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Non-invasive whole-body imaging of adult zebrafish with optoacoustic tomography

Rui Ma, Martin Distel, X Luís Deán-Ben, Vasilis Ntziachristos and Daniel Razansky

Institute for Biological and Medical Imaging, Helmholtz Center Munich and Technical University of Munich, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany

E-mail: *dr@tum.de

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Abstract
Zebrafish has emerged as an excellent vertebrate model organism for studies of evolution, development and disease. Due to its external development and optical transparency in embryonic stages, zebrafish offers a major advantage over other vertebrate model organisms by being amenable for microscopic studies of biological processes within their natural environment directly in the living organism. However, commonly used zebrafish strains lose their transparency within their first two weeks of development and thus are no longer accessible for optical imaging approaches at juvenile or adult stages. In this study we successfully apply optoacoustic imaging for non-invasive three-dimensional imaging of adult zebrafish. Since optoacoustics does not necessarilly require labeling, but can instead rely on the intrinsic tissue contrast, this imaging method has the potential to become a versatile tool for developmental studies from juvenile to adult stages in the intact zebrafish.

Online supplementary data available from stacks.iop.org/PMB/57/7227/mmedia
(Some figures may appear in colour only in the online journal)

1. Introduction
Over the last two decades zebrafish has received increasing attention as a model organism for studies of vertebrate evolution and development (Postlethwait et al 1998, Hove et al 2003). Since zebrafish produce a large number of offspring and show a comparatively fast development, it has proven to be a cost-effective model organism to model human diseases and perform high-throughput screens for novel therapeutics (Langenau et al 2003, North et al 2007, Yeh et al 2009). The zebrafish genome is almost completely sequenced and several methods for its genetic manipulation are readily available. These methods include e.g. Gal4/UAS- and Cre/loxP-based systems, allowing for spatially and temporally controlled transgene expression
(Asakawa and Kawakami 2008, Distel et al 2009, Hans et al 2009), morpholino-based gene knockdown and even recently established knock-out and knock-in technology using zinc finger nucleases (Doyon et al 2008). The establishment of the Tol2 transgenesis system has especially facilitated the generation of transgenic zebrafish by dramatically increasing the frequency of chromosomal integration and germ-line transmission of exogenous DNA (Kawakami 2004). In this way transgenic zebrafish can now be generated within approximately 3 months.

Most importantly, due to its external development and transparency during embryonic stages, zebrafish is an ideal model organism for optical imaging approaches, offering the opportunity to observe developmental processes at high resolution in a living organism. For example, using living transgenic zebrafish, it has been shown that hematopoietic stem cells derive directly from the endothelium of the dorsal aorta (Bertrand et al 2010, Kissa and Herbomel 2010, Lam et al 2010). Confocal or two-photon microscopy has been extensively employed for imaging zebrafish a few days post-fertilization (dpf) (Isogai et al 2001, Megason and Fraser 2003, Das et al 2003), requiring injection of a fluorescent probe or the use of fluorescent reporters. Other label-free optical imaging techniques, such as optical coherence tomography (Kagemann et al 2008), selective plane illumination microscopy (Arrenberg et al 2010) or optical projection tomography (Bassi et al 2011), have also been recently used for imaging zebrafish at early stages. Although one zebrafish mutant strain has been recently established, which stays transparent throughout its life (White et al 2008), other commonly used zebrafish strains lose their transparency starting at approximately 14 dpf and are thus not accessible for high-resolution optical imaging approaches at adult stages.

In order to study dynamic processes, such as tumorigenesis or regeneration, a non-invasive imaging method for juvenile and adult zebrafish would be extremely valuable. Toward this goal, two non-invasive methods using intrinsic contrast have been successfully applied to zebrafish so far: magnetic resonance microscopy (MRM) also termed magnetic resonance microimaging (μMRI) and ultrasound biomicroscopy (Kabli et al 2006, 2010, Goessling et al 2007). MRM relies on the same fundamental principles as magnetic resonance imaging (MRI), exploiting the nuclear characteristics of the protons abundant in tissue (Kabli et al 2006). Kabli et al applied MRM at a 9.4 T magnetic field to image adult zebrafish and were able to visualize structures including the brain, intestine, swim bladder and myoseptum at a spatial resolution of 137 μm in ex vivo studies. The same group also reported the visualization of tumors within a living adult zebrafish applying a 17.6 T ultra-high magnetic field (Kabli et al 2010). By using rapid acquisition with relaxation enhancement (RARE), they could improve the resolution to 78 μm in in vivo studies (Kabli et al 2006). Furthermore, μMRI has been successfully applied to visualize the complex rearrangements during gastrulation in Xenopus (Papan et al 2007).

Ultrasound biomicroscopy was used by Goessling et al to visualize liver tumors in living adult zebrafish. This method could achieve 68.2 μm lateral and 38.5 μm axial resolution by using a 40 MHz scanhead (Goessling et al 2007). However, speckle artifacts arising from interference of acoustic waves hamper the image quality, while a low contrast between different biological tissues is overall a limiting factor for application of ultrasound biomicroscopy in zebrafish.

Herein, we present optoacoustic tomography as an alternative tool for whole-body high-resolution imaging of adult zebrafish. The method is based upon the optoacoustic effect, which comprises emission of acoustic waves due to thermal expansion, caused by absorption of short pulses of light in tissue (Wang and Wu 2007). Thereby, the emitted acoustic signal depends upon light absorption within the biological sample, which makes it possible to visualize and distinguish between tissues with different optical absorption coefficients. With blood being the main absorber in mammalian tissues, optoacoustic tomography has been successfully applied to visualize vascular structures and disease-related neovasculature in small animals and humans.
Non-invasive whole-body imaging of adult zebrafish with optoacoustic tomography

Figure 1. Simplified scheme of 3D optoacoustic imaging of zebrafish. M1 and M2 form a mirror set composed of two mirrors with a 45° angle facing each other. BS is a beam splitter, which splits the beam into two parts and M3 and M4 are mirrors that reflect the laser beams to the fish. T stands for the ultrasound transducer.

(Wang et al 2006, Zhang et al 2006, Gamelin et al 2008, Lao et al 2008, Zhang et al 2009, Heijblom et al 2012). Much like ultrasonic imaging, optoacoustics can form images in real time and track dynamic events, such as perfusion, currently in 2D (Buehler et al 2010, Razansky et al 2011) but potentially also in 3D. Furthermore, in multi-spectral optoacoustic tomography (MSOT), pulses of different wavelengths are used in a time-shared fashion (Ntziachristos and Razansky 2010). In this way, specific tissue bio-markers and reporter agents, having changes in the absorption spectrum with characteristic differences from the absorption of background tissue, can be efficiently resolved. Nanoparticles and reporter molecules with steep absorption spectra changes are optimal for MSOT as they can be resolved by scanning narrow spectral bands (Razansky et al 2011, Ntziachristos and Razansky 2010, Ma et al 2009, Razansky et al 2009b, Yang et al 2009, Taruttis et al 2010, Glatz et al 2011). In particular, we have previously reported on visualization of fluorescent proteins in zebrafish by MSOT (Razansky et al 2009a).

Optoacoustic imaging of zebrafish was also performed with integrating line detectors (Gratt et al 2012) and with optical resolution optoacoustic microscopy (Ye et al 2012), the latest technique providing penetration depths in the same order as laser-scanning microscopy.

2. Materials and methods

2.1. Experimental setup and data acquisition

A simplified scheme of zebrafish optoacoustic imaging is illustrated in figure 1. It was adopted from the experimental setup used in (Ma et al 2009). Briefly, the nanosecond excitation light beam is produced via an optical parametric oscillator (MOPO-700 series, Newport Corp., Mountain View, CA), pumped by a Q-switched Nd:YAG (Quanta-Ray Lab-Series 190-30 Newport). The pulse duration is less than 10 ns and the repetition rate is 15 Hz. The beam at a wavelength of 587 nm passes through a slit as a thin beam and is then split into two beams to cover the periphery of the zebrafish. The laser intensity used was below the laser safety limit, established at 20 mJ cm⁻² (American Laser Institute 2000). For optoacoustic signal detection, a single-element cylindrically focused PZT transducer was used with a 15 MHz...
central frequency and a bandwidth up to approximately 20 MHz, 19.05 mm focal length and 13 mm element diameter (V319, Panametrics-NDT, Waltam, MA).

Cross-sectional 2D slices were acquired by rotating the fish on a rotation stage over 360°, while image reconstruction was done by the filtered backprojection algorithm (Xu and Wang 2006) together with a statistical correction approach to reduce the image distortion due to acoustic scattering (Dean-Ben et al. 2011). 3D data were acquired by scanning along the $z$-axis the mirror M2, the beam splitter (BS) and the transducer (T) with a step size of 150 μm and 68 scan steps. The mirrors M3 and M4 are stationary mirrors with a size much larger than the scanning length. The total acquisition time was 62 min for whole-body imaging, due to the low repetition rate of the laser and single-element ultrasound acquisition we are currently using. 3D image reconstruction and segmentation was performed using Amira software (Visage Imaging GmbH) by stacking the 2D slices.

2.2. Zebrafish handling

Intact dead zebrafish samples were obtained from the Institute for Developmental Genetics, Helmholtz Zentrum Munich. For imaging, the samples were embedded in a cylindrically shaped agar phantom. After the agarose was solidified, the phantom containing intact zebrafish was transferred to the optoacoustic imaging setup. The phantom was subsequently mounted onto the rotation stage and the tank was filled with water to ensure transmission of the ultrasound signal to the transducer. Procedures involving animals and their care were conducted in conformity with Bavarian government guidelines, which comply with national and international laws and regulations.

2.3. Histology

For H&E staining of histological sections, adult zebrafish were fixed in 4% PFA over night. Paraffin sections were cut at 10 μm and stained with hematoxylin and eosin. Images were taken using a Leica MZ16 microscope.

3. Results

3.1. 2D optoacoustic imaging in correlation with histology

Representative anatomical cross-sectional imaging slices in different regions are shown in figure 2 (left column). For correlation, histological sections (thickness 10 μm) of an adult female zebrafish stained with hematoxylin and eosin were further made and are also shown in figure 2 (right column). Comparison of the different sections revealed good concurrence between optoacoustic and histological sections. Major organs and tissues, such as brain, eyes (figures 2(A) and (C)), myotome, swim bladder, kidney, intestine and pectoral and pelvic fins (figures 2(E) and (G)), can readily be identified in the optoacoustic images, which in this case have a spatial resolution of about 35 μm as shown in (Ma et al. 2009), limited by the useful bandwidth of the ultrasonic detection utilized in the experiments.

3.2. Sagittal and coronal views and three-dimensional reconstructions

For 3D rendering, we recorded a stack of 68 slices starting at the posterior end of the eye of the adult female fish, moving posterior with steps of 150 μm. Movie 1 (available from stacks.iop.org/PMB/57/7227/mmedia) demonstrates a volumetric transverse scan through the entire fish. Figures 3(A),(C) and 3(B),(D) are selective reconstructed sagittal and coronal views,
Figure 2. Cross-sectional optoacoustic reconstructions of an adult intact female zebrafish (left column) with corresponding paraffin sections of the fish stained with hematoxilin and eosin (right column). Abbreviations: brain (B), eye (E), optic tectum (OT), torus semicircularis (TS), gill filaments (GF), sternohyoid (St), myotome (My), vertebrae (V), kidney (K), swim bladder (SB), intestinal bulb (IB), posterior intestine (PI), mid intestine (MI), fin (F), ovaries (O).
respectively, with the main anatomical structures readily identifiable (3D rendering of the sagittal sections can be found in movie 2 available from stacks.iop.org/PMB/57/7227/mmedia).

Using Amira software (Visage Imaging GmbH) we performed manual segmentation of organs based on the 68 cross-sectional 2D slices. Figure 4 demonstrates a reconstructed 3D image of the zebrafish section ranging from the anterior end of the eye to the pelvic fin. The entire 3D segmentation can be found in movie 3 (available from stacks.iop.org/PMB/57/7227/mmedia).

3.3. Comparative study using different modalities

The advantage of optoacoustic imaging is mainly due to its high soft tissue contrast based on optical absorption, and also due to the high spatial resolution (Wang and Wu 2007) and real-time imaging capabilities (Razansky et al 2011). It is known that the optical absorption coefficient of tissue structures varies by two to three orders of magnitude in the visible and near-infrared ranges (Wang and Wu 2007), which leads to a vast change of optoacoustic signals upon different tissue structures. Figure 5 illustrates the differences between the images obtained from an adult (1-year-old) wild-type zebrafish using micro-CT, ultrasound and optoacoustic tomography. The x-ray CT image was acquired using a commercial micro-CT (eXplore Locus, General Electric HealthCare, London, Ontario, Canada) while the ultrasound image was acquired using a commercial ultrasound system (Terson 2000+, Terson Ultrasound, Division of Teratech Corporation), which uses a linear array transducer with a central frequency of 7.5 MHz. Without the introduction of any extrinsic contrast, one could clearly visualize the inner structures of adult zebrafish using optoacoustic tomography with both good contrast and good spatial resolution (figure 5(C)). On the other hand, even though micro-CT performs well
Figure 4. 3D segmentation of zebrafish, performed from volumetric optoacoustic scans at different perspectives, with several main organs marked in pseudo colors. The segmentation was produced in Amira (Visage Imaging GmbH). Abbreviations: brain (B, yellow), eye (E, magenta), dilator operculi (DO, blue), liver (L, brown), part of the kidney (K, yellow), swim bladder (SB, white), skin (Su, light blue), cardiac ventricle (CV, red), intestinal bulb (IB, purple), mid intestine (MI, green), posterior intestine (PI, light green), pectoral fin musculature (PFM, light yellow), fin (F, blue).

Figure 5. Comparison between micro-CT (A), ultrasound (B) and optoacoustic imaging (C) of an adult (1-year-old) wild-type zebrafish.

with distinguishing bones and other hard structures (figure 5(A)), due to the small variations in tissue densities, it attains poor contrast in soft tissues, such as brain, cardiac ventricles, intestines, etc. Likewise, without the introduction of contrast agents, the ultrasound imaging
Table 1. Performance comparison between different imaging modalities suitable for whole-body visualization of zebrafish.

<table>
<thead>
<tr>
<th>Modality</th>
<th>Optoacoustic</th>
<th>Ultrasound</th>
<th>X-ray CT</th>
<th>MRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contrast mechanisms</td>
<td>Anatomical, functional, molecular</td>
<td>Anatomical, functional</td>
<td>Anatomical</td>
<td>Anatomical</td>
</tr>
<tr>
<td>Safety</td>
<td>Good</td>
<td>Good</td>
<td>Fair</td>
<td>Good</td>
</tr>
<tr>
<td>Soft tissue contrast</td>
<td>0.2–200 μm</td>
<td>50 μm–3 mm</td>
<td>1 μm–1 mm</td>
<td>20 μm–1 mm</td>
</tr>
<tr>
<td>Imaging depth</td>
<td>0.5–5 cm</td>
<td>1–25 cm</td>
<td>Whole body</td>
<td>Whole body</td>
</tr>
<tr>
<td>Speckle noise</td>
<td>None</td>
<td>Strong</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cost</td>
<td>Medium</td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
</tr>
</tbody>
</table>

attains low contrast variations in soft tissues of up to 10% (Angelsen 2000), while speckle noises further reduce the image quality and spatial resolution (figure 5(B)).

Table 1 further summarizes performance characteristics of various imaging modalities suitable for whole-body visualization of zebrafish.

4. Discussion

Zebrafish is a powerful model organism, easily accessible by optical in vivo microscopy techniques at embryonic stages. However, at juvenile and adult stages increasing opacity hinders such imaging approaches. Thus, imaging methods, not limited by light scattering in biological tissues, could become valuable tools to study development, tumorigenesis, angiogenesis and regeneration in vivo.

In this work, we have evaluated optoacoustic tomography as a high-resolution tool for whole-body imaging of adult zebrafish. It was found that this new imaging approach can effectively utilize the intrinsic absorption of biological tissues; therefore, application of agents for contrast enhancement can be avoided. In optoacoustics, the excitation is achieved by light while the generated information is carried by acoustic waves, which have insignificant scattering as compared to photons. Hence, as opposed to conventional optical imaging and microscopy approaches, optoacoustic imaging can provide high-resolution information from deep tissues. In this way, the contrast advantages of light can be utilized without being hampered by the limitations imposed by diffusion of photons in biological tissues. The in-plane spatial resolution in optoacoustic tomography is mainly determined by the effective bandwidth of the acoustic transducer employed, so that in principle this technique provides isotropic resolution in the imaging plane. A higher resolution is achieved by using a transducer capable of measuring the higher frequency components of the optoacoustic signals. However, the propagation of high-frequency optoacoustic waves is strongly affected by acoustic scattering (Deán-Ben et al 2011a, 2011b) and acoustic attenuation (Deán-Ben et al 2011c), which cause distortion and broadening of the signals and ultimately affect the resolution of the images. Such acoustic phenomena are more prominent for signals originated at deep locations inside tissue, so that the image quality is reduced with depth when imaging at higher frequencies. In any case, the loss of resolution with depth in optoacoustic imaging is much less than that in pure optical techniques beyond the optical transport mean free path. On the other hand, the out-of-plane resolution is conditioned by the sensitivity field of the acoustic transducer and the illuminated region. The height of the sensitivity field of the transducer is determined by acoustic diffraction. Provided the optoacoustic sources are confined within the depth of focus of the transducer, an approximately uniform out-of-plane resolution can be achieved.
The out-of-plane resolution may be increased if the laser beam is shaped in order to selectively illuminate the imaging plane. In such a case, the height of the illuminated region is determined by optical diffraction at the surface of the sample and by optical scattering within tissue for deep locations. Using a self-manufactured automated imaging setup, we recorded volumetric optoacoustic imaging data with a cross-sectional resolution of 35 μm, which allowed clear visualization of organs within an intact adult zebrafish, such as brain, intestine, swim bladder and kidney. As compared to the other imaging modalities that were applied for whole-body imaging of adult zebrafish, i.e. μMRI and ultrasound biomicroscopy, optoacoustics can provide a better spatial resolution and intrinsic contrast. Furthermore, optoacoustics and other light-based imaging methods operate on contrast mechanisms that offer a highly versatile ability to visualize cellular and subcellular functions and structures. Correspondingly, fluorescence microscopy and imaging are overwhelmingly utilized in biomedical research, for example in immunohistochemistry, in vitro assays, or cellular imaging in vivo. Since MSOT methods have already been shown capable of resolving optical molecular agents, fluorescent proteins and other types of reporter agents, the above molecular assays can now be effectively applied to an entire living model organism. Technological advances, such as the use of high frequency multi-element ultrasonic arrays for optoacoustic detection, will further improve spatial resolution and image acquisition speed and allow for building even higher resolution three-dimensional imaging capabilities for adult zebrafish using optoacoustic tomographic approaches.

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