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George J. Tserevelakis
George Filippidis
Evgenia V. Megalou
Costas Fotakis
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George J. Tserevelakis,[a,b] George Filippidis,[a] Evgenia V. Megalou,[c] Costas Fotakis,[a,b] and Nektarios Tavernarakis[c]
[a]Foundation of Research and Technology-Hellas, Institute of Electronic Structure and Laser, P.O. Box 1385, Heraklion, Crete, 71110, Greece
[b]University of Crete, Department of Physics, 71110, Heraklion, Crete, Greece
[c]Foundation of Research and Technology, Institute of Molecular Biology and Biotechnology, Heraklion, Crete, 71110, Greece

Abstract. In this study, we demonstrate the potential of employing third harmonic generation (THG) imaging microscopy measurements for cell tracking studies in live *Caenorhabditis elegans* (*C. elegans*) embryos. A 1028-nm femtosecond laser was used for the excitation of unstained *C. elegans* samples. Different *C. elegans* embryonic stages (from two-cell to threefold) were imaged. Live biological specimens were irradiated for prolonged periods of time (up to 7 h), testifying to the nondestructive nature of this nonlinear imaging technique. Thus, THG image contrast modality is a powerful diagnostic tool for probing *in vivo* cell division during early embryogenesis.
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Keywords: third harmonic generation; imaging; *Caenorhabditis elegans*; cell tracking; embryogenesis.

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1 Introduction

An animal starts its life as a single cell—a fertilized egg. During development, this cell divides repeatedly to produce many different cells in a final pattern of spectacular complexity and precision. Ultimately, the genome determines the pattern. Understanding the specialization of cells during embryogenesis can provide information on how stem cells specialize into specific tissues and organs.

Two photon excitation fluorescence (TPEF), second and third harmonic generation (SHG and THG, respectively) are nonlinear imaging techniques that have been used as diagnostic tools for the *in vivo* delineation and mapping of subcellular biological structures and processes. By employing these imaging modalities, valuable and complementary information from various biological samples at the microscopic level have been extracted.[1–3] These nondestructive techniques have the potential to offer new insights into complex developmental processes of embryogenesis in many biological settings. The main features of the three (TPEF–SHG–THG) nonlinear imaging modalities are summarized in Table 1.

Specifically, the THG process represents a nonlinear 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.[4] THG is sensitive to local differences in third-order nonlinear susceptibility $\chi^{(3)}$, refractive index, and dispersion.[5] In THG imaging microscopy the contrast arises from interfaces and optical heterogeneities of size comparable to the beam focus. In other words, no THG signal is collected when the laser beam is focused inside a homogeneous, normally dispersive medium.[4,6] In contrast to SHG modality that requires a medium without inversion symmetry, THG is allowed in any medium. Moreover, a main advantage of using THG compared to TPEF imaging modality is that no fluorescent markers are needed for staining the samples. Therefore, common issues of dye availability, or dye toxicity of the biological specimen are eliminated. Consequently, THG comprises an ideal diagnostic tool that provides unique structural, anatomical, and morphological information from various biological samples.

Several imaging techniques have been employed in embryology in order to track cell division stage of biological specimens, such as confocal laser scanning microscopy[7] and differential interference contrast (DIC-Nomarski).[8] Additionally, the nonlinear imaging modality of THG has been used as an alternative method to provide information related to morphological changes and complex developmental processes of Zebrafish,[9,10] *Drosophila*,[11] *Xenopus laevis*,[12] *Caenorhabditis elegans (C. elegans)* [13,14] and mouse[15] embryos.

Recently, quantitative cell tracking studies were performed at early stage zebrafish embryos through the detection of higher harmonic signals (SHG, THG).[16] Furthermore, different developmental stages of *C. elegans* embryos have been discriminated and individual embryonic cells have been identified via THG imaging measurements.[13,14] In the current work, for the first time to the best of our knowledge, cell tracking studies were performed in *C. elegans* embryos *in vivo* using high-resolution THG imaging measurements. *C. elegans* embryos were continually imaged across early development ranging from 2-cell to 3-fold stage. We extracted valuable information related to the

Address all correspondence to: George Filippidis, Institute of Electronic Structure and Laser, Foundation of Research and Technology–Hellas, P.O. Box 1385, Heraklion, Crete, 71110 Greece. Tel: 0030 2810 391320; Fax: 0030 2810 391305; E-mail: filip@iesl.forth.gr.

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Table 1 Main advantages of three nonlinear imaging microscopy techniques (TPEF-SHG-THG).

Non-linear modalities		
TPEF	SHG	THG
√ Deeper imaging	√ Coherent process	√ Coherent process
√ Limitation of the excitation region	√ No light absorption	√ No light absorption
√ Less photobleaching and phototoxicity	√ Allowed in non-centrosymmetric molecules only	√ Allowed in any medium
√ intrinsic three-dimensionality	√ Probing structures with high degree of orientation and organization	√ Arising from interfaces and optical inhomogenities
	√ No sample staining required	√ No sample staining required

complex developmental process of *C. elegans* embryogenesis. Specifically, via the collection of THG signals, we achieve to monitor the exact movement of cells and cell nuclei during mitosis at the early stages of *C. elegans* embryogenesis.

2 Materials and Methods

2.1 *Experimental Apparatus*

The use of femtosecond (fs) lasers enables high peak powers for efficient nonlinear excitation, but at low enough energies so that the biological samples are not damaged. In our study, an amplitude system t-pulse laser (1028 nm, 50 MHz, 1 W, 200 fs) has been used as an excitation source. The femtosecond laser beam was directed into a modified Nikon upright microscope (Nikon Eclipse ME600D). THG signals were detected in the forward direction. A 32×,0.85 numerical aperture (NA) objective lens (Carl Zeiss, C Achroplan, water immersion) was employed for tight focusing of the laser beam onto the sample and a 100×,1.4 NA condenser lens (Carl Zeiss, Plan Apochromat, oil immersion) was used for recording THG signals. The lateral resolution of our system was 0.6 μm. 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). LabView interface controlled both scanning and data acquisition procedures. Biological samples were placed between two very thin (∼70 μm) round glass slides (Marienfeld). The glass slides were separated with a 100-μm thick spacer 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). A single two-dimensional (2D) THG image of 300×300 number of pixels was recorded in less than 400 ms. In order to improve the signal-to-noise ratio (SNR), one 2D optical section was obtained from 20 average scans. The total time for obtaining an image was 8 s. The field of view used to obtain the THG images was the square of 68×68 μm^2. The pixel size was equal to 0.23 μm. The optical power density on the specimen was 14.1×10^5 W/cm^2 (16 mW average laser power, 0.32 nJ per pulse). The calculated typical range of values for SNR of the obtained THG images was 15 to 20. Our experimental apparatus allows the collection of two nonlinear optical signals simultaneously (one in the reflection and the other in the transmission mode). In the current study only the forward path of detection has been used.

2.2 *Biological Sample*

We used wild-type (N2) *C. elegans* embryos to perform the THG cell tracking experiments. *C. elegans* is a model system with several advantages. It is a small (1 mm), hermaphroditic, soil nematode, which grows in 2.5 days at 25°C to be an adult worm that lays 200 to 300 eggs. Worms are easily cultivated and maintained in the lab. Their transparency permits microscopic visualization of specific cells and subcellular structures. The lineage of the 959 cells in the adult is fully described and invariable among animals, which greatly facilitates studies of development. *C. elegans* embryogenesis is divided into three major stages and lasts 14 h at the incubation temperature of 20°C. Embryogenesis begins with the formation of the zygote followed by the establishment of the embryonic axes and the determination of the somatic and germ-line cell pathways. In the second stage, gastrulation takes place, most of the cell proliferation completes, and cell differentiation and organogenesis commences. During the final stage, morphogenesis and organogenesis completes and the egg subsequently hatches into a worm. As embryos advance through the various stages of embryogenesis, major morphological landmarks, as it grows from a zygote into a worm, are the bean, comma, 1.5-fold, 2-fold and finally the 3-fold stage. Specifically early in development, the embryo undergoes four

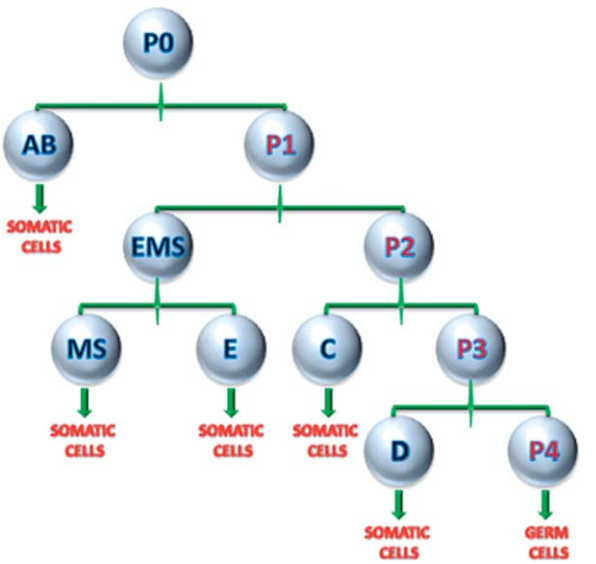


Fig. 1 Cell lineage tree of early development in the *C. elegans* embryo. Horizontal line indicates cell divisions. P0 denotes the zygote. The germ-line or P cells divide unequally to form the five somatic founder cells (AB, MS, E, C and D) and the primordial germ cell P4. The type of cells generated by each founder cell is indicated.

unequal divisions to produce five somatic founder cells (AB, E, MS, C, D) and the primordial germ cell P4. These divisions occur within 100 min following the first cleavage and the embryo at this time is at the 28-cell stage (Fig. 1). Embryogenesis concludes with hatching and at this stage the embryo contains 550 cells.

Worms were maintained at 20°C on standard nematode growth medium plates seeded with *Escherichia coli* as previously described.[14] Synchronized young adults were collected from their plates and were subsequently bleached in order to obtain eggs at early developmental stages. Eggs were subsequently mounted on glass slides for viewing.

3 Results and Discussion

In a previous study we demonstrated the ability of THG measurements to image *C. elegans* embryos.[14] Figure 2 depicts a 2D THG image (300×300 pixels) of an early-stage (16 cells) *C. elegans* embryo. Scanning was performed in a specific z position where the THG signal that arises from the sample was maximal. Individual embryonic cells can be clearly identified. Round dark regions represent cell nuclei. The cell nucleus fails to emit detectable THG signal due to its homogeneous constitution.[13,14,17] In contrast, high THG signals are generated by discontinuities in the region around the cell nucleus. The main contributors of the high THG signals are the endoplasmic reticulum, different organelles (e.g., mitochondria),[18] lipid depositions,[17] and other formations of discontinuous refractive index or $\chi^{(3)}$ values.[4,19] By performing THG imaging measurements the precise delineation of the 16 different cells that constitute the *C. elegans* embryo was easily derived. The detailed structure and the morphology of each cell were observed.

To demonstrate the ability of our system to image the morphological changes that occur early in development, we followed the same embryo for several hours. We took continual images at 1 or 3 min intervals and captured the unequal cell divisions that occur during the first few hours of embryogenesis. Figure 3 depicts the cell division process in a *C. elegans* embryo *in vivo* from two- to four-cell stage. The scanning was performed at a specific z position where the collected THG signal derived from the embryo was maximum. Nine sequential THG 2D images (300×300 pixels) are presented. The time interval between consecutive images was 2 m. The duration of data acquisition was sixteen minutes. Figures 3(a) and 3(b) show an embryo at two-cell stage. The next five Figs. 3(c)–3(g) depict the mitosis process until the four-cell stage. Figures 3(h) and 3(i) represent the *C. elegans* embryo at the four-cell stage. The time needed to complete cell division from two- to four-cell stage was 10 min. Cell nuclei appear as round dark regions since homogeneous areas fail to emit a THG signal. It is thus feasible to monitor the proliferation process inside a live *C. elegans* embryo. The exact movement of cells during mitosis is detectable via the collection of THG signals. We have to note that, due to the very fast data acquisition time (8 s) the micro movements of the *C. elegans* embryos are negligible. This results in the collection of high quality THG images from the embryos.

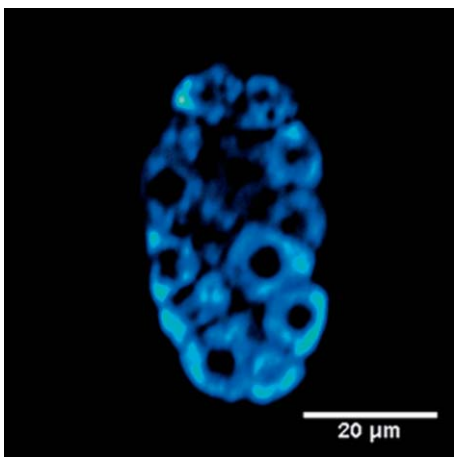
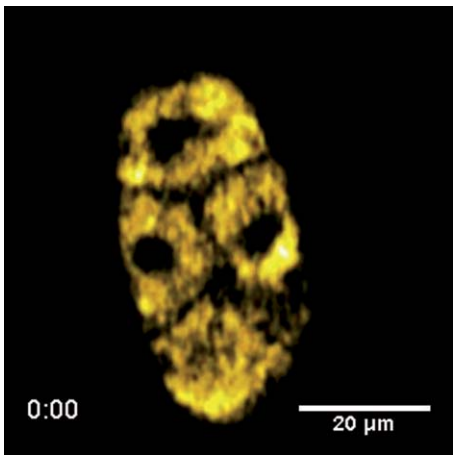


Fig. 2 THG image from an early-stage *C. elegans* embryo. Maximum THG signal is depicted in blue color, while minimal THG signal is depicted in black color.



Video 1 THG imaging measurements for cell tracking at early cell division stages of a *C. elegans* embryo. The whole procedure of data acquisition lasted 3 h. Time-lapse between images is 1 min. THG signal is depicted in yellow. (QuickTime 1.7 MB)
[URL: http://dx.doi.org/10.1117/1.3569615.1]

Figure 4 shows the division process in a *C. elegans* embryo *in vivo* from four- to multiple-cell stage. Six THG 2D images (300×300 pixels) at different developmental stages are presented. Figure 4(a) shows the initial four-cell stage of the embryo. Figures 4(b)–4(f) represent later multiple-cell developmental stages. The THG signal is observed from all the embryonic cells in all figures. Cell nuclei are visible as dark regions. Every cell from four- to multiple-cell stage can be outlined with satisfactory analysis. Video 1 depicts an animated time-lapse sequence of 2D THG signals from the same embryo. The whole procedure of data acquisition needed for each THG image lasted 8 s. The sample was imaged for 3 h and the time-lapse between images was 1 min. By performing THG measurements (see Video 1) it is feasible to monitor *in vivo* cell divisions at the early stage of *C. elegans* embryos. One of the main advantages of the application of THG imaging modality to *C. elegans* embryogenesis studies, compared with other well established optical techniques such as DIC, is that the morphology and the structure of cell nuclei at the early developmental stages of embryos are extremely perspicuous and well determined. The cell loses the nuclear integrity when it is entering the mitotic phase. The disappearance of the cell nucleus indicates the beginning of the

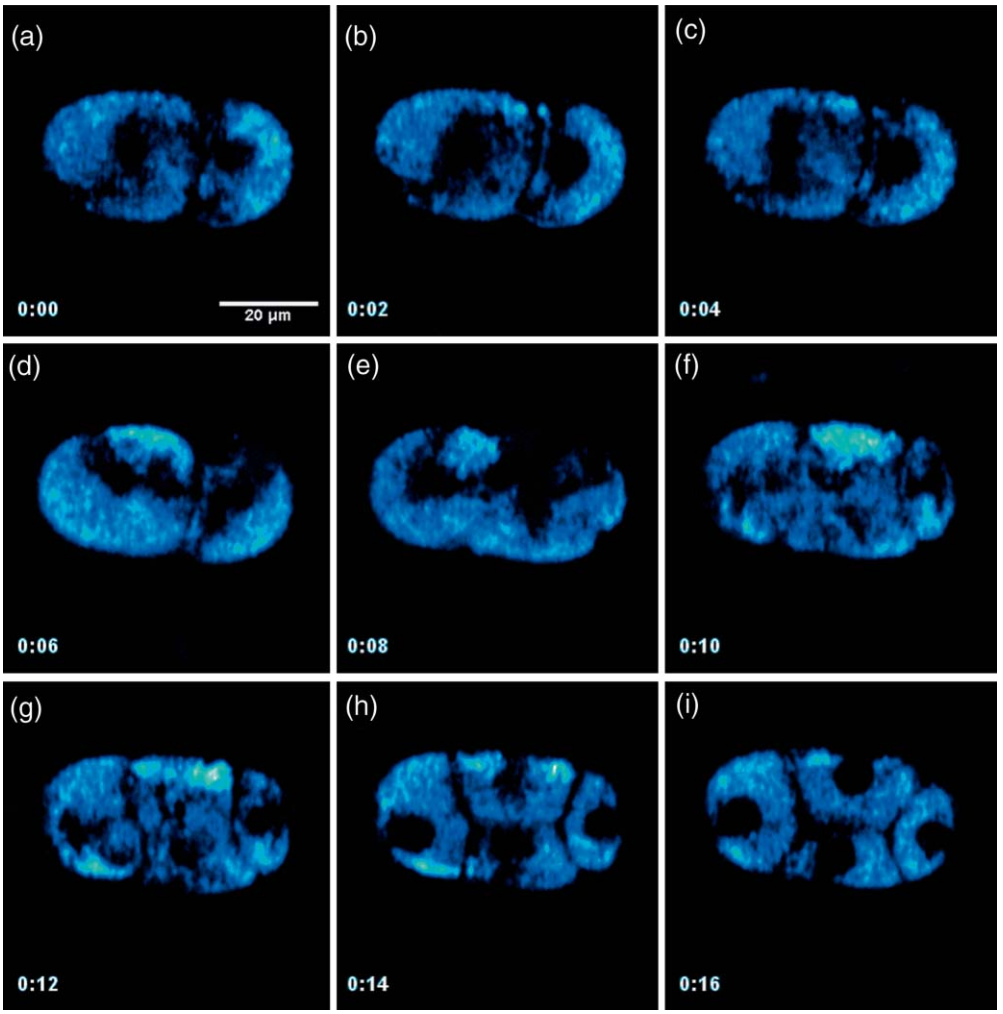


Fig. 3 THG images taken from a *C. elegans* embryo. Images show the *in vivo* asymmetric cell division from two- to four-cell stage.

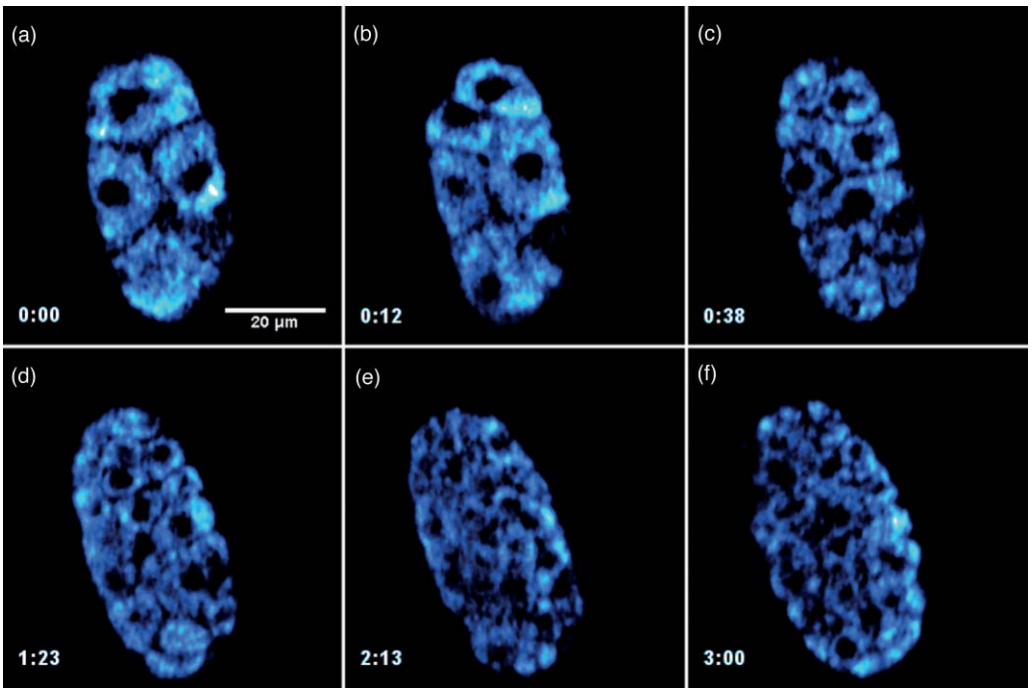


Fig. 4 THG images recorded from an early stage *C. elegans* embryo. Images depict the *in vivo* cell divisions from four- to multiple-cell stage.

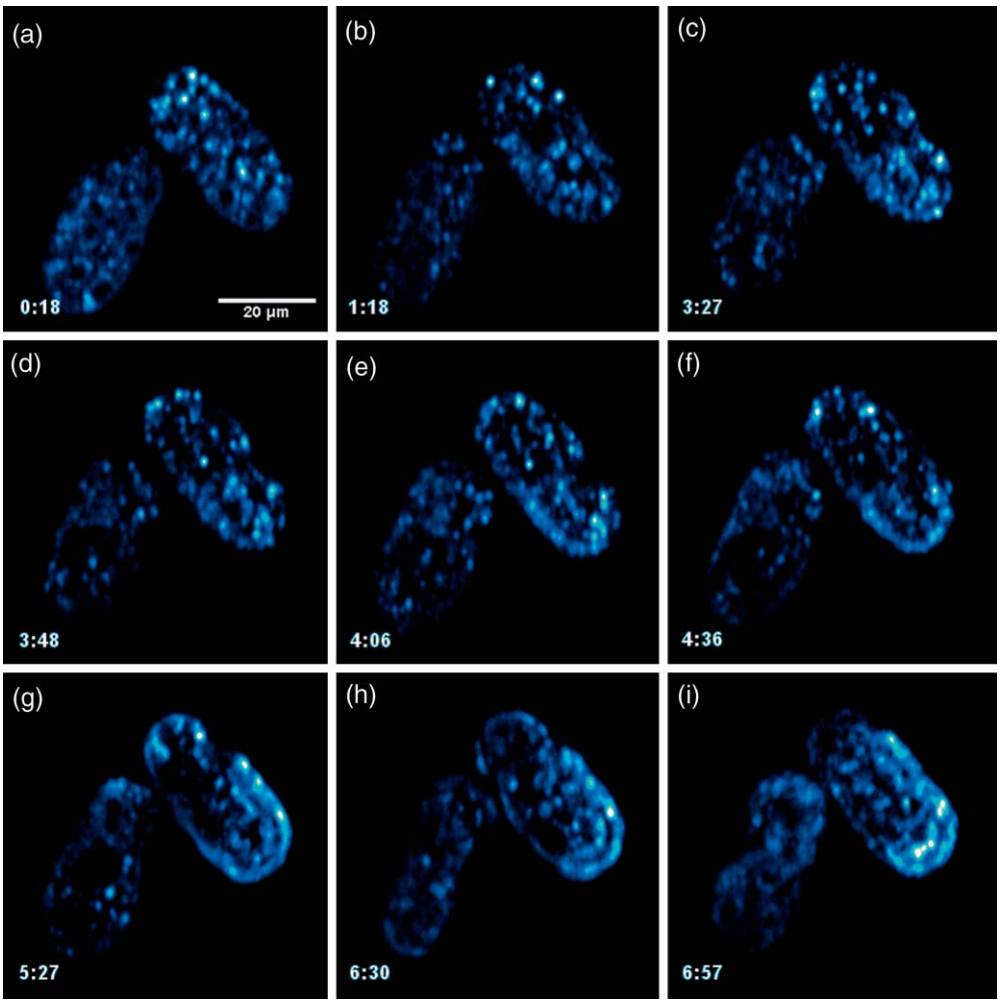


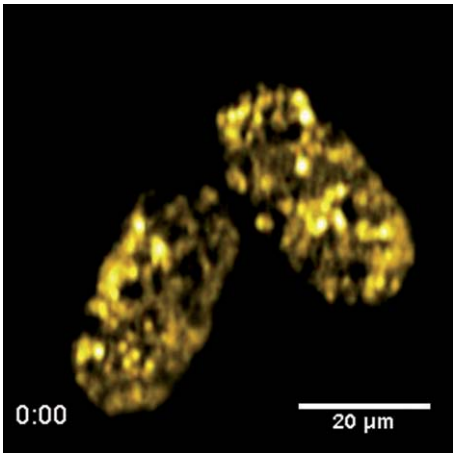
Fig. 5 THG images obtained from two live *C. elegans* embryos. Images show the embryonic development from multiple-cell to the threefold stage (right embryo).

mitotic procedure. This highly important biological procedure can be monitored precisely via THG imaging measurements since cell nuclei appear as black holes in the recorded images, creating very high contrast with the surrounding structures of the cytoplasm. Moreover, we can follow *C. elegans* embryonic growth across the different developmental stages.

Figure 5 shows the division process in two live *C. elegans* embryos from multiple-cell to threefold stage. Nine THG 2D images (300×300 pixels) at different developmental stages are depicted. Figure 5(a) shows two multiple-cell stage embryos. Figures 5(b)–5(d), depict a mid *C. elegans* embryonic stage (especially the right embryo of the figure). At this developmental stage, many cell divisions have already occurred. In Figs. 5(e) and 5(f) the right embryo is at the comma stage of *C. elegans* embryogenesis. At this developmental stage, the elongation of the new embryo has initiated and is progressing toward the first folding. The number of individual cells is increased and it is difficult to distinguish cell nuclei. In Figs. 5(g)–5(i), the right embryo is at the fold stages of embryogenesis. At these stages of embryogenesis, the embryo is elongated and folded inside the egg. The THG imaging modality provides information relevant to the overall contour of the animal. However, the movement of the new worm inside the egg is very rapid making it difficult to precisely monitor this motion with THG measurements. The whole procedure of cell division from multiple-cell to the threefold stage can be observed in the right *C. elegans* embryo in Video 2. Video 2 shows an animated time-lapse sequence of 2D THG signals arising from two embryos. The whole procedure of data acquisition needed for each THG image lasted 8 s. The samples were imaged for 7 h and the time-lapse between images was 3 min.

4 Conclusions

To the best of our knowledge, this is the first time that cell tracking studies have been performed with live *C. elegans* embryos by employing THG imaging microscopy measurements. THG imaging technique is an ideal diagnostic tool for tracking cell divisions, morphological, and structural processes in live *C. elegans* embryos. The movement of cells and cell nuclei during mitosis at the early stages of *C. elegans* embryogenesis is detectable. Furthermore, structural information from later developmental stages (fold stages) has been extracted. By employing this non-linear modality, no fluorescent markers are required for staining the samples. Due to the nature of the THG phenomenon (nonlinear coherent scattering effect), it is feasible to irradiate



Video 2 THG imaging measurements at late embryonic stages of two *C. elegans* embryos. The right embryo clearly shows the development from multiple-cell to the threefold stage. The whole procedure of data acquisition lasted 7 h. Time-lapse between images is 3 min. THG signal is depicted in yellow. (QuickTime 1.1 MB)
[URL: http://dx.doi.org/10.1117/1.3569615.2]

live *C. elegans* embryos for extended periods of time (for more than 7 h). Additionally, this imaging technique has the potential to provide exact temporal information for the different developmental processes (cell division, transition from an earlier to a later developmental stage, etc.) that occur during *C. elegans* embryogenesis.

The identification of healthy and unhealthy embryos is a possible application of this imaging technique. Mitochondria and lipid bodies have been considered as the most prominent structures of the THG signal from cells.[17,18] It can be assumed that they both give information related to the energetic profile of the cell. Consequently, THG imaging modality has the potential to be used as a novel diagnostic tool for monitoring embryonic health.

Eventually, we anticipate that THG modality can be utilized in combination with other optical techniques, such as DIC and fluorescence (confocal or multiphoton), to obtain complementary information in the research field of cell tracking of various biological samples (*C. elegans* embryos, *Drosophila* embryos, etc).

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