Cell division stage in *C. elegans* imaged using third harmonic generation microscopy

R. Aviles-Espinosa,[1] G. J. Tserevelakis,[2] S. I. C. O. Santos,[1] G. Filippidis,[2] A. J. Krmpot,[2] M. Vlachos,[3] N. Tavernarakis,[3] A. Brodschelm,[4] W. Kaenders,[4] D. Artigas,[1,5] and P. Loza-Alvarez, [1*]

[1]*ICFO-The Institute of Photonic Sciences, Mediterranean Technology Park, Av. Canal Olimpic s/n, 08860 Castelldefels (Barcelona), Spain.*
[2] *Institute of Electronic Structure and Laser, Foundation of Research and Technology-Hellas, P.O. Box 1385, 71110 Heraklion, Crete, Greece .*
[3] *Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology, 71110 Heraklion, Crete, Greece*
[4] *TOPTICA Photonics AG, Lochhamer Schlag 19, D-82166 Graefelfing, Germany*
[5]*Universitat Politècnica de Catalunya, c/ Jordi Girona 31, 08034 Barcelona, Spain*
[*]*pablo.loza@icfo.es*

Abstract: *C. elegans* embryogenesis, at the cell division stage, was imaged using third harmonic generation microscopy employing ultrashort pulsed lasers at 1028nm and 1550nm. This technique could be used for cell tracking studies without fluorescent markers.

OCIS codes: (190.4180) Multiphoton processes; (180.4315) Nonlinear microscopy; (140.3510)

1. Introduction

DIC (Differential Interference Contrast), confocal laser scanning microscopy (CLSM) and multiphoton laser scanning microscopy, have enabled the researchers to analyze different biological specimens providing different information (emitted signal) according to each used method. Given this, different sample preparation protocols are required to be able to have an optimum signal.

In embryology, there have been several works that have attempted to track the cell division stage using DIC microscopy of biological specimens. Given that, the contrast in this kind of microscopy is generated by the phase shifts induced to the light passing through the different sample structures. Therefore its applicability is limited if thicker and diffusing specimens are to be imaged. Additionally, for these studies, complex algorithms are used to track the cells. In this case, these algorithms might also use the differences in smoothness of the image texture between the nucleus and the surrounding structures of the sample [1].

CLSM has been proposed as an alternative to the previous technique [2]. This gives and improved resolution, optical sectioning capabilities and selective observation (i.e. fluorescently marked structures). However, it has still several disadvantages. Fist of all, the sample needs to be labeled to allow for a selective observation. Additionally the wavelengths used in this type of microscopy mainly lie in the UV range. All this compromises the sample viability producing phototoxic and photo damaging effects to the sample. Therefore, to perform cell tracking studies the excitation beam must be dynamically adjusted to preserve sample properties and viability. This excitation beam adjustments must be performed during the whole study. As a consequence, the acquired signal will differ according to time and, as in the previous technique, will require the use of complex algorithms that take into account these changes.

Nonlinear microscopy (NLM) has proved to be an alternative method with several advantages (compared with the previous techniques). These advantages arise from its nonlinear dependence on the excitation intensity, well known to reduce photodamage and phototoxic effects. The use of Two Photon Excited Fluorescence (TPEF) in embryology is benefited from the previously mentioned advantages; however the use of fluorescent markers remains as a limitation. Within the nonlinear imaging techniques the use of fluorescent markers can be avoided by Third Harmonic Generation (THG) microscopy.

In THG microscopy, the contrast is based either on a change in third order susceptibility or in the dispersion properties of the sample (within the focal volume). All this means that the signal is built up in the interfaces inside the sample [3]. Because biological samples have different structures that are made of different materials, THG microscopy can be use to obtain high contrast images without having to add any external maker. This condition is extremely important as it enables the study of biological samples in a less invasive way.

In this work, we employ THG microscopy to obtain high resolution images of C. elegans embryos at the cell division stage. This is done by employing two compact ultrashort pulsed lasers with different central wavelengths, one at 1028nm and the second one at 1550nm. In these cases, the generated THG signal of the employed lasers falls in the 340nm (accepted by most microscope objectives) and 515nm range (easily detected by most photomultipliers), respectively. These lasers represent an alternative to the commonly used Ti:sapphire lasers which are expensive, bulky and the emitted THG signal, lays deep in the UV region of the spectrum.

2. Materials and methods

2.1 Microscopy setup

Two different microscopy setups were employed to perform THG imaging of *C. elegans* embryos at the cell division stage. The employed laser wavelengths were 1028nm (Amplitude systems, t-pulse laser) and 1550nm (Toptica, Photonics, FemtoFiber FFS®). For the 1028nm system, a 20x objective (0.8 Numerical Aperture NA) was used for excitation and a 1.4NA condenser was used for collecting the THG signal. In addition, a lock-in amplification scheme was used for detection. The total time for acquiring a 300x300 pixel image is two minutes [4]. The 1550nm system had a 40x objective (1.3NA) and a 1.4NA condenser. In this case, signal was collected without any additional amplification scheme but the final image is the result of averaging five consecutive frames. The total time to produce one final 500x500 pixel image is five seconds [5]. Both setups were based on modified Nikon inverted microscopes in which a forward detecting mount was coupled to the system to be able to detect the THG signal [4, 5]. A schematic of both systems is displayed on Figure 1. Table 1 depicts the parameters that were employed in each system. The average power of each system is measured at the sample plane.

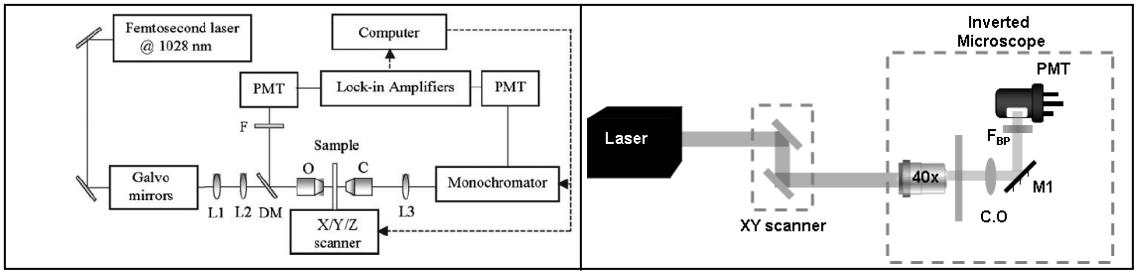

Fig. 1. Schematic representations of both THG setups. Left panel shows the experimental set-up for 1028nm PMT: photomultiplier tube, DM: dichroic mirror, O: objective, C: condenser, F: filter and L1/L2: telescope. Right panel shows the experimental set-up for 1550nm. Laser is the FemtoFiber FFS laser, C.O is the condenser optics; M1 is a silver mirror; FBP is the set of band pass filters (transmittance = 512 – 521nm); and PMT is the photo multiplier tube.

Table 1. Laser parameters used to perform THG microscopy

System	Magnification	Objective NA	Condenser NA	Wavelength [nm]	Rep rate [MHz]	P. Duration [fs]	Avg pow S.Plane [mW]	Peak power [kW]	Intensity [W/cm2]
T pulse laser	20x	0.8	1.4	1028	50	200	35	3.5	1.88E+10
FemtoFiber FFS	40x	1.3	1.4	1550	107	100	4.9	0.46	3.05E+09

2.2 Sample preparation

N2 wild type *C. elegans* were grown in nematode growth media and feed with OP50 (*Escherichia coli*). Worms were synchronized and embryos were attained in order to get a large number of specimens for the imaging experiments. The embryos were mounted between two thin cover slips (No. 0 thickness) and 10 µl M9 buffer. Both coverslips were separated with custom made spacers. Samples were imaged at constant room temperature (20 ºC).

3. Results

The THG signal, either at 1028nm (left panel of figure 2) or 1550nm (right panel of figure 2), was observed in the whole egg of all the imaged samples. In any case the generated images were similar. The cells comprising the embryo were well defined and clearly identified, showing different features inside them. In both images, it is possible to observe a well defined nucleus that was displayed as round dark region. No THG signal is generated here as the nucleus has a homogeneous constitution. In contrast, around the nucleus and inside each cell, many structures are capable of generating a bright THG signal. These might be composed of cytoplasm and other organelles. The exact identification of the THG signal sources are beyond the scope of this work.

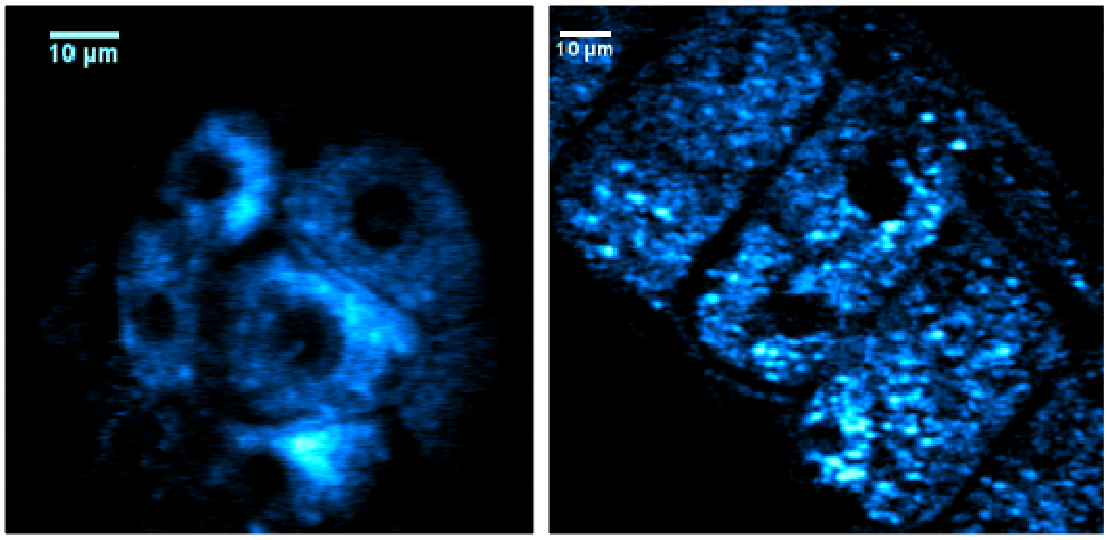

Fig. 2. THG signal from *C. elegans* embryos at the cell division stage. This image shows each cell and a clearly defined nucleus (dark round areas). The left panel is the image taken with the 1028nm system and the right panel is the image taken with the 1550nm system. THG signal is depicted in blue.

4. Discussion and conclusion

In cell linage studies it is fundamental to distinguish the nucleus from each cell to be able to follow it during the cell division stage. This premise has been achieved by the two setups here presented. Both systems show that a dark nucleus can be identified from its surroundings. In addition, the limiting boundary of each cell can be identified. The fact that the two different microscopy setups give the same qualitative information indicates that this method is intrinsic to the THG nature and not to the particular experimental set up or laser wavelength used. This study suggests that by using a fast (sub-second) scanning system, THG can be used for cell tracking studies in embryogenesis, helping to minimize the complexity of the currently used tracking algorithms. This can be achieved without any fluorescent marker, at very high resolution and increasing, at the same time, cell viability during imaging. Finally if the structures that generate the THG signal are identified, this technique will comprise a valuable tool for label free embryogenesis studies.

Acknowledgements

This work is supported by the Generalitat de Catalunya grant 2009-SGR-159, the Spanish government grant TEC2009-09698 and the EU FAST DOT program FP7-ICT-2007-2 (224338). This research has been partially supported by Fundació Cellex Barcelona.

5. References

[1] S. Hamahashi and S. Onami “Objective Measurement of spindle orientation in early Caenorhabditis elegans embryo,” Genome Informatics **16**, 86-93 (2005).

[2] Z. Bao, J. I. Murray, T. Boyle, S. L. Ooi, M. J. Sandel, and R. H. Waterson, “Automated cell linage tracing in Caenorhabditis elegans,” PNAS **103**, 2707-2712 (2006).

[3] P. Török and F. Kao, *Optical imaging and microscopy* (Springer series 2004), Chap. 8.

[4] G. Filippidis, E.J. Gualda, M. Mari, K. Troulinaki, C. Fotakis, N. Tavernarakis, "In vivo imaging of cell morphology and cellular processes in Caenorhabditis elegans, using non-linear phenomena," Micron **40**, 876-880 (2009).

[5] M. Mathew, S. I. C. O. Santos, D. Zalvidea, P. Loza-Alvarez, "Multimodal optical workstation for simultaneous linear, nonlinear microscopy and nanomanipulation: Upgrading a commercial confocal inverted microscope" Rev. Sci. Instrum. **80**, 073701 (2009)