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 immunohistochemisty, 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., 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.

[1]Blankenship *et al.* (2001), [2]Girao *et al.* (2005); [3]Alizadeh *et al.* (2003); [4]Reed *et al.* (2001); [5]Ma *et al.* (2001); [6]Jacobs *et al.* (2004); [7]Grey *et al.* (2003); [8]Jacobs *et al.* (2003).
* Precise duration to be determined experimentally.
Cryo-, cryosetioning; 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 ^{o}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 ^{o}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 ^{o}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

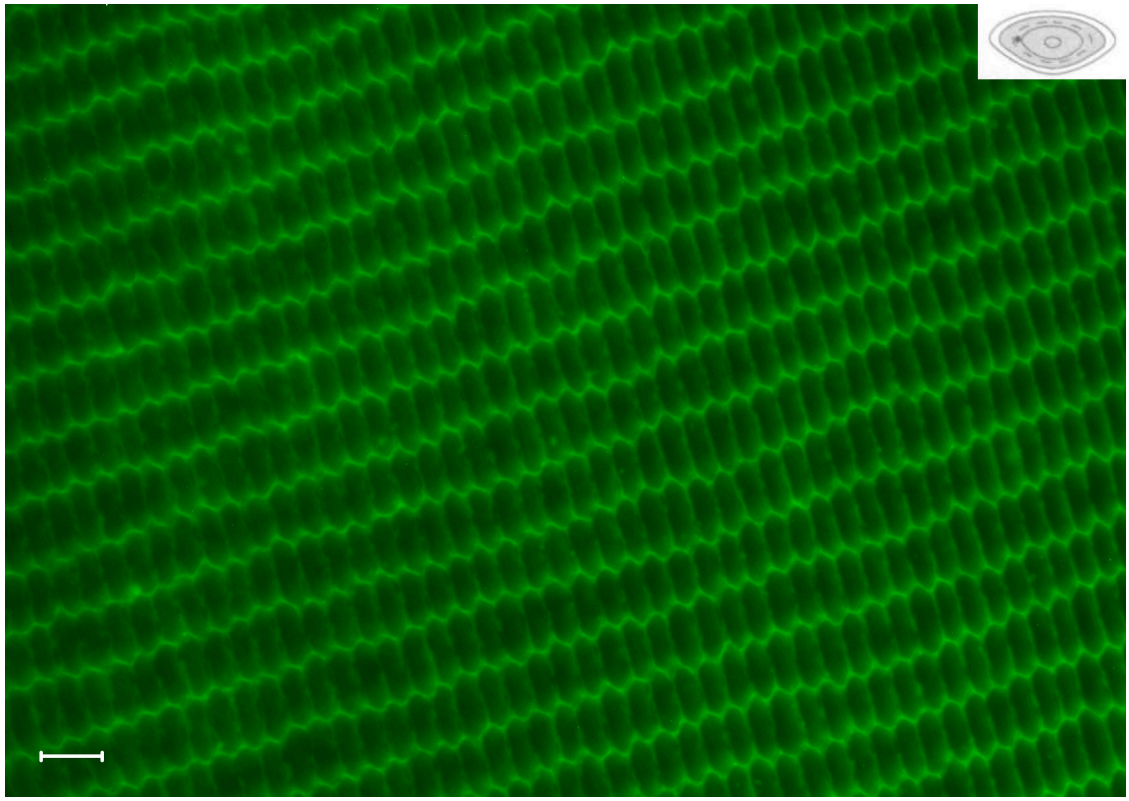
Fixation was initially carried out using lenses either whole or cut in halves or quarters along the equatorial or longitudinal axis, so as to allow better fixative penetration. It was found that there was little advantage gained from fixing lenses that had been halved or quartered. The cutting itself 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.

a)



b)

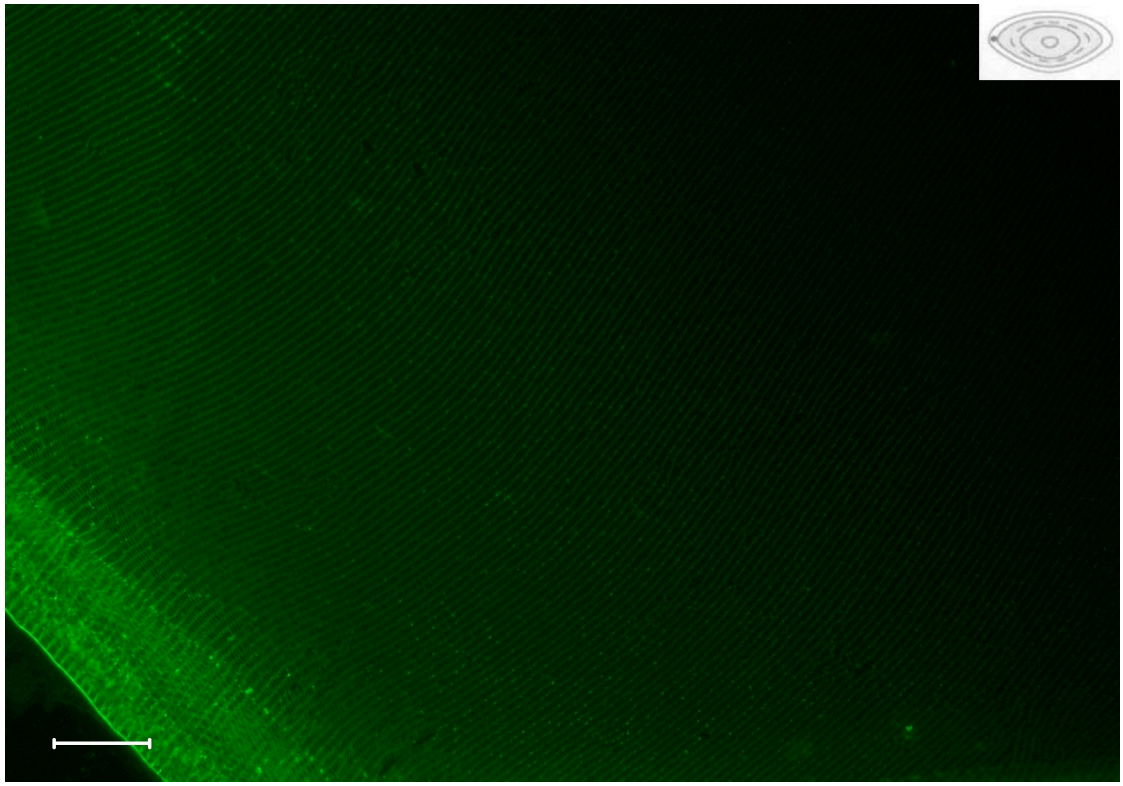


Figure 3.3.1 *Paraffin Embedded Lenses*
Cortical lens fibre cells in lenses obtained from sheep less than 12 months old that underwent paraffin processing were seen to remain intact across large portions of the lens tissue (a, 600x, b, 100x). Inconsistent FITC-lectin staining of fibre cell membranes 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

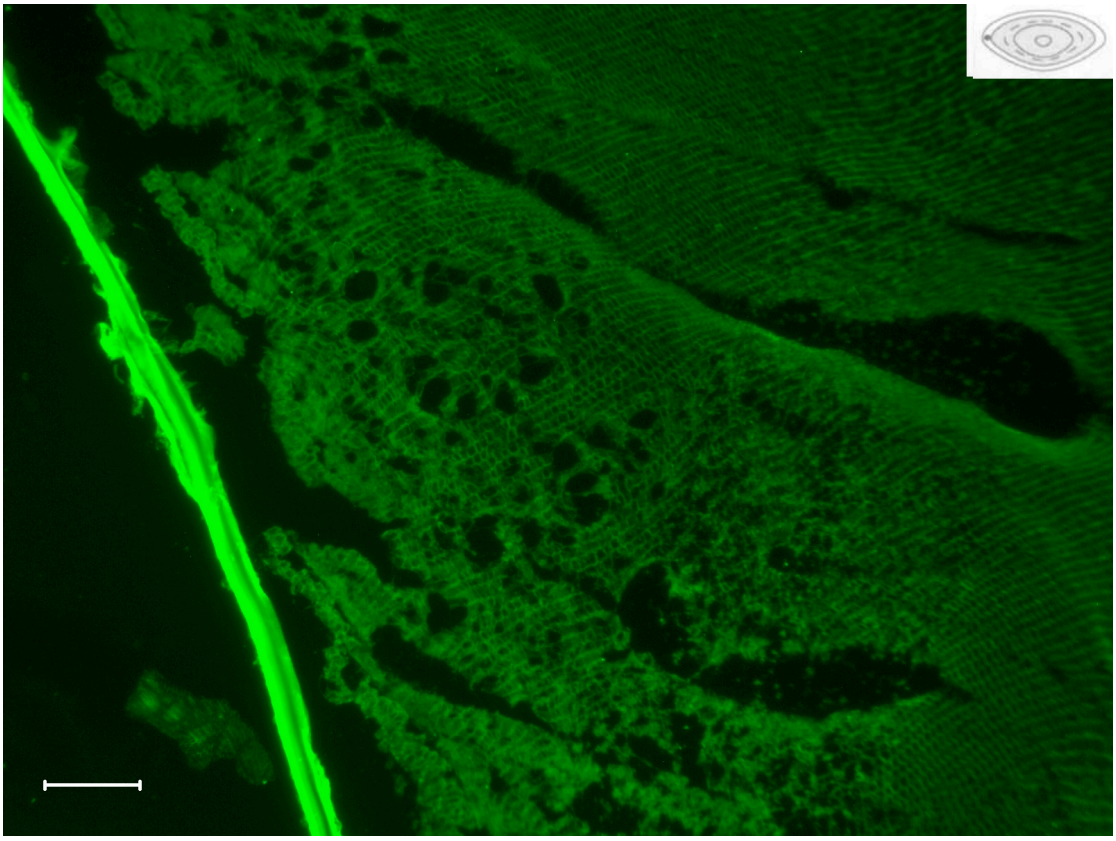
Following an adapted version of the method devised by Reed *et al.* (2001), dissected intact lenses were immediately mounted in *Gurr*® O.C.T. compound (BDH, Poole, UK) at 4^{o}C on pre-chilled cryosectioning chucks. Lenses were then frozen in embedding media in liquid nitrogen. Lenses were stored at -80 ^{o}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 ^{o}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 ^{o}C prior to fixing in pre-chilled (-20 ^{o}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.

a)



b)

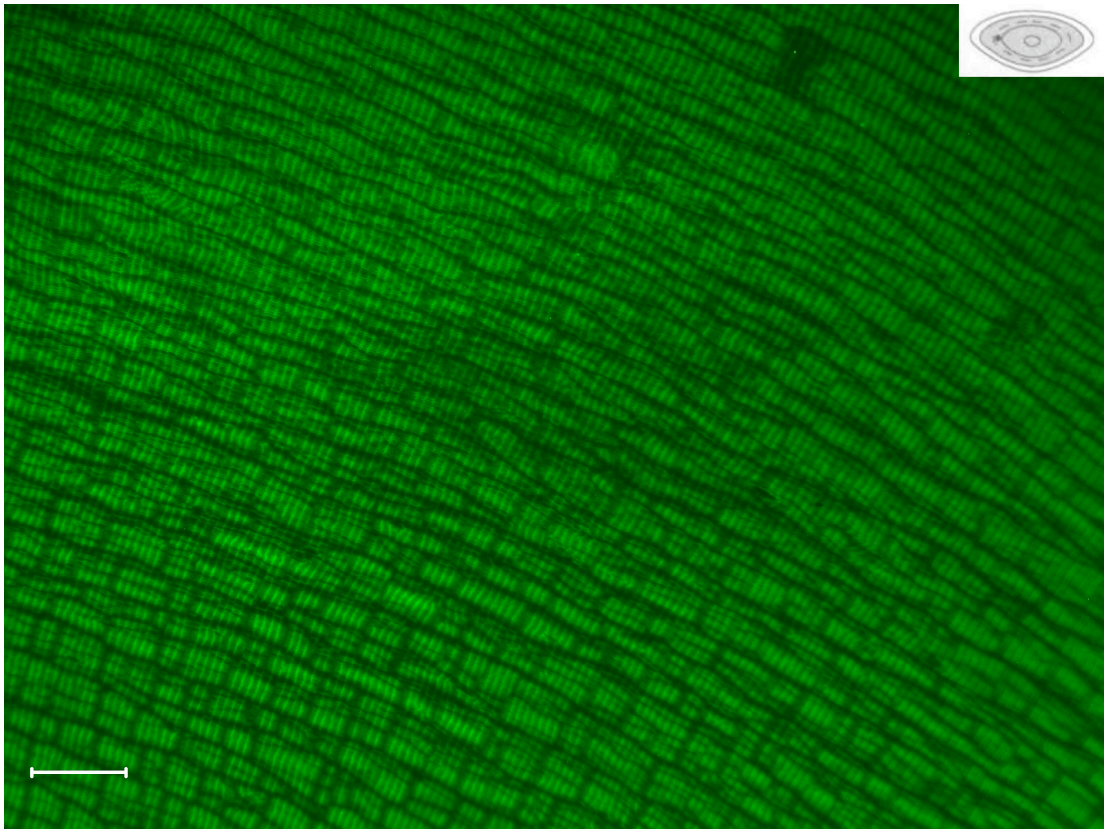
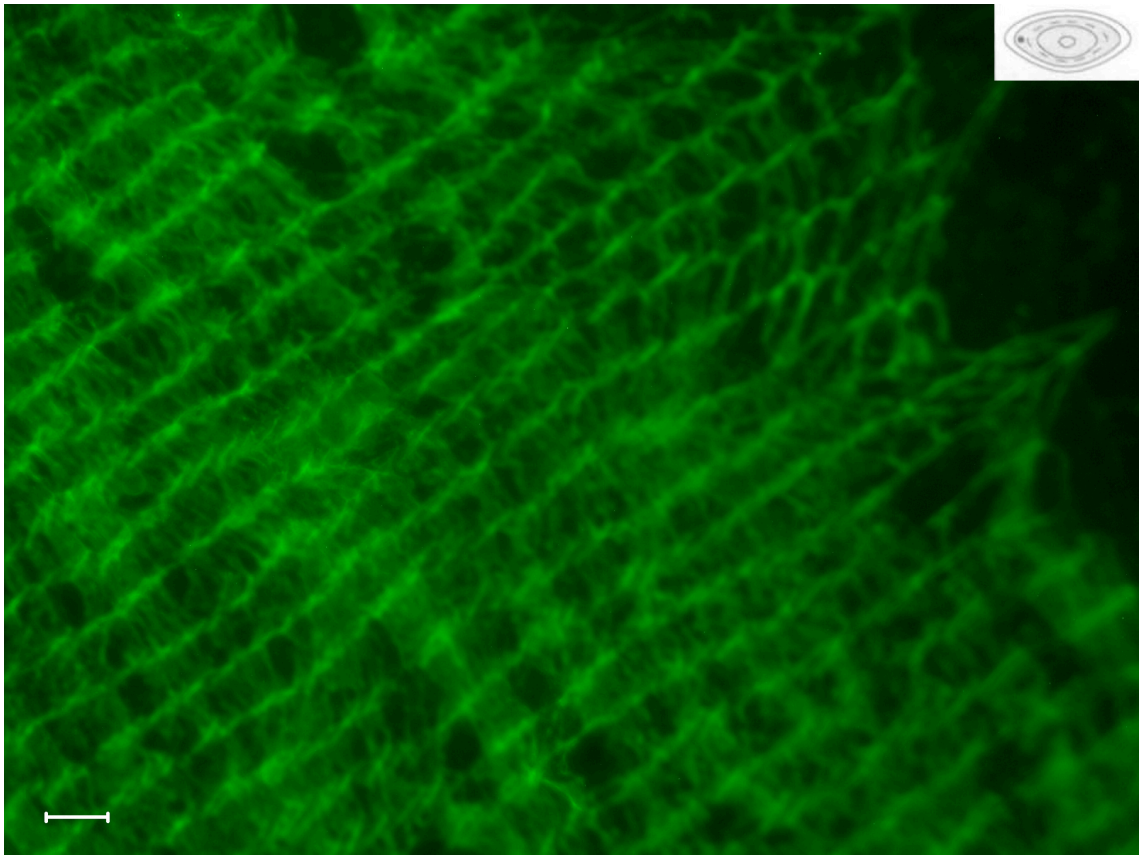


Figure 3.4.1 *Fresh Frozen Lenses*
a) Separation of capsule and cortical lens tissue in fresh frozen lens sections from sheep <12 months of age; b) Chattering pattern observed occasionally infresh frozen lenses. *Inset* schematic illustrating approximate location within the lens where the picture was taken (after Taylor *et al*., 1997). Both pictures are at 100x magnification and Scale bars are 100 μm.

c)



d)

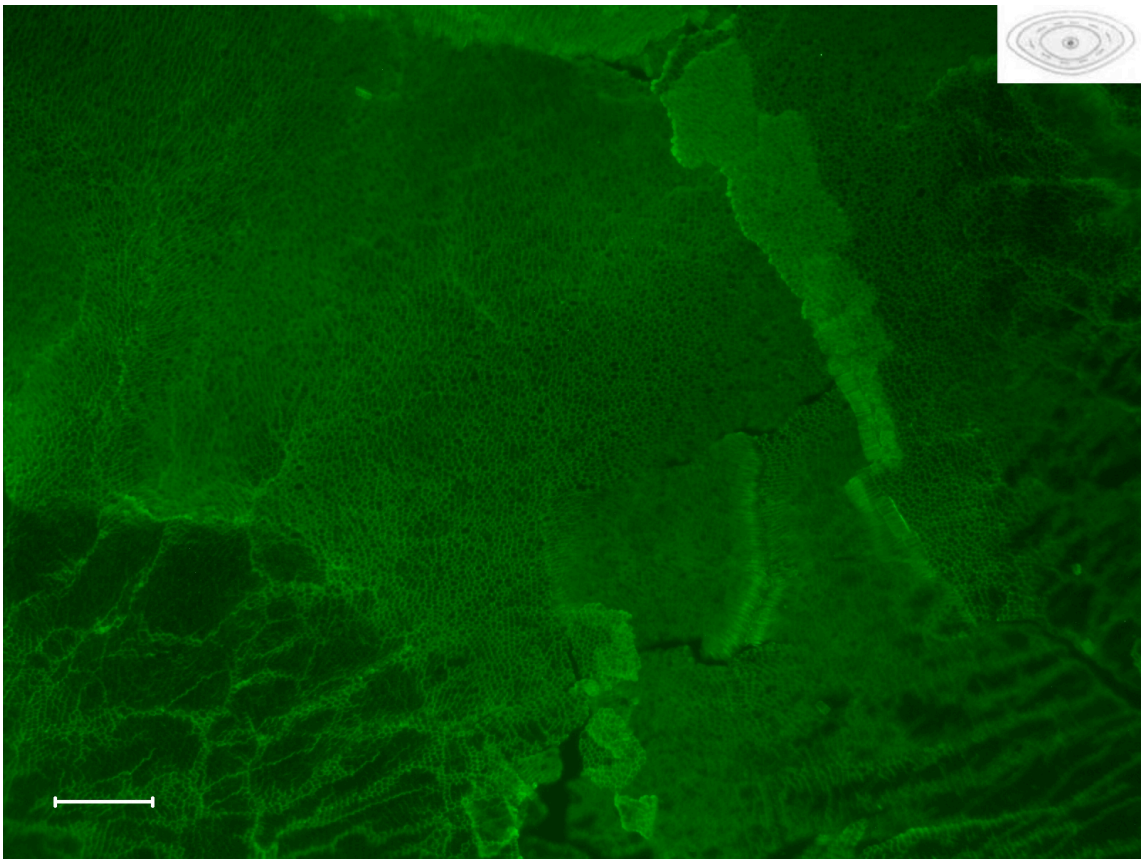


Figure 3.4.1 *Fresh Frozen Lenses*
Low quality morphological preservation of cortex fibre cells seen at high 400x magnification in normal lenses; d) largely intact core section illustrative of level of preservation of this part of the lens when processed using the fresh frozen method compared with alternative methods (100x magnication). *Inset* as above. Scale bars: c, 10 μm; d, 100 μm.