MULTIPHOTON MICROSCOPY FOR INTRAVITAL IMAGING IN MICE

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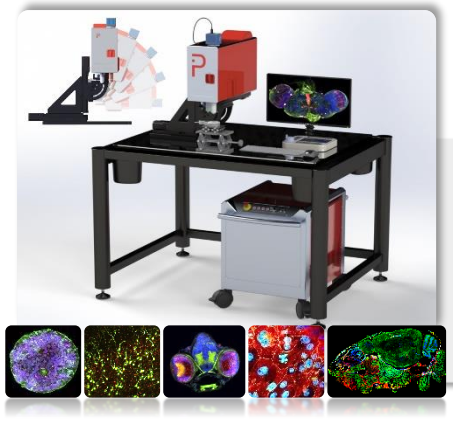
Intravital imaging in mice has significantly expanded our possibilities to study the in-vivo structural and functional relationships in tissue, overcoming many of the limitations that exist with both postmortem and in vitro preparations. In addition, the use of genetically modified mouse models and targeted labeling techniques allows the study of specific cell types, pathways, or disease-related processes. Advanced imaging techniques like multimodal multiphoton microscopy help to unravel fundamental relationships and researchers to explore tissues with unprecedented detail and precision.

The tremendous complexity of tissue like the brain makes it challenging to study, requiring advanced 3D imaging techniques to unravel its complex structure and dynamic functions. In the past few years, intravital imaging in mice has emerged as a powerful tool that allows researchers to explore the inner workings of tissues with unprecedented detail and precision.

Different methods are used for intravital imaging in mice, each offering insights into different aspects of tissue structures, function, and blood flow dynamics. Multiphoton Excitation Fluorescence Microscopy (MPEF), Confocal Microscopy, and Optical Coherence Tomography are among the leading methods used to study neuronal activity, vascular dynamics, and blood flow patterns as well as skin imaging in living mice. These techniques have revolutionized our ability to observe cellular and subcellular processes and provide insight into the complex architecture and functionality of the brain.

Multiphoton microscopy uses excitation wavelengths in the near infrared (NIR) range, providing similar resolution as conventional confocal microscopy, but with much higher tissue penetration depths [1,2]. Here, nonlinear optical effects based on the interaction of multiple photons arriving simultaneously at a molecule are produced using a highly focused laser beam. Therefore, the intensity of the generated signal does not increase linearly with the number of irradiated photons, but with the square (for two-photon effects) or the third power (for three-photon effects), making the signal is intrinsically confocal [3].



"MPX Neuroexplorer – the World´s first Turn-key, Portable and Easy-to-use Multimodal Multiphoton Microscope for Intravital Imaging Applications"

The MPX combines different imaging techniques in one easy-to-use and portable device: non-linear MP (two-photon, SHG & THG) and widefield epi-fluorescence and fluorescence lifetime imaging to maximize informational content, ranging from single cells up to living animals in upright and inverted configuration.

Experimental Setup for Intravital Multiphoton Imaging

Depending on the research objectives, the dimensions of an experimental setup and space requirements for mouse experiments vary greatly. Different experiments require different accessories and supportive devices like a heating mat, stage, stage holder for head-fixation air-floated cage for awake experiments, manipulators, and pumps and many more. Here we will demonstrate easy and straightforward intravital mouse imaging experiments using the MPX microscope using a wavelength tunable, dual-output high-performance ultrafast fiber laser engine, permanently aligned and integrated into the free moving 360-frontend (scanhead), which is lightweight, rugged, connected to the controller via a flexible two meter long umbilical, allows ultimate imaging flexibility. A large working distance as well as the opportunity for upright, inverted or oblique imaging on a precision 5-axis motion system covers all the requirements for mouse imaging, even enabling mesoscale imaging.

The entire system is powered by a handy and compact air-cooled mobile controller box that easily fits underneath any bench or optical table. The controller incorporates a powerfully PC workstation and all other electronic and laser related components, thus allowing a rapid assembly, turn-key, plug-and-play operation in any environment by non-experts.

In order to distinguish different cell types or cells from extracellular matrix such as collagen or to study their interaction, it is relevant to image at least 2 channels simultaneously. Due to the advanced optical design in the MPX, it can be easily changed between multi-channel multiphoton – or epi-fluorescence widefield imaging. Up to 4 independent channels can be imaged simultaneously during two-photon imaging. The built-in high-power multi-channel widefield epi-fluorescence engine allows multi-spectral functional imaging of a large region at a wider scale. Moreover, the fully equipped epi-fluorescence widefield modality facilitates for the broad focusing to the region of interest (ROI) instead of searing the right ROI based on 2P only, which is for some samples not feasible.

"Different Experimental Setups Require Different Working Spaces – From Macro to Mesoscale Imaging."

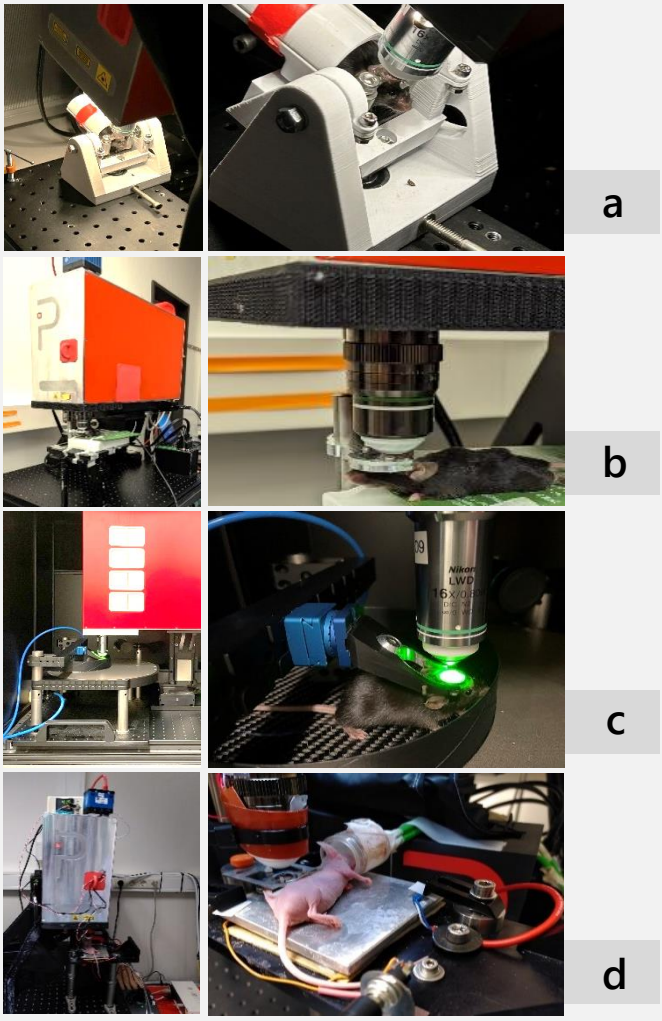
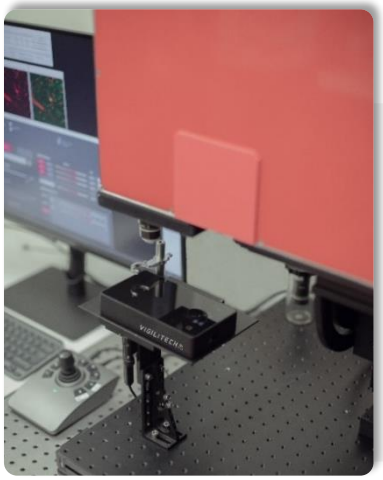


Figure 1: Space requirements of different intravital mouse imaging setups. a) Intracranial imaging of an awake mouse in oblique angles – head fixed with a 3D printed home-built stage, b) Intracranial imaging of an anesthetized mouse, c) Intracranial imaging of an awake mouse - head fixed on an air-floated stage mimicking movement for the mouse, d) intravital skin imaging of an

Monitoring Vital Signs Enhances Data Reliability and Prevents Animal Suffering in Research

Working with living animals is challenging for both: animals and researchers. Constant monitoring of vital signs reduces data variability between experiments, monitors the animal's wellbeing and enables researchers to intervene if needed. Moreover, tracking important metadata is essential for the comparison of highly complex intravital experiments to ensure data quality and reliability due to the impact of welfare on scientific outcomes. MARTA Pad maintains the temperature of the anesthetized mouse and tracks heart rate, breathing rate and the body temperature at all times. MARTA Pad operates remotely through fur without any electrodes. Hence, you can place animals on MARTA Pad and start real time monitoring instantly.



"MARTA: to minimize experiment variety and maximize reliability"

MARTA Pad constantly monitors vital signs during experiments, for animals' welfare and to add vital parameters to your data set. MARTA Pad is well suited for in vivo 2P MPEF imaging when combined with MARTA App, since it can be easily mounted under the MPX scan head. It can be turned into the "microscopy mode" by turning off all indication lights and visualizing vital signs on your computer.

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Intracranial Mouse Imaging

Multiphoton microscopy has greatly improved understanding of neuronal pathways and brain activity, structural and functional relationship of neuronal networks and cranial blood flow in the mouse brain. The integration of genetically encoded markers provides insight into the morphological and dynamic features of cells, their extracellular matrix and fundamental mechanisms underlying neuronal computation, information processing and metabolic behavior.

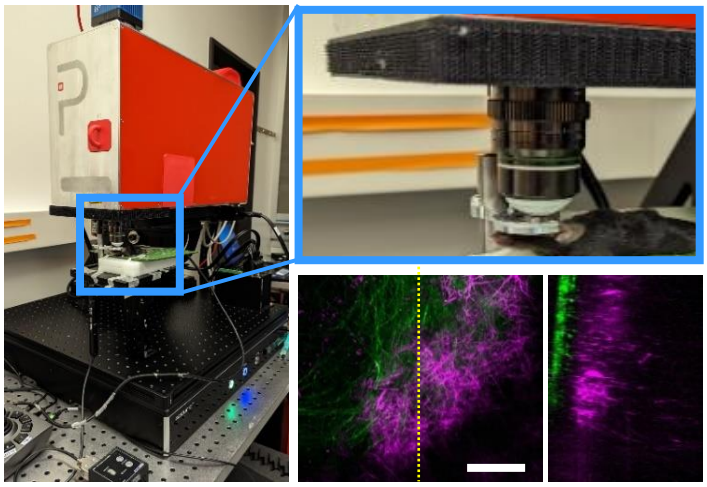

Figure 2: Experimental Setup for cranial intravital imaging in mice. A) MPX microscope 360-frontend (scanhead) on table-top anti-vibration table. B) Anesthetized mouse with a cranial window under the microscope. C) Multiphoton image (left) and orthogonal view (right) of a cranial intravital volume scan within the brain. Label-free SHG of the collagen (green) and tdTomato expression Oligodendrocytes (magenta). Scalebar 20 µm

Imaging of cranial blood flow in mice is one important aspect of in vivo imaging to study the vascular dynamics that support neuronal function. Blood flow imaging provides valuable information about blood flow patterns and changes in cerebral blood flow in this context. By studying the regulation of blood flow under different physiological and pathological conditions, these techniques improve our understanding of brain hemodynamics and its impact on neuronal activity and health.

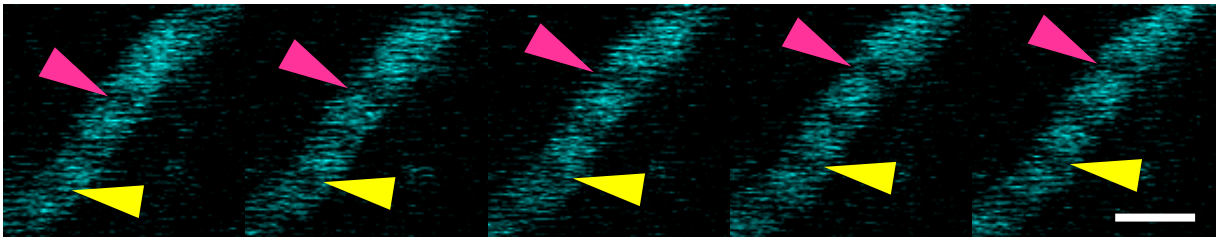

Figure 3: Intravital blood flow in a mouse brain capillary. Resonant scanning with 100 Hz shows single erythrocytes running through a brain capillary in a living mouse. Injected Texas Red in the blood (cyan) as an indicator was imaged and pink and yellow arrows indicate the travel distance of single erythrocytes. Scalebar 10 µm.

Intravital Imaging of Cancer Cell Invasion in the Mouse Dermis

Cancer cell migration and metastasis are hallmarks of cancer, which transform a locally growing tumor into a systemic and live threatening disease. Cancer cell invasion is regarded as a heterogeneous and adaptive process in which the interplay between tumor cells and their tumor micro-environment drives migration and dissemination of tumor cells forward, even under adverse microenvironmental conditions [4]. To further understand cancer cell invasion, tumor cell motility, the molecular and physical interactions of tumor cells with their surrounding microenvironment must be studied in time and three-dimensional space. Intravital microscopy, especially the combination of window models and multiphoton microscopy and fulfills these requirements by offering three-dimensional imaging with molecular specificity at subcellular resolution in live organs [3,5,6]. In addition, label free second and third harmonics generation imaging modalities bring fluorescently tagged cells into morphological context of the local tissue organization [3].

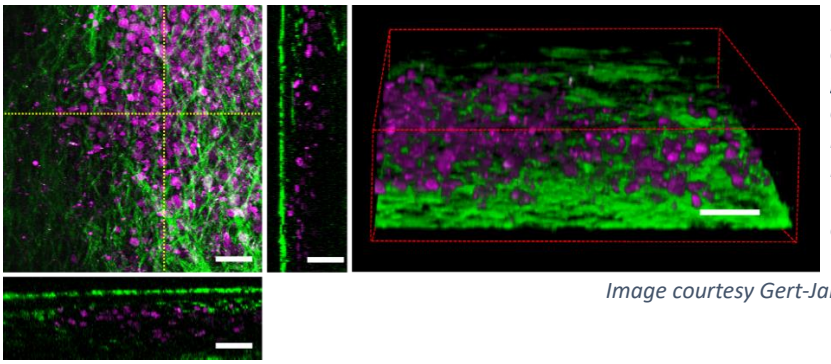

Figure 4: Intravital mouse skin imaging of the epidermal collagen (SHG - label-free) and mApple expressing melanoma cells (mApple-53BP1 transduced, located in the cell nuclei). a) Maximum intensity Projection of a volume scan with the respective XZ and YZ orthogonal views. b) 3D render of the volume scan. Scalebar 20 µm.

Image courtesy Gert-Jan Bakker, Radboud University, Nijmegen

A dorsal skinfold chamber model was applied to a BalbC mouse on day 0 as described in Haeger et. Al (2020) [7]. MV3 melanoma cells were injected into the mouse dermis at day 1, and Two-photon imaging was performed on day 3 using Prospective's MPX Neuroexplorer, a 25x WMP2 objective, 512x512 pixels and a z increment of 5 µm. Figure 4 shows a 3-dimensional stack of the mouse dermis, with fluorescent melanoma cells (magenta, mApple-53BP1 transduced, located in cell nuclei) and collagen type 1 (green, label free imaging SHG signal).

Summary and Outlook

The experimental requirements for intravital imaging are versatile and varying setups of the microscope itself but also of the accessories are necessary are needed. The MPX allows a large number of different experiments due its multimodality, which provides two-photon, SHG and epi-widefield fluorescence in the same region of the sample. The resonant scanning option can track single erythrocytes in brain capillaries and the multi-channel approach allows multiplexing to distinguish between different cell types and the extracellular matrix. The large working space provided by the 360-frontend allows intravital microscopy from any angle and positioning of any required accessories such as stages, heating, and monitoring pads.

Future developments will include improvements for even higher imaging resolutions and depth by implementing adaptive optics techniques and three-photon microscopy.

Furthermore, additional imaging techniques like Fluorescence Lifetime (FLIM) imaging or other contrast building techniques like CARS, SRS or PARS could maximize informational content and yield complementary data sets.

The implementation of easy-to-use optogenetic stimulation capability through the availability of an integrated powerful femtosecond laser will also further expand the range of use of the MPX imaging platform.

Acknowledgements

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