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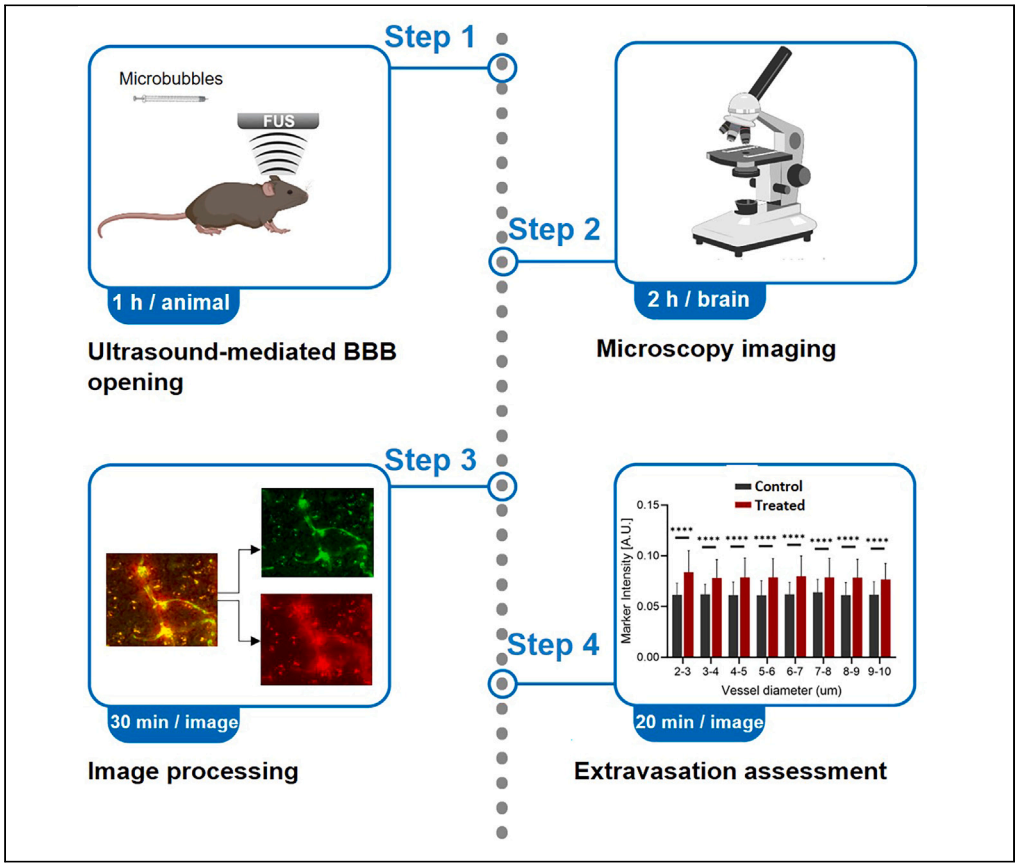
Protocol to assess extravasation of fluorescent molecules in mice after ultrasound-mediated blood-brain barrier opening



Blood-brain barrier disruption (BBBD) using focused ultrasound (FUS) and microbubbles (MBs) is an effective tool for therapeutic delivery to the brain. Here, we present an optimized protocol for quantifying fluorescent molecules extravasation in mice. We describe steps for ultrasound treatment, injection of MBs and fluorescent dyes, brain harvesting, microscopy imaging, and image postprocessing algorithm. Our protocol has proven to successfully conduct a diameter-dependent analysis that measures vascular leakage following FUS-mediated BBBD at a single blood vessel resolution.

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

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Highlights

Quantify BBB opening as a function of vessel diameter at single blood vessel resolution

High resolution of 1 μm for vessels with a diameter <10 μm is achieved

Microscopy imaging of fluorescent molecules extravasation across the BBB

Peko et al., STAR Protocols 5, 102770
March 15, 2024
https://doi.org/10.1016/j.xpro.2023.102770

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Protocol to assess extravasation of fluorescent molecules in mice after ultrasound-mediated blood-brain barrier opening

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https://doi.org/10.1016/j.xpro.2023.102770

SUMMARY

Blood-brain barrier disruption (BBBD) using focused ultrasound (FUS) and microbubbles (MBs) is an effective tool for therapeutic delivery to the brain. Here, we present an optimized protocol for quantifying fluorescent molecules extravasation in mice. We describe steps for ultrasound treatment, injection of MBs and fluorescent dyes, brain harvesting, microscopy imaging, and image postprocessing algorithm. Our protocol has proven to successfully conduct a diameter-dependent analysis that measures vascular leakage following FUS-mediated BBBD at a single blood vessel resolution.

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

BEFORE YOU BEGIN

Safe and localized blood-brain barrier disruption (BBBD) is essential for therapeutic delivery to the brain for a variety of brain diseases, because the BBB is an obstacle for effective drug delivery.[2,3] When low-energy ultrasound (US) is combined with intravenously injected MBs, the BBB opens in a noninvasive and localized manner,[4,5] enabling efficient drug delivery to the brain.[6–9] Here, we present a robust and high-resolution method for fluorescent molecules extravasation assessment across the BBB after FUS-mediated BBBD at a single blood vessel resolution. Our method utilizes two fluorescent dyes; the first is Evans blue (EB), a standard BBB impermeable dye for BBB leakage identification and quantification,[10] and a large 2000 kDa Fluorescein isothiocyanate–Dextran (FITC-Dextran) for blood vessels segmentation.[11,12] In this protocol, we describe all of the steps that are associated with the method, including *in vivo* procedures such as mice preparation, US treatment, and systemic injections of MBs and the two fluorescent dyes at specific time points that are carefully designed for localized extravasation across the BBB. Further, we describe the brain tissue processing steps including slicing and fluorescence microscopy imaging, followed by the usage of an automated image processing algorithm that was developed to quantify the extent of EB extravasation as function of microvasculature diameter.

Our method enables to evaluate BBB opening at a single blood vessel resolution and assess for microvascular-dependent variations.[1] It uses advanced fluorescent imaging of molecule extravasation across the BBB after FUS-mediated BBBD, detecting differences between vessels with a diameter of less than 10 μm. This approach enables to probe BBB opening at a higher resolution, and identify conditions that affect BBBD leading to the development of new strategies to improve BBBD.

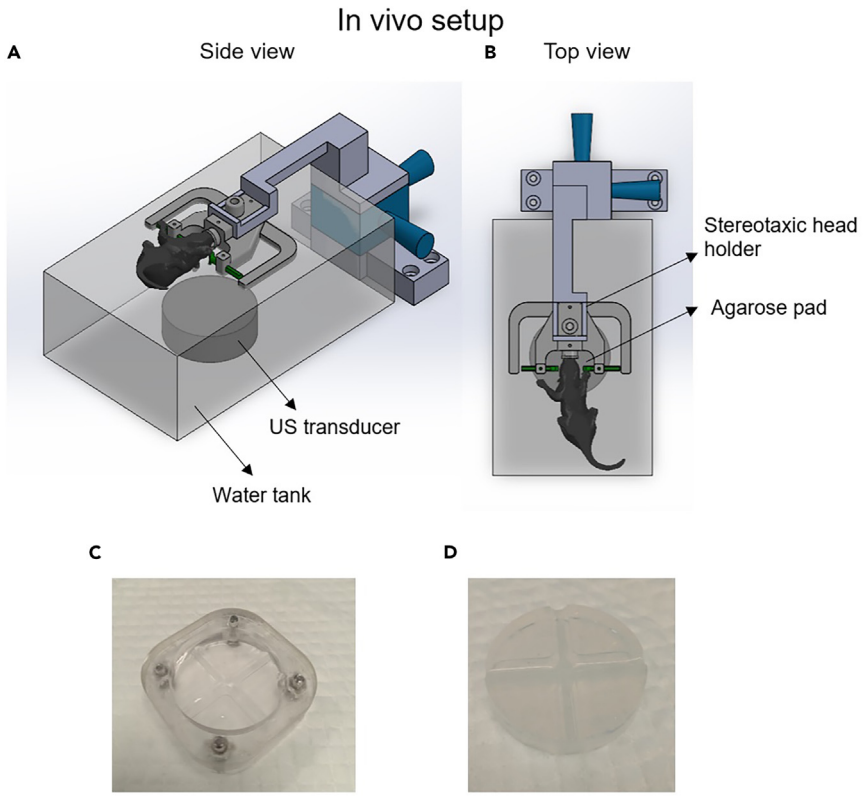

Figure 1. *In vivo* setup for US-mediated BBB opening in mice

(A and B) Illustration of the *in vivo* set up. The mouse is mechanically aligned at the focal spot of an US transducer, located at the bottom of a water tank, using a custom 3D printed stereotaxic head frame.

(C and D) A laser cut mold (c) for the agarose pad (d), used as an acoustic impedance matching between the water tank and the US gel applied at the mouse.

Institutional permissions

Female C57BL/6 mice (10–11 week old, 16–21 *g*, Envigo, Jerusalem, Israel) were used in the *in vivo* experiments, yet the method is not gender specific and could be used with male mice as well. All the animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Tel-Aviv University. Standard autoclaved lab diet and water were provided *ad libitum*. Mice were housed in an individually ventilated cage housing system under a 12-h light/ dark schedule at 22°C, in the presence of 2–5 mice per cage in total.

Stereotaxic frame positioning

Timing: 15 min

A 3D printed stereotaxic head frame is used to mechanically position the mice during the application of US treatment. It is important to use a stereotaxic frame that supports supine positioning, while the animal's snout is connected to the anesthesia machine tube.

1. Connect the stereotaxic frame to the US setup, as illustrated in Figures 1A and 1B.

⚠ CRITICAL: Position the animal's head exactly above the focal spot of the US transducer, as will be described in the step-by-step method details section.

Agarose pad

Timing: 1 h

Agarose pad is used for acoustic coupling between the water tank and the mouse, and served as a solid substrate that the mouse head could be positioned on. The agarose pad is prepared by mixing 1.5% agarose powder and deionized water.

2. Weigh agar (1.5 *g*) and deionized water (100 mL). Mix together in an Erlenmeyer flask.
3. Boil the solution in the microwave until all the powder has completely dissolved. Repeat the process until all the bubbles are removed.
4. Transfer the solution into the laser-cut mold (Figure 1C).
5. Allow it to congeal in room temperature (Figure 1D).
6. If not used within 24 h, store it in a box filled with distilled water at 4°C for up to a week.

⚠ CRITICAL: The agarose powder should be completely dissolved to obtain a homogeneous solution. No bubbles should be visible by eye in the suspension.

Note: Custom laser-cut mold measures 60 mm × 60 mm × 17 mm (length × width × height) and was made to shape the agarose pad with a hole in the middle to fit the mice head in a supine position (Figure 1D).

Note: We use one agarose pad per *in vivo* experiment; however, we usually prepare at least two agarose pads.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Perfluorobutane (C_4F_{10})	F2 Chemicals Ltd.	CAS: 355-25-9
Distearoylphosphatidylcholine (DSPC)	Avanti Polar Lipids	850365C, CAS: 816-94-4
18:0 PEG2000 PE	Avanti Polar Lipids	880120, CAS: 474922-77-5
Glycerol	Sigma-Aldrich	G5516, CAS: 56-81-5
Propylene glycol	Sigma-Aldrich	BP868, CAS: 57-55-6
Saline	Fisher Scientific	Z1376, CAS: 7647-14-5
Agarose powder	Holland Moran	400402500, CAS: 9002-18-0
Dulbecco's phosphate-buffered saline	Biological Industries Israel	02-023-1A
Isoflurane	United States Pharmacopeia	1349003, CAS: 26675-46-7
Evans blue dye	Sigma-Aldrich	E2129, CAS: 314-13-6
Fluorescein isothiocyanate–Dextran 2000	Sigma-Aldrich	52471, CAS: 60842-46-8
OCT cryo compound	Leica Biosystems	14020108926
2-Methylbutane	Merck-Sigma	M32631
Deposited data		
Example microscopy data	This paper	https://zenodo.org/badge/latestdoi/637521961
Experimental models: Organisms/strains		
Female C57BL/6 mice (10–11 week old, 16-21gr)	Envigo	C57BL/6JOlaHsd
Software and algorithms		
MATLAB	MathWorks, Inc.	https://www.mathworks.com/products/matlab.html
Rapid Editable Analysis of Vessel Elements Routine (REAVER)	Corliss, B.A. et al.	https://github.com/uva-peirce-cottler-lab/public_REAVER

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Image processing and data analysis software	This paper	https://zenodo.org/badge/latestdoi/637521961
Prism	GraphPad	https://www.graphpad.com/
Other		
AccuSizer FX-Nano, particle sizing systems	Entegris	A9000 AD
250 kHz, single element, focused ultrasound transducer	Sonic Concepts	H115
Arbitrary/Function generator	Tektronix, Inc.	AFG1012
Laboratory linear power amplifier	E&I	2100L
Transducer Power Output	Sonic Concepts	TPO-200
Needle hydrophone	Precision Acoustics	NH0500
Phased array ultrasound transducer	Philips	ATL P4-1
Vantage research ultrasound system	Verasonics, Inc.	Vantage 256
Low-flow electronic vaporizer	Kent Scientific	SomnoFlo
Cryostat microtome	Leica Biosystems	CM1950
Automated fluorescence microscope	Olympus Life Science	BX63
20x microscope objective	Olympus Life Science	UPLFLN 20X

MATERIALS AND EQUIPMENT

Degassed water

Degas distilled water in a volume that matches the US setup water tank. The water can be degassed by connecting a 4 L bottle of distilled water to house vacuum at least 24 h before usage.

Evans blue dye solution

EB is a fluorescent dye used for the BBB leakage observation and quantification.

Dilute 2% of EB in phosphate-buffered saline PBS (e.g., 40 mg of EB powder in 2 mL of PBS). Vortex briefly the solution to avoid sediments. Transfer the EB solution through 0.22 μm filter to a 3 mL cryogenic storage vial. Store at 4°C for up to a week.

> *Note:* Work with a lab coat and cover a working station with a liquid-absorbent pad, because EB may stain the clothes and the working station.

On the day of the *in vivo* experiment

Degassed PBS

Degas 50 mL of PBS −/− for the experiment under house vacuum for at least 2 h.

MB preparation and activation

MB are prepared and measured as described in our recently published study.[1,13,14]

Shortly, the MBs are composed of a phospholipid shell and a perfluorobutane (C_4F_{10}) gas core. The lipids (2.5 mg per 1 mL), distearoylphosphatidylcholine (DSPC; 850365C) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000PE; 880120) are combined at a molar ratio of 90:10. A buffer (mixture of glycerol (10%), propylene glycol (10%) and saline (80%) (pH 7.4)) is then added to the lipids and sonicated at 62°C . The MB precursor solution is aliquoted into vials with liquid volume of 1 mL and saturated with perfluorobutane. Before usage, the vials must be shaken for 45 s in a vial shaker (Bristol-Myers Squibb Medical Imaging Inc., N. Billerica, MA), and then purified via centrifugation to remove MBs smaller than 0.5 μm in radii. Further, to remove MBs larger than 5 μm in diameter, the size selection process is applied. Size selection includes adding PBS to a volume of 2.5 mL. Centrifuge for 1 min at 7*g*. Two layers will be observed; collect the lower layer, and half of the upper layer composed of dense while foam. For this step, put a needle inside a new syringe and pour the wanted layer to this new syringe. Repeat the process again, this time by centrifuging at 10 *g*. Finally, the size and

concentration of the MBs are measured twice with a particle counter system (AccuSizer FX-Nano, Particle Sizing Systems, Entegris, MA, USA).

⚠ CRITICAL: The bubbles are used within 3 h of their preparation. Should the MB concentration differ by more than 10% between the two measurements, incorporate a third measurement.

FITC-dextran dye solution

2000 kDa FITC-Dextran dye is used for blood vessel labeling.

Dilute 0.6% of FITC-Dextran (6 mg/mL) in normal saline in a 5 mL Eppendorf tube. Vortex the solution briefly and store at 4°C until use, while protecting the tube with aluminum foil.

⚠ CRITICAL: It must be freshly prepared and used on the day of an *in vivo* experiment.

US set-up

The US setup (illustrated in Figure 1A) is composed of a spherical focused single element FUS transducer (H115, Sonic Concepts, Bothell, WA, USA), operating at a center frequency of 250 kHz. The transducer has a focal distance of 45 mm and an aperture diameter of 64 mm. The transducer is located at the bottom of a water tank, facing upwards. This transducer is operated by a transducer power output system (TPO-200, Sonic Concepts, Bothell, WA, USA). The peak negative pressure (PNP) at the focal spot was calibrated using a needle hydrophone (NH0500, Precision Acoustics, UK).

Surgical tools

Prepare all surgical tools for harvesting the brains, including Iris scissors, dissecting scissors, surgical tweezers. The same surgical tools can be used for multiple mice, but make sure to extensively clean them with water and subsequently rinse them with 70% ethanol before each individual surgery.

Ice box

Fill a styrofoam box with ice for the storage of the fluorescent dyes.

Liquid nitrogen

Fill the liquid nitrogen tank for the post-operative brain preservation.

Dry ice

Prepare a styrofoam box with dry ice for the transportation of the brain.

Microscopy

For the microscopic observation described in this protocol, we use a motorized upright fluorescence microscope (BX63, Olympus life science, Waltham, Massachusetts, USA) with 4x and 20x objectives (UPLFLN 20X, Olympus life science). This objective has a focal depth of 1.1 mm. The excitation wavelengths and exposure times were 508 nm and 1150 ms for FITC-Dextran, and 615 nm and 115 ms for EB. All the imaging parameters were consistent for all sections. The final images were 1920 × 1200 pixels in size, with a pixel size of 0.29 mm in the imaging plane.

STEP-BY-STEP METHOD DETAILS

US set-up procedures

Timing: 15 min

This section will explain how to set up the US set-up for the BBB experiment.

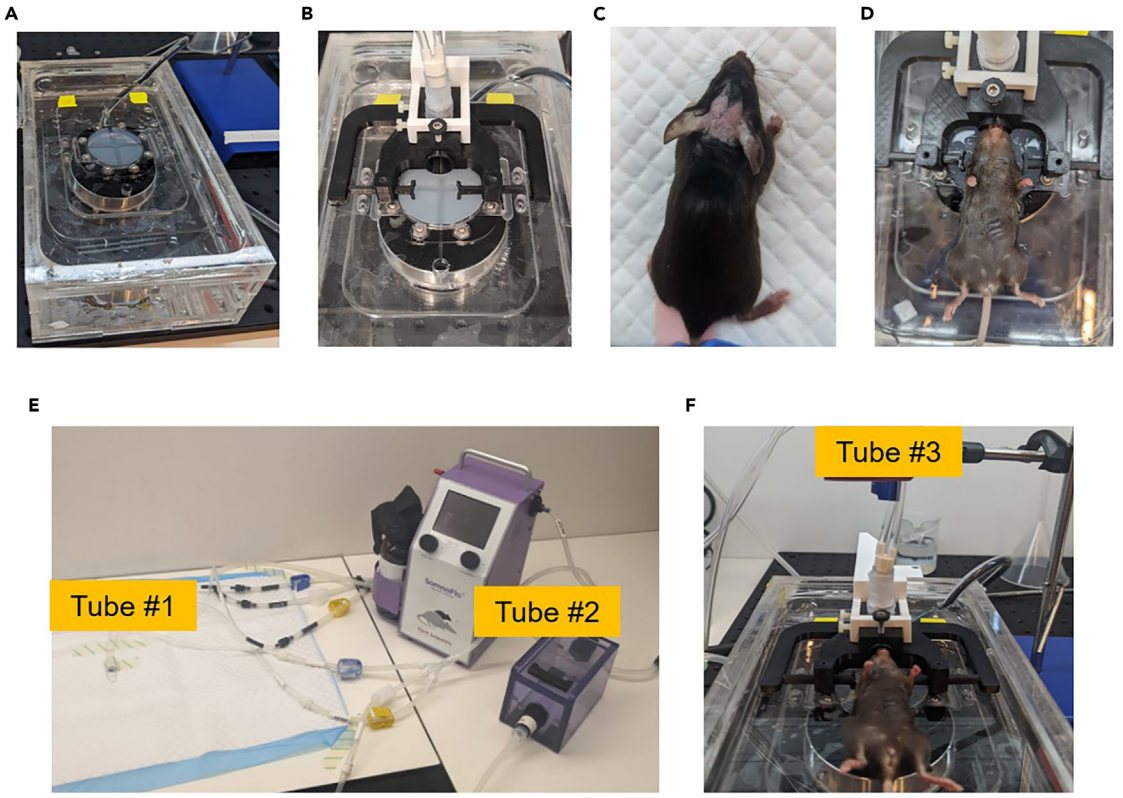

Figure 2. Experimental setup and animal positioning

Preparation of US setup and mice: (A) Fill the water tank with degassed water.
(B) Place an agarose pad at the agarose spacer.
(C) Animal shaving at the top of the head.
(D) Place the mouse in a supine position on top of the agarose pad using the head holder.
(E and F) Connect the anesthesia machine to three tube sets: one sedation tube on the liquid absorbing pad (tube #1), the second tube to the anesthesia induction box (tube #2), and the third tube-set to the head holder on top of the US setup (tube #3).

Note: Prepare a tank of degassed water, agarose pads, liquid-absorbable pads, anesthesia machine, US set-up and TPO.

1. Fill the water tank with degassed water. Do not fill the water above the setup cover (Figure 2A).
   a. Make sure that there are no air bubbles are present in the water tank.
2. Fill water below the agarose spacer, and then place the agarose pad and add water until the agarose pad is completely covered (Figure 2B).
3. Connect the head holder.

⚠ CRITICAL: Make sure there are no air bubbles beneath the agarose pad.

4. At the working station, place the heating mat beneath a liquid-absorbing pad.
5. Connect the anesthesia machine to three tube sets (Figures 2E and 2F).
   a. Place the sedation tube on the liquid absorbing pad (tube #1).
   b. Connect the second tube to the anesthesia induction box (tube #2).
   c. Connect the third tube-set to the head holder on top of the US setup (tube #3).
6. Connect the TPO to the matching network input socket.
7. Connect the matching network output to the transducer.
8. Connect the matching network to the powerline.
9. Set US parameters as follows: Frequency = 250 kHz, period = 1s, burst = 1 ms, PNP = 1.35.

a. Operate US at a high level of power to confirm that the transducer functions properly by observing a splash of water at the focus. For the TPO that we are using, a power of 70 W is typically used for this task.

Note: Sonication PNP, frequency and duration were optimized to induce safe and mild BBBD that is visible in fluorescent microscopy, without signs of microhemorrhage in histology.[1]

Mouse preparation

Timing: 15 min/animal

This section will explain how to anesthetize and prepare the mice for the BBB experiment.

Note: Prepare a permanent marker, shaver, cream for the shaving, sterile disposable cotton swabs and eye cream.

10. Sedation.
    a. Weigh and mark the animal.
    b. Place the mouse in the anesthesia induction box, and sedate it with 2.5% isoflurane using a high flow vaporizer system (200 mL/min, SomnoFlo, Kent Scientific).

⚠ CRITICAL: Make sure that the mouse is fully anesthetized; for that, pinch the toes. If fully asleep, the mouse will not react.

11. Animal shaving.
    a. Place the mouse on the working station and connect to the sedation tube with a 1.5% isoflurane low flow (50 mL/min).
        i. Make sure the other sedation tubes are closed.
    b. Apply eye ointment.
    c. Shave the fur on the top of the head (Figure 2C).
    d. Apply depilatory cream to the shaved area using a sterile cotton swab.
    e. After 2 min, remove the cream with a wet gauze.

In vivo BBB opening

Timing: 50–60 min/animal

This section will explain how to conduct the *in vivo* FUS-mediated BBB opening experiments.

Note: Prepare MB solution, fluorescent dyes stored in an ice box, US gel, recovery carton box for the mice and a heating lamp.

12. MB injection.
    MB dose is $2 * 10^7$ in a total volume of 50 μL for a 20 g mouse.
    a. Prepare a suitable dose of MB in a 1 mL syringe using the following formula:

$$MB\,[\mu l] = 3\mu l * \frac{2 * 10^7}{[MB]\left[\frac{\#}{\mu l}\right]}$$

    b. Add PBS −/− to a final volume of 150 μL.
    c. Using an insulin syringe, collect 50 μL of MB solution, and remove the air bubbles.
    d. Additionally, prepare an insulin syringe containing 4 mL/kg of EB solution.

⚠ CRITICAL: Make sure that the mouse is fully anesthetized.

Note: The following steps 12.e-13.e are relevant only for the treated group of mice.

e. Inject 50 μL of the MB solution retro-orbitally to the mouse.

⚠ CRITICAL: 30 s post MB injection, a treated group of mice should receive the FUS treatment. To do so, position the mouse's head in the stereotaxic frame with its face pointing downward. The focal point of the transducer is indicated by the center of the + sign on the agarose pad. Ensure the midpoint of the agarose pad aligns with the line extending between the mouse's ears and eyes.

13. Sonication and FUS treatment.
    a. Pour water on the agarose pad if it is dry.
    b. Apply US gel to the agarose spacer.
    c. Place the mouse in a supine position on top of the agarose pad using the head holder, as shown in Figure 2D.
        i. Ensure the continuous isoflurane inhalation anesthesia through the sedation tube connected to the head holder.
        ii. Make sure the other sedation tubes are closed.
    d. Wait 30 s from the MB injection and activate the US. Parameters could vary between transducers. For the 250 kHz transducer used here, US parameters included a 1 ms burst, pulse repetition frequency of 1 Hz and a total duration of 60 s.
    e. After US application, return the mouse back to the heating pad. Ensure the continuous isoflurane inhalation anesthesia through a nose mask.
14. EB injection.
    a. Inject 4 mL/kg of the EB solution retro-orbitally to the mouse. EB circulation time is 28 min, as was optimized previously.

⚠ CRITICAL: To verify that the EB injection is successful, mice feet and tail should turn blue within 1–2 minutes, and observed by eye.

    b. Dry the mouse and place it in the recovery box, under the heating lamp.
    c. Monitor the recovery.

Note: EB circulation time was optimized to obtain a mild EB leakage, as in.[1]

15. FITC-Dextran injection.
    a. Prepare an insulin syringe containing 4 mL/kg of FITC-Dextran solution.
        i. Keep the syringe protected from the light until use.
    b. 13 min post EB injection (e.g., 5 min prior to FITC injection), place the mouse in a plastic box and sedate it with 1.5% isoflurane.

⚠ CRITICAL: Make sure that the mouse is fully anesthetized.

    c. 18 min post EB injection, inject 4 mL/kg of FITC-Dextran solution retro-orbitally to the mouse. FITC-Dextran circulation time is 10 min.
    d. Place the mouse in the recovery box, under the heating lamp.
    e. Monitor the recovery.

Note: FITC-Dextran circulation time was optimized to maintain a strong fluorescence signal within the blood vessels, without leakage across the BBB.[1]

16. Brain Harvesting.

Note: Prepare surgical tools for the brain extraction, a tank of liquid nitrogen, 2-methylbutanal, OCT and disposable base molds (size 24 × 24 × 5 mm) for the freezing and dry ice for the transportation of the brains.

⚠ CRITICAL: Brains must be quickly harvested, covered in tissue freezing medium and flash-frozen using liquid nitrogen.

a. Ten minutes post FITC injection, euthanize the mouse.
b. Harvest the brain and place it in a disposable base mold, covered in tissue freezing medium (OCT) and flash-frozen using liquid nitrogen.
c. After flash-freezing, transfer the brain to a −80°C refrigerator for storage.

Microscopy imaging

Timing: 2 h/brain

Prior to microscopy imaging, the frozen brains are transferred to a −20°C cryostat microtome (CM1950, Leica Biosystems) and coronally cut to slices of thicknesses of 20 μm. For the image acquisition, we use a motorized upright fluorescence microscope (BX63, Olympus life science, Waltham, Massachusetts, USA) with an 20 objective (UPLFLN 20X, Olympus life science), as described in the section 'materials and equipment'.

⚠ CRITICAL: The brain slides should be imaged within 1 h of sectioning to avoid further EB diffusion.

17. Turn on the laser scanning microscope and image acquisition software.
18. Select a 4x objective lens to scan the entire slice.
    a. Focus the image using bright-field mode.
    b. Save the overview of the slice as .jpg file.
19. Select a 20x objective lens and choose a region of interest (ROI) in the sample.
    a. Focus the image using bright-field mode.
    b. For the acquisition of fluorescence images, set two channels with the excitation wavelength and exposure time of 508 nm and 1150 ms for FITC-Dextran (channel GFP), and 615 nm and 115 ms for EB (channel RFP).
    c. Set image resolution to 1920 × 1020 pixels with pixel size of 0.29 μm in the imaging plane.
20. Click 'Live' to display fluorescence signals.
    a. Set all channels (GFP and RFP) to show substantial levels of fluorescence signals without saturation, by adjusting the laser powers to the appropriate values.
    b. Search for ROIs with the visible blood vessels, by using the green channel (Figures 3A and 3D).
    c. Move to red channel (Figures 3B and 3E).
    d. In FUS treated brains, search for the widespread extravasation of EB (red) around the blood vessels (Figure 3E), compared to the control brains (Figure 3B). Acquire an image and save it as .tif files.
21. Acquire fluorescent images from the multiple brain regions in both treated and control brains.
22. Export images as .tif files and save it as *name_brain region_number.*

⚠ CRITICAL: Save control and tested files to be analyzed in separate folders. Exposure time might need to be adjusted when using a different microscope

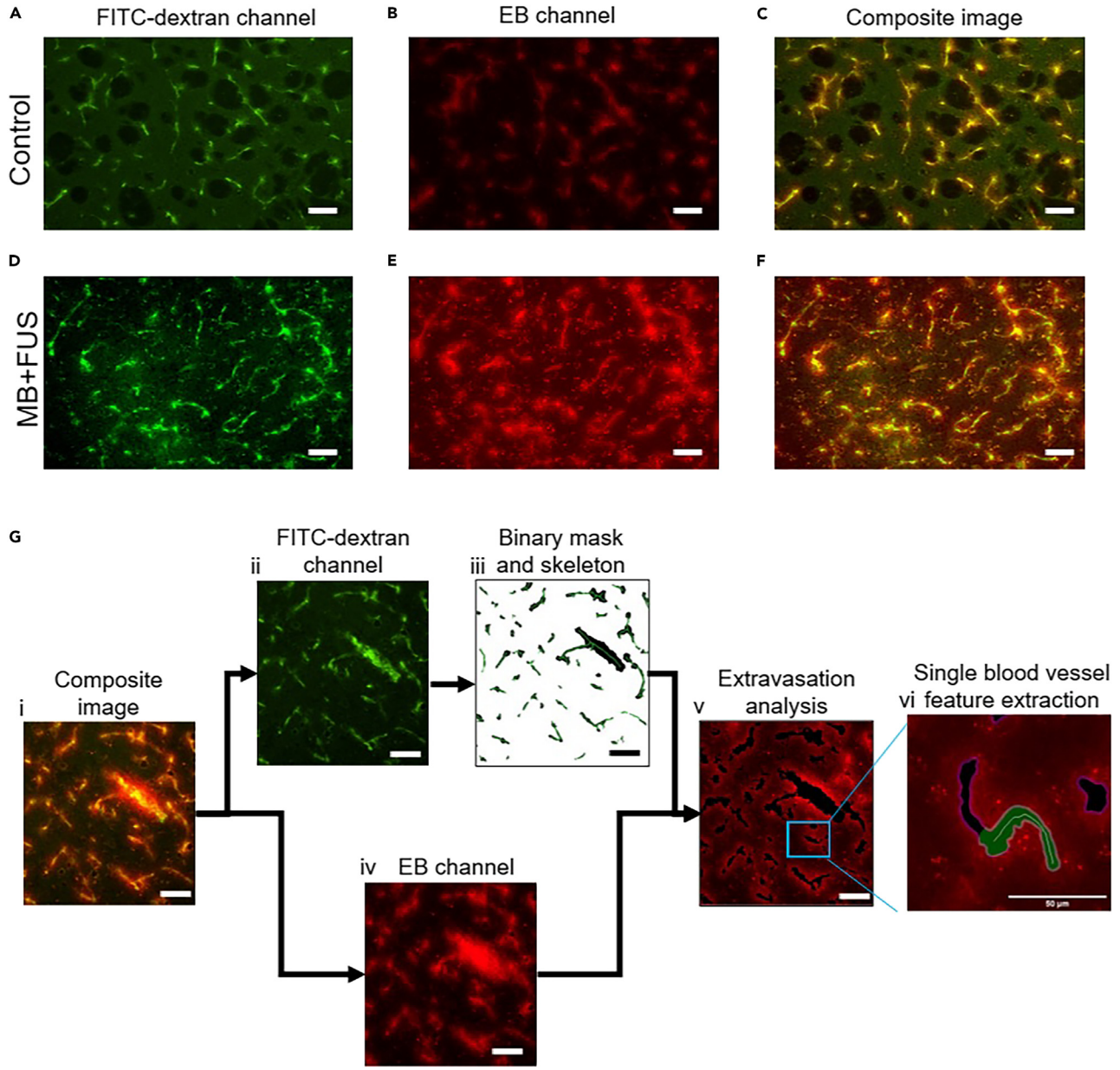

Figure 3. Microscopy imaging and image processing scheme

(A–F) Microscopy images of brain slices: Blood vessels visualization appears in green. EB extravasation appears in red. Control brain without BBBD (A, B), where the green and red channels overlap (C). FUS + MB treated brain (D and E) with the EB leakage around the blood vessels (F).

(G) Image processing scheme, as reported in our related work: (i) The original composite image is split to two channels: (ii) The green (FITC-Dextran) channel marking the blood vessels, and (iii) the red, EB channel. (iv) Morphological operations were applied to (ii) to perform vessel segmentation and skeleton extraction. (v) The binary mask in (d) was applied to the EB channel in (iii). (vi) The resulting image was used to determine the EB intensity in the perivascular ROI surrounding each vessel, and the vessel skeleton was used to extract the morphological features of each vessel. Finally, the median pixel intensity was quantified as a function of blood vessel diameter for the entire microvasculature population. Scale bars are 50 μm in all subfigures.

Image processing

Timing: 30 min/image

Microscopy image processing was performed using MATLAB. Briefly, the original image is split into two channels: the green channel is used for the vessel segmentation and morphological feature

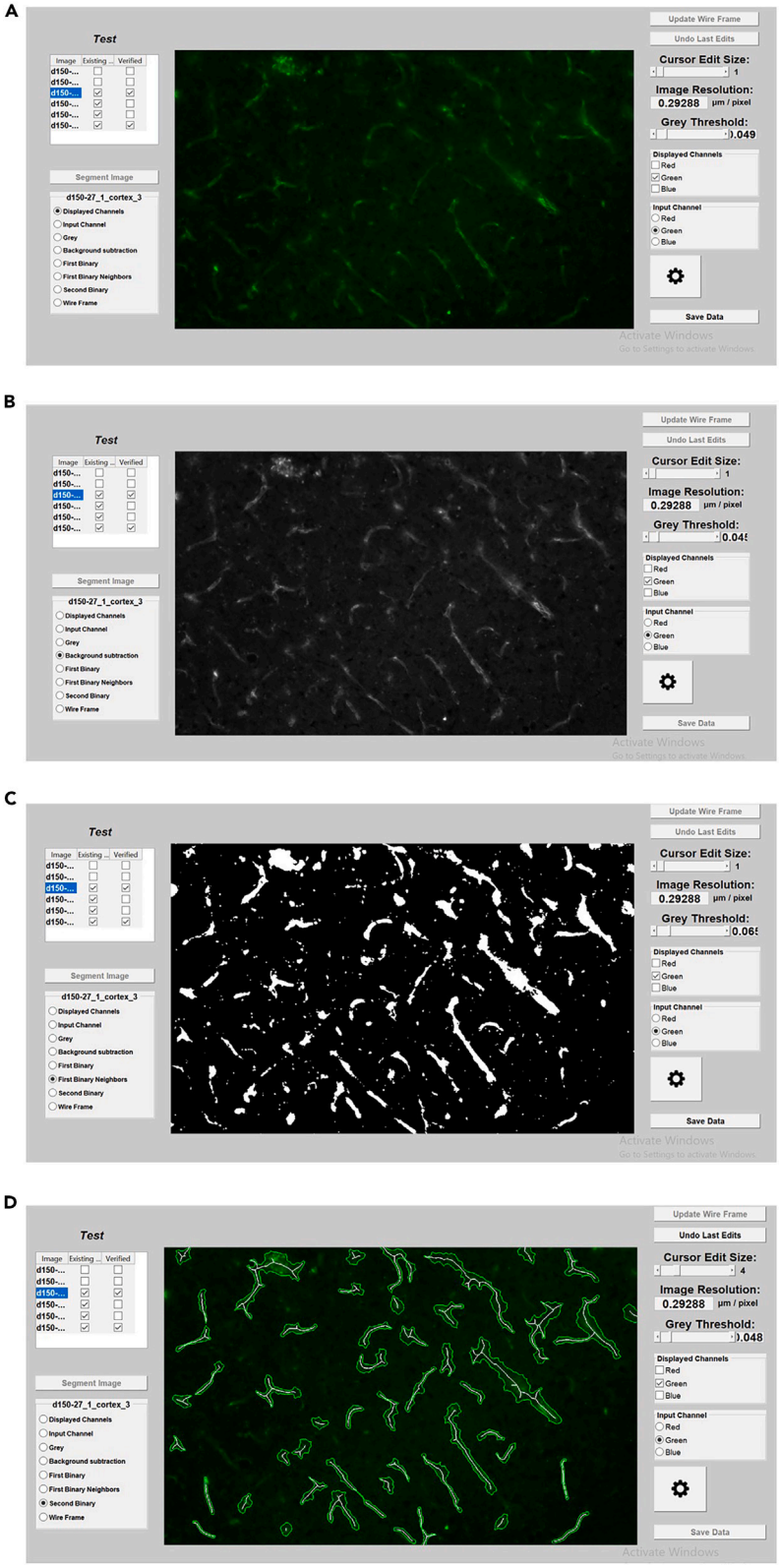
A
Test
Update Wire Frame
Undo Last Edits
Cursor Edit Size:
Image Resolution:
0.29288 µm / pixel
Grey Threshold:
Displayed Channels
Red
Green
Blue
Input Channel
Red
Green
Blue
Save Data
Segment Image
d150-27_1_cortex_3
Displayed Channels
Input Channel
Grey
Background subtraction
First Binary
First Binary Neighbors
Second Binary
Wire Frame
B
C
D

Figure 4. Blood-vessel segmentation and characterization is carried using a modified version of the Rapid Editable Analysis of Vessel Elements Routine (REAVER) tool

(A) Set the green channel as an input channel in REAVER GUI, used for the vessel segmentation.

(B) Use background subtraction image to improve signal to noise ratio.

(C) Use first binary neighbors' image to remove scattered noise in the image.

(D) An example of the final vessel segmentation.

extraction, whereas the red channel is used to quantify EB intensity around each blood vessel (Figure 3G).[1]

Note: All the MATLAB codes used for image processing can be found in the following GitHub repository https://github.com/ThellovitshLab/BBBD-REAVER.

⚠ CRITICAL: The MATLAB "Image Processing Toolbox" and "Statistics and Machine Learning Toolbox" should be installed in order to properly run the scripts.

23. Post-processing: blood-vessel segmentation and characterization.

Note: This was carried out using a modified version of the Rapid Editable Analysis of Vessel Elements Routine (REAVER) tool.[1]

a. Download the entire set of MATLAB files, which can be found in the following GitHub repository: https://github.com/ThellovitshLab/BBBD-REAVER/tree/master.
b. Activate MATLAB Software and run 'BBBDreaverApp' script.
c. Click 'Open REAVER GUI for segmentation'.

Note: Follow detailed instructions described in the following GitHub repository: https://github.com/ThellovitshLab/BBBD-REAVER/blob/master/REAVER%20GUI/REAVER%20GUI%20workflow.md

d. Load the tiff files (File > Load directory).
e. Open a representative image (by pressing the image name in the files menu on the left).
f. Choose the green (FITC-Dextran) channel as an input channel (right menu) (Figure 4A).
g. Open the gearbox tool and specify the default hyperparameters as following: averaging filter size: ~1.5 times more than diameter of the largest vessel (in px); minimum connected components area: slightly less than the expected smallest vessel cross-section (in px); wire dilation = 0; vessel thickness threshold: 0–5; background subtraction factor: 0.4–0.8. Choose an initial gray threshold = 0.06 (right menu). These values were found empirically in order to improve the contrast and facilitate the primary segmentation (which then manually curated by the user). They can be adjusted per frame.
h. Segment the image (click the left-side button).

Note: The above steps will create an initial segmentation, which can be improved iteratively (a new segmentation should be done for each iteration).

i. Look at the gray and the background subtraction image (left radio-button menu) (Figure 4B).
   i. The background subtraction image should be better than the gray image by having an enhanced SNR, sharper vessel edges and more uniform vessel intensity across different regions of the frame.
j. Look at the first binary and first binary neighbors image (left radio-button menu) (Figure 4C).
   i. The vessels should all be segmented in white. Likewise, a small amount of the scattered noise should also be segmented in white.
k. Look at the secondary binary image (Figure 4D). This is the final segmentation of the blood vessels.

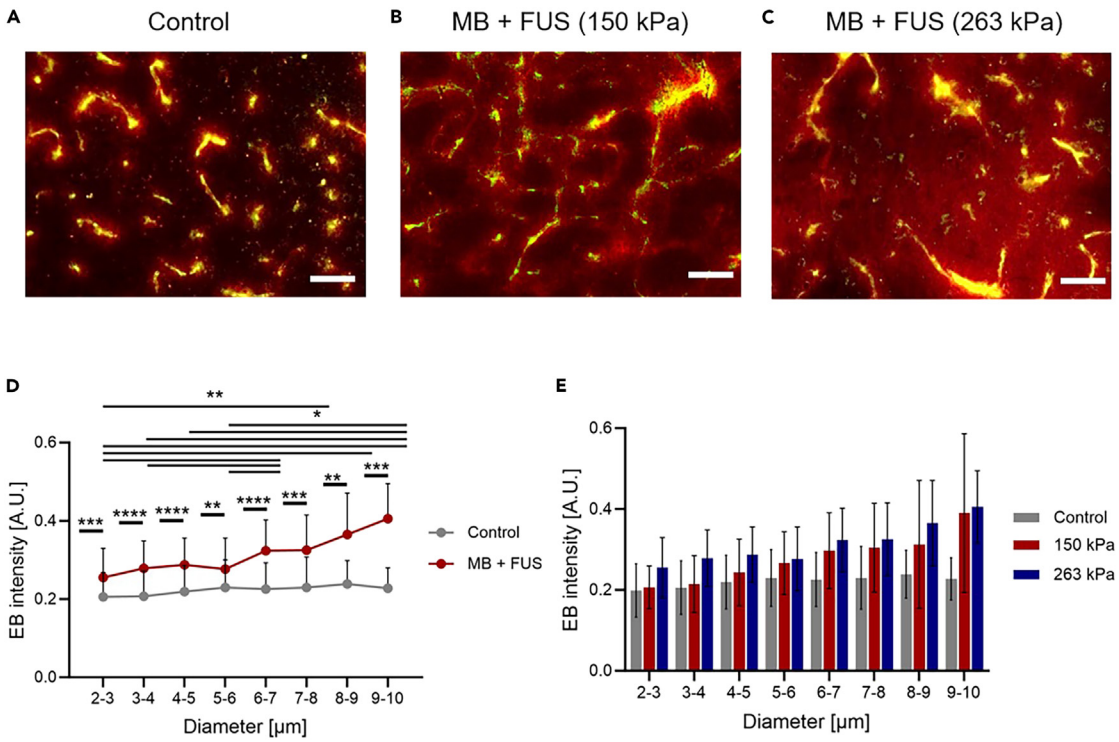

Figure 5. Expected outcomes: Microscopy images of brain slices

Blood vessels appear in green, whereas extravasated EB is red.

(A) Control brain without BBBD, where the green and red channels overlap.

(B and C) MB + FUS treated brain demonstrating increasing levels of EB extravasation to the perivascular area as a function of applied PNP: 150 kPa, and 263 kPa. Scale bars are 50 μm. EB extravasation quantification: (D) as a function of microvasculature diameter; (E) as a function of microvasculature diameter and applied PNP. The p values were *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The data in (E) is plotted as mean +- SD.

l. If needed, manually do the segmentation for desired blood vessels, by using "Cursor size edit" followed by "Update wire frame".

EXPECTED OUTCOMES

The protocol presented here provides a robust and large-scale method for fluorescent molecules extravasation assessment across the BBB following FUS-mediated BBBD at a single blood vessel resolution. Microscopy imaging of brain slices and an automated image processing algorithm conduct the segmentation of the blood capillaries and extract morphological features (green channel). These parameters are subsequently used for BBB opening identification and quantification as a function of blood vessel diameter (red channel). This method provides a high resolution of <1 μm, enabling to detect abnormalities and variations as a function of blood vessel diameter in small capillaries, and will aid in exploring drug delivery to the brain. This is important for many brain pathologies, such as Alzheimer's and Parkinson's diseases which are the most common type of age-related neurodegenerative diseases.[15–17] Our approach can quantify the EB diffusion into to the perivascular area surrounding blood vessels, and can assess multiple parameters, including the EB diffusion length, vascular density, and microvasculature diameter. Furthermore, it can be used to monitor additional key BBBD parameters, such as duration of BBB opening, and the ability to deliver different sized molecules across the BBB. In addition, it can detect variations in BBBD in all of the brain regions, without the need for complicated *in vivo* procedures such as craniotomies.

In this study, we present microscopy images of brain slices following BBB opening using US and MBs, which reveal clear and reliable blood vessel staining in green, as well as widespread extravasation of EB dye in red around the blood vessels (Figures 5B and 5C). Control brains (Figure 5A) show

expected overlap of EB with the green channel. Our analysis focuses on the relationship between EB extravasation and blood vessel diameter. Notably, in the treated brains, EB extravasation increases proportionally with the blood vessel diameter, resulting in significantly higher EB concentration compared to the control group (Figure 5D). However, in the control group (Figure 5D), EB extravasation remains consistent across different blood vessel diameters. We also examined the diameter-dependent EB extravasation as a function of peak negative pressure (PNP). Figure 5E illustrates the evaluation of EB intensity in microvasculature using two applied insonations: 150 and 263 kPa. At lower PNPs (150 kPa), no significant difference is observed compared to the control group for diameters up to 5–6 μm. However, in treated brains subjected to 263 kPa, EB intensity is significantly higher than in all other treated groups, including controls, for diameters larger than 5–6 μm (Figure 5E). For larger diameters, EB intensity is consistently higher in all treated brains relative to the controls.

Our method provides an effective platform for studying BBBD variations and identifying crucial parameters that influence FUS-mediated BBBD. The clinical implications of this research are substantial, particularly in the context of brain therapies for conditions such as brain cancer, inflammation, and neurodegenerative diseases. The findings hold promise for developing a vascular-size-dependent BBB opening platform, further enhancing the efficacy and precision of brain treatments.

QUANTIFICATION AND STATISTICAL ANALYSIS

Leakage quantification

EB quantification was performed for individual blood vessels. We excluded the vessels above 10 μm, since the majority of blood vessels in the mouse brain are smaller than 10 μm. Here, we present the procedure for the leakage quantification using custom-made MATLAB codes.

Note: Follow detailed instructions described in the following GitHub repository: https://github.com/TheIlovitshLab/BBBD-REAVER/tree/master.

1. In EB reaver window, define control and test directory.
2. Set parameters 'perivascular width' as 10, and 'distance from vessels wall' as 2.

Note: Perivascular area around the blood vessel and distance from the blood vessels wall were optimized and chosen as described in our published paper.[1]

3. Press 'Process folders'.

⚠ CRITICAL: Wait until all the control and treated images are being processed.

4. Further, press 'Create analysis object', and choose a generated file 'EB_analysis_from_2px__to_12px_N' for the control and treated group. a. This will generate a summary object of class 'Ext_analysis' and save it to the open MATLAB workspace as 'results'.

Note: The EB intensity results are divided into groups based on the vessel diameter. Vessels with diameters of 2–10 mm are sorted into diameter groups at 1 mm intervals (2–3, 3–4, ..., 9–10 mm).

5. Present results with desired graph format.
   a. Create a bar plot for the EB extravasation.

```
>results.barplot([2:10])
```

   b. Plot the fraction of strong BBBD blood vessels.

```
>results.openedHist([2:10],0,'on')
```

Note: The percentage of BBBD blood vessels for each diameter group was assessed by setting a threshold of two standard deviations above the mean control EB intensity. In the treated group, a strong BBBD was considered in all the blood vessels whose EB intensity exceeded the corresponding threshold.

Statistics

Statistical analysis was performed using MATLAB and GraphPad Prism. The results are presented as the mean ± SD. p values of less than 0.05 were considered significant.

LIMITATIONS

The first limitation of this study is that our transducer's focal spot is large, such that the most of the mouse brain was insonified. In order to induce BBBD more precisely, we could use a smaller focal spot by replacing the tranducer to one with a larger aperture and optimize US parameters for each brain region separately. In terms of microscopy imaging limitations, the images that are taken here are two dimensional, whereas the brain and blood vessels architecture are three-dimensional, potentially affecting the classification and accuracy. By implementing 3D-microscopy techniques, such as light sheet microscopy, we may assess BBBD volumetric effects as a function of blood vessel diameter.

TROUBLESHOOTING

Problem 1

Air bubbles within the water tank and/or beneath the agarose pad (major steps 1 and 2).

Potential solution

Carefully remove the agarose pad and refill the water tank with degassed water. Place the agarose pad, making sure that there are no air bubbles within the water tank and/or beneath the agarose pad (Figures 2A and 2B).

Problem 2

During the flash-freezing or slicing process, the brain got broken (major step 16b) (Figure 6A).

Potential solution

This brain could still be sliced and analyzed, with careful consideration. Pay careful attention to the flash freezing in the next experiments.

Problem 3

The microscopy images are showing long stripes or fractures (major step 20).

Potential solution

The frozen brain might have been too cold during the slicing. Make sure to use a correct cryostat temperature for brain slicing (−20 C).

Problem 4

The fluorescent signal is weak (major step 20) (Figure 6B).

Potential solution

This can be dependent on the expression level of the signal, and/or the localization. Increase the expression level of the corresponding signal; however, keep the signal-to-noise ratio.

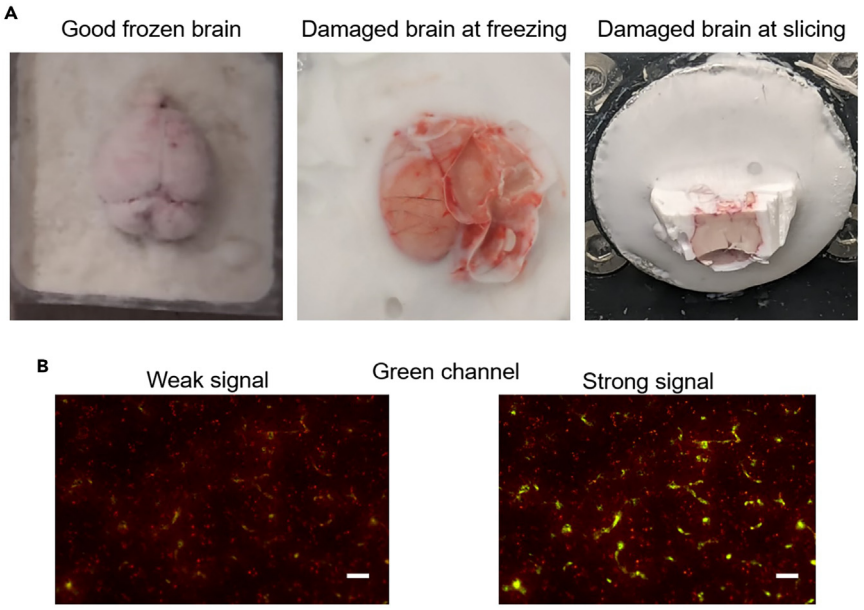

Figure 6. Troubleshooting during brain slicing and imaging

Troubleshooting: (A) During the flash-freezing or slicing process, the brain got broken (mid and right frames). (B) The fluorescent signal is weak (left frame). Scale bars are 50 μm.

Alternatively, this can be related to the failed injection of the corresponding dye in the mice (major steps 14a and /or 15c).

Problem 5

The automatic segmentation is not accurate to all brain regions (major step 23h) (Figure 4).

Potential solution

It is recommended to set the segmentation parameters per each brain region, separately. To do so, avoid the "Process All Images" option, instead note the optimal segmentation parameters for each region and apply the segmentation to each image separately.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Tali Ilovitsh (ilovitsh@tauex.tau.ac.il).

Technical contact

Information and technical requests for resources and reagents should be directed to and fulfilled by Dr. Lea Peko (lea.peko@gmail.com), Sharon Katz (sharonkats510@gmail.com) and Roni Gattegno (ronigattegno@mail.tau.ac.il).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All the codes used for image processing and analysis are described in our recently published paper[1] and can be found in the following GitHub repository https://github.com/TheIlovitshLab/BBBD-REAVER.

ACKNOWLEDGMENTS

This work was supported by funding from Insightec Ltd., the Israel Science Foundation (grant numbers 192/ 22 and 3450/20), the Israel Ministry of Science and Technology (grant number 101716), an ERC StG grant (no. 101041118) (NanoBubbleBrain), and a Zimin Institute grant, and was partially supported by a grant from the Nicholas and Elizabeth Slezak Super Center for Cardiac Research and Biomedical Engineering at Tel Aviv University.

AUTHOR CONTRIBUTIONS

L.P. assisted with the *in vivo* experiments, microscopy, and image processing and wrote this manuscript. S.K. designed and performed the research. R.G. revised the manuscript. T.I. advised and designed the research. All authors have approved the final version of this manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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