Study of Precorneal Tear Film Thickness and Structure by Interferometry and Confocal Microscopy

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Past measurements of precorneal tear film thickness in rabbits and humans gave values of 4–7 μm. These have been the accepted values. However, measurements presented in this report are much higher. Earlier techniques may have changed film thickness or not have fully included the layer containing mucus. Little is known about the mucus component. Thickness was measured in freshly killed animals of 10 species using two independent optical techniques. Coherent light was reflected from eyes and thickness was determined from spacing of interference fringes. Clear images of tissue structure were obtained with confocal microscopy, the tear film was visually identified, and thickness measured. Measurements by the two methods were closely correlated in each animal. No film was detected in trout or carp. In other species, thickness ranged from 9 μm in frogs to 15 μm in gerbils. Values are significantly larger than earlier estimates. Film thickness in three living rabbits was not significantly different from that measured shortly after death. There was no variation in thickness at five positions across the cornea in three species. Mucus content of rat tear film was examined by measuring thinning after application of 4, 8, and 20% (weight/volume) acetylcysteine, a mucolytic agent. Thickness was considerably reduced compared to controls, and the film almost completely removed by the highest concentration. The authors propose that the film is largely composed of mucus, not free aqueous solution. Our methods include such layers, and measurements are correspondingly larger than earlier ones. Invest Ophthalmol Vis Sci 33:1996–2005, 1992

In previous studies, tear film thickness was measured by several methods that gave similar results. Mishima used glass fibers that were laid in the tear film of rabbits. Thickness was taken to be the diameter of fiber that appeared level with the surface, about 7 μm. Fluorescein also was used by Mishima to measure film thickness. Rabbit eyes were vigorously washed and tear fluid was replaced with fluorescein solution. Thickness was determined from fluorescence to be 6 μm. The addition of fluorescein, without removal of the existing film, was employed by Benedetto in human eyes. Dye lost through drainage was estimated, and based on dilution of the remaining dye, thickness was calculated to be 4 μm. Ehlers soaked up human tear film with filter paper disks and measured the weight absorbed, from which volume and thickness of film fluid were calculated. Thickness was estimated to be about 7 μm.

A structure and composition of the tear film was proposed by Wolff from histologic studies of the eye surface and observations of the tear film with a slit lamp. The film seemed to be composed of three layers: (1) a lipid layer at the air surface; (2) a middle aqueous layer; and (3) a mucus layer on the epithelial surface. McDonald, using the colored interference fringes formed in reflected white light, measured the surface lipid layer to be less than 0.5 μm. Nichols, using electron microscopy of frozen sections, measured the inner mucus layer in guinea pigs to be 1 μm. Holly proposed a mucus concentration gradient, with concentration rising near the epithelium and becoming more dilute toward the tear film surface.

The above measurements of tear film, mucus, and lipid thickness have established a view that the film is about 7 μm thick and is a three layered structure in which free aqueous fluid is the main component. We set out to review this critically because the methods reported may have associated problems. When glass fibers are used for film thickness measurement, surface tension needs to be overcome to place the fiber beneath the surface. Sufficient pressure must be maintained to ensure that the full tear film thickness is occupied by the fiber. Otherwise, measurements would be smaller than the space available. It has been observed recently that such measurements leave a dent in the film surface for up to 10 sec (observation by Professor D. M. Maurice, Stanford University Medical Center, Stanford, CA, and Dr. 1996
J. I. Prydal, University of Cambridge, UK). Thus, this method can be considered to change the structure being measured. Indentation may be of underlying epithelial cells, but it also is possible that the film is a visco-elastic structure and that fibers are too flexible to be pressed through mucus, although sufficiently stiff to deform it.

Any method that seeks to replace the surface tear film with a measurable dye solution must fully replace the original film. Fluid exchange with a hydrophilic film with a measurable dye solution may be slow and may not replace all fluid with dye solution. However, if washing is too vigorous, these layers may be detached. If only part of the film is replaced, measured values will be too small. Dye dilution methods assume that dye has access to all water space and distributes evenly throughout the film. But most of the water in a mucus gel is intramolecular and fluorescein may be excluded from a significant volume.

Similar considerations apply to the use of filter paper for absorbing tear fluid and the weighing of it to determine thickness. Removing all free water from the film or all intramolecular water in mucus may not be possible. However, if removal is too thorough, excess fluid as well as cells may be removed.

Measurements of mucus thickness also need to be reconsidered. Preparation of specimens for microscopy can remove much mucus. This may be a special problem and it is considered further in this report.

As a result of these considerations and the availability of new technologies, we have examined two questions: How thick is the tear film and how much mucus does it contain? New methods for imaging and recording have become available, making possible measurements that could not have been performed reliably in the past. Two independent optical methods were used.

The first method used coherent light reflected from the eye to form interference fringes. Thickness was measured from fringe separation. Interferometry was used earlier to measure corneal thickness. The technique was modified to be more sensitive and reliable in the range for measurement of tear film thickness. Advantages of the fringe technique were that film structure was not disturbed, it could be used in living, conscious animals, and continuous recordings enabled measurement of changes with time. However, the specific identity of layers contributing to fringes could not be determined from fringe spacings alone. To identify these layers, a second technique, confocal microscopy, was used to measure thickness in the same eyes.

Confocal microscopy can be considered to be more 'direct.' Images were thin optical 'sections' through the cornea, and this enabled visual identification and accurate localization of tear film and epithelial surfaces. Thus, the separation between surfaces included all mucus. Measurements were made with the microscope focus control. This required very stable, immobile tissues, such as those in freshly killed animals.

Ten species were examined with both methods. They were from different habitats, with different environments impinging the eye, and had a variety of eye sizes and structures to test the methods. The range of tear film thickness was investigated, but statistical distribution or population data was not sought.

Information about the mucus content of the tear film was obtained by measuring the reduction in thickness caused by a mucolytic agent.

Materials and Methods

Interferometry

Interference between two reflections forms a pattern of fringes. Reflections from various corneal layers with different refractive indices give rise to sets of interference fringes, each with a spacing that corresponds to the separation between two surfaces. The largest change in refractive index and thus the brightest reflection is from air/tear interface. Fringes formed between this reflection and reflections from deeper layers are the brightest, although of low contrast because the two reflections are of different intensities. Fringes formed between reflections from two layers within cornea are obscured by brighter fringes. Thus, measurements are of the separation between external tear surface and deeper layers. A small peripheral part of circular fringe patterns was formed with our optical system, and fringes appeared as almost straight parallel lines. Thickness can be measured from fringe separation by calibration with artificial layers of known thickness, or calculated with theoretical equations.

The apparatus is illustrated in Figure 1. A 2 mW Helium-Neon laser beam was focussed with a 10X microscope objective (Leitz, Wetzlar, Germany) in a 20 μm pin hole. This spatial filter removed irregular variations in intensity across the beam and reduced noise. The resulting diverging beam was focussed by a 40X microscope objective (Nikon SLWD, numerical aperture 0.40; Tokyo, Japan) in front of the eye and animals were positioned so that light formed a spot 1 mm in diameter on the tear film surface. Use of a slightly defocused beam reduced irregularities in interference patterns from dust on the tear film surface. Also, light energy was distributed over a wider area to minimize warming of tissues. Reflected light was collected by a 20X microscope objective (Nikon SLWD, numerical aperture 0.35) and imaged with a CCD video camera (Sony AVC-DSC, Shinagawa-ku, Ja-
Interference fringes were formed on the light sensitive surface of the camera, so their focus was not altered by changes in lens or camera position. However, the size of video images was sensitive to changes in focus of the light spot on the eye surface. This was used to confirm that the spot was 1 mm in diameter. Images were recorded with a VHS video recorder with digital frame store (Sony SLV 401UB; Alasce, France). Continuous recordings could be reviewed later to select individual frames that showed clear regular fringes. This significantly improved the accuracy of the technique.

Thin layers produced 'widely' spaced fringes, and thicker layers gave narrower spacings. Lenses were of relatively large numerical aperture to increase the total number of fringes across the image. This made the apparatus more sensitive in the range of tear film thickness. Lenses with a larger numerical aperture could not be used because of their shorter working distance. The widest spacing that could be measured corresponded to a thickness of about 4-5 μm. Resolution of video recordings determined the upper limit of measurements, about 200 μm.

Video recordings of 10-30 sec were made. Fringe spacing was determined using Fourier analysis. There were patches in images where fringes were straight and regularly spaced. In other parts, however, they were not clear. Some were obscured by noise and others were of low contrast. Fourier analysis of whole images gave several peaks in plots of power against fringe spacing. Therefore, a specific spacing could not be determined by this method.

Analysis of the parts of images with clear regular fringes consistently gave single sharp peaks in the Fourier transform. An independent standardized procedure was used to select the area with the clearest fringes for measurement. The whole image was divided into sections. Each was analyzed and a measure of the contrast and regularity of fringes was used to determine the one from which measurements were to be made. Disturbances in fringe patterns may have resulted from irregularities in reflecting surfaces. Dust and bubbles on the tear film surface altered fringe patterns. Also, epithelial surfaces probably were not optically smooth, particularly were cells were desquamating.

Four frames of recordings from each animal were selected and digitized at a resolution of 512 × 512 pixels and 256 gray levels (Imaging Technology Inc., Overlay Frame Grabber; Woburn, MA). Images were divided into sections the full width of the image and 40 pixels high. Adjacent strips were overlapped by 50% to prevent a patch of clear fringes from being split between two sections. All sections were analyzed. One dimensional Fourier analysis of horizontal lines was done perpendicular to vertical fringes. The Fourier transform of the whole section was determined by selecting the maximum power at each spacing from all lines of the section. Sections with clear fringes gave a single peak in a plot of power against fringe spacing. Height of the peak above a standard width was determined for all sections of an image, and fringe spacing was determined from the section with the highest value. Measurements also were made directly from the video monitor and were not significantly different (95% level) from values obtained using Fourier analysis. The widest fringe spacing that could be measured by Fourier analysis corresponded to a thickness of 4-5 μm, and the finest spacing corresponded to about 250 μm. The frame grabber digitized lines of images into 512 pixels and this determined the resolution of measurements, about 1 μm. Measurements from the 4 frames from each animal were averaged.

Calibration was done by measuring fringe separation from cylindrical sheets of known thickness, and the radius was matched to the cornea studied. Eight sheets of polyethylene terephthalate (refractive index, n = 1.65) or polyester ester (n = 1.65) with wall thickness of 6-120 μm were used. Thickness was measured with a microscope with calibrated graticule to view the edge of flat sheets (standard error of the mean, 0-0.35 μm).

Fringe spacing was determined by Fourier analysis of four frames from each cylinder. Figure 2 shows the calibration data, fitted curve, and confidence limits for one species. Thickness is linearly related to frequency of fringes, the reciprocal of spacing:

\[ t = a + \frac{b}{x} \]

where \( t \) = thickness (μm), and \( x \) = fringe separation (pixels).
Fig. 2. Sample calibration for interferometry measurements of *Rana pipiens*. Wall thickness of calibration cylinders is plotted against frequency of fringes (reciprocal of spacing) measured by Fourier analysis of four interference images from each cylinder. Circles = data points. Solid line = linear regression used to calculate tear film thickness. Gradient 733.87, intercept with ordinate 2.25, correlation coefficient greater than 0.99. Values were corrected for the different refractive indices of tear fluid and calibration material. Dotted lines = 95% confidence limits, ±2.5 μm at 12-μm thickness.

Constant, a, and coefficient, b, were calculated by linear regression analysis, eg, \( a = 1.89, b = 773.121 \). Correlation coefficients were greater than 0.998 and at thickness 12 μm, confidence limits were ±1.1–3.2 μm. Thus, the method of calibration was sufficiently accurate for measuring tear film thickness. Thickness calculated from this equation was corrected for the different refractive indices of tear fluid and calibration material:

\[
t' = t(n_1/n_2)
\]

where \( t' \) = corrected thickness, \( n_1 \) = refractive index of tear fluid, \( 1.337 \), and \( n_2 \) = refractive index of calibration material.

Two additional calibrations were performed with dry hydroxyethylmethacrylate contact lenses (\( n = 1.5 \)) 60–200 μm thick, measured with a microscope with graticule to view the edge of a section of lens. Values measured using these calibrations were within ±5% of those obtained using cylinders. Thus, they supported the accuracy of using cylinders to calibrate for spherical surfaces.

**Confocal Microscopy**

Confocal microscopy is a significant modification of scanning reflected light microscopy. The specimen is illuminated by a scanning point of light. Light reflected from each illuminated point is collected to build up the image, while light reflected from above and below the plane of focus is excluded. Images are high-resolution, thin optical sections through the specimen. With conventional microscopy, resolution of structures below the surface is greatly reduced by other reflections.

Oil immersion microscopy was needed to reduce reflections from the tear film surface and obtain good images of epithelium. A tandem scanning reflected light microscope (constructed at Charles University, Plzen, Czechoslovakia) was used with a 10× eye piece and 40× oil immersion objective (Zeiss [Oberkochen, Germany] Plan Apochromat, numerical aperture 1.0; Zeiss 518C immersion oil, \( n = 1.515 \)). Illumination was provided by a xenon arc lamp with a broad band yellow filter to reduce chromatic aberration.

The animal's head was put under a 4× dry objective with the cornea center in the center of the field. The objective lens was changed, immersion oil was placed on it, and the microscope stage was raised until there was contact between oil and tear film. The microscope fine focus was used to measure separations between surfaces. Eye position was adjusted so that images were circular. Optical sections then were tangential to surfaces, so measurements were of radial...
thickness. Optical sections were 1–2 μm thick and the size of circular images could be varied while focussed on a surface. They became larger as sections were moved deeper into the layer. Markings on the eye piece were used to ensure that images were the same diameter when focussed on the tear film and epithelial surfaces. Thus, optical sections were at the same depth in each layer, although edges of images were not sharp. This limited the accuracy with which a surface could be located. Ten measurements of tear film thickness were made, followed by 6–10 measurements of epithelial thickness.

The focussing mechanism was calibrated using eight sheets of polyethylene terephthalate or polyester ester, from 6–120 μm. The equation relating thickness to graduations of fine focus was determined by linear regression analysis (correlation coefficient 0.99; confidence limits at thickness 12 μm, ±1.8). Refractive index correction was made using the same equation used for correction of thickness measured by interferometry (tear film 1.337, epithelium 1.435).

Mucous Component

Mucus content was estimated by measuring the change in film thickness after a mucolytic agent was applied. Acetylcysteine reduces disulfide bonds, breaks down glycoproteins into smaller molecules, and reduces viscosity of mucus. Aqueous solutions of 4, 8, and 20% (w/v) were applied for 5 min to one of the eyes of three rats. Normal saline controls were applied to one of the eyes of two rats. Solutions were at room temperature (23–26°C) and were stirred manually throughout this period. Eyes then were irrigated for 30 sec with 30 ml normal saline to remove denatured mucus. Measurements of thickness were made by interferometry before the procedure and after by interferometry and then confocal microscopy. Microscopy was not used before application of acetylcysteine because oil immersion may have altered the action of the mucolytic agent.

Procedure

Interferometry and confocal microscopy were done consecutively in the same eye to measure tear film thickness. Measurements were made as soon as possible after death, with the eye undisturbed in the orbit. Interferometry was within 1–2 min, and confocal microscopy 2–10 min after death. This sequence was necessary because confocal microscopy had to be done with oil immersion, which would have altered measurements by interferometry. Measurements were made in the center of the cornea. Details about the species studied are given in Table 1. Eyes of chickens and pigeons were held open with cyanoacrylate.

Table 1. Species examined using interferometry and confocal microscopy, in order of increasing tear film thickness

<table>
<thead>
<tr>
<th>Species (number)</th>
<th>Weight/age</th>
<th>Preparation</th>
<th>Tear film thickness by interferometry</th>
<th>Confocal tear film thickness</th>
<th>Confocal epithelial thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainbow trout: Salmo gairdneri (7)</td>
<td>250–410 g</td>
<td>(a)</td>
<td>0</td>
<td>0</td>
<td>46.7 ± 4.8</td>
</tr>
<tr>
<td>Mirror carp: Cyprinus carpio (5)</td>
<td>16–24 g</td>
<td>(a)</td>
<td>0</td>
<td>0</td>
<td>46.3 ± 3.8</td>
</tr>
<tr>
<td>Frog: Rana temporaria (4)</td>
<td>22–33 g</td>
<td>(a)</td>
<td>10.1 ± 0.5</td>
<td>8.0 ± 0.4</td>
<td>31.0 ± 1.5</td>
</tr>
<tr>
<td>New Zealand white rabbit: Oryctalagus cuniculus (4)</td>
<td>3.8–5.8 kg</td>
<td>(c)</td>
<td>11.8 ± 0.4</td>
<td>9.6 ± 0.7</td>
<td>41.7 ± 1.3</td>
</tr>
<tr>
<td>Rhode island red cross chicken: Gallus galus (3)</td>
<td>2.4–2.9 kg</td>
<td>(a)</td>
<td>11.3 ± 1.1</td>
<td>10.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Wistar rat: Rattus norvegicus (8)</td>
<td>220–350 g</td>
<td>(b)</td>
<td>11.4 ± 0.6</td>
<td>11.4 ± 0.4</td>
<td>44.4 ± 2.0</td>
</tr>
<tr>
<td>Frog: Rana pipiens (4)</td>
<td>22–34 g</td>
<td>(a)</td>
<td>12.9 ± 0.8</td>
<td>12.2 ± 1.3</td>
<td>29.8 ± 1.7</td>
</tr>
<tr>
<td>Duck: Anas platyrhynchos (3)</td>
<td>2.2–2.8 kg</td>
<td>(a)</td>
<td>13.3 ± 0.5</td>
<td>12.2 ± 0.4</td>
<td>38.3 ± 1.0</td>
</tr>
<tr>
<td>Pigeon: Columba livia (5)</td>
<td>320–520 g</td>
<td>(b)</td>
<td>12.6 ± 0.4</td>
<td>15.9 ± 0.9</td>
<td>28.5 ± 1.7</td>
</tr>
<tr>
<td>Mongolian gerbil: Gerbilinace gribulus (4)</td>
<td>45–54 g</td>
<td>(b)</td>
<td>14.1 ± 1.0</td>
<td>15.6 ± 1.8</td>
<td>24.6 ± 0.4</td>
</tr>
</tbody>
</table>

Animals sacrificed by: (a) decapitation, (b) cervical dislocation, (c) intravenous pentobarbitone, 150 mg/kg. Results are mean values in each species ± standard error. Epithelial thickness could not be measured in chicken eyes.
glue between the lower eyelid and skin below the eye. *Rana pipiens* and *Rana temporaria* had been stored at 3°C for more than 2 wk. Two of each family were kept at 18°C, closer to normal habitat, for 72 hr before measurement.

Variation of tear film thickness across the cornea was examined at five positions in two rats, two rabbits and one chicken by the fringe method (at center, midway from it to limbus, above, below, left, and right). With the rats, animals were in addition to those used for measurements by interferometry and microscopy.

To test whether values were significantly different from those in living animals, measurements were made before and after death in eyes of three additional rabbits. Living animals were examined with interferometry. Confocal microscopy was not used because the technique required that tissues were immobile. Thus, accurate measurements were not possible. Rabbits then were killed with an overdose of intravenous pentobarbitone (150 mg/kg). Measurements were repeated, first using interferometry and then confocal microscopy. As in earlier experiments, interferometry was done within 1–2 min, and confocal microscopy was done 2–10 min after death.

All procedures conformed to the ARVO Resolution on the Use of Animals in Research. Experiments were performed at room temperatures of 21–27°C and relative humidities of 48–69%. Student's t-test was used to test statistical significance at the 95% level. Confidence limits were calculated at 95%.

**Results**

Interference patterns consisted of three sets of fringes, each of specific spacing. Fringes formed by thin layers were more widely spaced than those from thicker layers. Thus, the set of the most widely spaced fringes corresponded to the thinnest layer that could be detected. This also was the most superficial layer, because fringes were formed by interference between bright reflections from tear film surface and reflections from a deeper layer. Thus, the set of the most widely spaced fringes probably corresponded to tear film thickness. Such fringes were obtained from all animals studied except fish. They were seen in patches of images, and their regularity and contrast varied with changes in eye position and between animals. Use of continuous video recordings allowed images to be reviewed and the clearest frames to be chosen for processing. This made reliable and accurate measurements possible. Images from trout and carp eyes were ‘speckle’ patterns without fringes. Speckle is produced by interference in multiple scattered reflections from uneven surfaces.

Fourier analysis consistently gave measurements of the most widely spaced set of fringes. There was a single sharp peak in Fourier transforms corresponding to spacings of 38–85 pixels (image diameter 512 pixels), thickness 8–18 μm. A sample image and its Fourier transform are shown in Figure 3. The transform is of the section of the image selected by computer analysis as containing the most regularly spaced and highest contrast fringes. There was some varia-

![Image](https://via.placeholder.com/150)

Fig. 3. (A) Photograph of interference pattern from rat eye, showing fringes corresponding to tear film thickness. Fringes are vertical; three are indicated with arrows. Section marked is that selected by Fourier processing to contain most regularly spaced and highest contrast fringes. Section is the full width of the image, 512 pixels, and 40 pixels high. (B) Fourier transform of section marked in (A). Power (arbitrary units) is plotted against fringe spacing. Position of the peak gives fringe spacing of 64 pixels, corresponding to 11-μm thickness.
tion in spacings across individual lines of pixels, but peaks in Fourier transforms were sufficiently sharp and narrow to determine a specific spacing from all images.

Linear regression of measurements against time indicated a significant change in thickness during the measurement period in only two animals. Both were Rana temporaria and film thickness decreased at about 4 μm/min.

The second set of more finely spaced fringes were of low contrast and rarely seen. Their spacing occasionally could be measured directly from the video monitor, but there were no peaks corresponding to these fringes in Fourier transforms. They corresponded to approximately 50 μm and were probably formed by the combined layers of tear film plus epithelium.

The third set of fringes were very finely spaced. In some images from gerbils and pigeons, this set gave a peak in Fourier transforms at spacing 3–4 pixels, which corresponded to thickness 170–220 μm, probably full corneal thickness. However, spacings were too close to the resolution of video recordings for reliable measurements.

Images obtained by confocal microscopy were clear. In optical sections tangential to the eye, the tear film surface was seen as a bright circular reflection. A sample image is shown in Figure 4A. As the optical section was moved deeper into the film, below the highest point on the film surface, images became larger open circles. Optical sections oblique to the eye surface showed a cross-section through tear film and epithelium. Film thickness appeared constant across such images and no areas of tear film 'break-up' were seen. Occasional desquamated cells were present in the film of most species. Cell outlines and nuclei of superficial epithelial cells were clear, and the surface could be identified in all species studied. Figure 4B is an image tangential to the epithelial surface of rat cornea. There was some variation in cell size and in clarity of cell detail in different species, but appearances were otherwise similar. Deeper cells were progressively less distinct and basal epithelial cells could not be seen. Stroma gave a brighter reflection, but its surface could not be localized well and measurements of epithelial thickness were less accurate than of tear film thickness. The standard deviation of measurements of epithelium were approximately double that of measurements of tear film thickness.

Measurements of tear film thickness were made in all animals studied. Values remained approximately constant throughout examination by microscopy. Linear regression of results against time were calculated for rats. Slopes were not significantly different from zero, indicating that there was no ongoing change in thickness.

Desquamated epithelial cells were more numerous in the tear film of Rana pipiens at 3°C and were often in clusters of 3–6 cells between 3 and 7 μm from the epithelial surface. Those at 18°C did not show these clusters, although desquamated cells were more common than in other species. Desquamated cells were not seen in the film of Rana temporaria. Tear film thickness was not significantly different in frogs at 3°C and at 18°C. Stromal surfaces in chickens could not be identified and epithelial thickness was not measured.

**Measurements by Interferometry and Confocal Microscopy**

Table 1 shows results obtained in each species. The difference between measurements of tear film thickness by interferometry and by confocal microscopy in all animals varied from −6.5 μm to +4.5 μm, mean 0.1 μm (confidence limits of mean: −0.5 to +0.7 μm).
Error in measurements by interferometry was mainly a result of irregularities of fringe spacing. Typical standard deviation was about 1.5 μm. Measurements of tear film thickness by confocal microscopy were less accurate—optical sections were 1 μm–2 μm thick and standard deviation was typically about 2.5 μm.

Figure 5 shows the results obtained in each animal. Mean tear film thickness measured by confocal microscopy is plotted against measurements by interferometry. The linear regression line has a correlation coefficient of 0.93 and gradient of 0.995. It passes through the abscissa at 0.05 μm. No tear film was found by both methods in the 12 fish. If these measurements are excluded, the gradient is 1.06 and the intercept is 0.9. Both regression lines are not significantly different from one passing through the origin with a gradient of 1, as would be the case if the two measurements were identical.

In species other than trout and carp, thickness varied from about 9 μm in Rana temporaria to about 15 μm in gerbil eyes. Confocal images of epithelium of fish eyes were clear and no tear film was present. This was consistent with the interference speckle pattern, which indicated a surface roughness.

Variations in Thickness Across the Cornea
There was no significant difference between any pair of measurements in all animals studied.

Examination of Living Animals
There was no significant difference in measurements of thickness between living and freshly killed rabbits. The mean difference in values determined by interferometry before and after death was −0.5 μm (confidence limits −3.0 to +1.9 μm). The difference between measurements in living rabbits with interferometry, and in freshly killed animals with confocal microscopy was −0.6 μm (confidence limits −4.5 to +3.3 μm).

Mucus Component
Images obtained using interferometry after application of acetylcysteine were speckle patterns without fringes. This did not necessarily imply that no tear

![Graph showing tear film thickness measured by confocal microscopy plotted against measurements by interferometry. Values obtained in all animal and the linear regression line are plotted; correlation coefficient 0.93, gradient 0.995, intercept with abscissa 0.05 μm. Points at the origin indicate that no tear film was found by either method in the 12 fish examined. These measurements were included in regression analysis, but the parameters are not significantly different if they are excluded. In both cases the line is not significantly different to one passing through the origin with a gradient of 1.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933168/ on 09/23/2017)
film was present, as in fish, but indicated that the mucolytic action of acetylcysteine was irregular and resulted in an uneven film surface. Tear film could be identified and measured with confocal microscopy. Surface irregularities were too fine to be resolved.

The results are shown in Figure 6. The film thinned by 1.8 µm and 3.1 µm in the two control experiments. Greater thinning was measured after application of acetylcysteine, and the film was almost completely removed by 20% concentration. Occasional areas of fluid, less than 2 µm thick, were seen on the epithelial surface. Application of acetylcysteine did not change epithelial thickness. Measurements were not significantly different from those obtained in rat eyes that had not been exposed to acetylcysteine.

Discussion

Our two independent methods gave values that were substantially larger than earlier measurements in most species. Previous methods may not have been sensitive to the portion of the film that contained mucus, but measurements using confocal microscopy were of the full thickness between external tear surface and tear/epithelial interface, thereby including mucus. Values correlated closely with measurements obtained by interferometry. Thus, this method also gave full film thickness. In addition, both techniques were optical and did not interfere with the film or change its thickness, as probably was the case with earlier methods. Measurements in living animals were not significantly different from those made after death, and measurements in each animal remained approximately constant throughout recordings of fringes and confocal microscopy. Mucus is non-Newtonian. It is more viscous when motionless, and the film seems to be predominately a stable viscous gel. There was no evidence of a significant change in its structure during the brief period after death when animals were examined. Also, application of oil for microscopy did not change film thickness. The surface lipid layer may have been dispersed, but it is less than 0.5 µm thick. This could not account for significant error.

Evidence for a substantial mucus component was provided by the action of a mucolytic agent, which considerably reduced film thickness compared to controls. It is possible that the main component is mucus, not free aqueous fluid, and that electron microscopy measurements reported by Nicholls under-estimated mucus thickness. The methods used may have removed more dilute mucus in which glycoproteins are expanded with water molecules and have weaker intermolecular bonds.

Some species not included in the present study have considerably thicker films with a large mucus compo-

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![Figure 6](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933168/fig6.png)
The authors of the present study, using interferometry, have measured tear film thickness in humans to be about 40 μm. Even this may be much exceeded in dolphin eyes, as reported by Waller, who did not make measurements but described a viscous gel clearly visible to the naked eye.

Our measurements provided no further information about the structure of the film. There may be a gradient of mucus concentration, as proposed by Holly. A superficial aqueous layer could not be measured or detected by our methods. It may be present, but is too thin, or the graduation of refractive index may be too fine to be detected by our current techniques. Earlier methods, which interacted mechanically or chemically with the film, may have increased or even created an apparent mucus-free aqueous space as an artifact, although there is no clear evidence of this. It is possible that mucus is present throughout the film and that there is no distinct free aqueous layer, but rather an interlacing free fluid space within a loose network of dilute mucus. It is not possible to clarify this further at this time.

Examination of two fresh water fish, trout and carp, found there to be no tear film. Lacrimal and hardonian glands are not present in fish, although there are conjunctival goblet cells. There may be no tear film under normal conditions or it may have been removed at some stage of transportation, storage, or by other unknown factors.

Measurements of thickness by optical methods require a correction for the difference in refractive index of tear fluid and calibration materials. However, the range of possible values is small and would not account for a significant error in our results. Also, comparison of the results by the two methods is independent of the refractive index. The value used in calculations, 1.337, was measured in human tear samples with an Abbe refractometer. An upper limit was estimated to be 1.459 based on studies of internal reflections in probes of various refractive indices applied to the eye. Thickness calculated using this value would be about 8% larger than the values presented. Because the film is an aqueous solution, the index is unlikely to be less than that of pure water, 1.333. Application of a corresponding correction would lower values by just 0.3%.

Key words: tear film, mucus, interferometry, confocal microscopy, acetylcysteine.

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