In vivo hybrid microscopy of small model organisms

Markus Seeger[1,2], Gil Westmeyer[1,2,3], Vasilis Ntziachristos[1,2,*]

[1]Chair of Biological Imaging and TranslaTUM, Technische Universität München, Munich, Germany
[2]Institute of Biological and Medical Imaging, Helmholtz Zentrum München, Neuherberg, Germany
[3]Institute of Developmental Genetics, Helmholtz Zentrum München, Neuherberg, Germany
*Correspondence to v.ntziachristos@tum.de

Abstract

We present the investigation of *in vivo* small model organisms, which are well established in biological and biomedical research, using a hybrid multiphoton and optoacoustic microscope (HyMPOM). The unique capabilities of HyMPOM for multimodal and potentially label-free signal acquisition, high resolution, as well as deep and fast imaging allow extraction of detailed information across large areas of living tissue on the microscale. Applying HyMPOM to living zebrafish-like fish larvae allowed exploration of the structural composition of the entire brain, including the brain vasculature and the neuronal network. Applying HyMPOM to the ears of living mice enabled accurate imaging of vasculature, connective tissue, keratinocytes, and sebaceous glands. The hybrid microscope proposed here constitutes a novel approach to explore small model organisms *in vivo* in great detail by revealing the spatial distribution and interplay of various tissue compartments on the microscale.

Introduction

Precise examination of the composition and structure of biological tissues as well as their mutual interaction can provide new general knowledge about biological systems and pathophysiological hallmarks that may be useful for theranostics. Optical microscopy can straightforwardly access single cells, but small model organisms such as fish larvae or mice require more advanced microscopes. Hence, powerful imaging tools on the microscopic scale are necessary to reveal the underlying structural and functional information of living specimens, which can help link *in vitro* studies on single cells to *in vivo* studies using small model organisms. For this purpose, high resolution, deep imaging, comprehensive contrast, as well as potentially label-free sensing are desired. In this project, we therefore merged several microscopy modalities into a single optical device to create a hybrid multiphoton and optoacoustic microscope (HyMPOM). HyMPOM offers unique imaging capabilities to examine biological and biomedical specimens at high spatial and temporal resolution. [1–4]

Methods

The custom-built hybrid microscope as schematically depicted in Fig. 1 and comprehensively described previously [5–8] is based on raster-scanning diffraction-limited optical excitations across a sample using a set of galvanometric mirrors (6215H Galvanometer Scanners, Cambridge Technologies). For the optical-resolution optoacoustic subsystem (OR-OA), a 532-nm laser emitting 1.4-ns pulses running at 50 kHz (SPOT-10-200-532, Elforlight Ltd) is utilized to generate optoacoustic signals that are detected by a spherically focused piezo-electric transducer (HFM23, Sonaxis) above the sample in transmission mode coupled to the sample by a buffer droplet. The multiphoton (MP) modalities of two-photon excitation fluorescence (TPEF) as well as second and third harmonic generation (SHG, THG) are based on a 1043-nm laser emitting 170-fs pulses (YBIX, Time-Bandwidth), which is co-aligned with the OR-OA subsystem. MP signals are detected by highly sensitive photomultiplier tubes (H9305-03, Hamamatsu). To achieve high-speed scanning, both a streaming-like acquisition mode (ADQ412, SP Devices; PCIe 6363, National Instruments) as well as an automated field-of-view tiling using sample-holding stages (MLS203-2 & MZS500-E, Thorlabs) integrated into an inverted microscope (AxioObserver.D1, Zeiss) is enabled. The microscope contains a CCD camera (AxioCam ICc 1, Zeiss) for brightfield validation of HyMPOM images. The HyMPOM system is fully controlled using Matlab (Matlab 2014a, Mathworks) [1,7].

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To showcase the feasibility of studying *in vivo* animal models, we applied HyMPOM to zebrafish-like fish larvae. We paralyzed a STIII medaka larva, which lacks pigmentation, at 5 days post-fertilization (5-dpf) with ~1% (v/v) of the muscle relaxant Flexeril (C4542-5G, Sigma-Aldrich), then embedded it in low-melting-point agar (A9414, Sigma-Aldrich) prepared with eggwater. Furthermore, we labeled the neurons by intraventricular injection of CaSPA-550 [9]. We also applied HyMPOM to ears of living athymic nude Hsd Foxn1 mice anesthetized with ~2% isoflurane (Isoflurane CP 1 ml/ml, cp-pharma). The physiological condition (PhysioSuite, Kent Scientific) of the animal was monitored throughout the measurement and body temperature was controlled. All animal procedures were approved by the Government of Upper Bavaria.

Results

The system achieved resolutions of ~0.5 µm laterally and ~6 µm axially for all equipped modalities at a maximum depth of up to 500 µm. It sensed a broad variety of biologically relevant compounds, e.g. vasculature, collagen, elastin, keratinocytes, hair follicle, sebaceous glands, and lipid embeddings [2,7,10–12]. This allowed us to study small model organisms such as fish larvae and mouse ears *in vivo* (see Fig. 2). Here we obtained single-cell resolution, allowing investigations of the vasculature and comprehensive imaging of the microstructural composition. Fig. 2a shows the multimodal image of a 5-dpf medaka larva with fluorescently-labeled neurons blended on top of an inverted brightfield image. HyMPOM enabled the extraction of detailed information about the brain vasculature using OR-OA (see Fig. 2b), the neuronal network using TPEF (see Fig. 2c), and lipid droplets surrounding the eyes using THG (see Fig. 2d). All these details are hidden in the corresponding brightfield image (see Fig. 2e). Furthermore, 3D rendering (see Fig. 2f) of the data reveals high spatial complementation of e.g. the microvasculature embedded in brain furrows. Fig. 2g depicts the multimodal and label-free assessment of a mouse ear with the associated brightfield image. Here, HyMPOM revealed the entire vasculature down to the microcapillary level using OR-OA (see Fig. 2h), the extracellular collagen matrix using SHG (see Fig. 2i), and sebaceous glands, keratinocytes, as well as tissue morphology using THG (see Fig. 2j). Such details are not visible in the corresponding brightfield image (see Fig. 2k), whereas the overlaid multimodal image (see Fig. 2l) allows microscale exploration of the spatial composition of the tissue.

Discussion

In this work, we showcase a novel approach for imaging biological specimens with cellular resolution and a multimodal contrast mechanism at speeds and penetration depths that allow extensive examination of biological and biomedical processes on the microscale. This allowed us to not only enlarge the knowledge of the composition and structure of biological tissue but also to gain new insights into structural and functional interplay among the most important tissues. This illustrates the potential of this approach for pathophysiology studies and diagnostic testing. Our work clearly establishes the suitability of hybrid multiphoton and optoacoustic microscopy to investigate small model organisms *in vivo,* making it a highly promising tool for basic and translational research.

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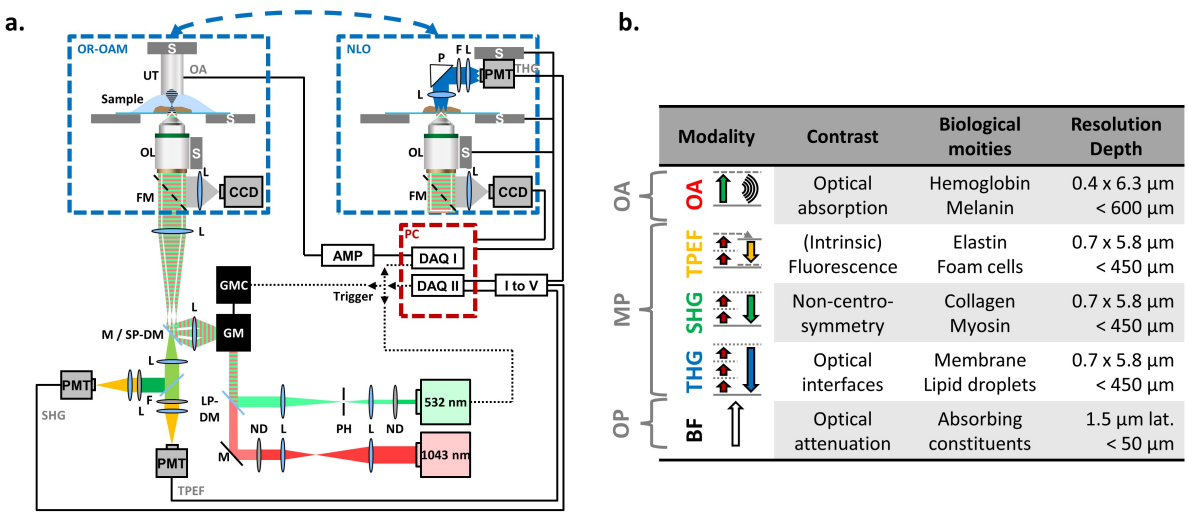

Figure 1 Schematic depiction of HyMPOM consisting of **(a)** two interchangeable microscopy systems, namely OR-OAM and MPM. Abbreviations: AMP, amplifier; DAQ, data acquisition card; F, optical filter; FM, flippable mirror; GM, galvanometric mirrors; GMC, GM control; L, lens; LP-DM, longpass dichroic mirror; M, mirror; ND, neutral density filter; OA, optoacoustic signal; OL, microscope objective lens; P, prism; PH, pinhole; PMT, photomultiplier tube; S, *xyz* stage; SHG, second harmonic generation signal; SP-DM, shortpass dichroic mirror; UT, ultrasound transducer; THG, third harmonic generation signal; TPEF, two-photon excitation fluorescence signal. **(b)** Overview of imaging capabilities of HyMPOM. Abbreviations: MP, multiphoton; OA, optoacoustic; OP, optical.

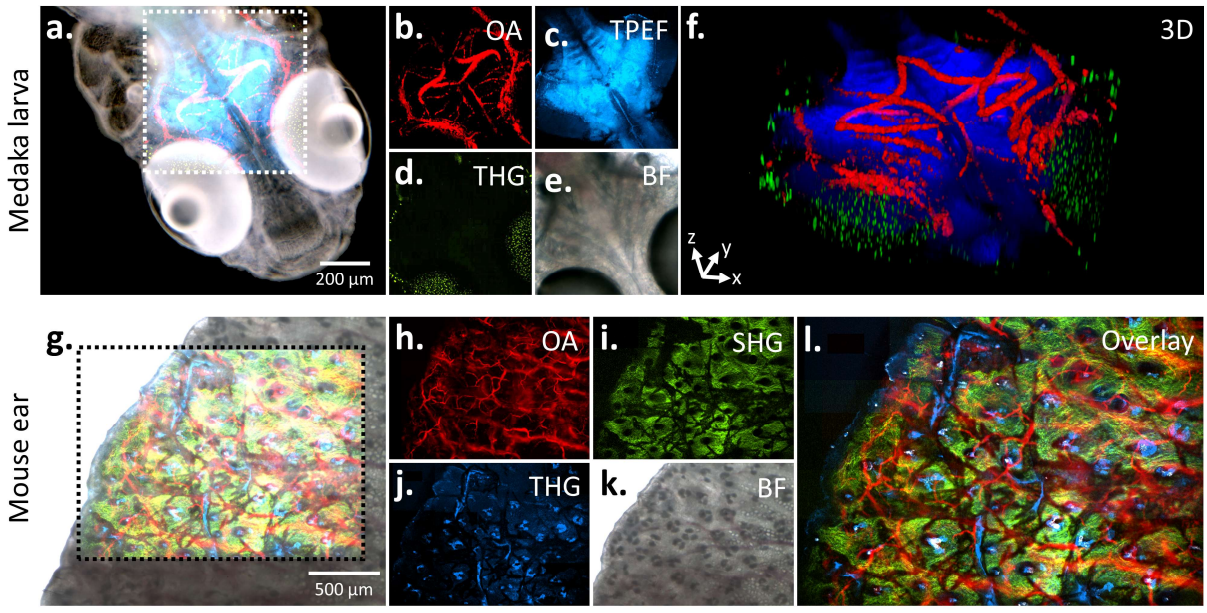

Figure 2. Hybrid optoacoustic and multiphoton microscopy (HyMPOM) imaging of *in vivo* medaka fish larva and mouse ear. Exemplary imaging capabilities of the hybrid microscopy to investigate living small model organisms. **(a)** Multimodal image blended onto the inverted brightfield image of a paralyzed 5-dpf medaka larva; separate depiction of **(b)** OA, **(c)** 2PEF, **(d)** THG, and **(e)** BF, as well as **(f)** a 3D rendering. **(g)** Multimodal image blended onto the brightfield image of the ear of an anesthetized mouse; separate depiction of **(h)** OA, **(i)** SHG, **(j)** THG, and **(k)** BF, as well as their **(j)** overlay. Abbreviations: BF, brightfield; OA, optoacoustic; SHG, second harmonic generation; THG, third harmonic generation; TPEF, two-photon excitation fluorescence.