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Pablo Artal, Francisco Ávila, Juan Bueno, "Second harmonic generation microscopy of the living human cornea," Proc. SPIE 10498, Multiphoton Microscopy in the Biomedical Sciences XVIII, 1049810 (23 February 2018); doi: 10.1117/12.2290470

SPIE.

Event: SPIE BiOS, 2018, San Francisco, California, United States

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Pablo Artal*, Francisco Ávila and Juan Bueno
Laboratorio de Óptica, Centro de Investigación en Óptica y Nanofísica,
Universidad de Murcia, 30100, Murcia, Spain.
*pablo@um.es

ABSTRACT

Second Harmonic Generation (SHG) microscopy provides high-resolution structural imaging of the corneal stroma without the need of labelling techniques. This powerful tool has never been applied to living human eyes so far. Here, we present a new compact SHG microscope specifically developed to image the structural organization of the corneal lamellae in living healthy human volunteers. The research prototype incorporates a long-working distance dry objective that allows non-contact three-dimensional SHG imaging of the cornea. Safety assessment and effectiveness of the system were firstly tested in ex-vivo fresh eyes. The maximum average power of the used illumination laser was 20 mW, more than 10 times below the maximum permissible exposure (according to ANSI Z136.1-2000). The instrument was successfully employed to obtain non-contact and non-invasive SHG of the living human eye within well-established light safety limits. This represents the first recording of *in vivo* SHG images of the human cornea using a compact multiphoton microscope. This might become an important tool in Ophthalmology for early diagnosis and tracking ocular pathologies.

Keywords: Second Harmonic Generation, collagen, in vivo corneal imaging.

1. INTRODUCTION

Second Harmonic Generation microscopy (SHG) arises from the interaction between high-repetition pulsed lasers and non-centrosymmetric structures, such as those molecules arranged in a crystalline fashion. In particular, SHG is a powerful microscopy tool to visualize type-I collagen structures, such as the skin and the cornea with submicron resolution[1]. This technique has been employed to analyze the organization of collagen in ex-vivo healthy corneas in vitro[2,3], to detect morphology changes in corneal infections[4], to characterize the disorders caused by certain pathologies (keratoconus)[5] or to detect the microscopic effects of laser intrastromal surgery[6]. The structural organization of collagen plays an important role in corneal transparency and biomechanical stability[7]. In this work, we report for the first time a prototype of a SHG microscope able to image the structural organization of corneal lamellae in the living human eye.

2. METHODS

2.1 Compact SHG-microscope

A clinical prototype of a SHG microscope has been developed. As illumination source a mode-locked Ti:Saphire laser is used. This provides pulses of 800 nm with a repetition rate of 76 MHz and duration of ~400 fs. The experimental system is a compact device mounted on a 40x25 cm^2 platform. It incorporates a non-resonant dual-axis galvo system and a stepper motor connected to a long-working distance microscope objective (NA=0.5) that allows non-contact SHG imaging. A dichroic mirror separates the illumination from the emitted light. SHG signal from the cornea under study is isolated after passing a narrow-band filter and reaches the recording unit (PMT). A XYZ chinrest attached to the platform allows the aligning of the subject's eye and facilitates stability during measurements. The exposure time for SHG image acquisition is controlled by an electro-mechanical shutter. An additional camera is used to control the position of the laser spot. This camera, as well as the scanners, the axial-focus positioning and the acquisition time are synchronized through a data acquisition card and custom C++ software. First measurements were carried out in two healthy normal volunteers. During the imaging session, the volunteer was asked to stare at a fixation target to avoid ocular movements. A schematic diagram of the experimental system is shown in Figure 1.

Multiphoton Microscopy in the Biomedical Sciences XVIII, edited by Ammasi Periasamy,
Peter T. C. So, Karsten König, Xiaoliang S. Xie, Proc. of SPIE Vol. 10498, 1049810

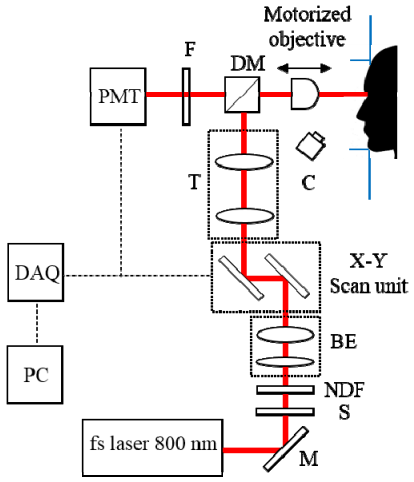

Figure 1. Optical layout of the SHG microscope. M, mirror; S, shutter; NDF, neutral density filter; BE, beam expander; T, telescope; DM, dichroic mirror; C, control camera; F, spectral filter; PMT, detection unit; DAQ, data acquisition card; PC, computer.

2.2 Safety considerations and experimental imaging

For SHG corneal imaging microscopy, the high-repetition pulsed laser is focused on corneal tissue (i.e. stroma). The propagation of the beam along the eye produces a projection on the retina. The area of this patch of light depends on the numerical aperture of the objective and the axial length of the eye. We have considered the maximum permissible exposure (MPE) to avoid both thermal retinal damage and the cornea and crystalline lens. According to International Commission on Non-Ionizing Radiation Protection[8], for the herein experimental conditions the maximum permissible irradiance at the corneal plane for an exposure time of 0.5 s is 195 mW. Considering that the area over the retina is ~7 mm^2, the MPE according to the ANSI Z136.1-2000[9] corresponds to ~430 mW. These data suppose a stationary spot, which corresponds to the worst scenario. However, our system was designed to provide a permanent scanning movement of the beam during light exposure. Then, these stationary safety limits are overestimated. The experimental image recording parameters established for the instrument are shown in Table 1.

Table 1. Technical specifications for SHG image acquisition.

SHG imaging parameters	
Image size	200 x 200 px
Scanned area	300 x 300 µm
Exposure time	0.5 s
Average power	20 mW

3. RESULTS

3.1 SHG imaging microscopy of ex-vivo tissues: a preliminary test for threshold measurement

Thermal damage implies both changes in the structure because of collagen denaturation and a SHG signal decrease[10]. A preliminary experiment in corneal tissue damage threshold was carried out. For this aim, four ex-vivo fresh porcine corneas (#1-#4) were imaged according to the imaging parameters of Table 1, but using 100 mW (instead of 20 mW) at the objective image plane. The first SHG image of each specimen (apex location) was used as control. Then, the same area was irradiated (i.e. scanned without image acquisition) during 60 (#1), 180 (#2), 240 (#3) and 300 (#4) seconds, respectively, and imaged again. Figure 2 compares the total SHG intensity values before and after irradiation. No significant changes in SHG intensity were found in the four specimens. Differences among the samples are due to the random choice of the imaged plane, since deeper locations provide lower signal levels. In addition, the structure of the

collagen distribution seems not to be modified after 300 s of irradiation as shown in the SHG images of Figure 2 (panels on the right). The similarity of the measured SHG intensity and the absence of changes in the fiber arrangement indicate a lack of thermally-induced damage. This experimental test corroborates that even using 5-times the pre-determined incident laser power, the SHG image recording settings are within safety limits.

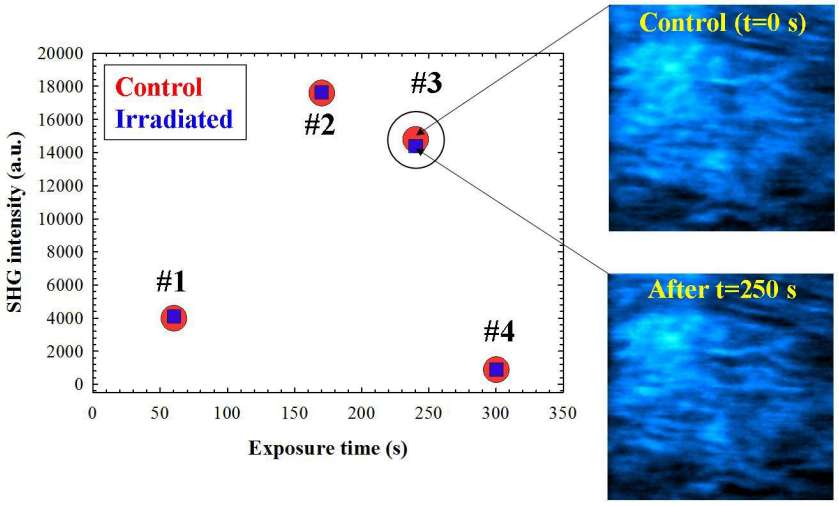

Figure 2. SHG intensity values for control (red) and irradiated samples (blue). The specimens corresponded to four ex-vivo porcine corneas. Panels at the right are the SHG images for one of the corneas as indicated.

3.2 *In vivo* SHG corneal microscopy

Figure 3 shows backscattered SHG images acquired from volunteer #1 around the corneal apex at two different depth locations. Images were acquired according to the experimental conditions of Table 1. Figures 3a and 3b correspond to the adjacent layer to the Bowman's membrane (i.e. anterior stroma) and to the mid stroma respectively. Unlike forward SHG emission, backscattered SHG signal provides more diffuse and regular information about the collagen patter within the lamellae[11], however with contrast enough to make clearly visible the stromal fibers, which distribution agrees well with previous experiments in ex-vivo human corneas[2]. The collagen fibers appear densely packed with high-organized arrangement, this configuration is critical to preserve optical transparency and corneal biomechanics[12].

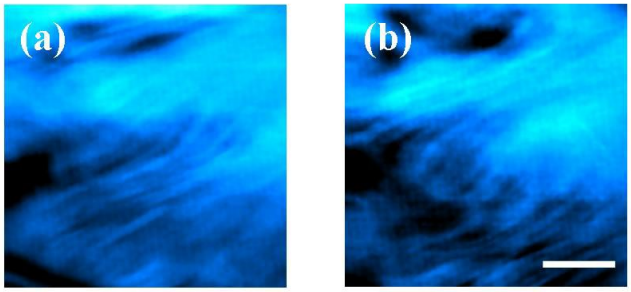

Figure 3. SHG images from the corneal apex of volunteer #1 at two depths locations within the stroma. Scale bar: 50 µm.

The cornea of volunteer #2 was also imaged with the same experimental conditions. SHG images of Figure 4 were recorded at two different locations, the apex (Figure 4a) and the peripheral cornea (Figure 4b). The depth location of both images is similar, a layer of the anterior stroma close to the Bowman's membrane. It can be observed that the collagen distribution at the apex is similar to that found in volunteer #1 (Figure 3a). However, this organization changes towards the periphery, decreasing the degree of interweaving and becoming in aligned distribution.

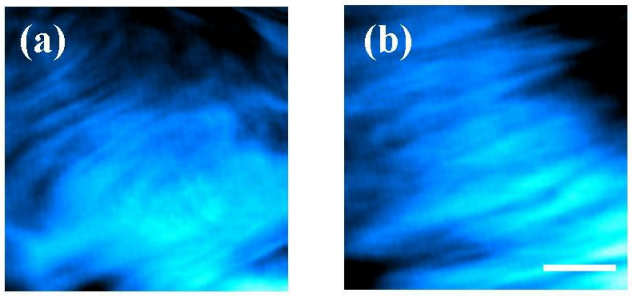

Figure 4. SHG images from volunteer #2 corresponding to the anterior stroma of the corneal apex (a) and the peripheral cornea (b). Scale bar: 50 µm.

4. DISCUSSION

We have developed a SHG microscope to analyze the stroma of the living human cornea with 3D high resolution for the first time. This instrument uses a non-contact imaging system to improve subject's comfort over the immersion objectives used in commercial linear microscopes to visualize the different layers of the cornea.

Fresh ex-vivo porcine ocular globes from a pig slaughterhouse were used in a preliminary test to measure the minimum average laser power and exposure time required to integrate enough and efficient signal to provide high contrast SHG imaging. This process also involved the evaluation of possible photo-damage effects by irradiating the tissues at the maximum available average power during different exposures. No significant changes in SHG signal intensity were found.

The experimental conditions, emphasizing the exposure to high-power pulsed lasers, were tested and stablished within the safety limits to protect the human cornea and crystalline lens, as well as the retina, according to the standards[8,9].

Living SHG images revealed similar structures to those previously obtained in ex-vivo human corneas[2]. The collagen distribution corresponding to the central part of the anterior corneal stroma showed an interwoven spatial arrangement, which has implications in corneal biomechanics.

SHG images comparing the corneal apex and the peripheral cornea also showed interesting results. At eccentric corneal locations, a lower the degree of cross-linking (i.e. the higher the degree of alignment) appears. These results are consistent with those reported by Boote et al.[13] related the collagen organization in peripheral human cornea. They reported how outside the central 6 mm of the cornea, the collagen becomes aligned to reinforce the cornea and limbus.

The present study brings out the potential of SHG microscopy to visualize the collagen matrix of the living human eye and its usefulness in the early detection of ocular pathologies, as well as in the analysis of its impact in corneal biomechanics and transparency.

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ACKNOWLEDGMENTS

This research has been supported by the Secretaría de Estado e Investigación, Desarrollo e Innovación (SEIDI) FIS2016-76163-R, the European Research Council Advanced Grant ERC-2013-AdG-339228 (SEECAT), Fundación Séneca-Agencia de Ciencia y Tecnología de la Región de Murcia (grant 19897/GERM/15) and the European Regional Development Fund (EU-FEDER).