New Light Choice for Virtual Skin Biopsy

Multi-Photon Microscopy Driven by Fiber-Based Sources

Harmonic generation microscopy (HGM) is one of the most important techniques for optical virtual skin biopsy. The occurrence of optical harmonics mainly relies on the interaction between ultrafast laser pulses and the skin tissue. Conventionally, the burden to drive HGM falls on the shoulder of solid-state lasers. Here we demonstrate a novel fiber-based solution that combines ultrafast fiber laser technology and fiber-optic nonlinear wavelength conversion harnessing self-phase modulation (SPM).

Accurate diagnostics of skin disease mainly relies on the pathohistological microscopic analysis of skin biopsies, which usually includes removing the suspected lesion, fixing, embedding, sectioning, H&E stain, etc. To avoid such an invasive modality, noninvasive optical techniques—optical coherence tomography [1], confocal microscopy [2], two-photon excitation fluorescence microscopy (2PEF) [3], and HGM [4]—are developed to achieve virtual skin biopsy. Among these label-free methods, HGM offers sub-μm optical resolution as well as the least invasive feature since the optical harmonic signal originates from the intrinsic sample geometry instead of fluorophores compared with 2PEF. For example, structures like collagen fibers with non-centrosymmetry allow second-harmonic generation (SHG); interfaces with optical inhomogeneity such as epidermal cell membrane can lead to third-harmonic generation (THG). Thus, one can visualize different strata and structures in epidermis and upper dermis by SHG/THG imaging.

Ultrafast lasers are the key to drive HGM. Illumination in the transmission window of 1100-1350 nm for less optical attenuation is usually adopted considering the light scattering and the water absorption within the tissue [5]. In this excitation wavelength range, the resulting SHG and THG have the wavelength corresponding to 1/2 and 1/3 of the excitation wavelength, and therefore can be efficiently detected by photomultiplier tubes. The conventional solution to produce femtosecond pulses in 1100-1350 nm relies on a mode-locked Ti:sapphire laser plus a synchronously pumped optical parametric oscillator (OPO). Since wavelength tuning is not required for HGM, a mode-locked Cr:forsterite laser directly generating pulses at ~1250 nm constitutes another option.
In general, these “black boxes” operate at the repetition rate of tens of MHz and produce ~100 fs pulses with ~10 nJ pulse energy. Compared with solid-state lasers, ultrafast fiber lasers emerge to be a robust workhorse for driving multi-photon microscopy (MPM). The use of optical fibers for light transmission makes fiber lasers less sensitive to environmental fluctuations. Other attractive features include reduced free-space alignment, absence of water cooling, and cost effective. Unfortunately, no ultrafast fiber lasers are demonstrated to emit femtosecond pulses in 1100-1350 nm.

**Material & Methods**

Recently we have demonstrated an approach to achieve wavelength widely tunable femtosecond pulses based on Yb-doped/Er-doped fiber lasers for driving multi-photon microscopy [6-10]. We employ SPM-dominated fiber-optic nonlinearities to broaden an input narrow spectrum provided by an ultrafast Yb-doped or Er-doped fiber laser; the resulting broadened spectrum features multiple well-isolated spectral lobes. We then use suitable optical filters to select the leftmost/rightmost spectral lobes [6]. Surprisingly the filtered pulses are nearly transform-limited, and the center wavelength can be tuned by simply adjusting the pulse energy coupled into the optical fiber. This technique is dubbed SPM-enabled spectral selection (SESS). Implementing SESS in large-mode-area fibers pumped by an ultrafast Yb-doped fiber laser (center wavelength: 1030 nm) allows us to achieve >10 nJ femtosecond pulses tunable in 1030-1215 nm [7]. While the approach is applied to dispersion-shifted fibers pumped by an ultrafast Er-doped fiber laser (center wavelength: 1550 nm), we can generate >10 nJ femtosecond pulses tunable in 1150-1350 nm [10]. We also show that SESS exhibits excellent energy scalability, which can produce femtosecond pulses with MW peak power at 1300 or 1700 nm [9] — two important transmission windows for three-photon excitation fluorescence (3PEF) of green/red fluorescent protein (GFP/RFP) [11, 12].
Results

We conduct HGM in human skin tissue ex vivo under a laser scanning microscope driven by a SESS source derived from an ultrafast Er-doped fiber laser operating at 31 MHz repetition rate [8-10]. The SESS source is tuned to 1250 nm with up to ~15 nJ pulse energy (corresponding to ~0.5 W average power) and 70 fs pulse duration. The illumination power after a water immersive objective lens (25×, 1.05 NA) is less than 50 mW (corresponding to ~1.6 nJ pulse energy). Different strata and structures in epidermis and upper dermis are representatively shown in figures 1-3. The field of view (FOV) is 500 µm × 500 µm. We use pseudo-colors to present the imaging: SHG in red hot and THG in cyan hot.

The imaging achieved by HGM is parallel to the skin surface, which provides a perpendicular orientation of view compared with conventional biopsy. As we increase the imaging depth, different strata in epidermis can be visualized in the sequence of stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB). These strata can be distinguished by the THG imaging contrast depicting the cell outline. For example, at 30 µm imaging depth in the skin tissue, SC with its nucleless feature appears at the top and the lower right corner of figure 1, whereas SG consisting of cells with keratohyalin granules locates in the center of the FOV. The nuclear area of the epidermal cells exhibits weaker THG contrast, which serves as a basis to calculate the nuclear-cytoplasmic (NC) ratio. In comparison with granular cells, spinous cells display higher NC ratio and constitute most part of the epidermis (fig. 2). At the junction of epidermis and upper dermis we can observe dermal papillae (DP) in figure 3. DP is a structure of collagen fibers (visualized by SHG) surrounded by SB (visualized by THG) featuring increasing surface area for nutrition from the capillary. The vacancies surrounded by collagen fibers act as capillaries, in which we can find red blood cells (white arrows in fig. 3) and lipids.

Conclusion

We demonstrate a fiber laser based SESS source for optical virtual skin biopsy enabled by HGM. Such versatile fiber-based ultrafast sources can be applied to many multi-photon microscopy modalities, such as 2PEF or 3PEF for deep-tissue imaging.

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**References**


