Second Harmonic Generation Microscopy of the Living Human Eye

Visualizing *In Vivo* Human Ocular Tissues with Two-Photon Microscopy

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Visualization and analysis of transparent living ocular tissues at microscopic scale have been a challenge during the last decades. Nowadays clinical devices only allow imaging cellular corneal structures; however, collagen fibers of the stroma (90 % of the corneal thickness) cannot be imaged. It has been recently reported a compact Second Harmonic Generation (SHG) microscope able to image the living human cornea and the sclera for the very first time.

Introduction

Second Harmonic Generation (SHG) microscopy is a nonlinear imaging tool that provides information on non-centrosymmetric and chiral structures [1]. The phenomenon is based on the interaction of two excitation infrared photons from a femtosecond laser with the sample under analysis. These turn into an emitted single photon that has always half the excitation wavelength (no energy loss). This is a sub-micron resolution technique with inherent confocal properties, used to visualize type-I collagen tissues without labeling procedures.

The cornea and the sclera of the eye are paradigmatic examples of collagen-based tissues [2]. From a clinical point of view, the visualization of the transparent collagen fibers of the corneal stroma has been challenging. Actual clinical devices are only effective for cellular imaging within the different corneal layers. To our knowledge, a commercially available instrument able to show the corneal collagen fibers at micrometric resolution does not exit.

In 2002, Yeh and co-authors reported the use of SHG microscopy for imaging the corneal stroma [3]. Since then, many authors have centered their research in exploring the fiber distribution in corneas of both humans and animal models [2], not only in healthy conditions but also under pathological ones [4]. Although SHG microscopy of the sclera can also be found in the literature, the interest in this part of the eye has been significantly reduced.

However, the use of this powerful technique has been mainly limited to the study of ex vivo tissues and was never applied in living human eyes before. Some experiments tried to image the in vivo cornea of animal models, but the success was very limited. The animals were always anesthetized and immobilized. Moreover, fluorescent viable dyes were also used to get two-photon fluorescent signal from stromal cellular layers and some nerves [5]. Only Latour et al. were able to observe collagen fibers in rats, using an immersion objective together with an ophthalmic gel and an aplanation device to minimize eye movements [6].

At the Laboratorio de Óptica of the Universidad de Murcia in Spain it has been able, for the very first time, to build a prototype of a SHG microscope to successfully image the collagen fibers of both the cornea and the sclera, in the living human eye [7]. Although our main interest was collagen visualization, 2-photon fluorescence images from other ocular structures were also obtained (corneal nerves, trabecular meshwork, individual cells within the juxta-canalicular tissue).

Methods

Clinically-oriented Second Harmonic Imaging Microscope

For the purpose of this work, a compact prototype of a SHG microscope has been developed. The clinical instrument was mounted on a 40x25 cm^2 platform and allows measurements in living humans. Figure 1 shows a schematic diagram. A mode-locked (76 MHz repetition rate) Ti:Sapphire laser was used as illumination source (λ=800 nm). The beam passes a XY scanning system and a dichroic mirror (to split incoming and outgoing pathways), before reaching the eye through a long-working distance objective (20x, NA=0.5). This type of non-immersion objective avoids eye-contact operation and overpasses the limitation of using aplanation devices and ophthalmic gels. The beam scans the ocular area of interest, and the emitted nonlinear signal is directed towards a spectral filter and the detection unit. A XYZ adjustable chinrest was attached to the platform to ensure the comfort for the subject, to minimize movements during assessment and to facilitate the eye's alignment operation. During measurements the subjects were also asked to stare at a fixation target. Two additional cameras were used to control for the correct position of the eye and the incidence point of the laser spot. The entire device was controlled through a data acquisition card and custom C++ software.

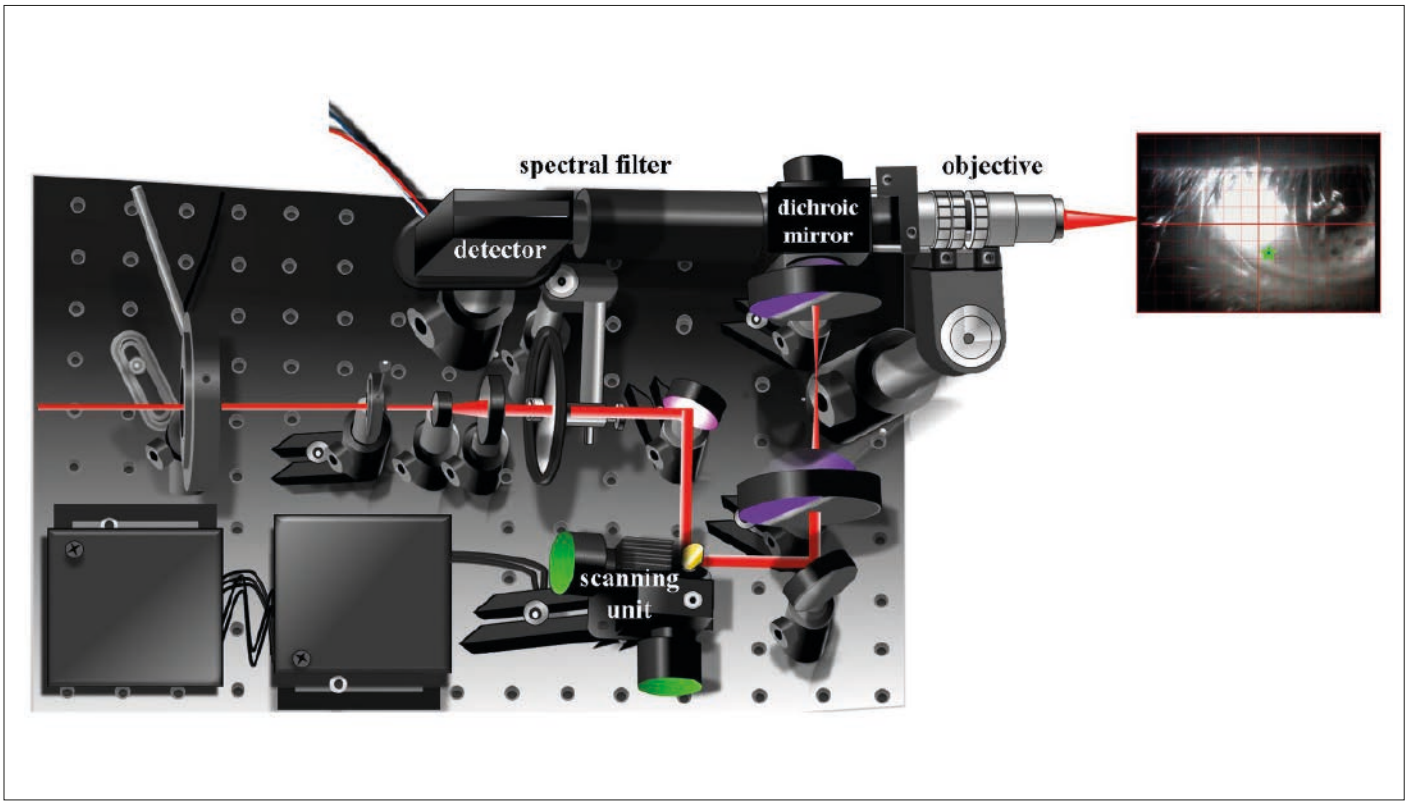

Fig. 1: Scheme of the SHG microscope used for in vivo human eyes.

Safety Considerations for Experimental Imaging

To ensure safe image acquisition, both exposure time and image size must verify certain conditions that were extensively reported in reference [7]. In brief, since safety limits for this kind of experiments were not clearly established, we proceed to compute the irradiance limits to protect the ocular structures. Both the International Commission on Non-Ionizing Radiation Protection and the ANSI Z136.1-2000 standards were considered and the maximum permissible exposure (MPE) determined. The worst scenario was assumed: a stationary spot instead of a dynamic (scanned) one. The final image settings used for the actual experiment allowed being several orders of magnitude below the MPE: 0.5 s for an image size of 300x300 µm, with an incident laser power of 20 mW at the sample's plane.

Results

SHG images of the corneal stroma in one of the volunteers involved in the experiment are shown in figure 2. Locations correspond to the corneal apex (fig. 2a) and the periphery (fig. 2b). Images were acquired according to the experimental conditions referred above. The distribution of collagen fibers at both locations is clearly different. Whereas at the apex certain interweaving is present, this pattern turns into a more aligned distribution at peripheral areas. It can be observed that in vivo SHG images provide enough contrast to make clearly visible the collagen fibers. Moreover, the distribution seen is coherent with that previously reported in ex vivo human corneas [2].

Apart from the corneal stroma, other collagen-based ocular structures were also imaged under the same living experimental conditions. SHG images of figure 3 were recorded at the corneal limbus (border between the transparent cornea and opaque sclera, figure 3a) and the sclera (fig. 3b). Details of the collagen architecture of human corneal limbal area were elucidated. The limbus contains stem cells. Despite the opacity of the scleral tissue, the fibers could also be seen.

Conclusions

A clinically-oriented non-contact SHG microscope has been developed to image collagen-based tissues in the living human eye. It provides non-invasive high-resolution structural imaging without labeling techniques. The damage thresholds for safe performance of the device have been accurately established and defined. Results presented here represent the first recording of in vivo SHG images of the human eye. These bring out the potential of this technique as a tool in clinical environments for early diagnosis and tracking of ocular pathologies, and surgery follow-ups.

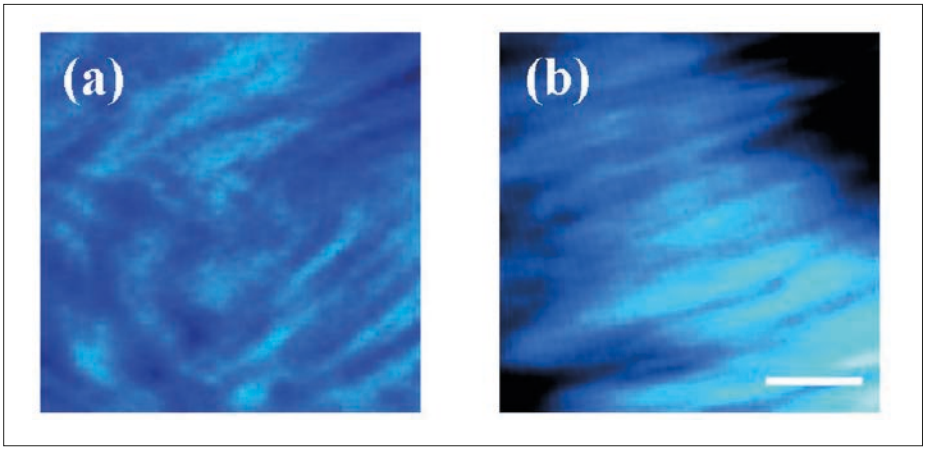

Fig. 2: SHG images from volunteer #2 corresponding to the anterior stroma of the corneal apex (a) and the peripheral cornea (b). Scale bar: 50 µm.

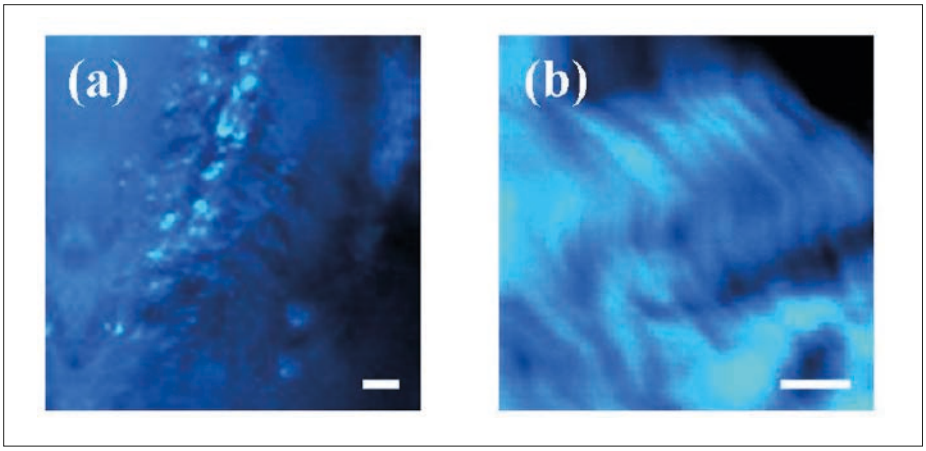

Fig. 3: SHG images from volunteer #1 corresponding to the corneal limbus (a) and the sclera (b). Scale bar: 50 µm.

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More on 2-photon-microscopy: http://bit.ly/WAS-2PM

[1] References: http://bit.ly/IM-Bueno2