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TITLE:

Positron emission tomography imaging for *in vivo* measuring of myelin content in the lysolecithin rat model of multiple sclerosis

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Keywords:^{11}C-PIB; PET imaging; myelin; multiple sclerosis; molecular imaging; lysolecithin rat model; VOI analysis

Summary:

This protocol has the aim of monitoring *in vivo* myelin changes (demyelination and remyelination) by positron emission tomography (PET) imaging in an animal model of multiple sclerosis.

Abstract

Multiple sclerosis (MS) is a neuroinflammatory disease with expanding axonal and neuronal degeneration and demyelination in the central nervous system, leading to motor dysfunctions, psychical disability, and cognitive impairment during MS progression. Positron emission tomography (PET) is an imaging technique able to quantify *in vivo* cellular and molecular alterations.

Radiotracers with affinity to intact myelin can be used for *in vivo* imaging of myelin content changes over time. It is possible to detect either an increase or decrease in myelin content, what means this imaging technique can detect demyelination and remyelination processes of the central nervous system. In this protocol we demonstrate how to use PET imaging to detect myelin changes in the lysolecithin rat model, which is a model of focal demyelination lesion (induced by stereotactic injection) (i.e., a model of multiple sclerosis disease). ^{11}C-PIB PET imaging was performed at baseline, and 1 week and 4 weeks after stereotaxic injection of lysolecithin 1% in the right striatum (4 µL) and corpus callosum (3 µL) of the rat brain,

allowing quantification of focal demyelination (injection site after 1 week) and the remyelination process (injection site at 4 weeks).

Myelin PET imaging is an interesting tool for monitoring *in vivo* changes in myelin content which could be useful for monitoring demyelinating disease progression and therapeutic response.

Introduction

Multiple sclerosis (MS) is a neuroinflammatory disease that affects the central nervous system, characterized by inflammation, demyelination, and axonal loss[1]. The prognosis of this disease is variable even with advances in treatment, and it is one of the most common causes of neurological deficits in young people[1]. The diagnosis of MS is based on the criteria of clinical manifestation and visualization of characteristic lesions by magnetic resonance imaging (MRI)[2,3].

Positron emission tomography (PET) can be a useful tool for *in vivo* monitoring of MS progression and therapeutic effects. The Pittsburgh compound B radiotracer (PIB) labeled with carbon-11 (^{11}C-PIB) is widely used to quantify β-amyloid plaques; however, in the last decade, it has been studied to quantify myelin content and show dynamic demyelination and remyelination[4-6].

Different amyloid PET tracers (^{11}C-PIB, ^{18}F-florbetaben,^{18}F-florbetapir, ^{18}F-flutemetamol) can be used to quantify myelin and provide important information about disease progression and therapeutic response, allowing identification of demyelination and remyelination processes, without the interference of neuroinflammation, which can occur with conventional magnetic resonance images (MRI)[7]. Amyloid PET imaging showed decreased tracer uptake in active MS patients compared to non-active patients which could be explained by early white matter damage in the active patients[8]. Lower amyloid tracer uptake was also associated with cognitive decline in a follow-up study, showing this technique to be a valuable tool for studying the pathophysiology of the disease and clinical outcomes[9].

The lysolecithin (LPC) rat model is a chemical induced model of multiple sclerosis, where the injected toxin, LPC, induces a high response of macrophages that results in increased inflammation and, consequently, demyelination[10,11]. The demyelination is rapidly reversed, in approximately 4 weeks, which makes this a good model for evaluating demyelination and remyelination processes in rodents. This model has already been evaluated using PET imaging, with good results and correlation with post-mortem essays[12].

Here we present the protocol for myelin PET imaging with ^{11}C-PIB in the lysolecithin rat model, showing this imaging technique to be a useful tool for *in vivo* measurement of myelin content.

Protocol

All procedures were conducted in accordance with the guidelines of the National Council for the control of Animal Experimentation (CONCEA, Brazil) and were approved by the Ethics

Committee for Animal Research of the Medical School of the University of Sao Paulo (CEUA-FMUSP, Brazil - protocol number: 25/15).

NOTE: In this protocol, we show how to induce a lysolecithin rat model of multiple sclerosis and how to acquire and analyze the myelin PET images.

1. Lysolecithin solution preparation

1.1. Weigh lysolecithin (L-α-Lysophosphatidylcholine from egg yolk) on an analytical scale using a conic plastic tube (1.5 mL).

1.2. Add sterile saline to the tube to make 1% solution (for example: weigh 1 mg of lysolecithin and dissolve it with 100 µL of saline) and dissolve the lysolecithin by shaking the tube (shaking from side to side and not turning from top to bottom).

1.3. Prepare the solution just before starting the animal model induction (do not stock the final ready solution).

2. Lysolecithin rat model – Stereotaxic surgery

2.1. Use male Wistar rats weighing between 220 – 270 g. Use gloves and mask during all procedures.

2.2. Induce anesthesia with 5% isoflurane mixed in 100% O_2 (1 L/min) using an induction box. Check if the animal is anesthetized by observing absence of movement (only breathing should be observed).

2.3. Place the animal in stereotaxic apparatus on a heating pad. Fix the nose and ears of the animal to the equipment.

NOTE: Details of how to perform stereotaxic surgery can be found in JoVE Science Education Database. *Neuroscience.* Rodent Stereotaxic Surgery. JoVE, Cambridge, MA, 2021.

2.3.1. Monitor the anesthesia for the whole procedure (including surgery and image acquisition). To adjust the isoflurane concentration, observe the animal breathing rate. A fast breathing rate requires higher concentration and a slow breathing rate requires lower anesthetic concentration.

2.4. Inject the analgesic (ketoprofen – 5 mg/kg) subcutaneously (dilute the medication to 1 mL in saline, this will help to hydrate the animal).

2.5. Put eye cream in the animal's eyes to protect against dehydration.

2.6. Use a 0.5% chlorhexidine solution to clean the incision area.

2.7. Inject 100 µL of lidocaine hydrochloride 2% subcutaneously in the region of incision.

2.8. Shave the skull area.

2.9. Use sterile instruments for this procedure.

2.10. Using a scalpel, make an incision of about 2 cm in the skin over the skull.

2.11. Expose the skull with Bulldog clamps.

2.12. Use a swab to clean the skull area with 1% hydrogen peroxidase.

2.13. Locate and mark the bregma (a stereotaxic rat brain atlas can help to identity the Bregma).

2.14. Position the Hamilton syringe (µL neuros syringes) at the bregma.

2.15. Using the bregma and a stereotaxic atlas as reference, position the Hamilton syringe at the following coordinates: Antero-posterior: -0.30 mm, latero-lateral: -3.0 mm, and ventral until it touches skull bone, and mark the skull and note the coordinates orientation on paper.

2.16. Drill the skull at the marked coordinate. Take care with dura mater (damaging dura mater can cause a lot of bleeding).

2.17. Fill the Hamilton syringe with 7 µL of lysolecithin solution (1% in saline). A greater volume can be placed in the syringe, but only 7 µL will be injected (take the bubbles out of the syringe to measure the volume correctly).

2.18. Position the Hamilton syringe at the previous coordinates to start the drug injection (total of 7 µL into 3 different ventral stereotactic coordinates). Considering the previously noted coordinates, lower the Hamilton syringe to ventral coordinate -5.0 mm.

2.19. Very slowly inject 2 µL of lysolecithin solution (1 µL/10 min). Wait 3 min.

2.20. Take the needle up to the next ventral stereotaxic coordinate (-4.2 mm) and slowly inject 2µL of lysolecithin solution (1 µL/ 10 min). Wait 3 min.

2.21. Inject the third ventral coordinate -3.0 mm (3 µL of lysolecithin solution - 1 µL/ 10 min).

2.22. Wait 5 min and then remove the Hamilton syringe from the brain.

2.23. Suture the skin.

2.24. Remove the animal from the stereotactic apparatus and allow the animal to awaken.

2.25. Return the animal to the home cage, keep the animal alone and under supervision to check for signs of discomfort.

2.26. Inject subcutaneous analgesic (ketoprofen – 5 mg/kg) diluted in saline, at 24 h and 48 h after the surgery.

3. PET acquisition

3.1. Take 7 to 20 MBq of ^{11}C-PIB radioactivity in a syringe (1 mL is the maximum volume allowed for intravenous injection in rats).

3.2. Anesthetize the animal with 5% isoflurane mixed in 100% O_2 (1 L/min) using the induction box.

3.3. Inject ^{11}C-PIB (radioactivity defined in item 4.1 above) into the penile vein, or tail vein of the rat (both administration sites are fine, the decision is a personal choice. We only advice not to use a retro-orbital injection since the location is too close to the region of interest (the brain) and can impair image quality).

NOTE: Image acquisition of baseline time point is performed before stereotaxic injection and the other 2 time points at 1 week and 4 weeks after surgery.

3.4. Remove the animal from anesthesia to allow the animal to awaken (leave the animal on a warm pad until it is completely awake).

3.5. Return the animal to the home cage.

3.6. Wait 30 min for the next step.

3.7. Anesthetize the rats with 5% isoflurane mixed in 100% O_2 (1 L/min) using an induction box.

3.8. Open PET scanner software.

3.9. Select **Scan > Pet Ready**.

3.10. Complete principal investigator details, Study ID, Series ID, Animal ID, Animal weight (g), and Additional notes. Select **Next**.

NOTE: Use dot (.) for decimal number in the animal weight.

3.11. Edit ROI area for scanning (usually between 3 and 8). This is the area that will be included in the image (areas between the numbers 3 and 8 of the bed cover will appear in the final image).

3.12. Position the anaesthetized rat on a standard rat bed of the PET scanner and turn the anesthesia on (3% isoflurane in 100% O_2 is a good start, and then the anesthesia rate should be adjusted as necessary). To adjust the anesthesia, check the monitoring data of the scanner software to verify that the animal is breathing in a constant and slow rhythm.

NOTE: Turn on the vacuum pump coupled to the activated carbon filter to collect isoflurane gas excess (if the pump is available).

3.13. Apply eye cream to protect the animal's eyes.

3.14. Push the animal bed inside the PET scanner.

3.15. Complete Activity details, Activity Calibration Time, Isotope (C-11), and Scan duration (20 minutes). Select **Start Scan.**

NOTE: A **Pet Moving Bed** message will appear, and the animal bed will move into the equipment and stop at the previously selected ROI position. Time of acquisition will start count down and number of counts per seconds will appear (CPS).

3.16. In the scanner software, navigate to the **Monitor** tab on the left of the screen, check **Change Threshold** to Respiratory parameters change (BPM).

NOTE: A window will pop up, complete with threshold parameter (500). Select **OK**.

3.17. Navigate to **0% POWER** to change temperature parameters to heat the animal during scan. A window will pop up; complete the parameter (80 - 100). Select **OK**.

NOTE: Choose between 80 and 100% power based on room temperature conditions (the more power, the warmer it will get).

3.18. Navigate back to **SCAN** tab.

3.19. Navigate to configuration tool (gear icon) and complete **Injection Time, Remaining Activity, Remaining Activity Calibration Time**. Select **Save.**

NOTE: A window will pop up "Are you sure you want to modify the protocol metadata?"> YES

3.20. Navigate back to the **Monitor** tab (check the animal's respiratory parameters, and if necessary, increase or decrease isoflurane concentration – usually 3% in 100% O_2 is enough).

3.21. When PET acquisition is finished, **Pet Ready** message will appear on the **Scan** tab, check **Arrow Out Icon** (left of configuration tool) to move out the animal bed.

3.22. Remove the animal from anesthesia and allow to awaken on a warm pad.

3.23. For reconstructing the image**,** navigate to **Reconstruct** tab.

3.24. Check plus icon at bottom left of the screen, and select the study file which will be reconstructed. Select **Next.**

3.25. Navigate to **Energy Resolution** tool. A window will pop up. Configure the energy peak (keV) to the equipment parameter (based on monthly quality control). Select **Close.**

NOTE: Energy peak can change every month when performing monthly equipment calibration.

3.26. Keep all other parameters as the default (Isometric voxel size: 400 mm; number of iterations: 30; Energy resolution: 30%; Save last iteration only; uncheck Keep binary data)**.**

3.27. Check **Add**.

NOTE: A window will pop up "The reconstruction was added to the queue and will start once the others have finished". Select **OK**. A file will appear in the reconstruction list on the Reconstruct tab and the status will appear as waiting or % (progress) or finished.

4. Image Analysis

NOTE: Perform image analysis using dedicated image analysis software. In the current protocol the demonstration uses a specific software program, but if it is not available, other options can be used.

4.1. Open **PMOD > Fuse it**.

4.2. Navigate to the Matching tab at the top of the screen.

4.3. Open the **Load Input** menu in the right middle of the screen and select Autodetect files, select PET file (DICOM, Interfile or NifTI), add to selected, click with Operations, Reorient to Standard Orientation, click **Load** and Close.

4.4. Verify if the animal **Species** is correct at the bottom of the screen (**RAT**).

4.5. Check **Crop** box at the bottom right of the screen.

4.6. Adjust the yellow box size in the PET image to take the whole brain.

4.7. Click the **Rigid** button on the right of the screen. At confirmation window click **Yes**.

4.8. Click on the brain tool at the right middle of the screen to open Reference Atlas. Select Px Rat (W.Schiffer) – T2.

4.9. Select **Match Results** from the top right tab (Select processing stage tab).

4.10. Click **Data reslicing** (4^{th}tab at top right menu).

4.11. Align the PET image with reference template using the rotate tool (white icon at center of PET image). Check alignment for 3 anatomical planes.

4.12. Save the co-registration file (right side menu of the screen).

4.13. Select output format (DICOMor NifTI), directory and file prefix. Click **Save**.

NOTE: If co-registered output is saved as NifTi the header image information will be lost.

4.14. Click **VOIs** menu at the bottom of the screen.

4.15. Navigate to **Template > Atlas** at the bottom of the screen (below VOIs window).

4.16. Select Px Rat(W.Schiffer) from the drop-down menu.

4.17. NOTE: If you need to analyze only specific VOIs you can select only the brain areas of interest. If you want all brain areas of the brain atlas, no action is needed.

4.18. Click **Outline** at the bottom of the screen.

NOTE: VOIs of brain areas will appear in VOIs window.

4.19. Manually draw the VOI in the lesion site and contralateral brain hemisphere area (^{11}C-PIB low uptake area and contralateral brain hemisphere, respectively).

4.20. Click in the area of PET image with ^{11}C-PIB low uptake.

4.21. Select **New VOI > OK**.

NOTE: A spreadsheet will appear

4.22. Navigate to **SPHERE** icon in the middle part of the screen (to the left of the VOIs window)

NOTE: A spreadsheet will appear.

4.23. Choose the **VOI name: lesion** and select **Apply**.

NOTE: A sphere will appear in the co-registered image.

4.24. Align the sphere in the lesion area and adjust the VOI for all anatomical planes.

4.25. Click **Close** (at the bottom part of the spreadsheet).

4.26. Select the lesion VOI (from VOIs list).

4.27. Navigate to **VOI mirroring operations** icon (at the top right of the VOIs window) and select **Clone and mirror left/right.**

4.28. Navigate to **Calculate Selected VOI Statistics** at the top of the VOIs list window. Navigate to **Select statistics to be calculated** icon to choose the output data (to the right side of the VOI statistics icon). Usually for myelin content check Statistics for group of VOIs, Average, SD, Min, Max).

NOTE: A spreadsheet will appear. The default setting Data Unit is kBq/cc. On the left top of the spreadsheet (VOI Statistics) you can check the Data Unit as SUV (Standardized Uptake Value). If you completed the radiotracer administration and image acquisition details correctly in the scanner software, as described previously, the SUV data will be calculated automatically by PMOD software, if not, you can edit the details.

4.29. Navigate to **Copy using system locale number format.**

NOTE: Verify if all statistics are selected to copy as output (Check icon at the right top of the screen). The data that will be copied depends on the Data Unit selected.

4.30. Paste the output data in a notepad or spreadsheet.

NOTE: Take care with software settings for dot and comma symbols between numbers. This can be different between language configurations.

4.31. Save the file with study name and details.

Representative Results

Figure 1 shows illustrative ^{11}C-PIB PET images with myelin changes over time. In the baseline scan, no differences can be seen in myelin content (i.e., no demyelination is present). In the 1-week time-point image, it is possible to see the focal demyelinated lesion (in the right hemisphere) as indicated by the white arrow. Images are presented in the 3 anatomical planes (coronal, axial, and sagittal) and it is possible to identify the demyelinated lesion in all of them. The 1-week image is the illustration of a well delimited lesion at the injection site, representing the correct model induction and image detection. In the 4 weeks image, no lesion is visible anymore, indicating that remyelination has occurred and myelin content is back to normal (or close to it).

The representative graphs show the quantification of the images of 4 animals in the 3 different time points. The first graph shows the results from the quantification of the lesion (manual VOI) to contralateral side ratio demonstrating more focal myelin changes, where the lysolecithin injection was performed. The second graph shows the same quantification, but in the striatum (injected striatum to contralateral ratio) and in this case the difference is not statistically significant, which can be explained by the small sample size and because the VOI is bigger and the radioactivity concentration is measured not only where the lysolecithin was injected.

Differences between groups were analyzed by the Kruskal Wallis test, followed by Dunn´s test for multiple comparisons and the results are presented as mean ± SD. In the lesion VOI (H = 7.063; P=0.017), in the 1-week image, the tracer uptake ratio (0.90±0.07) was 16% lower than baseline (1.07±0.06), with statistical significance (p=0.024). No significant differences were found in the 4-week image (1.01±0.06).

In the striatum, no statistical differences were found (H =1.412; P=0.5393). The uptake ratios for the images were 1.07±0.07 for baseline, 1.02±0.07 for 1 week, and 1.01±0.08 for 4 weeks.

The third graph (bottom line, left graph) presents the quantification of the contralateral striatum (non-injected side). In this graph it is possible to observe that there was no difference (P=9397) among time points, meaning that the variation in the injected side is due to myelin changes and not due to tracer uptake variation over time.

The final graph, in the bottom right, shows the quantification of the injected site (lesion VOI) in animals where the model was not well induced (probably due to fast lysolecithin injection, wrong stereotaxic manipulation, and/or incorrect solution preparation). In this case, the lower uptake is not seen in the 1 week time point, meaning no demyelination process has occurred, and the low uptake at 4 weeks can be related to a later demyelination process or tissue damage, both situations are related to bad animal model induction. This graph was added to the protocol to exemplify the appearance of the results when the animal induction is not well performed and to emphasize the importance of each step of the protocol, from the beginning to the end. There are no differences in the tracer uptake (H = 2.745, P = 0.267) with uptake ratios of 1.06±0.05, 1.02±0.14, and 0.96±0.10 for baseline, 1 week, and 4 week PET images.

Figure 2 adds more information to the results, where **Figure 2A** details where the manual VOI was drawn, based on the MRI template reference and **Figure 2B** shows the luxol fast blue staining (for details about the luxol fast blue staining protocol, see De Paula Faria et al.[13]) from the injected side and non-injected side at 7 days post stereotaxic injection.

Figure legend

Figure 1: Illustrative ^{11}C-PIB PET images showing images of baseline, 1 week, and 4 weeks after stereotactic injection. The graphs at the bottom of the figure represent the quantification of tracer uptake (n=4) at different time points. The first two graphs represent the uptake ratio in the injected side to contralateral side in the lesion and in the striatum in a well induced model (i.e., rats presenting lesion after lysolecithin injection). The third graph (bottom left) shows the quantification of non-injected striatum (negative control), and the final graph (bottom right) represents the ^{11}C-PIB uptake at the injection site of animals that did not present demyelinated lesion (badly induced model). Results are presented as mean±SD.

Figure 2: Lesion location details. A) Illustrative VOIs of injected side (dashed line) and non-injected side (white line) drawn manually based on the MRI template (region of corpus callosum and striatum). **B**) Luxol fast blue staining showing demyelination in the injected

hemisphere compared to the non-injected side (Top: 40x magnification, bottom: 100x magnification).

Discussion

The biggest advantage of using the lysolecithin model to study multiple sclerosis is the fast timeline for demyelination (about 1 week) and remyelination (about 4 weeks) to occur[14]. This model can also be induced in mice[15], however, induction in rats is more advantageous for *in vivo* PET imaging due to the larger size of the rat brain compared to mice.

The first step of the induction model is to be extremely cautious. This model was validated for myelin PET imaging by de Paula Faria et al.[10] in 2014 and it was shown that the speed of the lysolecithin injection inside the brain is crucial for a well induced model. The injection must be performed very slowly, 1 µL each 10 min, as a way of avoiding tissue damage. The lysolecithin solution should also be prepared on the same day as the stereotactic injection, preferably just before starting the surgery procedure. If the model will be used for the first time in a research group, we recommend that the model should be validated before performing any myelin quantification by PET imaging. The validation needs to include post-mortem tissue analysis (myelin staining, for example: Luxol fast blue histology (as shown in **Figure 2**) and myelin basic protein (MPB) immunohistochemistry) in the different time points intended to be used in *in vivo* analysis. In the results section we showed a quantification of radiotracer uptake where lesion induction was not well succeeded and, therefore, the differences were not detected by ^{11}C-PIB PET imaging.

The lesion to be quantified by this technique must be bigger than the PET scanner resolution (about 1 mm in preclinical equipment and about 5 mm in clinical equipment).

Once the model is well induced, the imaging procedure must be well planned, due to the radiotracer labeled with carbon-11, which has a short half-life of 20 minutes. The preclinical imaging laboratory personnel need to prepare all necessary material, fill the anesthesia system, check if everything is working properly, and print the forms to be completed during the experiment. The PET scanner should also be verified prior to the experiment, when all quality controls necessary in the equipment (dependent on each country) must be performed to check the scanner is well functioning. After receiving the tracer for injection, the measurement of activity must also be measured in a calibrated dose calibrator to guarantee the correct injected dose, and the information (activity in the syringe, before and after injection) written on the form, as well as the respective time when the measurement was performed. Establish which watch is going to be used, as the right time is the time on the workstation of the PET scanner, the time that will be considered in the decay correction of the images, therefore, any watches used during the experiment should be synchronized to the scanner workstation time.

During animal image acquisition, temperature and animal breathing should be monitored and the anesthesia adjusted, as necessary. Temperature is location dependent and should be adjusted for the animal well-being. After the image acquisition is finished, it is important to keep the animal on a warm pad to recover before being returned to the cage.

Image processing is crucial for getting reliable results from the experiments using PET imaging. The ideal is that the analyzer is not aware of the animal groups and/or treatment and that he/she already has experience in PET images with the PET tracer used in such a way as to guarantee perfect registration between the PET imaging and MRI template. We used the PMOD software in this protocol, but if this software is not available, alternative image quantification software can be used, although attention must be given to achieving good brain region definition and quantification. For the definition of the lesion location, extra care must be taken to ensure that the injected site is inside the drawn lesion VOI (a knowledge of rat brain anatomy is necessary).

It is important to say that myelin PET imaging can also be performed in other MS animal models, displaying unpredictable lesions, as already shown by our group in the Experimental Autoimmune Encephalomyelitis (EAE) marmoset model[5]. As already stated, the important parameter to consider in lesion quantification is the PET scanner resolution, which is the limitation for detection of lesions that are too small. PET imaging is a poor resolution imaging technique when compared to other techniques such as MRI, however it is a highly specific modality and, because of this, quantification of the PET images uses an anatomical template, such as the MRI, for helping to draw the region of interest (as shown in the above protocol).

Although the manual drawing of VOIs is operator dependent, it is the best option for the LPC animal model, since the lesion can be variable between animals. To diminish bias in the quantification process, it is important to perform a mirror VOI (as explained in the protocol), which will be in the same region and of the same size as the injected side. It is also important to have the stereotaxic coordinates in mind when drawing the VOI in the MRI template to guarantee that the correct brain region is considered. Using the myelin staining as a guide to identify the demyelinated area can also help in the drawing, as explained in de Paula Faria[12].

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Disclosures

None.

References

1 Oh, J., Vidal-Jordana, A., Montalban, X. Multiple sclerosis: clinical aspects. *Current Opinion in Neurology.* **31** (6), 752-759, doi:10.1097/wco.0000000000000622 (2018).

2 Sand, I. K. Classification, diagnosis, and differential diagnosis of multiple sclerosis. *Current Opinion in Neurology.* **28** (3), 193-205, doi:10.1097/wco.0000000000000206 (2015).

3 Thompson, A. J. et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurology.* **17** (2), 162-173, doi:10.1016/s1474-4422(17)30470-2 (2018).

4 Veronese, M. et al. Quantification of C-11 PIB PET for imaging myelin in the human brain: a test-retest reproducibility study in high-resolution research tomography. *Journal of*

Cerebral Blood Flow and Metabolism. **35** (11), 1771-1782, doi:10.1038/jcbfm.2015.120 (2015).

5 Carvalho, R. H. F. et al. C-11 PIB PET imaging can detect white and grey matter demyelination in a non-human primate model of progressive multiple sclerosis. *Multiple Sclerosis and Related Disorders.* **35**, 108-115, doi:10.1016/j.msard.2019.07.020 (2019).

6 Stankoff, B. et al. Imaging central nervous system myelin by positron emission tomography in multiple sclerosis using [methyl-(1)(1)C]-2-(4'-methylaminophenyl)- 6-hydroxybenzothiazole. *Annals of Neurology.* **69** (4), 673-680, doi:10.1002/ana.22320 (2011).

7 Faria, D. D. Myelin positron emission tomography (PET) imaging in multiple sclerosis. *Neural Regeneration Research.* **15** (10), 1842-1843, doi:10.4103/1673-5374.280311 (2020).

8 Pietroboni, A. M. et al. Amyloid PET as a marker of normal-appearing white matter early damage in multiple sclerosis: correlation with CSF -amyloid levels and brain volumes. *European Journal of Nuclear Medicine and Molecular Imaging.* **46** (2), 280-287, doi:10.1007/s00259-018-4182-1 (2019).

9 Pytel, V. et al. Amyloid PET findings in multiple sclerosis are associated with cognitive decline at 18 months. *Multiple Sclerosis and Related Disorders.* **39**, doi:10.1016/j.msard.2020.101926 (2020).

10 Faria, D. d. P. et al. PET imaging of glucose metabolism, neuroinflammation and demyelination in the lysolecithin rat model for multiple sclerosis. *Multiple Sclerosis Journal.* **20** (11), 1443-1452, doi:10.1177/1352458514526941 (2014).

11 Rinaldi, M. et al. Galectin-1 circumvents lysolecithin-induced demyelination through the modulation of microglial polarization/phagocytosis and oligodendroglial differentiation. *Neurobiology of Disease.* **96**, 127-143, doi:10.1016/j.nbd.2016.09.003 (2016).

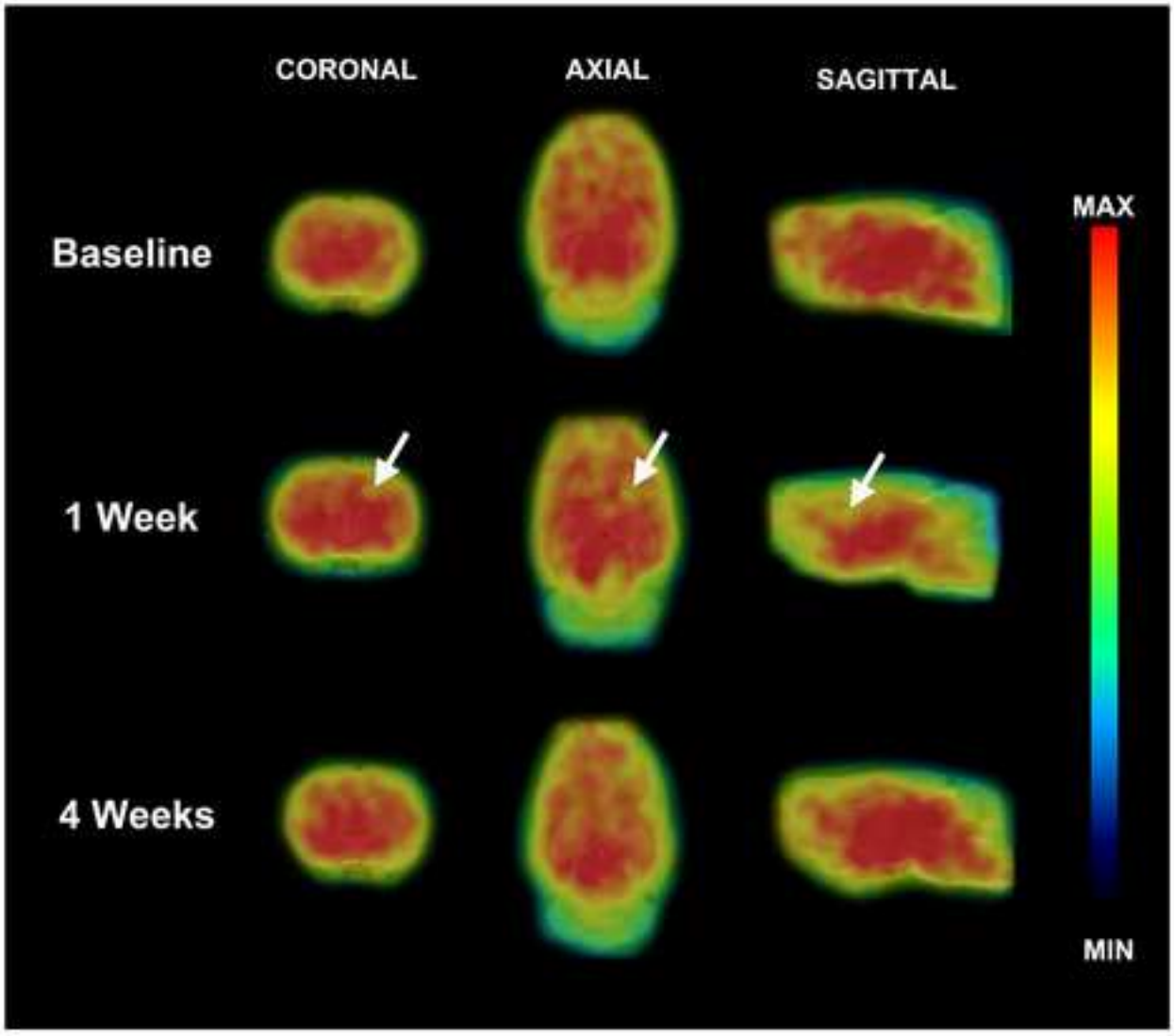
12 Faria, D. d. P. et al. PET imaging of focal demyelination and remyelination in a rat model of multiple sclerosis: comparison of [C-11]MeDAS, [C-11]CIC and [C-11]PIB. *European Journal of Nuclear Medicine and Molecular Imaging.* **41** (5), 995-1003, doi:10.1007/s00259-013-2682-6 (2014).

13 Faria Dde, P. et al. PET imaging of focal demyelination and remyelination in a rat model of multiple sclerosis: comparison of [11C]MeDAS, [11C]CIC and [11C]PIB. *European Journal of Nuclear Medicine and Molecular Imaging.* **41** (5), 995-1003, doi:10.1007/s00259-013-2682-6 (2014).

14 van der Star, B. J. et al. In Vitro and In Vivo Models of Multiple Sclerosis. *CNS & Neurological Disorders-Drug Targets.* **11** (5), 570-588, doi:10.2174/187152712801661284 (2012).

15 Najm, F. J. et al. Drug-based modulation of endogenous stem cells promotes functional remyelination in vivo. *Nature.* **522** (7555), 216-+, doi:10.1038/nature14335 (2015).

Figure 1



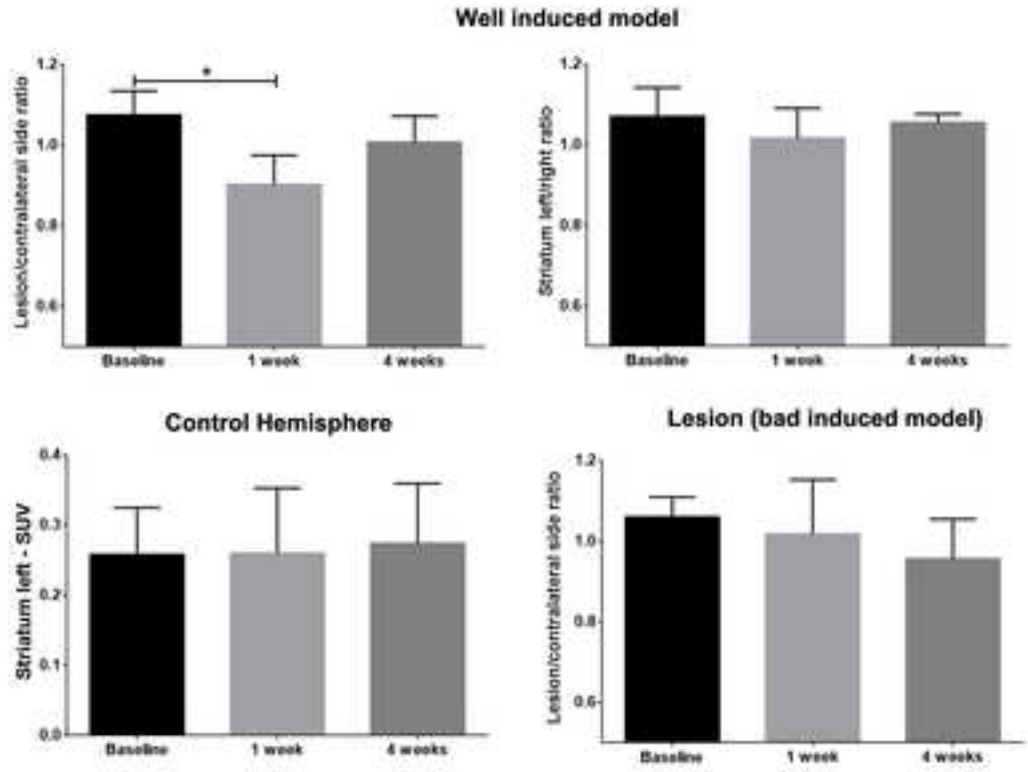


Figure 2

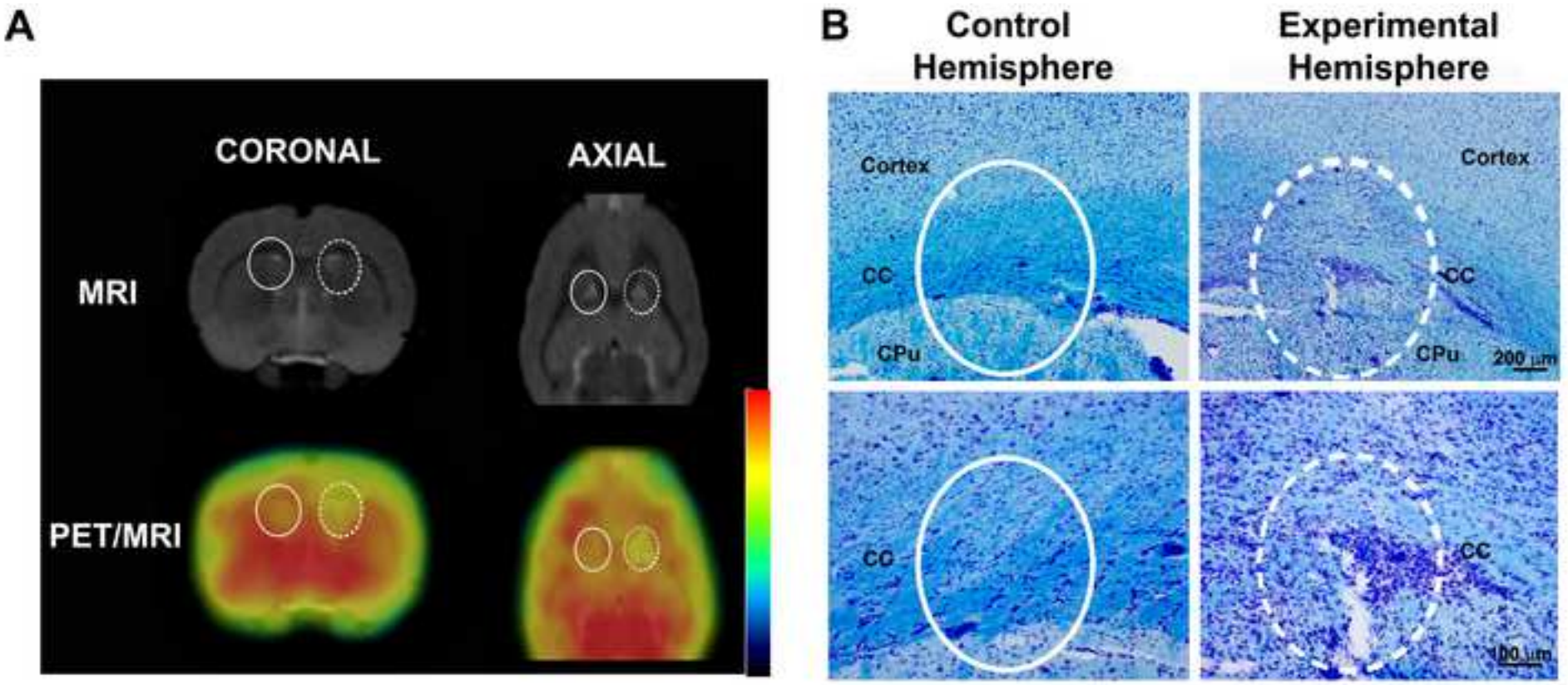


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_filled.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Analytical Balance	Marte	AUWZZOD	max: 220 g- min: 1 mg
Anestesia vaporizer	Nanitech	15800	
Beta-cube	Molecubes		
Bulldog clamp	Stoelting	5212043P	
clorexidine	Rioquimica		0.5%/100 mL
Cotton swabs	johnson e johnson		
Dose calibrator	Capintech		
Drill	Kinzo powertools	352901	Model Q0M-DC3C
Eppendorf tube	Eppendorf	30125150	1.5 mL
Eye lubricant	ADVFARMA	30049099	vaseline 15 g (pharmaceutical purity)
Fine forceps	Stoelting	52102-38P	
Gloves	Descarpack	212101	6.5 size
Heating pad	Softhear		
Injection Syringe	Hamilton	80314	10µ, 32ga, model 701
Insuline syringe	BD	328328	1 mL insulin syringes with needle
Isoflurane	Cristália	410525	100 mL , concentration 1 mL/1 mL
Ketoprofen or other analgesic	Sanofi		100 mg/2 mL
lidocaine	Hipolabor	1.1343.0102.001-5	2%/20mL
L-α-Lysophosphatidylcholine from egg yolk	Sigma-aldrich	L-4129	25 mg - ≥99%, Type I, powder
Needle holder	Stoelting	5212290P	
Oxygen	White Martins	7782-44-7	Compressed gas
PMOD software	PMOD technologies	Version 4.1	module fuse it
Rat anesthesia mask	KOPF	Model 906	
Saline	Farmace	0543325/ 14-8	0.9% sodium chloride for injection, 10 mL
Scapel blades	Stoelting	52173-10	
Scapel handles	Stoelting	52171P	
Scissor	Stoelting	52136-50P	
Semi-analytical Balance	Quimis	BK-3000	max:3,100 g; min:0.2 g
shaver	Mega profissional		AT200 model
Stereotactic Apparatus	KOPF	Nodel 900	
Universal holder with needle support	KOPF	Model 1772-F1	Hamilton support for 5 and 10 µL

Reviewer´s reply

Thank you for your comments and considerations!
Below we addressed each point (reply in red) and the changes in the manuscript are highlighted in yellow.

Editorial and production comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. For example:
Line 82: Weigh lysolecithin
Line 85: dissolve with
Response: The manuscript has been revised by the authors and also by a native English speaker.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Sigma Aldrich, Eppendorf, etc.
Response: Done

3. How is sufficient depth of anesthesia determined?
Response: The animal breathing rate needs to be monitored for the whole procedure and the isoflurane concentration adjusted accordingly (fast breathing needs anesthesia to be increased and vice versa).
This information was added to the written protocol.

4. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?
Response: Done

5. 2.5: What is the concentration of the chlorhexidine solution?
Response: It is 0.5% chlorhexidine solution. The concentration was added to the protocol.

6. 2.7: How much lidocaine is injected?
Response: It is injected 100 µL of lidocaine hydrochloride 2% subcutaneously. Information added to the written manuscript.

7. 3.3: How much is injected?
Response: The amount of 11C-PIB injected was defined in item 3.1 (in the revised version is item 4.1). We have referred to that in item 4.3 now.

8. Figure 1: Please define the error bars.
Response: The error bars are standard deviation from the mean (it is now defined in the results section and in the figure legend).

Changes to be made by the Author(s) regarding the video:
1. Please increase the homogeneity between the video and the written manuscript. For example, step 5 of the protocol in the video is image analysis but Step 5 of the written

protocol is image acquisition. Please unify the video and the written manuscript so users can follow along easily.
Response: The written manuscript was revised for the same sequence appearing in the video.

2. Audio from 0:00 to 3:01 and 8:00 to the end is way too loud, and audio in between is slightly too low. Turn down the too loud sections, and slightly raise the too quiet section so they match.
Response: The audio was adjusted.

3. Remove background music
Response: Done

4. Add animal ethics statement at the beginning of the video
Response: Done

5. Video doesn't fill the entire frame at 2:59
Response: It was adjusted.

6. Also display the speaker's name and instituion graphic during the conclusion
Response: Done

7. Missed blurring out the face at 2:43
Response: Done

Reviewers' comments:

Reviewer #1:

1. I would suggest to expand the Introduction section about the clinical application in humans of amyloid tracers for myelin imaging (e.g.Pytel et al. Amyloid PET findings in multiple sclerosis are associated with cognitive decline at 18 months. MSARD 2020;39;101926, showing association with cognitive decline and more brain atrophy over time; Pietroboni et al. Amyloid PET as a marker of normal-appearing white matter early damage in multiple sclerosis: correlation with CSF beta-amyloid levels and brain volumes. EJNMMI 2019;46:280-287, ...).
Response: The introduction was expanded, and the suggested references were included (Third paragraph of introduction section).

Reviewer #2:

1. Clarity of the result: The resolution of the result image provided by the author barely allows to visualize the absence of labeling at the lesion site. Better quality is required to clearly distinguish the absence of labeling. Moreover, the injections of LPC took place in striatum and corpus callosum, thus it would be great to be able to identify these areas in the image. However, in the provided image, the point designated by the arrow looks like cortex, making unclear whether what can be seen is actual demyelination after LPC injection. Enhanced images and comparison with stereotaxic atlas are mandatory here.
Response: The characteristic of PET is the low spatial resolution compared to anatomic imaging, as MRI, and, therefore, the definition of small regions as striatum and corpus

callosum is difficult; therefore, an anatomic template was used for helping the ROI drawing. We have added a figure (figure 2) with a more precise region of interest representation, and we have also adjusted the arrows in figure 1.

2. The quantified lesion site was manually drawn by the authors, and only this area was quantified. Although certainly the best way to quantify a precise region of interest, this subjective method could induce a strong bias. It would be of interest to also quantify non-injected brains regions at the different time points as negative control to make sure the variation of labeling in the region of interest is actually due to demyelination in the injection areas.
Response: We have added a figure (figure 2) where we present more precisely how the lesion VOI was drawn and an illustrative image of LFB staining showing demyelinated lesion in the injected side compared to the contralateral side.
We have also included a graph in figure 1 with the requested data: quantification of non-injected side (negative control) showing no variation in ^{11}C-PIB uptake over the time points. The limitation of manually drawn was added in the discussion section.

3. Finally, this method needs to be confirmed by the use of another model displaying unpredictable lesions, like Cuprizone feeding or EAE. Indeed, in the LPC injection model, the injection site allows to precisely determine where demyelination occurs. However, the usefulness of this PET technique to detect new and unknown demyelination areas requires to be confirmed by blind study on another model of demyelination, especially knowing that C-11 PIB radiotracer does not specifically bind myelin. This complementary experiment will also allow to determine the smallest demyelination lesion detectable, which is of highest interest in regard to already existing demyelination imaging techniques.
Response: Our group has already used ^{11}C-PIB for detecting demyelination in an EAE marmoset model (Carvalho, R. H. F. *et al.* C-11 PIB PET imaging can detect white and grey matter demyelination in a non-human primate model of progressive multiple sclerosis. *Multiple Sclerosis and Related Disorders.* **35** 108-115, doi:10.1016/j.msard.2019.07.020, 2019) with satisfactory results.
It is important to clarify that the limitation of lesion size detection is due to the PET scanner resolution. Preclinical scanners have the spatial resolution of about 1 mm (old scanners around 1.5 mm and new scanners with about 0.8 mm).
These informations were added to the manuscript (discussion section).

4. What is the upside of penile vein injection compared to tail vein or retro-orbital injections? Since gender is an important parameter in demyelinating diseases such as multiple sclerosis, most studies will likely include females, rending impossible penile vein injection.
Response: The administration site is a personal choice. In general, penile vein injection is easier than the tail vein, but that depends on personal training. We understand and agree with the reviewer concern about using female in the studies. Therefore, we make more explicit in the written manuscript, and video, that the either penile or tail injection are fine for tracer injection.
We have also included an observation about the retro-orbital injection, which would be less recommended in the case of brain PET image, since the injected material is radioactive, and the retro-orbital region will be close to the brain region (same region of the PET detectors) this can decrease image quality.

5. I would suggest to the authors to add a few screenshots of the data analysis process to facilitate following the analysis protocol.

Response: We appreciate the reviewer suggestion, but we believe that the sequence of analysis can be followed with the help of the video protocol (the import screenshots are there).