Non-invasiveness, High Cell Viability, and High Penetration of Multi-Harmonic Generation Microscopy

Chi-Kuang Sun[1], Shi-Wei Chu[1], Szu-Yu Chen[1], Tsung-Han Tsai[1], Tzu-Ming Liu[1], Chung-Yung Lin[2], and Huai-Jen Tsai[2]

[1] *Graduate Institute of Electro-Optical Engineering, National Taiwan University, Taipei, 10617, TAIWAN, ROC. Tel: +886-2-33665085. FAX: +886-2-23677467. E-mail: sun@cc.ee.ntu.edu.tw*

[2] *Institute of Molecular and Cell Biology, National Taiwan University, Taipei, 10617, TAIWAN, ROC.*

Summary: Highly penetrative and non-invasive *in vivo* microscopy with <500-nm resolution has the potential of offering new insight into the embryonic morphological changes and the complex developmental processes. Optical harmonic-generations, including second-harmonic-generation (SHG) and third-harmonic-generation (THG), leave no energy deposition to the interacted matters due to their energy-conservation characteristic, providing the optical "non-invasiveness" nature desirable for biological microscopy. By using endogenous harmonic-generations as the contrast agents, no fluorescence is required and the common issues of photodamage, phototoxicity, photobleaching, or dye availability for fluorescence microscopy can all be eliminated. Based on a Cr:forsterite femtosecond laser centred at 1230-nm, which is the transparency window of most biological specimens, we realize a non-invasive and highly penetrative multi-harmonic-generation microscopy. The complicated development within a >1-mm-thick zebrafish *(Danio rerio)* embryo from initial cell proliferation, gastrulation, to the tissue formation can all be observed clearly *in vivo* without any treatment on the live specimens. The excellent three-dimensional resolution of nonlinear harmonic generation microscopy (~0.3-μm with THG) allows us to capture the subtle developmental information on the cellular or sub-cellular levels occurred at deep (>700μm) inside the live embryos and larvae.

The virtual-transition-based SHG or THG has been used to measure membrane potential, to locate bio-crystalline structure, and to image endogenous structural proteins, cell morphology [1,2], and Ca^{2+} intracellular dynamics. Compared with other imaging techniques applicable to biological studies such as magnetic resonance imaging, ultrasound imaging, optical coherence tomography, confocal fluorescence microscopy, and two-photon fluorescence microscopy, virtual-transition based harmonic generation microscopy is expected to provide the advantages including sub-μm three-dimensional resolution and non-invasiveness. Due to its nonlinear nature, the generated SHG/THG intensities depend on square/cubic of the incident light intensity, thus allowing localized excitation and being ideal for intrinsic optical sectioning in scanning laser microscopy. The energy conservation characteristic of harmonic generations release no energy into the interacted medium, thus strongly increases the cell viability. Since the generation of optical harmonics has a weak dependency on the excitation wavelength, we are thus allowed to choose the desirable light source that can provide high penetration through turbid specimens with minimized unwanted light-tissue interactions including scattering, absorption, and photodamages. Also due to the virtual-transition nature of the harmonic generation process, no electron transition occurs after the harmonic generation process, the repetition rate of the excitation source is thus not restricted by the electron relaxation time and signal strength as well as cell viability can both be improved [3] by increasing the repetition rate of the laser source while reducing the pulse energy applied upon the live specimen.

0-7803-7888-1/03/$17.00©2003 IEEE

However, noninvasive and high penetrative harmonic generation microscopy, that can provide high cell viability and spatial resolution all at the same time, has not been fully realized. Previous studies using high rep.-rate Ti:sapphire lasers or fiber lasers as the light sources for harmonic generation microscopy caused either high nonlinear absorption (800 nm) or water absorption (1550nm) thus limited the illumination power and penetration depth with cell vitality penalty. Other studies using amplifier systems were facing the high pulse energy problem (thus cell damage) while the signals were limited by the low rep.-rate. Due to the combination of diminishing scattering cross-section and avoiding resonant molecular absorption of common tissue constituents such as water, light attenuation in live biological specimen reaches a minimum around 1200-1350 nm wavelength region. We choose 1200-1350 nm excitation wavelength regimes not only to provide high penetration and low photodamages but also to allow both SHG/THG within the visible spectrum. An all-solid-state Cr:forsterite femtosecond laser centred at 1230-nm with a 110 MHz rep.rate was thus implemented as the light source for our study, to minimize all light-tissue interaction while maximize the signal strength of SHG/THG.

Fig. 1a shows an *in vivo* optical sectioning taken by our harmonic generation microscope in a live zebrafish embryo at a depth of 700-μm from the chorion, showing the developing polster and tail bud (chorion are outside the viewing area). The yolk granule membranes and the semicrystalline membrane proteins in the yolk cells can be picked up by THG and SHG signals, respectively. The optical sectioning power and non-invasive nature of SHG and THG also allow us to observe the 3D cell proliferation processes inside embryo *in vivo* (Figs. 1b-g). In this presentation, we will use live zebrafish embryos as examples to discuss the penetration, cell viability, and non-invasiveness issues of harmonic generation microscopy. No optical damage can be observed during the whole proliferation process in our study even with 100-mW incident average power on the embryo after long-term (>12 hrs) observations.

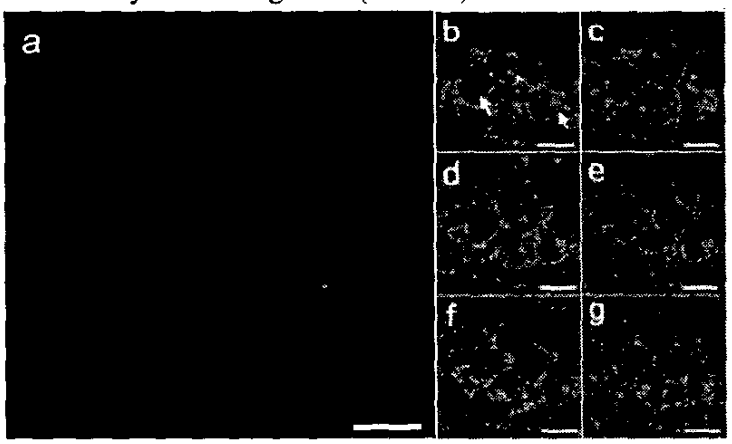

Fig. 1 (a) *In vivo* harmonic generation sectioning inside a live zebrafish embryo at the 2-somite stage, showing the whole embryo at a depth of 700-μm from the chorion surface (ventral view). Scale bar: 100-μm. (b)-(g) are time series of the mitosis processes inside a live zebrafish embryo *in vivo* monitored with multi-harmonic generation microscopy with a 40-s interval between each image. Scale bar: 20-μm.

Reference:

[1] D. Yelin & Y. Silberberg, *Optics Express* **5**, 169 (1999).
[2] M. Muller, J. Squier, K. R. Wilson, & G. J. Brakenhoff, *J. Microscopy* **191**, 266 (1998).
[3] S.-W. Chu, T.-M. Liu, C.-K. Sun, C.-Y. Lin, & H.-J. Tsai, *Optics Express* **11**, 933 (2003).