

1 **Studying magnetic susceptibility,**
2 **microstructural compartmentalisation and**
3 **chemical exchange in a formalin-fixed ex vivo**
4 **human brain specimen**

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14 **Running title**

15 Probing white matter phase mechanisms using formalin-fixed tissue

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25 **Word count**

26 3834

27

28 **Abstract**

29 **Purpose:** Ex vivo imaging is a preferable method to study the biophysical mechanism of white
30 matter orientation-dependent signal phase evolution. Yet, how formalin fixation, commonly
31 used for tissue preservation, affects the phase measurement is not fully known. We, therefore,
32 study the impacts of formalin fixation on magnetic susceptibility, microstructural
33 compartmentalisation and chemical exchange measurement on human brain tissue.

34
35 **Methods:** A formalin-fixed, post-mortem human brain specimen was scanned with multiple
36 orientations with respect to the main magnetic field direction for robust bulk magnetic
37 susceptibility measurement with conventional quantitative susceptibility imaging models.
38 Homogeneous white matter tissues were subsequently excised from the whole-brain
39 specimen and scanned in multiple rotations on an MRI scanner to measure the anisotropic
40 magnetic susceptibility and microstructure-related contributions in the signal phase. Electron
41 microscopy was used to validate the MRI findings.

42
43 **Results:** The bulk isotropic magnetic susceptibility of ex vivo whole-brain imaging is
44 comparable to in vivo imaging, with noticeable enhanced non-susceptibility contributions. The
45 excised specimen experiment reveals that anisotropic magnetic susceptibility and
46 compartmentalisation phase effect were considerably reduced in formalin-fixed white matter
47 tissue.

48
49 **Conclusions:** Despite formalin-fixed white matter tissue has comparable bulk isotropic
50 magnetic susceptibility to those measured via in vivo imaging, its orientation-dependent
51 components in the signal phase related to the tissue microstructure is substantially weaker,
52 making it less favourable in white matter microstructure studies using phase imaging.

53
54 **Keywords:** Quantitative susceptibility imaging, phase imaging, ex vivo imaging,
55 microstructure, white matter

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59 Introduction

60 Quantitative susceptibility mapping (QSM) is a physics-driven method to the study magnetic
61 properties of biological tissues (1). Some features that differentiated it from conventional MR
62 relaxometry include the field strength independence of the derived maps, relying on spatial
63 deconvolution and its ability to distinguish paramagnetic and diamagnetic substances since
64 they produce opposite contrasts. QSM is commonly performed on gradient echo phase data
65 owing to its direct relationship to magnetic field variations (2,3).

66
67 One major QSM research challenge is to understand the mechanism of phase evolution in
68 white matter (WM) (4,5). In deep grey matter (GM), strong correlations between QSM and iron
69 concentration have been demonstrated (6). Yet, in WM, the abundance of diamagnetic myelin
70 (relative to water) would have suggested a strong QSM contrast relative to cerebrospinal fluid
71 (CSF) (4,5). The lack of this strong contrast has been attributed to various biophysical
72 phenomena (7,8). The lipid-rich myelin bilayer sheath encapsulating the highly-ordered axons
73 in WM results in anisotropic susceptibility (7–12). Additionally, water protons exist in various
74 microstructural environments (13), namely myelin water, intra-axonal water and extra-axonal
75 water, which can have different signal decay rates and frequency shifts depending on the
76 composition of the tissue and the fibre orientation with respect to the main magnetic field (B_0)
77 (14–17), which also make the signal phase not representing the average magnetic field in a
78 voxel. The chemical exchange of protons between macromolecules and water can also
79 introduce a further frequency shift of the MR signal (18,19). Disentangling the origins of WM
80 phase contrast can improve our understanding of QSM and provide new means to account for
81 their impact in QSM.

82
83 As WM phase contrast is orientation-dependent, studying its properties requires data acquired
84 with different orientations to B_0 . Subject compliance limits the range of angles that can be
85 obtained in vivo (unnatural posture inside the scanner). Experiments with ex vivo samples, on
86 the other hand, do not suffer from this limitation, allowing long scanning sessions without data
87 degradation caused by motion and for histology to be performed as a means of validation of
88 any microstructural findings (20,21). One shortcoming of ex vivo experiments is that MR
89 measured parameters in tissues undergone formalin fixation (a common practice to preserve
90 human post-mortem tissue) show substantial differences to those found in vivo. Those
91 differences have been seen both on single- and multi-compartment relaxometry, and diffusion-
92 weighted imaging (22,23). Yet, a previous study showed that the bulk magnetic susceptibility
93 of brain tissues measured by QSM did not change significantly between in vivo and ex vivo

94 conditions, and also during a 6-week fixation period (24). This finding is in agreement with the
95 experiment results when studying the microstructural effect in phase imaging (25), where
96 comparable bulk magnetic susceptibilities were observed between fresh and fixed rat optic
97 nerves but the origins of the susceptibility contrast are different.

98

99 In this study, we investigate the magnetic susceptibility, compartmentalisation and chemical
100 shift effects on the MR phase using a formalin-fixed, post-mortem human brain specimen for
101 WM phase-contrast mechanism studies at 3T, providing comprehensive insights for the use
102 of fixed tissue in future QSM methodology studies. We performed multiple orientation
103 experiments in both whole-brain and excised tissue samples (26). This enabled us to obtain
104 both traditional QSM maps and ground truth bulk magnetic susceptibility measurements of the
105 excised samples, as well as a separate estimation of microstructure compartmentalisation
106 information. The samples were then studied using electron microscopy (EM) to further
107 evaluate microstructural correlates between MR and histology.

108

109 **Methods**

110 The study is divided into 3 parts:

111 1) a post-mortem, formalin-fixed human brain specimen was scanned on an MRI scanner in
112 multiple orientations with respect to B_0 for robust magnetic susceptibility measurements;

113 2) homogeneous WM specimens were excised from the whole-brain specimen, embedded in
114 agar and subsequently scanned again in various orientations in respect of B_0 to measure their
115 magnetic susceptibility and microstructure-induced field, similar to the experiment conducted
116 by (26);

117 3) the WM specimens used in the second part of the study were imaged by 3D EM, allowing
118 MRI data to be compared to histology.

119

120 **Tissue processing**

121 A post-mortem human brain specimen from a deceased male (aged 78 years old) with no
122 history of neurological disorder (cause of death: myocardial infarction) was used for this
123 research in accordance with the local ethics committee and the Anatomy Department of
124 Radboud University Medical Center (Radboudumc, Nijmegen, the Netherlands). The brain
125 specimen was immersed in 10% formalin for tissue fixation after being extracted from the skull.

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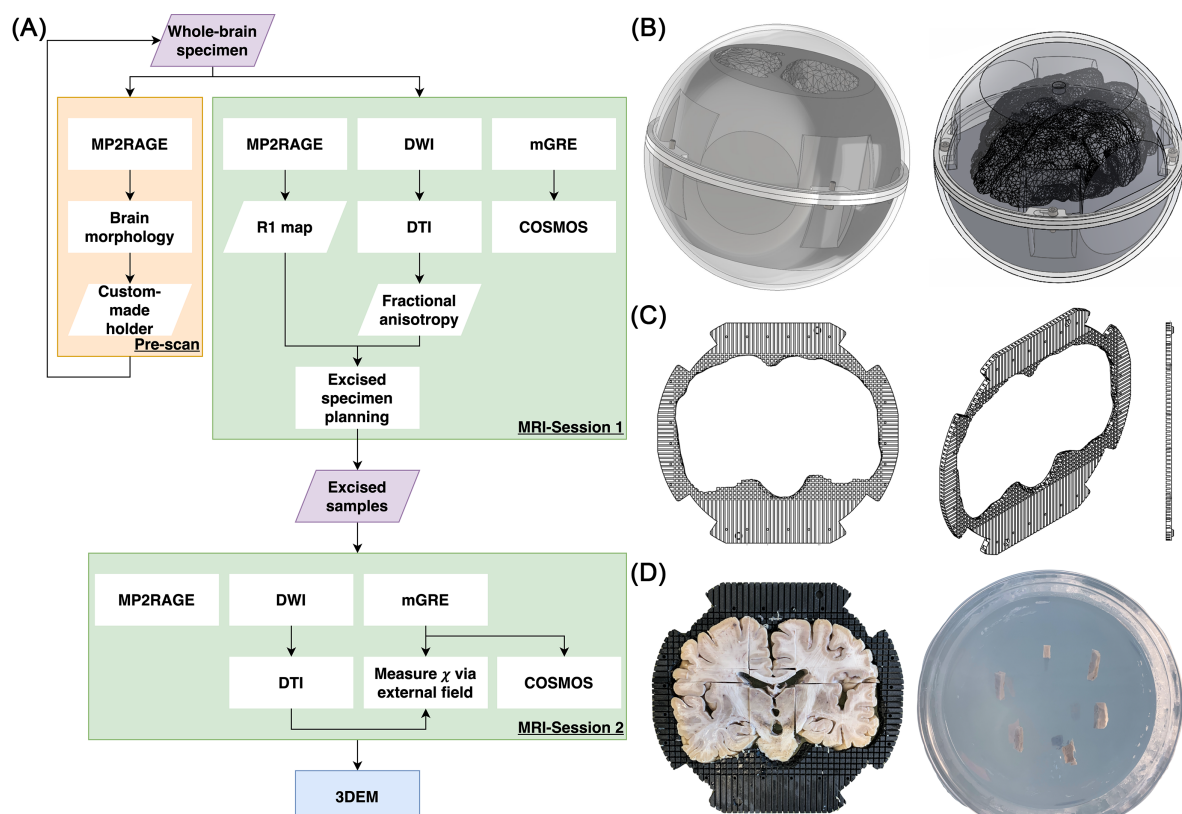
127 After one month of fixation, the specimen was scanned on an MRI scanner (imaging details in
128 section 2.2) to obtain the brain morphology for creating a tailor-made holder. The holder was
129 made of a stack of 35 4-mm thick, 3D-printed plastic plates having space with the same shape
130 of the specimen in the centre, covering most of the brain (see Figure 1) where the specimen
131 can be fitted tightly inside the holder and a surrounding spherical container. Each plate has a
132 grid layout with 4 mm x 4 mm elements, providing landmarks in MRI images for planning and
133 guidance of tissue excision for the validation experiment in the second MRI session.

134

135 After the first MRI session, 10 homogeneous WM regions of interest (ROIs) were identified
136 (DTI fractional anisotropy ≥ 0.45) with excisable volume (≥ 1 element of the holder plate grid,
137 i.e., 64 mm³). These WM tissues and 2 additional deep GM tissues (one from globus pallidus
138 and one from putamen) were then excised and embedded in 1% low-gelling temperature
139 agarose (A9414, Sigma Aldrich, Germany; with demineralised water) to ensure no extra tissue
140 protein denaturation would occur between the two acquisitions. The samples were positioned
141 in a polymethyl methacrylate cylindrical container with the main fibre orientation perpendicular
142 to the cylindrical axis. Imaging was performed about 1 day after the excised specimens were
143 acquired and 5 days after the whole brain scan.

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EM was utilised to provide an additional reference to understand and explain the MRI findings. Two days after the second MRI session, the WM specimens were sectioned to 100 μm on a vibratome (VT1000S, Leica Biosystems, Nussloch, Germany) before being immersed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for overnight incubation at 4°C. The specimens were then transferred to 0.25% glutaraldehyde in 0.1M sodium cacodylate buffer for storage at 4°C, and then delivered to the EM facility at the University of Oxford for imaging. The workflow of this study is summarised in Figure 1.



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Figure 1: (A) A summary of this study, consisting of a pre-scan, two MRI sessions and a 3D EM session. (B) Schematic of the experimental setup used in the first MRI session. The setup is made of two parts: an outer transparent sphere allowing free rotation of the specimen and a tailor-made inner holder to ensure the specimen was in a fixed position inside the sphere. (C) Schematic of the plate which forms the inner holder. The centre of the plate is a space with the shape of the specimen, surrounded by a grid structure providing location reference in MRI image (the gaps filled with water while the plate material gave no detectable signal), and guidance in sample excision for the second imaging session. (D) An illustration of how homogenous samples were acquired with the aids of the plate (right) and the excised specimens were embedded in the agar inside the cylindrical container (left).

164 **MRI experiments**

165 **Data acquisition**

166 The study was approved by the local ethics committee. All MRI data were acquired on a 3T
167 scanner (Prisma, Siemens, Erlangen, Germany) at room temperature (20°C) using a 64-
168 channel array head/neck coil (with only 48 head channels were enabled). The experiment
169 consisted of two imaging sessions: the first session was conducted on the whole-brain
170 specimen and the second session was conducted on the excised brain tissues. The following
171 protocol was used for the first session:

- 172 (1) MP2RAGE adopted to sensitise for T_1 values between 250 ms and 1000 ms, 1 mm
173 isotropic resolution, $T11/T12/TR=311/1600/3000$ ms, flip angle (α) #1/#2 = $4^\circ/6^\circ$, Tacq
174 = 5 min;
- 175 (2) 2D spin-echo EPI DWI, 1.6 mm isotropic, $TR/TE=15241/77.6$ ms, 2-shell
176 ($b=0/1250/2500$ s/mm², 17/120/120 measurements with 7 $b=0$ measurements
177 collected with reversed phase-encode blips for distortion correction), 20 repetitions,
178 Tacq = 5.6 hours;
- 179 (3) Monopolar, 3D multi-echo GRE, 1 mm isotropic resolution,
180 $TR/TE1/\Delta TE/TE6=40/3.45/6.27/34.8$ ms, $\alpha=20^\circ$ (optimised for WM T_1), 10 orientations
181 with respect to B_0 , chosen to optimise microstructural information decoding (27); Tacq
182 = 1.7 hours.

183
184 In the second scanning session, the excised specimens were scanned with the following
185 protocol:

- 186 (1) MP2RAGE sequence with the same parameters as above;
- 187 (2) 2D spin-echo EPI DWI, 1 mm isotropic, $TR/TE=15241/77.6$ ms, 2-shell
188 ($b=0/1250/2500$ s/mm², 17/120/120 measurements with 7 $b=0$ measurements), 9
189 repetitions, Tacq = 9 hours;
- 190 (3) Monopolar, 3D multi-echo GRE, 0.7 mm isotropic resolution,
191 $TR/TE1/dTE/TE6=40/3.45/6.27/34.8$ ms, $FA=15^\circ$ (optimised for agar T_1), 10
192 orientations with respect to B_0 (acquisition order was randomised); Tacq = 5.3 hours.

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194 **Data processing**

195 Each DWI repetition was pre-processed separately with MP-PCA denoising (28),
196 susceptibility-induced distortion correction (29,30) and eddy current-induced distortion

197 correction (31). DTI model was then utilised on the DWI data after averaging all repetitions.
198 R_1 maps and DTI results were then linearly registered to the GRE data using ANTs (32).

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200 GRE data with all orientations were first corrected for gradient nonlinearity induced geometric
201 distortion and then linearly registered to a common space, independent of the experiment
202 orientations. R_2^* maps were computed using a closed-form solution (33). Field maps were
203 computed using SEGUE (34) spatial phase unwrapping with optimum-weighted echo
204 combination (35) and tissue field maps were computed using LBV (36) in SEPIA (37). For the
205 whole-brain data, bulk isotropic magnetic susceptibility was derived using COSMOS (38).
206 Additionally, the QUASAR algorithm for multi-orientations was also applied to test if the bulk
207 isotropic magnetic susceptibility measurement improved when the field generated by non-
208 susceptibility contributions (f_ρ) were simultaneously estimated (39):

$$209 \quad f_N = d_N * \chi + f_\rho \text{ [Eq. 1]}$$

210 where f_N and d_N are the tissue field and a unit dipole field associated with the acquisition at
211 orientation N and * is the convolution operator.

212

213 For the excised specimen data, in addition to the computation of the bulk isotropic magnetic
214 susceptibility using COSMOS, quantification of isotropic and anisotropic magnetic
215 susceptibility (χ_i and χ_a) of the sample without confounding with non-susceptibility
216 microstructural contributions was performed by fitting the measured field f_N on the agar
217 surrounding the specimen in the following manner (26):

$$218 \quad \min_{\chi_i \& \chi_a} \|M_{\text{agar}}(f_N - \chi_i \delta f_{i,N} - \chi_a \delta f_{a,N} - C_N)\| \text{ [Eq. 2]}$$

219 where: M_{agar} is the binary mask on agar with inner and outer boundaries 1 and 5 voxels away
220 from the specimen tissue boundary in all directions; $\delta f_{i,N}$ and $\delta f_{a,N}$ are the frequency
221 perturbations generated by a homogeneous specimen per unit of isotropic and anisotropic
222 magnetic susceptibility at orientation N, with its orientation of tensor derived from obtained
223 from DTI; and C_N accounts for any baseline frequency differences in agar due to either
224 chemical exchange in the agar compartment or any residuals remaining after background field
225 removal for a particular orientation. Linear regression analysis was then conducted to compare
226 the magnetic susceptibility measurements between the COSMOS and external field method,
227 and between the two imaging sessions to determine if the excised specimens agree with the
228 ROIs in the whole-brain imaging data.

229

230 Tissue compartmentalisation contributions to the MR phase can be measured as the residual
231 field (f_R) inside the specimen with mask M_{specimen} :

232
$$f_{R,N} = \overline{M_{specimen}(f_N - \chi_l \delta f_{l,N} - \chi_a \delta f_{a,N})} [Eq. 3].$$

233 which is expected to vary with the angle θ between the fibre direction and B_0 as

234
$$f_{R,N} = A \sin^2 \theta_N + B [Eq. 4]$$

235 where A accounts for the microstructure orientation-dependent effect of the specimen and B
236 is orientation-invariant, related to both magnetisation exchange and microstructure (26).

237

238 **Electron microscopy**

239 Two corpus callosum (CC) specimens from the second MRI session (CC4 and CC5) having
240 the greatest discrepancy of the microstructural compartmentalisation effect underwent 3D EM
241 (40) to provide histology data for the MRI experiment validation. Each of the EM images has
242 a matrix size of 8000×8000 with an in-plane resolution of 13.7×13.7 nm (~0.11x0.11mm FOV)
243 and slice thickness of 100 nm. In total, 651 and 623 slices were acquired for CC4 and CC5
244 respectively.

245

246 Our EM data showed similar myelin sheath damage (splitting and swelling) as illustrated in
247 (41), resulting in unsatisfactory compartmental classification (intra-axonal, myelin and extra-
248 axonal compartments) from standard segmentation tools. To facilitate high-quality 3-
249 compartment classification, we first performed a semi-automatic intra-axonal segmentation
250 using ITK-snap (42) on down-sampled 3D EM data (87.7×87.7 nm and 100 consecutive slices).
251 The myelin sheath of each axon was initially defined by expanding the axon assuming a g-
252 ratio = 0.5 (for axon diameters < 1.2μm) or 0.6 (otherwise), followed by intensity thresholding
253 using the EM images. The resulting myelin mask is clearly influenced by the chosen threshold,
254 therefore, myelin volume fractions (MVF) derived from 5 threshold values range from 125 to
255 145 (step size of 5, most frequent intensities in the myelin mask are 104 (CC4) and 88 (CC5))
256 were reported. To account for the enlarged myelin volume due to swelling, we also computed
257 the image intensity corrected myelin probability as:

258
$$Myelin\ probability = \begin{cases} 1, & I < M \\ \frac{A - I}{A - M}, & M < I \leq A \\ 0, & I > A \end{cases} [Eq. 5]$$

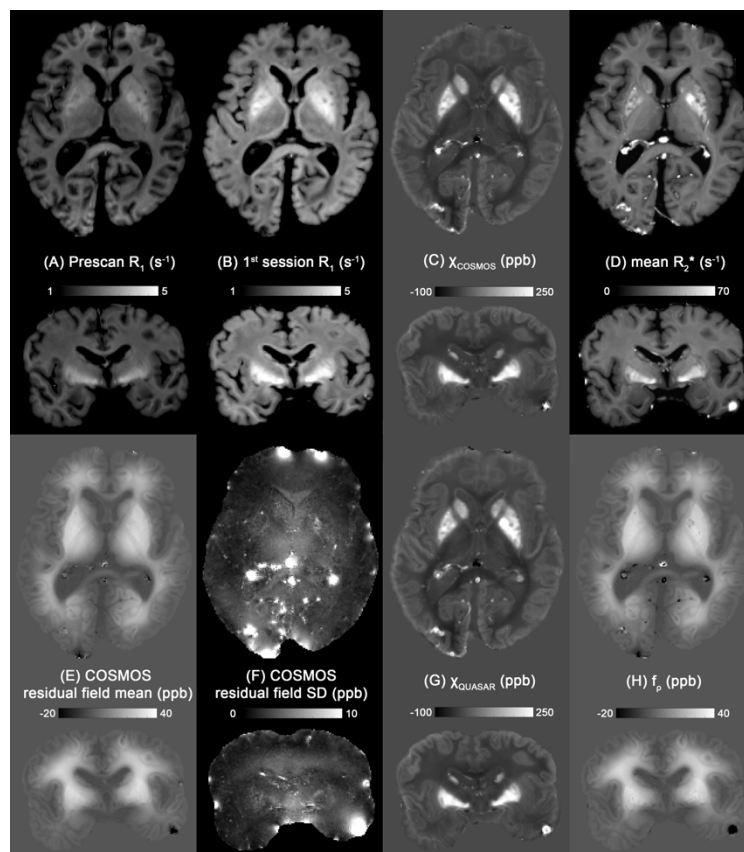
259 where A and M are the most frequent values inside the axonal and myelin masks and I is the
260 intensity of a voxel. The axon and myelin masks of each myelinated axon were eventually
261 combined in a single classification map to avoid repeated counts due to overlapping between
262 neighbouring axons.

263

264 Axonal volume fraction (AVF) and MVF were computed by counting the total number of voxels
265 of each compartment in the classification map, and were used to derive the sample g-ratio
266 (20). Effective axonal diameter was defined as the square root of the product of the 2nd and
267 3rd principal axis lengths of the axons obtained from the regionprop3 function of MATLAB
268 (Mathworks, Natick, US), from which the median and the skewness of the axonal diameter
269 distribution were computed. Fibre dispersion was computed from the axonal volume-weighted
270 average squared dot product between the axon main principal direction and the average
271 orientation of the entire sample (27). To further investigate the effect of realistic myelin sheath
272 geometry on the compartmental frequency shifts, field perturbations induced from myelin χ_i
273 was simulated in two scenarios: when the segmented sample axons were parallel or
274 perpendicular to B_0 . The frequency shift distribution in the extracellular space was
275 subsequently analysed.
276

277 Results

278 Whole-brain imaging results are shown in Figure 2. The R_1 maps obtained from the first
279 session (5-month fixation) show faster relaxation rates than those from the pre-scan (1-month
280 fixation), with the contrast between WM and cortical GM being clearly reduced, and DGM
281 showing increased R_1 (Figure 2A and 2B). The COSMOS derived magnetic susceptibilities
282 are in reasonable agreement with previously published in vivo data (see supplementary Figure
283 S1), where opposite magnetic susceptibility between WM and GM can be observed (Figure
284 2C). However, the residual field of the COSMOS estimation shows a slowly-varying pattern
285 across the brain that cannot be explained by the isotropic dipole field (Figure 2E) and is
286 relatively stable across orientations (see Figure 2F). This residual map shares similar contrast
287 and values with the QUASAR non-susceptibility contributions map (Figure 2H). Hence, not
288 surprisingly, the bulk magnetic susceptibilities derived from QUASAR (Figure 2G) and
289 COSMOS (Figure 2c) are comparable. Susceptibility tensor imaging was also performed, yet
290 the results beyond the mean susceptibility tensor were found not informative.

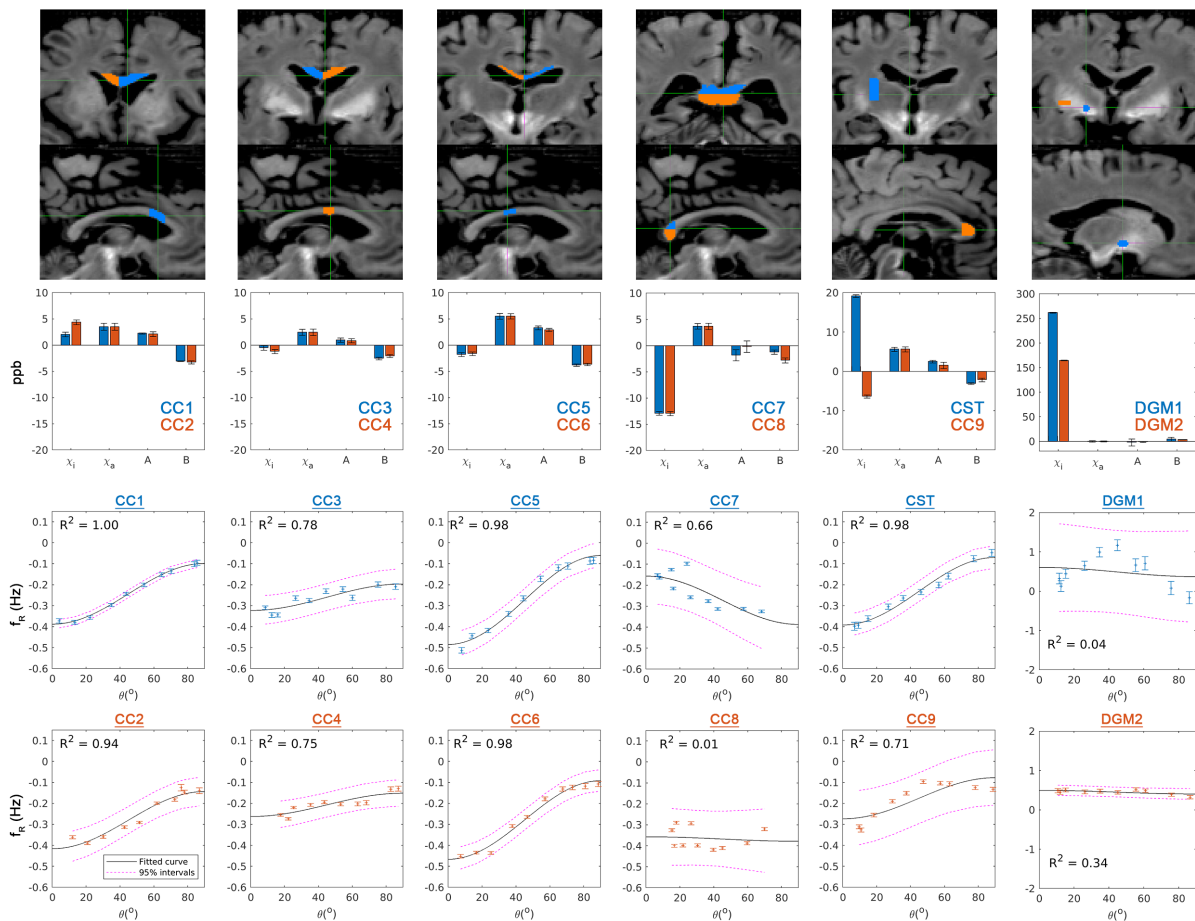


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292 Figure 2: Quantitative maps of the whole-brain specimens in transverse and coronal directions. (A) R_1
293 map obtained from a pre-scan after 1-month of formalin fixation and (B) R_1 map obtained from the 1st
294 imaging session while the brain was fixed for 5 months. (C) COSMOS derived bulk magnetic
295 susceptibility, (D) mean R_2^* map across the 10 rotations, (E and F) mean and standard deviation of the

296 residual fields from COSMOS across orientations, (G and H) QUASAR derived bulk magnetic
 297 susceptibility map and non-susceptibility contribution map.

298

299 Figure 3 shows the magnetic susceptibility of the excised specimens measured via the
 300 external field on agar with their ROIs illustrated in the whole-brain R_1 map. The mean χ_i and
 301 χ_a are -1.17 ± 9.18 ppb and 4.03 ± 1.63 ppb across WM specimens. A relatively strong positive
 302 χ_i is found in the corticospinal tract specimen (CST; 19.17 ppb), which was found in retrospect
 303 to be due to some DGM residual in one end of the excised sample. The coefficient A of $\sin^2\theta$
 304 dependence reflecting the WM microstructure effect has a mean of 1.46 ± 1.55 ppb with a mean
 305 intercept B of -2.75 ± 0.79 ppb in WM. However, the R^2 of the specimen residual field fitting
 306 suggests that not all WM specimens fit the $\sin^2\theta$ function equally well, particularly for samples
 307 obtained from the genu and splenium of the CC (CC7-CC9; R^2 ranging from 0.01 to 0.71).
 308 Therefore, we focused on the 6 WM specimens obtained from the body of the CC (CC1-CC6)
 309 in comparison to the whole-brain data in the linear regression analysis.



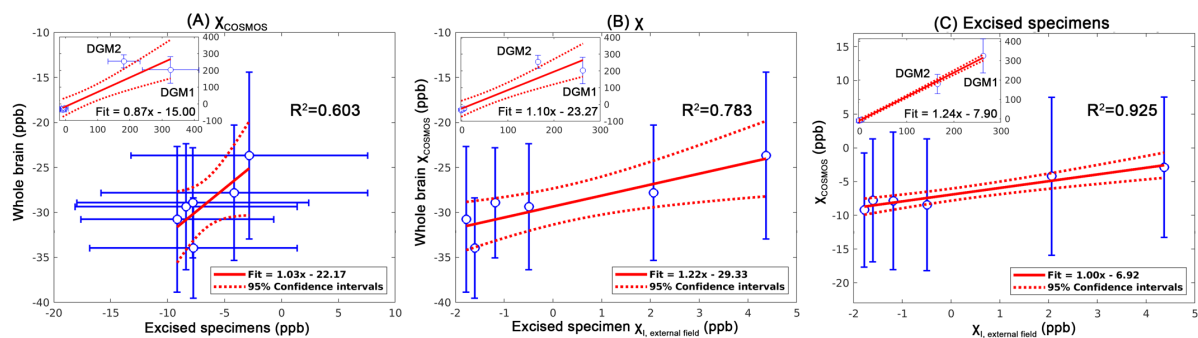
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311 Figure 3: (Middle row) Barplots of isotropic and anisotropic magnetic susceptibility, and coefficients A
 312 and B of the fitting of $\sin^2\theta$. (Bottom two rows) Fittings of $\sin^2\theta$ reflecting microstructure
 313 compartmentalisation of the excised specimens. Each column shows the results of two specimens with
 314 their ROIs illustrated in the whole-brain R_1 map (top two rows). Error bar indicates the standard error

315 (except for the coefficients A and B in the barplots; 95% confidence intervals in this case). CC: corpus
 316 callosum; CST: corticospinal tract; DGM1: globus pallidus; DGM2: putamen.

317

318 Strong linear relations were found in mean susceptibility estimated by COSMOS between the
 319 excised specimens and the corresponding ROI in the whole-brain data (cross-session;
 320 $R^2=0.603$, Figure 4A), between the χ_i from external field measurement and the mean
 321 COSMOS susceptibility in the whole-brain data (cross-session, cross-method; $R^2=0.783$,
 322 Figure 4B), and between the χ_i from external field measurement and the mean COSMOS
 323 susceptibility on the excised specimens (cross-method; $R^2=0.925$, Figure 4C). All the slopes
 324 of the linear regressions are close to 1, whereas the relatively large intercepts in Figure 4A-B
 325 reflect the different reference medium in the scans (1st session: water; 2nd session: 1% agar).



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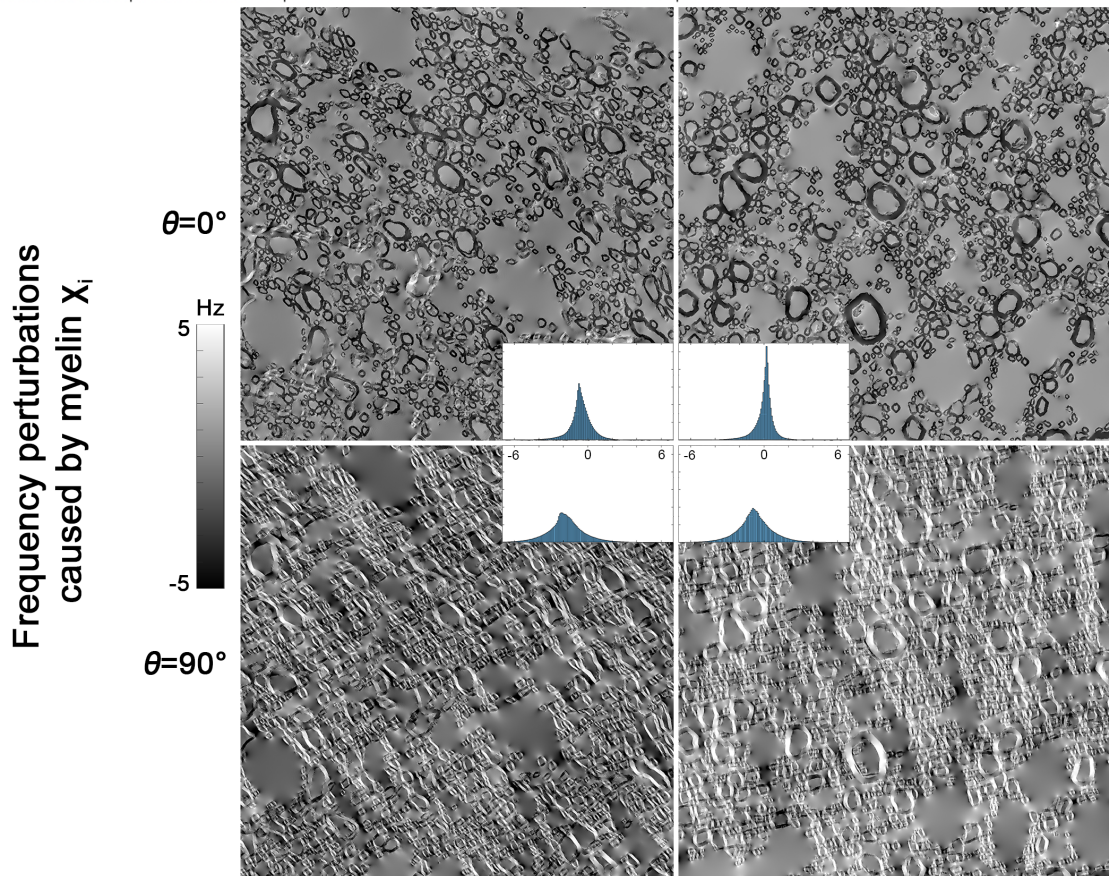
327 Figure 4: Linear regression analyses on the 6 WM specimens of (A) the magnetic susceptibility
 328 measured between two imaging sessions using COSMOS, (B) between the bulk magnetic susceptibility
 329 measured by COSMOS on the first session and the χ_i from external field measurement on the second
 330 session, and (C) the excised specimens between COSMOS magnetic susceptibility and external field
 331 derived χ_i . Blue points: measurement data; solid red line: fitted line; dash line: 95% confidence interval;
 332 error bar: standard deviation. The subplot of each panel shows the regression result when the DGM
 333 specimens were included.

334

335 The microstructural properties of two WM specimens (CC4: relatively weaker microstructural
 336 phase; CC5: strong microstructural phase) derived from 3D EM data are summarised in Figure
 337 5. Both specimens have similar MVF (8% difference), AVF (4% difference) and axonal
 338 diameter (1% difference). Noticeable differences are observed in fibre dispersion (50%
 339 difference) and in the FWHM of the extra-axonal frequency distributions when the fibre
 340 direction is parallel to B_0 (63% difference), the latter being attributed to the differences of both
 341 fibre dispersion and the spatial distribution of myelinated axons.

342

		CC4	CC5
# of Axons		1607	1565
Effective axonal diameter	Median, μm	0.944	0.957
	Skewness	1.798	2.058
Dispersion		0.135	0.081
AVF		0.183	0.192
MVF		0.272, 0.304, 0.328*, 0.345, 0.357	0.283, 0.304, 0.321*, 0.334, 0.344
MVF corrected		0.245, 0.265, 0.278*, 0.285, 0.289	0.238, 0.249, 0.257*, 0.261, 0.264
g-ratio		0.599	0.611
g-ratio corrected		0.630	0.654
Peak of extracellular frequency shift caused by myelin χ_1 (FWHM), Hz	$\theta=0^\circ$	-0.735 (0.829)	0.194 (0.434)
	$\theta=90^\circ$	-2.189 (1.85)	-0.919 (1.834)



343

344 Figure 5: (Top) Summary of the 3D EM derived CC4 and CC5 microstructural properties. MVF was
 345 separately probed using 5 different intensity thresholds (only the middle value indicated by * was
 346 showed in g-ratio). The corrected MVF was derived using Eq. [5]. (Bottom) Frequency induced by the
 347 myelin χ_1 at two orientations to B_0 ($\theta=0^\circ$ & 90°). Sub-figures show the frequency distributions in
 348 extracellular space, where the locations and the FWHM of the peaks are shown in the above summary.

349 Discussion

350 In this study, we examined the magnetic susceptibility and microstructural
351 compartmentalisation effect on MRI phase data on a formalin-fixed, post-mortem human brain
352 specimen. The bulk magnetic susceptibility of the whole-brain specimen shows comparable
353 contrast to those in the previous ex vivo studies (6,24), as well as to in vivo imaging: WM is
354 slightly diamagnetic, whereas cortical and deep GM are paramagnetic. Further investigation
355 reveals the residual fields of COSMOS have a gradient-like appearance varying from the
356 surface toward the centre of the specimen (Figure 2E), which is similar to the expected way
357 of how the solutions (fixative or water) diffused into the specimen. Since these residual fields
358 are relatively constant across different rotations, it is likely to be caused by the exchange effect
359 (17–19). These fields were captured as the non-susceptibility contributions by QUASAR and
360 they did not have a significant impact on the bulk magnetic susceptibility measurement (Figure
361 2C, 2G). This result is distinct from in vivo imaging results (Figure S1), where the susceptibility
362 differences in WM are more noticeable, suggesting that the effect of (sub)cellular structure of
363 WM is considerably reduced and the sphere of Lorentz inclusion utilised in COSMOS is
364 already a good approximation on formalin-fixed tissue.

365
366 While the bulk susceptibility of the WM samples in this study is similar to in vivo imaging, the
367 residual field analysis inside the excised homogenous tissue confirmed that the microstructure
368 compartmental frequency (parameter A in Eq. 4) is notably weaker in our samples than in vivo
369 and reported by others. In a similar experiment (26), the amplitude of the microstructure
370 frequency of a fresh bovine optic nerve at 7T was -18.75 ppb, significantly larger in magnitude
371 and with an opposite sign to what we have obtained in our CC samples, 1.46 ppb. Additional
372 analysis was performed to consolidate this result (see supplementary Figure S2). A reduction
373 of the microstructural compartmentalisation effect had already been reported in the literature
374 when studying fresh vs fixed rat optic nerves (25). One possible explanation is the structural
375 alteration of the myelin sheath in fixed tissues. In our 3D EM images, we observed myelin
376 sheath spitting and swelling in some of the myelinated axons, similar to the observation
377 reported in the previous study (41), and such phenomena appeared more frequently in larger
378 axons than small axons. Based on the general Lorentzian tensor approach (43), the increase
379 of the aqueous space of the myelin sheath can result in the amplitude reduction of the induced
380 frequency shifts inside the myelin sheath and the intra-axonal space. Microstructural
381 differences related to structures (bovine optic nerve vs human CC) and age-associated
382 demyelination (45,46), together with the tissue preparation methods can also contribute to the

383 differences observed in this study, as all these factors modulate the relative water
384 concentration in the three WM compartments.

385

386 All six specimens obtained from the body of the CC have similar magnetic susceptibility
387 anisotropy, suggesting that they have similar MVF based on the HCM approximation (Eq. S25
388 in (17)), and this is supported by the EM analysis (0.278 & 0.257 between the two samples).
389 The amplitude of the residual field inside the specimens is, on the other hand, subject to
390 various properties including MVF, AVF and the aggregate g-ratio of the sample (Eq. A14 in
391 (16)). Interestingly, the realistic geometry of the WM fibre also plays an important role in the
392 compartmental frequency shifts (27,44). This effect is clearly illustrated in the frequency
393 perturbation simulations in Figure 5: not only the centres but also the FWHM of the extra-
394 cellular frequency distribution of the two samples are different, despite the two specimens
395 having virtually identical MVF and AVF. The broader frequency spectrum of CC4 induces a
396 faster R_2^* decay in the extra-axonal space and the specimen also has a more disperse fibre
397 arrangement. These two factors together reduce the amplitude discrepancy between the slow
398 R_2^* (intra- and extra-axonal water) and the fast R_2^* (myelin water) compartments throughout
399 the echo time as well as the frequency difference between those compartments that could
400 result in a reduced compartmentalization effect.

401

402 The experimental setup, particularly the 3D-printed holder, is an effective tool also for other
403 ex vivo studies that involve histology. The grid on the 3D-printed plates not only facilitates
404 tissue excision with high precision but also provides landmarks in MRI images for experiment
405 planning and sample matching. The close to unity linear relations of the susceptibility
406 measurements between the two sessions (Figure 4A and 4B) support that the ROIs drawn on
407 the whole-brain images and the excised specimens are corresponding to each other.

408

409 One limitation of this study is the relatively long fixation time of the specimen. The first imaging
410 session happened after 5 months of fixation instead of the scheduled 2 months because of
411 the coronavirus measures that took place in the Netherlands. The prolonged fixation time
412 results in a substantial change of specimen R_1 between the pre-scan and the first session
413 (Figure 2A, 2B; average R_1 across the brain increased from $1.95 \pm 0.47 \text{ s}^{-1}$ to $2.67 \pm 0.74 \text{ s}^{-1}$).
414 The enhanced R_1 in deep GM suggests the contribution of iron to the R_1 was more pronounced.
415 An alternative and more likely explanation would be that the myelin contribution to R_1 could
416 be reduced by the fixation process, which would also explain the diminished cortical GM and
417 WM contrast observed at 5 months. Experiments conducted with a shorter fixation time can
418 potentially reduce the fixation effects on the signal phase. However, progressive changes in

419 relaxation parameters begin in the early stage of fixation (23) and it is likely that fixation
420 induced phase differences could also happen simultaneously. Lastly, the EM analysis can be
421 subject to sampling bias due to the limited field-of-view. Preliminary results of a recent study
422 suggest that large axons in the WM tissue can be under-represented in EM compared to light
423 microscopy when larger field of view is available (45). Such under-representation may have
424 an impact on the realistic geometry of the myelinated axons on the phase data.
425

426 **Conclusion**

427 The contributions of MR phase contrast observed in the formalin-foxed brain specimen are
428 substantially different from fresh tissue, despite the QSM maps derived from in vivo and ex
429 vivo imaging sharing similar contrasts and values. Particularly, the reductions of magnetic
430 susceptibility anisotropy and compartmentalisation are observed in the fixed WM tissue. An
431 increase of non-susceptibility contributions to phase contrast can also be found in fixed tissue,
432 which is potentially introduced by formalin fixation. Therefore, WM magnetic susceptibility and
433 microstructural quantification findings in studies using formalin-fixed tissue should be
434 interpreted with care. Our study suggests that the microstructural effects observed in our
435 samples encode information regarding WM arrangements such as dispersion and packing
436 while susceptibility anisotropy encodes myelin volume as was predicted from theory.

437

438 **Acknowledgement**

439 This work was funded by the Netherlands Organisation for Scientific Research (NWO) with
440 project number FOM-N-31/16PR1056. The authors would like to thank Ms. Sibrecht Bouwstra
441 for the 3D-printed holder construction, Ms. Shaghoyegh Abghari for laboratory support, Dr.
442 Errin Johnson for 3D EM data acquisition, and Dr. Ferdinand Schweser for the fruitful
443 discussion on magnetic susceptibility analysis including chemical exchange terms.

444

445 **References**

446

447 1. Rochefort L de, Liu T, Kressler B, et al. Quantitative susceptibility map reconstruction from
448 MR phase data using bayesian regularization: Validation and application to brain imaging.
449 *Magnetic resonance in medicine* 2010;63:194–206 doi: 10.1002/mrm.22187.

450 2. Deistung A, Schweser F, Reichenbach JR. Overview of quantitative susceptibility mapping.
451 *NMR in biomedicine* 2016;30 doi: 10.1002/nbm.3569.

452 3. Wang Y, Liu T. Quantitative susceptibility mapping (QSM): Decoding MRI data for a tissue
453 magnetic biomarker. *Magnetic Resonance in Medicine* 2014;73:82–101 doi:
454 10.1002/mrm.25358.

455 4. Duyn JH, Gelderen P van, Li T-Q, Zwart JA de, Koretsky AP, Fukunaga M. High-field MRI
456 of brain cortical substructure based on signal phase. *Proceedings of the National Academy of*
457 *Sciences of the United States of America* 2007;104:11796–11801 doi:
458 10.1073/pnas.0610821104.

459 5. He X, Yablonskiy DA. Biophysical mechanisms of phase contrast in gradient echo MRI.
460 *Proceedings of the National Academy of Sciences* 2009;106:13558–13563 doi:
461 10.1073/pnas.0904899106.

462 6. Langkammer C, Schweser F, Krebs N, et al. Quantitative susceptibility mapping (QSM) as
463 a means to measure brain iron? A post mortem validation study. *Neuroimage* 2012;62:1593–
464 1599 doi: 10.1016/j.neuroimage.2012.05.049.

465 7. Li W, Wu B, Avram AV, Liu C. Magnetic susceptibility anisotropy of human brain in vivo and
466 its molecular underpinnings. *Neuroimage* 2012;59:2088–2097 doi:
467 10.1016/j.neuroimage.2011.10.038.

468 8. Yablonskiy DA, He X, Luo J, Sukstanskii AL. Lorentz sphere versus generalized Lorentzian
469 approach: What would lorentz say about it? *Magnetic Resonance in Medicine* 2014;72:4–7
470 doi: 10.1002/mrm.25230.

471 9. Yablonskiy DA, Sukstanskii AL. Generalized Lorentzian Tensor Approach (GLTA) as a
472 biophysical background for quantitative susceptibility mapping. *Magnetic Resonance in*
473 *Medicine* 2015;73:757–764 doi: 10.1002/mrm.25538.

474 10. Yablonskiy DA, Sukstanskii AL. Effects of biological tissue structural anisotropy and
475 anisotropy of magnetic susceptibility on the gradient echo MRI signal phase: theoretical
476 background Liu C, Bowtell Richard, Schenck J, editors. *NMR in Biomedicine* 2017;30:e3655
477 doi: 10.1002/nbm.3655.

478 11. Liu C. Susceptibility tensor imaging. *Magnetic resonance in medicine* 2010;63:1471–1477
479 doi: 10.1002/mrm.22482.

- 480 12. Lee J, Shmueli K, Fukunaga M, et al. Sensitivity of MRI resonance frequency to the
481 orientation of brain tissue microstructure. *Proceedings of the National Academy of Sciences*
482 of the United States of America 2010;107:5130–5135 doi: 10.1073/pnas.0910222107.
- 483 13. MacKay A, Whittall K, Adler J, Li D, Paty D, Graeb D. In vivo visualization of myelin water
484 in brain by magnetic resonance. *Magnetic resonance in medicine* 1994;31:673–677.
- 485 14. Sati P, Gelderen P van, Silva AC, et al. Micro-compartment specific T2* relaxation in the
486 brain. *Neuroimage* 2013;77:268–278 doi: 10.1016/j.neuroimage.2013.03.005.
- 487 15. Gelderen P van, Zwart JA de, Lee J, Sati P, Reich DS, Duyn JH. Nonexponential T2*
488 decay in white matter. *Magnetic resonance in medicine* 2011;67:110–117 doi:
489 10.1002/mrm.22990.
- 490 16. Wharton S, Bowtell R. Gradient echo based fiber orientation mapping using R2* and
491 frequency difference measurements. *NeuroImage* 2013;83:1011–1023 doi:
492 10.1016/j.neuroimage.2013.07.054.
- 493 17. Wharton S, Bowtell R. Fiber orientation-dependent white matter contrast in gradient echo
494 MRI. *Proceedings of the National Academy of Sciences* 2012;109:18559–18564 doi:
495 10.1073/pnas.1211075109.
- 496 18. Luo J, He X, d'Avignon DA, Ackerman JJH, Yablonskiy DA. Protein-induced water 1H MR
497 frequency shifts: Contributions from magnetic susceptibility and exchange effects. *J Magn*
498 *Reson* 2010;202:102–108 doi: 10.1016/j.jmr.2009.10.005.
- 499 19. Shmueli K, Dodd SJ, Gelderen P van, Duyn JH. Investigating lipids as a source of chemical
500 exchange-induced MRI frequency shifts. Liu C, Bowtell Richard, Schenck J, editors. *NMR in*
501 *biomedicine* 2017;30:e3525 doi: 10.1002/nbm.3525.
- 502 20. Stikov N, Campbell JSW, Stroh T, et al. In vivo histology of the myelin g-ratio with magnetic
503 resonance imaging. *NeuroImage* 2015;118:397–405 doi: 10.1016/j.neuroimage.2015.05.023.
- 504 21. Langkammer C, Krebs N, Goessler W, et al. Quantitative MR Imaging of Brain Iron: A
505 Postmortem Validation Study. *Radiology* 2010;257:455–462 doi: 10.1148/radiol.10100495.
- 506 22. Seifert AC, Umphlett M, Hefti M, Fowkes M, Xu J. Formalin tissue fixation biases myelin-
507 sensitive MRI. *Magnetic resonance in medicine* 2019;82:1504–1517 doi: 10.1002/mrm.27821.
- 508 23. Shatil AS, Uddin MN, Matsuda KM, Figley CR. Quantitative Ex Vivo MRI Changes due to
509 Progressive Formalin Fixation in Whole Human Brain Specimens: Longitudinal
510 Characterization of Diffusion, Relaxometry, and Myelin Water Fraction Measurements at 3T.
511 *Frontiers in medicine* 2018;5:31 doi: 10.3389/fmed.2018.00031.
- 512 24. Evia AM, Kotrotsou A, Tamhane AA, et al. Ex-vivo quantitative susceptibility mapping of
513 human brain hemispheres. Connor JR, editor. *PLOS ONE* 2017;12:e0188395 doi:
514 10.1371/journal.pone.0188395.

- 515 25. Luo J, He X, Yablonskiy DA. Magnetic susceptibility induced white matter MR signal
516 frequency shifts—experimental comparison between Lorentzian sphere and generalized
517 Lorentzian approaches. *Magnetic Resonance in Medicine* 2014;71:1251–1263 doi:
518 10.1002/mrm.24762.
- 519 26. Wharton S, Bowtell R. Effects of white matter microstructure on phase and susceptibility
520 maps. *Magnetic Resonance in Medicine* 2015;73:1258–1269 doi: 10.1002/mrm.25189.
- 521 27. Hédouin R, Metere R, Chan K-S, et al. Decoding the microstructural properties of white
522 matter using realistic models. *Neuroimage* 2021;237:118138 doi:
523 10.1016/j.neuroimage.2021.118138.
- 524 28. Veraart J, Novikov DS, Christiaens D, Ades-aron B, Sijbers J, Fieremans E. Denoising of
525 diffusion MRI using random matrix theory. *Neuroimage* 2016;142:394–406 doi:
526 10.1016/j.neuroimage.2016.08.016.
- 527 29. Andersson JLR, Skare S, Ashburner J. How to correct susceptibility distortions in spin-
528 echo echo-planar images: application to diffusion tensor imaging. *Neuroimage* 2003;20:870–
529 888 doi: 10.1016/s1053-8119(03)00336-7.
- 530 30. Smith SM, Jenkinson M, Woolrich MW, et al. Advances in functional and structural MR
531 image analysis and implementation as FSL. *Neuroimage* 2004;23:S208–S219 doi:
532 10.1016/j.neuroimage.2004.07.051.
- 533 31. Andersson JLR, Sotiropoulos SN. An integrated approach to correction for off-resonance
534 effects and subject movement in diffusion MR imaging. *Neuroimage* 2016;125:1063–1078 doi:
535 10.1016/j.neuroimage.2015.10.019.
- 536 32. Avants BB, Tustison NJ, Song G, Cook PA, Klein A, Gee JC. A reproducible evaluation of
537 ANTs similarity metric performance in brain image registration. *Neuroimage* 2011;54:2033–
538 2044 doi: 10.1016/j.neuroimage.2010.09.025.
- 539 33. Khabipova D, Wiaux Y, Gruetter R, Marques JP. A modulated closed form solution for
540 quantitative susceptibility mapping—a thorough evaluation and comparison to iterative
541 methods based on edge prior knowledge. *Neuroimage* 2015;107:163–174 doi:
542 10.1016/j.neuroimage.2014.11.038.
- 543 34. Karsa A, Shmueli K. SEGUE: a Speedy rEgion-Growing algorithm for Unwrapping
544 Estimated phase. *IEEE Transactions on Medical Imaging* 2018;38:1347–1357 doi:
545 10.1109/tmi.2018.2884093.
- 546 35. Robinson SD, Bredies K, Khabipova D, Dymerska B, Marques JP, Schweser F. An
547 illustrated comparison of processing methods for MR phase imaging and QSM: combining
548 array coil signals and phase unwrapping. Liu C, Bowtell Richard, Schenck J, editors. *NMR in*
549 *biomedicine* 2017;30:e3601 doi: 10.1002/nbm.3601.

- 550 36. Zhou D, Liu T, Spincemaille P, Wang Y. Background field removal by solving the Laplacian
551 boundary value problem. *NMR in biomedicine* 2014;27:312–319 doi: 10.1002/nbm.3064.
- 552 37. Chan K-S, Marques JP. SEPIA—Susceptibility mapping pipeline tool for phase images.
553 *Neuroimage* 2021;227:117611 doi: 10.1016/j.neuroimage.2020.117611.
- 554 38. Liu T, Spincemaille P, Rochefort L de, Kressler B, Wang Y. Calculation of susceptibility
555 through multiple orientation sampling (COSMOS): A method for conditioning the inverse
556 problem from measured magnetic field map to susceptibility source image in MRI. *Magnetic
557 resonance in medicine* 2009;61:196–204 doi: 10.1002/mrm.21828.
- 558 39. Schweser F, Zivadinov R. Quantitative susceptibility mapping (QSM) with an extended
559 physical model for MRI frequency contrast in the brain: a proof-of-concept of quantitative
560 susceptibility and residual (QUASAR) mapping. *NMR in Biomedicine* 2018;31:e3999 doi:
561 10.1002/nbm.3999.
- 562 40. Kleinnijenhuis M, Johnson E, Mollink J, Jbabdi S, Miller KL. A semi-automated approach
563 to dense segmentation of 3D white matter electron microscopy. *Biorxiv*
564 2020:2020.03.19.979393 doi: 10.1101/2020.03.19.979393.
- 565 41. Duijn S van, Nabuurs RJA, Rooden S van, et al. MRI artifacts in human brain tissue after
566 prolonged formalin storage. *Magnetic resonance in medicine* 2011;65:1750–1758 doi:
567 10.1002/mrm.22758.
- 568 42. Yushkevich PA, Piven J, Hazlett HC, et al. User-guided 3D active contour segmentation
569 of anatomical structures: Significantly improved efficiency and reliability. *Neuroimage*
570 2006;31:1116–1128 doi: 10.1016/j.neuroimage.2006.01.015.
- 571 43. Yablonskiy DA, Sukstanskii AL. Lorentzian effects in magnetic susceptibility mapping of
572 anisotropic biological tissues. *Journal of Magnetic Resonance* 2018;292:129–136 doi:
573 10.1016/j.jmr.2018.04.014.
- 574 44. Xu T, Foxley S, Kleinnijenhuis M, Chen WC, Miller KL. The effect of realistic geometries
575 on the susceptibility-weighted MR signal in white matter. *Magnetic resonance in medicine*
576 2017;4:153–500 doi: 10.1002/mrm.26689.
- 577 45. Mordhorst L, Morozova M, Papazoglou S, et al. A representative reference for MRI-based
578 human axon radius assessment using light microscopy. *Biorxiv* 2021:2021.06.03.446739 doi:
579 10.1101/2021.06.03.446739.
- 580