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# Nonlinear Optical Crosslinking (NLO CXL) for Correcting Refractive Errors.

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## Abstract

Ultraviolet A (UVA) light-based photoactivation of riboflavin (Rf) to induce corneal crosslinking (CXL) and mechanical stiffening is now a well-known treatment for corneal ectasia and Keratoconus that is being used in a topographically guided photorefractive intrastromal CXL (PiXL) procedure to treat low degrees of refractive errors. Alternative approaches for non-invasive treatment of refractive errors have also been proposed that use femtosecond lasers (FS) that provide much faster, more precise, and safer results than UVA CXL. One such treatment, nonlinear optical crosslinking (NLO CXL), has been able to replicate the effects of UVA CXL, while producing a smaller area of cellular damage and requiring a shorter procedure time. Unlike UVA CXL, the treatment volume of NLO CXL only occurs within the focal volume of the laser, which can be placed at any depth and scanned into any pattern for true topographically guided refractive correction. This review presents our experience with using FS lasers to photoactivate Rf and perform highly controlled corneal CXL that leads to mechanical stiffening and changes in corneal shape.

### Keywords

Cornea; Crosslinking; Femtosecond Laser; Nonlinear

## 1. Introduction

During the last twenty years, laser assisted in situ keratomileusis (LASIK) surgery has become the standard of care to correct refractive errors of the human eye, including near and farsightedness. (Krueger et al., 2010; Pidro et al., 2019) The procedure consists of cutting a corneal flap and ablating the corneal stroma under the flap with an excimer laser. Due to its invasiveness, frequently pain and complications are associated with this surgery. For example, both epithelial ingrowth and inoperative epithelial abrasion occur in 1% of

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patients, (Toda, 2008) while 1/3000 cases may develop post LASIK ectasia that require corneal transplantation.(Bohac et al., 2018) Additionally dry eye is a common postoperative complaint, affecting 50% of patients at 1 week, 40% at one month, and 20–40% at 6 months, (Toda, 2008) and is a major contribution to patient dissatisfaction.(Golas and Manche, 2011) While the performance of LASIK is justified for the correction of medium and high refractive errors, the procedure is not ideal for the correction of low refractive errors, due to patient concerns regarding invasiveness and risk for potential complications.

While refractive errors are the most common vision-related disorder affecting over 200 million Americans, (Wittenborn and Rein, 2013) over 60 million people suffer from low degrees of myopia (between 0 and 2 diopters), another 60 million suffer from astigmatism (between 0 and 2 diopters) (Vitale et al., 2008), while an additional 20 million individuals have low degrees of presbyopia requiring <2 diopters of addition correction. (Lindstrom et al., 2013) Potentially, there are over 140 million patients with refractive errors that are mostly treated with glasses and contact lenses. Additionally, it was estimated that the yearly economic burden of refractive correction specifically for patients under the age of 40 in the United States is approximately $4.9 billion for visual aids such as glasses and contact lenses, and $7.3 billion for optometry visits as of the year 2012.(Wittenborn and Rein, 2013) When considering all age ranges and an entire lifetime, this cost could be a significant economic burden for patients. It is widely recognized that no single method for correcting refractive errors is either appropriate for or appealing to all patients, (Riley and Chalmers, 2005) indicating that a novel, noninvasive strategy for modifying the corneal refraction may provide a more cost effective and life style compatible alternative to improving and enhancing visual acuity in these patients.

Recently, several alternative strategies have been proposed that include both ultraviolet (UVA) and femtosecond laser (FS) light-based approaches to modifying corneal refractive power. The most commercially advanced procedure takes advantage of UVA photoactivation of riboflavin (Rf) to induce corneal crosslinking (UVA CXL). (Sporl et al., 1997; Wollensak et al., 2003) UVA CXL is a popular photodynamic therapy used to treat corneal ectasia and Keratoconus by increasing the corneal mechanical stiffness. (Spoerl et al., 1998; Wollensak et al., 2003) Standard UVA CXL requires debridement of the corneal epithelium, followed by a 30 minute soak with 0.1% Rf eye drops to saturate the stroma with the photoinitiator. The cornea is then irradiated for 30 minutes with 5.4 mJ of 370 nm UVA light with an irradiance of 3 $mW/cm^2$. (Sporl et al., 1997; Wollensak et al., 2003) By altering this procedure to utilize customized topographically guided UVA irradiation patterns, regional CXL can be produced. (Elling et al., 2018) This derivative CXL procedure, known as photorefractive intrastromal crosslinking (PiXL), has been shown capable of treating low degrees of both myopia and hyperopia without resulting in the dry eye or corneal flap related complications associated with LASIK. (Lim et al., 2017; Nordstrom et al., 2017; Sachdev et al., 2020; Stodulka et al., 2020; Taneri et al., 2013) Additionally, to reduce the 30 minute time of treatment, accelerated CXL has been proposed based on the Bunsen-Roscoe law of reciprocity using a higher UVA irradiance to reduce UVA exposure time but maintaining the same energy exposure of 5.4 mJ. (Mita et al., 2014)

Besides procedure time for UVA CXL, another important concern has been the method of Rf application to the corneal stroma. Since the corneal epithelium serves as a barrier to the penetration of water and solutes into the corneal stroma, traditional UVA CXL uses epithelial debridement to enhance rapid and efficient stromal imbibement of Rf. This procedure has many drawbacks including patient discomfort, prolonged visual recovery time, and potential for corneal infection. (Koller et al., 2009; Shalchi et al.,2015) To avoid debridement of the epithelium, transepithelial applications of Rf have been intensively studied, with many different methods having been tested in the lab and clinic. Most successful approaches rely on the addition of chemicals that break down the epithelial barrier, such as benzalkonium chloride (BAK),(Akbar et al., 2017; Aldahlawi et al., 2016; Chow et al., 2018; Gore et al., 2015a; Hayes et al., 2016; Lim et al., 2017) the addition of vitamins E or C,(Koc et al., 2017; Ostacolo et al., 2013) or iontophoresis.(Cassagne et al., 2016; Gore et al., 2015b; Mastropasqua et al., 2014; Vinciguerra et al., 2014) Whatever the method of Rf application, however, transepithelial UVA CXL has yet to reproduce the results of traditional UVA CXL.(Bottos et al., 2011; Chow et al., 2018; Kobashi et al., 2018; Rush and Rush, 2017; Shalchi et al., 2015) This could be due to the reduced concentration of Rf within the corneal stroma when the epithelium is left intact. It could also be due, in part, to the nature of the single photon Rf photoactivation. Since the reaction must begin at the surface, the corneal epithelium serves not only as a barrier to Rf penetration but also absorbs UVA irradiation reducing the UVA irradiance within the corneal stroma.

Recently, alternatives to UVA CXL have been proposed for refractive correction that use FS lasers to take advantage of nonlinear optical (NLO) laser tissue interactions that occur through multiphoton processes within the focal volume of the laser light. (Kuetemeyer et al., 2011; Kwok et al., 2016; Zipfel et al., 2003) Briefly, UVA CXL operates based on a linear process in which a single photon entering the tissue interacts with a single molecule, causing a reaction. In contrast, FS lasers have the ability to focus high concentrations of photons within very small volumes of tissue allowing for very low probability events, such as the simultaneous absorption of multiple photons by the same molecule to occur. One such NLO approach has been the use of 400 nm (blue) FS laser light to produce a localized change in the refractive index of the corneal stroma (Blue-IRIS) as defined by the focal volume. (Wozniak et al., 2017; Xu et al., 2011) While the underlying principle of this technique remains poorly understood, denaturation of the fibrillar collagen seems likely given the finding that the non-centrosymmetric structure of collagen is lost following Blue-IRIS, as evidenced by the loss of second harmonic generated signals from fibrillar collagen. (Yu et al., 2019) How this affects the long-term stability of the change in refractive index, and what other components of the extracellular matrix are thermally denatured remains to be determined.

Since the corneal shape is controlled by the structure and biomechanics of the stromal collagen, (Koudouna et al., 2018; Meek and Knupp, 2015) our laboratory has focused on combining the precision of FS lasers with the known mechanical stiffening effects of Rf photoactivation, which we have termed NLO CXL. While UVA CXL utilizes a single photon excitation to photoactivate Rf, NLO CXL requires two-photons with a combined energy equivalent to one UVA photon to simultaneously activate Rf and generate free electrons leading to the formation of free radicals. The statistical likelihood of this event occurring is

greatly increased within the two-photon focal volume of an extremely fast pulsed laser. For this reason, this method can be used to induce crosslinking within a highly precise and controlled volume of tissue to modify the mechanical stiffness in localized regions with customizable patterns.

Thus far our research has shown that NLO CXL can provide the same increase in corneal stiffness and flattening as that achieved by standard UVA CXL therapy. (Bradford et al., 2019; Bradford et al., 2017) Also, since UVA CXL is limited to irradiation of the outermost corneal tissue, it will never be able to avoid damaging the epithelium, or treat the posterior collagen structure. As reported by Bueno et al. tissue with a low level of initial organization experiences a greater CXL effect, such as the anterior versus posterior stroma, (Bueno et al., 2019) and it is unknown how this may affect the resulting corneal shape if different combinations of patterns and depths of CXL are performed. In contrast to UVA CXL, the precise nature of NLO CXL allows for a high level of control over the placement of the crosslinked volume. Not only can the activated volume of Rf be positioned at any depth within the cornea, its axial and lateral dimensions are highly adjustable depending on the focusing optics used. This opens the door to both posterior stromal crosslinking and truly transepithelial crosslinking. We have also developed a novel approach to enhance transepithelial Rf penetration into the cornea using FS laser micromachining of the cornea to form 3 μm diameter by 25 μm long microchannels. These advances and their implication for treating low degrees of refractive error are presented in this review.

## 2. Preliminary Studies: Proof of Concept

Chai et al. was the first to establish that FS lasers can be used to photoactivate Rf and induce mechanical stiffening of fibrillar collagen. His study tested the hypothesis that multiphoton, nonlinear, activation of Rf produced using a focused FS laser is capable of producing crosslinking within collagen in the same manner as single photon activation produced using UVA irradiation. (Chai et al., 2013) In this study Chai et al. established the optimal wavelength necessary to induce two-photon photoactivation of Rf. This was accomplished by imaging regions within a tank of Rf solution using a Zeiss LSM 510 confocal microscope coupled to a 75 MHz FS laser (Chameleon, Coherent Inc.) tuned to a range of infrared wavelengths from 740 to 960 nm. The highest Rf fluorescence was measured using an FS excitation wavelength of 760 nm, while intensity sharply declined using wavelengths above 780 nm.

Using the same microscope and laser setup, 100 mW of 760 nm laser light was focused through a 0.75 numerical aperture (NA), 20x objective (Carl Zeiss) into a Rf soaked sheet of compressed collagen hydrogel and scanned through multiple tiles at multiple layers to create a continuous volume of irradiated sample. Indentation testing was then used to evaluate the mechanical stiffening effect of the treatment. Indentation was used in this study, and in later studies, because NLO CXL was performed in such a small volume of tissue that the change in material stiffness would be impractical to measure using tensiometry, the more commonly used measure of UVA CXL effectiveness. While indentation testing is most useful for measuring the stiffening effect of tissue directly in contact with the probe, the resistance force can be somewhat dampened by under or overlying regions of uncrosslinked tissue.

Both treated and control samples were probed with a force transducer and the resultant force measurements were used to calculate the change in elastic modulus of each sample. The elastic modulus post treatment was significantly higher in both UVA CXL and NLO CXL treatment groups compared to control compressed collagen hydrogels but were not significantly different between each other.

This was the first report to demonstrate the ability of NLO photoactivation of Rf to produce a similar collagen crosslinking effect compared to UVA CXL, but the multiple hours required to perform a single treatment were not clinically practical.

## 3. Ex Vivo Experiments

After proof of concept was established in compressed collagen sheets, the issue of the clinically impractical procedure time needed to be addressed. A second study was performed to evaluate approaches to shorten the procedure time of NLO CXL using ex vivo rabbit corneas treated using a Zeiss LSM 510 confocal microscope and 75 MHz, 760 nm FS laser. (Bradford et al., 2016) In contrast to the previous experiment, which took several hours to perform NLO CXL on collagen hydrogels, the goal of this study was to perform NLO CXL within the same time frame as traditional UVA CXL (< 30 minutes). Specifically, this experiment tested the hypothesis that lowering the NA of the scanning objective to 0.1 would greatly increase the focal volume size, allowing for a similar depth of NLO CXL to conventional UVA CXL using only one pass of the FS beam. Theoretically the 0.75 NA utilized by Chai et al. was capable of producing a focal volume that was approximately 1.7 μm axial by 0.5 μm lateral. (Chai et al., 2013) By decreasing the NA to 0.1, the theoretical focal volume should be exponentially increased to 114 μm by 3.4 μm. (Zipfel et al., 2003)

Previous studies have established that UVA CXL of the cornea generates an enhanced collagen autofluorescence (CAF) within the range of 400–450 nm that can be used as a marker for the location of mechanical stiffening by Rf photoactivation, since this wavelength is outside the two-photon emission spectra for Rf (460nm-682nm).(Chai et al., 2011) For this reason, crosslinking in this experiment was microscopically assessed by evaluating CAF after the procedure. In under 30 minutes NLO CXL using an enlarged focal volume was able to produce an enhanced region of CAF, comparable in both depth and intensity to CAF produced using UVA CXL. Figure 1 shows a representative CAF image, displayed in green for better visual contrast, from each treatment protocol. The control sample (Figure 1A), treated with imbibition of Rf without crosslinking, showed no enhanced CAF, while both UVA CXL samples (Figure 1B) and NLO CXL samples (Figure 1C) showed enhanced CAF within the expected region of treatment. Furthermore it was found that the resultant CAF intensity was linearly related to decreasing laser scanning speed and approached the CAF of UVA CXL treated eyes at a speed of 8.9 mm/s, allowing for the treatment of a 3 mm diameter area in under 4 minutes. Compressed collagen hydrogels treated with NLO CXL or UVA CXL also resulted in the loss of lower molecular weight α1/α2 chains consistent with molecular crosslinking of collagen. This rapidly achieved crosslinking was used as further proof of concept and justification for the development of a custom NLO CXL delivery device.

Further ex vivo studies were performed with the goal of developing a custom delivery device and testing the mechanical stiffening effect of NLO CXL in rabbit corneas. (Bradford et al., 2017) As shown in Figure 2A, the 760 nm FS laser was directed first into a variable beam expander, allowing for control of the NA of the device. The expanded beam was then directed onto software controlled x, y scanning mirrors (GSI Lumonics, Bedford, MA) which control the placement of the focal volume within the cornea during treatment. After the mirrors, the beam is then sent through a second fixed expander to widen it again before it is directed onto the back aperture of the focusing objective. A software-controlled motor moves the objective to change the depth of the focal plane and a removable cone with contact glass serves to applanate the cornea and set a zero plane. The resultant device was capable of delivering and scanning a FS focal volume of continuously variable NA between approximately 0.1 and 0.2. The measured axial length of the focal volume ranged from 28.6 to 79.5 μm in a water tank containing Rf and could be placed at any depth up to 500 μm below the contact glass (Figure 2B). Treatment of ex vivo rabbit corneas using this system resulted in a 2.6 fold increase in mechanical stiffness using indentation testing and more than a 5 fold increase in CAF intensity within the treated region, Figure 3. While these results were comparable to UVA CXL controls, 900 mW of laser power was required to achieve CXL, well above the 46.1 mW retinal thermal limit set by the American National Standards Institute (ANSI).(Delori et al., 2007)

## 4. Amplified Pulse NLO CXL

While using a FS laser oscillator was effective in producing crosslinking comparable to UVA CXL in the lab, NLO CXL as described above has clinically impractical power requirements. To address this limitation, it was hypothesized that the power requirement could be reduced if the pulse energy was amplified. As amplified pulses are not commonly used for nondestructive therapies, it needed to be determined if crosslinking was even possible using such pulses. As proof of concept 5 kHz, amplified pulses at 1 μJ per pulse and 15 mm/s scanning speed were tested. CAF could be detected with zero pulse overlap and just 5 mW of total power, well below the nearly 1 W used in previous experiments, as seen in Figure 4.

Because amplified pulse lasers are not commercially available at the required wavelength of 760 nm, a new system was developed which converted 1030 nm, 50–150 kHz laser to 760 nm. Both ex vivo and in vivo rabbit eyes were then treated with the new system using 0.3 μJ pulse energy and 30 mW total energy. (Bradford et al., 2019) Using ex vivo eyes, it was determined that CAF intensity scaled downward logarithmically with increasing scanning speed, producing a comparable intensity to UVA CXL at 15.5 mm/s. A significant increase in corneal stiffness was also measured in ex vivo eyes. An in vivo study using amplified pulses also revealed significant corneal flattening in the treated eye of 1.2 diopters after two months, comparable to UVA CXL, Figure 5.(De Bernardo et al., 2015; Elling et al., 2017; Hersh et al., 2017; Kanellopoulos and Asimellis, 2015; Malik et al., 2017; Raiskup-Wolf et al., 2008; Shalchi et al., 2015; Vinciguerra et al., 2009)

## 5. Transepithelial NLO CXL

Traditional corneal crosslinking requires removal of the corneal epithelium to facilitate penetration of Rf into the corneal stroma. Removal of the corneal epithelium is painful, leads to delayed visual recovery and increases the risk of corneal infection. (Koller et al., 2009; Shalchi et al., 2015) Because of this, transepithelial UVA CXL is a highly active field of research. While numerous methods have been tested in an attempt to get enough Rf to penetrate an intact epithelium to perform UVA CXL, no current attempt at transepithelial UVA CXL has been as successful as the traditional method. (Bottos et al., 2011; Chow et al., 2018; Kobashi et al., 2018; Rush and Rush, 2017; Shalchi et al., 2015) Furthermore, using excipients to break down the corneal epithelial barrier such as BAK or ethylenediaminetetraacetic acid may have confounding toxic effects leading to additional pain and delayed recovery. (Saarinen-Savolainen et al., 1998; Vitoux et al., 2020) Finally, even if a sufficient stromal Rf concentration could be achieved, the subsequent UVA irradiation would likely also damage the epithelium or Rf soaked epithelium may even block the UVA irradiation, reducing the stromal crosslinking effect. In contrast, NLO CXL has the ability to bypass the epithelium entirely.

To address this problem, we assessed the ability of FS laser-induced optical breakdown, normally used to cut the corneal stroma, to form microchannels through the intact corneal epithelial layer to enhance Rf penetration without using excipients. To test this hypothesis, a study was conducted in ex vivo rabbit eyes in which the efficiency of Rf penetration across an FS laser micromachined epithelial channels was compared to the more common method of using BAK.

Prior to soaking the stroma of ex vivo rabbit eyes with Rf solution we used the same amplified NLO CXL system with 5–10 μJ pulses to produce 3 μm wide channels within the surface epithelium at a density of 100–400 channels per $mm^2$, as shown in Figure 6. Using 5 μJ pulses at the highest channel density, and a 1% Rf solution, we measured a stromal Rf concentration equivalent to eyes treated with epithelial debridement and 0.5% Rf solution (used for standard NLO CXL). Eyes treated with 0.01% BAK, as opposed to michrochannels, only achieved 25–35% of the level achieved when the epithelium was removed. Exposure times of Rf solutions remained constant at 30 minutes in all groups. Furthermore, tissue culture of eyes treated with BAK and subsequent NLO CXL showed significantly more cellular damage to the epithelium and anterior stroma compared to eyes treated with epithelial microchannels and NLO CXL, Figure 7A. In the case of epithelial microchannels paired with NLO CXL, cellular damage is restricted to the region of crosslinking, Figure 7B. This indicates that NLO CXL, unlike UVA CXL, has the ability to effectively crosslink corneal collagen below an intact epithelium without causing further damage that may lead to patient pain and discomfort and delayed visual recovery.

## 6. Summary

This review reviews our experience with developing a novel approach to corneal crosslinking using precisely guided, FS laser NLO photoactivation of Rf. Through five main studies the technique has evolved from the detection of mechanical stiffening within

collagen hydrogels to the generation of crosslinking using a single amplified pulse, and finally to the transepithelial penetration of Rf for a minimally destructive crosslinking therapy.(Bradford et al., 2019; Bradford et al., 2016; Bradford et al., 2017; Chai et al., 2013) NLO CXL using amplified FS pulses has thus far produced comparable results to standard UVA CXL in every manner tested. When transepithelial crosslinking is considered, however, it is likely to have an advantage since the focal volume can be positioned precisely at any depth underneath the epithelium, leading to minimal or no damage to the corneal epithelium that could lead to later pain and discomfort or delayed visual recovery. This advantage, combined with the high level of precision inherent to NLO CXL makes for a technique that has unprecedented potential to provide a safe, non-invasive, and highly customizable procedure for modifying corneal shape and, hence, refractive power to treat patients with low refractive errors including myopia, hyperopia, astigmatism and presbyopia.

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**Research Highlights:**

- NLO CXL produces a detectable increase in mechanical stiffness in collagen hydrogels.
- Rapid NLO CXL using an enlarged focal volume produced both an increase in mechanical stiffness and intensity of CAF in ex vivo rabbit eyes.
- A custom built NLO CXL device was able to deliver an adjustable focal volume and produce crosslinking comparable to UVA CXL.
- Amplified pulses are capable of producing NLO CXL with zero pulse overlap while staying within the ANSI power limits.
- Microchannels cut into the surface epithelium enable higher riboflavin penetration for transepithelial crosslinking while causing less epithelial damage than BAK.

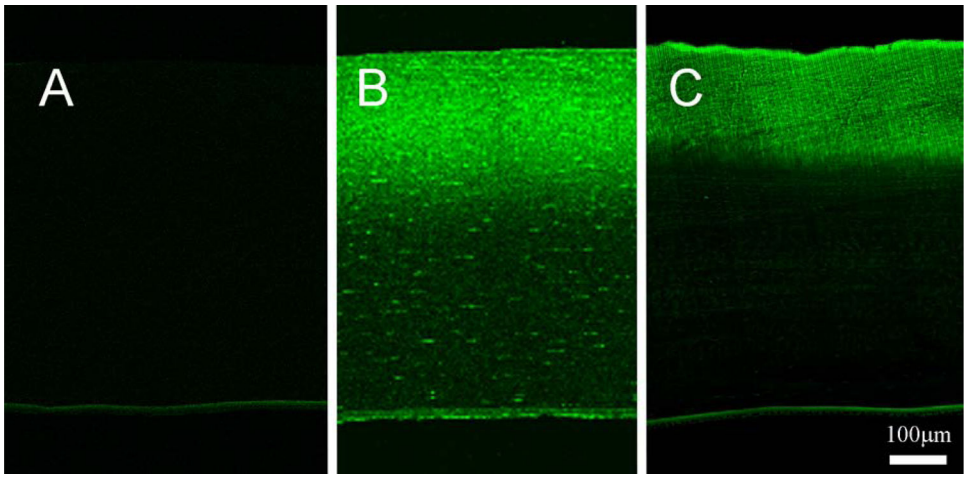


**Figure 1: CAF Images.**

(A) shows a representative CAF image of a control cornea with no crosslinking, (B) shows a cornea treated with UVA CXL, and (C) shows a cornea treated with NLO CXL using a Zeiss LSM 510 confocal microscope. CAF was detected in the 400–450 nm range, but images are shown in green to enhance contrast.

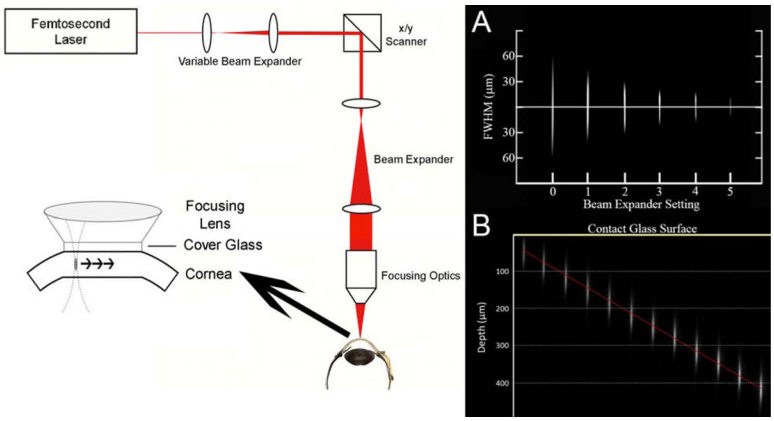


**Figure 2: Delivery Device.**

Schematic of designed delivery device with software controlled x, y scanners, variable beam expander, objective, and cone with contact glass. (A) Images taken of the two-photon focal volume within a tank of Rf solution as the focus is (A) changed in NA or (B) changed in depth beneath the contact glass.

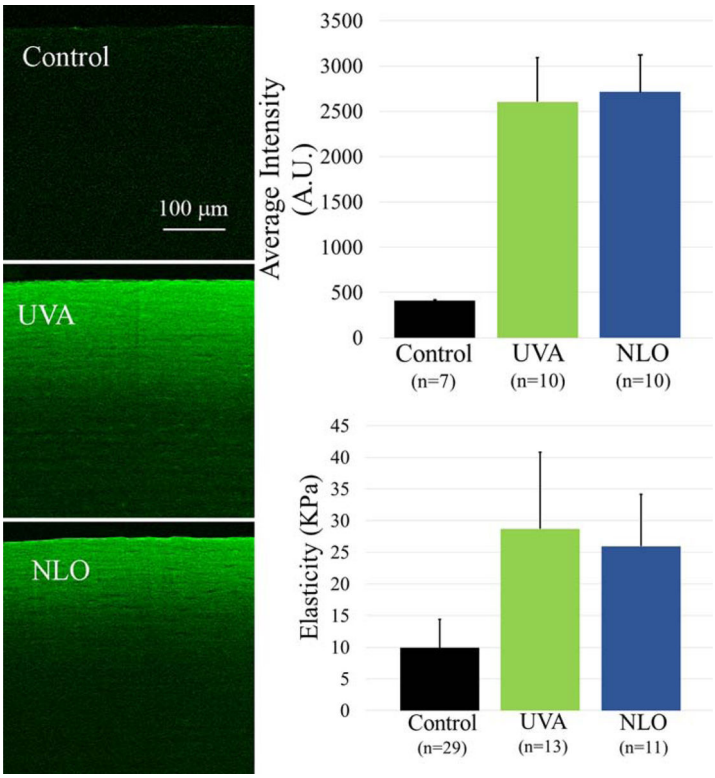


**Figure 3: CAF Intensity and Mechanical Stiffening.**

To the left are representative CAF images of control, UVA CXL, and NLO CXL treated corneas respectively. To the right are graphs showing the significanly increased CAF intensity and elasticity of corneas treated with either crosslinking method which are not significanly different from each other. CAF was detected in the 400–450 nm range, but images are shown in green to enhance contrast.

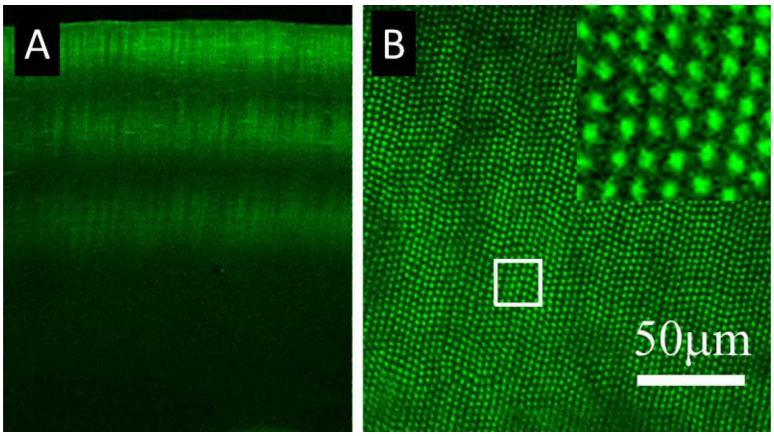


**Figure 4: En Face CAF after a Single Amplified Pulse.**

(A) A cross-sectional CAF image of a cornea treated with amplified FS pulses. (B) An enface CAF image of a cornea treated with single pulse NLO CXL as proof of concept for amplified pulse crosslinking. No pulses overlapped, and the non-crosslinked space between pulses is clearly visible as a drop in CAF intensity. CAF was detected in the 400–450 nm range, but images are shown in green to enhance contrast.

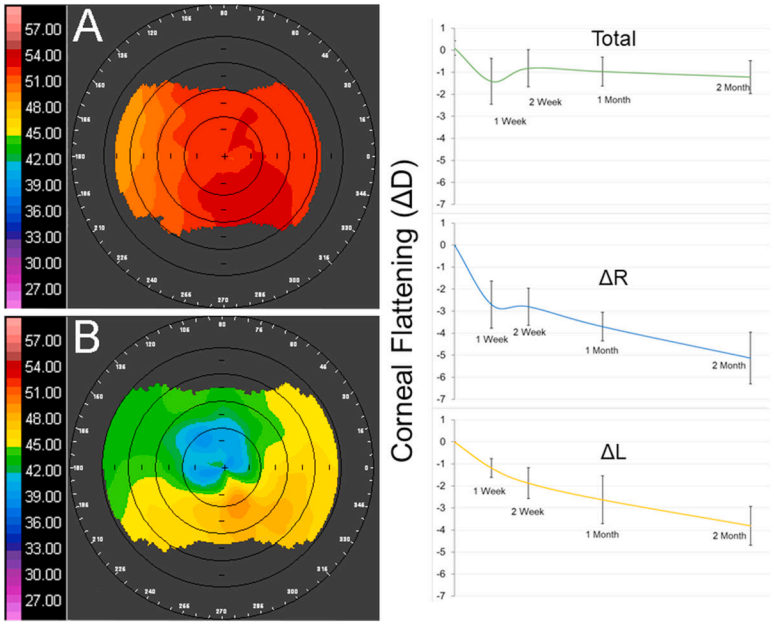


**Figure 5: In Vivo Topography.**

The corneal flattening of an in vivo rabbit cornea between baseline measurements (A) and two months post NLO CXL treatment (B) is localized to the region of treatment, a 4 mm circular region within the central cornea. The graphs indicate the total corneal flattening (ΔD), flattening within the treated right eye (ΔR), or flattening within the control left eye (ΔL).

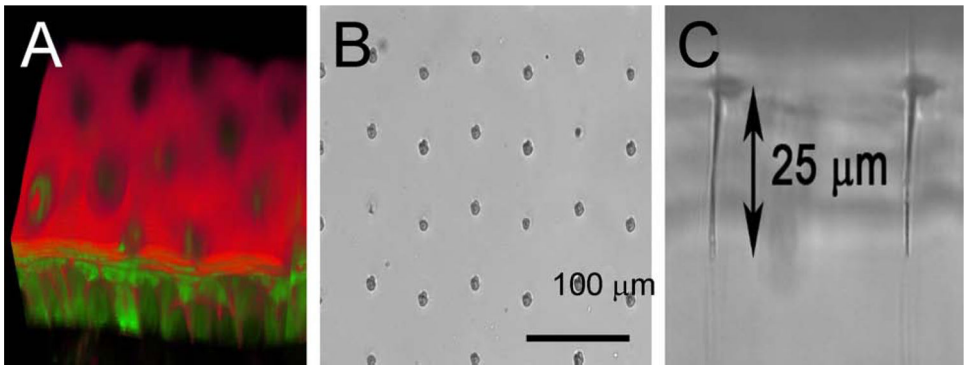


**Figure 6: Channel Spacing in Silicone and Corneal Epithelium.**

(A) A 3D reconstruction of a vibratome section of cornea from a microchannel treated corneal epithelium stained with Phalloidin and Propidium Iodide. Reconstruction based on a 3D stack of images taken with the laser scanning confocal microscope. (B and C) A grid of microchannels spaced 50 μm apart and 25 μm deep is shown is shown cut into a silicone sheet for demonstration.

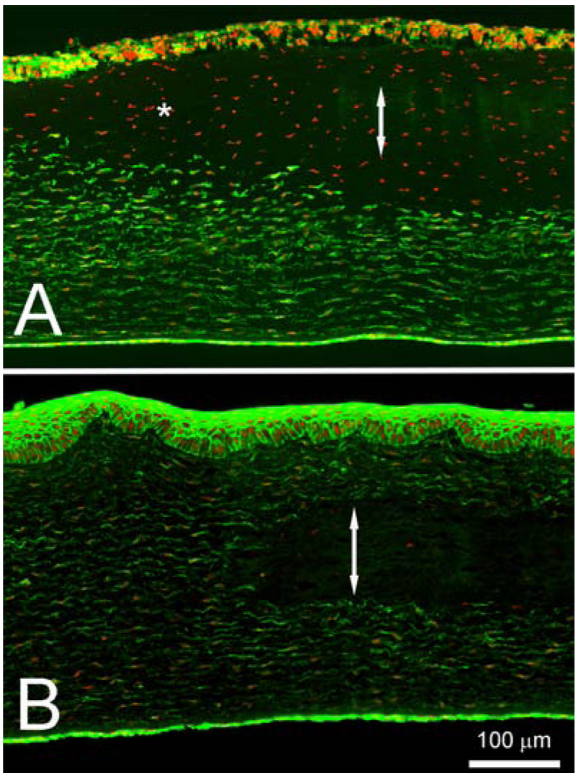


**Figure 7: Cellular Staining After Riboflavin Administration.**

(A) shows a representative image of Phalloidin and Propidium Iodide stained cornea treated with transepithelial BAK riboflavin administration followed by NLO CXL and 24 hour culture. The cornea in (B) however was treated with microchannel riboflavin administration and NLO CXL. Double headed arrows represent the region of crosslinking treatment while the asterisk represents cellular damage outside of treatment region.