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Custom Fit 3D-Printed Brain Holders for Comparison of Histology with MRI in Marmosets

Joseph R. Guy^a, Pascal Sati^a, Emily Leibovitch^a, Steven Jacobson^a, Afonso C. Silva^b, and Daniel S. Reich^a

Joseph R. Guy: joseph.guy@nih.gov; Pascal Sati: pascal.sati@nih.gov; Emily Leibovitch: leibovitchel@ninds.nih.gov; Steven Jacobson: JacobsonS@ninds.nih.gov; Afonso C. Silva: SilvaA@ninds.nih.gov

^aDivision of Neuroimmunology and Neurovirology, National Institute of Neurologic Disorders and Stroke, Bldg 10, Rm 5C103, 10 Center Drive MSC 1400, Bethesda, MD, 20892, United States

^bLaboratory of Functional and Molecular Imaging, National Institute of Neurologic Disorders and Stroke, 49 Convent Drive MSC 1065 Building 49 Room 3A72, Bethesda MD, 20892, United States

Abstract

Background—MRI has the advantage of sampling large areas of tissue and locating areas of interest in 3D space in both living and ex vivo systems, whereas histology has the ability to examine thin slices of ex vivo tissue with high detail and specificity. Although both are valuable tools, it is currently difficult to make high-precision comparisons between MRI and histology due to large differences inherent to the techniques. A method combining the advantages would be an asset to understanding the pathological correlates of MRI.

New Method—3D-printed brain holders were used to maintain marmoset brains in the same orientation during acquisition of ex vivo MRI and pathologic cutting of the tissue.

Results—The results of maintaining this same orientation show that sub-millimeter, discrete neuropathological features in marmoset brain consistently share size, shape, and location between histology and ex vivo MRI, which facilitates comparison with serial imaging acquired in vivo.

Comparison with Existing Methods—Existing methods use computational approaches sensitive to data input in order to warp histologic images to match large-scale features on MRI, but the new method requires no warping of images, due to a preregistration accomplished in the technique, and is insensitive to data formatting and artifacts in both MRI and histology.

Conclusions—The simple method of using 3D-printed brain holders to match brain orientation during pathologic sectioning and MRI acquisition enables rapid and precise comparison of small features seen on MRI to their underlying histology.

Corresponding Author: Daniel S. Reich (reichds@ninds.nih.gov), Bldg 10, Rm 5C103, 10 Center Drive MSC 1400, Bethesda, MD, 20892, United States, Phone: 301-496-1801, Fax: 301-402-0373.

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1. Introduction

In preclinical studies, in vivo MRI¹ is routinely used as a noninvasive method to examine tissue damage caused by neurological diseases (Boretius et al. 2006, Gaitán et al. 2014, Leibovitch et al. 2012, Rausch et al. 2003). While MRI is able to provide three-dimensional tissue representation of brain abnormalities caused by a disease, it may yield limited insight into the underlying pathological substrates due to both substantially lower resolution than histology and relative nonspecificity of intensity changes. When biopsy or autopsy is possible, histological techniques allow the investigation of underlying pathology initially detected by MRI ('t Hart et al. 1998, Boretius et al. 2006, Kap et al. 2010, Kobayashi et al. 2014, Rausch et al. 2003). However, it is often complicated to compare histology to MRI as the two techniques produce images at different dimension scales (hundreds of nanometers for histology using high-performance digital camera and microscope versus hundreds of micrometers for MRI at ultra-high fields). Moreover, it is often an imprecise and excessively laborious process to match the two-dimensional histological slides on a three-dimensional MRI volume due to variations in alignment and orientation between the two techniques.

Prior attempts at solving the inherent mismatch between MRI and histology have been made using computational deformation methods to match histological sections with MRI volumes for both human (Budde and Annese 2013, Bürgel et al. 1999, Schormann et al. 1995, Schormann and Zilles 1998) and laboratory animal (Dauguet et al. 2007, Ju et al. 2006, Nikou et al. 2013, Palm et al. 2008) brains. However, these methods rely on complicated computational approaches that are naturally highly sensitive to data formatting, input parameters, and image artifacts. Additionally, the registration process of these approaches that reconstruct the MRI and histology in the same data space distorts the original data, which can be undesirable and prevent higher precision comparisons.

An alternative approach is to improve the image correspondence between the two techniques during the acquisition of both the MRI and histological data. This strategy would eliminate issues related to data registration failures in the MRI to histology matching, and additionally, would be insensitive to the presence of artifacts in either dataset. Our laboratory developed a method to help resolve the pathological data mismatch with the use of 3D-printed custom-fit cutting boxes to assist in the sectioning of human brains (Absinta et al. 2014). This approach enabled high quality matching of histologic images of the gyrencephalic brains to MRI without the use of whole-brain sectioning or computational approaches and greatly eased the localization of focal areas of interest in histology using the MRI. We were also motivated to develop an approach similar to this that could be applied to marmosets due to their value as a preclinical neurologic disease model ('t Hart et al. 2000). The large inter-individual variation in brain size and shape in marmosets compared to rodents limits the utility of a standardized cutting block, which increases the value of a custom 3D-printed device to fit each brain. Furthermore, marmoset brain lissencephaly and perfusion fixation following euthanasia prevents postmortem deformations of the brain, thereby increasing the precision that a 3D-printed cutting block would provide. Here, we introduce a simple method that

¹**Abbreviations:** MRI (magnetic resonance imaging), EAE (experimental allergic encephalomyelitis), TE (echo time), TR (repetition time)

improves the pathologic cutting of tissue and also maintains alignment and orientation of common marmoset brains across the pathologic sectioning and the ex vivo MRI, thus facilitating comparison to in vivo MRI.

2. Methods

2.1 Tissue

Brains from five marmosets that underwent perfusion formalin fixation at the time of euthanasia (one healthy animal and four affected by an experimental allergic encephalomyelitis (EAE) (Gaitán et al. 2014)) were stored in 4% formaldehyde for 1-2 years prior to use for this project. The variable time stored in 4% formaldehyde did not affect MR signal.

2.2 In vivo MRI

The in vivo MRIs were done on four of the marmosets using a 2D spin-echo proton-density (PD) weighted acquisition on a 7T 30cm USR/AVIII (Bruker Biospin) MRI platform with a home-built 5-channel phased array surface coil (Gaitán et al. 2014). Marmoset anesthesia, positioning, and physiologic monitoring were in line with prior studies (Sati et al. 2012). The main parameters of the acquisition were: $150 \times 150 \mu\text{m}$ in plane resolution, slice thickness = $600 \mu\text{m}$, number of slices = 54, TE = 15 ms, TR = 1962 ms, flip angle = 180° , total acquisition time = 10 m 28 s.

2.3 Initial ex vivo MRI acquisition

For the first ex vivo MRI, each brain (Figure 1A) was immersed in a 50 mL Falcon tube filled with an MR-invisible fluid (Fomblin, Solvay S.A.) and firmly padded with gauze to control brain position within the tube. Care was taken to reduce the number of air bubbles inside the tube, as these cause artifacts on MRI and in the generated 3D model. The 50 mL Falcon tube was fitted inside a 38 mm volume coil (Bruker Biospin) and scanned using the same 7T platform. A 2D spin echo T_2 weighted sequence (Figure 1B) was chosen to minimize artifacts from magnetic field inhomogeneities due to residual air bubbles. Importantly, positioning of the sequence was done to match the alignment of the in vivo images. Sequence parameters were: $150 \mu\text{m}$ isotropic resolution, number of slices = 220, TE = 48 ms, TR = 23000 ms, flip angle = 180° , number of averages = 8, total acquisition time = 7 hr 22 m 6 s. Following the scan, the brain was returned to 4% formaldehyde solution for storage.

2.4 Creation of brain 3D models

Postmortem MR images were manipulated in MIPAV 7.2 (mipav.cit.nih.gov, RRID nif-0000-00329). To smooth over noise and to allow finer editing of details, the 2D spin-echo T_2 MRIs were upsampled to $100 \mu\text{m}$ isotropic resolution using a trilinear interpolation transformation. In order to enhance automated detection of the brain surface, a nonlinear noise reduction spatial filter algorithm was applied. A threshold-based segmentation was applied using an intensity value between the voxel values for brain and the background noise. This created binary images where 1 = brain and 0 = background noise, resulting in a generally artifact-free binary image representing the brain volume (Figure 1C). If artifacts

were present, these were manually removed with the brush tool. Importantly, this step can be avoided entirely if imaging artifacts are negligible and the signal to noise ratio is sufficient on the initial ex vivo MRI. Care was also taken to manually remove any sharp points on the lower surface of the brain, whether anatomical or due to artifact. This was done because these points may puncture the brain tissue, and dulling of them allows easy insertion of the brain tissue into a 3D-printed brain-shaped cavity. The final step was to apply a 2.5 D morphological hole-filling algorithm that removed ventricles and any interior brain surface or remaining artifacts (not pictured). A surface extraction was then applied to the final binary image using a marching cubes algorithm, and the resulting surface file was used to generate 3D models for printing.

2.5 Creation of 3D-printed brain holder models

The 3D file of the brain surface was opened in the 3D modeling program netfabb Professional 5.0 (netfabb GmbH). In the program's repair utility, any non-brain shells were removed by selecting the brain, inverting the selection, and deleting the newly selected shells. Algorithms were applied first for smoothing and then for triangle reduction. An automatic extended repair was applied to the brain model to fix any remaining defects. The completed brain model (Figure 1D) was subtracted in a Boolean operation from two different pre-built models:

1. The **brain cradle** is a volume representing a cutaway of the internal shape and size of the 50 mL Falcon tube. Figure 2A shows the brain cradle from side (A1), top (A2), perspective (A3), and front (A4). This was created by taking internal measurements of the 50 mL Falcon tube and generating 3D models of a cylinder and a cone of the same diameter, then adding them in a Boolean operation. Planar cuts were made in the completed shape to allow the cradle to fit in the tube and the brain to be inserted and rigidly held in place.
2. The **brain slicer** is carved from a $5 \times 5 \times 4.8$ cm beveled rectangular prism model that allows manual placement of gaps for the cutting blade. Figure 2B shows the brain slicer from perspective (B1), side (B2), front (B3), and top (B4). These gaps were created by Boolean subtractions of rectangular prism models of 50 μm thickness, which approximated the thickness of the blades used. These slice gaps could be strategically placed to create slabs of tissue targeted to only the region of interest. Blade holders were also modeled to assist with slicing by covering the ends of the blades (Figure 3).

The brain cradle and the brain slicer models were aligned with each other such that the marmoset brain model could be subtracted from both in a Boolean operation without further adjustments. The brain cradle model was placed to center the brain in the volume coil's field of view, and the brain slicer was placed to center the brain within it. Each brain model was then loaded into netfabb Professional, and new models of the brain cradle and brain slicer were generated with Boolean subtractions for each brain. Thus, a total of ten brain holder models were generated from five brain models. In order to avoid confusion, the appropriate marmoset's name was printed onto both the brain cradle and the brain slicer models.

The completed brain cradle (Figure 2D) was printed solid using a Stratasys Fortus 360. A 3D model of the brain cradle with (D1) and without (D2) the brain inserted are shown in Figure 2. Figure 2D3 shows a photograph of the 50 mL Falcon tube, brain cradle, and brain assembly. The completed brain slicer (Figure 2C) was printed solid using a 3D Systems Projet 3510, which was necessary for high resolution detail and the rigidity and strength of the blade-dividers. Perspective renderings of the brain slicer shown with the brain inserted (C1) and absent (C3) are also shown in Figure 2. Figure 2C2 shows an interior cutaway view of the brain slicer model with brain in place, and Figure 2C4 shows a perspective photograph of the brain in its brain slicer. The printing time for these models ranged from about 4 hours for the brain cradles and about 8 hours for the brain slicers.

2.6 Final ex vivo MRI acquisition

For the final ex vivo MRI, each brain was positioned in its custom-fit 3D-printed cradle and inserted snugly into a 50 mL Falcon tube filled with Fomblin. The use of the cradle reduced the volume of Fomblin required to immerse the marmoset brain and also reduced the dependency of gauze to position the brain, thus reducing places for air bubbles to become trapped. It also reduced the setup time by ideally positioning the brain centered inside the field of view, thus minimizing the amount of adjustments necessary before acquiring the images. The Fomblin-immersed brain, brain cradle, and 50 mL Falcon tube assembly (Figure 2D1) was placed in the same volume coil and scanner as the initial ex vivo MRI. The brain was imaged using a 3D T_2^* weighted sequence (MR images in Figures 3 - 7). The sequence parameters were: 100 μm isotropic resolution, TE = 10 ms, TR = 50 ms, flip angle 12.4°, averages = 36, total acquisition time = 33 hours.

2.7 Pathological sectioning

Following the second ex vivo MRI, the brain was positioned in its custom-fit slicer (Figure 2C4). Accu-Edge low profile microtome blades (Sakura Finetek, USA) were fitted between two 3D-printed blade holders (Figure 3). The blades and blade-holder assembly was placed into the 3D-printed brain slicer, cutting three of the five brains into all slabs simultaneously (Figure 3A); in two brains, these were used to extract a single slab of interest (Figure 3B). The brain slabs were individually removed from the slicer and were then stored in separate labeled containers filled with 4% formaldehyde.

2.8 Histological sectioning

Five slabs of brain tissue from three of the marmoset brains (marmosets 1, 2, and 3) were sent to a histological services company (Histoserv, Inc.) to be sliced in a cryostat microtome using the company's standard operating procedure, with instructions that the 10 μm thick histological sections should be parallel to the tissue surface. Each 10 μm section was positioned 100-125 μm apart, generating a total of 146 slides for the five slabs of tissue.

2.9 Slide staining

Histological staining was done using standard hematoxylin and eosin (H&E) overlaying Luxol fast blue (LFB). A modified method was used to give comparable lesion-to-white/

gray matter contrast on both the H&E/LFB and MR images. For staining, the following protocol in Table 1 was used.

2.10 Digitalization of slides and matching of images

The slides were digitalized using a Ventana iScan™ automated slide scanner. The digital slides were opened in Ventana Image Viewer and compared with coronally oriented MR images in MIPAV using the anatomy as large-scale landmarks and veins and lesions as small-scale landmarks. If lesions and other small features did not align, the MR images were iteratively rotated 0.5 degrees in MIPAV, with a transformation algorithm in the X and/or Y plane using windowed sinc interpolation, until the two images showed matching distant features. No rotations were done in the Z plane due to variations in the Z orientation between each histology slide.

3. Results

The high-resolution isotropic 100 μm T_2^* weighted MRI demonstrated high sensitivity to small discrete lesions and small anatomic features, as pictured in Figures 4 – 7. In Figure 4, MRI slices were selected based on the distance between the cutting blades and the caudal/rostral surfaces measured directly on the 3D model and the ex vivo MRI. As a result, the photographs of the surfaces of the brain slabs matched the gross morphology observed in the MRI slices.

Four of the five tissue slabs were examined histologically in three of the five marmoset brains, producing 146 histology slides. One of these four slabs of tissue (representing 27 slides) was not included due to inverted positioning of the brain hemispheres. Of the 119 remaining histology slides, 104 (87%) were determined to be free from large-scale artifacts that would prevent sufficient visual matching. The 15/119 histology slides with significant artifact were from the extreme anterior or posterior faces of the tissue slabs, reflecting the difficulty of sectioning a flat tissue surface. In order to precisely match small-scale features seen on the histology, the ex vivo MRI volumes were rotated by an average of 1.9° in the X-Y dimensions in order to precisely match small-scale features in the histology (rotations of 0° for slab 1 from marmoset 1, 3.6° for slab 2 from marmoset 1, 0° for marmoset 2, and 4.1° for marmoset 3). Following these rotations, 100% (104/104) of the investigated histology slides matched their corresponding slices from the ex vivo MRI as illustrated by examples chosen approximately every 600 μm in Figure 5.

Once the MRI and histology images were matched, the matching of small-scale features (such as white matter tracts, blood vessels and lesions) could be investigated as demonstrated in Figure 6. These small features still shared precise shape, position, and alignment on both ex vivo MRI and histology images (Figure 6 A4 and A5). Because the ex vivo MRI was set up using the same anatomical landmarks and field-of-view orientation as the in vivo MRI, the matching histology could also be extended to the in vivo images (Figure 6 A1 and B1). However, the degree of precision was lower due to the lower image resolution used for the in vivo MRI (Figure 6 B4).

Interestingly, very fine details not seen on in vivo MRI, such as lesions less than 200 μm in diameter, can still be verified by histology as demonstrated in Figure 7. The focal areas of increased signal intensity seen on MRI consistently matched areas of demyelination, as indicated by the absence of LFB stain (pink areas), and cellular infiltration, as indicated by the hematoxylin stain (purple areas). The exception to this is in marmoset 3, where MRI signal voids in a minority of lesions appeared to correspond to iron deposition from hemosiderin-laden macrophages (Figure 7 panels 10B, 11B, 12B, 13B).

4. Discussion

In this study, we propose a novel approach based on 3D printing technology to match MRI and histology images. Custom-fit marmoset brain holders were 3D-printed, which allowed MRI and pathologic cutting to be performed on brain tissues while preserving their alignment and orientation.

The advantage of this new approach is to quickly and precisely correlate ex vivo MRI findings to the underlying cellular architecture and relate these findings to in vivo MRI. The “blank” 3D models for the brain cradle and brain slicer (Figure 2A and 2B) only need to be generated once, while the Boolean operation subtracting brain volumes from these models can be done any number of times and only takes a few minutes per brain. The cost of raw material to print the brain cradles was about \$8 each, while the higher-detail brain slicers cost about \$35 each. Thus, the technique here would be well suited to comparing histology and MRI in studies with a large number of brains.

Additionally, the alignment of histology was precise enough to match 100 μm isotropic voxels on the ex vivo MRI. Therefore, our method can be used to perform histological identification of sparsely found points of interest or very small features detected on ultra-high resolution MR images. This has the potential to be used in preclinical studies such as in marmoset EAE where small, early developing lesions can be identified by MRI and investigated pathologically as demonstrated here.

Because of the familiarity of our laboratory with marmosets, only marmoset brains were used here to develop this new approach. Due to the genetically heterogeneous (outbred) nature of our marmoset colony, the brains have variable size and shape, though they are small enough to fit on a standard 1×3 inch glass slide. Therefore, in our view, this method is particularly advantageous over a standard one-size-fits-all cutting block. Although an approach such as the one described here is less advantageous when dealing with more homogenous brains such as those of age and weight-matched rodents, there is no technical reason why it could not be adapted for that application. However, applying a similar technique to much larger brains of human or higher primates results in its own unique set of challenges. Indeed, our laboratory has published a separate paper about using a 3D-printed cutting box to align ex vivo MRI and histology in a conceptually similar manner (Absinta et al. 2014).

We did not directly compare the results from our customized 3D-printed brain slicers to those derived from a standardized cutting block. The custom-fit brain slicers were designed to align the brains with the same positioning of in vivo scans, whereas use of a standardized

cutting block would require that the in vivo scans be tailored to the pathology rather than to each animal's distinct anatomy. Additionally, due to the large variation in size of marmoset brains in our colony, some brains are simply too large for the use of the cutting block in our inventory. Furthermore, to our knowledge there are no commercially available marmoset brain cutting blocks, which would severely limit the usefulness of such a comparison to other laboratories.

It should be noted that we only used histological slices that were cut using a cryostat microtome, and we did not investigate paraffin-embedded sections. It may be possible that additional artifacts introduced by paraffin-embedding of tissue may distort tissue and decrease the effectiveness of maintaining alignment with the 3D printed brain cradle and brain slicer. Although any deformations caused by freezing and embedding the tissue in a frozen medium for sectioning in the cryostat microtome were not enough to significantly distort the histology vs MRI matching, investigation of the impact of paraffin embedding on the radiologic matching is indicated. We intend to investigate this issue directly in future work.

Despite the care we took match the size of each holder to its corresponding brain, we found that one of the five brains did not fit well in its 3D-printed holder. This situation did not arise with the other four brains by a slight increase in size due to blurring that occurred during the upsampling of the initial ex vivo MRI data (section 2.4) and in the smoothing step of the 3D model (section 2.5). However, one brain model (marmoset 1) still needed to be increased in size by an additional 5% in order to accommodate insertion of the brain into its respective 3D-printed holder.

The most significant limitation was the need to rotate the MR images in order to match the histologic images. This is because this method only preserves the alignment between the techniques up to the point after pathologic sectioning. Histologic sectioning of the tissue slabs that is not done exactly parallel to the slab surfaces can introduce rotations into the histologic images. This is why it was necessary to minimally rotate two sets of MR images in order to match two of the four slabs of tissue investigated with histology; indeed, we were surprised that such rotation was not required in all cases. In the two cases where manual rotation was required, the process was time consuming (up to 3 hours per case), which highlights the importance of carefully performing histologic sectioning. However, due to the coregistration of the tissue slabs to the ex vivo MRI prior to histological sectioning, the rotations introduced by non-parallel sectioning in the cryostat microtome were minimized. If an apparatus or technique were used to ensure that histologic slicing was done parallel to the tissue surface, this situation could be avoided.

In summary, we present here a novel method for rapid and accurate histologic investigation of MRI findings in marmoset brains. The use of 3D-printed brain holders to maintain brain alignment and position greatly reduces the time burden and inherent inaccuracy of manually matching histologic sections with imaging findings. We suggest use of this method in studies that correlate new MRI techniques to underlying histology, that perform precision MRI to histology correlations in large study groups, or that require histology from sparsely found points of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- MRI and histopathology are complementary techniques, and data from each can inform the interpretation of the other.
- A method is described to facilitate comparison between MRI and histopathology in individual animals.
- Ex vivo MRI was used to generate 3D models of brains and 3D-printed brain holders.
- The 3D-printed brain holders maintained brain positioning between ultra-high resolution MRI and pathologic cutting.
- Preservation of brain position between techniques enabled high precision comparisons of ex vivo MRI to histology and facilitated comparisons with in vivo MRI.

Figure 1.

Generation of a marmoset brain 3D model. After a marmoset brain has been fixed in paraformaldehyde (A), a T_2 -weighted MRI is performed at a 150 μm isotropic resolution (B). The images are upscaled and thresholded to produce a binary volumetric image (C), the surface of which is used to generate a 3D model of the brain (D). Note: Brain pictured in B, C, and D (marmoset 1) is from a different animal than the brain pictured in A.

Figure 2.

The brain model is used to build custom-fit brain holders. This figure shows the transition from the brain 3D model and blank objects to the final 3D models ready to print. The brain cradle (A) is based on the internal volume of a 50 mL Falcon tube. Views from the side (A1), top (A2), perspective (A3), and front (A4) are provided. The brain slicer is built to contain the brain and guide the blades during pathologic cutting. Perspective (B1), side (B2), front (B3), and top (B4) views are provided. The 3D marmoset brain model is centered on both the cradle (D1) and the slicer (C1,C2) before a Boolean subtraction is applied (D2,C3). Photographs of the brain cradle and slicer are shown with the brain in place in D3 and C4.

Figure 3.

Brains from Marmoset 2 (A) and Marmoset 3 (B) are shown with their respective brain slicers. The blade holders with their blade inserts are shown in a configuration to cut all 2.5 mm thick brain slabs simultaneously (A) or to extract a single 5.0 mm thick slab of tissue containing a region of interest. Slicing downward and then retracting the blade-holder assembly extracted the tissue slab of interest (B).

Figure 4.

Tissue slab photographs with corresponding MRI slices. Ex vivo MRI (column B) slices were predicted based on blade position in the 3D model and in vivo slices were visually matched (column A). After pathologic cutting of the tissue, photographs (column C) were found to be consistent with ex vivo MRI slices and comparable to in vivo MRI across different slabs of tissue in marmoset 1.

Figure 5.

Whole slice matches between histology and ex vivo T_2^* MRI. Histology slices without significant artifact were selected approximately every 600 μm from each slab of tissue and displayed to the left of their corresponding MRI slice match. Two slabs from animal 1 (slab 1 A1-A4, slab 2 B1-B4), and one slab each from animal 2 (C1) and animal 3 (D1-D7) are shown. Whole-volume rotations of the MRI in the X and Y dimensions were necessary to increase precision: 0° for slab 1 animal 1, 3.6° for slab 2 animal 1, 0° for animal 2, 4.1° for animal 3. Close inspection reveals consistently matching small features (white matter lesions and small white matter tracts that change position from slice to slice) as well as overall anatomic matching.

Figure 6.

Selected MRI and histology slices show the correspondence of matching in both whole-slice and magnified views of EAE lesions. Two different marmoset brains and slices from different areas within those brains (A,B) were selected to show the high-detail preservation between in vivo MRI (A1, B1), final ex vivo MRI (A2, B2) and histology (A3, B3). In the first animal (panel A), the magnified views show that ex vivo MRI (A4) can be correlated on the voxel level to histology (A5). The location and shape of demyelinated EAE lesions (hyperintense areas on MRI and absence of blue LFB stain and increased cell density on histology) in the optic tract are matching, as indicated by the arrows pointing at the lesion borders. In the second animal (panel B), similar features can be observed on in vivo MRI (B1) ex vivo MRI (B2) and histology (B3). In the magnified views, the same EAE lesion was visualized on in vivo MRI (B4) in addition to the ex vivo MRI (B5) and histology (B6). Note the lesser degree of precision of the in vivo MRI due to its lower image resolution.

Note also the hypointense focal signal (green asterisk) within the lesion observed only on the T_2^* ex vivo MRI, which most likely represents an area of local iron (hemosiderin) deposition.

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Figure 7.

High-magnification views of corresponding MRI and histology. Magnified views were selected from images in Figure 5 to show that the high-precision matching between the H&E +LFB histology stain (A series) and the T_2^* ex vivo MRI (B series) is consistent throughout different areas of different brains. MRI images were windowed to best display lesion borders and histology images were rotated and resized to match. Arrows delineate lesions of high intensity on the MRI and their correspondence to absence of LFB staining (pink) and cellular infiltration (purple) on the histology images. Green asterisks indicate exceptionally low intensity regions within lesions that may represent local iron (hemosiderin) deposition.

Table 1

H&E + LFB staining protocol. Times are approximate.

Solution	Time
95% ethanol	5 minutes (twice)
Luxol fast blue	12-18 hours
95% ethanol	5 minutes
deionized water	3 minutes
lithium carbonate	5-7 minutes
95% ethanol	5 minutes
deionized water	3 minutes
Mayer's hematoxylin	3 minutes
tap water	15 minutes
eosin Y-B	10 seconds
95% ethanol	5 minutes
100% ethanol	5 minutes (twice)