

# Corneal collagen fibrile contraction under 2.1 $\mu\text{m}$ holmium laser irradiation

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We present a new method to measure the collagen fibrils' contraction velocity due to collagen coagulation under 2.1  $\mu\text{m}$  wavelength laser irradiation. The experiment was performed in vitro on porcine corneal tissue. The tissue marked with fluorescent collagen antibody was placed under the microscope objective and irradiated at  $\approx 100 \text{ W/cm}^2$  power density with 488nm wavelength. This irradiation was made through an optical setup that produces light and dark fringes on tissues surface resulting a stable photobleaching pattern with  $\approx 63 \mu\text{m}$  interfringe distance. The tissue contraction follows the laser pulse irradiation at 2.1  $\mu\text{m}$  wavelength. The shrinkage of the corneal tissue, without epithelial destruction, is obtained by irradiation with laser pulses that ranges from 15 mJ to 100 mJ. A photomultiplier attached to the fluorescence microscope detects the changes of the bleached pattern position and the resulted signal is analyzed allowing the calculation of the contraction's speed of irradiated collagen fibrils. Contraction's velocity of 0.57 mm/sec for 50 mJ pulse irradiation was measured.

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## 1. Introduction

The corneal reshaping technology called Laser Thermal Keratoplasty (LTK) is a non-invasive technique and very effective for the persons who require low to moderate correction (1-3 diopters) of farsighted and astigmatism. It is also very effective for surgically-induced hyperopia, especially for overcorrected post-PRK (photorefractive keratoplasty) patients.

LTK technique use a Ho:YAG or diode laser beam to heat a small area of the corneal tissue. Well-defined coagulation is thereby induced in the corneal stroma by strongly absorbed IR laser radiation. In the case of hyperopic correction these coagulations are equidistantly applied in a ring pattern in the corneal periphery [1]. Because the posterior surface of the cornea is bounded by the aqueous humor, whose index of refraction is slightly different of that of the cornea, it is the front surface of the cornea that provides  $\sim 90\%$  of the eye's refraction.

The human cornea consists of five layers: the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium. The stroma makes up about 90% of the thickness of the cornea [2]. It is divided into layers of collagenous material- the stromal lamellae, which run essentially parallel to the surface of the cornea uninterrupted from limbus to limbus. A lamella is made up of almost parallel fibers and each fiber consists of very fine fibrils. It is known that collagen has intermolecular cross-links that provide the fibers the unique properties of high tensile strength and good elasticity. An important characteristic of collagen is the fact that it undergoes

thermal shrinkage [3] of its fibers when they reach a rupturing of the stabilizing cross-links. The result is an immediate contraction (and increase in thickness) of the fibers to approximately 1/3 of their original length [4]. The resulting contractive forces due to shrinkage of collagen fibrils induces an approximately annular stress zone and increases the curvature of the cornea. This property of the collagen fibrils is used by LTK to change the curvature of the cornea.

In order to optimize laser pulse parameters, the temperature at which maximal shrinkage of collagen fibrils occurs is of major interest. Various studies show [5] that in the temperature range 60  $^{\circ}\text{C}$  to 95  $^{\circ}\text{C}$  an exponential relation between the denaturation time and temperature occurs. The denaturation for long time exposure of corneal collagen has reported to be between 55  $^{\circ}\text{C}$  and 58  $^{\circ}\text{C}$  [3,5]. In the time domain up to several seconds, higher temperatures are needed to achieve the same thermal damage degree.

The biomechanical properties of cornea were determined by irradiating with Holmium laser, the cornea probes were fixed into the grips of a material-testing machine [5]. Another method used to track structural changes in collagenous tissue during Ho-laser - induced denaturation is linear birefringence method [6]. This study shows that at low fluency region, denaturation is both thermally and mechanically induced. Also the ocular dose-response relationships were experimentally determined for selected exposure conditions at the erbium and holmium laser irradiation [7]. The main problem within refractive surgery, especially in the LTK, is the selection of the

optimal treatment modalities to correct the individual refractive error out of a vast variety of possible parameters.

This paper presents a new method for a better evaluation of laser parameters and exposure geometry of laser-collagen interaction. The purpose of the experiment is to determine the velocity of the contraction of collagen fibrils after Holmium laser irradiation and the amplitude of this contraction. The fluorescence photobleaching techniques [8-10] and histological techniques were combined to give a useful method for laser-collagen interaction study.

## 2. Methods and experiments

The main tissue chromophore in the IR part of the electromagnetic spectrum is the water, which is present in bound and free form. The fundamental symmetric and asymmetric vibration stretching made by water molecules produces strong absorption peaks at 2.94  $\mu\text{m}$  and 1.94  $\mu\text{m}$  wavelengths. The laser chosen to take advantage of these absorption peaks was the Cr:Tm:Ho:YAG (CTH) laser ( $\lambda = 2.1 \mu\text{m}$ ) which was driven by various applications as laser angioplasty, arthroscopic knee surgery, herniated disc surgery, cornea reshaping. This wavelength is easily transmittable through standard, low OH optical fibers, which is a requirement for use in minimally invasive clinical procedures. The absorption coefficient is 25-35  $\text{cm}^{-1}$  corresponding to a 1/e penetration depth of 285 – 400  $\mu\text{m}$ . This value is very appropriate for making coagulations in corneal stroma since its maximum thickness is less than 800  $\mu\text{m}$ . Because of the small size of the coagulated collagen volume we expect that the shrinkage of the collagen fibril is small enough to need a microscopic observation.

For precise measurement of the corneal collagen shrinkage we adapted the periodic pattern photobleaching technique [10] to our specific conditions. In order to observe the contraction of the collagen fibrils under IR laser irradiation we treat the corneal surface by fluorescence substances. There are around 19 different types of collagen in the human body and the corneal collagen is known as being of type I. The antibody for the collagen type I is monoclonal Anti-collagen Type I which recognizes the native (helical) form of collagen type I. The product does not react with the thermally denaturated molecule. The antibody shows no cross-reactivity with collagen types II – XI. Monoclonal Anti-Collagen Type I may be used for the localization of type I collagen using various immunochemical assays, 1:3000 minimum dilution. Secondary antibody marked with fluorescein isothiocyanate (FITC) produced by DAKO Co. was used in the technique known as indirect immunofluorescence. Fluorescein isothiocyanate (FITC) is a fluorochrome with the maximum absorption at 495 nm. Its excitation by 488 nm light leads to a fluorescence emission maximum around 520 nm. Using a 530 +/- 15 nm bandpass (BP) filter will give optimum detection for this fluorochrome. FITC is the most widely used form for conjugation to antibodies and proteins. FITC has a high quantum yield (efficiency of energy transfer from absorption to emission

fluorescence) and approximately half of the absorbed photons are emitted as fluorescent light. Other important property of FITC is the *permanent bleaching* when receive 488 nm laser irradiation at power density over  $\approx 50 \text{ W/cm}^2$ .

### 2.1 Preparation of corneal samples

Freshly enucleated porcine eyes were used for the study. The eyes were transported in Phosphates Buffer solution at temperature 0-4  $^{\circ}\text{C}$  and between the experiments the cornea samples were kept at 0-4  $^{\circ}\text{C}$ . Prior to the experiments the corneas were excised as whole. All cornea specimens received the incubation treatment at room temperature. In the first stage, after excision, the cornea was incubated with Monoclonal Anticollagen I (Sigma C-2456), 1:1000 dilution. Then, during the second stage of incubation, the cornea was treated with antibody IgG labeled with FITC (Dako F0479). The samples were incubated with the first antibody monoclonal collagen for 90 minutes and then were incubated with the second antibody for the same period. The entire process was performed in sterile atmosphere. The second antibody non-attached to the sample is removed by washing.

### 2.2 Experimental setup and results

There are three important tasks to be solved by the experimental setup. First we induce a permanent pattern on the cornea surface by using the property of FITC to bleach when it is irradiated by high intensity laser beam at 488nm wavelength.

The second task is to make an IR laser irradiation on the cornea sample and obtain the coagulation in a small area of the sample and, consequently, the contraction of the collagen fibril. The third task is to detect the collagen's shrinkage velocity.

The experimental setup, Fig. 1, uses the fluorescence microscope Zeiss Axioscope 2, the argon laser Stabilite 2017 SpectraPhysics, a CTH:YAG laser connected with a low OH content optical fiber with 400  $\mu\text{m}$  core diameter, the scope LeCroy LT342, CCD camera, videorecorder, image analyzer system LEICA-Image Database Matrox.

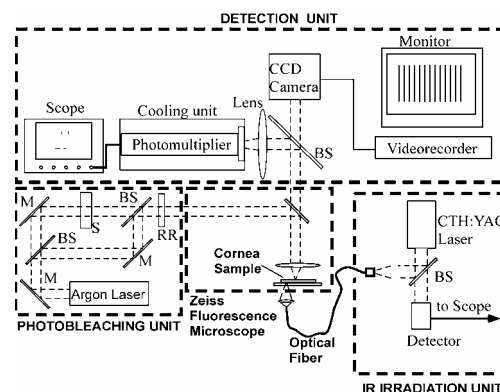


Fig. 1. Experimental setup for collagen speed contraction measurement. It has four units: ZFM- Zeiss Fluorescence Microscope; Photobleaching unit (RR-Ronchi Ruling, BS- beam splitter, S-shutter, M-mirror); IR irradiation unit; Detection unit.

As one can observe from Fig. 1 the setup has four functional parts: the first unit is the fluorescence microscope which keeps the corneal sample in the focal plane of its objective; the second is the photobleaching unit that induces a fringe pattern on the corneal surface placed at the microscope observation field; the third part irradiates the cornea by IR laser pulses delivered by optical fiber; the fourth stage detects and analyses the changes occurred during the IR laser irradiation. The corneal sample, previously treated with FITC, is fixed at one side on a glass support and placed in the field of the microscope, Fig. 2. The cornea is fixed at one side and the fringe pattern is induced in the center of the sample. The IR laser pulse is applied between the fixture point and pattern bleached region and induces the coagulation of the collagen fibril. Due to this coagulation the fibrils' contraction occurs and the patterned region will be displaced.

a) *Generation of the fringe pattern on the sample.* As one can see from the Fig. 1 the Argon laser can irradiate the sample by using two optical ways controlled by a shutter. The difference between the two ways consists in the laser power. The 488 nm wavelength low power laser is used to observe the fluorescence of the corneal surface. By opening the shutter the sample receives a brief intense laser irradiation at 488 nm wavelength. The laser beam pass through the Ronchi Ruling (periodic pattern printed on glass, with alternate black lines and transparent spaces of equal widths) placed at the rear focal plane of the microscope. The Ronchi Ruling with the period of  $L=50$  lines/cm produces a fringe pattern period of  $63 \mu\text{m}$  on the corneal sample located under 10X objective. On the corneal sample, placed in the focal plane of the microscope objective, it is obtained an image formed by light and dark fringes. The grating orientation produces fringes perpendicular on the axis, Ox, Fig. 2.

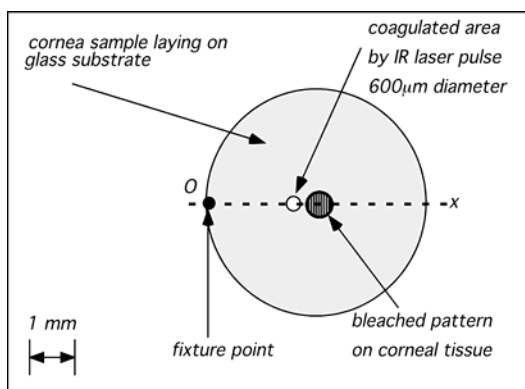


Fig. 2. Corneal tissue arrangement: the fixture point, IR laser irradiated region and fringe pattern area are in line.

The fluorescence pattern on the corneal surface is obtained by irradiating the sample with 5 W Argon laser power. The irradiation time varies from few seconds to one minute depending on the quantity of FITC bonded on the corneal surface and on the microscope objective used

to focus it. During the experiments we determine that irradiation with laser power density over  $100 \text{ W/cm}^2$  produces the photobleaching effect. Because of the high intensity of the laser beam (488 nm) the fluorescence of FITC is destroyed in the region of the light fringes. Fig. 3 shows the fluorescence image of the cornea taken before the high intensity irradiation when the 488 nm laser beam is set at low power level. Fig. 4 shows the same region after bleach, the image was taken in the same conditions as before.

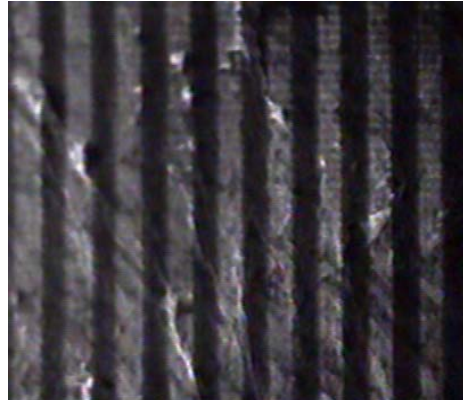


Fig. 3. The microscopical image of the corneal sample before FITC bleaching by the blue argon laser irradiation.



Fig. 4. The microscopical image of the corneal sample after FITC bleaching.

One can observe that almost entire fluorescence in the irradiated area was bleached, the thin vertical light fringes are visible because we intentionally displaced few microns the bleached pattern. After this short irradiation the shutter is closed and the sample is illuminated by a low intensity laser beam which produces the same image pattern on the corneal sample but at low power density to avoid any unnecessary bleaching. Any region of the corneal sample where the FITC was not bleached will emit photons by fluorescence if it is irradiated with low intensity laser beam at 488 nm wavelength. The bleached pattern is stable attached to the corneal sample.

b) *IR laser irradiation of the sample.* For all experiments we used a CTH laser operated in the free running pulse mode with pulse duration of about 200  $\mu\text{s}$ . The pulse length was measured by IA 020H – Electrooptical Systems detector. The laser beam was delivered to the probe by a 400  $\mu\text{m}$  diameter low OH optical fiber. The laser can deliver up to 4 J pulse energy but for our experiment we find out that pulses between 15 mJ and 100 mJ produce collagen's coagulation and avoid epithelial destruction. The repetition rate can be varied from 0.5 Hz to 30 Hz.

c) *Fringe pattern's movement measurements.* The bleached fringe pattern and the illumination fringe pattern are superimposed and the photomultiplier will give a signal proportional with the total fluorescence of the field. The IR laser pulse irradiation produces the collagen's coagulation, consequently it induces a shrinkage of the fibrils and a movement of the bleached fringe pattern toward the fixture point. The fluorescence emission is modulated as the bleached pattern and the illumination pattern fall into and out of phase. Any movement of the sample will produce an alternate between the dark field, Fig. 4 and light field Fig. 3. These variations are detected by the photomultiplier and the scope, Fig. 5.

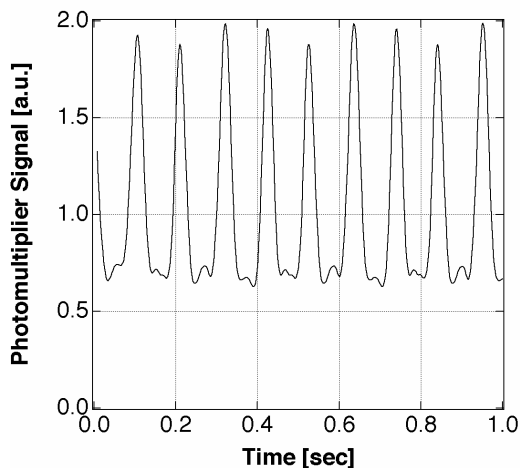


Fig. 5. The photomultiplier signal showing the movement of the corneal sample during the shrinking after CTH:YAG laser irradiation.

From the Fig. 5 one can measure the speed of the contraction and the contraction length. In this particular case we measured a contraction velocity of 570  $\mu\text{m}/\text{sec}$ . with 50 mJ IR laser pulses irradiated on the cornea sample. This method offers a very precise way to measure these two parameters. Depending on the fringe number of Ronchi Ruling and the microscope objective one can obtain fringes from 63  $\mu\text{m}$  to submicron dimensions. Some questions may arise about the direction of the collagen lamellae contraction. As the corneal sample is fixed at one point and inside the stroma the lamellae are disposed randomly the coagulation into a volume will produce contraction of any lamellae passing through that volume.

Taking into account the amount of fibrils aligned along the axis Ox Fig. 3 a relationship between the coagulated volume and contraction velocity can be derived.

In order to establish the relations between the laser parameters and the coagulated volume we made irradiations of the cornea samples by using different laser pulse energies. After irradiations, the samples were fixed in neutral formaline 10%, embedded in the parafine, cut in slices of 6  $\mu\text{m}$  thickness and treated with hematoxiline Harris and eosine. The treatment produces on the probe enough contrast to allow measurements by using a microscope and the image analyzer system LEICA – Image Database Matrox. The results are presented in Figs. 6 and 7. One can notice that the laser pulse energy of 100 mJ is too large because the epithelial layer is damaged. The future experiments will follow this procedure in order to reconstruct from the surface measurement the entire coagulated volume and correlate it with the laser pulse parameters. The first results of this measurement show that when 50 mJ IR laser pulse irradiates the corneal sample as much as 0.054  $\text{mm}^3$  volume is coagulated.

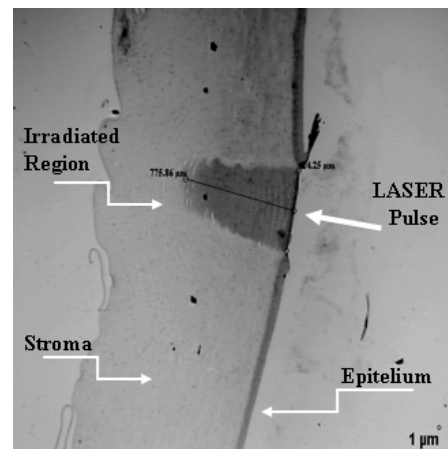


Fig. 6. The transversal view of the corneal irradiated area by CTH:YAG laser pulse,  $E=80$  mJ,  $t=200$   $\mu\text{s}$ .

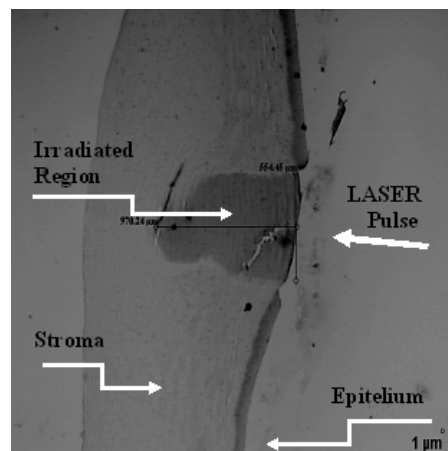


Fig. 7. The transversal view of the corneal irradiated area by CTH:YAG laser pulse,  $E=200$  mJ,  $t=200$   $\mu\text{s}$ .

### 3. Conclusion

A new method to study the corneal collagen fibrils contraction under IR laser irradiation is presented. Combining the immunochemical methods with optical methods we are able to measure the contraction speed and the total contraction length. The experiments made in vitro on corneal porcine samples show that the measurement of collagen fibril contraction velocity can be done and first result shows 0.57 mm/sec at 50 mJ IR laser pulse irradiation which produce 0.054 mm<sup>3</sup> coagulated volume. Future experiments will use this method combined with histological study to develop a contraction model for collagen fibril irradiated by laser.

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