

Simple fixation and storage protocol for preserving the internal structure of intact human donor lenses and extracted human nuclear cataract specimens

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Purpose: Increased use of phacoemulsification procedures for cataract surgeries has resulted in a dramatic decrease in the availability of cataractous nuclear specimens for basic research into the mechanism of human cataract formation. To overcome such difficulties, a fixation protocol was developed to provide good initial fixation of human donor lenses and extracted nuclei, when available, and is suitable for storing or shipping cataracts to laboratories where structural studies could be completed.

Methods: Cataractous lens nuclei (n=19, ages 12 to 74 years) were obtained from operating suites after extracapsular extraction. Transparent human donor lenses (n=27, ages 22 to 92 years) were obtained from the Ramayamma International Eye Bank. After the dimensions were measured with a digital caliper, samples were preserved in 10% formalin (neutral buffered) for 24 h and followed by fixation in 4% paraformaldehyde (pH 7.2) for 48 h. Samples were stored cold (4 °C) in buffer until shipped. Samples were photographed and measured before further processing for transmission electron microscopy.

Results: The dimensions of the samples varied slightly after short fixation followed by 1 to 5 months' storage before transmission electron microscopy processing. The mean change in the axial thickness of the donor lenses was 0.15±0.21 mm or 3.0±5.4%, while that of the extracted nuclei was 0.05±0.24 mm or 1.8±7.6%. Because the initial concern was whether the nuclear core was preserved, thin sections were examined from the embryonic and fetal nuclear regions. All cellular structures were preserved, including the cytoplasm, complex edge processes, membranes, and junctions. The preservation quality was excellent and nearly equivalent to preservation of fresh lenses even for the lens cortex. Cell damage characteristic of specific nuclear cataract types was easily recognized.

Conclusions: The novel fixation protocol appears effective in preserving whole donor lenses and cataractous nuclei over a wide age range. Dimensions varied only 2%–3%, and fiber cell damage correlated well with standard fixation. These methods enable researchers and clinicians in remote settings to preserve donor lenses and rare examples of extracapsular extractions for detailed examination at later times.

The human ocular lens is a continuously growing tissue. Newly produced fiber cells form at the equator, elongate toward the poles, and accumulate as layers over the older fiber cells in the center that continue to change with age [1,2]. This biphasic growth pattern of the lens generates two distinct regions, the nucleus and the cortex, with substantially different properties [3,4]. The outer cortex grows throughout life, whereas the inner nucleus becomes gradually more compacted and harder [5,6]. This is important for understanding and correcting age-related defects, such as presbyopia and the many types of cataracts associated with the nucleus [7,8].

Whole human lenses or intact lens nuclei are normally difficult to preserve [9,10]. Some fixation procedures have

been reported to cause significant swelling or shrinking that could alter internal structures [11]. Properly preserved lens material is essential for studies into the mechanism of aging and nuclear cataract formation, and sources are becoming rare due to the popularity of the phacoemulsification surgical technique for extracting lenses. However, in some instances cataractous nuclei are readily available, such as during corneal transplantation or removal of extremely hard nuclei.

The Vibratome sectioning technique, which the authors have used extensively for fresh lens specimens [10,12,13], works well but is technically challenging and cannot be performed at many locations. Minor modifications of standard fixation protocols have been developed to give initial fixation that will allow long-term storage and shipment followed by standard processing for transmission electron microscopy (TEM) ultrastructural analysis. Here the initial analysis of the changes in the specimen dimensions and preservation of internal cellular structure are reported.

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METHODS

Specimens: Cataractous lens nuclei (n=19, ages 12 to 74 years) were obtained, within 2 h of extracapsular cataract extraction or small incision cataract surgery, from the operating suites at L V Prasad Eye Institute (LVPEI), Hyderabad, India. Transparent human donor lenses (n=27, ages 22 to 92 years) were obtained, within 24 h of death, from the Ramayamma International Eye Bank, LVPEI. The dimensions (diameter and thickness) of the specimens were measured using a digital Vernier caliper (INOX IP54 Caliper, Micro Precision Calibration, Grass Valley, CA). All samples were obtained following procedures approved by the Institutional Review Board for protecting human subjects, and the study adhered to the tenets of the Declaration of Helsinki.

Sample fixation: The specimens were fixed initially with the following protocol at room temperature using small glass vials with gentle agitation: 10% formalin (neutral phosphate buffered solution containing 1%–2% methanol, cat. no. 15,740, EMS, Hatfield, PA) 5 to 10 ml per sample for 24 h and 4% paraformaldehyde (granular, cat. no. 19,208, EMS) in 0.2 M cacodylate buffer, pH 7.2 (cat. no. 11,653, EMS) for 48 h. (Cacodylate is a sodium dimethyl arsiniate buffer that must be handled carefully with personal protection and be disposed of as a hazardous waste.) Fresh paraformaldehyde fixative was prepared for each 48 h period. After fixation, the specimens were transferred to 0.2 M phosphate buffer and stored in 1 dram vials at 4 °C until shipped to the University of North Carolina (UNC) 1 to 5 months later.

Transmission electron microscopy: At the UNC laboratories, the specimens were measured with digital calipers, photographed on a grid, and processed for thin section TEM as described previously [12-15]. This procedure has been used so extensively that it has become the standard for Vibratome sections of fresh lenses and serves as a control for comparison to lenses fixed initially using the new protocol and stored for an extended time. Briefly, 200 μ m Vibratome sections were immersion fixed for 12 h in 2.5% glutaraldehyde, 2% paraformaldehyde, and 1% tannic acid in 0.1 M cacodylate buffer (pH 7.2). Fixed sections were treated with cold 0.5% osmium tetroxide for 60 min, stained in 2% uranyl acetate (in 50% ethanol) in the dark for 30 min, and dehydrated through a graded ethanol series. Sections were infiltrated and embedded in an epoxy resin (Epon 812, EMS). Thin sections were cut to 70 nm and imaged digitally with a FEI Tecnai 12 TEM at 80 kV. Some sections were grid stained with uranyl acetate and lead citrate.

RESULTS

Specimens and measurements: The representative photographed images of the donor lenses and extracted nuclear specimens (four of each) are shown in Figure 1A–D (donor lenses) and Figure 1E–H (extracted nuclei). The photographs were taken over a 0.5 mm grid faintly visible in Figures 1A,G. Figure 1E shows the specimen of an idiopathic developmental cataract that showed extensive white scattering rendering the nucleus opaque. An amber-colored nucleus was seen in Figure 1F that barely allowed any light transmission. The sample in Figure 1G displayed dark yellow pigmentation and sufficient opacity to be classified as an advanced or brunescient age-related nuclear cataract [10,15]. The opaque nucleus shown in Figure 1H was so hard that attempted phacoemulsification would potentially produce large damaging projectiles. When the nuclei had irregular shapes, such as the oblong shape in Figure 1G, the short and long axes were averaged. The nuclei excluded mainly the softer cortex that typically was about 750 μ m thick in adult donor lenses [4]. The average diameter of all adult donor lenses was 9.4 mm and that of the extracted adult nuclei was 7.1 mm, indicating the large size of the numerous nuclei available for ultrastructural analysis using the new fixation protocol.

Box-whisker plots (Figure 2) show the percentage changes in the dimensions (thickness and diameter) of the specimens following fixation and storage. The thickness values (Figure 2A) had a median change of –2.6% (inter-quartile range, IQR, –5.3% to –0.4%) for the donor lenses and a median change of –0.6% (IQR, –4.2% to 5.9%) for the extracted nuclei. The median values for the diameter (Figure 2B) were 0.9% (IQR, –1.3% to 1.9%) and –0.3% (IQR, –2.9% to 1.7%) for the donor lenses and extracted nuclei, respectively.

Preservation of nuclear fiber cells: Low-magnification views of fetal and embryonic nuclear fiber cells are shown in Figure 3. The large size of the cells, the irregular borders, the thin electron lucent bands between cells, and the smooth cytoplasm are notable features. There was no obvious structural damage in these well-preserved fiber cells from the nuclear core, consistent with previous comparison of transparent donor and age-related nuclear cataracts prepared from Vibratome sections of fresh lenses without prefixation [15,16]. The fetal nuclear fiber cells from a donor lens shown in Figure 3A were irregularly shaped and separated by complex interdigitations at cell interfaces. Similar complex interfaces are shown in Figure 3B, which includes three adjacent fiber cells from the embryonic nucleus of an extracted cataract specimen. A gap junction is located between cells 2 and 3 but requires higher magnification to be visualized.

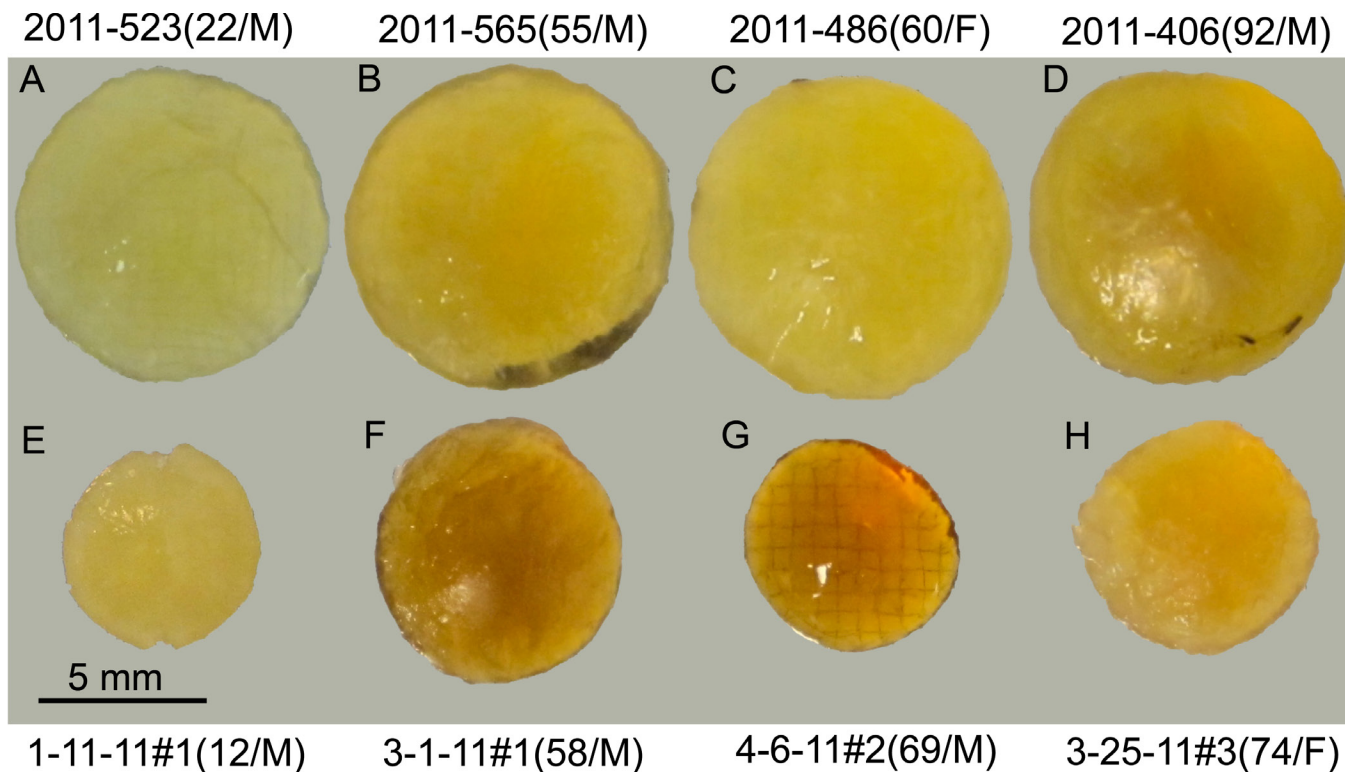


Figure 1. Examples of donor lenses and cataractous nuclei examined in detail by TEM. **A-D**: Donor lenses after fixation and storage. **E-H**: Extracted nuclei after fixation and storage. Donor lens identification and dimensions are **A**: 2011-523: 22/M (D=9.16 mm, T=3.41 mm); **B**: 2011-565: 55/M (D=9.60 mm, T=4.33 mm); **C**: 2011-486: 60/F (D=9.68 mm, T=3.94 mm); **D**: 2011-406: 92/M (D=9.28 mm, T=4.44 mm). Extracted cataractous nuclei identification and dimensions are **E**: 1-11-11#1:12/M (D=5.99 mm, T=2.27 mm); **F**: 3-1-11#1: 58/M (D=7.59 mm, T=3.42 mm); **G**: 4-6-11#2: 69/M (D=6.30 mm, T=2.44 mm); **H**: 3-25-11#3: 74/F (D=6.70 mm, T=3.22 mm). [Key: Sample ID: age in years/gender (D=final diameter, T=final thickness)].

Figure 4 shows high-magnification images of fiber cell interfaces from fetal and embryonic regions of nuclear cataracts. Figure 4A shows a well-preserved long gap junction, about 16 nm wide (as shown in Figure 3B) in an age-related nuclear cataract sample as is typically seen at the proper rotation of the specimen in the microscope stage to give an edge-on view. A short gap junction and undulating

membranes with extracellular space deposits (arrowheads) in a developmental cataract specimen are shown in Figure 4B. Extracellular space deposits stain similarly to proteins and are located on curved membranes in Figure 4C (arrowheads), which is an intercellular projection covered by paired membranes (arrows). These membrane features are consistent with those reported previously for Vibratome sections

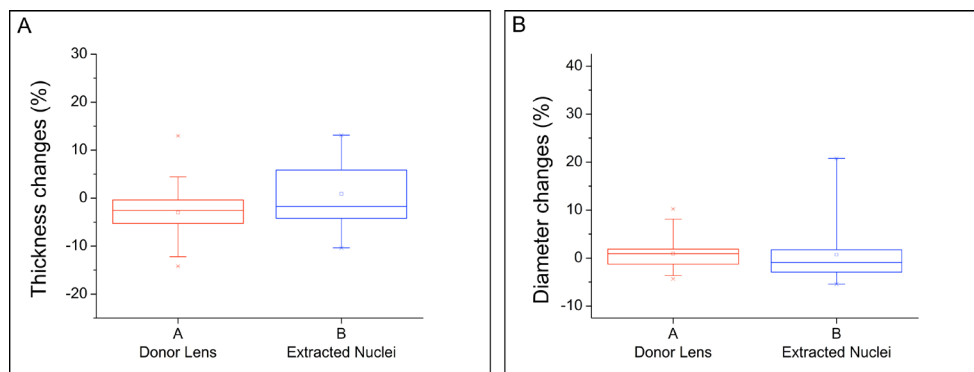


Figure 2. The percentage changes in the dimensions of the specimens following fixation and storage are shown as box-whisker plots. **A**: Changes in thickness expressed in box-whisker plots for donor lenses (red) and extracted nuclei (blue). **B**: Changes in diameter expressed as box-whisker plots for donor lenses (red) and extracted nuclei (blue).

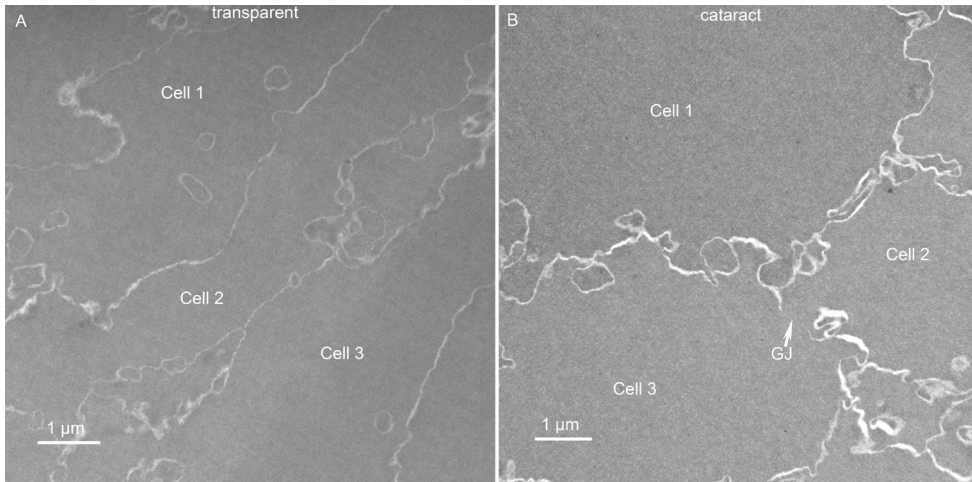


Figure 3. Low magnification electron micrographs of fetal and embryonic nuclear fiber cells. **A:** Donor lens image is from the fetal nucleus close to the embryonic nucleus: 2011–406 (92/M). **B:** Cataract nucleus image is from an opaque region of the embryonic nucleus: 3–25–11#3 (74/F). The open space between cells 2 and 3 is typically the location of a gap junction (GJ, arrow). [Key, here and subsequent figures: Sample ID (age in years/gender)].

immediately fixed from fresh lenses [17]. Multilamellar bodies were observed in all nuclear cataracts and most transparent lenses (Figure 5). These multilamellar bodies consistently have a dark core surrounded by membranes that are sometimes absent (Figure 5A) and are often only partially preserved (Figure 5B). Maturation of the multilamellar bodies accounts for the absence of multiple lipid layers [13]. Nuclear cataracts have more multilamellar bodies compared to donor lens nuclei, as reported previously [12-14,18,19].

Preservation of the lens cortex: Immersion fixation of the whole donor lens preserves the structural details of young fiber cells (Figure 6). Intermediate magnification showed

details of the capsule, epithelium, elongating fiber cells, and regularly spaced fiber cells of the outer cortex (Figure 6A). At high magnification, the ultrastructural details of membranous organelles within the epithelium can be visualized (Figure 6B), such as the nuclear envelope and a nuclear pore (arrow) and endoplasmic reticulum decorated with ribosomes (arrowheads). Intact mitochondria are recognized (Figure 6C) from the outer membrane and pronounced cristae membranes (arrow). Just posterior to the equatorial plane, the intact capsule about 10 μm thick (double arrow) can be seen adjacent to elongating fiber cells (Figure 6D). Gap junctions are visible at low magnification as a dark line (arrowhead) and at high magnification as pentalamellar structures (Figure

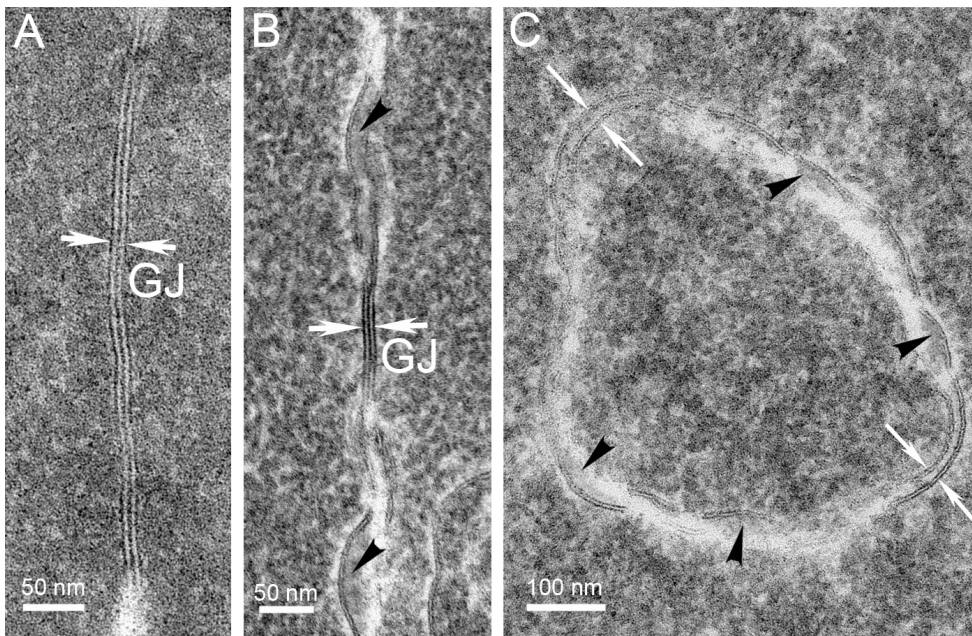


Figure 4. High magnification images from fetal and embryonic regions of nuclear cataracts. **A:** Displayed is a long gap junction (GJ, arrows) about 16 nm wide from a cataract: 3–25–11#3 (74/F). **B:** Displayed is a short gap junction (arrow) and undulating membranes with extracellular space deposits (arrowheads) from a cataract: 1–11–11#1 (12/M). **C:** Circular profile, which is a cross-section of an intercellular projection, is encased by a membrane pair (arrows) where several extracellular space deposits are visible (arrowheads) from a cataract: 4–6–11#2 (69/M).

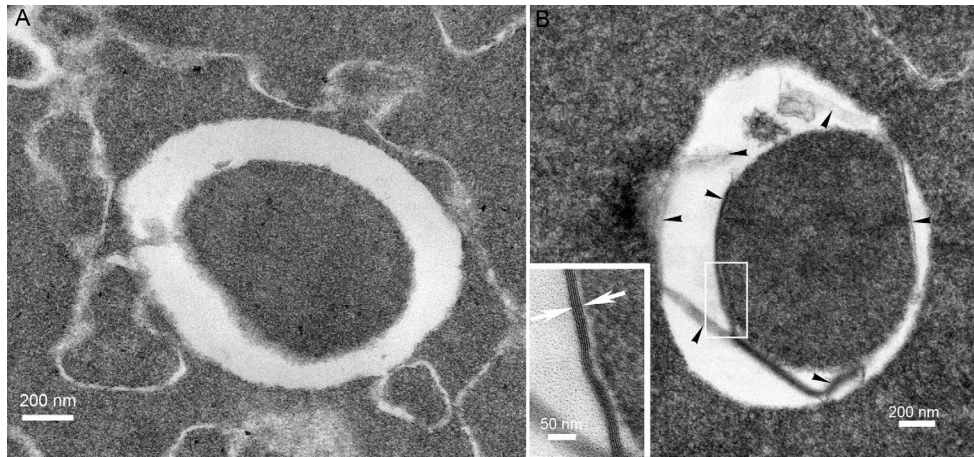


Figure 5. Electron micrographs of multilamellar bodies display their essential features. **A:** A multi-lamellar body 1.4 μm in diameter was observed in a donor lens: 2011-406 (92/M). Note the absence of membrane layers and the large electron lucent rim indicating a mature particle. **B:** A multilamellar body 1.7 μm in diameter observed in a cataractous nucleus: 4-6-11#2 (69/M). Note the prominent multi-lamellar membranes, which are partially disrupted (arrowheads)

indicating an intermediate stage of maturation. The repeat period of the bilayer membranes is about 5 nm for the four bilayers shown at high magnification (inset).

6E,F) between elongating fiber cells. The 16-nm-thick junctions in the outer cortex are similar to those observed in the nucleus (Figure 4) suggesting that once the junctions form, they retain structural integrity with age and cataract formation [10,15]. These images confirm that the membranes and junctions were well preserved at cell interfaces using the new fixation protocol.

DISCUSSION

It is essential to preserve the oldest cells in the ocular lens intact to understand the cellular mechanisms of aging and age-related nuclear cataracts. Such useful information can be obtained from donor lenses and extracted lens nuclei from operating rooms. Because of the popularity of phacoemulsification surgery for treating cataracts, extracapsular cataracts are rarely extracted, particularly in developed countries, unless otherwise indicated. This has led to a dearth of tissues available for structural studies, although facilities necessary to perform extractions are accessible. To find a solution to this problem, a simple fixation and storage protocol was developed.

The protocol involves immediate fixation, for 72 h, of the tissues upon receipt using commonly available fixatives and long-term storage, following which the specimens are ready for ultrastructural analysis where the facilities are available. Our results show that long-term storage in phosphate buffer following fixation with paraformaldehyde did not significantly alter the dimensions of the specimens. The median thickness and diameter of the donor lenses changed slightly, 2.6% and 0.9%, respectively, whereas changes in the median thickness and diameter of the extracted nuclei were

0.6% and 0.3%, respectively. The highly compacted nature of the lens nucleus may be responsible for the smaller change in the dimensions of the extracted nuclei compared to the donor lenses, which, in addition, have a cortical region.

The new fixation protocol is based on the successful fixation method developed for scanning electron microscope (SEM) studies of human and animal lenses using short fixation in neutral buffered formalin followed by long fixation in glutaraldehyde [20]. Glutaraldehyde is often used for ultrastructural studies; however, this fixative can create problems for subsequent studies using antibody labeling or fluorescence. The fixation in paraformaldehyde appears to be sufficient for ultrastructural analysis by TEM throughout the lens. The initial fixation in formalin opens minute passages, perhaps through membranes without causing membrane disruption, such that small molecule fixatives can penetrate to the lens center. This is consistent with the limited penetration of aldehyde fixatives even in small mouse lenses [21]. The initial fixation in 10% commercial formalin seems to be superior to fixation in 1.5% paraformaldehyde, which showed rapid water loss (averaging about 25%), lens shrinkage, and significant changes in the lens dimensions [11]. This study of preserved donor lenses in paraformaldehyde alone serves as a control for the present study with initial formalin fixation [11]. The changes with paraformaldehyde alone were so consistent among many lenses of different ages that the authors concluded that structural studies using paraformaldehyde fixative, such as [5,22,23], must be viewed with caution especially when reporting dimensions of fiber cells [11]. The fixations used in the structural studies referred to were different from the 1.5% paraformaldehyde used in [11]. The SEM study used the fixation noted above, as short fixation in

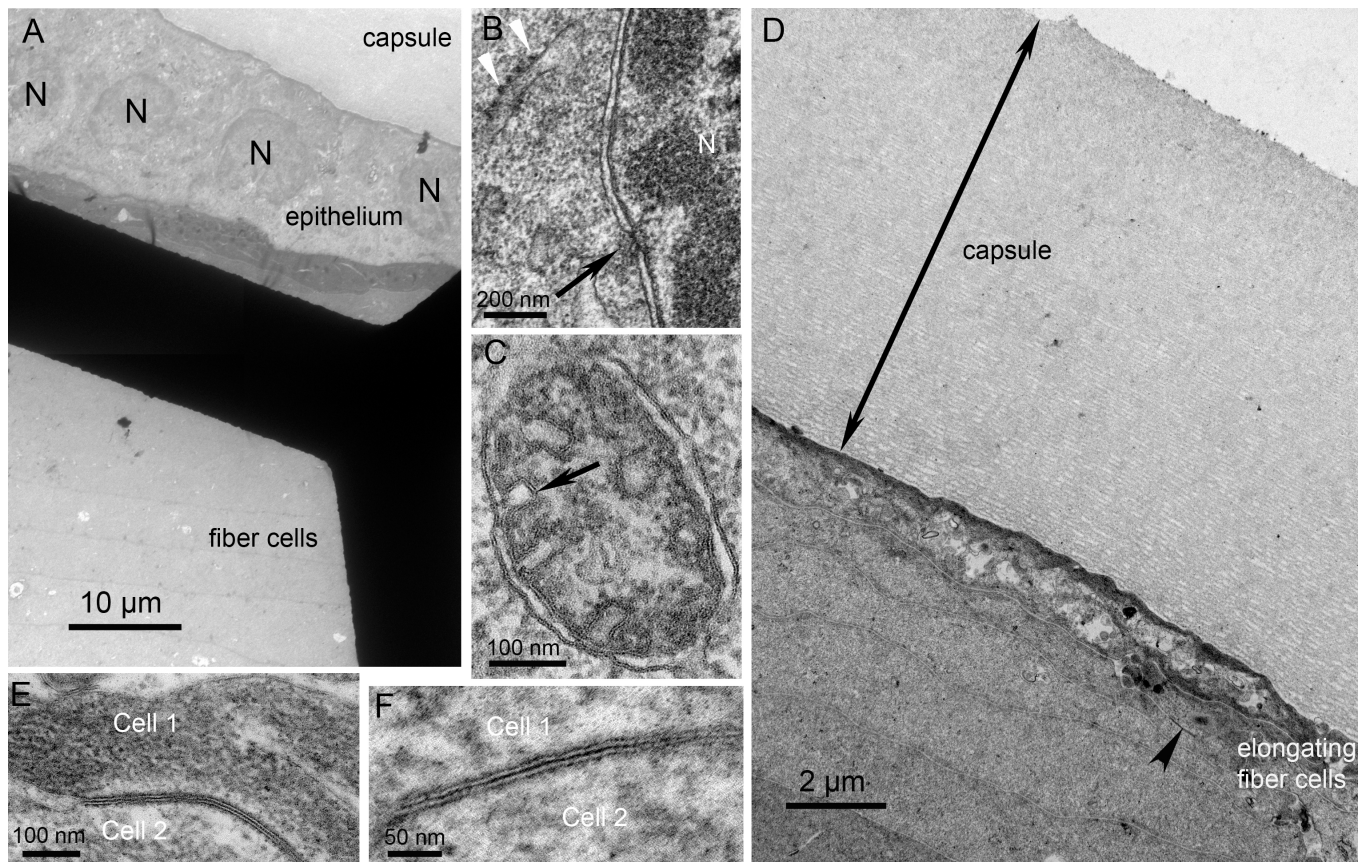


Figure 6. Structural details of cortical fiber cells. **A:** Displayed is a low magnification overview of the capsule, epithelium (N, nuclei), and outer cortex from 2011-406 (92/M). **B:** A high magnification image shows an epithelial cell nuclear envelope with a nuclear pore (arrow) and endoplasmic reticulum with adherent ribosomes (arrowheads) from 2011- 523 (22/M). **C:** High magnification image of a mitochondrion with a cristae membrane labeled (arrow) from 2011-523 (22/M). **D:** An intermediate magnification image shows elongating fiber cells and regular fiber cells of the outer cortex, just posterior to the equatorial plane. A gap junction between elongating fiber cells is marked (arrowhead). A 10- μ m-thick intact and undistorted capsule is shown (double arrow) from 2011-565 (55/M). **E:** A well-preserved interface is shown between Cell 1, a dark staining elongating fiber cell, and Cell 2 showing a gap junction (paired membranes) from 2011-565 (55/M). **F:** A similar gap junction (paired membranes) between two fiber cells (cells 1 and 2) is shown deeper within the outer cortex from 2011-565 (55/M).

formalin followed by long fixation in glutaraldehyde [5], and the TEM studies used the immediate fixation of Vibratome sections from fresh lenses in a glutaraldehyde/paraformaldehyde mixture [22,23]. We have no explanation why fixation in commercial formalin gives better preservation of lens dimensions, but the improvement may be due to the higher concentration of paraformaldehyde, the hydrated form of formaldehyde as methylene glycol [24], and the presence of a small percentage of methanol, which may help minimize the influence of osmotic barriers during early fixation. The improved fixation without major changes in cell dimensions is demonstrated in the good preservation of the cortex where the capsule is in close contact with the epithelium and fiber cells, the fiber cells are not swollen or distorted, and the interfaces between fiber cells are smooth (see Figure 6). Furthermore, using an entirely different fixation protocol, confocal

imaging of fluorescent labels was used to confirm the cellular dimensions reported in the TEM studies [25].

The fixation protocol used in this study appears to preserve the morphology of nuclear fiber cells. Fiber cells in the embryonic and fetal nuclear regions of normal aged lenses and age-related nuclear cataracts are intact, and their indistinguishable morphologies at low magnifications appear similar to previous descriptions [15-17]. The Vibratome sectioning of fresh lenses followed by mixed aldehyde fixation has become the standard for preserving fine structural details, and the previous studies serve as a control for the current study [15-17]. The difference here is that the Vibratome processing was preceded by rapid initial fixation and long-term storage. The important comparison is thus the preservation of the lens core, which must be stabilized with initial fixation to

provide preservation comparable to Vibratome sectioning of fresh lenses. The ultrastructural features of the fiber cells in these regions, including cytoplasmic texture, cell-cell interdigitations, circular profiles in cytoplasm representing finger-like projections from one fiber cell to an adjacent cell, and membrane undulations, noted in this study are consistent with earlier observations [10,16,17,22]. Gap junctions, formed by closely opposing membrane pairs, are also prominent throughout the nuclei of all specimens. Similar to earlier reports [10,16], these junctions are typically 16 nm wide with symmetric staining and almost no extracellular space. These appear as discontinuities in the white line seen separating adjacent fiber cells in low-magnification TEM images (see Figure 3) and are well appreciated at higher magnifications (see Figure 4) [16,17]. This visibility of the membrane pairs demonstrates the excellent preservation of the internal structure of the lens by the protocol described in this study. Protein-like deposits (see Figure 4), as found earlier, are also common in the extracellular space between fiber cells in nuclear cataracts [16,17]. These deposits stain similarly to the adjacent cytoplasm and are specifically located on the curved membrane arrays of aquaporin-0 [17].

Multilamellar bodies were observed in all nuclear cataracts and most transparent lenses. These are 1- to 4- μ m-diameter (average 2.4 μ m) globular particles that typically have a central core of cytoplasm and a low-density rim surrounding the core that initially contained multiple layers (three to ten) of lipid with thin interlayer spacing of 5 nm [12]. These particles are randomly distributed throughout the inner nuclear regions of age-related nuclear cataracts [14,26]. The core has variable staining density, thus variable protein density, compared to the adjacent fiber cell cytoplasm [12,13]. In aged particles, the membranes are often lost, the core is densely packed with protein (see Figure 5), and the refractive index fluctuations across the particles suggest that they are potentially strong sources of light scattering [13,14,18,19]. All features of multilamellar bodies reported in previous studies are preserved here using the new protocol.

The ultrastructure of the outer cortex is also preserved in donor lenses with the protocol used in this study. The capsule and the epithelium are intact. The regular (uniform size and shape) fiber cells of the outer cortex are arranged in radial cell columns, as described previously [25]. These cells are differentiated from the elongating fiber cells by differences in the staining pattern. The elongating cells stain dark, and this is believed to be due to the amount and organization of the cytoskeleton that control elongation (see Figure 6A,D). The quality of the preservation of the epithelium and the outer

cortex is at least as good as in previous studies of human lenses.

In summary, initial immersion fixation of whole donor lenses or extracted nuclei in formalin appears to enhance subsequent fixation in paraformaldehyde. Long-term storage in phosphate buffer after paraformaldehyde fixation results in only minor alterations in dimensions and gives similar preservation to standard fixation methods. The fixation protocols are sufficiently simple to be performed in most medical settings where the equipment and personnel needed for structural studies may not be available. These methods enable researchers and clinicians in remote settings to preserve donor lenses and rare examples of extracapsular extractions for detailed examination at later times.

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