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In vivo Polarization Dependant Second and Third Harmonic Generation Imaging of *Caenorhabditis elegans* Pharyngeal Muscles[1]

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Abstract—In this study Second and Third harmonic generation (SHG–THG) imaging measurements were performed to the pharyngeal muscles of the nematode *Caenorhabditis elegans*, in vivo with linearly polarized laser beam. Complementary information about the anatomy of the pharynx and the morphology of the anterior part of the worm were extracted. THG signals proved to have no dependence on incident light polarization, while SHG images are highly sensitive to the changes of the incident linearly polarized light.

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INTRODUCTION

Second and Third harmonic generation processes are scattering phenomena. Optical higher harmonic generation, including SHG and THG, does not deposit energy to specimens due to its energy-conservation characteristics, providing minimum sample disturbance (e.g., thermal, mechanical side-effects) which is desirable for biological studies. The main advantages of using SHG and THG as microscopic contrast mechanisms for in vivo biological studies are that minimum preparation and no staining of the samples is required. The common issues of photodamage, phototoxicity, photobleaching, dye availability, or dye toxicity can be eliminated. Moreover, in vivo cellular processes can be monitored for prolonged period of time.

SHG imaging modality provide information related to stacked membranes [1, 2] and arranged proteins with organized structures, such as collagen [3–7]. In addition, SHG is a useful technique for probing membrane potential induced alignment of dipolar molecules [8, 9]. THG is proven to be generated from regions with optical inhomogeneity [10] and is used for probing structural and anatomical changes of biological samples at cellular or subcellular level [11–13]. THG imaging technique has also proved to be very useful diagnostic tool providing information related to neurodegeneration phenomena [14]. Consequently, these two non linear contrast modes that produced simultaneously from the focal volume when a femtosecond laser pulse is tightly focused onto the specimen; attribute complementary information related to the biological sample.

Polarization dependant measurements obtain valuable information related to the optical anisotropy of the sample. A number of structural orientation studies accomplished using SHG imaging with controlled illumination polarization [15, 16].

Furthermore, polarization dependant SHG imaging studies have been performed to muscular structures of live biological specimens determining the mean orientation and the degree of organization of the main harmonophores of the tissue [17, 18]. On the other hand, biogenic crystal orientation has been extracted with the detection of THG signals induced by a tightly focused circularly polarized laser beam [19].

The target of the current study is to identify if the combination of second and third harmonic generation polarization dependant measurements can provide complementary information related to live biological samples. Specifically, our aim is to check the feasibility of THG modality to facilitate the extraction of precise and reliable structural orientation results, obtained via SHG polarization dependant imaging measurements, from biological specimens. In this work we present polarization dependant second and third harmonic generation imaging of pharyngeal muscles of *Caenorhabditis elegans* (*C. elegans*) samples in vivo. *C. elegans* larva and adult animals were irradiated. By employing linearly polarized laser beam, no polarization dependency was found in the recorded THG images. Therefore, by detecting both nonlinear signals

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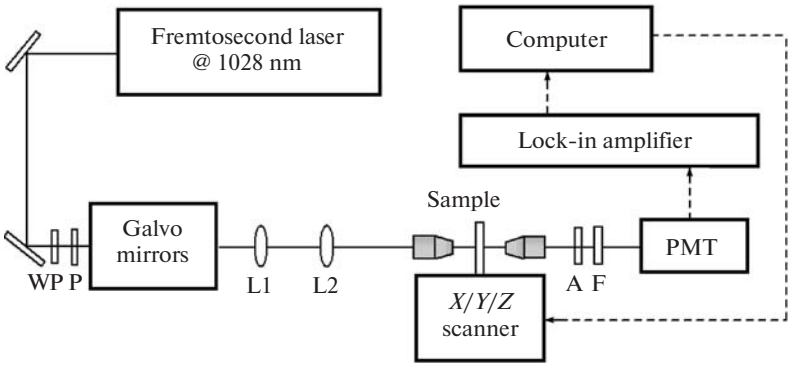

Fig. 1. Schematic of the experimental set-up. WP is a variable wave plate, P is a linear polarizer, L1 and L2 are lenses, A is an analyzer, F is a colored glass or an interference filter, and PMT is a photomultiplier tube.

simultaneously (SHG and THG) is feasible to have a framework of reference (through THG measurements) for the precise identification of the sacromeres and the orientation of the harmonophores of the muscles of the nematode (via SHG measurements).

EXPERIMENTAL APPARATUS

The experimental set-up is outlined in Fig. 1. A femtosecond t-pulse laser (Amplitude Systems) has been used to excite the biological samples at 1028 nm. The pulse duration is 200 fs and the repetition rate 50 MHz. A variable wave plate (Berek 5540-New Focus) and a linear polarizer (Glan-Thompson CLPG 10 670-1064-CVI) are employed in order to set the desired linear polarization of the excitation light. The beam is directed to a modified optical microscope (Eclipse ME600D-Nikon) using suitable dichroic mirrors, and is focused tightly onto the sample by an objective lens with high numerical aperture (50X N.A. 0.8-Nikon). To ensure that the back aperture of the objective is fulfilled a telescope system has been used.

The samples are placed between two round glass slides (0.06–0.08 mm-Marienfeld) that fit into a motorized *xyz* translation stage (8MT167-100-Standa). The minimum step of the stages in each direction is 1 μm. The scanning of the sample is performed by a pair of galvanometric mirrors (6210H-Cambridge Tech.) computer-controlled by specially designed software (Labview 7.1-National Instruments). A CCD camera (PLA662-PixeLINK) is used for the observation of the biological samples. The average laser power on the specimen is 30 mW (0.6 nJ per pulse).

The high-order harmonic signals are collected and collimated in transmission mode by a condenser lens (Plan-Apochromat 100X N.A. 1.4 oil immersion-Carl Zeiss). After passing through an analyzer (Glan-Taylor GT10A–Thorlabs) the signals are sent to a photomultiplier tube (Hamamatsu H9305-04) connected to a Lock-in Amplifier (SR810-Stanford Research Systems). A 340 nm colored glass filter (U 340-Hoya) and a 514 nm interference filter (F03-514.5-CVI) are employed for the detection of THG and SHG signals respectively. With this configuration, it is possible to record SHG and THG images in distinct sets of measurements.

BIOLOGICAL SAMPLE

We have used the nematode *Caenorhabditis elegans* (*C. elegans*), which is a particularly powerful organism for this kind of studies. *C. elegans* is a small (adults are approximately 1mm long with a diameter of 100 μm) free-living hermaphroditic nematode that completes a reproductive life cycle in 2.5 days at 25°C, progressing from a fertilized embryo through four larval stages to become an egg-laying adult. Each animal can give more than 200 progeny and lives for up to 2–3 weeks. The body of adults is comprised of 959 cells that can be easily recognized under a differential interference contrast (DIC) microscope, since the nematode has the great advantage of transparency.

C. elegans feeds with *Escherichia coli* in the laboratory, growing either on agar plates or in liquid medium. The worm uptakes the bacteria from its mouth and moves them to the intestine for degradation, through the pharynx. The pharynx is a bilobed, linear tube encased in a basement membrane. It involves seven cell types, including muscles, epithelia and neurons. Along the longitudinal axis there are eight sections of muscles that participate to the structure of the bulk of the pharynx. Radially, the muscles are organized with three-fold symmetry around the pharyngeal lumen. Posteriorly there is a last pharyngeal muscle that is connected to the intestine. Pharyngeal muscle, on the contrary to body muscle, is radial and not striated, with only a few sarcomeres per cell. While an animal is in the presence of food, this muscle contracts constitutively to draw food into the pharynx. For this reason almost all muscle cells of the pharynx are innervated by pharyngeal motor neurons that are responsible for the coordination of the movement of muscles.

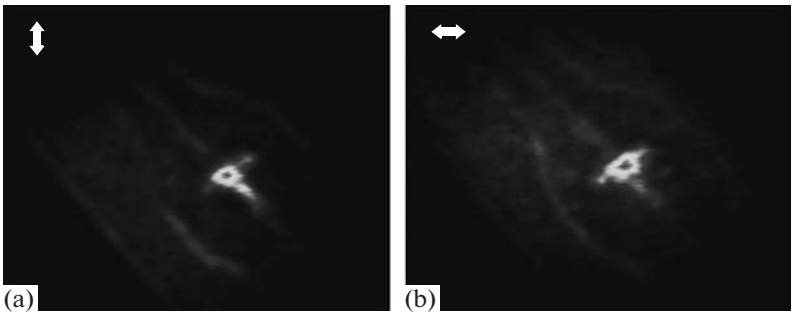

Fig. 2. THG images from the pharynx region of a wild type *C. elegans* larva. The arrows indicate the direction of the incident illumination polarization.

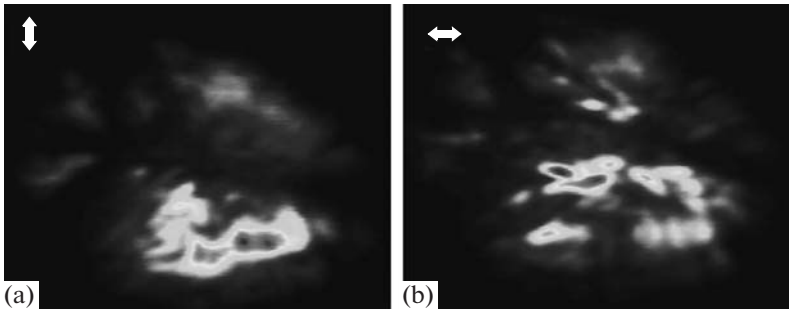

Fig. 3. SHG images from the pharyngeal muscles of a wild type adult *C. elegans* sample. The arrows show the direction of the incident light polarization.

We followed standard procedures for *C. elegans* strain maintenance. Nematode rearing temperature was kept at 20°C. Before each experiment, nematodes at the L1 stage of development or adults were anesthetized by immersing to 20 mM of sodium azide (NaN_3), and subsequently mounted on glass slides. The strain we used for this study was the N_2 (wild type) worm.

RESULTS

Figure 2 depicts THG images from the pharynx region of a wild type *C. elegans* larva. The dimensions of the scanned area are 15×15 μm^2 with a resolution of 300×300 points. Scanning was performed in a specific z position where the THG signal that arises from the sample was maximal. The arrows in Figs. 2a and 2b indicate the direction of illumination polarization. The source of high intensity THG signals are the formations of discontinuous refractive index in the *C. elegans* anterior body segment [12]. It is feasible the collection of strong THG signals originate from the body surface, the pseudocoelomic cavity and the boundaries between internal organs of the worm, due to changes of the refractive index values. The collected THG signals from the pharynx (optical homogeny tissue) are minimal. It is worth to mention that, weaker dependence on the incident light polarization is expected for the THG, since this modality is implemented to image interfaces. In our measurements, no polarization dependency is detected in the recorded THG images (Figs. 2a–2b).

Figure 3 presents SHG images from the pharyngeal muscles of a wild type adult nematode. The dimensions of the scanned area are 15×15 μm^2 with a resolution of 300×300 points. Scanning was performed at a specific z position where the collected SHG signal derived from the pharynx became maximum. The arrows in Figs. 3a and 3b indicate the direction of illumination polarization. Previous polarization anisotropy studies shown that, SHG signals are strongly polarized parallel to the laser fundamental polarization in *C. elegans* muscles [4]. The endogenous structures of well-ordered protein assemblies in the pharyngeal muscles, such as myosin thick filaments [17], are the main contributors to the recorded SHG signals. The strength of the SHG signals change according to the orientation of the structures. This fact makes SHG imaging modality highly sensitive to the changes of the incident linearly polarized light. By varying the incident light polarization (Figs. 3a and 3b) different mor-

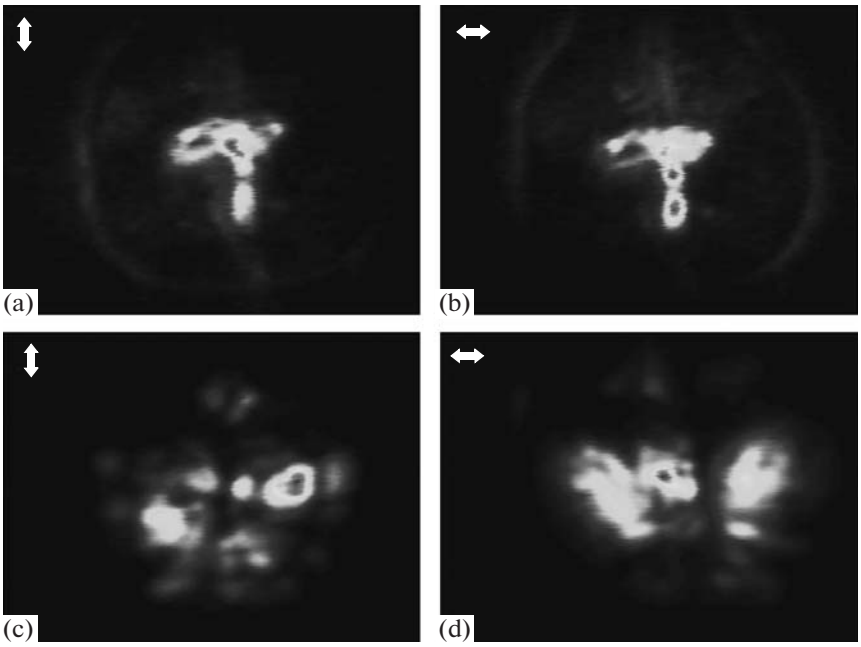

Fig. 4. (a, b) THG and (c, d) SHG images, respectively, recorded from the pharyngeal region of a wild type *C. elegans* larva. The arrows present the direction of the incident linearly polarized laser beam.

phological information's from the muscles of the terminal bulb of the animal are revealed.

In order to demonstrate the different dependence of second and third harmonic signals to changes of the incident linear polarized beam, images from the same sample were collected. Figures 4a and 4b show THG images while Figs. 4c and 4d the corresponding SHG signals from the pharyngeal region of a wild type *C. elegans* larva. The arrows indicate the direction of illumination polarization. The scanning was performed at a specific z position where the collected SHG signal derived from the pharynx became maximum and the dimensions of the scanned area are 15×15 μm^2 with a resolution of 300×300 points. The THG images give a detailed morphological and anatomical delineation of the terminal bulb of the larva and present a very weak dependence on incident light polarization (Figs. 4a and 4b). The respective SHG images reproduce the interior of the pharyngeal muscles and the strength of these signals is strongly sensitive to the changes of the incident linearly polarized light (Figs. 4c and 4d).

CONCLUSIONS

Second and Third harmonic generation imaging techniques are useful, non-destructive diagnostic tools that provide complementary and specific information about the structure and function of tissues and individual cells of live biological specimens. A number of polarization dependant SHG studies have been performed in *C. elegans* muscular structures [4, 17, 18] obtaining valuable data about the sacromeres and the mean orientation of the harmonophores of the muscles. In the current work, by employing linearly polarized laser beam, we combined second and third harmonic generation in vivo imaging measurements, arising from the pharyngeal muscles of the nematode. No polarization dependency was detected in THG images, while the strength of the recorded SHG signals is sensitive to the incident light polarization. The two non linear signals (SHG and THG) are generated simultaneously from the focal volume at the sample plane. Consequently, the detection of THG images gives a detailed and unique frame of anatomical reference which facilitates the structural orientation studies through SHG polarization dependant measurements. Moreover, while in this study we performed polarization dependence second and third harmonic generation measurements to the pharyngeal muscles of the worm, this approach can be readily adapted for work with diverse model organisms and a variety of tissues and muscular structures.

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