Electronic Supplementary Material

Title: Transcranial Photoacoustic Detection of Blood-Brain Barrier Disruption following Focused Ultrasound-Mediated Nanoparticle Delivery

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Material and Methods

Preparation of multiple components nanoparticles

FNP is a scalable block-copolymer driven self-assembly process that has been used to encapsulate organic actives in polymeric nanoparticles. Hydrophobic dyes were dissolved in the presence of an amphiphilic block copolymer and rapidly micro-mixed with water, leading to dye precipitation. The hydrophobic portion of the block copolymer then absorbs the precipitating dye, while the hydrophilic portion sterically crowds the surface, arresting further precipitation and growth to form stable water-dispersible dye NPs suitable for *in vivo* PAI applications (Fig. 1a). To complement our understanding of NP spatial distribution within brain tissues, NPs of three different diameters were labeled with three different fluorophores (Alexa-488, Alexa-405 and Alexa-647, SIGMA, USA) using FNP with fluorophore conjugated block-copolymers.

Ultrasound mediated BBBD

Briefly, animals were anaesthetized with isoflurane (2-2.5 % maintenance) and depilatory cream was used to remove hair from their heads when necessary. Following tail vein catheterization, animals were placed in supine position on a custom-built MR compatible bed and baseline T1-weighted MR images (in-plane resolution: 250 µm x 250 µm, Thk = 1.5 mm) were acquired using a 7T magnet (BioSpec 70/30 USR, Bruker, Billerica, MA, USA). The animal in the MR-compatible bed was then transferred from the magnet to a custom-made FUS system and positioned with the animal head coupled to a degassed water bag over the FUS positioning system (Fig. 1b). Microbubbles (0.2 ml/kg, Definity) followed by a saline flush (0.2 ml) and immediately after by NPs or NPs tagged with a fluorophore (Table S1 (ESM) reports volumes of each type of NP injected, n = 6) and/or indocyanine green (10, 30 or 50 mg/kg, cardiogreen (ICG), SIGMA, USA, n=5) were injected *i.v.* in the mouse tail vein as boluses at the time of sonication with the following parameters, kept standard for all

treatments: ultrasound frequency = 1.68 MHz; burst = 10 ms; PRF = 1 Hz; sonification duration = 2 min; estimated in situ negative peak pressure = 0.2-0.34 MPa. To confirm BBB openings, a gadolinium based contrast agent (0.2 ml/kg, Gadovist) was injected, after treatment, followed by a saline flush (0.2 ml) and the T1-w MRI sequence repeated with the animal positioned at the same location in the 7T magnet. During MR scanning and FUS procedures, bags filled with warm water maintained animals' body temperature at about 37 °C. Animals recovered from anaesthesia and were transferred to another room to proceed with PAI assessment.

3DCD and PAI

This external fibre bundle was preferred to the longer 20 x 1.25 mm embedded fibre bundle as standard in 15 MHz probe because the shorter and thicker fibre bundle provides increased fluence, and the laser light penetrates deeper into brain tissues through the animals' skull. The photoacoustic probe was rotated 90° from the side view shown in Fig. 1c before starting a scan. In addition, the photoacoustic system was calibrated using the energy sensor provided with the system at the beginning of each experiment as per supplier guidelines. Also, the photoacoustic system constantly monitored the laser energy output. For the *in vivo* 3DCD and 3D PAI scans, the transducer was operated at 12.5 MHz transmit frequency (PRF = 3kHz) and used to acquire 3D scans along a 15 mm to 17 mm distance. Each CD image frame took 0.9 s, and a 15 mm long scan took 135 s (step size = 100 µm). The photoacoustic image volumes were acquired with the 3D nanostepper mode to obtain photoacoustic image slices at selected NIR wavelengths (680; 700; 730; 760; 785; 810; 850; 875; 900 nm) and with 200 µm step size; these took 11 s per image slice and 13.83 min for 15 mm scan length.

The 3D Doppler and 3D PAI were acquired prior to FUS treatment, again about 2h after the intervention, and for n = 2 animals, 4 days post intervention. For the photoacoustic and ultrasound imaging procedures, animals were anaesthetized with isoflurane (2-2.5 %

maintenance) and positioned on a prone position on a heated table. Depilatory cream was then used to remove hair from their heads. The mouse heads were scanned from about the cerebellum to the olfactive bulb using the photoacoustic system with parameters listed above. After acquisition of baseline ultrasound and photoacoustic images, mice were recovered. MRI and stereotactic ultrasound mediated BBBD were performed, as described in ESM, on subsequent days. The animals were allowed to recover, then anesthetized about 2 h post treatment and ultrasound and photoacoustic images post intervention acquired using the same PAI protocol.

At the conclusion of experiments with an individual mouse, the anaesthetic (isoflurane) was increased to 5%, skin was removed and the same 3D photoacoustic scan was run. Shortly after, animals were perfused transcardially with saline solution followed by 10% formalin, the skull was removed and an identical 3D photoacoustic scan was run. The mouse brain could then be collected at the end of the scan. After 1-day fixation in 10% formalin, the excised brain was submerged in degassed ultrasound gel. To more precisely locate the FUS induced NP diffusion and guide histologic work, *ex vivo* PAI was performed at 40 MHz using the LZ550 photoacoustic probe. A 3D photoacoustic scan over a 19 mm range was performed on the whole brain with a 100 μm step size. The brains were then returned into a solution of 10% formalin in preparation for histology.

The photoacoustic spectra were obtained in the wavelength range of 680−970 nm in 5 nm increments for each NP for image processing. Briefly, polyurethane tubes (PU-033-25, SAI Infusion Technologies, Lake Villa, Illinois, USA) were filled with NPs solutions, submerged in degassed water and imaged with the photoacoustic system with similar configuration to the *in vivo* protocol.

FI from histology

Fixed brains were cryoprotected in 30% sucrose overnight at 4 ºC and then rinsed in 0.1 M phosphate-buffered saline (PBS, pH 7.4), snap-frozen in n-pentane and stored in a freezer (-80 ºC). Subsequently, the brains were cryosectioned (5-6 µm, Cryostat LEICA CM3050 S, USA) for FI and then stored in a -80 ºC freezer. Slides were then observed using an Olympus microscope (Olympus VS120 Virtual Slide Microscope, VS120-L100-W, ON, Canada) with identical acquisition parameters. Images were stored in the proprietary format, and later, visualized on a personal computer using OlyVIA (OLYMPUS OlyVIA 2.9). For each brain, up to four consecutive sections were observed.

Imaging time frame

The animal was first scanned with the PAI system to obtain baseline data, then recovered from anaesthesia. One or two days later, MRI and stereotactic ultrasound mediated BBBD and the intravenous injection of dyes were performed. Within 2 h of treatment, the animal was scanned with PAI using identical settings to the initial photoacoustic scan. Some animals (n = 2) were let to recover from anesthesia or perfused for histology. Four days later, the recovered animals were then scanned with PAI to analyze any changes over this period of time and euthanized at the end of the experiment. *Ex vivo* PAI of animals' brain was performed within a week after cardiac perfusion, and fluorescence imaging was performed two weeks following brain collection.

Data analysis

The composite images or multiplexed photoacoustic images are layers of spectrally unmixed photoacoustic images (SUPAI) superimposed on US B-mode images. Other photoacoustic images are single light wavelength images at the maximum of ICG or NPs' spectrum. All photoacoustic signal intensities were calculated by averaging pixel values in photoacoustic images by manually drawing region of interest (ROIs) covering the left and right hemispheres

(LH, RH). The maximum length within the brain in the depth (z) direction between two averaged photoacoustic signal peaks and the maximum lateral (x) spreading of dyes (ICG and NPs) at the locations of BBBD were both measured in mm in *ex vivo* coronal SUPAI, with display options in the default setting (Fig. S1, photoacoustic images). In addition, the width of each opening in the scanning (y) direction was calculated according to the step size of the 3D acquisition to yield the average BBB opening size in the scanning direction in mm. For the study of the spatial distribution of small, medium and large NPs around the locations of BBBD, the injected dose of each NP in an individual mouse was calculated so that absorbance or dyes' concentration was similar (Table S1). Photoacoustic images acquired can then be compared without additional correction(s), assuming that other factors such as attenuation, backscattering and NPs concentration that may impact the photoacoustic signals have the same effect on each studied NP. These photoacoustic images are referred to as normalized for dye concentration or normalized for maximum absorbance thereafter.

Statistical analyses were performed using NCSS 11 (NCSS, LLC. Kaysville, Utah, USA). Data are expressed as mean ± standard error of the mean (SEM) where n is the number of measured diameters (n = 40) in the CD study. The measured diameter of the blood vessels in LH and RH at baseline and 2 h post FUS were analyzed using the paired sample T-Test. An alpha error level of 5 % was used for the tests.

Table S1

Type, size and volume of dyes injected and type of fluorophore tagged to NPs in each animal

CD1 Mouse	M1	M2	M3		M4		M5		M6			M7			M8	
Type of NPs or dye	ICG10	ICG50	867	ICG30	784	867	ICG50	867	ICG50	867	784	774	867	784	774	867
Size (nm)	-	-	100	-	40	100	-	100	-	100	40	240	100	40	240	100
Volume (µL)	250	250	150	200	40	40	150	80	150	40	80	30	40	100	15	20
Fluorophore (Alexa-)	-	-	-	-	-	-	-	488	-	488	405	647	488	405	647	488

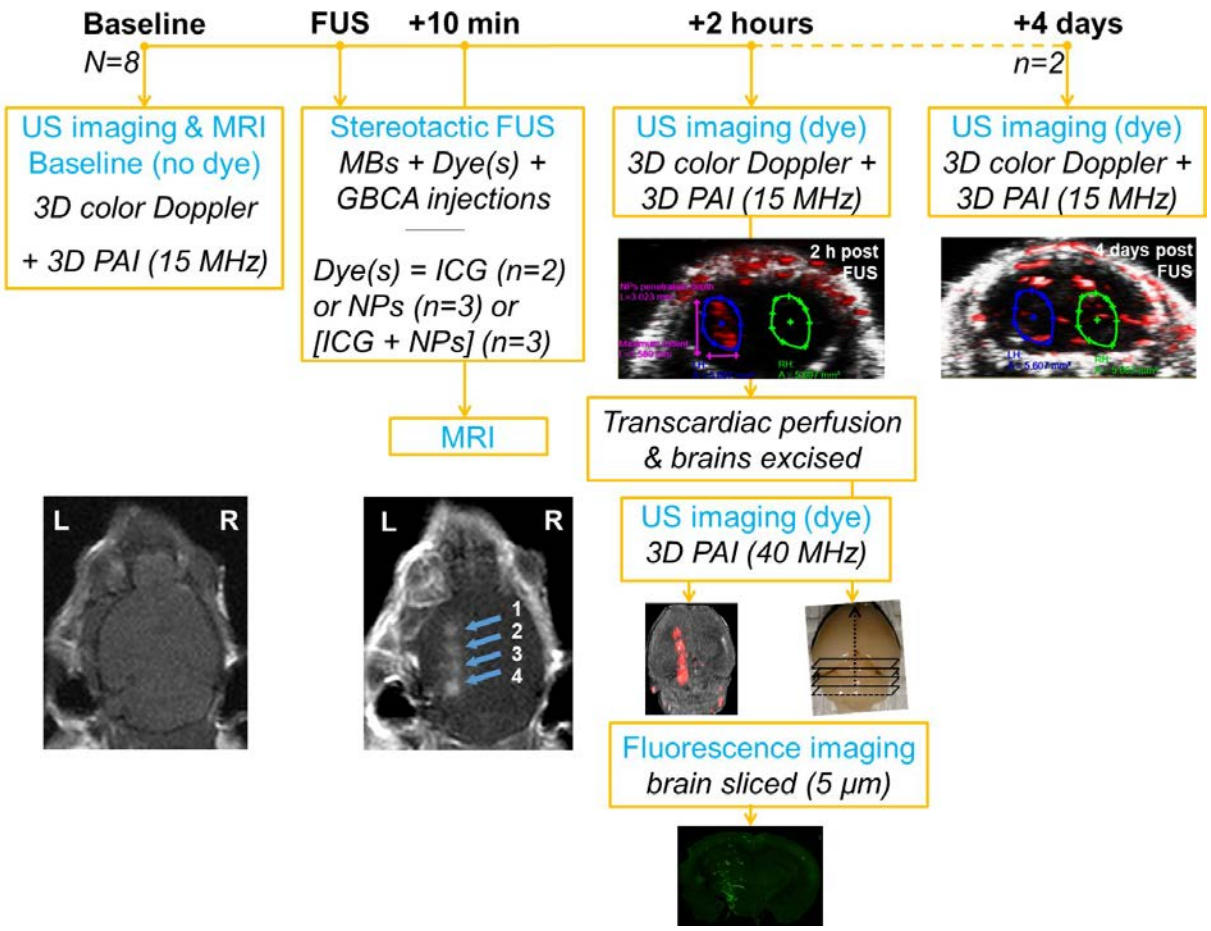

Fig. S1 Experimental timeline for stereotactic ultrasound mediated BBBD, MRI, 3D US, 3D PAI and FI. Left: MR images pre and post FUS to confirm BBB openings in the left hemisphere (LH) and an undisrupted right hemisphere (RH). Top right: photoacoustic coronal images 2 h and 4 days following FUS. Manually drawn region of interest in LH (blue) and RH (green). Maximum length and lateral spread of BBB opening were measured (purple lines). Middle right 3D surface rendering shown at 875 nm to detect the presence of NPs and illustration of 3D *ex vivo* US/photoacoustic acquisition. Black arrow shows the scanning direction over the top of an excised mouse brain. Black rectangles illustrate the recording of 2D US/photoacoustic planes for the US probe locations. Bottom right: FI of brain to detect any signal intensity variations from fluorophore-tagged NPs. Bright green signal in LH originates from NPs. Refer to “Imaging time frame” paragraph for timeline of histology.

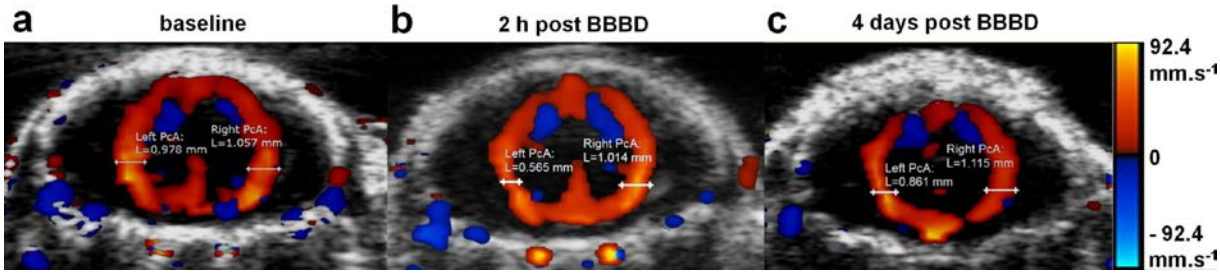

Fig. S2 2D Color Doppler cross sections at (**a**) baseline, (**b**) 2h post FUS and (**c**) 4 days post FUS allowing PCA diameter measurements (straight white lines) at studied time points.

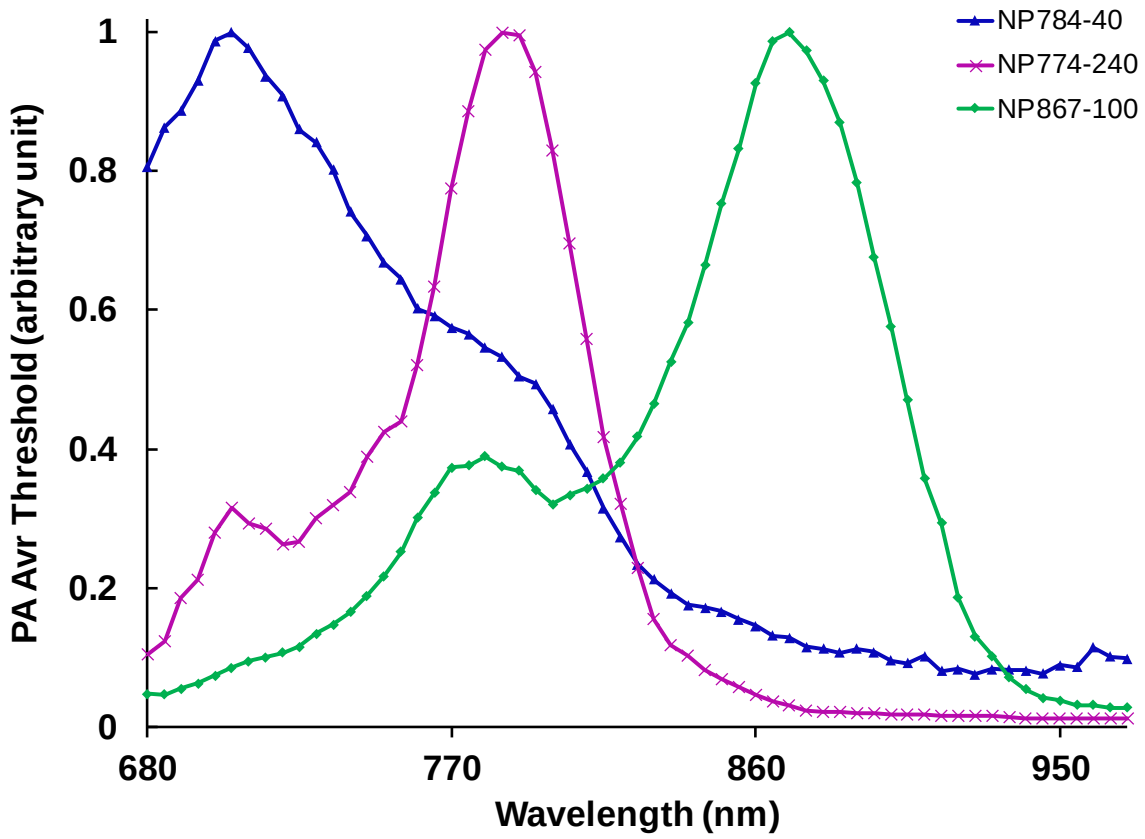

Fig. S3 Normalized spectra of dyes contained inside the nanoparticles designed for photoacoustc imaging. Note the well-separated peaks of the three NP's spectra.

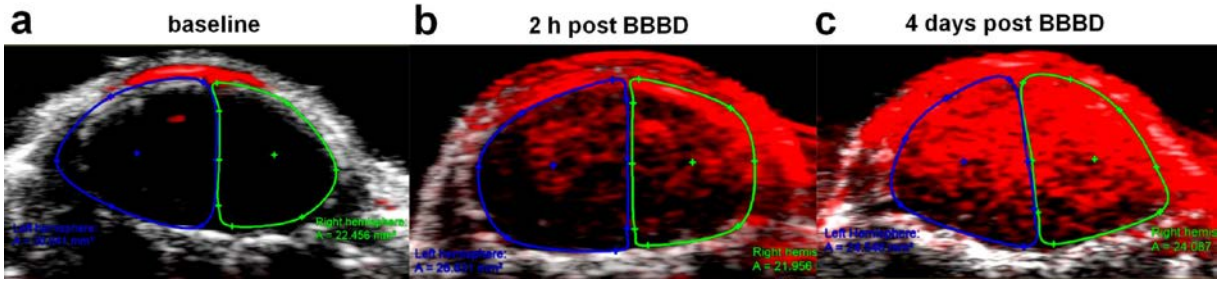

Fig. S4 *In vivo* photoacoustic images of NPs spatial distribution at (**a**) baseline, (**b)** 2h post FUS and (**c)** 4 days post treatment following NP867-100 injection. NPs accumulated and remained in brain tissues for at least 4 days post treatment.

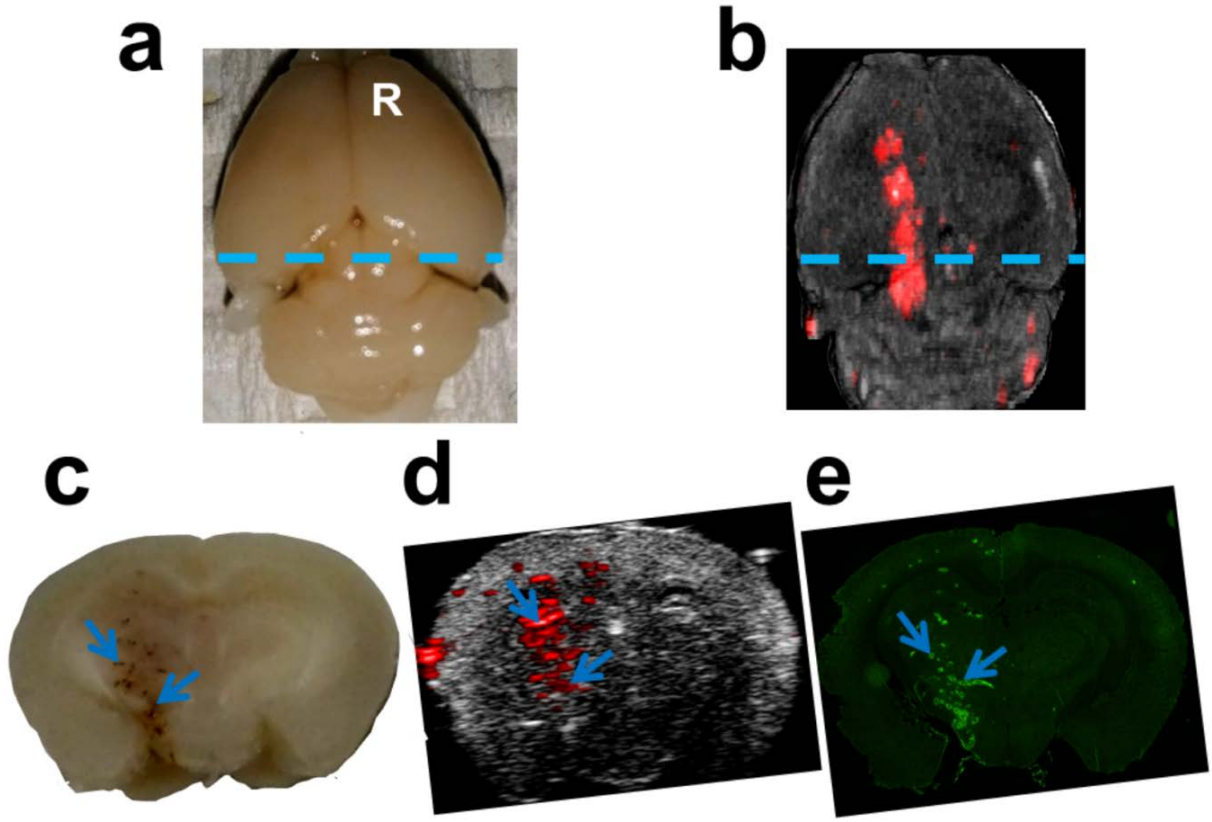

Fig. S5 a Excised mouse brain, (**b**) 3D photoacoustic rendering of *ex vivo* brain, (**c**) mouse brain tissues block, (**d**) photoacoustic image overlaid on 40 MHz ultrasound B-mode image and (**e**) fluorescence imaging. Blue dashed line indicates the location of the brain tissues block-face. Blue arrows highlights the presence of Alexa488 tagged NP867-100.

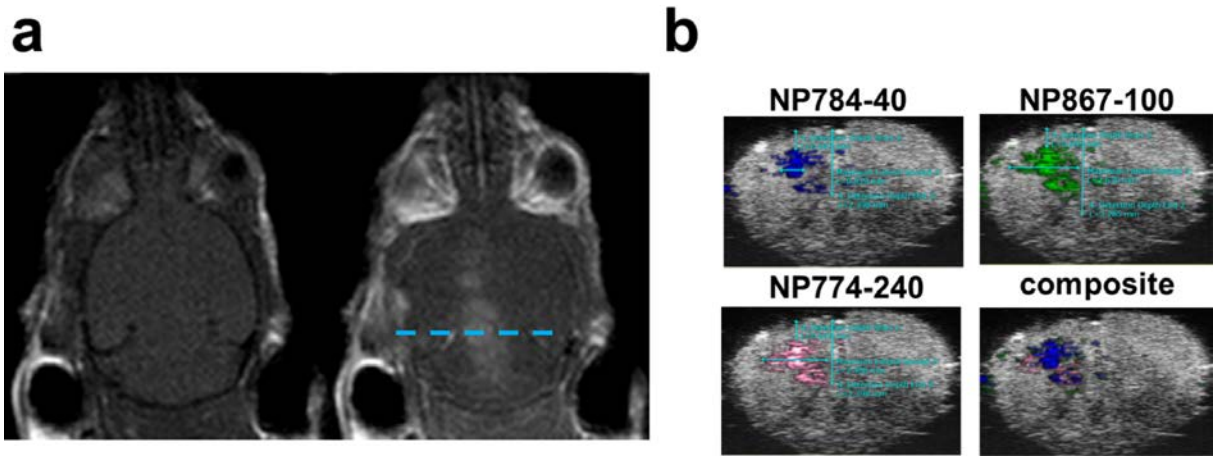

Fig. S6 *In vivo* MRI and *ex vivo* SUPAI normalized for dye concentration and superimposed on B-mode US images **a** MRI a few minutes before (left) and after (right) FUS treatment. Gadolinium based contrast agent-enhancement shows the spatial extent of BBB openings. Blue dashed line indicates the location of the PAI cross section. **b** *Ex vivo* SUPAI overlaid on 40 MHz US B-mode showing the variation of the spatial distribution of the three sizes of NPs at a given depth.

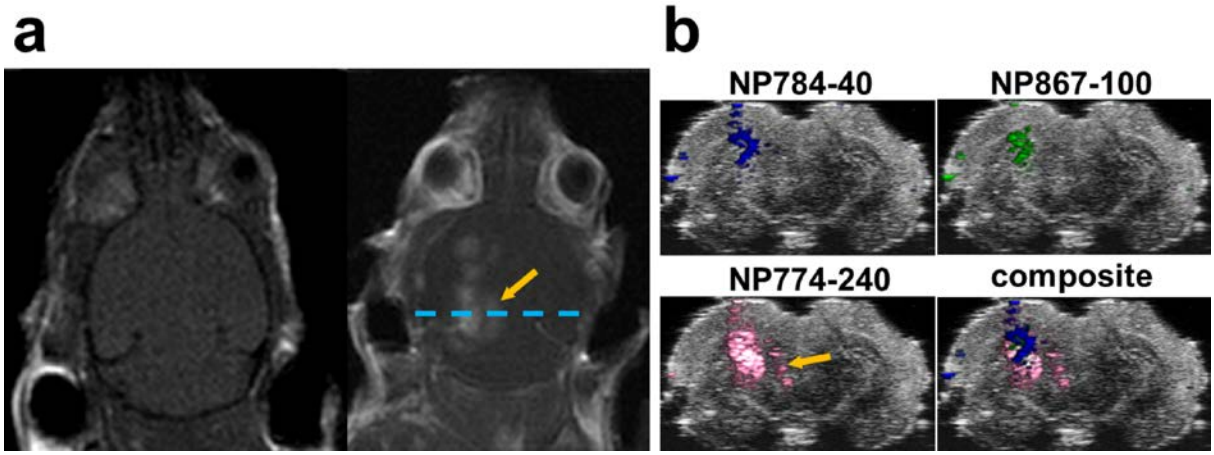

Fig. S7 *In vivo* MRI and *ex vivo* SUPAI normalized for maximum absorbance and superimposed on B-mode US images **a** MRI a few minutes before (left) and after (right) FUS treatment. Gadolinium based contrast agent-enhancement shows the spatial extent of BBB openings. **b** *Ex vivo* SUPAI overlaid on 40 MHz US B-mode. Gadolinium based contrast agent-enhancement shows the spatial extent of BBB openings. Blue dashed line indicates the location of the PAI cross section. Orange arrows points to signal enhancement in the left ventricle, indicative of increase permeability due to FUS, where only NP774-240 population was detected.