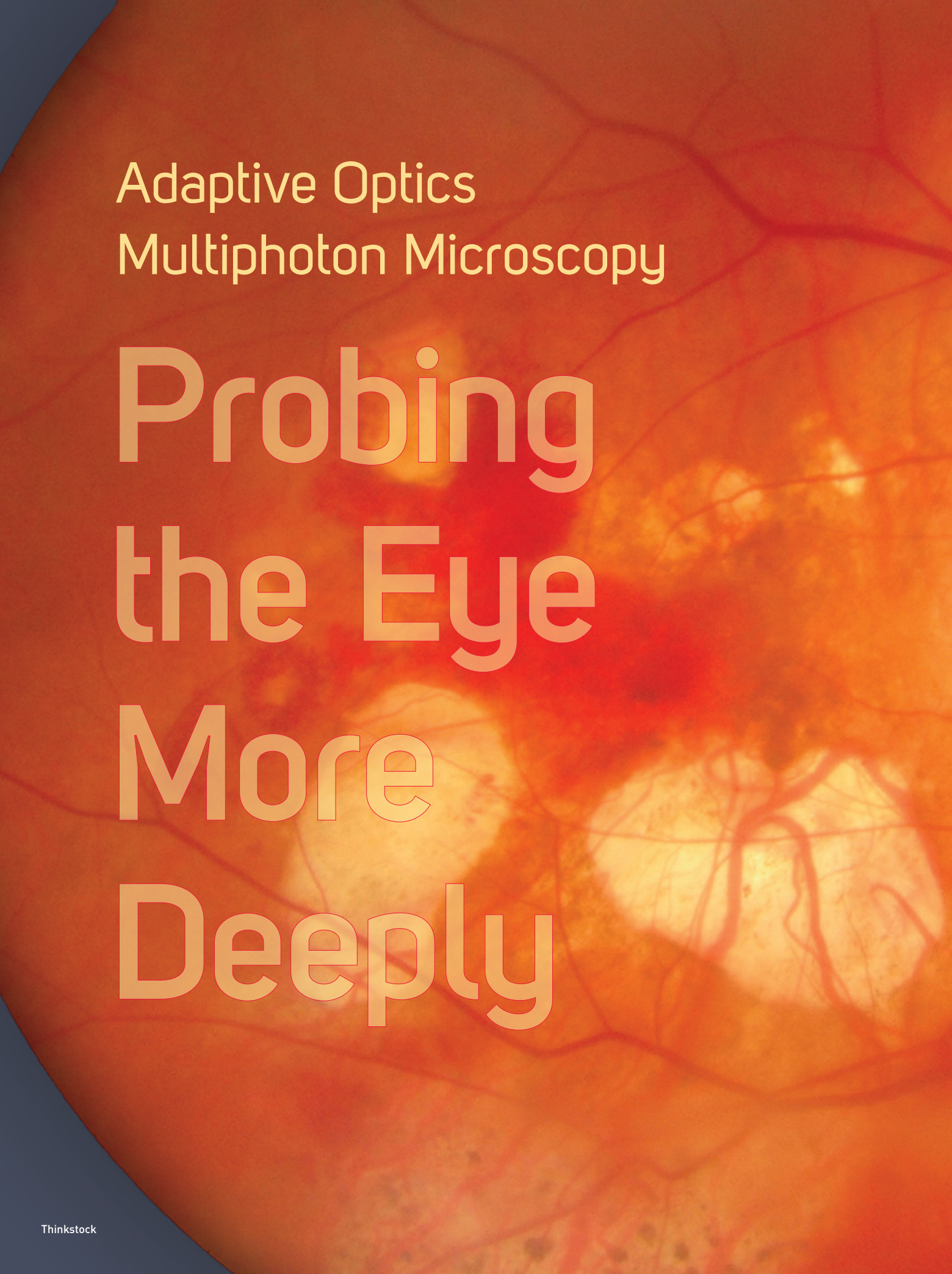

Adaptive Optics
Multiphoton Microscopy

Probing the Eye More Deeply

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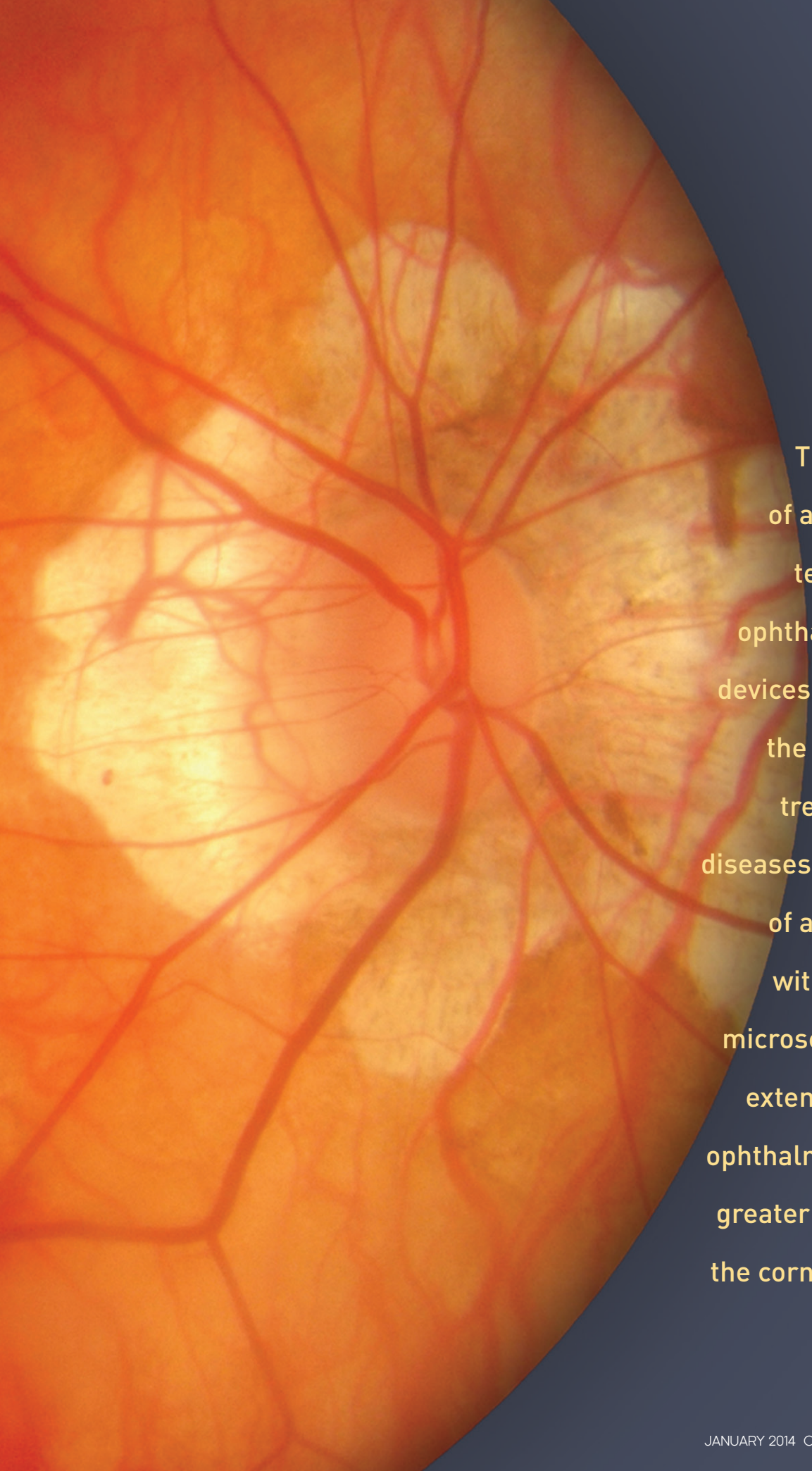
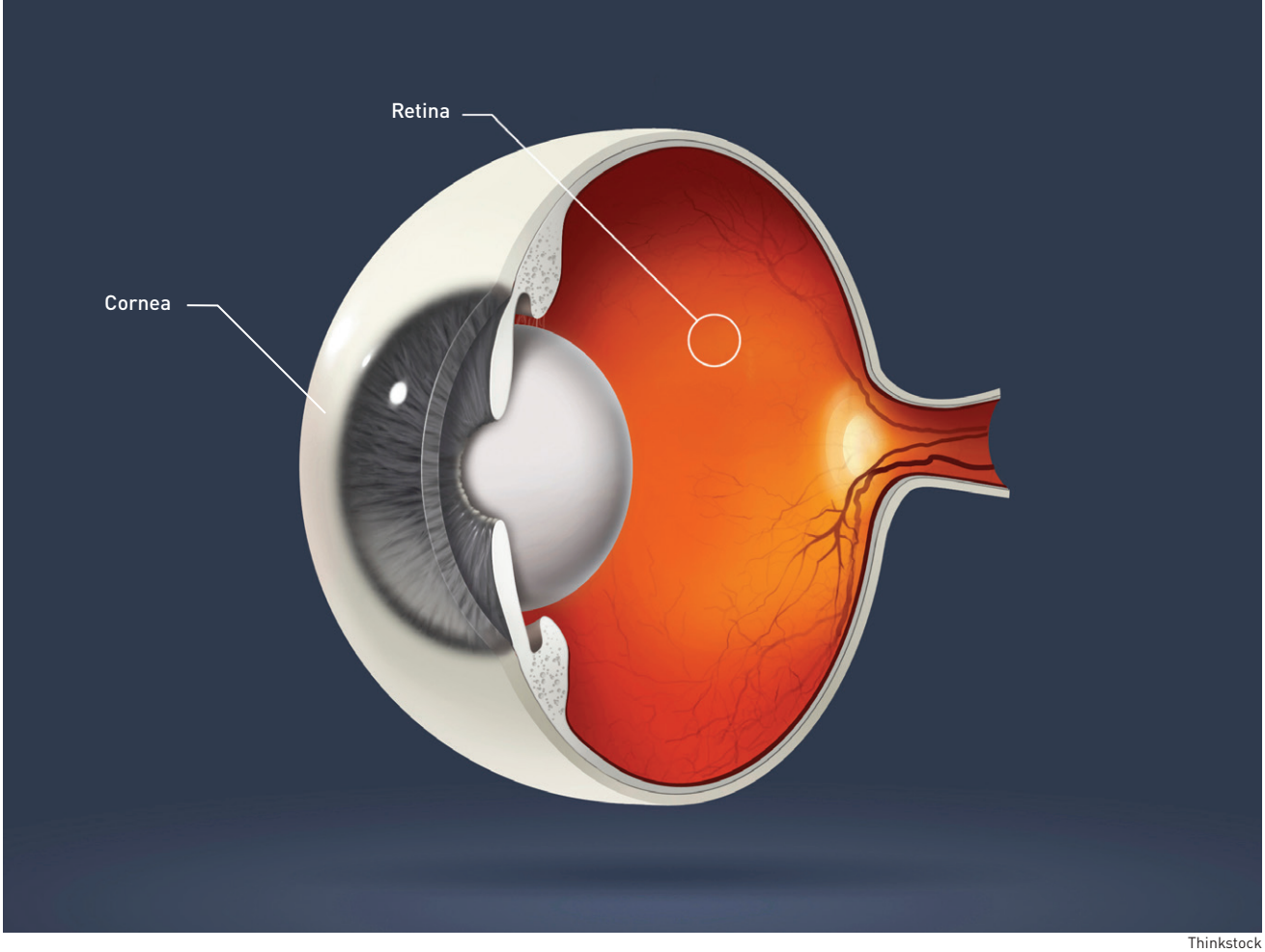


JUAN M. BUENO

The integration of adaptive optics techniques into ophthalmic imaging devices has improved the diagnosis and treatment of eye diseases. The coupling of adaptive optics with multiphoton microscopy may help extend the reach of ophthalmic imaging to greater depths within the cornea and retina.



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Detection and diagnosis of ocular pathologies and genetic anomalies require accurate visualization of the structures of the eye. However, the different layers of the cornea and the retina are transparent and often hard to image under natural conditions with clinical devices. Several imaging modalities improve a clinician's ability to view the cornea and retina, including specular microscopy and systems that integrate adaptive optics (AO) techniques.

By combining AO and multiphoton microscopy (MPM), it is possible to improve imaging of thick specimens, especially at deeper locations.

Devices based on specular microscopy image and analyze epithelial and endothelial cells as well as keratocytes in the cornea. Such instruments often use an immersion microscope objective that directly contacts the cornea. This approach, however, can be problematic because the cornea scratches easily and the most important corneal structure, the stroma, cannot be visualized.

During the last 15 years the integration of AO techniques—which were originally developed for astronomy applications—into scanning laser ophthalmoscopes (SLOs) and optical coherence tomography (OCT) instruments has expanded the frontiers of ocular imaging and improved the diagnosis and treatment of eye diseases. AO-equipped devices use the cornea and lens as a microscope objective and rely on AO techniques to correct their associated aberrations. An almost diffraction-limited beam focused at the rear of the eye significantly improves the kinds of details clinicians can visualize. In particular, some AO-SLO prototypes have visualized photoreceptors and, under experimental conditions, ganglion cells and retinal nerve fibers.

In parallel with the development of AO ophthalmic devices, different microscopy modalities have used AO approaches to avoid the detrimental effect of aberrations on the image quality of the specimens

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under study. By combining AO and multiphoton microscopy (MPM), it is possible to improve imaging of thick specimens, especially at deeper locations. This advance makes the technology an attractive approach for problems associated with imaging the eye, with its thick, complex structures. In this sense, alternative techniques such as AO-MPM might open doors to new methods to improve imaging the eye.

Multiphoton microscopy and ocular tissues

The development of femtosecond (fs) lasers as excitation sources led to the invention of MPM, including two-photon excitation fluorescence (TPEF) and second-harmonic generation (SHG). The basic principle underlying MPM is that for tightly focused ultrashort laser pulses, the photon density is high enough to induce multiphoton absorption within the focal volume, providing intrinsic optical sectioning (auto-confocality). During the last two decades, MPM has brought innovative solutions into biophotonics research.

Both TPEF and SHG are intrinsic signals and powerful tools for visualizing ocular tissues at high resolution with minimal disturbance and without labeling procedures. These techniques allow researchers to explore ocular structures to understand the origin and development of certain pathologies.

Overcoming aberrations in multiphoton microscopy

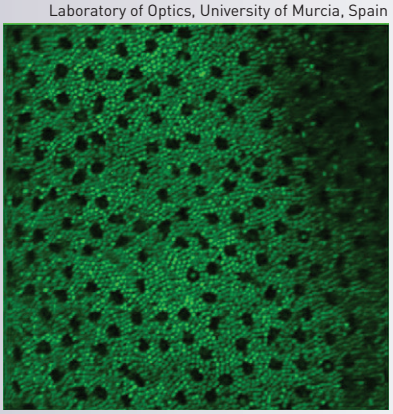
When the features of interest are close to the sample surface, MPM often provides high quality images. However, when the image plane is located at a deeper position within a thick specimen, image quality diminishes. Scientists using MPM are faced with the typical problem that occurs when imaging thick (even transparent) samples: the greater the depth, the less signal returned. Difficulties increase if nonlinear signals are recorded in the backward direction. Despite MPM's auto-confocal nature and its 3-D imaging capabilities, the modality is limited by the aberrations of the laser beam, the microscope optics and the specimen itself. Aberrations (especially higher-order ones) increase due to the cumulative effect of the index of refraction with depth. Independent of their origin, these aberrations enlarge the focal spot size and reduce imaging efficiency, both in contrast and resolution.

Thick samples such as the cornea (about 550 µm in humans) are very difficult to image in backscattered

Imaging Structures of the Eye with Multiphoton Microscopy

The retina

Laboratory of Optics, University of Murcia, Spain



Ex vivo TPEF image of a retinal area containing the photoreceptor layer of a human retina.

Recently, MPM identified retinal layers from the nerve fibers to the outer segments in both flat-mounted and *ex vivo* retinas. Retinal cells provided TPEF signal (i.e., autofluorescence) with a maximum signal at the photoreceptor layer. Whereas most structures were missing in regular bright field microscopy, TPEF images showed improved image contrast. Because individual cells were visualized, density, spatial distribution and inter-cell distance could be computed. Moreover, single-cell imaging improves disease detection and treatment evaluation because it identifies localized retinal dysfunction. Data collection of accurate cell distribution will be useful to explore anatomical-based visual functions, myopia models and sight-correction processes.

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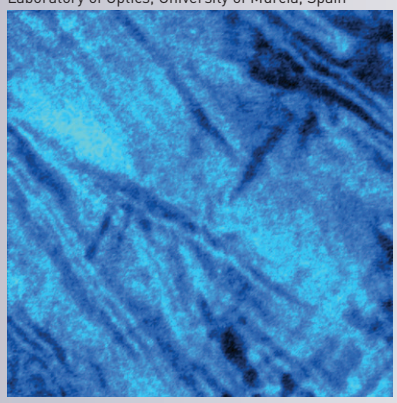

SHG image corresponding to the human corneal stroma.

The cornea

The stroma makes up over 90 percent of the cornea's thickness. Confocal clinical devices and MPM can visualize the cornea's epithelium, endothelium and keratocytes. The cells provide TPEF signals for MPM. However, the corneal stroma cannot be visualized with regular microscopy. The stroma's collagen fibers' natural non-centro-symmetric organization generates an SHG signal. Correct visualization will be essential for the analysis and comparison of both normal and diseased corneas.

Individual retinal cells and corneal stroma fibers are seen with enhanced contrast without noticeable photodamage.

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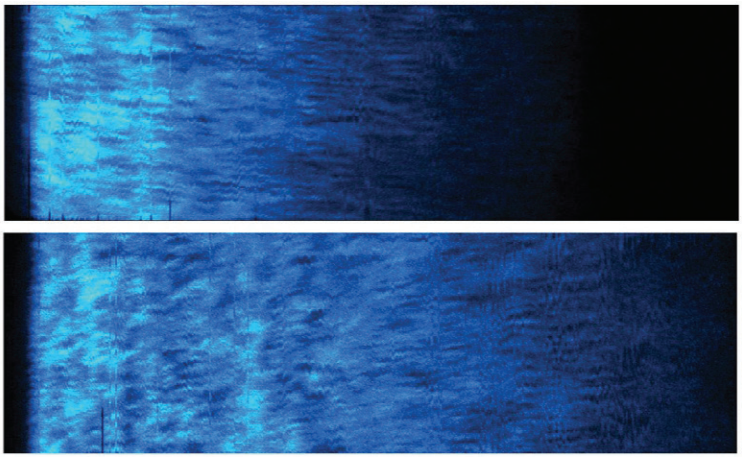

SHG tomography (XZ) of *ex vivo* porcine corneal stroma without (top) and with AO (bottom). This was used to compensate for the laser beam and microscope optics aberrations. Corneal depth increases from left to right. AO allows a deeper penetration within the specimen. Without AO, corneal thickness is underestimated.

MPM, especially at deeper layers. In our lab, we compensated for the laser beam and microscope optics aberrations in closed-loop by combining an adaptive element (deformable mirror or spatial light modulator) and a Hartmann-Shack sensor. This configuration noticeably enhanced SHG images corresponding to deeper corneal layers. For most samples the total stromal thickness was imaged with enough contrast and resolution to observe the collagen bundles. Moreover, retinal samples also benefit from this configuration because it provides better visualization of the retina's layered structure.

Unlike UV light, a near-infrared light beam from a fs-laser can pass through corneal tissue without significant one-photon absorption, going deeper and focusing on a tiny spot.

This AO implementation represents an effective solution for ophthalmological imaging. It provides morphological information on the global collagen orientation of the entire cornea and details on some inter-layer retinal connections. However, this operation might not be the most appropriate for all samples.

When compensating for the aberrations of the both the microscope optics and the laser source, the imaged sample presents a unique plane with the highest nonlinear signal. Due to the non-corrected specimen's aberrations, the rest of the planes are imaged with lower quality since the efficiency of the nonlinear phenomena decreases when imaging deep into the sample. Ideally, an AO-MPM device would accurately measure and correct for the corresponding aberrations at each location within the sample. This, however, would involve a plane-by-plane wavefront assessment, which is a complex operation in thick samples.

Instead, researchers use sensorless techniques. Such schemes offer an accurate strategy to compensate for an aberration pattern per imaged plane. Different strategies are based on algorithms modifying the incoming wavefront (i.e., aberration pre-compensation) to improve the recorded image according to different quality metrics, such as intensity or sharpness. Often, modal Zernike corrections combined with hill-climbing and stochastic algorithms are used. However, recent techniques involving iterative compensations of aberration and random scattering provide improved imaging at unprecedented depths (hundreds of microns).

These procedures provide enhanced images through an indirect measurement of the actual aberrations within the sample. Although robust, they are highly time-consuming and their performance depends on a set of image-based merit functions. In spite of this, sensorless techniques have provided interesting results on both TPEF and SHG imaging of ocular tissues.

Deeper-layer multiphoton imaging

Although aberrations are specimen-dependent, they tend to be dominated by a small number of Zernike aberration modes. If these terms are known, the time required for a sensorless procedure decreases significantly. Spherical aberration (SA) has been reported to be the

most important aberration term when trying to image deeper layers within a specimen.

SA in MPM is expected due to the mismatch of refractive indices between the tissue and the objective's immersion medium. The deeper the microscope objective is focused, the higher the SA's influence. This effect leads to image degradation that is more noticeable at increasing depths. The usual technique to correct for this unwanted SA is the use of an objective correction collar.

In our lab, we recently developed an alternative approach to increase penetration depth in 3-D MPM imaging. These results demonstrate that plane-by-plane SA compensation is not always necessary. A unique (customized) SA value can experimentally be measured for each sample to improve the image quality of deeper planes. This operation is based on the manipulation of the SA of the incident beam while performing fast tomographic (cross-sectional) multiphoton imaging. In particular, the AO system combines a deformable mirror and a Hartmann-Shack sensor to produce controlled amounts of SA. This approach leads to an increase in imaging depth, independent of the specimen-induced aberrations in backscattered MPM imaging with non-immersion objectives.

To manipulate the SA, a series of nonlinear tomography images for different amounts of SA is sequentially recorded. The SA value corresponding to the highest averaged intensity profile is chosen as the "optimum value." This operation takes only a few seconds. Once the AO module is set to produce this SA value, a regular stack of MPM images is acquired at different depth locations across the sample. As a result, when the optimum SA is induced, the image at best focus slightly worsens, but the image quality from deeper planes improves. This procedure partially overcomes the limits imposed by the aberrations and represents a compromise that improves 3-D MPM imaging. The technique has been proven in both retinal and corneal tissues and represents a new method to better visualize deeper layers through volume rendering reconstructions.

Improving femtosecond ablation performance

The imaging of biological structures in general, and ocular tissues in particular, benefits from the use of ultrafast lasers to produce multiphoton processes. However, the high power levels provided by these lasers can modify the optical properties and appearance of samples by delivering high power densities (above a certain threshold) to particular locations. This phenomenon, often used for material micromachining (ablation), can also be used as a tool for ophthalmic surgery.

Since the 1970s researchers have tested different laser sources for corneal refractive surgery, but most of the sources were based on UV one-photon absorption.

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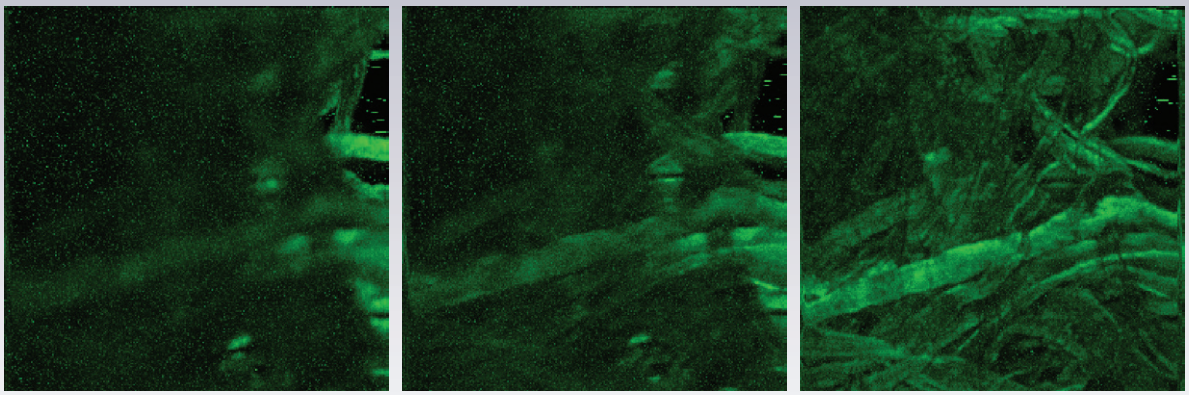

TPEF images of an *ex vivo* human epi-retinal membrane without AO (left), after correcting for the aberrations of the laser beam and the microscope (middle), and after correcting for the specimen aberrations at the imaged layer (right).

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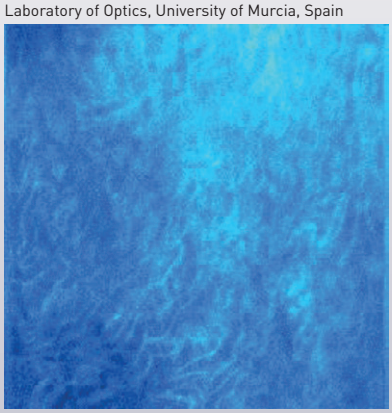
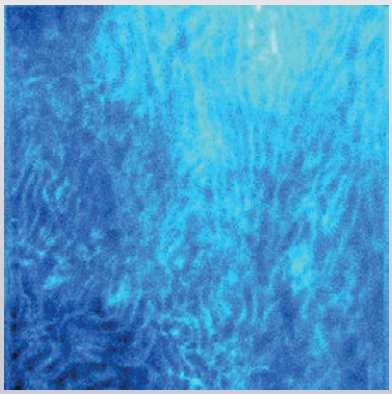
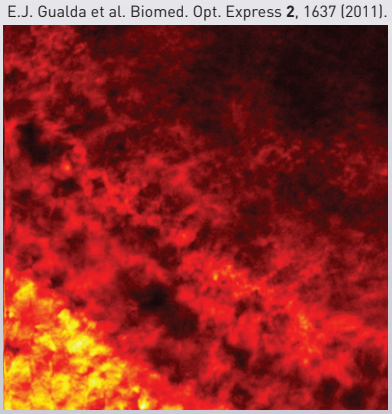




SHG images of an *ex vivo* porcine cornea (100 µm depth) acquired without (left) and with (right) SA induced.

E.J. Gualda et al. Biomed. Opt. Express **2**, 1637 (2011).



MPM images of an ablated chicken cornea showing an intrastromal pattern composed of a set of cavities.

Unlike UV light, a near-infrared light beam from a fs-laser can pass through corneal tissue without significant one-photon absorption, going deeper and focusing on a tiny spot. If the power is high enough, optical breakdown is produced at a well-defined volume within the cornea.

Although fs-lasers are currently used in clinical environments for cataract surgery and corneal flap creation, various researchers have used fs-lasers to produce photodisruption effects inside the cornea. The generated (intrastromal) patterns modify the local properties of the cornea (basically a change in the index of refraction) and can potentially be used to correct for refractive errors. Since these effects are highly localized, the irradiation hardly affects the surrounding regions outside the focal volume. Results in *ex vivo* corneas show that the external appearance and transparency remain intact.

Further advances in AO-MPM will help to obtain images of living corneas in a backscattered configuration and to decrease illumination power.

Because fs-ablation procedures and MPM imaging are based on the same multiphoton absorption, AO approaches might also improve intrastromal ablation performance. Aberration correction leads to smaller focal spots and enhanced sectioning capabilities, increasing multiphoton efficiency. Future AO fs-ablation devices could produce more accurate intrastromal patterns at deeper locations within the cornea. The combination of customized patterns created at different corneal stromal depth positions might potentially modify the corneal optical properties, leading to a predefined refractive power.

Prospects and conclusions

The combination of ultrafast laser systems and microscopy has become increasingly important in the field of biomedicine during the last two decades. Recent efforts to implement AO into MPM have extended the technique's sectioning capabilities and produced 3-D images with overall increased quality. AO-MPM of label-free, ocular tissues exploits the properties of molecules and structures in the eye when they interact with photons from short-pulsed lasers. This technique provides an alternative visualization method of ocular components.

Currently, AO-MPM can image different layers of the *ex vivo* ocular components with

enhanced contrast without noticeable photodamage. In retinal tissues, morphological studies can be performed as a function of location, which accurately elucidate the structure. AO-MPM clearly identifies most retinal cells with a high spatial resolution, exclusively due to local endogenous fluorescence (autofluorescence). Knowing the cell density of the retina's different layers might help to explain and quantify the loss of cells as a consequence of retinal pathologies or changes that occur when ocular refractive errors develop (i.e., myopia models).

In corneas, MPM can visualize the stroma organization. AO approaches restore contrast and resolution at deeper layers within the sample. Since the structure of the posterior stroma is clearly visible, researchers can track changes in corneal morphology due to a number of pathologies.

Key challenges

Despite the fact that MPM is useful in a wide range of ocular imaging applications, its implementation in living eyes is still challenging. Issues such as safety limits, acquisition times and image stability must be addressed before MPM can record images in living ocular structures in a regular manner. In addition, although the prices of fs-laser sources are lower than they were a decade ago, they are still very high. This is probably one of the biggest drawbacks to the use of MPM, not only for labs involved in basic research, but also for its use in clinical devices.

However, the main goal for researchers using MPM to study ocular tissues is using the technique to explore the structures of living eyes. Although some authors have reported on preliminary *in vivo* animal experiments, their results are not entirely satisfactory and require a noticeable improvement of the device.

Retinal images of living eyes are limited not only by the aberrations of the eye, but also by the low TPEF retinal emission (which travels through the ocular structures in a second pass), and the numerical aperture (NA) of the ocular optics. The eye's NA limits the maximum resolution of the multiphoton images of retinal structures. Continual ocular motion and the long integration times make MPM almost unsuitable for imaging the retina of living eyes.

Corneal imaging appears the most feasible application of MPM. Further advances in AO-MPM will help to obtain images of living corneas in a backscattered configuration and to decrease illumination power. Such changes will retain signal quality while avoiding local tissue damage. Accurate light exposure limits combined with known AO approaches and new optimized, real-time registration procedures will help the technique gain widespread acceptance in clinical environments.

Further refinements, such as combining AO-MPM *in vivo* imaging with AO-fs intrastromal surgery, offer the potential for surgical monitoring applications. Optimized for a backscattering open-air configuration, such an instrument could help establish new techniques for label-free, real-time tracking of corneal surgery.

Although some say the development of a "dual" device is just a matter of time, device designers must address the main obstacles facing such a device: the generation of customized changes in the cornea's optical properties and understanding how long the changes will endure. Nevertheless, despite the fact that the methods for controlling and assessing the changes produced in the corneas are still "a work in progress," it is likely that due to the advantages of an AO-MPM device compared to what is currently available, the AO-MPM approach will gain acceptance as a promising new refractive surgery tool.

AO-MPM's ability to perform non-contact, high-resolution, cross-sectional imaging of the cornea, as well as to modify the cornea's optical properties in a controlled manner, might enable the technique to revolutionize the field of ophthalmology in the near future. OPN

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