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Variations in the endogenous fluorescence of rabbit corneas after mechanical property alterations

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Abstract. Keratoconus is an eye disease in which the cornea progressively deforms due to loss of cornea mechanical rigidity, and thus causes deterioration of visual acuity. Techniques to characterize the mechanical characteristics of the cornea are important to better monitor changes and response to treatments. To investigate the feasibility of using the endogenous fluorescence of cornea for monitoring alterations of its mechanical rigidity, linear tensiometry was used to quantitate stiffness and Young's modulus (YM) after treatments that increase cornea stiffness (collagen photocross-linking) or decrease stiffness (enzymatic digestion). The endogenous ultraviolet fluorescence of cornea was also measured before and after these treatments. The fluorescence excitation/emission spectral ranges were 280 to 430/390 to 520 nm, respectively. A correlation analysis was carried out to identify fluorescence excitation/emission pairs whose intensity changes correlated with the stiffness. A positive correlation was found between variations in fluorescence intensity of the 415-/485-nm excitation/emission pair and YM of photocross-linked corneas. After treatment of corneas with pepsin, the YM decreased as the fluorescence intensity at 290-/390-nm wavelengths decreased. For weakening of corneas with collagenase, only qualitative changes in the fluorescence spectrum were observed. Changes in the concentration of native or newly created fluorescent molecular species contain information that may be directly or indirectly related to the mechanical structure of the cornea. [DOI: 10.1117/1.JBO.22.9.095005]

Keywords: UV fluorescence; stiffness; Young's modulus; cornea; rabbit eyes; keratoconus; rose bengal; collagen cross-links; fluorescence correlation matrix.

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1 Introduction

The cornea provides most of the power of refraction of the eye. It is transparent and composed of distinct layers with different compositions and structures. The stroma provides ~90% of the corneal thickness and is mainly composed of interwoven type I collagen fibers regularly arranged in multiple layers. The arrangement of the fibers is important for both the transparency and the rigidity of the cornea.

The biomechanical properties of the cornea have been linked to various ocular disorders; for example, keratoconus and corneal ectasia are associated with altered corneal rigidity. Keratoconus is a disease that causes the cornea to progressively acquire a cone-shaped appearance, producing image artifacts and reducing visual acuity. The etiology of keratoconus has been associated with a decrease of corneal rigidity.[1,2] Corneal cross-linking (CXL) is an established treatment for keratoconus.[3] CXL involves the production of chemical cross-links between the collagen and proteoglycan molecules in the cornea thereby increasing its mechanical stability.[4] There are treatments to alter the biomechanical properties of the cornea. However, current clinical methods for analysis of the eye are limited to structural measurements. Traditional mechanical tests are destructive and cannot be employed in clinical settings.

Current methods to measure corneal stiffness in patients include the air puff method that injects an air jet stream onto the eye surface to measure the displacement response. Promising methods currently in development are OCT elastography[5] and Brillouin scattering.[6] In this exploratory study, we evaluate fluorescence as an alternative noninvasive, nondisruptive method to monitor the mechanical stability of the cornea. We propose that fluorescence can be used to probe changes in molecular composition that relate to mechanical stability. Some fluorescent molecules associated with mechanical rigidity are inherently present in tissue, for example, collagen and elastin.[7–9] The fluorescence intensity produced at a given wavelength is proportional to the concentration of the fluorescent substance or fluorophore. This makes it possible to use fluorescence to identify or quantify the concentration of fluorescent materials.

2 Objectives

The objectives are (i) to identify the excitation/emission wavelength pairs whose fluorescence intensity varies when treatments alter the mechanical rigidity of the cornea and (ii) to determine whether or not these variations in fluorescence correlate with alterations in its mechanical rigidity. We evaluated the fluorescence intensity and rigidity relationship by increasing corneal stiffness by forming collagen cross-links with photocross-linking and reducing corneal stiffness by enzymatic digestion.

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3 Study Design and Methods

3.1 *Eye Model*

Young rabbit eyes (Pel-Freez Biologicals, Rogers, Arkansas) were used. The rabbit cornea has a similar diameter, curvature, and thickness to those of human eyes. Furthermore, the radius of curvature of the rabbit eye changes during their first months of life,[10] which serves as a better model of the unstable cornea in keratoconus than mature rabbit eyes.

3.2 *Cornea Treatments*

Different treatments were applied to the corneas to alter their stiffness. While corneas themselves have naturally varying levels of stiffness, manipulating the concentration of collagen cross-links allowed us to have larger biomechanical variations. Ten experimental groups were formed, each comprised of 10 corneas. The control group had no treatment of any type. Three groups were subjected to different levels of photocross-linking to increase cornea stiffness. The remaining six groups were subjected to proteolytic enzymatic digestion to reduce stiffness: three were exposed to collagenase and three to pepsin.

3.2.1 *Photocross-linking*

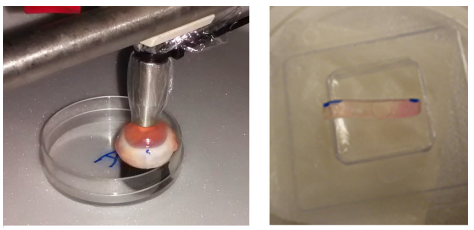
The epithelium was removed by immersing the cornea in 30% ethanol for 15 s and scraping the cell layer off with a blade. Next, the cornea was immersed (cornea facing down) in a 0.1% rose bengal (Sigma-Aldrich, St. Louis, Missouri) solution for 2 min. Afterward, the cornea was sprayed with phosphate buffered saline (PBS) and cleaned with tissue (KimWipe, Kimberly Clark, Dallas, Texas). The first group was exposed to green 532-nm laser light at constant power of 250 $\mathrm{mW/cm^2}$ for 800 s (200 $\mathrm{J/cm^2}$), the second group for 1200 s (300 $\mathrm{J/cm^2}$), and the third one for 1600 s (400 $\mathrm{J/cm^2}$). R200, R300, and R400 refer to these photocross-linked groups, respectively. In the Results and Discussion section the described photocross-linking method using rose bengal is referenced to as RGX.

3.2.2 *Proteolytic enzymatic digestion*

Three groups of corneas were submerged in either 0.1% collagenase (lyophilized collagenase, Advanced Biomatrix, San Diego, California) in PBS, and three groups were submerged in 1% pepsin (pepsin from porcine gastric mucosa, Sigma-Aldrich, St. Louis, Missouri) in 0.1 M HCl for 2, 4, and 8 h at ambient temperature. These groups are Pep2, Col2, Pep4, Col4, Pep8, and Col8, respectively.

3.3 *Biomechanics*

After treatment, strips were prepared from the middle of the corneas using a cutting tool comprised of two razor blades separated by 2 mm. The 2-mm-wide corneal strip is shown in Fig. 1. Next, each corneal strip was placed on a tensiometer (eXpert 4000 Admet, Norwood, Massachusetts) as shown in Fig. 2(a). This instrument has a built in initial subroutine that prestretches the material in order to determine the zero-strain state of the material before running a tensile test. During the tensile test, the instrument stretched the cornea strip at a constant 16.7 μm/s (~1 mm/ min) rate while recording the applied force as a function of displacement. Since the cornea is viscoelastic (nonlinear), the mechanical properties of the cornea were



(a) (b)

Fig. 1 (a) Fluorometer probe placed atop the rabbit eye. The probe was covered with a plastic film to avoid contamination. (b) Corneal strip excised after staining and irradiating the eye.

calculated from the slope at a tangent (strain of 0.1) of the experimental curves. The length, width, and thickness of the strip were measured with a caliper. The thickness and the force versus displacement curves were used to calculate the Young's modulus (YM).

Stiffness and YM characterize how resistant an object is to deformation when an external force is applied. For linear materials, stiffness is defined as the proportionality constant between the force applied (F) to an object and the elongation (or compression) produced by that force (δ). Analogously, for linear materials, the YM is defined as the ratio of the stress (σ) in a material to the strain (ε) exerted on it. The stress is defined as

$$\sigma = \frac{F}{wt},$$

where F is the force applied to the object and wt is its cross-section area, in this case w being the width of the corneal strip and t is the thickness of the corneal strip. Strain is defined as

$$\epsilon = \frac{\delta - L_0}{L_0},$$

where δ is the deformed length of the corneal strip at force F and L_0 is its initial length. Biological materials follow a J-shaped curve in response to deformation rather than a straight line.[11] Calculation of YM requires estimating a linear region of the curve (described as an amount of stretching before the sample is deformed irreversibly). We employed an algorithm that analyzes the stress–strain curve around a strain of 0.1 (deformation of 10% of the initial length) and finds the slope at that point with a two-point approximation (9% and 11% approximately). The slope is an approximation of the YM (Fig. 2).

3.4 *Fluorescence*

A randomized fiber optic Spectrofluorometer (SkinSkan, Horiba Scientific, Edison, New Jersey) was used to measure the endogenous fluorescence of the corneas. The instrument is comprised of two independent monochromators and a Xenon lamp, allowing both excitation and emission fluorescence measurements. A plastic film that did not affect measurements was used to protect the probe, as shown in Fig. 1(a). The probe is a bundle of randomized emission and detection fiber, and it is placed on top of the sample to measure backscattered fluorescence. Assuming a corneal thickness of 500 μm, 80% to 90% of the UV radiation emitted by the fluorometer is absorbed within the cornea,[12] that is, the fluorescence contribution from deeper eye structures is negligible. The device has detectors associated with both monochromators that measure both the excitation and the emission intensity. Fluorescence excitation–emission

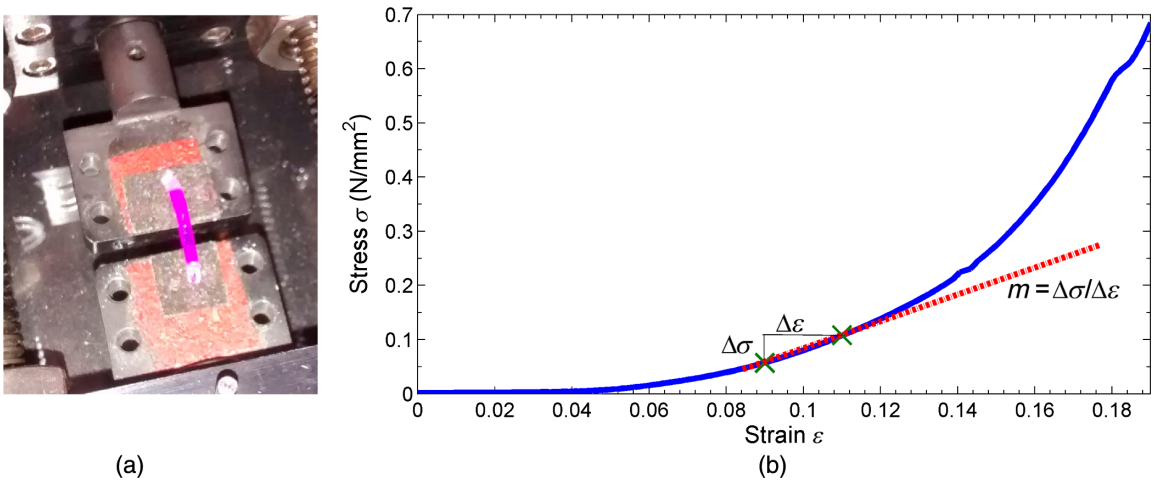


Fig. 2 (a) Corneal strip on grips of tensiometer. (b) Typical stress–strain curve produced by the tensiometer. Two points on the curve were selected around a 10% extension of the original length (0.1 strain) and the YM was calculated as the slope between them.

matrices (EEMs) in the 280- to 430-nm excitation and 390- to 520-nm emission ranges were acquired before and after photo-cross-linking and digestion treatments. The separation among scanned wavelengths was 4 nm. Each fluorescence emission spectrum was normalized to the measured lamp excitation spectrum, to compensate for differences in lamp spectral emission.

3.5 *Correlation Analysis*

The different fluorescence emission curves for each sample were stitched together to form an EEM. An EEM can be plotted in two dimensions by representing the excitation wavelength in one Cartesian axis, the emission wavelength in the other, and the fluorescence intensity associated with that pair represented in a color scale. This is a convenient visual way to identify fluorescent excitation/emission pairs (E/E pairs) that could be related to the stiffness of the corneal strip.

In total, 220 EEMs were obtained from scanning the corneas at different wavelengths. While it is possible to observe changes in the EEMs induced by the treatments, the sheer amount of information makes it difficult to determine which E/E pairs change the most just from visual evaluation of the EEMs or to identify the changes in fluorescence that are associated with changes in the mechanical properties. Thus, we calculated the correlation coefficients between the YM of a given experimental group and the fluorescence intensity of every E/E pair (837 different E/E pairs). The 837 correlation coefficients were arranged in a matrix whose rows correspond to the emission wavelength and its columns to the excitation wavelength. Each element of the matrix represents how correlated the YM is with the fluorescence at the corresponding wavelength pair. By repeating this procedure for each experimental group, we obtain 10 correlation matrices of YM and fluorescence intensity. These matrices were plotted as level curves to identify correlation coefficient maxima and minima.

The Spearman method was used to calculate these correlation coefficients. This method is best at identifying a variable that is changing monotonically (ever increasing/decreasing) with respect to another, even if that change is nonlinear. It is also more robust in the presence of outliers and nonnormal data than the Pearson's correlation. A correlation coefficient of 0 means no correlation, 1 is a direct correlation, and −1 is an inverse correlation. Intermediate numbers are associated with a possible correlation, direct or inverse according to the sign. The closer the coefficient is to 1, the more likely the correlation is statistically significant. A t-value can be calculated

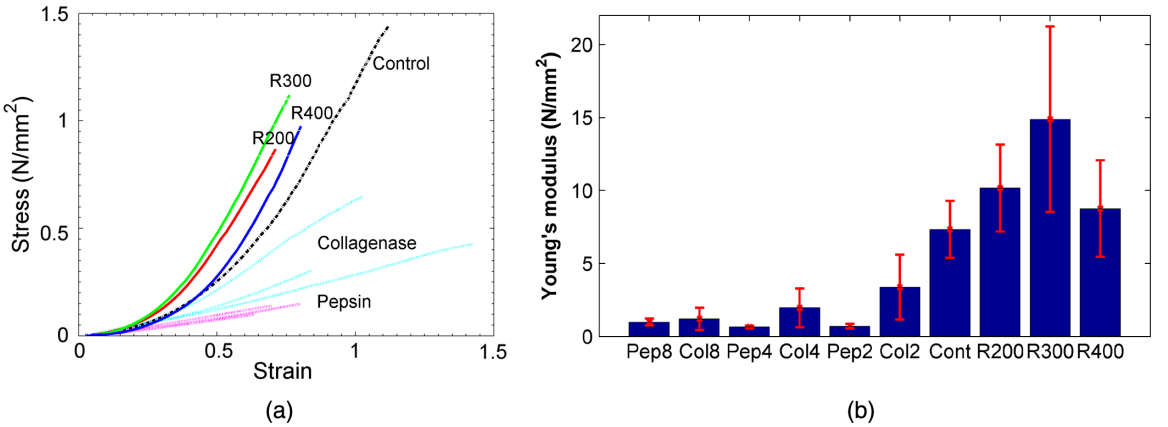


Fig. 3 (a) Mean stress–strain curves, obtained by averaging the stress–strain curves of each experimental group. (b) Mean YM for all experimental groups, with their corresponding standard deviation.

from the correlation coefficient ρ and the degrees of freedom (which is equal to the number of samples minus two, $n-2$) with the equation

$$t = \frac{\rho\sqrt{n-2}}{\sqrt{1-\rho^2}}.$$

A p-value (the probability that, if the null hypothesis was to be true, the same or larger correlation would be observed if the experiment is repeated) can be found from the t-value using the two-tailed cumulative student's t-distribution with $n-2$ degrees of freedom. Since $n = 10$ for our experimental groups, the correlation coefficient needs to reach an absolute value of 0.63 or more to reach $p \leq 0.05$, corresponding to $t = 2.3$.

All the data processing was performed in MATLAB® (MATLAB® R2014a, MathWorks, Natick, Massachusetts).

4 Results

4.1 *Experimental Measurements*

Average stress as a function of strain is shown for all groups in Fig. 3(a). The curves clearly indicate that relatively low forces were required to stretch the corneas subjected to enzymatic digestion while relatively larger forces were required to stretch the corneas subjected to cross-linking. The average YM at 10% deformation for each experimental group is shown in Fig. 3(b). In general, digested samples had a lower YM than the control

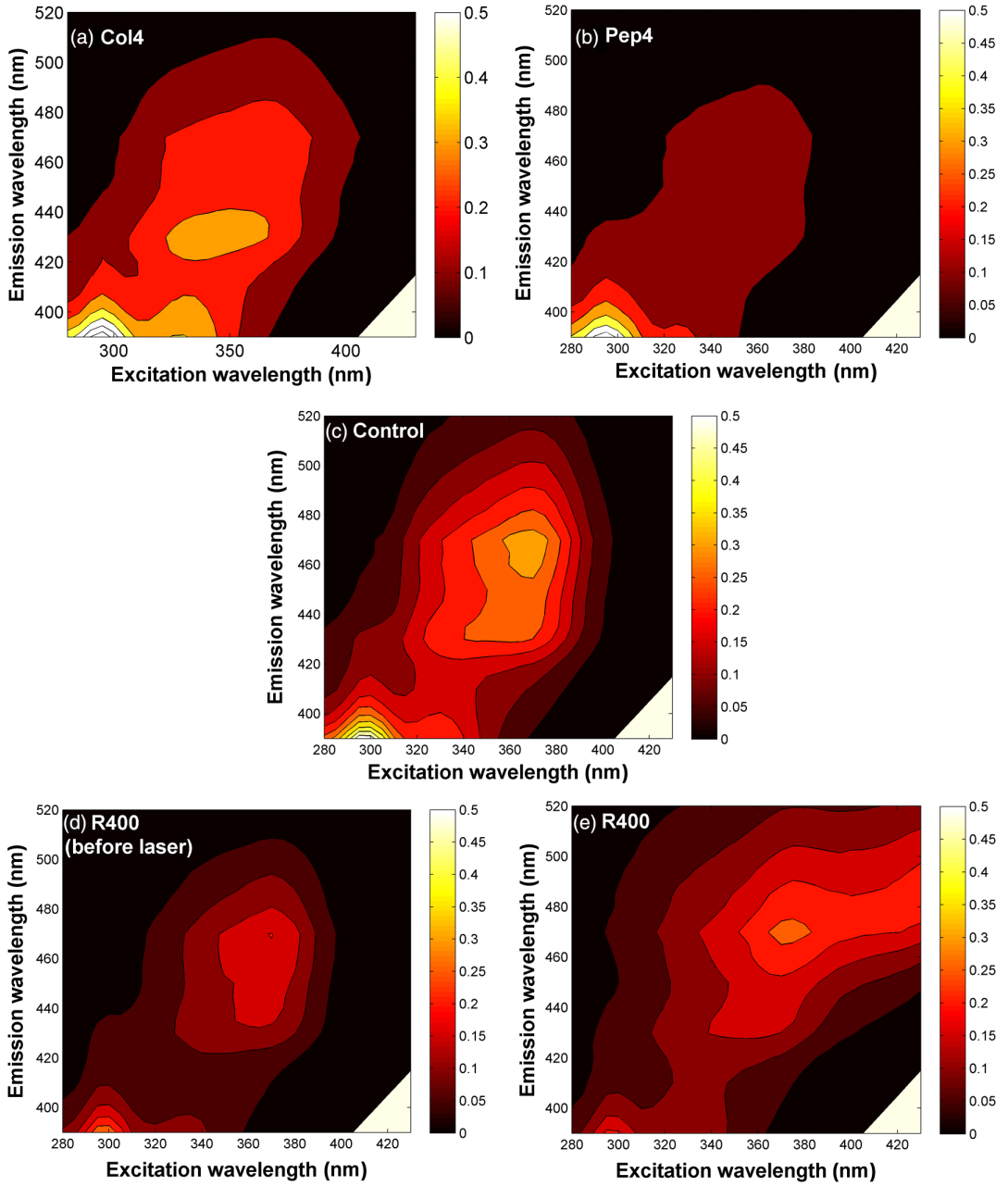


Fig. 4 Average EEMs for five representative cases of the experimental groups: (a) collagenase-treated samples, 4 h; (b) pepsin-treated samples, 4 h; (c) control untreated samples; corneas after staining with rose bengal, (d) before irradiation, and (e) after 400 J/cm^2 irradiation with green laser light.

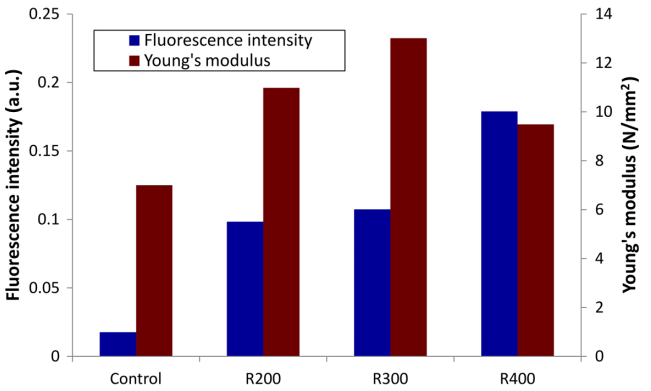


Fig. 5 Average YM (right scale) and mean fluorescence intensity (left scale) for the four RGX groups at the E/E pair of 415/480 nm. This fluorophore appears after the eyes have been treated with RGX, and its fluorescence intensity (correlated with its concentration) increases with the amount of laser treatment.

group, while the cross-linked groups had a higher YM (*t*-tested, $p < 0.05$, except for the R400 group).

Average fluorescence intensity maps (visual representation of EEM) for representative corneal treatments are shown in Fig. 4. Visual inspection clearly shows that different treatments resulted in different fluorescence patterns. Figures 4(a) and 4(b) show the fluorescence of corneas digested with collagenase (4 h) and pepsin (4 h), respectively. In these figures, the fluorescence peaks at 370/470 and 340/430 nm of the control [as shown in Fig. 4(c)] were severely attenuated after digestion with pepsin [Fig. 4(b)]. Digestion with collagenase resulted in small variations, increasing slightly the fluorescence intensity across all wavelengths except for the 290-/390-nm band [Fig. 4(a)]. Figure 4(d) shows the fluorescence intensity of corneas after exposure to rose bengal solution, and Fig. 4(e) shows the intensity after exposure to rose bengal and laser light (400 J/cm^2, right). Other RGX-treated groups displayed almost identical fluorescence patterns. Treating the cornea with RGX [Fig. 4(e)] created a fluorescent region centered around 415/480 nm. This fluorescent region is not the fluorescence of the rose bengal itself, since it is not present in the stained but nonirradiated eye [Fig. 4(d)], suggesting that it is a fluorescent species created during the cross-linking treatment.

4.2 *Correlation of Fluorescence to Decreased Corneal Stiffness*

Enzymatic digestion was used to decrease cornea stiffness. The corresponding fluorescence intensity of corneas treated with collagenase increased, and the fluorescence intensity of corneas treated with pepsin decreased relative to control (Fig. 4). The correlation analysis of Col4 and Pep4 suggested a positive correlation for excitation wavelengths shorter than 300 nm. For Pep4, there is a statistically significant correlation peak at 290/390 nm (correlation coefficient of 0.64, $p = 0.046$). This correlation peak also exists on Pep2 ($\rho = 0.62$, $p = 0.56$) and Pep8 ($\rho = 0.52$, $p = 0.12$), albeit with a nonsignificant p-value. In the case of collagenase, the only correlated pair that reaches statistical significance ($\rho = 0.68$, $p = 0.03$ at 280/410 nm) is present only in Col4 but not in Col2 ($\rho = -0.13$, $p = 0.72$) or Col8 ($\rho = -0.19$, $p = 0.60$) and is in a spectral region with relatively low fluorescence, suggesting this peak is meaningless. This corresponds to the fluorescence region of known skin fluorophores.[13,14]

4.3 *Correlation of Fluorescence to Increased Corneal Stiffness*

RGX was used to increase the stiffness of the corneas. Correlation analysis yielded a high correlation coefficient of around 0.7 ($p = 0.023$) at 415/480 nm for R200, which corresponds to a region of new fluorescence. Other neighboring pairs also show similar correlation coefficients; however, the intensity of the fluorescence signal of this pair was the highest [Fig. 4(e)]. There are other neighboring pairs ($415 \pm 15/480 \pm 15$ nm) that fulfill this correlation/intensity criteria and therefore could be discussed and potentially used likewise. A very similar result was found for the R300 group (not shown). However, the R400 group yielded a correlation coefficient close to zero. In Fig. 5, the bar graph of the fluorescence intensity of the 415/480 nm as a function of RGX treatment shows that the fluorescence increases with the fluence, relative to the control. Figure 5 also shows the values for YM, which increase only for R100 and R200 relative to control, but not R400. This is consistent with the slight negative correlation found from the R400 correlation matrix.

5 Discussion

We evaluated potential correlations between variations in fluorescence of the cornea (from both native molecules and newly created species) and its mechanical rigidity. Changes in fluorescence, relative to controls, within the 280- to 440/380- to 520-nm excitation/emission wavelengths correlate with increased stiffness by corneal photocross-linking of collagen, and with reduced stiffness in response to enzymatic digestion of collagen cross-links. The EEMs show that quantitative (intensity) and qualitative (maps distribution or shape) variations in fluorescence correlate with the rigidity of the cornea changes, suggesting that it is feasible to establish correlations. The magnitude, distribution, and direction of these changes are a function of the treatment type because, most likely, each treatment affects different molecular species. Having the fluorescence intensity directly or indirectly correlating to mechanical properties could be used in several ways, for example, a UV fluorescence photography system[15,16] to image and monitor treatment in corneas.

For corneal enzymatic digestion with pepsin, the YM decreased as the fluorescence intensity at 290-/390-nm wavelengths decreased ($\rho = 0.64$ and $p = 0.046$). Based on published fluorescence excitation and emission spectra of molecules innate to cell and tissue, photons of 290-nm wavelength can excite the fluorescence of nicotinamide adenine dinucleotide, pyridoxine, collagen cross-link, elastin cross-link, and tryptophan molecules.[17] At this excitation wavelength, only pyridoxine, collagen cross-link, elastin cross-link, and tryptophan molecules emit photons at the 390-nm wavelength. The fluorescence emission peak of tryptophan is at 340 nm, that is, tryptophan fluorescence at 390 nm is weak. The fluorescence emission peaks of pyridoxine, collagen cross-link, and elastin cross-link molecules are at 390, 390, and 420 nm, respectively, while their respective excitation peaks are at 310, 340, and 350 nm. It follows that a spectral analysis considering known fluorophores suggests that the spectral features of pyridoxine are the closest to the 290-/390-nm fluorescence observed herein.

Although the molecules mentioned above are in principle present in the cornea,[18] the literature on eye autofluorescence that we are aware of studied the lens only from 350- to 430-nm excitation wavelengths or the cornea at 446-nm excitation wavelength.[14,19,20] These studies concentrated on the relationship between eye autofluorescence and metabolism, for example, the accumulation of lipofuscin in age-related macular degeneration. Further studies are warranted to establish the molecular source of the 290-/390-nm fluorescence.

For corneal enzymatic digestion with collagenase, the YM decreased as the digestion time increased [Fig. 3(b)]; however, the fluorescence intensity slightly increased statistically uncorrelated with the YM (data not shown). The slight increase in intensity may be explained by superficial changes that allowed more photons either to enter the cornea or return from the cornea to the detector, or both. No fluorescence pair correlated with mechanical properties when using collagenase, but there was a change in shape of the fluorescence matrix, which could help identify the presence of collagenase in the eye. A limitation of enzymatic digestion is that the cornea was unevenly degraded because the spatial distribution of the enzyme is driven by diffusion, that is, a nonuniform distribution may result in undigested areas. For enzymatic digestion treatments, it is also a possibility that the variations in fluorescence may not be directly linked to mechanical properties through molecules present before treatment. The fluorescent cross-links of collagen may diffuse to the surface of the cornea as the enzymes release them, then the fluorescence signal could increase as more cross-links surface. This release process may also be a metric of the digestion process and, thus, an indirect metric of mechanical properties.

For corneal photocross-linking with RGX, changes in the fluorescence intensity of the 415-/480-nm EE pair are correlated with stiffening of corneas exposed to 200- and 300-J/cm^2 laser fluence. A laser dose of 400 J/cm^2 resulted in overexposure: the fluorescence intensity was higher than that of corneas exposed to lower fluence but the YM was similar to the modulus of corneas exposed to 200 J/cm^2. A possible explanation is that a prolonged exposure may cause structural weakening of the corneas, which is suggested by the negative correlation coefficient obtained at this wavelength band. This overexposure phenomenon has also been observed in the treatment of porcine eyes with riboflavin and ultraviolet A, doubling the time of exposure resulted in a decreased YM similar to that of nonexposed controls.[21] The reason for this change is not understood. It should also be noted that in the literature RGX has been studied up to a maximum laser dosage of only 200 J/cm^2 and thus higher amounts might not be clinically relevant. However, knowing that a very long exposure reduces the efficacy of the treatment, and that the fluorescence is proportional to exposure might be used by physicians as a guide to provide the optimal amount of treatment. The fluorescence maps of controls show no fluorescence at this EE pair. Therefore, either a new molecular species was created during treatment or an existing molecular species was unquenched. The former seems more likely than the latter as none of the known fluorescent molecules in tissue has a 415-/480-nm excitation/emission band. For RGX treatments, it is also possible that the variations in fluorescence may not be directly linked to stiffness through molecules present before treatment. An alternative interpretation for the RGX treatments is that variations in fluorescence are related to RGX photoproducts—as opposed to collagen cross-links—which in turn may be related to the efficiency of the treatment photochemistry and, thus, to the mechanical properties.

The YM ranges from 1 to 25 MPa, which is consistent with magnitudes reported in the literature for similar tests and cornea models. Scarcelli et al.[6] reported 3.7 to 31.7 MPa in a study that applied RGX to enucleated rabbit eyes. Cherfan et al.[22] and Wollensak and Iomdina[23] reported 11 to 20 MPa in a study that induced collagen cross-linking with riboflavin and UV applied to rabbit eyes *in vivo*. In a related fluorescence study using UV-initiated photocross-linking with riboflavin to increase corneal stiffness in enucleated rabbit eyes, the YM ranged from 10 to 30 MPa.[24] In this study, a dose-dependent decrease in the 290-/340-nm excitation/emission fluorescence pair with an increase in corneal stiffening after treatment was found. The source of UV fluorescence at these wavelengths is also endogenous to the cornea—and almost every tissue—and ascribed to the aromatic amino acid tryptophan.

6 Summary, Conclusions, and Significance

Changes in the concentration of native or newly created fluorescent molecular species contain information that may be directly or indirectly related to the mechanical structure of the cornea, and the relationship or not between variations in fluorescence and mechanical stiffness is treatment dependent. In this study, correlation analysis and signal levels show that there are clusters of excitation/emission wavelengths that could potentially be used for monitoring treatment response to corneal photocross-linking; for instance, fluorescence at 485 nm upon excitation at 415-nm blue light. The 290-/390-nm fluorescence of eyes with reduced stiffness correlated with mechanical properties with pepsin enzymatic digestion. Further comprehensive optical and mechanical studies focusing in these pairs are warranted to establish definitive correlations.

Disclosures

The authors have no relevant financial interests in this article and no potential conflicts of interest to disclose.

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References

1. R. Ambekar, K. C. Toussaint, and A. W. Johnson, "The effect of keratoconus on the structural, mechanical, and optical properties of the cornea," *J. Mech. Behav. Biomed. Mater.* **4**(3), 223–236 (2011).
2. I. S. Nash, P. R. Greene, and C. S. Foster, "Comparison of mechanical properties of keratoconus and normal corneas," *Exp. Eye Res.* **35**(5), 413–424 (1982).
3. A. Alhayek and P.-R. Lu, "Corneal collagen crosslinking in keratoconus and other eye disease," *Int. J. Ophthalmol.* **8**(2), 407–418 (2015).
4. C. Wittig-Silva et al., "A randomized, controlled trial of corneal collagen cross-linking in progressive keratoconus: three-year results," *Ophthalmology* **121**(4), 812–821 (2014).
5. M. R. Ford et al., "Method for optical coherence elastography of the cornea," *J. Biomed. Opt.* **16**(1), 016005 (2011).
6. G. Scarcelli et al., "Brillouin microscopy of collagen crosslinking: noncontact depth-dependent analysis of corneal elastic modulus," *Invest. Ophthalmol. Visual Sci.* **54**(2), 1418–1425 (2013).
7. R. Gillies et al., "Fluorescence excitation spectroscopy provides information about human skin *in vivo*," **115**, 704–707 (2000).

8. N. Kollias et al., "Endogenous skin fluorescence includes bands that may serve as quantitative markers of aging and photoaging," *J. Invest. Dermatol.* **111**(5), 776–780 (1998).
9. B. Hofmann et al., "Advanced glycation end product associated skin autofluorescence: a mirror of vascular function?" *Exp. Gerontol.* **48**(1), 38–44 (2013).
10. P. A. Tsonis, *Animal Models in Eye Research*, Academic Press, San Diego, California (2011).
11. University of Cambridge, "J-shaped curves," *DoITPoMS*, http://www.doitpoms.ac.uk/tlplib/bioelasticity/j-shaped-curves.php (15 September 2015).
12. L. Kolozsva et al., "UV absorbance of the human cornea in the 240- to 400-nm range," *Invest. Ophthalmol. Visual Sci.* **43**, 2165–2168 (2017).
13. M. Monici, "Cell and tissue autofluorescence research and diagnostic applications," *Biotechnol. Annu. Rev.* **11**, 227–256 (2005).
14. A. Chorvatova and D. Chorvat, "Tissue fluorophores and their spectroscopic characteristics," in *Fluorescence Lifetime Spectroscopy and Imaging*, L. Marcu, P. W. M. French, and D. S. Elson, Eds., pp. 47–84, CRC Press, Boca Raton, Florida (2015).
15. W. Franco et al., "Review of applications of fluorescence excitation spectroscopy to dermatology," *Br. J. Dermatol.* **174**, 499–504 (2015).
16. E. Gutierrez-Herrera et al., "Fluorescence excitation photography of epidermal cellular proliferation," *Br. J. Dermatol.* **174**(5), 1086–1091 (2016).
17. G. A. Wagnieres, W. M. Star, and B. C. Wilson, "*In vivo* fluorescence spectroscopy and imaging for oncological applications," *Photochem. Photobiol.* **68**(5), 603–632 (1998).
18. M. Xuan et al., "Proteins of the corneal stroma: importance in visual function," *Cell Tissue Res.* **364**(1), 9–16 (2016).
19. J. A. Zuclich, R. D. Glickman, and A. R. Menendez, "In situ measurements of lens fluorescence and its interference with visual function," *Invest. Ophthalmol. Visual Sci.* **33**(2), 410–415 (1992).
20. D. Schweitzer et al., "Towards metabolic mapping of the human retina," *Microsc. Res. Tech.* **70**(5), 410–419 (2007).
21. E. Lanchares et al., "Biomechanical property analysis after corneal collagen cross-linking in relation to ultraviolet A irradiation time," *Graefe's Arch. Clin. Exp. Ophthalmol.* **249**(8), 1223–1227 (2011).
22. D. Cherfan et al., "Collagen cross-linking using rose bengal and green light to increase corneal stiffness," *Invest. Ophthalmol. Visual Sci.* **54**(5), 3426–3433 (2013).
23. G. Wollensak and E. Iomdina, "Long-term biomechanical properties of rabbit cornea after photodynamic collagen crosslinking," *Acta Ophthalmol.* **87**(1), 48–51 (2009).
24. M. Williams, W. Lewis, and W. Franco, "Loss of tryptophan fluorescence correlates with mechanical stiffness following photo-crosslinking treatment of rabbit cornea," *Invest. Ophthalmol. Visual Sci.* **58**(2), 1110–1115 (2017).

Biographies for the authors are not available.