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Magnetic Resonance Microscopy of the Adult Zebrafish

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ABSTRACT

Magnetic resonance microscopy (MRM) is an imaging modality that allows for noninvasive acquisition of high-resolution images in intact opaque animals. 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 the opacity of the juvenile and adult stages. Application of high resolution MRM has not yet been explored in adult zebrafish. In this study we applied and optimized high resolution MRM methods to examine anatomical structures noninvasively in adult zebrafish. Clear morphological proton images were obtained by T_2-weighted spin echo and rapid acquisition with rapid acquisition with relaxation enhancement (RARE) sequences which revealed many anatomical details in the entire intact zebrafish at a magnetic field strength of 9.4 T. In addition, *in vivo* imaging of adult zebrafish revealed sufficient anatomical details. To our knowledge this is the first report of the application of high resolution MRM to study detailed anatomical structures in adult zebrafish.

INTRODUCTION

The zebrafish has emerged as an excellent model organism for studies of vertebrate evolution, diseases, biological pathways, and toxicologic mechanisms.[1,2] The near-completion of the zebrafish genome sequence and the expressed sequence tags (EST) sequencing project allow the use of the versatile morpholino-based antisense knockdown approach to rapidly analyze the function of many different gene products within the first 5 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 the opacity of the juvenile and adult stages, traditional optical microscopic methods are not suitable for the study of developmental processes in adult zebrafish.

Alternative methods of analysis are invasive, and fish are sacrificed and processed to reveal specific information such as morphology, histology, gene expression patterns, and physiological parameters during normal conditions and under stress or infection. Development or progression cannot 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/adult histology of the zebrafish are available either in internet-accessible databases or in the print literature. Imaging the larger areas of interest presents difficulties, with deformation of the specimen during the preparation process causing distortion, artifacts, and degradation of spatial resolution.[3–5] There is need for a rapid, sensitive, and noninvasive imaging method to follow developmental and other processes, not only of the embryonic phase but also of juvenile and adult stages,

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and to establish an anatomical atlas of adult zebrafish.

Magnetic resonance microscopy (MRM) is an imaging technique that explores the nuclear magnetic characteristics of the protons abundant in tissue.[6] It is a noninvasive, non-ionizing imaging modality that has unique three-dimensional capabilities and allows acquisition of high-resolution images noninvasively in intact opaque animals.[7–10]

MRM is founded on the same fundamental principles as magnetic resonance imaging (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 (a three-dimensional pixel) can be achieved. Combining high resolution MRM with 3D image reconstruction can give possibility to rapidly construct 3D atlas of zebrafish. MRM offers the possibility to image a live adult fish noninvasively, which is impossible using other imaging techniques. With MRM, it is possible to study developmental processes over an extended period of time in an individual fish, from embryo through adulthood. MRM can also be attractive and suitable for functional imaging investigations in zebrafish. With the help of MRM it is also possible to image the blood flow and to do local molecular spectroscopy, such as measuring ATP/ADP concentrations.[6,14] *In vivo* visualization of gene expression by MRI in combination with contrast agents in a living embryo of *Xenopus laevis* has been demonstrated.[5]

High resolution MRM has not yet been exploited for imaging adult zebrafish. The objective of this study was to optimize the MR setup and MRM sequences to visualize high-resolution structural details in adult zebrafish *in vitro*. Because of their very small size compared to a mouse or a rat, imaging adult zebrafish demands high resolution. To achieve this, we used a high magnetic field of 9.4 T. In addition to *ex vivo* studies, a flow-through setup was designed for *in vivo* MRM studies of adult zebrafish. We present the first results of *in vivo* MRM and demonstrate that high magnetic field MRM provides sufficient resolution to get rapid access to anatomical details in adult zebrafish. This paves the way for noninvasively studying disease development, biological pathways, and toxicologic mechanisms during the various developmental stages in individual living zebrafish.

RESULTS

In proton MRM, 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, spin-lattice relaxation time; T_2, spin-spin relaxation time).[15] Local variations in these parameters provide the vivid contrast seen in the images obtained by MRM, and various anatomical details can be clearly seen.

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 × 35 mm, we obtained a spatial resolution of 137 μm. Structures including the brain, intestine, swim

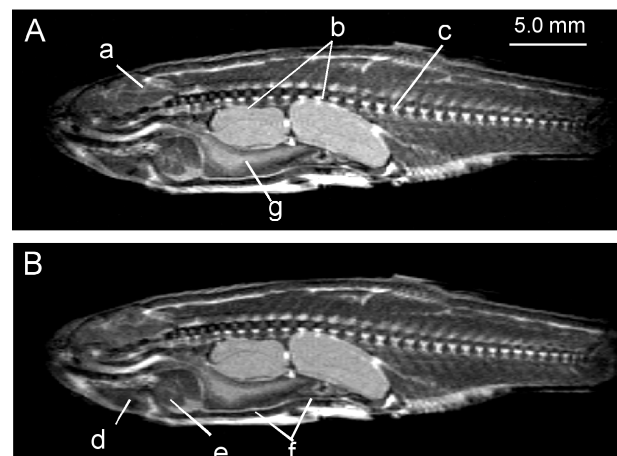

FIG. 1. High resolution images of adult zebrafish at a magnetic field strength of 9.4 Tesla. (**A**) Slices in the sagittal plane were obtained using the multislice multiecho (MSME) pulse sequence (echo time, 15 ms; repetition time, 2000 ms; ns, 4; total scan time, 27 min): a, brain; b, swim bladder; c, horizontal myoseptum; g, ovary. **B**. Slices in the sagittal plane obtained with the rapid acquisition with rapid acquisition with relaxation enhancement (RARE) sequence (echo time, 15 ms with effective echo time, 33.6 ms; repetition time, 2000 ms; number of scan, 4; total scan time, 8 min). The image resolution is 137 μm and slice thickness is 0.2 mm. Legend key: d, heart; e, stomach; f, intestine.

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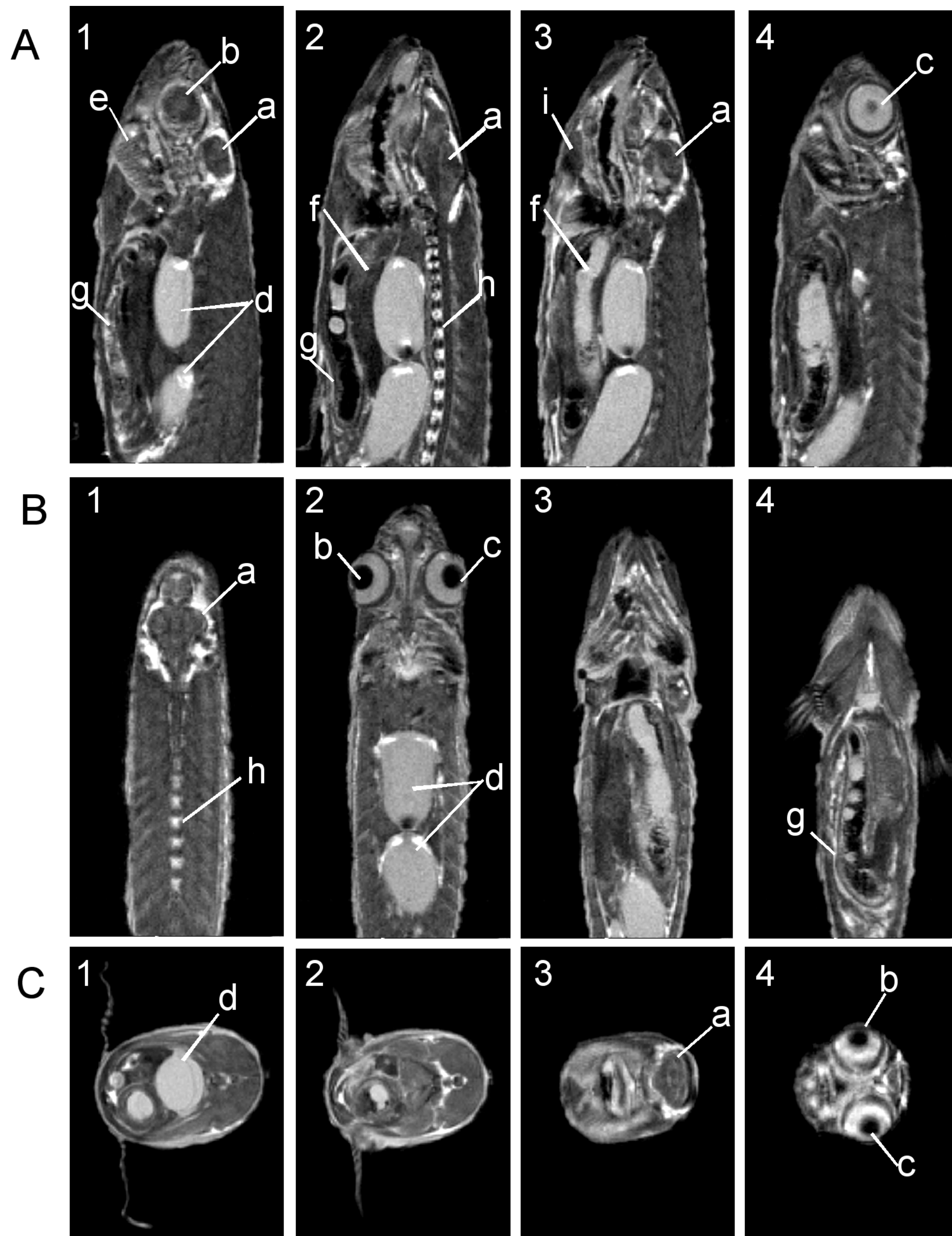

FIG. 2. High resolution images of adult zebrafish at 9.4 Tesla. Successive slices in (**A**) sagittal, (**B**) coronal, and (**C**) axial planes were obtained using the rapid acquisition with relaxation enhancement (RARE) pulse sequence (echo time, 15 ms with effective echo time, 33.6 ms; repetition time, 2000 ms; number of scan, 4; total scan time, 8 min). The image resolution is 78 μm and slice thickness is 0.2 mm. Legend key: a, brain; b, left eye; c, right eye; d, swim bladder; e, gills; f, ovary; g, intestine; h, horizontal myoseptum; i, heart.

bladder, and myoseptum can be clearly recognized (Fig. 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 MRM 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 single-phase encoding value per repetition time (T_R). The primary contrast is T_2-based. Sagittal images of adult zebrafish were obtained with the RARE sequence (Fig. 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 number of averages for imaging living zebrafish.

Four successive slices of adult zebrafish were obtained in the sagittal (Fig. 2A), coronal (Fig. 2B), and axial planes (Fig. 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 1 and 2 were obtained using formalin-fixed adult zebrafish. The formalin fixation process alters tissue characteristics by forming crosslinks between proteins or protein and nucleic acids, and by creating hydroxymethylene bridges and binding of calcium ions.[16] 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 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. 1) and unfixed (Fig. 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 3 was obtained using the same parameters as the images of the fixed fish, with an image resolution of 78 μm.

In vivo *imaging study*

On the basis of these optimized protocols for *ex vivo* imaging of zebrafish, we extended our

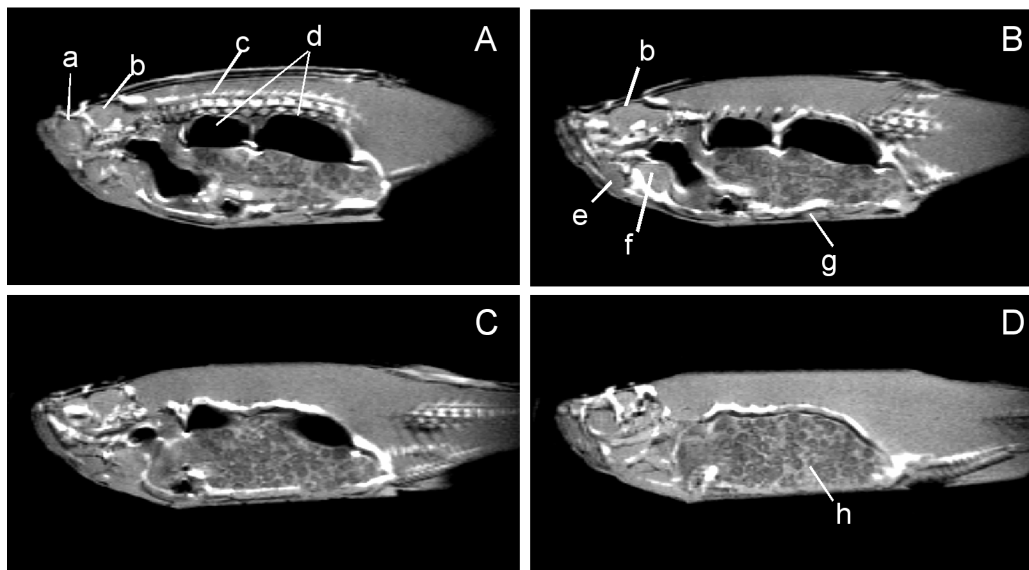

FIG. 3. High resolution images of successive slices (**A–D**) in the sagittal plane through the head of a fresh *ex vivo* unfixed adult zebrafish obtained by rapid acquisition with relaxation enhancement (RARE) pulse sequence at a magnetic field strength of 9.4 Tesla. The image resolution is 78 μm. Slice thickness 0.2 mm. Legend key: a, eye; b, brain; c, horizontal myoseptum; d, swim bladder; e, heart; f, stomach; g, intestine; h, eggs.

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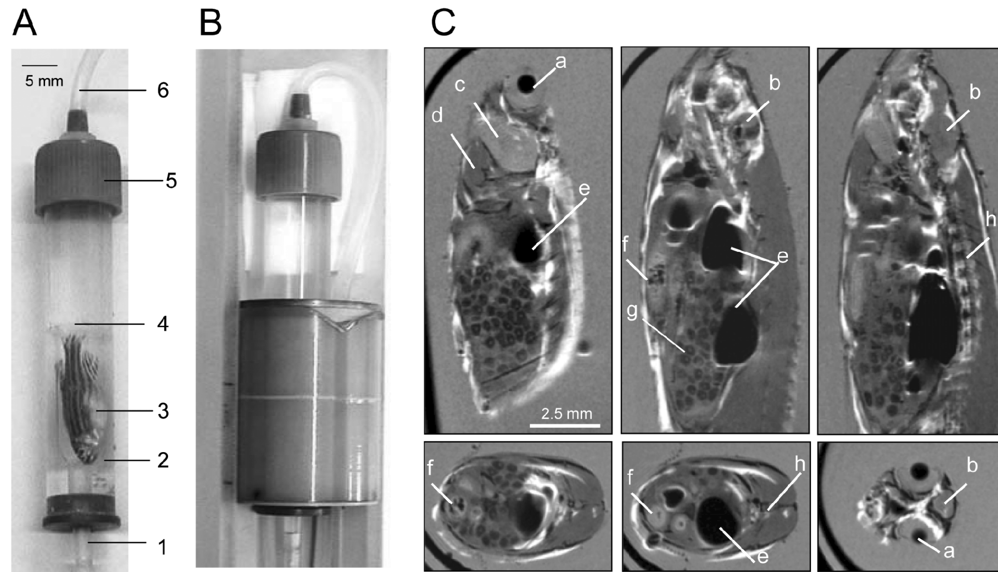

FIG. 4. (**A**) Design of flow-through chamber for *in vivo* MRI measurements of living adult zebrafish. 1, water inlet; 2, U-shaped PVC holding chamber into which water enters from the bottom near the mouth of the fish; 3, a specimen adult zebrafish; 4, a variable slide barrier with a hole at the bottom, to fit the size of the fish; 5, chamber closet; 6, water outlet. (**B**) Flow-through chamber fitted into the volume coil of a microimaging probe. (**C**) MRI images of anaesthetized living adult zebrafish obtained at 9.4 Tesla. Slices in sagittal (upper row) and coronal (lower row) planes were obtained using the rapid acquisition with relaxation enhancement (RARE) pulse sequence (echo train length, 4; echo time, 10.5 ms with effective echo time, 22.5 ms; repetition time, 1000 ms; number of scan, 2; total scan time, 128 sec). The image resolution is 78 μm. Slice thickness is 0.5 mm. Legend key: a, eye; b, brain; c, gills; d, heart; e, swim bladder; f, intestine; g, eggs; h, horizontal myoseptum.

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studies to image living adult zebrafish using MRM. 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.[17] 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.[17–20]

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 can be excluded if the flow rate of outside water is higher in comparison to the repetition time used for spin echo sequences.[19] 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. 4A. The chamber was fitted into a cylindrical resonator for a homogeneous excitation profile (Fig. 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. 4C). The flow of water to irrigate the gills of the zebrafish was kept low (20 mL/min) during the measure-

ments. 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 fishes.

Three-dimensional image reconstruction

In order to emphasize various anatomical structural components and the 3D continuity of these structures, the images are annotated from MRM image slices and as a result a 3D model is derived. Figure 5 shows a reconstructed three-dimensional image of zebrafish obtained from a series of two-dimensional MR slices using TDR-3Dbase software.[21] 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-3Dbase from histological sections is available,[22,23] 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* MRM images.

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.[24] 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 MRM.

Our results presented in this paper show that high field MRM provides sufficient resolution to get rapid anatomical details in adult zebrafish *ex vivo* as well as *in vivo*. Thus high-resolution MRM 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 MRM 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.

MATERIALS AND METHODS

Zebrafish

Adult wild-type zebrafish (*Danio rerio*) were maintained in recirculating aquarium systems according to established rearing proce-

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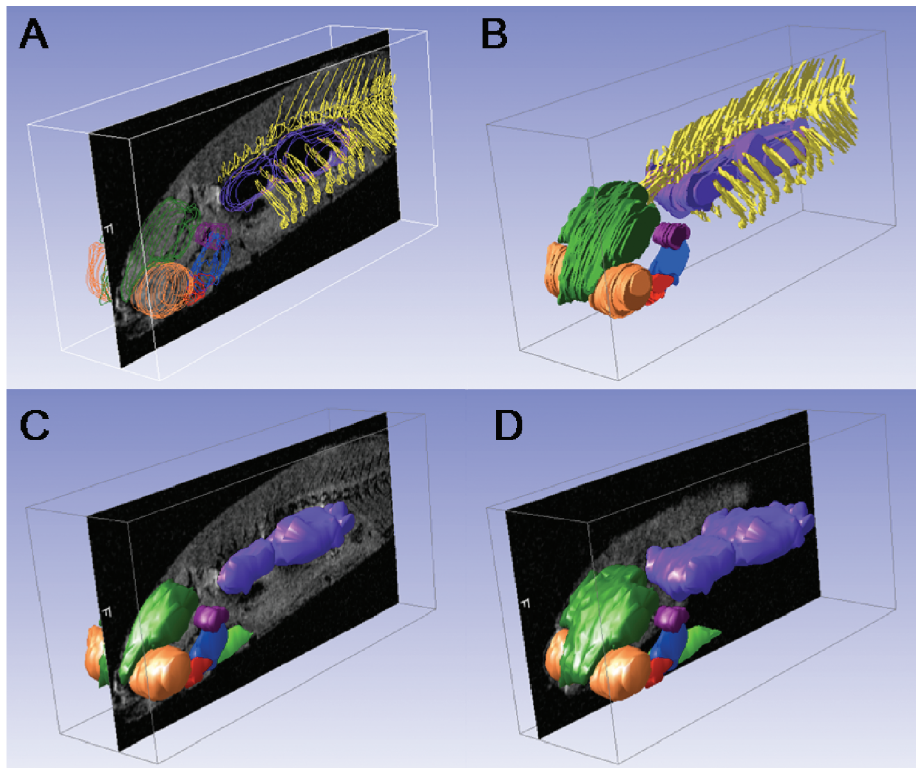

FIG. 5. Three-dimensional visualization of parts of the anatomy of the adult zebrafish reconstructed from 25 magnetic resonance (MR) slices (0.2 mm thick). The slices in the three-dimensional image were annotated with TDR-3Dbase. (**A**) The original contours are shown as projected on a slice through the midline of the fish. (**B**) The major anatomical structures are visualized as pseudo-voxels. (**C**) A surface visualization is projected on a mid-sagittal slice. (**D**) A surface visualization is projected on a lateral sagittal slice. Pleural ribs and vertebral hemal and neural arches (yellow); brain (green); swim bladder (purple); otic capsule (violet); eye (salmon); heart (red); liver (blue); gut (light green).

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dures.[25,26] The water temperature was maintained at 28°C with a flow rate of 150 L/min, with day/night light cycles (12 h dark, 12 h light). The fish were fed twice daily with commercial flake food.[26] 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, the fish was anesthetized by adding 0.01% MS222 (ethyl meta aminobenzoate metanesulfonic acid salt; Sigma Chemical Co.) to pH-controlled water. Subsequently the fish was transferred to a closed flow-through chamber, which was designed for continuous flow of aerated water to support a live zebrafish inside the magnet. The flow-through setup was then inserted in the center 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 anesthetic was pumped from a temperature-controlled aquarium to a tube fixed on the lower end of the flow-through cell close to the mouth of the fish. After passing through the chamber, the water was transported back to the aquarium. The setup allowed direct *in vivo* MR measurements at a constant flow speed (20 mL/min) that was regulated by a STEPDOS 03/08 pump (KNF Flodos AG, Switzerland). After the MRI measurements, the zebrafish was transferred back to an aquarium without anesthetic, where the fish recovered uneventfully from the experimental treatment.

Magnetic resonance microscopy

MR imaging was performed using a 400 MHz (9.4 T) vertical bore system, using a 20

mm volume coil and a 1 Tm^{-1} gradient insert (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 to determine position and select the desired region for subsequent experiments.

Various MRI scan protocols were used and optimized for high resolution imaging of zebrafish. T_2-weighted MSME and RARE[27] sequences were used for the *ex vivo* imaging of adult zebrafish. For *in vivo* imaging, RARE sequence was used. The FOV was varied from 1 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 Linux XWinNMR 3.2.

Three-dimensional reconstruction

A three-dimensional image of adult zebrafish was reconstructed from a series of two-dimensional MR slices with the help of TDR-3Dbase 3-D reconstruction software.[21] The images are imported into the TDR-3Dbase software as separate two-dimensional images while keeping the coherence of the stack. Using the TDR-3Dbase software allows building a contour model by delineating the structures of interest either manually by digitizer tablet or through an 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 into TDR-3Dbase software. A selection of anatomical domains, including brain, heart, liver, and swim bladder, was delineated in the images. The contours in the stack make up the three-dimensional geometrical model that is reconstructed and visualized as a three-dimensional rendering.

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