

# Changes in Rat Scleral Collagen Structure Induced by UVA–Riboflavin Crosslinking at Various Tissue Depths in Whole Globe Versus Scleral Patch

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**Purpose:** To investigate structural changes in scleral collagen fibers at various tissue depths before and after photosensitized crosslinking (CXL) both isolated scleral patch versus whole globe using second-harmonic generation (SHG) imaging.

**Methods:** Scleral tissues were harvested from Sprague–Dawley rats and separated into three groups: untreated sclera (control), full-thickness scleral patch for CXL (Free Scleral CXL group), and sclera in intact globe for CXL (Globe CXL group). The CXL groups were soaked in 0.1% riboflavin and irradiated with 365 nm ultraviolet-A light (power, 0.45 mW/cm<sup>2</sup>) for 30 minutes. SHG images were acquired every 5 μm between 10 and 60 μm from the outer scleral surface. Collagen fiber waviness was calculated as the ratio of the total length of a traced fiber and the length of a straight path between the fiber ends.

**Results:** In the Free Scleral CXL group, collagen waviness was significantly increased compared to the control group at 35 to 50 μm ( $P < 0.05$ ). In the Globe CXL group, collagen waviness was decreased compared to control at all depths with statistical significance ( $P < 0.05$ ) achieved from 10 to 45 μm.

**Conclusions:** Depending upon its initial state (i.e., free scleral patch versus mechanically loaded intact globe under pressure), collagen may experience different structural changes after CXL. In addition, the extent of the CXL effects may vary at different depths away from the surface.

**Translational Relevance:** Understanding the CXL effects on collagen structure may be important in optimizing the scleral crosslinking protocol for future clinical applications such as preventing myopic progression.

## Introduction

Ocular crosslinking (CXL) has been studied for more than 20 years, and ultraviolet-A light–riboflavin-induced CXL (UVA–riboflavin CXL) is a well-established method to strengthen collagen tissue and increase tissue biomechanical performance in both cornea and sclera.<sup>1,2</sup> Corneal CXL has been used clinically to safely halt the progression of kerato-

conus and other corneal ectasia with long-term efficacy.<sup>3</sup>

Scleral CXL has been shown to elicit a response similar to corneal CXL and may have important clinical applications in the future as well.<sup>4</sup> For example, it may be beneficial for scleral reinforcement in cases of posterior staphyloma or high pathologic myopia.<sup>5</sup> Theoretically, crosslinking the posterior sclera may flatten and reinforce tissue, ultimately preventing further myopic progression.

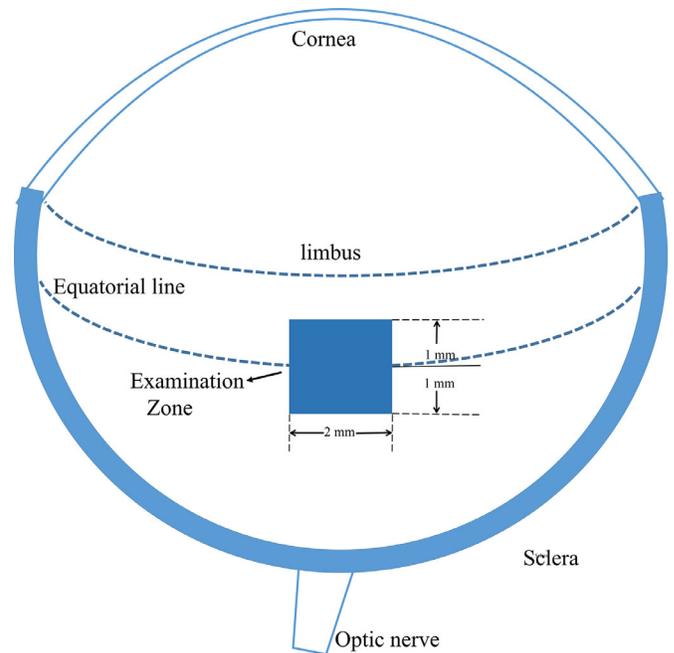
Historically, most CXL studies have used conventional histology with light microscopy to study tissue changes and various biophysical and biomechanical methods to measure tissue strength after CXL.<sup>6</sup> In recent years, more studies have emerged that use second-harmonic generation (SHG) microscopy to study corneal and scleral collagens directly after CXL. The unique advantage SHG offers is its ability to dissect and study different deep layers of tissue without having to cut and section the tissue after CXL, thus providing images of collagen in their natural state without chemical fixation. In 2013, Tan et al.<sup>7</sup> used SHG to study porcine corneal tissue after UVA-riboflavin CXL. They found an increase in collagen waviness and tissue stiffness after CXL, despite an apparent lack of corneal tissue alterations on histology with Masson's Trichrome staining. However, in 2015, Babar et al.<sup>8</sup> studied chemical CXL with sub-Tenon injection of sodium hydroxymethylglycinate (SMG) in cadaveric rabbit heads and found that collagen became less wavy. In 2018, Bradford et al.<sup>9</sup> performed UVA-riboflavin CXL on rabbit corneas *in vivo*; SHG microscopy revealed that collagen fibers became less wavy, and the difference was presumed to be due to sustained intraocular pressure in the intact globe during the CXL procedure. These studies highlight the inconsistent results reported regarding waviness changes with SHG after CXL treatment, possibly depending on the mechanical loading state of the scleral tissue prior to procedure.

The purpose of this study is to investigate and clarify CXL effects on sclera in both intact globes and free scleral patch tissue using SHG microscopy, as well as to observe the effects of CXL at different scleral depths from the irradiated outer surface. These results will enhance our understanding of the subtleties of collagen fiber changes after CXL for future potential clinical applications in various collagen-related diseases.

## Materials and Methods

### Tissue Preparation

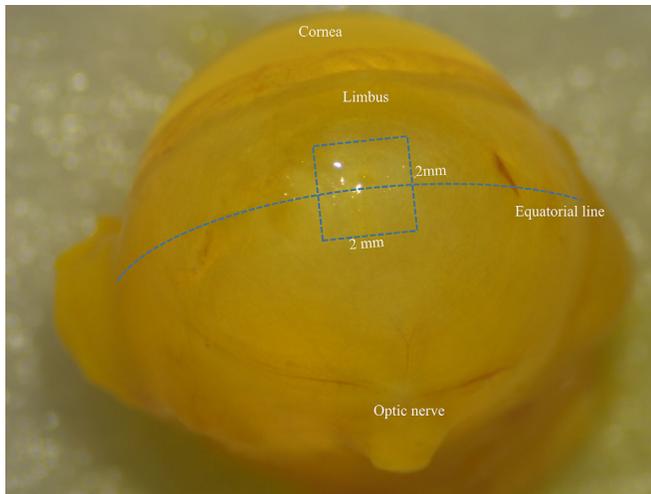
Eighteen right eyes were freshly harvested from 6-week-old female Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA, USA) following euthanization by carbon dioxide (CO<sub>2</sub>) overdose. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Institute of Animal Care and Use Committee, Albert Einstein College of Medicine (Protocol No. 20171205).



**Figure 1.** SHG images were obtained from the central  $2 \times 2$ -mm<sup>2</sup> sclera tissue as indicated (examination zone).

The 18 eyes were divided into three groups, with six eyes in each group, and treated as follows:

1. Control group (isolated scleral patch without CXL): The globes were immediately rinsed in phosphate-buffered saline (PBS) for 30 minutes. After the outer surface was cleaned, the conjunctiva and extraocular muscles were dissected and removed. Following removal of the entire cornea, the retinal and choroidal tissues were also removed. The orientation of the scleral cup was carefully noted, and the scleral cup was halved horizontally. Only the superior half of the cup without optic nerve was used for the experiment. For SHG imaging, a  $2 \times 2$ -mm<sup>2</sup> examination zone was selected (Fig. 1) from the center of equatorial area, 1 mm above and below the equator, 1.8 mm away from the limbus.<sup>10</sup>
2. Free Scleral CXL: After the globes were harvested, the conjunctiva and extraocular muscles were carefully dissected and removed. The globes were then soaked in 0.1% riboflavin (riboflavin 5'-phosphate salt hydrate powder; Merck KGA, Darmstadt, Germany) in PBS for 30 minutes. The superior half of the scleral cup was harvested in the same fashion as the control group, and CXL was performed on the half of the sclera with irradiation immediately above the center of the equatorial zone at the outer surface. After CXL, the  $2 \times 2$ -mm<sup>2</sup> examination zone



**Figure 2.** An intact globe after riboflavin treatment. CXL was performed on the intact globe with superior half facing up and SHG imaging was captured from the center of a  $2 \times 2\text{-mm}^2$  area at a similar location to the other two groups.

was similarly dissected and used for the SHG imaging as above.

3. Whole-globe CXL (Globe CXL): After the intact globe was harvested, cleaned, and soaked in riboflavin as above, attention was given to the orientation of the globes. The superior half of the whole globe was directly exposed to UVA light for the CXL procedure (Fig. 2). After CXL, the crosslinked sclera was dissected and a  $2 \times 2\text{-mm}^2$  examination zone of similar location was used for SHG imaging (Fig. 2, square box in the figure).

## Crosslinking Procedure

The crosslinking procedures were performed as previously published.<sup>11</sup> Briefly, the whole globes were soaked in either PBS (control) or 0.1% riboflavin in PBS for 30 minutes. In the Free Scleral CXL group, the half scleral cups were prepared as described above and were exposed to UVA radiation (Analytik Jena, Jena, Germany) with a peak wavelength of 365 nm from a mercury vapor ultraviolet lamp tube at a distance of 5 cm. The total radiation density of the UVA light source was measured 5 cm away from the light source ( $0.45 \text{ mW/cm}^2$ ). In the Globe CXL group, the UVA radiation was applied superiorly perpendicular to the center of the equatorial region, also at a distance of 5 cm.

## Second-Harmonic Generation Imaging

SHG sample preparation and imaging were performed as previously described.<sup>12</sup> Briefly, SHG images were generated with an inverted Olympus IX81 two-photon excited fluorescence microscope

(FluoView FV-1000; Olympus Corporation of the Americas, Central Valley, PA, USA). The tissue ( $2 \times 2 \text{ mm}^2$ ) was placed on a glass-bottom plate (Thermo Fisher Scientific, Waltham, MA, USA) with the outer scleral surface facing downward and cover-slipped with PBS. A square image ( $512 \times 512 \mu\text{m}^2$ ) was acquired at a resolution of  $1024 \times 1024$  pixels in multiple, consecutive, image stacks (z-stacks). All tissues were scanned in  $5\text{-}\mu\text{m}$  steps along the z-axis. The  $0\text{-}\mu\text{m}$  position in depth corresponds to the first z-position where the SHG signal is detected, and the last images were obtained at a depth of  $60 \mu\text{m}$ , as the images of collagen fibers beyond this depth became blurry.

## Collagen Fiber Waviness Measurement and Analysis

Collagen fiber waviness was measured using a previously described method.<sup>11</sup> For quantification of waviness from the SHG images, each collagen fiber was traced along its entire length using ImageJ (National Institutes of Health, Bethesda, MD, USA). Waviness was calculated as a ratio of the length of the traced fiber to the length of a straight path between the endpoints. This was done on 10 randomly selected fibers in each image ( $512 \times 512 \mu\text{m}^2$ ) from the experimental area at 10 to  $60 \mu\text{m}$  in depth. The collagen waviness was reported as the mean  $\pm$  standard deviation and analyzed using SPSS Statistics 24.0 (IBM, Armonk, NY, USA). Each treated CXL group was compared to the untreated control group at the same depth at similar corresponding locations, using independent sample *t*-tests. Statistical significance of all tests was defined as  $P < 0.05$ .

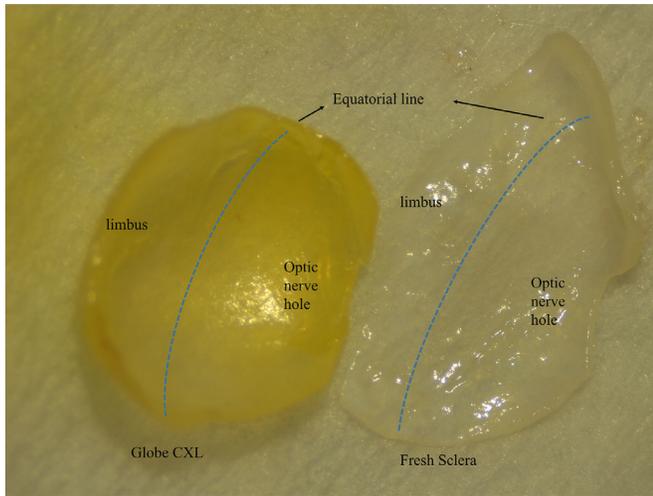
## Results

### Gross Tissue Examination after CXL Procedure

After whole-globe CXL treatment, dissected superior half sclera from the Globe CXL group was able to maintain its rigid structure and curvature (Fig. 3, left), whereas the normal control tissue collapsed flat (Fig. 3, right), which is similar to a previously reported observation in porcine cornea.<sup>7</sup> Similarly, the half scleral cup in the Free Scleral CXL group also maintained gross rigidity and curvature after CXL (not shown).

### SHG Study after CXL

SHG images were taken every  $5 \mu\text{m}$  in depth from 0 to  $60 \mu\text{m}$ ; representative images at 20, 40, and 60



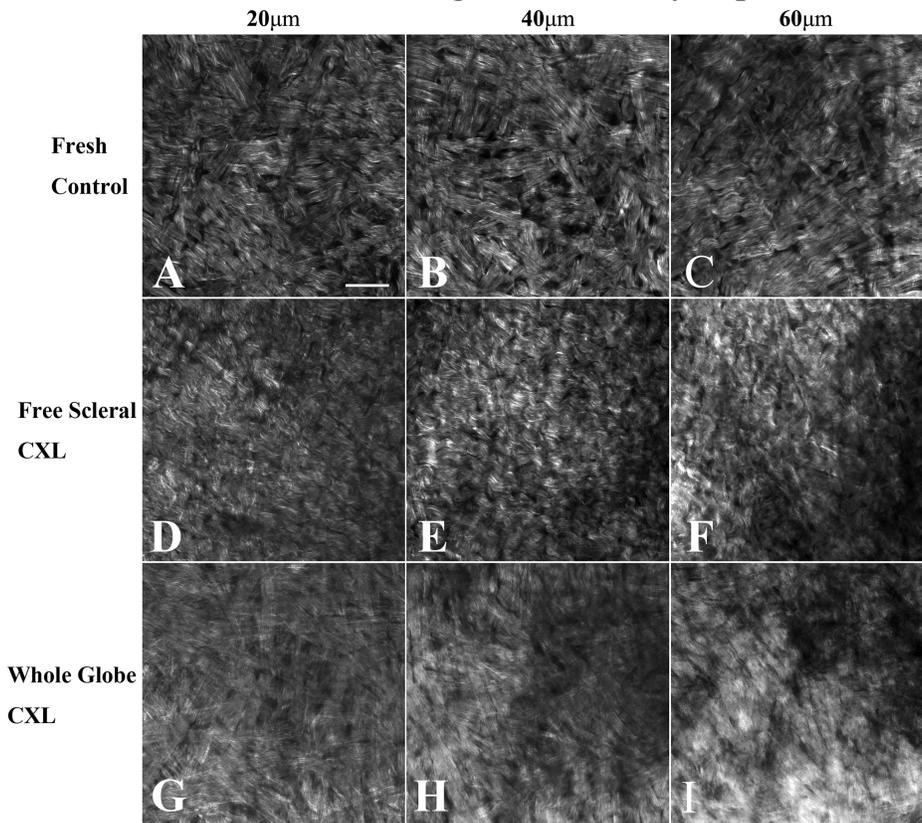
**Figure 3.** Half scleral cup from limbus to optic nerve in the whole-globe CXL group (*left*) versus fresh tissue control (*right*). Crosslinked sclera (*left*) was more rigid and maintained its curvature compared to the collapsed fresh scleral cup tissue (*right*).

μm deep are shown in [Figure 4](#). In the normal control scleral tissue, moderately wavy collagen bundles were arranged randomly with crossing. As depth increased, the collagen fiber bundles became thicker ([Figs. 4A–C](#), 20 to 60 μm), which is consistent with a previous study.<sup>13</sup> In the Free Scleral CXL group ([Figs. 4D–F](#)), the collagen bundles became much wavier and lost their original bundle arrangement, which was most apparent 40 μm deep ([Fig. 4E](#)). However, in the Globe CXL group ([Figs. 4G–I](#)), the collagen bundles lost waviness and became straighter after CXL treatment.

### Collagen Fiber Waviness Analysis

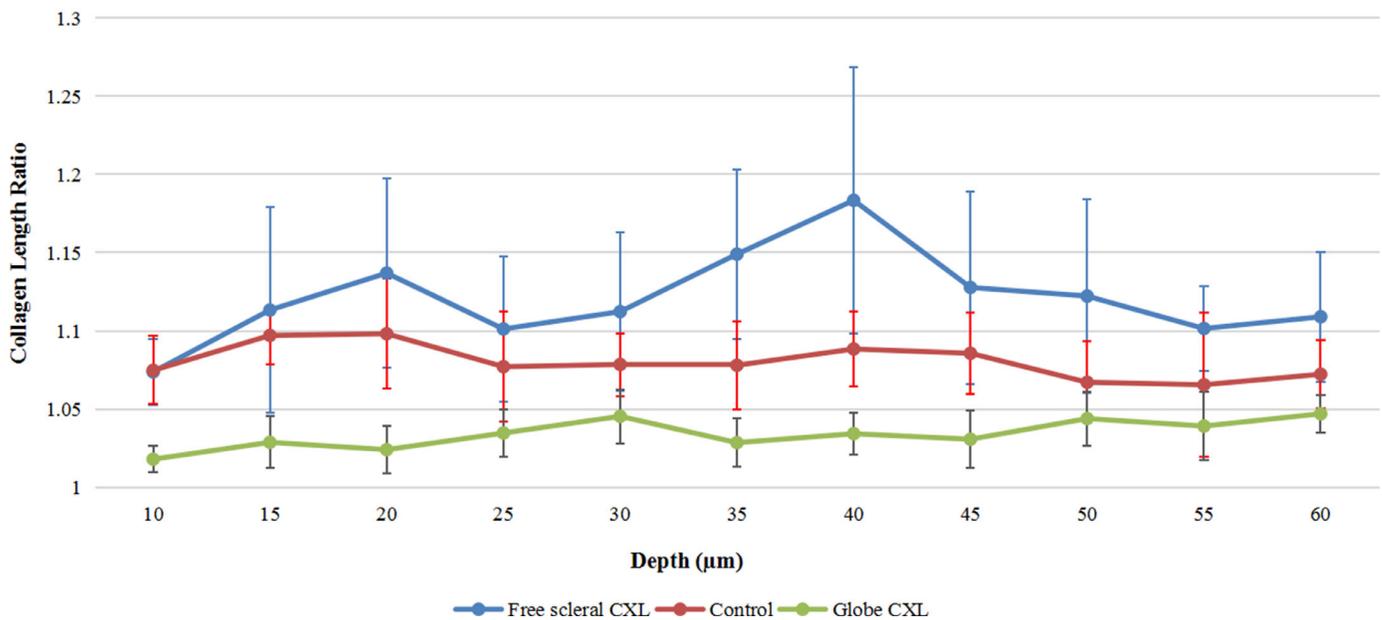
In the control group, no significant change in collagen waviness was seen at any depth (10–60 μm). In the Free Scleral CXL group ([Fig. 5](#)), collagen waviness was increased significantly compared to control at 35 to 50 μm deep ( $P < 0.05$ ), reaching peak waviness at 40 μm and gradually decreasing afterward. In the Globe CXL group ([Fig. 5](#)), the collagen fibers showed decreased waviness compared to control from 10 to

### SHG images of sclera by depth



**Figure 4.** SHG images acquired at 20, 40, and 60 μm from the outer scleral surface to deeper layers in three experimental groups. In the fresh sclera control group (A–C), the collagen bundles became thicker at deeper layers. After CXL, the collagen bundles became wavier in the Free Scleral CXL group (D–F), with the most pronounced effect at 40 μm deep, but straighter in the Globe CXL group (G–I). Bar: 50 μm.

### Waviness of Free Scleral CXL vs Whole Globe CXL vs Control



**Figure 5.** Collagen fiber waviness analysis among experimental groups at different scleral depths. In the Free Scleral CXL group, waviness was increased with most pronounced effect at 40 µm. In the Globe CXL group, waviness was decreased at all depths.

45 µm deep ( $P < 0.05$ ), followed by a slight decrease after 50 µm. In both groups with CXL treatment, the collagen waviness showed no significant change at depths over 50 µm.

## Discussion

In our study, we demonstrated significant collagen fiber waviness changes after scleral CXL with SHG microscopy. In dissected free scleral patch tissue, collagen waviness increased after CXL; however, in intact whole-globe CXL, collagen waviness decreased after treatment. The most significant changes in waviness were detected 35 to 50 µm deep from the scleral surface in the Free Scleral CXL group compared to control and 10 to 45 µm deep in the Globe CXL group. In the deeper layers of the sclera, the observed collagen fiber morphologic changes gradually disappeared in both CXL groups (after 45 µm or 50 µm depth, respectively). This may be due to a decrease in radiation energy levels reaching the deeper layers and decreased riboflavin gradient concentration.<sup>14</sup> Normal scleral collagen fibrils are interwoven and parallel-aligned to form collagen fibers, which then form fiber bundles.<sup>15</sup> Our SHG study also found collagen fibers inside a bundle are consistently parallel, while the collagen bundles are randomly crossed and interwoven to

form the web-like structure of collagen lamellae. We further observed that bundles became thicker as tissue depth increased, which is similar to a previous study.<sup>13</sup> Thicker bundles have been shown to require more energy for CXL, which may partly explain why we observed a lesser effect of CXL in deeper layers.<sup>2</sup>

Crosslinking studies by Spoerl et al.<sup>1</sup> investigated the effects of two chemicals (0.1% glutaraldehyde and 0.1% Karnovsky fixative) and UVA-riboflavin CXL on mechanical stiffness of corneal tissue. Anterior corneal stromal fluorescence has also been reported after chemical CXL, consistent with another study of UVA-riboflavin CXL in rabbit cornea.<sup>1,9</sup> In 2014, Zhang et al.<sup>16</sup> studied the effect of 365 nm UVA light (3 mW/cm<sup>2</sup>) with 0.1% riboflavin CXL of rabbit sclera in vivo and found that 40 minutes of radiation was the optimal time to achieve biomechanical changes without retinal damage. In our current study, we observed opposite effects on scleral collagen fibril waviness depending on the mechanical loading state of tissue during CXL. The increased waviness of Free Scleral CXL and the decreased waviness of Globe CXL suggest that the state of mechanical loading during CXL treatment affects collagen fibril morphology. However, both CXL groups appeared to grossly demonstrate increased rigidity enough to maintain native curvature after CXL. These conflicting results in changes of collagen fibril waviness have been described in previous studies: decreased collagen fiber

waviness after UVA–riboflavin CXL has been reported in porcine cornea<sup>17</sup> and rabbit sclera,<sup>18</sup> while Tan et al.<sup>7</sup> reported increased collagen fiber waviness. One potential reason for these differences is the state of collagen structure and tissue tension before CXL.<sup>18</sup> Interestingly these studies also showed similar increased tissue strength after CXL effects (i.e., tension resistance, thermal denaturation temperature) irrespective of the mechanical loading state despite displaying opposite collagen fiber waviness changes.<sup>7,18</sup> Thus, if the tissue is pressure “loaded” (like in whole-globe CXL), the collagen fibers may be in a more “tense state” and result in a “straightened” architecture after CXL.<sup>17,18</sup> On the other hand, if the tissue is “pressure free” (i.e., free scleral patch CXL in this study), the collagen fibers may be in a less “tense state,” resulting in greater waviness after CXL.<sup>7</sup> Besides, in our previous study (Miao Y, et al. *IOVS* 2019;60:ARVO E-Abstract 4651), we found the collagen structural change in scleral patch tissue with chemical CXL agents, including formaldehyde-releasing agents, such as glutaraldehyde and paraformaldehyde. The collagen waviness of the free scleral tissue also increased after chemical CXL even with very low concentrations, which is consistent with our current Free Scleral CXL results. Although further studies will be necessary to confirm these findings, the different effects on collagen fibers depending on the initial mechanical loading state may indicate future ability to utilize varying tissue pressure or other methods to modify the effects of CXL on collagen structure architecture.<sup>19</sup> Clinically, scleral CXL in both intact globe and scleral patch tissues has been used for posterior scleral enforcement surgery,<sup>20,21</sup> and UVA–riboflavin CXL–treated donor corneas have also been used for penetrating keratoplasty.<sup>22</sup>

This study demonstrated collagen fiber morphologic changes after UVA–riboflavin CXL with SHG in either whole-globe with normal intraocular pressure or free scleral patch tissue without mechanical loading, and this increases our understanding of how different collagen structural changes can be seen despite similarly increased gross tissue rigidity. In the future, we will further investigate changes in collagen morphology and biomechanical effects under various loading conditions with different tissue pressures, which may potentially have broader clinical applications. Currently, UVA–riboflavin CXL has been widely used for treatment of corneal ectasia in clinical practice with a well-documented safety profile and durable long-term effects. Building on our knowledge of corneal CXL, the transition to crosslinking sclera with ultraviolet light and riboflavin may have broader clinical applications.

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