REVIEW ARTICLE

**Correlation of histology and linear and nonlinear microscopy of the living human cornea**

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**Key words:** confocal microscopy, optical low-coherence reflectometry (OLCR), multiphoton excitation microscopy, second-harmonic generation (SHG) microscopy, third-harmonic generation (THG) microscopy, coherent anti-Stokes Raman scattering (CARS) microscopy, human cornea, corneal folds

The morphology and the function of cellular and noncellular structures in the living human cornea can be determined with modern correlative linear and nonlinear optical microscopic techniques and histology. Correlative microscopy is based on the use of different optical techniques to study the same specimen, ideally at the same location within the specimen, in order to increase the functional and/or morphological understanding of the specimen. A case study to assess the effect of overnight lid-closure on in vivo human corneal morphology is presented to illustrate correlative linear microscopy and optical low-coherence reflectometry. Nonlinear multiphoton excitation microscopy provides functional information on cellular metabolism based on the intrinsic fluorescence from the reduced pyridine nucleotides and the oxidized flavoproteins. Second-harmonic generation microscopy, a scattering process that does not deposit net energy into the tissue, provides structural information on corneal collagen organization. Molecular third-harmonic generation microscopy generates a signal in all materials and it an emerging technique. Coherent anti-Stokes Raman scattering microscopy provides chemical imaging for biology and medicine. The comparison and limitations of these microscopic modalities, linear and nonlinear microscopy applied to the cornea, and a review of some key findings is analyzed. A correlative integration and correlation of linear and nonlinear microscopies to study corneal function and structure is proposed to validate the clinical interpretation of microscopic images of the cornea.

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1. Introduction

The history of the linear and nonlinear optical microscope is inextricably linked to the development of new clinical instruments that play important roles in our understanding of cells and tissues and in the early diagnosis and the prevention of disease. In the domain of linear optics the response of the induced electromagnetic field is linear with the amplitude of the field. Linear optical effects include one-photon absorption, scattering, and fluorescence. Exchange of energy and momentum can occur in linear interactions, e.g. absorption and scattering. Linear optical phenomena are characterized by a induced polarization \( P(t) \) in the material that depends in a linear manner on the electric field strength \( E(t) \):

\[
P(t) = \varepsilon_0 \chi^{(1)} E(t),
\]

the constant of proportionality \( \chi^{(1)} \) is known as the linear susceptibility and \( \varepsilon_0 \) is the permittivity of free space.

Nonlinear optical effects are characterized by the nonlinear response of the induced polarization to the incident electromagnetic field; the response is nonlinear in the amplitude of the field [1, 2]. For the case of nonlinear optics, the optical response is described by expressing the induced polarization \( P(t) \) as a power series of the electric field strength:

\[
P(t) = \varepsilon_0 \chi^{(1)} E(t) + \chi^{(2)} E^2(t) + \chi^{(3)} E^3(t) + \ldots.
\]

The quantities \( \chi^{(2)} \) and \( \chi^{(3)} \) are known as the second- and the third-order nonlinear optical susceptibilities. In writing these two equations an important assumption is made: the polarization of the material at time \( t \), only depends on the instantaneous value of the electric field strength. It follows from this assumption, through the Kramers-Kronig relations, that the medium must be lossless and dispersion less. Typically nonlinear optical phenomena occur in the presence of laser-matter interactions in the presence of very high electric field strengths. At high light intensities, the incident light modifies the induced polarization such that light waves can interact and exchange energy and momentum. Examples of nonlinear optical effects include the Pockels and Kerr electro-optic effects, multiphoton excitation (MPE) [3], second-harmonic generation (SHG), third-harmonic generation (THG) [4], and coherent anti-Stokes Raman scattering (CARS) [5].

Correlative microscopy is based on the use of different optical techniques to study the same specimen, ideally at the same location within the specimen, in order to increase the functional and/or morphological understanding of the specimen. Typically, correlative microscopy could include linear (confocal microscopy, optical low-coherence reflectometry) together with nonlinear microscopy (MPE, SHG, THG, and CARS).

The purpose and the emphasis of this paper is to point out the limitations, and the multiple confounding factors that make the interpretation of corneal functional and morphological images difficult.

1.1 Confocal microscopy

In the last decades there have been improvements in both the resolution and the contrast of optical microscopes. The invention of various types of confocal microscopes, that generate contrast from the processes of linear absorption, scattering, and fluorescence, provides researchers in cell biology with extremely useful tools to investigate the biology of cells in thick, highly scattering tissues. Confocal microscopes are based on the principle of spatial filtering and permit live cell imaging of cells and thick, highly scattering tissues with improved “optical sectioning” and improved contrast as compared to traditional epi-fluorescence microscopes [6].

The first applications of confocal microscopy were applied to cells and tissues that were fixed, and stained with immunohistochemical stains of high specificity to delineate the fine structures in cells such as the cytoskeleton. Only later did researchers apply confocal microscopy to live cells and tissues both ex vivo and in vivo, i.e. three-dimensional imaging in vivo human cornea [7] and in vivo human skin [8].

Two modes of contrast are implemented in confocal microscopy. The first mode generates contrast in the images with reflected and scattered light that occurs at the interfaces with different refractive indexes [6]. A registered stack of optical sections could then be transformed in a digital computer into a three-dimensional volume that visualized the structure of cells and tissues, and these computer generated structures could be visualized from any arbitrary orientation. The second mode generates the image contrast by exciting the fluorescence from either intrinsic naturally occurring fluorescent molecules, for example, reduced pyridine nucleotides, NAD(P)H, and oxidized flavoproteins [6]. Both the reduced nicotinamide adenine dinucleotide, NADH, and the reduced nicotinamide adenine dinucleotide phosphate, NADPH, are denoted as NAD(P)H. The fluorescence of NAD(P)H is in the range of 400–500 nm. Alternatively, contrast in confocal microscopy could be based on fluorescent probes that are genetically over-expressed such as green fluorescent proteins (GFP), or molecular probes that were externally applies to the cells and tissues [6].
1.2 Confocal microscopy of the eye

The application of confocal microscopes to the anterior segment of the eye, specifically the avascular cornea, a highly transparent and organized layered structure provided a new generation of diagnostic instruments [7, 9–12]. The human cornea consists of superficial epithelial cells, intermediate epithelial cells, basal epithelial cells, limbal stem cells, Bowman’s layer, stromal keratocytes, alternating layers of approximately orthogonal bundles of collagen fibers, nerve fibers, and on the posterior side, Descemet’s membrane and a single layer of endothelial cells [11]. There is an interesting history of instrument development and innovation in the design of instruments for corneal biomicroscopy [13].

The development of the scanning slit, clinical confocal microscope is not a new paradigm, but the result of a continuous series of interlinked technical advances starting from the early work of Goldmann to Thaer’s development of the clinical scanning-slit confocal microscope [13]. There were many parallel developments in the advancement of confocal instruments for clinical use and the key figures include Goldmann, Maurice, Svishchev, Baer, Koester, Masters, and Thaer [7, 14]. The major problems that had to be solved in the development of a scanning slit confocal microscope for the clinical examination of the living human eye include how to acquire images from the moving eye, and how to obtain a sufficient signal-to-noise ratio to form high contrast images that are acquired at video rates [15]. A recent book details the story of these clinical developments that resulted in the modern scanning slit clinical confocal microscope [16]. All of these instruments use an incoherent lamp as the light source.

A recent design and development of a laser scanning confocal microscope that is applicable to the in vivo human cornea is based on the Rostock Cornea Module (RCM). This module attaches to a commercial Heidelberg Engineering scanning laser ophthalmoscope that is typically used for retinal imaging [17]. The attached module shifts the focus of the laser beam from the retinal surface to the cornea and the focal position is operator controlled by the motion of an secondary movable lens. This scanning laser instrument has the capacity to produce high contrast confocal images over a 400 × 400 μm field of view. It can also operate in a noncontact mode which may have several clinical advantages over the other types of clinical confocal microscopes that contact the cornea.

Both the scanning slit confocal microscope, manufactured by Nidek, and the RCM unit manufactured by Heidelberg Engineering, have limitations and differences in their performance. Both instruments can operate in the noncontact mode which is important to prevent transmission of microbes from patient to patient, and they avoid the use of the index matching gel. Both instruments can image scars, opacities, and calcifications in the stroma; however, superficial scars and opacities will scatter the incident light and cause a shadow effect on the images from the deeper cellular layers.

All types of clinical microscopes that are designed to image the full thickness of the human cornea have the capability to acquire images from the anterior surface (superficial corneal epithelial cells) to the posterior single layer of corneal endothelial cells. Within the stroma on the anterior side the acellular Bowman’s layer provides a landmark of depth within the stroma. Similarly on the posterior side of the stroma the appearance of the acellular Descemet’s membrane and the easily recognized layer polygonal endothelium provides a depth landmark. What is confounding is the ability to accurately determine the depth of focus within the corneal stroma. Although some clinical instruments provide a movable intermediate lens that can be positioned and moved with high accuracy; that does not necessarily correspond to the accurate depth of the focal plane of the microscope objective within the cornea. Since the keratocyte density and the organization of the corneal collagen fibers varies with depth within the corneal stroma it is important to solve this problem in the next generation of clinical confocal microscopes.

2. Review of key clinical applications with linear confocal microscopy

2.1 Correlative light microscopy of tangential sections and in vivo confocal microscopy

The foundations of confocal microscopy, the history of the biomicroscopy of the eye, a tutorial on the practical techniques of clinical confocal microscopy of the eye, and a review of studies on the clinical examination of the cornea with the confocal microscope for the cases of aging, contact lens wear, corneas with known pathologies, and studies of the morphological changes in the post surgical cornea were reviewed by Böhne and Masters [11]. Many examples of correlative light microscopy of tangential sections from paraffin-embedded ex vivo human corneas that were stained with PAS reagent are shown adjacent to scanning-slit confocal images of similar sections of the living in vivo human cornea. These multiple examples serve to illustrate the value of correlative light microscopy of ex vivo sections of the cornea and the equivalent sections as observed
with confocal microscopy of the in vivo human cornea. It is important to emphasize the comparison of in vivo confocal images of the human cornea and histological studies of ex vivo human corneas in order to minimize errors of interpretation of the clinical images. At the same time, investigators should be aware of the morphological differences between in vivo and ex vivo corneas, as well as the fixation and the staining artifacts of classical histology.

2.2 Long-term effects of contact lens wear on the human cornea: a new corneal degeneration

This paper illustrates the confounding effect of acquiring clinical confocal images of the human cornea with inappropriate optical resolution [18]. We posed the following question: what are the long-term effects of contact lens wear on the human cornea? We answered this question by first acquiring confocal images through the full thickness of the in vivo human cornea, and then by a frame by frame analysis to determine morphological changes within the cornea that are correlated with long-term contact lens wear, and do not occur in subjects that do not wear contact lenses.

The scanning-slit confocal microscope with a 50X/1.0 NA water immersion objective was used to investigate the corneal morphology in long-term contact lens wearers [18]. The authors investigated 13 patients with a history of up to 26 years of soft contact lens wear, 11 patients with a history of up to 25 years of rigid gas permeable contact lens wear, and a control group of 29 normal subjects without a history of contact lens wear. For contact lens wearers epithelial microcystic changes and alterations of endothelial cell morphology were found as described previously. The significant new finding was there were highly reflective panstromal microdot (submicron) deposits in the entire thickness of the stroma for the contact lens wearers. It was concluded that this newly observed stromal microdot degeneration scales with the years of contact lens wear and may be the early stage of a significant corneal disease. Further correlative microscopic studies involved electron microscopy of ex vivo human corneas, and spectroscopic studies of the microdots.

Initially, other groups were not able to observe the stromal microdot deposits because they used a Nipkow disk confocal microscope with a low magnification (20X) and a low NA microscope objective (0.5). The low magnification, low NA microscope objective did not provide the necessary resolution to image the stromal microdot deposits. Alternatively, the Nipkow disk tandem-scanning confocal microscope did not provide sufficient illumination to image the submicron stromal microdot deposits in the cornea.

This study illustrates the requirement of the appropriate optical resolution in studies with the clinical confocal microscope, as well as correlative electron microscopy to validate the clinical conclusions.

2.3 Overnight lid closure and the reversible micro-folds in the stroma of the human cornea

The next study shows the importance of correct experimental protocol to investigate transient phenomena in the in vivo human cornea. This case report illustrates the use of linear confocal microscopy (back scattered and reflected incoherent light) together with optical low-coherence reflectometry (OLCR) to study the physiological question: does overnight lid closure affect the structure of the cornea stroma?

This is an example of correlative studies involves a case report on the investigation of overnight lid closure and the reversible micro-folds in the stroma of the human cornea. We used clinical confocal microscopy and parallel reversible changes in corneal thickness as measured with optical low-coherence reflectometry (OLCR) to validate the interpretation of the confocal images of reversible micro-folds in the human cornea stroma.

The cornea is a specialized tissue, which maintains its normal optical properties from the fluid control exerted by the endothelial pumping function in the presence of an intact epithelial barrier. Failure of either one of these results in corneal fluid accumulation, which can be observed with the slit lamp as corneal haze and possibly epithelial edema. A large increase of corneal thickness may lead to the appearance of corneal folds, which can be easily visualized with the slit lamp. These folds are reversible and may disappear if corneal hydration control has been re-established [19].

On a daily basis the normal human cornea is subjected to hypoxic conditions under the closed lid during sleep [20–21]. It is well known that overnight lid closure results in a reversible, transient, increase of central corneal thickness. A diurnal variation in corneal sensitivity and thickness was studied: the overnight mean corneal swelling was 2.9 percent, and after two hours of open eye conditions with normal blinking, the cornea had deswelled to the same thickness as the previous night [22]. Another study of diurnal variations in human corneal thickness found a mean overnight increase of central corneal thickness to be 5.5 percent [23].
Micro-folds have previously been reported in the human cornea under a variety of conditions and pathology. When one observes human eye bank corneas with the confocal microscope there are numerous micro-folds present in the highly swollen corneas. Also confocal microscopy has verified the presence of micro-folds in keratoconus [24].

The subject underwent a complete ocular examination prior to and after the completion of the study. The examination consisted of biomicroscopy with a slit lamp, and with a clinical confocal microscope. Normal corneal morphology was observed in all corneal layers. The subject, aged 60, had no prior or present history of contact lens wear, no use of topical or systemic medication, nor any prior history of eye or corneal disease.

The method to induce corneal swelling was overnight lid closure. Prior to the onset of the overnight sleep period, one eyelid was bandaged to maintain the closed lid condition overnight. The surgeon carefully applied a no-pressure surgical bandage. Upon waking, the no-pressure surgical bandage was removed immediately before the slit lamp observation, and measurements of corneal thickness and confocal imaging of corneal morphology as described below were performed.

The noninvasive, custom-built, optical low-coherence reflectometer (OLCR) was mounted on a clinical slit lamp from Haag Streit, Switzerland. OLCR has high accuracy and precision to measure corneal thickness [25, 26]. Optical low-coherence reflectometry is a noninvasive interferometric optical technique that forms measures the optical pathlength of ocular tissue such as the cornea [27]. The contrast is formed from regions of tissue that show strong gradients of refractive index such as the air–cornea interface and the posterior cornea-aqueous interface. The design of the optical reflectometer is based on a Michelson interferometer constructed with single-mode optical fibers. Details of the design of the rapid optical pachometer have previously been reported [28]. The OLCR measurements of corneal thickness can be performed with high precision of about one micron and high intra- and inter-session reproducibility [29–32].

Immediately upon opening the patched eye and observing the renewal of normal blinking the corneal thickness was determined at preset intervals with OLCR. Within five minutes of the first determination of corneal thickness the cornea was observed with the scanning slit confocal microscope. It is critical to follow this protocol as the micro-folds begin to disappear with the onset of normal blinking.

Clinical confocal microscopy of the full thickness of the cornea was performed with a scanning slit confocal microscope (Confoscan 2) manufactured by Tomey, Germany. This microscope is based on the scanning slit confocal microscope previously described [6, 7, 11]. The 50X, NA 1.0 water immersion microscope objective was used. The protocol for the corneal examination with the clinical confocal microscope has been previously described [10, 11]. The key point is that in order to observe the transient microfolds within the corneal stroma it is necessary to make the confocal microscopic observations within a few minutes of the initiation of normal blinking. This is critical since the folds rapidly disappear with time and are completely absent within one hour.

The no-pressure surgical bandage which closed the eyelid was tolerated for the lid closure period from 10:00 PM to 8:00 AM. Upon removal of the no-pressure surgical bandage, normal anterior segment morphology was found together with an intact tear film and a normal blinking reflex was observed by slit-lamp examination. The corneal pachometry with OLCR yielded an overnight increase in corneal thickness of about 13 μm, and then a consecutive deswelling in both experiments (see Table 1).

The confocal microscopy examination performed prior to overnight lid closure showed a normal corneal structure in all layers from the corneal surface to the corneal endothelium. At five minutes after opening the lid, following overnight lid closure, confocal microscopy of the central cornea was again performed, and micro-folds were observed in all corneal stromal layers (Figure 1 A, B, C).

When there are micro-folds present in the corneal stroma they appear as a dark band when observed with a clinical confocal microscope. The appearance of a dark band may be explained by the changed orientation of the keratocyte nuclei within the micro-folds. Localized tilting of the stromal lamellae may also change other optical properties. All of these combined changes may result in a decrease in the amount of light that enters the collection cone of the microscope objective; thus we observe the appearance of dark bands.

After the closed lids are opened and normal blinking and tear film is re-established we found two concomitant changes: the corneal thickness returns to its normal values, and the micro-folds are no longer observed with confocal microscopy. The fact that the micro-folds were not present in the normal cornea prior to overnight lid closure, and were observed

<table>
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<tr>
<th>Table 1 Corneal thickness in overnight lid closure.</th>
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<td>Corneal thickness (μm)</td>
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<td>------------------------</td>
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<tr>
<td>Before overnight lid closure</td>
</tr>
<tr>
<td>At lid opening</td>
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<tr>
<td>After 1 hour</td>
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<td>After 4 hours</td>
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*After a two day rest period
immediately following this condition suggests that their origin is related to this condition. Overnight lid closure results in the formation of micro-folds in the stroma.

It is postulated that cyclic process of corneal thickening over the period of sleep with the concomitant formation of micro-folds in the stroma, presumably due to the corneal thickening, and the reversal of the corneal thickening and the removal of the folds in the stroma may present the cornea with the daily mechanical insult or stress.

If the subject resumed normal blinking and then after 30 minutes came to the eye clinic for the confocal eye examination and the measurements of corneal thickness with OLCR the transient morphological changes in the stroma would have been missed. Again, the correct experimental protocol is critical for human studies.

This study demonstrates the correlation between the temporal course of the overnight corneal swelling and the appearance of folds in the stroma, and the concomitant deswelling and loss of folds in the stroma following lid opening and normal blinking, as observed with clinical confocal microscopy and OLCR. Corneal thickness was measured to show the temporal link (not causality) between the observations of the folds and the increased thickness of the cornea. It is posited that the reduced oxygen concentration, changes lactate levels, results in corneal thickness increases, and these thickness changes result in the observed micro-folds. It provides another example of the limitations of confocal microscopy applied to the human subject. If the confocal microscopy was performed too late to observe the stromal folds, as explained in the previous paragraph, the results would be negative.

2.4 Other linear optical studies to study corneal function and morphology

Another optical technique to investigate tissue metabolism in the cornea is the measurement of the autofluorescence of NAD(P)H [33, 34]. This field of cornea research was developed by Masters and Chance and has since emerged as a widespread technique to measure corneal cellular respiratory function [35]. There are many confounding problems in both the measurements and their interpretation and they have been previously discussed [36]. In particular, there is the problem of validation between the optical measurements of autofluorescence and independent analytical studies of the cellular concentrations of the molecular concentrations that cause the autofluorescence [37–39]. Another confounding problem is that the cellular metabolism and the respiratory function of the various cell layers that comprise the corneal epithelium differ. Therefore, it is necessary to use an optical technique with the optical sectioning capability to image the autofluorescence from each of the cell layers that form the epithelium [40]. Typically, this is not done in may investigations of corneal autofluorescence and that confounds the in-
terpretation of the experimental results since the results are averaged over the various layers of the epithelium. Typical applications of these measurements have been used to measure the corneal oxygen distribution with contact lens wear [40].

Additionally, the measurement of autofluorescence of diabetic and healthy human corneas in vivo at different excitation wavelengths may hold diagnostic potential [41]. The authors of this study found that autofluorescence is increased in diabetes mellitus patients with retinopathy as compared to normal subjects. Fluorescence excitation was measured in the range 365 nm to 480 nm, and the fluorescence emission was measured in the range 532 nm to 630 nm. The study is confounded by the lack of clear identification of the molecules involved in the autofluorescence (the results suggest but do not confirm the involvement of flavins, NAD(P)H and another unidentified fluorophore). Perhaps the study of fluorescent lifetimes could help to identify the sources of the autofluorescence. Fluorescence lifetime measurement are less prone to the artifacts that plague measurements of fluorescence intensity.

Corneal autofluorescence, based on NAD(P)H fluorescence, can be used to measure the oxygen concentrations, oxygen flux, and the oxygen consumption under a variety of contact lenses made from different materials and with different oxygen diffusion coefficients and thicknesses [36]. As a correlative approach to the corneal cellular fluorescence studies cited above there are theoretical models that may help to interpret the fluorescence studies of corneal oxygen concentrations and cellular respiration. For example, in a recent investigation, the authors used a two-dimensional axi-symmetric finite element analysis (FEA) model for the contact lens on the cornea to model the corneal oxygen distribution with contact lens wear. From their FEA approach, the authors could model profiles of oxygen partial pressure, oxygen flux, and oxygen consumption form the central corneal to the limbal junction [42]. The results of the modeling are highly dependent on the details of the FEA model as well as the numerical values used in the simulation.

3. Introduction to nonlinear optical microscopy

3.1 Multiphoton excitation fluorescence functional imaging of the cornea

While the rate of one-photon absorption is proportional to the incident light intensity, the rate of two-photon absorption is proportional to the square of the incident light intensity. The quantum mechanical theoretical basis for multiphoton excitation processes in which the probability for the absorption of two photons from the ground state, to an excited state, via a virtual set of intermediate states, was first described in the 1931 dissertation of Maria Goeppert-Mayer [30]. Masters has recently published an English translation of her 1931 dissertation paper in the Handbook of Biomedical Nonlinear Optical Microscopy [31].

The development of multiphoton excitation microscopy solved critical problems for imaging live cells and tissues; the near-infrared light is less damaging than ultraviolet light for the excitation of fluorescent molecules with absorption bands in the ultraviolet, and the near-infrared light can penetrate deeper into tissues than ultraviolet light.

The small focal volume of multiphoton excitation is a result of the physics of the excitation [32]. Only in the small focal volume of the high aperture microscope objective is the intensity sufficient to result in multiphoton excitation. There is no excitation outside of the focal volume, therefore, no spatial confocal filtering is necessary, and there is no out-of-focus photobleaching and photodamage.

Cellular metabolism can be monitored by imaging the intrinsic fluorescence of the reduced pyridine nucleotides, and the oxidized flavoproteins with two-photon excitation microscopy [43]. It is critical to validate the interpretation of NAD(P)H images acquired with multiphoton excitation microscopy. In a previous study of multiphoton imaging of the basal cells in the ex vivo rabbit cornea, the source of the fluorescence was demonstrated to be NAD(P)H by the use of cyanide to block oxidative phosphorylation in the mitochondrial electron transport chain. In other investigations of NAD(P)H fluorescence both the emission spectra of the tissue independently the fluorescence lifetimes of the emission were measured [36]. The combination of emission spectra and fluorescence lifetime were used to validate the source of the fluorescence as NAD(P)H. It is strongly recommended that these validation procedures be followed in order to reduce the ambiguity of the interpretation of the non-linear linear fluorescent images of cells and tissues.

The subject of photodamage with femtosecond laser pulses is still an active area of research on multiphoton excitation microscopy of live cells [44]. A recent paper demonstrated the use of a pulse-picker to mitigate tissue damage and perhaps this technique could find use in clinical multiphoton excitation microscopes [45].

3.2 Second-harmonic and third-harmonic generation microscopy of the cornea

In 1961 Franken et al. experimentally demonstrated the generation of optical harmonics [46]. In second-
harmonic generation (SHG) an incident wave of frequency $\omega$ generates a new signal at the frequency $2\omega$. With the proper conditions the efficiency of this process can exceed 50 percent. SHG can only occur in media that does not contain inversion symmetry because all even-order nonlinear susceptibilities are zero in centrosymmetric media.

SHG and third-harmonic generation (THG) can be explained by the theory of the nonlinear susceptibility in which for the time domain the polarization is expanded in a power series of the sum of products of the linear susceptibility and the electric field, the second-order susceptibility and the square of the electric field, and the third-order susceptibility and the cube of the electric field and higher-order terms. SHG is described by the second-order susceptibility, and THG is described by the third-order susceptibility [47]. The complete theoretical description of SHG and THG requires that the polarization and the vector properties of the electromagnetic field be accounted for in the analysis.

THG has a broad potential for tissue imaging since it occurs in all materials, including dielectric materials with inversion symmetry [48]. In general THG is a weak process; however, it is dipole allowed. It occurs at all interfaces free from the constraint of phase-matching. Third-harmonic generation microscopy has a large potential for cell and tissue imaging. As new techniques are developed for the surface-enhanced THG at interfaces the technique may become useful for imaging the cornea.

SHG microscopy in tissues can be used to image microtubules, oriented protein structures and stacked membranes [49–51]. Another emerging development is the use of THG microscopy with nano-gold particles to utilize the surface-plasmon-resonance effect [52].

Both SHG and THG imaging do not exhibit the saturation effects or the photobleaching effects that are associated with multiphoton excitation fluorescence microscopy. Therefore, SHG and THG microscopy do not require either intrinsic nor extrinsic fluorescent probes. These microscopic techniques can penetrate into millimeters of tissue and provide sub-micron three-dimensional optical sectioning.

### 3.3 Review of some key applications with nonlinear optical microscopy of the cornea

In another investigation, the structure of the ex vivo rabbit cornea was studied with a multiphoton microscope that employed both SGH imaging and two-photon excited fluorescence (TPF) in a back scattering geometry [53]. Endogenous TPF and SHG signals from corneal cells and the extracellular matrix, respectively, were observed without exogenous dyes. The polarization dependence of the collagen SHG was used to study fiber orientation in the cornea. The authors demonstrated the spectra of the TPF fluorescence signal as well as the SHG signal in the collagen matrix of the cornea. The SHG signal had a quadratic dependence on the incident laser power. The authors compared the images from the TPF and the SHG modes with Hematoxylin and Eosin (H & E stain) stained en face formalin-fixed sections of the corneal stroma. Thus, they demonstrated the utility of correlative microscopy to interpret the images. Histology is fixed, stained tissue specimens is a useful comparison for optical imaging; however, the user should be cautious and knowledgeable about the artifacts of histology such as tissue shrinkage.

Another study of corneal pathology in a transgenic mouse model shows the importance of measuring the emission spectra of the cornea to validate the interpretation of the nonlinear microscopy [54]. The authors used nonlinear optical microscopy (two photon excited fluorescence, and second harmonic generation signals) to image the corneas of a transgenic mouse model. Images were presented of the combined nonlinear signals from the cornea that consisted of TPF and SHG combined signals. To validate the images, H & E stained sections of the transgenic mouse cornea were presented. Metabolic activity in both the epithelial (the authors did not specify which cell layer of the epithelium was imaged) and the endothelial cellular layers were imaged by co-localizing the reduced pyridine nucleotide, NAD(P)H images and the flavin adenine dinucleotide (FAD) images in different colors. The authors also presented emission spectra for NAD(P)H and FAD in both the corneal epithelial and endothelial layers of the transgenic mouse. Unfortunately, the emission spectra were not corrected for the instrument response.

Second-harmonic generation microscopy is another emerging area of nonlinear microscopy that is being applied to the cornea. SHG imaging of the excused porcine cornea after intrastromal femtosecond laser ablation was investigated to evaluate the next generation of laser surgical techniques [55]. This is an important approach since NIR fs laser surgery avoids the risks of mutagenicity and toxicity that accompanies the UV radiation of excimer lasers. Collagen has a noncentrosymmetric structure and can convert the incident ultrashort laser pulse to its second harmonic, thus providing a noninvasive imaging modality for the cornea. Since the SHG signal from the collagen in the cornea is emitted predominately in the forward direction (there was only a very weak signal in the back scattered path), it was detected in the transmission mode. The authors demonstrated that the SHG signal is proportional to the second power of the normalized illumination intensity. High
contrast SHG images of corneal collagen fibers were obtained from the anterior corneal surface to about 200 micron depth within the cornea. The problem of forward and back scattered higher-harmonic signals is the subject of another paper [56]. Collagen is the most abundant protein in the human body and exists in connective tissues including tendons, the skin, the cornea and the sclera. SHG imaging was used to image the porcine corneal collagen fibrils and to demonstrate their regular arrangement that accounts for corneal transparency. The sclera collagen fibrils are in a more inhomogeneous arrangement and that results in a lack of transparency. The SHG microscope was set up to acquire both the forward scattered SHG signal and also the backward scattered SHG signal.

It should be stressed that only electron microscopy can image the collagen fibrils, since the diameters and the spacing of the individual fibrils are far below the diffraction limit of the microscope. The SHG emission field is a function of the size and the shape of the collagen fibrils, and is highly asymmetric due to the phase matching condition. It was previously demonstrated that objects with an axial metric due to the phase matching condition. It was previously demonstrated that objects with an axial size smaller than 10 produce equal SHG signals in both the forward and the backward directions [57].

Since electron microscopy demonstrates that the diameter of corneal collagen fibrils is approximately 30 nm, there should be significant backward SHG signal. Actual imaging showed the backward SHG signal from the cornea was extremely weak and there was no connection between the signals seen in the forward and the backward SHG imaging. In contrast to the cornea, the SHG images of the scleral collagen fibrils are similar in the forward and the backward scattering directions. The authors suggest that backward SHG imaging may provide a sensitive clinical method to study corneal haze or cloudiness.

The next study shows how SHG microscopy is used to investigate collagen structure in the ex vivo human cornea. Second-harmonic generation imaging was used to identify differences in corneal stromal collagen between normal human and keratoconus corneas [58]. The authors examined six normal corneas from eye bank donors and 13 corneas of patients with keratoconus that were obtained after penetrating keratoplasty. A femtosecond titanium-sapphire laser with 800 nm output was used to generate second-harmonic signals at 400 nm from the central and the paracentral corneas. The authors found in keratoconus corneas there was less lamellar interweaving and a significant reduction or loss of lamellae inserting into Bowman’s layer. They conclude that compared with normal adult corneas, there were marked abnormalities in the structure of the anterior corneal collagen lamellae of keratoconus corneas.

Several groups have demonstrated the efficacy of correlative microscopy in which multiphoton excitation microscopy and higher harmonic generation microscopy are used to image the same cornea [59, 60]. Multiphoton autofluorescence and second-harmonic generation images were obtained from whole ex vivo porcine eyes. These images were obtained from the cornea, the limbal region, the conjunctiva, and the sclera. The 780 nm output of a femtosecond, titanium-sapphire laser was used to induce autofluorescence in the region (435–700 nm) as well as second-generation signals at 390 nm. The SHG signals were used to image the collagen structures within the corneal stroma and the sclera. The authors obtained very weak SHG signals of the collagen, but they were able to determine that the collagen in the cornea is organized in a depth-dependent fashion, whereas the scleral collagen is more randomly packed.

Other important applications of femtosecond lasers are in the area of using ultrashort laser pulses for refractive surgery. The use of ultrashort laser pulses (100–200 fs) can be used to generate microplasmas inside the cornea stromas [61, 62]. These plasmas can cut inside the tissue while leaving the anterior corneal layers intact. The authors found that the extent of thermal and mechanical damage to adjacent tissue is limited to 1 μm. Therefore, these lasers have a potential for use in intrastromal refractive surgery.

4. Discussion

The problems of interpretation of all types of microscopic images are formidable. There are optical aberrations in the imaging system and in the human eye, as well as artifacts due to specimen preparation of ex vivo specimens. One solution to the problem of aberrations is to incorporate an adaptive optics system (consisting of a wavefront sensor, a deformable mirror, and a feedback control circuit) to minimize the spherical aberrations from the eye [61].

A second problem is how to minimize the error of interpretation of corneal images. It is suggested that the use of correlative microscopy, in which similar specimens are imaged with multiple microscopic techniques, is helpful. An exemplar of this technique is a study of the structure of the lens fibers in ex vivo human lenses containing cataracts. A correlation of both reflected light confocal microscopy on a freshly excised human lens and the subsequent fixing, embedding, and microtome sectioning for scanning electron microscopy of the identical regions of the ocular lens, confirmed the interpretation of the confocal images [63]. Another example is the comparison of confocal images from the in vivo images of the cornea with ex vivo corneal whole mounts that
are fixed, stained with specific fluorescent probes, embedded and sectioned.

Finally, it is proposed that correlative microscopy become the standard procedure to validate the images that are obtained with linear and nonlinear microscopy. It is proposed to develop a new clinical imaging system that may be based on several new types of linear and nonlinear microscopies; i.e. confocal reflected light microscopy with multispectral sources based on arrays of LEDs, multiphoton excitation microscopy based on cellular NAD(P)H fluorescence, and second-harmonic microscopy based on signals from collagen fibers. During the development and validation with both ex vivo specimens, in vivo animal corneas, and finally clinical studies that have prior approval from institutional review boards (IRB) it is necessary that both the safety and the validity of the imaging techniques be subjected to strict scrutiny.

In the transition from the laboratory to the clinic there are several cautions to be addressed. It is important to compare the images of the cornea that are obtained with confocal microscopy, and those obtained with multiphoton excitation microscopy and second-harmonic generation microscopy. The clinical in vivo reflected light confocal microscopy images show a resolution and a signal-to-noise ratio that is similar to the those from ex vivo specimens and have been fairly well validated with both optical microscopy of sectioned whole mounts of human cornea as well as with electron microscopy studies of ex vivo human cornea.

Much more work is required to validate the ex vivo and the in vivo corneal images that are acquired with the nonlinear microscopic techniques such as multiphoton excitation microscopy and second-harmonic generation microscopy. It is proposed to use correlative microscopy that consists of imaging the same corneal specimen with reflected light confocal microscopy, multiphoton excitation microscopy, second-harmonic generation microscopy, and electron microscopy, in order to validate and to reduce the ambiguity of spurious claims of structural interpretation.

The various linear and nonlinear optical microscopic techniques differ in both the physics of contrast generation, the deposition of energy in the specimen, and the potential for cell damage during image acquisition [44]. Confocal microscopy that operates in the reflected light mode generates contrast from spatial variation in the refractive index within the specimen. Boundaries with large differences of refractive index, such as scar in the cornea are highly scattering and large signals are generated in the back-scattering direction which is optimal for a non-invasive instrument to image the in vivo cornea.

Before nonlinear optical techniques can be approved for routine clinical examination of the patient’s cornea there are several concerns that must be addressed. The first concern is patient safety. While two photon excitation microscopy of the cornea can provide functional imaging the process does deposit energy into the tissue. Studies of animal corneas, and ex vivo human corneas are needed to address safety issues. Cell and tissue damage as a functions of pulse width, laser repetition rate, laser frequency, and peak pulse power are required to meet FDA approval for a new medical device. Nonlinear harmonic generation may prove to be the least damaging to cells and tissue since there is no net deposition of energy into the tissue.

While the linear corneal imaging systems use either incoherent lamps or coherent continuous lasers as the light source, nonlinear imaging systems typically require expensive femtosecond lasers. Therefore, nonlinear optical microscopes are much more expensive.

Much of the work with nonlinear optical microscopy is based on two techniques: (1) multiphoton excitation microscopy that is based on two-photon excitation of either intrinsic fluorescent molecules such as NAD(P)H [43], or genetically over expressed green fluorescent, or extrinsic fluorescent molecules, or (2) second-harmonic generation microscopy. Multiphoton excitation microscopy is based on the nonlinear absorption of incident energy to form excited electronic states and the subsequent emission of fluorescence. One the other hand, second-harmonic generation microscopy is a nonlinear scattering process (predominately in the forward direction) and therefore, there is no energy deposition in the specimen. If second-harmonic generation microscopy is to become a clinical imaging modality for imaging the in vivo cornea, then it is imperative that new techniques be developed to enhance the back-scattered component of the SHG signal. Furthermore, while multiphoton excitation microscopy is useful for corneal functional imaging based on the fluorescence of the naturally occurring NAD(P)H, SHG microscopy is more restrictive. In SGH microscopy an incipient wave of frequency \( \omega \) generates a signal at the frequency \( 2\omega \). The physics of the process shows that all even-order nonlinear susceptibilities vanish in centrosymmetric materials; therefore, SHG occurs in materials with no inversion symmetry. SHG microscopy is especially suitable for the imaging of collagen fibers.

Because the back scattered SHG signal from the cornea is often weak the interpretation of SHG corneal images is often ambiguous. Published papers contain SHG images of the cornea with putative interpretations of structures within the cornea. Correlative microscopy that compares SHG microscopy and electron microscopy would serve to reduce the ambiguity of image interpretation.

The interpretation of the SHG microscopy of collagen 1 fibrils was investigated by Williams, Zipfel
and Webb [64]. In their paper they discussed in detail the principles governing SHG phase matching in the tightly focused microscopic optics. These principles will find useful application in the design of new clinical corneal nonlinear microscopes.

A new development with great potential for live cell and tissue imaging as well as clinical nonlinear imaging is third-harmonic generation (THG) microscopy through the technique of resonance enhancement with an absorbing dye as developed in the laboratory of C.-K. Sun [65]. This is the first report that demonstrates molecular THG microscopy with a nonfluorescent absorbing dye. This innovative contrast technique provides molecular labels that are specific and the signal is from the THG. The advantages of this high resolution technique include the lack of electron transitions, no induced photobleaching, no induced photodamage, as compared to fluorescence or absorption based microscopy.

Previously, the same group showed how metal nanoparticles can be used as an exogenous contrast agent for THG microscopy [65]. The technique is based on plasmon-resonance enhanced THG microscopy [66].

Another nonlinear imaging technique with great biological and medical potential is coherent anti-Stokes Raman scattering (CARS) microscopy [67, 68]. CARS may find useful applications in ocular tissue. CARS is a label-free imaging technique to image live cells and tissues based on molecular vibrational spectroscopy. Modern CARS instruments can acquire images at video-rates which permit imaging of cell migration and other dynamic processes. When the CARS microscope is tuned to specific molecular vibrational bands the images can provide specific chemical information and that property differs from all of the imaging techniques that were discussed previously.

5. Conclusion

Further progress in the development of new clinical optical instruments to investigate corneal function and structure is dependent on the need for improved signal detection in the back scattered direction of SHG imaging. It is imperative that correlative microscopy, that compares collagen structure as seen with SHG microscopy and with electron microscopy, be used in order to validate the nonlinear optical microscopy findings. New developments such as molecular THG microscopy and plasmon-resonance-enhanced THG microscopy are evidence of the rapid and innovative progress in nonlinear microscopic developments for live cell and tissue imaging, and eventually we shall see the development of clinical instruments that are based on these noninvasive techniques [66].

Finally, there is the necessity for a strong clinical collaboration in order that the questions posed in a particular study are either important to gain new understanding of the cell biology of the cornea or from the diagnostic side of corneal imaging. Nonlinear optical microscopy, such as multiphoton excitation and higher-harmonic generation, used in conjunction with linear confocal microscopy, when appropriately applied to the in vivo human cornea, with the prior safety studies documented, and with full validation in animal studies and on ex vivo human corneas, could provide clinicians and researchers with new diagnostic instruments.

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References

[56] M. Han, G. Giese, and J. F. Bille, Optics Express 13, 5791 (2005).