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Research article

Simultaneous ultra-high frequency photoacoustic microscopy and photoacoustic radiometry of zebrafish larvae *in vivo*

Michael J. Moore[a,b,c], Suzan El-Rass[c,d,e], Yongliang Xiao[c,d,e], Youdong Wang[c,d,e], Xiao-Yan Wen[b,c,d,e], Michael C. Kolios[a,b,c,*]

[a] Department of Physics, Ryerson University, Toronto, M5B 2K3, Canada
[b] Institute for Biomedical Engineering and Science Technology, A Partnership Between Ryerson University and St. Michael's Hospital, Toronto, M5B 1W8, Canada
[c] Keenan Research Center for Biomedical Science, Li Ka Shing Knowledge Institute, St Michael's Hospital, Toronto, M5B 1W8, Canada
[d] Zebrafish Centre for Advanced Drug Discovery, Li Ka Shing Knowledge Institute, St Michael's Hospital, Toronto, M5B 1W8, Canada
[e] Institute of Medical Science, Departments of Medicine, Laboratory Medicine and Pathobiology & Physiology, University of Toronto, Toronto, M5S 1A1, Canada

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ABSTRACT

With their optically transparent appearance, zebrafish larvae are readily imaged with optical-resolution photoacoustic (PA) microscopy (OR-PAM). Previous OR-PAM studies have mapped endogenous chromophores (e.g. melanin and hemoglobin) within larvae; however, anatomical features cannot be imaged with OR-PAM alone due to insufficient optical absorption. We have previously reported on the photoacoustic radiometry (PAR) technique, which can be used simultaneously with OR-PAM to generate images dependent upon the optical attenuation properties of a sample. Here we demonstrate application of the duplex PAR/PA technique for label-free imaging of the anatomy and vasculature of zebrafish larvae in vivo at 200 and 400 MHz ultrasound detection frequencies. We then use the technique to assess the effects of anti-angiogenic drugs on the development of the larval vasculature. Our results demonstrate the effectiveness of simultaneous PAR/PA for acquiring anatomical images of optically transparent samples in vivo, and its potential applications in assessing drug efficacy and embryonic development.

1. Introduction

In recent years, there has been a rapid increase in the use of zebrafish as specimens for biomedical research [1]. The rapid adoption of zebrafish as an in vivo biological model has been facilitated by several factors, including their low cost, high fecundity, and short maturation time [2,3]. As small animal models, zebrafish have been used in studies investigating the progression of disease [4], drug screening and discovery [1,5], and embryonic development [5–7]. While adult wild-type zebrafish exhibit a characteristic striped patterning dictated by the three variants of pigmentation cells in their bodies (melanophores, xanthophores, and iridophores) [8,9], the larval fish are largely transparent, and their internal structures are easily viewed using conventional microscopy techniques. To further enhance optical clarity, chemicals such as 1phenyl 2-thiourea (PTU) can be added early in the developmental cycle to inhibit melanophore production [10], or mutant fish which express limited pigmentation into adulthood are used [11].

Coupled with high optical transparency and abundant endogenous chromophores (i.e. melanin and hemoglobin) zebrafish larvae are also excellent candidates for imaging with photoacoustic microscopy (PAM). Conventional transmission-mode optical-resolution photoacoustic microscopy (OR-PAM) approaches have been used to image the eye, vascular system, and heart [12] as well as cardio-cerebrovascular development [13] in vivo in pigment-supressed zebrafish larvae. Other techniques, such as spatial resolution-invariant PAM (SIRPAM) have been used to acquire whole-body images of pigmentation in 3 day-post-fertilization (dpf) larvae over a lateral resolution-invariant axial range of 1.8 mm [14]. Multiview imaging techniques have also been employed in both PAM [15] and photoacoustic mesoscopy [16] setups to generate images with more isotropic resolution; however, these studies have only been performed with *ex vivo* specimens.

While PAM techniques yield high resolution maps of endogenous absorption, they contain no information pertaining to the gross anatomical tissue of the zebrafish, which has negligible absorption at illumination wavelengths typically used in PAM. For this reason, recent work has focused on creating hybrid systems combining several types of imaging modalities (e.g. as is commonly done with fluorescence and brightfield microscopy) with PAM to provide additional context for the

* Corresponding author at: Department of Physics, Ryerson University, Toronto, M5B 2K3, Canada.
E-mail address: mkolios@ryerson.ca (M.C. Kolios).

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acquired images. Rao et al. used an integrated photoacoustic, confocal, and two-photon microscope to acquire confocal fluorescence images of the spinal cord and posterior lateral line nerve as well as PAM images of vasculature in vivo in a transgenic larvae [17]. Soliman et al. used an integrated multi-photon and multi-scale photoacoustic microscope to acquire label free OR-PAM, as well as brightfield, and second, and third harmonic generation microscopy images of a 6 dpf wild-type zebrafish larvae [18]. The hybrid system was able to resolve melanin spots with PAM, as well as individual muscle fibrils and connective tissue within the trunk of the embryo with second and third harmonic generation microscopy, respectively. Finally, PAM systems incorporating optical coherence tomography detection have been used to image features including the larval eye, yolk, and swim bladder [19]. While each of these systems is capable of producing striking composite images depicting several unique anatomical features, each system requires specialized optical setups and the use of more than one laser. Furthermore, due to the different illumination pathways employed, no system was capable of simultaneous image acquisition.

We recently reported on a technique, termed photoacoustic radiometry (PAR), that can be used to simultaneously generate PA images as well as optical attenuation images using conventional transmission style OR-PAM [20,21]. In brief, in PAR imaging each laser pulse that is used to generate a PA signal is attenuated as it propagates through the sample. The attenuated optical pulse then impinges upon metallic components of the ultrasound (US) transducer - generating an additional PA signal within it that can be gated and used to reconstruct images. The amplitude of the PAR signal is directly proportional to the amount of light which is transmitted through the sample and incident on the transducer [20,21]. Attenuation of the laser beam due to both optical absorption and scattering decreases the signal amplitude, creating unique contrast in the resultant PAR images. Furthermore, since both PAR and PA signals are generated from a single laser pulse, both signals are acquired in the same RF-line and yield simultaneous co-registered images.

In this work, we demonstrate simultaneous label-free PAR and PA imaging of live zebrafish larvae. PAR images reveal structural features of the gross anatomy, while volumetric PA images depicted single red blood cells (RBC) and the vascular morphology. When merged, the PAR images provide useful landmarks for identifying the vasculature in the PA image. Finally, we applied the technique to study the effects of an anti-angiogenic drug that hinders the development of larval vasculature.

2. Methods

2.1. System setup

A schematic of the experimental system is shown in Fig. 1. A SASAM (SAarland Scanning Acoustic Microscope) photoacoustic microscope (Kibero, Saarbruken) built on an Olympus IX-81 microscope modified to include a pulsed 532 nm laser with pulse repetition frequency (PRF) of 4 kHz and pulse width of 330 ps (TeemPhotonics, France) was used for sample scanning. The laser was directed through a variable optical density neutral density (ND) filter and coupled into a single-mode fiber with a 2.5 μm core diameter and NA of 0.13 (Coastal Connections, USA). The collimated laser beam at the fiber output was spatially filtered by an iris and passed through a second ND filter. A portion of the beam was sampled using a 70/30 (T/R) beamsplitter (Thorlabs, USA) and directed to a joulemeter for pulse-to-pulse energy measurement (Gentec-EO, Canada). The transmitted portion of the beam was directed through the right side port of the SASAM and reflected off a dichroic mirror (Chroma Technology Corp., USA) housed in the IX-81 fluorescence cube turret. It was then focused through a 4X optical objective with a NA of 0.1 (Olympus, Japan) onto the sample. The laser pulse energy after the objective was approximately 50 nJ. The profile of the laser beam was measured using PAR with a knife-edge technique (Supplementary Fig. 1), and the full-width at half-maximum (FWHM) of the beam at the focal spot was found to be 5.3 μm. Fluorescence images of the larvae were acquired by rotating the turret to a fluorescence cube with excitation and emission wavelengths of 480 nm and 520 nm, respectively, and using a CCD camera affixed to the IX-81 left side port for image acquisition. Two different single element transducers were used in this work: a 200 MHz transducer with -6 dB bandwidth of 120 MHz, and a 400 MHz transducer with a -6 dB bandwidth of 180 MHz. The lateral/axial resolution of the transducers were calculated to be 8 μm/11 μm for the 200 MHz transducer, and 4 μm/7.5 μm for the 400 MHz transducer. The zebrafish larvae were placed on a motorized sample stage (Marzhauser Wetzlar, Germany), and scanned through the overlapping laser/transducer focal spots in a raster pattern. The step size in both the fast and slow scanning directions was 2 μm. PAR signals generated within the transducer and PA signals arriving from the sample were acquired in the same RF-line. Acquired RF-lines were digitized at a rate of 8 Gs/s using a 10 bit digitizer (Acquiris, USA), and were averaged 150 times to increase the SNR.

2.2. Preparation of zebrafish larvae

The zebrafish were raised using the techniques outlined in [22]. For the present study, two variants of zebrafish larvae were used: the transgenic *Tg* (*flk1*:GFP) line, which exhibits green fluorescent protein (GFP) expressing endothelial cells [23]; and the mutant *casper* line, which is largely devoid of melanophores and iridophores [11]. All zebrafish strains were housed and maintained under standard husbandry conditions [3]. To prevent excess melanin production in the *Tg* (*flk1*:GFP) fish, the embryos were treated with 200 μM of PTU [10] at 10 h post fertilization (hpf). For fish treated with the anti-angiogenic drug indirubin-3′-monoxime (I3M, or IRO), 4 μM was added to the zebrafish egg water at 8 hpf. All zebrafish experiments were conducted in accordance with St. Michael's Hospital Animal Care Committee approved protocol ACC403.

A glass-bottom petri dish (MatTek, USA) was filled with 300 μL of molten 1.5% low melting point agarose (Sigma, USA) at 40 °C. The agarose was allowed to set in a 4 °C fridge for 15 min. The zebrafish larvae were anesthetized using a 0.003% (w/v) solution of tricane (Sigma, USA), and pipetted onto the agarose filled petri dishes. Excess egg water was aspirated, and the fish was covered with 20 μL of the molten 40 °C agarose. The dishes containing larvae were left to set at room temperature for 30 min prior to imaging.

2.3. Image formation

The acquired 3D scan datasets were time gated to isolate the PAR and PA signals. The signal envelope for each RF-line was computed, and maximum amplitude projection (MAP) images were generated separately for both the PAR and PA data. For display purposes, the MAP images were smoothed using a 2D Gaussian smoothing filter and interpolated. Prior to smoothing, the PA MAP images from scans of *Tg* (*flk1*:GFP) fish were log compressed, thresholded, and a dilate mask (disk structuring element, radius 1 pixel) was applied to enhance visualization of the vasculature.

3. Results & discussion

3.1. In vivo imaging of transgenic zebrafish

To our best knowledge, there has been no direct comparison of PA images of the developing zebrafish vasculature with fluorescence microscopy images of the vasculature from the same larva. Towards this end, we acquired fluorescence and PAM images of the same 4 dpf *Tg* (*flk1*:GFP) zebrafish larvae. Fig. 2a shows the GFP expressing endothelial cells in the larval zebrafish trunk. The vessels have been pseudo-coloured red to aid with comparison to the PA image. At this