D.

Discussion

In this study, we have successfully adapted the senescence-associated β-galactosidase histochemistry protocol of Dimri et al. for use in the posteroir pole of the primate eye. The indigogenic method using X-Gal forms a granular precipitate that can be at least partially visualized in the presence of melanin pigment. Our procedure yields uniform staining of the RPE at pH 4.0, which would be expected for lysosomal β-galactosidase. It is of interest that the lysosomes of other cell types were much more weakly stained with this protocol. This finding undoubtedly reflects the very active lysosomal compartment of the RPE in comparison to other cell types in the retina/choroid. Staining at pH 4.0 for prolonged periods of time did reveal the presence of lysosomal β-galactosidase in other cell types (data not shown). Staining for senescence-associated β-galactosidase at pH 6 was uniformly negative in eyes from animals 1 or 2 years of age and yielded intermittent staining of the RPE monolayer in eyes from animals 16 and 29 years of age. It was especially interesting that RPE cells adjacent to cuticular drusen stained for senescence-associated β-galactosidase. A rhesus macaque at 29 years is equivalent to an 85- to 90-year-old human. Quantitative data for the appearance of senescence-associated β-galactosidase-positive cells as a function of chronological age or topographical distribution in the posterior pole were not provided in this brief study because of the very limited number of globes that were examined.

Tissue fixation and embedment protocols proved to be a critical aspect of our method. We found, for example, that postmortem times to fixation in excess of 6 hours yielded RPE lysis and inconsistent staining results (data not shown). Our data suggest that both well-controlled fixation and cryoprotection improved the reliability of our results. These observations are consistent with comments in standard histochemistry texts on the sensitivity of lysosomal β-galactosidase to aldehyde fixation. Fixation and tissue processing for the senescence-associated β-galactosidase activity at pH 6.0 may have identical or differing sensitivity to tissue-processing protocols. Currently it is not known how the pH 6.0 senescence-associated β-galactosidase activity is related to the pH 4.0 lysosomal β-galactosidase activity. These activities may be a function of identical or differing posttranslational modifications of the same gene product or may arise from different gene products.

Our permanganate-bleaching protocol resulted in a method that might prove useful for applications other than our histochemical procedure. By extensively fixing sections after the enzyme histochemistry step, we were able to bleach a very substantial percentage of all pigment with the retention of both adequate morphology and chromogenic product. Because color-based alkaline phosphatase protocols for in situ hybridization and immunohistochemistry can use chromogens that are resistant to bleaching, our protocol may be directly applicable to these methods.

This study has identified tissue processing as a very significant issue that must be carefully controlled in any planned studies of the human eye. It will be important next to apply these procedures to the human eye for an investigation of the presence of senescence-associated β-galactosidase activity in the RPE.

References


A New Video Pachometer

Jay W. McLaren and William M. Bourne

Purpose. Many studies of the cornea would benefit from a simple, objective method to measure corneal thickness. In this study, a new optical pachometer based on video and computer technology was designed and tested.

Methods. The slit beam of a photographic slit lamp was monitored with a video camera through one half of the biomicroscope. When the slit was properly aligned with the cornea, the operator triggered a flash, and one video

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Methods. The slit beam of a photographic slit lamp was monitored with a video camera through one half of the biomicroscope. When the slit was properly aligned with the cornea, the operator triggered a flash, and one video frame that included the flash was captured. A custom software package detected epithelial and endothelial edges. Corneal thickness was calculated from the median corneal image width and image widths from similar measurements of contact lenses with known thicknesses. Corneal thickness was measured in 25 subjects by using this new instrument and was compared to thickness measured by using a conventional Haag-Streit pachometer.

Results. Corneal thickness in the 25 subjects measured on the new instrument was $512 \pm 20 \mu m$ and $515 \pm 21 \mu m$ in the right and left eyes, respectively (mean ± SD). Thickness of the same corneas measured on the Haag-
Accurate and precise measurements of corneal thickness are critical in studies of normal corneal physiology and in assessing corneal diseases and managing treatment. The wide acceptance of keratoreflective procedures also demonstrates a need for simple, repeatable, and accurate measurement of corneal thickness.

Several optical pachometers have been used in studies of corneal thickness. One of the simplest is manufactured by Haag-Streit and attaches to their slitlamp. A trained observer can measure thickness to within 5 μm to 10 μm, but because the end point is subjective, this method can have considerable systematic errors between observers and between measurements by the same observer at different times. Another optical instrument, the Orbscan Topography System, recently has been used to map thickness of the entire cornea. This system is objective and is based on video images of a slit that is scanned across the cornea. Corneal thickness also has been measured by noncontact specular microscopes. Recently, Siu described a video pachometer, although little information was given on precision and accuracy.

Ultrasonic pachometers and contact specular and confocal microscopes also have been used to measure corneal thickness. These instruments are objective, and variability between operators is low. However, they must contact the cornea after topical anesthetic, a practice that in many studies may directly affect the thickness being measured and may mask or enhance physiological changes that are being studied. Accurate and precise measurements of corneal thickness are critical in studies of normal corneal physiology and in assessing corneal diseases and managing treatment.

In this study, we designed a noncontact pachometer that is simple to use and provides precision equal to that of the Haag-Streit pachometer. (Invest Ophthalmol Vis Sci. 1999;40: 1593–1598)

**METHODS**

**Instrument Description**

A low-level video camera (CCD 72; Dage MTI, Michigan City, IN) was mounted on the camera arm of a photographic slit lamp (Zeiss, Thornwood, NY). This camera monitored the view through the left optical element of the binocular biomicroscope. The microscope was adjusted so that the axis of this view was aligned perpendicular to the cornea. The slit beam entered the cornea at an angle of 55° to this axis, similar to the arrangement described by Mandell and Pole (Fig. 1). The beam width was set to 0.1 mm, and the magnification of the microscope was set to ×25.

Three small lights assisted in aligning the microscope and slit beam with the cornea. A dim green fixation light, 2 mm in diameter, was mounted between the left and right objective lenses. Two brighter red alignment lights were mounted at the vertical center, one on each side of the objective lens assembly, approximately 60 mm from the cornea. The subject fixated the green target while the operator positioned the image of the slit in the cornea between the Purkinje images of the two red lights. This condition ensured that the optical axis of the camera was perpendicular to the cornea. A cyan filter in the right eyepiece blocked the reflected image of the red lights to make their image visible only to the operator’s left eye, the same view as monitored by the camera. A reticle in the left eyepiece served as a reference for vertical alignment.

When the microscope was properly aligned, the operator initiated recording a single video frame by stepping on a foot switch. This set the camera to an integrate mode, triggered the photoflash, and immediately collected and transferred one video frame into the memory of a dedicated laboratory computer. The program saved the image on magnetic medium, normalized it, and compared corneal thicknesses as measured by the new instrument and a modified Haag-Streit pachometer.

**Corneal Thickness from Each Image**

Each image was stored as an array of pixels (460 pixels high by 640 pixels wide); each pixel (8 bits, or 256 intensity units) represented the brightness of its respective location on the image. Corneal thickness on any horizontal line can be determined from the number of pixels that represent the bright image of the slit beam within the cornea, if the distance represented by each pixel and the angle between the beam and the surface of the cornea are known.

On each horizontal scan line the intensity of the corneal image increased to a peak at the epithelium and then dropped to a plateau in the stroma. On most scan lines, intensity rose to a second smaller peak before dropping at the endothelial edge. The edges on each line were determined by the intersection of a line fitted to the maximum slope of the rise (epithelial) or fall (endothelial) of intensity and the respective background intensity. The background intensity was assumed to be the mean intensity in a 20-pixel by 20-pixel area centered on the hori-
FIGURE 1. Instrument design. A video camera monitored the slit image in the cornea through one half of a Zeiss biomicroscope. When the slit lamp was properly aligned with the cornea, the operator pressed a foot-switch that triggered the flash and captured one video frame. The video digitizer transferred the image to a laboratory computer that determined the width of the cornea image and cornea thickness. Two red lights were used for alignment, one mounted on each side of the objective lens. The operator positioned the slit in the cornea between the Purkinje images of these lights, to assure that the cornea was perpendicular to the optical axis of the microscope. A red-blocking filter in the right eyepiece made these lights visible only through the left half of the microscope.

In most images corneal widths (number of pixels between the epithelial edge and the endothelial edge) from the 460 scan lines were normally distributed, with several outliers that originated from regions where the beam passed through and illuminated debris in the tear film. The median width better represented the center of this distribution than the mean did, and the median was used as a representative image width to calculate cornea thickness. The field of view in the vertical direction included 2.2 mm of the central cornea, and our measurement represented the median of this entire section.

In early experiments, we found that the apparent corneal thickness increased slightly as the peak intensity at the epithelial edge brightened, a common effect of most video systems. This relationship was approximately linear, and a simple transformation gave an image width \( w \) that was independent of image intensity:

\[
w = m_0 \left( P_0 - P_{\text{ep}} \right) + w_0
\]  

where \( P_{\text{ep}} \) is the mean intensity of the epithelial peak, \( w_0 \) is the original median image width, and \( m_0 \) and \( P_0 \) are constants determined by calculating the image width of 10 corneas of 5 subjects at several flash intensities. \( P_0 \) was arbitrarily set to 100, a typical peak intensity. The power supply of the flash was set to either level I or level II during measurements.

Calibration

The instrument was calibrated by measuring the image width of 6 polymethylmethacrylate contact lenses with known thicknesses. The linear relationship between image width and the known thickness was used to convert corneal image width to corneal thickness, \( q \):

\[
q = r(m_0 \cdot w + b_0)
\]  

where \( m_0 \) and \( b_0 \) are the slope and intercept, respectively, of the line fitted through the contact lens thicknesses in micrometers, graphed as a function of image width in pixels, and \( r \) is a factor that adjusts for the difference in refractive index between the cornea and the contact lens.

The factor \( r \) is numerically equal to the ratio of the image width of the cornea to the image width of the contact lens when the true thicknesses are equal and is dependent on the angle between the slit and the optical axis of the microscope, the curvature and thickness of the cornea, and the indices of refraction of the cornea and the contact lens. If we assume a corneal radius of curvature of 8 mm, an index of refraction of 1.376, and the angle of incidence of the video pachometer of 55°, then the ratio \( r \) is equal to 0.90 for corneal thicknesses between 400 \( \mu \)m and 650 \( \mu \)m. When the angle of incidence of the slit was 45°, as it was in the Haag-Streit pachometer, this ratio was equal to 0.91.

Corneal Thickness in Human Subjects

Corneal thicknesses in 25 normal human subjects were measured by using the video pachometer and by using a modified Haag-Streit pachometer. Each subject gave consent to participate after the nature of the study was explained according to a protocol approved by the Institutional Review Board of Mayo Clinic.

Each subject was seated at the chinrest and fixated the green target light. The microscope and slit were aligned with the center of the left cornea, and 10 video images were collected within 2 minutes or less. The process then was repeated on the right eye. Corneal thickness was determined from each image by using Equations 1 and 2. Within the next 30 minutes, corneal thickness was measured by using the modified Haag-Streit pachometer. Thickness of each cornea was measured 10 times, and the mean and SD of these measurements were
calculated. One operator measured thickness of all corneas on the video pachometer, and a second operator measured all corneas on the Haag-Streit pachometer. The second operator was experienced in using this instrument. Corneal thicknesses measured on the Haag-Streit pachometer were increased by 8% to compensate for systematic differences between the operator's measurements and the same corneal thicknesses measured on a specular microscope in a different study.

RESULTS

Images of the Slit in the Cornea
The reflex from the anterior surface of the cornea appeared as a bright line along the epithelial image of the slit (Fig. 2). On the endothelial surface, a reflex also was visible, but it was approximately half as bright as the peak reflex from the epithelial surface and was slightly brighter than scatter from the stroma (Fig. 3). Most frames also contained the dim Purkinje images of the alignment lights, evidence of proper or improper alignment (Fig. 2).

Blooming Correction and Calibration
The width of the corneal image increased as brightness of the epithelial peak increased. The mean coefficient of Equation 1 determined from 10 eyes was \( m_b = 0.063 \pm 0.01 \) (mean ± SD). Thus, if the brightness increased from 100 units to 150 units, the measured width would have been reduced by 3.2 pixels to compensate for blooming.

Image widths of the contact lenses increased linearly with thickness. The coefficients of Equation 2, as determined by least-squares regression were \( m_{cl} = 6.3 \mu m/pixel \) and \( b_{cl} = -136 \mu m \).

Corneal Thickness in Human Subjects
Mean corneal thickness in 50 eyes of 25 subjects as measured with the Haag-Streit pachometer was 532 ± 21 \( \mu m \) (Table 1). When measured with the video pachometer, mean thickness was 514 ± 20 \( \mu m \), approximately 18 \( \mu m \) thinner than it was when measured with the Haag-Streit instrument. Differences between instruments in both eyes were statistically significant \( (P < 0.001, \text{ paired } t\text{-test}, \text{ right} \).
FIGURE 3. Intensity profile through cornea (top) and contact lens (bottom). Each profile represents the mean of 460 scan lines aligned on the anterior peak. The bright image of the cornea illuminated by the slit originated from scattering and reflection at the tear film, scattering in the stroma, and weaker scatter at the endothelial surface. The anterior peak and slope is dominated by the bright reflex from the air–cornea interface. In contrast, the posterior slope is from the weak reflex from the cornea-aqueous humor interface superimposed on scatter from the decreasing width of illuminated stroma and has a lesser slope than at the anterior surface. Edges were assumed to be at the intersection of the line fitted through the slope of the changing signal at each surface with the local background. This method identified edges of the image more consistently than did methods that used position of peak intensities. Images of contact lenses that were used for calibration also had an anterior and posterior peak, but the posterior peak was brighter and had a greater slope than the endothelial edge of corneas. Very little light was scattered from the center of the contact lens compared to light scattered from the stroma.

and Wilcoxon signed rank test, left; differences in left eyes failed a normality test). The average variability of measurements, as indicated by the mean SD of the 10 consecutive measurements, was $6.6 \pm 1.5 \mu m$ and $6.7 \pm 3.3 \mu m$ for the Haag-Streit and video pachometers, respectively ($P = 0.85$, paired $t$-test).

The mean and SD of differences between the two methods was $18 \pm 13 \mu m$. The limits of agreement (mean ± 2 SDs
of the difference)\(^ 3\) was \(-7 \, \mu m\) to \(44 \, \mu m\). A regression of differences on means was not significant \((r = 0.05, P = 0.36)\).

**DISCUSSION**

The video pachometer is a practical tool for studying thickness of the cornea. Measurement is fast, the cornea is free from contact, and the procedure is well accepted by study subjects. The binocular view and alignment lights make the device easy to align consistently without fatigue that often accompanies peering through a monocular eyepiece. Precision and accuracy are improved by capturing the video image during an interval of a few microseconds of the photographic flash. Unlike optical pachometers that rely on subjective alignment of two images, the edges of the cornea and corneal thickness are determined objectively by a computer algorithm and should be consistent from session to session and between operators. Precision is comparable with precision of the Haag-Streit pachometer and is acceptable for most studies.

Corneal thicknesses in normal subjects reported by other investigators have typically been \(515 \, \mu m\) to \(530 \, \mu m\), although some have found thicker corneas.\(^4\) Population SDs have typically been \(20 \, \mu m\). Mean corneal thicknesses in our sample of 25 subjects measured with the video pachometer were \(515 \pm 21 \, \mu m\) and \(513 \pm 20 \, \mu m\) (left and right eyes, respectively) and were consistent with these figures, although slightly lower than most measurements. In the same group of subjects, the Haag-Streit pachometer indicated corneal thicknesses of \(534 \pm 20 \, \mu m\) and \(530 \pm 22 \, \mu m\) in right and left eyes, respectively, approximately \(18 \, \mu m\) greater than thicknesses indicated by the video pachometer, but within the range reported by others.

Differences between the two instruments may arise from systematic errors in either instrument in reporting true thickness. Alignment of the two images of the cornea when using the Haag–Streit pachometer is subjective, and variability of measurements and systematic differences have been attributed to subtle differences in this end point from operator to operator or from one operator on different occasions. Mandell et al.\(^5\) found that the SD of 10 measurements decreased from \(15 \, \mu m\) to \(4 \, \mu m\) after several sessions of training. Differences in absolute thickness measured by these two instruments also may be attributed to the video pachometer and the algorithm used to detect edges on each video scan line. The criterion for determining an edge was designed to be independent of the intensity slope, which may be different in the contact lens, where the slope is dominated by the bright reflex from the surface, compared with the cornea, particularly at the endothelium where the slope is dominated by the decrease in scatter from the stroma. This method was applied consistently, although it may have introduced small systematic errors that could appear in any pachometer.

Precision and objectivity of this video instrument also are comparable to that of the Orbscan pachometer,\(^6\) an instrument dedicated to corneal topography and pachometry. The video pachometer described here was made by modifying a photographic slit lamp that may already be available and may continue to be used for slit lamp photography as needed. Access to custom software that operates the video pachometer would allow flexibility of this instrument in other measurements, such as corneal curvature, corneal shape, or anterior chamber depth, if needed. This instrument will be the basis of studies of endothelial cell function and other processes that are revealed through changes in thickness of the cornea. Its use in patients and study subjects will allow rapid central corneal thickness measurements to examine changes during an experimental event or to monitor thickness as part of a corneal examination.

**References**