Confocal Imaging: *In Vivo* and Clinical Applications

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Confocal microscopy is a collection of optical techniques that are applied in a variety of hardware configurations. Design strategies for the application of these techniques have generally used laser light (Pawley, 1990). In most laboratories, basic research use has employed laser light in conjunction with a fluorescent substrate to generate an optical signal, either through direct application of a fluorescent material to cells or by the stimulation of a chromophore associated with an antibody which will identify a cellular protein under some specified experimental conditions. The use of confocal microscopy in this type of situation generally requires a standard research microscope, and the tissue may be situated on a slide or other type of container that will provide a stable, controlled environment. Extensive computer analysis of the image, and often computer control of the confocal, is part of this approach. Although widely used in basic research settings, there have been some early attempts to apply white light confocal microscopy to human tissue, but only recently has it progressed to the level where it has become feasible to understand pathological changes associated with clinical problems (Petran and Hadravsky, 1968; Boyde, *1985;* Cavanagh *et al*, 1990; Masters, 1992; Beuerman, 1995).

As seen as follows, there are other considerations and trade-offs that must be made when developing an instrument for *in vivo* applications. An important first order consideration is the stability of the image due to the constant motion that occurs *in vivo*, even in the presence of anesthesia. Movements from sources such as pulse pressure and respiration move the field of view in the skin and other organs. In addition, saccadic movements of the eye would lead to blurring of the images if there was not some relaxation of the optical parameters such as optical section thickness and magnification. These movements are in the order of tens to hundreds of microns in amplitude and frequencies of several to more than 50 Hz. Thus, another primary consideration is the time for image acquisition. The potential for movement artifacts make methods for improving signal to noise ratios such as on chip integration, difficult to implement, and real-time, 30 fps, imaging or faster than real-time image acquisition, becomes the most feasible practical approach. In human use, observation time is generally restricted to a few minutes; however, in experimental settings with animals, considerably longer times can be used without adverse effects.

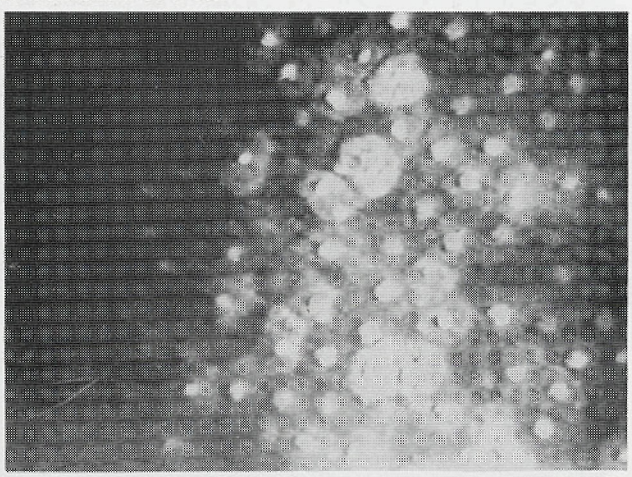

Figure 1 -- Confocal micrograph of the human corneal epithelium. The epithelial surface is seen as a mosaic of hexagonal cells. Prominent bright nuclei are seen throughout all of the cells and indicate the position of the surface cells. Some cells slightly below the focal plane are less clearly seen. Original magnification, x540.

Resolution is also a consideration in clinical confocal applications compared to its use in basic research with tissue specimens. For *in vivo* animal and human use, xenon and halogen light sources work well. However, resolution using broad spectrum white light will be less when compared with monochromatic laser light. Generally, *in vivo* applications have not used fluorescence and so the value of color imaging is questionable. The use of fluorescent materials may not be safe in humans. The qualities of confocal imaging also depend to a great extent on the aperture size that is used in either a slit scanning or a Nipkow spinning disk mechanism. Basic research applications may seek an optical section of only 1-3 µm. However, this would be difficult to accommodate in a living animal or patient due to motion problems. Increasing the aperture size has resulted in the use of an optical section thickness of about of 8-15 µm which still provides cellular views but makes motion artifacts tolerable.

Due to the favorable optical properties of the cornea of the eye, a great deal of progress has been made in imaging this tissue, in both normal and abnormal states. The confocal offers improvements over conventional methods for imaging the human cornea in the ophthalmology clinic (Beuerman, 1995). The conventional method, the slit-lamp, images the cornea as a blur and is based on an optical design that has changed little for many years. The low-power, about x40 magnification produces images that are degraded in resolution and contrast, and consequently limited in information. In *in vivo* applications, the only light that is available for viewing is reflected light and the challenge in applying confocal optics to the cornea is to provide sufficient contrast in a grey scale image in a real-time mode. Depending on the characteristics of the Nipkow disk, only a small fraction (less than 1% to 2%) of light incident on the upper surface is available for imaging.

Resolution of the confocal microscope has made it roughly equivalent to low power scanning and transmission electron microscopy. In the normal cornea, the cellular surface is seen as an array of cells with prominent nuclei, and in fact, the appearance of this cell layer was shown to be similar to that seen in fixed tissue in the scanning electron microscope (Cavanagh *et al*, 1990)(Figure 1). The advantage, of course, is that the tissue does not have to be removed and can be observed again at later times. The cornea can be viewed directly through a contact lens and the innervation of the epithelial layer seen as slender filaments lying roughly parallel to the surface (Figure 2). These sensory nerve endings cannot be seen by conventional light microscopy. The cornea in the human is about 500 microns thick and another cell layer on this inner surface has been seen with the confocal microscope and the appearance in the living eye agrees

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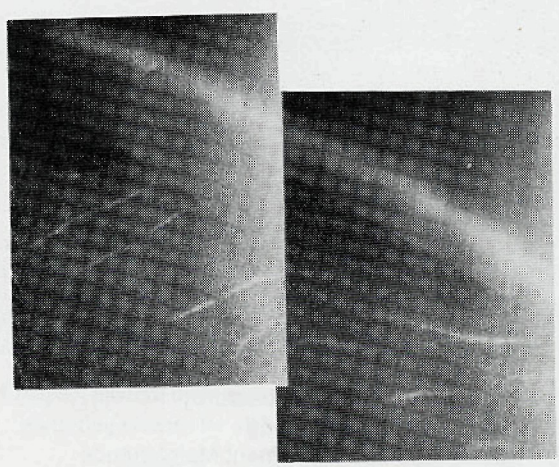

Figure 2 -- Confocal micrograph of the human corneal epithelium taken through a soft contact lens. The bright overlying band represents a cross-sectional view of the contact lens. The small bright, reflective fibrous structures are free nerve endings along the basal surface of the corneal epithelium. Remarkably, these small free nerve endings have been shown by transmission electron microscopy to be about 1 µm in diameter. Original magnification, x540.

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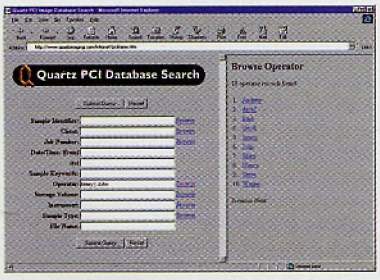

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well with that seen by scanning electron microscopy (Figure 3). Moreover, quantitative techniques have been developed to produce information about important cell parameters such as density and nearest neighbor relationships in the both cell layers (Laird *et al* 1996).

With the emphasis in modern medicine on rapid, non-invasive diagnosis, the clinical confocal microscope may have an important role. In addition to visualizing the normal cellular constituents of the cornea, it can also reveal cells of immune origin that are associated with an infection or inflammatory process. In an experimental study of corneal transplants, a common type of surgery performed in humans to prevent blindness, rejection of the transplanted tissue is encouraged to be able to understand this process in humans. As seen in Figure 4, when this experiment was carried out in rabbits, the cellular constituents of the inflammatory reaction associated with rejection and growth of blood vessels into a cornea could be directly observed. Investigators have shown that infectious organisms can be seen in the cornea *in vivo*. Fungal organisms are particularly easy to observe by confocal microscopy and this may eventually replace the need for biopsies (Figure 5). *Acanthamoeba*, an organism that has been found in the cornea of contact lens wearers and constitutes a serious threat to vision is usually cultured from a biopsy for positive identification.

In summary, the confocal microscope may have other clinical uses in addition to the eye. Fungal infections of the skin and nails, as well as various types of malignant conditions, may be amenable to confocal observation and diagnosis. Direct visualization of a pathological organism or condition may help to decrease the number of biopsies, decrease the time needed for a diagnosis while increasing the precision. ■

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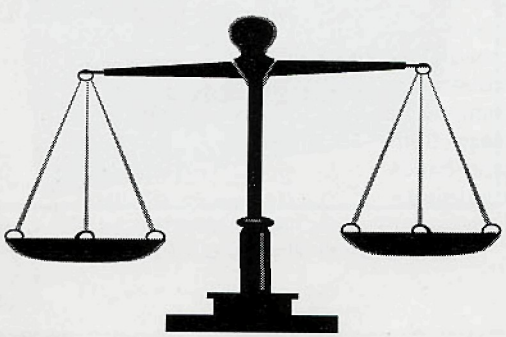

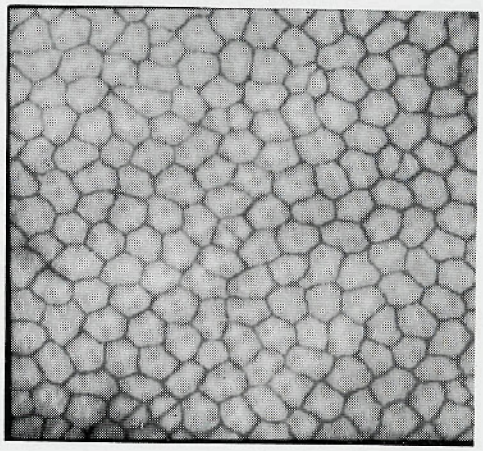

Figure 3-- Confocal micrograph of the hexagonal mosaic of the endothelium of the rabbit cornea. The normal organization is somewhat rearranged probably due to the age of the rabbit so that some cells have more than six adjacent cells. However, the confocal microscope clearly portrays the outlines of these cells. The thickness of these cells is about 5-7 µm. Original magnification, x540.

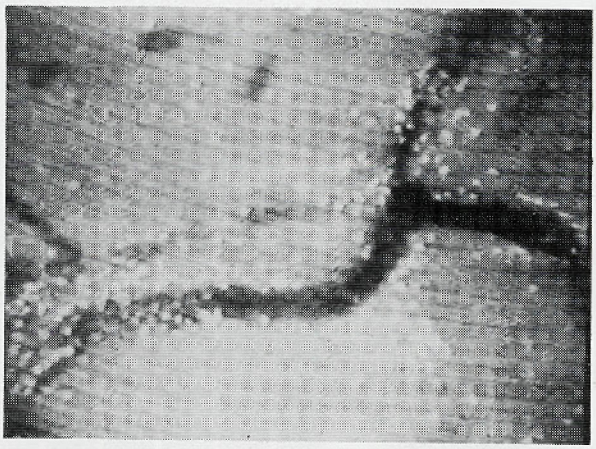

Figure 4 - Confocal micrograph from an experimental study of neovascularlization in the rabbit cornea. The background is the collagenous stroma of the cornea. The dark tubular structures are newly developed blood vessels and their dark appearance originates from the red blood cells and cells of immune origin flowing through them. After several days, these leaky vessels allow invasion of numerous cells of immune origin into adjacent tissue which forms a halo of cells around the vessel. Original magnification, x540.

Figure 5 -- Confocal micrograph of a human cornea. The bright, reflective filaments were tentatively identified as fungal and probably from *Aspergillus*. This was confirmed by growing the organism in culture. Original magnification, x540.

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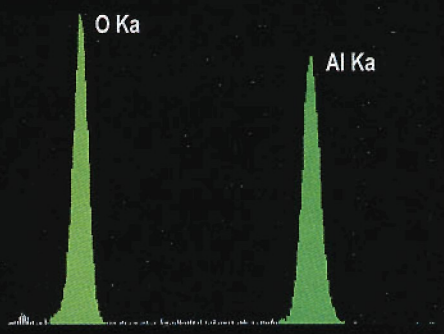

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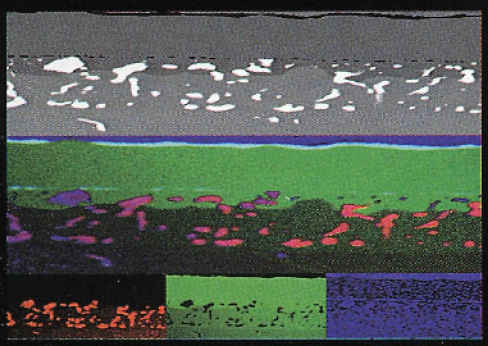

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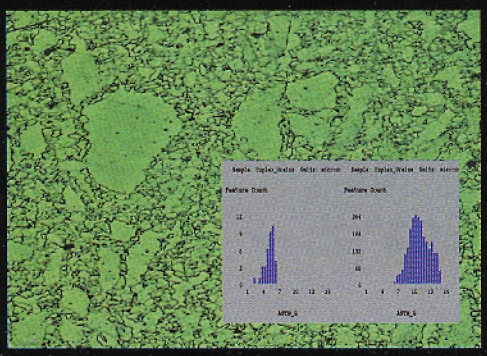

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