Crosslinking involves the formation of bonds between polymer chains, such as proteins. In biological tissues, these bonds tend to stiffen the tissue, making it more resistant to mechanical degradation and deformation. In ophthalmology, the crosslinking phenomenon is being increasingly harnessed and explored as a treatment strategy for treating corneal ectasias, keratitis, degenerative myopia, and glaucoma. This review surveys the multitude of exogenous crosslinking strategies reported in the literature, both “light” (involving light energy) and “dark” (involving non-photic chemical processes), and explores their mechanisms, cytotoxicity, and stage of translational development. The spectrum of ophthalmic applications described in the literature is then discussed, with particular attention to proposed therapeutic mechanisms in the cornea and sclera. The mechanical effects of crosslinking are then discussed in the context of their proposed site and scale of action. Biomechanical characterization of the crosslinking effect is needed to more thoroughly address knowledge gaps in this area, and a review of reported methods for biomechanical characterization is presented with an attempt to assess the sensitivity of each method to crosslinking-mediated changes using data from the experimental and clinical literature. Biomechanical measurement methods differ in spatial resolution, mechanical sensitivity, suitability for detecting crosslinking subtypes, and translational readiness and are central to the effort to understand the mechanistic link between crosslinking methods and clinical outcomes of candidate therapies. Data on differences in the biomechanical effect of different crosslinking protocols and their correspondence to clinical outcomes are reviewed, and strategies for leveraging measurement advances predicting clinical outcomes of crosslinking procedures are discussed. Advancing the understanding of ophthalmic crosslinking, its biomechanical underpinnings, and its applications supports the development of next-generation crosslinking procedures that optimize therapeutic effect while reducing complications.
Crosslinking can be thought of in two forms, endogenous and exogenous. Endogenous crosslinks are a natural part of tissue structure, maintenance, aging, or disease, and include lysyl oxidase-mediated crosslinking with age and advanced glycation end (AGE) product-mediated crosslinking in diabetes. Endogenous crosslinking will not be discussed in this review. Exogenous crosslinks are intentionally induced in certain ophthalmic treatments, and we will summarize a variety of exogenous crosslinking methods described for ophthalmic applications.

We will consider ophthalmic applications of crosslinking in two groups: First, the “light” methods, which induce crosslinks through irradiation of the target tissue with light (photochemical crosslinking). This irradiation is paired with administration of a photosensitizing agent, which increases the efficiency of the crosslinking process by absorbing photons and indirectly transferring the resulting energy to crosslink formation. The second group includes the “dark” crosslinking methods, which are solely chemically induced and require no photoactivation.

“Light” Methods

“Light” (photochemical) methods of crosslinking rely on energy delivered in the form of light to induce crosslinks between adjacent molecules. The advantage of this method is that light can be focused in a selective manner so that only the region or tissue of interest is crosslinked.

UV-A / Riboflavin

The most commonly used method of ophthalmic crosslinking is UV-A / riboflavin crosslinking. U-VA / riboflavin crosslinking is most commonly used to crosslink the cornea (the application for which it is US Food and Drug Administration [FDA] approved), but may also be used on other ocular tissues. The most widely used protocol for cornea crosslinking, the Dresden protocol, requires removal of the corneal epithelium, instillation of a 0.1% riboflavin / 20% dextran solution onto the surface of the cornea for 30 minutes, followed by 30 minutes of 370 nm UV-A irradiation at 3 mW/cm² with continued intermittent administration of riboflavin solution.

Although it has achieved widespread clinical adoption, modifications to the UV-A / riboflavin crosslinking protocol are under investigation. For instance, there is on-going development of protocols which do not require removal of the corneal epithelium (transepithelial crosslinking) for corneal crosslinking. The Dresden protocol calls for removal of the corneal epithelium prior to instillation of riboflavin. Removing the epithelium allows better penetration of the riboflavin, but it also causes patient discomfort, increases the risk of infection, and causes prolonged changes to function and morphology of the corneal nerves. Less disruption to the corneal nerves is observed in transepithelial CXL procedures and the risk of infection is reduced.

The most straightforward transepithelial method is to omit the epithelial debridement from the Dresden protocol and rely on the small amount of diffused riboflavin into the stroma to serve as photoactivator. However, most transepithelial protocols have reduced effectiveness compared to epithelium-off CXL.

Another method to address the issue is to partially perforate the corneal epithelium to minimize disruption while increasing the ability of riboflavin to cross into the stroma. Other methods of investigation include chemically weakening the tight junctions in the epithelium, iontophoresis across the cornea during riboflavin application, creation of a intrastromal “pocket” in which to inject the riboflavin, and the addition of solutions beyond riboflavin to enhance the riboflavin penetration.

Adjacent to the matter of transepithelial riboflavin penetration, there has been discussion in the field on the time course of riboflavin application. First, characterizing or reducing duration of instillation needed for the riboflavin to sufficiently penetrate into the desired tissue (commonly, the sclera or corneal stroma) for optimal crosslinking is a question of continued interest. Second, if riboflavin is instilled continuously (as called for in the Dresden protocol), a layer of riboflavin is left on top of the tissue surface, and may strongly absorb the UV-A rather than allowing for deeper penetration of the UV-A into the stroma. Washing the surface prior to UV-A exposure may allow for more complete crosslinking.

A third area of investigation for improvement of the riboflavin / UV-A CXL method is reducing the duration of the UV-A irradiation. This is primarily motivated by concerns of patients’ comfort and reducing overall procedure time. With 30 minutes of 3 mW/cm² 370 nm light as a baseline (a total energy delivery of 5.4 J/cm²), the use of higher intensity light (9–30 mW/cm²) for shorter periods of time (3 to 10 minutes) has been widely investigated. However, the kinetics of the crosslinking reaction are an important (and potentially limiting) factor in high intensity/short duration modifications. The crosslinking reaction
can be broken down into two types: type 1 (oxygen independent) and type 2 (oxygen dependent). In Dresden UV-A riboflavin CXL, in the first 15 seconds of UV-A irradiation, photochemically generated reactive oxygen species drive the crosslinking process by oxidizing the proteoglycan core proteins and collagen of the stroma.26 This is representative of a type II crosslinking reaction. After approximately 15 seconds, the endogenous oxygen is depleted and the crosslinking is driven by slower type I mechanisms, in which the energized riboflavin directly interacts with the proteoglycan and collagen molecules in the stroma.

During the 30-minute irradiation of Dresden protocol corneal crosslinking, oxygen continuously diffuses into the stroma (~400 um thick) and resupplies the oxygen within the stroma, allowing some type 2 reaction to continue occurring over 30 minutes. However, in accelerated protocols, the time for this oxygen replenishment is limited. Accordingly, it has generally been found that accelerated protocols (which rely on higher intensity light for shorter durations) result in more superficial stiffening of the stroma. Although short-term outcome studies have shown equal efficacy between Dresden CXL and many accelerated protocols in halting keratoconus, it is not known if the lesser penetration of the stiffening effect elevates the risk of late disease progression.

Several methods have been attempted to counteract this oxygen-limited superficial crosslinking result. One method is to perfuse the cornea with oxygen during UV-A irradiation, which has been shown to increase the stiffening effect of accelerated crosslinking.27 Another method is to use higher intensity light, but pulse the light in a manner which allows oxygen to be replenished in between pulses.28

### Other Photosensitizer Methods

Whereas generally hailed as a safe and effective treatment, riboflavin / UV-A crosslinking does have some drawbacks. Notably, the high-energy UV-A radiation presents phototoxicity concerns for long-duration tissue exposure, particularly for the cells of the corneal endothelium or retina.29 For this reason, corneal thickness is an important consideration in riboflavin / UV-A crosslinking, as the bulk of the riboflavin-infused stroma will absorb the UV-A light effectively shielding the endothelial cells. If the cornea is too thin, shielding may not be sufficient and damage may be done to endothelial cells. For this reason, patients with thin corneas are not given Dresden riboflavin / UV-A crosslinking, and instead may be administered a modified CXL protocol.30 Thus, other pairs of photosensitizers and excitation wavelengths have been explored for ophthalmic crosslinking.

These methods (see Table 1A) operate on a similar theory as riboflavin / UV-A crosslinking, but the photosensitizers may have different absorption wavelengths, different properties with respect to tissue penetration, and different energy dose requirements.

### “Dark” Methods

Dark methods of crosslinking rely solely on crosslinking by a chemical agent with no photoactivation. The lack of photoactivation can be an advantage if the tissue targeted for crosslinking is adjacent to photosensitive tissues (for instance, the sclera is adjacent to the photosensitive retina). However, without photoinitiation, spatial selectivity is reliant on the diffusion of the crosslinking agent itself. This diffusion can be difficult to control and cause unintended
effects by crosslinking adjacent tissues. Examples of ophthalmic “dark” crosslinkers are shown in Table 1B.

### Ophthalmic Targets for Crosslinking

Ophthalmic crosslinking has found a number of target applications in the eye. The only FDA-approved crosslinking treatment as of this writing is UV-A / riboflavin crosslinking for stabilization of progressive keratoconus or postrefractive surgery ectasia. However, several methods and applications have been trialed in research settings for a variety of ophthalmic conditions.

### Cornea

The most common use of corneal crosslinking is to mechanically stabilize ectatic corneas (Fig. 1). Keratoconus is the most common cause of ectasia and many clinical studies have emphasized the effectiveness of corneal CXL to halt the progression of keratoconus. In some cases, the stiffening induced by crosslinking even results in mild flattening of the cornea, effectively reducing the adverse morphological and optical effects of keratoconus to a small degree. In another case of degenerative disease, CXL has been shown to be useful in the treatment of pellucid marginal degeneration. CXL is similarly used to mechanically stabilize corneas in cases of postsurgical ectasia.

Additionally, crosslinking has been explored as a preventative measure in refractive surgery – essentially reinforcing corneas which may otherwise be too thin or weak to safely receive refractive procedures. In addition, CXL itself, if done in a spatially selective manner, can induce subtle and specific changes in cornea geometry, which can provide refractive correction. Further, CXL has been shown to be effective in improving corneal clarity and comfort for patients with corneal edema secondary to endothelial dysfunction by imparting some resistance to stromal swelling.

In a different class of procedures, CXL has been explored for its generation of reactive oxygen species, which may inhibit microbial infection. It has been...
Biomechanical aspects of crosslinking 

**Figure 1.** Overview of ophthalmic crosslinking for disease stabilization. Crosslinked regions of tissue are shown in green. Dotted lines indicate the progression of disease if crosslinking had not been applied. (Top) In corneal crosslinking (CXL) for keratoconus stabilization, the stiffening of the cornea prevents the progression of corneal steepening (dotted line). (Middle) In scleral crosslinking (SXL) for myopia stabilization, the stiffening of the sclera prevents further axial elongation of the globe (dotted line). (Bottom) In scleral crosslinking for glaucoma stabilization, the stiffening of the peripapillary sclera reduces strain on the lamina cribrosa and prevents further distention of the lamina cribrosa (dotted line).

Figure 2. An explanation of various measurement parameters.

Evidence from biomolecular studies suggests that not all crosslinking protocols form crosslinks in the same position. Even within photochemical crosslinking techniques, the crosslink position may differ. For example, studies suggest that riboflavin / UV-A and WST / NIR form crosslinks in different locations throughout the collagen hierarchical structure. For nonphotochemical crosslinkers, like decoron or transglutaminases, different crosslinking mechanisms are suggested. Crosslinking does not modify all mechanical parameters in the same way. For instance, crosslinking may significantly increase tensile strength, while not increasing viscosity. Further, different types of crosslinking may modify mechanical properties differently.

To understand why, it is necessary to refer to the hierarchical microstructure of collagen in the cornea. Individual collagen molecules form helical tropocollagen, which in turn make up microfibrils.
Figure 2. Overview of metrics used to quantify mechanical changes due to crosslinking the cornea or sclera. (Top) A sample which is wholly elastic will immediately deform when a load is added or removed. A sample which is viscous will continue to deform over time if a load is present. A sample which is viscoelastic, such as the cornea, will have both a viscous and elastic component in its deformation response to load. (Middle) Many different mechanical moduli are reported in ocular biomechanics literature. Young’s modulus, also known as the uniaxial elastic modulus, is the resistance to deformation from a uniaxial load. Shear modulus is resistance to deformation due to a shear load. Bulk modulus, also known as the volumetric elastic modulus, is the resistance to deformation given a volumetric compression. The tangent modulus is the instantaneous slope of the stress-strain curve at a given load (stress) when the curve is no longer linear (if the stress-strain curve is linear, tangent modulus is the same as Young’s modulus). Dynamic viscosity and shear viscosity are the time-dependent (rate-dependent) equivalents of Young’s modulus and shear modulus, respectively. Acoustic velocity is the propagation speed of a pressure wave and is dependent on the material’s bulk modulus, shear modulus, and density. Shear wave velocity is the propagation speed of a shear wave, and is dependent on shear modulus and density. (Bottom) Methods of assessing even smaller-scale mechanics include: adhesion force, which is the force required to retract an atomic force microscopy cantilever embedded in the sample; temporal decorrelation, as measured by DLS or OCT, is a measure of the quasi-Brownian displacements which result from random thermal energy within the sample; bond strength can be measured by the time required for the sample to be digested by enzymes.

(coated in proteoglycans), which make up fibers, which make up lamella, which interweave through the bulk of the corneal stroma. The location of crosslinks within this hierarchy is structurally important. For instance, if crosslinks are only formed within corneal collagen microfibrils (as may be the case in enzymatic crosslinking), it could be surmised that the cornea will better withstand tensile stresses. However, if no additional
Figure 3. Crosslinks within the collagen hierarchical microstructure (Referencing figures and text1,79–81) From right to left: Chemical crosslinks can be formed between residues in the primary collagen molecule, between collagen molecules in the tropocollagen triple helix, between tropocollagen in the microfibril, and within or between components of the ECM which surround the collagen fibrils (which are mostly proteoglycans82,83). Interlamellar crosslinks are not formed by chemical crosslinking.76 Enzymatic crosslinks are generally formed between tropocollagen in the microfibril or can be formed among the proteoglycans surrounding the fibrils.

Crosslinks have been formed between collagen fibers, they may slide over each other as easily as before crosslinking, resulting in a relatively unchanged shear modulus, as has been shown to be the case in CXL for keratoconus.76 Conversely, if chemical crosslinking increased binding between proteoglycans surrounding the collagen fibers, essentially increasing gelation, shear modulus may increase significantly but tensile strength may be relatively unchanged. Understanding and modeling the mechanical effects of various crosslinks in a hierarchical collagen structure has remained challenging.75

Thus, when choosing a biomechanical measurement to assess crosslinking efficiency, consideration should be given both to the anticipated mechanical change and desired outcome. For instance, if the desired clinical outcome is inhibiting keratoconus progression, enzymatic digestion may be the most appropriate metric. However, if the desired outcome is reducing the risk of postsurgical ectasia, measuring the tensile strength of the cornea may be the best indicator. Similarly, if the crosslink formation is anticipated to occur between proteoglycans, a shear modulus measurement may be most sensitive to crosslink formation. If the crosslink formation is anticipated to occur within microfibrils, a tensile strength measurement may again be best.

The crimping and tensioning of collagen fibrils is also an important consideration. The tension on collagen fibrils during crosslinking may affect the number and type of crosslinks formed.77,78 This is an important consideration if crosslinking is being induced in tissues ex vivo where IOP is exogenously maintained or tissue which is dissected and therefore has no tension. Collagen molecules are still slightly “crimped” in their natural state. Under tension, they straighten. Different crosslink formation may differently affect this uncirping process and other deformation mechanics.78

Finally, biological tissues, and particularly the cornea, exhibit mechanical properties which are highly dependent on environmental factors, such as hydration state of the tissue, tissue boundary conditions, pre-stress, and other pretreatment effects. Thus, even for studies using the same protocol and reporting the same mechanical parameter, results may not be comparable.

Overview of Methods

A variety of methods have been devised to measure the mechanical properties of the cornea and sclera. A brief description of each major method of assessing ocular biomechanics is given in Table 2. (With no significance of order listed.) They are listed below for cornea (Table 3) and sclera (Table 4) and organized by modulus reported. Some methods report multiple moduli. The t-value expresses the magnitude of the crosslink-induced change relative to the variation in the data (the difference in units of standard error, where higher values indicate a larger difference). This gives a sense of how sensitive each method is specifically to the change induced by crosslinking. Although individual experimental setups may impact the t-value, overall, examining t-value across methods and moduli may yield insight into which characteristics of the cornea fundamentally change as a result of crosslinking.

However, not all methods are equally sensitive to crosslink-induced changes in the ocular tissue. In Tables 3 and 4, methods are grouped by the modulus they report. Both tables are limited to riboflavin crosslinking (either UV-A or blue light) to avoid a confounding comparison of various crosslinking methods. For each, the t-value is shown for differentiating untreated samples from crosslinked samples. When choosing a mechanical assessment method to determine the efficacy of crosslinking treatment, a method which has previously shown high t-values
Table 2. Methods of Assessing Ocular Biomechanics

| Method Name                                      | In Vivo? | Brief Description                                                                                                                                                                                                 |
|-------------------------------------------------|----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Enzymatic digestion\(^{84}\)                    | No       | Samples incubated with enzyme, degradation rate measured                                                                                                                                                           |
| Atomic force microscopy (AFM)\(^{85}\)          | No       | Micro-cantilever tip impacts sample repeatedly, recording forces during contact and withdrawal.                                                                                                                    |
| Brillouin microspectroscopy\(^{86}\)            | Yes      | Optically detects acoustic velocity within a sample using a precise spectrometer.                                                                                                                                  |
| Acoustic microscopy\(^{87}\)                    | No       | Acoustic waves are focused and transmitted through coupling fluid to the sectioned sample, and reflected back from both the sample surface and substrate behind the sample, allowing the acoustic velocity within the sample to be measured. |
| Phase-decorrelation OCT\(^{88}\)                | Yes      | Using optical coherence tomography (OCT), measures random displacements of scatterers due to thermal energy fluctuations.                                                                                         |
| Ultrasound acoustic velocity\(^{89}\)          | Yes      | Using ultrasound, measures the acoustic velocity across a sample.                                                                                                                                                    |
| Ultrasound shear velocity (supersonic shear imaging)\(^{90}\) | No       | Using ultrasound, measures the shear velocity across a sample.                                                                                                                                                     |
| Optical coherence elastography (quasi-static)\(^{91}\) | Yes      | Using OCT, observes internal deformations of tissue as compressional loading (from ocular pulse, compression lens, etc.) is applied. Internal deformations are related to local mechanical properties. |
| Shear wave optical coherence elastography\(^{92}\) | Yes      | Using OCT, observes shear wave propagation (induced by air puff, ultrasound, ocular pulse, etc.) through tissue. Wave velocity is related to local mechanical properties.                                                   |
| Dynamic shear rheology\(^{93}\)                 | No       | A piece of tissue is removed, usually with a biopsy punch, and subjected to shear forces at different frequencies. The resistance to shear at each frequency is recorded, characterizing the viscoelasticity of the sample. |
| Strip extensiometry\(^{94}\)                    | No       | A strip of tissue is fixtured at the edges or ends, and resistance to mechanical loading is recorded, yielding stress-strain curves. The curves typically provide Young’s modulus, creep, and hysteresis. |
| Inflation testing\(^{95}\)                      | No       | An *ex vivo* globe is mounted and the relationship between globe expansion and change in intraocular pressure is observed, over short\(^{95}\) or long\(^{96}\) periods of time. |
| Thermal shrinkage\(^{51}\)                      | No       | As collagenous tissue is heated, tropocollagen denatures, resulting in significant tissue shrinkage. The threshold temperature of this denaturation indicated the stability (and crosslinking) of the collagen structure. |
Table 3. Corneal Crosslinking (CXL) Mechanical Properties Sensitivity to Riboflavin / UV-A Crosslinking

| Property Measured          | Method                                      | In Vivo? | t-Value, Untreated or Pretreated to CXL Corneas |
|----------------------------|---------------------------------------------|----------|-------------------------------------------------|
| Young’s modulus            | Atomic force microscopy                     | No       | 17.89<sup>97</sup>                              |
|                            |                                             |          | 2.43<sup>98</sup>                               |
|                            |                                             |          | 5.39<sup>99</sup>                               |
| Shear wave optical coherence elastography | Inflation testing                          | Yes      | 7.29<sup>100</sup>                             |
|                            | Supersonic shear imaging                   | No       | 6.36<sup>101</sup>                             |
| Tangent modulus            | Strip extensiometry                        | No       | 2.98<sup>102</sup>                             |
| Shear modulus              | Shear rheometry                            | No       | 2.79<sup>102</sup>                             |
| Shear viscosity            | Shear wave optical coherence elastography  | Yes      | 5.37<sup>100</sup>                             |
| Acoustic velocity          | Brillouin microspectroscopy                | Yes      | 5.38<sup>103</sup>                             |
|                            | Ultrasound                                  | No       | 3.64<sup>104</sup>                             |
|                            | Acoustic microscopy                         | No       | 4.38<sup>107</sup>                             |
| Brownian dynamics          | Phase-decorrelation OCT                    | No       | 18.38<sup>105</sup>                           |
| Molecular bond strength    | Enzymatic digestion time (collagenase, pepsin, matrix metalloproteinases, or trypsin) | No       | 29.79<sup>106</sup>                           |
| Adhesion force             | Atomic force microscopy                    | No       | 3.99<sup>99</sup>                              |
|                            | Strip cleavage                             | No       | Not significant<sup>76</sup>               |
| Corneal resistance factor (CRF) | Ocular response analyzer                | Yes      | 1.65<sup>107</sup>                            |
|                            |                                             |          | 2.27<sup>108</sup>                             |
|                            |                                             |          | No change<sup>109</sup>                        |
|                            |                                             |          | No change<sup>110</sup>                        |
|                            |                                             |          | 1.06<sup>111</sup>                            |
|                            |                                             |          | *2.09<sup>112</sup>                           |
|                            |                                             |          | *0.82<sup>113</sup>                           |
|                            |                                             |          | Not significant<sup>114</sup>                 |
| P2area                     |                                             | Yes      | 3.25<sup>109</sup>                             |
| Lateral to imposed axial displacement ratio (posterior central) | Optical coherence elastography (quasi-static) | No       | 0.75<sup>115</sup>                             |
| L2                         | Corvis STFor many more parameters, see references | Yes      | 3.31<sup>112</sup>                             |
| SP-A1                      |                                             | Yes      | Significant<sup>116</sup>                     |
| Integrated concave radius  |                                             | Yes      | Significant<sup>114</sup>                     |

*Indicates that the change detected was the opposite of the direction the other listed studies detected.

Note that for studies where the data provided was not sufficient to calculate the t-value, but the change was significant \((P < 0.05)\), "significant" maybe listed instead. "No change" indicates that the t-value was <0.1.

Table 4. Scleral Crosslinking (SXL) Mechanical Property Sensitivity to Riboflavin Crosslinking

| Property Measured           | Method                                    | In Vivo? | t-Value, Untreated or Pretreated to SXL Sclera |
|-----------------------------|-------------------------------------------|----------|------------------------------------------------|
| Young’s modulus             | Inflation testing                         | No       | ~19.2<sup>117</sup>                             |
|                            | Strip extensiometry                       | No       | 16.9<sup>118</sup>                             |
|                            |                                           |          | ~8.3<sup>119</sup>                             |
| Shear viscosity             | Dynamic shear rheology                    | No       | 11.52<sup>120</sup>                             |

may be preferred. However, several caveats are noted. (1) Methods which report changes relative to a paired control or as a percent change to a pre-test value are likely to report higher t-values than studies which report on unpaired samples without a self-control. (2) Studies which observe crosslinking treatment on diseased tissue may report a larger difference than studies which report the effect of crosslinking treatment of healthy tissue. (3) Although these studies all nominally use riboflavin / UV-A, small differences in protocol, such as hydration and pre-tensioning, may affect the amount of crosslinking. (4) Crosslinking may have a greater stiffening effect on human corneas than porcine corneas, although porcine corneas are commonly used in CXL research, and are included in Table 3 below. With
Table 5. Methods of Spatial Resolution of Mechanical Changes in the Cornea or Sclera

| Method Name                              | Comments on Ability to Spatially Resolve Mechanics                                                                 | Spatial Dimensions | Mechanical Components |
|------------------------------------------|---------------------------------------------------------------------------------------------------------------|--------------------|-----------------------|
| Acoustic microscopy                      | Can laterally resolve down to 1 μm, however, this requires good mechanical coupling and a smooth sample surface. 87,126 | 2                  | 1                     |
| Atomic force microscopy (AFM)            | May resolve with high precision (down to 10 nm, depending on tip size) in a prepared, ex vivo lateral (2D) cross-section 127 | 2                  | 1                     |
| Brillouin microspectroscopy              | Can resolve, non-contact and without perturbation in vivo, across three dimensions with approximately 2 μm resolution. The resolution is dependent on the optical system and sample properties. 128 Note that Brillouin spectroscopy is noisy in turbid media, making scleral measurements challenging. 129 | 3                  | 1-                    |
| Phase-decorrelation OCT                  | Can resolve, non-contact and without perturbation in vivo, across three dimensions with approximately 40 μm resolution. 126 | 2+                 | 1                     |
| Ultrasound (supersonic shear wave imaging) | Can resolve, across the lateral (2D) extent of the cornea in vivo, acoustic properties with a resolution of 400 μm. 117 | 2                  | 1-                    |
| Optical coherence elastography (OCE) – quasi-static | Resolution is highly dependent on sample contrast, method of perturbation, scan pattern, and processing. Capable of resolution across three dimensions in vivo, ranging between 10 and 200 μm. 130 | 2+                 | 2-                    |
| shear wave optical coherence elastography (SW-OCE) | More capable of resolving in the plane of wave propagation, as opposed to the transverse direction. Similar to OCE, resolution is highly dependent on sample contrast, method of perturbation, scan pattern, and processing. Given an appropriate setup, SW-OCE is capable of resolving over a volume, in vivo, approximately 400 μm resolution. 131,132 | 2+                 | 2                     |
| Inflation strain mapping                 | While this method may be possible in vivo, 12  current ex vivo results from cornea show a resolution of 26 μm axial and 112 μm lateral. 134 Could theoretically be applied over a volume. | 2+                 | 2-                    |

A “+” indicates that higher dimensionality is not typically reported, but may result from a natural extension of the technique (e.g. adjusting scan pattern or collection angle).

these caveats in mind, it appears that enzymatic digestion, phase-decorrelation optical coherence tomography (OCT), supersonic shear imaging, and shear wave optical coherence elastography are the three most sensitive methods to crosslinking-induced change in corneal mechanical properties (given the experimental conditions and regions of interest specified by their accompanying studies). Note that the bottom half of the table summarizes methods which report parameters rather than properties. These parameters do not have an inherent physical meaning in the same manner as, for instance, Young’s modulus. Additionally, a large family of similar yet different parameters may be synthesized from the same original set of measurements (as in waveform analysis for the ocular response analyzer [ORA]). For these reasons, only selected parameters are reported in the table below. More parameters can be found in individual articles.

Similarly, Table 4 reviews methods of SXL assessment, which has considerable overlap with CXL assessment techniques. Fewer studies overall have been reported for scleral crosslinking mechanics, both because SXL is less-studied and because dark crosslinkers are more popular in SXL studies. Fewer methods are represented here because fewer studies have been conducted using riboflavin crosslinking.

Resolving Mechanical Changes After Crosslinking

Choosing methods with appropriate resolve mechanical properties is important to detecting the mechanical changes induced by crosslinking. Because the cornea is biomechanically complex, exhibiting nonlinear viscoelastic behavior, which also varies over spatial and temporal scales, it is not straightforward to summarize the biomechanical effects of crosslinking.
Table 6. Biomechanical Studies of Accelerated Riboflavin / UV-A CXL versus Dresden.

| Study                        | Results                                                                 |
|------------------------------|-------------------------------------------------------------------------|
| Enzymatic digestion          | Dresden CXL provided better resistance to enzymatic digestion than accelerated or pulsed methods. However, another study found that standard and accelerated protocols have similar resistance to enzymatic digestion. |
| CorVis ST                    | Corvis ST SP A1 shows weaker stiffening effect for increasingly accelerated protocols. |
| Acoustic microscopy          | No clear difference in acoustic velocity found between Dresden and accelerated |
| Strip extensiometry          | Strip extensiometry shows a weaker stiffening effect for increasingly accelerated protocols |
| Brillouin microspectroscopy  | Brillouin microspectroscopy showed more superficial stiffening of the cornea for increasingly accelerated protocols. |

For instance, the Reichert Ocular Response Analyzer (ORA) — a pneumotonometer which reports corneal biomechanical properties — was used to study the effects of CXL on corneal hysteresis, and in the majority of studies, no significant change in corneal hysteresis was found after crosslinking. This led some to incorrectly conclude that crosslinking was not inducing a significant mechanical change in the cornea. However, more specific studies of corneal mechanics, including different parameters derived from the ORA, have shown that there is indeed evidence of a significant stiffening effect of crosslinking on the cornea.

Further, crosslinking may not occur evenly over a tissue, even if treatment is applied evenly. The spatial distribution of crosslinking formation is an important indicator of the efficacy of a method and useful for predicting what effect this will have on the morphology of the tissue. The various methods of assessing tissue biomechanics are not equivalently capable in this regard, and so the varying ability to resolve spatial information with each method may be an additional consideration when selecting an assessment method.

Comments on various methods which allow for spatially resolving the crosslinking effect may be found in Table 5, in order of decreasing resolving power. Apart from tissue sectioning, these methods generally do not provide any spatial resolution of crosslinking effects: enzymatic digestion, thermal shrinkage, strip extensiometry, inflation testing, and shear rheology. It is also important to highlight that some methods differ not only in capacity for 1, 2, or 3-dimensional spatial sampling but also in 1, 2, or 3-dimensional directional sensing (for example, capturing mechanical behaviors in-plane and out-of-plane). Additionally, the time period over which the mechanical response is recorded may be of interest, as long-time methods may probe different aspects of mechanical properties than short-time methods.

Biomechanical Differences Between Crosslink Protocols and their Correspondence to Clinical Outcomes

Some studies have used the same measurement methods to study the efficacy of various crosslinking protocols in a head-to-head comparison. Given the wide array of potentially confounding factors, notably hydration state of the tissue, and general disagreement between biomechanical parameters as measured by different setups, it is not advisable to compare absolute numbers between unrelated studies.

Given the wide array of crosslinking protocols proposed, there are naturally many studies comparing methods, both clinically and with benchtop methods. In nearly all cases, a proposed protocol is compared to the Dresden protocol.

Accelerated Crosslinking

Many clinical studies have sought to determine the efficacy of various accelerated (higher-intensity light for a shorter period of irradiation) protocols for riboflavin / UV-A crosslinking for keratoconus. Accordingly, meta-analyses address the question of clinical effectiveness in terms of long-term morphological outcomes.

A few studies have assessed biomechanical parameters in vivo after Dresden versus accelerated crosslinking. However, they have produced mixed results on the mechanical efficacy of accelerated crosslinking treatments. Two recent meta analyses concluded that the evidence was slightly in favor of Dresden crosslinking yielding a more significant increase in corneal...
Transepithelial Crosslinking

In addition to the study of accelerated crosslinking protocols, there has been considerable study in the field on the comparative effects of epithelial-on (transepithelial) versus epithelial-off (de-epithelialized) riboflavin / UV-A crosslinking protocols. These studies have concluded that although transepithelial CXL results in reduced healing time and improved best-corrected visual acuity, it is less effective (in most implementations studied) at halting the progression of keratoconus.

A few clinical studies (summarized elsewhere ref. 148) have specifically looked at biomechanical parameters of transepithelial CXL. Table 7 highlights published studies which assess the direct mechanical effects of epithelial-on versus epithelial-off (de-epithelialized) riboflavin / UV-A crosslinking.

Alternative Crosslinking Methods

Finally, there have been a limited number of studies reporting the efficacy of non-riboflavin methods of crosslinking to riboflavin / UV-A crosslinking. To our knowledge, no clinical trials have been completed assessing Dresden CXL to these alternative methods. Biomechanical results are summarized in Table 8. In addition, an important consideration of alternative protocols is reduced cytotoxicity.
Predicting the Mechanical and Morphological Outcomes of Crosslinking

In some cases, such as keratoconus, the desired outcome of crosslinking is primarily the stiffening effect. However, in other cases, such as myopia and refractive correction, the desired outcome is a morphological change. In some ways, this is an easier output metric to measure than stiffening – corneal topographers and tomographers are commonplace in refractive clinics, and can be used to determine the morphology of the cornea to a high degree of precision. However, titrating treatment for a desired morphological outcome is more difficult than titrating treatment simply to prevent keratoconus progression, where a broad range of outcomes may be equally acceptable. Visual acuity is sensitive to the morphology of the cornea on the order of 40 nm. Thus, morphological outcomes must be very tightly controlled to produce the desired improvement in visual acuity.

To help close this gap, a number of mechanical models of the cornea and ocular globe have been devised. These models allow for changes to the mechanical properties of constituent tissues to be modeled and the predicted morphological outcomes analyzed. To produce useful results, this type of modeling relies on a thorough understanding of the mechanical changes induced by crosslinking. If changes in mechanical properties of the tissues as a result of crosslinking are well-characterized, the morphological outcomes may be predicted and optimized for a desired effect.

Examples of work in this direction include a 2013 study which demonstrated an inverse, finite-element driven model for predicting morphological changes in the cornea due to riboflavin / UV-A crosslinking. A further paper demonstrated that laterally patterning the crosslinking can be used to fine-tune the refractive effect, helping to reduce aberrations. A 2017 study demonstrated an algorithm which predicts the stiffening effect of corneal crosslinking given riboflavin diffusion and irradiation parameters. Such modeling may serve as useful input to finite element-driven modeling of corneal morphology, to prescribe a CXL treatment pattern and protocol meeting the required stiffening profile to generate a given morphology.

Conclusion

Crosslinking using exogenous methods of “light” and “dark” varieties is a rapidly evolving area of translational interest. These approaches are being applied to an increasing number of vision-related diseases, such as keratoconus, progressive myopia, and glaucoma. Biomechanical measurement methods, which differ in spatial resolution, mechanical sensitivity, suitability for detecting crosslinking subtypes, and translational readiness, are central to the effort to understand the mechanistic link between crosslinking methods, the desired effects in tissue, and clinical outcomes of candidate therapies.

Acknowledgments

Supported by grants NIH R01EY028667, T32EB007509, T32EY007157, and R01HL126747, an Unrestricted Grant Award from Research to Prevent Blindness to the Department of Ophthalmology Cole Eye Institute (RPB1508DM), Foundation Fighting Blindness Center Grant to the Cole Eye Institute (CCMM08120584CCF), NEI/NIH P30 Core Center Grant (IP30EY025585), and the Sara J. Chehey Fund for Ocular Biomechanics Research at the Cole Eye Institute. Although the research reported in this presentation was supported by the NIH, the content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

B.J. Blackburn, A.M. Rollins, and W.J. Dupps are named on patents related to OCT-based biomechanical measurement held by Case Western Reserve University and Cleveland Clinic.

Disclosure: B.J. Blackburn, named on intellectual property related to biomechanical measurement held by Case Western Reserve University and Cleveland Clinic (P); A.M. Rollins, named on intellectual property related to biomechanical measurement held by Case Western Reserve University and Cleveland Clinic (P); W.J. Dupps, Glaikos (C), Alcon (C), named on intellectual property related to biomechanical measurement and modeling held by Case Western Reserve University and Cleveland Clinic (P)

References

1. McKay TB, Priyadarsini S, Karamichos D. Mechanisms of collagen crosslinking in diabetes and keratoconus. Cells. 2019;8(10):1239.
2. Wollensak G, Spoerl E, Seiler T. Riboflavin/ultraviolet-a-induced collagen crosslinking for the treatment of keratoconus. Am J Ophthalmol. 2003;135(5):620–627.
3. Spadea L, Salvatore S, Paroli MP, Vingolo EM. Recovery of corneal sensitivity after collagen crosslinking with and without epithelial debridement in eyes with keratoconus. J Cataract Refract Surg. 2015;41(3):527–532.

4. Kontadakis GA, Kymionis GD, Kankariya VP, Pallikaris AI. Effect of corneal collagen crosslinking on corneal innervation, corneal sensitivity, and tear function of patients with keratoconus. Ophthalmology. 2013;120(5):917–922.

5. Cifariello F, Minicucci M, Di Renzo F, et al. Epi-off versus epi-on corneal collagen crosslinking in keratoconus patients: a comparative study through 2-year follow-up. J Ophthalmol. 2018;2018:4947983.

6. Nawaz S, Gupta S, Gogia V, Sasikala NK, Panda A. Trans-epithelial versus conventional corneal collagen crosslinking: A randomized trial in keratoconus. Oman J Ophthalmol. 2015;8(1):9.

7. Abdel-Radi M, Eldaly Z, Abdelmotaal H, Abdelrahman R, Sayed M, Soliman K. Correlation between corneal demarcation line depth in epithelium-off and trans-epithelium accelerated corneal cross linking and keratoconus progression. Int J Ophthalmol. 2020;13(6):907.

8. Zhang X, Sun L, Shen Y, et al. Biomechanical and histopathologic effects of pulsed-light accelerated epithelium-on/-off corneal collagen cross-linking. Cornea. 2017;36(7):854–859.

9. Rubinfeld RS, Stulting RD, Gum GG, Talamo JH. Quantitative analysis of corneal stromal riboflavin concentration without epithelial removal. J Cataract Refract Surg. 2018;44(2):237–242.

10. Bradford S, Mikula E, Xie Y, Juhasz T, Brown DJ, Jester J V. Enhanced transepithelial riboflavin delivery using femtosecond laser-machined epithelial microchannels. Transl Vis Sci Technol. 2020;9(6):1.

11. Lim WK, Da Soh Z, Choi HKY, Theng JTS. Epithelium-on photorefractive intrastromal cross-linking (PiXL) for reduction of low myopia. Clin Ophthalmol. 2017;11:1205–1211.

12. Gore DM, O’Brart D, French P, Dunsby C, Allan BD. Transepithelial riboflavin absorption in an ex vivo rabbit corneal model. Invest Ophthalmol Vis Sci. 2015;56(8):5006–5011.

13. Hayes S, Morgan SR, O’Brart DP, O’Brart N, Meek KM. A study of stromal riboflavin absorption in ex vivo porcine corneas using new and existing delivery protocols for corneal crosslinking. Acta Ophthalmol. 2016;94(2):e109–e117.

14. Aldahlawi NH, Hayes S, O’Brart DPS, O’Brart ND, Meek KM. An investigation into corneal enzymatic resistance following epithelium-off and epithelium-on corneal cross-linking protocols. Exp Eye Res. 2016;153:141–151.

15. Rong S, Wang C, Han B, et al. Iontophoresis-assisted accelerated riboflavin/ultraviolet A scleral cross-linking: A potential treatment for pathologic myopia. Exp Eye Res. 2017;162:37–47.

16. Cassagne M, Laurent C, Rodrigues M, et al. Iontophoresis transcorneal delivery technique for transepithelial corneal collagen crosslinking with riboflavin in a rabbit model. Invest Ophthalmol Vis Sci. 2016;57(2):594–603.

17. Vinciguerra P, Mencucci R, Romano V, et al. Imaging Mass Spectrometry by Matrix-Assisted Laser Desorption/Ionization and Stress-Strain Measurements in Iontophoresis Transepithelial Corneal Collagen Cross-Linking. Biomed Res Int. 2014;2014:405487.

18. Dong Z, Zhou X. Collagen cross-linking with riboflavin in a femtosecond laser–created pocket in rabbit corneas: 6-month results. Am J Ophthalmol. 2011;152(1):22–27.

19. Caruso C, Ostacolo C, Epstein RL, Barbaro G, Troisi S, Capobianco D. Transepithelial corneal cross-linking with vitamin E-enhanced riboflavin solution and abbreviated, low-dose UV-A: 24-month clinical outcomes. Cornea. 2016;35(2):145.

20. Wang M, Zhang F, Qian X, Zhao X. Regional biomechanical properties of human sclera after cross-linking by riboflavin/ultraviolet A. J Refract Surg. 2012;28(10):723–728.

21. Scarcelli G, Kling S, Quijano E, Pineda R, Marcos S, Yun SH. Brillouin microscopy of collagen cross-linking: noncontact depth-dependent analysis of corneal elastic modulus. Invest Ophthalmol Vis Sci. 2013;54(2):1418–1425.

22. Arboleda A, Kowalczyk L, Savoldelli M, et al. Evaluating in vivo delivery of riboflavin with coumboll-controlled iontophoresis for corneal collagen cross-linking: a pilot study. Invest Ophthalmol Vis Sci. 2014;55(4):2731–2738.

23. Chow VWS, Chan TCY, Yu M, Wong VWY, Jhanji V. One-year outcomes of conventional and accelerated collagen crosslinking in progressive keratoconus. Sci Rep. 2015;5(1):1–7.

24. Tomita M, Mita M, Huseynova T. Accelerated versus conventional corneal collagen crosslinking. J Cataract Refract Surg. 2014;40(6):1013–1020.

25. Pahuja N, Kumar NR, Francis M, et al. Correlation of clinical and biomechanical outcomes of accelerated crosslinking (9 mW/cm² in 10 minutes) in keratoconus with molecular expression of ectasia-related genes. Curr Eye Res. 2016;41(11):1419–1423.
26. Kamaev P, Friedman MD, Sherr E, Muller D. Photochemical Kinetics of Corneal Cross-Linking with Riboflavin. *Investig Ophthalmol Vis Sci*. 2012;53(4):2360.

27. Seiler TG, Komninou MA, Nambiar MH, Schuerch K, Frueh BE, Büchler P. Oxygen Kinetics During Corneal Cross-linking With and Without Supplementary Oxygen. *Am J Ophthalmol*. 2021;223:368–376.

28. Mazotta C, Traversi C, Paradiso AL, Latronico ME, Rechichi M. Pulsed light accelerated crosslinking versus continuous light accelerated crosslinking: one-year results. *J Ophthalmol*. 2014;2014:604731.

29. Mooren P, Gobin L, Bostan N, et al. Evaluation of UVA cytotoxicity for human endothelium in an ex vivo corneal crosslinking experimental setting. *J Refract Surg*. 2016;32(1):41–46.

30. Iseli HP, Körber N, Karl A, et al. Damage threshold in adult rabbit eyes after scleral cross-linking by riboflavin/blue light application. *Exp Eye Res*. 2015;139:37–47.

31. Yildiz E, Anwaar Nazeer M, Bayraktutur B, Zibandeh N, Kizilel S, Sahin A. Novel corneal crosslinking technique with eosin-Y and visible light. *Acta Ophthalmal*. 2019;97.

32. Li Y, Zhang F, Sun M, et al. Safety and long-term scleral biomechanical stability of rhesus eyes after scleral cross-linking by blue light. *Curr Eye Res*. 2021;46(7):1061–1070.

33. Liu T-X, Luo X, Gu Y-W, Yang B, Wang Z. Correlation of discoloration and biomechanical properties in porcine sclera induced by genipin. *Int J Ophthalmol*. 2014;7(4):621–625.
reaction and applications. *Catalysts*. 2019;9(12):1035.

50. Campbell IC, Hannon BG, Read AT, Sherwood JM, Schwaner SA, Ethier CR. Quantification of the efficacy of collagen cross-linking agents to induce stiffening of rat sclera. *J R Soc Interface*. 2017;14(129):20170014.

51. Paik DC, Wen Q, Braunstein RE, Airiani S, Trokel SL. Initial studies using aliphatic β-nitro alcohols for therapeutic corneal cross-linking. *Investig Ophthalmol Vis Sci*. 2009;50(3):1098–1105.

52. Paik DC, Wen Q, Braunstein RE, Trokel SL. Aliphatic β-nitro alcohols for non-enzymatic collagen cross-linking of scleral tissue. *Exp Eye Res*. 2008;87(3):279–285.

53. Paik DC, Wen Q, Braunstein RE, Trokel SL. Short Chain Aliphatic-Nitro Alcohols for Corneoscleral Cross-Linking: Corneal Endothelial Toxicity Studies. *J Refract Surg*. 2008;24(7):S741–S747.

54. Babar N, Kim M, Cao K, et al. Cosmetic preservatives as therapeutic corneal and scleral tissue cross-linking agents. *Investig Ophthalmol Vis Sci*. 2015;56(2):1274–1282.

55. Pappa CS, Nguyen BA, Mahmoud AM, Agarwal G, Roberts CJ. Effect of penetration enhancer with novel corneal cross-linking using recombinant human decoron in porcine eyes. *Exp Eye Res*. 2021;206:108542.

56. Wu Y, Song W, Tang Y, Elsheikh A, Shao Y, Yan X. Efficacy and safety of transglutaminase-induced corneal stiffening in rabbits. *Transl Vis Sci Technol*. 2019;8(6):27.

57. Sun X, Chen D, Liu X, Yan X, Wu Y. Effect of enzyme-induced collagen cross-linking on porcine sclera. *Biochem Biophys Res Commun*. 2020;528(1):134–139.

58. Kopsachilis N, Tsaousis KT, Tsinopoulos IT, Kruse FE, Welge-Luessen U. A novel mechanism of UV-A and riboflavin-mediated corneal cross-linking through induction of tissue transglutaminases. *Cornea*. 2013;32(7):1034–1039.

59. Kobashi H, Rong SS. Corneal Collagen Cross-Linking for Keratoconus: Systematic Review. *Biomed Res Int*. 2017;2017:8145651.

60. Bayraktar S, Cebeci Z, Oray M, Alparslan N. Corneal collagen cross-linking in pellucid marginal degeneration: 2 patients, 4 eyes. *Case Rep Ophthalmol Med*. 2015;2015:840687.

61. Hersh PS, Stulting RD, Muller D, et al. U.S. Multicenter Clinical Trial of Corneal Collagen Crosslinking for Treatment of Corneal Ectasia after Refractive Surgery. *Ophthalmology*. 2017;124(10):1475–1484.

62. Lim EWL, Lim L. Review of Laser Vision Correction (LASIK, PRK and SMILE) with Simultaneous Accelerated Corneal Crosslinking—Long-term Results. *Curr Eye Res*. 2019;44(11):1171–1180.

63. Roy AS, Dupps WJ. Patient-specific modeling of corneal refractive surgery outcomes and inverse estimation of elastic property changes. *J Biomech Eng*. 2011;133(1):011002.

64. Seven I, Roy AS, Dupps WJ Patterned corneal collagen crosslinking for astigmatism: computational modeling study. *J Cataract Refract Surg*. 2014;40(6):943–953.

65. Bettis DI, Hsu M, Moshirfar M. Corneal collagen cross-linking for nonectatic disorders: A systematic review. *J Refract Surg*. 2012;28(11):798–807.

66. Richoz O, Kling S, Hoogewoud F, et al. Antibacterial efficacy of accelerated photoactivated chromophore for keratitis–corneal collagen cross-linking (PACK-CXL). *J Refract Surg*. 2014;30(12):850–854.

67. Ting DSJ, Henein C, Said DG, Dua HS. Photoactivated chromophore for infectious keratitis–Corneal cross-linking (PACK-CXL): A systematic review and meta-analysis. *Ocul Surf*. 2019;17(4):624–634.

68. Wollensak G, Spoerl E. Collagen crosslinking of human and porcine sclera. *J Cataract Refract Surg*. 2004;30(3):689–695.

69. Burgoyne CF, Downs JC, Bellezza AJ, Suh J-KF, Hart RT. The optic nerve head as a biomechanical structure: a new paradigm for understanding the role of IOP-related stress and strain in the pathophysiology of glaucomatous optic nerve head damage. *Prog Retin Eye Res*. 2005;24(1):39–73.

70. Downs JC, Roberts MD, Burgoyne CF. The mechanical environment of the optic nerve head in glaucoma. *Optom Vis Sci Off Publ Am Acad Optom*. 2008;85(6):425.

71. Thornton IL, Dupps WJ, Roy AS, Krueger RR. Biomechanical effects of intraocular pressure elevation on optic nerve/lamina cribosa before and after peripapillary scleral collagen cross-linking. *Invest Ophthalmol Vis Sci*. 2009;50(3):1227–1233.

72. Korneva A, Nguyen C, Schaub J, Nguyen TD, Quigley HA. Biomechanical effects on the mouse optic nerve head in experimental scleral crosslinking in glaucoma. *Invest Ophthalmol Vis Sci*. 2019;60(9):6188.

73. Hayes S, Aldahlawi N, Markovich AL, et al. The effect of bacteriochlorophyll derivative WST-D
and near infrared light on the molecular and fibrillar architecture of the corneal stroma. Sci Rep. 2020;10(1):1–11.
74. Liu T, Shen M, Li H, et al. Changes and quantitative characterization of hyper-viscoelastic biomechanical properties for young corneal stroma after standard corneal cross-linking treatment with different ultraviolet-A energies. Acta Biomater. 2020;113:438–451.
75. Depalle B, Qin Z, Shefelbine SJ, Buehler MJ. Influence of cross-link structure, density and mechanical properties in the mesoscale deformation mechanisms of collagen fibrils. J Mech Behav Biomed Mater. 2015;52:1–13.
76. Wollensak G, Spörl E, Mazzotta C, Kalinski T, Sel S. Interlamellar cohesion after corneal cross-linking using riboflavin and ultraviolet A light. Br J Ophthalmol. 2011;95(6):876–880.
77. Tonge TK, Ruberti JW, Nguyen TD. Micromechanical Modeling Study of Mechanical Inhibition of Enzymatic Degradation of Collagen Tissues. Biophys J. 2015;109(12):2689–2700.
78. Bell JS, Hayes S, Whitford C, et al. The hierarchical response of human corneal collagen to load. Acta Biomater. 2018;65:216–225.
79. Orgel JPRO, Irving TC, Miller A, Wess TJ. Microfibrillar structure of type I collagen in situ. Proc Natl Acad Sci USA. 2006;103(24):9001–9005.
80. Walimbe T, Panitch A. Best of both hydrogel worlds: Harnessing bioactivity and tunability by incorporating glycosaminoglycans in collagen hydrogels. Bioengineering. 2020;7(4):1–24.
81. Majumdar S, Wang X, Sommerfeld SD, et al. Cyclodextrin Modulated Type I Collagen Self-Assembly to Engineer Biomimetic Cornea Implants. Adv Funct Mater. 2018;28(41):1804076.
82. Parfitt GJ, Pinali C, Young RD, Quantock AJ, Knupp C. Three-dimensional reconstruction of collagen-proteoglycan interactions in the mouse corneal stroma by electron tomography. J Struct Biol. 2010;170(2):392–397.
83. Zhang Y, Conrad AH, Conrad GW. Effects of ultraviolet-A and riboflavin on the interaction of collagen and proteoglycans during corneal cross-linking. J Biol Chem. 2011;286(15):13011–13022.
84. Spoerl E, Wollensak G, Seiler T. Increased resistance of crosslinked cornea against enzymatic digestion. Curr Eye Res. 2004;29(1):35–40.
85. Meller D, Peters K, Meller K. Human cornea and sclera studied by atomic force microscopy. Cell Tissue Res. 1997;288(1):111–118.
86. Scarcelli G, Pineda R, Yun SH. Brillouin optical microscopy for corneal biomechanics. Invest Ophthalmol Vis Sci. 2012;53(1):185–190.
87. Beshtawi IM, Akhtar R, Hillarby MC, et al. Scanning acoustic microscopy for mapping the microelastic properties of human corneal tissue. Curr Eye Res. 2013;38(4):437–444.
88. Blackburn BJ, Gu S, Ford MR, et al. Noninvasive Assessment of Corneal Crosslinking with Phase-Decorrelation Optical Coherence Tomography. Invest Ophthalmol Vis Sci. 2019;60(1):41–51.
89. Dupps WJ, Netto MV, Herekar S. Surface wave elastometry of the cornea in porcine and human donor eyes. J Refract Surg. 2007;23(1):66–75.
90. Tanter M, Touboul D, Gennisson J-L, Bercoff J, Fink M. High-resolution quantitative imaging of cornea elasticity using supersonic shear imaging. IEEE Trans Med Imaging. 2009;28(12):1881–1893.
91. Ford MR, Dupps WJ, Rollins AM, Roy AS, Hu Z. Method for optical coherence elastography of the cornea. J Biomed Opt. 2011;16(1):16005.
92. Ford MR, Rollins AM, Dupps WJ. Quantitative In Vivo Corneal Elastography by Doppler Shear Wave Imaging. Invest Ophthalmol Vis Sci. 2014;55(13):3724.
93. Hatami-Marbini H. Viscoelastic shear properties of the corneal stroma. J Biomech. 2014;47(3):723–728.
94. Hoeltzel DA, Altman P, Buzard K, Choe K. Strip extensiometry for comparison of the mechanical response of bovine, rabbit, and human corneas. J Biomech Eng. 1992;114(2):202–215.
95. Elsheikh A, Anderson K. Comparative study of corneal strip extensiometry and inflation tests. J R Soc Interface. 2005;2(3):177–185.
96. Mattsson MS, Huynh J, Wiseman M, Coassin M, Kornfeld JA, Schwartz DM. An in vitro intact globe expansion method for evaluation of cross-linking treatments. Invest Ophthalmol Vis Sci. 2010;51(6):3120–3128.
97. Seifert J, Hammer CM, Rheinlaender J, et al. Distribution of Young’s modulus in porcine corneas after riboflavin/UVA-induced collagen cross-linking as measured by atomic force microscopy. PLoS One. 2014;9(1):e88186.
98. Matteolli S, Virga A, Paladini I, Mencucci R, Corvi A. Investigation into the elastic properties of ex vivo porcine corneas subjected to inflation test after cross-linking treatment. J Appl Biomater Funct Mater. 2016;14(2):163–170.
photooxidative collagen cross-linking with photosensitizer riboflavin and 370 nm UVA light on human corneoscleral tissues. *Microsc Microanal.* 2013;19(5):1334.

100. Han Z, Li J, Singh M, et al. Optical coherence elastography assessment of corneal viscoelasticity with a modified Rayleigh-Lamb wave model. *J Mech Behav Biomed Mater.* 2017;66:87–94.

101. Chang SH, Mohammadvali A, Chen KJ, et al. The Relationship Between Mechanical Properties, Ultrastructural Changes, and Intrafibrillar Bond Formation in Corneal UVA/Riboflavin Cross-linking Treatment for Keratoconus. *J Refract Surg.* 2018;34(4):264–272.

102. Søndergaard AP, Ivarsen A, Hjortdal J. Corneal Resistance to Shear Force After UVA-Riboflavin Cross-Linking Increased Shear Resistance After CXL. *Invest Ophthalmol Vis Sci.* 2013;54(7):5059–5069.

103. Shao P, Eltony AM, Seiler TG, et al. Spatially-resolved Brillouin spectroscopy reveals biomechanical abnormalities in mild to advanced keratoconus in vivo. *Sci Rep.* 2019;9(1):1–12.

104. He X, Spoerl E, Tang J, Liu J. Measurement of corneal changes after collagen crosslinking using a noninvasive ultrasound system. *J Cataract Refract Surg.* 2010;36(7):1207–1212.

105. Blackburn BJ, Gu S, Ford MR, et al. Noninvasive Assessment of Corneal Crosslinking With Phase-Decorrelation Optical Coherence Tomography. *Investig Ophthalmology Vis Sci.* 2019;60(1):41.

106. Aldahlawi NH, Hayes S, O’Braitt DPs, Meek KM. Standard versus accelerated riboflavin–ultraviolet corneal collagen crosslinking: Resistance against enzymatic digestion. *J Cataract Refract Surg.* 2015;41(9):1989–1996.

107. Viswanathan D, Kumar NL, Males JJ, Graham SL. Relationship of structural characteristics to biomechanical profile in normal, keratoconic, and crosslinked eyes. *Cornea.* 2015;34(7):791–796.

108. Gkika M, Labiris G, Giarmoukakis A, Koutsogianni A, Kozobolis V. Evaluation of corneal hysteresis and corneal resistance factor after corneal cross-linking for keratoconus. *Graefe’s Arch Clin Exp Ophthalmol.* 2012;250(4):565–573.

109. Spoerl E, Terai N, Scholz F, Raiskup F, Pillunat LE. Detection of biomechanical changes after corneal cross-linking using Ocular Response Analyzer software. *J Refract Surg.* 2011;27(6):452–457.

110. Goldich Y, Barkana Y, Morad Y, Hartstein M, Avni I, Zadok D. Can We Measure Corneal Biomechanical Changes After Collagen Cross-linking in Eyes With Keratoconus? - A Pilot Study. *Cornea.* 2009;28(5):498–502.

111. Greenstein SA, Fry KL, Hersh PS. In Vivo Biomechanical Changes After Corneal Collagen Cross-linking for Keratoconus and Corneal Ectasia: 1-Year Analysis of a Randomized, Controlled, Clinical Trial. *Cornea.* 2012;31(1):21–25.

112. Salouti R, Khalili MR, Zamani M, Ghoreyshi M, Nowroozzadeh MH. Assessment of the changes in corneal biomechanical properties after collagen cross-linking in patients with keratoconus. *J Curr Ophthalmol.* 2019;31(3):262–267.

113. De Bernardo M, Capasso L, Lanza M, et al. Long-term results of corneal collagen crosslinking for progressive keratoconus. *J Optom.* 2015;8(3):180–186.

114. Sedaghat MR, Momeni-Moghaddam H, Ambrósi R, et al. Long-term evaluation of corneal biomechanical properties after corneal cross-linking for keratoconus: A 4-year longitudinal study. *J Refract Surg.* 2018;34(12):849–856.

115. Ford MR, A Sinha Roy, Rollins AM, Dupps WJ. Serial biomechanical comparison of edematous, normal, and collagen crosslinked human donor corneas using optical coherence elastography. *J Cataract Refract Surg.* 2014;40(6):1041–1047.

116. Jabbarvand M, Moravej Z, Shahrazi K, et al. Corneal biomechanical outcome of collagen cross-linking in keratoconic patients evaluated by Corvis ST. *Eur J Ophthalmol.* https://doi.org/10.1177/112067212094798. Online ahead of print.

117. Wong FF, Lari DR, Schultz DS, Stewart JM. Whole globe inflation testing of exogenously crosslinked sclera using genipin and methylglyoxal. *Exp Eye Res.* 2012;103:17–21.

118. Wollensak G, Iomdina E. Long-term biomechanical properties of rabbit sclera after collagen crosslinking using riboflavin and ultraviolet A (UVA). *Acta Ophthalmol.* 2009;87(2):193–198.

119. Gawargious BA, Le A, Lesgart M, Ugradar S, Demer JL. Differential Regional Stiffening of Sclera by Collagen Cross-linking. *Curr Eye Res.* 2020;45(6):718–725.

120. Schuldt C, Karl A, Körber N, et al. Dose-dependent collagen cross-linking of rabbit scleral tissue by blue light and riboflavin treatment probed by dynamic shear rheology. *Acta Ophthalmol.* 2015;93(5):e328–e336.

121. Asri D, Touboul D, Fournié P, et al. Corneal collagen crosslinking in progressive keratoconus: Multicenter results from the French National Reference Center for Keratoconus. *J Cataract Refract Surg.* 2011;37(12):2137–2143.
122. Spoerl E, Terai N, Scholz F, Raiskup F, Pillunat LE. Detection of biomechanical changes after corneal cross-linking using ocular response analyzer software. *J Refract Surg.* 2011;27(6):452–457.

123. Vinciguerra P, Albè E, Mahmoud AM, Trazzia S, Hafezi F, Roberts CJ. Intra- and postoperative variation in ocular response analyzer parameters in keratoconic eyes after corneal cross-linking. *J Refract Surg.* 2010;26(9):669–676.

124. Goldich Y, Marcovich AL, Barkana Y, et al. Clinical and Corneal Biomechanical Changes After Collagen Cross-Linking With Riboflavin and UV Irradiation in Patients With Progressive Keratoconus. *Cornea.* 2012;31(6):609–614.

125. Blackburn BJ, Jenkins MW, Rollins AM, Dupps WJ. A Review of Structural and Biomechanical Changes in the Cornea in Aging, Disease, and Photochemical Crosslinking. *Front Bioeng Biotechnol.* 2019;7:66.

126. Rohrbach D, Silverman RH, Chun D, Lloyd HO, Urs R, Mamou J. Improved high-frequency ultrasound corneal biometric accuracy by micrometer-resolution acoustic-property maps of the cornea. *Transl Vis Sci Technol.* 2018;7(2):21–21.

127. Di Mundo R, Recchia G, Parekh M, Ruzz A, Ferrari S, Carbone G. Sensing inhomo geneous mechanical properties of human corneal Descemet’s membrane with AFM nanoindentation. *J Mech Behav Biomed Mater.* 2017;74:21–27.

128. Caponi S, Fioretto D, Mattarelli M. On the actual spatial resolution of Brillouin Imaging. *Opt Lett.* 2020;45(5):1063–1066.

129. Shao P, Besner S, Zhang J, Scarcelli G, Yun S-H. Etalon filters for Brillouin microscopy of highly scattering tissues. *Opt Express.* 2016;24(19):22232–22238.

130. Nguyen T-M, Aubry J-F, Fink M, Bercoff J, Tanter M. In vivo evidence of porcine cornea anisotropy using supersonic shear wave imaging. *Invest Ophthalmol Vis Sci.* 2014;55(11):7545–7552.

131. Hepburn MS, Wijesinghe P, Chin L, Kennedy BF. Analysis of spatial resolution in phase-sensitive compression optical coherence elastography. *Biomed Opt Express.* 2019;10(3):1496–1513.

132. Kirby MA, Zhou K, Pitre JJ, et al. Spatial resolution in dynamic optical coherence elastography. *J Biomed Opt.* 2019;24(9):96006.

133. Kling S. Optical coherence elastography by ambient pressure modulation for high-resolution strain mapping applied to patterned cross-linking. *J R Soc Interface.* 2020;17(162):20190786.

134. Kling S, Torres-Netto EA, Spiru B, Sekundo W, Hafezi F. Quasi-static optical coherence elastography to characterize human corneal biomechanical properties. *Invest Ophthalmol Vis Sci.* 2020;61(6):29.

135. Woo JH, Iyer JV, Lim L, et al. Conventional versus accelerated collagen cross-linking for keratoconus: a comparison of visual, refractive, topographic and biomechanical outcomes. *Open Ophthalmol J.* 2017;11:262.

136. Sadoughi MM, Einollahi B, Baradarani-Rafii A, Roshandel D, Hasani H, Nazari M. Accelerated versus conventional corneal collagen cross-linking in patients with keratoconus: an intrapatient comparative study. *Int Ophthalmol.* 2018;38(1):67–74.

137. Sherif AM. Accelerated versus conventional corneal collagen cross-linking in the treatment of mild keratoconus: a comparative study. *Clin Ophthalmol (Auckland, NZ).* 2014;8:1435.

138. Wen D, Li Q, Song B, et al. Comparison of standard versus accelerated corneal collagen cross-linking for keratoconus: a meta-analysis. *Invest Ophthalmol Vis Sci.* 2018;59(10):3920–3931.

139. Faramarzi A, Hassanpour K, Rahmani B, Yazdani S, Kheiri B, Sadoughi M-M. Systemic supplemental oxygen therapy during accelerated corneal cross-linking for progressive keratoconus; a randomized clinical trial. *J Cataract Refract Surg.* 2021;47(6):773–779.

140. Herber R, Ramm L, Spoerl E, Raiskup F, Pillunat LE, Terai N. Assessment of corneal biomechanical parameters in healthy and keratoconic eyes using dynamic bidirectional applanation device and dynamic Scheimpflug analyzer. *J Cataract Refract Surg.* 2019;45(6):778–788.

141. Aldahlawi NH, Hayes S, O’Brart DPS, Akhbanbetova A, Littlechild SL, Meek KM. Enzymatic resistance of corneas cross-linked using riboflavin in conjunction with low energy, high energy, and pulsed UVA irradiation modes. *Invest Ophthalmol Vis Sci.* 2016;57(4):1547–1552.

142. Herber R, Francis M, Spoerl E, Pillunat LE, Raiskup F, Roy AS. Comparison of waveform-derived corneal stiffness and stress-strain extensometry-derived corneal stiffness using different cross-linking irradiances: an experimental study with air-puff applanation of ex vivo porcine eyes. *Graefe’s Arch Clin Exp Ophthalmol.* 2020;258(10):2173–2184.

143. Beshtawi IM, Akhtar R, Hillarby MC, et al. Biomechanical properties of human corneas...
following low-and high-intensity collagen cross-linking determined with scanning acoustic microscopy. *Invest Ophthalmol Vis Sci.* 2013;54(8):5273–5280.

144. Hammer A, Richoz O, Mosquera S, Tabibian D, Hoogewoud F, Hafezi F. Corneal biomechanical properties at different corneal collagen cross-linking 2 (CXL) Irradiances 3. *Invest Ophthalmol Vis Sci.* 2014;55(5):2881–2884.

145. Hammer A, Richoz O, Mosquera SA, Tabibian D, Hoogewoud F, Hafezi F. Corneal biomechanical properties at different corneal cross-linking (CXL) irradiances. *Invest Ophthalmol Vis Sci.* 2014;55(5):2881–2884.

146. Webb JN, Su JP, Scarcelli G. Mechanical outcome of accelerated corneal crosslinking evaluated by Brillouin microscopy. *J Cataract Refract Surg.* 2017;43(11):1458–1463.

147. Nath S, Shen C, Koziarz A, et al. Transepithelial versus epithelium-off corneal collagen crosslinking for corneal ectasia: a systematic review and meta-analysis. *Ophthalmology.* [https://doi.org/10.1016/j.ophtha.2020.12.023](https://doi.org/10.1016/j.ophtha.2020.12.023). Online ahead of print.

148. Kobashi H, Rong SS, Ciolino JB. Transepithelial versus epithelium-off corneal crosslinking for corneal ectasia. *J Cataract Refract Surg.* 2018;44(12):1507–1516.

149. Aldahlawi NH, Hayes S, O’Brart DPS, O’Brart ND, Meek KM. An investigation into corneal enzymatic resistance following epithelium-off and epithelium-on corneal cross-linking protocols. *Exp Eye Res.* 2016;153:141–151.

150. Armstrong BK, Lin MP, Ford MR, et al. Biological and biomechanical responses to traditional epithelium-off and transepithelial riboflavin-UVA CXL techniques in rabbits. *J Refrat Surg.* 2013;29(5):332–341.

151. Scarcelli G, Kling S, Quijano E, Pineda R, Marcos S, Yun SH. Brillouin microscopy of collagen crosslinking: Noncontact depth-dependent analysis of corneal elastic modulus. *Investig Ophthalmol Vis Sci.* 2013;54(2):1418–1425.

152. Bekesi N, Gallego-Munoz P, Ibares-Frias L, et al. Biomechanical changes after in vivo collagen cross-linking with rose Bengal–green light and riboflavin-UVA. *Invest Ophthalmol Vis Sci.* 2017;58(3):1612–1620.

153. Brekelmans J, Veugen J, Rieff K, et al. Enzymatic digestion of porcine corneas cross-linked by hypo-and hyperosmolar formulations of riboflavin/ultraviolet A or WST11/near-infrared light. *Transl Vis Sci Technol.* 2020;9(10):4.

154. Roy AS, Dupps WJ. Patient-specific computational modeling of keratoconus progression and differential responses to collagen cross-linking. *Invest Ophthalmol Vis Sci.* 2011;52(12):9174–9187.

155. Legras R, Chateau N, Charman WN. Assessment of Just-Noticeable Differences for Refractive Errors and Spherical Aberration Using Visual Simulation. *Optom Vis Sci.* 2004;81(9):718–728.

156. Hepfer RG, Shi C, Wu Y, Waring IV GO, Yao H. Corneal Cross-Linking: Engineering a Predictable Model. *Crit Rev Biomed Eng.* 2014;42(3-4):229–248.

157. A Sinha Roy, Rocha KM, Randleman JB, Stulting RD, Dupps WJ. Inverse computational analysis of in vivo corneal elastic modulus change after collagen crosslinking for keratoconus. *Exp Eye Res.* 2013;113:92–104.

158. Kling S, Hafezi F. An algorithm to predict the biomechanical stiffening effect in corneal cross-linking. *J Refract Surg.* 2017;33(2):128–136.