**Supplementary material**

**Incorporating the effect of white matter microstructure in the estimation of magnetic susceptibility in ex-vivo mouse brain**

1. **Larmor frequency from WM with axons and uniform iron content**

In this section we investigate the Larmor frequency caused by the magnetic susceptibility of the sample when white matter (WM) contains both myelinated axons and iron. We assume that iron content varies very little across WM. We consider here for simplicity a porous media of WM, gray matter (GM) and Cerebral-spinal fluid (W). We describe the macroscopic tissue regions by the non-overlapping indicator functions for inside the brain. The WM compartment includes myelinated axons with susceptibility . Iron complexes with are found in both GM and WM, while water with are in both WM, GM and W. The microscopic susceptibility thus becomes where are non-overlapping microscopic indicator functions fulfilling . Here we neglected susceptibility anisotropy of myelin for simplicity. The microscopic Larmor frequency offset thus becomes

As a first step we rewrite Equation such that the tissue susceptibilities are referenced to the susceptibility

where . To describe the measured frequency shift, we consider the mesoscopically averaged frequency shift of Equation in terms of its mesoscopic and macroscopic contributions

We assume that iron is homogenously distributed in each voxel in both WM and GM such that . The iron WM susceptibility is further rewritten as the deviation from the mean across , i.e., . If the variation in the mesoscopically averaged bulk susceptibility in WM is sufficiently small compared to from myelin, such that we may neglect it as a first order approximation, similar to why we neglected susceptibility anisotropy (Wharton & Bowtell, 2015) (see simulation in Figure 5 in the main text), then the macroscopic contribution from iron becomes

Using Equations , and (4), the mesoscopically averaged frequency shift becomes

The macroscopic contribution of the constant susceptibility defines the reference frequency in Equation (4) and is removed by the background field removal algorithm (Schweser et al., 2017). We see that when referencing to water after susceptibility fitting, WM is described by both myelin and WM iron through , while GM describes its iron content , all in reference to water.

This is demonstrated in Figure S1A depicting a simple brain phantom with uniform susceptibilities , , . Susceptibility in ventricles is set to zero as we referenced to water . This defines the ground truth susceptibility , which we wish to estimate. We also get a susceptibility component across the whole sample after referencing to water and we also include a uniform external susceptibility . The Larmor frequency was computed using Equation (4) with including mesoscopic frequency shifts from myelin. We computed from 10 unique sample orientations made using electrostatic repulsion(Jones et al., 1999) to avoid magic-angle artifacts. Figure S1B shows the corresponding Larmor frequency including mesoscopic frequency shifts. We removed the reference frequency and external field contribution using LBV (Zhou et al., 2014), as is seen in Figure S1C. We then estimated the susceptibility using Equation (9) and referenced it to the mean susceptibility in the ventricles. This referencing removes any constant susceptibility component across the sample caused by LBV when removing external fields. Figure S1D shows the difference in susceptibility to ground truth . In WM, we find a mean susceptibility -0.98±0.35 in agreement with , in GM we find 4.93±0.41 corresponding to and in CSF 0±0.14 corresponding to .

A picture containing shape

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**Figure S1 - Susceptibility phantom WM iron and myelin including external fields: A** shows the ground truth susceptibility from WM iron, WM myelin, GM iron, while the ventricles have zero susceptibility. **B** shows the corresponding frequency shift from including frequency shifts from a uniform external and internal susceptibility. **C** shows after background field removal, which removes both contributions from the internal and external uniform susceptibility. **D** Shows the difference between the fitted susceptibility (after referencing to CSF in ventricles) and ground truth susceptibility .

1. **Indicator function for multilayer cylinder**

In this section we derive the mesoscopic demagnetization tensor (Sandgaard, Shemesh, et al., 2022), , for multi-layer cylinders with arbitrary orientations (see Figure 2) to extend our model for solid cylinders. As described in previous work, the mesoscopic demagnetization tensor (Sandgaard, Shemesh, et al., 2022) depends only on structural correlations,

Here is the structural correlation function, whose generic form in Fourier space is

and zero for . When susceptibility is uniform, the product defines the mesoscopic Lorentzian tensor (Kiselev, 2019; Sandgaard, Shemesh, et al., 2022) and characterizes (cf. Equation ). The indicator function for an infinitely long cylinder consisting of concentric shells is a superposition of solid infinite cylinders(Sandgaard, Kiselev, et al., 2022)

Here defines the indicator function in the 2D plane transverse to the orientation , where denotes the outer and inner radii of the *q*’th layer. Consider N multilayer cylinders as conceptualized in Figure 2. They are randomly positioned and exhibit arbitrary orientation dispersion independent of their size. Summing over all N multilayer cylinders the total correlation function , Equation , splits into a sum over autocorrelation and cross-correlation

where

and

The total mesoscopic demagnetization tensor relates to each of the two correlation functions by the sum , where each contribution is computed like in Equation using either Equation or . Using Equations and , we find for in Equation

.

The form of the autocorrelation is identical to that of solid cylinders considered previously (Sandgaard, Shemesh, et al., 2022), i.e., it described by a 2D correlation function in the plane perpendicular to . Using Equation , the contribution from autocorrelations ,

is given be the radial and angular integrals (Sandgaard, Shemesh, et al., 2022), respectively

=

We thus obtain for the autocorrelation contribution

Here is the scatter matrix (Fisher et al., 1987), which was rewritten in terms of , the Laplace expansion coefficients of the fODF. is the symmetric trace-free tensors (STF) corresponding to an irreducible rank-2 representation of SO(3) (Thorne, 1980). The cross correlation , Equation , corresponds to a sum of cross-correlations from solid cylinders, which we previously found not to contribute (Sandgaard, Shemesh, et al., 2022). We can thus set resulting in . This then yields the same mesoscopic dipole tensor as for solid cylinders

1. *Compartmental average Larmor frequency*

Here we briefly outline why Equation also corresponds to the mesoscopic dipole tensor in each of the three major water compartments. This means that the mesoscopic contribution to the average field in the extra-cylindrical compartment is the same as the intra-cylindrical compartment, and across bi-layers. Instead of relating the water indicator function directly to the negated indicator function of the cylinder, we may also characterize each major water compartment by their total indicator functions , and , respectively

(intra-cylindrical)

(bi-layers)

, (extra-cylindrical)

Hence, the structural correlation function is . From this we can define the mesoscopic contribution to the compartmental Larmor frequency :

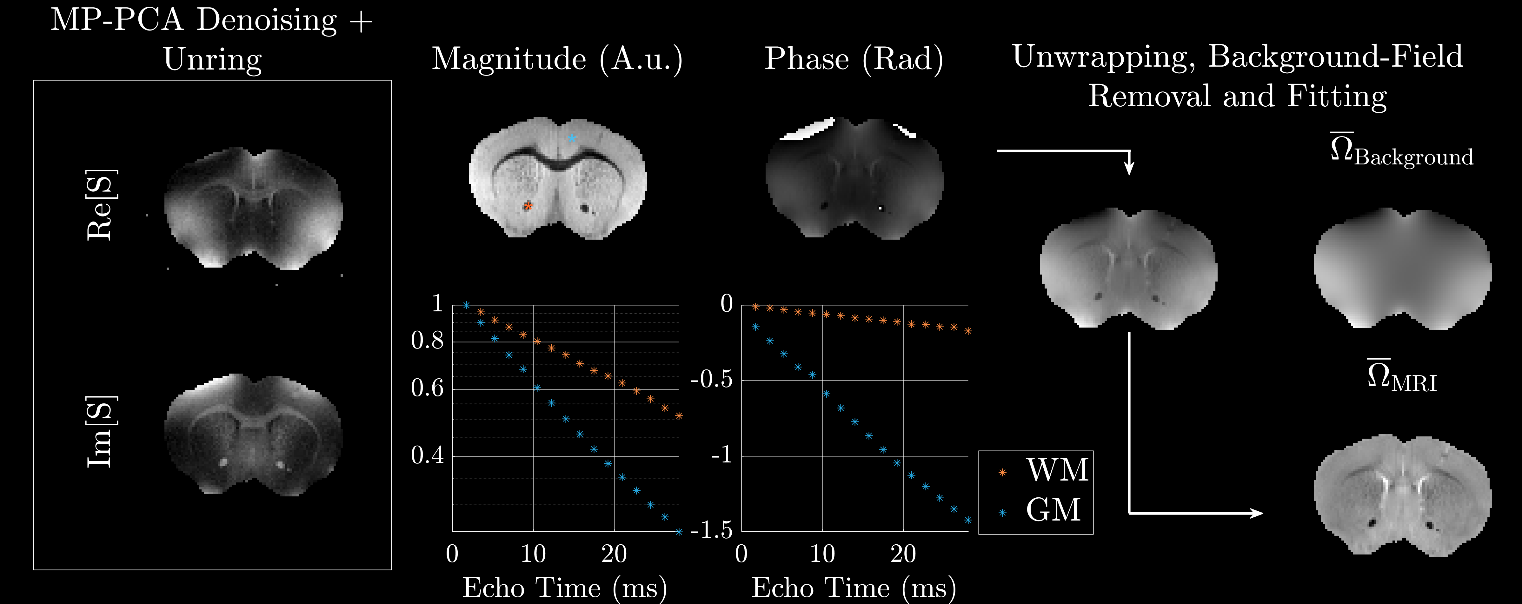
where the compartmental mesoscopic demagnetization tensor depends on the compartmental correlation functions

is a cross-correlation as it describes correlations between the water compartment defined by with volume fraction and the microstructure with indicator function , Equation , with volume fraction . Using Equations - in Equation , and that , yields identical mesoscopic dipole tensors for all compartments:

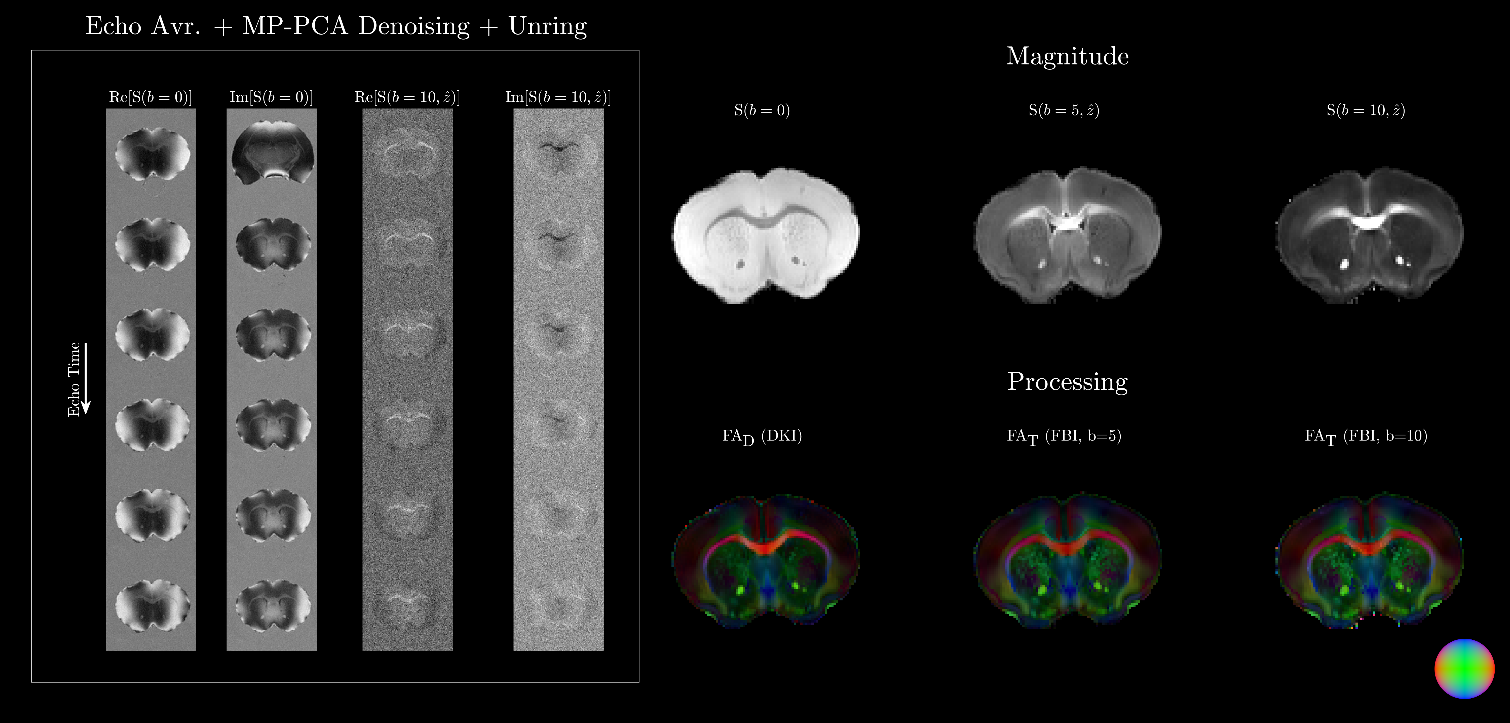
Thus every compartment experiences the same average magnetic field, and the weighted sum corresponds to Equation as expected. This means that if we filter the signal through diffusion weighting to isolate intra-cylindrical signals, we do not gain any new information about the magnetic microstructure.

**S3) Supplementary Figures**

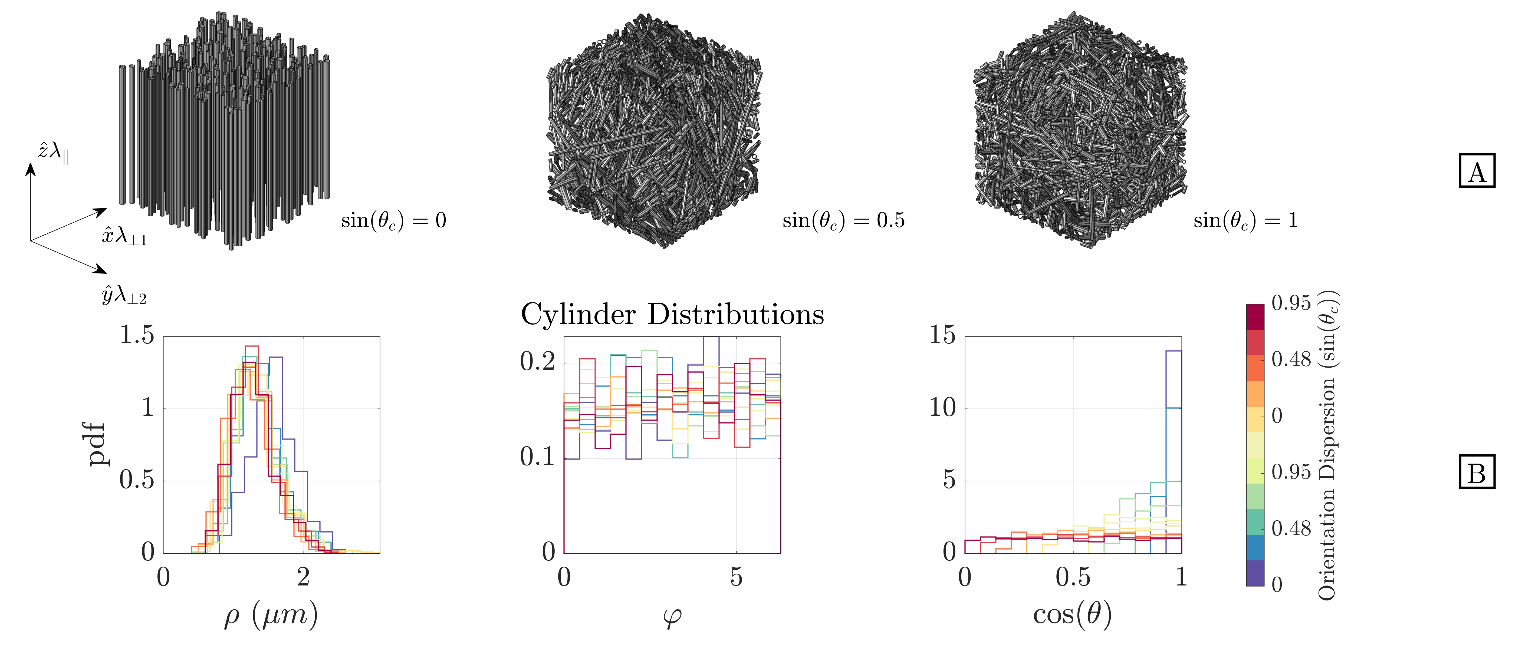
Here we present supplementary figures for the article.



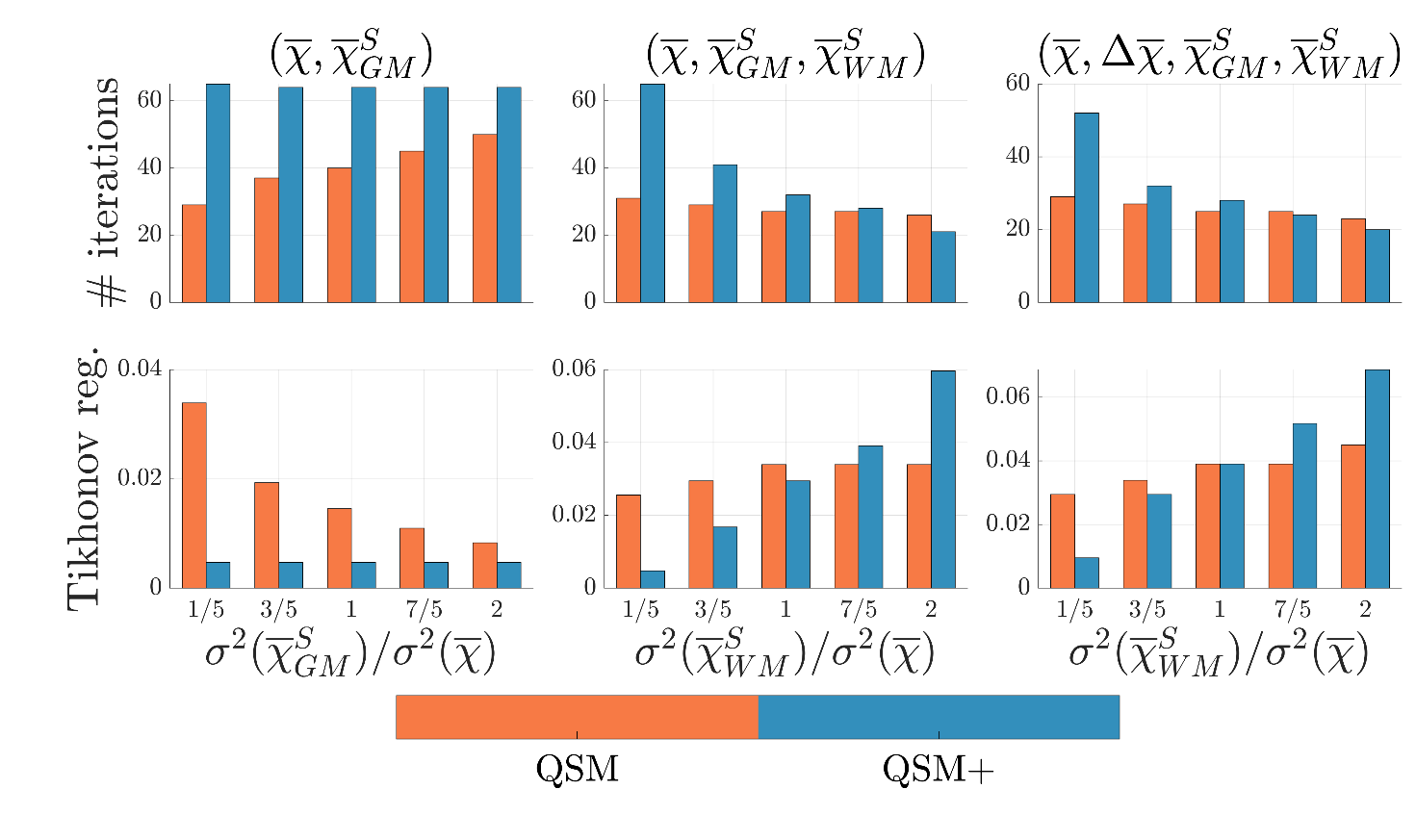
**Figure S2 - Overview of pipeline for MGE processing:** All the complex MGE images were denoised using MP-PCA followed by Gibbs-unringing. The complex phase was extracted, unwrapped and background-field corrected, and subsequently fitted to extract . shows the subtracted background frequency, using a *depth* and *peel* set to 3 to erode field errors from fluid accumulated on the surface of brain. Representative signal magnitude (left plot) and unwrapped and background-field corrected phase (right plot) are plotted for a white matter (cingulum in blue) and gray matter (thalamus in orange) voxel, respectively. Magnitude is shown in semi-log scale to illustrate the mono-exponential behavior of both signals are predominantly mono exponential. The phase behaves linearly in both WM and GM.



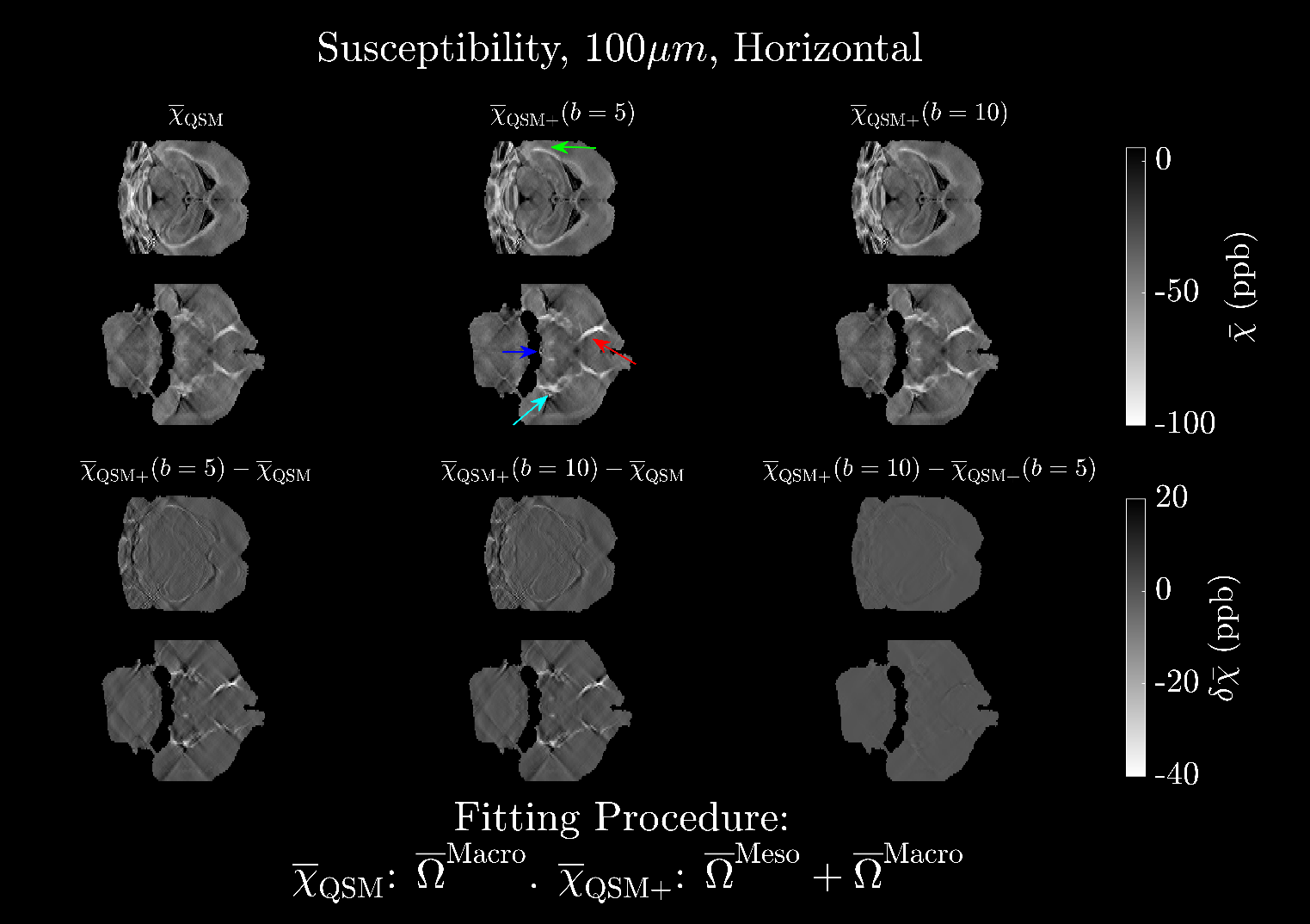
**Figure S3 - Overview of dMRI pipeline for data processing:** The Complex dMRI images were tensor MP-PCA denoised for each echo time individually followed by Gibbs-unringing. The signal magnitudes were then averaged over echo times using SVD, and the resulting images were then fitted with DKI or FBI for tensor or fODF estimation. Color-coded FA maps from diffusion tensor and scatter matrices (, cf. Equation in appendix A) from FBI are shown for various protocols. denotes the dMRI signal with b-value along , here the in-plane direction (green on sphere).



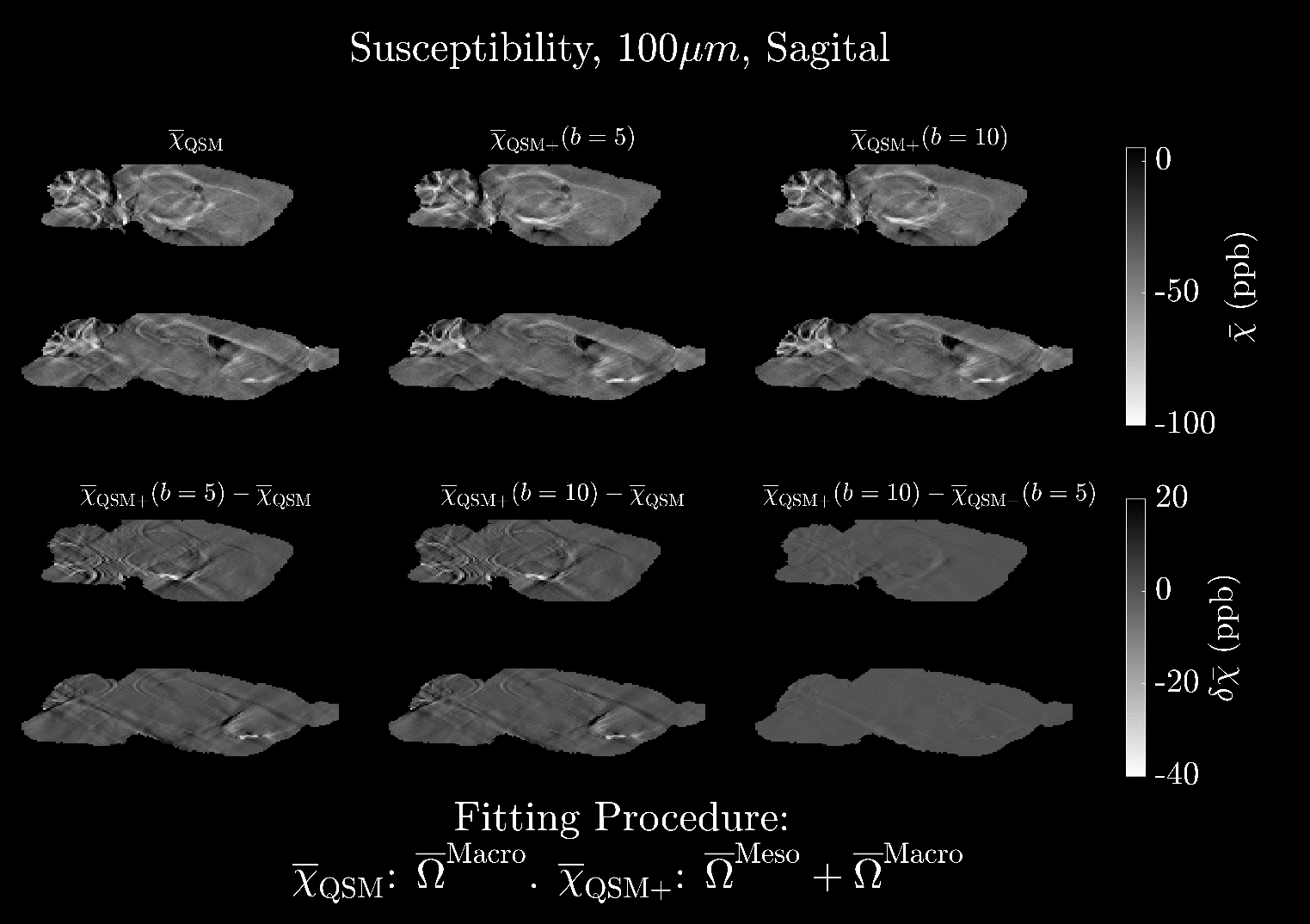
**Figure S4 - Populations of cylinders with different levels of orientation dispersion are shown in A. B** shows the probability density function (pdf) of the resulting cylinder parameters for each configuration. The cylinder radius is gamma-distributed, while and are uniformly distribution in the full range of azimuthal angle and from zero to the maximum polar angle , respectively. Colors are used to represent different populations with orientation dispersion indicated by the colorbar.



**Figure S5 – Optimal number of iterations and Tikhonov in phantom simulation:** The optimal number of iterations and Tikhonov regularization are shown for each algorithm and for each configuration of magnetic susceptibility. QSM+ requires more iterations to converge to the optimal solution but requires less regularization than QSM.



**Figure S6 - Susceptibility maps of mouse brain at 100 µm isotropic resolution:** Horizontal slices from the medial and anterior parts of the brain are shown. corresponds to zero mesoscopic contribution (analogous to QSM), and corresponds to a non-zero mesoscopic contribution calculated using this method.



**Figure S7 - Susceptibility maps of mouse brain at 100 µm isotropic resolution:** Sagittal slices from the medial and anterior parts of the brain are shown. corresponds to zero mesoscopic contribution (analogous to QSM), and corresponds to a non-zero mesoscopic contribution calculated using this method.

**S4) Susceptibility fitting with a single orientation versus multiple orientations in an ex vivo rat brain**

In this supplementary section we investigate the QSM quality from single orientation susceptibility fitting compared to a COSMOS fit (Liu et al., 2009), which means multiple directions are included in the QSM fit to overdetermine the inverse problem. We consider fitting without and with the addition of the mesoscopic frequency shift described by Equations (8) (QSM) and (9) (QSM+) in the main text, respectively.

Here we demonstrate that COSMOS including a mesoscopic frequency shift in WM offers the lowest residuals between the measured Larmor frequency and the predicted from fitting. This is also the case for single orientation fitting, but the improvement is very small due to the fitting algorithm being too sensitive to noise after only a few iterations.

**Methods**

*Ex vivo brain imaging*

All animal experiments were preapproved by the competent institutional and national authorities and carried out according to European Directive 2010/63.

*Animal preparation*

The Animal experiment were performed on a perfusion-fixed rat brain. Briefly, a rat was euthanized prior to the experiment with pentobarbital, transcardially perfused with phosphate-buffered saline (PBS) followed by a 4% paraformaldehyde (PFA) solution. The brain was then extracted and stored in 4% PFA in a fridge at 4 degrees Celsius for 24 hours. The brain was washed with PBS for at least 48 hours before imaging to minimize relaxation-effects induced by the fixative (Birkl et al., 2016). The brain was subsequently placed in a plastic cylinder filled with Fluorinert (Sigma Aldrich, Lisbon, Portugal).

*MRI experiments*

Experiments were performed on a 9.4 T Bruker Biospec (Bruker, Karlsruhe, Germany) interfaced with an Avance IIIHD console and equipped with a single-channel volume coil. Remmi sequences ([Remmi](https://remmi-toolbox.github.io/)) were used to acquire 3D gradient-recalled multi-echo images (MGE) and 3D dMRI images. For all acquisitions, repetition time was kept at 250 ms and the flip angle at 45 degrees. The Field-of-View (FOV) for these 3D acquisitions was 22.5×15.0 ×16.5 mm3, matrix size 150×100×110 which resulted in an isotropic resolution of (150 µm)3. For the MGE, the echo times were 4, 8.5,…, 26.50 ms, while dMRI was acquired at 20 ms. One average was acquired for the MGE and dMRI leading to an SNR around 40 in WM and 50 in GM for MGE, and an SNR around 4 in WM and 2 in GM for dMRI acquired with a b-value of 8 ms/µm2 and along 75 directions. Diffusion times for the dMRI experiment was 7/9 ms. The sample was scanned at room temperature. Acquisition time was 45 minutes for MGE and 17 hours for dMRI. MGE was acquired at 5 different orientations described by yaw = 90, 45, 0, 0, 90 degrees and pitch = 0, 0, 0, 45, 45 degrees. FOV was only permuted when yaw or pitch was 90 degrees to keep the longest dimension parallel to the sagittal direction of the brain.

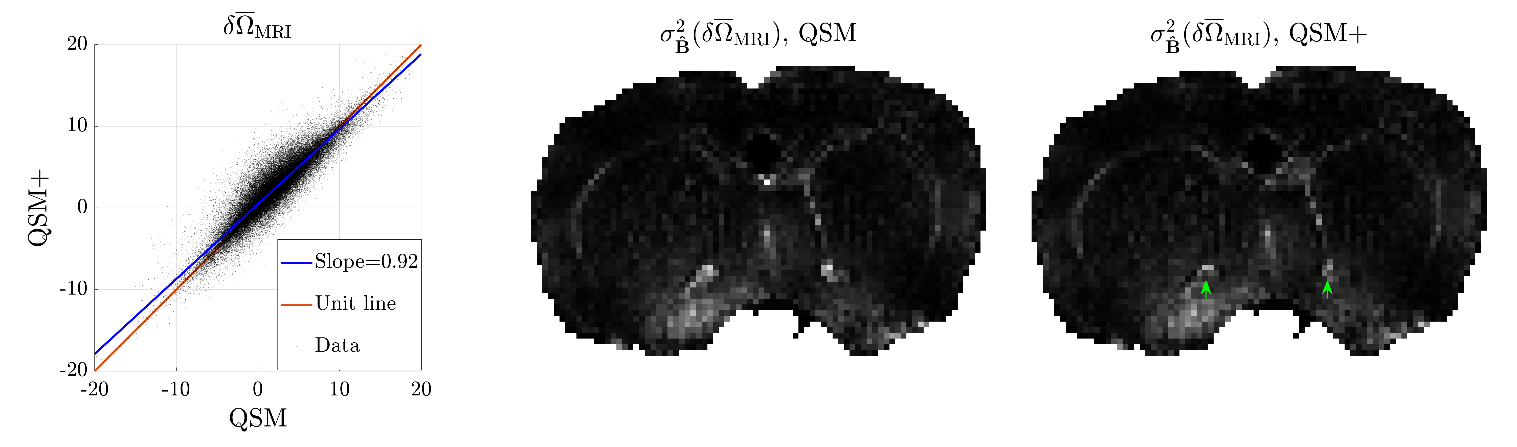
*Data processing*

MGE and dMRI processing was done as described in the manuscript. Images were further co-registered using an affine transformation to the brain positioned with yaw = 0 and pitch = 0, as this is the orientation where dMRI was acquired and the fODF was estimated. The rotation matrix from each co-registration was used to determine the direction of external field to the brain. Susceptiblity fitting was done using Equations (8) and (9) corresponding to QSM and QSM+ (with mesoscopic frequency shift), respectively. We estimated the susceptibility for QSM and QSM+ for every direction using the LSMR algorithm. Tikhonov regularization was applied for each orientation and fitting algorithm based on L-curve optimization. Lastly, we estimated the susceptibility for QSM and QSM+ including all directions, corresponding to the COSMOS method.

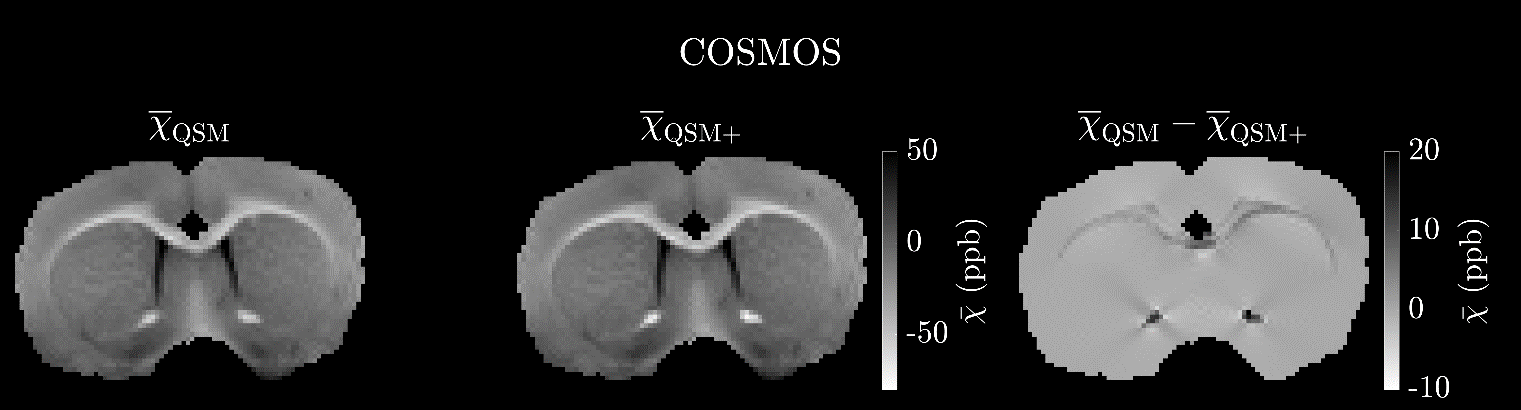
**Results**

*Multi orientation QSM (COSMOS)*

Figure S7 plots the residuals from COSMOS and a voxel-by-voxel comparison of the values between QSM and QSM+. Figure S8 shows the susceptibility fits for QSM and QSM+. Here we find a lower residual for QSM+ compared to QSM, i.e., when we account for the mesoscopic frequency shift, and the variance seems visually less biased in the anterior commissure, where the largest (most anisotropic axons) mesoscopic frequency shift was found. This demonstrates the difference between QSM and QSM+ when the ill-posed dipole inversion is overdetermined and does not corrupt fitting performance.



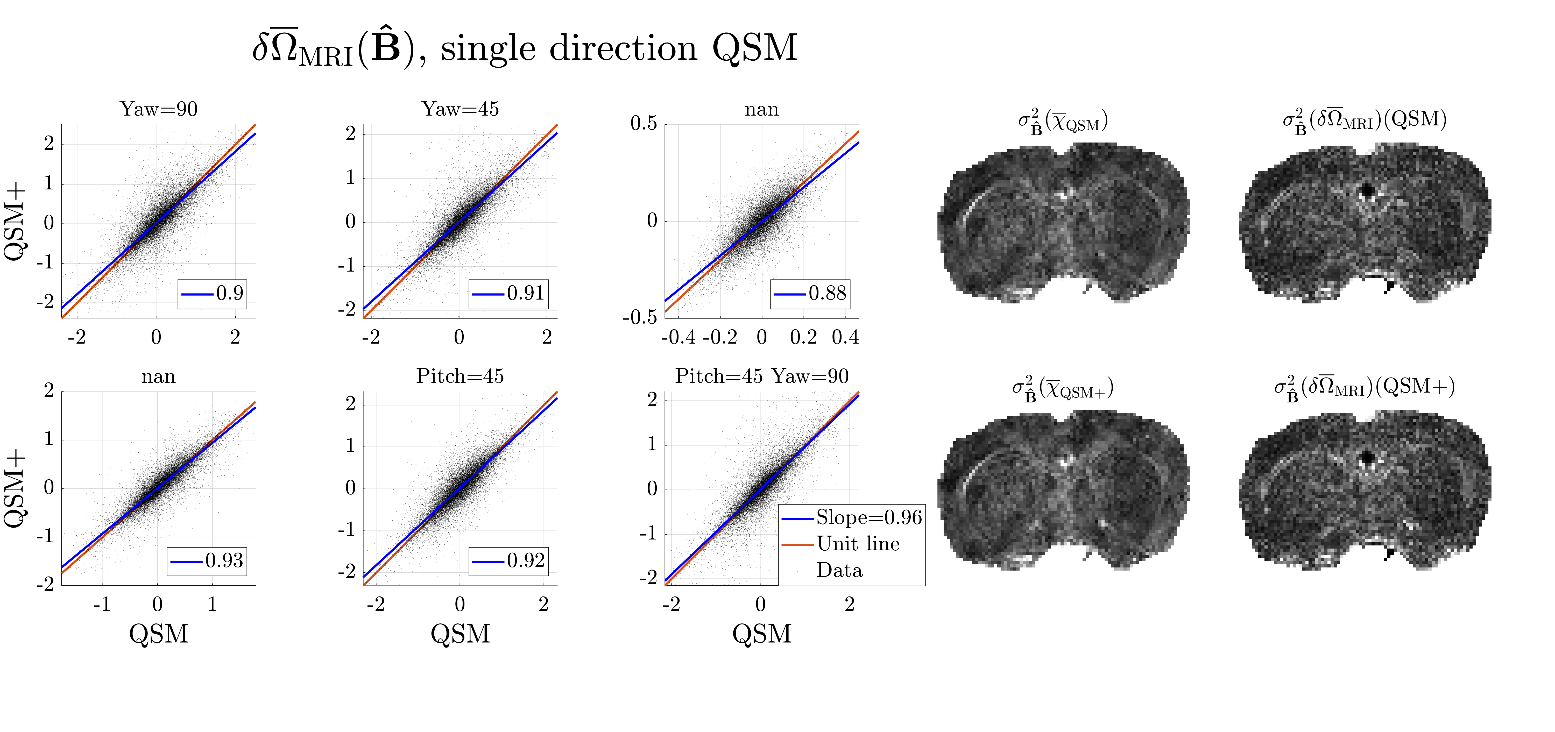
**Figure S8 – COSMOS Susceptibility fitting of rat brain at 150 µm isotropic resolution:** The plot to the left show voxel-by-voxel comparison of the residuals for fitting including all orientations. The red line corresponds to the unit line, while the blue shows a linear fit, with slope below 1, indicating lower residuals with QSM+. shows the variance in the residuals for a coronal slice of the rat brain in the anterior part of the brain.



**Figure S9 – COSMOS Susceptibility maps of rat brain at 150 µm isotropic resolution:** coronal slices from the anterior part of the brain are shown. corresponds to zero mesoscopic contribution (conventional COSMOS), and includes a non-zero mesoscopic contribution calculated using this method.

*Single orientation QSM*

Figure S9 shows the residual in Larmor frequency for each orientation, specified in the title. The figures are plotted voxel-by-voxel to compare QSM (x-axis) against QSM+ (y-axis). We find that the slope is slightly less than one, indicating a lower residual with QSM+. Figure S9 also illustrates the variance in and . Only a slight improvement is found with QSM+. Nevertheless, the susceptibility found via QSM and QSM+ are different. Importantly, QSM+ for a single direction predicts a more negative magnetic susceptibility in WM, in agreement with the COSMOS QSM+ fit.



**Figure S10 - Susceptibility fitting of rat brain at 150 µm isotropic resolution at 5 different orientations:** The plots to the left show voxel-by-voxel comparison of the residuals for each sample orientation labeled in the title. Nan corresponds to no rotation (two individual experiments are shown), and here the field is along the sagittal orientation of the brain. The red line corresponds to the unit line, while the blue shows a linear fit, with slope slightly below 1, indicating lower residuals with QSM+. and show the variance in the residuals and susceptibility fits, respectively, for a coronal slice of the rat brain in the anterior part of the brain.