

Nonlinear Optical Spectroscopy

And Imaging of Structural Proteins in Living Tissues

Paul Campagnola and William A. Mohler Several traditional nonlinear optical tools, such as second harmonic generation (SHG), third harmonic generation (THG) and coherent anti-Stokes Raman spectroscopy (CARS), have recently been used to image the morphology and organization of protein arrays in live cells and tissues. These methods provide more detailed molecular information than the more conventional fluorescence methods that are widely used in biology. The authors have used SHG microscopy to image collagen, actomyosin and tubulin structures in a variety of species including *C. elegans* nematodes, mammalian muscle tissue and fish scales.

n the past decade there has been extensive integration of nonlinear optical spectroscopic tools and biological microscopy. This comes at a time when microscopy itself has experienced significant technical advances that have revolutionized the area of biological imaging, especially in terms of fluorescence acquisition. Historically, optical microscopy was largely performed in a wide-field configuration and thus had little axial discrimination without the use of post-data-acquisition deconvolution. This difficulty has been eliminated by the advent of laser scanning confocal microscopy (LSCM), which provides axial discrimination through the use of a pinhole aperture in front of the detector that rejects out-of-focus light. Although initially described and patented in the 1950s, confocal detection was not implemented in commercial microscopes until the late 1980s. The delay was in part due to the need for the development of fast scanning optics and fast data acquisition. Since then, laser scanning confocal microscopy has become the tool of choice for imaging live cells and tissues by fluorescence, where three-dimensional (3D) data is acquired one focal plane at a time and can then be reconstructed.

Although LSCM provides axial sectioning in terms of data acquisition, the excitation occurs through the entire sample volume. Perhaps the most limiting feature of fluorescence microscopy is photobleaching of the fluorescent dyes that are used to enhance contrast. The negative effects of bleaching are manifested in two ways: first, upon sectioning into 3D samples by loss of signal intensity because of bleaching of regions out of the plane of focus; second, by the toxicity that arises from the free radicals generated during the photoprocess. This toxicity typically arises from the production of singlet oxygen that occurs following intersystem crossing to the triplet state. In 1990, Watt Webb and coworkers realized that these adverse effects of dves could be greatly reduced by the use of two-photon excitation.¹ Because multiphoton processes require very high peak power, through careful adjustment of the laser power, excitation can be confined precisely to the plane of focus. Confining the excitation to the plane of focus provides intrinsic sectioning; in addition, undesirable out-of-plane photoeffects are greatly









Figure 1. (a) SHG image of two adult *C. elegans* nematodes at 850-nm excitation. Whole organisms are approximately 1 mm long and 100 μ m thick. (b) SHG "spectra" of *C. elegans* pharyngeal muscle. The SHG appears exclusively at the expected wavelength and tracks the excitation wavelength. (c) and (d) two slices of mouse leg muscle tissue, approximately 100 and 300 μ m into a 550- μ m thick stack, showing the regular sarcomere pattern.

diminished. It should be noted, however, that photobleaching and phototoxicity still occur in the plane of focus. An additional advantage of this approach is that because the laser wavelengths are typically in the near IR (700-1000 nm), the reduced Rayleigh scattering allows for greater penetration into thick tissue samples than is possible by means of visible excitation. An enabling technological advance of multiphoton excited (MPE) fluorescence microscopy has been the commercial availability of robust, mode-locked titanium sapphire lasers. Ti:sapphire oscillators—with their high repetition rate (~80 MHz) and broad tunability (700-1000 nm)-are ideal for multiphoton fluorescence excitation. The nanoJoule pulse energies of these lasers, when focused with a high numerical aperture lens, provide peak powers sufficiently high for efficient two-photon absorption of most fluorescent dyes.

For these reasons, multiphoton excitation represents a major advance in laser scanning microscopy and has become, over the course of the past few years, a popular alternative to "ordinary," or linear, LSCM. A number of applications in research areas including cell biology, neuroscience and genetics have greatly benefited from the reduced photobleaching (and thus greater cell viability) in live cells and tissues. Applications that are even more powerful exploit the intrinsic three dimensionality of MPE to provide more detailed data in thick tissues. Such applications have included fluorescence recovery after photobleaching (FRAP) and fluorescence resonance energy transfer (FRET), where FRAP is used to measure diffusion constants and FRET is used to extract intermolecular distances between chromophores. While these schemes have long been implemented by means of one-photon excitation, the resulting data often needs to be deconvolved since absorption occurs through a volume, rather than a slice.

While multiphoton excited LSCM has added great value to biological fluorescence imaging (and is the subject of many papers and reviews), it only begins to scratch the surface of the power of nonlinear optics in biological imaging. In this article we will focus on other forms of nonlinear optics that not only possess the same enabling features as multiphoton excited fluorescence but also provide



more detailed molecular level data. In general, the nonlinear polarization for a material can be expressed as:

$$P = \chi^{(1)} E^1 + \chi^{(2)} E^2 + \chi^{(3)} E^3 + \dots$$

where *P* is the induced polarization, $\chi^{(n)}$ is the *n*th order nonlinear susceptibility, and *E* is the electric field vector of the incident light. The first term describes normal absorption and reflection of light; the second term describes second harmonic generation (SHG), hyper-Rayleigh scattering, sum and difference frequency generation; and the third term covers multiphoton absorption, third harmonic generation (THG), coherent anti-Stokes Raman scattering (CARS) and the optical Kerr effect (OKE). Our group has focused on SHG microscopy. Here we provide a summary of our own work in this area as well as an overview of the recent results of other research groups using THG, CARS and OKE.

A major difference between SHG and multiphoton absorption is that SHG requires a non-centrosymmetric environment to produce signal. This constraint is readily understood by examining the second term in the above equation. Since the SHG wave is a vector quantity, the induced polarization in a centrosymmetric sample (such as dye in a cuvette) from all directions would be equal and opposite and thus vector sum to zero. This noncentrosymmetric constraint can be satisfied in the form of a birefringent crystal, an ordered array or an interface. In terms of biological specimens, appropriate environments include cell membranes and structural protein arrays. Our earlier work² in high-resolution SHG imaging involved labeling cell membranes with voltage-sensitive styryl dyes that possessed large second order susceptibilities, $\chi^{(2)}$.



Figure 2. Simultaneous SHG and twophoton excited GFP image of pregnant *C. elegans* nematode expressing GFP::tubulin. (a) SHG channel shows both body wall components and microtubule assemblies corresponding to first embryonic cell divisions; (b) analogous two-photon excited GFP image with arrows denoting embryos. Note bright autofluorescence from granules in the gut; (c) two color overlap, where SHG is violet, GFP is green and overlap is represented in white. [Courtesy *Biophysical Journal*]

While this leads to high spatial specificity for cell membranes, to obtain sufficient contrast through SHG resonance enhancement, the laser wavelength had to overlap with the two-photon absorption band of the dye.

Unfortunately, working on resonance ultimately leads to photobleaching, just as in the case of fluorescence excitation. More recently, we observed that very large second harmonic signals are obtainable from several structural protein arrays in cells and tissues without the addition of exogenous labels.3 Not only does the possibility of obtaining these signals lead to the virtual elimination of photobleaching, more detailed molecular information is readily elucidated than is possible by use of fluorescent labels. This is because dyes only can infer details of protein assembly and motion, while SHG directly visualizes the submicrometerand micrometer-scale assemblies.

These structural proteins include: collagen, actomysosin complexes and tubulin, among others. Collagen, which exists in almost 20 distinct forms, is the most abundant protein *in vivo* and forms major portions of tissue including skin, bone, blood vessels and tendon. Actomyosin comprises smooth and striated muscle tissue, whereas tubulin polymerizes into microtubule assemblies that are involved



in many cellular processes including division and transport. Historically, these protein structures have been studied by other imaging modalities including electron microscopy and polarization microscopy, and it is well known that they form highly ordered, birefringent arrays. These proteins occur at very high concentrations (mM), and since SHG intensities scale as the square of the molecular density, (in conjunction with the birefringence), these arrays provide very high SHG contrast. Here we will focus on probing the details of the morphology, symmetry and organization of some examples of these arrays by means of SHG imaging microscopy.

SHG imaging microscopy

The SHG/MPE fluorescence microscope primarily consists of a modified LSCM and a titanium sapphire femtosecond laser oscillator. This instrument can simultaneously collect multiphoton excited fluorescence and SHG images, allowing the direct comparison of the contrast mechanisms. A 500 x 500 frame is acquired in one second, and at high numerical aperture, resolution of approximately 500 nm is achieved. The fluorescence is collected in the epi-illumination geometry in a non-descanned geometry, as this greatly increases the sensitivity. Since SHG is a coherent process, the signal copropagates with the laser and is detected in the forward configuration. A fiber optic based spectrometer is used to verify the wavelength of the SHG for spectral purity relative to autofluorescence or residual laser fundamental. Waveplates and polarizers in the excitation and collection paths, respectively, provide control over laser and SHG signal polarization.

Much of our work on SHG imaging has used C. elegans nematodes. These specimens are ideal for microscopy since they are optically clear, their morphology is well known from electron microscopy and they are genetically compliant, allowing for the facile introduction of mutations. Figure 1(a) shows the SHG image of two adult nematodes, where the contrast arises from muscle tissues, including the body wall muscle, the very bright pharynx and the chewing mechanism. The SHG spectrum of the pharynx as a function of excitation wavelength over the range of 800-890 nm is plotted in Fig. 1(b). It is observed that as the excitation wavelength is scanned, the wavelength of the SHG signal appropriately tracks and has the appropriate spectral width for excitation with 100 fs pulses, i.e., ~ 10 nm. A major advantage of nonlinear optical imaging is the coupling of intrinsic sectioning with the available depth of penetration by means of near infrared (NIR) excitation. Figures 1(c) and 1(d) show two slices of mouse muscle tissue revealing the sarcomere repeat pattern that is characteristic of striated muscle, where these bands are separated by approximately 2 µm. Muscle is quite turbid and these slices were acquired with high contrast from regions approximately 100 and 300 µm deep within a stack of tissue 550 µm thick. Indeed, only a loss of 4 fold in intensity was observed from the top to the bottom, thus demonstrating the effectiveness of SHG to probe deep within highly scattering tissue.

A major advantage of SHG imaging over fluorescence is the ability to directly probe molecular organization on the submicrometer- and micrometer-size ranges. Before SHG can be effectively used to study biological function, the physical nature of the SHG contrast in these protein structures must be wellunderstood. To this end, we combined simultaneous SHG and two-photon excited green fluorescent protein (GFP) imaging. GFP, a naturally occurring fluorescent protein from the jellyfish A. Victoria, has an amazing property: the gene that encodes for it can be linked to the gene of virtually any cellular protein

of interest. The resulting fusion is then placed in cells or a whole organism and the desired protein is expressed with the GFP label. Figure 2 shows the result for a *C. elegans* nematode that is expressing GFP:: tubulin. These nematodes are hermaphroditic and are always reproducing, providing the opportunity to look at early embryonic development. Tubulin polymerizes into microtubules that assemble into the highly organized centrosomes and mitotic spindle structures during the interphase and metaphase stages of cell division. The left panel in Fig. 2 shows the SHG image of part of an adult, where both the body wall and the microtubule structures can be observed. The middle panel is the two-photon excited GFP::tubulin showing primarily these latter structures, along with some autofluorescence from the gut. The right



Figure 3. (a) SHG and b) MPE GFP (right), of two interphase centrosomes in early embryonic cells are shown at high magnification. In the GFP channel, centrosomes are uniformly labeled with fluorescent tubulin. In SHG, the centrosomes yield double crescent profiles with a strong angular dependence that is derived from the laser polarization. (c) and (d) Simultaneous SHG and TPEF (GFP::β tubulin) of an embryo during the first mitosis. In (c), bright SHG in the spindle is interrupted by a discrete dark space at the spindle midzone, which is due to a symmetry cancellation effect. In (d), fluorescent microtubules compose the entire dense spindle array, as there are no symmetry constraints on the fluorescence.

panel is the two-color overlap, where the SHG and two-photon GFP, and spatial overlap, are shown in violet, green and white, respectively. As is clearly seen, the contrast in the two mechanisms is quite different. For example, the GFP image is devoid of any of the muscle components seen in the SHG channel. Higher magnification images of the microtubule assemblies, shown in Fig. 3, display more significant differences in the two channels. The SHG and GFP images of two interphase centrosomes are depicted, respectively, in Figs. 3(a) and 3(b). In the SHG channel, these radially symmetric structures appear as two diametrically opposed arcs. This is consistent with a signal arising from the electric dipole interaction, since only the subset of molecules whose dipoles are aligned with the laser polarization will produce signal.

By contrast, the GFP signal appears as a uniform disk. Although twophoton excitation is also a dipolar process, the GFP tag is at the end of the tubulin domain and can freely rotate, so that absorption and emission can occur at all angles. This demonstrates that while GFP provides a specific molecular marker, SHG contains molecular level data on organization that cannot be obtained by means of fluorescence.

A further aspect of SHG imaging that can be exploited for structural studies lies in the coherent nature of the process: oppositely oriented structures on the size scale of the coherence length (~excitation wavelength) vector sum to zero and do not produce observable SHG signals. An example of this behavior is demonstrated in Figs. 3(c) and 3(d),

which show the details of the SHG and GFP contrast in a mitotic spindle. The spindle (consisting of highly organized microtubules) aligns the chromosomes during division. Microtubules, which have a polarity, are known to interdigitate anti-parallel in the spindle midzone around the chromosomes, resulting in no net alignment of the dipoles. The expectation that this orientation will provide SHG symmetry cancellation is indeed borne out in the SHG image, because there is no signal in the region indicated by the arrow. By contrast, fluorescence is an incoherent process, and bright fluorescence is observed throughout this region. Such information about the evolution of the morphology of centrosomes and mitotic spindles during the cell division process would be of interest to researchers in the area of reproductive biology.

Historically, these structural protein arrays have been studied in situ by polarization microscopy. Contrast arises from linear birefringence in the sample and is only observed between orthogonal polarizers in the excitation and collection paths. Because of this mechanism, the technique does not readily lend itself to providing quantitative molecular level properties. By use of the appropriate combinations of laser polarization and signal polarizations, in contrast, all the relevant matrix elements of $\chi^{(2)}$ can be determined. Here we demonstrate the use of SHG polarization analysis to determine the alignment of collagen fibers in a fish scale. Collagen forms highly ordered assemblies of fibrils (scales that are 50-500 nm in size), which then organize into higher order micrometer-sized structures (fibers). While some fibrils can be resolved, in SHG microscopy collagen fibers are more typically visualized. Figures 4(a) and 4(b) show the collagen fibers that resulted when orthogonal laser excitation polarizations were used and the entire SHG intensity was collected. It is readily observed that the fibers are highly oriented and that the only ones that produce SHG contrast are oriented parallel with the laser polarization. This is equivalent to the case of the SHG contrast in the nematode interphase centrosomes in Fig. 3. Further, only residual vestiges of oppositely directed fibers appear with the polarization in the orthogonal direction, showing the highly oriented nature of the collagen fibers. The other approach to polarization analysis is more comparable to fluorescence anisotropy, in which the laser polarization is kept fixed and the SHG signal is analyzed with a Glan laser polarizer both parallel [Fig. 4(c)] and perpendicular [Fig. 4(d)] to the laser fundamental. To interpret this data, we use the anisotropy parameter β , given by:

$$\beta = \frac{I_{\text{par}} - I_{\text{perp}}}{I_{\text{par}} + 2 I_{\text{perp}}}$$



Figure 4. Polarization dependencies of SHG contrast. (a) and (b) SHG of collagen fibers in a tetrafish (*Gymnocorymbus ternetzi*), scale with the optimal laser polarization (a) and orthogonal polarization (b). The polarization dependence reveals the highly oriented nature of the fibers. (c) and (d) SHG polarization anisotropy measurements of an isolated scale where (c) and (d) result from the Glan laser polarizer oriented parallel and perpendicular, respectively, to the laser fundamental.

where I_{par} and I_{perp} are the intensities of the signals whose polarizations are parallel and perpendicular to the polarization of the incident laser. This parameter can vary between -0.5 and 1. The special case of zero represents the isotropic situation where I_{par} and I_{perp} are equal and would physically correspond to having complete randomization-or no order-of the dipoles. In Figs. 4(c) and 4(d), it is seen that the majority of the SHG polarization is parallel to that of the laser. Integration of the entire image intensities yields an anisotropy parameter, $\beta=0.7$, which indicates that the dipoles in the collagen fibers form well-aligned structures. Further, the anisotropy also addresses the nature of SHG in these proteins. While the protein arrays form crystalline-like structures, the frequency doubling is not completely analogous to that in more familiar uniaxial crystals, such as KDP and BBO. In the case of such true crystals, the SHG polarization is rigorously perpendicular to that of the laser because this polarization is the only non-vanishing matrix element. This form of SHG in proteins is best viewed as arising from the electric dipole interaction but should not be considered as a phase matched process; in other words, frequency doubling only arises in the focal zone from the molecules with appropriately aligned dipoles. An additional contribution likely arises from the fact that these protein structures are helical and thus chiral. It is known through our previous work and that of other groups that chirality enhances second harmonic intensities.

Second harmonic imaging of tissues has distinct advantages over imaging specimens stained with fluorescent dyes. The most obvious one lies in the fact that no staining preparation is necessary. Extensive processing (fixation, staining, sectioning) has typically been required to image the fine 3D structure of complex biological specimens. Similarly, there are no cytotoxic or phototoxic effects from the addition of exogenous labels. Furthermore, fluorescence anisotropy of a chemical label bound to protein is not equivalent to examining the anisotropy of the protein structure itself, as it is in the case of SHG (as shown in Fig. 3). We have indeed shown (see Fig. 4) that polarization analyses can yield data regarding the

molecular organization and symmetry of these matrices. We expect that these methods will have significant impact on in vivo studies in various fields of biology, including tissue organization, woundhealing, myofilament assembly, muscle development and disease, and the division cycle of normal and cancerous cells in situ. We have already begun to extend these methods to the analysis of fibrillar species in connective tissue and to studies of skin and muscle pathology. We view SHG as complimentary to ultraresolution structural techniques such as electron microscopy or x-ray diffraction. SHG does not approach the resolution of these methods, but specimens can be imaged in situ to study dynamical events such as development or response to drugs.

Other nonlinear optical microscopy modalities

We have provided a summary of our recent work on SHG imaging microscopy of endogenous structural protein arrays. Now we will provide an overview of other nonlinear optical schemes that are being used to image biological specimens without the use of exogenous labels.

Third harmonic generation

Third harmonic generation (THG) is perhaps the most closely related contrast mechanism to SHG. While THG has long been used as a tool to upconvert lasers to shorter wavelengths, only recently has it been applied to microscopic imaging. Unlike SHG, THG does not require a non-centrosymmetric environment to produce signal; it has been shown, however, that THG intensities are much higher in the vicinity of an interface, such as a cell membrane. Wilson and Squier recently demonstrated high resolution, high contrast THG imaging of specimens including plant leafs and red blood cells.⁴ In several ways, this scheme has parallels to our work on SHG imaging. However, THG is governed by $\chi^{(3)}$ and thus arises from different molecular properties than SHG, which is a $\chi^{(2)}$ process. For example, THG probes the volume around a membrane, whereas SHG probes the membrane itself. Currently, THG suffers somewhat from the fact that it is not compatible with titanium sapphire excitation: even with 1000 nm excitation, the



Figure 5. CARS image of 3T3 fibroblasts during apoptosis. The CH₂ protein symmetric stretch was imaged and the image collected in 8.5 s. [Courtesy of Prof. X. Sunney Xie and Dr. Ji-xin Cheng.]

THG signal is too far in the UV to be efficiently collected by conventional optics.

Coherent anti-Stokes Raman scattering (CARS)

CARS spectroscopy has also been recently introduced as a tool in biological imaging. The CARS signal is observed at $2\omega_{\rm p}$ - $\omega_{\rm s}$, where $\omega_{\rm p}$ and $\omega_{\rm s}$ are the pump and Stokes frequencies, respectively. In this scheme, two lasers are tuned so that their energy difference corresponds to a vibrational frequency. In terms of cellular imaging, this corresponds to normal modes of proteins. For example, the Xie group has used CARS to image cells during both interphase and metaphase, by tuning to the C-H aliphatic stretch of the lipid membrane and the PO₂- symmetric stretch of the DNA backbone, respectively.⁵ They were also able to monitor apoptosis, i.e., programmed cell death. This process includes events such as shrinking and loss of integrity of the cell membrane, as well as vesicle formation in the cytoplasm. As shown in Fig. 5, they observed these apoptotic effects in 3T3 fibroblast cells by imaging the aliphatic CH₂ stretch at 2845 cm⁻¹. In related work, Müller used CARS to image lipid multilamellar vesicles in urea and clearly observed three distinct peaks arising from the lipid and one peak from the urea, demonstrating the chemical specificity of the method.6 CARS thus has great potential to move from a traditional role in spectroscopy to that of a

high resolution imaging modality for biological applications.

Optical Kerr effect

Historically, lateral diffusion dynamics in cells have been studied by fluorescence recovery after photobleaching (FRAP) techniques. In this method, a fluorescent dye is bleached by high intensity light and the timescale for the recovery of fluorescence is used to measure diffusion constants and infer conclusions about the motion of macromolecules. In 2001, the Wiersma group elegantly demonstrated the use of the optical Kerr effect to directly probe the viscosity of cytoplasm.7 They determined that the Kerr response decay was 1.7 times slower in cell cytoplasm than in pure water, indicating that water motion is hindered in the former, presumably due to macromolecules and other solutes. These factors underlie the mechanics of transport in cells. The authors proposed to extend this work to probing solvent dynamics in subcellular domains and organelles.

Outlook

The past few years have seen the rise in the domain of biological imaging of several traditional spectroscopic nonlinear tools, including SHG, THG, CARS and the OKE. The groundwork has now been laid for application of this array of tools in probing real-life biological problems such as cancer and musculoskeletal diseases that arise from genetic defects.

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