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Imaging Corneal Cells Using Low Power Laser Beams

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Abstract. A simple method of characterising epithelial and endothelial corneal cells using laser light is presented. A continuous wave helium-neon laser emitting at a wavelength of 632.8 nm and a continuous wave argon laser emitting at 488 and 514.5 nm were used. The cell images obtained were used to calculate the dimensions of the endothelial corneal cells, a typical value being 34–36 μm diameter, with overall measurement errors of ± 21%. An optical mechanism for obtaining images is proposed and comments are made about the possibility of using this method as a diagnostic tool in corneal disease.

Keywords: Cell dimensions; Corneal endothelium; Corneal epithelium; Laser imaging

INTRODUCTION

This paper presents a method for the investigation of corneal structure which uses a He-Ne laser to obtain images of corneal cells as well as information about the dimensions of the basal epithelial and endothelial cells.

The endothelium is the deepest cellular layer of the cornea. It is in direct contact with the aqueous humour and represents the most important contributing layer to corneal transparency. The endothelial cells lack significant mitotic activity and damage may alter corneal hydration and thus its clarity [1]. The external surface of the endothelium is limited by Descemet's membrane. It is a unicellular layer with a refractive index of 1.376, and in a young adult consists of half a million flat, regular, hexagonal cells 6 μm deep and 18–20 μm across [2]. The cells become progressively more irregular towards the periphery.

Clinically, study of the endothelium is important since it consists of a cellular puzzle of hexagonal cells which lack the power of replication; with age there is a gradual diminution in the number of cells and this has implications for continued good sight. Detailed imaging of this layer gives information on the shape and density of the cells.

The most important imaging techniques for corneal investigations rely on specular and confocal microscopy. The specular microscope is an optical system which relies on the reflected light from an incident beam projected onto the corneal surface which shows the cellular shape and configuration at a magnification determined by the optics of the system. In the confocal microscope, the focal point of the light source is at the same place as the objective and information relating to corneal structure is obtained by scanning over the axis of interest. The method presented here offers the advantage of simplicity over these well-established methods, requiring neither an optical magnifying system nor a scanning one.

METHODS

The principle of the method presented here relies on the reflection of laser light at the interface between ocular media which possess different refractive indices.

A laser beam impinging on the cornea is split into several smaller beams by successive reflections from several layers. Because of the coherent properties of the laser light, the resulting beams may interfere with each other in the plane of observation, generating a very

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complicated pattern of interference fringes. Considering only the interference between the beam reflected at the air–lachrymal fluid interface (6% of the incident energy) and the beam reflected by one of the corneal cell layers (less than 0.02% of the incident energy), the conditions for obtaining a Gabor hologram exist, as follows.

1. The optical path difference between the two beams is much smaller than the coherence length of the incident beam ($l_c \sim 15$–20 cm);
2. The beam reflected at the air–lachrymal fluid interface is characterised by a spherical wavefront and may be considered as a reference beam, whereas the beam reflected by a corneal cell layer acts as the object. This second beam contains information about the particular cell layer since it is characterised by phase modifications originating in the variations in refractive index at the cellular level.

The resulting interference fringes contain all the visual information about the object; the fringe contrast contains the amplitude and the interfringe dimension contains the phase. In this way the information contained in the object wave is transferred to the carrying wave. The angle between the two reflected beams is very small (practically this generates an 'on-axis' Gabor hologram) so that the interference consists of very narrow fringes. Practically, each cell layer contributes to the formation of the interference pattern and a complex system of micro-fringes is obtained.

The first requirement for obtaining a good recording of the interferogram is good mechanical stability of the entire experimental instrumentation, since continuous small amplitude vibrations will make the interference microfringe image move on the photodetector surface. This produces a constant average illumination of the detector which degrades the image quality. Such ideal experimental conditions are difficult to achieve.

The laser beam incident on the cornea is multiply reflected and refracted within it, since the cornea consists of a superposition of several transparent layers with different refractive indices. If observed on a screen, the light reflected by a particular corneal layer forms the image of the corresponding corneal cell structure. The reflected beam becomes strongly divergent because of corneal curvature and the cell images formed by the experimental setup undergo a certain amount of magnification so that they become visible to the eye, but are not localised by the optical mechanisms used.

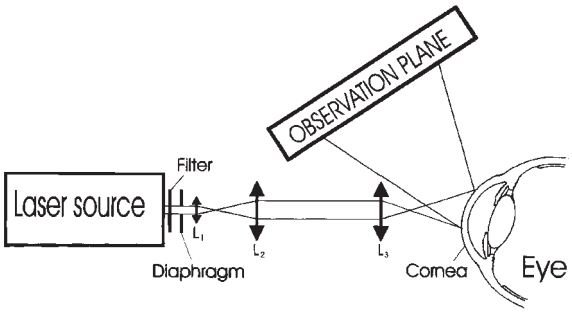

Fig. 1. Experimental set-up. L_1–L_2, collimating system; L_3, lens controlling the divergence of the incident beam.

Mathematical modelling of the image formation which takes place within the cornea allows the computation of the magnification factor of the system as a function of beam divergence. This allows the actual diameter of a corneal cell, within the experimental limits, to be deduced from the diameter of its projected image.

EXPERIMENTAL SET-UP

A He-Ne laser (632.8 nm) was used as the main light source, operating in TEM_{00} mode and the beam was attenuated by a filter so that its output did not exceed 0.05 mW in order to avoid retinal damage. An argon ion laser was also used at wavelengths of 495 and 514.5 nm, and the power output was also set at less than 0.05 mW. An iris diaphragm was mounted at the laser output aperture and adjustment of this allowed the number of cells to be investigated to be modified. The experimental arrangement used to obtain images is shown in Fig. 1.

The L_1–L_2 lens system was used to create a parallel laser beam (about 0.1 mrad divergence). A set of convergent lenses L_3 with focal distances ranging from 4 mm to 150 mm was used to alter the degree of divergence; L_3 was mounted in such a way as to localise the focal spot in front of the cornea with a maximum divergence of 0.4 rad. A pinhole was initially inserted between L_1 and L_2 in order to exclude any non-parallel rays from the collimated beam, but the improvement in contrast was negligible. In order to improve the images noticeably further stabilisation would have

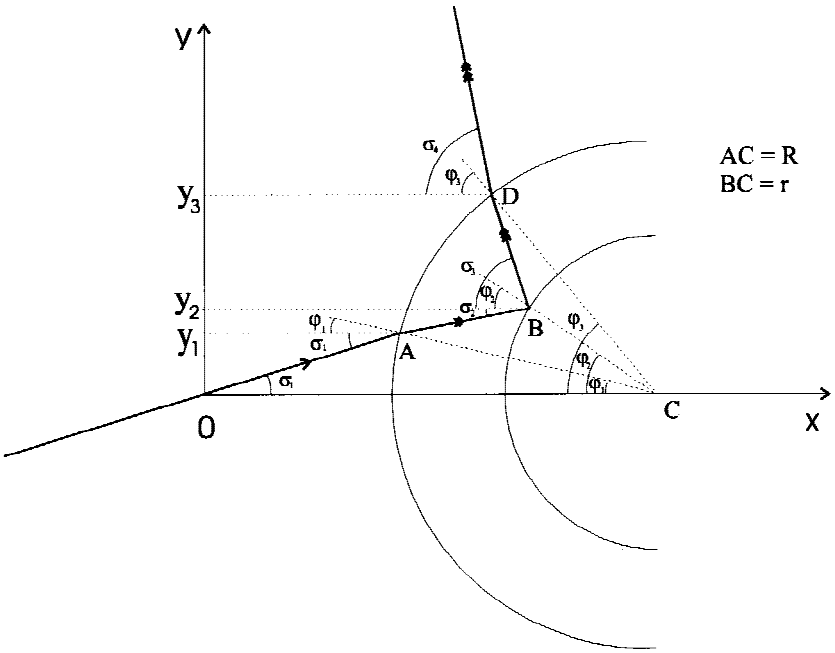

Fig. 2. Two-dimensional optical scheme for image formation.

been necessary which would have detracted from the simplicity of the set-up, and this approach was therefore abandoned. The reflected beam projected images of the corneal cells onto a screen which could be mounted at a variable distance from the eye being examined in order to obtain the best image quality in the observation plane (Fig. 1).

Studies were carried out in vivo on anaesthetised rabbits, cared for in accordance with EC guidleines [3]. Observed images could be recorded in the observation plane either using a still camera or by video.

RESULTS

Theoretical Considerations

The images obtained and the measurements derived from them are based on the multiple reflections of laser light at the interfaces from corneal layers possessing different refractive indices.

The incident laser beam is multiply reflected and refracted within the several layers of the cornea, and the radiation reflected by any particular corneal layer forms an image of its cells on the viewing screen. In the rabbit eye, the measured radius of a section through the cornea at epithelial level is 8.08 mm in the vertical plane and 8.36 mm in the horizontal plane. The reflected beams containing information about corneal structure become strongly divergent because of the curvature of the cornea.

Magnification depends on the depth of the reflecting layer and the divergence of the incident beam. The reflection coefficient of a reflection from the interface between two media of refractive indices n_1 and n_2 (for normal incidence) is:

$$r = \left(\frac{n_2 - n_1}{n_2 + n_1} \right)^2 \qquad (1)$$

Thus, the greater the difference between refractive indices, the more the laser beam is reflected.

The images of the greatest interest obtained from the cornea in vivo originated from the lachrymal fluid–epithelium and endothelium–aqueous humour interfaces. The best images obtained were, therefore, of the epithelium and endothelium.

In order to find a theoretical formula for the optical magnification which defines the ratio between the real cell diameter and that of its magnified image, the cornea was assumed to be an homogeneous spherical shell with concentric inner and outer surfaces. This is an acceptable approximation when considering the central corneal region alone [4].

The optical path of a laser beam within a vertical section of cornea is shown in Fig. 2, where the outer surface has a radius of

curvature R and the inner surface a radius of curvature $r = R - e$ (where e is the thickness of the cornea). In Fig. 2 a light ray incident on the corneal surface and its subsequent path through the cornea is considered. Figure 2 shows only the ray produced by reflection from the inner corneal surface. O is the point at which the ray intersects the optical axis of the eye and this is the origin of a rectangular coordinate system XOY where the optical axis of the eye is the abscissa. C is the centre of curvature of both the inner and outer corneal surfaces. The points where the incident ray intersects the two surfaces are given as A, B and D. σ_k ($k = 1 \ldots . 4$) is defined as the angles between the rays and the optical axis of the eye, i_j ($j = 1 \ldots . 5$) as the angles between the rays and their respective normals at the surfaces and φ_i ($i = 1 \ldots . 3$) as the angles between the normals of the surfaces (at the incident points) and the optical axis. The values Y_k ($k = 1 \ldots . 3$) are the ordinates of the incident points.

The first task is to establish, within the small angles approximation, a relationship between the tilt angle of the incident ray (σ_1, Fig. 2) and that of the emerging ray (σ_4) [4]. From the refraction low applied to points A, B and D it can be shown that

$$\sigma_4 = \sigma_1 + 2 \cdot n \cdot \varphi_2 - (n - 1) \cdot (\varphi_1 + \varphi_3) \qquad (2)$$

Assuming $e \ll R$ and $r/R \cong 1$ and differentiating the relation becomes

$$d\sigma_4 = d\sigma_1 + 2 \cdot d\varphi_2 \qquad (3)$$

Integrating eq. (3) over an interval corresponding to the diameter of a single cell, on the inner surface of the cornea

$$\Delta\sigma_4 = \Delta\sigma_1 + 2 \cdot \Delta\varphi_2 \qquad (4)$$

Keeping the small angles approximation (Fig. 3):

$$\Delta\varphi_2 = \frac{\Delta y}{r}$$
$$\Delta\sigma_4 = \frac{\Delta Y}{L} \qquad (5)$$

where Δy is the real cell diameter, ΔY is the diameter of the projected image of the cell and L is the distance between the eye and the screen. $\Delta\sigma_1$ is the divergence of the beam illuminating a single cell which may be

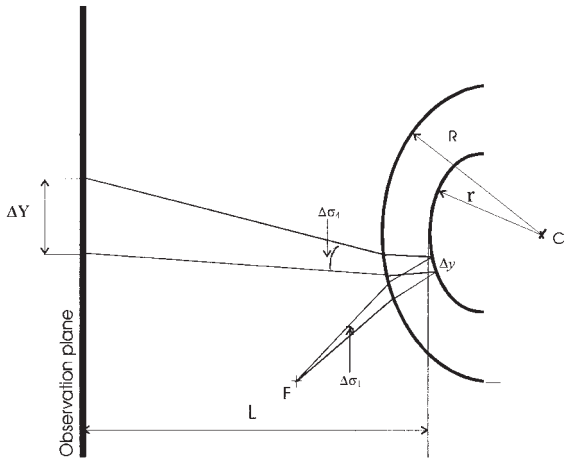

Fig. 3. The formation of a single cell image. ΔY, Δy are the dimensions of the image and the cell, respectively.

expressed as a function of the divergence of the incident beam, α:

$$\Delta\sigma_1 = \frac{\Delta y}{\omega} \cdot \alpha \qquad (6)$$

where ω is the diameter of the incident beam at the corneal surface.

The second task is to find an expression which defines the optical magnification β. By definition:

$$\beta = \frac{\Delta Y}{\Delta y} \qquad (7)$$

and consequently, for the endothelial cell, the magnification β_{en} is:

$$\beta_{en} = \frac{L}{\omega} \cdot \alpha + \frac{2 \cdot L}{r} \qquad (8)$$

An analogous expression found for the epithelial cell is:

$$\beta_{ep} = \frac{L}{\omega} \cdot \alpha + \frac{2 \cdot L}{R} \qquad (9)$$

Equations (8) and (9) show the dependence of the magnification on both the distance L between the screen and the corneal surface and the divergence of the incident laser beam.

Figure 4 demonstrates this dual dependence; the solid circles represent the experimental points in this study.

For $L = 40$ cm (a typical value), the minimum value for the magnification was obtained using a collimated He-Ne beam ($\beta = 114$). The maximum magnification obtained was 247 for a divergence of about 0.4 mrad.

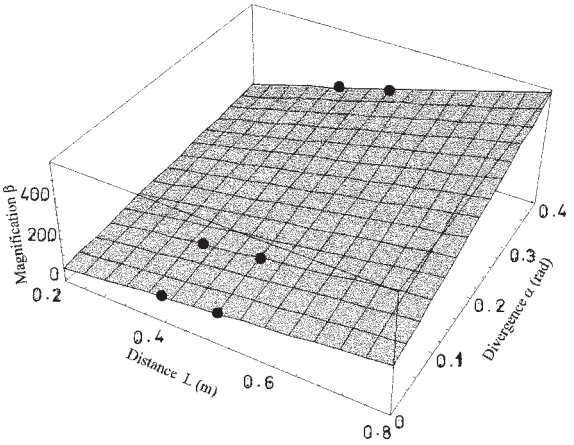

Fig. 4. The dependence of the magnification on distance and divergence (theoretically obtained).

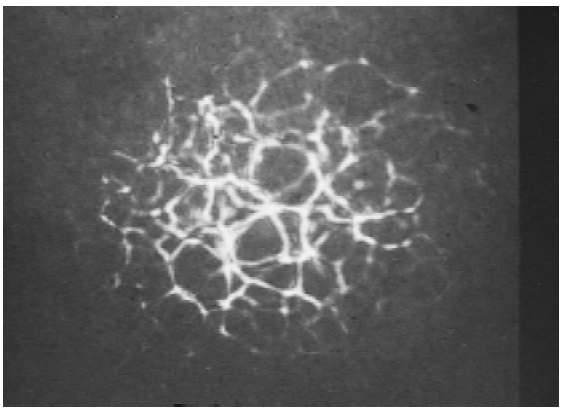

Fig. 5. Image obtained using a collimated He-Ne laser beam.

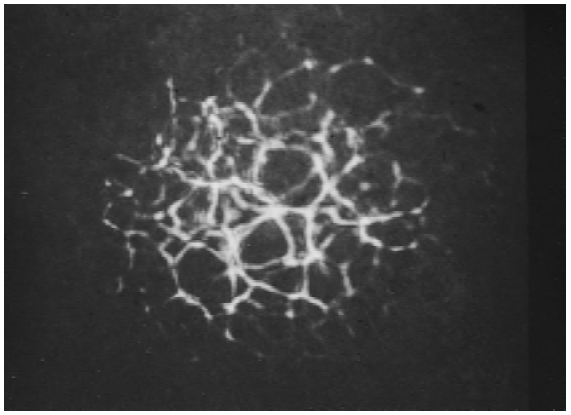

Fig. 6. Corneal cell image obtained with 0.2 rad divergence He-Ne laser beam.

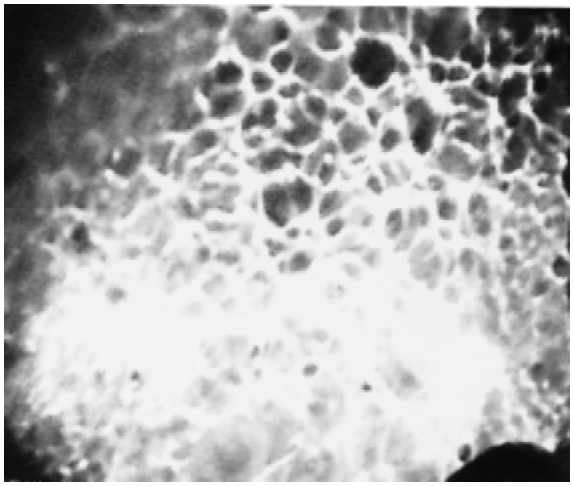

Fig. 7. Corneal epithelium image obtained with a 488 nm argon laser beam.

Experimental Results

Figure 5 shows an image using a He-Ne laser with a narrow collimated beam. The reflection from the the epithelial and endothelial layers gives rise to a superposition of images originating in these two corneal layers. In the central zone of the image the epithelial cells predominate and a less regular structure is revealed. In the image periphery, endothelial cells predominate and the intracellular membrane can be imaged at lower levels of illumination. Endothelial cells appear more regular and are more clearly separated. Reflected light from the peripheral zone of the cornea originated predominantly from the endothelial cells because the angle of incidence is greater, approximating to the reflection angle limit, which, according to our calculations, is approximately 78°. Figure 6 shows the endothelial image obtained using a He-Ne laser beam with a divergence of 0.2 rad. In this case the increased incident angle of 45° produced a better image of these more localised cells. A central overlap of the epithelial and endothelial cell images is still visible, but this is not as marked as that seen in Fig. 5.

Figures 7 and 8 show the results obtained when using a 488 nm argon ion laser with a larger beam diameter than that of the He-Ne laser. The multilayered structure of the cornea is responsible for the overlap of images seen in these pictures. Figure 8 shows the more regular hexagonal shapes of the endothelium, particularly in the peripheral zone.

A basic epithelial picture is shown in Fig. 9, with superimposed images. This image was obtained using the He-Ne laser with the beam at normal incidence to the cornea. The lachrymal film may overlap the other structures of the cornea, giving rise to the predominant image; this depends on the angle of incidence

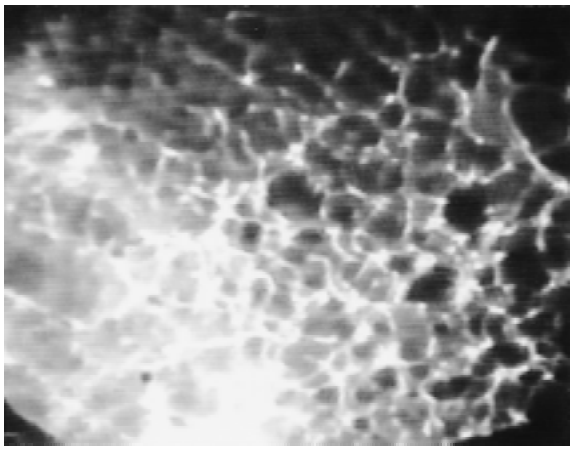

Fig. 8. Image obtained using a argon laser beam. The endothelium is more clearly seen at the peripheral zone.

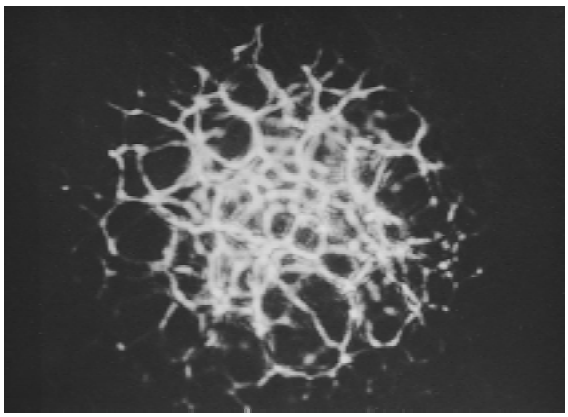

Fig. 9. Epithelial superposition obtained with a He-Ne laser beam at a normal incidence.

of the laser beam. The lachrymal film lacunae are clearly seen when imaged with the argon laser at 514.5 nm (Fig. 10).

This imaging technique allows us to measure the size of the epithelial and endothelial cells from the images obtained. These measurements were taken from the projection of the reflected beam on the screen. The diameter of any given cell can be derived approxi-

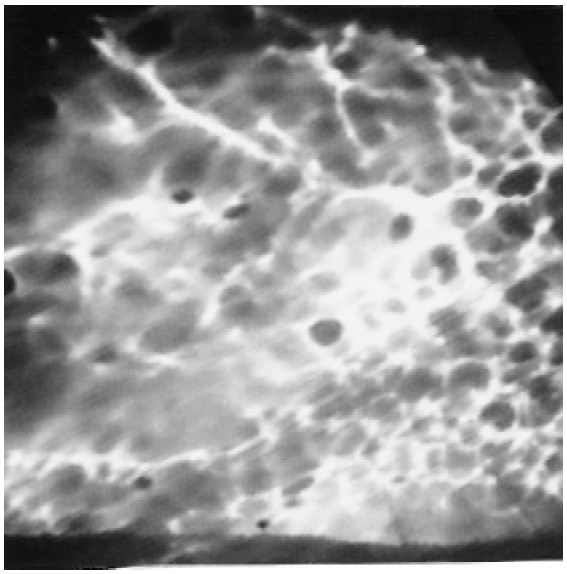

Fig. 10. Lachrymal film image obtained using a 514.5 nm argon laser beam.

mately using the magnification parameter β calculated as described above. The calculation was carried out for several values of incident beam divergence and distance. Several cells were randomly chosen and an average cell diameter was calculated as an arithmetical mean value. These data are presented in Table 1.

The main source of error in these calculations is lack of precision in the experimental measurement of beam divergence and the angle of incidence of the incoming beam. Although divergence can be measured in the far field with a fair degree of precision, it is difficult to get an accurate measurement of the angle of incidence of the incoming beam on the rabbit cornea. The errors involved are in the range ± 10% and the majority of this error comes from incidence angle inaccuracies. A subsidiary source of error lies in the measurement of the distance from the rabbit eye to the observation plane and this in turn affects the

Table 1. Endothelial cell diameters determined using He-Ne laser beam

Distance D (cm)	Divergence α (mrad)	Magnification β	Image diameter ΔY (mm)	Real diameter Δy (μm)
40	0	114	4	35
40	86	149	5	34
40	400	247	9	36
50	0	143	5	35
50	86	186	6.5	35
50	400	342	12	36

accuracy of cell diameter measurement within the range ± 10%.

There are also computational errors. The most important of these concerns the approximations made in calculating the magnification factor β which contributes a further error of ± 1%.

The overall error in calculating cell size shown in Table 1 is therefore in the range of ± 21%.

DISCUSSION AND CONCLUSIONS

The images of the inner corneal layers shown in Figs 5–10 provide information concerning corneal structure. Images from different depths of the cornea can be obtained by varying the laser beam divergence and angle of incidence. Images of the endothelium are most important clinically; these are shown in Figs 5, 6 and 8. The images are specific to the illumination conditions.

The problem of image stability needs to be considered. The eye is subject to rapid rotational movement on two axes and this leads to instability and lack of clarity of the images obtained. It is possible that this problem could be overcome by the use of a short pulsed laser.

The production of reliable images depends on the relationship between the incident beam divergence, the distance at which the image is measured and the magnification factor (equations 8 and 9). Using these parameters an average value of 34–36 μm for the diameter of the imaged cells was obtained. The error of ± 21% in this measurement arises from errors in divergence measurement, cornea-to-image distance and the calculation of β as outlined above.

The authors believe that there are no other reports in the literature relating to the measurement of the dimensions of corneal epithelial and endothelial cells in the rabbit so the accuracy of our measurements cannot be compared with other methods [5]. The results given by Garcia-Elskamp et al. [6] suggest that rabbit corneal cells are similar in size to human ones and if so, this means that our measurements are reasonably accurate within the limits of experimental accuracy. The method we have described should enable investigators to measure differences in cell size within the various zones of the cornea.

The main advantage of this method over others in common use is that images can be obtained non-invasively over a wide corneal field (limited only by the size of the incident beam) without contact with the cornea, without the use of additional magnification and without the necessity for electronic reconstruction of the images obtained.

We would hope that, when applied to the human eye, this method of measuring corneal cell size will give rise to better images and hence more accurate results because of the ability of the patient to co-operate with the investigator.

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