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Harmonic generation microscopy of bone microenvironment *in vivo*

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ABSTRACT

Here we report and review the investigation of bone microenvironment *in vivo* with harmonic generation microscopy. Excited by an infrared femtosecond laser, the second harmonic generation images can reveal the bone structures and boundaries. The third harmonic generation images can reveal the osteocytes, connecting canaliculi, and granular bone marrow cells. These imaging features can be used to observe and analyze the histology of the bone microenvironment in the future.

1. Introduction

Label-free second harmonic generation (SHG) and third harmonic generation (THG) contrasts have been widely used for the sectioning imaging of biomedical tissues both *ex vivo* and *in vivo*. The imaging signals depend nonlinearly on the excitation intensity of femtosecond lasers and can only be efficiently generated around the focal point. The out-of-focus background interference can thus be greatly reduced. Compared with the reflectance confocal microscopy excited at the same wavelength, the spatial resolution can also be improved by a factor of $\sqrt{2}$ and $\sqrt{3}$ for SHG and THG microscopy, respectively [1]. By scanning the laser beam, the sectioning images can be point-by-point obtained without a confocal pinhole. Compared with two-photon [2] or three-photon fluorescence [3] microscopy, the SHG and THG contrasts has less molecular but more morphological information. Since they are virtual state transitions, the signals can be excited at a near-infrared wavelength, which falls within the penetration window of most biological tissues and does not photobleach. The SHG contrast needs a non-central symmetry of materials and can effectively reveal the extracellular collagen networks [4–12]. Bone, as mineralized collagens, also have strong SHG signals [11]. On the other hand, SHG signals are usually undetectable within cells unless there are structured proteins like spindle fibers [13]. This modality can be used to identify collagen remodeling in tumor microenvironment [6], fibrosis in liver tissues [7], structural alteration in corneal edema [8], calcified plaques in atherosclerosis [9], the diagnosis of atrial fibrillation [10], the deformation of cartilage microstructures in osteoarthritis [11], and the boundaries of bone marrow cavities [12]. The THG contrast, different from SHG, can be generated even in homogeneous media. But due to a Gouy phase shift, the third-harmonic electric field generated before and after the focus will cancel each other. Therefore, only tight focusing at the interface of materials with different refractive indices or $\chi^{(3)}$ can generate detectable THG signals. With excitation at around 1200–1300 nm, the THG microscopy can reveal cellular morphology, subcellular organelles, and melanin distributions in deep tissues for the application of developmental biology [13,14] and clinical diagnosis [15–17] without labeling. In deep tissues, this technique has less background interference than reflectance confocal microscopy and better transverse resolution (~500 nm) [17] than infrared-laser based μ-optical coherence tomography (OCT) [18] and spectrally encoded confocal microscopy [19]. Recently, our group demonstrated that 30 Hz frame-rate THG microscopy at 1200–1300 nm excitation wavelength can *in vivo* capture the images of human blood cells and resolve their dynamic morphologies [20]. Simply using the cross-sectional area and the average THG intensity in the 2D THG microscopy, three major types of human leukocytes (neutrophils, monocytes, and lymphocytes) can be differentiated with high sensitivity and specificity [21]. This is because THG microscopy is a modality sensitive to intracellular lipids, which is a characteristic feature of leukocytes [21]. These results indicated that harmonic generation microscopy is a powerful method for label-free imaging of tissue both *ex vivo* and *in vivo*, such that

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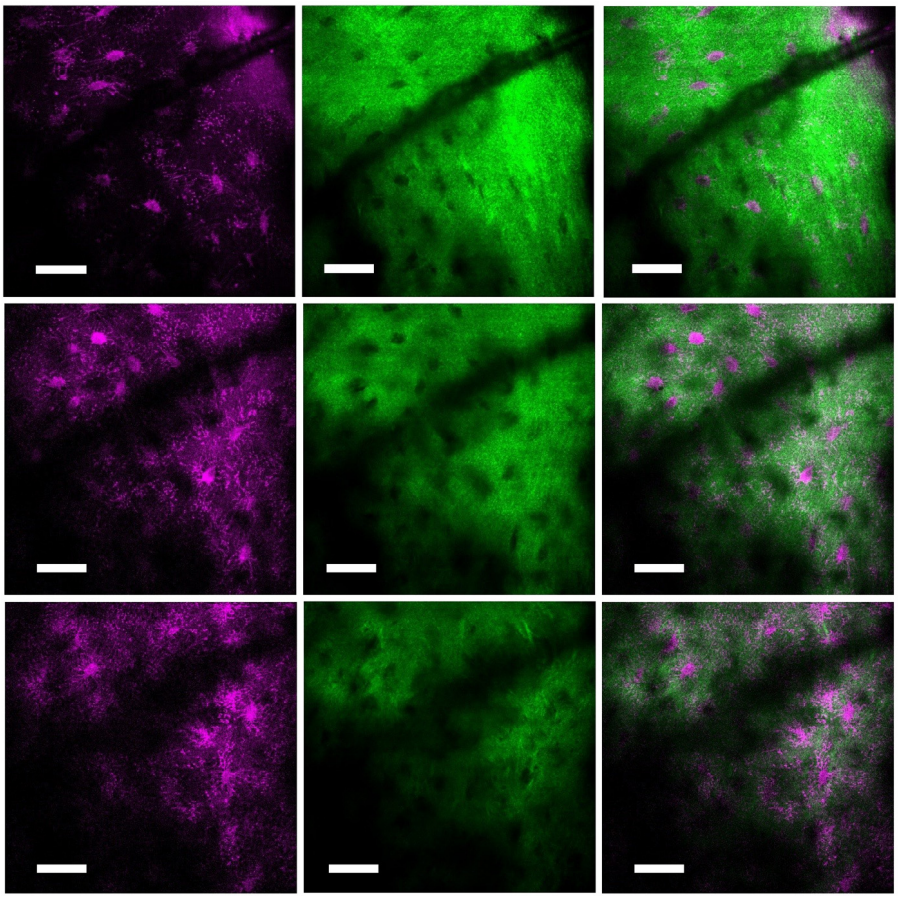

Fig. 1. Third harmonic generation (left column), second harmonic generation (middle column), and combined (right column) images of the calvarial bone of a mouse at the imaging depth of 20 μm (first row), 40 μm (second row), and 60 μm (the bottom row). Fields of view: 240 × 240 μm. Scale bars: 40 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cellular and structural information can be preserved at its original state and the morphodynamics can be analyzed in real time [20]. For the studies of bone tissues, the 1200–1300 nm excitation wavelength has the advantages of low scattering and absorption coefficients compared with visible and 800–1100 nm excitation [22]. Besides, THG modality provides label-free contrasts for the analysis of mineralization in bone development [23], cracks in human enamel [24], dentin tubules in fossil teeth [25], chondrocytes in mice ears [26], porosity and interfaces of bones [27], and osteocyte densities [28]. Most of these previous studies used *ex vivo* samples, and the information about cells may be lost. Here, we report and review the use of harmonic generation imaging of bone microenvironment *in vivo*. The star-shaped osteocytes and extended canaliculi can be easily observed in bone by THG contrast. In the bone marrow, we found THG contrast can highlight the granular leukocytes and the osteoblast on the wall of bone marrow cavities. Both bone repair process and hematopoietic stem cells niche can be studied based on this live imaging platform in the future.

2. Experimental setup

The laser source and imaging setup of this work have been mentioned in our previous report [6]. Basically, the laser source is a home-build femtosecond Cr:forsterite laser operating at 1250 nm. The pulse width is 100 fs, and the repetition rate is about 110 MHz. To initiate the mode-locking, the laser cavity uses a semiconductor saturable mirrors (SESAM). The SESAM consisted of 25 periods of GaAs/AlAs quarter-wave layers, followed by an $Al_{0.48}In_{0.52}As$ quarter wave layer with two embedded $Ga_{0.47}In_{0.53}As$ quantum wells. The laser beam was two-dimensionally scanned by a commercialized scanning unit (FV300, Olympus, Tokyo, Japan). A telescope lens tube was inserted between scanner and objective to form a $4f$ scanning structure. The scanning rays of the laser beam were converged to the back aperture of the objective, and the beam size was expanded to fit that of the back aperture. Before the objective, the laser beam transmitted through a multiphoton dichroic beam splitter with 865-nm edge wavelength. Finally, the laser beam was focused by a 60× water-immersion objective with a numerical aperture of 1.2 (UPLANSAPO, Olympus, Tokyo, Japan). The laser power after the objective was around 80 mW. The generated two-photon fluorescence (TPF), SHG, and THG signals were epi-collected by the same objective. The SHG and THG signals were reflected by the multiphoton dichroic beam splitter, separated by 490-nm edged dichroic beam splitters, and detected by photomultiplier tubes (PMT: R4220P for THG and R943-02 for SHG, Hamamatsu). The TPF signals of indocyanine green (ICG), an angiography dye, transmitted the multiphoton dichroic beam splitter, returned to the scanner and detected by the built-in PMT in the FV300 scanner (R928, Hamamatsu). The photocurrents from PMTs were converted to voltages and digitized by the analog to digital converter in the imaging system (Fluoview, Olympus). The assembled TPF, SHG, and THG images have 512 × 512 pixels at a 3 Hz frame rate.

3. Harmonic generation microscopy of mouse bone marrow in vivo

To image the bone microenvironment *in vivo*, we chose the calvarial bone marrow of an ICR mouse as the imaging site. The ICR mouse is a strain of albino mice originating in Switzerland and selected by Dr. Hauschka. The experimental procedure met the criteria outlined by the Institutional Animal Care and Use Committee of National Taiwan

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