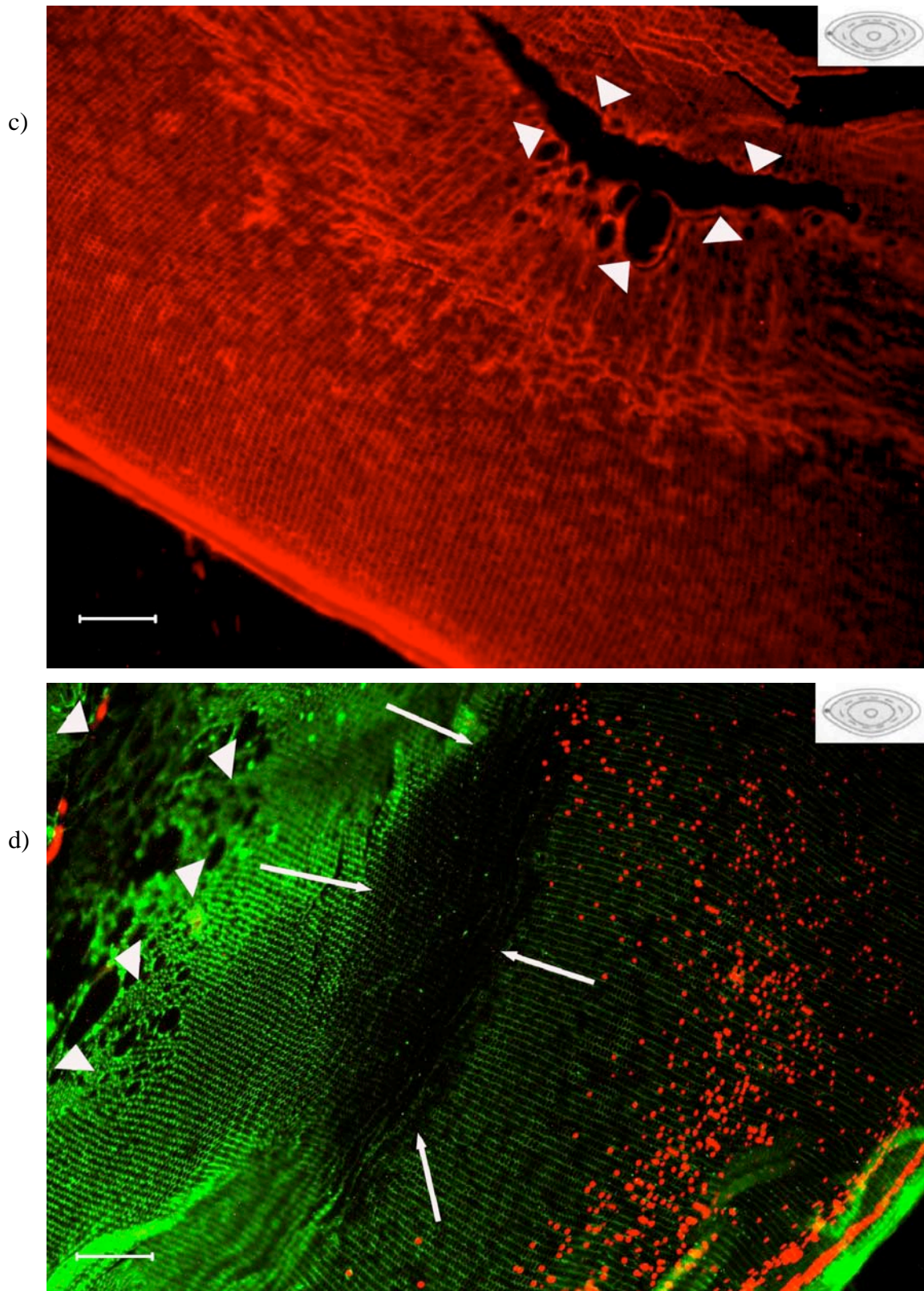


Figure 3.6.1 *Cataractous Lenses*

The above images (c, TRITC, red; d, green, FITC) show lens sections obtained from lenses that scored 2 on the cataract scale. It can be seen that the cataract damage has amplified, with large areas of atrophied cells (indicated by arrow-heads) appearing beneath the area where cataract damage first became apparent in stage 1 cataracts (as indicated by the arrows in *fig. 3.6.1 a* and *b*). Punctuate staining of fibre cell membranes was observed in cataract lens sections (as indicated by the arrows in *fig. 3.6.1 d*). Scale bars and insets are as for *fig. 3.6.1. (a)* and *(b)*.

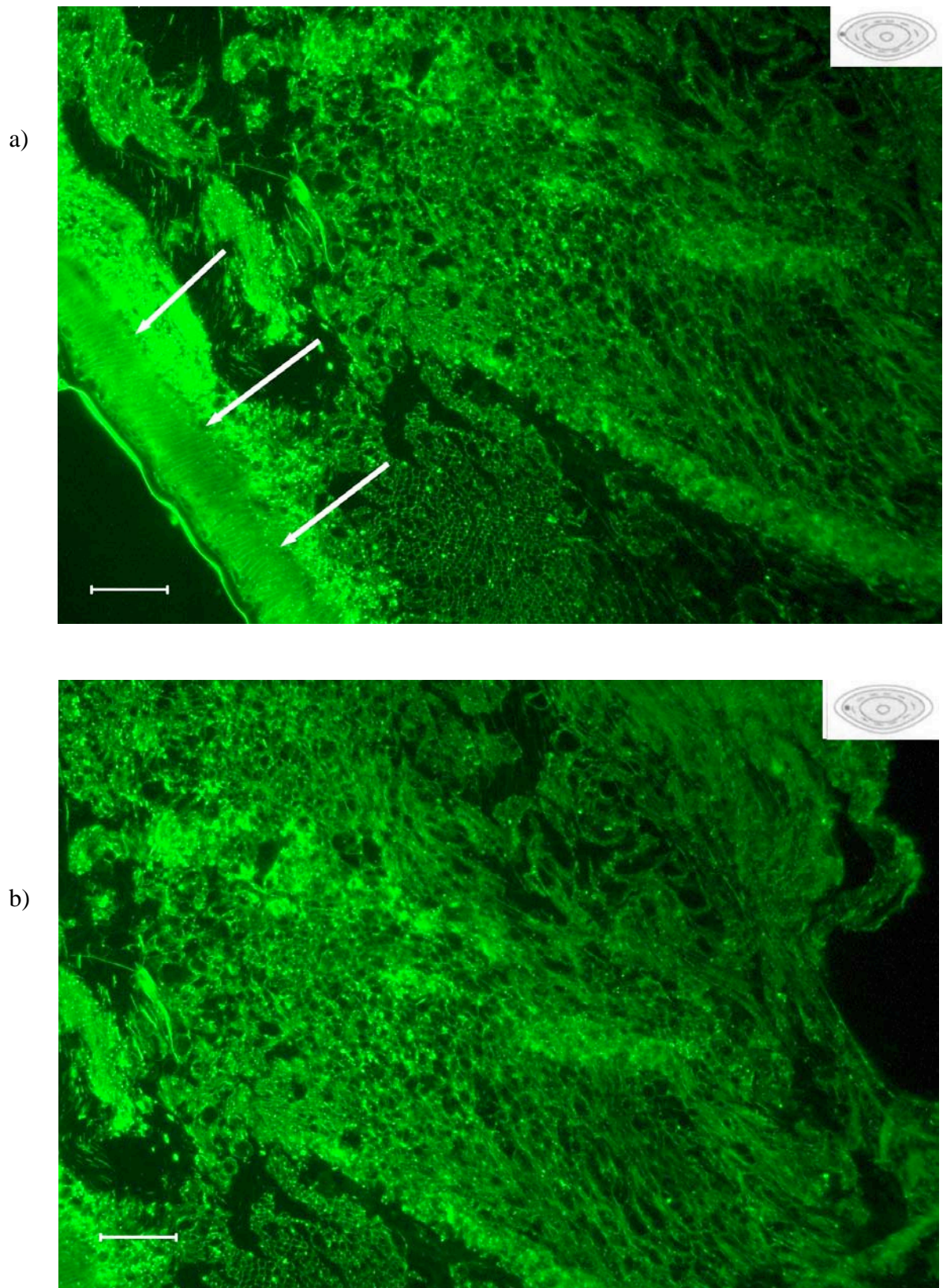


Figure 3.6.2 *Late Cataract Lens Morphology*

Figures a) and b) show the general pattern of staining that was observed in sheep aged less than 12 months afflicted with cataracts that were scored at greater than 2. Little intact lens fibre cell morphology can be observed. The arrows in (a) indicate the last vestiges of the intact outer cortex (as referred to in the main text). FITC staining was very weak in these lenses, and the above images have been enhanced. Scale bars are 100 μm and images obtained at 100x magnification.

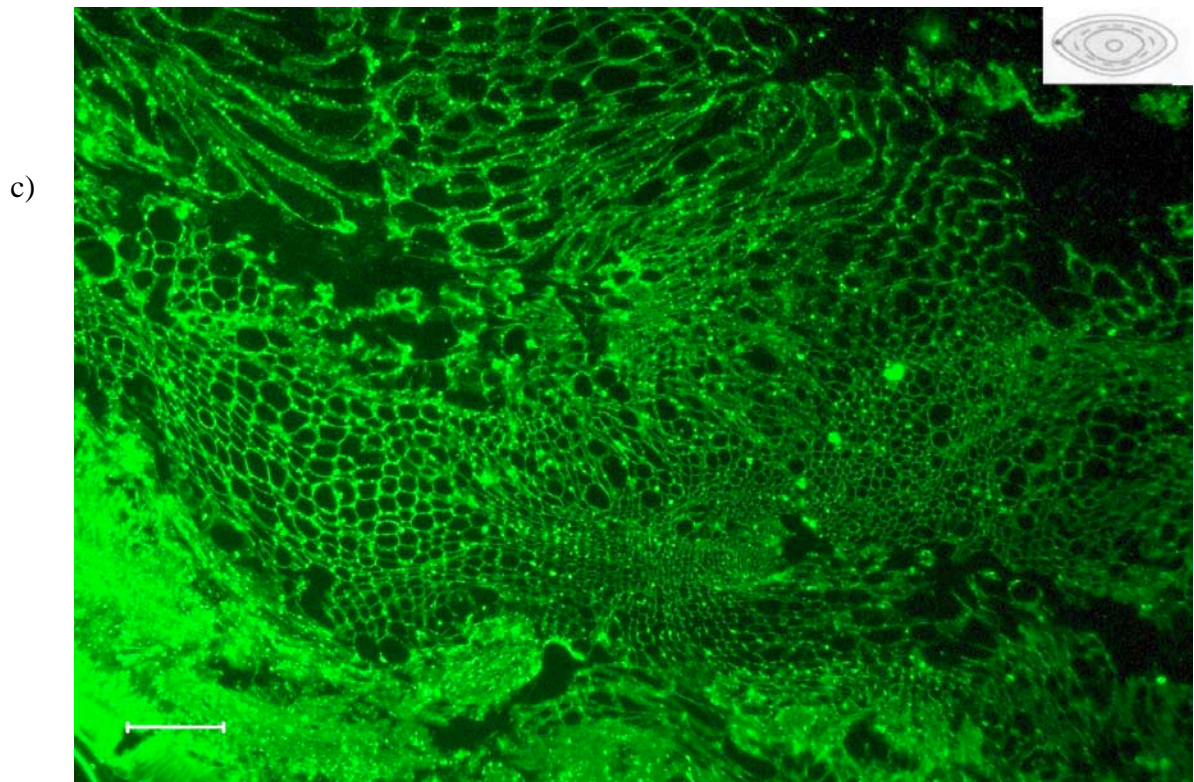


Figure 3.6.2 Late Cataract Lens Morphology

Few sections obtained from lenses that were affected with late stage cataract made it to the staining stage of the procedure, and the ones that did displayed degradation of the lenses internal structure with areas becoming isolated from the surrounding tissue, and a loss of connectivity between fibre cells. Staining was very weak in these lenses, and the above images have been enhanced. Scale bars are 100 μm , and insets are as for *fig. 3.6.1*. All images are at 100x magnification.

3.7 Discussion

There is currently no specifically optimised method available for processing normal or cataractous ovine lenses for immunohistochemistry. In light of this, three methodological paradigms were investigated in the attempt to find a suitable protocol for fixing sheep lenses. These were lenses fixed in paraformaldehyde then embedded in paraffin for sectioning, fresh frozen lenses fixed following cryosectioning (after Girao *et al.*, 2005; Alizadeh *et al.*, 2003; Reed *et al.*, 2003; and Reed *et al.*, 1999) and finally lenses fixed in paraformaldehyde, followed by cryoprotection and frozen sectioning (after Grey *et al.*, 2003 and Jacobs *et al.*, 2003). Each of the methods was evaluated on the basis of a set of criteria that revolved around preservation of tissue morphology primarily, as well as the ability to successfully replicate the procedure, and the overall ease of the procedure.

Immunohistochemistry on paraffin-processed tissue sections was initially investigated, as this method is known to offer several advantages over frozen tissue processing, including long term storage of tissue, the ability to obtain multiple sections from the same tissue sample at the user's discretion over an extended period, superior long-term preservation of cellular features compared to frozen sections and enhanced durability of both the tissue and sections taken there of (Walker, 2006; Krenacs *et al.*, 1999; Bratthauer, 1999). While paraffin processed tissue is generally thought to offer the advantage of being conducive to retrospective studies for potentially several decades owing to the apparent stability of proteins in wax, there is some evidence to suggest there is some deterioration of protein once the sections have been cut from the main block (Walker, 2006; Bertheau *et al.*, 1998). Also paraffin sections, and in particular those taken from the lens (Reed *et al.*, 2001), can be affected by autofluorescence (Niki *et al.*, 2004; Nuemann & Gabel, 2002) that could potentially interfere with fluorescence microscopy used in this investigation.

Following the “standard” technique (Krenacs *et al.*, 1999) for fixing and embedding lenses prior to sectioning for immunohistochemistry, lenses were fixed in 4% PFA followed by dehydration and embedding in paraffin. To begin with, 4 hours fixation of lenses was tested and found to be inadequate, resulting in an incompletely fixed lens where the outer most edges of the lens would “fix”, while the inner regions of sections typically would fall out at some stage during the remaining procedure. The fixed edges of the lens would section from the tissue block as an entire intact section and attach to a coated microscope slides completely, and displayed good morphology with FITC-lectin staining. The nucleus region in contrast would not section evenly and did not display good morphology, if only for the fact that this part of the lens would usually become detached from the slide and be lost from further analysis.

Increasing the length of fixation to approximately 120 hours in approximately 8 to 12 hour increments (essentially increasing fixation by the length of a workday or overnight) had little effect up until approximately 96 hours, at which time sectioning of the lens would produce the most complete sections. It was found that this length of fixation did not always produce uniform sections between tissue samples, possibly as a result of differences in lens size, and further increases in fixation did not prove beneficial in this regard. The use of “Plus” slides (ColorFrost/Plus Microscope Slides, Fischer Scientific, catalogue number 12-550-17) as mentioned in Reed *et al.* (2001) to overcome the loss of lens nucleus was also investigated

when it became obvious that further alteration of the fixation procedure was not practicable. According to the manufacturer, these slides are treated so as to provide electrostatic attraction of tissue for better adhesion to the glass microscope slides, and indeed sections did stick better to these types of slides. Controlled tests were not carried out however, so no definitive conclusion can be made in that regard, nonetheless the “*Plus*” slides were persisted with for the collection of paraffin sections.

Morphology of sections was evaluated by comparison with other light microscopic studies of the lens from the literature (particularly Jacobs *et al.*, 2003; Grey *et al.*, 2003; Young *et al.*, 2000; Bond *et al.*, 1996; Bassnett, 1995), as well from experience derived from sectioning several lenses that gave one some appreciation of what may be deemed as “good” morphology. It has been noted that the preservation of morphological detail in histological sections is deemed “good”, in somewhat of a circular manner, in that it is only what has come to be expected from a particular histological specimen. This is an accepted feature however of semi-quantitative observational methods such as immunohistochemistry (Cregger *et al.*, 2006; Taylor & Levenson, 2006; Berod *et al.*, 1981). The morphological preservation was thus deemed good for lenses processed using paraffin, with no noticeable deterioration in the shape of fibre cells. No problems were encountered with regards to autofluorescence. This was possibly owing to the fact that the FITC-lectin stain used was a robust and bright marker of cell membranes and was able to overcome any background autofluorescence.

Cryosectioning was tested in this investigation, as it is the favoured method for immunohistochemical labelling of lenses in other species (Girao *et al.*, 2005; Grey *et al.*, 2003; Jacobs *et al.*, 2003; Reed *et al.*, 2001; Ma *et al.*, 2001). The method of Grey *et al.* (2003) and Jacobs *et al.* (2003) was adapted, incorporating elements of the procedure devised by Reed *et al.*, (2001). The method of Reed *et al.* (2001) was also attempted. For the Jacobs and Grey *et al.* (2003) method, lenses were fixed in a PFA fixative, prior to embedding in O.C.T and freezing. As for PFA/paraffin lenses, fixation duration was altered as necessary to improve the level of penetration and therefore tissue fixation. It was found 120 hours fixation provided adequate fixation, however again the nucleus region of the lens would not section consistently. This is possibly due to differences in lens size and fixative penetration. Another possibility is that differences in the cryostat temperature during sectioning may have added to difficulties in obtaining consistent sections (Jacobs *et al.*, 2003). Sections were cut with the cryostat temperature set to -20 °C to maintain an optimum cutting temperature of -18 °C. The

chamber temperature would however fluctuate between -20 °C and -15 °C depending on ambient conditions, and on particularly hot days would require the cryostat temperature to be set at its lower limit of -30 °C in an attempt to maintain an ideal cutting temperature.

Morphological preservation of cryosectioned lenses was acceptable following fixation for approximately 120 hours coupled with cryoprotection. Cryoprotection did not require optimisation from what was originally set out in the method, and was effective in preventing the appearance of damage seen in sections that were not cryoprotected prior to freezing that tended to affect the outer-most cells in the lens (see for example *fig. 3.4.1, a*). The method of Jacobs *et al.* (2003) stated 1 hour cryoprotection only was necessary for rat lenses, however it was assumed that as fixation of the ovine lens took up to 5 times longer than in the rat lens, over night cryoprotection would be necessary. Potentially this length of cryoprotection was excessive, and some sections did appear to have degraded morphological preservation (compare the fibre cell morphology of *fig. 3.5.1, c*, with *3.3.1, a*), but it was not outside of what was to be expected when treating lenses in this manner (freezing etc). The alternative of foregoing cryoprotection altogether definitely produced sections of a lesser quality as witnessed by the results of using the fresh frozen lenses fixed post sectioning (compare *fig. 3.4.1, a-c* with *fig. 3.5.1, a-c*).

The fresh frozen lenses with fixation post-cryosectioning method (Reed *et al.*, 2001) was found to be unsuitable for the ovine lens, as a host of morphological abnormalities related to freezing damage were encountered in sections obtained in this manner (see *fig. 3.4.1, a-d*). Apparently the fresh frozen lenses with fixation post-cryosectioning method has been used with success on larger lens types such as the human lens (Ma *et al.*, 2001), and it was surprising that it did not transfer successfully to the ovine lens as they are also a large lens – that is, both human and ovine lenses are larger than the rodent lenses that are typically fixed this way. The ovine lenses (1000-1200 mg; Augusteyn & Cake, 2005) are much larger in terms of their wet weight than human lenses 200-270 mg (Augusteyn & Cake, 2005; Sanderson *et al.*, 2000), which could explain why this method did not transfer as was originally anticipated. As a result of the poor degree of morphological preservation, as well as discussions with other researchers in the field of lens immunohistochemistry, the fresh frozen lenses with fixation post cryosectioning method was not persisted with. Elements of the Reed *et al.* (2001) protocol were however incorporated into the overall protocol developed, such as the use of BSA for blocking sections, the use “Plus” microscope slides for superior section

adherence to slides over that encountered with the use of poly-L-lysine coated slides, and sectioning lenses at 16 μm .

Incidentally, a similar method to that developed by Reed *et al.* (2001) involving fresh frozen lenses with fixation post-cryosectioning was used for research in the ovine lens by Kistler and co-workers in a series of papers published in the 1980s (Kistler *et al.*, 1986; Kistler *et al.*, 1986a; Kistler *et al.*, 1985). Sheep lens sections used in these papers were fixed and sectioned following a protocol that appears to have been developed for rodent lenses covered in the earlier of the papers (i.e. Kistler *et al.*, 1985) and were sectioned with a cryostat followed by fixation in 2% PFA fixative. Fixation and processing of ovine lenses in this manner did not produce sections of a high quality (see Kistler *et al.*, 1986), in much the same way as was encountered here when sectioning fresh frozen lenses following the Reed *et al.* (2001) protocol. Cataractous lenses were also investigated in one of the papers by Kistler and co-workers (Kistler *et al.*, 1986), but these were processed for immunohistochemistry in JB-4 plastic, (JB-4 Embedding Kit[®], Polysciences Inc, Warrington, USA; see for example Choy *et al.*, 2005), a method which was not investigated here.

The PFA/cryoprotection method was applied to cataract-affected lenses with some success. Cataract lenses are fragile and consequently difficult to handle and obtain intact sections from for immunohistochemistry. They often require the use of thick vibrotome sections and confocal microscopy (see for example Bond *et al.* 1996). Pre-fixed cryosectioning of cataract lenses allowed for the collection of thin sections that could be observed with non-confocal microscopy. Early stage cataracts were fixed for the same duration as normal lenses, and sectioned similarly to normal lenses in that they could be collected such that sections remained largely intact throughout the rest of the staining procedure.

The appearance of cataract damage in early cataract lenses (see *fig. 3.6.1*) at approximately 600 μm from the lens surface may coincide with the boundary in the lens between the differentiating outer-fibre cells and the more mature, differentiated fibre cells. Interestingly, this boundary is known to be the location of lens fibre cell membrane-protein redistribution, which involves the processing and cleavage of membrane proteins including members of the connexin family (Grey *et al.*, 2003). The coincident appearance of cataract damage at around this area in the lens where processing and cleavage of proteins is known to take place might indicate the involvement of aberrant protease activity associated with normal lens fibre cell

differentiation being involved in the early stages of cataract. This is however entirely speculative, based only on proximity, and further investigations would be required to bear out this conjecture.

Cataract lenses fixed with surrounding eye tissue due to extensive cataract were fixed for 120 hours and tended to be full of ice that rapidly melted and washed away a lot of tissue following collection of sections. Liquefaction of cortical tissue is a common occurrence in cataract-affected lenses (Bond *et al.*, 1996), and was likely responsible for the large deposits of ice experienced in these lenses during sectioning. Often it was found in higher scoring cataract lenses that the vitreous humour that resides behind the lens was opaque, the result possibly of leakage of lens material, and was indicative of the delicate nature of lens in late stage cataract. It is likely that little lens tissue that could be visualised with FITC/TRITC or PI persisted in these regions in late stage cataracts, though cytoplasmic debris has been identified in the fluid-filled spaces of cataracts induced in rats (Bond *et al.*, 1996).

For the methods that required fixation prior to embedding, lenses were usually fixed whole, fixing lenses that cut in half along the equatorial or longitudinal axis was also attempted with mixed results. This alternative concept emerged from other reports (e.g. Alizadeh *et al.*, 2003), as well as personal experience, which suggested fixative penetration in the lens was very slow. Slow and/or a low degree of fixation can consequently make it difficult to investigate the nucleus of the lens due to improper fixation, and it was thought that reducing the size of the tissue to be fixed so as to allow better fixative penetration would result in a more workable tissue block with regards to section collection. This is also the general consensus in the literature surrounding fixation and processing of tissue for histology in general (e.g. Taylor & Levenson, 2006; Bratthauer, 1999).

All sections were cut from lenses through the equatorial plane. This allowed observation of lens cells at all stages of differentiation, maturation and ageing, given that lens cells are not turned over or lost from the lens, and differentiation occurs throughout the life of a lens with outside cells gradually becoming internalised to the lens nucleus (Jacobs *et al.*, 2004; Ireland *et al.*, 2000; Lee *et al.*, 2000). Jacobs *et al.* (2004) mention that lenses cut in the axial orientation display epithelial cell nuclei extending over the anterior surface to the “bow region” located at the equator, whereupon they begin to differentiate and can be seen to concentrate and track in toward the lens nucleus. Fibre cells in this region appear to curve back on themselves, in addition to becoming smaller with depth into the lens (see for example

fig. 3.6.1, d). In occasional sections obtained through the equatorial axis the epithelial cells fitted this description of axially obtained sections. This was due to cells present in slightly different orientations and indicated that sectioning was not precisely through the equatorial plane (Blankenship *et al.*, 2001); however this (imprecise equatorial sectioning) cannot be determined until after the sections have been collected and stained. Simple vetting of sections with a light microscope at the point of collection and appropriate adjustment of the microtome has been suggested (Marc Jacobs, personal communication, November 2005), however the microtome used to collection sections lacked the appropriate contrivances to alter the angle of sectioning in the first instance, and instead melting and resetting of the lens would have been required to alter the sectioning angle. This would have been time consuming and potentially pointless as it would have required several attempts before exact equatorial sectioning was possible, all the while to the detriment of the tissue and for little practical gain. Consequently this facet of sectioning was disregarded.

Due to the time constraints it was decided that some degree of tissue degradation was to be expected during the fixation and sectioning process, and indeed could not be avoided in most cases. It is apparent from a review of the literature that the lens generally is a difficult tissue for immunohistochemical analysis owing to slow or low tissue penetration of fixatives combined with the compacted nature of the lens nucleus (Alizideh *et al.*, 2003). Berod *et al.* (1981) suggest that the most systematic means of obtaining optimal tissue sections for immunohistochemistry is to compare a series of sections with a graded degree of fixation, as opposed to using a standard fixation procedure as a starting point and proceeding by altering the degree of fixation based on the interpretation of these results. It must be pointed out that the latter approach was used in this investigation, and future investigations may be remiss not to carry out optimisation of fixation of the lens using this strategy. The following passage from Taylor and Levenson (2006) illustrates there is generally a large amount of uncertainty regarding the length of fixation for any tissue:

“... the ideal time for fixation of a 5 mm thick tissue block is perhaps 12–24 hrs (no uniform agreement here; Leong, 2004; Dapson et al., 2005), in practice, the total time in fixative is very variable ... Fixation time in reality is almost entirely uncontrolled, varying anywhere from 6 to 24 hrs, or more [for a 5 mm thick tissue block]. Add to this, questions as to whether the formalin is freshly prepared and adequately buffered, plus variability in the rate of penetration of formalin in different types of tissues and into differently sized blocks, and the

result is a major impediment to standardization of an IHC [immunohistochemistry] stain and an obstacle to quantification.”

The fickle nature of the fixation and sectioning process thus lends itself to a small amount of what may be described as luck, and while immunohistochemistry may be a valuable scientific research and diagnostic procedure, there appear to be concerns in the literature regarding its reproducibility as well as lack of standardisation and quantification that must be taken into account when interpreting an immunohistochemical stain (Cregger *et al.*, 2006; Walker, 2006).

A summary of the key elements of each procedure used in these investigations is shown in **Table 3.2**. Preservation both the microscopic detail of the tissue as well as the lower-level gross preservation as it is sectioned is related to the fixation treatment and sectioning method used to obtain sections. PFA fixed paraffin-embedded sections provided a high degree of preservation of tissue, although the overall procedure itself was time consuming and somewhat inconsistent with regards to fixation of the deeper regions of the lens. In contrast, the method of cryosectioning lenses that were not fixed prior to sectioning was quick but was found to produce sections of poor quality. PFA fixation, cryoprotection and cryosectioning was a simple procedure to carry out that resulted in good morphological preservation and the ability to obtain sections with the lens nucleus region (generally) intact.

Table 3.2 *Summary of Results of Fixation and Sectioning Protocols Attempted with Adult Ovine Lenses*

Procedure	Findings
1. PFA fixed paraffin-embedded sections	+ High degree of morphological preservation in cortical fibre cells + Sturdy sections – Parts of the lens nucleus not fixed, frangible – Reliant on third parties for parts of the procedure
2. Fresh frozen, fixed post cryosectioning	+ Cells in the lens nucleus well preserved + Quick procedure compared to others attempted – Poor preservation of cortical fibre cell morphology – Inconsistent sectioning, – “Chattered” appearance of sections on slides
3. PFA fixation-cryoprotection-cryosection	+ Acceptable degree of morphological preservation + Easily completed on-site without having to rely on third parties + Transferred to cataract lenses – Fragile sections – Time consuming

Nb. “+” indicates a desirable quality of the method, while “-” indicates an undesirable feature

3.7.1 Final Protocol

A method based on cryosectioning with lenses prefixed in paraformaldehyde fixative was developed, and was desirable for several reasons. Among these was that cryosectioning based processing appears to be the favoured method for immunohistochemical labelling of lenses in other species (Girao *et al.*, 2005; Grey *et al.*, 2003; Jacobs *et al.*, 2003; Reed *et al.*, 2001; Ma *et al.*, 2001). Additionally, this method lent itself to convenience as the equipment to perform this type of procedure was readily accessible and the procedure itself was slightly less time-consuming overall compared to paraffin processing, in that all parts of the procedure could be carried out on site without having to rely on third parties for sectioning. This had the added bonus of allowing for better control of the entire procedure.

The developed protocol is for the processing of ovine lenses for immunohistochemistry is as follows:

1. *Remove lens from eye via posterior approach and fix whole for 120 hours in 4% (w/v) PFA fixative in PBS.*

2. *Cryoprotect lens in 10% (w/v) sucrose in PBS for between 4 and 8 hours, followed by 20% and 30% sucrose in PBS each overnight.*
3. *Embed lens in chilled O.C.T. tissue freezing medium on pre-chilled chucks, and freeze for 25 seconds in liquid nitrogen.*
4. *Transfer frozen embedded lenses to -20 °C cryostat and section lenses at 16 µm. Collect sections on HistoBond® adhesion microscope slides (Marienfeld GmbH & Co., Germany), briefly air dry, and then cover with 180 µL PBS in CoverWell™ chamber gaskets to prevent drying.*

SECTION 4 – CALPAIN-INDUCED PROTEOLYSIS OF THE LENS CYTOSKELETON

4.1 Introduction

Currently there is no effective treatment against cataract, and the only medical solution for the condition is lens replacement surgery (Carragher, 2006). Lens replacement surgery is an expensive procedure and is thus unavailable to many patients, in addition to being a time consuming and burdensome treatment (Olson *et al.*, 2003). Widespread “reticence” toward surgical procedures makes this form of treatment unappealing, and the need of less invasive treatments is thus desirable (Biswas *et al.*, 2004). It is estimated that a delay in cataract formation of about 10 years would reduce the prevalence of visually disabling cataract by about 45% (Kyselova *et al.*, 2004).

While preventative treatments for cataract involving antioxidant micronutrients remain controversial (Asbell *et al.*, 2005; Olson *et al.*, 2003; AREDS, 2001), inhibitors of calpain activity have long been of interest to researchers as non-surgical therapeutic agents for diseases including cataract. This stems from the large amount of evidence that points to a role for calpain in cataract development, and also in the pathology of other diseases such as diabetes, muscular dystrophy and conditions associated with ischemic injury and neurodegeneration (Cuerrier *et al.*, 2007; Carragher, 2006; Robertson *et al.*, 2005; Zatz & Starling, 2005; Biswas *et al.*, 2004; Nakamura *et al.*, 2003; Perrin & Huttenlocher, 2002; Fukiage *et al.*, 1997; Sanderson *et al.*, 1996; Lampi *et al.*, 1992). Peptide inhibitors such as calpain inhibitor *SJA6017* developed by Senju Pharmaceutical (Kobe, Japan) have emerged in recent times as potential cataract treatments (Robertson *et al.*, 2005), and *Cat0059* and inhibitor *Cat811* are examples of two novel calpain inhibitor compounds that have been developed locally for application in the same way. The objectives of calpain inhibitor development are to increase solubility and cell permeability of the inhibitor, in addition to addressing concerns regarding the reactivity of the aldehyde functional group with other molecules and proteins under physiological conditions (Carragher, 2006; Abell *et al.*, 2005; Biswas *et al.*, 2004; Nakamura *et al.*, 2003).

No calpain-like cleavage sites have been detected on human lens crystallins in aged human lenses (Nakajima *et al.*, 2006; Ma *et al.*, 2005), which suggests that for calpain to play a role in human cataract as has been postulated (or indeed accepted by some as the following quote indicates: “*The proteolytic action of calpain 2 on lens crystallin proteins causes cataracts in humans.*”; Saez *et al.*, 2006), proteolysis of proteins other than crystallin may be significant. Cytoskeletal proteins, particularly vimentin and spectrin, are prime substrates for calpain degradation. A decreased content of cytoskeletal proteins is among the biochemical changes observed in human cataracts (Matsushima *et al.*, 1997). These proteins are among the earliest proteins degraded during cataractogenesis, and are excellent biochemical markers for calpain activity. Other researchers found that cytoskeletal proteins were not protected from proteolysis in lenses treated with calpain inhibitors (specifically, E64; Matsushima *et al.*, 1997; Sanderson *et al.*, 1996), and consequently this aspect of calpain inhibition warrants further investigation for potential cataract treatments that are based on calpain inhibitors.

In this set of experiments the goal was to evaluate the role of the cytoskeleton and calpain in the ovine lens, as part of the investigation into the ovine cataract model. This was to be achieved by investigating the proteolysis of lens cytoskeletal proteins by calpain using a lens based cell-free (LBCF) method adapted from Sanderson *et al.* (1996). The efficacy of a selection of novel calpain inhibitors in preventing proteolysis of lens cytoskeletal proteins by calpain was then investigated in order to confirm calpain proteolysis and compare the potency of the inhibitors. Calpain inhibitors developed using *SJA6017* as a lead compound have been developed in collaboration between a group researching the role of calpain in cataract development at Lincoln University and synthetic chemists at the University of Canterbury. A selection of these novel inhibitors, as well as *SJA6017*, were assayed with urea-soluble fraction (USF) lens extracts that contain cytoskeletal proteins in the presence of calpain and calcium.

4.2 Materials and Methods

Sample Collection

Eyes were obtained from a local abattoir from sheep of unknown age and breed. Lenses were removed using a posterior approach and weighed. Lenses were separated into cortex and

nucleus/core fractions, on the basis of discernable differences in consistency and texture between the soft outer cortex and the hard nucleus sections (e.g. Robertson *et al.*, 2005; Grey *et al.*, 2003; Matsushima *et al.*, 1997). The outer cortex was removed from the solidified nucleus tissue using forceps, and yielded cortex tissue samples of an average weight of 495 ± 0.01 mg (n=6) from lenses of an average weight of 1545 ± 0.06 mg (values are means \pm standard error of the mean).

Extraction of Urea-soluble Protein Fraction (USF) from Lens Homogenates

The separated cortex tissue was then homogenised using an EppendorfTM homogeniser (Eppendorf, Hamburg, Germany) in 1x volume of Buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 2 mM DTT) containing Complete Mini/MinicompleteTM (Roche, Germany) protease inhibitor cocktail, prepared as per company instructions. Further, finer homogenisation was necessary and was carried out using sonication applied in three 5-second bursts at medium power to prevent overheating the sample. Homogenised samples were then separated by centrifuge (1 hour at 15,000 g at 1°C) into soluble and insoluble protein fractions. The supernatant containing the soluble fraction proteins was discarded, and the pellet containing insoluble proteins was re-suspended in 1 mL Buffer A minus protease inhibitor and centrifuged at 15,000 g for an hour at 1°C to remove any residual soluble protein, and this procedure was repeated twice (see *fig. 4.1*; Robertson *et al.*, 2005).

The pellet was resuspended in a small volume of 6 M urea (typically 100 μ L), homogenised by sonication for 5 seconds three times, and then centrifuged again as above. Following centrifugation, the supernatant containing the urea soluble protein fraction (USF) was collected, then the pellet resuspended in 6 M urea and centrifuged again, and once more the supernatant containing the USF was collected.

Cortex USF samples were then pooled and a sample from this pool assayed for protein content using the BCA Protein Assay Kit (Pierce, Rockford IL USA), as per manufacturers instructions. Briefly, a 10 μ L sample of USF protein diluted 20 times its original volume in purified water was assayed, and protein concentrations determined against a standard curve constructed using BSA as a standard (0, 0.0625, 0.125, 0.25, 0.5 and 1.0 mg/ml). The protein

concentration of the USF pooled fractions was then adjusted to 10 mg/ml, and stored at -20°C. A summary of USF extraction from lenses is given in *fig. 4.1*.

Band Identification by Western Immunoblot

USF protein (10 µg) was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in *NuPAGE*[®] LDS buffer containing β-mercaptoethanol carried out on *NuPAGE*[®] 4-12% Bis-Tris Gels (Invitrogen, Carlsbad, USA). Separation was at 200V for 1 hour, and was followed by transfer to polyvinylidene difluoride (PVDF) membrane at 100V for 1 hour in ice-cold transfer buffer (25mM Tris(hydroxymethyl)methylamine, 192 mM glycine, pH 8.3; Robertson *et al.*, 2005).

Monoclonal filensin antibody (Sigma, Missouri, USA) directed against full length and a 53 kDa filensin fragment (see Masaki & Quinlan, 1997) immunogenically derived from human and bovine lens, rabbit polyclonal anti-α-spectrin (fodrin), monoclonal anti-vimentin (V9 clone; e.g. Fujita *et al.*, 2003), and goat polyclonal anti-actin (C-11) raised against an carboxy terminus peptide of human actin, (all Santa Cruz Biotechnology, California, USA) were initially diluted 1:1000 in 5% non-fat dairy milk (NFDM) powder in Tris-HCl buffered saline (TBS) with 0.05% Tween-20 (TTBS) added, and incubated at separate times with the PVDF membrane for 1 hour at room temperature. The membrane was then washed 3 times for 10 minutes each in TTBS following antibody incubations.

Alkaline phosphatase conjugated secondary antibodies directed against the primary species of origin (rabbit, goat, and mouse; Sigma, Missouri, USA) were used at 1:2000 dilution in 5% NFDM in TTBS for 1 hour at room temperature, or alternatively overnight at 4°C, followed by two 10 minute washes in TTBS, one 10 min wash in TBS. Molecular weight standards were visualised by incubation with *Precision*[™] StrepTactin alkaline phosphatase conjugate diluted in the secondary antibody wash at 1:5000. Colour development of the alkaline phosphatase conjugate was achieved using an alkaline phosphatase *Bio-Rad Conjugate Substrate Kit* (Bio-Rad Laboratories, California, USA) as per manufacturer's instructions. The development step was run for several minutes then stopped by washing for several minutes through several changes of distilled water.

Calpain Inhibitor Assay

Cortex USF protein (1 mg) was incubated in microcentrifuge tubes with 100 μ L of m-calpain purified from sheep lung using a combination of hydrophobic, ion exchange, Reactive Red and Mono Q chromatography, which resulted in a pure sample of m-calpain with very few contaminants observed in SDS-PAGE-separated extract. To test the efficacy of calpain inhibitors in the USF assay, 12mM calcium and one of a selection of calpain inhibitors, including *SJA6017* (N-[4-fluorophenylsulfonyl]-L-valyl-L-leucinal), and two novel calpain inhibitors "*Cat0059*", and "*Cat811*" were included in the assay. All inhibitors were added to the assay at concentrations ranging from 0.1-10 mM. Assays were run twice, except for the *Cat0059* assays that were run three times.

Inhibitor assays were carried out at room temperature for 1 hour, timed from when the calcium necessary for calpain activation was added. Sample buffer for SDS-PAGE containing *NuPage*[®] LDS sample buffer (4x) and 5% β -mercapto ethanol was then added to stop the assay after 1 hour. USF proteins were then separated by SDS-PAGE as for Western blots. Gels that the proteins were separated on were fixed in gel fixative solution (50% methanol and 7% acetic acid in nanopure water), as per manufacturers instructions, and stained with *GelCode*[®] *Blue Stain* (Pierce, Rockford, USA) for visualisation and optical density analysis. *Precision Plus Protein*[™] standards (Bio-Rad Laboratories, California, USA) were run alongside lens USF samples to determine the mass of protein bands.

Separated Protein Band Optical Density Analysis

Separated proteins were analysed using Gel Doc EQ[™] (Bio-Rad Laboratories, USA) gel imaging hardware and *Quantity One*[®] (Bio-Rad Laboratories, USA) imaging and analysis software. Analysis of the proteins included measuring the intensity (peak and average intensity) of bands separated by electrophoresis. The band intensity was based on measurements of the Optical Density (O.D.) of bands, where a band is defined as the area where the signal intensity of a cluster of pixels is higher than the intensity of pixels that make up the background (*Quantity One*[®] User Guide; see *fig. 4.1* below for theoretical explanation). Molecular weights of separated protein bands on the gels were also analysed using the *Quantity One*[®] software pre-defined standards feature for estimating molecular weight from the standards run concurrently with assay samples. Raw band optical density data were

normalized to the average band intensity of USF^{-/-} across 5 gels, so as to adjust for differences in intensity measurements that resulted from variations in the degree of staining of protein bands or changes in light conditions when optical density measurements were taken.

To assess the sensitivity of optical density measurements, an SDS-PAGE gel with 0.625, 1.25, 2.5, 5 and 10 μg of USF protein was loaded and imaged. Optical intensity measurements were obtained using the *Gel Doc EQ*TM and *Quantity One*[®] imaging and analysis software, and using these data a curve illustrating the change in optical density for particular protein bands versus change in the amount of protein loaded was constructed.

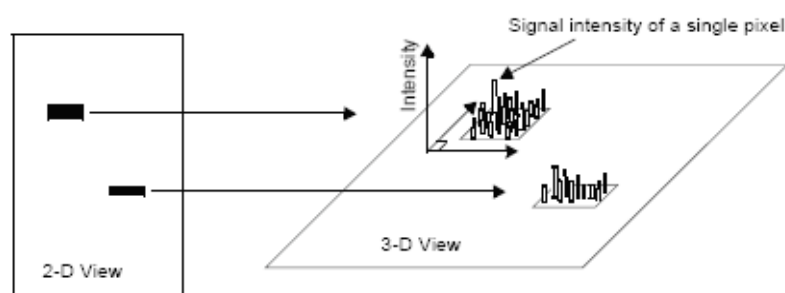


Figure 4.1 *Theoretical basis of Optical Density measurements.*

On the left (“2-D View”) is a schematic of an electrophoresis gel that shows two bands of different intensity that have been separated. On the right (“3-D View”) is shown a representation of the pixels in those bands following digitally imaging. It can be seen that the larger band on the left transfers in a digital image to a cluster of higher intensity pixels compared to the smaller band on the right, and this is the basis of the optical density measurements from which the presence of particular protein and the extent of cleavage was estimated (image from the *Quantity One*[®] User Guide).

In determining the efficacy of calpain inhibitors in inhibiting proteolysis of lens cytoskeletal proteins, the normalised optical density value was first converted to amount of protein using the regression equations derived from the dilution series (*fig. 4.3*), where the optical density value was substituted for the ordinate and the equation solved for the x -coordinate – i.e. the amount of protein. The same volume of assay-extract was always applied to a gel, while each assay contained different components by virtue of the activity of calpain, thus the amount of proteolysis for a specific protein between two gels could be deduced by dividing the amount of protein for a specific band in any assay by that in the control (USF^{-/-}) gel. The result was a ratio that expressed the amount of a particular protein in the inhibitor assay that was remaining relative to the control (after Sanderson *et al.*, 1996). The mean band

density resulting in the inhibitor assays was compared with the equivalent band in control assays using the t-test for significance.

Controls run in tandem with inhibitor assays included the following: a negative control that contained USF alone (**USF -/-**), as well as a positive control that contained USF with 12 mM calcium and added calpain but without inhibitor (**USF +/+**). Controls containing USF with 12 mM calcium but without added calpain (**USF +/-**), and USF without 12 mM calcium but with added calpain (**USF -/+**) were also run carried out and were found not to differ from the results gained for the USF-/- control.

Graphs were produced using GenStat graphing software and *Excel*[®] (Microsoft, Seattle, Washington) and t-tests were carried out using *Excel*[®]. Images were edited using *Adobe*[®] *Photoshop CS*[®] 8 and *ImageReady*[®] CS 8 (Adobe Systems Inc., San Jose, California) image editing software. A summary of the overall procedure applied to the lenses for extraction and calpain proteolysis is outlined in *fig. 4.2*.

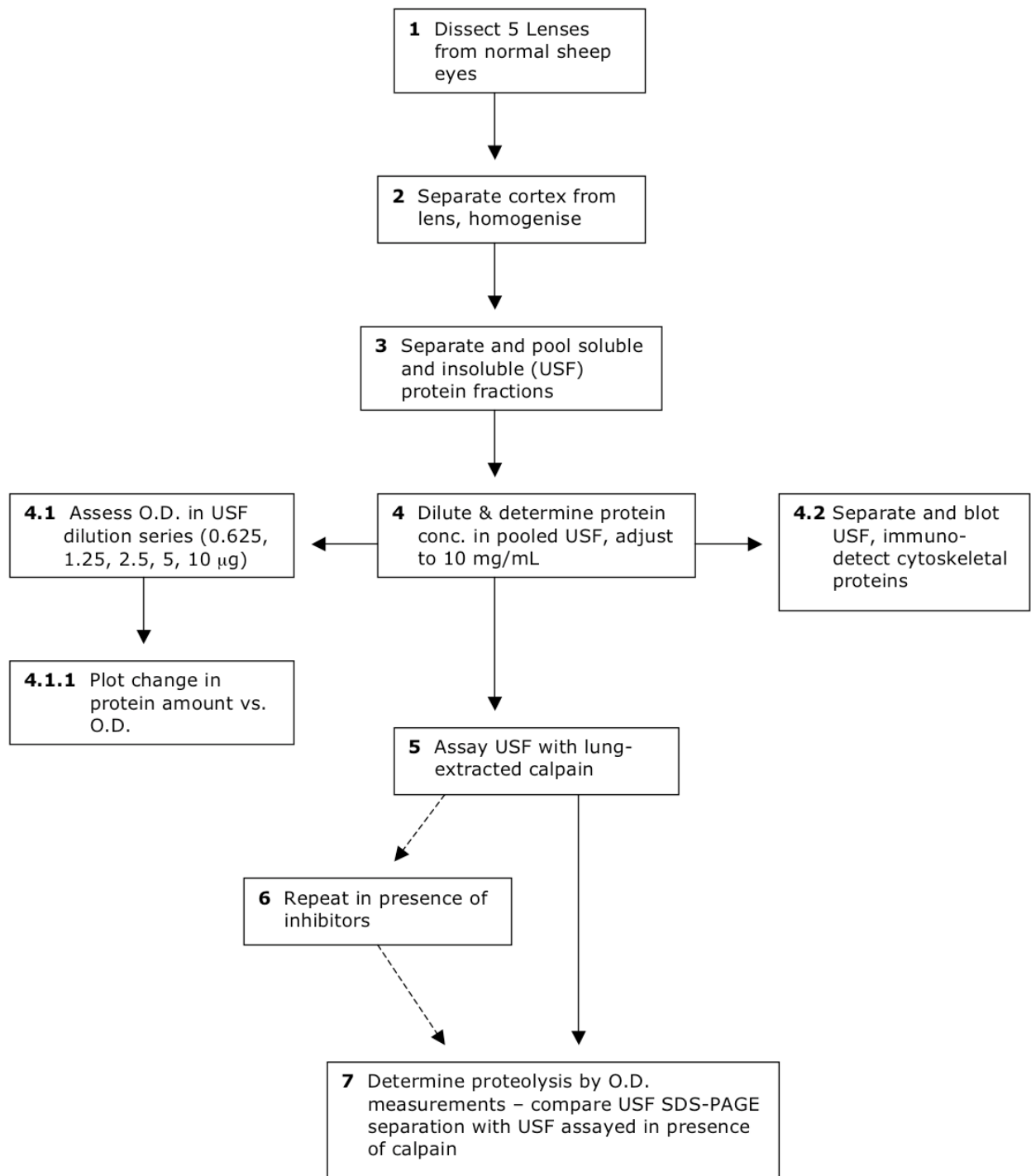


Figure 4.2 Flow chart outlining general procedure employed in this set of experiments.

O.D., optical density; USF, urea soluble fraction (insoluble fraction proteins from lens – see method for further details).

4.3 Results

Extraction of USF Proteins

The protein profile from sheep lens USF includes spectrin (>250 kDa; Robertson *et al.*, 2005), filensin and a filensin breakdown product (115 kDa and 53 kDa, respectively; Masaki & Quinlan, 1997), vimentin (57 kDa; Perng & Quinlan, 2005; Colucci-Gyon *et al.*, 1994), tubulin (55 kDa; Padgaonkar *et al.*, 1999) and actin (42 kDa; Rao & Maddala, 2006; Lee *et al.*, 2000), and these proteins were confirmed in the USF profile by immunoblotting (see *fig 4.4*). Other bands putatively identified on the basis of their mass but were not positively identified by immunoblotting included several crystallin proteins between 20 and 30 kDa.

Optical Density Assessment

All the protein bands measured displayed a linear increase in optical density as the amount of protein applied to the gel increased (*fig. 4.3*). The R^2 values for spectrin, filensin and vimentin were all 0.97 or greater, indicative of high degree of correlation between increasing protein amount and the change in optical density of the band. Spectrin could not be detected when the amount of protein loaded fell below 2.5 μg . The regression equation for each protein dilution was used later on to estimate the relative amount of protein in a given band from the intensity measurements taken following calpain/inhibitor assay.

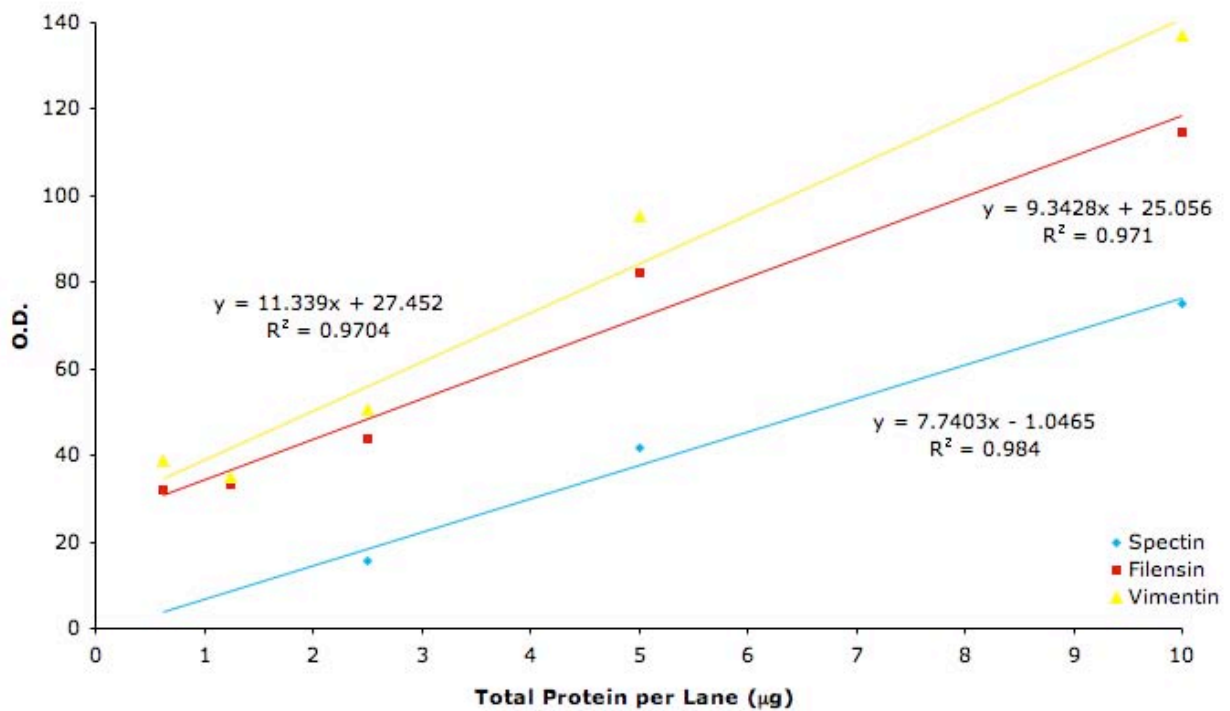


Figure 4.3 Dilution Series Optical Density Measurements.

Optical density measurements for spectrin, filensin and vimentin bands were obtained at a range of protein loading concentrations (0.625, 1.25, 2.5, 5 and 10 µg of USF protein). The unit of signal intensity plotted on the ordinate is optical density (O.D.).

USF Optical Density Measurements

An assay of USF+/+ was run where aliquots were removed at regular time intervals over an hour (*fig. 4.4*, in minutes) and placed immediately in SDS-PAGE LDS running buffer to denature the calpain. Later the proteins were separated by SDS-PAGE with controls so as to investigate the relative amount of protein degradation. This was necessary to both verify that calpain sourced from lung tissue was capable of proteolysis of lens USF protein in a manner that was both reproducible and controllable, as well as to aid in the overall goal of investigating the role of calpain proteolysis on lens cytoskeletal proteins.

A positive control that contained USF with 12 mM calcium and 1 mg of extracted calpain (i.e., USF +/+) was assayed over an hour, with aliquots removed as 1, 2, 10, 30 and 60 minutes and placed in SDS-PAGE running buffer to immediately halt calpain action. Lanes 5-8 (*fig 4.4*) contained protein from this assay. Controls containing USF without 12 mM calcium but with added calpain (USF -/+), USF with 12 mM calcium but without added

calpain (USF +/-), and a negative control of USF extract alone (USF -/-) were also assayed and run in the SDS-PAGE alongside the time controlled USF+/+ assays (*fig 4.4*, lanes 2-4, respectively). The control assays (USF-/+, USF+/-) were carried out for 1 hour at room temperature and revealed a minor change in band intensity, with spectrin, filensin, and vimentin present at a ratio of 0.98 or more relative to amounts in the USF-/- control (*fig. 4.4*, lane 9, “USF”).

A decrease in band intensity for spectrin, filensin, and vimentin was detected over the 60 minutes incubation period (lane 2, “+/+”), compared to corresponding bands in the USF-/- control (i.e. USF incubated without calpain), indicative of calpain-induced proteolysis

Vimentin was present at a ratio of 0.8 at 1 minute and below 0.6 at 10 minutes in the USF+/+ assay, and could not be found after this time. The ratio of vimentin did not change in the control assays over the course of an hour. The removal of vimentin from the assay by proteolysis is clear in *fig. 4.4*. The intense band at approximately 55 kDa in control assays confirmed by immunoblot to be vimentin is seen to slightly diminish in intensity at 1 and 2 minutes following calcium addition, then can be seen to further diminish in intensity at 10 minutes, and is not detectable after 60 minutes (lane 2, “+/+”).

At 60 minutes, 30% of spectrin was present compared to USF-/. Accumulation of several spectrin breakdown products of between approximately 148 and 162 kDa was detected almost immediately after calcium addition, and are visible as unlabeled bands at 1 minute. These bands were confirmed as spectrin products by immunoblotting, and were clearly absent in the USF-/- control assay. By 30 minutes these breakdown associated bands were found to have increased in their intensity 1.4 times over what was present after 10 minutes when they were first seen to appear at levels detectable with the instruments used (they were visible after 1 minute, *fig 4.4*).

A filensin band at approximately 100 kDa in mass was present at a ratio of 0.7 relative to USF-/- after 30 minutes of assay, and this ratio remained essentially unchanged after 60 minutes. This indicated a somewhat reduced degree of calpain proteolysis on filensin compared with vimentin and spectrin.

Other proteins identified immunologically or on the basis of their mass in the SDS-PAGE separation of lens USF protein included actin. Actin is known to be resistant to calpain proteolysis (Goll *et al.*, 2003), and this was born out in these results where its ratio after 1 minute was 1.08, and this value did not drop below 0.9 after an hour.

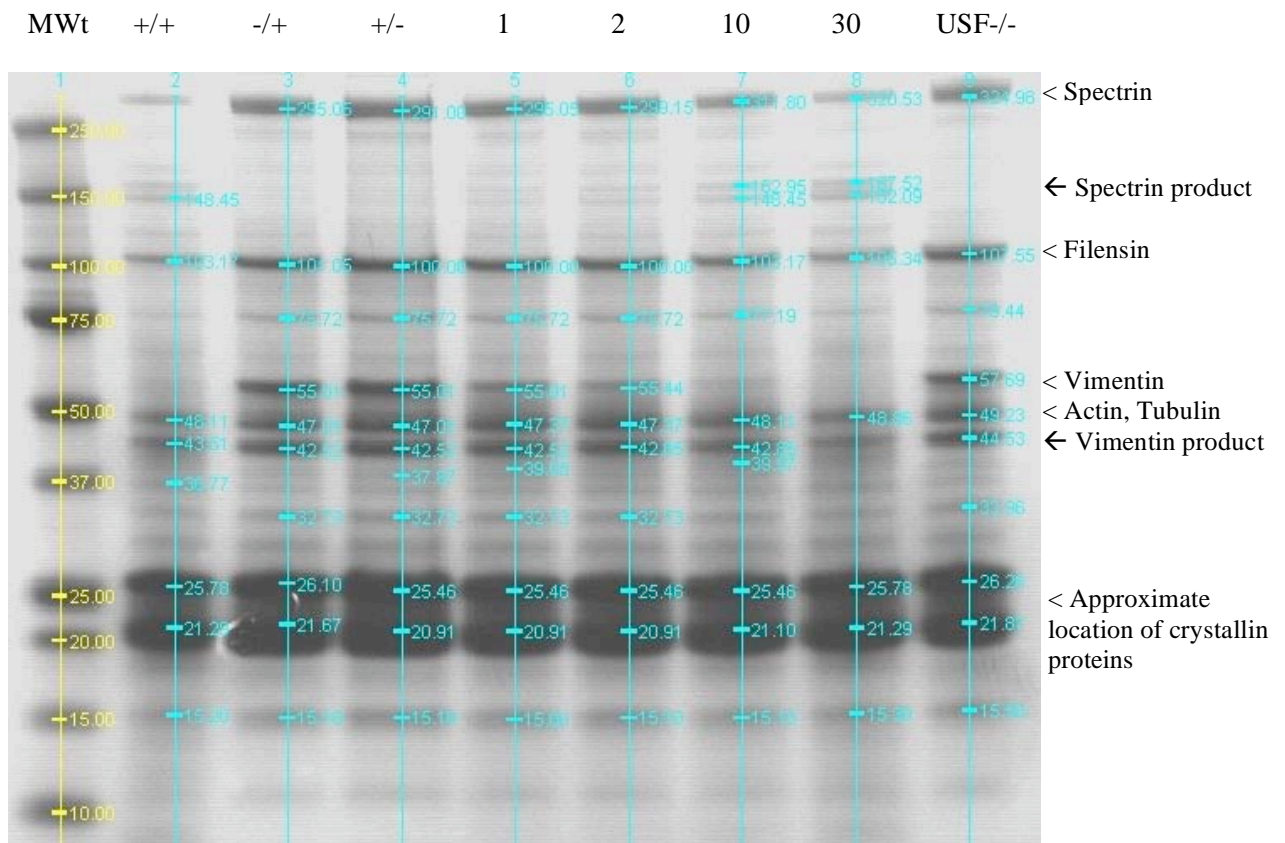


Figure 4.4 SDS-PAGE of USF Time-series Assay.

Bands that were identified by Western blots are labelled along the right hand side, with “←” indicating extra bands associated with calpain activity that emerged over an hour in the presence of calpain. Estimated molecular mass values (in kDa) are shown in blue, yellow values are molecular masses of the molecular weight marker from which the estimates were made. Lanes are numbered at the top (in blue) *MW_t*, molecular weight marker; +/+, USF with calpain + calcium; -/+, USF control without calcium + added calpain; +/-, USF with calcium but without calpain; 1, 2, 10, 30, time in minutes. +/+ equates to 60 a minute assay of USF with added calpain and calcium.

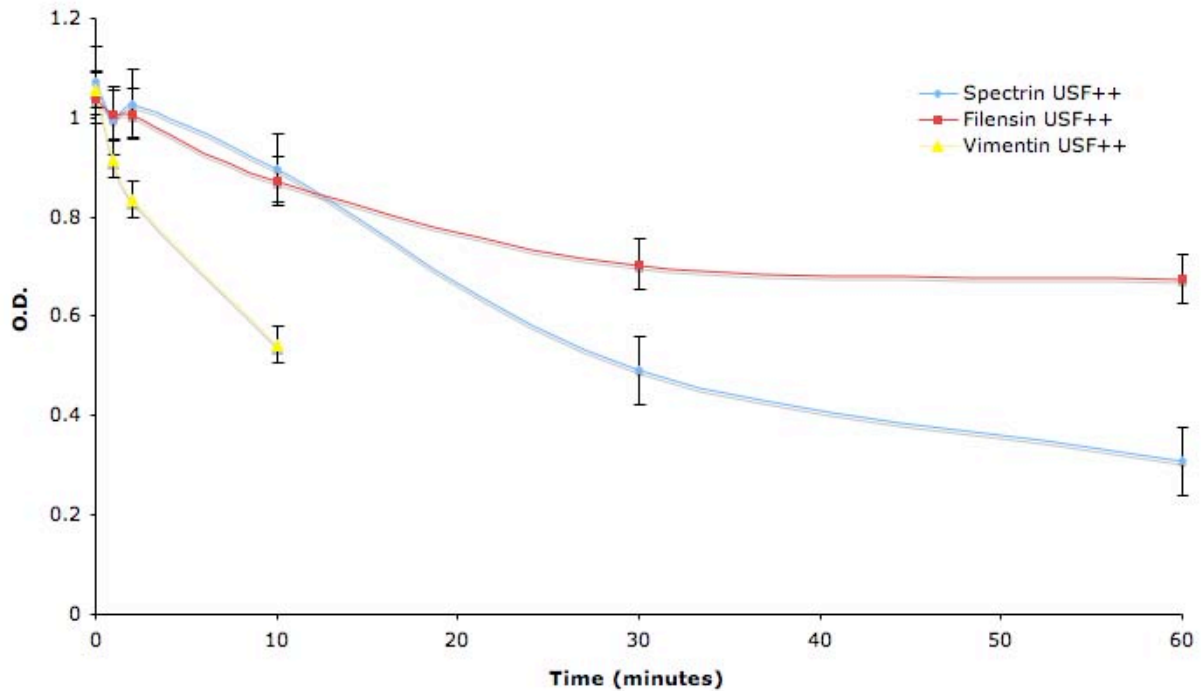
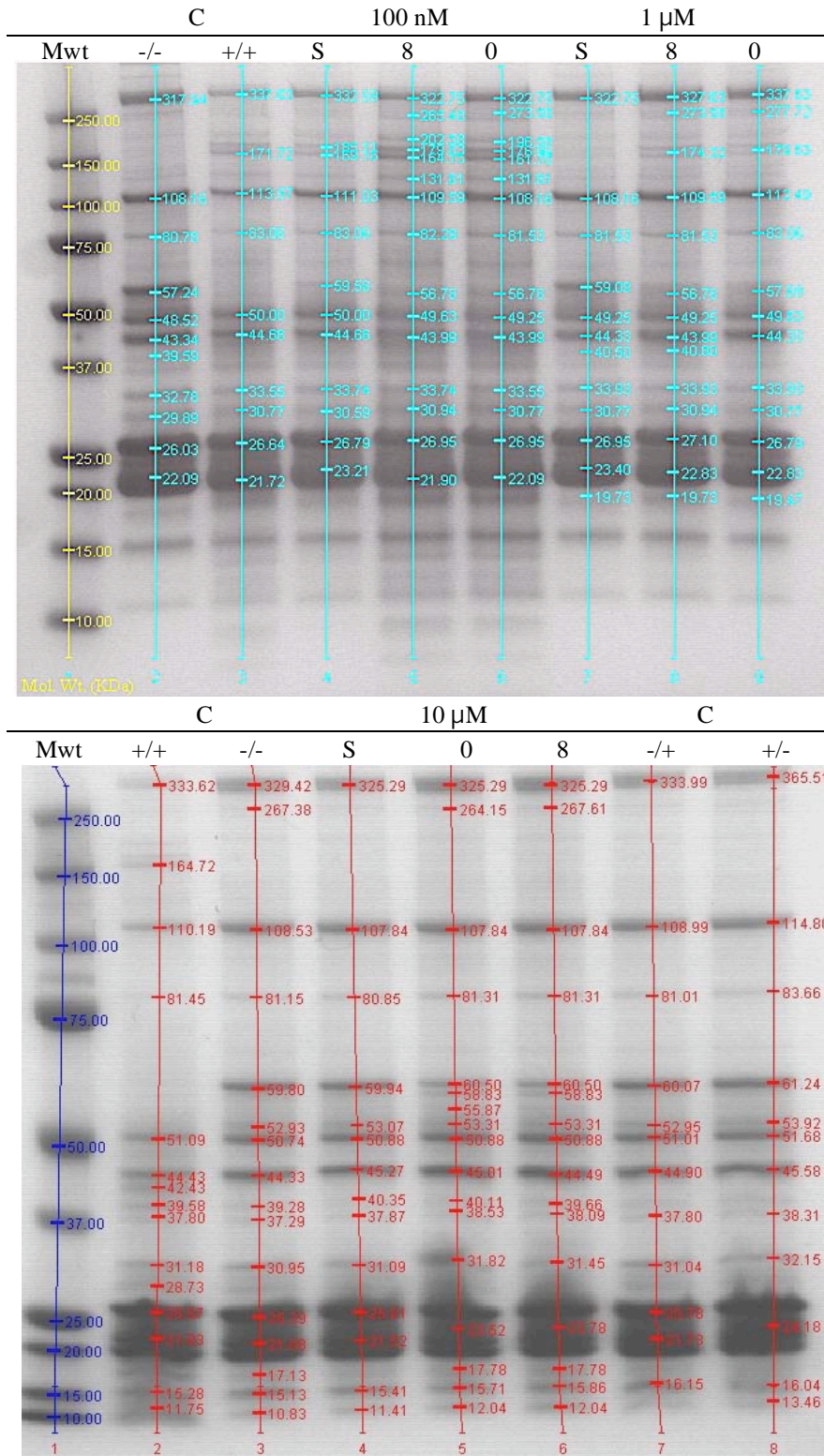


Figure 4.5. *USF Time Course Assay – Change in Cytoskeletal Proteins over an Hour in the Presence of Calpain*

Optical density values as seen in *figure 4.4*, plotted against time. Vimentin could not be detected after 10 minutes, and it can be seen clearly that filensin was the least sensitive to the action of calpain of the three cytoskeletal proteins followed in the time course assays. Values are mean band density \pm SEM, $n = 5$.

Calpain Inhibitor Assay

Generally speaking, proteolysis of USF protein constituents was effectively inhibited using inhibitors, and all of the inhibitors had comparable effects in the cell free system. In the majority of cases the addition of inhibitor had some perceptible positive effect that resulted in a larger amount of the selected protein remaining after 1 hour in the presence of calpain and calcium plus inhibitor when compared to the positive control assay (USF+/+). This indicated the lens based cell free assay itself was successful and validated its usage here as an assay of inhibitor efficacy.



- The presence/absence of spectrin, (>250 kDa) is an obvious indicator of calpain activity. In the USF+/+ and at 100 nM of all inhibitors, spectrin breakdown products are visible between 160-200 kDa. The absence of these bands at higher concentrations of inhibitor and in the USF-/- control indicates absence/inhibition of calpain. *Nb.* spectrin breakdown products are not visible at 1 μM SJA6017.

- A slight change in the appearance of the filensin band (107-110 kDa), between the USF+/+ and the inhibitor/USF-/- lanes is seen.

- The presence of vimentin (between 59-61 kDa) seen at 10 μM inhibitor, strongly suggests calpain inhibition. The complete absence of vimentin in USF+/+ lane contrasts with the other lanes.

Figure 4.6 SDS-PAGE Illustrating the Efficacy of Calpain Inhibitors at 0.1-1 μM (top) and 10 μM (bottom).

The concentration of inhibitors in the respective assay is as shown (in μM). Molecular weight standards are indicated in kDa on the left of each gel in yellow and blue, respectively. C, controls (positive and negative, as shown); S, SJA6017; 8, inhibitor *Cat811*; 0, inhibitor *Cat0059*.

- *Cat0059*

The presence of 100 nM of *Cat0059* resulted in 0.83 ± 0.08 and 0.94 ± 0.07 (values are means \pm SEM) of spectrin and filensin respectively remaining relative to the negative control (USF^{-/-}) assay, while vimentin was detected at 0.62 in one assay only but was absent in other replicates (n = 3; see *fig. 4.7*). At 1 μ M, *Cat0059* educed a significant (p < 0.01) degree of protection upon spectrin (0.95 ± 0.05). Presence of any of the inhibitors at 10 μ M was found to significantly increase the ratio of protein to what was found in the USF^{+/+} control (p < 0.05). Filensin bands from assays containing 10 μ M of inhibitor *Cat0059* were present at an average ratio of 0.91 ± 0.05 of that which was found in the negative control and this was significantly higher than what was found in the positive control (p < 0.05). Vimentin was present at a ratio of 0.90 ± 0.07 , and this was a significant (p < 0.05) increase.

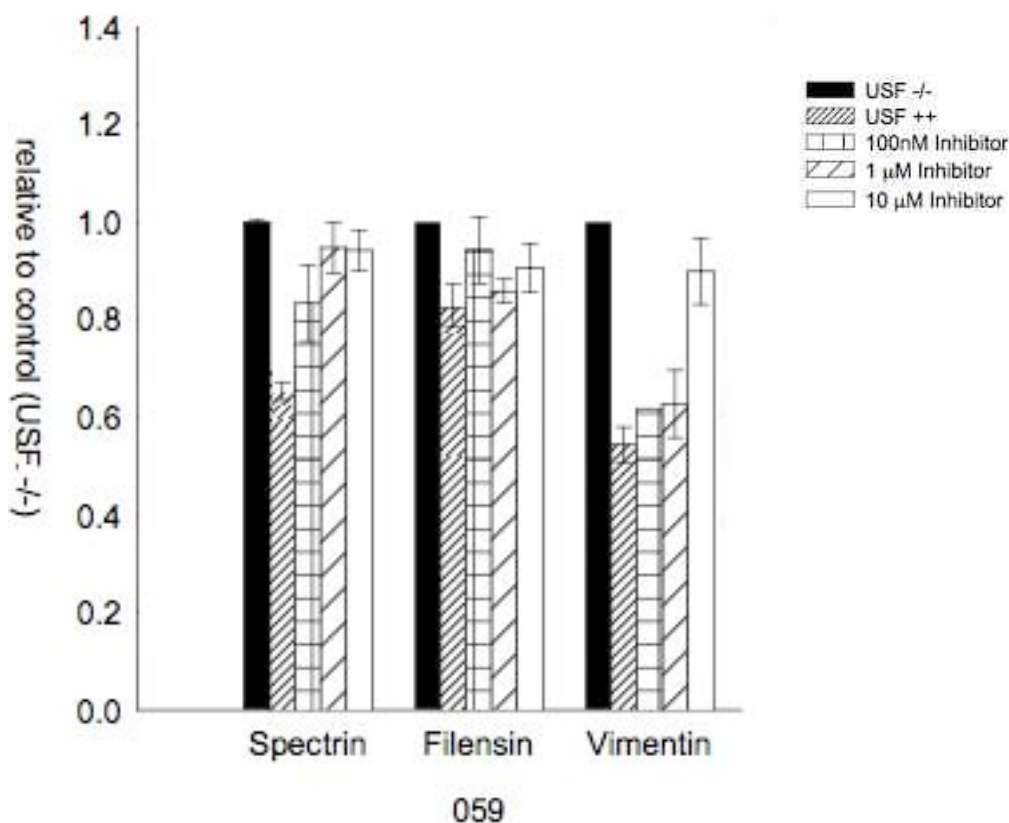


Figure 4.7 *Relative Lens Cytoskeletal Protein Proteolysis in the Presence of Calpain and Calcium – Processed Dose-Response Data for Cat0059.*

Inhibitors were tested in the lens based cell free inhibitor assay, over a period of 1 hour. The amount of selected protein remaining in an inhibitor assay is shown relative to the amount found in the control assay, i.e. USF^{-/-}, and is expressed as a ratio where the amount of any protein in USF^{-/-} is 1. Data are means \pm SEM. USF^{+/+} produced in all cases spectrin (n = 4), filensin (n = 4) and vimentin (n = 2) bands that were significantly less intense than what was found in USF^{-/-} (p < 0.01)

- *Cat811*

Assays where 0.1 μM (100 nM) of calpain inhibitor *Cat811* was applied, spectrin and filensin remained present at respective ratios of 0.76 ± 0.15 and 0.87 ± 0.02 , while vimentin could not be detected in any assay that contained this concentration of *Cat811* (see *fig. 4.8*). One μM of *Cat811* also was associated with a very significant ($p < 0.01$) degree of protection upon spectrin 1.11 ± 0.15 . At 1 μM concentration, filensin was found to have decreased compared to the amount found in the 100 nM assay, but as can be seen in the graph, there was extreme variation experienced in the amount of filensin found at 1 μM *Cat811*. Ten μM of *Cat811* inhibitor prevented calpain proteolysis of filensin such that a ratio of 1.07 ± 0.01 was observed, again significantly higher than that found in the positive control ($p < 0.05$). Vimentin was present at a ratio of 1.00 ± 0.08 when assayed with 10 μM of inhibitor *Cat811*, which was a significant ($p < 0.05$) increase over what was found in the positive control.

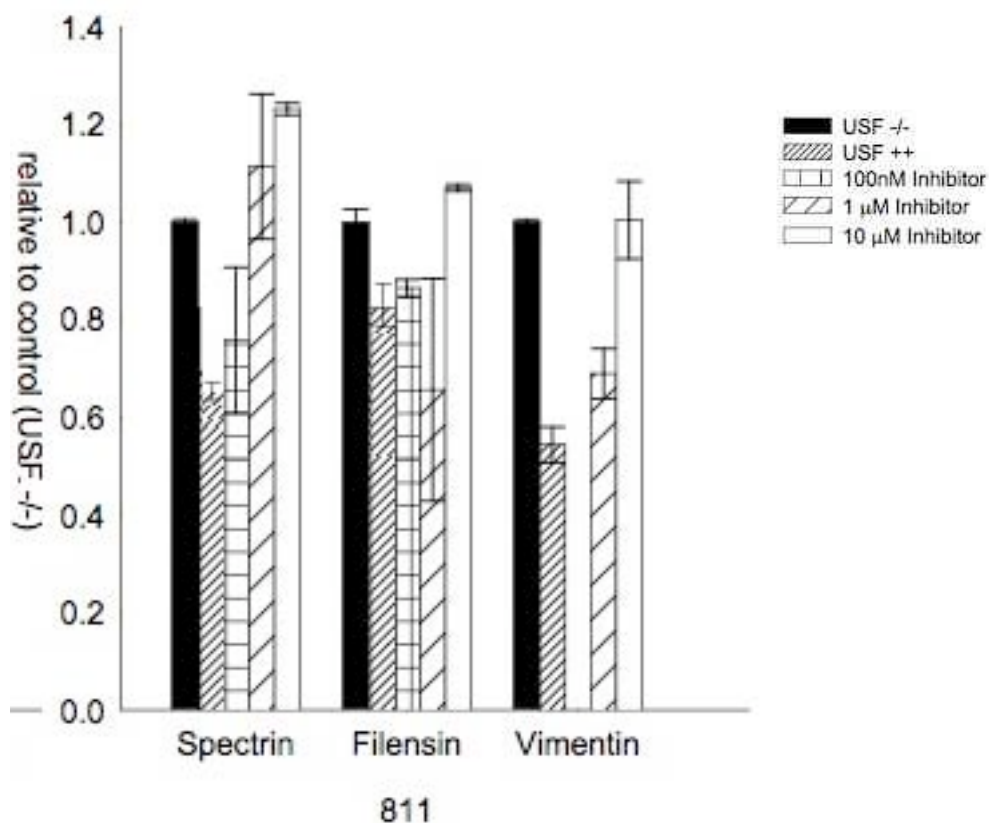


Figure 4.8 *Relative Lens Cytoskeletal Protein Proteolysis in the Presence of Calpain and Calcium. – Processed Dose-Response Data for Cat811.*

Assay conditions were as for *Cat0059* inhibitor. No vimentin band could be detected in replicates ($n = 3$) for vimentin at 100 nM of *Cat0059*.

- *SJA6017*

SJA6017 applied to USF+/+ at 100 nM resulted in spectrin and filensin remaining at a ratio of 0.79 ± 0.06 and 0.86 ± 0.06 respectively, and vimentin again was detected in only a single assay at 0.57 of that detected in the UFS-/- control (see *fig. 4.9*). There was a very significant increase ($p < 0.01$) in the band intensity ratio for spectrin with 1 μ M *SJA6017* added (0.89 ± 0.05). Vimentin was detected at a significantly higher ($p < 0.05$) level in the presence of 1 μ M of *SJA6017* 0.76 ± 0.02 , however the others inhibitors failed to generate a significant increase in vimentin relative to the USF+/+ control.

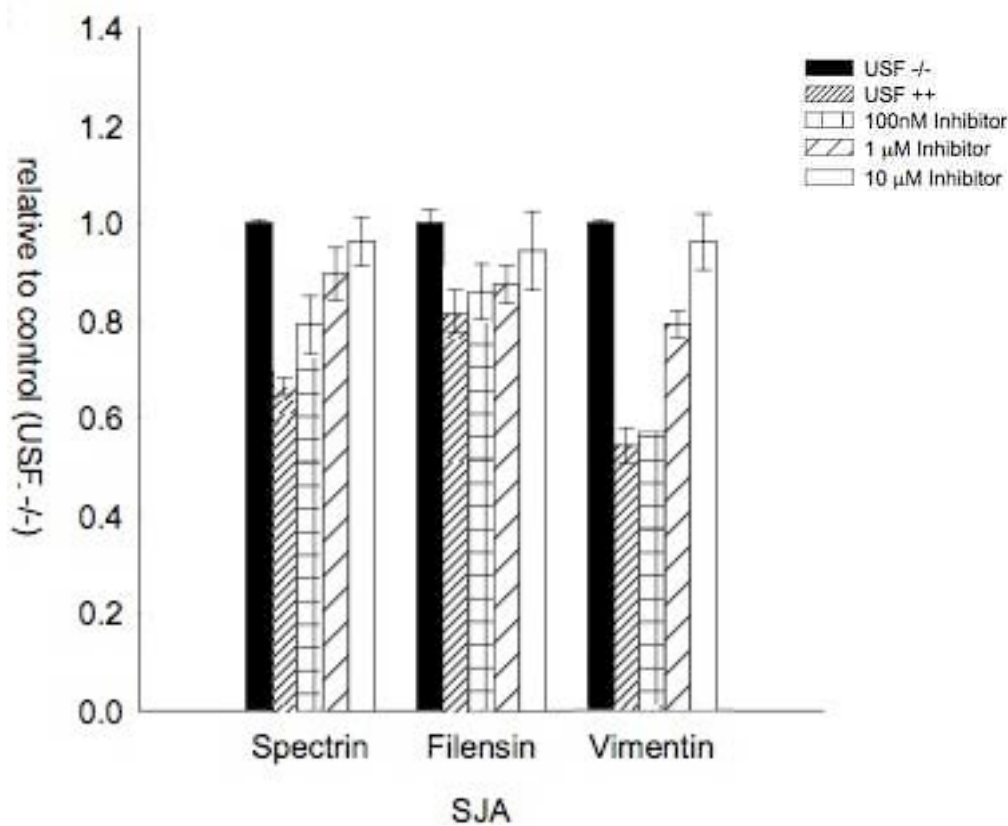


Figure 4.9 *Relative Lens Cytoskeletal Protein Proteolysis in the Presence of Calpain and Calcium – Processed Dose-Response Data for SJA6017*

Assay was carried out as for the other inhibitor assays.

In all cases where there was protein in the assay that could be measured (i.e. all selected proteins in all assays except for vimentin in the *Cat811* assay) there was an increase in the

ratio of protein remaining relative to the positive control. Addition of 100 nM of *SJA6017* or *Cat0059* was found to produce a significant ($p < 0.05$) difference in average band intensity ratio for spectrin and filensin (*Cat0059* only) over the USF+/+ control.

Addition of *Cat811* and *Cat0059* at 1 μM to the cell free assay resulted in vimentin bands that were 0.69 ± 0.07 and 0.63 ± 0.07 , respectively, which, while higher than what was observed in the positive (USF+/+) control, was a non-significant increase ($p > 0.05$). A significant change ($p < 0.05$) was detected for filensin with inhibitor *Cat0059* (0.86 ± 0.02), while the ratio was increased for *SJA6017* (0.87 ± 0.04) but this was not found to be a significant increase over the positive control. The average ratio of filensin in the presence of *Cat811* was $0.66 (\pm 0.22; n = 2$ for *SJA6017* and *Cat811*, $n = 3$ for *Cat0059*).

Ten micromole of *SJA6017* prevented calpain proteolysis of filensin with 0.94 ± 0.08 of the negative control, again significantly higher than in the positive control ($p < 0.05$). Spectrin in all cases was well protected by the inhibitors, present at levels that were significantly higher than that which was observed in the positive control ($p < 0.01$). Vimentin was present at a ratio of 0.96 ± 0.05 when assayed with *SJA6017* inhibitor, which was a very significant ($p < 0.01$) increase over what was found in the positive control. There was no significant difference between bands in assays containing 10 μM of inhibitors and the negative control, except for 10 μM of *Cat811* which was associated with an extremely significant ($p < 0.001$) increase of band intensity ratio to the negative control (1.23 ± 0.01).

Table 4.1 shows the cumulative inhibitor data collected in these experiments. In all but a few cases (filensin in the presence of *Cat0059* between 0.1 and 1.0 μM and spectrin between 1.0 and 10 μM of the same inhibitor) it may be seen that the amount of protein detected in a particular band increases with increasing concentration of inhibitor. Additionally the effect of the inhibitor on preventing vimentin proteolysis is obvious, as is the resistance of filensin to the action of calpain.

Table 4.1 Summary Data: Efficacy of Calpain Inhibitors in Preventing Proteolysis of Lens Cytoskeletal Proteins in the Presence of Calpain

	<i>0.1 μM</i>	<i>1.0 μM</i>	<i>10 μM</i>	<i>USF+/+</i>	<i>0 μM USF-/-</i>
Vimentin					
<i>SJA6017</i>	0.57	0.79 ±0.03*	0.96 ±0.06*	0.54 ±0.04	1.00 ±0.01
<i>Cat0059</i>	0.62	0.63 ±0.07	0.90 ±0.07*		
<i>Cat811</i>	N/A	0.69 ±0.07	1.00 ±0.08*		
Spectrin	<i>0.1 μM</i>	<i>1.0 μM</i>	<i>10 μM</i>	<i>0 μM</i>	
<i>SJA6017</i>	0.79 ±0.06*	0.89 ±0.05**	0.96 ±0.05**	0.65 ±0.04	1.00 ±0.01
<i>Cat0059</i>	0.83 ±0.08*	0.95 ±0.05*	0.94 ±0.04**		
<i>Cat811</i>	0.76 ±0.15	0.94 ±0.04*	1.23 ±0.01**		
Filensin	<i>0.1 μM</i>	<i>1.0 μM</i>	<i>10 μM</i>	<i>0 μM</i>	
<i>SJA6017</i>	0.86 ±0.06	0.87 ±0.04	0.94 ±0.08*	0.82 ±0.08	1.00 ±0.03
<i>Cat0059</i>	0.94 ±0.07*	0.86 ±0.02*	0.91 ±0.05*		
<i>Cat811</i>	0.87 ±0.02	0.91 ±0.05	1.07 ±0.01*		

Data are means (± SEM) band intensity relative to the negative control (USF-/-), inhibitor concentrations are listed in italics in μM.

* Significantly different to the applicable +/+ (plus calcium plus calpain – positive control) control for that protein (p < 0.05)

** Significantly different to the applicable +/+ control for that protein (p < 0.005)

4.4 Discussion

The aim of these experiments was to investigate the effect of calpain activity on cytoskeletal proteins as part of understanding the role of these proteases and the breakdown of the lens cytoskeleton in the ovine cataract model, as well as to investigate the efficacy of calpain inhibitors in preventing proteolysis of cytoskeletal proteins from the lens. Incubation of lens USF proteins in the presence of calcium and calpain lead to breakdown of lens cytoskeleton proteins spectrin, vimentin and filensin, with spectrin and vimentin particularly susceptible to calpain-induced proteolysis.

To test the ability of calpain inhibitors to inhibit the proteolysis of lens proteins, a lens based cell-free method was used that was adapted from Sanderson *et al.* (1996). Of the inhibitors used (*SJA6017*, *Cat0059* and *Cat811*), all offered some degree of protection of cytoskeletal proteins from calcium-induced calpain proteolysis. A significant amount of protection was detected when the inhibitors were present at 10 and 1 μM. All inhibitors were effective at the latter concentration, while *SJA6017* was found to provide significant

protection to spectrin and vimentin at 1 μM . One μM of *Cat0059* was found to protect spectrin and filensin, but not vimentin, while inhibitor *Cat811* was found to protect spectrin only at this concentration, with filensin protected in some replicates but experiencing wide variation such that no statistically significant protection was found at this concentration. *SJA6017* added to assays at 100 nM offered significant protection to spectrin, and *Cat0059* was found to protect filensin and spectrin to a significant degree at 100 nM.

On the basis of these results it may be deduced that the novel calpain inhibitor *Cat0059* was the most effective inhibitor by virtue of its superior quantitative protection of lens cytoskeletal proteins to a statistically significant level at the lowest concentration tested in this investigation. However, the *SJA6017* inhibitor seemed to offer the best overall protection of lens cytoskeletal proteins, due to its ability to inhibit proteolysis of vimentin, the most sensitive indicator of calpain proteolysis (Goll *et al.*, 2003; Sanderson *et al.*, 1996), at the lowest comparative concentration. *SJA6017* also offered the greatest absolute protection of spectrin and filensin at the lowest concentration (100 nM), although only the latter was protected at a level that was statistically significant. Additionally, spectrin breakdown products were not observed in assays that contained 1 μM of *SJA6017*, while they were perceptible in the other inhibitor assays at this concentration. Future investigations may benefit from testing *SJA6017* and *Cat0059* inhibitors together, so as to gainfully utilize the vimentin-protective benefits of *SJA6017* at low (1 μM) concentrations with the protection afforded to filensin and spectrin at 100 nM (and potentially less) of inhibitor *Cat0059*, although a mechanism explaining these findings is not immediately apparent.

Sanderson *et al.* (1996) carried out a similar investigation with calpain inhibitors and USF extracts from bovine lenses. In this investigation the inhibitors cBz-Val Phe, E64c and calpeptin were used and were found to provide maximal protection of spectrin filensin and vimentin when they were used at 20 μM , while 2 μM of calpeptin provided protection to spectrin and filensin, with vimentin “75%” protected. These results are in line with what was experienced in this investigation, where vimentin typically was found to be the most sensitive indicator of calpain activity, while spectrin and filensin were more resilient to calpain. Vimentin is known to be a prime substrate for calpain, and protection of vimentin similar to that achieved by Sanderson *et al.* (1996) was possible at 1 μM of *SJA6017* and in isolated cases with *Cat811* and *Cat0059*.

Filensin was particularly resistant to calpain proteolysis, and would typically be present at a high ratio (greater than 0.8) in the positive control compared to filensin in the negative control. The resistance to calpain induced proteolysis found in this investigation had the side effect that inhibition of calpain proteolysis in the *Cat811* and *SJA6017* assay was not found to have a statistically significant effect, even though there was an increase in the amount of filensin found in the inhibitor assay relative to the positive control. The resistance that filensin displays toward calpain proteolysis may be linked to its longevity in the lens, where it is found in at all stages of differentiation (Lee *et al.*, 2000; Sandilands *et al.*, 1995; Quinlan *et al.*, 1996), although it must be noted that filensin is known to undergo proteolytic processing during lens fibre cell differentiation, producing a range of stable fragments between 51 and 62 kDa that suggest multiple processing pathways for this protein in the lens (Perng & Quinlan, 2005; Sandilands *et al.*, 1995a). Vimentin and spectrin were found in the current research to be comparatively more susceptible to calpain proteolysis, as they have been elsewhere (Sanderson *et al.*, 1996; Marcantonio & Duncan, 1991). In contrast to the localisation of filensin, spectrin and vimentin are found mostly in the outer regions of the lens with fragments surviving in the older fibre cells as these proteins are proteolysed during differentiation (Lee *et al.*, 2001; Blankenship *et al.*, 2001). To this end, the lens based cell free assay that was used in this investigation made use of protein from the lens cortex in part due to the fact that the sensitive markers of calpain proteolysis, spectrin and vimentin, are difficult to isolate in the rest of the lens mass – specifically, the lens nucleus. This was confirmed in Western blots of the lens core region in which vimentin could not be identified, and spectrin was present only as proteolysed bands (see Section 5 *fig. 5.3.1*). Also, calpain activity (specifically, m-calpain) in the human lens has been found to be at its highest in the cortex and lowest in the nucleus (Sanderson *et al.*, 2000; David *et al.*, 1989), coinciding with the disappearance of spectrin and vimentin, while filensin remains present.

Robertson *et al.* (2005) carried out an investigation into the ovine cataract wherein cataractous lenses were compared with normal lenses for signs of proteolysis. Their investigation identified several bands that they associated with calpain activity arising from spectrin and vimentin, and several of these bands were encountered in this investigation. The most obvious was the appearance of spectrin products of between approximately 148 and 162 kDa in cataractous ovine lenses. These products have been quoted as 145 and 150 kDa (Robertson *et al.*, 2005), but are certainly the same bands that were reported in the current

investigation. The larger of the two has also been linked to caspase activity and is known to be present in the normal lens (Lee *et al.*, 2001), while the smaller of the spectrin breakdown products has been attributed to calpain activity specifically (Robertson *et al.*, 2005; Goll *et al.*, 2003; Wang *et al.*, 1998). Neither of the spectrin-product bands was apparent in the negative control that contained only lens USF extract, however incubation of that extract with calpain and calcium was found to produce those very bands that can be seen in *fig. 4.4*, and so can be attributed to the activity of calpain with a reasonable degree of certainty. Additionally a decrease in a vimentin breakdown product of approximately 40 kDa was also detected in the positive control (i.e. USF+/+, *fig. 4.4*), again similar to what has been seen in the cataractous ovine lens (Robertson *et al.*, 2005). The current set of results therefore demonstrates that the degradation of isolated ovine lens cytoskeletal proteins by exogenous calpain in the presence of calcium follows essentially the same pattern witnessed in the ovine cataract.

Thus it may be that calpain is responsible for the proteolysis of spectrin and vimentin that was witnessed in the lens during differentiation and cataract, while filensin remains largely intact throughout the lens owing to its increased comparative resistance to calpain-induced proteolysis. As has been noted, other proteases including caspase-3, caspase-6 and caspase-7 also exist in the lens. The caspases are known to be calpain substrates with caspase 3 cleavage by calpain involved in the activation of apoptosis (Raynaud & Marcilhac, 2006; Saez *et al.*, 2006), and in common with the calpains have been implicated in the lens fibre cell differentiation events that share some similarities with apoptosis such as organelle loss and proteolysis (Weber & Menko, 2005; Zandy *et al.*, 2005). Additionally, caspases have been found to play a role in differentiation in several other cell types (Fernando *et al.*, 2002; Sordet *et al.*, 2002; Weil *et al.*, 1999). The caspases thus cannot be ruled out as responsible for the cytoskeletal breakdown encountered during normal lens differentiation. One weakness of this argument would be that there has been few caspase substrates identified in the lens, and aside from their possible role in the degradation of the fibre cell nucleus, only spectrin and connexin 45.6 are commonly regarded caspase substrates in the lens (Lee *et al.*, 2001; Yin *et al.*, 2001).

In addition to the calpains and caspases, the ubiquitin-proteasome pathway is also known to be operational in the lens (Huang *et al.*, 1993), as well as other proteolytic systems including trypsin-like protease, proteasome and aminopeptidases (Girao *et al.*, 2005). The

ubiquitin-proteasome pathway appears to be the most specific of the proteolytic systems in the lens, and it is thought that this pathway is involved in regulating cell cycle events in the lens (Guo *et al.*, 2004). As such its targets involve chiefly cell cycle regulators such as p21^{WAF} and p27^{Kip}, cyclins, and transcription factors (Girao *et al.*, 2005; Guo *et al.*, 2004). Inhibition of the ubiquitin-proteasome pathway for example has been found to decrease the expression of CP49, filensin and major intrinsic protein MIP26 in cultured lens, giving an indication of its proteolytic effect in the lens (Guo *et al.*, 2006). Decreased proteasome (as distinct from the ubiquitin-proteasome pathway) activity has been identified in the lenses of elderly patients suffering cataract (Zetterberg *et al.*, 2003), indicating perhaps that reduced proteasome activity may be involved with cataract development as a result of the inability to remove damaged proteins (Viteri *et al.*, 2004). The evidence would seem to suggest that inhibition of some of these other proteolytic systems would be detrimental to the lens. While this might appear consequently to lend favour to the calpain-induced model of cataract development, it could also be construed as a caveat in the inhibition of proteolytic systems in the lens as a treatment or preventative measure.

In future studies it would be worthwhile to complete a similar investigation to the current research making use of cataractous lenses. In particular, it would be useful to compare directly the proteolysis witnessed in cataract with that induced in the cell free lens based calpain assay, in order to further substantiate the role of calpain proteolysis in the ovine cataract that is apparent from comparison with other studies. Further investigations with the inhibitors used in these experiments, in order to obtain more precise data regarding the working concentrations and limits for these compounds would substantiate the significance of these findings. In the current study, a concentration of 10 μM appears certain to inhibit the breakdown of cytoskeletal proteins for all of the inhibitors tested, while lower concentrations of inhibitors were not consistently effective. Some promising results were obtained at lower concentrations with *Cat0059* and *SJA6017* in particular, and future investigation may also benefit from examining the benefits of using these inhibitors in together, combining the protection provided by *SJA6017* at low concentrations for vimentin with that achieved when using *Cat0059* at 100 nM where it was found to have some protective effect on filensin and spectrin.

SECTION 5 – IMMUNOHISTOCHEMICAL LOCALISATION OF CYTOSKELETAL PROTEINS IN THE OVINE LENS

5.1 Introduction

Immunohistochemical observation of lens proteins in histological sections has assisted in elucidating the function or functions for a protein or proteins, on the basis of cellular localisation or temporal distribution (Donaldson *et al.*, 2004). Immunohistochemistry has been used to examine the lens cytoskeleton qualitatively in a wide variety of species. For example, studies in the rodent and bovine lens have described the localisation of vimentin and beaded filament proteins CP49 and filensin (Lindsey-Rose *et al.*, 2006; Alizideh *et al.*, 2003; Blankenship *et al.*, 2001; Ireland *et al.*, 2000; Sandilands *et al.*, 1995; Colucci-Guyon *et al.*, 1994), spectrin (Beebe *et al.*, 2001), and actin (Lee *et al.*, 2000; Woo *et al.*, 2000). Most of these studies observed changes in the cytoskeleton of lens cells as they differentiated from nucleated epithelial cells into fibre cells during growth of the lens. There is a dearth of literature with respect to the ovine lens and in particular the ovine lens cytoskeleton. Furthermore, the examination of cytostructure and cytoskeletal components in the ovine lens by means of immunohistochemical localisation may be helpful in further establishing the ovine lens as a large animal model for studying cataract.

The cytoskeleton has essential roles in the normal growth, maturation, differentiation, integrity, and function of cells in the human lens (Bozniac *et al.*, 2006). Its main components are actin microfilaments, intermediate filaments and microtubules. Actin is a major cytoskeletal protein in the lens, participating in and regulating events associated with fibre cell differentiation and it possibly plays a role in lens accommodation through stabilisation of epithelium under tension with myosin (Rao & Maddala, 2006; Kivela & Uusitalo, 1998). Actin, along with associated actin binding proteins such as spectrin, has been localised in the lens in association with the lens fibre cell plasma membrane, where actin has been detected with an increased presence at the short sides of these cells (Struab *et al.*, 2003). Electron micrographs indicate that the majority of actin in the lens exists in a network configuration associated with the cell membranes of lens fibre cells (Lo *et al.*, 1997). Little appears to be known about actin in the nucleus of the lens, although Lee *et al.* (2000) located actin in this

region of the lens that was associated with the fibre cell membrane without preference toward any particular region of membrane. Spectrin has been found to be associated with the plasma membrane in the outer epithelial cells of the lens (Beebe *et al.*, 2001), and the onset of organelle loss in lens fibre cells is known to coincide with spectrin cleavage (Zandy *et al.*, 2005). As a result, spectrin fragments have been detected in the organelle-free fibre cells in the late embryonic stage, and are restricted mostly to the cells of the nucleus in adult lenses where they are known to accumulate in a range of species (Lee *et al.*, 2001). In fibre cells of the lens cortex, spectrin has been localized by immunohistochemistry to the plasma membrane in bovine and chicken lenses (Woo *et al.*, 2000). Less is known about the localisation of spectrin in the nuclear fibre cells using the same technique, possibly due to difficulty associated with obtaining adequate tissue samples from this region of the lens, but it is thought the spectrin-actin cytoskeleton maintains its role in these cells (Alizadeh *et al.*, 2003; Lee *et al.*, 2000). There is some histological evidence that suggests that spectrin remains localized to the membrane of nuclear fibre cells, and other techniques such as Western blotting corroborate these findings (Lee *et al.*, 2001; Lee *et al.*, 2000; Matsushima *et al.*, 1997).

Intermediate filaments are known to sequentially appear and disappear during the developmental progression of cells in both rodents and humans (Boznic *et al.*, 2006). Of the intermediate filament proteins found in the lens fibre cells, vimentin makes the earliest appearance, as it is localised in epithelial cells and early differentiating fibre cells. In mature fibre cells deeper within the lens vimentin is absent with its disappearance defining a specific stage of differentiation (Perng & Quinlan, 2005; Quinlan *et al.*, 1996; Sandilands *et al.*, 1995). The lens specific intermediate filament protein filensin is distributed in the fibre cell in a manner that is dependent on the age of the lens. In mature lenses, filensin is found predominantly associated with the fibre cell plasma membrane, with its distribution becoming cytoplasmic toward the lens nucleus, while still being associated with the plasma membrane (Lee *et al.*, 2000; Sandilands *et al.*, 1995a). In the young lens however, the distribution of filensin is localised primarily in the cytoplasm (Blankenship *et al.*, 2001; Ireland *et al.*, 2000; Sandilands *et al.*, 1995).

The aim of these experiments was to carry out immunohistochemical labelling of a selection of targets based around the cytoskeleton in the ovine lens. The intention of which

was to study the structure of the lens cytoskeleton and examine the subcellular localisation of these proteins, and to compare ovine lens with lenses of other species. A variety of proteins of the lens cytoskeleton were selected as targets in this study, including vimentin, filensin, spectrin, and actin. These proteins were chosen on the basis that there is a reasonable amount known about their distribution within the lens in other species (e.g. Bozniac *et al.*, 2006; Alizadeh *et al.*, 2003; Beebe *et al.*, 2001; Ireland *et al.*, 2000; Lee *et al.*, 2000; Woo *et al.*, 2000; Sandilands *et al.*, 1995; Sandilands *et al.*, 1995a). Other cytoskeleton associated proteins such as tubulin were not investigated in the current study as there was little research to be had in the literature regarding the distribution of these proteins. Actin, spectrin, vimentin, and filensin thus represent a logical choice for an immunohistochemical investigation of the ovine lens cytoskeleton, and allow comparison with what has been found in other species. Furthermore, several of the selected proteins are representative of those that are generally degraded early on in cataract development in other species, and are known calpain substrates (Goll *et al.*, 2003; Lee *et al.*, 2001; Matsushima *et al.*, 1997).

The experimental approach taken in this investigation of the ovine lens cytoskeleton revolved around the use of immunohistochemistry coupled with Western immunoblotting of separated lens USF extracts. Immunoblots were used to identify the presence of target proteins and fragments, and as a proxy for the results obtained by immunohistochemistry, with the combined aim of isolating and localising lens cytoskeletal proteins to discrete regions of the lens.

5.2 Methods

Tissue Collection

Eyes from sheep of unknown breed and that were 1 year old or less were obtained from a local abattoir, and the lenses removed using a posterior approach (Robertson *et al.*, 2005). Lenses to be processed for Western blotting had their capsule containing epithelial cells removed and collected, and the lens mass was separated into cortex and nucleus as for Section 4 (see **Materials and Methods 4.2 Sample Collection**). Lenses were processed for immunohistochemistry whole.

Western Immunoblotting

USF proteins were separated from collected lens fractions as for Section 4 (see **Materials and Methods 4.2 Extraction of Urea-soluble Protein Fraction (USF) from Lens Homogenates and Band Identification by Western Immunoblot**). The protocol was modified for the lens capsule containing epithelial cells (referred to here on as capsule/epithelial cells) due to the negligible mass of this lens fraction. Collected capsule/epithelial cells were placed in 1 mL of Buffer A prior to homogenisation by sonication only (applied in three 5-second bursts at medium power to prevent overheating the sample). All subsequent steps for Western blotting of capsule/epithelial cells were carried out as for lens cortex and nucleus samples (see Section 4 – **Materials and Methods 4.2 Extraction of Urea-soluble Protein Fraction (USF) From Lens Homogenates**).

USF protein (10 µg) was separated by SDS-PAGE for all proteins that were immunoblotted. Western immunoblotting was carried out for each of the chosen proteins in this study (spectrin, actin, vimentin and filensin) using the same antibodies as used for immunohistochemical localisation (see *Immunohistochemical Localisation*, below), and lens fractions (capsule/epithelial cells, cortex, nucleus) were separated and blotted in duplicate.

Processing of lenses for immunohistochemistry

Lenses in all cases were processed for immunohistochemistry following the protocol developed in Section 3 – *Optimisation of a Method for the Processing of Ovine Lenses for Immunohistochemistry* (see Section 3 – 3.6.1 *Final Protocol* for details). Briefly, lenses were fixed whole in 4% PFA in PBS for 120 hours, followed by cryoprotection in 10-30% sucrose in PBS solution overnight. Lenses were frozen and embedded in O.C.T., frozen in liquid nitrogen and sectioned with a cryostat microtome (Leica Microsystems, Nussloch, Germany) set at -20 °C. Sections were cut at 16 µm and collected on HistoBond® adhesion microscope slides (Marienfeld GmbH & Co., Germany).

Immunohistochemical Localisation

The method for immunohistochemical staining of proteins and other cellular material was adapted from Reed *et al.*, (2001), and personal communication (Marc Jacobs, Department of

Physiology, School of Medical Science at Auckland University, November 2005; see also Jacobs *et al.*, 2003, 2004 and Grey *et al.*, 2003 for relevant details). In all cases non-ovine derived antibody specificity for the ovine antigens was specified initially with immunoblotting.

All incubations with antibody were performed either at room temperature for 2 hours, or at 4°C overnight, in a humid box. Antibodies were applied to blocked sections following sectioning and two washes in distilled water to remove O.C.T. freezing medium. For primary antibody labelling, sections were stained at various dilutions between 1:100 and 1:1000 in blocking solution. This was necessary to determine optimum staining of target proteins. Antibodies were visualised with *Alexa Fluor 488*[®] tagged secondary antibodies directed against the primary antibody species as necessary. Secondary antibodies were diluted from a 2 mg/mL stock solution that had been stored in aliquots at -20°C protected from light, and dilution was at 1:200 in blocking solution. Incubation in the presence of antibodies was in all cases followed by three five minute washes with 250 µL of PBS and thorough drying of the sections using dust-free paper towels followed each PBS wash. Prior to viewing, lens sections were mounted in a single drop of anti-fade fluorescent mounting medium (DakoCytomation, USA) and cover slipped. Sections were viewed and images collected as for sections in Section 3 using a Leica DMIRB microscope equipped with a mercury light source, filters, and digital RT camera. Exposure time for image collection was set to “auto”, and some image manipulation was necessary post collection (brightness/contrast).

Control staining of all antibodies was carried out as follows: minus primary antibody and positive for the equivalent secondary being investigated, and minus secondary antibody but positive for primary antibody. For example if actin was being investigated, the equivalent primary negative control would have first been incubated in blocking solution alone followed by incubation with *Alexa Fluor 488*[®] labelled chicken anti-goat IgG antibody, and in the case of the secondary negative control, the lens section initially would have been incubated in the presence of anti-actin antibody, followed by a blank incubation of blocking solution in place of the chicken anti-goat IgG. In addition to antibody controls, randomly selected sections were stained with FITC and/or PI, so as to assess lens morphology in a batch of sections.

In a selection of sections, TRITC conjugated *Triticum vulgare* lectin (Sigma, Missouri, USA) was employed as counter-stain on lens sections. Propidium iodide (PI) was also used as a counter stain for detecting cell nuclei. While primarily used to assess morphology of section

in batches of lens sections, membrane and organelle identification with TRITC-lectin and PI also allowed distinction of cellular components and confirmation of the localisation of target proteins (Reed *et al.*, 1999). TRITC-lectin was used a dilution of 1:200 of a 1 mg/mL solution, and PI at 1:3000 of a 1 mg/ml stock solution. PI was applied to sections diluted in secondary antibody incubation solution, and was thus applied to sections at room temperature for 2 hours, or at 4°C overnight in a humid box (as described in Section 3). PI and TRITC-lectin were visualised with mercury lamp excitation at 535 nm and emitted light was filtered at 615 nm.

5.3 Experiment 1 - Spectrin

5.3.1 Methods

Spectrin was detected in immunoblots and lens sections using a polyclonal anti α -spectrin (fodrin) raised in rabbit (Santa Cruz Biotechnology, California, USA). Spectrin was visualised in sections using *Alexa Fluor 488*[®] labelled goat anti-rabbit IgG secondary antibody (Molecular Probes, Oregon, USA). The *Alexa Fluor 488*[®] fluorescent dye used to tag the goat anti-rabbit IgG had an absorption maximum of 495 nm and an emission maximum of 519 nm. The fluorophore was excited with a mercury lamp and filtered at 520 nm for detection under the microscope with the digital camera.

5.3.2 Results

Immunoblotting

Spectrin was detected predominantly in the lens cortex by immunoblotting as whole spectrin at greater than 250 kDa (the limit of the molecular weight markers used in the immunoblot), as well as break-down products at just over 150 kDa and between 100 and 75 kDa (labelled bands in *fig. 5.3.1*), the latter of which are known to accumulate during normal lens aging (Lee *et al.*, 2001). Additionally, a weak band was observed at approximately 145 kDa in the

cortex samples. Spectrin was expected to be present in the lens nucleus, as it is known to accumulate there as has been found in other studies on lenses in different species (Lee *et al.*, 2001; Lee *et al.*, 2000; Matsushima *et al.*, 1997). A very faint band corresponding to full-length spectrin was detected in the nucleus extract, more so in one of the replicates. A 150 kDa spectrin fragment was detected in nucleus samples. No spectrin was detected in the capsule/epithelial cell extracts.

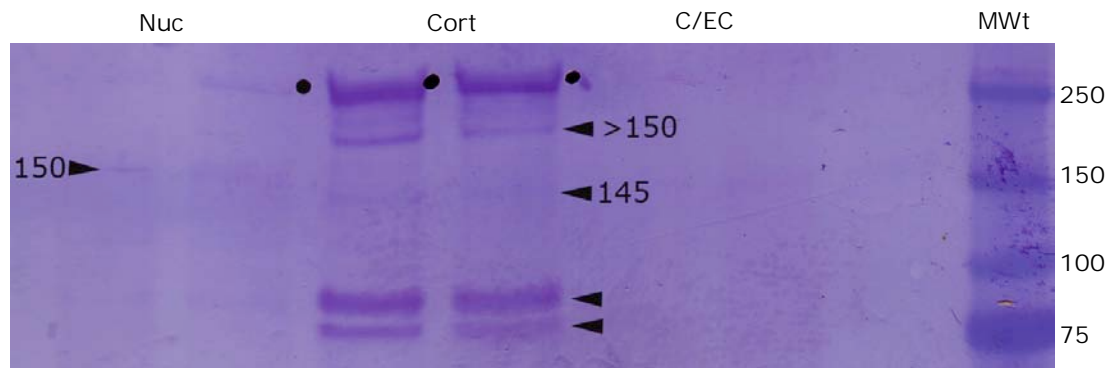


Figure 5.3.1 *Spectrin Immunoblot*

Full-length and fragments of spectrin were detected in the cortex samples only, with a faint full length band detected in one of the nucleus samples. An extremely weak band was observed at around 145 kDa in the cortex sample, corresponding to a specific calpain II product (Robertson *et al.*, 2005), and at 150 kDa a band can be seen in nucleus samples. •, full length spectrin; ◄, spectrin fragments (as identified by molecular mass in kDa); MWt, molecular weight marker (in kDa); C/EC, capsule/epithelial cells; Cort, cortex; Nuc, nucleus.

Immunohistochemical localisation

Spectrin primary antibodies were initially tested at a dilution of 1:100 in blocking solution, and this dilution was gainfully increased to 1:500, while further dilution above this level was found to be inadequate for detection with the fluorescent secondary antibody. In sections that were treated as controls, negligible fluorescence was detected at the wavelength used for detection of spectrin.

In the outer cortex fluorescence associated with spectrin staining could be identified throughout the membrane, and it was generally a very weak signal that was obtained regardless of the antibody concentration above 1:500, hence the decision to use 1:500 dilution. While some sections appeared to favour spectrin in either the long sides or short sides (see *fig. 5.3.2, b*, and *fig. 5.3.3, c-d*), the majority of sections stained showed spectrin

signal at all sides of the fibre cells. There did not appear to be any difference in the distribution of spectrin on/in the membrane across the cortex – i.e. between fibre cells in early maturity that were located near the periphery of the lens section, and the older cells deeper in the cortex.

The lens nucleus showed positive signs for spectrin on essentially all sides of the fibre cells and followed the irregular contours of the membranes of these cells (*fig. 5.3.2, e and d*). Areas of increased intensity for spectrin labelling were detectable in the nucleus that perhaps coincided preferentially at junctions of two or more cells, as opposed to the margin bordering two proximate cells that generally displayed spectrin localisation but at a less intense level.

Occasional sections immunostained for spectrin displayed a signal that was difficult to interpret owing to its seeming intense cytoplasmic localisation (see *fig. 5.3.3, a-b*). Again these results were likely due either to sectioning plane difficulties outlined in Section 3, non-specific staining by secondary antibodies, or unexpected cytoplasmic localisation of proteins. Section (b) presented in *fig. 5.3.3*, is a noticeable example of the plane of sectioning along fibre cells rather than through the cells as was originally desired (discussed in Section 3).

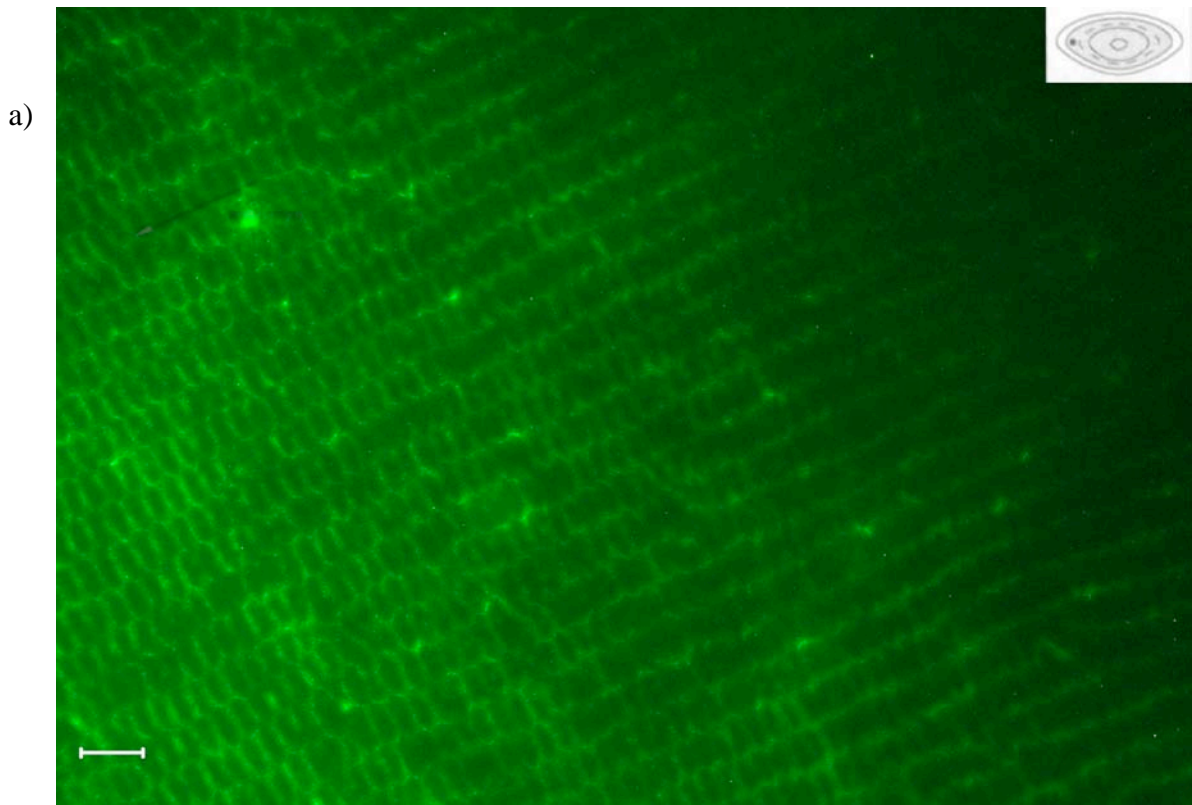


Figure 5.3.2. *Spectrin Distribution in the Ovine Lens Cortex.*

Spectrin was found to be distributed evenly about the cortical fibre cell membrane. *Inset* schematic illustrating approximate location within the lens where the picture was taken (after Taylor *et al.*, 1997). Scale bar is 10 μm .

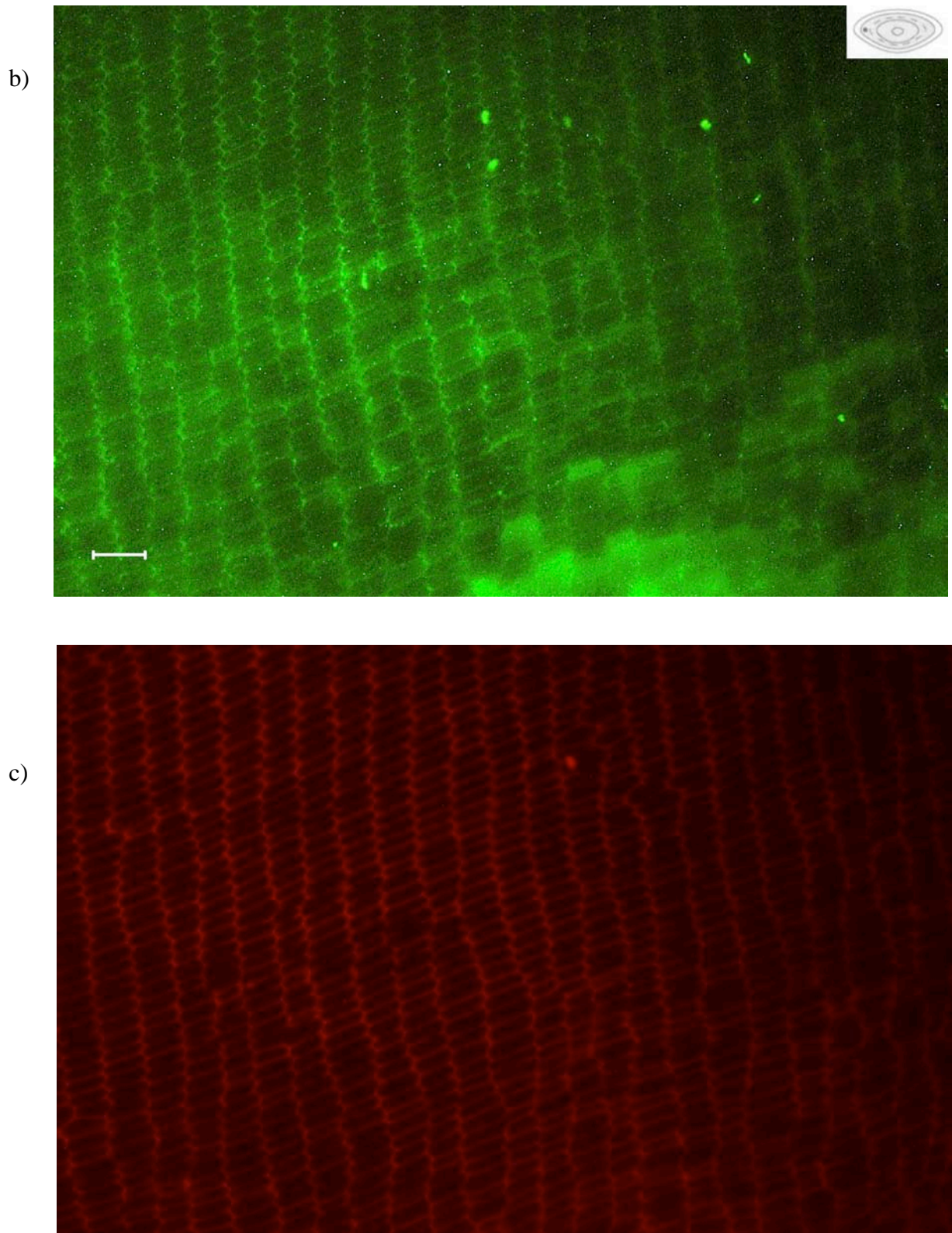


Figure 5.3.2. *Spectrin Distribution in the Ovine Lens - Cortex.*

Section (b) gives the impression of a slight increase in the presence of spectrin in the short sides compared with the long sides of the fibre cell (contrast with *fig. 5.3.2 a*). The membranous spectrin was confirmed TRITC-lectin (red, c) matching closely the spectrin signal detected on a double labelled section (i.e., the section presented in *fig. 5.3.2 b*) *Inset* as for *fig. 5.3.2*. Scale bar is 10 μm .

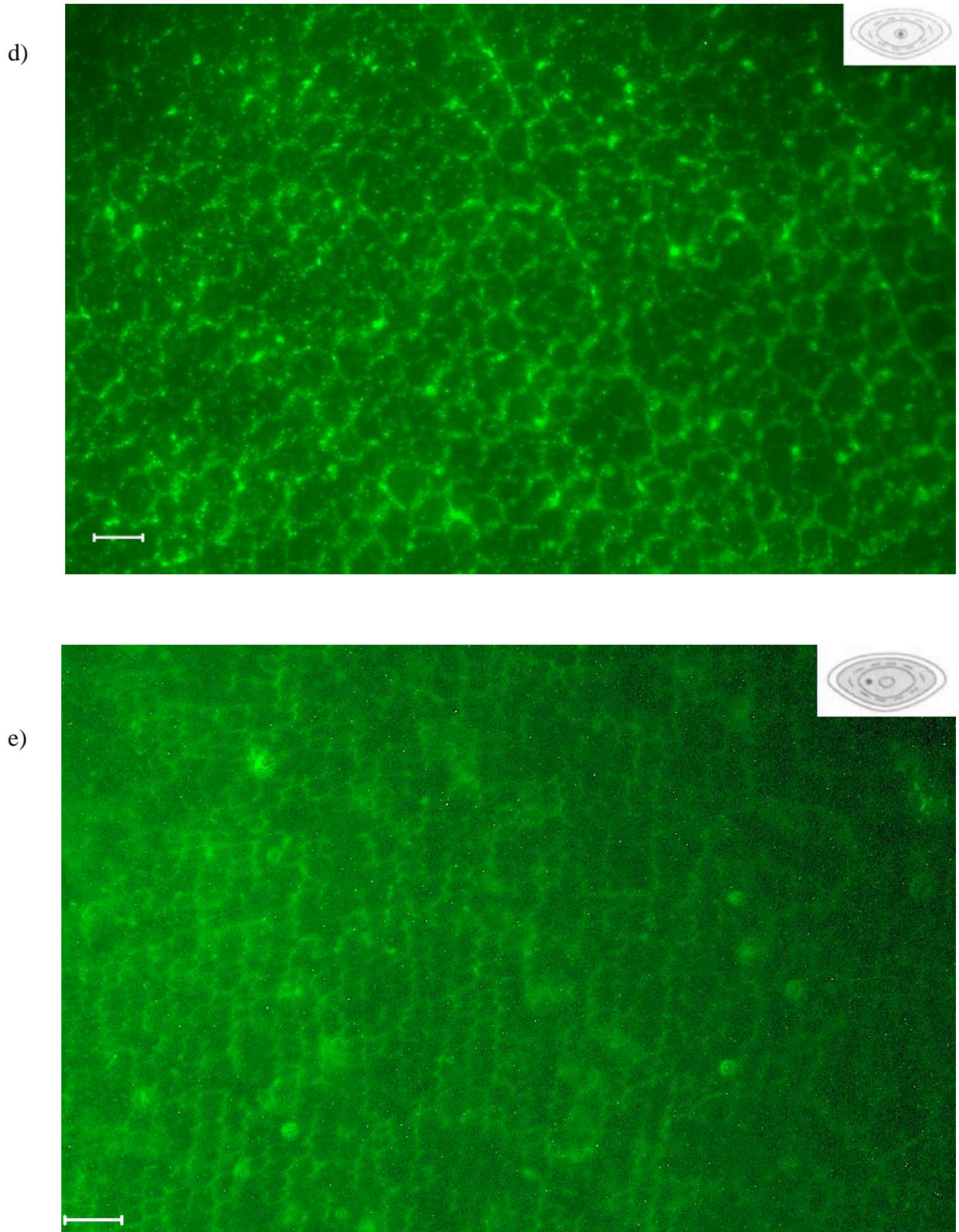


Figure 5.3.2. *Spectrin Distribution in the Ovine Lens - Nucleus.*

(d, e) Spectrin signal detected in the lens nucleus showing again essentially equal distribution around the cell periphery in the membrane, with random areas of intense signal seem at presumed junctional regions. (e, 630x magnification, scale bar is 10 μm ; f, 400x magnification, 10 μm scale). *Inset* as above.

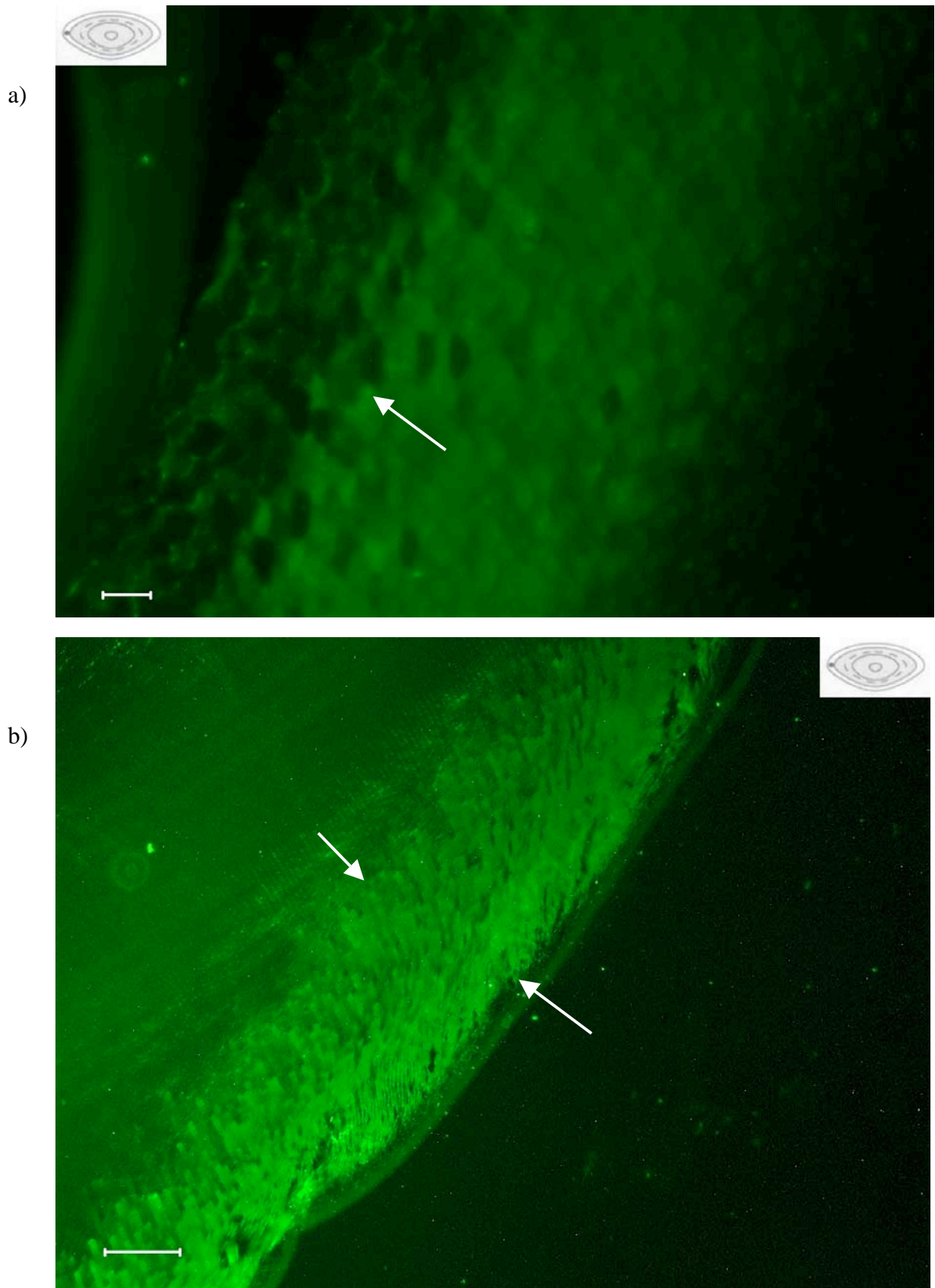


Figure 5.3.3 *Other Spectrin Localisation Results.*

Anomalous spectrin signal detected in the cytoplasm of fibre cells in the outer cortex was observed, which at high magnification (a) was not distinguishable between fibre cell cortex or membrane (see arrow in *fig. 5.3.3 a*), while at lower magnification spectin was observed preferentially at the extreme edge of the cortex between the areas specified by arrows in *fig. 5.3.3 b*. Scale bars: a, 10 μm scale bar; b, 100 μm

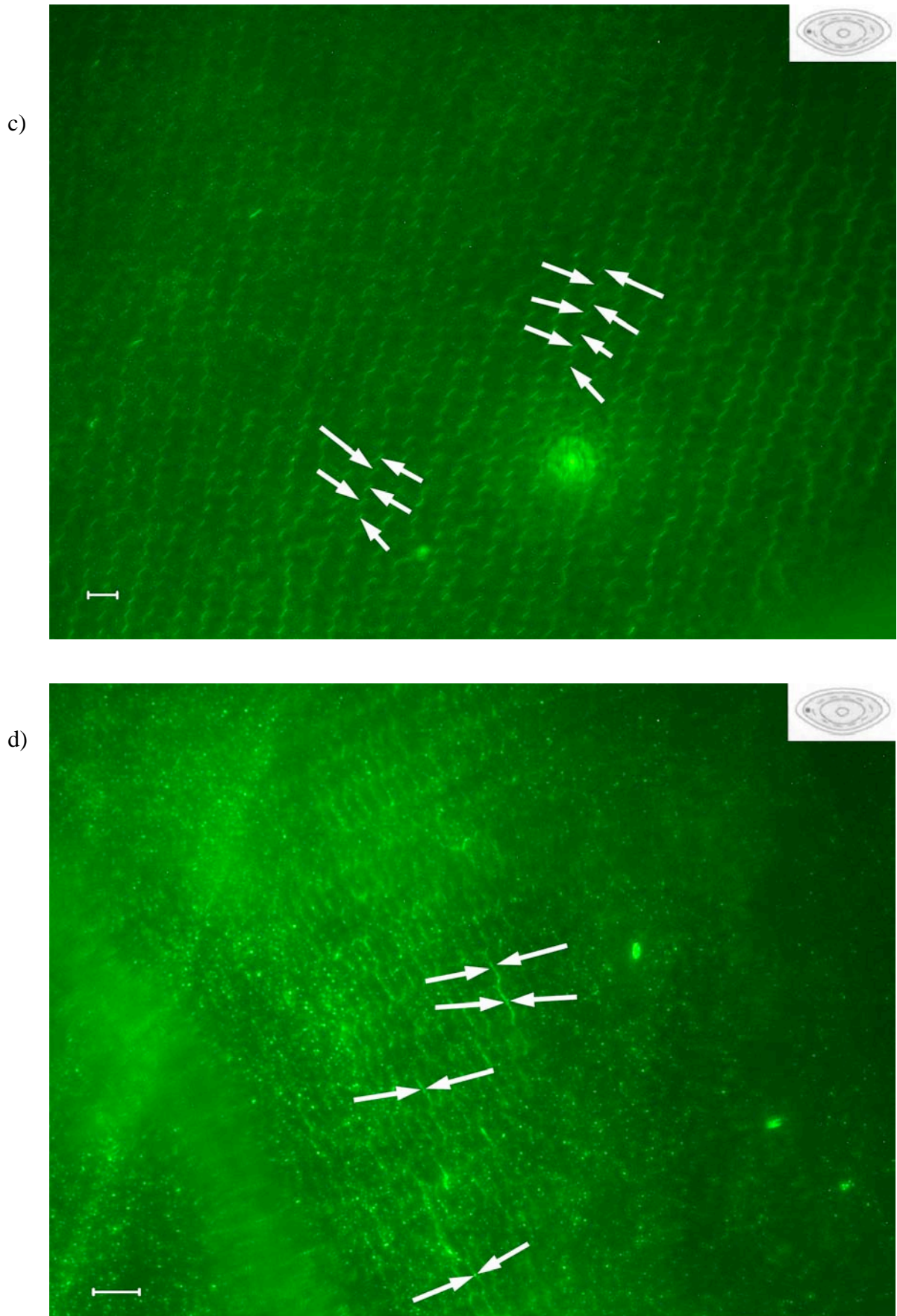


Figure 5.3.3 *Other Spectrin Localisation Results.*

Some sections additionally displayed non-typical staining for spectrin that suggested alternative distribution that favoured either the short or long sides of fibre cells, as indicated with arrows in *fig. 5.3.3 c and d*, respectively. *Inset* as above. Scale bars: 10 μm (c 400x magnification, d 630x magnification).

5.4 Experiment 2 – Actin

5.4.1 Methods

Goat polyclonal anti-actin (C-11; Santa Cruz Biotechnology, California, USA) raised against a carboxy terminus peptide of human actin was used in immunoblots of separated lens extracts and in histological lens sections of sheep to detect the presence of actin. The anti-actin antibody was diluted at between 1:100 and 1:1000 in blocking solution for incubations carried out on lens sections, and at 1:1000 for Western blots. Actin was visualised on lens sections using *Alexa Fluor 488*[®] labelled chicken anti-goat IgG secondary antibody (Molecular Probes, Oregon, USA) at 1:200 dilution in blocking solution.

5.4.2 Results

Immunoblotting

An intense band corresponding to actin was detected by immunoblot at approximately 42 kDa in separated lens cortex USF homogenates (see *fig. 5.4.1*). Meanwhile, extremely faint bands corresponding to actin were detected in separated lens nucleus, and immunoreactivity was absent in separated capsule/epithelial cell USF homogenates. No breakdown products were observed in actin Western blots.

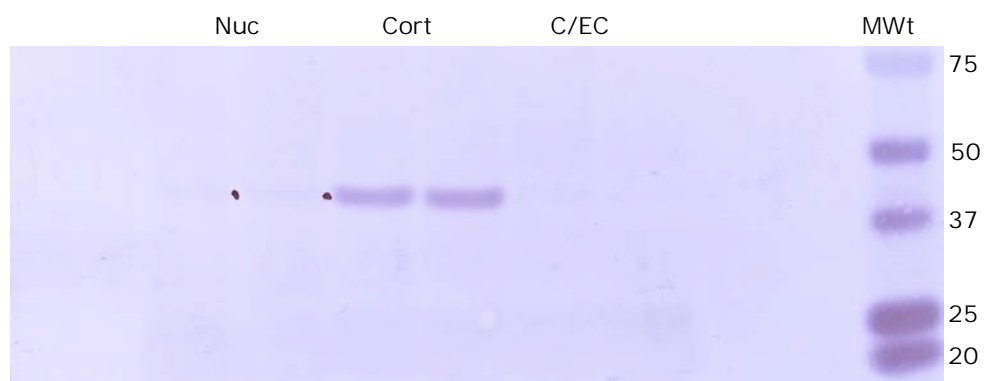


Figure 5.4.1 *Actin Immunoblot*

Blotted PVDF membrane showing reactivity to the actin antibody in lens the lens cortex, and somewhat less reactivity was present in the lens nucleus (as indicated by the • marks). It is possible that the detected presence of actin in the nucleus samples was due to contamination arising during the separation of the lens fractions. Abbreviations are as for *fig. 5.3.1*.

Immunohistochemical Localisation

Goat polyclonal anti-actin was capable of detecting actin at 1:1000 dilution in blocking solution, lowered from initial assay at 1:100, and there was little difference between the signal detected at either concentration. Actin was detected in cortical fibre cells and displayed a pattern of distribution that indicated actin was enriched at the short sides of the fibre cell membranes, in addition to a general presence at all parts of the membrane at reduced intensity to that seen in the short sides. Fibre cells generally displayed an evenly distributed signal along the short edge of the fibre cell (*fig. 5.4.2, b-d*), however some sections displayed a slightly altered distribution from that just described (see section *fig. 5.4.2, a*) in that there appeared to be a more intense signal at the corners of the fibre cells.

Actin could not be identified in the lens nucleus, but actin detected in the cortex nearer the lens nucleus appeared to be diminishing in intensity (see arrow *fig. 5.4.2, d*). Control sections (minus primary or minus secondary antibody) were negative for actin.

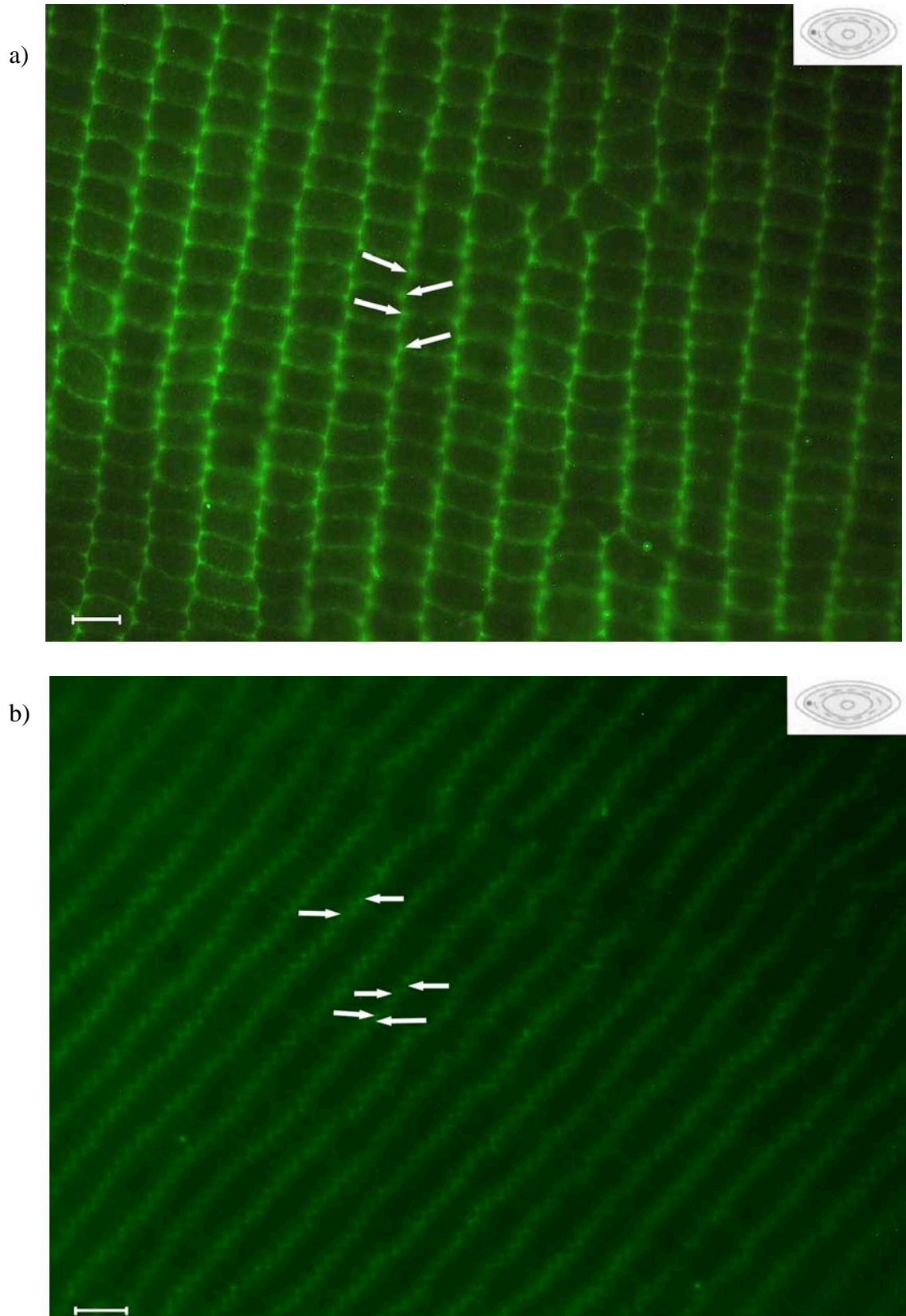


Figure 5.4.2 *Cross-sections of Cortical Fibre Cells Showing Actin Distribution.*

Distinct dots can be seen in section (a) at the corners of the fibre cells (see arrows, a), while actin was also found to be evenly distributed along the short sides (see arrows in b). Actin signal can also be seen as on the longer sides of the membrane in all cases, however at a reduced level to that in the short sides. *Inset*: schematic illustrating approximate location within the lens where the picture was taken (after Taylor *et al.*, 1997). Scale bar 10 μm .

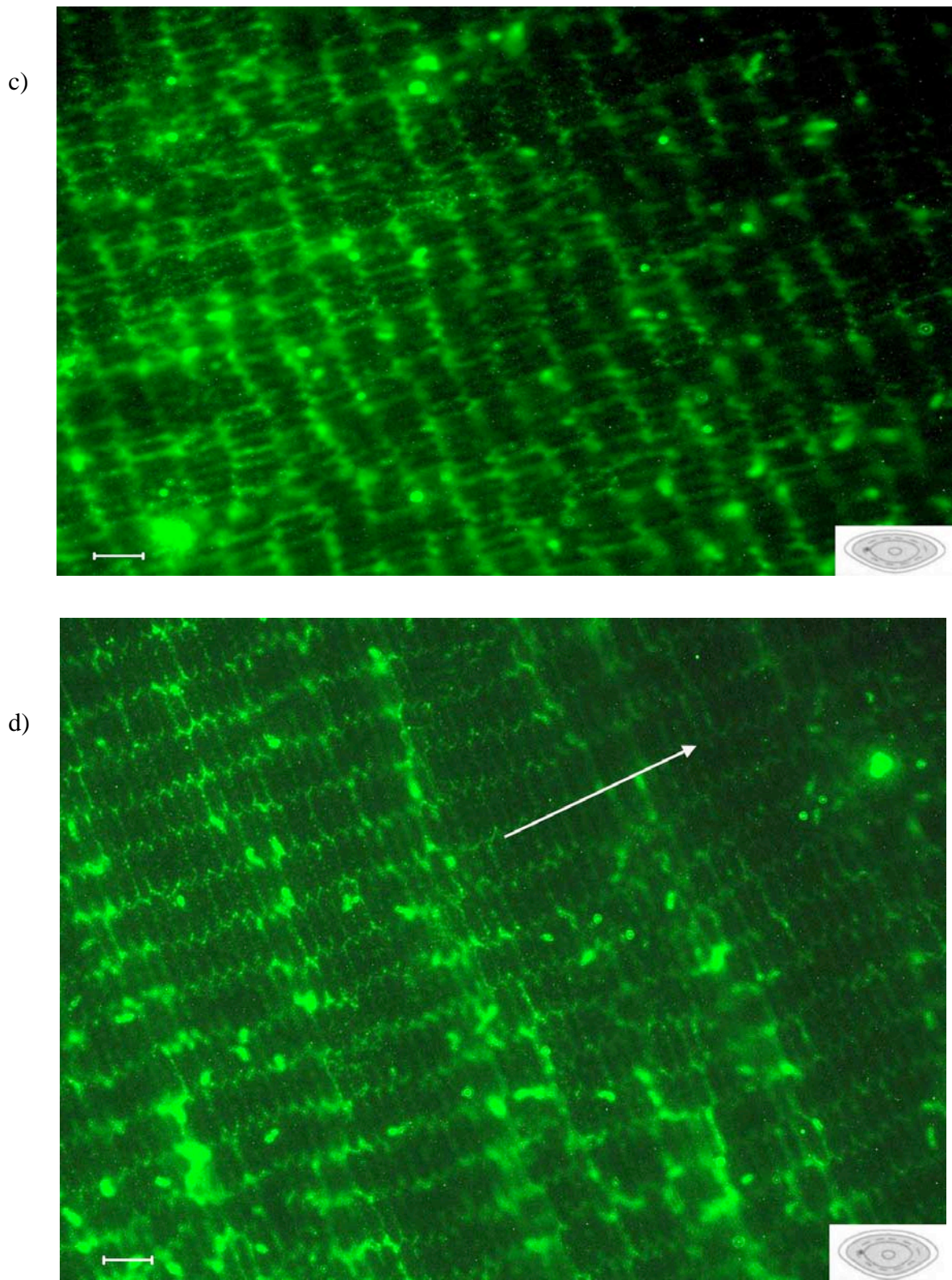


Figure 5.4.2 *Actin in the Deep Cortex.*

The above sections show the actin distribution deeper in the cortex. Deep cortex images were optimised with regards to brightness/contrast ratios as staining was very weak in this region and could not be improved during collection. The actin signal diminished from that seen in the outer cortex as depicted in sections (a) and (b), and this is particularly evident in (d), where the staining is decreased to the right of the picture, toward the nucleus (as indicated by the direction of the arrow). The fluctuating signal depicted was the result of presumably inconsistent sectioning which was often experienced at depth in the lens. *Inset* as above. Scale bar 10 μm .

5.5 Experiment 3 - Vimentin

5.5.1 Methods

Mouse polyclonal anti-vimentin (Santa Cruz Biotechnology, California, USA) was used as a primary stain to identify vimentin in separated USF extracts with immunoblotting and in lens sections. Vimentin antibodies were detected in lens sections by *Alexa Fluor 488*[®] fluorochrome tagged goat anti-mouse IgG antibody (Molecular Probes, Oregon, USA) diluted 1:200 in blocking solution.

5.5.2 Results

Immunoblot

Intense bands were detected for vimentin in the cortex fractions and capsule/epithelial cells (see *fig. 5.5.1*), at approximately 56 kDa, while no vimentin reactivity was to be found in lens nucleus extracts. In addition to the intense band that correlated to intact vimentin in the cortex, several bands of a lower intensity and molecular mass were detected that corresponded to naturally occurring vimentin products known to occur naturally in the lens (Robertson *et al.*, 2005). These included a band of approximately 54 kDa, as well as bands of around 50 kDa and 46 kDa (as indicated by arrows in *fig. 5.5.1*).

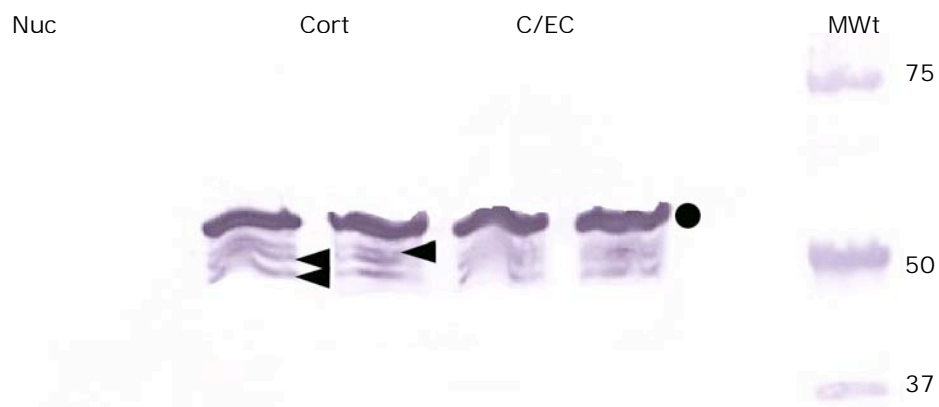


Figure 5.5.1 *Vimentin Immunoblot*

Intense reactivity was detected for a band of approximately 56 kDa in the cortex and capsule/epithelial cells. There was a dramatic disappearance of vimentin reactivity in the lens nucleus. •, complete/intact vimentin; ◄, vimentin fragments; Abbreviations are as for *fig. 5.3.1*

Immunohistochemical Localisation

Using mouse polyclonal anti-vimentin, vimentin was successfully detected in ovine lens sections at 1:500 dilution. Vimentin in the outer cortex was located with some intensity at the fibre cell membranes, and a positive vimentin signal could be identified in the fibre cell cytoplasm of the outer cortex (*fig. 5.5.2, a and b*). The intensity of the signal rapidly diminished in the deeper cortex in both the membrane and cytoplasm (*fig. 5.5.2, d-e*). Cytoplasmic vimentin appeared to diminish earlier, while the membrane signal for vimentin could be observed deeper in the cortex after the cytoplasmic signal was lost (see arrows *fig. 5.5.2, e*).

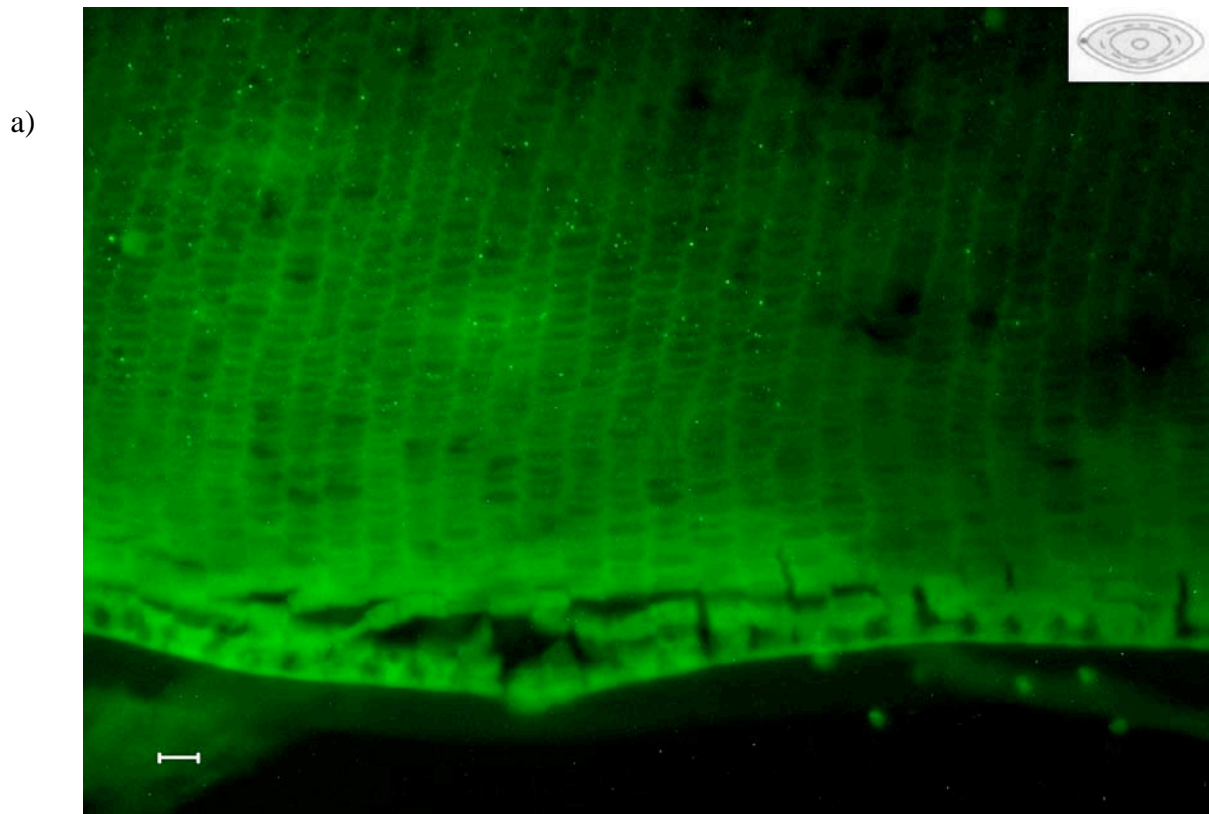


Figure 5.5.2 *Vimentin Distribution in the Cortex of the Ovine Lens.*

Above is shown the pattern for vimentin that was witnessed at the very edge of the lens in the outer cortex. The fibre cell membranes display the familiar pattern of these cells. The edges of the photographed sections show some tearing acquired during sectioning. *Inset* schematic illustrating approximate location within the lens where the picture was taken (after Taylor *et al.*, 1997). Scale bar 10 μm .

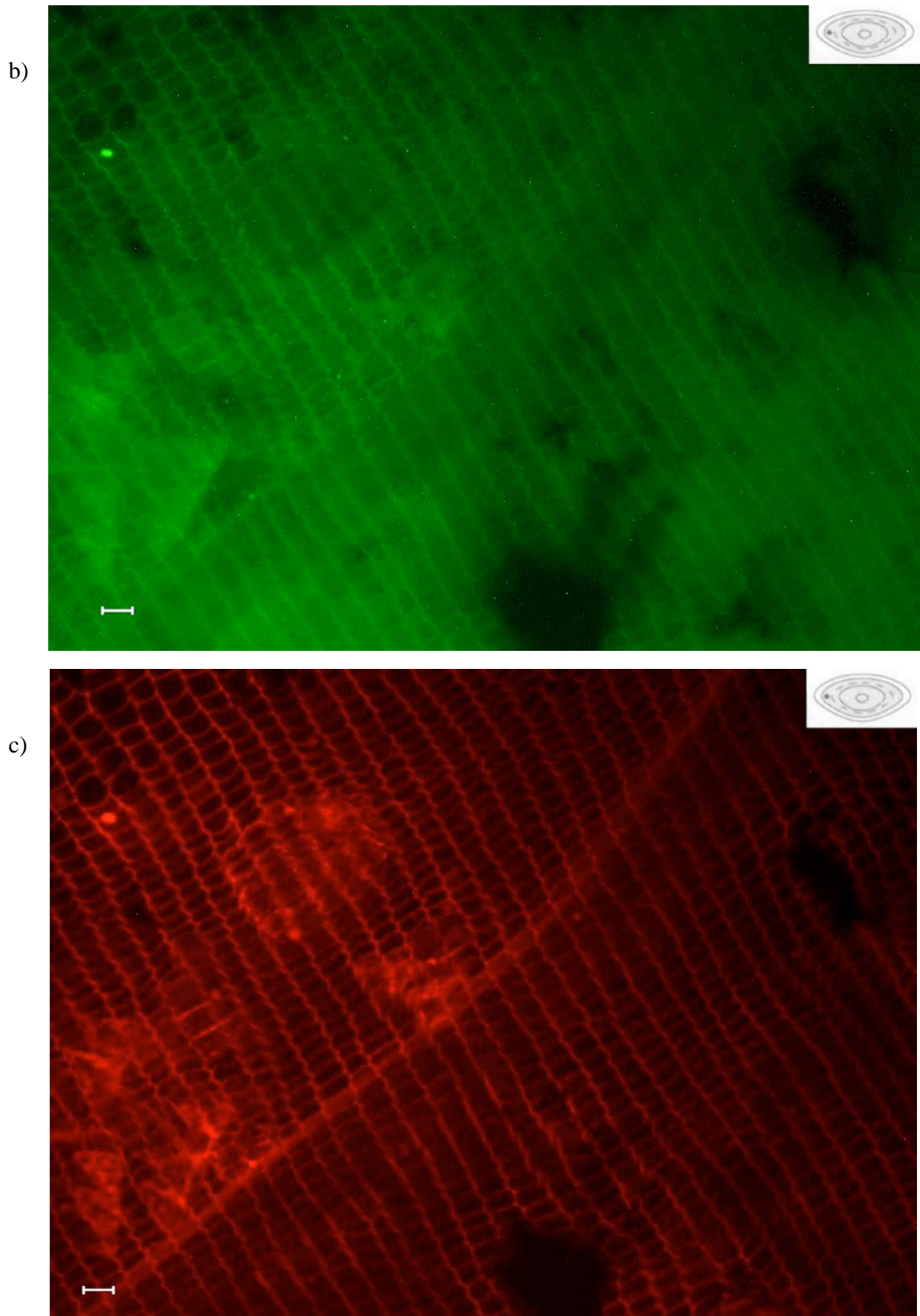


Figure 5.5.2 *Vimentin Distribution in the Cortex of the Ovine Lens.* Membrane and cytoplasmic signal found for vimentin (c), with the cytoplasmic staining being detected as fluorescence within the boundary of the fibre cell membrane, while (d) confirms membrane staining for vimentin. Scale bar 10 μm . *Inset* as above.

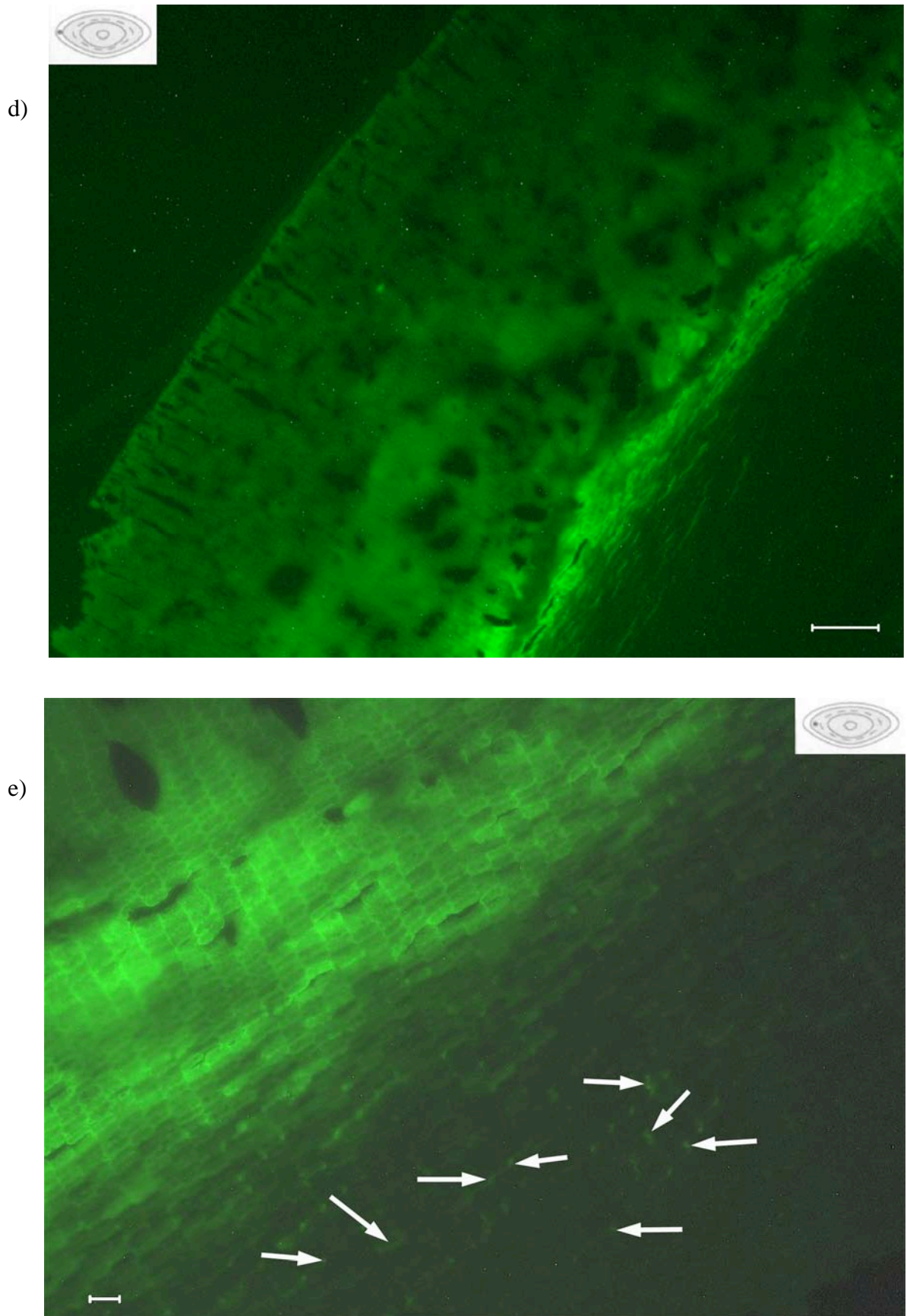


Figure 5.5.2 *Vimentin Distribution in the Cortex of the Ovine Lens.*

The above pictures show the vimentin signal rapidly diminishing as the cells track in toward the lens nucleus. The arrows in (e) are pointing out faintly detected membranous vimentin present after the cytoplasmic signal had mostly vanished. Scale bar: d, 100 μm ; e, 10 μm . *Inset* as above.

It was originally thought that no vimentin was detected in the lens nucleus, as it appeared that staining for vimentin was absent here (*fig. 5.5.3, a*). With some manipulation (brightness and contrast) of images however it was possible to detect a faint green signal in the lens nucleus (*fig. 5.5.3, b*). Few sections that were used as controls managed to retain the nucleus region intact throughout the staining procedure, and displayed a small amount of perceptible fluorescence, although at a level reduced from in positive sections. Consequently it was difficult to determine with a high degree of confidence if the green signal encountered in the lens nucleus was due to non-specific binding of the secondary antibody, vimentin induced, or due to inadequate filtration of TRITC used as a membrane stain in these sections. Sections were not stained for TRITC in a controlled manner; that is, in the way that the antibody staining was controlled by staining sections without one or the other antibodies used; instead TRITC was added to all sections as counterstain (including antibody controls). Consequently TRITC cannot be ruled out as responsible for the signal witnessed in positive sections.

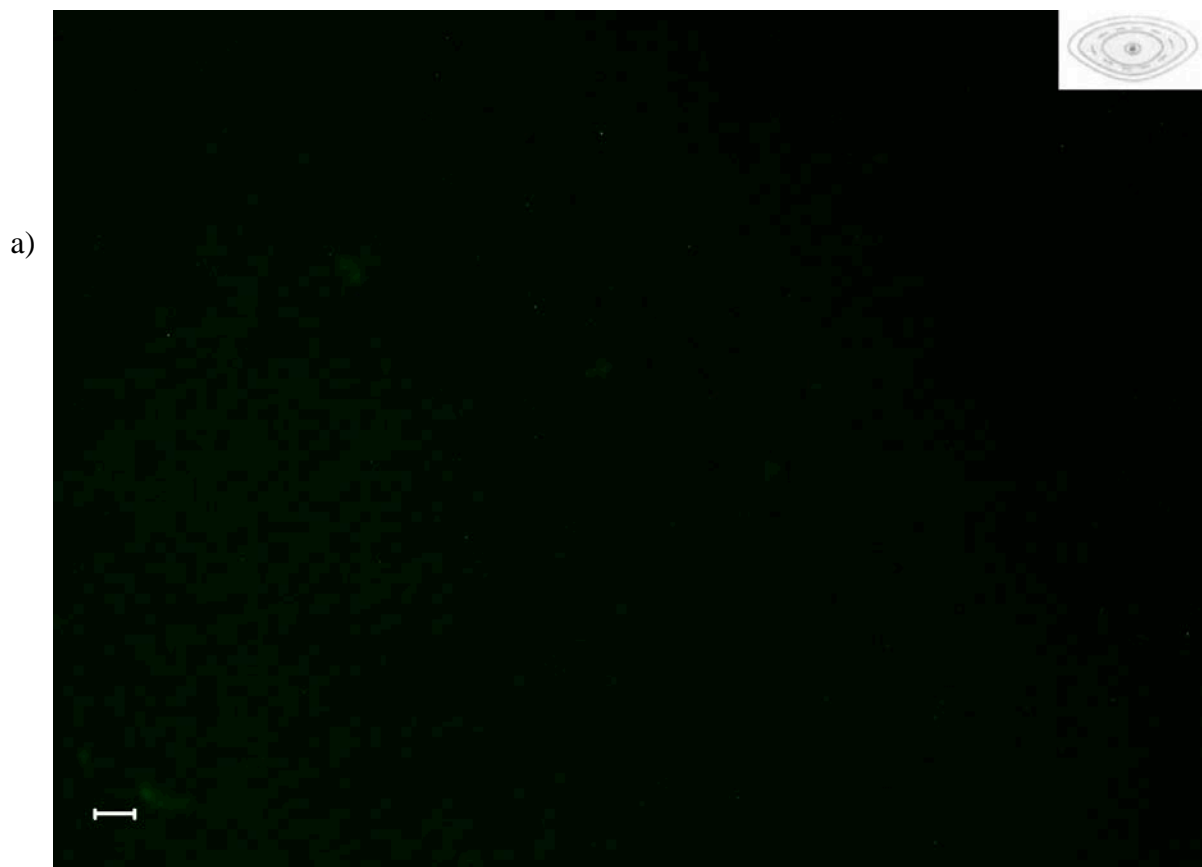


Figure 5.5.3. *Vimentin in the Lens Nucleus.*

Nucleus section of a lens stained for vimentin. No vimentin signal could be detected, either by naked observation or on original images (see following pictures). Scale bar 10 μm .

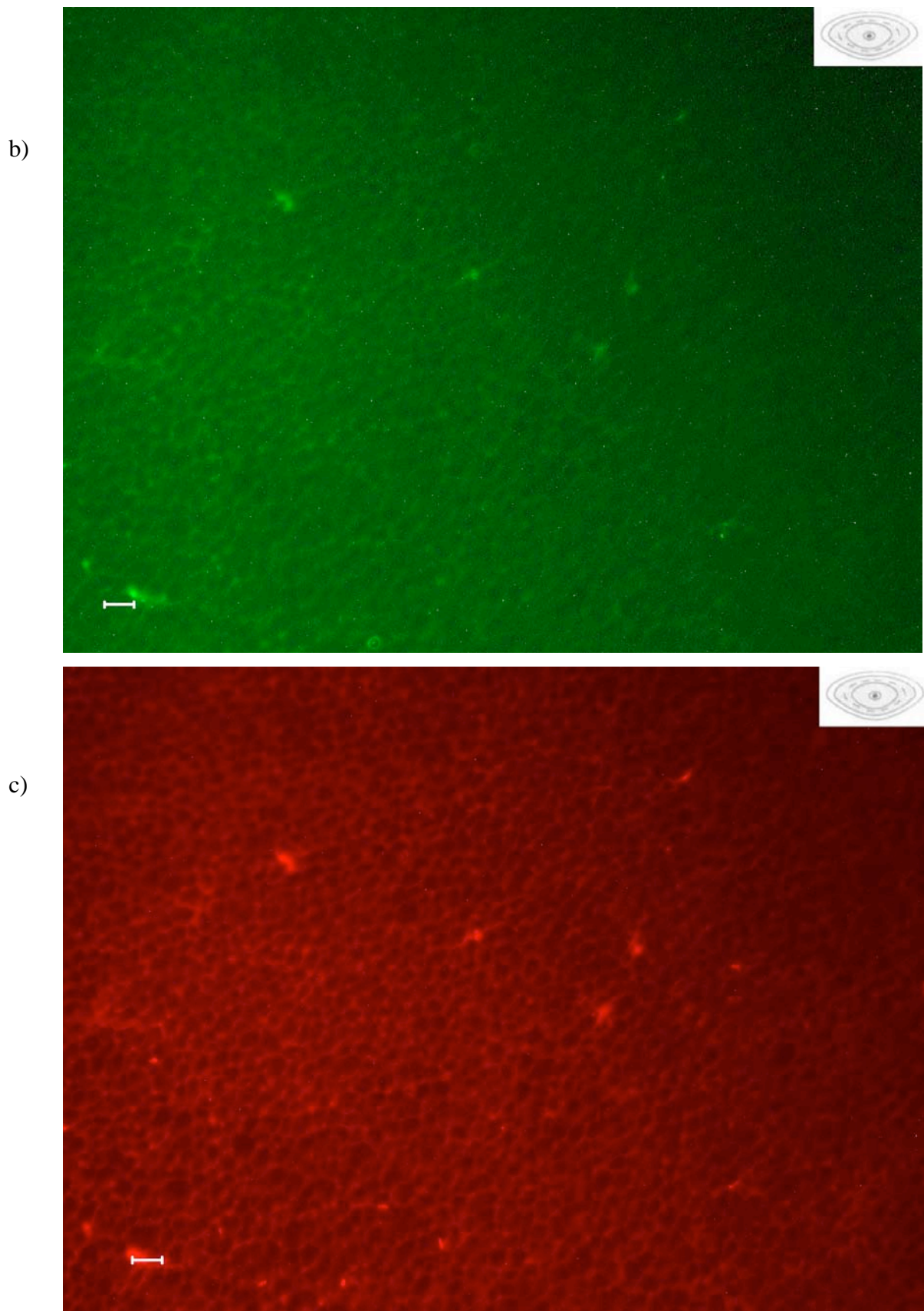


Figure 5.5.3. *Vimentin in the Lens Nucleus.*

Section (b) is the same as that presented in *fig. 5.5.3 (a)* with brightness and contrast parameters altered, revealing faint vimentin signal. (c) Fibre cell membranes in lens core from pictures (a/b) as confirmed by TRITC-lectin stain. *Inset* schematic illustrating approximate location within the lens where the picture was taken (after Taylor *et al.*, 1997). Scale bar 10 μm .

5.6 Experiment 4 - Filensin

5.6.1 Methods

Monoclonal filensin antibody directed against full-length filensin and fragments between 28-69 kDa (see Masaki & Quinlan, 1997; Sandilands *et al.*, 1995a) immunogenically derived from human and bovine lens (Sigma, Missouri, USA) was used to detect filensin in ovine lens sections and immunoblots. The monoclonal filensin antibody was diluted initially at 1:100, and was found to work best at a dilution of 1:500 in blocking solution. Filensin antibodies were identified in sections with *Alexa Fluor 488*[®] tagged goat anti-mouse IgG secondary (Molecular Probes, Oregon, USA).

5.6.2 Results

Immunoblot

Immunoblots using the filensin antibody were difficult to interpret, as several bands were found to be reactive in separated protein. Bands were clearly distinguished using this antibody when only 5 µg of protein was loaded to the SDS-PAGE, rather than using 10 µg as was the case for immunoblots of other proteins in this investigation (*fig. 5.6.1*). Full-length filensin was identified in immunoblots of lens extracts from all fractions. Intense bands at approximately 115 kDa and 53 kDa (bands labelled in *fig. 5.6.1* by • and ◀, respectively), as well as some poorly resolved bands at around 100 kDa and between 37 and 50 kDa were observed in nucleus and cortex extracts (as denoted by →, ◀, and > respectively in *fig. 5.6.1*), while only the 115 kDa band was observed in capsule/epithelial cells. Cortex samples contained the most intense 115 kDa filensin bands, while in the nucleus the 53 kDa fragment band appeared marginally larger than in the cortex or nucleus samples, possibly corresponding to accumulation of this filensin fragment in the nucleus. Additionally, bands of ~50 kDa (as labelled with † in *fig. 5.6.1*) were visible in nucleus samples only. One of the capsule/epithelial cells replicates had a faint band at 53 kDa, the position of an expected filensin fragment.

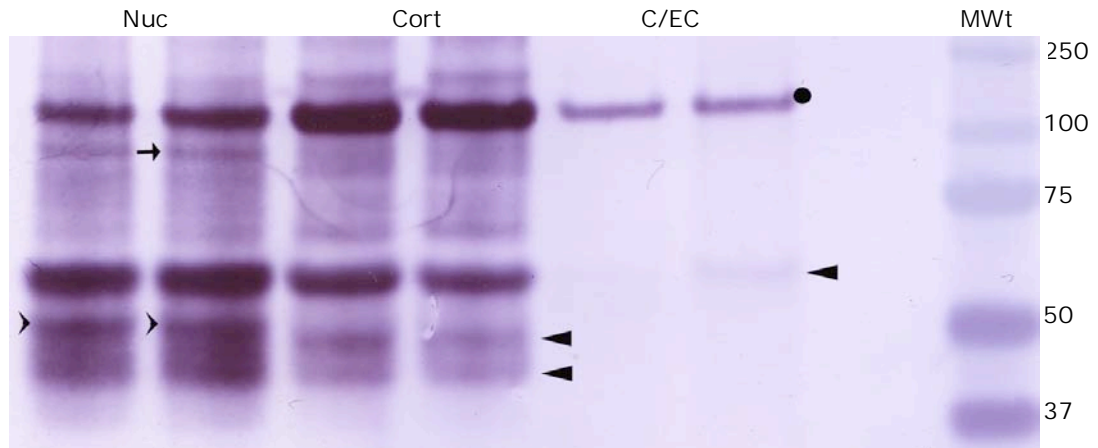


Figure 5.6.1 *Filensin Immunoblot*

Several bands corresponding to full length and fragmented filensin were detected by immunoblot in separated lens fractions. Clearly discernable is an intense band at 115 kDa corresponding to full length filensin, and another at 53 kDa. •, full length filensin; →, 100 kDa fragment; ◄, filensin fragments; >, ~50 kDa fragment observed in nucleus extracts only. Abbreviations are as for *fig. 5.3.1*

Immunohistochemical Localisation

The filensin signal detected was predominantly cytoplasmic (*fig. 5.6.2, a*), with the pattern of staining changing toward the lens nucleus to a seemingly confined area of the cytoplasm (see arrows, *fig. 5.6.2 b*). The staining pattern detected in the sections shown in *fig. 5.6.2, (b)* in the deeper cortex was more or less comparable to what has been found in this region in the lenses of other species (e.g. Sandilands *et al.*, 1995), with filensin beginning to be detected in the fibre cell cytoplasm. The outer cortex filensin signal did not show any defined pattern that would be associated with the membrane or cytoplasm, rather it was detected as nebulous fluorescence that followed the general lens gross structure without revealing any microscopic detail of filensin in this area (*fig. 5.6.2, a*), nor the membranous localisation that was expected. Filensin immunoblots (*fig. 5.6.1*) displayed multiple reactive bands for filensin antibody, and it may be that multiple filensin fragments were responsible for the immunohistochemical results observed.

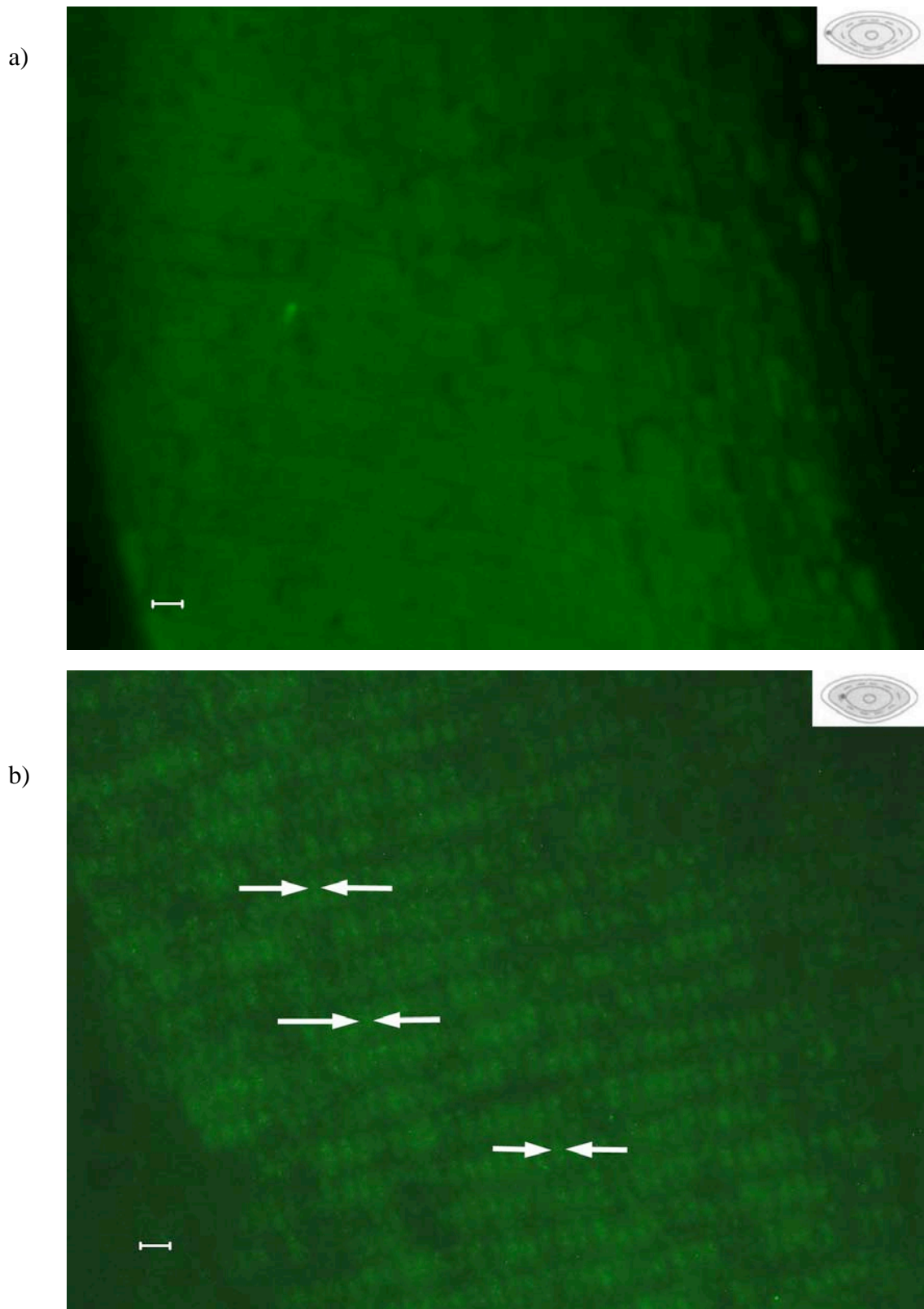


Figure 5.6.2 *Cytoplasmic Filensin.*

Beginning in the outer cortex (a), filensin was seen throughout the fibre cell (arrows in *fig. 5.6.2 a*), changing in the inner cortex (b) to a centralised region of the fibre cell (arrows in *fig. 5.6.2 b*). *Inset* schematic illustrating approximate location within the lens where the picture was taken (after Taylor *et al.*, 1997). Scale bar is 10 µm.

The images presented in *fig. 5.6.3*, (a) show filensin distribution in the outer cortex of the lens with the filensin pattern visible associated with the fibre cell membrane. The filensin signal detected was very weak and stained the fibre cell membranes dimly, and background fluorescence was distinguishable in all sections stained for filensin, as well as in both primary and secondary negative controls (see *fig. 5.6.3*, c), and may have hampered detection of filensin staining. There did not appear to be any obvious differences in the distribution of filensin between early fibre cells in the extreme outer of the cortex and the more mature fibre cells deeper in the cortex.

Little filensin appears to be present in the ovine lens nucleus as staining of this region produced inappreciable fluorescence (*fig. 5.6.3*, d and f). TRITC-lectin labelling of this region of the lens (*fig. 5.6.3*, e, g, h) revealed the familiar pattern of membrane staining for this region typified by irregular squashed semi-hexagonal cells (for clear example see in Section 3, *fig. 3.4.1*). Intense TRITC-lectin staining was detected at the fibre cell junctions in this region (see arrow-heads, *fig. 5.6.3*, e), which has been reported previously in ovine lenses (Kistler *et al.*, 1986). Viewing of the same area of the lens using the 520 nm filter revealed some green signal that did not follow any obvious pattern (*fig. 5.6.3*, d), such as following the membrane as illustrated by TRITC-lectin (*fig. 5.6.3*, e) labelling or within the cytoplasm of fibre cells as has been seen elsewhere (e.g. Blankenship *et al.*, 2001; Ireland *et al.*, 2000; Sandilands *et al.*, 1995). However some isolated areas of coincident fluorescence were shared between the TRITC-lectin and labelled filensin images (see arrows, *fig. 5.6.3*, d and e) suggesting inadequate filtration of fluorescence. Thus it may have been that the poorly focused signal detected in this region (i.e. *fig. 5.6.3*, d) was merely non-specific background fluorescence resulting from inadequate washing of the section following staining, or extraneous fluorescent materials.

The section shown in *fig. 5.6.3*, f, appeared to display membrane staining of filensin and TRITC-lectin such as that seen in the nucleus region of lens sections with irregular hexagonal structures visible, but again this was poorly focussed. This section was found in a lens section that suffered the common frailty associated with fixing the lens nucleus (see Section 3 – *Optimisation of a Protocol for Processing of Ovine Lenses for Immunohistochemistry*), resulting in a chunk of lens nucleus being exposed without surrounding cortex tissue, which explains the exposed area of lens nucleus.

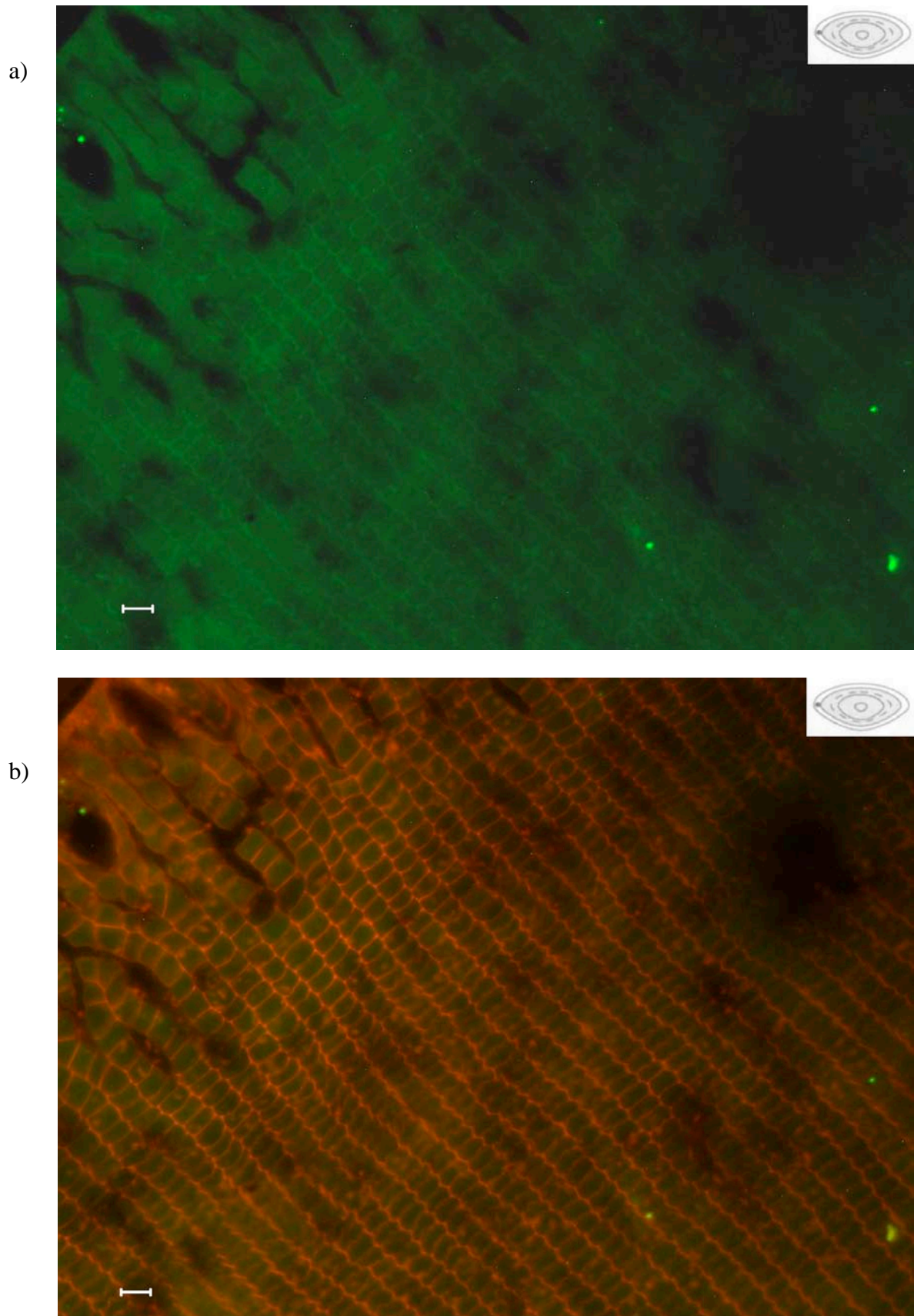


Figure 5.6.3 - Filensin Distribution in the Outer Cortex and Nucleus

Filensin in the outer cortex (a). Merge of (a) and TRTIC-lectin membrane counter stained section (b), confirming association of filensin with the fibre cell membrane in the outer cortex cells, in addition to a cytoplasmic signal. *Inset* schematic illustrating approximate location within the lens where the picture was taken (after Taylor *et al.*, 1997). Scale bar is 10 μm .

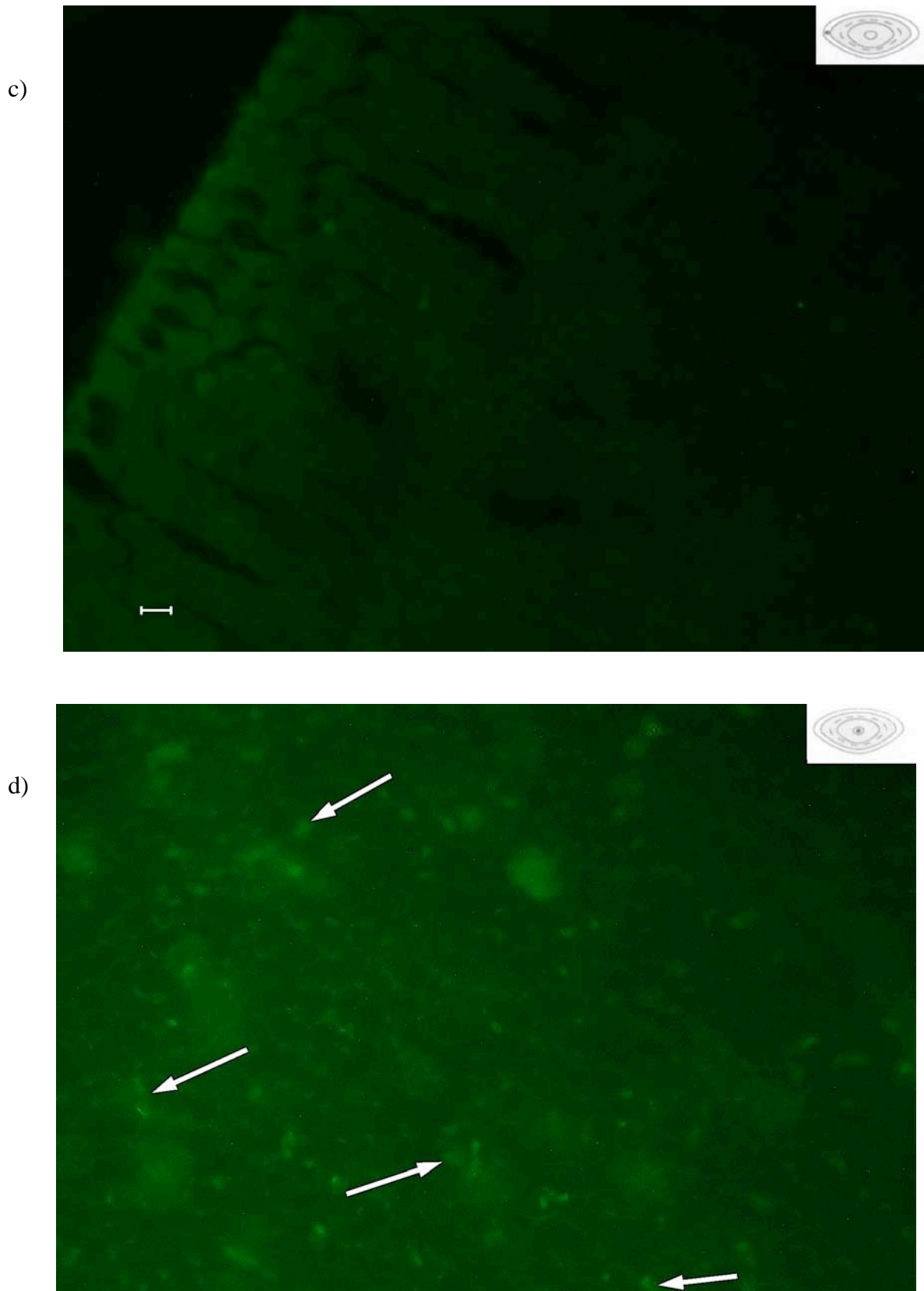


Figure 5.6.3 *Filensin Distribution in the Outer Cortex and Nucleus*

Primary negative control for filensin (c), showing slight background fluorescence signal that was detected in these control sections. (d) Filensin detected in the nucleus region, with arrows indicating areas of fluorescence that can be observed at 520 nm (i.e. the green filter to detect the Alexa 488 dye used to tag filensin antibodies) and with TRITC staining (see *fig. 5.6.3 e*). *Inset* as above. Scale bar is 10 μm .

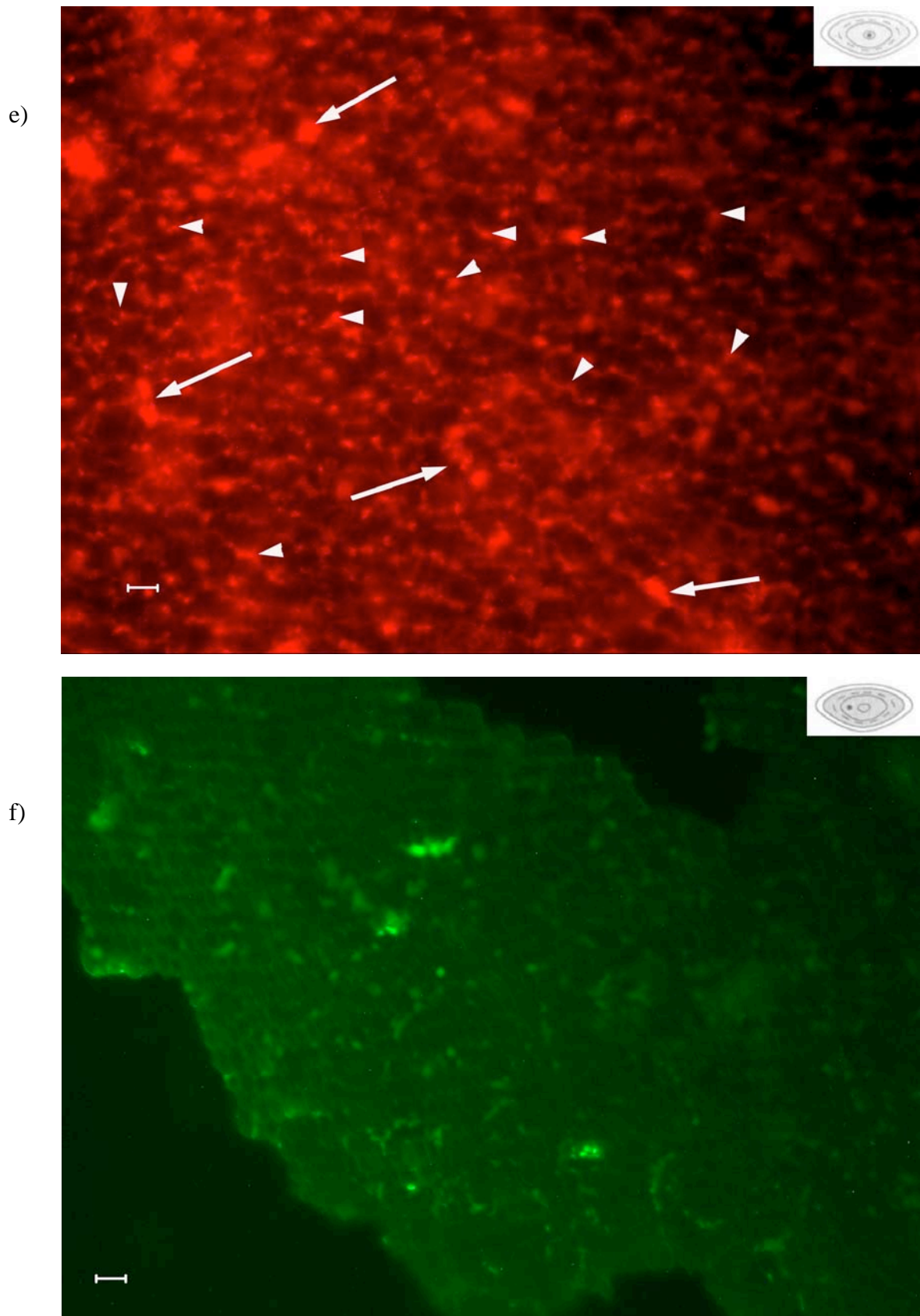


Figure 5.6.3 *Filensin Distribution in the Outer Cortex and Nucleus*

(e) TRTIC-lectin of the section shown in (fig. 5.6.3 d), with the same arrows indicating areas of synchronous fluorescence between the two pictures that were viewed with different filters, suggesting inadequate filtration of fluorescence. Arrow-heads in fig. 5.6.3 (e) point out areas of increased signal at membrane junctions (see main text). (f) Exposed section of lens nucleus staining positively for membrane associated filensin. *Insets* as above. Scale bar is 10 μm .

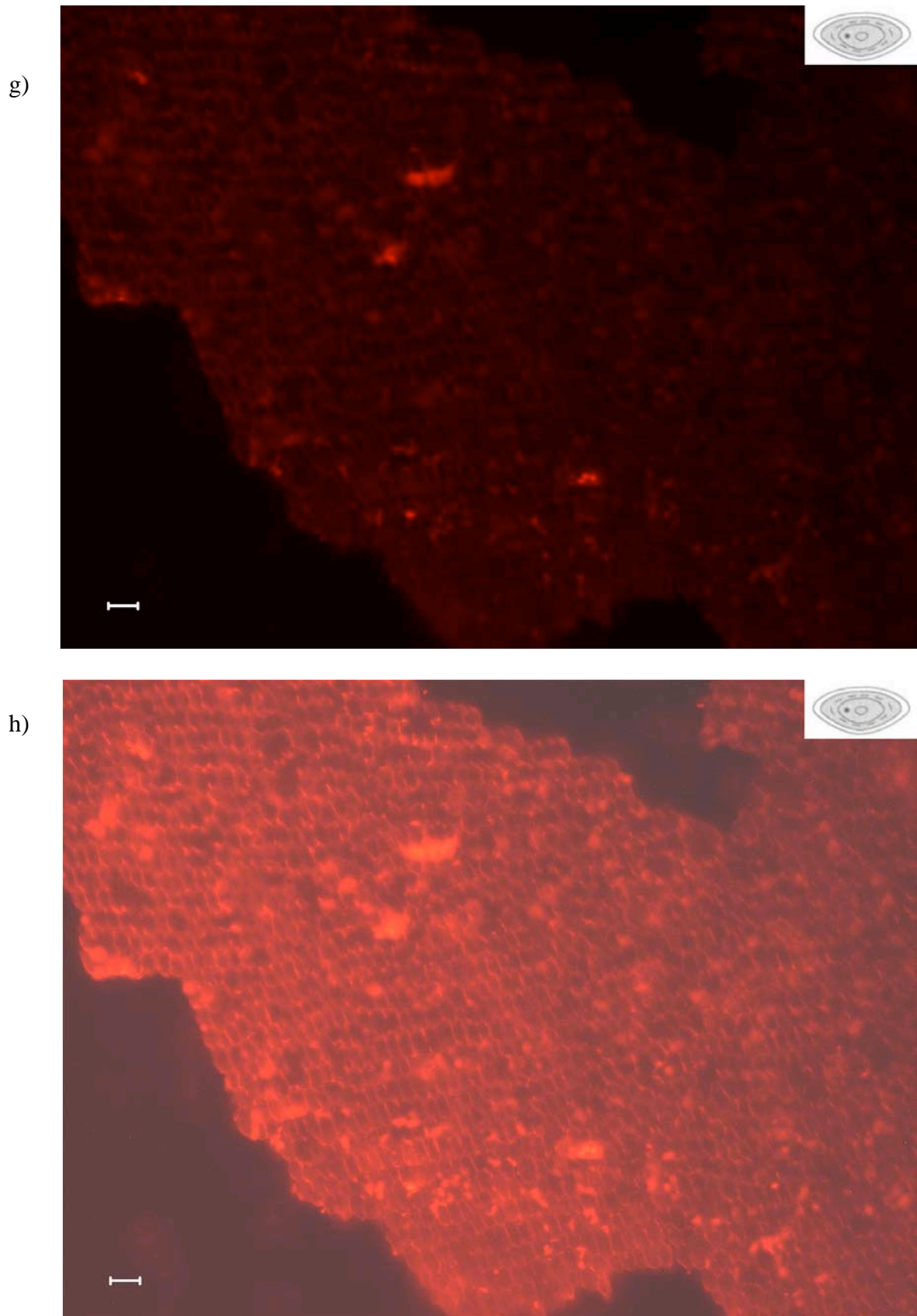


Figure 5.6.3 *Filensin Distribution in the Outer Cortex and Nucleus*

(e) TRITC-lectin membrane counterstain of the section presented in *fig. 5.6.3 (f)*, and then enhanced in (h). The pattern of TRITC-lectin staining confirmed the membrane distribution of filensin seen in *fig. 5.6.3 (f)*. *Inset* is as above. Scale bar is 10 μm .

5.7 Discussion

The major objective of this investigation was to specifically label cytoskeletal proteins of the ovine lens. A selection of proteins were chosen which were representative of those which were generally degraded early on in cataract development in other species (Lee *et al.*, 2001; Matsushima *et al.*, 1997), were known calpain substrates, and had a reasonable amount known about their distribution within the lens in other species (e.g. Alizadeh *et al.*, 2003; Beebe *et al.*, 2001; Ireland *et al.*, 2000; Lee *et al.*, 2000; Woo *et al.*, 2000). **Table 5.7.1** is a summary of significant findings from the immunohistochemical investigation.

Table 5.7.1 Summary of findings relating to immunohistochemical localisation of cytoskeletal proteins in the ovine lens

Target	Location in lens		
	Outer Cortex (<i>o.c.</i>)	Inner Cortex (<i>i.c.</i>)	Nucleus (<i>n.</i>)
Spectrin	Weak non-specific* membrane pattern.	No change between <i>i.c.</i> and <i>o.c.</i>	Membrane, possible accumulation at <i>f.c.</i> junctions.
Actin	Increased localisation at cell junctions, short sides of <i>f.c.</i> membrane, long sides reduced actin signal	Present at a reduced level to the <i>o.c.</i> , signal diminishes at depth in cortex.	Not detected.
Vimentin	Membrane and cytoplasmic localisation. Difficult to discern vimentin localisation due to background interference.	Diminished vimentin presence with depth, membrane localisation detected deeper in <i>i.c.</i> after loss of cytoplasmic signal.	Potentially detected in the <i>n.</i> , extremely faint signal; possible interference from TRITC hampered confirmation.
Filensin	Predominantly cytoplasmic, difficult to discern between cytoplasm and membrane.	Membrane only.	Potentially membranous.

* Non-specific with respect to any particular region of the fibre cell, and instead found at all points in these cells (*c.f.* increased actin at short sides).
f.c., fibre cell

Spectrin

Spectrin is a vital component of the cytoskeleton and is found throughout the lens, and is known to alter in its distribution in the fibre cell during lens growth and the differentiation of fibre cells (Zandy *et al.*, 2005; Beebe *et al.*, 2001; Woo *et al.*, 2000). Immunoblotting of lens USF extracts in this investigation revealed the presence of spectrin in the lens cortex, with the existence of full length (>250 kDa) spectrin detected, in addition to several breakdown products (see *fig. 5.3.1*). Immunohistochemical localisation of spectrin confirmed the immunoblot results, with positive staining for spectrin in the fibre cells of the inner and outer cortex. Spectrin was found to be associated with the plasma membrane of fibre cells and did not show any preference for either the long or short sides of the fibre cells in this region of the lens, as was the case for other proteins investigated (such as actin). The pattern of spectrin localisation in cortical fibre cells was in common with what has been found in other species such as chicken (Lee *et al.*, 2000; Beebe *et al.*, 2001). It may have been expected that spectrin would be distributed favouring the short sides of the fibre cells, as it is known to associate with actin in the cell (Straub *et al.*, 2003) and actin itself was identified in this part of the lens. Some sections displayed preferential localisation of spectrin along either edge of the fibre cell, but this may have been the result of variation in sectioning angle or thickness of the section itself (Lo *et al.*, 1997), since it was not seen in all samples.

Immunohistochemical staining also revealed the presence of spectrin in the lens nucleus. In this part of the lens spectrin was distributed on the membranes of fibre cells and followed the irregular membrane contour of these cells, again with positive staining for this protein at all points around the fibre cell(s) membrane. Immunoblotting of USF extracts from the lens nucleus revealed a much-reduced presence of spectrin compared to the cortex. Spectrin has been identified in lens nucleus extracts obtained from rat (Lee *et al.*, 2001; Matshshima *et al.*, 1997). Full-length spectrin, as well as fragments of the type that was identified in the cortex in this investigation (see fragments between 75 and 100 kDa in *fig. 5.3.1*), have been identified elsewhere in the lens nucleus. Notably, Lee *et al.* (2000) carried out a separation of the lens into sections similar to that completed in this investigation, however the separation of the lens was into cortex, outer nucleus and inner nucleus, whereas lenses were separated in this investigation into cortex and one nuclear fraction only. Thus the differences seen between the two studies may be due to the determination of the cortex/nucleus fraction between the two studies or that Lee *et al.* (2001) studied chicken and rat lenses. The sheep lens is much larger

and softer than both the chicken and rat, and as such makes complete separation of nucleus and cortex material difficult.

The immunohistochemical localisation of spectrin in the nuclear fibre cells of the lens has not been covered to a great extent in the literature, possibly due to difficulty associated with obtaining adequate tissue samples from this region of the lens for immunohistochemistry (Alizadeh *et al.*, 2003; Lee *et al.*, 2000). It is known that during the course of normal lens fibre cell differentiation spectrin is proteolytically processed by a range of proteases to fragments ranging in mass from 60 to 160 kDa, with a highly sensitive cleavage site producing a spectrin fragment of 150 kDa (Lee *et al.*, 2001; Lee *et al.*, 2000; Fukiage *et al.*, 1997). There is some histological evidence that suggests that spectrin remains localized to the membrane of nuclear fibre cells, and other techniques such as Western blotting corroborate this hypothesis (Lee *et al.*, 2001; Lee *et al.*, 2000; Matsushima *et al.*, 1997). It was found in this investigation that spectrin in the lens nucleus was associated with the plasma membrane of fibre cells, with of increased signal detected that appeared to be located preferentially at junctions of two or more cells, possibly indicating increased presence of spectrin in these regions. The finding of increased presence of spectrin in particular regions of lens fibre cells in the nucleus is not without precedent. Lee *et al.* (2000) described a similar pattern of distribution of spectrin in the lens nucleus, describing these observed regions on increased signal as “*ball-like foci and protrusions*” on the membranes of nuclear fibre cells, and it would appear that these are the same as what was seen in the present study. Tropomodulin, actin and tropomyosin were also distributed in this way in the nucleus of chicken lenses, and the explanation given for these foci/protrusions by Lee *et al.* (2000) was that they are the location of membrane interdigitations, as confirmed by scanning electron microscopy.

Actin

Actin is an important cytoskeletal protein in the lens, participating in and regulating events associated with fibre cell differentiation and possibly with a role in lens accommodation through stabilisation of epithelium under tension with myosin (Rao & Maddala, 2006; Kivela & Uusitalo, 1998). Histological sections obtained from lenses in this investigation conclusively presented an actin pattern that was localised preferentially to the short sides of the fibre cell in the lens cortex. Actin was detected in the lens nucleus in immunoblots of USF extracts from this region (*fig. 5.4.1*), although at very minor amounts compared to the cortex.

No actin breakdown products were observed by immunoblotting, and this was not unexpected, as actin is known to be highly resistant to proteolysis by proteases such as calpain (Goll *et al.*, 2003).

The pattern of actin localisation detected in the ovine lens was similar to that reported in other species including rat (Lo *et al.*, 1997), chicken (Lee *et al.*, 2000), and quail (Weber & Menko, 2006). It is thought this pattern of staining may be associated with a population of actin filaments additional to those of the membrane cytoskeleton (Lee *et al.*, 2000). Actin was observed at the long sides of the fibre cell, albeit at a much reduced intensity to that detected in fibre cell short sides. It has been suggested that increased intensity of protein detected at particular regions of lens fibre cells, as has been found for actin, is the result merely of increased membrane density at the short sides and apices of fibre cells (Lindsey-Rose *et al.*, 2006). This explanation does not account for the increased signal that was detected for actin only in this investigation. Other proteins studied in this investigation did not equally show any propensity for these presumed areas of increased membrane density even though they were found similarly throughout the rest of the membrane – i.e., to satisfy the explanation of increased membrane density at the corners, all proteins that have been detected in the fibre cell membrane would presumably have an increased presence in this part of the fibre cell also.

Selected sections stained for actin appeared to display a slightly altered pattern of actin localisation in the fibre cells. Most prominently, these fibre cells displayed intense “dots” of actin concentrated in the corners of the cells (see section *fig. 5.4.2, a*), instead of evenly distributed actin along the short sides that was the case in other lens sections observed. Lo *et al.* (1997) encountered similar such localisation of actin in rat lenses, and explained that each dot may be a composite of up to three actin bundles located at the intersection where the fibre cells meet.

Actin could not be identified in the nucleus of lenses in the current study by immunohistochemistry. Actin that was detected in the cortex nearer the lens nucleus appeared to be diminishing in intensity (see *fig. 5.4.2, d*). This possibly indicates that the presence of actin reduces in fibre cells as they enter the lens nucleus in ovine lenses, however others have had success in detecting actin in the lens nucleus in other species. Lee *et al.* (2000) detected actin in the nucleus of bovine lenses, and suggested that it was indeed the first time that actin had been identified in the lens nucleus in any species. It was found by Lee *et al.* (2000) that actin remained associated with the fibre cell membrane in the nucleus, following closely the irregular contours of these highly compacted cells rather than displaying enrichment at any

particular region (as is the case in the cortical fibre cells). Actin was detected using immunoblot in lens nucleus extracts in the current study, albeit at a much reduced level to that seen in the cortex. It may be that the actin detected was the result of contamination during separation of cortex and nucleus material, which is a distinct possibility given the difficulty of completely separating the two (see also Grey *et al.*, 2003). However given that actin has been observed in the nucleus of lenses in other species, there is no reason to think that the case would be any different in the sheep, and consequently the absence of actin by immunohistochemical localisation may have been the result of inadequate technique. As such, further work is required to determine if this is so.

Another possible reason that actin was not detected by immunohistochemistry in the lens nucleus was that the antibody employed in this investigation may not have been capable of detecting actin that was processed (i.e. proteolysed/truncated) during normal lens growth, as is known to be the case for other proteins in the lens such as filensin (Sandilands *et al.*, 1995, 1995a). The antibody used was raised against a peptide mapping at the carboxy terminus of actin (actin (C-11); Santa Cruz Biotechnology, California, USA). Should this carboxy-terminus peptide have been removed as the result of proteolytic processing during normal lens growth and differentiation, any actin in the lens nucleus would have been for all intents and purposes invisible to the antibodies used here to detect it. Lee and co-workers (2000) used monoclonal antibodies to actin (C-4) to detect actin in the lens nucleus, thus their finding of actin in the lens nucleus may be the result of the use of the different antibody. Incidentally, the “C-4” actin antibody apparently is named after the “*original wells in which they [the antibody] were detected*” (Lessard, 1988), which does not give any clue as to the specific difference between the C-4 and C-11 epitopes. Indeed, the localisation of actin as well as the other proteins of interest in this investigation would benefit from the use of a wide range of antibodies directed toward a variety of epitopes on the chosen target protein in order to further clarify their localisation in the lens, and would be of interest in future investigations.

Interestingly, actin has been found to show some association with gap junctions in the lens fibre cell of human and monkeys, a feature that may be exclusive to primates and could be related to the highly accommodating nature of primate lenses (Lo *et al.*, 1994). This feature was not investigated here, but may be of interest in future investigations of the ovine lens, particularly in establishing the usefulness of the ovine lens as a model for human cataract.

Vimentin

Vimentin is known to exist in cells in a network that extends out from the cell nucleus in a radial organisation (Clarke & Allan, 2002). Vimentin is found in the lens in epithelial cells and early differentiating fibre cells, but is absent from mature lens fibre cells, with its disappearance defining a specific stage of differentiation (Quinlan *et al.*, 1996). Using immunohistochemical localisation, vimentin was found in this investigation to be located in the lens cortex with both a membrane and cytoplasmic localisation, with first cytoplasmic vimentin disappearing from the deeper cortex followed by membranous vimentin. In the nucleus of lens little vimentin was present as observed by immunoblotting and immunohistochemistry. Several bands of less than 56 kDa, the approximate molecular mass of vimentin (Robertson *et al.*, 2005; Guest *et al.*, 2006), that were reactive to the vimentin antibody were detected in cortex extracts.

The immunohistochemical results obtained in the current experiments were in agreement with what has been found in other investigations in both lens extracts obtained from the lens nucleus (Fleschner, 2002; Matsushima *et al.*, 1997) as well as other immunohistochemical investigations (Bozanic *et al.*, 2006; Sandilands *et al.*, 1995), where vimentin has been found to diminish in its presence as epithelial cells begin to elongate during differentiation into fibre cells, resulting in decreased presence in the deep cortex and nucleus. Sandilands *et al.* (1995) reported peripheral and membrane localisation for vimentin as observed by immunohistochemistry, but no mention is made regarding cytoplasmic localisation of vimentin of the type that was observed in the outer cortex in this investigation. What is shown by Sandilands *et al.* (1995), as well as Blankenship *et al.* (2001), is that the pattern for vimentin labelling changes deeper in the lens, becoming associated with the membrane clearly, rather than between both the membrane and cytoplasm. The findings of Sandilands *et al.* (1995) and Blankenship *et al.* (2001) therefore provisionally confirm the results found in this experiment in this regard (see *figs. 5.5.2*).

The conclusion that vimentin was absent in the lens nucleus using immunohistochemistry was made with some obvious weaknesses in the evidence presented. Weak fluorescence was detected in the lens nucleus of control sections at the same wavelength as the fluorescent tag. The photos presented in *fig. 5.5.3* show an example of this weak signal detected that could only be readily observed following manipulation of the images with photo editing software, in

particular altering the brightness and contrast parameters. It is possible that the green signal observed in these sections was again due to defective filtration of the TRITC signal that was also included in these sections, the likes of which were observed in filensin sections. More robust images of control sections were required to determine the quality of vimentin signal detected in lens sections, but were not collected in abundance at the time of section viewing under the microscope as no signal could be detected and photography was considered neither useful, nor even possible. As such, comparatively few images were taken of controls compared to those of positive sections.

A systematic approach for image collection of lens sections stained for vimentin with TRITC counter-stain and control sections may be beneficial in discounting the effects of non-specific background fluorescence. A proposed approach would involve all sections regardless of their staining being photographed with the green filter in place and the camera set up to detect green fluorescence, followed by the red filter in place and the camera set for detecting green fluorescence, followed by the red filter and red detection, and finally green filter and red detection. The addition of a further set of controls for TRITC in future investigations may also be required, in which sections are stained alternatively with or without TRITC for each batch of the current used sections – i.e. vimentin positive+TRITC, vimentin positive-TRITC, minus primary/plus secondary+TRITC, minus primary/plus secondary-TRITC, and plus primary/minus secondary+TRITC, plus primary/minus secondary-TRITC. This approach would allow for the collection of conclusive evidence of inadequate filtration of a particular wavelength of light, as well as confirmation/rejection of the hypothesis that the fluorescent secondary antibody used in these sections was causing background fluorescence that could be detected in some sections. Some image collection was attempted following this routine and tentatively confirmed the detection of green fluorescence through the red filter and the camera set to detect light at the green wavelength (see *fig. 5.7.1*), again at a very low level but all the same casting some doubt on the results collected in this investigation for vimentin. TRITC-lectin might be abandoned as a counterstain in future investigations of this type.

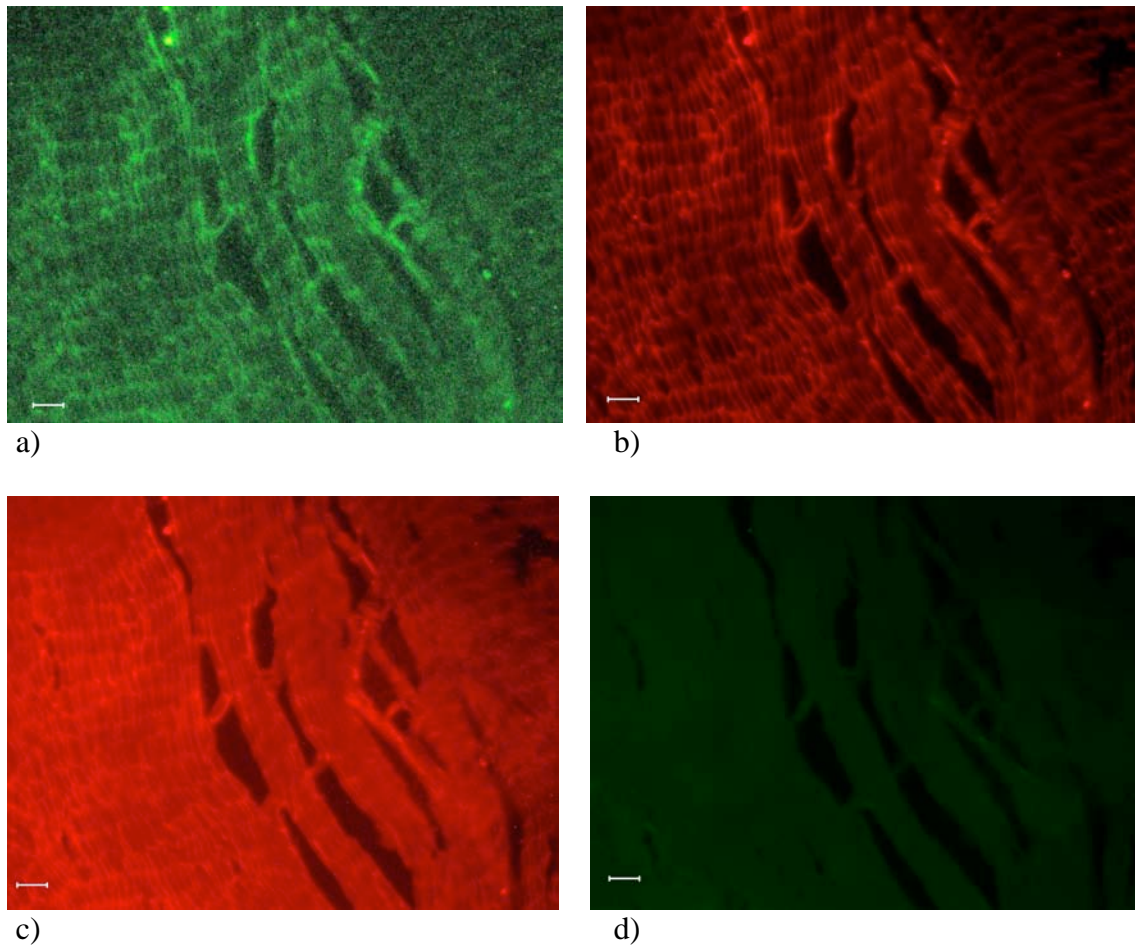


Figure 5.7.1 *TRITC/488 Signal*

A section of lens immunostained for vimentin and with TRITC-lectin photographed as follows: a) Red filter, camera set to detect green (b) red filter, red camera (c) green filter, red camera (d) green filter, green camera. These images confirm the detection of green signal when the red filter is in place (a), thus lending confusion to the interpretation of staining for that particular protein – (d) would otherwise be assumed to be depicting positive signal for vimentin (pending antibody controls) that may actually be the result of the TRITC-lectin counterstain.

Filensin

It was difficult to obtain a clear picture of filensin distribution in the ovine lens from immunohistochemical sections. The observed filensin localisation in the outer cortex in the plasma membrane as well as in the cytoplasm was in contrast to what has been described in other species (e.g. Lindsey-Rose *et al.*, 2006; Ireland *et al.*, 2000; Sandilands *et al.*, 1995; Merdes *et al.*, 1993). Filensin could not be conclusively identified by immunohistochemical localisation in the lens nucleus, although a membrane-associated signal was identified in selected sections that could not be verified as filensin due to background fluorescence in control sections.

The immunoblot results presented for filensin in *fig. 5.6.1* show the filensin antibody used in this investigation was highly reactive with several bands in lens extracts from all fractions of the lens, particularly the lens cortex and nucleus. In extracts of these parts of the lens, intense bands were observed for full length (115 kDa) and a 53 kDa filensin cleavage product fragment (Lindsey-Rose *et al.*, 2006; Masaki & Quinlan, 1997; Sandilands *et al.*, 1995a), in addition to 100 and 50 kDa band in nucleus extracts, as well as several bands between 37 and 50 kDa that have also been identified in research on lenses carried out elsewhere (Guest *et al.*, 2006; Sandilands *et al.*, 1995a). Filensin is known to change its presence in the fibre cells at different times in different regions in the lens (e.g. Sandilands *et al.*, 1995, 1995a), with full length filensin found at lower amounts toward the nucleus of the lens as fibre cells become more differentiated, and this was observed in immunoblots produced in this investigation where the filensin band was seen to be reduced in the nucleus compared to the cortex, while other bands, notably the one corresponding to the 53 kDa fragment, were seen to be increased in the nucleus, corresponding to the build up of filensin processing products in the nucleus.

Consequently, filensin immunoblots may offer some explanation for the poor immunohistochemical results obtained in this set of experiments. The immunoblots were characterised by an abundance of reactive bands, similar in some ways to the results of the immunohistochemical sections where the abundance of fluorescence detected made interpretation difficult. What the immunoblot results show is that filensin is found in the ovine lens in multiple forms, all of which are identified by the antibody used. The numerous filensin bands seen are due to extensive proteolysis and processing of this filensin that takes place during growth and differentiation of the lens (Lindsey-Rose *et al.*, 2006). Given that there was no way to discern between full length and processed fragments of filensin using immunohistochemistry without fragment-specific antibodies (as carried out for example by Sandilands *et al.*, 1995a), it may have been that the fluorescence witnessed in sections stained for filensin was the result of several of the bands identified by immunoblot reacting *in situ* with the antibody as proteolytic fragments that have been released into the cytoplasm from the membrane (Blankenship *et al.*, 2001). Indeed it has been shown elsewhere (Sandilands *et al.*, 1995a) that the various fragments are found in distinct compartments in the cell by immunohistochemical localisation. Another interesting facet of the extensive presence of filensin products is that the work carried out in the previous section (**Section 4**) found that filensin was somewhat resistant to calpain-induced proteolysis, and it might have been expected that there would not be an abundance of filensin products detected in the lens on the

basis of the results of incubation of filensin with calpain. Thus calpain may not be responsible for filensin proteolysis resulting in the breakdown products seen in the current work that are found in the lens during normal growth and differentiation. As mentioned in the discussion in Section 4, several other proteolytic systems have been identified in the lens that may be responsible.

The work of Lindsey-Rose *et al* (2006) investigated the interaction of filensin and aquaporin 0 in the bovine lens, and reported filensin localisation in both the fibre cell membrane and cytoplasm, but failed to mention specifically where (i.e. in the cortex, nucleus, or both) in lens the cytoplasmic filensin was detected. Images presented by Lindsey-Rose *et al* (2006) appear to show filensin with a membrane localisation only, and nothing is shown with regards to the presence of filensin in the cytoplasm of fibre cells. It is interesting to note that they found filensin and CP49 to be more apparent in the short sides of the fibre cells when co-localised with aquaporin 0, which they put down to the likelihood that membrane density was increased in these regions, rather than any specific requirement for either protein there. Sandilands *et al* (1995a) described a similar pattern of filensin staining in the short sides preferentially in mature lens fibre cells also, but if this was the case in the current investigation it was not distinguishable from the general membrane pattern witnessed. Lo *et al* (1997) have suggested that lens specific filensin and CP49 are related to the unique actin bundles seen that localise in the short sides and corners of the fibre cells also, but did not elaborate on this statement, and the only link that could be made was that they are both found only in the lens.

Straub *et al* (2003) detected CP49 (phakinin) in the cytoplasm of lens fibre cells in the cortex in bovine lenses. Their description of “*the notorious intense positivity for [intermediate filament] proteins such as vimentin and phakinin*” in this region suggests that this is a common finding. The CP49 distribution Straub *et al* (2003) found was comparable to the signal detected in sections stained for filensin presented in this investigation. As filensin and CP49 co-localize in the lens (Blankenship *et al.*, 2001), their findings would be in agreement with what was describe here for the ovine lens.

Rodent lens sections stained for filensin display an abundance of filamentous structures in the cytoplasm when observed with an electron microscope (Alizadeh *et al.*, 2003). Suffice to say, magnification at this magnitude was not attempted in this study and it is not known whether filensin visible at the magnification afforded by electron microscope would

necessarily transfer to something that was visible at the level of magnification used in this study.

Ireland *et al* (2000) detected filensin in chicken lenses that transitioned from a membrane to cytoplasmic distribution as fibre cells progressed toward the nucleus, which is also the case for bovine lenses as seen in the work completed by Sandilands *et al* (1995). Blankenship *et al* (2001) investigated the distribution of filensin in the developing lens, and gave a thorough account of the distribution of filensin from embryonic stages through to adulthood. In this account they describe that during the early postnatal stage filensin is not found to be principally membrane associated, as is the case in older lenses, and rather is located chiefly in the cytoplasm. Since the sheep lenses used for the filensin localisation in this investigation were not considered to be early postnatal, it seems unlikely that this would explain the potential filensin pattern observed in this investigation.

A common theme has emerged from reviewing the literature regarding the distribution of cytoskeletal proteins covered in this study. All have been found to be localised at one time or another in the fibre cells of the lens with an increased presence at the plasma membrane along the short sides of these cells preferentially. For example vimentin has been observed by Blankenship *et al.* (2001) in pre- and early postnatal mice lenses “*in cross sections [of lens] ... as though concentrated at the corners of this roughly hexagonal cell*”. The same localisation and distribution in fibre cells has been reported for filensin in bovine lenses (Lindsey-Rose *et al.*, 2006; Sandilands *et al.*, 1995a), and actin in rat, chicken, mouse, and quail (Lo *et al.*, 1997; Lee *et al.*, 2000; Blankenship *et al.*, 2001; Weber & Menko, 2006, respectively). The exception was spectrin for which no studies could be found in the literature that suggested anything other than an even distribution of spectrin around the fibre cell membrane (Lee *et al.*, 2000; Beebe *et al.*, 2001) and which was observed in this study. Conflicting reports showing localisation not favouring the short sides for filensin (Ireland *et al.*, 2000; Merdes *et al.*, 1993) show that there is variation in the localisation of these proteins in the lens between studies. It may be that preferential localisation of cytoskeletal proteins in the corners/short sides of fibre cells is an artefact related to sectioning or staining lenses this way. Species differences would not appear to explain the different findings of cytoskeletal distribution and localisation, as Merdes *et al.* (1993) observed an even distribution of filensin about the lens fibre cell in bovine species, the same animal used by Lindsey-Rose *et al.* (2006) when they observed filensin localisation preferentially at the short sides of the fibre cell.

Immunological detection of proteins in this study was carried out using an indirect method, whereby two antibodies were used to localise an antigen; a specific antibody directed toward the antigen of interest is detected by means of a secondary antibody that has been conjugated with either a fluorescent dye or an enzyme tag that is visible under a microscope under a particular wavelength of light in the case of fluorescent dyes, or following treatment with the enzyme substrates (Mao *et al.*, 1999). Enzymatic tags used in immunohistochemistry are typically alkaline phosphatase or peroxidase, while fluorescent tags are often fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) based (Cregger *et al.*, 2006; Mullins, 1999). The relative advantages and disadvantages of the two types of tagging are controversial, as some authors suggest enzymatic methods are difficult and can be hampered by high background, low resolution and/or lower sensitivity, and as staining depends on an enzyme reaction, endogenous enzymatic activity also may be a problem (Reed *et al.*, 2001; Mao *et al.*, 1999). Others argue that background fluorescence or autofluorescence can make analysis of fluorescent labelled specimens difficult (Niki *et al.*, 2004). Various methods are available to block non-target enzymatic activity or fluorescence deal. Direct staining of antigens is possible, and indeed is faster owing to its use of a single antibody, however direct staining results in lower signal intensity and is more expensive as each primary antibody must be labelled rather than being detected with a secondary (Javois, 1999). As there appear to have been problems associated with background fluorescence in this investigation, future studies of this type would probably benefit using enzymatic tags either instead of or supplementarily to fluorescent tags, in order to clarify labelling of antibody targets in the lens. Additionally, problems associated with sectioning plane that may have prohibited localisation of target proteins in this investigation, as well as with thick sections of the type used in this investigation, could be mitigated with the use of confocal microscopy (Reed *et al.*, 2001). One other point to make about the antibodies used in this examination of the ovine lens was that none of them were sheep specific, and instead were purchased from various antibody manufacturers. All were confirmed to work in the case of the ovine lens by Western blotting, however the development of sheep specific antibodies might be beneficial in optimising this procedure to achieve better results than were obtained.

To conclude this section, actin and spectrin have been conclusively localised in the ovine lens using immunohistochemical localisation. These results were provisionally confirmed via immunoblotting of separated lens extracts from the capsule/epithelial cells, cortex and

nucleus. Actin was found to display preferential distribution in the short sides of the fibre cells in the cortex of the lens, while spectrin was found in the cortex associated with the fibre cell membrane in common with what has been described in the literature in other species. In the lens nucleus, spectrin was not found to change its association with the fibre cell membrane, but actin could not be detected in this region of the lens by immunohistochemistry.

The distribution of filensin and vimentin in the lens was more difficult to ascertain with certainty. Filensin and several of its proteolytic products were identified in immunoblots, and immunohistochemical localisation revealed a general pattern of membrane association and cytoplasmic localisation in the outer cortex, however the pattern of localisation was indistinct due to the abundance of filensin products in the lens. Finally vimentin appeared to display both membrane and cytoplasmic association in the outer cortex, gradually diminishing with depth in the lens with membrane associated vimentin found only weakly in the deeper regions of the cortex and nucleus.

These results show proteins from the lens cytoskeleton as they change during growth and differentiation from the outer regions of the lens to the nucleus region, and provide the basis for future studies on the lens and cataract. Of particular interest for future research is the role of calpain in normal lens development and fibre differentiation, as well as the role of cytoskeletal proteolysis in the process of cataractogenesis. Immunohistochemical characterisation and localisation of calpain, similar to that completed by Ma *et al.* (2001), and cytoskeletal proteins in normal and cataract lenses may offer some insight into these processes in the ovine lens. While actin may not be useful in cataract studies as it is relatively resistant to proteolysis, as was in Section 4 of this thesis, filensin may present a useful target should proteolysis-product specific antibodies be employed (e.g. Sandilands *et al.*, 1995a) as it undergoes extensive proteolysis during normal differentiation. Vimentin potentially would be an interesting target for this type of study as it has been shown to be susceptible to proteolysis and is known to undergo changes in cataract models (e.g. Sanderson *et al.*, 2000; Robertson *et al.*, 2005). The immunohistochemical localisation of vimentin as it stands in the current investigation would require optimisation however as it was not entirely conclusive. The ideal cytoskeletal targets to follow as it emerges from this investigation would be spectrin, as it has been shown to undergo proteolysis by calpain during cataract development elsewhere (e.g. Robertson *et al.*, 2005), and was successfully localised in the current study.

SECTION 6 – CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Overall Conclusion

The cytoskeleton comprises a small proportion of the total protein in the lens, but is thought to be important in the development and maintenance of transparency. Calpain plays a role in cataract in several species (Biswas *et al.*, 2005; Robertson *et al.*, 2005; Inomata *et al.*, 2002), however calpain-like cleavage sites have not been detected in human lens crystallins *in vivo* (Nakajima *et al.*, 2006; Ma *et al.*, 2005). If calpain is important in human cataract, proteolysis of proteins other than crystallin must be occurring. The cytoskeletal proteins are known calpain targets in the lens, and proteolysis of cytoskeletal proteins may play an important role in the development of cataract. The ovine lens has been used in a model for cataract development, and there is little to be found in the literature regarding cytoskeletal proteins in lenses from this species.

***Hypothesis i:** Inhibition of calpain with novel inhibitors will result in the absence of proteolytic products associated with calpain.*

The research presented in Section 3 shows clearly that the cytoskeletal profile changes when lens extracts were incubated in the presence of calpain and calcium compared to extracts incubated in the presence of calpain and calcium plus inhibitor. Novel calpain inhibitors were successful in preventing the appearance of calpain proteolysis products for spectrin, vimentin and filensin, with the novel inhibitor *Cat0059* the most potent of the novel calpain inhibitors investigated, inhibiting calpain-induced proteolysis at 100 nM.

***Hypothesis ii:** Lens cytoskeletal proteins in the ovine lens will display a characteristic distribution for that selected protein, and will have a similar distribution to that seen in other species.*

Research presented has shown that for a selection of cytoskeletal proteins in the ovine lens there exists a characteristic pattern of expression that closely resembles that seen in other species (Section 5). Specifically, actin was found to display preferential distribution in the short sides of the fibre cells in the cortex of the lens but was absent in the nucleus, while

spectrin was found in the cortex and nucleus associated with the fibre cell membrane. Filensin was observed in the outer cortex of lens sections associated with the fibre cell membrane and cytoplasm, however the pattern of localisation was indistinct due to the abundance of filensin products in the lens. Vimentin displayed both a membrane and cytoplasmic association in the outer cortex that gradually diminished toward the lens nucleus, with membrane vimentin found only in the deeper regions of the cortex and nucleus.

Taken together, the evidence presented in this thesis shows the cytoskeletal proteins as crucial elements of the lens by way of their pervasive presence (as seen in lens sections presented in Section 5). This, coupled with evidence that lens cytoskeletal proteins are sensitive to calpain-induced proteolysis and novel calpain inhibitors inhibit this proteolysis (Section 4), suggests that these proteins may be useful targets in cataract prevention. With research suggesting calpain-induced proteolysis is crucial in the pathology of cataract (Cuerrier *et al.*, 2006; Robertson *et al.*, 2005; Shearer *et al.*, 1999), the cytoskeletal proteins represent susceptible targets for that proteolysis in the lens, and inhibition of proteolysis of lens cytoskeletal proteins as has been achieved in the current study supports the approach of inhibiting calpain activity to prevent cataract development.

6.2 Future Directions

Building upon the work in this thesis, the obvious way forward would be to carry out similar work focusing on the development and characteristics of the ovine cataract, as was originally proposed. Of particular interest would be a comparison of the USF protein profile of normal and cataract lenses using the methods applied in Section 3 in this investigation. This would allow for the approximate determination of the degree of proteolysis of lens cytoskeletal proteins in the cataract lens, and this coupled with the application of cataract inhibitors directly to cataract sheep eyes, comparing the protein profile of lens USF extracts between these eyes and cataract eyes would give an indication of the applicability of these inhibitors as a treatment for cataract.

Also of interest for future investigations would be following the development of the lens during growth and cataract by immunohistochemistry. In particular, the role of calpain in normal lens development and fibre differentiation, as well as the role of cytoskeletal proteolysis in the process of cataractogenesis. The appearance of cataract damage in early

cataract lenses (see *fig. 3.6.1*) at the approximate boundary in the lens between the differentiating outer-fibre cells and the more mature, differentiated fibre cells might indicate the involvement of aberrant activity of protease(s) associated with normal lens fibre cell differentiation in the early stages of cataract. Immunohistochemical characterisation and localisation of calpain, similar to that completed by Ma *et al.* (2001), and cytoskeletal proteins in normal and cataract lenses may offer some insight into these processes in the ovine lens.

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PUBLICATIONS ARISING FROM THIS THESIS

McDermott JD, Morton JD (2006). Changes in lens structure during development of an inherited ovine cataract. *Australia and New Zealand Society for Biochemistry and Molecular Biology (ComBio 2006)*. Brisbane, Australia, September 2006.

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