An Ex Vivo Imaging Pipeline for Producing High-Quality and High-Resolution Diffusion-Weighted Imaging Datasets

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Abstract: Diffusion tensor (DT) imaging and related multifiber reconstruction algorithms allow the study of in vivo microstructure and, by means of tractography, structural connectivity. Although reconstruction algorithms are promising imaging tools, high-quality diffusion-weighted imaging (DWI) datasets for verification and validation of postprocessing and analysis methods are lacking. Clinical in vivo DWI is limited by, for example, physiological noise and low signal-to-noise ratio. Here, we performed a series of DWI measurements on postmortem pig brains, which resemble the human brain in neuroanatomical complexity, to establish an ex vivo imaging pipeline for generating high-quality DWI datasets. Perfusion fixation ensured that tissue characteristics were comparable to in vivo conditions. There were three main results: (i) heat conduction and unstable tissue mechanics accounted for time-varying artefacts in the DWI dataset, which were present for up to 15 h after positioning brain tissue in the scanner; (ii) using fitted DT, q-ball, and persistent angular structure magnetic resonance imaging algorithms, any *b*-value between ~2,000 and ~8,000 s/mm², with an optimal value around 4,000 s/mm², allowed for consistent reconstruction of fiber directions; (iii) diffusivity measures in the postmortem brain tissue were stable over a 3-year period. On the basis of these results, we established an optimized ex vivo pipeline for high-quality and high-resolution DWI. The pipeline produces DWI datasets with a high level of tissue structure detail showing for example two parallel horizontal rims in the cerebral cortex and multiple rims in the hippocampus. We conclude that high-quality ex vivo DWI can be used to validate fiber reconstruction algorithms and to complement histological studies. *Hum Brain Mapp* 32:544–563, 2011. © 2010 Wiley-Liss, Inc.

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INTRODUCTION

Diffusion-weighted magnetic resonance imaging (DWI) of the brain is a unique method for assessing structural connectivity and for characterizing the microstructure of tissue in the brain by mapping the regional diffusion properties of water. Diffusion tensor imaging (DTI) [Basser et al., 1994] and related multifiber reconstruction algorithms [Alexander, 2005; Assaf et al., 2004; Frank, 2002; Jansons and Alexander, 2003; Tournier et al., 2004; Tuch, 2004; Tuch et al., 2002] use the directional variation of water diffusion to estimate the orientations of fibers, here referred to as fiber directions, in each image voxel. Tractography algorithms [Behrens et al., 2003; Conturo et al., 1999; Mori et al., 1999; Parker et al., 2003] then use the estimates of fiber orientations to reconstruct and visualize brain structural connectivity. The potential of tractography as a noninvasive tracer is promising; however, its reliability depends on the quality of the DWI dataset. Image quality is determined by the signal-to-noise ratio (SNR) and imaging parameters such as angular resolution [Jones, 2004] and the selected b -value [Alexander and Barker, 2005; Frank, 2002]. Moreover, in vivo DWI suffers from time-varying artefacts including motion and physiological noise [Skare and Andersson, 2001] as well as geometric distortions, related to the fast echo-planar imaging (EPI) sequences typically used in the clinic (see Le Bihan et al. [2006] for a review).

Several different approaches have been pursued to validate and test, for example, fiber reconstruction algorithms. Artificial datasets [Alexander, 2005; Lazar and Alexander, 2003; Leemans et al., 2005] or phantoms [Lin et al., 2003; Perrin et al., 2005; Poupon et al., 2008; Tournier et al., 2008] are often used for initial validation. Recently, the applicability of ex vivo DWI of postmortem tissue for anatomical visualization [Aravamuthan et al., 2009; Zhang et al., 2002], brain development [D'Arceuil et al., 2008; Zhang et al., 2005], and validation purposes [Dyrby et al., 2007; McNab et al., 2009b; Schmahmann et al., 2007] has been explored. In contrast to artificial datasets, DWI data-obtained ex vivo has the same neuroanatomical complexity as seen in vivo, but without the artefacts that are inherent to in vivo measurements. Ex vivo DWI of postmortem brain tissue allows for prolonged scanning sessions with stronger gradients in high-field experimental MR scanners and for acquiring DWI datasets with increased image resolution and SNR and higher b -values. Because time is less of an issue, slower sequences such as conventional diffusion-weighted spin-echo (DW-SE) sequences with single-line read-out can be used, thereby ensuring minimal image distortions compared to EPI.

Despite its great potential to produce high-quality datasets ex vivo DWI measurements face several challenges. Time-varying artefacts can occur as a result of scanner instabilities such as B_0 drift, heating of scanner hardware, heat conduction, and/or tissue mechanics. Heat conduction takes place when the temperature of the fixed tissue is higher (or lower) than the environmental temperature inside the magnet while scanning. The use of strong and fast-switching gradients during scanning potentially may heat up the gradient coils and hence increase the environmental temperature. Molecular motion is highly sensitive to changes in the environmental temperature and such changes should therefore be avoided while acquiring DWI datasets [Le Bihan et al., 1989]: for example, an increase in tissue temperature increases diffusivity and consequently the MR signal decreases. The mechanical instabilities in the tissue during scanning can introduce physical deformations and a change in position of the tissue. Such unstable tissue mechanics might result from, for example, the physical handling of the fixed tissue in the preparation process before it is placed in the scanner (shaking, squeezing, positioning in the coil, etc.) and the physical setup used to stabilize the brain in a given position in the coil. Another challenge is autolysis. Autolysis is a self-degeneration process of the cells due to the action of their own autogenous enzymes, which significantly degrades the tissue within the first 24 h [von Smolinski et al., 2005]. It determines how well postmortem tissue will represent the structural characteristics of in vivo tissue [Hukkanen and Roytta, 1987] and consequently will affect tractography [D'Arceuil et al., 2007a]. Fortunately, the destructive autolytic process can be stopped by fixation. Perfusion-fixation is the preferred procedure in animal models [Adickes et al., 1997; Monfils et al., 2005], whereas immersion fixation is used for human postmortem brain tissue [Yong-Hing et al., 2005].

The most widely used fixative is formaldehyde [Srinivasan et al., 2002], which stops all cellular activity by cross-linking proteins (for details, see Werner et al. [2000]) and slows down molecular motion. The reduced water diffusivity significantly impacts on the choice of imaging parameters [Thelwall et al., 2006]. Both the T1 and T2 relaxation constants decrease [Tovi and Ericsson, 1992; Yong-Hing et al., 2005], the latter with negative effect on SNR. Importantly, formaldehyde does not cause dehydration, and tissue shrinkage is generally only around 3% [Fox et al., 1985]. Well-fixed tissue is crucial when establishing a brain bank suitable for future DWI projects. However, the stability of diffusivity measures of fixed tissue, which has been stored for several years, has yet to be established. The aim of this study was to establish an ex

vivo imaging pipeline to generate high-quality DWI datasets characterized by the following criteria: (1) high SNR when compared with clinical human DWI datasets, which typically have a SNR between 15 and 20 in the non-DWI images in deep white matter (WM), (2) appropriate image resolution to distinguish anatomical structures of interest, (3) isotropic voxels to ensure rotational invariance, (4) absence of time-varying artefacts, (5) absence of geometrical distortions in the image volume, (6) optimal selected b -value to extract anatomical information of interest, and, especially for tractography, (7) the use of high-angular resolution diffusion imaging to ensure robust multifiber reconstruction. The ex vivo imaging pipeline consisted of a fixation, storage, preparation, and MR scanning stage as illustrated in Figure 1.

Three experiments were performed to test and optimize the ex vivo imaging pipeline especially for later use of tractography [Dyrby et al., 2007]. In experiment 1, we tested for possible time-varying artefacts in DWI datasets. In experiment 2, we empirically determined an optimal b -value range for consistent reconstruction of fiber directions, using three well-established fiber-reconstruction algorithms: fitting the diffusion tensor (DT) model [Basser et al., 1994], persistent angular structure magnetic resonance imaging (PASMRI) [Jansons and Alexander, 2003], and q-ball [Tuch, 2004]. In experiment 3, we tested the longitudinal stability of diffusivity in fixed tissue stored for years. Other choices that were made in setting up the ex vivo imaging pipeline were based on findings from earlier studies, for example, perfusion fixation and using a conventional DW-SE with single-line read-out. On the basis on our experiments and the optimal settings, they suggested for the ex vivo imaging pipeline, we then acquired a high-quality whole brain DWI dataset and a high-resolution regional DWI dataset. Finally, we performed a qualitative assessment of the anatomical accuracy of the high-quality and high-resolution DWI datasets by comparing tissue microstructure as reflected in fiber directional information with known anatomical features in WM, cerebral cortex, and the hippocampus. DWI datasets are publicly accessible at www.drcmr.dk.

METHODS

Tissue Fixation and Storage

The pig brain was used as a model, because its complex neuroanatomy closely resembles that of the human brain [Lind et al., 2007]. Moreover, the pig brain is large enough to allow for good anatomical resolution in an experimental MR scanner.

In total, eight normal pigs were included in this study: seven young Göttingen minipigs (P1–P7; 3 months of age, 5–6 kg in body weight; Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark) and one (P8) fully grown landrace pig (>12 months and body weight 40 kg). The animals were sedated using a Zoletile® mixture and perfusion-fixed transcardially in 4% paraformaldehyde (PFA) [Dyrby

Figure 1.

Flowchart of the four stages in the ex vivo imaging pipeline.

et al., 2007]. This fixation procedure was chosen as it ensured minimal autolysis in the tissue. The brains were removed and postfixed for at least 12 h in 1% PFA in

phosphate-buffered saline (0.1 mol/l and pH 7.4) at room temperature. Finally, they were cooled to 5°C for long-term storage. All procedures followed Guidelines for the Care and Use of Experimental Animals and approved by the Danish Animal Experiments Inspectorate.

Preparation for MR Scanning

Before the scanning procedure, the pig brains were kept at room temperature for at least 6 h to minimize heat conduction while scanning. Unless otherwise stated, each brain was placed in a sealed plastic bag with a minimal amount of fluid (as used for storage) surrounding the tissue to reduce the strong unwanted water proton MR signal and diminish the noise produced outside the tissue of interest. Before being placed in the coil, soft foam was gently wrapped around the prepared brain to stabilize the position of the tissue in the scanner.

MR Scanning

Ex vivo DWI scans of the fixed pig brains were performed on an experimental horizontal 4.7 T Varian Inova MR scanner with a bore size of 154 mm and gradients with a slew rate of 560 mT/m/ms and a maximum strength of 140 mT/m. The B_0 field was highly stable over time with a B_0 drift of about 0.1 Hz/h. For DWI, we used a single-channel volume RF coil, a conventional DWI-SE sequence with single-line read-out (2DFT), and a typical bandwidth (BW) of 50 kHz for an image matrix of 128×128 . The sequence is relatively slow compared to EPI, but gives minimal geometrical distortions. Because of the stable B_0 field and the optimized DWI-SE sequence, it was not necessary to correct for spatial image distortions during data preprocessing.

Testing and Optimizing Pipeline Components

Three experiments were performed to optimize the components in the ex vivo imaging pipeline: (1) testing for time-varying artefacts, (2) empirical determination of optimal b -value ranges for fiber reconstruction, and finally (3) evaluate the longitudinal stability of diffusivity in fixed tissue.

Experiment 1: Testing for time-varying artefacts

Three pig brains (P1–P3) were used to explore the presence of time-varying artefacts within a time period of 22 h from positioning the brain in the scanner, and, in this experiment, fluid used for storage surrounded the tissue. To reveal the time dependence of the signal, we plotted the ratio of the mean intensity between two time points as a function of time.

The DWI datasets used in this experiment consisted of two non-DW whole brain image volumes and 42 DW

whole brain image volumes acquired with evenly distributed noncollinear DW gradient directions. Each whole brain image volume consisted of two image volumes containing all the even or all the odd slices obtained at different points in time. The scan parameters for each image volume were as follows: TR = 3,500 ms, TE = 62 ms, matrix = 128×128 , 28 slices, field-of-view (FOV) = 65×65 mm², slice thickness = 0.5 mm, slice gap = 0.5 mm, b -value = 865 s/mm² (diffusion sensitization gradient duration δ = 5 ms, time between gradient pulse onsets Δ = 50 ms, and G = 100 mT/m). Although somewhat arbitrary, a relatively low b -value was chosen to ensure a DW-signal from the surrounding fluid with good SNR. We acquired four repetitions of the whole-brain DWI dataset (number of excitations NEX = 4). First, four repetitions of image volumes with even slices were acquired. This resulted in 176 time points [$4 \times (2 \text{ non-DW} + 42 \text{ DW volumes}) = 176$]. Next, four repetitions of image volumes with odd slices were acquired. Given that it took 7.5 min (128 phase encoding steps $\times$ 3.5 s TR) to obtain an image, that is, per DW volume, the time lag between even and odd slices constituting a whole brain image volume was 22 h (176×7.5 min).

Two regions-of-interest (ROI) were used: one tissue ROI including all brain tissue (i.e., a brain mask) and one ROI contains the surrounding fluid outside the brain. The brain mask for the tissue ROI was semiautomatic generated by adding up all non-DW and DW image volumes, applying a manually selected intensity threshold and final manual editing based on visual inspection. The fluid ROI was manually drawn in each brain and covered in total 11–14 neighboring even and odd slices. All ROIs in this study were created using the MIPAV program (<http://mipav.cit.nih.gov>) [Bazin et al., 2007].

To visualize any time-varying artefacts, only DW-image volumes were used because of their temperature dependency. At each of the 168 time points, we calculated the ratio of the mean intensity at a time point as the ratio between the mean intensity of the even and odd slices within predefined ROIs. Because the fluid ROI contains no structural information any time-varying artefacts observed in this ROI are solely related to temperature fluctuations. For enabling interbrain comparison of the time curves, a common baseline of zero was ensured by subtracting the mean of the 10 last time points from the ratio of the mean intensity at all time points for each brain. It should be noted that examining only a single-DW direction instead of 42 would have been sufficient to pick up time-varying artefacts, yet it would have been impossible to test whether time-varying artefacts vary with DW directions. Finally, the high-anatomical contrast in the whole-brain non-DW images allowed us to visually evaluate the presence of unstable tissue mechanics, for example, positional differences of tissue over time. We visually compared a non-DWI at respectively NEX = 1, 2, and 3 with a non-DWI of NEX = 4 using the image overlay functionality in the MIPAV program.

Experiment 2: Determination of optimal b -value ranges

Because the actual organization of the underlying fibers in the brain is unknown, we empirically explored the effects of different b -values on the reconstruction of fiber directions in postmortem tissue by using the DT model, PASMRI, and q-ball. All fiber-reconstruction algorithms are implemented in the Camino toolkit (<http://www.camino.org.uk>).

Eight DWI datasets with different b -values were acquired in one continuous scanning session on brain P4 (one hemisphere) 613 days post mortem. This set-up ensured spatial agreement between the eight DWI datasets. Using the DT model in a pilot study, we found an “optimal” b -value to be $\sim 4,000$ s/mm² as was also reported by D’Arceuil et al. [2007b]. We then tested a pseudologarithmic distribution of b -values around 4,000 s/mm² by varying the gradient strength (G), hence the q -value while keeping the diffusion time constant (acquisition schemes I–VIII, Table I). Each DWI data consisted of four repetitions during which three non-DW images and 61 DW-directions with evenly distributed gradient directions were acquired [Cook et al., 2006]. The number of DW directions selected ensured a sufficiently high-angular resolution for the estimation of anisotropy [Jones, 2004] and robust multifiber reconstruction needed for tractography. Sequence parameters were given as follows: TE = 67.8 ms, TR = 2,500 ms, δ = 27 ms, Δ = 33.5 ms, five axial 0.5 mm slices, slice gap = 3.5 mm, FOV = 65 × 32.5 mm², and matrix = 128 × 64. The SNR was, unless otherwise stated, calculated from a non-DWI image in a large ROI drawn in deep WM [Sijbers et al., 1998]. If more repetitions were acquired, then the magnitude images from each NEX were averaged off-line, before any further processing, and the final SNR was calculated as the SNR of the single NEX multiplied with the square root of NEX.

For evaluating how different b -values affected the reconstruction of fiber directions for a given fiber reconstruction algorithm, we calculated a generalization error based on the leave-one-out analysis [Hastie et al., 2001]. As generalization error, we used the mean angular discrepancy of fiber directions reconstructed across b -values. The mean angular discrepancy was calculated as a dot-product between a fiber direction reconstructed using DT, PASMRI, or q-ball at one b -value and those from the same algorithm at all other b -values. For the s^{th} b -value this can be expressed as

$$\langle \text{Angular discrepancy} \rangle^s = \frac{1}{N_t} \sum_{t=1 \neq s}^K \sum_{n=1}^{N_t} \left(\cos^{-1}(|x_n^s \bullet y_n^t|) \cdot \frac{180}{\pi} \right) [\text{degree}] \quad (1)$$

where N_t is the total number of voxels within a ROI and K is the number of different b -values (in our case, eight)

TABLE I. DWI acquisition schemes with increasing gradient strength (G) and corresponding b -values

Acquisition scheme	G (mT/m)	b -value (s/mm ²)
I	29	1,018
II	44	2,475
III	49	3,069
IV	56	4,009
V	68	5,911
VI	80	8,181
VII	84	11,295
VIII	111	15,750

from which fiber directions are reconstructed (Table I). Hence, x_n^s is the fiber direction for b -value s in voxel n and y_n^t is the corresponding fiber direction for b -value t in voxel n .

Minimizing Eq. (1) identified the b -value that was most consistent with the consensus of reconstructed fiber directions for a given fiber reconstruction algorithm. This gave a reasonable choice for the best option under the assumption that the range of b -values considered included those b -values that produced sensible results for each of the separate reconstruction algorithms. For those voxels where multiple fiber directions were detected, fiber directions were sorted by strength. Therefore, the first fiber direction x_n^s has the strongest peak. The sum over $n = 1, \dots, N_t$ in Eq. (1) include contributions from all voxels with at least one fiber direction and, for voxels with multiple fiber directions, y_n^t is the fiber direction that matches x_n^s most closely in orientation. For the second fiber direction, x_n^s is the second strongest peak, and the term in Eq. (1) has contributions only from voxels with at least two fiber directions. Hence, y_n^t is the peak fiber direction that matches x_n^s most closely in orientation and has not been used as the best match for the first fiber direction in x_n^s .

For q-ball and PASMRI, fiber directions and their related strengths were identified by means of the sfpeak-function implemented in Camino, which locates peaks in the output spherical functions using the algorithm in Jansons et al., 2003. We used default settings for q-ball, however, for PASMRI, the “-stdsfrommean” setting was increased from 0 to 25 because of the spikiness of generated peaks.

The mean angular discrepancy was calculated in three manually drawn ROIs defined on a single-axial slice as shown in Figure 3, left. One ROI contained all voxels with FA values larger than 0.3 (based on the DWI dataset with $b = 4009$ s/mm²), a second ROI was placed in the corpus callosum (CC) region containing voxels with high anisotropy, and a third ROI covered a more complex region in the rostral semiovale center and internal capsule, which included single, crossing as well as fanning fibers.

Finally, we visualized and evaluated the coherence in the spatial distribution of clusters containing voxels with one, two, or three fiber directions. This was done because fiber bundles extend over larger regions and one would

therefore expect consistency in fiber directions between neighboring voxels.

Experiment 3: Longitudinal stability of diffusivity in fixed tissue

To evaluate the inter- and intrabrain longitudinal stability of diffusivity measures in fixed brain tissue, we calculated the mean diffusivity (MD) and the (ordered) eigenvalues (λ_1 , λ_2 , and λ_3) derived from the DT model in a WM and a cortical gray matter (GM) ROI. By covering rather large brain regions, the longitudinal results were robust to minor differences in ROI placement across DWI datasets. The GM and WM ROIs were semiautomatically delineated on a slab of axial slices extending from below the inferior part of the CC to above the centrum semiovale. Tissues were segmented by means of intensity thresholding of the non-DWI images and subsequent manual editing. To avoid inclusion of heterogeneous regions of crossing fibers not correctly reconstructed by the DT model, only voxels with a FA value greater than 0.3 were included in the WM ROI. The cortical GM ROI included voxels within cerebral cortex only. ROIs were drawn on each DWI dataset. The evaluation of interbrain longitudinal stability was based on DWI datasets of brain P4, P5, P6, P7, and P8 scanned at 613, 65, 18, 35, and 392 days post mortem, respectively. DWI datasets included three acquisitions of brain P4 between days 166 and 942, four acquisitions of brain P5 between days 24 and 65 and, finally, five acquisitions of brain P8 between days 6 and 1,106. For P4–P7, scanning parameters were as described under experiment 2. Moreover, based on the results of experiment 2, a b -value of 4,009 s/mm² was used (acquisition scheme IV, Table I). For P8, the following settings were used: b -value = 4,090 s/mm²; TE = 80.1 ms; G = 63 mT/m; δ = 16.5 ms; Δ = 58.4 ms; FOV: 80 × 80 mm²; slice thickness = 0.6 mm; and matrix = 128 × 128.

Acquisition of High-Quality and High-Resolution DWI Data

On the basis of three experiments, we established an optimized ex vivo imaging pipeline to obtain a high-quality and a high-resolution DWI dataset. A single high-quality whole brain DWI dataset was obtained in the interleaved fashion as was used in experiment 1 using brain P5 (65 days postmortem). Eight repetitions were acquired over 10 days of continuous scanning with a final SNR of 60 in deep WM. Based on the results obtained in experiment 2, a b -value of 4,009 s/mm² was used (TE = 67.8 ms; TR = 6,500 ms; δ = 27 ms; Δ = 33.5 ms; three non-DW image volumes; 61 volumes with evenly distributed gradient directions, and with a final image resolution of 0.51 × 0.51 × 0.50 mm³ voxels, 70 slices, and no interslice gap). Using a weight ratio, the image resolution of the pig brain was ensured anatomically similar to clinical in vivo DWI datasets of the human

brain with an image resolution of about 1.7–2.5 mm isotropic voxels [Dyrby et al., 2007].

An additional high-resolution DWI data set was obtained using brain P8 (57 days postmortem). The DWI dataset consisted of 15 coronal slices with a high-in-plane image resolution of 0.23 × 0.23 mm and a slice thickness of 1.0 mm. A b -value of 4,095 s/mm² was used (δ = 16.57 ms, Δ = 57 ms, and G = 63 mT/m; three non-DW image volumes; 61 volumes with evenly distributed gradient directions; TE = 80.1 ms; TR = 2000 ms; NEX = 4). The final SNR of the four repetitions obtained was 15 in deep WM. For visualizing the anatomical position of the coronal slices of the high-resolution DWI dataset, a high-resolution whole brain T_1 -weighted structural image was obtained using a magnetization prepared gradient echo (MPRAGE) sequence (echo spacing = 5.6 ms, TE = 3 ms, TI = 800 ms, flip angle = 9°, image matrix = 256 × 256 × 256, with a voxel size of 0.31 × 0.31 × 0.31 mm³, and NEX = 36). The MPRAGE was obtained separately and therefore coregistered to a non-DW image volume in the high-resolution DWI dataset using SPM5 (<http://www.fil.ion.ucl.ac.uk/spm/software/spm5>).

In addition to WM, we focused on two anisotropic structures (neocortex and hippocampus) in the high-quality and high-resolution DWI datasets using MD, the FA map, and PASMRI. Two ROIs were manually drawn on the FA map in a small region of the frontal part of cortex. One ROI was placed in GM just at the border with WM and included voxels with low-FA values and no apparent coherent fiber direction as identified on directionally color-coded FA maps [Pajevic and Pierpaoli, 1999]. The second ROI was placed in GM underlying the cortical surface and included voxels with higher FA values and a coherent fiber direction that was vertically orientated toward the surface of the cortex. Furthermore, we measured anisotropy in the layers of the hippocampal cortex. Five ROIs were drawn on separate layers in the hippocampus that ran parallel to its surface. Layers were identified on the directionally color-coded FA map as a stripe of neighboring voxels with a similar fiber direction. The ROIs were drawn on the coronal slice shown in Figure 8.

RESULTS

Testing and Optimizing Pipeline Components

Experiment 1: Testing for time-varying artefacts

The observed time-dependent instabilities (time-varying artefacts) in the DWI dataset within the first 22 h of scanning are shown in Figure 2. The different starting time points in Figure 2A reflect the time it took from placing the brain in the scanner (time = 0) to starting the experiment. This included tuning, shimming, and choosing FOV. For the ROI-containing brain tissue, the ratio of the mean intensity at time point one was 8, 16, and 23% for P1, P2, and P3, respectively. In all three brains, the ratio of the

Figure 2.

Visualization of a time-varying artefact introduced in DWI datasets acquired immediately after placing/positioning the brain in the scanner. The ratio of the mean intensity was calculated in a tissue ROI (A) and a fluid ROI (A, top right). Results in (A) are shown for pig brain P1 (black, dashed), P2 (blue, striped), and P3 (red, solid). The time-varying artefact introduced in the DWI

dataset of P2 is shown in coronal (B–D) and sagittal (E–G) orientation at three time-points indicated in A (circle). The time-varying artefact is seen as an intensity difference between odd and even slices within tissue (arrow) and in surrounding fluid (double arrow). All images are intensity scaled equally.

mean intensity exponentially decreased to less than 5% after 7 h. Time-varying artefacts were largely absent after 15 h of scanning (Fig. 2A). The fluid ROI, sensitive to temperature fluctuations only, had a SNR of 15 measured in a DW-image, and time-dependent instabilities were observed for P2 and P3. However, the time-dependent signal leveled out faster than in tissue and was absent after 3 h for P2 and 7 h for P3 (Fig. 2A). For P1, the ratio of the mean intensity within the fluid ROI was zero at all time-points, indicating the absence of temperature fluctuations. This suggests that the tissue temperature of P1 before scanning matched the environmental temperature inside the magnet while P2 and P3 started at higher temperatures.

Because consecutive data points in Figure 2A are based on DW datasets obtained with different DW-directions the

smoothness of the curve (e.g., no visible outliers) indicate that the time-varying artefacts were independent of DW-direction.

Because of the data acquisition mode, even slices had lower DW signal intensities than odd slices (Fig. 2B–G). In the fluid surrounding the brain, maximal signal differences were observed at time point one (after 30 min) (Fig. 2B,E). However, after 2.5 h, the signal intensity of even slices in the fluid ROI increased and visually matched the signal intensities of odd slices (Fig. 2C,F), and, after 15 h, the signal intensities also matched for the tissue ROI (Fig. 2D,G). Finally, when inspecting the image overlay of the non-DW volume acquired at time point one with one, taken some hours later, subtle differences were noticeable in the position of the brain suggestive of unstable tissue mechanics in the first about 11 hours.

Figure 3.

Mean angular discrepancy, a generalization error calculated across b -values for DT (circle), q-ball (square), and PASMRI (triangle), was obtained in three regions of interest. On the left, a FA map of an axial slice shows the corpus callosum (CC; red) and the posterior centrum semiovale (Complex; blue) ROIs. A third ROI included all voxels within the slice with FA values > 0.3 . Results for the first fiber direction were obtained for the

CC ROI (red, striped), the complex ROI (blue, solid), and the FA > 0.3 ROI (black, dotted). Results for the second fiber direction were only obtained for the FA > 0.3 ROI using q-ball (green square) and PASMRI (green triangle). Generally, for both the first and the second fiber direction, mean angular discrepancy was maximal at the lowest b -values and minimal in the 4,000 s/mm^2 range.

Based on the above results, all subsequent DWI datasets were obtained at least 15 h after positioning a brain in the scanner, and during this “resting period,” the scanner was actively acquiring dummy runs of DWI or T1 weighted MPRAGEs.

Experiment 2: Determination of optimal b -value ranges

Before experiment 2, the temperature stability across b -values was monitored in a separate but similar DW acquisition where a wider range of b -values ranging from 1,000 s/mm^2 and up to 24,306 s/mm^2 were used. The temperature probe was placed close to the tissue. During scanning, there were no fluctuations in temperature depending on the b -values, and a typical and maximal temperature of 18.5 and 19°C, respectively, was measured.

For all 3 ROIs and each fiber reconstruction algorithm, the mean angular discrepancy for the single-fiber direction (DT, q-ball, and PASMRI) as well as for the second fiber direction (q-ball and PASMRI) was largest at the lowest b -value (1,018 s/mm^2) and smallest for b -values in the 3,000–8,000 s/mm^2 range (see Fig. 3). For the multifiber reconstruction algorithms, the mean angular discrepancy for the first fiber direction was, except for the lowest b -values, equivalent and a few degrees higher than for DT. For the second fiber, direction PASMRI had much lower mean angular discrepancies across all b -values when compared with q-ball. However, when compared with the first fiber direction, the mean angular discrepancy was generally

twice as high for the second fiber direction. In the CC and the complex ROIs, mean angular discrepancy for PASMRI and q-ball stabilized at a b -value $\geq 2,474 \text{ s/mm}^2$ (see Fig. 3). In the “FA > 0.3 ” ROI, however, mean angular discrepancy for both the first and second fiber direction in all three algorithms slightly increased with b -values above the 6,000 s/mm^2 range.

The visualizations of the PAS function and the q-ball orientation distribution function (ODF) in Figure 4 illustrate why the mean angular discrepancy was largest at the lowest b -value, as shown in Figure 3. Indeed, the PAS peak directions and q-ball ODF (single as well as multiple fibers) at the lowest b -value (1,075 s/mm^2 , Fig. 4A,F) were less well defined and showed lower spatial coherence than at higher b -values (Fig. 4B–E,G–J). Notably, changes in both the PAS function and q-ball ODF for b -values larger than 2,475 s/mm^2 appeared to be minimal for the fiber direction(s) as well as the shape of the functions.

Figure 5 shows maps of the numbers of PAS and ODF peaks at different b -values. By visual inspection, the spatial coherence of clusters in WM, with one and two fiber directions, was more consistent across b -values for the PAS than the q-ball ODF. A correspondence in the spatial distribution of single and two fiber populations in WM was seen when comparing PASMRI at a b -value of 2,475 s/mm^2 with q-ball at a high b -value of 15,750 s/mm^2 (Fig. 5C,L). At high b -values, PASMRI tended to identify two fiber directions in regions where only a single-fiber direction was reconstructed at low b -values. This was especially evident in voxels located close to clusters with two fiber

Figure 4.

Reconstructed fiber distributions were generated with PASMRI and q-ball at different b -values. The depicted region is indicated in Figure 5A (red square). Results for PASMRI (A–E) and q-ball (F–J) are shown for b -values of 1,075 (A, F), 2,475 (B, G), 4,009 (C, H), 8,181 (D, I), and 15,750 s/mm^2 (E, J). Color coding of fiber directions: red, Left–Right; green, Anterior–Posterior; blue, Superior–Inferior. Our results indicate that the spatial coherence in fiber directions improves for b -values $> 1,075 \text{ s/mm}^2$.

directions, suggesting an increased sensitivity of PASMRI to identify complex fiber architectures at higher b -value. Q-ball showed a similar but weaker trend at the two highest b -values. In accordance with known anatomy, the CC was consistently characterized by single-fiber directions, whereas clusters of multiple fiber directions characterized the rostral semiovale center and internal capsule regions (see Fig. 5). For both PASMRI and q-ball, clusters with three fiber directions were typically found in the cortex, deep GM, and hippocampus at higher b -values (Fig. 5B–F, K–L) and in fluid for most b -values (Fig. 5B–F, J–L).

Based on the above empirical results for the reconstruction of fiber directions, an optimal b -value for ex vivo DWI was chosen in the 4,000 s/mm^2 range and used in the following experiments.

Experiment 3: Longitudinal stability of diffusivity in fixed tissue

Across brains, no significant differences were observed in MD or in eigenvalues, in either the WM or the cortical GM ROI (Tables II and III). Diffusivity in cortical GM, when compared with WM, tended to be higher along the second principal direction (λ_2) and was significantly higher along the third principal direction (λ_3) (Tables II and III).

Repeated measurements of diffusivity within storage periods of up to 1,106 days did not reveal any significant intrabrain difference in WM or cortical GM longitudinal diffusivity for P4, P5, and P8 (see Fig. 6). When averaging the repeated measurements for each of the examined brains, the mean and standard deviation ($\pm$ SD) of MD in WM and cortical GM were, respectively, 0.23 ($\pm$ 0.03) and 0.28 ($\pm$ 0.03) $\times 10^{-3} \text{ mm}^2/\text{s}$ for P4, 0.23 ($\pm$ 0.05) and 0.25 ($\pm$ 0.03) $\times 10^{-3} \text{ mm}^2/\text{s}$ for P5, and 0.21 ($\pm$ 0.04) and 0.25 ($\pm$ 0.04) $\times 10^{-3} \text{ mm}^2/\text{s}$ for P8. Hence, the observed intrabrain variation in MD over longer time periods of storage was less than the variation observed across brains (Tables II and III). When comparing the last time points for P4 and P8, diffusivity seemed stabilized with only minor variations in both the WM and cortical GM ROIs.

The Optimized Ex Vivo Imaging Pipeline

Given the results from the three experiments together with previous research, we derived the following optimized ex vivo imaging pipeline:

- Stage 1 (fixation): treat tissue with perfusion fixation to minimize autolysis effect and for minimal tissue shrinkage use, for example, formaldehyde as fixative.
- Stage II (storage): postfixate in 1% formaldehyde and place the tissue at in a cooled environment (5°C) for long time storage.
- Stage III (preparation): for temperature stabilization, keep the brain tissue for several hours (here at least 6 h)

Figure 5.

The spatial distribution of voxels containing one (dark gray), two (light gray), or three (white) fiber directions, as detected by PASMRI (B–F), and q-ball (H–L) is depicted in an axial slice. The corresponding non-DW image and FA map are shown in (A) and (G). Results are presented for b -values 1,075 (B, H), 2,475 (C, I), 4,009 (D, J), 11,295 (E, K), and 15,750 s/mm^2 (F, L). The gray scale in (G) depicts FA values ranging from 0 to 0.8 and the region of CC (G, arrow) and the hippocampus (G,

circle) are indicated. For b -values lower than 11,295 s/mm^2 q-ball, in contrast to PASMRI detects fewer and smaller clusters containing two fiber directions within WM [e.g., internal capsule (H, ellipse) and centrum semiovale (I, circle)]. For both PASMRI and q-ball clusters with three fiber directions were typically located in the ventricles (C, K, arrow) where some remaining fluid can be found (A, arrow) and in cerebral cortex (F, L, arrow).

in an environmental temperature as close as possible to the temperature inside the MR magnet while scanning. Then, place and fix the position of the tissue in the scanner and run a prescan for a defined time period (in the current setup at least 15 h) to minimize the introduction of time-varying artefacts in the DWI dataset.

- Stage IV (MR scanning): select optimal b -values for the given aim (here fiber directions) and use a conventional DW-SE with single-line read-out for minimal image distortions. Probe and ensure a stabile temperature inside the magnet while acquiring the DWI dataset.

- Stage V (data processing): preprocess DWI data if needed (in current study: not needed) and choose the optimal reconstruction processing strategy for the given aim. In this study, we have chosen the DT model and PAS-MRI.

Acquisition of High-Quality and High-Resolution DWI Data

The high-quality whole-brain DWI dataset (see Fig. 7) and high-resolution DWI dataset (see Fig. 8) revealed

TABLE II. Interbrain variation in diffusivity and fractional anisotropy within the WM ROI for pig brains P4–P8*

Brains	Diffusivity				FA
	λ_1	λ_2	λ_3	MD	
P4	0.32 ± 0.05	0.20 ± 0.04	0.14 ± 0.03	0.22 ± 0.03	0.44 ± 0.02
P5	0.35 ± 0.07	0.20 ± 0.05	0.14 ± 0.04	0.23 ± 0.04	0.45 ± 0.13
P6	0.28 ± 0.04	0.16 ± 0.04	0.11 ± 0.03	0.18 ± 0.03	0.44 ± 0.11
P7	0.24 ± 0.08	0.13 ± 0.05	0.09 ± 0.04	0.16 ± 0.05	0.47 ± 0.12
P8	0.28 ± 0.06	0.16 ± 0.05	0.10 ± 0.04	0.18 ± 0.04	0.49 ± 0.02
All brains	0.29 ± 0.04	0.17 ± 0.03	0.12 ± 0.02	0.19 ± 0.03	0.46 ± 0.02

*Values are mean $\pm$ SD. Diffusivity measures shown are for the mean diffusivity (MD), and the three eigenvalues (λ_1 , λ_2 , and λ_3) and the results are in (10^{-3} mm²/s). DWI datasets were acquired with a b -value of 4,009 s/mm² at 613, 65, 18, 35, and 392 days postmortem for pig brains P4–P8, respectively. Only voxels with FA > 0.3 were included in the analysis.

detailed information concerning anatomical structure and fiber orientation on color-coded FA-maps (Figs. 7A,C, and 8E) and high contrast between GM and WM on MD maps (Figs. 7B,D and 8D). Fiber tracts such as the sub-callosum fasciculus (FSC) and callosal fibers were clearly visible in both DWI datasets (Figs. 7A–C and 8E). PASMRI was used for multifiber reconstruction, because it allowed the consistent detection of single as well as multiple fiber directions for b -values in the 4,000 s/mm² range.

Although the b -value was optimized for WM, two parallel and highly structured horizontal rims on the color-coded FA map could be distinguished in cortical GM (Fig. 7A,C). Notably, the rims were also discernible in datasets with lower SNR or datasets acquired at different b -values. The inner rim, defined by low-FA values ($\pm$ SD) 0.09 ($\pm$ 0.02), was located close to WM with a width of $1 - 1\frac{1}{2}$ voxels. The outer rim, defined by higher mean FA values 0.21 ($\pm$ 0.04) had a width of $1\frac{1}{2} - 2$ voxels. A similar pattern was apparent on the high-resolution FA map (Fig. 8E). The width of the rims seen on the high-resolution FA map was comparable to the rim width on the high-quality FA map. Note that because of the higher in-plane image resolution, rims on the high-resolution FA map extend over more voxels (Fig. 8E). Although the color-coded FA

maps suggested no preferred fiber direction in the inner rim, PASMRI, generally detected two fiber directions: one being vertically oriented toward the surface of the cortex and the other being parallel with the direction of the short association fibers just beneath the cortex. Note, however, that voxels in the inner rim with circular (more round) PAS functions indicated a higher uncertainty in the determination of fiber direction (Fig. 7E,F). Based on the color-coded FA maps (Figs. 7A,C and 8E), it was evident that the fiber direction of the outer rim was vertically oriented perpendicular to the surface throughout the cerebral cortex. In agreement, PASMRI generally detected a single-fiber orientation in the outer rim (Fig. 7E,F).

Similarly, on the high-resolution DWI dataset (Fig. 8E) rims of low (R3 and R5) and high (R1, R2, and R4) FA values were observed along the longitudinal axis of the hippocampus. This was also apparent in the high-quality DWI dataset (Fig. 7C). The color-coded FA maps in Figure 8E suggest that each rim had a general preferred orientation with respect to the surface of the hippocampus. A parallel orientation was apparent in rim 1, 3, and 5 with FA values ($\pm$ SD) of 0.43 ($\pm$ 0.13), 0.18 ($\pm$ 0.04), and 0.17 ($\pm$ 0.04), respectively. A vertical orientation was seen in rim 2 and 4 with FA values of 0.31 ($\pm$ 0.04) and 0.30 ($\pm$ 0.05). In agreement with the color-coded FA maps,

TABLE III. Interbrain variation in diffusivity and fractional anisotropy within the cortical GM ROI for pig brains P4–P8*

Brains	Diffusivity				FA
	λ_1	λ_2	λ_3	MD	
P4	0.32 ± 0.04	0.26 ± 0.04	0.22 ± 0.03	0.27 ± 0.03	0.20 ± 0.07
P5	0.31 ± 0.04	0.25 ± 0.03	0.21 ± 0.03	0.26 ± 0.03	0.18 ± 0.07
P6	0.26 ± 0.05	0.22 ± 0.04	0.19 ± 0.04	0.22 ± 0.04	0.16 ± 0.07
P7	0.26 ± 0.06	0.21 ± 0.05	0.17 ± 0.05	0.21 ± 0.05	0.21 ± 0.08
P8	0.28 ± 0.05	0.22 ± 0.04	0.18 ± 0.04	0.23 ± 0.04	0.21 ± 0.08
All brains	0.29 ± 0.03	0.23 ± 0.03	0.20 ± 0.02	0.24 ± 0.02	0.19 ± 0.02

*Values are mean $\pm$ SD. Diffusivity measures shown are for the mean diffusivity (MD), and the three eigenvalues (λ_1 , λ_2 , and λ_3) and the results are in (10^{-3} mm²/s). DWI datasets were acquired with a b -value of 4,009 s/mm² at 613, 65, 18, 35, and 392 days postmortem for pig brains P4–P8, respectively.

Figure 6.

Longitudinal stability of diffusivity within cortical GM (red) and WM (black) ROI as function of time post mortem for MD (A), the first (B), second (C), and the third eigenvalue (D) (λ_{1-3}). The b -value was 4,009 s/mm^2 for pig brain P4 (circle) and P5 (square) and 4,090 s/mm^2 for P8 (triangle).

PASMRI found single-fiber directions in rims 1, 2, and 4. However, rims 3 and 5 also contained crossing fibers. The latter probably explains the low-FA values in these two

rims (Fig. 8F). The transitions between rims were characterized by very low-FA values (<0.12) and crossing fibers as indicated by PASMRI (Fig. 8F).

Figure 7.

Fiber reconstruction algorithms were applied to the high-quality whole brain ex vivo DWI dataset. Directionally color-coded FA maps (A, C; red, Left–Right; green, Anterior–Posterior; blue, Superior–Inferior) and MD (B, D) are shown in coronal (A, B) and axial (C, D) views. The gray scale bar in (B) depicts the MD range from 0 to $0.8 \times 10^{-3} \text{ mm}^2/\text{s}$. The scale bar in (A) equals 10 mm. Because most free fluid was removed to increase SNR, the MD in some regions in the ventricles was zero (B, D arrow head) and some nonzero (D, double arrow head). The larger fiber tract projections are visible in great anatomical detail, for example, the fornix (Fx), the fiber bundles projecting through the capsule interna (CI) and the dense horizontal oriented anterior commissural (AC) fibers bundles (A, C). The anterior–posterior oriented subcallosal fasciculus (FSC) was the only larger fiber bundle in WM with high MD when, for example, compared

with CC (A, C, B, and D). In GM, two continuous parallel horizontal rims are visible in the cerebral cortex; an inner rim with values close to WM (A, C arrow head) and an outer rim with higher FA values placed close to the surface of cortex (A, C double arrow heads). In (E, F), which represents the squared region in a gyrus and the waved region in a sulcus (C), PASMRI demonstrates that the inner rim generally contains crossing fibers and includes fiber directions with higher uncertainty (arrow). The outer rim included only single fibers that were vertically and perpendicular oriented with respect to the surface of cortex. Similarly, several rims with low and higher FA values were observed in the hippocampus (C, within ellipse), which were not as obvious in the MD map (D). Deep GM structures such as the putamen (PUT) and the caudate (CD) had low-FA values (A, C) and high-MD values when compared with WM.

Figure 7.

Figure 8.

Fiber reconstruction algorithms were applied to the high-resolution ex vivo DWI dataset. The Red Cross hairs on high-resolution structural TIW images in axial (A), coronal (B), and sagittal (C) views indicate the position of the coronal slice of the high-resolution DWI dataset depicted in (D), (E), and (F). The MD (D) represents the squared region shown in (B) and the directionally color coded FA map in (E) a zoomed version of the region in (B). PASMRI results overlaid on a FA map in (F) represent the striped rectangular region in (E). The gray scale bar in (D) depicts the range of the MD from 0 to $0.8 \times 10^{-3} \text{ mm}^2/\text{s}$. In the MD map vessels penetrating from the surface of cerebral cortex into WM (D, *) as well as larger vessel in WM (D, **) appear with high-MD values (less-restricted diffusion). Large vessels in WM were also visible as low-FA regions

in the FA map (E, **). Because of the high resolution, low-FA regions of one voxel wide in WM probably signify partial volume effects as for example apparent on the border of the callosal fibers (CC) and the subcallosal fasciculus (FSC). In cerebral cortex, the inner and outer rim with, respectively, low and high-FA values were consistently several voxels wide (E, single and double arrowhead). In the hippocampus, several rims with low (E, R3, and R5) and high-FA values (E, R1, R2, and R4) were visible. Rims 1, 3, and 5 were generally parallel oriented with respect to the surface of hippocampus (E, green color), and rims 2 and 4 were vertically oriented (E, red/blue color). The close-up image of the PASMRI results in (F) demonstrates single fibers in rim 1, 2, and 4, whereas crossing fibers dominated rims 3 and 5.

In cerebral cortex and hippocampus, not all rims were apparent in the MD map, and no structured anisotropic diffusivity was observed in any of the deep GM nuclei such as putamen, caudate, or thalamus (Fig. 8D). Large blood vessels penetrating the surface of the cortex and extending into WM, characterized by a high-MD and low-FA values (isotropic diffusivity) were visible on the high-resolution DWI images in the hippocampus and deep WM (Fig. 8D,E). Note that the apparent structure in the MD image (Fig. 8D, white-striped box) is due to Gibbs ringing (an intensity variation between neighboring voxels), which is an imaging artefact affecting mainly the non-DWI image. Gibbs ringing is typically seen as high-intensity difference at the discontinuities or transitions from one tissue type to another (here between fluid in the ventricle and tissue in CC). Gibbs ringing rarely appears in anisotropy maps such as FA, because these estimates express the variance of the principle directions and hence are independent of the non-DW image.

DISCUSSION

In this study, we designed, tested, and optimized an ex vivo imaging pipeline, which included four stages: (I) fixation, (II) storage, (III) preparation, and (IV) MR scanning of the pig brain. Subsequently, we successfully generated a high-quality whole brain and a high-resolution regional DWI dataset. The time-varying artefact observed in ex vivo DWI datasets that originated in stage III gradually leveled out within the first 15 h of scanning. Using an empirical approach, we demonstrated that a broad range of b -values, with an optimum around 4,000 s/mm², ensured consistent fiber direction reconstruction in stage IV. Finally, we demonstrated longitudinal stability of diffusivity measures from fixed tissue that had been stored for years (stage II). The high-anatomical detail and fiber direction information evident in the high-quality and high-resolution DWI datasets underscore the potential and suitability of such an optimized ex vivo imaging pipeline in a wide range of neuroscience applications besides the verification and testing of tractography (Dyrby et al., 2007).

Minimizing Time-Varying Artefacts in the Preparation Stage

To our knowledge, this is the first study addressing time-varying artefacts in ex vivo DWI. Heat conduction and unstable tissue mechanics are the most likely sources for the observed time-varying artefacts. Heat conduction only affects diffusivity, whereas unstable tissue mechanics affect fiber directional information. The presences of time-varying artefacts were evidenced by the decrease in the mean intensity ratio, implying a gradual decrease in diffusivity and consequently increase in DW signal intensities. DW signal intensities gradually stabilized after 8 and 15 h

for fluid and tissue, respectively. Because artefacts in surrounding fluid are solely due to heat conduction, the increase in DW signal intensities over time indicates that tissue and surrounding fluid before scanning had a higher temperature than the temperature inside the scanner. Although heat conduction is more prolonged in tissue than fluid because of its larger volume and higher viscosity, the longer time for time-varying artefacts to disappear in tissue is most likely due to both heat conduction and unstable tissue mechanics. This interpretation is supported by the observed weak positional shifts in tissue over time and the simultaneous absence and presence of time-varying artefact in fluid and tissue, respectively, for pig brain P1. The absence of artefacts in fluid for P1 indicates that the temperature of the fluid was similar to that in the scanner bore. Note that this study was not designed to disentangle the relative contributions of heat conduction and unstable tissue mechanics. This issue needs to be addressed in future studies.

In our setup, where heating up of hardware was virtually absent and the temperature in the scanner was highly stable during the long scan sessions, the effect of heat conduction could be minimized by more carefully matching the tissue temperature and the temperature in the scanner bore. The contribution of unstable tissue mechanics could possibly be minimized by replacing the foam that was used to fix the brain in the coil, with a mechanically stable frame. Obviously, the specific strategies to minimize time-varying artefacts strongly depend on the particular setup. Nevertheless—and significantly—our findings indicate the importance of testing for possible time-varying artefacts when setting up an ex vivo imaging pipeline.

Determination of Optimal b -Value Ranges for the MR Scanning Stage

Traditionally, the selection of an optimal b -value has been based on optimizing the fit of the DT model to the DWI dataset [Jones et al., 1999]. For tractography, however, multifiber methods are preferable [Behrens et al., 2007] and the DT-based optimization is not appropriate. Compared to DT-based approaches, the mean angular discrepancy approach introduced here has several benefits as it can handle multiple fiber directions and allows including and evaluating more fiber reconstruction methods.

Our empirical results based on the reconstruction of fiber directions is in agreement with those of D'Arceuil et al. [2007b] who determined an optimal b -value based on the DT approach. In their study, using (immersion) fixed monkey brain, the b -value was initially increased to 4,025 s/mm² as a consequence of an observed 25% decrease in MD ex vivo as compared to in vivo. However, following final analysis, the optimal b -value for WM was determined to be 5,200 s/mm². Other DT-based studies have used the lower range of b -values tested in this study [Guilfoyle et al., 2003; Sun et al., 2005; Zhang et al., 2005]. In this

study, only the q -value was varied in the search for an “optimal” b -value, generally meaning that the same microstructural environment is probed at different length scales. It is possible that further refinement may come from varying the diffusion time, however, due to practical limitations in total scanning time, a single diffusion time, sufficient for the molecules to probe the microstructural environment, was selected.

The range of optimal b -values for multifiber reconstruction depended on the reconstruction algorithm used which agrees recent observations in phantom-based studies [Lin et al., 2003; Perrin et al., 2005; Poupon et al., 2008; Tournier et al., 2008]. For q-ball to consistently detect clusters of multiple fibers, b -values higher than 8,000 s/mm² were needed. For PASMRI b -values as low as ~2,000 s/mm² could be used. It is important to note that selecting too low b -value diminishes the ability to robustly discriminate crossing fibers within a voxel and lowers the spatial coherence of reconstructed fibers (a similar effect was observed for DT). Finally, although the range of b -values that was used to identify “optimal” b -values in terms of reconstructing fiber direction was somewhat coarse, our finding of a relatively broad range of “optimal” b -values (2,000–8,000 s/mm²) suggests that probably not much is gained by employing a finer grained sampling of b -values.

Longitudinal Stability of DWI Measurements Using Fixed Tissue

A 3-year period of tissue storage did not result in statistical differences within or across postmortem brains in either the cortical GM or WM ROIs. This finding confirms the longitudinal stability of DWI measurements for at least 3 years post mortem. The pre-mortem perfusion fixation ensures a fast, efficient, and homogeneous distribution of the fixative in the tissue in contrast to postmortem immersion fixation where formalin diffuses into the tissue at a rate of about 1 mm/h [Fox et al., 1985; Yong-Hing et al., 2005]. Ongoing cross-binding of proteins continues within the first weeks postmortem, which might lead to decreased viscosity within the cellular spaces until final preservation. Accordingly, the general tendency of lowered diffusivity observed following 3–5 weeks of storage could reflect final preservation of the tissue. The minor variation in diffusivity observed at the later time points (years) might also be due to chemical reactions in the tissue. It should be noted that, in this study, we have chosen to use formaldehyde, because it is a widely used and validated fixative that ensures a rapid fixation of tissue. However, other fixatives to stabilize cellular structure are available and may further improve long-term stability.

The observed MD and FA values are comparable to other studies on postmortem animal brains reporting decreased diffusivity in GM and WM of about 60 and 80% compared to in vivo [D’Arceuil et al., 2007b; Guilfoyle et al., 2003; Sun et al., 2005]. Differences observed are

likely related to the choice of ROIs as well as the used imaging parameters. Despite decreased diffusivity, no statistically significant differences in anisotropy have been observed between postmortem and in vivo brain tissue, suggesting that the structural information probed by DWI is preserved postmortem. This means that FA values of postmortem tissue can be used to assess the quality of the tissue in question. Indeed, a delayed start of the fixation procedure postmortem risks introducing autolysis, which might lead to abnormal FA values compared to in vivo as observed in human postmortem brain tissue [Gouw et al., 2008; McNab et al., 2009a; Pfefferbaum et al., 2004; Schmierer et al., 2007].

Anatomical Observations in the High-Quality and High-Resolution DWI Datasets

Using a multifiber reconstruction algorithm, color-coded FA maps of the high-quality whole brain and the high-resolution DWI datasets showed two horizontal parallel rims in cerebral cortex: an inner and outer rim. Anisotropy in cerebral cortex has also been noticed in postmortem mice [Zhang et al., 2003], monkey [D’Arceuil et al., 2007b], rabbit [D’Arceuil et al., 2008], and recently in the human brain [McNab et al., 2009a]. However, to the best of our knowledge, no previous studies have described the presence of two rims with different anisotropy combined with a multifiber reconstruction algorithm to elucidate the underlying cortical neuroanatomical structures known to exist but not usually visualized in standard clinical in vivo DTI.

The inner rim had a low anisotropy but appeared with PASMRI to include two crossing fibers with one fiber direction vertically oriented to the surface of the cortex and one horizontal fiber direction parallel to the underlying association fibers in WM [Mountcastle, 1997]. It is possible that the inner rim may reflect the lower cortical layers IV, V, and VI. Indeed, myelin stained cross sections of cortical tissue indicate an abundance of crossing fibers in these layers. The outer rim, defined by a relative low anisotropy (FA = 0.21) might represent the relatively wide supragranular layers II–III. Although it is generally accepted that these mid-layers include both vertical and parallel oriented fibers, we only detected a single vertical oriented fiber direction, probably because vertically oriented fibers are much denser organized and overshadow a “weaker” anisotropic signal of less coherent organized parallel fibers. However, the vertically oriented organization detected by DWI across both (horizontal) rims agrees with histology studies describing an (vertical) organization of cell columns throughout the cortex [Horton and Adams, 2005; Rockland and Ichinohe, 2004]. The low-FA value in the outer rim, which was low when compared with WM FA, may reflect a low-fiber density, an incoherent parallel fiber organization, as well as a high-cell body density. Finally, we were not able, even on the coronal high-

resolution DWI dataset, to clearly visualize layer I, which is known to contain dense crossing fibers, that is, fibers oriented parallel to the cortical surface connecting neighboring cortical regions and fibers vertically oriented harboring into the lower cortical layers.

In the hippocampus, we observed several parallel rims corresponding to the well-known structural organization of the hippocampus [O'Keefe and Nadel, 1978]. On the high-resolution DWI dataset, at least five rims with higher FA values were apparent with a lamellar organization as a sandwich along the longitudinal axis of the hippocampus [Andersen et al., 2000]. Each rim represented a general fiber direction orientation, either parallel (rim 1, 3, and 5) or vertical (rim 2 and 4) with respect to the surface of the hippocampus. The parallel oriented fibers seem to correlate with layers of interconnecting axons that constitute the hippocampal fiber pathways, that is, rim 1 reflects the Alveus, rim 3 Schaffer collaterals from the pyramidal cell layer and rim 5 the mossy fibers. Rim 1 had higher FA values than rims 3 and 5 and contained a single-fiber direction, whereas crossing fibers were detected in rims 3 and 5. This is in agreement with histological findings showing dense and spatial coherent myelinated axons in the Alveus (rim 1), whereas the unmyelinated mossy fibers and Schaffer collateral include a fraction of crossing dendrites of the vertical oriented pyramid cells (rims 3 and 5) [Mark et al., 1995; O'Keefe et al., 1978]. The vertically oriented fibers found in rims 2 and 4 had similar FA values and included single-fiber directions only. These rims seem to represent the vertically oriented pyramid cells and their dendrites distributed through the layers of Cornu Ammonis. Hence the broadest rim 2 may include a mixture of the oriens and pyramidal layer and rim 4 the radiatum layer.

Our findings on fiber orientation and anisotropy in the hippocampus agree well with results from (very) high-resolution (μm) ex vivo DWI studies of the hippocampus in the human [Shepherd et al., 2007] and mice [Zhang et al., 2002], acquired at 14.1T and 9.4 T, respectively, using a simple tensor model. Therefore, we argue that a highly accurate reconstruction of the well-described hippocampal microstructure is possible even at a relative lower image resolution, if a multifiber reconstruction algorithm such as PASMRI is applied.

The anatomical observations were made in two rather different DWI datasets: one high-quality with isotropic voxels and one high in-plane resolution with anisotropic voxels. The use of isotropic voxels ensures rotational invariance of anisotropic structures, which is important in, for example, tractography. Although isotropic voxels generally should be preferred, the use of isotropic voxels might not be optimal when a very high-image resolution is needed to study small anatomical structures, because SNR is linear proportional with voxel volume. In this case, anisotropic voxels with a higher in-plane resolution than slice thickness can be used to achieve an acceptable SNR. Note that, in this study, $0.25 \times 0.25 \times 1.0 \text{ mm}^3$ voxels

were used. Although a similar SNR could have been obtained by using $\sim 0.395 \text{ mm}^3$ isotropic voxels, in our experience, the in-plan resolution should be at least 0.31 mm^2 for visualization of the hippocampal layers. Importantly, the structure of interest must be assumed spatially constant over the length scale of a slice thickness to prevent partial volume effects (PVEs). Although we checked that the outer most layers of hippocampus were generally constant over the length scale of a slice in the high-resolution DWI dataset, we cannot exclude the possibility that the very low FA values observed between, for example, rims 1 and 2 in Figure 8(E,F) reflect PVE. The risk for PVE is higher in the cerebral cortex of the pig brain, because its highly convoluted nature makes it more likely that voxels cover different neighboring cortical regions. The latter might explain why no more detailed structure in cortical layers was observed.

Taking the weight ratio into consideration, the image resolution of the high-quality DWI dataset of the pig brain corresponds anatomically to in vivo human DWI datasets [Dyrby et al., 2007]. However, the anatomical details observed in the cerebral cortex and the hippocampus of the fixed pig brain is typically not detectable from in vivo human DWI datasets. Besides the obvious improvement in the quality of ex vivo versus in vivo DWI datasets, some variation may also be related to interspecies differences. For example, the volume fraction of GM compared to the total brain volume is generally larger in animals than in the human brain and thus benefits GM microstructural imaging [Zhang and Sejnowski, 2000].

Despite the fact that the ex vivo imaging pipeline was optimized for WM, detailed anatomical structure was observed in neocortex and hippocampus. The basic architecture of cortex is complex compared to WM, which generally is simplified as parallel tubes. Specific optimization of the b -value for DWI of structurally more complex regions such as the neocortex or hippocampus might further improve the structural contrast in brain GM.

Notably, diffusion MRI in postmortem tissue not only probes nerve fibers but also probes the vascular system, because the latter contains nonflowing fixative with water molecules performing Brownian motion. Indeed, relatively large blood vessels were visible on the MD image in the high-resolution DWI dataset. Because we used a b -value of $4,009 \text{ s/mm}^2$ with a q -value sensitive to molecular displacements within the $15.5\text{-}\mu\text{m}$ range, the acquisition was in principle sensitive to small vessels in the capillary bed of cerebral cortex, which have an average diameter of $7.5 \mu\text{m}$ in the monkey [Weber et al., 2008]. Despite the seemingly structured distribution of blood vessels at the macroscopic level, at the microscopic level, blood vessels do not seem to have a spatial dominant orientation as evidenced on electron micrographs [Harrison et al., 2002; Weber et al., 2008] and are therefore expected to produce a purely isotropic diffusivity component. As a consequence, it is unlikely that the observed cortical rims reflect the cortical vascular bed. Moreover, the mean volume fraction of

vessels in the cerebral cortex is only 2.14% [Weber et al., 2008], suggesting the isotropic diffusion component of the capillary bed to be a minor contributor to the overall diffusivity measured in cerebral cortex. We therefore suggest that observed FA values and fiber directions in the cerebral cortex as well as in the hippocampus represent fiber connections and their orientation with a diminutive bias from the microvascular system.

CONCLUSION

We tested and optimized an ex vivo imaging pipeline to obtain high-quality DWI datasets on fixed tissue. Future ex vivo DWI studies are recommended to test their setup for time-varying artefacts resulting mainly from heat conduction and unstable tissue mechanics. Our findings show that a wide range of “optimal” b -values existed, with a general optimum around $4,000 \text{ s/mm}^2$, for consistent reconstruction of single and multiple-fiber directions. Despite minor variations in diffusivity, collecting DWI data on fixed tissue stored for years seems feasible at least within the 3-year period of the study. The high-anatomical detail and fiber direction information evident in the high-quality and high-resolution DWI datasets underline the enormous potential of ex vivo DWI. Not only can it be used for development, testing, and validation of tractography algorithms within a true anatomical environment but also as a supportive modality to classical histology for the study of fiber organization, anatomical connectivity, and microstructure at the macroscopic level.

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