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Resolution enhancement of 2-photon microscopy using high-refractive index microspheres

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ABSTRACT

Intravital microscopy using multiphoton processes is the standard tool for deep tissue imaging inside of biological specimens. Usually, near-infrared and infrared light is used to excite the sample, which enables imaging several mean free path inside a scattering tissues. Using longer wavelengths, however, increases the width of the effective multiphoton Point Spread Function (PSF). Many features inside of cells and tissues are smaller than the diffraction limit, and therefore not possible to distinguish using a large PSF. Microscopy using high refractive index microspheres has shown promise to increase the numerical aperture of an imaging system and enhance the resolution. It has been shown that microspheres can image features ~λ/7 using single photon process fluorescence. In this work, we investigate resolution enhancement for Second Harmonic Generation (SHG) and 2-photon fluorescence microscopy. We used Barium Titanate glass microspheres with diameters ~20–30 μm and refractive index ~1.9–2.1. We show microsphere-assisted SHG imaging in bone collagen fibers. Since bone is a very dense tissue constructed of bundles of collagen fibers, it is non-trivial to image individual fibers. We placed microspheres on a dense area of the mouse cranial bone, and achieved imaging of individual fibers. We found that microsphere assisted SHG imaging resolves features of the bone fibers that are not readily visible in conventional SHG imaging. We extended this work to 2-photon microscopy of mitochondria in mouse soleus muscle, and with the help of microsphere resolving power, we were able to trace individual mitochondrion from their ensemble.

Keywords: Multiphoton microscopy, harmonics generation, microsphere, tissue analysis

1. INTRODUCTION

Microsphere-assisted microscopy has gained attention for near-field ultra-resolution imaging for potential applications in life and material sciences [1-10]. This method exploits high refractive index microspheres to improve the optical transfer function of a microscope by locally enhancing its numerical aperture (NA). Early implementations of this technique used silica microspheres with refractive index of about 1.46 and diameters in the range 2-9 μm. The microsphere was placed on top of the specimen to generate a magnified virtual image in the far-field [1]. For near-field imaging using microspheres another phenomenon called a photonic nanojet achieves resolving power smaller than the diffraction-limit of conventional microscopes [11]. A photonic nanojet is essentially a mode of electromagnetic beam propagation in the vicinity of the microsphere. The photonic nanojet produces a sharp and intense focus in the near-field which reduces the

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width of the Point Spread Function (PSF) of the microscope and increases the size of its optical transfer function. Other researchers have tried dielectric microspheres with higher refractive index such as Barium Titanate glass (index between 1.9 and 2.1), as a way to further improve the resolution [12]. The microsphere is usually placed in contact with the sample and has been used both with air and water immersion objective lenses. It has been shown that the immersion medium affects the photonic nanojet properties of the microspheres and the effective optical transfer function of the microscope [10, 13].

In this paper our focus is on the far-field process, using Barium Titanante glass microspheres, for multi-photon scanning microscopy. Imaging into highly scattering tissues has been a challenge for many biological studies. Multi-photon microscopy takes advantage of long wavelength excitation light to penetrate deeper in to the sample. One of the uses of this method of microscopy is in characterization of tissues by methods such as Second Harmonic Generation (SHG). SHG has been used to study collagen fibers in muscles [14]. However, applying this method to denser tissues such as bone is non-trivial due to its highly scattering characteristics [15]. SHG is sensitive to the orientation of the fibers relative to the polarization of the excitation light. Changing the state of polarization therefore gives comprehensive understanding of the tissue organization. However scattering tissues cause the polarization state to become distorted when reaches deeper planes, and increases error of polarimetry. Bessel beams have the potential to overcome this issue due to their self-healing characteristics and longer axial range [16, 17]. In this work, we produce a Bessel beam by focusing light into a micro-sphere [18], and demonstrate possibility of polarimetric analysis of collagen fibers in highly scattering bone tissue. This provides access to individual fibers at the depth of about 35 μm, and improves the accuracy of polarimetry. We further apply this method of microscopy to 2-photon microscopy of mitochondria deep in mouse soleus muscle. These magnified images provide spatial information about mitochondria and their interactions.

2. SIMULATION

We performed Finite-Difference Time-Domain (FDTD) modeling of the microsphere-assisted microscopy to show propagation of light through the microsphere and generation of a Bessel beam. We used a commercial-grade FDTD [19] numerical method to give a full-wave vectorial analysis of a Gaussian electromagnetic beam focused on a microsphere. For this simulation we used exact details from our microscope (*e.g.*, NA, wavelength, immersion medium) to model the light propagation. The FDTD simulation has a uniform spatial discretization of $\Delta\ell = \lambda_{\mathrm{op}}/(10n_{\mathrm{max}})$, where $\lambda_{\mathrm{op}} = 775$ nm is the operation free-space wavelength with 30 nm bandwidth, centered at λ_{op} and beam's focus waist diameter ($1/e^2$ power) is $w = 326$ nm. Due to symmetrical nature of the microsphere and to simplify the process, we simulated a micro-cylinder ($n_{MC} = 2.0$) with radius of $25\,\mu m$, placed on top of the sample and is assumed to be immersed in a Phosphate Buffered Saline (PBS) solution with refractive index $n_{\mathrm{bg}} = 1.33$ (Figure 1(a)). Perfectly Matched Layer (PML) is used on all computational domain boundaries to avoid reflection at the boundary.

The temporal simulation results are recorded by two monitor planes at the sample surface (laterally, $Z = 0$) and along the center of the sample (axially, $X = 0$). Stationary response is obtained by performing a Fourier transform at λ_{op}, the electric field intensity $\left|E(\lambda_{\mathrm{op}})\right|^2$ are depicted in Fig. 1. We placed the focus of the beam at $Z = 45\,\mu m$ and observed a relayed extended axially PSF with a peak at the depth of $Z = -19\,\mu m$. The

extended axial range starts from $Z = -13\ \mu m$ and continues further than $Z = -40\ \mu m$, see Figure 1 (b,c). The monitor at the sample (Figure 1(d)) shows the rings of the generated Bessel beam at the sample surface.

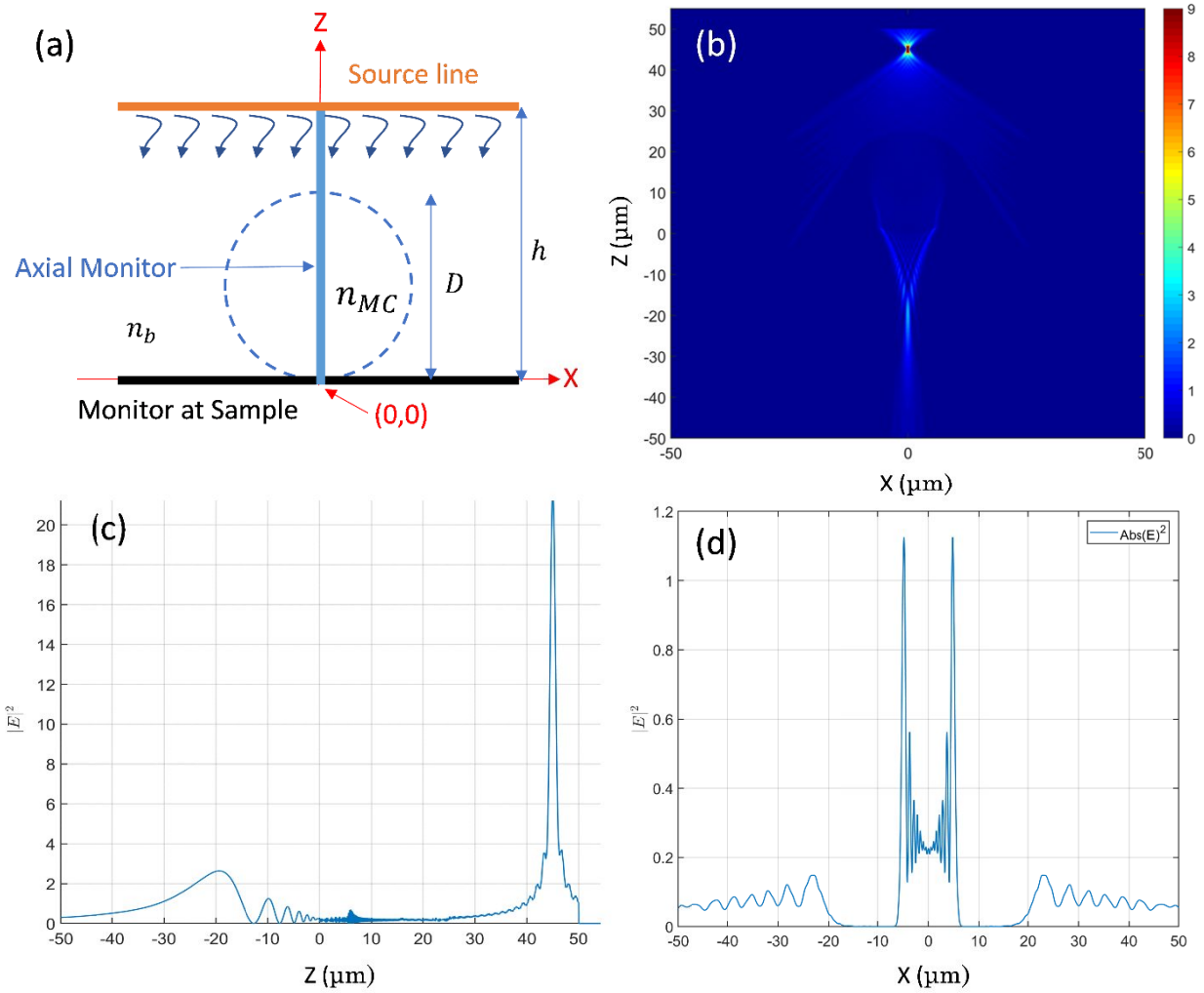

Figure 1- FDTD simulation of light propagation in a micro-cylinder. (a) Simulation setup. (b) Simulation results of 775 nm light propagation in a micro-cylinder and immersion media with indices of refraction 2.0 and 1.33, respectively. (c) Axial monitor, shows the focus of the objective lens with higher peak and the relayed focus inside of the sample. (d) Monitor at the sample surface.

3. METHODS

3.1 Imaging Apparatus

Our microscope is a home built open platform system modeled after our previous design [20-22] (Figure 2). A 1550 nm, 370 femtosecond pulsed fiber laser (Calmar Cazadero) with wavelength of 1550 nm and repetition rate of 10 MHz was used. The beam was frequency doubled with a SHG crystal (Newlight Photonics) to produce a 775 nm beam for two-photon excitation of the sample. Power was modulated using a Pockels cell (Conoptics) and scanned over the sample by a resonant-galvanometer (fast axis – slow axis) scanner (Sutter instruments MDR-R). A 60x Olympus (LUMFLN60x) water immersion objective with NA of 1.1 was used for imaging. Z-scanning was performed using an X-Y-Z stage from the Sutter Instruments (MPC-200). Photon multiplier tubes (PMTs) from Hamamatsu (H10770-40) were used for collection of the

emitted signals from the sample. Each signal was amplified using a transimpedance amplifier (Edmund Optics 59-178). The system is capable of simultaneous 4-color imaging, but for these experiments we only used two channels for acquisition of SHG (using a 390/18 nm Semrock filter) and TMRE (using a 585/40 nm filter from Semrock). A National Instruments DAQ card and FPGA module were used for control and synchronization of the system, and digitizing of the amplified SHG signal. The MATLAB-based Open-source software, Scanimage, [23] was employed to control the microscope.

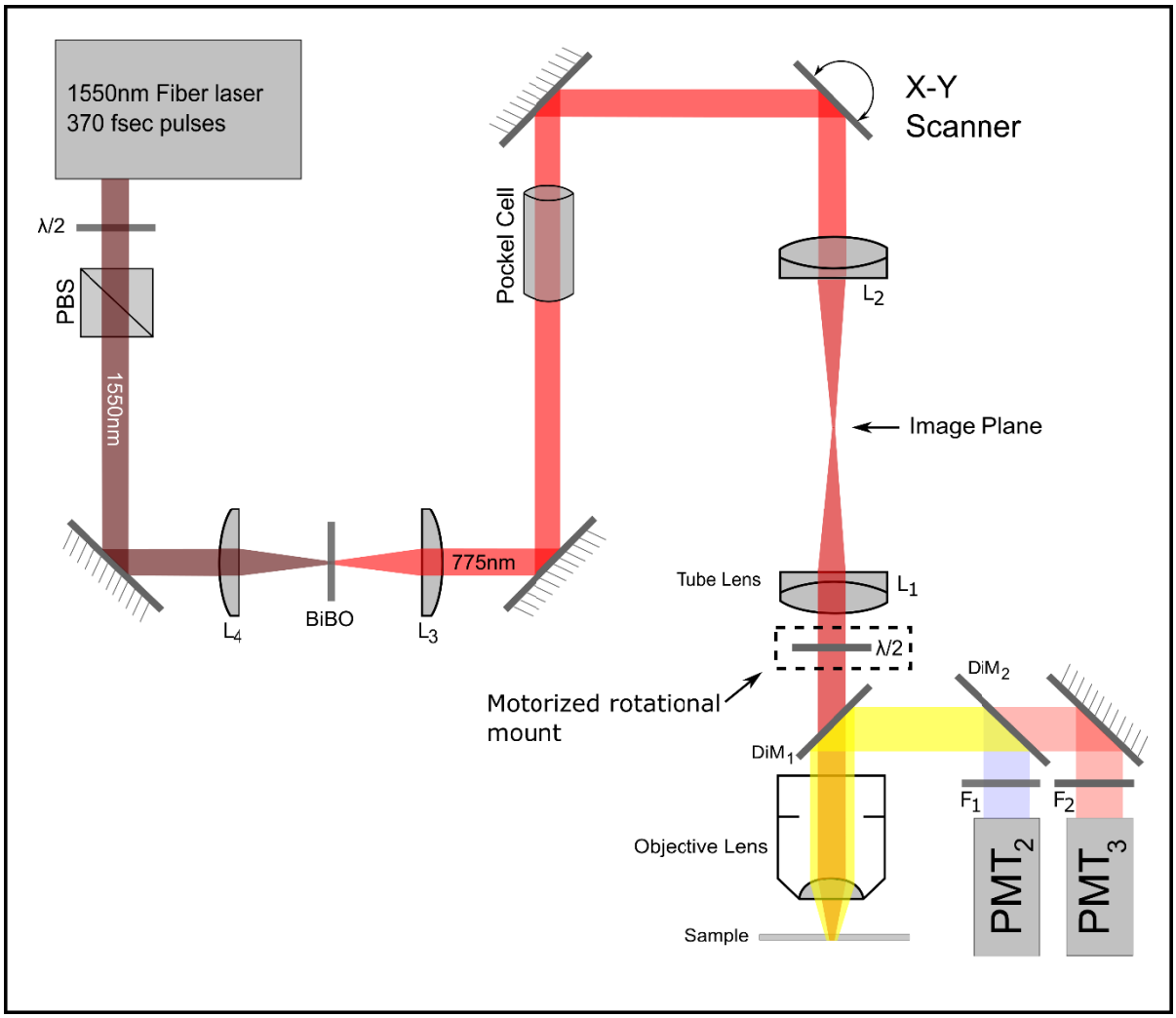

Figure 2 - Optical system diagram. PBS is Polarized Beam Splitter, DiM is Dichroic mirror, and PMT is Photon Multiplier Tube,

3.2 Sample preparation

For the experiments on the mouse skull we used a cranium collected at 12 weeks of age and fixed using 2% paraformaldehyde for 48 hours at 4°C. The mouse soleus muscle was prepared according to the protocol explained in [24]. The muscle was removed from a C57BL/6 mouse 1 hour before imaging. The muscle's mitochondria were stained using Tetramethylrhodamine ethyl (TMRE) for 30 minutes prior to imaging. All animal experiments were done in accordance with UGA IACUC.

4. RESULTS

We first tested effect of microsphere imaging on SHG imaging of bone collagen fibers. We show in Fig. 3 images from a Z-stack acquired at 30 μm beneath the surface, at the surface, and 35 μm above the surface of the sample where we start to see a magnified version of the sample. The produced image is a factor of 4 magnified image of focal planes at around -30 μm. These results show a great agreement with our FDTD modeling.

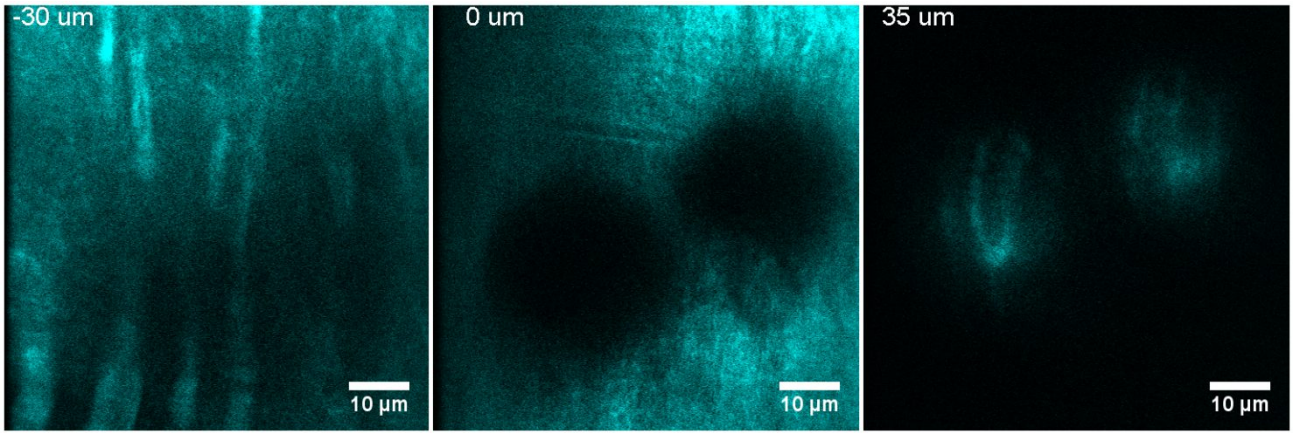

Figure 3 - Second Harmonic Generation in bone using microsphere. Z position relative to the surface of the sample is labelled on each panel, with negative number indicating focused inside of the sample.

The second experiment is 2-photon microsphere assisted imaging of mouse soleus muscle. The soleus muscle is a posterior crural muscle located in the mouse hindlimb that contributes to plantar flexion torque about the ankle joint. The soleus muscle is a mitochondria-rich slow-twitch, oxidative tissue that primarily uses oxidative phosphorylation to generate Adenosine Triphosphate (ATP). The muscle mitochondria are stained using TMRE, and placed in buffered suitable for protection of mitochondria. Here we show a Z- stack from -25 μm to the surface of the muscle and an image generated at 50 μm above the surface which corresponds to focal planes about -25 μm. Comparing the images at 50 μm and -25 μm shows many details that were not otherwise possible to capture in standard 2-photon images and have been revealed in the microsphere assisted microscopy.

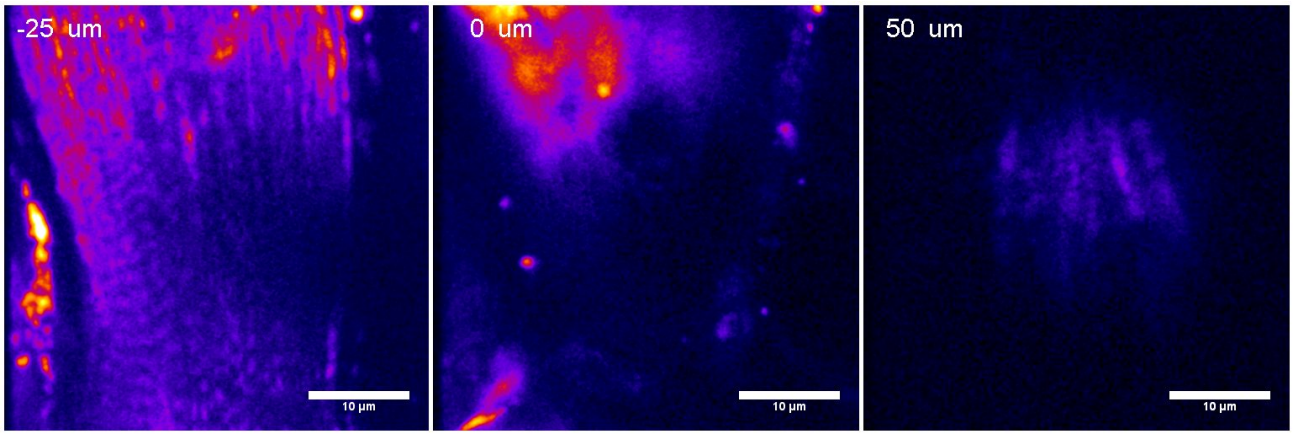

Figure 4 - 2-photon fluorescence imaging of mouse soleus muscle. The labels on the top-left of each panel indicates the location of focal plane relative to the surface of the sample.

5. CONCLUSIONS

Diffraction-limited imaging through highly scattering tissues such as bone or muscle is challenging. Even using long wavelength excitation, acquiring high-resolution images deep inside of the sample is a non-trivial task. For many biological studies however, it is important to image sub-cellular features that are usually close-to or sub-diffraction-limit. In this research, we demonstrated two experiments that benefitted from extra-resolution achieved by microsphere-assisted 2-photon microscopy.

Our FDTD simulations indicate generation of a Bessel like beam with extended axial range that enables 2D acquisition with improved lateral resolution and extended axial depth. We performed simulations at different distances between the microsphere and the objective lens. Our simulations show that an extended depth of about 10 μm could be achieved.

We experimentally showed microsphere-assisted enhancement of SHG polarimetry for bone characterization. Because bone is such a dense environment, it is extremely challenging to acquire accurate polarimetric information especially at depth.

We also experimentally demonstrated 2-photon microsphere-assisted microscopy of mouse soleus muscle's mitochondria. Mitochondria are organelles with a diameter of about 500 nm, which is very close to our 2-photon PSF size (316 nm). Here we showed imaging of individual mitochondria resolved in thick tissue, using microsphere assisted 2-photon microscopy.

In future we would perform scanning of the microsphere over the sample using both mechanical methods as well as optical tweezers to increase the imaging field-of-view.

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