In Vivo Harmonic Generation Microscopy for Least Invasive Virtual Biopsy

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Abstract
Harmonic generations leave no energy in the interacted matters and is ideal for in vivo clinical applications. With a submicron spatial resolution, here we will present thr first-ever clinical trial resuilts on harmonic generation biopsy.

Introduction
Biopsy is a medical procedure involving the removal of tissue samples from a living subject for pathological examination to determine the presence or extent of a disease, especially life-threatening diseases like cancer. The tissue is generally examined under a microscope by a pathologist, after extensive preparation procedure, including time-consuming fixing, embedding, sectioning and staining. For example, the current methods of cancer and precancer differential diagnosis require invasive tissue removal followed by pathological examination based on their staining pattern and morphological criteria of the cellular and nuclear features in the tissue sections. This biopsy procedure is not only invasive and painful, but also risk the sampling error as only few representative areas in a given lesion were taken for examine and only few sections were observed. Differential diagnosis for some lesions is technical difficult and requires experienced pathologist to make the final decision. Artifact due to operation errors during biopsy and tissue processing may also lead to misdiagnosis. Besides, clinical complications and side effects also occur. These include bleeding, scar formation, and the risk of cancer cell spreading.

Optical virtual biopsy techniques, for imaging cells and tissues at microscopic details capable of differentiating benign from malignant lesions non-invasively, are thus highly desirable. Without removing tissues, in vivo virtual biopsy not only avoids or minimizes the abovementioned disadvantages associated with the physically invasive biopsy procedure, but also reduces the cost and time for traditional pathological processing. Optical virtual biopsy could also potentially provide a more comprehensive bedside non-invasive total lesion scanning for improved clinical disease classification and therapeutic guidelines, and a feasible way for continuous disease monitoring during and after treatment.

Recently, optical imaging techniques such as optical coherence tomography (OCT) [1], confocal microscopy [2], reflection confocal microscopy [3], two-photon fluorescence (2PF) microscopy [4], and second harmonic generation (SHG) microscopy [5] have been applied for in vivo clinical imaging, aiming for virtual biopsy applications. OCT can provide millimeter penetrability but is limited by lower spatial resolution. The spatial resolution of confocal microscopy could be greatly improved with a pinhole, but the off-focus photodamages still exist. Based on nonlinear excitation, 2PF and SHG signals are limited in the focal volume and the high resolution optical imaging can be achieved with reduced off-focus photodamages [6]. Under the near-infrared (NIR) excitation (730~900nm) commonly used for 2PF and SHG microscopy [7], the imaging depth is limited by lower allowable excitation power, higher scattering and higher absorption in the skin [8].

Recently we have successfully developed a state-of-the-art optical virtual biopsy system based on least invasive harmonic generation microscop. The system aimed to detect subclinical life-threatening disease and to assist diagnostic decision making for clinically evident diseases. Pre-scanning of the lesion with this system also reduces sampling errors in physical biopsy and helps surgeons to determine the safety margins. Our preliminary in vivo clinical study indicated that the harmonic generation biopsy system outperforms current confocal and two-photon based techniques in providing higher penetration depth, higher spatial resolution, minimized photodamage and phototoxicity, reduced dye-toxicity due to minimized use of external fluorophores, and lowest photobleaching. This system enables 3D subsurface imaging in vivo without the need for surgical approach

In this presentation, the *in vivo* infrared (IR)-based nonlinear optical skin imaging of yellow race human (phototypes IV or V) is reported with combined epi second harmonic geneeration (SHG) and epi third harmonic generation (THG) modalities. To the best of our knowledge, this is the first report regarding the clinical trial of THG imaging. Under 1230 nm IR excitation, harmonic generation signals (SHG and THG) are generally more abundant than 2PF [9] in animal tissues. In this *in vivo* study, THG imaging contrast in the skin was found to be mainly provided by the interfaces [10] between lipid and corneocytes and the cytoplasmic laminated organelles [11]. In epidermis, the cellular morphology at different layers could be clearly

distinguished by epi-THG microscopy, and the THG contrast could also be found in dermis to show the fibroblasts, erythrocytes, collagen fiber bundles, and elastin fibers [12]. In dermis, collagen-sensitive SHG [13] was found to reveal the diverse collagenous structures in papillary and reticular dermis, and extend the imaging depth to more than 300μm, where contrast adjustment may be needed. A syringe-pump objective is designed to diminish the image blurring resulted from breathing and heart beating. Even with the image blurring, the sub-micron spatial resolution due to high nonlinearity [14] was still preserved within the whole imaging depth. In contrast to the previous imaging of the fixed skin [15,16], a higher penetrability and less resolution degradation were observed in the *in vivo* imaging. This in vivo study also provided the dynamic information like blood flow, which could not be obtained from fixed samples, for further *in vivo* optical virtual biopsy investigation and diagnoses.

Conclusions

In conclusion, the *in vivo* IR-based multimodal epi-SHG and epi-THG imaging of human's skin has been demonstrated. Under 1230nm excitation, the photodamages, scattering and combined linear and nonlinear absorption in the human skin could all be reduced. In human skin imaging, THG contrast arises from the interfaces between lipid and corneocytes, cytoplasmic organelles, and hemoglobin, while SHG contrast is provided by the collagen fibers in dermis. In epidermis, THG helps showing the multilayer structure of the stratum corneum and revealing the gradually changed cellular morphology from the stratum granulosum to the stratum granulosum by the strong THG contrast between the cytoplasm and nuclei. In addition, the average nuclear diameter and intra-nuclear distance of the keratinocytes could be analyzed from the in vivo THG images, and THG microscopy would have the ability for diagnosing the skin diseases with abnormal nuclear diameter and intra-nuclear distance like skin cancer. In dermis, SHG revealed both papillary dermis and reticular dermis and the distinct collagenous structures in two regions. Besides, the erythrocytes and the fibroblasts in dermis could be shown by THG and be easily distinguished from collagen fibers due to different imaging contrasts. Based on the SHG contrast provided by the collagen fibers in dermis, the imaging depth could be extended to more than 300μm. A syringe-pump objective was designed and used to stabilize the imaging plan and improve the imaging quality. Even with the image blurring resulting from vibrations, a submicron lateral resolution of THG microscopy was still preserved at a depth of ~300μm. In contrast to the previous fixed skin imaging, *in vivo* HGB of skin showed much reduced resolution degradation while providing the dynamic information in the live tissues. Through the damage evaluation, the noninvasiveness and safety of this imaging tool have also been preliminarily proved. Combined with the high spatial resolution, high penetrability, and the various imaging capabilities, the IR-based multimodal SHG and THG imaging will be a promising tool for future noninvasive virtual biopsy diagnosis of skin diseases. We acknowledge the assistance from Dr. Yi-Hua Liao and Dr. Wen-Jeng Lee of National Taiwan University Hospital. This research is sponsored by National Health Research Institute of Taiwan (NHRI-EX98-9201EI), National Taiwan University Research Center for Medical Excellence, and NTU grant 98R0036-01

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