

# Quantitative Analysis of Tear Film Fluorescence and Discomfort During Tear Film Instability and Thinning

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**PURPOSE.** The purpose of this study was to test the association between tear film fluorescence changes during tear break-up (TBU) or thinning and the concurrent ocular sensory response.

**METHODS.** Sixteen subjects kept one eye open as long as possible (MBI), indicated their discomfort level continuously, and rated ocular sensations of irritation, stinging, burning, pricking, and cooling using visual analog scales (VAS). Fluorescence of the tear film was quantified by a pixel-based analysis of the median pixel intensity (PI), TBU, and percentage of dark pixels (DarkPix) over time. A cutoff of 5% TBU was used to divide subjects into either break-up (BU) or minimal break-up (BU<sub>min</sub>) groups.

**RESULTS.** Tear film fluorescence decreased (median PI) and the percentage of TBU and DarkPix increased in all trials, with the rate significantly greater in the BU than the BU<sub>min</sub> group (Mann-Whitney *U* test,  $P < 0.05$ ). The rate of increasing discomfort during trials was highly correlated with the rate of decrease in median PI and developing TBU (Spearman's,  $r \geq 0.70$ ). Significant correlations were found between corneal fluorescence, MBI, and sensory measures.

**CONCLUSIONS.** Concentration quenching of fluorescein dye with tear film thinning best explains decreasing tear film fluorescence during trials. This was highly correlated with increasing ocular discomfort, suggesting that both tear film thinning and TBU stimulate underlying corneal nerves, although TBU produced more rapid stimulation. Slow increases in tear film hyperosmolarity may cause the gradual increase in discomfort during slow tear film thinning, whereas the sharp increases in discomfort during TBU suggest a more complex stimulus.

**Keywords:** tear film instability, tear film break-up, tear film thinning, evaporation, fluorescein concentration quenching, corneal sensory nerves, nociception

Dry eye is a common condition, affecting millions in the United States and elsewhere,<sup>1–5</sup> affecting quality of life,<sup>6–8</sup> and potentially engendering high health care costs now and in the future.<sup>9</sup> However, despite its prevalence and impact, the underlying etiology of the condition and the basis of dry eye symptoms remain subject to speculation.<sup>10,11</sup>

The recent international Dry Eye Workshop report identified the core mechanisms of dry eye as tear film hyperosmolarity and instability, which are thought to generate dry eye symptoms through repeated stress to the ocular surface, inflammation, and activation of corneal sensory nociceptors.<sup>10</sup> In support of this hypothesis, tear hyperosmolarity has been shown to stress the surface and trigger ocular surface inflammation,<sup>12,13</sup> and recent reports have associated tear film hyperosmolarity with dry eye symptoms.<sup>14</sup> However, while tear film osmolarity is likely to fluctuate markedly or spike<sup>15</sup> in the interblink interval due to evaporation or other factors,<sup>16–18</sup> it cannot be measured directly over the cornea with current techniques of sampling the tears from the inferior meniscus.<sup>19</sup> Thus, changing conditions within the tear film during instability that may provide stress to the underlying cornea and ocular surface can be difficult to assess.

The purpose of this study was to investigate ocular surface stresses associated with the dynamically changing tear film and their impact on ocular sensory responses using a human-based in vivo model. Previously, we have shown that tear film break-up (TBU) is associated with sensations of burning and stinging and results in increased symptoms of ocular irritation, implying that increased tear film hyperosmolarity or other adverse conditions may occur during TBU, stimulating ocular surface nerves.<sup>20–22</sup> In this study, we quantified global thinning and regional TBU by employing a pixel-based analysis of fluorescence and compared the concurrent sensory responses of TBU versus tear thinning during the interblink period. We hypothesize that changes in corneal fluorescence during the interblink period are due tear film thinning and/or TBU, and that both are associated with sensory stimulation to the cornea.

## METHODS

### Subjects

The study was conducted at the Indiana University School of Optometry. Informed consent was obtained from all subjects

and the study adhered to the tenets of the Declaration of Helsinki.

Considering that the purpose of the study was to investigate the sensory response to a wide range of tear instabilities, both dry eye and normal subjects were recruited to obtain the range. Sample size estimates were not available because few preliminary data were available for primary outcome variables. Sixteen subjects over 18 years of age who were non-contact lens wearers and free of systemic and ocular diseases aside from dry eye symptoms, based on the Dry Eye Questionnaire (DEQ)-5 score,<sup>23</sup> were enrolled in the study. However, subjects who showed significant corneal staining (greater than grade 2, Oxford Scale<sup>24</sup>) were excluded because corneal staining could potentially be a confounding factor affecting the ocular surface sensory response to tear film instability.<sup>11,25,26</sup>

## Procedures

The DEQ<sup>27</sup> was used to record habitual symptoms of dry eye, and a short form DEQ-5 score was calculated.<sup>23</sup> Subjects were then seated behind a slit lamp biomicroscope fitted with a digital video camera, and 2  $\mu$ L of sodium fluorescein (2%) was pipetted into the tested eye while the other eye was patched shut. As in previous studies, subjects were asked to keep the tested eye open as long as possible<sup>20,21</sup> while they turned a knob (potentiometer) to indicate the level of discomfort in the tested eye on a 0 to 10 scale (0 = no discomfort and 10 = very uncomfortable).<sup>22</sup> Following each trial, subjects rated their overall discomfort on separate integer scale of 1 (no discomfort) to 10 (very uncomfortable) and then indicated the level of irritation, stinging, burning, pricking, and cooling on visual analogue scales (VAS). The fluorescent tear film was recorded digitally (30 frames/s) using a cobalt blue excitation filter over the light source and a Wratten no. 8 barrier filter over the observation/imaging axes.

## Image Analysis

Ten images were extracted from each trial. The first image was taken immediately after eye opening and the last image was the frame just before the blink that ended the trial. The difference in time between the first and last image during each trial was termed the maximum blink interval (MBI).<sup>28</sup> The other eight images were extracted to coincide with increases in the discomfort level, or if the potentiometer was turned infrequently, image extraction was spaced evenly across the trial.

A custom MATLAB (The MathWorks, Inc., Natick, MA) program was used to convert images to grayscale, isolate the cornea as the region of interest in each frame, and count pixels of varying intensities within the corneal portion of the image. The camera gain was held constant during trials. Pixel intensity (PI) values ranged from 0 (black) to 255 (white) binned in units of five (i.e., 0–4, 5–9, etc.). The percentage of pixels within each bin was then calculated to obtain a PI distribution for the exposed cornea in each image.

Overall changes in corneal fluorescence over each trial were evaluated by quantification of PI distribution statistics. Median PI was used to track overall changes in the PI of distributions over time during each trial. However, we noted that some PI distributions became bimodal, skewed, or otherwise irregularly shaped as TBU formed, which was not necessarily captured by the median PI. Thus, we developed a second PI distribution metric, called DarkPix, which equaled the number of pixels with intensities less than or equal to the intensity of the first percentile of the intensity distribution at time zero. Thus, as the trial progressed, DarkPix recorded the number of image pixels with intensities lower than the darkest pixels observed at the beginning or first image in each trial.

We quantified the area of TBU using previous methods.<sup>20,21</sup> For this analysis, TBU was defined in the traditional clinical sense, as areas of dark spots, streaks, or amorphous areas in the fluorescent corneal tear film.<sup>29,30</sup> A clinically trained observer masked to subject and trial information identified the threshold PI that included all areas of TBU in the final image of each trial. This threshold PI value was then used to quantify TBU regions in all earlier images in that trial. The thresholding procedure for TBU was necessary for each trial because tear film fluorescence and reflection from the iris varied widely, so the range of PI values within a trial differed among subjects and trials.

## Data Analysis

Statistical analyses were performed using SPSS 20 (IBM SPSS, Armonk, NY). Data were represented by median and interquartile range and nonparametric tests were applied due to small sample size and lack of normality of some outcome variables.

In order to compare the sensory response to TBU versus tear thinning, subjects were divided into two groups based on the amount of TBU measured in the final image of each trial for data analysis: those with little TBU during the trial and those with extensive TBU. Given that there should theoretically be little fluid exchange from the corneal compartment of the tear film during the interblink interval,<sup>31,32</sup> we assumed that changes in tear film fluorescence during trials were largely due to evaporation.<sup>18</sup> Other forces, such as lateral flow of fluid or osmotic permeability into the surface epithelial cells were considered to play a minor role and thus were not considered.<sup>33</sup> Thus, in the absence of significant TBU, we attributed changes in tear film fluorescence to tear film thinning.

We used a cutoff of 5% TBU to separate these groups into those who exhibited >5% TBU (BU group) and those who showed  $\leq$ 5% or minimal TBU over the trial (BU<sub>min</sub>). We chose 5% TBU as a criterion for low or minimal TBU in the BU<sub>min</sub> group, rather than no TBU, because small dots of TBU that did not increase much in size often appeared in trials showing an otherwise stable tear film. Mann-Whitney *U* tests were used to compare MBI and tear film parameters (TBU, DarkPix, and the median of the PI distribution), including rates of change and VAS sensory data between BU and BU<sub>min</sub> groups.

Correlations among tear film parameters and between sensory responses were evaluated using Spearman's  $\rho$  correlation coefficient and Bonferroni correction for type I errors was used wherever applicable. In addition, an exploratory multiple factor analysis was conducted using R (The R foundation for Statistical Computing; <http://www.r-project.org/foundation/>) to uncover associations between fluorescence and sensory data.

## RESULTS

Table 1 shows age, sex, and symptom profile of subjects who participated in this study. Schirmer I scores are not listed in Table 1, but only one subject had a score (4 mm) below the least stringent cutoff for dry eye of 10 mm.<sup>34</sup> As Table 1 shows, subjects in this study can be classified as no signs or symptoms of dry eye as well as those with mild to moderate dry eye, with symptoms ranging from none to mild or moderate levels based on DEQ-5 score<sup>25</sup> and minimal corneal and conjunctival staining.<sup>24</sup> Less than half of the subjects had been diagnosed with dry eye, but half thought they had dry eye, which is a gestalt question associated with greater dry eye symptoms.<sup>35</sup>

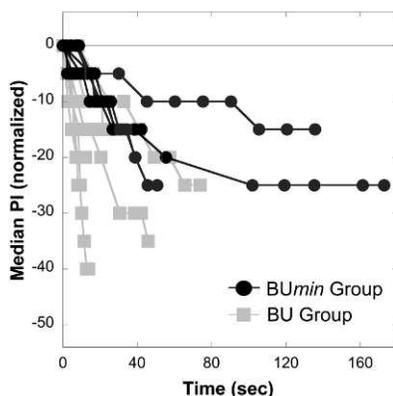
**TABLE 1.** Subject Demographics and Entering Habitual Symptoms and Signs of Dry Eye

Factor	
Sex, <i>N</i> (%)	
Female	10 (62.5)
Male	6 (37.5)
Age, y	
Mean (SD)	30.0 (10)
Range	22 to 53
DEQ, <i>N</i> (%)	
Discomfort	
Sometimes to constant	8 (50.0)
PM, moderate to intense	9 (56.3)
Dryness	
Sometimes to constant	7 (43.7)
PM, moderate to intense	9 (56.3)
DEQ-5 score <sup>23</sup>	
Mean (SD)	8.2 (4.9)
Range	0 to 16
Ocular surface staining (Oxford scale), <sup>24</sup> <i>N</i> (%)	
Corneal fluorescein	
Grade 1 or less	15 (93.7)
Grade 2	1 (6.3)
Conjunctival lissamine green	
Grade 1 or less	10 (62.5)
Grade 2	5 (31.3)
Grade 4	1 (6.3)
Dry eye, <i>N</i> (%)	
Diagnosed	6 (37.5)
"Do you think you have dry eye?"	8 (50.0)

PM, post meridiem.

### Pixel-Based Analysis of Fluorescence Changes After a Blink

Figure 1 shows the change in normalized median PI over time for each subject. The median PI decreased for all subjects and in all trials, regardless of whether subjects were in the BU or BUmin group. Subjects in the BU group tended to show more rapid declines in median PI values and blinked sooner, whereas

**FIGURE 1.** Change in median PI over trials for subjects in the BU and BUmin groups. The median PI was normalized from the initial median PI values so that changes can be easily visualized across subjects.

those in the BUmin group tended to display slower changes over time and were able to keep their eyes open longer.

Figures 2 and 3 show sample images, PI histograms for each of the 10 grayscale images during the trial, and changes in DarkPix and TBU over time for four individual subjects in the BUmin and BU groups, respectively. Subjects in BUmin group developed little TBU over the trial as seen in the images from the beginning (Figs. 2A, 2E) and at the end of a trial (Figs. 2B, 2F). The amount of TBU quantified by PI was minimal or negligible (Figs. 2D, 2H) over time. PI distributions (Figs. 2C, 2G) remained similar in shape as the trial progressed despite an increase in the number of pixels below the DarkPix cutoff that was determined by the left tail of the PI distribution ending at zero second of the first trial (e.g., 80 and 40 for subjects 1 and 2, respectively). The increase of percentages DarkPix (Figs. 2D, 2H) was slow over time (linear slope 0.43%/s and 0.35%/s for subjects 1 and 2, respectively), even though subject 2 showed a high percentage of DarkPix (approximately 60% of the corneal area) due to globally lost fluorescence of the tear film, without discrete areas of TBU. These data suggest that regional tear film thinning but not TBU was the primary cause of overall decreased fluorescence for these subjects.

In contrast, subjects in the BU group developed extensive TBU rapidly over short trials (Figs. 3A, 3E for the beginning; Figs. 3B, 3F for the end of the trial). As the trials progressed, PI distributions shifted rapidly to the left and tended to spread out as areas of TBU appeared and grew (Figs. 3C, 3G). In this case, the pixels identified as TBU were the same ones identified as DarkPix. The rates of increase in DarkPix (Figs. 3D, 3H) were high (linear slope 3.42%/s and 2.2%/s for subjects 3 and 4, respectively). For these two cases, TBU appears to have accounted for most of the corneal fluorescence loss in the trial. However, some subjects, usually those with longer MBIs, showed a more rapid increase in DarkPix than TBU (data not shown). These data suggest that TBU was a primary cause of decreased fluorescence for BU subjects, but that some global tear film thinning may have contributed.

Table 2 shows aggregate results (medians and interquartile ranges) for MBI and tear film fluorescence measures for subjects in the study. The MBI varied widely among subjects, and thus the difference between groups was not statistically significant. However, as Figure 1 shows, subjects in the BUmin group tended to be able to keep their eyes open longer (longer MBIs). The change in median PI was actually a negative number because all trials decreased in fluorescence, but it is listed as an absolute value in Table 2 for ease of presentation. The rate of change in fluorescence measures (TBU, DarkPix and Median) across the trials was significantly higher in the BU group (Table 2). When medians from each group were compared, the rate of increase in DarkPix (%/s) was more than 5 times greater in the BU than in the BUmin group (Mann-Whitney *U* test,  $P = 0.016$ ), the rate of change of the median PI was 4 times greater in the BU group (Mann-Whitney *U* test,  $P = 0.005$ ), and the rate of increase in TBU over time was 34 times faster in the BU group (Mann-Whitney *U* test,  $P = 0.002$ ).

Many of the metrics in Table 2 were significantly correlated. The rates of development of TBU, DarkPix, and the median of the PI distribution were all significantly positively correlated with each other ( $0.77 < r > 0.91$ ,  $P < 0.0001$ ) and the rate of TBU and PI median were negatively correlated with the MBI ( $r = -0.77$ ,  $-0.82$ , respectively,  $P < 0.0001$ ). These data suggest that rapidly increasing tear instability or thinning negatively impacts the ability to keep the eye open.

### Sensory Measures

Table 3 shows averages and standard deviations for all sensory measures in this study and separately for BU and BUmin

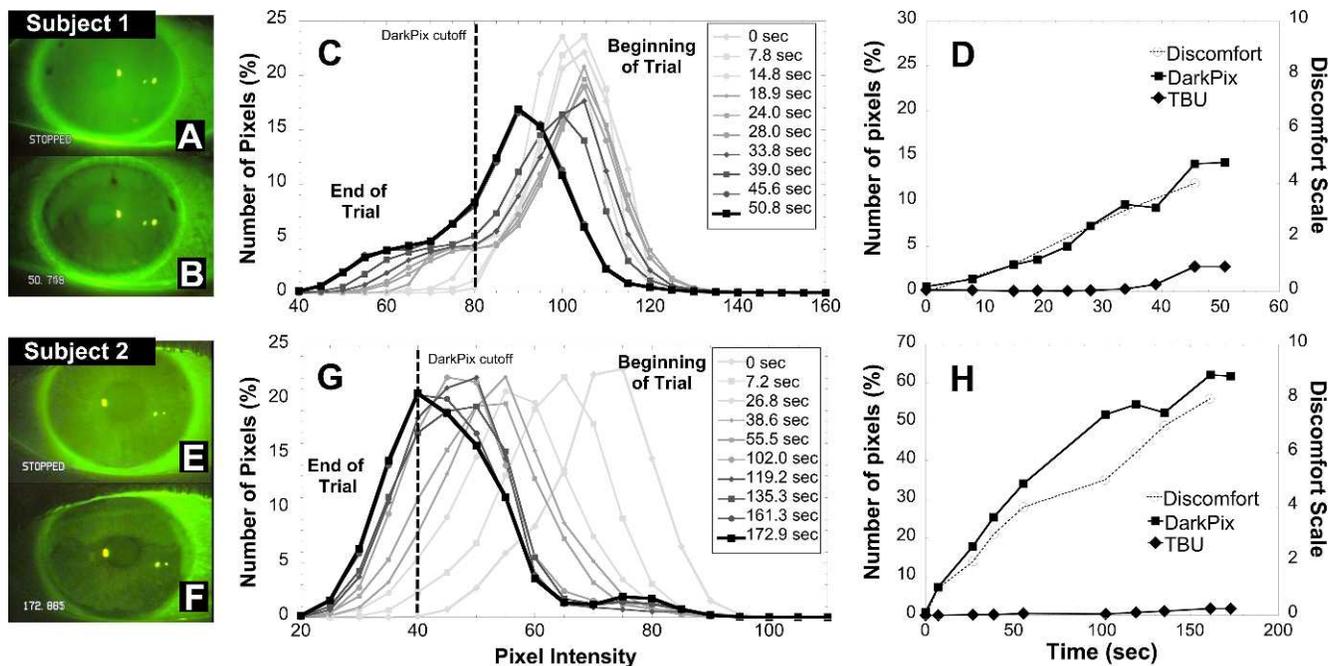


FIGURE 2. Images from the beginning (A, E) and end (B, F) of trials for subjects 1 and 2 in the BU<sub>min</sub> group. Changes in the distribution of PI over the trial are shown in (C) and (G), with the DarkPix cutoff at time 0 indicated by the dashed line for each trial. The change in discomfort, TBU, and Dark Pix over time is shown in (D) and (H).

groups. The rate of increase in discomfort during the trials, and the posttrial scores for each sensory scale were generally higher in the BU group. The differences between groups reached statistical significance (Mann-Whitney *U* test,  $P < 0.05$ ) for “irritation” and “pricking.” These results agree with our previous studies, which indicate that TBU in eye opening trials was associated with irritation, stinging, and burning,<sup>15,20,22</sup> but also suggest that diminishing corneal fluores-

cence over time without TBU is associated with an irritative affective response.

The difference in discomfort experienced by subjects in the BU and BU<sub>min</sub> groups is illustrated in Figures 2 and 3, which show individual data for the change in discomfort during trials reported by four of the subjects in this study. BU subjects 3 and 4 (Figs. 3D, 3H), who experienced extensive TBU, showed very rapid increases in discomfort during their short trials (0.77

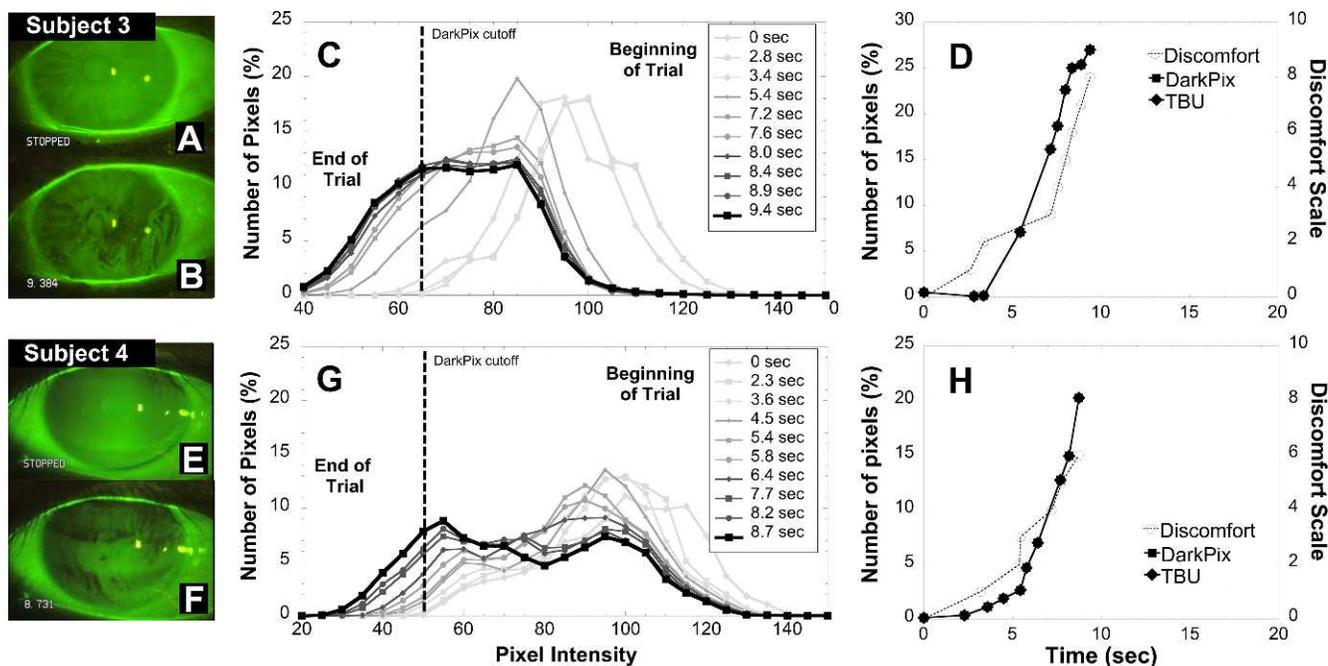


FIGURE 3. Images from the beginning (A, E) and end (B, F) of trials for subjects 3 and 4 in the BU group. Changes in the distribution of pixel intensity over the trial are shown in (C) and (G), with the DarkPix cutoff at time 0 indicated by the dashed line for each trial. The change in discomfort, TBU, and Dark Pix over time is shown in (D) and (H).

**TABLE 2.** The Median and the Interquartile Range for the MBI, TBU, and PI Data for BU<sub>min</sub> and BU Groups

Factor	BU <sub>min</sub> Group, ≤5% TBU, 7 Subjects	BU Group, >5% TBU, 9 Subjects
MBI	42.26 (28.10–114.46)	14.48 (11.52–29.59)
TBU (%) of corneal area		
Change over trial: median	2.75 (1.88–3.00)	18.53* (13.07–24.99)
Median	<b>0.03</b> (0.01–0.06)	<b>0.93</b> † (0.40–2.52)
DarkPix (%) of corneal area		
Change over trial: median	12.59 (4.07–42.89)	20.25 (14.77–45.61)
Rate of change	<b>0.21</b> (0.09–0.41)	<b>1.16</b> † (0.55–3.46)
Median of PI distribution		
Change over trial: median	<b>15</b> (11.25–23.75)	<b>25</b> † (18.75–36.25)
Rate of change: median	<b>0.41</b> (0.21–0.51)	<b>1.63</b> † (0.73–2.71)

Bold indicates variables that are significantly different from each other.

\* Statistical analysis not applicable because groups divided by this parameter.

† Significant difference between groups ( $P < 0.05$ , Mann-Whitney  $U$  test).

and 0.69 discomfort grades per second, respectively), whereas subjects 1 and 2 (Figs. 2D, 2H) reported much slower increases in the rate of discomfort (0.09 and 0.05 discomfort grades per second, respectively). Of note is that the levels of discomfort in the BU<sub>min</sub> examples increased approximately linearly and showed no evidence of a period of stable low discomfort at the beginning of each trial. In addition, although the rates of increase in discomfort were often much faster in the BU examples (Fig. 3), the level of discomfort at end of trial was similar for these examples (Fig. 3D compared with Fig. 2H).

Many of the sensory measures were significantly correlated with each other. The rate of discomfort over trials was significantly correlated with the discomfort grade after the trial and VAS scales of stinging and pricking ( $0.61 < r > 0.67$ ,  $P < 0.004$ , Spearman's  $\rho$ ). The VAS irritation score was significantly correlated with stinging, burning, and pricking ( $0.62 < r > 0.68$ ,  $P < 0.01$ , Spearman's  $\rho$ ). Cooling showed no significant correlations with any other measure.

### Relationship Between Tear Film Fluorescence and Sensory Measures

Figure 4A shows the relationship between changes in the median PI per second as a function of the changes in discomfort per second during trials for the BU and BU<sub>min</sub> groups. Both groups showed increasing discomfort as the median PI decreased, but the BU<sub>min</sub> group progressed at a slower rate than the BU group. These data strongly support the hypothesis that the rate of increasing discomfort during

extended eye opening trials<sup>22</sup> is highly correlated with the rate of diminishing fluorescence, whether associated with TBU or not.

The MBI could be considered a sensory measure because it represents the time the eye can be kept open before discomfort or pain caused the subject to blink.<sup>28</sup> In this study, the MBI was inversely associated with the rate of increasing discomfort in both groups (Fig. 4B). The correlations in Figures 4A and 4B support the hypothesis that rapidly developing TBU or tear thinning leads to rapidly increasing discomfort and the need to blink.

We used exploratory factor analysis in order to further understand more complex relationships among fluorescence and sensory variables tested in this study. This analysis yielded two main factors or dimensions that accounted for more than 60% of the variability in the data. The relationship between these two dimensions is illustrated by the correlation circle pictured in Figure 5, in which dimension 1 (accounting for 48.7% of the variability in the data) is plotted against dimension 2 (accounting for 12.7% of the variability in the data). Each variable component is a vector, and components that are close to each other indicate high correlations with each other, such as the rate of change in DarkPix, median PI, and TBU rate (previously discussed). These fluorescence measures are in close proximity to some sensory measures, including discomfort rate during trials and VAS scores of pricking and irritation after trials, indicating high correlations among these measures. The MBI opposes other data, due to its high negative correlation with other variables. Thus, the Figure 5 correlation circle provides a simplified summary image of the complex correlations among fluorescence and sensory data. These correlations support the hypothesis underlying this study, suggesting that changes in PI intensity over time during eye opening trials are associated with sensory stimulation of the ocular surface.

**TABLE 3.** The Median and Interquartile Ranges for Sensory Data

Factor	BU <sub>min</sub> Group (≤5% TBU)	BU Group (>5% TBU)
Discomfort		
Median rate during trial	0.22 (0.06–0.29)	0.45 (0.14–0.71)
Median grade after trial	5 (5–8)	7 (6–8)
Sensory VAS scales (10-mm scale), Median (interquartile range)		
Irritation	3.2 (1.1–5.9)	6.7 (4.4–7.4)*
Stinging	5.5 (2.6–6.8)	7.0 (6.1–7.5)
Burning	4.8 (3.3–5.4)	7.5 (4.6–7.5)
Pricking	1.1 (0.2–1.8)	3.5 (2.5–4.7)*
Cooling	0.6 (0.2–5.9)	2.2 (0.2–3.3)

\* Significant difference between groups ( $P < 0.05$ , Mann-Whitney  $U$  test).

### DISCUSSION

In this study, we used a model of extended eye opening with a pixel-based image analysis of the tear film, which revealed that both TBU and tear film thinning stimulate ocular surface sensory neurons. These data suggest that both global thinning or regional thinning of the tear film can stress the ocular surface, supporting a connection between ocular irritation and changes in tear film thickness or instability.<sup>10</sup>

All extended eye opening trials in this study resulted in some level of discomfort. While both tear thinning and TBU

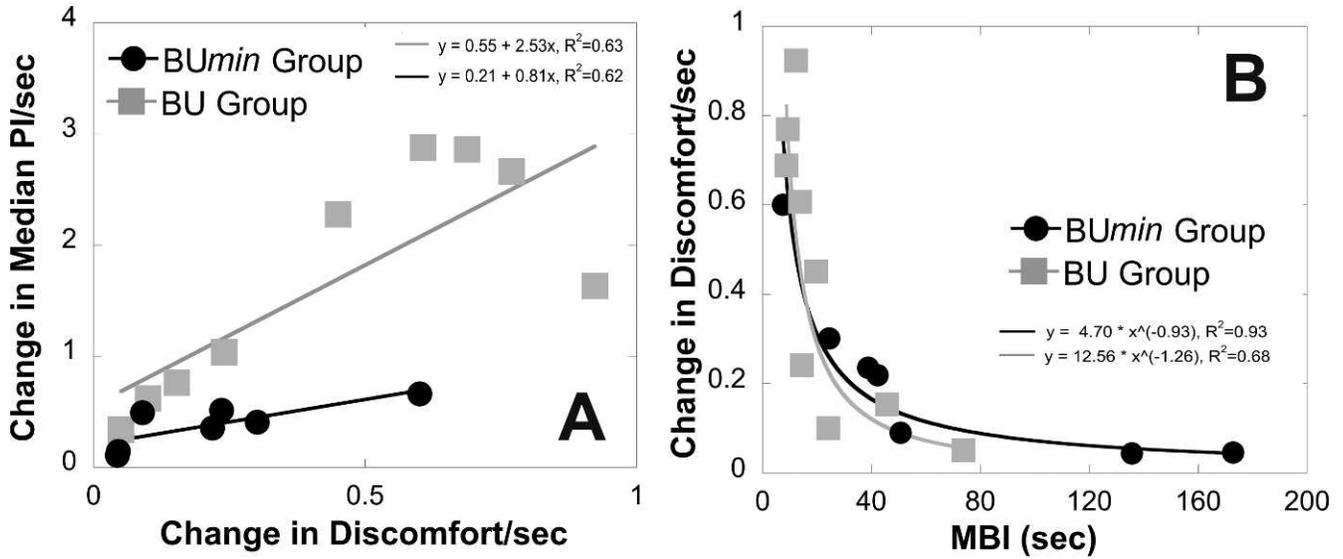


FIGURE 4. (A) Change in median PI versus change in discomfort over trials and (B) change in discomfort versus MBI in the BU and BUmin groups.

### Correlation circle

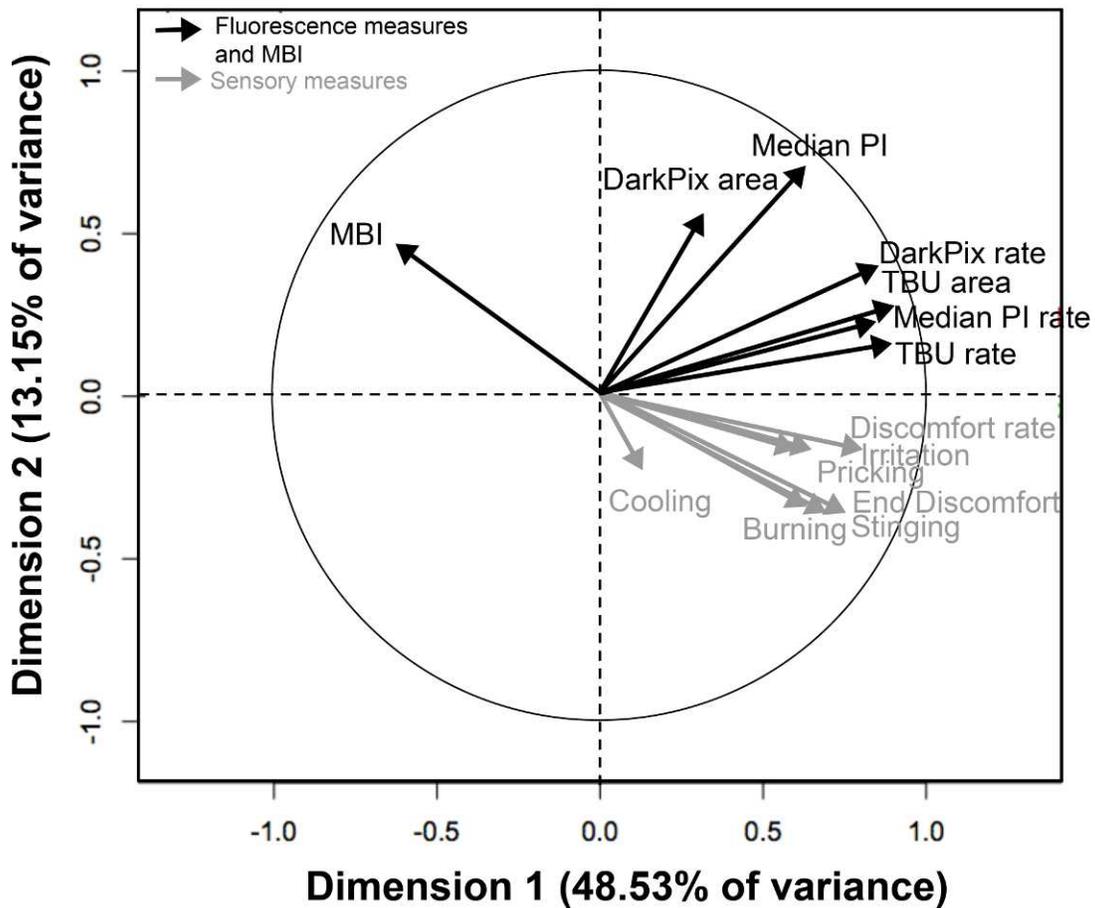


FIGURE 5. Correlation circle representing the two dimensions that account for approximately 62% of the variance of the data. Fluorescence measures and the MBI are indicated by black lines and the sensory data group are delineated by gray lines. The length of each vector indicates the strength of the correlation.

were associated with stimulation of ocular surface sensory neurons, the cause of the stimulation was not well understood. Subjects felt more stinging and burning than cooling and pricking, suggesting that the irritation during trials could have been, in part, a chemical stimulus.<sup>36</sup> Many have speculated that tear thinning leads to increases in hyperosmolarity,<sup>14,16,32</sup> providing a chemical stimulus for surface polymodal nociceptors<sup>11</sup> and increasing the inflammatory response.<sup>10,12,37</sup> Recently, we showed, using a combination of imaging and psychophysical and cell biology methods, that tear film hyperosmolarity may transiently spike during eye opening trials, perhaps reaching levels as high as 800 to 900 mOsm/kg.<sup>15</sup> Thus, given tear film evaporation, thinning and turnover rates,<sup>38-40</sup> it appears reasonable to attribute the slow increases in discomfort during trials for subjects 1 and 2 (Fig. 2) to thinning of the tear film with concurrent slow increases in hyperosmolarity.

However, the level of discomfort during TBU was often higher and increased much more quickly than with tear thinning (Table 2) and significantly more pricking sensations were associated with BU trials (Table 3). This suggests that TBU involves rapid increases in the stimulation of polymodal nociceptors, perhaps due to sharp local increases in hyperosmolarity.<sup>15</sup> Although the actual events within areas of TBU are unknown, this result also suggests that TBU may involve in rapid evaporation. This could lead to bound mucin layer<sup>41</sup> drying in the TBU area, which may slightly deform the ocular surface and result in mechanical stimulation to the nociceptors.<sup>36</sup> In addition, activation of cold-sensitive afferents may play a role, but their potential contribution to discomfort during TBU or tear thinning is less clear.<sup>25,36,42,43</sup>

Regardless of the exact nature of the stimulus provided by the tear film during TBU, these results strongly support the hypothesis that both TBU and tear thinning provide stress to the ocular surface in the form of sensory stimulation of underlying corneal nerves. However, some subjects in this study withheld from blinking for extended periods that would not be realistic under daily conditions. Thus, considering an average blink rate of 12 blinks/min,<sup>44</sup> slow thinning of a stable tear film (as in subjects 1 and 2) would be unlikely to produce much discomfort. However, with rapid and extensive TBU, the discomfort in the interblink interval increased quickly in a short period of time (subjects 3 and 4), so that these subjects presumably must blink frequently to avoid the discomfort associated with rapid and extensive TBU.

In a recent study, we showed associations between the discomfort generated by TBU, tear film hyperosmolarity and the production of pro-inflammatory mediators by corneal epithelial cells.<sup>15</sup> We and others have shown that dry eye patients often repeatedly display TBU during tasks such as computer use, during which the blink rate is known to slow markedly.<sup>45,46</sup> Thus, in patients with extensive and rapidly developing TBU (as in subjects 3 and 4), it appears likely that inadequate blinking could lead to discomfort and repeated stimulation of surface nociceptors over the day. This may explain, in part, the origin of dry eye symptoms because repeated nociceptor stimulation is associated with increased inflammation and hyperalgesia.<sup>11</sup>

Although keeping the eye open for extended periods occurs infrequently in daily life, we employ it here as a human-based in vivo model to study the stress that tear instability provides to the ocular surface. The need to blink at the end of the trial, defined by the MBI,<sup>28</sup> could be considered to provide information about both the tear film stimulus and the sensory integrity of the cornea as it responds to the pain or discomfort provided by TBU or tear thinning. In combination with spatiotemporal image analysis of the tear film, our methods allow study of the associations between tear film changes and

the sensory response providing much more information than the traditional tear break-up time (TBUT) clinical test, which employs time as the single metric for tear instability. In addition, while the TBUT has been used clinically for more than 40 years,<sup>30</sup> interpretation of tear film fluorescence changes remains poorly understood. For example, areas of TBU are often referred to as “dry spots,”<sup>30</sup> but their cause and composition remain poorly understood.

To address this issue and improve the understanding of events associated with TBU and thinning, we quantified tear film fluorescence changes over time within eye opening trials. Theoretically, the total corneal fluorescence (as measured in this study) during a trial could decrease by two possible mechanisms, either by fluorescein molecules exiting the tear film or by decreases in emitted fluorescence. However, current theory suggests that there is little fluid exchange in or out of the corneal compartment of the tear film during the interblink interval,<sup>31,32</sup> so that most of the fluid lost as the tear film thins over time occurs by evaporation.<sup>18,33,47</sup> This should act to increase the concentration of fluorescein molecules,<sup>40</sup> which are well known to exhibit quenching once a “critical concentration” is reached.<sup>48</sup> Concentration quenching of fluorescein molecules occurs when the fluorescein concentration is high enough to increase collisions between adjacent molecules, cause dye dimerization, or promote energy transfer to nonfluorescent dimers, all of which reduce emitted fluorescence.<sup>49</sup>

Recently, tear film thickness was measured directly with changes in fluorescence over time, demonstrating that fluorescein dye quenching occurred during tear film thinning.<sup>40,50</sup> Nichols et al.<sup>50</sup> showed that decreasing tear film thickness with extended eye opening was proportional to the square root of the declining fluorescence intensity. Adapting these methods to our pixel-based data, we obtained a mean fluorescein intensity decay rate of  $2.98 \pm 4.29$  per second (data not shown), which is consistent with their average results of  $4.11 \pm 6.78$  per second for 2% fluorescein. These data further support the hypothesis that diminishing fluorescence during eye opening trials in this study was due to fluorescein dye quenching as the tear film thinned.

In this study, we used fluorescence imaging and psychophysical methods to infer the tear film changes that produce an ocular surface sensory response to TBU and thinning. We found that both processes stimulated surface sensory neurons, and the rate of change was highly correlated with the rate of discomfort. Although the conditions in this study were not intended to mimic daily life, the results strongly suggest that tear instability can directly stimulate ocular surface neurons. If repeated over the day, this recurring stress and surface neural stimulation could explain the daily increase in dry eye symptoms of ocular discomfort.<sup>27,51</sup>

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