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Targeted delivery of therapeutic agents to the mouse brain using a stereotactic-guided focused ultrasound device

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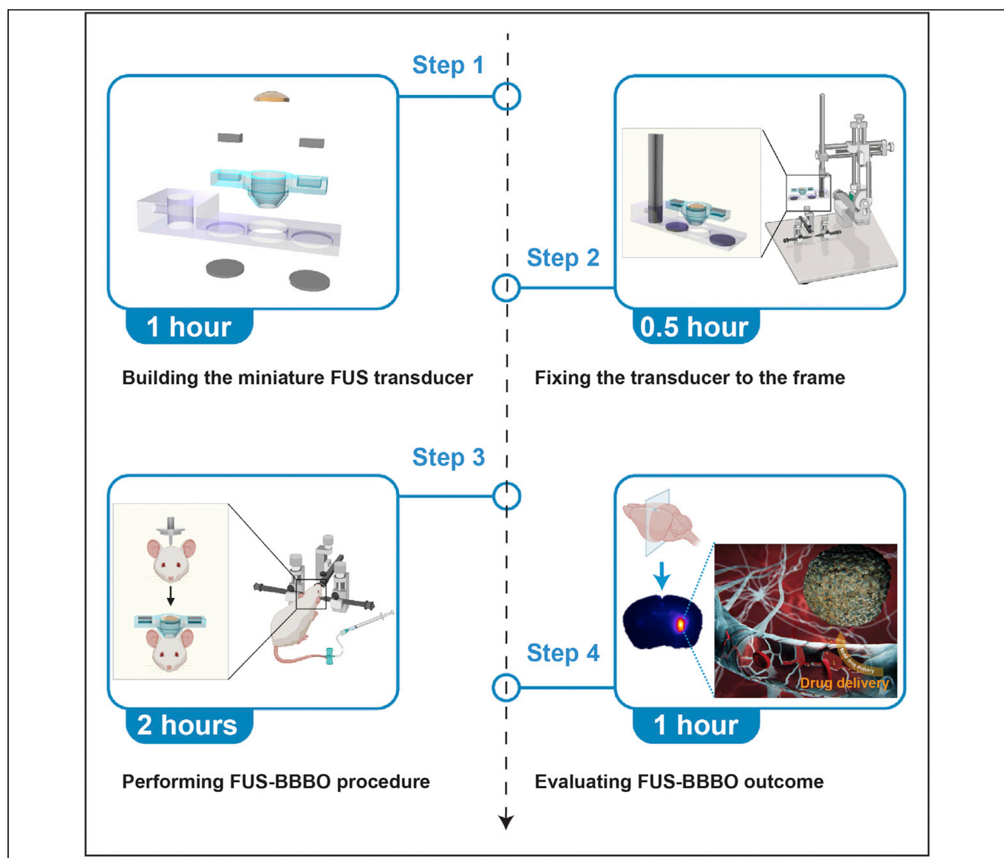

Existing protocols of focused ultrasound (FUS) combined with microbubble-mediated blood-brain barrier (BBB) opening (FUS-BBBO) in preclinical research require expensive ultrasound equipment and complex operating procedures. We developed a low-cost, easy-to-use, and precise FUS device for small animal models in preclinical research. Here, we provide a detailed protocol for building the FUS transducer, attaching the transducer to a stereotactic frame for precise brain targeting, applying the integrated FUS device to perform FUS-BBBO in mice, and evaluating the FUS-BBBO outcome.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for building a low-cost, easy-to-use, and precise focused ultrasound device

Steps for integrating the device with a stereotactic frame for precise brain targeting

Highly precise brain targeting of the built device on the mouse model

Example of using this device to perform brain drug delivery in mice

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Targeted delivery of therapeutic agents to the mouse brain using a stereotactic-guided focused ultrasound device

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SUMMARY

Existing protocols of focused ultrasound (FUS) combined with microbubble-mediated blood-brain barrier (BBB) opening (FUS-BBBO) in preclinical research require expensive ultrasound equipment and complex operating procedures. We developed a low-cost, easy-to-use, and precise FUS device for small animal models in preclinical research. Here, we provide a detailed protocol for building the FUS transducer, attaching the transducer to a stereotactic frame for precise brain targeting, applying the integrated FUS device to perform FUS-BBBO in mice, and evaluating the FUS-BBBO outcome.

For complete details on the use and execution of this protocol, please refer to Hu et al. (2022).[1]

BEFORE YOU BEGIN

FUS-BBBO is not only a promising technique for clinical applications but also a powerful preclinical research tool that can be adopted by a broad research community, including but not limited to neuroscience, neuro-oncology, and neurology. We provide a general protocol for performing FUS-BBBO in the mouse brain using a miniature FUS device made with a low-cost ultrasound transducer element (~$80 per element). This device is attached to a stereotactic frame using a 3D-printed adapter. The open-source 3D printing files are freely available at https://github.com/ChenUltrasoundLabWUSTL/stereotactic-system.git. Our protocol describes manufacturing the FUS device and conducting the FUS-BBBO experiment. Our protocol can be adapted to deliver various agents to the brain, such as chemotherapeutic agents, antibodies, nanoparticles, and gene vectors. In this protocol, Evans blue was used as a model drug to demonstrate the FUS-BBBO procedure.

Institutional permissions (if applicable)

All the procedures in animal studies must be approved by the Institutional Animal Care and Use Committee according to established regulations and guidelines. In this protocol, all procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Washington University in St. Louis in accordance with the National Institutes of Health Guidelines for Animal Research.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Evans blue	Sigma-Aldrich	Cat. # E2129-10G
Microbubbles	Lantheus Medical Imaging	Cat. # 515176-0690
Hair removal cream	Veet	Cat. # LL0247
Ultrasound gel	Aquasonics	Cat. # 03-50
Epoxy	Devcon	Cat. # 21045-6PK
0.9% saline	Fresenius Kabi	Cat. # 63323-186-03
Ophthalmic ointment	AVENTIX	Cat. # 065441
Experimental models: Organisms/strains		
Swiss-Webster mouse (6–10 weeks old of age, female)	Charles River Laboratories	Cat. # 024
Software and algorithms		
MATLAB	The MathWorks, Inc., MA, USA	https://www.mathworks.com/products/matlab.html
Image Studio	LI-COR Biosciences	https://www.licor.com/bio/image-studio/
GraphPad Prism	Dotmatics	https://www.graphpad.com/scientific-software/prism/
Other		
Hydrophone	Onda Corporation	Cat. # HGL-200
VialMix shaker	Lantheus Medical Imaging	Cat. # 515030-0508
Electrical heating pad	UL Standards	Cat. # 60730-1
Lead zirconate titanates	Del Piezo Specialties LLC	Cat. # DL-47
Epoxy	Devcon	Cat. # DA005
Stereotactic apparatus	David Kopf Instruments	Cat. # 940
Function generator	Keysight Technologies Inc.	Cat. # 33500B
Power amplifier	Electronics & Innovation	Cat. # 1020L
3D printer	Ultimaker Ltd.	Cat. # 62368-1
Neodymium magnets	McMaster-Carr	Cat. # 7048T32
Hamilton syringe	Fisher Scientific	Cat. # 1825
Small animal hair clipper	WAHL	Cat. # 78089
Solders	Weller	Cat. # 84032-12205
BMC cable	Thorlabs	Cat. # CA2124
Polyethylene tubing	INTRAMEDICTM	Cat. # 427405
LI-COR imaging system	Pearl Trilogy	Cat. # 9430-01
Fluorescence microscope	Keyence	Cat. # BZ-X810
Mouse brain matrix	World Precision Instruments	Cat. # RBMS-200C
DEFINITY microbubbles stored at 4°C.		

STEP-BY-STEP METHOD DETAILS

Building the miniature FUS transducer

Timing: 0.5–1 h

This step outlines how to build the miniature FUS transducer and calibrate the built transducer. After this step, a functional FUS transducer will be built.

1. Prepare lead zirconate titanate (PZT) ceramic piezo material (Figure 1A).

   Note: This can be purchased from the manufacturers (e.g., Del Piezo Specialties LLC.) at ~$80 per element. The PZT used in our study has a diameter of 13 mm and a radius of curvature of 10 mm.

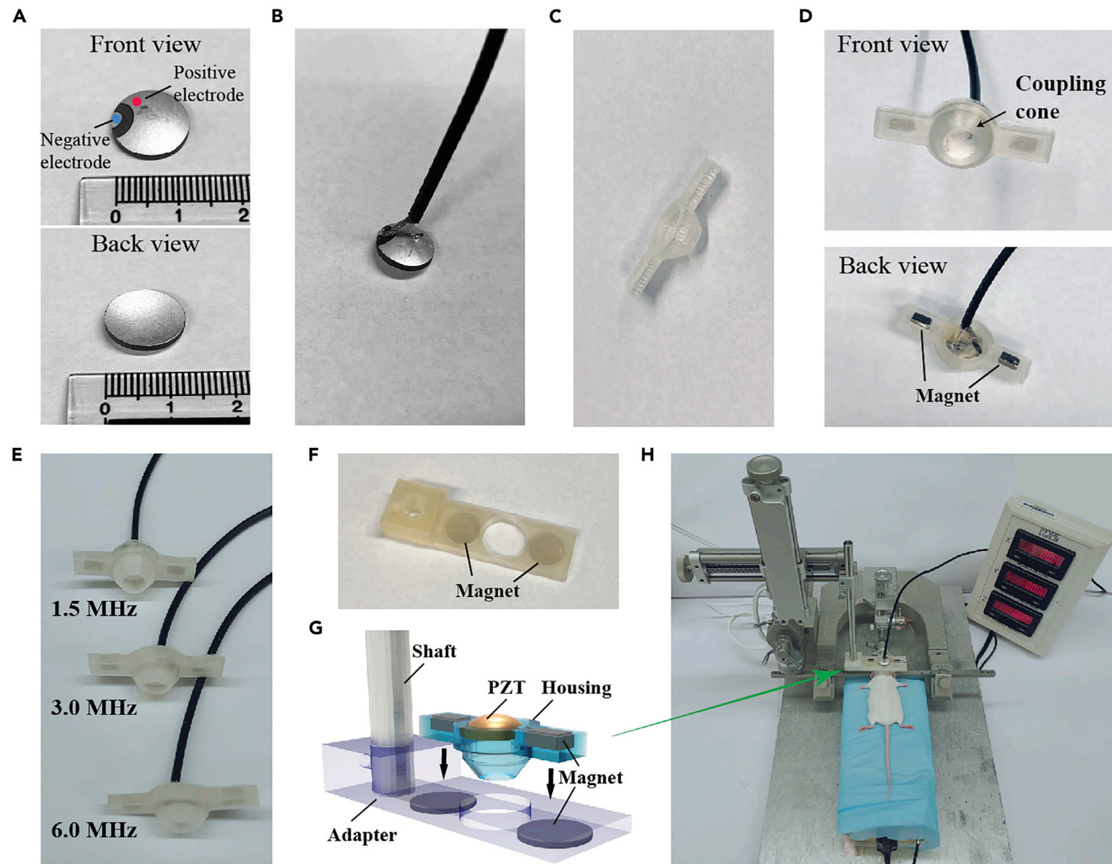

Figure 1. Miniature FUS device
(A) Lead zirconate titanate (PZT) elements.
(B) The coaxial cable/wires soldered to the PZT.
(C) 3D-printed housing.
(D) PZT in the 3D-printed housing. Two magnets are installed in the adapter.
(E) Fabricated transducers with different frequencies.
(F) 3D-printed adapter with two magnets.
(G) Adapter to connect the FUS transducer to the shaft of the stereotactic frame.
(H) Overview of the setup of the miniature FUS device for mice.

2. Split a 50-ohm impedance coaxial cable into positive and negative ports.
3. Connect the positive and negative ports to the positive and negative electrodes of the PZT by soldering (Figure 1B).

⚠ CRITICAL: After the cable wires are soldered to the electrodes, verify that the wires are connected to the electrodes in a closed circuit using a multimeter.

4. Cover the connection points of the soldered electrodes with epoxy.

Note: This procedure creates a waterproof covering and strengthens the attachment of the wires to the PZT element.

5. Fabricate the housing for the PZT using a commercial 3D printer (e.g., Ultimaker, Formlab, etc.) (Figure 1C).

Note: The 3D printing files of the housing design are available at https://github.com/ChenUltrasoundLabWUSTL/stereotactic-system.git. The 3D-printing material used in this study is polylactic acid (PLA).

6. Encase the PZT in the 3D-printed housing unit and use epoxy to seal the housing, and the FUS transducer will be ready (Figure 1D).

Note: No electrical impedance matching is needed because the real part of the transducer impedance at the resonance frequency is in the range of 31–59 ohms, which is close to the 50 ohms needed for a perfect impedance match.

⚠ CRITICAL: Use a multimeter to verify that the wires are connected to the electrodes using a multimeter during the assembly process until the PZT is completely secured to the housing.

7. Fill the transducer housing with degassed ultrasound gel in the coupling cone for acoustic coupling each time the device is used to generate ultrasound for FUS-BBBO.

Note: Ultrasound gel can be degassed using a centrifuge.

⚠ CRITICAL: Slowly inject degassed ultrasound gel into the coupling cone with a syringe to avoid gas trapped in the cone. Attention needs to be paid during this process to avoid trapping any bubbles.

8. Degas water by filling a side-arm flask with deionized and distilled water and connecting the side arm to the vacuum.

Note: The degassing process can be accelerated by placing the flask on a magnetic stirrer with a magnetic stirring bar set inside the flask.

9. Calibrate the pressure field generated by the FUS transducer using a needle hydrophone as a receiver in a degassed water tank.

Note: The transmitter system composes the FUS transducer, function generator, and power amplifier.

10. Use the function generator to generate an electric waveform that is amplified by the power amplifier and input into the transducer.

Note: During calibration, the transmitter system should be the same as the one used for the FUS-BBBO experiment. The pressures reported in our previous study were the peak negative pressures measured with three mouse skulls. Three FUS transducers with frequencies of 1.5, 3.0, and 6.0 MHz are shown in Figure 1E. The FUS transmission coefficients were 81.0% $\pm$ 4.2% at 1.5 MHz, 65.2% $\pm$ 2.1% at 3.0 MHz, and 38.6% $\pm$ 2.2% at 6.0 MHz based on the experimental calibration results. As the FUS frequency increased from 1.5 MHz to 3.0 MHz and 6.0 MHz, the full width at half maximum (FWHM) of the focal region decreased from 1.8 $\pm$ 0.1 mm to 1.0 $\pm$ 0.1 mm and 0.5 $\pm$ 0.0 mm, respectively. Correspondingly, the full length at half maximum (FLHM) decreased from 5.9 $\pm$ 0.1 mm to 3.6 $\pm$ 0.2 mm and 3.0 $\pm$ 0.1 mm. Users can select the frequency of the FUS transducer based on their specific applications.

⚠ CRITICAL: The waveform is recommended to have short pulses (approximately 10 cycles) to avoid reflection interference and potential cavitation damage on the hydrophone surface.

Attaching the FUS transducer to a stereotactic frame

Timing: 0.5 h

The purpose of this step is to attach the FUS transducer to the stereotactic frame. If this step is performed, the integrated device can precisely target a desired brain location for FUS treatment.

11. Fabricate the adapter for the FUS transducer using a 3D printer (Figure 1F).

Note: The design of the adaptor is available for download at https://github.com/ChenUltrasoundLabWUSTL/stereotactic-system.git.

12. Insert magnets in both the transducer housing and adapter to enable simple magnetic attachment and detachment of the transducer to the adapter.
13. Fix the adapter to the stereotactic frame via the shaft (Figure 1G).

Note: The transducer can then be easily attached to the adapter by the magnets. The FUS transducer can be moved to target a desired brain location in reference to the mouse brain atlas, as described in the next stop. The stereotactic frame has a 10-micron movement resolution for all axes and an easy-to-read compact digital display console (Figure 1H).

Performing the FUS-BBBO procedure

Timing: 1–2 h

The purpose of this step is to perform the FUS-BBBO experiment for drug delivery to the brain. Here, Evans blue is used as a model drug.

14. Prepare the microbubbles.
    a. Take the DEFINITY microbubbles out from the 4°C refrigerator.
    b. Activate the microbubbles via mechanical agitation using a VialMix shaker for a preset time of 45 s.
    c. Slowly withdraw the activated microbubbles from the vial using an 18 G needle attached to a 1 mL syringe following the manufacturer's instruction.

⚠ CRITICAL: As microbubbles can easily rupture, effort should be made to handle microbubbles carefully following manufacturer's instruction.

15. Anesthetize the mouse using isoflurane.
    a. Place the mouse in an anesthetic induction chamber. The chamber is connected to 1.5%–2% isoflurane with oxygen as the carrier gas.
    b. Move the mouse to the stereotaxic apparatus; the apparatus should have two ear bars and one biting bar.
    c. Hold the mouse's head flat and steady using the biting bar and ear bars.
    d. Connect the nose cone to the mouse's nose to deliver isoflurane (1%–2%) to maintain anesthetization.
    e. Apply ophthalmic ointment in each eye to protect the cornea from desiccation or other injuries.
    f. Place the animal on a heating pad to maintain body temperature at ∼37°C.

⚠ CRITICAL: Throughout this process, carefully monitor the mouse's breathing by observing the movement of the chest wall and adjusting the anesthesia level accordingly.

16. Remove the fur on the mouse head (Figure 2A).
    a. Trim the mouse fur from head to neck using a hair clipper.
    b. Apply hair removal cream to the mouse head and allow the cream to stay on for 2–4 min.
    c. Clean the area with gauze and alcohol pads.

Note: Ensure that no fur remains on the head and that the scalp is intact. Residual fur will block ultrasound propagation and attenuate the acoustic pressure in the target region.

⚠ CRITICAL: Be cautious when applying hair removal cream, as leaving hair removal cream for too long can burn the scalp.

17. Place a tail vein catheter for microbubble and drug injections.
    a. Warm the tail with a hand warmer if necessary; then, wipe the tail with an alcohol wipe and insert a 27 G × $^{1}/_{2}$" butterfly needle into the tail vein, with the other end of the needle connected to a polyethylene tubing.
    b. Connect the tubing to a 1 mL plastic syringe filled with saline.

Note: Approximately 10 μL saline can be injected into the mouse tail vein to ensure that tail vein catheterization is successful. If the injection is successful, secure the catheter using surgical tape.

18. Align the miniature transducer with the lambda on the mouse skull.
    a. Draw a dot using a marker pen on the mouse's scalp to indicate the location of the lambda on the mouse's skull (Figure 2B).

Note: Lambda is selected as a landmark because it is a point on the mouse skull that is visible through the scalp.

    b. 3D print a pointer that can fit into the transducer adapter. The tip of the pointer represents the geometric focus of the transduce (Figure 2C).
    c. Place the pointer in the holder and adjust its position using the stereotactic frame to align the pointer with the dot (Figure 2D).
    d. Fill the entire cavity of the coupling cone of the transducer housing with degassed ultrasound gel (Figure 2E).
    e. Apply ultrasound gel to the exposed skin above the skull (Figure 2F).

⚠ CRITICAL: Ensure that there are no air bubbles in the ultrasound gel by visual inspection when filling the cavity of the coupling cone of the transducer housing or placing the gel on the mouse head. The presence of air bubbles will interfere with the acoustic wave propagation through the gel.

    f. Switch the pointer to the FUS transducer (Figure 2G).
    g. Move the FUS transducer from the lambda to the target brain location using the coordinates of the target relative to the lambda (Figure 2H).

Note: The coordinates can be determined using the mouse brain atlas.[2,3] For example, if the desired brain target is in the left thalamus, according to the mouse brain atlas, its coordinates relative to lambda are: +2.26 mm in the anterior-posterior (AP) direction, -1.50 mm in the medial-lateral (ML) direction, and -3.30 mm in the dorsal-ventral (DV) direction.

19. Connect the ultrasound system according to the following sequence: Function generator → power amplifier → FUS transducer.
20. Set the ultrasound parameters as follows: 10 ms in pulse length, 1 Hz in pulse repetition frequency, and 120 s in total duration.

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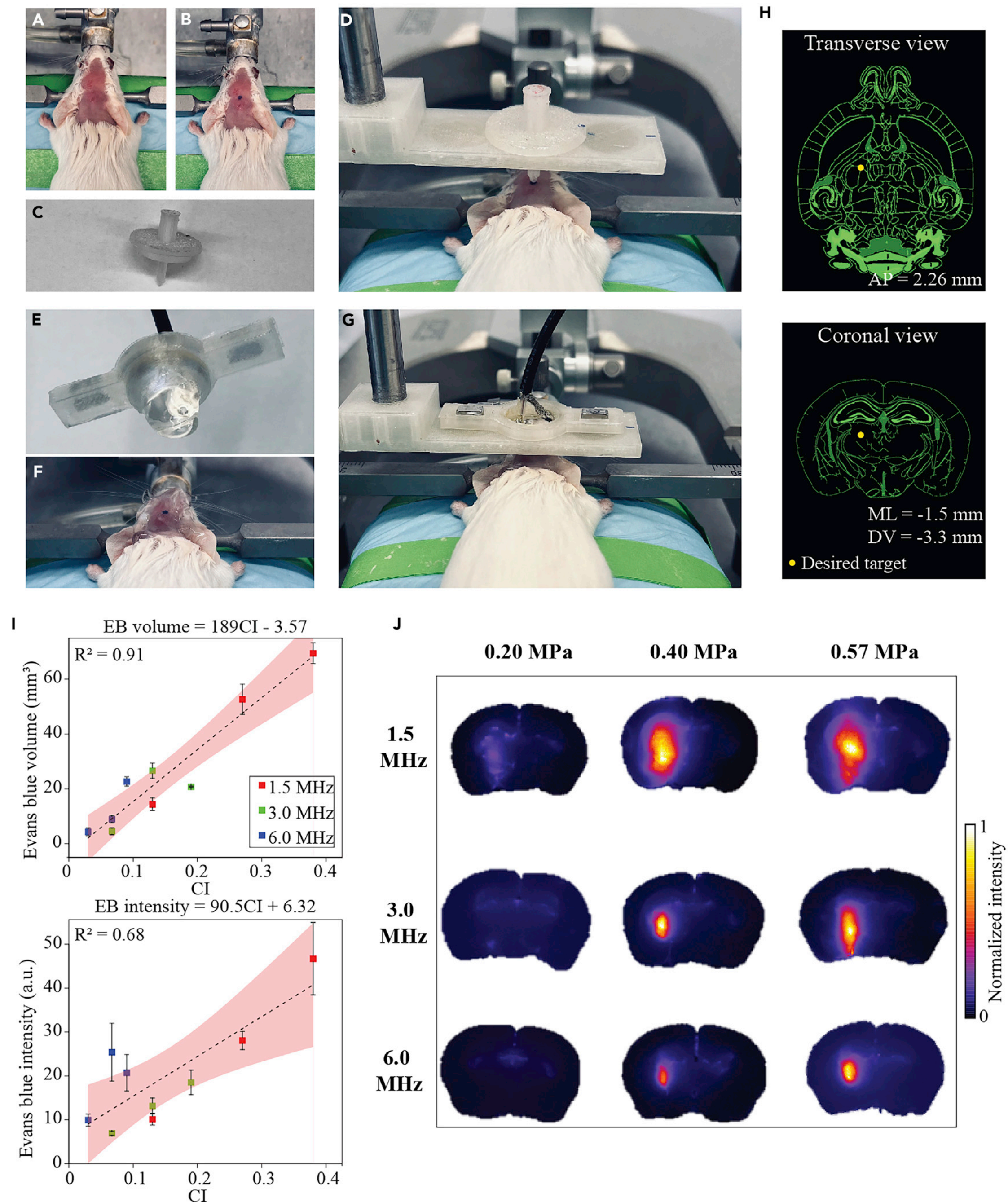

Figure 2. Application of the miniature FUS device

(A) Place the mouse on the stereotactic frame.
(B) Draw a dot on the lambda in the mouse skull.
(C) Picture of the 3D printed pointer.
(D) Align the 3D-printed pointer with lambda.
(E) Fill the coupling cone of the FUS transducer with degassed ultrasound gel.
(F) Place ultrasound gel on the mouse head.
(G) Replace the pointer with the FUS transducer.
(H) Navigate the transducer to the targeted brain location using the stereotactic frame according to the coordinates of the target relative to lambda. The coordinates are determined using the mouse brain atlas.
(I) Correlations between drug (here, Evans blue was used as a model drug) delivery volume and intensity with the cavitation index (CI).
(J) Fluorescence images for visualizing the Evans blue delivery outcome.[1]

Note: The frequency is determined by the center frequency of the FUS transducer (e.g., 1.5 MHz, 3.0 MHz, and 6.0 MHz), and the amplitude of the signal is set according to the hydrophone calibration result.

21. Inject the microbubbles.
    a. Use a Hamilton syringe to withdraw the microbubble solution. The dose of the DEFINITY microbubbles used in our study was 10 × the clinical dose, which was 100 μL/kg.

Note: Lower doses of microbubbles can be used, but the acoustic pressure will need to be increased to achieve a similar outcome.

    b. Replace the 1 mL plastic syringe that is connected to the tail vein tubing with the Hamilton syringe.
    c. Inject the microbubbles slowly into the tail vein.
    d. Change back to the plastic syringe.
    e. Inject saline (~10 μL) to flush the tail vein tubing.

⚠ CRITICAL: During the process of changing syringes, caution needs to be taken to avoid introducing any air bubbles into the catheter.

22. Perform FUS sonication.
    a. Before turning on the FUS, check the parameter settings are correct and the cable connection is correct (Figure 2I).
    b. Turn on FUS immediately following the microbubble injection.

Note: The applied FUS pressure and frequency depend on the user's application and desired delivery outcome. Based on our previous study, the FUS-BBBO outcome (volume and intensity) can be tuned according to the cavitation index (CI), which is defined by the ratio between the acoustic pressure (P) and frequency (f), ($CI = P/f$).

23. Deliver Evans blue (other agents also work).

Note: Administer Evans blue (60 μL of 2% Evans Blue) immediately after FUS sonication.

    a. Fill the Hamilton syringe with 60 μL of Evans blue.
    b. Place the Hamilton syringe into the tail vein tubing and slowly inject the Evans blue.
    c. Inject saline (10 μL) to flush Evans blue.
24. Recovery.
    a. Gently remove the tail vein catheter and apply pressure with gauze until the blood stops flowing from the puncture site.
    b. For acute studies, the animal is sacrificed by transcranial perfusion.
    c. For survival studies, place the mouse on a heating pad for recovery. Return the mouse to its home cage after the mouse wakes up.
25. Clean the transducer.
    a. Remove the ultrasound gel inside the coupling cone of the FUS transducer, flush the transducer with alcohol, and wipe and dry the FUS transducer.

Evaluating FUS-BBBO outcome

Timing: 0.5–1 h

The purpose of this step is to evaluate the FUS-BBBO outcome. If the FUS-BBBO procedure is successful, the delivered Evans blue can be clearly observed on the brain slices by visual observation.

26. To evaluate the FUS-BBBD outcome, mice are transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA).
    a. Harvest the brain and fix it in the 4% PFA solution.
    b. After fixation, cut the brains into 1-mm thick coronal sections using a mouse brain matrix.
27. Acquire fluorescence images of brain sections using the LI-COR imaging system to visualize the Evans blue delivery outcome (Figure 2J).
28. Quantify the delivery using the Image Studio by comparing the FUS-treated and nontreated sides of the brain on each fluorescence image.

Note: As an example, we choose the left and right sides of the mouse brain as the treated and nontreated sides, respectively.

    a. Draw a circle to cover the nontreated right side of the brain on each brain section.

Note: Three times the standard deviation above the mean pixel intensity within the non-treated region is calculated to represent the background signal intensity.

    b. Draw a circle to cover the FUS-treated left side of the brain.
    c. Identify the pixels with intensities greater than the background signal intensity.
    d. Calculate the number of pixels with enhanced fluorescence signals in all brain sections to determine the Evans blue delivery volume.
    e. Calculate the mean fluorescence intensity of pixels with enhanced fluorescence signals in all brain sections to determine the signal intensity of the delivered Evans blue.
29. Perform histologic examination using hematoxylin and eosin (H&E) staining for FUS-BBBO safety analysis.
    a. Cryoprotect the brain slices with the highest Evans blue fluorescence signals using sucrose solution and embed the slices at −20°C.
    b. Section the mouse brain tissue into 10 μm coronal sections.
    c. Dip the sections to water for 1 min.
    d. Rehydrate the sections by dipping them into 100%, 90%, and 70% ethanol, respectively, for 1 min each. Dip the sections in water for 1 min.
    e. Conduct hematoxylin staining for 2 min. Flush the section with water for 3 min. Dip the sections in 70% ethanol for 1 min.
    f. Conduct eosin staining for 10 s. Wash with 70% ethanol for 1 min.
    g. Dehydrate the sections by dipping them into 70%, 90%, and 100%, respectively, for 1 min each. Dip the sections in Xylene for 2 min.
    h. Place the stained sections on glass slides. Add a drop of seal solution to each section and apply a cover slide on the section.
    i. Obtain bright-field images of the stained sections using a fluorescence microscope with 2× and 20× objectives.
30. Perform statistical analyses to check safety.
    a. Import the bright-field images into MATLAB.
    b. Quantify the red blood cell extravasation using MATLAB by comparing the FUS-treated and nontreated sides of the brain on each bright-field image.
    c. Evaluate the statistical significance via the unpaired two-tailed Student's t-test using GraphPad Prism.

EXPECTED OUTCOMES

This protocol can be applied to build a low-cost, easy-to-use, and precise FUS device that can be used in preclinical research with minimal training. We use the FUS device combined with microbubbles to open the BBB. Evans blue served as a model drug in this protocol to evaluate the FUS-BBBO outcome. Strong linear correlations were found between the Evans blue delivery outcome (volume and intensity) and the cavitation index, as reported in our previous study.[1] The FUS-BBBO drug delivery volume and intensity) can be tuned by the CI. Other agents can be delivered to the brain by the same technique based on the user's desired applications. We have applied this protocol to deliver adeno-associated viral vectors (AAV) to the brain[4] and study the mechanical manipulation of glymphatic transportation.[5] In the future, we will combine the device with an acoustic hologram to produce an arbitrary acoustic pattern to increase the flexibility of this protocol.[6]

LIMITATIONS

This protocol is designed for FUS-BBBO in mice and is unsuitable for large animals. However, mice are the most common species used in brain-related research. Additionally, the FUS parameters (frequency and pressure) may need to be optimized for specific applications to prevent tissue damage and maximize agent delivery efficiency.

TROUBLESHOOTING

Problem 1

The miniature FUS transducer fabrication fails due to issues such as no signal output or the PZT element not fitting into the housing unit (Building the miniature FUS transducer, 4).

Potential solution

This issue may be caused by a soldering problem. Before soldering, please clear the surface of the PZT element using 100% alcohol to ensure a strong attachment between the wires and the PZT element. Furthermore, the ultrasound element may not fit in the 3D-printed housing due to variations during 3D printing. In this case, the dimensions of the designed 3D housing need to be slightly adjusted.

Problem 2

The location of the FUS-BBBO is not accurate (Performing FUS-BBBO procedure, 18d and 18g).

Potential solution

Carefully calibrate the FUS transducer to ensure that the focal position is correct. Attention should always be paid to the transducer coupling, as some air bubbles may be trapped in the coupling gel. Trapped air bubbles may result in failed delivery of ultrasound energy to the brain at the target position. Moreover, verify that the ear bar and biting bar are in the same plane during the experiments. Adjust the mouse head's position to ensure it is positioned horizontally without tilting.

Problem 3

The drug delivery outcome is not as expected (Performing FUS-BBBO procedure, 17, 21).

Potential solution

Tail vein injection needs to be performed carefully to ensure that the drug and microbubbles are delivered into the bloodstream through the tail vein. Ensure that the FUS can generate sufficient power before the experiments.

Problem 4

Brain damage occurs after the procedure (Performing FUS-BBBO procedure, 22).

Potential solution

This problem may be caused by using ultrasound pressures higher than needed and can be prevented by using a lower ultrasound pressure. The cavitation index should be within the safety range reported in our previous study (CI < 0.38).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to Hong Chen (hongchen@wustl.edu).

Materials availability

This protocol does not include any unique reagents.

Data and code availability

This protocol does not include any code or dataset.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Conceptualization, Z.H., Y.Y., H.C.; methodology, Z.H., Y.Y., S.C., H.C.; investigation, Z.H., Y.Y., D.Y., Y.G., C.C.; writing – original draft, Z.H., Y.Y., D.Y., Y.G., C.C., H.C.; writing – review & editing, Z.H., Y.Y., H.C.; funding acquisition, H.C.; resources, Z.H., Y.Y., H.C.; supervision, H.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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