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In vivo imaging of cellular structures and processes in *Caenorhabditis elegans*, using non-linear microscopy

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Abstract

We present the detailed imaging of structures and processes of the nematode *Caenorhabditis elegans* (*C. elegans*) using non-linear microscopy (TPEF-SHG-THG). Consummative and specific information about the anatomy of the nematode was collected by implementing a combination of THG, SHG and TPEF image contrast modalities on the same microscope. Additionally, 3-D reconstruction of TPEF and THG images was performed in order to achieve the three dimensional delineation of the outline, and specific cell types (neurons) of the worm. Moreover, THG imaging has been proved to be a very useful, non-destructive diagnostic tool for monitoring cellular processes *in vivo* (neuronal degeneration).

Keywords: THG, SHG, TPEF, imaging, microscopy, *Caenorhabditis elegans*

In the last few years, non-linear optical measurements used in conjunction with microscopy observations, have created new opportunities of research and technological achievements in the field of biology. Non linear imaging methodologies such as Two-Photon Excitation Fluorescence (TPEF) Second-Harmonic Generation (SHG) and Third Harmonic Generation (THG) are well-established non destructive techniques that have been recently used as tools for the *in vivo* imaging and mapping of sub-cellular biological structures and processes [1-4]. Their main advantages in comparison with the conventional one-photon confocal microscopy are the capability of intrinsic three-dimensionality, the increased axial resolution, the ability to section deep within tissues and the reduction of "out of focal plane" photobleaching and phototoxicity in the biological specimens. Additionally, the use of femtosecond (*fs*) lasers enables high peak powers for efficient nonlinear excitation, but at low enough energies so that biological specimens are not damaged. By employing these non linear modalities, *in vivo* cellular processes can be monitored with high spatial and axial resolution continuously for a long period of time, since the unwanted interactions (e.g., thermal, mechanical side-effects) are minimized. Furthermore, the combination of non linear image-contrast modes in a single instrument has the potential to provide complementary information about the structure and function of tissues and individual cells of live biological specimens.

C. elegans is a small (1 mm) free-living hermaphroditic, nematode worm that completes a life cycle in 2.5 days at 25°C. The simple body plan and transparent nature of both the egg and the cuticle have facilitated the exceptionally detailed developmental and anatomical characterization of this animal. The broad range of genetic and molecular techniques applicable in *C. elegans* allows a unique line of investigation into fundamental problems in biology.

The experimental set-up consists from an Amplitude Systems femtosecond laser operating at 1028 nm. This source is a compact diode- pumped femtosecond laser oscillator delivering a train of high peak power, 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 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 combined movement of the three stages is computer-controlled by specially designed software (National Instruments, Labview 7.1), allowing the implementation of a complicated scanning scheme. The average accumulation time in every step is 30ms. Alternative, by performing the scanning procedure with the use of a pair of computer-controlled galvanometric mirrors (Cambridge Tech. 6210H), high resolution images can be obtained.

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 (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.

Figure 1 depicts the non linear imaging (TPEF-SHG-THG) of the *C. elegans* anterior part. In these transgenic animals, GFP is expressed in the cytoplasm of the pharyngeal muscle cells. The dimensions of the scanned region were 51 x 21 μm^2. Scanning was performed at a specific z position where the collected TPEF signal derived from the pharynx became maximum. Figure 1(a) shows a TPEF image, obtained by backward detection, while figures 1(b) and 1(c) show the SHG and THG measurements respectively, detected in the forward direction. The combination of the three contributions is shown in figure 1(d) (TPEF in green, SHG in red and THG in blue). By TPEF imaging of the GFP molecules in pharyngeal muscles, we are able to visualize the inner part of the pharynx. 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]. GFP is expressed under the control of the myo-2 promoter in animals used for this experiment. The myo-2 promoter is specific to the pharynx and GFP expression is limited to the cytoplasm of pharyngeal muscle cells. GFP molecules, due to their random orientation in the pharynx region do not contribute to the SHG signal. 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. Consequently, the three image modalities provide complementary information about the biological sample.

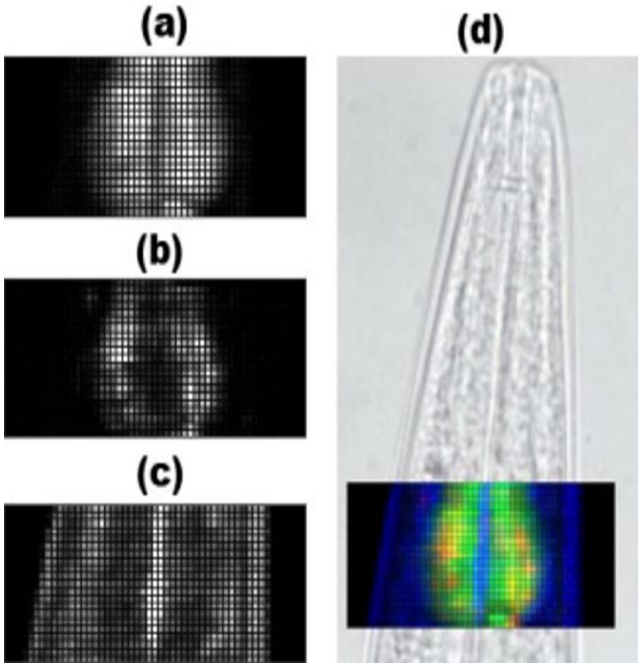

Figure 1: Non-linear signals generated in the pharynx region of a worm expressing GFP in the pharyngeal muscles: (a) TPEF (b) SHG (c) THG and (d) multimodal image obtained by the combination of the previous three: TPEF (green), SHG (red) and THG (blue).

The 3-D reconstruction of THG and TPEF images, recorded from the posterior part of the nematode is shown in Figure 2. We used strains that express GFP in the cytoplasm of the six mechanoreceptor neurons of *C. elegans*. The dimensions of the scanned region were 41 x 21 μm^2. Eight optical sections, 5 μm apart, were obtained. Figure 2(a) shows the z-projection of TPEF images, figure 2(b) depicts the corresponding THG images and figure 2(c) is the superposition of the two (TPEF and THG) 3-D reconstructed images. The precise 3-D localization of the two touch receptor neurons which are positioned near the tail of the animal is easily derived (red surface) from the reconstruction of TPEF images (due to fluorescence of the cytoplasmic GFP molecules). Moreover, detailed morphological information concerning the shape (gray surface) and the inner structure of the animal tail (blue surface) can be obtained from the reconstruction of THG images.

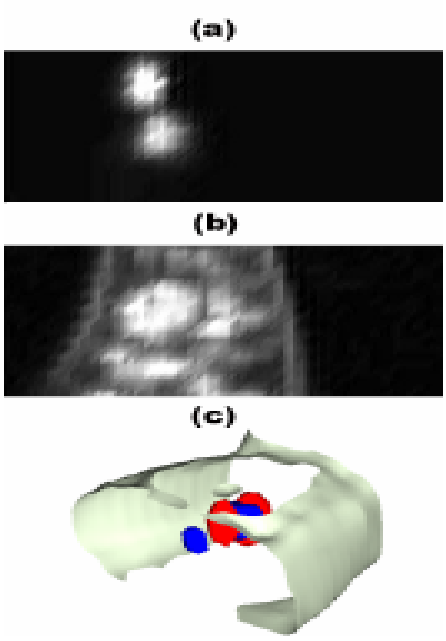

Figure 2: Z-projection of the non-linear signals generated in the tail of a worm expressing GFP in the mechanoreceptor neurons. The projection corresponds to 8 slices every 5 μm: (a) TPEF (b) THG. The 3D reconstruction is shown in (c).

To demonstrate the potential of THG as a diagnostic tool for neurodegeneration studies, the evolution of morphological alterations in degenerating neurons of *C. elegans* larvae was tracked over extended periods of time. In figure 3 can be seen the changes suffered from a dying neuron over 2 hours. The scanning area was 15x15 μm^2. The specimens were kept in darkness at intervals between the THG measurements in order to minimize unwanted photodamage effects. The first image (Fig. 3a) corresponds to the initial observation of the biological sample (time zero) and the neuron presents a great degree of degeneration. After one hour (Fig. 3b) a reduction of the volume of the swollen, degenerating neuron is apparent. This effect becomes more obvious with time (Fig. 3c, after 2 hours). An elongation of the neuron is also observed. In Figures 3a and 3b the THG signal arising from the sub-cellular organelles (e.g. nucleus) inside the neuron cell is detectable. Thus, THG imaging measurements permit not only the detection of structural features but also the identification of biological processes occurring *in vivo* in degenerating neurons. In Fig. 3c (2 hours after) the elimination of the THG signal arising from the inner fast moving organelles inside the cell, can be observed.

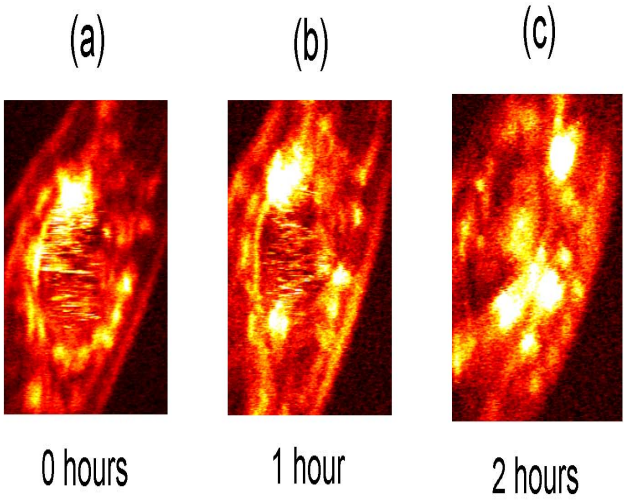

Figure 3: THG signal from the posterior part of a transgenic *C. elegans* larva. The time lapse between images is 1 hour and the scanned area is 15x15 μm^2.

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References

1. P. J. Campagnola, and L. M. Loew, "Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms", Nature Biotechnology 21 pp. 1356-1360, 2003.

2. E.J. Gualda, G. Filippidis, G. Voglis, M. Mari, C. Fotakis, and N. Tavernarakis "In vivo imaging of cellular structures in Caenorhabditis elegans by combined TPEF, SHG and THG microscopy" Journal of Microscopy 229, pp. 141-150, 2008.

3. C.K. Sun, S.W. Chu, S. Y. Chen, T. H. Tsai, T. M. Liu, C. Y. Lin and H. J. Tsai, "Higher harmonic generation microscopy for developmental biology" Journal of Structural Biology, 147, pp. 19-30, 2004.

4. D. Debarre, W. Supatto, A. M. Pena, A. Fabre, T. Tordjmann, L. Combettes, M. C. Schanne-Klein, and E. Beaurepaire, "Imaging lipid bodies in cells and tissues using third-harmonic generation microscopy'' Nature Methods 3 pp. 47-53, 2006.

5. S.V. Plotnikov, A.C. Millard, P.J. Campagnola, and W.A. Mohler, "Characterization of the myosin-based source for second-harmonic generation from muscle sarcomeres" Biophysical Journal 90, pp. 693-703 2006.