Label-free imaging of zebrafish larvae in vivo by photoacoustic microscopy

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Abstract: Zebrafish play an important role in biological and biomedical research. Traditional in vivo imaging methods for studying zebrafish larvae primarily require fluorescence labeling. In this work, relying on tissue intrinsic optical absorption contrast, we acquired high resolution label-free 3D images of zebrafish larvae by using photoacoustic microscopy (PAM) in vivo. The spatial resolution reaches several microns, allowing the study of microstructures in various living organs. We demonstrated that our method has the potential to be a powerful non-invasive imaging method for studying various small animal models, including zebrafish larvae, Caenorhabditis elegans, frogs and drosophila larvae.

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1. Introduction

Zebrafish, having 87% homologous gene and similar early development with human, is considered to be an important animal model in comparative biology and diseases researches for vertebrates [1]. It has been used for a long time to study gene expression, nervous and circulatory systems, cardiovascular disease and cancer, and thus have received intense attention [2–4]. Although the zebrafish can live for 2–3 years and grow to 6 cm in length, many important research focus on its first several days after fertilization, when the body is partially optically transparent. This optical clarity allows multiple optical microscopic imaging modalities, such as confocal microscopy, to play important roles in the study of zebrafish. However, tissue intrinsic scattering characteristics all over the body lead to low contrast under optical microscopy. Thus, pure optical microscopy generally require fluorescence labeling or exogenous contrast agent [5–7], which has the potential to interfere natural physiological progresses. Besides various optical microscopic methods, the optical projection tomography (OPT) and the optical coherence tomography (OCT) have been also used for imaging of zebra fishes. Recently, a flow OPT has been developed to image the genetically pigmentation-free zebrafish [8] in vivo and without labeling, which took advantage of the movement of cells present in bloodstream. However, many tissues and organs have no prominent moving components. Relying on the light scattering properties of tissue, OCT is successfully used to image different tissues and moving scatterers in the zebrafish [9–11]. Similar to optical microscopic methods, both OPT and OCT methods are significantly affected by the background scattering signal. Thus a novel method less affected by tissue scattering and can provide high resolution, label-free and in vivo imaging for the study of various tissues in zebrafish larvae is highly desired.

Photoacoustic (PA) imaging is an emerging hybrid biomedical imaging method that combines optical contrast with ultrasonic detection [12–15]. Unlike other pure optical microscopic imaging modalities, PA imaging detects ultrasound signals generated through thermal expansion after the tissue absorbs the exciting laser energy. PA imaging is highly sensitive to tissue optical absorption contrast. Over the past decade, PA imaging has made significant progresses and been implemented into a vast range of biomedical research fields. Many modalities such as photoacoustic computed tomography (PACT) and photoacoustic microscopy (PAM) have been developed [15,16].

Adult zebra fishes have been imaged non-invasively using reconstruction-based PACT methods [16,17]. In this study, we implemented another method by using a high-resolution PAM method, optical-resolution photoacoustic microscopy (OR-PAM), to image microstructures in zebrafish larvae. The lateral and axial resolutions of OR-PAM are determined by optical focusing and time-resolved ultrasonic detection, respectively [18–20].
Although the imaging depth of OR-PAM is similar to that of pure optical microscopes, OR-PAM provides much higher imaging contrast for optically absorbing tissues [21]. In the following, we described our imaging system and the animal experiments. Our results demonstrated that high-contrast, whole-body, and label-free in vivo imaging of zebrafish larvae can be obtained by OR-PAM, and this technique has a great potential to study various embryos or larvae of other animal models.

2. Methods and imaging system

The experimental setup of our imaging system is shown in Fig. 1. It employed a transmission mode configuration, where the light source was under the sample and the ultrasonic detection was set above the sample. Light illumination and ultrasonic detection were aligned confocally and coaxially. The irradiation laser pulses (532 nm) generated by a pulsed laser system (Elforlight SPOT-10-100-532, pulse width < 1.8 ns) was coupled into a single-mode fiber, then focused by a 4X objective lens (GCO-2111, China Daheng Group, Inc., NA: 0.1). The laser pulse energy reaching the object surface was measured to be 40 nJ. A LiNbO₃ focused transducer (focal length: 4.5 mm, center frequency: 40 MHz, NA: 0.33, fabricated at University Southern California, Los Angeles, CA) was used to detect PA signal. The ultrasonic signal was amplified by two amplifiers (Mini-Circuits ZFL-500), then recorded by a 14-bit high speed acquisition card (GAGE CompuScope 14200, sampling rate: 200 MS/s). During the imaging process, the culture dish holding zebrafish was fixed while the lens tube and transducer performed a 2D raster scanning without signal averaging.

The resolution of the system was measured by imaging a thin layer of diluted carbon nanoparticles (particle diameter < 100 nm) on a glass slide. Figure 2 shows the lateral and axial cross-section profile of a nanoparticle by PAM, respectively. According to the full-width
half-maximum (FWHM) of the Gaussian fitting results, the lateral and axial resolutions of the system within the focal zone of the illumination light are estimated to be 6 μm and 32 μm, respectively. The phase distortion in the optical lens and glass slide caused the lateral resolution to be lower than its diffraction limit set by the lens. The axial resolution is determined by the bandwidth of the transducer (~110% in receiving-only mode), which can be further improved by using a transducer with higher center frequency.

3. In vivo animal experiments

We raised the wild type zebrafish embryos in E3 water at 28°C, and added PTU (0.003% 1-phenyl-2-thiourea in 10% Hank's saline) to the medium ~24 hours post fertilization to prevent pigmentation. Several pigment-null zebrafish embryos aged between 3 - 4 days post fertilization (dpf) were imaged. Before each experiment, zebrafish larvae were placed in a culture dish previously coated with 1% Agarose (l. m. p.) and containing a small amount of E3 water. The fish were then lightly covered with 1 or 2 drops of melted Agarose, and oriented so that they were lying on their sides (sagittal view) as the Agarose cooled and solidified. The Agarose minimized the movement of the zebrafish larvae during the experiments, while keeping them alive.

Figure 3 shows a comparison between a PAM image (Fig. 3(a)) and pure optical image by optical microscope (Fig. 3(b)) and OCT (Fig. 3(c)). The distribution of pigments, structure of the eye, blood vessels, and the heart can be clearly seen in the PAM image without the aid of exogenous labeling. The boundary of the heart appears less focused due to the beating motion caused by the live heart during scanning. An animation to show the 3D reconstruction result of Fig. 3(a) is also provided online (Media 1). Comparison of Figs. 3(a) and 3(b) clearly shows that it is more challenging to recognize the circulatory system in the optical photograph due to its much less sensitivity of the tissue optical absorption. Our imaging results coincide nicely with previously published drawings of the vascular anatomy of a 3.5 dpf zebrafish larva [22]. Nevertheless, there are several small vessels overlapped with the eye that cannot be readily distinguished due to the presence of pigments in the brain and eyes, whose signal had one to two orders of magnitude stronger than that from the small blood vessels. The signal-to-noise ratio of the dorsal aorta or large pigments is about 30 dB, but only 6 dB for several small intersegmental vessels. Thus, in Fig. 3(a) we used the logarithmic value of the original maximum amplitude projection (MAP) data in order to demonstrate both large and small vessels and pigments. Figure 3(c) is a slice image from a 3D OCT result of a 3 dpf zebrafish larva [10]. By comparison between Figs. 3(a) and 3(c), PAM has a much less background
signal since it is less affected by tissue light scattering. However, OCT reveals more various organ structures, such as the ventricle, liver and spinal cord.

A series of 4 dpf zebrafish image slices were taken at different depths (50, 125, 200, and 275 μm) to demonstrate the capability of 3D imaging, as shown in Fig. 4(a). Due to the anatomical symmetry, only slices showing one half of the fish are provided, although the total depth of the field of view can reach more than 400 μm for our system. Figure 4(b) shows a 3D image of the zebrafish (created by VolView software, Kitware), and its 3D animation is available online (Media 2). This process allows the separation of overlapping vessels in MAP images, such as intersegmental vessels seen in Fig. 3a. For example, the two intersegmental vessels can be spatially separated and readily appreciated in Fig. 4(b). Due to the relative less axial resolution, some fine microvasculatures parallel to the illumination direction have not been distinguished, such as the bifurcation pattern of the dorsal longitudinal anastomotic vessels. A higher frequency ultrasonic transducer is needed to improve the axial resolution. The larva continued to live for more than two days after experiments, and microscopic

![Fig. 4. 3D PAM image. (a) Imaging slices at different depths. (b) 3D image of the zebrafish larva (Media 2).](image)

![Fig. 5. Imaging an eye of a 3 dpf zebrafish larva. (a) Imaging result using an optical microscope. (b) 3D image of the zebrafish larva by PAM (Media 3). (c) Demonstrated histology image of the eye of a 3 dpf zebrafish larva [23], (from http://zfatlas.psu.edu/view.php?w=494&x=2&c=1460.552&atlas=21, which is supported by NIH grant 5R24 RR01744, Jake Gittlen Cancer Research Foundation, and PA Tobacco Settlement Fund). (d) A lateral cross-section slice of the PAM 3D image in (b). The scale bar is 100 μm.](image)
examination showed that there was no observable damage due to the exposure of laser light or the methodology. Nevertheless, potential laser damage to eye needs further investigation. In addition, owing to the very strong PA signal from eye, the laser power can be significantly reduced when scanning that area.

Besides the microcirculation system, 3D PAM imaging provides more micro-structural information of other organs in the larva. Figure 5 gives the imaging result of one 3 dpf zebrafish eye by PAM, as well as its optical microscopic and histology counterpart. There is a hole indicating the location of lens as shown in the 3D view of the eye (Fig. 5(b)). It is because the optically transparent lens does not absorb the illumination light at 532 nm, and thus generates much less PA signals. However, retinal pigment epithelium (RPE) is intrinsically highly optical-absorbing, generating strong PA signals. Therefore, RPE in PAM images express bright color, opposite to its optically microscopic counterpart in Fig. 5(a). It can be visualized more readily in the online animation (Media 3) for Fig. 5(b). The “broken edge” in Figs. 5(b) and 5(d) are due to the shadowing effect by other strong light absorbers between the light source and the RPE tissue. In Fig. 5(d), we also provided a cross section of the 3D PAM image to demonstrate the inner structure of eye. Multi-layered structure can be found in the PAM image, which probably represents the plexiform layers as in the histology result, as shown in Fig. 5(c) (a sagittal histology slide).

4. Discussions and conclusion

We successfully obtained high-resolution and high-contrast images of living zebrafish larva, including blood vessels, pigments and eyes, based on their intrinsic contrast. This work demonstrates PAM has a great potential in the study of zebrafish animal models. Without the multiple wavelengths’ laser, we currently cannot differentiate various tissue components, such as melanin, lipid, or water, spectroscopic PA imaging based on their spectral-dependent absorption characteristics. Moreover, spectroscopic PA imaging also enables functional PA imaging, such as the mapping of oxygen saturation in the circulatory system. Functional imaging will be a focus of our future work. For imaging speed, it took 30 to 40 minutes to finish a whole-body scanning of a 3.5 dpf larva (~0.7 mm wide and ~2.7 mm long, with a step size of 2.5 μm), and this limitation is primarily set by the mechanical scanning speed of the translational stage. The imaging speed can be improved by either using a faster stage, such as the one based on a voice coil [24], or using an optical scanning method [25].

In addition, our results do contain some artifacts, which are primarily due to the ultrasonic reflection from organs. Optical shadowing effect also exists, which is caused by the presence of strong absorbers in front of another absorber along the illumination path. In this condition, the PA signals from targets behind a strong optical absorber can be significantly reduced. Both artifacts and shadowing effect need further investigation.

PA imaging has limitations in imaging tissues with less optical absorption (such as the cornea, lens, and lymphatic system), and organs with air cavities (such as the lung). Combining PA imaging with other imaging modalities, such as OCT, can provide complementary information. Recently, OCT and PA imaging have been combined successfully [25,26] in in vivo animal studies, and we expect this multimodal imaging method to be used for the study of animal larvae in future.

Overall, we present a novel method to study zebrafish larva that relies on intrinsic optical absorption contrast without labeling. However, PA imaging is by no means exclusive to exogenous contrast agent, such as highly optically-absorbing dyes and nanoparticles, which will be used if necessary. Our method can be implemented in a straightforward manner to study various animal models, such as C. elegans and larvae of frogs and drosophila.

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