Developing formalin-based fixative agents for post mortem brain MRI at 9.4 T

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Abstract

Purpose: To develop fixative agents for high-field MRI with suitable dielectric properties and measure MR properties in immersion-fixed brain tissue.

Methods: Dielectric properties of formalin-based agents were assessed (100 MHz–4.5 GHz), and four candidate fixatives with/without polyvinylpyrrolidone (PVP) and different salt concentrations were formulated. B1 field and MR properties (T1, R∗2, R2, and magnetic susceptibility [QSM]) were observed in white and gray matter of pig brain samples during 0.5–35 days of immersion fixation. The kinetics were fitted using exponential functions. The immersion time required to reach maximum R∗2 values at different tissue depths was used to estimate the Medawar coefficient for fixative penetration. The effect of replacing the fixatives with Fluoroinert and phosphate-buffered saline as embedding media was also evaluated.

Results: The dielectric properties of formalin were nonlinearly modified by increasing amounts of additives. With 5% PVP and 0.04% NaCl, the dielectric properties and B1 field reflected in vivo conditions. The highest B1 values were found in white matter with PVP and varied significantly with tissue depth and embedding media, but not with immersion time. The MR properties depended on PVP yielding lower T1, higher R∗2, more paramagnetic QSM values, and a lower Medawar coefficient (0.9 mm/√h; without PVP: 1.5). Regardless of fixative, switching to phosphate-buffered saline as embedder caused a paramagnetic shift in QSM and decreased R∗2, which progressed during 1 month of storage, whereas no differences were found with Fluoroinert.

Conclusion: In vivo-like B1 fields can be achieved in formalin fixatives using PVP and a low salt concentration, yielding lower T1, higher R∗2, and more para- magnetic QSM than without additives. The kinetics of R∗2 allowed estimation of fixative tissue penetration.

Keywords

B1 homogeneity, brain, dielectric properties, formalin, post mortem MRI
1 | INTRODUCTION

Ultrasound field MRI of whole post mortem brains permit long imaging sessions without motion artifacts, which brings about enhanced image quality at a high spatial resolution. The obtained data are complementary to histology and pathology studies and can be useful for tissue characterization, sectioning, 3D reconstruction of sectioned tissue data, and for diagnosis and investigation of neurodegenerative diseases.1–6

Ex vivo brain samples are preserved by perfusion and/or immersion in a fixative agent to slow down tissue decomposition.7–9 To optimize MRI, fixatives must be MR-compatible, suitable for tissue storage, and yield reproducible measurement results. To be MR-compatible means to have dielectric properties that ensure a homogeneous $B_1$ field, a magnetic susceptibility matching the tissue to improve $B_0$ homogeneity, and preferably a singulet $^1$H-NMR spectra to prevent chemical-shift artifacts.

Dielectric properties (electric conductivity, $\sigma$, measured in Siemens per meter [S/m]; and permittivity, $\epsilon$, a unit-less measure) of the fixative can influence ex vivo whole human-brain MRI image quality at 9.4 T, considering their large size.10 Using the same RF coils and clinical MRI protocols as those used in vivo, choosing in vivo–like properties may represent a viable way to maintain image homogeneity and brain-tissue contrast. Analytical solutions of Maxwell's equations governing RF-field transmission inside a sphere demonstrate how spatial field focusing and dielectric resonance can affect $B_1$ field homogeneity at high frequencies due to the dielectric properties of the sample.11 Radiofrequency penetration, power deposition, and the spatial shape of the electromagnetic fields therefore directly influence MRI quality. Such effects may be particularly evident in $T_1$-weighted and $T_2$-weighted MRI acquisitions using 180° pulses, when spin inversion and echo refocusing may fail, leading to strong contrast differences between areas where different flip angles are played out.

The most well-established chemical fixation agent is formaldehyde (formalin), although currently there is an ongoing quest for alternative fixative agents, considering its recent classification as a carcinogen (type 1B in Europe and group 1 of International Agency for Research on Cancer).12 Even at low concentrations, formalin can achieve its biocide action and stabilize tissue structure by cross-linking proteins.7–9 The rate of penetration of formalin can be quantified by the Medawar coefficient8 and can be facilitated through the use of phosphate-buffered formalin.9 This motivates its use for ex vivo MRI, but the dielectric properties of standardized formalin are not sufficient to yield good image quality at 9.4 T.10 Due to changes in water mobility and chemical exchange,13,14 formalin causes a shortening of relaxation times.15,16 This can be reversed by washing out the formalin and replacing it with PBS, which has been proposed as a method to increase the SNR.17 Nevertheless, washing out is not complete after 24 h for whole human brains.18 Phosphate-buffered saline ($c = 76.4, \sigma = 1.73$ S/m) also causes $B_1$ inhomogeneity at 9.4 T.10 Because fixative penetration and tissue fixation at all depths is a time-consuming process for large specimens like whole post mortem human brains, knowledge regarding these processes is required to ensure stable conditions and reproducible MRI measurements.

Here we investigated the possibility of developing formalin-based fixatives with additives to improve the dielectric properties, while assuring adequate tissue penetration, good fixation quality, and known MR properties. Additives were selected to achieve appropriate (1) dielectric properties that enable sufficient RF penetration and $B_1$ homogeneity; (2) magnetic susceptibility that matches the tissue to obtain good $B_0$ homogeneity and avoid susceptibility difference–related effects; and (3) chemical shift to ensure absence of offset artifacts. Additives like salt, polyvinylpyrrolidone (PVP), and low percentages of ethanol were used to decrease permittivity while salts, like NaCl and KCl, were used to adjust tonicity and conductivity. One PVP-based compound is known to have slight fixative properties.19 It also has interesting properties for MRI, due to its complex pattern in $^1$H-NMR spectra that avoids chemical-shift artifacts even at high temperatures, and its peculiar dielectric properties that allows tuning permittivity.20 The $B_1$-field homogeneity for MRI at 9.4 T was assessed in solutions and in pig brain tissue samples during 0.5–35 days of immersion fixation. We also investigated the MR properties ($T_1$, $R_1^*$, $R_2$, $R_2^*$, and magnetic susceptibility [QSM]) at different tissue depths in white and gray matter of pig brain samples. The kinetics were fitted using exponential functions similar to previous studies.15,21 The immersion time required to reach maximum $R_2^*$ values was used to estimate the Medawar coefficient for fixative penetration.8 The impact of embedding samples in Fluoroinert or PBS was also evaluated.17

2 | METHODS

2.1 | Preparation of fixatives and assessment of chemical shift–offset artifacts

Ready-to-use formalin (Roti-Histofix 4%, phosphate-buffered formaldehyde solution; pH 7; Carl Roth, Karlsruhe, Germany), polyvinylpyrrolidone (PVP; average molecular weight = 40 000 g/mol), ethanol (absolute for high-performance liquid chromatography; ≥ 99.8%),
sodium (NaCl; BioXtra; ≥ 99.5%), and potassium chloride (KCl, anhydrous, American Chemical Society reagent; ≥ 99.5%) (Sigma-Aldrich Chemie, Merck [Taufkirchen, Germany]) were used.

The $^1$H-NMR spectra of additives were generated using an online NMR predictor tool on the nmrdb.org website (Supporting Information Figure S1). Chemical shift–offset artifacts of ethanol (1%, 5%, 10%, 20%, and 40% [vol/vol]) in water with 0.9% NaCl were measured in five vials positioned inside a 2.8-L container using a holder. The outer container was filled with water, salt (0.9% [wt/vol]), and Dotarem (0.65 mM) as a $T_1$ modifier. The phantom was scanned at 9.4 T using single-slice gradient-echo MRI with different readout bandwidths (90, 120, 240, and 600 Hz/pixel). Due to visual inspection and the criteria of ghost/signal ratio less than 1/40, an acceptable allowance of 5% was determined for the ethanol concentration (Supporting Information Figure S2).

### 2.2 Measurements of dielectric properties

The DAK (Dielectric Assessment Kit) probe DAK-12 (Schmid & Partner Engineering [SPEAG], Zürich, Switzerland) connected to a Vector Network Analyzer (Keysight ENA 5071C; Agilent, Santa Clara, CA) was used to measure $\epsilon$ and $\sigma$ within the range of 100 MHz to 4.5 GHz at room temperature.

Formalin samples (250 ml) prepared with different combinations of NaCl (0%, 0.01%, 0.04%, 0.08% [wt/vol]) and PVP (0%, 2%, 5%, 10%, 20% [wt/vol]) concentrations (Figure 1) or with NaCl and ethanol (1%, 3%, 5% [vol/vol]) (Supporting Information Figure S3) were prepared and measured with DAK. The dependence of permittivity and conductivity on the concentrations of the additives was fitted with a polynomial in MATLAB (MathWorks, Natick, MA).

Depending on our selection criteria (see Section 3), four recipes for the fixative agents were defined, prepared four times at a 1-L volume to assess reproducibility and measured with DAK:

- Fix01: Formalin + 0.04% NaCl + 5% PVP + 5% ethanol
- Fix02: Formalin + 0.9% NaCl + 5% PVP + 5% ethanol
- Fix03: Formalin + 0.9% NaCl
- Fix04: Formalin + 0.8% NaCl + 0.2% KCl (corresponding to 140 and 2.7 mM, respectively).

For MRI, each fixative solution was prepared to be measured in an elliptic container (polycarbonate, 2.5 L) and in one of the chambers used for tissue samples.

### 2.3 Fixation and embedding of tissue samples

Two measurement-series were performed with pig brain hemispheres from the local slaughter center (post mortem interval < 5 h). Samples were kept at room temperature at all times and were placed in a four-chamber elliptic container (each chamber ~0.65 L, 3D-printed using polylactic acid with polycarbonate lids), after removal of dura and arachnoid to improve fixation and avoid artifacts from superficial blood vessels. Each chamber was filled with an embedding media and the samples immobilized by sterile gauze soaked in the same liquid. Air bubbles were removed using a desiccator.

In the first measurement series, four hemispheres, one per fixative, were embedded in their own fixative. The four samples were scanned at 9.4 T simultaneously at 12 h, 1, 2, 3, 4, 13, 19, 28, and 35 days of immersion fixation. Next, three hemispheres fixed with the same fixative were placed in three of the chambers with fixative embedder, with the fixative alone in the fourth. These experiments were performed after 38, 40, 41, and 42 days of fixation for Fix04, Fix03, Fix01 and Fix02, respectively, to check reproducibility.
In the second measurement series, four hemispheres (one for each fixative) were immersed for 28 days and then embedded in Fluorinert (FC-770) for MRI. Next, after 2 months of fixation, washing by immersion in PBS, replaced after 4, 8 and 12 h, was performed before final embedding in PBS. The MRI was repeated after keeping the samples in the chambers at room temperature for 1 month of PBS storage.

### 2.4 Magnetic resonance imaging measurements

The MRI data were acquired at 9.4 Tesla (T) using a whole-body system (Siemens, Erlangen, Germany) with a 16-channel-transmit/31-element receive array. B1-maps were obtained from actual flip-angle images (TE/TR1/TR2 = 7/20/100 ms; flip angle = 60°, voxel size = 3.1 x 3.1 x 5.0 mm³, FOV = 200 x 162.6 x 36 mm³), scaled with the transmitter voltage and a conversion factor to achieve the unit nT/V.

Parametric T1 maps were obtained from actual flip-angle image-corrected MP2RAGE measurements (TE/TR1/TR2 = 900/3500 ms; TR = 6 ms; TE = 2.3 ms; volume TR = 9 s; flip angle = 4/6°; voxel size = 0.8 x 0.8 x 0.8 mm³), also used to generate background-free MP2RAGE beta images. The R2* and QSM were obtained from a monopolar multi-echo gradient echo (TR = 34 ms; TE = 6.03, 12, 18, 24, 30 ms; flip angle = 15°; voxel size = 0.4 x 0.4 x 0.4 mm³; FOV = 204 x 165.8 x 46 mm³). The R2* maps were obtained using nonlinear least-squares fitting, and QSM images were generated from the third gradient echo after Laplacian unwrapping RESHARP (kernel, 1.2 mm; Tikhonov regularization, 10^-12) and dipole-inversion with iLSQR (http://people.eecs.berkeley.edu/~chunlei.liu/software.html; v. 3.0, 201905). Phase referencing was performed by setting the median value across all four samples to zero.

The R2 maps were obtained from single-slice Carr-Purcell-Meiboom-Gill spin-echo images; (TR = 3 s; 32 echoes in steps of 7.2 ms; TE1 = 7.2; TE32 = 227.6 ms; voxel size = 0.47 x 0.47 x 2 mm³; FOV = 192 x 192 x 2 mm³) followed by analysis using the extended phase graph model, while taking into account the actual B1 distribution as described previously. The R2 maps were generated by subtracting the R1 from the R2 maps. Based on the B1-field maps, the T1 and R2 measurements were repeated twice, with different settings of the reference voltage to achieve the nominal flip angle in the center of each brain sample (200 V for Fix02/03/04; 133 V for Fix01).

### 2.5 Data analyses

Data analysis was performed using MATLAB commands after affine co-registration and nearest-neighbor resampling to match the R2* image at 24 h in SPM12 (https://www.fil.ion.ucl.ac.uk/spm/software/spm12/). Whole brain masks were obtained from the MP2RAGE contrast image using activecontour and imclose. The function distancemap from https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSL yielded the tissue depth from the whole brain mask. Automatic whole brain gray-matter (GM)/white-matter (WM) segmentation was performed on R2*/T1-ratio maps using histeq and thresholding at 0.98. The GM and WM masks were also carefully outlined on the most central 10 slices using activecontour, followed by manual correction if deemed necessary.

The MR parameters were extracted from WM and GM voxels to generate median values. Nonlinear curve fittings of the time evolutions from 10 slices between 0.5 and 35 days were made with cftool. A bi-exponential function was fitted for T1, mono-exponential for QSM, and a sum of a mono-exponential decay and mono-exponential recovery for R2* (Table 2), in line with previous studies. Median values for the depth-dependent B1 values and DMAX, the day at which the R2* maximum was reached determined from pixelwise fits, were extracted from whole-brain GM, WM masks whenever ≥ 50 voxels were available within a certain depth bin, dbin going from 0.5 to 15.5 mm with a bin width of 1 mm. The DMAX was only extracted if the goodness-of-fit in terms of the adjusted regression coefficient R2_adj > 0.8, and if DMAX < 25 days. The Medawar coefficient K was obtained from dbin = K√t, where t = DMAX * 24 is the median DMAX expressed in hours.

The 95% confidence intervals for the prediction error of the exponential fits (predint) were generated and used to verify significant deviations of the MR properties at day 38–42, and for different embedding media. For B1, the coefficient of variation across three samples on day 38–42 was used to evaluate significance, as no variation with immersion time was observed. Statistical analyses using three-way analysis of variance (ANOVA) comparing tissue (WM-GM), fixatives (Fix01–04), and immersion time (0.5–35 days, treated as a continuous variable), and two-way ANOVA to evaluate WM-GM contrast across fixatives and immersion time, were performed using anovan, requiring p < 0.05 for significance. For the change in R2 (day 1 vs. 28) and R2 (day 4 vs. 28), two-tailed t-tests were performed.

### 3 RESULTS

#### 3.1 Dielectric properties of formalin-based solutions and selection of tissue fixatives

Both conductivity and permittivity of formalin decreased with increasing PVP concentrations, occurring at a higher rate in the presence of NaCl (Figure 1). The change in...
conductivity could be described with a third-order polynomial dependence on PVP, and a second on NaCl concentrations, with a RMS error of 0.016 and \( R^2 = 0.989 \). Likewise, permittivity showed a third-order polynomial dependence on the PVP and the NaCl concentrations, with RMS error = 0.37 and \( R^2 = 0.997 \). Effects of adding different amounts of NaCl and ethanol are shown in Supporting Information Figure S3. By adding either NaCl, PVP, or ethanol to the formalin, the permittivity decreased, with the greatest effect obtained by ethanol (Table 1). While adding NaCl led to an increase in conductivity, ethanol slightly reduced it. The addition of all three components did not lead to a linear decrease in permittivity.

### Table 1 Effect of additives on the dielectric properties of formalin (Roti Histofix 4%)

| Chemicals                              | \( \Delta \sigma \) (S/m) | \( \Delta \varepsilon \) |
|----------------------------------------|---------------------------|--------------------------|
| Formalin (reference)                   | 0                         | 0                        |
| Formalin + 0.9% NaCl                   | +1.33                     | −3.5                     |
| Formalin + 5% PVP                      | −0.06                     | −4.4                     |
| Formalin + 5% ethanol                  | −0.13                     | −5.3                     |
| Formalin + 0.9% NaCl + 5% PVP + 5% ethanol (Fix02) | +0.83                     | −8                       |

Note: The change in dielectric properties owing to all additives used for Fix02 is not simply a linear combination of the differences caused by adding each single material. Abbreviations: \( \Delta \sigma \) (S/m), conductivity difference; \( \Delta \varepsilon \), permittivity difference.

Additives were selected to achieve near to brain-equivalent dielectric properties: 0.04% NaCl, 5% PVP, and 5% ethanol (Fix01). Besides further reducing permittivity, ethanol facilitated tissue penetration. For Fix02 we used the same additives but increased the NaCl concentration to isotonic levels (0.9%), to minimize the risk of osmosis and cellular swelling. Fix03 was defined to study effects in absence of PVP and ethanol. Fix04 contained the exact physiological concentration of the two most common salts in tissue cells and was evaluated for consistency with previous measurements. The conductivity at 400 MHz and room temperature was 0.60 ± 0.01, 1.55 ± 0.08, 1.98 ± 0.02, and 1.89 ± 0.01 S/m for Fix01, Fix02, Fix03 and Fix04, respectively, and the permittivity was 71.2 ± 0.24, 70.3 ± 0.16, 73.8 ± 0.05, and 74.0 ± 0.23, respectively. The values obtained at other MRI operating frequencies are listed in Supporting Information Table S1.

### 3.2 | \( B_1 \)-transmit field mapping of the fixatives

The \( B_1 \) field of each fixative was measured in the absence of tissue in a 2.5-L container (Figure 2, upper row) and in one of the four chambers, while the remaining chambers contained brain samples immersed in the corresponding fixative (Figure 2, lower row). For Fix01, a nonuniform \( B_1 \) field with a central hot spot enclosed by a region where the \( B_1 \) field was about 90% weaker was observed (Supporting Information Figure S2).
Information Figure S4). The other fixatives yielded a more homogeneous $B_1$ field. The corresponding histogram (Figure 3A) was wider for Fix01, encompassing central voxels with high efficiency. The remaining fixatives more closely adhered to a Gaussian distribution. Ideally, to achieve the best performance for MRI, the fixatives should yield histograms with a narrow peak at high $B_1$. The SD of the $B_1$ field across the whole volume was 10.36, 6.38, 6.80, and 7.36 nT/V, respectively, for Fix01–Fix04. In the smaller container, histograms (Figure 3B) suggest that besides the difference in size and loading by tissue samples, the material of the container itself also affected the $B_1$ field, with SDs of 24.81, 8.59, 6.29, and 7.93 nT/V, respectively, for Fix01–Fix04.

3.3 | $B_1$-transmit field mapping of brain-tissue samples in different fixatives

Comparison of $B_1$ maps obtained in absence and in presence of brain tissue demonstrates that the sample itself modifies $B_1$ (Figure 2).

A three-way ANOVA analysis of extracted $B_1$ values revealed significant main effects of tissue, fixative, and duration of fixation ($F[56,1] ≥ 16, p ≤ 0.0002$) and a significant tissue-by-fixative interaction ($F[56,3] = 4.36, p = 0.0079$). The highest $B_1$ values were found in WM with Fix01 at early and late time points (Figure 3C,D). At increasing tissue depths, prominent effects that outsized the effect of immersion time were observed (Figure 3E,F).

3.4 | Effect of embedding media on $B_1$ in fixed brain tissue

The advantage of using a fixative with optimized dielectric properties in terms of obtaining high $B_1$ within the tissue was diminished when the surrounding fixative was replaced by other embedding media, but did not completely disappear (Figure 4). The $B_1$ in GM measured with fixative embedding was 40% higher for Fix01. With Fluorinert as an embedder, this number reduced to 5%–20%, and with PBS, Fix01 was still 15%–25% better. Likewise, in WM, Fix01 had 2%–15% higher $B_1$ in Fluorinert and 20%–25% in PBS. The embedding-induced change in $B_1$ of Fix02 exceeded the coefficient of variation on day 38–42 in WM,
whereas in GM the changes were just within. On the contrary, in the PVP-free fixatives, higher $B_1$ values were observed with both alternative embedding media. No effect of 1-month storage in PBS was observed.

3.5 Magnetic resonance properties in fixed brain tissue

Example maps of tissue $R_2^*$, $T_1$, and QSM during immersion are shown in Figure 5 together with the exponential fits of the time evolution in GM/WM. The ($T_1^*$) QSM values diminished (bi-)exponentially with time, whereas $R_2^*$ showed an increase followed by a decrease (Table 2). The reproducibility data at day 38–42 were not always within the 95% confidence intervals of the fits (Supporting Information Figure S5), especially for Fix03, which had several remaining air bubbles. For QSM and $R_2^*$, effects due to fiber orientation in WM could also have contributed to the variance. The three-way ANOVA showed significant main effects of tissue type and immersion time ($F[56,1] = 6.66; p \leq 0.0125$). For QSM there was also a significant difference between fixatives ($F[56,1] = 8.09; p = 0.0001$) and a significant tissue-by-fixative interaction effect ($F[56,3] = 3.31; p = 0.026$). Although the GM in Fix02 was the most paramagnetic, the WM in Fix04 was the most diamagnetic. The two-way ANOVA showed a significantly different QSM difference between GM-WM ($F[28,3] = 7.66$, $p = 0.0007$), being greater in the absence of PVP. The GM- WM contrast in $R_2^*$ changed significantly with immersion time, reaching the highest difference in Fix02 (26 s$^{-1}$) on day 4 ($F[28,1] = 7.52; p = 0.010$), although the effect of fixative was not significant ($F[28,3] = 0.33; p = 0.805$). The $T_1$ contrast was significantly different between fixatives ($F[28,3] = 13.61; p < 0.0001$) and across time ($F[28,1] = 18.32; p = 0.0002$), being greater in the absence of PVP.

The effects on $R_2^*$ were further investigated using single-slice $R_2^*$ maps, and the derived field-induced relaxation effects, $R_2'$. The maps acquired on day 1–4 could be compared directly, revealing a frontline of the penetrating fixative at 24 h in the $R_2$ map. After 28 days, a strong GM- WM contrast in $R_2'$ was found (Figure 6A,B). Repositioning the single slice at identical anatomical positions on day 4 and later was not perfect, causing a high variability of the values across time (Figure 6C–F). The $R_2$ increase from day 1 to 28 was significant in WM ($T = 3.18; p = 0.0155$), whereas $R_2^*$ in GM decreased significantly between day 4–28 ($T = 3.18; p = 0.0155$). Generally, the relative contribution of $R_2$ to the tissue $R_2^*$ was greater than the field-induced effects, especially close to the surface in GM voxels (Supporting Information Figure S6). This finding is in line with the properties of the fixative solutions (Supporting Information Table S2).

The change in relaxation times was accompanied by a 5%–12% decrease in tissue volume, which was also prominent in the pixel-by-pixel $R_2^*$ and $T_1$-difference maps between 12 h and day 28 (Supporting Information Figure S7).

3.6 Depth-dependence of $R_2^*$ kinetics and Medawar coefficient

The number of days required to reach maximal $R_2^*$ values depended on tissue type, depth, and fixative (Figure 7A–F). The depth dependence of all fitting coefficients is shown in Supporting Information Figure S8. Without PVP, DMAX was below 5 days and with $\leq 10$ days. Fitting of the Medawar equation (Figure 7G,H) yielded coefficients of 0.93/0.88/1.45/1.51 mm/h$^{1/2}$ in WM for
3.7 | Effect of embedding media on MR properties

No dependence on embedding media was found for T$_1$ in WM or GM (Figure 8). Magnetic resonance imaging using either the fixative or Fluorinert yielded R$_2^*$ within the 95% confidence intervals obtained from the exponential fits, whereas PBS-embedded samples had significantly lower R$_2^*$.

After 1 month of PBS storage, this value was further reduced, as a likely indication of washing out of the formalin fixatives. In GM, QSM was within bounds, whereas a tendency toward more diamagnetic values could be observed in WM, which is outside the 95% intervals for Fix02 and Fix03.

4 | DISCUSSION

We report on how the dielectric properties of formalin-based fixatives can be optimized by additives to obtain in vivo–like conditions at 9.4 T. As a starting point, off the shelf, phosphate-buffered formalin was chosen to
improve reproducibility, tissue penetration,⁹ and minimize the effect of chemical exchange.¹³,¹⁴ Four fixatives were formulated and evaluated in terms of transmit $B_1$ values in solution and in ex vivo pig-brain tissue. For further characterization, the MR properties in WM and GM brain tissue during 0.5–35 days of immersion fixation were investigated. The $R_2^*$ was found to be dominated by $R_ε$ effects, allowing comparisons with previous studies.¹⁵,²¹,³⁰ The kinetics of $R_2^*$ during immersion fixation was found to vary with tissue depth, allowing us to estimate the Medawar coefficient for tissue penetration. Finally, the effect on $B_1$ and MR properties in brain tissue by two alternative embedding media often used in MRI, Fluorinert, and PBS were investigated.

Studies proposing recipes for dielectric and tissue-equivalent phantoms for MRI typically use salt in combination with sugar or ethanol to adjust conductivity and permittivity.³¹ However, ethanol and sugar may become problematic for MRI, due to the large amounts required to diminish permittivity and multiplet proton resonances leading to image artifacts. We found that more than 5% ethanol introduced ghost artifacts and was not compatible with MRI at 9.4 T. The NVP compounds (polyvinylpyrrolidone) with slight fixative properties¹⁹ have proven useful to achieve in vivo–like dielectric properties at high magnetic field strengths.³²,³³ Like ethanol, PVP diminishes permittivity but causes less issues with chemical shift–dependent artifacts due to its complex spectral pattern.²⁰

Polyvinylpyrrolidone and NaCl can be used to modify permittivity and conductivity, respectively, of formalin, in a nonlinear and independent manner. Higher concentrations of NaCl increases conductivity and reduces permittivity slightly, while higher PVP concentrations reduce both properties with a greater effect on permittivity. These findings for formalin are therefore similar to previously published results for water-based solutions.²⁰ Polyvinylpyrrolidone increased the fixative viscosity, which limits tissue penetration; therefore, ethanol (5%) was added as a fluidifier. The fixative with the most brain tissue–like dielectric properties (Fix01) yielded a $B_1$ field reminiscent of previously published maps obtained in vivo at 9.4 T,²⁶ with a central spot of increased efficiency surrounded by a region with lower values. Increasing the salt concentration to isotonic values yielded a flatter transmit field (Fix01 vs. Fix02). This result is in line with analytical solutions of Maxwell’s equation for a sphere, demonstrating diminished effects of RF focusing and dielectric resonance¹¹ with higher salt concentrations; however, this comes at the cost of a lower efficiency (lower $B_1$ values). Reducing permittivity through the addition of PVP and ethanol also improved $B_1$ homogeneity, with the advantage of a slightly better efficiency (Fix02 vs. Fix03).

### Table 2: Exponential functions describing the change in MR properties ($T_1$, $R_2^*$, and QSM) in GM and WM tissue voxels during immersion fixation with four different fixative solutions

| Fixative | Tissue | $T_1$ [ms] | $R_2^*$ [a] | QSM [ppb] | $R_2^*$ [a] | $R_2^*$ [ppb] |
|----------|--------|----------|----------|----------|----------|----------|
| Fix01, GM | WM    | 379      | 0.53     | 0.005    | 11.18    | 4.46     |
| Fix01, GM | WM    | 436      | 0.48     | 0.002    | 9.64     | 3.55     |
| Fix03, GM | WM    | 501      | 0.75     | 0.004    | 12.61    | 6.98     |
| Fix04, GM | WM    | 390      | 0.88     | 0.002    | 10.56    | 4.62     |

Note: The exponential rate constants are expressed in units per day [$d^{-1}$]. For a graphical view, see Figure 5 and Supporting Information Figure S5.
FIGURE 6  Maps of $R_2$ (A) and $R'_2$ (B) investigated in single slices after 24 h and 28 days of immersion fixation. At 24 h, a continuous line with increased $R_2$ values appears close the tissue surface, indicating the location of the frontline for formalin penetration. After 28 days, the increase in $R_2$ had spread to the entire sample, indicating completion of fixation. The time course for $R_2$ (C,D) and $R'_2$ (E,F) in GM (C,E) and WM (D,F) show variability due to difficulties to place the single slice in exactly the same anatomical location on different days. A significant $R_2$ increase between day 1 to 28 was found in WM, and a $R'_2$ decrease between day 4–28 in GM. Generally, the relative contribution of $R_2$ to the tissue $R^*_2$ was greater than the field-induced effects (see Supporting Information Figure S6).

FIGURE 7  Depth dependence of $R^*_2$ kinetics during immersion fixation with different fixatives. The spatial locations of different tissue depths are shown in a sagittal slice through the pig-brain hemisphere (A) with corresponding curves fitted to the experimental data (B). The curves from the first 1–4 mm are located in the GM, while the remaining curves are dominated by WM. The day at which maximum $R^*_2$ is reached ($DMAX$) is shown as maps obtained from pixel-wise fits for Fix01 (C), Fix02 (D), Fix03 (E), and Fix04 (F). Median values for $\sqrt{DMAX * 24}$ in GM (G) and WM (H) at different tissue depths are shown as dots. Data points fulfilling three criteria (≥50 voxels within a certain depth bin, $d_{bin}$, going from 0.5 to 15.5 mm with a bin width of 1 mm; adjusted regression coefficient $R_2 > 0.8$; and $DMAX < 25$) were used to fit the Medawar coefficient, $K$, obtained from $d_{bin} = K \sqrt{t}$, where $t = DMAX * 24$ is expressed in hours.
the NaCl by KCl, an intermediate efficiency could be obtained (Fix04 vs. Fix03), thus deserving more attention in future studies.

$B_1$ values in brain-tissue samples were stable across 35 days. We found similar results at 6.5 and 8.5 months of storage in these fixatives (unpublished observation). The highest values were found for the in vivo–like fixative Fix01. The $B_1$ in WM was significantly higher than in GM, evidencing how tissue-specific properties besides formalin additives also influence transmit efficiency ex vivo. This effect was particularly prominent in the presence of PVP. The presence of myelin lipids in WM could potentially lead to higher concentrations of this lipophilic agent there. Generally, the $B_1$ values varied strongly with tissue depth, which may be linked to the well-understood RF-focusing effects.\(^{11}\) Numerical modeling of electromagnetic fields for the complex brain geometry of these single pig hemispheres would be required to fully demonstrate such an effect.

Several researchers have shown that the embedding media can influence the measured MR properties.\(^{17,34}\) Here we could show that it also influences the $B_1$ field itself. The use of either proton-free agents or PBS as embedders decreased $B_1$ for the PVP-containing fixatives, reaching significance in WM, and for Fix01 also in GM. There was a slight improvement in $B_1$ using fluorine for the PVP-free fixative Fix04, which also had the highest total salt concentration (1%). Although the advantage of optimized dielectric properties of the fixatives became less prominent in the presence of alternative embedders, the highest $B_1$ values was still obtained in the brain tissue with the in vivo–like fixative, Fix01. Its superior performance in our setting may be due to the use of a RF array coil designed and optimized for in vivo measurements of the human brain.\(^{21}\) In clinical settings, the availability of coils optimized for the in vivo condition is the rule; therefore, our results could be of value for such situations in general. Alternatively, the transmit field could be tuned to the dielectric properties of the sample. Previously, a coil with the same design as ours has been used at 9.4 T with kT-points parallel transmit, yielding excellent homogeneity within an 8-cm FOV covering a human sample from the occipital cortex.\(^{35}\) It would be interesting to evaluate the possibility of achieving good homogeneity within the 16-cm FOV required for whole brain ex vivo measurements as well. At 7 T, an RF coil designed to perfectly match the size of a whole-brain sample with a fluorine compound as embedder yielded superb images but still required repeated measurements at different flip angles to mitigate $B_1$.\(^{15}\) Therefore, the relative advantage of modifying the hardware and their use during spin excitation, refocusing and inversion, on the one hand, and modifying the dielectric properties of fixatives and/or embedding media, on the other hand, deserves further studies. Perhaps both

**FIGURE 8** Effect of embedding media on MR properties in GM (A,C,E) and WM (B,D,F) voxels. Pig-brain samples were immersed for 28 days in each fixative before measurements in its own fixative (blue, Fix) or in proton-free media (orange, Fluor). After 2 months of fixation, the samples measured in Fluorinert were washed with PBS for 24 h before embedding in PBS and MRI (yellow, PBS2). After 1 month of storage in PBS, MRI was performed again (PBS3, violet). Significance ($p < 0.05$, indicated by a star) was assessed based on the 95% confidence intervals determined from the exponential fits given in Table 2 and in Supporting Information Figure S5
types of action may help to achieve high-quality MRI at high fields.

In vivo, MR properties arise from the combined effect of the interstitial fluid and the tissue itself to reveal microstructure, whereas in fixed samples the CSR must be replaced to achieve preservation. This replacement must occur rapidly to minimize the occurrence of tissue alterations. Formalin-based tissue fixation itself is slow and biphasic, encompassing penetration and fixation. Mostly hydrated formaldehyde (methylene glycol monomers) molecules penetrate the tissue before dynamic equilibrium of formaldehyde is re-established locally, and stable fixation in terms of cross-linking between molecular groups in the tissue occurs. Formalin fixation inevitably affects MR properties, and we found that the addition of PVP yields shorter $T_1$, higher $R_2^*$, and more paramagnetic QSM values than formalin alone. Kinetic changes of MR properties may furnish additional information regarding the fixation process. We found that $T_1$ decreases bi-exponentially, whereas QSM decreases exponentially toward more di-magnetic values, reaching stable levels after about 4 days. $R_2^*$ increases exponentially to a maximum before being reduced again with progressive fixation time. These observations are in line with previous studies. We used pig-brain hemispheres, which have several similarities with the human brain, including a folded cortex and myelinated axons. With maximal tissue depths of 15 mm, a clear front line possibly reflecting formalin penetration could be observed at a tissue depth of about 4–5 mm in single-slice $R_2$ maps already after 24 h of immersion fixation. The $R_2^*$ values that could be investigated in whole brain tissue masks continued to increase for 5–10 days. The quality of the $R_2^*$ maps was sufficient to allow pixel-wise fitting of the kinetics, revealing a close relationship between the immersion time required to reach maximum $R_2^*$ values and tissue depth, reflecting the expected penetration of the fixatives into the tissue samples. The multifaceted kinetics of $T_1$ during immersion fixation has been modeled previously as contributions from fixed, unfixed, and decomposed tissue in varying fractions during fixation. Similarly, we found that the $R_2^*$ kinetics could be fitted well by a sum of a mono-exponential recovery and mono-exponential decay, even at the level of single voxels. The maximum $R_2^*$ observed could mark a turning point when tissue decomposition that drives the $R_2^*$ up is counteracted by the formalin solution with its low $R_2^*$ which penetrates the tissue. Indeed, with increasing tissue depths, this turning point was achieved at time points that increased as the square root of the immersion time. Such changes are expected for diffusion processes, and our estimates of the Medawar coefficient were lower than for the coagulated chick blood plasma used in the original publication, but faster than those measured in the human spleen. Similar analyses using larger tissue samples are warranted to verify these preliminary findings. Nevertheless, the values found comply with prior studies showing that MR properties can be quantified at 3 T and below, yielding homogeneous WM-GM contrast in human-brain specimens after a few months of formalin fixation. A common way to improve the SNR for ex vivo MRI is to wash out the fixative and replace it by PBS. It leads to in vivo–like $T_1$ times, whereas $T_2$ is less affected by washing. We found similar results in tissue fixed for 28 days after PBS washing and PBS embedding. Unchanged $T_1$, and slight QSM changes with a large decrease in $R_2^*$ that was further decreased after 1 month of PBS storage, were observed. These observations demonstrate that the substitution of the fixative with PBS is not immediate, and that kinetic changes of MR properties during PBS storage occurs. Such factors can be considered to allow reproducible measurements in ex vivo MRI studies. Without washout of the fixative, after replacing the fixative embedding by the proton-free Fluorinert, yielded no change in the MR properties, contrary to the $B_1$ effects.

5 | CONCLUSIONS

In vivo–like $B_1$ fields can be achieved in formalin solutions using PVP and a low-salt concentration as additives. The exact composition of the fixative agent and the immersion time will influence the MR properties of WM and GM. $R_2^*$ is a highly sensitive parameter and can be used as a proxy for tissue penetration, fixation, and washout of the fixatives. In the presence of PVP, lower $T_1$, higher $R_2^*$, and more paramagnetic QSM values were observed. This additive also yielded a smaller estimate of the Medawar coefficient and a more pronounced tissue shrinkage.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

**FIGURE S1** Predicted 1H-NMR spectra for ethanol, C2H5OH, composed of two multiplet resonances. One triplet from the hydrogens in the CH3 group resonate at 1.187 ppm, and a doublet of doublets from the CH2 group at 3.314 ppm

**FIGURE S2** Chemical-shift artifacts originating from ethanol in 1%, 5%, 10%, 20%, and 40% (vol/vol) concentrations for bandwidths per pixel (BW) of 90 Hz and 600 Hz. The first and second ghosts, related to the multiplet resonances in the ethanol 1H-NMR spectra, are obviously apparent in the bottle containing 40% of ethanol and BW of 90 Hz, which become less recognizable at lower concentrations and higher BW

**FIGURE S3** Conductivity (S/m) and permittivity (unitless) of formalin with 5% polyvinylpyrrolidone (PVP) after adding increasing amounts of ethanol and NaCl at 400 MHz. The change could be described with a second order polynomial dependence, with a RMS error of 0.012 and a $R^2$ equal to 0.919 for conductivity, and RMS error = 0.336 and $R^2$ equal to 0.930 for permittivity

**FIGURE S4** Profiles showing the variation in $B_1$-transmit field across a 2.5-L phantom for four fixatives

**FIGURE S5** Time evolution of $T_1$ (A,B), $R_2^*$ (C,D), and QSM (E,F) in gray-matter (GM) (A,C,E) and white-matter (WM) (B,D,F) voxels in the central slices of single pig-brain hemispheres, fitted with exponential functions between day 0.5–35. Shown are the 95% confidence intervals with predictions for the reproducibility measurements performed on day 38–42, indicated by crosses

**FIGURE S6** Median $R_2$ ($R_2^*$) values as a function of $R_2^*$ for four fixatives in WM and GM voxels. The contribution of $R_2$ to $R_2^*$ is greater than the field-dependent $R_2^*$ effect in GM voxels close to the brain surface. In WM, greater contributions of $R_2^*$ are observed, consistent with effects caused by tissue microstructure

**FIGURE S7** $R_2$ (A) and $T_1$ (B) difference maps between measurements performed 12 h and 28 days of immersion fixation in Fix04 (a.), Fix02 (b.), Fix03 (c.), and Fix01 (d.). The results show a prominent increase in WM $R_2$ for the brains immersed in Fix01 and Fix02, whereas GM and WM $T_1$ is more reduced with these two fixatives than with Fix03 and Fix04. Some tissue shrinkage can be identified and is further evaluated at all investigated timepoints, as shown in (C)

**FIGURE S8** Tissue-depth dependence of the coefficients for the pixel-wise fits of $R_2^*$ changes with immersion time, $t$ (expressed in days) in four fixatives according to $R_2^* = R_2^{a^*} . e^{-a t} + R_2^{b^*} . (1 − e^{-b t})$. Shown are median values extracted at different tissue depths between 0.5 and 15.5 mm, in 1-mm bins, which are selected when ≥50 voxels had a goodness of fit (adjusted regression coefficient) $R^2 > 0.8$. White-matter tissue is located approximately 5 mm or more from the surface, yielding a step-wise change in $R_2^*$ which is particularly prominent for Fix01 and Fix02

**TABLE S1** Dielectric properties of four candidate fixatives for different Larmor frequencies at different MR field strengths

Note: Mean and SD for four repetitions of mixing fixatives are listed. The recipe of Fix01 was purposely defined to have near-to-brain-equivalent dielectric properties at 9.4 T. Fix02 had an isotonic salt concentration and thus higher conductivity, which may be beneficial in terms of avoiding dielectric resonance and field focusing artifacts

**TABLE S2** Magnetic resonance properties for the fixative solutions

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