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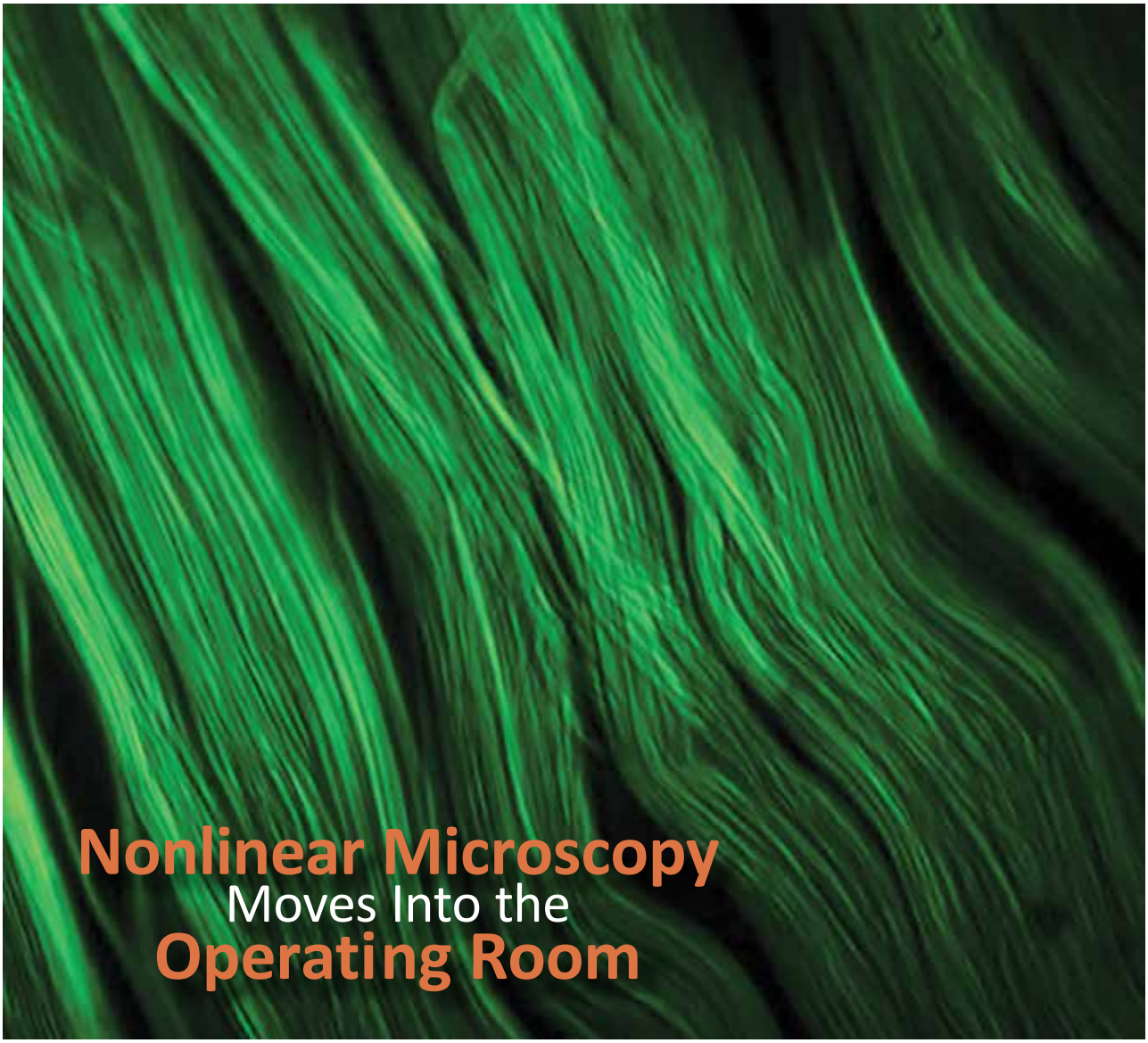

Nonlinear Microscopy Moves Into the Operating Room

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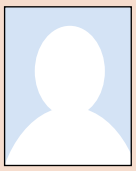
CONTRIBUTORS

Arthur DiMartino is the president of TechEn in Milford, Mass. **Page 26.**

Wolfgang Drexler is the head of the Center for Medical Physics and Biomedical Engineering at the Medical University of Vienna in Austria. **Page 35.**



Jason Ensher is the CTO and vice president of strategic marketing of Insight Photonic Solutions in Lafayette, Colo. **Page 35.**

Contributing editor **Marie Freebody** is a freelance journalist with a master's degree in physics from the University of Surrey, England. **Page 21.**

Thomas Hellerer is a professor of biophotonics at the University of Applied Sciences in Munich. **Page 30.**

Benjamin Miller is a professor of dermatology, biochemistry and biophysics and biomedical engineering at the University of Rochester Medical Center in Rochester, N.Y., as well as leading the roadmapping effort for the American Institute for Manufacturing Photonics. **Page 7.**

Michael Minneman is the president of Insight Photonic Solutions in Lafayette, Colo. **Page 35.**

Mojtaba Mohseni is a Ph.D. student at at the University of Applied Sciences in Munich. **Page 30.**

Christoph Polzer is a Ph.D. student at at the University of Applied Sciences in Munich. **Page 30.**

Nonlinear Microscopy Moves Into the Operating Room

Nonlinear optical microscopy is becoming a powerful tool in clinical research. Second-harmonic generation, for example, is capable of extracting fiber orientation and discriminating muscle and cartilage tissue.

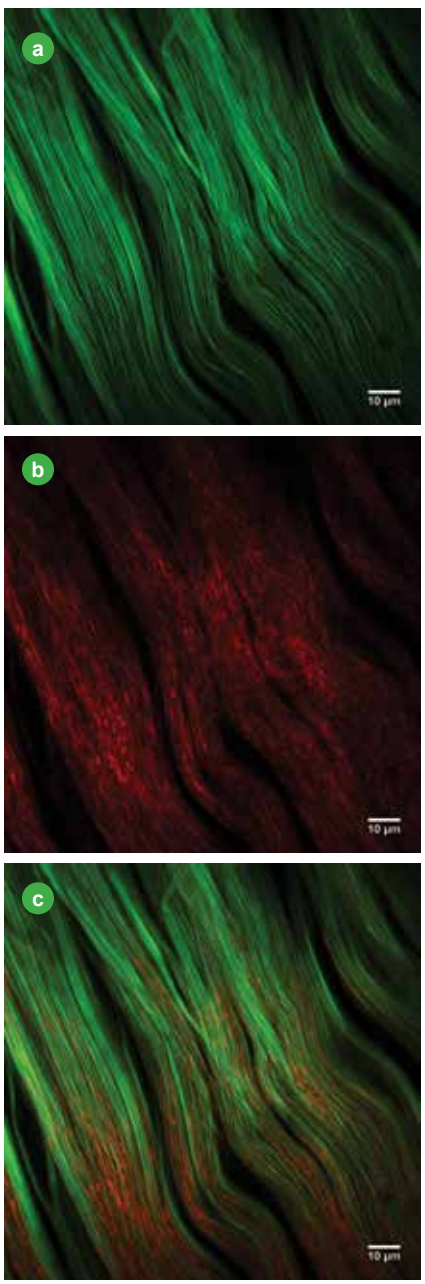


Figure 1. Forward- **(a)** and backward- **(b)** detected second-harmonic generation (SHG) signal and the composite **(c)** of **(a)** and **(b)** from a mouse knee. The elongated structure of collagen fibers is clearly visible in forward detection. Image taken at the Multiphoton Imaging Lab. Courtesy of Munich University of Applied Sciences.

BY THOMAS HELLERER, CHRISTOPH POLZER AND MOJTABA MOHSENI,
MUNICH UNIVERSITY OF APPLIED SCIENCES

The quality of a person's vision can influence life-and-death situations, and this is especially true for a surgeon cutting with the scalpel. Consequently, over the years, researchers have developed several techniques to improve and enhance the vision in the operating room (OR). Nonoptical imaging methods, such as ultrasound, magnetic resonance or computed tomography, leave much wanting. While these techniques see through the body, they lack high — submillimeter — resolution. But that is a level within the grasp of optical imaging methods.

Optical enhancement starts with "simple" magnifying glasses and continues with surgical microscopes and endoscopes. They often comprise a digital camera enabling sophisticated image processing. White light illumination and naturally colored vision are still predominant in the OR whereas modern techniques, such as fluorescence microscopy or optical coherence tomography (OCT), are limited to niche applications.

New imaging modalities beyond fluorescence are being investigated to provide specific contrast. Until now, this capability was the realm of fluorescence, where markers attach to cell constituents and light up against the dark background in the image. Chemical bonding ensures that the markers label only specifically to certain biological receptors. For example, fluorescence is very successful in making tumors or metastases of the bladder clearly visible. After treatment with levulinic acid, cancerous tissue accumulates porphyrin and appears red against a bluish background in fluorescence endoscopy.

Although its value is without question, fluorescence has some significant drawbacks: It relies on staining with extrinsic markers that are infused into the human body. They can do harm and need the approval of the FDA. Also, the markers bleach over time and have to be replaced continually. Otherwise, the fluorescence signal fades away with observation time. Novel imaging techniques based on nonlinear optical effects can overcome these and other drawbacks of labeling. Taken together, they form a multimodal tool kit for vision improvement inside the OR.

New imaging techniques

Nonlinear effects only become significant at very high light intensities. Consequently, lasers play an integral role in new developments. Avoiding direct damage to tissue, the lasers don't emit continuously but only ultrashort light pulses of very high peak powers. With the appropriate laser parameters, the average intensity remains moderate and the tissue is imaged without being damaged. The high peak intensities lead to nonlinear optical effects and generate different signals while interacting with the polarizability of the molecules inside the tissue. Because virtually every molecule is polarizable, nonlinear effects can address any part of the cell and yield the desired contrast in the image. There is no need for staining or other special preparation before imaging. In contrast to fluorescent-based methods, where bleaching is inherent, nonlinear effects do not change the molecular polarizability over time. Here, bleaching does not occur at all.

Coherent optical effects

The most prominent effects used for nonlinear imaging are second- and third-harmonic generation (SHG and THG) or coherent Raman scattering (CRS). They have the coherent nature of signal generation in common. The molecules act in these processes like catalytic promoters in a chemical reaction. For efficient and spe-

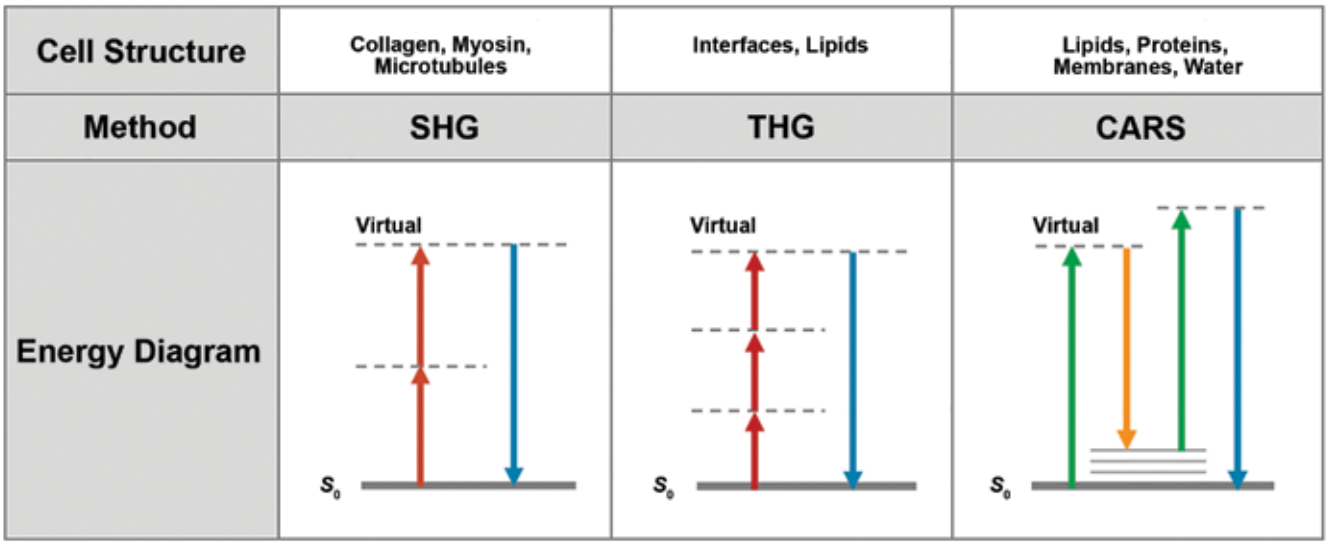


Figure 2. Depending on the type of cell structures, different nonlinear imaging methods are applicable. Together they form a tool kit for multimodal imaging. Courtesy of Munich University of Applied Sciences.

cific contrast generation, their presence is required, but the light fields interact and mix with themselves. For example, coherent anti-Stokes Raman scattering (CARS) is a special type of four-wave mixing. There is no transfer of energy or momentum from the light fields to the interacting molecules. This imposes restrictions on the interaction itself: In addition to energy conservation, the light fields must fulfill momentum conservation by themselves, leading to the so-called phase-matching condition. The consequence of this process is the forward emission of the signal along the direction of the illumination. This is a major difference to fluorescence spreading isotropically in all directions.

For pathology, a second microscope objective directly opposed to the illuminating objective collects the forward-generated signal inside finely sliced biopsies. The objectives form a 4Pi-geometry because of the full solid angle that they fill out in the case of forward detection. During surgery, the forward emission is definitely the most severe disadvantage of coherent techniques because the 4Pi-geometry is impracticable and the light needs to be collected in backward direction. Fortunately, biological tissue is highly scattering, and in this way many photons from the forward-generated SHG signal are scattered back and finally

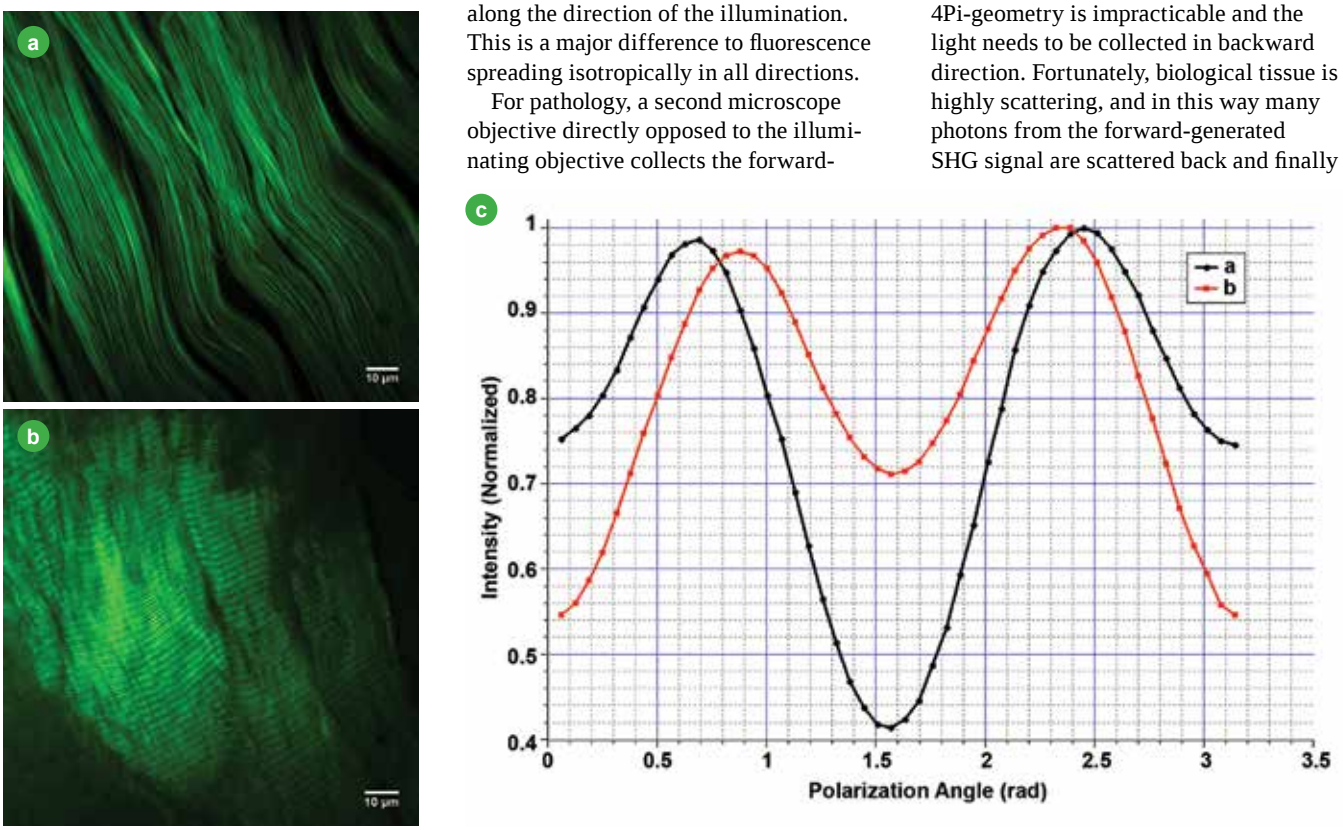


Figure 3. SHG images of different tissue types of a mouse sample: **(a)** cartilage (collagen fibers), **(b)** muscle fibers (myosin), and **(c)** second-harmonic generation intensity dependence on incident laser polarization at different locations. The analysis of the curves reveals the orientation and type of fibers. Images taken at the Multiphoton Imaging Lab. Courtesy of Munich University of Applied Sciences.

re-emitted in the direction of the light source.

The backscattered signal is collected by the very same lens that focuses the laser onto the tissue placed at the end of the endoscope. Some structures inside the tissue are prone to scatter light more effectively backward than others. These variances give rise to different appearances between forward- and backward-collected images of the same area of finely sliced samples (Figure 1).

SHG imaging

In the SHG process, two photons of the same laser pulse interact and generate a third photon with twice the energy (Figure 2). Consequently, the wavelength of the SHG signal is exactly half the laser wavelength and can be separated easily from the laser or surrounding light by several narrowband spectral filters. As the upper energy level is only of virtual nature, the SHG photon is generated simultaneously with the annihilation of the two incoming photons of the laser. Placing a very short and synchronized time window onto the time trace of the recording separates the weak SHG signal from the random background noise of the detector. These efforts enable nonlinear imaging inside the bright OR in the first place because otherwise the entire room has to be shaded as it is usually the case in laboratories of nonlinear optics.

Major advantages of SHG imaging

Virtually any femtosecond laser generates an SHG signal and only practical considerations are important for its implementation in a clinical environment. Fiber lasers, such as Toptica Photonics' FemtoFiber models, are leading the field because of their robustness, compactness and easy operation. Other than in CRS or fluorescence, the SHG process does not require any vibronic or electronic energy level that must be matched according to quantum mechanics. The free choice of the wavelength is one of the major advantages of SHG.

Wavelengths for SHG imaging are located in the near-infrared (NIR) spectral range, typically around 1 µm or even longer for two reasons. On the one hand, high penetration depths of approximately 1 or 2 millimeters can be achieved with NIR light in biological tissue. Both scattering and tissue absorption diminish drastically when shifting the wavelength

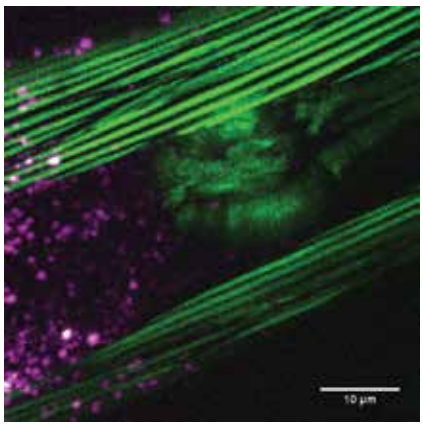


Figure 4. Projection of the three-dimensional multimodal image of the nematode *Ceanorhabditis elegans*. **Green:** second-harmonic generation of the body wall muscles and the pharynx. **Magenta:** third-harmonic generation (THG) of lipids. Image taken at the SLN facility at ICFO Barcelona. Courtesy of C. Polzer.

from the visible into the NIR region referred to as the "optical window" of tissue. This allows looking beneath the surface and visualizing, for example, the layer composition of a blood vessel. On the other hand, using a NIR laser beam generates an SHG signal that lies in the visible due to frequency doubling. This is very convenient because most standard optics and detectors are designed and optimized for visible light.

Furthermore, the combination with other well-established techniques, such as confocal fluorescence microscopy, is straightforward because the latter operates in the visible range too. In contrast to CRS, the microscopy or endoscopy setup for SHG is also more simple and robust because it requires just one laser wavelength instead of two. Therefore, it is free of complicated temporal or spatial alignments of different optical paths.

Limitations of SHG imaging

As mentioned before, nonlinear effects require very high intensities, which occur only in the focal volume of the focused ultrashort laser beam. For image acquisition, the laser focus scans through the tissue, covering typically about 100 µm in each direction while the signal is collected at each position. The image is built up pixel by pixel and reconstructed by the computer software. In consequence, the field of view is much smaller than what surgeons are accustomed to seeing. Additionally, only digital images are available instead of the usual direct view through microscope optics. In the case of weak signals, the image is not refreshed with video rate, but the image acquisition can take several seconds.

Careful consideration of the surgeon's demands results mostly in the following implementation: After taking an overview with "ordinary" imaging methods, the surgeon chooses a few critical spots where nonlinear imaging should take place for an optical inspection corresponding to a biopsy. At the chosen spots, several layers at different depths are imaged in a row, which software stacks together for rendering. This results in a 3D reconstruction of the tissue and allows turning it around and watching it from different angles. Additionally, digital images can be post-processed to enhance image quality and their information content is further enriched with sophisticated image analysis.

In particular, SHG provides structural information and molecular features of the specimen. For example, it shows that the SHG signal of collagen fibers is dependent on the polarization of the incident beam. By rotating the incoming polarization and collecting the signal at the different polarization states, it is possible to obtain the fiber orientation as well as the helical pitch angle (Figure 3). This information can be used for discrimination between healthy and pathological tissue and it allows determining the elasticity of cartilage, for example.

Multimodal imaging

With nonlinear optics, nature provides label-free imaging. However, not every type of tissue or biological sample produces a signal sufficiently strong for imaging. A molecule's capability to produce nonlinear signals depends on its properties like atomic composition, orientation and the overall 3D structure. In biological material, there are just two predominant sources for SHG: collagen fibers and muscle tissue (myosin). Fortunately, label-free imaging of other tissue types is possible based on the various mechanisms of different nonlinear effects.

It seems that for every type of tissue or biological structure, there is a nonlinear microscopy technique (Figures 2 and 4). The power of nonlinear microscopy lies in the combination of various techniques by means of multimodal imaging. The method of choice thereby depends on the molecular structure of interest. For example, THG signals can be produced at any interface where the nonlinear refractive index changes. The signal strength of

THG is proportional to the index change. Hence, this method is suitable for imaging the margins of lipid droplets in an aqueous environment. Lipids and membranes also give rise to strong CRS signals.

In contrast to harmonic generation, the CRS process involves a molecular state, which varies the signal strength significantly when tuning the laser wavelengths. Therefore, CRS is the only technique that is capable of addressing specifically different types of tissue. Despite its general applicability, CRS is limited as well regarding biological samples. The sensitivity of CRS is inferior to that of fluorescence and requires high concentrations of the substances being visualized. Nevertheless, the advantage of label-free imaging with specific contrast outweighs its limitations because it is minimally invasive and most suitable for in-vivo imaging. It is clear that multimodal imaging will become an indispensable tool for improved vision inside the OR.

Meet the authors

Thomas Hellerer is a professor of biophotonics at the Munich University of Applied Sciences (MUAS) in Munich; email: hellerer@hm.edu. Christoph Polzer is a Ph.D. student at MUAS; email: christoph.polzer@hm.edu. Mojtaba Mohseni is a Ph.D. student at MUAS; email: mojtaba.mohseni@hm.edu.

A Key Term in This Article

• **second-harmonic generation microscopy:** A nonlinear label-free imaging technique commonly used during surgical procedures for the visualization of collagen fibers and muscle tissue (myosin) with submillimeter resolution. During the second-harmonic generation (SHG) process, the two photons of the same laser pulse interact and generate a third photon with twice the energy, resulting in a signal that is half the laser's wavelength. Through the use of narrowband spectral filters, the SHG signal is distinguishable from the laser and surrounding light.

See **EDU.Photonics.com** for this and other definitions in the *Photonics Dictionary* and more information in the *Photonics Handbook*.