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Imaging *Caenorhabditis elegans* embryogenesis by third-harmonic generation microscopy

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ABSTRACT

In this study, third-harmonic generation (THG) imaging measurements were performed to characterize different developmental stages of the nematode *Caenorhabditis elegans* (*C. elegans*) embryos. Femtosecond laser pulses (1028 nm) were utilized for excitation. THG image contrast modality proved as a powerful diagnostic tool, providing valuable information and offering new insights into the complex developmental process of *C. elegans* embryogenesis.

1. Introduction

In vivo imaging of sub-cellular biological structures and processes is essential in biomedical sciences. Non-linear imaging microscopy techniques (two photon excitation fluorescence (TPEF), second-harmonic generation (SHG), and third-harmonic generation (THG)) comprise unique and extremely powerful tools for the extraction of valuable and unique information from a variety of biological samples *in vivo* (Campagnola and Loew, 2003; Filippidis et al., 2009; Mathew et al., 2009; Reshak et al., 2009; Zipfel et al., 2003).

In embryology there have been employed several techniques, such as confocal laser scanning microscopy (CLSM) (Cockell et al., 2004) and Differential Interference Contrast (DIC-Nomarski) (Hamahashi et al., 2005), in order to track cell division stage of biological specimens. Recently, the non-linear imaging modality of third-harmonic generation (THG) has been used, as an alternative method, providing information related to morphological changes and complex developmental processes of Zebrafish (Chen et al., 2006; Chu et al., 2003; Hsieh et al., 2008; Sun et al., 2004), Drosophila (Debarre et al., 2005, 2006; Supatto et al., 2005) and *Xenopus laevis* (Oron et al., 2004) embryos.

Non-linear imaging microscopy presents several advantages compared with other microscopic techniques, such as the capability of intrinsic three-dimensionality, the ability to section deep within tissues and the reduction of "out of focal plane" photobleaching and phototoxicity in the biological specimens. Non-linear microscopy imaging methodologies do not exhibit higher lateral and axial resolutions compared to the confocal one-photon microscopy. It must be emphasized, however, that the 3D axial resolution of the non-linear modalities is obtained by the limitation of the region of excitation, and not of the region of observation, as in the confocal one-photon microscopy (by placing a pinhole).

Third-harmonic generation (THG) process represents a non-linear scattering (coherent) phenomenon. Three photons of angular frequency ω are destroyed and a photon of angular frequency 3ω is simultaneously created in a single-quantum mechanical process (Barad et al., 1997; Muller et al., 1998). The use of femtosecond (fs) lasers as excitation sources enables high peak powers for efficient non-linear excitation, but at low enough energies (far below the ablation threshold) so that biological specimens are not damaged. Moreover, optical higher harmonic generation, including THG, does not deposit energy to specimens due to its energy-conservation characteristics, providing minimum sample disturbance which is desirable for biological studies. By employing THG as a microscopic contrast mechanism for *in vivo* biological studies, minimum preparation and no staining of the samples are required. Another advantage is that cellular and sub-cellular processes can be monitored for prolonged period of time.

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THG is sensitive to local differences in third-order non-linear susceptibility $\chi^{(3)}$, refractive index and dispersion (Debarre et al., 2006; Debarre and Beaurepaire, 2007; Oron et al., 2004). In THG imaging microscopy, the contrast arising from interfaces and optical inhomogeneities of size comparable to the beam focus (Debarre et al., 2005). No THG signal is collected when the laser beam is focused inside a homogeneous, normally dispersive medium (Barad et al., 1997; Squier and Muller, 2001). This non-invasive and non-destructive imaging modality can be used for the imaging of structural features and molecular processes of biological samples (Gualda et al., 2008). Differences in THG coefficients at different locations in the biological sample are translated into THG intensity differences that can be measured. In contrast to second-harmonic generation (SHG) modality that requires a medium without inversion symmetry, THG is allowed in any medium. The information obtained through THG imaging measurements are recorded without the need of adding exogenous fluorescent markers to the samples.

In this study, THG microscopy measurements were employed to get high-resolution images of different embryonic stages of *Caenorhabditis elegans* development. Precise structural and morphological information for different embryonic stages were collected. Due to the several advantages that this non-linear technique presents, it can be used as an alternative, new method for monitoring the developmental process of embryogenesis.

2. Experimental apparatus

The experimental set-up was based on the one used for our previous works (Filippidis et al., 2009; Gualda et al., 2008). An Amplitude systems t-pulse laser (1028 nm, 50 MHz, 1 W, and 200 fs) has been used as an excitation source. A modified Nikon upright microscope (Eclipse ME600D) was employed. THG signals were detected in the forward direction. A 20×, 0.8 numerical aperture (NA) objective lens (Carl Zeiss, Plan Apochromat, 0.55 mm working distance, air immersion) was employed for tight focusing of the laser beam and a 100×, 1.4 NA condenser lens (Carl Zeiss, Plan Apochromat, 0.17 mm working distance, oil immersion) was used for recording THG signals. The scanning procedure was performed with a pair of galvanometric mirrors (Cambridge Tech. 6210H) and the focal plane was selected with a motorized translation stage (Standa 8MT167-100). Lab View interface controlled both scanning and data acquisition procedures. Biological samples were placed between two very thin (∼50 μm) round glass slides (Marienfeld). The glass slides were separated with a 100 μm thick spacer in order to avoid damaging the embryos.

After filtering with a 340 nm color glass filter (Hoya U 340), THG was detected by a photomultiplier tube (PMT Hamamatsu H9305-04). Phase-sensitive detection (lock-in amplifier SR810 Stanford Research Systems) was used for the amplification of the collected THG signal and enhancement of signal-to-noise ratio. The total time for obtaining a 300 × 300 pixel image was around 2 min. The average laser power on the specimen was 35 mW (0.7 nJ per pulse).

3. Sample preparation

We have utilized *C. elegans*, which has emerged as a convenient and powerful model organism for our analysis. *C. elegans* is a small, hermaphroditic, free-living soil nematode (adults are approximately 1 mm long with a diameter of ∼80 μm). Its reproductive life cycle is very short (2.5 days at 25 °C). Fertilized embryos progress through 4 larval stages to become egg-laying adults that can produce a large number of progeny (more than 200 individuals). Animals feed with *Escherichia coli* (under laboratory conditions), on agar plates or in liquid medium. One major advantage of *C. elegans* is the transparency of its body which has allowed easy visualization and monitoring of cellular processes throughout its life cycle.

C. elegans embryogenesis can be divided into two consecutive phases: proliferation and organogenesis/morphogenesis. The first phase includes cell divisions from a single cell to a mass of about 550 undifferentiated cells. Considerable cell rearrangements, cell-specific migrations and synchronous stem-cell divisions can occur during this phase of embryogenesis. During the second phase, cells are terminally differentiated (very few additional cell divisions occur). As a final outcome of embryogenesis, the embryo elongates threefold and takes form as an animal. The main characteristic stages of this phase are the (i) bean, (ii) comma, (iii) 1.5-fold, (iv) 2-fold and (v) 3-fold stage. Animal's main body plan is already established at the end of embryogenesis, and remains unchanged during postembryonic development.

We followed standard procedures for *C. elegans* strain maintenance. Nematode rearing temperature was kept at 20 °C. Before each experiment, synchronized gravid adult animals were collected from their cultivation plates with H_2O and were bleached (bleach solution: 1% hypochloride and 0.5N NaOH diluted in H_2O). Through this procedure, eggs of different developmental stages were obtained from their parents' uteri. After being washed several times with H_2O, eggs were subsequently mounted on glass slides. The strain we used for this study was the N2 (wild type).

4. Results–discussion

A femtosecond laser system, emitting at 1028 nm, is employed for the realization of non-linear optical imaging of *C. elegans* embryos, which provides the advantage that third-harmonic signals are located in the near ultraviolet (UV) range of the spectrum (∼343 nm). Consequently, there is no need to use far-UV optics with special coatings for the collection of the THG signal. Moreover, by employing this excitation wavelength (1028 nm), unwanted thermal heating of the biological samples due to absorption by water, is moderated.

Fig. 1(a) depicts a 2D, THG image (300 × 300 points resolution) of an early-stage *C. elegans* embryo. Individual embryonic cells can be clearly identified. Round dark regions represent cell nuclei. The cell nucleus does not emit THG signal, due to its homogeneous constitution. Thus, there are no significant changes either to refraction index values or to the third-order non-linear susceptibility $\chi^{(3)}$ that would efficiently produce detectable THG signals (Debarre et al., 2006). In contrast, high THG signals are generated by discontinuities in the region around the cell nucleus. The main contributors to these non-linear signals are the cytoplasm, different organelles (e.g. mitochondria), lipid depositions (Debarre et al., 2006) and other formations of discontinuous refractive index. The overall structure of each cell can be identified with satisfactory analysis from the recorded THG signals. Fig. 1(b) represents a 3D reconstruction of THG images recorded from the same *C. elegans* embryo. Seven adjacent optical sections (2 μm apart) are used for the reconstruction. The precise 3D delineation of the different cells that constitute the embryo is easily derived from the reconstruction of THG images. The detailed morphology and the different anatomical features of each cell can be observed. We note here that no fluorescent markers have been used. The whole procedure of data acquisition needed for the 3D reconstruction of THG images lasted around 20 min. Image J (Java-based program) was used for processing of the obtained data and for creation of the 3D reconstruction images.

Fig. 2(a) depicts a 2D, THG image derived from an early-to-mid *C. elegans* embryonic stage. Fig. 2(b) represents the 3D reconstruction of the non-linear signals. The width of the embryo is around 20 μm. Five optical sections (2 μm apart) are used for the reconstruction. These sections represent the central part of the sample.

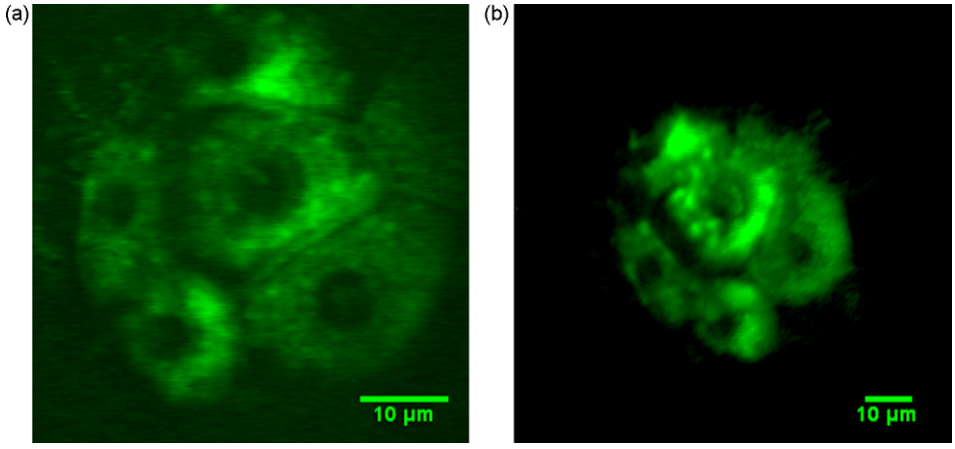

Fig. 1. THG images from an early *C. elegans* embryonic stage: (a) a slice image and (b) 3D reconstruction. Maximum THG signal is shown with green color while minimal THG signal is presented with black color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

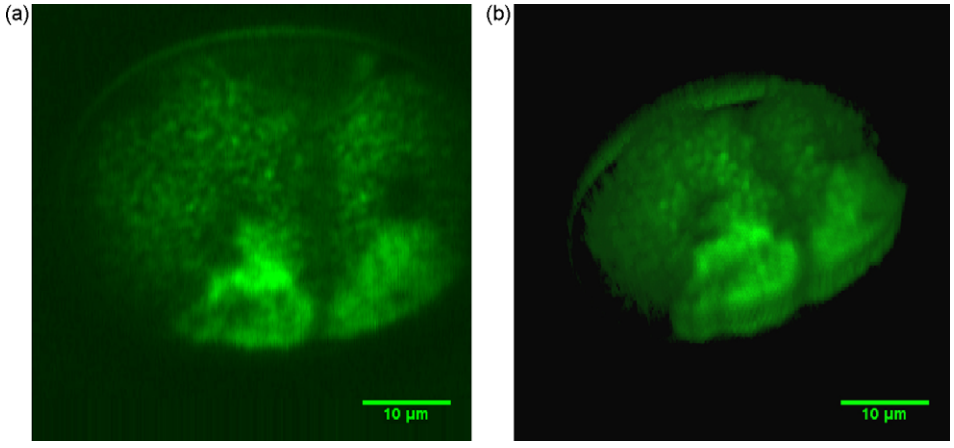

Fig. 2. THG images from an early to mid *C. elegans* embryonic stage: (a) a slice image and (b) 3D reconstruction. THG signal is shown with green color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The resolution of each image is 400×400 points. Individual cells and internal structures of the *C. elegans* embryo are detectable.

Fig. 3 depicts a 2D (300×300 points resolution) THG image derived from the comma stage of *C. elegans* embryogenesis. At this developmental stage, many cell divisions have already occurred and the number of individual cells has been increased. In addition, the elongation of the embryo has initiated and is progressing towards the first folding. Scanning was performed in a specific z position where the THG signal that arises from the sample was maximal. Different cells are detectable via THG measurements although it is difficult to distinguish cell nuclei.

Fig. 4 depicts a 3D reconstruction of THG images recorded from a 3-fold stage *C. elegans* embryo, just before egg hatching. At this late stage of embryogenesis, the embryo has fully elongated and is folded inside the egg before hatching into a stage 1 larva. The structure and the morphology of the folding embryo inside the egg can be observed through the collection of THG signals. Furthermore, the cuticle of the embryo is clearly discernible using the THG modality, providing information relevant to the overall contour of the animal. Five optical sections (2 μm apart) are used for the reconstruction. The resolution of each image is 400×400 points.

Multiphoton microscopes can be easily adapted for measuring THG signals (Chu et al., 2003). We have to note that, the non-linear imaging technique of two photon excitation fluorescence (TPEF) has been already employed as diagnostic tool for monitoring the development of Zebrafish embryos (Tsai et al., 2006). Moreover,

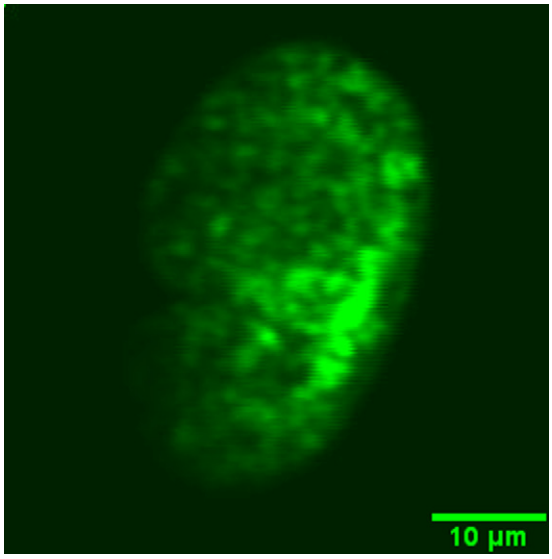

Fig. 3. 2D, THG image from the comma stage of *C. elegans* embryogenesis. THG signal is shown with green color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

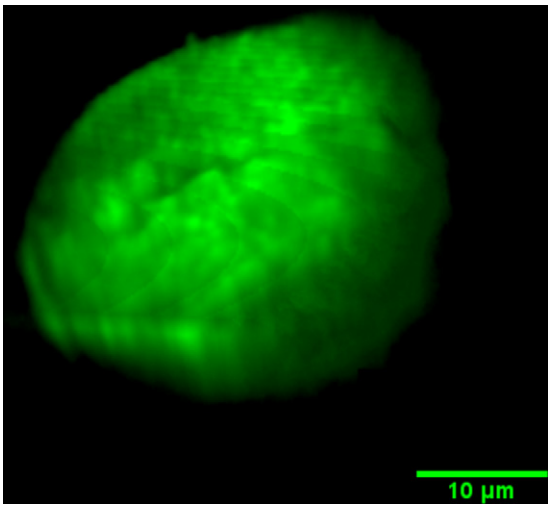

Fig. 4. 3D reconstruction of THG images recorded from a 3-fold stage of *C. elegans* embryogenesis. THG signal is shown with green color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

complementary information about the development of Drosophila embryos have been extracted by implementing a combination of TPEF and THG image contrast modes (Debarre et al., 2004; Supatto et al., 2005). While two photon excitation fluorescence images provide information about specifically labelled sites within the sample, THG provides structural and morphological information (interfaces, inclusions). The main advantages of using THG as microscopic contrast mechanism for embryogenesis studies, compared to TPEF imaging modality, are that minimum preparation and no staining of the samples is required. In addition, the common issues of dye availability, or dye toxicity to the specimen can be eliminated.

In our study the exact separation of different developmental stages and the accurate morphological delineation of *C. elegans* embryos, through the collection of THG signals, have been achieved. We believe that, THG modality can be utilized in combination with other optical techniques, such as TPEF, CLSM and DIC, in order to obtain complementary unique information in the research field of embryology.

5. Conclusions

In the present work, the THG imaging modality has proved to be very useful as a diagnostic tool in providing information related to *C. elegans* embryogenesis. The different developmental stages can be easily discriminated via THG imaging measurements. By employing this non-destructive modality, no fluorescent markers are required for staining the samples. Additionally, the percentage of cell viability during the imaging procedure is dramatically increased, in comparison with other optical techniques, due to the nature of the THG phenomenon (non-linear coherent scattering effect) (Chen et al., 2006; Hsieh et al., 2008).

We anticipate that this methodology has the potential to provide unique information into the complex process of embryogenesis. Cell tracking studies can be performed into a variety of embryos, by employing more advanced equipment that can minimize the time for data collection.

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