Supplement:

Three-dimensional NMR Microscopy of Zebrafish Specimens

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Fixation

To assess fixation completeness we used imaging and spectroscopy. The fish swim bladder, normally filled with gas, in the course of fixation fills with liquid (Fig. S1 left). To make sure the fixative has penetrated the bladder we recorded 1H PRESS spectrum (Fig. S1 right). The swim bladder completely fills with liquid within 4 days (Fig. S1 left) and narrow lines in the 1H spectrum at 3.2 ppm and 8.3 ppm show presence of fixative (Fig S1. right). Since the swim bladder is in the middle of the fish body it is reasonable to assume that if fixative arrived to the bladder then it also reached all other tissues.

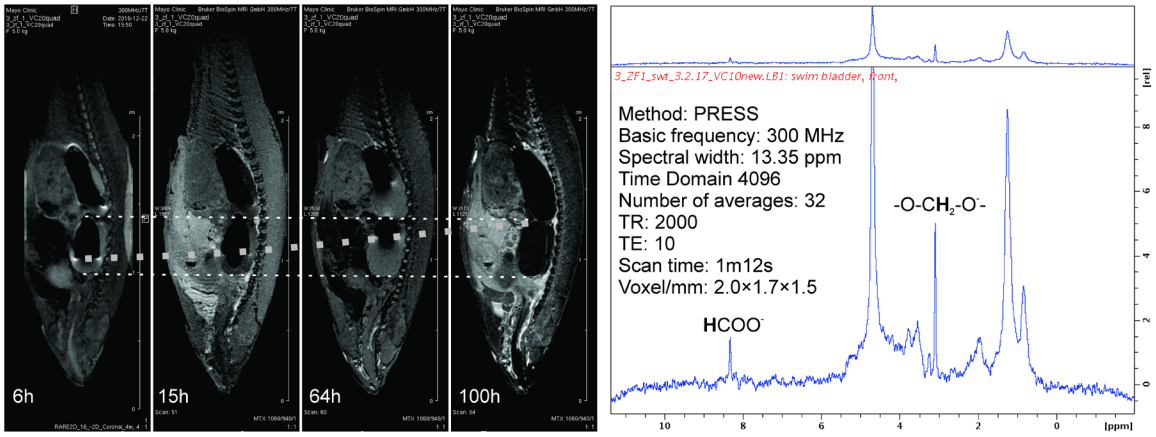

Figure S1 Left: central sagittal slice through a series of 3D images recorded at an average time from start of the fixation indicated at the bottom left in each panel. After ~ 4 days the bladder is filled with liquid. Dashed lines indicate top and bottom of the bladder and dotted line the rise of liquid meniscus. Images are recorded with different methods and resolution, therefore contrast/resolution are incomparable. **Right:** 1H PRESS (300 MHz) of fixed zebrafish swim bladder: Sharp lines at 3.2 ppm and 8.3 ppm are from fixative (-CH2- and formate, respectively). The fixative hydroxyls (~ 5.2 ppm) are not clearly visible because of their fast exchange and proximity to the water line.

Formalin fixative is rather complex mixture of various formaldehyde hydrate derivatives, (monomer hydrates (methylene glycol, HO-CH_2-OH) partially polymerized hydrates (polymethylene glycol, paraformaldehyde, $HO(CH_2O)_nH$) methylated, oxidized (formic acid)etc.). Most of the derivatives are in dynamic equilibrium, thus identification of any within the specimen could be used as an indicator of the fixative penetration. In the PRESS spectrum shown in Fig. S1 right, one can easily identify the formate

line (8.3 ppm), and the methylene line (3.2 ppm) which confirms that the fixative has reached the middle of the specimen (swim bladder).

Specimen mounting

To improve shimming the formalin fixed (FF) specimens (Fig S2A) are scanned using 10 mm thin wall NMR tubes (Fig. S2B, C). A Teflon plug is inserted at the bottom of the NMR tube to keep the specimen in the center. The specimen is further secured within the tube by a custom cut U-shaped Teflon sheet (0.7 mm thick) (Fig. S2B, C) and then the tube is filled with Fluorinert. With such an arrangement we were able to achieve the water linewidth better than 150 Hz across the specimen (linewidth within the swim bladder: 20 Hz for water and 7 Hz for CH_2 groups from fixative).

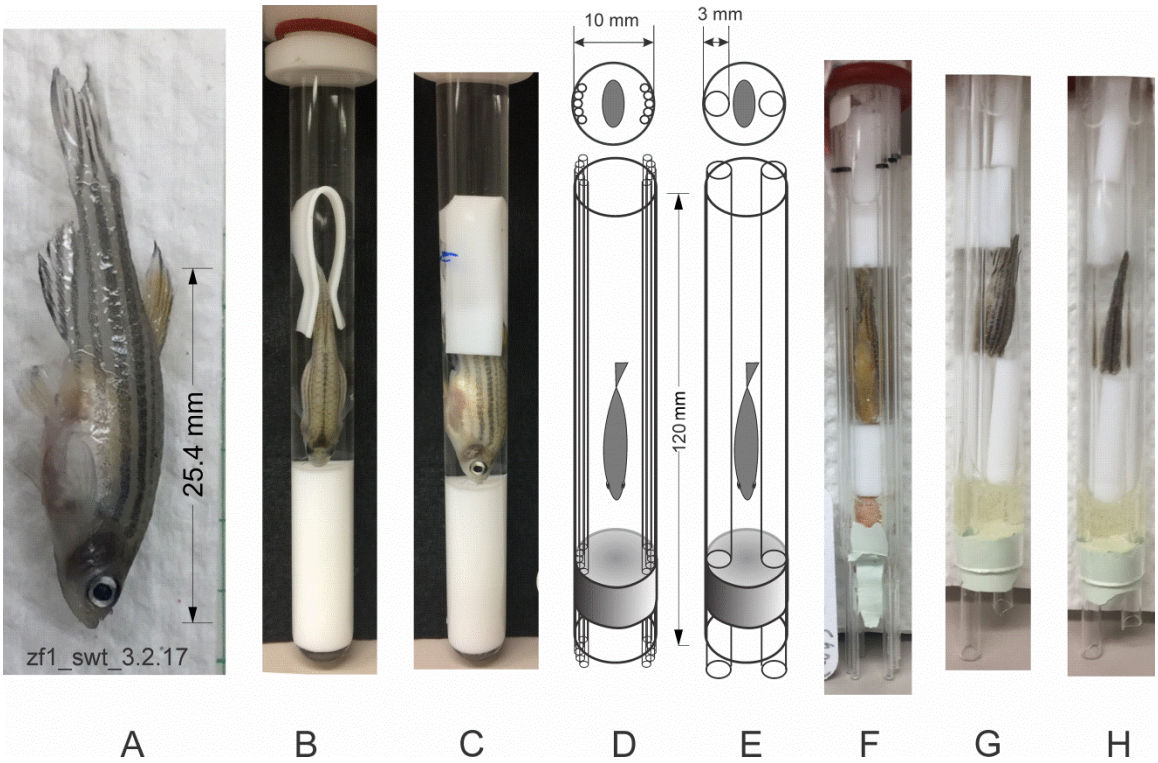

Figure S2 Fixed specimen (A) was placed into 10 mm NMR tube (B, C) above the Teflon plug which serves as a spacer to keep the specimen away from the air/ tube border. To enhance airflow for temperature control, for imaging the FFPE specimens (at 60° C) or unfixed specimen (at 4° C) the two variable temperature chambers are designed (D, E). Panels F,G,H show photos of the chambers with FFPE specimens inserted.

The formalin fixed paraffin embedded (FFPE) specimens need to be scanned above 55 C. To enable flow of conditioned air around the specimen when placed in 10 mm RF coil, custom made chambers are used. From 10 mm NMR tube bottom is cut out and a series of capillaries (diameter 1.4 mm) five at each side are glued to the inner wall of the tube (Fig. S2D,F). The bottom of the chamber is sealed with Vinyl Polysiloxane (AquaSilUltra, dental impression composite) and additionally leak-proof with cyanoacrylate glue. Much easier to manufacture is a chamber with just two (3 mm) capillaries, Fig. S2E,G,H. This

chamber provides much higher airflow, is easier to make leak proof but due to smaller working volume can't accommodate big specimens. Before inserting into the chamber, the FFPE specimen is warmed up and while melted released of excessive paraffin. Then, like FF specimen it is placed into the chamber above the Teflon plug. Few Teflon pieces inserted above the specimen kept it from floating upon addition of high temperature Fluorinert (FC-40, 3M). Besides improving magnetic field homogeneity Fluorinert accelerates heat transfer from conditioned air to the FFPE specimen. Figures S2F,G,H show photos of the chambers with FFPE specimens inserted.