

Scanning Microscopy

Volume 1992
Number 6 *Signal and Image Processing in
Microscopy and Microanalysis*

Article 6

1992

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Masters, Barry R. (1992) "Confocal Microscopy and Three-Dimensional Reconstruction of Thick, Transparent, Vital Tissue," *Scanning Microscopy*: Vol. 1992 : No. 6 , Article 6.

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CONFOCAL MICROSCOPY AND THREE-DIMENSIONAL RECONSTRUCTION OF THICK, TRANSPARENT, VITAL TISSUE

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Abstract

The three-dimensional visualization of the 400 micron thick, transparent, *in situ* cornea is described to demonstrate the use of confocal light microscopy for noninvasive imaging of living cells and thick tissues in their normal, vital conditions. Specimen preparation and physiological stability, as well as light attenuation corrections are critical to data acquisition. The technique to provide mechanical stability of the specimen during the duration of the image acquisition is explained. A laser scanning confocal light microscope (LSCM) was used to obtain optical serial sections from rabbit eyes that were freshly removed and placed in a physiological Ringer's solution. This study demonstrates the capability of the confocal light microscope to obtain a series of high contrast images, with a depth resolution of one micron, across the full thickness of living, transparent tissue. The problems of nonisotropic sampling and the limited eight-bit dynamic range are discussed. The three-dimensional reconstructions were obtained by computer graphics using the volume visualization projection technique. The three-dimensional visualization of the cornea in the *in situ* eye is presented as an example of image understanding of thick, viable biological cells and tissues. Finally, the criterion of image fidelity is explained. The techniques of confocal light microscopy with its enhanced lateral and axial resolution, improved image contrast, and volume visualization provides microscopists with new techniques for the observation of vital cells and tissues, both *in vivo* and *in vitro*.

Key Words: Confocal microscopy, cornea, image fidelity, three-dimensional reconstruction, volume rendering.

Introduction

The eye is a uniquely difficult specimen for microscopic observation. The cornea is a transparent, low contrast structure whose physiological function is the transmission and refraction of visible light. The theory of corneal transparency has been developed. (Benedek, 1971; McCally, Farrell, 1990). Eye motions in the head, linear movement along the optic axis and rotational motion, contributes to the difficulty of microscopic observation. The standard optical instrument for ocular observation is the slit lamp. The slit lamp, which is actually a microscope, permits the observation in real-time of an oblique optical section across the full thickness of the cornea (Berliner, 1949). The slit lamp can not provide the observer with an image of the cornea which is situated in the plane of the cornea.

This study covers the three-dimensional visualization of the cornea from optical sections obtained with a laser scanning confocal microscope (LSCM). The basic principles of confocal light microscopes have been previously described (Pawley, 1990; Wilson, 1990). In order to investigate the problems associated with three-dimensional reconstructions of confocal optical serial sections from the cornea, we have made the microscopic observations of corneas from *in vitro* eyes. These observations were performed under conditions described in the **Methods** section which preserved the normal cellular structure and the physiological functions of the cornea during periods of image acquisition. The rationale for the use of an *in vitro* eye was to eliminate the problem of eye motion, and therefore determine the ultimate limitations of image quality (resolution and contrast). The three-dimensional reconstruction of the stack of two-dimensional confocal images could then

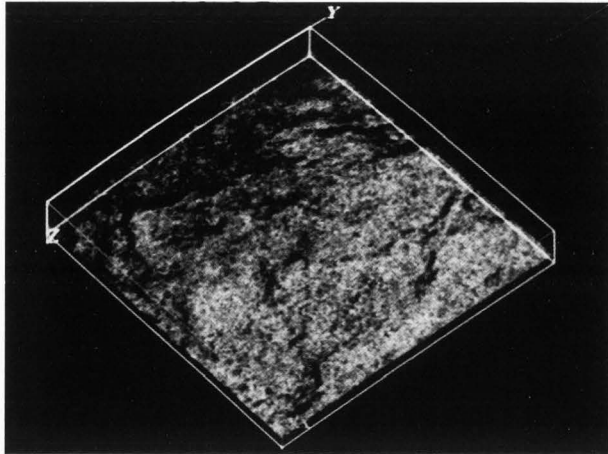


Fig. 1. The three-dimensional reconstruction of the basal lamina of the *in situ* cornea. The frame is 100 microns on a side. This figure is a reconstruction of four sections, each of one micron thickness.

be visualized and serve as a standard upon which to compare other studies made *in vivo*. The three-dimensional visualizations of the *in situ* cornea are intended to demonstrate the quality of three-dimensional ocular visualization (Masters, Paddock, 1990a,b; Masters, 1991a).

Four types of confocal ocular microscopes now exist as clinical prototypes for the observation of the *in vitro* and the *in vivo* eye (Masters, 1990a,b; Masters, 1991b). The tandem scanning confocal microscope was originally developed for *in vivo* microscopy of thick tissues (Petran et al., 1985). This microscope has been arranged in a horizontal position coupled to a head rest for *in vitro* and *in vivo* ocular observation (Lemp et al., 1986; Dilly, 1988; Jester et al., 1988; Jester et al., 1991). A new real time confocal microscope has been designed based on a one-sided tandem scanning microscope, and has been applied to ocular imaging (Xiao et al., 1990; Masters, Kino, 1990; Masters, Kino, 1993). A scanning slit clinical confocal microscope with the capability of serial optical sectioning the living *in vivo* human eye and measuring the reduced pyridine nucleotide fluorescence (NADH) from the cells has been developed (Masters et al., 1991). A modified wide-field corneal specular microscope based on two sets of slits and a oscillating prism represents another type of corneal confocal microscope (Koester, Roberts, 1990). All of these

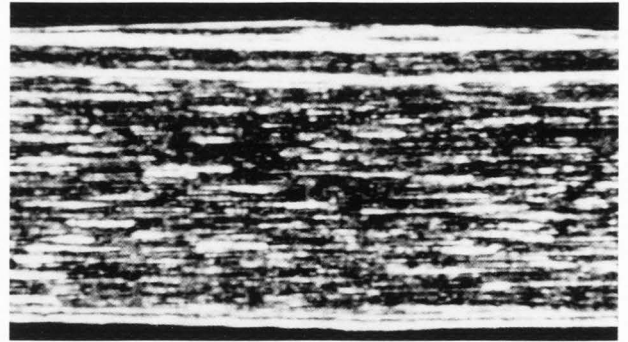


Fig. 2. The three-dimensional reconstruction of the full thickness of the *in situ* cornea. The edge on view is shown in the plane of the cornea. The cornea is 400 microns thick. The anterior portion of the cornea is on the top of the figure and the posterior region is on the bottom. The upper bright line is the superficial epithelial cells (highly reflective). The next lower bright line is the basal lamina. The distance between these two regions is the corneal epithelium. The bright linear segments represent the nuclei of the keratocytes. The posterior region of the cornea shows two bright lines. The first line is Descemet's membrane, and the line at the lower portion of the cornea is the layer of corneal endothelial cells. The distance from the top to the bottom of the figure is 400 microns.

instruments have the capability to obtain stacks of two-dimensional optical serial sections of the *in vitro* and the *in vivo* cornea, but they have not been used for three-dimensional reconstruction of the cornea. The three-dimensional visualization of the cornea and ocular lens in the *in situ* eye was presented as a demonstration of three-dimensional image understanding of thick, viable biological cells and tissues (Kriete, Masters, 1990; Masters, 1990a; Masters, Paddock, 1990b; Masters, 1991a).

Materials and Methods

Specimen preparation

The eyes were obtained from male New Zealand albino rabbits weighing about 3 kg. The rabbits were maintained and handled in accordance with the Association of Researchers in Vision and Ophthalmology Resolution on the Use of Animals in Research. The rabbits were anesthetized with an intramuscular injection of ketamine HCL (40

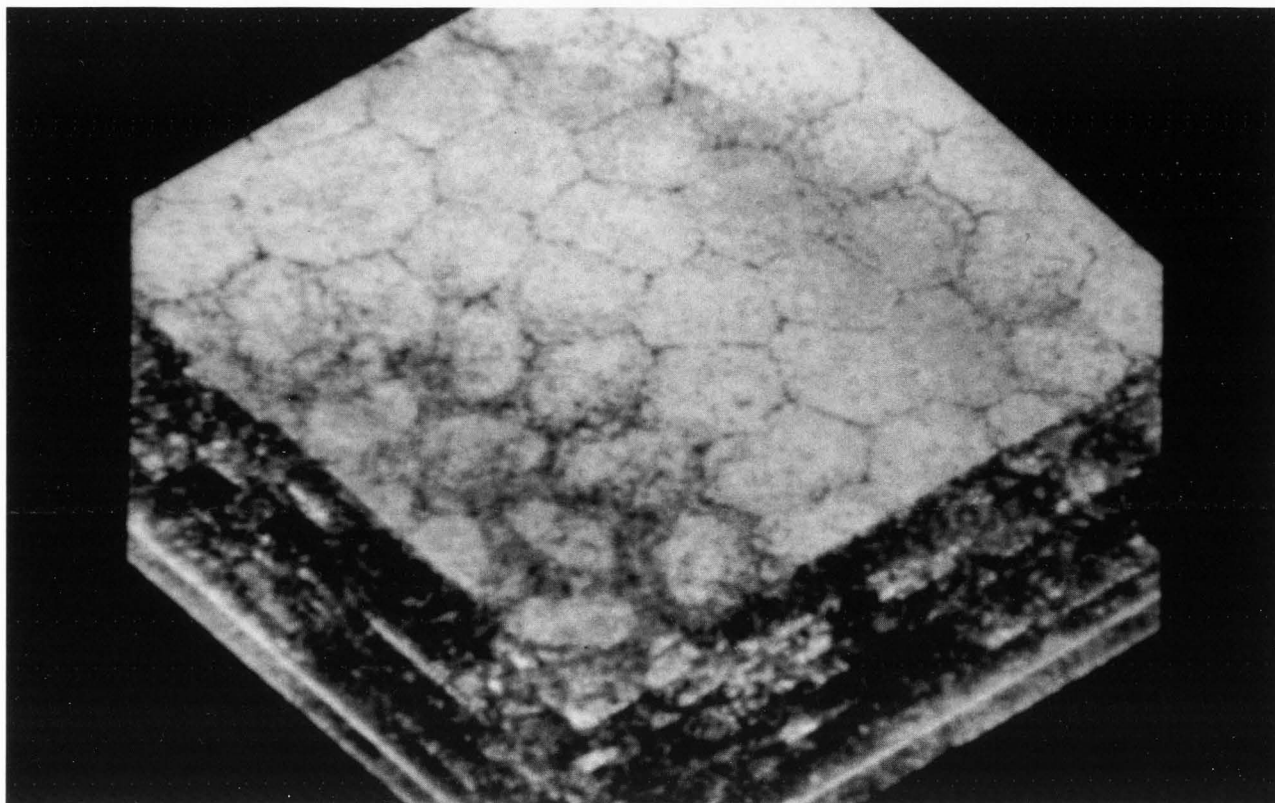


Fig. 3. The three-dimensional reconstruction of the full thickness of the *in situ* cornea. The polygonal endothelial cells are shown at the top of the figure. These cells are 400 microns posterior to the front surface of the cornea. These cells form the single layer of endothelial cells on the posterior region of the cornea. The cornea is rotated with respect to Fig. 2. and shows the posterior endothelial cell layer on the top of the figure.

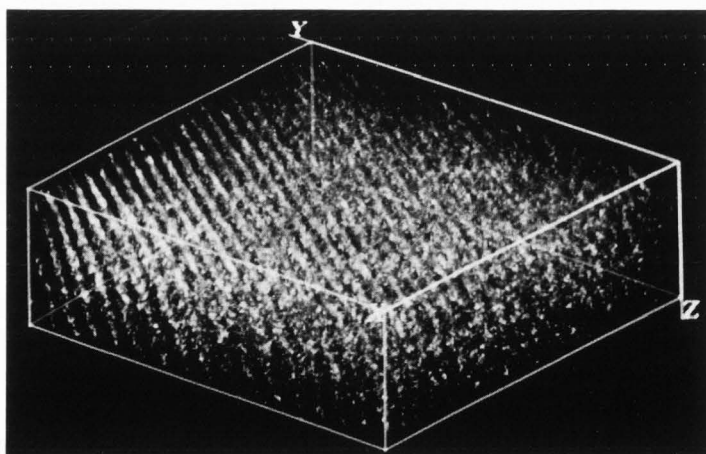


Fig. 4. The three-dimensional reconstruction of a region of the ocular lens situated about 100 microns posterior to the front surface of the ocular lens. The image was made in the *in situ* eye. The linear structures are the aligned lenticular fibers within the ocular lens. The distance between the individual fibers is 7 microns. The top of the figure is the anterior region of the lens and the bottom of the figure is the posterior side of the lens volume.

mg/kg) and xylazine (5 mg/kg). The eyes were freed of adhering tissue and swiftly enucleated. The intact eyes were placed in a container filled with aerated, bicarbonate

Ringer's solution which contained 5 mM glucose and 2 mM calcium at 30°C. The solution was changed every ten minutes in order to maintain the eyes in a physiologically vital state.

Image acquisition

The corneas were observed *in situ* with a BioRad® MRC-600 laser scanning confocal microscope. The microscope was operated in the back scattered light mode. The laser excitation was a 25 mW argon ion laser with excitation lines at 488 nm and 514 nm. The combined lines were used for confocal image acquisition.

The LSCM detector aperture was 2 mm. The light detector was a photomultiplier (Thorn EMI 9828B) with an S-20 photocathode. Photon counting was used to increase the signal-to-noise ratio. The photomultiplier output was digitized to eight bits which resulted in images with a dynamic range of 256 gray levels.

A Nikon Optiphot microscope with an 8x ocular was coupled to the LSCM. The microscope objective was a Leitz 25X water immersion objective with an NA of 0.6. The objective had a free working distance of 1.7 mm. The long working distance of the microscope objective permits the observation of the full thickness of the rabbit cornea which is 0.4 mm. This long distance microscope objective was critical for the *in situ* confocal observation of the ocular lens (Masters, 1992). The fine focus of the LSCM was incriminated in 3 micron steps except for studies of the basal lamina which used 1 micron steps.

The eyes were placed in a container that gently provided mechanical stability and minimized the mechanical motion of the eye. The eye was covered with a bicarbonate Ringer's solution which was frequently exchanged. The tip of the microscope objective was dipped into the Ringer's solution, but never made physical contact with the eye.

The light attenuation effects of observing a 400 micron thick transparent sample were corrected in the following manner. The light rays made two passes through the thickness of the cornea in the image acquisition from each of the focal planes. The light was attenuated by two processes: light absorption and scattering. Since the tissue is transparent in the visible region, the main source of light attenuation with depth in the cornea specimen was light scattering. Therefore, images acquired near the surface of the cornea were brighter than images acquired at greater depths. The BioRad® confocal microscope only has a dynamic range of 256 gray levels. The first

slice of the image acquisition was at the surface of the cornea, and the last slice situated at a depth of 400 microns. If the gain was set at the start of the image acquisition, then the first few optical sections would have sufficient contrast and signal to noise ratios, but the deeper optical sections would not have sufficient contrast and appear dark due to the insufficient signal intensity. To compensate for the problem of light attenuation, the gain for each optical section was manually adjusted to maximize the contrast and the signal to noise ratio.

In addition to the problem of light attenuation, the limited dynamic range of the BioRad® confocal microscope truncated the intensity range between the highest and the lowest intensities into only 256 gray levels. There are anatomical structures within the cornea which have intense reflectivity and others with extremely weak reflectivities. The gain had to be manually adjusted to detect the weakly reflecting structures without saturating the highly reflecting structures. Because of the limited dynamic range of the 8-bit digitization of the BioRad® system (and other commercial LSCM systems), we can not adequately compensate for this problem.

Three-dimensional reconstruction by the volume rendering technique

The stacks of two-dimensional optical sections were collected by computer control of the Nikon Optiphot microscope equipped with a stepping motor. A number of frames, usually eight, were summed for each optical section and the image was stored in a file on the hard disk of the computer. Then the stepping motor moved the stage by a three micron increment. This operation was under computer control. The computer collected and stored 134 optical sections, each 256 x 256 pixels in size.

The data sets were transferred to a Silicon Graphics Personal Iris 4D/35 via an Ethernet connection. The data sets were all converted in to a readable format by running an extract program to remove the BioRad® header files. The data sets were reconstructed using Voxel View Software (Vital Images, Fairfield, IA). The Voxel View software uses a fast implementation of the volume rendering technique (Levoy, 1989). The volume rendering used the default settings threshold, opacity and contrast. The lighting function was not implemented.

Results

The following figures are three-dimensional reconstructions of the *in situ* rabbit cornea which were generated according to the methods described in this manuscript. **Fig. 1** shows the volume reconstruction of basal lamina situated 40 microns below the surface of the cornea. **Figs. 2** and **3** show the three-dimensional reconstruction of the full thickness of the *in situ* cornea from serial sections. **Fig. 4** is a three-dimensional reconstruction of the *in situ* ocular lens in the rabbit eye. This reconstruction is only of a region of the ocular lens and is provided to demonstrate that even at the full working distance of the microscope objective (1.57 mm), it is possible to obtain two-dimensional serial sections with sufficient contrast from the transparent, thick ocular lens.

These results validate the use of reflected light confocal microscopy to acquire stacks of images of unfixed, unstained, vital ocular tissue. A stack of registered two-dimensional optical sections can be visualized as a three-dimensional object with computer assisted three-dimensional reconstruction software. This paper illustrates the three-dimensional visualizations by showing single views of the reconstructed objects. However, the three-dimensional reconstructions can be viewed from any angle with the use of a computer work station.

Discussion

The image acquisition used the Argon laser as a light source for strong lines at both 488 nm and 514 nm. It is preferable to use a single wavelength of light to minimize the chromatic aberrations of the optical system. The filter sets used in the BioRad® system in the reflected light mode use dual wavelengths. We have used single wavelengths of either 488 nm or 514 nm in the Zeiss confocal system and obtained reflected light images of the cornea and the ocular lens which are similar to those obtained with the dual wavelengths.

The cornea was scanned by the laser beam more than 100 times during the image acquisition of the stack of two-dimensional images from the full thickness of the tissue. There is a possibility of photodamage or phototoxicity of the tissue during image acquisition. The cornea and lens of the rabbit

is almost transparent to light of wavelengths 488 nm and 514 nm. To test for structural changes in the cornea due to the laser light scanning we obtained single images of various optical sections were acquired in random sequence. These images are similar to the full stack of sequential images acquired through the full thickness of the cornea. While these observations do not rule out the possibility of photodamage or phototoxicity to the tissue, confocal microscopy in the reflected light mode did not detect such effects.

The image acquisition of a stack of two-dimensional optical sections from a 400 micron thick object, that is nearly transparent to visible light and has little intrinsic contrast, is a difficult task. Within the stromal region, there is continuous light attenuation due to light scattering along the optical path. We compensated for this attenuation by manually increasing the gain between each optical section within the stroma to maintain the contrast and the signal to noise ratio.

The full thickness of the cornea has several highly reflecting regions. The surface of the cornea is highly reflective due to the large difference in refractive index between the superficial epithelial cells and the tear film (*in vivo*) or the of Bicarbonate Ringer's solution (*in vitro*). The basal lamina shown in **Fig. 1** is highly reflecting due to the difference in refractive index between the stroma and basal lamina. The single layer of corneal endothelial cells on the posterior surface of the cornea is highly reflecting due to the difference in refractive index between these cells and the aqueous humour of the eye.

Since the detection system of the confocal microscope is limited to an eight bit analog-to-digital converter, the dynamic range of the confocal microscope is only 256 gray levels. This limited dynamic range is insufficient to cover the wide range of reflected light signal intensities obtained from the stack of optical sections of the cornea and the ocular lens. The weakest signals were obtained from the posterior regions of the stroma. The strongest signals were obtained from the superficial layer of epithelial cells, the basal lamina, and the layer of endothelial cells. In order to image the complete stack of two-dimensional optical sections, the gain of the amplifier was manually adjusted to cover the wide dynamic

range of signal intensities. This approach is reasonable if the confocal microscope is used in the reflected light mode in order to study corneal morphology. For the case of quantitative fluorescence confocal microscopy, this arbitrary manual gain adjustment is not suitable.

There are several aspects of digital image processing that are relevant to the three-dimensional reconstructions from optical serial sections. They include preprocessing operations, processing stacks of two-dimensional images, image segmentation, and image analysis. Although these digital image processing operations were not performed on the three-dimensional reconstructions discussed in this article they are important for increased image understanding. Finally, the criterion of image fidelity is explained with respect to three-dimensional reconstructions.

Prior to the computer assisted three-dimensional visualization, segmentation and analysis of ocular images, it is necessary to perform preprocessing on the individual two-dimensional images. Some of these operations involve the two-dimensional images and its nearest neighbors, while others operate only in the individual two-dimensional images.

The preprocessing may include some or all of the following: digital deblurring, registration, geometrical warping, and filtering. Spatial geometrical warping is a method to compensate for spatial distortion caused by the optical system. The microscope objectives used with confocal microscopes were designed for a specific tube length and refractive index of the coupling material. When the LSCM is used with live biological specimens, such as the eye, the refractive index is usually both unknown and variable from optical section to optical section. This results in optical aberrations and a reduction in image quality (Keller, 1990). The preprocessing operations listed above can be used to partially compensate for these aberrations. The theory of three-dimensional image formation in the confocal microscope in terms of the point spread function has been developed (Sheppard, Cogwell, 1990). The detailed experimental and theoretical aspects of the the loss of image definition and light fall off with depth in the specimen in confocal microscopy had been analyzed (Carlsson, Lundahl, 1991).

The processing operations on the individual optical sections yields stacks of two-dimensional images. Registration is the alignment of a set or stack of two-dimensional images using operations of translation, rotation, and "elastic bending" or geometrical warping of the individual images to produce a common alignment based on a set of fiducial points. These are points that are common to each image. If there is eye movement on axis or rotations of the eye during the collection of the stack of two-dimensional images, then it is necessary to perform operations to preserve image registration of the stack. In the ideal case for three-dimensional reconstruction, the resolution in three orthogonal directions is equal. Then each volume element in the reconstructed three-dimensional image produced from the two-dimensional slices would be a cube. In practice, this is not the case and the volume elements, or voxels are not cubes but cuboids. The image processing operation of interpolation can be used to resize the slices and produce a volume in that the voxels are cubes.

The next step is the operation of image filtering. The purpose of filtering is to smooth or enhance the individual two-dimensional images. If the point spread function of the microscope is known or can be closely approximated, then Wiener or inverse filtering can be used to sharpen the images. The success of many of these inverse filtering operations is strongly coupled to the amount of noise in the images. Various two-dimensional filters may be employed to enhance the edges of individual objects in the image.

The two-dimensional stack of confocal images from the eye is the input data set for the three-dimensional visualization. A simple method to give the illusion of three dimensions is the preparation of stereo pairs. In this method, the stack is separated into two images with an angular separation of about six degrees. Each eye of the viewer is presented with one image of the pair. Usually, the images are color coded and red-green glasses are used to separate the two images.

Another method of three-dimensional reconstruction is volume rendering or volume visualization. The technique of volume rendering is a projection method that permits the viewer to visualize the stacks of two-dimensional images without first fitting geometric primitives to the data. This is of

great use when the boundaries of the objects are fuzzy and not sharply defined in space, i.e. the images in back scattered light from the living eye. The advantage of this technique over other methods is high image quality and the lack of a requirement to precisely define the surface geometry (Levoy, 1989).

The principles of volume rendering techniques are as follows (Levoy, 1989; van der Voort et al., 1989). The method is based on the assignment of a color and a partial opacity to each voxel. The three-dimensional rendering is formed by merging voxels that project to the same pixel on the image plane. This technique has merit as compared to surface rendering methods since there is no requirement for making a binary classification of the data. The method also has problems; mainly high computational costs and difficulties to merge surface and volume data in a composite visualization.

Animations of the three-dimensional visualizations of the eye can be prepared and displayed. A set of views of the rendered three-dimensional cornea or ocular lens can be prepared with one degree rotations along several axes. These animations can be transferred to videotape for display at scientific meetings. The use of motion is a very important visual cue to represent the three-dimensional eye. The human brain can enhance the three-dimensional illusion of the object on the two-dimensional display device when motion of the visualization occurs.

We define segmentation as the classification or identification of individual elements in the three-dimensional volume composed of discrete voxels. In order to separate the various ocular structures, it is necessary to use three-dimensional image processing operations. These operations on the voxels or volume elements are analogous to the point and group operations that operate on pixels or picture elements in the two-dimensional images. A complete set of three-dimensional image processing routines is a valuable addition to standard image processing libraries. The use of three-dimensional edge detectors can now be used to separate various structures. The segmented objects may now be color coded for increased image understanding.

In addition to image classification and image understanding, it is often necessary to analyze the three-dimensional image. Image analysis is the quantitative evaluation of the three-dimensional images. The measurements

include the following: lengths, areas, surfaces, angles, and volumes. Prior to analysis, it is usually necessary to define the objects in the image (segmentation) and to have a standard calibration scale or system. While three-dimensional visualization is important for image understanding, the analysis of these images is of equal importance.

What is the relationship of the three-dimensional reconstruction from the stack of serial optical sections to the original specimen? Fidelity is the accuracy that the three-dimensional rendered object corresponds to the original object. The concept of image fidelity may also be applied to two-dimensional digital images. The fidelity of a three-dimensional rendered object may be described at several levels.

The first criterion may be qualitative. The three-dimensional volume visualization should contain the information on the structure and shape that occurs in the original object. A second level of image fidelity may state that the three-dimensional visualization should not contain any shape or structures that are not present in the original object.

Many digital processing operations change the image. For example, edge detectors may detect the edges, but they also may move the edges in the process. For example, there are edge shifts produced by median filters. The information content of a digital image may be modified (decreased) in the process of visualization or other digital image processing operations.

A higher level of image fidelity are the quantitative aspects. This refers to the quantitative mapping between the objects and the three-dimensional volume visualization. For example, lengths, angles, surfaces, and volume in the object should have a close correspondence in the three-dimensional visualization. The fidelity of the three-dimensional visualization is a function of the fidelity of the two-dimensional images.

The three-dimensional reconstructions of the *in vitro* cornea and *in situ* ocular lens provide a new method of visualization of unstained, unfixed ocular tissue. The two-dimensional imaging of the cornea and the ocular lens cited in the references provides a contrast and resolution that is not possible with the slit lamp. Several laboratories are currently engaged in the development and clinical use of *in vivo* confocal microscopes (Lemp et al., 1992; Wegener et al., 1992;

Beuerman et al., 1992). This paper is limited to a demonstration of three-dimensional visualization of *in vitro* ocular tissues.

Conclusions

The noninvasive visualization of living human tissue and cells is now a reality. The application of confocal microscopy and computer graphics presents the clinical and basic vision scientist with a new tool, a tool of great utility. This new tool is called-confocal ocular microscopy, and it represents a new paradigm for ocular visualization. Now we can visualize the living, *in situ* eye in three dimensions, and investigate the temporal changes of the three-dimensional structures (four-dimensional visualization). This paper demonstrates the feasibility of the technology on the *in situ* eye; the application to the *in vivo* eye is part of our current studies. The concomitant real-time digital collection of a set of two-dimensional images will provide the basis for three-dimensional visualization of these ocular structures.

The applications of the new paradigm include the following: functional stereology of the corneal cells, stereology of nerve trunks and fibers, dynamics of wound healing in three-dimensional, and four-dimensional imaging of corneal morphology. Functional stereology is the three-dimensional or four dimensional visualization of structure and cellular function in the living eye.

Acknowledgements

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

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.