Scanning Microscopy

Volume 7 | Number 2 Article 20

6-7-1993

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Barry R. Masters
Uniformed Services University of the Health Sciences

Scanning Microscopy, Vol. 7, No. 2, 1993 (Pages 645-651)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/93$5.00+.00

SPECIMEN PREPARATION AND CHAMBER FOR CONFOCAL MICROSCOPY OF THE *EX VIVO* EYE

Barry R. Masters

Department of Anatomy and Cell Biology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

Phone No.: (301)-295-3204 / FAX No.: (301)-295-1715 / E-mail: BMASTERS@USUHS

(Received for publication September 22, 1992, and in revised form June 7, 1993)

Abstract

A chamber is described for maintaining the mechanical and physiological stability of the *ex vivo* eye during observation with confocal microscopy. The mechanical stability is provided by a plastic ring situated on the limbal region of the eye. The ring and supporting chamber are designed to reduce mechanical motion of the specimen. The ring and chamber size vary with the species and size of the eye under examination. The physiological stability over a period of approximately one hour is provided by immersing the eye in a bicarbonate Ringer's solution that is exchanged every five minutes. This fluid exchange is made between periods of microscopic observation. The suggested method for confocal microscopic observation of cornea and ocular lens in an *ex vivo* eye is to use a non-contact water immersion microscopic objective with a high numerical aperture. This is a non-invasive, non-applanating system for the confocal microscopical observation of *ex vivo* rabbit or human eye.

Sample preparation and the specimen chamber are described. Optical sections of the cornea and lens obtained with a confocal microscope from a freshly removed *ex vivo* rabbit eye are presented as examples of applications of this technique.

Key Words: Confocal microscopy, *ex vivo* eye, cornea, ocular lens, eye chamber.

Introduction

Confocal imaging of the *ex vitro* eye involves special procedures that are necessary to maintain its mechanical, structural, biochemical and osmotic integrity. The microscopist must work with a sample that is very different from the typical monolayer of cells grown on a cover slip. The rabbit and human eye are roughly of spherical shape, easily deformable, and therefore, difficult to stabilize in position on the microscope stage. The eye has unique optical, mechanical, and geometrical properties that must be accounted for to achieve optimal results with a confocal microscope. In addition, the cornea must remain hydrated, and supplied with oxygen and other nutrients (Dikstein and Maurice, 1972).

The human cornea consists of a series of layered structures: epithelium, Bowman's membrane, stroma, Descemet's membrane, and an endothelium (Klyce and Bauerman, 1988). The thickness of the central region of the human cornea is 500 μm. There are gross structural differences between the human and rabbit corneas. The rabbit cornea is 400 μm thick in the center region. The rabbit cornea does not have a Bowman's membrane.

The epithelium consists of five to seven layers of cells. The rabbit epithelial thickness is 40 μm. Within the stroma, which is about 355 μm thick, are keratocytes situated between layers of collagen fibrils. Descemet's membrane is an acellular region that separates the stroma from the endothelium. The corneal endothelium, a single layer of cells, forms the most posterior layer of the cornea and functions to maintain corneal transparency (Dikstein and Maurice, 1972).

The unique optical properties of the cornea and ocular lens correspond to its function: the collection of light external to the eye, and imaging that light on the retina. Light rays are refracted by the anterior and posterior layers of the cornea and the ocular lens. The light rays must pass through a series of transparent media: cornea, aqueous humor, ocular lens, and vitreous humor before interacting with photoreceptors in the retina.

The transparency of the cornea and the lens as well as the specimen thickness, makes it difficult to image these specimens with conventional microscopy (McCally and Farrell, 1990). When observed with

conventional light microscopy, the light collected from the out of focus planes tends to degrade the image by reducing contrast and resolution. The contrast observed in an image in confocal microscopy is due to differences in the refractive index that differentially scatter light.

The small amount of light reflected from the cornea and ocular lens (usually less than 1%) is due to small differences in refractive index combined with other losses of light intensity such as absorption and scatter along the optical path. Therefore, confocal microscopy of the cornea and ocular lens in the reflected and back scattered mode results in images that are quite noisy. To reduce the noise, it is usually advisable to average several images. The BioRad® confocal microscope system employs Kalman averaging to improve the signal to noise ratio; this results in improved image contrast. If noise is random, then averaging several video frames will increase the signal to noise ratio. To average several frames, usually four to eight frames, it is imperative that the sample remains stable mechanically. If this is not so, the resulting averaged image will appear blurred. This is a good test for the mechanical stability of the microscope-specimen combination!

The wavelength of the light used for illumination of the specimen is related to penetration depth when observing the deeper regions of the ocular lens. While the cornea and the aqueous are practically transparent to light below 400 nm, the human ocular lens contains pigments that absorb this light. This absorption is important if one is using a laser scanning microscope to observe the nuclear or posterior regions of the ocular lens. Since the scatter of light has an inverse fourth power dependence on wavelength, longer wavelengths should be used to reduce light scatter; also, longer wavelengths of light result in deeper penetration and the capability to image deeper regions of the lens. On the other hand, the resolution of the confocal microscope depends on the wavelength used for the illumination of the object; shorter wavelengths yield increased resolution.

The eye must be supported mechanically to keep from sagging under its own weight. Serial optical sections suitable for three-dimensional reconstruction are unobtainable if there is mechanical movement. The confocal microscope should be isolated from mechanical disturbance. The effect of mechanical movement is especially critical in confocal instruments that are not real-time, i.e., those requiring several seconds of scanning to form the image. The eye specimen should be stable during the scanning interval. However, for real-time confocal systems, and *in vivo* confocal microscopy, the image is recorded at video rates (33 milliseconds per frame). The video images can be directly recorded to video tape or a digital disk. Alternatively, the video signals can be digitized by a frame grabber as part of a digital image processor. Any motion that occurs within the 33 ms period will degrade the quality of the image.

Furthermore, the tissue must be kept hydrated, and supplied with glucose and oxygen (Dikstein and Maurice, 1972; Bachman and Wilson, 1985). If the globe is not properly hydrated, it will slowly lose water by evaporation, and this will continuously reduce the diameter of the eye. If the ionic and osmotic conditions of the solution surrounding the cornea are inappropriate, then the cornea will undergo spontaneous swelling. Mechanical trauma to the cornea from the objective, i.e., an applanating system, as well as incorrect composition of the bathing solution could result in epithelial swelling. Various degrees of both epithelial and corneal swelling may occur in the absence of an appropriate environment for the cornea.

With proper precautions and correct technique it is possible to obtain submicron resolution confocal images from the *ex vivo* eye. Regions of ocular tissue in an *ex vivo* eye that have been imaged with confocal microscopy including the cornea (Lemp *et al.*, 1986; Masters, 1989; Masters and Kino, 1990; Masters and Paddock, 1990) and the ocular lens (Masters, 1992).

Materials and Methods

Specimen preparation

Eyes were obtained from male New Zealand white rabbits weighing 2.5 kilograms. The rabbits were maintained and handled according to State and Federal regulations and the ARVO Resolution on the Use of Animals in Research. The rabbits were euthanized with an overdose of pentobarbital. After death, the eyes were removed and freed of adhering tissue. The intact eyes were placed in a container filled with bicarbonate Ringer's solution. A series of confocal images was taken immediately.

Bicarbonate Ringer's solution

The composition of the epithelial bathing solution mimicked the composition of the aqueous humor of the rabbit eye. The Ringer's solution has the following composition: (all concentrations expressed in mmole/liter) sodium 160.0; potassium 5.0; calcium 1.0; magnesium 1.0; chloride 130; bicarbonate 25.0; phosphate 3.0; glucose 5.0; glutathione 0.3; and 7.4 pH, and 305 MOsm osmolarity.

Confocal imaging

The technique described is based on a BioRad® MRC-500 laser scanning confocal microscope. The author has also worked with other confocal microscopes to image ocular tissue and obtained comparable results. While the examples in the figures were made with the laser confocal microscope, the techniques are general and can be applied to other confocal microscopes.

The globe was gently placed in a black plastic container and immersed up to the limbus in freshly aerated bicarbonate Ringer's solution. The plastic ring shown in Figure 1 was gently placed over the eye.

The dimensions of the ring and the holder vary with the size and the weight of the rabbit or the size of the eye. Studies of the human eye require a larger chamber and ring than that required for an *ex vivo* rabbit eye. For a typical eye obtained from a New Zealand

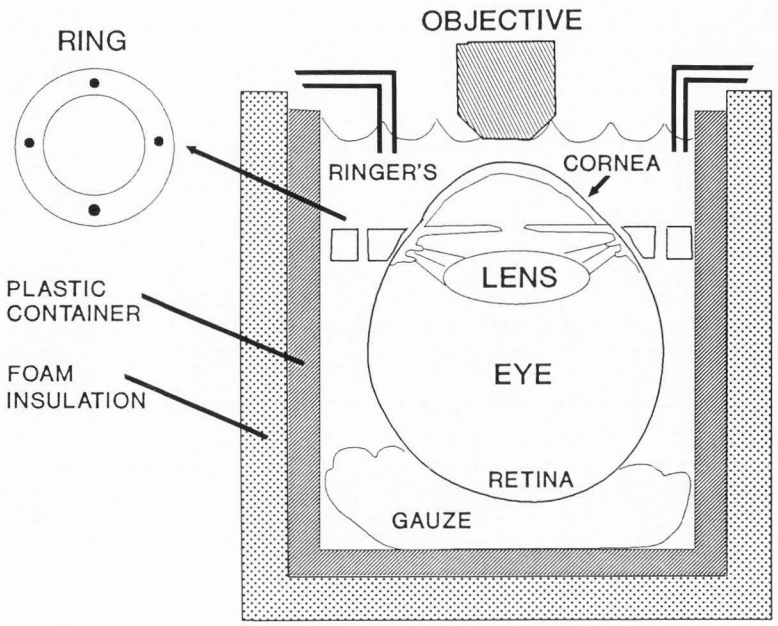

Figure 1. A schematic diagram of the holder which supports the *ex vivo* eye under stable physiological and mechanical conditions for confocal imaging. The eye globe is placed in a container which provides mechanical stability and hydration control. The container is inside a foam jacket for temperature control. The eye rests on a gauze pad under Ringer's solution. A plastic ring with four holes to allow fluid movement is inserted to stabilize the eye. Aerated bicarbonate Ringer's solution surrounds the entire eye. Every five minutes, within image acquisition, the Ringer's solution is exchanged. The dimensions of the ring and chamber vary with the size of the eye under observation. For observation of the human eye-bank eye, a larger ring is required.

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rabbit weighing 2.5 kg, the following dimensions are suggested. The inside diameter of the container is 31 mm, the outside diameter of the plastic ring 30.5 mm, and the diameter of the hole centered in the ring 15 mm.

The four holes in the plastic retaining ring are required for proper operation of the chamber. Two holes allow passage of fluid, and two holes are used to grab the ring during placement. The passage of fluid is necessary during the initial placement of the ring onto the eye in the chamber. A small pair of forceps is used to grab the ring in the holes across the central hole, and to gently place the ring over the eye globe. The cornea protruded through the central hole and the ring made even contact with the eye at the limbal region. For the initial focusing, a drop of bicarbonate Ringer's solution was suspended from the microscope objective. This drop optically coupled the tip of the objective to the surface of the cornea. Once the cornea was observed in the microscope, a small volume of bicarbonate Ringer's was added to the container to completely immerse the globe and the tip of the microscope objective. Every five minutes, freshly aerated bicarbonate Ringer's solution was exchanged. A two-piece plastic cover rested on top of the container and surrounded the microscope objective. This plastic cover was used to retard evaporation of the Ringer's solution. The plastic cover (not shown in Fig. 1) did not make contact with the microscope objective.

Light from an argon laser using the combined wavelengths of 488 and 514 nm was used to illuminate the object. A Leitz 25x water immersion objective with an numerical aperture (N.A.) of 0.6 was used to obtain the images. The objective never contacted the cornea.

The low reflectivity of the cornea required maximum amplification of the signal from the detector. At this high amplification, a bright spot of stray light appeared in the center of each image. A gray disk was used to partially mask this reflection and to designate its position (see centers of Figures 3 and 6). Images devoid of the black square or disk were cropped from the total image. A method, based on the polarization of the incident laser light that reduces the intensity of the reflection artifact, has been developed (Szarowski *et al.*, 1992). However, this method also results in a reduction of the intensity of reflected light from the eye that reaches the photodetector in the microscope.

The confocal images represented an average of 16 images. The images were made from the central region of the cornea, that is, the optic zone, and represent many fields in the central cornea.

Non-contact confocal microscopy

The most important aspect of the eye-microscope interaction is that there is no direct physical contact of the tip of the microscope objective with the surface of the eye. Some confocal systems use applanating objectives that flatten the cornea. The stated purpose is to flatten the cornea and to provide a flat, large field of view. A second purpose of an applanating microscope objective is to reduce the motion of the *in vivo* eye along the optic axis due to the heart beat induced pulse in the blood vessels (Lemp *et al.*, 1986). Of course, this is not a problem in the observation of the *ex vivo* eye. The system described in this paper does not use such an applanating system. In fact, the applanating system has clear disadvantages. The fluid is not in contact with the cells that are in direct contact with the applanating tip of the microscope objective. The direct contact deforms the cornea and the resulting forces may tend to remove some superficial cells during the process of moving the objective to move to a different position on the eye.

Microscope objectives for confocal imaging

The choice of a microscope objective depends on several different requirements (Keller, 1990). To collect light with high efficiency, a high N.A. is desirable.

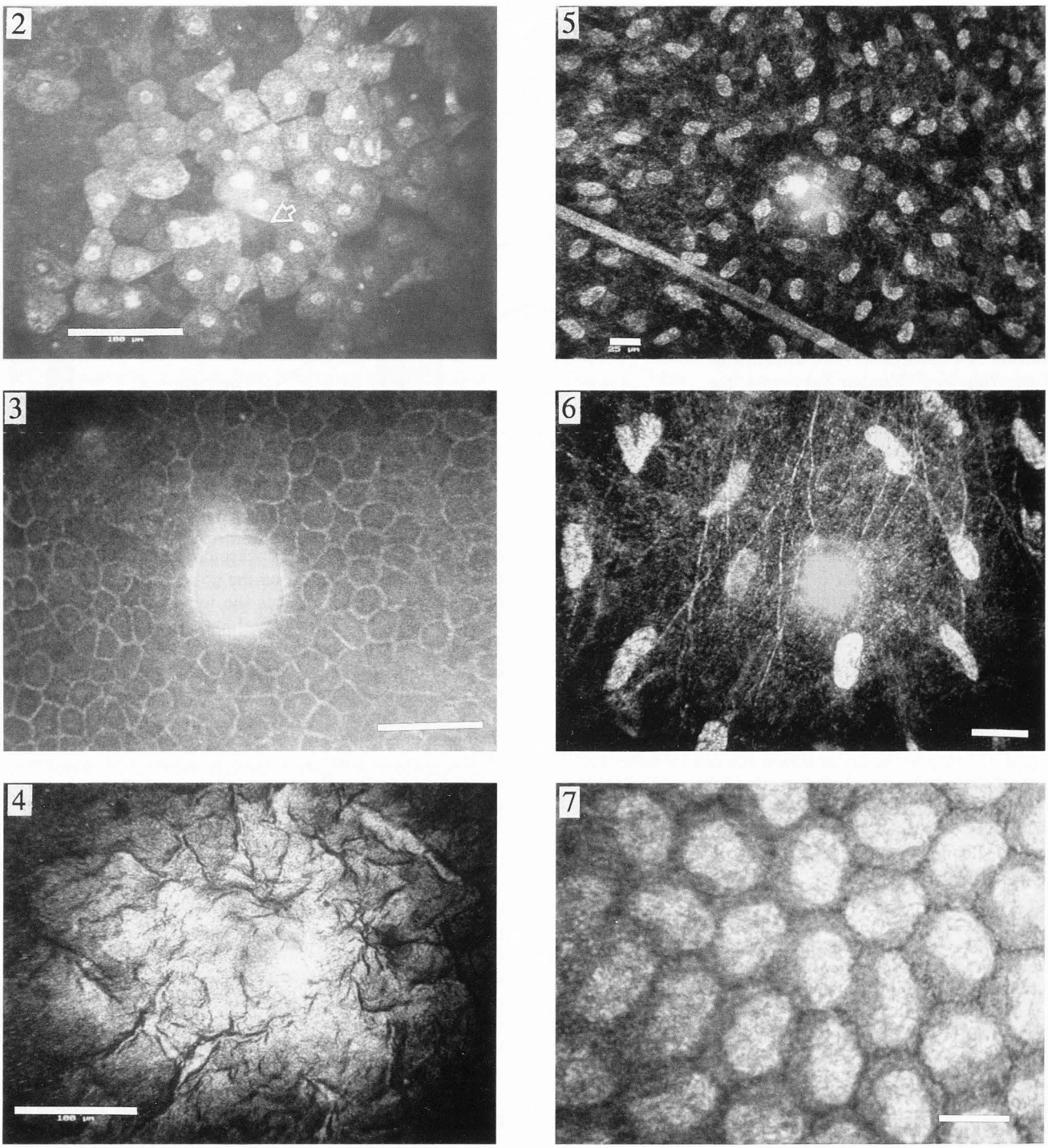


Figure 5. Confocal back scattered light micrograph of a nerve fiber in the anterior stroma of *ex vivo* rabbit eye. The nuclei of stromal keratocyte cells are shown in the focal plane. Bar = 25 μm.

Figure 6. Confocal back scattered light micrograph of nuclei from stromal keratocytes in the anterior stroma in an *ex vivo* rabbit eye. The linear fibers between adjacent keratocytes are shown. Bar = 25 μm.

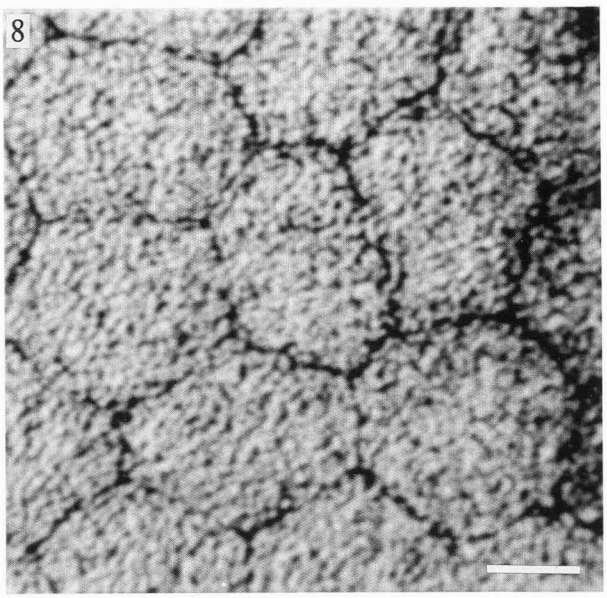
Figure 7. Confocal back scattered light micrograph of nuclei from endothelial cells in an *ex vivo* rabbit eye. The bright oval or bean shaped objects are the cell nuclei. The darker regions are the endothelial cells. Bar = 25 μm.

Figure 2 (at left). Confocal back scattered light micrograph of the superficial cells of the rabbit corneal epithelium. The polygonal cells with borders in contact are shown. The bright nuclei are seen. The brightest cells are in the focal plane and darker cells are below the focal plane. This is a planar intersection of a curved surface (the globe) and therefore in the non-contact mode used in our method not all of the cells are in the focal plane. The arrow points to a superficial cell that is slightly below the plane of focus. Bar = 100 μm.

Figure 3 (at left). Confocal back scattered light micrograph of the basal epithelial cells of an *ex vivo* rabbit eye. The bright cell borders are shown. Bar = 50 μm.

Figure 4 (at left). Confocal back scattered light micrograph of the basal lamina of the *ex vivo* rabbit cornea. Bar = 100 μm.

Figure 8 (at right). Confocal back scattered light micrograph of endothelial cells in an *ex vivo* rabbit eye. The focal plane is a the interface of the posterior region of the cornea and the aqueous humor. The linear borders of the endothelial cells are shown. Bar = 10 μm.



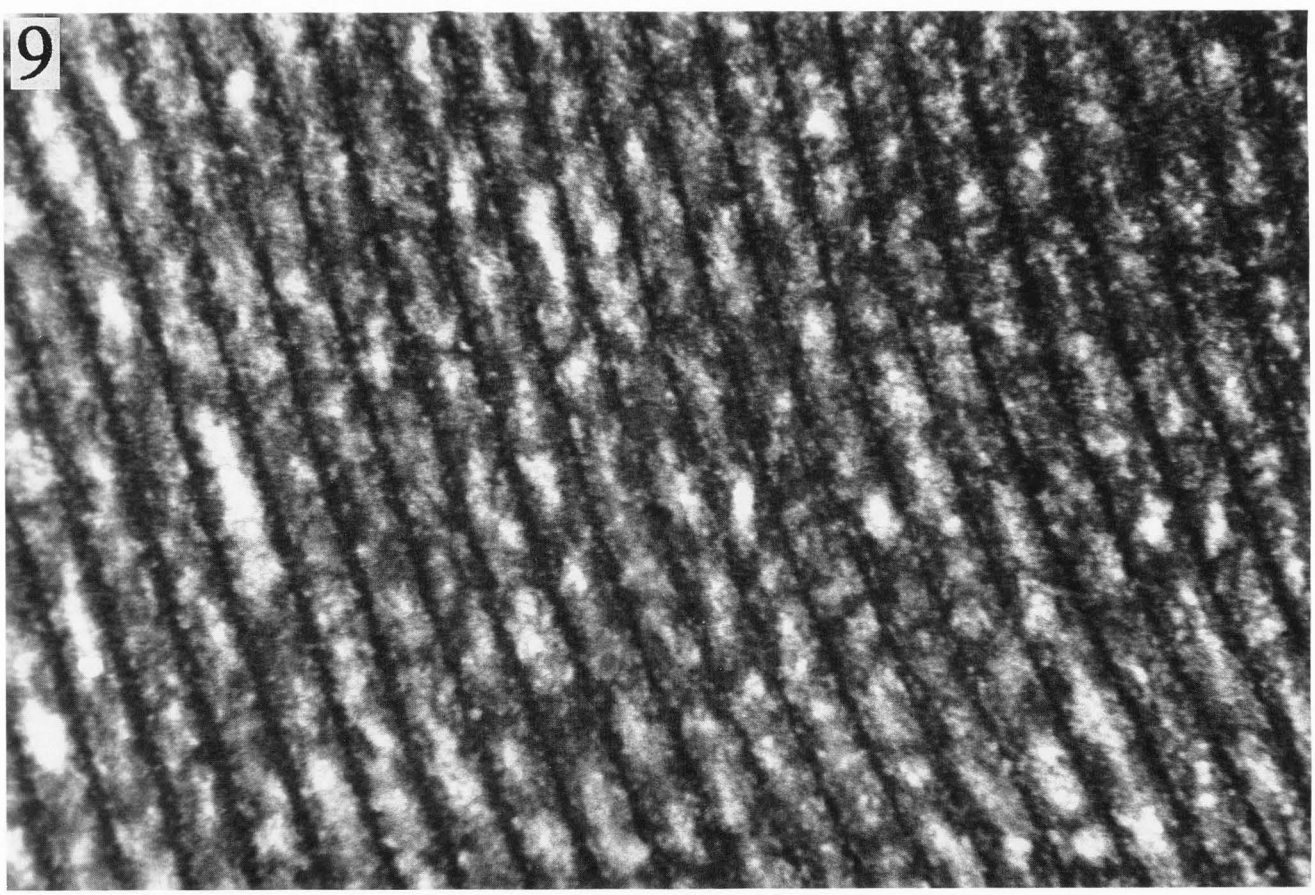


Figure 9. Confocal back scattered light micrograph of the *in situ* ocular lens in an *ex vivo* rabbit eye. The width of the parallel lenticular fibers is 7 μm. The image is from the central portion of the pupil and the image plane is about 100 μm posterior to the anterior surface of the lens.

Microscope objectives with large N.A. have reduced free working distance. To image the full thickness of the rabbit cornea, a free working distance of about 400 μm is necessary. To image the anterior region of the ocular lens in the intact eye, a low power objective (i.e., 25 X) could be used.

Since a variety of laser wavelengths are available for laser scanning confocal microscopes the choice should include considerations of specimen penetration depth and light transmission of the objective. It is important to determine the transmission of the objective at the wavelength used in the measurement. Monochromatic illumination will produce clearer images than those made with white light illumination due to chromatic aberrations of the optics and the objective.

The Leitz, 50X, N.A. 1.0 water immersion objective is also suitable for use with the BioRad® laser scanning microscope. A higher power objective may not be an improvement since the exit pupil may be smaller than the illuminating laser beam. Furthermore, the resulting reduction of the free working distance only permits confocal imaging of the anterior region of the cornea in an intact eye.

Results and Discussion

A series of optical sections was made of a freshly removed *ex vivo* rabbit eye from the anterior surface of the eye (Fig. 1) across the full thickness of the cornea, across the aqueous humor, and into the fibers of the crystalline lens (Figures 2-9). The normal ocular surface is shown in Figure 2. In reflected light confocal microscopy with water immersion microscope objectives, the nuclei appear as bright ovals. The curvature of the eye limits the field of view. Cells slightly below the focal plane appear darker. About 40 μm below the optical sections of Figure 2 are the basal epithelial cells (Fig. 3); their bright borders are visible. In the center of each basal epithelial cell is a brighter cell nucleus. With the techniques described in this paper, the cell borders of the basal epithelial cells are always observed. The highly reflecting and rough surface of the basal lamina (the membrane that separates the corneal epithelium from the corneal stroma) is shown in Figure 4. Figure 5 shows an optical section in the anterior region of the stroma; the long linear structure in the lower left-hand region is a nerve fiber. The bright oval objects are the nuclei of the stromal keratocytes. Figure 6 is an optical section of the posterior stromal just anterior to Descemet's membrane. The oval bright objects are the nuclei of the stromal keratocytes. The linear fibers in the plane of the nuclei are fibrils that appear to connect the cell bodies of the keratocytes. These linear structures may be extracellular and are not related to the keratocytes (Scharenberg, 1955). The endothelium is the posterior surface of the cornea. Figure 7 is an optical section of the corneal endothelial cells. The focal plane is two μm into the thickness of the endothelial cells (4 μm thick). The bright regions are the nuclei of the endothelial cells. Figure 8 shows an optical section of the posterior surface of the corneal endothelium; the linear cell borders are shown with the rough surface of the endothelium.

If the free working distance is sufficient to optically section the *in situ* ocular lens, then it is possible to focus across the cornea and obtain confocal images of the lenticular fibers. An example of a confocal image of *in situ* lenticular lens fibers is shown in Figure 9.

The images shown in this paper are typical images that are obtained with the microscope objectives described with the sample chamber. Early work, before the final development of the present techniques, resulted in images of poorer quality (Masters, 1989; Masters and Kino, 1990; Masters and Paddock, 1990).

Confocal images obtained from human eye-bank material are different from *in vivo* human confocal microscopy since the storage of the eye-bank eyes usually results in corneal swelling. For example, Figure 10.13 in our previous publication (Masters and Kino, 1990) shows the cell bodies of stromal keratocytes of swollen human eye-bank eyes. That figure, which shows keratocyte cell bodies, was actually made from a swollen human eye-bank eye. Also, some confusion may have resulted by the mislabeling of another figure in another publication [Figure 6 in Masters (1989)] as keratocyte cell bodies in normal rabbit corneas; actually the figure is of a swollen human eye-bank cornea. This mislabeling of two figures in my previous works has resulted in some confusion in the literature (Jester *et al.*, 1992).

The cell bodies of keratocytes are not usually observed in eyes in which the corneas are of normal thickness and hydration. In the fresh normal *ex vivo* eye, we usually observe only the nuclei of the stromal keratocytes (Figs. 5 and 6). In summary, the normal cornea shows nuclei of stromal keratocytes in its normal state of hydration. If the cornea swells, then the cell bodies of the stromal keratocytes are observed.

Conclusions

A chamber is described for maintaining the mechanical and physiological stability of the *ex vivo* eye during observation with confocal microscopy. Optical sections obtained with a confocal microscope from a freshly removed *ex vivo* rabbit eye are presented as examples of the technique. The techniques presented result in improved contrast, sharpness, and detail as compared to previous works (Masters, 1989; Masters and Kino, 1990; Masters and Paddock, 1990).

Acknowledgments

This work was supported by a grant from NIH EY-06958.

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Discussion with Reviewers

J. Auran: In relation to Figure 6, you state "These linear structures may be extracellular and are not related to the keratocytes (Scharenberg, 1955)"; please elaborate.
Author: The Scharenberg work refers to the histology of the human cornea. A silver carbonate technique was used to study cells and nerves in fixed material with light microscopy. The results in the present paper were obtained on fresh, unstained, unfixed, *ex vivo* rabbit eyes. The optical thickness of the confocal section in Figure 6 is about 2 μm. Although the linear fibers appear to be in the plane of the keratocyte nuclei, it is not demonstrated that they are part of the keratocyte cell bodies. In this confocal microscopic image only the keratocyte nuclei are observed; the keratocyte cell bodies are not observed.