Hybrid label-free multiphoton and optoacoustic microscopy (MPOM)

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ABSTRACT

Many biological applications require a simultaneous observation of different anatomical features. However, unless potentially harmful staining of the specimens is employed, individual microscopy techniques do generally not provide multi-contrast capabilities. We present a hybrid microscope integrating optoacoustic microscopy and multiphoton microscopy, including second-harmonic generation, into a single device. This combined multiphoton and optoacoustic microscope (MPOM) offers visualization of a broad range of structures by employing different contrast mechanisms and at the same time enables pure label-free imaging of biological systems. We investigate the relative performance of the two microscopy modalities and demonstrate their multi-contrast abilities through the label-free imaging of a zebrafish larva *ex vivo*, simultaneously visualizing muscles and pigments. This hybrid microscopy application bears great potential for developmental biology studies, enabling more comprehensive information to be obtained from biological specimens without the necessity of staining.

Keywords: Microscopy, Nonlinear microscopy, Photoacoustic imaging, Multimodal imaging, Label-free imaging

1. INTRODUCTION

The recent breakthrough discoveries and the high impact applications generated through contemporary biomedical research have triggered the need for more sophisticated imaging methods. Current optical microscopy approaches based on different contrast mechanisms have played a significant role in terms of obtaining structural and functional information in biological specimens at the cellular and sub-cellular level. However, a comprehensive understanding of biological systems requires simultaneous observation of different anatomical structures [1], a feature that is usually not provided by a single label-free imaging modality. Therefore, the integration of several microscopy modalities into a single hybrid device has the potential to provide complementary contrast over a wide range of biological structures, thus enhancing the understanding of complex biological properties [2]. Furthermore, most of the commonly employed microscopy techniques rely on staining of the imaged specimens. However, artificially added fluorescent molecules can interfere with physiological functions [3] or can be even toxic for biological microenvironments (phototoxicity) [4].

Herein, we report on the development of an integrated multiphoton and optoacoustic microscope (MPOM) [5], which provides label-free multi-contrast imaging of biological samples. Multiphoton microscopy represents a powerful modality, offering optical-diffraction limited resolution at an increased imaging depth compared to other fluorescence microscopy techniques. Furthermore, the utilization of Second Harmonic Generation (SHG) contrast enables label-free imaging of birefringent biological structures, such as collagen [6] and myosin [7]. On the other hand, Optoacoustic Microscopy (OM) visualizes optical absorption of endogenous molecules including hemoglobin and melanin by measuring ultrasonic waves that are induced by tightly focused pulsed laser radiation [8]. We characterize the relative performance of the two integrated microscopy modalities and investigate their multi-contrast capabilities by the label-free imaging of a zebrafish larva *ex vivo*.

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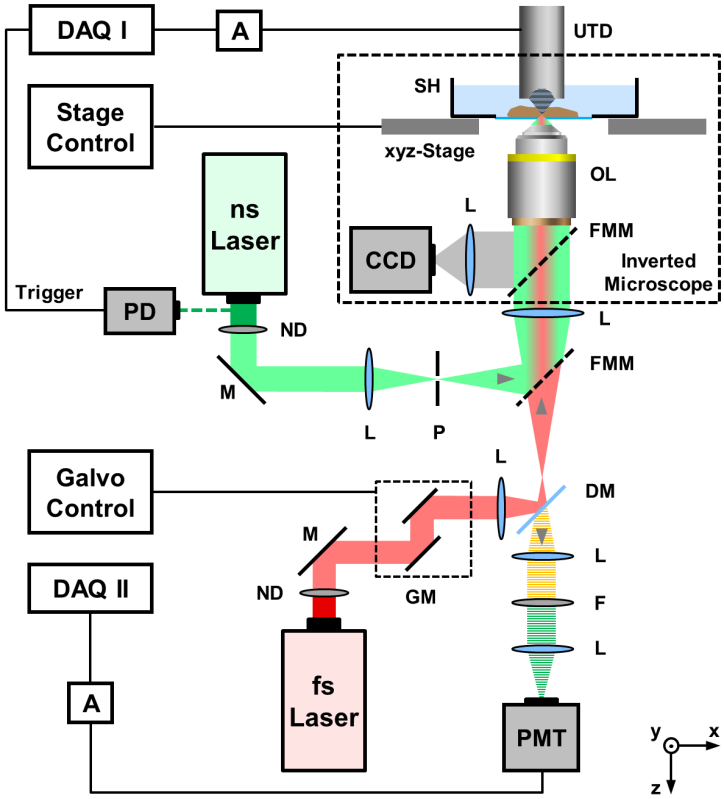

Figure 1. Schematic of the MPOM setup. (ND) Neutral density filter. (L) Lens. (M) Mirror. (P) 25 µm pinhole. (FMM) Flip-mount mirror. (PD) Photo diode. (GM) Galvanometric mirror set. (DM) Dichroic mirror. (SH) Sample holder. (F) Optical filter. (PMT) Photomultiplier tube. (OL) Objective. (UTD) Ultrasound transducer. (A) Amplifier. (DAQ) Data acquisition card.

2. METHODS

2.1 Experimental setup

Figure 1 illustrates the custom-designed MPOM system, incorporating multiphoton and optoacoustic microscopy. Two different laser sources are used for the different modalities, which are both coupled into a modified inverted microscope (AxioObserver.D1, Zeiss, Jena, Germany) to irradiate the sample.

For two-photon (TPEF) and SHG microscopy, a femtosecond laser (YBIX, Time-Bandwidth, Zurich, Switzerland; pulse repetition rate: 84.4 MHz; pulse width: 170 fs; average output power: 2.8 W) is used for non-linear excitation of the sample at 1043 nm. After attenuation, the laser beam is guided onto a set of galvanometric mirrors (6215 H, Cambridge Technology, Bedford, Massachusetts, USA), which performs raster scanning of the beam at the xy-plane within the sample. Afterwards, the excitation beam is reflected from a dichroic mirror, expanded by a pair of lenses and tightly focused into the specimen by a high NA objective (Plan Apochromat 20X, Zeiss, Jena, Germany; numerical aperture: 0.8; air immersion). The positioning of the focal plane in the sample along the z-direction is achieved by means of a high-precision motorized piezo-stage (MZS500-E, Thorlabs). A motorized stage (MLS203-2, Thorlabs), which is mounted to the z-stage, is used in order to move and position the sample in the xy-direction. The non-linear radiation generated at the optical focus (TPEF or SHG) is collected in back-reflection mode through the objective. The dichroic mirror is transparent in the visible range and transmits the emitted light, whereas and an optical bandpass filter is employed for wavelength selection. The non-linear signals are detected by a photomultiplier tube (H9305-03, Hamamatsu, Hamamatsu City, Japan), amplified and subsequently recorded by a digitizer card (PCIe-6363, National Instruments, Austin, Texas, USA; vertical resolution: 16-bit).

In case of optoacoustic microscopy, the excitation source is a pulsed solid-state laser (Flare HP PQ Green 2 k-500, Innolight GmbH, Hannover, Germany; energy per pulse: 570 μJ; repetition rate: 1.2 kHz) with 515 nm wavelength and 1.8 ns pulse duration. The laser beam is attenuated and spatially filtered by a pinhole for proper focusing. In order to fill the back aperture of the employed objective (PLN 10X, Olympus, Hamburg, Germany, numerical aperture: 0.25) the beam is expanded and coupled into the inverted microscope by means of a flip mount mirror. The acoustic signals that are generated mainly in the focus region of the optical illumination are detected by a spherically focused ultrasound transducer (SONAXIS, Besancon, France; central frequency: 78 MHz). Detector and illumination are aligned coaxially and confocally with respect to each other for SNR maximization. The optoacoustic signals are amplified (AU 1291, Miteq, New York, USA; gain: 63 dB) and recorded by a fast 8-bit digitizer card (Cobra Max CS23G8, Gage Applied, Lockport, USA; max. sampling rate per channel: 1.5 GS/s). Reflected light at the laser output is detected by a photo diode (DET36A, Thorlabs) and provides the trigger signal for the data acquisition. Scanning of the sample is achieved by translating the sample holder in discrete steps via the xy-stage, while illumination and transducer are kept stationary. The samples are placed on a thin glass slide, fixed with plastic foil and coupled to the transducer with a drop of water.

A CCD camera (AxioCam ICc 1, Zeiss, Jena, Germany) is utilized for the brightfield imaging of the specimen. Synchronization of the scanning devices and data acquisition are controlled through LabVIEW for multiphoton microscopy and via Matlab in case of optoacoustic microscopy.

2.2 Image processing

The optoacoustic signals were bandpass filtered between 25 MHz and 125 MHz in order to reject noise and cropped around the focus region to discard out-of-focus signals. Finally, the Hilbert transform was used to calculate the envelopes of the signals in order to restore the axial extent of the imaged structures.

The images of both modalities were processed and co-registered with imageJ. Standard image processing operations were applied homogeneously to the entire images, including windowing, interpolation, Gaussian blurring and median filtering.

2.3 Spatial resolution characterization

The spatial resolution of the multiphoton microscope was characterized by measuring fluorescent nanobeads with 100 nm diameter (TetraSpeck Fluorescent Microspheres Size Kit, Invitrogen, Carlsbad, California, USA). Because the beads acted as point-like signal sources compared to the achievable resolution of the multiphoton modality, the 3D measurement of a single bead directly represented the 3D point spread function (PSF) of the system.

For the optoacoustic microscopy modality, the lateral resolution was estimated by imaging black 2.8 μm polystyrene microspheres (Polybead, Polysciences Inc., Warrington, Pennsylvania). The microspheres were embedded in pure agar for fixation purposes. A single microsphere was imaged in steps of 0.2 μm. The signals were averaged five times for SNR improvement. Since in this case, the sphere size is comparable to the achievable spatial resolution of the optoacoustic modality, the PSF could not be directly obtained from a single measurement. In order to derive the lateral resolution, we assumed a Gaussian shape for the real cross-sectional profile of the sphere, where the nominal diameter of 2.8 μm corresponds to ±3σ of the Gaussian. We further assumed that the measurement process can be described by a convolution of the real microsphere profile with the Gaussian shaped beam waist of the laser focus. The lateral resolution R_{OM} could therefore be estimated by using the equation

$$R_{OM} = \sqrt{d_{\exp}^2 - \left(\frac{2}{6}\sqrt{2\ln(2)}\, d_{nom}\right)^2}, \qquad (1)$$

where d_{exp} is the full width at half maximum (FWHM) of the measured microsphere profile and d_{nom} the nominal diameter of the sphere.

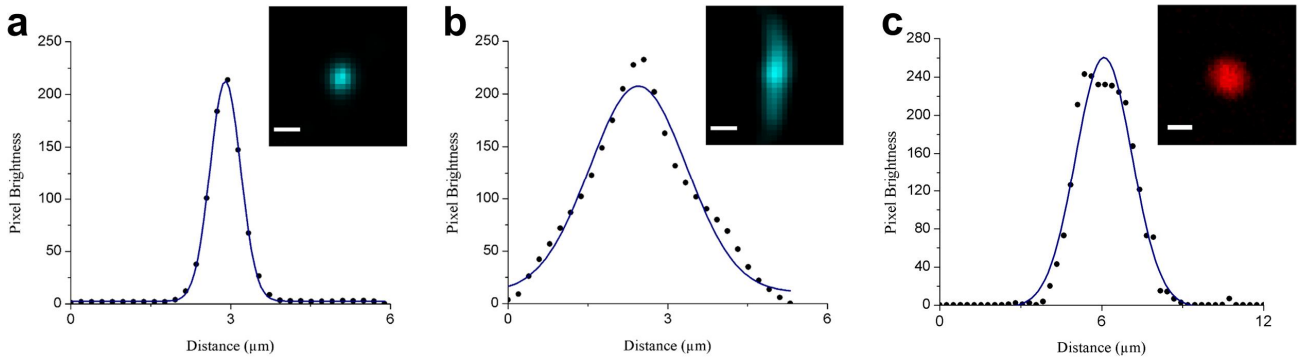

Figure 2. Spatial resolution characterization of the MPOM modalities. (a,b) Multiphoton microscope. The graphs show the lateral (a) and axial (b) profiles of a single 100 nm fluorescent bead, fitted with Gaussian curves (blue). The insets illustrate lateral and axial views of the nanobead, respectively. Scale bars: 1 μm. (c) Optoacoustic Microscope. The blue curve represents the Gaussian fit of the measured profile of a 2.8 μm microsphere. The inset shows the corresponding top view of the sphere, represented by a maximum amplitude projection (MAP) along the z-axis. Scale bar: 2 μm.

2.4 Hybrid zebrafish imaging

We studied the multi-contrast imaging capability of the hybrid microscope by imaging an 11-days-old zebrafish larva (huC::GCamP5G) *ex vivo*. The SHG and OM images were obtained from the same 200 μm × 200 μm of the fish body. The optoacoustic scan was performed within a single plane with a 2 μm step size and five times signal averaging. The pulse energy at the specimen was measured to be ~6 nJ. For the multiphoton scan, the pulse energy at the focal plane was reduced to ~0.8 nJ, while at each z-position, 5 frames were averaged for SNR improvement. Additionally, a brightfield image was captured by the CCD camera as a reference. Saturated pixels were removed from the SHG image to improve the visibility.

3. RESULTS

3.1 Spatial resolution characterization

The curves in Figure 2(a,b) show the Gaussian fitted lateral and axial profiles of the nanobead measured by recording TPEF signals via the multiphoton microscope. From the FWHM of the fits, the lateral resolution of the multiphoton modality was determined to be ~674 nm, whereas the axial resolution was ~2.15 μm. Top and side views of the nanobead are provided by the insets in Figure 2(a,b), which correspond to lateral and axial projections of the system's PSF, respectively.

Figure 2(c) illustrates the lateral profile of the microsphere imaged with the OM system. From the FWHM of the fit and the evaluation of Equation (1), the lateral resolution of the OM modality was calculated to be ~2.2 μm. The inset shows the maximum amplitude projection (MAP) of the imaged sphere along the z-dimension. On the other hand, the axial resolution of the optoacoustic system depends on the detection characteristics of the transducer and was previously measured to be ~7 μm in [9].

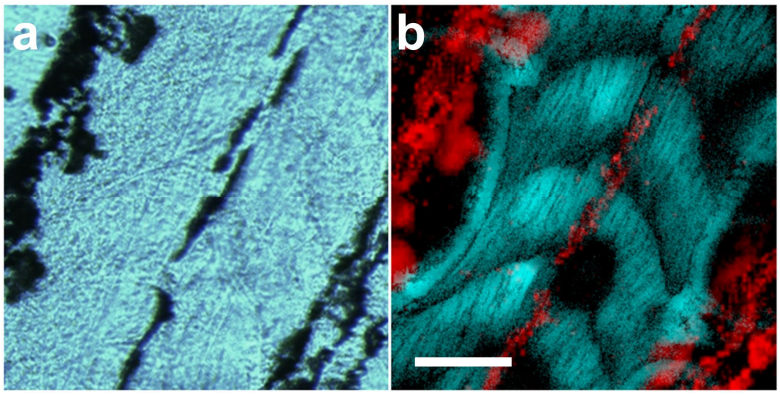

Figure 3. Hybrid imaging of a zebrafish larva tail *ex vivo*. (a) Brightfield image of a 200 µm × 200 µm region of the zebrafish body. (b) Hybrid image of the same region showing melanocyte stripes (red) measured with the optoacoustic microscope and the fish musculature (cyan) imaged with the multiphoton microscope by recording SHG signals. Scale bar: 50 µm.

3.2 Hybrid zebrafish imaging

Figure 3(a) represents the brightfield image of the selected region at the fish body, showing the pigmented lateral and central stripes of the fish. An overlay of the SHG and OM measurements of the same region is provided by Figure 3(b). The SHG signals are shown in cyan, whereas the red color represents the MAP of the optoacoustic scan. The optoacoustic signals originate from the strongly absorbing melanocytes of the lateral and central stripes of the fish tail and match with the pigment patterns shown in the brightfield image. On the other hand, the SHG signals obtained by the multiphoton microscope were generated through strong non-linear responses in the fish muscles. Consequently, the SHG image visualizes the musculature of the zebrafish, whereas even single myofibrils can be distinguished.

4. DISCUSSION

In summary, the developed hybrid multiphoton and optoacoustic microscope (MPOM) presented in this work demonstrates promising label-free imaging capabilities and contrast complementarity. The recorded multimodal zebrafish image successfully illustrates the capabilities of the MPOM system to simultaneously visualize distinct biological structures in unstained specimens with high contrast. This technique could prove useful for biological examinations that require concurrent label-free visualization of different anatomical features in biological specimens, such as developmental biology studies. The range of detectable molecules can be readily extended by employing exogenous contrast agents, such as fluorescent molecules, gold nanorods or other nanoparticles. A future implementation could be the addition of different excitation wavelengths to enable the differentiation of other tissue structures and extraction of functional parameters such as tissue oxygenation based on distinct characteristics of the respective absorption spectra. Furthermore, the integration of Third Harmonic Generation (THG) microscopy, which uses the same laser as the other multiphoton modalities, could provide access to the label-free imaging of different structures, including lipid droplets or cell membranes.

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REFERENCES

[1] Keller, P. J., "Imaging Morphogenesis: Technological Advances and Biological Insights," Science 340(6137), 1184-1194 (2013).

[2] Vinegoni, C., Ralston, T., Tan, W., Luo, W., Marks, D. L. and Boppart, S. A., "Integrated structural and functional optical imaging combining spectral-domain optical coherence and multiphoton microscopy," Appl. Phys. Lett. 88(5), 053901 (2006).

[3] Zhang, X. and Kiechle, F.L., "Hoechst 33342 alters luciferase gene expression in transfected BC3H-1 myocytes," Arch. Pathol. Lab. Med. 127(9), 1124-1132 (2003).

[4] Ouédraogo, G. D. and Redmond, R. W., "Secondary Reactive Oxygen Species Extend the Range of Photosensitization Effects in Cells: DNA Damage Produced Via Initial Membrane Photosensitization," Photochem. Photobiol 77(2), 192-203 (2003).

[5] Tserevelakis, G. J., Soliman, D., Omar, M. and Ntziachristos, V., "Hybrid multiphoton and optoacoustic microscope," Opt. Lett. 39(7), 1819-1822 (2014)

[6] Cicchi, R., Vogler, N., Kapsokalyvas, D., Dietzek, B., Popp, J., and Pavone, F. S., "From molecular structure to tissue architecture: collagen organization probed by SHG microscopy," J. Biomed. Opt. 6(2), 129-142 (2013).

[7] Campagnola, P. J., Millard, A. C., Terasaki, M., Hoppe, P. E., Malone, C. J., and Mohler, W. A., "Three-dimensional high-resolution second-harmonic generation imaging of endogenous structural proteins in biological tissues," Biophys. J. 82(1), 493-508 (2002).

[8] Ntziachristos, V., "Going deeper than microscopy: The optical imaging frontier in biology," Nat. Methods 7(8), 603-614 (2010).

[9] Omar, M., Gateau, J. and Ntziachristos, V., "Raster-scan optoacoustic mesoscopy in the 25-125 MHz range," Opt. Lett. 38(14), 2472-2474 (2013).