Chapter 16

Models of Neurodegenerative Disease – Alzheimer's Anatomical and Amyloid Plaque Imaging

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Abstract

Alzheimer's disease (AD) is an important social and economic issue for our societies. The development of therapeutics against this severe dementia requires assessing the effects of new drugs in animal models thanks to dedicated biomarkers. According to the amyloid cascade hypothesis, β-amyloid deposits are at the origin of most of the lesions associated with AD. These extracellular deposits are therefore one of the main targets in therapeutical strategies. Aβ peptides can be revealed histologically with specific dyes or antibodies, or by magnetic resonance microscopy (μMRI) that uses their association with iron as a source of signal. The microscopic size of the lesions necessitates the development of specific imaging protocols. Most protocols use T_2-weighted sequences that reveal the aggregates as hypointense spots. This chapter describes histological methods that reveal amyloid plaques with specific stains and MR-imaging protocols for in vivo and ex vivo MR imaging of AD mice.

Key words: Animal model, mouse, amyloid, APP, PS1, imaging, MRI.

1. Introduction

Alzheimer's disease (AD) is a severe dementia with critical social and economic consequences. Senile plaques are one of the hallmarks of this disease. They are microscopic lesions that measure less than 20 μm in humans (1). These lesions are constituted of aggregated extracellular deposition of β-amyloid (Aβ) peptides. Amyloid deposits are believed to occur in the brain a long time, maybe decades, before the occurrence of clinical AD (2) and according to the amyloid cascade hypothesis, amyloid is at the origin of most of the pathological processes associated with AD (3).

To date, there is no curative treatment against AD, but many disease-modifying treatments are under investigation (4). The

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development of these treatments relies on the use of animals such as transgenic mouse models of amyloidosis (5). Most of these models are based on the overexpression of mutated forms of APP alone or with an additional mutation of presenilin (PS1 or PS2) genes (6). The generation and use of these models require the ability to phenotype these animals and to evaluate AD-like pathologies.

MRI can play a critical role to follow up these models. First, MRI can be used to follow up cerebral atrophy in transgenic mouse models of AD (**Fig. 1**) (7). This biomarker is critical because in humans, cerebral atrophy appears progressively during the evolution of AD (8) and it is associated with disease progression in clinical trials (9). Imaging amyloid plaques would also be critical to follow up the AD pathology in animals. Today, in humans, amyloid plaque imaging relies mainly on positron emission tomography and on specific ligands such as PIB (2). Such ligands are however difficult to use in small rodents (10).

MR methods using MRI are under development to either quantify some parameters that reflect amyloid load (11, 12) or to directly detect amyloid plaques (13, 14).

This chapter describes methods to detect amyloid plaques in vivo and ex vivo in the brains of transgenic mouse models of AD.

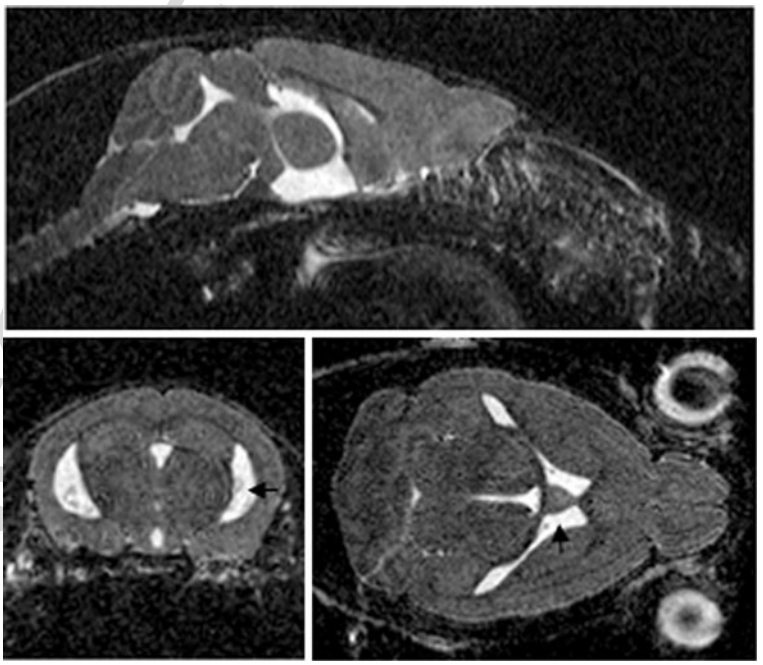

Fig. 1. In vivo 3D acquisition of a mouse brain: sagittal (*top*), coronal (*left*), and axial (*right*) views. Cerebral ventricles filled with CSF appear hyperintense on these images (*arrows*). The measure of their volumes can be used as an index of atrophy.

2. Material

2.1. Mouse Models

APP/PS1dE9 mouse models of AD can be purchased from the Jackson Laboratory (http://www.jax.org/; 600 Main Street Bar Harbor, Maine 04609 USA) (*see* **Note 1**).

2.2. MR Imaging Systems for In Vivo Imaging

1. MRI spectrometer and MR probes: 7-T spectrometer (Pharmascan, Bruker Biospin GmbH) equipped with a 9-cm inner diameter gradient system (760 mT/m strength and 6836 T/m/s slew rate) and interfaced to a console running Paravision 5.0. birdcage coil (Bruker) of 38 mm diameter for power transmission–reception. Other high-field MRI systems can also be used for in vivo imaging of mouse models of AD.
2. Mouse holder and monitoring (**Fig. 2**): the head of the animal is stabilized in a head holder using ear bars and a bite bar built in a dedicated cradle (available from RAPID Biomedical GmbH, Germany). The head holder is inserted into the radio frequency (rf) coils during the imaging session. This setup prevents movements of the animals during the long imaging acquisitions. Monitoring devices, such as the MR-compatible small animal monitoring and gating system (respiration/IBP module) from SA Instruments Inc (Stony Brook, NY 11790, USA), are used to follow the animal's physiological parameters. The animals are warmed with a water-filled heating blanket connected to a thermoregulated water bath (circulating thermostat system from Bruker).

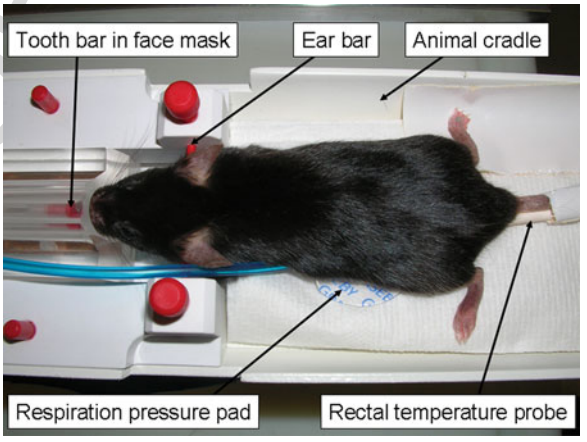

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Fig. 2. Mouse setup for in vivo MR imaging. The animal is held still with tooth and ear bars. The isoflurane anesthesia is delivered via a face mask. Respiration is recorded through a pressure pad and body temperature is recorded through a rectal probe.

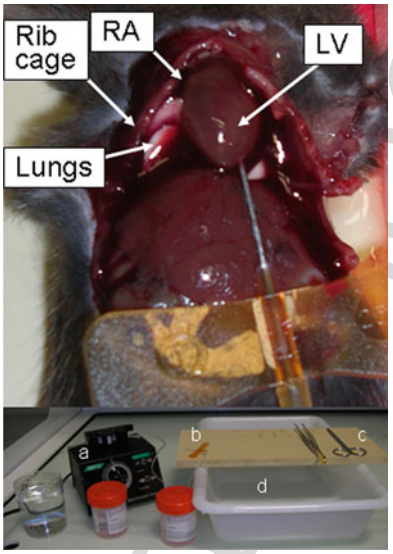

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Fig. 3. Mouse intracardiac perfusion. The *top picture* shows the rib cage cut off and the butterfly needle inserted into the left ventricle (LV). The *bottom picture* shows the material used: formalin beakers and PBS beaker, peristaltic pump (**a**), butterfly needle (**b**), forceps, scissors (**c**), surgical board and draining bucket (**d**). RA, right atrium.

3. Anesthetic devices: isoflurane vaporizer connected to several debitmeters for O_2/N_2O/Air and one cage dedicated to anesthesia induction (Minerve, Esternay, France).
4. Isoflurane gas (Belamont, Paris, France) is used for anesthesia.
5. Air, O_2, N_2O tanks.

2.3. Animal Sacrifice

1. Peristaltic pump for intracardiac perfusion: Mini-peristaltic pump II by Harvard Apparatus (**Fig. 3**). AQ2
2. Surgical supplies such as scissors (available from WPI Europe (http://www.wpi-europe.com), dressing forceps (10 cm long), micro bulldog clamp (3 cm long), butterfly needle (25 gauge).
3. Perfusion fluids: phosphate-buffered saline, and 10% buffered formalin at room temperature.
4. Surgery board and draining bucket.

2.4. MR Imaging Systems for Ex Vivo Imaging

1. Clinical 7-T spectrometer (Syngo MR, VB15, Siemens), equipped with an AC84 head gradient set with 36-cm available bore (80 mT/m strength and slew rate of 333 mT/m/s). Birdcage coils can be used (inner diameter = 24 mm) for signal transmit–receive. Other MR systems can also be used for ex vivo MRI. For example, we performed

previous studies on a 4.7-T spectrometer (Bruker) (13) or on the 7-T spectrometer used for in vivo imaging.

2. 10-mL syringes can be used to make containers that allow keeping the brain still in place and soaked.
3. Fluorinert™ Electronic Liquid FC-4(3 M™, Cergy-Pontoise, France), a fully fluorinated liquid is used to embed the sample before MRI and to remove any background signal. AQ3

2.5. Histological Analysis

2.5.1. General Histology

1. Sliding freezing microtome (e.g., LEICA SM2400).
2. Homemade baskets to rinse tissue. Commercial systems (15-mm Netwell insert with 74-μm mesh size polyester membrane from Corning Life Sciences) are available as an alternative.
3. Slow orbital agitator and routine small equipment for histology lab.
4. Phosphate buffer (PB).
5. Dimethyl sulfoxide.
6. Glycerol.
7. Dry ice.
8. Superfrost+ glass slides.

2.5.2. β-Amyloid Staining with BAM10 Antibody

1. Usual glassware and small equipment for histology lab.
2. Phosphate-buffered saline (PBS).
3. Hydrogen peroxide 30% (Sigma H 0904).
4. Octylphenol ethylene oxide condensate 0.2% (Triton X-100™, Sigma).
5. Normal rabbit serum, can be aliquoted at −20°C (Vector® Labs S 5000).
6. Monoclonal BAM10 clone A3981 (Sigma).
7. Biotinylated IgG anti-mouse, BA-9200 (Vector®).
8. Sodium azide 8%, stored at room temperature (Sigma S 8032).
9. Avidin–biotin complex (ABC VECTASTAIN kit, Elite PK 6100).
10. Tyramin biotin reagent, stored at 4°C (Blast PC 2815-0897)
11. Peroxidase substrate kit (VIP SK 4600 substrate kit for peroxidase, Vector®).

2.5.3. β-Amyloid Staining with Congo Red

Labeling of amyloid deposits is done by standard Congo red staining (adapted from Ref. (15)).

1. Usual glassware and small equipment for histology lab.
2. Solution S1: 80° ethanol saturated with NaCl.
3. Solution S2: Saturated solution of Congo red (Fluka, Ref. 60910) made in saline ethanol.
   S1 and S2 solutions are stable for weeks/months but it is recommended to prepare them immediately before staining.
4. Sodium hydroxide.

2.5.4. Iron Staining with Perls-DAB Method

Staining of iron deposits is performed by means of the Perls' method with diaminobenzidine intensification (16).

1. Usual glassware and small equipment for histology lab.
2. Potassium ferrocyanide (Sigma ref. P 9387).
3. Tris buffer.
4. Diaminobenzidine (DAB) dissolved in distilled water (1 g/1000 ml). DAB solution can be prepared and aliquoted at −20°C before use.
5. Methanol.
6. Hydrogen peroxide 35%.
7. Hydrochloric acid 35%.

2.5.5. Analysis and Quantification of Histological Stainings

1. Slide scanner with high optical resolution (e.g., Super CoolScan 8000 ED scanner, Nikon, Champigny sur Marne, France) (*see* **Note 2**). Use a scanner that has a 4000 dpi in-plane digitization resolution (pixel size 6.35 μm^2) to allow quantification of large objects (e.g., plaques and big focal iron deposits). Work under calibrated and constant illumination conditions.
2. Optical microscope equipped with a digital camera (optional).
3. ImageJ freeware (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2005).
4. Adobe Photoshop® software or any image manipulation program (e.g., The Gimp, http://www.gimp.org/, can be a good freeware alternative).

3. Methods

3.1. Mouse Preparation for In Vivo MRI

1. Place the mouse in the induction cage.
2. Turn the oxygen or air tank on.
3. Turn the flowmeter up to 1–1.5 L/min.

Table 1
Example of acquisition parameters for the fast spin echo sequence used to record T_2-weighted MR images in vivo

Parameter		Value	Unit
Repetition time	TR	2500	ms
Echo time	TE	92.3	ms
Field of view	FOVx FOVy FOVz	15 30 15	mm
Matrix	MATx MATy MATz	128 256 128	
Rare factor		16	

4. Induce anesthesia by turning the isoflurane level to 5% until the animal is in lateral decubitus for 2 min.
5. Maintain anesthesia at a concentration of 1.0–1.5% (*see* **Note 3**).
6. Place the mouse prone in the animal cradle of the MR scanner; insert the teeth into the tooth bar and the ear bars into the ear canal; put the respiration and temperature probes in place; cover the animal with the warming blanket.
7. Insert the head of the animal in the rf coil and slide the animal into the magnet for imaging.

3.2. In Vivo MR Brain Imaging

The parameters for a typical T_2-weighted spin echo sequence are presented in **Table 1**. The resolution with these parameters is about 117 μm isotropic and the acquisition time is about 42 min.

3.3. Mouse Sacrifice and Preparation for Ex Vivo MRI

3.3.1. Mouse Sacrifice and Fixation

The entire perfusion fixation procedure should be performed under a hood, possibly equipped with a sink connected to ad hoc disposal to evacuate perfused fluids.

1. Prepare the pump: pour about 500 mL of PBS in a beaker and 500 mL of formalin in another beaker. Connect a butterfly needle to the outflow end of the peristaltic pump and drop the inflow end of the pump into the PBS beaker. Run the pump until no more air bubbles are visible in the perfusion line.
2. Anesthetize the mouse with an intraperitoneal injection of pentobarbital sodium (at a dose of 100 mg/kg).
3. When the animal is deeply anesthetized and all reflexes are lost (toe- and tail-pinch checks), place the animal in supine

position on a dissecting board placed over a draining bucket. Tape or pushpin the limbs away from the body to hold the animal still. Proceed fairly quickly to begin the perfusion before the heart stops beating.

4. Perform a bilateral thoracotomy: reach the sternum with the tooth forceps and, just under the ribs, make a bilateral incision into the skin and through the pleural cavity. Use scissors to cut the ribs towards the shoulders and remove them to expose the heart cavity. Cut through the diaphragm then through the pericardium to expose the heart. Be careful not to puncture any organ. Make sure you can clearly identify the left and right ventricles (that are colored with slightly contrasted red nuances) and the right atrium.
5. Hold the heart with smooth forceps or thumb–index soft pinch and insert the butterfly needle into the left ventricle from the apex up, without piercing into the right ventricle. When the needle is in place (**Fig. 3**), turn the pump on (flow rate ~2 mL/min), and immediately make an incision into the right atrium with a pair of fine scissors to let the blood flow out. Flush the blood out with PBS until the perfusate runs clear. If the perfusion is properly performed, all organs should turn white and the tail should briefly stiffen up.
6. When all the blood is flushed out, turn the pump off and switch the perfusate line to formalin. Turn the pump back on and perfuse the fixative for about 5 min or until the mouse limb are stiff.
7. Upon completion of the fixation, turn the pump off and remove the needle from the heart.
8. Release the mouse, cut its head off, dispose the carcass in a biological hazard bag, and then proceed to brain dissection.

3.3.2. Brain Dissection

After the mouse has been perfusion fixed and the head has been cut off, quickly proceed to brain dissection to limit tissue degradation.

1. Make a medial incision through the skin of the head from the base of the neck to the nose to expose the skull.
2. Localize the olfactory bulbs through the skull, between the two eyes. Insert the tip of sharp scissors through the skull on the medial line at the tip of the olfactory bulbs. Open the inserted scissors to crack open the skull and to separate it in two halves.
3. With tooth forceps, pull away the two skull halves. Then remove the skull from around the cerebellum and other pieces that might still be covering the brain. Be very careful not to pull apart, squeeze, or slice the brain while removing the skull (*see* **Note 4**).

4. The brain should now be free from the skull. With a small spatula, reach under the brain and gently release it from its cup; the cranial nerves should easily break.
5. Drop the brain in a container with formalin for a 24-h post-fixation at 4°C, and dispose of the rest of the head carcass in a biological hazard bag.

3.3.3. Passive Staining

We adapted and optimized previously published "staining" protocols (13, 17) to Gd-stain the fixed brains.

1. After a post-fixation of at least 24 h, soak the brain sample in a solution of phosphate buffered saline (PBS) and 0.5 M gadoterate meglumine at a dilution of 1:200 (2.5 M) (*see* **Note 5**).
2. Store at 4°C for at least 24 h prior to imaging.

3.3.4. Imaging Holder

Imaging holders can easily be home built with 10-mL syringes (**Fig. 4**).

1. Use two syringe pistons to close each end of the holder made with the syringe. You can use plastic pieces to hold the brain tight in place in the holder but be careful not to squeeze it (*see* **Note 6**).
2. Place the brain in the imaging holder and fill it half way (up to the beginning of the brain) with Fluorinert®.
3. Close the holder and remove all air bubbles. To do so, insert a 26-gauge needle filled with Fluorinert® between the cap and the wall of the holder. Push in some fluid; the air bubbles will exit from the small gap created by the needle. Slowly remove the needle while still pushing in some fluid. All air bubbles should be gone. If not, insert the needle again and repeat Step 3.

3.4. Ex Vivo MR Brain Imaging

A 3D gradient echo sequence can be used (FLASH) to acquire T_2*-weighted images. **Table 2** gives typical parameters to acquire 72 images in about 14 h at a resolution of about 23 × 23 × 90 μm^3.

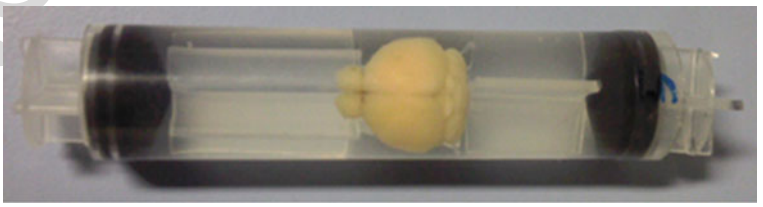

Fig. 4. Mouse brain holder for ex vivo imaging. The brain sample is held still in a 10-mL syringe filled with Fluorinert.

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Table 2
Example of acquisition parameters for the 3D gradient echo sequence used to record T_2*-weighted MR images ex vivo

Parameter		Value	Unit
Repetition time	TR	100	ms
Echo time	TE	21	ms
Field of view	FOVx FOVy	24 20.25	mm
Matrix	MATx MATy	1024 864	
Slice thickness		0.09	mm
Number of slices		72	
Flip angle	FA	25	0

3.5. Histological Studies

3.5.1. General Histology

1. Section whole brains or single hemispheres (frontal 40 μm-thick sections) on a freezing microtome after a 1-week fixation in 10% formalin (*see* **Note 7**) and subsequent cryoprotection in 20% glycerin and 2% DMSO in 0.1 M PB.
2. Collect 12 batches of serial sections (ranging from the frontal pole to the end of the caudal part of the hippocampus). Immediately rinse in 0.1 M PB the series of free floating sections to be stained and mount them on Superfrost+ glass slides before drying them overnight at room temperature (or in an oven at 40°C). Remaining tissue can be stored at −20°C in cryoprotectant as backup material.
3. For each mouse, it is suggested to perform a Nissl (thionin) stain to control for tissue quality and/or cytoarchitectonic anomalies before processing Congo red and Perls-DAB stains.

3.5.2. β-Amyloid Staining with BAM10 Antibody

Follow these steps for free-floating sections (total solution volume of 5 mL) (**Fig. 5**) AQ4

Day 1

1. Rinse the sections six times in PBS for 5 min.
2. Incubate in H_2O_2 (0.3%) for 20 min.
3. Rinse three times in PBS for 10 min.
4. To the (PBS + 0.2% Triton) solution, add 4.5% of normal rabbit serum. Incubate for 30 min.
5. To the (PBS + 0.2% Triton) solution, add 3% of normal rabbit serum and 0.1% of the primary antibody. Incubate for 48 h at room temperature (or 3 days at 4°C).

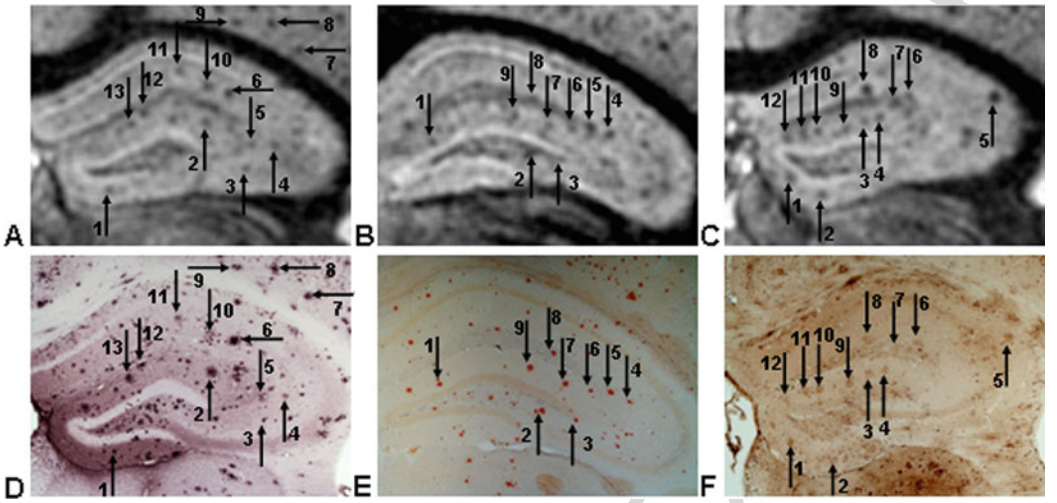

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Fig. 5. Hypointense spots detected at the level of the hippocampus with MRI (*top*, **a**–**c**) match anti-Aβ staining (**d**), Congo red staining (**e**), and Perls' staining (**f**). This indicates that, in the mouse model that we used, these spots correspond to amyloid plaques and that they are loaded with iron.

Day 3

1. Rinse three times in PBS for 10 min.
2. To the (PBS + 0.2% Triton) solution, add 3% of normal rabbit serum and 0.1% of the secondary antibody and incubate for 1 h.
3. Rinse three times in PBS for 10 min.
4. To the (PBS + 0.2% Triton) solution, add kit reagent A (avidin DH) at dilution 1/250 and kit reagent B (biotinylated enzyme) at dilution 1/250. Incubate in this avidin–biotin complex for 1 h.
5. Rinse three times in PBS for 10 min.
6. Revelation with VIP substrate: to 5 mL of PBS, add three drops of each of the kit reagents (reagents 1, 2, 3, and hydrogen peroxide) and mix well. Incubate the free-floating sections in this solution for about 2 min.

3.5.3. β̃-Amyloid Staining with Congo Red

Labeling of amyloid deposits is done by standard Congo red staining (adapted from Ref. (15)) (**Fig. 5**).

1. Prepare S1 and S2; filter S2 before use.
2. Put slides under running tap water for 20 min.
3. Add NaOH [10^{-4}] to S1 and incubate the slides for 30 min.
4. Add NaOH [10^{-4}] to S2 and incubate the slides for 30 min.
5. Rinse under tap water.
6. Dehydrate in alcohols, clear in xylene, and coverslip with Eukitt.

3.5.4. Iron Staining with Perls-DAB Method

Staining of iron deposits is performed by means of the Perls' method with diaminobenzidine intensification (16) (**Fig. 5**).

1. Rehydrate the slides under running tap water for 20 min.
2. Inactivate endogenous peroxidase activity by immersing the tissue in a methanol (20%)/H_2O_2 (3%) solution made in distilled water for 10 min.
3. Rinse in distilled water twice for 5 min.
4. Incubate in acid potassium ferrocyanide solution (for 100 mL of distilled water, add 1 g of potassium ferrocyanide and 1 mL of 35% HCl) for 20 min.
5. Rinse in distilled water for 5 min and then twice in 0.1 M Tris buffer for 5 min.
6. Dilute DAB 2X in 0.2 M Tris; add 30–40 μL of H_20_2 per 100 mL of final volume just before reaction.
7. Incubate the slides in DAB until a good signal-to-noise ratio is obtained (reaction is monitored under the microscope).
8. Rinse in distilled water. Store DAB in waste container or detoxify it.
9. Dehydrate in alcohols, clear in xylene and coverslip with Eukitt.
   (*see* **Notes 8** and **9**).

3.5.5. Analysis and Quantification of Histological Stainings

If the purpose of the study is to register MR images with histologically assessed topography of plaques, no additional processing is required (13).

3.5.5.1. Amyloid Deposits

For quantitative analysis, amyloid loads are evaluated using computer-based thresholding methods. Scans are prepared using Photoshop software to outline selected regions of interest (ROI). Images are then processed with ImageJ freeware using a dedicated macrocommand that extracts amyloid deposits from background tissue (18, 19). Briefly, image processing to detect plaques relies on RGB color component adjustment, global-automated threshold based on entropy criterion, and morphometric filtering according to Feret's diameter. Macro is available on demand.

The step-by-step instructions are as follows:

1. Run ImageJ and open the macrocommand.
2. Select the folder containing the images to be analyzed (tiff format).
3. Automatic processing of all images is then initiated (this can take few minutes in case numerous files have to be processed).
4. After batch processing, a new subdirectory named "processed" is automatically created in the parent directory and contains (1) thresholded images allowing quick visual inspection of the results of plaque segmentation and (2) a

text file that can be imported (tab format) in a spreadsheet program and that contains all morphological data (e.g., total surface, thresholded surface) required to calculate amyloid loads for each image.

Regional amyloid loads are expressed as percent of tissue surface stained by the Congo red dye that corresponds to the proportion of plaque volume according to Delesse's principle (20). Evaluation of amyloid loads can be performed in multiple ROIs. Quantitative analyses are usually performed on several serial sections to sample the whole rostro-caudal extent of each ROI.

3.5.5.2. Iron Deposits

If the purpose of the study is to register MR images with histologically assessed topography of iron deposits, no additional processing is required (14).

Considering now more quantitative aspects (e.g., measuring iron loads), it is known that there is a relationship between "true" tissue iron content (as assessed for instance by atomic absorption spectroscopy) and intensity of Perls' staining (21, 22). We and others perform an analysis of Perls-stained brain tissue by means of optical densitometry, OD (11, 23, 24).

OD determines levels of iron deposition on the basis of transmitted light in the stained tissue. OD can be automatically calculated in selected ROIs using ImageJ or Photoshop. The optical density of each pixel is derived from its gray level and the mean iron load is defined as the mean OD from all pixels of the ROI. Standards to assess absolute iron quantities are not easily available for the analysis of Perls-stained material; therefore only relative quantities of iron deposition can be calculated, still allowing inter-group comparisons.

The step-by-step instructions are as follows:

1. Run image J and set measurements to include the mean gray level variable as output.
2. Open image to be analyzed.
3. Outline the region of interest using the polygon tool.
4. Run the measure command (shortcut: ctrl+M).
5. Uncalibrated optical density of Perls staining is calculated using the following formula: $OD = \log_{10}(255 - M_{GL})$ with M_{GL} as mean gray level of the region of interest.

4. Notes

1. The APP/PS1dE9 double transgenic mice express a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1

(PS1-dE9) both directed to CNS neurons. Both mutations are associated with early onset of AD. The "humanized" Mo/HuAPP695swe transgene allows the mice to secrete a human Aβ peptide. The included Swedish mutations (K595N/M596L) elevate the amount of Aβ produced from the transgene by favoring processing through the β-secretase pathway. The PS1 mutation elevates the amount of Aβ produced from the transgene by favoring processing through the γ-secretase pathway. We used the strain referenced as "Stock Number: 004462." This strain is maintained as a hemizygote line by crossing transgenic mice to B6C3F1/J mice. The strain referenced as "Stock Number: 005864" can also be used. These mice are based on the 004462 strain but were backcrossed to C57BL/6 J for at least eight generations.

2. If the resolution of the scanner appears to be insufficient (e.g., tiny deposits are not detected or with very blurred edges), it is recommended to digitize material at higher resolution using a microscope coupled to a camera. For optimal results, images should be stored as tiff files (jpeg compression has detrimental effects when images are further processed).
3. If O_2 is used as a pushing gas for isoflurane, the percentage of isoflurane will have to be increased to achieve the same level of anesthesia.
4. To avoid pressing against the brain and damaging the tissue during dissection, you can fold the skin around the inferior (ventral) part of the head and hold it between your fingers; this way you should not touch the brain itself at all.
5. By soaking the brain sample in a PBS solution (and 0.5 M gadoterate meglumine) for at least 24 h prior to MR imaging, you will regain some signal due to tissue rehydration.
6. The length of the holder should be at least four times as long as the brain so that the brain sits away from the ends to avoid imaging artifacts that might arise from the pistons. It is also recommended to wipe off the syringe graduation marks with ethanol as they might also cause imaging artifacts.
7. As a general rule, if the animal is fixed via a perimortem intracardiac perfusion, no further tissue fixation is necessary. For fresh brains or brains perfused with saline/PBS only, a 1-week fixation should be done in 10% formalin. However, in the ex vivo protocol for MR imaging, the brains are soaked for days in the staining solution containing PBS instead of formalin. It is therefore recommended to soak the brains again in formalin before sectioning them for histology evaluation.

8. Perls-DAB staining can alternatively be performed on free-floating sections to maximize penetration of reagents.
9. Before dehydrating tissue, a nuclear counterstain can be applied using thionin, nuclear red, or Harris hematoxylin. Do not counterstain when quantification of iron deposition has to be performed by optical densitometry.

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Chapter 16

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