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Letter

Highly effective 525 nm femtosecond laser crosslinking of collagen and strengthening of a human donor cornea

B S Shavkuta[1], M Y Gerasimov[2], N V Minaev[1,7], D S Kuznetsova[3], V V Dudenkova[3], I A Mushkova[2], B E Malyugin[2], S L Kotova[4,5], P S Timashev[1,5], S V Kostenev[2], B N Chichkov[1,6] and V N Bagratashvili[1]

[1] Institute of Photonic Technologies, Research Center "Crystallography and Photonics" RAS, 108840, Moscow, Troitsk, Russia
[2] S. Fyodorov Eye Microsurgery State Institution, Moscow, 127486, Russia
[3] Institute of Biomedical Technologies, Nizhny Novgorod State Medical Academy, Nizhny Novgorod, 603005, Russia
[4] N.N. Semenov Institute of Chemical Physics RAS, Moscow, 119991, Moscow, Russia
[5] Institute for Regenerative Medicine, Sechenov First Moscow State Medical University, 119991, Moscow, Russia
[6] Leibniz Universität Hannover and Laser Zentrum Hannover e.V., 30419 Hannover, Germany

E-mail: minaevn@gmail.com (N Minaev)

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Abstract

A two-photon laser femtosecond crosslinking process at the wavelength of 525 nm was studied in a human donor cornea in the presence of riboflavin using two-photon optical microscopy and nanoindentation. It was shown that such an approach results in efficient crosslinking of the corneal collagen and a significant (three-fold) increase in the Young's modulus of the corneal structure. Application of a femtosecond laser with the wavelength of 525 nm allows a drastic enhancement of efficiency in the presence of riboflavin on human corneas and a 50-fold reduction of the laser treatment duration in comparison with the use of a femtosecond laser with the wavelength of 760 nm. We relate this effect to a significant growth in the coefficient of two-photon absorption due to the laser wavelength falling within the edge of the photoinitiator (riboflavin) absorption band. Our studies on a donor human cornea demonstrate the good potential for the clinical application of a femtosecond laser with the wavelength of 525 nm for increasing the cornea rigidity using the two-photon laser femtosecond crosslinking technique.

Keywords: 525 nm femtosecond laser, human cornea, two-photon collagen crosslinking, keratoconus, riboflavin, micromechanical properties

(Some figures may appear in colour only in the online journal)

1. Introduction

Femtosecond laser technologies are widely used in medicine and, in particular, in ophthalmology for vision correction and treatment of a number of eye diseases [1–4]. There exist several dystrophic diseases of the cornea, among which the most wide-spread one is keratoconus [5–7], a pathological process in which the cornea thins out and its biomechanical properties deteriorate, leading to its distortion and serious vision loss. The key approaches to the treatment of keratoconus are based

[7] Author to whom any correspondence should be addressed.

on the enhancement of the cornea elasticity [7]. Here, a technique of UV-induced corneal collagen crosslinking is developed [8], which is especially efficient when riboflavin is used as a photoinitiator, for initiating the processes of 3D corneal collagen crosslinking, increasing its elasticity [9, 10]. Note, that many biological tissues may be strengthened by crosslinking of the collagen they contain [11, 12].

The mechanism of 3D collagen crosslinking consists in UV-excitation of riboflavin molecules, causing the formation of free radicals out of aminoacid residues [13] and highly active singlet oxygen [14]. As a result, crosslinking occurs between tyrosine, threonine and histidine residues of aminoacids of collagen fibers [14]. Such a treatment of a cornea allows significant enhancement of its strength characteristics without damage to endothelial cells [7]. In contrast to swine samples, whose rigidity increased by 1.8 times, this technique showed a higher efficiency towards human corneas with more than a three-fold increase in its rigidity [15]. However, such a method has drawbacks, in particular: difficulty of precise regulation of the cornea irradiation and, as a consequence, damage to healthy regions [16]; impossibility of crosslinking fibers through the whole thickness [17].

A photochemical approach to cornea strengthening based on the combined application of femtosecond laser radiation with the wavelength of 1064 nm (creation of 'pockets' for riboflavin inside the stroma) and UV radiation (irradiation of riboflavin in a 'pocket' and initiation of crosslinks in collagen) was suggested in the study [18] and realized on a rabbit cornea [19]. The clinical applicability of such combined crosslinking was shown in [20] in the experimental treatment of patients. At the same time, it is worth noting that such a process is unavoidably associated with the creation of a defect inside the stroma. In the study [21], a two-fold increase in the elastic modulus of a collagen hydrogel was achieved when using a femtosecond laser at the wavelength of 760 nm for realization of two-photon laser femtosecond crosslinking (2P-CXL) in a collagen gel impregnated with riboflavin. The experiments on 2P-CXL with the wavelength of 810 nm, conducted in [22] on bovine cornea (without application of UV radiation), which utilized two-photon and Brillouin microscopies, directly showed the presence of crosslinks and enhancement of the cornea rigidity with a high spatial resolution. A nonlinear optical device was demonstrated in [23] to produce effective 2P-CXL within the cornea of rabbit eyes *ex vivo* using a 760 nm femtosecond laser.

The most important advantage of 2P-CXL as compared to the traditional method is its extremely local effect: 0.5–5 μm along the X and Y axes (the width of the laser radiation caustic), 2–20 μm along the Z axis (the caustic length), determined by the laser radiation divergence and numerical aperture of the NA micro-objective used for the radiation focusing. This locality allows the action on the cornea precisely inside the stroma (with a thickness of ~500 μm), without damaging the endothelial and epithelial corneal layers, and, as a consequence, reduction of the risk of infectious complications for the eroded corneal surface, and reduction of the visual discomfort and of the rehabilitation time [20].

Here, we have studied the 2P-CXL process as applied to a human donor cornea with the use of femtosecond laser radiation at the wavelength of 525 nm; using two-photon optical microscopy and nanoindentation, we have shown that such an approach results in efficient crosslinking of the corneal collagen and a significant increase in the Young's modulus of the corneal structure.

2. Materials and methods

2.1. Human donor cornea

To conduct the experiment on 2P-CXL, we used a human cornea excluded from clinical application by the morpho-functional criteria. The experiment was approved by the S. Fyodorov Eye Microsurgery State Institution Ethical Committee (Statement No 77.10 of 21.02.2017). Four samples of human cornea were obtained from the eye tissue bank of the S. Fyodorov Eye Microsurgery State Institution. After excision the corneal buttons without epithelium were stored overnight in Borzenok–Moroz cornea storage [24] solution at +4 °C for 16 h.

2.2. 2P-CXL of a human cornea

The 2P-CXL process in our study consists in 3D crosslinking of collagen molecules in the human cornea; a sketch of the set-up is presented in figure 1(a). For laser irradiation, we applied a micro-three-dimensional structuring system (Laser Center Hannover) using the second harmonic of a TeMa-100 femtosecond laser (Avesta Project). For controlling and setting the laser power, we used a half-wave plate mounted on a motorized rotary stage in combination with a polarization cube. A power meter was arranged on the lateral side of the polarization cube to be used for the continuous control of the laser radiation power. An acousto-optic modulator was used as an optical gate capable of working at frequencies higher than 1 MHz. For the high-speed movement of laser radiation through the volume of the cornea, we used a Galvo scanner with a mounted planar microscopic objective (Olympus PLAN 4× , with the numeric aperture of NA = 0.1), which was installed on a precision Z-stage (Aerotech ABL-1000). We used a CCD camera with an objective, which makes it possible to focus the laser light precisely within the cornea volume of several microns in size.

The region in which the 2P-CXL process takes place (voxel size) represents an ellipsoid elongated along the Z axis, restricted by the region of the objective's focal waist due to localization of the two-photon action [25, 26]. The size of this region (voxel size) depends on the optical system parameters (especially on the objective numerical aperture, in our case NA = 0.1), laser light power and parameters of the irradiated material (in our case, on the riboflavin concentration). In our experiment, we selected these parameters in a way that provided the highest capacity when filling the interior space according to the preset 3D model on a millimeter scale, not requiring submicron resolutions typical for this method when using objectives with high numerical apertures. The parameters of the volume 'filling' were selected taking into account the obvious existence of overlay of the fields where the two-photon absorption took place and also the highest process productivity. The maximum voxel size (in respect to the

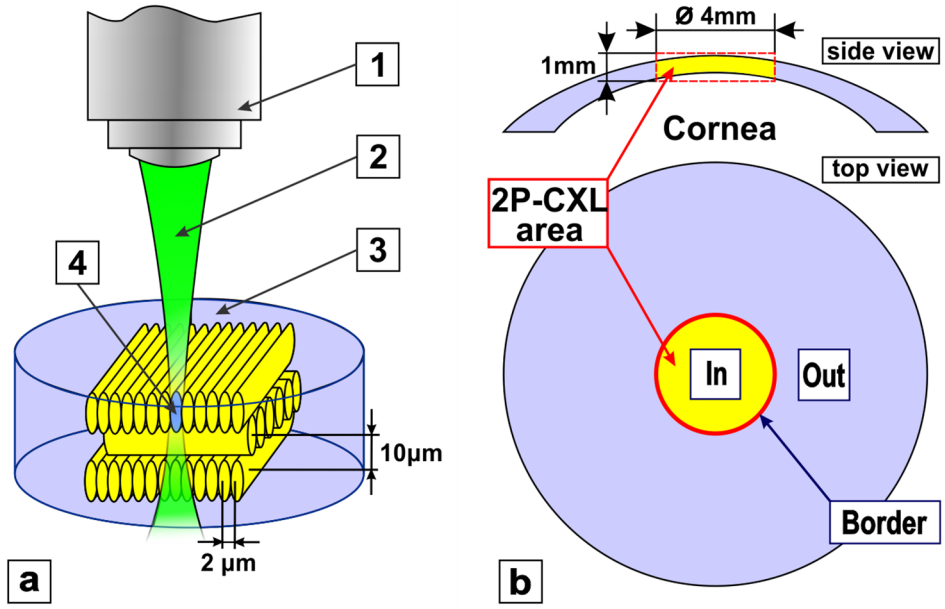

Figure 1. (a) Scheme of the process of donor human cornea 3D crosslinking using the two-photon polymerization setup: (1) planar microobjective (4×, NA 0.1), providing focusing of the femtosecond laser; (2) radiation into the interior space of the cornea; (3), (4) region where the 2P-CXL process takes place when radiation is moved through the cornea volume with a galvo scanner located above the objective. (b) Schematic image of the cornea, with the designated regions in which the following was performed: femtosecond treatment, visualization of the structural packing of collagen by multiphoton microscopy and measuring the effective Young's modulus by nanoindentaion. In yellow (In), the region subjected to the femtosecond treatment is highlighted, in blue (Out) the intact cornea region is colored, the visualization and measurements of the mechanical properties were also conducted at the border (Border) between the regions.

parameters of the optical scheme caustic) was ~4 μm in the vertical plane and the diameter in the horizontal plane was ~3 μm.

A circular cylindrical region with a diameter of 4 mm was irradiated in the central cornea area installed in an artificial anterior chamber imitating the natural cornea curvature. The sample was treated with a 0.1% riboflavin solution for 30 min prior to laser irradiation. The corneal surface was also moistened with the riboflavin solution in the course of experiment every 2 min using a pipette. The riboflavin concentration in the cornea measured by the corresponding absorption spectra was 3.25×10^{-6} mol ml^{-1}.

The cornea was irradiated layer by layer (see figure 1(a)) by moving the objective with a precision mover. The femtosecond laser light was focused into the cornea volume, followed by moving the focal point in the horizontal plane with the speed of 0.5 m s^{-1} using the galvo scanner, filling the contour of each layer with separate lines with a distance of 2 μm between the lines. The distance between separate planes was 10 μm.

The microscopic planar objective with a low numerical aperture we used allowed us to irradiate a large area with an extremely high speed of the laser beam movement, with the irradiation of the whole volume of the cylindrical 3D region with a diameter of 4 mm and height of 1 mm taking no more than 20 min.

The average laser power in the course of the 2P-CXL process of the cornea was 100 mW, pulse frequency was 70 MHz, and pulse duration was 200 fs. The energy in a pulse was 1.4 nJ, the energy density per pulse was 0.03 J cm^{-2}. The whole dose of the laser energy for the total time (1200 s) of the cylindrical corneal area (see figure 1(b)) irradiation was ~63 J. Thus, the treatment of 1 mm^3 of the cornea took no more than 100 s on average.

2.3. Nanoindentation of the human cornea

The local mechanical characteristics of the donor cornea were measured with a Piuma Nanoindenter (Optics11), which included a controller, an optical fiber and a spherical probe for force–displacement curve acquisition. The probe was attached to a flexible cantilever, the displacement of which after the contact with a surface was measured using an interferometer via an optical fiber.

To measure the Young's modulus of the cornea, the probe was immersed for 5 μm into the sample at each point of measurement. The Young's modulus for each point was computed according to the Hertzian contact mechanics model for a spherical body indenting a flat surface, using the built-in Piuma software [27, 28].

For the study of the eye cornea's mechanical characteristics, we used a cantilever with the spring constant of 2.94 N m^{-1} and a probe with the 45 μm radius of curvature. The measurements were conducted in a solution for the cornea storage heated to 34 °C corresponding to the normal temperature of the human cornea. The samples were immobilized at the bottom of a Petri dish on a support repeating the shape of the cornea, using a weight. During the measurements the probe was always located inside the fluid medium at a sufficient depth, in order to avoid measurement errors due to adhesion forces at the air–water boundary. The area of the Young's modulus mapping was 2000 × 2000 μm with a step of 200 μm

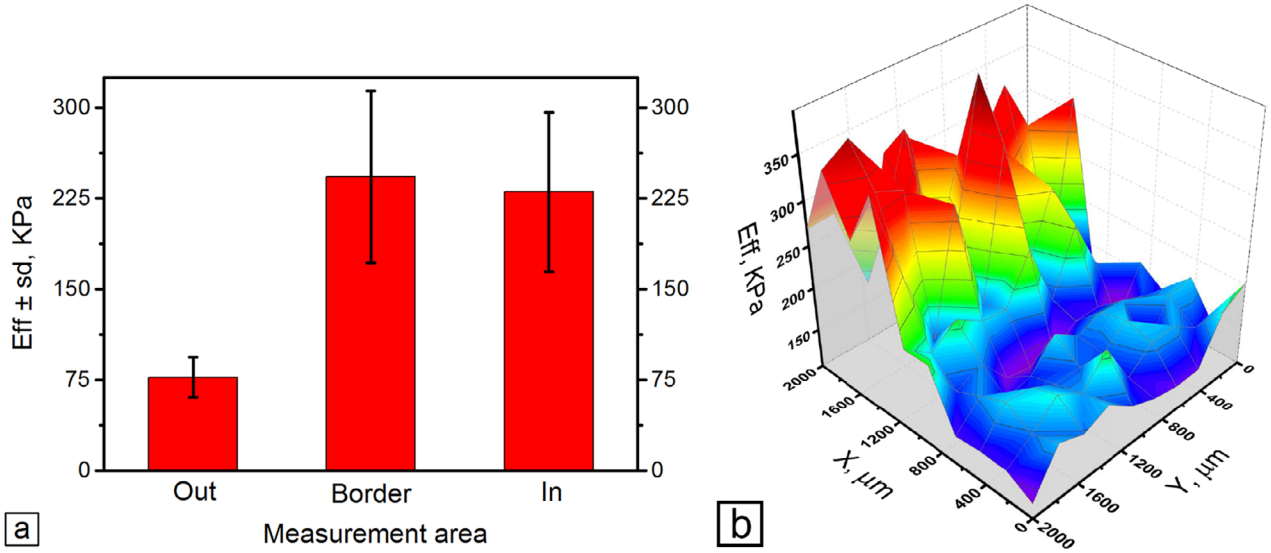

Figure 2. (a) The surface Young's moduli for the three corneal areas, in accordance with figure 1(b); (b) Young's moduli distribution over the corneal surface at the border of the treatment zone.

by the *X* and *Y* axes. Based on the results of the measurements, the effective Young's modulus was computed and its distribution over the surface was plotted.

2.4. Two-photon scanning laser microscopy of the human cornea

To determine the effect of laser irradiation on the structure and packing of collagen fibers in the cornea, we studied the samples of the cornea by two-photon laser scanning microscopy in the second harmonics generation (SHG) regime, using an LSM 880 apparatus (Carl Zeiss). The cornea was moistened with a 0.9% sodium chloride solution for a better adhesion to the glass of the apparatus, as well as to avoid it drying. The following parameters were determined: the SHG signal intensity of collagen (green channel in the images) and the intensity of riboflavin autofluorescence (red channel in the images).

3. Results

The treatment of the cornea was conducted according to the concept presented in figures 1(a) and (b). After the treatment the cornea was immersed into a conservation medium at +4 °C for the measurements of the effective Young's modulus and visualization of the collagen fibers' structure. The local Young's moduli of the corneal surface were computed with the exclusion of obviously false measurement points falling out of the supposed range, and for the border and inner regions they were computed only for the parts subjected to irradiation. In figure 2(a), a histogram of the effective Young's moduli of the corneal surface is displayed for the three regions of measurement according to figure 1(b). This demonstrates that the value of the Young's modulus at the border of the treatment and within the area of the femtosecond treatment grows by about three times (figure 2(a)). In figure 2(b), a typical 3D Young's modulus plot is presented, obtained in the region of the border between the treated and intact zones.

The analysis of the SHG and autofluorescence signal variation with the cornea depth revealed that the levels of the SHG signal in the control and treated regions decreased with depth in both cases, however, the SHG signal level of collagen was twice as high in the treated region as it was in the control region. The level of collagen autofluorescence significantly increased within the laser-treated region through the entire depth of the sample. To compare the differences in the aurofluorescence between the superficial layers of the irradiated and non-irradiated cornea area, we analyzed a fragment at their border (see figure 3).

To obtain absorption spectra, we cut the de-epithelized cornea crosswise up to a depth of 4 mm from each side towards the center and pressed it between two silica glasses (KU-1, transparent in the UV region) so that no air bubbles would appear between the cornea and the glass. For saturation with riboflavin, the cornea was soaked in the 0.1% riboflavin solution for 10 min. Then, the cornea was flattened between two silica glasses so that it had a planar shape without distortion, and absorption spectra were acquired using a Cary 50 (Agilent) spectrophotometer with the baseline correction in respect to the silica glasses themselves. To avoid premature drying before the absorption spectrum acquisition, the cornea was moistened with the storage fluid, which was characterized by appearance of the absorption peak in the region of 560 nm.

To determine the coefficients of two-photon absorption in the initial cornea and in the cornea saturated with riboflavin, we used the Z-scanning technique [29]. A power meter was placed under a silica plate supporting the cornea to continuously register the femtosecond laser radiation passing through the cornea (figure 5). The laser light with the parameters corresponding to the crosslinking experiments was focused either in the cornea volume, or above the cornea, or below the cornea at a significant distance.

As our experiments showed, some increase in the absorption of the radiation power occurred when focusing the laser radiation inside the cornea, which is evidently related to the two-photon absorption contribution (table 1).

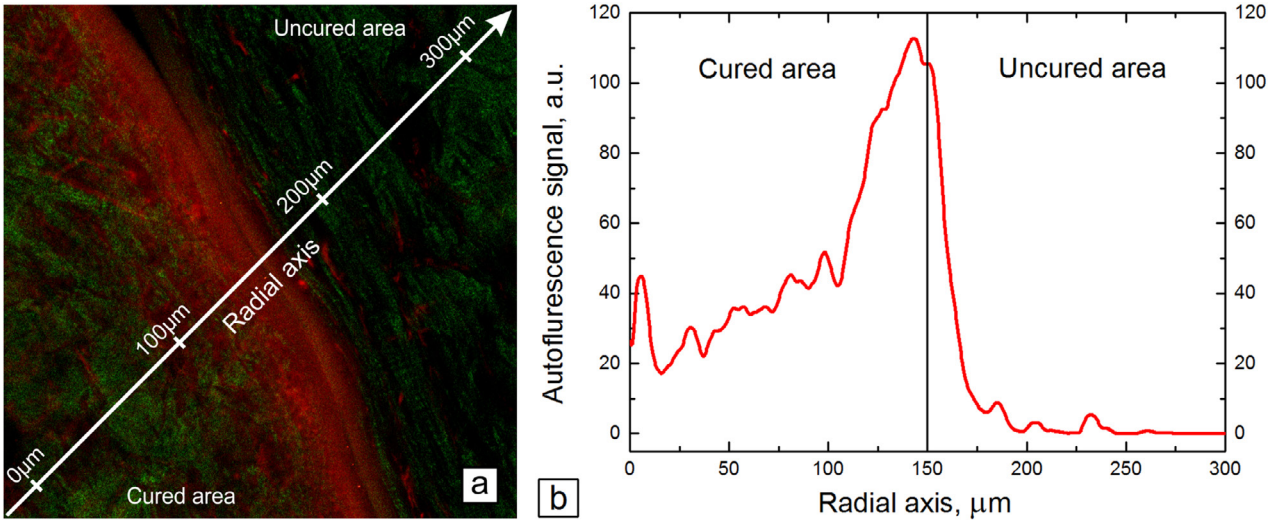

Figure 3. Combined SHG and autofluorescence image at the corneal surface in the region of the irradiated area border, obtained by multiphoton tomography (a) and a profile of the autofluorescence signal intensity along the corneal surface (b).

Table 1. Relative variations in the absorbed power when focusing the laser radiation inside the cornea ($Z = 0$ nm) and outside the cornea ($Z = -3000$ μm and $Z = +3000$ μm), at the laser radiation intensity of 1.8×10^6 W cm^{-2}.

	$Z = -3000$ nm	$Z = 0$ nm	$Z = +3000$ nm
Initial cornea	$0.0 \pm 0.8\%$	$2.0 \pm 0.8\%$	$0.0 \pm 0.8\%$
Riboflavin-saturated cornea	$0.0 \pm 0.8\%$	$4.0 \pm 0.8\%$	$0.0 \pm 0.8\%$

As seen from table 1, a relative value of the absorbed power is essentially higher in the cornea saturated with riboflavin than that in the initial cornea due to two-photon absorption.

4. Discussion

Our experiments on 2P-CXL using a femtosecond laser at the wavelength of 525 nm demonstrated high efficiency of the cornea crosslinking process. The Young's moduli for irradiated regions grew by more than three times as compared to the initial values, which was also observed in [15] for crosslinking via a one-photon process. At the same time, the time required for efficient treatment is essentially lower when using a laser source with the wavelength of 525 nm. In particular, it is 50 times lower than that for a laser with the wavelength of 760 nm in the same application [21].

Let us make an estimation for the coefficient of two-photon absorption in riboflavin at the wavelength of 525 nm based on the results of our Z-scanning measurements (table 1). A relative absorbed power of the laser radiation in riboflavin is $2.0 \pm 0.8\%$. Taking into account the voxel length of the laser radiation caustic being ~14 μm, we obtain an estimated coefficient of laser light absorption (at the wavelength of 525 nm) $k \approx 14\,\mathrm{cm}^{-1}$. One may explain such an unexpectedly high value of the two-photon absorption coefficient in riboflavin by the fact that the wavelength of 525 nm falls within the edge of the absorption band of riboflavin, thus absorption in riboflavin is essentially a two-step process. It is the high coefficient of absorption at the wavelength of 525 nm that is probably a reason for the observed extremely high efficiency of the 2P-CXL process.

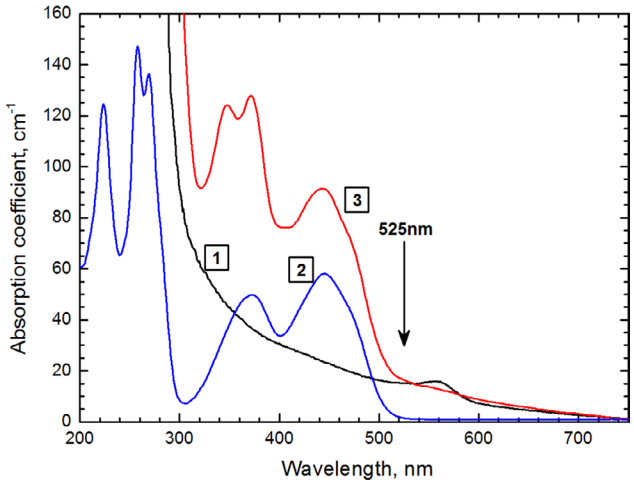

Figure 4. Absorption spectra: (1) original cornea; (2) 0.1% aqueous ribovlavin solution; (3) cornea saturated with riboflavin.

The effect of the cornea autofluorescence increase by more than 40 times is worth mentioning, which is observed in the spectral range 450–650 nm during visualization of the collagen matrix by laser scanning microscopy. Such an effect is related to crosslinking of collagen fibers under the action of reactive oxygen species (first of all, singlet oxygen) [13, 14], which form as a result of riboflavin excitation under UV radiation. At the same time, autofluorescence of the irradiated area of the cornea is associated not only with collagen crosslinking [17, 30], but also with binding of the products of the side reaction of riboflavin under UV irradiation (flavins) with corneal collagen [31]. In figure 4, the border of the irradiated side is depicted, and, as seen from the analysis of the signal intensity, the autofluorescence signal is two orders of magnitude higher at the border than that within the irradiated area. We believe that this effect is related to the fact that the reaction of 3D photoinduced

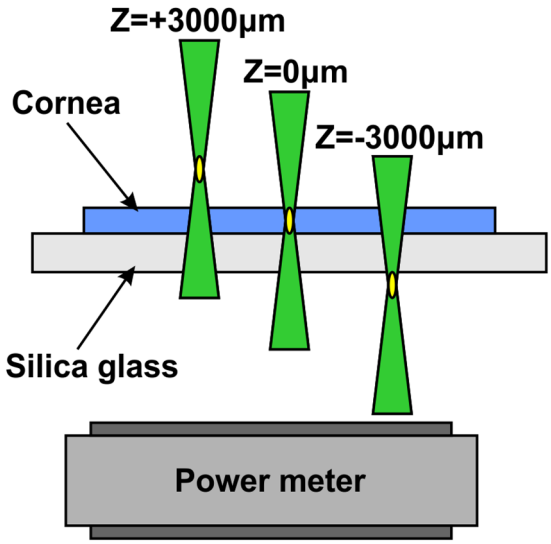

Figure 5. Principle of two-photon absorption measurement by Z-scanning.

crosslinking proceeds most efficiently at the border of the laser treatment, since the concentration of oxygen reaches a maximum in that region due to its diffusion from the non-irradiated area.

Note that the suggested approach to 2P-CXL with the use of a laser with the wavelength of 525 nm allows a significant reduction in the time of the cornea treatment when translating the 2P-CXL method into clinical practice, due to the fact that the UV radiation action on the unit volume of cornea is extremely short, crosslinking of collagen fibers does not require additional diffusion of oxygen from the outside [13], which allows decreasing the cornea damage from UV irradiation [13, 31].

5. Conclusion

In this study, we have shown that application of a femtosecond laser with the wavelength of 525 nm allows a drastic enhancement of the 2P-CXL process efficiency in the presence of riboflavin on a human cornea and a 50-fold reduction in the laser treatment duration in comparison with the use of a laser with the wavelength of 760 nm. We relate this effect to a significant growth of the coefficient of two-photon absorption due to the radiation wavelength falling within the edge of the photoinitiator (riboflavin) absorption band. It is of importance that the Young's moduli for the surface of the cornea that underwent the 2P-CXL process increased by more than three times, which indicated that photoinduced collagen crosslinking took place. The autofluorescence intensity of collagen structures also grew dramatically (up to two orders of magnitude), which testifies to an efficient process of photoinduced collagen crosslinking. The performed studies on a donor human cornea demonstrate a good potential of clinical application of a femtosecond laser with the wavelength of 525 nm for increasing the cornea rigidity using the 2P-CXL technique.

Acknowledgments

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