Dark-field third-harmonic imaging

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Coherent cancellation of third-harmonic generation (THG) in a tightly focused laser beam is shown to enable a label-free imaging of individual neurons in representative brain tissues. The intrinsic coherence of third-harmonic buildup and cancellation combined with the nonlinear nature of the process enhances the locality of the dark signal in THG, translating into a remarkable sharpness of dark-field THG images. Unique advantages of this technique for high-contrast subcellular-resolution neuroimaging are demonstrated by comparing THG images of hippocampus and somatosensory cortex in a mouse brain with images visualizing fluorescent protein biomarkers. © 2013 AIP Publishing LLC.

Over the past years, ultrafast nonlinear optics has shown a tremendous progress in addressing the most urgent needs of bioimaging toward the development of a unique arsenal of methods and tools for chemically selective, label-free, deep-tissue imaging with an unprecedented spatial resolution. Since the seminal work on two-photon microscopy,1 which paved the way for the nonlinear-optical breakthrough in bioimaging,2,3 a broad variety of nonlinear-optical approaches have been developed to confront the most challenging problems in the vast area of biosciences. Microscopy based on second- and third-harmonic generation (SHG and THG) has been shown to suggest attractive methods for exploring the structural properties of biotissues, allowing fine details in the morphology of biotissues to be visualized with a high spatial resolution. Chemically selective bioimaging has become possible through the use of coherent Raman scattering (CARS and SRS), which enable a three-dimensional label-free subcellular-resolution imaging of bio-objects, including brain structures.13,14 Stimulated emission depletion (STED) and related techniques are capable of providing a spatial resolution well below the 100 nm level, pushing the frontiers of bioimaging.

Nonlinear-optical processes used for imaging are divided into two classes with regard to their coherence. Processes of the first class, such as multiphoton-absorption-induced fluorescence, SRS, and STED, are insensitive to the phase of the nonlinear signal relative to the phase of the relevant nonlinear polarization of the medium. For the processes of the second class, which includes SHG, THG, and CARS, nonlinear signal generation is highly sensitive to the phase mismatch between the nonlinear signal and the nonlinear polarization.17 While the importance of the nonlinear nature of signal generation for the high spatial resolution and larger penetration depths in nonlinear-optical imaging is commonly appreciated, the role of coherence is much less articulate in the literature.

Here, we show that coherent effects can significantly enhance the potential of nonlinear-optical processes as imaging methods, suggesting powerful approaches for bioimaging. As a particular application, we demonstrate that coherent cancellation of THG in a tightly focused laser beam enables a label-free high-sharpness imaging of individual neurons in representative brain tissues. Experiments presented below in this paper verify the high contrast, morphology-related content, and subcellular resolution of dark-field THG neuroimaging of representative brain tissues, such as the hippocampus and somatosensory cortex.

The efficiency of THG is controlled by the third-order nonlinear-optical susceptibility of the material and the mismatch of the phase of the harmonic field, $\phi_h$, and the phase $\phi_p$ of the third-order polarization induced in the medium. This phase mismatch, $\Delta\phi = \phi_h - \phi_p$, consists of two parts, $\Delta\phi = \Delta\phi_m + \Delta\phi_g$, where $\Delta\phi_m = \Delta k z$ is the phase mismatch due to the dispersion of the medium (is the coordinate along the propagation path, $\Delta k = 6\pi\Delta n/\lambda_p$, $\lambda_p$ is pump wavelength and $\Delta n$ is difference of the refractive indices of the medium at the pump and third-harmonic frequencies) and $\Delta\phi_g = \phi_h - \phi_p$ includes the geometric (Gouy) phase shift $\phi_p$ of the focused beam and the corresponding phase shift $\phi_h$ of the THG nonlinear polarization. In a paraxial Gaussian-beam approximation, $\phi_p = -\arctan(2b/\lambda)$, where $b = 2\pi w_0^2/\lambda_p$ is the confocal parameter, $w_0$ is the beam waist diameter, and $n$ is the refractive index.

Phase-matching effects may have a dramatic impact on THG. In Fig. 1(a), we present the intensity of the third harmonic (the left axis) and the Gouy phase shift of the pump field (the right axis) calculated as functions of the propagation path $z$, measured from the beam focus, for a Gaussian beam tightly focused into a homogeneous nondispersive medium using the rigorous Green-function analysis (solid lines) and the standard paraxial approximation (dashed lines). The third-harmonic signal is seen to vanish at the
output of an extended medium (large $z$ in Fig. 1(a)), no matter how high the laser intensity and optical nonlinearity of the medium are. This third-harmonic cancellation is coherent in its nature and is caused by the Gouy phase of the focused beam, which is close to $\pm \pi/2$ for $|z| \gg b$ (Fig. 1(a)), translating into a geometric phase mismatch $\Delta \phi_g \approx \pm \pi$ everywhere except the beam waist region of length $b$. If, however, a tightly focused laser beam comes across an optical inhomogeneity, the phase-mismatch-induced suppression of the third-harmonic output is no longer perfect, giving rise to a nonvanishing third-harmonic signal. This effect, which enables three-dimensional imaging based on THG, contrasts third-harmonic generation with CARS, where $\Delta \phi_g \approx 0$, and, hence, the sensitivity of the process to phase matching does not translate into a complete coherent cancellation of the signal, which confines THG to the beam-waist region.

We now consider a medium with isolated spherical inclusions of diameter $D$. In Fig. 1(b), we show the third-harmonic intensity calculated as a function of $D$ using the Green-function method for a pump beam with $\lambda = 1.25 \mu m$ focused by an objective with a numerical aperture NA = 0.65, yielding $b \approx 6.7 \mu m$. Results of calculations performed for a medium without the material part of the phase mismatch, $\Delta \phi_m = 0$ (dashed line), are compared in this figure with calculations for a medium with a typical dispersion of lipid bodies (solid line). Due to the larger overall phase mismatch $\Delta \phi$, translating into shorter coherence lengths, material dispersion is seen to confine THG to an even smaller region. The maximum third-harmonic signal is now observed for particles with $D \approx 4 \mu m$, while the signal for inclusions with $D > 8 \mu m$ totally suppressed. When embedded in an optically inhomogeneous medium where the size of inhomogeneities is on the order of the laser wavelength, such that the net third-harmonic signal is nonzero, spherical inclusions can be visualized as dark fields in the maps of the third harmonic. Experiments presented below in this paper show that this model explains the key features of third-harmonic images of representative brain tissues. Neuron bodies in such tissues are, of course, not homogeneous, but consist of randomly distributed small-scale organelles. However, since the sizes of organelles (50–200 nm (Ref. 20)) are much smaller than $\lambda$, the nonlinear susceptibility of neuron bodies is still a meaningful parameter, which can be defined in terms of standard effective-medium models.

In experiments, we studied brain tissue samples taken from the hippocampus and somatosensory cortex of transgenic mice whose genome was modified to include an enhanced green fluorescent protein (eGFP) gene under the sequence of genes encoding zinc finger (zif) early growth response proteins. These two types of brain tissues exhibit distinctly different densities of neurons, allowing a meaningful validation of the potential of dark-field THG imaging against imaging based on the fluorescent response from GFP. The slices of brain tissues studied in our experiments were extracted from the Tg(Egr1-EGFP)60Gsat/Mmcd (Stock Number: 014709-UCD) male mice aged from 4 to 6 months with a weight of 25–35 g.

As a coherent source of ultrashort laser pulses, necessary for efficient THG, we used a home-built ytterbium-fiber-laser-pumped mode-locked Cr: forsterite laser oscillator, which delivered laser pulses with a central wavelength of 1.25 $\mu m$ and a pulse width of 40 fs. The extended-cavity design of the Cr: forsterite laser used in our experiments provides an increased laser energy output, yielding laser pulses with an energy up to 20 nJ at a pulse repetition rate of 20 MHz. Such a combination of the laser pulse energy and repetition rate is ideal for high-speed THG imaging. The unamplified output of the Cr: forsterite laser was directly used as a pump in third-harmonic imaging. Due to the reduced attenuation of biotissues at 1.25 $\mu m$, the central wavelength of this laser is instrumental in probing deeper layers and thicker samples.

Cr: forsterite laser radiation was focused on a sample by a 40× objective with a numerical aperture NA = 0.65. The third harmonic was filtered from the laser beam with the use of filters and dichroic mirrors and was detected with the use of a photomultiplier. The signal-to-noise ratio for the third harmonic was improved by using optical heterodyning. To this end, the pump beam was modulated with a chopper at a reference frequency of 379 Hz. The photomultiplier output produced at this frequency in response to the third-harmonic signal was read out by a lock-in amplifier. The time required to record one pixel with this reference frequency, providing efficient noise suppression, was about 100 ms. This time can be reduced to a microsecond level by applying megahertz reference frequencies supported by commercially available lock-in amplifiers. Third-harmonic images were synthesized through point-by-point measurements with the sample.
scanned in the transverse plane with respect to the laser beam by stepper motors with a step of 1 μm along both coordinates. A typical time required to record images shown in Figs. 2(a) and 2(b) is 30 min.

The confocal parameter of the laser beam focused with an NA = 0.65 objective (b = 6.7 μm) was much smaller than the typical sizes of neuron bodies in the studied brain tissues (8–12 μm), providing coherent cancellation of the third harmonic from neuron cells (see Fig. 1(b)). In Figs. 2(a) and 2(b), we present the images of a hippocampus tissue taken for 20-μm-thick brain-tissue slices with the use of THG (Fig. 2(a)) and fluorescence response from eGFP (Fig. 2(b)). The areas of minimum intensity in third-harmonic images perfectly match the areas of the highest brightness of GFP fluorescence, confirming that the dark fields in third-harmonic images visualize neuron bodies. Moreover, due to its coherent and nonlinear nature, THG yields much sharper and much better resolved images of neurons, whereas the fluorescence images suffer from blurriness, which is due to the lower locality of the fluorescent response, making signals from the adjacent groups of neurons overlap, thus lowering the image quality.

For somatosensory cortex tissues, where the density of neurons is much lower, isolated neurons can be reliably visualized using both THG and the fluorescence technique (Figs. 3(a) and 3(b)). Here, the higher sharpness and better spatial resolution of THG images can be appreciated from the clear and sharp boundaries of individual neurons. This higher quality of THG images is adequately reproduced by our model of optical response of brain tissues (Figs. 3(c) and 3(d)), where the neuron bodies are modeled as spherical inhomogeneities in the spatial distribution of third-order susceptibility χ(3)(r), while neuron axons and dendrites, as well as the network of neurofilaments are included through randomly oriented inhomogeneities in χ(3)(r), whose size distribution mimics that of axons and dendrites in mouse somatosensory cortex tissues. The model provides a good fit for THG images, where the contribution of out-of-focus neurons is negligible, effectively reducing the reconstruction of neuron positions in space to a two-dimensional problem. Our model, however, fails to reproduce the details of blurriness in fluorescent images, where much of the signal comes from out-of-focus neurons, which makes it difficult to reconstruct the neuron coordinates in a full three-dimensional problem. This increased blurriness of fluorescence images along with the lower spatial resolution prevents the detection of important morphological features of neuron bodies, as well as visualization of neuron axons and dendrites. Third-harmonic generation, on the other hand, can clearly resolve, as can be seen from Figs. 3(a) and 3(c), the shape features of some of the well-isolated individual neurons and provide a high-sharpness detection of neuron body boundaries. Smaller, submicron-scale features of neurons can be resolved, as suggested by tentative experiments and simulations, by THG with a tighter beam focusing.

In summary, we have demonstrated that coherent cancellation of THG in a tightly focused laser beam enables a high-sharpness, label-free imaging of individual neurons in representative brain tissues. The combination of nonlinearity and coherence has been shown to be the key factor behind the remarkably high sharpness of dark-field THG images. Unique advantages of this technique for high-contrast subcellular-resolution neuroimaging have been demonstrated by comparing THG images of hippocampus and somatosensory cortex in a mouse brain with images visualizing fluorescent protein biomarkers.

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