Technical Note

Enhanced multiphoton imaging with a customized laser scanning microscope using three-photon excitation at 1650 nm

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Introduction

Multiphoton microscopy, at present mainly two-photon microscopy and particularly when using femtosecond laser sources, has become an essential technique in biomedical imaging due to its ability to achieve deep-tissue visualization with subcellular resolution. Unlike traditional confocal microscopy, which is restricted to near-surface layers or cultured cell monolayers, multiphoton microscopy enables imaging depths of up to 1 mm in biological samples. In both basic and applied and clinical research, the technique contributes to solving many scientific questions, such as in understanding Alzheimer's and Parkinson's disease or in cancer research. However, further improvements in penetration depth would be desirable. One approach to improving imaging depth is three-photon microscopy, which enhances contrast and resolution; particularly in the wavelength range around 1700 nm, several groups have demonstrated imaging with significantly increased penetration depth [1,2]. Within the framework of the funding projects Eurostars - MARS and ZIM - MultiHero, LLS ROWIAK developed a multiphoton microscope setup specifically designed for three-photon microscopy at wavelengths around 1700 nm.
The LLS laser dissection and multiphoton imaging system "CellSurgeon" comes standard with a laser wavelength in the near infrared (700-1100 nm) as an excitation light source. The aim of the study was to examine the three-photon microscopy efficiency of the microscope when using a femtosecond laser source with a wavelength of 1650 nm.

Method / Material

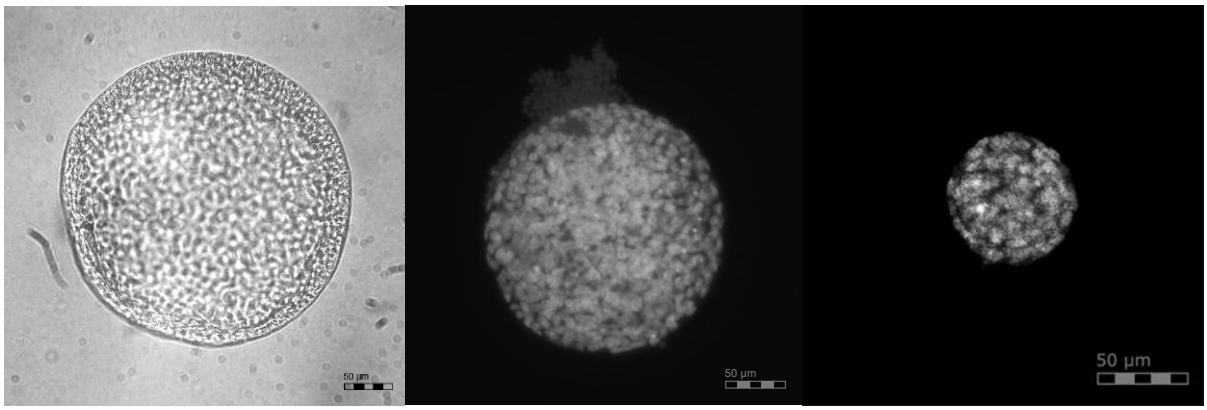
For this study the laser scanning microscope is based on a Carl ZEISS Axio Imager.Z2 Vario, integrated with a Fianium high power femtosecond fiber laser (FP1060-fs) with a center wavelength of 1064 nm for two-photon microscopy and a Carl ZEISS W Plan-Apochromat 20x/1.0 DIC VIS-IR objective lens for wavelengths up to 1700 nm. The system was controlled using LLS CellSurgeon software. LLS ROWIAK had the opportunity to test a Thorlabs - FSLOPAX1 Optical Parametric Amplifier (OPA) with integrated 1035 nm Ytterbium Femtosecond Fiber Laser. The Thorlabs OPA has tunable repetition rate from 1 to 4 MHz and an integrated ytterbium fiber pump laser that converts femtosecond pulses at 1035 nm into pulses at 1650 nm. To evaluate the performance of the customized laser scanning microscope when using a femtosecond laser source with a wavelength of 1650 nm, imaging was applied to different biological samples.
Airway organoids derived from adult murine epithelial cells [3] (provided by PD Dr. S. Kalies and his group, Institute for Quantum Optics, Leibniz University Hannover, Germany) labeled with Hoechst dye were scanned in z-direction to determine the maximum imaging depth in which the sample structure is still clearly identifiable using multiphoton imaging. The multiphoton images of the organoids were then compared with brightfield images (Carl ZEISS Axio Imager.Z2 Vario) and fluorescence microscope images (Carl ZEISS Axiovert 5) of the sample.
To study the ability of three-photon imaging to visualize structural patterns, calcified cartilage samples (provided by Prof. Dr. G. van Osch and her group, Erasmus MC, University Medical Center Rotterdam, Netherlands, in cooperation with Experimental Orthopedics, Department of Orthopedic Surgery, Otto-von-Guericke University Magdeburg, Germany) and mouse artery samples (provided by Institute for Laboratory Animal Science, Hannover Medical School, Germany) were analyzed and compared with two-photon imaging at a wavelength of 1064 nm. In addition, rose leaves with and without mycosis (provided by Prof. Dr. D. Heinemann and his group,

Institute of Botany, Leibniz University Hannover, Germany) were examined using three-photon microscopy.

Results

Imaging depth and sample structure

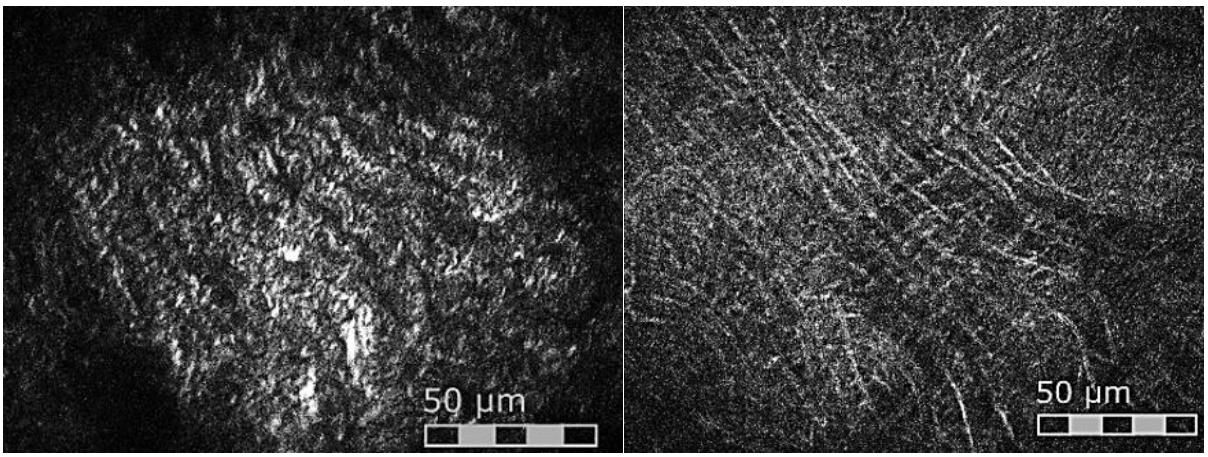
Organoids labeled with Hoechst dye were scanned in z-direction to determine multiphoton-imaging depth. At a laser power of 250 mW, the imaging depth reached 144 µm. Comparing the multiphoton images of the organoid samples with brightfield images and fluorescence microscope images, a clearly defined sample structure is visible in the multiphoton images.



Airway organoids labeled with Hoechst dye. Left: Brightfield image. Center: Fluorescence image at an excitation wavelength of 352 nm. Right: Multi-photon image at a wavelength of 1650 nm.

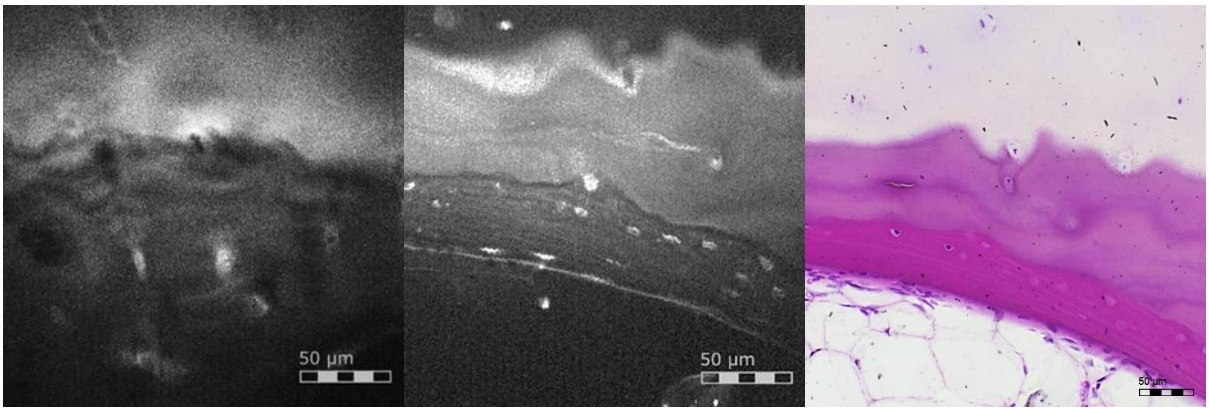
Visualization of structural patterns in various biological tissues

In mouse arteries, three-photon imaging achieved an imaging depth of 200 µm at a laser power of 300 mW, whereas two-photon imaging reached 140 µm despite a higher laser power of 400 mW. Compared to two-photon imaging, three-photon microscopy offers greater imaging depth, improved structural contrast, and lower image noise. In two-photon microscopy, the detected signal is primarily caused by second-harmonic generation (SHG) signal, whereas in three-photon microscopy, both autofluorescence and third-harmonic generation (THG) signals can be excited and detected.



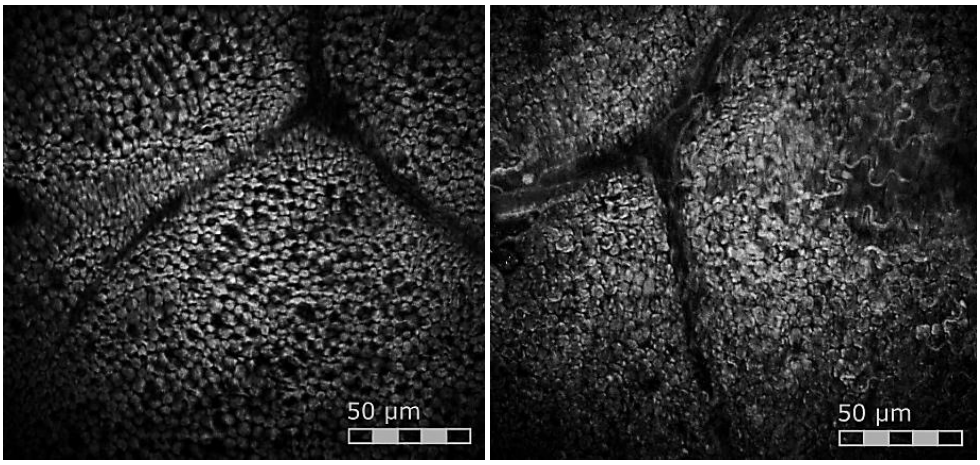
Mouse artery. Left: Two-photon image at a wavelength of 1064 nm. Right: Three-photon image at a wavelength of 1650 nm.

Calcified cartilage samples showed particularly strong results for three-photon imaging. With maximum laser power of 385 mW, a depth of up to 200 µm was achieved. Three-photon images presented detailed visualization of single cells, interfaces, and other microstructural patterns with minimal image noise compared to two-photon imaging.



Calcified cartilage. Left: Two-photon image at a wavelength of 1064 nm. Center: Three-photon image at a wavelength of 1650 nm. Right: Histological staining, Haematoxylin & Eosin

Rose leaves - with mycosis and without were scanned at 300 mW, reaching an imaging depth of 100 µm. The infected leaves showed noticeable structural changes compared to the healthy control samples. This study also demonstrated that three-photon microscopy can visualize structural differences clearly and show details in high resolution for botanical specimen.



Rose leaves three-photon images at a wavelength of 1650 nm. Left: Rose leaf without mycosis. Right: Rose leaf with mycosis.

Conclusion

The examination of the three-photon microscopy efficiency of the customized laser scanning microscope when using a femtosecond laser source with a wavelength of 1650 nm demonstrated that the existing LLS multiphoton microscope setup enables reliable imaging of various biological samples. Compared to two-photon imaging, three-photon imaging achieved greater imaging depths, higher contrast, and lower background noise. The excitation wavelength of 1650 nm further improved tissue penetration due to reduced scattering in biological tissues. The LLS three-photon microscope setup with integrated femtosecond laser source with a wavelength of 1650 nm successfully imaged complex biological and plant tissues with high image quality and increased imaging depth, demonstrating its potential for advanced biological research.

For inquiries regarding complete three-photon microscopy setups, subsystem integration, individual components, or customized hardware modifications for specific experimental or technical requirements, please contact us.

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References

[1] Horton N.G, Wang K, Kobat D, Clark C.G, Wise F.W, Schaffer C.B, Xu C. In vivo three-photon microscopy of subcortical structures within an intact mouse brain. *Nature Photonics*. **7** (3), 205-209 (2013).

[2] Wang, T, Xu, C. Three-photon neuronal imaging in deep mouse brain. *Optica*. **7** (8), 947-960 (2020).

[3] Gentemann L, Donath S, Seidler A.E, Patyk L, Buettner M, Heisterkamp A and Kalies S. Mimicking acute airway tissue damage using femtosecond laser nanosurgery in airway organoids. Front. Cell Dev. Biol. 11:1268621. doi: 10.3389/fcell.2023.1268621 (2023).