Tear Film Images and Breakup Analyzed Using Fluorescent Quenching

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PURPOSE. Tear evaporation should increase fluorescein concentration, causing fluorescence dimming from self-quenching for high but not low fluorescein concentration. This prediction was tested and compared to the predicted effect of “tangential flow” that fluorescence dimming should be similar for high and low concentrations.

METHODS. A custom optical system was used for video recording of tear film fluorescence in 30 subjects. The subjects were asked to blink at the start of the recording and try to keep their eyes open for the rest of the 60-second recording. An initial recording was made after instillation of 1 μ L 0.1% fluorescein followed by further recordings at 5-minute intervals using 0.5% and 5% fluorescein.

RESULTS. Decay of fluorescence was considerably greater for the high (5%) concentration condition than for the low (0.1%) concentration. This is shown by “ratio images” (ratio of the intensity of a fluorescence image at a later time divided by that of an earlier image), fluorescence decay curves, fluorescence decay rates, and histograms of estimated tear thickness decrease. For example, for the high concentration condition, decay rates were higher than for the low concentration for all 30 subjects ($P < 0.0001$, binomial test). Additionally, breakup time was significantly reduced for the high compared to the low concentration condition.

CONCLUSIONS. The greater fluorescence decay and more rapid breakup for the high concentration condition are the results expected if thinning and breakup are mainly due to evaporation, hence causing self-quenching. Fluorescence decay rate for the low concentration condition was not significantly greater than zero.

Keywords: breakup, fluorescence, quenching, evaporation, tangential flow

Fluorescein has been used in many ways for the study of tear film and ocular surface physiology and for clinical evaluation of dry eye disorders. For example, it may be used to study the volume of tears in the eye and the rate of tear turnover.^{1–4} It can also be used to study epithelial permeability.⁵ For dry eye disorders, it is used for corneal staining.^{1,6} It is commonly used clinically for the measurement of fluorescein breakup time,^{1,7} as well as the characteristics and mechanisms of tear film breakup.^{8–11}

An important factor contributing to fluorescent intensity is the phenomenon of self-quenching,^{4,5,12–14} which greatly reduces the efficiency of fluorescence for high fluorescein concentrations. For low fluorescein concentrations, considerably less than a “critical concentration” of approximately 0.2%, fluorescent efficiency (ratio of emitted to absorbed photons) is high and largely independent of concentration. For high fluorescein concentrations, considerably above the critical concentration, fluorescent efficiency falls inversely with the square of concentration¹³; thus, at these high concentrations, doubling the concentration of fluorescein reduces efficiency by a factor of four. For some quantitative applications of fluorescent intensity measurements, it is important to minimize the effect of quenching by using sufficiently low fluorescein concentration.^{4,5,12}

Nichols et al.¹³ have shown how fluorescent quenching can be used as a tool to study the relative contribution of two major mechanisms to tear film thinning (thickness reduction) between blinks and breakup. The two mechanisms are evaporation and “tangential flow,” that is, flow of tears along the corneal surface.^{15,16} “Divergent tangential flow,” in which more tears flow out of a small area than into it, can cause thinning of the tear film. Evaporation increases fluorescein concentration, so quenching should reduce fluorescent efficiency and intensity for high fluorescein concentrations but not for low fluorescein concentrations. Divergent tangential flow should reduce fluorescent intensities roughly equally (in percentage terms) at both concentrations. Based on these principles, Nichols et al.¹³ analyzed the mechanisms of thinning of the tear film after a blink, using an optical system that simultaneously recorded fluorescent intensity and tear film thickness from a central area of the cornea. The fluorescent decay rate was found to be 4-fold faster using a high fluorescein concentration compared to a low fluorescein concentration, despite comparable tear film thinning rates for the two concentrations. This is the result expected if evaporation contributes more to tear film thinning than tangential flow, and supports similar conclusions about the

dominant contribution of evaporation from other studies by our group.^{15,17–20}

The purpose of this study was to apply the characteristics of quenching to the analysis of video recordings of tear fluorescence images using both low and high concentrations of fluorescein. The results were interpreted in terms of contributions from evaporation and tangential flow. It is argued that they help explain the mechanisms involved in tear film breakup.

METHODS

Optical System

A purpose-built optical system was used for fluorescent video imaging of the ocular surface. The illumination system and camera were mounted on a rigid base plate attached to a slit-lamp base. The principle of the illumination system is illustrated in Figure 1. The cornea and part of the surrounding conjunctiva were illuminated by two beams at equal angles of 34° on either side of the visual axis. Each beam covered an elliptical area of 15-mm width by 12-mm height. In Figure 1, one edge of each beam is represented by a thick line because it strikes the conjunctiva near normal incidence and provides strong illumination, whereas the other beam strikes at a glancing incidence, providing less illumination and so is represented by a thin line. The use of these two beams provides relatively uniform illumination over the cornea and conjunctiva without the subject's needing to look straight at a bright light source. The illumination system is also used as a focusing aid; for example, if the optical system is too close to the eye, the bright edges (from the thick rays in Fig. 1) will move outward and the strongly illuminated area will increase. The operator therefore tried to maintain the strongly illuminated area at a width of 15 mm (approximately 90% of image width), as in Figure 1, and the camera was adjusted to be in focus in this condition. A light-emitting diode, reflected by a glass plate, was used as a fixation point. A Basler (Ahrensburg, Germany) sCA640-74fm video camera was positioned along the visual axis and recorded an image of 656 × 494 pixels, covering a width of 16.5 mm. Fifteen images per second were recorded with a 12-bit intensity resolution (range, 0–4095 units). Interference filters (490 nm) were placed in the illumination beams, and a 535-nm interference filter was used in the camera lens system to transmit fluorescent light while blocking most reflection from the cornea.

Video Analysis

Video recordings were analyzed with a custom program that detected the occurrence and duration of blinks (recorded at lower left of images). To display weak fluorescence, the range of recorded intensity on the displayed image could be varied, and this was recorded at the lower right of the images (e.g., Fig. 2). To improve signal-to-noise ratio, images could be averaged after alignment of a selected area (typically 50 × 100 pixels) of conjunctiva based on a cross-correlation algorithm.¹⁸ The conjunctiva was used for this alignment, partly because the fluorescence image over the conjunctiva has a higher contrast initially than that over the cornea, particularly for low fluorescein concentration (e.g., Fig. 3A). The image pattern over the conjunctiva is probably largely due to surface roughness of the conjunctival surface (this would explain the high contrast over the conjunctiva in Fig. 3A, compared to the low contrast over the relatively smooth cornea). As expected from this, the conjunctival image was also found to show less change than the corneal image (compare Figs. 2A and 2B). A

FIGURE 1. Principle of the illumination system for fluorescent imaging. The cornea and part of the surrounding conjunctiva are illuminated by two beams at equal angles on either side of the foveal axis (see text for details).

pixel-by-pixel ratio of two images (or two averages of images, e.g., Fig. 2C) could be displayed, again after alignment of the images or averages with the cross-correlation algorithm. Thus the ratio image, $R(x,y)$, was given by

$$R(x,y) = I_2(x,y)/I_1(x+dx,y+dy) \quad (1)$$

where x and y are horizontal and vertical position in pixels, I_1 and I_2 are intensities of the first and second images, and dx and dy are the horizontal and vertical shifts needed to bring the first image into alignment with the second image. The ratio image thus shows the fractional dimming of fluorescence at every position of the exposed corneal surface. For the low concentration condition, in which quenching is unimportant, any dimming should be largely due to divergent tangential flow. For the high concentration condition, in addition to the effect of any tangential flow, evaporation should cause dimming due to quenching from increasing fluorescein concentration.

Subjects

The study protocol was approved by the Institutional Review Board in accordance with the Declaration of Helsinki. Informed consent was obtained from all subjects prior to enrollment. This study included 30 subjects, 17 female, average age 47 ± 17 years, who were screened to be healthy non-contact lens wearers age 18 years or older with no significant medical or ocular history except dry eye symptomatology. Female participants who were pregnant or lactating were excluded. To extend the range of observations, dry eye patients were not excluded, but the study was not designed to analyze differences between dry eye and normal subject populations. Presence or absence of dry eye symptoms was assessed using the Ocular Surface Disease Index (OSDI) survey (Allergan, Inc., Irvine, CA). Based on OSDI scores, 12 subjects were classified as having dry eye.

Procedure

Initially 1 μ L 0.1% fluorescein solution was instilled in the right eye of subjects. Each was asked to blink a few times to mix the fluorescein into the tear film. Subjects were positioned in a head/chin-rest assembly and asked to fixate on the light-emitting diode. Video recordings were then made for 60

FIGURE 2. Images obtained using high (5%) fluorescein concentration. (A) Average of 15 aligned images taken approximately 2 (1.5–2.5) seconds after a blink. (B) Average of 15 images taken 10 seconds later. (C) “Ratio image,” that is, ratio of intensity in later image (B) divided by the intensity in the earlier image (A) after alignment of the two images. Vertical arrows in (A) and (C) indicate that features of the breakup pattern were already present 2 seconds after the blink. Dry eye condition, 26-year-old female.

seconds, with the subjects asked to blink 1 second after the start of the recording and to try to hold their eyes open for the remaining time. Subsequently, 5 and 10 minutes after the first instillation, 1 μ L 0.5% and 5% fluorescein, respectively, was

FIGURE 3. Images obtained using low (0.1%) fluorescein concentration for comparison with Figure 2 (same subject). (A) 2 seconds after the blink. Vertical arrow shows fluorescence from the crystalline lens, whereas horizontal arrows show reflections from the illumination sources. (B) 10 seconds later. (C) Ratio of image B to image A. Diagonal arrows in (A), (B), and (C) indicate a developing arcuate region of breakup under the upper lid.

instilled and recordings were made as above. Only the results for 0.1% and 5% fluorescein are presented here; after dilution by the tear volume,² the fluorescein concentration in the tear film would be expected to be, respectively, considerably above and below the critical concentration of 0.19%.

Breakup Time Measurements

The video recordings for 0.1% and 5% fluorescein were randomly intermixed and relabeled following removal of subject identifiers and concentration of fluorescein information. A masked clinician reviewed these video files frame by frame to determine specific frame numbers capturing the occurrence of a blink and occurrence of the first breakup. From this information, breakup time was determined as the time to the observation of the first black spot following complete eyelid opening.

RESULTS

As already noted, quenching of fluorescence from the tear film occurs at high fluorescein concentrations, considerably above approximately 0.2% but not considerably below 0.2%. Thus evaporation, which increases fluorescein concentration, should cause greater dimming of fluorescence for the high compared to the low fluorescein concentration condition. Divergent tangential flow should cause roughly equal effects for both concentrations.

Figure 2 illustrates images using high (5%) fluorescein concentration in a dry eye subject, whereas Figure 3 shows corresponding images for the low (0.1%) concentration in the same subject. Figures 2A and 3A show an average of 15 images (after the alignment described in Methods) taken approximately 2 (1.5–2.5) seconds after the blink, whereas Figures 2B and 3B show an average taken 10 seconds later. (The first 1.5 seconds after the blink was excluded from this analysis to avoid the effects of the strong tangential [upward] flow of lipid after the blink.^{18–20}) Figures 2C and 3C present a “ratio image,” that is, the ratio of the intensity in the later image divided by the intensity in the earlier image (again after alignment). A striking difference between high (Fig. 2) and low (Fig. 3) concentration conditions is that there is considerable dimming over the cornea for the high concentration condition (compare Figs. 2B and 2A) but little overall dimming in the low concentration condition (compare Figs. 3B and 3A). This difference is also shown in the ratio images, with the ratio over the cornea low for the high concentration case (Fig. 2C) but close to one for the low concentration case (Fig. 3C). Because evaporation is expected to cause dimming by quenching in the high concentration condition but much less in the low concentration condition,¹³ these observations are consistent with evaporation as the main cause of dimming over the cornea for the high concentration condition of Fig. 2 (divergent tangential flow should cause equal dimming in both conditions). (It may be noted that the images of Fig. 3 were obtained 10 minutes after those of Fig. 2 and that some variation in conditions may have occurred, in addition to change in fluorescein concentration; however, reflex tears were observed entering the lower meniscus at approximately the same time for both conditions, after 17 and 14 seconds for 5% and 0.1%, respectively, suggesting that breakup was similar in the two recordings.)

Some further observations on Figures 2 and 3 may be noted. First, for the high concentration condition, much of the pattern of tear thinning and breakup shown in Figures 2B and 2C was already evident at 2 seconds after the blink (Fig. 2A). For example, two dark, roughly horizontal lines indicated by the arrow in Figure 2A are precursors to corresponding strong thinning and breakup (Fig. 2C, arrow). However, for the low concentration condition, there was no indication after 2 seconds (Fig. 3A) of where most breakup would occur. Second, an exception to this general absence

of breakup indication after 2 seconds for the low concentration condition was an arcuate region of dimming under the upper lid (slanting arrow in Fig. 3A), which developed into a larger, stronger dimming in Figures 3B and 3C (slanting arrows). Third, for the high concentration condition, the ratio intensity distribution (Fig. 2C) may be described as a smoothly varying function covering the whole corneal area, whereas for the low concentration condition, the ratio image (Fig. 3C) may be described as small dark regions within a rather uniform light background (ratio of approximately 1.0).

Ratio images, comparing 14 with 2 seconds after the blink for a second dry eye patient, are shown in Figure 4A (high concentration) and Figure 4B (low concentration). These show many of the same features seen in Figures 2 and 3; for example, for the high concentration condition (Fig. 4A) there is considerable dimming over much of the cornea, but little dimming over much of the cornea in the low concentration condition (Fig. 4B). The star in Figure 4A indicates probable reflex tears that could be seen drifting down the cornea in the video recording. The star in Figure 4B indicates the region where a small partial blink had occurred.

If tear film thinning after a blink is mainly due to evaporation rather than tangential flow, quenching due to evaporation should cause more rapid dimming for high than for low fluorescein concentration. Figure 5 shows decays of fluorescent intensities over a central area of the images 2.5 mm in diameter; the high and low concentration conditions are represented by blue and red curves, respectively. Figure 5A is for the subject of Figures 2 and 3; Figure 5B is for the subject of Figure 4; and Figure 5C is for a normal subject. On these log-linear plots, the initial parts of the high concentration curves, between 2 and 7 seconds after the blink (i.e., omitting any initial transient), is fairly linear, implying an exponential decay of fluorescence; dashed lines in Figure 5 show least-squares fits to the data in this interval. Slopes for the low concentration condition (red curves) were much smaller, namely 6%, 6%, and 26% of the slopes for high concentration in Figures 5A, 5B, and 5C, respectively. This is consistent with the lack of dimming for the low concentration condition in Figures 3C and 4B.

The straight line fits to the log-linear plots in Figure 5 correspond to an exponential decay of the form

$$f(t) = f_0 e^{-kt} \quad (2)$$

where $f(t)$ is fluorescence intensity as a function of time, t , and f_0 and k are constants. The decay rate constant, k , is a measure of how rapidly the intensity decays; it was determined by fitting the exponential curve between 2 and 7 seconds after the blink, as indicated by the dashed lines in Figure 5. Dots in Figure 6 give $100k$ for the low fluorescein concentration condition as a function of $100k$ for the high concentration condition. The dots corresponding to the three subjects in Figures 5A, 5B, and 5C have been circled and labeled. The dashed line represents equality of decay rates for the two conditions; it is seen that the decay rate for the low concentration condition is less than for the high concentration condition for all 30 subjects ($P < 0.0001$, binomial test). The triangle gives mean decay rates, namely 4.71%/s for high fluorescein concentration and 0.25%/s for low concentration. The decay rates for high concentration were all greater than zero ($P < 0.0001$, binomial test), whereas those for low concentration did not differ significantly from zero ($P = 0.123$, Wilcoxon signed rank test).

Figure 7 shows histograms for estimated decrease in tear thickness, for the high fluorescein concentration condition, between 2 and 7 seconds after a blink for the three subjects of

FIGURE 4. Ratio of intensity at 14 compared to 2 seconds after a blink for a second dry eye subject (53-year-old female). (A) High concentration condition. The *star* marks a region of possible reflex tears drifting down the cornea. (B) Low concentration condition. The *star* marks the region covered by a partial blink.

Figure 5. Each vertical bar in the histogram shows the number of pixels, within an analysis area of approximately 31,000 pixels, which correspond to a decrease in thickness within the range given by the x -axis. These estimates are based on the assumption, supported by the results in Figure 6, that most of the tear film thinning between these times is due to evaporation (rather than tangential flow). It is further assumed that the concentration of fluorescein in the tear film was

considerably above the critical concentration of 0.19% and so fluorescent intensity, f , should be inversely proportional to the square of fluorescein concentration, c .¹³ Thus

$$f(t) = A/c(t)^2 = Bb(t)^2 \quad (3)$$

where A and B are constants and $b(t)$ is tear thickness; the right-hand side of the equation is based on the observation that concentration will be inversely proportional to tear thickness if

FIGURE 5. Decay of fluorescent intensity after a blink. *Blue and red curves* correspond to high and low fluorescein concentration conditions. **(A)** Dry eye subject of Figures 2 and 3. **(B)** Dry eye subject of Figure 4. **(C)** Normal subject, 21-year-old female.

thinning is due to evaporation rather than tangential flow. Thus for any point in the image, thickness is proportional to the square root of fluorescence, and the percentage decrease in tear thickness will be given by

$$100\{b(2) - b(7)\}/b(2) = 100\{\sqrt{f(2)} - \sqrt{f(7)}\}/\sqrt{f(2)} \quad (4)$$

The histograms in Figure 7 were derived, after the image alignment described in Methods, using a 5-mm-diameter central area chosen to avoid obstruction from eyelashes. Figure 7A shows a broad histogram of rapid thinning with a peak at over 30% thinning compared to the narrow histogram for the normal subject in Figure 7C with a peak at less than 6% thinning. Both these histograms have a single peak, whereas the histogram in Figure 7B appears to have three peaks and is intermediate in thinning rates between those of Figures 7A and 7C.

Figure 8 shows breakup times derived from masked examiner analysis of the video recordings. Breakup time for the high concentration condition is plotted as a function of breakup time for the low concentration. The masked clinician who determined breakup times found that two videos for the low concentration condition were too dim to estimate breakup reliably, so only 28 subjects are included in Figure 8. The dashed line represents equality of breakup times for high and

low concentrations. Points for the three subjects in Figure 5 have been circled and labeled. For the low concentration condition and two subjects, including the subject of Figure 5C, breakup was not observed within the 60-second video recording. Median breakup times for the low and high concentration conditions were 5.70 and 3.05 seconds, respectively, with significantly longer times for the low concentration ($P < 0.001$, Wilcoxon signed rank test). It is notable that all breakup times for the high concentration condition were less than 13 seconds, but 8 of 28 times for the low concentration were greater than 13 seconds.

DISCUSSION

Previous studies from our laboratory have indicated that evaporation is generally more important than divergent tangential flow in causing tear thinning between blinks, except in some special cases.¹⁵ Earlier studies include evidence that, first, there is little tangential flow over most of the exposed cornea except for the upward flow just after blink¹⁸; second, there is a large reduction in tear thinning rate when one is wearing tight-fitting goggles that prevent or reduce evaporation¹⁷; third, there is an inverse correlation between thinning rate and lipid thickness, which is expected if thinning is mainly due to evaporation and the lipid layer is a barrier to

FIGURE 6. Fluorescence decay rate (k in equation 2) for low fluorescein concentration versus rate for high fluorescein concentration. *Dots* corresponding to the three subjects in Figure 5 (A–C) have been circled and labeled. The *triangle* represents average rates for the 30 subjects, and the *dashed line* represents equal thinning rates at low and high concentrations.

FIGURE 7. Histograms of estimated decrease in tear film thickness for the three subjects of Figure 5 (A–C). See text for details.

evaporation²¹; and fourth, tear breakup tends to occur in regions where the lipid layer is particularly thin.²² In concordance with the dominant role of evaporation in tear thinning, Liu et al.¹⁰ interpreted their measurements of development of ocular discomfort after a blink in terms of response to tear osmolarity increase caused by evaporation.

The current studies extend the use of self-quenching developed by Nichols et al.¹³ as a way of analyzing tear thinning in terms of contributions from evaporation and tangential flow. In the previous study, the decay of fluorescence over a central small region of the cornea was shown to be approximately four times greater with use of a high concentration of fluorescein compared to a low concentration. That is, the result expected if fluorescence decay is due to evaporation and self-quenching (which occurs at high but not at low fluorescein concentration). For comparison, tangential flow would be expected to cause roughly equal changes in fluorescence (in percentage terms) at high and low fluorescein concentration. Begley et al.,⁸ in a study relating fluorescence dimming with corneal sensation, have also interpreted their observations in terms of self-quenching.

The current studies provide two types of evidence that the fluorescence decay observed here is mainly due to evaporation rather than tangential flow. The first evidence is to note that tangential flow would be expected to cause a redistribution of the tear film, rather than an overall thinning. Thus divergent tangential flow would cause tears to flow out of some areas, causing thinning, but correspondingly convergent tangential flow would cause tear thickening in other areas. However, the observed results for the high concentration condition in Figures 2C, 4A, 5, 6, and 7 indicate an overall thinning of the tear film rather than a redistribution, as expected from evaporation rather than tangential flow.

The second piece of evidence that the fluorescence dimming is mainly due to evaporation comes from comparison of results for the high concentration condition (greater self-quenching) with the low concentration condition (less self-quenching). For the high concentration condition, the ratio images of Figures 2C and 4A show considerable dimming over a 10- or 12-second period, whereas for the low concentration condition, there is little overall dimming for the corresponding ratio images, Figures 3C and 4B. The same conclusion is evident from the fluorescence decay curves of Figure 5, where decay for the high concentration condition (blue curves) is much more rapid than for low concentration (red curves). Similarly, Figure 6 shows that fluorescence decay rate (in %/s) for high concentration is more than that for low concentration for all 30 subjects. For the high concentration, all subjects had a positive decay rate, whereas for the low concentration, the average decay rate was not significantly different from zero, indicating that, on average, tangential flow caused little tear film thinning in these studies. All these findings support the conclusion that tear film thinning between blinks (after the initial transient upward flow) is mainly due to evaporation rather than divergent tangential flow. Previous studies by others have also emphasized the importance of evaporation and the lipid layer in determining tear film breakup.^{23,24}

A limitation of the clinical utility of breakup time is that this measure ignores much of the information available in the dynamic fluorescent images. For example, two patients may have the same breakup time, such as 5 seconds, but one patient who is developing breakup over an extensive area may have a more severe deficiency than the other whose breakup is limited to a small region. Therefore, other measures of breakup have been proposed that may better represent the severity of dry eye.^{8,25,26} Likewise, it is suggested that measures such as the fluorescence decay plots of Figure 5, the fluorescence decay rates of Figure 6, and the thinning histograms of Figure 7 may provide a better description of dry eye severity than fluorescein breakup time. Breakup time measurement has been criticized for its lack of reproducibility.²⁷ However,

FIGURE 8. Breakup times for the high fluorescein condition plotted as a function of breakup times for the low concentration condition. (A–C) Plots corresponding to the three subjects in Figure 5. The dashed line represents equality of breakup times for high and low concentrations.

Nichols et al.²⁸ reported that breakup time repeatability was better than for the Schirmer test and for fluorescein and rose bengal staining. Reduction in the amount of fluorescein instilled may help to improve reproducibility.^{29–31} Additionally, we are currently studying whether the measures illustrated in Figures 5, 6, and 7 may provide greater reproducibility than breakup time.

As expected from the dominant effect of evaporation and self-quenching, Figure 8 shows that fluorescein breakup times for the high concentration condition were significantly less than for the low concentration. This corroborates results of Shim et al.,³² who reported that breakup time was significantly and inversely related to fluorescein concentration. In apparent contradiction, Johnson and Murphy³³ reported that increasing the volume of instilled fluorescein from 1 to 2.7 μ L increased breakup time significantly; however, they reduced the fluorescein concentration for the larger volume so that the expected concentration in the tear film was the same for both volumes, and their result might have been due simply to the increased volume of the aqueous layer for the 2.7- μ L instillation.

It may be noted that the breakup time analysis in Figure 8 provided poorer discrimination between results from high and low concentrations than the fluorescence decay rate analysis in Figure 6. This may be related to the suggestion above that thinning rates are a more reliable measure of dry eye severity than breakup time measurements.

In conclusion, our results show how video recordings involving self-quenching of fluorescence can add further evidence that tear film thinning and breakup are largely due to evaporation rather than divergent tangential flow. It is suggested that fluorescein breakup time is an inadequate measure of the information contained in dynamic fluorescein images, and suggestions are made for measures that may be more reproducible and provide better information about disease severity.

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