Journal of Visualized Experiments

A Benchtop Approach to the Location Specific Blood Brain Barrier Opening Using Focused Ultrasound in a Rat Model

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61113R2
Full Title:	A Benchtop Approach to the Location Specific Blood Brain Barrier Opening Using Focused Ultrasound in a Rat Model
Section/Category:	JoVE Neuroscience
Keywords:	Focused Ultrasound; blood brain barrier; noninvasive neuromodulation; Drug delivery; noninvasive drug delivery; stereotaxic surgery
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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.	Birmingham, AL, USA

UAB MEDICINE
Department of Radiology

December 10, 2019

Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA 02140

JoVE protocol manuscript submission

To Whom It May Concern:

I am pleased to submit a manuscript with the title "A benchtop approach to location specific blood brain barrier opening using focused ultrasound in a rat model" for consideration for publication in the Journal of Visualized Experiments.

Focused ultrasound (FUS) mediated blood brain barrier (BBB) opening is a noninvasive method for localized delivery of agents to the brain. Therefore, it is a more translational alternative to stereotaxic intracranial surgery and can be used for a wide range of applications. The purpose of this manuscript is to provide an accessible approach to FUS BBB opening in order to accelerate its use in neurobiological research. This manuscript is therefore in alignment with the missions of JoVE.

Thank you very much for your consideration.

Sincerely,

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TITLE:

A Benchtop Approach to the Location Specific Blood Brain Barrier Opening Using Focused Ultrasound in a Rat Model

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KEYWORDS:

Focused ultrasound, Blood brain barrier, noninvasive neuromodulation, drug delivery, noninvasive drug delivery, stereotaxic surgery

SUMMARY:

Focused ultrasound with microbubble agents can open the blood brain barrier focally and transiently. This technique has been used to deliver a wide range of agents across the blood brain barrier. This article provides a detailed protocol for the localized delivery to the rodent brain with or without MRI guidance.

ABSTRACT:

Stereotaxic surgery is the gold standard for localized drug and gene delivery to the rodent brain. This technique has many advantages over systemic delivery including precise localization to a target brain region and reduction of off target side effects. However, stereotaxic surgery is highly invasive which limits its translational efficacy, requires long recovery times, and provides challenges when targeting multiple brain regions. Focused ultrasound (FUS) can be used in combination with circulating microbubbles to transiently open the blood brain barrier (BBB) in millimeter sized regions. This allows intracranial localization of systemically delivered agents that cannot normally cross the BBB. This technique provides a noninvasive alternative to stereotaxic surgery. However, to date this technique has yet to be widely adopted in neuroscience laboratories due to the limited access to equipment and standardized methods. The overall goal of this protocol is to provide a benchtop approach to FUS BBB opening (BBBO) that is affordable and reproducible and can therefore be easily adopted by any laboratory.

INTRODUCTION:

Despite the many discoveries in basic neuroscience, the number of emerging treatments for neurodevelopmental and neurodegenerative disorders remains relatively limited[1,2]. A deeper understanding of the genes, molecules and cellular circuitry involved in neurological disorders has suggested promising treatments unrealizable in humans with current techniques[3]. Effective treatments are often limited by the need to be brain penetrable and site-specific[4–8]. However, existing methods of localized drug delivery to specific brain regions (e.g., delivery via injection or cannula) are invasive and require an opening to be made in the skull[9]. The invasiveness of this surgery prevents the routine use of localized delivery into the human brain. Additionally, tissue damage and the resulting inflammatory responses are ubiquitous confounds for basic and preclinical studies that rely on intracerebral injection[10]. The ability to noninvasively deliver agents across the blood brain barrier (BBB) and target them to specific brain regions could have a tremendous impact on treatments for neurological disorders, while simultaneously providing a powerful investigational tool for preclinical research.

One method of targeted transport across the BBB with minimal tissue damage is transcranial focused ultrasound (FUS) together with microbubbles to focally and transiently open the BBB[11-16]. FUS BBB opening has gained recent attention for the treatment of neurodegenerative disorders, stroke and glioma by localizing therapeutics to target brain regions such as neurotrophic factors[17–19], gene therapies[20–22], antibodies[23], neurotransmitters[24], and nanoparticles[25,26,27–29]. With its wide range of applications and its noninvasive nature[30,31], FUS BBB opening is an ideal alternative to routine stereotaxic intracranial injections. Furthermore, due to its current use in humans[30,32], preclinical investigations using this technique can be considered highly translational. However, FUS BBB opening has yet to be a widely established technique in basic science and preclinical research due to lack of accessibility. Therefore, we provide a detailed protocol for a benchtop approach to FUS BBB opening as a starting point for labs interested in establishing this technique.

These studies were conducted with either a high-power air backed FUS specific ultrasound transducer or a low power damped focused ultrasonic immersion transducer. The transducers were driven by an RF power amplifier designed for reactive loads and a standard benchtop function generator. Details for these items can be found in the **Table of Materials**.

PROTOCOL:

All experimental procedures were done in accordance with UAB Institutional Animal Care and Use Committee (IACUC) guidelines.

1. Focused ultrasound driving equipment setup

1.1. Use 50 Ohm coaxial BNC cables to connect (1) the input of the ultrasound transducer to the output of the RF amplifier and (2) the input of the RF amplifier to the output of the function generator.

1.2. Set the function generator mode to a sinusoid burst once per second with a 1% duty cycle.

1.2.1. For the damped 1 MHz low-power immersion transducer with a 0.8 inch focal distance used with a 50 dB RF amplifier, set the starting settings to: 1 MHz sine wave, 1 V peak to peak, 10k cycle, 1 s interval (or period).

1.2.2. For the air backed, 1.1 MHz high-power transducer, set the initial settings to: 1.1 MHz sine wave, 50 mV peak to peak, 11k cycles, 1 s interval.

NOTE: Do not operate the transducers unless they are submerged. Do not place a hand or other body part at the ultrasound focus or touch the transducer face while it is operating.

2. Focused ultrasound benchtop setup

2.1. 3D-print the stereotaxic frame and the stereotactic frame holder.

2.2. Connect the transducer to the XYZ positioner using a clamp holding a PVC pipe (**Figure 1a**). Bolt the clamp onto the X-axis slide and lock with a wing nut.

2.2.1. If using the high-power ultrasound transducer, attach it to the PVC pipe using a matched pair of magnets. Ensure that one magnet has a hole and the other has a matching protrusion. Cap the bottom of the PVC pipe and attach one of the magnets to it using epoxy (see **Figure 1b**).

2.2.2. Attach the second magnet to the top center of the high-power ultrasound transducer using epoxy. Make sure it is at the very center of the transducer (**Figure 1c**).

2.3. If using the high-power ultrasound transducer, make a pointer for nulling the XYZ positioner. The tip of this pointer indicates the location in space of the center of the transducer focus when the ultrasound transducer is attached to the XYZ positioner. Make a pointer out of an 18 G needle cut to be the length of the focal distance of the transducer plus the thickness of the transducer (**Figure 1d)**.

2.3.1. Apply epoxy to a third magnet (this magnet also pairs with the PVC cap magnet) and attach it to the top of the pointer. The pointer will then be able to attach to the magnet on the PVC pipe for XYZ nulling (**Figure 1d**).

2.4. If using the low-power immersion transducer, 3D-print the file for the pointer and the mounting clip.

2.4.1. Attach the low-power immersion transducer to the PVC pipe by clipping the mounting clip onto to the PVC pipe and insert the transducer into the ring (**Figure 1f**).

2.3. Make a water bath by gluing together pieces cut from acrylic sheet that will be able to rest on top of the animal's head above the stereotactic frame (**Figure 1e**).

2.3.1. Cut an opening in the bottom of the bath that is about the size of the animal's head. Cover the hole in the water bath with a polyimide tape.

NOTE: Care must be taken to ensure that water does not leak around the polyimide tape as it can wet the rat's fur and cause hypothermia.

2.4. Make an MRI fiducial by filling a 4 mm diameter thin-shelled plastic or glass sphere with an MRI visible fluid (e.g., Vitamin E oil) and seal it. Place it in the MRI fiducial holder on the right side of the 3D-printed stereotaxic frame (**Figure 2a**).

2.5. Firmly secure the 3D-printed frame holder to the XYZ positioner at a good location for animal positioning. Slide the tab on the rostral end of the frame holder into the matching channel on the Y-axis rail and securing with set screws (**Figure 1h**, red arrows).

2.6. For driving the XYZ positioner, install the USB to serial converter onto a computer by following the manufacturer's directions and plug in the converter. Install the runtime environment and motor controller software onto the PC.

2.6.1. Ensure that the proper serial port is selected in the software by selecting the USB to serial converter in the port selection dropdown control on the front panel of the controller software. Connect the USB to serial converter to the stepper motor controller box using a 9-pin serial crossover cable (e.g., RS232 null modem cable).

2.6.2. Run the controller software to test that the stepper motors can be driven under software control. This step may require the assistance of local IT support.

3. Intracranial targeting procedure

NOTE: Male Sprague Dawley rats weighing 250-350 g were used for these experiments. Animals had free access to water and rat chow, and were maintained on a 12:12 h light:dark cycle.

3.1. Put the animal under anesthesia (3% isoflurane with oxygen) and check for the lack of response to the toe pinch. Then insert the catheter as described below.

3.1.1. Warm the tail with a lamp to make is easier to hit the vein. Be careful not to overheat the animal or to burn the tail.

3.1.2. Once the animal is asleep (does not respond to a toe pinch), insert the 24 G tail vein catheter that will be used to deliver microbubbles, Evans blue dye (EBD), gadobutrol MRI contrast if using MRI, and the experimental agent of interest. Once the vein is hit, blood will fill the sheath, slowly remove the inner needle while pushing the sheath further into the vein.

NOTE: See a guide like Stuart and Schroeder[33] if doing the rat tail vein injections for the first time.

3.1.3. If there is no blood flow, slowly move the catheter sheath out of the vein to test that the needle may have poked through the vein. If blood flows when the catheter is pulled back slightly, then first poke went through the vein and the catheter placement will need to be restarted at another location on the tail that is rostral to the previous location.

3.2. Fill the catheter plug with saline and screw the catheter plug into the end of the catheter port as soon as the port has filled with blood. Carefully wrap the lab tape around the catheter and the tail to keep it in place. Start with a small piece at the top and work in the caudal direction, leaving the very end of the catheter plug exposed.

3.3. Plug the anesthesia line onto the anesthesia connector on the stereotaxic frame (**Figure 2a**) and fix the animals head into the frame by placing the mouth onto the bite bar and by guiding the ear bars into both ear canals, then tighten the set screws. Make sure the animals head is secure and level.

3.4. Move the animal into the MRI bed and connect the anesthesia line to the nose cone. In this protocol a 9.4 T small bore animal MRI was used.

3.5. Collect coronal and axial T2-weighted images that capture the whole brain as well as the MRI fiducial (**Figure 2b**) for coordinate measurements. Provide the local MRI physicist or tech the following information so that they can build the MRI protocol.

3.5.1. For coronal images (**Figure 2b** top), use the following parameters: number of images: 27, width: 62.2 mm, height: 62.2 mm, depth: 37.97 mm, Voxel size: 0.24 x 0.24 x 1.41 mm^3.

3.5.2. For axial images (Figure 2b bottom), use the following parameters: number of images: 13, Width: 61.47 mm, Height: 53.81 mm, Depth: 16.7 mm, Voxel size: 0.41 x 0.21 x 1.29 mm^3.

NOTE: It is not necessary to have these exact parameters as long as the coronal in plane resolution is close to 0.25 mm and the images cover the whole brain and fiducial.

3.6. Collect coordinate measurements from the above images by recording the distance from the MRI fiducial to the brain region that will be targeted with FUS.

3.6.1. At the scanner, on the coronal images collected in step 3.5, find the image in which the fiducial is the largest, indicating the center of the fiducial. Record the distance from the top of the fiducial to the brain region of interest in mm (the MRI software will have a scale or point measurement tool, consult local MRI tech or physicist on how to do this) in both the medial/lateral direction and in the dorsal ventral direction (**Figure 2b**, top).

3.6.2. At the scanner, on the axial images collected in step 3.5, find the image that shows the very top of the fiducial and measure the distance from the center of the fiducial to the target brain region in both the dorsal/ventral direction and in the medial/lateral direction (**Figure 2b**, bottom).

3.6.3. Compare the two medial/lateral measurements and if they are different use the average. These coordinate measurements will be used later in step 4.3 for guiding the FUS focal point to the target brain region with the XYZ positioner.

3.7. Collect MRI prescan images. Compare these images to the images collected after FUS BBB opening (**Figure 4**). T1-weighted images will later be used to visualize BBB opening, T2-weighted images will later be used to ensure no tissue damage has occurred following FUS treatment[34].

3.7.1. For T1-weighted axial images, use the following parameters: width: 30 mm, height: 51.2 mm, depth: 3.0 mm, voxel size: 0.23 x 0.2 x 0.23 mm^3, number of images: 13.

3.7.2. For T2-weighted axial images, use the following parameters: width: 30 mm, height: 51.2 mm, depth: 2.6 mm, voxel size: 0.2 x 0.2 x 0.2 mm^3, number of images: 13.

3.7.3. For T1-weighted coronal images, use the following parameters: width: 30 mm, height: 30 mm, depth: 27 mm, voxel size: 0.16 x 0.16 x 1 mm^3, number of images: 27.

3.7.4. For T2-weighted coronal images, use the following parameters: width: 30 mm, height: 30 mm, depth: 27 mm, voxel size: 0.12 x 0.12 x 1 mm^3, number of images: 27.

NOTE: As in step 3.5, these imaging parameters need not be exactly the same as listed. These images have a smaller FOV and higher resolution than the ones collected in step 3.5.

3.8. Keeping the animal in the stereotaxic frame, quickly transport the animal from the MRI bed to the benchtop FUS setup. Ensure that the animal remain asleep for the transfer under the effect of anesthesia.

3.9. For longer transfer times, use a box for transfer that is large enough to fit the animal and the frame. With the anesthesia plug still attached, place the animal and frame inside the box and allow the excess isoflurane to fill the box for a few minutes. Unplug the anesthesia line and quickly transfer.

4. Focused ultrasound procedure

4.1. Upon arriving to the FUS benchtop setup, immediately plug the anesthesia line into the nose cone and continue to run 1.5-3% isoflurane with oxygen. Do this as quickly as possible to avoid the animal waking up.

4.2. Slide the frame into the frame holder and snap it into place firmly. Use clippers to shave the animal's head. Brush away excess hair and apply hair remover cream to the scalp. Let sit for 3 min and wipe away with water and gauze.

4.3. If MRI is not available for targeting, use a standard (not 3D-printed) stereotactic frame to

null the pointer position to bregma by touching the pointer tip to bregma (a scalp incision will be needed for this) and nulling the software or recording the coordinates. Move the XYZ carriage up by 50 mm by clicking the **up 50** button in the software and swapping the pointer for the transducer. Based on a rat brain atlas as described previously[35], move to the desired brain coordinates using the stepping buttons in the software. If using this method instead of MRI, skip down to section 4.6.

4.4. If using MRI guidance, attach the pointer and move the pointer to the location of the MRI fiducial (**Figure 1d**,**g**). Position the pointer at the very top and center of the MRI fiducial (a small hole in the top of the fiducial holder is provided for pointing). Click the null position button which is the point from which all distances in the MRI image were calculated.

4.5. Remove the pointer and move the positioner to the medial/lateral coordinates and the rostral/caudal coordinates. Raise the positioner up by pressing the **up 50** button to allow for the placement of the water bath and ultrasound gel. If the top of the Z-axis travel is reached, the nulling location will be invalidated. The dorsal/ventral coordinate will be set after the transducer is added.

4.6. Apply ultrasound gel to the animal's scalp and place the water bath over the animal with the polyimide tape window pressed onto the gel. Make sure that there are no air bubbles in the ultrasound gel.

4.7. Fill the water bath with degassed water.

4.8. If using the high-power transducer, lower the positioner so that the magnet is just above the water. Attach the transducer to the positioner by carefully lowering the transducer into the water at an angle to prevent air bubbles from getting trapped underneath the face and connect the magnets.

4.9. If using the low-power immersion transducer, lower the positioner into the water just above the transducer clip. Then clip the transducer in place by slowly lowering it into the water at an angle to prevent air bubbles from getting trapped underneath the face.

NOTE: A transparent bath is helpful when looking under the transducer face for bubbles.

4.10. Lower the positioner to the dorsal/ventral coordinate.

4.11. Turn on the RF power amp.

4.12. Inject 1 mL/kg of 3% Evans blue dye (EBD) by sticking the needle tip into the catheter plug and injecting. Allow it to circulate for 5 min.

4.13. Activate the microbubbles by shaking them violently with the with the bubble shaker.

4.13.1. Prepare 5x the dose of 30 µL/kg of microbubbles (bubble conc. 1.2 x 10^{10}/mL) in 0.2 mL of saline to account for the 2 FUS treatments and the tubing in the 18 G winged infusion set. For example, if the rat weighs 200 g, then fill the syringe containing 18 G needle tip with 30 µL of microbubbles in 1 mL of saline.

NOTE: Make sure to use 18 G needle tips for both up taking and injecting with the winged infusion set.

4.13.2. Invert the syringe several times to get a uniform distribution of microbubbles. Then attach and fill the winged infusion set. Position the syringe on the infusion pump and set the infusion pump to deliver 0.2 mL at a rate of 6 mL/min. This will provide slow infusion of the microbubbles over the 2-min FUS exposure.

4.13.3. Insert the winged needle into the catheter plug.

4.14. First, run the infusion pump, wait 3 s and start the FUS treatment by pressing the output enable button on the function generator (labeled "**on**" on the function generator in the **Table of Materials**). Repeat these two times per region with 5 min in between to allow the microbubbles to clear.

4.14.1. Press the **on** button again on the function generator to stop the FUS treatment when the infusion pump stops at 2 min.

4.14.2. Wait for 5 min for the microbubbles to clear. Then start the infusion and the second FUS treatment.

4.14.3. Immediately after the second FUS treatment, inject gadobutrol contrast (if using MRI) and the agent of interest, for example, viral particles. Total delivered volume of all agents should not exceed 5 mL/kg.

NOTE: The timing of delivery (e.g., before or after FUS BBB opening) of the agent of interest may differ depending on the agent used.

4.15. Turn off the RF power amp and immediately transport animal back to the MRI.

5. MRI confirmation of BBB opening

5.1. If MRI is not available, skip to section 6 and use EBD expression for confirmation of BBB opening.

5.2. Place the animal back onto the MRI bed at the exact same location as in step 3.7 and plug in the anesthesia line.

5.3. Collect the MRI post scans with the same imaging parameters used in step 3.7 to visualize

gadobutrol MRI enhancement in the region of BBB opening (**Figure 3b**,**e**).

6. Perfusion and tissue collection

6.1. Perfuse the animal with cold 4% formalin until the blood runs completely clear.

6.2. Remove the brain and place in 4% formalin or PFA at 4 °C overnight. Next, place brain in 30% sucrose solution until the brain sinks (about 2-3 days). Finally, flash freeze in liquid nitrogen or on dry ice and store at -80°C until cryosectioning.

6.3. Freeze the brain in OCT and take cryosections.

6.4. Fix and coverslip sections for fluorescence microscopy. EBD excitation peaks at 470 and 540 nm and emission peaks at 680 nm. Coverslip with DAPI mounting medium in order to visualize overall cellular morphology.

REPRESENTATIVE RESULTS:

Here, we demonstrate that focused ultrasound with microbubbles can induce localized BBB opening using the parameters specified above with both the low-power immersion transducer (**Figure 3**) and the FUS transducer (**Figure 4**). First, in early experiments, the low-power immersion transducer was targeted to one brain hemisphere either anterior (**Figure 3b**) or medial **(Figure 3a**). Animals were then sacrificed 2 hours later with perfusion (**Figure 3a**) or without perfusion (**Figure 3b**) and 10 µm frozen brain sections were collected. FUS BBB opening was evident by EBD autofluorescence (excitation: 470 and 540 nm, emission: 680 nm) in the target hemisphere (white arrows **Figure 3a and 3b**).

We have found it best to perfuse the animals for clear visualization of BBB opening with EBD autofluorescence. However, BBB opening can still be visualized without clearing the blood vessels (**Figure 3b**). Cellular uptake and clearance of EBD following BBB opening begins as soon as 30 minutes after BBB opening and increases over 24 hours[37]. For evaluation of BBB opening with EBD autofluorescence, it is best to sacrifice the animal between 15 minutes and 3 hours of BBB opening. Though ultimately, the time of sacrifice will depend on the agent that was delivered. For example, in an AAV study, 3 weeks post BBB opening and AAV delivery (**Figure 5c**) may be appropriate.

In later experiments, the FUS transducer was targeted to either the hippocampus (**Figure 4a-c**) or the anterior cingulate cortex (ACC) (**Figure 4 d-f**) and in addition to EBD, the MRI contrast agent gadobutrol (0.1 mL/kg) was IV injected to verify targeted opening of the BBB in vivo. **Figure 4b**,**e** show enhanced MRI contrast where the gadobutrol contrast has entered the tissue 1 hour after BBB opening and contrast agent injection. This contrast change is evident when comparing to the MRI prescans taken before the FUS procedure (**Figure 4a,d**). Animals were then sacrificed by perfusion 1.5 hours after BBB opening and 10 µm cryosections were collected. EBD autofluorescence is evident in FUS targeted regions further indicating location of BBB opening (**Figure 4c,f**). This figure highlights how MRI contrast can sometimes be difficult to see (as in the

difference between **Figure 4b** and **Figure 4e**); therefore, it is helpful to confirm BBB opening with visualization of EBD autofluorescence as in the fluorescence micrograph in **Figure 4f**.

To assess whether this technique could be used for the targeted gene delivery AAV9-hsyn-GFP and gadobutrol contrast were injected IV (titer: 1.32×10^{14} GC/mL, 0.05 mL/kg) immediately after BBB opening in the hippocampus. The animal was then MR imaged 30 minutes after BBB opening and sacrificed 3 weeks later by perfusion. 10 µm cryosections were collected for fluorescent imaging of GFP expression. BBB opening was evident by gadobutrol contrast in the target hippocampus (**Figure 5a,b**). In addition, gene delivery was confirmed by GFP expression in the target hippocampus evident by green fluorescence (**Figure 5c**). Note that at this time point EBD has cleared out and is only evident in the ventricles (**Figure 5c**).

FIGURE AND TABLE LEGENDS:

Figure 1: FUS benchtop setup. (**a**) FUS setup including the XYZ positioner, a 30 mm diameter PVC pipe for attachment of the transducer, the 3D-printed stereotaxic frame, and the infusion pump. (**b**) The end of the PVC pipe is capped, and a magnet is attached to it with epoxy. (**c**) Another matching magnet is attached to the top center of the high-power transducer with epoxy. (**d**) In addition, another matching magnet is attached to the pointer for nulling the positioner at the top and center of the MRI fiducial. (**e**) The pointer is eventually replaced with the high-power transducer and a water bath is coupled to the animal's head with ultrasound gel. (**f**) The low-power immersion transducer can be attached to the PVC pipe with the 3D-printed transducer clip. (**g**) For positioning, the transducer is replaced with the 3D-printed pointer and the positioner is nulled at the top and center of the MRI fiducial. (**h**) The stationary 3D-printed frame holder allows for the animal to be returned to the same position after MRI if multiple FUS treatments are needed.

Figure 2: Stereotaxic frame with MRI fiducial for MRI-guided coordinates. (**a**) Animals are first positioned in a 3D-printed stereotaxic frame equipped with an MRI fiducial. (**b**) The frame is then placed inside the MRI bed and distance from the fiducial (dotted circle) to the target brain region is measured using both coronal images for the dorsal/ventral (D/V) measurements and axial images for the rostral/caudal (R/C) measurements, medial/lateral (M/L) measurement can be collected from both axes. Animals are kept in the frame and transferred to the FUS station where a pointer is used to null the XYZ positioner at the location of the fiducial. The pointer is then replaced with the transducer and which can then be moved based on the coordinates collected.

Figure 3: Evans blue dye (EBD) confirms FUS BBB opening both with and without perfusion. (**a**) Micrograph of a 10 µm brain section 2 hours after FUS BBB opening with the low-power immersion transducer targeted to the medial left hemisphere. This is a representative image of an animal that was perfused with 4% buffered formalin prior to tissue collection. BBB opening is evident by EBD red autofluorescence (arrow). (b) Micrograph of a 10 µm brain section 2 hours after FUS BBB opening targeted to the anterior left hemisphere. This is a representative image of an animal who was not perfused prior to tissue collection therefore, EBD remains in the blood vessels. BBB opening is evident where EBD has leaked out of the blood vessels (arrow).

Figure 4: FUS BBB opening confirmed with Gadobutrol MRI contrast and EBD expression. MR images before (**a** and **d**) and after (**b** and **e**) BBB opening. gadobutrol contrast enhancement confirms location of BBB opening in vivo (**b** and **e**, arrows). (**c**) 10 µm brain section showing further confirmation of BBB opening with EBD autofluorescence (red) in the hippocampus (blue DAPI nuclear stain). Scale bar; 500 µm. (**f**) Micrograph of a 10 µm brain section following BBB opening in the anterior cingulate cortex evident by EBD red autofluorescence (arrow).

Figure 5: Localized delivery of AAV9-hsyn-GFP to the hippocampus via FUS BBB opening. MRI confirmation of BBB opening with MRI contrast agent, MRI contrast (arrows) in both coronal (**a**) and axial (**b**) T1-weighted images. (**c**) histological confirmation of GFP expression in the FUS targeted hippocampus (green) 3 weeks after FUS BBB opening and AAV9-hsyn-GFP IV injection. Blue indicates DAPI nuclear stain for overall cellular morphology.

DISCUSSION:

Here we described a benchtop approach to microbubble assisted FUS BBB opening with alternative approaches including, two different transducers and methods for intracranial targeting with and without MRI guidance. Currently, in order to establish MRI-guided FUS BBB opening in the lab, there is the option to purchase excellent ready-to use devices that provide highly standardized and reproducible results with user-friendly interfaces. However, many labs are not prepared for the cost of such instruments. Therefore, the main goal of this protocol is to provide a starting point that any lab could establish in order to build their expertise in the technique.

FUS BBB opening is now a widely used technique and it is often the case that different groups utilize a variety of agents and anesthetics, each of which can affect the degree of BBB opening and extravasation. Importantly, the particular anesthetic used can affect the magnitude of BBB opening so it is important to consider this when executing this protocol[38]. Here, the anesthetic isoflurane is used because animals can be maintained under isoflurane for the length of the protocol and levels of isoflurane gas can be easily adjusted based on the animal's respiration rate and heart rate. In addition, we delivered isoflurane with oxygen because it was more accessible than medical air; however, medical air may allow more extensive BBB opening[39]. Some MRI contrast agents are more appropriate for this protocol than others. For example, in our hands, gadoteridol produced no contrast enhancement even when EBD leakage was clearly present in tissue postmortem. Microbubble formulation is also important. Here we use perflutren lipid microspheres. Other microbubble formulations such as perflutren protein-type A microbubbles are readily available, but the type of microbubble used will affect the results[15].

Controls of function generators can vary quite a bit, so refer to the manual for instructions on how to enter the settings listed in step 1. The appropriate command voltage (V peak to peak on the function generator) depends strongly on the transducer properties, RF amplifier gain, RF amplifier to transducer matching, the age and size of the animal, the microbubble type and concentration, and the desired treatment effect. The peak to peak V will need to be determined by trial and error. Start with the settings suggested in step 1 and determine the effect histologically. If there is tissue damage, lower the peak to peak V by 10% and try again. Likewise,

484 if there is no BBBO then raise the peak to peak V by 10% and try again. Setting too high of a V can
485 damage the low power immersion transducer. This will be apparent as a cracking or distortion of
486 the transducer face. The manufacturing lead times on transducers can be long, so when starting,
487 we suggest purchasing more than one transducer as a backup. Ultrasound transducers can also
488 damage amplifiers if they are improperly matched. For simplicity and reliability, we suggest using
489 a rugged power amplifier that can drive complex loads (such as the RF power amplifier in the
490 materials table). Note that while the high-power transducer comes with a matching circuit, the
491 low power immersion transducer does not. The suggested RF amplifier can handle the reflected
492 power from the poorly matched transducer, but some amplifiers may be damaged in this
493 configuration. Also, if step 2.7 proves problematic after installing the driver and software, double
494 check that the proper serial port is selected in the software. Next try a different serial cable. If
495 that fails, then seek local IT support.
496
497 From experience, it will take practice and multiple adjustments to achieve tight accuracy in brain
498 region targeting. This can be seen in the differences of targeting between the early experiments
499 (**Figure 3**) and the most recent experiments (**Figure 5**). We began the experiments using a classic
500 stereotaxic frame and we include it as an option here if there is no access to a 3D printer or if
501 there is no access to rodent MRI. However, MRI-guidance with MRI fiducials and the 3D printable
502 frame provided (or of a custom design) is the ideal method. First, it accounts for individual
503 differences between animals by gathering coordinates within the animal rather than relying on
504 an averaged rat brain atlas. In addition, the use of MRI allows confirmation of FUS location
505 targeting in vivo rather than relying on postmortem EBD expression. This is important when
506 delivering agents that may require more than 24 hours to take effect such as AAVs (**Figure 5**).
507 Lastly, the frame holder provided allows returning the animal back to the same position after
508 MRI to fix any targeting errors or to repeat FUS after insufficient BBB opening without having to
509 redo the coordinates. The portable 3D-printed stereotaxic frame can be used in any MRI with a
510 clear bore of 200 mm or wider.
511
512 Depending on the blood vessel distribution in the target location, skull thickness[40], the presence
513 of ventricles, and other factors the degree of BBB opening can vary. For this reason, we provide
514 a method for repeated targeting with the frame and frame holder. Accuracy of targeting depends
515 critically on keeping the transducer focus at a consistent location with respect to the targeting
516 pointer. The tip of this pointer should indicate the location in space of the center of the
517 transducer focus when the transducer is attached to the XYZ positioner. The magnets allow the
518 pointer and transducer to be easily swapped while maintaining this colocalization. The hole and
519 protrusion in the magnets should match as precisely as possible. Any variability in this connection
520 reduces the repeatability of the FUS focus targeting. However, there will be a spatial offset
521 between the pointer tip and the ultrasound focus. Once it is confirmed that the offset is
522 consistent, it can be corrected by using the MR images to calculate the difference in mm of the
523 resulting BBB opening location (location of MRI contrast) and the intended target location. This
524 difference can then be factored into the null location. Accuracy and repeatability also benefit
525 greatly from using the same ultrasound frequency and using rats of a similar size and age in a
526 given set of experiments. Ultrasound attenuation by the rat brain and rat skull varies with
527 frequency and skull size and skull thickness varies with age. The skull is also a small cavity with

respect to the ultrasound pulse and the incident ultrasound interacts with reflections inside the skull to produce a complex sound field that is dependent on the tissue, skull, frequency and the position of the transducer[40].

As stated elsewhere, this protocol is intended to provide a low investment alternative to excellent MRI compatible commercial solutions that already available for purchase. There are important limitations that result from keeping the cost low. There are also limitations inherent to the technique given physics and the current state of the art. Remarkably, despite these limitations, and as shown in the representative results, we can achieve consistent delivery of dyes, particles, and viruses to the hippocampus of rats with submillimeter accuracy. The most important limitations are that (1) the shape of the FUS focus depends on the intervening tissue and especially the shape and thickness of the skull. The need to remove the animal from the MRI to perform the FUS treatment prevents real-time feedback on the localization and intensity of the FUS focus. Without this real-time feedback several experiments need to be done to confirm setting for each combination of targeting location. Once the settings are "dialed in", we have found good repeatability. (2) The XYZ positioning and fiducial system as constructed, while precise, does not provide accuracy in the coordinate frame of the positioner from experiment to experiment. The relative locations of the XYZ home, the rodent's skull, and the frame can move relative to each other from experiment to experiment. This is obviated by using an MRI image for targeting, a targeting pointer and fiducial with known location in space relative to the FUS focus, ensuring the MRI coordinate system is parallel to the XYZ positioner system, by doing test treatments prior to the set of real treatments and by doing the entire procedure within one session so that the animal does not need to be repositioned into the frame. Note that, because only one fiducial is used, frame rotations are not correctable, so it is critical to ensure the frame is level with respect to the MRI bed and bore. In summary, the pointer location does not indicate the true FUS focus, but we have found the offset is consistent for a given brain location provided the skull does not rotate relative to the ultrasound transducer. Also note that consistent timing of gadobutrol delivery relative to the BBB opening and the imaging is crucial for consistent results especially if MRI contrast change is used as a proxy for the amount of BBB opening (see [36]).

We first began FUS BBB opening in the lab with the low-power immersion transducer described above. We found that it is an affordable option for getting started with this technique. Most importantly, adaptation of this protocol can provide a noninvasive alternative to intracranial stereotaxic surgery and preclinical research performed with this technique can be considered highly translational due to the current use of transcranial FUS in humans[30,32,41]. Once established in a lab, this technique can be used as a noninvasive alternative to stereotaxic surgery. Thus, transforming a strictly investigative tool into a highly translational tool. Our lab will use this technique for the MRI guided, localized delivery of viruses and nanoparticles to develop novel, non-invasive neuromodulation techniques that can be used in freely behaving awake rodents and non-human primates. The current work focuses on Designer Drugs Exclusively Designed for Designer Receptors (DREADDs) and the sensitization of neurons to low doses of high energy particles such as x-rays. The lab is also working on a new version of this protocol that can be performed in a human 3 T scanner to eliminate the need to move the animal during the treatment and to allow real-time targeting feedback.

ACKNOWLEDGMENTS:

This research was supported in part by an NSF EPSCoR Research Infrastructure grant to Clemson University (1632881). In addition, this research was supported in part by the Civitan International Research Center, Birmingham, AL. The authors gratefully acknowledge the use of the services and facilities of the University of Alabama at Birmingham Small Animal Imaging Shared Facility.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

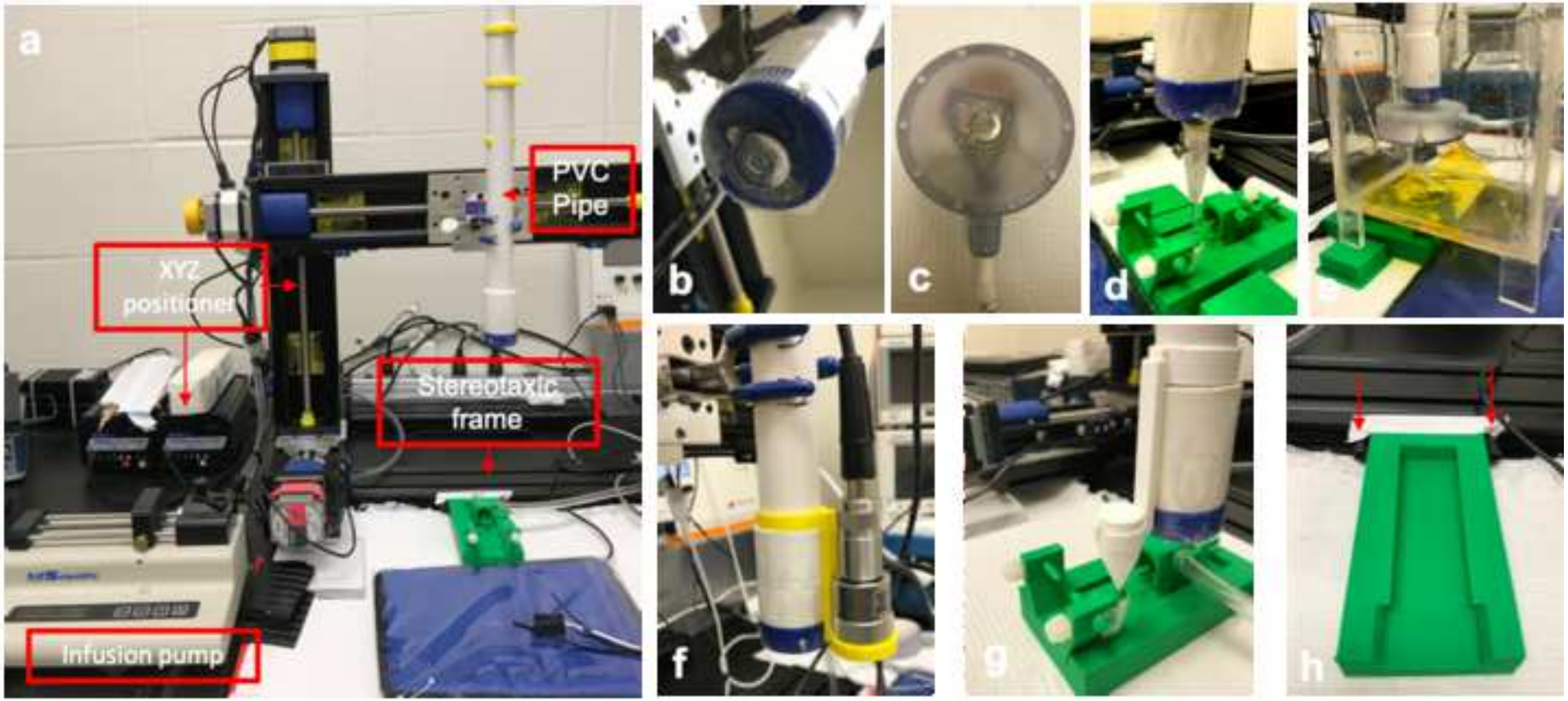

Figure 2

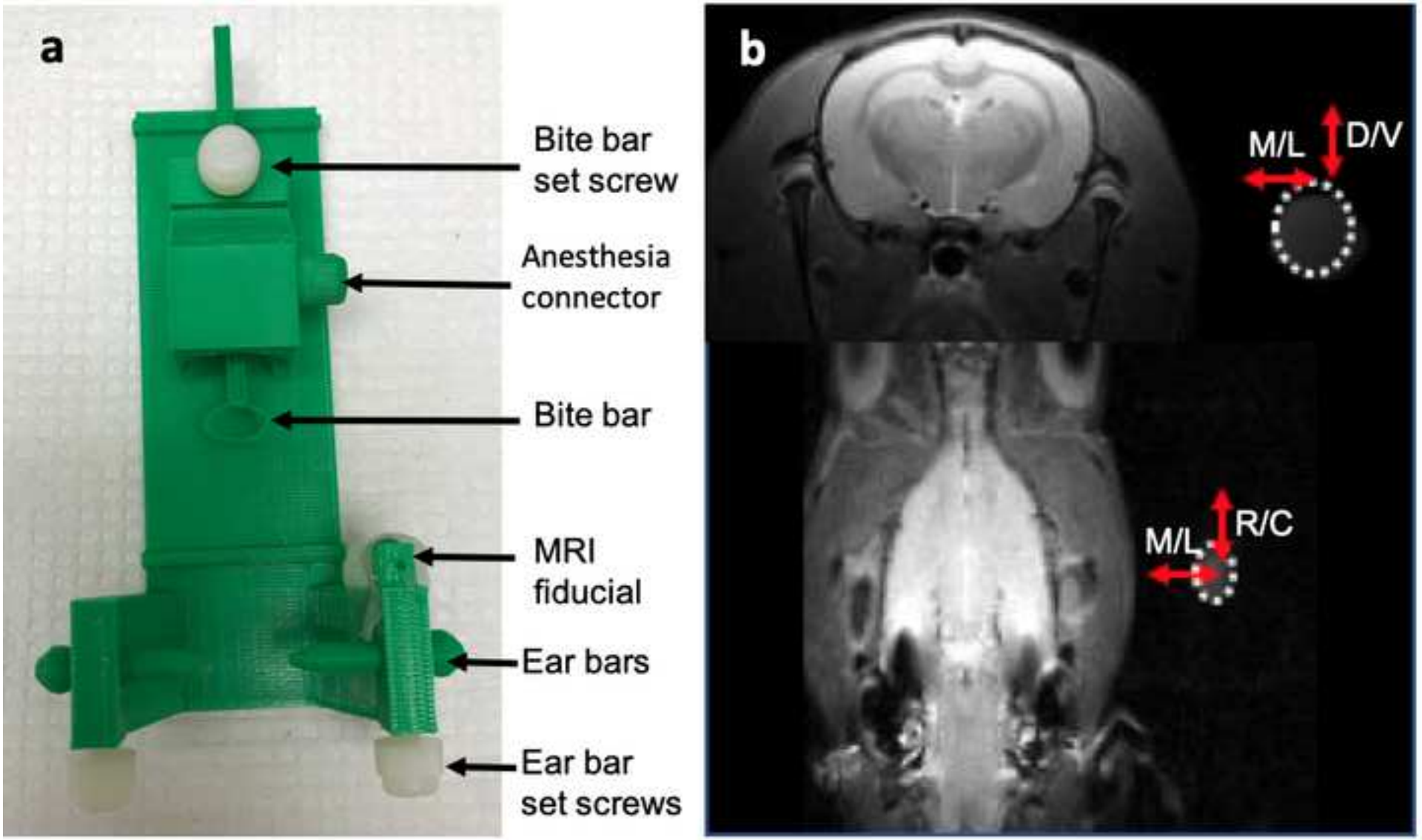

Figure 3

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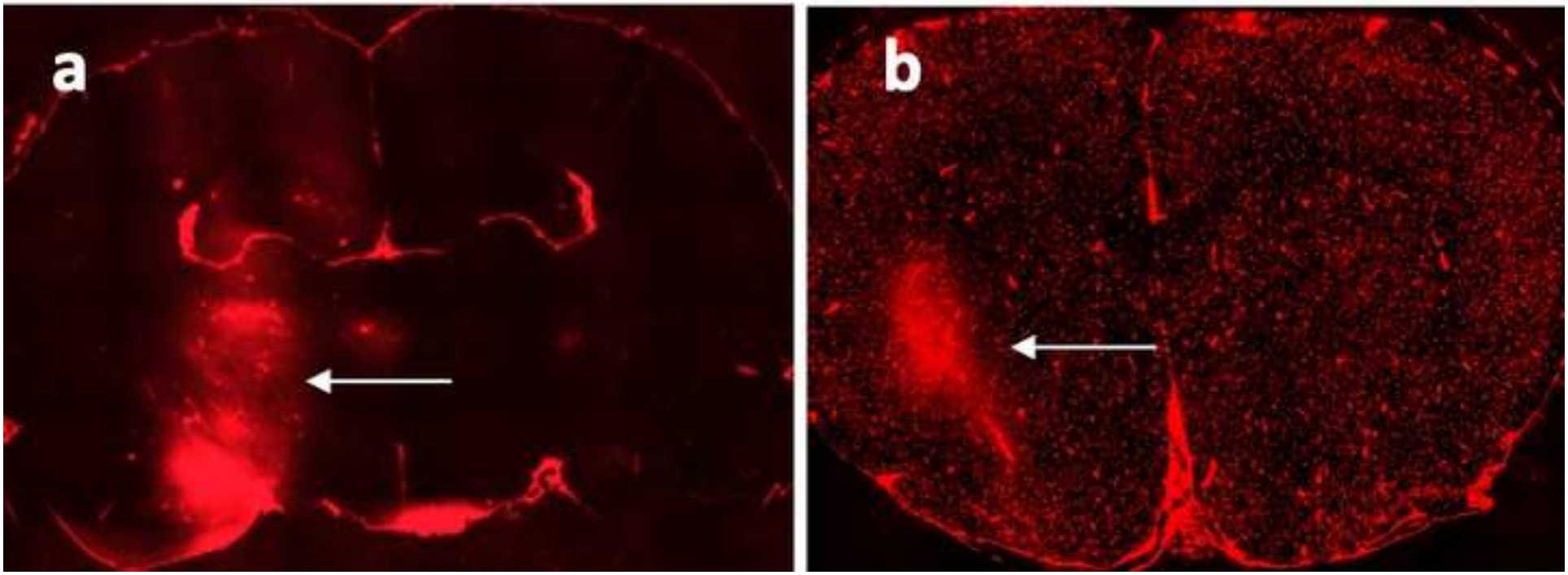

Figure 4

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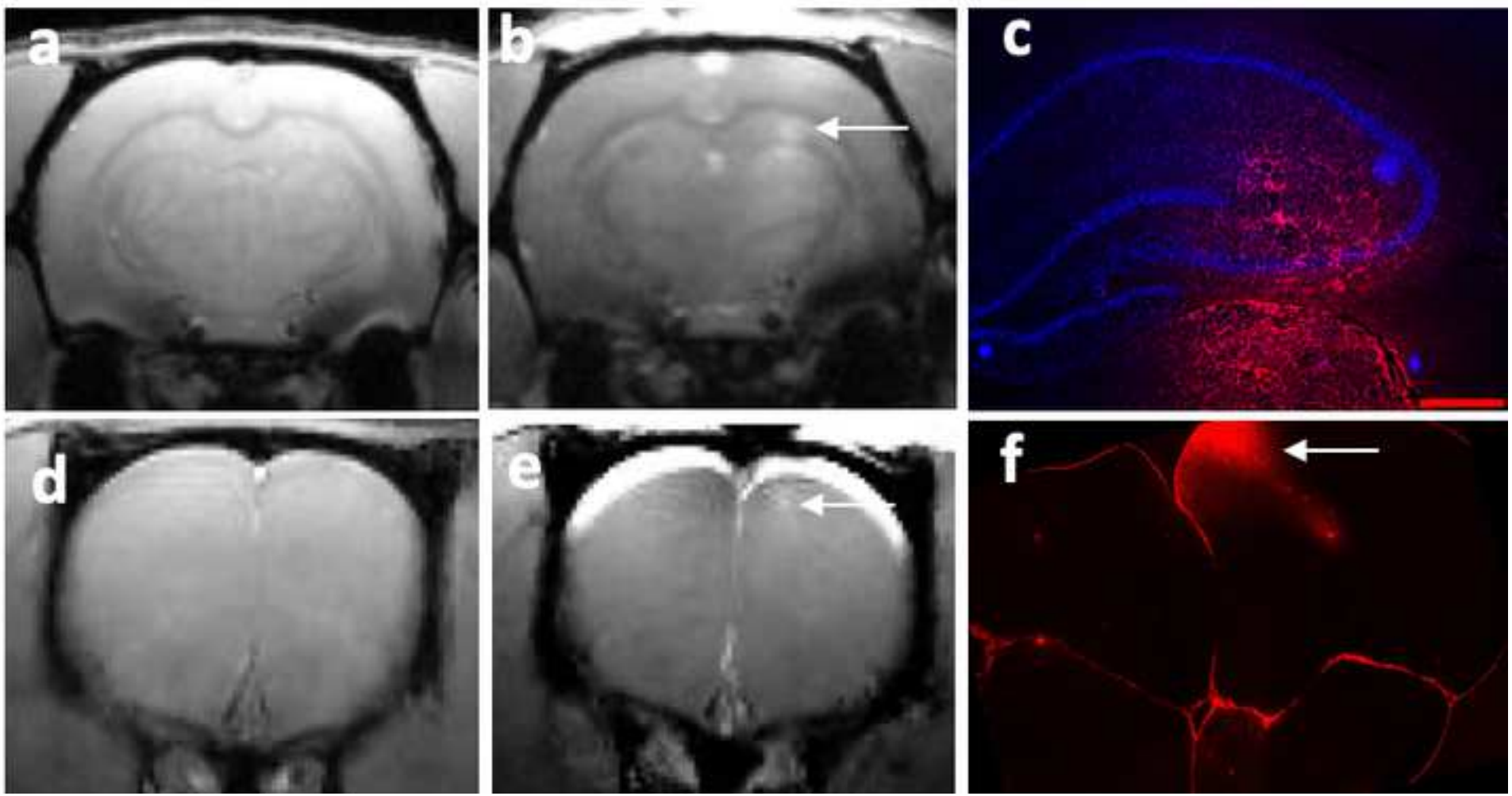

Figure 5

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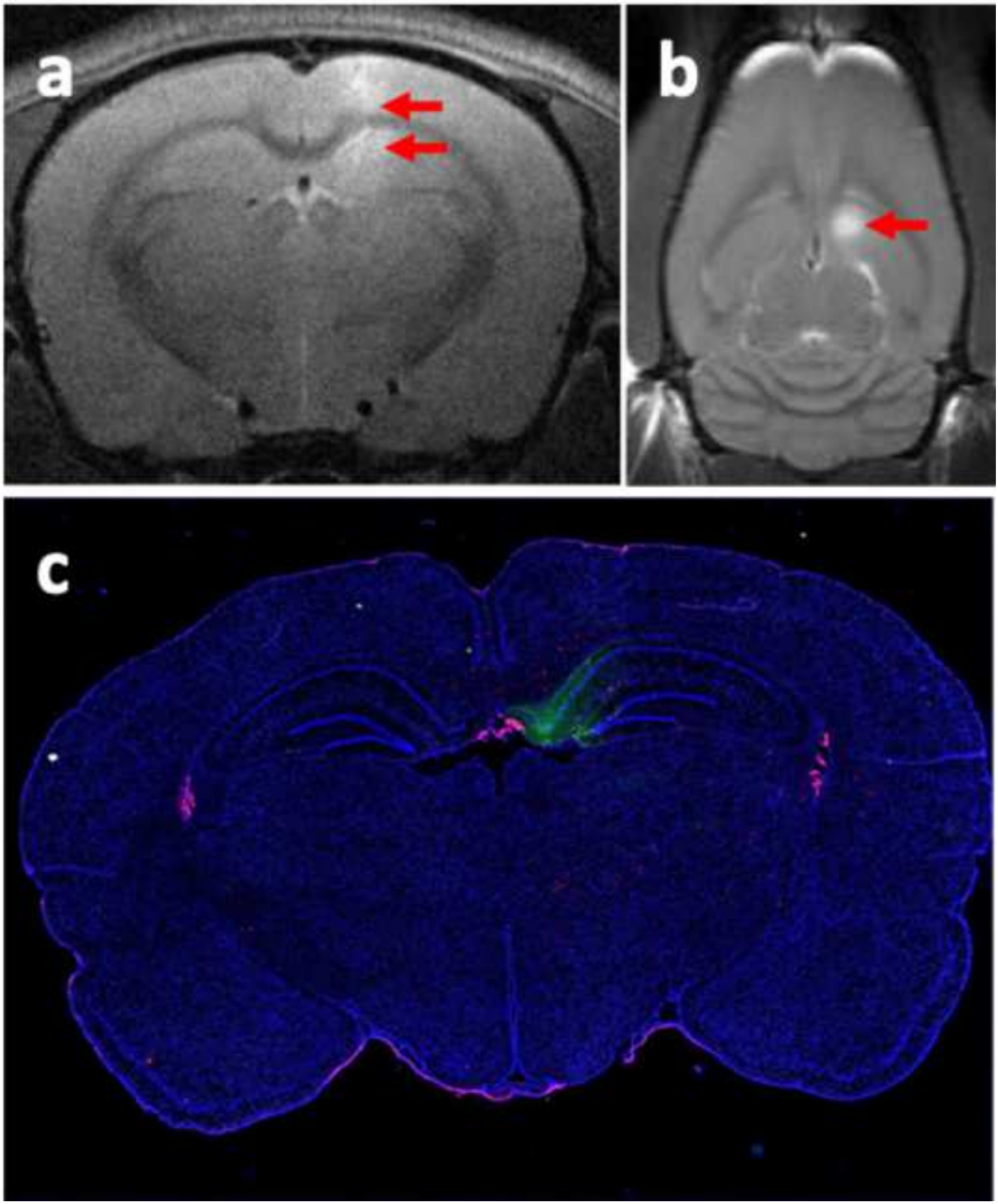

Name of Material/ Equipment	Company	Catalog Number
Bubble shaker	Lantheus Medical Imaging	VMIX
Catheter plug/ Injection cap	SAI infusion technologies	Part Number: IC
Evans blue dye	Sigma	E2129-10G
Function generator	Tektronix	AFG3022B
FUS transducer, 1.1MHz	FUS Instruments	TX-110
Heating pad for Mice and Rats	Kent Scientific	PS-03
Infusion pump	KD Scientific	780100
Kapton tape	Gizmo Dorks	https://www.amazon.com/dp/B01
Low power immersion transducer, 1MHz	Olympus	V303-SU
Magnet sets	WINOMO	https://www.amazon.com/dp/B01
RF amplifier	E&I	A075
Tail vein catheter	BD	382512/ Fisher Item: NC122851
Ultrasound contrast microbubbles	Lantheus Medical Imaging	DE4, DE16
Ultrasound gel	Aquasonic	https://www.amazon.com/dp/B07
Winged infusion sets, 22ga.	Fisher Healthcare	22-258087
motor controller software	N/A	N/A
runtime environment for the motor controll	National Instruments	LabView runtime engine version
3 axis Linear stage actuator (XYZ positioner)	Velmex	
bolts	Velmex	MB-1
motor controller	Velmex	VXM-3
mounting cleats	Velmex	MC-2
mounting cleats	Velmex	MC-2
usb to serial converter	Velmex	VXM-USB-RS232
x-axis linear stage	Velmex	MN10-0100-M02-21
x-axis stepper motor	Velmex	PK266-03A-P1
y-axis linear stage	Velmex	MN10-0100-M02-21
y-axis stepper motor	Velmex	PK266-03A-P1
z-axis damper	Velmex	D6CL-6.3F
z-axis linear stage	Velmex	MN10-0100-M02-21
z-axis stepper motor	Velmex	PK266-03B-P2

3D printable files

Immersion transducer mount and pointer
Stereotaxic frame
Stereotaxic frame holder

9.4T small bore animal MRI	Bruker	Bruker BioSpec 94/20
AAV9-hsyn-GFP	Addgene	
Cream hair remover	Church & Dwight	
gadobutrol MRI contrast agent	Bayer	
Stereotactic frame	Stoelting	#51500
turnkey FUS delivery device	FUS Instruments	RK-300

Comments/Description

VIALMIX, actiation device used to activate Definity microbubbles
Catheter plug/ Injection cap
Evans blue dye
Dual channel, 250MS/s, 25MHz
1 MHz MRI-compatible spherically focused ultrasound transducer with a hydrophone
Heating pad- PhysioSuite for Mice and Rats
KDS 100 Legacy Single Syringe Infusion Pump
Gizmo Dorks Kapton Tape (Polyimide) for 3D Printers and Printing, 8 x 8 inches, 10 Sheets per Pack
Immersion Transducer, 1 MHz, 0.50 in. Element Diameter, Standard Case Style, Straight UHF Connector, F=0.80IN PTF
WINOMO 15mm Sew In Magnetic Bag Clasps for Sewing Scrapbooking - 10 Sets
75W
24g BD Insyte Autoguard shielded IV catheters (non-winged)
DEFINITY (Perflutren Lipid Microsphere)
Ultrasound Gel Aquasonic 100 Transmission 1 Liter Squeeze Bottle
Terumo Surflo Winged Infusion Sets

custom software written in LabView for controlling the Velmex motor controller
https://www.ni.com/en-us/support/downloads/software-products/download.labview.html

BiSlide Bolt 1/4-20x3/4" Socket cap screw (10 pack), Qty:3
Control,3 axis programmable stepping motor control, Qty:1
Cleat, 2 hole BiSlide, Qty:6
Cleat, 2 hole BiSlide, Qty:2
USB to RS232 Serial Communication Cable 10ft, Qty:1
BiSlide, travel=10 inch, 2 mm/rev, limits, NEMA 23, Qty:1
Vexta Type 23T2, Single Shaft Stepper Motor, Qty:1
BiSlide, travel=10 inch, 2 mm/rev, limits, NEMA 23, Qty:1
Vexta Type 23T2, Single Shaft Stepper Motor, Qty:1
D6CL Damper for Type 23 Double Shaft Stepper Motor, Qty:1
BiSlide, travel=10 inch, 2 mm/rev, limits, NEMA 23, Qty:1
Vexta Type 23T2, Double Shaft Stepper Motor, Qty:1

https://www.tinkercad.com/things/cRgTthGXSRq
https://www.tinkercad.com/things/ilynoQcdqlH
https://www.tinkercad.com/things/aZNgqhBOHAX

ParaVision version 5.1

Nair cream
Gadavist (Gadobutrol, 1mM/mL)
not MRI compatible
ready to use MRI compatible FUS for rodents

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.

The manuscript has been thoroughly proofread and any spelling or grammar issues have been corrected.

2. Please provide affiliations for each author in the manuscript doc.

Author affiliations have been added.

3. Please provide an email address for each author.

Email addresses for each author have been added.

4. Please ensure that the Summary clearly describes the protocol and its applications in complete sentences between 10-50 words. Presently it is more than 50-word limit.

The summary has been edited and reduced to 49 words.

5. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

The abstract is within the word limit and has been edited to clearly state the goal of the protocol.

6. JoVE cannot publish manuscripts containing commercial language. 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: FUS Instruments (Toronto, ON), Olympus (NDT Instruments, Immersion transducer), power amplifier (Electronics and Innovation, LTD, Rochester, NY), function generator (Tektronix, Beaverton, OR), BNC cables, Olympus Immersion transducer, XYZ positioner (Velmex, Bloomfield, NY), X Bislide, LabVIEW, kapton tape, etc.

All commercial language has been removed and all commercial products have been added to the materials table.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

The numbering of the protocol has been edited to follow JoVE Instructions for Authors.

8. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

The ethics statement has been moved, it is now just before the first protocol step.

9. 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.”

All the text in the protocol has been edited and is now in the imperative tense. Any text that could not be written in the imperative has been added as a “note”.

10. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

The text has been revised to no longer include any personal pronouns.

11. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Intro/Discussion.

Any discussion items in the protocol section have been moved to either the Introduction section or the Discussion section.

12. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

The protocol steps have been edited to include only 2-3 actions per step.

13. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step.

The protocol has been edited to include notes only when absolutely necessary. The remaining notes have been edited to be brief and concise. Details about how to perform a step were either made very concise and added to the step itself or were added as a substep.

14. Please ensure you answer the “how” question, i.e., how is the step performed? Please include all the button clicks in the software, knob turns, hard experimental steps etc.

Each protocol step has been edited to answer the question; “how was this done”.

15. 1: How is this done?

A more detailed explanation of step 1 has been added.

16. 2.1: Please include the CAD Files for 3D printing.

The links were made public so they will not expire and have been added to the materials table. In addition, we have included the stl files.

17. 2.4: How is this done?

A more detailed explanation of step 2.4 has been added.

18. Please use micro symbol from the insert symbol function. Please do not use u.

Any “u” has been replaced with a micro symbol.

19. 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.

The highlighted text amounts to 2 pages including headings and spacing.

20. 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].”

All figures in the manuscript are original.

21. As we are a methods journal, please revise 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

The Discussion section had been edited to include only the above listed items. Any extensive notes that had previously been in the protocol section have been moved to the Discussion section.

Reviewer 1

Pg. 4, Sec 2.2: Discuss the accuracy of using magnet. Particularly, any means to prevent from focal beam tilting problem? I suggest to more some description.

Discussion of the importance of magnet placement for accuracy has been discussed. Furthermore, the accuracy of using the MRI and single-fiducial system for the FUS focus targeting and the inherent limitations of this system has been added to the Discussion section.

Pg. 4, Sec. 2.3: How to confirm the pointer indicates the true energy focus of the focused ultrasound transducer? Geometrically measurement may not be sufficient. I suggest to give more detailed description.

A more detailed description of how to calibrate the pointer to match the FUS focus has been provided.

Pg. 4. Sec. 2.4: State how to make a MR fiducial.

A description of how to make an MR fiducial has been added in sec. 2.3.

Pg. 6, Sec. 3.6: List MRI T1 and T2 parameters

Both T1 and T2 MRI parameters have been included.

Pg. 6, Sec. 3.7: Describe clearer how to correlate MR T2 anatomical imaging and utilize it to perform precision guidance of the FUS beam to specific brain region.

A clearer description of how to use the MR anatomical images to guide the FUS focus to specific brain regions has been added.

P. 6, Sec. 3.7: How to adjust the focal depth of the FUS energy in the animal brain?

A description of how to adjust the FUS focus has been added.

Reviewer 2

1. It would be helpful to discuss the choice of anesthetic, whether ketamine/xylazine, isoflurane as that choice affects the BBBO magnitude as noted by McDannold et al., (PMC3129385). When we choose isoflurane, it is essential to give details about the carrier gas, oxygen versus medical air as that has a significant effect on the BBBO threshold and the BBBO reproducibility, as in McDannold et al., (PMC5191922).

The choice of isoflurane with oxygen and how this can affect BBBO was discussed with the references mentioned.

2. Given that the magnitude of BBBO decays exponentially with time, there should be no or minimal delay in the timing between MR-contrast injection and T1-weighted MRI (e.g. see Marty et al., Journal of Cerebral Blood Flow & Metabolism. 2012). At the least, the need to be consistent regarding this timing should be emphasized. An ideal time to inject the MR-contrast agent is right before the T1-weighted MRI starts and to take at least 3-T1 sequences to see the Gadolinium kinetics during peak plasma concentration of Gd (Fig. 1C in Aryal et al., 2013).

The timing between FUS BBB opening and MR imaging has been included.

3. The ultrasound attenuation by the rat brain and rat skull at different frequencies should be discussed, as should the uncertainty from standing waves within the skull given the spatial-pulse length of the ultrasound pulses used in these procedures. The relationship between ultrasound attenuation and skull thickness with different ages should be specifically noted (O'Reilly et al., PMC3228246).

Ultrasound attenuation by the skull and the differing skull thickness with age has been added to the Discussion section with the reference mentioned.

4. In this review, the paper that demonstrated FUS-mediated BBBO for the first time should be included (Hynynen et al., PMID: 11526261).

Hynynen et al., PMID: 11526261 has been referenced in the introduction.

*What gauge of catheter is used for tail vein injection? This should be indicated in the part number and description.

The 24g catheter has been added to the protocol text and to the materials table.

*Suggest providing the dose of microbubbles/kg in addition to the volume used here, as different microbubble vendors used different concentrations of bubbles in their formulations.

The dose of the microbubbles was made more specific.

*The inclusion of links for the 3D printed files is and will be greatly appreciated.

The links were made public so they will not expire and have been added to the materials table. In addition, we have included the stl files.

A Benchtop Approach to the Location Specific Blood Brain Barrier Opening Using Focused Ultrasound in a Rat Model, Rebuttal 2, 26 Feb 2020

Editorial comments:

1. The editor has formatted the manuscript to match the journal style. Please retain and use the attached version for revision.

The attached version was used for revision with track changes.

2. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

The reference style was updated as requested.

3. Please address specific comments marked in the manuscript.

Specific comments in the manuscript were addressed using track changes.

4. Once done please ensure that the highlighted text is no more than 2.75 pages including headings and spacings.

We have confirmed that the highlighted text is less than 2.75 pages.

Small immersion transducer mount and pointer stl file

Stereotactic frame holder

Stereotactic frame