Journal of Visualized Experiments

Delivery of antibodies into the brain using focused scanning ultrasound

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61372R2
Full Title:	Delivery of antibodies into the brain using focused scanning ultrasound
Keywords:	Focused ultrasound, Scanning ultrasound, Antibodies, Neuroscience, Microglia, Alzheimer's disease, Drug delivery
Corresponding Author:	Jürgen Götz AUSTRALIA
Corresponding Author's Institution:	
Corresponding Author E-Mail:	jgoetz@med.usyd.edu.au;j.goetz@uq.edu.au
Order of Authors:	Jürgen Götz
	Gerhard Leinenga
	Liviu-Gabriel Bodea
	Wee Kiat Koh
	Rebecca M. Nisbet
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Brisbane, QLD 4072, Australia

TITLE:

Delivery of Antibodies into the Brain Using Focused Scanning Ultrasound

AUTHORS AND AFFILIATIONS:

Gerhard Leinenga[1], Liviu-Gabriel Bodea[1], Wee Kiat Koh[1], Rebecca M. Nisbet[1], Jürgen Götz[1]

[1]Clem Jones Centre for Ageing Dementia Research, Queensland Brain Institute,
The University of Queensland, Brisbane, QLD, Australia

Corresponding Author:
Jürgen Götz (j.goetz@uq.edu.au)

Email Addresses of Co-Author:
Gerhard Leinenga (g.leinenga@uq.edu.au)
Liviu-Gabriel Bodea (l.bodea@uq.edu.au)
Wee Kiat Koh (weekiat.koh@uq.edu.au)
Rebecca M. Nisbet (r.nisbet@uq.edu.au)

KEYWORDS:

focused ultrasound, scanning ultrasound, antibodies, neuroscience, microglia, Alzheimer's disease, drug delivery

SUMMARY:

Presented here is a protocol to transiently open the blood-brain barrier (BBB) either focally or throughout a mouse brain to deliver fluorescently-labeled antibodies and activate microglia. Also presented is a method to detect the delivery of antibodies and microglia activation by histology.

ABSTRACT:

Only a small fraction of therapeutic antibodies targeting brain diseases are taken up by the brain. Focused ultrasound offers a possibility to increase uptake of antibodies and engagement through transient opening of the blood-brain barrier (BBB). In our laboratory, we are developing therapeutic approaches for neurodegenerative diseases in which an antibody in various formats is delivered across the BBB using microbubbles, concomitant with focused ultrasound application through the skull targeting multiple spots, an approach we refer to as scanning ultrasound (SUS). The mechanical effects of microbubbles and ultrasound on blood vessels increases paracellular transport across the BBB by transiently separating tight junctions and enhances vesicle- mediated transcytosis, allowing antibodies and therapeutic agents to effectively cross. Moreover, ultrasound also facilitates the uptake of antibodies from the interstitial brain into brain cells such as neurons where the antibody distributes throughout the cell body and even into neuritic processes. In our studies, fluorescently labeled antibodies are prepared, mixed with in-house prepared lipid-based microbubbles and injected into mice immediately before SUS is applied to the brain. The increased antibody concentration in the brain is then quantified. To account for alterations in normal brain homeostasis, microglial phagocytosis can be used as a cellular marker. The generated data suggest that ultrasound delivery of antibodies is an attractive approach to

treat neurodegenerative diseases.

INTRODUCTION:

Therapeutic ultrasound is an emerging technology aimed at treating brain diseases in a noninvasive manner, in part by facilitating access of therapeutic agents to the brain[1-3]. As only a small fraction of therapeutic antibodies targeting brain diseases are taken up by and retained in the brain[4], therapeutic ultrasound offers the possibility to increase their uptake and target engagement[5,6].

In our laboratory, we are developing therapeutic approaches for neurodegenerative diseases in which an antibody in various formats is delivered across the blood-brain barrier (BBB) using microbubbles. To achieve this, ultrasound is applied through the skull into the brain in multiple spots using a scanning mode we refer to as scanning ultrasound (SUS)[7]. The mechanical interaction between the ultrasound energy, the intravenously injected microbubbles and the brain vasculature transiently separates the tight junctions of the BBB in a given sonication volume, allowing antibodies and other cargoes including therapeutic agents to effectively cross this barrier[7-9]. Moreover, ultrasound has been shown to facilitate the uptake of antibodies from the interstitial brain into brain cells, such as neurons, where the antibody distributes throughout the cell body and even into neuritic processes[5,10].

Alzheimer's disease is characterized by an amyloid-β and tau pathology[11], and a host of animal models is available to dissect pathogenic mechanisms and validate therapeutic strategies. An SUS approach, by which ultrasound is applied in a sequential pattern across the entire brain, when repeated over several treatment sessions, can reduce amyloid plaque pathology in the brains of amyloid-β-depositing amyloid precursor protein (APP) mutant mice and activate microglia which take up the amyloid, leading to improvement in cognitive function[7]. BBB opening with ultrasound and microbubbles also reduces tau pathology in pR5, K3 and rTg4510 tau transgenic mice[12-14]. Importantly, whilst microglia remove extracellular protein deposits, one of the underlying clearance mechanisms for intraneuronal pathologies induced by SUS is the activation of neuronal autophagy[12].

Here, we outline an experimental process, by which fluorescently labeled antibodies are prepared, and then mixed with in-house lipid-based microbubbles, followed by retroorbital injection into anesthetized mice. Retroorbital injection is an alternative to tail vein injection which we have found to be equally efficacious and simpler to repeatedly perform. This is immediately followed by applying SUS to the brain. To determine the therapeutic antibody uptake, mice are sacrificed and the increased antibody concentration in the brain is then quantified. As a proxy of the change in brain homeostasis, microglial phagocytic activity is determined by histology and volumetric 3D reconstruction.

The generated data suggest that ultrasound delivery of antibodies is a potentially attractive approach to treat neurodegenerative diseases. The protocol can be similarly applied to other drug candidates, as well as model cargos such as fluorescently labeled dextrans of defined sizes[15].

PROTOCOL:

All animal experiments were approved by the animal ethics committee of the University of Queensland.

1. In-house microbubble preparation

1.1. Weigh out a 9:1 molar ratio of 1,2-distearoyl-sn-glycero-3-phosphocholine and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)-2000] (ammonium salt). 0.5 mg of lipid mixture is required per 1 mL of microbubble solution. Alternatively, lipids can be bought already in chloroform, if using pre-dissolved lipids proceed to step 1.3.

1.2. Dissolve the lipid in a small volume of chloroform in a glass beaker.

1.3. Evaporate the chloroform with an evaporator or a nitrogen stream.

1.4. Rehydrate the dried lipid film with 10 mL of PBS + 10% glycerol + 10% propylene glycol solution that has been filtered through 0.22 µm filter.

1.5. Place the rehydrated lipid solution in a water bath sonicator set to 55 ˚C (above the melting temperature of the lipids) and sonicate until fully dissolved.

1.6. Aliquot lipid solution into autoclaved 1.5 mL HPLC vials and screw on the septa caps.

1.7. Aspirate all of the air in the vial with a 5 mL syringe equipped with a 27 G needle and create a vacuum in the vial.

1.8. Add octofluoropropane to the vial with the included syringe, drawing up the gas from the canister. Fill the vial with 1-2 mL of octafluoropropane by reading the volume in the syringe.

1.9. Seal each vial with paraffin film and refrigerate.

1.10. On the day of the experiment, bring the vial to room temperature, add 0.5 mL of 0.9% NaCl solution to the vial, then place the vial in an amalgamator and agitate for 45 s (preset time) to produce the microbubbles.

2. Microbubble quality control using a coulter counter

2.1. Take the microbubble solution out of the amalgamator and vent the gas from the vial by piercing the septa with a 27 G needle.

2.2. Dilute the microbubble solution by performing two-step 1:5,000 serial dilutions by adding 100 µL of microbubble solution into 5 mL of filtered flow solution, and then taking 100 µL of 1:50 diluted microbubble solution and pipetting into 10 mL filtered flow solution in a cuvette. Use a 1 mL syringe with a 19 G needle.

2.3. Check that the electrolyte tank has sufficient flow solution and that the waste tank is empty.

2.4. Place the cuvette in the coulter counter platform and lock it into place. Use a 30 µm aperture for sample acquisition.

2.5. In the software, load the standard operating method (SOM), then select **Edit SOM | concentration.** Enter 5000x dilution.

2.6. In the software, choose a suitable file name. For example, microbubble_1_date.

2.7. Load and secure the cuvette into the platform.

2.8. In the software select **Run| Preview** and verify that the sample concentration is less than 10%. If this number is higher than 10%, perform a new dilution of the microbubbles with a higher dilution factor.

2.9. Select '**Start**' to begin an acquisition of the sample to obtain the initial readout.

2.10. Rinse the aperture of the Coulter counter with filtered flow solution after each measurement. Repeat steps 2.2-2.9 to obtain 3 replicates.

2.11. Place a cuvette with diluted microbubble solution into a sonicator water bath and sonicate for 30 s. Solution will change from opaque to clear as the microbubbles are destroyed.

2.12. Measure the solution with sonicated microbubbles and label as blank.

2.13. In the software, subtract the final readout from the initial readout. This subtracts any particles that are not microbubbles and do not contain gas.

2.14. Select **Display Results** in the software, to display the microbubble concentration, size distribution, average size, and volume concentration.

3. Fluorescent antibody labeling

3.1. Obtain a 1 mg/mL solution of mouse IgG in PBS without any additives.

3.2. Label 1 mg of mouse IgG with AlexaFluor 647 in 0.1 M sodium bicarbonate buffer by following the manufacturers' instructions located in the kit. This amount of fluorescently labeled IgG is sufficient to perform this procedure on 5-7 adult mice where a 5 mg/kg dose of antibody is administered.

3.3. Add the dye to the solution of IgG in 0.1 M sodium bicarbonate buffer and incubate for

15 min at room temperature.

3.4. Purify the fluorescently labeled antibody by pipetting the antibody solution into a spin column and centrifuging at 1,000 *x g* for 5 min. Free dye will remain in the column bed.

3.5. Use a spectrophotometer to measure the protein concentration. Measure the absorbance of the conjugate solution at 280 nm and 650 nm (A280 and A650). Calculate the concentration of protein in the sample using the equation:

$$Protein\ concentration\ (M)\ =\ [A280 - (A650\ x\ 0.03)]\ x\ dilution\ factor/203{,}000.$$

3.6. Use a spectrophotometer to calculate the degree of labeling using the equation:

$$moles\ dye\ per\ mole\ protein = A650\ x\ dilution\ factor\ /\ 239\ 000\ x\ protein\ concentration\ (M).$$

NOTE: An acceptable degree of labeling is 3-7 moles dye per mole protein and typically obtained degree of labeling is around 6.

4. Ultrasound set-up

4.1. Using the focused ultrasound system, add the 5 mm spacer to the water bolus to position the ultrasound focus 9 mm below the bottom of the water bolus.

4.2. Fill the water bolus with approximately 300 mL of deionized water that has been degassed with an inline degasser for 20 min (oxygen content should be below 3 ppm). Place the annular array into the filled water bolus and use a dental mirror to check that there are no air bubbles on the surface. If present on the surface, remove the annular array and replace it in the water bolus.

4.3. Launch the application software. In the waveform menu, select **Set waveform duty cycle**. Settings are PRF (Hz) 10, duty cycle 10%, focus 80 mm, center frequency 1 MHz, amplitude (MPa peak negative pressure) 0.65 MPa, mechanical index = 0.65. Press **Set** to define the waveform and store it in memory.

NOTE: The focused ultrasound system is pre-calibrated by the manufacturer from measurements taken by a calibrated hydrophone.

4.4. In the focused ultrasound system software, define a treatment plan. This requires defining a treatment region consisting of multiple individual treatments sites, and defining actions to be taken at each of those treatment sites. In this case, the treatment zone is one hemisphere of the mouse brain.

4.5. In the motion controller window, go to the **Scan** tab and **Enter** start, stop and increment value for motion in the x dimension, and start, stop and increment value for motion in the y

direction. Enter values for X: start -4, stop 3.50 and Y: -3.00, stop 3, Increment 1.5, # loops: 1.

4.6. Define the actions for treatment sites. In the motion controller window, select the **Event** button. In the **Script Editing Window**, select a list of actions that will be executed in the order selected at each treatment site. Set **Movement Type** to raster grid at the top of the script window. In the events tab select **Add Actions** to move them to the script panel, and add **Move Synchronously**, **Start** trigger arb, wait, **Stop** trigger arb. Click on the wait action and select a wait time of 6, 000 ms.

NOTE: These settings will make the treat a 6 x 5 grid of treatment spots spaced 1.5 mm apart, with each spot having a treatment duration of 6 s. The total duration to sonicate a mouse brain is approximately 3 min. This size treatment grid is suitable for adult C57/Bl6 mice weighing approximately 30 g. The size of the grid of treatment spots can be adjusted up or down depending on the size of the mouse.

5. Animal preparation

5.1. Weigh the mouse with a balance accurate to 0.1g.

5.2. Anesthetize mouse with 90 mg/kg ketamine and 6 mg/kg xylazine. Test for absence of reflexes with a toe pinch. Alternatively, mice can be anesthetized with isoflurane using an appropriate inhalational anesthetic apparatus.

5.3. Use an electric razor to shave the hair from the head of the animal, then apply hair removal cream with a cotton bud, leave on for 2-3 min or until the hair is wiped away clean with a damp piece of gauze.

5.4. Mark the center of the mouse's head with a permanent marker. The transducer has a hole in its center and the transducer focus and focal spot can be aligned visually.

5.5. Fill a small weigh boat that has previously had the bottom cut off and replaced with plastic wrap glued to the bottom of the weigh boat with ultrasound gel. This serves as an 8 mm spacer and provides good coupling to the head of the mouse and allows visual inspection of the focus of the transducer aligned with the head of the mouse.

6. Microbubble preparation

6.1. Warm a microbubble vial to room temperature. To activate, add 0.5 mL of 0.9% NaCl solution to the vial and place in an amalgamator to agitate for 45 s to produce the microbubbles.

6.2. Vent the vial by piercing the septa with a 27 G needle.

7. Ultrasound treatment

7.1. Invert the vial of microbubbles and gently draw up 1 µL/g bodyweight of the solution. To this add solution of fluorescently labeled antibody and mix gently in the syringe. The maximum volume injected is 150 µL.

NOTE: In-house prepared microbubbles are approximately 60-fold less concentrated than the clinically used (e.g., Definity microbubbles). Adjust the concentration such that the number of microbubbles injected are similar to those clinically used.

7.2. Inject the microbubble and antibody solution retroorbitally taking care to inject gently and slowly.

7.3. Place the mouse in the head holder and fix the nose of the mouse. Then place the ultrasound gel-filled small weigh boat on top of the head.

7.4. Lower the water bolus until it sits on top of the ultrasound gel in the weigh boat.

7.5. Use the joystick to move the transducer focus to the center of the head. Select **Reset Origin** in the motion tab.

7.6. Select complete scan. Steps 7.3-7.6 will take 2 min.

7.7. For consistency, set a timer to ensure a 2 min delay between injecting microbubbles and selecting complete scan.

7.8. After the treatment is complete, move the mouse to a warmed recovery chamber and apply ophthalmic ointment to the eyes.

8. Tissue harvesting and processing

8.1. At the timepoint of interest after ultrasound delivery (at least 1 h to detect high levels of antibody in the brain) deeply anesthetize the mouse and perfused it with PBS.

8.2. Collect the brain and fix by immersion in 4% PFA for 24 h at 4 °C, then wash with PBS.

8.3. Image the brain in an infrared scanner by placing the brain on the tray and by acquiring an image in the 700 nm channel.

NOTE: After fixation, the brain is removed from the PFA, washed in PBS and sectioned. Alternatively, it can be placed in ethylene glycol cryoprotectant solution for 24 h at 4 °C or until submerged, and then moved to a new cryoprotectant containing vial and placed at -20 °C for long term storage.

8.4. Section the brain cold in PBS using a vibratome. Glue the brain to the platform and cut 30-40 µm sections and collect into PBS.

NOTE: lysosomal autofluorescence (prevalent in sections from animals older than about 12 months) should be bleached by overnight illumination of the sections in a light chamber box, at room temperature and in PBS containing azide (0.02%) to block bacterial growth.

9. Tissue staining and image acquisition

9.1. Transfer sections to blocking solution (5% BSA in 0.2% Triton/PBS) for 2 h at room temperature , then wash the sections 3x by replacing the solution with 0.2% Triton/PBS.

9.2. Incubate sections at 4 °C overnight with the primary antibodies against Iba1 (dilution 1:1,000) and CD68 (dilution 1:500), in 0.2% Triton/PBS, followed by 3x washes with 0.2% Triton/PBS.

9.3. Incubate sections for 2 h at room temperature with fluorescent secondary antibodies (dilution 1:500), followed by 3x washes with 0.2% Triton/PBS only.

NOTE: The nuclei can also be stained using DAPI solution (0.5 µg/mL).

9.4. Transfer the sections to a slide and coverslip with hard-set mounting media. Allow sufficient time for the mounting medium to solidify before visualization and image acquisition (overnight).

9.5. With a confocal microscope obtain z-stack images (at least 10 µm depth and 0.3 µm step size, 10 images per animal) of the ultrasound targeted brain area by using a confocal microscope and an objective of at least 40x magnification.

NOTE: Be careful to acquire images within the signal dynamic range, avoiding under-/over-exposure. Save the image files in the corresponding software format used by the microscope.

10. Image analysis

10.1. Convert the z-stack microscopy files using the image analysis software file importer.

10.2. Open the converted files into the software and adjust the intensity of the Iba1 channel to observe microglial cells.

10.3. Crop a single microglial cell by drawing a box around it, select ‘crop’ and save the new file.

10.4. Build the 3D surface rendering of the IBA1 signal by:

10.4.1. Open the single cell Imaris file generated in the previous step.

10.4.2. Add a surface to the file.

10.4.3. Select the channel corresponding to the Iba1 staining.

10.4.4. Apply a threshold that should overlap the Iba1 staining

10.4.5. Select only the structure of interest that is forming the microglial cell of interest

10.5. Finalize the process

10.5.1. Obtain and record the volume of the Iba1 staining

10.5.2. Build the 3D surface rendering of the CD68 staining

10.5.3. Inside the IBA1 surface subfile, mask the CD68 channel and remove the voxels outside of the IBA1 staining

10.5.4. Add a new surface to the file

10.5.5. Select the channel corresponding to the CD68 staining

10.5.6. Apply a threshold that should overlap the CD68 staining and is as closely as possible matching the staining volumes

10.5.7. Finalize the process, as only the intra-microglial CD68 structures will be present in this file and, thus, they do not require another threshold filtering step

10.5.8. Obtain and record the number and average volume of the CD68 positive structures

10.5.9. Calculate the relative volume of CD68 structures per its corresponding IBA1 structures as a measure of microglial activation in a phagocytic state.

REPRESENTATIVE RESULTS:

Using this protocol fluorescently-labeled antibodies are delivered to the brain and can be detected, along with microglia activation. The conclusion that can be drawn is the use of focused ultrasound and microbubbles markedly enhances brain uptake of antibodies and can deliver antibodies to the whole brain or hemisphere of a mouse when used in a scanning mode. **Figure 1** shows the TIPS ultrasound application device (different components labeled) that is used to open the BBB. **Figure 2** shows the representative results from Coulter counter measurements of size and concentration which should be obtained when the microbubbles are produced correctly. To easily visualize the delivery, the antibodies were labeled with a far-red fluorescent dye. Antibody uptake by the brain can be easily visualized in whole brain or sections using an infrared scanner or using fluorescent microscopy on brain sections. Brain sections show the location of the fluorescently labeled antibody at a microscopic level. Representative results for scanning

ultrasound delivery to the hippocampus of fluorescently labeled anti-tau antibody RN2N is shown in **Figure 3**. To observe any alteration of normal brain homeostasis as a consequence of SUS and antibody delivery, one read-out was the microglial lysosomal content in relation to phagocytosis. **Figure 4** shows representative staining for microglia using Iba1 and the microglial lysosome specific marker CD68 to determine whether microglia become more phagocytic following the delivery of the antibody.

FIGURE AND TABLE LEGENDS:

Figure 1: The focused ultrasound system used for delivering ultrasound with critical components being labeled. (**A**) The home-made gel holder serving as an 8 mm spacer. (**B**) View through the transducer showing how the target is aligned with the focus visually (**C**).

Figure 2: Quality control measurements of in-house prepared microbubbles. (**A**) Coulter counter equipment used to obtain summary statistics (**B**) and size distribution of microbubbles in number (**C**) and volume distribution (**D**).

Figure 3: Delivery of fluorescently-labeled antibody into the brain using SUS. (**A**) A fluorescently labeled antibody fragment specific for a tau isoform delivered on its own, SUS on its own, and a combination treatment revealed increased uptake of the antibody by a sonicated hemisphere when combined with SUS using near infrared fluorescent imaging of one hemisphere of the brain. A look-up-table (LUT) was applied, with higher fluorescence intensity in arbitrary units displayed in warmer colors. (**B**) Quantification of fluorescence was done without subtracting the SUS-only control levels of background fluorescence. Mean ± SEM shown. (**C**) Increased uptake of the fluorescently labeled antibody by hippocampal neurons shown in low and high magnification images of brain sections. In the combination treatment, the antibody distributes into cell bodies and even dendrites as shown for hippocampal neurons. Blue=DAPI, magenta=antibody fragment. Scale bar: 50 µm.

Figure 4: Representative immunofluorescence labeling for microglia, showing the expected morphology of microglia and rendered in 3D with the image analysis software. Microglia morphology is observed in green and levels and distribution of CD68 is observed in red. Scale bar 10 µm. Green=Iba1, red=CD68.

DISCUSSION:

Fluorescently labeled antibodies can be delivered to the brain using focused ultrasound together with microbubbles applied in a scanning mode. Antibody delivery, microglial morphology and lysosomal enlargement can be detected by fluorescence microscopy following scanning ultrasound. Microglia can take up into their lysosomes antibodies and antigens that the antibodies have bound to in an Fc-receptor-mediated process[4].

There are a number of critical steps to achieve repeatable BBB opening and antibody delivery using this method. It is critical to ensure good coupling between the ultrasound transducer and the head of the mouse. Remove all of the hair on the head of the mouse and ensure that there

are no air bubbles in the coupling. The characteristics of the in-house prepared microbubbles are critical to success. Microbubbles must have a sufficient concentration of 10^8 microbubbles/mL and a size distribution such that over 90% of the microbubbles are below 10 µm in diameter. This is because larger microbubbles are known to be filtered out of the circulation by the lungs. An acceptable median size is between 1 -3 µm. Microbubbles must be handled and injected gently to avoid destroying them in the syringe. It is important that the ultrasound be applied no later than 2 min following the injection of the microbubbles which can be achieved with practice. Targeting of a whole brain or entire hemisphere is easily achieved with the SUS approach and accuracy of targeting is unlikely to be a problem when targeting large regions. A smaller brain region such as the hippocampus or striatum can also be successfully targeted but in this case it is important that the focus overlaps the targeted region. The height of a mouse brain is similar to the axial length of the focused ultrasound beam at 1 MHz using a typical ultrasound transducer, so that the transducer needs only be moved in the x and y dimension and not the z dimension. This can be determined by the knowledge of stereotaxic coordinates for a particular brain structure and by viewing of the lambdoid and sagittal sutures through the depilated skin of the mouse.

Here we demonstrate a technique that uses retroorbital injections to deliver microbubbles and antibody. An alternative to retroorbital injections is tail vein injections which is also an effective technique to deliver microbubbles and antibodies. The advantages of retroorbital injection is it is technically less challenging than tail vein injections, and can be repeated multiple times (alternating eye of injection) with very minimal risk of tissue damage.

If no fluorescently labeled antibody is detected in the brain it is probable that the BBB did not open. Troubleshooting should focus on obtaining a concentrated microbubble solution and injecting it so as not to destroy the microbubbles and delivering the ultrasound within two minutes of the injection time. If no BBB opening occurs, the peak negative pressure setting can be increased, with the caveat that higher peak negative pressures increase the chance of causing microhemorrhages which we do not detect at a peak negative pressure setting of 0.65 MPa using the settings described. Depending on the antigen specificity of the antibody the staining pattern will be different. The staining pattern obtained when injecting an anti-tau antibody is shown in **Figure 2**.

This technique can be applied to a range of antibodies and as long as consistent BBB opening is obtained, binding of the antibody to a target in the brain can be assessed. The scanning ultrasound approach achieves opening of the BBB across an entire mouse brain in a reproducible manner.

A limitation of this technique is that the occurrence and extent of the BBB opening is not observed while the mouse is alive. This limitation could be overcome by including MRI imaging with gadolinium contrast agent to the procedure, but this significantly increases the time and cost of the procedure.

Described here is a single sonication and single administration of antibody protocol which can be

used to determine how much increased uptake of antibodies can be achieved, as well as where they are located in the brain after delivery. The protocol can also be used in a longitudinal study to assess therapeutic effects of antibody delivery. In a treatment study the protocol can be repeated with an inter-treatment interval of one week or longer in order to evaluate the therapeutic potential of an antibody delivered to the brain. The therapeutic potential of the antibody delivered by ultrasound can be assessed in transgenic mouse models of neurodegenerative diseases. Read-outs of therapeutic effect could include behavioral tests, and histology and biochemistry for the levels of pathological proteins for example tau, amyloid-β or synuclein.

In conclusion, we have outlined a method to open the blood brain barrier in mice to deliver fluorescently-labeled antibodies. This method will be of interest to researchers evaluating therapeutic approaches for neurodegenerative diseases.

ACKNOWLEDGMENTS:

We acknowledge support by the Estate of Dr Clem Jones AO, the National Health and Medical Research Council of Australia [GNT1145580, GNT1176326], the Metal Foundation, and the State Government of Queensland (DSITI, Department of Science, Information Technology and Innovation).

DISCLOSURES:

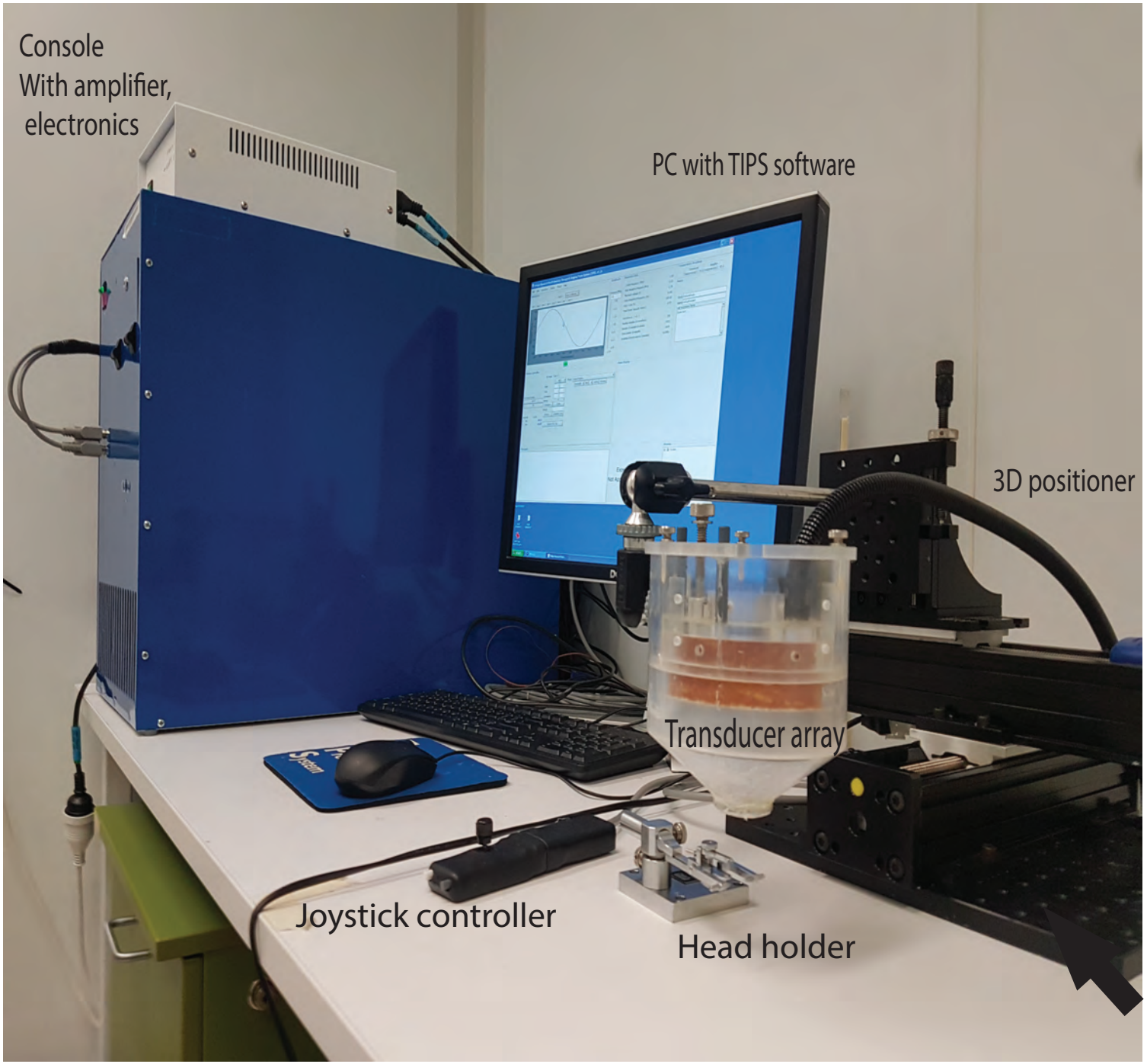
We have nothing to disclose.

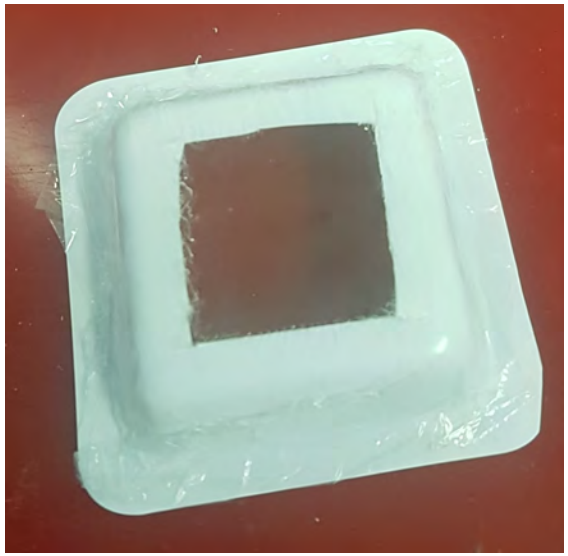
REFERENCES:

1. Choi, J. J. et al. Noninvasive and transient blood-brain barrier opening in the hippocampus of Alzheimer's double transgenic mice using focused ultrasound. *Ultrasonic Imaging.* **30** (3), 189-200 (2008).
2. Lipsman, N. et al. Blood-brain barrier opening in Alzheimer's disease using MR-guided focused ultrasound. *Nature Communications.* **9** (1), 2336 (2018).
3. Pandit, R., Chen, L., Götz, J. The blood-brain barrier: physiology and strategies for drug delivery. *Advanced Drug Delivery Reviews.* (19), 30238-8 (2019).
4. Golde, T. E. Open questions for Alzheimer's disease immunotherapy. *Alzheimers Research & Therapy.* **6** (1), 3 (2014).
5. Nisbet, R. M. et al. Combined effects of scanning ultrasound and a tau-specific single chain antibody in a tau transgenic mouse model. *Brain.* **140** (5), 1220-1230 (2017).
6. Janowicz, P. W., Leinenga, G., Götz, J., Nisbet, R. M. Ultrasound-mediated blood-brain barrier opening enhances delivery of therapeutically relevant formats of a tau-specific antibody. *Scientific Reports.* **9** (1), 9255 (2019).
7. Leinenga, G., Götz, J. Scanning ultrasound removes amyloid-beta and restores memory in an Alzheimer's disease mouse model. *Science Translational Medicine.* **7** (278), 278ra233 (2015).
8. Burgess, A. et al. Targeted delivery of neural stem cells to the brain using MRI-guided focused ultrasound to disrupt the blood-brain barrier. *PLoS One.* **6** (11), e27877 (2011).

9. Chen, H. et al. Focused ultrasound-enhanced intranasal brain delivery of brain-derived neurotrophic factor. *Scientific Reports.* **6**, 28599 (2016).
10. Leinenga, G., Langton, C., Nisbet, R., Götz, J. Ultrasound treatment of neurological diseases - current and emerging applications. *Nature Reviews Neurology.* **12** (3), 161-174 (2016).
11. Götz, J., Halliday, G. & Nisbet, R. M. Molecular Pathogenesis of the Tauopathies. *Annual Reviews of Pathology.* **14**, 239-261 (2019).
12. Pandit, R., Leinenga, G., Götz, J. Repeated ultrasound treatment of tau transgenic mice clears neuronal tau by autophagy and improves behavioral functions. *Theranostics.* **9** (13), 3754-3767 (2019).
13. Nisbet, R. M. et al. Combined effects of scanning ultrasound and a tau-specific single chain antibody in a tau transgenic mouse model. *Brain.* **140** (5), 1220-1230 (2017).
14. Karakatsani, M. E. et al. Unilateral Focused Ultrasound-Induced Blood-Brain Barrier Opening Reduces Phosphorylated Tau from The rTg4510 Mouse Model. *Theranostics.* **9** (18), 5396-5411 (2019).
15. Valdez, M. A., Fernandez, E., Matsunaga, T., Erickson, R. P., Trouard, T. P. Distribution and Diffusion of Macromolecule Delivery to the Brain via Focused Ultrasound using Magnetic Resonance and Multispectral Fluorescence Imaging. *Ultrasound in Medicine and Biology.* **46** (1), 122-136 (2020).

Figure 1





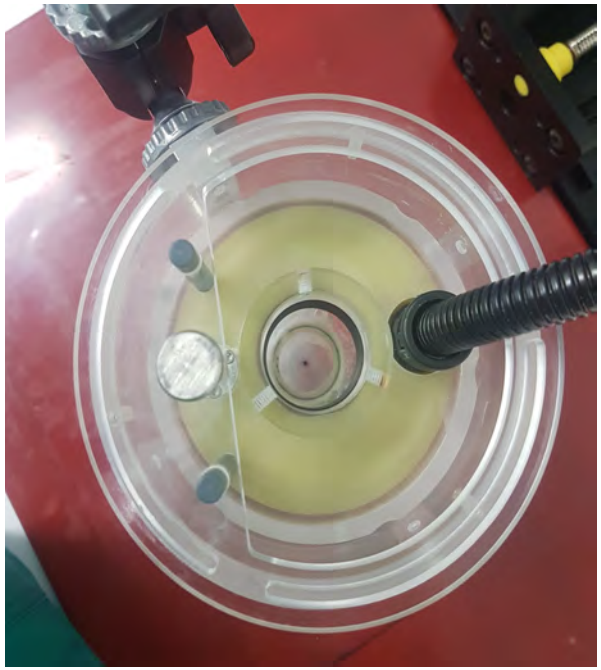


Figure 2

A

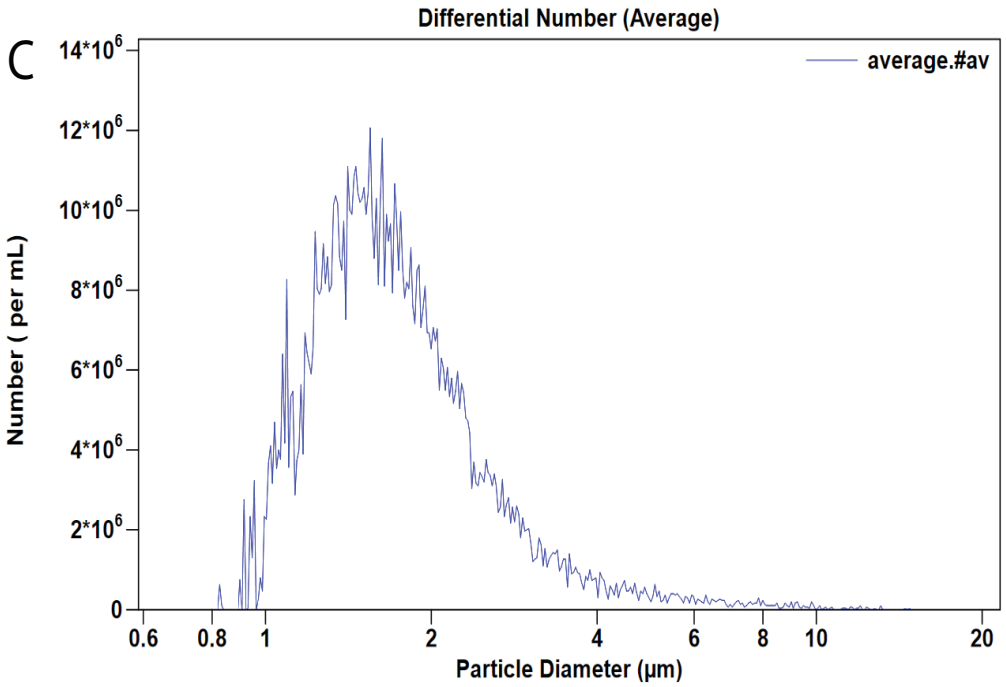
B

Calculations from 0.600 µm to 18.00 µm

Number: 912.6e6 per ml
Mean: 1.885 µm S.D 0.98 µm
Median 1.637 µm

< 2µm
71.6%

C



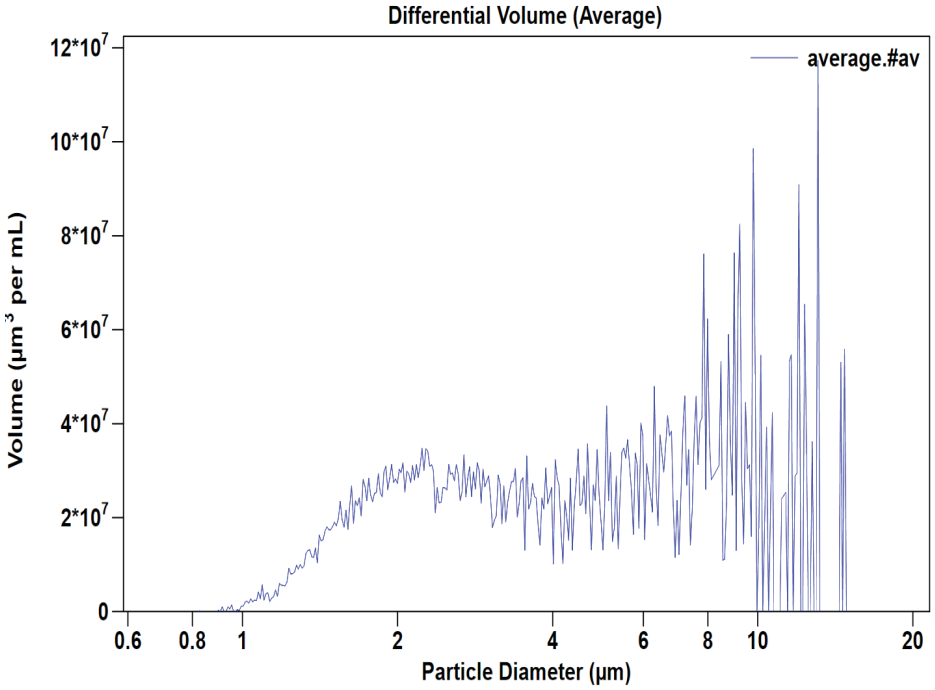


Figure 3

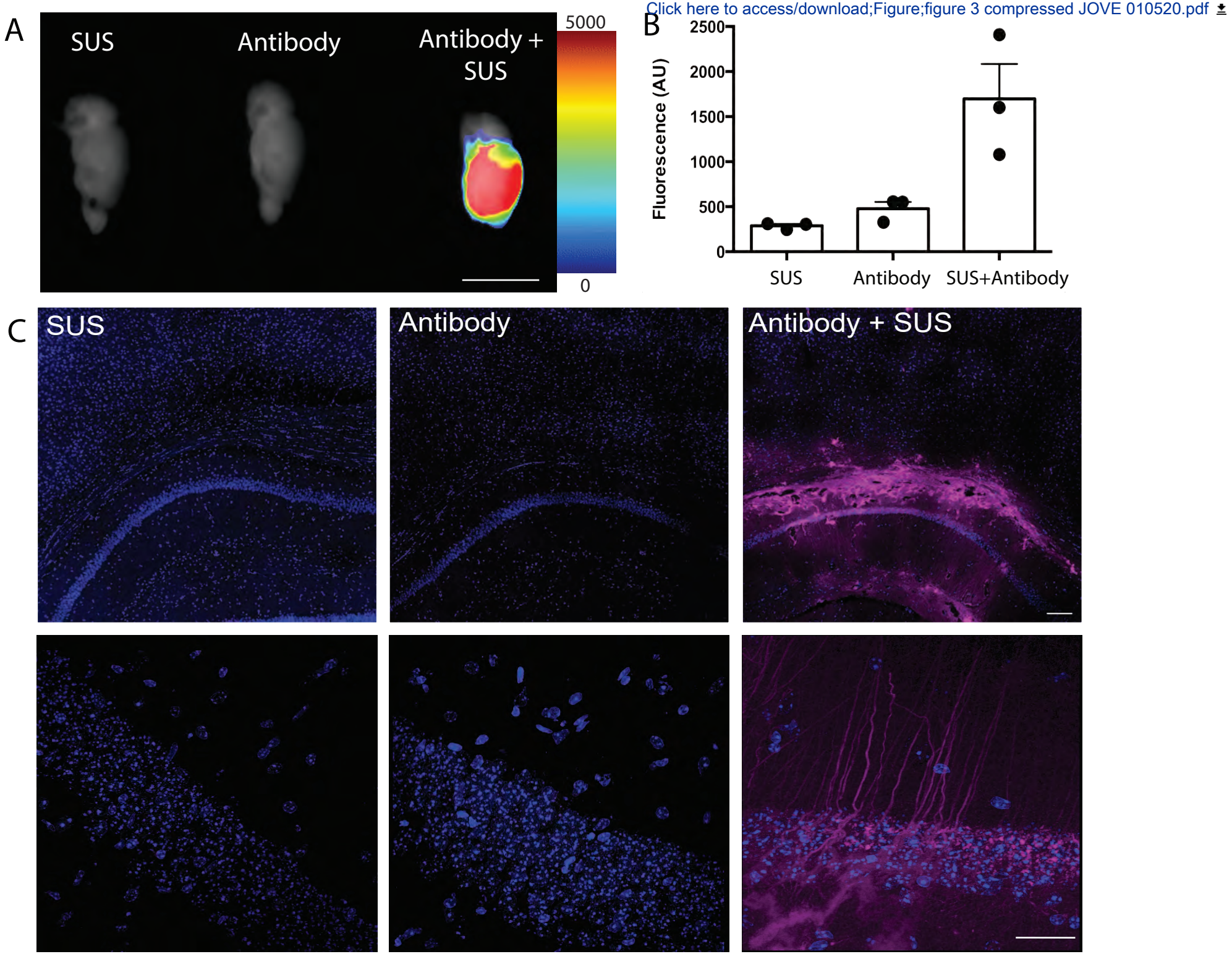
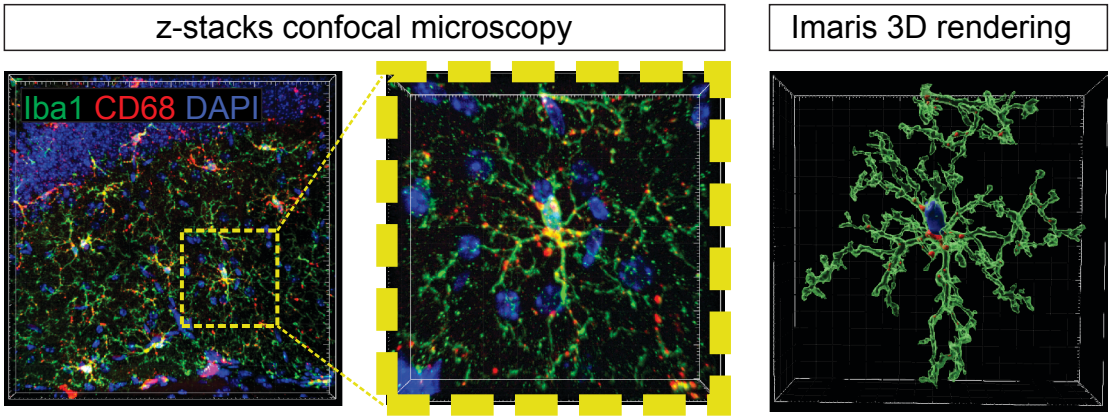


Figure 4



Name of Material/Equipment	Company	Catalog Number	Comments/Description
1,2-distearoyl-sn-glycero-3-phosph	Avanti	850365C	
1,2-distearoyl-sn-glycero-3-phosph	Avanti	880128C	
AlexaFluor 647 antibody labeling kit	Thermo Fisher	A20186	
CD68 antibody	AbD Serotec	MCA1957GA	Use 1:1000 dilution
Chloroform	Sigma-Aldrich	372978	
Coulter Counter (Multisizer 4e)			
Glycerol	Sigma-Aldrich	G5516	
Goat anti-rabbit IgG, Alexa Fluor 488	Thermo FIsher	A-11008	Use 1:500 dilution
Goat anti-rabbit IgG, Alexa Fluor 488	Thermo Fisher	A-11077	Use 1:500 dilution
Iba1 antibody	Wako	019-19741	Use 1:1000 dilution
Image analysis software	Beckman Coulter	#8547008	
Isoflow flow solution	Beckman Coulter	B43905	
Near infrared imaging system Odyssey Fc	Licor	2800-03	
Octafluoropropane	Arcadophta	0229NC	
Propylene Glycol	Sigma-Aldrich	P4347	
TIPS (Therapy Imaging Probe System)	Philips Research	TIPS_007	
	Bitplane		

CLEM JONES CENTRE FOR AGEING DEMENTIA RESEARCH (CJCADR) at the
QUEENSLAND BRAIN INSTITUTE

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

Professor Jürgen Götz
Foundation Chair of Dementia Research
The University of Queensland
Building 79
Brisbane QLD 4072
Telephone: +61 7 3346 6329
Facsimile: +61 7 3346 6301
Email: j.goetz@qbi.edu.au

May 1, 2020

JoVe Methods Collection invitation - FUS brain therapies

Dr Ronald Myers
JOVE

Dear Dr Ronald Myers, dear Dr Tao Sun,

Please find our updated version in track changes mode attached.
We have considered all comments and made all requested revisions.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. - done
2. Please use American English throughout. – all checked and corrected
3. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points - done
4. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …" - done
5. 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 and Reagents
For example: Multisizer 4e, IsoFlow, paraffin film, Acuvette, Nanodrop, Philips Therapy and Imaging Probe System, Imaris, Vialmix Amalgamator, Licor Odyssey Fc infrared scanner, Beckman Multisizer 4e, BD, etc . - done
6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be"

throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." – done, in several instances the 'Note' option was used.
7. The Protocol should contain only action items that direct the reader to do something. - done
8. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step. - considered
9. Please ensure you answer the "how" question, i.e., how is the step performed? - considered
10. Please use complete sentences throughout. - considered
11. 2.10: How is this done? - edited
12. 4: Please include all the button clicks and knob turns in this case to show the action. – provided as detailed as possible
13. 10: Please include all the button clicks in the software to show how this is done. – provided as detailed as possible
14. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol - highlighted
15. Please ensure the result is described with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. - done
16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." – N.A. as no reuse
17. As we are a methods journal, please expand the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
- all done
18. Please do not abbreviate the journal titles in the references section. – all done
19. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns. – extensive list provided

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Leinenga et al report a method to deliver antibodies through the blood-brain barrier using a non-invasive ultrasound technique. They describe the process of producing and counting microbubbles, performing the ultrasound treatment in mice, and analyzing the

brain tissue post-mortem. I believe this manuscript provides a complete protocol that can be used by multiple groups interested in antibody delivery into the brain. I suggest it for publication in the Journal of Visualized experiments following minor revision.

Major Concerns:
None.

Minor Concerns:
Title: I suggest rephrasing to "Delivery of antibodies into the brain using therapeutic scanning ultrasound". – title has been changed
L. 30-31: Tight junction disruption is not the only mechanism of drug transport across the BBB. – has been edited
L. 50: SUS does not stand for scanning mode. Please define the acronym SUS appropriately. Also, "scanning" is not a necessary element of BBB opening using focused ultrasound (FUS) and microbubbles, but rather a mechanistic way of targeting multiple spots within the brain. I suggest defining that clearly to avoid confusion. – thanks, edited in abstract and introduction
L. 54: Blood-borne factors may be confused with blood-borne pathogens. I suggest rewording to "therapeutic agents". – we now use the term ‘agents’
L. 62: Please define APPsw mouse model. Also, this is not the only transgenic mouse model in which AD clearance has been observed after FUS treatments. Please include more examples from literature (such as rTg4510, etc). – expanded and now also including tTg 4510, pR5 and K3 mice
L. 69: Why retro-orbital and not intravenous? Is there evidence that this method is more effective for microbubble/antibody administration? – explanation provided lines 124-5
L. 77: Authors are encouraged to cite published papers that can be tracked by readers. There are loads of recent studies using Dextran as a drug model (e.g. Valdez et al, 2019, UMB or Morse et al, 2019, Radiology). – now citing Valdez.
L. 81: Do lipids have to be in powder form? – edited lines 180,181
L. 88: What are the melting points of the two lipids? – added: 55˚C (above the melting temperature of the lipids)
L. 92: How do you replace the air from the vial? How can you measure 1-2ml of octafluoropropane gas? – changed to: Add octofluoropropane to the vial with the included syringe, drawing up the gas from the canister. Fill the vial with 1-2ml of octafluoropropane by reading the volume in the syringe. It is also explained how a vacuum is produced in the vial
L. 94: I suppose authors mean to activate the prepared lipid solution and L. 95: What is the purpose of dilution prior to activation? - 10. On the day of the experiment, bring the vial to room temperature, add 0.5 ml 0.9% NaCl solution to the vial, then place the vial in an amalgamator and agitate for 45 seconds (preset time) to produce the microbubbles.
L. 129: Is there a reference for this equation?- no, but information is provided in the kit and the kit has details and catalog number in table of materials.
L. 141: What is an acceptable level of oxygen saturation? – added: (oxygen content should be below 3 ppm)
L. 144: Please replace "waveform duty cycle" with "ultrasound treatment parameters". Center frequency or pressure are not part of the duty cycle definition. - corrected
L. 145: Please clarify that this is the center frequency and amplitude stands for peak-

negative pressure. – corrected
L. 158: Authors should mention that mice can be also anesthetized with inhalable anesthetics (e.g. isoflurane). – added: Alternatively, mice can be anaesthetized with isoflurane using an appropriate inhalational anaesthetic apparatus.
L. 175: What is this dose compared to the clinically-approved dose of Definity or SonoVue microbubbles? Please provide the dose also in number of microbubbles per ml of blood. – added: The maximum volume that should be injected is 150 µl. Note: In-house prepared microbubbles are approximately 60-fold less concentrated than the clinically used Definity microbubbles, such that the number of microbubbles injected are similar to those clinically used.
L. 177: Please provide a reference that this administration route is equally effective with the intravenous route. – unpublished based on extensive in-house expertise
L. 186-187: Why would there be such a delay between injecting microbubbles and initiating sonication? How much time is required for microbubbles to reach circulation following retro-orbital administration? Microbubble half-life in circulation is less than two minutes, so a two-minute delay would incur a 50% drop in the microbubble concentration in the blood, at least after intravenous administration. – only done for consistency (as added).
L. 191: How long should be the delay between ultrasound treatment (not delivery) and perfusion? Should this be adjusted according to the circulation time of each antibody? – added: After ultrasound delivery at the timepoint of interest post sonication (at least one hour to detect high levels of antibody in the brain) deeply anesthetise the mouse and perfused it with PBS.
L. 216: Please replace "lysososmal" with "lysosomal". - fixed
L. 227: Please replace "care full" with "careful". - fixed
L. 299: Please remove "are critical steps" to avoid repetition. – entire discussion edited
L. 300: Too high microbubble concentration will not be tolerated by mice. and L. 301: Is there a desirable mean size or size range? If so, please include the acceptable size range and/or mean/median size. – Added: Microbubbles must have a sufficient concentration of 108 /ml and a size distribution such that over 90% of the microbubbles are below ten micrometers in diameter. This is because larger microbubbles are known to be filtered out of the circulation by the lungs. An acceptable median size is between one and three micrometers.
L. 306: I believe authors should mention the limited axial specificity of BBB opening, given the high axial-to-lateral ratio of single-element ultrasound transducers or annular arrays in this case. – Added: The height of a mouse brain is similar to the axial length of the focused ultrasound beam at 1 MHz using a typical ultrasound transducer, so that the transducer needs only be moved in the x and y dimension and not the z dimension.
L. 321-322: MR-guided systems can open the BBB in multiple spots as well. – agreed. We have edited this sentence.
L. 323: This is not true. BBB opening can be achieved outside the MRI and still be confirmed using MRI right after. Sacrifice is not necessary.
Fig. 1: The arrow for the transducer points to the wall.
Fig. 3: Why is only half the brain shown here? What is the colormap showing? A color bar is necessary in Figure 3A. Also, it looks like there is absolutely no fluorescence in the antibody only condition, whereas there is clearly higher fluorescence intensity in the quantification figure 3B. Is there a threshold or ROI applied? – This has been fixed and

a new figure has been uploaded.
Table: I suggest adding more details in the compounds, such as dilution factors, total quantity used etc. – Dilution factors etc have been added.

Reviewer #2:
Manuscript Summary:
This is a well written protocol that clearly described the procedure of drug-delivery using the TIPS ultrasound system. Steps are in good details.

Minor Concerns:
Page 3, line 137: the calibration of the system shall be specified. – The following has been added: Note: The focused ultrasound system is pre-calibrated by the manufacturer from measurements taken by a calibrated hydrophone.
Page 3, line 147: Is the grid settings the same for all mice or they are adjusted for different size of mice? – This has been edited and two additional steps have been added.
Page 4, line 182: authors should specify how to achieve geometrically alignment between the ultrasound probe focus and the marker on mouse's head. Is that by visual or some alignment tools are used? – It is now described in text that we use visual alignment and it is also added to figure 1 and figure 1 figure legend

Reviewer #3:
Manuscript Summary:
This manuscript describes experimental procedures for delivering antibodies to the brain using microbubbles and ultrasound to temporarily permeabilize the blood brain barrier. There is increasing interest in such methods and thus this manuscript is important and can be very useful for laboratories involved in this work or interested in starting such research. The manuscript is well written and provides very useful information to the field. Some questions that should be addressed are provided below.

Major Concerns:
1. While retroorbital injection is used in these studies, it seems that tail vein catheterization would allow a more consistent delivery of microbubbles and allow the timing between injection and administration of the ultrasound to be shorter and more easily controlled. This should be discussed. – discussed: new lines 1028-1032
2. If being used in a therapeutic study that requires multiple treatments of antibody, how is the BBB opening validated? The procedures described seem appropriate for terminal studies only. Some guidance for how a longitudinal study would be carried out would be very helpful. – this is discussed in lines 1049-1057

Minor Concerns:
3. L67. "Here we are outlining an…" Should be changed to "Here we outline an…" - fixed
4. L74. "…antibodies is an attractive approach" should be written "…antibodies is a potentially attractive approach…" These methods are still in development. – term added
5. L120. Should reference where the manufacturer's instructions can be located. -

referenced
6. L145. For completeness, the mechanical index should be provided. – has been added
7. L176. How much fluorescently labeled antibody is drawn up? How much can be drawn up? – 150 ul
8. L180. Because the ultrasound gel-filled weighboat is homebuilt, a photograph of it would be very useful. – added to figure 1
9. L185. It is a little confusing where the 2-minute delay comes into the procedure. Steps 3-6 should take two minutes. Does that mean a timer should be set to make sure that the scanning starts 2 minutes after injection? This should be clarified. – has been clarified
10. L227. "Be care full …" should be changed to "Be careful …" – thank you. fixed
11. L258. The first sentence of results should be removed. It belongs in the discussion. – sentence removed and edited
12. L298. Much of the discussion should be moved into the protocol section. The first sentence of the results could be moved to the beginning of the discussion. As mentioned previously, some discussion of how these techniques could be used in longitudinal studies would greatly increase the relevance of the manuscript. – we reorganised the ms slightly in line with this suggestion
13. Fig. 1. A close up image of the gel-filled weighboat could be added as a separate panel to this figure. – added to figure 1
14. Fig. 3. Are the Licor images of whole brains, or are they sectioned? - Brain hemispheres.

Thank for your consideration.

Kind regards,

Jürgen Götz