In vivo imaging of anatomical features of the nematode *Caenorhabditis elegans* using non-linear (TPEF-SHG-THG) microscopy.

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ABSTRACT

In this study, we present the detailed imaging of the nematode *Caenorhabditis elegans* (*C. elegans*) at microscopic level by performing Two-Photon Excitation Fluorescence (TPEF), Second-Harmonic Generation (SHG) and Third Harmonic Generation (THG) measurements. Due to their inherent advantages in comparison with the conventional microscopy (increased resolution, ability to section deep within tissues, minimization of photodamage and photobleaching effects), the non-linear microscopy techniques comprise a unique and extremely powerful tool for the extraction of valuable and unique information from biological samples. We developed a compact, reliable, inexpensive non-linear imaging system, utilizing femtosecond laser pulses (1028nm) for the excitation of biological samples. The use of 1028nm wavelength as excitation source minimizes photodamage effects and unwanted heating (due to the water absorption) of the biological specimens. The emitted THG signal lies in the near UV part of the spectrum (343nm). Detailed and specific structural and anatomical features of the worm were collected by recording THG signals. Consummative, unique information concerning the morphology and the functions of the nematode was obtained by implementing the combination of THG, SHG and TPEF image contrast modalities on the same microscope.

Keywords: Cellular and molecular imaging, Scanning microscopy, Multiphoton processes

1. INTRODUCTION

C. elegans is a soil dwelling non-parasitic nematode, 1 mm in length that can be cultured in large numbers on agar plates and feed on bacteria. Under standard conditions wild type *C. elegans* has an average life span of approximately 3 weeks while it reaches adulthood within 3 days. Numerous advantages make the nematode an ideal platform in which to dissect complex biological phenomena by combining both genetic and molecular imaging approaches. The hermaphroditic nature of *C. elegans* offers ease of genetic analysis and allows the investigation of various physiological processes. Complex cellular mechanisms have been identified through mutagenesis approaches and many mutant genes have been cloned and characterized. It is also the first multicellular organism with a fully sequenced genome and the only one in which all cellular divisions has been monitored [1]. The complete cell lineage diagram reveals that the adult hermaphrodite worm consists of 959 somatic cells while the adult male consists of 1031 [2]. Electron microscopy analysis revealed that 302 of these somatic cells comprise worm's nervous system for which the full wiring diagram is available [3]. All neuronal cells have been identified and classified in terms of their function, position, connectivity and morphological features and the ~8000 chemical connections within *C. elegans* nervous system have been mapped.

All these features together with the transparency of the animal make *C. elegans* an attractive model for neurobiology studies. Different genetic and microscopy approaches allow the selective inactivation or ablation of specific *C. elegans* neurons, allowing the identification of their function in the animal's physiology and behaviour. Considering that only few neurons are required for *C. elegans* viability, mutations affecting nervous system function that would probably result in lethality in other organisms, can be studied effectively in the worm.

C. elegans is also well suited for quantitative, *in vivo* microscopy studies, aimed to understand metabolism, behavior, drug delivery processes and disease related changes in the body. Therefore, it is necessary, to develop techniques with noninvasive characteristics. In the nematode, cells can be identified by light microscopy because of the worm

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transparency. This makes the observation and quantification of cellular events and physiological processes using bright field techniques such as differential interference contrast microscopy (DIC) possible.

Methods for introduction of exogenous DNA in worms and the expression of a great variety of fluorescent markers in specific cells make the application of both DIC and fluorescence microscopy, as well as confocal microscopy and Two-Photon Excitation Fluorescence (TPEF) possible. It is important to note that non-linear imaging has several advantages compared to one-photon confocal imaging. In contrast to conventional one-photon confocal microscopy, the use of infrared light instead of visible or ultraviolet light, allows TPEF to image deep within tissues, reducing ultraviolet induced damage. Due to the quadratic intensity dependence of the non-linear effects, fluorescence is only generated at the focal plane of the sample, providing the capability of intrinsic optical sectioning without the use of a pin-hole, and reducing dramatically "out of focal plane" photobleaching and phototoxicity in the biological specimens [4]. Such properties make TPEF suitable for long experiments, such as developmental studies, where the sample has to be under observation for several hours. Additionally, some other non-linear effects, such higher harmonic generation (Second Harmonic (SHG) and Third Harmonic Generation (THG)) can be simultaneously exploited in order to get combined information.

Despite similarities, SHG, THG and TPEF are based on fundamentally different phenomena. TPEF relies on non-linear absorption followed by fluorescence emission whereas the first two are coherent processes and rely on non-linear scattering. The endogenous oriented structural proteins, such as actomyosin complexes and collagen generate SHG signals [5]. SHG is a useful technique for probing structures and functions of membranes and the membrane potential induced alignment of dipolar molecules [6-8]. THG is generated near the interface between two media [9]. Thus, THG can be used to image the different organs of transparent biological samples with a 3-D microscopy capability [10-12]. Moreover, strong THG signal has been proved to arise from lipids [13]. A specific advantage of THG is that, while SHG requires a medium without inversion symmetry, third-order processes such as THG originate in any medium. Furthermore, optical higher harmonic generation, including SHG and THG, does not deposit energy to specimens due to its energy-conservation characteristics, providing minimum sample disturbance which is desirable for biological studies.

As reported previously, [9-12] a non-linear microscope can be easily adapted for measuring THG signals. Several research groups have performed detailed imaging of the *C. elegans* body, by recording non-linear phenomena (TPEF and SHG) at the microscopic level [14-17], obtaining valuable information concerning the biology of the nematode. In this study THG modality is employed to image *C. elegans* samples. By detecting THG signals from the worm, specific and detailed anatomical information can be retrieved. Furthermore, detailed imaging and mapping of the nematode *Caenorhabditis elegans* (*C. elegans*) using the combination of three non-linear image-contrast modalities (TPEF, SHG, THG) has been achieved. Wild type worms and transgenic animals, which express Green Fluorescence Protein (GFP) in the pharyngeal muscle cells, have been imaged. Additionally, mutants that express GFP in mechanosensory neurons and transgenic expressing DsRed in motor neurons have been investigated. The combination of three non-linear image-contrast modalities (TPEF, SHG, THG) in a single microscope can provide unique and complementary information allowing a three dimensional (3D) reconstruction of animal body structures. New insights into embryonic morphological changes and complex developmental processes are expected to be analysed by exploiting all three techniques simultaneously in a unique tool of non-linear imaging microscopy.

In this paper, we first describe the experimental apparatus and then use it to map several regions of the nematode body, such as the tail and the pharynx, in combination with THG, SHG and TPEF measurements. Finally, we also briefly analyze the possibilities of using THG for the analysis of embryological studies focusing on the uterus region and embryos.

2. EXPERIMENTAL APPARATUS

The experimental set-up is outlined in figure 1. In our study we used a femtosecond laser oscillator emitting at 1028nm as an excitation source. The laser source (Amplitude Systems t-pulse) is a compact diode- pumped femtosecond laser oscillator delivering a train of high energy, short duration pulses. The average power of the laser is 1 Watt, with pulse duration of less than 200fs and a repetition rate of 50MHz. The emitted SHG signal lies at 514 nm whereas the THG signal lies in the near ultraviolet (UV) part of the spectrum near 343nm. Therefore, common condenser lenses without special coatings (Carl Zeiss Plan-Apochromat 100X/1.4 oil) can be used for the detection of the signal. By using the 1028nm as excitation source, photodamage effects onto the biological specimens are reduced (due to the lower power per photon) compared with the typical wavelength of around the 800nm. Moreover, at 800 nm the THG signal is completely

absorbed by standard microscope slides or even from thin cover slips, imposing an inverted configuration of the microscope [10]. Unwanted thermal heating of the sample are also avoided due to the lower absorption coefficient of water for this wavelength, compared with systems using 1.2 μm wavelength and above [11].

The beam is directed to a modified optical microscope (Nikon Eclipse ME600D) using suitable dichroic mirrors, and is focused tightly onto the sample by an objective lens with high numerical aperture (Nikon 50X N.A. 0.8). To ensure that the back aperture of the objective is fulfilled a telescope system has been used. The average laser power on the specimen is 30mWatt (0.6 nJ per pulse). No damage in the sample is observed at this power. A CCD camera (PixeLINK PL A662) is used for observation. Biological samples are placed between two round glass slides (Marienfeld 0.06mm-0.08mm) that fit into a motorized xyz translation stage (Standa 8MT167-100) by employing a special holder. The minimum step of the stages in each direction is 1 μm. The induced losses of the emitted THG signals, due to the thin cover slips which are used to mount the biological samples, are constrained. In order to get high resolution images the scanning is performed by a pair of galvanometric mirrors (Cambridge Tech. 6210H) computer-controlled by specially designed software (National Instruments, Labview 7.1). Alternatively, low resolution image can be performed moving directly the sample with the xy stage. This configuration limits the resolution to 1 μm, but allows scanning bigger areas, without aberration due to the edges of the aperture of the objective.

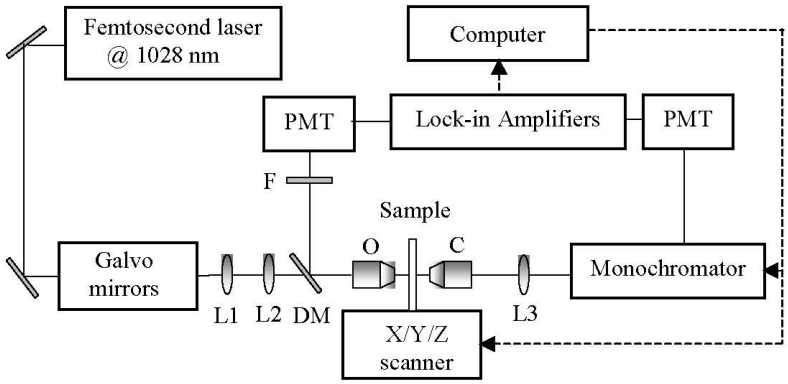

Fig. 1. Schematic representation of the experimental set-up. Two signals are collected simultaneously, TPEF in the backward and THG or SHG in the forward direction, by tuning the monocrhomator. The system is completely automated and controlled with a LabView interface. PMT: photomultiplier tube, DM: Dicrhoic mirror, O: objective, C: condenser, F: filter and L1/L2: telescope lenses, L3: lens.

TPEF signals are collected in the backward direction using a photomultiplier tube (PMT Hamamatsu R4220) connected to a Lock in Amplifier (SR810 Stanford Research Systems). The use of a Lock in Amplifier provides a very good noise rejection, and measurements can be performed in less stringent lighting conditions. The photomultiplier tube is attached at the position of the microscope eye-piece. A short pass filter (SPF 650nm CVI) is placed at the photomultiplier input to cut off the reflected laser light. Furthermore, a long pass filter (LP 520nm CVI) is used for the elimination of reflected SHG and THG signals. For thin samples, most of SHG and THG signals propagate with the laser and are collected and collimated by employing a common condenser lens (Carl Zeiss Plan-Apochromat 100X N.A. 1.4 oil immersion). A dichroic mirror (Enhanced Aluminium Mirror, Thorlabs) is used to reflect the transmitted beam. The signals are focused by means of a lens (UV Fused Silica Plano-Convex, Thorlabs) into the slit (100μm) of a monochromator. The focal length of the lens is 3 cm. The monochromator (Digikrom CM110 CVI) consists of two gratings (1200 grooves/mm each). The first one is suitable for visible light (maximum efficiency 80% at 500nm) and the second is appropriate for ultraviolet light (maximum efficiency 72% at 300nm). The resolution of the monochromator is 1nm. For the detection of signals, a photomultiplier tube (PMT Hamamatsu R636-10) connected to another Lock in Amplifier is used. With this configuration, it is possible to record SHG and THG signals in distinct sets of measurements by tuning the monochromator in different spectral regions. By detecting SHG or THG images in the forward direction and TPEF images in the backward direction, our experimental apparatus allows the collection of two non-linear optical signals simultaneously.

Five different strains and transgenic *C. elegans* samples have been used: wild type, expressing GFP (membrane localized and cytoplasmic) in mechanoreceptor neurons, expressing diffuse GFP in the pharyngeal cytoplasm and expressing DSRED in the head and motor neurons. We followed standard procedures for *C. elegans* strain maintenance, crosses and other genetic manipulations [18]. Nematode rearing temperature was kept at 20^{o}C. Before each experiment, young adult animals were anaesthetized by immersing to 0,5M of sodium azide (NaN_3), and subsequently mounted on glass slides.

3. POSTERIOR PART

C. elegans has been established as an ideal model organism for neurobiology studies. The nematode nervous system comprises 302 neurons whose pattern of connectivity has been completely mapped out, and shown to be a small-world network [3]. This relative simplicity has been used for studying sensory processing and the neural coding of behaviour. Most studies in this area have concentrated on identifying the specific neurons and molecules involved in generating behavioural responses to chemical, mechanical and thermal stimuli. Individual neurons can be easily identified by transgenic animals using cell-specific markers such as fluorescent proteins. By recording simultaneously the three nonlinear signals described above (TPEF-SHG-THG) cumulative information about the biological sample can be obtained. Due to their specific nature, are generated by different structures and molecules in the animals. Of special interest is the capacity for three-dimensional (3D) reconstruction of a live organism that should facilitate the detailed description of anatomical features of both wild type and genetically modified animals. Such reconstructions may contribute to the understanding of the molecular mechanisms pertinent to specific genetic alterations. The use of a non-linear microscope with excitation wavelength of 1028 nm allows us to record all three signals with a single laser, simplifying the layout of the set-up. Additionally, with this configuration, we ensure that produced signals correspond to the same optical section.

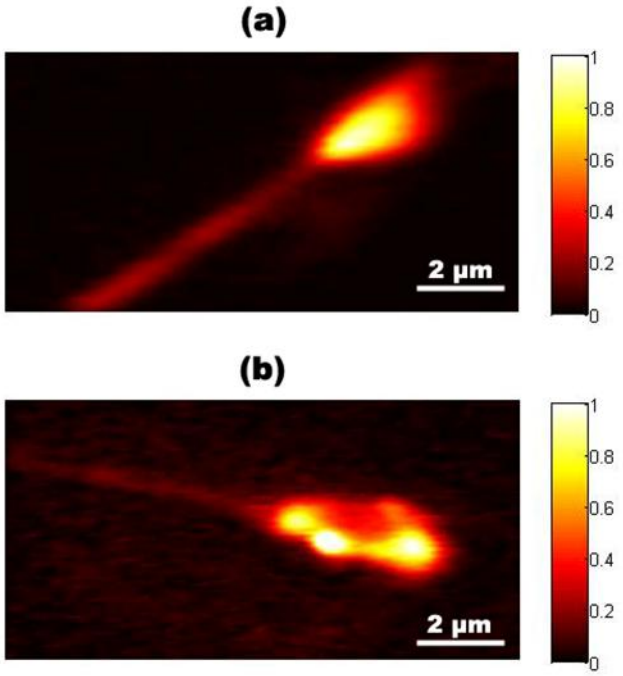

Fig. 2. TPEF images from mechanosensory neurons of *C. elegans* marked with (a) diffused GFP (b) oriented GFP.

In this section transgenic animals that express GFP under the *mec-4* touch-cell specific promoter in two out of the six mechanoreceptor neurons are imaged. The PLM(L/R) touch receptor neurons are located in the posterior part of the worm, close to the tip of the tail, away from the gut autofluorescence. In figure 2 TPEF signal arising from these neurons are shown. Scanning was performed at a specific z position, where the TPEF signal emanating from the neuron axon was

maximal. The dimensions of the scanned region were 12 x 6 μm^2 with a resolution of 60 nm/pixel. Two different kinds of GFP markers have been used diffuse GFP (figure 1(a)) and oriented GFP (figure 1(b)). The first one is localized in the cytoplasm of the mechanoreceptor neurons of the animal, when the second is attached to the membrane of the neuron. The absorption and emission maxima for GFP are 489 and 508, respectively. Although the wavelength of our system is not optimal excite GFP molecules (only 20% is absorbed at 514 nm) not only the neuronal cell bodies (2-3 microns) but others anatomical components such as neuronal axons (~200 nm) are detectable.

Since THG mainly provides information about boundaries between different media, it is feasible to reconstruct the animal outer surface and extract structural information from several inner organs by performing scan at different z positions. To this end, we utilized the Matlab software package (MathWorks.Inc) to design appropriate routines. The 3-D reconstruction of THG and TPEF images, recorded from the posterior part of the nematode is shown in Figure 3(a). We used strains that express GFP in the cytoplasm of the six mechanoreceptor neurons of *C. elegans*. Twelve optical sections, 1 μm apart, were obtained. The precise 3-D localization of the two touch receptor neurons which are positioned near the tail of the animal is easily derived (green surface) from the reconstruction of TPEF images (due to fluorescence of the cytoplasmic GFP molecules). High THG signals were generated by discontinuities in the region of the tail (red surface). These discontinuities are likely due to lipid depositions [13] or formations of discontinuous refractive index in the tail. Figure 3(b) depicts non linear imaging (TPEF-SHG-THG) of the posterior part of a sample. TPEF image was obtained in backward detection, while SHG and THG measurements were detected in the forward direction. SHG and THG measurements were performed in different sets by tuning the monochromator at 514 nm and 343 nm, respectively. There are main peaks at 514 nm (SHG) and 343 nm (THG) which abruptly reduce, as the monochromator setting was changed 3-4 nm around these wavelengths. These observations are in perfect agreement with the spectral distributions, which present the non-linear phenomena of SHG and THG.

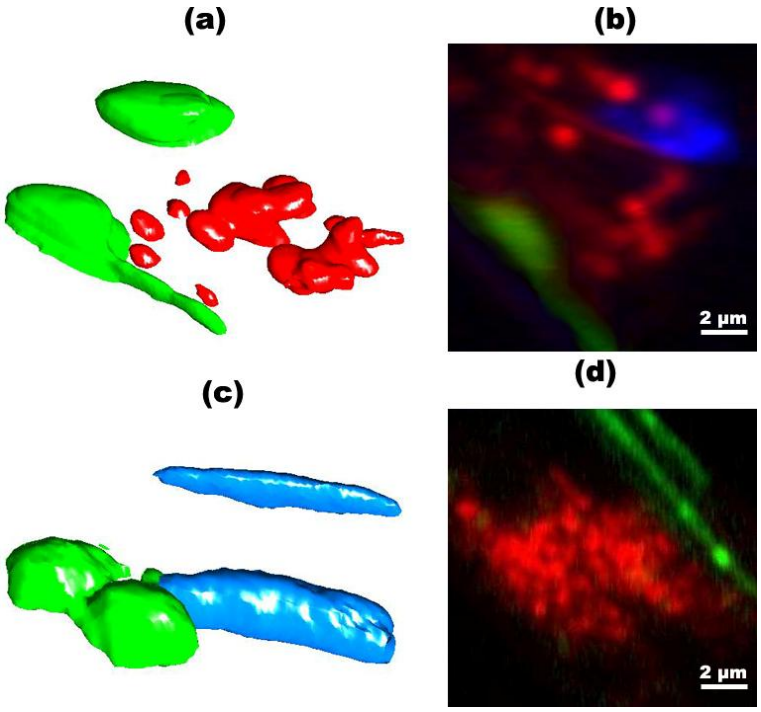

Fig. 3. Non-linear signals (TPEF (green), THG (red) and SHG (blue)) generated at the tail of an animal expressing GFP in the mechanoreceptor neurons: (a) 3D reconstruction with diffuse GFP (b) Multimodal images from the posterior part (c) 3D reconstruction with oriented GFP (d) the mid-body section showing the neuronal axon and the intestines. Images obtained with the high resolution set-up.

The recorded TPEF signal arises from cytoplasmic GFP molecules, which are expressed in these two touch receptor neurons. It is clear that the precise localization of a touch receptor neuron and its axon (green). The SHG image enabled us to detect somatic muscles close to the tail of the worm. Endogenous structural proteins (actomyosin complexes) are the main contributors to the recorded SHG signals [5, 16]. GFP molecules in the neuronal cell are symmetrically distributed. Therefore, their contribution to the observed SHG signal is expected to be minimal. The morphology and the contour of the worm can be observed through THG and SHG measurements, the last one due to its composition of collagen. Rather, discontinuities (such as sub-cellular organelles, inclusions, cavities, lipid depositions, etc) in the *C. elegans* posterior body segment are the source of high intensity THG signals. Figure 3(c) shows the superposition of the 3D reconstruction of TPEF (green) of an neuron with oriented GFP and SHG (blue) arising from somatic muscles. The image is form from 15 slices obtained every 1 μm. The neuronal axon of these neurons travels all along the mid body of the worm. In fig 3(d) can be clearly distinguish the axon in the area of the intestine presenting some clusters of GFP molecules (green). Intestinal cells are filled with numerous granules that likely contain lipids, proteins and carbohydrates. Due to this concentration of lipids, a big signal of THG signal is detected from intestine (red). The dimensions of the images are 13 x 13 μm^2, with a 65 nm/pixel resolution.

4. ANTERIOR PART

The high degree of cell differentiation renders *C. elegans* a perfect experimental system for studies of differentiation, cell biology, and cell physiology. For example the alimentary system of the nematode is made up of a large variety of tissues and cell types. In this section we concentrate in the foregut, including by the buccal cavity and the pharynx.

The pharynx is a muscular organ that pumps food into the pharyngeal lumen, grinds it up, and then moves it into the intestine. A pharyngeal-intestinal valve connects the pharynx to the intestine. Figure 3 depicts the non linear imaging (TPEF-SHG-THG) of the *C. elegans* anterior part. In these transgenic animals, GFP is expressed under the control of the *myo-2* promoter. The *myo-2* promoter is specific to the pharynx and GFP expression is limited to the cytoplasm of pharyngeal muscle cells. Images were recorder with the low resolution configuration of the set-up, i.e., by moving the sample with the xy stages. The dimensions of the scanned region were 60 x 25 μm^2, 60 x 30 μm^2 and 60 x 25 μm^2, with 1 μm resolution. In order to get valuable information scanning was performed over different animals at specific positions.

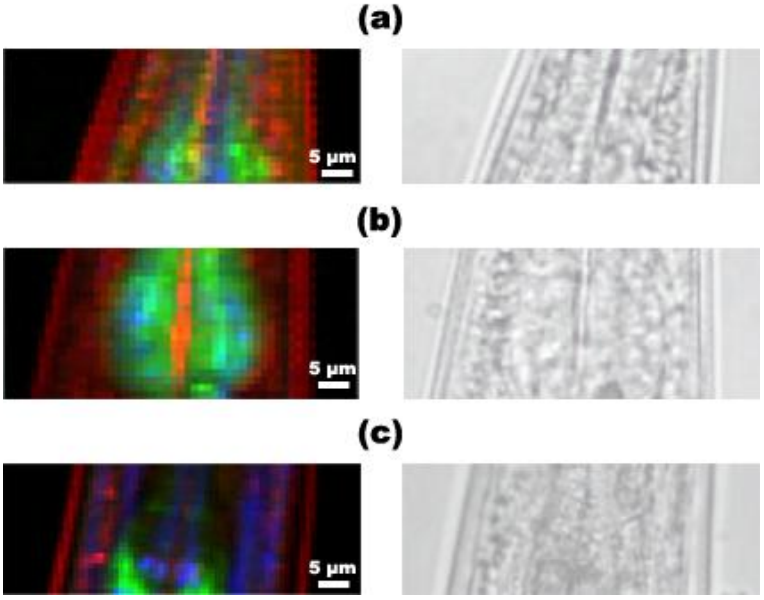

Fig. 4. Multimodal image obtained by the combination of TPEF (green), THG (red) and SHG (blue) from *C. elegans* expressing GFP in the pharynx cytoplasm. Images obtained with the low resolution set-up (1μm).

On the left part, the combination of the three contributions (TPEF in green, SHG in blue and THG in red) is shown. On the right hand, we also include the light-transmission picture corresponding to the same region. That will allow us to detect the sources of the different effects. By TPEF imaging of the GFP molecules in pharyngeal muscles, it is possible to visualize the inner part of the pharynx. Although the spatial resolution of this set-up is limited by the minimum step of the scanning stage (1 μm), the body wall of the worm as well as the internal tissue and organ arrangement, can be observed with satisfactory resolution, through THG signal. The body wall consists of tough collagenous cuticle underlain by hypodermis, muscles, and nerves. The worm skin or hypodermis provides a storage site for lipids and other molecules producing a big THG signal. In contrast, the fluid filled body cavity that separates the body wall from internal organs does not contribute to the THG. Then, the contour and shape of the worm can be observed through THG measurements. Furthermore, high THG intensity signals were collected from the linings of the animal pseudocoelomic cavity (figure 3(a), (b)), while no signal arise from the pharyngeal muscle. Food particles trapped in the sieve (figure 3(b)) in the metacorpus or anterior bulb and the grinder (figure 3 (c)) in the terminal bulb also generates great signal. The sieve traps food particles while fluid is expelled by the pharyngeal channels. The grinder has a function of macerating the food and as a valve to regulate one-way traffic of food into the intestine.

Highly ordered structures generate a high level of SHG signal. The endogenous structures of well-ordered protein assemblies in the pharyngeal muscles, such as actomyosin complexes, are the main contributors to recorded SHG signals [5, 16, 17] in figure 3(c), where the structure of the longitudinal somatic muscles can be clearly visualized. The obliquely striated somatic muscles contain many sarcomeres repeating in regular order in one cell. In figure 3(a) and 3 (b) the SHG arise from the interior of the pharyngeal muscle. Pharyngeal muscles are form with non striated muscles contain a single sarcomere. The first five layers of muscles (pm1-5) are radial oriented filaments attach medially to the cuticle of the lumen and laterally to the pharyngeal basal lamina.

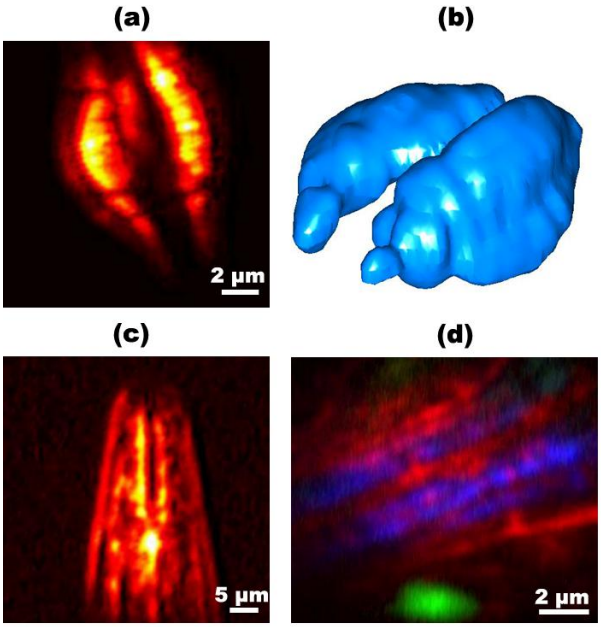

Fig. 5. Higher harmonic signals from wild type *C. elegans*: (a) SHG from the anterior bulb of the pharynx (b) 3D reconstruction of the SHG signal from the anterior bulb of the pharynx (c) THG signal from the buccal cavity. (d) Multimodal image obtained by the combination of TPEF (green), THG (red) and SHG (blue) from *C. elegans* expressing DsRed in CEP neurons. Images obtained with the high resolution set-up.

In figure 5 we present the some images with increased resolution, scanning the sample by means of the galvo mirrors. Figure 5(a) shows the SHG signal recorded from the pm4 muscle of the anterior bulb of the pharynx of a wild type *C. elegans*. The image is 200 x 200 points with a resolution of 80 nm/pixel, approximately. In Figure 5(b) the obtained 3-D

reconstruction of SHG images is shown. No fluorescent markers have been used, reducing the photodamage. Twelve optical sections, 1 μm apart, were collected. In figure 5(c) we image the buccal cavity of a larva through THG. The image dimensions are 50 x 50 μm^2. The structure of the pharyngeal epithelium can clearly distinguish. In figure 5 (d) we show a high resolution picture (12 x 12 μm^2 with 60 nm/pixel) of the isthmus region between the two pharyngeal bulbs. In this case transgenic animals were genetically modified to express DsRed strain marker in the ADE, CEPD and CEPV neurons in the head, PDE in the mid body and PVQ in the tail. In the anterior part of the worm the nervous system includes twenty neurons that innervate and regulate the activity of the pharynx. The three classes of dopaminergic neurons (CEPs, ADEs, and PDEs) function redundantly to sense the mechanosensory stimulus from bacteria and mediate the motor circuit to control the behavioral known as basal slowing response. In the image it is visible the CEPDL neuron (CEP neurons are directly exposed to the pseudocoelomic body fluid), through TPEF measurement (green). DsRed has significant advantages in this setting, such as bright red fluorescence and resistance against photobleaching. The absorption and emission maxima are 557 and 579, respectively. The wavelength of our system is optimal to excite DsRed absorbing more than 50% at 514 nm, and emitting far away from the wavelength of SHG. SHG signal (blue), like in figure 4 (c), arise from longitudinal somatic muscles, whereas THG signal show the boundaries of the pharyngeal muscles due to abrupt changes of the refractive index values. This indicates constitution differences between different tissues. This information provides a frame of reference, which facilitates the precise localization of specific stained markers by fluorescence measurements and allowing structural orientation studies through SHG experiments.

5. MID-BODY AND EMBRYOS

Recently a combined two-photon-excited fluorescence and THG microscope have been used for the characterization of morphogenetic movements in developing *Drosophila melanogaster* embryos [13], showing that THG imaging does not perturb sensitive developmental dynamics.

C. elegans embryos are particularly suitable for differentiation and morphogenesis analysis. Fertilized eggs move down the uterus and out through the vulva, continuing development outside the uterus. Newly hatch larvae have 588 cells and additional divisions of somatic blast cells occur during the four larval stages eventually giving rise to 959 somatic cells. Besides the reproducibility and speed of its development, additional advantages of *C. elegans* are its transparency and its small size (approximately 60 x 30 mm^2), so it fits within a high magnification microscope field. To identify the role of a specific cell type, targeted killing has been used extensively. This process can be performed by laser ablation microsurgery or by genetic means (cell-specific expression of cytotoxic agents). By combining the THG capabilities of 3D imaging with long term measurements (3 to 6 hours) it is possible to visualize and map cell division and development in living embryos, larvae and adults.

Figure 6 shows two image of low resolution (1 μm) of embryos. In the first one the combined image of SHG (blue) and THG (red) has been obtained for an embryo in a late developmental stage, just before hatching. The dimensions of the scanning region were 60 x 40 μm^2. The eggshell is clearly recognized by SHG signal, although signal from THG harmonic it is also observed. The eggshell consists of an external vitelline layer, a tough central layer made principally of chitin, and an internal lipid layer. THG shows the body of the larva, rolled over it. Strong signal of SHG arise from specific location of the nematode.

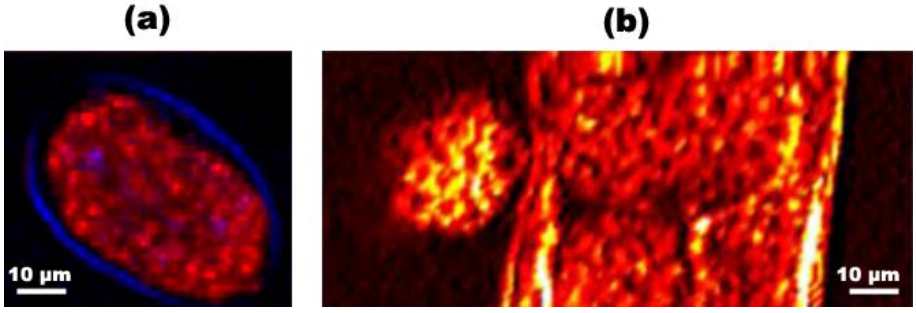

Fig. 6. (a) Multimodal image of an embryo before hatching obtained by the combination of SHG (blue) and THG (red) (b) THG signal of the mid-body section of wild type C. elegans, showing and laid egg. Images obtained with the low resolution set-up (1μm).

The central part of the worm is shown in figure 6 (b). The dimensions of the scanned area were and 121 x 55 μm^2. Three eggs are visible, two inside the uterus and another in the vicinity of the animal. The contours of individual cells inside the laid egg are detectable through THG measurements. We note that the signals are obtained without the use of labeling molecules so a quantitative description of the dynamics of internal structures can be obtained not only in mutant embryos but also in unstained wild-type using THG imaging.

6. CONCLUSIONS

In this study, we demonstrate the potential of combining three different non-linear imaging techniques in a single microscope for the analysis of specific cellular and anatomical features of *C. elegans*. Femtosecond laser pulses (1028 nm) were utilized for the excitation of biological samples. We find that THG is a powerful tool for probing structural and anatomical changes of biological samples and processes at the microscopic level. Common condenser lenses without special coatings can be used since the emitted THG signal lies in the near UV part of the spectrum (343nm). Complementary information about the anatomy of the nematode adult and embryos were collected simultaneously by implementing a combination of THG, SHG and TPEF image contrast modalities on the same microscope. Additionally, 3-D reconstruction of TPEF, SHG and THG images was performed in order to achieve the three dimensional delineation of organs (pharynx) and specific cell types (neurons) of the worm.

We note that in *C. elegans* the ability to perform simultaneous THG, SHG and TPEF imaging allows coupling of structural information extraction with monitoring of specific cellular physiological parameters, such as membrane potential in neurons [14]. Given the wealth of information on the biology of this model organism and the broad range of genetic and molecular techniques applicable, this system will aid in-depth investigations of how genes specify and control neuron function to generate behavior. Also, these non-invasive techniques should have a significant effect on developmental biology, visualizing and quantifying morphogenetic movements *in vivo*.

7. ACKNOWLEDGMENTS

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