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Corresponding Author:	Rianne Haumann VU medisch centrum Amsterdam, Noord-Holland NETHERLANDS
Corresponding Author's Institution:	VU medisch centrum
Corresponding Author E-Mail:	r.haumann1@amsterdamumc.nl
Order of Authors:	Rianne Haumann
	Elvin 't Hart
	Marc P. P. Derieppe
	Helena C. Besse
	Gertjan J. L. Kaspers
	Eelco Hoving
	Dannis G. van Vuurden
	Esther Hulleman
	Mario Ries
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TITLE:

A High-Throughput Image-Guided Stereotactic Neuronavigation and Focused Ultrasound System for Blood-Brain Barrier Opening in Rodents

AUTHORS AND AFFILIATIONS:

Rianne Haumann[1,2*], Elvin 't Hart[2*], Marc P. P. Derieppe[2], Helena C. Besse[3], Gertjan J. L. Kaspers[1,2], Eelco Hoving[2], Dannis G. van Vuurden[1,2], Esther Hulleman[1,2], Mario Ries[3]

[1] Amsterdam UMC, Vrije Universiteit Amsterdam, Pediatric Oncology, Cancer Center Amsterdam, The Netherlands

[2] Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands

[3] Imaging Division, Utrecht University, Utrecht, The Netherlands

* Co-authors contributed equally to this study

Email addresses of co-authors:

Rianne Haumann	(r.haumann1@amsterdamumc.nl)
Helena C. Besse	(H.C.Besse-3@prinsesmaximacentrum.nl)
Marc P. P. Derieppe	(M.P.P.Derieppe-3@prinsesmaximacentrum.nl)
Elvin 't Hart	(E.tHart-6@prinsesmaximacentrum.nl)
Eelco Hoving	(E.W.Hoving@umcutrecht.nl)
Esther Hulleman	(E.Hulleman@prinsesmaximacentrum.nl)
Gertjan J.L. Kaspers	(G.J.L.Kaspers@prinsesmaximacentrum.nl)
Mario Ries	(M.Ries@umcutrecht.nl)
Dannis G. van Vuurden	(D.G.vanVuurden@prinsesmaximacentrum.nl)

Corresponding author:
Esther Hulleman
E.Hulleman@prinsesmaximacentrum.nl

KEYWORDS:

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SUMMARY:

The blood-brain barrier (BBB) can be temporarily disrupted with microbubble-mediated focused ultrasound (FUS). Here, we describe a step-by-step protocol for high-throughput BBB opening in vivo using a modular FUS system accessible for non-ultrasound experts.

ABSTRACT:

The blood-brain barrier (BBB) has been a major hurdle for the treatment of various brain diseases. Endothelial cells, connected by tight junctions, form a physiological barrier preventing large molecules (>500 Da) from entering the brain tissue. Microbubble-mediated focused ultrasound (FUS) can be used to induce a transient local BBB opening, allowing larger drugs to

enter the brain parenchyma.

In addition to large-scale clinical devices for clinical translation, preclinical research for therapy response assessment of drug candidates requires dedicated small animal ultrasound setups for targeted BBB opening. Preferably, these systems allow high-throughput workflows with both high-spatial precision as well as integrated cavitation monitoring, while still being cost effective in both initial investment and running costs.

Here, we present a bioluminescence and X-ray guided stereotactic small animal FUS system that is based on commercially available components and fulfills the aforementioned requirements. A particular emphasis has been placed on a high degree of automation facilitating the challenges typically encountered in high-volume preclinical drug evaluation studies. Examples of these challenges are the need for standardization in order to ensure data reproducibility, reduce intra-group variability, reduce sample size and thus comply with ethical requirements and decrease unnecessary workload. The proposed BBB system has been validated in the scope of BBB opening facilitated drug delivery trials on patient-derived xenograft models of glioblastoma multiforme and diffuse midline glioma.

INTRODUCTION:

The blood-brain barrier (BBB) is a major obstacle for drug delivery into the brain parenchyma. Most therapeutic drugs that have been developed do not cross the BBB due to their physicochemical parameters (e.g., lipophilicity, molecular weight, hydrogen bond acceptors and donors) or are not retained due to their affinity for efflux transporters in the brain[1,2]. The small group of drugs that can cross the BBB are typically small lipophilic molecules, which are only effective in a limited number of brain diseases[1,2]. As a consequence, for the majority of brain diseases, pharmacological treatment options are limited and new drug delivery strategies are need[3,4].

Therapeutic ultrasound is an emerging technique that can be used for different neurological applications such as BBB disruption (BBBD), neuromodulation, and ablation[4-7]. In order to achieve a BBB opening with an extracorporeal ultrasound emitter through the cranium, focused ultrasound (FUS) is combined with microbubbles. Microbubble-mediated FUS results in increased bioavailability of drugs in the brain parenchyma[5,8,9]. In the presence of sound waves, microbubbles start to oscillate initiating transcytosis and disruption of the tight junctions between the endothelial cells of the BBB, enabling paracellular transport of larger molecules[10]. Previous studies confirmed the correlation between the intensity of the acoustic emission and the biological impact on the BBB opening[11-14]. FUS in combination with microbubbles has already been used in clinical trials for the treatment of glioblastoma using temozolomide or liposomal doxorubicin as the chemotherapeutic agent, or for therapy of Alzheimer's disease and amyotrophic lateral sclerosis[5,9,15,16].

Since ultrasound mediated BBB opening results in entirely new possibilities for pharmacotherapy, preclinical research for clinical translation is needed to assess the therapy response of selected drug candidates. This typically requires a high-throughput workflow with both high-spatial

precision and preferably an integrated cavitation detection for monitoring of targeted BBB opening with a high reproducibility. If possible, these systems need to be cost effective in both initial investment and running costs in order to be scalable according to the study size. Most preclinical FUS systems are combined with MRI for image-guidance and treatment planning[15,17-19]. Although MRI gives detailed information about the tumor anatomy and volume, it is an expensive technique, which is generally performed by trained/skilled operators. In addition, high-resolution MRI may not always be available for researchers in preclinical facilities and requires long scanning times per animal, making it less suitable for high-throughput pharmacological studies. Noteworthy is that, for preclinical research in the field of neuro-oncology, in particular infiltrative tumor models, the possibility to visualize and target the tumor is essential for treatment success[20]. Currently, this requirement is only fulfilled by MRI or by tumors transduced with a photoprotein, enabling visualization with bioluminescence imaging (BLI) in combination with administration of the photoprotein substrate.

MRI-guided FUS systems often use a water bath to ensure ultrasound wave propagation for transcranial applications, whereby the head of the animal is partly submerged in the water, the so called ''bottom-up'' systems[15,17,18]. While these designs work generally well in smaller animal studies, they are a compromise between animal preparation times, portability and realistically maintainable hygienic standards during usage. As an alternative to MRI, other guidance methods for stereotactic navigation encompass the use of a rodent anatomical atlas[21-23], laser pointer assisted visual siting[24], pinhole-assisted mechanical scanning device[25], or BLI[26]. Most of these designs are “top-down” systems in which the transducer is placed on top of the animal’s head, with the animal in a natural position. The ''top-down'' workflow consists either of a water bath[22,25,26] or a water-filled cone[21,24]. The benefit of using a transducer inside a closed cone is the more compact footprint, shorter setup time and straight-forward decontamination possibilities simplifying the entire workflow.

The interaction of the acoustic field with the microbubbles is pressure dependent and ranges from low-amplitude oscillations (referred to as stable cavitation) to transient bubble collapse (referred to as inertial cavitation)[27,28]. There is an established consensus that ultrasound-BBBD requires an acoustic pressure well above the stable cavitation threshold to achieve successful BBBD, but below the inertial cavitation threshold, which is generally associated with vascular/neuronal damage[29]. The most common form of monitoring and control is the analysis of the (back-)scattered acoustic signal using passive cavitation detection (PCD), as suggested by McDannold et al.[12]. PCD relies on the analysis of the Fourier spectra of microbubble emission signals, in which the strength and appearance of stable cavitation hallmarks (harmonics, subharmonics, and ultraharmonics) and inertial cavitation markers (broadband response) can be measured in real-time.

A “one size fits all” PCD-analysis for precise pressure control is complicated due to the polydispersity of the microbubble formulation (the oscillation amplitude depends strongly on the bubble diameter), the differences in bubble shell properties between brands, and the acoustic oscilation, which depends strongly on frequency and pressure[30-32]. As a consequence, many different PCD detection protocols have been suggested, which have been adapted to particular

combinations of all these parameters and have been used in various application scenarios (ranging from in vitro experimentation over small animal protocols to PCD for clinical usage) for robust cavitation detection and even for retroactive feedback control of the pressure[11,14,30-35]. The PCD protocol employed in the scope of this study is derived directly from McDannold et al.[12] and monitors the harmonic emission for the presence of stable cavitation and broadband noise for inertial cavitation detection.

We have developed an image-guided neuronavigation FUS system for transient opening of the BBB to increase drug delivery into the brain parenchyma. The system is based on commercially available components and can be easily adapted to several different imaging modalities, depending on the available imaging techniques in the animal facility. Since we require a high-throughput workflow, we have opted to use X-ray and BLI for image-guidance and treatment planning. Tumor cells transduced with a photoprotein (e.g., luciferase) are suitable for BLI imaging[20]. After administration of the photoprotein substrate, tumor cells can be monitored in vivo and tumor growth and location can be determined[20,36]. BLI is a low-cost imaging modality, it enables to follow the tumor growth over time, it has fast scanning times and it correlates well with tumor growth measured with MRI[36,37]. We have opted to replace the water bath with a water-filled cone attached to the transducer to enable flexibility to freely move the platform on which the rodent is mounted[8,24]. The design is based on a detachable platform equipped with integration of (I) small-animal stereotactic platform (II) fiducial markers with both X-ray and optical-image compatibility (III) rapid-detachable anesthesia mask, and (IV) integrated temperature regulated animal heating system. After the initial induction of anesthesia, the animal is mounted in a precise position on the platform where it remains during the entire procedure. Consequently, the entire platform passes all stations of the workflow of the entire intervention, while maintaining an accurate and reproducible positioning and sustained anesthesia. The control software allows the automatic detection of the fiducial markers and automatically registers all types of images and image modalities (i.e., micro-CT, X-ray, BLI and fluorescence imaging) into the frame of reference of the stereotactic platform. With help of an automatic calibration procedure, the focal length of the ultrasound transducer is precisely known within, which enables the automatic fusion of interventional planning, acoustic delivery and follow-up imaging analysis. As shown in **Figure 1** and **Figure 2**, this setup provides a high degree of flexibility to design dedicated experimental workflows and allows interleaved handling of the animal at different stations, which in-turn facilitates high-throughput experiments. We have used this technique for successful drug delivery in mouse xenografts of high-grade glioma such as diffuse midline glioma.

PROTOCOL:

All in vivo experiments were approved by the Dutch ethical committee (license permit number AVD114002017841) and the Animal Welfare Body of the Vrije Universiteit Amsterdam, the Netherlands. The investigators were trained in the basics of the FUS system in order to minimize the discomfort of the animals.

1. Focused ultrasound system

NOTE: The described setup is an inhouse built BBB disruption system based on commercially available components and includes a 3D-printed custom-made cone and detachable stereotactic platform. The system is designed modular, which facilitates modifications according to available equipment and specific use. The protocol describes the procedure for the sonoporation of a larger area in the pontine region of the mouse brain. By adjusting the target location, different parts of the brain could be targeted. In this study a 1 MHz mono-element transducer with a, focal length of 75 mm, an aperture of 60 mm and a focal area of 1.5 x 1.5 x 5 mm (FWHM of peak pressure) was used. The focal plane of the transducer is positioned through the cranium of the animal in the horizontal plane intersecting with the ear bars.

1.1. Select an appropriate transducer for BBB opening in rodents.

NOTE: Based on the properties of the microbubbles and the employed frequency, the acoustic settings, in particular the mechanical index (MI), are subject to change[13,38].

1.2. Place the transducer in the 3D-printed cone.

1.3. Employ an acoustically transparent mylar membrane at the bottom-end of the cone to achieve acoustic coupling of the beam propagation path, and fill the cone with degassed water.

1.4. Mount the transducer above the animal on a motorized linear stage as shown in **Figure 1** allowing automatic vertical positioning of the transducer.

1.5. Design a detachable stereotactic platform based on the requirements of the study, which includes temperature regulated heating, bite and ear bars, anesthesia and multi-modality fiducial markers, as shown in **Figure 1** and **Figure 2**. The mounting of the stereotactic platform consists of a 2D linear stage system, which allows precise automatic positioning (< 0.1 mm) of the animal under the beam.

1.6. Connect the transducer to the acoustic emission chain shown in **Figure 1** consisting of a transducer, a function generator and a power amplifier.

1.7. Devise an image-processing pipeline to detect the multi-modality fiducial markers that allows precise sonoporation targeting of the brain area of interest and collection of the cavitation data detected by the needle hydrophone.

1.8. Calibrate the system and determine the focus point of the transducer in correspondence to vertical positioning of the animal on the stereotactic platform.

2. Animal preparation

NOTE: The following protocol is specified for mice but can be adapted for rats. For these experiments female athymic nude Foxn1-/- mice (6-8 week old) were used.

2.1. Allow the animal to acclimatize for at least one week in the animal facility and weigh the animal regularly.

2.2. Administer buprenorphine (0.05 mg/kg) via subcutaneous (s.c.) injection 30 min prior to FUS treatment to start analgesic treatment.

2.3. Anesthetize the animal with 3% isoflurane, 2 L/min O_2 and verify that the animal is deeply anesthetized. Keep the animals anesthetized during the whole procedure and monitor the breathing frequency and heart rate to adjust the concentration of isoflurane as required.

2.4. Apply eye ointment to prevent dry eyes and avoid possible injury.

2.5. Remove hair on the top of the head with a razor and depilatory cream and wash afterwards with water to remove any residues to avoid irritation to the skin.

2.6. For experiments with BLI tumor models, inject 150 µL of D-luciferin (30 mg/mL) intraperitoneal (i.p.) with a 29 G insulin syringe for BLI image-guidance.

2.7. Insert a 26-30 G tail vein catheter and flush the catheter and vein with a small volume of heparin solution (5 UI/mL). Fill the catheter with heparin solution to avoid blood clotting.

NOTE: Good catheterization is seen when there is a reflux of blood into the catheter. Avoid air bubbles in the catheter to prevent emboli. To avoid excessive injection pressure, make sure the length of the catheter is as short as possible.

2.8. Place the animal on the temperature regulated stereotactic platform to avoid hypothermia.

NOTE: Hypothermia reduces blood circulation, which can affect the injection/circulation of microbubbles and the pharmacokinetics of the drugs[39].

2.9. Immobilize and fix the head of the animal on the stereotactic platform using ear bars and a bite bar. Fixate the body with a strap and tape the tail of the animal to the platform.

3. In vivo image-guided focused ultrasound

NOTE: For this protocol a 1 MHz mono-element transducer with a tone-burst pulse with a 10 ms duration, a MI of 0.4 and a pulse repetition frequency of 1.6 Hz with 40 cycles for 240 s was used. The protocol is optimized for microbubbles stabilized by phospholipids containing sulphur hexafluoride (SF_6) as an innocuous gas, whereby the mean bubble diameter is 2.5 µm and more than 90% of the bubbles are smaller than 8 µm.

3.1. Place the stereotactic platform with the mounted animal in the imaging modality (e.g., BLI or X-ray) and take image(s) of the animal.

3.2. Use the multi-modality fiducial markers in combination with the image-processing pipeline to mark the position of the animal according to the focus point of the transducer.

3.3. Determine the target area by placing a brain outline over the acquired X-ray image or using BLI images to determine the center of the tumor (**Figure 2**). The position of specific parts of the brain are specified in the Paxinos Brain Atlas[40] using the skull markings bregma and lambda as reference points. For example the pons is located x=-1.0, y=-0.8 and z=-4.5 from lambda.

3.4. Shield the animal's nostrils and mouth with adhesive tape to prevent ultrasound gel interfering with breathing.

3.5. Apply ultrasound gel on top of the animal's head.

3.6. Retract the skin of the animals' neck, lubricate the needle hydrophone with ultrasound gel and place the needle hydrophone in the direct vicinity of the occipital bone.

3.7. Guide the transducer to the correct position using the image-processing pipeline and the focus point.

3.8. Apply the preconfigured settings to all attached devices and target the brain region of interest.

NOTE: Depending on the research question, tumor or brain regions can be sonoporated as a single focal point or as volumetric shape, as shown in **Figure 2**.

3.9. Activate microbubbles as described by the manufacturer. Inject one bolus of 120 µL (5.4 µg) of microbubbles.

3.10. Flush the tail vein catheter with saline to check the opening of the catheter.

3.11. Inject the microbubbles and start the insonation.

3.12. Record microbubble cavitation with the needle hydrophone.

3.13. Administer an intravascular contrast agent or drug after sonoporation. The dose, timing and planning are dependent on the purpose of the study and the drug.

NOTE: Evans blue is a common color agent to assess BBB opening[41].

3.14. Monitor the animal until the predetermined time point or before the humane endpoint.

4. Analysis of microbubble cavitation

NOTE: Here the applied procedure is described, which is suitable for in vivo experimentation for SF_6-phospholipid microbubbles with an average diameter of 2.5 µm (80% of the bubbles below 8 µm) excited with a burst-tone pulse of 10 ms duration at a frequency of 1 MHz, as originally suggested by McDannold et al.[12].

4.1 Fourier-transform the recorded PCD signal from the time-domain into the frequency domain.

4.2 Integrate the resulting spectral power for stable cavitation detection around the 2nd and 3rd harmonic (± 50 kHz), as shown in **Figure 3** (green box at 2 and 3 MHz).

4.3 Integrate the spectral power for inertial cavitation detection, between principal frequency, the 2nd, 3rd harmonic, the 1st and 2nd ultraharmonic and the first sub-harmonic (± 150 kHz), as shown in **Figure 3** (red boxes).

4.4 Integrate the spectral power around the principle frequency (1 MHz ± 50 kHz) for the normalization of both previously obtained PCD signals.

NOTE: The PCD signal, for SF_6-phospholipid microbubbles in vivo experiments at 1 MHz, does not display ultraharmonics or subharmonics before inertial cavitation sets in, as shown in **Figure 3**.

REPRESENTATIVE RESULTS:

The described FUS system (**Figure 1** and **Figure 2**) and the associated workflow have been used in over a 100 animals and produced reproducible data on both healthy and tumor bearing mice. Based on the recorded cavitation and the spectral density at the harmonics at the peak moment of the microbubble bolus injection, the spectral power of each frequency can be calculated using the Fourier analysis as explained in step 4 of the Protocol. Based on the acoustic protocol (1 MHz, 10 ms pulse duration) with a MI of 0.4 in combination with microbubbles, the normalized integrated power spectrum at the 2nd and 3rd harmonics normalized the integrated power spectrum of the excitation frequency observed in **Figure 3**. This provided a very sensitive and reliable means of stable cavitation detection, in comparison to no detection of subharmonics when no microbubbles were injected or the observation of inertial cavitation when a MI of 0.6 was applied. In case of inertial cavitation, an increased broad-band noise floor of up to 25 dB was detected as well as the appearance of ultra-harmonics and subharmonics. Although an acoustic pressure of an MI of 0.4 and 0.6 resulted in no macroscopic damage, microscopic damage was evidenced histologically at a MI of 0.6, as shown in **Figure 4**. A further increase of the pressure amplitude up to a MI of 0.8 resulted in a macroscopic brain hemorrhage of larger vessels and wide-spread tissue lysis with the extravasation of erythrocytes. The histological findings corresponded to the acoustic data from the passive cavitation sensor, as shown in **Figure 3**, confirming the damaging properties of inertial cavitation of the brain tissue. As a consequence, a MI of 0.4 was chosen as the safe pressure amplitude that provided very reproducible BBB-opening, while providing a safe margin to the inertial cavitation regime, as observed before[11].

Intravenous Evans blue was injected to validate the opening of the BBB in the pontine region. The strong albumin-binding of Evans blue leads to a large molecule of more than 66 kDa[42]. At the

level of the pons and partly the cerebellum, extravasation of Evans blue-conjugated albumin was observed in the mouse treated with FUS and microbubbles in contrast to the mouse without microbubbles (**Figure 5**). This emphasizes the precise targeting of the region of interest based on image-guided stereotactic navigation with the in-house build FUS system and the described protocol.

FIGURE AND TABLE LEGENDS:

Figure 1. Focused ultrasound setup. (**A**) Schematic representation of the focused ultrasound set up. (**B**) Picture of the focused ultrasound setup. The system consists of a top-down mounted transducer on a 1D linear stage over a second 2D stage for automatic 3D positioning. The transducer is built in a water filled beam-cone, closed at the bottom with an acoustically transparent mylar membrane, which conducts the sound to the cranium of the animal. The transducer is connected to a power amplifier, which is in-turn connected to an arbitrary waveform generator (AWG) for signal generation. For cavitation detection a detachable hydrophone in combination with a low-noise voltage amplifier is used. The hydrophone is placed in the direct vicinity of the occipital bone. The external hydrophone has a 2 mm active surface and is acoustically coupled with ultrasound gel. Both the high-voltage signal of the excitation pulse as well as the recorded cavitation signal are digitalized by a standard 200 MHz oscilloscope and relayed to a control computer (not shown) for on-the-fly processing and real-time control.

Figure 2. Focused ultrasound workflow. The proposed workflow of the focused ultrasound system starts with (**A**) the initial positioning of animal on a detachable stereotactic platform, note the application of the acoustic coupling gel (applied post BLI/X-ray). Simultaneously multimodal imaging can be conducted for targeting. (**B**) At first X-ray imaging is a possibility, whereas a region of interest can be targeted with the help of an outline of the brain (which in turn is referenced to the mouse brain atlas[40], adapted to the size and posture of the skull). (**C**) Alternatively, a BLI image of a luciferase transfected diffuse midline glioma tumor overlaid on an X-ray maximum intensity projection can be applied for targeting. (**D**) Subsequently, the stereotactic platform is mounted with the animal in therapy position with both hydrophone and transducer attached. The transducer automatically drives in therapy position and sonicates the chosen trajectory post bolus injection. The system is optimized for high-throughput experiments, whereby multiple platforms allow interleaved work, as shown on top.

Figure 3. Cavitation monitoring. (**A**) Frequency spectrum of an in vivo experiment in the absence of microbubble administation at a MI of 0.4 at 1 MHz. (**B**) Shown is the corresponding spectrum at peak-bolus after injection of microbubbles. Note the increase of the higher harmonics, which is indicative for stable cavitation of the microbubbles. (**C**) Corresponding spectrum observed at a higher MI of 0.6 in combination with microbubble injection, within the transition band to the onset of inertial cavitation, leading to an increase in noise floor up to 25 dB and the appearance of ultraharmonics and subharmonics.

Figure 4. BBB opening and associated histology. (**A**) Stable cavitation using an MI of 0.4 evidenced an intact brain parenchyma in both white light macroscopy and HE stained microscopy. (**B**) After a MI of 0.6 first signs of local irreversible tissue damage of the brain

parenchyma is becoming apparent in the HE stained histological data. (**C**) For even higher mechanical pressure of MI 0.8, macroscopic hemorrhaging is apparent as well as wide-spread tissue lysis of the brain parenchyma and the extravasation of erythrocytes due to micro-hemorrhaging. The blue hue in the white light macroscopy is indicative for the extravasation of the co-injected intra-vascular contrast agent Evans blue indicating BBB opening (see **Figure 5** for a sagittal view).

Figure 5. **Validation of BBB opening.** Demonstration of successful BBB opening in the stable cavitation regime (**B**) compared to the control (**A**), no microbubbles injected. In this case Evans blue has been used as an intravascular contrast agent. The strong albumin-binding of Evans blue leads to a large molecule of more than 66 kDa. As a consequence, evidence of the Evans blue extravasation is indicative for paracellular transport across the BBB due to a (partial) opening of the tight junctions.

DISCUSSION:

In this study, we developed a cost-effective image guided based FUS system for transient BBB disruption for increased drug delivery into the brain parenchyma. The system was largely built with commercially available components and in conjunction with X-ray and BLI. The modularity of the proposed design allows the use of several imaging modalities for planning and assessment in high-throughput workflows. The system can be combined with more comprehensive high-resolution 3D imaging modalities, for example high-resolution MRI or micro-CT, while for the bulk of the study 2D imaging modalities such as 2D X-ray and/or BLI are used. 2D X-ray and/or BLI are both considerably more cost effective as well as ideal for high-volume studies due to their respective short acquisition times. The transducer described here is well suited to produce BBBD in larger areas (on the scale of a mouse brain) in deeper parts of the brain (f number of 1.25). We have used the system for diffusely growing tumors in the pontine region[43,44]. For these regions a larger volume needs to be sonoporated that encompasses the entire tumor region in the pons. The modular system can easily be adjusted for other types of brain tumors in more supratentorial parts of the brain. In order to decide on the transducer type one should hold into account the f-number, focal length and frequency.

The overall design proposes thereby two refinements compared to previously suggested designs. (I) Frequently a water bath is used for ultrasound wave transmission of therapeutic systems. For transcranial applications in small animals this type of design results in larger and inverted setups, whereby the animal is partially submerged[11,22,25]. While these designs work generally very well in the scope of smaller animal studies, they are a compromise with respect to setup times, portability and realistically maintainable hygienic standards during usage. In particular the latter is of considerable importance in the scope studies encompassing immunocompromised animals and thus strict hygienic standards. As a consequence, in order to design a system with a more compact footprint, shorter setup time, easy decontamination possibilities and a natural position of the animal during the entire workflow, a “top-down” design was chosen. (II) The second design choice that differs from several previously described designs was to omit the direct integration of the acoustic delivery system into a medical imaging system such as an MRI or a micro-CT[15,17-19,45]. While fully integrated systems are ideal for longitudinal pharmacokinetic studies or

explorative research on a limited number of animals, such setups are generally less suitable for high-volume pharmacological studies due to considerably increased complexity, high running-costs and need for trained/skilled operators. Furthermore, such systems are generally limited to only one imaging modality. As a consequence, the proposed design here relies on a modular detachable stereotactic platform, which is compatible with several imaging modalities (micro-CT, small animal MRI, a variety of BLI/fluorescence cameras, these with or without integrated X-ray imaging) and provides also multi-modality fiducial markers for automatic fusion of all image data in a common frame of reference for both interventional planning and the follow-up post BBB opening.

With respect to practical considerations, the most critical point of failure in the procedure is the stability of the microbubbles due to their limited lifetime and their fragile nature. We would like to emphasize that the following discussion concerns microbubbles stabilized by phospholipids and containing sulphur hexafluoride (SF_6) as an innocuous gas[46,47], while other microbubble formulations will generally display different properties.

Timing before microbubble injection: The advertised lifespan of commercially available microbubbles after re-hydration is between as 3 and 4 hours. While this is suitable for diagnostic ultrasound applications, it should be noted that during this entire period the microbubbles continuously lose gas and consequently the mean bubble diameter is subject to a continuous downward-drift from the initial average size of 2.5 µm. For therapeutic applications such as ultrasound-mediated BBBD this implies much stricter timing-imperatives, since the oscillation amplitude of stable cavitation (at a given frequency and pressure) and the onset-threshold of inertial cavitation are as a direct consequence also subject to a continuous drift. In our experience, we have observed that microbubbles are best used within 30 minutes after rehydration in order to obtain reproducible results, similar to previous reportings[48].

Timing after microbubble injection: In larger primates, commercially available SF_6-phospholipid microbubbles display a blood-plasma elimination half-life of about 6 minutes and more than 80% of the administered gas is exhaled via the lungs after only 11 minutes[48]. In small mammals such as mice and rats the blood-plasma elimination half-life of this type of microbubbles in vivo is with 90-120 seconds considerably shorter due to the higher heartrate[20]. As a consequence, the rapid dynamic of the microbubble concentration directly after bolus injection and the fast subsequent plasma elimination combined with the continuous gas volume loss of the bubbles imposes strict timing requirements on the sonication/injection protocol in order to obtain reproducible results within the short duration of 3-4 minutes post-injection. Longer procedures or more extensive volumes of BBBD require preferably a continuous administration of microbubbles. However, such an approach is complicated by the buoyancy of the bubbles in both the syringe and the feeding-system and also introduces a considerably increased dead volume by the required infusion tubing. In our experience the simpler solution of splitting the total injection volume into 2 to 3 smaller sub-doses provided a robust and reproducible results.

In addition, microbubbles are very pressure sensitive and high hydrostatic pressures during injection are therefore not recommended. As a consequence, large needles (>19 G) are

recommended for the transfer of microbubbles into a plastic tube or to draw up microbubbles with a syringe[49]. For i.v. injection in mice 26-30 G needles are recommended; since larger needles are more difficult to insert into the tail vein. The 26 G needle is recommended since the hydrostatic pressure is lower with this needle. However, in case of difficult venous access the 30 G needle is recommended.

The cranium of the mouse is an important attenuator of the pressure amplitude that significantly lowers the pressure amplitude at the focus. Attenuation is determined by the frequency of the transducer and the density of the medium the ultrasound wave propagates. Higher ultrasound frequencies and high tissue densities, like bone results in high attenuation. The pressure amplitude is partially absorbed by bone and some pressure amplitude is lost by reflection and scattering[50]. In our experiments we have determined in mouse cadavers that the attenuation at 1 MHz is 14.5 ± 1.3 dB/cm with an average skull thickness of 0.9 mm as shown before[21,50]. Cavitation monitoring is highly recommended since microbubbles reflect distinct acoustic emissions during stable cavitation and inertial cavitation. Wideband emission is a distinct acoustic emission for inertial cavitation[12]. Real-time monitoring makes it possible to detect inertial cavitation and lower the pressure amplitude accordingly to avoid tissue damage.

Previous reports described the influence of the type of anesthesia on the achieved BBB permeability[11,31]. For isoflurane based anesthesia, a vasodilation occurs shortly after anesthesia initiation, which is associated with a slight reduction of the cerebral blood flow. Furthermore, anesthesia over extended durations, in particular in absence of a temperature stabilization, leads to a reduced heart rate. Since both factors can potentially lead to a larger variance of the cerebral concentration of both microbubbles or co-administered drugs, a strict anesthesia protocol is advisable to achieve reproducible results[51]. Anesthesia with 1.5% v/v isoflurane in 2 L/min oxygen for 35 to 45 minutes was not problematic, as advised by Constantinides et al.[51]. In contrast to McDannold et al. who showed that this gas mixture in combination with the specific type of their microbubbles was problematic[52], we have not observed noteworthy problems with this type of microbubbles. Alternatively, the animals can be anesthetized with a mix of ketamine/xylazine, which has no known vasoactive effects[53].

In summary, the imaging-guided BBB-opening technique described here has been used for high-volume preclinical drug evaluation studies that demonstrated the efficiency of the suggested workflow. The system could thereby be operated by non-technical personnel after a short training due to the high degree of automation. This in combination with the simplicity of the setup resulted in a high degree of standardization, which in turn ensures experimental reproducibility, reduced intra-group variability and thus allows to reduce the required sample size.

ACKNOWLEDGMENTS:
This project was funded by the KWF-STW (Drug Delivery by Sonoporation in Childhood Diffuse Intrinsic Pontine Glioma and High-grade Glioma). We thank Ilya Skachkov and Charles Mougenot for their input in the development of the system.

DISCLOSURES:

The authors have nothing to disclose.

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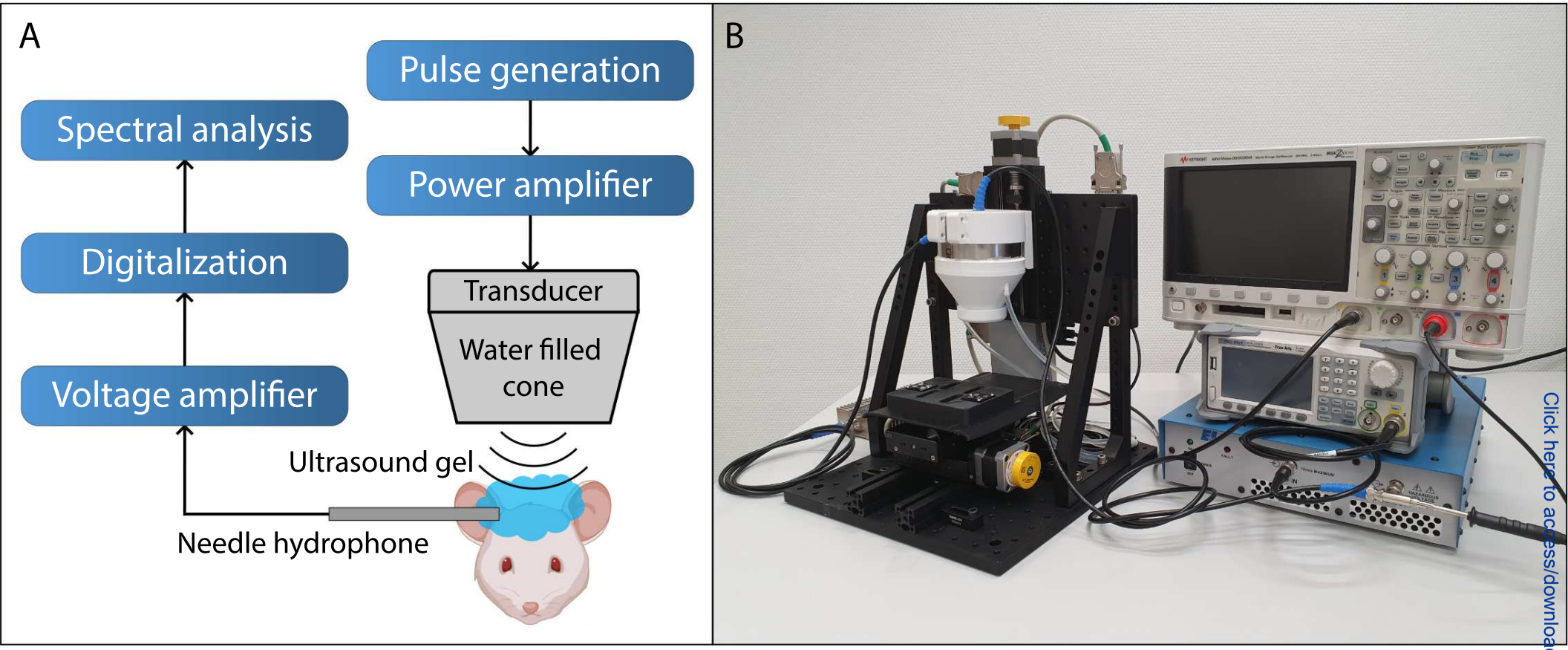
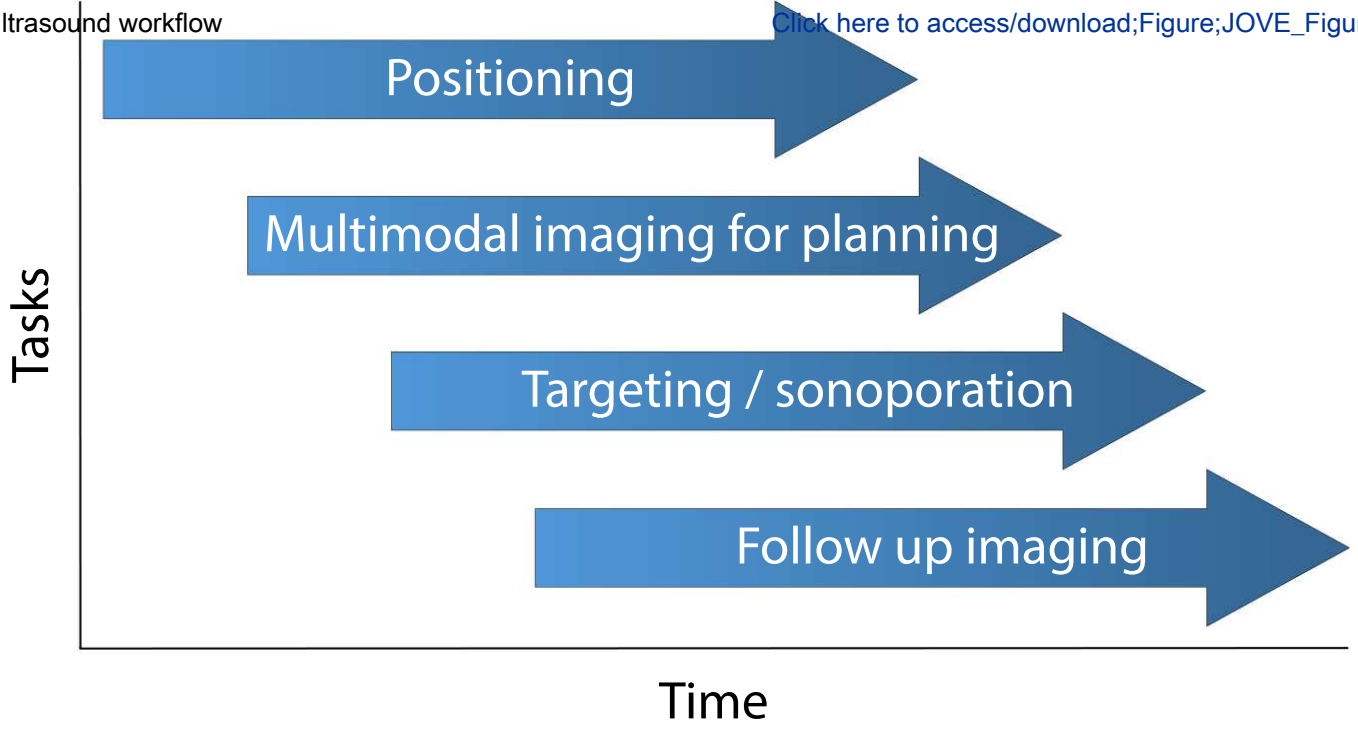
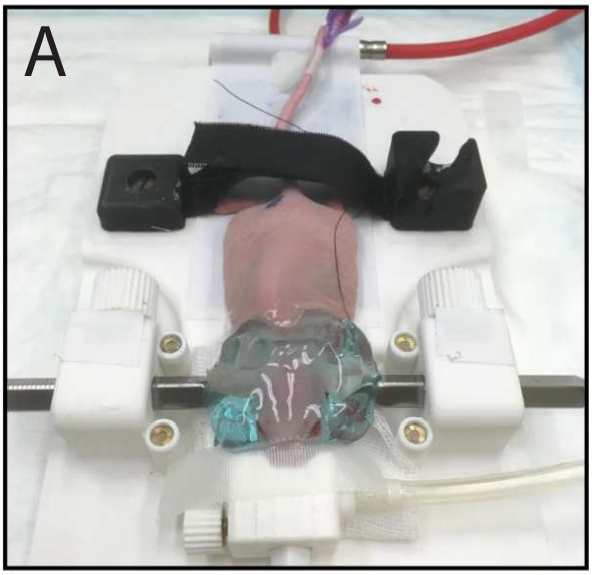
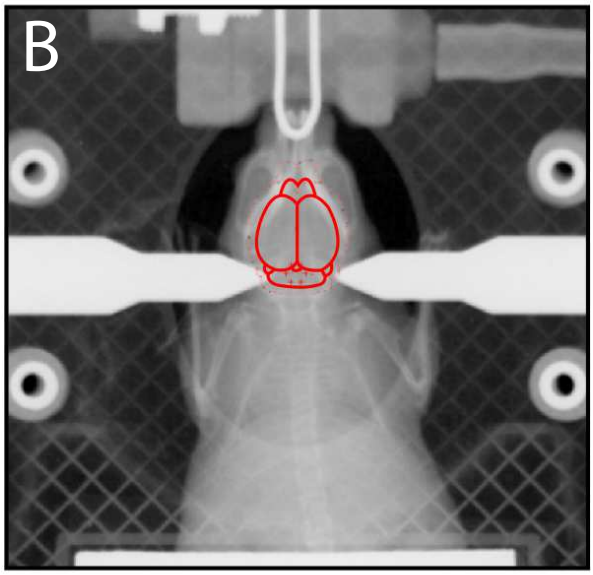
Focused ultrasound setup



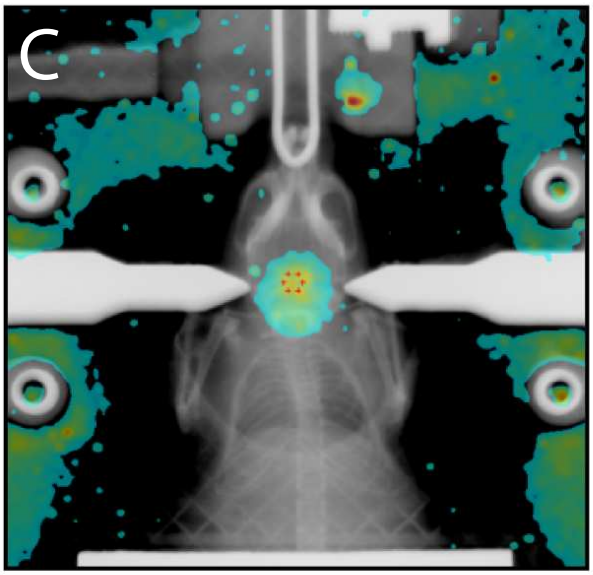




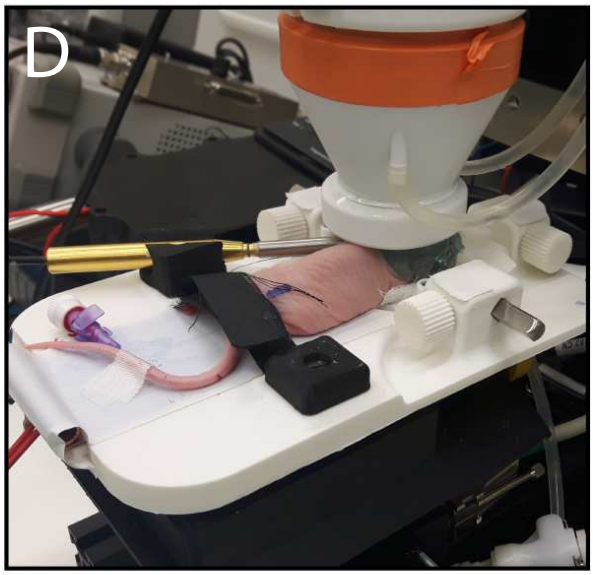
Positioning



X-ray imaging



BLI imaging



Targeting / sonication

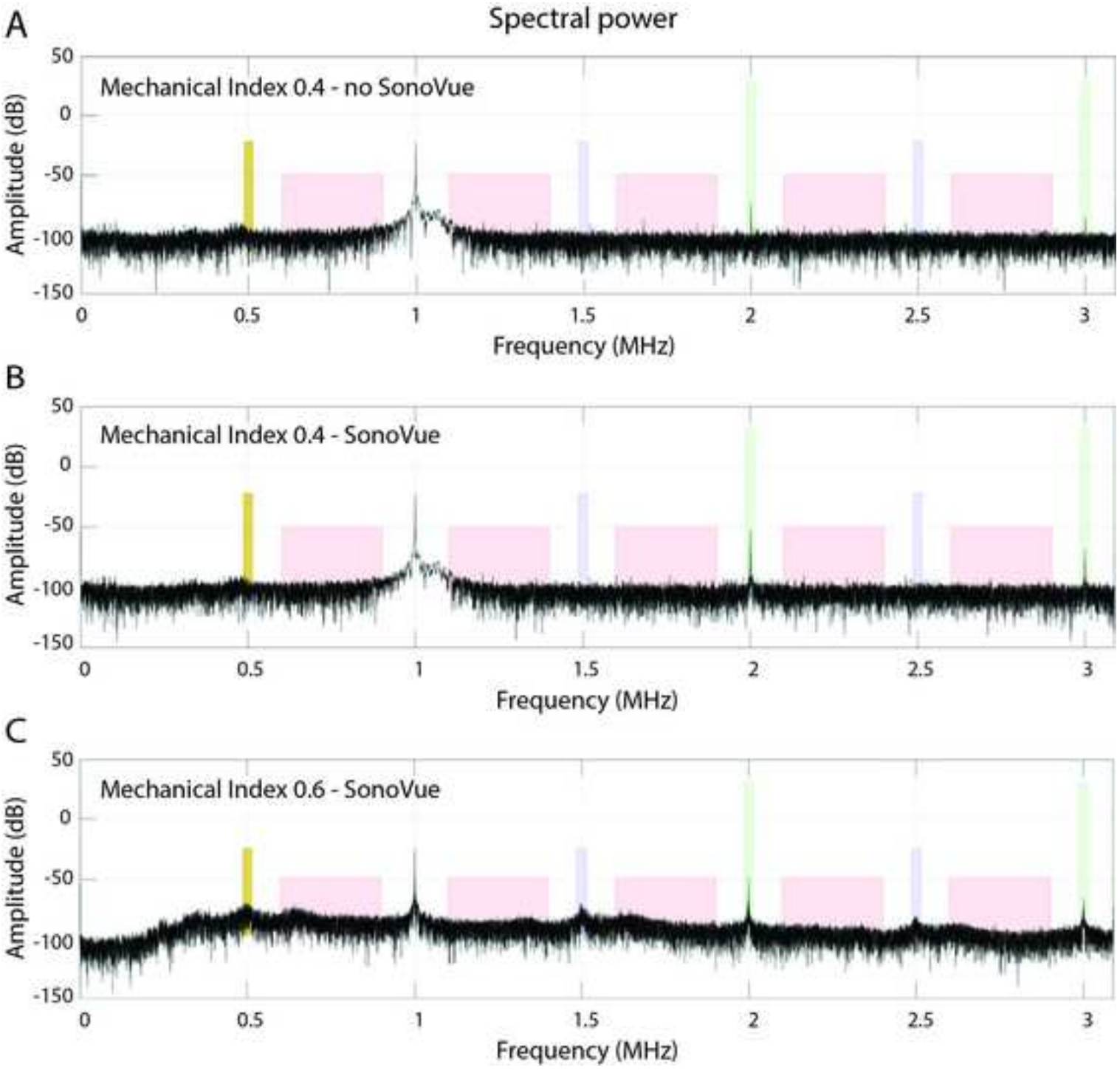


Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 mL luer-lock syringe	Becton Dickinson	309628	Plastipak
19 G needle	Terumo Agani	8AN1938R1	
23 G needle	Terumo Agani	8AN2316R1	
3M Transpore surgical tape	Science applied to life	7000032707	or similar
Arbitrary waveform generator	Siglent	n.a.	SDG1025, 25 MHz, 125 Msa/s
Automated stereotact	in-house built	n.a.	Stereotact with all elements were in-house built
Bruker In-Vivo Xtreme	Bruker	n.a.	Includes software
Buffered NaCl solution	B. Braun Melsungen AG	220/12257974/110	
Buprenorfine hydrochloride	Indivior UK limitd	n.a.	0.324 mg
Cage enrichment: paper-pulp smart home	Bio services	n.a.	
Carbon filter	Bickford	NC0111395	Omnicon f/air
Ceramic spoon	n.a	n.a.	
Cotton swabs	n.a.	n.a.	
D-luciferin, potassium salt	Gold Biotechnology	LUCK-1	
Ethanol	VUmc pharmacy	n.a.	70%
Evans Blue	Sigma Aldrich	E2129	
Fresenius NaCl 0.9%	Fresenius Kabi	n.a.	NaCl 0.9 %, 1000 mL
Histoacryl	Braun Surgical	n.a.	Histoacryl 0.5 mL
Hydrophone	Precision Acoustics	n.a.	
Insulin syringe	Becton Dickinson	324825/324826	0.5 mL and 0.3 mL
Isoflurane	TEVA Pharmachemie BV	8711218013196	250 mL
Ketamine	Alfasan	n.a.	10 %, 10 mL
Mouse food: Teklad global 18% protein rodent diet	Envigo	2918-11416M	
Neoflon catheter	Becton Dickinson	391349	26 GA 0.6 x 19 mm
Oscilloscope	Keysight technologies	n.a.	InfiniiVision DSOX024A

Plastic tubes	Greiner bio-one	210261	50 mL
Power amplifier	Electronics & Innovation Ltd	210L	Model 210L
Preamplifier DC Coupler	Precision Acoustics	n..	Serial number: DCPS94
Scissors	Sigma Aldrich	S3146-1EA	or similar
Sedazine	AST Farma	n.a.	2%
SonoVue microbubbles	Bracco	n.a.	8 µl/ml
Sterile water	Fresenius Kabi	n.a.	1000 mL
Syringe	n.a.	n.a.	various syringes can be used
Temgesic	Indivior UK limitd	n.a.	0.3 mg/ml
Transducer	Precision Acoustics	n.a.	1 MHz
Tweezers	Sigma Aldrich	F4142-1EA	or similar
Ultrasound gel	Parker Laboratories Inc.	01-02	Aquasonic 100
Vidisic gel	Bausch + Lomb	n.a.	10 g

Rebuttal: A high-throughput image-guided stereotactic neuronavigation and focused ultrasound system for blood-brain barrier opening in rodents

JoVe collections: Rianne Haumann & Elvin 't Hart

Manuscript number: 61269

Letter to the Editor:

We would like to thank the editor and the reviewers for the constructive comments. Based on the helpful suggestions, several changes have been made and we feel that they clearly improved the quality of our manuscript. We thereby hope that the revised manuscript is acceptable for publication.

Editorial comments:

Changes to be made by the Author(s):

E1. 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.

As suggested, the authors have proofread the article and corrected spelling and grammar issues where needed, see annotated manuscript.

E2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

As requested, all commercial language has been removed and replaced by generic terms. Commercial products are named in the Table of Materials and Reagents according to the JoVe guidelines. The changes are highlighted in the following sections of the annotated document:

- Line 168: "Protocol".
- Line 285: "Representative results".
- Line 314: "Figure and Table legends".
- Line 364: "Discussion".

E3. Presumably, the unlabeled image file is Figure 2?

The authors agree; Figure 2 has been updated, see new image uploaded.

- Line 327: "Figure 2. Focused ultrasound workflow".

E4. 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." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

As requested the protocol is written in the imperative tense and the usage of the "Notes" is more concise. The changes are highlighted in the document in the "Protocol" section of the annotated manuscript.

- Line 168: "Protocol".

E5. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

As suggested, the "Protocol" section has been updated with action statements, and the following discussion points have been moved to the discussion (see highlighted content in the annotated manuscript):

- Line 405-433: "With respect to […] and reproducible results".
- Line 434-438: "In addition microbubbles […] the tail vein".
- Line 440-446: "The cranium of […] as shown before".
- Line 447-450: "Cavitation monitoring is […] avoid tissue damage".

E6. Please specify the age/gender/strain of the mouse.

As requested, the age/gender/strain of the mouse has been specified in the "Protocol" section:

- Line 208-209: "For these experiments female athymic nude Foxn1-/- mice (6-8 week old) were used".

E7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

The authors agree to add more details in the "Protocol" section, and explain how the step is performed. Additional reference are highlighted in the annotated manuscript:

- Line 245-248: "3.3 Determine the target […] z=-4.5 from lambda".
- Line 269-272 " Here the applied […] McDannold et al".

E8. How far above the animal is the transducer mounted?

The focal distance of our transducer is 75 mm, therefore the transducer is positioned 75 mm above the animal (from the horizontal plane intersecting with the ear bars). This information is added in the annotated manuscript:

- Line 180-183: "In this study […] the ear bars".

E9. How is the animal mounted? Are the limbs taped?

The head of the animal is fixated in a stereotactic frame that includes ear bars and a bite bar. In addition, the body is strapped and the tail taped to the platform. This information is added in the "Protocol" section:

- Line 231-232: "2.9. Immobilize and fix […] to the frame".

E10. 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. Please consider moving some of the notes about the protocol to the discussion section.

The authors agree with the editor, and the usage of "Notes" has been modified to be more concise. The changes are highlighted in the annotated manuscript:

- Line 440-446: "The cranium of […] similar to others".
- Line 447-450: "Cavitation monitoring is […] avoid tissue damage".

E11. Figure 4: Please provide scale bars for the microscopic images.

As indicated, the authors have added scale bars to the microscopic images, see updated Figure 4:

- Line 348: "Figure 4. BBB opening and associated histology".

Reviewers' comments:

Reviewer #1:

Manuscript Summary: This manuscript describes a stereotactic neuronavigation based system for ultrasound mediated BBB opening in small animals. There is limited referencing to relevant prior work. Given that there are multiple small animal FUS platforms, some now commercial (e.g. FUS Instruments and Image-Guided Therapy), that have been built there requires more discussion from the authors re: the place of their proposed system in the field. Additional procedural details are also required.

The authors would like to thank the reviewer 1 for the feedback and suggestions.

Major Concerns:

R1.1. Work by others should be compared and contrasted. There are systems in existence that do not require submersion of the animal in a water bath (as the authors describe in the discussion) and some permit movement between imaging modalities.

The authors agree with the reviewer and have added representative examples of ultrasound setups in the annotated manuscript to set our FUS platform in the context of published contributions in the community. An emphasis has been put at other setups that 1) do not require any submersion of the animal in a water bath, but also 2) the lack of high-throughput with MRI and 3) other guidance methods for stereotactic navigation. This information has been added in the "introduction" section of the annotated manuscript:

- Line 91-114: "Most preclinical FUS […] the entire workflow".

R1.2. Throughout there should be additional references. Eg. "… BBB due to their physicochemical parameters (e.g. lipophilicity, molecular weight, hydrogen bond acceptors and donors) or are not retained due to their affinity for transporters in the brain." Add reference. The entire protocol should

be reviewed for appropriate referencing. These protocols are intended to help others duplicate the work so must guide readers to the necessary additional resources.

As indicated, additional and appropriate references are added in the article. The changes are highlighted in the following sections of the annotated document:

- Line 63: “Introduction”.
- Line 285: “Representative results”.
- Line 314: “Figure and Table legends”.
- Line 364: “Discussion”.

R1.3. "… pulse in real-time to a dedicated control computer running in-house software written with Matlab. NOTE: Real-time monitoring of cavitation is recommended to determine the safety of the procedure. Inertial cavitation can lead to severe irreversible tissue damage, as shown in Figure 3 and 4." How are the waveforms analyzed? This is critical for people to be able to reproduce the work.

The information, concerning cavitation and waveform analysis are added in the “introduction” and “protocol” section of the annotated manuscript:

- Line 121-138: “The most common […] inertial caviatation detection”.
- Line 268-283: “4. Analysis of microbubble […] in Figure 3”.

R1.4. With the above comment, there is not a single reference to the abundance of work on monitoring and controlling therapy base on cavitation signals. At a minimum the 2006 study showing the correlation of BBB opening (McDannold) with changes in harmonic emissions should be mentioned, but there are many studies correlating bioeffects to spectra, and then also those that use these to control the exposures (O'Reilly 2012, Arvanitis 2013, Sun 2017, etc)

That is indeed an embarrassing omission. More as an explanation than an excuse: This part had been originally in the article, which got somehow chopped in the last-minute edits. This is now (rightfully so) back and as requested, 9 references have been added to document monitoring of cavitation signals in our workflow, among those: O’Reilly et al 2012, McDannold et al 2006, McDannold et al 2008 and Sun et al, 2017. See highlighted references in the “Introduction” section:

- Line 79-81: “Alongside, previous studies […] on the BBB opening”.
- Line 116-121: “The interaction of […] with vascular/neuronal damage”.
- Line 268-283: “4. Analysis of microbubble […] in Figure 3”.

R1.5. As already discussed by O'Reilly et al.8 the type of anesthesia has an effect on the BBB disruption. Vasodilation occurs after the initiation of isoflurane, with potentially a reduction of the blood flow and the circulation of microbubbles. A stable anesthesia protocol is therefore advisable to achieve reproducible results. Alternatively, the animals can be anesthetized with a 340 mix of ketamine/xylazine, which has no known vasoactive effects" Ref 15, not 8 was the first to highlight this issue. It's worth noting a key issue with isofluorane is the carrier gas. If oxygen is used the bubbles rapidly dissolve. With medical air they are stable much longer and BBB opening is effective. I believe that Ref 8 does comment on this, although it has been anecdotally reported by many studies.

As mentioned, the authors appreciate the comment of reviewer and share his opinion with respect to the impact of anesthesia on the variance of the obtained BBBD. The anesthesia/bubbles are indeed one key aspect to reproducibility, which is frequently glossed

over. More concise information concerning the anesthesia has therefore added in the document. Some of the “pitfalls” (which we can substantiate based on our data / direct experience) are added/emphasized in the “Discussion” section of the annotated manuscript:

- Line 452-461: “Previous reports described […] known vasoactive effects”.

A remark to the reviewer:

- We keep fairly strict anesthesia protocols and did with respect to the vaso-activity of isoflurane not experience any problems. But indeed, we underlined the importance to do so in order to obtain reproducible results.
- We added another important point, which we found also crucial with respect to reproducibility of the results: The gas mix (i.e. the amount of isoflurane in the breathing gas, the resulting depth of anesthesia and the associated body-core temperature of the animals). This really changes the heartrate of the animals a lot and thus leads to a large variation of cerebral blood flow (with all associated variation with respect to BBBD and subsequent drug delivery). We extended the paper accordingly and gave references evidencing/investigating this influence.
- With respect to N_2/O_2 mixture in the anesthesia gas: phospholipid SF_6 bubbles loose in an adult human in the first 8 min after injection 80 % of their gas volume (reference has been added to the manuscript) and in a mouse display a plasma evacuation half-life of 60-120 sec only. We respectfully doubt if the N_2/O_2 gas mixture has a significant influence on this already very dynamic behavior. However, the reviewer is absolutely right: Taking into consideration the very short life-time of these bubbles in-vivo into consideration is crucial for reproducible results. The manuscript has been extended in the discussion section accordingly.

Minor Concerns:

R1.1.1. Requires copy-editing for typos and small grammatical errors.

As mentioned at E1, the authors have proofread the manuscript to ensure that there are no spelling or grammar issues, see annotated manuscript.

Reviewer #2:

Manuscript Summary:

The authors describe a convenient method and protocol for performing BBB opening using stereotactic guidance and image-registration. This should be of interest to people considering research in this area. The authors describe the system and the method for using it to open the BBB in rodents. Overall the manuscript is well written, but I'm not sure it can serve as a completely independent guide for readers in the absence of video. A couple of the areas where further clarification would help are listed below. As currently presented, this represents more of a 'see what we can do' paper rather than a visualized experiment designed for others to replicate.

The authors thank the review for the comments and suggestions. The protocol has been changed and in our opinion we feel comfortable now that readers are able to duplicate the work and design a FUS system based on their own requirements. Here after the video will be made.

Major Concerns:

R2.1. The registration of skull landmarks should be explained better. In section 3, the authors describe putting the animal on an imaging system

As mentioned, the authors have added additional information regarding the targeting of the animal. The chances are highlighted in the "Protocol" section of the annotated manuscript:

- Line 245-248: "3.3 Determine the traget […] z=-4.5 from lambda".

R2.2. The animal prep should specify details for mice and rats separately where relevant. For example, I am doubtful a 26g tail vein catheter can be placed in a mouse by most researchers. More commonly used would be 27-30g, and then some comments on the need to inject slowly to avoid bubble destruction should be provided. Also, some comments on keeping the length of catheter reasonable to avoid excessive injection pressure is important too.

The authors agree with the reviewer and updated the document. The changes are highlighted in the annotated manuscript:

- Line 223-227 "2.7. Insert a 26-30 […] short as possible".

R2.3. The authors display frequency spectra from acquired hydrophone signals, but little to no details are provided describing the signal chain/filtering to get this result. As written it's unlikely anyone without expertise in this specific area could reproduce these acoustic emission results.

As mentioned in our reply R1.4 (to the other reviewer), the authors agree with the statement of the reviewer. The requested information regarding the analysis of the frequency spectra by passive cavitation detection and the Fourier transformation has been added. And a (brief) overview of the corresponding literature has been added in the introduction. The changes are highlighted in the annotated manuscript:

- Line 121-138: "The most common […] inertial cavitation detection".
- Line 268-283: "4. Analysis of microbubble […] in Figure 3".

R2.4. The authors should refer to other top-down stereotactic FUS systems in research use - at least 3 groups are using such a system (konofagou in Columbia, Chopra in texas, and Liu in Taiwan). There are probably others.

As requested by the reviewer, we added a much more comprehensive overview over the existing prior-art. Indeed, multiple groups using top-down FUS systems have been mentioned in the "introduction" section. The changes and references are highlighted in the annotated manuscript:

- Line 107-114: " Alternative to MRI […] the entire workflow".

Rebuttal 2: A high-throughput image-guided stereotactic neuronavigation and focused ultrasound system for blood-brain barrier opening in rodents

JoVe collections: Rianne Haumann & Elvin ’t Hart

Manuscript number: 61269_R1

Letter to the Editor:

We would like to thank the editor and the reviewers for the constructive comments. The comments were addressed and changes in the manuscript were made accordingly. We hope that the revisions improved the manuscript and that the manuscript is acceptable for publication.

Editorial comments:

E1. Please copy-edit the manuscript. There are many awkward phrases and typographical errors throughout the manuscript.

> As suggested, the authors have copy-edit the article and corrected spelling and grammar errors where needed, see annotated manuscript (E1).

Reviewers' comments:

Reviewer #1:

Manuscript Summary: The revised manuscript is greatly improved, however I have a few remaining comments. Most are minor, but a couple, including the discussion of catheter gauge and carrier gas are important changes for those wishing to follow this protocol to be successful.

Major Concerns:

R1.1. Line 483: I would recommend suggesting that the readers use the larger end of that range (ie. 26G) as with the smaller 30G there is likely to be substantial MB destruction during injection, adding variability. Perhaps recommend the 26-30G but suggest the 30G should used only if venous access has been an issue.

> The first submission of the manuscript indeed recommended the use of a 26G needle for i.v. injection of microbubbles. However, the reviewer was doubtful that a 26G needle could be placed by researchers in the field. Therefore, we addressed this issue and recommended a needle between 26-30G. We agree that 26 G is recommended. Therefore, we have addressed this issue in the current version.
> - Line 484-487

R1.2. Line 504: It should be mentioned that the reference to 1.5% isofluorane and 2% oxygen for 35-40 mins in mice is for their general well-being and not a BBB study. The authors should cite McDannold et al, 2017 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5191922/ who describe a reduced BBB opening effect with oxygen vs medical air as the carrier gas for isoflurane, and recommend medical air or lab air for use. This is important as readers trying to implement this protocol with isofluorane and oxygen will struggle with inconsistency in results. This point was raise in the last review and has not be adequately addressed in this revision

We have in fact included this point already in the last revision (L507-509) with the advised reference of McDannold et al. We like to underline, that in our experience we really have no indication in our experiments that this aspect posed any problems. We certainly do not doubt McDannolds' work, but we have (see for example Figure 4 in McDannolds' paper) simply not reproduced it, nor did we run into similar problems. We achieve with isofluorane+O_2 plenty of extravasation of Evans Blue (>99% Albumin bound, so more than 66kDa of molecular weight), our PCD signal evidences clearly stable cavitation, etc.
However, we like to underline that plasma clearance and loss of gas volume of the bubbles is both entirely dependent on the design of bubbles (inert gas, coating, shell material,etc). McDannolds used Optison back then, while we use Sonovue and herein lies most likely the solution to the riddle, but at the end of the day, this is merely a hypothesis, which is hard to validate since Optison is off the market.
In the current version of the manuscript we have underlined this *potential* problem and therefore as an important point to take into account (or to avoid altogether and use Ketamine instead), but we'd like to keep it - with a reference to McDannolds' work - as an advice, but not present it as if our findings represent an independent reconfirmation of McDannolds' findings.

- Line 507-511.

R1.3. In the results/discussion it is worth noting that the step size between MIs (0.4 vs 0.6 vs. 0.8) is quite large and different levels of tissue effects and acoustic signatures (e.g. sub or ultraharmonics in the absence of inertial cavitation) might be observed at intermediate pressures.

This is indeed true, but maybe it is important to consider that it is a manuscript for JoVE which is a methods paper. As a consequence, we have chosen to present three representative pressure regimes ((I) stable cavitation at the upper limit of the transition to inertial cavitation, (II) inertial cavitation, and (III) deep in the inertial cavitation regime, where wide-spread tissue lysis occurs). We hope this helps the reader to adjust their respective experimental set-ups accordingly.
We also considered more fine-grained data, too, even analysed with short-time Fourier transform (STFT) and Gabor Wavelets across the 10 ms burst tone and in the transition regime. But at the end we decided against it, since we felt for the purpose of this paper, we should keep things simple (and reproducible) for the reader. Therefore we have chosen to use only three pressure regimes.

More interesting is indeed the aspect of a lack of ultraharmonics in the stable cavitation regime. The second point (potential appearance/lack of ultra-harmonics) is addressed in detail below (point 3.1 reviewer #3).

Minor Concerns:

R1.1.1. Line 84: Add ref to: Carpentier, A.; Canney, M.; Vignot, A.; Reina, V.; Beccaria, K.; Horodyckid, C.; Karachi, C.; Leclercq, D.; Lafon, C.; Chapelon, J.-Y.; Capelle, L.; Cornu, P.; Sanson, M.; Hoang-Xuan, K.; Delattre, J.-Y. & Idbaih, A. (2016), 'Clinical trial of blood-brain barrier disruption by pulsed ultrasound', Science Translational Medicine 8(343), 343re2

Thank you for the suggestion. We have added the reference.

R1.1.2. Line 100: 'enabaling' -> 'enabling'

The authors have corrected the grammatical error.

R1.1.3. Line 132: 'osccilation' -> 'oscillation'

The authors have corrected the grammatical error.

R1.1.4. Line 296: Replace 'sonoporation' with 'insonation' since you are referring to starting the ultrasound exposure, not necessarily the start of the bioeffect (sonoporation)

Indeed, it is correct to use the term insonation in this line. We have replaced sonoporation with insonation.

- Line 296

Reviewer #3:

Manuscript Summary: This manuscript presents a high-throughput image guided method for opening rodent's blood-brain barrier by focused ultrasound system. It will help in drug delivery and screen in preclinical settings.

Major Concerns:

R3.1. In Fig. 3 showing the spectral power for FU with different mechanical indexes in the presence and absence of microbubbles, not visible changes are observed for Figs. 3A and 3B, in the presence and absence of microbubbles. Is this true? In addition, the scale for the vertical axis (Amplitude) is different for Figs. 3A (from -200 to 100) and 3B (from -150 to 100). Please make a change.

First of all, sincere thanks for pointing the scale problem out, because in the retouching of the figures, the scale of Figure 3c was incorrectly set and this has been rectified.

With respect to the second point:

1) There is difference between Figure 3a (no bubbles present) and Figure 3b (bubbles present, and same pressure): Shown is the spectral power, so the increase of >20dB of for example the second harmonic at 2 MHz corresponds to a > 100x increase of the signal at this frequency, we have found typical for bubble cavitation (of Sonovue at 1MHz, 400kPa). Therefore we conclude that there is indeed a difference and it is substantial (but does not appear so large when shown in decibel).

2) However, we are aware that the reviewer (intuitively) expects subharmonics and and/or ultraharmonics (besides the harmonics, which are present).

We have repeated these experiments *in vitro* in a large cylinder/capillary (100-200 um diameter). Great care has been taken, that the experiment is similar, then one does indeed get subharmonics and ultraharmonics, too (at the same frequency, pressure, using the same bubbles, whatnot).
As soon as we go *in vivo* (same pressure including the compensation for the cranial absorption), the sub- and ultraharmonics are gone. This is indeed surprising. However, we have replicated this with two different PC-detectors independently and observed this in over 80 animal experiments with Sonovue: We never ever once had sub- or ultraharmonics with these bubbles at this frequency (before inertial cavitation sets in). Naturally this is bubble specific (most papers use Definity or similar bubbles), but this effect has to our knowledge not been really reported in the literature with Sonovue.
I hope that the reviewer agrees, that in the scope of this methods paper, we would like to report this as a phenomenological finding. Because if people increase the pressure in the

hope for finding sub- or ultraharmonics (at this frequency and with these bubbles) as it has been reported for example for Definity bubbles, they will encounter wide-spread haemorrhaging.
But indeed, beyond these practical implications (which is the purpose of JoVe), this is a rather peculiar finding that merits further investigation.
We have meanwhile (as a small side project) dug into the theory of what potentially causes this effect that in the stable cavitation regime *in vitro* ultraharmonics are present and i*n vivo* they are not. We believe meanwhile that this is essentially caused by viscoelastic damping in the much smaller capillaries (10-30 um), which reduces the oscillation amplitude of the bubbles considerably.
While this is certainly an interesting/noteworthy topic, it meanders rapidly beyond the scope of this paper, since this would really require (and merit!) the appropriate theoretical fundament and more specific experiments to explain/investigate. In summary, we believe that we underlined the complexity of the PCD signal for specific bubble types (at specific pressures and frequency) in the introduction (L128-138) and in the M&M part (L326) sufficiently.

R3.2. For validating the BBB opening shown in Lines 401-406, it was claimed that the molecule as large as albumin (66kDa) can be extravasated because Evans blue is bound to albumin in the blood. Therefore the observed blue (Evans blue) spreading in the brain is Evans blue bound albumin. The molecular weight of Evans blue is ~960 Da, much smaller than albumin and much easier to extravasate. If authors just want to claim that FU improved the extravasation of Evans blue, it is fine. But if authors want to claim that FU improved albumin extravasation, new experiments using albumin need to be performed. In addition, it also said that the Evans blue extravasation is due to partial opening of the tight junction in the paracellular pathway across the BBB. This needs to be justified.

We respectfully like to indicate that this is apparently a misconception of the reviewer.
The established literature (reaching back to the 1950s) is with respect to this clear, please have for example a look at: Yao et al "Evans Blue Dye: A Revisit of Its Applications in Biomedicine", Molecular Imaging in Targeted Therapeutics, Volume 2018, Article ID 7628037 https://www.hindawi.com/journals/cmmi/2018/7628037/ from which we'd like to quote:

"In various species, the binding capacity of albumin appears to be strongest in humans and dogs as 8–14 moles of Evans blue [28].When injected intravenously, EB becomes fully albumin-bound and only a ***small proportion of free EB (0.11%–0.31%)*** *can be found in the blood. The fact that EB dye is confined to the blood makes it unique among other dyes, for example, sodium fluorescein, which evenly distributes between the blood and organs such as the liver [10]."*

In human plasma, Evans blue is >99.7% protein bound (to all kind of proteins). Exactly this is also the reason, why it established itself since 1910 as a very reliable intravascular contrast agent (which it would be with a molecular weight of 960kDa alone/unbound clearly not). Albumin is thereby the (a) most prevalent protein (60% of the total plasma proteins in the plasma) as well as (b) the smallest of these plasma proteins (plasma proteins can be as big as 2MDa, whereby Albumin with 66kDa is the smallest). After flushing the vasculature post BBBD, the remainder is indeed to >99% protein bound Evans Blue, with an aggregate molecular weight of > 66kDa (and – but to a much lesser extent, even heavier).
We respectfully hope that the reviewer agrees that this fact is maybe frequently overlooked by scientists with a technical background, but is literally text-book knowledge among the more physiology/medicine/pharmacology trained community, who are both working in this

field of BBBD research. We added a sentence with a clarification and the reference, to the manuscript, but keep this aspect apart from this modification at a minimum.

- Line: 379

R3.3. For FU induced BBB opening, not only the ultrasound intensity but also the exposure duration matters. Not sure how the duration is incorporated in the study. Please clarify.

That is entirely correct. However, it is not only the duration of each individual burst-tone pulse which determines the final BBBD result, but also the number of pulse repetitions and the bubble concentration in the plasma that influences this.

Mouse models are ideal for cost effective drug-screening studies and also have the possibility to use genetically modified animals. While mouse models are in these aspects very favourable, this type of model also has severe limitations (compared to the clinical situation). One of these limitations is the high heart-rate and the very rapid clearance of the microbubbles.

Because this means in practice that the entire experiment is limited to 60-120s and that during this time a highly dynamic bubble concentration is present in the plasma (the initial bolus is very rapidly diluted and subsequently cleared. The bubble concentration varies during this time by almost two magnitudes!). Not one of the given acoustic pulses excites the same bubble concentration in the target area.
This renders the mouse model with respect to the aspect that the reviewer refers to a rather unfavourable - or rather: difficult - model.
For research that aims to study the interdependence of (a) pulse-length, (b) number of pulses, (c) PRF, (d) bubble concentration, larger animals, such as rats or preferably rabbits (or even bigger species) are much more suitable for this, since the pharmacokinetics of the bubbles resembles much more a clinical situation.
But this is also not the purpose of the described experimental set-up. Here we describe “a cooking book recipe” of exploiting US-BBBD for high throughput pharma research on mice. For that we simply chose a pragmatic way: Can we bring the US-BBBD into saturation? Hynynnen et al. showed burst-tone pulses that exceed 10ms do not show additional benefit of additional BBB permeability. So we simply try to cover the BBBD-target volume with 10ms pulses as often and as “randomly” (to provide equal spatial averaging during the bolus passage and the subsequent washout) as possible. With this strategy we found empirically, that more than 40 burst-tone pulses per location did not lead to a detectable increase of the extravasation of the intravascular tracers, i.e. saturation is reached.

Minor Concerns:

R3.1.1. The grammar needs to be carefully checked.

We would like to refer to E1 with detailed description of the changes made.

Reviewer #4:

Manuscript Summary: This manuscript suggested experimental set-up of focused ultrasound for rodent. Focused ultrasound for brain has been recently introduced in clinical field, and accordingly, there were also much needs of pre-clinical research using animal model of brain diseases. However, fully commercialized system is expensive, so many researchers cannot attempt to perform experiments using focused ultrasound technique. Considering this situation, this manuscript can offer valuable experience of experimental set-up with low cost.

Major Concerns:

R4.1. Line 150 1.7.: Exact location of needle hydrophone probe should be described. It is confusing whether needle hydrophone was inserted into brain or other space.

We have described in line 280 and line 366-367 the position of the needle hydrophone.

R4.2. Line 222 REPRESENTATIVE RESULTS: Please add detail description about microbubble injection. (dose, injection method, etc.) Injection doses and methods will also contribute to efficacy of BBB opening by FUS.

In the protocol, we have included the volume, concentration and dose method (Line 291-292).

R4.3. Line 238 : The authors used MI to indicate acoustic power. But, many researcher also use MPa for estimating acoustic pressure for brain FUS. So, it will be better to add MPa for indicating acoustic power for indicating intensity of ultrasound energy.

Due to a lack of consensus of the standards for describing the acoustic amplitudes, the therapeutic ultrasound community uses the pressure directly (in Pascal), while the diagnostic ultrasound community normalizes by the frequency and uses the mechanical index. In this manuscript we cite ''McDannold et al. Blood-brain barrier disruption induced by focused ultrasound and circulating preformed microbubbles appears to be characterized by the mechanical index. *Ultrasound Med Biol.* **34** (5), 834-840, (2008)''. Here, they describe that the mechanical index is a meaningful metric to describe the BBB disruption and correlate this to tissue damage. Therefore, we have opted for the use of mechnical index since we connect the tissue damage to the acoustic pressure.

R4.3. Line 288 DISCUSSION : Safety issue is one of the major concerns for FUS application for brain diseases. Referencing below articles, degree of tissue injury and BBB opening at 0.4 MI need to be described.

1. **Shin, Jaewoo, et al. "Focused ultrasound-mediated noninvasive blood-brain barrier modulation: preclinical examination of efficacy and safety in various sonication parameters." Neurosurgical focus 44.2 (2018): E15.**
2. **O'Reilly, Meaghan A., Olivia Hough, and Kullervo Hynynen. "Blood-brain barrier closure time after controlled ultrasound-induced opening is independent of opening volume." Journal of Ultrasound in Medicine 36.3 (2017): 475-483.**

Thank you for the suggested references. We have added the references.

And I wonder whether the authors used feed-back system. If yes, it will be better to be added details such as input peak negative pressure.

No real-time feedback system was used in this setup. When done properly (i.e. with intercranial pressure calibration on 2-3 cadavers as an initial calibration) the settings are very reproducible across an experimental series (which is however checked with the PCD on an interindividual basis). What experiments are concerned that rely on bolus injection in mice, feed-back (or feed forward) control in combination in with the (a) very rapid heart rate and (b) the very fast plasma clearance rate of the bubbles turns out in practice more a burden than a boon. Consequently, within the scope of a JoVe paper we had opted to suggest what

works well in practice.However, for experiments in larger species (or at higher frequencies) this is a completely different matter and real-time pressure control is naturally a clear asset.