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## Aldehyde Fixative Solutions Alter the Water Relaxation and Diffusion Properties of Nervous Tissue

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### Abstract

Chemically-fixed nervous tissues are well-suited for high-resolution, time-intensive MRI acquisitions without motion artifacts, such as those required for brain atlas projects, but the aldehyde fixatives used may significantly alter tissue MRI properties. To test this hypothesis, this study characterized the impact of common aldehyde fixatives on the MRI properties of a rat brain slice model. Rat cortical slices immersion-fixed in 4% formaldehyde demonstrated 21% and 81% reductions in tissue  $T_1$  and  $T_2$ , respectively ( $P < 0.001$ ). The  $T_2$  reduction was reversed by washing slices with phosphate-buffered saline (PBS) for 12 h to remove free formaldehyde solution. Diffusion MRI of cortical slices analyzed with a two-compartment analytical model of water diffusion demonstrated 88% and 30% increases in extracellular apparent diffusion coefficient ( $ADC_{EX}$ ) and apparent restriction size, respectively, when slices were chemically-fixed with 4% formaldehyde ( $P \leq 0.021$ ). Further, fixation with 4% formaldehyde increased the transmembrane water exchange rate 239% ( $P < 0.001$ ), indicating increased membrane permeability. Karnovsky's and 4% glutaraldehyde fixative solutions also changed the MRI properties of cortical slices, but significant differences were noted between the different fixative treatments ( $P < 0.05$ ). The observed water relaxation and diffusion changes help better define the validity and limitations of using chemically-fixed nervous tissue for MRI investigations.

### Keywords

formaldehyde; glutaraldehyde; water exchange; brain slice; mathematical model

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Magnetic resonance imaging (MRI) or microscopy studies of chemically-fixed biological samples have become a common experimental paradigm (1). The aldehyde fixative typically used for MRI investigations, formaldehyde, achieves tissue fixation by cross-linking protein amine groups via methylene bridges to render tissues metabolically and structurally stable

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(2). These sample properties permit long scan times and thus, chemically-fixed samples are well-suited to high-resolution, multidimensional MRI acquisition schemes that are devoid of motion or flow artifacts. Further, with chemically-fixed samples, it is often possible to isolate tissues or organs of interest, then employ high-field magnets with smaller, sample-specific radiofrequency coils to improve signal-to-noise ratio (SNR) per unit time. These advantages have been exploited for the creation of MRI rodent brain atlases (3–5).

Despite the widespread use of chemically-fixed tissue samples for high-resolution MRI studies, there remains no consensus in the MRI community on proper or acceptable fixation methods, nor is much known about how aldehyde chemical fixative solutions alter the tissue microstructures responsible for the MRI properties of tissue. By cross-linking proteins throughout the intra- and extracellular spaces of tissues, aldehyde fixatives may alter the local chemical environment sampled by water molecules during the MRI acquisition. In addition, although aldehyde fixatives do not react avidly with most lipid molecules, aldehydes will cross-link membrane proteins to each other and/or to adjacent proteins located in the intra- and extracellular spaces (2); this may have a significant impact on the integrity of the cellular membranes. These biophysical changes to the tissue microstructure may alter MRI contrast mechanisms and could lead to erroneous extrapolations of fixative-based data to in vivo MRI studies.

Previous studies, for example, have demonstrated reductions to rat and human nervous tissue  $T_1$  or  $T_2$  after immersion into 4% formaldehyde solutions (6–13). Several reports also have suggested that aldehyde fixatives significantly decrease mean water diffusivity in nervous tissue (14–17). There are conflicting reports whether aldehyde fixatives significantly reduce (16) or do not affect diffusion anisotropy in nervous tissue (11,14,15,17). However, with one exception, these prior studies were confounded by the collection of MRI data from fixed tissue samples that remained in their respective aldehyde fixative solutions, thus failing to distinguish the MRI effects of the free fixative solution vs. the additive chemical reactions of aldehyde fixatives on the tissue itself (the results of the present study demonstrate the importance of this distinction). Previous diffusion MRI studies of fixative effects compared MRI of in vivo samples (i.e., at 37°C) to ex vivo fixed tissues imaged at room temperature (11,14–17), but sample temperature differences alone may reduce tissue water diffusion significantly (18). In addition, studies of how fixative affects tissue MRI properties that have involved human tissue may be complicated by the variable postmortem interval prior to tissue fixation (19,20) and from the possible autolysis that may occur (21) during the significant delay for fixative penetration into gross human nervous tissue samples (2,10,13). Given these limitations to the previous literature, there is still a need for well-controlled, hypothesis-driven investigations into how fixative affects the MRI properties of nervous tissue.

In an attempt to address these concerns, we recently demonstrated that aldehyde fixative solutions significantly alter the relaxation and diffusion properties of a tissue model composed of gel-immobilized erythrocyte ghosts (18). This simplified tissue model reproduced the restrictive effects of cellular membranes, but does not model other aspects of in situ nervous tissue, including the intracellular and extracellular components, as well as the complex three-dimensional (3D) arrangement of heterogeneous cell shapes, sizes, and types normally found within nervous tissue. To further investigate the effects of aldehyde fixatives on the MRI properties of tissue, we now have extended our investigation to a rat cortical brain slice model of nervous tissue (22,23) using aldehyde fixative solutions commonly employed in MRI and histopathology studies. This study characterized the  $T_1$ ,  $T_2$ , and water diffusion properties of rat cortical brain slices before and after chemical fixation with 4% formaldehyde, 4% glutaraldehyde, or a modified Karnovsky's solution (2% formaldehyde and 2% glutaraldehyde) (24). The study results indicate that commonly-employed aldehyde

fixative solutions significantly alter the MRI properties of nervous tissue, albeit in different ways from the changes reported in the erythrocyte ghosts tissue model or in the previous literature. The characterization of these changes in rat cortical brain slices improves our understanding of the validity (and limitations) for MRI studies of chemically-fixed nervous tissue samples.

## Materials and Methods

### Rat Cortical Slice Procurement

The use of laboratory rats for this study was approved by the University of Florida Institutional Animal Care and Use Committee. Rat cortical slice procurement has been described previously (22). Male P<sub>30</sub> Long-Evans rats (75–100 g) were anesthetized with isoflurane, then decapitated. The brain was removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) (120 mM NaCl, 3 mM KCl, 10 mM glucose, 26 mM NaHCO<sub>3</sub>, 2 mM CaCl<sub>2</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, and 1.4 mM MgSO<sub>4</sub>) gas-equilibrated with 95% O<sub>2</sub>/5% CO<sub>2</sub> to maintain a pH of 7.4. The ACSF osmolarity was 290 ± 1 mOsm/kg as determined by Osmette A freezing point depression osmometer (Precision Systems, Inc., Natick, MA, USA). The rostral and caudal ends of the brain were removed with gross coronal cuts, then 500- $\mu$ m-thick coronal sections were cut using a Vibratome (VT 1000S; Leica Microsystems, Wetzlar, Germany). The cortical ribbons from each hemisphere were trimmed with a scalpel (using cuts perpendicular to the convexity of the cortical surface) into 3 × 4-mm segments.

These cortical slices remained immersed in ice-cold ACSF for 1 h after rat sacrifice to minimize procurement-induced ischemic damage (25). Slices then were equilibrated gradually to room temperature and placed into a multislice perfusion chamber for MRI measurements. To maintain tissue viability, slices were perfused continuously with fresh ACSF (2 ml/min) while the perfusion chamber was lowered into the magnet, during pilot image acquisition and between MRI measurements (26). No perfusion occurred during data collection.

### Aldehyde Fixation of Rat Cortical Slices

After procured rat cortical slices had equilibrated with room temperature, some slices were used for viable imaging experiments (described below) and some slices from each rat were immersed in various aldehyde fixative solutions. This study design used immersion fixation methods to control for the effects of slice procurement in the viable, unfixed treatment group and to model the treatment of human autopsy or biopsy samples. However, it should be noted that perfusion and immersion fixation methods may affect the MRI properties of tissue differently. The fixative solutions consisted of phosphate-buffered saline (PBS) (290 mOsm/kg) with 4% formaldehyde, 4% glutaraldehyde, or 2% formaldehyde plus 2% glutaraldehyde (referred to as Karnovsky's solution) (24). All solutions had a pH of 7.4. The cortical slice samples were immersed in a volume excess of their respective fixative solutions (>100:1) at room temperature for 3–4 h, then stored in a similar volume of fresh fixative solution at 4°C for 10+ days to complete the chemical reactions of fixation. After this period, the slices were gradually equilibrated to room temperature, then imaged in the perfusion chamber while immersed in their respective fixative solutions. After these MRI measurements (described below), the samples were washed over 12 h with four to five PBS solution changes at room temperature and then reimaged using the perfusion chamber setup while immersed in PBS.

### MRI Experiments in Rat Cortical Slices

MRI data were obtained at room temperature using a 10-mm birdcage coil interfaced to a Bruker 17.6-T vertical magnet and console with 1000 mT/m imaging gradients. Pilot multislice axial, sagittal, and coronal T<sub>1</sub>- and diffusion-weighted imaging sequences were

used to optimize the positions of 300- $\mu\text{m}$ -thick axial MR-defined slices through the center of the 500- $\mu\text{m}$ -thick rat cortical slices. The imaging protocol consisted of water diffusion measurements at four diffusion times ( $T_d$ ) along with  $T_1$  and  $T_2$  measurements (described below). All images for MRI measurements had limited in-plane resolution (matrix size =  $128 \times 64$ , FOV = 15 mm) to maximize the SNR while reducing the time required per scan. This resolution was sufficient to manually draw rectilinear regions-of-interest (ROIs) for each cortical slice that excluded ACSF perfusate, the molecular layer of the cortex, and any apposed white matter from the corpus callosum or external capsule. Water diffusion measurements in cortical slices employed a pulsed-gradient spin-echo multislice sequence with 12 diffusion-weighted images using diffusion gradients oriented parallel to the tissue surface (0–950 mT/m) and  $T_d$ s of 10, 20, 35, and 50 ms ( $\delta = 3$  ms). Diffusion gradient strengths were employed so that each  $T_d$  measurement yielded images with  $b$ -values between 7 and 15,000  $\text{s}/\text{mm}^2$  (including imaging cross-terms). Diffusion measurements had two averages with a 1.5-s repetition time and echo time (TE) was minimized with respect to  $T_d$  (TE = 24, 34, 49, and 64 ms, respectively). The measured diffusion coefficient of water ( $1.94 \pm 0.04 \text{ ms}^{-1}\mu\text{m}^2$ ) in the ACSF or PBS solutions using this protocol was similar to published values at room temperature (27) indicating accurate gradient calibrations for the diffusion MRI measurements.

$T_1$  values were determined from a partial saturation experiment using 10 logarithmically-spaced repetition times between 75 ms and 10 s (TE = 10 ms).  $T_2$  values were estimated with a multiecho sequence (TR = 10 s) using 30 consecutive 10-ms echo images. Preliminary experiments demonstrated that this method of  $T_2$  measurement is not significantly influenced by water diffusion at room temperature. Further, the  $T_1$  and  $T_2$  relaxation rates were similar to previously unpublished values for water in PBS or ACSF solutions at 17.6-T. SNR for the relatively proton-density-weighted images (TR = 10 s, TE = 10 ms) obtained were calculated as the mean signal in the cortical slice (minus the mean noise signal) divided by the standard deviation (SD) of the noise signal. Note, these “relative proton density” determinations may be confounded by fixative-induced changes to spin populations with very short  $T_2$ s.

Measurement for each diffusion time took 38 min for completion, the  $T_1$  measurements required 50 min, and the  $T_2$  measurements required 10 min. Viable slices were perfused with fresh ACSF equilibrated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  for 8–10 min between each measurement (26). Additional multislice sagittal and coronal diffusion-weighted pilot images were acquired between MRI measurements to monitor for slice movement due to ACSF perfusion. Rat cortical slices that were chemically-fixed in aldehyde solutions did not require perfusion.

### Analytical Diffusion Model

Diffusion and  $T_2$  MRI data were analyzed using a two-compartment model of water diffusion with transmembrane water exchange that was validated previously in bovine optic nerve, human erythrocyte ghosts, and rat and human cortical brain slices (28–32). In this model, the cortical tissue was represented as a system consisting of cells surrounded by extracellular water. Diffusion inside cells was assumed to be restricted with apparent restriction size,  $a$ ; this results in a reduced water mobility with the apparent diffusion coefficient,  $\text{ADC}_{\text{IN}}$ . Water diffusion in the extracellular compartment ( $\text{ADC}_{\text{EX}}$ ) was mediated by tortuosity. The transverse relaxation times of intra- and extracellular water were allowed to differ. Water exchange between the intra- and extracellular compartments was described with the exchange rate constants  $k_{\text{IE}}$  and  $k_{\text{EI}}$ , respectively. The intracellular magnetization fraction ( $M_{\text{IN}}$ ) could also be determined by the model fitting—it should be noted that this is not equivalent to the intracellular volume fraction because of intra- and extracellular differences between proton concentrations and  $T_2$  decay rates. A more detailed

explanation of the model can be found elsewhere (31). In very high SNR datasets, cortical slices demonstrate minimal diffusion anisotropy (fractional anisotropy [FA]  $\approx 0.1$ ) (33). This anisotropy is within the noise of the diffusion data collected here (SNR  $\approx 15:1$ ) such that it will not influence the model parameter fits obtained (as discussed in Ref. 31). All model fitting was performed using the sequential quadratic algorithm of the MATLAB software package (The MathWorks, Inc., Natick, MA, USA).

### Statistical Analysis of MRI Data

The sample number in the different treatment groups for characterizing water relaxation in rat cortical slices were sufficient for statistical analysis: control ( $N = 18$ ), immersed in 4% formaldehyde ( $N = 13$ ), immersion in Karnovsky's solution ( $N = 12$ ), immersion in 4% glutaraldehyde ( $N = 12$ ), washed after fixation in 4% formaldehyde ( $N = 13$ ), washed after fixation in Karnovsky's solution ( $N = 11$ ), and washed after fixation in 4% glutaraldehyde ( $N = 12$ ). Sample numbers for the diffusion MRI experiments are reported in Table 1. Three of the 58 cortical slices analyzed with the two-compartment diffusion model had extreme outlying fits that were excluded from further analysis using Chauvenet's criterion (one exclusion each from the control, 4% formaldehyde, and Karnovsky's solution treatment groups). The various MRI parameters then were compared statistically between the treatment groups using a one-way analysis of variance (ANOVA) with post-hoc Tukey multiple comparisons tests using SigmaStat 2.03 (Systat Software Inc., San Rafael, CA, USA). Statistical significance was predetermined at  $P < 0.05$ .

## Results

Chemical fixation with solutions containing glutaraldehyde led to a pale yellow color change in the rat brain slices within 1 h of slice immersion. Samples fixed in glutaraldehyde solutions (even after overnight washing in PBS) remained rigid and dense relative to viable slices or slices fixed only with formaldehyde. Prior to immersion fixation, the molecular layer appeared distinct compared to the remaining cortical layers of the rat cortical slices (Fig. 1). The lateral edges of individual slices also demonstrated increased water diffusivity and decreased  $T_2$  due to the procurement process. Note that fixation in any of the aldehyde solutions obscured these visual differences (Fig. 1). These regions were excluded in the ROI used for quantitative analysis of the data. Otherwise, there were limited visual MRI image differences for cortical slices imaged prior to fixation or after fixation with the various solutions and overnight washing with PBS. The subsequent MRI property differences due to fixative treatments became more readily apparent through quantitative analysis.

The  $T_1$  values for viable cortical slices ( $2.020 \pm 0.050$  s) were statistically different from all other treatment groups ( $P < 0.001$ ) (Fig. 2). After immersion fixation, the  $T_1$  decreased 21% with 4% formaldehyde fixation, 26% for Karnovsky's solution fixation, and 25% for 4% glutaraldehyde fixation (all comparisons,  $P < 0.001$ ). Slices immersed in 4% formaldehyde had  $T_1$  values postfixation ( $1.60 \pm 0.04$  s) that were 6% higher than slices fixed in Karnovsky's solution ( $P < 0.001$ ) and 5% higher than slices fixed in 4% glutaraldehyde ( $P = 0.001$ ). The  $T_1$  of slices fixed in Karnovsky's solution did not differ significantly from slices fixed in 4% glutaraldehyde. Washing slices fixed in 4% glutaraldehyde overnight with PBS increased the slice  $T_1$  by 10% to  $1.66 \pm 0.03$  s ( $P < 0.001$ ). Washing overnight did not significantly change the  $T_1$  for slices fixed in 4% formaldehyde or Karnovsky's solution ( $P > 0.05$ ). After washing, the  $T_1$  of slices fixed in Karnovsky's solution ( $1.53 \pm 0.06$ ) were 8% lower than slices treated with both the other fixatives ( $P < 0.001$ ).

The  $T_2$  values for viable cortical slices ( $0.098 \pm 0.006$  s) also were statistically different from all other treatment groups ( $P \leq 0.01$ ) except compared to samples fixed in Karnovsky's solution and washed with PBS overnight ( $P = 0.987$ ) (Fig. 3). Immersion fixation reduced

rat cortical slice  $T_2$  by 81% for slices in 4% formaldehyde, 64% for slices in Karnovsky's solution and 54% for slices in 4% glutaraldehyde (all comparisons,  $P < 0.001$ ). The  $T_2$  of slices immersed in the three different fixative solutions were all statistically different from each other ( $P < 0.01$ ) such that  $T_2$  decreased approximately 6.5 ms for each 1% addition of formaldehyde to the final fixative solution (range = 0–4% formaldehyde,  $R^2 = 0.983$ ). Hence, slices in 4% formaldehyde had the lowest  $T_2$  ( $0.019 \pm 0.002$  s). Washing immersion-fixed cortical slices overnight in PBS significantly increased  $T_2$  in all fixative treatment groups (all comparisons,  $P < 0.001$ ) such that the  $T_2$  of slices fixed in Karnovsky's solution and washed overnight in PBS no longer differed significantly from unfixed, viable slices ( $P = 0.987$ ). However, the  $T_2$  of washed slices originally fixed in 4% formaldehyde ( $0.117 \pm 0.015$  s) or 4% glutaraldehyde ( $0.107 \pm 0.003$  s) were 19% and 9% higher than the  $T_2$  of viable cortical slices, respectively (both comparisons,  $P \leq 0.01$ ). After washing, slices originally immersed in the three different fixative solutions were again all statistically different from each other ( $P < 0.01$ ).

Aldehyde fixation also reduced the SNR measured in proton-density-weighted images. For example, SNR was reduced 37% by immersion in 4% formaldehyde ( $P < 0.001$ ), while subsequent washing of the slices fixed with 4% formaldehyde restored SNR to 86% of prefixation values ( $P < 0.001$ ). Similar SNR reductions were observed in slices immersed in glutaraldehyde solutions (e.g., 32% decrease with Karnovsky's solution,  $P < 0.001$ ). Unlike slices fixed in 4% formaldehyde, however, there was no significant SNR improvement after washing the glutaraldehyde-fixed samples overnight in PBS. This low SNR observed in the proton density images and the short  $T_2$  of slices still immersed in their respective fixation solutions was most evident in diffusion-weighted images, especially at high  $b$ -values and long diffusion times. Hence, the SNRs of diffusion MRI data of rat brain slices immersed in the three fixative solutions were not sufficient for acceptable fits of the two-compartment diffusion model. Similar SNR limitations for diffusion analysis were reported previously in a study of a single human brain immersed in fixative (12).

The two-compartment model of water diffusion fit the data well (Fig. 4) and indicated that aldehyde fixation had several significant effects on the cortical slice microstructures sampled by water diffusion (Table 1). It was not possible to accurately determine the intracellular diffusion coefficient due the effects of restriction in cortical slices even at relatively short diffusion times of 10 ms (31,33). Fixation with 4% formaldehyde increased the  $ADC_{EX}$  of cortical slices by 88% compared to viable, unfixed tissue ( $P = 0.019$ ). Slice fixation with Karnovsky's solution also increased  $ADC_{EX}$  by 47%, although this change was not statistically significant ( $P = 0.377$ ), probably due to variability in the fits of this model parameter. Slice fixation in 4% glutaraldehyde also did not significantly change  $ADC_{EX}$ . Fixation with all three solutions increased the apparent restriction size ( $a$ ) in the rat cortical slices 30% to 40% from viable tissue values ( $P \leq 0.021$ , all comparisons). The cortical slice transmembrane water exchange rate ( $k_{IE}$ ) was increased by fixation in 4% formaldehyde and Karnovsky's solutions by 239% and 244%, respectively, from control tissues ( $P < 0.001$ , both comparisons). This corresponded to a reduction in the mean intracellular residence time of water (equivalent to  $1/k_{IE}$ ) from 61 ms in viable slices to 18 ms in cortical slices fixed using formaldehyde-containing solutions. The exchange rate in slices fixed with 4% glutaraldehyde, however, did not differ significantly from control slices, but was statistically different from the other two fixation treatments ( $P < 0.001$ , both comparisons). In contrast, 4% glutaraldehyde significantly reduced the intracellular magnetization fraction ( $M_{IN}$ ) by 25% from viable, unfixed cortical slices ( $P = 0.009$ ) whereas the other two fixation solutions did not alter  $M_{IN}$  significantly compared to viable slices (although these too differed significantly from the 4% glutaraldehyde-treated slices).

## Discussion

### Aldehyde Fixatives Alter Water Relaxation in Rat Cortical Slices

The protocol for fixation of cortical slices was designed to achieve rapid cessation of metabolic activity (initial room temperature immersion) and irreversible chemical fixation prior to the MRI experiments (10+ days of fixation). Previous studies (10,13) indicated that formaldehyde immersion fixation may require several weeks prior to human nervous tissue reaching steady-state  $T_1$  and  $T_2$  values. However, brain slices are likely to reach steady-state rapidly given the inherent rates of aldehyde fixative penetration (0.5 to 1 mm/h at room temperature) (34) and the thinness of brain slices (500  $\mu\text{m}$ ) compared to gross human brain samples. Penetration of aldehydes into the rat cortical slices was indicated by the pale-yellow color change observed rapidly after immersion (34). The hemiacetal-like adducts formed when formaldehyde reacts with the  $\epsilon$ -amine groups of lysine and the amide nitrogen in peptide linkages of the tissue are reversible, especially if the tissue is washed within 24 h of initial fixation, but these adducts then form methylene bridges that are chemically stable (2,34). In contrast, mid-chain aldehydes of the polyglutaraldehyde molecule in glutaraldehyde fixation solutions react with proteins to form stronger, irreversible crosslinks immediately. Glutaraldehyde also completes fixation faster than formaldehyde despite its more retarded penetration into the tissue (2). Hence, 10+ days of immersion fixation in the various fixative solutions was considered sufficient to assure stability of the cross-links formed. The subsequent washing of the cortical slices in PBS removed bulk fixative, but did not reverse these additive chemical reactions of fixation.

$T_2$  of the rat cortical slices was reduced 54% to 81% after immersion in aldehyde fixative solutions (Fig. 3). In the formaldehyde concentration range studied, the  $T_2$  decrease correlated with increasing formaldehyde (10–15 ms decrease per 2%).  $T_2$  reductions have been reported previously (6,8). However, this study demonstrates that this reduction is due largely to bulk fixative solution within nervous tissue since  $T_2$  returned to pre-fixation values after the sample was washed overnight in PBS. Similar observations were recently reported in erythrocyte ghosts (18) and in a single macaque brain slice (17). This conclusion is supported by experiments that demonstrated the addition of 4% formaldehyde reduced the  $T_2$  of PBS from 1.94 s to 37 ms, while 4% glutaraldehyde reduced the  $T_2$  of PBS to 61 ms (18).  $T_2$  shortening in aldehyde fixative solutions has been attributed to chemical exchange (35). It was surprising that after overnight washing in PBS,  $T_2$  was prolonged 19% and 9% in cortical slices fixed in 4% formaldehyde or 4% glutaraldehyde, respectively. The biophysical origins of these statistically significant increases are difficult to determine in the present study, but the observed  $T_2$  prolongation may relate to chemical changes to the tissue or possibly to increases in transmembrane water exchange between compartments with different intrinsic water relaxation properties (see below).

Immersion in aldehyde fixative solutions also reduced rat cortical slice  $T_1$  approximately 21% to 26% (Fig. 2). The  $T_1$  of cortical slices fixed in the three fixative solutions only differed by 6%. Similar  $T_1$  reductions after immersion in formaldehyde have been reported previously for tissue samples (6) and for the animal pole of *Xenopus* oocytes (36). Washing out free fixative solution did not substantially increase cortical slice  $T_1$ . Further, the  $T_1$  of a PBS phantom was not significantly affected by the addition of aldehyde fixatives (18). These results suggest the  $T_1$  changes from aldehyde fixation are not due to bulk fixative solution, but from the additive chemical reactions of the aldehyde fixatives. These reactions may increase spins with slower correlation times by increasing the proportion of aliphatic protons and/or increasing hydration layer water (9). Steric restrictions from aldehyde cross-linking reactions also may decrease the correlation time of bulk water (7).

This study demonstrates the SNR benefits of washing free fixative solution from chemically-fixed nervous tissue samples. The 32% to 37% reduction in the SNR of relative proton density MRI of slices immersed in 4% formaldehyde is partially due to the significant  $T_2$  shortening (98 to 19 ms) relative to the echo time of these measurements ( $TE = 10$  ms). The  $T_2$  reductions from the three fixative solutions were largely mitigated by washing cortical slices overnight in PBS solutions. However, relative proton density SNR in 4% formaldehyde-fixed slices was not fully returned after washing when  $T_2$  was restored to near prefixation values. Further, relative proton density SNRs in slices fixed with Karnovsky's solution or 4% glutaraldehyde were unchanged by washing, despite the observed increases in tissue  $T_2$ . This suggests glutaraldehyde-based fixatives may cause larger reductions in proton density for the cortical slices (or differentially affect tissue spin populations with very short  $T_2$ s unobserved by current methods). Thickman et al. (6) previously suggested dehydration as the cause for  $T_1$  and  $T_2$  shortening in tissues fixed only in formaldehyde solutions. Overall, washing chemically-fixed nervous tissue samples in PBS to remove free fixative should increase SNR for multiple MRI contrast methods by lengthening  $T_2$  relaxation times (and increasing relative proton density in 4% formaldehyde-fixed samples). Similar results were observed when formaldehyde-fixed tissue samples were washed in gadolinium-containing PBS solutions (17). After the MRI experiments are completed, however, it probably remains prudent to replace tissue samples into fixative solutions prior to correlative histology studies.

### Aldehyde Fixatives Alter Water Diffusion Behavior in Rat Cortical Slices

Increases to apparent restriction size and cell membrane water exchange observed with diffusion MRI may relate to aldehyde fixative-induced membrane changes that have perhaps not been fully appreciated outside the electron microscopy community. Both formaldehyde and glutaraldehyde will react with membrane phospholipids containing free amine groups (e.g., phosphatidyl ethanolamine) (2,34). Formaldehyde also reacts with carbon double bonds on unsaturated fatty acids and can deplete the lipid membrane over long periods of fixation (34). Cross-links between membrane proteins to nearby proteins in the intra- or extracellular space also may sterically disrupt cellular membranes. These various chemical reactions may reorganize the physical structure of the native cell membrane.

The electrochemical gradient across cellular membranes also will be disrupted by formaldehyde or glutaraldehyde fixation. Aldehyde fixatives bind the positive amine groups of proteins, which then increases the fixed negative charge in the membrane and cytoplasm, and increases pore size in the membranes (34). Further, aldehyde fixation changes the conformation and impairs activity of sodium-potassium adenosine triphosphatase (ATPase) (34) (activity of this ATPase also will be reduced by fixative-induced cellular energy failure). These membrane changes were reflected by the marked increase in transmembrane water exchange observed by the two-compartment model in this study (e.g., 240% increase for cortical slices fixed in 4% formaldehyde). Formaldehyde-induced increases in membrane permeability also have been demonstrated recently for *Xenopus* oocytes (36). Such membrane changes allow sodium and chloride to move more freely into the intracellular space along their concentration gradients. Water that passively follows these ions then swells glial and neuronal processes and reduces the size of the extracellular space from 20% to 5% (37). This change was reflected in the observed 30% to 40% increase in apparent intracellular restriction size of the rat cortical slices induced by the three fixative solutions ( $P \leq 0.021$ ). Despite this change, the intracellular magnetization fraction was not significantly increased by any of the three fixatives—this may reflect a disproportionate increase in the volume occupied by formaldehyde methylene bridges or polyglutaraldehyde cross-links within the intracellular space. To preserve normal *in vivo* cellular compartmentation, it may be necessary to alter the fixation protocol (38), although these

methods are not commonly employed by investigators outside the electron microscopy community.

The impact of aldehyde fixatives on extracellular ADC in the rat cortical slices appeared less robust with more variability compared to the observed impact of fixation on membranes and apparent restriction size. The cortical slice extracellular ADC was increased by 4% formaldehyde, but was not changed significantly by the other two fixative solutions. The observed increases to  $ADC_{EX}$  with formaldehyde fixation are likely due to changes in the extracellular environment and/or changes to cell geometry. Unfortunately, restriction effects prevent accurate determination of the intracellular diffusion coefficient ( $D_{IN}$ ) by the two-compartment model (31). Alternatively, previous studies have suggested that formaldehyde fixation decreased mean water diffusivity in nervous tissue by 30% or more (14–17). The cortical slice preparation technique may contribute to the diffusion differences observed here with formaldehyde fixation. However, previous studies also compared water diffusion in formaldehyde-immersed tissue imaged at room temperature to *in vivo* tissue. This temperature reduction alone (37° to 20°C) reduced extracellular ADC by 25% in an erythrocyte ghost tissue model ( $P < 0.05$ )(18) suggesting temperature reductions may underlie some of the reductions in ADC described in previous reports (17). With consistent temperature, a study of water diffusion in *Xenopus* oocytes found only a 6% reduction in ADC after the formaldehyde-fixed oocytes were washed (36).

Comparison between the analytical model water diffusion parameters and monoexponential fits to diffusion-weighted signal attenuation in prior reports is limited. Because the latter method is common, the initial slope of the cortical slice data also was determined. As described previously (31), the ADC of the initial slope ( $ADC_{TOT}$ ) at low  $b$ -values is a function of two-pool model parameters governed by the equation:

$$ADC_{TOT} = M_{IN} * ADC_{IN} + (1 - M_{IN}) * ADC_{EX} \quad (1)$$

The first terms contribute little because the intracellular ADC ( $ADC_{IN}$ ) in cortical slices determined by the analytical model fits was quite low due to restriction effects ( $0.016 \pm 0.003 \mu\text{m}^2/\text{ms}$ ). Thus, when formaldehyde increased  $ADC_{EX}$ , without altering  $M_{IN}$  substantially, it also increased  $ADC_{TOT}$  of the cortical slices approximately 50%, from  $0.20 \pm 0.02$  to  $0.31 \pm 0.11 \mu\text{m}^2/\text{ms}$  (when  $T_d = 50$  ms). Similar increases to  $ADC_{TOT}$  occur with Karnovsky's solution or 4% glutaraldehyde ( $0.32 \pm 0.07$  and  $0.32 \pm 0.08 \mu\text{m}^2/\text{ms}$ , respectively), although the latter were due to reductions in  $M_{IN}$  and not increased  $ADC_{EX}$ . Further study in other tissue models should be done to confirm these results.

Orientation dependent restriction effects from cellular membranes appear responsible for water diffusion anisotropy observed in nervous tissue (reviewed in Ref. 39). The significant observed increase in water membrane exchange induced by aldehyde fixation may be considered equivalent to a reduction in membrane restriction of water. This then suggests the additional hypothesis that water diffusion anisotropy may be altered by aldehyde fixation. Two previous studies that attempted to test this hypothesis using rat spinal cords led to somewhat contradictory results with one study reporting a 25% reduction in anisotropy (16), whereas the other study failed to demonstrate statistically significant changes to diffusion anisotropy after formaldehyde fixation (15). Another study did not detect significant anisotropy changes in the corpus callosum of macaque brain with formaldehyde fixation (17). Again, however, diffusion MRI measurements in these studies were confounded by comparing *in vivo* data to *ex vivo* data obtained at room temperature. This temperature reduction alone (37° to 20°C) reduced transmembrane water exchange

rates 66% in an erythrocyte ghost tissue model ( $P < 0.05$ ) (18). Hence, temperature differences in these studies may have masked the impact of aldehyde fixatives on membrane water exchange (or restriction) underlying diffusion anisotropy. Further, the validity of the latter spinal cord study was limited by the use of different diffusion MRI acquisition protocols for the two treatment groups (15). Unfortunately, rat cortical slices do not provide an effective tissue model of in vivo diffusion anisotropy. A better experiment to test this important hypothesis would be to again characterize diffusion anisotropy in rat white matter in vivo and ex vivo after aldehyde fixation (and washing with PBS), but using the same MRI acquisition parameters and temperatures.

### Use of Aldehyde-Fixed Nervous Tissue for MRI Studies

In contrast to other recent studies, these experiments: 1) evaluated the impact of three common aldehyde fixative solutions on nervous tissue MRI properties while controlling for temperature, 2) separated the MRI impact of bulk fixative versus additive chemical reactions, and 3) used an analytical two-compartment model of water diffusion to estimate tissue biophysical changes from fixatives. Aldehyde fixatives altered the tissue water relaxation and diffusion properties significantly. Overnight washing in PBS reversed significant  $T_2$  shortening in the tissue samples from the free fixative solution and should prove helpful in most MRI studies to increase image SNR per unit time. The observed permanent changes to the MRI properties of aldehyde-fixed nervous tissue may not affect studies of tissue cytoarchitecture or gross anatomical structure greatly; however, acquisition parameters may be better optimized for aldehyde-fixed samples. In contrast, investigations that rely on aldehyde-fixed tissue to model in vivo tissue biophysics (e.g., compartmental water exchange or diffusion contrast mechanisms) may be confounded significantly. The net impact of fixation on diffusion anisotropy (16) may not be as substantial as the significant increase in membrane permeability and changes to tissue compartment size suggested by the two-compartment model for cortical slices. However, the impact of fixative on diffusion anisotropy is still not well characterized given the confounding temperature differences in previous studies. In general, further study will be needed to determine whether the lack of statistical difference observed in some MRI properties of the cortical slices (or other tissue models) represents a fixative's ability to preserve the relevant tissue microstructures sampled by water vs. reproduction of the unfixed tissue MRI properties by a juxtaposition of different fixative-induced structural changes unobserved by present methods.

The results of the cortical slice model should be valid for other anatomic regions or species of nervous tissue chemically-fixed with aldehyde solutions for MRI investigations. Unfixed, viable brain slices imaged in this fashion appear normal by histology, sustain normal electrophysiological responses to stimuli and have stable MRI properties for 10 to 12 h after procurement (26,32). Rat and human brain slices also share similar water diffusion properties (32). However, tissue slice procurement may alter the baseline  $T_1$ ,  $T_2$ , and water diffusion in rat cortical slices from in vivo water behavior. MRI measurements of cortical slices were obtained at room temperature to minimize such procurement-induced changes and because the relative hypothermia helps to maintain the viability of unfixed cortical slices in the perfusion chamber (26). The lower temperatures also are typical for MRI studies of fixed tissue.

The three fixative solutions studied are commonly used for research, but all changed water diffusion behavior and all reduced SNR,  $T_1$ , and  $T_2$  in the cortical slice model of nervous tissue. Hence, when aldehyde tissue fixation is to be used, choosing a particular aldehyde fixative solution may need to be tailored to preserve or minimize change to the specific MRI properties to be investigated. Similarly, histologists select the fixative solution that best preserves the aspect of the cell to be studied (34). In the past, the majority of MRI

investigations have relied on tissue samples prepared with 4% formaldehyde solutions, which in the present study induced the most MRI property changes in cortical slices!

In contrast, glutaraldehyde fixative solutions are considered more appropriate for electron microscopy studies of the same nervous tissue microstructures (2,34) that water samples during the MRI experiment. Unlike formaldehyde, glutaraldehyde chemical reactions will not reverse over time under any conditions (2). Further, studies of perfusion-fixed spinal cords demonstrate better preservation of the axonal cytoskeleton with glutaraldehyde fixative solutions (40). Unfortunately, glutaraldehyde-based fixatives mask antibody recognition sites in tissue so the needs of any subsequent correlative histology should also be considered. The slow penetration of glutaraldehyde into tissue (2,34) does not impact 500- $\mu\text{m}$ -thick brain slices, but may lead to central tissue autolysis (21) and significant delays to complete fixation in larger samples (10,13). Electron microscopists use Karnovsky's solution in thick samples to overcome this particular problem since the formaldehyde component will penetrate quickly to preserve the tissue without autolysis until glutaraldehyde reaches the tissue core (24,34). Nervous tissue samples fixed with Karnovsky's solution had restored  $T_2$  and intracellular spin density compared to samples fixed solely with glutaraldehyde (suggesting higher potential SNR for structural MRI studies), although the transmembrane water exchange in slices fixed in Karnovsky's solution was elevated similar to 4% formaldehyde fixation.

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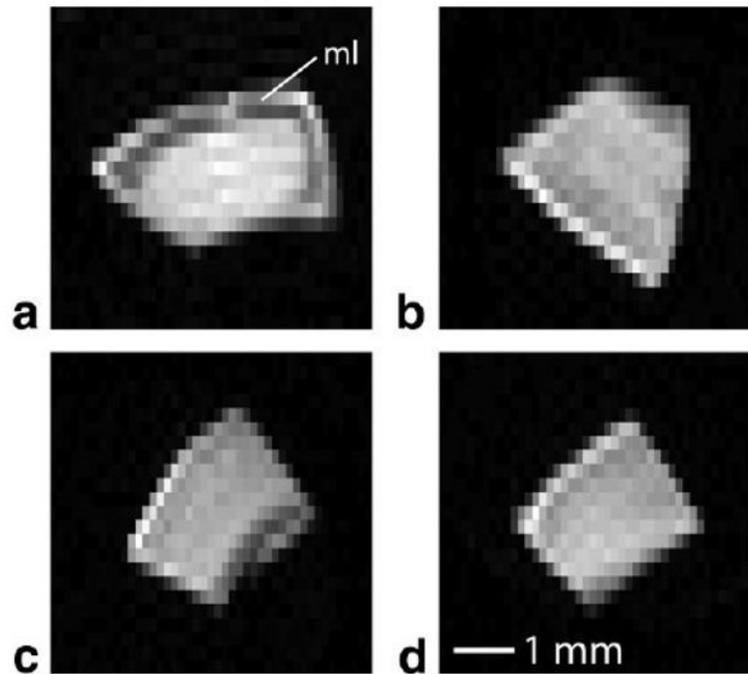
Grant Sponsor: National Institutes of Health (NIH); Grant numbers: RO1 NS36992 and P41 RR16105.

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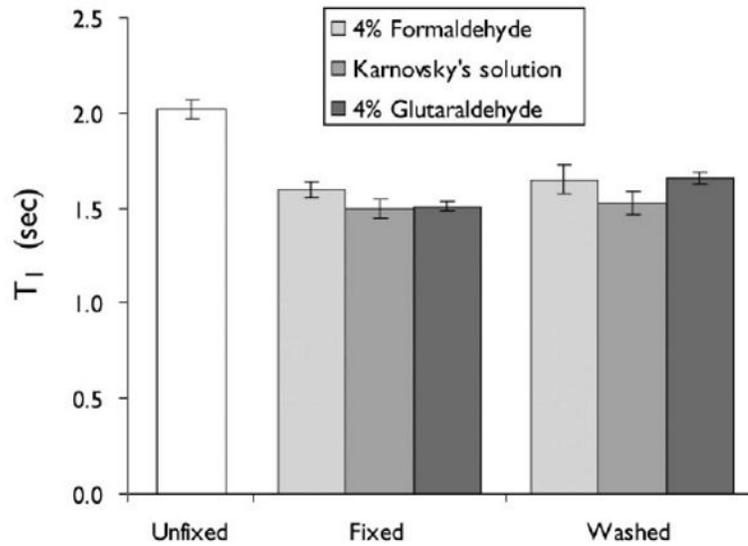
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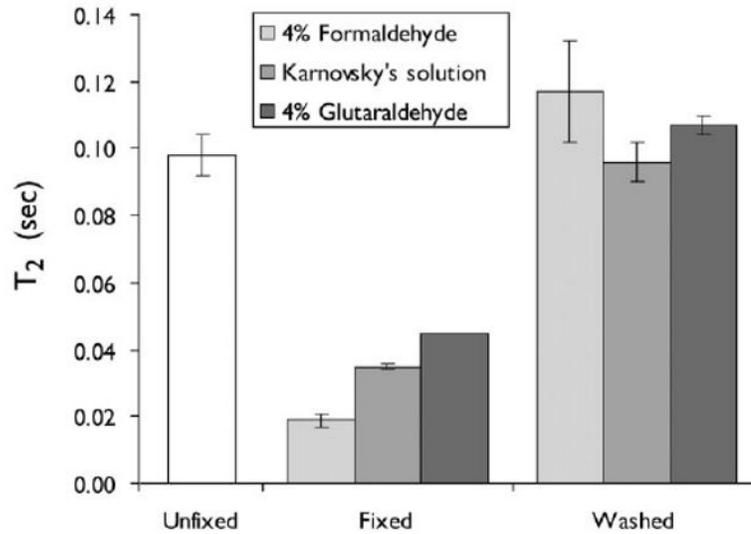
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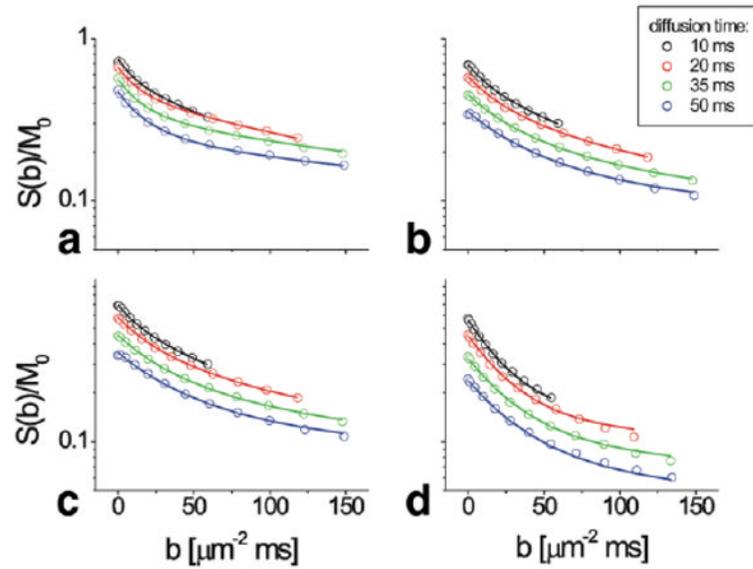
**FIG. 1.** Representative diffusion-weighted MR images of viable rat cortical slices (**a**), cortical slices after fixation in 4% formaldehyde (**b**), rat cortical slices after fixation in Karnovsky's solution (2% formaldehyde and 2% glutaraldehyde) (**c**) and rat cortical slices after fixation in 4% glutaraldehyde (**d**) (all slices:  $117 \times 234 \mu\text{m}$  in-plane resolution,  $T_d/\delta = 20/3$  ms,  $b = 2451 \mu\text{m}^{-2}\text{ms}$ ). Fixed slices were washed in PBS for 12 h prior to imaging to minimize tissue SNR and  $T_2$  reductions observed when slices remained immersed in the aldehyde fixative solutions. The acellular molecular layer (ml) was excluded from the ROI used for quantitative analysis of the data.

**FIG. 2.**

Bar graph demonstrating the effects of aldehyde chemical fixation on the  $T_1$  relaxation properties of rat cortical slices (mean  $\pm$  SD). Fixation with 4% formaldehyde, 4% glutaraldehyde, or Karnovsky's solution (2% formaldehyde and 2% glutaraldehyde) reduced cortical slice  $T_1$  significantly ( $P < 0.001$ ). The  $T_1$  relaxation effects of fixation were only marginally restored compared to controls when the fixed samples were removed from the fixative solution and washed multiple times overnight in PBS, indicating permanent microstructural changes to the tissue lattice from fixation.

**FIG. 3.**

Bar graph demonstrating the effects of aldehyde chemical fixation on the  $T_2$  relaxation properties of rat cortical slices (mean  $\pm$  SD). Fixation with 4% formaldehyde, 4% glutaraldehyde, or Karnovsky's solution (2% formaldehyde and 2% glutaraldehyde) resulted in statistically significant reductions of cortical slice  $T_2$  ( $P < 0.001$ ).  $T_2$  relaxation effects of fixation were reversed ( $P < 0.001$ ) when fixed samples were washed multiple times overnight in PBS to remove free fixative solution. This suggests the significantly shortened  $T_2$  relaxation times observed in the fixative-immersed samples were largely due to the effects of bulk fixative solution.



**FIG. 4.**

Representative semi-log diffusion-weighted signal attenuation plots for viable, perfused rat cortical slices (**a**), and cortical slices fixed in 4% formaldehyde (**b**), Karnovsky's (2% formaldehyde and 2% glutaraldehyde) (**c**), or 4% glutaraldehyde solutions (**d**). Chemically-fixed slices had to be washed after fixation to obtain sufficient SNR in the diffusion-weighted images for model fitting. Data from diffusion times of 10, 20, 35, and 50 ms at different  $b$ -values are shown for each sample. The lines demonstrate the two-compartment analytical model fits. The impact of fixative solutions on the fitted model parameters is shown in Table 1. Note, the data was not normalized such that signal decrease with increasing diffusion time at  $b = 0 \mu\text{m}^{-2}\text{ms}$  in each plot reflects  $T_2$  relaxation effects.

**Table 1**  
Impact of Chemical Fixation on Nervous Tissue Microstructure Parameters Estimated From Fitting a Two-Compartment Analytical Model to MRI Data From Rat Cortical Slices\*

Treatment Group	N	ADC-EX ( $\mu\text{m}^2\text{ms}^{-1}$ )	a ( $\mu\text{m}$ )	$k_{IE}$ ( $\text{s}^{-1}$ )	$M_{IN}$ (no units)
Artificial cerebrospinal fluid	14	$0.64 \pm 0.19^a$	$2.43 \pm 0.39^{a,b,c}$	$16.36 \pm 7.79^{a,b}$	$0.69 \pm 0.04^a$
4% Formaldehyde	13	$1.20 \pm 0.84^{a,b}$	$3.15 \pm 0.24^a$	$55.42 \pm 8.86^{a,c}$	$0.75 \pm 0.13^b$
Karnovsky's solution	12	$0.94 \pm 0.43$	$3.19 \pm 0.26^b$	$56.33 \pm 6.51^{b,d}$	$0.67 \pm 0.13^c$
4% Glutaraldehyde	16	$0.64 \pm 0.13^b$	$3.40 \pm 1.04^c$	$22.89 \pm 12.26^{c,d}$	$0.52 \pm 0.19^{a,b,c}$
One-way ANOVA (P value)		0.008	<0.001	<0.001	<0.001

\* Values are mean  $\pm$  SD. Note: Cortical slices treated with fixative solutions were washed with phosphate-buffered saline for 12 h prior to MRI data collection.

<sup>a-d</sup> Statistically significant differences ( $P < 0.05$ ) between individual treatment groups as determined by post-hoc Tukey pairwise multiple comparisons tests.