Multimodal nonlinear imaging of the human cornea

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Abstract
Purpose: To evaluate the potential of third-harmonic generation (THG) microscopy combined with second-harmonic generation (SHG) and two-photon excited fluorescence (2PEF) microscopies for visualizing the microstructure of the human cornea and trabecular meshwork based on their intrinsic nonlinear properties.

Methods: Fresh human corneal buttons and corneoscleral discs from an eye bank were observed using a multiphoton microscope incorporating a titanium:sapphire laser and an optical parametric oscillator for the excitation and equipped with detection channels in the forward and backward directions.

Results: We elucidate original contrast mechanisms of THG signals in cornea with physiological relevance. THG microscopy with circular incident polarization detects microscopic anisotropy and reveals the stacking and distribution of stromal collagen lamellae. THG imaging with linear incident polarization also reveals cellular and anchoring structures with micrometer resolution. In edematous tissue, a strong THG signal around cells indicates the local presence of water. Additionally, SHG signals reflect the distribution of fibrillar collagen and 2PEF imaging reveals the elastic component of the trabecular meshwork and the fluorescence of metabolically active cells.

Conclusions: The combined imaging modalities of THG, SHG, and 2PEF provide key information about the physiological state and microstructure of the anterior segment over its entire thickness, with remarkable contrast and specificity. This imaging method should prove particularly useful for assessing glaucoma and corneal physiopathologies.

Introduction
Non-invasive optical methods that enable in vivo or in situ visualization of tissue components are of particular relevance in ophthalmology because they provide key information about the physiology and diseases of the eye. Optical coherence tomography (OCT) 1 and confocal reflectance microscopy 2 are two commonly used techniques for obtaining in situ images of the anterior segment of the eye. These two techniques detect scattered light, and provide three-dimensional cell-scale information. However, as their contrast mechanism relies on spatial variations of refractive indices, they may sometimes offer limited contrast and specificity. An alternative promising method for obtaining virtual biopsies from intact tissue is multiphoton microscopy (MPM) 3. MPM relies on the nonlinear excitation of fluorescent molecules or on harmonic generation: two or three photons from a pulsed infrared focused laser beam interact simultaneously with a molecule or structure to produce one photon in the visible range (Fig. 1).

Signal generation in MPM occurs in a confined volume, resulting in three-dimensional sub-cellular spatial resolution. A specific advantage of MPM is that fluorescence and harmonic images can be recorded using the same excitation source but separate detectors to visualize several endogenous sources of contrast. This multimodal capability provides structural and biochemical information on unstained samples which is not accessible using other noninvasive methods.

2PEF imaging with 700-900nm excitation can detect the distribution of endogenous chromophores such as nicotinamide adenine dinucleotide (phosphate) (NAD(P)H), flavins, retinoids, lipofuscin, elastin, and others 4. Previous studies have reported the use of 2PEF for visualizing corneal epithelial cells, limbus stem cells, and stromal keratocytes 5,6. SHG occurs at exactly half the excitation wavelength, and is obtained only from dense noncentrosymmetrical structures 4,7 such as fibrillar...
collagen. SHG microscopy has been shown to be a sensitive probe of the structural organization of collagen in tissues \[^{10-12}\] and is therefore an effective approach for imaging collagen lamellae in the corneal stroma \[^{7,13-16}\].

Third harmonic generation (THG) is an additional contrast mechanism\[^{17,18}\] that is easily combined with SHG/2PEF imaging. THG does not require molecular asymmetry and can be produced by any medium, unlike SHG. However no signal is generally observed from homogeneous media, because of destructive interference resulting from the axial phase shift experienced by the excitation beam near focus (Gouy shift) \[^{17}\]. Instead, THG is obtained from optical inhomogeneities whose sizes are comparable to the beam focus. When the beam is focused at the interface between two media (a) and (b), the signal approximately scales as \(|\alpha_a - \alpha_b|^2\), where \(\alpha = x^{(3)} / n_{3\omega} (n_{3\omega} - n_{\omega})\), \(x^{(3)}\) is the third-order nonlinear susceptibility, \(n_{3\omega}\) is the refractive index at the harmonic frequency, and \((n_{3\omega} - n_{\omega})\) is the refractive index dispersion \[^{19}\]. This nonlinear contrast mechanism produces highly contrasted images, and in particular a strong signal is observed at the interface between an aqueous medium (e.g. cell cytoplasm) and a lipidic, mineralized or absorbing organelle a few 100s of nm in size \[^{19}\]. Recent studies have shown that THG can be used to visualize the morphology of unstained tissues \[^{20-22}\], and to image embryo morphogenesis in small organisms \[^{23}\]. Besides this basic contrast mechanism, THG can also detect birefringence using appropriate polarization of the excitation beam \[^{24}\].

In this study we evaluate the combination of THG, SHG and 2PEF microscopy for imaging intact human eye tissue and we unravel some original nonlinear optical properties of the cornea and of the trabecular meshwork. We show that these contrast mechanisms can provide three-dimensional (3D) images with micrometer resolution of several key tissue components over the entire thickness of the anterior eye segment, which should prove interesting in ophthalmologic research.

**Methods**

*Multimodal multiphoton microscopy*

Imaging was performed on a custom-built laser scanning 2PEF-SHG-THG microscope equipped with detection channels in the backward (epi) and forward (trans) directions (see Fig.1) \[^{19}\]. Excitation was performed using a titanium:sapphire oscillator (Coherent Inc., USA) and a synchronously pumped optical parametric oscillator (OPO) (APE, Germany) delivering 100-150 fs pulses at the focus of the objective. The microscope incorporated galvanometer mirrors (GSI Lumonics), motorized water-immersion objectives (20× 0.95 numerical aperture (NA) and 60× 1.2 NA, Olympus, Japan), photon-counting photomultiplier modules (ET Enterprises Ltd., UK), lab-designed counting electronics, dichroic mirrors and filters (Chroma Technology Corp, USA, and Semrock, USA). For THG imaging, red-shifted excitation wavelengths were used (typ. 1200 nm) so that two-photon absorption by endogenous absorbers was minimized and higher pulse energies could be used while preserving cell viability \[^{18}\]. Simultaneous THG/SHG imaging was performed with trans-detection of both signals, unless otherwise stated. The incident polarization was controlled by inserting a polarizer and a quarter wave plate before the scanners. 2PEF imaging was performed with 730 nm excitation to excite cellular fluorescence, except for trabeculum imaging where combined 2PEF/SHG imaging was performed with 860 nm excitation to enhance elastin contrast compared to cellular contrast. 2PEF was usually epidetected, and 390-450 nm bandpass (resp. GG455 filter) was used upon 730 nm (resp. 860 nm) excitation. High-resolution images were recorded using a 1.2 NA 60× (water immersion) objective resulting in 1.2 µm axial resolution in THG images at the tissue surface. Unless otherwise specified, larger-scale images were recorded using the 20× objective with its pupil under-filled (resulting in 0.75 effective NA) providing 3.5 µm axial (and sub-µm lateral) resolution in THG images. Power after the objective was typically 100 mW with 1200 nm excitation and 20-60 mW with 730-860 nm excitation, and was adjusted with imaging depth to compensate for signal attenuation. Acquisition time was 3-20 µs/pixel (i.e. typically 1-3s for a 512×512 image).

*Samples preparation*

The study was conducted according to the tenets of the Declaration of Helsinki and the French legislation for scientific use of human corneas, and was approved by the French Society of Ophthalmology Ethics Committee. We analyzed fresh human corneal buttons obtained from patients that underwent penetrating keratoplasty mainly for post-traumatic localized corneal scarring, allowing us to image healthy portions of the cornea. Immediately after their removal, the whole trephined corneal buttons were placed in Hanks medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% Dextran T500 (Sigma-Aldrich) to avoid edema. Corneal buttons were maintained between two 150µm-thick glass coverslips to flatten the corneal surface and imaged from the epithelium side or the
endothelium side, depending of the experiment. When estimating epidetected SHG and THG signals, the bottom glass lamella was covered with black tape to minimize light reflection at the glass-liquid interface.

Trabeculum was imaged in human corneas obtained from the Banque Française des Yeux (French Eye Bank) of Paris that were unsuitable for transplantation mostly because of low endothelial cell density. Corneoscleral discs were stored using the organ culture technique in CorneaMax medium (Eurobio, Courtaboeuf, France). For imaging, they were maintained in a custom-made acrylic glass dish filled with Hanks medium and observed from the endothelium side.

Results

Corneal epithelium

Representative multiphoton images of the corneal epithelium in slightly edematous corneas are presented in Fig.2 and in Movie 1. High NA THG imaging with 1.2 µm excitation provides a detailed view of the epithelium architecture, by revealing cells and nuclei boundaries. Since no THG can be obtained from a homogeneous isotropic medium with normal dispersion, the observed signals result from sub-µm bulk heterogeneity. Their origin can be understood using numerical calculations of THG by model geometries. We calculated the relative THG signal obtained from interfaces and slabs of various thicknesses and orientations with respect to the laser beam (Fig.2g,h), using the general model described in ref 25. Nuclear membrane visibility results from optical contrast between nuclei and cytoplasm, creating an interface between two bulk media. Cell-cell junction contrast is of a different nature and can only be explained by the presence of a sizable "slab" of intercellular medium. This contrast makes it possible to readily distinguish squamous cells, wing cells, and deeper basal cells based on their size and morphology. Figure 2f shows an XZ reprojected THG image of the epithelium and anterior stroma, where the epithelial stratification is clearly visualized. We note that this signal depends on the size of intercellular space, so that osmotic stress may enhance cell visibility. Additional punctuate THG signals in the cytoplasm originate from lipidic or dense organelles. In a complementary manner, 2PEF imaging with 730 nm excitation using 390-450 nm bandpass detection reveals fluorescent cytoplasmic organelles emerging over a diffuse background. These signals most likely correspond to NAD(P)H fluorescence as discussed in earlier studies. We note that these combined THG/2PEF images provide an immediate diagnosis of epithelium quality.

Figure 1. Principles of multiphoton/multiharmonic imaging. (a) SHG/2PEF with excitation in the 730-860 nm range. (b) THG/SHG with 1200 nm excitation. (c) Experimental arrangement and signal directionality. Acquisition is performed parallel to the surface of the cornea. Images presented in this article are either the XY acquisitions, or XZ-reprojections. (d) Histological slice of a human cornea, shown for comparison with the nonlinear microscopy images. Scale bar: 100µm.

Figure 2. Representative multiphoton images of the corneal epithelium. (a) XY view of an edematous cornea. (b) XZ-reconstruction showing the epithelial stratification. (c) Corresponding 2PEF image. (d) Thinner epithelium in a non-edematous cornea. (e) XZ-reconstruction showing the epithelial stratification. (f) Corresponding 2PEF image.
Figure 2. THG/SHG/2PEF imaging of epithelium and epithelium-stroma junction in an unstained human cornea with light edema. (a-d) large-scale imaging of an unstained cornea. See Movie1 for the full stacks. (a,b) THG/2PEF images of the epithelium recorded 38 µm below the surface. (c),(d) THG/SHG images of the epithelium-stroma junction recorded 43 µm below the surface. (e-f) 1.2 NA THG imaging of a fresh epithelium: (e) representative XY image from a Z-stack; (f) representative XZ reprojection from the same data. (g,h) Numerical calculations of THG from lateral and axial structures excited by a focused Gaussian beam (1.2 NA): (g) Signal from slabs of variable thickness, mimicking cell-cell interfaces; (h) Signal from interfaces between bulk media as a function of interface distance from the focus, mimicking cytoplasm-nucleus interface. Scale bars 50 µm.

Epithelial-stromal junction
Multimodal images of the epithelial-stromal junction are shown in Fig.2 and in movie 1. Basal epithelial cells are adjacent to the fluorescent Bowman’s layer beyond which a 20-30 µm thick region appears dark in the THG images. This layer exhibits a relatively uniform or speckle-like SHG signal, consistent with the disruption of lamellar organization of the collagen at the anterior stroma. As seen in Fig.2, this region also contains ribbon-like anchoring structures visible in the THG images that assemble into fascicles connecting the epithelium to the stroma. Since these structures are visible in the THG images but not in SHG, they likely exhibit a density contrast compared to collagen-I fibrils in the stroma, which may be consistently explained by the presence of other collagen types or extracellular matrix components. Combined SHG/THG imaging of the epithelial-stroma junction could therefore be an effective way to study abnormal adhesion complexes involved in common corneal pathologies. Finally, subbasal nerve fibers are readily visible in THG images (Fig.3b) and in 2PEF images with a lower contrast.

Harmonic imaging of stromal organization
In non edematous (transparent) human corneas, multimodal images can be recorded over the entire corneal thickness with little loss in resolution. An example of integral SHG/THG imaging is shown in Fig.3 and in movies 2 and 3. As mentioned in previous studies, corneal SHG signals originate from the
collagen fibrils that compose the ≈2 µm-thick orthogonally stacked stromal lamellae. Since individual fibrils are not resolved because of their small diameter (35 nm) and dense packing, harmonic emission results from interference processes governed by coherence lengths that are different in the forward and backward directions. Forward-detected SHG images exhibit striated features that likely reflect the orientation and distribution of the fibrils (see Fig.3b). Backward-SHG (B-SHG) images result from a shorter coherence length and appear as relatively uniform or speckle-like at all depths. THG images provide complementary information. As discussed later, stromal THG arise from differences in anisotropy between successive lamellae, so that XZ-projected THG images reveal the stacked organization of the stroma with sub-lamellar resolution (Fig.3c). Combined SHG-THG imaging provides a rich description of the lamellar organization of the intact stroma over its entire thickness. One striking feature of these images is that they reveal the different large-scale organizations of collagen lamellae at successive depths, as exemplified in Fig.3b. Tissue-scale heterogeneity is more pronounced in the anterior stroma, whereas the posterior stroma exhibits a more regular, long-range stacked organization (Fig.3c). Defects are superimposed to this contrast in THG images, suggesting the presence of folds or cracks throughout the entire stroma which may be intrinsic or due to the flattening of the cornea during the measurement. They appear to be a continuation of the anchoring structures discussed above (movie4) and are also visible in B-SHG images (Fig.3d).

![Figure 3. THG/SHG imaging of stroma organization at different depths. (See also Movie2, Movie3, and Movie4.)](image)

(a) XZ reprojection of a series of THG / SHG images recorded with the epithelium (top) facing the objective. NA≈0.75, scale bars 20 µm(X)×100 µm(Z), Z-step size: 2 µm. (b) XY images recorded at depths indicated in (a). Scale bar 100 µm. (c) XZ reprojections of THG stacks showing lamellae organization in the anterior and posterior stroma. NA=1.2, scale bars 20 µm. (d) Combined THG / B-SHG images recorded in the posterior stroma. NA=1.2, scale bar 50 µm.
**THG with circular polarization detects micron-scale corneal anisotropy**

The above THG imaging experiments were performed with linearly polarized laser light. Indeed, no THG is obtained from isotropic media illuminated with circularly polarized light, even in the presence of heterogeneities. Conversely, TH with circular incident polarization can be used to specifically detect anisotropy with micrometer resolution. For example, media with strong linear anisotropy (birefringence) can efficiently generate TH with circularly polarized excitation. The case of layered stromal lamellae bears additional subtlety because adjacent lamellae exhibit perpendicular fibril orientations, i.e., they may be viewed as stacked slabs with alternate anisotropy directions (noted x and y). When the excitation beam (propagating along direction z) is focused near the interface between two x-y lamellae, harmonic light from the first lamella emerges with a polarization state different from the one from the second lamella. This creates an effective heterogeneity, so that even in the case of weak birefringence, the Gouy-shift-induced destructive interference is not complete and a THG signal is observed at the interface. Excitation with circular polarization is therefore a means to specifically detect anisotropy variations. In Fig.4 and movie5, a section of the anterior stroma was imaged using SHG, THG with linear incident polarization (lin-THG), and THG with circular incident polarization (circ-THG). Lin-THG reveals optical heterogeneities such as epithelial cell boundaries and stromal keratocytes (discussed below), whereas circ-THG is obtained specifically from lamellae interfaces. When going from linear to circular excitation, cell signals are decreased by a factor of about 20 while lamellae signals are increased 2-3 times, providing a convenient means to distinguish the two contributions. We point out that circ-THG and SHG signal maxima are generally not correlated, suggesting that strongest SHG is obtained inside lamellae whereas circ-THG is observed at interfaces.

![Figure 4. THG/SHG cornea imaging with linear and circular incident polarization.](image)

**Visualization of keratocytes network and of edema**

In non edematous fresh corneas, relatively weak THG signals are also obtained from the stromal keratocytes (see e.g. Fig.5a). The ratio of peak keratocytes THG over peak stromal THG is typically $\rho = 1.7 \pm 0.5$. However, this signal is significantly enhanced in edematous stroma. This is illustrated in Fig.5b, showing THG/2PEF images recorded in the same cornea as in Fig.5a after 24h storage in a hypotonic culture medium in order to induce corneal swelling (Hanks medium with 1% dextran...
supplementation). Cell-to-stroma signal ratio increases to $\rho \approx 4-9$, and the keratocytes network and interconnections become readily visible. This THG signal increase is not consistently correlated with changes in cell fluorescence, as shown in Fig. 5b. This indicates that the THG signal reflects the local swelling of the tissue around cells at the onset of edema, and probably detects a water layer around cells according to the contrast mechanisms illustrated by the numerical calculations in Fig. 2g, h. In strongly edematous corneas however (Fig. 5c, movie6, movie7), keratocytes exhibit strong fluorescence reflecting their metabolic activity besides strong THG signal. Under such conditions, THG/SHG/2PEF images indicate that 10-100 $\mu$m large vacuoles devoid of fibrillar collagen are present between stromal lamellae, and THG/2PEF imaging reveal the presence of cells within these regions, forming bridges between the disconnected lamellae. We note that a strong 2PEF signal is observed from the Descemet’s membrane that can be attributed to elastin.

We also observed that THG from keratocytes is enhanced to a lesser extent in corneas that have been kept in a storage medium containing phenol red. In this commonly used protocol, the observed increased THG from cellular structures may be attributed to resonant enhancement through two- or three-photon absorption\(^{28}\), since phenol red linear absorption peaks at 430 nm and 560 nm. We point out that although this protocol may be used to enhance keratocytes visibility in THG images, it prevents 2PEF imaging because of increased background fluorescence and of photodamage onset.

![Figure 5. THG/2PEF imaging of keratocytes and water accumulation in edematous corneas. (a) Fresh non-edematous cornea. (b1, b2) Same cornea after 24h in 1% dextran solution. Strong THG is observed around keratocytes. (c1-3) THG-2PEF–SHG imaging of a cornea with strong edema. Top images are XY images and bottom images are XZ reprojections. Images (a), (b1) and (c1) are presented with similar color scales. Excitation wavelength for 2PEF: 730 nm. Scale bars 50 $\mu$m. Z-step size: 0.5 $\mu$m. See also Movie6 and Movie7.](image)

**Endothelium**

Fig. 6 shows typical THG and 2PEF images of the endothelium, and a THG image of the endothelial-stromal junction (right). THG is mainly observed from cytoplasmic organelles and, to a lesser extent, from nuclear membranes. Like in the case of the epithelium, fluorescence may be attributed mostly to mitochondria, delineating the cell nucleus and boundary as dark regions. Both image modalities exhibit heterogeneity in cell-to-cell signal level, suggesting that they may be used to assess cellular metabolism. When imaging the endothelium at a larger scale, the detailed morphological information present in THG/2PEF images provides a direct diagnosis of tissue quality with potential relevance in
e.g. grafting operations. Finally, THG images acquired 10 µm above the endothelial cells reveal a hexagonal array of fibrous patches (Fig. 6c), producing a signal typ. 5±2× dimmer than cell components. These structures likely correspond to Descemet's membrane excrescences that connect the endothelial cells to the stroma.

**Figure 6.** THG/2PEF imaging of endothelial structures. (a,b) representative THG/2PEF images of endothelial cells. (c) THG images of junction structures located between the endothelium and the Descemet's membrane. Scale bars 20 µm.

**Trabecular meshwork**

Representative 2PEF/SHG images of the trabecular meshwork are shown in Fig. 7. They take advantage of the strong endogenous fluorescence of elastin, which is readily detected along with collagen SHG 4, 29. Fig. 7 shows a prominent Schwalbe's line composed of fibrillar collagen oriented parallel to the limbus and covered by endothelial-like cells. The anterior edge of the meshwork shows numerous intermingled cord-like structures composed of elastic fibers and a few collagen fibrils. In the inner corneoscleral meshwork located closest to the anterior chamber, the cord-like trabecular lamellae form a loose three-dimensional network. In the juxtacanalicular meshwork, multiphoton imaging is somewhat restricted by the presence of pigmented structures exhibiting low photodamage threshold that may correspond to melanin granules within giant macrophages. We note that THG images also reflect the meshwork structure, without additional specificity.

**Figure 7.** 2PEF/SHG imaging of the trabeculum. Excitation wavelength, 860 nm. The schematic depicts the imaging geometry. Visible structures include endothelial cells (ec), trabecular meshwork (m), and Schwalbe's line (dashed line). Scale bar 100 µm.

**Directionality of harmonic emission**

We analyzed the directionality of the multiphoton signals in order to assess the applicability of this methodology for in vivo imaging. 2PEF light is emitted isotropically at the focus of the objective and epidetection is easily achieved. The situation is different in the case of harmonic generation, that predominantly occurs in the direction of propagation of the laser beam. Here, epidetection generally relies on the detection of the weaker backward-emitted component, or on internal backscattering of the forward-emitted component within the tissue 27.
In transparent (non edematous) corneas, we found that the ratio of forward- to backward- SHG signals was 2-3. However SHG and B-SHG signals are qualitatively different, because they are characterized by different coherence lengths and probe different scales within the sample. B-SHG images are more homogeneous and do not reflect the collagen fibrils orientation like forward SHG images. The ratio of forward- to backward- THG emission was significantly higher (>20), so that epi-THG imaging was generally not practical under our usual conditions. Finally, on edematous corneas (which are more scattering) and when using an objective with a large field-of-view to enhance the collection of diffuse light, we estimated that at least 10% of the forward-emitted THG/SHG signals could be detected in the epi-channel, depending of the degree of edema.

Discussion
This study shows that multimodal THG-SHG-2PEF imaging is a powerful approach for visualizing the microstructure of the human cornea and trabecular meshwork. In particular, we report original findings concerning the application of THG microscopy to human eye tissue. Subtle nonlinear optical effects provide insight into the stromal microstructure as well as information on local swelling. We note that harmonic imaging provides superior contrast from isotropic and/or anisotropic media compared to techniques relying on linear scattering, owing to the coherent nonlinear nature of the signal generation process. Moreover cornea is only weakly fluorescent, and THG is an interesting complement/alternative to fluorescence for morphological imaging. THG reveals heterogeneities with sizes of a few 100 nm (fig 1g), such as non-aqueous cytoplasmic organelles (fig. 6), interfaces, fibrillar structures and cell processes (figs. 2 and 5), whereas 2PEF specifically reveals fluorescent organelles. Harmonic generation does not involve absorption by the sample, and excitation wavelengths larger than 1 µm can be used for THG/SHG imaging in order to minimize photodamage, and to increase imaging depth e.g. in edematous corneas.

Combined THG/SHG imaging provides a 3D description of the stroma lamellar organization and keratocytes network that should enable their extensive investigation (fig. 3). A remarkable feature is the ability to probe corneal birefringence and to visualize collagen lamellae inserting into Bowman's layer. Changes of these structures are involved in corneal disorders such as keratoconus. Similarly, ultrastructural changes of the adhesion complexes between epithelial basal cell layer and the underlying stroma are found in recurrent corneal erosion and Cogan's dystrophy.

Combined THG/2PEF imaging provides indications on keratocyte physiology and three-dimensional distribution (fig. 5). Previous studies relying on reflectance imaging and immunohistochemical investigations have reported that the keratocyte connection network is a key factor in corneal transparency and wound healing process. In particular, keratocyte migration and activation through the intercellular connections could be involved in refractive surgery regression or haze formation and corneal allografts wound healing. Multiphoton studies of intact tissues could therefore be used to obtain additional information about corneal pathologies.

Our study also shows that nonlinear imaging may provide insight into the mechanisms of transparency loss in swollen corneas. Based on electron micrographs, the loss of short-range ordering of collagen fibrils has been proposed as a possible mechanism for increased light scattering and decreased transparency. Other studies reported the presence of regions devoid of collagen fibrils in edematous corneas that have been called lakes. A previous study based on confocal reflectance microscopy reported that an increase in corneal hydration could result in a better visualization of the kerocytes. Changes in the refractive index of the surrounding tissue was advanced to explain this change. Our THG/2PEF observations performed on intact corneas confirm that water first accumulates around the keratocytes as the cornea swells, and that larger water accumulation regions form between lamellae in strongly edematous corneas. Such changes may be involved in refractive disorder and increased light scattering.

Assessment of endothelial viability is of crucial importance in cornea explants stored in eye banks, as it is the key criterion for the aptness for corneal grafting. Because vital cell tests would require tissue preparation, the evaluation of endothelial cell density and morphology is usually done under standard optical microscopy, often after having exposed the endothelial cells to a mild osmotic shock to improve the otherwise very weak contrast. 2PEF/THG could prove particularly valuable for this purpose by providing superior contrast and additional information about tissue integrity.
Similarly, the pathogenesis of glaucoma should benefit from MPM studies. Most glaucomas are due to an increase in the resistance to aqueous humor drainage through the trabecular meshwork (TM) because of intercellular space narrowing. However, several mechanisms have been proposed to explain this structural change. The effects of glaucoma-targeting drugs and laser trabeculoplasty on the TM also remain poorly understood. Studies based on light or electron microscopy do not allow the accurate evaluation of the intercellular space size or the volume of connective and cellular components volume because of possible preparation artifacts. In contrast, MPM allows in situ measurements of the intercellular spaces size and isolation of the cellular, collagenous and elastic components of the TM.

As a perspective, the applicability of harmonic/fluorescence imaging for in vivo diagnostic applications would be of great interest. Although the backward-detected harmonic signals are weak (particularly in the case of THG), in vivo epidetection will be enhanced by reflections at intraocular interfaces, which should redirect ≈4-10% of the forward-directed emission toward the objective. This prediction is corroborated by the recent report of epidetected THG signals from a fixed mouse eye. This effect will be even more pronounced in opaque corneas, so that epidetection may be a direct indication of edema. Epi-THG can be further enhanced by using large-field optics and shorter pulses, since third-order signals are inversely proportional to the pulse duration squared. Extensive studies will be necessary however to determine which experimental conditions can be safely used in vivo. We also point out that multiphoton imaging is easily combined with femtosecond pulse-induced ablation, so that THG-SHG-2PEF imaging may be used to visualize the cornea microstructure after laser ablations.

In conclusion, our study unravels original nonlinear optical properties of the anterior segment of the human eye with physiological relevance, and elucidates contrast mechanisms involved in THG microscopy of the cornea. THG/SHG imaging provides a detailed view of stromal microarchitecture. Also of particular relevance are the THG/2PEF signals reflecting the status of the keratocyte network, an essential actor in corneal inflammatory responses and wound healing processes. Finally, in situ imaging of the connective and cellular components of intact trabecular meshwork may provide crucial information about glaucoma physiopathology. The contrast and specificity provided by nonlinear imaging and the ability to image the entire thickness of the anterior segment should generally prove relevant for biomedical research.

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References


Movies

Movie 1. Epithelium and stroma (human cornea with edema): 2PEF+THG+SHG 400×534×300 µm stack. 2PEF excitation wavelength: 730 nm. THG/SHG excitation wavelength: 1200 nm. Numerical aperture ≈0.75 (20× objective). Scale bar 100 µm.

Movie 2. Entire cornea: THG+SHG 315×620×590 µm stack showing successive organizations of the stroma. THG/SHG excitation wavelength: 1200 nm. Z-step size: 2 µm. Numerical aperture ≈0.75 (20× objective). Scale bar 100 µm.


Movie 4. THG imaging of epithelium-stroma junction and stroma, showing anchoring fibers followed by "folds" in the stroma. 470×490×170 µm stack. Z-step size: 1 µm. Numerical aperture ≈0.75 (20× objective). Scale bar 100 µm.

Movie 5. Changing the polarization of the excitation beam from linear to circular in THG imaging suppresses the signal from isotropic (non-birefringent) media, and can be used to isolate the signal from birefringent structures. Z-stack of epithelium-stromal junction imaged using THG with linear incident polarization (Lin-THG, blue), THG with circular incident polarization (Circ-THG, red), and SHG with circular incident polarization (green). 630×390×86 µm stack. Z-step size: 2 µm. Numerical aperture ≈0.75 (20× objective).
