2 Magnetic Resonance Microimaging of the Adult Zebrafish*

2.1 Abstract

Magnetic resonance microimaging (μMRI) is an imaging modality that allows acquisition of high-resolution images in intact opaque animals non-invasively. The zebrafish (*Danio rerio*) is an important model organism for the study of vertebrate biology. However, optical *in vivo* studies in zebrafish are restricted to very early developmental stages due to opaqueness of the juvenile and the adult stages. Application of high resolution μMRI has not yet been explored in adult zebrafish. In this study we applied and optimized high resolution μMRI methods to examine anatomical structures non-invasively in adult zebrafish. Clear morphological proton images were obtained by *T₂*-weighted spin echo and rapid acquisition with relaxation enhancement (RARE) sequences revealing many anatomical details in the entire intact zebrafish at 9.4T. In addition, *in vivo* imaging of adult zebrafish revealed sufficient anatomical details. To our knowledge this is the first report for the application of high resolution μMRI to study detail anatomical structures in adult zebrafish.

*This chapter was published in Zebrafish 2006; 3:431-439*
Chapter 2

2.2 Introduction

The zebrafish has emerged as an excellent model organism for studies of vertebrate evolution, diseases, biological pathways, and toxicological mechanisms (1, 2). The near completion of the zebrafish genome sequence and the EST sequencing project allow the use of the versatile morpholino-based antisense knock-down approach to rapidly analyse the function of many different gene products within the first five days of zebrafish development. External development and optical clarity during embryogenesis allow for visual analysis of early developmental processes, and high fecundity and short generation times facilitate genetic analysis. However, due to opaqueness of juvenile and adult stages, traditional optical microscopic methods are not suitable to study various developmental processes in adult zebrafish. Alternative methods of analysis are invasive; fish are sacrificed and processed to reveal specific information like morphology, histology, gene expression pattern and physiological parameters during normal and abnormal conditions such as stress and infection. In addition, development and progression can not be studied over time in the same fish and analysis has to be repeated on different individuals. At present, no high resolution images of the complete juvenile and adult histology of the zebrafish are available, neither in internet accessible databases nor in the literature. Unavailability of such resources is amongst other things due to difficulties in the imaging of larger areas of interest and an extra complication is experienced in deformation of the specimen in the preparation process causing distortion, artefacts and degradation of spatial resolution (3-5). These raise a need for a rapid sensitive and non-invasive imaging methods to follow developmental or other processes, not only of the embryonic phase but also of juvenile and adult stages and to establish an anatomical atlas of adult zebrafish.
Magnetic resonance microimaging (μMRI) is an imaging technique that explores the nuclear magnetic characteristics of the abundant protons in tissue (6). It is a non-invasive, non-ionizing imaging modality that has unique three-dimensional capabilities and allows acquisition of high-resolution images non-invasively in intact opaque animals (7-10). μMRI is founded on the same fundamental principles as MRI but produces images with higher spatial resolution because of the use of strong magnetic field gradients (200-1000 mT/m) and specialized radio frequency (RF) coils (11-13). For example, a spatial resolution of 20 μm$^3$ per voxel can be achieved. μMRI offers the possibility to image a live adult fish non-invasively, which is impossible using other imaging techniques. With μMRI, it is possible to study developmental processes over an extended period of time in the same fish from embryo till adulthood. μMRI can also be very attractive and suitable for functional imaging investigations in zebrafish. With the help of μMRI it is also possible to image the blood flow and to do local molecular spectroscopy like measurement of ATP and ADP concentration (6, 14). In vivo visualization of gene expression by MRI in combination of contrast agents in a living embryo of X. laevis has been demonstrated earlier (5).

High resolution μMRI has not yet been exploited for imaging adult zebrafish. The objective of the present study is to optimize the MR setup and μMRI sequences to visualize high resolution structural details in adult zebrafish in vitro which can be extended later to an in vivo system. Because of the very small size compared to a mouse or a rat, imaging of adult zebrafish needs high resolution. To achieve this, we used a high field of 9.4T for μMRI study of adult zebrafish. In addition to ex vivo studies, a flow-through setup has been designed for in vivo μMRI studies of adult
zebrafish and first results of in vivo μMRI are presented. We demonstrate that high field μMRI provides sufficient resolution to get rapid access to anatomical details in adult zebrafish in a short time. This paves the way for studying disease development, biological pathways, and toxicological mechanisms during various developmental stages in individual living zebrafish.

2.3 Materials and methods

Zebrafish

Adult Wild-type zebrafish (Danio rerio) were maintained in recirculating aquarium systems according to established rearing procedures (15, 16). The water temperature was maintained at 28 °C with a flow rate of 150 L/min, with day/night light cycles (12h dark versus 12h light). The fish were fed twice daily with commercial flake food according to Westerfield (2000) (16). All fish were handled according to Institutional Animal Care and Use Committee guidelines. For ex-vivo imaging, adult zebrafish were euthanized and immediately embedded in Fomblin (Perfluoropolyether). Alternatively, the fish were fixed in 4% buffered paraformaldehyde (Zinc Formal-Fixx, ThermoShandon, UK) for 2 days and subsequently embedded in Fomblin.

Experimental setup for in vivo imaging

For in vivo MRI measurements, fish was anesthetised by adding 0.01% MS222 (ethyl meta aminobenzoate metanesulfonic acid salt; Sigma chemical co.) to pH controlled water. Subsequently fish was transferred to a closed flow-through chamber, which was designed for continuous flow of aerated water to support living zebrafish inside the magnet. The flow-
through setup was then inserted in the centre of the volume coil (2 cm diameter) inside the microimaging probe, which was then inserted in the bore of the vertical MR magnet (400 MHz). Aerated water with anaesthetic was pumped from a temperature controlled aquarium to a tube fixed on lower end of the flow-through cell, which was close to the mouth of the fish. After passing the chamber the water was transported back to the aquarium. The setup allowed direct \textit{in vivo} NMR measurements at constant flow speeds (20 ml/min) which were regulated by a STEPDOS 03/08 pump (KNF Flodos AG, Switzerland). After the MRI measurements, zebrafish were transferred back to normal aquarium without anaesthetic where fish recovered uneventfully from the experimental treatment.

\textit{Magnetic resonance microimaging}

MR imaging was performed using a 400 MHz (9.4T) vertical bore system, using a 20 mm volume coil and a 1 Tm\textsuperscript{-1} gradient insert from Bruker Analytic, Germany. Before each measurement the magnetic field homogeneity was optimized by shimming. Each session of measurements began with a multislice orthogonal gradient-echo sequence for position determination and selection of the desired region for subsequent experiments.

Various MRI scan protocols were used and optimized for high resolution imaging of zebrafish. \textit{T}_2-weighted multi slice multi echo (MSME) and Rapid Acquisition with Relaxation Enhancement (RARE) (17) sequences were used for the \textit{ex vivo} imaging of adult zebrafish. For \textit{in vivo} imaging, the RARE sequence was used. The FOV was varied from 1 cm to 3.5 cm with an image matrix of 256 × 256. Data acquisition and processing were performed with Para Vision 3.02pl (Bruker Biospin, Germany) running on a
Silicon Graphics 02 workstation with the Irix 6.5.3 operating system and using a Linux pc running XWinNMR 3.2.

3D reconstruction

A 3D image of adult zebrafish has been reconstructed from a series of 2D MR slices with the help of 3D reconstruction software, TDR-3D base (18). The images are imported in the TDR-3D base software as separate 2D images while keeping the coherence of the stack. Using the TDR-3D base software allows to build a contour model using by delineating the structures of interest either manually by digitizer tablet or through automated procedure. The contours are stored in a geometrical database from which other geometrical representations can be generated. MR image files of adult zebrafish containing 25 slices of 0.2 mm thickness with calibrated scale markers were imported in TDR-3D base software. A selection of anatomical domains, i.e., brain, heart, liver and swim bladder, etc. was delineated in the images. The contours in the stack make up the 3D geometrical model that is reconstructed and visualized as a 3D rendering.

2.4 Results

In proton μMRI, the signal intensity arising from any element (voxel) in the three-dimensional image is typically a function of the water concentration and relaxation time (\(T_1\), spinlattice relaxation time; \(T_2\), spin-spin relaxation time) (19). Local variations in these parameters provide the vivid contrast seen in the images obtained by μMRI, and various anatomical details can be clearly seen.
2.4.1 *Ex vivo* studies

Sagittal slice images of a fixed adult zebrafish were obtained by multislice multiecho (MSME) pulse sequences at 9.4 T using a repetition time of 2000 ms and an echo time of 15 ms. With an anisotropic field of view (FOV) of 20 mm X 35 mm, we obtained a spatial resolution of 137 μm. Structures including the brain, intestine, swim bladder, and myoseptum can be clearly recognized (Fig. 2.1A). The total scan time used for this set of experiments was 27 minutes. Although this scan time is adequate for high-resolution *ex vivo* imaging, it is not reasonable for imaging living adult zebrafish, which requires very short scan times to ensure the survival of the zebrafish during and after μMRI measurements.

To reduce the total acquisition time, we explored rapid acquisition using the rapid acquisition with relaxation enhancement (RARE) sequence. RARE is a multiecho imaging sequence in which each refocused echo is acquired after having experienced a different phase-encoding value. This reduces the total imaging time. Because $B_0$ refocusing is inherent in the sequence, it is less vulnerable to susceptibility-induced dephasing than, for example, gradient echo sequences. In addition, it is substantially faster to apply than a spin-echo sequence with a singlephase encoding value per repetition time (TR). The primary contrast is $T_2$-based. Sagittal images of adult zebrafish were obtained with the RARE sequence (Fig. 2.1B). With a RARE factor (echo train length) of 4, a repetition time of 2000 ms, and an echo time of 15 ms (effective, 33.7 ms), we obtained high resolution images in a period as short as 8 minutes, with 4 number of averages resulting in a clear image of head and abdominal structures. Because of very high signal- to-noise ratios in these images, the scan time can be further reduced by taking a smaller
Figure 2.1 High resolution images of adult zebrafish at 9.4T. Slices in the sagittal plane were obtained using the MSME pulse sequence (TE= 15 ms; TR= 2000 ms; ns=4; T. Scantime 27 min) (A) or RARE sequence (TE=15 ms with effective TE 33.6 ms; TR=2000 ms; ns=4, T. Scantime 8 min) (B). The image resolution is 137 μm. Slice thickness 0.2 mm. (a) brain; (b) swim bladder; (c) horizontal myoseptum; (d) heart; (e) stomach; (f) intestine; (g) ovary

number of averages for imaging living zebrafish. Four successive slices of adult zebrafish were obtained in the sagittal (Fig. 2.2A), coronal (Fig. 2.2B), and axial planes (Fig. 2.2C) using the RARE sequence with an image resolution of 78 μm. Many anatomical details are clearly visible. The shape of the brain is nicely visible in all three planes. Figures 2.1 and 2.2 were obtained using formalin-fixed adult zebrafish. The formalin fixation process alters tissue characteristics by forming crosslinks between proteins or
Figure 2.2 High resolution images of adult zebrafish at 9.4T. Successive Slices (1-4) in sagittal (A), coronal (B) and axial (C) planes were obtained using the RARE pulse sequence (TE= 15 ms with effective TE 33.6 ms; TR= 2000 ms; ns=4; T. Scantime 8 min). The image resolution is 78 μm. Slice thickness 0.2 mm. (a) brain; (b) left eye; (c) right eye; (d) swim bladder; (e) gills; (f) ovary; (g) intestine; (h) horizontal myoseptum; (i) heart.
Figure 2.3 High resolution images through the head of a fresh unfixed adult zebrafish obtained by a RARE pulse sequence at 9.4T. Successive slices (A-D) in sagittal plane. The image resolution is 78 μm. Slice thickness 0.2 mm (a) eye; (b) brain; (c) horizontal myoseptum; (d) swim bladder; (e) heart; (f) stomach; (g) intestine; (h) eggs.

protein and nucleic acids, and by creating hydroxymethylene bridges and binding of calcium ions (20). For MR imaging, the fixation process enhances contrast. However, to open the possibility of in vivo imaging of zebrafish, MR methods were also optimized and applied to zebrafish without fixation. Figure 2.3 shows four slices through the sagittal planes of adult zebrafish taken immediately after death without formalin fixation. Clear differences can be recognized in fixed (Fig. 2.1) and unfixed (Fig. 2.3) zebrafish. For instance, the swim bladder appears black in fresh unfixed zebrafish while in fixed zebrafish they appear white due to penetration of formalin in the swim bladder. Eggs in the ovary are more clearly seen in fresh unfixed zebrafish. Although the overall contrast in MR images of
unfixed zebrafish was less than for fixed zebrafish, it is sufficient to obtain morphological and anatomical details. Figure 2.3 was obtained using the same parameters as the images of the fixed fish, with an image resolution of 78 μm.

2.4.2  *In vivo* studies

On the basis of these optimized protocols for *ex vivo* imaging of zebrafish, we extended our studies to image living adult zebrafish using μMRI. Although *in vivo* MRI has become an approved tool in medicine and pharmacologic research, very few studies have used this method to uncover physiology in aquatic organisms (21). Aquatic animals require special setups and several precautions for supporting *in vivo* imaging. For example, fish need a continuous flow of aerated water to irrigate their gills during the MRI measurements. This requires a special watertight flow-through chamber to support the fish and to prevent any contact of water with the RF coil and gradient insert. The fish needs to be immobilized to prevent motion artifacts, either by restraining or using anesthetic. In addition, imaging artifacts due to the surrounding water flow should be minimized. *In vivo* MRI studies in a few aquatic animals such as teleosts (*e.g.*, carp), eelpout, and *Gadus morhua* have been successfully demonstrated (21-24). Due to the small size of zebrafish, additional precautions are needed for *in vivo* imaging. For example, a high-resolution microimaging magnet, needed to get good resolution with small fish, has limited space for a flow-through chamber. The small flow-through chamber cannot support a high flow of water that would be needed if unwanted signal from surrounding water is to be avoided. It has been shown that unwanted signal from surrounded water...
**Figure 2.4** (A) Design of flow-through chamber for in vivo MRI measurements of living adult zebrafish. 1, water inlet; 2, U-shaped PVC chamber to hold the fish into which water inlet enters from the bottom near the mouth of the fish; 3, a specimen of adult zebrafish; 4, a variable slide barrier to fit the size of the fish with a hole at the bottom; 5, chamber closet; 6, water outlet. (B) Flow-through chamber fitted into the volume coil of microimaging probe. (C) MRI images of anaesthetized living adult zebrafish obtained at 9.4T. Slices in sagittal (upper row) and coronal (lower row) planes were obtained using the RARE pulse sequence (echo train length = 4; TE = 10.5 ms with effective TE 22.5 ms; TR = 1000 ms; ns=2; T. Scan time 2 min 8s). The image resolution is 78 μm. Slice thickness 0.5 mm. (a) eye; (b) brain; (c) gills; (d) heart; (e) swim bladder; (f) intestine; (g) eggs; (h) horizontal myoseptum.

can be excluded if the flow rate of outside water is higher in comparison to the repetition time used for spin echo sequences (23). Furthermore zebrafish cannot tolerate a high flow of water. The experimental time should be kept as short as possible since the zebrafish has a lower tolerance to anesthetic than bigger fish such as carp.
A small flow-through chamber designed to support imaging of living zebrafish is shown in Fig. 2.4A. The chamber was fitted into a cylindrical resonator for a homogeneous excitation profile (Fig. 2.4B). Sagittal and coronal anatomical MR images of living zebrafish were obtained using a RARE pulse sequence with an image resolution of 78 μm (Fig. 2.4C). The flow of water to irrigate the gills of the zebrafish was kept low (20 mL/min) during the measurements. Although the dynamic intensity range of the image was slightly affected by the signal from the surrounding water, sufficient signal-to-noise ratio and image contrast was achieved to distinguish various anatomical details in the images. Several structures such as brain, heart, gills, swim bladder, and intestine can be nicely resolved. The total scan time was 128 seconds. The fish remained under anesthesia in the magnet for up to 15 minutes and recovered uneventfully from the experimental setup. Experiments with live fish were repeated with 5 different animals.

2.4.3 Three-dimensional image reconstruction

In order to emphasize various anatomical structural components and the 3D continuity of these structures, the images are annotated from μMRI image slices and as a result a 3D model is derived. Figure 2.5 shows a reconstructed three dimensional image of zebrafish obtained from a series of two-dimensional MR slices using TDR-3D base software (25). Here complete three-dimensional models of various structures such as brain, heart, liver, and swim bladder are constructed. While a three-dimensional atlas of zebrafish development produced using TDR-3D base from histological sections is available (18, 26), at this time there is no atlas of the adult zebrafish. We consider this work as a start that will pave the way for
building a high-resolution anatomical atlas of adult zebrafish using both *ex vivo* and *in vivo* μMRI images.

**Figure 2.5** 3D Visualization of parts of the anatomy of the adult zebrafish reconstructed from 25 MR slices (0.2 mm thick). The slices in the 3D image were annotated with TDR-3Dbase. In panel A the original contours are shown as projected on a slice through the midline of the fish. In panel B the major anatomical structures are visualized as pseudo voxels whereas in panel C and D a surface visualization is shown, projected on a mid-sagittal and lateral sagittal slice. Pleural ribs and vertebral haemal and neural arches (yellow), Brain (green), Swim-bladder (purple), Otic capsule (violet), Eye (salmon), Heart (red), Liver (blue) and Gut (light green).
2.5 Discussion

Zebrafish is rapidly becoming one of the most important vertebrate animal models used in genetic analysis. However, in order to move beyond functional genomic analyses and towards therapies, drugs, and other useful applications, we need to understand the data that these studies generate within the framework of the functional structure of the zebrafish organism in sufficiently intimate detail. Genomics, bioinformatics, proteomics, and other high throughput experimental design paradigms seek to unravel the organism’s genetic program by charting its intricately entwined regulatory, sensory, and metabolic pathways, ultimately resulting in a description of life as a system in “information space.”

Many such studies analyze correlations of gene expression levels without taking the state of the organism into account. Since the genetic program of an organism is only meaningful in the context of its functional physical structure, imaging is essential to uncover in real space the anatomy that underlies this system of life, its variation between individual organisms with the same or similar genotype, and its structural plasticity under the influence of life processes. Only by understanding the intimate physical structure of life can we gain insight into how the collections of inanimate molecules in living organisms interact with each other to constitute, maintain, and perpetuate the living state—the ultimate goal of life science research (27). For adult zebrafish, profound insight into anatomical and developmental details is missing since analysis with the traditional methods of light microscopy and electron microscopy is difficult, due to distortion, artifacts, and degradation of spatial resolution (3-5). This raises the need for a rapid, sensitive, and noninvasive imaging method such as μMRI. Our results presented in this chapter show that high field μMRI provides sufficient
resolution to get rapid anatomical details in adult zebrafish ex vivo as well as in vivo. Thus high-resolution μMRI can be applied in vivo to study disease development, biological pathways, toxicologic mechanisms, and possible drug screening during various developmental stages in individual living zebrafish noninvasively.

The signal-to-noise ratio of the μMRI increases linearly with the field strength and since an adult zebrafish is small compared to a mouse or a rat, it would be highly beneficial to further improve resolution by using an ultrahigh magnetic field (7). The optimization of the pulse sequence to image zebrafish at an ultrahigh field of 17.5 T is presently underway.

Acknowledgements

We thank Suzanne Kiihne and Fons Lefeber for technical advice and help concerning the μMRI and Annemarie Meijer for advice and providing the facility to work with the zebrafish. This work was partly supported by grants from Centre for Medical Systems Biology (CMSB), CYTTRON within the Bsik program (Besluit subsidies investeringen kennisinfrastructuur) and a European Communities 6th framework grant (contract LSHG-CT-2003-503496).

References


