Combined Nonlinear and Femtosecond Confocal Laser-Scanning Microscopy of Rabbit Corneas after Photochemical Cross-Linking

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PURPOSE. Photochemical cross-linking of corneal stromal collagen using riboflavin and ultraviolet irradiation is an evolving treatment for keratoconus. The purpose of the present study was to investigate the wound-healing process in rabbit corneas after cross-linking.

METHODS. Photochemical cross-linking was performed according to a standard protocol on the right eyes of eight male New Zealand White rabbits; the left eyes served as controls. Untreated controls and cross-linked rabbit corneas were imaged 3 days, 6 days, and 6 weeks after treatment using a customized setup for three-dimensional nonlinear microscopy and confocal laser-scanning microscopy of reflected femtosecond light (fs-CLSM).

RESULTS. The combination of fs-CLSM in reflective mode and two-photon–excited fluorescence permitted differentiation of the following zones in the lamina propria of treated corneas 3 and 6 days after cross-linking: (1) an anterior zone with postapoptotic keratocyte debris, visible only on fs-CLSM in reflective mode; (2) a posterior zone with activated kerocytes with strong autofluorescence; and (3) surviving or restored keratocytes with moderate autofluorescence beyond the intermediate zone. Repopulation with normal keratocytes was achieved by 6 weeks. Bi-directional, second-harmonic generation (SHG) imaging showed no global differences in the fiber orientation and lamellar structure of stromal collagen at any time point. A relatively strong additional two-photon excited fluorescence occurred in the treated corneas with a diffuse three-dimensional spatial distribution.

CONCLUSIONS. This combination of imaging modalities has the potential to become a new clinical instrument capable of visualizing corneal changes at the cellular and extracellular level. (Invest Ophthalmol Vis Sci. 2011;52:4247–4255) DOI: 10.1167/iovs.10-7112

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Keratoconus is a noninflammatory disorder of the cornea characterized by progressive conical protrusion and central stromal thinning. In the past, contact lens intolerance and other complications meant that keratoplasty was the only realistic surgical option.1

More recently, corneal cross-linking, a stromal stiffening procedure that aims to halt the progression of keratoconus, has been reported to be a less invasive alternative to keratoplasty.2 Apart from mechanical stabilization, cross-linking also reduces the enzymatic digestion of corneal collagen.3 Collagen I microfibrils are the major constituents of corneal stroma and their partially interwoven lamellar arrangement is responsible for the mechanical stability and convex shape of the cornea.4 Within the collagen lamellae, keratocytes form a three-dimensional (3D) cellular network that can be visualized in 3D by in vivo confocal microscopy (slit scanning and laser scanning).5,6 Photochemical cross-linking treatment of keratoconus by application of riboflavin (vitamin B2) and UVA light (370 nm) introduces additional bonds, leading to mechanical stiffening of the cornea.6–9 Like every relatively new treatment approach, corneal collagen cross-linking is a method with potential complications since many aspects have still to be elucidated. Moreover, the cross-linking procedure itself is continually being modified.10–12 Consequently, high levels of interest surround the meticulous investigation of wound healing after collagen cross-linking.

Dose-dependent apoptotic cell death after cross-linking has been demonstrated in animal and human studies.13–15 In many instances, keratocyte apoptosis has been shown to be a natural and nondestructive part of wound healing.16–18 Complete cell replacement by activated keratocytes after collagen cross-linking has been demonstrated in numerous experimental and clinical studies.18–22 The wound-healing process after collagen cross-linking has been investigated mostly at the cellular level, but the events occurring in collagen matrix also require accurate examination.

One clinically established method of corneal imaging is in vivo confocal laser-scanning microscopy (CLSM) in reflective mode, with continuous-wave diode laser light. Without the use of exogenous dyes, cellular structures are rendered visible through their light-scattering properties. Cell membranes, nucleus, and nerve fibers scatter laser light, which is detected confocally to yield depth-resolved information. The normal anatomy of the cornea as well as various corneal abnormalities, disorders, and diseases have been characterized using CLSM in reflective mode and slit lamp confocal microscopy.5,6,23–26

Owing to corneal transparency, the collagen matrix is scarcely visible with CLSM in reflective mode. It is at this point that nonlinear microscopy comes into play. Whereas two-photon–excited fluorescence (TPEF) microscopy, as originally devised by Denk et al.27 can probe the autofluorescence of cells, it has been shown that the collagen in the corneal stroma...
can be visualized with second-harmonic generation (SHG) imaging. In SHG microscopy the noncentrosymmetric, hyperpolarizable bonds in the highly abundant stromal collagen I molecules give rise to nonlinear frequency doubling.29,30

Cell visibility is accomplished through TPEF microscopy of reduced nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine disphosphonucleotide (NADPH), together abbreviated as NAD(P)H. As an advantage over conventional fluorescence microscopy, TPEF shows no photo-bleaching of NAD(P)H in the out-of-focus planes.31 The epithelial cell layer of the cornea can be visualized clearly with this autofluorescence.32–35 The level of NAD(P)H autofluorescence in the stromal keratocytes depends on their metabolic activity.31 Several corneal disorders, postoperative changes, and diseases, including keratoconus, have been studied with nonlinear microscopy.33–35

Because the cross-linking process affects both cells and collagen matrix, a combination of the linear back-scattering contrast in reflective fs-CLSM and the nonlinear contrasts of SHG and TPEF was felt to be advantageous for the present study. Such a setup, which combines back-scattering and two-photon contrast for corneal imaging, has been presented previously by various groups, showing the complementary image information yielded by a multimodal approach.36–38 Very recently, a study has also described the integration of nonlinear microscopy into the CLSM (HRT, Heidelberg Retina Tomograph; Heidelberg Engineering, Heidelberg, Germany) for in vivo imaging.39

Materials and Methods

Animals

The rabbits were handled in full compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the animal experimentation committees of the University of Rostock and of Hannover Medical School.

Eight albino New Zealand White rabbits (Crl:KBL; NZW), Charles River Laboratories, Sulzfeld, Germany) weighing approximately 1.5 to 1.7 kg underwent photochemical cross-linking. Three rabbits were used for the day 3 group, three for the day 6 group, and two for the week 6 group.

Cross-Linking Procedure

Photochemical cross-linking was performed on the right eye of each rabbit, as described elsewhere.53,54 The left eye in each case served as the control.

Briefly, the animals were anesthetized with xylazine hydrochloride (5 mg/kg intramuscularly; Rompun, Bayer, Leverkusen, Germany) and ketamine hydrochloride (50 mg/kg intramuscularly; Ketavet, Pharma- cia, Erlangen, Germany). A central area of corneal epithelium (diameter 6.5 mm) was removed, and premoistening with 0.1% riboflavin solution in 20% dextran (Peschke GmbH, Nürnberg, Germany) was performed 5 minutes before cross-linking. Irradiation with UVA light (wavelength: 370 nm; UV-X irradiation system: Peschke GmbH, Nürnberg, Germany) was then combined with riboflavin drops every 5 minutes for 30 minutes. After treatment, bandage contact lenses were fitted on the cross-linked eyes. Anti-inflammatory eye drops (Floxal containing 3 mg ofoxacin/ml. Floxal solution; Mann Pharma, Berlin, Germany) were applied to the cross-linked eyes five times daily until termination of the experiment for the day 3 and day 6 groups and for 1 week for the week 6 group. The left eyes remained untreated and served as intact controls.

The rabbits were killed with an overdose of sodium pentobarbital (Sigma-Aldrich Chemie GmbH, Munich, Germany). The enucleated globes were imaged with fs-CLSM in reflective mode, backward SHG and TPEF. Trephined corneal buttons from one animal in each group were used to perform forward SHG imaging in addition to backward SHG, TPEF, and fs-CLSM in reflective mode. The globes and corneal buttons were hydrated with buffered saline solution during microscopy.

Multimode Microscopy

A retinal tomograph (HRT; in conjunction with the Rostock Cornea Module; Heidelberg Engineering GmbH; Fig. 1A) was used to acquire in vivo reference images of the corneas for comparison with femtosecond laser scattering contrast. A detailed description of this commercial instrument can be found elsewhere.25

The customized two-photon microscope (Fig. 1B) uses a femtosecond laser (Chameleon-XR; Coherent Inc., Santa Clara, CA) and a scanning system (modified Tissue Surgeon; Rowia GmbH, Hannover, Germany), which is coupled to the laser port of an inverted microscope (Axio Observer; Carl Zeiss, Jena, Germany) with a long-distance water-immersion objective (LD C-Apochromat 40×/NA 1.1; Carl Zeiss Meditec, Jena, Germany). The laser was tuned to a central wavelength of 800 nm for SHG and to 750 nm for the two-photon excitation of NAD(P)H. The fluorescent blue light and the backward SHG at 400 nm were separated from laser light through a dichroic mirror (HC 670/SP; Semrock, Rochester, NY) and a laser blocker (HC 680/SP; Semrock) and color filtered with a blue bandpass filter (BrightLine HC 590/40; Semrock). Finally, the filtered light was collected onto a photomultiplier tube (Fig. 1B; PMT 1; R6537; Hamamatsu Photonics, Herrsching, Germany). Forward SHG light was collected by a plano convex lens.

FIGURE 1. Microscopy setups. (A) Inset: optical setup of the commercial confocal laser scanning microscope in reflective mode for in vivo corneal imaging consisting of a diode laser; pmt: photomultiplier; bs: beam splitter. (B) Customized multiphoton microscope: fs laser; femtosecond oscillator; hwp, half-wave plate; pbs, polarizing beam splitter cube; bs, nonpolarizing beamsplitter; sm, galvanometer-mounted scanning mirrors; ii, 12, scanning telescope; dm: dichroic mirror (HR>680 nm, HT<670 nm); obj, 40× objective with numerical aperture of 1.1; gbd, glass bottom dish; 13–17 collecting and focusing lenses for photomultipliers; pmt 1–3, photomultiplier tubes for backward SHG and TPEF, forward SHG and reflective confocal detection, respectively; lp1, lp2, short pass filters λ>670 nm; bp1, broad bandpass filter (417 ± 50 nm) for backward SHG and NAD(P)H autofluorescence; bp3, narrow bandpass filters for second-harmonic detection at a wavelength of 400 ± 10 nm; bp2, bandpass for green autofluorescence emission; fw, motorized filter wheel.
FIGURE 2. (A) Large-scale mosaic built with reflective mode confocal images using the femtosecond laser at 800 nm showing the stromal keratocyte network. White frame: position of image (B). (B) In close-up the typical keratocyte shape is visible. Note the qualitative agreement with a representative reflective CLSM image acquired at \( \lambda = 670 \) nm (continuous-wave diode laser) of stromal keratocytes in (C).

Epithelial Layer

Figure 3 demonstrates merged images of fs-CLSM in reflective mode and TPEF. The three images (Figs. 3A–C) were acquired at increasing depth within the epithelium of a cross-linked rabbit cornea 6 days after treatment. The natural color of the NAD(P)H autofluorescence is in the blue spectral range, but is pseudo colored green here. The cell borders and cell nuclei remain dark in the fluorescence. Epithelial imaging by fs-CLSM in reflective mode showed back-scattering from cell membranes, which is pseudo colored red here. The layered structure of wing cells (Figs. 3A, 3B) and basal cells (Fig. 3C) was almost fully restored by day 6 after cross-linking.

Differences in Stromal Backward SHG between the Central and Peripheral Regions

Mapping of the cornea with a mosaic of adjacent frames covering one quadrant of the cornea helped to locate the central region of the cornea (Fig. 4A) before commencing stack acquisition. The peripheral region displayed a tangentially oriented, parallel pattern (Fig. 4B), whereas the central cornea had a more irregular pattern with fluctuating orientation (Fig. 4C).

Cross-Linking Signatures in SHG Signals

In the control corneas, the layered structure of the collagen lamellae resulted in a layered intensity pattern of the forward SHG (Fig. 5A) and backward SHG (Fig. 5F) cross-sectional views. Six weeks after treatment the cross-sectional views showed an evident cross-linking signature. In the \( x-z \) plane of the backward signal (Fig. 6H), in particular, two distinct zones could be differentiated. The first zone above 200 \( \mu m \) displayed a more intensive and more densely textured structure by comparison with the control (Fig. 5F). Below 200 \( \mu m \) a normal backward SHG pattern was observed (Fig. 6H).

The forward SHG images of the untreated stroma (Figs. 5B, 5C) and their spatial frequency distribution in the Fourier-
transformed images (Figs. 5D, 5E) revealed that the length of the parallel streaks in the forward SHG images increases with depth from 20 μm in the anterior stroma to more than 100 μm in the posterior stroma. The orientation of the streaks changes in steps of 60° or 90°, leading to spatial frequencies that are grouped in narrow intersecting bundles of lines in the Fourier-transformed images. In the anterior stroma, several domains of differently oriented streaks are present, whereas in the posterior stroma, one or two major axes of orientation can be seen in a single image. These findings are in accordance with the anatomic structure of the stromal collagen matrix, with short, interwoven microfibrils in the anterior stroma and long, stacked lamellar microfibrils in the posterior stroma. In the cross-linked stroma (Fig. 6C) the parallel streaks of the forward SHG image appeared straighter than the rippled streaks (Fig. 5E) in the untreated cornea. A possible explanation for this difference is the release of intraocular pressure, resulting in rippling of the relaxed fibrillar lamellae. In contrast, the cross-linked fibrils remained photochemically fixed in their straight arrangement. In the cross-linked corneas, there was an additional signal in the backward SHG image, starting from the anterior and persisting down to an intermediate zone in the middle stroma. The contrast in the typical patterns of backward SHG is covered by a brighter signal in the anterior stroma. This strong additional signal was observed at all three study time points (3 days, 6 days, and 6 weeks) after cross-linking. A similar signal was also noted at an excitation wavelength of 750 nm (Fig. 7B), using an emission filter that blocks the second harmonic of 750 at 375 nm. SHG can therefore be excluded as the source of the signal at 750 nm excitation. A new broadband fluorescence introduced by the cross-linking process is potentially the most likely explanation for both additional signals.

Differentiating between Living Keratocytes and Cell Debris

In a control cornea, a keratocyte network was visualized on fs-CLSM in reflective mode (Fig. 7F) and characterized as metabolically active by colocalized autofluorescence of NAD(P)H (Fig. 7G). SHG imaging demonstrated fibrillar organization on forward SHG imaging (Fig. 7I) and a typical backward SHG pattern (Fig. 7H).

Six days after treatment, the cross-linked cornea revealed differences with all four imaging modalities (Figs. 7A–D), when compared with the control cornea in Figures 7F to 7I. Only a diffuse, indistinct scattering signal was obtained with fs-CLSM
in reflective mode (Fig. 7A). No cell-associated autofluorescence signal was detectable in Figure 7B, where an unstructured autofluorescence background covered the image. The backward SHG image of cross-linked stroma (Fig. 7C) showed only negative (dark) footprints of cell debris within a homogenous signal. The forward SHG image (Fig. 7D) exhibited straight streaks with poor contrast. The merged image (Fig. 7E) displayed a noisy, color-mixed background with cell debris as the most structured feature.

**Repopulation of Stromal Keratocytes**

Figure 8 illustrates maximum intensity projections along the y-axis of the TPEF signal in the imaging volume. The row of images shows the cross-linked corneas (Figs. 8A-C) at 3 days, 6 days, and 6 weeks, respectively, after treatment, alongside a control cornea (Fig. 8D) that displayed spots of autofluorescence throughout its entire stromal thickness. In contrast, Figure 8A shows the cross-linked cornea 3 days after treatment, with bright unstructured autofluorescence in the anterior stroma. Only at depths below 200 to 250 μm did spots of autofluorescence reappear, indicating metabolically active cells. After 6 days (Fig. 8B) the stroma was unpopulated down to an imaging depth of 250 μm; in the posterior stroma, however, a weak autofluorescence signal from sparsely present keratocytes can be identified. After 6 weeks (Fig. 8C) keratocyte repopulation occurred throughout the stroma, but cell density was still less than that in the control cornea (Fig. 8D).

**Postapoptotic, Activated, and Normal Keratocytes**

Figures 9A–C present the single detection channels (fs-CLSM in reflective mode, TPEF, backward SHG) of a distinct layer of the anterior stromal zone 3 days after cross-linking, revealing postapoptotic keratocyte debris in the fs-CLSM reflective contrast (Fig. 9A), absence of keratocyte autofluorescence but only strong diffuse fluorescence (Fig. 9B), and an unstructured backward SHG signal (Fig. 9C).

In an intermediate stromal zone (Figs. 9E–G), elongated autofluorescent structures (Fig. 9F) with colocalized scattering (Fig. 9E) were detected 3 days after cross-linking, most probably representing activated keratocytes. The backward SHG signal (Fig. 9G) carries the negative footprints of the activated keratocytes contrasted on a homogenous background.

**FIGURE 6.** Forward SHG (A–D) and backward SHG (H–K) images of a cross-linked cornea 6 weeks after treatment. By contrast with the control cornea (Figs. 5A–C), the forward SHG patterns show much straighter collagen fiber signals in (C) down as far as an intermediate zone at ~220 μm, returning to a wavy pattern at 250 μm (D), which is similar to the control (Fig. 5C). The straight fibers in (C) correspond to the Fourier transformed image (F) with less spreading of fiber orientations. The backward signal (I) is much less structured above the intermediate zone, and its intensity is much higher than in the control (Fig. 5G). Below the intermediate zone, the image patterns of forward and backward SHG in (D), (G) and (K) become similar to those in Figure 5C, 5E, and 5H for the control cornea.

**FIGURE 7.** Multimodal microscopic comparison of a cross-linked (A–E) versus a control (F–J) cornea. The fs-CLSM images in (A) versus (F) are red in (E) versus (J), the autofluorescence images in (B) versus (G) are green in (E) versus (J), the backward SHG images in (C) versus (H) are blue in (E) versus (J), and the forward SHG images in (D) versus (I) are gray in (E) versus (J). The most evident difference is the loss of structure in the cross-linked cornea, as seen in the backward SHG images (C) versus (H) and the autofluorescence images (B) versus (G).
In the images of the most posterior zone (Figs. 9I–L) the stroma was not affected by cross-linking: Keratocytes of normal shape were seen on fs-CLSM in reflective mode (Fig. 9I), colocalized and partly surrounded by NAD(P)H autofluorescence (Fig. 9J).

Three-Dimensional Visualization

The 3D visualizations presented in Figure 10 summarize the above observations. The control cornea (Fig. 10A) shows an epithelium with strong NAD(P)H autofluorescence and a patchy, yet dense population of metabolically active keratocytes. The cross-linked cornea also has a closed epithelium 3 days after treatment, but the stroma shows a clear signature of treatment. The additional fluorescence in the backward SHG and fluorescence signals in the cross-linked cornea (mixed to a cyanlike color in Fig. 10B) appear to indicate the penetration of treatment. The reflective signal is more prominent after cross-linking, the patchy NAD(P)H signal indicating the autofluorescence of cytoplasm is absent in the first 200 μm of the stroma, and only isolated areas of NAD(P)H autofluorescence excited at λ = 750 nm, present at the early time points, decreased after 6 weeks.
D(P)H autofluorescence are visible in the deeper stroma (Fig. 10B, white arrows).

**DISCUSSION**

Several advantages of entirely fs-laser-based linear and nonlinear microscopy in a single setup were demonstrated in the present study. Scattering contrast was linked to the nonlinear contrast of TPEF and SHG imaging for pixel-wise comparison. Colocalization of NAD(P)H in the cytoplasm and the scattering contrast of cells were found to be complementary in the epithelial layers, as has been reported by fluorescence and scattering contrast of cells were found to be physiological state represented by NAD(P)H-related TPEF. The information generated by fs-CLSM in reflective mode with the fs-CLSM; green, TPEF of NAD(P)H; blue, backward SHG. (A) Untreated control cornea. (B) Treated cornea 3 days after cross-linking. Dimensions (x, y, z) of the volumes are 125, 125, and 300 μm. Note the decreasing intensity of additional fluorescence with increasing depth in the treated cornea. Below the fluorescent zone there are keratocytes showing NAD(P)H fluorescence (white arrows). For individual slices of (B), refer to Figure 9.

FIGURE 10. Ray-cast, multicolor, 3D image stacks of corneas in the globe. Color coding: red, fs-CLSM; green, TPEF of NAD(P)H; blue, backward SHG. (A) Untreated control cornea. (B) Treated cornea 3 days after cross-linking. Dimensions (x, y, z) of the volumes are 125, 125, and 300 μm.

D(P)H autofluorescence are visible in the deeper stroma (Fig. 10B, white arrows).

The differences in forward versus backward SHG have been addressed in detail by Williams et al., both theoretically and experimentally. In the case of corneal stroma, however, a strong forward SHG signal is observed and it is only this forward image that reproduces fibril orientation. The dependence of backward SHG on the lamellar arrangement of corneal collagen has been demonstrated experimentally by observing that extracted corneal collagen microfibrils in aqueous solution lead to a much stronger backward signal. Native corneal backward SHG is much weaker and does not reproduce fibril orientation. As a consequence, fibril length and domains of equal orientation cannot be deduced from backward SHG images of the native cornea. As soon as the corneal...
After cross-linking, an increase in collagen fibril diameter and spacing of the order of several nanometers has been measured by electron microscopy.48 This change is well below the resolution limit of SHG imaging, and so this thickening cannot be observed. The change in forward SHG of cross-linked corneas toward less wavy and straighter streaks is an indicator of cross-linking in the excised corneal button, but it is not clear whether forward SHG of the control cornea might not show the same straightforward if it could be detected in vivo under physiological pressure. Subtraction of the autofluorescence background in the group 6 weeks after treatment suggests that backward SHG is not influenced in a characteristic way by cross-linking. We conclude that the fluorescence observed after cross-linking, which caused spectral interference with the SHG images, does not reflect major structural changes to the fibrillar arrangement but might be an indicator of photochemically induced changes at a molecular level. On the other hand, compared with any changes in the SHG pattern, this fluorescence is more strongly related to the cross-linked zone, and it can be detected more easily. It may therefore be possible to use this signal to monitor treatment outcomes.

Slit lamp images of treated patients’ eyes have revealed a demarcation line between the cross-linked anterior stroma and untreated posterior stroma.49 A similar demarcation line could be drawn in the cross-sectional resin and in our images, separating the zone of homogenous fluorescence from the more structured zone below. The origin of this stromal autofluorescence in the cross-linked cornea could not be clarified. The spatial distribution of the fluorescence strongly suggests that it matches the volume that is infiltrated with riboflavin and irradiated with UVA light. Very recently, evidence has emerged from fluorescence lifetime imaging to support the hypothesis that the signal may arise from enhanced collagen autofluorescence originating from additional cross-links in the treated corneas.50 Our findings in the corneas 6 weeks after cross-linking reveal less pronounced autofluorescence than at the earlier time points. This result suggests that the fluorescence is a transient change, and therefore it cannot be regarded as a persistent indicator of cross-linking. We conclude that further investigations are needed to clarify whether this fluorescence is a reliable measure of the degree and quality of cross-linking.

Concerning the cellular processes of wound healing after cross-linking, keratocyte demise and repopulation were observed in the present study, supporting findings previously made with a variety of methods.19 One slight difference compared with earlier studies using the same cross-linking protocol is the presence of living keratocytes as early as day 3 after treatment, possibly due to the younger age of the rabbits (at day 3 and day 6) used in our study. In particular, our multimodal approach permitted in situ differentiation of postapoptotic stellate structures visible in CLSM or scanning slit microscopy images from living keratocytes. Of greatest interest here is the observed presence of metabolically active elongated cells at the frontier of repopulation. Whereas previously the classification of these elongated structures in the CLSM image as activated keratocytes was in some doubt, this conclusion is now strengthened by the colocalized, enhanced NAD(P)H signal.

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