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Abstract. Advancing the practical utility of nonlinear optical microscopy requires continued improvement in imaging depth and contrast. We evaluated second-harmonic generation (SHG) and third-harmonic generation images from *ex vivo* human skin and showed that a sub-40 fs, 1060-nm Yb-fiber laser can enhance SHG penetration depth by up to 80% compared to a >100 fs, 800 nm Ti:sapphire source. These results demonstrate the potential of fiber-based laser systems to address a key performance limitation related to nonlinear optical microscopy (NLOM) technology while providing a low-barrier-to-access alternative to Ti:sapphire sources that could help accelerate the movement of NLOM into clinical practice. © The Authors. Published by SPIE under a Creative Commons Attribution 3.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI. [DOI: [10.1117/1.JBO.20.12.120501](https://doi.org/10.1117/1.JBO.20.12.120501)]

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In vivo, label-free nonlinear optical microscopy (NLOM) of human skin is under investigation for a broad range of clinical applications spanning from skin cancer detection and diagnosis^{1–4} to characterizing and understanding keratinocyte metabolism,⁵ skin aging,^{6,7} pigment biology,^{8,9} and cosmetic treatments.^{10–12} NLOM signals are derived from several sources including cellular cofactors, melanin, and extracellular matrix proteins. Although exceptionally rich in both anatomic and functional contrast, NLOM has relatively limited penetration depth in turbid materials. This is due to the fact that multiple

light scattering diminishes the instantaneous excitation intensity and nonlinear signal generation in the focused laser beam. As a result, there is considerable interest in exploring how light source performance can be optimized to improve imaging depth. Ti:sapphire lasers, commonly used in NLOM imaging, are generally able to access the superficial dermis of human skin to depths of 150 to 200 μm . Penetration depth primarily depends on the material scattering length at the excitation wavelength, the efficiency of the nonlinear excitation process, the excitation average power, repetition rate, pulse width, and the detection geometry.^{13,14} Adjusting these parameters in order to improve the penetration depth has been explored in several studies using Ti:sapphire and optical parametric oscillator-based femto-second lasers as excitation light sources.^{15–21} Sun et al. have shown that reduced light scattering using a Cr:Forsterite 1230 to 1250 nm source can increase penetration depth up to 300 μm for harmonic generation imaging of human skin.²² Improvements in penetration depth can also be achieved when using shorter laser pulse widths.¹⁶ Depth resolved imaging studies require higher average laser powers and thermal damage to tissue becomes an issue of concern.^{23,24} Photothermal absorption of tissue is wavelength dependent, and so is the damage threshold. Heating following laser exposure at 800 nm is five times greater than at 1060 nm, and the damage threshold at 800 nm is three times lower than at 1060 nm.²⁵ With the development of next-generation fiber lasers, it is possible to imagine combining these technical features with more compact, portable, and inexpensive light sources that could facilitate clinical translation of NLOM technology.

Fiber-based laser sources have been used for NLOM imaging of thin tissue cross-sections,^{26–28} mouse brain,²⁹ and human skin tissue³⁰ using fluorescence labeling. In this work, we evaluate the performance of a sub-40 fs, 1060-nm Yb-fiber laser for label-free NLOM imaging of human skin. The effect of excitation wavelength and pulse width on penetration depth in thick, turbid tissues is determined by comparing the fiber laser to an 800 nm Ti:sapphire laser source. We employ the depth-dependent decay of second-harmonic generation (SHG) signals as a standard metric for evaluating performance.

The excitation laser sources used were a Ti:sapphire oscillator (MIRA 900; Coherent Inc.; 220 fs, 76 MHz, 600 mW output power, tuning wavelength 720 to 980 nm) tuned to 800 nm for this study and a Yb-fiber laser (BioPhotonic Solutions Inc., 1060 nm, sub-40 fs, 39.2 MHz, 200 mW compressed output power). The prototype Yb-fiber laser, with self-similar pulse evolution,²⁸ has an integrated adaptive phase-amplitude pulse shaper (MIIPS-HD, BioPhotonic Solutions Inc.) based on a 4f configuration with a two-dimensional spatial light modulator. The purpose of the pulse shaper was to control high-order phase distortions introduced by the high numerical-aperture (NA) objective and other dispersive elements in the beam path. The 1060-nm pulses were compressed to nearly transform limited duration using multiphoton intrapulse interference phase scan (MIIPS),³¹ and their full-width half maximum duration was measured by interferometric autocorrelation using the microscope detection unit (BioPhotonic Solutions Inc.) at the focal plane. Each of the two excitation beams (800 and 1060 nm) was directed toward our home-built laser-scanning microscope and focused into the sample by an Olympus objective (XLPL25XWMP, 25 \times /1.05 NA water). The nonlinear signals from the sample were epi-collected and directed toward two photomultiplier tubes (R3896, Hamamatsu) by a dichroic

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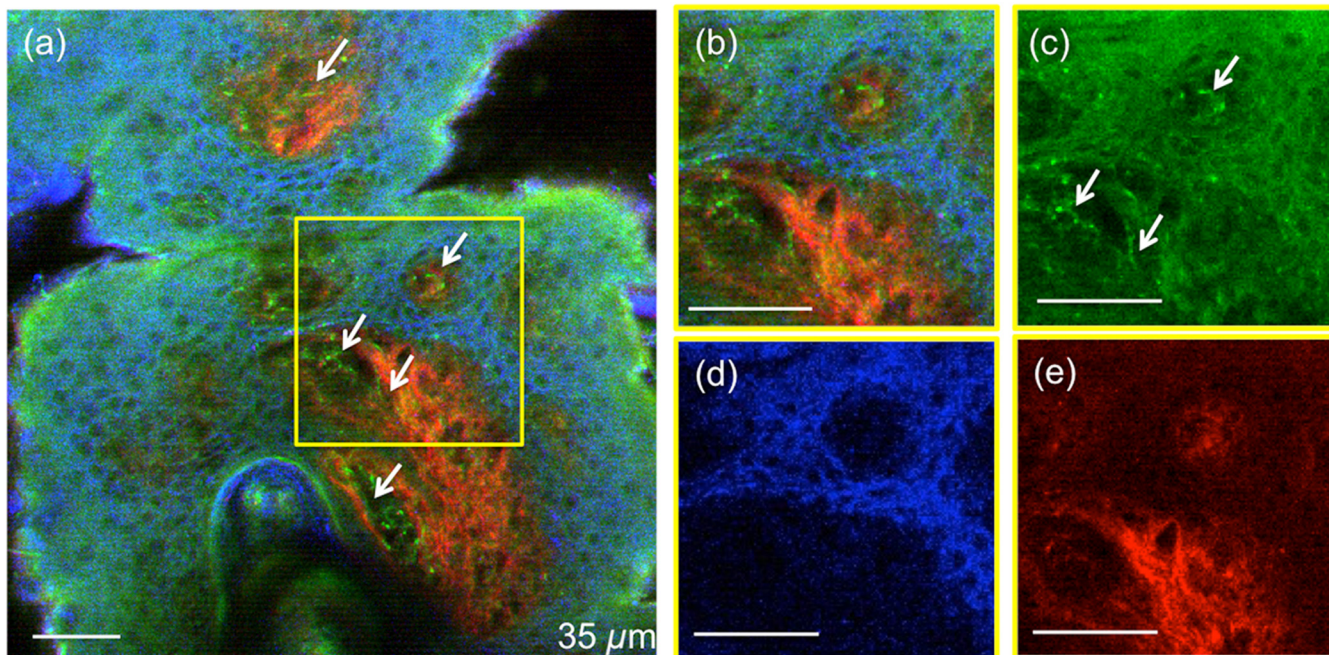


Fig. 1 Multimodal nonlinear optical microscopy (NLOM) images of human skin acquired with 800- and 1060-nm excitation wavelengths at the same depth. (a) Epidermal-dermal junction in human skin imaged by third-harmonic generation (THG) (blue) and second-harmonic generation (SHG) (red) using 1060 nm and by two-photon excited fluorescence (TPEF) (green) using 800 nm as excitation wavelengths ($z = 35 \mu\text{m}$). TPEF signal originates from keratin in the epidermal keratinocytes and from elastin fibers (arrows) in the superficial papillary dermis, while THG signal highlights the keratinocytes only; SHG signal originates from collagen fibers. (b) Multimodal NLOM image corresponding to the inset in (a) representing contribution from three channels: (c) TPEF signal from keratinocytes and elastin fibers (arrows), (d) THG signal from keratinocytes, and (e) SHG signal from collagen fibers. Scale bar is $50 \mu\text{m}$.

mirror (Semrock, Inc., 510 LP). The dichroic mirror was used to split the emission signal into two spectral channels defined by the emission filters: 440 SP; 375/110 BP and 720 SP; 535/150 BP (Semrock Inc.). We used discarded human skin tissue (fixed in formalin) to test the effect of sub-40 fs, 1060-nm excitation laser pulses on depth penetration in this sample. For each excitation wavelength (800 and 1060 nm), we acquired five stacks of images as optical sections of $\sim 430 \times 430 \mu\text{m}^2$ (512×512 pixels) at different depths ranging from 0 to $200 \mu\text{m}$ ($2 \mu\text{m}$ step). In the sample studied in this work, the main contrast mechanisms for 800-nm excitation are based on two-photon excited fluorescence (TPEF) signals from keratin, melanin, and elastin fibers and on SHG signals from collagen fibers. When using 1060 nm as excitation, the epidermis is visualized by third-harmonic generation (THG) contrast derived from refractive index discontinuities at interfaces, while dermal contrast is derived from collagen fiber SHG.

Figure 1 shows merged images of human skin acquired at the same depth with 800- and 1060-nm excitation. THG imaging of the keratinocyte structure in human skin epidermis using 1230 nm as excitation has been reported by Sun et al. in several studies.^{6,22} THG is not generated by elastin fibers in human dermis, although signals from elastic cartilage have been observed.³²

Figure 2 shows representative images corresponding to one of the stacks acquired in the same location of the sample by using 800 and 1060 nm as excitation. The images in Figs. 2(a)–2(c) and 2(f)–2(h) represent en-face (x - y plane) images acquired at different depths. The cross-sectional (x - z plane) images shown in Figs. 2(d) and 2(e) were obtained

from three-dimensional (3-D) image reconstruction of en-face stacks using Amira (FEI Inc.).

To compare the penetration depth attained by each excitation wavelength, we adjusted the laser powers (40 mW for 800 nm and 20 mW for 1060 nm) such that the average intensity of the SHG signal corresponding to the sample surface ($z = 0$) was similar for both wavelengths. The laser power and all the other experimental parameters were kept the same during the data acquisition. The SHG signals measured in the dermis of the skin sample are plotted versus depth in Fig. 2 on log scale. The signal calculated at each depth represents the average of the pixel intensities in the SHG images at that particular depth. The SHG intensity decay curve was normalized to the maximum intensity value for each wavelength.

The SHG intensity decays as a function of depth z according to $I_{\text{SHG}} \sim \exp(-Az)$, where A is the attenuation coefficient that includes the sample absorption and scattering properties at both the excitation and emission wavelengths. The inverse of A yields a $1/e$ attenuation length of $49 \mu\text{m}$ for 800 nm and $88 \mu\text{m}$ for 1060 nm, an increase of $\sim 80\%$ for the Yb-fiber laser source. Similar results were obtained for all five stacks acquired in the sample, which shows that 1060 nm, sub-40 fs pulses can provide deeper penetration in highly scattering samples, such as skin.

In summary, these results demonstrate the potential of fiber-based laser systems to be used as excitation light sources for NLOM imaging of highly turbid media. Despite their current lack of tunability, short-pulse, $>1 \mu\text{m}$ wavelength fiber lasers can provide a low-barrier-to-access alternative to conventional Ti:sapphire lasers. They are of particular interest in applications

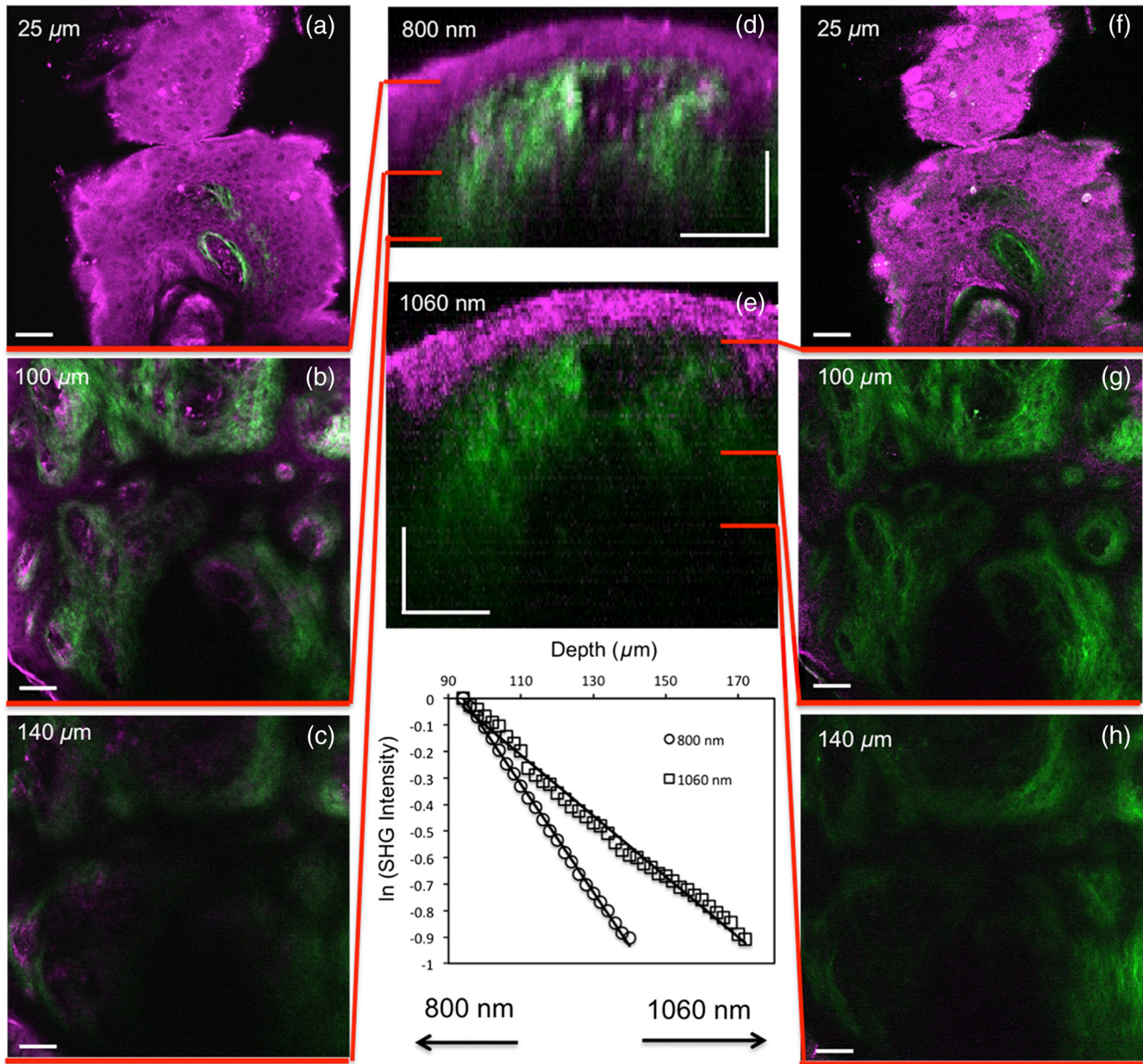


Fig. 2 *Ex vivo* imaging of human skin using 800 nm (Ti:sapphire laser) and 1060 nm (Yb-fiber laser). (a–c) Horizontal sections (x - y scans) at different depths corresponding to 800-nm excitation wavelength. The optical sections show images of the epidermal cells through the TPEF signal (magenta, $z = 25 \mu\text{m}$); collagen fibers (green; SHG signal) and elastin fibers (magenta, TPEF signal) ($z = 100 \mu\text{m}$; $140 \mu\text{m}$). Vertical sections were obtained from three-dimensional reconstruction for (d) 800-nm and (e) 1060-nm excitation wavelengths (40 mW for 800 nm and 20 mW for 1060 nm). Horizontal sections (x - y scans) at different depths corresponding to 800- and 1060-nm excitation wavelengths are shown in (a–c), (f–h), respectively. The optical sections show images of the epidermal cells through the THG signal (magenta, $z = 25 \mu\text{m}$) and collagen fibers (green; SHG signal) ($z = 100 \mu\text{m}$; $140 \mu\text{m}$). Scale bar is $50 \mu\text{m}$. The plot represents the SHG signal attenuation (logarithmic scale) with depth, for 800- and 1060-nm excitation wavelengths.

related to *in vivo* imaging of human skin as they can deliver up to 80% improvement in SHG imaging depth compared to conventionally used Ti:sapphire lasers. An additional benefit for *in vivo* human skin imaging is related to the THG contrast mechanism which, unlike TPEF, does not involve absorption and might allow for the use of higher excitation powers. With continued development of expanded wavelengths, powers, and pulse characteristics, these systems are expected to increase in

use, particularly in skin studies where assessment of 3-D morphology is important.

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