

Depth-resolved multiphoton polarization microscopy by third-harmonic generation

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We achieve depth-resolved polarization microscopy by measuring third-harmonic generation induced by a tightly focused circularly polarized beam. In crystals exhibiting strong birefringence this signal is dominated by positively phase-matched third-harmonic generation. This process occurs in only optically anisotropic media, in which the birefringence compensates for the phase mismatch between the fundamental and the third harmonic induced by dispersion. Both the intensity and the polarization of the emitted signal provide information on the local optical anisotropy. We demonstrate the technique by imaging biogenic crystals in sea urchin larval spicules. © 2003 Optical Society of America

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The field of multiphoton microscopy has been rapidly developing since the development of compact femto-second lasers. Two-photon¹ and three-photon² fluorescence microscopy show better axial resolution and improved signal-to-background ratios than standard laser-scanning fluorescence microscopy. Coherent multiphoton techniques such as second-harmonic generation,³ third-harmonic generation^{4–8} (THG), and coherent anti-Stokes Raman spectroscopy^{9–12} have recently become an attractive alternative for depth-resolved imaging of unstained samples. Since the measured signal depends not on the refractive index but on the nonlinear susceptibility, such techniques provide, in addition to the intrinsic depth resolution, rich information on the samples that is unattainable even by advanced linear microscopy techniques, such as phase microscopy or differential-interference contrast microscopy. In this Letter we present a novel multiphoton microscopy technique, based on the THG process, that provides detailed depth-resolved information on the sample optical anisotropy.

Polarization microscopy, in which the sample is placed between two crossed polarizers, has long been used to image samples that exhibit birefringence. Numerous techniques have been developed to reduce the background caused by birefringence of the microscope optics and depolarization of the illuminating beam in the sample and to circumvent the need to rotate the sample in order to measure the birefringence properties.^{13,14} Recently, laser-scanning polarization microscopes were introduced. By rotation of both the input polarization and the analyzed signal polarization it is possible to obtain the Stokes parameters in each pixel. However, this requires the use of computer-controlled polarization modulators, resulting in a rather complex microscope system.¹⁵

THG microscopy has been used until now to characterize isotropic transparent specimens.^{4–8} In THG microscopy third-harmonic light is generated at the focal point of a tightly focused short-pulse laser beam. Assuming illumination by a Gaussian beam and following the derivation given in Ref. 16, the total THG signal amplitude is

$$A_3 = \frac{i2\pi g\omega}{nc} A_1^3 \int \frac{dz \chi^{(3)}(z)\exp(i\Delta kz)}{(1 + 2iz/b)^2}, \quad (1)$$

where b is the focal depth, $\chi^{(3)}$ is the respective third-order susceptibility, A_1 is the amplitude of the illuminating beam, and $\Delta k = 3k_1 - k_3$ is the wave vector mismatch between the fundamental and the third harmonic. Integrating Eq. (1) shows that for materials with normal dispersion, where the phase mismatch Δk is negative (or even zero), no signal is generated from a homogeneous bulk medium. In this case the symmetry along the optical axis leads to complete destructive interference between the signal generated before and after the focus. No net THG is thus observed unless inhomogeneities, such as material interfaces, break the symmetry along the optical axis. A measurable amount of third-harmonic light is thus generated by only inhomogeneities on the scale of the focal depth. This fact has its basis in standard THG microscopy techniques.^{4–8}

However, this situation can be strikingly different for birefringent media, such as most crystals. As in standard phase matching of second-order nonlinear processes, birefringence can be used to compensate for unfavorable phase mismatches. When the birefringence is strong enough, a positive phase mismatch is achieved. Integrating Eq. (1) for this case generates a THG signal that scales as

$$A_3 \propto b\Delta k \exp(-b\Delta k/2), \quad (2)$$

and which is polarized along the fast axis even from a bulk medium. According to relation (2), the intensity of the signal depends on both the birefringence (and thus on the crystal orientation) and the focal depth (i.e., the illumination geometry). The THG intensity varies with the polarization of the illuminating beam, depending on the type of phase matching (type I: slow, slow, slow \rightarrow fast, type II: slow, slow, fast \rightarrow fast, or type III: slow, fast, fast \rightarrow fast). Which of types three types of process is dominant depends on the crystal properties.

To utilize THG for depth-resolved polarization microscopy, the THG generated at inhomogeneities within isotropic media must be suppressed. This can be accomplished with a circularly polarized light fundamental excitation beam. In this case the THG signal is proportional to $|\chi_{xxxx} - \chi_{xyyy} - \chi_{xyyx} - \chi_{xyxy}|^2$, which vanishes for an isotropic medium.¹⁷ For type II and type III phase matching, circular polarization is close to the optimal polarization for maximizing the THG signal.

It should be noted that in passing through a birefringent medium the polarization state of the fundamental excitation beam changes. Moreover, when the beam is focused at an interface between a birefringent medium and an isotropic one, some THG is generated even with circularly polarized illumination. This signal, unlike the positively phase-mismatched THG, is generated even from weakly birefringent media, because a full compensation of the phase mismatch by birefringence is not required. It vanishes, however, when the beam is focused within the birefringent medium. Indeed, a comparison between the THG from circularly and linearly polarized light has recently been used for mapping anisotropy of interfaces and surface layers.¹⁸

We study depth-resolved polarization THG in calcite, a negative uniaxial crystal exhibiting strong birefringence. Calcite is interesting because of its important role as a biogenic crystal.¹⁹ In the following we demonstrate the utility of this technique by observing calcite crystal within sea urchin larval spicules.

By measurement of angle phase-matched THG from a thick calcite crystal it has been established that the dominant process is type II ($ooe \rightarrow e$).²⁰ As our first experimental demonstration of positive phase-mismatched THG by a tightly focused beam, we consider THG from a single calcite crystal. The illumination source in our experiments is an optical parametric oscillator that delivers 100-fs pulses at 1.5 μm and an energy of 0.5 nJ (Spectra Physics OPAL). These are focused with a N.A. = 0.85 objective,²¹ either at the interface or into a thick (2 mm) x -cut calcite crystal. The THG is collected by a condensing lens, filtered, and measured by a photomultiplier tube. Shown in Fig. 1a is the total THG signal as a function of the angle between the input linear polarization and the ordinary (y) axis of the crystal, either at the interface (dashed curve) or within the crystal (solid curve). As can be seen, the signal from the interface, which does not require phase matching, is dominated by the diagonal terms χ_{oooo} or χ_{eeee} and thus peaks when the input beam is polarized along one of the crystal axes. In contrast, when the beam is focused within the crystal, the signal peaks at an angle of $\sim 35^\circ$ from the ordinary axis and vanishes when the input beam is polarized along the crystal axes, corresponding to type II phase matching. To further characterize the THG signal, we illuminate the crystal with circularly polarized light and add an analyzer between the sample and the photomultiplier. Figure 1b shows the THG signal as a function of the angle between the analyzer and the ordinary axis of the crystal, both at the interface and within the

crystal. In the phase-matched case this confirms that the THG signal is indeed linearly polarized along the extraordinary axis.

For the THG microscopy experiments we use a Zeiss Axiovert-135 microscope that is modified into a scanning THG microscope. A detailed description of the system can be found in Ref. 22. Briefly, laser light is coupled through one of the microscope ports and focused into the sample with the same N.A. = 0.85 objective lens. The focal spot is scanned with two computer-controlled optical scanners. The input polarization is controlled by a zeroth-order quarter-wave plate. THG light is collected by a N.A. = 0.63 condenser, filtered by a bandpass filter at 500 nm, passed (when applicable) through a thin-film polarizer serving as an analyzer, and measured by a photomultiplier and a rf lock-in amplifier. An integration time of $\sim 300 \mu\text{s}$ per pixel is used. The sample, shown in Fig. 2, consists of individual sea urchin larval spicules spread on a glass microscope slide and immersed in an $n = 1.61$ index-matching oil.

We first show in Fig. 2a the observed THG signal with linearly polarized illumination. As can be seen, a strong background is generated over the entire frame, on top of which a slightly stronger signal is observed from the spicule. The strong background is caused by THG from the glass-immersion-oil interface. This background is, however, completely eliminated when the illuminating beam is circularly polarized, as shown in Fig. 2b. The strong signal generated from the spicule is in this case predominantly caused by the positively phase-mismatched THG process in calcite. Figures 2c and 2d show the image obtained when an analyzer allowing either vertical or horizontal polarization is inserted between the sample and the detector. Although the image is maintained for one polarization orientation, it completely disappears for the other. This indicates that throughout the larval spicule the crystal orientation is maintained. This is in agreement with x-ray diffraction measurements, indicating the indeed the entire larval spicule is made of a single calcite crystal.¹⁵

The depth-resolved laser-scanning multiphoton polarization microscopy scheme described above requires

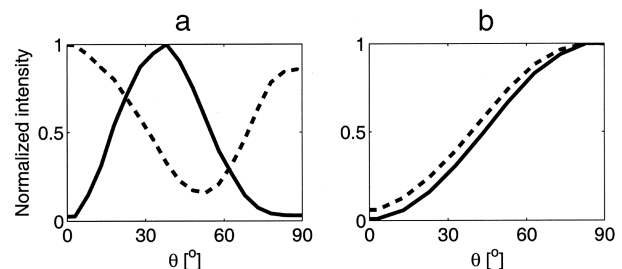


Fig. 1. Experimental THG measurements from a thick x -cut calcite crystal when the input beam is focused either at the air-calcite interface (dashed curve) or within the calcite crystal (solid curve). a, THG intensity as a function of the relative angle between the input (linear) polarization and the ordinary axis of the crystal. b, THG intensity observed with circularly polarized illumination as a function of the relative angle between the analyzer polarization and the crystal ordinary axis.

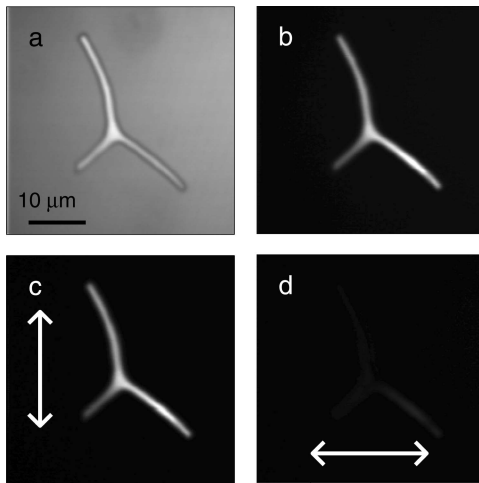


Fig. 2. Images of a single larval spicule spread on a glass slide. a, Image obtained with linearly polarized light, showing THG from both bulk birefringent media and interfaces. b, Image obtained with circularly polarized light, showing THG from only bulk birefringent media. c, Image obtained with circularly polarized light, with an analyzer oriented along the vertical direction. d, same as c, with an analyzer oriented along the horizontal direction. Images c and d, which are given on the same intensity scale, indicate that throughout the larval spicule the crystal orientation along the horizontal axis is maintained.

a simple optical setup, with no need for highly accurate polarization characterization of both the input beam and the output signal, as in advanced linear polarization microscopy techniques. Moreover, it exhibits intrinsically all the inherent advantages of multiphoton microscopy: a superb depth resolution and an ability to accurately measure the properties of highly scattering samples. It is especially suited for microscopy of biogenic crystals, such as calcite and aragonite, which exhibit strong birefringence. Requiring nothing but a quarter-wave plate and an analyzer in the optical path, this method can be easily combined with other multiphoton microscopy techniques.²³

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21. Note that because of the large positive phase mismatch in calcite, the THG signal as derived in relation (2) becomes exceedingly small when objectives with a small numerical aperture are used. We have verified this experimentally by using N.A. = 0.25 and N.A. = 0.57 objectives.
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