Multimodal Nonlinear Imaging of the Human Cornea

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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 under 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. Original contrast mechanisms of THG signals in cornea with physiological relevance were elucidated. THG microscopy with circular incident polarization detected microscopic anisotropy and revealed the stacking and distribution of stromal collagen lamellae. THG imaging with linear incident polarization also revealed cellular and anchoring structures with micrometer resolution. In edematous tissue, a strong THG signal around cells indicated the local presence of water. Additionally, SHG signals reflected the distribution of fibrillar collagen, and 2PEF imaging revealed 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. (Invest Ophthalmol Vis Sci. 2010;51:2459–2465) DOI:10.1167/iovs.09-4586

Noninvasive 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 and confocal reflectance microscopy 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, because 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). 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 subcellular 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 that is not accessible using other noninvasive methods.

Two-photon excited fluorescence (2PEF) imaging with 700- to 900-nm excitation can detect the distribution of endogenous chromophores such as nicotinamide adenine dinucleotide (phosphate) (NAD(P)H), flavins, retinoids, lipofuscin, elastin, and others. Previous studies have reported the use of 2PEF for visualizing corneal epithelial cells, limbus stem cells, and stromal keratocytes. Second-harmonic generation (SHG) occurs at exactly half the excitation wavelength and is obtained only from dense noncentrosymmetric structures, such as fibrillar collagen. SHG microscopy has been shown to be a sensitive probe of the structural organization of collagen in tissues and is therefore an effective approach for imaging collagen lamellae in the corneal stroma.

Third-harmonic generation (THG) is an additional contrast mechanism that is easily combined with SHG/2PEF imaging. THG does not require molecular asymmetry and can, unlike SHG, be produced by any medium. 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). Instead, THG is obtained from optical inhomogeneities whose sizes are comparable to that of the beam focus. When the beam is focused at the interface between two media (a) and (b), the signal approximately scales as \( \chi^3 / n_{ab} (n_{aw} - n_o) \), where \( \chi^3 \) is the third-order nonlinear susceptibility, \( n_{aw} \) is the refractive index at the harmonic frequency, and \( n_{aw} - n_o \) is the refractive index dispersion. This nonlinear contrast mechanism produces highly contrasted images; 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 measuring a few hundred nanometers. Recent studies have shown that THG can be used to visualize the morphology of unstained tissues and to image embryo morphogenesis in small organisms. Besides this basic contrast mechanism, THG can also detect birefringence using appropriate polarization of the excitation beam.
In this study, we evaluated the combination of THG, SHG, and 2PEF microscopy for imaging intact human eye tissue, and we unraveled 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 ophthalmic 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). Excitation was performed using a titanium-sapphire oscillator (Coherent Inc., Santa Clara, CA) and a synchronously pumped optical parametric oscillator (APE, Berlin, Germany) delivering 100- to 150-fs pulses at the focus of the objective. The microscope incorporated galvanometer mirrors (GSI Lumonics, Bedford, MA), motorized water-immersion objectives (20× NA; Olympus, Tokyo, Japan), photon-counting photomultiplier modules (ET Enterprises Ltd., Uxbridge, Middlesex, UK), laboratory-designed counting electronics, dichroic mirrors and filters (Chroma Technology Corp., Bellows Falls, VT; Semrock, Rochester, NY). For THG imaging, red-shifted excitation wavelengths were used (typically 1200 nm) so that two-photon absorption by endogenous absorbers was minimized and higher pulse energies could be used while preserving cell viability. Simultaneous THG/SHG imaging was performed with transdetection of both signals, unless otherwise stated. Incident polarization was controlled by inserting a polarizer and a quarter-wave plate before the scanners. 2PEF imaging was performed with 740-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 with cellular contrast. 2PEF was usually epiphotographed, and 390- to 450-nm bandpass (respectively GG455 filter) was used with 730-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 underfilled (resulting in 0.75 effective NA), providing 3.5-μm axial (and submicrometer lateral) resolution in THG images. Power after the objective was typically 100 mW with 1200 nm excitation and 20 to 60 mW with 730 to 860 nm excitation, and it was adjusted with imaging depth to compensate for signal attenuation. Acquisition time was 3 to 20 μs/pixel (i.e., typically 1–3 seconds for a 512 × 512 image).

**Sample Preparation**

The study was conducted according to the tenets of the Declaration of Helsinki and French legislation for scientific use of human corneas, and it was approved by the French Society of Ophthalmology Ethics Committee. We analyzed fresh human corneal buttons obtained from patients who underwent penetrating keratoplasty primarily for posttraumatic localized corneal scarring, allowing us to image healthy portions of the cornea. Immediately after their removal, 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 150-μm-thick glass coverslips to flatten the corneal surface and were imaged from the epithelium side or the endothelium side, depending on the experiment. When estimating epidelected 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 French Eye Bank (Paris, France) that were unsuitable for transplantation primarily because of low endothelial cell density. Corneoscleral discs were stored in medium (CorneaMax; Eurobio, Courtaboeuf, France) using the organ culture technique. For imaging, they were maintained in a custom-made acrylic glass dish filled with Hanks medium and were observed from the endothelium side.

**RESULTS**

**Corneal Epithelium**

Representative multiphoton images of the corneal epithelium in slightly edematous corneas are presented in Figure 2 and in Movie S1 (all Movies available at http://www iovs .org/cgi/content/full/51/5/2459/DC1). High numerical aperture THG imaging with 1.2-μm excitation provides a detailed view of the epithelium architecture by revealing cells and nuclei boundaries. Because no THG can be obtained from a homogenous isotropic medium with normal dispersion, the observed signals result from submicrometer bulk heterogeneity. Their origin can be understood using numerical calculations of...
NAD(P)H fluorescence, as discussed in earlier studies. We observed a diffuse background. These signals most likely correspond to cellular organelles. Nuclear membrane visibility results from optical contrast between nuclei and cytoplasm, creating an interface between two bulk media. This contrast makes it possible to readily distinguish epithelial stratification is clearly visualized. We note that this could therefore be an effective way to study abnormal adhesion complexes involved in common corneal abnormalities. Finally, subbasal nerve fibers are readily visible in THG images (Fig. 3b) and in 2PEF images with a lower contrast.

Epithelial-Stromal Junction
Multimodal images of the epithelial-stromal junction are shown in Figure 2 and in Movie S1. Basal epithelial cells are adjacent to the fluorescent Bowman’s layer, beyond which a 20- to 30-μm-thick region appears dark in the THG images. This layer exhibits a relatively uniform or specklelike SHG signal, consistent with the disruption of lamellar organization of the collagen at the anterior stroma. As seen in Figure 2, this region also contains ribbonlike anchoring structures visible in the THG images that assemble into fascicles connecting the epithelium to the stroma. Because these structures are visible in the THG but not in the SHG images, they are likely composed of extra-cellular matrix components other than collagen I fibrils. Combined SHG/THG imaging of the epithelial-stroma junction could therefore be an effective way to study abnormal adhesion complexes involved in common corneal abnormalities.

Harmonic Imaging of Stromal Organization
In nonedematous (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 Figure 3 and in Movies S2 and S3. As mentioned in previous studies, corneal SHG signals originate from the collagen fibrils that compose the ~2-μm-thick orthogonally stacked stromal lamellae. Given that individual fibrils are not resolved because of their small diameters (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 relatively uniform or specklelike at all depths.

THG images provide complementary information. As discussed later, stromal THG arises from differences in anisotropy between successive lamellae so that XZ-projected THG images reveal the stacked organization of the stroma with sublamellar 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 Figure 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 that may be intrinsic or caused by flattening of the cornea during measurement. They appear to be a continuation of the anchoring structures discussed previously in Epithelial-Stromal Junction (Movie S4) and are also visible in B-SHG images (Fig. 3d).

Detection of Micrometer-Scale Corneal Anisotropy by THG with Circular Polarization
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 circularly polarized detection 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. Layered stromal
lamellae bear additional subtlety because adjacent lamellae exhibit perpendicular fibril orientations; that is, 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 a polarization state different from the one from the second 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 Figure 4 and Movie S5, 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, whereas circ-THG is obtained specifically from lamellae interfaces. When going from linear to circular excitation, cell signals are decreased by a factor of ~20, whereas lamellae signals are increased two to three 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 the strongest SHG is obtained inside lamellae, whereas circ-THG is observed at interfaces.

Visualization of Keratocyte Network and of Edema

In nonedematous fresh corneas, relatively weak THG signals were also obtained from the stromal keratocytes (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 Figure 5b, showing THG/2PEF images recorded in the same cornea as in Figure 5a after 24-hour storage in a hypotonic culture medium to induce corneal swelling (Hanks medium with 1% dextran supplementation). Cell-to-stroma signal ratio increases to \( \rho \approx 4-9 \), and the keratocyte network and interconnections become readily visible. This THG signal increase is not consistently correlated with changes in cell fluorescence, as shown in Figure 5b, indicating 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 Figures 2g and 2h. In strongly edematous corneas, however (Fig. 5c; Movie S6, Movie S7), keratocytes exhibit both strong fluorescence reflecting their metabolic activity and strong THG signal. Under such conditions, THG/SHG/2PEF images indicate that 10- to 100-μm large vacuoles devoid of fibrillar collagen are present between stromal lamellae, and THG/2PEF imaging reveals 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 because phenol red linear absorption peaks at 430 nm and 560 nm. We point out that although this protocol may be used to enhance keratocyte visibility in THG images, it prevents 2PEF imaging because of increased background fluorescence and of photodamage onset.

### Endothelium

Figure 6 shows typical THG and 2PEF images of the endothelium and a THG image of the endothelial-stromal junction (right). THG is primarily observed from cytoplasmic organelles and, to a lesser extent, from nuclear membranes. As in the epithelium, fluorescence may be attributed primarily to mitochondria, delineating the cell nucleus and boundary as dark regions. Both image modalities exhibit heterogeneity in the 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 morphologic information present in THG/2PEF images provides a direct diagnosis of tissue quality with potential relevance in, for example, grafting operations. Finally, THG images acquired 10 μm above the endothelial cells reveal a hexagonal array of fibrous patches (Fig. 6c), producing a signal typically 5–2× dimmer than cell components. These structures likely correspond to Descemet’s membrane excrescences that connect the endothelial cells to the stroma.

### Trabecular Meshwork

Representative 2PEF/SHG images of the trabecular meshwork are shown in Figure 7. They take advantage of the strong endogenous fluorescence of elastin, which is readily detected along with collagen SHG.4,29 Figure 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 cordlike structures composed of elastic fibers and a few collagen fibrils. In the inner corneoscleral meshwork located closest to the anterior chamber, the cordlike trabecular lamellae form a loose three-dimensional network. In the juxtacanalicular meshwork, multiphoton imaging is somewhat restricted by the presence of pigmented structures exhibiting a 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.

### Directionality of Harmonic Emission

We analyzed the directionality of the multiphoton signals 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, which 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 (nonedematous) 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 homogeneous15 and do not reflect collagen fibril orientation as
do 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 on 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 and information on local swelling. We note that harmonic imaging provides superior contrast from isotropic and anisotropic media compared with techniques relying on linear scattering because of 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 morphologic imaging. THG reveals heterogeneities with sizes of a few 100 nm (Fig. 1g), such as nonaqueous cytoplasmic organelles (Fig. 6), interfaces, fibrillar structures, and cell processes (Figs. 2, 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 to minimize photodamage and to increase imaging depth (e.g., in edematous corneas).

Combined THG/SHG imaging provides a 3-D description of the stroma lamellar organization and keratocyte 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 in 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 3-D 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 allograft wound healing. Multiphoton studies of intact tissues could therefore be used to obtain additional information about corneal abnormalities.

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 better visualization of the keratocytes. A change 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 because it is the key criterion for the aptness for corneal grafting. Given that vital cell tests would require tissue preparation, the evaluation of endothelial cell density and morphology is usually conducted using 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 types of glaucoma are caused by an increase in the resistance to aqueous humor drainage through the trabecular meshwork because of intercellular space narrowing. Several mechanisms have been proposed to explain this structural change. The effects of glaucoma-targeting drugs and laser trabeculoplasty on the trabecular meshwork also remain poorly understood. Studies based on light or electron microscopy do not allow accurate evaluation of intercellular space size or volume of connective and cellular components because of possible preparation artifacts. In contrast, MPM allows in situ measurements of intercellular space size and isolation of the cellular, collagenous, and elastic components of the trabecular meshwork.

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 can be enhanced by reflections at intraocular interfaces, which should direct ~4% to 10% of the forward-directed emission toward the objective. This prediction is corroborated by the recent report of epideected 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 the use of large-field optics and shorter pulses because 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 corneal microstructure after laser surgery.

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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