Turnkey Multiphoton Microscopy for 3D Samples

Label-Free and 4D Imaging in Life-Science and Medicine

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Compared to epi-fluorescence wide-field (WF) and laser scanning confocal techniques, multiphoton microscopy (MP) is highly superior when it comes to imaging depth or the identification of label-free biomarkers. Here, excitation with a femtosecond laser typically between 780–1300 nm produces nonlinear optical fluorescence. The intensity of the generated signal increases with the square of the laser peak power (for 2-photon) or the third power (for 3-photon). This phenomenon is confined to a very tight focal volume, significantly reducing the absorption cross-section from out-of-focus planes. Moreover, using wavelengths in the NIR range leads to lower scattering in tissue and thus yields higher penetration depth and lower photodamage compared to linear confocal techniques [1]. By engaging different imaging modalities, a broad range of applications including 3D, label-free, deep tissue, live-animal, whole organ, and whole slide imaging can be addressed. Here, we present high-quality imaging of 3D and 4D samples as well as a label-free and non-destructive imaging of unique biomarkers to understand structural and functional relationships in healthy and diseased tissue with time-saving, multimodal MP microscopy delivering orthogonal informational content and enhanced imaging depth.

One Size Fits All: From the Micro to the Macro Scale

3D and 4D *ex vivo* and *in vivo* imaging have gained increasing importance. The key element here was the understanding that cells behave differently in a 3D surrounding than in standard 2D cell culture [2]. As a result, experiments are becoming more complex, i.e., using awake animals or *in vitro* 3D cell cultures. The future requires a next-generation microscope that is agile and adaptable with features that lower the user barrier, expand on diagnostics with multiple modes, and save resources and time while improving results. With this concept in mind, Prospective's MPX was engineered to be suitable for label-free as well as 3D/4D imaging from the macro to the micro-scale. The MPX-multiphoton microscope is a turnkey, compact, and fully integrated next-generation multimodal microscope, combining different imaging techniques in one easy-to-use and portable device: non-linear MP microscopy (two-photon, higher harmonics - SHG & THG) and linear WF microscopy epi-fluorescence and fluorescence lifetime (FLIM) to maximize informational content and to yield complementary data sets, ranging from single cells up to living animals as shown in figure 1 [3]. Here, the MPX addresses a broad variety of samples from the micro to the macro scale, starting with *ex vivo* 3D cellular models comprising so-called spheroids and organoids.

However, to mimic a healthy 3D surrounding, these models are limited in size because of arising hypoxia in the core when growing bigger than ~400 um in diameter [4]. To overcome size limitations because of necrotic cores 3D models with a vascularization system such as tumors grown on a CAM membrane, 3D printed cell constructs, or even 4D *in vivo* imaging can be used to evaluate samples on the macro-scale as shown in figure 2. However, when it comes to samples in the mm-cm macro scale, additional sample processing techniques such as tissue clearing can be applied to enhance imaging depth and increase the informational 3D volumetric content of these samples, as shown in figures 1 and 2.

Tissue Clearing for Whole Organ Imaging

Tissue and 3D cell constructs are highly inhomogeneous, making them challenging to image due to light scattering and absorption. Increasing the optical translucency by tissue clearing techniques can enhance the imaging depth by a factor >10. However, tissue-clearing methods suffer from some drawbacks: organic solvent-based clearing methods shrink the sample, whereas water-based methods often don't yield the same clearing effect [5]. Moreover, only a few reagents are known for tissue clearing in living organisms, so clearing protocols are mostly related to fixed samples. 3D imaging in fixed samples provides the advantage that there are many staining and processing methods to improve the image quality, signal intensity, or penetration depth, however, they only represent a snapshot of the tissue. Figure 2 shows a whole cleared mouse brain stained for neurons (green). The penetration depth here was limited by the working distance of the objective (4 mm) and could be increased by tissue clearing by a factor of >10.

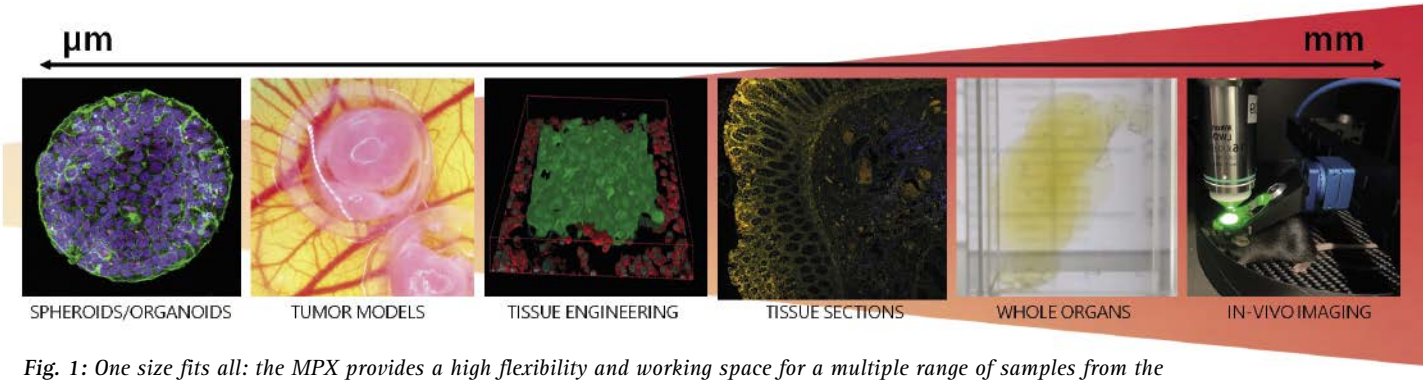

Fig. 1: One size fits all: the MPX provides a high flexibility and working space for a multiple range of samples from the macroscale down to the microscale: key applications are 3D and 4D, label-free, deep tissue, whole slide, whole organ, and live-animal imaging.

Intravital 4D Deep Tissue Imaging

To investigate not only structural but also functional relationships, living tissue and cells must be imaged in 4D time series. However, as mentioned before, optical clearing methods are mostly used for fixed samples. To do 4D timelapse deep tissue imaging of living cells and animals, the right sample must be chosen. In figure 3 we demonstrate zebrafish 4D time-lapse imaging. Zebrafish have long served as invaluable subjects in the fields of metabolic pathology, developmental biology, and neuroscience. Its status as a vertebrate and its sustained transparency in the larval stage makes it a highly practical and expedient live model for research. Figure 3 shows 4D time-lapse neuronal tracking of retina cells and the migration of neurons in the spinal cord of 4-day-old zebrafish larvae.

Another prominent example of 4D *in vivo* imaging is the investigation of neuronal activity in mouse brain using standard markers such as GCaMP as shown in figure 2. GCaMP is a fluorophore-tagged and genetically modified calcium indicator that responds to the efflux of calcium (Ca^{2+}) [6]. Overall, higher penetration depths combined with lower photodamage are major advantages of MP microscopy.

Label-Free Biomarkers for Translational Diagnostics

The identification of label-free biomarkers using multimodal MP imaging has rapidly spread throughout biomedical research in the past few years. A few nonlinear optical modalities are being extensively considered for clinical purposes. For example, two-photon, three-photon, second-harmonic generation (SHG), third-harmonic generation (THG), coherent anti-stokes Raman spectroscopy (CARS), stimulated Raman spectroscopy (SRS), and fluorescence lifetime imaging (FLIM) are all used in research [7,8]. They all have unique advantages and disadvantages. However, combining modalities to maximize informational content and yield complementary data is necessary when it comes to native biological samples since they are highly heterogeneous [1]. As described above this heterogeneity on the one hand making it challenging to image these samples, but on the other hand allows to identify label-free intrinsic biomarkers. Here, timesaving, depth-resolved, sample-saving and non-invasive data acquisition is highly beneficial. Label-free biomarkers can originate from optical or structural properties and phenotype of the sample. Currently, the workflow for diagnosis from a biopsy or resection is tissue fixation followed by paraffin sectioning and histological staining e.g., H&E staining. This is time-consuming, requires experienced technicians, and includes toxic staining reagents. The use of label-free biomarkers in pathology would provide a faster and easier diagnosis. Here, we demonstrate SHG and two-photon imaging as powerful diagnostic modalities providing endogenous molecular and chemical distinction for differentiating healthy from diseased tissue as shown in figure 4. The combination of both modalities provides a label-free contrast originating from molecules such as NAHD, FAD, elastin, proflavine, and hemoglobin, providing intrinsic endogenous fluorescence or structural properties from non-centrosymmetric molecules like fibrous collagen [9]. Morphological and structural changes in the cell or the surrounding matrix e.g. the nucleus shape in cancerous tissue or the alignment and amount of collagen fibers in wound healing, fibrosis, and tumors can serve as valuable diagnostic tools.

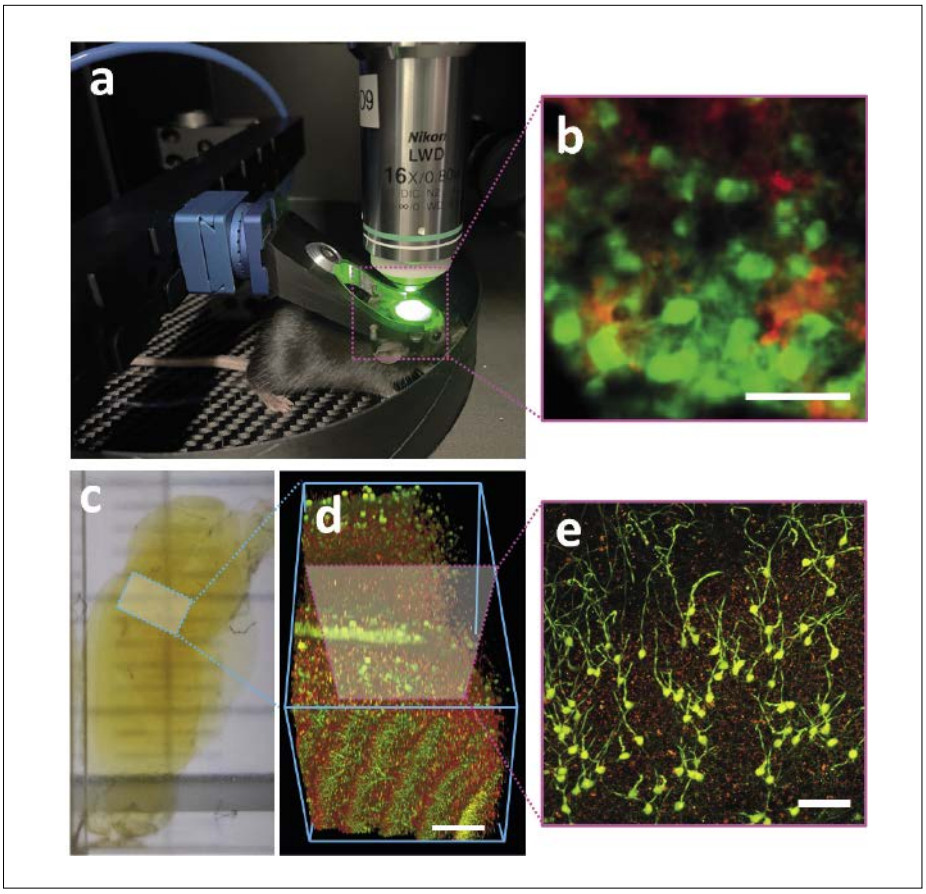

Fig. 2: Intravital MP mouse brain imaging of GCaMP-expressing neurons in the cortex and deep tissue imaging of a fixed and cleared mouse brain. a) Microscope setup of live mouse in-vivo brain imaging and b) close-up of the cortex at ~300 µm z-position in depth, c) whole cleared mouse brain, d) volume scan of ~3.5 mm in depth, and e) one exemplary z-plane showing stained neurons (green). Scalebars: 100 µm

Engage Imaging Speed and Resolution – Epi-Fluorescence Guided MP Microscopy

Even though non-linear microscopy techniques are highly beneficial for deep tissue and label-free imaging they are scanning-based techniques with a limited acquisition speed making them slower than epi-WF microscopy. However, engaging the speed of WF and the confocal depth resolving properties of non-linear microscopy can be used for epi-fluorescence WF-guided MP microscopy. Here, a WF overview fast scan serves as guidance to the region of interest (ROI) which is then imaged in detail using MP microscopy [3]. The capability to perform multimodal analysis, on the same region of interest without the inconvenience of moving the sample to another system, maximizes content, provides orthogonal data, and saves time as shown in figure 4.

Conclusion and Outlook

High-quality imaging of 3D and 4D samples as well as label-free and non-destructive imaging of unique biomarkers is becoming more important in understanding cellular mechanisms and their structural and functional relationship. Engaging different imaging modalities, a broad range of key ap-

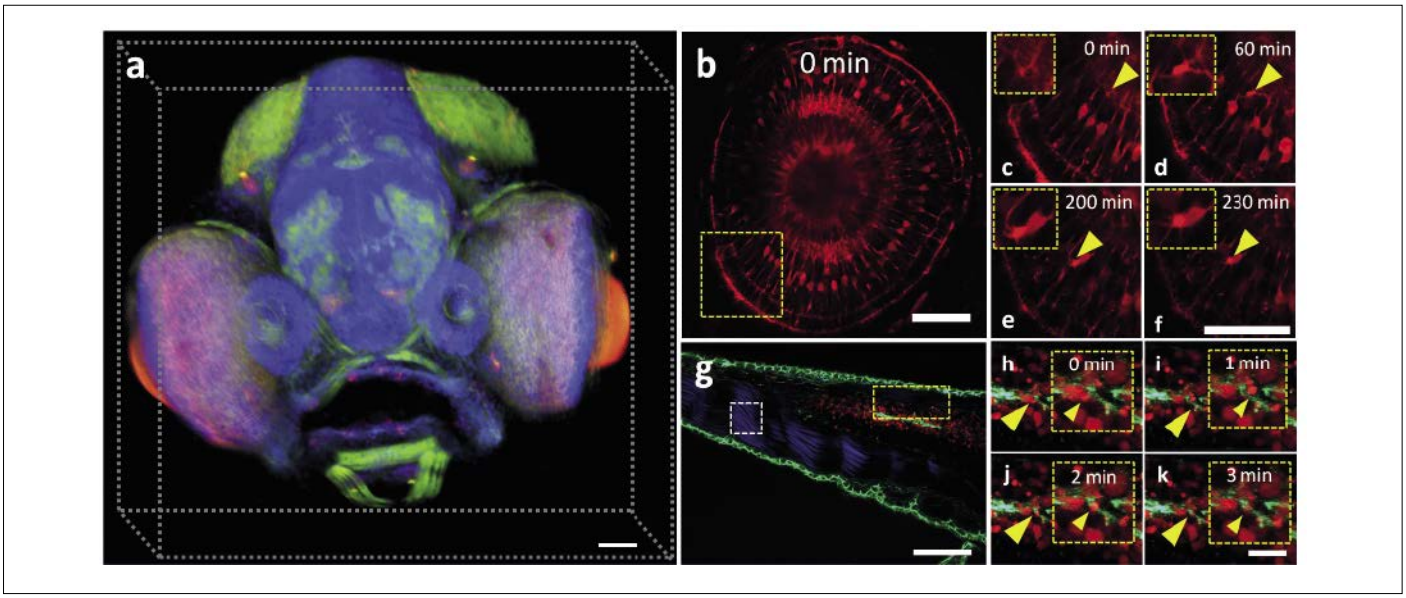

Fig. 3: MP 3D volume scan (a) and timelapse imaging (b-k) of zebrafish larvae. a) 3D projection of a fixed zebrafish larvae expressing prox1:TagRFP and stained with Hoechst (cell nuclei – blue) and Phalloidin (actin – green). b-f) Migration of retina cells in a living zebrafish eye imaged by time-lapse microscopy. 4-day-old zebrafish larvae expressing tp1:LifeAct-mCherry (red) with the respective close-ups. Scalebar: 50 µm (b) and 20 µm (c-f). g-k) Neuronal tracking of cells in the spinal cord in a living 4 day old zebrafish larvae alpha-catenin-YFP (green), tp1:mCherry-NLS (red), and the SHG signal from the muscle (blue) with the respective close-ups. Scalebar: 100 µm (g) and 20 µm (h-k).

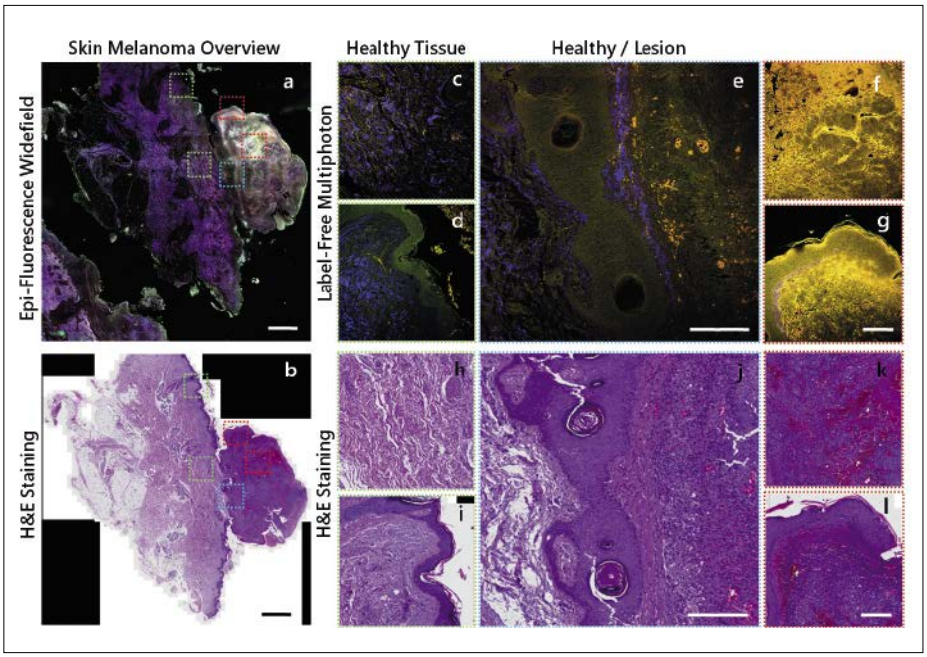

Fig. 4: Epi-fluorescence widefield guided multiphoton microscopy of a human label-free skin melanoma FFPE section. Fast WF overview scan (a) and H&E stained ground truth (b) for orientation and respective close-ups of healthy tissue (d,c and h,i), interface between healthy and diseased tissue (e and j), and regions in the lesion (j,g and k,l). a) Endogenous fluorescence collected by epi-fluorescence λex./λem. 390/432 nm (magenta), 475/515 nm (green), and 555/595 nm (orange). c-g) Two-photon endogenous fluorescence collected at λex./λem 1040/542nm (green) and 1040/595 nm (red) and the SHG signal at 520 nm (blue). Scalebar: 1 mm (a&b) and 200 µm (c-l).

plications including 3D imaging, label-free, deep tissue, live-animal, whole organ, and whole slide imaging can be addressed.

Here, we demonstrated the use of multimodal MP microscopy to gain orthogonal informational content, enhance imaging depth, and save time in 3D *in vivo* and *ex vivo* samples as well as large area tissue sections. Higher penetration depth can be achieved by tissue-clearing techniques to enhance optical translucency in fixed samples. We showed that using different microscopy techniques such as WF-guided MP microscopy can save time and provide additional data by delivering fast overview scans. Future developments include improvements for even higher imaging resolutions and depth by implementing adaptive optics (AO) techniques and three-phtonon microscopy.

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[1] References: https://bit.ly/IM-Kiderlen